Probing the Structure of Supported Membranes and Tethered Oligonucleotides by Fluorescence Interference Contrast Microscopy

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Fluorescence interference contrast microscopy (FLIC) is a powerful method to structurally characterize fluorescent objects with nanometer-scale resolution in the z direction. Here we use FLIC to characterize the water layer underlying supported membranes and membrane-tethered double-stranded oligonucleotides. FLIC measurements of supported membranes containing the lipid-anchored fluorescent dye DiI in both leaflets indicate the thickness of the water layer separating the solid support and the lower lipid leaflet is 1.3 ± 0.2 nm. Addition of cobalt(II) chloride to a DiI-supported membrane quenches the fluorescence in the top leaflet of the supported membrane; FLIC measurements of this system precisely locate the DiI to the bottom leaflet. These experiments confirm the accuracy of the model and parameters used to determine the water layer thickness, demonstrate the ability to differentiate between fluorescent objects whose average position differs by ~1.9 nm, and provide a widely applicable method to test the resolution of other high-z-resolution fluorescent microscopies. FLIC measurements of Alexa-labeled double-stranded oligonucleotides tethered to a supported membrane indicate that the DNA double helix is oriented perpendicular to the surface. Complications that arise from uncertainty in the orientation of the fluorophore are discussed. Several improvements in FLIC methodology are described. These stringent tests of the resolution of FLIC and the ability to unambiguously determine fluorescent lipid distribution provide structural insight on assemblies at membrane interfaces and permit the detection of even subtle changes at such interfaces.

Introduction

Supported membranes have found wide applications as mimics of cell membranes1–3 and in studies of the phase behavior of multicomponent lipid membranes.4–6 Supported membranes are formed by self-assembly of lipid bilayers onto a solid support, typically silica (see Figure 1A). A thin water layer separates the bottom leaflet of the supported membrane from the solid support; different reports6–10 suggest that this layer is between 0.2 and 4 nm in thickness. Importantly, the lipids in supported membranes maintain the lateral fluidity associated with lipid membranes in vesicles and cells. The water layer separating the supported membrane from the solid support is believed to be critical for the lateral fluidity of both leaflets. Ideally, transmembrane proteins in supported membranes would show similar mobility; however, in the few studies reported, transmembrane proteins have been found to be largely immobile, at least on the length scale sampled by fluorescence recovery after photobleaching (FRAP) measurements.11,12 Because transmembrane pro-

Figure 1. (A) Schematic diagram of a supported membrane. The position of fluorophores in the bottom and top leaflet relative to the aqueous layer is described by $d_1$ and $d_2$, respectively. (B) Schematic diagram of oligonucleotide-tethered vesicles. This diagram is not to scale; the approximate sizes of the oligonucleotide tether and the vesicle diameter are indicated. (C) Schematic diagram of the FLIC experiment. To determine the distance between a fluorescent object and the SiO$_2$ surface, identical objects are assembled on SiO$_2$ steps of different thicknesses on Si. The Si surface acts as mirror so that the incoming and reflected excitation light interfere, creating a standing wave of excitation light near the surface. Thus, the identical objects experience different light intensities on different SiO$_2$ steps. Although it is not shown for simplicity, the emitted fluorescence undergoes interference in a similar manner. For simplicity, the transition between SiO$_2$ steps is shown to be abrupt; however, this transition region between SiO$_2$ steps is actually quite gradual, with a slope of ~1.
teins often have soluble domains which protrude from the membrane, the immobility of transmembrane proteins is frequently attributed to contact of the protein with the solid support. It is often found that function is reduced or eliminated in immobile proteins; therefore, this poses severe restrictions in using supported membrane as a model system for studying transmembrane protein function in this format. To overcome these limitations, many investigators have attempted to separate the membrane from the solid support by a greater distance by tethering the membrane to the surface using spacers of fixed length or by incorporation of a polymer6,12,53 or protein layer14 to provide a cushion between the solid support and the membrane.

A different and promising approach to study interactions of membrane proteins in a native-like environment utilizes two-dimensional arrays of vesicles tethered to the surface of a fluid-supported membrane by hybridization of complementary oligonucleotides (Figure 1B),15 by chelation of oligohistidine by nickel nitritetriacetic acid,16 or by biotin—streptavidin coupling.17 In the system developed in our lab,17 small unilamellar vesicles displaying a short antisense oligonucleotide are exposed to a supported membrane that displays the sense oligonucleotides on its surface, resulting in tethering of intact and mobile vesicles in a sequence-specific manner to the supported membrane. Because it is straightforward to incorporate integral membrane proteins into vesicles and because the oligonucleotide-tethered vesicles are mobile and can be individually visualized, this system is potentially well suited for studying interactions between transmembrane proteins in different vesicles displaced from the solid support but retaining many of the advantages of the planar format.

