CARS and Confocal – a Successful Affair
Leica TCS CARS Opens New Ways for Research

Sniffing Out the Secrets of Social Behavior
The New Understanding of Olfactory Neurosensorics

Avoid Confounding, Improve Accuracy
Leica Angle Two for Precise 3D Brain Access
Dear Readers,

The cell – the elementary unit of all living creatures and the object of desire of biomedical research all around the globe. A human adult consists of a hundred thousand billion cells, and there are roughly 220 different types of cell. About 100 billion nerve cells perform their task in the human brain. How the smallest sub-units and molecular complexes of a cell work, how it communicates with other cells via the cell membrane, how changes on molecular level are connected with pathogenesis and how neuronal networks are capable of fascinating achievements such as learning and memory – there is still a long road to travel before these mysteries are fully solved. On the way, scientists are encountering many exciting questions, whose answers they are putting together like the pieces of a puzzle.

We have therefore devoted this special issue of reSOLUTION to neuroscience and cell biology. We introduce you to new technologies such as CARS Microscopy, the OPO and the 2C STED. Users report on their research approaches and results. Altogether, we want this issue to give you an idea of our spectrum of system solutions and products that are helping biologists and neuroscientists make new discoveries.

We, Leica Microsystems, are playing our part by cooperating closely with scientists and users to provide scientists with technologies and methods in the form of user-friendly systems that deliver fast and reproducible results.

Have fun reading!

Anja Schué
Communications & Corporate Identity

Didier Goor
European Marketing Manager Research

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Leica TCS CARS Opens New Ways for Research

CARS and Confocal – a Successful Affair

Dr. Stefanie Landwehr and Vanessa Lurquin, Ph.D., Leica Microsystems

Since the advent of confocal microscopy, scientists have gained many new insights that lead to a deeper understanding of how life works. The most important drawback of single-photon and multiphoton confocal microscopy is the need to label the specimen. CARS (Coherent Anti-Stokes Raman Spectroscopy) addresses this issue because it is non-toxic, non-destructive, and minimally invasive. With the implementation of CARS into the Leica TCS SP5 broadband confocal system, Leica Microsystems adds new innovation to its product portfolio for live cell applications and small animal studies.

Staining processes have constraints: Dyes bleach with time, can be phototoxic, and can influence subsequent research methods and camouflage subsequent research information. Processes with stained specimens are not suitable for long term analysis: Atmospheric conditions destroy dyes, and dyes alter when in contact with air or moisture – any change to environmental condition will influence the experimental result. Also, the staining process is time consuming. Some samples cannot be stained at all, because the attachment of a fluorophore or the presence of the dye changes the functionality of the molecule or the organism.

Coherent Anti-Stokes Raman Spectroscopy – the technique

CARS is a third-order, nonlinear process that involves a pump beam at a frequency of ωp and a Stokes beam at a frequency of ωs. The specimen is stimulated through a wave-mixing process. The anti-Stokes signal at ωas=2ωp−ωs is generated in the phase matching direction as vibrational contrast at the frequency difference Δν=vsp−vs between the pump beam and the Stokes beam. This equals the frequency of the vibrational energy of a particular chemical bond.

Live molecular profiling with CARS

The main applications of CARS microscopy are found in biological, pharmaceutical, and dermatological research, biomedical imaging, food processing, and materials science. Its potential has been demonstrated for various biomedical applications, such as the imaging of lipid transport, protein concentrations, DNA, RNA, tissue in a living organism, and order in liquid crystals. By integrating CARS technology into the Leica TCS SP5, Leica Microsystems offers the latest technology combined with an easy-to-use confocal system.

High resolution images

The conventional scanner is optimized for morphological studies as in brain and skin, or for imaging subcellular features such as the cytoskeleton. It allows sampling up to 8196 x 8196 pixels per image, combining a large field of view with high resolution. Also, Fig. 1: CARS energy diagram
the speed of the scanner can be adapted from 400 Hz to 2800 Hz in bidirectional mode.

At the skin surface (Fig. 2), long filaments corresponding to the hairs with chromospheres can be seen with a very strong signal because hairs are covered with oil. At the skin surface, a bright polygonal pattern outlines the corneocytes forming the top layer of the skin, the corneum. The signal arises from the intercellular space rich in lipids, cholesterol, and ceramides. In a second layer, bright structures surrounding the roots of hair are detected, the sebaceous glands. They are multicellular compartments packed with sebum reservoirs containing triglycerides and wax esters. At 70–80 µm of the surface, adipocytes, rich in fat, are found in the dermis. Because the CARS signal is generated only at the focal point, the 3D imaging capability is shown with the maximum projection.

**CARS at video rate**

The resonant scanner on the Leica TCS CARS system provides the benefits of compact design and fast frame recording. The resonant scanner is based on the true confocal concept of point-illumination and point-observation. The resonant scanner allows a speed of 16000 Hz frequency in bidirectional mode. At a frame size of 512 x 512 pixels, the system acquires 29 images per second. With lower sampling, the speed can be increased up to 290 frames per second at a resolution of 512 x 32 pixels. Dynamic processes with high time resolution can be imaged and measured or a linescan can be taken at full rate.

An averaging function is available on the Leica TCS CARS system. Applying averaging improves the signal-to-noise ratio, which is especially useful in the case of the resonant scanner. It is possible to find the right compromise between image quality and acquisition speed, depending on the imaging requirements. As with the Leica TCS CARS the amplitude of the confocal resonant scanner is tunable, which allows it to zoom in by applying smaller amplitudes. With this feature it is possible to focus into regions of interest while acquiring images at video-rate. The pan function – another helpful device to quickly move into interesting areas, which are not necessarily in the center of the microscopic field – is also available with the resonant scanner.

Subsequently CARS microscopy opens new ways to visualize structures based on intrinsic vibrational properties without staining or labeling the specimen. The specimen does not suffer from perturbation by the dye or photo-bleaching. CARS opens new methods of research, especially in cell biology, neurosciences, pharmacology, dermatology, and medical imaging.
Making CARS Microscopy Accessible

“Many research areas, whether from a life science or materials science background, require fast, non-invasive imaging with high spatial resolution, high molecular specificity, and high sensitivity. As an optical method by which contrast is generated on the basis of spectroscopic properties intrinsic to the sample, CARS microscopy can offer all of this. In neuroscience, for example, CARS microscopy can have an impact comparable to two-photon microscopy, except that it does not rely on the introduction of fluorescent labels.

The advent of commercial CARS microscopy systems is certainly a major step toward making the technique accessible to researchers interested in its application. The Leica TCS CARS is especially attractive, since it offers hands-off operation while allowing the full potential of CARS microscopy to be used. Its complete integration into the well-established Leica TCS SP5 environment allows every user familiar with confocal microscopy to rapidly take advantage of all new possibilities offered by CARS microscopy.”

Prof. Andreas Zumbusch, Department of Chemistry, University of Konstanz, Germany, heads a research group for Physical Chemistry. He focuses on single molecule fluorescence spectroscopy and microscopy, as well as on the development and application of non-linear optical microscopy.

Fig. 5: The deviation of fatty components in food can be shown with Leica TCS CARS. The maximum projection shows that lipid components (red) are located in all regions of a potato chip but not only at the surface. Green parts in the chip provide structural information taken with only an multiphoton laser.

Fig. 6: Deviation of lipid droplets in cream, overlay image. The green background shows the water (acquired at 3150 cm\(^{-1}\)) while the red dots are fatty components (acquired at 2850 cm\(^{-1}\)).

Fig. 7: These CARS pictures show lipid-rich adipocytes of the subcutaneous fat layer of mouse skin. The left image is taken at full speed of the resonant scanner, acquiring images at a rate of 29 images / second in bi-directional mode, i.e., at video rate. Within the pixel dwell time of 120 ns only a few CARS photons are detected, resulting in a noisy image. The image on the right corresponds to averaging over 30 images taken with the resonant scanner, i.e., corresponds to the image quality of the non-resonant scanner.
Embryonic development relies on genetic coding and non-coding informations. In particular, physical forces generated by blood flow are critical for proper development of the cardiovascular system. To gain new insight into the fundamental control of cell response to physical changes and to study the dynamics and roles of biological flow during the development of the zebrafish, Dr. Julien Vermot established his lab last year at the Institute of Genetics and Molecular and Cellular Biology (IGBMC) in Strasbourg, France. He belongs to the first lab to use the Leica DM6000 CFS equipped with an OPO/Ti:Sa infrared source for deep tissue imaging and infrared excitation wavelengths up to 1300 nm.

**OPO IR Laser in Developmental Biology**

Deep Tissue Imaging – From Visible to IR Wavelengths

Dr. Andrea Pfeifer and Dr. Bernd Sägmüller, Leica Microsystems

Embryonic development relies on genetic coding and non-coding informations. In particular, physical forces generated by blood flow are critical for proper development of the cardiovascular system. To gain new insight into the fundamental control of cell response to physical changes and to study the dynamics and roles of biological flow during the development of the zebrafish, Dr. Julien Vermot established his lab last year at the Institute of Genetics and Molecular and Cellular Biology (IGBMC) in Strasbourg, France. He belongs to the first lab to use the Leica DM6000 CFS equipped with an OPO/Ti:Sa infrared source for deep tissue imaging and infrared excitation wavelengths up to 1300 nm.

The IGBMC, one of the leading European centers of biomedical research, is devoted to the study of higher eukaryotic genomes, the control of genetic expression, and the analysis of gene and protein functions. Dr. Vermot, group leader and scientific coordinator of the IGBMC imaging facility, coordinates the development of the light imaging techniques scientific program in collaboration with the board of users and the imaging facility. His research focuses on the roles of fluid flow during embryogenesis. He is interested in characterizing fluid motion at a detailed level, such as watching blood cells flow, using resonant point scanning. Dr. Vermot explains why the zebrafish is the optimal organism for studying in vivo fluid mechanics, as well as why he chose IR imaging to accomplish this and gives us an outlook for the future.

**Dr. Vermot, can you give us an overview of your current research?**

We are interested in addressing embryonic development; in particular, we try to understand what are the roles of biological flows during organogenesis and their connections to the developmental program encoded in DNA. More precisely, we want to understand the effects of biological flow at the cellular and tissue scale and find out how cells interpret the physical information provided by their environments, which is dominated by mechanical stress. We principally use zebrafish as a model organism and are keen to use and develop quantitative approaches based on live cell imaging. “How are flows generated in embryonic cavities?” is another question we try to answer. We usually deal with micrometer size structures and need high-speed imaging that is safe for the animal.

**In particular, you are interested in fluid dynamics from embryonic development to adulthood. What drives your research?**

Basically, we explore the limits of the models, proposing that genes are the only driver of morphogenesis. More and more, we see that emerging complexity is dependent on the physical environments of the cells, flow being one of them. Practically, we look at the role of blood flow during cardiovascular development because it is related to human diseases, but there are many other organs whose development strictly depends on biological flows.

