

Cell biology beyond the diffraction limit: near-field scanning optical microscopy

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Summary

Throughout the years, fluorescence microscopy has proven to be an extremely versatile tool for cell biologists to study live cells. Its high sensitivity and non-invasiveness, together with the ever-growing spectrum of sophisticated fluorescent indicators, ensure that it will continue to have a prominent role in the future. A drawback of light microscopy is the fundamental limit of the attainable spatial resolution – ~250 nm – dictated by the laws of diffraction. The challenge to break this diffraction limit has led to the development of several novel imaging techniques. One of them, near-field scanning optical microscopy (NSOM), allows fluorescence imaging at a resolution of only a few tens of nanometers

and, because of the extremely small near-field excitation volume, reduces background fluorescence from the cytoplasm to the extent that single-molecule detection sensitivity becomes within reach. NSOM allows detection of individual fluorescent proteins as part of multimolecular complexes on the surface of fixed cells, and similar results should be achievable under physiological conditions in the near future.

Key words: Super-resolution, Near-field scanning optical microscopy, Single-molecule detection, Distribution, Cell surface, Membrane, GFP

Introduction

Fluorescence microscopy has gained a firm position among the most important research tools in modern cell biology. It offers high chemically specific contrast, and is non-invasive, and sample handling is non-destructive and therefore allows the study of living cells. Moreover, optical detection is very fast, providing picosecond time resolution, and the very nature of light offers additional contrast mechanisms (e.g. based on polarization or phase discrimination) that yield additional information. Furthermore, imaging can be performed in three dimensions by multi-photon excitation, confocal detection or deconvolution techniques. Finally, a wide range of ultra-sensitive detectors – for example, cooled CCD (charge-coupled device) cameras and photon-counting devices – that make optical detection extremely sensitive, even down to the single-molecule level, are available.

The ability to tailor fluorescent fusion proteins, exploiting the strong autofluorescence of the green fluorescent protein (GFP) family (Patterson et al., 2001), has fueled interest in fluorescence microscopy even further. The most successful applications of these fluorescent proteins are in gene expression, protein targeting and trafficking, and protein-protein interaction studies (Tsien, 1998; Lippincott-Schwartz et al., 2001). Employing various forms of these fluorescent proteins, in most cases using fluorescence resonance energy transfer (FRET) as a read-out (Wouters et al., 2001), highly potent cellular indicators for calcium (Miyawaki et al., 1997; Miyawaki et al., 1999), pH (Lopes et al., 1998), cyclic AMP (Zaccolo et al., 2000), cyclic GMP (Honda et al., 2001), caspases (Harpur, 2001) and small G protein activity (Mochizuki et al., 2001), among others, have been developed.

The limit to the resolution that can be reached in optical

imaging techniques is directly related to the wavelength of the light. This diffraction limit originates from the fact that it is impossible to focus light to a spot smaller than half its wavelength. In practice this means that the maximal resolution in optical microscopy is ~250-300 nm. Since a large body of evidence indicates that dynamic cell-signaling events start by oligomerization and interaction of individual proteins (i.e. on the molecular scale), the need for imaging techniques that have a higher resolution is growing. Traditionally, high-resolution cell biology (Table 1) is the arena of electron microscopy, which offers superb resolution but lacks the above-mentioned advantages of fluorescence microscopy. The advent of scanning probe microscopy (Table 1), and especially atomic force microscopy (AFM), in which an atomically sharp probe attached to a cantilever is scanned over the surface of interest, has made nanometer resolution also attainable on living cells (Hansma et al., 1994; Putman et al., 1994). However, although AFM produces a high-resolution topographical picture of the sample, it lacks chemical specificity. Hence, although individual molecules can be seen, their identities cannot be defined. This seriously limits the usefulness of AFM for high-resolution imaging on cells. Initially, this contrast problem was tackled by the use of immunogold-labeling approaches (Damjanovich et al., 1995; Neagu et al., 1994). A promising new way around the problem comes from work on the specific labeling of the AFM probe with biomolecules (e.g. with antibodies or ligands). This introduces a contrast mechanism based on specific interactions between the probe and certain types of molecule in the specimen (Willemsen et al., 2000). Other attempts to enhance AFM contrast involve the modification of the probe by fluorescent molecules, which introduces an optical contrast

