# Supporting Information

## Proteo-Lipobeads for the oriented encapsulation of membrane proteins

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## Materials and Methods

### *Materials*

Di-8-butyl-amino-naphtyl-ethylene-pyridinium-propyl-sulfonate (di-8-ANEPPS) and 1,2-dihexadecanoyl-sn-glycero-3-phospho-(N-[4-nitrobenz-2-oxa-1,3-diazolyl)ethanolamine (NBD-PE) were from Invitrogen. 4-(1-[2-(di-*n*-butylamino)-6-naphthyl]-4-butadienyl)-1-(4-butyllsulfonate) quinolinium betaine (di-4-ANBDQBS) was synthesized according to Matiukas *et al*. [1] Cytochrome *c* oxidase (C*c*O) from *Paracoccus denitrificans* with a His-tag engineered to the C-terminus of the subunit I was expressed and purified according to Dürr *et al.* [2] Agarose beads, Thermo Scientific HisPur Ni-NTA Resin (PI-88221), 50-150µm were purchased from Fisher Scientific. 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DiPhyPC, > 99%) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(1-pyrenesulfonyl) (18:1 Pyrene DOPE) were both purchased from Avanti Polar Lipids. Fluorescein DHPE (1-(8-((3',6'-dihydroxy-3-oxo-spiro(isobenzofuran-1(3H),9'-(9H)xanthen)-5-yl)amino)-3-hydroxy-8-thioxo-2,4-dioxa-7-aza-3-phosphaoct-1-yl)-1,2-ethanediyl ester, P-oxide) were purchased from Invitrogen. Aniline (ACS reagent, ≥99.5%) , 3-carboxy-PROXYL (3-(Carboxy)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, 3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, free radical ) (98%, 3-CP), valinomycin (1 mg/mL in DMSO, 0.2 µm filtered), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, ≥98%) were purchased from Sigma-Aldrich. The ruthenium complex $[(bpy)_2Ru(diphen)Ru(bpy)_2](PF_6)_4$ ($Ru_2C$) was prepared by the nickel catalyzed coupling of $[Ru(bpy)_2(Cl\text{-}phen)](PF_6)_2$ using similar conditions to those described

by Johansson et. al.[3] In the present case commercially (Sigma-Aldrich) available 5-chlorophenanthroline was used in place of 4-chlorobipyridine. Purity (specifically the absence of monomers) was assessed with ESI-MS.

**Preparation of the Proteo-Lipo-Beads (PLBs)**

*Immobilization of the Protein*

0.5 ml of the deposit of the agarose beads (HisPur Ni-NTA Resin), slurried in 20% ethyl alcohol were repeatedly rinsed, centrifuged (Heraeus Fresco Microcentrifuge, Thermo Scientific) for 1 min at $5x10^3$ rpm and resuspended, first in ultrapure water, then DPK buffer solution (0.05 M $K_2HPO_4$, 0.1 M KCl, pH = 8) and finally in DDM DPK buffer (0.05 M $K_2HPO_4$, 0.1 M KCl, pH = 8, 0.1% DDM). Thereafter, C*c*O dissolved in DDM DPK buffer was adsorbed to the Ni-NTA-functionalized surface of the agarose beads present within 0.5 ml of the deposit to a final concentration of 197 nM. After 2h adsorption time the beads were rinsed again, centrifuged and resuspended in DDM DPK buffer to remove unspecifically adsorbed proteins. Centrifuging for a last time the liquid is fully removed and only the remaining pellets are used for further steps.

*Dialysis*

The Spectra/Por Float-A-Lyzer (MWCO: 500-1000 Da) (volume 5 ml), obtained from Carl Roth was filled with 4 ml of a DiPhyPC-Pyrene DOPE solution (10:1, final concentration 40 µM DiPhyPC, 4µM Pyrene DOPE in DDM-DPK buffer) and the agarose beads loaded with C*c*O. In the case of NBD-PE labeled proteo-lipo-beads, NBD-PE was added to the DiPhyPC-Pyrene DOPE solution before filling the Float-A-Lyze to a final NBD-PE concentration of 12.2 µM. The sample was dialyzed in 1L of DPK buffer solution at room temperature for 24 hours with 6 complete dialysate changes after 2, 4, 14 and 16 hours.

