Published in final edited form as:

Methods Mol Biol. 2010 ; 654: 141–168. doi:10.1007/978-1-60761-762-4_8.

Toward drug design: recent progress in the structure determination of GPCRs, a membrane protein family with high potential as pharmaceutical targets

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Summary

G protein-coupled receptors (GPCRs) constitute a highly diverse and ubiquitous family of integral membrane proteins, transmitting signals inside the cells in response to an assortment of disparate extracellular stimuli. Their strategic location on the cell surface and their involvement in crucial cellular and physiological processes turn these receptors into highly important pharmaceutical targets. Recent technological developments aimed at stabilization and crystallization of these receptors have led to significant breakthroughs in GPCR structure determination efforts. One of the successful approaches involved receptor stabilization with the help of a fusion partner combined with crystallization in lipidic cubic phase (LCP). The success of using LCP matrix for crystallization is generally attributed to the creation of a more native, membrane-like stabilizing environment for GPCRs just prior to nucleation and to the formation of type I crystal lattices, thus, generating highly ordered and strongly diffracting crystals. Here we describe protocols for reconstituting purified GPCRs in LCP, performing pre-crystallization assays, setting up crystallization trials in manual mode, detecting crystallization hits, optimizing crystallization conditions, harvesting, and collecting crystallographic data. The protocols provide a sensible framework for approaching crystallization of stabilized GPCRs in LCP, however, as in any crystallization experiment extensive screening and optimization of crystallization conditions as well as optimization of protein construct and purification steps are required. The process remains risky and these protocols do not necessarily guarantee success.

Keywords

GPCR; lipidic cubic phase; membrane proteins; fluorescence recovery after photobleaching; crystallization; crystallography; minibeam

1. Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of integral membrane proteins in the human genome, with ~800 GPCR family members identified (1). These receptors communicate signals across the plasma membrane in response to a variety of extracellular stimuli, ranging from photons, ions and small molecules to peptides and proteins. The signals are amplified and transmitted to downstream effectors inside the cells primarily through coupling to heterotrimeric guanine nucleotide binding proteins (G proteins). Upon activation, receptors trigger complex cascades of reactions controlling crucial physiological and cellular processes. As such, GPCRs are implicated in multitude of diseases, making them

important pharmaceutical targets. In fact, about 40% of drugs on the market act on the G protein-coupled receptors (2).

GPCR family members share common architecture of a 7 transmembrane α -helical bundle (3). It remains intriguing how such a relatively simple scaffold has evolved to selectively bind to thousands of diverse ligands transmitting signals to dozens of different effectors. The primary questions in the GPCR field include understanding mechanisms of signal transduction and aspects of ligand selectivity and specificity. These and other essential questions can only be answered when a variety of biophysical and biochemical data are combined with high-resolution structures of family members stabilized in different functional states.

Despite the great importance of GPCRs and immense worldwide efforts aimed at obtaining their structures, very little progress has been achieved until recently. Indeed, until late 2007 a high resolution structure of only one highly atypical GPCR member, visual rhodopsin, was known (4). Rhodopsin is unusual in that it is highly abundant in the eye's retina and contains a covalently bound ligand, 11-cis retinal, keeping the receptor conformationally stable in a fully inactive state. However, all other receptors are expressed in miniscule amounts in native tissues and activated by diffusible ligands. For structural studies, it has therefore become necessary that these proteins be heterologously overexpressed, solubilized, and purified. Human integral membrane proteins, including GPCRs, are notoriously difficult to express and they are highly unstable when solubilized by detergents. What makes GPCRs even more challenging is their observed conformational heterogeneity highlighting dynamic equilibrium between multiple functional states (5), obviously vital for signal transduction; however, a formidable roadblock for structural studies. For example, most GPCRs possess a certain level of basal activity in the apo-, or unliganded, state. Ligands that increase activity are called agonists, those that bind to the receptor and block it without changing its activity are called antagonists, and ligands that decrease the activity below basal are called inverse agonists. Typically there is a whole range of intermediate states between completely inactive and fully active receptor, and ligands that induce or stabilize them are called partial inverse agonist and partial agonists. In general, receptors are more stable and less conformationally heterogeneous in the inactive states and become progressively less stable and more heterogeneous as they are converted into more active states.

Thus, it is now generally accepted that in order to crystallize a GPCR, it has to be stabilized in a single conformational state. Last year an explosion of technological breakthroughs have made it possible to stabilize the conformation and determine the high-resolution structures of several members of the GPCR family. One of the successful approaches involved developing a monoclonal antibody toward a structural epitope on the third intracellular loop of the human β_2 -adrenergic receptor (β_2 AR) (6). The antibody/receptor complex was crystallized in bicelles and the structure was determined at a moderate resolution of 3.4–3.7 Å (7). In the second approach a highly flexible intracellular loop between helices 5 and 6 of the human β_2 AR was replaced by a compact and stable protein, lysozyme from T4 bacteriophage (T4L) (8). The chimeric receptor (β_2 AR-T4L) was crystallized in lipidic cubic phase (LCP) and the structure solved to a resolution of 2.4 Å (9). The same approach was recently used to obtain the structure of the human adenosine A_{2A} receptor at 2.6 Å resolution (10). Finally, the third approach toward receptor stabilization involved a systematic mutagenesis strategy (11,12). This strategy helped to identify 6 mutations in turkey β_1 -adrenergic receptor that increased its melting temperature in detergent solution by 21 °C. The thermally stabilized receptor was crystallized by a traditional vapor diffusion technique and the structure was solved to 2.8 Å (13). All of these studies were done on receptors with bound ligands, typically inverse agonists or antagonists, locking them in a stable conformation. Additional stabilization was achieved by crystallizing β_2 AR/Fab complex in bicelles (7), and both T4L fusion receptors, the β_2 AR-T4L and A_{2A} R-T4L, in the lipidic cubic phase (9,10,14).

Comparison of these new structures highlights important details related to the plasticity of the GPCR 7TM core and the involvement of the extracellular loops in the ligand binding (15,16). In addition, the new structures provided superior templates for homology-based modeling of other GPCRs and for performing virtual ligand screening and structure based drug design (17). While new approaches have enabled GPCR structure determination, more remains to be done to address crucial questions centering on mechanisms of signal transduction upon receptor activation. To gain insights into these mechanisms, it is essential to obtain a high-resolution structure of an activated receptor with an agonist bound. Recently a crystal structure of the bovine opsin bound to a transducin fragment shed some light on the receptor activation mechanism (18). However, the atypical mode of the rhodopsin activation and the lack of an agonist in the structure prevent generalizations applicable to other GPCRs responding to diffusible ligands. It is conceivable that a similar approach of stabilizing an activated receptor by a G protein, $G\alpha$ subunit or by a fragment of $G\alpha$ will yield a high resolution structure in the near future.