For both supported membranes and oligonucleotide-tethered vesicles, the separation between the membrane surface including transmembrane proteins and nearest surface is presumed to dictate whether membrane components are mobile or not. For supported membranes, this distance corresponds to the water layer thickness, schematically illustrated as \( d_{\text{water}} \) in Figure 1A. For oligonucleotide-tethered vesicles, this distance is the shortest distance from the vesicle to the supported membrane, shown as \( d_{\text{dsDNA-vesicle}} \) in Figure 1B. To gain insight into what forces modulate these separations—ultimately so that they can be controlled—measuring \( d_{\text{water}} \) and \( d_{\text{dsDNA-vesicle}} \) with high precision is desirable. In addition to the value of more precisely measuring the water layer underlying supported membranes, distributional information would allow us to ask whether the water layer thickness is invariant or can be tuned by other parameters, such as lipid charge, ionic strength, and pH. In the same vein, we would like to characterize the distance separating tethered vesicles from the supported membrane. Because the persistence length of double-stranded DNA is many times larger than the length of the oligonucleotides used in the tethered vesicle system, the double-stranded oligonucleotide acts as a rigid rod, yet the linker which connects the lipid headgroup to the 5’ end of the sense strand has the potential to be quite flexible. Knowledge of \( d_{\text{dsDNA-vesicle}} \) would serve to additionally characterize the system of oligonucleotide-tethered vesicles, would suggest if significant interactions may be present between membrane components of the supporting membrane and the oligonucleotides or tethered vesicle and would inform interpretations of vesicle-vesicle interactions.

Measurements of \( d_{\text{water}} \) and \( d_{\text{dsDNA-vesicle}} \) both require high precision information on the structure in the direction perpendicular to the surface, the \( z \) direction. Fluorescence interference contrast microscopy (FLIC), developed extensively by Fromherz and co-workers for biological microscopy,20-22 uses a relatively simple experimental setup to attain high-resolution \( z \) information. To date, FLIC has been very effective in studies of biomimetic membranes, such as Dil-labeled cell membranes,20-22 lipid components in tethered supported membranes,10 and intermembrane junctions between a membrane patch and a supported membrane.23-25 In FLIC (Figure 1C), the sample is assembled on a series of SiO\(_2\) steps of different thickness on a highly reflective and flat Si surface, a FLIC chip. As shown schematically in Figure 1C, when a sample is imaged using a conventional epi-fluorescence microscope, interference between incoming and reflected excitation light creates a very well-defined variation in the excitation intensity in \( z \). The emitted fluorescence interferes in a similar manner, and the different SiO\(_2\) step thicknesses modulate the position of the fluorescent molecules relative to the overall interference pattern. Thus, the position of the fluorophore relative to the SiO\(_2\) surface can be determined by fitting the observed fluorescence intensity as a function of the SiO\(_2\) thickness to an appropriate model. In general, as the distance of the object from the SiO\(_2\) surface increases, the maximum fluorescence intensity occurs at a smaller SiO\(_2\) thickness. An analysis by Braun and Fromherz estimates the accuracy of FLIC to be \( \sim 1 \) nm;24 however, this estimation assumes the distribution of fluorophores between the leaflets of the bilayer is known. Without such knowledge, distances determined by FLIC potentially have an additional systematic error of as much as \( \pm 1.8 \) nm.

The major drawback to FLIC is that the structure of interest must be assembled identically over all the SiO\(_2\) steps.24 Fortunately, this requirement poses no difficulty for supported membrane samples. However, this requirement poses a severe restriction for a population of oligonucleotides-tethered vesicles because the vesicle reference Distribution function, the distribution function, would allow us to ask whether the water layer thickness is invariant or can be tuned by other parameters, such as lipid charge, ionic strength, and pH. In the same vein, we would like to characterize the distance separating tethered vesicles from the supported membrane. Because the persistence length of double-stranded DNA is many times larger than the length of the oligonucleotides used in the tethered vesicle system, the double-stranded oligonucleotide acts as a rigid rod, yet the linker which connects the lipid headgroup to the 5’ end of the sense strand has the potential to be quite flexible. Knowledge of \( d_{\text{dsDNA-vesicle}} \) would serve to additionally characterize the system of oligonucleotide-tethered vesicles, would suggest if significant interactions may be present between membrane components of the supporting membrane and the oligonucleotides or tethered vesicle and would inform interpretations of vesicle-vesicle interactions.

