CRYSTALLIZATION OF MEMBRANE PROTEINS IN MICROBATCH

Chayen, N.E. and Saridakis, E. (2008) *Nature Methods,* 5, 147-153.

An increasing number of membrane proteins in a variety of different detergents have been crystallised in microbatch under oil. Some of these had failed to crystallise by all methods other than microbatch. Dispensing is quick and simple even when performed manually and the drops in oil do not spread out as they do in vapour diffusion over the siliconised coverslips. Using robots thousands of microbatch trials can be dispensed in high throughput mode in nanolitre volumes.

The microbatch can be used for both screening and for optimisation of membrane proteins. The protocol for setting up microbatch experiments containing membrane proteins is identical to that described in exercise 2.

Exercise 3: Harvesting and mounting crystals from microbatch

Chayen N.E. (1998) *Acta Cryst* D54, 8-15

Harvesting crystals from microbatch is slightly more difficult than harvesting from coverslips or from standard sitting drops. However after some practice it can be achieved easily.

Two alternative ways of harvesting crystals from microbatch:

Materials required

- Cryoprotectant solution
- Precipitate solution at ~ 5 % higher concentration than that in the drops
- Micro tools (Hampton Research)
- Standard pipette
- Scalpel
- Loops
- Depression plates

Method

1. Add a few microlitres of cryo-protectant solution to the drop containing the crystals.
2. After several minutes check that the crystals are not cracked or dissolved by looking at them under the microscope. If they crack/dissolve, adjust the concentration of cryo-protectant or change cryo-protectant.
3. Take the crystals directly out of the oil using a loop and freeze.

If the above protocol proves tricky, harvest in the following way:

1. Add harvest solution (of ~5% higher concentration of precipitant than that in the drop) into the well containing the crystals. If you have a 1 l drop, add 5-10 l of harvest solution.
2. Wait a short while (up to 15 minutes) to allow the crystals to equilibrate.
3. Withdraw the enlarged drop using a standard 10-100 l pipette which had its tip cut off with a scalpel in order to widen its bore.
4. If the crystals stick to the vessel, loosen them gently inside the drop using micro tools or very thin strips of filter paper (the edge of the strip that will touch thecrystal is best torn rather than 'cleanly' cut with scissors).
5. Transfer the drop into a depression well containing more harvest solution.
6. From this stage onwards, handle the crystals as you would be from a standard diffusion trial.

Exercise 4: Containerless crystallization

Chayen N.E. (1996) *Protein Engineering* 9, 927-929.
Chayen N.E. and Saridakis E. (2002) Lead Article Acta Cryst. D. 58, 921-927.

Purpose of this experiment: (1) to aid harvesting **(2)** to reduce the amount of nucleation in a trial by eliminating the effects of surface contact between the crystallization trial and its supporting vessel.

Materials required

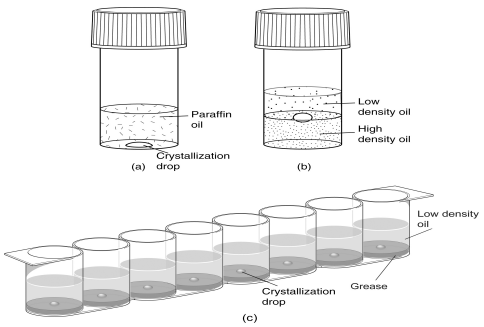
- "Gelled Surface" kit from Molecular Dimensions, UK (figure c)
- Paraffin oil
- Lysozyme 40 mg/ml in Na acetate, pH 4.7, 12% NaCl from the previous experiments

Procedure

- Pipette the paraffin oil into the wells of the Gelled-Surface plate.
- Prepare the protein/precipitant drops as described for microbatch experiments.
- Insert the pipette tip into the well, under the surface of the paraffin oil, and dispense the drop.
- Experiment with different size drops e.g. 1-20 l.

You can also try dispensing a drop under oil (figure a) and compare with a drop dispended between two oils of different densities (Figure b).