Two of the best resolution GPCR structures to date, not including rhodopsin, have been obtained using crystallization in LCP, also referred to as *in meso* crystallization (19). The success of *in meso* crystallization can be attributed primarily to two factors: stabilization of highly flexible receptors in a membrane-like LCP environment and formation of type I crystal lattice leading to highly ordered and strongly diffracting crystals. Here we describe methods related to reconstitution of purified receptors in LCP, performing pre-crystallization assays, setting up crystallization trials, harvesting crystals and acquiring crystallographic data. We recommend the reader to get familiar with more general and detailed protocols for *in meso* crystallization recently published in ref. 20. In this chapter the emphasis is on working with highly unstable GPCRs, which require pre-crystallization analysis of multiple constructs, extensive crystallization optimization and crystallographic data collection on multiple microcrystals using a highly collimated synchrotron beam.

2. Materials

2.1. Reconstitution of protein in LCP

2.1.1. Lipid mixing

1. Monoolein (1-oleoyl-rac-glycerol) (Sigma or Nu-Chek Prep).
2. Additive lipids (Sigma or Avanti Polar Lipids).
3. Chloroform (HPLC grade, Sigma).

2.1.2. Mixing lipid with protein

1. Purified protein solution (*see* Note 1).
2. Two 100 μ L gas-tight removable needle syringes without a needle (Hamilton, cat.# 81065).
3. 25 or 50 μ L gas-tight syringe with a flat tip (point style 3) removable needle (gauge 26s) (Hamilton, cat.# 80265).

¹Protein prepared for crystallization trials or pre-crystallization assays should run on an analytical size exclusion chromatography column as a single sharp monomer peak without significant contribution from dimers or larger oligomeric aggregates. Protein solution should not contain excessive amounts of detergent as high concentration of detergents could destroy the lipidic cubic phase (35,36). If the high detergent concentration creates a problem, try reducing the detergent content as low as possible during purification steps. For example, for His-tagged proteins use saturated binding to a Ni sepharose resin and elution with a minimal amount of buffer to concentrate the protein without increasing the detergent content, and finally use the largest possible cutoff centrifugal concentrators.

4. Syringe coupler made of two gauge 26 removable needles (Hamilton, cat.# 7768-02) and two removable needle (RN) nuts (Hamilton, cat.# 30902) as shown in Fig. 1 and described in ref. 21.

2.2. LCP-FRAP crystallization pre-screening assays

2.2.1. Protein labeling

1. Purified protein solution at 1 – 5 mg/mL concentration.
2. Stock solution of 5 mg/mL Cy3 monofunctional N-hydroxysuccinimide ester (Cy3 NHS ester) (GE Healthcare) in dimethylformamide (DMF) (Sigma). Can be stored at –20 °C for up to two weeks (*see* Note 2).
3. Labeling buffer: 50 mM Hepes pH 7.2, 150 mM NaCl, 0.05 % w/v n-dodecyl- β -D-maltopyranoside (DDM), 0.01 % w/v cholesterol hemi-succinate (CHS), ligand.
4. Wash buffer: 50 mM Hepes pH 7.5, 150 mM NaCl, 0.05 % w/v DDM, 0.01 % w/v CHS, 20 mM imidazole, ligand.
5. Elution buffer: 50 mM Hepes pH 7.5, 150 mM NaCl, 0.05 % w/v DDM, 0.01 % w/v CHS, 200 mM imidazole, ligand.
6. Desalting column PD-10 (GE Healthcare).
7. Ni Sepharose resin (GE Healthcare).
8. Empty 2 mL gravity-flow columns (Thermo Scientific).

2.2.2. Sample setup

1. Glass slides, 127.8 × 85.5 mm, 1 mm thick (Erie Scientific) (*see* Note 3)
2. Cover slips, 112 × 77 mm, 0.2 mm thick (Erie Scientific) (*see* Note 3)
3. Perforated double stick spacer (3M 9492MP double stick tape, 60 μ m thick, cut to 112 × 77 mm, with 96 punched 7 mm in diameter holes making 12 columns and 8 rows with 9 mm distance between the centers of the adjacent holes) (Saunders Corp.) (*see* Note 3).
4. Screening solutions (*see* Note 4).

²Protein can be labeled with a variety of fluorescent dyes, readily available from Invitrogen, GE Healthcare and other companies. We have tried Fluorescein, Rhodamine 6G, Tetramethylrhodamine and Cy3. Our preferable choice is Cy3 due to its good solubility, low environmental sensitivity and suitable bleaching properties. There are two common conjugation chemistries for protein labeling: thiol-reactive and amino-reactive. The advantage of using amino-reactive dyes is that virtually any protein can be labeled and that these dyes impose a minimal disturbance to the protein when attached predominantly to the N-terminus. The drawback of the amino-labeling is that co-purified with the protein free amino group-containing lipids, such as phosphatidylethanolamines, are also labeled. This introduces unwanted background signal, which is difficult to separate from the protein signal. The thiol-reactive dyes do not label lipids, however, they rely on availability of free cysteines exposed on the protein surface. Additionally we have found that cysteine labeling often destabilizes GPCRs. In this protocol we describe universal protein labeling at its N-terminus with succinimidyl ester derivatives of fluorescent dyes. We also recommend trying cysteine labeling if it does not disturb the properties of the protein. Labeling with thiol-reactive dyes can be performed according to manufacturer's instructions.

³The 96-well plate is designed primarily for compatibility with robotic crystallization setup and imaging (Fig. 4H). In case of manual operations it is more convenient to make glass sandwich plates of standard 25 × 75 mm² microscope slides, spacers with punched holes arranged in 8 rows and 2 columns (Fig. 4F) or 9 rows and 3 columns (Fig. 4G), and 18 × 18 mm² (Fig. 4F and H) or 25 × 25 mm² (Fig. 4G) glass cover slips depending on the wells configuration.

⁴Screening solutions for LCP-FRAP should be selected to represent a range of common precipitants. We found that PEG-ion-pH and ion-pH screens with 30 % v/v PEG 400 or 20 % w/v PEG 4000, variety of salts and pH ranging from 5 to 8 are good starting points in screening for diffusion of GPCRs with and without fused lysozyme.

