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Lipidic cubic phases as matrices for membrane protein crystallization

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Abstract

This review provides detailed procedures for the crystallization of membrane proteins via the lipidic cubic phase method. Bacteriorhodopsin-specific, hands-on protocols are given for (i) the preparation of bacteriorhodopsin from purple membrane by monomerization in octylglucoside and gel filtration chromatography or by selective extraction after pre-treatment with dodecyl-tri-methylammonium bromide, (ii) the incorporation of bacteriorhodopsin into lipidic cubic phases by mixing in vials or within coupled syringes and, (iii) the crystallization of bacteriorhodopsin in the lipidic matrix by adding a solid salt or an overlaying with a solution. References for further useful procedures and materials are listed in order to provide biochemists and crystallographers with all information that is necessary to grow crystals of the membrane protein bacteriorhodopsin.

1. Introduction

It is the purpose of this review to familiarize the experimental scientist with the practicalities involved in the use of lipidic cubic phases for membrane protein crystallization purposes. The detailed protocols given here enable the reader to readily generate crystals of the membrane protein bacteriorhodopsin. After mastering the crystallization of bacteriorhodopsin, further resources may be consulted, e.g., those listed in Table 1. It lists references that describe useful procedures and lipidic cubic phase related crystallization reports which may be instrumental in designing crystallization screens, handling of non-colored proteins, or the retrieval of crystals for X-ray diffraction purposes. It is hoped that the simple protocols in this review serve as a basis for benchmark crystallization experiments that embolden the crystallographer to experiment with more difficult proteins, in particular those that have not yielded crystals employing conventional crystallization methods.

The track record of the lipidic cubic phase crystallization method is summarized in Table 2. It has been particularly successful for membrane proteins with seven transmembrane α -helices (bacteriorhodopsin, halorhodopsin, sensory rhodopsin II, and sensory rhodopsin II with its transducer domain). It is therefore believed that heptahelical membrane proteins that are of non-bacterial origin, namely G-protein coupled receptors (GPCR), may be appropriate targets for this crystallization method. After its inception and description in 1996 [9], the crystallization of bacteriorhodopsin has been repeated in many laboratories (Table 2). The reproduction of well-diffracting bacteriorhodopsin crystals, the application of the method to other membrane proteins, and the continuing development of lipidic cubic phase-based crystallization technology demonstrate the value and future promise of this approach. Numerous original research reports and reviews have been published on the topic of lipidic cubic phase-based protein crystallization. For an introduction to this subject, consult Table 1.

2. Protocols: crystallization of bacteriorhodopsin in practice

In general, lipidic cubic phase-based crystallizations are often carried out as a two-step process. At first the protein solution is mixed with the lipid, and a lipidic cubic phase forms spontaneously. This first step is considered complete when the material is transparent, non-birefringent, and very viscous. Then the second step, crystallization, is started by the addition of a crystallant. The latter may be a solid salt mix or a solution. Depending on the precise conditions, crystals typically appear within days or weeks [1].

Bacteriorhodopsin is the major protein component in ‘purple membrane,’ a membrane fraction obtained from *Halobacterium salinarum*. Purple membrane preparations may be obtained from cultivated *H. salinarum* strain ET1001 or strain S9 [20]. Alternatively, they are available from several commercial sources, i.e., as lyophilized powders from Sigma (Product No. B0184) Table 3. A total of 10 mg of purple membrane should be sufficient for initial crystallization experiments. Purple membrane is fairly stable when exposed to daylight and room temperature. In contrast, detergent solubilized bacteriorhodopsin should be kept cold and in the dark whenever possible.

2.1. Bacteriorhodopsin preparation from purple membrane

2.1.1. Avoiding the detergent solubilization step—Bacteriorhodopsin crystals may be grown directly from purple membrane which is mixed with monoolein, without any pretreatment [14]. Though conceptually simple, the timeframe for this crystallization strategy, many months, is prohibitively long to be considered as a benchmark experiment.

2.1.2. Monomerization in β -octylglucoside and gel filtration chromatography—Purple membrane may be solubilized directly with β -octylglucoside. To obtain monomeric bacteriorhodopsin, a gel filtration chromatography step is required [6].

