

Protein Crystallization Using Microfluidic Technologies Based on Valves, Droplets, and SlipChip

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free interface diffusion, high throughput, screening, phase diagram, in situ diffraction

Abstract

To obtain protein crystals, researchers must search for conditions in multidimensional chemical space. Empirically, thousands of crystallization experiments are carried out to screen various precipitants at multiple concentrations. Microfluidics can manipulate fluids on a nanoliter scale, and it affects crystallization twofold. First, it miniaturizes the experiments that can currently be done on a larger scale and enables crystallization of proteins that are available only in small amounts. Second, it offers unique experimental approaches that are difficult or impossible to implement on a larger scale. Ongoing development of microfluidic techniques and their integration with protein production, characterization, and in situ diffraction promises to accelerate the progress of structural biology.

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Nucleation: a process in which a small number of molecules become arranged in the pattern of a crystalline solid, forming a site where additional particles are deposited as the crystal grows

Phase diagram: used to show conditions under which thermodynamically distinct phases can occur at equilibrium, and used to predict conditions for crystallization of a protein or to narrow the range of conditions that must be screened in trials

INTRODUCTION

This review discusses the use of microfluidics in protein crystallization for structural determination using single-crystal X-ray diffraction. Elucidation of three-dimensional protein structures via X-ray crystallography has been the basis for understanding biophysical and biochemical mechanisms (17, 22). However, identifying the correct conditions for protein crystallization is complex because a large chemical space must be explored (3, 6, 25, 57). Microfluidics is a technology that manipulates small (nanoliter to femtoliter) amounts of fluids (2, 21, 24, 35, 39, 52, 69, 72, 78, 81–85, 87), and it affects the field of protein crystallization in two ways. First, it allows miniaturization of experiments that are already possible on a larger scale. Because samples of many proteins are rare and reagents

can be expensive, it is advantageous to perform these trials with small volumes. Second, microfluidics provides unique experimental approaches that are difficult or impossible to implement on a larger scale, such as precise control of diffusion of molecules and nucleation of crystals.

In the first part of this review, we discuss protein crystallization in terms of a simple protein-precipitant phase diagram. We discuss four traditional methods for protein crystallization: microbatch, vapor diffusion, dialysis, and free interface diffusion (FID). We then turn our attention to microfluidic approaches, first looking at established microfluidic approaches to implement microbatch, vapor diffusion, and FID methods, and then looking at emerging microfluidic approaches. We then consider the membrane protein crystallization using microfluidic methods and discuss the diffraction of crystals obtained from microfluidic experiments. Finally, we briefly explore the future of protein crystallization and the use of microfluidics to further this field.

PROTEIN CRYSTALLIZATION: PHASE DIAGRAM AND TRADITIONAL METHODS

Obtaining protein crystals remains an obstacle to solving their structures (6, 7, 19, 29, 74, 88), thus hindering investigators from elucidating their functions at the molecular level (17, 22, 28, 41–43, 53, 61). Because protein crystallization is generally an empirical process, obtaining high-quality crystals for X-ray diffraction requires exploration of multidimensional chemical space, an ambitious undertaking that can involve screening multiple precipitants of various concentrations in hundreds or thousands of individual crystallization trials (18, 40, 54, 55, 68, 77). This exploration of chemical space can be represented by using a phase diagram. In the simplest version, a phase diagram graphically shows whether a protein is soluble or not under various conditions.

Here we illustrate the concept of the phase diagram by using a two-dimensional version

(Figure 1). In this diagram (5), conditions for crystallization are described by two parameters: protein concentration and precipitant concentration. In more realistic situations, many other factors, such as protein purity, pH, temperature, ionic strength, types of buffer, additives, and precipitants, must be considered, and the phase diagram may have dozens of dimensions and discontinuous regions (3, 7, 57). The phase diagram plots the solubility curve of the protein (see Figure 1 for more details).

Within the zone of supersaturation there are three other zones: nucleation, precipitation, and metastable (Figure 1). In the nucleation zone, the concentration of protein is high enough for nuclei to form spontaneously and to reach a critical size to support subsequent crystal growth. In the precipitation zone, the concentration of the protein is too high and nucleation and growth occur too rapidly, resulting in disordered structures such as aggregates and precipitates. In the metastable zone, the concentration of the protein is too low and no new nuclei will form, although existing crystals can continue to grow. Because these zones are related to kinetic phenomena, the boundaries between them are not well defined. For example, under the same conditions, incubation lasting a few seconds may lead to nucleation, whereas hours-long incubation may lead to precipitation. Chemical space in crystallization experiments is multidimensional, and several zones may correspond to nucleation and growth of different crystal forms and polymorphs. For membrane proteins, the search for conditions for crystallization is more complicated. Detergents, lipids, or both may be required to solubilize and stabilize membrane proteins of interest; their addition inevitably requires a revised phase diagram involving the detergents or lipids (14, 56, 64, 74, 89). Nevertheless, the zones can serve as guide when searching for the appropriate conditions to crystallize a particular protein.

Microbatch methods (9, 10) are based on finding a starting point on the phase diagram where crystallization occurs. The starting point must be in the nucleation zone (Figure 1). Supersaturation occurs upon mixing the

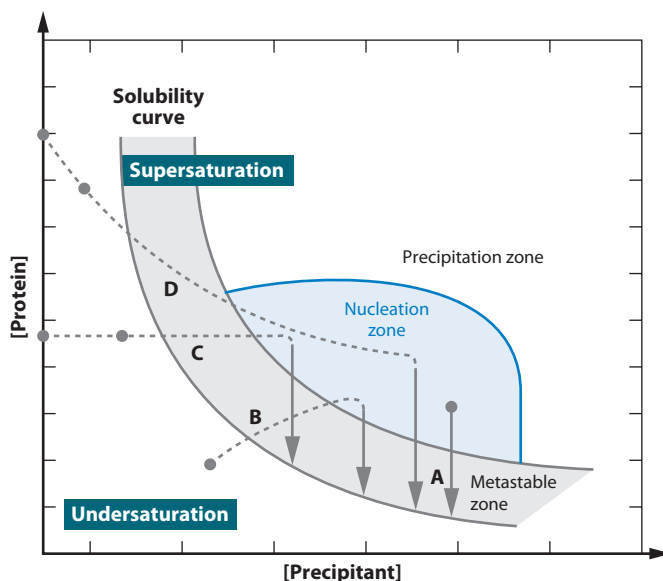


Figure 1

Simplified phase diagram for protein crystallization. Paths for microbatch (A), vapor diffusion (B), dialysis (C), and free interface diffusion (D) are shown. Crystals only form in the zone of supersaturation (right of the solubility curve), where the concentration of the protein in the precipitant solution is above its solubility. In the zone of undersaturation (left of the solubility curve), crystals dissolve and cannot form. (Figure and caption reproduced with permission of the International Union of Crystallography, Reference 5, Copyright © 1998.)

protein and the crystallizing agents in a small droplet, and the conditions in the droplet change only as the protein comes out of solution. If the conditions are correct, nuclei will form and crystals will begin to grow on the nuclei. As crystals grow, the concentration of protein begins to fall rapidly. Once the line reaches the metastable zone, no new nuclei will form, but the ordered growth of crystals will continue on the existing nuclei until the line intersects the solubility curve.

