SUPPORTING INFORMATION

Ultrabright Förster Resonance Energy Transfer nanovesicles: the role of the dye diffusion

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Experimental Procedures

1. Materials

5-Cholesten-3β-ol (purity 95%) was purchased from Panreac (Barcelona, Spain). Hexadecyltrimethylammonium bromide (CTAB, BioUltra for molecular biology, purity ≥99.0%) was obtained from Sigma-Aldrich. 1,1'-dioctadecyl-3,3,3’,3'-tetramethyl-indocarbocyanine perchlorate (DiI) and 1,1'-dioctadecyl-3,3,3’,3'-tetramethyl-indodicarbocyanine perchlorate (DiD) were purchased from Life Technologies (Carlsbad, USA). MilliQ water was used for all the sample preparation (Millipore Ibérica, Madrid, Spain). Ethanol (EtOH) was from Teknocroma (Sant Cugat del Vallès, Spain). Carbon dioxide (CO₂, 99.9% purity) was purchased from Carburos Metálicos S.A. (Barcelona, Spain). All reagents and solvents purchased from commercial suppliers were used without further purification.

2. Methods for Molecular Dynamics Simulations

Protocols for MD simulation. All MD simulations were performed using the NAMD program. The equations of motion were solved using a 2 fs time step. Electrostatic interactions were computed using the PME method (PME) with usual settings in NAMD (1 Å resolution, updated each 2-time steps). Lennard-Jones interactions were truncated at 1.2 nm employing a switching function starting at 1.0 nm. Periodic boundary conditions were employed in all directions. The temperature was kept constant at 298 K using a Langevin thermostat with a relaxation time of 1 ps. The pressure of 1 atm and zero surface tension were imposed using the anisotropic Nosé-Hoover-Langevin piston implemented in NAMD (oscillation period of 100 fs and decay time of 50 fs). In all cases, we performed an initial energy minimization and an equilibration run until temperature, pressure, and membrane area reach stable values (these runs were short since initial configurations were built from equilibrated ones). The production runs were 40 ns for each case.

Results for S1 and S2 simulations. For both the DiI-DiI and DiD-DiD dye cases (S1 and S2 in Table S7), both pairs are perfectly incorporated and interdigitated between QS components, as illustrated in the snapshots of Figure S10 and S11. As observed there, both dyes are located and oriented as expected, being the head groups at the water-surface interface and the tails completely immerse in the hydrophobic region of the membrane. Results from the DiI-DiI pair (S1 simulation) show that both dyes remain inside the QS membrane stable along simulation time, not deforming the membrane nor aggregating. The calculated thickness of the bilayer (measured from the peaks of the nitrogen-nitrogen atom distance from CTAB distribution) is 4.2 nm for S1 and 4.3 for S2. In our previous simulations with a single dye, we obtained 4.2 nm for both dyes. It is also close to the value 4.3 nm for the bilayer thickness obtained in our
previous work in absence of dyes.\textsuperscript{2} Note that MD simulations of Dil dyes in DPPC phospholipid bilayer\textsuperscript{6} also predict the absence of aggregation.

We have computed the distance between both dyes present in S1 and S2 simulations as a function of time. As seen in Figure S10, the Dil-Dil distance (measured between the central carbon atoms of both dyes) presents a wide distribution, roughly uniform, indicating an absence of Dil-Dil interaction. In the case of DiD-DiD (Figure S11), we do not observe aggregation but there is a larger tendency of both dyes to be one near the other instead of being fully separated, as seen in Figure S11 (right). As shown in that figure, there is a peak at a separation of about 3 nm between the two central carbons of each dye.

