Journal of Visualized Experiments

Optimisation of Crystal Growth for Neutron Macromolecular Crystallography

--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE61685R1
Full Title:	Optimisation of Crystal Growth for Neutron Macromolecular Crystallography
Corresponding Author:	Monika Budayova - Spano Institut de Biologie Structurale Grenoble, France FRANCE
Corresponding Author's Institution:	Institut de Biologie Structurale
Corresponding Author E-Mail:	monika.spano@ibs.fr
Order of Authors:	Elham Vahdatahar
	Niels Junius
	Monika Budayova - Spano
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Biology
Please provide any comments to the journal here.	All changes are highlighted in blue color in the revised version of the manuscript as well as in our letter to the editorial & production team and reviewers.

TITLE:

Optimization of Crystal Growth for Neutron Macromolecular Crystallography

AUTHORS AND AFFILIATIONS:

Elham Vahdatahar[1], Niels Junius[1,2], Monika Budayova-Spano[1]*

[1] Université Grenoble Alpes, CEA, CNRS, IBS, Grenoble, France
[2] Current address: ELVESYS SAS, Paris, France

Email addresses of co-authors:
Elham Vahdatahar (elham.vahdatahar@ibs.fr)
Niels Junius (niels.junius@laposte.net)

Corresponding author:
Monika Budayova-Spano: (monika.spano@ibs.fr)

KEYWORDS:

Dialysis; Neutron macromolecular crystallography; OptiCrys; Phase diagram; Temperature control; Crystal growth; Protein solubility.

SUMMARY:

Structural studies of biomacromolecules by crystallography require high-quality crystals. Here we demonstrate a protocol that can be used by OptiCrys (a fully automated instrument developed in our lab) and/or microdialysis buttons for growing large high-quality crystals based on knowledge of the crystallization phase diagram.

ABSTRACT:

The use of neutron macromolecular crystallography (NMX) is expanding rapidly with most structures determined in the last decade thanks to new NMX beamlines having been built and increased availability of structure refinement software. However, the neutron sources currently available for NMX are significantly weaker than equivalent sources for X-ray crystallography. Despite advances in this field, significantly larger crystals will always be required for neutron diffraction studies, particularly with the tendency to study ever-larger macromolecules and complexes. Further improvements in methods and instrumentation suited to growing larger crystals are therefore necessary for the use of NMX to expand.

In this work we introduce rational strategies and a crystal growth bench (OptiCrys) developed in our laboratory that combines real-time observation through a microscope-mounted video camera with precise automated control of crystallization solutions (e.g., precipitant concentration, pH, additive, temperature). We then demonstrate how this control of temperature and chemical composition facilitates the search for optimal crystallization conditions using model soluble proteins. Thorough knowledge of the crystallization phase diagram is crucial for selecting of the starting position and the kinetic path for any crystallization experiment. We show how a rational approach can control the size and number

of crystals generated based on knowledge of multidimensional phase diagrams.

INTRODUCTION:

Understanding the structure-function relationship of proteins and the mechanism of physiological pathways often relies on knowing the positions of hydrogen atoms (H) and how charge is transferred within a protein[1,2]. Since hydrogen atoms scatter X-rays weakly, their positions can only be determined with very high resolution X-ray diffraction data (>1 Å)[3,4]. Conversely, neutron crystallography can be used to obtain an accurate position of hydrogen atoms in biological macromolecules as hydrogen and deuterium (H^2, isotope of hydrogen) atoms have scattering lengths of roughly equal magnitude as oxygen, nitrogen and carbon[5]. However, neutron flux from available neutron sources is weaker than that of X-ray beams, so this must often be compensated for[2,3]. This can be achieved by exchanging H with H^2 and/or increasing the volume of crystals to reduce the incoherent scattering of hydrogens and increase the signal-to-noise ratio of diffraction images.

There are various crystallization approaches (the corresponding schematic phase diagram is shown in **Figure 1**) for obtaining large and high-quality crystals for both X-ray and neutron bio-macromolecular crystallography[6]. In vapor diffusion, a droplet prepared from a mixture of a protein and a crystallization solution is equilibrated over time, through evaporation of water or other volatile species, against a reservoir containing a higher concentration of precipitant of the same crystallization solution. The increase in concentration of protein and precipitant in the droplet leads to the supersaturation required for spontaneous nucleation followed by crystal growth at these nuclei[6,7]. Although vapor diffusion is the most frequently used technique for growing crystals[4], the crystallization process cannot be precisely controlled[8]. In the free interface diffusion method, crystallization solution diffuses into a concentrated protein solution, very slowly directing the system towards supersaturation. This method can be considered a batch method with a slow mixing rate[6,9–12]. In the batch method, the protein is rapidly mixed with a crystallization solution leading to rapid supersaturation and in turn uniform nucleation with many crystals[3,7]. This method accounts for approximately one third of all structures currently deposited in the Protein Data Bank. The dialysis method is also used for growing high-quality and well-diffracting protein crystals. In the dialysis method, molecules of precipitant diffuse from a reservoir through a semi-permeable membrane into a separate chamber with the protein solution. The kinetics of equilibration are dependent on various factors, such as temperature, membrane pore size and the volume and concentration of protein samples and crystallization agents[6].

Crystallization phase diagrams can be used to describe different states of a protein as a function of different physical or chemical variable[3]. As illustrated in **Figure 1**, each crystallization technique can be visualized as using a different kinetic trajectory to reach the nucleation and metastable zones of such a diagram[6,10,13]. This provides information about protein solubility and the protein concentration at which a thermodynamic equilibrium between crystal and solution is observed, thereby finding the optimal conditions for nucleation and growth[3,14]. In a two-dimensional phase diagram, the protein concentration is plotted as a function of one variable and the other variables are kept constant[15]. In such a phase diagram, when the protein

concentration is below the solubility curve, the solution is in the undersaturated region and no nucleation or crystal growth occurs. Above this curve is the supersaturation zone where the protein concentration is higher than the solubility limit[3,14]. This is further divided into three regions: the metastable zone, the spontaneous nucleation zone, and the precipitation zone. In the metastable zone, supersaturation is not sufficient for nucleation to occur within a reasonable time but growth of seeded crystals can take place. Aggregation and precipitation are favored in the precipitation zone, where supersaturation is too high[14,15].

When sufficient supersaturation for spontaneous nucleation is achieved, the first nuclei will appear[10]. The growth of crystals leads to a reduction in the protein concentration until the limit of solubility is reached. As long as supersaturation stays in the vicinity of the solubility curve, there will be no significant change in the size of crystals. However, it has been shown that variations in the temperature and chemical composition of crystallization solution (for example, the concentration of precipitant) will affect protein solubility and may lead to the initiation of further crystal growth[8,13,16].

As dialysis is advantageous for good quality crystal growth, the OptiCrys crystallization bench illustrated in **Figure 2**, was designed and developed in our laboratory to control crystallization in a fully automated manner[8]. For this purpose, software was written with LabVIEW that allows the control and monitoring of the temperature of a flowing reservoir dialysis setup in contact with Peltier elements, via an electronic controller and a chiller. The same software also automatically regulates the chemical composition of the crystallization solution (for example the exchange of crystallization agents) using a multichannel fluidic system. Additionally, a digital camera and an inverted microscope are used to visualize and record the crystallization process. Two crystallization chambers with 15 µL and 250 µL volumes are available for growing crystals for different purposes. As the crystallization process is reversible, screening for different conditions is possible with just a few microliters of the protein solution as long as the sample is not damaged[8]. As a result, using this method minimizes the amount of protein material used.

From previous work[8], it is apparent that during the crystal growth process, in situ observations need to be carried out at regular time intervals. These can range from a few seconds to several days, depending on the event under observation (precipitation, nucleation or crystal growth).

The optimization of crystal growth with OptiCrys is based on temperature-precipitant concentration phase diagrams. In the case of proteins with solubility as a direct function of temperature it is possible to make use of the salting-out regime[18]. This is where increasing the ionic strength of the solution, which can be visualized using protein-precipitant phase diagrams, decreases the solubility of the protein. Likewise, proteins with inverse solubility can make use of the salting-in regime[18]. Nucleation occurs in the nucleation zone, in the vicinity of the metastable zone, and crystal growth then takes place in the metastable zone of the phase diagram until the protein concentration reaches the solubility limit. As shown in **Figure 3A**, with constant chemical composition temperature can be decreased to keep the crystallization solution in the metastable zone to prevent new nucleation. Crystals grow until the second crystal/solution equilibrium is achieved and after that no further increase in the size of crystals

is observed. The temperature is decreased several times until the crystals reach the desired size. In **Figure 3B**, at constant temperature, increasing the precipitant concentration keeps the solution in the metastable zone. This process can then be repeated several times to obtain large crystals. Changing the temperature and manipulating the crystallization solution conditions, by controlling the supersaturation levels, are two powerful tools for separating nucleation and growth of crystals that are controlled precisely and automatically by OptiCrys[5,8,14].

Examples of protein crystals grown by temperature-controlled or temperature- and precipitant concentration-controlled crystallization as well as relative diffraction data obtained are available in the literature and PDB. Among them are human γ-crystallin E, PA-IIL lectin, yeast inorganic pyrophosphatase, urate oxidase, human carbonic anhydrase II, YchB kinase, and lactate dehydrogenase[5,14 17,18].

Although OptiCrys was commercialized by NatX-ray, there are many laboratories that do not have access to this instrument or to the serial approach it offers. The alternative to this technique is to use commercially available plastic microdialysis buttons with various volumes. Using these, temperature and chemical composition can be adjusted and varied manually. Inspection of microdialysis buttons cannot be done in situ and must instead be done manually with an optical microscope. Temperature control can be achieved by keeping the sample in a vibration-free temperature-controlled incubator. It is essential to keep the temperature constant to ensure that crystallization experiments are reproducible. Significant variation in temperature may also lead to damage or destruction of crystals[5].

Here we provide a detailed protocol describing sample preparation and the use of control software for the growth of large, high-quality crystals suitable for neutron protein crystallography. This step-by-step procedure was designed to take advantage of the crystallization phase diagram in order to select a starting position and kinetic path to control the size and the quality of the crystals generated. Additionally, a detailed protocol for growing crystals with microdialysis buttons is presented which uses the same rationale to obtain large, high-quality crystals.

PROTOCOL:

1. Dialysis method with microdialysis buttons

1.1. Sample preparation

1.1.1. Prepare protein solution by dissolving 30 mg of chicken egg-white lysozyme as a lyophilized powder in 1 mL of CH_3COONa buffer (100 mM sodium acetate, pH 4) in order to obtain a solution with a final concentration of 30 $mg \cdot mL^{-1}$.

1.1.2. Centrifuge the sample at the 13,000 × *g* for 10 min at 277 K. This process helps to remove any aggregation before starting the crystallization process.

1.1.3. Check the absorbance of the sample at 280 nm and calculate the protein concentration by using the Beer-Lambert equation (A= εcl).

NOTE: According to the Beer-Lambert equation, electronic absorbance (A) is directly proportional to the concentration (c, $mg \cdot mL^{-1}$) of an absorbing species given a constant optical pathlength (l, cm). The gradient of this linear relationship is the molar extinction coefficient (ε, for lysozyme at 280 nm is 2.64 mL mg^{-1} cm^{-1}) [19]. The sidechains of aromatic amino acids (tyrosine, tryptophan, and phenylalanine) and disulphide bonds between cysteine residues have strong absorbance at ~280 nm arising from spectroscopically-allowed $\pi - \pi^*$ transitions. As the majority of proteins contain these residues, protein concentration can typically be calculated easily by measuring the absorbance at 280 nm, given knowledge of the extinction coefficient.

1.1.4. Prepare crystallization solutions as shown in **Figure 4**. Filter all the stock solutions with 0.22 µm Millipore filters before preparing the crystallization solution.

1.2. Crystal growth

1.2.1. Cut a cellulose dialysis membrane with appropriate molecular weight cutoff (6-8 kDa) and soak it in distilled water.

NOTE: Dialysis membrane discs are commercially available for microdialysis buttons, but if the dialysis tubing is used, don't forget to cut the edges in order to separate the two layers of membrane from the tube to have only single layer membranes.

1.2.2. Fill wells of a 24- well tray with 2 mL of crystallization solution in the same order as shown in **Figure 4**.

NOTE: If buttons with larger volumes are used (e.g., 200 µL), fill 50 mL tubes with a minimum of 5 mL crystallization solution in order to ensure efficient exchange.

1.2.3. Add/Pipette 35 µL of lysozyme solution to the chamber of the microdialysis button as illustrated in **Figure 5A**.

NOTE: To avoid the formation of air bubbles in a 30 µL dialysis button when it is closed, an extra volume (dead volume) of 5 µL of additional protein must be added, which means a total of 35 µL of protein sample. This additional protein sample creates a slightly domed shape on top of the chamber, as shown in **Figure 5B**, which prevents the formation of air bubbles.

1.2.4. Take an applicator of the appropriate size and place the elastic O-ring at its extremity (**Figure 5C**). Then place the membrane, previously lightly wiped/drained using a piece of fibre-free paper, on top of the chamber of the dialysis button. Be careful not to put dust in the chamber when applying the paper. Set the dialysis membrane in place by transferring the elastic O-ring from the applicator to the groove of the dialysis button (**Figure 5C**).

NOTE: The critical moment of handling is fixing the dialysis membrane on top of the chamber by transferring the elastic O-ring from the applicator into the groove of the dialysis button. All movements must be perfectly synchronized to avoid enclosing air bubbles with the sample in the protein chamber. It is useful to practice stretching the elastic O-ring before its application to know its rigidity, use tweezers to hold part of the membrane during its application and carry out the first test experiments with a model protein.

1.2.5. Transfer the button to the well or to the 50 mL tube using tweezers (**Figure 5D**).

1.2.6. Cover the well with a coverslip, pressing it gently on the grease to seal the well (**Figure 5E**).

NOTE: If there is no grease on top of the wells, be sure to add this before starting the experiment or use a piece of tape instead of glass coverslip. The principle of protein crystallization using microdialysis buttons is illustrated in **Figure 5**.