2.2.3. FRAP data collection

1. FRAP station consisting of a Zeiss AxioImager A1 fluorescent microscope with an EC-Plan 10× objective (NA = 0.3), an HBO 100 epi-illumination and a Cy3 fluorescence filter set (excitation at 543 nm with 22 nm bandwidth, emission at 605 nm with 60 nm bandwidth); a tunable dye cell (set at 551 nm) MicroPoint laser system (Photonic Instruments); a CoolSnap HQ2, 14 Bit, cooled (−30 °C) CCD (1392 × 1040 pixels, 6.45 μm/pixel) monochrome FireWire camera (Photometrics); an XYZ automated microscope stage MS-2000 and an automated shutter with controller (Applied Scientific Instrumentation). All the FRAP station hardware is controlled by an ImagePro Advance Microscopy program suite (Media Cybernetics) (*see* Note 5).

2.3. Crystallization in lipidic mesophases

1. Protein reconstituted in LCP at 10 – 30 mg/mL final concentration prepared as described in section 3.1.
2. 10 μL gas-tight removable needle syringes without a needle (Hamilton, cat.# 80065).
3. Repetitive syringe dispenser (Hamilton, cat.# 83700) (*see* Note 6).
4. Short (0.375 inch), flat-tipped needle (point style 3, gauge 26, Hamilton, cat.# 7804-03).
5. Glass slides, 127.8 × 85.5 mm, 1 mm thick (Erie Scientific) (*see* Note 3)
6. Cover slips, 112 × 77 mm, 0.2 mm thick (Erie Scientific) (*see* Note 3)
7. Perforated double stick spacer (3M 9500PC double stick tape, 140 μm thick, cut to 112 × 77 mm, with 96 punched 5 mm in diameter holes making 12 columns and 8 rows with 9 mm distance between the centers of the adjacent holes) (Saunders Corp.) (*see* Note 3).
8. Crystallization screens (*see* Notes 7 and 8).
9. MiteGen MicroMounts (*see* Note 9).

3. Methods

3.1. Reconstitution of protein in LCP

3.1.1. Lipid mixing—The procedure described here can be used to supplement monoolein or any other LCP host lipid with lipophilic additives. Such additives can modulate properties of LCP or can preferentially interact with proteins reconstituted in LCP and therefore are useful for optimization of crystallization conditions. Cholesterol was found to be the best lipid additive significantly improving the β₂AR-T4L crystal size (9,14). Cholesterol was also used for

⁵To perform FRAP measurements we have used a simple and affordable system consisting of a fluorescent microscope with an attached laser. Alternatively, any dedicated commercial FRAP systems and most confocal microscopes can be used to run LCP-FRAP assays that are described in this protocol.

⁶The Hamilton syringe dispenser can be modified as described in ref. 37 to decrease the dispensing volume ~3 times, from 200 nL to 70 nL.

⁷It is convenient to use commercial sparse matrix screens for initial screening. On average, however, about 30 % of commercial screen conditions are not compatible with LCP crystallization, since they transform LCP into lamellar or hexagonal phase or completely dissolve the lipid (30). Such conditions can be diluted 2 times with water to increase the compatibility percentage. Additionally, more specific grid or sparse matrix screens can be prepared following results of the LCP-FRAP assays described in section 3.2.

⁸During *in meso* crystallization the soluble fraction of LCP is diluted ~50 times, due to vast excess of precipitant (1 μL) over the LCP bolus (50 nL), thus significantly depleting the ligand in the receptor vicinity. Therefore, depending on the ligand binding affinity, solubility and off-rate, it may be necessary to supplement crystallization screens with the ligand.

⁹We prefer MiTeGen MicroMounts over standard nylon loops for harvesting crystals from LCP due to their rigidity and thinner profile, which allow for easier penetration into LCP and for picking minimal amount of lipids along with the crystal.

crystallization of adenosine A_{2A} receptor (10). Compatibility of monoolein-based LCP with several lipid-like molecules was reported in ref. 22.

1. Weigh a small amount (few mgs) of an additive lipid in a small (1–2 mL) amber glass vial with a Teflon-lined cap.
2. Add an appropriate amount of monoolein to obtain required concentration of the additive lipid (*see* Note 10).
3. Dissolve the lipids in ~200 – 400 μ L of chloroform.
4. Evaporate bulk of the solvent using a gentle stream of dry, filtered nitrogen, keeping the vial warm (~37 °C) to prevent lipid from freezing.
5. Remove the last traces of chloroform under a vacuum (<100 mTorr) for at least 4 hours (preferably overnight).
6. Flush the vial with argon gas, close the cap and store at –20 °C or lower temperature until used.

3.1.2. Mixing lipid with protein

1. Transfer a necessary amount (15 – 50 mg) of a host cubic phase lipid (e.g. monoolein) or a lipid mixture prepared in section 3.1.1. into a small 0.5 mL plastic vial.
2. Melt lipid at ~40 °C (*see* Note 11).
3. Weigh a 100 μ L gas-tight syringe with attached coupler.
4. Remove the plunger and transfer molten lipid into the syringe barrel through the plunger end using an adjustable volume pipette (*see* Note 12) (Fig. 2A).
5. Insert the plunger back and slowly move the lipid up in the syringe to remove any trapped air bubbles. Stop when the lipid reaches the end of the coupler needle (Fig. 2B).
6. Weigh the syringe to determine the total mass of the lipid in the syringe.
7. Use a 25 or 50 μ L syringe with a flat tipped 26s gauge needle to transfer an appropriate amount of the protein solution in the second 100 μ L gas-tight syringe to achieve 40 wt% of protein solution in the final mixture (for example, for 30 mg of lipid use 20 μ L of protein solution) (Fig. 2C). Avoid trapping air bubbles during the syringe loading.
8. Move the protein solution with the plunger up as far as possible to minimize the air trapping after assembling the syringe mixer (Fig. 2D).
9. Screw the syringe containing protein solution to the open end of the coupler attached to the lipid syringe.
10. Move the protein solution and the lipid through the coupler inner needle back and forth from one syringe to another by pushing alternatively on the corresponding plungers until the lipid mesophase in the syringe mixer become homogeneous and transparent (Fig. 2E). This sequence of motions will mechanically mix the lipid with

¹⁰Do not exceed 100 – 200 mg of total lipid mixture per vial, since complete removal of solvent will be difficult. Use several vials or larger volume vial for making larger volume stocks of mixed lipids.

¹¹Melting temperature of monoolein is 37 °C. Different lipids can melt at higher or lower temperature. Adjust the incubation temperature to be few degrees above the lipid melting temperature. Incubation time should be no longer than necessary to melt the lipid in order to avoid possible degradation.

¹²After taking the molten lipid from incubator it remains liquid at room temperature for few minutes before it solidifies allowing for transfers with a pipette.

the protein solution through the action of shearing forces forming inside the narrow coupler needle. Upon mixing a lipidic cubic phase will form spontaneously and the protein will become inserted into the lipid bilayer of the LCP. Complete mixing requires a few hundred passages and typically takes less than 5 minutes (*see* Note 13).

