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Atomistic picture of fluorescent probes with hydrocarbon tails in lipid bilayer membranes: an investigation of selective affinities and fluorescent anisotropies in different environmental phases

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Abstract

By reverting to spectroscopy, changes in the biological environment of a fluorescent probe can be monitored and the presence of various phases of the surrounding lipid bilayer membranes can be detected. However, it is currently not always clear in which phase the probe resides. The well-known orange 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarboxyanine perchlorate (DiI-C18(5)) fluorophore for instance as well as the new, blue BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) derivative were experimentally seen to target and highlight identical parts of giant unilamellar vesicles of various compositions, comprising mixtures of dipalmitoyl phosphatidylcholine (DPPC), dioleoyl phosphatidylcholine (DOPC), sphingomyelin (SM) and cholesterol (Chol). However, it was not clear which of the coexisting membrane phases were visualized (Bacalum et al., Langmuir 32 (2016), 3495). The present study addresses this issue by utilizing large-scale molecular dynamics simulations and the z-constraint method, which allows evaluating Gibbs free energy profiles. The current calculations give an indication why, at room temperature, both BODIPY and DiI-C18(5) probes prefer the gel (S0) phase in DOPC/DPPC (2:3 molar ratio) and the liquid ordered (Lo) phase in DOPC/
SM/Chol (1:2:1 molar ratio) mixtures. This study highlights the important differences in orientation and location and therefore in efficiency between the probes when they are used in fluorescence microscopy to screen various lipid bilayer membrane phases. Dependent on the lipid composition, the angle between the transition state dipole moments of both probes and the normal to the membrane are found to deviate clearly from 90°. It is seen that the DiI-C18(5) probe is located in the headgroup region of the SM:Chol mixture, in close contact with water molecules. A fluorescence anisotropy study indicates also that DiI-C18(5) gives rise to a distinctive behavior in the SM:Chol membrane compared to the other considered membranes. The latter behavior has not been seen for the studied BODIPY probe, which is located deeper in the membrane.

**Introduction**

Molecular insight into the condition and properties of lipid membranes, which are fundamental components of living cells, is of utmost importance for various areas of biomedical research including drug design, drug pharmacology, or medical diagnosis and prognosis [1–4]. To give only one example of the crucial role of membranes, it has been shown that increased fluidity and polarity of cell membranes correlate with the metastasis in cancer cells [5]. Well-designed membrane-specific probes can picture biological membrane properties by means of optical imaging and suited spectroscopic techniques. Cholesterol (Chol) highly contributes to the structure of the membranes of many mammal cells [6]. For example, in hepatocellular carcinoma, which is the fifth most frequent cancer worldwide, high Chol levels were found to lead to tumor progression and malignancy [7–9]. The specific development of probe molecules which have an expressed affinity for Chol-abundant membrane regions is a particularly relevant and challenging topic. In the current work, computer modeling is used to investigate interactions of optically active probes with various membrane models and to evaluate whether they can identify the spectral fingerprints of specific biological conditions.

Natural membranes can be organized in different phases, with distinction between single-component and multicomponent membranes. For lipid systems of a single type, a gel phase (Sₒ) membrane is characterized by a high order of lipid packing. The liquid-crystalline or liquid disordered phase (Lₒ) of the membrane is characterized by a reduced lipid packing and higher diffusion coefficients. In complex lipid systems, Chol promotes phase segregation and gives access to the liquid-ordered state (Lₒ), a phase which is often also enriched in sphingomyelin.
The different ratios among the lipid components of a membrane are important parameters that determine its phase. Single component membranes made of dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC) or distearoylphosphatidylcholine (DSPC) have been extensively studied \([12,14,15]\). The phase in a single component system depends on the lipid chemical structure and the temperature. DOPC with its transition temperature of \(-17 \, ^\circ\text{C}\) adopts a liquid phase at room temperature; the DPPC membrane is in the \(S_0\) phase at room temperature but adopts the \(L_d\) phase above its transition temperature of \(41 \, ^\circ\text{C}\) \([16]\). Two and/or three components membranes have also been evaluated, \textit{e.g.}, made of DOPC/DPPC or DOPC/SM/Chol in studies, which highlighted that different ratios between the components modulate membrane properties \([10,17-21]\). Considering the number of possible combinations of membrane components, as well as possible lipid segregations, mixtures of phases are expected in biological membranes. The ternary mixtures can be schematically visualized along with their relevant tie lines in temperature dependent triangular diagrams, from which the phase compositions as well as their coexistence can be read \([22-24]\). There is finally the need of techniques capable of distinguishing and (locally) characterizing these different phases.

One of the most popular dyes to unravel this complex membrane structure is \(1,1'-\text{dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate (DiI-C18(5))}\). It is a dialkyl carbocyanine (see Figure 1), which is amphiphilic due to the positively-charged head chromophore consisting of two indole rings connected by 5-carbon cyanine moiety and the 18-carbon saturated alkyl chains, which are important for the phase-selective partitioning in the membrane \([25,26]\). DiI-C18(5) exhibits a high extinction coefficient and a high fluorescence quantum yield, and is highly fluorescent and photostable when incorporated into membranes \([27]\). Fluorescence spectroscopic analyses of the DiI-family have been used to investigate: membrane rotational lipid mobility \([28]\); membrane potential \([27]\); membrane fusion \([29]\); fluorescence resonance energy transfer \([30]\); phase separation \([31]\); lipid leaflet transmigration \([32]\); and the existence of lipid rafts \([33]\). As the precise location, orientation and lipid/phase selectivity of the dye is often unknown or only partially described, the interpretation of fluorescence lifetime, anisotropy and rotational dynamics may be complex. Gullapalli \textit{et al.} theoretically investigated the properties of two and four DiI-C18(3) probes, which have a cyanine backbone made of 3 carbon atoms, within a DPPC lipid bilayer in its \(L_d\) phase at 323 K, safely above the transition temperature \([34]\). The probes were found below the head group – water interface and report well the rotational and lateral diffusion components of the lipid dynamics. The calculations showed that the dye
causes minor changes at the interface in the ordering of the water dipoles and electrostatic potential.

Recently, the meso-amino substituted BODIPY probe 8-[(2-sulfonatoethyl)amino]-4,4-difluoro-3,5-dioctadecyl-4-bora-3a,4a-diaza-s-indacene (BNP, see Figure 1) was synthetized and optically characterized \[^{35}\]. This probe expresses similar behavior with respect to membranes as DiI-C18(5), but fluoresces in the blue part of the visible spectrum. The BODIPY dyes are known to combine outstanding spectroscopic and (photo)-physical properties, such as bright fluorescence with absorption and emission bands in the visible range, as well as stability toward light and chemicals. In particular, BNP was found to be excitable by either 1 or 2 photons in combination with a high fluorescence quantum yield; this probe was found to preferentially partition in the same lipid phase as DiI-C18(5) \[^{35}\]. In this experimental work by Bacalum \textit{et al}., BNP and DiI-C18(5) were studied in a 2:3 mixture of DOPC:DPPC (L\textsubscript{d}:S\textsubscript{o} phases) and a 1:2:1 mixture of DOPC:SM:Chol (L\textsubscript{d}:L\textsubscript{o} phases) at room temperature. Although we could expect a tiny contribution of DOPC to the L\textsubscript{o} phase, for simplicity it has been further omitted. Li and Cheng observed that the smaller DiI-C18(3) probe preferentially partitioned in the DPPC S\textsubscript{o} phase of the DOPC:DPPC binary mixture \[^{36}\]. With respect to the ternary mixture, Baumgart \textit{et al}. investigated a 50:27:23 ratio (DOPC:SM:Chol), and reported that DiI-C18(3) preferentially partitions in the DOPC L\textsubscript{d} phase \[^{17}\]. Fluorescence microscopy provided insights into the DiI-C18(3) probe embedded in a dozen ternary mixtures \[^{18}\]. However, neither for the larger DiI-C18(5) probe nor for BNP, the phase partitioning is known for the specific ratio of lipid systems considered in \[^{35}\].

It is currently a challenge to accurately evaluate optical properties of the probe within various lipid bilayers. This task first requires a correct and comprehensive evaluation of large scale structural features of the molecular assembly made of the probe and the lipid bilayer, which can be obtained by Molecular Dynamics (MD) simulations. For the current work therefore, MD simulations were performed to gain insight into the interactions of specifically both DiI-C18(5) and BNP probes within biological membranes and to understand their phase preference. Attention is paid to their locations and motions within the lipid bilayers and how this impacts on their spectroscopic features. \textit{In silico} membrane models have been constructed in the past and a vast development with increasing accuracy is noted \[^{37-42}\]. MD calculations have been used to accurately evaluate simultaneously equilibrium positions of xenobiotics in lipid bilayers, their partition and diffusion coefficients at subpicosecond and atomic resolution \[^{43-}\].
Focusing on the simulation of optically active probes opens the possibility towards the development of non-invasive techniques which provide insights into the impact of surrounding environment in (non) linear and fluorescence spectroscopy \cite{49,50}. Here, MD simulations are used to assess the interaction of both DiI-C18(5) and BNP in four different lipid bilayers and lipid phases. One of them is the DPPC membrane in its Ld phase, which is considered at the same temperature as in the study of Gullapalli \textit{et al.} \cite{34} to enable a direct comparison. The structural and physical-chemical properties of the four lipid bilayer models are discussed in terms of their areas per lipid, order parameters and non-bonding interaction energies. The Gibbs free energy profiles of DiI-C18(5) and BNP are investigated along the z-axis of the membrane, which is oriented perpendicular to the membrane surface. The differences between the equilibrium positions and orientations of both probes, and the variations of their transition dipole moments within the various environments are identified as being decisive for the linear and non-linear optical spectra \cite{51,52}. Finally, the fluorescence anisotropy of both probes is modelled and similarities as well as differences in the behavior of DiI-C18(5) and BNP are highlighted.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{molecular_structures.png}
\caption{Molecular structures of (a) DiI-C18(5) and (b) BNP. The red arrows are the transition state dipole moments for both probes. To describe the positions of the probes, the middle carbon atom of the \(-(\text{CH}=\text{CH})_2-\text{CH}=- \) bridge is considered for DiI-C18(5) and the Boron atom for BNP. Remark that the \(\pi\)-conjugated core in both molecules is confined to those parts of the molecules without tails and – in the case of BNP – without headgroup.}
\end{figure}

\textbf{Computational details}

The MD simulations were performed using the Gromacs 4.5.7 \cite{53,54} software and the Gromos 43A1-S3 force field \cite{55-58}. The lipid bilayer models consisted of 128 lipid molecules.
surrounded by at least 4500 explicit water molecules, which were described by the extended single point charge (SPC/E) model. Na\(^+\) and Cl\(^-\) ions were added to bulk water at a physiological concentration (0.9%). The spatial reference frame is such that the x- and y-axes are taken in the plane of the bilayer, whereas the z-axis is perpendicular to the membrane surface. Periodic boundary conditions were considered in 3 dimensions. Electrostatic interactions were treated by the particle-Mesh Ewald method \[^{59}\] and bonds were constrained by the LINCS algorithm \[^{60}\]. Electrostatics and van der Waals short-range interaction cutoffs were set to 1.6 nm. The NPT ensemble was used, with the Nosé–Hoover thermostat \[^{61,62}\], and a Parrinello–Rahman barostat \[^{63}\] for a semi-isotropic pressure coupling at 1 bar and compressibility of \(4.5 \times 10^{-5}\) bar\(^{-1}\). The simulation time step was set to 2 fs and the coordinates in the simulation were saved every 500 steps.

