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3 Fluorescence Imaging on the Nanoscale: Bioimaging Using Near-field Scanning Optical Microscopy

Linda J. Johnston

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Fluorescence microscopy is one of the most widely used tools for visualization of biological structures, despite the fact that diffraction of light limits the spatial resolution to several hundred nanometers for visible excitation. This review will focus on one method for overcoming the diffraction limit and achieving nanoscale spatial resolution in optical microscopy, namely near-field scanning optical microscopy. A brief overview of the technical details of various aperture and apertureless-based near field methods is presented, followed by examples that illustrate recent applications of near field techniques to cellular imaging. Finally, presepctives on new approaches and a comparison with recent developments in super-resolution fluorescence imaging are presented.

1 Introduction

Fluorescence microscopy has been widely used to visualize biological structures. It has a number of advantages, including the availability of a wide range of fluorescent probes with environment-sensitive properties, high sensitivity (down to the level of single molecules), high temporal resolution, straightforward implementation and low cost. Furthermore, the development of genetically encodable fluorescent proteins has greatly enhanced the possibilities for live cell fluorescence imaging. Nevertheless, diffraction restricts the maximum spatial resolution of optical microscopy to approximately half the wavelength of the excitation light, a very significant limitation for visualizing proteins and complexes, which are typically less than 10 nm in size, in the densely packed cellular environment. There have been many improvements since the first optical microscope designs over 400 years ago, including the use of a confocal geometry to reduce out-of-focus background and two-photon and total internal reflection methods to improve axial resolution; however, none has overcome the basic limitation imposed by diffraction of light. Alternative approaches such as scanning probe microscopy and electron microscopy do provide capabilities for high spatial resolution imaging, achieving molecular and even atomic scale resolution in some cases. However, they have significant issues with lack of chemical specificity (eg, with atomic force microscopy), harsh sample preparation requirements (eg, for electron microscopy) and incompatibility with imaging live cells. The last two decades have seen a revolution in optical microscopy with the development and application of two conceptually different strategies for achieving nanoscale spatial resolution.¹

The first of the approaches to overcome the fundamental limitation of diffraction of light was a family of near-field optical techniques that either utilize aperture-based probes to illuminate the sample or rely on the enhanced optical field in proximity to a sharp metallic tip to locally excite the sample.²⁻⁴ More recently the

development of far-field super-resolution methods based on the principle of
 45 switching fluorophores on and off sequentially in time has provided an alternative
 means of resolving subcellular structures.⁵ These include several ensemble methods
 based on targeted switching and readout; molecules are switched between on and off
 states (usually a fluorescent singlet state and a dark ground state) using a high
 intensity shaped laser beam to deplete “on” molecules at the edges of a diffraction
 50 limited excitation area. The first implementation, stimulated emission depletion
 microscopy (STED), uses one laser beam to excite fluorescence and a second
 doughnut-shaped high intensity beam at longer wavelength to stimulate emission
 from fluorophores at the edges of the excited region, thereby creating a sub-
 diffraction spot size.⁶ A second stochastic approach for achieving super-resolution
 55 is based on randomly switching on a low density of excited molecules such that
 individual molecules are always separated by greater than the diffraction limit.
 Individual molecules are imaged, the centers of their diffraction-limited spots are
 localized with nanoscale precision and images are created by repeating the
 activation/measure/bleach sequence many times. The two most common variations
 60 of the stochastic switching and readout approach are photoactivation localization
 microscopy (PALM) and stochastic optical reconstruction microscopy (STORM),
 which differ by the type of switchable probe used.²⁻⁴

This review covers recent progress in the application of near-field techniques to
 biological problems. The following section provides a brief overview of the near-
 65 field principle and the technical implementation of aperture-based near-field
 scanning optical microscopy (NSOM). The interested reader is referred to a number
 of excellent reviews that provide considerably more detail on various technical
 aspects of the development of NSOM microscopes and probes.⁷⁻¹⁴ Later sections
 discuss applications of near field methods for fluorescence imaging of biological
 70 samples, focusing primarily on results obtained since 2005. Much of the data
 reported in earlier studies was of the “proof-of-principle” variety, demonstrating
 impressive resolution, but often lacking in new biological insight. This review will
 emphasize detailed studies that put near-field imaging of biological samples on a
 quantitative footing and that attempt to correlate nanoscale organization with
 75 biological function. Recent advances in apertureless NSOM and combinations of
 NSOM with other methods are also presented, prior to concluding with a perspective
 on future directions and a comparison with super-resolution techniques.

2 Near-field Approaches to Overcome the Diffraction Limit

Diffraction limits the size of the spot to which light can be focused using
 80 conventional lenses. According to Abbe’s theory of diffraction and the Rayleigh
 criterion,¹⁵ two objects can be resolved when their separation, d , is greater than:

$$d = 0.61 \lambda / n \sin \alpha$$

where λ = wavelength and $n \sin \alpha$ is the numerical aperture, NA, of the focusing
 lens (n = refractive index of the medium, α = the angle of acceptance of the lens).
 85 When light is focused using a lens and the transmitted or emitted light is collected
 and imaged on a detector, only the far-field propagating component is collected
 because both the collection lens and detector are in the far-field (ie, at distances
 greater than many times the wavelength of the light). The non-propagating
 evanescent component that exists near the object at dimensions less than the

wavelength of light is not typically collected by the lens, hence the origin of Abbe's diffraction limit. Use of high numerical aperture objectives ($NA > 1$) leads to the general approximation that the lateral spatial resolution is limited to $\sim \lambda/2$ or 200-300 nm for visible light in the best case scenario with optimal optical components and good signal-to-noise. Although in principle one can improve resolution by going to shorter wavelength, this is not a practical solution for fluorescence microscopy.