3.2. LCP-FRAP crystallization pre-screening assay

LCP-FRAP assay is designed to measure diffusion properties of membrane proteins reconstituted in LCP at a variety of different screening conditions (23). The long-range diffusion of membrane proteins in LCP is essential for successful crystallization, however, the microstructure of LCP imposes spatial constraints on diffusion of large proteins or oligomeric protein aggregates. Our data indicate that one of the primary reasons for failure of the *in meso* crystallization trials with GPCRs is due to a fast nonspecific protein aggregation. While this event is equivalent to massive precipitation, and is easily recognizable in crystallization trials in solution, it does not produce any visual feedback in LCP, since the size of the non-diffusing stuck protein oligomeric aggregates is well below the optical resolution. It has been found that the aggregation behavior of a protein depends on the particular protein construct, host lipid and additives employed in crystallization trials.

The LCP-FRAP protocol, described below, involves labeling the protein of interest with a fluorescent dye, reconstituting the labeled protein into LCP, loading a 96-well glass sandwich plate with LCP and screening solutions, performing a FRAP data acquisition and analyzing the data. Work on automation of data acquisition and data analysis is in progress. The LCP-FRAP assay is useful for prescreening multiple protein constructs, for assessing the role of ligands and lipid additives and for identifying precipitants that are non-conductive to protein diffusion. The latter are then excluded from the subsequent crystallization screens.

3.2.1. Protein labeling

1. Bind the protein to a Ni Sepharose resin in a batch mode (*see* Note 14).
2. Exchange the protein buffer to labeling buffer (*see* Note 15).
3. Add Cy3 NHS ester stock solution in DMF to the resin to achieve Dye/Protein molar ratio of 2 – 5. Mix the resin with the dye by pipetting repetitively the slurry up and down with a 1 mL pipette.
4. Incubate at 4 °C in the dark for 2–3 hrs using a gentle rocking.
5. Wash out the bulk of unreacted dye and labeled lipids with 5 column volumes (CVs) of wash buffer.
6. Incubate the resin with 5 CVs of wash buffer at 4 °C in the dark overnight on a gentle rocker to dissociate tightly bound to the protein unreacted dye and labeled lipids.
7. Wash with 5 – 10 CVs of wash buffer until the flow-through does not contain any detectable dye.
8. Elute the protein with elution buffer.

¹³The syringe mixer can warm up due to a friction between the lipidic mesophase and the syringe coupler needle. To avoid heating up the sample, limit the mixing rate to ~1 stroke / s. A useful trick is to slightly cool down the mixer by putting it for few seconds on ice or in a refrigerator, which speeds up the process of achieving a homogeneous cubic phase. Do not overcool it, however, as below 18 °C monoolein-based LCP is unstable and can convert into a lamellar crystalline phase damaging the protein.

¹⁴We provide the protocol for labeling and cleaning proteins with C-terminal His-tag. Procedure for labeling untagged proteins should be modified accordingly to include either ion-exchange or size exclusion chromatography for removing the unreacted dye.

¹⁵The pH of the buffer should be between 7 and 7.5 to label predominately the free N-terminus of the protein. The buffer should not contain free amino groups.

9. Concentrate to 1 – 5 mg/mL using a Vivaspin concentrator with 100 kDa cutoff.
10. Measure absorbance at 280 nm (A_{280}) and 552 nm (A_{552}) and determine the protein labeling percentage using the following equation:

$$\frac{[dye]}{[prot]} = \frac{A_{552}}{\epsilon_{552}} \frac{\epsilon_{280}}{(A_{280} - k \times A_{552})},$$

where $\epsilon_{552} = 150,000 \text{ M}^{-1}\text{cm}^{-1}$ is the molar extinction coefficient of Cy3 at 552 nm; ϵ_{280} is the molar extinction coefficient of the protein at 280 nm; and $k = 0.08$ is the correction due to absorption of Cy3 at 280 nm.

The expected labeling percentage is between 2 – 10 %.

3.2.2. Sample setup

1. Mix the labeled protein with a lipid to form LCP using a syringe mixer as described in section 3.1.2.
2. Set up samples in a glass sandwich plate with a 60 μm thick spacer either manually (section 3.3.2.) or robotically (24) using FRAP screening solutions instead of crystallization screens.
3. Incubate the plate at 20 °C for at least 12 hours to allow for equilibration of screening solutions with LCP before starting the FRAP measurements.

3.2.3. FRAP data collection—The protocol describes data collection using a custom build FRAP station controlled by an ImagePro Advance Microscopy program suite (Fig. 3A). Most of the steps are automated using the scripting language of the ImagePro. Similar protocol can be implemented on any FRAP capable microscope system.

1. Place the sample on a microscope stage and focus on a homogeneous area of the sample through a 10 $\times$ objective.
2. Take 3 – 5 fluorescence images to capture the pre-bleached state of the sample using a CoolSnap HQ2 cooled to –30 °C CCD camera. Reduce the acquisition area to a 501 $\times$ 501 pixels central sensor area (corresponding to the sample area of 325 $\times$ 325 μm) to minimize the read-out and image transfer times.
3. Trigger the MicroPoint laser firing 10–20 short 5 ns pulses at 20 MHz repetition rate (see Note 16).
4. Immediately after triggering the laser start recording a fast post-bleached sequence of 200 images (100 – 500 ms exposure time per image) streaming the images as fast as possible into the computer memory.
5. Follow with a slow post-bleached sequence of 50 images selecting the delay time between images (1 – 20 s) depending on the diffusion rate of the protein. Close the shutter during the pauses between images to minimize sample bleaching by the incident light.
6. Save all recorded images into a single multiframe 16 bit tiff file for data analysis.

¹⁶Number of laser pulses depends on the dye, filters, laser power, sample thickness, etc. Adjust the number of pulses to achieve ~30–50 % bleaching.

3.2.4. Data analysis—FRAP data analysis steps described here are implemented using ImagePro (Media Cybernetics), Microsoft Excel and Prizm (GraphPad), however other image analysis and curve fitting packages can be employed to perform similar tasks.

