Supporting Information

Molecular Orientation Determination in Nanodiscs at the Single-Molecule Level

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Figure S1. Scheme for the conversion of incident polarization and the direction of the electric field at the sample plane.

Figure S2. Determination of the system reflectivity of s- and p-polarized excitation light for a 488 nm laser.

Figure S3. Absorbance of DiO-labeled Nanodiscs under s- and p-polarized light.

Supplementary method: Reagents, Calculation of the dichroic ratio, Absorbance-based linear dichroism, the relationship between the dichroic ratio and evanescent electric field components, and computer simulation of the sampling rate.

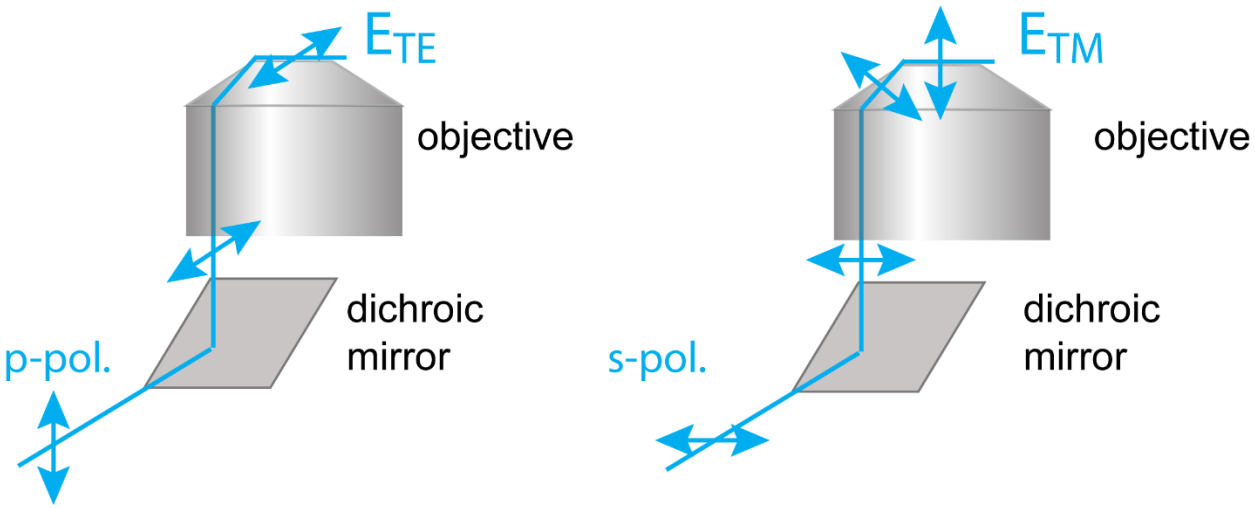

Figure S1: Scheme for the conversion of incident polarization and the direction of the electric field at the sample plane. A p- or s-polarized excitation light produces E_{TE} or E_{TM} excitation field at the sample plane, respectively.

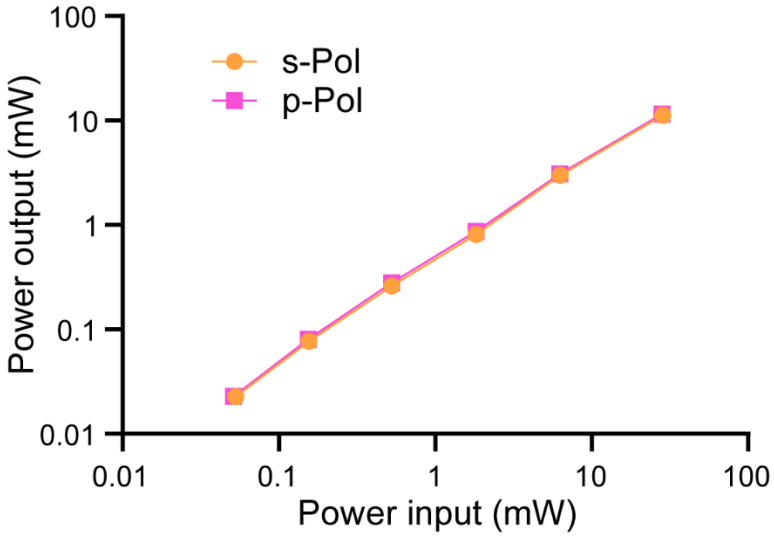

Figure S2: Determination of the system reflectivity of s- and p-polarized excitation light for 488 nm laser. Both s- and p-polarized light power were measured before the microscope and after the objective. No significant difference in the reflectivity was detected between s- and p-polarized light.