Vapor diffusion (34, 44, 58), dialysis (67, 90), and FID methods (71) are based on finding a path through the phase diagram that leads to crystallization. In vapor diffusion, the crystallization trial starts at a point in the undersaturation zone. A droplet containing a solution of protein and precipitant is allowed to equilibrate with a larger reservoir containing a solution of the precipitant, sometimes referred to as mother liquor, at a higher concentration.

Microbatch: a protein crystallization technique in which a small drop of protein sample is combined with the crystallization reagents and the mixture is maintained under constant concentration

Vapor diffusion: a protein crystallization technique in which a small drop containing the protein, buffer, and precipitant equilibrates with a large reservoir containing similar buffers and precipitants in higher concentration

Free interface

diffusion (FID): a protein crystallization technique in which pure solutions of proteins and precipitants gradually diffuse under the influence of a concentration gradient

Growth:

incorporation of protein molecules on the surface of nuclei in a specific orientation

SlipChip:

a microwell-based microfluidic device whose operation does not require instruments

Valves:

in microfluidic systems, the open or close states of the valves control flow of reagent solutions

Droplets:

compartments of one phase surrounded by another phase. The two phases are immiscible in one another

As water evaporates from the droplet, protein and precipitant concentrations in the droplet increase (**Figure 1**). Once the path reaches the nucleation zone, the protein in the droplet starts to nucleate. This nucleation is followed by crystal growth and a drop in the protein concentration, similar to the growth described for microbatch.

In dialysis, pure solutions of protein and precipitant are separated by a membrane that is impermeable to protein but that allows passage of the precipitant. The crystallization trial, on the side of the membrane containing the protein, starts at a low precipitant concentration. The precipitant diffuses across the dialysis membrane into the protein solution, causing an increase in precipitant concentration. Because the protein can not diffuse across the membrane, its concentration stays the same (**Figure 1**). Once the path reaches the nucleation zone, the protein starts to nucleate and then crystals start to grow, similar to the growth described for microbatch.

In FID, protein and precipitant gradually diffuse under the influence of a concentration gradient. Initially, pure solutions of protein and precipitant are connected by an interface. At this interface, the protein begins to diffuse into the precipitant solution, and the precipitant begins to diffuse into the protein solution. On the protein side, the amount of protein decreases as it diffuses into the precipitant solution and the amount of precipitant increases as it diffuses into the protein solution (**Figure 1**). When the path enters the nucleation zone, nuclei begin to form and support the following growth, similar to that described for microbatch. In counterdiffusion (30, 63), the precipitant is placed on one side of an elongated protein sample, usually in a capillary. Because counterdiffusion uses principles similar to FID to explore the crystallization path, we discuss it as an FID approach.

Any technology that explores the chemical space to identify conditions for crystallization should (*a*) be capable of carrying out many experiments simultaneously to provide sufficiently dense coverage of the multidimensional chemical space, (*b*) use small quantities of

samples to carry out these experiments, (*c*) precisely control mixing, interfaces, and time of contact between solutions, and (*d*) enable evaluation of a crystal's quality by X-ray diffraction. Microfluidic approaches satisfy these criteria and are thus attractive for crystallizing proteins.

MICROFLUIDIC APPROACHES

We describe three microfluidic approaches used to explore the chemical space to identify conditions for protein crystallization: (*a*) valve-based systems (37) (**Figure 2a**), (*b*) droplet-based systems (94) (**Figure 2b**), and (*c*) systems based on SlipChip and related well-based approaches (23, 98) (**Figure 2c**). All these systems define nanoliter volumes in which crystallization takes place, but they define these volumes differently. In valve-based systems (37) (**Figure 2a**), different aqueous reagents are loaded into different chambers. A protein sample and a precipitant can be mixed by opening the valve that connects the chambers to form crystallization trials. Loading different precipitants into chambers and mixing precipitants with protein are all initiated by separately operating different pneumatic valves in their open or closed states. In droplet-based systems (**Figure 2b**), crystallization takes place inside aqueous volumes surrounded by an immiscible carrier fluid. To optimize crystallization conditions, hundreds of droplets, each containing different crystallization conditions, can be generated in a single experiment by systematically changing the flow rates of the streams of aqueous reagent, protein, buffer, and precipitants, and by flowing these streams into fluorinated carrier fluid. In SlipChip (23) (**Figure 2c**), the solutions are loaded into wells in two plates that can move relative to one another. This motion of the plates brings protein and precipitant solutions into contact, initiating crystallization. The volumes of the precipitant solution and protein solution are defined by the volumes of the corresponding wells, without a need for metering each volume. Nanoliter wells are filled precisely with a protein solution as the protein solution is introduced through a series

