Visualization and measurement of the local absorption coefficients of single bilayer phospholipid membranes using scanning near-field optical microscopy

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Abstract: Here we report the results of shear-mode thicknesses and absorption coefficient measurements made on neat membranes using scanning near-field optical microscopy (SNOM). Biomimic neat membranes composed of two different types of phospholipid molecules: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were found to exhibit different absorption coefficients under the SNOM. The localization of the lipids could be identified and correlated to the morphology of the membrane domains indicating that SNOM can be an effective and accurate approach for the label-free characterization of the structure-function relationships in cell membranes.

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1. Introduction

The biological membrane defines the boundaries of a cell by forming a physical barrier between the biochemical machinery of cellular processes and the outside world. It has a highly heterogeneous structure, composed of many different types of lipids, proteins, and carbohydrates [1–4]. Specific membrane domains, e.g. rafts, are known to modulate protein function; therefore, the composition and factors driving such formation have been extensively studied [5–7]. It is believed that the regulatory properties of membrane domains are related to their unique physicochemical characteristics, which are determined by the thermodynamic phase and local composition of the bilayer. Hence, understanding the structure and composition of these domains is believed to provide important information about the regulatory function of lipid membranes.

Due to the complexity of biological membranes, biomimetic lipid membranes are used as model systems for studying membrane processes, including structural properties such as domain segregation [8,9], and biochemical membrane processes such as membrane protein function [10–12]. Phospholipid bilayer membranes exhibit a complex phase behaviour that is most frequently compared to liquid crystals; in each phase, constraints are imposed on lipid packing, yet the lipid molecules retain substantial lateral mobility [13–15]. This lateral mobility allows the spontaneous segregation of lipids into domains of different composition that are in dynamic equilibrium [15].

A range of opto-mechanical microscopy techniques have been used to study supported and unsupported lipid membranes, including atomic-force microscopy (AFM) [11,16], super-resolution
fluorescence microscopy [17], fluorescence correlation spectroscopy [18] and interferometric scattering microscopy [19,20]. The most success to date in imaging domain dynamics has been achieved with the AFM [11,16,21–23]. Whilst AFM can detect sub-nanometer changes in height it is only able to provide morphological maps of the sample surface and does not provide direct information regarding the membrane composition. Fluorescence microscopy can also be used to visualize membrane domains, however, the limited spatial resolution and the requirement for target-specific high-density labeling limit the applicability of such techniques [24]. The key issue with labelling in this scenario is that the fluorophores often have larger molecular weight than the lipid head groups. Therefore, one cannot expect that the molecule would have the same kind of diffusion or interaction behaviour with neighbouring molecules as it would without the label [25].

The identification and unambiguous characterization of domains within cell membranes requires a method capable of simultaneously imaging the morphology and estimating the composition of un-labeled lipid structures at the nanoscale. A technique capable of fulfilling these requirements is scanning near-field optical microscopy (SNOM). SNOM is an optical imaging technique which intrinsically provides images with spatial resolution below the diffraction limit [26]. In SNOM, near-field imaging is performed by probing evanescent (non-propagating) photons that are strictly localized in the near-field region. The evanescent photons are critical for near-field imaging, since they carry information about the fine structure of the sample [27,28]. Though SNOM is primarily an optical technique, however the instrument incorporates a mechanical feedback loop to trace the sample surface. Hence, it is possible to simultaneously generate a spatially resolved map of both the sample morphology and the optical absorption coefficient. Since its initial development, SNOM has proven to be a powerful tool for nanometer to micrometer scale imaging across a broad variety of samples in biology, materials science, and nanotechnology [29,30].

Depending on the structure of the probes, there are two types of SNOM: apertureless SNOM and aperture SNOM. In apertureless SNOM, the probe is a nanometer-size AFM metallic tip which is used as a scatter source. Apertureless SNOM is also known as scattering-type SNOM (s-SNOM) which can be combined with label-free vibrational spectroscopies to study a wide range of biomolecular systems such as imaging of homogeneous lipid bilayer membranes [31,32].

In contrast, the probe used in aperture SNOM is a waveguide with one end tapered and ending with a very small, subwavelength size aperture. The aperture is brought close to the sample in the near-field zone (5-10 nm from the sample surface) where it collects the near-field light, and then guides it through the waveguide to the detector. Although most of the applications of aperture SNOM are in the visible wavelength range however it can be also used in the other spectral region by using probes with different operating wavelengths. For instance, infrared range aperture SNOM (IR-SNOM) is previously being used to study the formation of locally ordered multiple bilayers lipid membranes [33]. Though there are some applications of SNOM to study the molecular organization of homogeneous lipid membranes however the ability of SNOM to perform local absorption imaging of lipid membranes is yet to be explored.

