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TIRF Microscopy Turned Upside Down

Fluorescence Microscopy
of the Apical Membrane of Polarized Epithelial Cells

Good Vibrations

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Where are Memories Stored?

Mapping Billions of Synapses with Microscopy and Mathematics

EDITORIAL

Dear Readers,

The possibilities now offered by light microscopic technologies would have been inconceivable 20 years ago. Super-resolution microscopy has provided bioscientists with many new ways of visualizing and analyzing the processes of life right down to the molecular scale. STED microscopy was the first commercial technology to show that it was possible to overcome the resolution limit – and in doing so, triggered a veritable technology race. Meanwhile, different approaches have established themselves for resolving detail below the diffraction limit of light. Yet an end to these developments is not in sight. In this issue of reSOLUTION, we feature a new super-resolution technology that has taken the leap from the physics labs to being a user-friendly marketable system. The Leica SR GSD is the latest addition to our portfolio of super-resolution products.

CARS microscopy is another relatively new technology that is gaining popularity not only among molecular biologists but also in material sciences. CARS uses molecular vibrational spectra for chemical contrast generation. For this reason it works without external labeling.

Further developments in laser technology, image recording and software are also contributing to the further sophistication of imaging techniques that will pave the way for new experiments. Examples include white light laser technology, new hybrid detectors, lambda square mapping software and multi application imaging platforms. You will also find other application and technology reports in this issue of reSOLUTION that we hope will be of interest to you.

Have fun reading!

Anja Schué
Corporate Communications

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Fluorescence Microscopy of the Apical Membrane of Polarized Epithelial Cells

TIRF Microscopy Turned Upside Down

Christoph Greb and Prof. Ralf Jacob, Philipps University Marburg

Application of TIRF microscopy (Total Internal Reflection Fluorescence) allows the visualization of structures at the apical surface of polarized epithelial cells that have been hidden in conventional fluorescence microscopy images. Hence, the approach reveals new insights into the composition of this characteristic cell pole that elucidate processes in apical protein trafficking.

MDCK cells as model for intracellular trafficking

The plasma membrane of polarized epithelial cells is divided into two clearly separate parts; an apical membrane domain facing the organ lumen or, in cell culture, the medium, and the basolateral domain, which is connected with adjacent cells or the extracellular matrix. The two membrane domains are separated by tight junctions and have their own specific protein and lipid content. A classic model system for studying the intracellular trafficking of these components into their particular target membrane is provided by MDCK cells (Madin-Darby-Canine-Kidney, which are used in this article). The study of intracellular sorting processes is of fundamental importance for understanding the cell's protein and lipid trafficking system. Wrong targeting of these components can cause organ defects such as those found in various diseases of the lungs, the intestinal tract and the kidney. Prominent examples of this are cystic fibrosis or congenital sucrase-isomaltase deficiency.

Physical phenomenon visualizes membrane proximal processes

The subject of our team's research at the Institute of Cell Biology and Cell Pathology of the Philipps University Marburg is the elucidation of protein sorting to the apical cytoplasmic membrane of MDCK cells. The application of TIRF microscopy (in short:

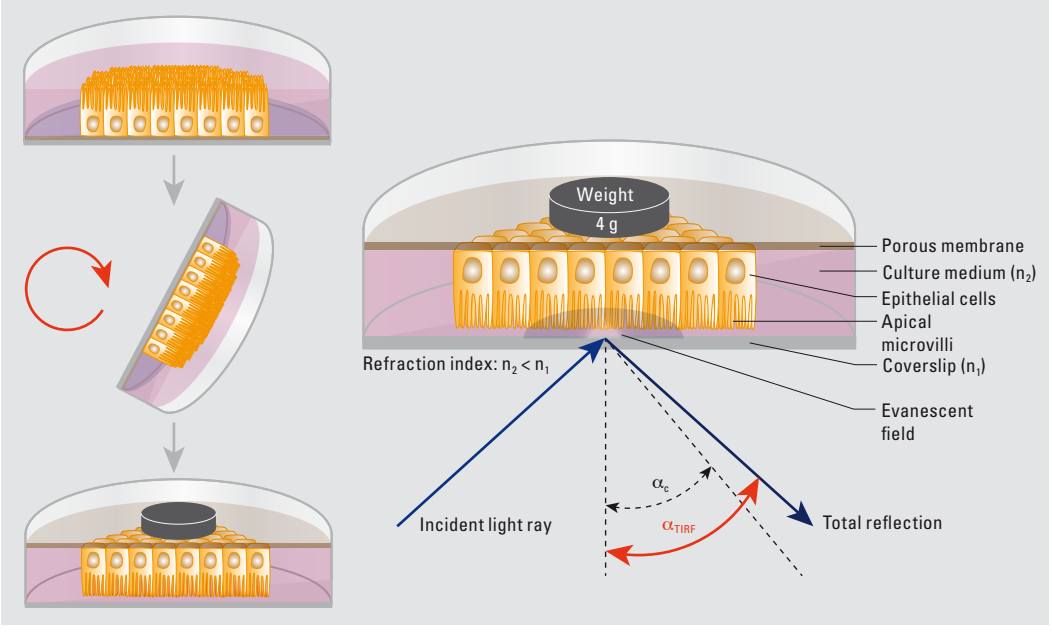

Fig. 1: Experimental setup for apical TIRFM: MDCK cells are sown on a porous membrane (PET filter) and incubated for several days. To view the apical part of the cells, the PET filter is removed from its plastic mount and placed upside down in a Bachofer chamber in close contact with the coverglass that is fitted there. To make sure the cells stay close to the coverglass, a 4 g weight is put on top of the PET filter. Due to this arrangement, the apical part of the MDCK cells that is lined with microvilli lies within the evanescent field produced at the surface of the coverglass by the total reflection of the laser beam. Only fluorescent particles within this field are excited and therefore visible.

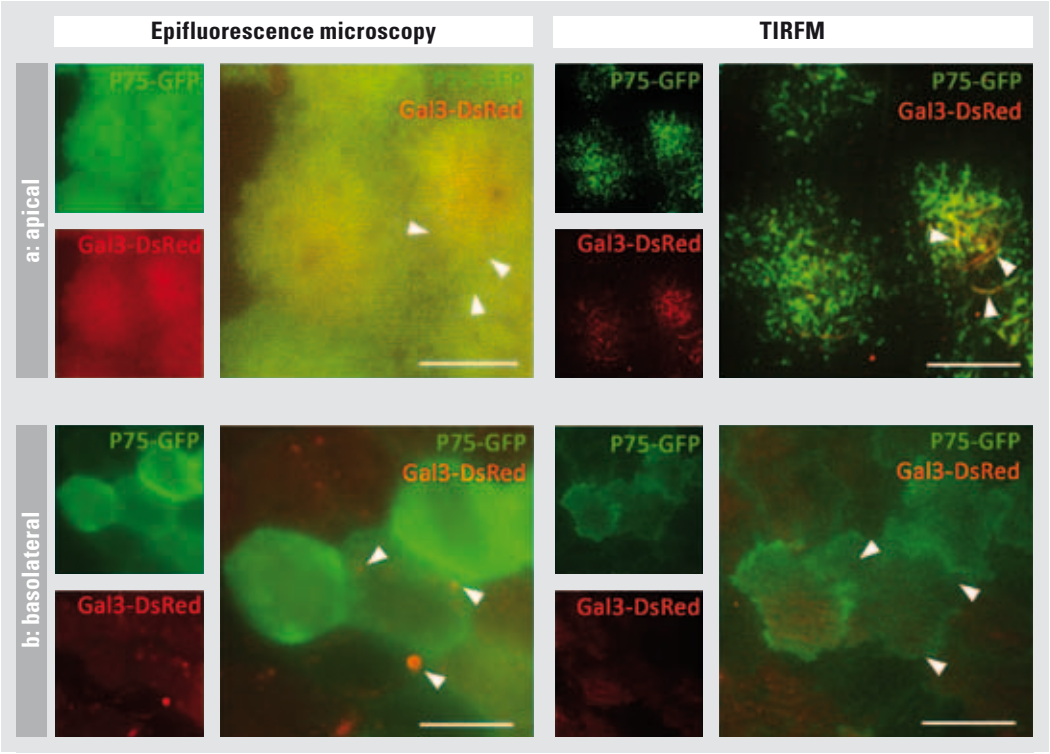

Fig. 2a–b: Comparison of epifluorescence microscopy and TIRFM,
a: For apical TIRFM, MDCK$_{p75\text{-}GFP/Gal3\text{-}DsRed}$ cells were cultivated on PET filters and viewed using the Leica AM TIRF MC based on the Leica DMI6000 B fluorescence microscope. Whereas the epifluorescence images (left) only show a diffuse light, the TIRF images of the same cells (right) are capable of resolving tiny structures (arrow tips). These are often tubular and exhibit the fluorescence of both markers used.
b: For the basolateral TIRFM, the same cell line was directly cultivated on the coverglass of a Bachofer chamber and also viewed in the TIRF microscope. Again, TIRF images delivered better resolution. Also, red signals far below the cell surface are not excited in the TIRFM (arrow tips). Scale bar: 10 µm

TIRFM) proved extremely useful for this work. TIRFM employ the physical phenomenon of total reflection to generate a so-called evanescent field. This field only extends over a penetration depth of a few hundred nanometers and excites fluorophores in its range near to the coverglass (Fig. 1).

With the Leica AM TIRF MC mentioned in this article and based on the Leica DMI6000 B fluorescence microscope, the penetration depth of the excitation light into the cell can be continuously adjusted from 70 to 300 nm. In practice, this can be used to excite fluorescence-tagged proteins in the immediate vicinity of the cytoplasmic membrane, in our case of polarized epithelial cells.

Sample preparation to investigate the apical cytoplasmic membrane

Observation of the apical membrane domains of MDCK cells is a particular technical challenge, because, unlike TIRF excitation of fluorophores in the basolateral membrane domain, for which cells can be directly cultivated on a coverglass, cells for inspecting the apical side first have to form a polarized epithelium in a petri dish. This is done by growing them on porous PET filters to obtain an epithelial monolayer. The filters with the polarized cell layer are then cut out of their mount and put upside down onto the coverglass of a Bachofer chamber. Physical proximity between the apical membrane of the cells and the evanescent field at the coverglass is achieved by putting an additional weight on the filter (Fig. 1).

TIRFM vs. epifluorescence

Figure 2a shows a comparison of conventional epifluorescence images and the image of various fluorophores on the apical membrane domain of MDCK cells made by TIRFM. Here, a predominantly apical membrane protein, the neurotrophin receptor (p75), was studied in comparison with its apical sorting receptor, galectin-3. The two proteins were labelled with fluorescence proteins, p75 with eGFP (p75-GFP) and galectin-3 with DsRed (Gal3-DsRed) for imaging in the fluorescence microscope. Whereas conventional epifluorescence images tend to produce a diffuse light over the whole cell body in both channels, the TIRF images reveal extremely distinct and clearly structured objects. Most of these are finger-like protuberances of the apical membrane, so-called microvilli, decorated with p75 or, in some cases, with galectin-3.

Looking at the basolateral side of the cells (Fig. 2b), the TIRF images again show clearer structures despite the smaller amounts of protein in this membrane domain. Cell borders are far more clearly demarcated and have a more amorphous line compared with the epifluorescence image. The image also shows the limited excitation range of the TIRFM in a narrow field around the cytoplasmic membrane. This is shown by intracellular Gal3-DsRed-positive

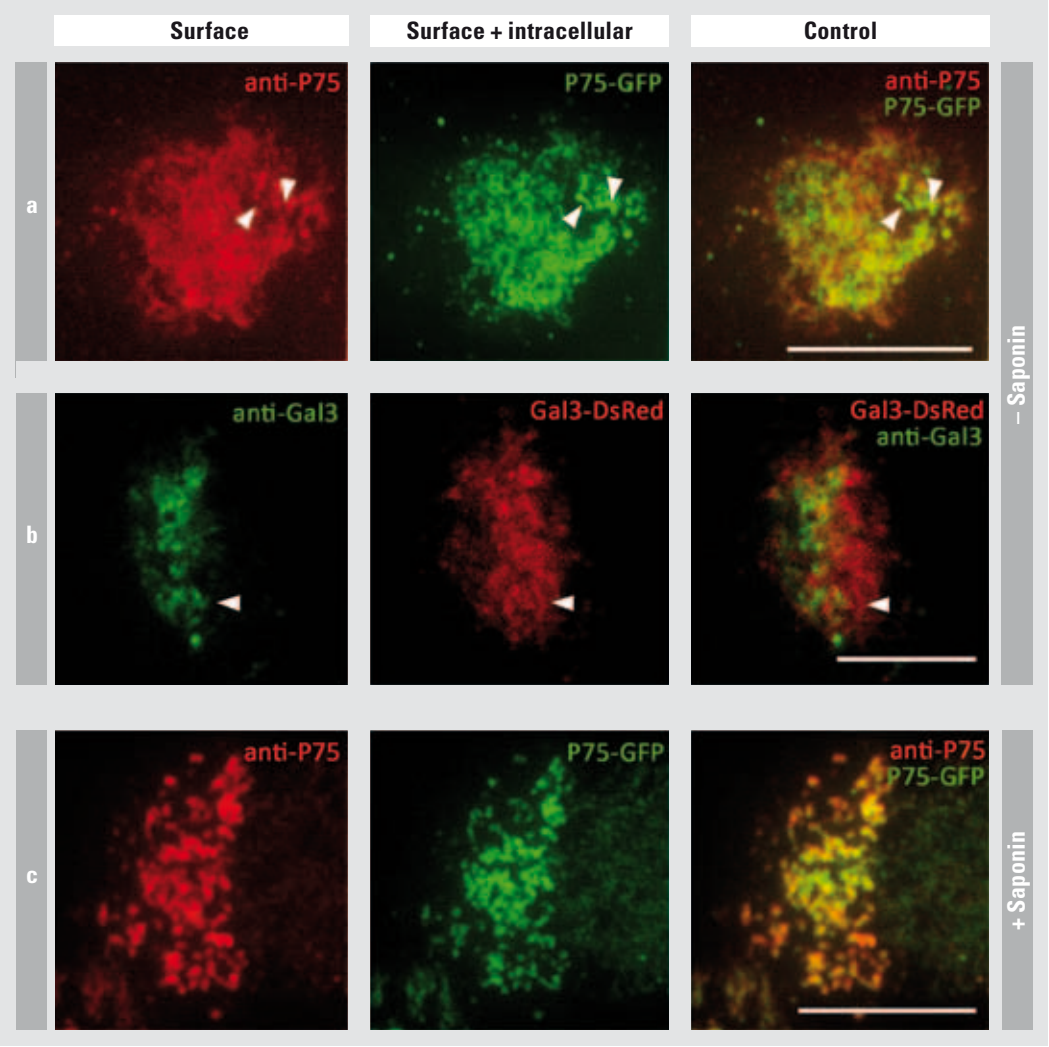

Fig. 3a–c: The TIRFM images show structures both on and just below the cytoplasmic membrane.
a: $MDCK_{p75\text{-}GFP}$ cells were cultivated on PET filters and subjected, without previous permeabilization, to immunofluorescence staining using an anti-p75 antibody. Thus only p75 structures on the cell surface were stained, not inside the cell (left). A parallel look at the p75-GFP signal shows that the TIRFM picks up structures on and inside the cytoplasmic membrane as well as in a small area below it. Here, tubular elements appear (arrow tips) that vary from the antibody signal on the surface.
b: A similar picture is obtained with $MDCK_{Gal3\text{-}DsRed}$ cells. Here too, intracellular Gal3-DsRed signals appear that were not picked up by the antibody on the surface (arrow tips).
c: In the control, $MDCK_{p75\text{-}GFP}$ cells were permeabilized with a detergent before immunofluorescence staining. Here, the two signals are identical. Scale bar: 10 µm

objects, which appear in epifluorescence mode, but are not detected by the evanescent field of the TIRFM.

TIRF visualizes apical intracellular structures

To find out whether the long structures on the apical side of the cells illustrated in Figure 2a are microvilli or intracellular structures, $MDCK_{p75\text{-}GFP}$ or $MDCK_{Gal3\text{-}DsRed}$ cell lines were subjected to immunofluorescence staining without permeabilizing the cells first. Antibodies therefore had no access to the cell interior, but were only able to stain structures on the cell surface. Here, it was evident that some objects were intracellular, probably endosomal compartments located immediately below the apical membrane of MDCK cells. These objects can only be visualized with fluorescing fusion proteins, but not with immunofluorescence. As a control, the cells were permeabilized with the detergent saponin before antibody treatment. In this case the immunofluorescence images were identical with those of the GFP or DsRed signal (Fig. 3c).

Outlook

TIRFM is a useful tool for viewing cellular processes in the plasma membrane or its immediate environment. It can be used for observing either fixed or living tissue, even in several fluorescence channels if necessary. These preconditions give reason to hope that apical membrane proximal processes or structures that could not be clearly detected with techniques available so far will soon be visualized with the TIRFM technique.