For example, we look at the role of cilia driven flow, which happens at a smaller scale compared to blood flow. Blood flow is controlled by heart contractions, which is about two orders of magnitude bigger than cilia. As a result, cilia generate a slower, smaller flow profile.

**For example, we look at the role of cilia driven flow, which happens at a smaller scale compared to blood flow. Blood flow is controlled by heart contractions, which is about two orders of magnitude bigger than cilia. As a result, cilia generate a slower, smaller flow profile.**

We found that the inner ear of zebrafish relies on motile cilia activity, which is important for the development of the sensory organ. Another example is the ‘left-right-organizer,’ present very early in development to break the embryonic left-right symmetry. Importantly, we can differentiate the different types of flow depending on the type of cilia beat. To see at this scale in 3D we need a very fast point scanning instrument.
How is the new Leica system used for your research?

Zebrafish is a very imaging friendly animal. The larvae are transparent and easy to culture under a microscope. However, structures that generate flows are often localized in deep, light scattering tissues. In this case two-photon imaging is the modality of choice because it allows deep imaging with limited phototoxicity. The Leica DM6000 CFS with an OPO helps to perform multicolor two-photon imaging using conventional fluorescent proteins, such as GFP and RFP. Second Harmonic Generation is possible. It also allows us to manipulate the tissue through femtosecond cell ablation where you can target single cells in the tissue and perform imaging. This technique is challenging, it may not work all the time but can give interesting results. Two-photon microscopy is usually used to look deep into the sample. Furthermore, as opposed to single-photon microscope techniques, two-photon imaging illuminates only the part of the sample you image, thereby it limits photobleaching and photodamage.

Today, many research projects are directed towards neurodegenerative, cancer, and lifestyle related diseases. How is your research connected with these diseases?

Most of us at IGBMC work on basic research. However, many of our projects are linked to human diseases. Most of the basic mechanisms in biology, when they go wrong, lead to such problems. We look at the origin of those diseases and do work that will lay the foundation for further and specific research to develop therapies. To do so, imaging is key and will be even more important in the future.

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Zebrafish embryo expressing the DsRed fluorescent protein under the control of a blood cell specific promoter. Fast imaging with resonant scanner at 167 frames per second at 512 x 64 pixels. Courtesy of Julien Vermot, IGBMC Imaging Center, Strasbourg, France.
What is an OPO?

Imaging thick tissue sections as well as whole animals plays a growing role in life science research. Obtaining spatial information in deep tissue areas is crucial to fully understanding biological processes. However, image quality decreases the deeper you image in the tissue, as light is scattered by biological specimens. Current methods allow light to reach about one hundred microns deep with standard widefield or confocal fluorescence microscopy by using excitation sources in the visible range. Unfortunately, it becomes impossible to penetrate hundreds of microns into the tissue while using visible light. Because light scattering is dependent on the wavelength, better tissue penetration can be achieved by using longer excitation wavelengths. This is where excitation with infrared light, two-photon processes, and the OPO (optical parameter oscillator) can dramatically improve image quality.

How do we get longer excitation wavelengths?

First, you need laser sources in the red and infrared. Normally, these sources, called Ti:Sa lasers (titanium-sapphire), start with red wavelengths, e.g., 680 nm, and range into the infrared, e.g., 1080 nm. Second, you need two photons to reach the fluorescent dye at approximately the same time. Then the two photons of, for example, 1000 nm, together equal the energy of an excitation wavelength of approximately 500 nm. This process is called multiphoton or two-photon imaging.

When the maximum wavelength of the IR laser is at 1080 nm, the longest reachable excitation in this two-photon process equals approximately 540 nm. However, many labels and dyes used in biological research need to be excited at longer wavelengths and cannot be used for two-photon imaging, unless an excitation wavelength longer than 1080 nm is used. With an optical parametric oscillator, or OPO, you can now use excitation wavelengths up to 1300 nm in the two-photon imaging process. This allows exciting dyes with an excitation maximum in standard one-photon microscopy of up to approximately 650 nm, which is a great improvement to confocal imaging. The more dyes that are possible and reachable with the two-photon process, the more information obtained from specimens with large imaging depths.

What are the applications for OPO?

If you look at the neurosciences, there is a field called connectomics, which is related to the connections between neurons, or between cells in general. To obtain a roadmap of connections between cells you need both a large overview and detailed resolution. The aim is to understand the function of the tissue — to look at how the circuits work. Many other research areas can benefit from the OPO. For example, in developmental biology it is crucial to protect tissue from photodamage during intravital embryo imaging as well as deep penetration of highly scattering tissues. Here, the longer excitation wavelengths generated by the OPO are optimal. Additionally, the OPO is useful for using red and far red dyes for multiphoton imaging. Even simultaneous excitation of two dyes at two different wavelengths is possible with the OPO.

How does an OPO work?

It is important to note that an OPO utilizes non-linear optics, which underlying physics are not easy to explain. However, think of single photons from a pump laser, which leave the IR source. In an optical resonator and a non-linear crystal, the pump photons overlap and produce a signal and an idler. Those three waves, the pump, the signal and the idler, interact in the non-linear crystal. The signal — which is what you want — gains power with every round trip in the resonator. This is called parametric amplification of the signal, and the pump loses power accordingly. The signal is then coupled out and used for IR imaging.

What does Leica Microsystems offer?

We have fully integrated the control of the Coherent Compact OPO with our Leica LAS AF software, which greatly facilitates its operation. The Leica TCS SP5 MP and the new Leica TCS MP, designed for infrared imaging with OPO, both extend the choice of excitation wavelengths from what was formerly up to 1080 nm, to now up to 1300 nm. The user has the choice of three operation modes: single or sequential excitation with 1040 to 1300 nm with the OPO alone; 680 to 1040 nm with the Ti:Sa IR laser alone; or simultaneous excitation with both excitation sources, at 740 to 880 nm with the Ti:Sa laser, and 1030 to 1300 nm with the OPO.

Tubulin stained with Atto647N. Two-photon excitation with OPO at 1200 nm.
How do real neural networks, composed of numerous different types of neurons, interconnected by complex arrangements of synapses, process information? Randy M. Bruno, Ph.D., Assistant Professor at the Department of Neuroscience, Columbia University, NY, USA is pursuing this question using the rodent whisker-barrel system. Here, anatomically and functionally distinct networks—barrels and barrel columns—are clearly identifiable, and the sensory transducers that provide input are directly controllable. With a variety of paired-recording techniques he investigates the mechanism for propagating information between thalamus and cortex, to study receptive field generation in excitatory and inhibitory neurons, and to demonstrate micro-organization of inputs to cortical columns. Using imaging techniques such as confocal and two-photon microscopy, Prof. Bruno visualizes neuronal dendritic arborization of neurons and their synaptic interconnections.

Leica TCS SP5 Supports Research of Synapses and Cortical Circuits

Exploring the Concert of Neuronal Activities

Myriam Gastard, Ph.D., Leica Microsystems

Prof. Bruno, can you describe your research interests?

We want to understand cortical circuitry—to know how this one circuit, iterated over the entire neocortex, solves tactical, visual, and cognitive problems. The outcome of many laboratories’ research is that you have the same cell types, arranged in the same laminar structures, and having the same general connectivity with each other and with other areas of the brain. It is as if nature reiterated this one circuit for many different tasks. Our goal is to reverse engineer that circuit.

Since I am a physiologist, we routinely record activity from individual neurons or groups of neurons to assess what the neuronal population is doing. But we all become anatomists in the process of doing this because we need to know how the neurons are connected, too.

We can use conventional tracers or newer methodologies like viral expression of fluorescence protein, label large groups of anatomical connections. And, in the course of doing the single cell recordings, we can label single axons. As we start to look at pairs of neurons, we’re trying to figure out the connectivity between individual cell types, or two particular cells, and get back to what the real circuit is.

Which model organisms do you use to investigate these different connectivities?

To study the barrel cortex we work with rat and mouse. These two common laboratory species rely heavily on their senses of touch and smell because they are nocturnally active. Rats have this very stereotypical pattern of whiskers on their faces, which they use for tactile sensation the way humans use finger tips. They swipe their whiskers back and forth over objects and textures as they explore their environments, and they do it with the same frequency of palpation that humans use when we stroke our finger tips across something. They have similar psycho-physical thresholds, so they can discriminate surfaces a little
better than humans can, but they are basically very similar. The information from whiskers is processed by the barrel cortex. Barrels are very easily identified anatomical structures in the cortex: each barrel, a group of thousands of neurons, maps on to one whisker. So now we have a discrete sensory organ that we can control — a whisker — and an identifiable network that is listening to it. We can control the input and take apart the network.

We use electrophysiology approaches on anesthetized, sedated, and conscious head-fixed animals. We are now getting into behavioral studies because ultimately sensation is an active process.

What are the technical approaches?

Everything we do is in vivo, although we are now starting to work in slices. We heavily rely on whole cell recording in vivo to actually patch into neurons and record intracellular membrane potential as well as action potentials. This approach is wonderful for looking at synaptic inputs and is key to the research.

We also use a lot of conventional physiology recording techniques like extracellular recording of single units and local field potentials. We do two-photon imaging of both voltage and calcium sensors. We also do a lot of anatomy, looking at the dendrites and axons of single cells we’ve recorded from. For examining large axonal tracks, we employ conventional tracers and viral mediated expression of GFP and many fluorophores.

What are your imaging techniques?

For anatomical purposes, we use a Leica TCS SP5 broadband confocal. On fixed tissues we image either GFP or dyes like Alexa. A custom two-photon microscope is for anesthetized and conscious in vivo experiments where we use a variety of synthetic dyes to measure voltage or calcium. We are also experimenting with different viruses in the lab for expressing different genetically encoded indicators.

Are there technical limitations that restrict your research projects?

I have never met a scientist who is completely happy with the technologies that are available. So, yes there are limitations. What we can do today is fabulous, but I think we are almost insatiable when it comes to technology. So when we use confocal imaging of fixed tissue to map out structures and detailed morphology, we don’t image for the purposes of getting nice pictures. We’re usually trying to obtain something we can quantify, and that often means that we scan large structures (hundreds of microns) in 3D, but we have limited resolution due to diffraction limits. Regarding the diffraction limit and the depth of recording, new technologies such as STED and the OPO laser can contribute toward solving these problems. But they probably cannot overcome all limitations. One field for new development is better dyes, especially with regard to sensitivity. These are problems that we desperately need molecular biologists and organic chemists to overcome.

To do good neuroscience these days, you have to frequently combine incredibly different skill sets. You need computer programming, molecular biology, physiology, anatomy, physics, and chemistry, just to mention the most important ones. That being said, this is really fun and exciting because there are so many distinct skill sets involved and so many people to collaborate with.

