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reSOLUTION

Observing Life's Nanostructures with STED

Superresolution Light Microscopy

New Standard in Electrophysiology and Deep Tissue Imaging

Confocal Fixed Stage System

From Gene to Cell – from Cell to Embryo

The World's First Macro Zoom Confocal



Dear Readers,

The World Health Organisation estimates that more than one billion people suffer from disorders of the central nervous system. In Europe, disorders of the brain account for approximately one third of all diseases. To concentrate on the progress in these fields, ERA-Net NEURON started funding almost 10 million Euro to twelve European projects on neurodegenerative diseases of the central nervous system in February 2009 – projects we could support with our technologies.

The coming months are full of activities in the neuroscience field, e.g. in Göttingen, Liverpool, or Sölden – with new opportunities to network, exchange experience and look for new solutions presented at these events. The content of this reSOLUTION has been selected bearing in mind these important upcoming events – from stereotaxic instrumentation to the new electrophysiology platform.

While the global economic crisis still holds the first page titles in the press, every organisation is watching closely to see what the impact will be on their own business. Health Care and Life Science Research will probably suffer less than other areas. But it will be vital both for the scientific community and for their industrial partners like Leica Microsystems to adapt to the changing environment. This reSOLUTION presents outstanding examples of collaboration between members of the scientific community and ourselves: the development of the first Large Scale Imaging Macro-Confocal and the super-resolution STED technology. Both products are applied in neurosciences, too.

Have fun reading!

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Superresolution Light Microscopy

Observing Life's Nanostructures with STED

Anja Schué, Leica Microsystems

The secrets of life and the causes of many diseases can only be fully explained if we understand the functions of the smallest components of organisms. Using the super high resolution STED microscope of Leica Microsystems, research scientists are now able to observe cellular proteins and molecular structures measuring only a few nanometres.



Fig. 1: Leica TCS STED: its superresolution capacity allows confocal imaging with a resolution two to three times higher than could ever be achieved in a conventional scanning microscope without compromising on usability – superresolution at a mouse click.

When Ernst Abbe formulated the theory in 1873 that the maximum resolution of a light microscope was limited to half the wavelength of visible light of about 200 to 350 nanometres, nobody was thinking of observing structures of a few nanometres with light microscopes. However, biomedical research scientists are particularly interested in far smaller proteins and intracellular structures. Watching the way proteins and other molecular complexes move, work and interact helps us understand life's processes – how diseases develop and can be treated.

The decisive breakthrough was achieved by the physicist Professor Stefan Hell, now Head of NanoBiophotonics at the Max-Planck Institute in Göttingen, Germany. He invented fluorescence microscopes with which he outwitted Abbe's law. The super high resolution STED microscope is capable of resolving details as small as 20 nanometres. The first STED systems, for the production and marketing of which Leica Microsystems has an exclusive license, are already in use – for researching signal transmission in nerve cells, for instance. Three scientists who are using STED microscopy for neuroscience research report on their experiences:

Prof. Dr. Stephan Sigrist, Institute of Biology Freie Universität Berlin and NeuroCluster of Excellence, Charité, Berlin, Germany

Dr. Silvio Rizzoli, European Neuro-science Institute (ENI) and Cluster of Excellence Microscopy at the Nanometer Range at the DFG Research Centre for Molecular Physiology of the Brain (CMPB), Göttingen, Germany

Dr. Gregorz Wilczynski, Laboratory of Molecular and Systemic Neuromorphology, Nencki Institute of Experimental Biology, Warsaw, Poland

What does STED show you that you couldn't see before?

Sigrist: For the first time, STED brings light into darkness in the field of synaptic proteins. We recognise

“Why are we interested in flies that turn into crash pilots?”

sub-structures of synapses and are able to localise proteins such as bruchpilot. Bruchpilot plays a key role in synaptic signal transmission in the nerve cells of the *Drosophila* fly by building up a specific structure there for supporting signal transmission. If the *Drosophila* fly does not have much bruchpilot, it cannot sustain flight, if it has none at all, it dies. The protein is found in similar form in humans, too, and could be connected with diseases of the nervous system. Studying animals helps to understand the functions of the protein in humans. Understanding biological signal transmission is not only important for science in general. It is probable that synaptic defects trigger a large number of neurodegenerative diseases. In addition, it is almost certain that memory and learning processes are organised at synapses.

“For the first time, we have filmed real-time movement of synaptic vesicles.”

Rizzoli: Measuring only 40 – 50 nm, vesicles, which stock neurotransmitters in the synapsis and release them for signal transmission to the cell membrane, are among the smallest organelles of the nerve cell. Only with the help of STED were we able to localise individual vesicles, which usually occur in groups of 100 – 300. And contrary to previous assumptions that they hardly moved, we see that they move to and fro all the time, extremely rapidly and seemingly at random. This was inconceivable for us, as signal transmission is a highly complex, controlled process. We managed to record a first live video of these processes. As well as this, our knowledge of the vesicle recycling process at the cell membrane has also been revolutionised by STED. We now know, for example, that after fusing with the cell membrane, vesicle molecules are connected like a drop of oil in water – which facilitates vesicle recycling.

“Whether mushroom-shaped, branched or stubby – with STED we hope to classify dendritic spines efficiently.”

Wilczynski: Most synapses are situated on tiny protrusions 200 nm to 2 µm in size called dendritic spines. Their different shapes and sizes are thought to have a crucial influence on signal transmission. Changes of the dendritic spines also play a role in diseases such as epilepsy and the congenital disease Fragile X syndrome. Conflicting evidence has been obtained on dendritic spine form variety so far, and STED enables us to examine it in much more detail than with conventional confocal microscopy and to perform much more analysis than with electron microscopy. With STED we can examine several thousands of dendritic spines in the time it takes us to do 200 to 300 with EM. STED gives our results far higher statistic relevance for a new classification of dendritic spines.

You were one of the first to work with STED. What was it like to see the first images?

Sigrist: Without exaggerating, I can say that I discovered a new world. I immediately realised that STED is a breakthrough for finding answers to our questions and that we had had extremely naïve ideas of what we could see with light microscopy. But, after all, that’s the beauty of science – that new discoveries always raise new questions.

Rizzoli: I well remember the day in 2005 when I took the first photos of vesicles on a STED prototype in Stefan Hell’s laboratory. In those days, it still took five to ten minutes to take a photo – now it only takes 28 milliseconds – and it took us all day to get a good picture. It was fascinating, like opening a new chapter in the book of science. Research scientists must have had similar feelings in the fifties when the first electrophysiological image of a synapsis was produced. Nobody even knew about vesicles then.

Wilczynski: I was naturally delighted with the greater resolution that STED provides. The images are not only a bit sharper, it’s a whole new class of imaging. Actually, I’d expected this new technology to offer higher resolution. But I also found out very quickly that the sample material and the subject of your research play a crucial role for whether you get the most out of the higher resolution.

How do you rate the future significance of STED in life sciences?

Sigrist: Very important indeed, as STED takes us into the realm of protein complexes and therefore gives us a really close up view of life. At present, we are able to resolve structures below the 100 nanometre mark. Professor Hell, who is working on the further development of STED, has already achieved far higher resolutions. If we can use resolutions of a few tens of nanometres, it will be possible to determine with light microscopy whether proteins are close together or further apart. This would constitute a further quantum leap in our understanding of protein functions.

Rizzoli: STED has proved that the former resolution limit can be overcome. Today, everyone who works with conventional high-end resolution wants to work with super high resolution. Meanwhile, many laboratories are trying to develop new, super high-res techniques. But STED has made the most progress and is the only technology that really works. STED was like a starting pistol for a real technology race.

It will be exciting to see what else happens in the next few years.

Wilczynski: I'm quite sure that the significance of super high resolution light microscopy will increase. And I'm also sure that even technologies like STED

will continue to improve in terms of resolution. I also see advantages in comparison with EM – morphological examinations of cellular structures that were only possible with complex EM in the past can now be realised much more quickly with STED, as our case shows.



