Microscope Technology and Biomedical Applications: A Fit?

Modern Microscopy for Biomedical Research

Biomedical research is increasingly targeting more than just the cell as the classical object of microscopy. On one side of the application spectrum investigations move towards the basic molecular interactions and on the other side large complex systems as model organisms are studied. Both directions and the exploration of function rather than structure provide challenges for the microscopic imaging techniques that - as direct observation methods - are and will remain extremely valuable enabling tools in modern biomedical research.

Advanced modern microscopes (LSM 510, Carl Zeiss MicroImaging, Germany).

From the very beginning of the microscope it was used to answer key questions of life. With one of the first microscopes Leeuwenhoek discovered at 270-fold magnification red blood cells, "crawling monsters" in the water, and investigated the structures of muscles, besides others. Another early microscopist, Hooke, established with the "Micrographia" in 1667 the foundations of microbiology and introduced the term "cell." After Schleiden and Schwann postulated in 1837 that the cell is the building block of all life, cell theory became one of the corner stones of biology. In modern times with the discovery of the genetic mechanisms, the breaking of the genetic code, the improved understanding of genetic expression and regulation as well as its role in establishing the organism and maintaining its health even smaller-scale processes are at the very centre of biomedical research. Under such circumstances it is obvious that microscopy's role in the biomedical sciences has not been diminished but continues to further increase. Adding to the trend of concentrating on ever more fundamental and therefore more microscopic events in biology, science has also realized that not only the understanding of the cell but an understanding of the complex interactions of cells in tissues and whole organisms is needed if the mysteries of life are to be solved. Thus the spatial and temporal scales to be studied have not only become finer but also wider. How does microscopic technology cope with such re-

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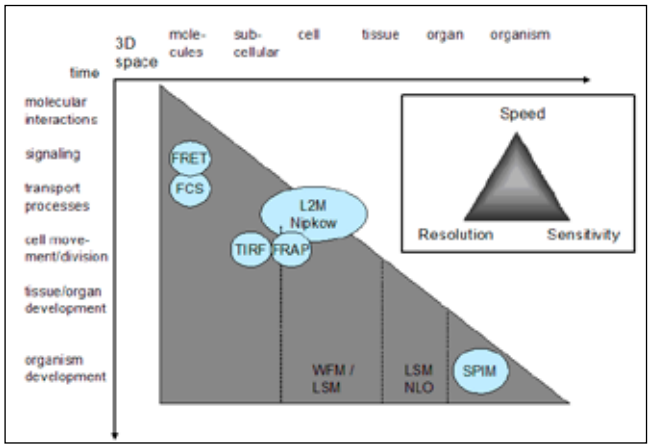

FIGURE 1: **Spatial and temporal scales for biomedical imaging and microscopic techniques employed. Only the range inside the triangle is relevant. The inset shows the "triangle of compromise" that is relevant for all imaging techniques.**

quirements? Is there an appropriate fit that drives developments in both areas, technology and biomedicine, or are there large discrepancies that will put microscopy on the sidelines of biomedical research? These are the questions we want to address in this article focusing on research activities in the areas cell biology, developmental biology, and neurobiology. We will show that microscopy remains an important tool in the biomedical field even though other, non-imaging techniques have become valuable tools to explore life as well.

Fundamental Trends in Biomedical Research

As has been mentioned above, biomedical research increasingly moves into areas that have not been addressed by classical microscopy. This becomes obvious when looking at the spatial and temporal scales involved, see Fig. 1.

One direction concerns the study of fundamental processes, involving the interactions of molecules, often proteins. But also the study of the dynamics of small cell compartments and cell domains is part of this trend. The synapses of nerve cells within our brains are one example of focused research interest. The release and uptake of neuro-transmitters from and into vesicles inside these sub-cellular structures is tightly controlled and modulated by preceding activity. The underlying regulatory mechanisms are key to such integrative functions as learning and memory. Here spatial scales in the sub-micron range are associated with very fast processes taking place in the sub-ms range.