References


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Superresolution Opens up New Perspectives in Neurobiology

The Missing Link to the Nanocosm of Life

Wernher Fouquet, Ph.D., NeuroCure Berlin

Fully understanding the functionality and complexity of the human central nervous system remains one of the major open questions in modern science. In order to address this, understanding the structural architecture of the chemical synapse, a cellular specialization responsible for proper signal transduction and communication between neurons, is of crucial importance. Considering the size of a synapse, e.g., Drosophila NMJ of roughly 500 nm in diameter, it appears logical that, in order to visualize its spatial assembly, the resolution of image acquisition methods needs to be accordingly high. Owing to their diffraction limited resolution, confocal and widefield fluorescence microscopes cannot properly display subsynaptic organization. Stimulated emission depletion microscopy (STED) can be the method to overcome this barrier.

In the past decades, there have been many significant scientific leaps to understand the nature of the human central nervous system. The most prominent of these findings involved the characterization of the chemical synapse, which describes a highly specialized compartment in neurons. Here, electrical impulses translate into a chemical signal to transmit information within a neuronal circuit onto a specific target cell. Through this signal translation, information can rapidly be modulated and processed by either strengthening or weakening the transmission efficiency.

Therefore, synapses and their corresponding neuronal networks are thought to coordinate adequate responses to an environmental stimulus, learn and store information from past experiences, and finally form the basis for such immensely intricate processes as behavior and cognition. The answer to how this is possible lies deeply buried beneath the complexity of neuronal wiring and the multitude of synaptic proteins with their numerous functions and interactions.

Revealing biological nanostructures

By dissecting the molecular composition of the synapse and its architecture, one can gather valuable information concerning the machinery of signal transduction and modulation. It is not surprising that the more that is known about the synapse, the higher the demand for visualizing small nuances that impact the structure’s functionality. Most synapses, though, are tiny cellular specializations. In order to explore the synaptic architecture using simple light microscopy, an image resolution that displays structures down to the molecular level cannot be achieved.

One widely used method for reaching higher resolution is to use an electron microscope (EM), which achieves higher resolution by irradiating the probes with considerably smaller wavelengths than used in light microscopy. Through EM and in combination with tomographic image processing, synaptic structures can be visualized with a resolution of only a few nanometers, which is many times higher than any light microscopy technique. A drawback is that

Fig. 1: Presynaptic T-bar structure depicted by different imaging techniques. (A) NMJ labeled with two antibodies for BRP, either for the C-term (Nc82) or N-term. Because of its spherical structure of single subdivisions of the Drosophila NMJ (boutons), single synapses (see inset) show a segregation of the BRPN-term label toward the outside of the bouton. As in these regions the synaptic membrane lies perpendicular to the focal plane, a polarized orientation of BRP from the membrane to cytoplasm may be implicated. (B) Same structures now imaged with the Leica TCS STED (green) and a confocal reference (red). In these images an architectural arrangement was described for BRP that depicts striking similarities to the T-bar observed in electron micrographs (C).
EM often involves quite elaborate dehydration and contrasting procedures. Even though very small structures are nicely displayed, attributing the visualized structures to the localization of one or more proteins via immuno-labeling remains tricky. Furthermore, time-consuming difficulties arise when high resolution images are needed from bigger or thicker samples, and several EM slices need to be merged or reconstructed.

Recent advances in light microscopy, such as the development of STED microscopy, greatly contribute to this issue by offering a revolutionary simple method of fluorescence visualization with image resolution ranging down to 30 nm, and creating the fully new concept of nanobiophotonics.

**Contributions of STED to neurobiology**

Conventional fluorescent microscopy is perfectly suited for analysis of a biological specimen, since the localization of fluorescent dyes is easily assessed in both fixed and living specimens. STED goes one step further by enabling the detailed discrimination of even smaller cellular organelles and sub-compartments. In neurobiology many considerable achievements have been made, as described in a few examples below:

Synaptic vesicles (50 – 80 nm) are transport units used by the cell to harvest neurotransmitters, which on demand, are fused with the presynaptic membrane and release their content into the synaptic cleft. Understanding the process of how such vesicles are formed, transported, and docked to the proper release site and how the endo/exocytosis vesicle recycling works is hugely important to the scientific community. Recently, video rate STED imaging of live specimens was used to describe vesicle mobility along axons. With the help of STED, the transport of vesicles was described more precisely, detecting even small changes in speed and direction otherwise unrecognizable in conventional image acquisition. In other experiments the localization of a synaptic vesicle’s associated protein (Synaptotagmin) was characterized upon vesicle fusion. Their findings contributed to an overall understanding of how vesicle-specific proteins may be retrieved from the plasma membrane during endocytosis.

Temporal aspects of how single components of the synapse are incorporated into the protein matrix throughout synapse maturation, e.g. via synaptic precursor vesicles, are not yet fully understood.

Studies on the Drosophila NMJ were performed to analyze the synapse structure and assembly. Presynaptic electron dense structures named “T-bars” (owing to their characteristic shape in electron micrographs) were shown to comprise Bruchpilot (BRP). BRP is thought to play a role in signal transduction by acting as a presynaptic scaffolding protein. Through the application of STED technology, in a synergistic combination with established imaging techniques, valuable information concerning the architecture of the roughly 250 nm size T-bar and adjacent structures was obtained (Fig. 1). Similar studies as in Drosophila were performed on murine retina cells, where the composition of presynaptic proteins associated to precursor vesicles, which are thought to promote synaptogenesis, was described.

In the examples above, STED microscopy revealed a very precise distribution of fluorescently-tagged synaptic proteins, which until then were unrecognizable via conventional confocal imaging. When compared to data from electron micrographs, a whole new set of information was retrieved. But unlike EM, STED, due to its simple methodology, allowed image acquisition not only on an uncomplicated and quick fashion, but also in a larger scale, thereby assisting in

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**Fig. 2:** Single synapses of different sizes show considerable structural rearrangement. STED images of GFP show DLiprin-αGFP as discrete dots arranged around the synapse core labeled by BRPNc82 (magenta), ranging from one to two dots at small, freshly assembled synapses and from four to five dots at mature ones. These architectural features weren’t stipulated in EM pictures nor in confocal images, demonstrating the importance of STED analysis for structural characterization at the Drosophila neuromuscular junction. Scale bar: 250 nm.
a more extensive understanding of the synaptic structure and its impact on the signal transduction (Fig. 2). Thus, STED can be generally described as a “missing link” between confocal and electron microscopy.

The STED findings concerning the characterization of the synaptic architecture broaden our understanding of the synapse function, which contributes to the general picture of how the central nervous system works and how complex processes such as learning and memory are accomplished.

Superresolution in live cell imaging

Live cell imaging, though, is where STED microscopy shows its most considerable strengths. Understanding small structural changes, protein localization, turn-over rate or redistribution in live cells, especially during neuronal activity, is crucial for the characterization of synapses. This not only holds true for neurobiological research, but concerns many fields in biological and medical sciences. Developments in STED technology, which were first limited to, above-average bright and stable fluorescent dyes, such as Atto®594 and Atto®647N, more recently allowed the visualization of fluorescent proteins in live specimens with both recently developed far-red fluorescent proteins and commonly used markers such as EGFP and EYFP.

With these improvements, the gain of resolution previously limited to fixed tissue, can now be achieved in live specimens. Also, STED is the most straightforward technique to visualize dynamic protein re-organization, since it can penetrate tissue considerably (15 – 20 µm is typical), allows fast image acquisition, and doesn’t depend on stochastic post-processing and reconstruction. STED, therefore, opens new possibilities of data acquisition including time-lapse and FRAP experiments. With the application of this method a whole new range of questions regarding dynamic aspects may be addressed, enabling super-resolution for live cell imaging (Fig. 3).

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Fig. 3: Live cell imaging with Leica TCS STED CW. Consecutive STED images (single confocal slices) of CitrineDiliprin-α in intact live Drosophila larvae demonstrate, in principle, the possibilities for time lapse and FRAP experiments.
Discovering Cellular Morphology Beyond the Diffraction Limit

Confocal Nanoscopy Goes Multicolor

Dr. Jochen J. Sieber, Leica Microsystems

Scientists strive to understand the architecture of life. They want to learn how biological structures are arranged in respect to one another. Do they co-localize within or are they excluded from the same superstructure? Does localization follow a special pattern and how does the overall arrangement reflect the biological function? Multicolor superresolution imaging allows these fundamental questions to be addressed by far-field fluorescence microscopy in unprecedented detail.

Exploring beyond the diffraction limit

Despite all the great insights obtained with conventional fluorescence microscopy, it is easy to become frustrated when studying subcellular structures, as the biological entities of interest are often significantly smaller than the diffraction limit. Under conventional far-field fluorescence microscopes, which are incapable of resolving objects closer to one another than about 200 nm, details of interest are lost in a blur. Several methods of far-field superresolution microscopy overcome this fundamental obstacle and allow morphological details to be studied far beyond the diffraction limit.

Stimulated Emission Depletion (STED) imaging\(^1\), as confocal laser scanning microscopy, moves a spot of excitation light over the sample, detects the emitted fluorescence and generates pixel by pixel the image of the observed optical section. To achieve superresolution, the diffraction-limited excitation spot is overlaid with a donut-shaped point spread function of a second laser: the STED laser. A process called stimulated emission prevents dyes from emitting fluorescence anywhere in the focal volume except for the very center of the donut. The amount by which the effective focus can be shrunk, i.e. the resolution that can be achieved, depends on the intensity of the STED laser as well as on the dye. With the Leica
TCS STED, sub 60 nm lateral resolution is routinely achieved with e.g., Atto 647N. Confocal and STED microscopy are a perfect match and can be readily implemented in the same setup. With the available commercial realizations such as the Leica TCS STED CW the user can toggle between confocal and STED resolution by a single mouse click.

Achieving 2C confocal superresolution

STED microscopy allows fast and unbiased information to be obtained on the structure and organization of a biological entity of interest. The next step is to put this information into context. Much can already be learned by exploring one structure with a resolution of e.g., 60 nm and imaging several confocal counterstains at the same time. Nevertheless, seeing how two structures are organized in relation to one another in subdiffraction-limited detail opens the door to a completely new world of co-localization studies (Fig. 2).