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Time Correlated Single Photon Counting

Choose Your Excitation Wavelength

Corentin Spriet[1], Aymeric Leray[1], Dave Trinel[1], Franck Riquet[2], Laurent Héliot[1]

Fluorescence Lifetime Imaging Microscopy (FLIM) is widely used to quantify protein-protein interaction by measuring the FRET (Förster Resonance Energy Transfer) occurring between two fluorophores spaced by a few nanometers. FLIM is also used in a large array of applications ranging from tissue imaging to fluorophore environment probing. Although time correlated single photon counting (TCSPC) is the method of choice for fluorescence lifetime quantification, it requires dedicated instrumentation including i) a pulsed laser source, ii) a photon counting card, and iii) a fast detector. Such technical requirements render TCSPC acquisitions difficult to perform and/or narrow down the choices of usable fluorophores.

The Leica TCS SP5 X overcomes these limitations since it combines a pulsed white light laser (WLL) with an ultrafast photo-multiplier (PMT), allowing tunable spectral detection. This system is highly versatile and user-friendly for FLIM experiments. In this paper, we illustrate its potential in two biological applications: interaction studies and autofluorescence multispectral imaging.

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2. EA4479 Laboratoire de Régulation des Signaux de Division, Science and Technology University of Lille, USR 3078 CNRS, Cell Signalomics Group, Parc de la Haute Borne, 59650 Villeneuve d'Ascq, France.

Technical description

Supercontinuum generation has attracted much attention since the first report of photonic crystal fibers in the late 1990s. It allows coherent white light production by pumping a highly non linear photonic crystal fiber with ultrashort laser pulses (picosecond or femtosecond). Due to its broad light spectrum, this source was commonly used for spectroscopic purposes and it was also recently implemented on a commercial microscope system: the Leica TCS SP5 X.

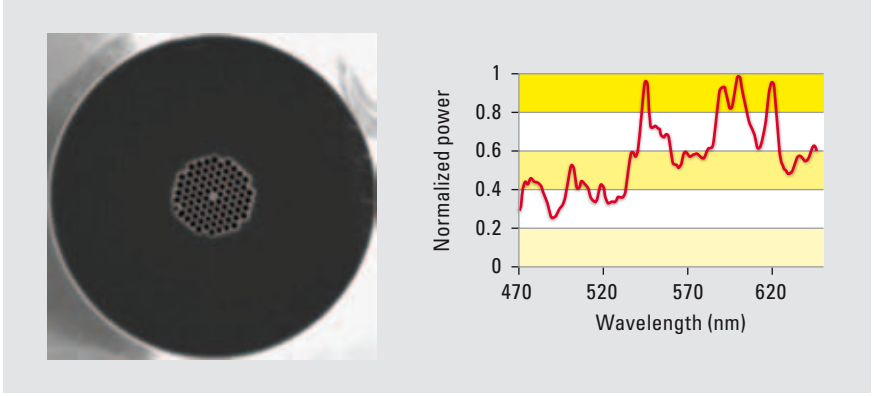

Fig. 1: Non linear photonic crystal fiber cross section (left) and white light spectrum between 470 and 670 nm measured on the Leica TCS SP5 X (A.U).

Equipped with an Acousto-Optical Beam Splitter (AOBS), this system provides a pulsed laser source easily tunable from 470 to 670 nm, allowing for the excitation of a large panel of fluorophores at their absorption maximum. In order to determine whether the WLL is a suitable source for FLIM experiments, we ran a calibration protocol based on the one validated for two-photon FLIM measurements[1]. Notably, we measured the system's instrumental response function and obtained a full width at half maximum around 300 ps, perfectly compatible with fluorescence lifetime measurements in cells and tissues. We then tested its capability on the two major fields of FLIM application: i) interaction studies by FRET and ii) tissue autofluorescence imaging. To address both situations, we applied the following experimental conditions:

- Lifetime acquisitions were performed using the Leica TCS SP5 X confocal and the internal PMT. Photon counting was performed using the Becker and Hickl SPC830 card and SPCImage was used for lifetime analysis.
- Two excitation sources were used for FRET measurements: the Cameleon ultra2 Ti:Sa laser at

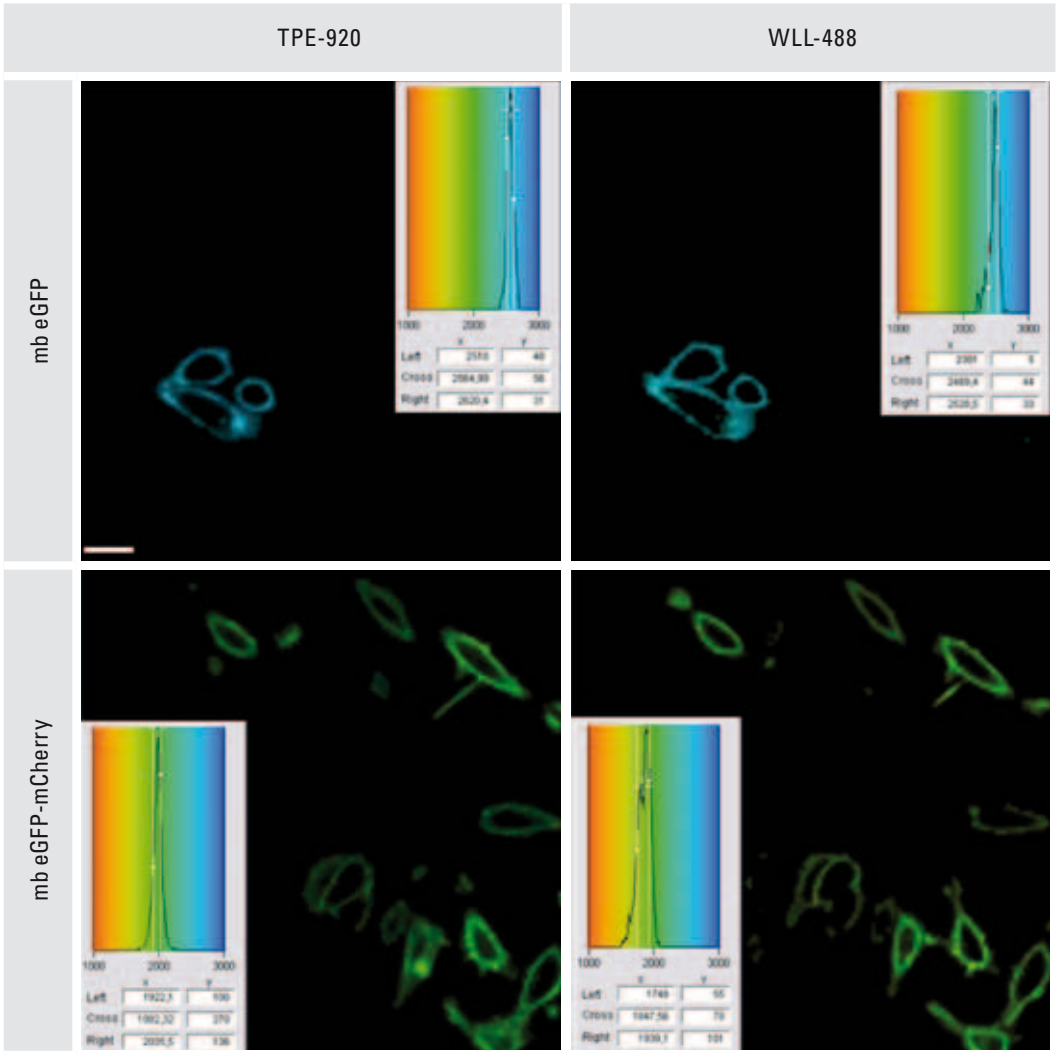

Fig. 2: Fluorescence lifetime imaging of both a negative (mb-eGFP) and positive (mb-eGFP-mCherry) FRET reference using either the WLL or the Ti:Sa laser. Acquisitions were performed using a 40x, NA 1.25 oil immersion objective. Scale bar: 40 µm.

920 nm (8 mW : objective back pupil) and the WLL at 488 nm. Detection bandpass was set between 500 and 540 nm and acquisition time to 300 s.

- For convalaria cross section imaging we also used two distinct excitation sources: the 405 pulsed laser diode and the WLL. The excitation and detection bandpass are reported in the third figure. The acquisition time was set to 60 s per image.

Versatility for FLIM-FRET experiments

The Leica TCS SP5 X presents both a pulsed WLL and an ultrafast detector with spectral selection. The system we developed is also equipped with a photon counting card (SPC830, Becker and Hickl) and a Ti:Sa laser (chameleon ultra II), a state-of-the-art source for TCSPC experiments. FLIM is particularly adapted for visualizing and measuring FRET occurring between interacting proteins at the nanometer scale in tissues or cells. Thus, in order to validate the WLL as a source for FLIM, we performed TCSPC experiments on positive and negative FRET references alternately using Ti:Sa and WLL as excitation laser source on the same cells. As shown in figure 2, both excitation sources give consistent results and allow quantitative FRET measurements. The WLL can then be used as a versatile source for FLIM-FRET experiments. Furthermore, in TCSPC mode, the lifetime accuracy is directly dependent on the number of acquired photons. Using this system, we can optimize the number of collected photons by i) optimizing the excitation wavelength to the fluorophore absorption maximum and ii) choosing the ideal detection range with maximum donor emission without acceptor fluorescence bleedthrough.

Multi-Spectral Lifetime Imaging Microscopy (MSLIM)

Combining AOBS and spectral selection using the internal PMT, the system allows high versatility regarding both excitation and emission wavelength selection. As previously shown, correlated spectral

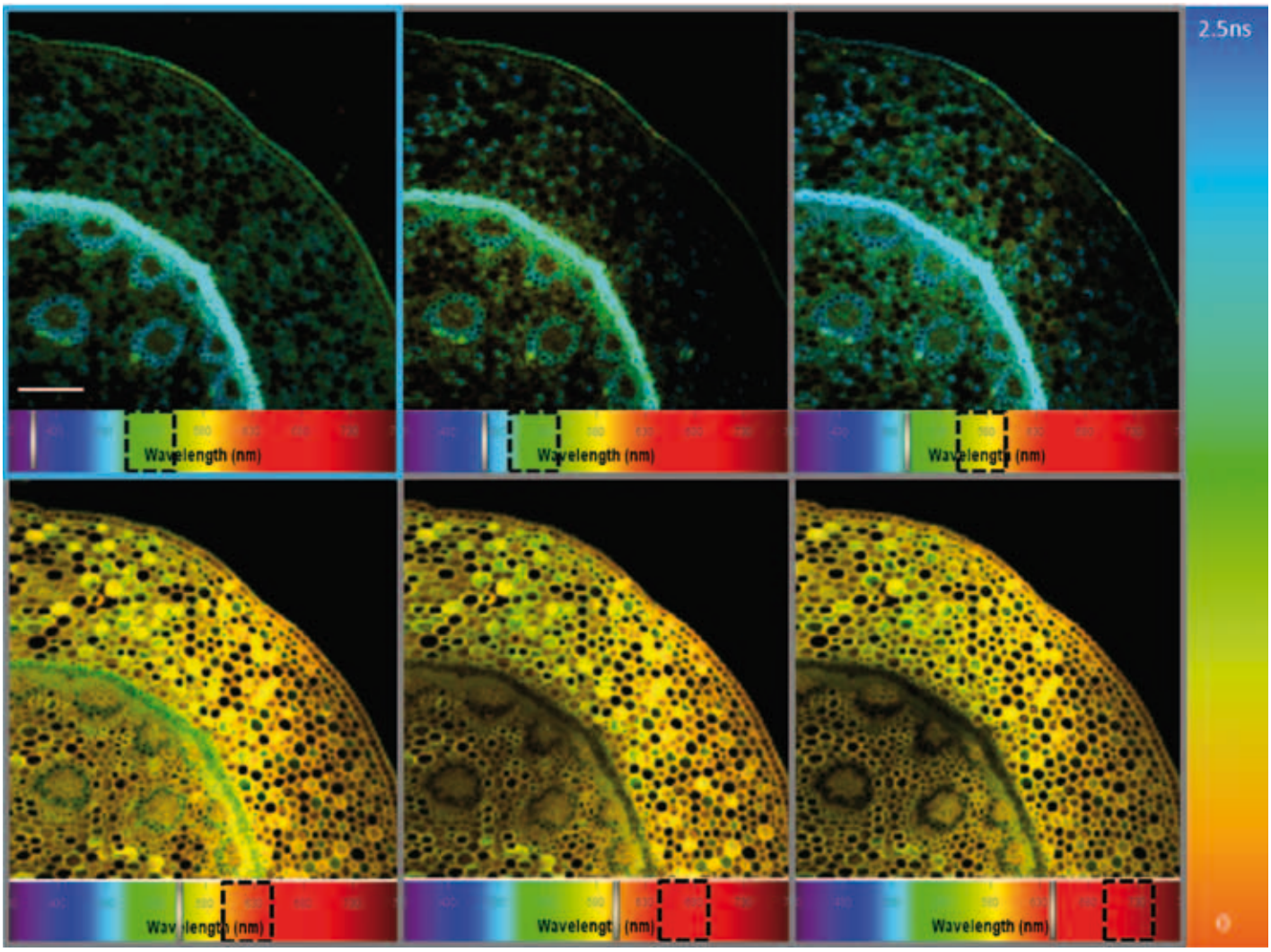

Fig. 3: Sequential fluorescence lifetime imaging of convalaria cross-section using various excitations (grey bar) and observation (dashed box) wavelength windows. Excitation is achieved using the WLL, except for 405 nm excitation, which is achieved by using a pulsed laser diode. Acquisitions were performed using a 10x, NA 0.3 dry objective. Scale bar: 500 µm.

and lifetime measurement (SLiM) is a promising and powerful technique for discriminating multi-labeled samples and for detecting molecular interactions inside heterogeneous and auto-fluorescent media such as tissues[2,3]. The use of a WLL further extends the possibilities for studying complex fluorescent signals. Indeed, one can now perform Multi-Spectral Lifetime Imaging Microscopy (MSLIM) by tuning both excitation wavelength (WLL through AOBS) and observation range (moving the slit in front of the internal PMT). Here, we present an example of autofluorescence MSLIM using a fixed convalaria slide.

Conclusion

Data reported here demonstrate the capability of the WLL for FRET imaging in living cells. Results obtained illustrate the usefulness of such a flexible and user-friendly source for molecular interaction studies and are perfectly consistent with those obtained using Ti:Sa. Furthermore, the combination of easily adjustable excitation and emission wavelength ranges achievable with the Leica TCS SP5 X can be used to dissect very complex fluorescence signals in a new acquisition technique combining spectral (excitation and emission) and lifetime imaging microscopy (MSLIM).

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Mapping Billions of Synapses with Microscopy and Mathematics

Where are Memories Stored?

Allison Paradise, Leica Microsystems

Information storage in the mammalian brain is a complex problem complicated by scale. It has long been understood that a few widely distributed synapses are responsible for encoding any one memory, but for decades visualizing and creating a memory map has been a nearly impossible task. That was before the laboratories of Drs. Gary Lynch and Christine Gall at the University of California, Irvine pioneered a technique that allows them to map learning-induced functional changes in individual synapses throughout the hippocampus. Their labs use a combination of widefield imaging techniques and image segmentation analysis to visualize these changes. Dr. Christopher Rex, Project Scientist, discusses the lab's findings, and their broader implications for the field of learning and memory.

Fig. 1: Dr. Christopher Rex, Project Scientist, Department of Anatomy & Neurobiology, University of California, Irvine

Dr. Rex, please describe your research interests.

Our lab studies learning and memory in the neocortex and hippocampus of rodents. We are interested in understanding how information is stored in the brain at the most fundamental levels. This has been a difficult question for the field to answer because not only is the information sparsely encoded (only a few synapses) but it is widely distributed as well (over many brain regions). The number of synapses required to encode any one memory is extremely small. For example, if there are 10^{15} synapses in your brain, there may only be tens to hundreds of disperse synapses that will encode any single piece of information. This is the worst needle-in-a-haystack situation you can imagine.

How are you addressing this question?

To address this problem our lab studies long-term potentiation (LTP). LTP is the physiological mechanism by which cells increase and maintain their synaptic strength within a short time period, in the order of seconds, but that can persist for days, months, or years. Our work began with slice recordings, but measuring physiological activity alone was not enough to distinguish one synapse from another. This prompted our lab to search for the biological correlates to the observed physiological activity. We identified important second messenger signaling cascades or phosphorylation events that occur at isolated synapses when we induce LTP. Specifically we focused on structural events such as cytoskeletal changes that occur at the processes of neurons. We identified and visualized enzymes that regulate these LTP-induced cytoskeletal-structural events. This finding is very important because it allows us to visualize the locations where information is encoded on the level of individual synapses.

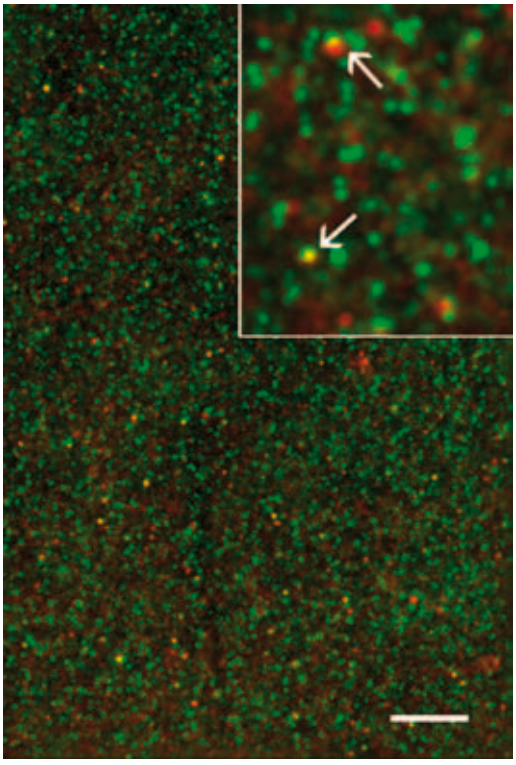

Fig. 2: A hippocampal brain slice was labeled with anti-PSD95 (green) and anti-phospho-p21-activated kinase (p-PAK; red). Low power image shows typical labeling in field CA1 stratum radiatum where quantitative analysis is performed. High magnification inset shows individually labeled puncta and the overlap of instances of PSD95- and p-PAK-positive profiles (indicated by the arrows). Scale bar: 10 µm for low magnification; 3 µm for high magnification.