The other direction has to do with the drive to better understand the complex interplay of processes on smaller and larger scales that give rise to the various functions and malfunctions in living organisms. It has been realized that a purely analytical approach is insufficient to understand the function of live. Systems biology tries to understand function from a more integrative point of view with substantial consequences for fundamental and medical research. For this approach the study of the details of tissue, organs, and organisms (e.g. so-called model organisms) in their native environment is needed often involving the combination of large-scale and small-scale spatial and temporal effects.

However, it is not only the scales that have changed. Even within the classical scale of microscopy biomedical research has moved from the study of structure in two dimensions (e.g. of adherent cells) to the study of structure and function in three-dimensional space. This means studying dynamic processes in their complexity as they enfold with and without active manipulation.

Microscope Technology: Driven by and a Driver of Biomedical Applications

How has the development of the technological basis of microscopy dealt with the requirements imposed by the above-sketched trends in biomedical research? Before addressing this question it is worthwhile to look at some fundamental issues arising at the technological level. Some of the most

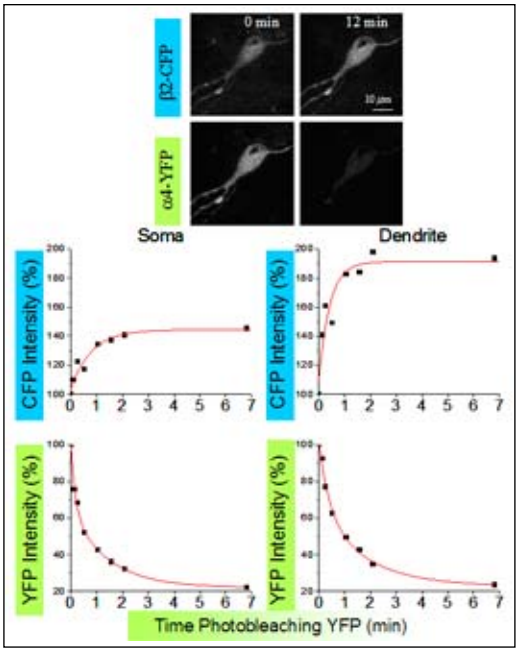

FIGURE 2: **FRET between YFP and CFP to study the Nicotine-induced upregulation in neurons by α4β2 receptors FRET occurs between (α4-YFP-M)(β2-CFP-M) subunits transfected in ventral midbrain neurons. The Images showing increases in CFP fluorescence over time, whereas YFP fluorescence decreases with photobleaching. The Plots of percentage of intensity change in CFP and YFP fluorescence over photobleaching time of YFP indicate greater dequenching of CFP between subunits expressed in dendrites as compared with soma. Therefore receptors in dendrites show a higher degree of assembly than in the soma (courtesy of Raad Nashmi, Caltech Institute of Technology, Pasadena USA; for further information see Nashmi et al. The Journal of Neuroscience, December 17, 2003 • 23(37):11554 –11567).**

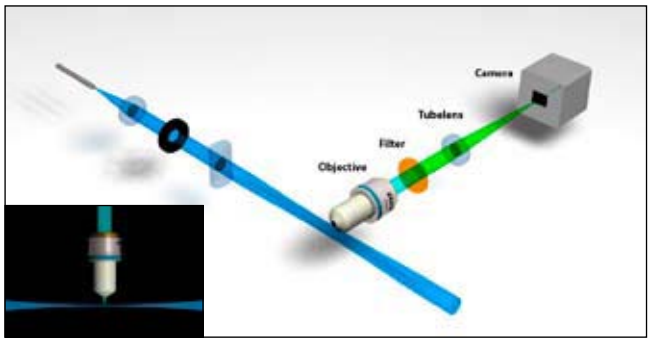

FIGURE 3: **Schematic setup of the illumination and the detection beam path in EMBL's single plane illumination microscope (SPIM, J. Huisken, E. Stelzer, EMBL Heidelberg).**

