

Exercises - instructions

SEEDING LAB EXERCISES

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In this lab session we will be doing two exercises:

1. Instantaneous streak seeding varying the protein concentration.
2. Comparison of jab and streak seeding with overnight equilibration.

Reference:

For a review article on seeding, see: Bergfors, T. "Seeds to Crystals" J. Structural Biol. 2003, vol. 142, 66-76

1. What is seeding?

The use of an existing nucleus (usually a small crystal or crystal fragment), introduced into a new drop, where it acts as a growth site.

2. Why seed?

- To separate nucleation from growth and bypass the need for spontaneous nucleation. It is easier to add onto an already existing nucleus than create one *de novo*. See Fig. 1. **Seeding and the phase diagram.**

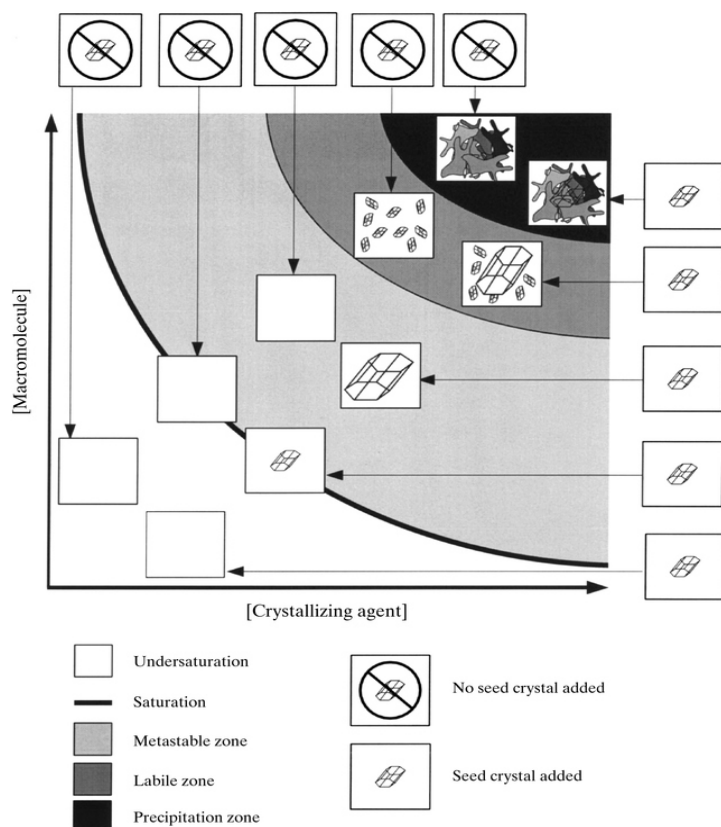


Figure 1: Seeding and the phase diagram. From Luft and DeTitta, Acta Cryst. (1999) D55, 988-993.

- Improve the size of the crystals or control the number of crystals
- Get more consistent results when crystals don't always appear in known conditions
- Speed up results if spontaneous nucleation is slow
- To obtain a wider range of crystal forms (polymorphs) by seeding into totally different precipitants

3. What are the types of seeding?

- **Macro seeding** is the transfer of a single, pre-grown, washed crystal.
- **Micro seeding** is the transfer of microscopic crystals, crushed up into fragments.
- **Streak seeding** is a form of micro seeding that transfers the microseeds by a stroking motion with a whisker or hair of some sort. See Fig. 2. **Streak seeding.** Can be used on its own or in combination with microseeds in a dilution series.
- **Jab seeding** is another variation on micro seeding where the new drop is "innoculated" with a single jab of the seed transfer tool.

