Journal of Visualized Experiments

Microcrystallography of protein crystals and in cellulo diffraction

--Manuscript Draft--

Manuscript Number:	JoVE55793R1
Full Title:	Microcrystallography of protein crystals and in cellulo diffraction
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Microcrystallography; microcrystals; X-ray diffraction; in cellulo diffraction; polyhedra; in vivo crystals
Manuscript Classifications:	5.5.196.309: Crystallography; 8.1.158.201: Biochemistry
Corresponding Author:	Fasseli Coulibaly Monash University Clayton, Victoria AUSTRALIA
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	fasseli.coulibaly@monash.edu
Corresponding Author's Institution:	Monash University
Corresponding Author's Secondary Institution:	
First Author:	Fasseli Coulibaly
First Author Secondary Information:	
Other Authors:	Marion Boudes
	Damia Garriga
Order of Authors Secondary Information:	
Abstract:	The advent of high-quality microfocus beamlines at many synchrotron facilities has permitted the routine analysis of crystals smaller than 10 µm in their largest dimension, which used to represent a challenge. We present two alternative workflows for the structure determination of protein microcrystals by X-ray crystallography with a particular focus on crystals grown in vivo. The microcrystals are either extracted from cells by sonication and purified by differential centrifugation, or analyzed in cellulo after cell sorting by flow cytometry of crystal-containing cells. Optionally, purified crystals or crystal-containing cells are soaked in heavy atom solutions for experimental phasing. These samples are then prepared for diffraction experiments in a similar way by application onto a micromesh support and flash cooling in liquid nitrogen. We briefly describe and compare serial diffraction experiments of isolated microcrystals and crystal-containing cells using a microfocus synchrotron beamline to produce datasets suitable for phasing, model building and refinement. These workflows are exemplified with crystals of the Bombyx mori cypovirus 1 (BmCPV1) polyhedrin produced by infection of insect cells with a recombinant baculovirus. In this case study, in cellulo analysis is more efficient than analysis of purified crystals and yields a structure in ~8 days from expression to refinement.
Author Comments:	Filming would involve three locations: the structural virology laboratory (19 innovation walk, Clayton campus), the FACS platform (15 innovation walk, Clayton campus) and the Australian Synchrotron (800 Blackburn Rd, Clayton). They are all in close proximity.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	02-15-2017

TITLE:
Microcrystallography of protein crystals and *in cellulo* diffraction

AUTHORS:
Marion Boudes*
Infection and Immunity Program
Monash Biomedicine Discovery Institute and Department of Biochemistry and Molecular Biology
Monash University
Melbourne, VIC, Australia
marion.boudes@monash.edu

Damià Garriga*
Infection and Immunity Program
Monash Biomedicine Discovery Institute and Department of Biochemistry and Molecular Biology
Monash University
Melbourne, VIC, Australia
damia.garriga@monash.edu

Fasséli Coulibaly
Infection and Immunity Program
Monash Biomedicine Discovery Institute and Department of Biochemistry and Molecular Biology
Monash University
Melbourne, VIC, Australia
fasseli.coulibaly@monash.edu

*Equal contribution

CORRESPONDING AUTHOR:
Fasséli Coulibaly (fasseli.coulibaly@monash.edu)

KEYWORDS:
Microcrystallography, microcrystals, X-ray diffraction, *in cellulo* diffraction, polyhedra, *in vivo* crystals

SHORT ABSTRACT:
A protocol is presented for X-ray crystallography using protein microcrystals. Two examples analyzing *in vivo*-grown microcrystals after purification or *in cellulo* are compared.

LONG ABSTRACT:
The advent of high-quality microfocus beamlines at many synchrotron facilities has permitted the routine analysis of crystals smaller than 10 µm in their largest dimension, which used to

represent a challenge. We present two alternative workflows for the structure determination of protein microcrystals by X-ray crystallography with a particular focus on crystals grown *in vivo*. The microcrystals are either extracted from cells by sonication and purified by differential centrifugation, or analyzed *in cellulo* after cell sorting by flow cytometry of crystal-containing cells. Optionally, purified crystals or crystal-containing cells are soaked in heavy atom solutions for experimental phasing. These samples are then prepared for diffraction experiments in a similar way by application onto a micromesh support and flash cooling in liquid nitrogen. We briefly describe and compare serial diffraction experiments of isolated microcrystals and crystal-containing cells using a microfocus synchrotron beamline to produce datasets suitable for phasing, model building and refinement.

These workflows are exemplified with crystals of the *Bombyx mori* cypovirus 1 (BmCPV1) polyhedrin produced by infection of insect cells with a recombinant baculovirus. In this case study, *in cellulo* analysis is more efficient than analysis of purified crystals and yields a structure in ~8 days from expression to refinement.

INTRODUCTION:

The use of X-ray crystallography for the determination of high-resolution structures of biological macromolecules has experienced a steady progression over the last two decades. The growing uptake of X-ray crystallography by non-expert researchers exemplifies the democratization of this approach in many fields of life sciences[1].

Historically, crystals with dimensions below ~10 µm have been considered as challenging, if not unusable, for structure determination. The increasing availability of dedicated microfocus beamlines at synchrotron radiation sources worldwide and technological advances, such as the development of tools to manipulate microcrystals, have removed much of these barriers that stymied the wide use of X-ray microcrystallography. Advances in serial X-ray microcrystallography[2,3] and micro electron diffraction[4] have shown that the use of micro- and nanocrystals for structure determination is not only feasible but also sometimes preferable to the use of large crystals[5-7].

These advances were first applied to the study of peptides[8] and natural crystals produced by insect viruses[9,10]. They are now used for a diverse range of biological macromolecules including the most difficult systems such as membrane proteins and large complexes[11]. To facilitate the analysis of these microcrystals, they have been analyzed *in meso*, particularly membrane proteins[12] and in microfluidics chips[13].

The availability of these novel microcrystallography methodologies has raised the possibility of using *in vivo* crystallization as a new route for structural biology[14-16] offering an alternative to classical *in vitro* crystallogenesis. Unfortunately, even when *in vivo* crystals can be produced, several obstacles remain such as the degradation or loss of ligands during the purification from cells, difficulty in the manipulation and visualization of the crystals at the synchrotron beamline and tedious X-ray diffraction experiments. As an alternative crystals have also been analyzed directly within the cell without any purification step[17-19]. A comparative analysis suggests that

such *in cellulo* approaches may be more efficient than the analysis of purified crystals and yield data of higher resolution[20].

This protocol is intended to assist researchers new to protein microcrystallography. It provides methodologies focusing on sample preparation and manipulation for X-ray diffraction experiments at a synchrotron beamline. Two options are proposed using isolated crystals for classical microcrystallography or crystal-containing cells sorted by flow cytometry for *in cellulo* analysis (Figure 1).

PROTOCOL:

Note: *In vivo* crystallization has been reported in many organisms including in bacteria, yeast, plants, insects and mammals [reviewed in [21]]. Crystallization of recombinant proteins has also been achieved in the laboratory using transient transfection of mammalian cells and baculovirus infection of insect cells. The following protocol has been developed using the *Bombyx mori* cypovirus 1 (BmCPV1) polyhedrin gene cloned in a recombinant baculovirus under the baculovirus polyhedrin promoter, generated as per instructions in reference [22]. Thus, although this protocol may be adapted to other cell types (e.g. mammalian cells), we describe here the procedures for insect cells. The protocol assumes that over-expression of the protein of interest has already been achieved. Methods for the production of a recombinant baculovirus and its use for expression of the protein of interest are available in references [23-27].

1. Identification of crystal-containing cells

1.1. If cells are grown in monolayer, directly inspect them in the flask. If cells are grown in liquid culture, use the following protocol.

1.1.1. Using a sterile serological pipette, transfer 100 µL of cells into a microcentrifuge tube. Take care to ensure that the sterility of the main culture is maintained; handle the cells in a Class II biosafety cabinet and follow standard aseptic techniques.

1.1.2. Pipet 5 µL of cells from the microcentrifuge tube onto a glass slide.

1.1.3. Carefully place a glass coverslip onto the liquid, avoiding the formation of air bubbles. If the slide cannot be visualized within 15 min, seal the coverslip with vacuum grease to avoid evaporation.

1.2. Image the flask or slide with an inverted microscope. If available, use phase contrast and/or differential interference contrast microscopy (DIC). Both techniques enhance the contrast in transparent samples and emphasize lines and edges, thus facilitating the identification of *in vivo* crystals.

1.3. Carefully examine the cells. Start at 200X magnification and zoom in at maximal magnification when detecting a potential crystal. The presence of crystals may be suggested indirectly by changes in the cell morphology (Figure 2). Look for sharp edges and changes in

refractivity (if using phase contrast or DIC). Known *in vivo* crystals adopt different shapes including rods, stacks of needles, cubes, pyramids and polyhedra (see Figure 2 and Table 1 for some examples).

