Supporting information for:

Picolitre-scale Crystallization of Membrane Proteins

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Materials and Methods

Materials

Monoolein (356.5 g/mole, lot M239-JA12-O) was purchased from Nu Chek Prep (Elysian, MN). Texas Red® 1,2-dihexadecanoyl-sn-glycero-3-phosphatidylethanolamine triethylammonium salt (Texas Red-PE, 1381.85 g/mole, lot 45B1-7) was from Molecular Probes, Inc. (Eugene, OR). Salts and buffers were obtained from Sigma-Aldrich (St. Lois, MO, USA). Water (resistivity > 18 MΩ·cm) was purified by using a Milli-Q Water System (Millipore Corporation, Bedford, MA) consisting of a carbon filter cartridge, two ion exchange filter cartridges, an organic removal cartridge, and a terminal 0.22 μm filter.

Bacteriorhodopsin (bR, 20.8 mg protein/mL, lot 8A) was solubilized from purple membranes of *Halobacterium salinarum* in 1.5 %(w/v) octylglucoside, 25 mM sodium/potassium phosphate, pH 5.6 buffer using standard protocols (Dencher & Heyn, 1982). Outer membrane cobalamin transporter, BtuB, in 10 mM Tris, pH 8.0, 0.1 M NaCl, 0.1 %(w/v) LDAO buffer was obtained from Prof. W. Cramer (Purdue University) and was concentrated to 11.5 mg protein/mL. Both proteins were stored at -70 °C for several months before being used in crystallization trials.

Methods

Crystallization *In meso* crystallization trials were set up using a combination of protein dispersed in a lipid (monoolein)/aqueous buffer mix and precipitant solution. The former was prepared by thawing the protein solutions on ice and combining them with monoolein using a lipid mixing device, as described (Cheng *et al.*, 1998; Cherezov & Caffrey, 2003). The weight ratio of protein solution to lipid was 2/3. In the case of BtuB, this protocol generates a homogenous, transparent cubic mesophase. However, with bR which had a relatively high detergent concentration, a mixture of lamellar and cubic phases emerged upon mixing. The former reverts to the cubic phase upon incubation with precipitant. This type of behavior is not unusual (Misquitta & Caffrey, 2003).

Crystallization trials were set up in 96-well glass plates using an *in meso* crystallization robot, as described (Cherezov *et al.*, 2004). In the case of bR, dim red light was used during all manipulations. Volumes of mesophase, 20 nL and above, were dispensed with a 100 μL gas-tight syringe. For smaller volumes, a 10 μL syringe was employed. The dispensing system was designed to have a very low dead volume. Unrecoverable material, after dispensing the mesophase, remains in two 10 mm-long sections of 26 gauge needle (0.26 mm inner diameter); one is part of the coupler and the other is the dispensing needle, with a total volume of ~1 μL. Each bolus of mesophase was overlaid with 1 μL precipitant solution and the wells were sealed with a glass coverslip. Plates were stored in a 20 °C incubator and examined intermittently for crystal growth over a 5-week period using an Eclipse E400-POL microscope (Nikon Inc., Melville NY). Digital images were recorded with a Nikon CoolPix 950 camera (Nikon Inc., Melville NY) attached to the microscope. Crystal dimensions were quantified using an eyepiece with calibrated micrometer scale and the values reported have an associated error of ~3 μm for crystal sizes larger than 10 μm and ~1 μm for crystal sizes smaller than 10 μm.

The precipitant solutions used were chosen based on past success with the test proteins (Cherezov *et al.*, 2004; Misquitta *et al.*, 2004). They included 2.1 - 2.8 M sodium potassium phosphate pH 5.6 for bR and 0.2 - 0.4 M ammonium formate, 8 - 12 %(v/v) 2-methyl-2,4-pentanediol (MPD), 0.1 M 2-morpholinoethanesulfonic acid/NaOH (MES) pH 6.5 for BtuB.

Delivered Volume Characterization The reproducibility with which volumes of lipid/protein mesophase were delivered was assessed by fluorescence using a sample that had been doped with a small amount of the highly fluorescent lipid, Texas Red-PE. For this purpose, monoolein with 0.1 mole% Texas Red-PE, prepared gravimetrically, was homogenized by mechanical mixing in the molten state at 40 °C. Cubic phase was prepared using the lipid-mixing device, as described above, with water in place of the protein solution.

