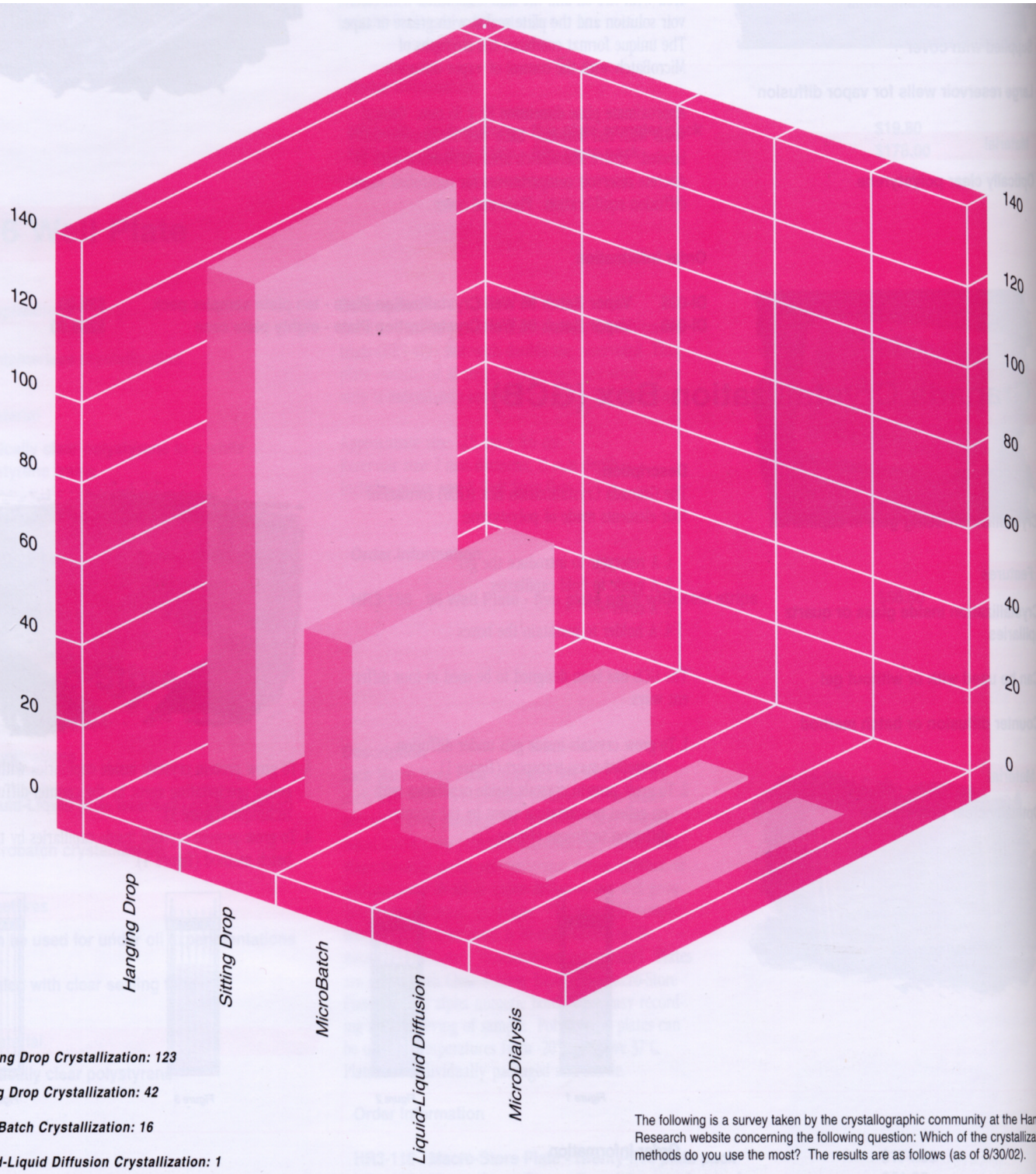


***Hanging Drop Crystallization: 123***

***Sitting Drop Crystallization: 42***

***MicroBatch Crystallization: 16***

***Liquid-Liquid Diffusion Crystallization: 1***

***MicroDialysis Crystallization: 0***

The following is a survey taken by the crystallographic community at the Hampton Research website concerning the following question: Which of the crystallization methods do you use the most? The results are as follows (as of 8/30/02).

