



Beyond Confocal, by Stephen Lockett, December 19 2009

1) Introduction

Over the past two years, there have been major and diverse advances in optical microscopy that are transforming experimentation in biology. On one front, three emerging technologies significantly break the diffraction barrier of light resulting in a two to ten-fold improvement in spatial resolution of optical microscopy. This provides much more detailed understanding of the spatial organization and interactions of individual fluorescence-labeled proteins in cells, with localization at the scale of the macro-molecule at the highest achievable resolutions (≈ 20 nm). On a second front, progressive improvements in sensitivity now allow for the repetitive detection of each individual, fluorescence-labeled molecule 1,000s of times over. This bestows unprecedented abilities for analyzing the dynamics of individual macro-molecules inside cells. Taken together, these advances result in tools for undertaking biochemistry at the level of individual macro-molecules within living cells or tissue. This is a revolution in biology that is significantly redirecting scientists away from in vitro experiments answering what proteins can do in the test tube, to in situ experiments answering what proteins do in their natural environment. Furthermore, advancements on several other image acquisition fronts are imparting: (i) improved techniques to image deeper into tissue samples, (ii) methods to correlate optical and electron microscope images, and (iii) detection of unlabeled, specific molecular species using vibrational spectroscopy. In parallel with advances in image acquisition, progress in computational image analysis enables extraction of much more information from images and automation of microscopy with increasing reliability. This document briefly outlines the major advances, highlighting their strengths and limitations while indicating some of their current and potential applications.

2) Breaking the Diffraction Barrier

2.1) Photo-Activated Light Microscopy (PALM)

PALM (Betzig et al, [Science 2006](#)), by design detects individual fluorescence-labeled molecules and has been demonstrated for elucidating cytoskeletal structure and focal adhesions. It achieves the highest lateral spatial resolution of the three technologies for breaking the diffraction barrier. For the commercial instrument (Carl Zeiss Inc), the lateral resolution is 20 nm (the size of a macro-molecule / protein). However, experimental systems requiring complex optics for interferometric detection achieve at least a factor of two improved resolution (≈ 8 nm). Consequently, the difference between PALM images and conventional optical microscope images is striking (figure 1). PALM has two principal limitations: (i) imaging is generally limited to the thin 200 nm layer of the sample juxtaposed to the supporting glass cover-slip, and (ii) samples need to be fixed to achieve the highest possible spatial resolution, because of very long exposure times for each image (≈ 20 minutes). Nevertheless the full potential of PALM is far from realized. For example, the ongoing development of more varieties of photo-activatable and photo-convertible fluorescence protein tags will enable the simultaneous detection of two or more proteins. Such a capability will provide greatly enhanced insight into the

interactions of individual proteins and the composition and heterogeneity of molecular complexes on location in the cell. Thus, while limited to the cell surface, diverse applications for understanding cell locomotion and the transmission of HIV particles are anticipated. Although PALM is not ideal for living cells, it will still significantly outperform established techniques when analyzing the dynamics of individual, slow-moving proteins.

