A new method for automated crystallization of membrane proteins

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High-throughput crystallization of membrane proteins

Approximately 30% of a mammalian genome encodes for membrane proteins. Membrane proteins are one of the most important protein classes and defects in membrane proteins are implicated in a number of serious diseases.

Membrane proteins constitute around 50% of possible novel drug targets. However, despite their essential functions, the information available on membrane protein structures is very limited.

Crystallization is the bottleneck for drug design based on membrane protein structures. Currently two techniques are used: Crystallization methods for soluble proteins that are often inadequate for membrane proteins in general; and crystallization in cubic phase.

The cubic phase of mono-olein (MO) is a bi-continuous lipidic phase resembling natural membranes. Its 3-dimensionality allows the membrane protein to diffuse as required to form 3D crystals. Currently, cubic phase crystallization is frequently performed in batch mode, requiring large amounts of protein.

There is a need for a new solution for membrane protein crystallization:

- Small amounts of folded, functional protein
- Automatable dispensing of protein for screening

96-well format crystallization plates can be used as a basis for automated nano-volume crystallization of membrane proteins. Wells should contain a pre-dispensed volume of MO that may be enriched by a precipitating solution pre-dispensed into the reservoir wells of the plate. Therefore, it is possible to have up to 96 simultaneous screening experiments. Furthermore, the effect of the precipitating solution on the cubic phase (forced induction of phase separation) and on the protein are controllable separately, which is impossible with the current batch method used.

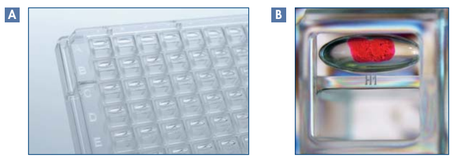
The NeXtal® CubicPhase System

The NeXtal CubicPhase system enables fully automatable, high-throughput membrane protein crystallization setup. Easy manual setup of the experiments is also possible using a multichannel pipet. The system utilizes the advantages of vapor diffusion crystallization together with those of *in meso* crystallization, which targets the phase transformation point from cubic to lamellar phase as well as crystallization in presence of excess water or sponge phase.

The NeXtal CubicPhase Kit consists of two components:

- Crystallization µplates:
  - Extra evaporation-tight NeXtal CubicPhase µplates delivered prefilled with mono-olein that enable automated setup of the meso-phase experiment upon hydration with membrane protein solution
- Two sets of 96 dedicated screening solutions optimized for in meso experiments and successfully used for membrane protein crystallization:
  - NeXtal CubicPhase I Suite: 96 variations of buffered solutions with differing added salts (it contains no other components, such as organics or PEGs). pH variation and the ionic strength are chosen to fit to the *in meso* phase experiments.
  - NeXtal CubicPhase II Suite: Uses different molecular weight PEGs at set pH as precipitating agents.

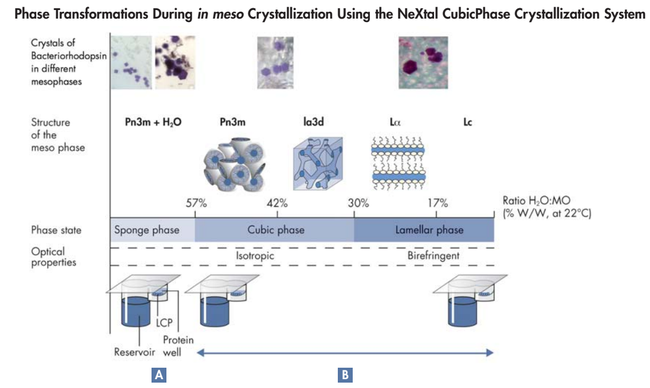
It is possible to use the CubicPhase Crystallization µPlate with other screening solutions.[1]



Features of the NeXtal Evolution Phase µplate. These include a groove for easy cutting of tape, a broad rim to avoid evaporation, and a unique "half American football" shaped protein well. A The NeXtal Cubic Phase µplate. B Top view of the oval-shaped well coated with MO. For better visualization, the MO has been colored with a red dye.