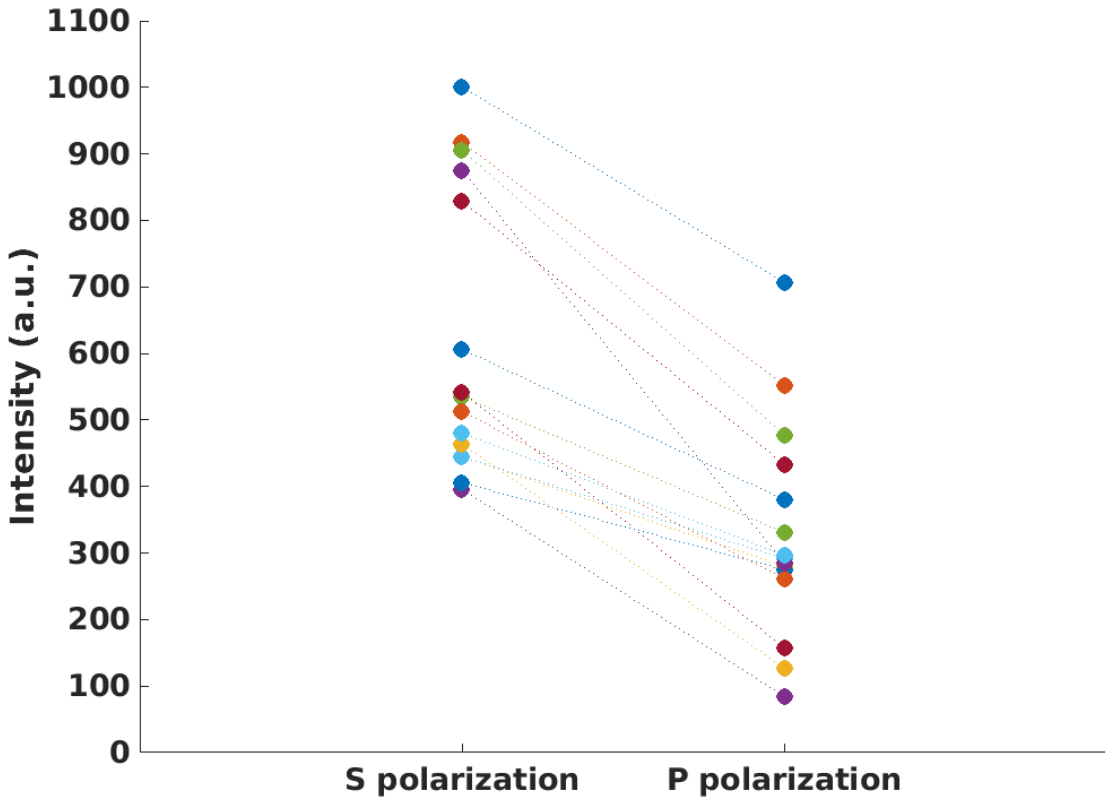

Figure S3: Absorbance of DiO-labeled Nanodiscs under s- and p-polarized light. The data were obtained using a prototyped version of our instrument with the half wave plate rotated by hand.

Supplementary Method

Reagents.

DiO'; DiOC18(3) (3,3'-dioctadecyloxacarbocyanine perchlorate) (Cat #: D275), BODIPY™ FL C12 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid) (Cat #: D3822) and BODIPY™ FL DHPE (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt) (Cat #: D3800) were purchased from Thermo Fisher Scientific.

Calculation of the dichroic ratio

Once it is known which peak corresponds to which polarization, the dichroic ratio can be calculated using the following formula:

$$F_{TM/TE} = \overline{S(t)} \pm 0.5 * A$$

where $\overline{S(t)}$ is the mean emission intensity of the particle, A is the amplitude of the Fourier peak, and the plus/minus corresponds to either TM or TE intensity. This formula yields the fluorescence intensity for both TM and TE radiation, which can be used to compute the dichroic ratio and thus the orientation angle as described in the results section.