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between the two leaflets. We improve the accuracy of this measurement by explicitly measuring and accounting for the fluorophore distribution between the two leaflets. We present a stringent test of the ability of FLIC to distinguish small changes in position by comparing measurements of a supported membrane with fluorophores in both leaflets versus one containing fluorescent species only in the lower leaflet. To increase the precision of our measurement, we avoid contributions of inhomogeneous illumination and use a smaller NA objective to increase the sharpness of the interference pattern. These results quantitatively demonstrate the ability to resolve objects whose average position is 1.9 nm apart in the z direction, and thus provide a versatile resolution test that can be applied to other high-\(z\)-resolution fluorescence microscopy techniques. We also present FLIC results for fluorescently labeled double-stranded oligonucleotide tethers that suggest the distal end of the oligonucleotide tether is separated from the supported membrane by close to the maximal amount.

### Materials and Methods

#### Fabrication and Characterization of FLIC Chips.

FLIC chips, composed of a series of 16 SiO\(_2\) steps on Si, were fabricated by standard photolithographic procedures. After RCA (Radio Corporation of America) cleaning, an approximately 150 nm thick layer of SiO\(_2\) was grown on Si using thermal oxidation in the presence of O\(_2\)(g). Four rounds of photoresist development and buffered HF etching were used to produce 16 5 mm depth of etching. Before use, the FLIC chips were soaked in 90% distilled water, and baked at 400 °C for 4 h. After use, the FLIC chips were sonicated in water, 2-propanol, and chloroform, cleaned in piranha solution (75 vol% concentrated H\(_2\)SO\(_4\) and 25 vol% 50% H\(_2\)O\(_2\), heated to 70 °C), and the SiO\(_2\) thickness for each step was measured by ellipsometry (Variable Angle Stokes Ellipsometer L116SP, Gaertner Scientific, Stokie, IL). The parameters \(n_0 = 3.882\), \(k_0 = -0.019\) at 632.8 nm were used to determine the SiO\(_2\) thickness. Unlike bulk SiO\(_2\), ultrathin layers of SiO\(_2\) less than 15 nm have a refractive index that depends on the presence of O\(_2\)(g). Four rounds of photoresist development and buffered HF etching were used to produce 16 5 mm depth of etching. Before use, the FLIC chips were soaked in 90% distilled water, and baked at 400 °C for 4 h. After use, the FLIC chips were sonicated in water, 2-propanol, and chloroform, cleaned in piranha solution (75 vol% concentrated H\(_2\)SO\(_4\) and 25 vol% 50% H\(_2\)O\(_2\), heated to 70 °C), and the SiO\(_2\) thickness for each step was measured by ellipsometry (Variable Angle Stokes Ellipsometer L116SP, Gaertner Scientific, Stokie, IL). The parameters \(n_0 = 3.882\), \(k_0 = -0.019\) at 632.8 nm were used to determine the SiO\(_2\) thickness. Unlike bulk SiO\(_2\), ultrathin layers of SiO\(_2\) less than 15 nm have a refractive index that depends on the SiO\(_2\) thickness:27–29 therefore, both the SiO\(_2\) thickness and the SiO\(_2\) refractive index were free parameters which were fit by the software. The resulting best-fit values of the SiO\(_2\) refractive index as a function of SiO\(_2\) thickness were consistent with values reported by Kalnitsky et al.27 Ellipsometric measurements of the SiO\(_2\) thickness were made at three locations on each step, with a resulting variation in thickness on the order of ±0.3 nm.

#### Formation and Imaging of Supported Bilayers.

Small unilamellar vesicles composed of 0.5 mol% 1,1′-di-octadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI, Molecular Probes, Eugene, OR) and 99.5 mol% egg phosphatidylcholine (egg PC, Avanti Polar Lipids, Alabaster, AL) were prepared in Millipore water (resistivity 18.2 MΩ/cm) by vesicle extrusion through 50 nm polycarbonate membranes. These vesicles were diluted to ~1 mg/mL in 10 mM phosphate 50 mM sodium chloride, pH 7.2 buffer immediately before use. Sulphhydryl-reactive vesicles composed of egg PC and 0.5 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[3-[2-pyridyldithio]propionate] (NPDP-PE, Avanti Polar Lipids, Alabaster, AL) were prepared at a lipid concentration of 25 mM in using 100 mM NaOH followed by the addition of 500 mM NaOH to 100 mM sodium chloride, 2 mM EDTA, pH 8.0 buffer (borate–citrate buffer) by vesicle extrusion through 50 nm polycarbonate membranes. To prepare oligonucleotide-modified vesicles, the sense oligonucleotide (5′-AGCGGATAACATTTCCACAGGA-3′) modified with a disulfide group on the 5′ end (C\(_3\) thiol modification, IDT DNA Technologies, Coralville, IA) was exposed to 10 mM excess tris(2-carboxyethyl)phosphine (TCEP) and added to the sulphydryl-reactive vesicles to a final DNA concentration of 50 \(\mu\)M. The resulting vesicles were isolated on a Sepharose CL-4B gel filtration column with the borate–citrate buffer as eluant.