Table 1. High-resolution imaging in cell biology

Type	Subtype	Remarks	Application	References	
Light microscopy	Wide field, phase-contrast, differential interference contrast (DIC), etc.	Max. resolution 250 nm Spectral, polarization and phase contrast	TIRF: cell-substrate contact zone, tunable penetration depth DIC: cell-substrate contacts, focal adhesion	Smilenov et al., 1999 (IRM) Lippincott-Schwartz et al., 2001 (FRAP)	
	Confocal laser scanning microscopy (CLSM)	Relatively easy to do Widely accessible	Intermolecular and intramolecular dynamics by FRET	Toomre and Manstein, 2001 (TIRF)	
	Total internal reflection fluorescence microscopy (TIRF) (evanescent wave microscopy)	Non invasive Possible on living cells	CLSM, FRAP	Johns et al., 2001 (TIRF)	
	Reflection contrast microscopy: interference reflection microscopy (IRM)	Dynamics; protein diffusion, targeting and trafficking	Various microscopy imaging Single molecule imaging (Table 2)	Richter et al., 2000 (IRM)	
	Electron microscopy	Scanning EM Transmission EM Scanning transmission EM	Max. resolution 0.1 nm Contrast based on density Requires expert knowledge Invasive Not possible on living cells	Cell surface Cell sections Immunogold labeling Mass determination Imaging of sub-molecular structure	Allen et al., 2000 Muller and Engel, 2001
*Scanning probe microscopy	Subtype	Contrast	Surface techniques	Cell volume changes	Korchev et al., 2000a; Korchev et al., 2000b (SICM, cell volume)
	SICM	Ionic current	Simultaneous topography imaging	Localization of single ion-channel on cell surface	
	SCM/ScAM	Dielectric properties	Resolution dependent on probe radius (tens of nm) and sample physical properties	Polymer science/tissue engineering	Willemsen et al., 2000 (AFM, Forces)
	SCPM	Chemical potential	Requires expert knowledge	Single-molecule imaging	Engel and Muller, 2000 (AFM, sub-molecular resolution)
	SThM	Thermocouple junction scanned across sample	Apart from AFM techniques still in experimental phase	Molecular interactions in cells (AFM force imaging)	Lehenkari et al., 2000 (AFM in cell biology)
AFM	Short-range interaction forces between tip and sample	Non invasive Possible on living cells	Sub-molecular resolution of proteins by carbon nanotubes (AFM)	Woolley et al., 2000 (AFM, nanotubes)	
NSOM/ PSTM	Optical, sub-wavelength optical fiber probe	NSOM: optical contrast combined with topography Optical resolution depending on probe aperture diameter (20-120 nm) and probe-sample distance (less than 5-10 nm) Possible on living cells (see text)	See Tables 2 and 3	See Tables 2 and 3	

*A detailed classification of scanning probe microscopies can be found in Friedbacher and Fuchs, 1999.

mechanism (Vickery and Dunn, 2001). Currently, however, the combination of high-resolution scanning probe and fluorescence microscopy is the realm of another scanning probe technique: near-field scanning optical microscopy (NSOM).

NSOM

In NSOM, as in the case of AFM, a sharp probe physically scans the sample surface (Fig. 1). Besides topography, NSOM also generates optical images. A typical NSOM configuration is shown in Fig. 2. The practical feasibility of this kind of NSOM was first demonstrated by Pohl et al., immediately following the advent of scanning probe microscopy and in fact before the introduction of the AFM (Pohl et al., 1984). The most generally applied near-field optical probe consists of a small aperture, typically 20-120 nm in diameter (i.e. much smaller than the wavelength of the excitation light), at the end of a metal-coated tapered optical fiber* (Fig. 3A,B). This probe is the most critical element of the technique and is difficult to produce reliably and in large quantities (Van Hulst et al., 2000).

