Practical Exercises, Protocols and References

FEBS INSTRUCT Course

Nove Hrady June 2014

Naomi Chayen
Imperial College London

n.chayen@imperial.ac.uk

Dear Participants,

I have written down some methods 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

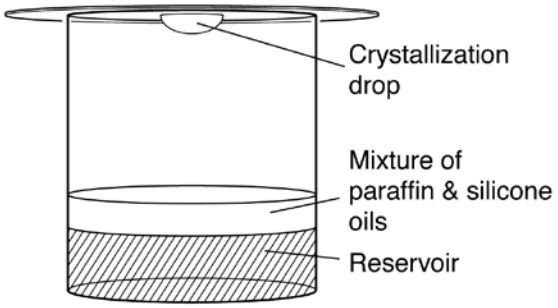
1. Use a Linbro type plate type for hanging drops.
2. Coat the lips of the reservoirs with grease or oil (unless your plates are pre-greased).
3. Pipette 0.6 - l ml of the reservoir solution which gives you the showers of crystals into each well.
4. Pipette volumes ranging from 0.1 ml to 0.5 ml of a 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 µl of the precipitant solution.
3. Insert the tip into the well under the surface of the oil and dispense the 1 µl 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 µl of protein solution to that well and mix gently with the pipette tip. The two (separate) 1 µl drops join and become a 2 µl 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.

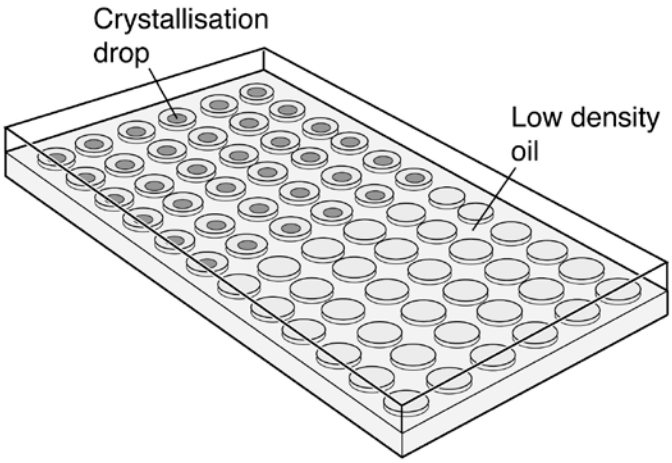
Example with lysozyme

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 µl 40 mg/ml lysozyme + 0.5 µl 12% NaCl.
3. In the second row, repeat, with 1 µl + 1 µl.
4. In the third row, repeat with 4 µl + 4 µl.
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 parafin: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 µl, 2 µl and 8 µl 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.



Method for setting up using a robot

There are several robots for setting up screening experiments 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. "Crystallization of Membrane Proteins in Oils" Chapter 8 in "Methods and Results in Crystallization of Membrane Proteins" Iwata, S. ed (International University Line, USA) 2003, 131-139.

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 μl drop, add 5-10 μl 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 μl 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: <u>Containerless crystallization</u>

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

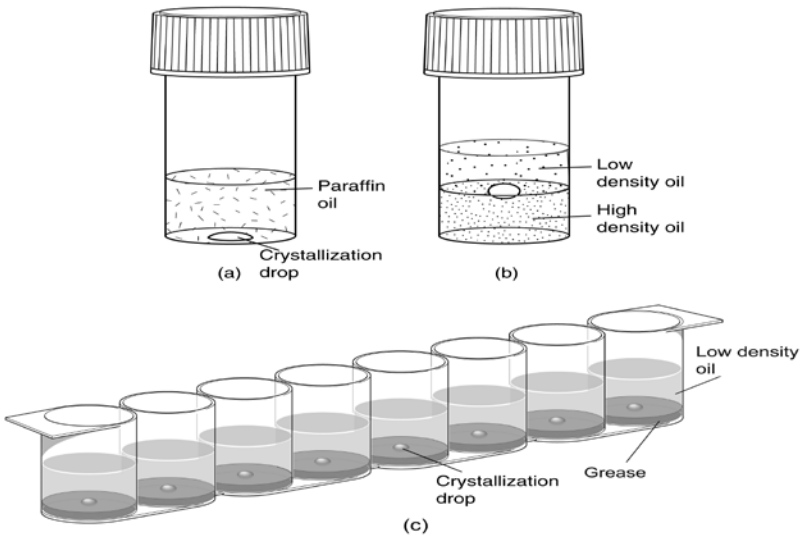
- "Gelled Surface" kit from Molecular Dimensions, UK (figure c)
- Paraffin oil
- Lysozyme 40 mg/ml in Na acetate, pH 4.7, 12% NaCl from the previous experiments

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 μl.