1.2.7. Keep the sample at 293 K in a thermoregulated incubator. Different temperatures may be necessary according to the protein and crystallization conditions used.

NOTE: The same grid of crystallization conditions shown in **Figure 4** (allowing the concentration of precipitant to be varied) can be screened as a function of temperature. In such a case, the same crystallization plate must be reproduced and each copy must be placed in an incubator regulated at a different temperature. This requires having several vibration-free thermoregulated incubators available.

1.2.8. Check the tray or tubes for crystals (**Figure 4**) and take notes on a regular basis, typically daily, to distinguish what is in each tray. Good notes are essential to avoid false positive results and to discriminate dust from crystals.

2. Crystal growth process using OptiCrys

2.1. Sample preparation

2.1.1. Prepare a protein solution and a dialysis membrane as described in section 1.1.

2.1.2. Prepare stock solutions of NaCl (4 M) and of CH_3COONa pH 4 (1 M) and filter them into 50 mL tubes.

2.1.3. Add the protein solution (15 µL of lysozyme with 30 $mg \cdot mL^{-1}$) to the dialysis chamber of the temperature-controlled flowing reservoir dialysis setup. Refer to **Figure 6** for details of the temperature-controlled flowing reservoir dialysis setup. OptiCrys has two dialysis chambers, the minimum volume is 15 µL and the maximum volume is 250 µL.

2.1.4. Cover the overchamber with a dialysis membrane and fix the membrane with the elastic O-ring (**Figure 6B**).

NOTE: This setup is different from microdialysis buttons where each chamber is sealed directly by a dialysis membrane. In the flow cell, the dialysis membrane is instead fixed to the overchamber allowing the mounting of crystals without its removal. Instead the overchamber can simple be unscrewed from the reservoir for this purpose.

2.1.5. Flip the overchamber and place it on top of the dialysis chamber. Slowly and gently press it to remove all the air trapped between the two pieces and avoid bubbles in the chamber (**Figure 6C**). Practice with a model protein can be advantageous when training to avoid air bubbles and sample loss.

2.1.6. Fix the Reservoir in its position by gently screwing it on top of the overchamber, again being mindful to avoid trapping air bubbles. Over-tightening of the reservoir can also form bubbles the crystallization chamber (**Figure 6D**).

2.1.7. Add the crystallization solution and cover the reservoir chamber with the airtight cap. (**Figure 6G**). Maximum volume of the reservoir is 1 mL.

2.1.8. Transfer this assembly and insert it into the brass support. This support is in contact with Peltier elements that are used to control the temperature.

2.1.9. As illustrated in **Figure 6**, the airtight cap is equipped with an optical window to allow top illumination of the dialysis chamber. Put the light source (**Figure 2**) on the window allowing light to pass through the chamber.

2.1.10. The reservoir chamber can be connected to a pump to allow it to function as a continuous flow cell. Connect the tubing on top of the 50 mL tubes that contain the stock solutions and distilled water to the rotary valve as illustrated in **Figure 7**.

NOTE: If automatic preparation and changing of crystallization solutions is not desired during the experiment, omit step 2.1.10. In such a case, the crystallization solution must be prepared and the reservoir pre-filled manually.

2.2. Software

2.2.1. Turn on the computer and launch the software **Croissance crystalline [Crystal growth]**. This control software is written with LabVIEW and offers a user-friendly graphical interface. It includes 4 different graphical interfaces (**Accueil [Welcome], Parametrage [Setting], Essai [Test],** and **Maintenance [Maintenance]**) (**Figure 8**).

NOTE: Translations from French are in brackets.

2.2.2. Select the **Maintenance** view by clicking the button as illustrated in **Figure 8**. This view is currently the most often used and allows users to control the majority of parameters during the experiment.

NOTE: After clicking on the **Maintenance** view, a new window with different sections for controlling parameters such as temperature or light will appear. In **Figure 8** different parts of this view are shown with arrows and frames. In the following steps, we demonstrate how each parameter is controlled using the software.

2.2.3. **Regulateur de temperature [Temperature controller]** section allows the control and monitoring of temperature. Click on the button, number one in **Figure 8**, to turn it on.

NOTE: Temperature range in OptiCrys is 233.0–353.0 $\pm$ 0.1 K.

2.2.4. Set the temperature on the **consigne [setpoint]** section and press enter (**Figure 8(2)**). Below this button, there is a graph with 2 traces (red and yellow). The red trace shows the final (ordered) temperature and the yellow trace shows the current temperature. As shown in **Figure 8(3)**, the temperature is set at 20 °C.

NOTE: In order to grow a crystal, as explained in the crystal growth section, many temperatures will need to be chosen. To change the temperature, add each new temperature in the **consigne [setpoint]** section and press the Enter button on the keyboard.

2.2.5. Turn on the light by increasing the luminosity from the **Lumières [Lights]** section in Figure 8. Luminosity ranges from 0 to 100, "0" indicates that the light is off and at "100" the light is set to maximum of intensity and brightness. By increasing the light, in the **Microscope** section, one can see inside the dialysis chamber. During the experiment, the brightness in the cell may vary; adjust the parameters to clearly visualize inside the dialysis chamber. Magnification can also be increased or decreased by using the + and – buttons in front of “zoom” for better viewing.

2.2.6. On the right-hand side of the **Microscope** section, there are several sections for storing relevant information for each crystallization experiment. Each user can create a folder to store information on crystallization conditions, protein names and the molecular weight cutoff of the dialysis membrane used (**Figure 8(4)**).

2.2.7. The user can define a name for the experiment by simply typing it on the **Nom Dossier [Folder name]**. Clicking on the Dossier [Folder button] (shown with a green frame in **Figure 8**) opens a new window. In this window, there will be a text file that contains all the information defined for the experiment. In addition, time-stamped images are saved in this folder for future processing.

2.2.8. From the **NB Images** section**,** select the number of images that should be taken during the course of the experiment. Specify the number of the images in the right-hand panel along with the desired time interval between these (e.g., min, hour, day). **Figure 8** shows the software

setup to record zero images in a minute.

NOTE: Use the **Pompe [Pump]** section for mixing stock solutions and injecting crystallization solution into the reservoir chamber. See section 2.1.10 and **Figure 7** for an explanation of the principle of the fluid mixing system.

2.2.9. Input concentrations of the stock solutions in **Etape 1 [Step 1]: solutions stocks.** For the crystallization experiment in the next section, NaCl 4 M and CH_3COONa 1 M pH4 will be used.

NOTE: Concentrations of stock solutions are in molar units.

2.2.10. Define the final concentration of each solution. For example 0.75 M for NaCl and 0.1 M for CH_3COONa pH4. Input these in the final concentration section (**Figure 8**) in the **Etape 2 [Step 2]: solution a preparer [solution to prepare].** Press the **Calcul [Compute]** button, which is shown with a red frame in Figure 8. The final volume of each stock solution that will be used in mixing will display in the volume panel in front of each concentration panel.

2.2.11. Press the **Lancer preparation [Launch preparation]** button (**Figure 8**). As illustrated in **Figure 7**, the rotary valve takes each stock solution and injects them to the mixing tube via a switch.

2.2.12. After the crystallization solution has been prepared, click on the **Entrée solution [Solution Entry]** button in **Etape 3 [Step 3]: Flux** of the pump section (yellow frame in **Figure 8**). The switch changes to inject the new crystallization solution from mixing tube into to the reservoir chamber. To stop the exchanging process, press the **Arret distribution [Distribution Stop]** button.

NOTE: Observe the crystallization process during the experiment and modify parameters such as temperature, crystallization solution and zoom, in the corresponding graphical interface of the supervision software. By using the software there is no need to remove the airtight cap or the flow cell during the experiment so the only variable will be the one that user changes through the software.

2.3. Large crystal growth

2.3.1. Add 15 µL of lysozyme with a concentration of 30 $mg \cdot mL^{-1}$ to the Dialysis chamber (**Figure 6A**).

NOTE: Prepare the protein sample as described in section 1.1.

2.3.2**.** Assemble the temperature-controlled flowing reservoir dialysis setup as described in section 2.1 and **Figure 6**.

2.3.3. Prepare the crystallization solution. Do not forget to filter all the stock solutions before

sample preparation with 0.22 µm filters. For this experiment, the crystallization solution contains 0.75 M NaCl and 0.1 M CH_3COONa pH 4. This can be added manually or by using the reservoir chambers and pumping system as described in sections 2.2.10 to 2.2.12.

2.3.4. Set the temperature to 295 K as explained in sections 2.2.3 and 2.2.4 and shown in Image 8. Under initial conditions, equilibrium between the dialysis chamber and the reservoir will be reached after approximately 90 minutes and the first visible nuclei will appear after 22 hours.

2.3.5. Allow crystals to grow until no more visible changes in the size of the crystals are observed (**Figure 9**, panel 1).

NOTE: In order to determine the nucleation time and to measure the variation in the size of the crystals, record images every 15 or 20 min, which is respectively 4 or 3 images per hour in the **NB Images** section. For in situ observation of protein denaturation, aggregation and precipitation or crystal dissolution or nucleation, typically between a few seconds to a few tens of minutes are required. However, for crystal growth this range is between a few minutes to a few hours.

2.3.6. After three days, lower the temperature to 291 K to restart crystal growth. Keep the temperature constant and let the crystal develop (**Figure 9**, panel 2). For this stage of the experiment, it will be sufficient to record images every 2 hours and check every 10 to 12 hours for any change in the size of the crystals. The experiment can be continued if no change in the size of the crystals is observed.

NOTE: Depending on the protein and precipitant concentrations in the crystallization solution and the volumes of protein used, the time needed to reach the equilibrium for each step may vary.

2.3.7. Decrease the temperature to 288 K to restart crystal growth. In the experimental condition of the case presented here, one day is enough to reach equilibrium (**Figure 9**, panel 3).

2.3.8. Check the size of the crystals and maintain a constant temperature as long as the crystal continues to grow.

2.3.9. After 4 days, decrease the temperature to 275 K in order to restart crystal growth (**Figure 9**, panel 4).

In the experimental conditions of the case presented, after around 10 days a crystal that is 500 µm in one dimension was be obtained (**Figure 9**).

2.4. Controlling the crystal size

2.4.1. Prepare a protein solution and the temperature-controlled flowing reservoir dialysis set

up as described in sections 2.3.1 and 2.3.2.

2.4.2. Prepare crystallization solutions with 0.9 M NaCl and 0.1 M CH_3COONa pH 4.

2.4.3. Set the temperature to 291 K and allow crystals to grow. Under initial conditions, the first nucleation event will start after around one hour and numerous crystals will grow in the dialysis chamber for three hours (**Figure 10**, steps: 1 and 2). Record images every 20 minutes to check the size of the crystals during the growth process.

NOTE: The optimization of crystallization conditions is crucial in controlling most of the final properties of generated crystals. The temperature and chemical composition of crystallization solutions can be changed to dissolve and re-grow crystals of uniform size. It should be also noted that a protein sample is not consumed in such an experiment, as conditions can be reversed to re-dissolve the sample as long as it is not denatured. When dissolving crystals by changing temperature and maintaining constant chemical composition, continue the experiment as follows:

2.4.4. Once crystals have grown at 291 K, these can be dissolved to re-grow fewer, larger crystals. Increase the temperature gradually over 20 min to reach 313 K. It takes around one hour to dissolve all the crystals inside the dialysis chamber (Figure 10, steps: 3-5). Record images every 5 to 15 minutes to monitor the dissolution process.

NOTE: Many proteins are sensitive to high temperatures. Be sure to work within the temperature range where the protein is stable to avoid any damage/denaturation. In addition to the protein solubility, temperature also affects the buffer solution. For example, the pH of the buffer can change with temperature, especially in the Tris buffer. I such a case, it is crucial to set the pH according to the temperature at which the experiment is performed[18]. It should also be noted that protein dissolution takes significantly less time (from a few minutes to a few hours) compared to protein crystal growth (from a few hours to a few days). In general, during the dissolution of the crystals, the temperature increases gradually and slowly (respecting the short total dissolution time), mainly in the case of partial dissolution of the crystals to avoid the increase of the crystal mosaicity. When the crystals are growing, the temperature can decrease quickly (in less than a minute) to the set temperature (respecting the long total growth time). Regular monitoring of the crystallization chamber by recording images is advisable to prevent damage to the protein and help define the optimal time for dissolution or growth of crystals for each protein studied.

2.4.5. After all crystals have dissolved set the temperature to 295 K to initiate a second nucleation event (Figure 10, steps: 6,7). Record images every 5 minutes to monitor the second nucleation process. In this step, the first nuclei appeared after around 18 minutes.

NOTE: At this temperature, the solution will be in the nucleation zone, in the vicinity of the metastable zone. As a result, only a few nuclei will appear in the crystallization chamber.

2.4.6. Continue the experiment repeating the optimization workflow described in section 2.3 for growing larger crystals. The total duration of the experiment represented in Figure 10 is only a few days.

NOTE: If during the nucleation phase the crystals appear at different times, crystals of different sizes are obtained in the crystallization chamber. In such a case, the increase in temperature (in the case of proteins with direct solubility) will result in quicker dissolution of the smaller crystals. Depending on the kinetic ripening effect, the extra protein (gained from dissolution) can then be used for the growth of the larger crystals.

When dissolving crystals at constant temperature by changing the chemical composition of the **crystallization solution,** continue the experiment as follows:

It is also possible to dissolve crystals grown previously by changing the chemical composition of the crystallization solution during the experiment to re-grow a population of uniformly sized crystals under new conditions.

2.4.7. Prepare the protein solution (2.3.1), the temperature-controlled flowing reservoir dialysis setup (2.1.) and the crystallization solution (2.4.2) as described above. Under initial conditions in the nucleation zone far from the metastable zone, numerous small crystals will appear in the crystallization chamber and begin to grow (Figure 11, steps: 1,2).