*Other types of probe exist; for example, aperture-less and non-metal-coated aperture type probes have been described. Applications of the former in biology have, to our knowledge, not yet been described; examples of the latter are reviewed below.

In fluorescence mode*, it serves as a constriction that funnels an incident light wave to dimensions that are substantially below the diffraction limit. This results in a light-source that has the size of the aperture. However, in contrast to common light sources such as lightbulbs and lasers, the light emitted by the probe is predominantly composed of evanescent waves rather than propagating waves. The intensity of the evanescent light decays exponentially and to insignificant levels ~100 nm from the aperture. Effectively, the probe can excite fluorophores only within a layer of <100 nm from the probe – that is, in the ‘near-field’ region (Fig. 3B). Sample fluorescence can subsequently be collected by conventional optics (Fig. 2) and transformed into an optical image of the sample surface in which the resolution is now primarily dictated by the aperture dimensions rather than by the wavelength of the light.

Since the near-field intensity decays so rapidly with distance from the probe, for efficient excitation it is essential to have accurate control of the probe-sample distance during scanning. As in AFM, this can be accomplished by using a (force) feedback loop. However, in contrast to regular AFM, which

*Here, we focus on fluorescence-mode NSOM. Applications of transmission-type NSOM to biological systems have also been described; however, for these the reader is referred to Van Hulst and Moers (Van Hulst and Moers, 1996).

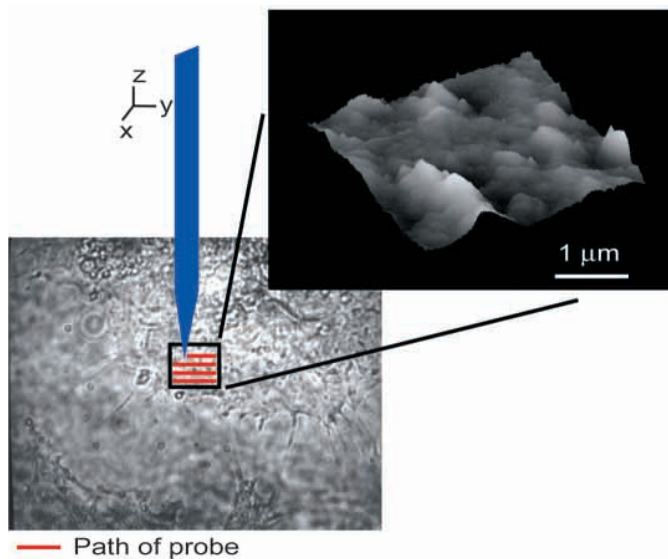


Fig. 1. The principle of scanning probe microscopy. In AFM and NSOM, a sharp probe is used to map the topographic features on the sample surface accurately. This is done by physically scanning the probe over the surface while maintaining a constant probe-sample distance by force feedback.