1. Open a multiframe FRAP recovery image sequence tiff file with ImagePro.
2. Locate the frame with the darkest bleached spot.
3. Select a circular region of interest (ROI) around the bleached spot.
4. Select four square ROIs in homogeneous regions of LCP at a distance from the bleached spot to serve as reference signals to compensate for decrease of fluorescence intensity due to bleaching during the image acquisition sequence.
5. Integrate total fluorescence intensities inside the selected ROIs in all frames and transfer all the data into an Excel spreadsheet.
6. Correct for the bleaching and any light intensity fluctuations during the acquisition by dividing the intensity inside the bleached spot by the averaged intensity of the reference squares.
7. Normalize the signal to make the pre-bleached intensity equals to 1 and intensity of the bleached spot equals to 0.
8. Fit the normalized intensity vs. time (extract exact time of acquiring each frame from the original multiframe tiff file), $F(t)$, with GraphPad Prizm using the following equation (25):

$$F(t) = M \times \exp\left(-\frac{2T}{t}\right) \times \left(I_0\left(\frac{2T}{t}\right) + I_1\left(\frac{2T}{t}\right)\right), \quad (\text{Eq. 1})$$

where M is the mobile fraction of diffusing molecules, T is the characteristic diffusion time, t is the real time of each recorded frame, and I_0 and I_1 are 0th and 1st orders modified Bessel functions (*see* Note 17) (Fig. 3B).

9. Determine the size of the bleached spot by radially integrating the spot intensity with the ImagePro and fitting the integrated intensity profile with the GraphPad Prizm using a Gaussian shape. Use the half width at half maximum (HWHM) of the Gaussian as a measure of the spot radius, R .
10. Calculate the diffusion coefficient, D , as:

$$D = \frac{R^2}{4T} \quad (\text{Eq. 2})$$

11. Compare the mobile fractions and diffusion coefficients obtained at different screening solutions.
12. Design new crystallization screens based on components facilitating protein diffusion. Exclude conditions for which protein diffusion were not observed from subsequent

¹⁷If the experimental recovery data do not follow Eq.1, it is likely that more than one population of diffusing molecules is present in the sample. The extra signals can originate from different protein oligomeric states or from labeled lipids. If two populations of molecules have substantially (more than an order of magnitude) different diffusion rates, then it is possible to fit the experimental recovery curve with a two component equation and extract both contributions. In practice, when amino-labeling is used, even after an extensive wash in an attempt to remove labeled lipids, there is a residual recovery signal of 5 to 15 % coming from the labeled lipids. The protein diffusion signal can in most cases be extracted using a two component diffusion equation with a fixed characteristic diffusion time for lipid molecules determined from a separate FRAP measurement on a sample containing only labeled lipids (23).

crystallization trials. If the protein did not diffuse in any of the screened conditions, consider broadening the screening space or trying a new protein construct.

3.2.5. High-throughput LCP-FRAP—Recording a complete fluorescence recovery curve is time consuming, taking 10 – 30 min per sample depending on the protein diffusion coefficient. This limits the number of samples that can be processed in a reasonable time. We noticed that in the case of GPCRs majority of screens result in no measurable recovery of signal. Thus, when a 96-well plate is used to set up FRAP samples, it is possible to sequentially bleach all the samples first and then to measure the total recovered intensity for each sample after 30 – 60 min incubation. This procedure provides protein mobile fractions for all 96 samples within ~2 hrs. Full recovery curves are then recorded only for the samples with significant protein mobility.

3.3. Crystallization in lipidic mesophases

In meso crystallization trials can be performed manually or robotically. Protocols for manual crystallization setup are provided in this section; using robotics for *in meso* crystallization is extensively discussed in refs. 24,²⁶. It is preferable to perform *in meso* crystallization trials in glass sandwich plates, described in refs. 24,²⁷. These plates have excellent optical properties for detection of very small colorless protein crystals growing in LCP matrix. Currently the glass sandwich plates are not available commercially and must be assembled from separately ordered glass slides and perforated spacers (section 3.3.1.). Alternatively, most commercial micro-batch, sitting or hanging drop plates could be used for the manual crystallization setup with the caveat that one may obtain less than optimal conditions for detection of small colorless crystals, primarily due to the scattering of light from a rough boundary between the LCP bolus and precipitant solution (Fig. 4A–C). The problem can be circumvented by sandwiching the LCP bolus with a 5 mm diameter glass coverslip (Warner Instruments, cat.# W2 64-0700) as shown in Fig. 4D–E and used in ref. 28.

Crystallization setup starts with mixing protein solution with lipid as described in section 3.1.2. It is advisable to have plates and screening solutions ready before starting protein reconstitution in LCP, and to proceed to crystallization setup (section 3.3.2.) immediately after forming protein-laden LCP, because some proteins may not be stable in LCP without added precipitant solution. The whole process of manual setting up a 96-well plate including mixing of protein and lipid takes about 1 hr. In addition to the description of the manual *in meso* crystallization setup, this section contains protocols for crystal detection, optimization and harvesting.

3.3.1. Assembling glass sandwich plates

1. Silanize both base glass slide and coverslip with AquaSil according to manufacturer's instructions.
2. Peel off protective liner from one side of a perforated spacer sheet.
3. Attach the spacer to a base glass slide, aligning them along their top and left sides.
4. Wrap the plate into a clean aluminum foil.
5. Use a brayer to apply a uniform pressure on the spacer for ensuring better adhesion.
6. Wrap the coverslip into a separate aluminum foil to protect it from dust.
7. The plate is now ready for crystallization setup. Plates can be stored at 20 °C for up to several months.

3.3.2. Manual crystallization setup

1. Attach a 10 μ L gas-tight syringe to a repetitive syringe dispenser.

2. Transfer protein-laden LCP, prepared in section 3.1.2., into the 10 μ L syringe affixed to the repetitive dispenser (Fig. 2F).
3. Attach a short removable needle to the 10 μ L syringe.
4. Push the plunger until the cubic phase starts coming out from the needle. Fix the plunger with a gripping nut of the repetitive dispenser.
5. Unwrap a glass sandwich plate (prepared in section 3.3.1.) and place it on a flat surface.
6. Position the syringe needle at the center of the first well ~200–300 μ m above the surface and press on the button of the dispenser to deliver 200 nL of the protein laden cubic phase bolus (*see* Note 18) (Fig. 2G).
7. Repeat Step 5 to dispense cubic phase into three more wells forming a 2 $\times$ 2 square.
8. Add 1 μ L of precipitant solutions on top of the cubic phase boluses in each of the four wells (Fig. 2H).
9. Cap the four loaded wells with a 18 mm square glass coverslip (Fig. 2I). Use a wooden toothpick to press on the coverslip around the wells to properly seal them.
10. Repeat Steps 5 – 8 until the whole plate is filled up.
11. Incubate the plate at 20 $^{\circ}$ C (*see* Note 19).

