SUCCEEDING WITH SEEDING: SOME PRACTICAL ADVICE

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Abstract: Seeding is a powerful and versatile method for optimizing crystal growth conditions. This article discusses, from a practical point of view, what seeding is, the selection and transfer of seeds, and into what conditions they should be transferred. The most common causes of failures in seeding experiments are also analyzed.

Keywords: crystallization; microseeding; optimization; seeding; streak seeding.

1. Introduction

Crystallization is the rate-limiting step in the process of determining a three-dimensional macromolecular structure by X-ray crystallography. Automation and miniaturization of the crystallization setup have greatly facilitated massive screening. However, screening in itself, no matter how extensive, is still no guarantee that crystals will be found or that they will provide diffraction-quality data. At least half of the crystals obtained in an initial screen cannot be used without further optimization [1]. Therefore, optimization methods are often crucial for the success of a crystallization project.

One powerful tool in the arsenal of optimization techniques is seeding. While it is not a universal solution to all optimization problems, seeding is relatively cheap, fast, and easy, which makes it worth trying at an early stage. Possible applications include:

- If spontaneous nucleation is slow, i.e., the drop stays clear for a long time (weeks to months) before crystals appear
- To reduce showers of crystals
- To increase the size of crystals
- To improve reproducibility due to erratic nucleation, i.e., supposedly identical drops do not consistently produce crystals
- If crystals grow in clusters rather than singly

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- To improve crystal quality
- As a diagnostic tool to determine if the drop is undersaturated or supersaturated.

2. What is seeding?

Seeding is the use of an ordered, solid phase which is introduced into an experiment to act as a growth surface for the crystals. Spontaneous nucleation (the generation of a stable, ordered nucleus) is a kinetically demanding step. Therefore, molecules prefer to accumulate on a ready-made template, if one is available.

2.1. HOMOGENEOUS VS HETEROGENEOUS NUCLEANTS

If the seed consists of the same molecules as the target to be seeded, it is said to be a homogeneous nucleant. The molecules need not be identical though crystals of one protein can be used as seeds for the crystallization of a related form of the protein, e.g., a homologue from a different species, the selenomethionyl-substituted form, a mutated or slightly different construct, or in complex with a ligand, heavy atom, or cofactor. Generally speaking, the more similar two proteins are, the more likely it is that crystals of one will be able to function as a template for the crystal growth of the other. (Were this not the case, lysozyme crystals would work as seeds for recalcitrant proteins that refuse to crystallize. Alas, things are never that easy.)

Nevertheless, heterogeneous nucleants, i.e., materials consisting of molecules unrelated to the target protein, can be extremely effective. Anyone who has encountered crystals growing on a clothing fiber in their drops [2] or on a scratch in the glass coverslip has already experienced heterogeneous nucleation. The inclusion of these nucleants is usually unintentional. A universal substance, capable of catalyzing the crystallization of all proteins, is the dream of every crystallographer, but like the philosopher's stone in alchemy, its existence remains elusive. Some of the many materials that have been tested as heterogeneous nucleants in protein crystallization include hair [2], sand [3, 4], lipid layers [5], polyvinylidene difluoride membranes [6], porous silicon wafers [7, 8], and Langmuir-Schaeffer films [9].

This article will focus chiefly on microseeding techniques using crystals of structurally related or identical proteins as the seeds.

3. What to use as seeds

Any of the following can be used as nucleants or seeds:

- Single, small crystal, typically 0.05–10 microns in the longest dimension.
- Slurry of microcrystals.

- Fragment chipped from a larger crystal.
- Crystal, or pieces thereof, that has already been used in the X-ray beam.
- Crystalline precipitate.
- In desperate cases, any solid phase of the protein, e.g., gels or oils [4, 10]. Gels and oils are not ideal starting material, but they do exhibit some short-range order and this may be enough to trigger the ordered growth of a crystal.

Seed quality is dependent on the quality of the parent crystals. Large crystals do not make good seeds because they have accumulated too many defects. It is therefore better to chip a fragment from the large crystal or to smash it into small fragments; this will generate fresh edges and surfaces for growth. Extremely small crystals and crystalline precipitates can be used without pulverization; crystals typically referred to as sea urchins, plates, etc. can be smashed or crushed in the drops where they have grown and used directly from there.