The four lipid bilayer models were built with a homemade script, they consisted out of one probe and in total 128 lipids (two leaflets of 64 lipids): pure DOPC at 298 K (L\(_d\) phase), pure DPPC at 298 K (S\(_o\) phase) and at 323 K (L\(_d\) phase), and a 2:1 SM:Chol mixture at 298 K (L\(_o\) phase). The SM acyl chains contain 17 and 15 methyl groups for the sn1 and sn2 acyl chains, respectively. Upon these systems, periodic boundary conditions in all directions have been applied. All membranes were equilibrated during 20 to 40 ns long free simulations, after which convergence of structural parameters (i.e., area per lipid, lipid order parameters...) were ensured. In line with previous work \[^{35}\], atom types were assigned by PRODRG \[^{64}\], while partial atomic charges have been used which result from the restrained fit of electrostatic potential (RESP) \[^{65}\]. They were calculated at the level of density functional theory (DFT) by means of the B3LYP functional \[^{66,67}\], Dunning’s correlation consistent cc-pVDZ basis set \[^{68}\], and a PCM model which was chosen to describe an implicit solvent model with a dielectric constant of diethyl ether (\(\varepsilon = 4.24\)) \[^{69}\]. The Lennard-Jones parameters of the boron atom for the BNP probe, which are not by default present in the applied force field, have been taken from reference \[^{70}\]. Further parametrizations for the bonded interactions of the Boron atom have been performed by means of previous DFT method.

The Gibbs free energy profiles for BNP and DiI-C18(5) were calculated by means of the \(z\)-constraint method \[^{71,72}\], in which bulk water was put as a reference. The distance between the centers of masses of the lipid bilayer and the Boron atom for BNP, or the middle carbon atom of the \(-(CH=CH)2–CH=\) bridge for DiI, was constrained, and the required force was monitored.
The averaged force was then used to calculate the Gibbs free energy profile, also called potential of the mean force \(^{72,73}\), as:

\[
\Delta G(z) = - \int_{\text{outside}} \langle F(z') \rangle_i \, dz',
\]

where \( \langle F(z) \rangle_i \) is the force which is needed to keep the molecule at a given depth \( z \). A series of windows was obtained every 0.1 nm for \( z \)-constraint simulations. The initial structures for each window were generated by merging probe and membrane coordinates, minimized to avoid steric clashes, and \textit{g_membed} \(^{74}\) was used to remove overlapping lipids when appropriate. In this process, the probes were oriented along the \( z \)-axis, with the lipid tails in the direction of membrane center. For the \( z \)-constraint process, 100 ns simulations were performed per window, ensuring convergence of Gibbs free energy profiles. The computational error was found to be \( \sim 1 \text{ kcal/mol} \). Starting from the minimum energy positions of the Gibbs free energy profiles, 300 ns long MD simulations were performed without applying additional constraints, of which the first 40 ns were discarded from the simulation window, as being the time required to equilibrate the system. The analysis of the structures of the membranes were performed on these unbiased simulations with GROMACS internal tools, area per lipid for individual lipid types was obtained by the \textit{FATSLiM} script \(^{75}\).

The transition state dipole moments of the BNP and DiI-C18(5) probes have been calculated using approximate second order coupled cluster theory (CC2) and the double zeta polarized (DZP) basis set.

In total, these simulations required a computational effort of more than 40 \( \mu \text{s} \). To perform these calculations, the \textit{Lindgren} cluster at the PDC Center for High Performance Computing in Stockholm (864 000 core hours, 2013-2014), the \textit{muk} tier-1 cluster of the Flemish Supercomputer Centre (VSC) (264 960 core hours, 2014-2015), as well as the \textit{Beskow}, \textit{Triolith} and \textit{Abisko} clusters with in total 105 000 core hours/month (2015) were used.

**Results and discussion**

**Characterization of the membranes**

If the simulated DOPC (L\(_d\)), DPPC (S\(_o\)), DPPC (L\(_d\)) and SM:Chol (L\(_o\)) bilayer membranes are expected to influence the distribution and dynamic behavior of the embedded probes, their inherent properties should be accurately modelled. The structure of lipid membranes can be
well described by the density plots of various membrane components along the normal axis to the membrane plane. The density distributions of the lipid constituents and of water from the center of the membrane were constant between 2 nm and 0.8 nm for SM:Chol (Figure 2). In the other three membranes, locally higher lipid density was found with a peak at around 1.7 nm from the center, followed by a rapid decrease to the center. This effect is explained by the presence of free volumes just beneath the aqueous interface in contact with the polar head group region \(^{34,76,77}\) and is manifestly seen in the SM:Chol membrane. Concomitantly, the thickness of the SM:Chol membrane was greater, as seen by a shifted point where the density of the water equals that of the lipids (i.e., crossing at 2.5 nm for SM:Chol with respect to 2.3 nm for the L_α-phase DOPC and DPPC, see Figure 2). As expected, a similar increase of the thickness was observed for DPPC (S_o).

The thickness, in terms of distance of the highest density peaks, agrees well with experimental data. We observed differences in thickness between the different membranes, namely 4 and 4.5 nm for the DPPC (L_d) and the SM:Chol bilayer, respectively. The latter simulated thickness agrees with the experimental value of 4.6-4.7 nm \(^{78}\). This value mainly depends on SM, as Chol is known not to significantly modify the conformation of SM molecules \(^{14}\). The thickness of the DOPC and DPPC (S_o) bilayers is found in between 4 and 4.5 nm.

![Figure 2: Density distributions of the lipid constituents (full line) and of water (dotted line) within the various membrane phases with respect to the center of the membrane](image)

Over the 300 ns of MD simulations, the area per lipid exhibited constant values, i.e., \(~0.45 \text{ nm}^2/\text{lipid}\) for SM:Chol (L_o, 0.40 nm\(^2/\)Chol and 0.48 nm\(^2/\)SM), \(~0.51-0.52 \text{ nm}^2/\text{lipid}\) for DPPC (S_o), 0.58 nm\(^2/\)lipid for DOPC and DPPC (L_d) (Figure S1). Although the calculated area per
lipid in the $L_d$ phase is lower than some of the reported experimental data [79]. The area/lipid values represent well the differences in the studied phases. A tighter packing and condensing effect were previously observed in $S_o$ phase as well as in the presence of Chol [80].

The potential energy of interaction between the lipid tails ($V_{tails}$) can be derived from the average sum of Lennard-Jones and short-range Coulomb potentials between all pairs of atoms in the lipid tail region [80,81]. Concerning Chol, all atoms but the hydroxyl group were included, whereas for phospholipids, all tail atoms up to the three glycerol carbon atoms were included. The potential energy was averaged from 180 to 280 ns. The $V_{tails}$ values per atom are very similar for all four membrane models (1.193, 1.127 and 1.110 kcal/mol for DPPC ($S_o$), SM:Chol and DPPC ($L_d$), respectively). The decreasing values from 1.193 to 1.110 kcal/mol mainly point the decrease of van der Waals contacts between lipid tails. The latter value is different from that of DOPC, which amounts to 1.182 kcal/mol, likely be due to the greater van der Waals interactions between the unsaturated bonds deep in the DOPC tails not present in the DPPC molecules. Finally, the value for DPPC ($S_o$) agrees with the one communicated by Wennberg et al. in 2012 [80].

To characterize the employed membrane models, the order parameter $|S_{CD}|$ is calculated, too. It is experimentally obtained using deuterium NMR by using the equation [34,82]

$$S_{CD} = \left[ \frac{3}{2} \left( \cos^2 \theta_{CD} \right) - \frac{1}{2} \right],$$

(2)

with $\theta_{CD}$ being the angle between C-H bond of the lipid tails and the z-axis. The brackets denote time averaging and corresponds to an ensemble averaging when experiments are performed. The value of the order parameter $S_{CD}$ can vary from -0.5 with $\theta_{CD} = 90^\circ$ (indicating full ordering of the C-H bonds perpendicular to the z-axis) to 1 with $\theta_{CD} = 0^\circ$ (indicating full ordering of the C-H bonds along the z-axis and the C-C bonds therefore more oriented perpendicular to the z-axis). Based on $S_{CD}$ values, we confirmed the typical differences between the membranes in the $L_d$, $S_o$ and $L_o$ phases: as reported in Figure S2, $|S_{CD}|$ values for the sn-1 and sn-2 tails amount maximally to ~0.40 for SM:Chol, ~0.35 for DPPC ($S_o$), and 0.25 for both DOPC and DPPC ($L_d$). These maxima are obtained at carbon C8 for SM:Chol and DPPC ($S_o$), while for DOPC and DPPC, the maxima are reached at C6. For C3, close to the headgroup and the glycerol moiety of the lipids, $S_{CD}$ amount to 0.27 for SM:Chol as well as for DPPC ($S_o$), and to 0.20 for both $L_d$
membranes. For SM:Chol, the quite strong increase in $|S_{CD}|$ towards the middle of the tail can be linked with the presence of Chol, which pushes the tails of SM deeper in the membrane, so as to accommodate the perpendicular orientation of the C-H bonds, diminishing hydrophobic effects. On the other hand, for DPPC ($S_o$), the high $|S_{CD}|$ values are related to the high packing, in agreement with $V_{tails}$ values, and with the higher amount of water present at the level of the glycerol group of the tails (Figure 2).

**Gibbs free energy profiles for Dil-C18(5)**

$z$-Dependent Gibbs free energy profiles provide information about partition and preferred positions (free-energy minima), as well as capacity of transfer from one to the other leaflet (Gibbs free energy barriers) independently from diffusion effects. The profile for Dil-C18(5) (Figure 3, left hand side) exhibits the deepest well (-38 kcal/mol) in the DPPC ($S_o$) membrane. The well is energetically less favorable by 5 kcal/mol in both the DPPC ($L_d$) and SM:Chol ($L_o$) bilayers; therefore based on the Gibbs free energy alone, one cannot distinguish any preferred affinity to both DPPC ($L_d$) and SM:Chol ($L_o$) bilayers. The affinity of Dil-C18(5) to DOPC ($L_d$) membrane is the least favorable one (potential well of -28 kcal/mol). The here presented data seem to answer therefore the question which membrane Dil-C18(5) prefers in a DOPC:DPPC ($L_d$: $S_o$) and a DOPC:SM:Chol ($L_d$: $L_o$) mixture, like has been used by Bacalum et al. in ref. [35].

Namely, the simulations indicate that in the former case, after equilibration of the biological environment, confocal microscopy will allow visualizing the DPPC ($S_o$) regions of the unilamellar vesicle, whereas in the latter case, the $L_o$ region of the SM:Chol mixture will be bright. For the concentrations used in the current study, Dil-C18(5) should thus be considered as a $L_o$ marker, and contrasts therefore with the findings of Baumgart et al. and Kahya et al. for Dil-C18(3) embedded in ternary lipid mixtures with other concentration ratios [17,18].

From the analysis given in Figure 3, the position of the global minima were similar except for SM:Chol (1.3, 1.3, 1.2 and 1.9 nm for DOPC ($L_d$), DPPC ($S_o$), DPPC ($L_d$) and SM:Chol ($L_o$), respectively). Although in this latter case, the bilayer thickness is greater, this makes Dil-C18(5) closer to the polar group region in SM:Chol with respect to the other membranes.

As we applied the $z$-constraint method from the center of the membrane and used a window for every Ångström, the barriers of transfer from one to the other leaflet have been obtained.
Significant differences are seen: the barrier at the middle of the bilayer is ~8 kcal/mol with DOPC and SM:Chol, and it is lower (4-5 kcal/mol) with DPPC (L_d) and DPPC (S_o). As repeatedly seen with amphiphilic compounds, the insertion into fluid bilayers requires small or even no energetic barriers in the polar head group region. Noncovalent interactions (electrostatic and H-bonding) mainly drive insertion and positioning, with little influence of size within the μs timescale.