3.3.3. Crystal detection—Typically crystals start appearing in the time frame between a few hours and a few weeks after setup. Majority of GPCR crystals are first detected between 12 hours and 7 days of incubation. Crystallization wells should be inspected periodically either under a microscope or with an automated imager. We use the RockImager line of incubator/imagers from Formulatrix which is compatible with imaging the glass sandwich plates. In this section we describe inspection of wells and detection of crystals using a microscope equipped with a polarizer and a rotating analyzer. An automatic imager adds a convenience of taking images of wells at scheduled time points and storing them in a database for later viewing. In difficult cases, however, it is useful to take the plate out of the imager and to inspect it under a microscope using a higher magnification than what is normally achievable with the imager.

1. Inspect each well under a microscope with a 10 $\times$ objective using a bright-field illumination and cross-polarizers. Under cross-polarizers the cubic phase is dark as it is isotropic while most crystals appear as bright objects. Make a note of any such birefringent object or any crystal-like object to detect if they grow up with time (Fig. 5A–B). Use 40 $\times$ or higher magnification objective to better see the shape of small objects. Sometimes the whole lipid phase bolus can turn birefringent meaning that it has transformed into a lamellar or hexagonal phase, both of which are unlikely to support growth of membrane protein crystals. Ref. 20 provides extensive examples of images one can encounter when following up *in meso* crystallization trials.
2. When a crystalline object is observed during the initial screening there are several options to establish whether this object contains protein or not:

¹⁸If the needle is too far from the glass surface the cubic phase bolus coming out of the needle as a tube curls back and sticks to the needle. If the needle is too close to the glass surface the cubic phase bolus balls up and sticks to the needle. Both of these cases result in no or incomplete delivery. The right distance and feeling for accurate dispensing come with practice and is relatively easy to achieve.

¹⁹Crystallization plates can be incubated at different temperatures, however, monoolein-based cubic phase is stable only at temperatures above 18 $^{\circ}$ C (38). Recently, several new lipids were developed specifically for low temperature *in meso* crystallization (39,40). It is important to avoid temperature fluctuations during plate incubation and imaging, because fluctuations by just few degrees can induce formation of liquid droplets inside the cubic phase. These droplets scatter light, making it difficult to detect crystals.

- a) Set up replicate trials using exactly the same components lacking the protein (*see* Note 20).
- b) Label a small fraction of the protein (typically less than 1 %) with a fluorescent dye to see if the generated crystals are fluorescent (29) (*See* Note 21) (Fig. 5C).
- c) Use a UV fluorescence microscope to distinguish between fluorescent protein crystals and non-fluorescent salt crystals (*See* Note 22).
- d) If the crystals are relatively large, harvest them and check for diffraction using a minibeam at APS or at other synchrotron sources.

3.3.4. Crystal optimization—Initial crystal hits in LCP often appears as showers of very small crystal and crystallization conditions have to be optimized. In general, *in meso* crystal optimization strategies are similar to optimization strategies for crystallization of macromolecules in solution. One difference is that along with tweaking the precipitant solutions composition and pH, one has the ability to optimize the LCP host lipid and lipid additives. When planning for optimization of *in meso* trials it is important to understand how LCP responds to changes in concentration of a given component. For example, salts at high concentration can transform LCP into a hexagonal phase, which is not conducive to crystallization, while some small organic molecules, like 2-methyl-2,4-pentanediol (MPD), can completely dissolve the lipids, usually inducing massive protein precipitation. Effects of soluble and lipid-like components on LCP are described in refs. 22,30,31. Other parameters that we found to be critical for optimization of crystal growth *in meso* are the size and the shape of LCP bolus and the protein concentration. In this section we describe steps that have proven to be useful for optimization of β_2 AR-T4L crystals (9).

1. After initial crystal hit is detected and verified to be the protein, initiate the first round of optimization by applying coarse concentration – pH grid screens. As an example, vary PEG 400 concentration between 10 and 40 % (v/v) with 5 % (v/v) steps; salt concentration between 0 and 500 mM with 100 mM steps and buffer pH between 6 and 8 with 0.5 steps.
2. Using the best conditions from step 1 as a guide, screen for the salt identity using a broad selection of salts as, for example, available in the Salt StockOptions kit from Hampton Research (*see* Note 23).
3. Optimize the buffer identity and concentration. Try several different buffers with the same pH. Try different buffer concentrations between 50 and 200 mM. (*see* Note 23).

²⁰Appearance of similar to original crystals in such control trials can confirm that something else apart from the protein is crystallizing. Control trials resulting in no crystals, however, do not prove that the original crystal was from the protein, since it is difficult to exactly reproduce protein-free conditions.

²¹Be aware that sometimes even low percentage labeling can prevent protein from crystallizing. Try different dyes and conjugation chemistries as discussed in Note 2.

²²Before attempting UV imaging check that material, from which crystallization plates are made, is sufficiently transparent to UV light. For example, for glass sandwich plates we use 1 mm slides made of electroverre glass (Erie Scientific) with transmittance of 40 % at 280 nm, allowing for efficient excitation of protein's tryptophans. The coverslips used in these plates are made of 0.2 mm borosilicate glass, which is essentially non-transparent to UV light.

²³When the described optimization procedure was applied to initial crystal hits of β_2 AR-T4L, improvements were achieved almost at each step. Salt in the initial hit, lithium sulfate, was replaced by sodium sulfate as a result of step 2. Initial Hepes buffer was replaced by bis tris propane, which consistently gave better crystal size and shape, on step 3. Additions of cholesterol and dioleoylphosphatidylethanolamine have improved crystal size on step 5. The optimum concentration of cholesterol was found to be 10 % (wt/wt). The best soluble additive was identified as 5 % of 1,4-butanediol on step 6. We found that increasing concentration of β_2 AR-T4L from 20–30 mg/mL, which was used in the initial screening to 50 mg/mL resulted in larger crystal size on step 8. Increasing protein concentration even further to 60 mg/mL destabilized the lipidic cubic phase and abolished crystal growth. Finally, we observed that decreasing the volume of cubic phase from 50 to 20 nL on step 9 improved the crystal size even further.

4. Set up fine grid-screens around the best conditions obtained during the first three steps. Useful increment for PEG 400 concentration is 1 – 2 % (v/v); for salt: 20 – 50 mM; for pH: 0.1 – 0.2.
5. Screen for lipid additives using the best fine grid screens from step 4. Lipid additives should be mixed with the host LCP lipid as described in section 3.7.2. A good starting concentration of lipid additives is 5 wt%. For most promising additives concentration should be optimized (*see* Note 23).
6. Screen for soluble additive. Commercial additives screens (for example, from Hampton Research or Emerald BioSystems) can be conveniently used for this purpose. Use 10 times dilution for initial screening. Optimize concentrations of promising additives (*see* Note 23).
7. Create fine pH – concentration screens including all components that improve crystallization.
8. Optimize protein concentration by setting up trials with protein concentration varied between 10 mg/mL and maximum achievable and using the screens prepared in Step 7 (*see* Note 23).
9. Optimize the volume of lipidic cubic phase bolus using the screens prepared in Step 7. (*see* Notes 23 and ²⁴).