3.1. PREPARING AND STORING SEED STOCK SOLUTIONS

It is often more convenient to work with the seeds in a microcentrifuge tube instead of working directly with them from the droplet. Among other things, the droplets tend to dry out or one may wish to make a quantitative dilution series (see Section 4) of the seeds and reuse them. Therefore, to make a seed stock, the parent crystals are transferred in some of their mother liquor to a microcentrifuge tube. Glass tissue homogenizers, Seed Beads (Hampton Research, Inc.), sonication, vortexing, acupuncture needles, dentist's tools, etc. can be used to crush the crystals into a crystal slurry [11]; the actual method of generating the seeds is not critical. What is important is that the seeds are collected and stored in a mother liquor where they are stable, i.e., do not dissolve or become contaminated by microbial growth. A common mistake is that seeds that have been grown at room temperature are transferred to a microcentrifuge tube and put in the refrigerator. Not all crystals tolerate moving back and forth between the refrigerator and the lab bench. Some people flash-cool their seed stock in liquid nitrogen [12]; here again, not all seeds may survive this treatment. Thus, for the storage and reuse of seed stocks, the following simple precautions should be taken:

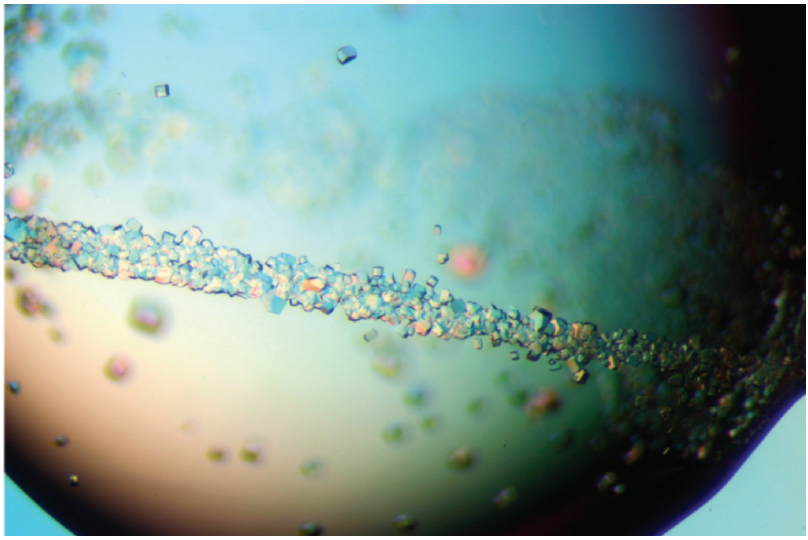
- Remove a 5–10 μL aliquot of the seed stock and examine it under the microscope at high magnification to verify that the seeds have indeed survived the storage conditions. This is especially important to do if temperature changes have been involved.
- Check the seed stock solution for the odor of bacterial contamination and if necessary, make new mother liquor and seeds.

4. How to transfer the seeds

The use of an intact, single crystal as a seed is referred to as macroseeding. This is a difficult technique. (For those who wish to attempt it anyway, an excellent how-to example is found in Mowbray [13].) Working with microseeds is much easier, especially by streak seeding [10, 14–16]. Streak seeding usually employs some kind of animal hair or whisker as the transfer tool (a so-called seeding wand) for the seeds. The surface of an intact parent crystal in a droplet is stroked with the hair to pick up microcrystals. Alternatively, the seeding wand can be dipped into a droplet or microcentrifuge tube containing a slurry of microcrystals. In either case, the microcrystals or seeds adhere to the hair, which is then swiped through the new crystallization drop whereby the seeds are transferred. The new crystals will grow along the streak line of deposited seeds (Figures 1a and b).

This is by far the simplest and fastest way of seed transfer, and the crystals that result may well be large enough to use without further refinement of the seeding protocol.

However, it may be necessary to reduce the number of seeds; this can be done, for example, by passing the seeding wand through several washes of mother liquor to remove the excess seeds. A more quantitative method is to make a dilution series of the seed stock. Two detailed descriptions of how to make seed dilutions can be found in Fitzgerald and Madsen [17] and Luft and DeTitta [11].



