Setup of a controlled *in meso* phase crystallization experiment using CubeCrystal Plates

Overview

This protocol describes the setup of a crystallization experiment in controlled *in meso* phase using the novel CubeCrystal Plates. This method combines the advantages of lipidic cubic phase (LCP) batch setups with vapor diffusion.
In contrast to other LCP methods, the setup can be done using standard nanoliter dispensing equipment capable of dispensing 100 nl volumes. The protein wells are precoated with lipid (e.g. mono-olein). Mixing of the protein suspension with the lipid is not required, but the protein diffuses passively into the lipid coating. In effect, CubeCrystal Plates allow for the setup of a vapour diffusion experiment in a lipidic cubic phase environment.
Because of the diversity of proteins, this protocol can only give first guidelines. Optimization factors include, but are not limited to,

Concentration of target protein:

typically 2-5 mg/ml, but concentrations up to 15 mg/ml have been successfully used.

Nature of lipid:

Standard plates contain mono-olein, but other modifications are possible. Contact us to learn more.

Choice of precipitant solution:

Care should be taken to avoid solutions containing alcohols, other organic solvents or concentrations of PEG higher than 20%. These dissolve the LCP phase.

Avoid pH>8.0 as this destabilizes the mono-olein.

Dilution of precipitant solution:

Dilutions from 1:1 to 1:10 or higher may be used. We recommend to use a 1:4 dilution as first starting point.

Dilution of the precipitant solution is a crucial parameter in the CIMP setup. As for any vapour diffusion experiment, precipitant dilutions have the advantage that almost never protein precipitants are obtained, and the exposure to salt is reduced. The major advantage however in connection with the CIMP technology is that you can influence the concentration of the protein at the end of the experiment, i.e. when the equilibrium is reached. The higher the salt concentration gradient between the reservoir and the protein well, the stronger the osmotic force within the vapour diffusion compartment. Strong osmotic forces lead to high concentration factors, and low water content within the protein well. The water content of the *in meso* phase, in turn, yields different phases within the phase diagram of the monoolein:water mixture. This way, you can reach different *in meso* phases by adjusting the water content of the protein well. See Fig. 1 for an overview.

Note that LCP formation is temperature-dependent. Therefore it is crucial to incubate the CubeCrystal Plate at a temperature of 18°C or above. If the protein of interest requires incubation at lower temperatures, consider using a different lipid or lipid mixture. Contact us to discuss possible alternatives.

All our protocols are available for free download at www.cube-biotech.com/protocols.
Please contact us at contact@cube-biotech.com if you have questions or need assistance in adapting the protocols for your target protein.

Equipment

- ☐ Liquid handling robot capable of pipetting 100 nl volumes
- ☐ Liquid handling robot capable of pipetting 50 µL volumes - alternatively Micropipettor
- ☐ Micropipettor (preferably 8-channel pipette) to dispense 200-800 µL volumes
- ☐ Micropipetting tips
- ☐ Temperature-controlled incubator for crystallization plates
- ☐ Microscope for visualization of protein crystals

Materials

- ☐ CubeCrystal 2-well MO Plates (e.g. 10 pc, Cube Biotech #10103)
- ☐ Crystallization screening solutions (e.g. 96x1.5mL)
- ☐ 2 sealing tapes per plate
- ☐ 1 empty deepwell block, holding 96 x 1.5 mL or 96 x 2 mL volumes (e.g. Corning 3961)