![Figure 3: Gibbs free energy surfaces of (left) DiI-C_{18}(5) and (right) BNP in function of the distance (in nm) from the center of the membrane along the z-axis, perpendicular to the membrane surface. The centers of mass of the DiI-C_{18}(5) and BNP cores have been constrained. The error bar is contained in the thickness of the line.](image)

**Analysis of the unconstrained trajectories for DiI-C_{18}(5) in the various membranes**

It is worth noting that the Gibbs free energy profiles are generated based upon a constrained movement of the core of the probe. To discuss the equilibrated positions and orientations of DiI-C_{18}(5) and to profoundly evaluate the influence of the finite temperature, a free production run of 300 ns was performed for each membrane in the presence of DiI-C_{18}(5), with the minima of the Gibbs free energy profile as starting geometries. Illustrations of the DiI-C_{18}(5) probe in the various membranes are given in Figure 4. As a measure for the position of DiI-C_{18}(5), the middle carbon atom of the cyanine-backbone was considered with respect to the membrane center. For SM:Chol, DiI-C_{18}(5) is situated at 1.75±0.11 nm from the membrane center, in close contact to the polar head group region (Figure 5). For both DPPC bilayers, DiI-C_{18}(5) is located deeper, at ~1.0 nm from the membrane center, *i.e.*, in contact with the lipid tails (the exact value for the S_o is 1.04±0.09 nm, while it is 1.09±0.09 nm for the L_d phase). Gullapalli et al. observed a value which was with its 1.26 nm a bit higher for DiI-C_{18}(3) in DPPC (S_o) [34]. In DOPC, the location is an intermediate of the other two, however with a broad distribution ranging from 1.3 to 1.8 nm (1.47±0.21). Except for SM:Chol, the mean positions in free
simulations were slightly deeper than the positions of the free energy minima, but these differences were found within errors and thermal motion. In all membranes, the probes have their light sensitive core embedded in lipid head groups and the lipophilic tails pointing towards the center of the membrane. We calculated the angles of the tails of Dil-C18(5) with the $z$-axis (Figure S3). These angles take the value $\sim 155^\circ$ for both $L_d$ membrane phases, $\sim 165^\circ$ for DPPC in the $S_o$ phase, and $\sim 170^\circ$ for SM:Chol in the $L_o$ phase.

**Figure 4: Illustrations of the Dil-C18(5) and BNP probes in the different environments under investigation in the current study.**

While in SM:Chol the chromophore moiety of Dil-C18(5) is located at the surface of the membrane in contact with bulk water, it is located significantly more deeply in the other membranes. The Dil-C18(5) $\pi$-conjugated core is located below the level of the phosphates at a distance of 0.5 nm in SM:Chol, 0.3-0.8 nm in the $L_d$ phase of DOPC, 1.0 nm in the $L_d$ phase of DPPC and 1.2 nm in So phase (DPPC). In the SM:Chol membrane one has to consider not only the average level of membrane surface, but also a local arrangement of the membrane. The chromophore moiety of Dil-C18(5) experiences here free volumes and induces a small cavity, in which water molecules are pulled (Figure S4). Indeed, due to this surface position and such re-arrangements, Dil-C18(5) is more surrounded by water molecules in the SM:Chol membrane than e.g. in the $S_o$ phase or the $L_d$ phases of DPPC. For SM:Chol, at the Dil-C18(5) preferred position, the density of water is still 45% of that of the pure water layer, while practically no water is left with DPPC both in $S_o$ and $L_d$ phases (Figure 2). It can also be remarked that the maximum density of water experienced by Dil-C18(5) in the DOPC ($L_d$) membrane amounts
to 20%. This effect is quantified by the radial distribution functions of DiI-C18(5) and the surrounding water molecules in the various membranes (Figure S5): the first maxima (at 0.45 nm from the DiI-C18(5) core) is very low for both phases of DPPC membranes (<0.2), slightly higher in DOPC (0.3) and significantly higher in SM:Chol (0.6). The water cavity experienced in the SM:Chol membrane is then responsible for the different behavior of DiI-C18(5) in this membrane.

Being decisive for the photoselection of the probe, the distribution of the angles between the transition dipole moment and the z-axis of the membrane is given in Figure 5. For DiI-C18(5), the transition dipole moment is oriented along the cyanine backbone and is displayed in Figure 1. Knowing that a perfect photoselection in confocal microscopy requires an angle of 90°, DiI-C18(5) in DOPC appears the most efficient with a most populated angle of ~85°. For SM:Chol and DPPC (S₀), the most abundant peak is seen at 72°. It can be remarked that for DPPC (S₀), the distribution of the angle is rather symmetric around its maximum, while for SM:Chol a slight asymmetry is seen together with a minor shoulder at higher values. The DPPC (L₀) lipid bilayer is characterized by a broad distribution of angles of a similar population, which are between 70° and 80°, which agrees with the angle of 77° reported for the smaller DiI compounds investigated by Gullapalli et al. [34] or with the range of ±10° around the perpendicular position with respect to the z-axis reported by Axelrod for erythrocyte ghosts [83].

The pronounced angles of the transition state dipole moments in the different membranes can be related to the differences in orientation between the sn-1 and sn-2 chains of the lipids and to the differences in position of the probe along the z-axis. To better describe the orientation of DiI-C18(5), the angle between the normal to the coplanar core and the z-axis was followed as well. A symmetric distribution was obtained centered at around 51° only for SM:Chol. In DOPC, essentially all values between 30° and 80° were observed, with only a slight preference for 35-40°. For DPPC (L₀), the angle increased from 30° to 80°. In DPPC (S₀), the angle distribution was ranging from 70° to 80°. Combining the analyses for both angles, S₀, and to a less extend L₀, restrain orientation to the probe.
Figure 5: DiI in various membrane phases — (top) the position of the middle atom of the cyanine backbone of DiI along the z-axis expressed in terms of the distance from the center of the membrane, the dotted vertical lines denote the most abundant position of the phosphor atoms; (center) the angle between the transition dipole moment and the z-axis; (bottom) angle between the axis perpendicular to the plane of the DiI-C18(5) molecule and the z-axis. These data are taken from a free MD run and are convoluted with Gaussian profile peaks with a full width half maximum of 8°. The errors are displayed in Figure S6.

The order parameter profiles of DiI-C18(5) show the same trend in all four domains, i.e., higher values close to the polar head group region which decrease when inserting deeper in the bilayer, as expected for lipid-type compounds (Figure 6). Close to the polar head, the highest $|S_{CD}|$ values (0.35-0.39) are observed in SM:Chol (L_o) and DPPC (S_o), whereas lower values (0.20-0.23) are observed in DOPC and DPPC (L_d). A further analysis can be performed making use
of the above definition of $S_{CD}$ which relates to the angles between C-H bonds of the lipid tails and the $z$-axis. Due to the free space which is available at the top of the SM/Chol bilayer and the high abundance of water molecules, the mid C-C bonds of the tails of DiI-C18(5) are seen to straightly enter further down towards the center of the membrane, parallel to the $z$-axis. In DPPC ($S_o$), the DiI-C18(5) $|S_{CD}|$ value is also high for the first bonds below the nitrogen atoms, but the curve flattens down and the slope diminishes due to the high packing between the lipid tails, assuring a well-defined and orientation of the last carbon-carbon bonds of the tails. As expected from the position of the probe and the characteristics of the subsequent lipid bilayers, $|S_{CD}|$ values are lower in both $L_d$-phase membrane models, while the typical decrease along the tails is less steep than for the other two lipid bilayers. In the DOPC membrane, in the middle of the tails of DiI-C18(5), a slight increase of the $S_{CD}$ value is further on observed, which even surpasses the corresponding values for DiI-C18(5) in the DPPC ($S_o$) environment, which is in DOPC attributed to the double bond.

![Figure 6: Order parameters for DiI-C18(5) in the various membranes. The carbon atom index points at the number of the carbon in one of the tails, starting from the carbons attached to each of the nitrogens.](image)

**Gibbs free energy profiles for BNP**

The Gibbs free energy profiles of BNP given in at the right hand side in Figure 3 show a well of ~33 kcal/mol in DOPC and SM:Chol; it is marginally deeper in DPPC ($S_o$) and significantly deeper in DPPC ($L_d$). Also, the most stable positions of BNP can to some extent be identified within the limits of the used $z$-constraint method. It mostly partitions at 1.2, 1.9, 1.1 and 1.5 nm in DOPC ($L_d$), DPPC ($S_o$), DPPC ($L_d$) and SM:Chol ($L_o$), respectively. The differences in the preferred position are however less clear than for DiI-C18(5). The markedly small Gibbs free energy differences in these profiles illustrate why within the constraints of the employed theories and simulations a comparison with DiI-C18(5) was needed to identify the lipid phases.
present in the bright areas which were seen in the confocal microscopy images published in [35]. Based upon our simulations for DiI-C18(5) and the related discussion above, it is subsequently safe to assume that BNP in the employed biological environments can be found in the $S_o$ phase when a mixture of DOPC ($L_d$) and DPPC ($S_o$) is considered and in the $L_o$ phase when a mixture of DOPC ($L_d$) and SM/Chol ($L_o$) is involved. We would like to stress here again the importance of the ratio of the employed mixture, as we employed DOPC:SM:Chol in a 1:2:1 ratio. By means of comparison, Baumgart et al. reported the $L_d$ phase as the preferred one for the DiI-C18(3) probe in a DOPC/SM/Chol mixture in basically a 2:1:1 ratio [17]. Other authors who reverted to the benchmark DiI-C18(5) probe, discussed the ternary mixtures in other ratios, too, without solving the issue for the mixture under investigation in the current study, but warning for the particular strong influence of the mixed lipid constituents when phase preferences are concerned [18–21].

The barrier for the transfer of BNP between the upper and lower leaflet amounts to ~10 kcal/mol for both DPPC membranes as well as for DOPC. It is calculated as the difference between the minimum of the potential energy surface and the maximum Gibbs free energy value found around the membrane center. The barrier amounts to ~14 kcal/mol for SM:Chol ($L_o$). The largest differences between both probes are therefore found for DPPC ($S_o$) and SM:Chol ($L_o$); the larger barriers are here reported for BNP and should be allocated to the influence of the Boron and Fluorine atoms.

**Analysis of the unconstrained trajectories for BNP in the various membranes**

As for DiI-C18(5), selecting the frames from the global minima of the Gibbs free energy profiles, a free production run was performed for 300 ns. Illustrations of the BNP probe in the various membranes are given in Figure 4. The boron atom of BNP was at $1.4\pm0.2$ nm from the membrane center in both the DOPC and SM:Chol membranes (Figure 7), while it was inserted deeper (at $1.2\pm0.2$ nm) in DPPC ($L_d$). Conversely, in DPPC ($S_o$), it was at $1.7\pm0.1$ nm, closer to the phosphorus atoms of the membrane surface, being located at 2.25 nm. It can be remarked that the boron atom is located rather close to the lipid tails, while the middle atom of the cyanine backbone of DiI-C18(5) is found higher in the molecule.

This difference in preferred position in the DPPC ($S_o$) and DPPC ($L_d$) environments is related to the difference in packing and area per lipid between both membranes. In DPPC ($S_o$), the packing in between lipid tails is likely to complicate insertion of BNP. Moreover, the core of
BNP has a weak zwitterionic charge distribution between the nitrogen and boron atoms, making them slightly positive and negative, respectively. This favors interactions with water molecules abundant in this region of DPPC (S_o) (up to 20% of the density of pure water). The similar position of BNP in DOPC and SM:Chol is a manifestation of the interaction with tail unsaturation and Chol.

The angle between the transition dipole moment of BNP and the z-axis amounts to 70°-75° in DOPC (Figure 7). With an angle of 85° (and a minor distribution at 50°), the photoselection was found to be stronger in DPPC (L_d). In the DPPC (S_o) bilayer, the maximum of the distribution is found at 67°, however a shoulder can also be seen at 86°. Rather in contrast to Dil-C18(5), the angle distribution in SM:Chol is very broad with many contributions between 30° and 60°, and a major peak at 73°.