important of these can be discussed using the so-called "triangle of compromise," see the inset in Fig. 1. The dimensions resolution and speed directly address the spatial and temporal scales of biomedical investigations. The third dimension, sensitivity, is a central feature of any imaging technique with direct consequences for the application. Even the optical technologies that are commonly hailed as "non-invasive" require some interaction with the sample observed. Interaction always implies interference and the sensitivity of the imaging system (besides other factors) determines the degree of unwanted interference needed. Related to sensitivity is the issue of specificity. Fluorescence labeling has provided remarkable specificity in biomedical imaging but often at the prize of considerable interference that becomes particularly obvious when fixed, i.e. death cells are imaged. The advent of fluorescent proteins represented a revolution in fluorescence imaging because it minimizes interference by allowing for genetically encoded labeling that also opens up new opportunities for functional imaging. Emission fingerprinting by spectrally resolved detection as realized for the laser scanning microscope by the LSM 510 META (Carl Zeiss MicroImaging, Germany) allows one to distinguish the various fluorescent proteins in 3D imaging and thereby ensuring high specificity despite strongly overlapping emission spectra. Other imaging techniques that convey molecular specificity even without labels, such as CARS microscopy, are in the coming but are still no match to the specificity and sensitivity of fluorescence techniques.

The "triangle of compromise" is to depict the basic dimensions of the imaging process that are counteracting in the sense that the improvement in one dimension can only be achieved by relaxing the requirements with regard to the other ones. At different levels this holds true for fundamental as well as technological reasons. At the molecular scale one clearly approaches some fundamental limits. These are discussed in Infobox 1. As one conclusion it is not for technological but for fundamental reasons that single-molecule fluorescence imaging in live cells at sub-ms time scales will be impossible. The observation at large scales entails limits as well. A multi-cellular organism as the worm C. elegans (consisting of about 2000 cells) has a size of 1 mm. Investigating such an organism in its entirety up to the molecular level requires the recording of $(1\ \text{mm}/10\ \text{nm})^3 = 10^{15}$ data points per time frame. If each data point is represented by 8 bits and the time resolution is 100 ms only, we have 10 PetaByte per second. Even assuming an enormous progress in data transfer, storage, and processing such a data stream will be hard to handle.

The development of microscopy for biomed-

THE COMPANY

Carl Zeiss MicroImaging GmbH

Carl Zeiss MicroImaging GmbH offers microscopy solutions and systems for research, laboratories, routine and industrial applications, as well as spectral sensors for the analysis market. The company develops, produces and markets instruments, software and accessories for microscope systems and associated techniques. Microscopy from Carl Zeiss is present in all major future-oriented markets. The spectrum of products ranges from traditional light and stereo microscopes to laser scanning systems and automated microscope systems, covering applications ranging from biotechnology, pharmaceutical research, health care and biomedicine to quality assurance and materials analysis. It is supplemented by an extensive line of single components such as gratings, spectral sensors, spectrometer units and specific solutions in process analysis.

Carl Zeiss MicroImaging GmbH emerged from the Microscopy Group as a 100% subsidiary of Carl Zeiss AG on March 1, 2006. During fiscal year 2005/06, the Microscopy Group at Carl Zeiss generated revenues of approximately EUR 340 million (USD 440 million) with a global workforce of about 1600. For more information on Carl Zeiss MicroImaging GmbH please visit www.zeiss.com/micro.