To prepare Alexa 594-labeled antisense oligonucleotides, the antisense oligonucleotide (5′-TCTCTGTGTGAATTGGTTATC-CCGT-3′) modified with a disulfide group on the 5′ end (C\(_3\) thiol modification, IDT DNA Technologies, Coralville, IA) was dissolved in 20 mM phosphate 140 mM sodium chloride, pH 7.2 buffer, exposed to 10 molar excess TCEP, and added to 13 molar excess Alexa Fluor 594 C\(_3\) maleimide (Molecular Probes, Eugene, OR) dissolved in dimethyl sulfoxide to a final oligonucleotide concentration of 0.1 mM. Alexa-labeled oligonucleotides were isolated from the excess Alexa 594 C\(_3\) maleimide using a standard ethanol precipitation with extensive washing. On the basis of the absorption spectrum and their extinction coefficients, the molar ratio of Alexa to oligonucleotide was 1:1.

The supported membranes were formed by vesicle fusion onto the FLIC chip, followed by extensive rinsing with Millipore water. For the FLIC experiments on Alexa-labeled oligonucleotide tethers, a solution of 0.1 mM Alexa-labeled oligonucleotide in borate–citrate buffer was incubated with the supported membrane displaying the sense oligonucleotide for 30 min followed by extensive rinsing of the surface with additional buffer. Two small strips of double-sided tape were used to seal the FLIC chip-supported membrane to a 22 mm × 40 mm glass coverslip (~0.1 nm thickness), trapping an approximately 100 µm thick buffer layer. This covered membrane, designated as the FLIC chip, demonstrated the ability to resolve objects whose average position is 1.9 nm apart in the z direction, and thus provide a versatile resolution test that can be applied to other high-\(z\)-resolution fluorescence microscopy techniques. We also present FLIC results for fluorescently labeled double-stranded oligonucleotide tethers that suggest the distal end of the oligonucleotide tether is separated from the supported membrane by close to the maximal amount.

#### Quenching by Cobalt(II) Chloride.

To determine the fraction of DiI in the top lipid leaflet of a supported membrane, we performed fluorescence-quenching studies of DiI in supported membranes.30 As additional controls, similar fluorescence-quenching experiments were performed on DiI-containing supported monolayers, micelles, and vesicles in solution. Micelles containing DiI were formed by addition of N,N-dimethyldecylamine-N-oxide (LDAO) to DiI-containing vesicles to ~1 vol%. Supported monolayers containing DiI were formed by vesicle fusion onto poly(dimethylsiloxane).31 For quenching experiments on supported membranes and supported monolayers, the fluorescence intensity was measured at three different locations on each step. This average and its corresponding standard deviation are the reported values in Figures 3–5. The image shown in Figure 3B was linearly contrast-adjusted using Adobe PhotoShop.

### Numerical Fitting, Modeling, and Parameters.

To quantitatively model and fit FLIC data, Fromherz and co-workers

Figure 2. (A) Calculations of the normalized fluorescence intensity as a function of SiO₂ thickness for a Dil-containing supported membrane with different distributions of fluorophores assuming a 1.5 nm thick water layer. The red line and blue line correspond to the relative intensity of supported membranes on a FLIC chip containing Dil only in the upper leaflet (d₂ = 3.69 nm, f₂ = 1) and only in the bottom leaflet (d₁ = 0.09 nm, f₁ = 1), respectively. Other parameters in this calculation include NA = 0.45, γ_α = 76.5°, γ_β = 80°. (B) Effect of varying the numerical aperture (NA = 0.45, blue line; NA = 1.0, black line) on the calculated fluorescence intensity as a function of SiO₂ thickness of a supported membrane with Dil only in the bottom leaflet. All other parameters are the same as in (A).

have developed an electromagnetical model that describes steady-state fluorescence near a multilayer interface that includes absorptive losses, nonradiative decay, and near-field losses. The equations derived by Lambacher and Fromherz[22] are more general cases of equations derived by Hellen and Axelrod,[32] Sullivan and Hall,[33] and Mertz[34] among others. A general discussion of the general principles of FLIC has been presented by Parthasarathy and Groves.[35] We use the model of Lambacher and Fromherz to model and fit FLIC data. The observed fluorescence intensity per unit time for a fluorophore near the surface, J, depends on the excitation rate of the fluorophore (k_{ex,ill}), the detection rate of emitted photons (k_{em,det}), the rate emitted by the fluorophore (k_{fl}), and rate of de-excitation due to nonradiative processes:

\[ J = \frac{k_{ex,ill}k_{em,det}k_{fl}}{k_{em,det}k_{fl} + \Phi_0} \]

