

Chapter 2

Scoring and Phases of Crystallization

Abstract The practice of scoring of protein crystallization screening results is more honored in the breach than in the observance. However, as we hope to show in the balance of this treatise, it can lead to a means for extracting more information than immediately apparent from a crystallization experiment. Scoring has advantages beyond simple good scientific note-keeping practice; the act of objectively examining one's results, with some thought added, can lead to a deeper appreciation of what led to those results, be it at the protein, screening solution, or mechanics of setting up the plate level. The first goal is to have a system which reflects an increase in the desirability of the results obtained with the numerical score. The scoring scale does not have to be complex or extensive; a 10-point scale is elaborated on herein. However, the scale should clearly distinguish between classes of desirable outcomes.

2.1 Introduction

The opening mantra of this chapter, and in fact for all successful protein crystallization experiments, is that there is no substitute for careful visual observation of crystallization plates. Even in the absence of a formal analysis methodology, such as those outlined in subsequent chapters, an alert and careful observer will note patterns emerging in the results, either from well to well or within the droplets of a given well if that approach is taken. This chapter is written to give examples of how we interpret crystallization results. Other interpretation schemes may be used, but the primary importance is that one develops a familiarity with the results that are, or could, be obtained.

Tracking protein crystallization results, particularly in smaller laboratory's, is often a matter of circling the found outcomes of interest with a Sharpie™. Notes may be taken, but since the outcome of interest is a nicely faceted crystal then why bother noting that this precipitation was gummy in appearance, that one was lightly granular, and the one next to it was heavy and brown, while in between were several clear wells? Thus, while over the years a number of scoring scales have been put forth, they are rarely used when all that was deemed necessary was to circle the hits on the plate with a Sharpie™.

2.2 Why Score Crystallization Drop Results?

There are several reasons why one should score their crystallization screening results, not the least of which is that it is good scientific note keeping. In the absence of any formal post screening analysis, knowing what happened as the protein was placed in solution with a number of different chemicals, over a range of concentrations and pH's, may still serve as a basis in guiding subsequent optimization strategies when a hit is finally obtained. Careful note taking, with respect to the solution compositions, can serve to rule out the inclusion of specific chemicals, or suggest changes in the protein concentrations used. However, the rationale that serves as the basis of this treatise is that the scores can be used in the analysis of the results obtained, for potentially extracting crystallization conditions where one previously had none, or for expanding on the known conditions and identifying those that can more reproducibly yield crystals. This latter point is of particular interest if one is going to extensively work with the protein, such as for binding studies, and reliable crystalline conditions are needed.

2.3 Our Scoring Scale

A practical scoring scale needs to reflect an improvement in outcomes with an increase in the score. Many scales begin with a score of 0 for a clear solution. Referring to Fig. 1.1, we see that clear solutions can occur on either side of the solubility line, and in fact are not the worst outcome that can occur. That distinction is reserved for a heavy precipitate, and even here there are two types that can occur; one where the protein is still “intact” and can be redissolved, and second where the protein is partially denatured and cannot be redissolved. Distinguishing between these two precipitant types is not always easy, although having a heavy brown precipitate is typically taken as an indicator of the second type. Regardless, it then is apparent that changes in solution conditions that take an outcome from a precipitate to a clear solution are not detrimental, but an improvement in the outcome. The scoring scale that we have found best is provided revised score column of Table 2.1. This scale is the same as given by [3]. Figures 2.1, 2.2, and 2.3 gives an illustration of these scores.

2.4 Our Scoring Procedure

We follow a defined procedure for scoring crystallization screening results. All plates use trace fluorescently labeled protein, which enables us to follow what the protein is doing in response to the crystallization screening solution being tested [8, 11]. We use Corning 3553 CrystalEX™ sitting drop crystallization plates having 3 drop

Table 2.1 Scores for protein crystallization images

General category	Hampton's score	Revised score as in [3]	
Non-crystals	1	2	Clear drop
	2	3	Phase separation
	3	0	Heavy precipitate
	3	1	Light precipitate
Likely leads	4	4	Birefringent precipitate or Microcrystals
	–	4	Bright spots (Not present in Hampton's category)
Crystals	5	5	Urchins, spheroids, dendrites - non-faceted crystals
	6	6	Needles
	7	7	Plates - 2D crystals
	8	8	3D crystals <μm
	9	9	3D crystals >200 μm

positions/precipitant well. The three positions are set up at protein:precipitant ratios of 1:1, 2:1, and 4:1 (vol:vol) or, alternatively, 1:2, 1:1, and 2:1. Assuming the same endpoint precipitant concentrations at equilibrium the varying drop ratio's give an indication of the effects of protein concentration on the outcome, and we frequently have results that progress from precipitate or small crystals to large single crystals across the three drops.

