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Near-Field Scanning Optical Microscopy for High-Resolution Membrane Studies

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Abstract

The desire to directly probe biological structures on the length scales that they exist has driven the steady development of various high-resolution microscopy techniques. Among these, optical microscopy and, in particular, fluorescence-based approaches continue to occupy dominant roles in biological studies given their favorable attributes. Fluorescence microscopy is both sensitive and specific, is generally noninvasive toward biological samples, has excellent temporal resolution for dynamic studies, and is relatively inexpensive. Light-based microscopies can also exploit a myriad of contrast mechanisms based on spectroscopic signatures, energy transfer, polarization, and lifetimes to further enhance the specificity or information content of a measurement. Historically, however, spatial resolution has been limited to approximately half the wavelength due to the diffraction of light. Near-field scanning optical microscopy (NSOM) is one of several optical approaches currently being developed that combines the favorable attributes of fluorescence microscopy with superior spatial resolution. NSOM is particularly well suited for studies of both model and biological membranes and application to these systems is discussed.

Keywords

NSOM; SNOM; Lipid membranes; High spatial resolution; Sub-diffraction-limited resolution; Fluorescence microscopy

Introduction

Near-field scanning optical microscopy (NSOM), also known as scanning near-field optical microscopy (SNOM), is a scanning probe technique developed to surpass the spatial resolution constraints that traditionally limit conventional optical microscopy (1–11). As shown in Fig. 1, NSOM uses fiber optic probes to funnel light down to the nanometric dimension. By scanning these probes near a sample surface, fluorescence measurements can be taken with a spatial resolution of tens of nanometers, which represents an order of magnitude improvement over that obtainable with conventional fluorescence microscopy (1–11). NSOM, therefore, helps bridge the gap in spatial resolution between far-field optical approaches and high-resolution techniques such as electron microscopy.

While electron microscopy has been extraordinarily successful at directly probing biological structures, the increased spatial resolution comes at the expense of working conditions, optical contrast mechanisms, and often very high costs. Fluorescence microscopy, on the other hand, is comparatively inexpensive and easily implemented on fixed or viable biological tissues. It also has an extensive history of fluorescent probe and method development, and exhibits spectroscopic, polarization, and dynamic capabilities. These attributes prove especially powerful for biological studies and it is these applications that have driven many of the technological developments experienced in fluorescence microscopy. A major goal in developing NSOM, therefore, was to create a fluorescence

method that retains the favorable attributes associated with conventional fluorescence microscopy but with a spatial resolution approaching that attainable with electron microscopy.

Figure 2 shows a NSOM fluorescence image which demonstrates the sub-diffraction-limited spatial resolution possible with this technique. Each bright spot in Fig. 2 represents fluorescence from a single dye molecule dispersed in a lipid monolayer. Since the individual dye molecules are much smaller than the NSOM aperture, the feature size reflects the aperture diameter of the particular NSOM probe used for imaging (see Note 1). Each single molecule emission feature in Fig. 2 has a full-width half-maximum of ~20–30 nm, illustrating the high spatial resolution capabilities of NSOM (7). This example, therefore, demonstrates both the single molecule fluorescence detection limits of NSOM and its high spatial resolution, which is an order of magnitude better than that obtained with conventional optical approaches (1, 7, 10, 11). Moreover, since a feedback mechanism is implemented to maintain the tip–sample gap during scanning, NSOM provides a force mapping of surface topography much like atomic force microscopy (AFM). For biological applications and especially those involving membranes, the simultaneously measured NSOM fluorescence and topography images provides unique capabilities for directly comparing the location of optical markers with sample structure. These attributes offer intriguing possibilities for studying nanometric biological structures such as lipid rafts in membranes.

While commercial instrumentation is marketed for NSOM measurements, it is straightforward to modify existing scanning probe microscopes to implement NSOM. Here we discuss in detail how our particular NSOM instrumentation is constructed and the methods that we have learned for optimizing NSOM performance. Before outlining the details of this approach, however, we discuss how NSOM probes are fabricated. Resolution is largely dictated by the quality of the NSOM probe and, in our view, it is very important that the NSOM tips be fabricated in-house. We, therefore, provide a short primer on the fiber optic probe fabrication and metal coating conditions that we have found consistently lead to NSOM probes with well-defined, small apertures.

2. Materials

There are many ways of incorporating NSOM capabilities onto existing APM platforms. Listed below are the major pieces of instrumentation used in our laboratory for this along with that needed for NSOM tip fabrication.

