Video Article

Crystallization of Membrane Proteins in Lipidic Mesophases

Wei Liu[1], Vadim Cherezov[1]

[1]Molecular Biology, The Scripps Research Institute

Correspondence to: Vadim Cherezov at vcherezo@scripps.edu

URL: https://www.jove.com/video/2501
DOI: doi:10.3791/2501

Keywords: Structural Biology, Issue 49, membrane protein, lipidic cubic phase, crystallization, Fluorescence recovery after photobleaching (FRAP), G protein-coupled receptors

Date Published: 3/28/2011

Citation: Liu, W., Cherezov, V. Crystallization of Membrane Proteins in Lipidic Mesophases. *J. Vis. Exp.* (49), e2501, doi:10.3791/2501 (2011).

Abstract

Membrane proteins perform critical functions in living cells related to signal transduction, transport and energy transformations, and, as such, are implicated in a multitude of malfunctions and diseases. However, a structural and functional understanding of membrane proteins is strongly lagging behind that of their soluble partners, mainly, due to difficulties associated with their solubilization and generation of diffraction quality crystals. Crystallization in lipidic mesophases (also known as *in meso* or LCP crystallization) is a promising technique which was successfully applied to obtain high resolution structures of microbial rhodopsins, photosynthetic proteins, outer membrane beta barrels and G protein-coupled receptors. In meso crystallization takes advantage of a native-like membrane environment and typically produces crystals with lower solvent content and better ordering as compared to traditional crystallization from detergent solutions. The method is not difficult, but requires an understanding of lipid phase behavior and practice in handling viscous mesophase materials. Here we demonstrate a simple and efficient way of making LCP and reconstituting a membrane protein in the lipid bilayer of LCP using a syringe mixer, followed by dispensing nanoliter portions of LCP into an assay or crystallization plate, conducting pre-crystallization assays and harvesting crystals from the LCP matrix. These protocols provide a basic guide for approaching *in meso* crystallization trials; however, as with any crystallization experiment, extensive screening and optimization are required, and a successful outcome is not necessarily guaranteed.

Video Link

The video component of this article can be found at https://www.jove.com/video/2501/

Protocol

A typical outline of an *in meso* crystallization experiment is shown in Fig.1[1,2]. Pre-crystallization LCP-FRAP assays are optional; however, they can significantly accelerate the process of searching for initial crystallization conditions, especially in the case of difficult membrane proteins[3].

1. Protein Reconstitution in LCP

1. Purify a membrane protein of interest in a detergent solution and concentrate the protein/detergent complexes to ~10 - 20 mg/mL, taking care not to over-concentrate the detergent[1,4].
2. Transfer ~25 mg of an LCP host lipid (typically monoolein) or a lipid mixture into a 1.5 mL plastic tube and incubate at 40 °C for few minutes until the lipid melts.
3. Attach a syringe coupler to a 100 µL gas-tight syringe.
4. Load the syringe with the molten lipid using an adjustable volume pipette. Record the volume of the lipid in the syringe.
5. Load another 100 µL syringe with the protein solution at a protein solution-to-lipid ratio 2/3 v/v.
6. Connect both syringes together through the syringe coupler.
7. Push the syringe plungers alternately to move the lipid and protein through the inner needle of the coupler, back and forth, until the lipid mesophase becomes homogeneous. LCP forms spontaneously upon mechanical mixing, and the protein becomes reconstituted in the lipid bilayer of LCP. Formation of LCP can be verified by its transparent and gel-like consistency and by the absence of birefringency when viewed under a microscope equipped with cross-polarizers, or, if possible, by using small-angle X-ray diffraction[1].

2. LCP-FRAP Pre-crystallization Assays

LCP-FRAP assays are designed to measure the diffusion properties of membrane proteins reconstituted in LCP at a variety of screening conditions[3]. The long-range diffusion of membrane proteins in LCP is essential for successful crystallization; however, the microstructure of LCP constrains diffusion of large proteins or oligomeric protein aggregates. A common reason for failure of an *in meso* crystallization experiment is a fast protein aggregation leading to a loss of diffusion. It has been shown that the aggregation behavior of a protein depends on the particular protein construct, the host lipid and the composition of the screening solution[3].