Two approaches for achieving two color (2C) STED images have been reported. The first is technically demanding and uses two separate sets of excitation and STED wavelengths for two spectrally separated dyes. The second solution (the ‘one donut approach’) uses a standard fluorophore (Stokes shift 10 – 30 nm) in combination with a large Stokes shift dye (e.g., >100 nm for Chromeo 494) of partially overlapping emission spectra. This allows the use of only one STED laser. The differences in the excitation spectra are exploited to distinguish the two dyes. To generate a 2C image, two frames are recorded with different excitation laser lines active in each frame. With the right dye combination and a balanced staining, brilliant 2C STED images are collected following this approach, even when the same band is detected for both channels. Of course, the differences in emission spectra can also be exploited to help distinguish the dyes. The advantage of applying the same STED donut for both superresolution channels not only reduces the overall cost and complexity of the system, but also avoids chromatic aberration problems, which other implementations of superresolution microscopy need to compensate.

Providing standard tools for co-localization studies at the nanoscale

Leica Microsystems implemented 2C STED based on the ‘one donut approach’. Both confocal superresolution systems, the Leica TCS STED and the Leica TCS STED CW, were enabled to perform co-localization studies in the sub 100 nm realm. To this end, a second excitation laser at 531 nm was introduced in addition to the 640 nm line for the Leica TCS STED which realizes confocal nanoscopy with pulsed la-

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**Fig. 2:** New quality of co-localization studies. Confocal (left) and STED image (right) of the same structures. Indicated intensity profiles of 860 nm shown below. Dyes: Chromeo 494 (green); Atto 647N (red).
sers in the deep red range. Also, a special filter cube for the highly sensitive avalanche photodiode detectors (APDs) was designed for the recommended dye pair Chromeo 494/Atto 647N. This assures optimal dye separation without the need for post processing (Fig. 1 and 2).

The Leica TCS STED CW uses continuous wave lasers for STED imaging in the visible range. It facilitates confocal nanoscopy with green standard dyes, e.g., Alexa 488, Oregon Green 488, FITC, Chromeo 488, and also autofluorescent proteins (FPs) e.g. eYFP, Citrin and Venus. A newly designed objective – the HCX PL APO 100x 1.40NA Oil STED Orange – allows the TCS STED CW user to freely choose any argon line (458, 476, 488, 496, 514 nm) for excitation together with the 592 nm laser for STED. So a lot of dye and or fluorescent protein combinations have become applicable to 2C confocal nanoscopy.

Excellent 2C images are obtained e.g. from samples stained with BD Horizon V500 and Oregon Green 488 without crosstalk between channels. Fluorophore combinations showing a bigger spectral overlap also yielded good results after dye separation with the appropriate software package in the Leica LAS AF software. With the multitude of available dyes and FPs, especially in the visible range, more and more fluorophore combinations will prove useful for multicolor confocal nanoscopy.

Conclusions

The optical sectioning capability of confocal microscopy has boosted co-localization studies to the next level. Nevertheless, diffraction-limited resolution often masks important subcellular details in the recorded z-stacks. Fully embedded in state-of-the-art confocal instruments, multicolor STED microscopy allows fast, unbiased and easy access to hidden morphologies within optical sections of cells, tissues or even organisms. The multicolor confocal nanoscopy systems Leica TCS STED and Leica TCS STED CW enable life science researchers to explore cellular architecture beyond the diffraction limit.

References


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New Insights Into the Dynamic Organization of Synapses

Restless Receptors

Anja Schué, Leica Microsystems

Synapses are the switch-points in our brain for information transmission, learning and memory. And there is evidence to suggest that changes and malfunctions in synapses are partly responsible for a number of neurological and psychological disorders. Neuroscientists already know a lot about how signals are transmitted from neuron to neuron. Yet many synaptic processes are still not fully understood.

Dr. Daniel Choquet, Research Director at the Institute for Interdisciplinary NeuroSciences (IINS) of the CNRS and Bordeaux 2 University and Head of the Bordeaux Imaging Center (BIC) is researching signal transmission in the postsynaptic membrane. His studies and his developments of imaging techniques have provided new insights into the dynamics of glutamate receptors, which are involved in synaptic transmission in 80% of excitatory cerebral nerve cells and play a key role in synaptic plasticity. The use of superresolution technologies is making an essential contribution to this research.

Dr. Choquet, how has the understanding of the role of postsynaptic receptors changed?

When I started working in Bordeaux Neuroscience Institute (INB) in 1997, receptors for neurotransmitters were believed to be stable and rather immobile molecules whose activity and regulation were purely based on phosphorylation and structural modification. However, my earlier experience of cell biology made me wonder why the dynamics of neuronal receptors should be any less complex than those of other cell components.

Various studies then proved that receptors are not firmly anchored in the membrane, but move in permanent exchange processes by endo- and exocytosis. Some years later, we were able to show that the receptors also move in the plane of the cell membrane by lateral diffusion and travel relatively long distances within the synapse. In the last few years our Laboratory ‘Cellular Physiology of the Synapse’ has been in close cooperation with the physics group of Brahim Lounis at the University of Bordeaux 1 to start characterizing this mobility and examining the way it is regulated. In doing so, we made an amazing discovery for the knowledge of the time: The movements of the receptors are regulated by the neuron activity which, in turn, is directly connected with learning and memory.

Today, we know that receptors move very rapidly and that this mobility plays an essential role in the signal transmission between neurons. In fact the mobility of receptors controls the reliability of neuronal transmission. We have revealed that a minor modification of the mobility has a major impact on high frequency transmission. In addition this mobility enables the replacement of desensitized receptors by naïve receptors within a few milliseconds. This reduces synaptic depression and allows the neuron to transmit information at a higher frequency.

These results have radically changed our understanding of neuronal physiology. Besides the electrophysiological techniques, the light microscopic techniques have played a decisive role. They have literally shed new light on signal transmission functionality and synaptic plasticity.

What issues are you working on at the moment?

We are consolidating this knowledge by pursuing two different lines of research. We are extending our experiments, which have concentrated up to now on cell cultures and brain sections, to include ex vivo Fig. 1: Dr. Daniel Choquet (front row, right) with his team of the Bordeaux Imaging Center
investigations and even studies of living organisms. Only then can we gain a better understanding of how learning and memory are actually influenced by the regulation of receptor movements.

On the other hand, we intend to research receptor mobility within the synapse on a nanometer scale down to the smallest detail in order to find out, for example, how scaffold proteins are involved in the regulation of receptor mobility. Our working hypothesis is: different information speeds and regulations are directly related to learning and memory.

What is the relevance of your work for the research of neuronal diseases?

Today, we assume that changes or malfunctions at synapses play a definite role in neuronal and psychological disorders. That’s why not only neurodegenerative diseases, but also epilepsy or autism are also called synaptopathies. Of course, our basic molecular research of the animal model is still far from being clinically relevant, but our work is already linked to that of our colleagues in pathology.

For instance, we have begun, using animal models, to examine the defects in receptor trafficking that are observed in Alzheimer’s and Parkinson’s disease. A whole department of the INB is occupied with neurodegenerative diseases.

Heading the BIC is no doubt an added advantage for your research?

In Bordeaux, I had the dual function as head of a research group and Head of the Imaging Facility right from the beginning. I have taken care to make the imaging tools we develop for our own experiments available to the entire community. Since then, the facility has steadily grown – like my research group. In its present size and function as core facility, the BIC has evolved from the fusion of the light microscopy (LM), electron microscopy (EM) and the plant imaging facility.

We are extremely successful with the further development of imaging technologies. In 2002, we managed to obtain the first live images of the movements of AMPA receptors (AMPAR), a sub-group of the glutamate receptors, in the cell membrane by using a relatively crude approach of tracking by video microscopy of micrometer-sized latex beads bound via antibodies to AMPAR subunits. However, this method is not suitable for tracking receptors inside the synapse. Together with the physics group of Bordeaux 1, we then started developing single molecule detection techniques. We were the first group in Europe to apply this technique successfully to living neurons. Another method we developed is the photothermal imaging of nanogold particles to track receptors in live neurons for long periods. Although the gold particles do not bleach or blink, and allowing theoretically infinite recording times, spontaneous photothermal signals from mitochondria may interfere with the tracking of gold particles.

Of course, we are currently devoting a lot of our attention to superresolution technologies, which are extremely important for neuroscientific research. Apart from Single Molecule Detection, we use STED microscopy and PALM. Beyond this, we have also developed a new superresolution technique in the last few years called PAINT. This point-accumulation-for-imaging-in-nanoscale-topography method allows dynamic superresolution imaging of arbitrary membrane proteins in living cells. In a recent paper we published a further development of this approach called uPAINT (universal PAINT). This method is based on continuously and stochastically labeling membrane surface.
Another avenue we are able to explore thanks to our LM and EM facilities is Correlative Light Electron Microscopy (CLEM). This enables EM images of three-dimensional cellular nanostructures to be linked to fluorescence microscopic information on receptor localization, movement and interaction. Our aim here is the application of Superresolution Photonic Imaging and EM on one sample – i.e. Super CLEM.

What other projects are you supervising apart from your research work?

The INB is one of Europe’s leading neuroscientific communities and the umbrella organization for various groups and laboratories of the CNRS, INSERM and INRA. The institute has grown tremendously in the last few years – today, about 600 people from a wide variety of disciplines are working on neuroscientific projects. A new building with roughly 12,000 square meters of lab space will be built within the next three years.

One of the many activities with which the INB has made an international name for itself is the Neurocampus project, which is receiving a 65 million euro grant from the Aquitaine region. We are also, for example, project partners in the ERA-Net NEURON (Network of EUROpean funding for Neuroscience research). We are also strongly involved in the European ESFRI infrastructure project Euro-Biomaging, whose French counterpart is France-Bioimaging that unifies the most advanced French imaging core facilities in a comprehensive network. Besides this, I am involved in fostering the participation of the INB in the national Excellence Initiative. And that’s not all by any means – it rarely gets boring here. In spite of all the work, it is extremely exciting to play a part in developing the achievement potential and the scientific reputation of the INB.

References

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Fig. 4: STED image of a cultured hippocampal neuron

Fig. 5: Superposed DIC and fluorescence image of a cultured hippocampal neuron expressing GFP
High Content Screening Automation for Confocal and Widefield Microscopes

Amplify the Power of Imaging

Peter Sendrowski, Leica Microsystems

High Content Screening is a rapidly growing approach in life science research to answer complex questions in a shorter amount of time. As the paradigm shifts from descriptive imaging to quantitative analysis, researchers benefit from non-biased results using automated imaging systems to efficiently discover relationships between cells and organisms.

Fig. 1: High Content Screening with interactive system control for fully automated mitosis acquisition: microtubule secondary screen; scrambled siRNA Tubulin (green), H2B (red). Leica TCS SPS, Objective: 63x oil (pre-scan); zoomed, maximum projection: 30 x 0.4 μm slices, 2 channels (high resolution). Courtesy of Christian Conrad, EMBL, Heidelberg, Germany. (1) Mitochek Project: www.mitocheck.org.
Leica Microsystems introduces a new automated concept to obtain imaging data: Leica HCS A High Content Screening Automation. The combination of a high resolution confocal point scanner or widefield system with LAS AF Matrix M3 software offers valuable benefits for biomedical research:

- The highest resolution provides maximum information and is the basis for precise analysis.
- Sophisticated automation of the imaging system enables far more experiments to be run in a shorter amount of time in a standardized way. Statistically relevant and unbiased datasets are generated.
- Leica HCS A provides flexibility and facilitates easy adaptation to the experiments of today and tomorrow.