Prof. Dr. S. Sigrist

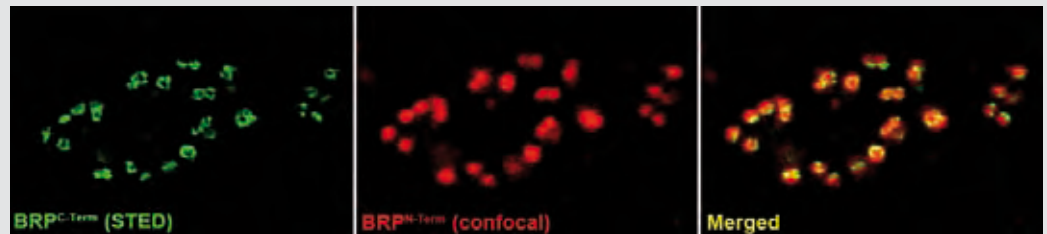


Fig. 2: Comparison between STED and conventional, confocal microscopy. Representation of a neuromuscular junction of the fruit fly *Drosophila melanogaster* that has been immunohistologically labelled with antibodies against the synaptic protein DLiprin-GFP. Substructures can now be visualised with the STED technology (resolution: 80 nm), which were otherwise unrecognisable in conventional confocal microscopes (resolution: 250 nm, see arrow heads in Fig. 3). Courtesy of S. Sigrist, Institute of Biology Freie Universität Berlin, Germany

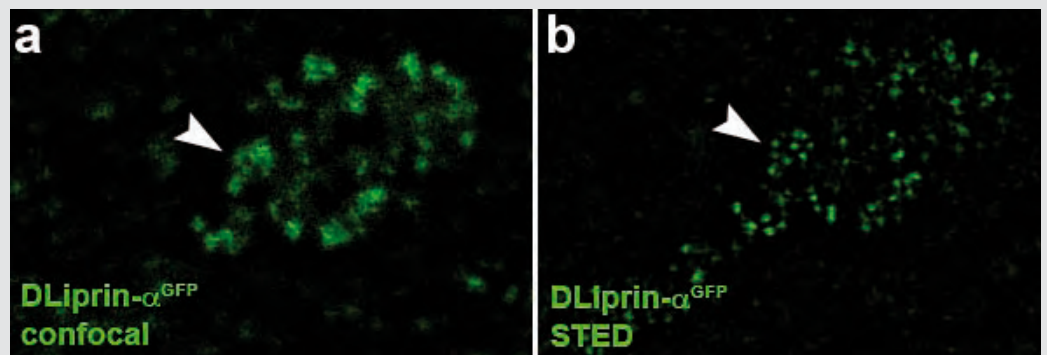


Fig. 3 a-b: Immunohistological co-staining of two antibodies which bind at different regions of the synaptic protein Bruchpilot (BRP). The increased resolution resulting from the STED technology (green, BRPC-Term) allows us to probe the spatial organisation of BRP at synapses. The overlay of the sequentially acquired confocal images (red, BRPN-Term) with the STED images clearly shows the higher resolution obtained by STED microscopy. Courtesy of S. Sigrist, Institute of Biology Freie Universität Berlin, Germany



Dr. S. Rizzoli

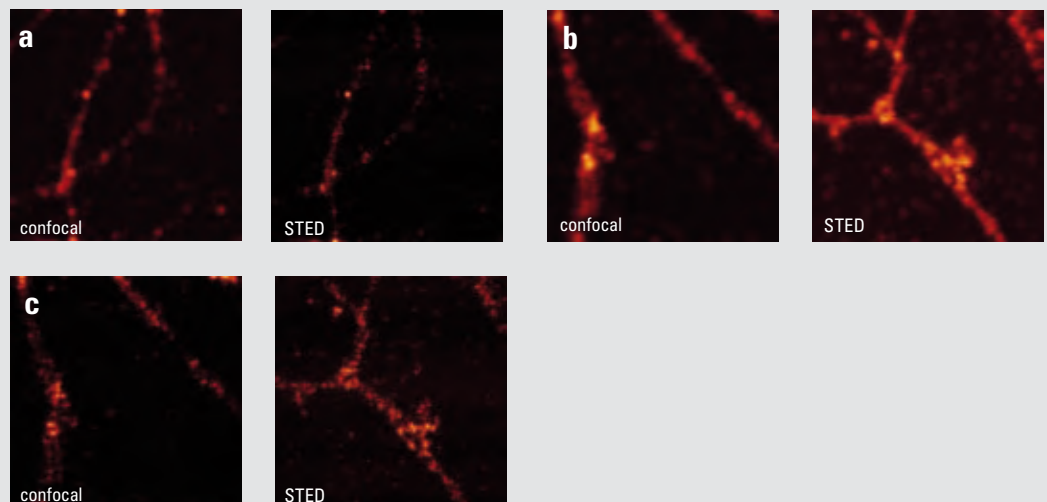


Fig. 4 a-c: The images show various areas in axons of neurons in cell culture. The cells were taken from the hippocampus of a rat (*Rattus norvegicus*). The synaptic protein Synaptotagmin 1 was labelled with a primary monoclonal mouse antibody and then with a secondary antibody carrying the fluorophore (Atto 647N). In STED mode, the synaptic vesicles can be clearly distinguished as individual points, which is not the case in confocal mode. Courtesy of S. Rizzoli, European Neuroscience Institute (ENI), Göttingen, Germany

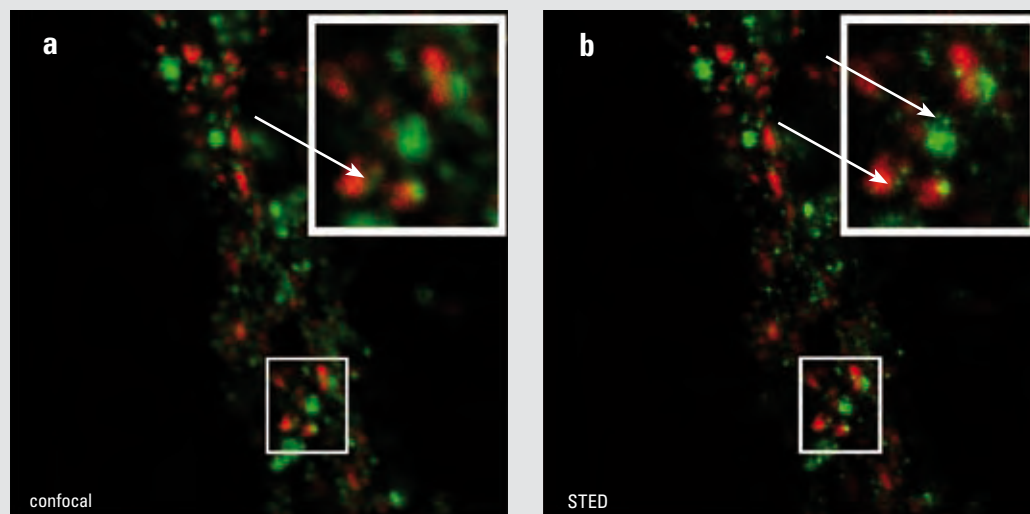


Fig. 5 a-b: Confocal (a) and STED images (b) of dentritic protrusions in cultured neurons. The inserts show the improved resolution with STED microscopy at the level of individual synapses. Green: Atto647-labelled GFP, red: synaptophysin. Courtesy of G. Wilczynski, Nencki Institute of Experimental Biology, Warsaw, Poland



Dr. G. Wilczynski (left) and his team

New *in vivo* Labelling Method for STED Microscopy

In the past, biologically relevant STED imaging was mainly limited to fixed immunolabelling techniques. The HaloTag® labelling technology developed by Promega is a new approach for live cell imaging in STED microscopy. This technology is comprised of the HaloTag® reporter protein, to which a gene of interest is fused, and the HaloTag® ligands. The reporter protein is an engineered, catalytically inactive derivative of a hydrolase that forms a covalent bond with the ligands. The ligands are diverse markers, such as fluorescent dyes, to which a linker designed to react covalently to the reporter protein is attached by simple chemistry. For this purpose, a membrane impermeant ligand was optimised for the Leica TCS STED microscope containing the fluorescent dye Atto655 and used to label live HeLa cells stably expressing human β -1 integrin-HaloTag® fusion protein. These cells were generated to study integrins; proteins

essential for cell adhesion and migration that are localised to both the cell surface and trafficking vesicles. STED imaging revealed localisation of the integrin fusion in unprecedented detail.

Reference

J. Schröder, H. Benink, M. Dyba, and G. V. Los; In Vivo Labelling Method Using a Genetic Construct for Nanoscale Resolution Microscopy; Biophysical Journal, Volume 96, Issue 1, 7 January 2009.

More information on the principle of the HaloTag® labelling technology:

<http://www.promega.com/paguide/animation/selector.htm?coreName=halotag01>

Confocal Fixed Stage System

New Standard in Electrophysiology and Deep Tissue Imaging

Irmtraud Steinmetz, Leica Microsystems

The function of nerve and muscle cells relies on ionic currents flowing through ion channels. These ion channels play a major role in cell physiology. One way to investigate ion channels is to use patch clamping. This method allows investigation of ion channels in detail and recording of the electric activity of different types of cells, mainly excitable cells like neurons, muscle fibres or beta cells of the pancreas. The patch clamping technique was developed by Erwin Neher and Bert Sakmann in the 1970s and 80s to study individual ion channels in living cells. In 1991 they received the Nobel Prize for Physiology and Medicine for their work. Today the patch clamping technique is one of the most important methods in the field of electrophysiology.