1. Digital Instruments Nanoscope IIIa AFM controller and work station (Now Bruker, <http://www.bruker-axs.com>): Bioscope backplane electronics module, signal access module, photon counting module.
2. Zeiss Axiovert 100 inverted fluorescence microscope equipped with high numerical aperture objective (FLUAR, 60×/1.45 oil) or equivalent.
3. X-Y closed-loop piezo sample scanner with central void to accommodate microscope objective (Mad City Labs, Nano H100, <http://www.madcitylabs.com>).
4. Avalanche photodiode (APD) single photon counting module (EG&G SPCM-AQR).
5. Interference filters and dichroic beamsplitter (Chroma, <http://www.chroma.com>).
6. Long working distance microscope (Infinity K2, <http://infinity-usa.com>) with CCD camera (Cohu, <http://www.cohu.com/>) or equivalent to monitor tip position above the sample.

7. Lock-in amplifier for detecting the shear-force feedback signal (Stanford Research Systems, SR830 DSP lock-in amplifier, <http://www.thinksrs.com>).
8. Sutter Instruments P-2000 micropipette puller or equivalent with arms modified to accept single-mode optical fiber (www.sutter.com).
9. Fiber strippers (www.thorlabs.com).
10. Thermal evaporation chamber with deposition monitor for coating aluminum onto probes.

3. Methods

3.1. Tip Fabrication

As with all scanning probe techniques, the quality of the measurement is largely dictated by the quality of the probe. This seems to be especially true for NSOM where the formation of a sub-wavelength aperture for the delivery of light can be problematic. There are two main approaches for fabricating tapers in the fiber optics used for NSOM tips: one is through chemical etching while the other utilizes a heating and pulling method (12, 13). Each technique offers distinct advantages and disadvantages. The chemical etching approach uses chemical etchants, such as hydrofluoric acid, to form tapers in the fiber optic probes. The main advantage of this technique is that very large taper angles can be fabricated which increases light throughput efficiency (13–17). This approach is also inexpensive and easily scaled for simultaneous probe production. However, even with the newer tube etching approaches, this technique often produces probes with rough, pitted, or jagged surfaces which degrade the quality of the metal coating used to confine light within the aperture. Since the quality of the metal coating is the key metric for NSOM tip performance, this is a major obstacle for the chemical etching approach. For this reason, we find that the heating and pulling method for tip formation remains the best approach for standard NSOM tip fabrication (18, 19). This is the most prevalent approach for fabricating NSOM probes and, in our experience, the easiest method for reproducibly fabricating probes once the proper procedures are in place. We will, therefore, limit our discussion to fiber optic probes heated and pulled to a taper and then coated with aluminum.

3.1.1. Heating and Pulling Fiber Optic Probes

The heating and pulling method utilizes a heat source to soften the fiber and a pulling mechanism to draw the fiber down to the point of rupture. This approach is simple and once the appropriate parameters are found, it is very repeatable. While the method does require additional instrumentation, it yields very reproducible tapers with exquisitely smooth and clean surfaces that enable deposition of high-quality metal films (see Note 2).

1. To fabricate NSOM probes we use single mode optical fiber with 125 μm cladding. We have used single mode fiber from a number of vendors and find no difference for probe fabrication.
2. Approximately 5 mm of the jacket is removed with fiber strippers and the exposed fiber optic cleaned with ethanol. The fiber is mounted in a Sutter Instruments P-2000 micropipette puller with arms modified to accept single-mode optical fiber. These pullers incorporate a CO₂ laser for the heat source and programmable control over all important parameters for taper formation such as laser power, pull strength, and pull delay. These parameters enable a large range of taper shapes to be fabricated in fiber optic probes. For example, high heat and hard pulls result in long tapers with very small tip apertures while low heat and soft pulls result in short tapers with larger tip apertures (18, 19). Unfortunately, this approach cannot create short tapers with small apertures, a combination that incorporates efficient light

throughput with high spatial resolution. Etching methods can produce this geometry, but as mentioned earlier have other issues that make metal coating, difficult. We have explored both approaches in our laboratory and believe the heating and pulling method produces quality probes with small apertures more reliably.

3. The exposed fiber is aligned in the path of the heating laser and clamped in place. The fiber is then heated and pulled using parameters that lead to the desired tip shape (see Note 3). It is important that the laser and fiber be aligned properly to obtain a symmetric taper. This should be checked from time to time by positioning a piece of thermal fax paper behind the fiber while momentarily firing the laser on low power. The laser spot burned on the fax paper should have a shadow from the fiber exactly bisecting the spot. If the line is high or low on the spot, asymmetric tapers can result.
4. To ensure tip to tip consistency, we only use pulled fibers from one arm of the puller and discard the other. The pulled fibers are immediately stored in an enclosed container to reduce dust contamination until loaded into the coating chamber.

