Advantages of the TOPAZ™ Approach for Protein Crystallization

A Theoretical Proposition

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Introduction

A knowledge of protein structure has become essential for today's drug discovery process and protein function studies. Industry and academia have therefore invested heavily into the development of high-throughput systems for protein crystallization. Despite impressive increases in the numbers of trials per day, the inefficiency of traditional methods continues to be an obstacle to obtaining crystals that are useful for solving structures. In this paper we discuss the principles of free interface diffusion (FID) and vapor diffusion and their application in the TOPAZ System for Protein Crystallization. At the core of this system are the TOPAZ screening chips, which require only sub nanoliter volumes of protein sample per experiment and provide a tighter control of experimental variables such that crystallization studies become more efficient and reproducible.

The Fundamentals of Protein Crystallization

Protein crystallization relies on manipulating the state of a crystallizable protein in solution so that the solution is gradually and uniformly driven to a supersaturated state. If the rate of concentration is optimal, crystal nucleation and crystal growth occurs. Nucleation is key to the process; however, if too many nuclei form in a crystallization reactor, the remaining protein material may be insufficient to grow crystals of a useful size.

Typically, a large number of experiments are required to find crystallization conditions. These studies test the effects of combining the protein solution with reagents that have different properties, such as pH, ionic strength, and dielectric constant. The effects of incubation times and temperatures are also studied. Through rounds of optimization, investigators attempt to grow

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crystals that are large and well-ordered so that they may be analyzed by X-ray diffraction to determine a three-dimensional structure with atomic resolution.

The literature frequently uses a two-dimensional plot to describe the solubility of a protein as a function of variables, such as temperature, concentration of precipitating agent, pH, or any other parameter that affects the solubility. (See Figure 1 on page 3 for an example of such a plot.) Two curved lines designated as solubility and supersolubility are superimposed on the plot to differentiate hypothetical regions of phase space:

- An undersaturated region, where the protein remains soluble indefinitely;
- A precipitation/nucleation region, where the protein solution becomes supersaturated enough to form a solid phase;
- A metastable region, which lies between the two, where the crystals can grow but where the flow of formation of new nuclei depends exponentially on the value of supersaturation.

The shapes and boundaries of these areas differ depending on protein characteristics. The system is influenced by the interplay of thermodynamic and kinetic factors, as well as by the geometry of the crystallization reactor. The time history of a given crystallization experiment can be represented as trajectories across these plots, representing the concentration of protein and the concentration of precipitating agent (or temperature or pH values) at progressive time points. We use phase space diagrams in this paper to illustrate differences between crystallization methods.

The Principles of Drop-based Methods

Currently, evaporation is the most widely used method for sampling crystallization conditions. This technique is commonly referred to as vapor diffusion or drop-based because reagents and precipitants are mixed instantaneously, and then a droplet of the mixture is gradually concentrated through equilibration with a reservoir solution of typically twice the concentration of the precipitant. The gradual loss of water from the drop brings the drop to the region of precipitation /nucleation. The formation of nuclei reduces the supersaturation value, bringing about metastability. This change is represented as a straight line passing through the origin of the plot because evaporation of solvent keeps the ratio of protein to reagent constant. (See Figure 1.) The rate at which supersaturation is achieved depends on the geometry of the reactor, the size of the drop, and the initial concentrations in the well and the drop.

A variation on vapor diffusion, microbatch experiments are set up under a layer of immiscible oil. The rate at which solvents diffuse from the system can be controlled through the use of oil that is more or less permeable; however, there is no control over the end point of evaporation.