3.3.5. Crystal harvesting—Due to a strong adhesion of the double sticky spacer to glass slides it is impossible to peel off the cover slip to expose a single well for crystal harvesting. Therefore a piece of the cover glass should be cut and removed first. When crystals are obtained in commercial plastic trays, in which well opening is a trivial procedure, skip Section 3.3.5.1. and proceed directly to crystal harvesting section 3.3.5.2.

3.3.5.1. Opening a well in the glass sandwich plate

1. Place a glass sandwich plate with crystals on a harvesting stereo microscope with a variable zoom.
2. Focus the microscope on a well with crystals, using a low zoom, so that the whole well is visible in the field of view (Fig. 6A).
3. Score the cover glass in four strokes forming a square inside the well boundaries using a sharp corner of a Hampton Research ceramic capillary cutting stone (Fig. 6B).
4. Using a strong sharp point tweezers press around the scored perimeter to propagate the scratches through the thickness of the cover glass (Fig. 6C).
5. Punch two small holes in the cover glass at opposite corners of the square.
6. Inject 2–3 μ L of precipitant solution into the well through one of the holes to reduce dehydration during the subsequent well opening steps (*see* Note 25) (Fig. 6D).

²⁴The rationale behind adjusting the LCP bolus volumes is that the larger the volume the longer it takes for the precipitants to diffuse in the LCP and establish an equilibrium. The transient gradients forming during this process may affect the nucleation and crystal growth rates. *In meso* crystallization robot (24) can be used to vary lipidic cubic phase volume between 20 and ~500 nL. With manual setup the cubic phase volume range is limited to 70 – 700 nL when using the modified syringe dispenser coupled to 10 and 100 μ L syringes (37).
²⁵Certain precipitants may transform LCP into a sponge phase (41). It is virtually impossible to harvest crystals grown in the sponge phase using the described technique. The sponge phase has a liquid-like properties and is drawn away from the well by the surface tension when the well is opened up. To overcome this problem the sponge phase can be transformed back into the cubic phase by lowering the concentration of the precipitant. The cubic phase has a gel-like consistency and stays in place when well is opened. For example, the common precipitant, PEG 400, at concentrations above 35 % (v/v) can induce sponge phase formation. Lowering PEG 400 concentration to 30 % (v/v) on this step will bring LCP back within few minutes. When lowering PEG 400 concentration keep concentrations of other ingredients unchanged.

7. Use an angled sharp needle probe to free one or two edges the glass square (Fig. 6E) and carefully lift it up (Fig. 6F). The cubic phase bolus can remain on the bottom of the well or can be lifted up with the glass square piece. In the latter case flip the glass piece over and place it inside the well.
8. Add an extra 5 μL of precipitant solution, supplemented with a cryo-protectant, if necessary, on top of the exposed cubic phase bolus (*see* Note 26) (Fig. 6G–H).

3.3.5.2. Harvesting

1. Increase magnification of the microscope to $\sim 100\times$, focus on a crystal and adjust the polarizer and analyzer angles so that the birefringent crystal has a good contrast with the background, while making sure that there is still enough light to see the harvesting loop.
2. Harvest the crystal by scooping it directly from the lipidic cubic phase using a MiteGen MicroMount with a diameter matching the size of the crystal (*see* Note 27).
3. Immediately plunge the MicroMount with the harvested crystal in a dewar with liquid nitrogen to flash freeze it and then transfer it into a storage or shipping dewar.

3.4. Data collection from microcrystals using a 10 μm minibeam

The protocol describes data collection strategies from small LCP grown GPCR crystals using a 10 μm minibeam on the GM/CA CAT beamlines at the Advance Photon Source (Argonne, IL). In most parts the protocol can be directly translated to data collection on any other microcrystallography beamline with beam size of 10 μm or less.

3.4.1. Alignment of invisible crystals with the minibeam—This part of the protocol was originally designed to align invisible in a frozen opaque lipidic mesophase crystals of $\beta_2\text{AR}$ -T4L with a 10 μm minibeam at the GM/CA CAT beamlines. Recently an automatic rastering was implemented at the GM/CA CAT to facilitate crystal centering, however, the original procedure described here can be useful at other microfocus beamlines.

1. Mount a cryo-loop with a crystal on the beamline goniometer.
2. Center the loop on the rotation axis.
3. Attenuate the beam 20 times (*see* Note 28).
4. If the area of the loop is smaller than $60\times 30\text{ }\mu\text{m}$ then go directly to Step 5. Otherwise, use $50\times 25\text{ }\mu\text{m}$ slits with a 300 μm collimator to scan the whole area of the loop taking 1 s exposures with 0.5° oscillation at each point.
5. When diffraction from a protein crystal is observed, switch to a 10 μm minibeam and start scanning within the area of $60\times 30\text{ }\mu\text{m}$ using 10 μm steps.
6. After detecting diffraction with the 10 μm minibeam, select a strong spot and use the total intensity in that spot to search for the best diffracting position by moving the

²⁶On this step it is possible to dissolve the lipid and free the crystals using enzymatic lipid digestion (42), detergent solutions (43), sponge-inducing compounds (41) or mineral oils. We have observed that any of these treatments were detrimental to the diffraction quality of harvested crystals of $\beta_2\text{AR}$ -T4L and A_2AR -T4L. These crystals diffracted the best when harvested directly from the cubic phase.

²⁷When harvesting a crystal from the cubic phase, try picking up as little lipid as possible to reduce scattering background from lipids during data collection. If the crystal is located deep inside the cubic phase bolus, use a micro-tool or an empty MiTeGen MicroMount to remove excess of the cubic phase from the top and expose the crystal to the surface. Then use a fresh MicroMount to harvest the crystal.

²⁸Beam attenuation will depend on the flux. Attenuate as much as possible to reduce the radiation damage while still having the ability to observe low resolution diffraction from the crystals.

crystal in the direction of increasing the intensity initially with 5 μm and then with 2 μm steps.

7. Rotate the crystals 90°.
8. Scan across the loop in the direction perpendicular to the rotation axis using 10 μm steps.
9. When diffraction is detected, fine tune the crystal position as described in Step 6.

3.4.2. Crystallographic data collection—Radiation damage limits high resolution data collection from small crystals. Collecting and merging data from multiple crystals is necessary to acquire a full data set. It is essential to optimize data collection strategy to obtain the best possible results. Here we describe the protocol used to collect high quality data from $\sim 30 \times 15 \times 5 \mu\text{m}^3$ $\beta_2\text{AR-T4L}$ crystals and $\sim 60 \times 10 \times 5 \mu\text{m}^3$ crystals of $\text{A}_2\text{AR-T4L}$.