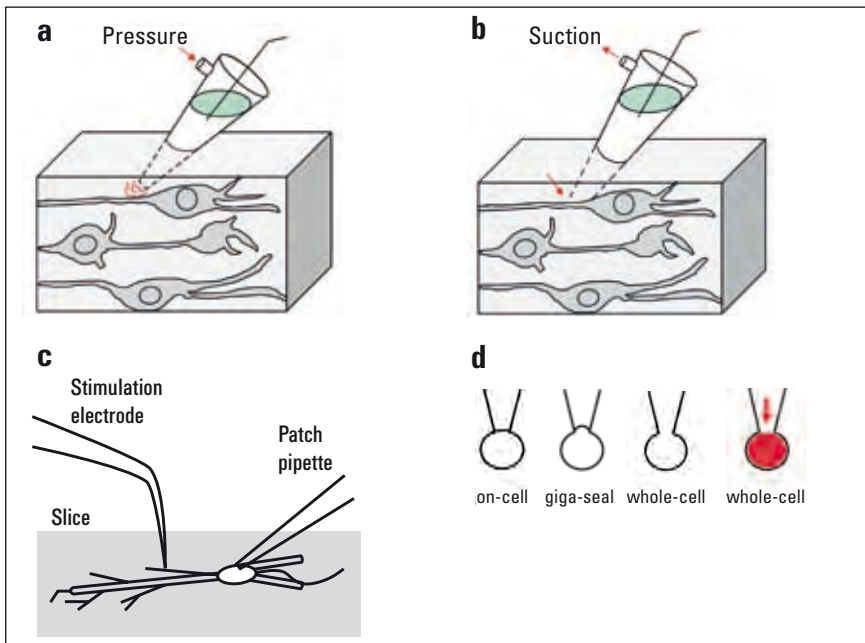


Fig. 1: The principle of the patch clamp technique. a) Pressure is applied to the pipette to penetrate through the tissue. b) Suction is applied to form a tight contact with the cell. a) and b) adapted from Stuart et al. 1993) c) A patch electrode is attached to a neuron to record voltage signals. A stimulation electrode can be placed close to a dendrite for extracellular stimulation by voltage pulses. d) Steps to get a whole cell configuration: the soma of the cell is approached (on cell), formation of a giga-seal by releasing pressure, brief suction disrupts the membrane (whole cell) cell can be filled with dyes or drugs. d) from PhD thesis of Th. Nevan: "Calcium dynamics in dendrites and spines in the somatosensory "barrel" cortex of the rat", 2003.



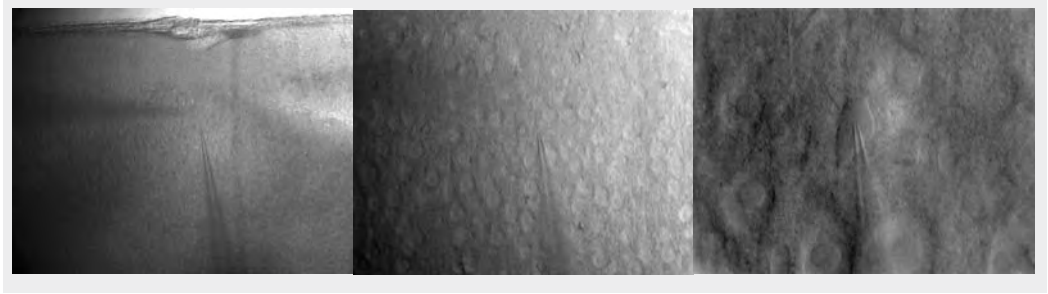
Fig. 2: The Confocal Fixed Stage System Leica DM6000 CFS integrates the Leica DM6000 fixed stage microscope into the Leica TCS SP5 confocal platform. The system helps to realise highly sophisticated experimental setups in physiology and electrophysiology from single cells to whole organisms.

Large field of view and high magnification

In patch clamp experiments a tight seal is formed between a glass pipette and the cell membrane. This arrangement allows the recording of small currents and voltages involved in the activity of neurons and other cells (Fig. 1). A large observation field is necessary in order to find the regions of interest within a specimen, for example in the brain of a living mouse, living brain slices, embryos, etc. In addition, it is important to be able to visualise detailed structures so that pipettes and electrodes in the specimen can be placed accurately.

The Leica DM6000 CFS (Fig. 2) combines the new high NA/low magnification objective lens HCX APO L 20x/1.0 W with the magnification changer in the CCD camera mode. This combination allows for a fast overview of the specimen while keeping a large observation field. Furthermore, the observation field can be changed easily by using different magnification changers. A large field of view is achieved

Fig. 3: Identification of cells: Dot gradient contrast transmitted light images of a mouse brain slice taken by a CCD camera (Leica DFC360 FX). To identify the layer of interest in a brain slice, overview imaging was performed using the magnification changers 0.35 and 1.0. Single cells were selected for the experiment when using the 4x magnification changer.



using the 20x objective. To identify individual cells, the 4x magnification changer can be used. Therefore, using both the objective and the magnification changer it is possible to easily position pipettes very close to the cell (Fig. 3). If a higher resolution is required, as for the investigation of neuronal spines, the system can be switched to confocal scan mode.

Single cell electroporation

To study neuronal networks in the brain, specific labelling of individual cells is required. A new method, called single cell electroporation (Nevian and Helmchen 2007), allows rapid and selective loading of cells. Brief voltage pulses are delivered, causing an electromagnetic field to be applied to the membrane. This results in the transient formation of small pores; the pores close within seconds. Charged molecules are transported in the direction of the electrochemical gradient during opening of the pores. This method can be used to load neurons with calcium-sensitive dyes and other dyes (Fig. 4).

Using the single cell electroporation technique, the background staining is lower compared to using the loading by intracellular recording electrodes or patch pipettes. The single cell electroporation technique is especially well suited for functional imaging of subcellular Ca^{2+} -dynamics *in vitro* and *in vivo*. Multiple substances can be loaded to obtain morphological and functional measurements at the same time. Furthermore, multiple cells can be loaded sequentially in order to image small neuronal networks by using the same pipette.

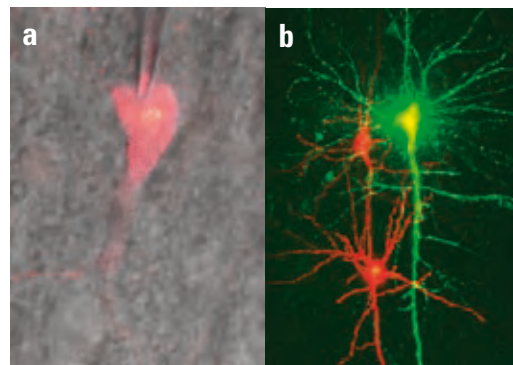


Fig. 4: a) A specific neuron was loaded with a calcium sensitive dye by single cell electroporation; overlay of simultaneous acquisition of fluorescence with a Non-Descanned Detector (NDD) and transmitted light; b) small neuronal network: rat brain slice, layer 5, red: Interneurons Alexa 594, green: Pyramidal Cell Oregon Green Bapta 1 (calcium sensitive), $Z = 123 \mu\text{m}$, two photon excitation; detection with a 2-channel NDD. Courtesy of Dr. Thomas Nevian, Inst. of Physiology, University of Bern, Switzerland

Correlating optical and electrical data – and images

In many physiological applications the reaction of cells to different types of stimuli is of interest. The reactions of the cells, as seen by electrical and fluorescence intensity data, need to be measured and displayed in a synchronised manner. The intensity data usually refer to the intracellular calcium concentration or pH-value. The Leica DM6000 CFS enables the synchronised correlation of electrical and optical data; voltage recordings are correlated in synchronisation with the fluorescence intensity data and are automatically displayed in graphs. This reveals a fast and direct overview of the experimental progress and online data evaluation. Furthermore, images are displayed below the graphs to get fast information about the morphology of the cell.

A data acquisition box and Leica trigger unit are integral components for viewing the intensity and voltage data correlation. Recorded signals, as from a neuron, are typically amplified, and then sent to a NI Data Acquisition Box (DAQ box) from National Instruments, which digitises the signals. The DAQ box is connected to the trigger unit that allows for the synchronisation with the scanning process. It is also connected to the PC so that correlated optical and electrical data, as well as images, can be displayed.