Typically, the plates are fluorescently imaged on a regular basis, and scoring is carried out between the 6th and 8th week after setup. The first step is to manually go through the plates well by well, using a standard low power microscope typically used for crystal plate viewing, and note down a score for each well on a scoring sheet. The score written down at this point is for what is observed under white light. The second step is to review the scores written down with respect to the most recent fluorescent images. The scores at this point are adjusted as necessary based upon what the fluorescent image reveals. Thus, objects that scored as a crystal are downgraded to what the background conditions show if they do not fluoresce, which indicates that they are not protein but salt crystals. It is at this point that we identify outcomes having a score of 4, the bright spots. The score for any given crystallization drop is that of the highest scoring object within that drop. This often necessitates careful examination of the drop contents, zooming in on features of interest and focusing through the solution. One small faceted crystal within a drop containing precipitate, granular precipitate, or apparently non-faceted crystals, will result in the drop being scored as an 8. Similarly, if a cluster of crystals or rods that might otherwise be scored as a 5 has one or more that protrude out sufficiently that it could be cleaved off and

mounted for diffraction as a single crystal then the score is that of the piece that can be cleaved off.

2.4.1 *What You See Is Not Always Simply Classified*

The first question when scoring a crystallization plate is “what is this”? While a faceted crystal, a long rod, or a spikey urchin may be obvious, there are other outcomes that are not so clearly defined. A good resource for interpreting one’s results are the images on the Terese Bergfors website: <http://xray.bmc.uu.se/terese>. A guide to the scoring used in our work is shown in Figs. 2.1, 2.2, and 2.3.

As can be inferred from Fig. 2.1, there are no hard and fast rules for scoring what one observes. While most outcomes are easily scored, this is not always the case. For example, as shown in Fig. 2.1, Panels A, B, and C, what distinguishes a heavy vs. a light precipitate? This particularly when there may be occasions where the

