Editorial for PROTOPLASMA — Special Issue on Microscopy

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Traditionally, two conceptual approaches underlying cell biology were structure and function, whereby microscopy was considered more or less synonymous with structure. These days, however, the advent of confocal microscopy and related fluorescence techniques has led to a new discipline: “functional imaging”. Advances in our understanding of the morphology of cells and their constituent organelles have always, and continue to do so, gone hand-in-hand with developments in microscope construction and sample preparation techniques. The introduction of molecular biological methods into cell biology beginning in the 1980s has been accompanied by a renaissance in light microscopy. Today, virtually every new issue of a cell biological journal has at least several articles containing data obtained using a confocal microscope for the detection of fluorescently labeled protein. This development was given a kick-start with the discovery of green fluorescent protein (GFP) and related fluorescent proteins in the 1990s and continues unabated. Unfortunately, the downside of this “get rich quick with GFP” trend was that after a few years interest in electron microscopy temporarily decreased, and fewer people were prepared to invest the necessary time required to get proficient in this method. As a consequence, trained electron microscopists were achieving the status of rare orchids at the beginning of this millennium. Not surprisingly, for a number of years, the quality of published electron micrographs, due to poorly trained users and the lack of qualified reviewers, was at a lower standard than in 1970. However, the gradual appreciation of the limits of GFP technology for subcellular protein localization has led to an

increased demand for high resolution immunogold localizations, a procedure which itself has profited enormously from the introduction of various cryotechniques since about 1985. GFP technology has also enabled the two disciplines of light and electron microscopy to be combined in the form of correlative light electron microscopy (CLEM). Further development and implementation of modern methods in light and electron microscopy now enable us to go beyond the improved morphological description of an organelle and allow now the detection and visualization of individual molecules.

Examples of such developments are the so-called superresolution and single molecule techniques, which are nowadays based on the available plethora of genetically encoded visible proteins, ultra-fast excitation light sources as well as detectors with highly improved photon sensitivity. The impact of advanced fluorescence-based imaging approaches has allowed for many new insights into biological processes. Fluorescence imaging in general offers a great opportunity to reveal dynamic processes in living cells, tissues, or whole organisms such as zebrafish, mice, or plants. Confocal microscopy has opened doors for biologists to unravel protein localization and, in combination with GFP technology, has enabled the visualization of multiple fluorescently labeled proteins simultaneously.

Although colocalization studies can reveal whether biomolecules coincide at the subcellular level, information about the molecular composition of different fluorescently labeled biomolecular complexes remains unknown. Since the early 1970s, many *in vitro* studies have been conducted which have revealed the molecular assembly of biomolecules. In these studies, Förster resonance energy transfer (FRET) methodology has been exploited to investigate, e.g., binding kinetics, protein complex formation, or lipid protein interactions. FRET is a dynamic spectroscopic ruler for unraveling distances between molecules. It took quite some years, starting from the end of the 1990s, to be able to visualize protein interactions in living cells. This was achieved by combining FRET with other fluorescence imaging techniques of which

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fluorescence lifetime imaging (FLIM) is one of the most robust methods for quantifying FRET. Since FLIM set-ups have been commercialized during the last decade, many biologists now have the possibility to access this technique via specialized imaging centers. This development has made FLIM a “standard” tool for the *in vivo* identification of protein interactions.

In this Special Issue, two reviews discuss FLIM applications; one is devoted to the medical sciences and discusses recent technical developments (Ebrecht et al.) and the second focuses on the potential of FLIM in plant systems (Bücherl et al.). FRET–FLIM measurements result in images with pixel information that is beyond the diffraction limit of visible light as FRET takes place at the nanometer scale. Alternative imaging approaches solving the diffraction-limited constraints associated with confocal microscopy has led to the development of superresolution imaging techniques such as stimulated emission depletion (STED) microscopy, structured illumination microscopy (SIM), and stochastic optical reconstruction microscopy (STORM)/ photoactivated localization microscopy (PALM). The latter two techniques are founded on the discovery of photoswitchable probes enabling the localization of fluorescent molecules with a precision far below the physical limit of conventional light microscopy. These recent cutting edge technologies are extensively discussed in the review of Hedde and Nienhaus.

Genetically encoded fluorescent proteins can also be used for the characterization of biological processes such as monitoring calcium fluxes by utilizing FRET-based biosensors. In the article of Hamers and co-workers, a detailed overview of several engineering strategies for generating and optimizing biosensor performance is given along with a discussion of biosensor applications in mammalian systems as well as in plants. FRET spectroscopy is further exploited by the application of single-molecule FRET (smFRET), a powerful tool for elucidating biological structures and mechanisms on the molecular level. The article of Farooq and co-workers illustrates how smFRET can be applied to precisely characterize dynamics and rarely occurring events of DNA/RNA polymerase activities at the single molecule level. In this Special Issue, there are two more reviews discussing single molecule applications, but with a focus on *in vivo* applications. The work of Langhans and Meckel elaborates on the application of single molecule detection and tracking in plant systems. A procedure, in which all the necessary steps required to perform a single molecule experiment, is described from data acquisition to analysis. Another single molecule-based technique is fluorescence fluctuation spectroscopy (FFS), which is extensively discussed by Mark Hink. This methodology can provide quantitative molecular information on fluorescently labeled biomolecules, e.g., diffusion rates, binding kinetics, and average number of molecules. Though mainly developed and used for *in vitro* systems, this technique has in the meanwhile been successfully applied *in vivo*. As FFS relies on monitoring differences of fluorescence fluctuations, this approach can only be performed at low nanomolar concentrations.