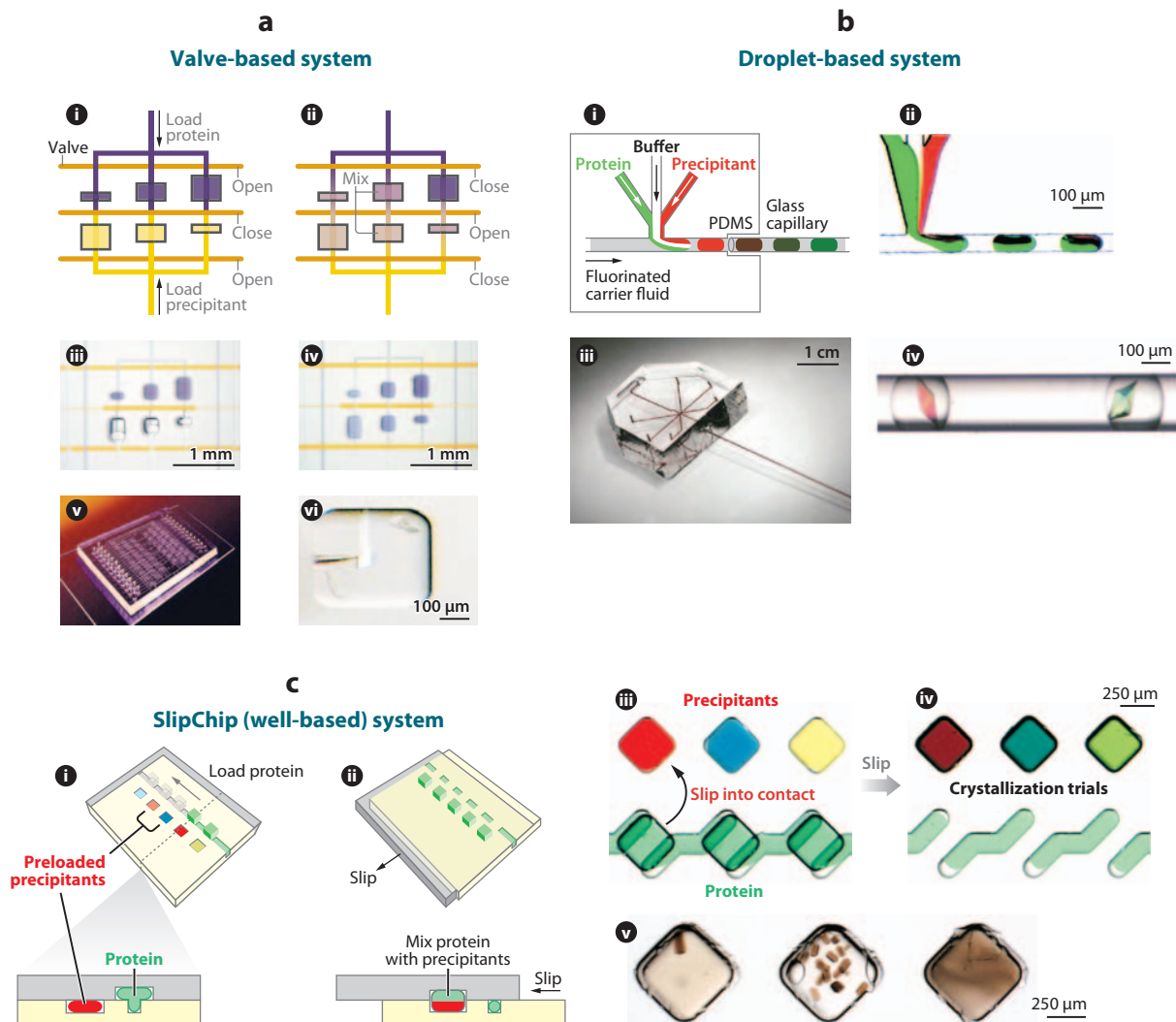


Figure 2

Examples of microfluidic devices applied to protein crystallization. (a) A valve-based system. Schematics showing (i) loading of precipitants and protein and (ii) the process of free interface diffusion (FID) on-chip. Microphotographs iii and iv corresponding to schematics i and ii, respectively. (v) Microphotograph of the entire device. (vi) Crystals of aquaporin obtained using this device. (Figure and caption reprinted from Reference 35 Copyright © 2003, with permission from Elsevier Ltd.) (b) A droplet-based system. (i) A schematic of the device. After the droplets containing the crystallization trials are formed, the trials are flowed into a glass capillary, flow is stopped, and crystallization occurs. (ii) A microphotograph illustrating droplet formation. (iii) A photograph of the device itself. (iv) Protein crystals obtained using this device. In situ X-ray diffraction can be performed on the crystals contained in the droplets. (Figure and caption reprinted with permission from Reference 97 Copyright © 2004 by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim and Reference 79 Copyright © 2003 by the American Chemical Society.) (c) A SlipChip (well-based) system. Schematics showing (i) loading of protein into a SlipChip that has already been preloaded with precipitants and (ii) slipping to combine protein and precipitants to form trials. (iii) Microphotograph of loading a green food dye (mimicking the protein) into a SlipChip that has already been preloaded with colored dyes (mimicking precipitants). (iv) Microphotograph of the SlipChip after slipping to combine the solutions. (v) Crystals of the photosynthetic reaction center from *Blastochloris viridis* obtained using this device. (Figure and caption reprinted with permission from Reference 23 Copyright © 2009 by the Royal Society of Chemistry.)

of wells and ducts. Movement of the plates disconnects all the wells from the ducts, isolates the wells containing the protein, and then brings them in contact with the wells containing preloaded precipitants, thus initiating crystallization.

PROTEIN CRYSTALLIZATION IN MICROFLUIDICS: IMPLEMENTING ESTABLISHED METHODS

In this section, we discuss how the traditional approaches to protein crystallization—

microbatch, vapor diffusion, and FID—have been implemented on a ~ 10 -nL scale using microfluidics.

Microfluidic Approaches to Implement Microbatch Methods

To implement the microbatch method in the droplet-based microfluidic system (92), the protein, buffer, and precipitant solutions flow in through different aqueous channels, meet at the junction, and form a droplet. A fluorinated carrier fluid transports the droplet (Figure 3). The carrier fluid is immiscible to the aqueous

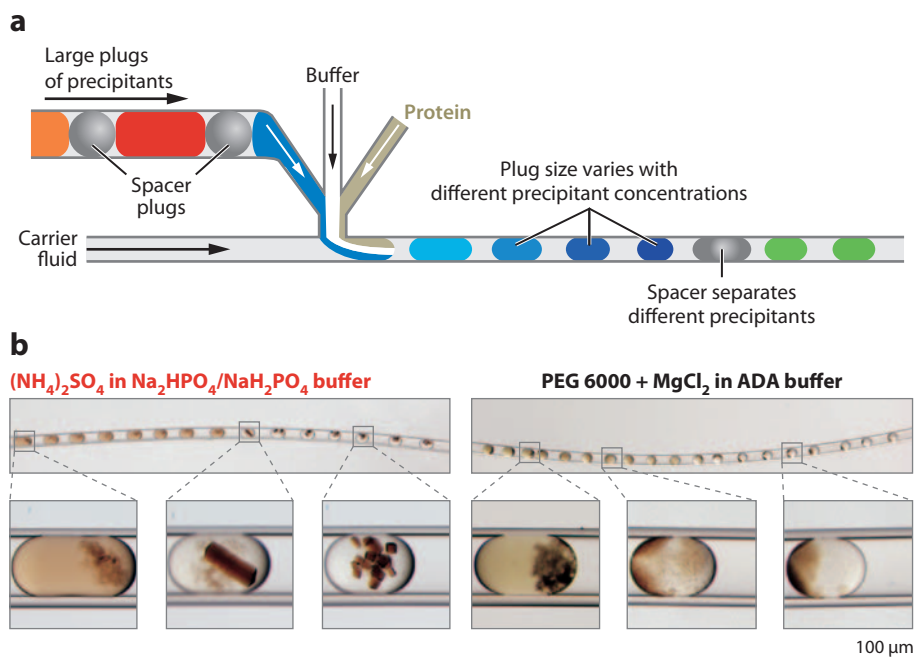


Figure 3

A droplet-based microfluidic hybrid approach to screen crystallization conditions using the microbatch method. (a) A schematic illustration of the hybrid approach. A preformed array of ≈ 140 -nL reagent droplets separated by ≈ 40 -nL spacers is flowed into the microfluidic channel. The reagent, buffer, and protein streams are combined as they are flowed into a stream of a fluorinated carrier fluid. For each reagent, ≈ 50 smaller (≈ 10 to 15 nL) droplets are formed, each potentially containing a different concentration of the reagent. This concentration may be deduced from the size of the droplet. (b) Microphotographs of two regions in a Teflon capillary containing droplets from a hybrid screen performed for the photosynthetic reaction center (RC) from *Blastochloris viridis*. As the concentration of one precipitant increased (left), a transition from slight precipitation, to large single crystals, to small microcrystals was observed. For another precipitant (right), a transition from precipitation to phase separation was observed. (Caption and figure reprinted with permission from Reference 50 Copyright © 2006 National Academy of Sciences, USA.) PEG, polyethylene glycol.

solutions and does not have chemical exchange with the droplets. As a result, all the droplets are separated and no evaporation or loss of chemicals occurs. Thus, each droplet is an individual microbatch crystallization trial. In this system, concentration gradients can be created by varying independently the flow rates of protein stream, buffer stream, and precipitant stream (94–96). The protein can be merged with a stream containing an array of droplets, each of which contains a different precipitant; as a result, a sparse matrix screening can be performed (12, 93). Furthermore, those two experiments can be combined into a hybrid method, in which different precipitants are screened and each precipitant is tested at multiple concentrations in one experiment. In such an experiment, one researcher can set up approximately 1300 crystallization trials using 10 μL protein within 20 min (50).

To implement the microbatch method in a well-based system (98), two glass plates containing wells are brought into close contact. Each well is individually user-loaded by a degassing method under a sacrificial polydimethylsiloxane (PDMS) slab; one plate is loaded with various precipitants, the other plate is loaded with the protein sample. The loaded plates are then aligned on top of each other to bring wells containing precipitants and wells containing protein samples into contact to form individual crystallization trials. The plates are stored under paraffin oil to prevent evaporation.