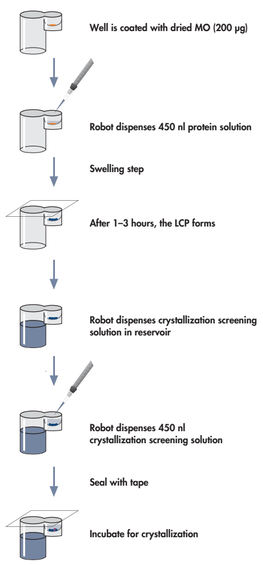
Phase transformations during *in meso* crystallization using the NeXtal CubicPhase Crystallization System

Phase transformation points vary according to the components of the crystallization solution



Phase transformations in meso-phase experiments. A Lipids such as MO have the ability to form complex phases with aqueous solutions, dependent on temperature and the water:mono-olein ratio. These phases are named based on the crystallographic characteristics. The starting point of the crystallization experiment is a mixture of MO and excess aqueous solution (protein and precipitating agent) called the sponge phase. B The vapor diffusion from the protein well to the reservoir increases the concentration of protein and precipitant within the drop and triggers a transformation of the meso phase. Depending on when the water pressure equilibrium between the protein well and the reservoir is reached, the structure of the meso-phase reached will be anywhere from sponge phase to lipidic-cubic phase (LCP) to lamellar phase. It is possible to distinguish which phase is reached by examining the optical properties of the protein well. The sponge and LCP structure are isotropic, whilst the lamellar phase displays birefringent properties. Adapted from Caffrey.[2]

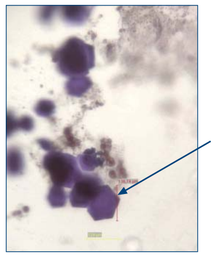
NeXtal CubicPhase workflow



These are some important points to consider before starting membrane protein crystallization with the NeXtal CubicPhase System.

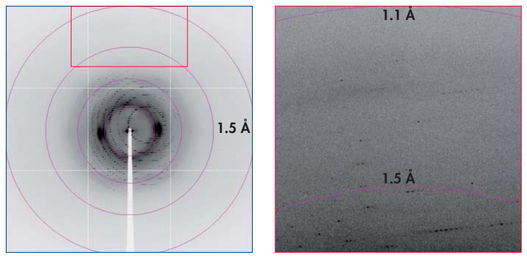
- Protein concentration 5–35 mg/mL. Large volumes with low concentration possible. The detergent must allow the formation of cubic phase
- Reduces chance of surface precipitation of protein
- The chosen mixing ratio must allow a sufficient volume reduction (dehydration) to reach the desired phase at the equilibrium point
- Is formation of cubic phase observed?
- Look for appearance of lamellar phase

Crystallization of Bacteriorhodpsin provides high-resolution data



High-resolution data measured in Grenoble ESRF.
Bacteriorhodopsin (BR) crystallization conditions: $Na/KPO_4/H_20$
Screen: 0.2–3.2 M Na/KPO_4; pH 4–10

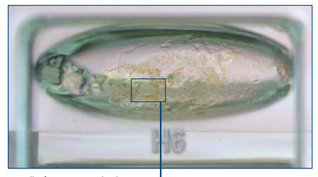
Purple BR protein crystal



Left: BR diffraction data

Right: Detail from left picture showing the diffraction limit at 1.1 Å

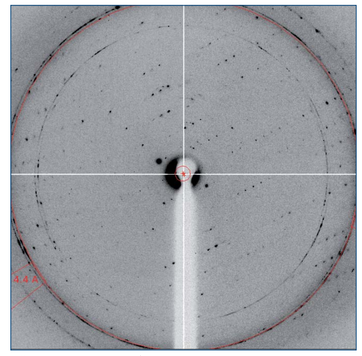
Structural analysis of a new Sensory Rhodopsin (SR): Crystallization and diffraction data



1 well of a Sensory Rhodopsin crystallization experiment in a 96-well NeXtal Evolution µplate.

SR crystals at microscopic magnification.

Reaction setup: 0.2 µL MO; 0.45 µL protein; 0.45 µL buffer; 70 µL precipitation reservoir.