The orientation of the molecular plane of BNP with respect to the z-axis showed that this probe is rather perpendicular to the surface, with an angle of ~85° for DPPC (S_o) and SM:Chol. In DOPC, the maximum is at ~71°, although a shoulder is noticed at 85°. In DPPC (L_d), the distribution is broader, with a shallow maximum at 59°.

Although it has been experimentally found that both Dil-C18(5) and BNP probes target the same membrane phases and in contradiction to the first assumptions [35], it can be concluded based upon the current MD simulations that BNP behaves rather differently from the relatively known Dil-C18(5) one in terms of its orientation and equilibrium position in the membrane.
Figure 7: BNP in various membrane phases – (top) the position of the boron atom along the z-axis expressed in terms of the distance from the center of the membrane; (center) the angle between the transition dipole moment and the z-axis; (bottom) angle between the axis perpendicular to the plane and the z-axis. These data are taken from a free MD run and are convoluted with Gaussian profile peaks with a full width half maximum of 8°. The errors for the angle distributions are given in Figure S6.

**Fluorescence anisotropy**

When polarized light is applied to a biological environment, the probability of excitation of the probe depends on the angle between the transition state dipole moment and the electric field vector of the incoming electromagnetic radiation. A smaller angle leads to a higher excitation
probability. As a consequence, the initial emission after pulsed excitation has a defined polarization. Rotational mobility within a time span determined by the fluorescence lifetime will reduce the fluorescence polarization. The fluorescence anisotropy $r$ is generally defined by means of the fluorescence intensities obtained parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the polarization of the excitation light via

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp},$$  \hspace{1cm} (3)

when the sample is excited with vertically excited light \[^{84}\].

For DiI-C18(5) and BNP in lipid bilayers of various composition, the relaxation of $r(t)$ after a $\delta$-pulse excitation was investigated. This relaxation depends on the rotational dynamics, the intrinsic anisotropy $r_0$ (corresponding to the anisotropy at $t=0$) and the conditions of the environment surrounding the light sensitive probe. In agreement with the study by Lipari and Szabo upon the effect of librational motion upon fluorescence depolarization \[^{85}\] and in line with the theoretical models advocated by Heyn, Jähnig and Ameloot \[^{86-88}\], the rotational correlation function $C(t)$ is an autocorrelation function and is given in terms of the second order Legendre polynomial $P_2(x) = (3x^2 - 1)/2$ and the orientation of the transition dipole moment at $t = 0, \mu(0)$, and time $t$ after excitation, $\mu(t)$ \[^{34,89}\]:

$$C(t) = \langle P_2(\mu(0)\mu(t)) \rangle,$$  \hspace{1cm} (4)

where the brackets denote the ensemble average, or equivalently, the average over all initial times in the MD calculations, and with $C(t) = \frac{r(t)}{r_0}$ \[^{85}\]. Since our quantum chemical calculations indicate that the absorption and emission dipoles of the probes under investigation are parallel to each other and as the intrinsic anisotropy $r_0$ or the anisotropy at time $t = 0$ for 1-photon excitation depends on the angle $\delta$ between both dipoles via \[^{84}\]:

$$r_0 = \frac{2}{5} P_2(\cos \delta),$$  \hspace{1cm} (5)

a maximum value of $r_0 = 0.4$ has been considered.
Being embedded in a lipid bilayer, the fluorophore has a limited rotational freedom. The fluorescence lifetime (ranging from hundreds of picoseconds to a few nanoseconds) sets a time window over which the rotational motions can be monitored in an experimental context. In line with previous theoretical and experimental analysis \cite{34,35}, a double exponential function is used to describe the rotational correlation function:

\[
C(t) = \beta_1 \exp(-t/\theta_1) + \beta_2 \exp(-t/\theta_2) + C_\infty, \tag{6}
\]

where \( \theta_1 \) and \( \theta_2 \) are correlation times. The \( C_\infty \) constant reflects that the rotational correlation function, and therefore the fluorescence anisotropy, does not decay to zero. One can define the mean correlation time \( \langle \theta \rangle \) as:

\[
\langle \theta \rangle = \frac{\sum \beta_i \theta_i^2}{\sum \beta_i \theta_i}.
\]

The results of the analysis are given in Table 1. The quality of the fit was tested by the \( \chi^2 \) analysis. As our fit leads here to a deviation in the order of barely \( 10^{-6} \), the high quality of the function used is ensured with a time window up to 25 ns. The \( C_\infty \) parameter in the \( S_o \) phase for both DiI-C18(5) and BNP are the highest ones in the range of investigated environments, pointing at a particularly confined freedom of rotation. The residual \( C_\infty \) for both compounds decreases when a more fluid-like lipid environment is considered. It can also be seen that the \( L_d \) phase of DPPC displays a slightly smaller constant than the one of DOPC in the same phase. From our analysis, it has been found that \( C_\infty (S_o) > C_\infty (DOPC, L_d) > C_\infty (DPPC at 323K, L_d) > C_\infty (L_o) \). These inequalities have to be put in relation to the nature and packing of the various membranes. For the difference between the results for the \( L_d \) and \( L_o \) phase, the particular position of DiI-C18(5) in the SM:Chol membrane and the presence of the free volumes with water can be recalled. The restricted motions of the probes are finally confirmed by the smaller (larger) relaxation time constants \( \theta_1 \) (\( \theta_2 \)). For DPPC (\( L_d \)) and DiI-C18(3), Gullapalli et al. reported \( \theta_1 = 0.99 \) ns and \( \theta_2 = 6.9 \) ns for the fast and slow components \cite{34}. These values have to be compared with the ones of 0.11 ns (\( \theta_1 \)) and 11.57 ns (\( \theta_2 \)) found for DiI-C18(5) in this study. The values reported by Ariola et al., who studied DiI-C12(3) in the DOPC (\( L_d \)) membrane, can be compared with the ones of Gullapalli et al. and amount to \( \theta_1 = 1.2 \) ns and \( \theta_2 = 9.6 \) ns \cite{90}. The obtained time constants for the SM:Chol membrane with not only a very low fast component but also a low slow component point at the special place of the DiI-C18(5) probe: a low steric hindrance of the chromophore is seen in the neighborhood of the top of the
lipid acyl chains, while also the collective motion of the lipids in the membrane does not stretch the decay of the rotational autocorrelation function.

A steady-state fluorescence anisotropy of ~0.35 has been measured for BNP in the DPPC $S_o$ phase, while it decreased to ~0.15 upon transition to the $L_a$ phase. The fluorescence lifetimes of this probe reaching up to $4.4 \pm 0.2$ ns were found to be independent of the phase and the temperature of the lipid system $^{[35]}$. Especially for BNP, changes in fluorescence anisotropy can consequently be entirely ascribed to restricted tumbling motions of the probe, which are described by Table 1 with the two relaxation times and the limiting anisotropy at long times. From the time constants, it can be seen that the mean relaxation times are larger for BNP than for DiI-C18(5). As the carbocyanines are known to have a shorter fluorescence lifetime of ~1.0 ns $^{[26]}$, the steady state fluorescence anisotropy of BNP is thereupon more sensitive to slower rotational motions than DiI-C18(5). The presented data confirm therefore successfully the assumptions made for BNP at the time of its synthesis $^{[35]}$.

The profoundly low value of 0.12 for $C_\infty$ in SM:Chol as well as the small associated average decay time of 0.43 ns found for DiI-C18(5) point at a strongly pronounced decay of the fluorescence anisotropy and might be another manifestation of the presence of free volumes and a high amount of water molecules in the top polar region of the lipid bilayer. As depicted in Figure 4, the tails of the probe are located along with the acyl tails of the lipids in the membrane. The tails of DiI in SM:Chol are almost parallel to the $z$-axis as can be deduced from the angle of ~170° between the $z$-axis and the vector described by the first and one of the last carbon atoms of the acyl tails of DiI (See Figure S3). Differences between the fitted parameters (e.g. $C_\infty \sim 0.62$ and 0.41 for DiI-C18(5) and BNP in DPPC($L_a$) – or 0.12 for DiI-C18(5) and 0.69 for BNP) for DiI-C18(5) and BNP can finally be related to the differences in position of the probes in the lipid bilayer. It is again an indication for the fundamental differences between the two probes. The anisotropy results, together with the Gibbs energy profiles of DiI-C18(5) embedded in the various lipid bilayers, correct and supplement the image for DiI-C18(5) provided in $^{[76]}$ as the probe is not found to perform surface dynamics in the water phase of the membrane but rather tumbles with two relaxation time constants at different distances from the center of the bilayer.
To give an interpretation to the $C_\infty$ parameter, Kinosita et al. proposed in 1977 a so-called ‘wobbling in a cone’ model, in which the transition dipole and the symmetry axis of the probe are assumed to move without restriction in a cone fixed with respect to the membrane [91]. The model relates the $C_\infty$ parameter to half the cone angle such that a large value of $C_\infty$ corresponds to a small cone angle. It can be remarked that the transition state dipole moments for DiI-C18(5) and for BNP are not oriented along the lipid tails of the respective membranes, which invalidates the ‘wobbling in a cone’ model [85].

When DiI-C18(5) is approximated to a rod which is oriented along the backbone of the probe, Kinosita’s other model of ‘wobbling outside the cone’ could be considered [91], which describes a spatial angle which is avoided by the transition state dipole moment. The analysis of the spherical coordinates (See Figure S7) gives a limited range for the angle between the transition dipole moment and the z-axis, which would be natural for any model describing a wobbling motion, *as well as* for the movement in the plane of the membrane described by the angle $\varphi$. It is this hindrance in $\varphi$ which invalidates the ‘wobbling outside the cone’ model as it assumes a free movement of the emission dipole moment for this angle. In the figure, it is also seen that the restriction of the motion of DiI-C18(5) in the plane is less severe for the $L_d$ phases than it is for the $S_o$ and $L_o$ phase. These plots are disentangled in Figures S8 and S9, in which the densities for the individual movements along the $\varphi$ and $\theta$ angles are given. All in all, for DOPC($L_d$) and DPPC ($L_o$), the probe can move in the plane of the membrane over angles of 1.4 and 1.2 radians (~80° and ~70°), respectively. For DPPC ($S_o$) and SM:Chol ($L_o$), the range of $\varphi$ amounts to 0.3 and 0.4 radians (~17° and ~22°), respectively. Discarding small artefacts due to a limited simulation time, these plots are found to be symmetric around 0° for $\varphi$ and 90° for $\theta$. For DiI-C18(5) embedded in SM:Chol, the theta angle is however exclusively restricted to the first quadrant.

Since the tails of the DiI-C18(5) probe can be compared to e.g. the two acyl chains of a DPPC lipid and making abstract of the flexibility of the upper bonds and the out-of-plane distortions of the upper dihedral angles in the tails, the tumbling motion of the backbone and therefore transition state dipole moment of DiI-C18(5) can be related to any wobbling motion of the neighboring lipids. The 3-dimensional movement of the transition state dipole moment is given in Figure 8, showing the specific and restricted movement of the dye up to a timescale of 100 ps. For DPPC ($L_d$), the movement of the probe can be read and a connection can be made with
the areas of high density in the plane of the molecule, as visualized by the angle $\varphi$ in Figure S8. The transition dipole moment of the probe describes zones in time with periods of $\sim$60 ns due to a rather constrained movement in phase with the neighboring lipids and exhibits herein a motion with a smaller solid angle. For DOPC, analogous solid areas are seen. For SM:Chol ($L_o$), the zones are described in $\sim$75 ns, while for DPPC ($S_o$), this period increases to almost 90 ns.