**Additional results for S3 simulations.** The main results for the S3 simulation are reported in the main paper, Figure 5. Here, as additional material, we report the DiD-Dil separation shown in Figure 5c calculated differently. In Figure 5c, the separation between dyes is calculated as the separation between the N atoms of each dye. Here, in Figure S12 we also report the separation between Dil and DiD during the S3 simulation computed as the donor-acceptor separation. The separation is computed as the distance between each nitrogen atom of the Dil (donor) and the centre of mass of the chromophore of the DiD dye (acceptor).
Figure S1. Representation of the Dil-DiD FRET pair in QS, named QS-I,D. a) Normalized absorption and emission spectra of Dil and DiD in ethanol. Dil and DiD are considered ideal for single-pair FRET measurements as they show well-separated absorption and emission spectra with a significant overlap (marked in orange) between the donor (Dil) emission and the acceptor (DiD) absorption. The two dyes have comparable quantum yields, and good photostability. b) Representation of a Quatsome loaded with the Dil-DiD FRET pair, called QS-I,D.
Figure S2. Equipment configuration of the small-scale reactor DELOS-susp (depressurization of an expanded liquid organic solution–suspension). Nanovesicles were prepared following the DELOS-susp procedure, schematically represented in the Figure S2 a. This method has been previously described\(^7\) and includes the depressurization of a CO\(_2\)-expanded organic liquid solution into an aqueous phase containing a solution or dispersion of a polar compound using mild conditions of temperature (308 K) and pressure (10 MPa). a) The general procedure includes: (a) Loading of the organic solution containing the membrane components (cholesterol and dyes); (b) Addition of liquid compressed CO\(_2\) and formation of a CO\(_2\)-expanded solution with all the membrane components dissolved; (c) Depressurization of the CO\(_2\)-expanded solution into an aqueous solution containing the free surfactant (CTAB). The equipment used for the preparation of QS by Delos-susp is schematized in this Figure S2 b. b) The configuration comprises a 7.3 mL high-pressure vessel (HPV), whose temperature is controlled by an external thermostatic bath; a syringe pump (model 260D, ISCO Inc, USA) (P) to introduce CO\(_2\) inside the HPV through valve V-4; a depressurization valve (V-7) from which the expanded liquid solution is depressurized into the aqueous phase placed in a collector (C) located after V-7, N\(_2\) is pumped into the vessel through V-6. A one-way valve is located after V-6 to prevent contamination of CO\(_2\) in the N\(_2\) line. V-2, V-3, and V-5 are dividing the CO\(_2\) and N\(_2\) pipelines. There is also a pressure indicator (PI) and another one-way valve before the vessel to prevent the backflow from HPV, which could lead to contamination of the gas lines.

The specific protocol for the preparation of the four QS-I,D formulations is detailed in Table S1.
Figure S3. Histograms obtained from cryo-TEM images of FRET nanoprobes. a) QS-I,D 143; b) QS-I,D 81; c) QS-I,D 17 and d) QS-I,D 2. The histograms show the distribution of the QS diameters, measured from n=150 nanovesicles.
Figure S4. STORM pictures and histograms of FRET nanoprobes. STORM pictures (scale bar = 5 µm) for the QS-I,D with different loadings. a) QS-I,D 2; b) QS-I,D 17; c) QS-I,D 81 and d) QS-I,D 143. The histograms show the distribution of the QS diameters, as obtained by STORM.
Figure S5. Absorption and excitation spectra of QS-I,D at different loadings. Diluted solutions were used for absorption and fluorescence measurements, with optical densities of ∼0.1. The spectra are normalized at 650 nm. Spectra were measured in aqueous media with 1 cm path length quartz cuvette, with excitation at \( \lambda_{em} = 710 \) nm.