The basic concept of using near-field optics to overcome the diffraction limit was originally developed by Syngue over 80 years ago (Fig. 1).¹⁶ He hypothesized that it was possible to deliver light through apertures with dimensions smaller than the wavelength of light. If the aperture was placed in close proximity to the sample (ie, in the near-field), then the light passing through the aperture could be used to image a specimen without diffraction degrading the resolution. Although the concept was relatively simple, significant advances in methods for fabricating nanometer-sized apertures and controlling the aperture-sample distance were necessary before the concept could be implemented. The first proof-of-principle experiments at optical wavelengths were reported simultaneously by two groups in 1984,^{17, 18} leading to the development of a family of NSOM methods.

Fig. 1. Basic principle of near-field illumination. Light passes through an aperture, a , with dimensions that are smaller than the wavelength (λ) of the incident light. The spatial resolution is determined by the aperture dimensions in the x-y plane and by the decay of the evanescent field in the z-dimension.

The most common method for implementing NSOM utilizes an optical fiber probe with a sub-wavelength aperture to illuminate the sample (Fig. 2). The probe is positioned close to the sample (within the near-field) and either fluorescence or transmitted light is collected using a high NA objective mounted underneath the sample. Images are obtained by raster-scanning the sample under the tip, giving simultaneous topographic and optical scans. One possible microscope configuration is shown in Fig. 3. A continuous laser beam is coupled into the optical fiber probe which is mounted in an AFM piezo scanner equipped with a position sensitive detector to regulate the tip-sample interaction. The probe and scanner are mounted on an x-y scanning stage supported on an inverted optical microscope. This has the advantage of allowing brightfield or epifluorescence measurements in order to locate specific areas of the sample prior to high resolution NSOM imaging. NSOM is also

Fig. 2. Schematic representation of the various approaches for near field scanning optical microscopy: A, aperture-based NSOM; B, tip-enhanced (apertureless) NSOM; c, Tip-on-aperture NSOM.

Fig. 3. Schematic of a near field fluorescence microscope based on an inverted optical microscope and using a cantilevered probe mounted on the z-scanner of an atomic force microscope. A bent NSOM probe is shown at top left.

frequently implemented on a laser scanning confocal microscope.

Although most applications for biological samples have employed an illumination geometry similar to that shown in Fig. 3, an alternate configuration employs sample irradiation through an objective and signal collection by an NSOM probe positioned

within the optical near-field. NSOM can also be configured in a reflection mode for non-transparent samples. In this case the probe may be used to both deliver and collect light from the sample. Alternately, the tip is used to illuminate the sample and collection optics are mounted to the side of the probe.

The two most important components of a near-field microscope are the probe and the system used to regulate the tip-sample distance; collectively they determine the attainable resolution and the ability to control the force exerted on the sample and to minimize damage to the sample. Various approaches have been used and the details are summarized in earlier reviews.^{9, 19} The first successful version of an NSOM probe was based on a tapered single mode optical fiber created by a laser heating and pulling method and then coated with aluminum.²⁰ Probes must be coated with metal, usually aluminum, to confine the light within the probe in regions where the probe dimensions are smaller than the wavelength of the light. Although metal coating is typically accomplished by vacuum evaporation using a shadow coating method to create an aperture, more recent approaches using focused ion beam milling give better-defined and more reproducible apertures. Chemical etching methods provide an alternate route to probe fabrication and can be used to create probes with a large cone angle, rather than the long tapered probe produced by laser heating/pulling.^{21,22} The shorter tapered region leads to increased light throughput. The transmission efficiency of NSOM probes decreases rapidly as the probe aperture decreases, which sets a practical limit of approximately 50 nm for the spatial resolution with an aperture based approach.

Two main methods have been used to regulate the tip-sample distance.^{9, 19} The first uses straight optical fiber probes with a shear-force feedback system based on either an optical or a tuning fork method. Alternately, bent cantilevered probes can be used in a regular tapping mode AFM operation (Fig. 3). The pros and cons of each method have been summarized.⁹ Fig. 4 shows a bent optical fiber probe created by a two-step chemical etching method and with a focused ion beam milled aperture. Probes of this design have been reported to have transmission efficiencies that are between 10 to 40 times higher than those for tapered optical fiber probes, and are compatible with cellular imaging and imaging in an aqueous environment.²²⁻²⁴ A typical image of a polymer sphere test sample is also presented in Fig. 4

Fig. 4. A scanning electron microscopy image of an aluminum-coated probe with an aperture (~ 90 nm in diameter) produced by focused ion beam milling (left). NSOM fluorescence image of 40-nm dye-loaded polymer spheres embedded in a thin polymer film and imaged with a 60-nm aperture probe (right).