Try the method using your problem proteins that are giving you many small unusable crystals.



Exercise 5: Filtration experiments

Chayen N.E., Saridakis E., Sear, R.P. (2006). *PNAS* 103: 597-601

Purpose of this experiment: Filtration will remove particles (dust, protein aggregates, fungi, bacteria, etc) and thus reduce the amount of nucleation in the trials leading to the growth of fewer larger crystals.

Materials required:

- Centrifugal filters: 0.2 µm 0.1µm
- http://www.milipore.com/catalogue/item/ufc30vv25 or
- http://www.millipore.com/catalogue/item/ufc30vv00
- Bench centrifuge.
- 40 mg/ml lysozyme stock
- 12% NaCl

Procedure:

1. Mix 150 l of lysozyme at 40 mg/ml with 150 l precipitant (12% NaCl in 10 mM acetate buffer pH 4.7) in a microcentrifuge tube.
2. Divide the mixture into 3 aliquots of 100 l each:
   (a) leave one aliquot unfiltered
   (b) filter the second aliquot through a 0.22 µm filter
   (c) filter the third aliquot through a 0.1 µm filter
   Filtration is performed by placing the filters in a bench centrifuge at 2-9 g for 1 – 2 minutes.
3. Dispense droplets of each aliquot for crystallization under the oil.

Expected results:

You will get a number of crystals in the unfiltered solution, a smaller number of larger crystals in the solution which was filtered with a 0.22µm filter, and very few or no crystals at all in the solution which was filtered through the 0.1 µm filter.

Warning:

- You need to filter a minimum of 40 µL in order not to lose any protein.
- Do not use the filtration method if your protein is sticky

Exercise 6: Improving crystal quality by separating nucleation and growth in hanging drops (not using oils!)

Saridakis, E. and Chayen, N.E. (2003) *Biophys J.* 84, 1218-1222.
Chayen, N.E. (2004) *Current Opinion in Structural Biology* 14, 577-583.
Chayen, N.E. (2005) *Progress in Biophysics and Molecular Biology* 88, 329-337.

This is a method to get nucleation "going" and then "back off" before the nucleation becomes excessive. It can be used for optimisation when small useless crystals are formed, and improvement can not be obtained neither by fine-tuning the concentrations of the protein and precipitating agents nor by addition of additives. In practice, cover-slips holding the drops are incubated for some time over reservoir solutions that normally give many small crystals and after given times the cover-slips are transferred over reservoirs with lower precipitant concentrations that would normally yield clear drops. This method can also be used for screening.

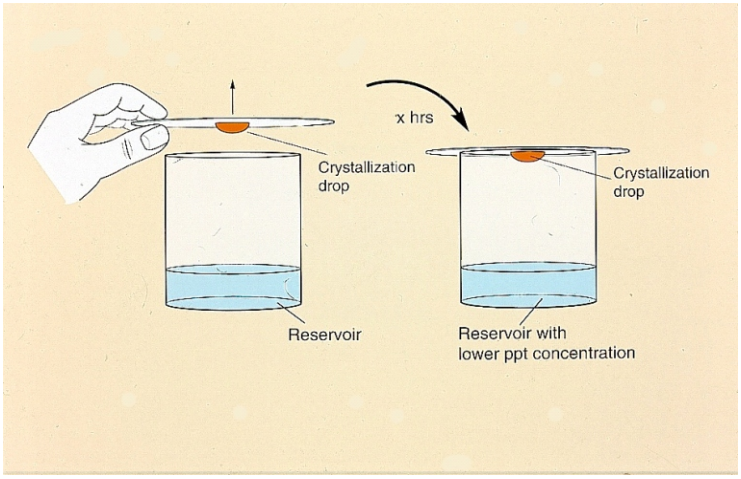
Example for optimisation:

Materials needed:
Trypsin from porcine pancreas(Sigma catalogue no: T-0134)dissolved in de-ionisd water at a concentration of 40 mg/ml.