The efficiency of energy transfer is defined as the fraction of photons absorbed by the donor (DiI) which are transferred to the acceptor (DiD). It is estimated experimentally comparing the absorption spectrum with the excitation spectrum, detected from the emission of the acceptor, as in Equation 1, where \( A_A(\lambda_A) \) and \( A_D(\lambda_D) \) are the maximum absorbance of donor and acceptor, at \( \lambda_A \) and \( \lambda_D \), respectively. The fluorescence intensity of the acceptor (\( I_A \)) is indicated with two wavelengths in parentheses: the first one is the excitation wavelength, and the second is the observation wavelength.

\[
\Phi_{FRET} = \frac{A_A(\lambda_A)}{A_D(\lambda_D)} \left[ \frac{I_A(\lambda_D, \lambda_A^{EM})}{I_A(\lambda_A, \lambda_A^{EM})} \right] - \frac{A_A(\lambda_D)}{A_A(\lambda_A)}
\]  
(1)
Figure S6. Normalized absorption spectra of fluorescent QS. Spectra were measured in aqueous media with 1 cm path length quartz cuvette. The spectra are normalized at Dil emission ($\lambda_{em} = 551$ nm).

Absorption spectra of QS-I,D contain two peaks, the characteristic absorption bands for the monomers Dil and DiD (~552 nm and ~649 nm, respectively). The small high energy shoulder (516 nm and 600 nm, for Dil and DiD respectively) can be safely assigned as the first the vibronic replica of the monomer band,$^8$ attributed to the symmetric C-C valence vibration of the polymethine chain in the electronic excited state.$^9$ Variations in the spectral profile of absorption spectra can be ascribed to different origins, including the presence of aggregates. Several studies on cyanine dyes have investigated the relationship between the absorption bands and a growing population of H-dimers in solution.$^{10-13}$ As shown in Figure S4, when the concentration of dyes entrapped at the QS membrane is augmented (from QS-I,D 2 to QS-I,D 143) the 600 nm band becomes more prominent pointing out the presence of H-aggregates, mainly related to the absorption band of H-dimers. Moreover, although the molar ratio of two dyes was kept constant (1:1, see Table S4), the ratio between maximum absorbance of DiD and Dil dyes in QS-I,D changes with increasing loading. This fact is also in line with the formation of DiD H-aggregates in QS membrane at increasing loadings,$^{14,15}$ due to the hypochromism expected for H aggregates.$^{16}$
**Figure S7. Procedure for FRET ratio estimation through TIRF-microscopy.** a) Raw TIRF images at 561 nm of the four formulations (scale bar = 5µm). b) Schematic representation of the procedure for the calculation of FRET ratio through TIRF-microscopy.

The TIRF-images were analysed following the next steps for all the formulations:

1. Subtract background from original images. Divide the far-red emission image by the full-emission image to obtain a ratiometric image.
2. Threshold the full-emission image to obtain a mask; QS = 1, Background = NaN (Not a Number).
3. Multiply the ratiometric image by the mask. (FRET ratio information)
4. Multiply the full-emission image by the mask. (Brightness Information)
5. Stack both masked images and measure both parameters in each QS. Individual QS were easily identified in the full-emission image (output of step 4), defining the delimiting area. This same area was used to measure the brightness (output of step 4) and the FRET ratio (output of step 3).
Figure S8. FRET ratio results represented for each nanoprobe (main values plotted in Figure 4e). TIRF-microscopy counts vs FRET ratio values. In the Table are presented the averaged values obtained per N nanovesicles studied (N=total number of nanoprobes interrogated, SD = Standard Deviation).
Figure S9. Brightness calculated from TIRF-microscopy. TIRF-microscopy counts vs total brightness intensity (DiI+DiD emission). In the Table are presented the averaged values obtained per N nanovesicles studied (N = total number of nanoprobes interrogated, SD = Standard Deviation).