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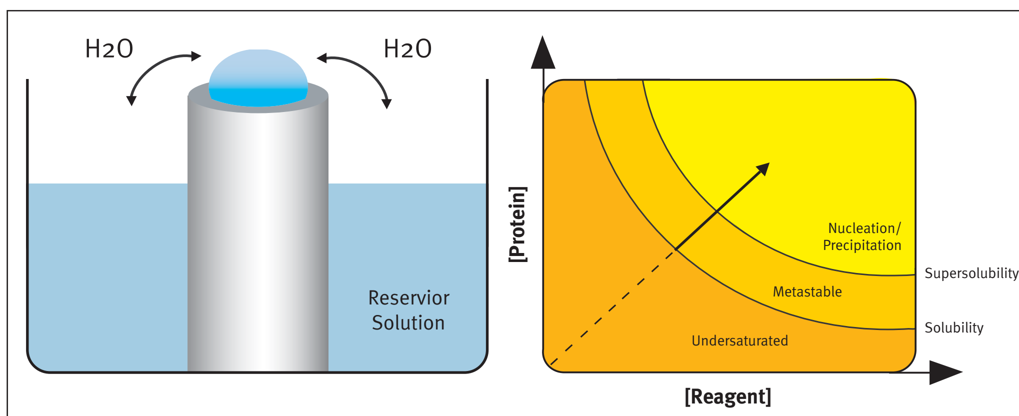

Figure 1: Vapor diffusion. The diagram on the left shows the setup of a sitting-drop vapor-diffusion experiment. A droplet of the precipitants and protein is concentrated through equilibration with a much more concentrated precipitant solution in the reservoir. The diagram on the right shows the increases in concentrations for a hypothetical starting concentration. The trajectory is a straight line through the origin because the ratio of protein solution to precipitant concentration within the droplet remains constant throughout the equilibration process.

While drop-based methods using microwell plates have been the *de facto* standard for many years, they suffer from fundamental limitations. First, the rapid rate of initial mixing may cause a reagent shock; consequently, the protein may be driven to precipitation before nucleation can occur. Second, liquid-dispensing technologies for drop-based methods lack well-to-well precision, especially when transferring solutions that have a range of viscosities. Third, when drop-based methods are performed at the nanoliter scale, the rapid dehydration of the drops tends to cause the formation of salt crystals instead of protein crystals. This latter tendency impedes the reliability of automated image analysis. The brute-force automation of drop-based methods fails to overcome the problem of managing the huge number of experiments required to complete a study.

The Principles of Classical Free Interface Diffusion

Free interface diffusion (FID) relies on slow, diffusive mixing of liquids, rather than evaporation, to achieve the critical supersaturation for nucleation. The interacting precipitants and protein solutions are placed one in front of the other in the reactor, in direct contact or, optionally, separated by an intermediate physical buffer. (See García-Ruiz, 2003 and references therein.) It is preferable to peform FID at minimal convection, a state in which fluid viscosity dampens inertial

1. J. M. García-Ruiz. Counterdiffusion methods for protein crystallisation. *Methods in Enzymology* 368 (2003) 130-154.

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forces (García-Ruiz *et al*., 2001[1,2]). This may be achieved by confining solutions in thin capillaries or by adding gels (Robert *et al*, 1999[3]), or by performing the experiment under microgravity conditions in space (Bosch *et al*., 1992[4] ; Ries-Kautt *et al*., 1998[5]). Thus, precipitant and protein mix gently by diffusion, with the rate of mixing depending on the diffusivity of the solution components – a function of their molecular weights, the viscosity of the solution, and the dimensions of the cell. In general, the precipitant solutes migrate to the protein side first, as they have a lower molecular weight. (See Figure 2.) Although the transport patterns are rather complex, they are much more efficient than drop-based methods for the screening of crystallization conditions. (See Figure 3 on page 5 and Otalora and García-Ruiz 1997[6].)