The SlipChip is another well-based system that uses two plates containing wells (23, 48). There are two ways to implement the microbatch method in the SlipChip: preloaded and user-loaded. In the preloaded SlipChip (**Figure 2c**) (23), the bottom plate contains an array of wells preloaded with different precipitants. These wells are covered by the top plate, which acts as a lid for the precipitant-containing wells. The chip also has a fluidic path, composed of an array of disconnected ducts in the bottom plate and an array of wells in the top plate (complementary to the array of wells in the bottom plate), that is connected only when the top and bottom plates are aligned in a specific

configuration. A protein sample can be introduced into the fluidic path, filling both wells and ducts. Then, the top plate is slipped, or moved, relative to the bottom plate to bring protein- and precipitant-containing wells in contact to form crystallization trials. Each corresponding set of wells forms a single crystallization trial. In the user-loaded SlipChip (**Figure 4**) (48), no precipitants are preloaded. The user-loaded SlipChip contains different fluidic paths for different precipitants and a separate fluidic path for the protein sample. By simply pipetting an aliquot of solution into a fluidic path, all the wells in that path can be filled. The respective wells of precipitants and the protein sample can be designed to have different volumes while the combined volume of each trial remains the same. As a result, different precipitants and different protein-precipitant ratios for each precipitant can be screened at the same time in one SlipChip. Less than 4 μL of a protein sample was used to screen 16 precipitants and 11 mixing ratios for each precipitant, totaling 176 crystallization trials on a single SlipChip.

Microfluidic Approaches to Implement Vapor Diffusion Methods

Three microfluidic methods have been developed to perform vapor diffusion. The first method, which is both valve and droplet based, relies on a formulator module to create mixtures of precipitants and a protein sample and on a two-phase injector to create nanoliter volume droplets encapsulated in an immiscible carrier fluid (46). Each droplet comprises an individual crystallization trial, and an osmotic bath dehydrates the droplets to mimic the vapor diffusion process (**Figure 5a**). The second method, which is droplet based, generates alternating droplets of crystallization trials (trial droplet) and solutions with high salt concentration (salt droplet) (**Figure 5b**). A fluorinated carrier fluid physically separates the droplets. Because the carrier fluid is water permeable, the salt droplet with high salt concentration dehydrates the adjacent trial droplets. This dehydration stops when the osmotic pressure in the

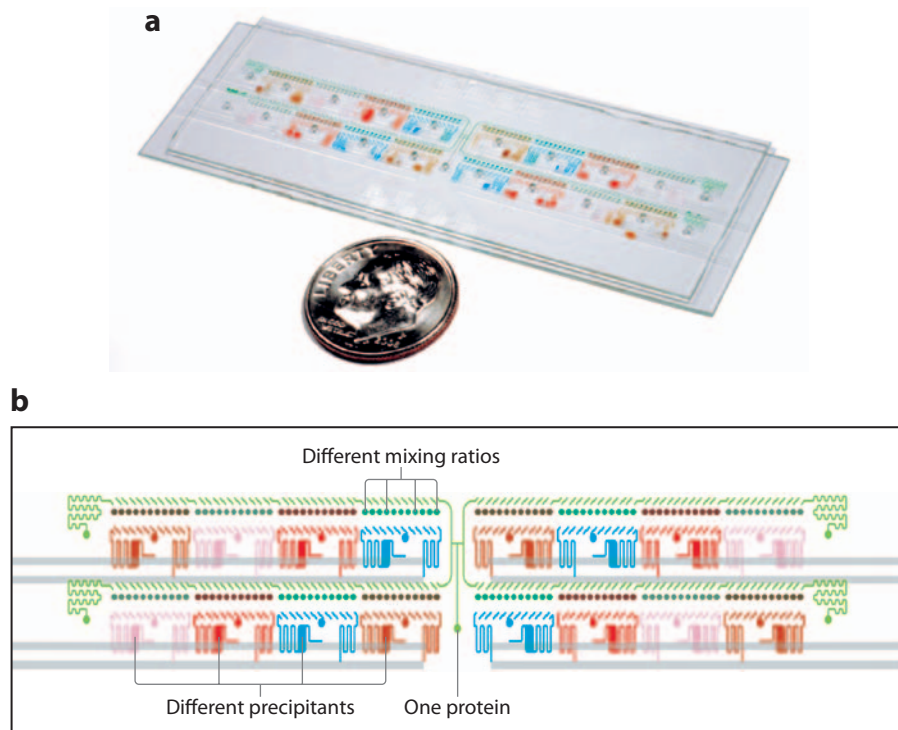


Figure 4

A user-loaded SlipChip to screen one protein sample against different precipitants at different concentrations using the microbatch method. (a) A photograph of a user-loaded SlipChip for screening one sample (shown with green dye) against many different reagents (shown with brown, pink, red, and blue dyes) at various concentrations (the different wells). (b) A schematic of the layout of the user-loaded SlipChip. (Caption and figure reprinted with permission from Reference 48. Copyright © 2009 by the American Chemical Society.)

trial and salt droplets becomes the same (97). The third method is also based on droplets (76) and is described in more detail below.

Microfluidic Approaches to Implement FID Methods

FID methods rely on diffusion of precipitant and protein. The path through the phase diagram for FID is different from that for vapor diffusion, (71), which resulted in a higher success rate for crystallization trials (37). However, setting up experiments for crystallization using FID requires careful manipulation of fluids and has only rarely been performed since its development in 1972. A microfluidics system using valves was developed to meter nanoliter volumes of fluids, which allowed FID

experiments to be performed in a robust way (37). By using a valve-based formulator, phase knowledge can be obtained (38) (Figure 6a–c) and this knowledge enables rational screens using on-chip FID (1) (Figure 6d–f). Moreover, due to the flexibility in the design of the chip, different diffusion times between the protein and precipitant are obtained by altering the length of connecting channels through which diffusion occurs (Figure 6g).

Another microfluidic system was recently developed to perform FID experiments based on SlipChip (23). In this system, all protein and precipitant solutions were loaded by pipetting, and the connection of protein to precipitant was initiated by slipping the connecting channels to bridge the protein wells and the precipitant wells, with FID taking place through the bridge