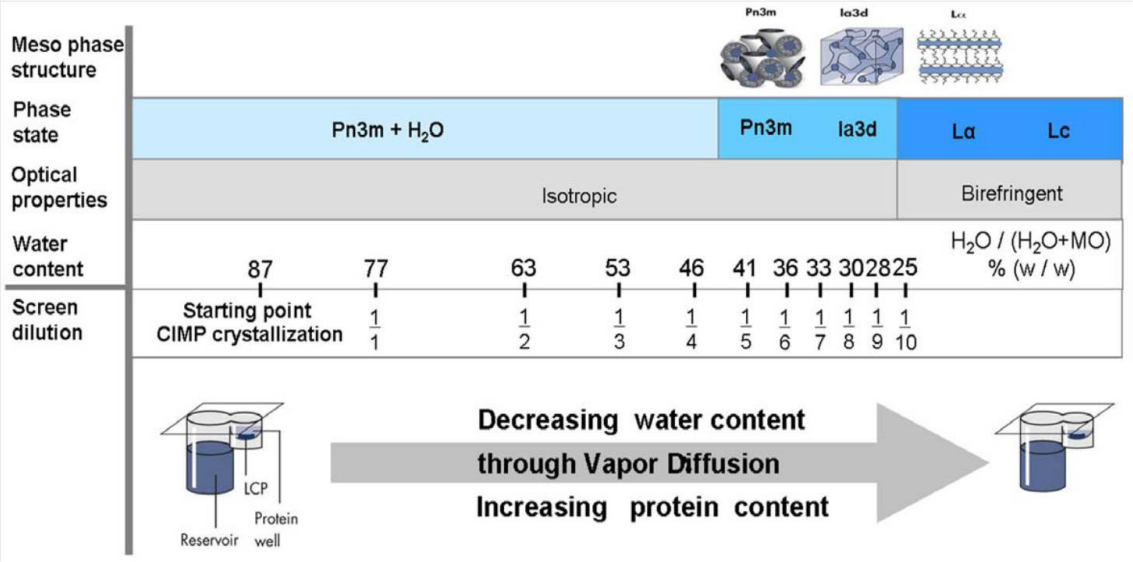

Fig. 1: Relationship between dilution of precipitant solution in the protein well, water content of the equilibrated protein drop, and the *in meso* phase that can be reached in a given experiment. Adapted from (1).

ratio	1:1	1:2	1:3	1:4	1:6	1:8	1:10
volume precipitant solution (µL)	500	333	250	200	143	111	91
volume water (µL)	500	667	750	800	857	889	909
	Instructions: Pipet suitable volume of precipitant solution into an empty 2 mL deepwell block and fill with double distilled water to 1 mL. Mix well. Scale up or down if necessary.						

Table. 1: Pipetting scheme for different dilutions of precipitant solutions.

Procedure

1. Equilibrate the CubeCrystal Plate to room temperature by incubation for at least 10 min.
2. Prepare protein solutions concentrated 2 mg/ml to 5 mg/ml for first trials.
3. Prepare suitable dilutions of the precipitant solutions according to Table 1 using a microliter dispenser or a micropipettor, into the 96 wells of the spare deepwell block. Mix by pipetting up and down.
4. Unpack the CubeCrystal Plate, and dispense 100 nL of the protein solution onto the dry lipid in each protein well using a nanoliter robot.
5. Seal with tape, and incubate at 18-22°C for 3 h.
6. Dispense 50 µL each of the 96 ***undiluted*** precipitant solutions into the reservoir wells of the CubeCrystal Plate using a micropipettor or pipetting robot.
7. Dispense 100 nL each of the 96 ***diluted*** precipitant solutions from the spare deepwell block into the corresponding protein wells of the CubeCrystal Plate using a nanoliter robot.
8. Seal with tape, and incubate at 18-22°C.
9. Monitor the plates on a regular basis using a microscope. During the first two weeks, a daily inspection is recommended.

Note: Do not unpack the plate before reaction setup.

Tip: For optimization, test two different protein concentrations in the two parallel wells.

Note: The CubeCrystal 2-well MO plate is based on the Swissci MRC 2-well plate, so that similar teaching protocols can be used.

Note: It is crucial to incubate at no less than 18°C in all steps.

References:
1. Kubicek J., Schlesinger, R., Baeken C., Büldt, G., Schäfer, F., Labahn, J. (2012) Controlled in meso phase crystallization - a method for the structural investigation of membrane proteins. PLoS One 7(4):e35458.