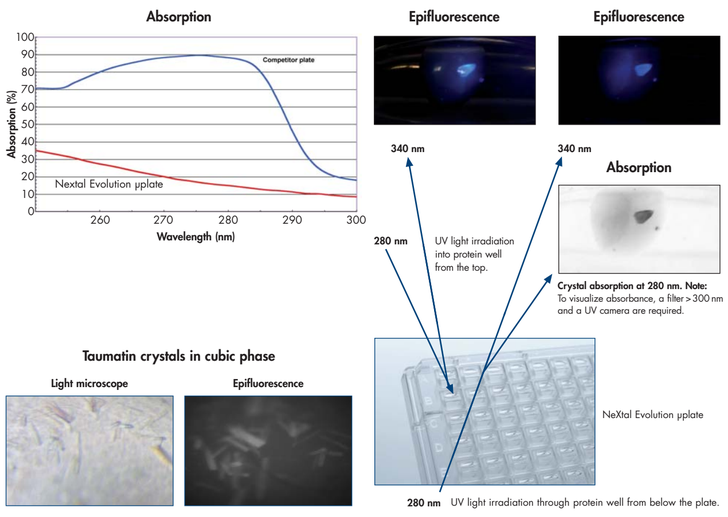
Diffraction data of SR at 3.6 Å resolution.

ERSF/ID14-1 031108 mx724-b2s4_3_001
sym: Immm
cell: a=42.6 A b=114.8 A c=134.1 A
310708-B12 cMO direct

Solving the structure is ongoing ← **3.6 Å resolution (Grenoble synchrotron)**

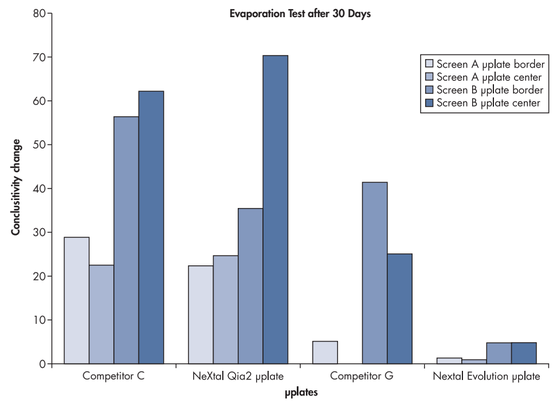
Crystal detection with the NeXtal Evolution µplate

The NeXtal Evolution µplate is transparent at UV 280 nm wavelength



Evolution µplate design eliminates evaporation

The NeXtal Evolution µplate design dramatically reduces evaporation in all 96 wells of the plate. This is in contrast to all other existing types of commercial crystallization plates. In the first crystallization experiments, we observed significantly increased reproducibility of protein crystal formation.



Buffers for evaporation test:

Screen A (Buffer 9): 0.1M Tris pH 8.5; 35% MPD; 0.2M ammonium sulfate

Screen B (Buffer 46): 0.1M sodium cacodylate pH 6.5; 35% isopropanol; 0.2M magnesium chloride

Conclusions

- We have developed a crystallization plate – the NeXtal Evolution µplate – with several advantages for vapor diffusion:
  - Broad rim to avoid evaporation
  - Groove for cutting of tape – enables one experiment to be opened without damaging surrounding experiments
  - Unique protein well shape – enables easy removal of small crystals
  - Transparency of plate at 280 nm
- We developed a new method for automated *in meso* crystallization (sponge-, cubic-, and lamellar-phase screening) :
  - Crystallization screens have been tested for compatibility with the different structures of the *in meso* phase
  - NeXtal CubicPhase µplates for simplified screening for the optimal crystallization conditions – compatible with all available liquid handlers
  - Two new crystallization screens have been tested for compatibility with the different structures of the meso phase
  - Tailored setup for both *in meso*- and sponge-phase crystallization
  - Crystallization and structural analysis of Sensory Rhodopsin – a new 7 transmembrane protein.

References

1. Cherezov, V., Fersi, H., Caffrey, M. (2001). Crystallization screens: compatibility with the lipidic cubic phase for in meso crystallization of membrane proteins. Biophys. J. **81**, 225.
2. Caffrey, M. (2008). On the mechanism of membrane protein crystallization in lipidic mesophases. Cryst. Growth Des. **8**, 4244.

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