# Preliminary Sample Preparation

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## Crystal Growth 101

### Lyophilization

Avoid lyophilization. Even though there are many examples of proteins which crystallize after lyophilization (lysozyme, thaumatin, hemoglobin, etc...), lyophilization is to be avoided when possible. If the protein is lyophilized the protein needs to be dialyzed before crystallization. Dialyze the protein against deionized water or a stabilization buffer before crystallization. Dialysis will remove non-volatile buffers and other chemicals that may have been present before lyophilization.

### Ammonium Sulfate Precipitation

Avoid using ammonium sulfate precipitation as a final purification and/or concentration step. It is often very difficult to completely remove all the ammonium sulfate by a desalting column of dialysis. The remaining trace amounts of ammonium sulfate can interfere with crystallization screening results and create reproducibility problems. It is not uncommon for trace amounts of ammonium sulfate in the sample to cause precipitation or excessive nucleation in screen conditions containing polyethylene glycol and salt.

### Batches

Avoid combining different purification batches for crystallization trials. Purification conditions and procedures are never identical so each batch should be screened separately.

### Profile the Protein

Ideally, you will purify your own protein, but this is not always reality. So, it is always a good idea to characterize your protein before beginning crystallization experiments. Profiling your protein before crystallization can often provide valuable clues during screening and optimization of crystallization conditions. Assay to seriously consider:

- SDS-PAGE
- Native PAGE or Dynamic Light Scattering
- IEF (Isoelectric Focusing) Gel
- Mass Spectroscopy

The results of these assays can:
- Determine the purity of the sample
- Determine the homogeneity of the sample
- Identify batch to batch variations
- Identify stability problems with the sample

### How Pure?

How pure should the protein sample be for crystallization trials? As pure as possible. That's some answer, is it not? Integrating common sense into the question, we might arrive at the following answer. For initial screening, the sample should be at least 90 to 95% pure on a Coomassie stained SDS-PAGE. Finally, it does no harm to screen an "impure sample" as one can always perform further purification. Remember, crystallization used to be considered a very powerful purification tool (and still is!).

If the initial screen does not produce crystals, any promising results, or it becomes next to impossible to improve crystal quality during optimization, one should consider further purification of the sample.

### Storing the Sample

Most proteins can be stored successfully at 4 degrees Celsius or -70 degrees Celsius. Check with the person preparing the protein or compare your protein to a similar protein in the literature for best storage temperature.

Ideally, one should assay the activity and stability of the protein before storage and then later on at various points in time to determine the sample storage stability.

Repeated freezing and thawing of the sample should be avoided. Aliquot the sample into multiple small microcentrifuge tubes. Make the aliquots small enough so that the entire aliquot can be consumed in the experiment after thawing.

Sometimes people like to add glycerol (10 to 50% v/v) to help proteins better tolerate freezing. Avoid this if possible since it is often difficult to remove glycerol by dialysis or filtration. The presence of glycerol is a crystallization variable. Glycerol can behave as a precipitant, an additive, or cryoprotectant and therefore can influence the outcome of a crystallization experiment.

In general, it is better to store proteins more concentrated than diluted. When too dilute, adsorption of the protein onto the storage container can lead to significant losses. However, precipitation can sometimes be a problem when the protein is stored too concentrated.

### Sample Handling

Be nice to your protein. Remember that proteins make an excellent food source for microbes. Protect your sample from microbes by storing the protein at less than 4° Celsius and not leaving the sample for extended periods of time at room temperature.

When thawing a sample or mixing a lyophilized sample into solution do not shake or vortex the protein. Avoid foaming the sample. Foam can be a sign of denaturation.

Allow the sample to equilibrate to the temperature where the crystallization experiments will be set up and/or incubated before setting the experiment.

The field of crystal growth is full of opinions and controversy. There are several opinions on what should be done with the sample just prior to setting the crystallization experiment. Let's have a look at those opinions.