Table 1 – Pre-exponential parameters $\beta$ and rotational correlation time $\theta$ for DiI-C18(5) and BNP in the four considered environments. All rotational correlation times are given in ns.$^a$

|          | $\beta_1$ | $\theta_1$ | $\beta_2$ | $\theta_2$ | $C_\infty$ | $\langle \theta \rangle$ |
|----------|-----------|------------|-----------|------------|-------------|--------------------------|
| **DiI-C18(5)** |           |            |           |            |             |                          |
| DOPC ($L_d$) | 0.02      | 0.07       | 0.09      | 2.93       | 0.89        | 2.91                     |
| DPPC ($S_o$) | 0.02      | 0.05       | 0.02      | 2.67       | 0.97        | 2.63                     |
| DPPC ($L_d$) | 0.04      | 0.11       | 0.34      | 11.57      | 0.62        | 11.55                    |
| SM:Chol ($L_o$) | 0.47     | 0.06       | 0.41      | 0.48       | 0.12        | 0.43                     |
| **BNP** |           |            |           |            |             |                          |
| DOPC ($L_d$) | 0.06      | 0.39       | 0.27      | 24.69      | 0.65        | 24.60                    |
| DPPC ($S_o$) | 0.04      | 0.08       | 0.03      | 7.97       | 0.93        | 7.83                     |
| DPPC ($L_d$) | 0.11      | 0.49       | 0.45      | 19.31      | 0.41        | 19.20                    |
| SM:Chol ($L_o$) | 0.06     | 0.05       | 0.25      | 15.57      | 0.69        | 15.56                    |

$^a$ The mean correlation time $\langle \theta \rangle$ and the $C_\infty$ are also reported.
Figure 8: The movement of the transition state dipole moment vector of DiI-C18(5) along the MD trajectory. All vectors have been translated to the origin. One dot corresponds to 100 ps; the time runs from 0 ns (black) to 300 ns (white), as indicated by the color bar.

Conclusions and outlook

The behavior of BNP and DiI-C18(5) molecular probes was investigated in various lipid bilayers in three different phases. By means of demanding MD simulations, the Gibbs free energy profiles of both probes showed that they preferentially partition into the S0 phase of the DPPC bilayer rather than in the Ld phase of the DOPC bilayer. The Lo phase of a 2:1 SM:Chol mixture was also preferred with respect to the Ld phase.

The positions and orientations of the probes are primordial to anticipate their optical properties in situ, e.g., in biological membranes. The depths of insertion differ depending on the phase, and that relative to this, the probes in the SM:Chol mixture are stabilized more towards the polar head group region of the membrane. The orientation of the transition dipole moment is
very different with the two probes: for DiI-C18(5), the angle between the transition dipole moment and the \( z \)-axis in DOPC \( (L_d) \) is closer to a perfect 90° value than for the rather new probe BNP. A striking difference is however seen for the molecules in the DPPC \( (L_d) \) phase, for which the distribution of the angle ranges from 70° to 80° for DiI-C18(5), while for BNP it peaks at around 85°. From investigations of the membrane density and supported by simulations of the fluorescence anisotropy, it follows that in the SM:Chol \( (L_o) \) phase, a high amount of water molecules is found in the vicinity of the probes and that the embedded probes are less restricted in their movement than when they are surrounded by the other membrane phases.

Although the blue fluorescing BNP probe has been introduced as an alternative for the older yellow DiI-C18(5) one, it has been proven that they may behave differently with respect to their interaction with membranes. It is expected that the differences in position and orientation in various biological membranes will affect the linear and more the non-linear absorption spectra. The current research opens therefore a gateway towards a better investigation of the properties of biological membranes and tissues using nonlinear and fluorescent properties of selective molecular probes.

**Supplementary information**

Area per lipid along the simulated trajectory for the various membranes; order-parameters for the various membrane phases for the sn-1 and sn-2 tails; illustrations of the DiI-C18(5) and BNP probes in the different environments under investigation in the current study; radial distribution functions of DiI-C18(5) and surrounding water molecules for the considered membranes; angle with the \( z \)-axis of the vector described by the first and fifteenth carbon atom of the acyl tails of DiI; distribution of the vector of the transition dipole moment in spherical coordinates \( \theta \) and \( \varphi \); density plots for the vector of the transition dipole moment in function of the azimuthal angle \( \varphi \); density plots for the vector of the transition dipole moment in function of the angle \( \theta \); .itp-files for DiI-C18(5) and BNP.

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TOC:

Movement of DiI-C18(5)
Supplementary information

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Figure S1: Area per lipid (given in nm$^2$/molecule) along the simulated trajectory for the various membranes. The moving average is given using 30 terms.

Figure S2: Order-parameters $|S_{cd}|$ for the various membrane phases for the sn-1 tail (top) and the sn-2 tail (bottom). The CH2 groups are numbered consecutively from 2 to the end (18 for DOPC and Sphingomyelin, and 16 for DPPC). Number 1 is the carbonyl carbon. Due to the nature of $|S_{cd}|$, the value for it as well as for the terminal CH$_3$ group cannot be obtained. The sn-2 tail can be distinguished as it is attached to the middle carbon of the glycerol backbone.
Figure S3: The angle with the z-axis of the vector described by the first and fifteenth carbon atom of the acyl tails of DiI. The two tails (top and down part of the figure) are separated out for clarity.
Figure S4: Side view and top view of final frames of free simulation of DiI-C18(5) in DOPC and SM/Chol membranes with highlighted surrounding water molecules. The DiI-C18(5) molecules are shown in magenta sticks, water within 8 Å are shown as red and white sticks, phosphates are displayed as orange balls, oxygen of cholesterol as red balls. In side view, lipid molecules are shown as green sticks (green – carbon, blue – nitrogen, red – oxygen), in top view lipids are displayed as semi-transparent grey surface. In both cases, DiI-C18(5) is in contact with lipid head groups, but in DOPC lipid head groups cover and hinder DiI, in SM/Chol DiI is in direct contact with bulk water and is therefore less hindered by lipid head groups.
Figure S5: Radial distribution functions of the core of the DiI probe and surrounding water molecules for the various membrane phases.

Figure S6: Top: Distribution of the angle between the transition dipole moment and the z-axis in various membrane phases; Bottom: angle between the axis perpendicular to the plane of the DiI-C18(5) (left) or BNP (right) molecule and the z-axis. Smoothed and rescaled plots are displayed in Figures 5 and 7. To obtain the error bars, the distribution plots were calculated for every 40 ns of simulations with bins of 2° (discarding the first 40 ns of the simulations). For each bin, the average and standard error were calculated.
Figure S7: Distribution of the vector of the transition dipole moment in spherical coordinates $\theta$ and $\phi$ for the four membranes. $\theta$ is the angle with the $z$-axis of the membrane, while $\phi$ denotes the angle in the plane of the membrane.
Figure S8: Density plots for the vector of the transition dipole moment in function of the azimuthal angle $\varphi$ for the four membranes. It denotes the angle in the plane of the membrane in radians. Since the x-axis in the plane of the membrane (to which is referred by the $\varphi$-angle) is not uniquely defined, in these plots 0 radians is taken as the midpoint of the sampled angles. For DOPC, the two symmetric peaks are covered within the maximum at $\varphi=0.0$ and 0.2 radians.
Figure S9: Density plots for the vector of the transition dipole moment in function of the angle $\theta$ for the four membranes. It denotes the angle between the transition dipole moment and the $z$-axis of the membrane in radians. The first peak for DPPC ($S_o$) (at $\theta = 1.4$ rad) and the second peak for DPPC ($L_d$) (at $\theta = 1.9$ rad) are less expressed due to a limited simulation time.
Table S1: diii.itp

; Charges were computed with RED and Duan method: b3lyp/cc-pVTZ
; SCRF(IEFPCM, Solvent=diethylether)
; This file was generated by PRODRG version AA100323.0717
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; Acta Crystallogr. D60, 1355--1363.
;

[moleculetype]
Name nrexcl
LIG  3

[atoms]
1  CH3  1  LIG  CBS  1  -0.0285  15.0350
2  CH2  1  LIG  CBR  1  0.0271  14.0270
3  CH2  1  LIG  CBQ  1  0.0076  14.0270
4  CH2  1  LIG  CBP  1  -0.0071  14.0270
5  CH2  1  LIG  CBO  1  -0.0019  14.0270
6  CH2  1  LIG  CBN  1  0.0056  14.0270
7  CH2  1  LIG  CBM  1  -0.0004  14.0270
8  CH2  1  LIG  CBL  1  -0.0012  14.0270
9  CH2  1  LIG  CBR  2  0.0004  14.0270
10 CH2  1  LIG  CBJ  2  0.0035  14.0270
11 CH2  1  LIG  CBI  2  -0.0004  14.0270
12 CH2  1  LIG  CBH  3  -0.0011  14.0270
13 CH2  1  LIG  CBG  3  0.0071  14.0270
14 CH2  1  LIG  CBF  3  0.0109  14.0270
15 CH2  1  LIG  CBE  3  0.0007  14.0270
16 CH2  1  LIG  CBD  3  0.0068  14.0270
17 CH2  1  LIG  CBC  4  0.0932  14.0270
18 CH2  1  LIG  CBB  4  0.1068  14.0270
19 NR  1  LIG  NBA  4  0.0572  14.0067
20 C   1  LIG  CAT  4  0.0180  12.0110
21 CR1 1  LIG  CAV  4  -0.1198 12.0110
22 HC  1  LIG  HAV  4  0.1042  1.0080
23 CR1 1  LIG  CAX  4  -0.1396 12.0110
24 HC  1  LIG  HAX  4  0.1405  1.0080
25 CR1 1  LIG  CAW  4  -0.1166 12.0110
26 HC  1  LIG  HAW  4  0.1311  1.0080
27 CR1 1  LIG  CAU  5  -0.1906 12.0110
28 HC  1  LIG  HAU  5  0.1593  1.0080
29 C   1  LIG  CAS  5  0.0317  12.0110
30 CR1 1  LIG  HAU  5  0.0792  13.0190
31 HC  1  LIG  CAY  5  -0.0711 12.0110
32 CR1 1  LIG  CAZ  5  0.0942  12.0110
33 C   1  LIG  CAQ  6  0.0321  12.0110
34 C   1  LIG  CAP  6  -0.0785 13.0190
35 C   1  LIG  CAO  6  0.0792  13.0190
36 C   1  LIG  CAN  7  -0.0032 13.0190
37 C   1  LIG  CAM  7  0.0792  13.0190
38 C   1  LIG  CAL  7  -0.0711 12.0110
39 C   1  LIG  CAH  7  0.0323  12.0110
40 CH2 1  LIG  CAG  7  0.0942  12.0110
41 CH3 1  LIG  CAJ  7  0.0284  15.0350
42 CH3 1  LIG  CAK  7  0.0316  15.0350
43 C   1  LIG  CAD  8  0.0394  12.0110
|   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|
|   |   |   |   |   |   |   |   |   |

**Table:**

|   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|
|   |   |   |   |   |   |   |   |   |

**Bonds:**

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
|   |   |   |   |   |   |   |