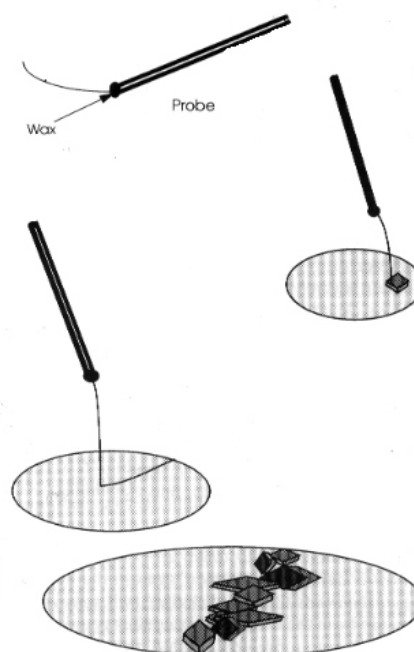


Figure 2: Streak seeding from Enrico Stura, Published in "Crystallization of Nucleic Acids and Proteins" Edited by Ducruix and Giege, 1992, Oxford University Press.



Experiment 1: Instant streak seeding

Reference: This exercise has been adapted from **Enrico Stura's** chapter "Seeding" in **Protein Crystallization: Strategies, Techniques, and Tips** edited by T. Bergfors 1999 International University Line, La Jolla Ca.

Purpose of this experiment:

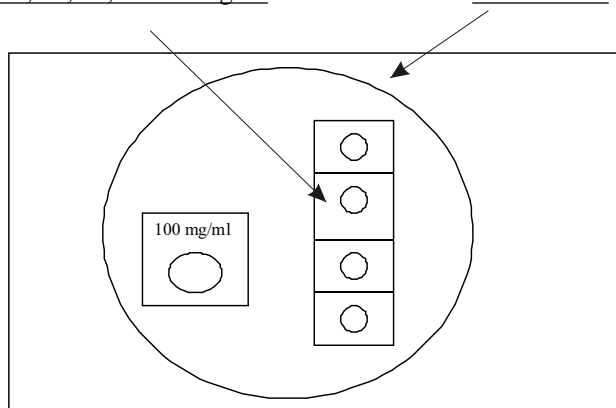
1. To learn how to generate new seeds by the easy, fast, and simple method of streak seeding.
2. To observe the effects of decreasing protein concentration on the nucleation rate.

Materials needed:

- a crystal wand — available from Hampton Research (HR8-133) or you can make your own from a cat whisker, human hair or horse tail hair, super glue or wax to affix the whisker to a yellow pipette tip, and a razor blade to cut the whisker.
- lysozyme 100 mg/ml stock solution.
- A dilution series of lysozyme at 80, 60, 40 and 20 mg/ml (already prepared for you.)
- 30% polyethylene glycol (PEG) 5000 or 6000 in 1 M NaCl, 50 mM Na acetate, pH 4.7 = the precipitant (ready-mixed for you today).
- A surface for making the drops, e.g., a Petri dish lid, cover slip, etc.

Method:

1. On the Petri dish lid, pipette 10 μ l of lysozyme (100 mg/ml). Now add an equal volume (i.e., 10 μ l) of the precipitant (= 30% PEG 6000 in 1M NaCl, 50 mM Na acetate, pH 4.7).
2. Watch the drop under the microscope. The first thing you should see is a phase separation. After that, the crystals should nucleate in 5-15 minutes. The fresher the lysozyme the longer time it will take to nucleate, so the time will vary. It can take some practice to recognize the nucleation in its initial stages. Note: If the protein precipitates immediately, the protein concentration is too high. Redo with a slightly lower protein concentration; try 80 mg/ml.
3. Once the parent crystals have formed in the drop with 100 mg/ml, you can set up the 10 μ l drops of lysozyme at 80, 60, 40, and 20 mg/ml on the inside of a Petri dish lid.



4. **Add 10 μ l of the precipitant to each of these 4 new drops and stir. Important!** These drops also need precipitant, not just protein, or the experiment does not work. Students often forget this step.

5. Now you can transfer the nuclei (from the parent drop) by streak seeding. Dip the seeding wand once into the drop of parent crystals to pick up the seeds, then streak the wand across the four new drops. You do not need to re-dip the wand into the parent drop for each new drop.

What to look for or think about:

- See if you can get the crystals to grow along a streak line. Because of the high protein concentrations you will also get spontaneous nucleation but many crystals will form preferentially along the streak line. The crystals will be extremely small because they grow so rapidly. In this experiment the protocol has been optimized for speed, rather than crystal size.
- You don't need to save these drops overnight, but we will use them in the next experiment. In the current experiment we demonstrated the method and the principles for streak seeding. In the next experiment, a more refined version will be presented.