Note: The proportion of cells containing crystals will be different in each case and can even vary between preparations but in general one should not expect to find crystals in all cells. Similarly, the number of crystals per cell is also variable (see Table 1), and more than one crystal may be found in one cell. These factors need to be optimized where possible by adjusting the multiplicity of infection, altering the length of expression and varying the protein construct. On one hand, conditions of protein expression and cell growth that maximize the number of crystal-containing cells will improve productivity (e.g. higher multiplicity of infection and late harvesting of cell culture). On the other hand, conditions where cells contain a single crystal facilitate data collection (e.g. low multiplicity of infection). Given the enrichment brought by flow sorting described in step 2.2, we recommend aiming for larger crystals (e.g. a single crystal per cell) even if the proportion of crystal-containing cells is low.

1.4. If crystals are identified, record their position (when imaging monolayer in a flask), and estimate the percentage of crystal-containing cells. If available, use a microscope equipped with a camera capable of tracking changes at a single cell level.

1.5. For cells in suspension, determine the degree of cell viability following the protocol detailed in reference [28]. For cells cultured in a monolayer, estimate by visual inspection the approximate degree of confluence and amount of detached cells.

1.6. Monitor the crystal growth, cell growth and viability twice a day.

1.7. Harvest the cells as soon as crystal growth appears to stop. For remarkably robust crystals like viral polyhedra, extended incubation times (>4 days) increase the number of large crystals. However, for typical protein crystals, we recommend to harvest cells when viability drops below 80% to prevent damage caused by cell lysis.

1.8. Remove the flask from the incubator and proceed to step 2.2 as soon as possible. Keep the cells on ice during the following steps, unless specified otherwise.

2. Sample purification

Note: We describe two methods of analysis of the *in vivo* crystals from purified crystals and *in cellulo*, respectively.

2.1. Purification of crystals

Note: Unless there is evidence that the crystals of interest are sensitive to low temperature, work on ice and use ice-cold buffers to avoid crystal degradation.

2.1.1. Pipet 50 mL of infected Sf9 cells in a 50 mL conical centrifuge tube. This represents ~4.10^8 cells in a typical experiment.

2.1.2. Pellet cells at 450 x g for 10 min at 4 °C.

2.1.3. Discard the supernatant and resuspend the pellet in 40 mL of chilled phosphate buffered saline (PBS), pH 7.4.

Note: PBS is proposed as a starting point for purification as a standard isotonic buffer with the aim of mimicking the cellular environment where the in vivo crystals grew. It has been used for most in vivo crystals grown in cell culture to date [9,14,29]. Alternatively, DMEM media has also been successfully used in the purification of *in vivo* crystals from mammalian cells [19]. If crystals visibly suffer from their transfer in PBS or present poor diffraction, this parameter should be investigated as a potential source of degradation.

2.1.4. Sonicate the resuspended pellet for 30 s at 10 mA using a sonicator equipped with a 19-mm probe. To avoid heating the sample, place it in a beaker filled with ice while sonicating. Chemical or mechanical cell disruption may be used as alternatives if crystals appear to be damaged by sonication as assessed in step 2.1.7.

2.1.5. Centrifuge at 450 x g for 10 min at 4 °C. After centrifugation, 2 layers are apparent: an upper layer of cellular debris, which is pale brown, and a lower pellet of polyhedrin crystals, which is white and chalky.

2.1.6. Discard the supernatant and remove the upper layer by cautious pipetting. Resupend the lower pellet in 40 mL of chilled PBS.

2.1.7. Assess the quality of the samples by imaging with a light microscope.

2.1.7.1. Pipet 5 µL of cells or purified crystals onto a glass slide. This can be done at room temperature.

2.1.7.2. Carefully place a glass coverslip onto the liquid, avoiding the formation of air bubbles.

2.1.7.3. Image the slide with an inverted microscope at 200X magnification. Image the sample within 15 min of its preparation on the slide. If the slide cannot be visualized within 15 min, seal the coverslip with vacuum grease to avoid evaporation.

Note: In the case of polyhedra, crystals will appear as refringent cubes of ~1-10 µm per side. Check the integrity of the crystals looking for signs of loss of sharpness (e.g. round edges), cracks or dissolution. Also monitor the presence of cell debris that will appear as clumps and objects of irregular size and shape.

2.1.8. Repeat steps 2.1.5 to 2.1.7, halving the volume of PBS used to resuspend the pellet at each cycle until the crystals appear free of debris. Resuspend the final pellet in 1 mL of chilled PBS and transfer the crystals to a microcentrifuge tube. Store the purified crystals at 4 °C.

2.1.9. Estimate the concentration of crystals by using a hemocytometer as described by the manufacturer. Count crystals as if counting cells.

[place Figure 3 here]

2.2. Isolation of crystal-containing cells by flow-cytometry

Note: To facilitate the definition of gates in flow cytometry, it is recommended to grow a control culture in parallel to the one expressing the protein of interest. This can be done by infecting a flask of Sf9 cells with a non-recombinant baculovirus, or by preparing mock-infected cells. Sorting and imaging of samples is usually done at room temperature.

CAUTION: Propidium iodide is a suspected carcinogen and should be handled with care.

2.2.1. Pipet 5 mL of infected Sf9 cells - representing ~4.10^7 cells - in a tube adapted to flow-cytometry analysis. Also, prepare a second tube with an equivalent number of Sf9 cells infected with a non-recombinant baculovirus.

2.2.2. Add propidium iodide (PI) to both samples at a final concentration of 1 µg/mL just prior to flow cytometric evaluation. This step is necessary to identify dead cells, cell clumps and debris that may alter or mask cell distribution on the forward- and side-scattering plot. Prepare PI stocks of 1 mg/mL in water and store at 4 °C protected from light.

2.2.3. Apply cells to a standard flow cytometer capable of cell sorting according to forward- (FSC) and side-scattering (SSC). Analyze at least 10,000 events of the control (i.e. non-crystal containing) sample. After discarding dead cells, cell clumps and debris from the analysis, generate the FSC to SSC plot.

2.2.4. Analyze at least 10,000 events of the crystal-containing sample. Compare the FSC to SSC plot with the mock one. Look for the appearance of a distinct population corresponding to crystal-containing cells. Typically, cells with intracellular crystals will have a higher SSC due to an increased in morphological complexity. The FSC may also increase if crystal growth causes an increase in the volume of the cell. Define the gates of the flow sorter to isolate cell populations corresponding to the areas of the scatter plot that differ from the mock plot. Image the sorted samples by light microscopy as described in step 1 to confirm which population is associated with crystal-containing cells.

2.2.5. Using the gates defined in step 2.2.4, sort crystal-containing cells in PBS, or in any other isotonic buffer. Record the number of crystal-containing cells sorted as well as the final volume to facilitate subsequent steps. Store the sorted cells on ice or at 4 °C.

Note: In an hour, >300,000 polyhedra-containing cells are sorted on a cell sorter with a 100 mm nozzle (138 kPa with a frequency of 39 kHz). This amount of cells is enough to prepare > 1,000 meshes.

2.2.6. Following the protocol detailed in step 2.1.7, image the cells with a light microscope to confirm the enrichment in crystal-containing cells. Sample preparation for data collection should be done as soon as possible after cell sorting since crystals may degrade over time or be released due to cell lysis.

[place Figure 4 here]

3. Sample preparation for data collection

Note: If cells or crystals are to be derivatized for experimental phasing follow protocol detailed in section 3.1. Otherwise, go directly to sections 3.2 for purified crystals or 3.3 for *in cellulo* analysis.

3.1. Derivatization of purified or *in cellulo* microcrystals

CAUTION: Consult all relevant material safety data sheets (MSDS) before use. Most of the chemicals used for experimental phasing are acutely toxic. Use all appropriate safety practices including the use of a chemical fume hood and personal protective equipment. Dispose of waste products in accordance with your institution policy.

Note: The nature of heavy atoms, their concentration and incubation time are given on an indicative basis for crystals of the BmCPV1 polyhedrin. These parameters must be determined experimentally for each new target. Subsequent staining with trypan blue in step 3.3.2 can be omitted if cells are readily colored by the chemical compound used for phasing.

3.1.1. Prepare saturated solutions of the desired heavy atoms (or solutions of other chemicals used for experimental phasing). Typically, volumes lower than 100 µL will be required for each heavy atom. Remove undissolved salts and debris by centrifugation at maximum speed in a microcentrifuge for 5 min at room temperature.

3.1.2. Prepare aliquots of ~100,000 crystals or sorted cells per heavy atom compound. If PBS is not compatible with the chemical used for derivatization (i.e. precipitate forms and the solution becomes cloudy), pellet the crystals at 4,000 x g for 3 min at room temperature in a microcentrifuge, or the cells at 150 x g for 3 min, remove the supernatant and gently resuspend the crystals or cells in 20 µL of a suitable buffer. Repeat pelleting and resuspension to ensure that no traces of PBS remain. Adjust the final volume to 20 µL for all aliquots.

3.1.3. Add 20 µL of heavy atom solution or other chemical compound of choice to the crystals or cells. The optimal concentration of heavy atom will depend on a combination of cellular parameters (permeability, off-target proteins …) and the nature of the crystal of the protein of interest. As a starting point, test 3 different concentrations: full saturation, 1/10e, 1/100e.