3.1.2. Aluminium-Coated NSOM Probes

Tapered fiber optic probes alone are not sufficient to act as NSOM probes since light escapes from the sides of the taper region. A reflective coating must be deposited on the taper to confine light within the probe until exiting a well-defined aperture at the distal end of the probe (3,18). Without the reflective film, escaping light can excite fluorescence in the sample which leads to complicated patterns in the fluorescence image (9).

1. To confine light within the taper until exiting a well-defined aperture at the probe end, a reflective film of 50–100 nm of aluminum is coated around the sides. Aluminum has superior reflectivity properties in the visible region of the spectrum and, thus, requires the least amount of material to confine excitation light (20). This is important as the overall dimensions of the NSOM probe affects how close the probe aperture can approach the sample surface. While aluminum has the best optical properties, it unfortunately has a propensity to form oxides during the coating process (21). The oxides tend to dramatically degrade both the reflective properties of the coated film and the resulting performance metrics of the NSOM tip (22–25). In our opinion, the metal-coating step in fabricating NSOM probes represents the largest single barrier that must be overcome before carrying out quality NSOM measurements. Fortunately, much is known about coating aluminum and with care, high-quality, thin reflective Al films can be easily deposited onto tapered fiber optics.
2. To reduce oxide formation during film deposition, the coating needs to be carried out under a good vacuum. Achieving vacuum quickly is also important to reduce the batch time required for tip fabrication. We use a custom built thermal evaporation chamber (see Fig. 3) that uses a turbo pump to achieve pressures of 10^{-6} Torr in a few hours (see Note 4). Turbo pumps are desirable over diffusion pumps since they are cleaner and capable of achieving vacuum quickly.
3. The pulled fibers are mounted in a custom-built spinning mechanism that rotates the probes while coating to evenly deposit the metal film around the taper. The fiber is mounted into the fiber chuck shown in Fig. 3a. The all metal chuck is vacuum compatible and contains a spring clamp that immobilizes the fiber and a spool where the excess fiber is wound within and covered with the ring shown.

Approximately 1 cm of the tapered probe extends from the top of the chuck for coating.

4. As shown in Figs. 3b, c, four fiber chucks can be mounted in the rotating platform. The rotating mounts spin the tips during coating to evenly expose the sides of the taper. The coating must be deposited such that only the sides of the taper are coated, leaving the aperture at the end of the tip free for light to exit. Given these constraints, thermal evaporative coating methods tend to work the best. Thermal evaporation can achieve very high deposition rates and since the deposition is line of sight, simply orienting the tips away from the aluminum source leaves the aperture uncoated. The tips, therefore, are tilted away from the aluminum source at an angle of approximately 35°. The fiber probes are held 20 cm from the aluminum source and a rotating feedthrough is used to spin the tips at a rate of approximately 5 Hz during deposition.
5. In-house fabricated tantalum boats are used to hold and heat the aluminum. We have tried various materials and geometries for holding and heating the aluminum and found tantalum works the best for us. Tantalum is malleable so boat fabrication is simple and it is able to handle the large currents necessary for fast evaporation rates without boat failure. The boats should be as small as possible to reduce chamber heating, which can also have deleterious effects on the Al coating quality. In our configuration, we use boats with dimensions ~1×5 cm cut from a 0.015 in.-thick tantalum sheet. Fresh boats are used for each coating and loaded with approximately 5 in. of 1.0 mm-diameter aluminum wire (99.999%, Aldrich).
6. It is essential that the chamber incorporate a shutter above the aluminum source. For quality films, the aluminum needs to be melted, degassed, and impurities burned-off before opening the shutter and exposing the tips to the aluminum. The aluminum should be heated sufficiently so that once the shutter is opened, a high deposition rate is immediately achieved.
7. We have found that enclosing the heating apparatus (electrodes, tantalum boat, and aluminum) in a metal housing helps reduce ambient heating and leads to smoother aluminum films on the probes.
8. Pinholes in the aluminum coating often indicate the presence of contamination on the pulled fiber (20). It is important to decrease the time between pulling the fiber and getting it into vacuum to reduce the chances of dust attaching to the surface.
9. As mentioned, several parameters are important for obtaining smooth, highly reflective aluminum coatings on NSOM probes. Obviously, obtaining a good vacuum is important since this will reduce the presence of oxygen which is known to degrade coating quality by forming aluminum oxide grains. As an example, Figs. 4a, b compare AFM topography images of NSOM probes coated at 1×10^{-3} Torr and 3×10^{-6} Torr, both coated at the same deposition rate. The $2 \mu\text{m} \times 2 \mu\text{m}$ images reveal a dramatic reduction in grain size as the ambient pressure is reduced. Similarly, deposition rate is found to significantly affect the final grain structure in the aluminum coating. Figure 4c, d compares AFM measurements from NSOM tips coated at low and high deposition rates. Clearly, higher rates correspond to smaller grains and smoother coatings which translate into higher film reflectivity (20, 26). These results are summarized in Table 1 which tabulates the mean surface roughness of the aluminum coating under various conditions.
10. Once coated with 50–100 nm of aluminum, a metal collar is glued onto the end of the NSOM probe as shown in Fig. 3d. The collar enables the probe to be firmly held in the NSOM head without damage. Since the tip will be oscillated for