Experiment 2: Comparing the effects of adding different amounts of seeds.

Purpose of this experiment:

- **To illustrate the effect of adding different amounts of seeds.**
- **To illustrate a very fast and easy way of creating a dilution series of seeds.**

In contrast to experiment 1 above, this experiment will be allowed to equilibrate overnight to permit slower growth of the crystals after seeds are added. Three rows of identical drops will be prepared. Seeds will be added by either *jabbing* the drops or *streak seeding* them. These drops will be compared to a control where no seeds have been added. An entire row of drops will be seeded sequentially to create a dilution of the seeds. Thus, the first drop will have the most seeds and the last drop in that row will have the least.

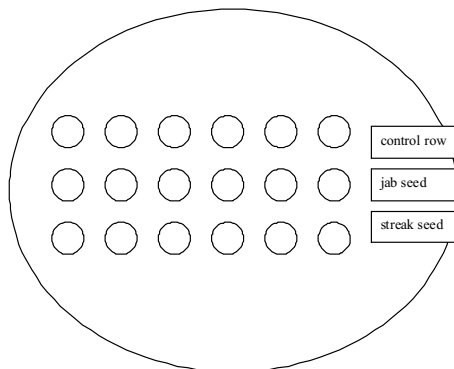
Materials required:

1. two 20 ml Petri dishes (the large size)
2. 5 ml of 6% NaCl in 50-100 mM sodium acetate buffer, pH 4.7
3. 5 ml of 12% NaCl in 50-100 mM sodium acetate buffer, pH 4.7
4. 100 microliters of 20 mg/ml lysozyme, dissolved in water
5. a seeding wand (you can use the same one from the previous experiment.)
6. two eppendorf tubes

Method:

1. In an eppendorf tube, mix 50 microliters of lysozyme (20 mg/ml) with 50 microliters of 6% NaCl in 50-100 mM sodium acetate buffer, pH 4.7 = MIXTURE A.

2. In another eppendorf tube (mark them, so you don't mix them up!), mix 50 microliters of lysozyme (20 mg/ml) with 50 microliters of 12% NaCl in 50-100 mM sodium acetate buffer, pH 4.7 = MIXTURE B.
3. In one Petri dish, pour approximately 5 ml of the buffered 6% NaCl solution in the bottom = PLATE A.
4. Repeat for the other Petri dish, using buffered 12% NaCl instead = PLATE B.
5. Using MIXTURE A, pipette 3 rows of 6 drops each onto the lid of PLATE A. Each drop will contain 5 microliters.



See picture:

6. Repeat for MIXTURE B and PLATE B.
7. Row 1 will be the control.
8. Row 2 will be *jab seeded*. Using the seed transfer tool, dip or stir it into the parent drop in the previous experiment (experiment 1) to pick up seeds.
9. Now jab all the drops in row 2 with the seed transfer tool. To do so, barely touch the outermost edge of the

drop with the tool = a quick jab. Rinse the tool in the reservoir solution between each jab to create a dilution series of the seeds.

10. Row 3 will be *streak seeded*. Rinse the seed transfer tool thoroughly and wipe it off with a tissue. Dip or stir it into the parent drop in the previous experiment (experiment 1) to pick up a new batch of seeds.
11. Now streak seed the drops in row 3 with the seed transfer tool. Rinse the tool in the reservoir solution between each streak to create a dilution series of the seeds.
12. Invert the lids of the Petri dishes over the correct, respective reservoirs and seal the rims with parafilm.
13. Wait 24 hours to observe the results.

Expected results:

1. After 24 hours, the control row in PLATE A will probably be still clear (but crystals will grow there after 48 hours, so it is best to examine the plates before that). In PLATE B, you probably will see sea urchin-type crystals by 24 hours.
2. The rows with jab seeding and streak seeding will give different effects. Jab seeding works best if the drop is very large, because there is a dilution effect across the surface of the drop. The drops here are only 5 microliters. Once back home, try the effect on large sitting drops (40 microliters) to make it more pronounced.
3. Jab and streak seeding several drops in a row (serial seeding) is a fast and easy way of making a dilution series of seeds in the new drops. The drops seeded last (by either method) should show the least number of crystals compared to the first drops in the row (**Figure 3**).