3.1.4. Keep the samples at 4 °C for up to 3 days. Crystals of the polyhedrin are very resistant and dense and require long soaks to incorporate the heavy atoms; for other types of crystals the incubation period and concentration of heavy atoms should be determined experimentally. Too short incubation times will not allow the incorporation of proper amounts of the heavy atom, while long incubation times can affect the integrity of the crystals, hence lowering their diffraction quality or even dissolving them. As a starting point, try 3 different incubation times for each heavy atom solution (e.g. 1 min, 1 h and overnight). Successful derivatization is assessed by analysis of diffraction data as briefly described in section 5 and references therein.

3.1.5. Wash away the excess of heavy atom solution by pelleting the crystals at 4,000 x g for 3 min at room temperature in a microcentrifuge, or the cells at 150 x g for 3 min, removing the supernatant and gently resuspending the crystals or cells in 10 µL of buffer.

3.2. Preparation of micromesh support with purified microcrystals

CAUTION: Use of liquid nitrogen is associated with hazards such as asphyxiation, cold burns, fire in oxygen-enriched atmosphere and explosion. Please consult the risk management and safe work practices of your institution.

3.2.1. Based on the crystal concentration calculated in step 2.1.9, adjust the concentration to 10^7 crystals/mL by diluting the sample; or by pelleting the crystals at 4,000 x g for 3 min at room temperature in a microcentrifuge, adjusting the final volume by removing supernatant, and gently resuspending the crystals.

3.2.2. If samples are meant to be frozen in advance, cool down the dry shipper or dewar with liquid nitrogen. Prepare the vials or pucks, as appropriate, and cool them down with liquid nitrogen.

3.2.3. Resuspend the crystal pellet and apply a 0.5 µL droplet of crystals onto a micromesh by pipetting. Use 700-µm meshes with 25-µm square holes as a starting point. The area of the mesh is not critical but a larger area provides more crystals per mesh and leads to slower evaporation during preparation. The size of holes may need to be optimized to match the size of crystals larger than 25 µm. If required for systematic studies, indexed meshes are available to facilitate a methodical grid scan during data collection.

3.2.4. Remove most of the excess liquid by blotting with a paper wick. The crystals should be spread evenly on the mesh, without touching each other. Repeat step 3.1.1. for optimization of sample concentration. Proceed quickly and do not remove too much of the liquid. In the absence of cryoprotectant, the liquid evaporates rapidly with the risk of salt crystal formation and/or loss of the film.

3.2.5. Add cryoprotectant: pipet 0.5 µL of a solution of 50% ethylene glycol in water onto the mesh (the composition of the cryobuffer is to be adapted to the sample; suggestions for optimization of cryoprotectant can be found in [30]). Remove the excess liquid by blotting with a paper wick. By contrast with the previous step, here the remaining film of solvent should be as

thin as possible to reduce parallax effects that complicate the process of alignment between the crystal, the beam path and the goniometer; and to minimize background caused by scattering of X-rays by liquid in the beam path.

3.2.6. Immediately flash-cool the micromesh in liquid nitrogen and transfer to a vial or robot puck, then to a dry shipper or dewar for storage.

3.3. **Preparation of micromesh with crystal-containing cells**

CAUTION: Use of liquid nitrogen is associated with hazards such as asphyxiation, cold burns, fire in oxygen-enriched atmosphere and explosion. Please consult the risk management and safe work practices of your institution.

3.3.1. Transfer the sorted cells to a 1.5 mL microcentrifuge tube. Based on the cell counts recorded by the cell sorter, adjust the concentration to 10^7 cells/mL by diluting the sample with PBS; or by pelleting the cells at 150 x g for 3 min at room temperature in a microcentrifuge, adjusting the final volume by removing supernatant, and gently resuspending the cells.

3.3.2. Mix the cells with an equal volume of trypan blue solution (0.4% w/v) to a final concentration of 0.2 % w/v.

3.3.3. If samples are meant to be frozen in advance, cool the dry shipper or dewar down with liquid nitrogen. Prepare the vials or pucks, as appropriate, and also cool them down with liquid nitrogen.

3.3.4. Gently resuspend the cells and pipet 0.5 µL of sorted cells onto a micromesh.

3.3.5. Remove the excess liquid by blotting with a paper wick. The cells should be spread evenly on the mesh, without touching each other. If not using cryoprotectant (step 3.3.6), the remaining film of solvent should be as thin as possible to reduce parallax effects that complicate the process of alignment between the crystal, the beam path and the goniometer; and to minimize background caused by scattering of X-rays by liquid in the beam path.

3.3.6. Optionally, add cryoprotectant to the cells: pipet 0.5 µL of 50% ethylene glycol, 50% PBS solution onto the mesh. The composition of the cryobuffer is to be adapted to the sample; suggestions for optimization of cryoprotectant can be found in reference [30]. Remove the excess liquid by blotting with a paper wick, leaving only a thin film of solvent.

Note: Omission of the cryoprotection step did not alter the quality of X-ray diffraction when crystals of the polyhedrin were analyzed *in cellulo*, while this step was required for purified crystals[20]. This only applies to the incubation of the cells with cryoprotectant solution; the sample should still be analyzed at 100K to minimize the effects of radiation damage on the crystals.

3.3.7. Immediately flash-cool the micromesh in liquid nitrogen and transfer to a vial or robot puck, then to a dry shipper or storage dewar, as appropriate.

4. Data collection

Note: Parameters used for data collection are given as a guide and should be optimized for each crystal type and synchrotron beamline.

4.1. Collect data on a microfocus crystallography beamline (cf. reference [7] for a list of microfocus beamlines and their characteristics). If available, use a collimated beam that matches the size of the crystals.

4.2. For experimental phasing by the Single-wavelength Anomalous Dispersion method (SAD), collect at an energy maximizing the anomalous signal of the heavy atom of choice. For phasing using the Multiple Isomorphous Replacement method, (MIR), collect above the energy of a suitable absorption edge for the heavy atom of choice (available at http://skuld.bmsc.washington.edu/scatter/AS_periodic.html).

4.3. After loading the mesh onto the goniometer, start by centering the micromesh with the beam path using the face-on (convex side) and side-on orientations. Then, with the micromesh face-on, fine-tune the alignment by aligning the center of a particular horizontal lane of the micromesh (for instance, start with the central one). Then collect data along this horizontal lane with only minor readjustments to the alignment. If available, use centering based on rastering at low-dose X-ray diffraction for fine-tuning the alignment (consult the local contact at the synchrotron beamline).

Note: As a limited amount of data can be collected on microcrystals due to radiation damage (typically, 10 to 30 images with 1 ° oscillation), in theory the crystal only needs to be aligned with the beam path for 10-30 °. However, it is critical that the alignment is accurate as the crystal can easily by missed by the microbeam (generally collimated to a diameter of ~10 µm or smaller).

4.4. Collect diffraction data until diffraction is lost due to radiation damage. For crystals of the polyhedrin, exposure time of 10 sec is typically used for a 1 ° oscillation for a flux of approximately 3.6 x 10^{11} photons/s at 13 keV with a CCD detector (total dose <30 MGy). Optimize these settings depending on the beamline and nature of the crystal used. In particular, low dose and fine slicing are recommended for pixel X-ray detectors.

Note: If using the *in cellulo* strategy, the cells stained with trypan blue will appear as blue dots against the support background, making them easier to align to the beam. If the collimated microbeam has approximately the same size as the cells (~10µm), all crystals contained in a cell will be illuminated simultaneously. For cells containing multiple microcrystals, this leads to the observation of multiple diffraction patterns, which can be difficult to process. However, diffraction from one of the crystals often tends to dominate the diffraction pattern and is preferentially indexed during data processing. For beamlines with an X-ray beam significantly

smaller that the cell, rastering at very low X-ray flux (>95% attenuation) should be used to center on a single crystal before data collection.

4.5. Proceed to the next crystal in lane.

4.6. Once all the crystals of the lane have been tested, proceed to the next lane and repeat the alignment procedure. Proceed until sufficient data has been collected or all crystals have been tested.

4.6.1. Make sure that the lanes that have been processed are clearly identified so that crystals are not missed or shot twice (if necessary, draw a sketch). Typically, trypan blue turns yellow after the cell has been irradiated, which may be used as a marker.

[place Figure 5 here]

5. Data processing

Note: Here, we only mention briefly the steps of data processing that are specific to serial microcrystallography, where data from multiple crystals are merged. Many excellent articles are available describing data processing in depth[31-36] and structure determination is beyond the scope of this protocol.

5.1. Select the crystal with the best diffraction resolution and quality as a reference.

5.2. Individually process each crystal using standard crystallography packages such as HKL-2000[31], Mosflm[32] or XDS[33]. To ensure that radiation damage does not deteriorate the quality of the dataset, carefully select the last image with acceptable radiation damage. Cut-off criteria indicating excessive radiation damage are specific of each crystal type, processing practice and the purpose of the experiment. For phasing of the BmCPV1 polyhedrin crystals, data was discarded once the signal-to-noise $\langle I \rangle / \langle \sigma(I) \rangle$ per image dropped below 2 or the R_{sym} exceeded 50% as reported by HKL-2000[31].