Some like to filter the sample through a 0.2 micron (or smaller, but be sure to compare the MW of your sample to the pore size of your filter so as not to stick your sample on the filter) pore size filter into a sterile container. Filtration can remove microbial contamination (but not the proteases) as well as sample aggregation. Turbid sample solutions with lots of precipitate should be solubilized or centrifuged before filtration to avoid the ugly experience of sticking the sample to a filter membrane. Use filters with the smallest possible dead volume to minimize sample loss. Some of the centrifugal microfilration devices are certainly worth consideration. Before filtering the sample, wash or flush the filter with a small amount of the sample buffer/storage solution before filtering the sample. This will test the filter for compatibility with your sample buffer and remove any trace glycerol which can sometimes be present from the manufacture. If possible, test filter a small aliquot of the sample, and measure the activity/OD before filtering the entire sample. Do this to test the adherence of the sample to the filter media. Read and follow the instructions supplied with the filter before introducing the sample to the filter.

Some like to centrifuge the sample. Centrifugation removes large sample aggregates

# Preliminary Sample Preparation

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and amorphous debris. Post centrifugation views can provide a visual clue of aggregation/precipitate for seemingly clear solutions. Following centrifugation use only the supernatant for crystallization trials.

Others prefer to avoid filtration or centrifugation before setting crystallization experiments. One view is that the presence of amorphous material or aggregates can enhance the changes for crystallization be acting as nucleants.

### To Azide or Not

Sodium azide ($NaN_3$) is an anti-microbial preservative that is sometimes used to protect samples and crystallization reagents from microbial contamination. Sodium azide is toxic and should be handled with care. Typical sodium azide concentrations are 1 mM or if you prefer % measurements, between 0.02% and 0.1% w/v.

If you choose to use sodium azide remember that:
- It is toxic to humans as well as microbes.
- It is an inhibitor for some proteins and may become an unintentional ligand for your sample.
- It can interfere with heavy atom derivatization.
- Some metal azides are explosive.
- There are reports where eliminating sodium azide from the experiment improved crystallization.

Alternatives to sodium azide include thymol and Thimerosal.

A final alternative to the use of antimicrobials is the use of proper sterile technique and materials. Sterile filter all samples and reagents into sterile containers. Store samples and reagents at 4 degrees Celsius or lower. Use sterile pipet tips. Keep your work area clean. Develop a sterile technique with your crystallization set ups. With common sense, sterile reagents and sample, good technique, and sterile pipet tips, one can successfully avoid the use of chemical antimicrobials in the crystallization lab.

### Label & Organize Samples

Label samples clearly with the sample identification, batch identification, and date of storage. Here small cryo labels can be very useful. Color coding samples can be a nice organization tool. For the sake of easy organization and identification it is sometimes more convenient to nest samples. For example, store batches of small microcentrifuge tubes in 10 ml of 50 ml centrifuge tubes and organize them by batch or sample.

It is prudent to write down and hold onto detailed notes concerning the purification, storage, and handling of the sample. It is obvious that one should also maintain records of crystallization trials which should include:

- Sample Information
- Name of sample
- Sample identification (batch, storage location, storage temperature, etc...)
- Sample buffer composition, additives, ligands, etc...
- Sample concentration
- Crystallization Experiment Information
- Method
- Drop size and composition
- Reagent composition
- Temperature
- Date
- Person performing experiment

### Questions to Ponder About the Sample

- Does a similar sample exist and has it been crystallized?
- Does the sample contain free cysteines?
- Does the sample contain additives such as sodium azide, ligands, inhibitors, or substrates?
- Is the protein glycosylated?
- Is the protein phosphorylated?
- Is the protein N-terminal methylated?
- At what temperature is the protein stable?
- How does sample solubility and stability change temperature?
- How does sample solubility and stability change with pH?
- Does the sample bind metals?
- Is the protein sensitive to proteolysis?
- What class of protein am I working with (antibody, virus, enzyme, membrane protein)?
- What have been the most successful approaches with my class of protein?
- What is the source of the sample?
- How was the sample purified and stored before it arrived into my hands?
- What is in the sample container besides the sample (buffer, additives, etc...)?
- Is the sample pooled purification aliquots or a single batch?
- How much sample do I have and how much more is available?
- How pure is the sample?
- How homogeneous is the sample?
- Does anyone possess any solubility information on this sample?
- What is unique about this protein?
- What is necessary chemically and physically to maintain a stable active sample?

