

Prologue

There is only one rule in the crystallization. And that rule is, there are no rules. Bob Cudney

Introduction

Optimization is the manipulation and evaluation of biochemical, chemical, and physical crystallization variables, towards producing a crystal, or crystals with the specific desired characteristics.¹

Table 1
Optimization Variable & Methods

Successive Grid Search Strategy – pH & Precipitant Concentration	Reductive Alkylation
Design of Experiment (DOE)	Chemical Modification
pH & Buffer	Complex Formation
Precipitant Concentration	Deglycosylation
Precipitant Type	Point Mutants
Protein Concentration	Truncations, Deletions, & Fusion Proteins
Converting Hits in nl Drops to μ l Drops	Surface Entropy Reduction
Drop Volume & Drop Ratio	Gel Growth
Seeding	Surfaces & Nucleating Agents
Additives	Tag On or Tag Off
Crystallization Method	Fresh Sample
Sample Buffer	Fresh PEG, Old PEG
Further Purification & Batch Variation	Change the Kinetics
Temperature	Pressure
To Centrifuge, Filter, or Not?	Magnetic & Electric Field
Additional Screening – When to Stop & Try Something Else	Microgravity
Modification of the Protein	Vibration
Proteolysis	Data & Literature Review

Once the appropriately prepared biological macromolecular sample (protein, sample, macromolecule) is in hand, the search for conditions that will produce crystals of the protein generally begins with a screen. Once crystals are obtained, the initial crystals frequently possess something less than the desired optimal characteristics. The crystals may be too small or too large, have unsuitable morphology, yield poor X-ray diffraction intensities, or possess non ideal formulation or therapeutic delivery properties. It is therefore generally necessary to improve upon these initial crystallization conditions in order to obtain crystals of sufficient quality for X-ray data collection, biological formulation and delivery, or meet whatever unique application is demanded of the crystals. Even when the initial samples are suitable, often marginally, refinement of conditions is recommended in order to obtain the highest quality crystals that can be grown. The quality and ease of an X-ray structure determination is directly correlated with the size and the perfection of the crystalline samples. The quality, efficacy, and stability of a crystalline biological formulation can be directly correlated with the characteristics and quality of the crystals. Thus, refinement of conditions should always be a primary component of the crystal growth process. The improvement process is referred to as optimization, and it entails sequential, incremental changes in the chemical parameters that influence crystallization, such as pH, ionic strength and reagent concentration, as well as physical parameters such as temperature, sample volume and overall methodology. Optimization can also entail the application of novel procedures and approaches that may enhance nucleation or crystal development. Here, an attempt is made to provide guidance on how optimization might best be applied to crystal growth problems, and what parameters and factors might most profitably be explored to accelerate and achieve success (Table 1).

Strategy

The fundamental strategy in optimization focuses on the incremental adjustment of crystallization parameters that, hopefully, converges on the best nucleation and growth conditions. But how does one choose which variable or set of variables to first evaluate? And how best does one prioritize the order in which to evaluate the variables? And once the primary variables have been evaluated, what other methods, strategies, techniques, and new variables should be considered and evaluated, and in what priority? The variables are numerous, diverse and often interdependent. Thus, multiple approaches and procedures can be applied to each of them, in parallel if possible. Some parameters may be addressed in a straightforward and systematic way, such as buffer pH or precipitant concentration. Others, such as additives or detergents may require a significant amount of trial and error, as well as a significant application of creativity and biochemical insight. Feeling overwhelmed before we've even picked up a pipette? Balderdash! There is nothing to fear but fear itself, and the reward is in the journey.

Sometimes one of the greatest obstacles to success in optimization is lack of effort and insufficient commitment of the experimenter. Optimization may also require a substantial amount of protein, and this is often limiting. Making protein can be expensive grunt work. As well, the formulation of a myriad of solutions, adjusting pH to exact values, and so on can be tedious. Many experimenters would rather struggle with marginal crystals grown from the first hit in a screen than undertake an optimization effort. But in the end, this approach may catch up to such experimenters as they feel the pain of crummy crystals or not quite good enough X-ray data. Fortunately, there are many time tested protocols and methods as well as ready to use reagents, plates, and tools to choose from that help reduce the activation energy associated with the optimization process. Many such protocols, methods, reagents, and tools will be presented here as part of this overview of optimization.

Screen Results

A screen result can be distilled down to one of three categories. First category, a hit, one or more crystals, microcrystals, or microcrystalline precipitate. Second category, a clear drop or solution. Third category, something not crystalline and not a clear drop; this would be precipitate, liquid-liquid phase separation, gel, aggregates, or some other phase behavior.

A Hit! Step One - Successive Grid Search Strategy – pH & Precipitant Concentration

If only a single reagent in a screen produces a hit (crystal), the initial optimization path could pursue a successive grid search strategy to evaluate pH and precipitant concentration.^{2,3} The initial grid search may screen around +/- 1 pH unit and +/- 20% of the precipitant concentration in a 24, 48, or 96 well crystallization plate. Using a 24 well plate, an initial grid screen of a hit in GRAS Screen 6 reagent 56(E8) 16% w/v Polyethylene glycol 3,350, 0.1 M HEPES pH 7.5 is shown in Figure 1 using the stock solutions in Table 2. The original hit reagent is positioned near the center of the grid and then expanded. One can use MakeTray (an application on the Hampton Research web site, located under Support) to generate grids and calculate volumes from stock solutions, and provide a printout of the formulation worksheet for your notebook.

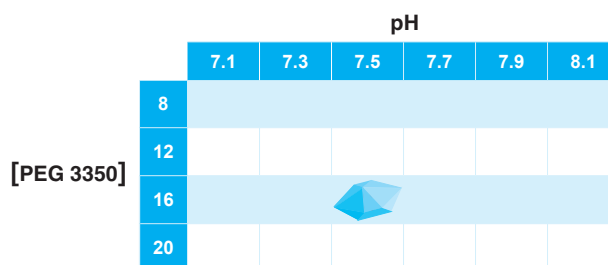
Table 2

Crystallization reagent that produced hit, and stock solutions for grid screen

Crystallization Reagent	Stock Solutions
16% w/v PEG 3,350 (precipitant: a polymer)	50% w/v PEG 3,350
0.1 M HEPES, pH 7.5 (buffer)	1.0 M HEPES, at 6 pH levels

Figure 1

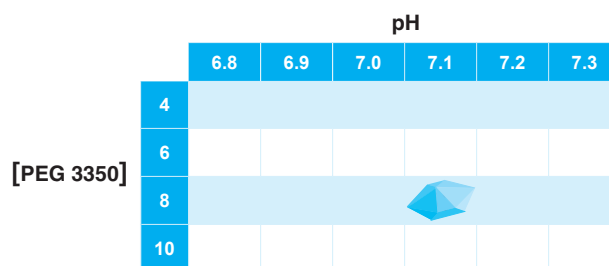
Grid screen for a hit in 16% w/v Polyethylene glycol 3,350, 0.1 M HEPES pH 7.5



Successive grid screens can be set as needed, based on the results of the previous grid screen. To generate a successive grid screen, choose the best result from the previous grid screen, place it near the center of the next, successive, and finer grid, and create another grid screen. For example, if the best result in the prior grid screen is 8% w/v Polyethylene glycol 3,350, 0.1 M pH 7.1, one might generate the successive, finer grid screen in Figure 2.