1. Collect 5 frames at 0 and 90 degrees from a single crystal to determine the space group, lattice parameters, mosaicity and resolution. Adjust the sample to detector distance and the oscillation width per frame to achieve the optimal data collection.
2. Estimate the susceptibility of crystals to radiation damage by collecting a sequence of exposures at the same crystal orientation. Determine the absorbed dose at which the total diffracting intensity drops 2 times and use this number as a guide of maximum dose for planning data collection in Step 3.
3. With a strongly diffracting crystal collect a full low resolution dataset using an attenuated 10 μm minibeam. After collecting the first 5 frames, run a Strategy as implemented, for example, in HKL2000 (32) or XDS (33) to optimize the starting phi angle and the range of rotation. Chose the beam attenuation and the exposure time taking into account the number of needed exposures and the maximum x-ray dose tolerated by the crystals determined in Step 2 (*see* Note 29).
4. Collect high-resolution wedges of data from several crystals. Chose the beam attenuation and exposure time to be able to collect at least 5–10 frames per crystal with the highest achievable resolution. Typically we used 1–2 s exposure and 1° oscillation per frame with unattenuated beam.
5. After collecting each new wedge merge it with previous data using the low resolution set as a reference for scaling. Discard the wedge of data if it does not scale well, significantly increasing the Rmerge factor (*see* Note 30).
6. Repeat Steps 4 and 5 until a complete data set at desired resolution is assembled. Remove the low resolution data from the final scaling step (*see* Note 31).

3.5. Conclusion

The *in meso* crystallization method was introduced more than a decade ago (34), and since that time it has proven successful in obtaining high resolution structures of difficult membrane proteins, such as human GPCRs (9,10,14). The method, however, has been in limited use mostly due to the difficulties in handling of sticky and viscous, gel-like LCP material. The

²⁹On this step it is important to collect a full dataset with a minimal damage to the crystal. Resolution is not critical as long as the dataset is complete and scales well, thus giving reasonable Rsym. Typical datasets we collected at this step had resolution $\sim 5 - 6 \text{ \AA}$ and Rsym $< 10\%$.

³⁰This method relies on a high isomorphism between crystals. The *in meso* grown crystals of $\beta_2\text{AR-T4L}$ and $\text{A}_2\text{AR-T4L}$ did have this quality. The data rejection rate due to poor merging was less than 20 %.

³¹To obtain a full dataset for $\beta_2\text{AR-T4L}$ in complex with carazolol we merged data from 27 crystals (9). For $\beta_2\text{AR-T4L/timolol}$ (14) and $\text{A}_2\text{AR-T4L/ZM241385}$ (10) 11 and 13 crystals were used respectively. Fewer crystals were required in the latter two cases because of the higher symmetry space group and slightly lower resolution.

protocols in this chapter provide directions for approaching *in meso* crystallization of GPCRs or any other membrane proteins. As with any crystallization experiment, the *in meso* crystallization approach requires substantial screening and optimization efforts, without being guaranteed success. Additionally, for many membrane proteins, and especially in the case of GPCRs, extensive protein engineering aimed at protein stabilization and elimination of highly flexible and disordered parts is an absolute pre-requisite before entering into crystallization trials. Lastly, although crystallography provides atomic resolution 3-d structures, these static snapshots of preferred stable conformations preclude full understanding of the dynamic nature of these molecules. With increases in the number of GPCR crystal structures other complimentary approaches, such as NMR and computer modeling, also covered in this volume, are becoming essential for determining the structural basis for ligand specificity and for deciphering the mechanisms of signal transduction.

Acknowledgments

This work was supported in part by the NIH Roadmap Initiative grant P50 GM073197 (JCIMPT), Protein Structure Initiative grant U54 GM074961 (ATCG3D), and NIH grant R21 RR025336. The authors acknowledge contributions from colleagues Michael A. Hanson, Wei Liu, Jeffrey Liu, Mark Griffith, Ellen Chien, Veli-Pekka Jaakola, Chris Roth and Peter Kuhn.

The authors acknowledge the support of Janet Smith, Robert Fischetti, and the GM/CA-CAT team at the Advanced Photon Source, for assistance in development and use of the minibeam and beamtime. The GM/CA-CAT beamline (23-ID) is supported by the National Cancer Institute (Y1-CO-1020) and the National Institute of General Medical Sciences (Y1-GM-1104).

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Figure 1.

A. Attaching the syringe coupler to a gas-tight syringe. After the coupler is attached the syringe is filled with lipid. Then a second syringe, filled with protein solution, is attached to the other end of the coupler. **B.** Section through the syringe coupler.

Figure 2.

Sequence of steps during manual set up of *in meso* crystallization trials. Reconstitution of protein in LCP (A – E) is described in section 3.1.2. Setting up trials in a glass sandwich plate (F – I) is described in section 3.3.2.

Figure 3.

A. The LCP-FRAP station. **B.** Fluorescence recovery curves obtained for β_2 AR-T4L reconstituted in monoolein based LCP after 12 hr incubation in the presence of 0.1 M Bis tris propane pH 7.0, 25 % (v/v) PEG 400, 5 % (v/v) 1,4-butanediol and different concentration of sodium sulfate: 0 M (triangles), 0.05 M (squares), 0.1 M (diamonds) and 0.4 M (circles).

Figure 4.

Different ways of setting up crystallization trials in LCP: **A.** Microbatch, **B.** Sitting drop, **C.** Hanging drop, **D.** Modified hanging drop, **E.** Modified microbatch. In (**D and E**) LCP bolus (black) is sandwiched using a small 5 mm in diameter glass coverslip in order to improve optical properties and facilitate detection of crystals growing in LCP. The best conditions for detecting small colorless crystals are achieved when crystallization trials are performed in glass sandwich plates. (**F – H**) show top views of the glass sandwich plates used in our laboratory. Plates (**F and G**) are based on a standard microscope slide, which are convenient to use for setting up crystallization trials manually. Plate (**H**) has the SBS footprint and is suitable for both robotic and manual setups.

Figure 5. Needle-like crystals of the engineered adenosine A2a receptor grown in LCP. **A.** Bright-field illumination. **B.** With cross-polarizers. **C.** Fluorescence. The protein was trace-labeled with Cy 3 NHS. Fluorescence picture in **(C)** was recorded using excitation at 543 nm (22 nm bandwidth) and emission at 605 (60 nm bandwidth).

Figure 6.
Sequence of steps illustrating opening of a well in the glass sandwich plate for crystal harvesting (see section 3.3.5.1. for details).