5.3. For each crystal, define the resolution limit and number of frames and reprocess with the correct parameters to avoid integrating data with little or no signal.

5.4. Scale and merge data from the best crystals to the reference crystal(s) using standard packages such as Scalepack in HKL-2000[31], SCALA in CCP4[34,35] or XSCALE in XDS[33]. Add the data from the different crystals progressively while monitoring the global quality of the dataset, ideally until the dataset reaches an acceptable completeness (>95%). Reject crystals that present a poor isomorphism with the best/reference crystal as detected by differences in unit cell parameters and poor R_{merge}/chi-square per image. If too many good quality datasets do not scale with the reference crystal(s), use a program like BLEND[36] that carries hierarchical clustering to define the best combination of datasets to merge.

Note: In some space groups, alternate indexing options need to be tested to match the

reference crystal.

5.5. Proceed to experimental phasing, molecular replacement or refinement using standard crystallography packages.

REPRESENTATIVE RESULTS:

An overview of both alternative methods for structure determination using *in vivo* microcrystals is presented (Figure 1). Polyhedra can easily be purified by sonication and centrifugation. Due to their density, they form a layer at the bottom of the tube underneath a layer of debris that can be removed by pipetting (Figures 3a and 3b). The sample is then subjected to several rounds of sonication and washes until a sufficient level of purity is reached as judged from the white and chalky aspect of the pellet (Figure 3c).

For the *in cellulo* approach, flow cytometry profile of non-infected cells is compared with profile of crystal-containing cells and used to determine which cell population should be selected to sort crystal-containing cells away from the other cells (Figure 4). In this example, cells containing polyhedra have a higher side-scattering than non-infected cells. Other differences in scattering patterns may be observed for other crystal types and gating should be modified accordingly.

Purified microcrystals or crystal-containing cells are pipetted onto a micromesh (Figure 5). Cells stained with trypan blue can be easily visualized using cameras available at most of synchrotron beamlines (Figure 5b), while purified crystals are laborious to identify and align with the X-ray beam due to their small size (Figure 5c). When collecting data, it is best to proceed in a grid pattern in order to minimize the centering procedures, and to avoid exposing twice the same cell or crystal, or missing any (Figure 5a). Data collected should be processed carefully to account for differences in diffraction limits, the effect of radiation damage and a possible lack of isomorphism.

Figure 1: Overview of two methods for structure determination using *in vivo* microcrystals. Microcrystals are produced in cell culture by over-expression of the protein of interest using a recombinant expression system. In specific cases, microcrystals may also be produced naturally by the cells in particular conditions. The top row shows the classical approach where cells are lysed and crystals are purified by differential centrifugation. Purified microcrystals are pipetted onto a micromesh. After removal of the excess liquid, a cryoprotectant solution is added, excess liquid is blotted again and the mesh is flash-cooled. For X-ray diffraction experiments, crystals are carefully aligned with the X-ray beam, which may be the rate limiting step in data collection. The bottom row presents the *in cellulo* approach. Cells are sorted by flow-cytometry to specifically select crystal-containing cells. Cells are stained with trypan blue to facilitate visualization and spread onto a micromesh. In this case, the addition of a cryoprotectant is optional. For X-ray diffraction experiments, cells are easily visualized using typical cameras available at synchrotron beamlines facilitating the centering process.
Image of automated liquid chromatography system courtesy of GE Healthcare AB, Uppsala, Sweden.

Figure 2: Examples of *in vivo* microcrystals.
Selected examples of in vivo crystals: a) Sf9 cells with cypovirus polyhedra; b) LD652 cells with entomopoxvirus spheroids; c) *Bacillus thuringiensis* with Cry3A crystals (arrow); d) Sf9 cells with *Trypanosoma brucei* cathepsin B crystals; e) Sf21 cells with calcineurin crystals (arrows); f) COS7 cells monolayer with PKA:Inka1 crystals; g) CHO cells with IgG crystals. Reproduced from [14,18,29,37,39,42], with permission. Scale bar: 10 µm.

Figure 3: Purified *in vivo* microcrystals.
Representative purification of crystals of the BmCPV1 polyhedrin from Sf9 insect cells showing (a) pelleted cell debris and crystals after sonication, and (b) close-up on the debris and crystal layers. Debris layer can be carefully resuspended in the supernatant and discarded for faster crystal purification. c) Optical microscopy image of purified polyhedra. Scale: 50 µm.

Figure 4: Cell-sorting.
Representative flow cytometry scatter plots from (a) non-infected cells (mock), which do not contain crystals, and (b) Sf9 cells infected by a recombinant baculovirus over-expressing for the BmCPV1 polyhedrin. Differences in the scattering pattern between the two populations allowed gating of the crystal-containing cell population (red gate, high SSC values). Each plot represents 20,000 sorting events. SSC: side light scattering; FSC: forward light scattering. c) Optical microscopy image of a representative population of the sorted cells, showing the presence of intracellular crystals. Scale: 50 µm.

Figure 5: Data collection strategy from samples on micromesh support.
(a) Strategy of data collection per lane; alignment is done at the beginning of each lane (dots) and data is collected along the lane. b) Close-up of a trypan blue-stained, crystal-containing cell on a mesh, as seen on the microcrystallography beamline screen. c) Close-up of a purified polyhedra on a mesh, as seen on the microcrystallography beamline screen. The mesh squares are 25 µm x 25 µm.

Table 1: Summary of *in vivo* crystals grown in cultured cells expressing recombinant protein.

DISCUSSION:
This protocol provides two approaches to analyze microcrystals with the aim of facilitating the analysis of very small crystals that would have been overlooked in the past.

Critical steps for microcrystal purification
The presented protocol has been optimized using *Bombyx mori* CPV1 polyhedrin expressed in Sf9 cells as a model system. However, *in vivo* microcrystals display a great variability in mechanical resistance. For instance, cathepsin B needle-like crystals grown in insect cells are rigid and highly resistant to mechanical stress, and could be purified using a protocol similar to the one described here [14]. On the other hand, firefly luciferase crystals, also grown in cultured insect cells and with a similar needle-like morphology, immediately dissolve upon cell lysis[38]. Thus, the protocol for extraction and purification of *in vivo* crystals will need to be adapted in a

trial-error basis for particular cases as described in steps 1.3, 2a.3-4, 2b.4, 3.1.3-4 and 3.2a.5.

For fragile crystals, (partial) separation from cell debris might be achieved by low speed centrifugation (i.e. 200 x g) or addition of a sucrose cushion at the bottom of the tube instead of repeated sonication. As a general rule, crystal samples prepared for diffraction data collection purposes do no need to be highly purified. Thus, rapid purification is preferable to cryo-cool the crystals before they might decay.

Application to *in vitro* and *in vivo* microcrystals

Although both protocols are here illustrated with the analysis of *in vivo* grown crystals, the methodology can be readily used for microcrystals grown in crystal trays from purified recombinant protein. In this case, the following modifications should be considered: i) the mesh support may be used to harvest the microcrystals directly from the crystallization drop, rather than transferring them by pipetting; ii) since the amount of crystals may be a limiting factor, special care will need to be taken when blotting the excess of liquid from the mesh support, to avoid excessive removal of crystals; iii) different cryoprotectant buffers will need to be tested, as done in conventional crystallography (see [30]).

On the other hand, for *in vivo*-grown crystals, the *in cellulo* approach is strongly recommended. Because cells are easier to visualize and manipulate than purified crystals, this approach is more efficient in terms of beamtime usage and more accessible to researchers with little or no experience in microcrystallography. It is also more suitable for fragile crystals that may be affected by the extraction and purification from the cell due to changes in the chemical environment, dilution of the surrounding proteins and mechanical damage. Besides, cells provide a protective environment to the crystals, alleviating the need for the addition of a cryoprotectant. Omission of the cryoprotection treatment has been previously shown to prevent accumulation of defects and improved the isomorphism between individual crystals, for data collection at room temperature[40]. The *in cellulo* protocol also bypasses the requirement for incubation in cryoprotectant solution, while still allowing data collection at cryogenic temperature. Overall, *in cellulo* data collection has two main advantages: 1) it may produce data of better quality and higher resolution for a given period of beamtime[20]; and 2) it maintains the crystallized protein in a cellular environment increasing the chances of retaining a biologically-relevant ligand.

Limitations of the technique

An intrinsic complication of serial microcrystallography is the important number of partial datasets generated during data collection. Consequently, data processing becomes an increasingly time-consuming process. However, the basic workflow for data processing can largely be automated. For example, the optimal selection of isomorphous crystals can be determined by hierarchical cluster analysis[28], for instance implemented in BLEND [36]. In addition, programs developed for the analysis of X-ray free electron lasers (XFEL) can also be adapted to process serial microcrystallography data collected on synchrotron beamlines[2] and, at some beamlines automated rastering of mesh and crystal detection have been implemented greatly facilitating serial data collection[2,41].

