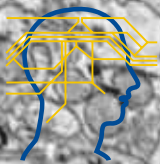
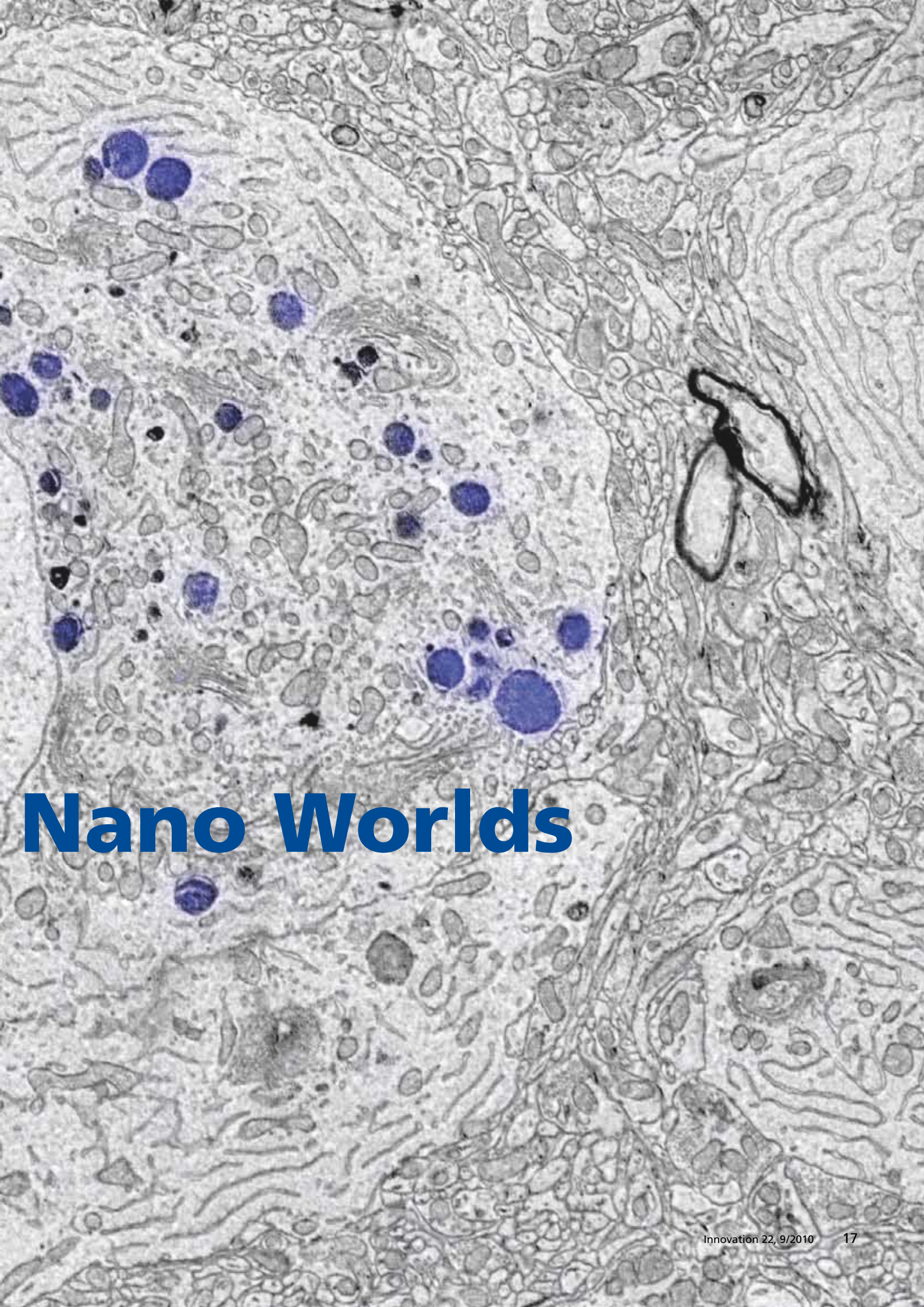


The rapid advances being made in the development of technical methods is providing researchers with insights into increasingly small details of living matter. Traditional light microscopy plays its part in helping scientists understand cellular processes. The electron microscope reveals structures down to the nano range. Correlative microscopy combines both techniques and therefore offers new possibilities to biomedical research. At the same time, super-resolution optical systems are advancing right to the molecular level.