| Sample   | N  | Mean    | SD      | Median  |
|----------|----|---------|---------|---------|
| QS-I,D 2 | 166| 3004.6  | 5556.1  | 1035    |
| QS-I,D 17| 299| 22742.0 | 38326.5 | 8432    |
| QS-I,D 81| 211| 15489.9 | 12360.3 | 11441   |
| QS-I,D 143| 202| 59289.5 | 104758.1| 17909   |
Figure S10. Stability over time and upon dilution of QS-I,D 81. (a) Fluorescence emission spectra, (b) FRET efficiency representation of the QS-I,D 81 after 1, 3, 6, 8 months and more than two years from the synthesis. (c) Micelles of CTAB loaded with Dil and DiD at same concentration as QS-I,D 81 were progressively diluted in aqueous media. Under 1 mM (the critical micellar concentration of CTAB micelles, referred as CMC), micelles become unstable indicated by the significant changes on the FRET ratio, otherwise, the FRET ratio of QS-I,D 81 is preserved at higher dilutions.
Figure S11. Geometry of Dil (left) and DiD (right) dyes in CPK representation with indication of dimensions and charge. The color scale indicates the partial charge of each atom in $e$ units.
Figure S12. Summary of results of simulation S1: two Dil molecules inside a Quatsome bilayer (Table S9, Supporting Information). The snapshot of the system (front view and top view) shows both QS components CTA+ and cholesterol in blue and cyan, respectively. Water is shown as red dots and ions are shown as pink spheres. The two Dil dyes are shown in licorice representation in magenta. The histogram shows the distribution of the distance between the centre of mass of the chromophoric part of the two dyes. In the top view a black line defines the distance between the dyes.
Figure S13. Summary of results of simulation S2: two DiD molecules inside a Quatsome bilayer (Table S9, Supporting Information). The snapshot of the system (front view and top view) shows both QS components CTA+ and cholesterol in grey and cyan, respectively. Water is shown as red dots and ions are shown as pink spheres. The two DiI dyes are shown in licorice representation in dark blue. The histogram shows the distribution of the distance between the centre of mass of the chromophore part of the two dyes. In the top view a black line defines the distance between the dyes.
Figure S14. Donor - Acceptor separation during simulation S3 as a function of time, computed as the distance between each of the two nitrogen atoms of the DiI (donor), indicated either as N1 or N2, and the centre of mass of the chromophore of the DiD dye (acceptor).
Table S1. Dil and DiD concentrations of the ethanolic solution used for the preparation of QS-I,D formulations by Delos-susp methodology.

| SAMPLE    | [Dil] (mM) [a] | [DiD] (mM) [a] |
|-----------|----------------|----------------|
| QS-I,D 2  | $2.27 \times 10^{-2}$ | $2.27 \times 10^{-2}$ |
| QS-I,D 17 | $2.27 \times 10^{-1}$ | $2.27 \times 10^{-1}$ |
| QS-I,D 81 | $4.54 \times 10^{-1}$ | $4.54 \times 10^{-1}$ |
| QS-I,D 143| $9.07 \times 10^{-1}$ | $9.07 \times 10^{-1}$ |

[a] Dye stock solutions were used for the preparation of the initial ethanolic solution. Dil and DiD were dissolved, separately, in EtOH at a high concentration (~5 mM) and the concentration of both solutions was determined through UV-Vis spectroscopy.

For the Delos-susp experiments, 3.11 mL of ethanolic solution at 7mM of cholesterol and with different concentrations of Dil and DiD ([Dil] and [DiD]), depending on the QS-I,D formulation, were prepared (see Table S1). The mixture was kept under stirring protected from light for 40 minutes. The ethanolic solution containing the cholesterol and dyes was loaded into a high-pressure vessel of 7.3 mL at atmospheric pressure and the working temperature ($T_w = 308$ K) (Figure S2). The solution was then volumetrically expanded with compressed CO$_2$ until a molar fraction ($X_{CO2}$) of 0.60, reaching a working pressure ($P_w$) of 10 MPa. The system was kept at 308 K and 10 MPa for approximately 1 hour to achieve a complete homogenization and to attain thermal equilibration. Afterwards the depressurization of the volumetric expanded organic phase was performed over 25.11 mL of an aqueous solution at 7mM of CTAB. In this step a flow of N$_2$ at the working pressure is used as a plunger to push down the CO$_2$ expanded solution from the vessel and to maintain a constant $P_w$ inside the vessel during depressurization. Details of the equipment configuration are given in Figure S2, Supporting Information.
Table S2. Characteristics of the dye-loaded nanoformulations.

