Biological Applications of Multiphoton NSOM with Multiple Spectroscopic Modes

Vinod Subramaniam, Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

Keywords: NSOM, SNOM, fluorescence spectroscopy, multiphoton excitation

INTRODUCTION

Near-field scanning optical microscopy (NSOM) combines the enhanced lateral and vertical resolution characteristics of scanning probe techniques with simultaneous measurements of optical signals, yielding resolutions beyond the limits of conventional diffraction optics; for a recent review see [1]. The ability of NSOM to simultaneously map topographic and optical properties with extremely high spatial resolution coupled with single-molecule fluorescence detection sensitivity has led to its development into an important technique for imaging biological systems.

The principle of NSOM is deceptively simple. The essence of the technique is the illumination of an object through a microscopic (sub-wavelength) aperture in an opaque screen positioned extremely close to the sample (which lies in the near-field of the electromagnetic radiation exiting the aperture, before diffraction effects are manifested; see Fig. 1). The resulting resolution is a function only of the aperture size and of the probe-sample distance, but not of the wavelength of the illuminating light. The probe is then scanned relative to the sample while maintaining a fixed probe-sample separation (thus generating a topographic image of the sample) and simultaneously producing optical information (absorption, scattering, and/or fluorescence).

The most commonly used near-field probe is an aluminum-coated tapered optical fibre with an aperture diameter ~100 nm. The light transmitted through, scattered or emitted from the sample is generally collected with an objective lens (Fig. 1b), and processed. Fluorescence is the prevalent optical contrast method for NSOM of biological samples. Fluorescence probes are specific, excessively sensitive, and exhibit photophysical processes and properties (spectra, lifetime, polarization, energy transfer) that can be exploited to yield information about the structure and dynamics of the molecule being studied. The unique capability of NSOM to provide a correlation between high-resolution surface topography and the fluorescence signal(s), not achievable in other optical microscopies, is of particular relevance in biological applications. Thus, for example, one can ask questions regarding the distribution of cell surface receptors for external signalling molecules such as growth factors and hormones. In combination with fluorescence resonance energy transfer (FRET) methods, NSOM can be operated with contrast modes dependent on intermolecular separations on the order of 2-10 nm, yielding an additional improvement in resolution.

Biological applications of NSOM pose several challenges, including the difficulty of imaging soft samples, tracking large topographical changes, accounting for tip-sample interactions, and rationalizing resolution and contrast mechanisms. In particular, operation under water or in physiological buffer solutions, a critical requirement for realistic biological studies, poses problems for implementation in bacteria, in fruitfly embryo cells, and in mammalian cells as a fusion protein with the epidermal growth factor (EGF) receptor [12,13]; distributions of transmembrane receptors for epidermal (EGF) and platelet-derived (PDGF) growth factors [14]; clustering of the erbB2 receptor tyrosine kinases [2]; interactions of cell surface proteins via FRET [13,15]; and multiphoton (two-, three-photon) simultaneous excitation of probes for different intracellular compartments (mitochondrial, nuclear) [16-17].

The instrument has been operated to date in a shared-aperture mode, i.e. with uncoated tips supplying a near-field mode for excitation as well as for detection of fluorescence signals (Fig. 1b). Such tips are formed in a highly...
reproducible single step heating and pulling process from telecommunication optical fibres. Although the illumination is not as well localized as with standard (metal-coated) aperture probes, the requirement for the excitation light and the collected fluorescence to pass through the fibre tip results in an automatic alignment of the excitation/emission paths with an effective lateral imaging resolution of 150-200 nm, i.e. considerably better than the diffraction limit of the conventional optical microscope. The fibre tip is mounted in a shear-force sensor head mounted above the piezoelectric tube scanner of the Digital Instruments SPM. The sample is laterally scanned beneath the fibre tip and a distance of 2-5 nm is maintained by monitoring shear-force interactions between the laterally vibrated tip and the sample surface. The scanning process is controlled by the electronics of the SPM system.

We have implemented multiple photophysical modes in our modular microscope, including: (i) multiple laser sources; (ii) dual detection channels; (iii) spectrograph operation for spectral acquisition; (iv) high-intensity continuous-wave (cw) or pulsed excitation for multiple photon (two-photon, three-photon) excitation (MPE); (v) various modalities for detection of fluorescence resonance energy transfer (FRET), and (vi) fluorescent lifetime detection in the frequency domain. A schematic of the SPM system depicting the multiple excitation and detection schemes is shown in Fig. 2.

APPLICATIONS
Green Fluorescent Proteins in Cells

Green fluorescent proteins (GFP) isolated from certain species of jellyfish have attracted enormous attention in recent years due to their use as reporter molecules in cell, developmental and molecular biology. GFP can be fused to a variety of proteins without affecting their function. These proteins are expressed in vivo and thus act as remarkably versatile indicators of structure and function within cells. The GFP fusion proteins can be visualized and localized in cells and embryos using standard microscopy techniques. We have expressed, imaged, and performed site-specific spectroscopy on various mutants of GFP in bacteria, Drosophila Schneider cells, and expressed in mammalian cells as a GFP-receptor construct.