1. Label the protein with a fluorescent dye (Cy3 or similar) at a protein/dye ratio of ~100/1, remove the unreacted dye and concentrate the protein to ~ 1 mg/mL. Label either free amines or free thiols. When labeling free amines, use pH between 7 and 7.5 to predominately label the free N-terminus. Be aware that amino labeling can also label lipids co-purified with the protein[2,3].
2. Reconstitute the labeled protein in LCP as described in section 1).
3. Set up assay plates as described in section 3) using LCP-FRAP screening solutions instead of crystallization screens[2].
4. Incubate the plates at 20 °C in the dark for at least 12 hours to achieve an equilibrium state.
5. Place one of the plates on the LCP-FRAP station and focus on the first well using a 10x objective.
6. Acquire 5 fluorescent images to capture the initial pre-bleached state.
7. Trigger the laser. The laser power and number of pulses should be adjusted to bleach ~30 - 70% of the labeled protein in the middle of the bleached spot.
8. Immediately after triggering the laser, start recording a fast post-bleaching sequence of ~200 images at the fastest possible rate.
9. Follow with recording of a slow post-bleach sequence of ~50 images, selecting the delay between images as 1-20 s, depending on the diffusion rate of the protein.
10. Integrate the intensity inside the bleach spot in all frames and correct it for bleaching and light intensity fluctuations during the acquisition by dividing the intensity inside the bleached spot by the averaged intensity of a reference spot outside of the laser bleached area.
11. Normalize the corrected intensity to make the pre-bleached intensity equal to 1 and the initial bleached intensity equal to 0.
12. Fit the curve of the normalized intensity vs. time, *F(t)*, using the following equation[5]:
    $F(t) = M \times \exp(-2T/t) \times (I_0(2T/t) + I_1(2T/t))$, (Eq.1)
    where M is the mobile fraction of diffusing molecules, T is the characteristic diffusion time, *t* is the real time of each recorded frame, I_0 and I_1 are the 0^{th} and 1^{st} order modified Bessel functions.
13. Calculate the diffusion coefficient, D, as:
    $D = R^2/4T$, (Eq.2)
    where R is the radius of the bleached spot.
14. Move to the next well and repeat steps 2.5) - 2.13).
15. Compare the mobile fractions and diffusion coefficients obtained for the different screening conditions. Design new crystallization screens based on the components that facilitated protein diffusion and excluding conditions for which protein diffusion was not observed. If the protein did not diffuse in any of the screened conditions, consider broadening the screening space or trying a new protein construct.

3. Setting Up LCP Crystallization Trials

1. Reconstitute the protein in LCP as described in section 1).
2. Transfer the protein-laden LCP into a 10 µL gas-tight syringe attached to a repetitive syringe dispenser.
3. Attach a short removable needle (gauge 26, 10 mm length) to the 10 µL syringe.
4. Dispense 200 nL boluses of LCP on the surface of four adjacent wells forming a 2x2 square.
5. Overlay each of the LCP boluses with 1 µL of corresponding crystallization screen solution.
6. Cap four loaded wells with an 18 mm square glass coverslip. Apply a gentle pressure on the coverslip to seal the wells.
7. Repeat steps 3.4)-3.6) with the next set of 4 wells until the whole plate is filled.
8. Incubate the plate at a constant temperature, periodically checking for crystal formation and growth.

4. Harvesting Crystals from LCP

1. Place a plate with protein crystals under a stereo microscope with variable zoom, equipped with a linear rotating polarizer and analyzer.
2. Focus on the well of interest using a low power zoom so that the whole well is placed within the field of view.
3. Score the coverslip glass in four strokes making a square inside the well boundaries using a sharp corner of a ceramic capillary cutting stone.
4. Press around the scored perimeter with strong sharp-point tweezers to propagate the scratches through the thickness of the coverslip glass.
5. Punch two small holes at opposite corners of the scored square.
6. Inject few µL of precipitant solution through one of the holes to reduce dehydration during the subsequent steps.
7. Using an angled sharp needle probe break up the glass along one or two sides to free the cut-out square.
8. Carefully lift up the glass square watching for the cubic phase bolus. If the bolus is stuck to the coverslip, then flip the glass square over and place on the bottom of the well.
9. Add an extra few µL of precipitant solution, supplemented with a cryo-protectant, if necessary, on top of the exposed cubic phase bolus in the well.
10. Increase magnification of the microscope and focus on a crystal.
11. Adjust the angle between the polarizer and the analyzer to increase the contrast between the birefringent crystal and the background, while keeping enough light to see the harvesting loop.
12. Select a MiTeGen MicroMount with a diameter matching the crystal size and then harvest the crystal directly from the LCP by scooping it into the MicroMount.
13. Flash freeze the MicroMount with the harvested crystal in liquid nitrogen, and ship it to a synchrotron source beamline for X-ray data collection[6].