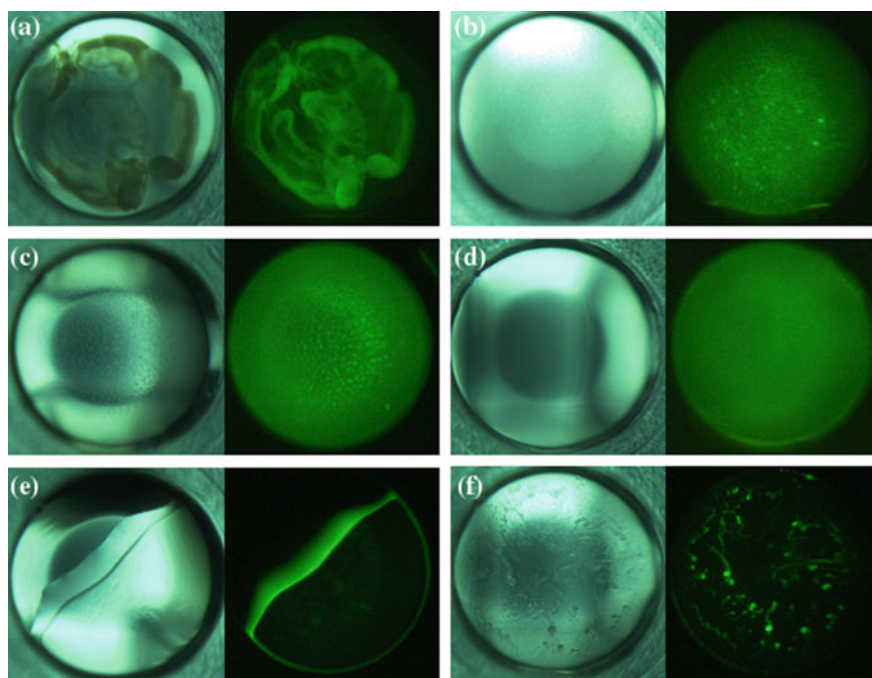


Fig. 2.1 Outcomes and their scores. Each panel has a white light and its corresponding fluorescence image. Panels A and B, score = 0, heavy precipitate, however note the presence of bright spots in Panel B, which would result in this being scored 4; Panel C, score = 1, light precipitate; Panel D, score = 2, clear solution; Panels E and F, score = 3, phase transition, although the presence of the bright spots in Panel F would result in this being scored 4. The protein in all images is canavalin, purified from Jack Bean

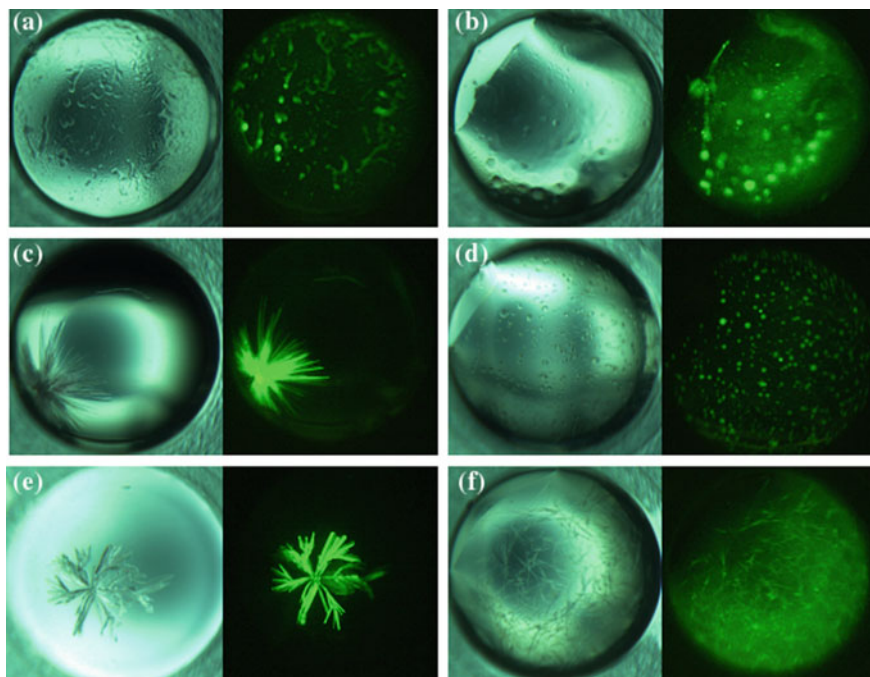


Fig. 2.2 Outcomes and their scores, each panel showing a white light and its corresponding fluorescence image. Panels A and B would have a starting score of 3, but due to the bright spots this would be increased to a score of 4. In the case of B the larger areas of intensity correspond to observable structures, and these may also be scored as 5. Panels C, D, and E, scores = 5, non-faceted crystals, with C being “urchins”, D being spheroids, and E dendrites. Panel F, score = 6, needles. Proteins are A, B - Canavalin; C, D, F - *Klebsiella pneumoniae* Inorganic pyrophosphatase, E - Tt36

precipitate is not clearly observable, unless one is using a non-white light imaging method. This is often a judgement call on the observers part. However, when making the distinction, one should attempt to be consistent in making that distinction for a given set of plates where the outcomes will be analyzed together. Also note that Panel A shows a heavy brown precipitate while B shows a heavy “white” precipitate. The nature of the precipitate in these cases is likely totally different, with one, B, likely being readily soluble while the other is likely not. Also note that the precipitate in B has a number of “bright spots” distributed throughout. As a result, the drop shown in panel B would be given a score of 0 during the manual examination, then that would be revised to a score of 4 during the fluorescence review.

The next difficulty comes in distinguishing a very light precipitate from a clear solution. While this is readily apparent in Fig. 2.1, Panels C and D, in many cases very light precipitates may not be visible. This is again a case where consistency in a set of plates is more important than accuracy. On occasion the results from other drops for that crystallization condition may suggest whether the solution is a

light precipitate or a clear solution. However, precipitate does often show up when observing the plates with fluorescent illumination.