feedback, it is important that the collar be glued firmly on the probe so the dithering amplitude gets transferred efficiently to the probe.

Figure 5 shows an electron micrograph of a NSOM probe coated with aluminum under optimal conditions given in Table 1. The reduced grain size in the aluminum coating increases film reflectivity, thus decreasing the amount needed to confine light within the taper region. This reduces the overall dimensions of the probe end which helps in positioning the probe near the sample surface, especially for samples with complicated morphology as often found in biological tissues.

3.2. Near-Field Scanning Optical Microscope

The major components needed for implementing NSOM measurements are already available and just need to be adapted. Most home-built and commercial NSOMs are built on optical microscopy platforms and use scan electronics developed for AFM. Commercial optical microscopes equipped with high numerical aperture optics aid in both sample visualization and signal collection. For biological applications, inverted fluorescence microscopes provide a very flexible and stable platform for NSOM measurements (27).

Figure 6 shows one of our NSOM instruments built on a Zeiss Axiovert 100 inverted fluorescence microscope equipped with a high numerical aperture objective (FLUAR, 60×/1.45 oil). The design shown in Fig. 6 shares many common features with the Digital Instruments Bioscope AFM with slight modifications made to accommodate NSOM measurements (27). The NSOM head holds the fiber optic probe and the necessary laser, detection, and piezo actuators to implement the tip-sample feedback control. It is held in a dovetail mount connected to a motorized z-motion stage, which is used to control the large-scale adjustment of the tip toward the sample. The coarse z-motion stage is used to bring the NSOM tip within the 5 μm travel of the z-piezo tube which holds the NSOM probe. The z-piezo tube controls the tip-sample gap while the sample itself is held on a separate x-y piezo stage that scans the sample under the NSOM tip. This arrangement keeps the NSOM probe centered on the microscope objective which ensures that the fluorescence signal remains aligned on the small active area of the APD detector. Shown in Fig. 6 is a long working distance microscope (Infinity K2) with CCD camera to monitor the approach of the NSOM probe toward the sample surface (see Note 5). The microscope is controlled using a Digital Instruments Nanoscope IIIa controller equipped with a Bioscope backplane electronics module, signal access module, and photon counting module. A lock-in amplifier is also used to detect the oscillating shear-force tip feedback signal (not shown in Fig. 6).

3.2.1. Shear-Force Feedback for Tip-Sample Gap Regulation

To maintain the tip close to the sample surface while scanning, a feedback mechanism must be implemented. Most schemes introduced for NSOM revolve around force measurements between an oscillating tip and the sample surface. A popular method is the shear-force approach where the tip is dithered laterally at its resonant frequency while the amplitude of the oscillation is monitored (28–30).

1. Figure 7 shows a NSOM head built for shear-force feedback. The tip is mounted on a piezo bimorph that is affixed to the end of a z-piezo tube and encased in a protective metal housing. The z-piezo tube is used to move the tip vertically with respect to the sample surface while the piezo bimorph oscillates the tip laterally at its resonant frequency.
2. The amplitude of the oscillating tip is monitored from the side using a fiber-coupled laser to shadow the tip onto a split photo-diode detector. The lateral dithering of the NSOM tip in the path of the feedback laser produces an oscillating signal on each side of the split photodiode detector. To reduce the length of the

NSOM tip extending from the bimorph, the housing of the split photodiode has been removed and the substrate ground flat to the edge of the active areas as seen in Fig. 7. Minimizing the length of the NSOM probe emerging from the bimorph is desirable since it increases the resonance frequency of the tip, thus producing more stable feedback conditions (see Note 6).