What were the series of experiments that helped you arrive at the understanding you have now?

The lab began its work in electrophysiology, stimulating neocortical and hippocampal slices with an electrode to induce LTP. We and others showed that the structural changes that occur with LTP appear

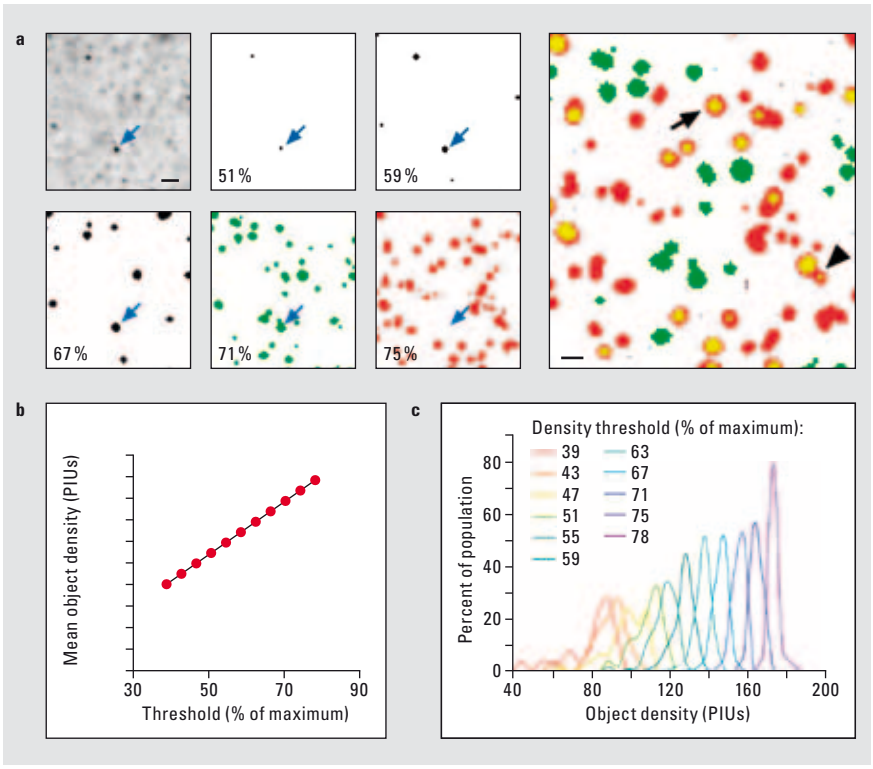

Fig. 3a–c: a: Photomicrograph (upper left) shows immunofluorescent labeling for PSD95 (intensity levels inverted so that signal shows dark). Other small panels show output from iterative automated image analysis for density thresholds at 51–75 % of maximum intensity. Blue arrow indicates detection of the same puncta across thresholds. Note, that puncta is not detected at 75 %. Larger panel at right shows overlay of analyses for 71 % (green) and 75 % (red) of maximum intensity (overlap shown in yellow). Note that many objects are detected by one threshold, but not the other. Arrow indicates an object detected by both thresholds but with differences between the detected object boundaries. Post-identification analysis of the same object across all thresholds was used to assess the appropriate boundary. Arrowhead indicates an object that is detected as two separate puncta at the 71 % threshold but as a single object at the 75% threshold. As described in Methods, objects detected across more than one threshold were counted. Scale bar: 2 µm for left panels, 0.5 µm for right panel.
b: Plot shows mean intensity values of identified elements from a representative image at incremental binarization thresholds.
c: Plot shows element raw intensity histograms for individual binarization thresholds.

to be permanent, but that the second messenger signals (phosphoprotein levels) are temporary. Immediately following stimulation, therefore, we fixed the tissue and labeled it with phosphoprotein-specific antibodies. In doing this we had a functional marker of LTP-like activity at individual synapses that had occurred within the last five minutes or so. We also counterstained the slices with an antibody that is specific for PSD95, a protein that is found only at the postsynaptic density of excitatory synapses. This allowed us to identify the locations of the synapses themselves. In this study we found that we had a very nice correlation between the number of synapses that had high levels of phophoproteins and LTP.

From there we bridged into the learning studies. Although we had found a very good marker for LTP with artificial stimulation we wanted to see if and where these same events occurred in the brains of animals under naturalistic learning circumstances.

To address this, our lab developed a learning paradigm called unsupervised learning. It is different from conditioned learning or associative learning in that the animals don't have explicit cues given by the experimenters; the animals are allowed to roam a complex environment for 30 minutes. Then we rapidly freeze the brain and prepare sections. Initially we targeted the hippocampus because we knew that it was involved in this form of learning, but we focused only on very small regions of the hippocampus because the task of mapping the information was so labor-intensive. We found that the animals that went through the learning environment had a greater number of synapses with dense levels of phosphoprotein indicating LTP-like activity at synapses within the hippocampus.

Since then we have developed something extremely exciting, in connection with the bigger question we had, namely where exactly is this information being stored? We have expanded the learning study to take contiguous regions across an entire section of hippocampus. For this study, we worked closely with Leica Microsystems to develop automated microscopy so that we could scan across an entire section at high resolution.

We combined this with a complex analytical system that we have developed in-house. To give you an idea of how many synapses we are examining, for the very first LTP and learning studies that we published we measured a few thousand synapses. For the subsequent papers we measured up to one million synapses. And now for one section of hippocampus we are measuring about 200 million synapses, and for a whole hippocampus we are measuring well over a billion. That's for one animal. So for one study hundreds of billions of synapses are being measured and mapped, which is really remarkable.

What changed in the technology that enabled you to expand your research to this degree?

We had been using an upright Leica DM6000 B epifluorescence microscope, acquiring dual channel, three micron-thick z-sections in order to reconstruct 3D immunofluorescent images. The challenge for us was to maintain our optical sections at varying depths in the tissue section to match the optimal plane of labeling. To do this we needed an autofocus that could focus on the micron-sized, individual spheres that are our labeled synapses. For this we found Metamorph's algorithms to be perfectly suited. Additionally this software provided the flexibility to go from adjacent locations and find the optimally labeled plane. We tried a number of programs, and we even tried to write our own software for this before we found Metamorph.

Now we are collecting images around the clock 24 hours a day in order to collect enough data to generate large scale maps. We found that using the internal filter turret slowed us down and caused too much wear and tear on the microscope, so we

invested in Leica Microsystems' fast external filter wheels. We now have assembled a system that has very low mechanical wear and tear and can run robustly and reliably for days on end.

Using this new system we developed a workflow where Metamorph performs the acquisition and as soon as the images are acquired we use custom-written software to send it to our cloud analytical system, which begins the analysis immediately. The images are deconvolved and then they are analyzed by image segmentation, which is all in-house custom designed software that we've built over the past three or four years.

In short we've gone from a study that originally took about six months to image and analyze to one that we can do in about a week. The increased speed makes it possible for us to map large brain regions.

How are the data analyzed?

In our image segmentation analysis we're looking for objects that are approximately 200 nm in diameter. We perform the image segmentation separately on each channel. We then identify colocalization, better termed coplacement, by comparing the boundaries that are identified for each object. We have found that the numbers of excitatory synapses containing dense phosphoprotein labeling is in the order or 2–5 %; it is a very sparse signal as we would expect from a high-capacity memory system.

Why not use confocal microscopy for this work?

We used confocal microscopy in our first publication, but we have since elected to use widefield microscopy coupled with deconvolution because the acquisition is much faster. We know that the resolution we are able to obtain is not as good as what we could get with a laser scanning system, but we are willing to accept that trade-off in order to have the speed and efficiency that our current system permits. We have also found that the elements we are looking at, dimly labeled elements that photobleach quickly, are not identifiable with confocal microscopy. Deconvolution, on the other hand, preserves the light making it easier to identify the synapses.

What does the future hold?

There are a number of things on the horizon for this project. We will be introducing multiple learning paradigms and expanding the number of brain regions we analyze. We are also always trying to increase the efficiency of the system.

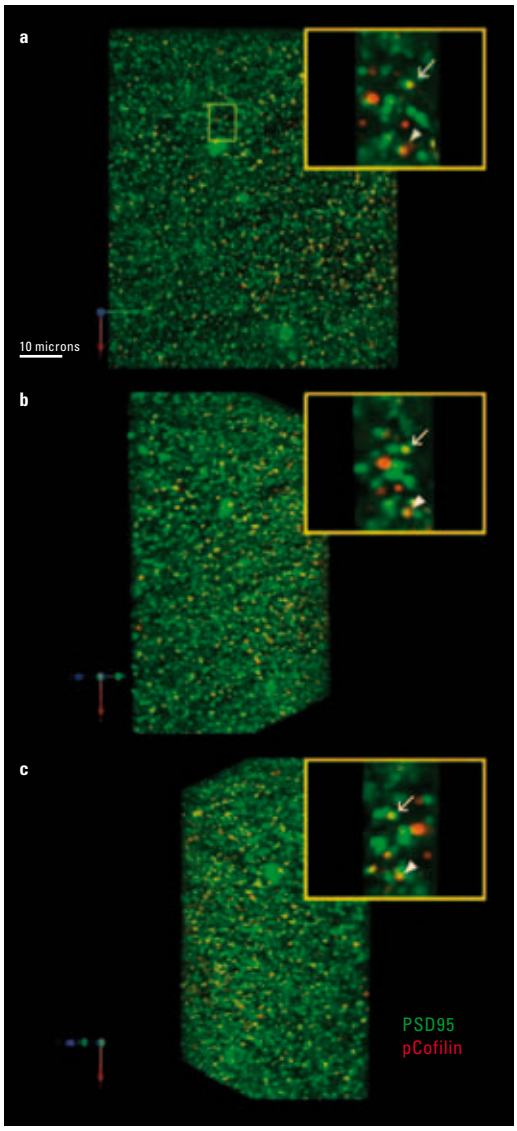

Fig. 4a–c: Deconvolution photomicrograph of a field of PSD95- (green) and pCofilin (red)-immunoreactive elements. Image Z-stack was reconstructed in 3D and views show 45 (panel b) and 135 (panel c) degree rotations. Inserts show high magnification view of zone outlined in a. Arrow and arrowhead indicate co-localized elements.

In conclusion …

For a long time there's been the belief, supported by evidence from lesion studies, that different brain regions are responsible for different forms of learning. But we did not know whether the information associated with that learning is stored in those brain regions, or whether those regions perform a function that is necessary either for the system or for that particular form of learning. Our lab will continue to strive to develop an empirically based understanding of how and where information is processed in the brain in order to address these questions.

Dr. Rex, thank you for the interview.

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Perspectives of CARS Microscopy in Life Science Research

Good Vibrations

Dr. Bernd Sägmüller, Leica Microsystems

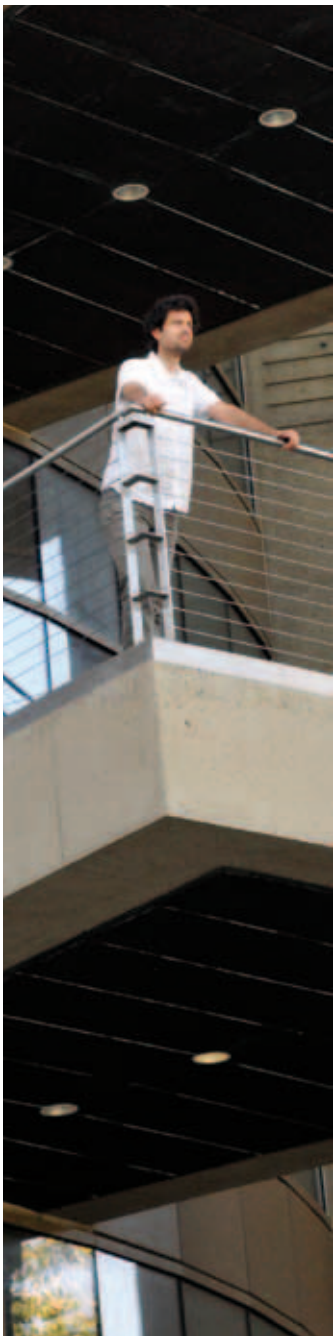

Fig. 1: Eric Potma, Ph.D., assistant professor at the University of California, Irvine, USA

The ability to see the cell's molecular machinery at work has contributed immensely to our understanding of cellular functioning. In recent years, new molecular imaging techniques, such as coherent anti-Stokes Raman scattering microscopy (CARS), have been developed for rapid vibrational imaging of living cells. The intrinsic molecular vibrations leave specific fingerprints in the vibrational spectrum. Eric Potma, Ph.D., assistant professor at the University of California, Irvine, USA and his team advance and apply this novel imaging technique for unveiling the molecular secrets of microscopic biological systems.

Professor Potma, how did you come across CARS microscopy?

When I was a graduate student in the Netherlands I did ultrafast spectroscopy. And my adviser suggested I apply those techniques to microscopy. This was a hot trend in the late '90s, when the two-photon microscopy technique was becoming increasingly popular. We also saw a bit of second harmonic microscopy in those days, but not a lot. I was playing with a laser system that had two colors, and thought about doing a pump-probe type of microscopy.

In 1999, I saw Andreas Zumbusch of the University of Konstanz, Germany (then a postdoc in the laboratory of X. Sunney Xie) and Michiel Müller of the University of Amsterdam, the Netherlands, showing their work on CARS microscopy at the FOM conference. I was very impressed with the capabilities and the contrast of CARS microscopy. I immediately went back to my laboratory in the Netherlands and lined up the instrument to do CARS. The next day we got the first CARS signal. I have been doing CARS microscopy ever since.

What was the first sample you used and how did you get involved in biology?

The first sample we looked at was Dictyostelium discoideum, an amoeba cell. We used these cells as model systems to learn about water distribution in cells. We focused on the water band OH-stretching and did dynamic measurements on flushing water through the cells to examine water diffusion in living cells. I was in a physical chemistry department and we focused on biophysical applications in those days. We collaborated with biologists and cell biologists and those people brought us the samples. Ever since it has been a very natural merger of the two fields.

It was great to work with biologists since they brought the real questions to the table, which made us tweak our instruments in such a way that we could see things that actually matter. There is a great collaborative spirit between biological researchers and those more involved in physics.

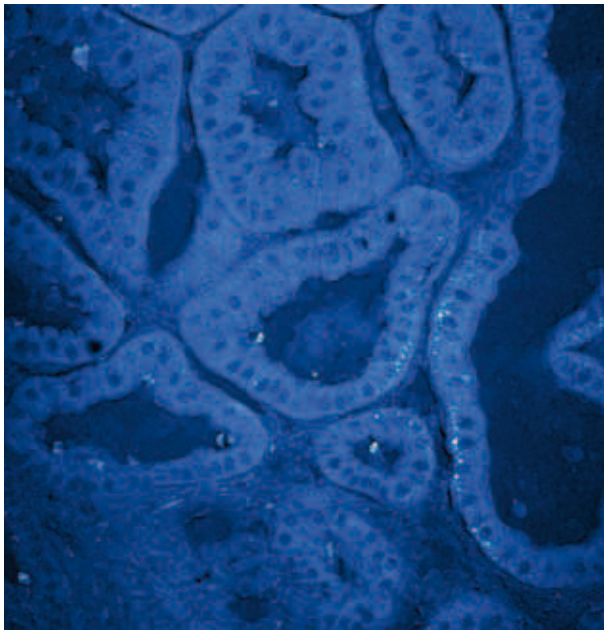

Fig. 2: Kidney tubules: CARS image at 2845 cm^{-1} of the mouse kidney, showing the tubules and intracellular lipid droplets.

Is the emphasis shifting back to physics?

CARS became popular on the biology side, but you can probe anything else with this particular type of contrast. You can probe vibrational and electronic features as well. We are looking at carbon nanotubes, and trying to understand the propagation and coherent evolution of primary excitations. We also study the nonlinear optical properties of plasmonic structures on a microscopic level. CARS is a great way to perform these applications. So, it is finding its way into material science as well.

How can CARS microscopy give us insight into fundamental cellular processes?

The real benefit of CARS microscopy is that you look at molecules just the way they are. There is no need to put labels on them, no need to dress them up in a certain way to make them fluorescent. That's really where the technique is advantageous. You can look at all molecules that have a good Raman signature and so there are a couple of biomolecular candidates that can be easily visualized using the CARS microscope.

Any question dealing with lipid metabolism is where CARS can make a difference, as well as any question dealing with the mobility of water molecules, membrane dynamics, and variations in protein density distributions. And CARS holds great promise for following extrinsic agents like drug molecules or any molecular compound with a strong vibrational signature in tissue. This is great, since these molecules are typically hard to visualize otherwise, as they cannot always be labeled. Usually they are too small – if you label them you don't get them into the cell or you change their functionalities.