Panels E and F of Fig. 2.1 show drops that would be scored as phase transitions during the initial white light scoring. Although not the case here, phase transitions are often spherical in shape, and can on occasion be mistaken for spheroids. In the case of Panel E the protein, which is fluorescent, has clearly separated from other components of the solution. This underscores the importance of having a means other than simple transmission microscopy for examining one's results. One may not know what solution components are separating out, whether they are the protein or some other (probably polymer) component. This distinction is important as we are most concerned with what the protein is doing in reaction to the solution conditions. Figure 2.1 Panel E shows the fluorescent image for a protein phase separation.

The white light image for Fig. 2.1, Panel F, shows an apparently “gummy” precipitate. We had initially scored this type of result as a heavy precipitate, but have shifted to scoring it as a phase transition. In large measure this is due to this type of precipitate often having bright spots, as shown in the fluorescent image, giving it a score of 4. As bright spot results can often be optimized to crystallization conditions, and as they are often associated with this type of precipitate, then we felt that it should be upgraded in the scoring to reflect the increased likelihood that these conditions may be on the path to crystallization.

The one novel scoring point, for our laboratory, is for the bright spots. Bright spots do not show crystalline features when viewed at higher magnification, and we currently assume them to be failed crystal nucleation's, where non-specific self-association kinetics have overtaken the orderly crystal self-assembly process. This is a score that is not made during the initial manual analysis phase but instead assigned when resolving those results with the fluorescent images. As we use trace fluorescent labeling for all of our crystallizations, the first pass image interpretation mantra is that intensity = structure. This is because the fluorescence intensity is a function of the density of the probe concentration, and the greatest protein, and thus probe, concentration will be had in the crystalline state. As shown in Fig. 2.2, Panels A and B, the bright spots can show up in a variety of “background” outcomes. We have found that in ~30% of the cases these conditions can be optimized to obtain crystals, and thus this score represents a major source of previously unknown lead conditions.

The most common crystalline outcome is often non-faceted crystals, which we assign a score of 5. Some of the most often observed of these are shown in Fig. 2.2, panels C, D, and E. Panel E shows what is typically referred to as an “urchin”. These are often also observed as a more linear spray of needles commonly referred to as a “shaving brush”. Panel D shows spheroids. Smaller spheroids can often be mistaken for phase separations. A distinguishing characteristic however is the presence of surface features or roughness on the spheroids. Panel E shows a dendritic crystal form. These may present as the stick-like crystal shown, as a snowflake-like feathery structure, or some intermediate form. This score is essentially a “catch-all” for any outcome that is crystalline, does not have clear facets, does not fit into one of the other categories, or includes clusters of crystals. The clusters can be stacks of 2D or agglomerations of 3D crystals. These sometimes have protruding single crystals,

and our rule of thumb is that if we think we can cleave a clear faceted region off then the structure, and thus the well, is given the score for that part.

Needles, a score of 6, often show up as shown in Fig. 2.2, Panel F. They can be clearly resolvable as individual needles, or they may be present as a dense cluster, or any outcome in between. Careful examination of the fluorescent image for Panel F shows an interesting light pipe phenomena often associate with needle (and rod) shaped crystals, where the ends fluoresce at a higher intensity than the body. This phenomenon is sometimes also found with 2D plates, and to a lesser extent with 3D crystals, where the edges are often more intense. We distinguish needles from rods by the presence, or not, of facets. If the ends of the crystals are clearly flat or have facets under higher magnification then they are scored as rods, 3D crystals, and not needles. This also holds true for the body of the crystal; if it is faceted then it is a rod, not a needle. Outcomes having a score of 5 or 6 are not suitable for diffraction analysis, but can be used as a source of material for seeded crystal growth [7].