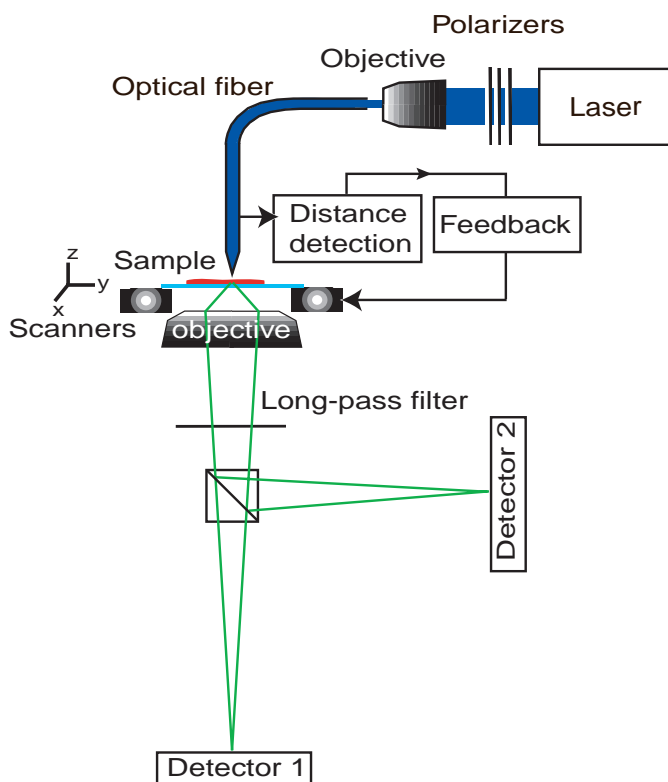


Fig. 2. Schematic lay out of a near-field scanning optical microscope. The NSOM probe is a tapered optical fiber (Fig. 3A). Laser light is coupled into the fiber and is used to excite fluorophores as the probe scans the sample surface. The probe-sample distance is maintained constant at <10 nm during scanning by shear-force-based distance detection in combination with an electronic feedback system controlling the piezoelectric scan stage. Fluorescence is collected by a conventional inverted microscope. Dual-channel optical detection allows wavelength and/or polarization discrimination.

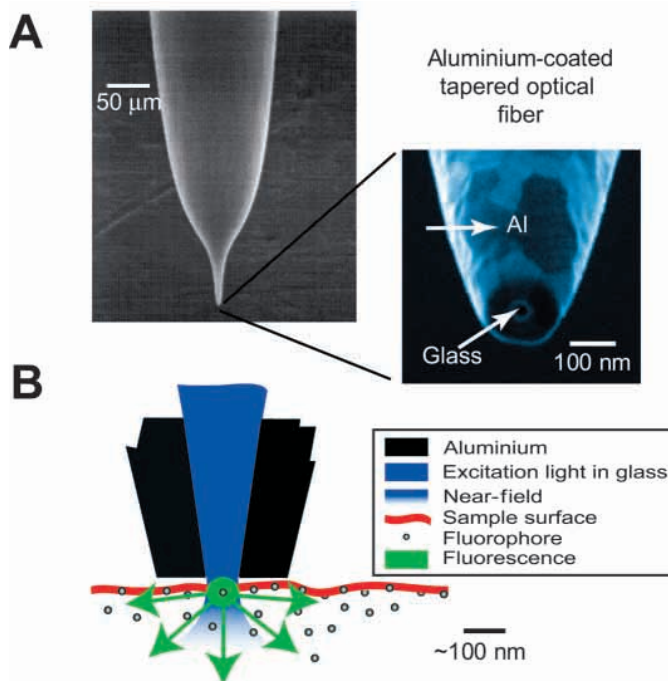


Fig. 3. The near-field optical probe. (A) An optical fiber is pulled to a final diameter of 20–120 nm and subsequently coated with aluminium. This coating serves to confine the light to the tip region. A subsequent etching step results in a flat and circular endpoint and aperture. The aperture functions as a miniature light source, and its diameter primarily determines the optical resolution of the microscope. (B) The principle of surface-specific excitation. The optical near-field generated at the aperture has significant intensity only in a layer of <100 nm from the aperture; lower lying fluorophores are therefore not excited. Hence, background fluorescence is effectively suppressed. This forms the basis for the high optical detection sensitivity of this technique.

exploits the bending of a cantilever attached to the probe as a direct measure of the probe-sample interaction force, in NSOM an indirect method, based on shear-force damping, is commonly used. For this, the NSOM probe, or a piezoelectric tuning fork attached to it, is oscillated at its resonance frequency in a lateral vibrational mode (with a <1 nm amplitude); when in proximity to the sample, shear-forces dampen this motion and induce measurable changes in the oscillation amplitude and phase. An electronic feedback system, controlling the probe-sample distance directly through the piezoelectric scan stage, is subsequently used to maintain a constant oscillation amplitude/phase during scanning. In this way, a constant probe-sample distance of <10 nm is realized (Fig. 2). The feedback signal itself, as in AFM, is used to generate a topographic map of the sample surface (Fig. 1). Of course, unique to NSOM is the fact that a corresponding fluorescence map is simultaneously generated.