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
Chayen NE (2009) *J. Appl. Cryst.* **42**, 743–744.

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.millipore.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
   © 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.
2. 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 40µL 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.

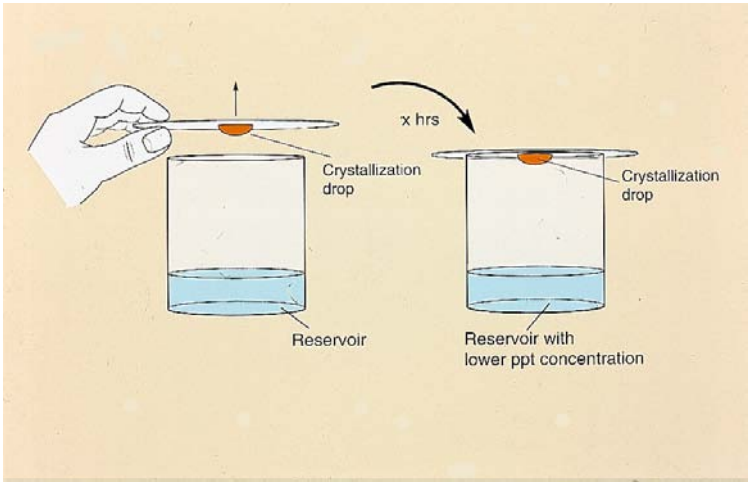
Procedure:

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: <u>What to do when you get no crystals?</u>

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.

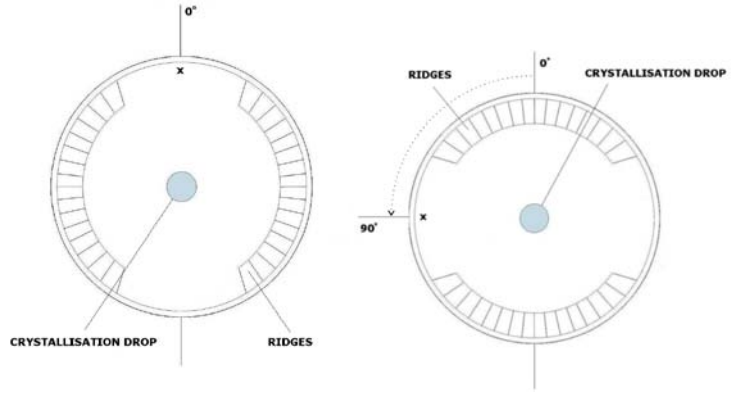
Materials required:

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.

<u>Expected results:</u>

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.



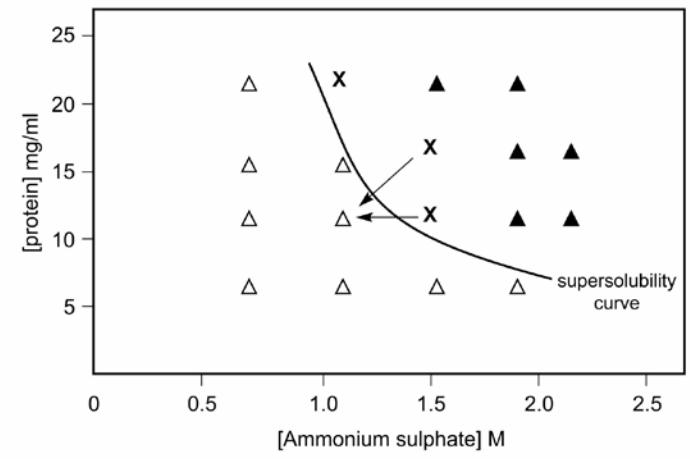
The method can also be used for optimization as described in Govada, L. and Chayen, N.E. (2009) *Crystal Growth & Design* 9, 1729-1732.

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.
Saridakis *et al.* (2011) *PNAS* 108, 11081-11086.
Kertis *et al*. (2012) *J. Mater. Chem.* **22**, 21928-21934.
Saridakis, E. and Chayen. N.E (2013) *Trends in Biotechnology* 31, 515-520.
Khurshid *et al.* (2014) *Nature Protocols*

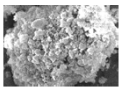
First, determine the metastable zone as follows:

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. □ 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.

Molecular Dimensions



moleculardimensions.com

Naomi's Nucleant MD2-07

Towards a 'universal' nucleant for protein crystallization,

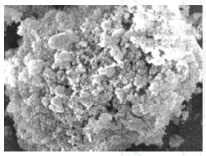
Developed and patented at Imperial College London, the most effective nucleant of any material tested.