Figure 2

Successive, finer grid screen for a hit in 8% w/v Polyethylene glycol 3,350, 0.1 M HEPES pH 7.1



To make the reformulation of hits, as well as the formulation of successive grid and custom screens convenient and consistent, Hampton Research offers Optimize™ polymers, non-volatile organics, salts, buffers, and other reagents in ready to use, concentrated stock solutions. Hampton Research also offers a portfolio of different StockOptions Buffer Kits; a set of ready to use, concentrated, titrated buffer stocks. For example, the StockOptions HEPES Buffer Kit is a set of 15 1.0 M HEPES buffers covering pH 6.8 – 8.2 in 0.1 pH increments. The StockOptions Buffer Kits are available for each of the buffers appearing in Hampton Research screens.

Design of Experiments – DOE

Before delving deeper, a brief mention of experimental design. Design of Experiments (DOE) is a technique that can be used to gain increased knowledge of, as well as improve processes. A number of different DOE approaches have been applied to protein crystallization in recent decades with varying levels of success. Incomplete factorial designs¹⁴, systematic grid screens¹⁵, orthogonal arrays¹⁶, sampling techniques¹⁷, Box-Wilson and Box-Bhenken¹⁸ central composite designs, and others have been described and used in an

effort to standardize, as well as apply statistical methods to crystallization. A variety of programs have been implemented and are available from liquid handling automation and imaging vendors, as well as shareware applications that appear and disappear from time to time through the Internet. While optimization methods could seemingly benefit from DOE, one of the issues is that one may not yet have uncovered the variable(s) key to crystal perfection. A screen may have produced a hit. Yet exhaustive exploration of hit variables may not lead to the desired result. If the key variable is not in the initial hit (box), then one needs to look outside the box in order to introduce and explore new variables, such as additives, seeding, protein modification, and others that may deliver the desired crystal.

Optimizing Multiple Hits

If the initial crystallization screen produced more than one hit, one needs to review and compare the different hit reagent formulations and look for commonalities and trends. Compare pH, buffer type, primary precipitant (precipitant), secondary reagent, and additives in an attempt to identify a common variable shared among the hit producing reagents. The buffer is typically present at a concentration of 0.1 M with an indicated pH such as 0.1 M HEPES pH 7.5. A primary precipitant is the chemical of the highest concentration and the secondary reagent is the chemical of next highest concentration in a multi chemical formulation. For example, in the Crystal Screen reagent 15, 0.2 M Ammonium sulfate, 0.1 M Sodium cacodylate trihydrate pH 6.5, 30% w/v Polyethylene glycol 8,000, the primary precipitant is 30% Polyethylene glycol 8,000 and the secondary reagent is 0.2 M Ammonium sulfate. A primary precipitant, as well as a secondary reagent can be a polymer, salt, volatile or non-volatile organic, or other chemical. The buffer is not typically considered the primary or secondary reagent. Another component found in crystallization reagent are additives. Additives may be metals, mono- and multivalent salts, amino acids, dissociation agents, linkers, polyamines, chaotropes, reducing agents, polymers, sugars, polyols, amphiphiles, detergents, volatile and non-volatile organics, ligands, co-factors, inhibitors, and so on. Additives are typically present in relatively low concentrations, perhaps 1 mM to 100 mM or 1 to 4% v/v.

Each component of a reagent producing a hit should be considered for work up. For example, for a hit in Crystal Screen reagent 15, 0.2 M Ammonium sulfate, 0.1 M Sodium cacodylate trihydrate pH 6.5, 30% w/v Polyethylene glycol 8,000, one would vary the concentration of Ammonium sulfate and the concentration of Polyethylene glycol 8,000, as well as vary the pH. One optimization experiment would vary pH (5.5 – 7.4) versus Polyethylene glycol 8,000 concentration (20 – 35% w/v) while holding the Ammonium sulfate concentration constant at 0.2 M. A second optimization experiment would vary pH (5.5 – 7.5) versus Ammonium sulfate concentration (0.05 – 0.25 M) while holding Polyethylene glycol 8,000 concentration constant at 30% w/v. If varying the concentration of the primary precipitant, secondary reagent, and pH do not achieve the desired results, one should consider add-

ing an additive, or try evaluating similar primary precipitants (Polyethylene glycol 3,350 – 10,000 and Polyethylene glycol monomethyl ether (PEG MME)) as well as evaluating similar secondary reagents (Ammonium salts and sulfate salts).

If the multiple hits have a similar pH and/or similar precipitant, such as pH 5.0, 5.5 and 6.0, and Polyethylene glycol 25% w/v 3,350, 20% w/v 4,000, and 15% w/v 6,000, one might focus a grid screen between pH 4.5 and 6.5 versus 15 to 25% w/v PEG 4,000. Polyethylene glycols of similar molecular weight often produce overlapping results. That being said, if the desired results are not achieved with PEG 4,000, then PEGs of similar molecular weight, as well as PEG MME should be worked up.

If all hits contain a polymer such as Polyethylene glycol (PEG) as the precipitant, and no crystals were grown from salt reagents, then one would focus on PEG, and not salts. If crystals appeared at both pH 5 and 8, then one should work up conditions around both pH 5 and 8. During such an optimization, one may pursue a successive grid strategy, but also consider additional screening. For example, if a hit is obtained in Index reagents with salts as the primary precipitant, one may set additional salt based screens such as SaltRx 1 and SaltRx 2. If a hit is obtained in Index reagents with polymers as the primary precipitant, one may set additional polymer based screens such as PEGRx 1 and PEGRx 2. If a hit is obtained in Index reagents with PEG as the primary precipitant and salt as the secondary reagent, one may set additional screens such as PEG/Ion and PEG/Ion 2.