With CARS we image such targets at a rate that is much faster than conventional vibrational imaging. We are talking about imaging in real-time, which is important for imaging all things biological, like living cells and tissues in vivo – these are the situations where CARS microscopy really helps.

Where would you focus with CARS in life science research?

There is a very important research direction that aims at visualizing endogenous molecules in living animals. The CARS microscope is great because of its speed, so you can monitor molecules in real-time. For instance, people have used CARS to look at myelin degradation – which is a way to investigate diseases like multiple sclerosis. There is no other way you could do this; visualizing myelin in real time in a living animal is really difficult. CARS is the only avenue for people to do this.

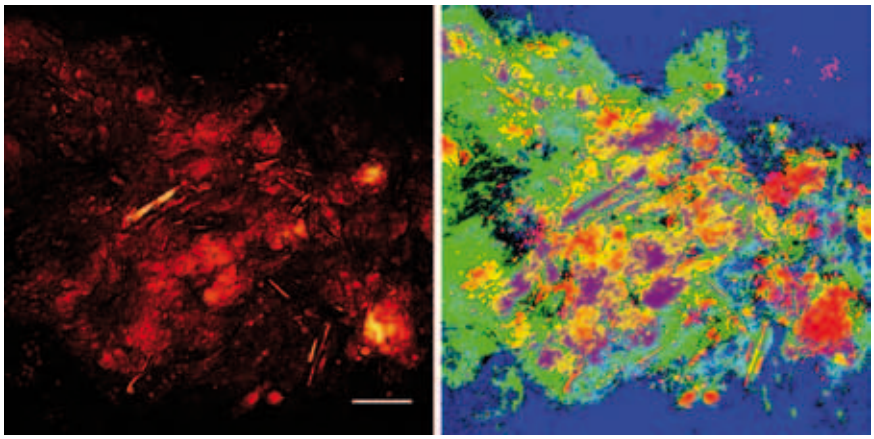

Fig. 3: AortaPCA: This is a CARS image of an atherosclerotic lesion in a mouse aorta. The left panel shows a regular CARS image of the lesion at 2845 cm^{-1}, highlighting the lipophilic components. The right panel shows a spectral decomposition (principal component analysis) of the CARS signal in the 2750–3050 cm^{-1} range, showing that different areas in the image correspond to different lipophilic compounds. Each color in the image corresponds to a different CARS spectrum. Scale bar is 50 microns.

Another example is the use of CARS for skin imaging: attempts are under way to do this with a system that is currently optimized such that you can put your arm under a microscope and look at tissue morphology and abnormalities. This has direct implications for improving human health.

What does a commercial CARS system need to have to be successful in the market?

Such a system needs to be easy to operate. People want to focus on the applications, they want to start an experiment first thing in the morning. They don't want to fiddle with lasers and microscope settings. Ease of use is very important. This includes biological applications, people with medical research pro-

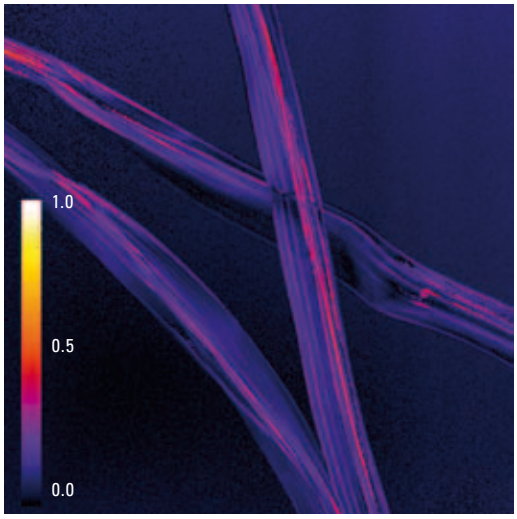

Fig. 4: Rayon anisotropy: CARS anisotropy image of rayon fibers. The anisotropy was determined at the 2880 cm^{-1} methylene stretching mode of rayon. The brightness in the image corresponds to the degree of orientation of the methylene mode in the fiber.

jects who want to look at their samples. The system must be very user friendly to tap into a huge number of user groups.

You need to be able to automatically tune to several laser wavelengths, and also address wider portions of the spectrum for deeper chemical analysis …

… which you are currently taking to its next stage, such as hyperspectral imaging with multivariate analysis?

You can combine that naturally. Once you have a hyperspectral data stack you can improve the data extraction using multivariate analysis – so I believe it's a powerful resource. Anything that can automate data extraction will attract a larger pool of users. Currently, it's confined to academic users with a lot of experience; optical and engineering expertise is essential to operate these systems.

What is your vision of CARS microscopy for the next 20 years?

I think we can anticipate that with CARS the same thing will happen as with confocal imaging. CARS is the latest edition of contrast methods. You want to inspect your samples, look at molecules, have depth-resolved imaging and do it with the least amount of perturbation to the sample – no harsh treatment or staining protocols – this is especially true for living systems.

So, having a system that adds this type of contrast to the microscope is an enormous step forward. It clearly widens the current landscape of scientific investigation. You can see more than was previously possible. It enables you to do more and to work on key applications. Just one breakthrough application is a tremendous success in itself.

For instance, people would have never foreseen the impact of being able to directly visualize lipid metabolism, an essential process in our bodies that was previously difficult to study without the use of perturbing labels.

By creating such new research avenues, CARS is a true asset to the label-free imaging approach. Other applications are waiting to be discovered. It is inevitable that CARS will continue to have a major impact in the biological and material sciences. It's the way forward.

Professor Potma, thank you for the interview.

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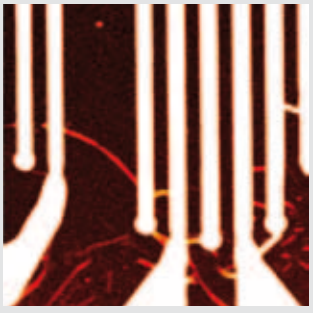

Fig. 5: Nanotubes: Electronic CARS signal from carbon nanotubes between Ti electrodes. The wider white lines correspond to the electrodes.

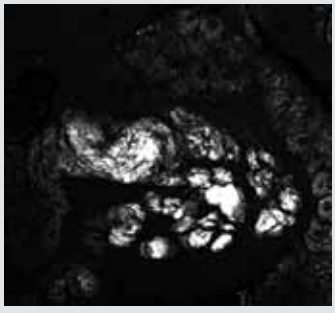

Fig. 6: Meibomian: Transversal cross section through a mouse meibomian gland. CARS signal was measured at 2845 cm^{-1} to visualize the lipids in the gland. The center portion of the image corresponds to the duct of the gland, whereas the peripheral regions show the lipid rich meibocytes.

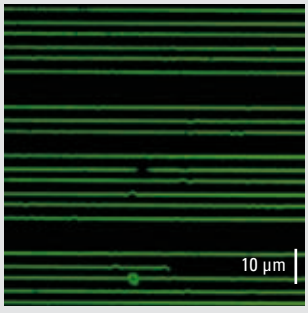

Fig. 7: Nanowires: Electronic CARS image of semiconducting CdSe nanowires using ps laser pulses. CdSe wires are 330 nm wide, 60 nm high and were lithographically fabricated on a glass coverslip.

Ten Years of Laser Microdissection by Leica Microsystems

Dissection Perfection!

Kerstin Pingel, Leica Microsystems

Laser microdissection is a relatively new technique, having first been developed in the 1990s. It makes it possible to obtain homogenous, ultrapure samples from heterogenous starting material. A researcher can selectively and routinely analyze regions of interest down to single cells to obtain results that are reproducible, and specific. Laser microdissection uses a microscope to visualize individual cells or cell clusters. Regions of interest are selected by software, excised from the surrounding tissue by a laser, and collected by gravity into specialized devices for analysis. There has been enormous progress in the development of laser microdissection instrumentation in recent years and Leica Microsystems is one of the major players.

Leica Microsystems was the first company to use an upright microscope for laser microdissection. The advantage is that material cut out for analysis, regardless of its size or shape, falls due to gravity and lands in a collection container – without additional steps and without contamination.

One of the technological advances that only Leica LMD systems feature is the adjustable laser that cuts even nonstandard specimens like wood, bones, teeth or tissue cultures. Dedicated objectives for the Leica LMD systems guarantee the highest possible laser power. As the laser beam movement is controlled by high precision optics, fast and precise cuts can be made. The microscope stage and the sample both remain fixed. Only Leica LMD systems enable specimens to be cut during fluorescence, because there are separate beam paths for the laser and fluorescence.

Fig. 1: The Leica LMD7000 laser microdissection system combines high laser power and high repetition rates.

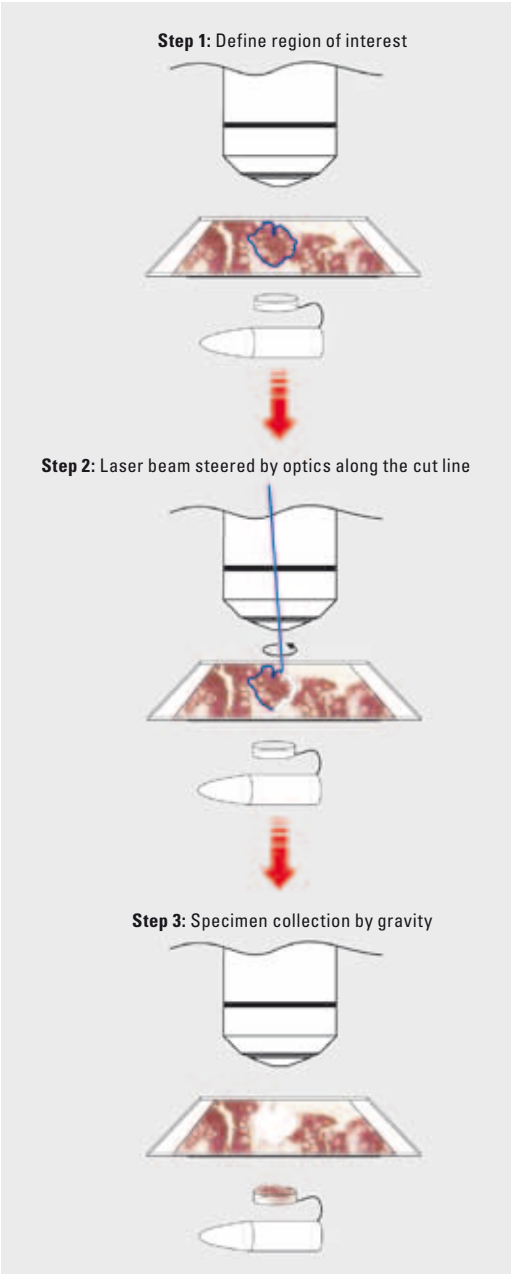

Fig. 2: The process of microdissection

In the year 2000 the first laser microdissection system from Leica Microsystems, the Leica AS LMD, was launched. With the Leica LMD7000, the third generation system and the most successful of Leica

Microsystems' widefield imaging products is now on the market, complementing the product portfolio in addition to Leica LMD6500. Laser microdissection plays a major role in a wide variety of applications. Here, Leica customers report on the research results they have attained by using laser microdissection.

Improving pharmacogenetic analyses of tumor cells

Dr. Niccola Funel from the Department of Surgery, Unit of Experimental Surgical Pathology, University of Pisa, studies the Pancreatic Ductal Adenocarcinoma (PDAC). A key focus of his research is identifying new techniques to tailor adequate treatments.

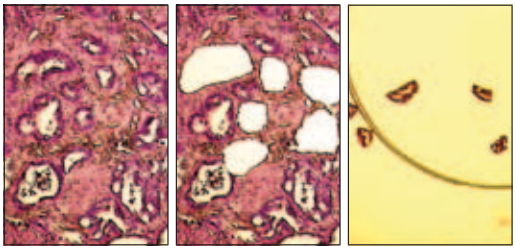

Fig. 3: Frozen section of Pancreatic ductaladenocarcinoma (PDAC) stained with hematoxylin and Eosin, objective 10x (before cutting, after cutting and inspection)

"PDAC is a very aggressive pancreatic tumor, and overall survival of PDAC patients is very poor. The PDAC tissue exhibits an intense desmoplastic reaction which can mask the true tumor expression. However, the target of drugs is referred to the epithelial cells and not the stromal cells (desmoplastic reaction). We have demonstrated that laser microdissection improves pharmacogenetic analyses on PDAC, as it helps the pathologist to harvest tumor cells only and thus establish their real genetic expression. We compared LMD tissues versus non LMD tissues and found a significant difference in terms of mRNA and miRNA expression. Finally, working with laser microdissection, we found a progressive upgrade of methodological approach, increasing the efficiency of molecular tests."

The Morbus Parkinson puzzle

Dr. Falk Schlaudraff researched Morbus Parkinson at the molecular level as a doctoral candidate under the supervision of Prof. Dr. Birgit Liss in the Molecular Neurophysiology team at the Institute of Applied Physiology, University of Ulm.

"There are several theories on how the disease originates. M. Parkinson can be compared to a puzzle.

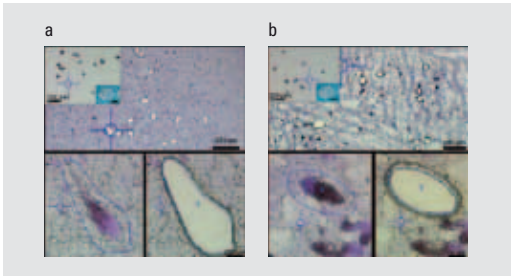

Fig. 4: Laser microdissection and mRNA-expression analysis of individual substantia nigra (SN) neurons from human PD and control postmortem brains. Pools of neuromelanin-positive [NM (+)] neurons were isolated via LMD of cresylviolet-stained horizontal midbrain cryosections from PD (a) and control brains (b) (published in: Gründemann et al., Nucleic Acids Research, 2008, 1–16 doi:10.1093/nar/gkn084, © Oxford University Press).

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 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: 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 were able to confirm the results for the whole tissue. However, we also examined microdissected cells in parallel. Here we found that the investigated microRNA expression is not changed on a single cell level. This tissue artifact was detected with the aid of laser microdissection."

Molecular network in Arabidopsis

The research of Prof. Lucia Colombo and Raffaella Battaglia, Ph.D. from the Department of Biology at the University of Milan aims at understanding the molecular mechanisms that control plant reproduction. The lab uses *Arabidopsis thaliana* as model species. Molecular information obtained from this small model species has often been fundamental to highlighting molecular networks in agronomically important species like rice.

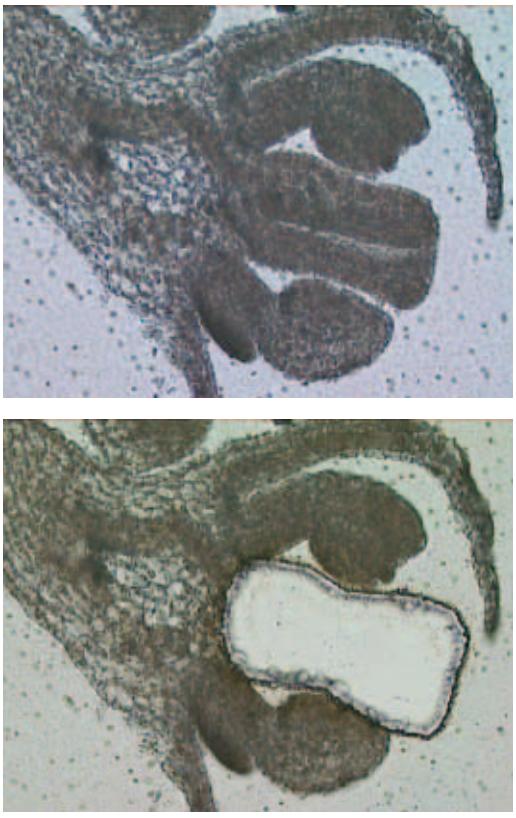

Fig. 5: Laser microdissection helps to identify and functionally characterize genes which play a role during female organ formation and fertilization in *Arabidopsis thaliana*.

"We want to identify and functionally characterize those genes which play a role during female organ formation and fertilization in *Arabidopsis thaliana*. Our lab has strongly contributed to the comprehension of the molecular mechanisms at the base of ovule differentiation. We participated in the identification and functional characterization of those genes, mainly encoding for transcription factors, which give important signals to start the ovule differentiation process.

MADS-box transcription factors represent an important group of key regulators which act as molecular switches to activate molecular cascades leading to mature organ formation. Despite the fact that the importance of MADS-box proteins for such functions as organ identity determination and flowering time control is well established, few direct target genes of MADS-box transcription factors have been identified in plants until now. Using laser microdissection, we have recently identified the first gene regulated by MADS-box transcription factors involved in ovule differentiation.

We know which genes are switched off as a consequence of the lack of activity of MADS-box factors. We have identified and functionally described the *VERDANDI (VDD)* gene as a direct target of ovule identity MADS-box factors but also playing a fundamental role in female gametophyte differentiation. The possibility to combine single cell analysis with genome-wide approaches is giving a powerful impulse to developmental biology."