### Technical Support

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# Crystal Growth Techniques

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## Crystal Growth 101

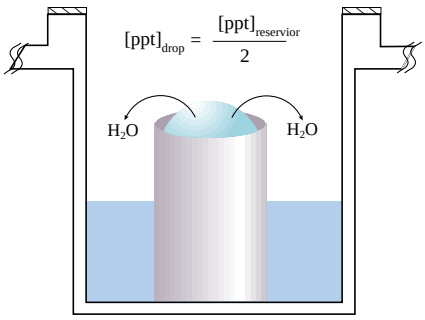
There are several techniques for setting up crystallization experiments (often termed "trials") including sitting drop vapor diffusion, hanging drop vapor diffusion, sandwich drop, batch, microbatch, under oil, microdialysis, and free interface diffusion. Here we offer an overview of these crystallization techniques.

### Sitting & Hanging Drop Crystallization

Sitting and hanging drop methodologies are very popular because they are easy to perform, require a small amount of sample, and allow only a large amount of flexibility during screening and optimization.

Using the sitting drop technique (figure 1) one places a small (1 to 40 microliters) droplet of the sample mixed with crystallization reagent on a platform in vapor equilibration with the reagent. The initial reagent concentration in the droplet is less than that in the reservoir. Over time the reservoir will pull water from the droplet in a vapor phase such that an equilibrium will exist between the drop and the reservoir. During this equilibration process the sample is also concentrated, increasing the relative supersaturation of the sample in the drop.

*figure 1*



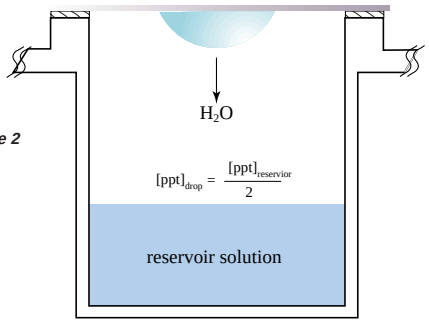

The advantages of the sitting drop technique include speed and simplicity. The disadvantages are that crystals can sometimes adhere to the sitting drop surface making removal difficult. This disadvantage can turn into an advantage where occasionally the surface of the sitting drop can assist in nucleation. The sitting drop is an excellent method for screening and optimization. During production, if sticking is a problem, sitting drops can be performed in the sandwich box set up.

Sitting drop crystallization may be performed using Micro-Bridges® or Glass Sitting Drop Rods™ with VDX or Linbro plates. Both plates can be sealed with clear sealing tape or plain cover slides for easy viewing and access. Sitting drop crystallization may also be preformed using the Cryschem Plate™. The Cryschem Plate is a specially designed plate with a post already in the center of the reservoir.

Using the hanging drop technique (figure 2) one places a small (1 to 40 microliters) droplet of the sample mixed with crystallization reagent on a siliconized glass cover slide inverted over the reservoir in vapor equilibration with the reagent. The initial reagent concentration in the droplet is less than that in the reservoir. Over time the reservoir will pull water from the droplet in a vapor phase such that an equilibrium will exist between the drop and the reservoir. During this equilibration process the sample is also concentrated, increasing the relative supersaturation of the sample in the drop.

The advantages of the hanging drop technique include the ability to view the drop through glass without the optical interference from plastic, flexibility, reduced chance of crystals sticking to the hardware, and easy access to the drop. The disadvantage is that a little extra time is required for set ups.

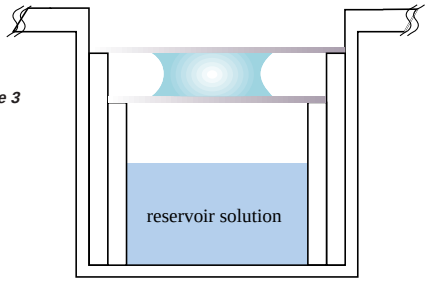
*figure 2*




### Sandwich Drop Crystallization

The Q Plate is specially designed to allow for hanging drop, sitting drop, and sandwich drop vapor diffusion. Here we will address only the sandwich drop feature (figure 3). For sandwich drop the sample solution mixed with the precipitant is placed in the middle of a lower 18 mm siliconized glass cover slide followed by one setting a larger 22 mm siliconized glass cover slide in position along an upper edge which allows for a small amount of space between the cover slides but is close enough such that the drop is sandwiched between the glass cover slides. The advantages to the techniques are an excellent optical pathway for microscopic examination and an alternate equilibration method. The disadvantages include tedious set up and the plate's large footprint.