1. Resuspend 10 mg lyophilized purple membrane (Sigma–Aldrich, B0184) in 10 ml of 150 mM KCl and collect the purple membrane by centrifugation (30 min, 18,000 rpm JA-20, 4 °C).
2. Resuspend pellet in 6 ml of 25 mM NaH₂PO₄, pH 6.9, and add 2 ml of 10% (w/v) β -octylglucoside.
3. Sonicate in bath sonicator for 1 min in the dark at room temperature and incubate overnight without stirring.
4. Adjust pH to 5.5 with 0.1 N HCl.
5. Centrifuge for 45 min at 15 °C at 55,000 rpm (Ti 70 rotor) and discard the pellet.
6. Concentrate with Centriprep YM-50 filtration device to less than 2 ml.
7. Apply concentrate on a BioGel A 0.5 m column or a comparable preparative gel filtration column (e.g., TSK G3000SW, Tosoh Bioscience, Montgomeryville, PA, USA,) with 1.2% β -octylglucoside in 25 mM NaH₂PO₄, pH 5.5, as the stationary phase. Discard the first and collect the second peak.
8. Concentrate the pooled bacteriorhodopsin fraction with a Centriprep YM-50 filtration device. Adjust to a final concentration of 9 mg/ml in 25 mM NaH₂PO₄, pH 5.5.

2.1.3. Selective extraction after DTAB pretreatment—Alternatively, bacteriorhodopsin may be extracted from purple membranes which have been pretreated with dodecyltrimethylammonium bromide (DTAB), a cationic detergent that is frequently used in detergent solubilization experiments [8].

1. Resuspend 10 mg lyophilized purple membrane (Sigma–Aldrich, B0184) in 10 ml of 150 mM KCl and collect the purple membrane by centrifugation (30 min, 18,000 rpm JA-20, 4 °C).
2. Resuspend pellet in 10 ml of 10 mM DTAB, 150 mM Na/K acetate. Tip-sonicate for a total of 1 min, chill on ice between 20 s pulses.
3. Discard supernatant after centrifugation (1 h, 18,000 rpm JA-20, 4 °C) and resuspend purple pellet in 12 ml of 1.2% (w/v) β -octylglucoside, 25 mM NaH_2PO_4 , pH 6.9.
4. Sonicate again as above and adjust pH to 5.5 with 0.1 N HCl.
5. Clear by centrifugation (1 h, 18,000 rpm JA-20, 4 °C) and discard pellet. The supernatant contains monomerized, β -octylglucoside solubilized bacteriorhodopsin.
6. Concentrate bacteriorhodopsin solution with a Centriprep YM-50 filtration device (Millipore, Billerica, MA, USA). Adjust the final concentration to 9 mg/ml in 25 mM NaH_2PO_4 , pH 5.6.

The concentration of bacteriorhodopsin is usually determined spectrophotometrically. The extinction coefficient of β -octylglucoside solubilized bacteriorhodopsin at 560 nm is ca. 63,000 cm^2/mmol ($MW_{\text{bacteriorhodopsin}}$ 26.8 kDa). An absorbance ratio A_{280}/A_{560} of 1.5–2.0 is acceptable. The concentrations of β -octylglucoside and those of residual purple membrane lipids in bacteriorhodopsin preparations are substantial. They depend on the type of pretreatment and they typically vary from batch to batch. This variation may be the source of slightly different outcomes of crystallization experiments with respect to crystal growth kinetics, crystal size, and X-ray diffraction quality.

2.2. Crystallization setup: mix protein and lipid, add crystallant

In the first step of the crystallization experiment, the bacteriorhodopsin solution is mixed with the lipid to form a lipidic cubic phase (ca. 3.5 mg/ml bacteriorhodopsin, ca. 60% monoolein). This procedure may be carried out in a glass vial (Fig. 1) or in two coupled syringes [15] as shown in Fig. 2. Protocols are given below for both options. For best results the crystallization experiments should be carried out under dim light at room temperature. The crystallization materials should be kept in total darkness after preparation.