Currents and calcium in heart muscle cells

With the calcium-sensitive dye Fluo4 labelled isolated cardiomyocytes from trout were stimulated by a trigger pulsing regime using patch clamping (HEKA EPC-10 double). Cardiomyocyte responses to intracellular calcium concentration and ionic currents were measured using fluorescence imaging and electrical recordings. The stimulation protocol on the patch clamp setup was synchronised with the confocal time lapse series using a trigger on the patch clamp setup to mark events in individual frames on the time axis. Line triggers are recorded in order to have the exact correlation of the image scan, fluorescence signal intensity, and electrical

response of the cell (Fig. 5). These triggers are automatically generated by the scan head. This means that whenever a line is scanned, its corresponding trigger pulse is recorded and displayed in the quantification chart. Synchronisation of images and triggered pulses is accomplished using the Leica DAQ box.

Multiphoton microscopy and external detectors

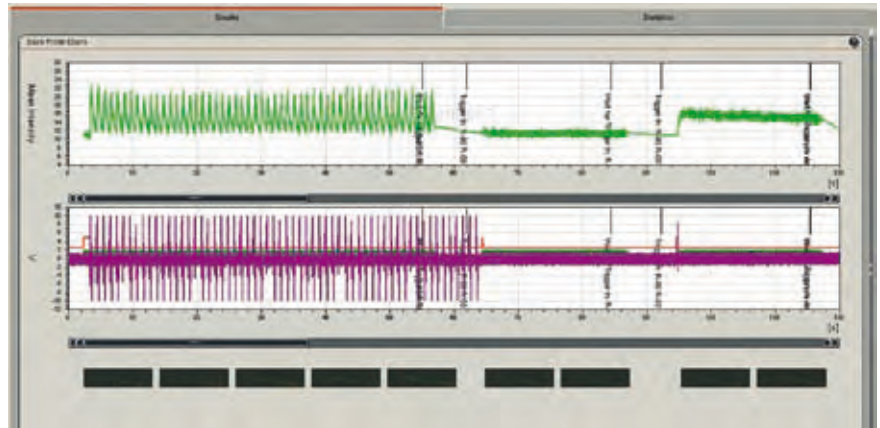
A major challenge in physiological applications is the study of cells in deeper layers of tissue. Multiphoton microscopy provides several advantages for solving this challenge. Lower light scattering, restricted excitation and bleaching to the focal plane, and reduced phototoxicity are the properties of multiphoton microscopy that enable visualisation of deep structures. Brain slices, for example, are highly scattering and are usually sliced several hundred micrometres thick. Therefore it is very difficult to image brain slices using a standard confocal microscope. Thicker specimens can be imaged using multiphoton microscopy due to an enhancement in photon collecting efficiency.

In confocal microscopy the pinhole aperture rejects the out-of-focus fluorescence light as well as the scattered (diffused) light. Since the scattered light is not 'seen' by the detector, it is not easy to image highly light scattering tissues such as thick brain slices. In multiphoton microscopy however, no confocal pinhole is required to reject the out-of-focus light because all fluorescent light originates from the focal spot. The emitted light from the specimen does not need to pass through the microscope again. Therefore, detectors can be placed as close as possible to the specimen so that scattered photons can be collected. In this way significantly higher photon collection efficiency is achieved compared to confocal microscopy.

The external Non-Descanned Detectors (NDDs) are PMTs for collecting emitted light from the focused and scattered light (Fig. 6). The NDDs are boxes containing PMTs for the detection of either two or four different fluorescence signals (4-channel NDD). To separate the signals, several filter cubes are available and can be introduced into the detector box.

Fluorescence and high contrast transmitted light imaging

Unstained neurons in thick brain slices are phase objects. To make them visible it is necessary to convert their phase gradients into amplitude gradients. There are different ways to achieve this. One is by using differential interference contrast (DIC),



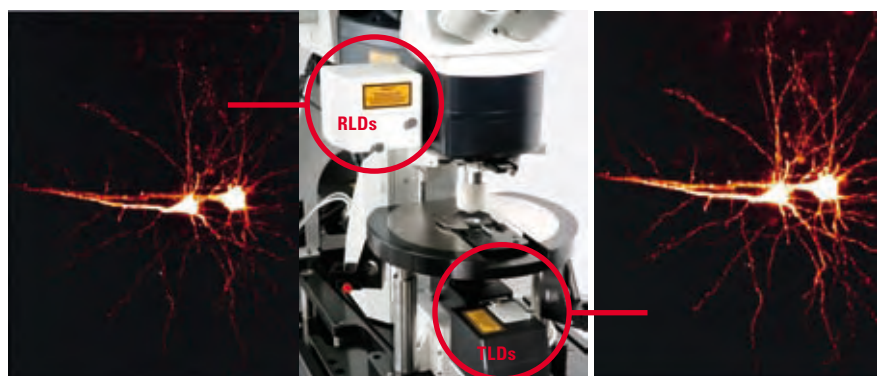
which requires prisms and polarisation filters in the beam path. Thus, when transmitted light and fluorescence are imaged together the photon collecting efficiency is reduced due to the optical components in the beam path.

Infrared Scanning Gradient Contrast (SGC imaging) – also referred to as Dotd Contrast – is another method used to visualise unstained cells in thick scattering tissue. This is a special optical system developed by Hans-Ulrich Dodt (Max-Planck Institute, Munich, Germany). In this technique the contrast can be specifically adjusted to enable different structures (horizontal or vertical) to be highlighted.

Using the Dodt gradient contrast method no optical components are needed in the beam path to get high-contrast and high-resolution images. Here, a lens system between the microscope stage and the lamp house re-images the aperture plane of the condenser. Spatial filtering is performed by a quarter annulus located in the illumination beam. After the annulus, a diffuser is placed to generate an oblique illumination across the condenser aperture. The light-stop blocks much of the illuminating light and only a part of the illuminating light cone is used. Therefore, less stray light is generated in the slice and an oil-immersion condenser, e.g. with 1.4 NA, can be used for high

Fig. 5: Quantification of the experiment described in the text. Intensity graph (top), electrical data (middle) and images (bottom) recorded during a patch clamp experiment in a cardiomyocyte. Cells have been stimulated by different pulsing protocols, and their reactions as indicated by ionic currents and calcium signal intensity were measured within an ROI. The lower graph shows the current recorded (purple), stimulation trigger pulses (red) and line trigger (green).

Fig. 6: A Leica DM6000 CFS System equipped with external detectors (NDDs). Images: neurons selectively marked in a 300 μm thick living mouse brain section excited with IR light. Two neurons in a living brain slice labelled with Oregon Green Bapta 1. Detection of the same fluorescence by RLD and TLD. The TLD shows a significantly brighter signal. Courtesy of Dr. Thomas Nevean, Inst. of Physiology, University of Bern, Switzerland



resolution images. Adjustments can be made to the direction of the spatial filter and to the distance of the diffuser with respect to the spatial filter.

The contrast created is very strong and images look similar to DIC images. The Dodt gradient contrast images are detected with a transmitted light detector or so called Dodt detector. The PMT of the Dodt detector has a higher IR-sensitivity (up to approx. 900 nm) compared to regular PMTs. A Leica DM6000 CFS system equipped with NDDs and a Dodt detector allows simultaneous detection of fluorescence and transmitted light when using IR excitation. It provides the highest photon collection efficiency as there are no optical components in the beam path. This also facilitates many applications in physiology, e.g. optically guided patch clamping (Fig. 7). The NDDs are also suitable for detection of second harmonic generation signals (SHG) (Fig. 8).

The Dodt gradient contrast works in camera and scanning mode when using IR excitation. It can also be used upon VIS excitation, but the contrasting effect can be less prominent.

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Fig. 7: Time lapse series of single cell electroporation of a pyramidal neuron with Ca^{2+} indicator Oregon Green Bapta-1: Overlay of fluorescence channel (non descanned detector) and transmission channel upon simultaneous acquisition by IR light. Images taken in intervals of 280 ms. In the first frame the schematic drawing illustrates the experimental setup: A single pulse of -15 V over 10 ms is delivered. (Nevian and Helmchen, 2007). The advantage of the scanning gradient contrast is obvious: It is easy to view simultaneously the cell structure and the loading process of the fluorescence dye.