(a)

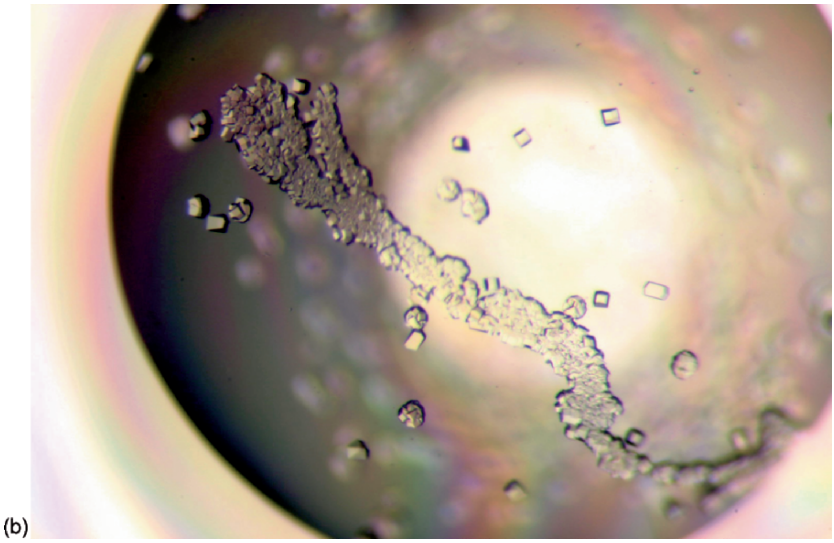


Figure 1. Lysozyme crystals growing on streak seeding lines: (a) vapor-diffusion setup; (b) microbatch-under-oil. The oil does not affect the seed transfer.

5. What to seed into: supersaturation levels in the new drop

Some understanding of seeding in terms of the phase diagram is helpful when deciding on the composition of the new drops where the seeds are to be deposited.

For a solution to crystallize, it must first be in a state of supersaturation. This in itself is no guarantee that crystals will form: crystallization also requires nucleation. This event is the formation of the first stable, ordered nucleus. The likelihood of this happening is related to the number of molecules in solution – the more there are, the greater the probability that any two or more of them will collide with each other, overcome the competing forces to drive them apart, and remain as an ordered nucleus.

Unfortunately, the levels of supersaturation that promote spontaneous nucleation are too high for the slow, accumulative growth that leads to well-ordered, large-sized crystals. For this reason, if nucleation does manage to occur, it often results in showers of small crystals rather than a few, single, and large ones. Seeding is an optimization technique that separates the nucleation event from the growth process: the seed crystals are removed from the original drop in which they nucleated and placed in a new experimental condition. The new drop should be equilibrated at a level of supersaturation high enough to support crystal growth, but low enough to prevent spontaneous nucleation. These different regions of supersaturation are represented in the phase diagram as the labile and metastable zones (Figure 2a).

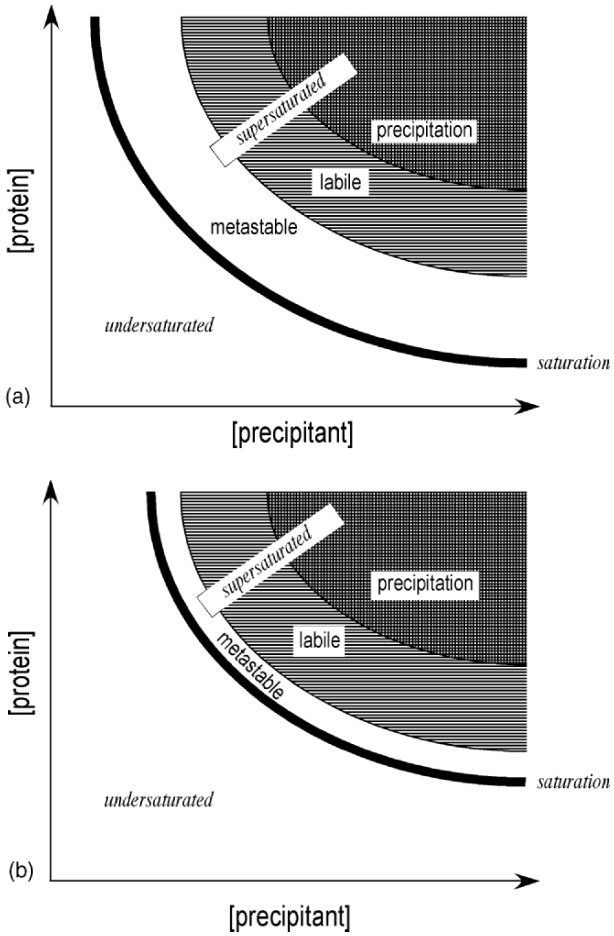


Figure 2. (a) A theoretical phase diagram. The thick black line represents the limit of solubility. Below this line, the solution is undersaturated and above it, supersaturated. Seeds placed in an undersaturated solution dissolve. Spontaneous nucleation occurs in the labile zone of the supersaturated phase, but the best crystal growth occurs in the metastable region. At the highest levels of supersaturation, the solid phase aggregates in a disordered fashion, seen as precipitate. (b) The same as part (a), but here the metastable region is very narrow, making it a poor candidate for seeding experiments.