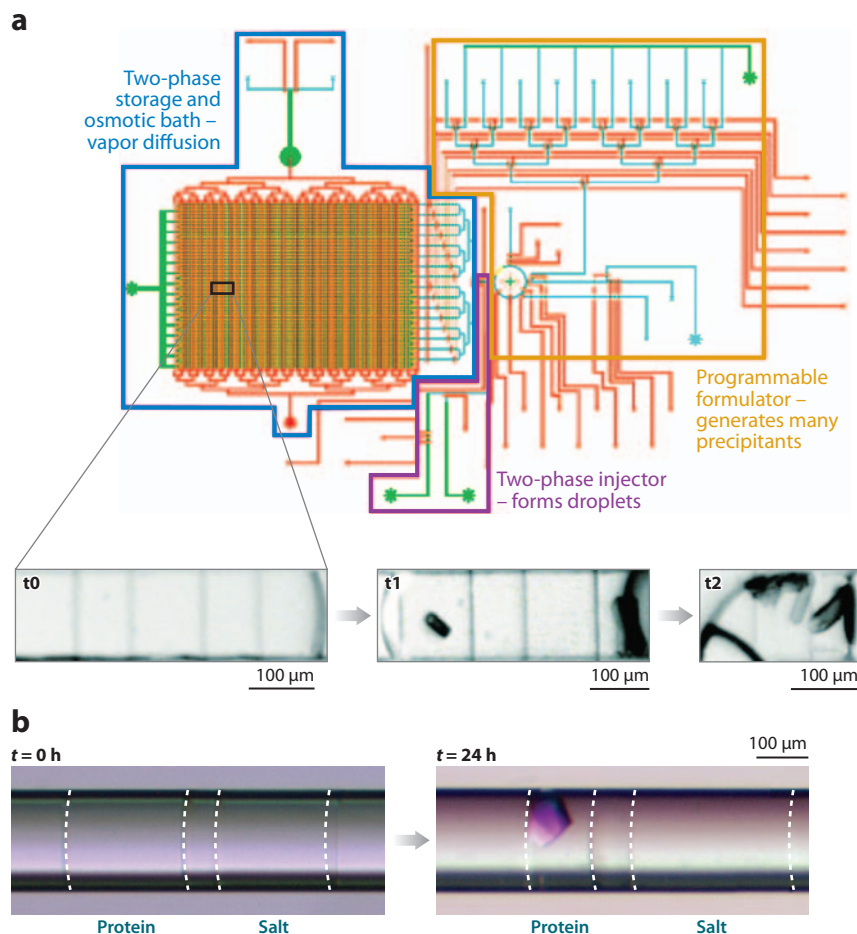


Figure 5

Examples of vapor diffusion methods implemented in microfluidic devices. (a) Reagent mixing, droplet formation, and droplet incubation to form crystals are accomplished on a single device consisting of three integrated modules. Schematic of the device shows the formulation module (right), droplet injector (center), and a two-phase storage module and osmotic bath for vapor diffusion (left). Micrographs of a crystallization trial show droplet concentration and resulting crystal growth. Images were taken immediately after droplet formation (t0), in equilibrium with a 1 M NaCl bath (t1), and in equilibrium with a 2 M NaCl bath (t2). (Figure and caption reprinted with permission from Reference 46 Copyright © 2007 by the American Chemical Society.) (b) Protein crystallization via alternating droplets of protein and precipitant (salt) solutions. Microphotographs of a pair of alternating droplets at 0 h (left) and at 24 h (right) after the droplets were transported into the capillary. A crystal formed within the droplet of the protein solution after the volume of the droplet decreased by 50%. Dashed lines indicate the interfaces between the aqueous droplets and the carrier fluid. (Figure and caption reprinted with permission from Reference 97 Copyright © 2004 by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.)

(47). For this process, no specialized equipment is necessary. This SlipChip allows one protein sample to be screened against multiple precipitants, as well as multiple diffusion times to be screened for each precipitant (Figure 7).

Approximately 12 μ L of a protein sample was screened against 48 precipitants on three SlipChips, totaling 480 experiments. Li et al. (47, 48) observed that when screening the same protein against the same set of precipitants,

microbatch SlipChips and FID SlipChips gave different hit rates. Consequently, a composite SlipChip was developed to perform both microbatch and FID in the same SlipChip (47). SlipChip-based crystallization was compared with current state-of-the-art technologies at the Seattle Structural Genomics Center for Infectious Disease (SSGCID) for two proteins: glutaryl-CoA dehydrogenase from *Burkholderia pseudomallei* (48) and dihydrofolate reductase/thymidylate synthase from *Babesia bovis* (47). SlipChip-based crystallization produced crystals of both proteins in space groups different from those produced at SSGCID. Furthermore, both crystal structures were solved at

higher resolutions higher than those for structures solved at SSGCID: glutaryl-CoA dehydrogenase at 1.73 Å (PDBid: 3II9) compared to 2.2 Å at SSGCID (PDBid: 3D6B), and dihydrofolate reductase/thymidylate synthase at 1.95 Å (PDBid: 3KJR) compared to 2.35 Å at SSGCID (PDBid: 3I3R).

In another version of FID, counterdiffusion experiments of protein crystallization are performed in channels that are centimeters long and hundreds of micrometers thin (20, 62). A protein solution is fully loaded into these long channels, and different precipitants are placed on top of the outlets of the channels. Diffusion occurs through the outlets and a gradient of

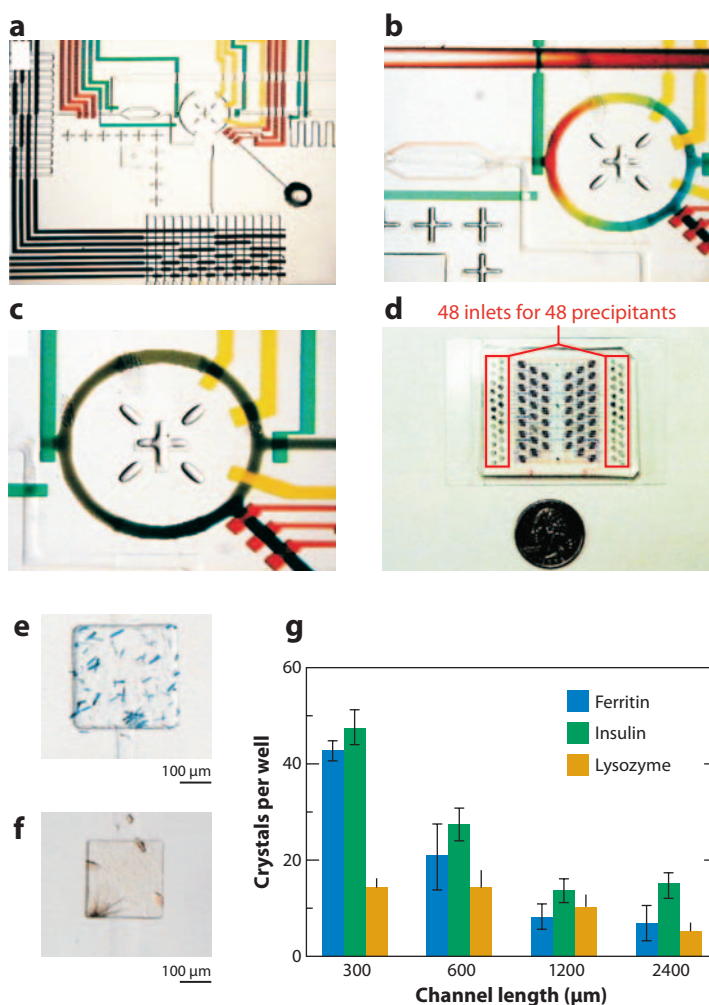


Figure 6

(a–c) Micrographs of combinatorial mixing of food dyes with a microfluidic formulation chip. In all images, the diameter of the mixing ring is 1.5 mm. (a) Integration of multiplexer (dark blue), peristaltic pumps (red), rotary mixer (yellow), and PCI junction (center; green) components for on-chip combinatorial formulation. (b) Color gradient formed by consecutive injections of blue, green, yellow, and red dyes into mixing ring. (c) Pumping around ring for 3 s results in complete mixing of dyes. (Caption and figure reprinted with permission from Reference 38 Copyright © 2004 National Academy of Sciences, USA.) (d) Microfluidic device with 144 parallel reaction chambers that implements simultaneous metering and mixing reactions in each chamber. (Caption and figure reprinted with permission from Reference 37 Copyright © 2002 National Academy of Sciences, USA.) (e, f) Crystals were successfully obtained by using the formulator and free interface diffusion (FID) screening device. (e) Bacteriorhodopsin D85S rod crystals were grown in 0.125 M potassium acetate, 0.1 M sodium citrate (pH 6.5), and 35% PEG 1500. (f) P450 1–12G plate crystals were grown in 0.28 M ammonium sulfate, 15% 1,3-propanediol, 0.1 M imidazole (pH 7.5), and 30% PEG 8000. (Caption and figure reprinted with permission from Reference 1 Copyright © 2006 National Academy of Sciences, USA.) (g) Shortening the connecting channels through which diffusion of protein and precipitants occurred resulted in fewer crystals per well for all three tested proteins (ferritin, insulin, and lysozyme). (Caption and figure reprinted with permission from Reference 36 Copyright © 2006 by the American Chemical Society.)