3 Cellular Imaging with Aperture-based NSOM

The quest for nanoscale fluorescence imaging of biological samples has been motivated by the inability of conventional fluorescence microscopy to resolve sub-cellular structures. Since NSOM is inherently a surface-sensitive technique, applications have typically been directed towards understanding the organization or compartmentalization of cellular membranes. The classical fluid mosaic view²⁵ of the cell plasma membrane as a sea of lipids with freely diffusing proteins has now been replaced by a model in which the lateral organization of membrane receptors is domains plays a critical role in controlling protein-protein interactions and the assembly of signaling complexes.²⁶⁻²⁹ This compartmentalization of cellular membranes provides an important element of spatial and temporal control of cell signaling and can also modulate the interaction of the cell with its environment, as for example in invasion of viruses and bacteria. Frequently the localization of proteins in specific membrane domains is at least partly controlled by the lipid environment. In particular, cholesterol and sphingolipid-enriched domains known as lipid or membrane rafts have been shown to sequester specific classes of proteins.²⁷ Estimates of the size of lipid rafts are typically less than 200 nm and their small size and dynamic nature make their direct detection, especially in live cells, a significant challenge.³⁰ NSOM and other super-resolution techniques are an obvious solution to this problem.

The cellular imaging examples presented in this section all deal with a common theme of assessing the distribution of membrane receptors and domains and elucidating their impact on biological function. Although there are a few reports of live cell imaging using NSOM,³¹⁻³⁴ most detailed studies have examined fixed cells, either dry or in physiological buffer, generally using immunofluorescence labeling with specific antibodies to detect the protein or membrane domain of interest.

Membrane receptors and signaling complexes

One of the first applications of NSOM for visualization of signaling complexes examined β -adrenergic receptors (β AR), a widely-studied member of the G-protein coupled receptor family, an important class of therapeutic targets. Functional β_2 AR receptors were shown to be organized in clusters of ~140 nm average diameter with a cluster density of $\sim 1/\mu\text{m}^2$ in both immortalized H9C2 cells and primary murine cardiomyocytes, Fig. 5. Quantification of the cluster intensities was combined with a calibration based on the average fluorescence intensity of the antibody used to label the receptor and assumptions on labeling stoichiometry to estimate that the packing density of proteins varied from 12-72 for cluster diameters of 120-160 nm. Two-color experiments demonstrated that approximately 20% of clusters are colocalized with caveolae membrane domains. Adrenergic stimulation with the ligand isoproterenol produced no change in either cluster size or intensity, indicating that the receptor is pre-associated in nanoscale signaling islands. The preorganization of the receptor provides a mechanism for rapid response to β_2 AR agonists without the requirement for large scale diffusion to form signaling complexes. The ability to monitor organization of signaling complexes on a length scale that bridges the gap between confocal microscopy and the nearest-neighbour interactions probed by fluorescence resonance energy transfer illustrates the potential of NSOM to determine the stoichiometry of various proteins within signaling domains and provides new insight on how GPCRS produce cellular responses.^{35, 36}

Fig. 5. NSOM imaging of β_2 AR in fixed murine neonatal cardiac myocytes and H9C2 cells. (A, B) Simultaneously recorded NSOM topography and fluorescence images from neonatal myocytes. (C) A small scale fluorescence image for β_2 AR in H9C2 cells. (D) Histogram showing the distribution of cluster sizes for the receptor in unstimulated H9C2 cells. Reproduced from *Nat. Chem. Biol.* 2005, **1**, 196, with permission of Macmillan Publishers Ltd.

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Although the initial study of β_2 AR provided significant insight, the quantification of the receptor based on fluorescence intensities from immunofluorescence staining has some limitations. These include the requirement for assumptions concerning the stoichiometry of antibody labeling and the possibility that the large size of the labeling primary-secondary antibody complex leads to an underestimate of the number of receptors/cluster. These constraints have been addressed by expressing a fluorescent protein fusion (Venus- β_2 AR) in HEK293 cells at variable expression levels.³⁷ The results showed that the number of small nanometer-sized clusters increased by a factor of ~ 3 with increased Venus- β_2 AR expression level, but that their size was roughly constant with an average diameter of ~ 150 nm and with a range of 2 to ~ 60 proteins/cluster. The constant cluster size independent of expression level may reflect a biological preference for localization of the receptor in nanoscale domains. The cluster size, density and number of β_2 AR/cluster are similar to those for β_2 AR clusters in H9C2 and primary cardiomyocytes. This suggests that the inducible expression of β_2 AR is representative of the normal physiological distribution of the receptor and that there is a biological preference for

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β_2 AR distribution in small nanometer-sized clusters.