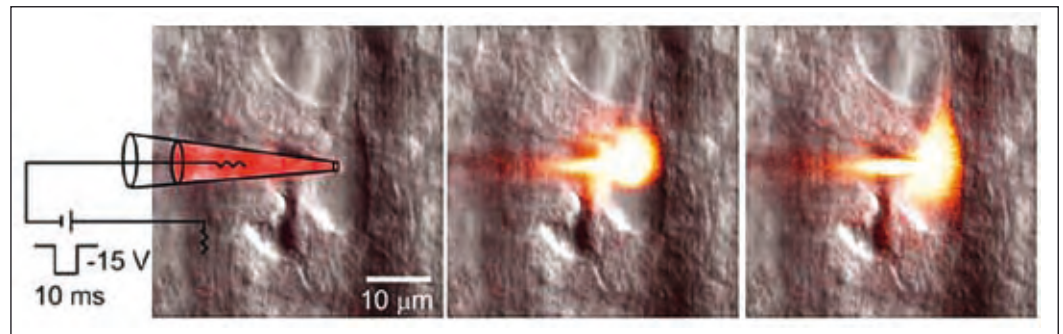
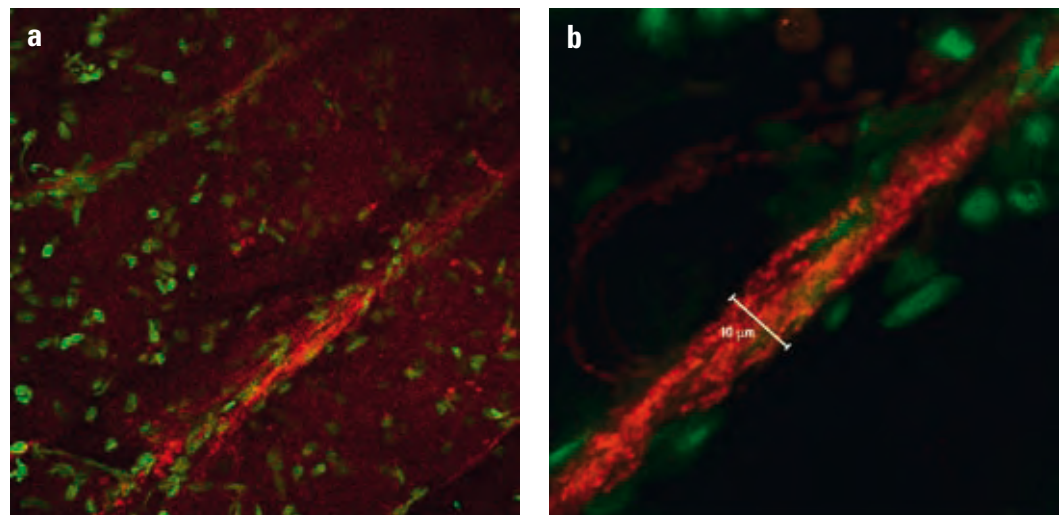


Fig. 8: The mouse heart was excised and a 2% agarose gel was subsequently cast around a 1 mm thick slice of the organ. Two-photon excitation was used with 840 nm. Fluorescent labelling of cell nuclei was done with Syto13 (green). a) Overview image, cell nuclei of cardiomyocytes are visible. The “threads” of nuclei belong to endothelial cells in the capillaries around the cardiomyocytes. In red the second harmonic generation signal of collagen in the capillaries is visible. The vague reddish background is auto-fluorescence of the cardiomyocytes. b) Zoomed image, second harmonic generation signal of collagen and its association with endothelial cells is more clearly visible. Courtesy of Dr. Marc van Zandvoort, Biophysics, Univ. of Maastricht, Netherlands.



Resolution & Magnification

Beware of “Empty” Magnification

Anja Schué, Leica Microsystems

In the simplest case, a microscope consists of one lens close to the specimen (objective) and one lens close to the eye (eyepiece). The magnification of a microscope is the product of the factors of both lenses. A 40x objective and a 10x eyepiece, for example, provide a 400x magnification. However, it is not only the magnification but also the resolution that indicates the performance capacity of a microscope.

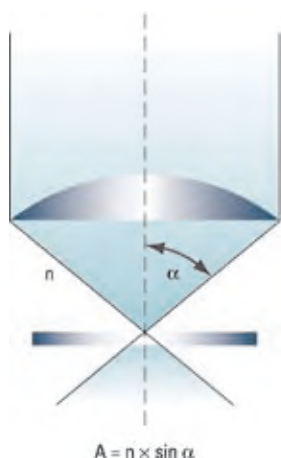


Fig. 1: The numerical aperture of the objective determines the detail resolution and brightness of the image.

Resolution is the ability to render two closely adjacent dots separately. According to the Rayleigh criterion, the minimum distance between two dots able to be separately imaged corresponds to approximately one-half the wavelength of the light.

$$d = 0.61 * \frac{\lambda}{n * \sin \alpha}$$

λ = light wavelength

n = refractive index of the medium between specimen and objective

α = half the aperture angle of the objective

Therefore, with blue light, the resolution limit is approximately $d = 0.2 \mu\text{m}$; with red light, around $d = 0.35 \mu\text{m}$. UV objectives attain a resolution just

under $0.2 \mu\text{m}$. With the naked eye, we are not able to differentiate structures smaller than 0.2 millimetres. The value $n \times \sin \alpha$ corresponds to the numerical aperture (NA), the measure of the light gathering capacity and the resolution of an objective. Because the aperture angle cannot exceed 90° and the refractive index is never less than 1 ($n_{\text{air}} = 1$), NA is always below 1 for air. When immersion oil is used ($n > 1$), the numerical aperture increases (to up to approx. 1.45) and, along with it, the resolution.

Immersion oil increases resolution

To make the microscopic resolution detectable to the eye, the image appears in the eyepiece with corresponding magnification. The resolution and magnification are always directly interdependent. An objective with low magnification has a low numerical aperture and thus a low resolution. For a high magnification objective, the numerical aperture is also high, typically 0.8 for a 40x dry objective. However, because the numerical aperture cannot be increased beyond a certain point, the usable magnification range is also limited in classic light microscopes. The “useful” magnification is between $500 \times \text{NA}$ and $1,000 \times \text{NA}$.

Everything beyond the “useful” magnification is called “empty” magnification. Though structures appear larger, no additional details are resolved. For high-resolution analysis of microstructures, empty magnification is not desirable. Nevertheless, empty magnification can sometimes be quite useful for making details more easily visible for the human eye. Examples of this are video microscopy and also digital microscopy, where greatly enlarged images are displayed on a monitor.



Fig. 2: Images taken with different numerical apertures.



Fig. 3: Different magnifications, same resolution – no additional details are visible.

The World's First Macro Zoom Confocal

From Gene to Cell – from Cell to Embryo

Anja Schué, Leica Microsystems

Modern developmental biology is an emerging field of research, studying the dynamics of cell growth, differentiation processes and the development of organs *in vivo*. Thus, an increasing number of scientists extend their focus of bio-research from single cell studies to entire organisms, analysing the complex interaction within whole animals. These studies require an imaging system which provides high resolution, a large workspace, and a large field of view. A pioneering imaging system provides all these features in one: the Leica TCS LSI.

In vivo – large scale imaging

The large scale imaging platform adapts perfectly to the experiment needs of living specimen analysis. True confocal technology is used to provide crystal clear images of highest spectral resolution, revealing finest details of the model organism, no matter if drosophila fly, mouse, zebra fish, plant or other model. An automated optical zoom system allows for seamless magnification change, easy switching from overview to details and free 3D navigation through the specimen. Comfortable specimen handling is provided by generous workspace.

High resolution from macro to micro

The time for preparation, pre-selection and orientation of the specimen is reduced enormously as macro and high-resolution confocal are combined in one *in vivo* system. Entire new experiments become possible. By avoiding the transportation between different imaging tools, stress to living specimens is reduced, the survival rate increased. The Leica TCS LSI visualises cell growth and the fascinating differentiation of cells into organs in real life from cell to embryo – from embryo to adult.

Making life visible in 4D

Time resolved 4D processes – e.g. protein interactions or influence of drugs in bio-medical research – can be easily studied at highest resolution as advanced time lapse software is provided. The Leica LAS AF Live Data Mode offers perfect automation for cell development studies.

A novelty in confocal imaging even for large specimens is the tremendous working distance of 97 mm and a field of view of 16 mm provided by the 1x apochromatic macro objective. Additionally, high resolution compound objectives can be adapted to use the Leica TCS LSI as a classical confocal.