5. Representative Results:

An engineered human beta 2 adrenergic G protein-coupled receptor (β_2AR-T4L) was expressed in baculovirus infected sf9 insect cells and purified in dodecylmaltoside (DDM)/ cholesteryl hemisuccinate (CHS) detergent solution bound to a partial inverse agonist carazolol[7]. The protein was labeled with Cy3 NHS ester and used in LCP-FRAP pre-crystallization assays (Figure 2). Coarse grid screens based on several conditions selected from the results of LCP-FRAP assays produced initial crystal-like hits (Figure 3). Further optimization of precipitant conditions yielded diffraction quality crystals (Figure 4).

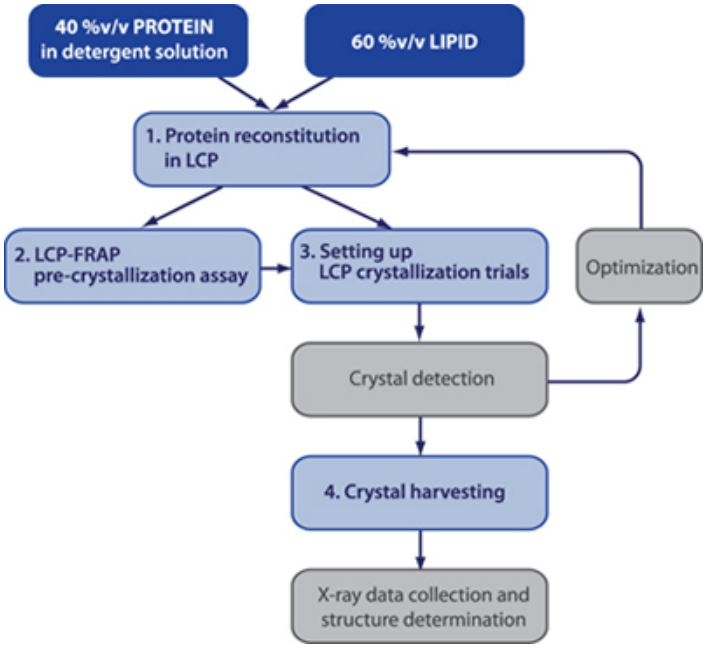

Figure 1. Flow-chart of a typical LCP crystallization experiment. Steps in the gray boxes are not described in the current protocols.