2.2.1. Step 1: Incorporation of bacteriorhodopsin into a lipidic cubic phase

1. Transfer 6 mg of dry monoolein (monooleoyl-*rac*-glycerol [C18:1c9]) into a vial (e.g., a PCR tube or a glass tube with an inner diameter of 3 mm).
 2. Add 4 μl of bacteriorhodopsin solution (9 mg/ml) from 2.1.2 or 2.1.3 to the lipid and seal the vial.
 3. Mix monoolein and bacteriorhodopsin solution by repeated centrifugation in a benchtop centrifuge. Rotate by 180° about the long axis between short runs to provide mixing. Continue until a homogeneous purple, transparent, non-birefringent, and gel-like material has formed.
1. Transfer 60 mg of dry monoolein (monooleoyl-*rac*-glycerol [C18:1c9]) into a 250 μl Hamilton syringe (gastight, RN).
 2. Add 40 μl of bacteriorhodopsin solution (9 mg/ml) from 1.2 or 1.3 into a second 250 μl Hamilton syringe (gastight, RN).
 3. Join both syringes via a coupler (Fig. 2).

4. Mix monoolein and bacteriorhodopsin solution by repeated transfer of the material from one syringe into the other. Continue until a homogeneous purple, transparent, non-birefringent, and gel-like material has formed.
5. Transfer all of the material into one syringe and attach a suitable dispenser, e.g., a manual ratchet dispenser with a needle (Fig. 2).
6. Dispense portions of 0.2 μl into a pre-filled well (see below) and cover with transparent tape.

2.2.2. Step 2: Crystal nucleation and growth—Bacteriorhodopsin nucleation and crystallization can be initiated by the addition of a mix of solid salt to the preformed lipidic cubic phase. Since weighing of very small amounts of salt is cumbersome, solutions have been formulated that can likewise trigger the crystallization process [18]. Typically such solutions are used for multi-well experiments, whereas salts are used primarily for ‘large-scale’ crystallizations in vials.

1. Prepare Sørensen salt mix by grinding 94.8 g KH_2PO_4 and 5.2 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$.
 2. Remove seal from vial (containing 10 mg of lipidic cubic phase, from 2.2.1.1) and place on balance.
 3. Add 2–3 mg of Sørensen phosphate. Seal vial.
 4. Centrifuge once in benchtop centrifuge until salt crystals combine in bottom of vial (Fig. 1A).
1. Prepare the crystallization solution, 27% (w/v) PEG 2000 in 100 mM Sørensen phosphate buffer, pH 5.6.
 2. Fill 1 μl of the crystallization solution into a single well and cover it with transparent tape. To this well-based setup, a portion of lipidic cubic phase is added according to Section 2.2.1.2. See Figs. 2 and 3.

2.3. Observation of bacteriorhodopsin crystallization setups

Bacteriorhodopsin crystallization setups should be stored in darkness at constant temperature (e.g., room temperature). Often crystals can be detected within a few days after setup. It is simple to spot bacteriorhodopsin crystals in setups because the crystals are colored purple (Figs. 1 and 3). Although large crystals can be seen by the naked eye, it is advisable to inspect crystallization setups with a dissecting stereo microscope. When investigating crystallization setups, care must be taken to keep the temperature constant, i.e., prevent warming the setups during manipulation and by microscope light. Otherwise liquid bubbles form inside the lipidic cubic phase [16,17]. These are detrimental to observation and disrupt the crystallization process.