The mitochondrial hypothesis of aging

Why do we grow old? Scientists have been looking for an answer to this question for many years – particularly against the background of the increase in neurodegenerative diseases among older people such as Morbus Parkinson. PD Dr. med. Andreas Bender from the Neurological Hospital of the University of Munich-Großhadern is researching the mitochondrial hypothesis of aging, which regards a vicious molecular circle of oxidative damage and mtDNA mutations as being a cause of neuronal functional disorders.

"Our aim is to investigate at single-cell level why some cell types are more susceptible to neurodegenerative diseases like Morbus Parkinson than others. After extracting these cells from human post mortem brain tissue by laser microdissection we subject them to molecular-biological examination. In particular, we are looking for damage to mitochondrial DNA (mtDNA).

Mitochondria have not been thoroughly researched with single cell analysis so far, although evidence is growing that they could play a major role in neurodegenerative diseases and in the aging process.

What surprised us was that there were a lot of mtDNA mutations in 60- to 70-year-old control patients as well – although distinctly fewer than in the Parkinson group. In a second step we therefore examined control samples of people of all ages, from a few months to a hundred years old. We found that the mtDNA deletions increase with age. We are born with no or extremely few deletions and at some stage in our life the critical threshold of 50 to 60 % of mutations is reached.

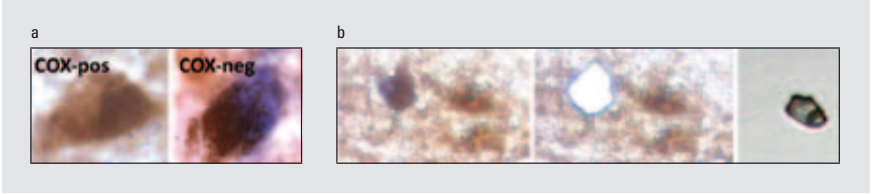

Fig. 6a–b: Cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) staining of dopaminergic substantia nigra neurons (a). Normal neurons are COX-positive (brown). Neurons with high levels of mtDNA deletions are COX-negative (blue). b: A COX-negative neuron is cut out with laser capture microdissection (middle) and identified in the lid of a PCR tube (right).

Laser microdissection plays an extremely important role, as the precise dissection of individual cells ensures that we really examine the cells that interest us. Before the age of laser microdissection, most examinations were done on homogenates. These contained different types of cells, so that the molecular connections we were researching sometimes disappeared in a great background noise. If a disease only affects one particular cell type, as in the case of Parkinson's, we only obtain meaningful results when we are able to analyze homogeneous cell material."

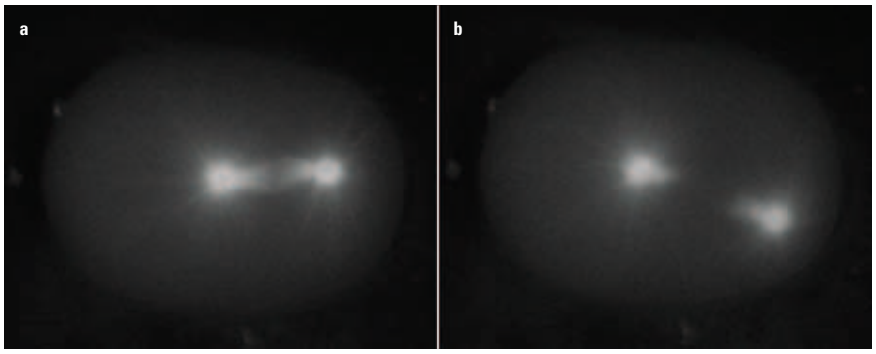

Fig. 7a–b: Mitotic spindle ablation in a wild type one-cell embryo. A *C. elegans* one-cell embryo expressing α-tubulin fused to GFP. The mitotic spindle just before (a) and after laser ablation (b). The two spindle poles separate fast after the ablation. (Grill, S. et al, 2001, Nature)

Understanding asymmetric stem cell division

Prof. Monica Gotta from the Department of Genetic Medicine and Development at the University of Geneva uses the LMD for the study of asymmetric cell division, which allows the generation of cell diversity during development and stem cell renewal.

"Establishment of cell polarity and positioning of the mitotic spindle are essential prerequisites for asymmetric cell division. While many players in these processes have been identified, the big challenge is now to understand how these regulate each other and how they regulate polarity and spindle positioning. My lab studies these processes using the *C. elegans* embryo as a model system. This is a great system for studying asymmetric cell division as the one cell embryo is polarized and the first division is asymmetric. Furthermore we can combine genetics, live cell imaging, biochemistry and genomics/proteomics.

One limitation of the embryo is the egg shell, which does not allow the use of inhibitors. To overcome this problem we use the LMD. It allows us to permeabilize the tough and impermeable eggshell by producing small holes that allow penetration of drugs dissolved in the embryo mounting medium and therefore inhibition of a pathway or protein of interest with high spatial and temporal resolution.

We also use the LMD for the mechanical destruction of other structures such as centrosomes and microtubules, which allows measurements of movement and velocities in wild type and mutant backgrounds. The system has also been used to ablate specific cells during development to study cell function and/or lineage."

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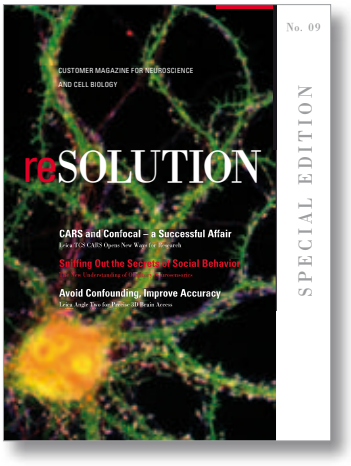

A Personal Perspective

Research Between Failure and Success

When Thomas Edison was asked why so many of his experiments failed he explained that they were not failures: with each failed experiment Edison discovered a method that did not work. Successful researchers have some things in common: passion, tenacity and the acceptance of failure as a part of success. In our new column, renowned scientists and Leica users talk about how they deal with setbacks.

Since 2008, **Stephan Sigrist** has been a Professor of Molecular Development and Biological Genetics in Animals at the Freie Universität Berlin. Sigrist's area of focus revolves around the question of what happens in the brain when learning takes place: Under the microscope, he studies the organization of synapses. Sigrist has been one of the first users of a Leica TCS STED.

Prof. Dr. Beth N. Orcutt is a postdoctoral researcher at the Center for Geomicrobiology at Aarhus University in Denmark. Her area of research is studying microbes at the bottom of the ocean. She works with a Leica laser microdissection system.

Could you briefly describe the last time you found yourself at a dead end, and how you felt?

Orcutt: As an environmental microbiologist working with natural samples from the bottom of the ocean, it is a regular experience to face obstacles in analyzing my samples. Most instruments on the market are primarily designed for working with biomedical samples, or maybe with water samples – how do I adapt these technologies to examine the life of tiny microbes living in sediment and rocks?

Sigrist: Dead ends, of course, are common. The real secret to being a good researcher is the ability to recognize what will work and what will not. The key is the ability to react flexibly to unscalable hurdles and to find alternative paths that lead to the same goal.

How do you overcome such difficulties? What do you do to get things moving again?

Orcutt: The ubiquity of these technological limitations is seemingly never-ending, but I try to stay positive. Developing new techniques is a rewarding endeavor, and awakes the hidden engineer and problem solver inside.

Sigrist: It is most important to keep the motivation of younger colleagues strong. They have to remember that they sometimes have to wait for successes. After nearly two decades of experience, I no longer need to be reminded of this. In addition, I work on many projects simultaneously, and there is always at least one that brings success.

What do you hope to achieve in your field of research?

Orcutt: The aim of my research is to understand how life exists in one of the most remote and energy-starved places on earth: below the ocean in sediment and oceanic crust. I want to know how microbes survive and thrive down there. Working with international teams of researchers, the next few decades will surely see numerous advances in our collective understanding of microbial life in the dark ocean.

Sigrist: Our big goal is to explain and replicate the organization of synapses. Important central mechanisms, however, are still generally unknown. Certain proteins obviously play a large role, and we know, for example, that mutations can cause autistic disorders.

Is it possible (or necessary) to learn how to deal with setbacks? What would be your advice for young scientists?

Orcutt: I do believe it is possible to learn about dealing with setbacks. I would advise young scientists to ask professors, postdocs, and supervisors for real-world examples from their own experiences. I would also recommend reading "self-help" books from the world of business. They offer a broad perspective on how to set goals, develop strategies to achieve goals, and evaluate successes and failures.

Sigrist: Most important is that you enjoy your job. If success is not the only focal point, but instead you can immerse yourself deeply into your research, the work itself can offer fulfilling gratification.

Leica SR GSD, Leica TCS STED and Leica TCS STED CW

The Full Spectrum of Super-Resolution

Dr. Tanjef Szellas, Dr. Christian May and Jo Fallowfield, Leica Microsystems

It is less than 20 years since super-resolution arrived on the light microscopy scene, but it already plays an important role, particularly in life sciences[1]. Applications are wide ranging – from dynamic vesicle movements in the sub-100 nm range to fluorescence images of sub-cellular structures, allowing researchers to see details only previously possible with electron microscopy. Leica Microsystems offers super-resolution systems that utilize different technologies for different applications. The Leica TCS STED and the Leica TCS STED CW are based on STimulated Emission Depletion (STED), whilst the new Leica SR GSD harnesses the principle of Ground State Depletion and Individual Molecule Return (GSDIM).

In light microscopy, the term super-resolution refers to methods that surpass the so-called diffraction limit. This limit, defined by Abbe's law, means that particles of the same type can no longer be optically separated – or resolved – if they are closer to each other than 200–250 nm. Their images simply overlap too much and thus prevent their identification as individual objects.

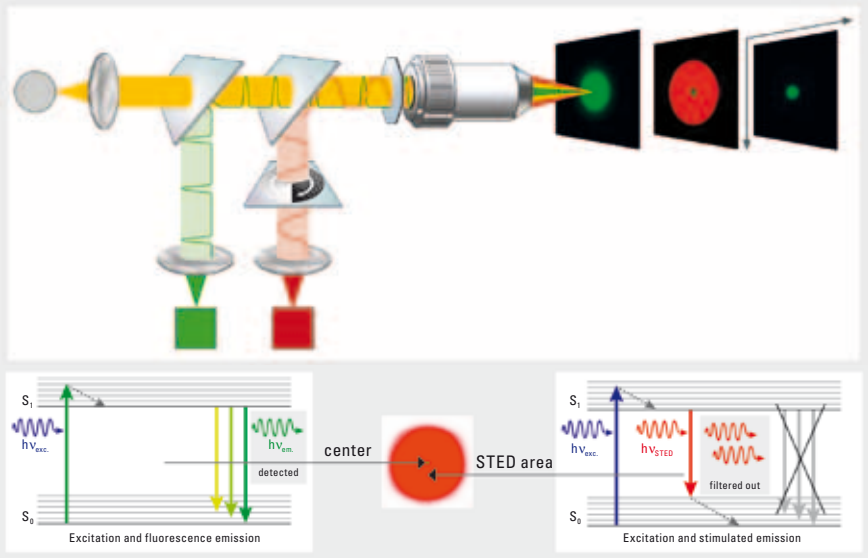

Fig. 1: Resolution enhancement in STED microscopy requires two different lasers, one for fluorophore excitation and one red shifted laser to annihilate excitation by stimulated emission. This applies to pulsed STED but also to STED with continuous wave lasers. Both laser beams are focused through the objective onto the sample and perfectly aligned by scanning mirrors (beam scanning). The intensity distribution of the STED beam features a ring shape with zero intensity in the center. Thus, no excitation annihilation occurs in the inside of the STED doughnut. This ring shape is generated by a highly efficient helical vortex phase filter so that the fluorescence spot is minimized. The involved photophysical processes are confined to different areas of the STED scanning spot. The conventional excitation of the fluorophores that is followed by spontaneous emission of photons with different energies (= wavelength) dominates inside the ring, where the STED intensity is close to zero. The STED laser depopulates the excited electronic state S_1 by inducing stimulated emission in the periphery. The released photons are indistinguishable from the STED laser photons and spectrally filtered out. The process is not related to bleaching and can be repeated many thousands of times.

STED microscopy

Conquering the limit of diffraction can be achieved with STED microscopy[2, 3, 4]. This method uses a confocal system to scan the sample with a fluorescence-exciting laser, while overlaying this laser beam with another, ring-shaped laser beam. The ring-shaped laser reduces the size of the scanning spot through induction of stimulated emission, i.e. the fluorophore molecules in the periphery of the excited area are prevented from emitting fluorescence. Increasing the laser power causes the spot to become smaller – in theory until infinity (Figure 1). The efficiency of a STED microscope crucially depends on the use of first-grade optics and mechanics, as well as the interplay between suitable fluorophores and the lasers. Due to the comparatively high intensity of the STED depletion lasers, an increase in resolution requires fluorophores with next to zero probability of excitation at the depletion wavelength. Otherwise, as well as displaying stimulated emission, they may also be excited and fluoresce in the conventional way.

There are different ways of achieving this. In the Leica TCS STED CW, continuous wave (CW) lasers are used to induce continuous fluorescence excitation (particularly the Ar lines 458, 488 and 514 nm) and depletion (592 nm). This allows the use of established fluorophores such as Alexa 488 and Oregon Green as well as fluorescent proteins such as YFP and GFP. The achievable optical resolutions range from 60–100 nm, depending on the depletion proper-

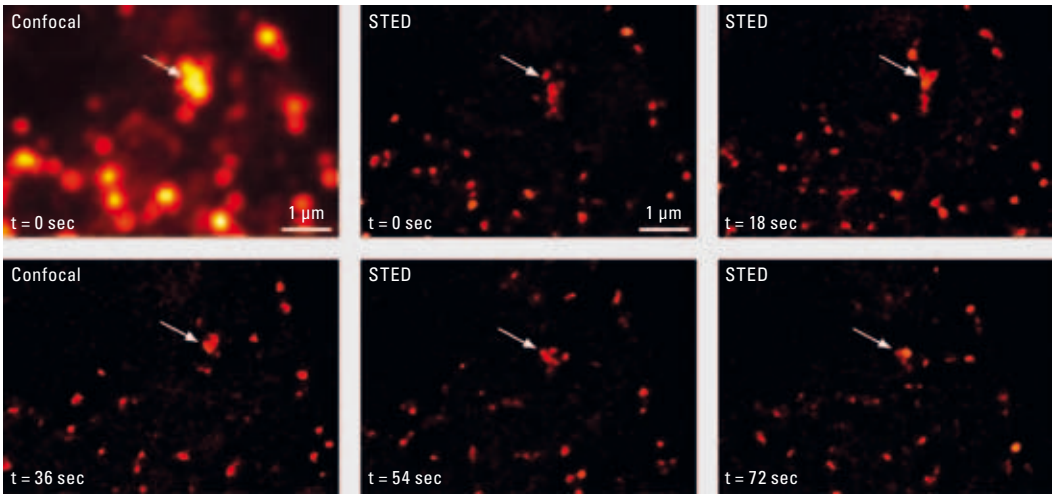

Fig. 2: STED CW time lapse experiment: movement of large dense core vesicles labeled with the fluorescent protein Venus inside of living PC12 cells.

ties of the standard fluorescent markers or fluorescent protein.

The resolution improvement by STED is maximized if pulsed lasers are used, as it is the case with the Leica TCS STED. This is because the power of the depletion laser is compressed into a short period of time. The achievable resolution is below 50 nm, but requires longer acquisition times in comparison with CW systems.

Super-resolution by localization

Scientists take a completely different approach with concepts based on localization microscopy. From experiments for identifying single molecules, it had been known for decades that molecules could be much more accurately localized than optically imaged. The center of the intensity distribution is determined in x and y, subject to a Gaussian distribution. The accuracy of this measurement is directly scalable with the number of detected photons (Figure 3). This allows the position of single molecules to be determined to an accuracy of a few nanometers. In fluorescence-labeled samples there are usually several thousands of fluorophore molecules in a diffraction-limited spot that are excited simultaneously. Their signals overlap, so that localization is no longer possible. To apply this process to super-resolution, a trick is used: Instead of switching on a large number of molecules at the same time, they are made to fluoresce sequentially. The individual signals can then be temporally resolved and localized with maximum accuracy. These individual images are then accumulated, resulting in a highly precise image of the actual structure, achieving an accuracy of tens of nanometers.

The way in which this temporal resolution is achieved is the key difference between the concepts of STORM or PALM and FPALM in comparison with GSDIM. The PALM technique, published by Betzig and Hess[5], relies on photo-switchable or activatable fluorophores or fluorescent proteins. These are first put in an excitable state with the aid of an activation laser, and then made to fluoresce by the read-out laser. By setting the activation laser at a low intensity, only a few fluorophore molecules are excited at one time, so that statistically, only one molecule per diffraction-limited spot is switched on and then excited. The difference between PALM and STORM is the type of fluorophores used. STORM is based on pairs of cyanin dyes connected via a linker. Both PALM and STORM therefore require special fluorophores or fluorescent proteins.