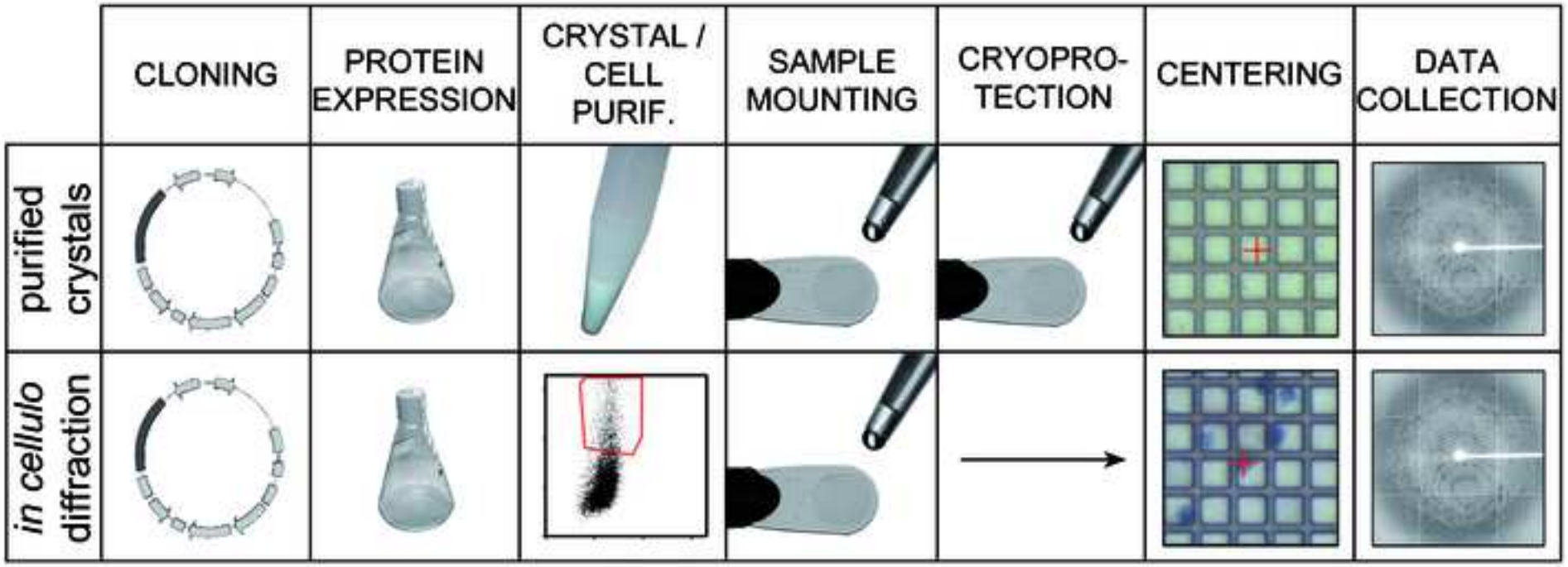
ACKNOWLEDGMENTS:
Authors would like to acknowledge Mr. Chan-Sei Lay for providing pictures of purified microcrystals. Diffraction data collection part of the protocol was optimized at MX2 beamline of the Australian Synchrotron, with invaluable assistance of the beamline staff. This project was supported by a Future Fellowship of the Australian Research Council to FC (FT1210893) and DP130104885.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Tari, L. W. The utility of structural biology in drug discovery. *Methods Mol Bio (Clifton, N.J.)* **841**, 1–27, doi:10.1007/978-1-61779-520-6_1 (2012).
2. Gati, C., *et al.* Serial crystallography on in vivo grown microcrystals using synchrotron radiation. *IUCrJ* **1** (2), 0–0, doi:10.1107/S2052252513033939 (2014).
3. Redecke, L., *et al.* Natively inhibited Trypanosoma brucei cathepsin B structure determined by using an X-ray laser. *Science* **339** (2013), 227–230, doi:10.1126/science.1229663 (2013).
4. Shi, D., Nannenga, B. L., Iadanza, M. G. & Gonen, T. Three-dimensional electron crystallography of protein microcrystals. *eLife* **2** (0), e01345–e01345, doi:10.7554/eLife.01345.019 (2013).
5. Evans, G., Axford, D., Waterman, D. & Owen, R. L. Macromolecular microcrystallography. *Crystallog. Rev.* **17** (2), 105–142, doi:10.1080/0889311X.2010.527964 (2011).
6. Smith, J. L., Fischetti, R. F. & Yamamoto, M. Micro-crystallography comes of age. *Curr Opin Struct Biol* **22** (5), 602–612, doi:10.1016/j.sbi.2012.09.001 (2012).
7. Boudes, M., Garriga, D. & Coulibaly, F. Reflections on the Many Facets of Protein Microcrystallography. *Aust J Chem* **67** (12), 1793–1806, doi:10.1071/CH14455 (2014).
8. Nelson, R., *et al.* Structure of the cross-beta spine of amyloid-like fibrils. *Nature* **435** (7043), 773–778, doi:10.1038/nature03680 (2005).
9. Coulibaly, F., *et al.* The molecular organization of cypovirus polyhedra. *Nature* **446** (7131), 97–101, doi:10.1038/nature05628 (2007).
10. Coulibaly, F., *et al.* The atomic structure of baculovirus polyhedra reveals the independent emergence of infectious crystals in DNA and RNA viruses. *Proc Natl Acad Sci U S A* **106** (52), 22205–22210, doi:10.1073/pnas.0910686106 (2009).
11. Johansson, L. C., *et al.* Structure of a photosynthetic reaction centre determined by serial femtosecond crystallography. *Nat Comm* **4**, doi:10.1038/ncomms3911 (2013).
12. Li, D., Boland, C., Aragao, D., Walsh, K. & Caffrey, M. Harvesting and Cryo-cooling Crystals of Membrane Proteins Grown in Lipidic Mesophases for Structure Determination by Macromolecular Crystallography. *J Vis Exp* (67), doi:10.3791/4001 (2012).
13. Roedig, P., *et al.* A micro-patterned silicon chip as sample holder for macromolecular crystallography experiments with minimal background scattering. *Sci Rep* **5**, 10451, doi:10.1038/srep10451 (2015).
14. Koopmann, R., *et al.* In vivo protein crystallization opens new routes in structural biology.

Nature Methods **9** (3), 259–262, doi:10.1038/nmeth.1859 (2012).
15. Gallat, F.-X., *et al.* In vivo crystallography at X-ray free-electron lasers: the next generation of structural biology? *Phil Trans R Soc B* **369** (1647), 20130497, doi:10.1098/rstb.2013.0497 (2014).
16. Duszenko, M., *et al.* In vivo protein crystallization in combination with highly brilliant radiation sources offers novel opportunities for the structural analysis of post-translationally modified eukaryotic proteins. *Acta Cryst. F***71** (Pt 8), 929–937, doi:10.1107/S2053230X15011450 (2015).
17. Axford, D., Ji, X., Stuart, D. I. & Sutton, G. In cellulo structure determination of a novel cypovirus polyhedrin. *Acta Cryst D* **70** (Pt 5), 1435–1441, doi:10.1107/S1399004714004714 (2014).
18. Sawaya, M. R., *et al.* Protein crystal structure obtained at 2.9 Å resolution from injecting bacterial cells into an X-ray free-electron laser beam. *Proc Natl Acad Sci U S A* **111** (35), 12769–12774, doi:10.1073/pnas.1413456111 (2014).
19. Tsutsui, H., *et al.* A Diffraction-Quality Protein Crystal Processed as an Autophagic Cargo. *Mol Cell* **58** (1), 186–193, doi:10.1016/j.molcel.2015.02.007 (2015).
20. Boudes, M., Garriga, D., Fryga, A., Caradoc-Davies, T. & Coulibaly, F. A pipeline for structure determination of in vivo-grown crystals using in cellulo diffraction.*Acta Cryst D**72, 576-585*, doi:10.1107/S2059798316002369 (2016).
21. Doye, J. P. K. & Poon, W. C. K. Protein crystallization in vivo. *Curr Opin Colloid Interface Sci* **11** (1), 40–46 (2006).
22. Mori, H., *et al.* Expression of Bombyx mori cytoplasmic polyhedrosis virus polyhedrin in insect cells by using a baculovirus expression vector, and its assembly into polyhedra. *J Gen Virol* **74** (Pt 1), 99–102 (1993).
23. Arevalo, M. T., Wong, T. M. & Ross, T. M. Expression and Purification of Virus-like Particles for Vaccination. *J Vis Exp* (112), doi:10.3791/54041 (2016).
24. Yates, L. A. & Gilbert, R. J. C. Efficient Production and Purification of Recombinant Murine Kindlin-3 from Insect Cells for Biophysical Studies. *J Vis Exp* (85), doi:10.3791/51206 (2014).
25. Berger, I., *et al.* The MultiBac Protein Complex Production Platform at the EMBL. *J Vis Exp* (77), doi:10.3791/50159 (2013).
26. Margine, I., Palese, P. & Krammer, F. Expression of Functional Recombinant Hemagglutinin and Neuraminidase Proteins from the Novel H7N9 Influenza Virus Using the Baculovirus Expression System. *J Vis Exp* (81), doi:10.3791/51112 (2013).
27. Khurana, A. & Kronenberg, M. A Method For Production of Recombinant mCD1d Protein in Insect Cells. *J Vis Exp* (10), doi:10.3791/556 (2007).
28. Ricardo, R. & Phelan, K. Counting and Determining the Viability of Cultured Cells. *J Vis Exp* (16), doi: 10.3791/752 (2008).
29. Baskaran, Y., *et al.* An in cellulo-derived structure of PAK4 in complex with its inhibitor Inka1. *Nat Comm* **6**, 1–11, doi:10.1038/ncomms9681 (2015).
30. Armour, B. L., *et al.* Multi-target Parallel Processing Approach for Gene-to-structure Determination of the Influenza Polymerase PB2 Subunit. *J Vis Exp* (76), doi:10.3791/4225 (2013).
31. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* **276**, 307–326 (1997).

32. Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. *Acta Cryst D***67** (Pt 4), 271–281, doi:10.1107/S0907444910048675 (2011).
33. Kabsch, W. XDS. *Acta Cryst D***66** (Pt 2), 125–132, doi:10.1107/S0907444909047337 (2010).
34. French, S. & Wilson, K. On the treatment of negative intensity observations. *Acta Cryst* A**34**, 517-525, doi:10.1107/S0567739478001114 (1978).
35. Winn, M. D., *et al.* Overview of the CCP4 suite and current developments. *Acta Cryst D***67** (Pt 4), 235–242, doi:10.1107/S0907444910045749 (2011).
36. Foadi, J., *et al.* Clustering procedures for the optimal selection of data sets from multiple crystals in macromolecular crystallography. *Acta Cryst D***69** (8), 1617–1632, doi:10.1107/S0907444913012274/dz5278sup1.pdf (2013).
37. Rey, F. A. Virology: holed up in a natural crystal. *Nature* **446** (7131), 35–37, doi:10.1038/446035a (2007).
38. Schönherr, R., *et al.* Real-time investigation of dynamic protein crystallization in living cells. *Struct Dyn* **2** (4), 041712, doi:10.1063/1.4921591.10 (2015).
39. Hasegawa, H., *et al.* In vivo crystallization of human IgG in the endoplasmic reticulum of engineered Chinese hamster ovary (CHO) cells. *J Biol Chem* **286** (22), 19917–19931, doi:10.1074/jbc.M110.204362 (2011).
40. Ishchenko, A., Cherezov, V. & Liu, W. Preparation and Delivery of Protein Microcrystals in Lipidic Cubic Phase for Serial Femtosecond Crystallography. *J Vis Exp* (115), e54463, doi:10.3791/54463 (2016).
41. Zander, U., *et al.* MeshAndCollect: an automated multi-crystal data-collection workflow for synchrotron macromolecular crystallography beamlines. *Acta Cryst. D**71, 2328-2343***, doi:10.1107/S1399004715017927 (2015).
42. Fan, G. Y., *et al.* In vivo calcineurin crystals formed using the baculovirus expression system. *Micros Res Tech* **34** (1), 77–86, doi:10.1002/(SICI)1097-0029(19960501) (1996).
43. Nass, K., *Investigation of protein structure determination using X-ray free-electron lasers* (2013). PhD dissertation. Hamburg University.