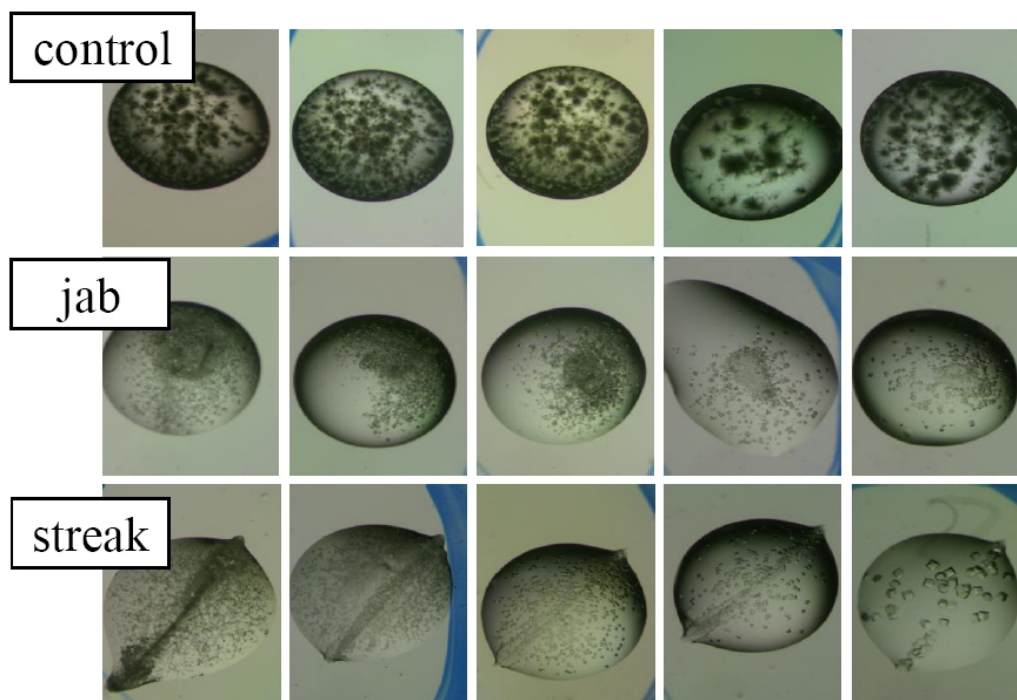


Figure 3. Comparison of drops that have been serially seeded with a control. These results were photographed 48 hours after setup. Row 1 is a control and has not been seeded. The lysozyme crystallizes in the form of sea urchins or spherulites. Row 2 has been jab seeded. The needle inoculated with seeds was jabbed into the center of the drops. Row 3 has been streak seeded. Notice that crystals tend to form along the seed line, but there is much spontaneous nucleation even outside the streak line in the first drops. However, the final drop in the row (drop 5 in the series) has nice, large crystals. This effect was achieved because the number of seeds added to the drop was diluted by passing the streak wand through the first drops in the row.



CRYSTALLIZATION UNDER OIL

Naomi Chayen

Imperial College London

Dear Course Participants,

I have written down some of the methods that I will talk about in the workshop in the form of exercises, to give you step by step protocols of setting up the experiments.

The methods are mostly for optimization – in cases that you get poor crystals that need improvement. Some can also be applied for screening as mentioned in the specific protocols.

These methods have been successful in yielding diffraction quality crystals of a variety of proteins in cases where conventional methodology failed. I have used lysozyme and trypsin as models in some of the protocols in order to have examples and results that you can see within a short time, but of course, the aim is to use these techniques with your problem proteins.

There is no magic bullet to solve all the crystallization problems however having a portfolio of different techniques is very helpful.

GOOD LUCK!