The GSDIM principle is based on switching regular fluorophores using their dark states[6]. Every fluorophore can be excited from the ground state (S_0) to a

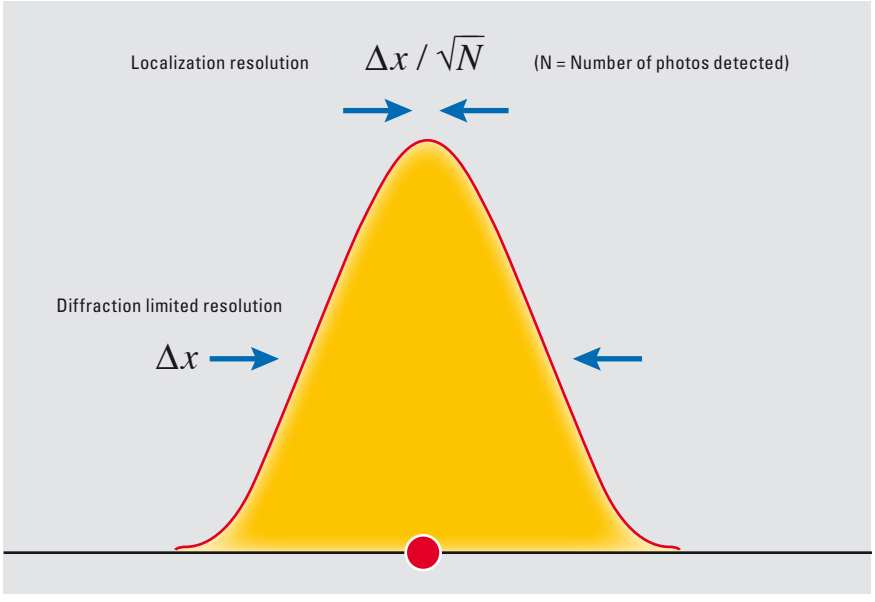

Fig. 3: In localization microscopy, the center of the intensity distribution of the photons is determined in x and y, subject to a Gaussian distribution. The accuracy of this measurement is directly scalable with the number of detected photons. Molecules can therefore be localized with a much greater degree of precision than they can be optically imaged. Drawing by J. Merz, Boston University

"A Large Gain in Resolution"

Dr. Alexandra Elli, Philipps University Marburg

Dr. Alexandra Elli, Coordinator of the Bio-Imaging facility at the Institute of Cell Biology and Cell Pathology of the Philipps University Marburg, reports on her first experiences with the new Leica SR GSD super-resolution system:

Why did you decide to participate in the beta test for the Leica SR GSD system?

Dr. Alexandra Elli: The decision to participate in the beta test for the Leica SR GSD system was based on a number of reasons. The most important reason was having the chance to be one of four laboratories worldwide to test a new super-resolution microscopy method using Leica equipment. This offers the opportunity to actively take part in the latest stages of development and contribute to the successful commercialization of this product. From a scientific point of view, being a beta tester for the Leica SR GSD system opens up a whole new range of experiments which were not possible before since this is the first super-resolution microscope in our bioimaging facility. Last but not least, our collaboration with Leica Microsystems has been very fruitful in the past.

What are the perspectives of GSD technology for your research focus?

Working on protein trafficking, it is of utmost importance to localize specific target proteins inside cellular compartments. Since the achievable resolution with the GSD system is far superior to the microscopy methods used in our lab, we feel confident that we can better resolve cellular components and image target proteins and hopefully their interactions within these compartments.

What is your impression of the first images you have taken, especially regarding the gain in resolution?

From the first images taken with the new system, it is clearly visible that there is a large gain in resolution. Structures become visible which were completely blurred before. This becomes obvious when you look at microtubules, for example. Here single subunits can be identified in the GSD image.

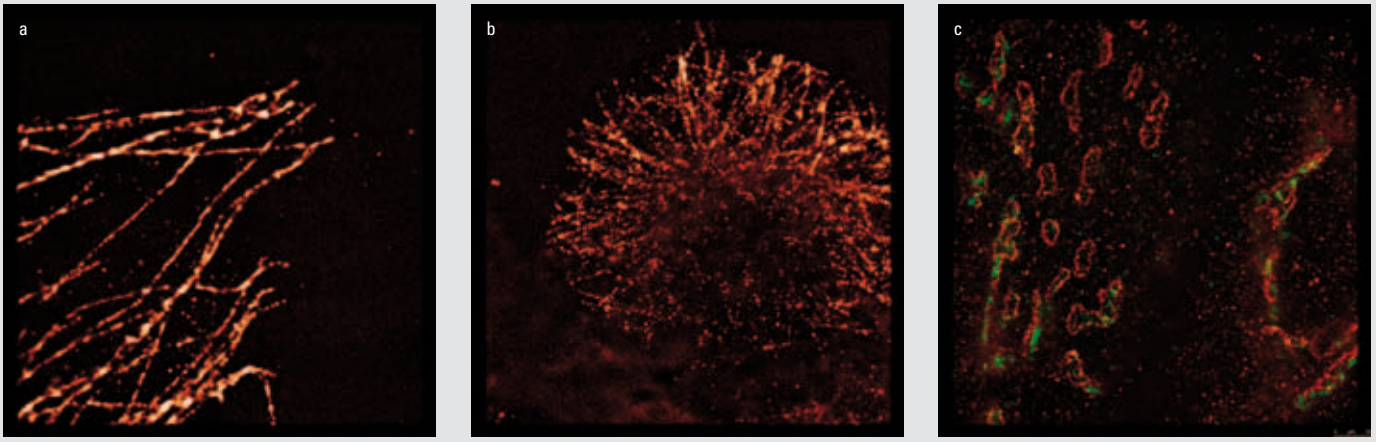

Fig. 1a–c: GSD images. a: Human clear cell renal cell carcinoma cells, immunofluorescence staining against α-tubulin with AF 647. b: Human kidney epithelial cells, immunofluorescence staining against α-tubulin with AF 647. c: Golgi membrane protein Giantin and Golgi matrix protein GM130, immunofluorescence staining with AF 647 and AF 488, respectively.

higher electronic state (S_1) and with the return of the electrons to the ground state, light of a higher wavelength is emitted. In addition to the excitation of electrons, there is the possibility to switch the electrons to a so-called dark state or triplet state (T_1). The lifetime of this triplet state is several magnitudes higher than the excited state, so with enough light, most of the electrons will accumulate in the dark state. From this, electrons will return spontaneously but as single events so they can be detected as single blinking spots in an image sequence (Figure 4). Using a separate laser, the proportion of molecules returning from T_1 to S_0 can be controlled to achieve maximum resolution and, at the same time, get enough molecules moving through the S_0–S_1 cycle as the number of molecules in the triplet state increases.

The new Leica SR GSD

The Leica SR GSD is the latest addition to our portfolio of super resolution products. Based on GSDIM technology, it surpasses the resolution limits

previously set by other widefield super resolution systems. GSDIM as well as STED are based on methods developed by Prof. Stefan Hell at the Max Planck Institute Göttingen, Germany, and exclusively licensed by MPI to Leica Microsystems. Following on from the successful technology transfer and commercialization of STED, the Leica SR GSD extends the range of cutting edge tools offered by Leica Microsystems, allowing the user to select the best working solutions for various applications. Standard fluorescent dyes can be used to achieve resolution of about 20 nm to explore sub cellular structures and molecular localization.

Results from the system have already revealed image detail that has never been seen using an optical microscope before. The Leica SR GSD is a multifunctional microscopy platform. Whilst featuring super-resolution it can also be used for TIRF and high-speed widefield live cell imaging applications. Thanks to the optimized workflow of the software, the user can quickly switch from a regular fluorescence overview to detailed super-resolution imaging during a single experiment. The Leica SR GSD is based on the tried and proven Leica AM TIRF MC system and has the possibility to combine GSD super-resolution with TIRF evanescent field illumination for improved resolution in all spatial dimensions. Three powerful lasers offer high flexibility of fluorophore selection. This allows a single experiment to yield a regular fluorescence overview alongside detailed super-resolution imaging.

Super-resolution techniques demand that the sample is as stable as possible. To guarantee best imaging conditions, the Leica SR GSD introduces a brand new technology in drift compensation. With this approach, it is now possible to directly observe the super-resolution image whilst it is being acquired. No offline drift compensation calculation is needed. This also simplifies sample preparation, as the addition of gold beads or other fiduciary marks is not needed.

Joined forces

The Leica TCS STED and Leica TCS STED CW confocal systems join forces with the widefield Leica SR GSD, giving Leica Microsystems a complete product offering for super-resolution. Working together with leading scientists to constantly challenge imaging boundaries, we surpass the previously accepted resolution limits to bring more biological details to light. With the possibility of using different super-resolution technologies to cater to varying applications, Leica Microsystems now helps researchers to bridge the gap between light and electron microscopy.

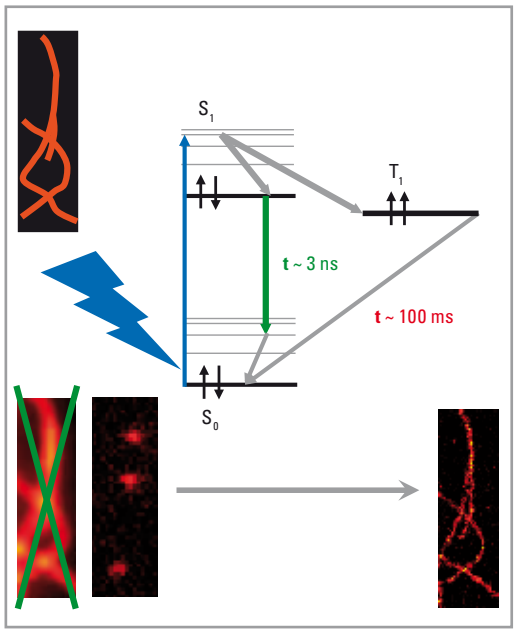

Fig. 4: The GSDIM principle is based on switching regular fluorophores using their dark states. Repetitive excitation of the singulet ground state S_0 to the excited singulet state S_1 elicits fluorescence emission and switches a molecule to a dark triplet state T_1 with long lifetime. Only a very few molecules ever return from a dark state to S_1, from which they are then excited and read out.

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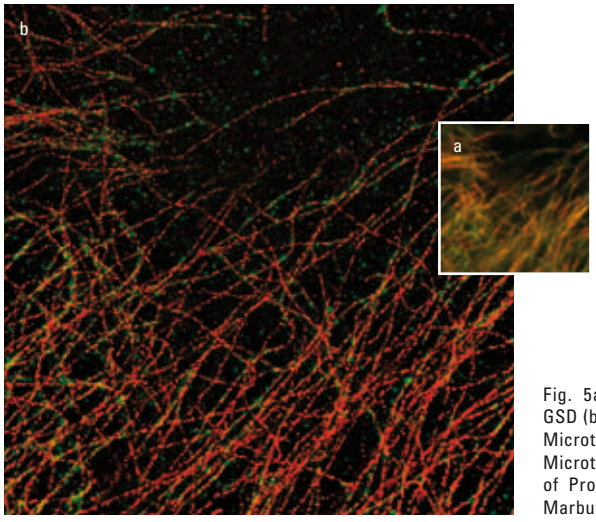


Fig. 5a–b: Resolution enhancement of GSD (b) versus widefield (a): MDCK cells: Microtubules, Alexa 642 (red) and Tyr-Microtubules, Alexa 488 (green). Courtesy of Prof. Ralf Jacob, Philipps University Marburg, Germany

Intravital Multiphoton Imaging at High Speed

Visualizing Cellular Networks

Dr. Andrea Pfeifer, Leica Microsystems

A common cause of the most life-threatening diseases is the disruption of complex biological networks. The importance of intercellular connections is an emerging feature of cancer, neurological and immunological diseases. Therefore, a growing number of research project focus on intact cellular networks. Specimens used for these studies are thick tissue sections or whole animals. Imaging cellular networks at high resolution deep within biological tissues is a demanding task. Visible light is strongly scattered by the specimens and cannot excite fluorophores in deep tissue sections. Here, multiphoton microscopy is the perfect tool as it uses infrared light for excitation which is less prone to scattering in tissues. The new Leica TCS MP5 is dedicated to the needs of multiphoton imaging. Optimal transmission up to 1300 nm and an integrated Optical Parametric Oscillator (OPO) solution allow researchers to reach even deeper into samples.

Fig. 1a–b: 3D reconstructions of representative 50 µm z-stacks from timelapse acquisitions. Excitation at 910 nm, spectral unmixing was performed using the LAS AF software.
a: Microglia labeled with GFP (green) are shown in relation to blood vessels injected with 655 nm quantum dots (red) residing in the brain parenchyma. Some microglia have their processes wrapped around blood vessels. Second harmonic generation (SHG) of skull bone (blue).
b: Spleen seven days following infection with lymphocytic choriomeningitis virus (LCMV). Anti-viral CD8-CFP (red) and CD4-YFP (green) T cells. Courtesy of Dr. Debasis Nayak, Dr. Bernd Zinselmeyer and Dr. Dorian McGavern, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD, USA

Intravital imaging of soft tissues

Gaining access to the tissue of interest without interrupting the studied process is crucial for the success of intravital imaging. In neurosciences, connections and interactions between neurons and other cell types of the nervous system are regularly studied in living animals by multiphoton microscopy.

More recently, intravital imaging of soft tissues provides exciting new insights into the biology of cancer. Repeated observation of tumor metastases and blood vessel formation over several days is possible with imaging windows implanted in the skin of the animal.

Multiphoton imaging – individually configured

As a dedicated multiphoton microscope, the Leica TCS MP5 is the perfect platform for intravital imaging. In combination with the Leica DM6000 CFS fixed stage microscope it offers the highest mechanical and electronical stability for demanding applications like electrophysiology. The Leica TCS MP5 equipped with the inverted microscope Leica DMI6000 B is optimal for observation through ventral imaging windows.

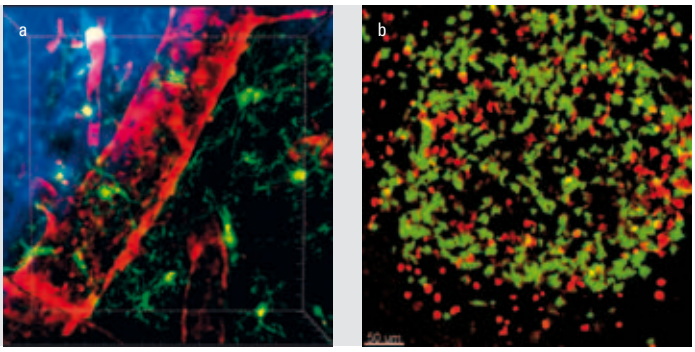
Many processes in living organisms are highly dynamic and need to be monitored with high temporal resolution. The Leica Tandem Scanner combines two scanners to offer the full range of scan speeds in one Leica TCS MP5 system. The resonant scanner allows recording of fast dynamics and faster acquisition of detailed z-stacks by high-speed scanning. For optimal spatial resolution the conventional scanner can be used with an exceptionally large field of view of 22 mm.

Explore the full range of fluorescent proteins

Interactions of proteins and different cell types are monitored by using specific fluorescent markers for each species. The Leica TCS MP5 can accommodate up to seven external detectors for simultaneous multichannel acquisition. This includes non-descanned detectors (NDDs) for reflected and

Dr. Dorian McGavern, NIH/NINDS, Bethesda, MD, USA:

"The Tandem Scanner adds impressive speed to our multiphoton microscope. Combining high-speed scanning with the Leica TCS MP5 offers a real advantage for intravital microscopy."



The Principle of Multiphoton Imaging

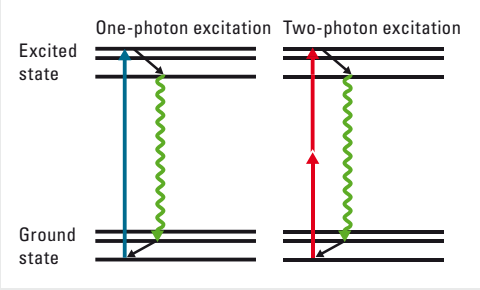
For multiphoton excitation, a fluorescent molecule has to absorb multiple photons almost simultaneously. When two photons are absorbed, we speak of two-photon excitation. Three-photon excitation is less common but sometimes used to improve resolution. For many fluorophores, the wavelength for two-photon excitation is roughly twice the wavelength for single-photon excitation while the emission spectrum usually remains the same.

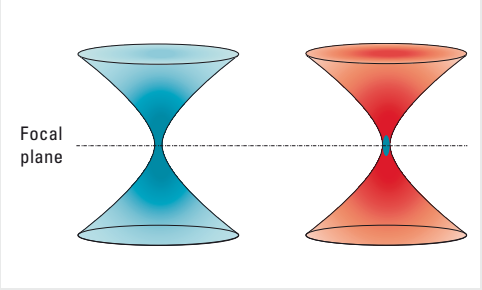
Reduced phototoxicity and bleaching by localized excitation

The high photon density necessary for multiphoton excitation is generated by pulsed IR lasers with high peak power. While peak powers of several hundred kW are reached, average power is still low enough to avoid damage to the sample. The probability of two photons reaching the fluorophore almost simultaneously is only sufficiently high in the vicinity of the focus and drops exponentially outside of the focus. This reduces photobleaching and phototoxicity outside of the focal plane.

Non-descanned detection (NDD) for optimal light collection

In multiphoton microscopy all emitted photons are a result of excitation in the focal plane. This results in optical sectioning without the need for a detection pinhole. Thus, it is possible to improve the efficiency of light collection by placing the detectors as close as possible to the source of emission.