For fluorescence intensity measurements use was made of a microarray scanning device. Accordingly, different volumes of doped-cubic phase was delivered onto standard 75 mm x 25 mm microscope glass slides at room temperature using the *in meso* robot described above. For each volume, 70 replicate boluses were delivered in rows with a bolus separation of 1 mm. The distance between the tip of the dispensing syringe needle and the glass plate surface was adjusted in the range from 75 to 175 μm to optimize delivery.

Fluorescence intensity measurements were performed using an Affymetrix 428 scanner (excitation, 535 nm; emission, 575 nm; spatial resolution, 10 μm; gain 10 dB). Scanned images were saved as 16-bit Tiff files and processed using the Fit2D program (Hammersley, 1989) to generate plots of projected intensity versus distance along a row on the plate. Integrated peak areas were corrected for background between peaks and Excel was used to calculate averages and coefficients of variance (CV).

Actual dispensed volumes were calculated assuming that all material was delivered to the glass slide and by knowing the advance per step (independently calibrated and reported on in Cherezov et al., 2004) of the motorized pump driving the dispensing syringe plunger. Thus, the nominal value of 528 pL delivered volume was determined when the smallest advance per step of 3.175 μm was used in combination with a 10 μL gas-tight Hamilton microsyringe. The latter has a bore corresponding to 10 μL/60 mm or 167 pL/μm.

References

Cheng, A., Hummel, B., Qiu, H. & Caffrey, M. (1998). *Chem. Phys. Lip.* **95**, 11-21.

Cherezov, V. & Caffrey, M. (2003). *J. Appl. Cryst.* **36**, 1373-1377.

Cherezov, V., Peddi, A., Muthusubramaniam, L., Zheng, Y. F. & Caffrey, M. (2004). *Acta Cryst.* **D60**, 1795-1807.

Dencher, N. A. & Heyn, M. P. (1982). *Methods Enzymol.* **88**, 5-10.

Hammersley, A. P. (1989). *Syn. Rad. News* **2**, 24-26.

Misquitta, Y. & Caffrey, M. (2003). *Biophys. J.* **85**, 3084-3096.

Misquitta, L. V., Misquitta, Y., Cherezov, V., Slattery, O., Mohan, J. M., Hart, D., Zhalnina, M., Cramer, W. A. & Caffrey, M. (2004). *Structure* **12**, 2113-2124.

Table S1. Dependence of the average maximum BtuB crystal size (μm) on mesophase bolus volume and precipitant composition[a].

Precipitant		Bolus Volume (nL)		
Amm. Formate (M)	**MPD (%(v/v))**	**0.53**	**20**	**50**
0.2	**8**	3	ND[b]	15
	10	7	25	43
	12	12	ND	40
0.3	**10**	13	20	42
	12	12	6	8
0.4	**8**	5	19	7
	10	ND	46	14
	12	14	19	47

[a] Data recorded on day 37 post setup.
[b] ND, not detectable.

Table S2. Dependence of BtuB crystallization success rate (percentage)[a] on mesophase volume and precipitant composition[b].

Precipitant		Bolus Volume (nL)		
Amm. Formate (M)	**MPD (%(v/v))**	**0.53**	**20**	**50**
0.2	**8**	29 (7)	0 (2)	50 (2)
	10	50 (6)	25 (4)	33 (6)
	12	75 (4)	0 (2)	67 (3)
0.3	**10**	86 (7)	83 (6)	100 (5)
	12	100 (9)	80 (5)	100 (2)
0.4	**8**	100 (7)	80 (5)	100 (6)
	10	0 (2)	100 (5)	100 (6)
	12	100 (5)	100 (5)	100 (6)

[a] Success rate is shown as a percentage calculated as 100-times the number of wells with crystals divided by the total number of wells in the trial (shown in brackets).
[b] Data recorded on day 37 post setup.

Table S3. Dependence of the average dispensed mesophase volume on needle tip-to-plate distance.