For PLBs suspendended in Tris-HCl/KCl buffer (5 mM/35 mM) pH = 8), the same procedure was applied replacing the DPK buffer by the Tris-HCl/KCl buffer in all of the preparation steps.

*Labeling with potential-sensitive fluorescent dyes*

Stock solution of PSFD di-4-ANBDQBS was prepared in pure ethanol solution at a concentration of 1 mg/5 ml. The stock solution of di-8-ANEPPS was prepared by dissolving the powder in DMSO at a ratio of 1 mg/5 ml. 20 µl of the stock solution was added to the suspension of PLBs loaded with C*c*O (40 µl in 1 ml DPK buffer solution) to a final concentration was 6.6 µM and 6.9 µM di-8-ANEPPS or di-4-ANBDQBS, respectively. After incubation for 15-20 min in DPK buffer solution the PLBs were washed three times in dye-free DPK buffer solution by rinsing, centrifugation and resuspension.

*Labeling with pH-sensitive fluorescent dye*

Stock solution of fluorescein DHPE was prepared in chloroform solution at a concentration of 1 mg/10 ml. 20 µl of the stock solution was added to the suspension of PLBs loaded with C*c*O to a final concentration of 3.3 µM fluorescein DHPE. After incubation for 15-20 min in Tris-HCl/KCl buffer solution the fluorescein labeled PLBs were separated from chloroform and then the PLBs were washed three times in dye-free Tris-HCl/KCl buffer solution by rinsing, centrifugation and resuspension.

**Laser Scanning Confocal Fluorescence Microscopy (LSM)**

Laser scanning confocal fluorescence microscopy measurements were carried out in an upright Leica TCS SP5 II microscope with a 10x dry objective (Leica, HC PL APO 10x/0.40 CS). The Proteo-lipo-beads were attached via the hydrophobic pyrene anchor group to the

hydrophobic surface of the flow cell (μ-slide upright, ibidi GmbH, Munich, Germany). The 488 nm line of a multi argon laser and the 633 nm line of the He-Ne laser were used for the excitation of di-8-ANEPPS and di-4-ANBDQBS, respectively. In case of di-8-ANEPPS and di-4-ANBDQBS labeled PLBs the emission bandwidth was detected at 600-670 nm, and 651-794 nm, respectively. SNARF-1 labeled PLBs were excited using the 514 nm line of a multi argon laser and the emission bandwidth was detected at two different channels: 550-610 nm and 620-700 nm. For fluorescein DHPE labeled PLBs the 488 nm line of a multi argon laser was used for the excitation and the emission bandwidth was detected at 492-610 nm. Images were taken every 1.2 s before, during and after 35 s continuous illumination with a halogen lamp (Fiber-lite DC 950, Dolan Jenner Industries) These Images were then used to analyze localized intensities in the immediate vicinity of the PLBs using the software Leica Application Suite Advanced Fluorescence.

**Table S1.** List of fluorescence dyes, systematic names and excitation and emission wavelengths.

| Fluorescence label | Systematic name | $\varepsilon_{ex}/\varepsilon_{em}$/nm |
|---|---|---|
| NBD-PE | (1,2-dihexadecanoyl-sn-glycero-3-phospho-(N-[4-nitrobenz-2-oxa-1,3-diazolyl)ethanolamine) | 458 / 530 |
| di-8-ANEPPS | 4-(1-[2-(di-n-octylamino)-6-naphthyl]-2-ethenyl)-1-(3-propylsulfonate) pyridinium betaine) | 488 / 620 |
| di-4-ANBDQBS | (4-(1-[2-(di-*n*-butylamino)-6-naphthyl]-4-butadienyl)-1-(4-butyllsulfonate) quinolinium betaine) | 633 / 740 |
| fluorescein DHPE | 1-(8-((3',6'-dihydroxy-3-oxo-spiro(isobenzofuran-1(3H),9'-(9H)xanthen)-5-yl)amino)-3-hydroxy-8-thioxo-2,4-dioxa-7-aza-3-phosphaoct-1-yl)-1,2-ethanediyl ester, P-oxide | 488 / 520 |
| SNARF-1 | Benzenedicarboxylic acid, 2(or 4)-[10-(dimethylamino)-3-oxo-3H-benzo[c]xanthene-7-yl] | 514 / 580, 650 |