If the reagent formulation of each the initial hits is chemically diverse and without an obvious commonality, then one could choose a hit that produced crystals with characteristics nearest to the desired end result. One could then work up a successive grid screen strategy and/or perform additional crystallization screens based on the best hit. If sufficient sample is available, and time is of the essence, it can be prudent to workup multiple hits simultaneously. Cast a wide a net as is allowed by the amount of protein available. Often times, after a second round of optimization, it will become more evident which conditions are worth pursuing and which are likely to remain problematic.

pH & Buffer

While evaluating pH as a crystallization variable, one should also consider the buffer. When reproducing and optimizing a condition, be sure to use the same buffer in the original screen producing the hit. If HEPES buffer was used, uses HEPES, do not at first, use HEPES sodium. The same is true for Tris and TRIS hydrochloride, Citric acid and Sodium citrate, and other buffer pairs. The measured conductivity of 1.0 M HEPES pH 7.5 is 13 mS/cm, whereas 1.0 M HEPES sodium pH 7.5 is 43 mS/cm. Different buffers require different volumes of the acid or base pair of the buffer, or HCl or NaOH to be titrated to a specific pH. This in turn can result in a reagent with a unique ionic strength.

This subtle change in ionic strength can influence crystallization and can make the difference in being able to successfully reproduce a hit or not.

The buffer molecule itself can be a crystallization variable. 1.0 M Tris pH 7.5 has a measured conductivity of 43 mS/cm where 1.0 M HEPES pH 7.5 13 mS/cm. While this difference in ionic strength could be masked in a high salt condition, it might be significant in a low ionic strength polymer based condition. While it is important to at first use the same buffer used to produce the hit, evaluating a different buffer is something that should be considered during optimization. Until evaluated, one does not know if varying ionic strength will worsen or improve crystal quality. The unique chemical structure of different buffers effective over the same pH can also be a crystallization variable.^{4,5} The buffers Citrate, Malate, Succinate, Phosphate, Cacodylate, MES, Bis-Tris, and ADA are all appropriate buffers at pH 6. The unique chemical properties and structure of each of these buffers may influence crystal quality. Screen both pH and buffer type during optimization.

The ionizable amino acid side chains in proteins are aspartic and glutamic acid (pKa values of about 4.5), histidine (pKa = 6.02), cysteine (pKa = 8.2), lysine (pKa = 10.5), tyrosine (pKa = 10.2), and arginine (pKa = 12.2). Although the pKa of an ionizable group on a protein may be strongly influenced by its chemical environment, it is worth keeping these pKa values in mind, as it is in their immediate neighborhoods that the charges on a protein, their distribution and their electrostatic consequences may be most sensitive. Buffer pH can determine the ionization state of the side chains, and that can determine sample-sample and sample-solvent interactions, as well as lattice interactions, all having the potential to manipulate crystal quality.

Keep in mind the actual, measured pH of the crystallization reagent (condition, cocktail) may be different from that of the buffer stock used in formulation. This is okay, as long as one can reproduce the result. Most screen reagents are made from 0.5 or 1.0 M titrated buffer stocks, diluted to a final concentration of 0.1 M or lower. Dilution alone can change the pH, as well as the effect of additional chemicals, including salts, polymers, non-volatile organics, and additives. Unless otherwise indicated, or a Grid Screen kit which are titrated to pH after buffer and chemicals are added, the pH indicated on a tube label or formulation is that of a 1.0 M stock prior to the addition of other chemicals. The measured pH of each screen reagent is available in the Support Materials section of the screen's web page at hamptonresearch.com. Formulation details for screen buffers is available in Crystal Growth 101 – Buffer Formulation.

Precipitant Concentration

A crystallization experiment containing microcrystalline precipitate, microcrystals, or numerous small crystals may indicate the concentration of the precipitant is too high, and lower concentrations should be explored. A crystallization experiment with precipitate is also associated with precipitant

concentration that is too high; lowering the precipitant concentration may lead to crystals. Clear drops indicate the precipitant concentration is too low and one should evaluate increasing concentrations of precipitant. The appropriate precipitant concentration is interdependent on variables such as protein concentration, salt concentration, pH, and temperature.

Precipitant Type

Polyethylene glycols (PEGs) are the most common precipitants used today for the crystallization of proteins. PEG molecular weight varies from 200-20,000 but the middleweight range of PEGs (3,350-8,000) are most effective when growing crystals for X-ray diffraction. Experience seems to show that PEGs in the range of 200-600 are similar, PEGs in the range of 1,000-2,000 are similar, PEGs in the range of 3,350-8,000 are similar and PEGs in the range of 10,000-20,000 are similar. For example, a protein that crystallizes in a particular molecular weight of PEG, such as 3,350, is likely to crystallize in PEG 4,000, 6,000, and 8,000 albeit at decreasing PEG concentration as the molecular weight of the PEG increases. When optimizing a PEG based reagent, consider varying the concentration of the PEG, using similar molecular weights, and substituting PEG MME for PEG (or vice versa) during optimization. When crystals appear in PEG, they often do so across a much broader concentration, range than salts. A crystal may appear in 5-20% PEG, although it may have an optimum within that range, so successive grid screening is warranted with PEGs. When optimizing a reagent with PEG as the primary precipitant with a secondary salt, one should not only evaluate similar PEGs, but also similar secondary salts. When screening the secondary salt, one generally holds the cation constant, screening various anions, then hold the anion constant and screening various cations. For example, for a hit in 20% w/v Polyethylene glycol 3,350, 0.2 M Ammonium sulfate, when evaluating the secondary salt, one would screen Ammonium salts (Ammonium acetate, chloride, citrate, fluoride, formate, nitrate, phosphate, and tartrate) as well as Sulfate salts (lithium, magnesium, potassium, and sodium). PEG conditions, free of salt, with buffer present are considered low ionic strength conditions. Low ionic strength conditions are typically more sensitive to pH and temperature, so be sure to evaluate a broad and fine (0.1 pH increments) pH, as well as a temperature range between 4 and 37° Celsius. Vapor diffusion equilibration rates are lower in PEG based reagents, especially low ionic strength, free of salt, which drives equilibration more strongly than PEGs.