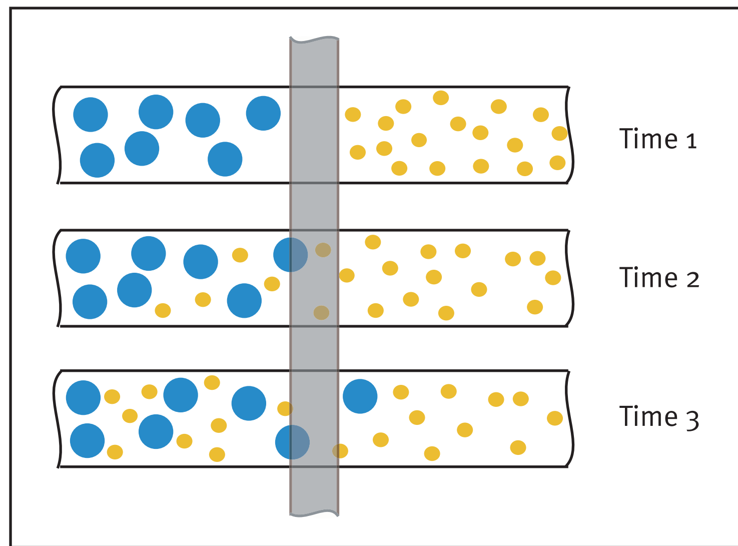

Figure 2: The effect of diffusivity on mixing. The diagram shows the smaller reagent molecules diffusing into the solution of larger protein molecules, at three successive time points.

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Classical FID experiments are performed close to equilibrium, at supersaturation values in the metastable region (Salemme 1985[7], Hansen *et al*. 2002[8]). Although slight increases in precipitant concentration may cause protein to precipitate out of solution in this region, in FID experiments, the gentle diffusion of solution components, relative to the rapid mixing of the same solutions in drop-based experiments, reduces the likelihood of precipitation and can result in higher supersaturation levels. This increases the chances of successful crystal nucleation.

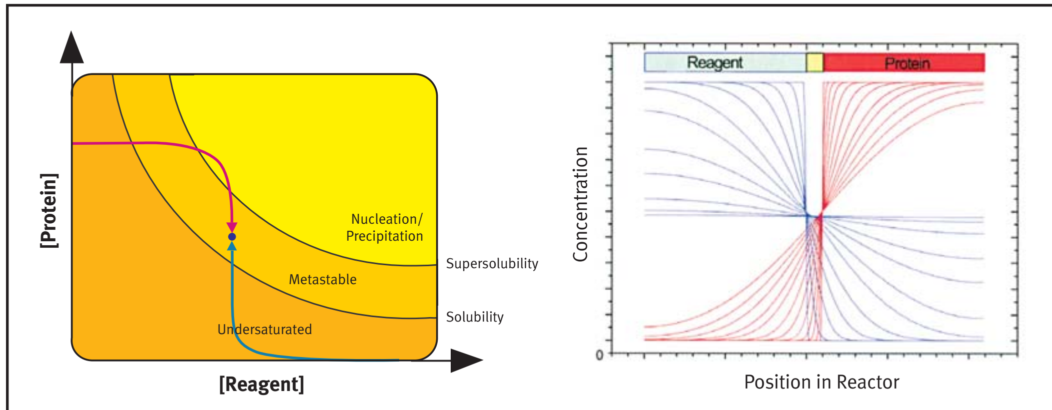

Figure 3: Free interface diffusion. The diagrams above illustrate changes in concentrations for equal volumes of protein solution (red) and reagent (blue) over time. The diagram on the left represents these dynamics as a single curved trace superimposed on the hypothetical regions of phase space. The diagram on the right shows these dynamics as a function of the position of the reactants in the reactor at successive time points. It is a gentle diffusion of components that creates a range of concentration ratios. (The diagram on the right provided by Prof. García- Ruiz, CSIC-Universidad de Granada, Granada, Spain.)