2D Plates, having a score of 7, are shown in Fig. 2.3, Panel A. The last questionable distinction is between plates and 3D crystals. Again, this is often a judgement call. In our hands, it becomes a 3D crystal when there are clearly visible faceted edges,

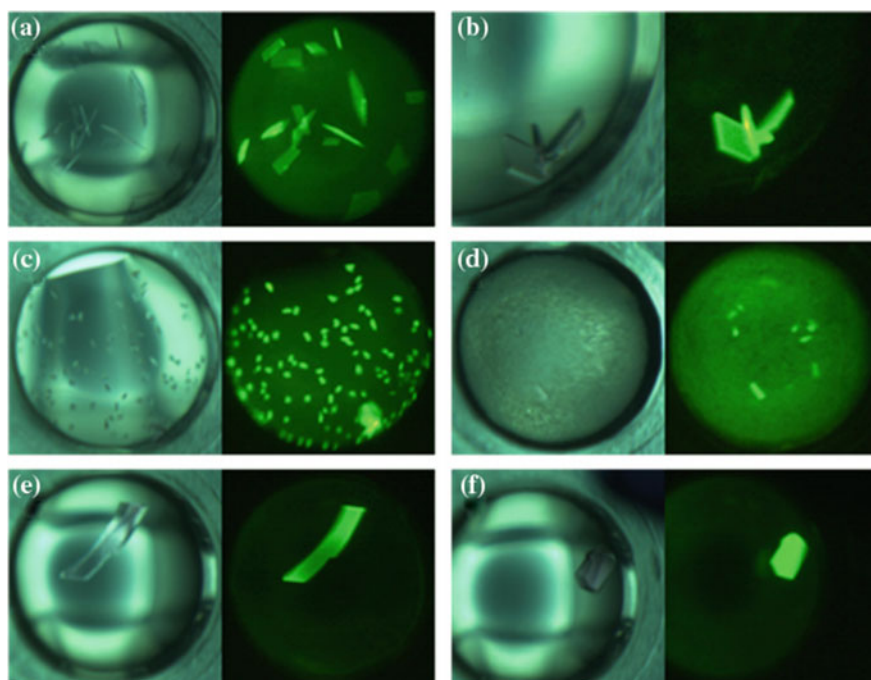


Fig. 2.3 White light and corresponding fluorescent images of scored plate outcomes. Panels A and B, score = 7, plate crystals. Panels C and D, score = 8, small 3D crystals size $\leq 200\ \mu\text{m}$, with the crystals in Panel D being surrounded by precipitated protein. Panels E and F, large 3D crystals, size $\geq 200\ \mu\text{m}$. All crystals are of *Klebsiella pneumoniae* inorganic pyrophosphatase

similar to the distinction between needles and rods. This is illustrated in Fig. 2.3, Panel B, where the plate-like crystals show a distinct edge, both in the white light and fluorescent image. Note that the body of the plate-like crystals, in both Panels A and B, fluoresces at a lower intensity. It is advantageous to have a microscope with a scale attached to gain some estimate of the thickness of the crystal. When imaging with TFL the edge typically shows up as a more intense fluorescence, particularly when it is partially or wholly oriented towards the viewing direction, while the body shows up with a lower fluorescence intensity when it is perpendicular to the viewing axis. However, the presence of straight fluorescent edges bordering a weaker fluorescence signals the presence of 2D plates where one may not have observed them using white light imaging.

Small 3D crystals, having a score of 8, are illustrated in Fig. 2.3, Panels C and D. Smaller crystals, or crystalline appearing material, should be examined under high magnification to look for the presence of faceted edges, which distinguishes them from non-faceted crystalline material having a score of 5. Panel D shows small crystals that are buried in precipitate. While they may not be apparent to cursory examination under transmission microscopy, they become readily apparent when viewed using fluorescence illumination.

The last category, and not surprisingly the least common, is large 3D crystals $\geq 200\mu\text{m}$ in size. Two examples of these are shown in Fig. 2.3, Panels E and F. Obtaining crystals of this size was once the goal of crystallographers several decades ago. However, as the X-ray technology has advanced these are not as desirable, except maybe as an experimental trophy. One potential benefit is that they do show that one can obtain larger crystals of that particular protein, thus suggesting that at some future date one could carry out neutron diffraction studies on it.