Method of use:

- Simply add a single grain* to a crystallization drop. *Easy to place with fine tweezers or a whisker.*
- Use in screening or optimization to nucleate supersaturated conditions.
- Use in optimization where excessive nucleation occurs (i.e. lots of tiny crystals). *Back off the precipitant concentration to the metastable zone and then use a grain to nucleate.*
- Negates twinning.
- Protein crystals are easily detached from the nucleant using a whisker or a cryo loop.

Introduction

This material (bio-glass) (CaO-P_2O_5-SiO_2) has a highly porous surface with cavities of similar sizes to proteins. It is hypothesised that the cavities entrap protein molecules, thereby encouraging nucleation and crystal formation.



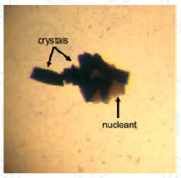
Scanning electron micrograph showing the highly porous nature of this material.

To date Naomi's Nucleant has facilitated the crystallization of 14 proteins, the highest number reported for any single nucleant. Many of these proteins have proven difficult to crystallize and some of these, including membrane proteins, have only been crystallized in the presence of Naomi's Nucleant.

In addition to test proteins those proteins that can be named are: multi drug resistance protein (a membrane protein), modified cyclodextrine, oxyntomodulin, myosin binding protein C, lobster shell α-crustacyanin, c-phycocyanin, α-actinin actin binding protein. Several other proteins have also been crystallized but cannot be named at this time.

Often the crystals obtained were of increased diffracting quality compared to those resulting from standard techniques. For example myosin binding protein C diffracted to 1.6Å compared to 3Å.

Crystals (arrowed) of β-lactamase grown on a grain of Naomi's Nucleant by Rosalida leone at imperial College, London.



Crystals of lobster shell α-crustacyanin grown on a grain of Naomi's Nucleant.

Contact Us

Molecular Dimensions Ltd. would be very grateful if investigators were prepared to provide feedback on their own experiences with Naomi's Nucleant. Crystallization reports or pictures can be e-mailed to enquiries@moleculardimensions.com

References:

Chayen, N.E., Saridakis, E. and Sear R. Experiment and theory for heterogeneous nucleation of protein crystals in a porous medium. PNAS (2006) 103, 597-601.

Saridakis, E. and Chayen N.E., Towards a 'universal' nucleant for protein crystallization. Trends in Biotechnology (2009) 27, 99-106

Eisenstein, M. The shape of things. *Nature Methods* (2007) 4, 95-101.

*** Please note:** There is a wide variety of grain sizes in each vial. Every grain is useable as a nucleant – even the very small ones (which are still much larger than a protein molecule!)

Ordering information

Naomi's Nucleants	1 vial	MD2-07
(approx 3mg)	(approx. 300 grains)	
Fine tweezers	1	MD9-25

References on crystallization methods in microbatch and vapour diffusion (and some in microgravity)

Chayen, Shaw Stewart, Maeder & Blow (1990). An automated system for microbatch protein crystallisation and screening. ***J. Appl. Cryst.*** **23**, 297-302.

Chayen, Shaw Stewart & Blow (1992). Microbatch crystallisation under oil - a new technique allowing many small-volume crystallisation trials. ***J. Crystal Growth,*** **122**, 176-180.

Chayen, Shaw Stewart & Baldock (1994). New developments of the IMPAX small-volume crystallisation system. ***Acta Cryst. D*** **. 50**, 456-458.

Chayen, Radcliffe & Blow (1993). Control of nucleation in the crystallisation of lysozyme. ***Protein Science,*** **2,** 113-118.

Normile (1995). Search for better crystals in inner and outer space. ***Science*** **270**, 1921-1922.

Baldock, Mills & Shaw Stewart (1996). A comparison of microbatch and vapour diffusion for initial screening of crystallization conditions. ***J. Crystal Growth,*** **168**, 170-174..

D'Arcy, Elmore, Stihle & Johnston (1996). A novel approach to crystallising proteins under oil. ***J. Crystal Growth,*** **168**, 175-180.

Chayen (1996). A novel technique for containerless protein crystallisation. ***Protein Engineering*** **9,** No. 927-929.

Lorber and Giege (1996). Containerless protein crystallisation in floating drops. ***J. Cryst. Growth,*** 168, 204-215.

Chayen *et al.* (1996). Trends and challanges in experimental macromolecular crystallography. **Q. Rev. Biophys. 29**, 227-278.

Chayen (1997). A novel technique to control the rate of vapour diffusion, giving larger protein crystals. ***J. Appl. Cryst.*** **30**, 198-202.