Here, we demonstrate the direct visualization and optical characterization of self-assembled domain structures in single bilayer phospholipid membranes using SNOM. The combined spatially resolved topography data and simultaneously acquired near-field optical intensity images reveal the spatial distribution of the two different lipids in the membrane. This enables label-free imaging and measurement of the morphology and optical properties of the nanodomains in which the constituent lipids can be clearly identified.

2. Materials and methods

2.1. Buffer preparation

Single bilayer membranes made of two different types of phospholipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were
prepared by the liposome deposition method. Phosphate buffered saline solution (20 mM) containing 100 mM sodium chloride (Merck) at pH 6.9 was used in all experiments. Potassium dihydrogen phosphate (KH$_2$PO$_4$) and dipotassium hydrogen phosphate (K$_2$HPO$_4$) were purchased from Fluka (Switzerland) at ACS Reagent grade. Ultrapure water purification system (Sartorius AG, Germany) was used to provide 18.2 MΩcm deionized water for all solutions.

### 2.2. Preparation of liposomes

1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC) and 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Chloroform (spectrophotometric grade >99.8%, Sigma–Aldrich, Castle Hill, NSW, Australia) was used to create individual stock solutions by dissolving lyophilized DOPC and DPPC in chloroform. The lipid thin-film method was used for liposome preparation. This method starts by dissolving the lipid in chloroform; then the solvent is evaporated under a stream of nitrogen gas with constant vortexing to yield a thin and dry lipid layer. The dry film is hydrated with buffer solution, followed by brief vortexing and sonication to remove lipid from the test tube wall and disperse aggregated liposomes. The liposomes were deposited on the glass slide used for SNOM imaging and incubated for 30 min. Finally, the fused membranes were rinsed with ultrapure water several times to remove any excess salt and unopened liposomes and dried for the measurements.

### 2.3. SNOM configuration

The SNOM used in this study operates in a transmission mode configuration and is a modified version of a shear-force based SNOM system (NT-MDT Ntegra Solaris platform, Zelenograd, Russia). In transmission mode, the SNOM can be used in both the ‘collection’ and ‘illumination’ mode of operation. In illumination mode, light is coupled into the broad end of the fibre and then conducted through to the tapered end, where it becomes concentrated towards the aperture. In collection mode a light source in the far-field is used to illuminate the sample; this gives rise to an evanescent field at the sample surface. By using a sub-wavelength sized aperture probe the evanescent field is converted into propagating waves inside the fibre [34].

Here, we firstly configured our SNOM to operate in collection mode and later in illumination mode for imaging the DOPC and DPPC membrane patches on a glass substrate. For imaging in collection mode, a spatially filtered and collimated 640 nm laser beam was directed onto the sample using an objective lens with a 0.3 Numerical Aperture (NA), 10X (Olympus) positioned in a defocused state for illuminating the sample. The biomimetic membrane sample on a glass substrate was dried and placed on the sample holder of a mechanical stage. Raster scans on the sample were performed to simultaneously acquire both topography and near-field transmission data. A commercially available Al-coated tapered fibre probe having an aperture diameter of ~125 ± 25 nm (MF113_NTF, TipsNano) was used for raster scanning and collection of the transmitted near-field data. Although probes with smaller aperture diameter can be used for higher spatial resolution however smaller aperture size will decrease the amount of light that can be transmitted through the probe. Thus, reducing the image contrast significantly due to the lower signal-to-noise ratio at the detector, which is not desirable for imaging thin layered membrane samples.

Images were acquired in SNOM transmission mode at a scanning rate of 0.01 Hz. The signal was then detected and converted into an electric signal by a photomultiplier tube (R2027 from Hamamatsu). Here, we define a “membrane patch” as an island of single bilayer phospholipid membranes on a glass support, whereas “domains” are areas of distinct morphology (as defined by a height and/or optical absorption difference) within a membrane patch.
2.4. Calibration

The single bilayer membrane patches are very thin (∼ 10 nm thick) and SNOM can potentially exhibit artefacts due to the motion of the probe in a direction normal to the sample surface ("z-motion"). To counter this effect, we carefully operated the equipment under the “constant height” mode. In addition, we performed SNOM scans on a calibration chevron sample to characterize the system’s sensitivity. The calibration sample has four platinum on carbon (Pt/C) bars with thickness values of approximately 3, 8, 13 and 18 nm (measured by AFM) respectively, comparable to the height of the single bilayer membrane patches. The SNOM scans (Fig. 1) shows detectable optical absorption from all four of the bars, although we note that in the case of the thinnest bar the signal is less clear due to unevenness of the background intensity from the substrate (Table 1). Also, it can be seen that no artefacts appear visible in the scans on the nanofabricated test object confirming that the optical transmission images correspond to actual sample features.