Absorbance-based linear dichroism

Absorbance-based linear dichroism in a slab waveguide was measured using a custom-built total internal reflection instrument described in our previous work.[1] The instrument consisted of a 488nm laser source, neutral density filter, polarizer, photoelastic modulator (PEM), sample chamber, and a photomultiplier tube (PMT) fitted with an integrating sphere for detection. The polarizer served to define the initially vertically polarized excitation, which was subsequently modulated between two orthogonal polarizations by the PEM. The modulated light was coupled into the sample chamber by a BK-Schott glass prism, where it was totally internally reflected between a flat ArrayIt SuperClean-2 substrate (ArrayIt Corporation) and buffer (20 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, pH 7.3). The light was coupled out of the sample chamber by another prism, and the light was scrambled by an integrating sphere before being detected by the photomultiplier. A reference PMT was used to correct for variations in the source intensity. Both PMTs were fitted with 488nm interference filters. The PEM generated a modulated linear dichroism signal at twice the modulator frequency, which was detected using a lock-in amplifier (SR850, Stanford Research Systems). The currents generated in the PMTs were converted voltages using preamplifiers (SR570, Stanford Research Systems) and read out by multimeters (HP 34401A, Hewlett-Packard Company). Data acquisition was done in LabVIEW, and analysis was done using a MATLAB script. Absorbance values were calculated using a reference of buffer alone. The sample absorbance was measured after loading a 350 µL sample of 500 nM Nanodiscs and waiting at least 5 min.

The relation between the dichroic ratio and evanescent electric field components

For totally internally reflected light, the projections of the evanescent excitation electric field on the three laboratory axes can be expressed as following:[2]

$$E_x = 2\frac{(\sin^2\beta - n_{21}^2)^{1/2}\cos\beta}{(1-n_{21}^2)^{1/2}[(1+n_{21}^2)\sin^2\beta - n_{21}^2]^{1/2}}$$

$$E_y = \frac{2\cos\beta}{(1-n_{21}^2)^{1/2}}$$

$$E_z = \frac{2\sin\beta\cos\beta}{(1-n_{21}^2)^{1/2}[(1+n_{21}^2)\sin^2\beta - n_{21}^2]^{1/2}}$$

where β is the incident angle of the excitation light. We estimate β = 79.58 degree given the numerical aperture N.A. = 1.49 for the objective and the refractive index of the immersion oil n = 1.515 and n_{21}= n(immersion oil)/ n(water) = 1.515/1.335. The absorbance of each molecule can be expressed in three laboratory axes as

$$A = kl(u_x E_x + u_y E_y + u_z E_z)^2$$

where k is a constant, l is the effective absorption path length, and μ is the transition dipole moment of the fluorophore within Nanodiscs. The absorption due to TE and TM radiation can be calculated separately.

$$A_{TE,x} = A_{TE,z} = 0$$

$$A_{TE,y} = kl\int_0^{2\pi} |E_y|^2|\mu|^2 d\phi = kl\int_0^{2\pi} |E_y|^2|\sin\theta\ \sin\phi|^2|\mu|^2 d\phi = \pi kl|E_y|^2|\sin\theta\,|^2|\mu|^2$$

$$A_{TM,x} = kl\int_0^{2\pi} |E_x|^2|\mu|^2 d\phi = kl\int_0^{2\pi} |E_x|^2|\sin\theta\ \cos\phi|^2|\mu|^2 d\phi = \pi kl|E_x|^2|\sin\theta\,|^2|\mu|^2$$

$$A_{TM,y} = 0$$

$$A_{TM,z} = kl\int_0^{2\pi} |E_z|^2|\mu|^2 d\phi = kl\int_0^{2\pi} |E_z|^2|\cos\theta|^2|\mu|^2 d\phi = 2\pi kl|E_z|^2|\cos\theta|^2|\mu|^2$$

$$\rho = \frac{F_{TE}}{F_{TM}} = \frac{QY * A_{TE}}{QY * A_{TM}} = \frac{|E_y|^2}{|E_x|^2 + 2|E_z|^2\cot^2\theta}$$

Computer simulation of sampling rate

Simulations were performed using MATLAB by adding Gaussian noise of varying magnitude to sinusoidal signals of amplitude and offset based on previously observed single-molecule fluorescence traces. For each noise magnitude or total signal length tested, 5000 trials with resampled noise were averaged.

REFERENCES

(1) Mustafa, G.; Nandekar, P. P.; Camp, T. J.; Bruce, N. J.; Gregory, M. C.; Sligar, S. G.; Wade, R. C., Influence of Transmembrane Helix Mutations on Cytochrome P450-Membrane Interactions and Function. *Biophys. J.* **2019,** *116*, 419-432.

(2) Cropek, D. M.; Bohn, P. W., Surface Molecular Orientations Determined by Electronic Linear Dichroism in Optical Wave-Guide Structures. *J. Phys. Chem.* **1990,** *94*, 6452-6457.