A second detailed quantitative study of membrane protein clustering visualized the distribution of a C-type lectin, DC-SIGN, that is involved in pathogen recognition for several viruses and other microorganisms.³⁸ DC-SIGN is expressed in dendritic cells that help to regulate immune responses and its spatial arrangement in the membrane is believed to be linked to its function as a viral receptor. NSOM studies established that >80% of the receptor was localized in nanodomains with a mean size of 185 nm and that the domains were randomly distributed across the membrane surface. Calibrations using the average intensity of single molecules indicated that there was an average of 5-10 proteins/cluster, but that the packing density was very heterogeneous (ie, the intensity did not scale linearly with cluster size as would be expected for a single packing density in both large and small clusters). An earlier study of the same receptor in fixed cells in an aqueous environment gave similar conclusions, although there were some differences in cluster size and protein density.³⁹ Finally, single molecule bleaching studies demonstrated that NSOM could be used to image individual proteins within a cluster at much higher density than is possible with confocal microscopy. The presence of DC-SIGN in domains with sizes that are similar to viruses and the heterogeneity of domain packing densities were hypothesized to be important factors for providing flexibility for receptor binding to a variety of different pathogens.

A third study from our group has probed the membrane distribution of the epidermal growth factor receptor, which is over-expressed in many solid tumors and thus an important therapeutic target.⁴⁰ The EGFR is organized in small clusters with an average size of 150 nm in both unstimulated and EGF-treated Hela cells, although the distribution of cluster sizes is slightly wider after EGF treatment, Fig. 6. The packing densities vary substantially from cluster to cluster, with anywhere from 1 or 2 proteins to greater than 100 proteins/cluster and with the peak of the intensity histograms typically having 5-15 molecules/cluster. The relatively modest changes in cluster size and density after EGF stimulation suggest that receptors are pre-organized in nanoscale domains on the cell surface, indicating that mechanisms for organization of the EGFR are independent of ligand activation on the length scale of 100 nm. Treatment with the tyrosine phosphorylation inhibitor, AG1478, led to a two-fold increase in cluster density and to a larger fraction of clusters with smaller diameters and fewer receptors. AG1478 also resulted in localization of small individual clusters in larger, but non-contiguous, membrane patches, illustrating the hierarchical arrangement of protein domains on the cell surface. Two-color colocalization experiments demonstrated that the fraction of EGFR that colocalizes with both lipid rafts and caveolae decreased in the presence of the inhibitor. Overall, this study demonstrates the utility of high resolution fluorescence imaging for understanding receptor clustering as a function of agonist and antagonist treatment, information that is relevant to design of therapeutic strategies based on modulation of EGFR signaling.

The organization of subunits of interleukin 2 (IL2) and interleukin 15 (IL15) cytokine receptors has been examined.⁴¹ These receptors are both involved in T-cell-mediated immunity and, although they share receptor subunits and have common cellular responses, they can also exhibit distinct and contrasting contributions to immunity. NSOM studies of the IL2 and IL15 α -subunits in a human leukemia T-cell line indicated that there were two different populations for both receptors, one comprised of relatively large clusters and one with either individual proteins or very

Fig. 6. NSOM imaging of EGFR in fixed HeLa cells. (A, B) Simultaneously recorded NSOM topography and fluorescence images. (C) A small scale fluorescence image for the boxed area in image B. The inset at the top right shows the region outlined at the bottom right on a different intensity scale, illustrating that some brighter regions correspond to multiple small clusters. (D) Bar chart shows the variation in the number of EGFR/clusters for control, EGF-stimulated and AG1478-treated cells. Reproduced from *J. Biol. Chem.* 2010, **285**, 3145, with permission of the American Society for Biochemistry and Molecular Biology Inc.

small clusters. Quantitative size and intensity analysis demonstrated that the monomer fraction was ~30% for IL15 but much lower for IL2. Interestingly, correlation of cluster size with intensity for each receptor indicated that there was a constant packing density for each receptor, but that the packing density for IL2 was approximately 10 times that of IL15, even though their average cluster diameters were quite similar (450 nm and 360 nm). This result provides evidence that domains may be assembled from “building blocks with repeated structures and composition”,⁴¹ in contrast to various other studies (see above) which found a wide range of packing densities for individual clusters of membrane proteins. There was also a significant degree of overlap of the two receptors based on an estimate of 40% colocalization. Previous FRET data for IL2 and IL15 had shown the presence of a significant fraction of dimeric or oligomeric receptor. Further experiments combining NSOM with FRET would be required to elucidate the relationship

between dimers/oligomers and the two populations of receptor and the constant cluster packing density observed by NSOM.

Two recent reports from the Chen group have used NSOM plus immunofluorescence with quantum dot labeling to examine the membrane distribution of various receptors on the surface of T-cells.^{42, 43} In one case they demonstrated that only 10% of the $\gamma\delta$ T-cell receptor (TCR) clusters were >90 nm in diameter, whereas 40% of $\alpha\beta$ -TCR clusters were larger than 90 nm.⁴² Significant changes in cluster size for V γ 2V δ 2 T cells were observed following antigen-induced in vivo clonal expansion of the cells. The clustering behavior of the $\gamma\delta$ TCR was maintained but in addition to nanoclusters (<250 nm), larger features assigned as nanodomains (> 250 nm) and microdomains (>1000 nm) were also observed. A second study showed that TCRs (CD3, CD4 and CD8) were found as either isolated receptors or small nanoclusters labeled with 2-4 quantum dots in unstimulated T cells with relatively low levels of colocalization (<10% of CD3 with either CD4 or CD8).⁴³ Activation of the cells led to significant changes in the distribution of the receptors with co-clustering of CD3 with CD4 or CD8 to give 200-500 nm nanodomains and >500 nm microdomains. In these studies the brightness and resistance to photobleaching of quantum dots provided an advantage over standard immunofluorescence with dye-labeled antibodies.