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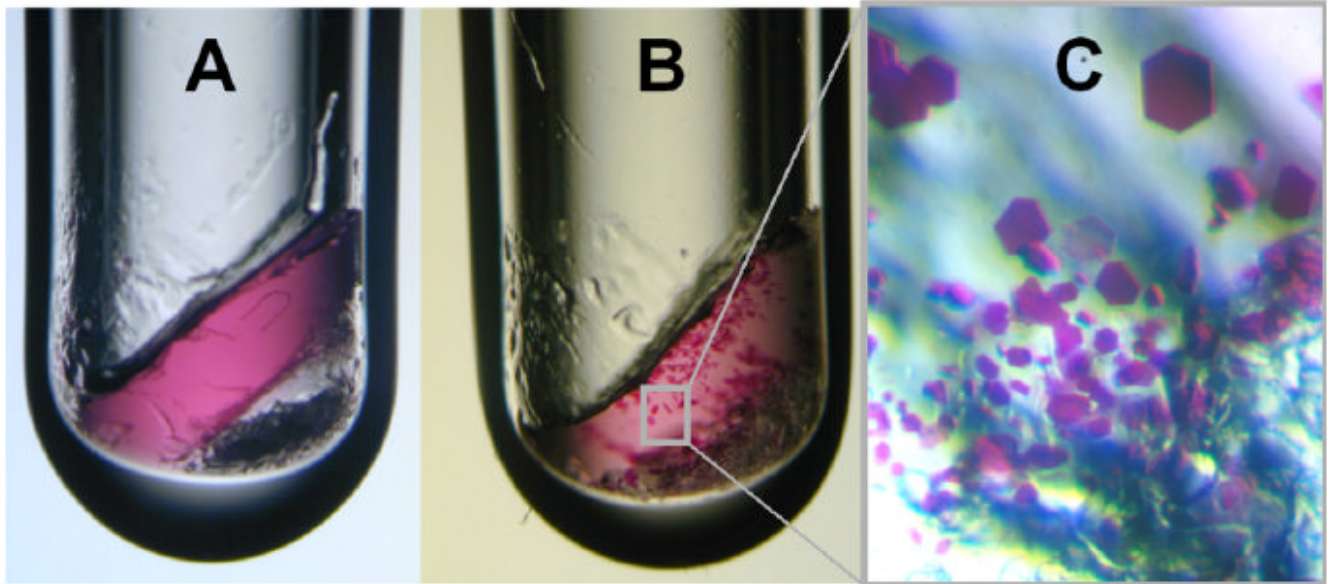


Fig. 1. Growth of bacteriorhodopsin crystals within a monoolein-based lipidic cubic phase matrix in a glass vial. (A) The 10 μ l crystallization experiment was set up in a glass vial as lined out in 2.2.1.1. At first the purple bacteriorhodopsin is distributed homogeneously in the lipidic cubic phase. The bottom of the 3 mm diameter tube is filled with solid Sørensen salt. (B) Bacteriorhodopsin microcrystals formed within 1 month. (C) Schematic close-up of purple bacteriorhodopsin crystals with sizes up to ca. 100 μ m along their longest dimension.