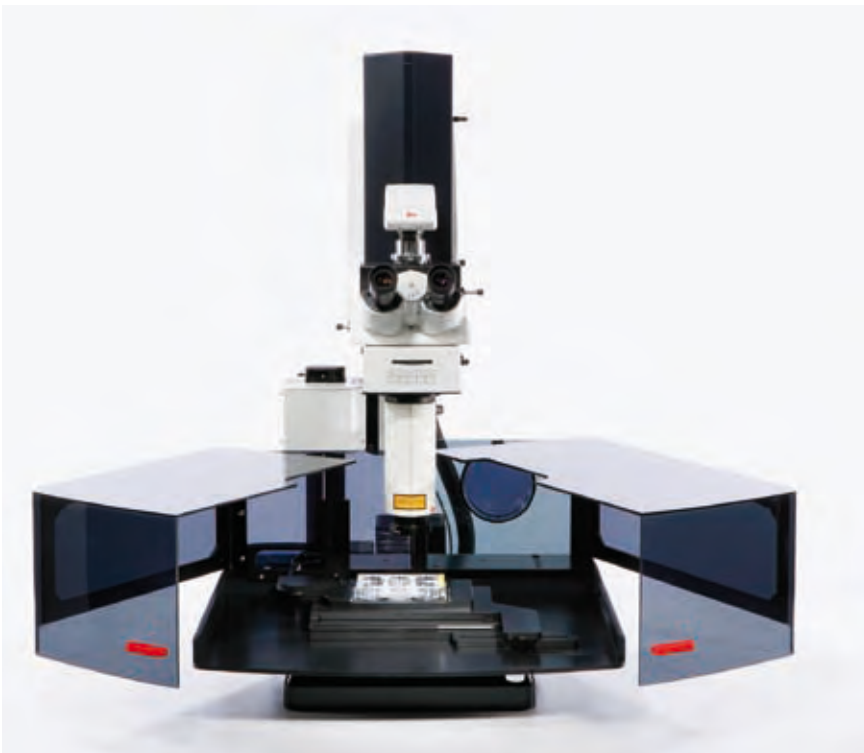


Fig. 1: The unique Leica TCS LSI combines high resolution from micro to macro, a large workspace, and a large field of view. It opens new pathways for *in vivo* studies.

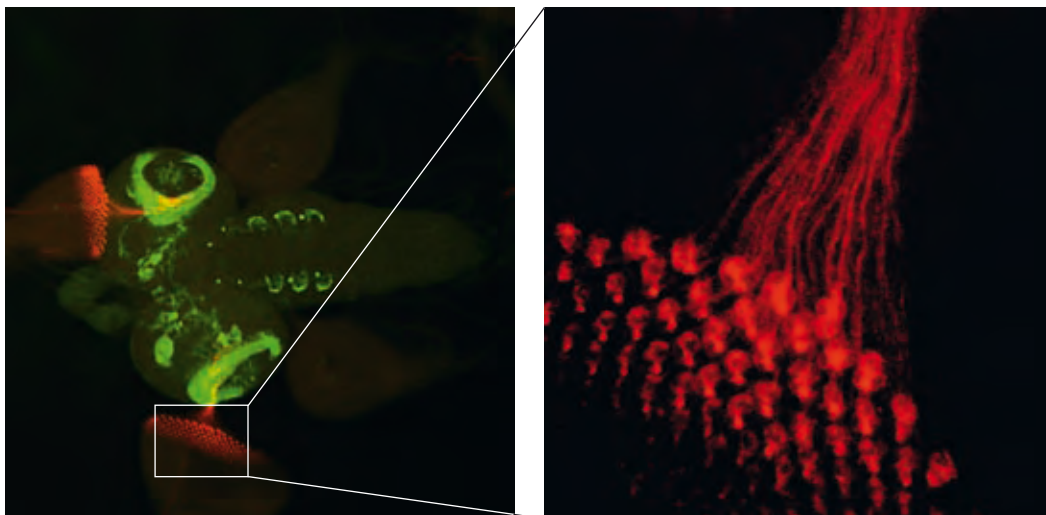


Fig. 2: The motorised optical zoom offers the advantage of flexible magnification to identify the finest details of model organisms, e.g. eye of the drosophila larva.

IGBMC and Leica Microsystems

Successful innovations need an idea to spark them off and professionals to put them into practice. In the case of the Leica TCS LSI it was the collaboration between the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire), one of Europe's leading biomedical research centres, and Leica Microsystems that enabled its development. For over ten years a close and productive relationship has existed between the IGBMC and Leica which has brought enormous benefits to both sides.

Combined expertise

The IGBMC Imaging Centre owns more than 400 microscopy systems. Its founder and manager, Dr. Jean-Luc Vonesch, buys all the light microscopes and confocal systems from Leica Microsystems. The institute is one of the first to use Leica Microsystems' latest technologies. This provides valuable feedback from experienced users so that products can be improved and further developed. The cooperation goes further than this, though. Eight years ago, Vonesch and microscopy specialist Didier Hentsch already had the idea of developing a microscope for *in vivo* studies that combined the advantages of macroscopy with those of high-resolution fluorescence technology.

The result was the Leica MacroFluo™. The special thing about the MacroFluo™ concept is the combination of the long working distances and object fields of a stereomicroscope with the vertical light path typical of microscopes. This guarantees absolutely parallax-free imaging and a maximum of precision for examining whole *in vivo* models.

From the idea to an integrated system

A few years later, the time was ripe for the next innovation: the combination of macroscopy and confocal technology. After three years' development time and three prototypes, the Leica TCS LSI was ready for the market. "Right from the start, Leica Microsystems helped us to realise our ideas," says Vonesch, "We discussed what we were envisaging and followed the project through together from the idea to the integrated system by excellent cooperation with product management." Today, the Leica TCS LSI is put to extremely successful use at the IGBMC, e.g. for exploring arteriosclerosis or the cause of thrombosis in mice. "And we're already working on improvements for the next generation of instruments," adds Vonesch.

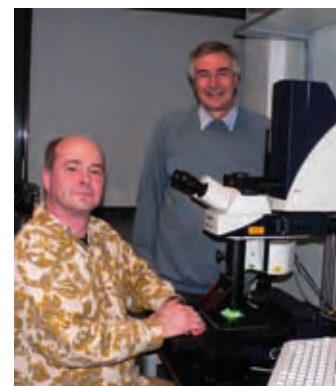


Fig. 3: The fathers of the first super-zoom confocal system: Didier Hentsch (left), microscope specialist at the IGBMC, and Dr. Jean-Luc Vonesch, Head of the IGBMC Imaging Centre in Strasbourg, France.

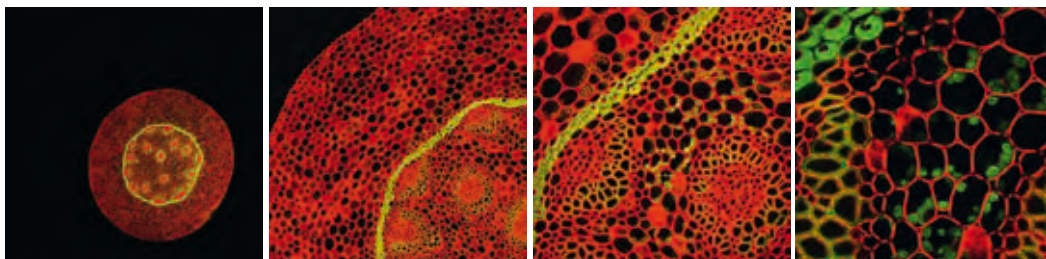


Fig. 4: Seamless zooming in and out. The 16:1 super zoom offers the largest magnification range from 0.57x to 9.2x. The Leica Z-zoom systems are fully apochromatic and allow continuous and parallax-free magnification adjustment.

New Leading-edge Laser Microdissection Systems

Perfection of Dissection

Kerstin Pingel, Leica Microsystems

Laser microdissection (LMD) allows researchers to selectively and routinely analyse microscopic regions of interest down to single cells. Applying this technique, scientists obtain results that are reproducible and specific. For laser microdissection, a microscope is used to visualise small structures. The individual cells or cell clusters are subsequently selected using software, excised from the surrounding tissue by a laser, and released into a collection device for analysis.

Leica Microsystems is a driving force of LMD instrumentation technology. For the first time, high laser power and high laser repetition rates are combined within one instrument – the Leica LMD7000 system. The laser’s high pulse repetition rate is ideal for the fast excision of regions of interest from thin and soft samples. Additionally, high laser power allows the dissection of thick and hard specimens. Both, laser repetition rate and power, can be adjusted to the sample.

The Leica LMD7000 as well as the Leica LMD6500 laser microdissection systems use gravity for specimen collection. The dissected material, independent of its size or shape, is collected in a contact-free, contamination-free manner for further analysis. No additional procedures are necessary for collection. The laser beam movement of the Leica LMD system

is controlled by high precision optics, whereas the microscope stage and the sample are both fixed. This allows high cutting speed at low magnifications as well as precise cutting accuracy at high magnifications, which is a prerequisite to obtain homogeneous material for downstream analysis and reliable results.

The Leica LMD7000 and Leica LMD6500 laser microdissection systems are the ideal instruments for dissecting live cells, single cells, and specific cell clusters for biomarker research, molecular pathology, and many more applications.

- Specimen collection by gravity – contact-free and contamination-free.
- Movement of the laser beam via optics – for the highest possible precision and cutting speed.
- Integration of a flexible, adjustable laser – for the highest feasible power and thinnest cutting lines at the same time.



Fig. 1: The full two-screen support of the Leica Microsystems LMD software offers new possibilities, such as a two screen solution with an optional pen-screen for drawing and a second screen for viewing with all the necessary controls.