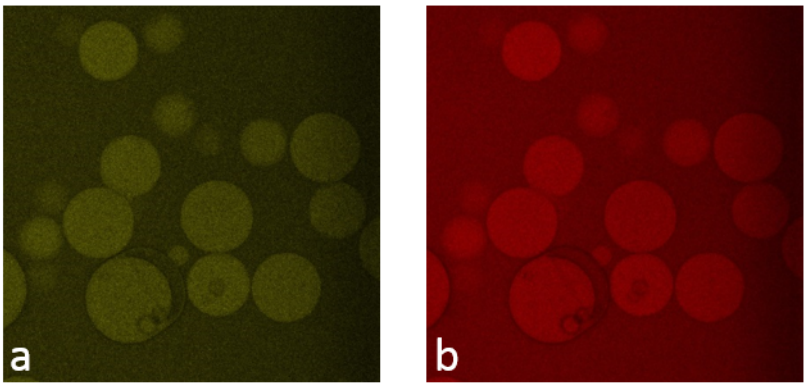


**Figure S2.** Laser scanning images of agarose-PLBs submersed in a 20 µM SNARF-1 solution in aqueous DPK buffer, measured at a) channel (550-610 nm) and b) channel (620-700 nm).

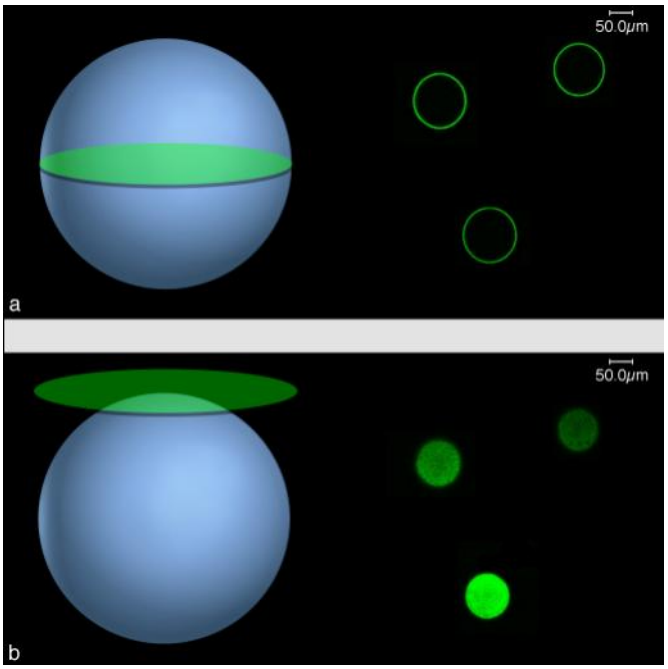


**Figure S3.** Laser scanning images taken at the a) equatorial plane and b) the pole of a CcO based PLB labelled with di-8-ANEPPS.

Concerning the use of PLBs for cell-free bioassays, stability over time is a key issue. PLBs were prepared as described and labelled with di-4-ANBDQBS. Images taken by LSM showed that lipid bilayers were stable after storage in the refrigerator for at least 12 days

(Figure S3a,b). PLBs after deep freezing and thawing were stable after resuspension in dimethylsulfoxide (Figure S3d) or glycerol (10%) buffer whereas they collapsed after deep freezing in aqueous buffer (Figure S3c). Stability also strongly depends on the ionic strength of the buffer solution used for incubation and dialysis. Images obtained from PLBs prepared in Tris-HCl/KCl (5 mM/35 mM) and DPK buffer appear to be similar. In comparison, PLBs prepared in Tris-HCl (5 mM), exhibit a weak di-4-ANBDQBS fluorescence emission (not shown). Proteoliposomes treated under the same conditions could not be used for LSM because they fused on the surface of the flow cell indicating the superior stability of our new biomimetic system.