Naomi

Exercise 1: Insertion of oil barrier to slow down vapor diffusion experiments

Chayen N.E. (1997) *J. Appl. Cryst.* 30, 198-202

The purpose of this experiment is to improve the size and quality of crystals which are obtained as showers of small useless crystals, twinned crystals or precipitate. The experiment also demonstrates the effect that oil-over-the-reservoir has on the rate of equilibration (and subsequent effects on crystal growth.)

Materials (can be purchased from Hampton Research, Molecular Dimensions and other companies selling crystallization kits and tools)

- Crystallization plates for hanging or sitting drops
- cover slips
- grease or sealing tape
- silicone oil
- paraffin oil
- Al's oil
- Protein solution
- Crystallization reagents

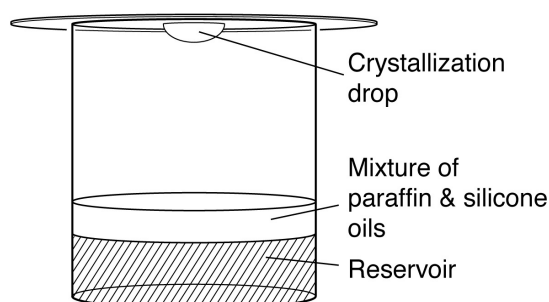
Method for Preparation of the oils

1. Mix paraffin and silicone oils in equal volumes.
2. Shake well and allow to stand for several minutes.
The oils are totally miscible once the bubbles have disappeared.

Method for setting up hanging drops

1. Use a Linbro type plate type for hanging drops.
2. Coat the lips of the reservoirs with grease or oil (unless your plates are pre-greased).

3. Pipette 0.6 - 1 ml of the reservoir solution which gives you the showers of crystals into each well.
4. Pipette volumes ranging from 0.1 ml to 0.5 ml of a mixture of paraffin and silicone over all the reservoirs except for one reservoir. The oil will form a layer above the reservoirs (figure).



5. Dispense the hanging drops on the coverslips as usual by mixing the protein solution with the reservoir solution. Use the reservoir without oil as your source of precipitant for all the drops.
6. Invert the cover slips and place over the wells containing the oil layer.
7. Place the last drop over the reservoir without the oil. This drop will act as your control.
8. Incubate at the temperature of your choice.
9. If the quality of the crystals is not sufficiently improved, repeat the protocol using different ratios of paraffin and silicon.

Method for sitting and sandwich drops

In the case of sitting and sandwich drops, set up the trials as you would normally do and place the layer of oil above the reservoir before sealing the plates with tape.

Warning: This technique does not work with PEG or MPD concentration above 13% but is very effective at concentrations below 13% and at all concentrations of all salts.

Expected results:

Wait patiently for the results because in trials containing an oil barrier, crystals require longer periods (e.g. 8-10 days compared to 12-24 hours) to grow to full size, but their quality is improved.

Exercise 2: Setting up microbatch trials

Chayen *et al* (1992) *J.Cryst. Growth* 122, 176-180.
D'Arcy *et al* (1996) *J.Cryst. Growth* 168, 175-180
Chayen N.E. (1997) *Structure* 5, 1269 - 1274.

Purpose of experiment: To employ a method of crystallization with different inherent qualities to that of diffusion methods. The method is used for screening and for optimisation.

Materials required:

1. Three microbatch plates; also known as HLA, Terasaki or microtiter plates available from Hampton Research, Nunc, Molecular Dimensions, etc.
2. Low density oils:
 - Paraffin oil
 - Silicone oil
 - Al's oil

Proteins and buffers required:

1. Lysozyme at 40 mg/ml in 10 mM sodium acetate buffer pH 4.7
2. Precipitant: 12% NaCl in 10 mM sodium acetate buffer pH 4.7

General procedure for setting up microbatch manually:

1. Pipette or dispense 5 ml of paraffin oil into a microbatch plate. The oil will spread over the plate and cover the wells.
2. Using a Gilson P2 or similar pipette, withdraw 1 μ l of the precipitant solution.
3. Insert the tip into the well under the surface of the oil and dispense the 1 μ l drop. If you find it difficult to hold the tip in mid-oil, you can rest the edge of the tip on the floor of the plate as you dispense. As you withdraw the tip from the oil, the drop will detach from it and fall to the bottom of the well.
3. Now add in the same way 1 μ l of protein solution to that well and mix gently with the pipette tip. The two (separate) 1 μ l drops join and become a 2 μ l drop.
4. Incubate at the temperature of your choice.
5. Observe trials regularly under a light microscope.