Since expression in living systems is sometimes difficult to tune, there are alternative strategies available for monitoring dynamics of molecules at higher expression levels. This method is known as fluorescence recovery after photobleaching (FRAP), which is discussed in detail by Malte Wachsmuth. An advantage of this methodology is that it can be performed on a regular confocal microscope. It allows revealing the mobility of biomolecules as well as the binding kinetics in living cells. All previous fluorescence-related techniques are also referred to as the “F” techniques. However, since the borders of different disciplines in science have become blurred over the last decades, a better term for describing “F” techniques is “functional imaging”. Finally, Gualda and co-workers present developments of new fluorescence imaging platforms such as light sheet imaging. This new imaging configuration has led to high-resolution images that can capture cell differentiation and proliferation of whole organisms such as zebrafish, plants *in vivo*, and real time. The great advantage of this approach is performing live cell/tissue imaging with reduced photodamage and fast acquisition rates. This new imaging concept is very promising, and initiatives such as OpenSPIM and OpenSpinMicroscopy have emerged as open access platforms for light sheet and optical projection imaging.

As already mentioned, the most significant advances in electron microscopy over the last 30 years have involved cryotechniques. Although not displacing classical chemical fixation procedures, especially when large numbers of specimens are to be screened, e.g., in pathology, they have become the gold standard for today’s ultrastructural investigations. In terms of the validity of the observations made, freeze-fixed specimens cannot be challenged. This does not mean that everything published before the cryo era is incorrect; indeed, from the structural point of view, many organelles can be well-identified and monitored in chemically fixed specimens. With cryo-methods not only is antigen preservation enormously increased leading to better immunogold labeling, but these have also provided higher quality images with improved fine structural details. Moreover, in the form of cryo-electron microscopy (Cryo-EM), macromolecular complexes can now be studied at near atomic detail. This Special Issue on Microscopy contains two articles devoted to cryo-methods in electron microscopy. In one, Kent McDonald not only gives a detailed account of the latest freezing techniques together with a hands-on account of how to prepare specimens and work with commercially available equipment, but he has also added a much appreciated historical perspective. The other article delivered by Andy Höninger is a comprehensive update on what could conveniently be termed macromolecular electron microscopy. Cryo-electron tomography figures prominently in this research area and is fueled by new developments in computing power and software as well as in the construction of super-

stable microscopy stages and electron detectors. The reader will soon realize that cryo-electron microscopy is very expensive to establish and requires highly trained personnel.

Correlative light electron microscopy (CLEM) is where GFP-technology meets light and electron microscopy, and, in terms of subcellular protein localization, is a goal that many groups are striving for. To our knowledge, until now, nobody has succeeded in getting this technique to work on plants, but it has been achieved with varying degrees of success on animal cells.

The article of Hodgson and co-workers presents a new way of performing CLEM by including the Tokoyasu method of cryosectioning for the immunogold labeling step in the procedure. In this regard, it should be noted that while the Tokoyasu method is widely used for conventional immunogold localizations on mammalian cells, it has rarely been performed on plant material.

Two methods which have only very recently come onto the scene are described in the articles of Hughes et al. and Carziniga et al. The technique known as serial block face scanning electron microscopy (SBEM) is a new 3D imaging set-up for which a special type of scanning electron microscope is required. This is realized by incorporating an ultra-microtome within the specimen chamber, which is again an expensive piece of equipment. It essentially involves imaging the cut surface of the specimen block rather than looking at the section and provides similar information that of serial section transmission electron microscopy. This technique has great

potential and may challenge electron tomography data obtained from plastic sections cut from high-pressure frozen-freeze substituted specimens. Unfortunately, such samples tend not to have sufficient contrast, so there is certainly room for improvement. A completely new method which does not involve electron microscopy, but can provide nanoscale 3D data from cryo-fixed, unstained whole cells is cryo-soft X-ray tomography (Cryo-SXT). Required is a source of soft X-rays—usually from a synchrotron and is therefore not universally available. In addition, the microscope needs a special cryogenically cooled device with tilting and specimen rotation capabilities. SXT data sets from rapidly frozen samples are then analyzed with software packages similar to those in use for electron tomography. The current best resolution with this new technique is around 20 nm. Immunogold labeling is possible but at its infancy and it will therefore be intriguing to see how Cryo-SXT develops.

The guest editors of this Special Issue of PROTOPLASMA are delighted to have had the opportunity of assembling a mixture of high quality scientific reviews, which highlight recent developments in the fields of electron and light microscopy. We hope that our readers appreciate this as well, and that these contributions will be inspiring and useful for their own future research.

Conflict of interest The authors declare that they have no conflict of interest.