*figure 3*




### Free Interface Diffusion

Free interface diffusion crystallization is less frequently used than sitting or hanging drop vapor diffusion but it is one of the methods used by NASA in microgravity crystallization experiments. Using this method one actually places the sample in liquid contact with the precipitant. When doing so one attempts to create a clearly defined interface between the sample and the precipitant. Over time the sample and precipitant diffuse into one another and crystallization may occur at the interface, or on the side of high sample/low precipitant or low sample/high precipitant. The technique allows one to screen a gradient of sample precipitant concentration combinations. The technique can readily be performed in small capillaries (figure 4).

### Batch

Batch crystallization is a method where the sample is mixed with the precipitant and appropriate additives creating a homogeneous crystallization medium requiring no equilibration with a reservoir. The technique is popular with small molecule crystallographers. The advantages to the technique are speed and simplicity but the disadvantage is that only a narrow space of precipitant/sample concentration can be

# Crystal Growth Techniques

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sampled in a single experiment. A batch experiment can be readily performed in a capillary, small container, or plate with a small reservoir such as the Macro-Store Plate™ (HR3-116). One must be very close to the conditions which promote crystal growth in order for this technique to be successful.

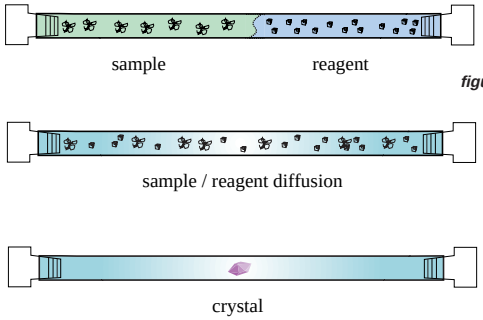


*figure 4*

### MicroBatch Under Oil

In this technique a small drop of the sample combined with the crystallization reagent is pipetted under a layer of oil. For a true MicroBatch, the drop is placed under Paraffin Oil (figure 5) which allows little to no evaporation nor concentration in the drop. A Modified MicroBatch can be performed when the drop is placed under a mixture of Paraffin Oil and Silicon Oil, or straight Silicon Oil (figure 6). Such oils allow water vapor to permeate from the drop and allow sample and reagent concentration. Unless the drop is equilibrated with a reservoir, water will leave the drop until that only solids remain.

The benefits of Crystallization Under Oil include the use of very small sample and reagent volumes with less concern for unwanted evaporation, the minimization of surface interaction with the sample, the ability to precisely control sample and reagent concentrations during the experiment, and the minimization of condensation during temperature fluctuations.

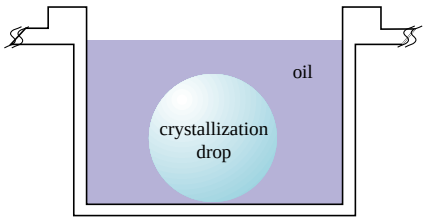


*figure 5*

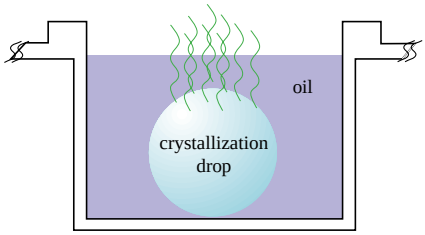


*figure 6*

### Microdialysis Crystallization

Dialysis crystallization involves placing the sample in a Dialysis Button which is sealed with a dialysis membrane. Water and some precipitants are then allowed to exchange while retaining the sample in the cell. The Dialysis Button is placed into a suitable container holding the precipitant or crystallization media (figure 7). For example, one might dialyze a sample requiring a high ionic strength for solubility against a solution of low ionic strength. The technique allows for salting in and salting out, as well as pH crystallization techniques

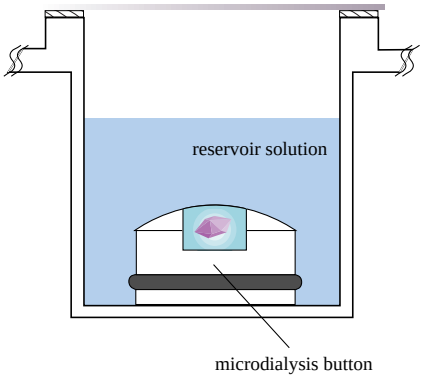


*figure 7*

### Technical Support

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# Viewing Crystallization Experiments