The optical detection sensitivity of NSOM depends largely on the extremely small excitation/detection volume set by the aperture dimensions as well as the depth of penetration of the near-field into the specimen. Together, these properties effectively reduce background fluorescence and thereby enhance detection sensitivity. Betzig and Chichester exploited this eight years ago, providing the first observation of single-molecule fluorescence under ambient conditions (Betzig and

Table 2. Single-molecule fluorescence microscopy

Technique	Application	Living cells?	Resolution	Localization accuracy	Max. no. of dyes per μm^2	References
Wide-field	Single molecule diffusion in cell and artificial membranes	Yes	<250 nm	<<40 nm	<10	Schmidt et al., 1996 Schütz et al., 2000 Harms et al., 2001
Confocal	Enzyme/DNA conformational dynamics studied by single pair FRET Single dye photophysics	Yes	~250 nm	~40 nm	10	Weiss, 1999 Ha et al., 1999 Moerner et al., 1999 Van Oijen et al., 1999 Deniz et al., 1999 Talaga et al., 2000
TIRF	Cell-substrate contact zone Inter- and intramolecular interactions and dynamics; FRET Single molecule diffusion	Yes	~250 nm	~40 nm	10	Ishijima et al., 1998 Kitamura et al., 1999 Ishii and Yanagida, 2000 Sako et al., 2000
NSOM	Single GFP/dye photophysics DNA conformation studied by single pair FRET Single molecule spectroscopy Lifetimes of single dyes	Not yet	>50 nm possible	>6 nm	100	Betzig et al., 1993 Xie and Dunn, 1994 Ambrose et al., 1994 Ha et al., 1996 Ruiter et al., 1997 Garcia-Parajo et al., 1999 Garcia-Parajo et al., 2000 Van Hulst et al., 2000

Chichester, 1993). Furthermore, employing the polarization characteristics of the near-field, they showed that it is possible to determine the full spatial orientation of fluorescent molecules by making use of polarization-sensitive fluorescence detection (see below). Subsequently, developments in instrumentation that greatly improved signal-to-background ratios allowed single-molecule fluorescence studies to come within reach of far-field methods such as total internal reflection, confocal and bright-field microscopy (see Table 2). The advantage of single-molecule studies is that they provide a way to monitor time-dependent processes and reaction pathways in non-equilibrated systems, which reveals the distribution of a given molecular property instead of a statistical average. This has already led to a whole new frontier in science, its applications ranging from basic photo-physics and material research to biology (Frontiers in Chemistry, 1999; Xie and Lu, 1999; Sako et al., 2000; Ishii and Yanagida, 2000). Nowadays almost all experiments in this field use far-field methods, mainly because they are relatively easy to use. However, the obvious disadvantage is that, because of the diffraction-limited system response, only scarcely labeled samples can be studied, but, in biology, molecules are usually present in close proximity. The combination of topographical information, optical super-resolution and single-molecule detection sensitivity therefore makes NSOM a unique tool for biological applications.

Biological applications of NSOM

A large body of evidence indicates that (induced) lateral organization in the plasma membrane is crucial to trigger cell signaling. One example is the early events in signal transduction by the epidermal growth factor receptor (EGFR), dimerization and phosphorylation following EGF binding (Yarden and Schlessinger, 1987a; Yarden and Schlessinger, 1987b). Another is the formation of focal adhesions, large signaling complexes involved in cell-adhesion and migration,

and their equivalent on leukocytes, 'adhesionomes'. An important first step in the assembly of such complexes appears to be clustering of integrin receptors (Balaban et al., 2001; Van Kooyk and Figdor, 2000). Understanding of the signaling capacities of these adhesion sites requires insight into their assembly and spatial organization on the molecular level. The concept of 'lipid rafts' provides yet another level of organization in the plasma membrane at the submicroscopic level (Simons and Toomre, 2000). So far, these phenomena have been studied by use of far-field optical techniques, such as TIRF, IRM and confocal microscopy (Table 1). Clearly, higher-resolution/sensitivity would produce a major step forward in our understanding of the underlying mechanisms.