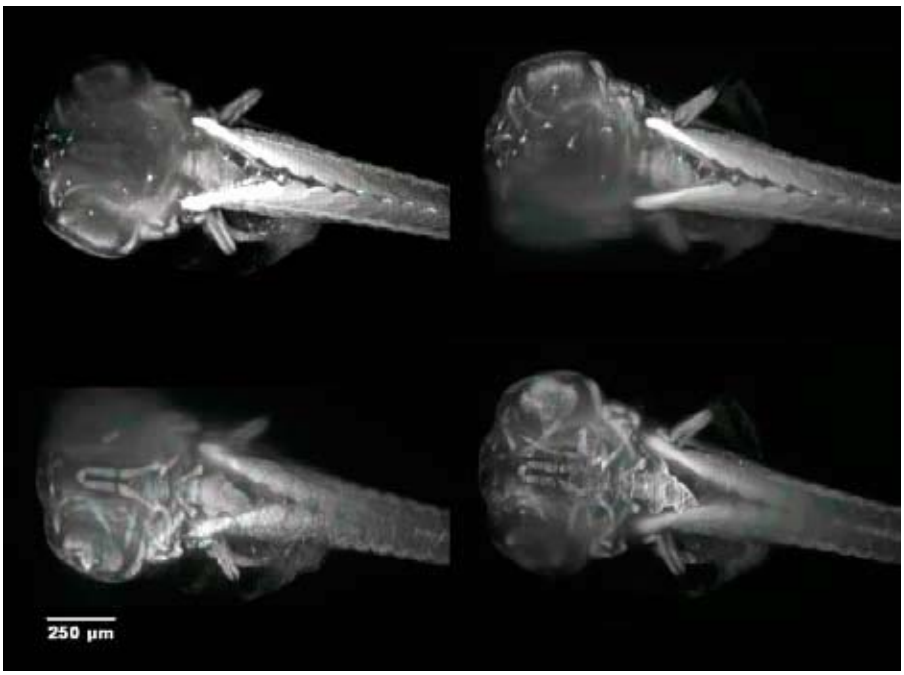

FIGURE 4: **Projections through SPIM images stacks of a Medaka (Ricefish) sample recorded independently along four directions. GFP labeled muscle tissue is excited at 488 nm while the fluorescence emission is detected between 500 - 550 nm with a Carl Zeiss Fluar lens 5x/0.25 (J. Swoger, J. Huisken, E. Stelzer, EMBL Heidelberg).**

MICROSCOPIC-TECHNIQUES: A GLOSSERY

FCS: fluorescence correlation spectroscopy
In a small diffraction limited spot the fluorescence is excited and confocally detected by time-correlated single-photon counting. Utilizing correlation processing the diffusion of a single or several fluorophores including binding processes can be studied. By combination with confocal imaging (as in the ConfoCor Systems by Carl Zeiss Microimaging, Germany) the location can be chosen arbitrarily in 3D within biological samples.

FLIM: fluorescence lifetime imaging
The FLIM image contains in addition to the fluorescence intensity also information about the fluorescence lifetime in each data point. This is achieved by time-resolved detection either by time-correlated single-photon counting or by frequency-domain techniques.

FRET: fluorescence resonant energy transfer
The change of fluorescence as energy transfer between a first fluorophor (the acceptor) and a second fluorophor (the donor) that is excited by excitation light are used as indicator for the proximity of the fluorophores to within the Förster radius (5-10 nm). The increase or reduction of fluorescence intensity or changes in the fluorescence lifetime are common signals that are measured, see also text.

FRAP: fluorescence recovery after photobleaching
By strong excitation the fluorescence within a certain region is bleached. The recovery of the fluorescence after this bleaching by transport processes (active, diffusion) is observed as a function of time.

L2M: line scanning confocal microscope (e.g. LSM 5 LIVE, Carl Zeiss Microimaging, Germany)
In contrast to the LSM the illumination and detection is done in a line-wise fashion using a slit as discriminator and a line camera as detector. Due to the parallel acquisition of a full line a several hundred-fold speed increase can be achieved while maintaining 3D imaging capabilities.

LMPC: laser micro-dissection and pressure catapulting
In laser catapulting parts of a sample under a microscope can be marked and catapulted into separate receptacles using laser light. This can be used to microscopically select and separate cells and sub-cellular compartments for further processing.

LSM: laser scanning microscope
Excitation of the fluorescence is achieved by focusing laser light to a diffraction limited spot. Only the fluorescence light that passes a variable pinhole conjugated to the spot in the sample can be detected, i.e. all out-of-focus light is rejected. The image is built up by raster scanning the sample in 3D. If the excitation is done by a NIR fs-Laser via a multi-photon process (typically two-photon) the excitation is restricted to the focal region and a 3D image can be built up without using a pinhole (LSM 510 NLO, Carl Zeiss Microimaging, Germany). This and the use of less-scattered NIR-light allows one to image deeper into samples.