Fig. 2: Frozen section (10 μm) of a mouse aorta (whole vessel) stained with cresyl violet on a POL frame slide.

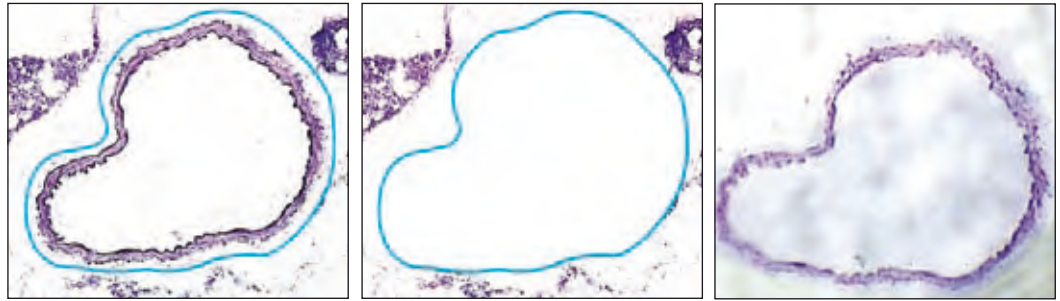
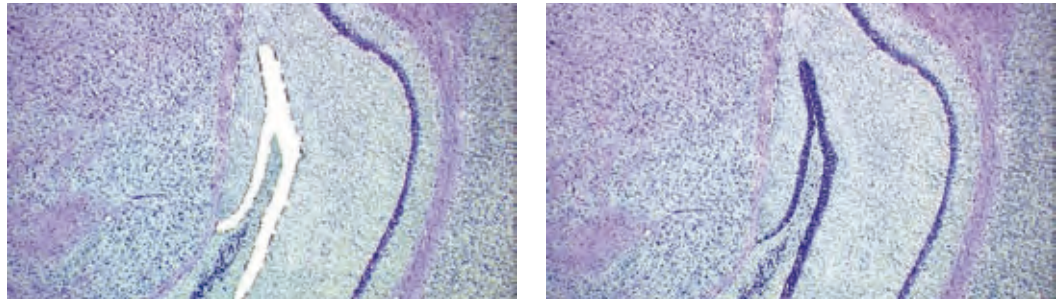


Fig. 3: Dissection of mouse hippocampus, 12 μm thick section, stained with Toluidin blue



Laser Microdissection in Neuroscience

At the Institute of General Physiology at Ulm University, Birgit Liss and her research team are studying the function and gene expression of individual midbrain neurons, which release the neurotransmitter dopamine. Dysfunctions of distinct subpopulations of dopamine midbrain neurons are associated e.g. with Parkinson's disease or Schizophrenia. By combining UV-LMD with real-time RT-PCR they quantify gene-expression of single dopamine neurons and of pools of dopamine neurons. For isolation of individual neurons from fixed mouse brain-sections or post mortem human midbrain sections, they use an UV laser microdissection system from Leica Microsystems. Read the summary of a current study by Jan Gründemann, Falk Schlaudraff, Olga Haeckel and Birgit Liss (*Nucleic Acids Res.* 2008 Apr;36(7):e38):

Elevated α -synuclein mRNA levels in individual UV-laser-microdissected dopaminergic substantia nigra neurons in idiopathic Parkinson's disease

The presynaptic protein α -synuclein is involved in several neurodegenerative diseases, including Parkinson's disease (PD). In rare familial forms of PD, causal mutations (PARK1) as well as multiplications (PARK4) of the α -synuclein gene have been identified. In sporadic, idiopathic PD, abnormal accumulation and deposition of α -synuclein might also cause degeneration of dopaminergic midbrain neurons, the clinically most relevant neuronal population in PD.

Thus, cell-specific quantification of α -synuclein expression levels in dopaminergic neurons from idiopathic PD patients in comparison to controls would provide essential information about contributions of α -synuclein to the etiology of PD. However, a number of previous studies addressing this question at the tissue level yielded varying results regarding α -synuclein expression.

To increase specificity, we developed a cell-specific approach for mRNA quantification that also took into account the important is-

sue of variable RNA integrities of the individual human post mortem brain samples.

We demonstrate that PCR-amplicon size can confound quantitative gene expression analysis, in particular of partly degraded RNA. By combining optimised UV laser microdissection and quantitative RT-PCR techniques with suitable PCR assays, we detected significantly elevated α -synuclein mRNA levels in individual, surviving neuromelanin- and tyrosine hydroxylase-positive substantia nigra dopaminergic neurons from idiopathic PD brains compared to controls. These results strengthen the pathophysiologic role of transcriptional dysregulation of the α -synuclein gene in sporadic PD.

The complete publication is available at the Journal's webpage: <http://nar.oxfordjournals.org/cgi/content/full/36/7/e38>

New Stereotaxic Instrument

Deep Brain Visualisation and Surgery in Animal Research

Charles W. Scouten, Leica Microsystems

Cellular events deep in the living brain have been monitored with electrical probes, but visual observation has not been possible. Two new instruments now enable visual observation of cellular level events deep in the living brain. For the first time, the laser microendoscope Leica FCM1000 permits visualisation at the cellular level. A very small probe, 300 microns or greater, is inserted into the brain, and laser microscopy allows observation of a small group of cells located at the tip. Movement of fluorescent-labelled substances into or out of cells, axonal sprouting after chemical or physical trauma, migration and fate of injected stem cells, micro vascularisation changes, and other features can be directly observed even as they are occurring. For effective use in brain research, it must be possible to get the tip of the microendoscope accurately to a target site in the brain, and even to be able to return to the same target at a later time.

Reaching any target with stereotaxis

The new small animal stereotaxic instrument Leica Angle Two significantly improves accuracy, and enables the operator to reach any target site in the brain from any chosen angle accurately and consistently. A stereotaxic instrument consists of a species-specific head holder mounted on a base plate and used to orient the animal's head in a defined position, and a manipulator with movement axes aligned with the head holder. The manipulator is used to move a probe to selected targets in the brain of the animal relative to chosen zero points, usually skull landmarks Bregma and Lambda, visible lines where skull plates have grown together. These points overlie the brain at consistent positions relative to brain structures in rodents. They are at a crossover of the midline suture, and the anterior (Bregma) and posterior (Lambda) coronal sutures across the skull perpendicular to the midline.

Precise orientation

The Angle Two manipulator features linear encoders on the three linear axes, and rotary encoders on tilt and rotation movements, five instrumented axes in all. These connect to a computer, into which a target point in conventional atlas coordinates relative

to Bregma and assuming "skull flat" – Bregma and Lambda at the same vertical coordinate– is entered either by typing, or by scrolling an onscreen atlas of coronal sections of the brain, and clicking on the desired target point with the mouse.

The animal is then anaesthetised and installed in the head holder, the skull landmarks exposed, and the manipulator tilted and/or rotated to any chosen complex angle. Moving the probe tip to Bregma, and clicking an onscreen button, informs the computer as to where Bregma is in the tilted and rotated and non-orthogonal coordinates, and enables it to instantly calculate how far to move along each linear axis to reach the chosen target, given the manipulator tilt and rotation. The atlas display on screen shows the current position of the probe tip above or in the brain as the operator moves it toward the target. The user can see what structures are being traversed as the probe moves toward the target.

No time-consuming adjustment

Adjusting the animal's head to skull flat is normally a trial-and-error and time-consuming process. Very small errors in head tilt give large errors in position reached, especially if the probe is being lowered to a position deep in the brain. The Leica Angle Two



Fig. 1: The Leica Angle Two Stereotaxic Instrument

includes the Virtual Skull Flat™ feature. Moving the probe to Lambda, and clicking a button on screen, after showing the computer where Bregma is located, enables the computer to calculate the degree of head tilt, and how that alters the target position. The path to the target is then recalculated given the head tilt, and a mathematically correct target position presented. It is no longer necessary to achieve actual skull flat adjustment.