(1)

where Φ₀ is the quantum yield of the fluorophore and k is the rate of fluorescence emission in the absence of the surface. The full expressions for k_{ex,ill}, k_{em,det}, and k_{fl} can be found in the work of Lambacher and Fromherz,[22] and we follow their notation. Because it occurs in the expressions for k_{em,det} and k_{fl}, cancels out and need not be explicitly calculated. Equation 1 gives J in units of energy/s; however, since the fluorescence intensity in measured in counts/s, the data is fit to

\[ J_{exp} = aJ \]

(2)

where a is simply a scaling factor that is constant for all the SiO₂ steps.

Because the value of d_{water} is determined by measuring the position of the Dil fluorophores relative to the SiO₂ surface, the fraction of fluorophores in the bottom and top leaflet of the membrane, f₁ and f₂, respectively, and their positions in the membrane, d₁ and d₂, respectively, (see Figure 1A) must be known. Alternatively, if d_{water}, f₁, and f₂ are known, then d₁ and d₂ can be determined. To fit for d_{water}—Alexa the Alexa fluorophores are assumed to be uniformly located at a fixed distance away from the surface. If a calculated FLIC curve, generated using a
model with a distribution of fluorophores, is fit with a model that has all the fluorophores located at a single position, then the resulting best fit value for the single position model is approximately equal to the weighted average of the original distribution. Interference in the z direction is caused by the difference in path length between the incoming and reflected rays. This path length difference for any z position depends on the wavelength of light, its incidence angle relative to the surface normal (θinc), the refractive indices of the layer system, and the thickness of each layer. Therefore, eq 1 includes the relative contributions of the light wavelengths involved in excitation, emission, and detection. The range and relative contribution of θinc’s is given by the numerical aperture of the objective (NA) and the aperture function, which describes the intensity of excitation light functioned by θinc. The model of Lambacher and Fromherz assumes that the intensity is a constant for all θinc’s and compensates for this approximation (which is incorrect for most microscopes) by treating the NA as a free parameter. For the narrow distribution of θinc’s used in these experiments, the aperture function is not expected to have a significant effect on the fitted value of dwater or dhydro-Alexa, so the data were fit using the specified NA and treating the aperture function as a constant. The terms in eq 1 account for the dependence of the absorption probability on the angle between the absorption transition dipole moment and the direction of the excitation light electric field and the probability of emission along a given θem’s given by the orientation of the emission transition dipole moment. The orientations of the absorption and emission transition dipole moments are described relative to the surface normal, γabs and γem.

The refractive indices of Si, SiO2, and H2O as a function of wavelength are taken from Palik. As mentioned above, ellipsometric measurements show the SiO2 refractive index to be a function of the SiO2 thickness for very thin films; however, use of a more complex model of SiO2 refractive index in the FLIC fitting routine did not significantly change the value of dwater or dhydro-Alexa. The highly hydrated headgroup regions of the lipid bilayer were assumed to have the same refractive index as water or ice and are thus treated as part of the aqueous layers. The refractive index of the hydrocarbon region of the membrane was taken to be 1.475, independent of wavelength. The thickness of the hydrocarbon layer of a hydrated egg PC bilayer is 3.78 nm. From fluorescence quenching studies of membrane probes that have a similar structure to Di using the parallax method, the Di fluorophore in methylated Alexa 594-C5 maleimide in water was obtained from the manufacturer. The quantum yield of Alexa 594 is approximately 0.55,40 and the quantum yield of Di is assumed to be 0.35 on the basis of the measured quantum yields of related compounds.41,42 Following the analysis by Sund et al.,42 the absorption and emission transition dipole moment of Di are aligned 76.5° and 80°, respectively, relative to its conjugated bridge axis, that this axis is parallel to the membrane plane, and that the membrane is perfectly parallel to the SiO2 surface, giving γabs = 76.5° and γem = 80°. The absorption and emission transition dipole moments of Alexa 594 are assumed to be parallel on the basis of the measured limiting anisotropy (r0 = 0.376) of the structurally related Alexa 488 fluorophore.44

The output spectrum of the mercury arc lamp and the transmission spectra of the excitation filters, emission filters, and dichroic mirrors were obtained from the respective manufacturers. The numerical modeling codes used to produce the calculations and fits were written in Matlab.