Text: Monika Etspüler

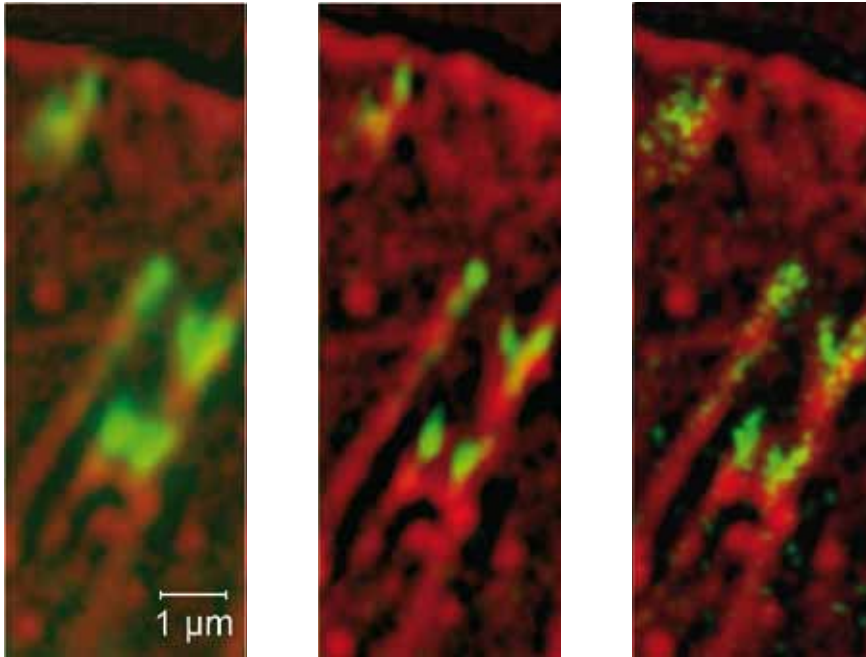


Bridging the **Micro and**



Nano Worlds

Advancing into Ever-smaller Dimensions



Paxillin staining of a connective tissue cell. The comparison with a traditional wide-field photo (left) demonstrates the additional resolving power possible with the new super resolution techniques SR-SIM (center) and PAL-M (right).



To develop a deeper understanding of the processes in living organisms, it is necessary to investigate the complexity of individual biological structures and functions. Two new systems from Carl Zeiss pave the way to ultra-high resolution and therefore also to the world of the nanocosm: on the one hand, correlative microscopy with the *Shuttle & Find* interface which enables easy relocation in the electron microscope of the specimen sections seen in the light microscope; on the other, super-resolution systems with which the diffraction limit of light microscopes can be overcome.

Correlative microscopy: In special applications specimens are first examined in a light microscope and then in an electron microscope. However, the switch from the micro to the nano world has its problems: finding the same specimen area at a magnification over one thousand times higher is like looking for the proverbial needle in a haystack. To simplify cross-system microscopy and enable fast specimen and image transfer, Carl Zeiss has developed *Shuttle & Find*. The world's only company to manufacture both light and electron microscopes launched *Shuttle & Find* on the market for materials analysis last year. Now, this interface is also available for correlative microscopy in the life sciences.

Specific properties. Light and electron microscopes have specific properties that determine for what applications they are used. Living specimens can only be examined

with light and laser scanning microscopes. These achieve a resolution limit up to roughly 200 nanometers (1 nanometer = 10^{-9} meters). Electron microscopes are used for advancing farther into the nano world. Their resolving power is more than two orders of magnitude above that of a light microscope. Due to the technology and functionality of the electron microscope, however, it can be used solely for static examinations on non-living material. Until now, a combination of the two techniques for the analysis of a defined region of interest was very complicated, if not impossible.

Use in materials analysis. Aalen University in Germany was one of the first facilities to use *Shuttle & Find* for its research activities. Here, Carmen Hafner and Timo Bernthaler are examining lithium-ion batteries from small electronic appliances. And as with all rechargeable batteries, the ravages of time do not make an exception here either. However, the issue of ageing processes and performance loss has become increasingly more urgent now that lithium-ion batteries have practically become an emblem of electro-mobility and an emission-free future. The composition and granularity of a structure allow conclusions to be drawn not only about the type of production used, but also about the material properties.

Timo Bernthaler attaches *Shuttle & Find* to the motorized specimen stage of the *Axio Imager.2* light microscope. The specimen holder with the specimen is calibrated via three markings on the surface, to which it first has to move. The cross-section of a battery appears with 25x magnification on the monitor. "One year's development work was necessary to produce such a good sample," he explains almost in passing. The spirally arranged separators between the anode and the cathode have definitely seen better days. Decomposition is already evident in some areas. Timo Bernthaler marks the "trouble spots" that are stored electronically to enable their subsequent relocation in the electron microscope. Another calibration is required before the ex-

*Previous double page:
Ultra-thin section through brain of zebra finch. Fluorescence-labeled vesicles in a neuron, overlaid with scanning electron microscope image..*

The Elyra PS.1 microscope system combines SR-SIM and PAL-M technology in a single system.



amination of the region of interest in the EM. The structural damage of the material is plain to see on the monitor – but at 16,000x magnification this time.