| SAMPLE  | [a] Dye concentration in bulk (µM) | [b] Ratio Dil:DiD | [b] Total dye loading (x10<sup>-3</sup>) | [c] Dye encapsulation efficiency (%) | [d] QS concentration (particle/mL) x10<sup>11</sup> | [d] Hydrodynamic diameter (nm) NTA | [d] Mean diameter (nm) STORM |
|---------|-----------------------------------|-------------------|---------------------------------|-------------------------------------|-----------------------------------------------|---------------------------------|-------------------------------|
| QS-I,D 2 | 2.5                                | 2 : 2             | 1.00 : 0.95                     | 1.0                                 | 100                                           | 88                             | 141 ± 5                        | 144 ± 1                       | 141 ± 44                       |
| QS-I,D 17 | 24.4                               | 21.1              | 1.00 : 0.89                     | 8.8                                 | 98                                            | 84                             | 137 ± 9                        | 156 ± 2                       | 159 ± 49                       |
| QS-I,D 81 | 55                                 | 53                | 1.00 : 0.88                     | 25.0                                | 100                                           | 96                             | 68 ± 2                         | 138 ± 3                       | 156 ± 58                       |
| QS-I,D 143 | 104                                | 99                | 1.00 : 0.98                     | 49.0                                | 95                                            | 90                             | 72 ± 4                         | 125 ± 2                       | 164 ± 49                       |

[a] The final concentration of dyes was determined measuring the absorbance of the diafiltrated fluorescent Quatsomes in ethanol and applying the Lambert-Beer Law (see Experimental Section).

[b] The dye loading indicates the relation in composition between the content of dye vs membrane components. It is calculated as [(mg dye/mL solution) / (mg membrane components<sub>CTAB+Chol</sub> /mL solution)]. The final concentration of membrane components was estimated lyophilizing the fluorescent Quatsomes after the diafiltration (see Experimental Section).

[c] The dye encapsulation efficiency is defined as the ratio between the amount of dye present in the final formulation of QS-I,D and the initial amount of dye loaded into the reactor.

[d] QS concentration and size distribution were measured by Nanoparticle Tracking Analysis (NTA). The averaged results are obtained from n ≥ 3 (error ± 7%).
Table S3. Geometric parameters of the fluorescent QS

| Geometric parameters of a Chol/CTAB QS |
|----------------------------------------|
| [a] Diameter outer (nm)                  | 120                        |
| Diameter inner (nm)                     | 110.6                      |
| [b] Thickness membrane (nm)              | 4.70                       |
| Volume vesicle exterior (nm$^3$)         | 904778.7                   |
| Volume vesicle interior (nm$^3$)         | 708376.3                   |
| Membrane Volume (nm$^3$)                 | 196402.4                   |
| Membrane Volume (dm$^3$)                 | 1.9 E-19                   |
| Membrane vs nanovesicle volume           | 22%                        |
| Surface outer vesicle (nm$^2$)           | 4.52E+04                   |
| Surface inner vesicle (nm$^2$)           | 3.84E+04                   |
| [c] Surface synthon (nm$^2$)             | 0.57                       |
| Number synthons at the outer QS surface  | 7.94E+04                   |
| Number synthons at the inner QS surface  | 6.74E+04                   |
| Number of total synthons per 1 QS        | 1.47E+05                   |

[a] Average size obtained from NTA measurement and cryo-TEM pictures.
[b] Obtained from previous works, based on AFM-FS experiments.
[c] Obtained from Molecular Dynamics simulations.
Table S4. Volume of QS membrane in relation to the total volume

| Number of QS per mL | [a] Membrane volume of QS per mL | [b] QSs membrane vs total volume (%) |
|---------------------|---------------------------------|-----------------------------------|
| QS-I,D 143          | 7.24E+12                        | 1.42E-06                          | 0.14%                             |
| QS-I,D 81           | 6.76E+12                        | 1.33E-06                          | 0.13%                             |
| QS-I,D 17           | 1.37E+13                        | 2.69E-06                          | 0.27%                             |
| QS-I,D 2            | 1.41E+13                        | 2.77E-06                          | 0.28%                             |

[a] Membrane volume of a QS obtained from Table S3 is multiplied by the number of quatsomes estimated in 1mL, determined by NTA.