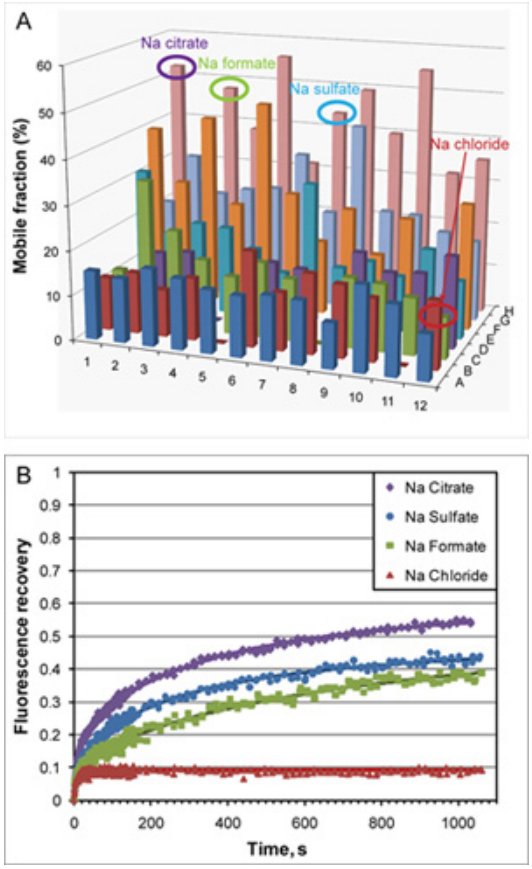

Figure 2. LCP-FRAP assay with β_2AR-T4L/carazolol in monoolein based LCP. A) Results of an LCP-FRAP assay performed in an automatic high-throughput mode, in which each sample of a 96-well plate is bleached sequentially and fluorescence recovery is measured after a 30 min incubation. The obtained fluorescence recoveries, which represent the mobile fraction in each sample, are plotted for all 96 samples. The screening solutions contain 0.1 M Tris pH 8, 30 % v/v PEG 400 combined with 48 different salts at two different concentrations. B) Fluorescence recovery profiles for several representative conditions. Solid line curves represent fits by Eq. 1.The mobile fractions and the diffusion coefficients are determined using Eqs. 1 and 2. Fast recovery of less than 10% in the sample containing Na chloride is due to fluorescently labeled lipids co-purified with the protein.

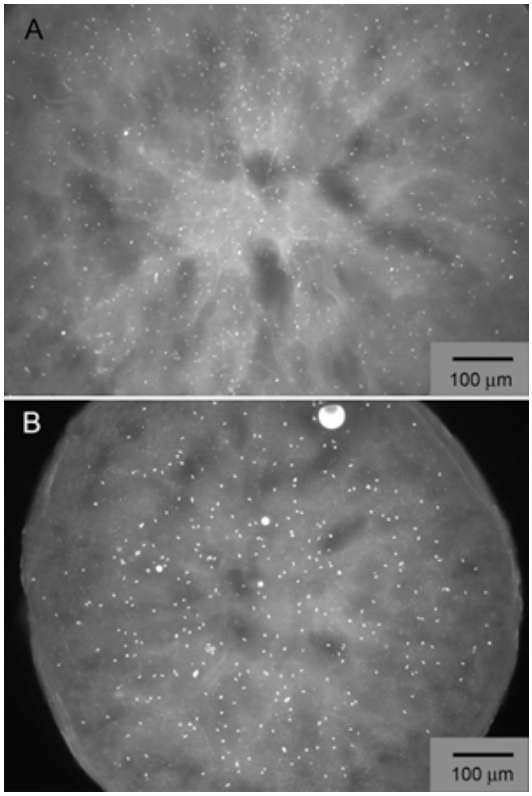

Figure 3. Initial crystal hits of β_2AR-T4L/carazolol obtained by a coarse grid screening around most promising conditions identified by LCP-FRAP, containing Na sulfate (panel A) and Na Formate (panel B). The protein is labeled with Cy3 NHS ester and the fluorescent images are taken using excitation at 543 nm and emission at 605 nm.

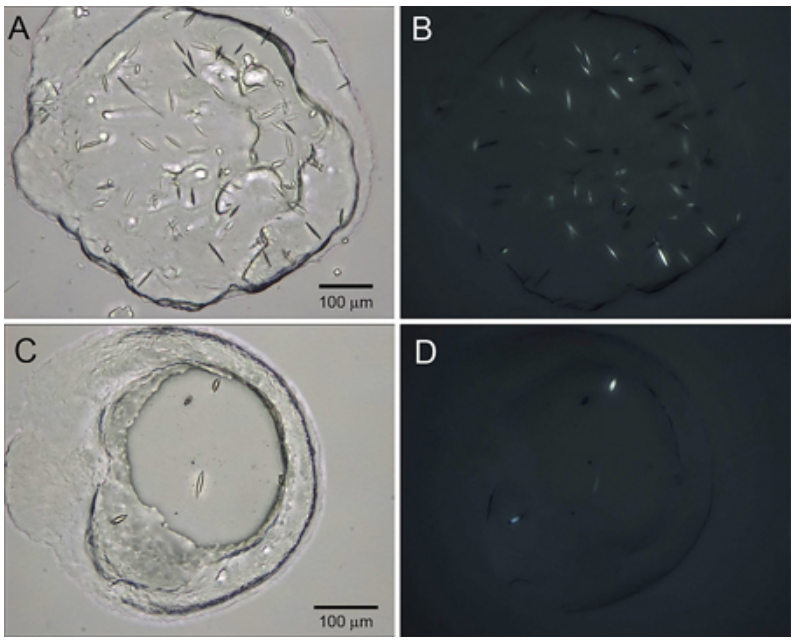

Figure 4. Optimized crystals of β_2AR-T4L/carazolol. The images of crystals grown in the presence of Na sulfate (panels A and B) and K Formate (panels C and D) are taken in the brightfield mode (panels A and C) and using cross-polarizers (panels B and D).