Visualizing membrane rafts

A large number of studies have used glycolipid or glycosylphosphatidylinositol (GPI)-linked proteins as markers for raft domains and the same approach has been used for super-resolution studies. In one example, Chen and coworkers have taken advantage of NSOM's capability to record topography and fluorescence to show that two different glycolipids, gangliosides GM1 and GM3, are localized in distinct, non-overlapping clusters on the plasma membrane of polarized MDCK cells.⁴⁴ Furthermore, the GM3 raft domains (mean size of 190 nm) were distributed predominantly on the microvilli-like protusions of the apical cell membrane whereas the GM1 nanodomains (mean size of 160 nm) were found on the edge of or between the microvilli. The same group has also examined the effects of temperature and fixation on the nanoscale distribution of two raft markers, GM1 and the protein CD59, in the membrane of T-cells.⁴⁵ Lower temperatures (4°C) resulted in the redistribution of raft clusters as well as their enlargement by a factor of 30-60% and a greater degree of colocalization of the two raft markers. The results also showed that raft markers were distributed at raised regions of the cell membrane. The changes in distribution of raft markers as a function of temperature are directly relevant to the widely-used approach of using detergent extraction at low temperature as a means to quantify the extent of liquid-ordered "raft" phases in cell membranes.⁴⁶

Similar clustering of asialo-GM1 was observed in HeLa cells, using antibody labeling to detect the glycolipid clusters.⁴⁷ Two populations of asialo-GM1 were observed, one that was clustered in domains with an average size of ~90 nm and a second diffuse level of glycolipid. Detergent extraction studies showed that asialo-GM1 was present in both soluble and insoluble membrane fractions leading to the hypothesis that the membrane insoluble fraction corresponded to the clustered glycolipid observed in the NSOM images. Colocalization studies showed that a fraction of the asialo-GM1 colocalized with caveolae, which are specialized domains with an invaginated membrane structure and a lipid composition similar to rafts. The

limited spatial resolution available with confocal microscopy gave apparent colocalization values that were over twice those obtained by NSOM, indicating the limitations of confocal for assessing overlap of small membrane domains.

A more detailed study of the distribution of GM1 has been published very recently.⁴⁸ Labeling GM1 with its fluorescent protein receptor, CtxB, in monocytes demonstrated the formation of densely packed clusters with a distribution of sizes (peaking at 123 nm) and numbers of protein-GM1 complexes (Fig. 7); clustering was significantly disrupted by M β CD treatment, although some heterogeneity was still observable, Fig. 7. Dendritic cells that express ~ 30 times less GM1 also showed clusters, typically with ~ 2 CtxB/cluster. Two-color NSOM studies showed that GM1-CtxB domains had low colocalization with both raft and non-raft proteins. However, the GM1 clusters were shown to be in close proximity to cluster of both LFA-1 and CD55, both of which are believed to localize in rafts, but to be randomly distributed with respect to the non-raft protein, CD71. The high resolution imaging results were combined with computational studies, leading to the conclusion that small nanoscale rafts must be interconnected in the plasma membrane. It was further hypothesized that this interconnectivity is a necessary prerequisite for larger scale coalescence upon cell activation.

Fig. 7. (A) Confocal image of CtxB-GM1 at the cell surface of a fixed monocyte. Scale bar: 5 μ m. (B, C) Confocal zoom-in and NSOM images of the area highlighted in A. Scale bars: 1 μ m. Insets in B and C highlight the increase in resolution afforded by NSOM. (D) Histogram of the nearest neighbour distribution (nnd) of CtxB-GM1 spots (bars) and simulations of random organization (line). (E) Size distribution of individual CtxB-GM1 spots (bars) and of individual CtxB on the glass (line) obtained with the same NSOM probe. (F) Intensity distribution of CtxB-GM1 for multiple cells (gray) and of single CtxB nonspecifically attached to glass (dash). Reproduced from *Proc. Natl. Acad. Sci.* 2010, **107**, 15437, with permission of National Academy of Sciences.