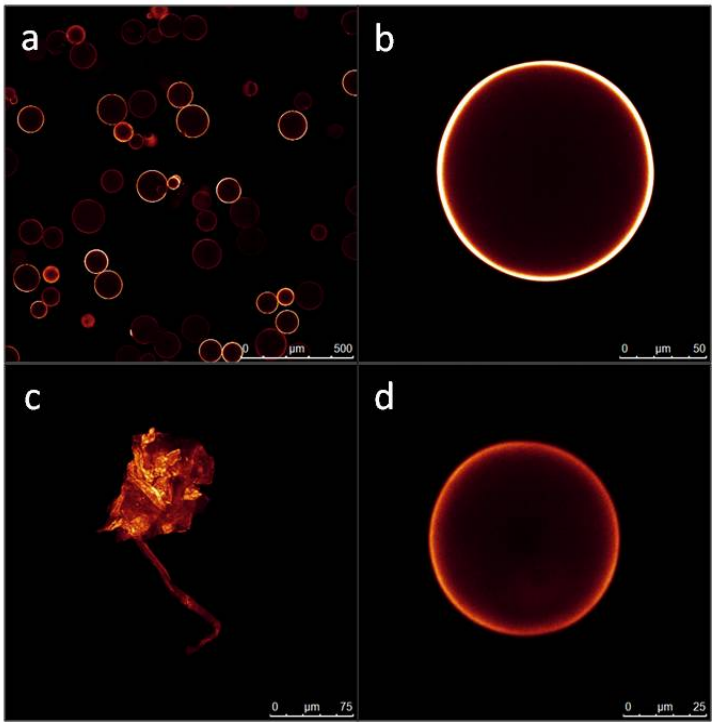


**Figure S4**. Laser scanning images of agarose-PLBs labeled with di-4-ANBDQBS : 10x magnified section of the flow cell (a), zoom on a single bead (b), bead after shock freezing without (c) and with added DMSO (d).

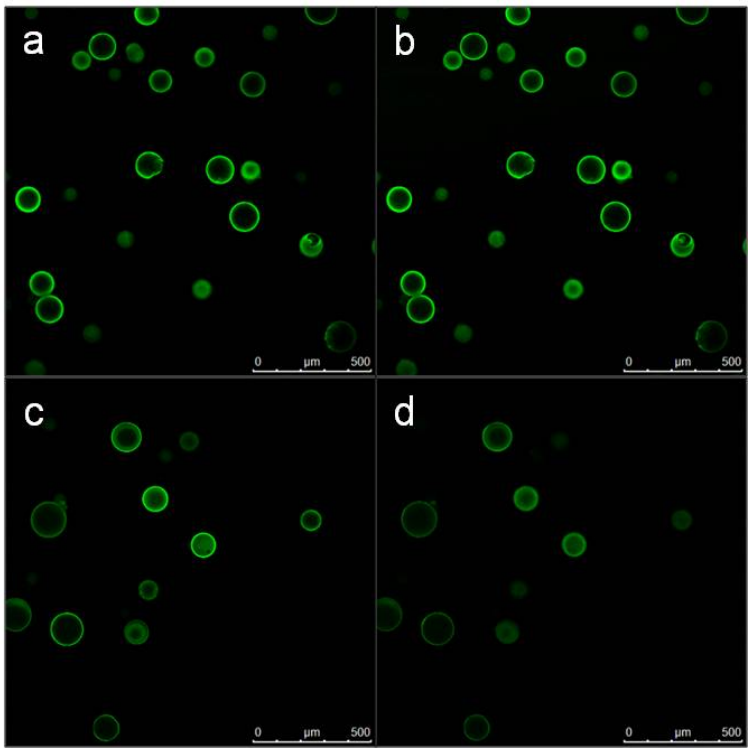


**Figure S5.** Laser scanning images of agarose-PLBs labeled with fluoresceine DHPE: without (a) and with (b) white light illumination, without (c) and with (d) white light illumination with ruthenium complexes, 3-carboxy-proxyl and aniline added to the Tris-HCl/KCl (5 mM/35 mM).

References

[1] a) A. Matiukas, B. G. Mitrea, A. M. Pertsov, J. P. Wuskell, M. D. Wei, J. Watras, A. C. Millard, L. M. Loew, *American Journal of Physiology-Heart and Circulatory Physiology* **2006**, *290*, H2633-H2643; b) J. P. Wuskell, D. Boudreau, M. D. Wei, L. Jin, R. Engl, R. Chebolu, A. Bullen, K. D. Hoffacker, J. Kerimo, L. B. Cohen, M. R. Zochowski, L. M. Loew, *Journal of Neuroscience Methods* **2006**, *151*, 200-215.

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[3] K. O. Johansson, J. A. Lotoski, C. C. Tong, G. S. Hanan, *Chemical Communications* **2000**, 819-820.