Discussion

The protocols provide a basic visual guidance for the main steps involved in performing *in meso* crystallization experiments. More in-depth details related to these protocols, emphasizing possible pitfalls, shortcomings or alternative routes are available elsewhere[1,2]. Optional LCP-FRAP assays can help at the earlier stages to select the most promising protein construct, LCP host lipid and lipid additives, as well as limit the range of possible precipitants and buffer conditions[3]. Once an initial crystallization hit is found, it should be optimized to obtain better quality

crystals. Optimization of *in meso* crystallization conditions is essentially similar to optimizing conditions for soluble proteins with the addition of extra parameters associated with the composition of LCP[1]. Membrane protein crystals grown in lipidic mesophase are typically smaller in size than crystals obtained in detergent solution, but more ordered, thus, benefitting strongly from using microfocus beamlines available at modern synchrotron sources[6].

Many of the procedures related to *in meso* crystallization, including setting up crystallization or assay plates, conducting LCP-FRAP assays and detecting crystals, have been semi- or fully automated[1,2,8,9], allowing screening of a large range of conditions while consuming small amounts of protein and lipid. On the other hand, protein reconstitutions in LCP and crystal harvesting remain manual and more tedious operations and, thus, have a need for improvement.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was funded in parts by the NIH grants GM073197 and RR025336.

References

1. Caffrey, M. & Cherezov, V. Crystallizing membrane proteins using lipidic mesophases. Nat. Protoc. 4, 706-731 (2009).
2. Cherezov, V., Abola, E. & Stevens, R.C. Recent progress in the structure determination of GPCRs, a membrane protein family with high potential as pharmaceutical targets. Methods Mol. Biol. 654, 141-170 (2010).
3. Cherezov, V., Liu, J., Hanson, M.A., Griffith, M.T. & Stevens, R.C. LCP-FRAP assay for pre-screening membrane proteins for *in meso* crystallization. J. Cryst. Growth Design 8, 4307-4315 (2008).
4. Misquitta, Y. & Caffrey, M. Detergents destabilize the cubic phase of monoolein: implications for membrane protein crystallization. Biophys. J. 79, 394-405 (2003).
5. Soumpasis, D.M. Theoretical analysis of fluorescence photobleaching recovery experiments. Biophys. J. 41, 95-97 (1983).
6. Cherezov, V., Hanson, M.A., Griffith, M.T., Hilgart, M.C., Sanishvili, R., Nagarajan, V., Stepanov, S., Fischetti, R.F., Kuhn, P. & Stevens, R.C. Rastering strategy for screening and centering of microcrystal samples of human membrane proteins with a sub-10 micrometer size X-ray synchrotron beam. J. R. Soc. Interface 6, Suppl. 5, S587-S597 (2009).
7. Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Kobilka, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Kobilka, B.K. & Stevens, R.C. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318, 1258-1265 (2007).
8. Cherezov, V., Peddi, A., Muthusubramaniam, L., Zheng, Y.F. & Caffrey, M. A robotic system for crystallizing membrane and soluble proteins in lipidic mesophases. Acta Crystallogr. D 60, 1795-1807 (2004).
9. Kissick, D.J., Gualtieri, E.J., Simpson, G.J. & Cherezov, V. Nonlinear optical imaging of integral membrane protein crystals in lipidic mesophases. Anal. Chem. 82, 491-497 (2010).
10. Cheng, A., Hummel, B., Qiu, H., & Caffrey, M. A simple mechanical mixer for small viscous lipid-containing samples. Chem. Phys. Lipids 95, 11-21 (1998).
11. Cherezov, V. & Caffrey, M. A simple and inexpensive nanoliter-volume dispenser for highly viscous materials used in membrane protein crystallization. J. Appl. Cryst. 38, 398-400 (2005).
12. Cherezov, V. & Caffrey, M. Nano-volume plates with excellent optical properties for fast, inexpensive crystallization screening of membrane proteins. J. Appl. Cryst. 36, 1372-1377 (2003).
13. Cherezov, V., Fersi, H. & Caffrey, M. Crystallization screens: compatibility with the lipidic cubic phase for *in meso* crystallization of membrane proteins. Biophys J. 81, 225-242 (2001).