Garcia-Parajo and coworkers have examined the distribution of the leukocyte-specific integrin, LFA-1, which mediates cell migration and cell adhesion and is involved in the formation of the immunological synapse.⁴⁹ This study aimed at
 400 addressing the role of lipid rafts in controlling integrin-mediated cell adhesion. Earlier confocal studies had demonstrated that LFA-1 colocalized with the glycolipid GM1 and that raft disruption interfered with ligand binding. Interestingly, the higher spatial resolution available with NSOM demonstrated that LFA-1 localized in small clusters (size <85 nm, limited by probe aperture size) but did not
 405 colocalize with GPI-AP, a raft marker. The latter showed a large population of monomers (70%) but also a small population of nanodomains (30%, 2-4 proteins/nanocluster) that were clustered in specific regions. Despite the absence of colocalization, it was observed that about half of the GPI-PA clusters were in close proximity to LFA-1 clusters in the absence of ligand activation, suggesting the
 410 formation of ligand-independent platforms. Ligand activation (with ICAM) increased the number of GPI-AP clusters to 80%, resulting in the formation of localized adhesion platforms with ICAM-LFA-1 clusters. These results led the authors to conclude that the formation of distinct but proximal nanodomains (or hotspots) of the two protein receptors occurs in the absence of ligand, probably as
 415 the result of interactions with the cytoskeleton. Ligand binding to the integrin receptor leads to larger functional platforms that promote adhesion. It should be noted that the use of two-color experiments, quantitative analysis of cluster size and intensity, and the presence of corroborating data from EM were all necessary in order to develop a complete picture of the compartmentalization of LFA-1.
 420 Furthermore, the compartmentalization is more complex than that observed at lower spatial resolution using confocal microscopy.

A common theme emerging from these studies is the formation of nanoscale membrane raft domains as assessed by labeling the commonly-employed glycolipid and protein raft markers. Although the NSOM studies are for fixed cells, another
 425 recent study has provided evidence for nanoscale lipid domains in live cells.⁵⁰ Interestingly, previous NSOM studies have also detected small nanoscale glycolipid clusters in supported lipid membranes.^{51, 52}

4 Antennas and Apertureless

The above examples highlight the utility of NSOM imaging with aperture probes for
 430 probing the nanoscale organization of membrane proteins and lipid domains in cellular membranes. Despite these advances, the reproducible fabrication of probes remains a significant challenge, with most groups making their own custom probes. Furthermore, the available resolution is limited by the light transmission through the optical fiber probe to ~50 nm; in fact, most published studies have used probes with
 435 aperture diameters between 70-90 nm. Several apertureless and optical antenna-based approaches have been developed in attempts to improve the spatial resolution.

Apertureless (ortip-enhanced) near-field approaches rely on the local enhancement of illuminated or scattered light in a small region adjacent to the end of a sharp metal or metal-coated tip (Fig. 2).⁵³⁻⁵⁵ Several effects contribute to the field
 440 enhancement, including excitation of surface plasmon resonances in the metal, an electrostatic lightning-rod effect associated with localized surface charge densities and antenna resonances.^{53, 55} Pointed metal tips are also referred to as antennas based on their similarity to electromagnetic antennas. Tip-enhanced Raman scattering

methods have been used successfully in a range of applications with signal
 445 enhancements of more than 10 orders of magnitude having been reported.⁵⁵
 However, tip-enhanced techniques for fluorescence microscopy are somewhat more
 complex. This arises primarily because the tip-induced fluorescence enhancement
 depends on several factors, including the tip material and shape, the polarity and
 wavelength of the illuminating light used, the tip-sample distance and reduction of
 450 the fluorophore lifetime/intensity by proximity to the metal tip. Early
 demonstrations of apertureless methods focused on single isolated molecules. For
 example, <10-nm resolution was demonstrated for imaging individual dye-labeled
 DNA molecules using a silicon tip.⁵⁶ A number of other examples have reported
 impressive spatial resolution for imaging single dye molecules or quantum dots
 455 deposited on glass substrates and for imaging carbon nanotubes, frequently using an
 antenna approach with a small metal nanoparticle attached to the end of the tip.^{55, 57}
 In one particularly interesting study, Sandoghdar and coworkers examined the
 effects of the gold nanoparticle diameter and the tip-sample distance on the
 achievable spatial resolution and fluorescence enhancement using a combination of
 460 computational work and imaging of dye molecules embedded in a thin film. These
 results clearly demonstrated the trade-off between fluorescence enhancement and
 spatial resolution. Optimal signal to noise with 20-nm resolution and an
 enhancement factor of 30 times was achieved with an 80-nm diameter nanoparticle.

Although the development of high spatial resolution is driven by the need for
 465 higher resolution biological imaging methods, most studies have not yet tackled
 biological samples in a physiological environment. However, a recent example from
 the Novotny group has shown that Ca^{2+} -ATPases can be imaged in isolated
 erythrocyte membrane patches in an aqueous environment using an 80-nm gold
 nanoparticle antenna (Fig. 8). This study demonstrated ~50 nm spatial resolution
 470 with a fluorescence enhancement of ~8 and the ability to resolve individual closely
 spaced proteins.⁵⁸

475 **Fig. 8.** Cartoon depicting the principle for antenna-based NSOM (left). NSOM
 image showing individual Ca^{2+} ATPase in the erythrocyte plasma membrane with a
 cross section through one of the fluorescence spots indicating ~50-nm resolution.
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Chemical Society.

