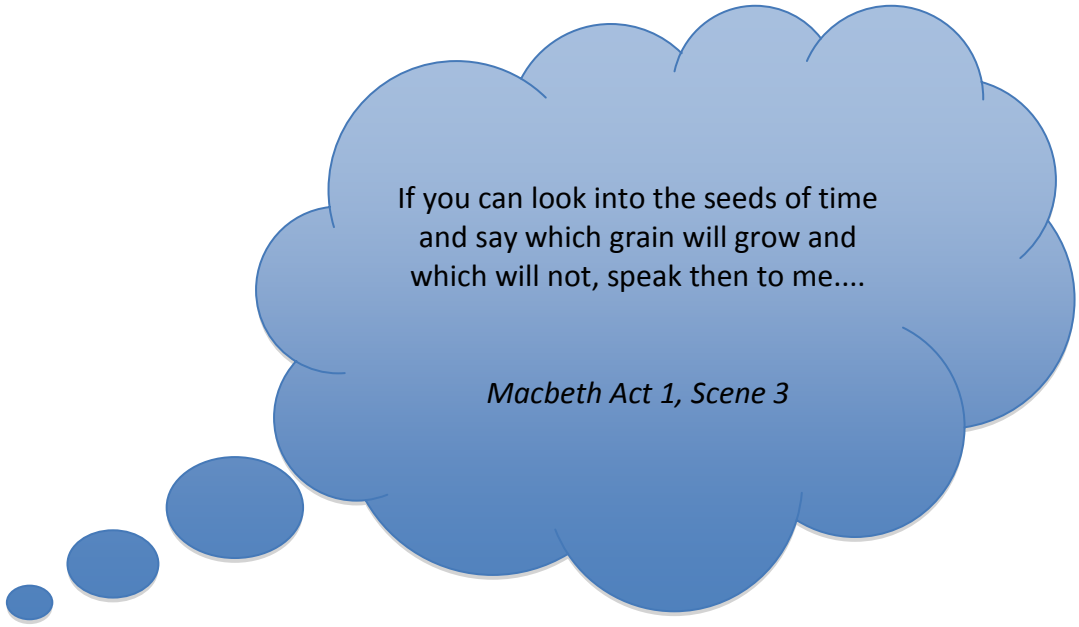


Seeding Lab Exercises Background

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.**
- 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?

- **Macroseeding** is the transfer of a single, pre-grown, washed crystal.

- **Microseeding** is the transfer of microscopic crystals, crushed up into fragments.
- **Streak seeding** is a form of microseeding 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 of microseeding where the new drop is "innoculated" with a single jab of the seed transfer tool.
- **Matrix microseeding** is when microseeds are placed into a screen of conditions totally unrelated to the ones where the seed crystals originated.