So far, most applications of NSOM in biology involve systems that are more or less isolated (Table 3) – for example, studies on fluorescently labeled chromosomes (Moers et al., 1996), DNA (Ha et al., 1996; Garcia-Parajo et al., 1998) and purified fluorescent proteins (Garcia-Parajo et al., 1999; Garcia-Parajo et al., 2000). Cell biological studies include fluorescence imaging of cytoskeletal components in 3T3 fibroblasts (Betzig et al., 1993) and colocalization of malarial and host skeletal proteins on malaria-infected erythrocytes (Enderle et al., 1997). Furthermore, sub-wavelength-sized membrane patches in human skin fibroblasts (Hwang et al., 1998) and activation-dependent receptor clustering on a human breast carcinoma cell line have also been studied (Nagy et al., 1999). Although these studies show a resolution well beyond that of a confocal microscope, to the best of our knowledge no study showing single-molecule detection sensitivity in a cell membrane by NSOM has been reported.

Single-molecule detection on cells by NSOM

To demonstrate the potential of near-field optical microscopy to study the distribution and orientation of cell surface

Table 3. Biological applications of NSOM

System	Mode	Type of analysis	Air	Liquid	References
Mouse fibroblast	Aperture type, metal-coated fiber	High-resolution imaging of actin cytoskeleton in a cell protrusion	+		Betzig et al., 1993
	Shared aperture (collection mode), non-coated probe	Study on the localized interactions between labeled conA molecules on the surface of 3T3 fibroblasts (FRET)	+		Kirsch et al., 1999
DNA	Aperture type, metal-coated fiber	DNA conformation probed using dual-labeled oligomers (FRET)	+		Ha et al., 1996
Human erythrocytes	Aperture type, metal-coated fiber	Imaging of molecular colocalization of malarial and host skeletal protein on the cell membrane	+		Enderle et al., 1997
Human skin fibroblast	Aperture type, metal-coated fiber	Investigation of cell membrane domains	+	+	Hwang et al., 1998
Human breast tumor cell	Shared aperture (collection mode), non-coated probe	Detection and characterization of ErbB2 clustering on the surface of quiescent and activated cells			Nagy et al., 1999
Chromosome	Aperture type, metal-coated fiber	High-resolution fluorescence in situ hybridization	+		Van Hulst and Moers, 1996 Hausmann et al., 2001

molecules, we investigated the distribution of LFA-1 (α L/ β 2 integrin) molecules in murine fibroblasts. Fig. 4A shows a bright-field image as well as a near-field 'zoom-in' on the surface of a fixed and critical-point-dried L-cell (a mouse fibroblast cell line) expressing human GFP-tagged LFA-1*.

The near-field image shows a number of interesting findings. First individual fluorescent LFA-1 molecules (circles, squares and hexagon) as well as clusters of molecules can be recognized (arrows). Secondly, one can observe different orientations of the GFP linked to the LFA-1 β chain, as determined by the polarization of the emitted light. The latter is of great value to discriminate LFA-1 molecules that are closer than half the tip-aperture diameter. Although these molecules cannot be discriminated by their fluorescence intensity signal, the difference in polarization contrast reveals them as separate entities (Van Hulst et al., 2000). Finally, the figure shows examples of photodissociation (circles) and the characteristic blinking behavior (squares and inset) of single molecule GFP fluorescence (Garcia-Parajo et al., 2000). Fig. 4B depicts a line trace through the feature marked with a hexagon, showing a signal-to-background ratio of 7 and a 90 nm full width at half maximum (FWHM; arrows). From the latter, one can calculate that the lateral resolution in this image is \sim 40 nm (Van Hulst et al., 2000). Taken together, the discrete photo-behavior, defined polarization characteristics and the number of counts per spot provide compelling evidence that individual molecules can be recognized on the cell surface by NSOM.

