# CRYSTALLIZATION UNDER OIL

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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**)**

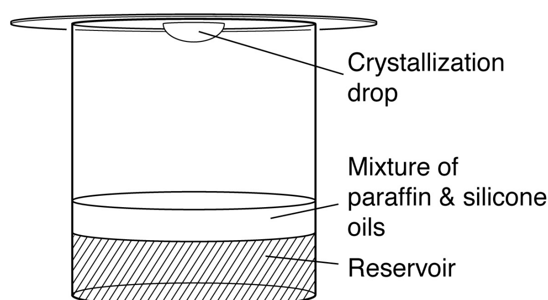
- Crystallization plates for hanging or sitting drops
- cover slips
- grease or sealing tape
- silicone oil
- paraffin oil
- Al's oil
- Protein solution
- Crystallization reagents

*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 reservoirsexcept 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.

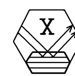
*Method for sitting and sandwich drops*

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.

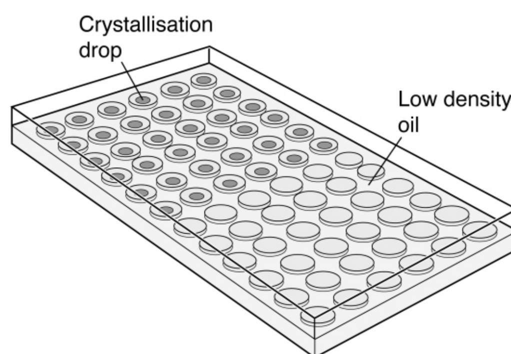
Microbatch is mechanically the simplest crystallization method and is therefore increasingly applied for high throughput trials especially for screening.

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




Method for setting up using a robot

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

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.