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

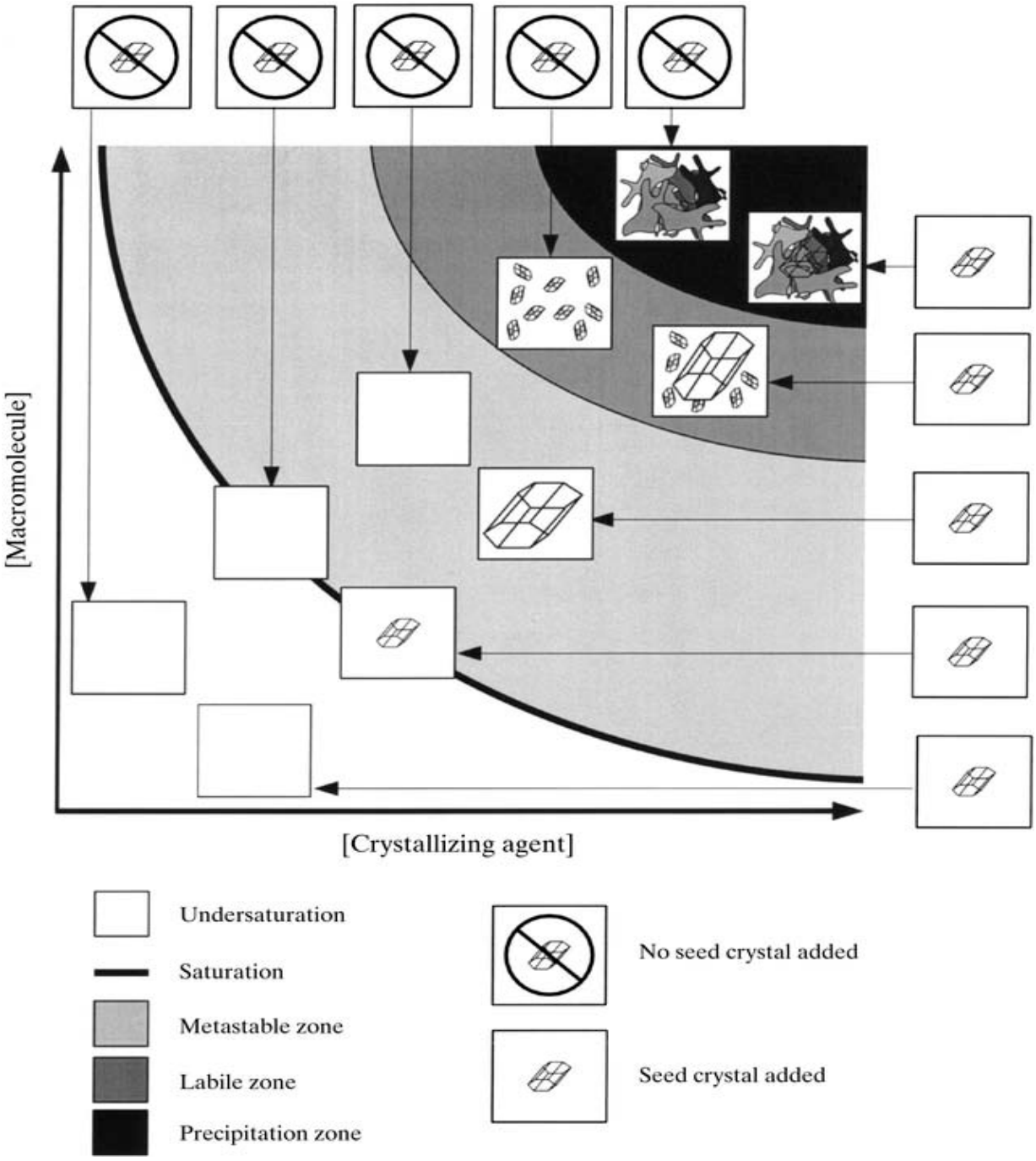
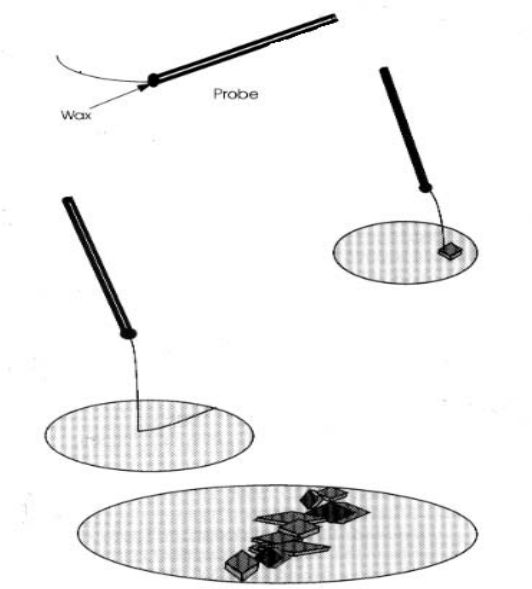


Figure 2: Streak seeding from Enrico Stura, Published in "Crystallization of Nucleic Acids and Proteins" Edited by Ducruix and Giege, 1992, Oxford University Press



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:

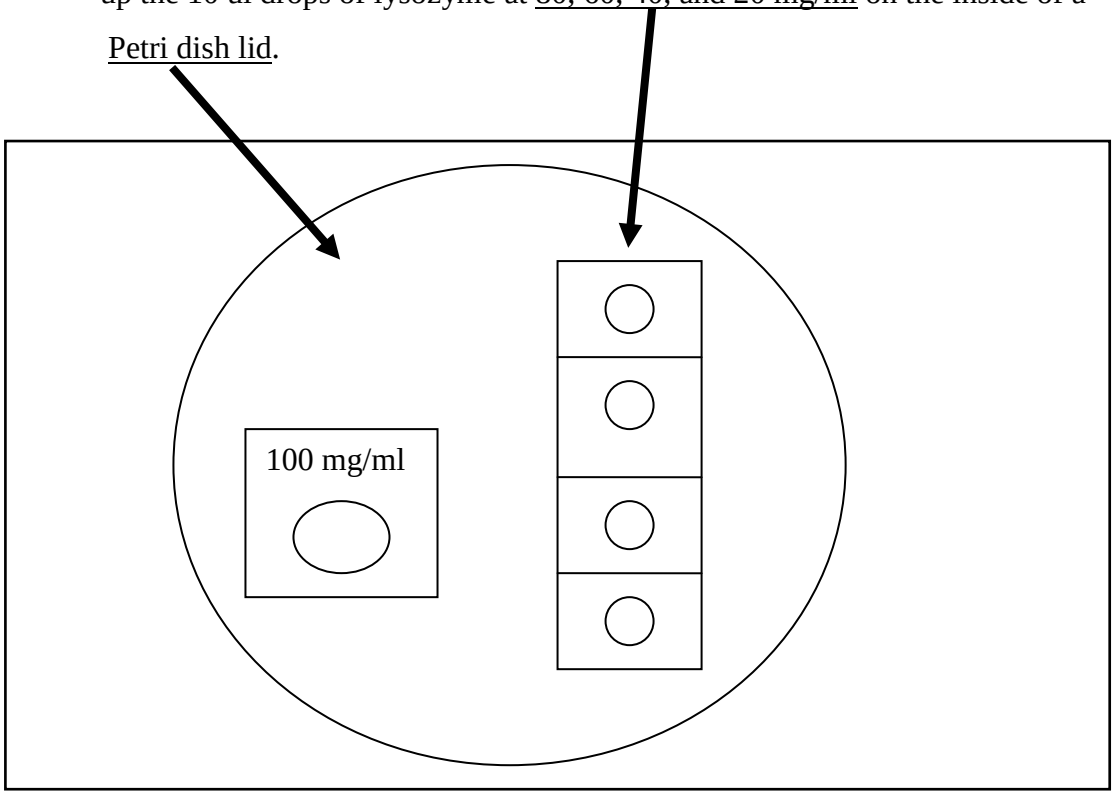
- a crystal wand --- available from Hampton Research (HR8-133) or you can make your own from a cat whisker, human hair or horse tail hair, super glue or wax to affix the whisker to a yellow pipette tip, and a razor blade to cut the whisker.
- lysozyme 100 mg/ml stock solution.
- A dilution series of 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:

- On the Petri dish lid, mix 10 ul of lysozyme (100 mg/ml) with an equal volume of the precipitant (= 30% PEG 5000 or 6000 in 1M NaCl, 50 mM Na acetate, pH 4.7). It is generally a good practice to always add the precipitant to the protein and not the other way around.

- Crystals should nucleate in 5-15 minutes. The fresher the lysozyme the longer time it will take to nucleate. Observe under the microscope. 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.

- Once the parent crystals have formed in the drop with 100 mg/ml, you can set up the 10 ul drops of lysozyme at 80, 60, 40, and 20 mg/ml on the inside of a Petri dish lid.



- **Add 10 ul 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.

- Now you can transfer by streak seeding the nuclei generated in step 3. 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.

1. The purpose of this experiment is to demonstrate the method and the principles. In the next experiments, a more refined version will be presented.
2. Recover the 20 microliter drop of 100 mg/ml lysozyme (the parent drop). Place the 20 microliters (or as much as you can recover) into an eppendorf tube and fill to about 50 microliters with the precipitant (30% peg, etc). This will be your Seed Stock in the next experiment.

Experiment 2. Comparing the effects of adding different amounts of seeds. The fast and dirty version.

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 the previous experiment, this one 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 fewest.