![Image](image_url)

**Fig. 1.** (a) Collection-mode SNOM imaging of the Pt/C chevron sample. Simultaneously acquired (a) topography (in nm) and corresponding (b) optical transmission images (in V) of the same sample. Scale bar length is 5 µm. (c) Representative topography (red) and transmission (blue) profiles along line #1 in the respective image pairs.

| Carbon Bar Thickness (nm) | Absorption Coefficient (× 10^5 cm⁻¹) | Average Near-Field Transmission |
|---------------------------|-------------------------------------|---------------------------------|
| 8.44 ± 0.64               | 3.57 ± 0.36                         | 0.74                            |
| 13.74 ± 0.42              | 3.85 ± 0.39                         | 0.60                            |
| 18.91 ± 1.45              | 3.81 ± 0.52                         | 0.49                            |

3. Results and discussion

3.1. Visualization of DPPC and DOPC membranes via topography and optical transmission

DPPC and DOPC are frequently used as model systems to mimic cell membranes [35–37]. These two phospholipids have very similar structures, with the same head group and identical acyl chain length. The difference between them is the presence of a double bond in each of the acyl chains of DOPC, whereas the chains of DPPC are saturated. The double bonds increase the effective volume of the acyl moieties and inhibit tight packing of the hydrophobic membrane core, hence reducing the van der Waals interactions between the adjacent molecules. As a result, DOPC has a main phase transition temperature of 16.5°C causing it to form a fluid phase (L_α) at room temperature. In contrast, the main transition temperature of DPPC is reported as 41.3°C,
and therefore it is in gel phase (L_β) at room temperature [38,39]. Hence, the two lipids have characteristically different packing properties.

Topography and optical transmission intensity images, along with the corresponding line profiles of membrane patches of DPPC (a, b and c) and DOPC (d, e and f) are shown in Fig. 2. The DPPC membrane patches were measured to be between 6.03 and 10.89 nm thick. For DOPC-based membranes, the heights of the individual patches were found to be somewhat lower, within the range of 4.44 to 6.32 nm (Fig. 3). These thicknesses are somewhat higher for single bilayer membranes compared to the corresponding AFM measurements [40,41]. Such variations between AFM and SNOM height profiles can be attributed to the different modes of operation e.g. contact or tapping mode in AFM versus shear force mode in SNOM, with the latter resulting in a lower force being applied to the membrane and thus a variation in the measured force envelope.

![Fig. 2.](image)

The presence of multiple bilayer membranes is also possible. Therefore in the followings the absorption coefficients are used to characterize the different lipids, where the layer height is already factored in, instead of absorbance or transmittance. Consistently variations between single bilayer and multiple bilayer membranes should not influence the experimental absorption coefficient results.

The optical intensity images (Fig. 2b and 2e) show a characteristically higher amount of absorption at the locations of the membrane patches also identified in the corresponding topography images. Such a high degree of absorption is unexpected based on the far-field extinction coefficients of the lipids; thus, it is attributed to the contrast enhancement resulting from near-field effects. Note that, in conventional far-field optical microscopy where imaging is performed by collecting only the propagating photons, single bilayer membranes do not produce detectable absorption. In contrast, near-field imaging is performed by probing evanescent photons that are non-propagating and strictly localised in the near-field region. Generally, this evanescent field decays exponentially as a function of the distance from the originating surface and subsequently provides subwavelength confinement. Also, the fast decay of the field intensity of the evanescent waves produces strong field gradients in the vicinity of the aperture by localizing the near-field to a volume wires ∼150 nm nanometers of the sample surface. Consequently, this can increase the absolute signal in the near-field and could enhance the image contrast
Fig. 3. Blue and red triangles represent the experimental measurements of the absorption coefficients of DOPC and DPPC-based membrane nanodomains, with respect to the thickness of the individual domains.

significantly. This probe induced near-field contrast enhancement mechanism is similar to what has been reported previously for scattering type SNOM imaging with normal mode of oscillation [42].