2.4.8. After three hours, when many medium-sized-crystals are visible in the crystallization chamber (**Figure 11**, steps: 3), decrease the NaCl concentration (0.9 M) gradually to reach zero. For this, prepare a new crystallization solution containing only buffer solution with 0.1 M CH_3COONa pH 4. Use the pumping system to exchange it with the crystallization solution in the reservoir chamber. Follow steps 2.2.10 to 2.2.12 for the preparation and injection of a new solution into the reservoir chamber. With this new solution, when solutions are exchanged the NaCl concentration decreases in the chamber until the final solution in the reservoir chamber contains no more than 0.1 M of CH_3COONa pH 4 and no NaCl. Record images every 10 minutes to record the dissolution process.

2.4.9. Allow the crystals to dissolve completely (**Figure 11**, steps: 4,5). Dissolution time is around two hours for this experiment. As previously mentioned, dissolution time is dependent on the protein system, crystallization conditions and dialysis chamber volume used. Regular observation of the crystallization chamber (see **Microscope** section) and recording images and notes during the experiment is essential.

2.4.10. When all crystals inside the chamber are dissolved (**Figure 11**, step 5), use the pumping system again to prepare a new crystallization solution by injecting NaCl at a lower concentration than previous (0.75 M NaCl in 0.1 M CH_3COONa pH 4).

2.4.11. Inject the new solution into the reservoir chamber (**Figure 11**, steps 6,7) and repeat the crystallization growth optimization workflow as described in section 2.3. A uniform population

of larger crystals will be generated. The results shown in **Figure 11** were obtained after a few days.

REPRESENTATIVE RESULTS:

In Sections 2.3 and 2.4, three examples of optimized crystal growth are presented, showing use of the instrument and an experimental design for growing large crystals. For this demonstration, we have used lysozyme as a model protein, although crystal growth experiments have been successfully performed with many other protein systems using this method (see above). By using and mastering the protocol presented here one can adapt it for other protein candidates.

In section 2.3 we demonstrated that established rational crystallization strategies could be beneficial in growing crystals with sufficient scattering volumes for neutron protein crystallography. Here, we demonstrate that the rational optimization strategies proposed also allow the generation of a uniform population of crystals of any specific size required for downstream structure determination approaches.

These two experiments are designed to emphasize the importance of phase diagrams in controlling crystal nucleation and growth. Here, control of the temperature and chemical composition of crystallization solutions in combination with monitoring the crystallization process in real time are used to study the qualitative phase diagram. Using this method, nucleation and crystal growth can be rationally optimized in a reversible manner. Use of such a serial approach also reduces the amount of protein and the time required to control the size and quality of the crystals.

In the dialysis method, a protein solution is separated from a crystallization solution by a semi-permeable membrane[6] (**Figure 5**). This dialysis membrane allows small molecules such as additives, buffer and ions to pass through the membrane but not macromolecules such as proteins[6, 20]. This feature allows the crystallization solution to be modified during the course of the experiment[6]. Exchange of the solution can be done manually, for example in microdialysis buttons, or in an automated manner using an instrument developed for this purpose, OptiCrys[8].

In the first set of experiments, microdialysis buttons were used for the crystallization of chicken egg-white lysozyme. Microdialysis buttons were immersed in crystallization solutions with different salt concentrations. In this simple crystallization grid experiment, the only variable is precipitant concentration whilst temperature is kept constant (293 K). As shown in **Figure 4**, slight variations in the salt concentration induce a change in the size and numbers of crystals observed, allowing investigation of the crystallization phase diagram. In **Figure 4** panel 1, the crystallization solution contains 0.7 M NaCl and a limited number of larger crystals have appeared in the buttons. By increasing salt concentration from 0.7 to 1.2 M, supersaturation increases and the solution in the nucleation zone moves away from the metastable zone (**Figure 4** panels 1 to 6). As a result, the number of crystals increases and their size decreases.

In the first experiment with a fully automated instrument enabling temperature-controlled

dialysis crystallization, OptiCrys (**Figure 9**), the crystal growth experiment was tailored to generate large crystal growth. The experiment was launched at an initial temperature of 295 K with a crystallization solution containing 0.75 M NaCl and 0.1 M Na acetate buffer pH 4. Under these experimental conditions, the crystallization solution reached the nucleation zone in the vicinity of the metastable zone of the phase diagram (**Figure 9**, arrow 1). As a result, only a few nuclei were generated during the first stage of the experiment. In order to grow selected crystals further (shown in **Figure 9**), the crystal growth optimization workflow was driven towards the metastable zone by varying temperature as soon as the crystal-solution equilibrium was reached.

Each time equilibrium between crystal and solution was reached, the temperature was lowered, first to 291 K, then to 288 K and finally to 275 K, to keep the crystallization solution in the metastable zone. The result of this experiment is a single large crystal suitable for both macromolecular X-ray and neutron crystallography.

For most proteins, the precise quantitative phase diagram (or just a qualitative diagram) has not yet been obtained due to the lack of experimental devices capable of accurately measuring protein concentration (or just of observing/detecting the crystallization process in real time) during crystallization experiments[18]. As a result, it is often not possible to design the experiment in such a way that crystallization begins in the optimal area of the phase diagram, in the vicinity of the metastable zone.

Therefore, a crystallization optimization study must take place before the experiment dedicated to the growth of a large volume crystal is undertaken. In this study, using temperature variations (at constant chemical composition) on the one hand and variations in chemical composition (at constant temperature) on the other hand, it is necessary to identify the metastable zone and to delineate the optimal conditions for starting a large crystal growth experiment.

To this end, two other experiments are presented which were tailored to demonstrate the reversibility of the temperature-controlled dialysis crystallization experiments with OptiCrys for nucleation, crystal growth, dissolution and re-growth. The crystal growth optimization workflow was controlled so that a uniform population of fewer, larger lysozyme crystals was grown, using variation of temperature or precipitant concentration.

In the second experiment with OptiCrys, the chemical composition of the crystallization solution was kept constant throughout the experiment (0.9 M NaCl in 0.1 M CH_3COONa pH 4) with variable temperature. The initial temperature was set at 291 K. The results of this experiment are summarized in **Figure 10**. Because of high supersaturation, a large number of small crystals appeared in the crystallization chamber (Figure 10, panels 1 and 2). In accordance with the concept of direct protein solubility, by gradually increasing the temperature to 313 K, all of the crystals were dissolved (**Figure 10** panels 3,4 and 5). Finally, by lowering the temperature to 295 K, the second nucleation was initiated in the vicinity of the metastable zone and allowed controlled formation of a lower number of nuclei. Further crystal growth resulted

in the uniform generation of a population of larger crystals (**Figure 1**0, panel 7).

As shown in **Figure 11**, variation of the chemical composition of the crystallization solution, at a constant temperature of 291 K, can likewise be used to obtain a uniform population of larger crystals. Similar to the previous experiment, the initial condition was 0.9 M NaCl in 0.1 M CH_3COONa pH 4. The NaCl concentration was then lowered gradually from 0.9 M to zero to dissolve the crystals (**Figure 11**, panels 4 and 5). At this point, NaCl was completely replaced by a buffer solution of 0.1 M CH_3COONa pH 4. Reducing the salt concentration keeps the solution in the undersaturated zone of the phase diagram, which leads to the dissolution of the crystals. Then, a new crystallization solution with lower ionic strength, at 0.75 M NaCl in 0.1 M CH_3COONa pH 4, was injected into the reservoir chamber. At this precipitant concentration, the first nuclei appeared (**Figure 11**, panel 6) after 90 minutes. The number of generated crystals was lower and the crystals reach a larger volume (**Figure 11**, panel 7) than before.

FIGURES AND TABLES:

Figure 1. Schematic phase diagram. Kinetic trajectories for four crystallization techniques are represented in a salting-out regime. Each method achieves nucleation and crystallization differently, visualized by a different kinetic pathway through the phase diagram to reach the nucleation and metastable zones. The solubility curve separates undersaturation and supersaturation regions. Supersaturation is divided into three zones: metastable, nucleation and precipitation. In the nucleation zone, spontaneous nucleation occurs while in metastable zone crystal growth takes place. This Figure is adapted from Junius *et al*.[8]

Figure 2. Schematic representation of the crystallization bench (OptiCrys). The LED light source is located on top of the temperature-controlled dialysis flow cell. An inverted microscope and the digital camera are shown at the top right of the image with the blue arrow. The red circle represents the location of the chiller tubing.

Figure 3. Schematic two-dimensional crystallization phase diagram as a function of temperature (A) and precipitant concentration (B). (A) In case of a protein with direct solubility, decreasing the temperature keeps the crystallization solution in the metastable zone. Temperature variation can be repeated several times to control the crystal growth process until crystals with the desired volume are obtained. (B) Changing the concentration of the precipitant solution can also be used to keep the crystallization solution in the metastable zone for growing crystals. This Figure is adapted from Junius *et al*.[8]

Figure 4. Crystals of lysozyme obtained using the dialysis method. This experiment was performed at a constant temperature in 0.1 M sodium acetate buffer pH 4. Increasing NaCl concentration from 0.7 M to 1.2 M increases the nucleation rate and results in a larger number of crystals.

Figure 5. **Overview of the protein crystallization process by the dialysis method.** (A) By adding the protein to the chamber of the dialysis button, (B) a dome shape is created on the top of the

chamber. C) An applicator is used to transfer the O-ring to the groove of the dialysis buttons in order to fix the dialysis membrane in place. (D) The dialysis button is ready for immersion in the reservoir solution. (E) Crystallization solution passes through the semipermeable membrane and crystals start to form inside the chamber.

Figure 6. Schematic view of the temperature-controlled flowing dialysis setup. (A) The protein sample is added to the dialysis chamber. (B) The dialysis membrane is fixed onto the overchamber with an O-ring by using an applicator. (C) The overchamber is turned and fixed onto the top of the dialysis chamber. White arrows indicate where a screw is placed on the overchamber. (D) The reservoir chamber is turned clockwise (E) and fixed on top of the overchamber. (F) The reservoir chamber is covered by an airtight cap with connectors to a pumping system and (G) the flow cell is placed in the brass support. This Figure is adapted from Junius *et al*.[8]

Figure 7. Preparation and injection of the crystallization solution in the reservoir by the fluidic system (A). Tubes containing salt and water are connected to the pressure/vacuum controller (B) and to the rotary valve (C). By using the pressure, pressure/vacuum controller creates a constant flow of the liquids from the tubes to the rotary valve. Each liquid passing through the flow meter (D) and the switch is injected into the mixing tube (F). Once all the liquids have been added to the mixing tube, the switch by some modifications injects the final solution from the mixing tube into the reservoir (G). The liquid flows through the system in the direction of the arrows in the diagram marked in ascending order (from 1 to 6).

Figure 8. Maintenance view of the supervision software. This view is used to control different parameters like temperature, light, crystallization solution and zoom.

Figure 9. The phase diagram as a function of the temperature. A single large lysozyme crystal is obtained by systematically changing the temperature from 295 K to 275 K. At each step, crystal growth is stopped upon reaching the solubility curve. Reducing the temperature by keeping the solution in the metastable zone restarts crystal growth. The images have different levels of magnification. This Figure is adapted from Junius *et al*.[8, 18]

Figure 10. Optimization of crystal growth at constant chemical composition using temperature control. Starting the nucleation process in the nucleation zone at 291 K, far from the metastable zone, results in the formation of numerous crystals. Increasing the temperature to 313 K then dissolves the crystals until no visible nuclei are seen in the dialysis chamber. Finally, decreasing the temperature to 295 K restarts the nucleation process for the second time leading to a limited number of larger crystals. This Figure is adapted from Junius *et al*.[8, 18]

Figure 11. Optimization of crystal growth at a constant temperature using variations in precipitant concentration. Decreasing the precipitant concentration from 0.9 M to 0 M dissolves the crystals obtained during the first nucleation event. The crystallization process is restarted by the injection of the same precipitant but at lower ionic strength, 0.75 M, which leads to the formation of a few larger crystals. This Figure is adapted from Junius *et al*.[8, 18]

DISCUSSION:

Different physical, chemical and biological variables affect protein crystallization by affecting protein solubility[21]. Among these variables, temperature and chemical composition of the crystallization solution are used here in combination with dialysis technique to improve and grow large high-quality crystals of biomacromolecules for neutron diffraction studies. By using knowledge of phase diagrams, crystallization is made more predictable. Although screening of different crystallization conditions in a serial approach is also possible, the main aim of using the rational approaches presented is to separate and control the kinetics of crystal nucleation and growth.

Similar to all crystallization studies, high quality pure and homogeneous protein samples, and dust-free crystallization solutions increase the success rate of the experiment. Filtration and centrifugation of solutions are essential steps in the described protocols. Knowing the physicochemical properties of the proteins studied such as the molecular weight (to choose the appropriate dialysis membrane), the isoelectric point, and the protein solubility are crucial for the design of an optimal crystal growth experiment. Also, consideration must be made for protein stability at different temperatures or with different chemicals to prevent sample loss and increase the likelihood of success. Considering the temperature range of OptiCrys (233.0–353.0 ± 0.1 K), a broad range of proteins can be crystallized using it. But it is worth to stress that proteins that are primarily thermo-stable, such as proteins from thermophilic sources, would benefit the most in temperature-controlled large-volume crystal growth experiments offered by this instrument.

Using a low-volume dialysis chamber (when using OptiCrys) or microdialysis buttons and screening several temperatures and crystallization conditions (e.g., grids of precipitant concentration or pH), it is possible to gain information on the location of the limit of the metastable zone (kinetic equilibrium between nucleation and metastable zones). This is invaluable when designing a successful crystal growth experiment especially for new protein candidates in crystallization. Without this information experiments can start from an area of the phase diagram with high supersaturation, too far from the limit of the metastable zone to easily control nucleation. Although dissolution of precipitate is possible by increasing temperature for example, for thermo-unstable proteins with direct solubility, maintaining the sample at high temperature for a long period can cause aggregation. Thus, the best strategy consists of using an initial condition with lower supersaturation located near the limit of metastability, where nucleation can be controlled. In line with this, crystallization prescreening decreases the chance of having aggregates in the dialysis chamber and increases the success rate.