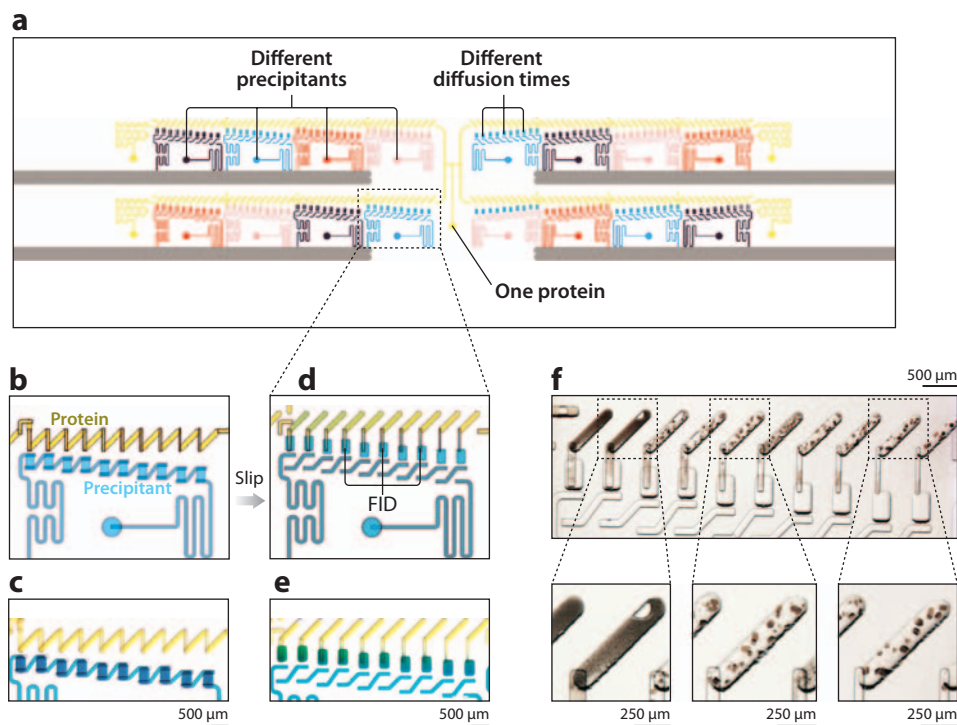


Figure 7

Free interface diffusion (FID) in SlipChip. (a) A schematic of the SlipChip. Multiple precipitants (purple, blue, red, and pink), as well as multiple diffusion times for mixing the protein (yellow) with each precipitant, can be screened on the same SlipChip. (b) A schematic of protein (yellow) and precipitant (blue) solutions after loading by pipetting. (c) A microphotograph of the food dye experiment corresponding to panel b. (d) A schematic of how protein and precipitant wells from one plate can be bridged by narrow channels from another plate due to slipping. (e) A microphotograph of the food dye experiment corresponding to panel d. (f) The effect of diffusion time on crystallization of reaction center from *Blastochloris viridis*. With shortest diffusion time, only precipitates were obtained (left inset). With increased diffusion time, fewer crystals were obtained (middle and right insets). The results were consistent with those in **Figure 6g**. (Caption and figure reprinted with permission from Reference 47 Copyright © 2009 by the American Chemical Society.)

precipitant concentrations can be formed along those channels. These and related traditional methods [such as gel acupuncture (31)] are attractive because of their simplicity, although they typically require larger volumes of protein than other methods described in this review.

PROTEIN CRYSTALLIZATION IN MICROFLUIDICS: EMERGING METHODS

Despite the success of the traditional approaches in crystallizing proteins described

above, many proteins are recalcitrant to crystallization. The optimal conditions for crystallization may be difficult to determine for many reasons. For example, the nucleation zone and the metastable zone may lie in different parts of the phase diagram, in some cases even discontinuous parts of the phase diagram. Traditional approaches for bridging this gap include inducing nucleation by adding minerals or synthetically designed nucleants (8, 59), altering concentrations of protein and reagents by using approaches such as FID, varying temperature, and seeding. Although these traditional approaches

have had some measure of success, microfluidic methods can provide additional capabilities for precise control of the time and position of each crystallization trial in the chemical space. Microfluidic approaches, such as precise control of mixing (11) and nucleation (32, 33, 45, 70, 76), may be difficult or impossible to implement on larger scales.

Proteins may be difficult to crystallize if the nucleation zone and the metastable zone on the phase diagram have different optimal conditions. Although FID can be used in some of these cases, microfluidic technologies can also bridge this gap by decoupling the nucleation and metastable regions of the phase diagram. It must be established whether the path from the nucleation zone into the

metastable zone is continuous or discontinuous. If the path is continuous, microfluidics can be used to control evaporation and rehydration to decouple nucleation and growth, which can be accomplished by using the Phase Chip (76) (**Figure 8**). This device is constructed from two PDMS layers that are sealed together. In the upper, thick (5-mm) layer there are flow channels and storage wells. In the lower, thin (40- μm) layer there is a reservoir sealed by a 15- μm -thick PDMS membrane. Water vapor flows primarily between the drop and the reservoir, but it can also flow through the thick PDMS layer. Initially, there is protein solution in the well and a reservoir below filled with 6 M NaCl. Then, the water flows out of the drop, owing to the osmotic pressure. This dehydration leads to the formation of crystal nuclei. Next, the reservoir is filled with a lower concentration of NaCl, which changes the osmotic pressure. The water flows back into the drop, and the path moves from the nucleation zone to the metastable zone on the phase diagram. At this point, the existing nuclei continue to grow into crystals, but no new nuclei form. If the path from the nucleation zone into the metastable zone is discontinuous, microfluidic approaches offer control of nucleation time with subsecond precision in nanoliter volumes. To take advantage of these features, Gerdt et al. (32, 33) have developed a droplet-based microfluidic system to separate and independently control the nucleation and growth stages of protein crystallization to allow the growth of single-protein crystals (**Figure 9**). This approach enables a jump from the nucleation region to the metastable region of the phase diagram.