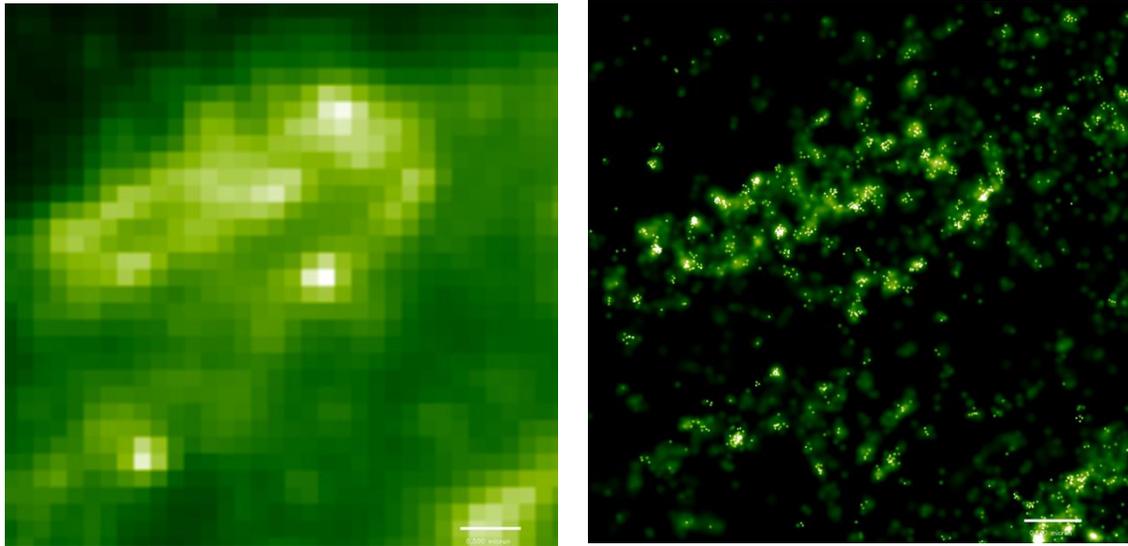


Figure 1: Left: Actin stress fibers tagged with the photo-convertible fluorescent protein, TDeosFP-actin and imaged with total internal reflection microscopy (TIRF), which has approximately 200 nm spatial resolution. Right: The same field of view imaged by PALM with a resolution of 20 nm.

Experimental PALM instruments are more advanced than the commercial system and can acquire 3D images, but with only a subset of fluorescent tagged proteins being detected. The most advanced experimental version of PALM (interference PALM) achieves an axial resolution down to 8 nm, but requires special sample preparation.

2.2) Stimulated Emission Depletion (STED) Microscopy

STED microscopy, in contrast to PALM, acquires 3D images, and sample scanning is drastically faster (seconds per image). This makes STED highly suitable for imaging fast protein dynamics in living cells ([Eggeling et al Nature 2008](#)) and it has been applied to understand the composition of lipid rafts. However, STED has lower lateral resolution than PALM (70 nm) and the commercial system (Leica Inc.) has the same axial resolution as confocal (500 nm), whereas experimental instruments ([in the lab of Stefan Hell](#)) demonstrate axial resolution of 100 nm. Currently, a major limitation of STED is the requirement to use the specific fluorescence dye, Atto647, but this restriction is expected to be lifted.



2.3) Structured Illumination Microscopy (SIM)

SIM is the third super-resolution technique ([Gustafsson et al, Biophysical Journal, 2008](#)). Its major advantage over the other two is the lack of restrictions on the choice of fluorescence labels, readily facilitating multi-color 3D imaging for localizing multiple proteins in cells. On the other hand, its resolution improvement is only a factor of two (130 nm laterally and 250 nm axially) (figure 2). While still a significant improvement over confocal, the ability to elucidate individual protein interactions is severely weakened compared to PALM. Light exposure and image acquisition times are intermediate between PALM and STED (\approx one minute per 2D image), leaving STED the preferred technique for living samples.