[b] Membrane Relation between the QS membrane volume and the total volume in 1mL.
Table S5. Calculation of the dye concentration at the QS membrane

| QS [a]  | Dye bulk concentration [b] | Dye per QS [c] | Concentration of dye per QS [d] |
|---------|---------------------------|----------------|---------------------------------|
|         | QS per mL Dil (mol/mL)    | Dil per QS (mol/QS) | DiD per QS (mol/QS) | Total dye (mol/QS) | Dil at the nanodomain (M) | DiD at the nanodomain (M) | Total conc. at the nanodomain (M) |
| QS-I,D 143 | 7.24E+12                  | 1.04E-07                | 9.90E-08          | 2.03E-07            | 1.44E-20              | 1.37E-20              | 2.80E-20            | 7.31E-02    | 6.96E-02    | 1.43E-01    |
| QS-I,D 81  | 6.76E+12                  | 5.50E-08                | 5.30E-08          | 1.08E-07            | 8.14E-21              | 7.84E-21              | 1.60E-20            | 4.14E-02    | 3.99E-02    | 8.13E-02    |
| QS-I,D 17  | 1.37E+13                  | 2.44E-08                | 2.11E-08          | 4.55E-08            | 1.78E-21              | 1.54E-21              | 3.32E-21            | 9.07E-03    | 7.84E-03    | 1.69E-02    |
| QS-I,D 2   | 1.41E+13                  | 2.50E-09                | 2.20E-09          | 4.70E-09            | 1.90E-22              | 1.67E-22              | 3.56E-22            | 9.65E-04    | 8.49E-04    | 1.81E-03    |

[a] Number of QS nanovesicles obtained from NTA measurements, the averaged results are obtained from n ≥ 3 per sample (error ± 7%).

[b] Obtained from UV-Vis spectra measured in bulk

[c] Obtained by: Dye (mol/mL) / QS per mL

[d] In order to obtain the concentration; dye per QS is divided by membrane volume (dm³) obtained from Table S3.
Table S6. Brightness per particle

| SAMPLE       | \( \varepsilon \) per particle | \( \phi_{\text{DiD}} \) (%) | Brightness \(_p\) (x 10^6) | \( \phi_{\text{DiD}} \) (%) | Brightness \(_p\) (x 10^6) |
|--------------|-------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| QS-I,D 143   | 1.05E+09                       | 5.6%                        | 58.52                       | 6.3%                        | 65.84                       |
| QS-I,D 81    | 6.77E+08                       | 10.9%                       | 73.83                       | 12.8%                       | 86.70                       |
| QS-I,D 17    | 1.74E+08                       | 16.5%                       | 28.69                       | 20.4%                       | 35.47                       |
| QS-I,D 2     | 4.65E+07                       | 18.1%                       | 8.42                        | 40.7%                       | 18.94                       |

[a] Brightness of the nanoparticle excited at DiI and emission recorded at DiD (\( \lambda_{\text{ex}} = 520 \) nm, \( \lambda_{\text{em}} = 670 \) nm)

[b] Brightness of the nanoparticle excited at DiD and emission recorded at DiD (\( \lambda_{\text{ex}} = 600 \) nm, \( \lambda_{\text{em}} = 670 \) nm)