Apertureless approaches have demonstrated a significant improvement in spatial resolution over aperture-based near field approaches and have the added advantage of avoiding the use of fragile-optical fiber based probes. However, they suffer from three significant restrictions that have so far prevented their wide-spread use, particularly for biological imaging. First, photobleaching is more problematic than in normal fluorescence microscopy, since the focused laser beam used to continuously irradiate the tip also irradiates the sample. Second, the background signal that results from far-field irradiation of the tip is relatively high in some cases, and may exceed the tip-enhanced signal for samples with a high chromophore density. Finally, quenching of fluorophores by the metallic tip reduces the tip-enhanced signal, but does not affect the far-field background signal. A background suppression method based on modulation of the feedback loop used to regulate tip-sample distance has been developed recently.⁵⁹ This method resulted in a ten-fold improvement in fluorescence enhancement for measurements of a thin layer of relatively closely spaced dye molecules measured using an 80-nm gold nanoparticle antenna. Considerably larger improvements in the enhancement factor were obtained when imaging an isolated membrane patch with a high density of calcium channels. One interesting observation was the wide variation in enhancement factors measured for individual molecules; this complication will preclude a quantitative intensity analysis of protein clusters, as has been successfully used with aperture-based NSOM (see above).

An elegant solution to the background and photobleaching issues associated with apertureless NSOM consists of placement of a metal tip in close proximity to a subwavelength aperture, Fig. 2.^{60, 61} In one example, the van Hulst group has constructed a nanoantenna on the end of an aluminum-coated optical fiber probe with a 100-nm diameter aperture.⁶² Excitation of the nanoantenna by the near-field aperture results in lower background illumination of the sample than can be obtained with a focused laser beam and minimizes photobleaching. Initial studies on dye molecules embedded in a thin polymer film demonstrated 25-nm spatial resolution with low background and examined the effect of nanoantenna length on the antenna performance. The tip-on-aperture approach has recently been applied to cellular imaging.¹² A resolution of 30 nm was demonstrated for imaging LFA-1 clusters on the surface of monocytes; clusters measured with the antenna probe had an average size of 72 $\pm$ 21 nm, whereas a conventional aperture NSOM probe was only able to set an approximate upper limit for the cluster size of 85 nm. The authors have argued that further optimization of the antenna geometry should result in improved signal to background levels and higher spatial resolution. However, it remains to be seen whether this approach will be of general utility since the probe fabrication is complex and the method retains the problems associated with a bulky and fragile optical fiber probe.

5 Hybrid NSOM Methods

Several recent studies have illustrated the potential of combining NSOM with dynamic measurements using fluorescence correlation spectroscopy (FCS). FCS relies on the temporal analysis of fluctuations of the fluorescence signal as fluorophores diffuse in and out of a small observation volume, and has been widely used to measure single molecule dynamics, yielding concentrations, diffusion

coefficients and association/dissociation constants for biomolecules.⁶³ FCS is usually implemented on a confocal microscope, with diffraction-limited optics resulting in femtoliter observation volumes. Reducing the size of the observation volume would provide several advantages, particularly for studies of membrane proteins. First, it would allow single molecule sensitivity even in the presence of high molecular concentrations; confocal FCS measurements are typically limited to concentrations between 100 pM and 100 nM, whereas typical concentrations of biomolecules in cell membranes are in the μM to mM range. Second, it would allow the detection of spatial heterogeneities in the dynamics of individual proteins or lipids confined to nanoscale membrane domains. Third, the shorter residence time of molecules in the smaller observation volume would minimize photobleaching. These advantages have motivated a number of approaches for reduction of the observation volume for FCS measurements. The most successful to date has been the use of sub-wavelength sized apertures (zero-mode waveguides) in thin metal films which produce attoliter (10^{-18} l) and zeptoliter (10^{-21} l) observation volumes.⁶⁴ Nanoapertures have significant promise for single molecule assays in solution and have also recently been employed to study diffusion in cell membranes.^{65, 66} However, their applicability is significantly limited by the requirements for growing cells on the metal films used to construct the apertures and for membrane invagination into the nanoapertures, which dramatically restricts the membrane area that can be probed.

The use of near field optical probes with nanometer-sized apertures has the potential to allow both nanoscale imaging and dynamic measurements using FCS in reduced observation volumes/areas. An initial proof-of-principle experiment employed a sub-wavelength NSOM probe to measure diffusion of a dye-labeled lipid in a supported lipid bilayer.⁶⁷ Using a probe with a 140-nm aperture diameter, FCS-NSOM data indicated a roughly 10-fold reduction in diffusion time compared to confocal FCS, Fig. 9. The FCS-NSOM diffusion time combined with the measured diffusion coefficient for Oregon Green DHPE gave an excitation radius of 73 nm in excellent agreement with the probe aperture size, and clearly demonstrating an order of magnitude reduction in observation volume for FCS-NSOM as compared to a confocal geometry. This study also demonstrated the need

Fig. 9. Cartoon (right) illustrating FCS-NSOM configuration for a supported lipid bilayer. Correlation curves (left) showing the change in diffusion time for confocal (a) and NSOM (b) FCS measurements for Oregon Green–DHPE in a DOPC membrane. Reproduced from *Appl. Phys. Lett.* 2008, **93**, 163904 with permission of

American Institute of Physics.

for further improvements to the probe design for more routine FCS measurements, particularly with smaller apertures.⁶⁷⁻⁶⁹ A similar proof-of-principle experiment with
565 a larger NSOM probe (300 nm diameter aperture) gave data with superior signal to noise for diffusion of fluorescent beads in solution.⁷⁰