By adding LAS AF Matrix M3, the value of an imaging system becomes more than the sum of its parts. Leica HCS A speeds up experimental throughput and amplifies laboratory capacity.

Intelligent microscopy

Leica Microsystems provides the technology to perfectly match the needs of researchers. From basic mosaic up to 3D or 4D time resolved multiwell analysis, extensive automation capability converts confocal and widefield systems into fully featured high content screening devices.

Five new autofocus routines plus z-drift compensation ensure that the target specimen remains in focus. Thus, high quality data is generated even during long term measurements. Automated tracking functions move even cells that escape during observation back into the central imaging field. A variety of multiple scan jobs can be freely combined at multiple positions, which offers maximum freedom for new experiment designs. With this, Leica HCS A offers the flexibility to easily combine low resolution pre-screens with high resolution secondary scans.

Fig. 2: Leica HCS A includes re-engineered mosaic algorithms for excellent results at the push of a button – to visualize the finest details as well as information overview: mouse diaphragm muscle stained against neuro filament 150. Mosaic: xyz: 5 x 5 x 101 images. (Green: secondary antibody coupled to Alexa Fluor 488) and acetylcholine receptors (Red: alpha-bungarotoxin coupled to AlexaFluor 647). Courtesy of Dr. Rüdiger Rudolf, Cellular Signaling in Skeletal Muscle, Karlsruhe Institute of Technology, Germany.

Fig. 3: Leica HCS A supports frequently used multiwell plate formats to automatically study multi-dimensional experiments: time resolved or concentration dependent tests unveil true biological context. Zebrafish, Danio rerio, Neurogenin - GFP. H2A Courtesy of J. Legradi, Dr. U. Liebel, KIT Karlsruhe Institute of Technology, Germany.
Based on LAS AF Matrix M3 software, the Leica HCS A package also offers top-level flexibility for defining screening patterns. It can be used for microtiter plates, chambered coverslips, spotted arrays, tissue micro arrays, petri dishes, and lab-on-a-chip applications. The single positions can combine with different recording parameters. All parameter settings can also be transferred by LAN for use by all other Leica HCS A screening systems. The software platform offers a number of modules and functions that greatly enhance the efficiency of the imaging system.

For system customization, Leica Microsystems created the Computer Aided Microscopy (CAM) interface, which allows remote control of the confocal or widefield system. The interface can be addressed by all modern programming languages including Matlab™ or Labview™. While data streams to local storage devices on the fly, the laboratory’s image analysis runs in parallel. As soon as a rare event is detected during a pre-screen, the microscope, via the CAM interface, can start a secondary scan at high resolution. The cells of interest are immediately analyzed, exactly when the rare events happen or hits are identified. Fast acquisition, on-the-fly analysis, and feedback system control add new dimensions to imaging automation.

Perfect integration

The open architecture of Leica HCS A matches existing data structures. The Open Microscopy Environment data structure OME.TIFF is compatible with most existing image analysis solutions like ImageJ 1, MetaMorph®, Leica MM AF or Definiens® and ensures full compatibility with modern analysis platforms. Researchers benefit from applying existing algorithms or can create new ones to automatically analyze data in 2, 3 or 4 dimensions. All information is present as the Data Exporter generates OME.TIFF plus the XML metadata structure on the fly.

Leica HCS A imaging formats are platform independent and can be shared on Apple MAC™ OS X, Microsoft Windows® or LINUX2 platforms. The scalable data model offers easy collaboration between local teams, and fast data and information exchange.

The Leica HCS A package is available for the Leica TCS SP5 broadband confocal, Leica TCS SPE personal confocal, Leica TSC LSI macro confocal, and Leica AF7000, AF6500, and AF6000 widefield systems.

A step ahead

Leica Microsystems develops high-end technologies for a wide range of image acquisition techniques. Based on this core competence, a joint partnership was developed to provide even more powerful customer solutions. Definiens AG established an entirely new perspective in image analysis with its Definiens Cognition Network Technology® to identify and measure biological specimens in 2D, 3D or 4D time lapse. Automated tracking of cellular movements up to the quantification of developmental processes in whole organisms is now possible.

Leica HCS A High Content Screening Automation empowers researchers to gain scientific advantage by obtaining more results in a shorter time – efficiently and statistically robust.

Annotations

MAC™ OS X is a registered trademark of Apple® Inc. Windows® is a registered trademark of the Microsoft® Corporation. (1) Linux is a free Unix-type operating system originally created by L. Torvalds with the assistance of developers around the world.

Definiens® is a Registered Trademark of Definiens AG. (2) ImageJ is a public domain Java image processing program inspired by National Institutes of Health, NIH Image for Windows®, Mac™ OS, Mac™ OS X and Linux. MetaMorph® is a Registered Trademark of MDS Analytical Technologies. (3) Open Microscopy Environment (OME) is a multi-site collaborative effort among academic laboratories and a number of commercial entities. OME is developed as a joint project between research-active laboratories at the Dundee, NIA Baltimore, and Harvard Medical School and LOCI. LabVIEW® is a registered trademark of NI National Instruments Inc. MATLAB™ is a registered trademark of The MathWorks™, Inc., Inc. C# is a programming language standardized by ISO. C# is a programming language developed by Microsoft, Inc.
One of the greatest challenges in high content screening (HCS) is the availability of effective technologies for image data analysis. HCS creates significant information management challenges as a wealth of data needs to be reliably, reproducibly and efficiently analyzed. Current image analysis tools are often unable to track the data from screens that contain more than a couple hundred genes. In a recent partnership, Leica Microsystems combined its HCS automation package with powerful Definiens software to offer more robust, efficient image analysis.

Definiens Cognition Network Technology®

When Nobel Laureate Prof. Gerd Binnig founded the company Definiens he made a radical departure from traditional, pixel-based image analysis approaches. Definiens Cognition Network Technology® does not simply identify the ‘objects of interest’ but all of the intermediate objects together with their interrelationships (context). In effect, a model is built that is represented by Definiens’ unique Cognition Network.

It stores all of the objects, sub-objects and their semantic relationships in a clear hierarchy. The difference in approach is profound. It is the contextual information contained in the Cognition Network that enables the automated extraction of information – in exactly the same way as a human being makes sense of an image.

2D, 3D, and time lapse applications

Definiens’ software suite is based on this unique technology. Definiens Developer XD is an integrated development environment designed for researchers, scientists, and image analysis experts to create and test solutions for the full range of biomedical image analysis. It can handle any imaging modality, 2D, 3D and time lapse applications. Cases that were previously only addressed by a human observer applying tedious manual segmentation can now be automated. This capability facilitates new experimental approaches and workflows, such as assay development. Definiens Developer XD provides researchers with 2D, 3D and time-based tools for tracking cellular processes and generates useful data regarding cell morphology and structure, including correlating cell speed changes and persistence of movement.

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Fig. 1: Four channel confocal image of Cell Culture (original data): Matrix M3, interactive plate view, ome.tiff format, approx. 7 mpx/stack, 4 channels, 1 stack per well

Fig. 2: Development of fully automated 3D analysis of primary rat brain cells in Definiens Developer XD
The combination of CYTOO’s HCA Platform with the Leica HCS A solution provides a highly reproducible high content analysis solution. Conventional cell culture conditions on uniform adhesive supports lead to high morphological variability and uncontrolled cell migration. This situation is in sharp contrast to what is found in tissue, where cells respond to external spatial information (both from the extracellular matrix and neighboring cells) and adopt a reproducible polarized architecture, necessary for proper tissue function.

**Normalize Cells**

Dr. Constantin Nelep and Dr. Joanne Young, CYTOO Cell Architects

Highly reproducible results

Advanced cell culture supports developed by CYTOO Cell Architects (Fig. 1) use proprietary concave adhesive micropatterns that control the cell adhesion microenvironment. As cells show a highly reproducible response to the geometry of the adhesive micropattern, this leads to the normalization of cell shape, internal architecture and orientation of division (Fig. 2) thereby overcoming a fundamental obstacle that plagues all current High Content studies.

Exquisite control over assay design

As micropattern features (shape, size, molecular composition) can be modulated and refined in myriad ways, live and endpoint analyses, both existing and novel ones, can be engineered for a wide range of cellular activities, processes and phenotypes, thereby enabling much greater High Content data depth.

**The Reference Cell™**

This dramatic decrease in cell variability enables highly reproducible quantification of the spatial distribution of cell compartments and individual proteins. Averaging the distribution observed across only a limited number of cells (20 – 50) leads to a Reference Cell, a highly robust representation of protein and organelle organization. The Reference Cell can be used to assess the significance of any perturbation introduced in and across large scale screening experiments and reveals any subtle changes in cellular phenotype. Moreover, the regular array of adhesive micropatterns significantly facilitates automated image capture microscopy both with fixed and live cells and resolves the cell segmentation headaches encountered with image analysis of cells grown on conventional cell culture supports. Integration of micropattern specific image processing with interactive microscope control can also provide powerful and flexible experimental designs such as on-the-fly image analysis for capturing rare events.

**References**


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**Fig. 1:** CYTOO micropatterned supports come in a range of: (A) CYTOOchips™ (up to 20,000 micropatterns per coverslip with special cell localization grid) and (B) CYTOOplates™ (up to 5000 micropattern per well) dedicated respectively for basic research and for High-Throughput Microscopy/HCS applications. Standard micropattern shapes include Crossbow, H, Y, L and full disks.

**Fig. 2:** Example of micropatterned HeLa single cells (seeded on 1100 µm² fibronectin crossbow-shape patterns providing cell polarization). CYTOOchip imaged with Leica TSC SPE / LAS AF Matrix. Objective 63x. Actin: Phalloidin-FITC; Nuclei: Hoechst. Only 20 – 50 cells are needed to obtain statistically relevant data.
Picovitro Plates and Leica HCS A

Looking for Rare Cells or Cellular Events?

Fredrik Hellborg, Ph.D., Picovitro

In many cell biology studies it is essential to identify and analyze a specific event or cell in order to understand the behavior of the cellular network or the whole organism. Unfortunately, these events or cells are usually rare. Today, the combination of Picovitro plates with the Leica HCS A solution enables researchers to grow and analyze large numbers of single physically isolated cells or clones of cells and overcome the obstacle of identifying and analyzing rare events or cells.