3. Each side of the detector is amplified and sent to the differential input of a lock-in amplifier which is referenced to the signal used to drive the bimorph. The DC output of the lock-in amplifier, therefore, is proportional to the oscillation amplitude of the NSOM probe which will decrease when the tip interacts with the sample. This approach is easily integrated with commercial AFM electronics that support tapping mode AFM measurements.
4. To generate the feedback signal, most commercial AFM instruments use an error signal. Therefore, the DC output of the lock-in amplifier must be subtracted from a software-controlled “set point” voltage that is set by the user to define where on the force curve the tip should be positioned. Most AFM manufacturers sell a signal access module that enables access and control of all the relevant signals necessary to implement a shear-force feedback scheme.

3.3. NSOM Fluorescence Imaging

Fluorescence continues to be the most prevalent and useful contrast mode utilized in NSOM measurements. For the arrangement shown in Fig. 6, excitation light exiting the NSOM probe excites fluorescence in the sample which is collected from below using a high NA objective. The collected light is passed through filters to remove the excitation light and then imaged onto the active area of a high quantum efficiency APD detector. The output of the photon counting module is sent to the AFM controller where a photon counting chip bins the counts in the appropriate pixel.

1. The NSOM head containing the NSOM probe is slowly lowered toward the sample using the coarse adjust-motorized z-stage. It is important to have light exiting the NSOM probe which can be seen from the CCD camera coupled with the long working distance microscope. As the tip nears the sample surface, a reflected spot from the surface will also be visible. Carefully adjusting the motorized z-stage to bring the spot exiting the NSOM probe and the reflected spot close together on the monitor enables quick positioning of the NSOM probe within the small travel of the z-piezo tube.
2. Once the NSOM probe is near the surface, the detector needs final alignment. The small active area of the APD presents some challenges in aligning the fluorescence signal. We use a flipper mirror to direct a red light source into the optical fiber and through the NSOM probe to mimic fluorescence. The red light exiting the NSOM probe enables the APD to be aligned with the optical filters. With stable feedback and careful movements, this also enables the focus to be adjusted while the tip is engaged on the sample surface which is critical for maximizing the fluorescence signal when using a high NA objective.
3. We use a closed-loop x - y piezo stage for sample scanning. Like all fluorescence measurements, sample photobleaching often proves limiting in NSOM studies. Open loop piezo scanners suffer from piezo creep and hysteresis which requires several “training” scans before collection of a reliable image. For AFM measurements this is usually not a problem, but for NSOM measurements repeatedly scanning the same area increases photobleaching and, therefore, degrades the signal. Closed-loop scanners that actively adjust for creep and hysteresis enable efficient collection of NSOM fluorescence signals without the

repeated prior scans required with open loop stages. Moreover, these capabilities are especially important for single point spectroscopic or dynamic measurements where the NSOM probe needs to be positioned and held above a precise location of the sample.

3.3.1. NSOM Fluorescence Imaging of Membranes

NSOM is well suited for structural studies on model membranes and biological membranes where the two-dimensional nature of the sample enables the close approach of the NSOM aperture (31–36). The high spatial resolution of NSOM combined with the simultaneous fluorescence and force signals proves especially informative in membrane systems. These signals can be directly correlated to understand membrane organization as a function of constituents and conditions.

For model membrane studies, the Langmuir-Blodgett (LB) technique has been used extensively to transfer lipid films onto solid supports where they are amenable for study with surface techniques such as NSOM. The LB technique enables precise control over the lipid film surface pressure, which is related to the first derivative of free energy with respect to surface area. As such, these studies provide a direct link between film properties and thermodynamics. Moreover, the LB technique enables films to be fabricated over a wide range of temperatures, pressures and film compositions which further enhances the properties that can be explored.

Figure 8 shows representative NSOM measurements on a DPPC monolayer transferred onto mica using the LB technique. This film was transferred in the phase coexistence region of the pressure isotherm where less ordered liquid-expanded (LE) phase coexists with the more ordered liquid-condensed (LC) domains. As studied extensively with AFM, these phase differences result in ~5–8 Å height changes which can be seen in the NSOM shear force image shown in Fig. 8a (31). The NSOM fluorescence image shown in Fig. 8b maps the location of the fluorescent lipid analog DiIC₁₈, which has been doped into the DPPC film at 0.25 mol% to stain LE regions of the membrane.

These measurements help illustrate the utility, and some of the advantages, of NSOM for studying the structure of lipid monolayers. First, force capabilities enable resolution of Angstrom level height changes associated with phase coexistence, while high lateral resolution in the fluorescence image can detect small domains beyond the resolution of conventional optical techniques. Second, simultaneous force and fluorescence mapping provides a direct, pixel by pixel comparison between surface topography and fluorescence properties. This enables the unambiguous assignment of domains to specific phases which avoids artifacts due to defects in the membrane. Third, the sub-diffraction-limited measurements afforded by using NSOM fluorescence can spatially map the locations of fluorescent probes or fluorescently tagged species with resolution reaching tens of nanometers. These capabilities can help in studies of biologically relevant membrane microdomains such as those encountered in lipid raft studies.