Salts, both inorganic and organic, are the second most common precipitants used today for the crystallization of proteins. The multivalent anions (SO_4^{2-} , PO_4^{3-} , Citrate₃⁻, etc.), yielding a higher ionic strength according to the square of their charge, are the most frequently utilized and productive salts. The type of cation or anion can affect protein solubility and stability. As just stated, when evaluating salts during optimization, one can hold the cation constant and vary the anion, and then hold the anion constant and vary the cation. This can help identify the optimal salt and also indicate if there is a

preference for a specific anion or cation. The concentration of salt suitable for promoting crystallization is typically much more narrow than for PEGs. Successive grid screens as fine as 0.05 M increments are often profitable and worth the effort. Vapor diffusion equilibration rates are higher in salt based reagents than PEG based reagent. The solubility dependence of most proteins are lessened by high salt concentration, so it is likely less rewarding to carry out experiments at multiple temperatures, although crystallization for purification and formulation would be an exception to this generalization.

Organic salts and acids, pH neutralized or not, can be effective crystallization reagents. Unless the measured pH of a salt is paired well with buffer pH, a salt based reagent can significantly influence the pH of the buffer, even pushing the solution pH outside the buffering capacity of the buffer. The use of neutralized organic acids makes pH pairing more obvious since the pH of the neutralized organic acid is consistent and indicated on the container, whereas the measured pH of some salts can vary significantly. Sodium malonate (malonic acid neutralized with Sodium hydroxide) has been shown to be highly effective in comparison to other commonly used salts such as ammonium sulfate.⁶ Sodium malonate is soluble across a broad pH, allowing the formulation of concentrated stocks of pH 4, 5, 6, 7, and 8, with the added advantage of being able to screen pH and salt concentration by blending two adjacent pH stocks (4/5, 5/6, 6/7, 7/8). Other neutralized organic acids such as Ammonium citrate, Malic acid, and Succinic acid have also shown to be unique and useful precipitants and secondary salts. This expanding portfolio of salt based precipitants requires more protein for optimization, and this prompted Hampton Research to develop Tacsimate™; a single reagent composed of seven organic salts, neutralized to pH 4, 5, 6, 7, 8, or 9, allowing one to screen seven different organic acids using a single reagent.

Mixtures of PEGs and salts have proven to be powerful crystallization reagent formulations. Formulating PEG as the primary precipitant with salts as the secondary salt, at a concentration of 0.2 M has demonstrated broad success in the crystallization of biological macromolecules. A 0.2 M divalent anion concentration is almost precisely the concentration that would be predicted from physical-chemical considerations to provide the optimal electrostatic shielding between proteins in a solution.^{7,8} This may explain why a 0.2 M salt concentration provides an optimal ionic strength for many proteins that crystallize in PEG and other non-salt precipitants. The PEG/Ion and PEG/Ion 2 screens, and a host of other conditions are predicated on this mixture of PEGs and salts. The diversity of salts in the PEG and salt mixture is key to the sampling of pH and ionic strength in the presence of PEG, without the need for an added buffer since the salt drives the pH of the reagent. Hits produced in PEG/Ion type reagents can be optimized for pH by adding a buffer to fine screen pH, as part of successive grid screening of PEG concentration and pH, as well as an evaluation of similar PEGs.

Protein Concentration

For most macromolecules, the optimal protein concentration is between 5 and 20 mg/ml, although there are, of course, many exceptions. Complexes, large assemblies, viruses, and proteins with limited solubility usually range lower at 2-5 mg/ml. Peptides and small proteins tend to range higher at 20-50 mg/ml. The Protein Data Bank⁹ and Biological Macromolecular Crystallization Database¹⁰⁻¹² report concentration ranges as low as 1 mg/ml and as high as 300 mg/ml.

To determine the appropriate protein concentration for crystallization screening, one can use a pre-crystallization test such as the Hampton Research PCT kit.

When evaluating crystallization experiments, clear drops indicate that either the relative supersaturation of the sample and reagent is too low or the drop has not yet completed equilibration. If the drop remains clear after 3 to 4 weeks, consider repeating the screen condition and doubling the sample concentration. If more than 70 of the 96 drops are clear, then consider doubling the sample concentration and repeating the entire screen.

Drops containing precipitate indicate either the relative supersaturation of the sample and reagent is too high, the sample has denatured, or the sample is heterogeneous. To reduce the relative supersaturation, dilute the sample twofold with sample buffer and repeat the screen condition. If more than 70 of the 96 drops contain precipitate and no crystals are present, then consider diluting the sample concentration in half by adding an equal volume of sample buffer to the sample and repeating the entire screen.

Below a certain protein concentration, the protein will simply not crystallize at all, producing a clear drop, or a drop with light precipitate. On the other hand, excessive protein concentration can favor uncontrolled nucleation, rapid and disordered growth, leading to numerous small crystals, or few to many crystals with visible and invisible defects. Note, beauty is only skin deep. External appearance has no relation to the quality of the crystal, in terms of X-ray diffraction, stability, or solubility.

Protein concentration and precipitant concentration work hand in hand. When presented with precipitate or an excessive number of small crystals, one may reduce the protein and/or the precipitant concentration to lower the relative supersaturation. Just the same, protein and/or precipitant concentration may be increased to raise the relative supersaturation, in attempt to make something happen in clear drops. Changing the protein may not have the same effect as changing the precipitant concentration, so consider both options during optimization.

Converting Hits in nl Drops to μ l Drops

Hits produced in nanoliter sized drops, such as 100 nl protein plus 100 nl reagent are typically scaled to larger drops during optimization. However, it is not unusual for a crystal producing reagent at the nanoliter scale to produce precipitate at the microliter scale. Current popular belief as to why this takes place is that the surface volume ratio is great for the smaller drops, meaning more protein is lost on the surface of smaller drops. In addition, the smaller drops equilibrate more rapidly with the reagent well. To correct for this when scaling to larger drops, the amount of protein needs to be decreased. One can accomplish this by diluting the protein concentration in half, or one can add half the amount of protein (at the original concentration) to the drop. For example, if the original drop was 100 nl of 10 mg/ml protein plus 100 nl of reagent, the scaled up drop could either be 1 μ l of 5 mg/ml protein plus 1 μ l of reagent or 0.5 μ l of 10 mg/ml protein plus 1.5 μ l of reagent. One may also need to reduce the precipitant concentration.

Drop Volume & Drop Ratio

Automated liquid handling systems (robots) allow one to set very small drops (100 nl protein plus 100 nl reagent) which conserves protein and allows one the option to screen more reagents, or have more protein available for optimization. Such small drops tend to produce small crystals. When larger crystals are necessary, the nanoliter sized drops are typically scaled to microliter drops. A compromise for those using crystals for X-ray diffraction analysis is to screen using 300 nl protein plus 300 nl reagent. This still saves protein compared to 1 μ l protein plus 1 μ l reagent drops, it increases the likelihood of producing crystals large enough for X-ray diffraction straight from the screen drop, and can prevent the need for scaling drop size.