After designing an experiment, preparing dialysis chambers (OptiCrys) or microdialysis buttons is another important step. Preventing air bubble formation in the dialysis chamber/button increases the chance of successful crystallization especially when small volumes are used. The presence of air bubbles in dialysis chamber may also change the kinetics of the crystallization process and reduce the reproducibility of the experiment (because the protein/solution contact

surface has been modified). Not only protein but also crystallization solution can affect the success of the experiment. Using new 50 mL tubes for the pumping system each time one wants to start a new experiment and washing tubing after each experiment decreases the chance of contamination and avoids the creation of salt crystals in the apparatus.

The use of microdialysis buttons is an alternative when OptiCrys is not available. The strategies for optimizing crystallization and monitoring crystal growth mentioned above, must be carried out manually. Typically this necessitates being outside a thermoregulated incubator, which can be problematic when temperature regulation is a critical step in the methodology described. This does not facilitate changing the chemical composition of the crystallization solutions, or monitoring crystal growth by imaging, so the crystal growth process cannot be controlled in real-time.

Knowledge of the phase diagram is the basis of using the crystallization bench, OptiCrys, to systematically grow large, high-quality crystals in an automated fashion. Control of physicochemical parameters like temperature, precipitant concentration, and pH during crystallization moves the protein-solution equilibrium in a well-defined kinetic trajectory across the phase diagram. This is complemented by the use of a dialysis membrane to adjust mass transport and create a controlled gradient in the crystallization chamber that affects the size and quality of the crystals. Therefore, using both thermodynamic data and kinetic trajectories is essential to control the crystallization process in order to grow high-quality crystals. Thanks to OptiCrys, systematic phase diagrams in a multidimensional space can be studied with a serial approach using significantly less material than before. To demonstrate this methodology, we provide here a case study with a model protein, chicken egg-white lysozyme. By using and mastering the protocol presented here one can adapt it for real protein systems[5, 14, 17, 18].

ACKNOWLEDGMENTS:

MBS acknowledges the support of the MI/CNRS under the contract Instrumentation at the limits 2014-2015. NJ acknowledges CEA's International Doctoral Research Program (Irtelis) for the PhD Fellowship. Authors acknowledge funding from the European Union's Horizon 2020 Research and Innovation Program under Marie Skłodowska-Curie grant agreement number 722687. Authors are also grateful to Dr Esko Oksanen (ESS, Lund) and Dr Jean-Luc Ferrer (IBS, Grenoble) for helpful conversations and insights. IBS acknowledges integration into the Interdisciplinary Research Institute of Grenoble (IRIG, CEA).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Blakeley, M.P., Langan, P., Niimura, N., Podjarny, A. Neutron crystallography: opportunities, challenges, and limitations. *Current Opinion in Structural Biology*. **18** (5), 593–600, doi: 10.1016/j.sbi.2008.06.009 (2008).
2. Snell, E.H., Van Der Woerd, M.J., Damon, M., Judge, R.A., Myles, D.A.A., Meilleur, F. Optimizing crystal volume for neutron diffraction: D-xylose isomerase. *European*

Biophysics Journal. **35** (7), 621–632, doi: 10.1007/s00249-006-0068-4 (2006).
3. Ng, J.D., Baird, J.K., Coates, L., Garcia-Ruiz, J.M., Hodge, T.A., Huang, S. Large-volume protein crystal growth for neutron macromolecular crystallography. *Acta Crystallographica Section F:Structural Biology Communications*. **71**, 358–370, doi: 10.1107/S2053230X15005348 (2015).
4. O'Dell, W.B., Bodenheimer, A.M., Meilleur, F. Neutron protein crystallography: A complementary tool for locating hydrogens in proteins. *Archives of Biochemistry and Biophysics*. **602**, 48–60, doi: 10.1016/j.abb.2015.11.033 (2016).
5. Oksanen, E., Blakeley, M.P., Bonneté, F., Dauvergne, M.T., Dauvergne, F., Budayova-Spano, M. Large crystal growth by thermal control allows combined X-ray and neutron crystallographic studies to elucidate the protonation states in Aspergillus flavus urate oxidase. *Journal of the Royal Society Interface*. **6** (SUPPL. 5), doi: 10.1098/rsif.2009.0162.focus (2009).
6. Krauss, I.R., Merlino, A., Vergara, A., Sica, F. An overview of biological macromolecule crystallization. *International Journal of Molecular Sciences*. **14** (6), 11643–11691, doi: 10.3390/ijms140611643 (2013).
7. Chayen, N.E. Comparative Studies of Protein Crystallization by Vapour-Diffusion and Microbatch Techniques. *REVIEW Acta Cryst* (1998).
8. Junius, N., Oksanen, E., Terrien, M., Berzin, C., Ferrer, J.L., Budayova-Spano, M. A crystallization apparatus for temperaturecontrolled flow-cell dialysis with real-time visualization. *Journal of Applied Crystallography*. **49**, 806–813, doi: 10.1107/S1600576716004635 (2016).
9. Salemme, F.R. A free interface diffusion technique for the crystallization of proteins for X-ray crystallography. *Archives of Biochemistry and Biophysics*. **151** (2), 533–539, doi: 10.1016/0003-9861(72)90530-9 (1972).
10. Chayen, N.E., Saridakis, E. Protein crystallization: From purified protein to diffraction-quality crystal. *Nature Methods*. **5** (2), 147–153, doi: 10.1038/nmeth.f.203 (2008).
11. Otálora, F., Gavira, J.A., Ng, J.D., García-Ruiz, J.M. Counterdiffusion methods applied to protein crystallization. *Progress in Biophysics and Molecular Biology*. **101** (1–3), 26–37, doi: 10.1016/j.pbiomolbio.2009.12.004 (2009).
12. Garciá-Ruiz, J.M. [8] Counterdiffusion Methods for Macromolecular Crystallization. *Methods in Enzymology*. **368**, 130–154, doi: 10.1016/S0076-6879(03)68008-0 (2003).
13. Budayova-Spano, M., Koruza, K., Fisher, Z. Large crystal growth for neutron protein crystallography. *Methods in Enzymology*. **634**, 21–46, doi: 10.1016/bs.mie.2019.11.015 (2020).
14. Budayova-Spano, M., Dauvergne, F., Audiffren, M., Bactivelane, T., Cusack, S. A methodology and an instrument for the temperature-controlled optimization of crystal growth. *Acta Crystallographica Section D: Biological Crystallography*. **63** (3), 339–347, doi: 10.1107/S0907444906054230 (2007).
15. Asherie, N. Protein crystallization and phase diagrams. *Methods*. **34** (3), 266–272, doi: 10.1016/j.ymeth.2004.03.028 (2004).
16. Astier, J.P., Veesler, S. Using temperature to crystallize proteins: A mini-review. *Crystal Growth and Design*. **8** (12), 4215–4219, doi: 10.1021/cg800665b (2008).
17. Budayova-Spano, M. *et al.* A preliminary neutron diffraction study of rasburicase, a

recombinant urate oxidase enzyme, complexed with 8-azaxanthin. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications*. **62** (3), 306–309, doi: 10.1107/S1744309106006439 (2006).
18. Junius, N., Vahdatahar, E., Oksanen, E., Ferrer, J.-L., Budayova-Spano, M. Optimization of crystallization of biological macromolecules using dialysis combined with temperature control. *Journal of Applied Crystallography*. **53** (3), doi: 10.1107/S1600576720003209 (2020).
19. Grimsley, G.R., Pace, C.N. Spectrophotometric Determination of Protein Concentration. *Current Protocols in Protein Science*. **33** (1), 3.1.1-3.1.9, doi: 10.1002/0471140864.ps0301s33 (2003).
20. McPherson, A., Gavira, J.A. Introduction to protein crystallization. *Acta Crystallographica Section F:Structural Biology Communications*. **70** (1), 2–20, doi: 10.1107/S2053230X13033141 (2014).
21. McPherson, A. Introduction to protein crystallization. *Methods*. **34** (3), 254–265, doi: 10.1016/j.ymeth.2004.03.019 (2004).