Needle Tip-to-Plate	Bolus Volume (nL) [a]				
Distance (μm)	**0.53**	**1.06**	**1.58**	**2.1**	**4.75**
75	0.63 (67.1)				
87.5	0.47 (29.6)				
100	0.63 (62.1)	1.10 (29.0)	1.47 (21.7)		4.37 (13.1)
112.5	0.51 (43.4)				
125	0.51 (89.8)	1.01 (42.3)		1.94 (27.3)	3.98 (12.2)
137.5	0.56 (62.5)				
150	0.54 (77.2)	1.05 (61.0)			3.65 (12.7)

[a] Volumes are reported as averages with the CV, expressed as a percentage, in brackets.

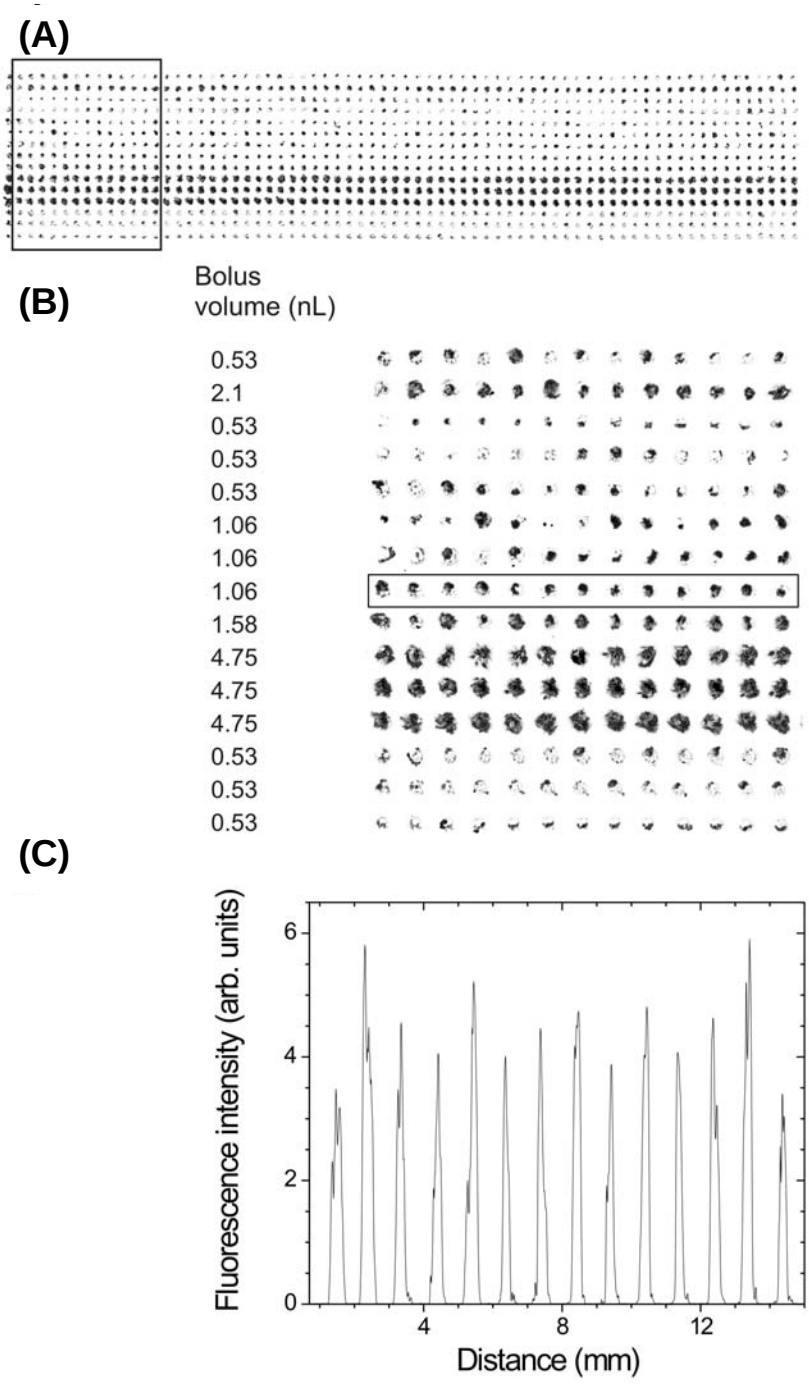

Fig. S1 Quantitative analysis of the lipidic cubic phase delivery. **(A)** Scanned image of fluorescence from a microscope glass slide (75 mm x 25 mm) with different volumes of the cubic phase. In-row bolus separation is 1 mm. There are 70 boluses in each row. An enlarged view of the boxed area in **(A)** is shown in **(B)**. A fluorescent scan along the boxed row in **(B)** is shown in **(C)**.