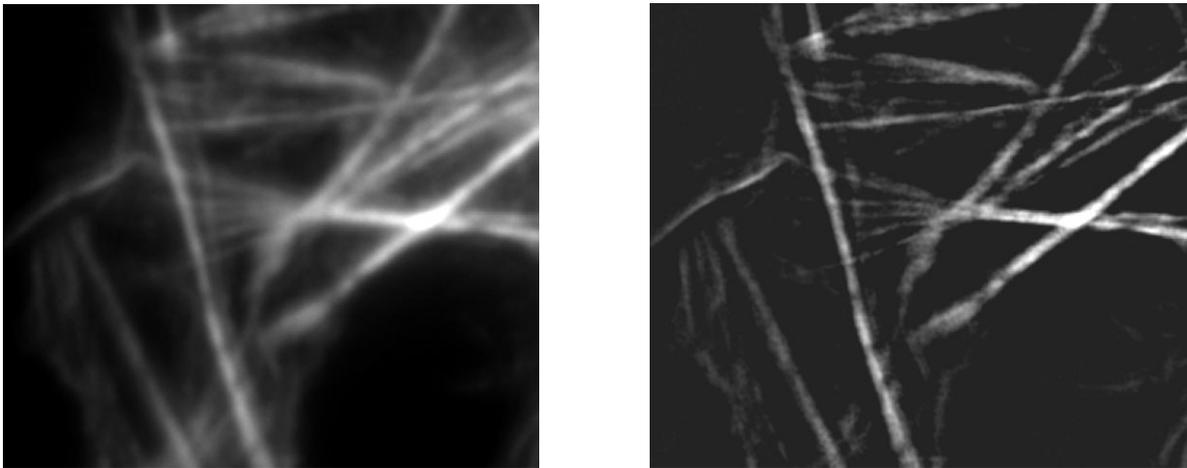


Figure 2: Left: conventional epi-fluorescence microscope image of actin stress fibers. Right: the same field of view imaged with SIM.

3) Non-Linear Optics

Non-linear optics opens up a range of new microscope techniques that provide inherent 3D imaging, increased penetration depth, detection of non-fluorescent compounds and increased spatial resolution ([link to 2.2 above](#)).

3.1) Multi-Photon Microscopy (MPM)

Now a well-established technique ([Denk et al, Science 1990](#)), MPM provides much greater penetration into to living tissue (up to 0.5 mm versus 0.2 mm for confocal) while maintaining high, sub-cellular spatial resolution.

3.2) Vibrational Spectroscopy

An important underlying issue with fluorescence microscopy is whether the fluorescence label perturbs the actions of the labeled molecular species of interest. It is generally



accepted that ligating fluorescent proteins (GFP and variants) to the protein of interest does not pose significant problems. However, fluorescence labeling of smaller molecules, candidate pharmaceuticals for example, is highly problematic. In contrast, labeling small organic molecules by replacing non-exchangeable hydrogen with deuterium is far less problematic and is widely done in NMR studies. Replacing hydrogen with deuterium in $-CH$ bonds (i.e. $-CH$ to $-CD$) significantly shifts the vibrational resonance of the bond, enabling it to be uniquely detected using Raman spectroscopy. Although, Raman spectroscopy has traditionally had very low sensitivity, non-linear optical methods such as Coherent anti-Stokes Raman Spectroscopy (CARS) (Holtom et al, Traffic 2001) and stimulated Raman spectroscopy (SRS) (Freudiger et al, Science 2008) hold the potential for detecting deuterated small molecules in living cells at micro-molar concentrations. However, more optics development is required and achieving this sensitivity is still several years away. Nevertheless, the implications for this are profound, potentially providing scientists with vastly improved capabilities to analyze drug-protein interactions on location in the living cell, alongside analyses of protein-protein interactions.

3.3) Stimulated Emission Microscopy

While fluorescence dyes are much more sensitive than absorption dyes for detecting low concentrations of specific molecular species of interest, many chromophores that absorb light have extremely low fluorescence. This is because relaxation from the excited state to the ground state is predominantly non-radiative. Recently Min et al (Nature, 2009) showed how using stimulated emission, the fluorescence of such chromophores can be detected with high sensitivity. Their paper shows detection of (i) non-fluorescent variants of green fluorescent protein in living E. coli cells; (ii) the indigo chromogenic substrate resulting from lacZ expression even without induction of the substrate; (iii) the drug toluidine blue O used in photodynamic therapy, and (iv) hemoglobin.

4) Other Advances in Optical Microscope Image Acquisition

4.1) Increasing Sensitivity

Ongoing improvements in the sensitivity of microscope cameras combined with increasing photo-stability of fluorescence labels now enables detection of each fluorescent molecule 10,000s of times under optimal conditions. Utilizing such repetitive detection, it is possible to analyze the movement of each tagged macro-molecule (protein), and correlating the movement of one macro-molecule with another tagged macro-molecule enables determination of the on / off binding kinetics of the interaction between the two species. In conjunction with techniques such as image cross-correlations spectroscopy (Digman et al, Biophysical Journal, 2009), such kinetics can be simultaneously calculated at all locations across the image, providing a unique and powerful method to analyze local chemistry in living cells. For example questions about the differences in the dynamics of a given protein complex at the cell membrane versus internalized in the cytoplasm can be addressed.