Figure 1

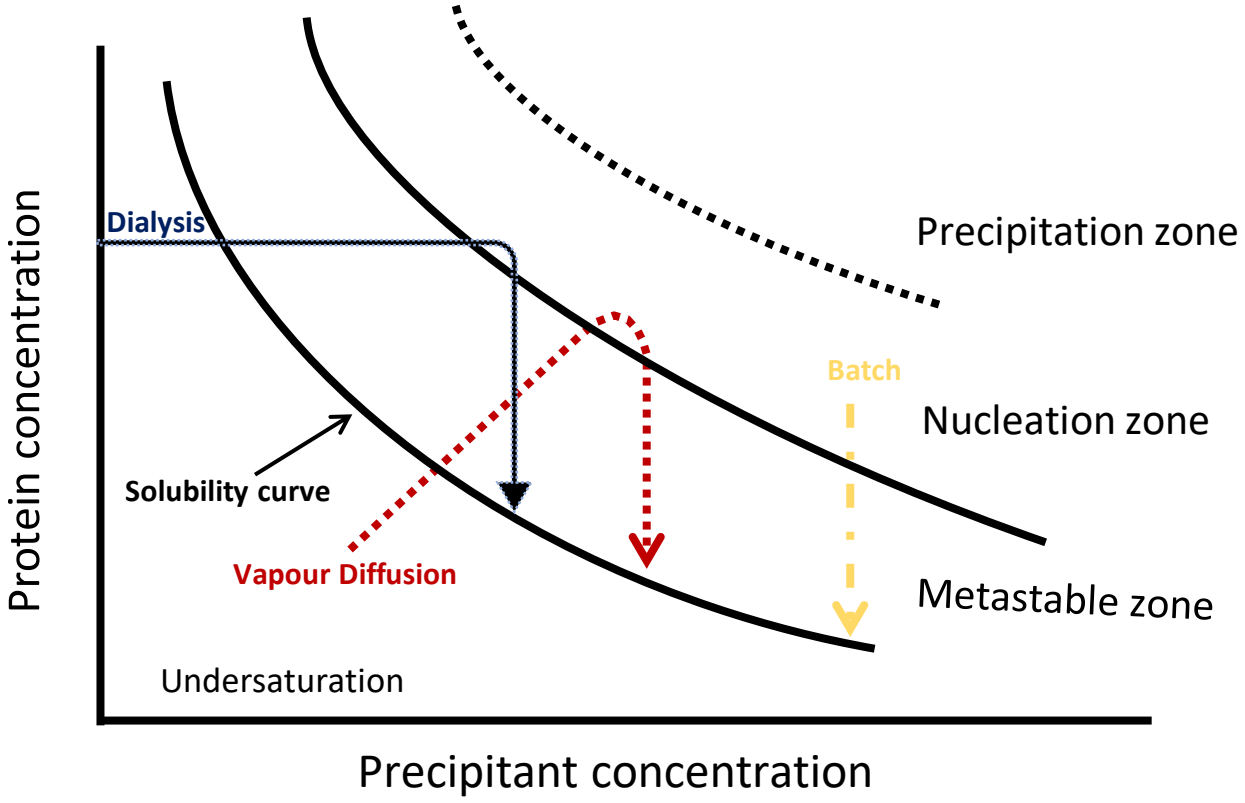


Figure 2

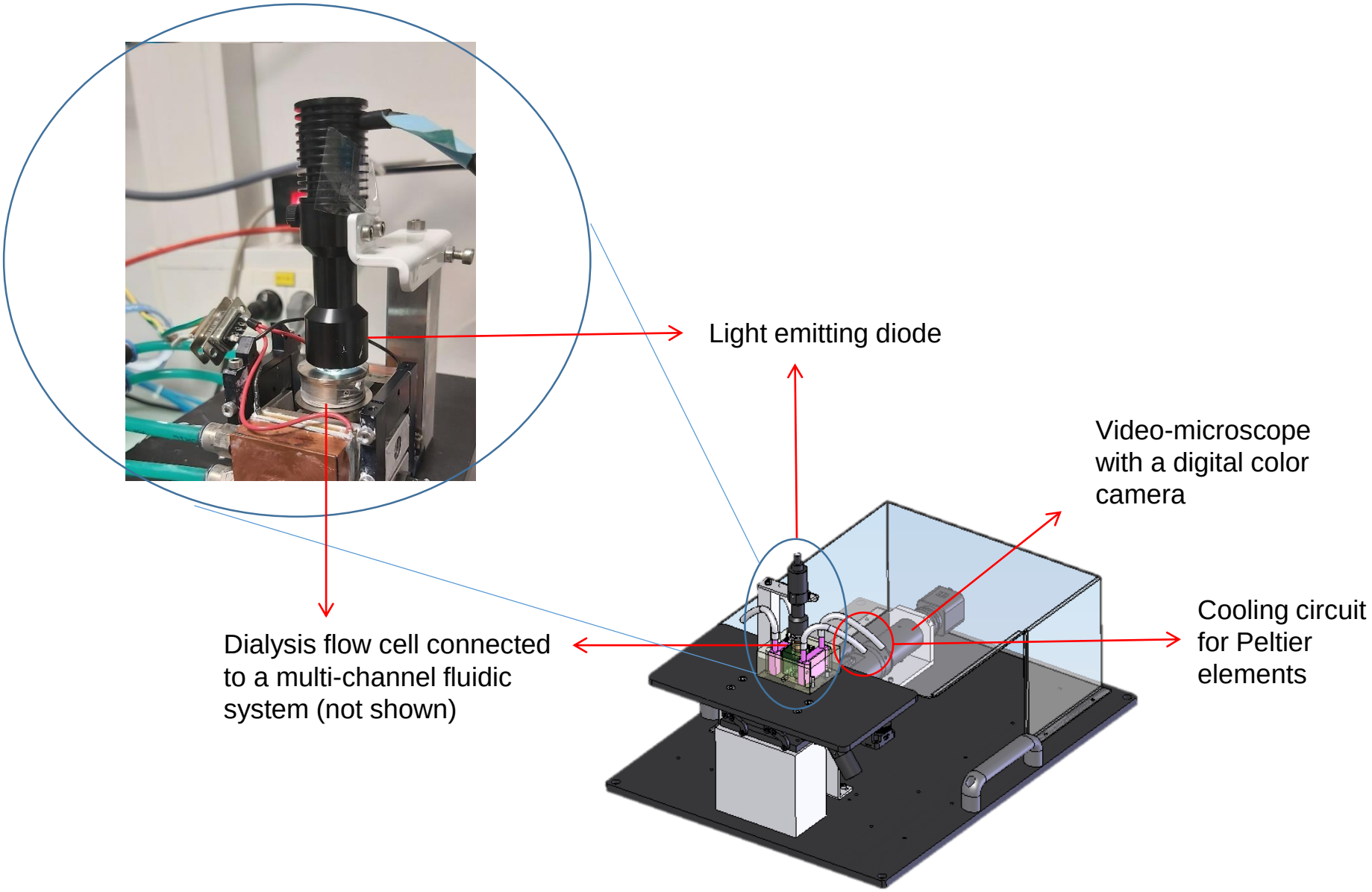


Figure 3

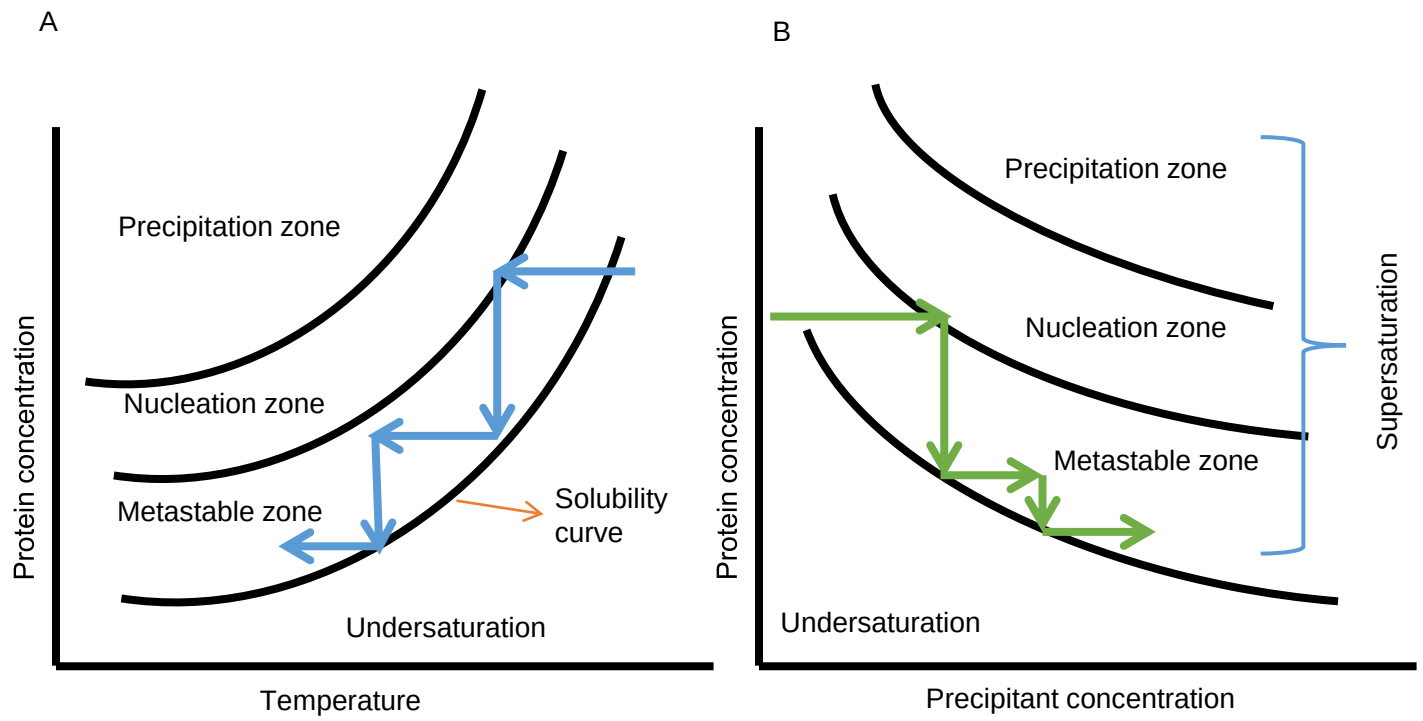
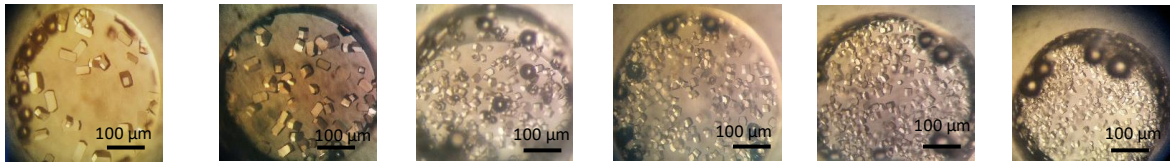


Figure 4



1	2	3	4	5	6
NaCl 0.7 M 0.1 M NaOAc pH 4	NaCl 0.8 M 0.1 M NaOAc pH 4	NaCl 0.9 M 0.1 M NaOAc pH 4	NaCl 1.0 M 0.1 M NaOAc pH 4	NaCl 1.1 M 0.1 M NaOAc pH 4	NaCl 1.2 M 0.1 M NaOAc pH 4