An alternative to the classical FID is the counterdiffusion method, in which the experiments are performed in a long capillary, using highly concentrated precipitating solutions to provoke successive nucleation events approaching equilibrium. As the two solutions equilibrate, a spatiotemporal gradient of supersaturation creates a progressive nucleation front that moves across the protein solution. Therefore, crystals may form at any position along the capillary where concentrations are favorable. In this way, a single experiment explores a broad range of protein and precipitant concentrations. As an additional advantage of both classical FID and counterdiffusion approaches, the experiments are implemented in diffusive media in the absence of convective flow, allowing the integration of molecules into the crystal in the most achievable ordered arrangement, mimicking microgravity experiments.

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Investigators have long recognized that the kinetic properties of classical FID and counterdiffusion increase the efficiency of discovering the conditions for crystallization either for small molecule compounds or for macromolecules (Henisch, 1970[9] ; Salemme, 1972[10] ; Zeppezauer *et al*., 1968[11]; García-Ruiz, 1991[12]; Koszelak *et al*., 1996[13]; Carter *et al*., 1999[14]; A. McPherson *et al*., 1999[15]; Barnes *et al*., 2002[16]; see also Ng *et al*., 2003[17] and references therein). It has been theorized and experimentally demonstrated that both classical FID and counterdiffusion techniques help to find quickly the optimal crystallization conditions, thus improving dramatically the quality of the crystals as seen from X-ray diffraction data (Maes *et al*., 2004[18]; Biertümpfel *et al*., 2002[19]). In most cases, these FID and counterdiffusion experiments have been implemented using glass capillaries; however, glass capillaries have drawbacks. Setting up capillaries for high-throughput screening is labor intensive, and the handling of glass capillaries with a diameter of less than 100 microns is a difficult task. Capillaries with larger diameters can be used, but this may give rise to convective motion, which can break the symmetry of the diffusive coupling and disrupt orderly crystal growth. Although adding agarose to the protein solution suppresses convection (García-Ruiz *et al*., 2001[20]), this remedy introduces additional steps into the experiment protocol and adds another chemical to the crystallization cocktail.

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TOPAZ Chips for Sub-nanoliter Volume FID

Fluidigm Corporation has introduced a novel approach that takes advantage of the inherent strengths of drop-based methods and classical FID while overcoming their limitations through an innovative technology for their implementation. Fluidigm's TOPAZ screening chips are configured for high-throughput experiments with fully integrated, high-density circuitry engineered in a compact device. The chip is designed as part of a system, which includes hardware, software, and reagents optimized for in-chip FID. (See Figure 4.) Unlike capillaries, which can be tedious and time-consuming to use, TOPAZ screening chips enable the automated setup of 96, 384 or 768 experiments: one, four, or eight proteins assayed against 96 reagent conditions. Each experiment takes place within a *diffusion cell*, which consists of a pair of chambers separated by an interface valve, to control diffusion. After the protein samples and reagents are automatically distributed into their respective inlets and chambers, interface valves are opened, and the fluids begin diffusive mixing. (See Figure 5.) The feasibility and utility of the Fluidigm technology was first demonstrated by Hansen *et al*., 2002[8].