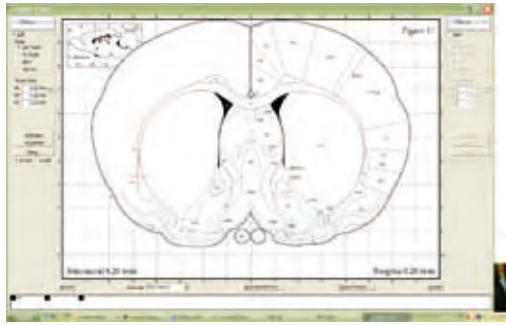


Fig. 2: Angle Two screen

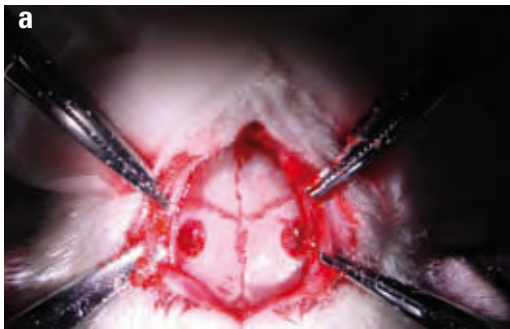


Fig. 3: a-b Intersections of Bregma and Lambda

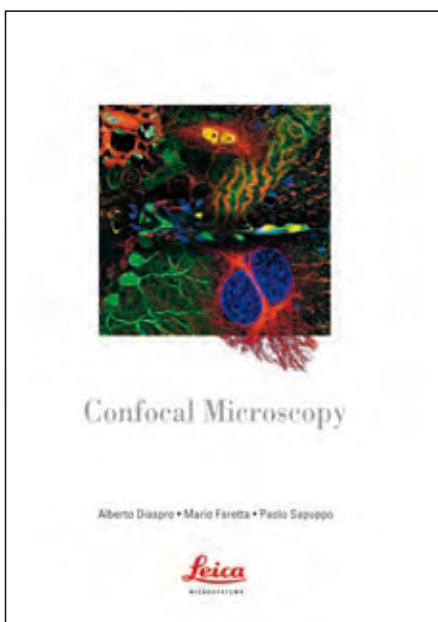
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www.leica-microsystems.com/EU-research



Christoph Thumser, Sales Manager Research Central Europe

Prepared for the Future

Anja Schué, Leica Microsystems

How does Leica Microsystems support scientists in their applications?

Besides our innovative products, the excellent quality of our advice and support is even more important. Our highly qualified sales staff are renowned for their in-depth understanding

“Together with scientists, we are constantly on the look-out for concepts that can advance biomedical research. We see ourselves more as consultants than as sales reps.”

of applications and techniques. Together with scientists, they are constantly on the look-out for concepts that can advance biomedical research. We see ourselves more as consultants than as sales reps. On top of this we regularly support scientific events such as the 8th Göttingen Meeting of the German Neuroscience Society.

What innovative technologies does Leica Microsystems have to offer for Life Science research?

Besides innovative solutions such as STED microscopy, supercontinuum white-light laser or multi-colour TIRF, our strength lies in the breadth of our product portfolio. Leica Microsystems offers high-end quality for all areas of research: the most powerful confocal microscope, the first super zoom 3D

“I don’t think biomedical research will be too badly affected by the crisis as it is mainly funded by the public sector.”

confocal system for in vivo imaging, our stereomicroscopes with FusionOptics™ or our highly innovative prod-

uct line in widefield microscopy – and all the systems are combined by a common software platform. Then there’s our complete product range in sample preparation and complementary products of the companies we have acquired. For example, we offer a special vibrotome and a stereotactic system for the neurosciences.

However, we are flexible to integrate products we don’t make ourselves, ranging from special cameras to specific software solutions. For instance, the Metamorph software we recently added to our product range has made a major contribution towards the quantification of microscope images.

What impact do you think the economic crisis will have on research activities?

I don’t think biomedical research will be too badly affected by the crisis as it is mainly funded by the public sector. For example, the German government gives high priority to investment in research and education, as seen by its support for elite universities or clusters of excellence. After all, therapies against previously incurable diseases can only be developed if first-class research facilities are available.

So far, we have not seen any signs of large research institutes having less money to spend. Neurosciences is one of the areas that receive particularly high funding in order to develop effective therapies for diseases such as Alzheimer’s, Parkinson’s, Multiple Sclerosis, Creutzfeld-Jakob etc.

On the other hand, the biotechnology business or pharmaceuticals industry could be economic risk areas. It is also uncertain how the budgets of the federal states will affect planned investments in view of declining tax revenues. We generally know that an economic crisis has a delayed effect on public sector spending. Nevertheless, I always look to the future with optimism. Leica Microsystems’ sales figures overstepped the billion US dollar mark last year – mainly through organic growth, but also through acquisitions. We’re better prepared for the future than ever before.



Christoph Thumser, Sales Manager Research for Germany, Switzerland and Austria

Leica Microsystems Reports Record Sales

Strategic Acquisitions and Organic Growth

Dr. Kirstin Henze, Leica Microsystems

For the first time in its history, Leica Microsystems' annual sales volume for 2008 exceeded the billion US dollar mark. "Over the last two years, we have seen a dramatic increase in the demand for our products throughout the world. In most of the markets in which we operate – including biomedical research, clinical applications, industry, microsurgery, and histopathology – we have achieved double-digit organic growth rates. Moreover, we have substantially expanded our product breadth through a number of strategic company acquisitions," comments Dr. David Martyr, President of Leica Microsystems.

Numerous company acquisitions expand product range and benefit customers

Leica Microsystems was purchased by Washington D.C.-based Danaher Corporation (NYSE: DHR) in the summer of 2005. Since that time, Leica Microsystems has acquired and integrated eight companies in Australia, Europe, the US, and Asia. With these acquisitions, Leica Microsystems has significantly broadened its product offering and now provides one of the most comprehensive ranges of microscopy and histopathology products on the market. Leica Microsystems' histology offering now includes consumables for use with its instruments. This allows histology customers to obtain all needed products from a single source. "I'm pleased to say that we've not only expanded our product portfolio through strategic acquisitions, but we have also gained significant market share as a result of innovation within our existing segments," says Martyr.

Innovative strength drives organic growth

An important pillar of the success of Leica Microsystems, according to Martyr, is its innovative strength. In 2008 alone, the Life Science, Biosystems, Industry and Surgical Divisions launched over 50 new, and in some cases, breakthrough products. As a result of its recent product launches, Leica Microsystems is now at the cutting edge of technology. Examples of innovation in life science include the super high-resolution STED technology, the macro confocal Leica TCS LSI, and stereomicroscopes with FusionOptics™.

Leica Microsystems is owner of the Leica brand

Leica Microsystems owns the rights to the Leica name and the Leica brand and controls its use through licensing agreements. Leica Microsystems, Leica Geosystems, and Leica Camera are financially, legally, and operatively independent companies, operate in different markets, and belong to different owners.



Dr. David Martyr, President of Leica Microsystems

Events

Please also visit our website on www.leica-microsystems.com for further information on Leica Research workshops in Europe.

2009

8th Göttingen German Neuroscience Meeting

March 25 – 29
Goettingen, Germany
www.nwg-goettingen.de/2009

11th International Neuroscience Winter Conference

March 31 – April 4
Sölden, Austria
www.inwc.sambax.com

FOM 2009, Focus on Microscopy

April 5 – 8
Krakow, Poland
www.focusonmicroscopy.org

20th National Meeting of the British Neuroscience Association

April 19 – 22
Liverpool, Great Britain
www.bna.org.uk/bna2009/

XII CLSM Course in the Spanish Confocal Microscopy School

April 20 – 24
Barcelona, Spain
Registration: http://sm.uab.es/preinscripcion_Leica.doc

9th Congress of the French Neuroscience Society

May 26 – 29
Bordeaux, France
www.neurosciences.asso.fr/Activites/colloques/SN09/

ISCOM, International Student Congress of Medical Sciences

June 2 – 5
Groningen, Netherlands
www.iscoms.nl

ELMI

June 9 – 12
Glasgow, Great Britain
www.elmi2009.org

X Italian Confocal Seminar

June 10 – 12
Rome, Italy
www.leica-microsystems.com/Seminario_Confocale

Leica French Confocal User Meeting

June 25 – 26
Bordeaux, France

7th European Biophysics Congress

July 11 – 15
Genoa, Italy
www.ebsa2009.org

6th European Zebrafish Genetics & Development Meeting

July 25 – 29
Rome, Italy
www.zebrafish2009.org

EMBO - ELSO meeting

August 29 – September 1
Amsterdam, Netherlands
www.the-embo-meeting.org

16th International Society of Developmental Biologists Congress

Sept 6 – 10
Edinburgh, Great Britain
www.isdb2009.com

2nd European Congress of Immunology

September 13 – 16
Berlin, Germany
www.eci-berlin2009.com

XIII Congreso de la SENC, Sociedad Española de Neurociencia

September 16 – 19
Tarragona, Spain
www.senc2009.com

National Congress of the Italian Society for Neuroscience (SINS)

October 2 – 5
Milano, Italy
www.sins2009.it

BIOTECHNICA

October 6 – 8
Hanover, Germany
www.biotechnica.de

World Conference on Regenerative Medicine

October 29 – 31
Leipzig, Germany
www.wcrm-leipzig.com

ScanLab 2009

November 25 – 27
Stockholm, Sweden
www.scanlab.nu

2010

7th FENS Forum of European Neuroscience

July 3 – 7
Amsterdam, Netherlands
fens2010.neurosciences.asso.fr

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