Figure 5

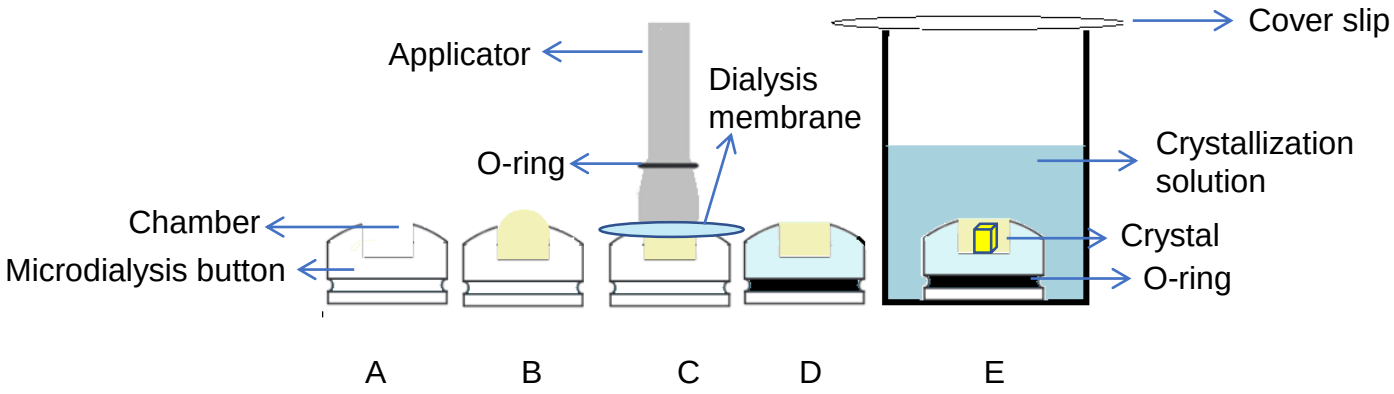


Figure 6

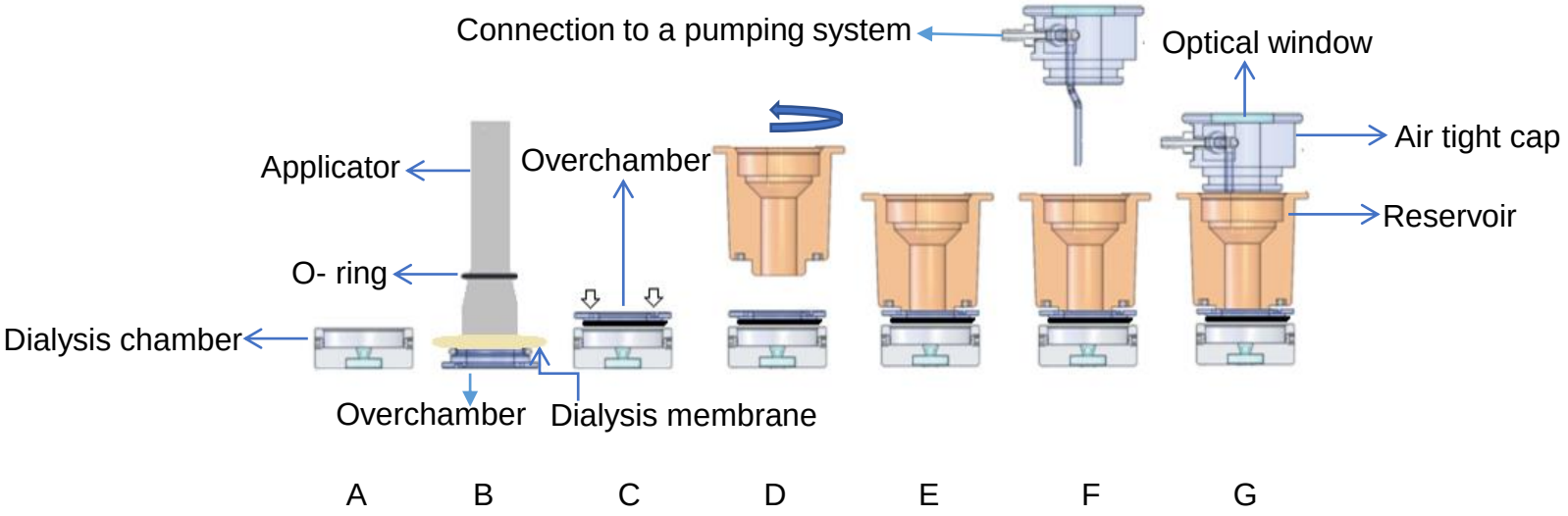


Figure 7

Click here to access/download;Figure;figure7.pdf

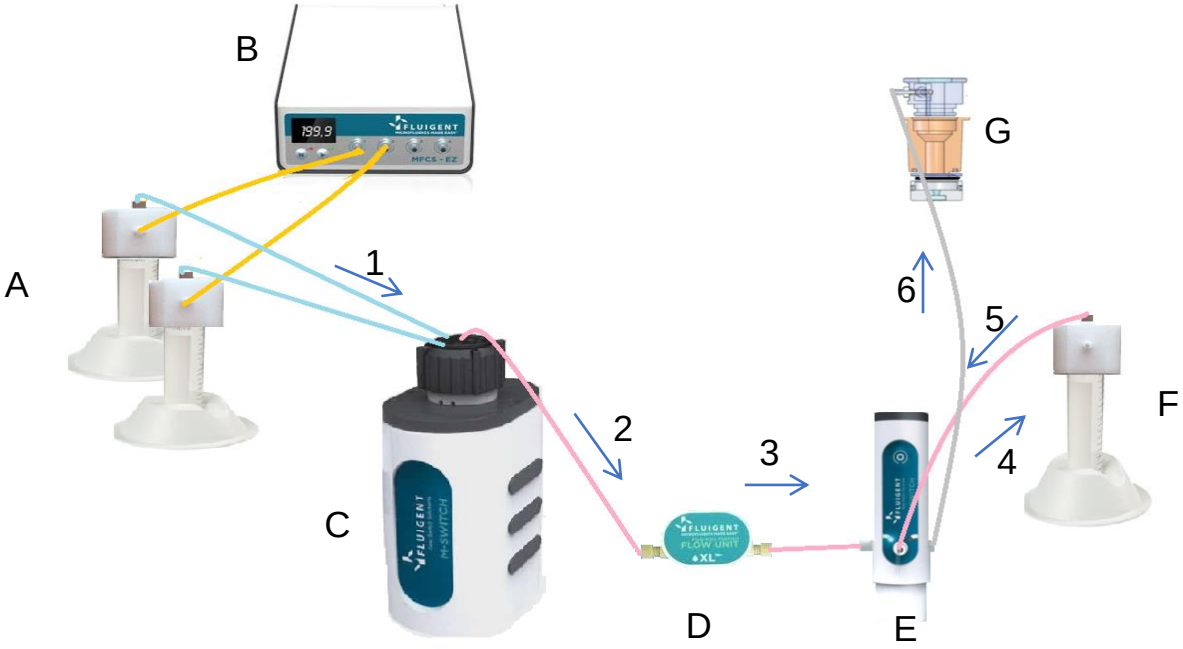


Figure 8

Click here to access/download;Figure;figure8.pdf

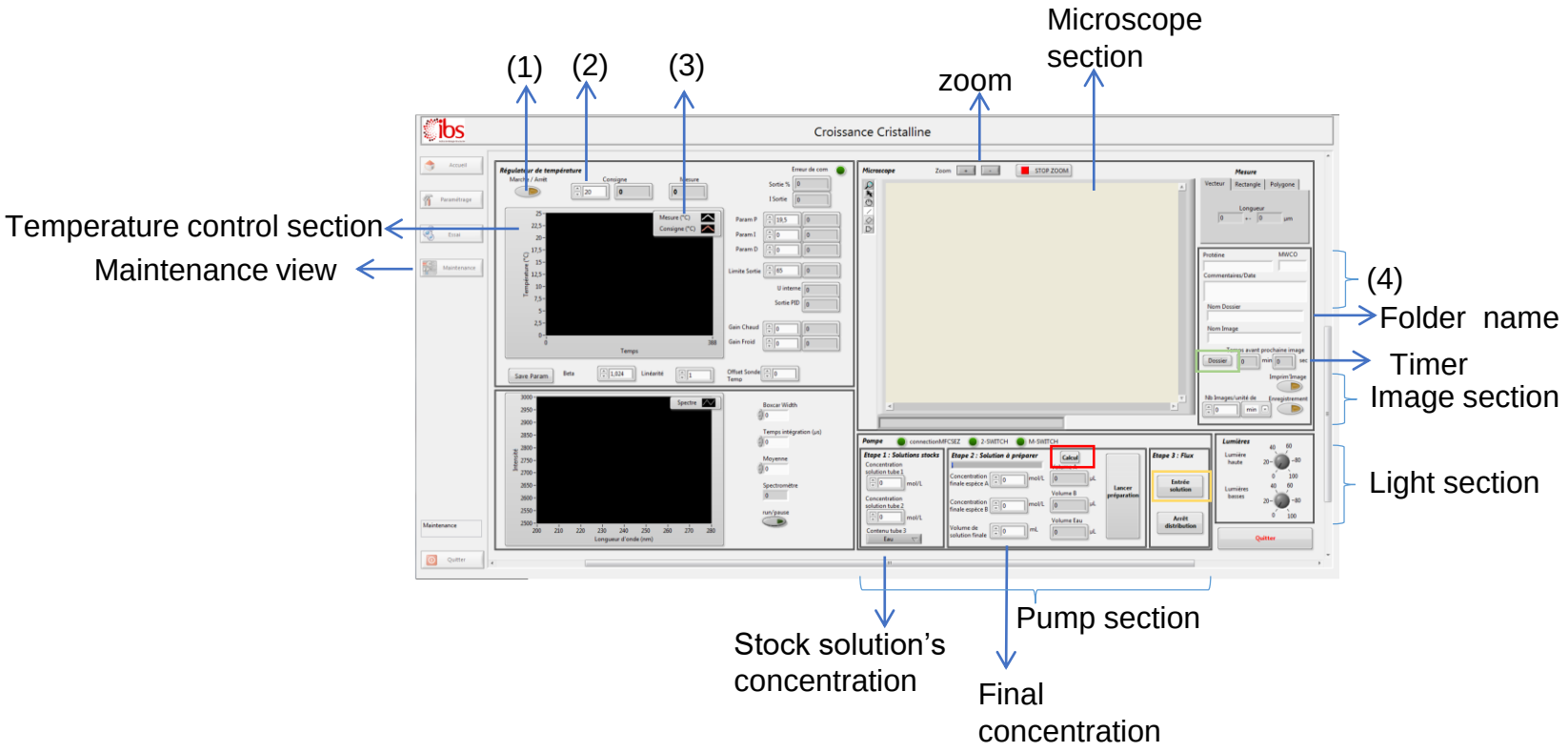
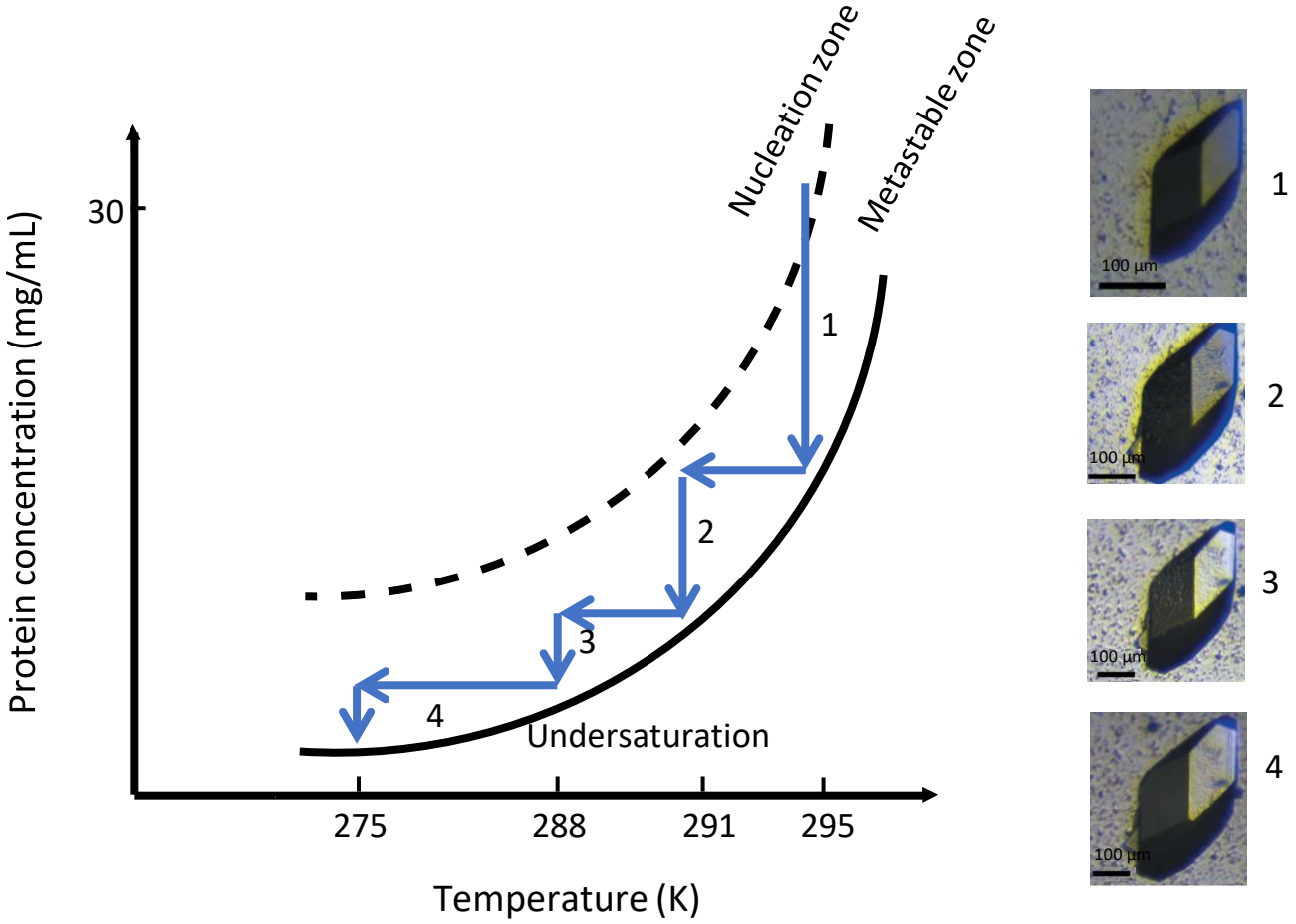


Figure 9



Zoom is not the same to fit the crystal size.

Figure 10

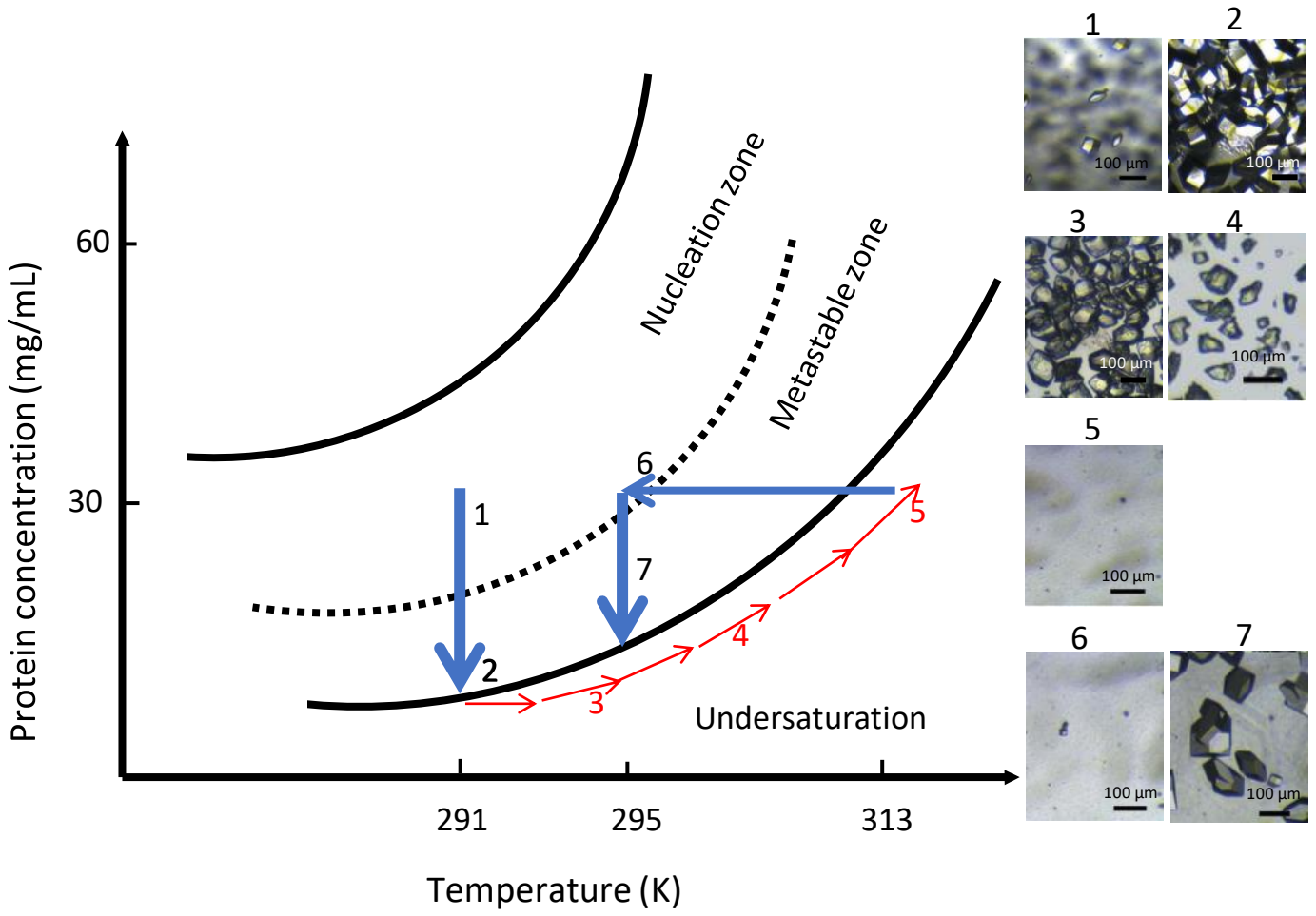
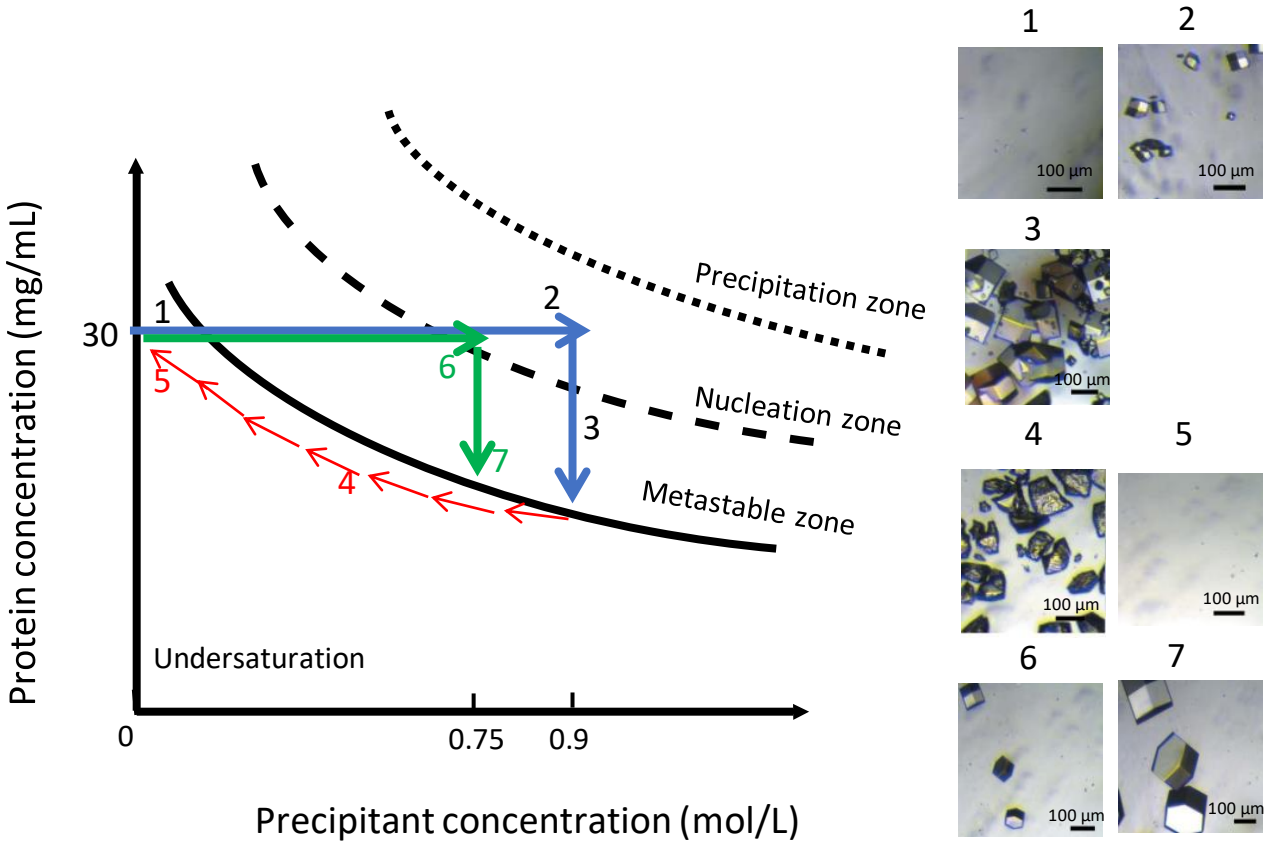


Figure 11



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
200 µl Dialysis Button	Hampton Research	HR3-330	Dialysis button
24 well plates	Jena Bioscience	CPL-132	Crystallization plate
2-Switch	FLUIGENT	2SW001	Switch
30 µl Dialysis Button	Hampton Research	HR3-324	Dialysis button
50 mL Corning Centrifuge tubes	Sigma-Aldrich	CLS430828-500EA	Centrifuge tubes
Acetic acid	Sigma-Aldrich	S2889	Chemical
Chicken Egg White Lysozyme	Sigma-Aldrich	L6876	Lyophilized protein powder
Dialysis Membrane Discs 6-8 kDa MWCO	Spectrum	132478	Dialysis membrane
Dialysis Membrane Tubing 6-8 kDa MWCO	Spectrum	132650T	Dialysis membrane
Microcentrifuge	Eppendorf	Minispin	Bench-top centrifuge
Flow Unit	FLUIGENT	FLU-XL	Flow meter
Flowboard	FLUIGENT	FLB	Flowboard
Microfluidic Flow Control System EZ	FLUIGENT	EZ-01000002	Pressure/vacuum controller
MilliporeSigma 0.22 µm syringe Filters	Millipore	GSWP04700	0.22 µm pore size filter
M-Switch	FLUIGENT	MSW002	Rotary valve
Opticrys	NatX-ray	PRT008	Crystallization bench
Siliconized circle cover slides	Hampton Research	HR3-231	Glass slides
Sodium Chloride ≥ 99%	Sigma-Aldrich	746398	Chemical
Switchboard	FLUIGENT	SWB002	Switchboard
Thermoregulated incubator	Memmert	IPP30	Thermoregulated incubator

Optimisation of Crystal Growth for Neutron Macromolecular Crystallography

Elham Vahdatahar[1], Niels Junius[1,2] and Monika Budayova-Spano[1]*

[1] Université Grenoble Alpes, CEA, CNRS, IBS, Grenoble, France
[2] Current address: ELVESYS SAS – 172 Rue de Charonne, 75011 Paris, France

Response to editorial, production and reviewers' comments:

Journal of Visualized Experiments – JoVE61685

Dear Editor,

We first thank all referees for their careful reading of our manuscript, their valuable remarks and for their relevant comments regarding our work. We are also pleased to read that most referees considered our work interesting and worth for publication. We list below our answers to all the points raised by editorial & production team and referees, as well as the changes done in our revised manuscript (highlighted below in blue and with the same color in the revised version of the manuscript). We now hope that this revised and improved version of the manuscript as well as the video will be suitable for publishing in Journal of Visualized Experiments as the Video Produced by Author.