Single-molecule localization accuracy by NSOM as compared to other microscopical techniques

A direct comparison between the localization accuracy of cell membrane receptor molecules of NSOM and far-field imaging techniques is instructive (Table 2). In confocal fluorescence studies, using a 1.3 numerical aperture objective, two groups have reported a lateral positioning accuracy of 30-40 nm (Schmidt et al., 1996; Van Oijen et al., 1998). For 10^4 collected photons and a 70 nm aperture probe, NSOM can be calculated to attain a localization accuracy of 6 nm – a level comparable to the FRET interaction-distance regime (0-10 nm). Given a

resolution of \sim 50 nm, NSOM should allow independent observation of several hundreds of molecules/ μm^2 , which is an order of magnitude more than that achievable by far-field methods. We expect the achievable level of sensitivity of near-field methodology to be sufficient to tackle questions related to the distribution of molecules that are packed at physiologically relevant densities on the cell surface. We therefore anticipate that NSOM will yield results comparable to those obtained by (immunogold) transmission electron microscopy but under physiological conditions.

These intrinsic advantages of NSOM might revolutionize the life sciences. But why is NSOM hardly used in cell biology, despite its development almost two decades ago? First, as an imaging tool, NSOM is a complex technique that requires well-trained operators. Second, as we point out above, the resolution and sensitivity of a near-field microscope depend strongly on the quality of the probe aperture and the accuracy of the feedback system, necessitating full control of the technology. Moreover, the top-grade near-field microscopes are still under development in experimental physics laboratories and are therefore not widely accessible. Perhaps most important is the fact that, despite great efforts of various groups including our own, the standards set by the initial work of Betzig and Chichester cannot yet be reached in a liquid and more physiological environment. This is the technical challenge that has to be faced in the coming years.

Conclusion/perspectives

NSOM combines the high resolution of scanning probe microscopy with the contrast of optical microscopy. It can achieve single-molecule detection sensitivity on the cell surface and allows independent observation of molecules at physiologically relevant packing densities. The level of detail and sensitivity offered by this technique will be of particular value in studies of the spatial organization of the plasma membrane. Co-localization studies, a common application of far-field fluorescence imaging in cell biology, when performed by NSOM will provide unprecedented detail and accuracy, which are impossible to obtain by diffraction-limited imaging techniques. Potentially of great importance will be the application of NSOM in single-pair FRET studies combined with high-resolution imaging to study intermolecular

*Sample preparation will be detailed elsewhere.