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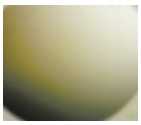
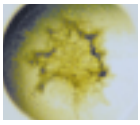
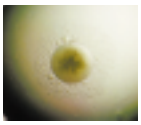
## Crystal Growth 101

***figure 4***
Typical observations in a crystallization experiment.


Clear Drop


Skin/Precipitate


Precipitate


Precipitate/Phase


Quasi Crystals


Microcrystals


Needle Cluster


Plates


Rod Cluster


Single Crystal

### Observing the Experiment

Gently set the plate onto the observation platform. If the platform is smooth and free of protrusions one may simply slide the plate in the X and Y directions on top of the viewing platform to view each of the drops. Use low magnification to view and center the drop in the field of view. Scan the drops at 20 to 40x magnification. When something suspicious appears increase the magnification to 80 or 100x for a better view. Scan the entire depth of the drop using the fine focus control of the microscope. Sometimes crystals will form at different depths of the drop because different areas of the drop can equilibrate at different rates. Also, crystals sometime forms at the top of a drop and as the crystal gains mass it will fall to a lower portion of the drop. Scrutinize everything until you are familiar with the differences between crystals, microcrystals, precipitate, and sweater fuzz. True crystals will feature edges. Precipitate does not have edges. Crystals can appear as needles, blades, walnuts, spherulites, plates, and various geometric shapes. Crystals vary in size anywhere from a barely observable 20 microns to 1 or more millimeters but most seem to fall in the range of 0.05 to 0.5 mm. Figure 1 shows typical examples of what one might observe in a crystallization experiment.

### Diffractable Crystals

Crystals useful for X-ray diffraction analysis are typically single, 0.05 mm or larger, and free of cracks and defects.

### Differentiating Between Microcrystals & Precipitate

Microcrystals (less than 0.02 mm) can be difficult to differentiate from precipitate, especially under low power or with a low to medium quality microscope. Differentiate microcrystals from amorphous precipitate by looking for birefringence (light colored shiny spots under a polarizer in dark field mode = crystals). Other tests to differentiate crystals from precipitate include streak seeding, or the use of a small amount (1 microliter) of colored, low molecular weight water soluble dye (crystal violet and methylene blue will often penetrate the solvent channels of macromolecular crystals and color them, where as precipitate will not be colored).

Precipitate can appear as clumps, fine wispy clouds, or anything in between and can range in color from white to yellow, brown or rust. During screening and very preliminary optimization one may wish to observe the drops immediately after set up, one day later and each day thereafter for the first week. Observations may be performed once a week thereafter until the drops turn into a crust of deceased sample and reagent. Never throw plates away until the drop is dead. Why? Most crystallization plates are made from polystyrene which allows for some evaporation over time. Evaporation leads to increased relative supersaturation and maybe crystals over time. Time also can lead to changes in the protein (denaturation of less stable forms, proteolytic cleavage, and other changes) which might promote crystallization. Take careful notes during observations and be especially conscious of changes that occur between observations. Most crystallization observations are done at room temperature since this is where one will find most microscopes and it is most comfortable.

### Cold Experiments

4°C experiments may be observed in a cold room by moving the microscope into the cold room. Allow time for the microscope to equilibrate to 4°C to prevent fogging of the optics as well as unnecessary temperature transfer from the warm microscope to the cold experiment. Wear a warm jacket with gloves to stay as comfortable as possible in the cold room. Excessive moisture in a cold room can be very destructive to a microscope so check with your maintenance group to keep the cold room as dry as possible. If a cold room is unavailable one is forced simply to work fast, moving plates from an incubator to the microscope carefully and making rapid yet thorough observations. Move only one plate at a time and gently close the incubator door between transfers since slamming the door will cause vibrations which can influence crystallization. Cold experiments tend to fog up rapidly especially if the light source is hot (if no infrared filters or light pipes are used). This is difficult to avoid and is one reason researchers prefer working in cold rooms.

### Technical Support

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