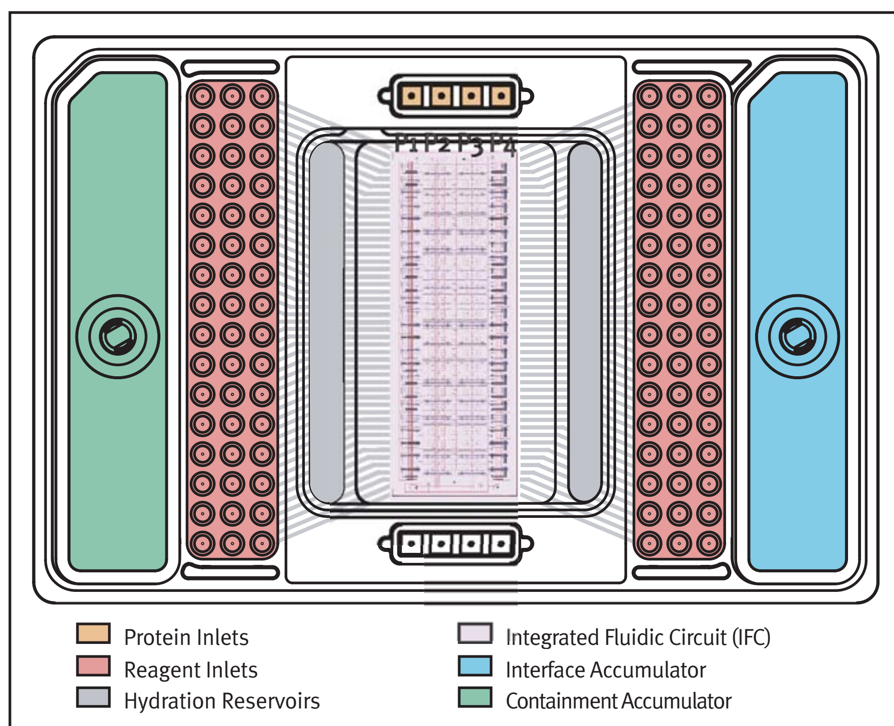

Figure 4: The TOPAZ screening chip for protein crystallization. The TOPAZ chip, manufactured to a SBS microtiter plate standard, houses the components required for the automated setup and maintenance of experiments. A 4.96 integrated fluidic circuit (IFC) is shown in the center of the chip. Continuing advances in IFC fabrication have sustained an exponential rate of increase in feature densities, with a corresponding increase in throughput.

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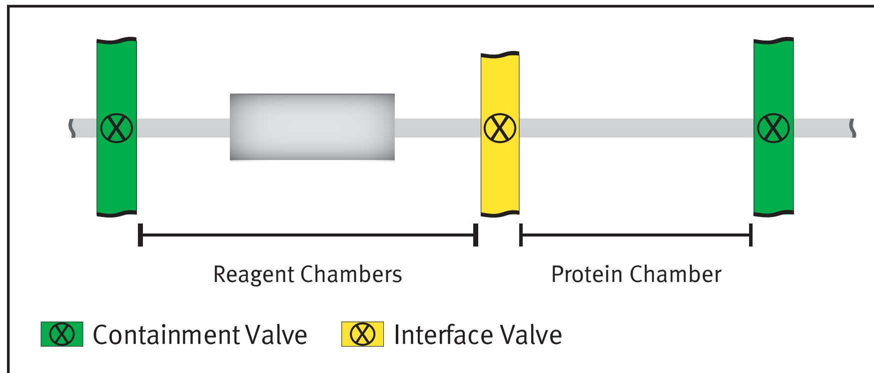

Figure 5: A diffusion cell within a TOPAZ screening chip.
Reagent and protein solutions are isolated in their respective chambers until the interface valve is opened to allow diffusion across a transfer channel.

Control of Experiments

Crystallization screening involves a series of low probability experiments and multiple test parameters, including temperature, humidity level, reagent and sample chemistry, and ultimately the mix of protein to reagent. TOPAZ screening chips and protocols are designed to create a reproducible experimental environment such that protein-reagent chemistry can be highlighted as the key test variable. (See Figure 6.)

TOPAZ screening chips are loaded via a distribution network of channels and chambers that control the final experimental volumes. Each protein chamber accommodates precisely 770 picoliters via this volumetric metering strategy. Unlike traditional dispensing, in-chip loading is uniform across a wide range of fluid viscosities, under one standard protocol. The net effect is a highly reproducible set of fixed-volume experiments.

The IFC device, where crystallization occurs, is made of a gas-permeable material and is subject to the transport of water vapor similar to a microbatch experiment. The chip provides a mechanism to control the rate and direction of water movement to reduce the effect of environmental differences between one experiment and the next. Using an onboard humidity chamber and hydration strips, relative humidity is maintained near 100 percent during the diffusion process and then follows a gradual dehydration curve until the end point of the experiment. The result is a dual screening technique: FID and vapor diffusion (i.e. evaporative concentration), which explores a broad range of conditions in a single experiment. (See Figure 7.)