Figure 3 shows shear force topography (A), feedback error signal (B), and fluorescence (C) images of a Chinese hamster ovary (CHO) cell stably transfected with a fusion construct of the epidermal growth factor receptor (EGFR) and GFP, allowing visualization of the distribution of cell surface EGFR receptors.

Multiphoton Excitation and Multicolour Imaging

The excitation spectra of several biologically relevant dyes lie in the near-ultraviolet region, thereby posing serious problems for laser scanning microscopy. High-energy UV light is generally scattered and absorbed strongly by biological tissue, deteriorating image quality and inducing photodamage of living cells.

Continuous-Wave Two-Photon Imaging

The 647 nm emission of an Ar-Kr mixed gas laser was coupled into the NSOM and used to excite the UV-absorbing DNA dyes DAPI, the bisbenzimidazole Hoechst 33342 (BBI-342), and the UV- and visible-absorbing intercalating dye ethidium bromide. Polytene chromosomes of Drosophila melanogaster and the nuclei of 3T3 Balb/c cells labeled with these dyes were readily imaged. Figure 4 depicts shear force topography and 2PE-fluorescence images of a Drosophila polytene chromosome. Note that in the topographic image cellular debris from the squashing procedure is clearly visible, but is absent from the fluorescence image, because the enhancement of DAPI fluorescence occurs only when the dye is bound to double-stranded DNA.

The multiphoton origin of the detected fluorescence was confirmed by measuring the fluorescence intensity as a function of excitation power, which showed the expected (2nd order) dependence on the excitation power in the range of 8 - 180 mW.

Picoscend Pulsed Multiphoton Imaging

We have also implemented pulsed picosecond multiphoton excitation in the NSOM using a solid-state Nd:YVO4 laser emitting at 1064 nm. With this system we have achieved simultaneous three-photon excitation of near-UV absorbing fluorophores and two-photon excitation of dyes excitable in the visible green range in the NSOM. This combination of multiphoton excitations offers the possibility of simultaneous dual-colour imaging. Additionally the use of a frequency-doubled cw Nd:YAG laser in this configuration allows us to compare one- and two-photon excitation of the same sample directly.

Figure 5 shows shear force topography (A), two-photon excitation fluorescence (B), and three-photon excitation fluorescence (C) images of dried MCF7 adenocarcinoma cells labelled with the mitochondrial specific dye MitoTracker Orange CM-H$_2$TMRos (M7511, Molecular Probes, Leiden, The Netherlands), and the nuclear stain BBI-342 (Calbiochem, Bad Soden, Germany). The contrast was excellent without any crosstalk between the emission signals. The fluorescence intensity showed the expected non-linear (2nd and 3rd order) dependence on the excitation power in the range of 5 - 50 mW. The smallest resolved structures had a width of &lt; 200 nm, a value - 1/6 of the fundamental wavelength or 1/3 the wavelength corresponding to the two-photon energy.

A prominent advantage of multiphoton excitation is the confinement of the excitation to the focal volume, yielding lower collateral damage to the sample due to photobleaching. Indeed we observe reduced photobleaching effects due to light leakage from the sides of the fibre tip in the case of multiphoton excitation. This result has important implications for the microscopy of light sensitive samples.
imaging of which by conventional line-scan 1PE can involve photodamage in neighboring as yet unscanned regions. The effect is apparently suppressed, or at least diminished significantly, in 2PE-NSOM.

Multiphoton excitation extends the accessible spectral range to the near-UV and also allows multicolor imaging of two different dyes free of crosstalk. In addition, the background signals are significantly reduced, constituting one of the primary advantages of multiphoton excitation. The thickness of the surface layer contributing to the optical signal and the lateral extent of the excitation are also reduced significantly.

PROSPECTS
NSOM in combination with fluorescence methodologies represents a powerful technique for the visualization of cellular systems and biological interactions with a spatial resolution not routinely achievable with far-field optical microscopy. The integration of spectral resolution, lifetime determination, and multiphoton excitation techniques with NSOM has significantly enhanced the capabilities of this microscopy mode. The extension of NSOM to the challenging task of performing near-field microspectroscopy on living cellular systems in aqueous environments promises to reveal new insights into the mechanisms and dynamics of receptor-ligand interactions, and in general, the physiology of the initial stages of receptor signal transduction pathways.

ACKNOWLEDGEMENTS
The author would like to thank Professors M. Aono and Y. Yamamoto for their invaluable discussion and support.

REFERENCES
9. Ha, T., et al. Probing the interaction between two single molecules. Fluorescence resonance energy transfer between a single donor and a single acceptor. PNAS 93, 6264-6268, 1996