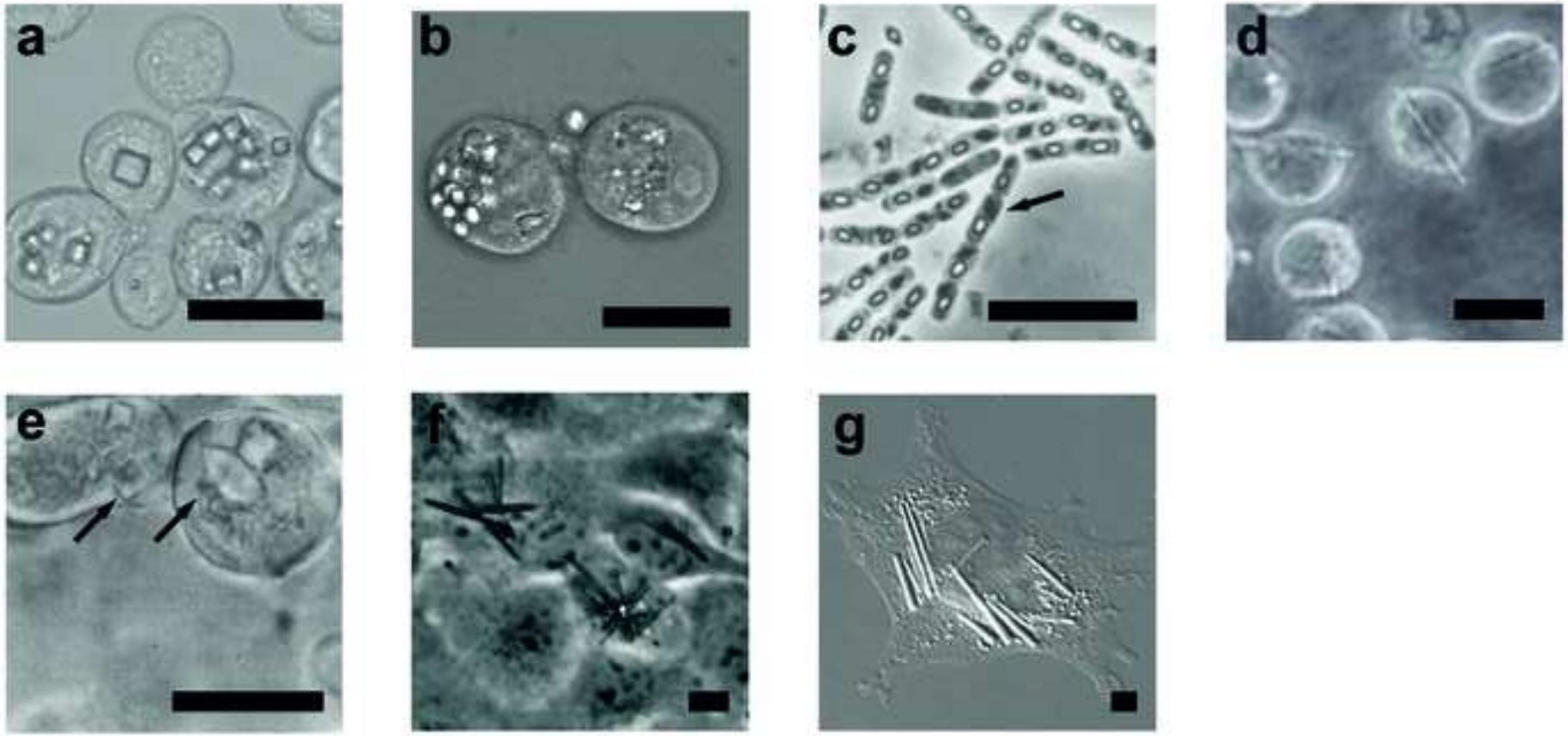


Figure3

Click here to download Figure 3.tif

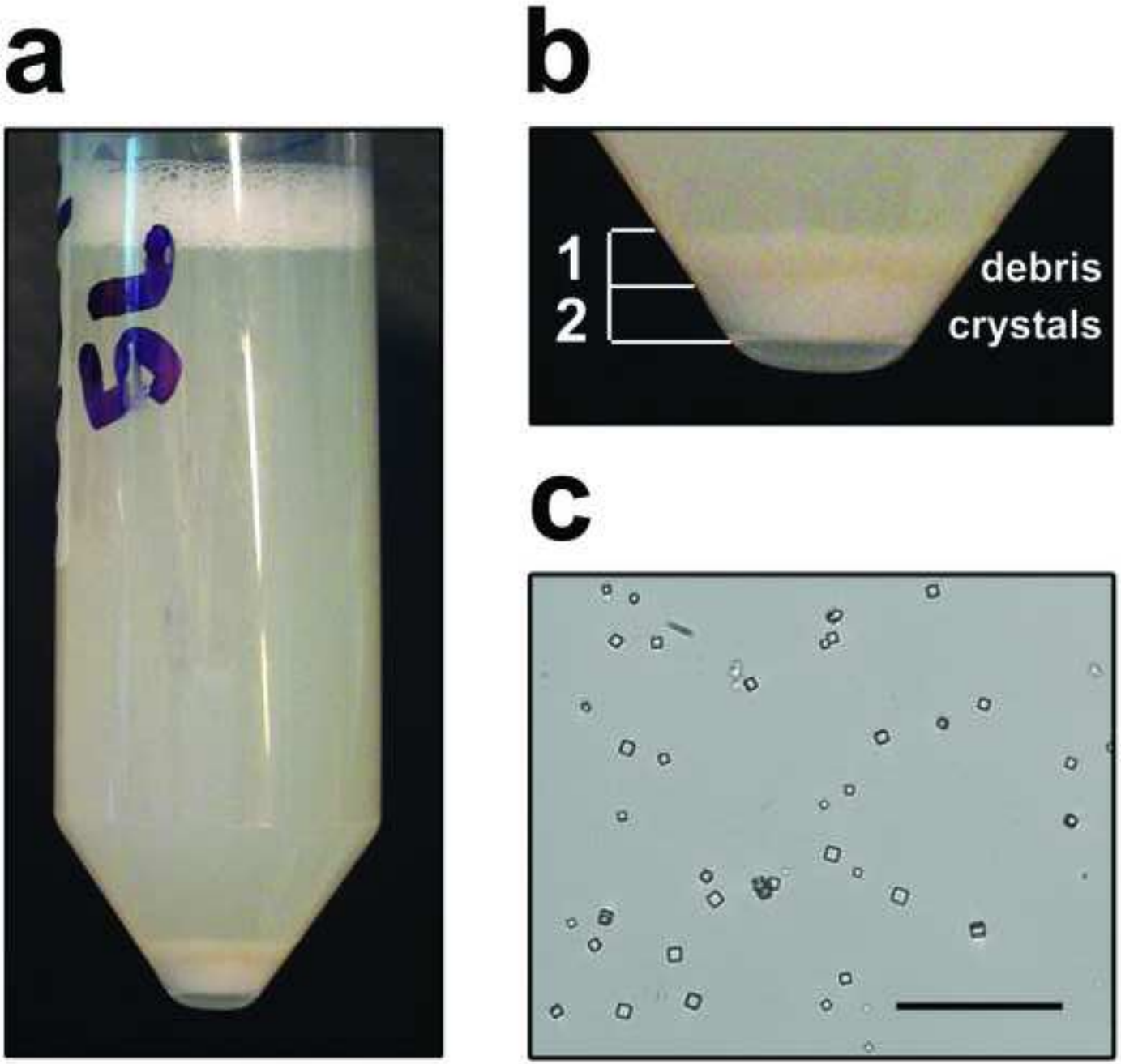


Figure4

Click here to download Figure 4.tif

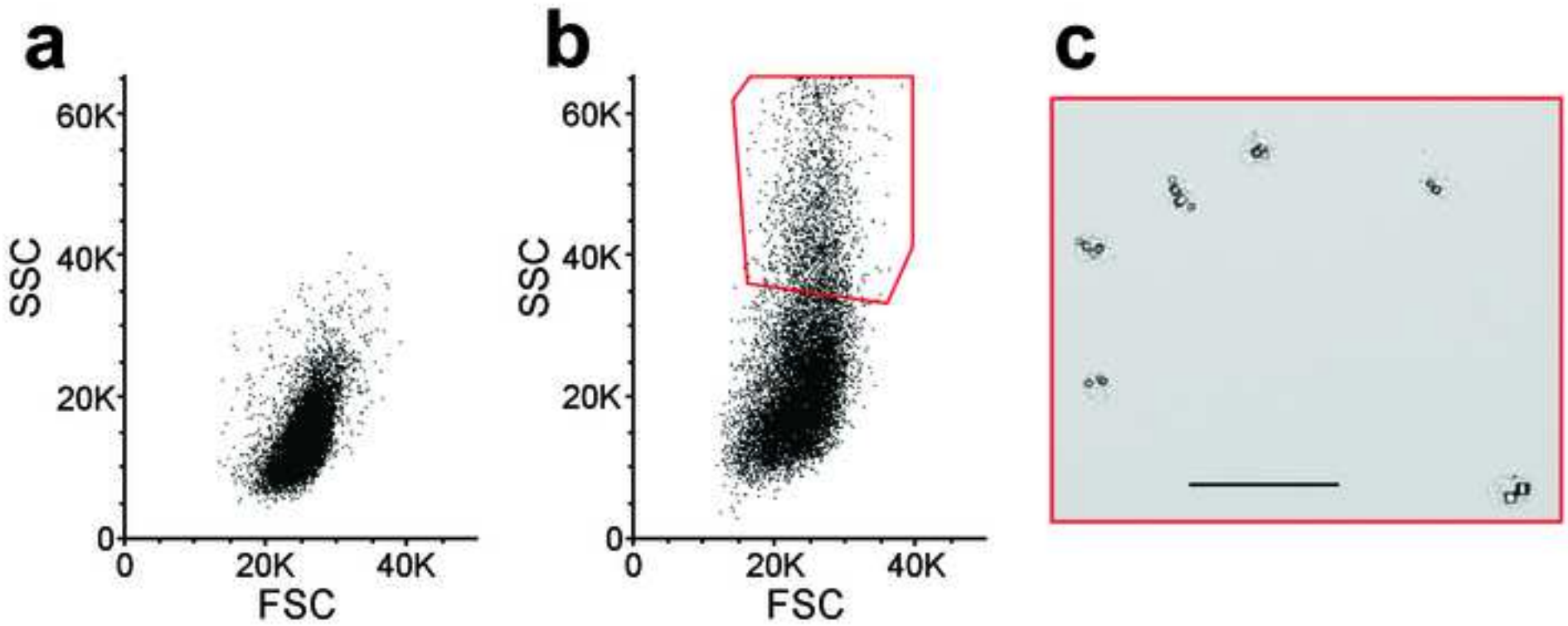
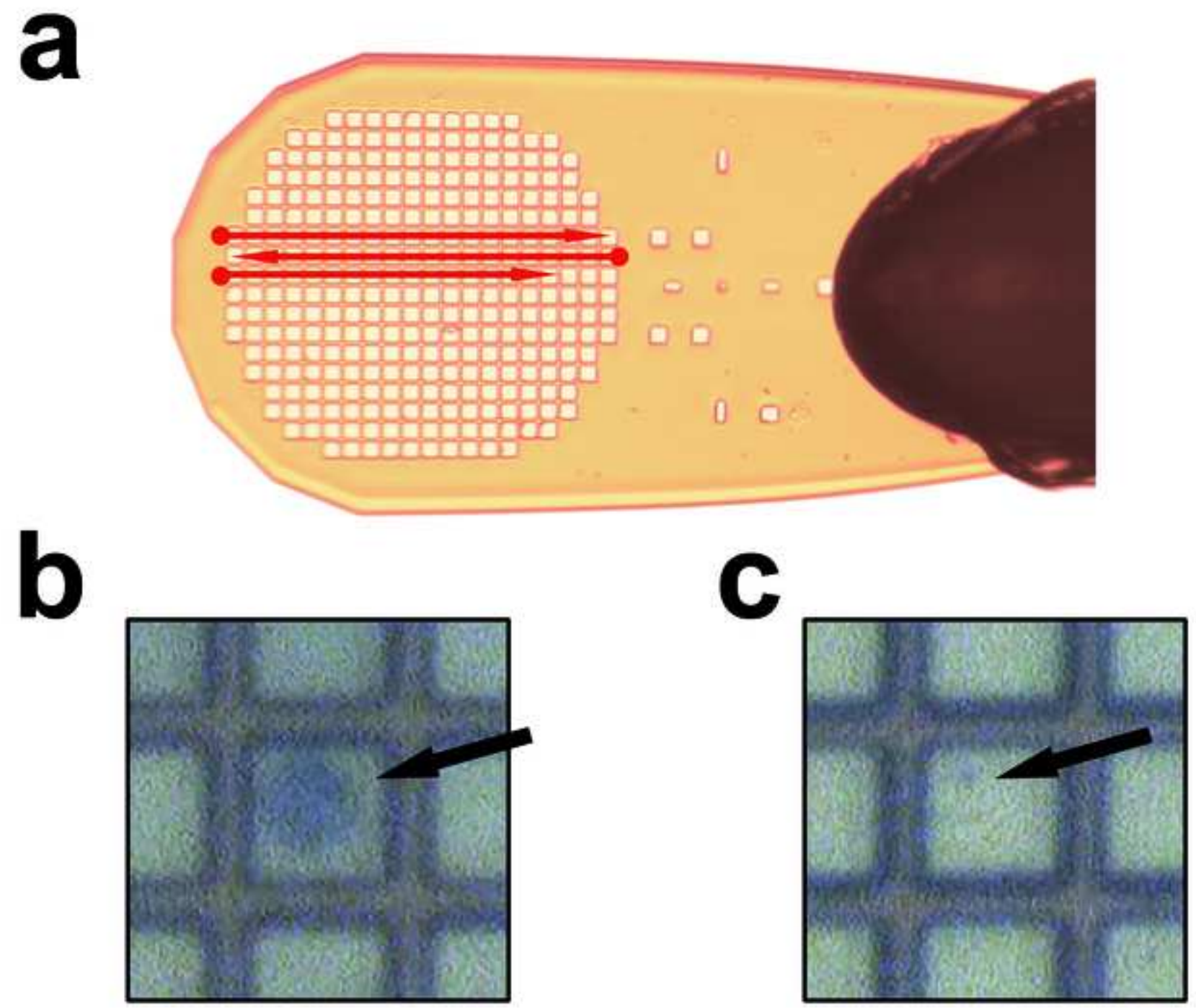