4.2) Correlative Optical and Electron Microscopy (EM)

The major benefit of fluorescence microscopy (as well as vibrational spectroscopy) is that detected signals are specifically from the molecular species of interest, enabling optical microscopy to provide information that directly relates to main stream in vitro biochemistry and molecular biology analyses of specific molecular species. However, structural context is lacking in optical microscope images, often requiring the use of additional fluorescence labels targeting cellular structures and organelles. Alternatively, correlation with electron micrographs can provide this context with the advantage of much higher resolution of structural detail. Consequently, EM also serves a vital role to validate the localization of observed molecular distributions detected with super-resolution optical microscopy.

5) Image Analysis

Currently, analysis of optical microscope images is very limited and is directed to answering a particular preconceived question of the investigator, leaving the vast majority of the potential information in images untapped. Furthermore, the problem of archiving, analyzing and annotating microscopy images is compounded because the size of data sets is growing drastically due to both improved spatial resolution and increasing dimensionality. Four dimensional imaging (three spatial dimensions and time) is the norm in optical microscopy, and is essential for understanding the dynamics of candidate pharmaceuticals and their molecular targets. Yet, 4D images are extremely large and, more significantly, the information contained within them cannot be assimilated by the 2D human visual system without real-time computational pre-processing. Moreover, the curse of dimensionality is not limited to four. Spectral analysis can add a further three dimensions (absorption spectrum, emission spectrum and excited-state lifetime), raising the dimensionality to seven, while performing studies across large libraries of compounds or cell lines adds yet another dimension. Given the enormous size of microscope images and the high dimensionality, only automatic and quantitative computational analysis has the potential to comprehensively, robustly and accurately extract information from the images. This information, through mathematical modeling will lead to much more in depth understandings of molecular mechanisms.

Given the sheer complexity of image analysis when applied biological samples, it is not surprising that image analysis and visualization technologies are currently lacking. Yet, the time for these technologies is now, as biology advances beyond bulk, in vitro based measurements into the analysis of individual molecules in their true environment.

5) Issues

In this final section, several key issues are raised that need to be considered and addressed in order for the recent advances in spatial resolution and sensitivity to maximally benefit human health.

i) Image analysis: While dramatic advancements in microscope image acquisition are underway, driven largely by the big four microscope companies (Zeiss, Leica, Olympus



and Nikon), post-processing of images for information extraction and modeling significantly lags behind and is rate limiting.

ii) Deeper imaging: Understanding carcinogenesis requires analysis of both individual cells and analysis of cells in the context of others at the tissue level. However, the aforementioned discussion about improved spatial resolution and improved sensitivity applies to the thin layer of the sample the thickness of one cell or less juxtaposed to the supporting cover-slip. These improvements are lost deeper into living samples, because of spherical aberration and light scattering and absorption, leaving confocal and multi-photon microscopy still the techniques of choice for deep imaging. It will be a question for the future whether techniques such as adaptive optics and single plane illumination microscopy (SPIM) (Keller PJ, Stelzer EH. *Curr Opin Neurobiol.* 2008) can supersede confocal for deep tissue imaging.

iii) Perturbation of living cells in optical microscopy: Fluorescence labeling and exposure to high light intensities (approximately one million fold more intense than the sun) are not natural. While cells usually survive these conditions and labeled proteins appear to function normally, we are not analyzing the endogenous protein of interest in its natural environment.

iv) Validation: Unlike other imaging modalities, we do not have access to the ground truth about the composition of the sample in optical and electron biological microscopy. Instead we estimate the truth by extrapolating from correlative biochemical and structural biology studies, and by acceptance of familiar appearing structures in images. Hence test phantoms manufactured at the nano-scale are needed for robust validation of both image acquisition and image analysis methods.

6) Conclusion

Breaking the diffraction barrier of light enables optical microscopy to image at the molecular scale, enabling biochemistry and molecular biology to be performed on location in the natural and complex environment of the cell. This is beginning to lead to a much more integrated and complete understanding of normal versus diseased cell mechanisms, and consequently will dramatically improve our ability at the National Cancer Institute to devise therapies against cancer and HIV infection.