**Results and Discussion**

**Quantitative Determination of Water Layer Thickness and Resolution Test.** Although the approach of Kiessling and Tamm inspired us to use FLIC to characterize the structure of supported membranes, here we refine the accuracy of the measurement of the thickness of the water layer, dwater, by characterizing the distribution of Di fluorophores in the supported membrane and explicitly accounting for this distribution in the model used to fit the data. Although it is typically assumed that a fluorescent lipid probe is equally partitioned between the two leaflets of the supported membrane, recent results have shown that the distribution can be highly asymmetric for charged lipid probes. As illustrated in Figure 2A, calculations of the same water layer thickness with different distributions of fluorophores give similar, yet distinguishable, FLIC curves. To a first approximation for our experimental conditions, the sum of the fitted water layer thickness (dwater) and the weighted average of the fluorophore position (dfluovavg; dfluovavg = f1d1 + f2d2), i.e., dfluovavg + dwater, is constant for a given set of data. That is, if the value of dfluovavg used as a known parameter is increased by 1.0 nm, then the fitted dwater value will decrease by 1.0 nm. As we demonstrate in experiments discussed below, incorrect assumptions of fluorophore distribution yield incorrect measurements of dwater.

Additionally, we sought high-precision FLIC measurements and a quantitative demonstration of FLIC’s z resolution. To accomplish this, the systematic error in intensities caused by inhomogeneous illumination across the field of view was eliminated by using larger SiO2 steps and by comparing intensities at the same position within the field of view as described above (see Materials and Methods). We also chose to use smaller NA objectives to image supported membranes using FLIC. Figure 2B shows calculations of the fluorescence intensity, normalized to the maximum intensity, as a function of SiO2 thickness for a small 0.45 NA objective and for a large 1.0 NA objective. The variation in intensity is steeper for the small NA; since each θinc has an intensity maximum that occurs at a slightly different SiO2 thickness, increasing the number of θinc’s broadens the FLIC curve. Assuming that the same signal-to-noise ratio can be produced with large NA and small NA objectives (a situation that is true for our experiments), the steeper FLIC curve associated with a smaller NA objective would provide higher precision z positions.

Quenching experiments using cobalt(II) chloride were performed to determine the Di distribution in egg PC supported membranes. Several observations indicate that 500 mM cobalt(II) chloride completely quenches only the fluorescence from Di in the top leaflet, i.e., the leaflet furthest from the solid support. In the presence of 500
mM cobalt(II) chloride, the steady-state DiI fluorescence from a solution of detergent–phospholipid micelles or from a DiI-containing supported monolayer is completely quenched (data not shown). Despite this relatively high concentration of cobalt(II) chloride required for complete quenching, vesicle and supported membrane experiments both suggest that only the DiI fluorophores accessible to bulk solution are quenched. Addition of 500 mM cobalt(II) chloride to 0.5 mol% DiI-containing 50-nm-diameter egg PC vesicles or egg PC glass-supported membranes results in immediate quenching of 60 ± 2% and 54 ± 4% of the steady-state fluorescence, respectively.49 Addition of higher concentrations of cobalt(II) chloride did not result in increased quenching of the fluorescence. Taken together, we interpret these results as indicative of the percentage of DiI in the top leaflet: for intact vesicles, 60% ± 2% of the DiI is present in the outer leaflet, and 54% ± 4% of the DiI is in top leaflet for supported membranes. Although the negatively charged Texas Red probe is asymmetrically distributed in egg PC supported membranes such that the bulk of the fluorophores are in the upper leaflet,45–47 i.e., the leaflet further away from the negatively charged SiO2 surface, the positively charged DiI lipid is almost equally distributed between the two leaflets in egg PC supported membranes.40

With this information in hand, FLIC was used to determine dwater, the thickness of the water layer between the SiO2 support and the bottom leaflet of the supported membrane. As shown in Figure 3B, vesicle fusion of egg PC vesicles containing 0.5 mol% DiI onto FLIC chips resulted in highly uniform fluorescence over a single SiO2 step, and that intensity varied with the SiO2 thickness. The results from these FLIC experiments have a good signal-to-noise ratios: the intensity standard deviation was ~1% across a given SiO2 step, while the maximum fluorescence intensity was ~400% that of a glass-supported membrane of identical composition. Spot photobleaching demonstrated that there was no detectable immobile fraction and that the DiI lipids could freely diffuse from one SiO2 step to the next (data not shown). Assuming the fluorophore distribution as previously determined, the FLIC data were fit with two free parameters as given by eq 2: a scaling parameter and dwater. Measurements of several supported membranes on different FLIC chips with sets of slightly varying SiO2 step thicknesses yield dwater = 1.3 ± 0.2 nm.