During optimization, it is common to scale drops to 1 μ l protein plus 1 μ l reagent or even larger volumes. The volume of the drop can have a significant effect on the final size of the crystals produced. Andrew Karplus and Kristin Fox demonstrated a 60 fold increase in drop volume produced a 730 fold increase in crystal volume, and a simultaneous increase of the effective diffraction limit of the crystals from near 2.5 angstrom to well beyond 2.0 angstrom resolution.¹³ The crystal volume increase may be due to the increased amount of protein and the lower equilibration rate associated with larger drops. Hanging drop vapor diffusion drops as large as 20 μ l are straightforward to set, Cryschem sitting drop vapor diffusion crystallization plates can accommodate drops as large as 40 μ l, and nine well glass plates and sandwich box setups can accommodate drops as large as 800 μ l for vapor diffusion.

Drop ratios allow one to explore varying levels of initial and final protein and reagent concentration, explore different equilibration paths, and cover a wider range of relative supersaturation. All by simply changing the amount of sample and reagent added to the drop. Multiple drops with varying drop ratios can be set during screening and/or optimization to explore the effects

that different initial protein and reagent concentration, different final protein concentration, and the equilibration path, may have on crystal nucleation, growth, and quality. For more information on drop ratios see Crystal Growth 101 – Drop Ratio.

Seeding

Seeding allows one to grow crystals in the Metastable Zone, where spontaneous homogeneous nucleation cannot occur, but crystal growth from seeds can occur. Why would one want to do this? For control, reproducibility, and to improve the likelihood of a successful crystallization experiment. For more information, see Crystal Growth 101 – Seeding.

Hampton Research offers several kits related to seeding, including the Crystal Crusher, Seeding Tool, and Seed Bead Kits.

Additives

Additives are considered chemicals in a crystallization reagent in addition to the primary precipitant, secondary reagent, and buffer. Additives can affect the solubility and crystallizability of biological macromolecules. These chemicals can perturb, manipulate, and stabilize sample-sample and sample-solvent interactions, as well as perturb water structure, which can alter and improve the solubility of crystallization of the protein. Additives can stabilize or engender conformity by specific interactions with the protein. Additives can also establish stabilizing, intermolecular, non-covalent cross-links in protein crystals and thereby promote lattice formation.

The most commonly useful class of additives, and the only class of which we have any real understanding, are those which may, for physiological reasons, be bound by the protein with consequent favorable change in the protein physical-chemical properties or conformation. These include co-enzymes and prosthetic groups, inhibitors, enzymatic products, ions, and other effector molecules. Often times the ligand bound form of the protein is structurally defined and stable, while the unliganded form is not, and often the former will crystallize when the latter will not.

Chaotropes, osmolytes, and cosolvents can perturb or stabilize the hydration of macromolecules by altering the physical relationship between the surface of the protein and water.²⁰⁻²³ Detergents and non-detergent sulfobetaines (NDSB) can manipulate hydrophobic interactions and alter the solubility of the macromolecule and reduce aggregation without directly affecting its physical-chemical properties.^{24,25} Multivalent cations such as the divalent Cadmium chloride, Calcium chloride, and Magnesium chloride, and the trivalent Yttrium chloride, Iron chloride, and Nickel chloride stabilize conformation and structure of macromolecules.²⁶ Volatile organic solvents alter the dielectric constant of water which can manipulate and stabilize the hydration of macromolecules. Salts alter the activity coefficient of water and help reinforce attractions among macromolecules. Biocompatible water-

soluble ionic liquids, organic salts and salts with melting points at or below room temperature have demonstrated usefulness as additives.²⁷ Chemical protectants, molecules that assure protein integrity such as the reductants DTT and TCEP, as well as metal atom scavengers such as EDTA are yet another class of additives. Dissociating agents (phenol), amino acids, linkers (6-aminohexanoic acid), polyamines (spermidine), polymers (polyethylene glycol), sugars (sucrose), amphiphiles (benzamidinium hydrochloride), and non-volatile organics ((+/-)-2-Methyl-2,4-pentanediol) are other additive classes. More recently, additives termed silver bullets, because of their ability to play a primary role in the crystallization process, have expanded the portfolio of additives. Silver bullet molecules, which play an active role in creating and maintaining the crystal lattice through the formation of intermolecular interactions of a reversible nature, i.e. they serve to crosslink, electrostatically or through hydrogen bonds and hydrophobic interactions, surface groups on neighboring molecules.²⁸

Additives are easy to use. They are simply added to the reagent producing the crystal. The additive can be placed directly into the drop, or added to the reagent well and mixed with the macromolecule. Additives are screened much like crystallization reagents, in sets of 24 or 96. Unless there is de novo knowledge clearly indicating an additive be included in the crystallization experiment, the general strategy is to screen a portfolio of 96 or more additives, search for an effect, and then refining the effect.

Hampton Research offers several different additive screens, including the Additive Screen, Silver Bullets, Detergent Screen, and Ionic Liquid Screen.

Crystallization Method

Sitting drop, hanging drop, sandwich drop, microbatch, liquid bridge, free interface diffusion, microdialysis, sequential extraction, and other methods of crystallization can present a unique start, equilibration path, and endpoint. Subtle or significant, these different methods can and should be explored when other optimization procedures do not deliver the goods.

Sample Buffer

It can often be the case where the sample buffer is the final purification buffer, or a routinely used favorite, “because that’s what we’ve always done before” or “it seemed okay last time”. An evaluation of pH, buffer type, and portfolio of additives and excipients using Dynamic Light Scattering (DLS) to assay for monodispersity and aggregation, and Differential Scanning Fluorimetry (DSF) to assay for stability, can help to identify an optimal buffer formulation for the protein. A homogenous, monodisperse, stable sample can offer a better chance for crystallization, as well as produce highly ordered crystals. The sample is the single most important variable in the crystallization experiment.^{18,19} For more information, see Crystal Growth 101 – Sample Preparation for Crystallization for more information.

Further Purification & Batch Variation

With few exceptions, the probability of growing large single crystals of high quality is always substantially increased with homogeneity of the sample. In some instances, minor impurities are the impediment to high quality crystals, or even preliminary crystals, and once the macromolecule is subjected to further purification, crystallization is improved. When in doubt, purify further. And do everything possible to ensure the purification process is consistent, batch to batch. When one encounters a problem reproducing a hit, always question if the batch of protein is the same or different. Many a time reproducibility is blamed on reagents, when in reality it is variability in the macromolecule.