In practice, the phase diagram of the protein is rarely available. The location of the different zones within the supersaturated phase has to be determined empirically unless the crystallization setup is equipped with some kind of instrumentation, e.g., a light-scattering instrument, to monitor the onset of nucleation [18]. When the problems are too much or too rapid nucleation, the concentration of the protein or the precipitant, or both, should be lowered

in the new set of drops to be seeded. The following rule of thumb can be used as a starting point in the search for the metastable zone: begin by halving the protein concentration in the new drops. Of course, precipitant concentration could also be manipulated, but lowering the protein concentration has a slight advantage in that it reduces the amount of protein sample needed. The ease with which the metastable zone can be localized will depend on how wide it is (Figure 2b). Some proteins have very narrow metastable zones. In these cases, it is almost impossible to pinpoint the right concentrations of protein or precipitant for adding the seeds and some other optimization method will be necessary.

When the problem is slow nucleation, i.e., the drops stay clear for weeks or months before the crystals appear, adding a seed will jump start the nucleation process. The same or even higher protein or precipitant concentrations can be used in the new drops because the goal is not to reduce nucleation but to encourage it.

6. Troubleshooting

When seeding fails to work as expected, it is often due to an improperly designed experiment or mishandling of the seeds. Some of the common problems that can sabotage the success of a seeding experiment are described below.

6.1. THE EXPERIMENT IS NOT EQUILIBRATED

Adding seeds to an undersaturated solution will cause them to dissolve. Many vapor-diffusion experiments begin in an undersaturated state and only reach supersaturation after a period of equilibration against their reservoirs. It is difficult to give a general rule for how long time one should wait before seeding the new drops: the time required for the drop to equilibrate with the reservoir is dependent upon many different factors (see Luft and DeTitta [19] for a review and for specific examples [20].) However, as a rough guide, the effects of seeding should be obvious within 2–7 days. If the drops that have been seeded are still clear after 1 week, the cause may well be that the seeds dissolved.

6.2. EVAPORATION HAS OCCURRED

The drop may have been properly equilibrated to, or have been begun at, the metastable zone, but when the experiment is reopened for the purpose of adding the seeds, some evaporation occurs and the level of supersaturation soars back into the labile zone. This is always a potential problem in vapor-diffusion

experiments and is more pronounced with hanging drops than sitting drops, and with extremely small drop volumes. In this respect, seeding into microbatch is more reproducible.

6.3. NO SEEDS ARE TRANSFERRED

Improper storage of the seeds, as discussed in Section 3.1, may cause the seeds to dissolve. Another scenario is that all the seeds have sunk to the bottom of the microcentrifuge tube. The seed stock should be vortexed immediately before use to redisperse the seeds. In transfers by streak seeding, the seeds may not have adhered to the seeding wand. The animal whiskers or hairs do wear out as seeding wands and need to be replaced when they no longer seem to be depositing seeds. Acupuncture needles are another popular tool for seed transfer, but it should be kept in mind that smooth surfaces like metal and glass do not trap the seeds nearly as well as hair.

6.4. TOO MANY HETEROGENEOUS NUCLEANTS ARE PRESENT

Too many heterogeneous nucleations already present in the drop will mask the effect of any intentionally added nucleant, i.e., the seeds. Typical extraneous sources of nucleation are dirt, dust, denatured protein molecules, and clothing fibers. If seeding is not giving the desired effect, filter all the components of the drops through a 0.22 μm filter immediately before setup. Wear a lab coat and work cleanly.

6.5. THE PROTEIN IS NOT PURE ENOUGH

Seeding is not particularly effective in improving crystal quality if the problem is microheterogeneity in the protein sample [21]. An additional purification step may be required instead of, or prior to, application of the seeding protocol. Microheterogeneity can be assayed, for example, with isoelectric focusing gels.

7. Summary

Although efforts to automate crystal optimization are now underway [1], a follow-up optimization experiment is often still designed and implemented manually. Microseeding, and especially streak seeding, are easy to perform which makes them attractive as optimization methods. Seeding has a wide range of applications and the recommendations presented here will hopefully encourage its implementation in the laboratory.

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