Editorial and production team:

Comment 1 & 2: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. There are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 27-37, 271-274, 414-419, 774-778.

Response: Accordingly, special attention has been paid to spelling and grammar, which have been considerably improved throughout the manuscript (all changes are highlighted in blue). The main modified paragraphs of text are following:

27-37: The use of neutron macromolecular crystallography (NMX) is expanding rapidly with most structures determined in the last decade thanks to new NMX beamlines having been built and increased availability of structure refinement software. However, the neutron sources currently available for NMX are significantly weaker than equivalent sources for X-ray crystallography. Despite advances in this field, significantly larger crystals will always be required for neutron diffraction studies, particularly with the tendency to study ever-larger macromolecules and complexes. Further improvements in methods and instrumentation suited to growing larger crystals are therefore necessary for the use of NMX to expand.

96-110: The increase in concentration of protein and precipitant in the droplet leads to the supersaturation required for spontaneous nucleation followed by crystal growth at these nuclei.[6, 7] Although vapor diffusion is the most frequently used technique for growing crystals[4], the crystallization process cannot be precisely controlled[8]. In the free interface diffusion method, crystallization solution diffuses into a concentrated protein solution, very slowly directing the system towards supersaturation. This method can be considered a batch method with a slow mixing rate[6, 9–12]. In the batch method, the protein is rapidly mixed with a crystallization solution leading to rapid supersaturation and in turn uniform nucleation with many crystals [3, 7]. This method accounts for approximately one third of all structures currently deposited in the Protein Data Bank. The dialysis method is also used for growing high-quality and well-diffracting protein crystals. In the dialysis method, molecules of precipitant diffuse from a reservoir through a semi-permeable membrane into a separate chamber with the protein solution. The kinetics of equilibration are dependent on various factors, such as temperature, membrane pore size and the volume and concentration of protein samples and crystallization agents[6].

271-274: In the flow cell, the dialysis membrane is instead fixed to the overchamber allowing the mounting of crystals without its removal. Instead the overchamber can simple be unscrewed from the reservoir for this purpose.

414-419: Note: In order to determine the nucleation time and to measure the variation in the size of the crystals, record images every 15 or 20 min, which is respectively 4 or 3 images per hour in the **NB Images** section. For *in situ* observation of protein denaturation, aggregation and precipitation or crystal dissolution or nucleation, typically between a few seconds to a few tens of minutes are required. However, for crystal growth this range is between a few minutes to a few hours.

770-781: Knowledge of the phase diagram is the basis of using the crystallization bench, OptiCrys, to systematically grow large, high-quality crystals in an automated fashion. Control of physicochemical parameters like temperature, precipitant concentration, and pH during crystallization moves the protein-solution equilibrium in a well-defined kinetic trajectory across the phase diagram. This is complemented by the use of a dialysis membrane to adjust mass transport and create a controlled gradient in the crystallization chamber that affects the size and quality of the crystals. Systematic phase diagrams in a multidimensional space can be studied with a serial approach using significantly less material than before. To demonstrate this methodology, we provide here a case study with a model protein, chicken egg-white lysozyme.

Comment 3: Please obtain explicit copyright permission to reuse any figures from a previous publication.

Response: The images we have reused in this manuscript are from two of our articles published in the Journal of Applied Crystallography. Both articles are open access. According to the copyright policy that can be found here: https://journals.iucr.org/services/copyrightpolicy.html, we can reuse the materials that are open access without getting permission just by citing them.

Statement from the website of the Journal concerned: "Open access:
Authors of open-access articles will not be asked to transfer copyright to the IUCr, but will instead be asked to agree during article submission to an open-access licence. This licence is a Creative Commons

Comment 4: The video may benefit from a slightly higher export bitrate. Consider using higher quality settings when submitting the next revision.

Response: Video sequences related to sample preparation for both dialysis buttons and the crystallization bench were added to the final video. Higher quality settings were used to improve the overall quality such as:
Resolution: 3849×2160 Ultra HD, frame raye: 24, quality: best
Beat rate strategy: Constant bitrate with data rate 192Kb/s and Bit depth 16.

Comment 5: Improvement of Narration Performance & Coverage

Response: We recorded all the audios again by using a microphone to improve the narration quality.

Reviewer #1:

Comment 1: Although the authors have mentioned the limited availability for the rational optimization strategies with automated OptiCrys and proposed the alternative microdialysis buttons manual approach, it still remains challenging to real-time control and monitor with microdialysis buttons, especially difficult to conduct the experiment of changing in the chemical composition.

Response: Thank you for this comment. We agree with the reviewer's concern about the use of microdialysis buttons as an alternative to OptiCrys and have highlighted these concerns in the discussion section (second last paragraph) of the manuscript. On the other hand, although it is not possible to change the temperature and chemical composition, nor to control the crystallization process automatically and the experiment is not as accurate as with the OptiCrys, with the knowledge of the phase diagram, we are convinced that in the case of robust proteins (often used for neutron diffraction studies), it is still possible to grow large and high quality crystals in the microdialysis buttons. Keeping the temperature constant during the experiment (using the thermoregulated incubator) and changing the crystallization solution manually (as accurately as possible) makes the experiment reproducible in the case of a robust protein system such as lysozyme. Using the rational reasoning behind the design of such experiments (knowledge of the phase diagram) is the key to growing large, high-quality crystals.

Reviewer 1 has reported several typos and grammatical errors. We have fixed all of them and proofread the manuscript to eliminate all such errors.

Page 2: Reference 9 has been replaced with 8.

Page 4: µL was replaced with µm.

Page 4: pour was replaced by add / pipette.

Page 5: paper replaced by fibre-free paper.

We changed the sentence in section 2.1.6 to emphasize avoiding the bubble creation in the crystallization chamber: Fix the Reservoir in its position by gently screwing it on top of the overchamber, again being mindful to avoid trapping air bubbles.

Reviewer 1 has reported several modifications in the images to be made. We have modified some images according to the comments and added more details to others in order to make them easy to understand:

In figure 4 fonts changed according to other text.

In figure 6 we changed the colour of dialysis membrane for better visibility. And we labelled the pump connection part to the airtight cap.

Figure 8 is a software interface so we cannot remove some part of it. But as suggested we added more details in the image and we eliminated some of the numbers to make following the protocol easier.

Table 1 has been deleted and this information has been added to figure 5.

Reviewer #2:

Comment 1: Although lysozyme is typically employed as model protein for development purposes, the paper would reach higher impact if results for at least another protein would be presented. This could be introduced in the discussion section just as a figure.

Respond: We agree with the reviewer that demonstrating results with proteins other than lysozyme has a greater impact on our work. As mentioned and quoted in our manuscript, crystal growth experiments have been successfully performed with real protein systems using the described method and our results have been successfully published (open access) in the Journal of Applied Crystallography (2020). These examples are cited in the introduction of our manuscript. In the work presented in the JoVE it is not a question of telling the same story. The

reason for the current manuscript is to bring readers the detailed protocol of controlled crystal growth with a model protein available in most laboratories. By using and mastering the protocol explained here for lysozyme, users will be able to easily reproduce it and then adapt it for other protein candidates. To underline this aspect, a sentence has been added:

- in section 2. Crystal growth process using OptiCrys (last paragraph 2.2.12): "By using and mastering the protocol presented here one can adapt it for other protein candidates."
- In Discussion (last paragraph, last sentence): "By using and mastering the protocol presented here one can adapt it for real protein systems[5, 14, 17, 18]"

Comment 2: This reviewer recommend the publication after revision of the English language mainly in the introduction and figure legends

Response: We have proofread the paper to eliminate all such errors and fixed all of them.

Comment 3: Please add the maximum and minimum volumes accepted by the batch container used with the OptiCrys.

Answer: The allusion of the reviewer is probably made in relation to the volume of the reservoir of our temperature-controlled flowing dialysis setup because the crystallization technique used here is dialysis and not batch. Maximum volume of the reservoir is 1 mL. We have added this information/sentence in section 2. Crystal growth process using OptiCrys (paragraph 2.1.7).

Question: what is the dimension of the dialysis button used for the OptiCrys.

Response: As mentioned in the Introduction, OptiCrys has two dialysis chambers, the minimum volume is 15µL and the maximum is 250 µL. To emphasize we added this information also to the section 2. Crystal growth process using OptiCrys (paragraph 2.1.3): "OptiCrys has two dialysis chambers, the minimum volume is 15µL and the maximum volume is 250µL."

Suggestion: mention figure 1 in the second paragraph. It would help in understanding the point being made in this paragraph

Response: Thank you for this suggestion. In agreement, for the better understanding of the readers, we have added the mention of the figure 1 in the second paragraph.

Examples of language revision:

The first sentence of the introduction has been changed to: Understanding the structure-function relationship and physiological pathways mechanisms relies on the precise position of the hydrogen network and the knowing how charge is transferred within a protein.

In order to better describe Beer-Lambert's equation, the following sentences have been added to the Section 1. Dialysis method with microdialysis buttons (paragraph 1.1.3): According to the Beer-Lambert equation, there is a correlation between absorbance and concentration. As the sidechain of aromatic amino acids (tyrosine, tryptophan, and phenylalanine) has strong absorbance at 280 nm, with knowing or calculating the excitation coefficient, protein concentration can be calculated by measuring the absorbance at 280 nm.

As already mentioned, in line 212 (page 4) the term pour has been replaced by the term add / pipette.

Legend of figure 1 has been updated as suggested by the reviewer.

Answer to the comment on the video: We agree with the reviewer on some aspects of the video. The real-time video sequences of sample preparation for OptiCrys and the dialysis method with microdialysis buttons were added to the final video.

Reviewer #3:

Comment 1: The abstract start with "… a neutron data set…" in the first sentence as an example of the lack of precision in the text. Explain kinetic path, salting-out/-in, correlation between source/crystal size, exposure time…

Response: Thank you for this comment. We understand the reviewer's concern to clarify certain terms in the document. But the purpose of this manuscript is to describe the detailed protocol for the controlled growth of crystals with a model protein available in virtually any laboratory. As we have not discussed data collection, we have also not discussed exposure time and crystallography statistics. The reason we have used the term "neutron data set" in the first paragraph of the abstract is to stress that the volume of the crystals is the limiting factor for the collection of the neutron crystallography data set. It should also be noted that our summary and the text of our manuscript have been significantly modified and clarifications have been added. In addition, it should be mentioned that our manuscript is part of the collection of methods "Neutron Scattering in Biological Sciences: Techniques and Applications". As such, it does not focus on the method of neutron protein crystallography. A specialised paper by another author will be devoted to these aspects in the mentioned collection.

Finally, in this manuscript we have taken care to avoid repetition of explanations of phenomena (such as direct/inverse solubility, in/out salting, kinetic pathway, Ostwald ripening...etc) already described in detail in recently published papers (notably the 2020 paper published in J. Appl. Cryst.) For readers in need of such explanations, we have regularly added references to the above-mentioned work.

As an example of changes made (in the introduction): “The optimization of crystal growth with OptiCrys is based on temperature-precipitant concentration phase diagrams. In the case of proteins with solubility as a direct function of temperature it is possible to make use of the salting-out regime[18]. This is where increasing the ionic strength of the solution, which can be visualized using protein-precipitant phase diagrams, decreases the solubility of the protein. Likewise, proteins with inverse solubility can make use of the salting-in regime[18].”

Comment 2: The authors should discuss in much greater how to approach experiments with a new protein/project. What should be tried - what is a sensible workflow. The key limitation with the number of concurrent experiments possible when using the OptiCrys setup needs to be addressed for this to be a relevant publication.

Response: Thank you very much for the very helpful comment. In the section of the Representative results, we mentioned that the phase diagram has not yet been obtained for most proteins. Therefore, it is not possible to design the experiment in such a way that crystallization begins in the optimal part of the phase diagram, near the metastable zone. Then, further down in the text, we presented two experiments with OptiCrys for the proteins that we do not know their phase diagram (by using nucleation, crystal growth, dissolution, and regrowth). In order to clarify, we have added a paragraph between the first statement and the experiments:

Therefore, a crystallization optimization study must take place before the experiment dedicated to the growth of a large volume crystal is undertaken. In this study, using temperature variations (at constant chemical composition) on the one hand and variations in chemical composition (at constant temperature) on the other hand, it is necessary to identify the metastable zone and to delineate the optimal conditions for starting a large crystal growth experiment.