2.4.2 Hierarchical Categories

Classifying protein crystallization trial images into a *number of categories* is one of the main tasks in analysis. However, the key point in such analysis is to determine the categories. The number of categories is usually determined based on the purpose of the analysis. In the literature, we have observed that typically the number of categories is between 2 and 10. Since the most common goal is to detect the presence of a crystal, the use of two categories as crystals and non-crystals is not rare (Zuk and Ward [18], Cumba et al. [6], Cumba et al. [4], Zhu et al. [17], Berry et al. [2], Pan et al. [9], Po and Laine [10]). Additional categories are typically obtained by using sub-categories of these two main categories and erroneous/mistake/unclear or doubtful categories. Clear, precipitate, and crystal categories are three categories used by Yang et al. [15]. The five categories analyzed by Bern et al. [1] are empty, clear, precipitate, microcrystal hit, and crystal categories. Another group of five categories is formed as clear drop, creamy precipitate, granulated precipitate, amorphous state precipitate, and crystal categories by Saitoh et al. [12]. An example of six categories includes experimental mistake, clear drop, homogeneous precipitant, inhomogeneous precip-

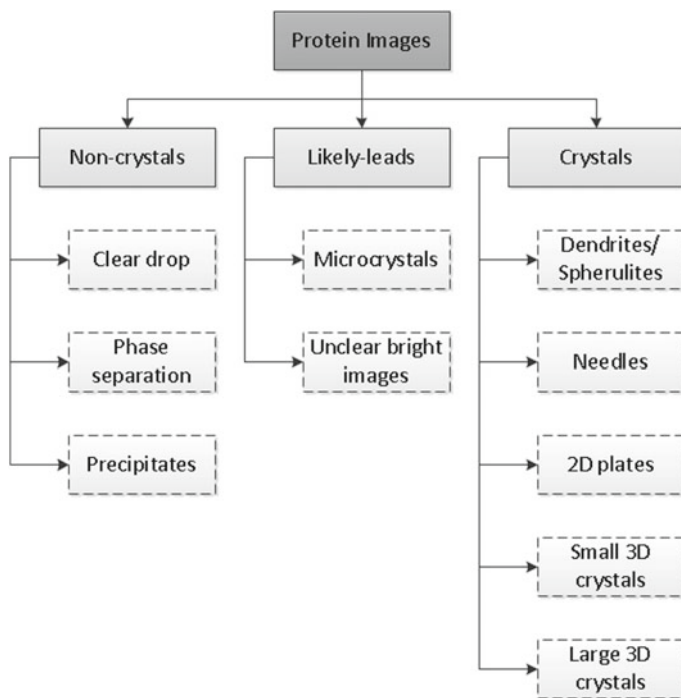


Fig. 2.4 Hierarchy of crystallization categories [13]

itant, microcrystals, and crystal categories (Spraggon et al. [14]). Another example of six categories includes phase separation, precipitate, skin effect, crystal, junk, and unsure categories (Cumba et al. [5]). 10 categories used by [16] are (clear, precipitate, crystal, phase, precipitate and crystal, precipitate and skin, phase and crystal, phase and precipitate, skin, and junk categories).

There is not a perfect system that would classify crystallization trial images into any number of categories. However, binary categorization as crystals vs. non-crystals should be avoided where possible. The costly misclassification occurs when a crystal is classified as a non-crystal. To detect such an error, the expert analyzes non-crystal images in addition to crystal images categorized by the system to avoid missing crystals. This would suggest checking all images in the experiment, thus losing the value of a classifier. In our work, we have added one more category in between crystals and non-crystals as likely-leads. Classifying crystals as likely-leads is not a major problem as long as crystals are not labeled as non-crystals and false positive rate where non-crystals labeled as crystals is low.