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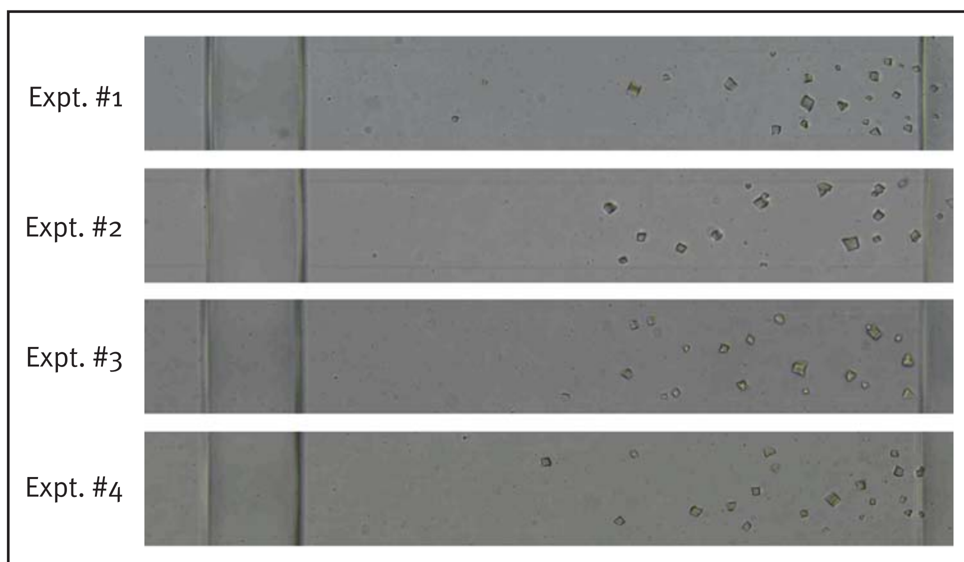

Figure 6: Reproducibility of Experiments. Shown are images of crystallization activity for four experiments with identical parameters. The nucleation gradients reflect the range of chemical space explored with classical FID. The replication of these gradients among experiments suggests reproducibility of results with TOPAZ screening chips.

A final control parameter is the amount of time that protein and reagents are permitted to diffuse across the transfer channel. By controlling the amount of time that the interface valves are open, the concentration of protein is preserved within the protein chamber. This is based on the diffusive properties of liquids in micro channels and the dependency on molecular size. In this scenario, the movement of protein macromolecules is significantly slower than the corresponding rate for smaller reagent molecules. FID times were empirically derived to maximize the extent of reagent equilibration in the diffusion cell while minimizing the corresponding decrease in protein cconcentration from the protein chamber. This protocol development was coupled with the determination of the optimal reagent-protein ratio used in the TOPAZ screening chips. (See Figure 8.) Both the ratio and the FID time were selected based on internal and external data showing the greatest frequency of hits.