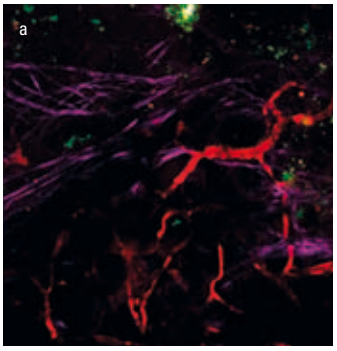


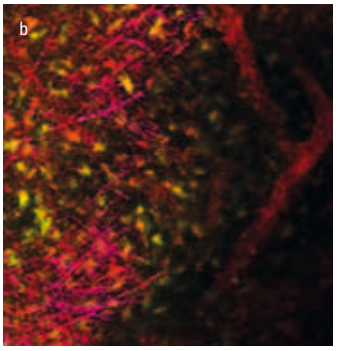


Left: Energy levels of a fluorophore in one-photon (left) and two-photon (right) excitation. Colors correspond to the absorbed and emitted wavelengths.
Right: The probability of excitation in one-photon excitation (left) linearly depends on the amount of photons from the light source. In two-photon excitation (right) it is proportional to the square of the intensity of the light source and limited to the vicinity of the focal plane.

transmitted light detection of fluorescent signals and a high-contrast brightfield detector to give researchers the full picture of their sample.

Excitation up to 1300 nm with an optical parametric oscillator (OPO) allows researchers to explore the full range of fluorescent proteins and dyes by multiphoton microscopy. With Leica's OPO solution simultaneous multiphoton excitation of green and red fluorophores is possible. The longer wavelengths of the OPO can reach deeper into tissue and are less harmful to samples. Leica Microsystems offers an OPO solution that is fully integrated in the software interface of LAS AF for easy tuning and setup of laser wavelengths. High transmission up to 1300 nm of the Leica TCS MP5 scan optics brings all the power from the OPO to the sample (see Research reSOLUTION No.9, p. 8).





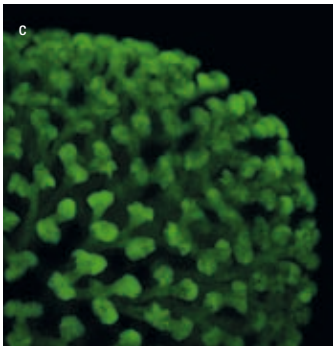


Fig. 2a–c: a, b: Mouse mammary gland (a) and spleen (b). Blood vessels labeled with 70 kD-Texas Red excited with OPO at 1150 nm (red). Simultaneous excitation at 800 nm results in second harmonic generation (SHG) signal of type I collagen (purple) and autofluorescence of single cells (green). Courtesy of Evelyne Beerling, Dr. Jacco van Rheenen, Hubrecht Institute, Utrecht, The Netherlands
c: Ureteric bud from an embryonic day 16 kidney from a HoxB7 EGFP mouse. Courtesy of Prof. Deborah Hyink, Mount Sinai School of Medicine, New York, NY, USA.

Leica CellReporter – The Multi Application Imaging Platform

Run Your Assay of Choice

Dr. Claire Bungard, Leica Microsystems

Traditionally, cell based assays have been time consuming, manual assays providing semi quantitative data. Until now High Content Screening systems were needed to get object-based high-content data. These systems require expert user input and analysis. Leica CellReporter offers a fully quantitative, rapid imaging platform that gives object based results. It is easy-to-use, flexible, and fits a wide range of cell- or bead-based assays.

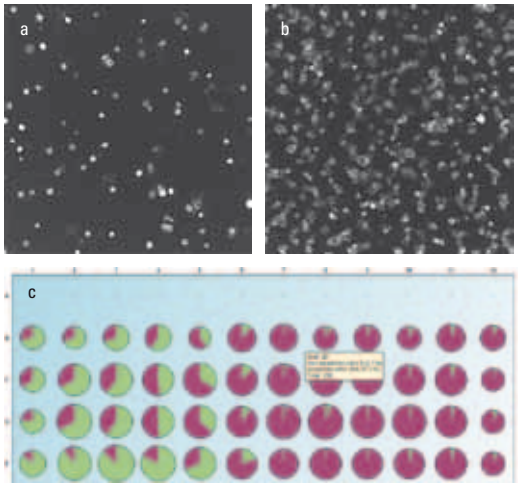


Fig. 1a–c: Image and analysis of Annexin V binding on the Leica CellReporter. Cells were treated with increased concentrations of a pro-apoptotic drug and stained with Hoechst, an all cell dye to identify each cell and TxRed labeled antibody against Annexin V (AbCam).
a: Image example for a low concentration of pro-apoptotic drug.
b: Image example for a high concentration of pro-apoptotic drug.
c: After applying a gate based on cells that were Annexin V positive and those that were Annexin V negative the system automatically presents the proportions per well as pie charts. The size of each pie chart relates to the number of objects (cells) per well.

Highly flexible and easy-to-use

The multi application imaging platform Leica CellReporter overcomes many of the challenges faced when working with conventional cell- and bead-based assays. It generates high-quality, quantitative data by utilizing state-of-the-art imaging technology and image analysis software to image, analyze and report on individual cells or beads.

One of the key advantages of the system is the ability to run such a wide range of cell based assays without having to make any change in hardware or software. The running of the system, and the software analysis, has been developed so that it is easy to use without the need for extensive training. With its multiple wavelength filters it is possible to multiplex a number of assays, improving greatly the speed and output of results. This flexibility and ease of use has been key to the interest and success of the imaging platform.

Quantitative and object based data

The Leica CellReporter is not only able to run a wide range of different cell based assays – such as cytotoxicity, viability, apoptosis, proliferation etc. – the set-up of the system allows the user to study one particular response in several different ways. Apoptosis is one example of this. Figure 1 shows the images of the cells when a labeled Annexin V antibody is used to distinguish cells that are apoptotic from those that are not. Figure 1a is a typically non-apoptotic well and 1b is a typical image for a highly apoptotic well. Using the gating in the software it is possible to quantitatively measure the proportion of cells that are apoptotic and therefore the efficacy of particular drugs or compounds on a given sample.

In combination with this and using the Hoechst nuclear dye it is also possible to determine cells that are in late apoptosis by looking for fragmentation of the nucleus. The Leica CellReporter has the advantage over a number of systems in that it not only detects a signal but it measures the standard deviation of the signal within the object – in this case the nucleus of the cell. Figure 2 is an example of the staining of the nucleus in apoptotic and non apoptotic cells, with the analysis that can be done using the cell reporter to quantitatively measure the proportions of each.

Labeled antibodies against different caspases can also be imaged so the complete activation chain from onset of apoptosis to cell death can be interrogated on the system. It offers the user the flexibility to run the assay which is most suitable to their particular study, which can be adapted and changed as

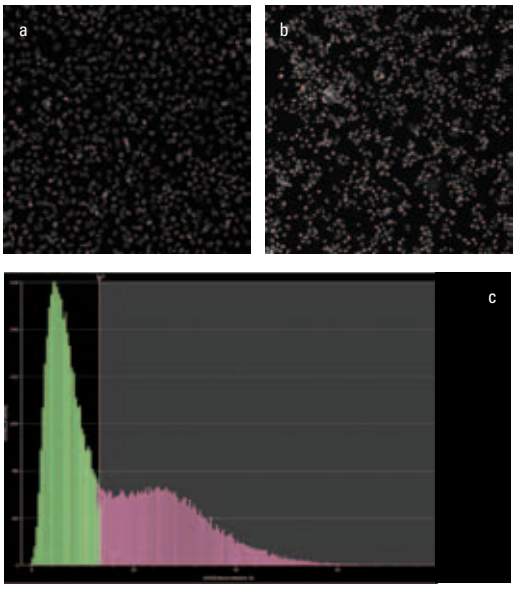


Fig. 2a–c: Image and analysis of Hoechst nuclear staining to identify apoptotic cells. a: Image from a control well, with low proportions of apoptotic cells. b: Image from a well treated with high concentrations of Staurosporine, a pro-apoptotic agent, with high proportions of apoptotic cells. c: Histogram showing the number of objects (cells) against the SD of signal within the DAPI channel. When a cell is in a late stage of apoptosis the nucleus condenses and fragments. The SD of the nuclear stain is then high. This parameter can then be used to separate apoptotic from non-apoptotic cells.

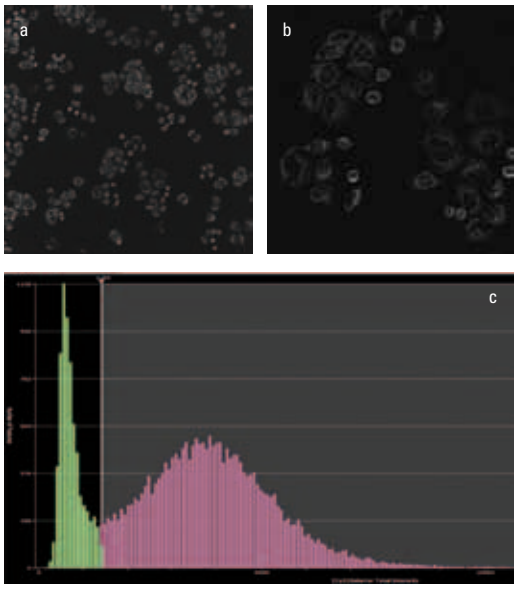


Fig. 3a–c: Image and analysis of cells with active and healthy mitochondria using TMRE accumulation as a measure. a and b: Image in the Cy3 channel, one zoomed, to show the speckled staining around the nucleus. This signal is the accumulated TMRE within the healthy mitochondria. c: Using the Cy3 exterior statistics (2 pixels around the nucleus) cells can be divided into two clear populations; healthy and proliferating and unhealthy or dying.

required. This highlights the flexibility of one given assay, a concept which applies to a whole host of cell based assays.

High resolution screening

The Leica CellReporter has a resolution of 0.8 µm, so if there is a stable fluorescent signal which can be seen at this resolution, the system can be used to measure it. However, the flexibility of the software allows certain organelles that cannot be resolved, such as mitochondria, to be analyzed regardless. Figure 3 shows the images and analysis run when looking at mitochondrial activation. Although individual mitochondria cannot be clearly resolved, the fluorescence around the nucleus of cells with active mitochondria can be clearly measured. This then allows a rapid and quantitative measurement of active mitochondria.

Leica CellReporter analysis tools also make it an ideal system for looking at translocation assays. The system takes fluorescent readings from within the nucleus (termed interior statistics) and also around the nucleus (termed exterior statistics). Users can then create their own statistics within the software. In the case of translocation, the ratio of staining from the cytoplasm (exterior) to the nucleus (interior) can be created as a statistic. Figure 4 gives an example of the image and analysis from the NFkappaB translocation assay. As the ratio moves to 1 or above, it is possible to quantitatively measure the proportion of activated cells. The examples presented here show the flexibility offered with the Leica CellReporter. The rapid, high resolution screening system with intelligent analysis is allowing laboratories to get fast quantitative results for a whole range of cell- and bead-based assays which were previously time consuming, and in some cases not quantifiable.

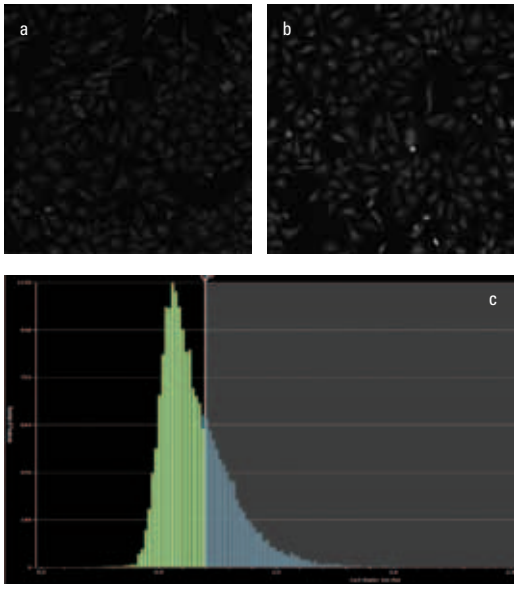


Fig. 4a–c: Image and analysis of NFkappaB translocation. Stimulated and unstimulated cells were incubated with a Cy3 labeled anti NFkappaB antibody. In unstimulated cells the NFkappaB remains in the cytoplasm only (a), upon stimulation it is translocated from the cytoplasm into the nucleus (b). Within the statistics manager area of the Leica CellReporter software a ratio of interior to exterior can be defined. When this ratio is 1 or above, the NFkappaB is equal or greater in the nucleus than the cytoplasm and therefore the cells are stimulated, as seen in c.

Integrated Confocal Spinning Disk Solution

Imaging Fast Biological Processes

Markus Schechter, Leica Microsystems

Many of today's questions in cell and developmental biology can only be answered by studying living cells and organisms. For continuous three-dimensional imaging of highly dynamic processes such as cell division, protein trafficking or interaction, and vesicle movement in living cells, Leica Microsystems offers the integrated multipoint-confocal spinning disk solution Leica SD AF. It combines key advantages for living cell studies: high-speed image recording, high sensitivity, resolution and contrast, yet low photo toxicity.

Fig. 1a–c: Application examples for spinning disk technology.
a: 3rd instar drosophila melanogaster larva. Right: Maximum projections of 81 optical planes (z distance 0.7 µm) using Yokogawa spinning disk, 20x IMM objective (NA = 0,7) and 488 nm laser line. Left: 3D reconstruction of widefield images. Courtesy of Prof. Stephan Sigrist, Freie Universität Berlin.
b: Primary cultured cortical neuonal cells. Maximum projection of 36 optical planes (z distance 0.2 µm) acquired with Yokogawa spinning disk and 100x Oil objective (NA = 1.47) using laser lines 405 nm, 488 nm, 561 nm and 642 nm). Red: Cy5 staining of bIII-tubulin, a neuron specific marker. Green: Cy2 staining of Nestin, a marker of neuronal stem cells. Purple: Cy3 staining of DCX, a marker of immature neurons. Blue: DAPI, stains the nuclei of the cells. Sample provided by FAN GmbH, Magdeburg, Germany.
c: 3D reconstruction of 260 optical planes (z distance 0.7 µm) of a Copepoda using Yokogawa spinning disk. Optical planes were imaged with a 20x IMM objective (NA = 0.7) and 488 nm laser line.

High light efficiency and maximum scanning speed

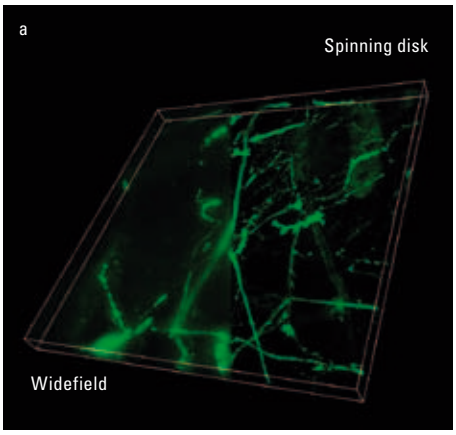
The Leica SD AF is based on state-of-the-art spinning disk technology from the Yokogawa Electric Corporation. The rotating tandem disk system consists of a diaphragm with 20,000 pinholes behind a collector disk with micro lenses, ensuring high efficiency of the excitation light and maximum scanning speed of up to 2000 fps. This makes the CSU-X1 scan head the preferred spinning disk for fast live cell imaging. The system can be equipped with a basic scanning-disk unit or a high-end unit offering top speed and fast filter wheels.

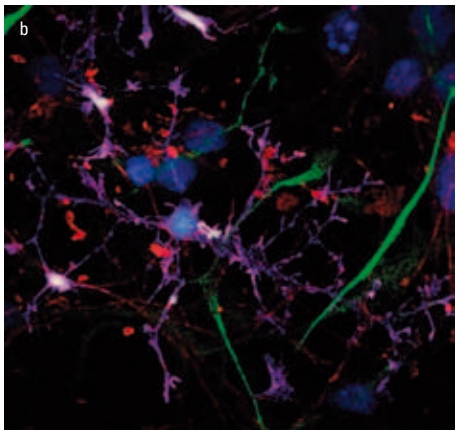
Excellent image and stability

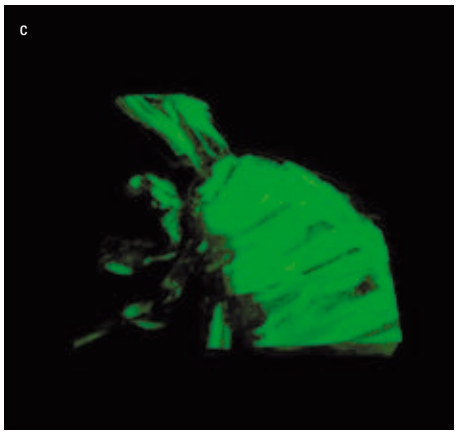
Thanks to Leica Microsystems' proprietary ACS technology (Advanced Correction System) with improved chromatic correction, the Leica SD AF also delivers excellent results, regardless of the wavelength imaged. The optimized optical adaption of the spinning disk head to Leica microscopes, improves confocality even at lower magnifications and provides homogenous illumination for the complete field of view. In long-term experiments, the water immersion micro dispenser developed by Leica Microsystems automatically supplies the objective with water, enabling aberration-free focusing in the sample without loss of contrast. Another useful option in the configuration with inverted Leica DMI6000 B is the Automated Focus Control (AFC), which keeps the sample in focus during time-lapse experiments.