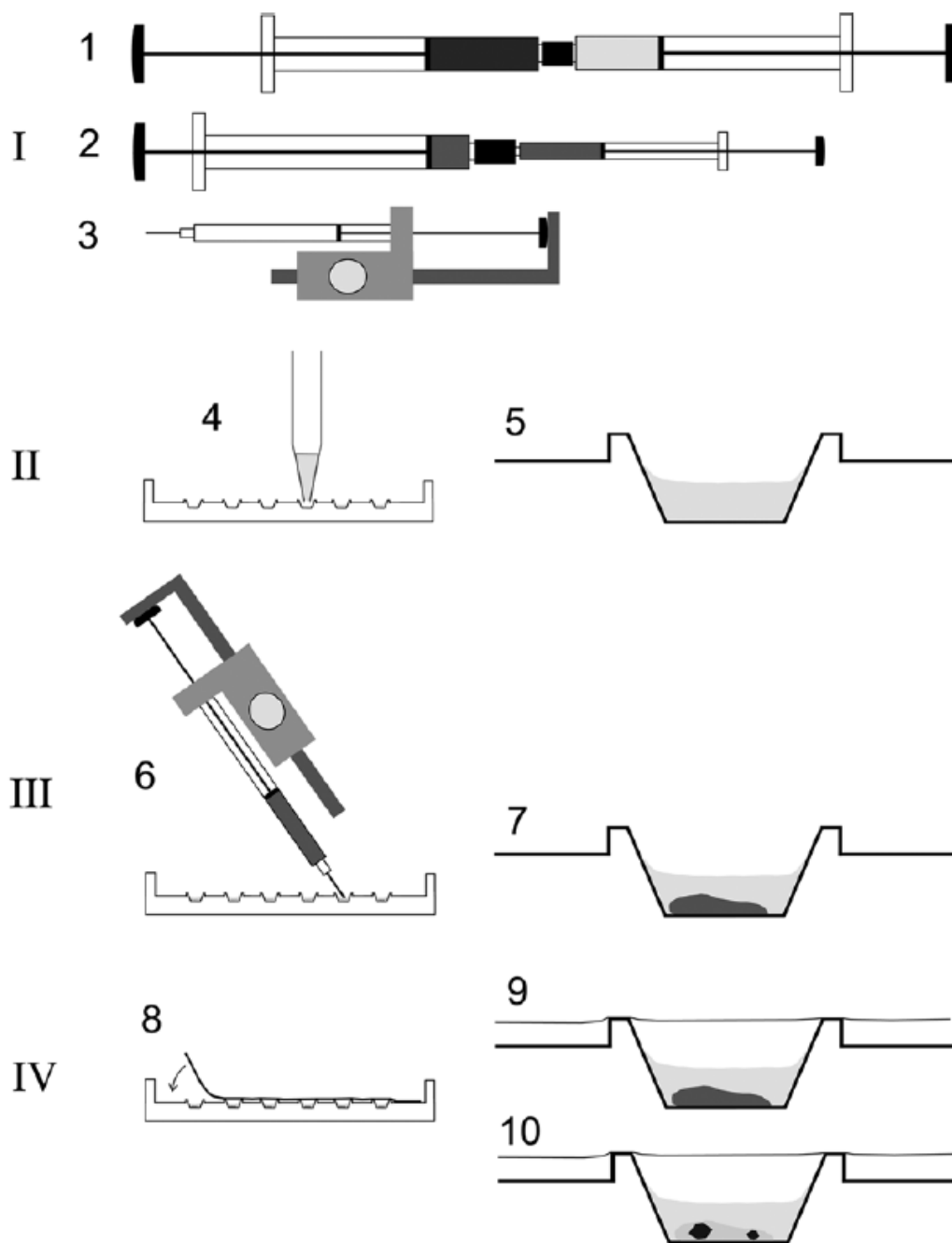


Fig. 2. Schematic procedure for the rapid preparation of micro crystallization experiments using the cubic phase method. Crystallization setups are prepared using syringes in combination with a semi-automatic dispenser-driven micro syringe and Terasaki plates. The procedure involves four basic steps: [I] Preparation of the proper lipidic cubic phase using two 250 μl gas-tight syringes coupled together as described [2] (1). Typically, the cubic phase consists of ca. 60% monoolein, ca. 40% water and protein—i.e., bacteriorhodopsin—at a concentration of ca. 3.5 mg/ml in 20 mM Sørensen phosphate buffer, pH 5.6. For dispensing into microwells, the material is transferred into a microsyringe with 10 μl total volume either directly into the syringe barrel or via an appropriate adapter (2). This microsyringe is assembled into a semi-

automatic dispenser (3) which allows dispensation in 50 steps of ca. 0.2 μl each. [II] Prior to dispensation of the lipid material, six microwells in one lane of a 72-well Terasaki plate are filled with 1 μl of the precipitant solutions (4) and dried optionally at 60 $^{\circ}\text{C}$ for 30 min. The latter resembles crystallization of bacteriorhodopsin using dry salt as the precipitation agent as described previously [9,11,23]. A single well is shown on the right (5). [III] Ca. 0.2 μl of the preformed lipidic material is added to each microwell by positioning the shortened syringe needle at an angle over the well bottom and by triggering the semi-automatic dispenser (6). The average weight of a single shot of 60% (w/v) monoolein containing cubic phase using the dispenser was determined to be $222 \pm 10.3 \mu\text{g}$. The cubic phase is stable in an excess of an overlaying liquid (7). [IV] The microwells are sealed with a clear transparent tape (8) and the plates may be stored (9) at different temperatures. Inspection of crystals (10) with a microscope can be carried out without disturbance from both sides simply by flipping the entire plate.