1 M Tris pH 8.4
3M ammonium sulphate
Mineral oil (viscosity of ~5) to put round the edge of the Linbro plates for sealing the cover-slips (not the thick Vaseline usually applied as a seal).
Standard Linbro plates or pre greased plates and siliconised cover-slips or EasyXtal Tools (From Qiagen).
Pasteur pipette with rubber squeezer or plastic squeezer pipettes.

Procedure:
1) Set up 6 trials under conditions that would give you low quality crystals, in this example it is reservoir solutions containing 1.7M ammonium sulphate and 0.1M Tris pH 8.4. Dispense those into 6 wells of a plate.

2) Prepare 6 reservoirs with solutions containing precipitant concentration that would result in producing a clear drop if crystallization drops were set up and left to incubate under these conditions. In this example it is reservoir solutions containing 1.3M ammonium sulphate and 0.1M Tris pH 8.4. Dispense those into 6 wells of another row in the same plate.



3) Set up hanging drops by taking equal volumes (recommended 1 microlitre + 1 microlitre) of the 40mg/ml protein solution and mixing it with the well solutions containing the 1.7 M ammonium sulphate.

4) After 2 hours transfer one of the cover-slips from over the wells containing the 1.7M ammonium sulphate and just place it over one of the wells which contain 1.3M ammonium Sulphate (Figure). The transfer should only take 1-2 seconds.

5) Repeat the same with the other drops after 3, 4 and 6 hours.

6) Leave 2 drops at the high concentration (1.7M) as controls

7) set two drops at the low concentration (1.3M) as controls.

8) Observe the results each day for two weeks.

Expected results:

The drops which are left over the reservoirs containing 1.7 M ammonum sulphate will have clusters of crystals overnight. The drops hung over the 1.3M should be clear. The drops transferred after 4 or 6 hours should produce large single crystals after 8-14 days.

Tip: The time of transfer is selected by reference to the time in which it took to see the first crystals in the trials producing the poor crystals. In the case of trypsin, crystals appeared within 24 hours meaning that nucleation would have occurred anytime between set up of the experiments to several hours before the crystals appeared. Hence transfer was done at intervals of 2 hours after set up. Trials that are transferred too soon will produce clear drops while those that are transferred too late will yield low quality crystals. In cases of other proteins where crystals take a few days to appear, the transfers should be performed at longer time intervals e.g. every 12 hours or so.

For screening:

3D structure screen by (Molecular Dimensions, UK, MD1-13)

This screen consists of two sets of solutions: 24 contain sparse matrix screening conditions; the other 24 are a 70% dilution of the precipitants in those solutions (buffer and additive concentrations remain the same). Using this screen, the hanging drops are first incubated for 3-6h over the solutions at high concentrations. The cover-slips holding the drops are then transferred over the reservoirs at 70% dilution.

Exercise 7: What to do when you get no crystals?

Khurshid, S., Govada, L. and Chayen, N.E. (2007) *Crystal Growth & Design* 7, 2171-2175.

This is a screening technique used in vapour diffusion hanging drops when trials remain persistently clear.

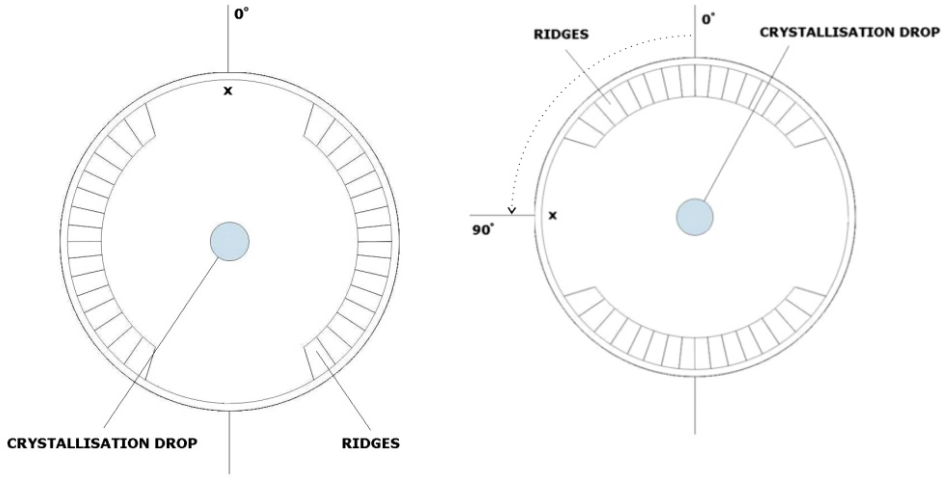
Materials required:

1. EasyXtal Tools Made by Qiagen
2. Screens of your choice
3. Your protein solution

1. Set up your screening as you would normally do
2. Loosen the screw caps of drops that remained clear after 2 weeks by 90° (see diagram). The angle of cap allows variable amounts of evaporation without exposing the drops
3. Observe the drops every 2 hours in the first day and re-seal the caps when the drops visibly shrink.

Expected results:

By loosening the cap you are allowing evaporation for a limited time. What you are effectively doing is inducing nucleation which you then arrest when re-sealing. This will drive the system into supersaturation and hopefully give you a hit which you can then proceed to optimise using the technique of your choice.



The method can also be used for optimization as described in Govada, L. and Chayen, N.E. (2009) *Crystal Growth & Design* 9, 1729-1732.

INTRODUCING NUCLEANTS INTO CRYSTALLIZATION TRIALS

Chayen N.E., Saridakis E., Sear, R.P. (2006) *PNAS* 103, 597-601
Saridakis, E. and Chayen, N.E. (2009) *Trends in Biotechnology* 27, 99-106
Chayen, N.E. and Saridakis, E. (2008) *Nature Methods*, 5, 147-153.

First, determine the metastable zone as follows:

1. Note the conditions in a 'hit' of a screen that have yielded poor crystals or a
crystalline precipitate.

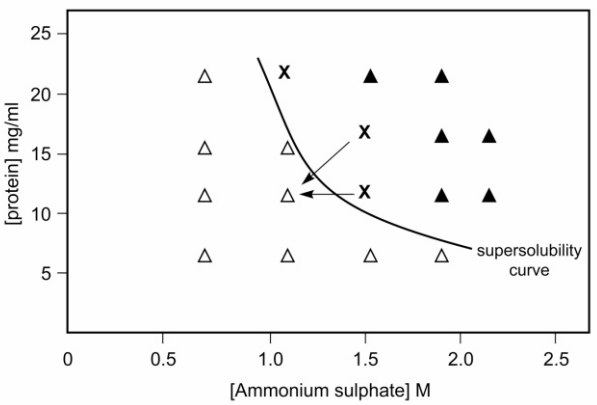
2. Dispense (manually or by a robot) 10-24 trials using a crystallization method of your choice, varying the protein and precipitant concentrations in steps (as instructed in point 3 below) on a matrix grid.

3. The concentrations of protein and precipitating agents should be above and below
the concentrations that gave the conditions of the hit. Alternatively the pH,
temperature, or another parameter to which solubility is sensitive can be varied.

4. Plot the results and you will obtain the supersolubilty curve. e.g. diagram below.:For example, if a screen produced 'hits' at conditions of 10-22 mg/ml of protein and 1-1.5 M Ammonium sulphate, the experiments are set at concentrations ranging from 5-25 mg/ml protein versus 0.5-2.2 M ammonium sulphate, thus covering a range of conditions above and below the 'hits. The area just below the curve is the metastable zone. **X** represent conditions obtained from screening 'hits', giving low quality crystals or crystalline precipitate. Δ represent clear drops; ▲ represent precipitate.

Insert your nucleant at conditions just below the curve (where the heads of the arrows are pointing) using fine tweezers and let the trial incubate as you would normally do.

Nucleant are used mostly for optimization but can also be used for screening. When nucleants are placed in supersaturated conditions, crystals will usually appear faster.



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