Temperature

While some proteins demonstrate temperature dependent solubility, others do not.⁸¹ So it is not surprising that temperature may be of appreciable importance for the crystallization of some macromolecules, or it may have no significance at all. Macromolecules may show normal or retrograde solubility, and this can be a function of ionic strength and pH.⁸² Macromolecular crystallization in reagents composed of low ionic strength, containing polyethylene glycol, polyol, volatile- or non-volatile organic, no or very low salt, and a buffer typically demonstrate more temperature dependence and should be evaluated at multiple temperatures, such as 10, 20, and 30° Celsius. Volatile organics such as 2-Propanol or tert-Butanol should be set at reduced temperatures for increased efficacy as well as to protect the stability of the macromolecule.^{83,84} When practical, crystallization screens should be set at two or three different temperatures between 4 and 37° degrees Celsius. 10, 20, and 30° Celsius is a popular choice. Even when crystals are not obtained, comparing results between reagents at multiple temperatures can give valuable insight into the temperature and reagent dependent solubility of the macromolecular sample. If sample is limited, or screening multiple temperatures simultaneously is not possible, set and incubate the experiments at one temperature, for a period of time, score, and then move the experiments to another temperature. Score, and then move to a third temperature and compare the results between temperatures. And do not base the evaluation of temperature simply on the appearance or not of crystals. Compare all experimental results, including clear, precipitate, phase separation, as well as the size, number and visible appearance of any crystals.

Incubating the crystallization experiment at an elevated temperature for a short period of time, and then moving the experiment to room temperature, or cooling the macromolecule, reagent and experiment during set up and incubation, as well as temperature ramping and/or cycling are all methodologies with demonstrated success.⁸⁵⁻⁸⁷

To Centrifuge, Filter, or Not?

Just before setting crystallization experiments, one may choose to centrifuge

or filter the sample to remove amorphous material, particulates, and aggregates, or not. One school of thought says that during initial screening, one should not centrifuge or filter the sample before the setup, as particulates and amorphous material may serve as a nucleating agent for crystallization. This school then suggest during optimization that the sample then be centrifuged or filtered to improve crystal quality. The OCD school of thought says be consistent, be clean, and always filter (0.22 micron) or centrifuge the sample. Centrifugation may be preferable to filtering, as there is no risk of sample loss due to the macromolecule binding to a filter, or dead volume, both wasting precious protein.

Additional Screening - When to Stop & Try Something Else

When the initial screen of 24, 48, or 96 reagents does not produce the desired macromolecular crystals, one may screen more reagents, with a focus on a different chemical space, using care not to duplicate conditions. If the land of clear and precipitated drops, free of crystals continues, how does one know when to say uncle and try something else? A well behaved, crystallizable protein, a trait we cannot assign the macromolecule until after it is crystallized, but only happens about 35% of the time, may crystallize in a chemically diverse screen composed of 96 to 384 reagents. Segelke, in 2000, analyzing a pool of structural genomics crystallization data, estimated that a protein with a success rate of only 2% would likely crystallize in a screen of 300 random reagents.⁶⁴ Crystallization efforts since that time, through global protein structure initiatives, indicate that perhaps a diverse chemical space of 384 reagents (4 x 96) is a reasonable number of reagents to screen before trying something else beyond screening. That being said, efforts developed and pursued since 2000 by Luft and DeTitta have focused on a screen composed of 1,536 reagents.⁶⁵ On more than one occasion the screen has successfully delivered the desired crystal on as few as 1 reagent, of 1,536. So when to stop screening and try something else? Somewhere between 384 and 1,536 reagents; your mileage may vary.

Modification of the Protein

When optimization of the primary crystallization variables, seeding, additives, and further screening do not provide the desired crystals, and the sample being the single most important variable in the crystallization experiment, a logical approach is to change the sample in some manner.

Proteolysis

Proteases can be used to trim floppy bits from a protein, as well as generate fragments or domains for crystallization screening. A proteolytic fragment or domain of a protein may crystallize more readily or form better diffracting crystals than the intact protein.²⁹⁻³³ Proteases can be used to generate small, active fragments or domains of the target protein for crystallization. The fragment or domain can be used directly for crystallization experiments. Or the proteolytic sample analyzed by gel electrophoresis and/or mass spectrometry for mass and sequence for subsequent cloning, expres-

sion, purification and crystallization. Using proteolysis to enhance sample crystallization, the current overall success rate for yielding a deposited crystal structure has been reported to be better than 12%.³¹ Hampton Research offers the Proti-Ace and Proti-Ace 2 kits for limited proteolysis, in situ proteolysis, and proteolytic screening of protein samples for crystallization and structure determination. Refer to the Proti-Ace User Guide for proteolysis methodologies.

Reductive Alkylation

Reductive alkylation of lysine residues to change protein properties (pI, solubility and hydrophathy) which may promote crystallization via improved crystal packing. Reductive methylation, ethylation, or isopropylation of lysine residues has been successfully applied to obtaining high quality crystals.³⁴⁻³⁷ Reductive alkylation does not change the intrinsic charge on a protein but may change the isoelectric point (pI) slightly. The N-terminal amino group on the backbone will also be reductively alkylated. In general, alkylated proteins retain their original biochemical function. Hampton Research offers this methodology in the Reductive Alkylation Kit. The protocol is designed with the goal of generating a high degree of modification with few side reactions, resulting in a homogeneous population of protein.

Chemical Modification

Despite the number of reagents available to modify particular sidechains of proteins, reductive alkylation is the method that appears most prominently in the crystallization literature. Although site specific chemical modification has been a useful tool in proteomics, the application has not transitioned into macromolecular crystallization. The options are numerous and include the modification of amino groups, histidine residues, arginine, carboxyl groups, cysteine, cystine, methionine, tyrosine, and tryptophan.³⁸

Complex Formation

Proteins that have not yet crystallized in their native state may be crystallized as complexes. Complex formation followed by crystallization screening can be performed with inhibitors, co-factors, and antibody fragments.³⁹⁻⁴¹ The conformational changes induced upon such ligand binding may be favorable to the crystallization process by exposing new crystal contacts or by stabilizing the protein. Complex formation can be combined with seeding, as demonstrated by the crystallization of antibody-antigen complexes, promoted by combining complexation with microseed matrix screening (MMS).⁴² See Crystal Growth 101 – Seeding for the microseed matrix screening procedure.