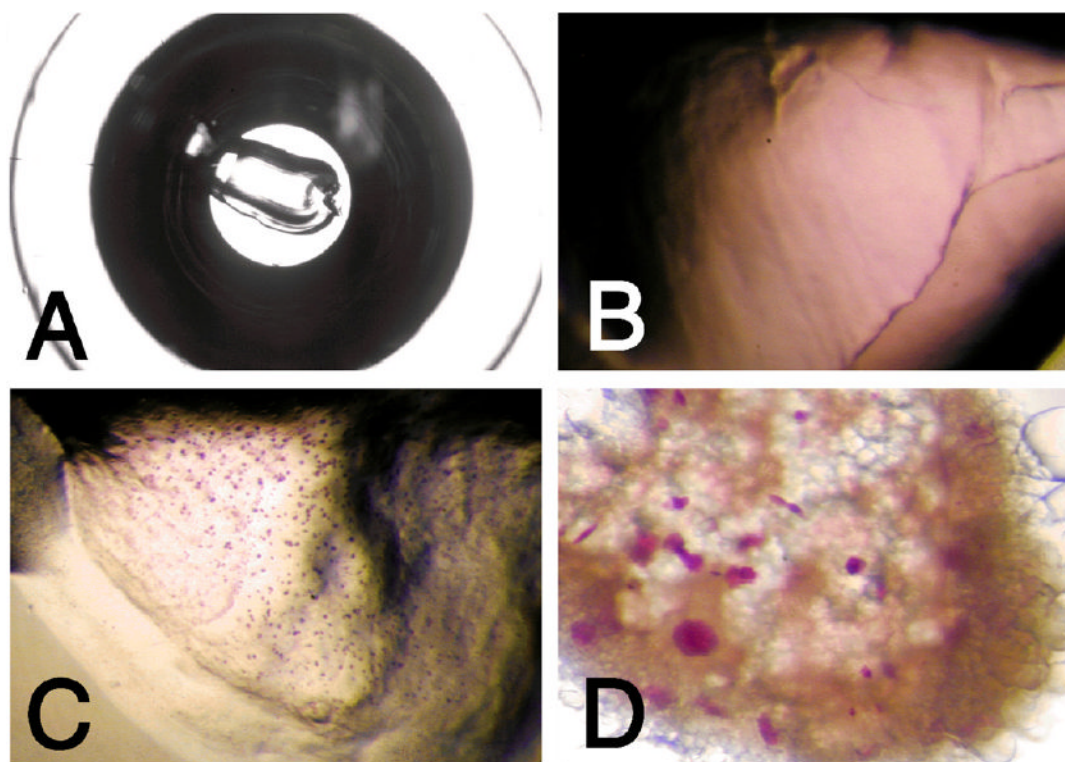


Fig. 3.

Growth of bacteriorhodopsin crystals using the syringe and microwell-based crystallization procedure. Shown are photographs of cubic phase crystallization setups in a microwell of a Terasaki plate. (A) Overview of well containing 0.2 μ l cubic phase dispensed with manual syringe dispenser. Well bottom diameter is ca. 1.2 mm. (B) Close-up view of crystallization setup prior to crystallization. The purple bacteriorhodopsin is homogeneously distributed in the lipidic cubic phase. (C) Bacteriorhodopsin microcrystals formed in 0.2 μ l of cubic phase when solid salt was used as a crystallization-inducing agent. (D) Purple bacteriorhodopsin crystals with sizes up to ca. 75 μ m along the longest dimension were grown by adding 0.2 μ l of initial monoolein cubic phase containing 3.5 mg/ml bacteriorhodopsin to 1 μ l of 27% PEG 2000 in 100 mM Sørensen phosphate buffer, pH 5.6.

Table 1

Useful methods and procedures have been developed to aid in various aspects of the lipidic cubic phase crystallization methodology

Topic	Description	Reference
Lipidic cubic phase based crystallization	Pioneering bacteriorhodopsin and lysozyme crystallization report, glass vials are employed	[9,10]
	Monoolein as matrix for crystallization of five different membrane proteins	[3]
	Review of the vial-based technique	[21]
Micro crystallization	Syringe and manual ratchet dispenser are employed, 200 nl volume; use of liquid instead of solid salt as crystallization inducing agent	[15]
	Reproduction of above procedure	[22]
	Refinement of above procedure and further reduction of volume	[5]
Observation of crystallization setups by microscopy	Observation options and guide to interpretation of crystallization results	[16]
	Demonstration that Hoffman modulation contrast and polarization microscopy are useful for the detection of small, colorless protein crystals	[17]
Crystal harvesting	Detergent mediated release	[12]
	Enzymatic release	[13]
	Overview with protocols similar to the ones given here	[18]
Cubic phase forming lipids	Lists of lipids and lipid mixes that support bacteriorhodopsin crystallization are given in Appendix A of the review	[11]
	Crystallization in absence of any detergent	[14]
Screening conditions	A list with compatible salts and solutions is given	[18]
	The destabilization effect of a set of crystallization solutions is investigated	[4]
	Boundaries on protein size and crystallization conditions are estimated from a crystallization model	[7]
	Temperature-dependent phase behaviour of monoolein-detergent mixes	[24]
	Lyo- and thermotropic monoolein phase diagram	[1]