Chayen (1997) The role of oil in macromolecular crystallization. ***Structure*** **5**, 1269-1274.

Chayen (1998) Comparative studies of protein crystallisation by vapour diffusion and microbatch. ***Acta Cryst. D*****54**, 8-15.

Chayen, N.E. and Helliwell, J.R., "Protein Crystallography: the Human Genome in 3 - D" ***Physics World*** 11 (1998), 43-48.

Chayen, N.E., "Novel Methodology in the Crystallisation of Biological Macromolecules**" J. *Cryst. Growth*** 198/199 (1999) 649-655.

Chayen, N.E. and Helliwell, J.R., "Space-grown Crystals May Prove their Worth" ***Nature*** 398, (1999) 20.

Chayen, N.E. "New Trends in Macromolecular Crystallisation" ***Research Trends*** (1999) 5, 141-149.

Saridakis, E. and Chayen, N.E. "Improving protein crystal quality by de coupling nucleation and growth in vapour diffusion". ***Protein Science*** (2000), 9 755-757.

Jovine L. (2000) A simple technique to control macromolecular crystal nucleation efficiently using a standard vapour-diffusion set up ***J. Appl. Cryst***. 33, 988-989.

Chayen, N.E. and Saridakis E. (2001) Is Lysozyme the ideal model protein? ***J. Cryst. Growth*** 232, 262-264.

Chayen, N.E., Saridakis, E., El-Bahar, R. and Nemirovsky, Y. (2001) Porous silicon: an effective nucleation-inducing material for protein crystallisation. ***J. Mol Biol.*** 312, 591-595.

Moreno, A., Saridakis, E. and Chayen, N.E. (2002) Combination of oils and gels for enhancing the growth of protein crystals. ***J Appl Cryst.*** 35, 140-142.

Chayen, N.E. (2002) Tackling the bottleneck of protein crystallization in the post-genomic era ***Trends in Biotechnol.*** 20, 98

Chayen, N.E. and Saridakis, E. (2002) Protein crystallization for Genomics: Towards High Throughput Optimisation Techniques. ***Lead Article Acta Cryst D*** 58, 921-927.

Chayen, N.E. and Helliwell, J.R., (2002) "Protein Crystallization in Microgravity: Are We Reaping the Full Benefit of Outer Space? ***Annal. New York Academy of Sciences*** 594, 591-597.

Cianci, M., Rizkallah, P.J., Olczak, A., Raftery, J., Chayen, N.E., Zagalsky, P.F. & Helliwell, J.R. (2002) "The Molecular Basis of the Coloration Mechanism in Lobster Shell: Beta Crustalcyanin at 3.2 A Resolution." ***PNAS*** 99, 9795-9800.

Chayen, NE. and Hilgenfeld, R. (2002) Crystallization Comes of Age ***Trends in Biotechnol***. 20, 320-321.

Saridakis, E., Dierks, K., Moreno, A., Dieckmann, M. W. M. and Chayen, N.E. (2002) Separating Nucleation and Growth in Protein Crystallization Using Dynamic Light Scattering ***Acta Cryst D*** 58, 1597-1600.

Nield, J., Rizkallah, P., Barber, J. and Chayen, N.E. (2003) The 1.45 Å Three-dimensional Structure of C-Phycocyanin from the Thermophilic Cyanobacterium Synechococcus Elongatus ***J. Struct. Biol.*** 141, 149-155 (and Front cover).

Dunlop, K.V. and Hazes B. (2003) When less is more: a more efficient vapour diffusion protocol ***Acta Cryst. D***59, 1797-1800.

Saridakis, E. and Chayen, N.E. (2003) Systematic Improvement of Protein Crystals by Determining the Supersolubility Curves of Phase Diagrams ***Biophys J.*** 84, 1218-1222.

Chayen N.E. (2003) Protein Crystallisation for Genomics: Throughput versus Output ***J. Structural and Functional Genomics*** 4, 115-120.

D'Arcy, A., MacSweeney, A. and Haber, A. (2004) Practical aspects of using the microbatch method in screening conditions for protein crystallization***. Methods*** 34 (3) 323-328.

Nneji, G.A. and Chayen, N.E. (2004) A crystallization plate for controlling evaporation in hanging drops ***J. Appl. Cryst.*** 37, 502-503.

Chayen, N.E. (2004) Turning Protein Crystallization from an Art into a Science ***Current Opinion in Structural Biology*** 14, 577-583.

Chayen, N.E. (2005) Methods for separating nucleation and growth in protein crystallization ***Progress in Biophysics and Molecular Biology*** 88, 329-337.