S10
| s11 | 37 | 38 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CAM CAL |
| s11 | 39 | 38 | 2 | 0.133 | 11800000.0 | 0.133 | 11800000.0 | ; | CAH CAL |
| s11 | 40 | 39 | 2 | 0.139 | 8660000.0 | 0.139 | 8660000.0 | ; | CAG CAH |
| s11 | 41 | 42 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CAG CAJ |
| s11 | 42 | 43 | 2 | 0.153 | 8660000.0 | 0.153 | 8660000.0 | ; | CAG CAD |
| s11 | 43 | 44 | 2 | 0.133 | 11800000.0 | 0.133 | 11800000.0 | ; | CAD CAE |
| s11 | 44 | 45 | 2 | 0.159 | 12300000.0 | 0.159 | 12300000.0 | ; | CAF HAF |
| s11 | 45 | 46 | 2 | 0.139 | 10800000.0 | 0.139 | 10800000.0 | ; | CAB HAB |
| s11 | 46 | 47 | 2 | 0.109 | 12300000.0 | 0.109 | 12300000.0 | ; | CAF CAA |
| s11 | 47 | 48 | 2 | 0.139 | 10800000.0 | 0.139 | 10800000.0 | ; | CAF CAB |
| s11 | 48 | 49 | 2 | 0.148 | 5730000.0 | 0.148 | 5730000.0 | ; | CBT CBU |
| s11 | 49 | 50 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 50 | 51 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBV |
| s11 | 51 | 52 | 2 | 0.153 | 11800000.0 | 0.153 | 11800000.0 | ; | CBT CBU |
| s11 | 52 | 53 | 2 | 0.153 | 11800000.0 | 0.153 | 11800000.0 | ; | CBT CBV |
| s11 | 53 | 54 | 2 | 0.109 | 12300000.0 | 0.109 | 12300000.0 | ; | CBT CBU |
| s11 | 54 | 55 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 55 | 56 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 56 | 57 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 57 | 58 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 58 | 59 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 59 | 60 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 60 | 61 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 61 | 62 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 62 | 63 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 63 | 64 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 64 | 65 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 65 | 66 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 66 | 67 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 67 | 68 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 68 | 69 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 69 | 70 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |
| s11 | 70 | 71 | 2 | 0.153 | 7150000.0 | 0.153 | 7150000.0 | ; | CBT CBU |

[pairs]

; ai aj fu c0, c1, ...

| s11 | 1 | 4 | 1 | ; | CBS CBP |
| s11 | 2 | 5 | 1 | ; | CBR CBS |
| s11 | 3 | 6 | 1 | ; | CBQ CBN |
| s11 | 4 | 7 | 1 | ; | CBP CBM |
| s11 | 5 | 8 | 1 | ; | CBO CBL |
| s11 | 6 | 9 | 1 | ; | CBN CBD |
| s11 | 7 | 10 | 1 | ; | CBM CBJ |
| s11 | 8 | 11 | 1 | ; | CBL CBI |
| s11 | 9 | 12 | 1 | ; | CBK CBD |
| s11 | 10 | 13 | 1 | ; | CBJ CBG |
| s11 | 11 | 14 | 1 | ; | CBI CBP |
| s11 | 12 | 15 | 1 | ; | CBH CBE |
| s11 | 13 | 16 | 1 | ; | CBG CBD |
| s11 | 14 | 17 | 1 | ; | CBF CBC |
| s11 | 15 | 18 | 1 | ; | CBE CBH |
| s11 | 16 | 19 | 1 | ; | CBB BAO |
| s11 | 17 | 20 | 1 | ; | CBC CAT |
| s11 | 18 | 21 | 1 | ; | CBC CAQ |
| s11 | 19 | 22 | 1 | ; | CBP CAP |
| s11 | 20 | 23 | 1 | ; | NBA HAV |
| s11 | 21 | 24 | 1 | ; | NBA CAU |
| s11 | 22 | 25 | 1 | ; | NBA CAY |
| s11 | 23 | 26 | 1 | ; | NBA CAX |
| s11 | 24 | 27 | 1 | ; | NBA CAO |
| s11 | 25 | 28 | 1 | ; | CAT MAX |
| s11 | 26 | 29 | 1 | ; | CAT CAU |
20  28  1 ;   CAT  HAU
20  31  1 ;   CAT  CAY
20  32  1 ;   CAT  CAZ
20  34  1 ;   CAT  CAP
21  26  1 ;   CAV  HAW
21  27  1 ;   CAV  CAU
21  30  1 ;   CAV  CAR
21  33  1 ;   CAV  CAQ
22  24  1 ;   HAV  HAX
22  25  1 ;   HAV  CAW
22  29  1 ;   HAV  CAS
23  28  1 ;   CAX  HAU
23  29  1 ;   CAX  CAS
24  26  1 ;   HAX  HAW
24  27  1 ;   HAX  CAU
25  30  1 ;   CAW  CAR
26  28  1 ;   HAW  HAU
26  29  1 ;   HAW  CAS
27  31  1 ;   CAU  CAY
27  32  1 ;   CAU  CAZ
28  30  1 ;   HAU  CAR
29  34  1 ;   CAS  CAP
30  35  1 ;   CAR  CAO
31  34  1 ;   CAY  CAP
32  34  1 ;   CAZ  CAP
33  36  1 ;   CAQ  CAN
34  37  1 ;   CAP  CAM
35  38  1 ;   CAO  CAL
36  39  1 ;   CAN  CAH
37  40  1 ;   CAM  CAG
37  53  1 ;   CAM  NAI
38  41  1 ;   CAL  CAJ
38  42  1 ;   CAL  CAG
38  43  1 ;   CAL  CAD
38  52  1 ;   CAL  CAC
38  54  1 ;   CAL  CBT
39  44  1 ;   CAH  CAE
39  50  1 ;   CAH  CAB
39  55  1 ;   CAH  CBU
40  45  1 ;   CAG  HAE
40  46  1 ;   CAG  CAP
40  50  1 ;   CAG  CAB
40  54  1 ;   CAG  CBT
41  44  1 ;   CAJ  CAE
41  52  1 ;   CAJ  CAC
41  53  1 ;   CAJ  NAI
42  44  1 ;   CAK  CAE
42  52  1 ;   CAK  CAC
42  53  1 ;   CAK  NAI
43  47  1 ;   CAD  HAP
43  48  1 ;   CAD  CAA
43  51  1 ;   CAD  HAB
43  54  1 ;   CAD  CBT
44  49  1 ;   CAE  HAA
44  50  1 ;   CAE  CAB
44  53  1 ;   CAE  NA1
45  47  1 ;   HAE  HAF
45  48  1 ;   HAE  CAA
45  52  1 ;   HAE  CAC
46  51  1 ;   CAF  HAB
46  52  1 ;   CAF  CAC
47  49  1 ;   HAF  HAA
47  50  1 ;   HAF  CAB
48  53  1 ;   CAA  NA1
49  51  1 ;   HAA  HAB
49  52  1 ;   HAA  CAC
50  54  1 ;   CAB  CBT
51  53  1 ;   HAB  NA1
52 55 1 ; CAC CBU
53 56 1 ; NAI CBV
54 57 1 ; CBT CBW
55 58 1 ; CBU CBX
56 59 1 ; CBV CBY
57 60 1 ; CBW CBZ
58 61 1 ; CBX CCA
59 62 1 ; CBY CCB
60 63 1 ; CBZ CCC
61 64 1 ; CCD CCG
62 65 1 ; CCE CCH
63 66 1 ; CCF CCI
64 67 1 ; CG CCG
65 68 1 ; CCH CCK

[ angles ]
; ai aj ak fu  c0, c1, ...
1  2  3  2  109.5  520.0  109.5  520.0 ; CBS CBR CBQ
2  3  4  2  109.5  520.0  109.5  520.0 ; CBR CBQ CBF
3  4  5  2  109.5  520.0  109.5  520.0 ; CBQ CBF CBO
4  5  6  2  109.5  520.0  109.5  520.0 ; CBF CBO CBM
5  6  7  2  109.5  520.0  109.5  520.0 ; CBO CBM CBM
6  7  8  2  109.5  520.0  109.5  520.0 ; CBM CBM CBM
7  8  9  2  109.5  520.0  109.5  520.0 ; CMB CBL CBL
8  9 10  2  109.5  520.0  109.5  520.0 ; CBL CBK CBK
9 10 11  2  109.5  520.0  109.5  520.0 ; CBK CBJ CBJ
10 11 12  2  109.5  520.0  109.5  520.0 ; CBJ CBI CBI
11 12 13  2  109.5  520.0  109.5  520.0 ; CBI CBB CBB
12 13 14  2  109.5  520.0  109.5  520.0 ; CBB CBG CBG
13 14 15  2  109.5  520.0  109.5  520.0 ; CBG CBF CBE
14 15 16  2  109.5  520.0  109.5  520.0 ; CBF CBE CBD
15 16 17  2  109.5  520.0  109.5  520.0 ; CBE CBM CBC
16 17 18  2  109.5  520.0  109.5  520.0 ; CBM CBC CBM
17 18 19  2  111.0  530.0  111.0  530.0 ; CBC CBB NBA
18 19 20  2  125.0  375.0  125.0  375.0 ; CBB NBA CAT
18 19 33  2  125.0  375.0  125.0  375.0 ; CBB NBA CAQ
20 19 33  2  108.0  465.0  108.0  465.0 ; CAT NBA CAQ
19 20 21  2  132.0  760.0  132.0  760.0 ; NBA CAT CAV
19 20 29  2  108.0  465.0  108.0  465.0 ; NBA CAT CAS
21 20 29  2  120.0  560.0  120.0  560.0 ; CAV CAT CAS
20 21 22  2  120.0  505.0  120.0  505.0 ; CAT CAV HAV
20 21 23  2  120.0  505.0  120.0  505.0 ; CAT CAV CAX
21 22 23  2  120.0  505.0  120.0  505.0 ; HAV CAV CAX
21 22 24  2  120.0  505.0  120.0  505.0 ; CAX CAX HAX
21 23 25  2  120.0  505.0  120.0  505.0 ; CAX CAX CAV
23 24 25  2  120.0  505.0  120.0  505.0 ; HAX CAX CAX
23 25 26  2  120.0  505.0  120.0  505.0 ; CAX CAX HAW
23 25 27  2  120.0  505.0  120.0  505.0 ; CAX CAX CAW
26 25 27  2  120.0  505.0  120.0  505.0 ; HAW CAX CAW
26 27 28  2  120.0  505.0  120.0  505.0 ; CAX CAX CAW
25 27 28  2  120.0  505.0  120.0  505.0 ; CWA CAX WAH
25 27 29  2  120.0  505.0  120.0  505.0 ; CWA CAX CAS
28 27 29  2  120.0  505.0  120.0  505.0 ; HAU HAU CAS
20 29 27  2  120.0  505.0  120.0  505.0 ; HAU CAX CAU
20 29 30  2  108.0  465.0  108.0  465.0 ; CAT CAS CAR
27 29 30  2  132.0  760.0  132.0  760.0 ; CAU CAS CAR
29 30 31  2  109.5  520.0  109.5  520.0 ; CAS CAR CAY
29 30 32  2  109.5  520.0  109.5  520.0 ; CAS CAR CAU
29 30 33  2  104.0  444.4  104.0  444.4 ; CAS CAR CAQ
31 30 32  2  109.5  520.0  109.5  520.0 ; CAY CAR CAY
31 30 33  2  109.5  520.0  109.5  520.0 ; CAY CAR CAQ
32 30 33  2  109.5  520.0  109.5  520.0 ; CAZ CAR CAQ
19 33 30  2  108.0  465.0  108.0  465.0 ; NBA CAQ CAR
19 33 34  2  120.0  560.0  120.0  560.0 ; NBA CAQ CAP
30 33 34  2  120.0  560.0  120.0  560.0 ; CAR CAQ CAP
33 34 35  2  115.0  610.0  115.0  610.0 ; CAQ CAP CAO
34 35 36  2  115.0  610.0  115.0  610.0 ; CAQ CAP CAN
[ dihedrals ]

; ai aj ak al fu co, cl, m, ...