Deglycosylation

Proteins with high or heterogeneous carbohydrate content can prove difficult to crystallize. The enzymatic removal of sugars to promote crystallization has been used successfully in numerous instances.⁴³⁻⁴⁶ The glycosylation problem can also be solved by expression of glycoproteins in mammalian

cells in the presence of N-glycosylation processing inhibitors.⁴⁷ This allows for correct glycoprotein folding, but leaves the macromolecule sensitive to enzymes like endoglycosidase H, that reduce the N-glycans to single residues, enhancing the chance for crystallization.

Point Mutations

Proteins have a natural potential to interact via hydrogen bonds, ionic or electrostatic, and Van der Waals contacts and these are the same interactions which occur in the intermolecular packing within a protein crystal. For a protein resistant to crystallization, point mutations that change unfavorable contacts to more favorable contacts can and has been a successful crystallization strategy. Mutation of lysines to arginine or glutamine is one such option.⁴⁸ There are a number of examples in which rational surface point mutations were used to engineer crystal contacts to generate a crystallizable protein.⁴⁹⁻⁵² When pondering point mutations, keep in mind that not all mutations that resulted in crystalline protein have been rational. In a number of cases serendipity has played a role in the ability of the protein to crystallize. Consider both rational and random point mutations.

Truncations, Deletions, & Fusion Proteins

Proteins are often composed of domains tethered by flexible linkers. Excessive flexibility can be a source of heterogeneity which can interfere with crystallization. Deleting or removing flexible regions at the termini or within a protein may promote crystallization by minimizing the interfering effects from heterogeneity. N-terminal and C-terminal truncations and deletions and well as insertions and deletions within a protein can be handled with recombinant protein expression.⁵³⁻⁵⁵ The creation of fusion proteins, using recombinant techniques where genes or gene fragments are spliced together to form gene fusions can be used to generate soluble protein for crystallization.⁵⁶⁻⁵⁸

Surface Entropy Reduction

The propensity of macromolecules to associate favorably and form crystals is largely defined by topology and physical chemistry of the surface of the macromolecule. Large, flexible amino acids such as Lysine, Glutamic acid and Glutamine, on the surface of the macromolecule can form an entropic shield and impede favorable intermolecular interactions and crystallization. Generating variants of the macromolecule free of the entropic shield, by replacing selected large and surface exposed residues with smaller residues such as Alanine is another way to promote a crystallizable sample. This crystal engineering strategy, based on surface entropy reduction (SER) has been extensively tested and shown to be another tool for handling macromolecules recalcitrant to crystallization.⁵⁹⁻⁶² The SER strategy can also be combined with other rational protein engineering strategies, such as using the fusion protein methodology, to encourage crystallization of less than well behaved macromolecules.⁵⁹

Gel Growth

The presence of gel in a crystallization experiment can reduce convection and sedimentation. Crystallization in gels can be performed by vapor diffusion, batch, microdialysis, and free interface diffusion. Gels composed of agarose as well as a silica hydrogel are well known as media for optimizing the size and quality of crystals.^{68,69} Dong, through X-ray diffraction measurements of crystals grown in gels versus those in standard liquid drops showed an improvement in crystal order.⁷⁰ Hampton Research offers the Silica Hydrogel Kit and a Low Melting Agarose Gel for macromolecular crystallization in a gel matrix. For more information about using gels for crystal growth, see the Silica Hydrogel and LM Agarose for Crystallization user guides.

Surfaces & Nucleating agents

The effect of surfaces on macromolecular crystallization is not yet well understood. Intuition indicates surfaces should be important. It has been rumored that the best crystal growers were bearded, as bits from the beard made their way into crystallization experiments, serving as nucleating agents for the macromolecules. Don't laugh, there's proof of concept to hair and other bits promoting crystallization.^{90,91} Crystals are often observed, attached to a glass or plastic surface, growing at an air-water-phase interface, on a fiber or some debris in the sample. Surfaces can be important for crystallization since nucleation seems to preferentially occur on a surface. It is proposed that macromolecules adsorbed onto a surface conformationally constrained and this may assist in the formation of an ordered array, eventually resulting in a crystal. Both epitaxial (close lattice match) and heterogeneous (poor lattice match) examples appear in the literature, together with other proposed surface and nucleating agents.^{92,93} Most of the time however, our experiments attempt to minimize the effect of surfaces by silicizing glass cover slides and plates or using plastic plates molded from more hydrophobic materials. Time will reveal if a holy grail nucleant truly exists, or not.

Tag On or Tag Off

Protein tags, sometimes called affinity tags, are amino acid sequences grafted onto a recombinant protein. The tags are attached to proteins for various applications, but affinity tags are added to proteins so that the protein can be purified away from other materials in the prep using an affinity column. The poly(His) tag, often referred to as a His-tag is widely used for protein purification, as it binds to metal chromatography matrices. Other tags include the maltose binding protein (MBP) and glutathione-S-transferase (GST) tags.

When it comes time to crystallize the purified, tagged protein, a common question is whether or not the tag should be removed for crystallization. The reason being, recall that crystallization is favored when the protein is homogeneous, monodisperse, and free of flexible domains or termini. Supersti-

tion leads us to believe that His-tags should be unstructured and flexible, and this could deter crystallization. Yet, in reality, depending upon the tag and the protein, the tag may or may not be a variable in the crystallization experiment. The current consensus is to screen the protein for crystallization with the tag in place. If there are no crystals, then remove the tag and screen again. Based on what we hear and read, most remove the tag before screening, so the tag is not a variable. That being said, there are reports where only the tag form of the protein crystallized, or the presence of the tag resulted in a crystal form different from the non-tagged protein because the tag participated in crystal packing. And when asked about the length of the tag, the audience says 6 is better than 10.

Fresh Sample

Protein degradation can occur over time. Some proteins are more sensitive to change, aggregation, degradation, and denaturation than others. It is often the case where the more interesting, troublesome, and costly the protein is to produce, the less stable it is. Using fresh protein seems to be an advantage for crystallization. Heterogeneous, degraded, aggregated, and denatured protein is less prone to crystallize than fresh, homogeneous, and monodisperse protein.