3.2. Absorption coefficients of DPPC and DOPC

In this work, we estimated localised absorption coefficients ($\alpha$) of individual membrane patches by rearranging Beer-Lambert’s law to get that

$$\alpha(x, y) = \log_e \left( \frac{I_m}{I(x,y)} \right) $$

where $I_m$ is the maximum light intensity measured in the absence of the sample, $I(x,y)$ is the transmitted light intensity through the sample, and $z(x,y)$ is the measured thickness of the sample. Given the same experimental conditions were maintained throughout the measurements we can assume that the measured values of the local absorption coefficients ($\alpha$) depend only on the material properties. Hence, in this work, we have performed measurements of the absorption coefficients ($\alpha$) of individual membrane patches from simultaneously acquired correlated topography and near-field optical intensity data.

The distribution of the resulting absorption coefficients of DPPC and DOPC-based membrane nanodomains is plotted in Fig. 3 in $cm^{-1}$, with respect to their individual heights. The mean and standard deviation of the measured absorption coefficient of the DPPC membranes was found to be $1.94 \pm 0.09 \times 10^5$ $cm^{-1}$, which is approximately 19% greater on average than the $1.61 \pm 0.09 \times 10^5$ $cm^{-1}$ measured for DOPC membranes. The average height of the DPPC-based membranes ($8.84 \pm 1.24$ nm) was also larger than that of the DOPC membranes ($5.49 \pm 0.55$ nm). Both of these differences may be attributed to the variations in the lipid packing order. As described above, DPPC has saturated acyl chains whereas DOPC is an unsaturated lipid. A double bond in the cis conformation located near the middle of a chain interferes with the packing of the hydrocarbon chains, resulting in weaker intermolecular forces due to steric hindrances and
increased spacing between molecules [43]. The measured thickness of the DOPC membrane is thus expected to be smaller than the DPPC membrane at the same temperature due to a combination of an actual difference in thickness and a lower elastic modulus. The difference in absorption coefficients is directly related to changes in the optical density of the material. Whilst, in general, the same moieties are present in both lipids, the presence of the double bond in the cis conformation of the DOPC membranes increases the effective volume of the acyl moieties thus inhibiting tighter packing of the hydrophobic membrane core which results in a lower optical density compared to the DPPC membranes. In Fig. 3, it can be seen that DOPC and DPPC can be distinguished by their absorption coefficients.

3.3. Illumination mode v.s. collection mode

Aside from the strong near-field absorption by the lipid molecules, the effect of scattering and reflection also needs to be taken into consideration. Supported lipid bilayers are highly transparent when viewed in the far-field, hence we assume that the effect of reflection will be minimal for these plasma membrane samples. In order to examine the possible effect of scattering on the optical transmission, we modified our SNOM setup to operate in illumination mode in order to compare the results to collection mode.

In illumination mode, light passes through the nanometer-sized aperture of the SNOM probe, and the detector is placed in the far-field (Fraunhofer region), thus collecting all of the transmitted light using an objective lens. This wide-area detection allows for the collection of both the scattered and transmitted light, whereas in collection mode the transmitted light dominates the signal due to the small angle, localized detection (Fresnel region) by the SNOM probe. It was determined that the “inverted" SNOM produced optical transmission images of comparable quality and yielded similar results, in terms of imaging, to the original collection-mode configuration.

In Fig. 4, the illumination and collection mode data are compared for a narrow range of membrane thicknesses distributed around the mean values. For DOPC, the average values of $\alpha$ in illumination and collection mode were estimated to be $1.32 \pm 0.05 \times 10^5$ cm$^{-1}$ and $1.40 \pm 0.06 \times 10^5$ cm$^{-1}$ respectively. For the DPPC membranes, the values were $1.75 \pm 0.01 \times 10^5$ cm$^{-1}$ and $1.94 \pm 0.09 \times 10^5$ cm$^{-1}$ for the illumination and collection mode respectively. In both cases, the average value of $\alpha$ in collection mode was found to be approximately 10% higher than the value obtained in illumination mode for both DPPC and DOPC membranes. This is not surprising as the transmitted intensity is expected to be higher in illumination mode due to the contribution of the scattering. This, in turn, means that the measured absorption coefficient is anticipated to be higher for collection mode which was confirmed in both cases. This result shows that the contribution of scattering is minimal compared to the transmitted signal due to the absorption. Hence we conclude, scattering does not have a significant influence on the observation that the near-field absorption of these membranes is orders of magnitude higher than their far-field absorption [44,45]. Other than scattering, another possible reason for the observed difference in the data could be the difference in the angle of incidence of the light impinging on the material. In collection mode imaging, an almost plane wave is used to illuminate the sample. On the other hand, a point source illumination (i.e. a range of angles) is used in illumination mode imaging. Since the transmittance of light is dependent on the angle of the incidence absorption within the same material could be different for the two different geometries.