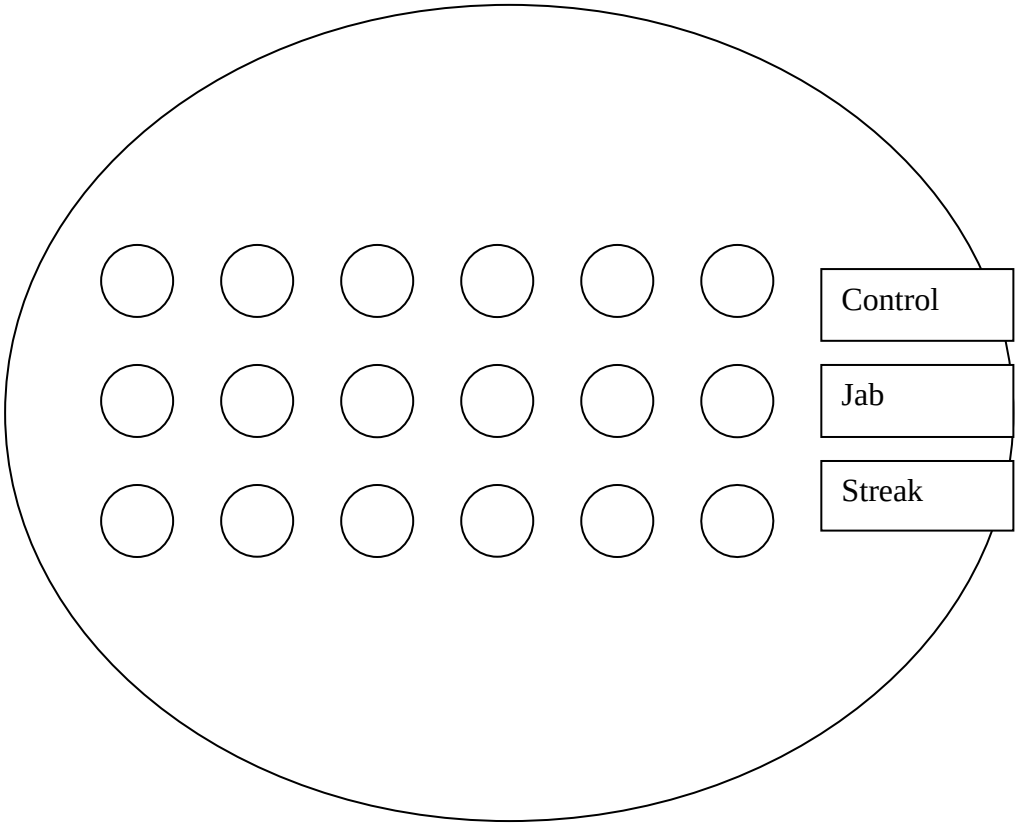
Materials required:

1. one Petri dish (the large size).
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. one eppendorf tube
7. seed stock, created with the microcrystals grown in the previous experiment. Collect the parent seed crystals (grown in the drop of 100 mg/ml plus precipitant). Top up the solution with precipitant so that you have a seed stock of about 50 microliters.

Method:

- In the eppendorf tube, mix 20 microliters of the stock solution of lysozyme (100 mg/ml) with 80 microliters water = 20 mg/ml lysozyme.
- Now add 100 microliters of 12% NaCl in 50-100 mM sodium acetate buffer, pH 4.7= 200 microliters of READY MIXTURE A (protein + precipitant). This is enough for both Experiments 2 and 3.

- Into the Petri dish, pour approximately 5 ml of the buffered 12% NaCl solution in the reservoir (bottom of the plate).
- Using READY MIXTURE A, pipette 3 rows of 6 identical drops each onto the lid of the Petri dish = a total of 18 drops. Each drop will contain 5 microliters. (This uses 90 microliters; the remainder will be for the next experiment.)



Petri dish for experiment 2

- Row 1 will be the control = no seeding.
- Row 2 will be *jab seeded.* Using the seed transfer tool, dip or stir it into your seed stock (from the previous experiment) to pick up seeds.
- 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. This will dilute the number of seeds attached to the seed transfer tool, thereby creating in a fast way a dilution series of the seeds.

- 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 seed stock to pick up a new batch of seeds.
- 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.
- Invert the lids of the Petri dishes over the reservoir and seal with Parafilm.
- Wait 24 hours to observe the results.