The precision in this measurement, 0.2 nm, is based on the variation in the fits across five different supported membranes. For a given data set of 16 intensities and SiO2 thicknesses, the uncertainty in the best-fit value of dwater was quite small, i.e. standard deviation of ~0.02 nm. The best-fit values of dwater for 30 different regions taken from a single FLIC chip-supported membrane varied by ~±0.05 nm. As an additional indication of the accuracy of the model and parameters used to fit the data and as a quantitative demonstration of precision of the FLIC experiments, we performed FLIC on 0.5 mol% DiI-containing egg PC supported membranes in the presence of 500 mM cobalt(II) chloride, thus creating a situation in which only DiI molecules in the bottom leaflet fluoresce.51 An example of the data from one such experiment is shown in Figure 3C. Using dwater = 1.3 nm, the FLIC curve can be fit to determine the position of the fluorophores in the bottom leaflet of the supported membrane, dt. Over several experiments, the average fluorophore position agrees well with the measurement from parallel quenching experiments: dt = 0.2 ± 0.5 nm vs. 0.09 ± 0.2 nm.50 This agreement, based on FLIC data from both the quenched and unquenched DiI PC membranes, provides additional confirmation that Co2+ quenches DiI only the top leaflet and of the water layer thickness.54 The FLIC curve for a DiI-supported membrane in the presence of Co2+ is shifted to higher SiO2 thicknesses relative to that of an identical membrane in the absence of Co2+. This shift is small, corresponding to ~2 nm, but significant and reproducible between multiple data sets with varying sets of SiO2 step heights. Figure 3D highlights this
difference by plotting the difference between the FLIC fits shown in Figure 3C, in which each fit was normalized to a maximum intensity of 1. If the two data sets were indistinguishable, the difference in normalized intensity would be a straight line at zero and the fitted value of $d_{\text{dial}}$ would be close to 2.0 nm, i.e., the average position of the DI when present in both leaflets. The absolute value of the difference in normalized intensity summed over all the steps ($\sum |\Delta \text{fit intensity}|$), i.e., the difference between the two data sets, is $\sim 20\%$ for the NA $= 0.45$ objective and would be $\sim 4\%$ for the NA $= 1.0$ objective. The signal-to-noise ratio for this experiment is $\sim 1\%$, which strongly suggests that distinction between the both leaflet and bottom leaflet cases could not be made with a NA $= 1.0$ objective. These results demonstrate that the FLIC experiment is exquisitely sensitive to changes in fluorophore position and distribution in supported membranes, especially when low-NA objectives can be used.

**Determination of Alexa-Labeled Oligonucleotide Tether Position.** A schematic diagram of the Alexa-labeled oligonucleotide tethers is shown in Figure 4A. The duplex DNA consists of 24 base pairs, corresponding to an end-to-end distance of 7.8 nm. The 5′ end of the sense oligonucleotide is covalently coupled to the lipid headgroup by a disulfide bond and flexible linker region that is $\sim 2$ nm in length if fully extended. Likewise, the Alexa fluorophore is connected to the 5′ end of antisense oligonucleotide strand by a thioether bond and $\sim 2.5$ nm flexible linker. The oligonucleotide tethers bind sequence-specifically; a supported membrane displaying the sense oligonucleotide binds the Alexa-labeled antisense oligonucleotide strand but not noncomplementary Alexa-labeled oligonucleotides (data not shown). The Alexa-labeled oligonucleotide tethers diffuse freely in two dimensions with a diffusion coefficient on the order of $\sim 1 \mu m^2/s$, and upon application of an electric field parallel to the surface, they move toward the positive electrode (data not shown). A combination of absorption spectroscopy to measure the lipid concentration and fluorimetry to measure the concentration of Alexa-labeled tethered oligonucleotides was used to measure the density of displayed oligonucleotides as $\sim 1$ or 2 per vesicle (data not shown). Thus, the oligonucleotide-modified lipid density corresponds to approximately 0.01 mol% of the supported membrane or 2 tethered oligonucleotides per square micrometer.

An example of FLIC data for the Alexa-labeled oligonucleotide tethers is shown in Figure 4B. For comparison, the FLIC curve for a supported membrane with Dil in both leaflets is shown as a solid red line. The intensity maximum occurs at significantly smaller SiO$_2$ thicknesses for the Alexa-labeled oligonucleotide tethers in comparison to the Dil-supported membrane, suggesting that the Alexa fluorophore is displaced further from the SiO$_2$ surface. Although anisotropy measurement of related compounds suggest that the absorption and emission transition dipole moment of Alexa fluorophore are nearly collinear, there is no independent information on the orientation of transition dipole moments of Alexa fluorophore relative to the surface. This uncertainty in the orientation complicates fitting the FLIC data.