3.4. Mixed DPPC and DOPC

Next, experiments were conducted on a mixed sample of 1:1 DPPC and DOPC lipids. The aim of this experiment is to probe separate sub domains in a mixed lipid sample by correlating their topographic information with the near-field optical absorption data. The absorption coefficient ($\alpha$) at a specific wavelength is normally used in quantitative analysis, to measure the concentration
of a sample. However, in a spatially and compositionally defined system such as single bilayer membranes, mapping the single wavelength absorption coefficient can be used for qualitative analysis, provided the values are characteristically different for the measured materials.

The corresponding topography and near-field intensity images of a mixed lipid (DPPC:DOPC) sample are shown in Fig. 5(a) and 5(b). Figure 5(c) shows the topography and absorption coefficient profiles along line #1 estimated from the respective topography and intensity images. In the topography and optical images (5(a) and 5(b)) the domains are marked with yellow and blue arrows for DPPC and DOPC lipids respectively. Since the SNOM probe oscillates laterally, it has a much broader convolution profile than is typical for an AFM, which normally produces a flat cross-sectional profile for these types of membrane patches. The size of the domains was found to be between ~230 nm to 600 nm. Here both the topography and intensity data show a heterogeneous profile with two distinct regions, with a step height and intensity difference of ~1.04 nm (Fig. 5(c)). The absorption coefficient of the regions consists of DPPC and DOPC domains are found to be approximately as $2.18 \times 10^5 \text{ cm}^{-1}$ and $1.71 \times 10^5 \text{ cm}^{-1}$ respectively which is consistent with the distribution of the measured values of the two lipids as presented in Fig. 3. Thus, indicating that under identical experimental conditions, we can potentially determine the type of lipids based on their absorption coefficients. The reduced contrast in the optical image compared to the topography image is due to the presence of electrical noise and beam inhomogeneity. However, spatially confined distinct domains can still be identified within the mixed membrane: one DOPC rich and two DPPC rich, which nevertheless exhibit some degree of intermixing, as expected for a dynamic system. The results are consistent with prior AFM work conducted on mixed DPPC:DOPC membranes that suggest a dynamic phase separated system where it was shown that, while DPPC/DOPC domains can have vastly different geometries, there is always a height difference of ~ 1 nm between DOPC and DPPC-rich areas [46–48]. However, the ability to identify the lipids within their respective domains is unique to SNOM measurements compared to AFM and, in combination with the topography information,
has not previously been reported. Hence, we have shown, it is possible to perform label-free chemical imaging with SNOM by quantitatively mapping the absorption coefficients of surface confined thin layer samples.

**Fig. 5.** Collection mode SNOM imaging of the mixed lipid (DPPC:DOPC) patches. (a) Topography and (b) SNOM intensity images showing the domains marked with the arrows within the DPPC:DOPC patches. (c) shows the corresponding profiles of topography and absorption coefficient along line #1 estimated from the respective topography and intensity images. (d) schematic of the lipid domains.

4. Conclusions

SNOM imaging has been successfully employed to visualize and characterize the domains of single bilayer phospholipid membranes quantitatively by directly estimating their near-field absorption coefficients from simultaneously acquired topography and near-field intensity data. The DOPC and DPPC-based phospholipid membranes exhibited characteristically different near-field absorption coefficients that may be explained in terms of the packing order of the hydrocarbon chains. This characteristic difference made it possible to identify the different lipids in the mixed membrane, which paves the way towards a new method for probing the distribution of lipids in biomimetic and biological membranes in future. Given the sensitivity of the measurements and to improve the signal to noise (S/N) ratio, dynamic optical elements such as a lock-in amplifier can be introduced as discussed in the literature [49]. In addition, it could be interesting to perform the measurements in other spectral range particularly in the infrared regime where DOPC and DPPC lipids tend to exhibit strong resonances [50]. However, conducting this type of SNOM measurements requires non-trivial new optical system and will be the subject of future investigation. To further enhance the potential applications of SNOM for more complex biological systems, simultaneous spectroscopic measurements can be carried out which will provide additional information about the samples’ chemical structures. In addition, water-based SNOM scanning is also possible with careful characterization of the control mechanism in the probe oscillation for more biocompatible samples [51,52].
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