Chayen N.E., Saridakis, E. and Sear, R.P. (2006). Experiment and theory for heterogeneous nucleation of protein crystals in a porous medium. ***Proc. Natl. Acad. Sci. USA***. 103: 597-601

Chayen, N.E. (2006) "Optimization Techniques for Automation and High throughput" Methods in Molecular Biology, 363, 1, 175-190.

Chayen, N.E. "Automation of non-conventional crystallization techniques for screening and optimization" Chapter 3 in "Macromolecular Crystallography, Conventional and High-throughput Methods" Sanderson, M. and Skelly, J. ed. (Oxford University Press) pp 4-58 2007.

Helliwell, J.R. and Chayen, N.E. (2007). “A down to-Earth Approach. ***Nature*** 448, 58-59.

D’Arcy, A., Villard, F. & Marsh, M. (2007). Acta Cryst. D63, 550–554.

Khurshid, S., Govada, L. and Chayen, N.E. (2007) ”Dynamic screening experiments to maximize hits for crystallization” ***Crystal Growth & Design*** 7, 2171-2175.

Chayen, N.E. and Saridakis, E. (2008) “Protein crystallization: From purified protein to diffraction-quality crystal” ***Nature Methods***, 5, 147-153.

Chayen, N.E. (2009) Rigorous Filtration for Protein Crystallization. ***J. Appl. Cryst.*** 42, 743–744.

Saridakis, E. and Chayen, N.E. (2009) “Towards a ‘Universal’ Nucleant for Protein Crystallization**”** ***Trends in Biotechnology*** 27, 99-106.

Govada, L. and Naomi E. Chayen, N.E. (2009) “Crystallization by Controlled Evaporation Leading to High Resolution Crystals of the C1 Domain of Cardiac Myosin Binding Protein-C (cMyBP-C)” **Crystal Growth & Design** 9, 1729-1732.

Chayen, N.E. (2009) High Throughput Protein Crystallization. ***Advances in Protein Chemistry and Structural Biology***, **77**, 1-22.

Bolanos-Garcia, V.M. and Chayen, N. E. (2010) New directions in conventional methods of protein crystallization. *Progress* ***in Biophysics and Molecular Biology*** **101,** 3-12.

Khurshid, S., Haire, L.F. and Chayen, N.E. (2010) Automated seeding for the optimization of crystal quality**.** ***J Appl. Cryst.*** **43**, 752–756.

Boudjemline, *et al.* (2011) Use of Dual Polarisation Interferometry as a diagnostic tool for protein crystallisation. *Anal. Chem.* **83**, 7881–7887.

Garcia-Caballero *et al.* (2011). Optimization of Protein Crystallization: The OptiCryst Project. *Cryst. Growth Des.* **11**, 2112–2121.

Shaw Stewart *et al.* (2011) Random Microseeding: A Theoretical and Practical Exploration of Seed Stability and Seeding Techniques for Successful Protein Crystallization. *Crystal Growth & Design* 11, 3432–3441.

Saridakis *et al.* (2011) Protein crystallization facilitated by molecularly imprinted polymers. *Proc. Natl. Acad. Sci. USA* **108**, 11081-11086.

Kertis *et al*. (2012) Heterogeneous nucleation of protein crystals using nanoporous gold nucleants. *J. Mater. Chem*. **22**, 21928-21934.

Saridakis, E. and Chayen. N.E (2013) Polymers assisting in Protein Crystallization. *Trends in Biotechnology* 31, 515-520.

Khurshid *et al.* (2014) *Nature Protocols*

Books

Protein Crystallisation Bergfors, T.M. ed. 2009 La Jolla, International University Line, USA.

“Macromolecular Crystallization and Crystal Perfection” 2010 N.E. Chayen, J.R. Helliwell, and E.H. Snell. Oxford University Press, Oxford, UK.

Crystallisation of Biological Macromolecules. 1999 McPherson, A. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA.

Crystallization of Nucleic Acids and Proteins. 1999 Ducruix, A. & Giegé, R., eds. Oxford University Press, Oxford.

Methods and Results in crystallization of Membrane Proteins. 2003 Iwata, S. ed La Jolla, International University Line, USA.

Macromolecular Crystallography Protocols 2006 Doublie, S. ed Humana Press, New Jersey.

Macromolecular Crystallography, Conventional and High-throughput Methods 2007 Sanderson, M.R. and Skelly, J. V. ed. Oxford University Press.

Protein Crystallization Strategies for Structural Genomics. 2007 Chayen, N. E. ed La Jolla, International University Line, USA.