Fully integrated, precise, and flexible

The spinning disk solution is compatible with a large number of microscopes – upright and inverted Leica research microscopes and the fixed-stage microscope Leica DM6000 FS – and can be adapted to individual requirements with a wide range of accessories. Features include an integrated laser safety concept, predefined settings for spinning disk applications and integration in Leica MM AF software powered by MetaMorph® for image acquisition and analysis. Due to the bypass mode of the spinning disk unit, the system can also be used to produce epifluorescence and brightfield images as well as multipoint confocal images. Speed, safety and a single point of contact make this a truly integrated system solution.





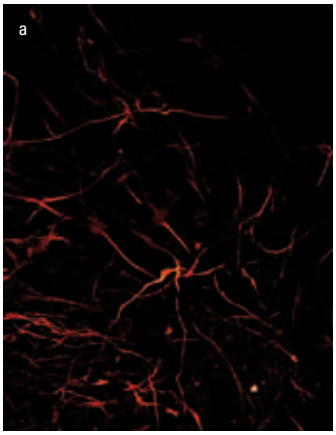


Hybrid Detector for Standard Confocal Platform

Quantum Leap in Photon Efficiency

Dr. Constantin Kappel, Leica Microsystems

Biological imaging is changing from a qualitative to a data-driven, quantitative science as demand shifts towards quantitative annotations of genes in vivo. The goal is to unravel the underlying functional interaction networks. Biological disciplines, such as neuroscience, developmental biology, cell biology and pharmacology, to name a few, rely on confocal imaging to gain insights into the spatio-temporal organization of live cells or living organisms. Today's imaging systems need to serve as a quantitative measurement device as well as to reproduce the finest details with high fidelity. Leica Microsystems' answer is a new detection concept, as implemented in its Leica HyD hybrid detector.



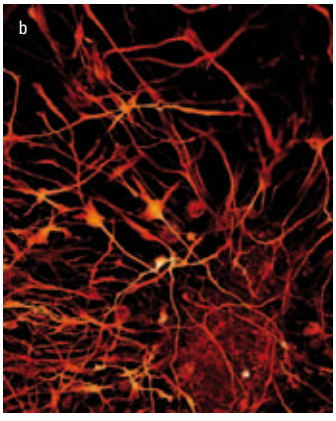
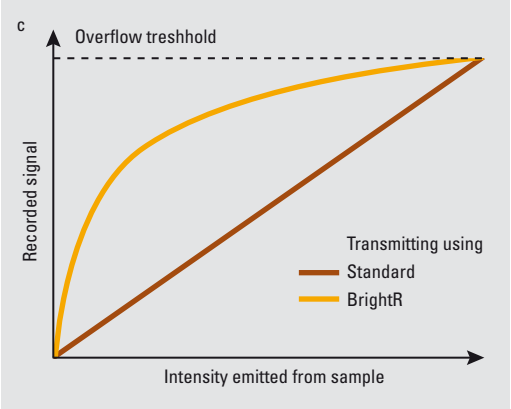


Figure 1a–c: BrightR (b) makes weak signals accessible and renders them in the same image by taking just a single exposure. This is particularly useful in situations with very dynamic samples (a). Dynamic in this case refers to the differential accumulation of dye or fluorescent protein in different structures or parts of the image. BrightR works very efficiently with neurons or similar material. Effectively, BrightR acts as a dynamics compressor for the signal (c).

Quantitative imaging made easy

With its unparalleled contrast Leica HyD delivers publication-ready images out of the box – there is no need for post-processing. All imaging tasks benefit from Leica HyD's low dark noise, high sensitivity and large dynamic range. The latter is increased even further by single photon counting. This represents the most attractive approach to image quantification, since the number of registered photons is in direct proportion to the concentration of molecules under study. Thus, biochemical information becomes accessible through single photon counting and in situ spectroscopy.



Thanks to its high quantum efficiency of typically 45% at 500 nm, its low noise and large dynamic range, the hybrid detector is the most versatile detector in our Leica TCS SP5 confocal platform. Along with Leica Microsystems' proprietary filter-free beam-splitting concept and waste-free spectral detection design not requiring any recycling loops in the beam-path, these properties make the Leica TCS SP5 ideally suited for quantitative measurements and all-purpose imaging alike.

BrightR reinforcement for highly dynamic samples

Some biological samples accumulate far more fluorescent labels in specific structures than others. Likewise, the physical size of labeled structures can vary greatly. Both result in a highly dynamic distribution of light intensity. Such samples are intrinsically difficult to record, because either the bright parts of the image are overexposed or the dim parts underexposed.

Leica Microsystems addresses this imaging problem with its innovative BrightR imaging mode (Brightness Reinforcement). With BrightR dim structures are amplified more than bright ones, resulting in an extended dynamic range capable of capturing very bright structures and intricate details in the same image. Unlike other image acquisition strategies involving a large dynamic range, this is accomplished in a single image recording. This way the sample is only exposed to light once.

Lambda Square Mapping – Explore Photonic Landscapes

Care for a Little More Color?

Dr. Lioba Kuschel, Leica Microsystems

Fluorescent labeling is a well-approved technology in microscopy to make colorless structures visible. Additionally, natural fluorescence can yield valuable information about organization and metabolic conditions within tissues and cells. Such fluorescent molecules can be identified and characterized by fluorescence spectra. For the first time – using the Leica TCS SP5 X – complete excitation emission spectra can be acquired with the local resolution of a confocal image. Constituents of complex fluorescent mixtures become recognizable and can be assigned to subcellular structures.

Fluorescence characterization

So far in confocal microscopy scientists discriminate fluorescent species of multiple stained samples by their characteristic emission spectra. To obtain such a spectrum, fluorescence is excited at a fixed wavelength and emission intensity is measured at different wavelengths.

However, if a sample contains several fluorescent species the emission spectra may overlap. The problem arises how to discriminate between them. In various cases, fluorophores with similar emission spectra show different excitation properties. Under such circumstances excitation spectra discriminate between these species: Fluorescence is excited at variable wavelengths and emission is detected at a fixed wavelength range.

Combined excitation emission spectra

If a sample consists of a complex mixture of fluorophores the identification of the single molecule species by looking at single excitation or emission spectra will get quite cumbersome. It would become much easier if the full spectral fluorescence characteristic were obvious at one glance. This is now given by the λ^2 plot, which shows the full excitation emission spectrum in one graph. Fluorescence intensity is displayed as color (2D) or as color and height (3D) over excitation and emission wavelength.

Full spectral information

To aquire a full excitation emission spectrum, both the excitation wavelength and the detection range need to be tuned. The Leica TCS SP5 X offers the prerequisite for both: the white light laser can be tuned in 1 nm steps in the range from 470 to 670 nm, and the detection range of the SP detector is tunable with a precision of 1 nm as well in the range from 400 to 800 nm. For each excitation emission pair a full confocal image is now acquired. For data analysis regions of interest can be defined in the confocal image. In the λ^2 plot the fluorescence intensity of these regions of interest is displayed over excitation and emission wavelength.

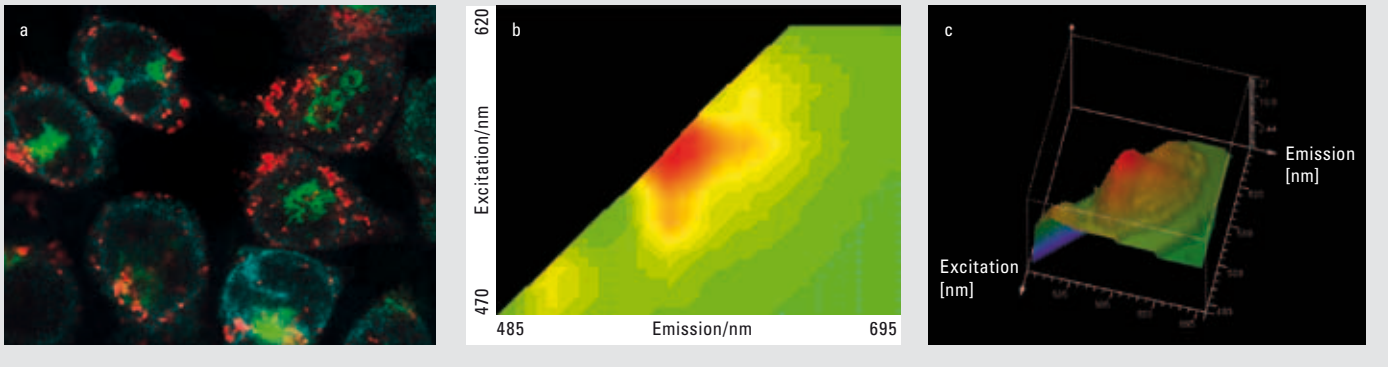
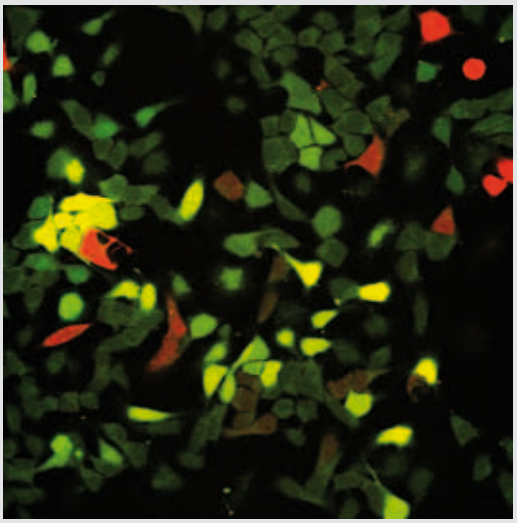


Fig. 1a–c: Fixed cells with triple staining a: GalNacT2-GFP (Golgi), LAMP-546 (Endosomes), Calnexin 594 (ER). Corresponding fluorescence peaks are visible in an logarithmic scaled λ^2 plot (b, c). Courtesy of Matthias Weiss, Cellular Biophysics Group, Bioquant, Heidelberg, Germany



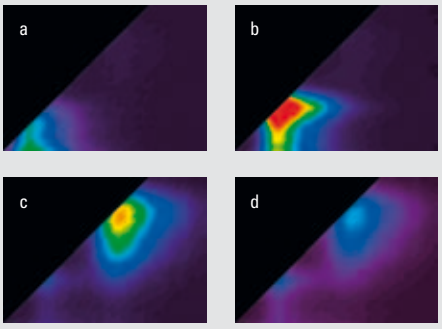
Top: Overview image – mixture of fixed cells expressing 4 different fluorescent proteins.

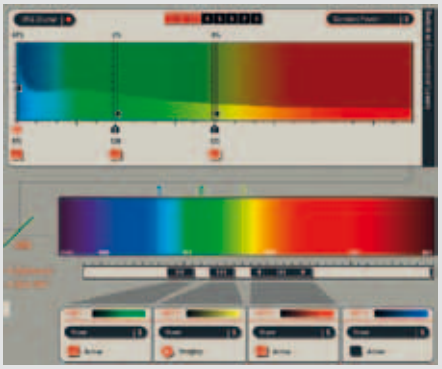
Scaling of λ^2 plots
- Excitation range: 470 to 620 nm
- Detection range: 480 to 700 nm

The different FPs can be identified by their characteristic spectrum. Excitation emission peaks are given in brackets. All cells show a small autofluorescence peak at (512, 533) nm.
a: GFP (470, 511)
b: YFP (515, 530)
c: mCherry (577, 605)
d: mKate (582, 620).

According to the λ^2 contour plots, instrument parameter settings are optimized. The different FPs can now be identified within one image. The fluorescence intensity of mKate transfected cells is much lower compared to cells labeled with mCherry.

λ^2 contour plots of single cells expressing different FPs





Sample: Courtesy of Kees Jalink, Department of Cell Biology, The Netherlands Cancer Institute Amsterdam, The Netherlands

Applications

In general, Lambda Square mapping is a very convenient tool to automatically characterize complex fluorescence characteristics of any sample. Modern research in biology typically targets fluorescence samples with five or more fluorochromes. Also, intrinsic fluorescence of samples, e.g. biofilms, shows a complex composition of fluorescent species.

In such complex samples the λ^2 plot still discriminates and identifies species with strongly overlapping spectral characteristics. For standard imaging the spectral information is useful to optimize instrument setting. This way, image contrast and quality will be enhanced.

Characterizing dyes

To improve and enhance the properties of artificial labels, new fluorescent dyes and proteins are permanently developed. Before releasing them to the market they need to be completely characterized. Developers need to specify their properties not only in the chemical pure form but also at their labeling target. That means properties need to be measured in living or fixed tissues or cells. Combining full spectral and image information, the Leica TCS SP5 X facilitates such studies.

This is also important for well known chemical dyes or fluorescent proteins, as the spectral characteristics depend significantly on environmental conditions of the molecule. These conditions often vary in different cell types, organelles, and physiological states. Using Lambda Square mapping, such modifications become obvious.

Reference

Borlinghaus RT, Gugel H, Albertano P and Seyfried V: Closing the spectral gap – the transition from fixed-parameter fluorescence to tunable devices in confocal microscopy. Proceedings of the SPIE 6090 (2006) 159–164.

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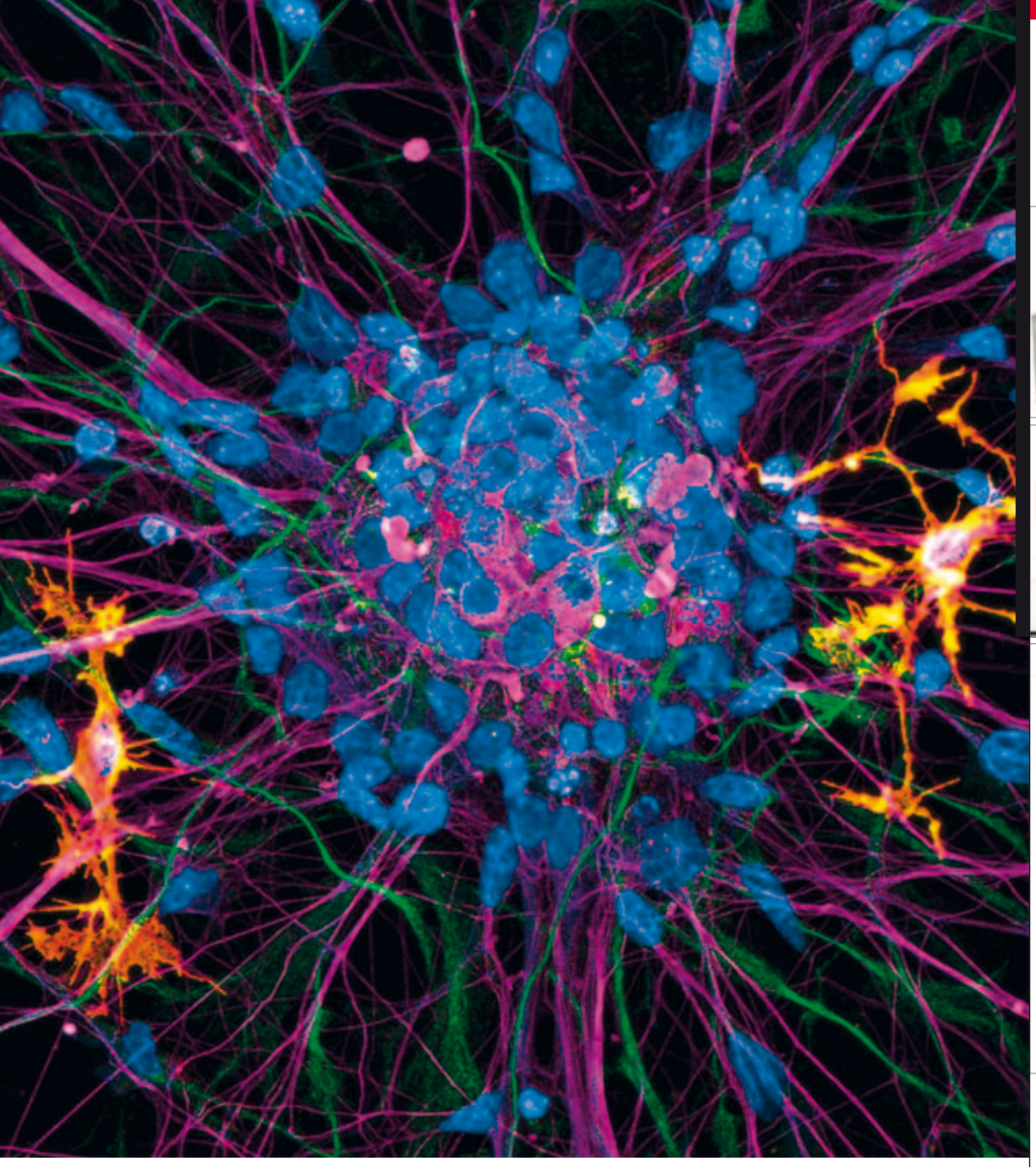
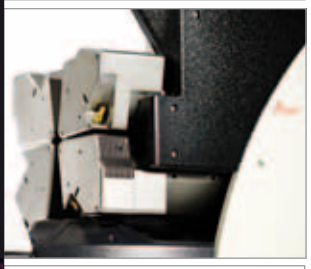
Backpage image: Primary culture of rat brain, labeled with DAPI, NG2-Cy3 and Tubulin-Cy5, respectively





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