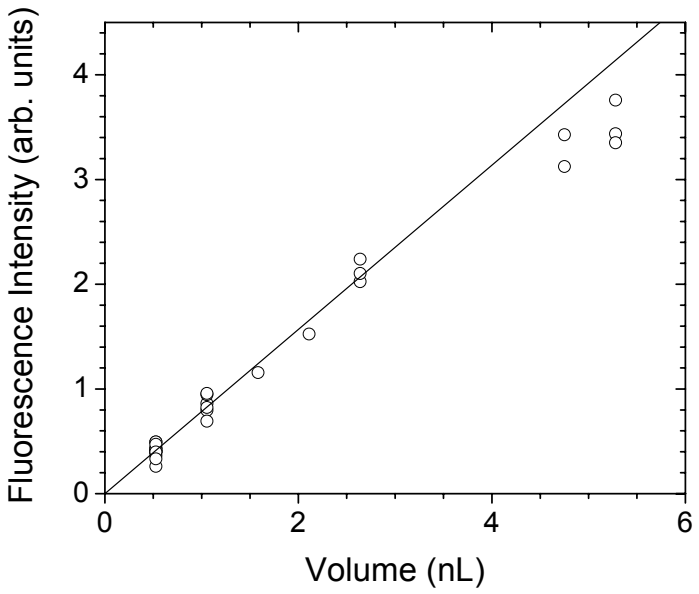

Fig. S2 Calibration plot relating fluorescence intensity to nominal volume of delivered lipidic cubic phase. Each point is the average of 70 replicate boluses. A linear best fit to the data below 3 nL is shown. Volumes ≥5 nL lie outside the linear range. Non-linearity is likely due to an inner filter effect and/or to scatter associated with these larger boluses.

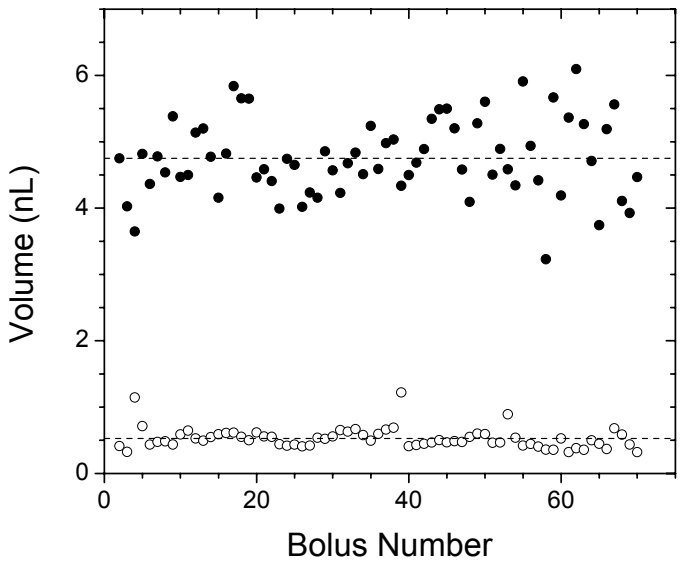

Fig. S3 Reproducibility of lipidic mesophase delivery. A total of 70 replicate 530 pL (open circles, CV = 29.6%) and 4.75 nL boluses (closed circles, CV = 12.2%)) were delivered to a glass plate and volume quantitation was by fluorescence intensity measurement. Average volumes are indicated by horizontal dotted lines. When outlier data points at bolus numbers 4, 39 and 53 are removed from the 530 pL data set the CV is 19.6%.