“One year’s development work was necessary to produce such a good sample”

Timo Bernthaler

Biological perspectives. Whether it is used for the life or materials sciences, *Shuttle & Find* works along the same principles in each case. The differences lie, above all, in the design of the specimen holder, which must fulfill much more stringent requirements for biological material.

- In light microscopy cover slips are used for living specimens. However, the electron beams of an electron microscope cannot penetrate the cover slip material. Therefore, the holder had to be

constructed so that the specimen can be examined from two sides in the microscope.

- In light microscopy immersion oil is used at very high magnifications. But, oil contaminates electron microscopes. Therefore, it must be possible to remove it in its entirety. For this purpose, Carl Zeiss has applied for a patent for a technique in which a thin film applied between the immersion oil and the cover slip can be simply “stripped off” with the oil.
- After all, in the specimen holder it should also be possible to prepare the object being examined, i.e. fixing, dyeing and embedding, without affecting the direct surrounding area with the markings.

Shuttle & Find opens up interesting perspectives in cell biology. One example: it enables the overlay of images taken with the light and electron microscopes. A light and laser scanning microscope makes it possible to observe how large viruses labeled with fluorescent dye penetrate a host cell. Electron microscopy delivers information about the surface morphology of the cell in the regions of interest. The fluorescence signals indicate whether and in what area a virus docks onto a cell or to what extent it has already invaded it.



Shuttle & Find being used in the specimen area of an electron microscope.

“SR-SIM allows the spatial imaging of structures. This is a huge benefit”

Martin Bastmeyer

Super-resolution systems. In the years ahead an important research goal will be to visualize the diversity of the workings of a cell down to the molecular level. This requires light microscopes with fluorescence-based technology and extremely high resolution, also known as “super resolution.” *Elyra* from Carl Zeiss combines these two quality features. *Elyra S.1* (SR-SIM), which stands for Structured Illumination Microscopy, has a resolution that is twice as high as that of traditional fluorescence microscopes. *Elyra P1* (PAL-M), also known as Photo Activated Localization Microscopy, achieves a resolution of 20 nanometers and therefore functions in a range in which single molecules can be localized.

Elyra PS.1, a combination of PAL-M und SR-SIM, is installed in the laboratory of Prof. Dr. Martin Bastmeyer, who holds the chair of Cell and Neurobiology at the Karlsruhe Institute of Technology (KIT). One of the applications for which the scientist uses SR-SIM is to examine the cytoskeleton which provides stability and elasticity to a cell. It consists of a network of actinium filaments and microtubules. These are protein fibers that ensure that the cell keeps its shape. In the SR-SIM these structures are discernible as fine threads. With the aid of PAL-M, Bastmeyer is

succeeding in localizing individual paxillin proteins. Paxillin, which appears as a green fluorescent dot in the specimen, is one of over 100 proteins that together form the docking point of the actinium filaments on the cell membrane.

The application determines which of the two super-resolution microscopy techniques is used. “SR-SIM allows the spatial imaging of structures. This is a huge benefit,” explains Martin Bastmeyer. “PAL-M requires a large amount of preparation time. In addition, the optical sections must be extremely thin,” he adds. For PAL-M, only fluorophores from special GFP mutants (Green Fluorescent Proteins) are used, while all fluorescent dyes can be utilized for SR-SIM.

Outwitting diffraction limits. Although the technology behind SR-SIM and PAL-M is totally different, the two systems nevertheless have one thing in common: to work at all, they have to circumvent a law of physics formulated by Ernst Abbe as far back as 1873. The general rule applies that the wave nature of light sets limits to the resolving power of a microscope. The maximum resolving power of even the best microscope lies at a minimum distance of 200 nanometers between two points. If more detail is required, the microscope and microscopic images must be modified in such a way that resolution beyond Abbe’s law is possible.

In structured illumination (SR-SIM), therefore, a defined grating structure is projected into the focal plane

of the fluorescence microscope. The generated modulation contrast between bright and dark areas in the image can then be used to distinguish between two closely adjacent points. To ensure that the super resolution is not only available in individual areas of the image, the grating is laterally displaced across several positions and rotated. This guarantees a uniform increase in resolution in all three spatial directions. The individual raw images generated in this process have little to do with “real” imaging. Subsequent software computation is needed to turn this into a realistic microscope image.

Photoactivated localization microscopy (PAL-M) uses switchable fluorophores to circumvent the diffraction limit. The specimen is irradiated with different wavelengths so that only a few molecules can be excited at any one time. This makes it possible to determine the exact position of an individual molecule with nanometer accuracy. This process is repeated in 20,000 to 40,000 single images, and the results are subsequently added to form an overall image.

More networking ahead. The future trend is heading toward further networking of the different microscopy techniques. It is not yet possible to “marry” super-resolution light microscope systems and electron microscopes via *Shuttle & Find*. If this is successful, there is nothing left to hinder the determination of the position of a single protein and its function in a highly resolved cell structure.