Nipkow: Nipkow disk scanning microscope
In contrast to the LSM the illumination and detection is done by an array of spots and conjugated pinholes, respectively. The illumination and detection are done through a rotating disk with small pinholes. The fluorescence can be observed by eye or imaged onto a camera.

PALM: photo-activation localisation microscopy
If chromophores in a sample are sparsely activated, localised and bleached an image can be built up successively with a resolution far beyond the diffraction limit. The technique is inevitably slow and therefore limited to fixed samples but achieves molecular resolution.

RESOLFT: reversible saturable optical transitions microscopy
In contrast to STED in this technique the reduction of the excitation volume is achieved by reversibly switching off the chromophore.

SPIM: single-plane illumination microscopy
Using a cylindrical optics light is focused to a thin "sheet of light" in a plane perpendicular to the axis of observation. The fluorescence excited in this sheet is imaged onto a camera. A 3D image is acquired by moving and rotating the object, typically embedded in Agarose. See also text and Fig. 3.

STED: stimulated emission depletion microscopy
A reduction of the excitation volume in a single diffraction limited spot is achieved by stimulated emission in an area surrounding the spot maximum. This reduction corresponds to an effective resolution improvement.

TIRF: total internal reflection fluorescence microscopy
Excitation of the fluorescence is achieved by illumination of a boundary glass-sample with light above the angle of total internal reflection. Only the evanescent field can penetrate the sample and excite fluorescence in a thin plane at the boundary (approx. 100 nm thick) that is typically imaged onto a camera. TIRF is used to observe molecules near the boundary background free.

WFM: wide-field microscope
In the conventional microscope the fluorescence is excited in the full field of view either in trans- or in epi-illumination. The fluorescence is observed by eye or imaged onto a camera. 3D imaging can be achieved by post-processing of a stack of images (deconvolution) or by structured illumination.

ical application is an interplay of technological advances in the attempt to fully cover the space within the fundamental limits and of biomedical developments that allow to better utilize existing or developing technologies. One such example is FRET. We already mentioned the fundamental limits of the observation of molecular interactions. With the present technology it is also difficult to image in the far-field beyond the diffraction limit of about 200 nm in lateral and 500 nm in axial direction. This is more than one order of magnitude away from the molecular scale. However, if one has an marker for molecular interactions, preferably the common fluorescence emission, one wouldn't need to see the interaction directly. The mechanism of energy transfer between fluorescing molecules if they approach each other within the Förster radius of a few nm can be such a marker. There are two processes where this type of marker can be used: a) two molecular species are interacting and are approaching each other to within the Förster radius and b) a molecule undergoes a conformation change as the result of an interaction with the environment. In case a) each molecular species is labeled with a chromophore while in b) the molecule is labeled with chromophores at places that are approaching or moving away from each other with the conformation change. The emission spectrum of one of the two chromophores (the donor) must overlap with the excitation spectrum of the other chromophore (the acceptor). Under these circumstances non-radiant energy transfer results in a diminished fluorescence of the donor (or reduced lifetime) and an increased fluorescence of the acceptor. As alternative imaging strategy, one can bleach the acceptor and record the fluorescence increase of the donor due to reduced FRET, see Fig. 2. Because of their spectra and their usefulness for live cell imaging, fluorescent proteins as CFP and YFP are ideal chromophore pairs. Here special constructs that can be genetically encoded are used to allow dynamic observation at the molecular level with microscopes utilizing emission fingerprinting or FLIM.

Another example is the observation of whole organisms in their spatio-temporal development. Classical microscopy and even multiphoton laser scanning microscopy have some limits in this regard. Two reasons are: 1) the classical observation of large specimen along one direction results in a poor image quality towards its far side, 2) the observation deep inside large specimens requires a long working distance that

FUNDAMENTAL LIMITATIONS OF IMAGING AT MOLECULAR SCALES

A single molecule that fluoresces or has a fluorophore attached can only emit at a limited rate given by the inverse of the fluorescence lifetime τ. Since τ is typically > 1 ns, the maximum rate of fluorescence emission is about 10^9 photons per second. These photons are emitted in all directions and only a fraction of those is captured by the detection system and converted into electrons that can be registered electronically. This fraction is realistically no more than about 10 per cent. The fundamental uncertainty of the measurement is the photon noise that scales with the square root of the number of photons registered. Therefore one needs about 100 registered photons to achieve a signal to noise ratio of 10.