For a population of Alexa fluorophores located a fixed distance from the supported membrane surface, the shape and position of the intensity maximum of the FLIC curve changes with the orientation of their absorption transition dipoles relative to the surface. Figure 4C shows several calculated FLIC curves corresponding to different fluorophore orientations in which the fluorophore is located 10 nm from the membrane surface and each curve is normalized to have a maximum intensity of 1. For $\gamma_{\text{abs}} = \gamma_{\text{em}} < 30°$, the shape of the calculated FLIC curves are incompatible with the data in Figure 4B and the fact that the Alexa fluorophores can be at most $\sim 12.5$ nm from the surface. However, for $\gamma_{\text{abs}} = \gamma_{\text{em}}$ ranging from 30° to 90°, the shape of the FLIC curves are similar to the data shown in Figure 4B; the main difference between these normalized curves is the shift of the intensity maximum to larger SiO$_2$ thickness with decreasing $\gamma_{\text{abs}}$. To bound the possible range of values for $d_{\text{oligo-Alexa}}$, the data were fit separately with two models assuming a single fixed orientation corresponding to $\gamma_{\text{abs}} = 90°$ or $\gamma_{\text{abs}} = 30°$, giving $d_{\text{oligo-Alexa}}$ of 11.1 ± 2.2 and 17.3 ± 2.2 nm, respectively. The sum of the residuals and the sum of the square of the residuals for the fits are greater for the $\gamma_{\text{abs}} = 30°$ model than the $\gamma_{\text{abs}} = 90°$ model. A more realistic model for the Alexa-labeled oligonucleotide tethers would include a

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(55) A fluorescence polarization experiment could be used to determine the average orientation of the Alexa 594 fluorophore relative to the surface normal. The surface coverage of fluorophores is on the order of 2 per square micrometer, so attaining sufficient signal-to-noise in such an experiment with our current setup would be extremely challenging.
distribution of orientations rather than a single orientation. Fitting the data to a model that assumes the Alexa fluorophore is randomly oriented yields $d_{\text{oligo-Alexa}} = 14.1 \pm 1.8 \text{ nm}$; the residuals and sum of the square of the residuals in this case are approximately the same as those in the $\gamma_{\text{abs}} = 90^\circ$ model. If the linkers associated with the Alexa-labeled oligonucleotide tether are fully extended and the axis of the duplex DNA is oriented normal to the surface, $d_{\text{oligo-Alexa}}$ would be its maximum possible value, $\sim 12.5 \text{ nm}$. Therefore, these results strongly suggest that the Alexa fluorophore is separated from the surface by close to the maximal distance. That the oligonucleotide tether is oriented nearly normal to the membrane surface, despite the presence of a relatively long and flexible linker between the 5’ end and the lipid headgroup, is interesting. It is possible that this orientation results from repulsion between the highly negatively charged duplex DNA and remaining $\sim 0.49 \text{ mol}\%$ unreacted and negatively charged N-PDP-PE lipids. It is observed that vesicles displaying noncomplementary oligonucleotides do not bind to the supported membrane, suggesting that there is not a strong attractive net force between these vesicles and the supported membrane. Taking these observations together, we suggest that the tethered vesicles are separated from the underlying supported membrane by close to the full oligonucleotide tether length.

**Conclusions**

In conclusion, we have measured the water layer thickness underlying supported egg PC membranes as $1.3 \pm 0.2 \text{ nm}$. This result closely agrees with measurements of the water layer thickness underlying phosphatidylcholine bilayers using NMR$^{10}$ ($1.7 \pm 0.5 \text{ nm}$) and using FLIC$^8$ ($1.7 \pm 1.0 \text{ nm}$) but is a more precise measurement. FLIC measurements of egg PC supported membranes in which fluorescence was emitted only from the bottom leaflet confirmed the accuracy of this measurement and quantitatively demonstrated the ability to distinguish fluorophores separated by $\sim 1.9 \text{ nm}$. Additionally, the distal 5’ end of the oligonucleotide tethers is separated from the supported membrane by close to the maximal distance.

Importantly, this set of experiments provides a straightforward resolution test and calibration standard for other high-$z$-resolution fluorescence microscopies. For these experiments, a small NA objective was used to increase the maximum resolution attainable with FLIC. For samples with more limited fluorescence intensities, use of a small NA objective is not desirable. Another approach to sharpen the interference pattern without decreasing the signal-to-noise ratio is to restrict the excitation $\theta_{\text{inc}}$’s, yet use the full NA of the objective to maximize the ratio of detected/emitted photons. This can be accomplished by closing the aperture diaphragm down to a small but known size, restricting the excitation $\theta_{\text{inc}}$’s to near $0^\circ$. Taken together, these results reinforce the value of using FLIC to characterize fluorescent structures at and near membranes.

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