19 18 33 20 2 0.0 167.4 0.0 167.4 ; imp NBA CBB CAQ CAT
20 19 21 29 2 0.0 167.4 0.0 167.4 ; imp CAT NBA CAV CAS
21 20 23 22 2 0.0 167.4 0.0 167.4 ; imp CAV CAT CAX HAV
23 21 25 24 2 0.0 167.4 0.0 167.4 ; imp CAX CAV CAW HAX
25 23 27 26 2 0.0 167.4 0.0 167.4 ; imp CAW CAX CAU HAW
27 25 29 28 2 0.0 167.4 0.0 167.4 ; imp CAU CAX CAS HAU
29 30 27 20 2 0.0 167.4 0.0 167.4 ; imp CAS CAX CAU CAT
30 29 32 31 2 0.0 167.4 0.0 167.4 ; imp CAX CAS CAZ CAY
33 34 30 19 2 0.0 167.4 0.0 167.4 ; imp CAQ CAP CAR NBA
38 39 40 53 2 0.0 167.4 0.0 167.4 ; imp CAH CAL CAG NAI
40 39 42 41 2 35.3 334.8 35.3 334.8 ; imp CAG CAH CAX CAJ
43 40 44 52 2 0.0 167.4 0.0 167.4 ; imp CAV CAX CAV CAS
44 46 45 45 2 0.0 167.4 0.0 167.4 ; imp CAV CAX CAV CAG
46 44 47 42 2 0.0 167.4 0.0 167.4 ; imp CAV CAX CAV CAF
48 46 50 49 2 0.0 167.4 0.0 167.4 ; imp CAV CAX CAV HAA
50 48 52 47 2 0.0 167.4 0.0 167.4 ; imp CAV CAX CAV HAB
52 53 50 43 2 0.0 167.4 0.0 167.4 ; imp CAC NAI CAB CAD
53 54 52 39 2 0.0 167.4 0.0 167.4 ; imp NAI CBT CAC CAH
43 44 48 49 2 0.0 209.3 0.0 209.3 ; imp CAD CAE CAF CAK
44 46 48 50 2 0.0 209.3 0.0 209.3 ; imp CAE CAF CAA CGB
46 48 50 52 2 0.0 209.3 0.0 209.3 ; imp CAF CAA CAG CAC
48 50 52 43 2 0.0 209.3 0.0 209.3 ; imp CAA CAG CAC CAI
50 52 43 44 2 0.0 209.3 0.0 209.3 ; imp CAB CAC CAD CAG
52 43 44 46 2 0.0 209.3 0.0 209.3 ; imp CAC CAD CAE CAF
20 21 23 25 2 0.0 209.3 0.0 209.3 ; imp CAT CAV CAX CAW
21 23 25 27 2 0.0 209.3 0.0 209.3 ; imp CAV CAX CAV CAU
23 25 27 29 2 0.0 209.3 0.0 209.3 ; imp CAX CAV CAU CAS
25 27 29 30 2 0.0 209.3 0.0 209.3 ; imp CAV CAU CAS CAT
27 29 30 31 2 0.0 209.3 0.0 209.3 ; imp CAU CAS CAT CAV
29 20 21 23 2 0.0 209.3 0.0 209.3 ; imp CAS CAT CAV CAX
4 3 2 1 1 0.0 5.93 0.0 5.93 ; dih CBP CBQ CBK CBS
5 4 3 2 1 0.0 5.93 0.0 5.93 ; dih CBO CBP CBQ CBR
6 5 4 3 1 0.0 5.93 0.0 5.93 ; dih CBN CBO CBP CBQ
7 6 5 4 1 0.0 5.93 0.0 5.93 ; dih CBM CBN CBO CBP
8 7 6 5 1 0.0 5.93 0.0 5.93 ; dih CBL CBM CBN CBO
9 8 7 6 1 0.0 5.93 0.0 5.93 ; dih CBK CBL CBM CBN
10 9 8 7 1 0.0 5.93 0.0 5.93 ; dih CBJ CKJ CBK CBL
12 11 10 9 1 0.0 5.93 0.0 5.93 ; dih CBH CBI CBJ CKB
13 12 11 10 1 0.0 5.93 0.0 5.93 ; dih CBG CBH CBI CBJ
14 13 12 11 1 0.0 5.93 0.0 5.93 ; dih CBF CBG CBH CBI
15 14 13 12 1 0.0 5.93 0.0 5.93 ; dih CBE CBF CBG CBH
16 15 14 13 1 0.0 5.93 0.0 5.93 ; dih CBD CBE CBF CBG
17 16 15 14 1 0.0 5.93 0.0 5.93 ; dih CBC CBD CBE CBF
18 17 16 15 1 0.0 5.93 0.0 5.93 ; dih CBB CBC CBD CBE
19 18 17 16 1 0.0 5.93 0.0 5.93 ; dih NBA CBB CBC CBD
17 18 19 33 1 0.0 1.06 0.0 1.06 ; dih CBC CBB NBA CAQ
29 20 19 18 1 180.0 33.5 2 180.0 33.5 2 ; imp CAS CAT NBA CBB
34 33 19 18 1 180.0 33.5 2 180.0 33.5 2 ; dih CAP CAG NBA CBB
33 30 29 20 1 180.0 33.5 2 180.0 33.5 2 ; dih CAQ CAR CAS CAT
set as double bond
29 30 33 34 1 180.0 33.5 2 180.0 33.5 2 ; dih CAS CAR CAQ CAQ
set as double bond
35 34 33 19 1 180.0 167.2 180.0 167.2 ; dih CAO CAP CAQ NBA
gd_12 from RTOL
36 35 34 33 1 180.0 33.5 2 180.0 33.5 2 ; dih CAN CAO CAQ CAQ
gd_14 from RTOL
37 36 35 34 1 180.0 167.2 180.0 167.2 ; dih CAM CAN CAO CAP
gd_12 from RTOL
38 37 36 35 1 180.0 33.5 2 180.0 33.5 2 ; dih CAL CAM CAN CAO
gd_14 from RTOL
39 38 37 36 1 180.0 167.2 180.0 167.2 ; dih CAH CAL CAM CAN
gd_12 from RTOL
43 40 39 38 1 180.0 33.5 2 180.0 33.5 2 ; dih CAD CAG CAH CAL
set as double bond
38 39 53 54 1 180.0 33.5 2 180.0 33.5 2 ; dih CAL CAH NAI CBT
set as double bond
39 40 43 52 1 180.0 33.5 2 180.0 33.5 2 ; dih CAH CAG CAD CAC
43 52 53 54 1 180.0 33.5 2 180.0 33.5 2 ; dih CAD CAC NAI CBT
55 54 53 39 1 0.0 1.06 0.0 1.06 ; dih CBU CBT NAI CAH
56 55 54 53 1 0.0 5.93 0.0 5.93 ; dih CBV CBU CBT NAI
57 56 55 54 1 0.0 5.93 0.0 5.93 ; dih CBW CBV CBU CBT
58 57 56 55 1 0.0 5.93 0.0 5.93 ; dih CBX CBW CBU CBT
59 58 57 56 1 0.0 5.93 0.0 5.93 ; dih CBY CBX CBW CBB
60 59 58 57 1 0.0 5.93 0.0 5.93 ; dih CBZ CBY CBX CBW
61 60 59 58 1 0.0 5.93 0.0 5.93 ; dih CCA CBZ CBY CBX
62 61 60 59 1 0.0 5.93 0.0 5.93 ; dih CCB CCA CBZ CBY
63 62 61 60 1 0.0 5.93 0.0 5.93 ; dih CCC CCB CCA CBZ
64 63 62 61 1 0.0 5.93 0.0 5.93 ; dih CDD CCF CCE CCA
65 64 63 62 1 0.0 5.93 0.0 5.93 ; dih CEE CCE CCD CCA
66 65 64 63 1 0.0 5.93 0.0 5.93 ; dih CCF CCE CCD CCA
67 66 65 64 1 0.0 5.93 0.0 5.93 ; dih CCG CCF CCE CCA
68 67 66 65 1 0.0 5.93 0.0 5.93 ; dih CCH CCG CCF CCE
Table S2: bodipy.itp

```plaintext
[ moleculetype ]

; Name nrexcl

[ atoms ]

| nr | type | resnr | resid | atom | cgrn | charge | mass |
|----|------|-------|-------|------|------|--------|------|
| 1  | CH3  | 1     | _2    | CBM  | 1    | 0.000  | 15.0350 |
| 2  | CH2  | 1     | _2    | CBL  | 2    | 0.000  | 14.0270 |
| 3  | CH2  | 1     | _2    | CBK  | 3    | 0.000  | 14.0270 |
| 4  | CH2  | 1     | _2    | CBJ  | 4    | 0.000  | 14.0270 |
| 5  | CH2  | 1     | _2    | CBI  | 5    | 0.000  | 14.0270 |
| 6  | CH2  | 1     | _2    | CBH  | 6    | 0.000  | 14.0270 |
| 7  | CH2  | 1     | _2    | CBG  | 7    | 0.000  | 14.0270 |
| 8  | CH2  | 1     | _2    | CBF  | 8    | 0.000  | 14.0270 |
| 9  | CH2  | 1     | _2    | CBE  | 9    | 0.000  | 14.0270 |
| 10 | CH2  | 1     | _2    | CBD  | 10   | 0.000  | 14.0270 |
| 11 | CH2  | 1     | _2    | CBC  | 11   | 0.000  | 14.0270 |
| 12 | CH2  | 1     | _2    | CBB  | 12   | 0.000  | 14.0270 |
| 13 | CH2  | 1     | _2    | CBA  | 13   | 0.000  | 14.0270 |
| 14 | CH2  | 1     | _2    | CAZ  | 14   | 0.000  | 14.0270 |
| 15 | CH2  | 1     | _2    | CAY  | 15   | 0.000  | 14.0270 |
| 16 | CH2  | 1     | _2    | CAX  | 16   | 0.000  | 14.0270 |
| 17 | CH2  | 1     | _2    | CAW  | 17   | 0.000  | 14.0270 |
| 18 | CH2  | 1     | _2    | CAV  | 18   | 0.000  | 14.0270 |
| 19 | C    | 1     | _2    | CAH  | 19   | 0.280  | 12.0110 |
| 20 | CR1  | 1     | _2    | CAG  | 19   | -0.311 | 12.0110 |
| 21 | HC   | 1     | _2    | HAG  | 19   | 0.170  | 1.0080  |
| 22 | CR1  | 1     | _2    | CAF  | 19   | -0.242 | 12.0110 |
| 23 | HC   | 1     | _2    | HAF  | 19   | 0.182  | 1.0080  |
| 24 | C    | 1     | _2    | CAJ  | 19   | 0.044  | 12.0110 |
| 25 | C    | 1     | _2    | CAK  | 20   | 0.134  | 12.0110 |
| 26 | N    | 1     | _2    | NAO  | 20   | -0.413 | 14.0067 |
| 27 | H    | 1     | _2    | HAO  | 20   | 0.287  | 1.0080  |
| 28 | CH2  | 1     | _2    | CAP  | 20   | 0.369  | 14.0270 |
| 29 | CH2  | 1     | _2    | CAQ  | 21   | -0.106 | 14.0270 |
| 30 | SDMSO| 1     | _2    | SAR  | 21   | 0.945  | 32.0600 |
| 31 | OM   | 1     | _2    | OAT  | 21   | -0.613 | 15.9994 |
| 32 | OM   | 1     | _2    | OAU  | 21   | -0.613 | 15.9994 |
| 33 | OM   | 1     | _2    | OAS  | 21   | -0.613 | 15.9994 |
| 34 | NR   | 1     | _2    | NAI  | 22   | -0.179 | 14.0067 |
| 35 | B    | 1     | _2    | BAL  | 22   | 0.494  | 10.8110 |
| 36 | F    | 1     | _2    | FAM  | 22   | -0.354 | 18.9984 |
| 37 | F    | 1     | _2    | FAN  | 22   | -0.354 | 18.9984 |
| 38 | NR   | 1     | _2    | NAE  | 22   | -0.179 | 14.0067 |
| 39 | C    | 1     | _2    | CAC  | 23   | 0.044  | 12.0110 |
| 40 | CR1  | 1     | _2    | CAB  | 23   | -0.242 | 12.0110 |
| 41 | HC   | 1     | _2    | HAB  | 23   | 0.182  | 1.0080  |
| 42 | CR1  | 1     | _2    | CAA  | 23   | -0.311 | 12.0110 |
| 43 | HC   | 1     | _2    | HAA  | 23   | 0.170  | 1.0080  |
| 44 | C    | 1     | _2    | CAD  | 23   | 0.229  | 12.0110 |
| 45 | CH2  | 1     | _2    | CBO  | 24   | 0.000  | 14.0270 |
| 46 | CH2  | 1     | _2    | CBP  | 24   | 0.000  | 14.0270 |
| 47 | CH2  | 1     | _2    | CBQ  | 24   | 0.000  | 14.0270 |
| 48 | CH2  | 1     | _2    | CBR  | 24   | 0.000  | 14.0270 |
| 49 | CH2  | 1     | _2    | CBS  | 24   | 0.000  | 14.0270 |
| 50 | CH2  | 1     | _2    | CBT  | 24   | 0.000  | 14.0270 |
```