Performing individual experiments in parallel

The Picovitro plate is a small cell culture plate with many wells and a glass bottom. Each well is 0.5 μl in volume, which is small enough for many cell types to survive as single cells but large enough to allow the cells to expand into clones of a few hundred cells if required. In this way it is possible to perform many individual experiments in parallel in a small format, and to find and study rare events of interest. Each Picovitro plate has 672 wells and is the same size as a standard microscope slide. The glass bottom makes the plate excellent for analysis by most types of microscopy and/or other imaging techniques.

Identifying events of interest

While Picovitro provides the platform for performing the experiments, Leica Microsystems provides the tools to analyze the experiments to find what is interesting. The automated high content screening solution from Leica Microsystems provides fully automated screens of Picovitro plates. The image analysis software identifies wells that contain events of interest such as cell division or chromosome condensation. These cells can be retrieved from the well or analyzed in more detail by, for example, imaging the particular well with higher magnification.

By combining the Picovitro plates with automated high content screening, research and biotechnology labs are able to perform new cellular experiments which have not been possible with commonly available resources until now. This new solution leads to new ways of investigating cell behavior and destinies – and studying cell cultures at a new level of detail.

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Design & dimensions of Picovitro plates

Picovitro plates are constructed of three layers: a glass bottom, a silicon microgrid that forms the wells, and a semipermeable membrane to facilitate gas exchange and avoid evaporation.

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Fig. 1: At the size of a conventional slide, 672 microwells are etched in silica body, optimized for single cell culturing.

Fig. 2: Semi-permeable silicone membrane prevents evaporation, facilitates gas exchange, and avoids contamination.
The New Understanding of Olfactory Neurosensorics

Sniffing Out the Secrets of Social Behavior

Anja Schué, Leica Microsystems

In his novel “Perfume – the Story of a Murderer”, Patrick Süskind put the power of odors into words better than anyone before him. It may be a fascinating idea, but no one will ever be able to create the perfect fragrance that makes a person irresistibly attractive. In the animal world, on the other hand, olfactory signals are an essential element of social communication. Yet we are only just beginning to understand the complexities and functional differences of the sense of smell in mammals. Prof. Marc Spehr, head of the Department of Chemosensation at RWTH Aachen University since 2009, is researching the neuronal mechanisms of olfactory perception and signal processing using the mouse model. He and his team are trying to find out how substances for social interaction are perceived and how this perception generates a specific type of behavior.

Fig. 1: Prof. Marc Spehr tested the new Leica DM6000 FS Fixed-Stage Microscope before the market launch.

“We are highly satisfied with the microscope’s optics and stability. We find the large working distance and the space around the specimen impressive, too. The accessibility to the specimen area is important to neurophysiologists, as we need a lot of peripheral equipment.”

Prof. Spehr, why are scientists becoming more and more interested in the nose?

You could say this was triggered by the discovery of the multigene family of olfactory receptors by Linda B. Buck und Richard Axel in 1991, for which both were awarded the Nobel Prize in 2004. Since then, the nose, in particular the subject of molecular chemosensation, is definitely "en vogue". We have only recently gained new and exciting information that puts some of our previous knowledge of the mammalian sense of smell in jeopardy. We used to think the olfactory system consists of only two anatomically and functionally separate components: the nasal membrane or main olfactory system that actually smells, and the vomeronasal organ (VNO) or accessory olfactory system that detects socially relevant chemosignals, often referred to as pheromones.

Today, two additional olfactory subsystems, the septal organ and the Grüneberg ganglion, have been discovered or rediscovered. And not only that – we now know that the functional structure of the sense of smell is far more complex and differentiated. For instance, some social signals are recognized via the main olfactory epithelium, just as some "conventional" odors are detected via the VNO. Within the subsystems, different neuron families are specialized for specific sensory stimuli and exhibit characteristic axonal projection patterns. In the last five years in particular, groundbreaking discoveries about such specialized nerve cells in the main olfactory
mucosa have revealed a totally different repertoire of signaling proteins than the canonical olfactory cells with which we are familiar. Recent publications have shown, for example, that sensors of the Grüneberg ganglion probably act as both smell and cold sensors.

What have you discovered about olfactory receptors?

Working together with Prof. Ivan Rodriguez from the University of Geneva, we showed in 2009 that the VNO not only comprises the two already known V1R and V2R receptor families, but also a third group, the formyl peptide receptors (FPR). Two other representatives of this receptor type are known from immunology, where they play a key role in the chemotactic reaction of immune cells toward the site of a bacterial infection or inflammation. Our results suggest that the FPRs can provide a mechanistic explanation for an already known sensory phenomenon in rodents: mice can smell whether other mice are healthy or sick.

We still need to find out a lot more about the molecular processes of the sense of smell and the coding logic of social chemical signals. However, our new understanding of the olfactory system also leads to new and exciting issues. If we can decipher the different receptor types and their signal transmission strategies within neuronal networks, we will better understand the neurophysiological basis of social behavior in mammals.

What issues are you working on at present?

One of our current research projects focuses on what happens in a vomeronasal nerve cell when an FPR has bound its ligand, i.e. how this chemical binding signal translates into action potentials by the neurons. The biochemical translation of olfactory stimulation into the language of the brain is a key focus of our experiments. Another topic we are currently working on is the processing of signals in the accessory olfactory bulb, the part of the olfactory brain that receives information from the VNO. We are trying to understand how excitatory and inhibitory neurons interact, and how the information originates that ultimately triggers changes in hormone levels and behavioral reactions.

Could this chemosensory knowledge be clinically relevant in future?

At the RWTH Aachen, we are in close contact with our colleagues in the Department of Psychiatry. One of the symptoms of neurodegenerative diseases such as Parkinson’s or Alzheimer’s is a damaged sense of smell. The exact link is not clear yet, but we know that this symptom is noticeable at an early stage of the disease. It would therefore be conceivable that this could play a role in early diagnostics. Another extremely futuristic idea, though maybe not entirely absurd, is to develop an artificial VNO, with FPRs acting as a biosensor for diagnosis of medical disorders.

Can the findings from the animal models be transferred to humans, too?

Only to a limited extent, as the sense of smell is even more important for many animal species than for humans. This can already be seen from the number of relevant genes. A mouse has around 1200 genes exclusively encoding odorant receptors. Only one third of these genes are still functional in humans. Humans no longer show a monomodality that is sometimes observed in animals. We simultaneously process different sensory stimuli, going primarily by visual impressions. Although olfactory cues obviously trigger specific types of behavior in humans as well, we are yet to identify a human pheromone on the molecular level.
Aggressive, territorial or sexual behavior is directly linked to olfactory perception in many mammals. In mice, we can control some behavioral phenotypes via specific chemical signals. I can’t imagine anything similar in humans. Besides, social communication on the basis of chemical signals mainly works conspecifically. So when we find something in a mouse, we get new ideas on how and where to look for something potentially similar in humans.

You use a wide variety of techniques for your experiments. How important is the microscope for your research?

We use electrophysiological recordings and activity measurements, different imaging methods, molecular-biological, biochemical, and behavior analysis techniques. Using a microscope, particularly for live cell imaging, is one of the central methods in our research. We mainly work with live tissue sections from the nose, the VNO, and the olfactory bulb. To correlate electrophysiological measurements with imaging data, we need a good fixed stage microscope equipped with the relevant optical techniques such as DIC (Differential Interference Contrast) and plenty of room for manipulation and perfusion systems, and patch clamp pre-amplifiers. For this type of upright microscopy, special water immersion objectives that show high transmission even in the near infrared and UV light range and fast, high-resolution cameras are essential. Apart from the optics and accessibility to the area around the specimen, another main criterion is absolute microscope stability. We also use inverted microscopes and confocal systems for our different imaging approaches.

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Single-cell Analysis After Laser Microdissection

The Morbus Parkinson Puzzle

Dr. Olaf Spörkel, LabScience Communications

After Morbus (M.) Alzheimer, M. Parkinson is the second most common progressive neurodegenerative disease. Before the first symptoms manifest themselves, up to 70 percent of dopamine-releasing neurons in the mid-brain have already died. Dr. biol. hum. Falk Schlaudraff from the Molecular Neurophysiology team at the Institute of Applied Physiology of the University of Ulm used modern laser microdissection methods to isolate and analyze cells from post mortem tissue specimens taken from M. Parkinson patients in order to gain molecular insight into the disease.

Dr. Schlaudraff, what is the current state of M. Parkinson research?

A characteristic sign of M. Parkinson is the deterioration of dopaminergic neurons in the mid-brain, specifically in the substantia nigra (SN, black substance). Different causes and forms of this disease have been identified. In the case of the genetic familial form, for example, it has been possible to identify various genes that have a causal influence for M. Parkinson. However, we still don’t know whether all the relevant genes have been identified, or exactly how they contribute to pathogenesis. There are several theories on how the disease originates. M. Parkinson can be compared to a puzzle. We have already found many of the pieces and can put some of them together, but we don’t know what the whole picture looks like. We haven’t worked out the significance of some of the pieces yet, neither do we know how many pieces of the puzzle we still have to find before we can present a full picture of M. Parkinson and actually understand the disease.

What was the focus of your research?

I concentrated on gene expression analysis of individual dopaminergic midbrain neurons of the substantia nigra. These cells selectively degenerate as the M. Parkinson disease progresses. Once a patient notices the cardinal symptoms of M. Parkinson, such as the resting tremor that is a characteristic of this disease, more than 70 percent of dopaminergic SN neurons have already died. One of the aims of my research was to develop optimized qPCR (quantitative polymerase chain reaction) assays. These enable valid comparison of the gene expression of promising gene candidates in individual neurons from human post mortem tissue of M. Parkinson patients with the gene expression of the same neurons from healthy control subjects. We developed a qPCR-based platform that could be used to isolate individual cells from native tissue and obtain highly comparable gene expression analyses. Our analysis, for example the RNA quality of the specimens, showed that the quality of the results is not influenced by the staining process and laser microdissection.

Why are dopaminergic neurons in substantia nigra particularly affected?

Analyses of the different post mortem specimens are like snapshots in a specific stage of M. Parkinson. I can compare these snapshots with each other. But I can’t use these specimens to detect whether a changed gene expression is the result or the cause of the disease. We
What other results did you obtain from your research?

I detected an orchestrated change of gene expression in the selective dopaminergic neurons of M. Parkinson patients. This change affects genes that are involved in the regulation of the dopamine metabolism as well as genes that code for ion channels. We showed, for example, a higher expression of several genes involved in the synthesis and provision of dopamine in the surviving dopaminergic neurons. We also looked at various gene expression patterns of ion channels that regulate the activity of the dopaminergic neurons. Here too, we noticed a change in the expression of some of the examined genes of M. Parkinson patients.

What do you use laser microdissection for?