Given the correspondence between the topography and fluorescence images seen in Fig. 8, one might argue that AFM measurements of topography are sufficient to measure phase distributions in these simple systems with high resolution. However, assigning phase based on height changes alone can be ambiguous in monolayer systems and becomes difficult to impossible in more complicated systems.

For example, Fig. 9 shows an AFM image of a DPPC bilayer formed through the sequential transfer of monolayers onto a mica substrate. Each DPPC monolayer was transferred at the same phase coexistence region of the pressure isotherm as that shown in Fig. 8. Three

quantized height level changes are observed in the AFM image (see Fig. 9a) which corresponds to the stacking of LE and LC domains from each monolayer that forms the bilayer. This convolution of height information from each monolayer forming the bilayer makes assigning phase distributions in each leaflet impossible. However, high-resolution NSOM fluorescence measurements can resolve the submicron domains present in the leaflets by simply controlling which leaflet incorporates the fluorescent dye. Figure 9b, c show the NSOM force and fluorescence from a DPPC bilayer in which only the top layer has been doped with the fluorescent probe. While the NSOM force image suffers the same limitation as the AFM measurements, the NSOM fluorescence image clearly reveals similar domain structure as that seen in monolayers. Similar measurements with the dye loaded in the bottom leaflet shows comparable behavior.

For model membrane studies, the simultaneous collection of both force and fluorescence information with NSOM can also provide valuable insights into membrane collapse and buckling. For example, Fig. 10 shows a monolayer of the replacement lung surfactant, Survanta, used in the treatment of respiratory distress syndrome. The monolayer has been compressed to the point of collapse where the monolayer has buckled into a multilayer structure. This is evident in the NSOM force and fluorescence images shown in Fig. 10 which reveal a transition in the film structure. Examination of the NSOM force image (see Fig. 10a) reveals a ~4–5 nm height change in the sample which coincides with a large change in the phase structure seen in the NSOM fluorescence image (see Fig. 10b) (37). These and similar measurements show that the collapse of these films results in the continuous folding of the monolayer into a multilayer structure. The complexity seen in the NSOM fluorescence image arises from the stacked monolayers in the folded regions. By comparing the structure seen in the NSOM fluorescence images at folded edges correlated with the force images, it was determined that the folds were formed from a continuous monolayer film and did not arise from the breaking and stacking of individual monolayer film units (37).

As illustrated in the previous examples, the high spatial resolution and simultaneous collection of both topographic and fluorescence signals with NSOM becomes increasingly informative and useful as the complexity of the membrane system increases. For natural biological membranes, these capabilities can help unravel complex structural details that would otherwise be hidden or ambiguous in studies relying solely on either fluorescence or topography information alone. To help illustrate this point, NSOM studies of the nuclear envelope isolated from the nuclei of *Xenopus laevis* oocytes are briefly discussed (38).

The nuclear envelope contains large protein complexes known as nuclear pore complexes (NPC) that form the only direct route of passage across the nuclear envelope. These NPCs are easily seen by AFM images such as that shown in Fig. 11. Each toroidal structure in the image is an individual NPC in the membrane. Close examination reveals that some NPCs have a mass located in the central pore region, the identity of which has been the subject of much speculation. Some studies suggested they represent vault complexes, which are large ribonucleoproteins whose specific functional roles remain elusive.

To directly probe the relationship between NPCs and vault complexes, NSOM studies were carried out with immuno-fluorescently labeled vault complexes. The NSOM images in Fig. 12 show the NSOM topography, fluorescence and overlay images from this study. The NSOM force image in Fig. 12a reveals the locations of NPCs in the nuclear membrane, albeit with lower resolution than comparable AFM measurements. This can be compared with the small fluorescent features seen in the NSOM fluorescence image (see Fig. 12b) which maps the locations of the individually labeled vault complexes. NSOM, therefore, provides a novel method for colocalization where the force image maps the locations of

NPCs and the NSOM fluorescence image localizes the vault complexes, all with ~50 nm spatial resolution (5, 38). Figure 12c displays an overlay of the NSOM force and fluorescence images which clearly shows that vault proteins are localized with NPCs.

The above studies help illustrate the utility of employing NSOM measurements for the analysis of biological membranes. While fluorescence microscopy has found widespread use in understanding membrane organization and structure, its limited spatial resolution restricts its ability to explore membrane properties at a more fundamental level. The small length scale and complexity of structures encountered in lipid monolayers and bilayers necessitate the use of high-resolution techniques such as NSOM. NSOM combines the favorable attributes of fluorescence microscopy and AFM which seems particularly well suited for deciphering the structure and organization of biological membranes at the nanometric level.