Microbatch is mechanically the simplest crystallization method and is therefore increasingly applied for high throughput trials especially for screening.

Example with lysozyme

1. Fill the microbatch dish with 5-6 ml of paraffin oil.
2. **Lysozyme:** Make 3 rows of drops. In the first row, the drop size will be 0.5 μ l 40 mg/ml lysozyme + 0.5 μ l 12% NaCl.
3. In the second row, repeat, with 1 μ l + 1 μ l.
4. In the third row, repeat with 4 μ l + 4 μ l.
5. Repeat the experiment in two identical dishes, one with 5 ml of silicone oil, one with 5 ml of a 50:50 mixture of paraffin:silicone and another with 5 ml of paraffin oil.

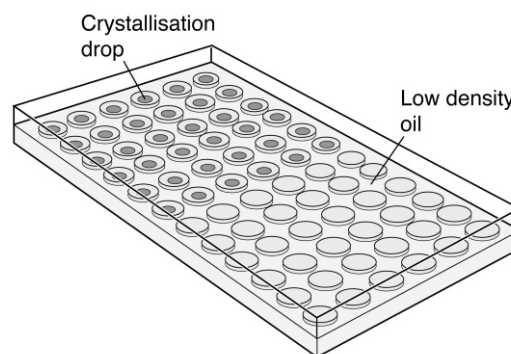
Tip: Instead of mixing the precipitant (NaCl) with the protein in the plate as described above in the general procedure, you can also pre-mix the two ingredients in an eppendorf tube then add the ready made drops of 1 μ l, 2 μ l and 8 μ l respectively to the plate.

Expected results:

The lysozyme crystals will appear over 2 to 7 days. See if the size of crystals varies as a function of drop size in the lysozyme experiment.

The silicone oil should give faster results and paraffin oil the slowest.

There are several robots for setting up screening experi-



Method for setting up using a robot

ments in microbatch. The precipitant solutions are transferred simultaneously from stock screening solutions to crystallization plates by any number of syringes depending on the robotic system. The drops are dispensed under oil and the protein is added to the precipitant drops using a dedicated syringe for the protein solution either simultaneously with the screening solutions or at a later stage. Some of the robots have a routine of mixing the drops.

Tip: For screening purposes it is preferable to use silicone oil or a mixture of paraffin and silicone oils. This allows some evaporation of the drops leading to a higher number of 'hits' and faster formation of crystals compared to trials which are set under paraffin oil. In the case of optimization, where the conditions need to be known and stable, the trials must be covered by paraffin oil.

N.B. Microbatch can be used for almost all the known precipitants, buffers and additives including detergents. The oils do not interfere with the common precipitants such as salts, polyethylene glycols (PEG), jeffamine MPD and even glycerol and ethanol. Microbatch, though, can not be used for crystallization trials containing small volatile organic molecules such as dioxane, phenol, or thymol since these molecules dissolve into the oil.



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 D* 54, 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 μ l drop, add 5-10 μ 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 μ 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 the crystal 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

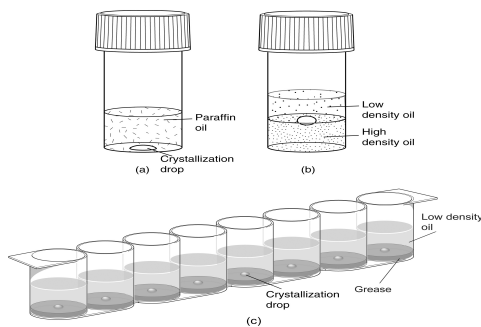
- "Gelled Surface" kit from Molecular Dimensions, UK (figure c)
- Paraffin oil
- Lysozyme 40 mg/ml in Na acetate, pH 4.7, 12% NaCl from the previous experiments

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 μ l.