A combination of NSOM and FCS was used to image closely packed nuclear pore protein complexes and to measure diffusion of dye-labeled nuclear transport factor.⁷¹ This impressive experiment was possible by suspending isolated nuclear envelope
570 membranes across 20 mm troughs in a regular grid so that pores in a free standing membrane could be imaged with the NSOM probe and the probe positioned over an individual pore for FCS measurements of transport through the pore. A light-emitting triangular aperture was used as the near-field probe and the probe-sample distance was maintained using only the elastic response of the membrane, resulting
575 in improved optical resolution over earlier studies using a fiber aperture probe and force feedback to image a similar sample.⁷² The diffusion measurements for the nuclear transport factor were consistent with interaction of the protein with amino acids on the edges of the channel during its transit through the pore.⁷¹ Note that the reduced observation volume is a significant advantage due to the high protein
580 density in the nuclear pore envelope.

The first example of FCS-NSOM measurements on live cells was recently reported.³⁴ This study used a 120-nm aperture probe to measure diffusion for two different dye-labeled lipids in Chinese hamster ovary cells. A phosphatidylethanolamine probe gave results consistent with free diffusion, but with
585 the diffusion time shifted to shorter times due to the reduced illumination area of the NSOM probe. By contrast, sphingomyelin showed slower diffusion and results typical of confined diffusion; confinement of sphingomyelin in cholesterol-enriched domains provides a plausible explanation for this observation. Importantly, this experiment demonstrates the potential to image cell membranes with nanoscale
590 resolution and to measure diffusion associated with selected nanoscale membrane compartments.³⁴

Finally, an apertureless near-field approach using a 40-nm gold nanoparticle as an optical antenna has also been used for FCS measurements. A reduction of four orders of magnitude in the observation volume was demonstrated by using the
595 enhanced far-field to near-field coupling of metallic nanoparticles upon surface plasmon excitation.⁷³ Proof-of-principle experiments with Rose Bengal dye in solution indicated that the method gave information on both the diffusion of the dye and its interaction with the nanoparticle surface.

In another hybrid NSOM approach, near field fluorescence imaging has been
600 combined with scanning electrochemical microscopy (SECM).³¹ This experiment used a specially designed bent, etched optical fiber probe with a 100-nm aperture, surrounded by a 250-nm gold ring electrode and an outer layer of insulating polymer. Operating in dynamic force mode, resolutions of 240, 380 and 180 nm were obtained in AFM, electrochemical and optical modes, respectively. The method
605 was used for simultaneous topography, fluorescence (using a Ca²⁺-sensitive dye) and electrochemical imaging of thin neurites in differentiated PC12 cells. It is worth noting, however, that a combination of AFM and SECM coupled with confocal or epifluorescence microscopy may provide comparable results, with a much more straightforward scanning probe design.

610 6 Conclusions and Outlook

The studies summarized in this review highlight the capabilities of NSOM for resolving the nanoscale organization of the plasma membrane. A quantitative approach has been implemented for examining the clustering of membrane receptors, their localization in membrane domains such as lipid rafts and the consequences of cellular activation (eg, by binding agonists or antagonists) on the nanoscale organization. Several common themes emerge: (1) there is a significant level of pre-organization of receptors in the membrane of resting cells, (2) there is a wide range of cluster sizes and packing densities for protein clusters and (3) there is frequently a hierarchial membrane organization. The nanoscale resolution provided by NSOM allows one to interrogate biological structures on a length scale that is intermediate between the diffraction-limited spatial resolution of confocal microscopy and the short-range, nearest-neighbour interactions probed by fluorescence resonance energy transfer and to correlate protein distribution with the cellular topography.

The above advances notwithstanding, NSOM remains a difficult technique that is still not widely accessible, especially to biological labs. A decade ago, a review by Edidin concluded that “Biological NSOM is still waiting to happen. The microscopes need applied physicists to make them work, while biologists are needed if the physicists are to do more than show possibilities. ...it seems that NSOM too will need 16 or so years to mature for biology”.¹⁰ Although more recent work has demonstrated that aperture-based NSOM has significant potential, the difficulties associated with probe fabrication, the slow scan speed and the restriction to sample interfaces still limit its widespread adoption. Apertureless methods have achieved superior resolution, but the complexities of optimizing the tip-induced fluorescence enhancement while minimizing background for specific probes are not straightforward to overcome. Ten years ago, few would have predicted the rapid progress that has been achieved in applying far-field super-resolution fluorescence imaging to biology. Capabilities for live cell imaging and the ability to probe both cell surfaces and internal structures are significant advantages of super-resolution approaches. Nevertheless, they bring a different set of challenges, including the requirement for specific fluorescence probes with photophysical properties optimized for switching, the high laser intensity needed for non-linear optical methods such as STED and the significant challenges associated with data acquisition/analysis for stochastic methods. In this respect NSOM is a more flexible technique, both with respect to the choice of fluorophore for labeling and the ease of implementing two-color experiments. What is most exciting today is the fact that researchers now have an arsenal of nanoscale imaging tools at their disposal; each will undoubtedly have its own niche, but collectively they open the door to great advances in our understanding of the nanoscale details of cellular organization.

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