Microfluidic approaches also address the problems that arise when there is a narrow window of conditions in which nucleation occurs. Microfluidic systems may overcome this problem by controlling interfaces to promote nucleation and extend this region of the phase diagram. Roach et al. (70) modified a previously developed fluorosurfactant with an oligoethylene glycol head group, RfOEG, to design a new fluorinated amphiphile,

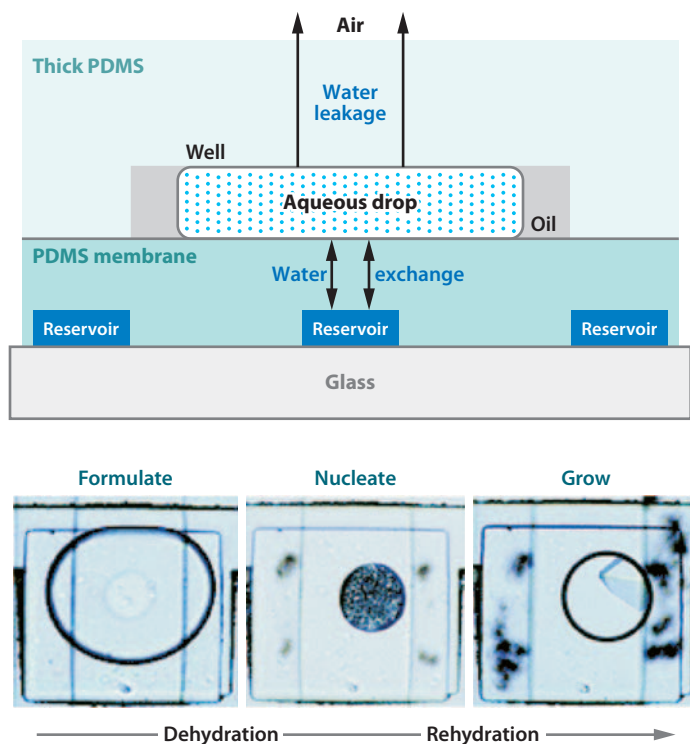


Figure 8

The Phase Chip is a microfluidic device designed to determine the phase diagram of multicomponent fluid mixtures. (*Top*) Schematic of a vertical section of the Phase Chip. (*Bottom*) Crystals at various time points. (Caption and figure reprinted with permission from Reference 76 Copyright © 2007 by the American Chemical Society.) PDMS, polydimethylsiloxane.

RfNTA, which introduces specific adsorption of His-tag proteins at the interface to create laterally mobile self-assembled monolayers. The formation of self-assembled monolayers increased the range of successful conditions, the success rate at a given condition, the rate of nucleation, and the quality of the crystals formed when applied to membrane protein crystallization (45). Microfluidic systems, with their high surface-to-volume ratios, are especially sensitive to interfacial effects.

PROTEIN CRYSTALLIZATION IN MICROFLUIDICS: MEMBRANE PROTEINS

There are additional considerations and methods for membrane protein crystallization because of the increased complexity of the process. First, using a lipidic mesophase, such as the lipidic cubic phase (LCP), is an attractive route to crystallize membrane proteins because the lipidic mesophase provides an environment similar to the natural environment of membrane proteins (4). Current developments in LCP-based microscale protein crystallization include robotic systems that allow for accurate handling of small amounts of LCP material (13). LCP is highly viscous and is challenging to handle inside microfluidic devices. Nevertheless, Perry et al. (65) developed a microfluidic system that used pneumatic valves to form a LCP on-chip at volumes below 20 nL to crystallize membrane proteins (**Figure 10**). Droplet-based microfluidic systems are suitable for handling viscous solutions—even suspensions of solids (75) and clotted blood (80)—because of the lubricating layer of carrier fluid that separates the sample inside the droplet from the channel wall. Li et al. (49) developed a droplet-based microfluidic system to dispense nanoliter-volume droplets of LCP material and subsequently merge the LCP droplets with aqueous droplets to form crystallization trials (**Figure 11**).

Second, a wide variety of detergents are commonly used to stabilize membrane proteins, but these detergents can interfere with

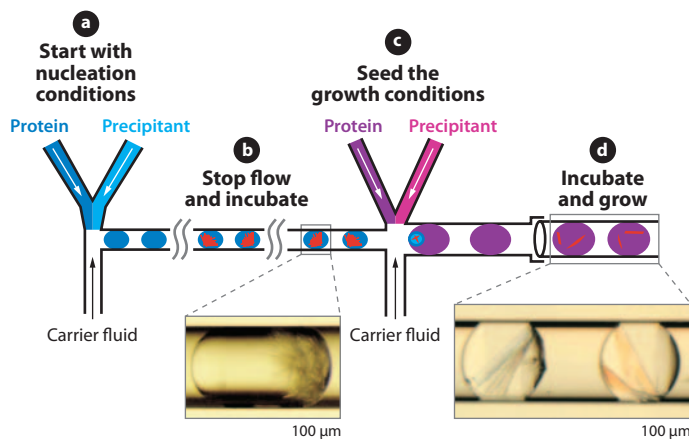


Figure 9

A microfluidic approach designed to separate the nucleation and growth stages in protein crystallization. (a) High-concentration protein and precipitant solutions are combined to form droplets. (b) The flow is stopped, and the droplets are incubated to generate seed crystals. (The left microphotograph is a typical SARS protein crystal grown in high-supersaturation solutions in which excess nucleation leads to clustered microcrystals.) (c) Lower-concentration protein and precipitant solutions are combined to form droplets containing lower-supersaturation solutions that lead to crystal growth. Each droplet containing seed crystals seeds multiple growth droplets. (d) Growth droplets that contain seed crystals are flowed into a glass capillary and incubated. (The right microphotograph depicts two typical droplets of low supersaturation that have been seeded with SARS protein microcrystals.) Figure and caption reprinted with permission from Reference 33 Copyright © 2006 by Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

crystallization. Li et al. (51) developed an approach that used cyclodextrin-based host-guest chemistry in a microfluidic device to capture and sequester detergent monomers to modulate membrane protein crystallization. This approach was used to simplify the process of protein concentration by removing free detergent micelles and to affect the packing of protein-detergent complexes by removing loosely bound detergent. Using host-guest chemistry, the detergent capture approach could be expanded to include time-controlled removal of loosely bound detergent or more controlled thermodynamics suitable for selective binding with different detergents. Incorporating various detergent exchange protocols into microfluidic screens is an attractive opportunity, especially alongside the development of designer detergents (60, 91).

LCP: lipidic cubic phase

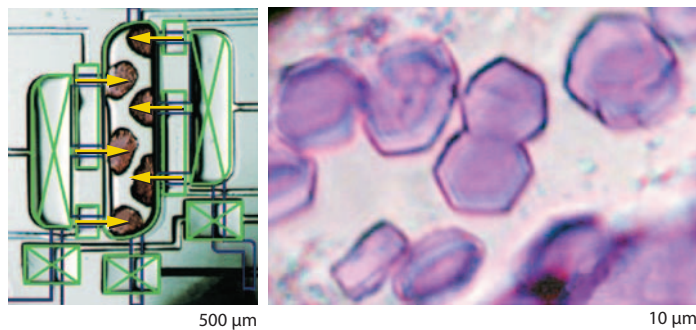


Figure 10

A microfluidic method for the formation of aqueous/lipid mesophases to enable screening of suitable crystallization conditions of membrane proteins from a membrane-like phase in volumes less than 20 nL. (*left*) Optical micrographs of an aqueous 13.5 mg ml^{-1} bacteriorhodopsin solution (*left and right chambers*) mixed with the lipid monoolein (*center chamber*) in a microfluidic chip. The blue lines delineate the edges of the fluidic channels. Chambers are filled with a protein solution and lipid through inlet channels (situated vertically below each chamber), a straight-line injection of protein is delivered into the lipid-containing center chamber (*arrows*), and then consecutive, chamber-to-chamber injections of the fluid mixture driven by valves are given through different sets of inlets to create a net circulatory motion to homogenize the mixture of protein and lipids. (*Right*) Crystals of the membrane protein bacteriorhodopsin obtained using this device. (Caption and figure reprinted with permission from Reference 65 Copyright © 2009 by the American Chemical Society.)