You can also try dispensing a drop under oil (figure a) and compare with a drop dispensed 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 0.1 μm
- <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 μl of lysozyme at 40 mg/ml with 150 μl precipitant (12% NaCl in 10 mM acetate buffer pH 4.7) in a microcentrifuge tube.
2. Divide the mixture into 3 aliquots of 100 μl 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 0.22 μm 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-ionised 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).

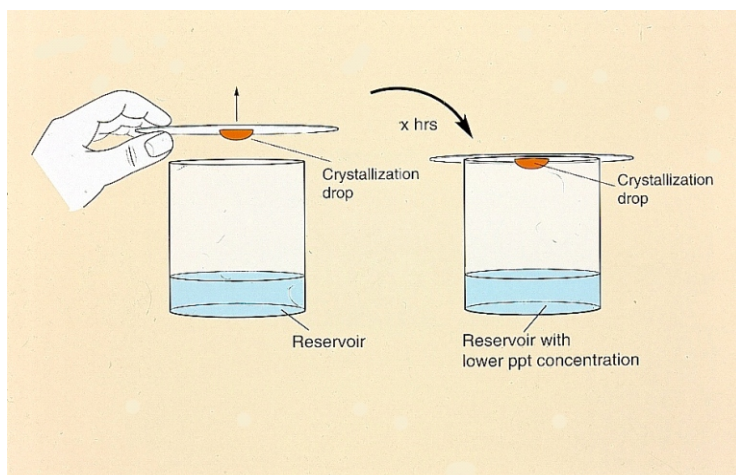
Standard Linbro plates or pre greased plates and siliconised cover-slips or EasyXtal Tools (From Qiagen).

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 ammonium 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.

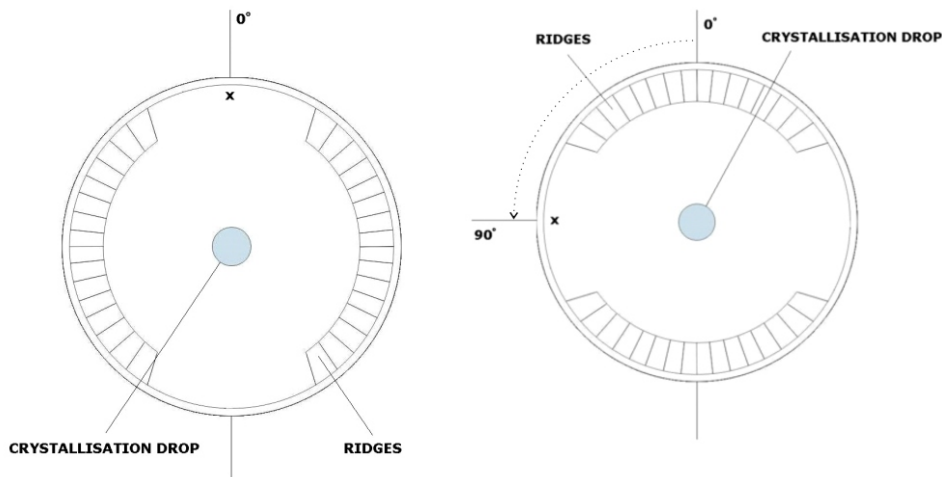
Materials required:

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.

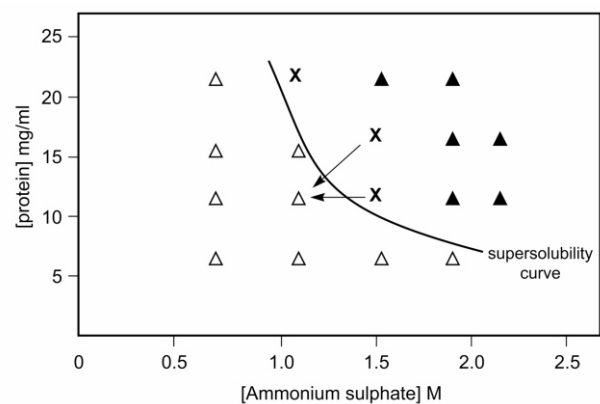
First, determine the metastable zone as follows:

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 supersolubility 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. ?? 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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