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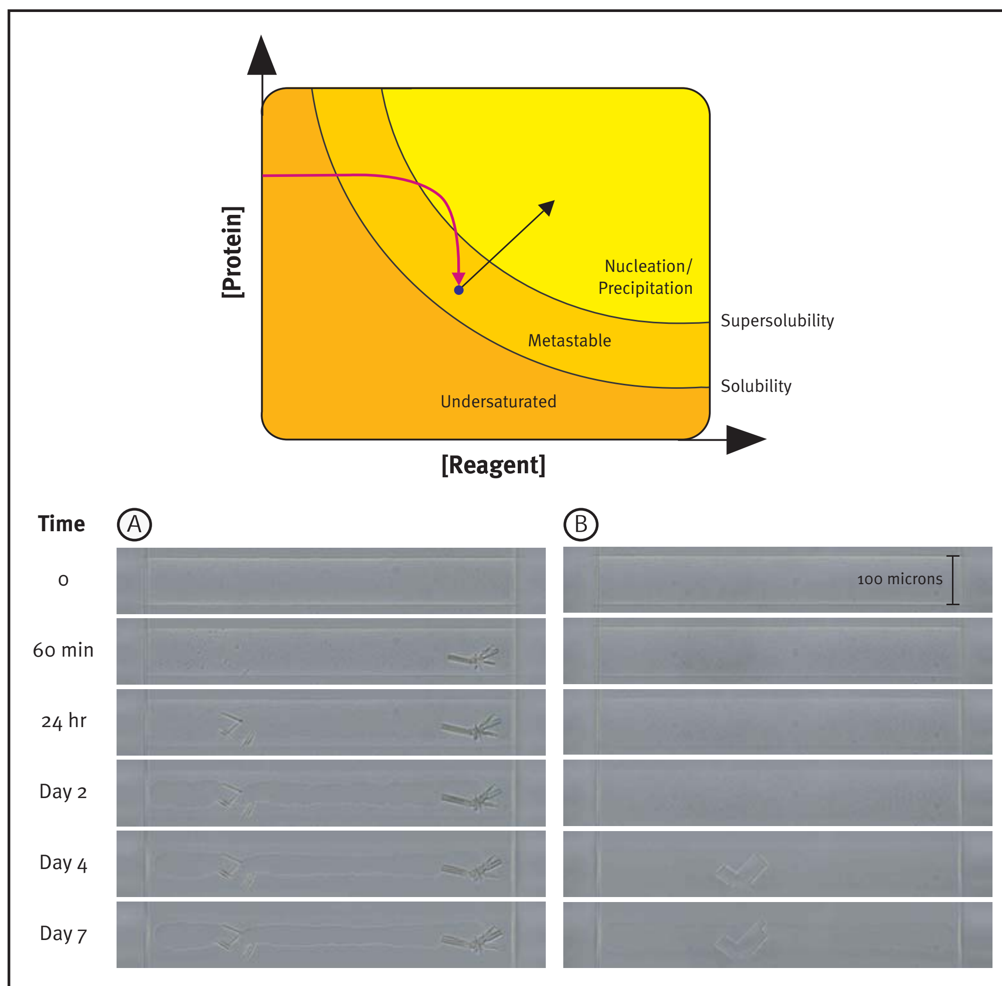

Figure 7: Free interface diffusion on TOPAZ chips. This diagram shows the change in protein concentration as a curved line with an end point in the metastable region, signifying controlled FID. The straight line represents the constant change in the ratio of protein solution to precipitant through controlled evaporation. The images on the bottom show the effects of evaporation within the channels, after FID has stopped (time = 0). Experiment A shows a slight increase in crystal size over time. Experiment B shows the appearance of one large crystal at day four.