Brightness per particle was determined as the product of the molar extinction coefficient per particle (\( \varepsilon_p \)) and the fluorescence quantum yield (\( \phi \)).\(^{17}\) The \( \varepsilon_p \) was determined by the Lambert-Beer Law (\( \varepsilon_p = \text{Abs}_{650}/C_{QS} \) (M)), where \( \text{Abs}_{650} \) was obtained by UV-Vis spectroscopy and the molar concentration of QS (\( C_{QS} \)) from the NTA values (Table S3). The \( \phi \) measurements were carried out using a Quantaurus-QY Plus (UV-NIR absolute PL quantum yield spectrometer C13534-11), Hamamatsu Photonics. The samples were diluted until absorbance values OD ≈ 0.1 were obtained, the excitation wavelength was 520nm and 600nm, for FRET and direct DiD emission determination, respectively, (the direct excitation of DiD at 520nm can be considered negligible).\(^{15}\) Illumination time was 0.9 seconds and the final \( \phi \) value come from an average of 20 repetitions.
Table S7. Optical characteristics of fluorescent inorganic and organic nanoparticles reported in bibliography.

| FLUORESCENT NANOFLUID | SIZE (nm) | FLUOROPHORE | λ_em (nm) | DYE (wt %) | BRIGHTNESS (M⁻¹ cm⁻¹) | REF |
|------------------------|-----------|-------------|-----------|------------|------------------------|-----|
| **FLUORESCENT INORGANIC NANOFLUIDS** | | | | | | |
| **QUANTUM DOTS** | | | | | | |
| QDot-585 | 15-20 | / | 585 | / | 1.2 x 10⁵ | 18,19 |
| QDot-605 | 11 | / | 605 | / | 5.8 x 10⁵ | 20 |
| QDot-655 | 15 | / | 655 | / | ~10⁶ | 21 |
| **DYE-LOADED SILICA NANOFLUIDS** | | | | | | |
| Silica core-shell NPs dye doped | 24 | Rhodamine B derivative | 588 | 0.36% | 7.5 x 10⁵ | 22 |
| Fluorophore-rich centre + siliceous shell | 20-30 | TRITC | 577 | [a] | ~ 2-3x QD585 | 23,24 |
| Mesoporous silica NP encapsulating dye | 28 | Polymethine cyanine dye LS277 | 815 | 1.5 | 6.2 x 10⁵ | 25 |
| Silica Core-Surfactant Shell NPs dye doped | 24 | Rhodamine B derivative | 590 | ~0.36% | 1.5 x 10⁵ | 19,26 |
| **FLUORESCENT ORGANIC NANOFLUIDS** | | | | | | |
| **DIRECT ASSEMBLY OF DYE NPs** | | | | | | |
| FONs built from Thienothiophene dyes | 27 | Derivative Ila | 570 | ~100% | 4 x 10⁷ | 27 |
| 39 | Derivative Ib | 711 | ~100% | 2 x 10⁷ | |
| FONs built from spirofluorene dyes | 14 | JD193 | 716 | ~100% | 9 x 10⁵ | 28 |
| **DENDRIMERS** | | | | | | |
| Phosphorous-based dendrimers (2-G₂) | Unknown | Quadrupolar 2P photosensitizers | 560 | [b] | 2.1 x 10⁵ | 29 |
| Phosphorous dendrimers (G1 – G4) | ~8 | TP-Chromophore | 423 - 445 | [b] | 0.75 x 10⁶ – 3.4 x 10⁶ | 30 |
| POLYMER-BASED FNP |
|-------------------|
| **PLGA NP**       |
| 40                |
| Rhodamine B (R18) |
| 590               |
| 5%                |
| 1.8 × 10^7        |
|                    |
| **PEMA-AspN3 NP** |
| 20                |
| Rhodamine B (R18) |
| 590               |
| 50%               |
| 5.1 × 10^7        |
|                    |
| **PLGA-PEG NP**   |
| 66                |
| DiD               |
| 665               |
| 0.5%              |
| 1.3 × 10^7        |
|                    |
| **PLGA-PEG, sample T3 (FRET cascade)**|
| 86                |
| DiO:Dil:DiD (1:1:0.5) |
| 672               |
| 2.10%             |
| ~10^7             |
|                    |
| **Carboxylated PSP**|
| 100               |
| Nile Red          |
| 635               |
| 0.74%             |
| 6.8 × 10^7        |
|                    |
| **PLGA NP**       |
| 38                |
| Lumogen Red       |
| 605               |
| 5%                |
| 7.5 × 10^6        |