Table 2

Track record of the lipidic cubic phase-assisted protein crystallization method

Protein	Resolution (Å)	PDB accession code or reference
Bacteriorhodopsin	3.7	[9]
Bacteriorhodopsin	2.35	1AP9
Bacteriorhodopsin	2.3	1BRX
Bacteriorhodopsin	1.55	1C3W
Bacteriorhodopsin D96M	2.0	1C8S
Bacteriorhodopsin, K-state	2.5	1DZE
Bacteriorhodopsin, K-state	2.6	1IXF
Bacteriorhodopsin, L-state	2.1	1EOP
Bacteriorhodopsin, L-state	2.3	1R3P
Bacteriorhodopsin E204Q	1.8	1F4Z
Bacteriorhodopsin E204Q	1.7	1F50
Bacteriorhodopsin	2.3	1IW6
Bacteriorhodopsin D85S/F219L	2.0	1JV6
Bacteriorhodopsin early M state	2.0	1KG8
Bacteriorhodopsin D85S O state	2.25	1JV7
Bacteriorhodopsin mock early M	1.81	1KG9
Bacteriorhodopsin	1.65	1KGB
Bacteriorhodopsin, K-state	1.43	1MOK
Bacteriorhodopsin	1.47	1MOL
Bacteriorhodopsin M-1	1.43	1MOM
Bacteriorhodopsin	1.9	1QHJ
Bacteriorhodopsin early state	2.1	1QKO
Bacteriorhodopsin early state	2.1	1QKP
Sensory rhodopsin II	2.4	1JGJ
Sensory rhodopsin II K state	2.27	1GU8
Sensory rhodopsin II K state	2.27	1GUE
Sensory rhodopsin II	2.1	1H68
Sensory rhodopsin II	2.4	1JGJ
SRII with transducer complex	1.93	1H2S
Halorhodopsin	1.8	1E12
Halorhodopsin	3.2	[3]
Photosynthetic reaction centre <i>Rhodospseudomonas viridis</i>	3.7	[3]
Photosynthetic reaction centre <i>Rhodobacter Sphaeroides</i>	2.35	1OGV
Photosynthetic reaction centre <i>Rhodobacter Sphaeroides</i>	ca. 6	[3]
Light harvesting complex 2, <i>Rp. acidophila</i>	ca. 25	[3]
Nicotinic acetylcholine receptor		[19]

Table 3

Materials that are used in the protocols for the preparation of bacteriorhodopsin from purple membrane and for the crystallization of bacteriorhodopsin in a monoolein lipidic cubic phase

Purpose	Item	Order information
Microcrystallization	250 μ l gas-tight syringe	Model 1725, Hamilton, Reno, NV, USA
	10 μ l microsyringe	Model 701-26s, Hamilton, Reno, NV, USA
	Semi-automatic ratchet dispenser	Model PB600, Hamilton, Reno, NV, USA
	Terasaki plate	Nunc, Rochester, NY, USA
	Clear transparent tape	Crystal Clear, Manco, USA
Cubic phase forming lipid	Monoolein, 1-monooleoyl- <i>rac</i> glycerol	NuCheck, MN, USA or Sigma-Aldrich, USA
Source of bacteriorhodopsin	Lyophilized purple membrane from <i>S9 Halobacterium salinarum</i>	Product No. B0184, Sigma-Aldrich, USA
Detergent to solubilize bacteriorhodopsin	β -Octylglucoside, <i>n</i> -octyl- β -D-glucopyranoside	No. O311 Anatrace, OH, USA
Cationic detergent for purple membrane pretreatment	DTAB, dodecyl-trimethylammonium bromide	Aldrich Chem. Metuchen, NJ, USA
Filtration device	Centriprep YM-50	Millipore, Billerica, MA, USA