Fresh PEG, Old PEG

Sometimes there is nothing more frustrating than being unable to reproduce a crystallization condition. A hit is obtained in a screen, yet reproducing the hit is evasive. Since there are so many crystallization variables, there can be many reasons for the irreproducibility. One that seems to rear its ugly head from time to time is the difference between fresh PEG (polyethylene glycol, poly(oligo)-oxyethylene-based compounds) and old PEG. Aging of PEG can alter the chemical properties of common polyethylene glycols, resulting in increased levels of aldehydes, carboxylates, and peroxides, increased ionic strength, as well as increased metal binding and a reduction of pH.^{94,95}

Hampton Research measured pH and conductivity of 8 different PEGs stored in a variety of typical laboratory storage settings over a period of 18 months.⁹⁶ pH and conductivity were selected as suitable indicators of PEG aging (reduced pH and increased ionic strength) because pH and ionic strength can be significant crystallization variables. pH and conductivity measurements were recorded at 25° Celsius. All PEG solutions were initially prepared at the same time, sterile filtered, filled into sterile PETG bottles, and purged with argon before closing the cap on the bottles. The results varied with PEG type and molecular weight, and how the PEG was packaged and stored. Data showed aging affects can be accelerated by warm temperature, light, and the presence of oxygen. PEG solutions appear most stable when stored frozen (-20° Celsius), and refrigerated (4° Celsius) PEG solutions are more stable than those stored at room temperature. Aging of solutions stored at room temperature can be further minimized by purging atmosphere (oxygen) from filled containers using argon. Finally, the aging

of PEGs can be further minimized by storing the sealed solutions protected from light. Protein crystals can be grown in fresh PEG solutions or aged PEG solutions. But crystals grown in aged solutions will sometimes not grow in fresh solutions and vice versa.

When it comes to PEG, both quality and consistency should be considered significant crystallization variables. Hampton Research Optimize Polyethylene glycol solutions are supplied in sterile, optically a clear PETG bottle, purged with argon before closure, and packaged in a protective carton. The carton helps protect the Polyethylene glycol from light. The optically clear PETG bottle has low oxygen permeability and also allows one to inspect the solution for color change or the appearance of amorphous material.

Use fresh PEG.

Change the Kinetics

It has been said the reward is in the journey. And when it comes to the crystallization of macromolecules, that means the geometry, path, and kinetics of the experiment can determine whether or not a crystal is grown, as well as the quality of the crystal. Various crystallization methods, sitting, hanging, and sandwich drop, microbatch, dialysis, free interface diffusion, and others, can each present a unique experimental geometry, path, and kinetics of equilibration. Not only the method, but the type, size, and shape of the plate or device can provide unique kinetics and with it, unique crystallization outcomes.^{97,98} So another option during optimization is to try a different crystallization method, plate, or device to evaluate different kinetics.

Drop ratios are another way to manipulate the kinetics of crystallization, and that is presented in Crystal Growth 101 – Drop Ratios.

The use of alternative reservoirs is another way to change the path and kinetics of a vapor diffusion experiment. In the early, medieval times of protein crystallization, it was typical to pipette a number of drops in vapor diffusion with a common reservoir (reagent well, dehydrant). For example, a sandwich box setup, where drops of protein and reagent are dispensed into a 9 well siliconized glass plate, and that plate placed inside a plastic sandwich box, partially filled with a reservoir such as concentrated Ammonium sulfate, and sealed with a greased cover. Later on, crystal growers purloined multiwell tissue culture plates in order to pipette reservoirs to match the reagent in the hanging drop. Today crystal growers can choose from dozens of different flavors of plates and devices for crystallization. Returning to kinetics, one may fill the reservoir of these plates with a common dehydrant, one that is an alternative to the drop paired reagent, in order to manipulate and evaluate the kinetics of vapor diffusion.⁹⁹⁻¹⁰²

Layering of oil over the reagent in the reservoir is yet another method to control kinetics, in this instance, slowing the rate of vapor diffusion between

the reagent and the drop, which can be fine-tuned by adjusting the volume and composition of the oil layer.¹⁰³

Pressure

Pressure has been explored as a crystallization parameter, as hydrostatic pressure can affect system uniformity and be rapidly altered.⁶⁶ Protein solubility and crystal growth rates for several proteins were found to be dependent upon pressure.⁶⁶⁻⁶⁷

Magnetic and Electric field

High magnetic fields have been applied for the purpose of improving crystal quality. Not only a homogeneous magnetic field but also a steep-gradient magnetic field (inhomogeneous magnetic field) have been shown to improve crystal growth.^{71,72} A magnetic force can counteract the gravitational force when applied in the opposite direction, and magnet-based quasi-microgravity environments have been exploited as an alternative to the space-based microgravity environment.⁷³ Experimental studies on protein crystallization using a high magnetic field have reported a reduction in the number of crystal nuclei,⁷⁴ as well as a relatively slow crystal growth rate,⁷⁵ when compared with those under normal-gravity conditions outside the magnetic field.

External electric fields have been shown to reduce the nucleation rate, produce fewer, larger crystals⁷⁶ as well as improved crystal quality.⁷⁷

Microgravity

Microgravity has been used as a crystallization variable to study the fundamental mechanism of macromolecular crystal growth, manipulate crystal nucleation, growth, size, and quality, as well as improve crystal quality.^{78,79} Overall, microgravity has delivered crystals of preferential size and of improved quality.⁸⁰

Vibration

Some level of mechanical vibration often takes place during the crystallization experiment. Work by Lu, looking at several different proteins crystallized with and without vibration demonstrated enhanced morphology and improved X-ray diffraction resolution with mechanical vibration, and proposed vibration may be a method for obtaining more hits during crystallization screening.^{88,89} Anecdotal observations related to vibration sometimes show a preference for setting the experiments aside in a still place like a fine spirit, for later analysis. Others report no difference between experiments set in vivacious, vibrating incubators and near motionless incubators or lab cupboards. In microgravity it is said crystal growth rates varied with a clear connection to when astronauts are exercising. At this time, the question “shaken or stirred”, when it comes to crystallization, remains an area for further exploration.

Data & Literature Review & Mining

Delve into the literature.¹⁻¹³⁷ George Santayana said that those who cannot remember the past are condemned to repeat it. Yet in protein crystallization, the past (literature) is so full of wisdom, techniques, and methods, that it seems difficult to repeat some of them, so dig in and discover from the past. Before beginning a crystallization project one should review the literature related to their macromolecule and any similarities. Search the Protein Data Bank (www.wwpdb.org) and Biological Macromolecule Crystallization Database (<http://xpdb.nist.gov:8060/BMCD4/index.faces>) and the Internet for purification, characterization, crystallization, and structural data, as these could provide useful insight, clues and answers, before beginning, as well as during crystallization efforts. Read, learn, and apply.

Epilogue

If no crystals form, dump the samples in the sink and curse the darkness.

Alexander McPherson

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