In addition, in the Discussion section we also added/accommodated an important related paragraph:

Using a low-volume dialysis chamber (when using OptiCrys) or microdialysis buttons and screening several temperatures and crystallization conditions (e.g. grids of precipitant concentration or pH), it is possible to gain information on the location of the limit of the metastable zone (kinetic equilibrium between nucleation and metastable zones). This is invaluable when designing a successful crystal growth experiment especially for new protein candidate. Without this information experiments can start from an area of the phase diagram with high supersaturation, too far from the limit of the metastable zone to easily control nucleation. Although dissolution of precipitate is possible by increasing temperature for example, for thermo-unstable proteins with direct solubility, maintaining the sample at high temperature for a long period can cause aggregation. Thus, the best strategy consists of using an initial condition with lower supersaturation located near the limit of metastability, where

nucleation can be controlled. In line with this, crystallization prescreening decreases the chance of having aggregates in the dialysis chamber and increases the success rate.

Finally, to answer the reviewer question: OptiCrys has only one temperature-controlled flowing setup (flow cell) so concurrent experiments are not possible.

To emphasize, a sentence to the last paragraph of the Discussion has been added: "Thanks to OptiCrys, systematic phase diagrams in a multidimensional space can be studied with a serial approach using significantly less material than before."

Comment 3: Figure 1: It is not clear which text relates to what. Use different font sizes or colours

Response: As a result, we changed the colours according to the arrows' colours.

Comment 4: Figure 2: Not really useful in current format - delete or add more detail

Response: More details were added to this image.

Comment 5: Figure 3: include headings for the two diagrams so it is clear what they illustrate.

Response: Thank you for the suggestion. Heading was added to this image.

Comment 6: Combine figures 4 and 6 into one figure

Response: Figure 4 illustrates the sample preparation for the dialysis method. In this method, the membrane is placed on top of the button and fixed by an elastic O-ring. However, as it is shown in figure 6 (flow cell of OptiCrys) the dialysis membrane is fixed on top of the overchamber. In the next step, overchamber is flipped and fixed on top of the dialysis chamber. For more clarification, we changed the colour of the dialysis membrane in image 6.

Comment 7: Figure 7: Not really useful in current format - delete or add more detail. As a minimum combine with figure 9

Response: Thank you for your comment. We deleted Figure 7 and added more details to figure 9.

Comment 8: Figure 8: delete 8A

Response: Accordingly, we deleted figure 8A.

Comment 9: Figures 10-12: Consider combining the figures. Be more precise with values on axes!

Response: Thank you for this helpful comment. Figures 10-12 (renumbered figures 9-11) describe various experimental results. Although it is a good idea to present them in a single figure to compare the results, the images will be small and the details will not be legible. The information will therefore be less clear and it will be difficult to understand the process. Therefore, the images have not been combined. However, the values for the protein concentration have been added on the axis as well as the direction of the axes.

Comment 10: Table 1 contains so little info that it could be written in a sentence instead.

Response: Information in table 1 was added to the image 5 and the table was deleted.

Comment 11: The Author produced video in its current format is not useful. It needs to include some actual live recordings from the lab of setting up OptiCrys experiments.

Response: We agree with the reviewer comment on the video. Therefore the real-time video of sample preparation for OptiCrys and the dialysis methods with microdialysis buttons were added to the final video.

Reviewer #4:

Comment 1: The authors describe a protocol to produce large volume and high-quality protein crystals. Nowhere in the study is crystal quality ever assessed or defined. Traditionally, the quality of protein crystals is determined by their diffraction properties, such as resolution limits (over noise ratio) or mosaicity. There is no demonstration here that shows the protein crystals obtained by the current protocol are suitable for X-ray or Neutron diffraction. Since the technique is for growing large volume crystals for neutron diffraction, having an example of a neutron diffraction image would be very helpful. Otherwise, it is recommended that obtaining protein crystal volume would be the focus of the method and define crystal quality as purely a visual attribute.

Response: Thanks for this comment. As you mentioned, in this paper we didn't talk about the quality of data we had obtained from these crystals. Focus of this manuscript is to develop a step-by-step protocol based on the knowledge of the phase diagram to control size and number of crystals generated for X-ray and Neutron crystallography. Therefore, we did not talk about data collection and crystallography statistics in this paper. For the crystal quality, we mentioned some examples and cited our previous works to this manuscript.

We modified the text of the introduction as follows:

Examples of protein crystals grown by temperature-controlled or temperature- and precipitant concentration-controlled crystallization as well as relative diffraction data obtained are available in the literature and PDB. Among them are human γ-crystallin E, PA-IIL lectin, yeast

inorganic pyrophosphatase, urate oxidase, human carbonic anhydrase II, YchB kinase, and lactate dehydrogenase [5, 14, 17, 18].

Comment 2: There are many cases that larger crystals can have more imperfection than those that are smaller for various reasons. The kinetics of the equilibration process can contribute greatly to the suitability of the protein crystals to X-ray and Neutron diffraction.

Response: We agree with this comment. As you mentioned in your comment, one reason for imperfection in larger crystals is the lack of controlling kinetics of the crystal growth. Growing large and high-quality crystals is challenging but OptiCrys uses both thermodynamic data and kinetic trajectories to control the crystallization process in order to grow high-quality crystals. Accordingly, we highlighted these aspects in the text of manuscript, mainly in the last paragraph of the discussion section:

Knowledge of the phase diagram is the basis of using the crystallization bench, OptiCrys, to systematically grow large, high-quality crystals in an automated fashion. Control of physicochemical parameters like temperature, precipitant concentration, and pH during crystallization moves the protein-solution equilibrium in a well-defined kinetic trajectory across the phase diagram. This is complemented by the use of a dialysis membrane to adjust mass transport and create a controlled gradient in the crystallization chamber that affects the size and quality of the crystals. Therefore, using both thermodynamic data and kinetic trajectories is essential to control the crystallization process in order to grow high-quality crystals. Thanks to OptiCrys, systematic phase diagrams in a multidimensional space can be studied with a serial approach using significantly less material than before. To demonstrate this methodology, we provide here a case study with a model protein, chicken egg-white lysozyme. By using and mastering the protocol presented here one can adapt it for real protein systems[5, 14, 17, 18].

Comment 3: The study says that the OptiCrys instrument can control temperature accurately and allow crystallization solutions to be controlled and changed in an automated manner. However, the automation component is not clear. It is not known whether the authors intend to say that the software Croissance cristalline can observe the crystal growth and change temperature or crystallization without human intervention or does it require the user to observe manually and change the crystallization parameters accordingly? The OptiCrys is presented as an automated instrument, but the degree of automation is not defined. Clarification on this would be very helpful.

Response: Thank you for your comment. User should observe the crystallization progress and change the parameter like temperature, crystallization solution, and zoom on the software. However, all the changing process will be done during the experiment without transferring the flow cell or removing the airtight cap, so the only variable will be the one that user changes through the software.

To clarify the degree of automation, we have added an additional paragraph to the section Software (paragraph 2.2.12):

Note: Observe the crystallisation process during the experiment and modify parameters such as temperature, crystallisation solution and zoom, in the corresponding graphical interface of the supervision software. By using the software there is no need to remove the airtight cap or the flow cell during the experiment so the only variable will be the one that user changes through the software.

Comment 4: Eventually, the software may be more universally used if the panel tabs are written in English- even though I personally think it sounds nicer in French.

Response: The prototype that has been developed in our lab has the panels in French but we plan to change them to English in the near future. In the commercial version of the instrument all the panels are in English.

Comment 5: Temperature changes can affect pH changes depending on the buffer used. For example, protein crystallization conditions that utilizes Tris buffer will have significant changes in pH as a function of temperature. It would be helpful for the authors to list or recommend buffers that can be used for the OptiCrys.

Response: As you mentioned, temperature can affect slightly the pH of the buffer especially at higher temperatures. For example in the case of water from 0 °C to 100°C (273 K to 373K), pH changes from 7.47 to 6.14. In our experiments, we use 35°C/308K or 40°C/ 313K just for dissolving the crystals and the crystallization process most of the time starts below 25°C/ 298K. But as we mentioned before, knowing the physicochemical properties of the protein and screening of the pH, temperature and crystallization buffer are useful to have a successful crystallization.

In general, we use the same buffers for crystallisation with OptiCrys as those used by other conventional crystallisation techniques. However, for proteins that are extremely sensitive to pH variation, we recommend using OptiCrys, taking advantage of the variation in chemical composition of the crystallisation media at constant temperature.

To point out this aspect in our manuscript, we added following sentences to the paragraph 2.4.4 (Controlling crystal size): "In addition to the protein solubility, temperature also affects the buffer solution. For example, the pH of the buffer can change with temperature, especially in the Tris buffer. I such a case, it is crucial to set the pH according to the temperature at which the experiment is performed[18]."

Comment 6: The technique presented here focuses on the crystallization process from mostly a thermodynamic approach. Kinetic effects significantly contribute to the growth and quality of protein crystals. It would be helpful to see the effects of the crystallization process with information on the rate of temperature changes. The current report only indicates the temperature adjustments but there is no information on how fast or slow the temperature jumps may be.

Response: The temperature changes happen in real-time unless we mention it in the protocol. For example in paragraph 2.4.4 for dissolving the crystals, we increased the temperature gradually over 20 min to reach 313 K. We also showed it with several arrows in figure 11. Apart from the sample that we presented here, changing the temperature greatly depends on the protein that is used. But generally for dissolving the crystals, temperature increases gradually and slowly, mainly in case of partial dissolution of crystals to avoid the increase of the crystal mosaicity. When growing the crystals, the temperature can decrease quite rapidly (in less then 1 minute) to the desired value. What is important is the monitoring of the crystallization chamber.

Accordingly to the comment, the notes in sections 2.3.5 and 2.4.4 of the manuscript have been adapted:

Note (2.3.5): In order to determine the nucleation time and to measure the variation in the size of the crystals, record images every 15 or 20 min, which is respectively 4 or 3 images per hour. For *in situ* observation of protein denaturation, aggregation and precipitation or crystal dissolution or nucleation, typically between a few seconds to a few tens of minutes are required. However, for crystal growth this range is between a few minutes to a few hours.

Note (2.4.4): It should also be noted that protein dissolution takes significantly less time (from a few minutes to a few hours) compared to protein crystal growth (from a few hours to a few days). In general, during the dissolution of the crystals, the temperature increases gradually and slowly (respecting the short total dissolution time), mainly in the case of partial dissolution of the crystals to avoid the increase of the crystal mosaicity. When the crystals are growing, the temperature can decrease quickly (in less than a minute) to the set temperature (respecting the long total growth time). Regular monitoring of the crystallization chamber by recording images is advisable to prevent damage to the protein and help define the optimal time for dissolution or growth of crystals for each protein studied.

Comment 7: It is important to stress that proteins that are primarily thermo-stable would be those that would benefit the most in large-volume crystal growth. Proteins from thermophilic sources would be ideal for this technique.

Response: Temperature range in OptiCrys is 233.0–353.0 $\pm$ 0.1 K. So a broad range of protein including proteins from thermophilic sources can be crystallized, using this instrument. Thank

you for your comment. For more clarity, the temperature range of the OptiCrys was added to the manuscript in Section2.2.3: Note: Temperature range in OptiCrys is 233.0–353.0 ± 0.1 K.

Accordingly, following sentences have been added to the Discussion section (in the second paragraph):

Considering the temperature range of OptiCrys (233.0–353.0 ± 0.1 K), a broad range of proteins can be crystallized using it. But it is worth to stress that proteins that are primarily thermo-stable, such as proteins from thermophilic sources, would benefit the most in temperature-controlled large-volume crystal growth experiments offered by this instrument.

Comment 8. The rational strategies using dialysis buttons or with the OptiCrys do not address ripening effects with temperature or even Oswald ripening. Since the dissolution and recrystallization protein crystals are the main processes in the reported protocol to obtain large-volume crystals, I think it is worthy of including ripening effects as a strategic discussion.

Response: Thank you for pointing this out. In the kinetic ripening, changing the temperature induce dissolution of the smaller crystals and consequently leads to the growth of larger crystals. In the method presented here, we completely dissolve all the crystals to start the crystallization in the nucleation zone near the metastable zone. We repeat this process until we obtain a small number of nuclei.

In order to take into account this comment we added a note to the section 2.4.6:

Note: If during the nucleation phase the crystals appear at different times, crystals of different sizes are obtained in the crystallization chamber. In such a case, the increase in temperature (in the case of proteins with direct solubility) will result in quicker dissolution of the smaller crystals. Depending on the kinetic ripening effect, the extra protein (gained from dissolution) can then be used for the growth of the larger crystals.

Comment 9. How much protein material is needed for a single optimization experiment using the method reported? It would be interesting to know if the current protocol can reduce the amount of protein expenditure compared to other methods for obtaining large-volume crystals. If it is a significant reduction, it can certainly be a bragging point.

Response: The minimum protein for an experiment is 15µl. Since the process is reversible, the sample can be used as long as it is not denatured. But the final volume of the protein needed depends on the protein nature and how the crystallization process proceeds.

In agreement, we added a following sentence to the Introduction section (fifth paragraph): "As a result, using this method minimizes the amount of protein material used."

Comment 10: There are minor grammatical problems throughout the paper that obscure important points. Carefully reviewing sentence constructs is recommended.

Response: Thank you for your comment. We have fixed all of them and proofread the manuscript to eliminate all such errors.

Comment 11: Figures are introduced out of order and that makes it hard to read or follow. For example, it seems that figure 6 and 8 are introduced in the text before 5 and 7 respectively.

Response: Thank you for pointing this out. We deleted some of the figures. New figures' numbers are in the correct order.

Comment 12: It would be helpful to have the number (100um in this case) written on top of the scale bar.

Response: Thank you for pointing this out. We added 100 µm to the scale bars.