| LIPID NP / NANOEMULSIONS |
|--------------------------|
| **Lipid nano-droplets containing Dil**|
| 87                        |
| Dil-TPB                   |
| 553                       |
| 8                         |
| 7.9 × 10^7                |
|                          |
| **Fluorescent nanoemulsion (in VEA oil)**|
| 45                        |
| Dioxaborine barbituryl styryl (DBS-C₈) |
| 544                       |
| 1.5                       |
| 30 × 10⁶                  |
|                          |
|                          |
|                          |
| 50                        |
| Nile Red derivative (NR668) |
| 563                       |
| 1.5                       |
| 15 × 10⁶                  |

| FLUORESCENT ORGANIC NANOPARTICLES BASED ON QUASTOMES |
|-----------------------------------------------------|
| **QS-D**                                            |
| 120                                                 |
| DiD                                                  |
| 673                                                  |
| 2.3%                                                 |
| 1.8 × 10^8                                           |
| **QS-I,D 81 (FRET)**                                |
| 150                                                 |
| Dil:DiD (1:1)                                       |
| 673                                                  |
| 4.8%                                                 |
| 7.4 × 10^7                                           |

[a] wt % not reported, however is given the concentration per particle [TRITC] = 1.43 nM/particle

[b] The photosensitisers are part of the structure of the dendrimers nanoprobe.
Table S8. Estimation of donor-acceptor averaged distance in the Quatsome bilayer for the four formulations.

| Molecules per QS | QS-I,D 143 | QS-I,D 81 | QS-I,D 17 | QS-I,D 2 |
|------------------|------------|------------|------------|------------|
| Dil concentration (µM) | 104,0 | 55,0 | 24,4 | 2,5 |
| DiD concentration (µM) | 99,0 | 53,0 | 21,1 | 2,2 |
| Dil molecules | 8652 | 4900 | 1073 | 107 |
| DiD molecules | 8236 | 4722 | 928 | 94 |
| Total dye molec. | 16888 | 9623 | 2000 | 201 |
| QS surface per dye (nm²) | 5 | 9 | 42 | 420 |
| [a] Theoretical average distance between two dyes in movement (nm) | 2 | 3 | 7 | 21 |

[a] Dyes are constantly diffusing through the QSs membrane, at higher loadings dye molecules are closer to each other due to the higher amount of molecules per volume, leading to shorter average distances.
Table S9. Composition of the systems considered in the MD simulations.

|                | Atoms (total) | Num molec. Dil/DiD//water/CTA/Chol | Sim. Time | Box Size     |
|----------------|---------------|-------------------------------------|-----------|--------------|
| S1 (DiI-DiI)   | 53892         | 2/0/12926/108/108                   | 41.5 ns   | 32.2 nm$^2$ x 16 nm |
| S2 (DiD-DiD)   | 53696         | 0/2/12858/108/108                   | 40.5 ns   | 32.1 nm$^2$ x 16 nm |
| S3 (DiI-DiD)   | 101248        | 1/1/23778/216/216                   | 40.6 ns   | 91.2 nm$^2$ x 16 nm |

Composition of the systems considered in the MD simulations, including the total number of atoms, number of molecules of each component, simulation time, and box size (for each dye molecule a Cl$^{-}$ anion is also present and for each CTA$^{+}$ surfactant a Br$^{-}$ counterion is also present).
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