4. Notes

1. Transmission measurements should never be used or trusted to quantify resolution of a NSOM tip. There is extensive literature illustrating the artifacts inherent in such measurements. The gold standard for characterizing resolution of the tip aperture remains single molecule NSOM fluorescence measurements such as that shown in Fig. 2.
2. It is very important to develop in-house fiber optic tip fabrication capabilities. Tailoring the tip shape and characteristics to the performance metrics desired is often required. Moreover, for biological samples, which often have complicated surface topography and a propensity to foul NSOM tips, several probe replacements are usually required before optimal imaging is obtained. This can quickly become cost prohibitive with commercial probes.
3. The particular shape of the NSOM probe is dictated by the performance characteristics sought. As mentioned, using high heat and hard pulling parameters yields the smallest tip apertures but very long taper regions. While small apertures are usually desirable, the long tapers dramatically reduce the efficiency of light delivered to the tip aperture. For NSOM measurements with resolution in the ~50–100 nm range, we find the two-stage taper shape shown in Fig. 1 to be the best option for obtaining quality results (7). The shallow taper angle near the distal end of the tip produces a small aperture while the larger taper angle before that enables efficient coupling of light toward the aperture.
4. The major components of the chamber include: (1) Leybold Turbovac 151 backed with an Alcatel mechanical roughing pump; (2) Inficon XTM/2 Deposition monitor positioned above the tips is essential for controlling both the rate of deposition and total film thickness.
5. The long working distance microscope is an important component of any NSOM microscope. By monitoring the light from the NSOM probe and its reflection off the sample surface, the NSOM probe can be quickly and conveniently positioned within the travel of the z-piezo tube. It is also useful for monitoring the condition of the NSOM probe during imaging.
6. Stable tip feedback is critical for obtaining high-quality NSOM fluorescence images. In addition to the usual approaches of adjusting feedback gains and scan angles to explore the best imaging conditions, adjusting power through the NSOM probe can also have a dramatic effect on tip feedback performance. Power-induced heating of the metal coating leads to NSOM tip expansion, which can sometimes be used to improve tracking of the sample topography (7, 39).

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Fig. 1.

A magnified view of a typical aluminum-coated NSOM probe fabricated using the heating and pulling method. A small spot of light can be seen emerging from the distal end of the probe. The probe has been fabricated with a double taper geometry which, for general NSOM imaging, strikes a balance between light throughput and small aperture size.

Fig. 2. NSOM fluorescence image of a $2\ \mu\text{m} \times 2\ \mu\text{m}$ region of a DPPC monolayer doped with the fluorescent lipid marker DilC₁₈. Each emission feature represents fluorescence from a single probe molecule in the membrane and has a FWHM of $\sim 20\text{--}30\ \text{nm}$. This illustrates the high spatial resolution and single molecule detection limits of NSOM. Because the reporter molecule is much smaller than the NSOM probe, the FWHM of each emission feature reflects the size of the particular NSOM aperture used in the imaging.

Fig. 3.

(a) A fiber-chuck is used to hold the NSOM probe in the evaporative coater. The extra length of fiber from the NSOM probe is sealed inside the spin-chuck with a removable metal ring. (b) Four rotating mounts position the fiber chucks above a central opening, housing the thermal aluminum evaporation element. Mounted above the rotating tips is a quartz crystal microbalance to measure deposition rate and total film thickness. (c) The aluminum evaporation element is surrounded by a metal housing to reduce heating experienced at the tips. A manual shutter is used to block the source when melting and degassing the Al before coating the tips. (d) A metal jacket is glued to an aluminum-coated NSOM probe to assist in mounting the tip in the NSOM head.

Fig 4. $\mu\text{m} \times 2 \mu\text{m}$ AFM images of the aluminum coatings applied to NSOM probes in vacuums of (a) 1×10^{-3} Torr and (b) 3×10^{-6} Torr. As seen in these images, a decrease in the chamber pressure results in a considerable decrease in roughness of the Al coating. Figures (c) and (d) show similar AFM measurements on NSOM tip coatings deposited at rates of $30 \text{ \AA/s}$ and $2,000 \text{ \AA/s}$, respectively. These results show that the quality of the Al coating also depends on the rate of deposition, with faster rates preferable over slow depositions. Adapted from ref. 20 with permission.

Fig. 5.
A scanning electron microscopy image of a NSOM probe fabricated using optimal conditions outlined in the text.

Fig. 6.