In the last few years there have been many studies comparing complete tissue specimens of substantia nigra of M. Parkinson patients with healthy tissue. However, this comparison is misleading, as in these patients, 70 percent of the neurons that are obviously involved in the disease are already degenerated at its onset. Also, the composition and the sectioning of the examined brain tissue are extremely heterogeneous. So the “tissue” approach we have adopted up to now is like comparing apples with pears.

We wanted to selectively view the midbrain dopaminergic neurons that are involved in the pathogenic process and used laser microdissection for validated comparison at the single-cell level. This technique makes it possible to accurately cut individual dopaminergic neurons out of complex tissue, without contact or contamination, and analyze the gene expression in individual cells. The most prevalent type of tissue in the brain is supporting tissue: Glial cells are ten to 50 times more common than the neurons we are interested in. Without laser microdissection, it would be almost impossible to clearly characterize the relatively rare nerve cells on a molecular level; they would not be distinguishable from background noise.

The analysis of single cells frequently leads to different results from those obtained from a complete tissue examination. Studies have shown that the expression of certain microRNAs is changed in the tissue of M. Parkinson patients. We followed up these statements and at first we were able to confirm the results for the whole tissue. However, we also examined microdissected cells in parallel. Here we found that the microRNA expression is not changed on a single cell level. This tissue artifact was detected with the aid of laser microdissection.

What are the benefits of the laser microdissection system you use?

We chose the Leica Microsystems LMD system, which enables contact-free dissection of single cells or, if necessary, larger areas of tissue. The dissected material is captured in the cap of a tube and can be processed immediately. Leica Microsystems also gave us excellent support. Their technical service provided swift, comprehensive, and reliable answers to all sorts of questions.

What developments do you expect?

At present, there are no tests for diagnosing M. Parkinson at an early stage, and there are many variants of the disease and diseases with similar symptoms. A certain percentage of M. Parkinson cases are therefore diagnosed wrongly or not diagnosed at all. The prerequisite for successful treatment of M. Parkinson would be effective early diagnosis. If the disease could be diagnosed at an initial stage in which the...
neurons are just beginning to degenerate, it might be possible to prevent progressive neuron degeneration so that the disease does not break out at all. The identification of biomarkers in blood or cerebral fluid is currently a major research focus. There are genes that are not only expressed in the brain, but ubiquitously—in all cells. If the expression of these genes were changed in the dopaminergic neurons of an M. Parkinson case, it would be possible to examine more easily accessible tissue for diagnostic purposes. The first steps have been taken, but there’s still a long way to go before such a test can be implemented, and further long-term studies need to be conducted.

What projects are you working on at the moment?

After obtaining my Ph.D. in July 2010, I started working in an R&D department of an industrial company, where I develop systems for analyzing nucleic acid.

References

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Fig. 3: LMD and mRNA-expression analysis of individual substantia nigra DA neurons from human PD and control postmortem brains. (A) and (B) Pools of neuromelanin-positive [NM+] neurons were isolated via LMD of cresylviolet-stained horizontal midbrain cryosections from PD (A) and control brains (B). Upper panel: PD (A) and control (B) cryosections after LMD of small pools of NM+ neurons from SNpc. Lower panels: Representative PD (A) and control (B) SNpc NM+(+) neurons before (left) and after dissection (right). Insert: cap control after UV-LMD. Scale bars: 250 mm, 20 mm, respectively. (C) Scatter plot of a-synuclein gene-expression levels in PD and control brains. a-Synuclein gene expression of each pool of 15 NM+(+) and TH(+) SNpc neurons is given as pg-equivalents of total cDNA derived from human SN-tissue per cell (standard curve quantification), determined via quantitative real-time PCR. Bars represent mean a-synuclein expression for 3Npc pools of each brains±SEM. (D) Scatter plot of the mean a-synuclein cDNA levels (±SEM) against the RNA integrity number for each brain. Brain Bank codes are indicated next to each dot. (E) Linear regression between mean a-synuclein expression and RNA integrity number for all individual analyzed control and PD brains showed no positive correlation between higher RNA quality of the tissue and detected a-synuclein expression levels. (controls: black dotted line, R²=0.0506; PD brains: red dotted line, R²=0.9950; all analyzed brains combined: black line, R²=0.4389). Please note that PD brains showed a strong inverse correlation between RNA integrity and detected a-synuclein expression levels (red dotted line, R²=0.9950). (F) Mean expression levels of a-SYN, TH and ENO2 were significantly higher in individual NM+(+) SN DA neurons from PD brains compared to controls. Published in: Nucleic Acids Research, 2008, 1–16 doi:10.1093/nar/gkn884, © Oxford University Press.
Humans and larger animals have great variation in brain size, and even in the relative size of different structures within brains of a given size. Fortunately for neuroscience research, rodent brains are made with more quality control; they are much more uniform animal to animal within a species and age range. This uniformity makes it possible to create coordinate maps or atlases of the brain, showing the three dimensional location of every structure in the brain relative to a zero point. For common age ranges among common laboratory species like rats, mice and hamsters, Bregma, a point on the skull where the bone plates have grown together, is usually used as the zero point. Together with a suitable manipulator and oriented head holder, such maps can be used to position probes at a preplanned site in the brains of several rodents. The instrument for moving a probe to a given coordinate in space — inside the brain — is a stereotaxic instrument.

Leica Angle Two for Precise 3D Brain Access

Avoid Confounding, Improve Accuracy

Charles W. Scouten, Ph.D., Leica Microsystems

The reason to place a probe at a known location in brain is to study localization of function in the brain. The brain is a very heterogeneous structure, and different sites and regions have different functional roles to play. Learning to understand that organization is one of the major goals of neuroscience, and requires regional studies of brain. Various stains and immune-markers can show regional chemical variation in brain. Stereotaxic insertion of probes — for stimulation, injection, recording, microdialysis, lesion or other functions — is an important technique to gain understanding of brain functional organization.

Traditional stereotaxic instruments consist of a head holder that is aligned with a manipulator, with the manipulator movements measured by vernier scales with a resolution of 100 microns. A newer idea is to use digital scales in place of vernier, and obtain easier, less error prone reading and resolution to 10 microns. Manipulators can be tilted and rotated, but doing so breaks the alignment with the head holder, and changes the distance that must be moved along each axis to reach the desired target. Sometimes an angled approach is necessary, to avoid arteries, or other structures important to the research. In this case, the investigator must perform math on the atlas coordinates, and usually pilot studies, to develop coordinates that work. Once that is done, the manipulator is locked in place, since further changes in tilt would again change the coordinates needed.

Fig. 1: The Leica Angle Two stereotaxic instrument improves accuracy in small animal brain surgery.
Straight up and down

Consequently, it is almost universal in stereotaxic research to do all surgeries for an experiment on several animals from the same angle of approach. This is usually straight down, 90 degrees from skull flat head alignment. This has been necessary given the instrumentation available, it is unfortunate because the resulting “action-at-target” variable is confounded with the “path to target” variable. Since they are linked, nothing distinguishes which one caused the change that is measured later.

Confounding

Imagine injecting deep in brain. Even with a glass pipette as a needle, some fluid will leak out the tip on the way down. When pressure is applied, some fluid will run up the side of the injection needle, where an opening is already made, rather than push back or flow into surrounding brain tissue. After an injection, when a pressurized bolus of fluid is at the tip, and the tip is withdrawn, the fluid will follow the needle back out. In the end, there is an intense injection at the target site, and a decreasingly intense injection along the needle track, usually all the way to the surface of the brain.

Later testing shows a behavioral change as a result of the injection. But was that change due to the injection at the target site, or the injection somewhere higher up in the brain? Two variables that change in lock step are termed “Confounded,” and one cannot discern which was responsible for an observed result. A similar analysis applies with lesions, the electrode creates damage coming down, and always comes down through the same structures. Did the lesion damage at target cause the outcome alone, or in conjunction with the damage getting to the target?

The solution: Leica Angle Two

The Leica Angle Two Stereotaxic Instrument, developed in part by an SBIR grant from the U.S. NINDS/NIH to MyNeuroLab, is designed to address and resolve this issue. With the Leica Angle Two, it is easy to do every surgery from a different angle, with no loss of accuracy. In fact, features described below result in enhanced accuracy together with less risk of human error, so that fewer lab animals are needed for each experiment. The Angle Two will pay for itself in reduced animal costs. This is accomplished by adding rotary encoders to the tilt and rotation movements, and feeding all five axes of position information to a computer. The computer does the complex three dimensional trigonometry, monitors the tip position, and can give the coordinates along the three linear axes to reach the target after taking into account any tilt or rotation of the manipulator. Connection to a computer allows several other significant features.

Atlas integration

The Leica Angle Two user can scroll through the plates of an on-screen atlas, and then click on the exact desired target location to set the target coordinates, assuming skull flat and Bregma zero point. Red cross-
stereotaxis

Virtual Skull Flat™

The head must be held in a standard position. Usually, this is skull flat, as defined by George Paxinos. With conventional stereotaxic instruments, the user touches the probe down at Bregma, takes a zero reading (or zeroes a digital display), then moves the probe caudally to the Lambda point and touches the tip down again. Hopefully, both points will be at the same vertical reading. If not, a repetitive trial and error adjustment of a dovetail supporting the animal’s teeth begins, in order to adjust head tilt to the zero plane. Eventually, the user decides it is close enough. In contrast, with the Virtual Skull Flat feature found only on the Leica Angle Two, the user touches down at Bregma and Lambda, and the computer calculates how tilted the head is and corrects the linear axes distance to the target for where it is, given the head tilt. There is no need to ever physically adjust the degree of head tilt; the computer directs the user to the correct target even though the head is tilted.

Named target icons

A user may save a set of target coordinates as an icon on the screen below the atlas. To reset those coordinates as the desired target, the user double clicks on the icon when in set target mode. This is very useful when there are multiple experiments in progress, and avoids mistakes in re-entering coordinates.

Bilateral surgeries

After performing a surgery on one side of the brain, to position a probe in the symmetrical opposite side of the brain, the user clicks a minus sign after the ML (medial to lateral) coordinate to reset the target to the same point on the other side. There is no need to return to Bregma or remeasure head tilt. The computer displays how to reach that point on the other side, even if the manipulator is rotated or tilted.

Final report

After each surgery, the user clicks on the export position button to save the present coordinates and tilts to a line in a file. The animal ID, date, and other information is typed in as needed. At the end of the experiment, the user can print a one page report with a line for every animal, and an accurate listing of the exact atlas coordinates, and the tilt and rotation, at which the manipulation was performed.