Depending on the depth of analysis of protein crystallization images, the classification could be performed roughly as non-crystals, crystals, and likely-leads or for the sub-categories of these categories as mentioned in the previous section. For analyzing protein crystallization trial images, we generally use two levels of hierarchical

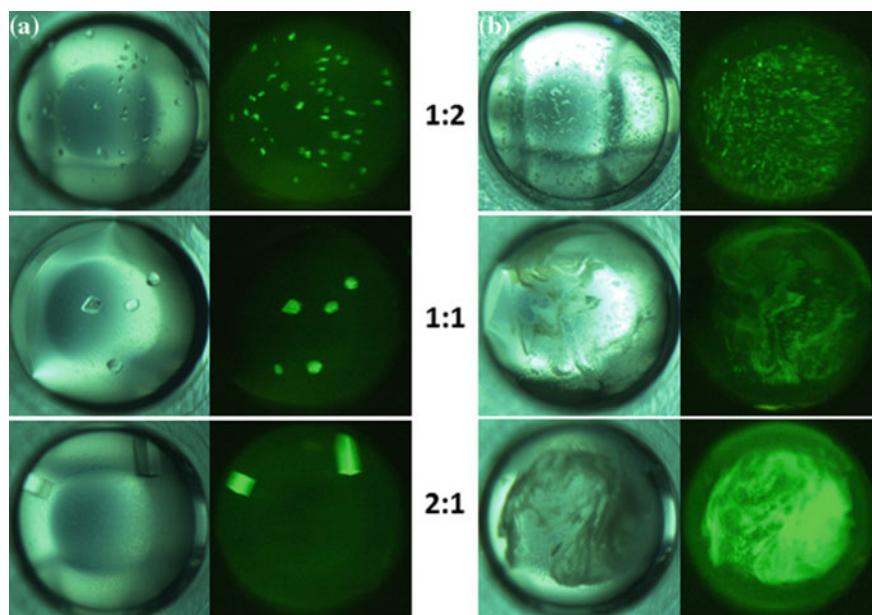


Fig. 2.5 Variations in crystallization drop outcome with changing protein:precipitant drop volume ratios. Column A shows a progression of smaller to larger, while column B shows a progression of crystals to precipitate

categorization as shown in Fig. 2.4. This hierarchy helps develop classifiers for the first level and then classifiers for each category of the first level. Such a hierarchy enables developing classifiers at two levels.

2.5 Even if You Are Not Going to Process Your Scored Data...

Careful scoring requires attention to and consideration of what is being observed. Insights beyond just the assignment of a numerical score can be obtained, which can be utilized to direct subsequent crystallization optimization experiments. For example, random, or limited grid, screens where one or two components are varied would be expected to show a trend in the results with the changes in the components. This is the reason for using such a screen, and justifies its utility. Similarly however we can also observe trends in screen set-ups where we vary the protein:precipitant drop ratio. Two examples of this are shown in Fig. 2.5, both coming from the same screening plate for the protein concanavalin A. In both cases the protein:precipitant drop volume ratios are 1:2, 1:1, and 2:1. A first pass assumption is that at equilibrium the drop and well precipitant concentrations are equivalent to those of the reservoir

solution for all three cases, although this assumption does not take into account the protein contribution to the crystallization drop vapor pressure. For the 1:2 drop the initial precipitant concentration is high relative to the protein and the equilibrium protein concentration would be low. In the results shown for Panel A we find that this results in many smaller crystals. At a 1:1 ratio, the “standard” crystallization drop, we find fewer but larger crystals, while at 2:1 the starting precipitant concentration is low and the equilibrium protein concentration higher, likely higher than the starting protein concentration. In this case fewer nucleation events occur and there is more protein available to feed the growth of larger crystals. These results can be mapped directly onto the phase diagram in Fig. 1.1. The results in Panel B show an opposite effect. Crystals are nucleated at higher precipitant and lower protein concentrations. As the protein:precipitant ratio increases the results go to increased precipitation and no crystals, the opposite of what is observed for the wells in Panel A. The precipitants in Panel A are ethylene glycol and Polyethylene glycol 8,000, while those in Panel B are sodium chloride and sodium citrate. The results give a good indication of how one should adjust the protein and/or the precipitant concentration in both cases for obtaining optimized crystallization results.

2.6 Summary

While it may appear to be tedious at the outset, the scoring of one’s crystallization screening experiments can be a very productive exercise. Firstly, one has a more detailed description of the results obtained for the experiment(s), which can always be referred to in subsequent work. Secondly, the act of scoring directs the mind towards considerations of how or why those results were obtained, possible new approaches that can be tried to obtain crystals, and insights into how one can optimize found crystallization conditions. Time spent carefully observing, and scoring, one’s crystallization results can speed up progress towards the ultimate goal, well diffracting crystals.

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