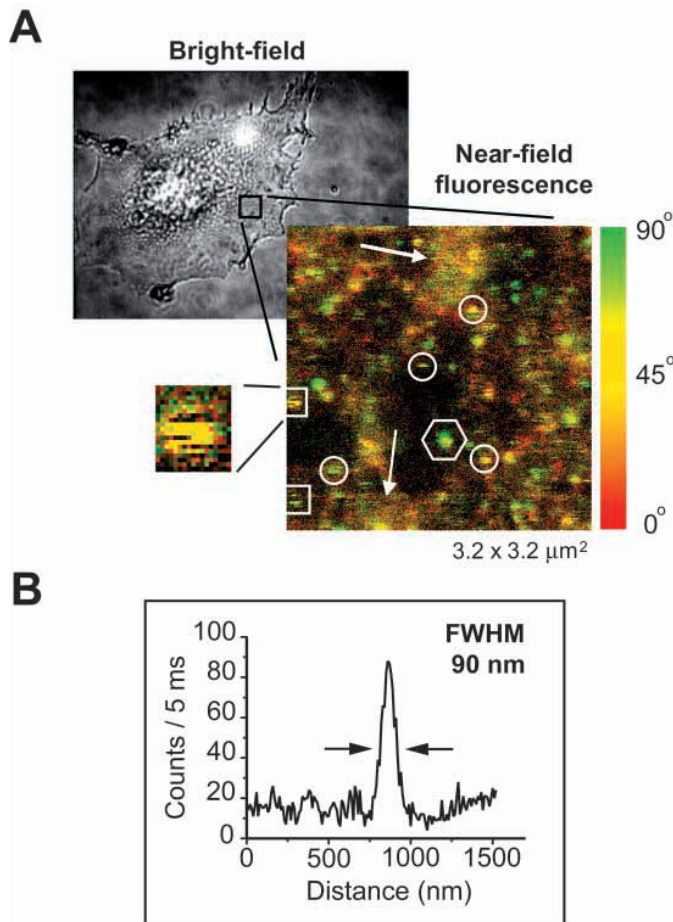


Fig. 4. (A) Single molecule detection on cells by NSOM. This figure shows a 40 nm optical resolution near-field 'zoom-in' on the indicated area ($3.2 \times 3.2 \mu\text{m}^2$) in the bright-field image of a fibroblast expressing LFA-1-GFP. GFP excitation is accomplished using 488 nm light (Ar-Kr laser line) linearly polarized along 90° . Fluorescence is collected by a 1.3 numerical aperture oil-immersion objective in combination with standard optical filters. A polarizing beam-splitter cube (Newport, Fountain Valley, CA) is used to split the fluorescence signal into two perpendicular polarized components (compare with Fig. 2). Both signals are then detected by photon-counting avalanche photodiode detectors (APD, SPCM-100, EG&G Electro optics, Quebec). The red/green color-coding of the signals reflects the orientation of the GFP molecules in the plane of the sample. Examples of clustered molecules (arrows) as well as examples showing clear single-molecule detection sensitivity are indicated (circles and squares). The squares show the fast-blinking behavior typical of single molecule GFP fluorescence. The circles present demonstrations of discrete photodissociation phenomena.

(B) Estimation of the resolution in the near-field image. This figure shows a line trace through the feature marked with the hexagon in the near-field image. The full width at half maximum (FWHM; arrows) of such traces can be used to obtain an estimate for the maximal resolution (half the FWHM) in the near-field image. On this basis, we estimate the resolution in the near-field image to be ~ 40 nm.

interactions or intramolecular dynamics at the single-molecule level on cells. The first such examples have already been reported (Kirsch et al., 1999); however, although clearly showing resolution below the diffraction limit, single-molecule detection sensitivity has not yet been reached. Importantly,

NSOM bridges the gap between the diffraction-limited response of normal light microscopy and the 5–10 nm distance sensitivity inherent in FRET and, as such, already provides important additional but otherwise unobtainable information.

In the past decade, especially the past two years, several other approaches to break the diffraction limit have been developed. These include interferometric microscopy methods such as 4Pi confocal microscopy (Hell and Stelzer, 1992), 15M (Gustafsson et al., 1999), standing-wave-total-internal-reflection fluorescence microscopy (Cragg and So, 2000) and harmonic excitation light microscopy (Frohn et al., 2000). These methods, however, do not have single-molecule detection sensitivity, and all require extended electronic post-processing of the images. Recently, a novel technique involving 'point spread function (PSF) engineering', which exploits stimulated emission depletion (STED), has been described (Klar et al., 2000). This method involves the induced quenching of fluorescence by stimulated emission at the rim of the diffraction-limited focal spot, thereby squeezing it to an almost spherical shape of ~ 100 nm in diameter. Thus far, this is the only technique that seriously rivals the small excitation/detection volume and therefore sensitivity of NSOM. The maximal resolution obtained with aperture-type NSOM relates to the limited energy throughput of the near-field probe. This limits the minimum size of the aperture to be used and hence the resolution of the microscope to ~ 20 nm. A possible way around this involves exploiting single-molecule emitters, attached to a scanning probe, which act as light source to excite molecules in the sample (Michaelis et al., 2000).

The most important technical challenge that remains is the construction of an NSOM instrument that operates under physiologically relevant conditions and allows the study of soft, rough and motile surfaces, such as the plasma membrane of living cells. When combined with single-molecule detection sensitivity and an optical resolution that is comparable to transmission electron microscopy, this will prove to be an invaluable tool in cell biology. These are truly exciting times.

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