References


To see a video of the Leica Angle Two demonstration, go to www.MyNeuroLab.com
New Tool to Study Traumatic Brain Injury

Impact Neurotrauma – From War to Sports

Charles W. Scouten, Ph.D., Leica Microsystems

Brain damage due to impact is the most common cause of permanent disability in children. Research into causes, treatments, and avoidance of impact damage has been hampered by cumbersome and inaccurate equipment for animal research. Large pneumatic, electromagnetic, or gravity driven devices, in which the animal is positioned underneath a descending probe, were the state-of-the-art. Leica Microsystems has introduced the Leica Impact One, with a remote actuator head small enough to be held in and positioned or angled precisely by a stereotaxic instrument. This device has greatly enhanced the accuracy and reproducibility, animal to animal, of controlled cortical or skull impact. Voice coil technology reduces overshoot related to impact velocity, and enables cleaner separation of the variables.

New focus on impact sports

Impact Neurotrauma is an increasingly urgent area of study. On the morning of the February 2010 Super Bowl, U.S. Football Championship, Face the Nation, a respected political news forum, did a special on impact sports that result in former players having specific brain defects and common behavioral sequelae. The magazine Science picked up the same topic in 2009 with the article, “A Late Hit for Pro Football Players,” from Greg Miller.

Today, the U.S. Department of Defense is investing money into neurotrauma research especially as a defense against IED’s (improvised explosive devices). Impact neurotrauma has long been the most common cause of child and infant long term morbidity. And new data reveals that sporting events cause more long term damage than previously thought, and in milder sports than the known problem frequently found in ex-boxers. Brain damage is commonly disabling, disruptive, and long term; and hence expensive.

Studies from the 1920s have documented the high risk of dementia and neurodegeneration in boxers. This effect is well known enough to have been given the name, “dementia pugilistica,” a name now changed to “Chronic Traumatic Encephalopathy” or CTE, to acknowledge that very similar brain damage is found prematurely in the brains of other impact subjects. CTE is characterized by behavior changes (mild cognitive impairment, emotional outbursts, depression), and recently, in microscopic autopsy studies, by tau protein tangles around blood vessels in certain parts of the brain. This pathology is rare in normal subjects, some is present in Alzheimer’s patients, but in different areas.

Arising questions

Since beginning careful studies to look at microscopic brain abnormalities, 11 out of 12 former pro football players coming to autopsy have had tau tangles that should not be present. A disturbing case was an 18 year old high school football player who had significant tau tangles. An 18 year old brain should not have any such abnormalities. At what age do such impacts begin to have significant effect? What about young children in sports?

Research has yet determine if multiple mild impacts can cause tau tangles, if area of impact is important, or if only severe concussive injury produces these tau tangles. Can helmets be devised to avoid the injury? Clearly, the football helmets available today may help, but do not solve the problem. The mechanism by which the tau tangles may influence behavior are not determined. Methods to diagnose the presence of tau tangles short of autopsy have not been developed.

More precision and reproducibility

Research funded by the U.S. NINDS/NIH (National Institute of Neurological Diseases/National Institutes of Health) through an SBIR (Small Business Innovative Research) grant to MyNeuroLab (now part of Leica Microsystems) for developing a blood test for brain injuries. The U.S. Army, in collaboration with Banyan Biomarkers, Inc., has announced the development of a blood test that detects proteins released into the blood as a result of any recent brain injuries. A Leica Impact One, Banyan is using for animal research, was involved in this development. The test has been validated in a small group of human patients at present.

The availability of a blood test for brain damage would greatly enhance diagnosis and treatment of mild traumatic brain injury, and facilitate animal research in further studies.
Microsystems) has led to development of a novel impact device with a remote actuator that can be mounted on a small animal stereotaxic instrument, and take advantage of the instrument’s precise positioning and angle capabilities.

Today, Leica Microsystems offers the new Leica Impact One, together with the most advanced stereotaxic instrument, Leica Angle Two. With the stereotaxic instrument, the extended impact tip may be positioned relative to skull landmarks, and at any angle. It is then retracted, and, again with the stereotaxic instrument, advanced toward the preplanned maximum penetration depth. The operator can then fire an impact upon either skull or the exposed brain or spinal cord.

The Leica Impact One, mounted on a stereotaxic instrument, can precisely control the position and angle of impact, dwell time (the time the tip remains in contact), and velocity of impact in a very reproducible way. This represents a significant advance in the precision and reproducibility of instrumentation available to study the results of impacts on neural tissue.

References

The new Leica Impact One – enhances accuracy and reproducibility of controlled cortical or skull impact

- Position and reproducible positioning of impact (via stereotaxic instrument)
- Mounts on an existing stereotaxic instrument
- Control of impact probe terminal velocity
- Control of dwell time
- Control (via stereotaxic) maximum penetration depth
Leica Vibratome Series

Cutting Edge Precision

Vibrating blade microtomes are used to produce monolayer or thick sections of fixed or fresh tissue under physiological conditions without freezing or embedding. Sectioning fresh tissue specimens with Leica Microsystems’ VT Series maintains the morphology, enzyme activity and cell viability of the tissue. Their use also minimizes artifacts, compression distortion, cell destruction and other inherent deleterious effects of sectioning. Freezing fractures cell membranes, and there is a loss of cytosol. This is avoided by Vibratome sectioning, so that immune or other staining of cellular free cytosol proteins is much more vivid with Vibratome cut sections. This includes natural proteins, and HRP and other markers. Other applications include cell culturing of different organs, sections for patch clamping, electrophysiology, free floating sections and many other applications in neuroscience.

In order to maintain physiological conditions while sectioning, chill the buffer and minimize the vertical deflection of the blade holder as well as the blade. During operation, the blade moves laterally and forward and the section thickness is achieved by vertically feeding the specimen stage. Other parameters including section thickness, amplitude, frequency, knife travel speed and blade angle influence section quality.

The Leica Vibratome Series of instruments offers a complete product range that controls some or all of these parameters. The features of each instrument vary in the degree of automation, ranging from the Leica VT1000 P to the fully automated Leica VT1200 S with optional Vibrocheck, for measuring and minimizing vertical blade deflection.

Leica Vibratomes have been developed in collaboration with renowned scientists throughout the world and suit every researcher’s application.

Online videos

See our online videos of the Leica VT1200 S, where physiologists show and explain how they work with the Leica Vibratome.

Go to: http://www.leica-microsystems.com and search for “Showcase Leica VT1200 S” or scan this QR code with your iPhone or Smart Phone
Leica DMI6000 B with Adaptive Focus Control

Explore Life in All Dimensions

Dr. Karin Schwab, Leica Microsystems

Monitoring and analyzing dynamic processes in living cells is challenging, even for the most precise and reliable microscope systems. The Leica DMI6000 B inverted microscope is the core component of Leica Microsystems’ widefield and confocal systems for live cell imaging, offering unsurpassed stability inherent to its design. It is capable of effortlessly maintaining the focus under live cell imaging conditions.

Improving the best

Even the most robust microscope is susceptible to sudden temperature fluctuations, for instance if a climate chamber needs to be opened to add a solution to the specimen during the course of an experiment. Therefore Leica Microsystems has developed the new Leica DMI6000 B with Adaptive Focus Control (AFC) for researchers that require consistent multidimensional imaging without loss of focus. Available for both widefield and confocal applications, the AFC dynamically regulates the focus position, whenever or wherever the experiment requires it. Tested and approved in collaboration with scientific partners, Leica’s Adaptive Focus Control ensures that the specimen remains in focus throughout the experiment (Fig. 1).

When experience counts

Designed with great emphasis on reliability, the AFC principle involves the reflection of a light beam at a suitable surface to keep the distance between the objective and specimen constant. The process is established, robust, and remarkably fast. It is applicable to a variety of glass and plastic substrates and can work with more than 25 objectives. The underlying technique works for all selected contrast methods and does not require images to be captured from the specimen. This ensures that cells remain viable for longer, and deliver reliable results even over long periods of time.

Smooth integration

To be truly effective for a wide range of experiments, Adaptive Focus Control is integrated into Leica Microsystems’ intuitive software workflow. For full flexibility, AFC can also be operated in stand-alone mode without PC connection using the microscope’s function keys. Remote control is possible via the Leica SmartMove or Leica STP6000 control panel. The Intelligent Automation of the Leica DMI6000 B offers the ultimate ease of use. A single push of the ‘hold focus’ button is all it takes – the system works automatically in the background, optimizing the results for each objective. The system focuses on the specimen, while you focus on getting results.

The need for speed

When moving from position to position within a specimen, small differences in focus are often observed as...
you look down the eyepieces. The impressive speed of the AFC is clearly demonstrated here, as it automatically corrects for these small focus differences in real time. This is of great benefit when imaging multi well plates. AFC can be used for fast process monitoring. Imagine you want to monitor vesicle movement in 3D over time. The challenge here is to keep the cells in focus while acquiring high-speed z-stacks. This is achieved with the Leica Super Z Galvo stage. You set up the time lapse experiment in combination with z-stacks, defining when and at which position AFC will be activated. The experiment benefits from faster, reliable results – no over-sampling, less stress to the specimen due to reduced exposure to light, and smaller data sets.

**Focusing on change**

Cells are dynamic structures – they change shape constantly and even round up before entering mitosis. What is of interest and sharply in focus right now, may be at a completely different z position a couple of hours later. Here you need the flexibility to dynamically adapt to changing cell positions or morphology. The solution is simple: the combined action of AFC with the Leica digital auto-focus for extra focusing versatility. The possibility to define exactly when and at which positions this focusing combination is activated ensures every event in the time lapse is captured.

"AFC is such a powerful tool for time lapse experiments. I am impressed how fast and reliable it is", states Prof. Dr. Ralf Jacob, University of Marburg, Department of Clinical Cytobiology and Cytopathology, Germany.

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**Fig. 2**: TIRF imaging with simultaneous AFC. MDCK cells expressing GFP-p75. Courtesy of Prof. Dr. Ralf Jacob and Dr. Alexandra Elli, Institute of Cytobiology and Cytopathology, University of Marburg, Germany.

**Fig. 3**: AFC switched on: COS cells were transfected with a Golgi specific GFP variant. The complete microscope system was placed in a climate chamber at 37°C. Shortly after starting the acquisition 1 ml of an ice-cold salt solution (PBS) was added (60 s). Throughout the entire acquisition sequence, the sample remains in the desired focal plane. Notice that over the complete acquisition time no focus drift could be measured. Courtesy of Prof. Dr. Ralf Jacob and Dr. Alexandra Elli, Institute of Cytobiology and Cytopathology, University of Marburg, Germany.
The Fast Track to Superresolution

Subdiffraction imaging goes confocal – with the new Leica TCS STED CW!

Explore high speed scanning on the nanoscale! Leica Microsystems has integrated the ingenious concept of STED into its high end confocal microscope Leica TCS SP5. The solution is highly effective and stunningly simple: easy to use, highly affordable and available as an upgrade. Featuring a 592 nm continuous wave depletion laser the new Leica TCS STED CW enables the use of a whole range of well known fluorophores and proteins. Find out what this can do for you!

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Living up to Life