Expected results:

- After 24 hours, you probably will see sea urchin-type crystals in the control row
- 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 the effect more pronounced.
- 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. **See an example in the next Figure with a series of 5 drops.** (Note that you have made a series of six drops although the figure shows 5 drops.)

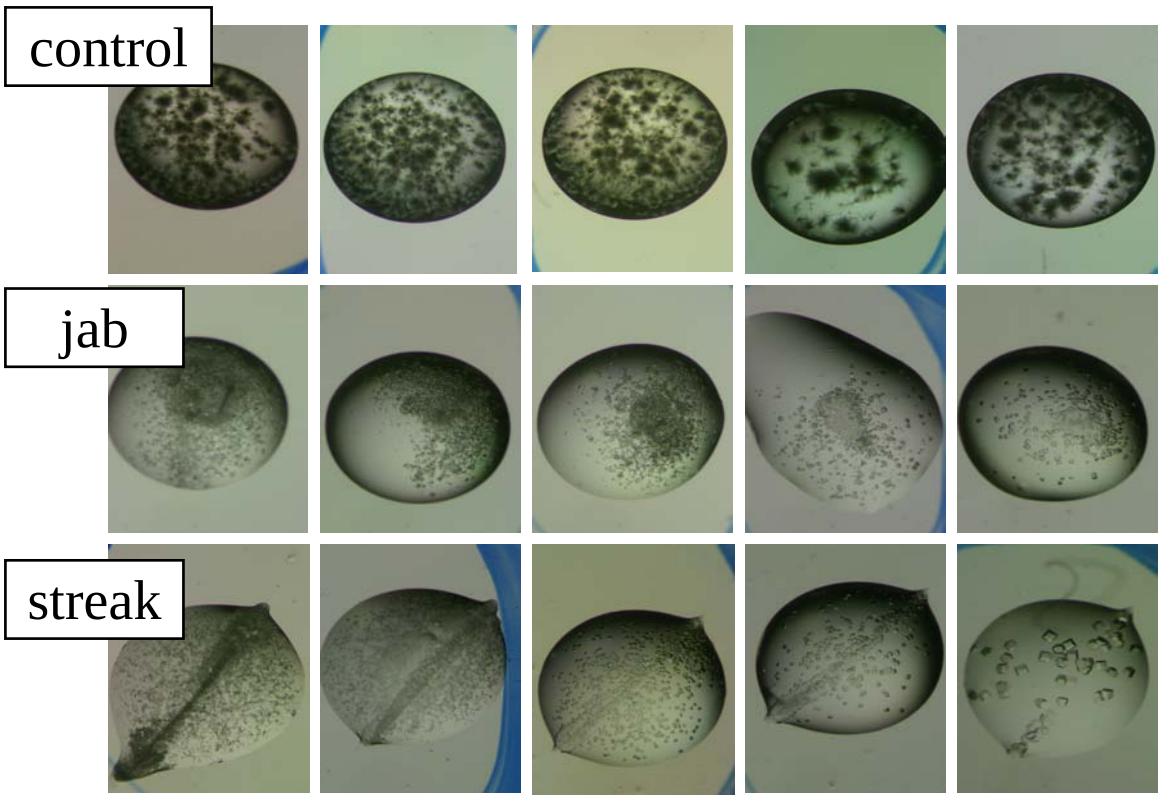


Figure 3. Comparison of drops that have been serially seeded in two different manners. These results were photographed 48 hours after setup. Row 1 is a control and has not been seeded. The lysozyme crystallizes in the form of sea urchins or spherulites. Row 2 has been jab seeded. The seed transfer tool, innoculated 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 seed transfer tool through the first drops in the row.

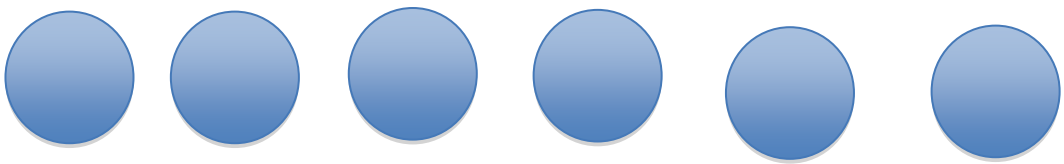
Experiment 3. Comparing the effects of adding different amounts of seeds after making a dilution series of them.