CONCLUSION AND FUTURE DIRECTIONS

Membrane proteins control many signaling pathways and are the targets of more than 50% of pharmaceutical drugs. If we understand the structures of the complexes formed between membrane proteins and their biological partners, we will enhance our knowledge of many basic cellular functions. Although many new structures of membrane proteins have been discovered, there remains a huge gap between the number of solved structures for membrane proteins and the number of solved structures for soluble proteins (86). Implementation of current microfluidic technologies has been focused on soluble proteins, but to address this gap, these technologies must now be focused on membrane proteins and their complexes. Some of the microfluidic technologies discussed in this review may be immediately applicable to crystallizing membrane proteins, and some

may require modifications before they can be applied to this problem. Although large surface-to-volume ratios of microfluidic devices could be useful (45), they also present a potential problem, especially for solutions used in crystallization of membrane proteins containing detergents and other amphiphiles. Unless surface properties are controlled, the surfaces of microfluidic devices would likely cause losses of these molecules and thus affect crystallization.

Although screening of crystallization conditions and the actual crystallization experiments to obtain crystals suitable for X-ray diffraction can be performed in small volumes, currently these experiments begin by producing a large volume of protein samples. In addition, the thousands of trials generated by microfluidic experiments require efficient imaging systems to monitor the results and reliable database systems to manage the results (66). By developing methods (15) to quickly prescreen crystallization conditions in small volumes so that only useful reagents are screened, fewer but more meaningful data points must be collected and managed. To affect how these problems are addressed requires the development of technologies for the production, purification, and biophysical characterization of proteins on small scales.

Once crystals are obtained from the microfluidic experiments, they need to be characterized by X-ray diffraction. The minimum trial volume that produces crystals large enough for X-ray diffraction is $\sim 10 \text{ nL}$ (37, 50), although advances in synchrotron facilities may enable analysis of smaller crystals (16, 73). Extraction of crystals from microfluidic devices is well established (37, 50). An exciting possibility is provided by X-ray diffraction of crystals in situ, inside the device, with many microfluidic methods compatible with both in situ diffraction to evaluate crystal quality (37, 50) and in situ diffraction to determine crystal structure (20, 36, 62, 97). Diffraction in situ is preferred because it eliminates the potential for researchers to damage the crystal during postcrystallization manipulation. Diffraction in situ is especially important for membrane

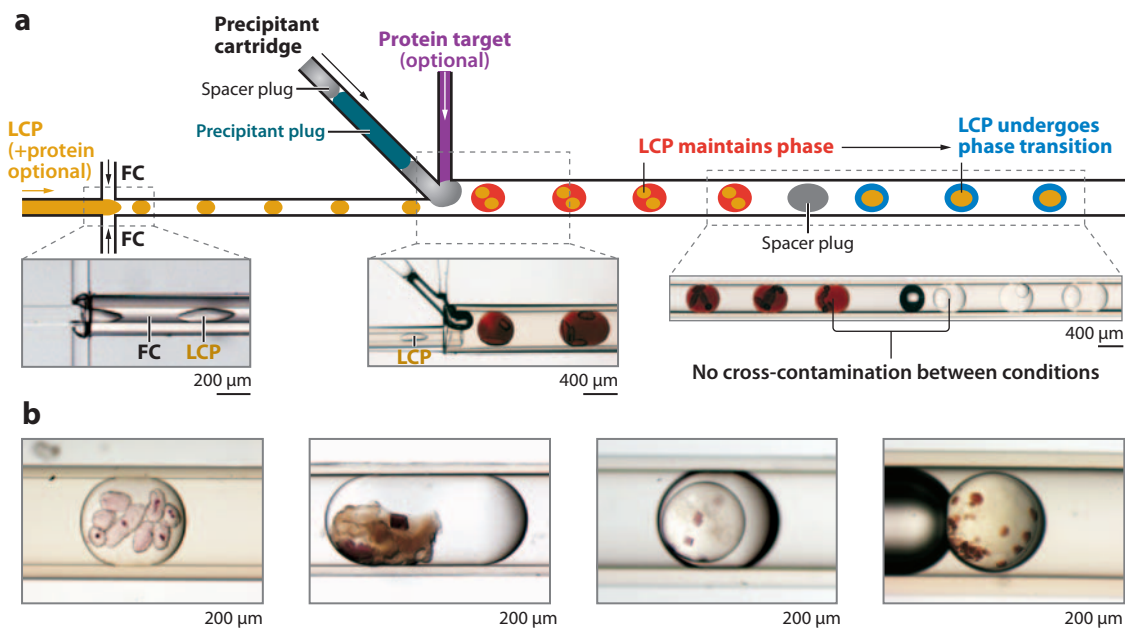


Figure 11

Droplet-based microfluidic system for membrane crystallization within lipidic mesophases. (a) A schematic of the droplet-based microfluidic system for dispensing lipidic cubic phase (LCP) material into droplets and merging the LCP droplets with aqueous droplets containing protein and precipitants. Small LCP droplets (~ 1 nL) were formed in a flow-focusing device using fluorinated carbon (FC) as a carrier fluid. The LCP droplets were transported in Teflon tubing, and then they merged downstream with the aqueous droplets (~ 80 nL), which were formed by combining a protein sample and various precipitants. Upon merging with droplets containing certain precipitants, the LCP material may undergo phase transition to form another lipidic mesophase material. The droplets of the crystallization trials were stored and incubated at 23°C in Teflon tubing to allow crystals to grow. The microphotographs show LCP droplets forming in the flow-focusing device, LCP droplets merging successfully with precipitant and protein solutions, and the absence of cross-contamination of aqueous droplets separated by air bubbles. (b) Membrane protein crystals can be obtained in this system by using two methods: (I) the protein is premixed in LCP material, and (II) LCP material is formed without the protein and the protein is added externally and allowed to diffuse into the LCP material. From left to right: A droplet with crystals (dark purple) of bacteriorhodopsin from *Halobacterium salinarum* obtained using method I. A droplet with crystals of carotenoid-containing reaction center (RC) from *Rhodobacter sphaeroides* obtained by using method II. A droplet with crystals of carotenoidless RC from *Rhodobacter sphaeroides* obtained by using method II. A droplet with crystals of RC from *Blastochloris viridis* obtained by using method II. (Caption and figure reprinted with permission from Reference 49 Copyright © 2009 by Springer.)

proteins, because membrane protein crystals have a higher solvent content and thus are more prone to damage during postcrystallization manipulation. Microfluidic systems and future ad-

vances described in this review, combined with ongoing dissemination of technologies (26, 27), are likely to accelerate further the progress of structural biology.

SUMMARY POINTS

1. To obtain protein crystals, a large chemical space must be searched to identify optimal conditions for crystallization. This requires thousands of crystallization experiments.

2. Microfluidic approaches are attractive for protein crystallization because they (a) carry out many experiments simultaneously to cover a dense, multidimensional chemical space; (b) use small quantities of samples; (c) precisely control mixing, interfaces, and time of contact between solutions; and (d) enable evaluation of crystal quality by X-ray diffraction.
3. The traditional approaches to protein crystallization—microbatch, vapor diffusion, and FID—have been carried out in valve-based, droplet-based, and well-based microfluidic devices.
4. Microfluidic approaches crystallize proteins that are recalcitrant to crystallization via traditional larger-scale approaches.
5. Membrane proteins add additional complexity to the crystallization process, but they have been crystallized in microfluidic devices by using a LCP material or detergents.

DISCLOSURE STATEMENT

The authors are listed as coinventors on University of Chicago patents or patent applications for some methods presented in this article. Rustem F. Ismagilov was a consultant to deCODE Biostructures.

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Errata

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