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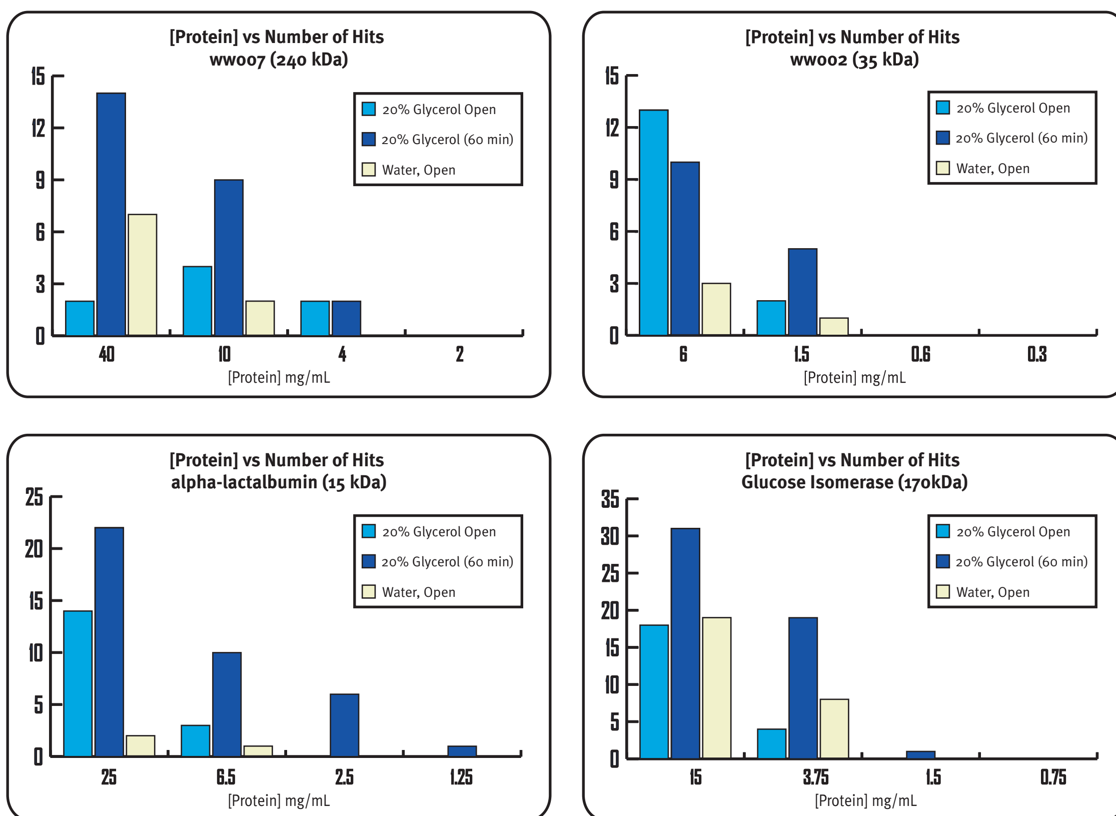

Figure 8: TOPAZ protocol development. These histograms show results of R&D studies to determine conditions that maximize the crystal hit rate. In summary, for samples of low concentration and low molecular weight, closing the valves early in the diffusion process and using a composite hydration fluid of glycerol and water versus water alone increases the hit rate.

Translation to Diffraction-Quality Crystals

Once crystals have been obtained in a TOPAZ screening chip, the next step is to optimize and translate the crystallization conditions to other formats, such as microbatch or drop based vapor diffusion. The goal of translation is to produce crystals that are of sufficient size and quality to collect X-ray diffraction data. Fluidigm provides the *Guide to Translating Screening Conditions* as an aid to designing translation experiments based on conditions discovered with TOPAZ nano-scale crystallization. In this model, a researcher assesses both the number and quality of crystals from a limited number of experiments, and then enters this information into the *Translation Workbook*. This workbook serves as a design engine for scaling up screening conditions to larger volumes and sets up the appropriate grid screen to be run. These tools have been validated against both model proteins and samples from independent laboratories.

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Conclusion

While the benefits of classical FID have been widely accepted for some time, implementation had been restricted to low-throughput operations. As a more practical alternative, TOPAZ screening chips provide a straightforward means of implementation–a method that explores a wide swath of chemical space by means of classical FID and subsequent vapor diffusion. In addition, TOPAZ screening chips require significantly less protein sample than either drop-based methods or FID in glass capillaries and reduce experimental variability by a more precise control of the chip environment and protein-reagent mix. This control of variables transforms protein crystallization from largely trial and error to a straightforward, reproducible process. Fluidigm Corporation has introduced software, hardware, and reagents that completely automate the use of TOPAZ screening chips: the FID Crystallizer, for experiment setup; the AutoInspeX Workstation, for image capture and analysis of crystallization activity; and OptiMix™ reagents. In all, the system is conservative in protein, simplified in operation, and consistent in result.

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