Regardless of the technique employed the diffraction limited volume probed is at best about 200^3 nm³. To achieve a molecular resolution of 10^3 nm³ one needs to sample the diffraction limited volume $(200/10)^3 \cong 10^4$ times.

As a consequence one needs about 10^{-9} x 10 x 100 x 10^4 s = 10 ms to image at the molecular level. However, molecules typically diffuse 50 nm in 10 – 50 ms (cytosol) or in 0.15 – 1.5 ms (membrane) counteracting the molecular resolution to be achieved.

Another limitation arises from the total number of photons that a fluorophore can emit before undergoing photochemical changes (bleaching). A typical number is about 10^5 meaning that the above described sampling to achieve molecular resolution will often be possible only if many (thousands) molecules are in the volume probed.

is generally associated with a low magnification and a low numerical aperture (NA). The latter results in a reduced lateral resolution of about $\lambda/(2NA)$, e.g. 2.5 µm (NA = 0.1, λ = 500 nm). Even worse, the axial resolution drops much faster with $2\lambda/NA^2$, i.e. to approximately 100 µm in the case above. Thus along the axial direction resolution and contrast tend to be extremely weak and result in poor 3D imaging.

The SPIM technology circumvents both problems. Again the sample preparation is an essential prerequisite to a reasonable imaging process. Applying an illumination from the side in a thin plane an axial resolution can be achieved that is decoupled from the NA of the observation lens (see Fig. 3).

By embedding the sample in a medium that is stiff and viable (such as Agarose) the sample can be rotated and stacks of images can be recorded independently along different directions. The SPIM technology is considerably faster than point scanning due to its use of cameras. Since the illumination light sheet exites fluorescence in the focal region of the detection system only it is much less invasive and reduces photo-bleaching significantly. The results are quite impressive, see Fig. 4.

Finally we would like to give an example of imaging within the classical spatial domain but at speeds that are typical for transport and signaling events (with a temporal resolution of 10 ms and less) in 3D. Here one must leave the point-scanning approach whereby it is not the speed of scanning but the number of photons that can be collected within the observation time from within the resolution volume that sets the limit. Approaches are a multiple point-wise scanning as realized in the Nipkow disk microscopes e.g. based on the Yokokawa scanning engines (e.g. Yokogawa CSU10, Yokogawa Electronic Corp., Japan) or line scanning L2M as realized in the LSM 5 LIVE (Carl Zeiss MicroImaging, Germany). Both approaches allow 3D imaging by confocal detection and have – as not entirely new ideas – greatly benefited from the development of the camera technology.

What will the future bring?

Most obviously there will be an evolutionary improvement in the technological basis of microscopy that will follow the technological advance in key components of modern microscopes as light sources, objective lenses, filters, cameras, displays, computers, and software. These advances must and will promote aspects of the biomedical applications by allowing

- less invasive observations (label-based and label-free)
- faster imaging (true 3D observations of intra-cellular processes)
- manipulation and observation at near-molecular scales
- easier and automated use of "more intelligent" instruments.

One key element for such progress will be improved sensitivity.

But judging from the past we can also expect some less evolutionary changes, i.e. the emergence of new methods. One factor for such discontinuous changes is the strong interaction of technology and application. New labels and the direct use of biochemical and photochemical processes by novel technologies may enable to extend the frontiers of the possible. However, this will often restrict the generality of such methods, since the application itself, the sample preparation, and the fluorescent probe become an integrated part of the technology. High-resolution techniques as STED, RESOLFT, or PALM are examples of such developments. Another example is the combination of manipulation and observation that will allow one to change intra-cellular processes for study but also for medical purposes. Already existing examples are LMPC and FRAP.

Thus - finally - the answer to the question raised in the title will be a Salomonic one even in the future. There is a fit and there is a gap between applications and technology. Both, fit and gap, drive the developments in technology and application and will continue to do so.