Materials required:

1. two Petri dishes (the large size).
2. 5 ml of 12% NaCl in 50-100 mM sodium acetate buffer, pH 4.7
3. 5 ml of 6% Nacl in 25-50 mM sodium acetate buffer, pH 4.7 (dilute the buffered 12% NaC lwith water.)
4. one eppendorf tube with a Seed Bead
5. seed stock, created with the microcrystals grown in the previous experiment. Collect the parent seed crystals (grown in the drop of 100 mg/ml plus precipitant). Top up the solution with precipitant so that you have a seed stock of about 50 microliters.

1. Return to the seed stock created in the previous experiments i.e., the eppendorf containing approximately 50 µL.
2. Transfer it to an eppendorf tube containing the Seed Bead™. (Note: a round-bottom 2 ml eppendorf tube actually works better than the 1.5 ml V-shaped eppendorfs supplied with the Seed Bead.) In today's experiment, the seeds are already microcrystals, but if you have larger crystals, a seed bead is useful for creating a homogeneous seed "soup" or "slurry".
3. Vortex the eppendorf tube containing the Seed Bead at full power for 30-60 seconds.
4. After the vortex step, add another 50 µL of precipitant (30% PEG, 1 M NaCl) to the microcentrifuge tube and mix the solution well with a pipette. This will give you about 100 microliters of **concentrated seed stock.**

5. Label four microcentrifuge tubes: **1:5, 1:25, 1:125, and 1:625.** This will reduce the likelihood of accidentally mislabeling the different dilutions later on.
6. **Prepare four 1:5 serial dilutions.** How? Make 50 µL of a 1:5 dilution by adding 10 µL of the concentrated stock to 40 µL of precipitant and mixing well with a pipette. Repeat this procedure exactly, using 10 µL of the 1:5 dilution and 40 µL of precipitant to give 50 µL of a 1:25 dilution, and so on. Serial dilution is a more accurate method than preparing each stock directly from the 100% solution because it does not require the pipetting of extremely small volumes. However, it does require thorough mixing of each stock solution before preparing the next dilution.
7. Into a Petri dish, pour approximately 5 ml of the buffered 12% NaCl solution in the reservoir (bottom of the plate).
8. Using **READY MIXTURE A** from the previous experiment, pipette a row of 6 identical drops each onto the lid of the Petri dish. Each drop will contain 5 microliters.
9. To each drop, add 0.2 µL from seed stock and then each seed dilution solution (1:5, 1:25, 1:125, and 1:625). The sixth drop will be a control (no seeds). Do not stir the drops. Seal the dish with parafilm.



seed stock **1:5** **1:25** **1:125** **1:625** **no seeds**

reservoir solution: 12% NaCl in sodium acetate buffer, pH 4.7

10. In a second Petri dish, add approximately 5 ml buffered 6% NaCl.

11. In an eppendorf tube, make 25 microliters of 20 mg/ml lysozyme (take 5 microliters of 100 mg/ml stock solution, add 20 microliters water.)

12. Add 25 microliters of the reservoir solution (buffered 6% NaCl).

13. You now have 50 microliters of **READY MIXTURE B.**

14. Using **READY MIXTURE B**, pipette a row of 6 identical drops onto the lid of the Petri dish. Each drop will contain 5 microliters.

15. To each drop, add 0.2 µL from the seed stock and then each seed dilution solution (1:5, 1:25, 1:125, and 1:625). The sixth drop will be a control (no seeds). Do not stir the drops. Seal the dish with parafilm.

seed stock **1:5** **1:25** **1:125** **1:625** **no seeds**

reservoir solution: 6% NaCl in sodium acetate buffer, pH 4.7

Expected results: Wait 6-24 hours. If the seeding has worked, the effect of the dilution series should be obvious, with successively fewer crystals for each dilution. For the lab discussion tomorrow, indicate how many crystals each drop contained.

Questions to answer for the discussion session:

1. Which dilution gave the best result, i.e., 3-4 large crystals?

2. Can you save the seed dilutions and re-use them?

3. Which delivery method works best: jab, streak, or aliquot?