S16
52       CH2     1    _2      CBU    31    0.000  14.0270
53       CH2     1    _2      CBV    32    0.000  14.0270
54       CH2     1    _2      CBW    33    0.000  14.0270
55       CH2     1    _2      CBX    34    0.000  14.0270
56       CH2     1    _2      CBY    35    0.000  14.0270
57       CH2     1    _2      CBZ    36    0.000  14.0270
58       CH2     1    _2      CCA    37    0.000  14.0270
59       CH2     1    _2      CCB    38    0.000  14.0270
60       CH2     1    _2      CCC    39    0.000  14.0270
61       CH2     1    _2      CCD    40    0.000  14.0270
62       CH3     1    _2      CCE    41    0.000  15.0350

[ bonds ]
; ai  aj  fu    c0, c1, ...
2    1    2    0.153   7150000.0    0.153   7150000.0 ;   CBL  CBM
3    2    0.153   7150000.0    0.153   7150000.0 ;   CBM  CBL
4    3    2    0.153   7150000.0    0.153   7150000.0 ;   CBJ  CBJ
5    4    2    0.153   7150000.0    0.153   7150000.0 ;   CBA  CBA
6    5    2    0.153   7150000.0    0.153   7150000.0 ;   CBB  CBB
7    6    2    0.153   7150000.0    0.153   7150000.0 ;   CCA  CCA
8    7    2    0.153   7150000.0    0.153   7150000.0 ;   CBC  CBC
9    8    2    0.153   7150000.0    0.153   7150000.0 ;   CCB  CCB
10   9    2    0.153   7150000.0    0.153   7150000.0 ;   CCC  CCC
11  10    2    0.153   7150000.0    0.153   7150000.0 ;   CCD  CCD
12  11    2    0.153   7150000.0    0.153   7150000.0 ;   CCE  CCE
[ pairs ]
; ai aj fu c0, c1, ...
1 4 1 ; CBM CBJ
2 5 1 ; CBL CBI
3 6 1 ; CBK CBH
4 7 1 ; CBJ CBG
5 8 1 ; CBI CBF
6 9 1 ; CBH CBE
7 10 1 ; CBG CBD
8 11 1 ; CBF CBC
9 12 1 ; CBE CBB
10 13 1 ; CBD CBA
11 14 1 ; CBC CAZ
12 15 1 ; CBB CAY
13 16 1 ; CBA CAZ
14 17 1 ; CAZ CAW
15 18 1 ; CAY CAV
16 19 1 ; CAX CAR
17 20 1 ; CAV CAG
18 21 1 ; CWA NAI
19 22 1 ; CAV CAF
19 24 1 ; CAV CAJ
19 35 1 ; CAV BAL
20 23 1 ; CAH HAF
20 24 1 ; CAI CAJ added manually
20 28 1 ; CAJ CAF added manually
21 25 1 ; CAH NAE
22 26 1 ; CAH CAK
23 27 1 ; CAH CAY
24 28 1 ; CAH CAB
25 29 1 ; CAH CAQ
26 30 1 ; CAH CAC
27 31 1 ; CAH HAO added manually
28 32 1 ; CAP CAY
29 33 1 ; CAP CAY
30 34 1 ; CAP CAB
31 35 1 ; CAP OAAT
32 36 1 ; CAP OAU
33 37 1 ; CAP OAS
| Angle  | Value  | Value  | Value  | Value  | Value  | Value  | Value  | Value  |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 1      | 2      | 3      | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBM    |
| 2      | 3      | 4      | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBL    |
| 3      | 4      | 5      | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBK    |
| 4      | 5      | 6      | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBJ    |
| 5      | 6      | 7      | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBI    |
| 6      | 7      | 8      | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBH    |
| 7      | 8      | 9      | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBG    |
| 8      | 9      | 10     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBF    |
| 9      | 10     | 11     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBE    |
| 10     | 11     | 12     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBD    |
| 11     | 12     | 13     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBC    |
| 12     | 13     | 14     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBB    |
| 13     | 14     | 15     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CBA    |
| 14     | 15     | 16     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CAZ    |
| 15     | 16     | 17     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CAY    |
| 16     | 17     | 18     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CAH    |
| 17     | 18     | 19     | 2      | 109.5  | 520.0  | 109.5  | 520.0  | CAX    |
| 18     | 19     | 20     | 2      | 120.0  | 560.0  | 120.0  | 560.0  | CAW    |
| 19     | 20     | 21     | 2      | 120.0  | 560.0  | 120.0  | 560.0  | CAV    |
| 20     | 19     | 21     | 2      | 120.0  | 560.0  | 120.0  | 560.0  | CAV    |
| 21     | 20     | 21     | 2      | 120.0  | 560.0  | 120.0  | 560.0  | CAV    |
| 22     | 20     | 21     | 2      | 120.0  | 560.0  | 120.0  | 560.0  | CAV    |
| 23     | 22     | 23     | 2      | 120.0  | 560.0  | 120.0  | 560.0  | CAV    |
| 24     | 23     | 24     | 2      | 120.0  | 560.0  | 120.0  | 560.0  | CAV    |
24 25 39 2 120.0 560.0 120.0 560.0 ; CAJ CAK CAC
26 25 39 2 115.0 610.0 115.0 610.0 ; NAO CAK CAC
25 26 28 2 128.9 700.0 128.9 700.0 ; CAF NAO CAP ga_30
modified to 128.9Å*
26 28 29 2 109.5 520.0 109.5 520.0 ; NAO CAP CAQ
27 26 25 2 115.8 415.0 115.8 415.0 ; HAO NAO CAK ga_31
modified to 115.8Å*
27 26 28 2 115.3 460.0 115.3 460.0 ; HAO NAO CAP ga_17
modified to 115.3Å*
28 29 30 2 109.5 520.0 109.5 520.0 ; CAP CAQ SAR
29 30 31 2 109.5 518.0 109.5 518.0 ; CAQ SAR OAT
30 30 32 2 109.5 518.0 109.5 518.0 ; CAQ SAR OAU
30 30 33 2 109.5 518.0 109.5 518.0 ; CAQ SAR OAS
31 30 32 2 109.5 518.0 109.5 518.0 ; OAT SAR OAU
31 30 33 2 109.5 518.0 109.5 518.0 ; OAU SAR OAS
32 30 33 2 109.5 518.0 109.5 518.0 ; OAU SAR OAS
19 34 24 2 108.0 465.0 108.0 465.0 ; CAH NAI CAJ
19 34 35 2 125.0 375.0 125.0 375.0 ; CAH NAI BAL C-N=B C
24 34 35 2 125.0 375.0 125.0 375.0 ; CAJ NAI BAL C-N=B C
34 35 36 2 110.1 696.4 110.1 696.4 ; NAI BAL FAM N-B-F
34 35 37 2 110.1 696.4 110.1 696.4 ; NAI BAL FAN N-B-F
34 35 38 2 109.5 447.3 109.5 447.3 ; NAI BAL NAE N-B-N C
36 35 37 2 110.7 788.2 110.7 788.2 ; FAM BAL FAN F-B-F
36 35 38 2 110.1 696.4 110.1 696.4 ; FAM BAL NAE F-B-N
37 35 38 2 110.1 696.4 110.1 696.4 ; FAM BAL NAE F-B-N
35 38 39 2 125.0 375.0 125.0 375.0 ; BAL NAE CAC B-N=C C
35 38 44 2 125.0 375.0 125.0 375.0 ; BAL NAE CAD B-N=C C
39 38 44 2 108.0 465.0 108.0 465.0 ; CAC NAE CAD
25 39 38 2 120.0 560.0 120.0 560.0 ; CAK CAC NAE
25 39 40 2 132.0 760.0 132.0 760.0 ; CAK CAC CAB
38 39 40 2 108.0 465.0 108.0 465.0 ; NAE CAC CAB
39 40 41 2 126.0 575.0 126.0 575.0 ; CAC CAB HAB
39 40 42 2 108.0 465.0 108.0 465.0 ; CAC CAB CAH
41 40 42 2 126.0 575.0 126.0 575.0 ; HAB CAB CAH
40 42 43 2 126.0 575.0 126.0 575.0 ; CAB CAA HAA
40 42 44 2 126.0 575.0 126.0 575.0 ; CAB CAA HAA
43 42 44 2 126.0 575.0 126.0 575.0 ; HAA CAA CAB
38 44 42 2 108.0 465.0 108.0 465.0 ; NAE CAD CAA
38 44 45 2 120.0 560.0 120.0 560.0 ; NAE CAD CBN
42 44 45 2 120.0 560.0 120.0 560.0 ; CAA CAD CBN
44 45 46 2 109.5 520.0 109.5 520.0 ; CAD CBN CBO
45 46 47 2 109.5 520.0 109.5 520.0 ; CBN CBO CBP
46 47 48 2 109.5 520.0 109.5 520.0 ; CBO CBP CBQ
47 48 49 2 109.5 520.0 109.5 520.0 ; CBP CBQ CBR
48 49 50 2 109.5 520.0 109.5 520.0 ; CBQ CBR CBS
49 50 51 2 109.5 520.0 109.5 520.0 ; CBS CBS CBT
50 51 52 2 109.5 520.0 109.5 520.0 ; CBT CBT BUU
51 52 53 2 109.5 520.0 109.5 520.0 ; CBT CBU CBV
52 53 54 2 109.5 520.0 109.5 520.0 ; CBU CBV CBW
53 54 55 2 109.5 520.0 109.5 520.0 ; CBV CBW CBX
54 55 56 2 109.5 520.0 109.5 520.0 ; CBW CBX CBY
55 56 57 2 109.5 520.0 109.5 520.0 ; CBX CBY CBZ
56 57 58 2 109.5 520.0 109.5 520.0 ; CBY CBZ CCA
57 58 59 2 109.5 520.0 109.5 520.0 ; CBZ CCA CCB
58 59 60 2 109.5 520.0 109.5 520.0 ; CCA CCB CCC
59 60 61 2 109.5 520.0 109.5 520.0 ; CCB CCC CCE
60 61 62 2 109.5 520.0 109.5 520.0 ; CCE CCE CCE

[ dihedrals ]

; ai aj ak al fu c0, c1, m, ...
19 18 34 20 2 0.0 167.4 0.0 167.4 ; imp CAH CAV NAI CAG
20 19 22 21 2 0.0 167.4 0.0 167.4 ; imp CAG CAH CAF HAG
22 20 24 23 2 0.0 167.4 0.0 167.4 ; imp CAF CAG CAJ HAF
24 34 25 22 2 0.0 167.4 0.0 167.4 ; imp CAJ NAI CAK CAF

S20
|    |    |    |    |   | 0.0 | 5.9 3 | 0.0 | 5.9 3 | dih | CCC | CCB | CCA | CB2 |
|----|----|----|----|---|-----|-------|-----|-------|-----|-----|-----|-----|-----|
| 60 | 59 | 58 | 57 | 1 |     |       |     |       |     |     |     |     |     |
| 61 | 60 | 59 | 58 | 1 |     |       |     |       |     |     |     |     |     |
| 62 | 61 | 60 | 59 | 1 |     |       |     |       |     |     |     |     |     |