A NSOM instrument constructed on an inverted fluorescence microscope. The NSOM head contains the z -piezo to control the tip-sample gap and the shear-force feedback detection system. The head is mounted in a motorized dovetail for coarse adjustment of the head toward the sample. The sample is mounted below on a closed-loop x - y piezo stage that scans the sample under the probe. Fluorescence from the sample is collected through the microscope, passed through emission filters, and imaged onto the active area of an avalanche photodiode (APD) detector.

Fig. 7.

The NSOM head is used to control the z -position of the near-field probe using a shear-force feedback mechanism. The probe is mounted onto a piezo bimorph held at the end of a z -piezo tube. The bimorph is used to dither the NSOM probe laterally at its resonance frequency. The probe oscillation amplitude is monitored from the side by using a fiber-coupled laser source that shadows the tip onto a split photodiode detector. Each region of the detector is amplified and sent to the differential input of a lock-in amplifier referenced to the tip oscillation frequency.

Fig. 8. Simultaneously collected NSOM topography (**a**) and fluorescence (**b**) images of a DPPC monolayer doped with the fluorescent membrane probe DiI C₁₈. Small ~8 Å level height differences observed in the topography image reflect the coexisting liquid-expanded (LE) and liquid-condensed (LC) phases. The bright regions in the NSOM fluorescence image mark the LE domains which incorporate the fluorescent lipid probe. Adapted from ref. 31 with permission.

Fig. 9.

(a) AFM topography image of a DPPC bilayer membrane on a mica substrate formed using the Langmuir-Blodgett/Langmuir-Schäfer (LB/LS) technique. The $10\ \mu\text{m} \times 10\ \mu\text{m}$ region shows three quantized height levels due to the stacking of liquid-expanded (LE) and liquid-condensed (LC) from each leaflet. The convolution of height information from each leaflet forming the bilayer makes it impossible to assign the phase structure present in each leaflet. NSOM topography (b) and fluorescence (c) images on a similar bilayer, however, can be used to measure the phase distribution in each side of the lipid bilayer by controlling which leaflet incorporates the fluorescent probe. While the NSOM topography (b) suffers the same convolution problem that AFM has, the NSOM fluorescence image (c) shows distinct structures reflective of coexisting lipid phases similar to the monolayer shown in Fig. 8. (All images are $10\ \mu\text{m} \times 10\ \mu\text{m}$.) Adapted from ref. 31 with permission

Fig. 10.

23 $\mu\text{m} \times 23 \mu\text{m}$ NSOM topography (**a**) and fluorescence (**b**) images of a monolayer of the replacement lung surfactant Survanta. The surfactant has been compressed beyond the collapse pressure which has resulted in buckling of the monolayer. This is evident from the change in topography in the force image and corresponding transition in phase structure observed in the NSOM fluorescence image. (Both images are 23 $\mu\text{m} \times 23 \mu\text{m}$.) Adapted from ref. 37 with permission.

Fig. 11.

$2\ \mu\text{m} \times 2\ \mu\text{m}$ AFM image of the nuclear envelope from oocytes of *Xenopus laevis*. The donut-like structures observed in the membrane represent nuclear pore complexes (NPC) which form the only direct pathway across the membrane. A mass is observed in the pore region of many of the NPCs. The identity of this mass is the subject of much speculation, but some believe it represents vault complexes localized to the pore. Adapted from ref. 38 with permission.

Fig. 12.

NSOM topography (a) fluorescence (b) and overlay (c) images of the nuclear envelope in which vault complexes have been immunofluorescently labeled. Individual NPCs are observed in the NSOM topography image, albeit with lower resolution than the AFM measurement shown in Fig. 11. Features in the NSOM fluorescence image reflect labeled vault complexes which can be compared directly with the location of NPCs observed in the force image. The overlay image shows the colocalization of NPCs and vault complexes with less than 100 nm resolution. Scale bar in each image is 1 μm . Adapted from ref. 5 with permission.

Table 1

Mean surface roughness of Al coatings of NSOM probes deposited under various conditions as measured by AFM

Coating condition	Surface roughness mean(Std. Dev.)(nm)
Pressure= 1×10^{-3} Torr	68.1(24.9)
Pressure= 1×10^{-4} Torr	34.6(2.7)
Pressure= 1×10^{-5} Torr	20.7(2.6)
Pressure= 3×10^{-6} Torr	16.1(2.2)
Rate=30 Å/s	36.3(7.6)
Rate=700 Å/s	18.3(2.0)
Rate=2,000 Å/s	10.5(2.0)

Smoother, high-quality Al coatings are achieved at lower pressures and faster deposition rates