Figure5

Click here to download Figure 5.tif



Table

PROTEIN NAME	PROTEIN SIZE	EXPRESSION SYSTEM	LOCATION IN CELL	FORMATION TIME	NUMBER OF CRYSTAL/CELL	CRYSTAL SHAPE	CRYSTAL SIZE	SPACE GROUP	REFERENCE
calcineurin	79 kDa	Sf21 insect cells	cytoplasm	3 days	1 to 3	cubic, rhomboid	<10 x 6 µm	n/d	Fan et al., *Micros Res Tech* (1996) [Ref 42]
cypovirus polyhedrin	29 kDa	Sf9 insect cells	cytoplasm	2-3 days	1 to 10+	cubic	~5 µm per side	I23	Coulibaly et al., Nature (2007) [Ref 9]
baculovirus polyhedrin	29 kDa	Sf21 insect cells	cytoplasm nucleus	2 - 3 days	>10	cubic	2-4 µm per side	I23	Coulibaly et al., *PNAS* (2009) [Ref 10]
cathepsin B	37 kDa	Sf9 insect cells	ER lumen	3 - 8 days	1 to 3	needle-like	10-15 µm length 0.5 - 1 µm width	$P4_22_12$	Koopmann et al., *Nature* (2012) [Ref 14] Redecke et al., *Science* (2013) [Ref 3]
inosine monophosphate dehydrogenase	57 kDa	Sf9 insect cells	ER lumen	3 - 6 days	1 to 3	needle-like	10-30 µm length 1 µm width	$P42_12$	Nass KJ. PhD Thesis (2013) [Ref 43]
firefly luciferase	62 kDa	Sf9 insect cells	peroxisomes	3 - 5 days	1 - 5	needle-like	<200 µm length 1-3 µm width	n/d	Schönherr et al., *Struct. Dyn.* (2015) [Ref 38]
GFP-µNS	45 kDa	Sf9 insect cells	ER lumen	4 days	1 to 10+	needle-like	<15 µm length <1-5 µm width	n/d	Schönherr et al., *Struct. Dyn.* (2015)[Ref 38]
immunoglobulin G	150 kDa	CHO cells	ER lumen	5 days	1 to 30+	needle-like	<60 µm length <7 µm width	n/d	Hasegawa et al., *J. Biol. Chem.* (2011) [Ref 39]
coral fluorescent Xpa protein	26 kDa	HEK293 (also rat neuron & mouse fibroblast)	cytoplasm	2 - 5 days	1	needle-like	~7 µm length 4 µm width	$P2_12_12$	Tsutui et al. *Molecular Cell* (2015) [Ref 19]
PAK4:Inka1 complex	41 kDa	COS-7 cells (also HeLa and HEK293)	cytoplasm	2 days	1 to 5	needle-like	50-100 µm in length <5 µm width	$P6_3$	Baskaran et al., *Nat Comm* (2015) [Ref 29]

Table of Materials/Equipment

Click here to download Table of Materials/Equipment jove_materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Sf9 cells	Life Technologies		
SF900-SFM insect medium	Life Technologies		
1L cell culture flask	Thermofisher Scientific		
Shaking incubator for insect cell culture	Eppendorf		
50mL conical tubes	Falcon		
Centrifuge with swing buckets for 50mL tubes	Eppendorf		
Sonicator equiped with a 19mm probe	MSE Soniprep 150		
Glass slides	Hampton Research		
Hemacytometer	Sigma-Aldrich		
Propidium iodide	Thermofisher Scientific		
BD Influx cell sorter	BD Biosciences		
Hampton Heavy atom screens	Hampton Research		
Microcentrifuge	Eppendorf		
Micromesh	Mitigen		700/25 meshes offer a larger surface. Indexed meshes can be purchased for systematic studies.

Paper wick	Mitigen	The size of the paper wick can be varied for optimal flow. This will largely depend on the nature of the crystals and cryoprotectant used.
Ethylene glycol	Sigma-Aldrich	
Trypan blue	Life Technologies	
MX2 microfocus beamline	Australian Synchrotron	A list of available microfocus beamlines can be found in Boudes et al. (2014) *Reflections on the Many Facets of Protein Microcrystallography.* Australian Journal of Chemistry 67 (12), 1793–1806, doi:10.1071/CH14455.

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Microcrystallography of protein crystals and in cellulo diffraction

Author(s): M. Boudes, D. Garriga and F. Coulibaly

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

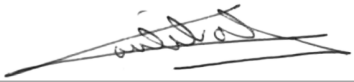
11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Fasseli Coulibaly
Department:	Biomedicine Discovery Institute
Institution:	Monash University
Article Title:	Microcrystallography of protein crystals and in cellulo diffraction
Signature:	 Date: Dec 2, 2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

MONASH University

A/Prof Fasséli Coulibaly
ARC Future Fellow
ph: +613 990 29225
email:

fasseli.coulibaly@monash.edu
Structural Virology laboratory
School of Biomedical Sciences
Faculty of Medicine, Nursing and Health Sciences
VIC3800 Australia

Melbourne, January 31, 2017

Editorial Office,
Journal of Visualized Experiments

Dear Dr Nam Nguyen,

We are grateful for the constructive comments of the Editor and Reviewers. We have revised the manuscript with the aim to answer them fully and provide a detailed account of the changes below.

As an overview, we have made these main modifications:

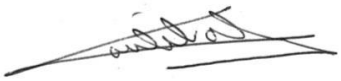
- The main concern raised by Reviewers #2 and #3 is that the manuscript was trying to cover too much to allow a detailed description of all the steps of the protocol. To address this, we removed all the initial section describing the expression of recombinant proteins in insect cells, given that this topic is already covered extensively in the JoVE archive and elsewhere (see JoVE references Arevalo et al. 2016; Yates et al. 2014; Berger et al. 2013; Margine et al. 2013; and Khurana et al. 2007). We also shortened the last section on data processing and structure determination.

- This allowed us to focus on methods specific to *in vivo*-grown microcrystals and their use in X-ray data collection. The revised version of the protocol now includes the following sections: identification, purification, preparation for data collection, data collection and general guidance for data processing.

- Following Reviewers #3' s recommendations, the paragraph on visualisation of *in vivo* crystals has been expanded to a full section, also including a new figure and a table, showing different examples of in vivo-grown microcrystals, to offer more in depth guidance on the identification of crystal-containing cells (which will be also complemented by the video protocol).

- As requested by the Editor, ~2 pages have been highlighted to indicate the steps of the protocol that should appear in the video protocol. We concur with Reviewer #2 that data processing and structure determination are beyond the scope of the video protocol and have not included any part of this last section for video production.

With kind regards,
A/Prof Fasseli Coulibaly



Detailed responses to the Editor and Reviewers' comments:

Editorial comments:

- The manuscript has been revised to remove as much as possible typos and errors, and to ensure all abbreviations are defined.
- Brand names and commercial language have been removed from the main text. Note: a brand name is still mentioned in Figure 1 legend, to acknowledge the use of an image subject to copyright.
- References have been updated, abbreviating the journal titles.
- The Discussion section has been rewritten to cover the requested topics.

Reviewer #1:

- "The authors should discuss the possibility/risk of dissolving the crystals during resuspension."

We agree with Reviewer #1 that the stability of crystals will vary on a case-by-case basis. This point is now mentioned in the Discussion section (lines 712-730).

> *"The presented protocol has been optimized using Bombyx mori CPV1 polyhedrin expressed in Sf9 cells as a model system. However, in vivo microcrystals display a great variability in mechanical resistance. For instance, cathepsin B needle-like crystals grown in insect cells are rigid and highly resistant to mechanical stress, and could be purified using a protocol similar to the one described here {Koopmann:2012bb}. On the other hand, firefly luciferase crystals, also grown in cultured insect cells and with a similar needle-like morphology, immediately dissolve upon cell lysis. Thus, the protocol for extraction and purification of in vivo crystals will need to be adapted in a trial-error basis for each particular case."*

Reviewer #2:

- Should steps 2a.3, 2a.4 and 2a.6 be done on ice? Same applies to 2b.2 and 2b.5.

Temperatures at which the work should be carried on are now indicated throughout the protocol.

- Please re-write step 2a.7.3) for clarity.

Step 2a.7.3) now reads:

> *"Image the slide with an inverted microscope at 200 X magnification. The sample should be imaged within 15min of its preparation on the slide. If the slide cannot be visualized within 15 min, ensure that the coverslip is sealed with vacuum grease to avoid evaporation.*
> *In the case of polyhedra, crystals will appear as refringent cubes of ~1-10 μm per side. Check for integrity of the crystals (roundness of the edges, cracks, etc). Also monitor the presence of cell debris that will appear as clumps and objects of irregular size and shape."*

- "Should the propidium iodide be prepared in water or in a buffer?"

This information has been included at step 2b.2:

> *"PI stocks of 1 mg/ml are prepared in water and stored at 4ºC protected from light."*

- "Could the force applied to crystals during centrifugation affect the crystals integrity i.e. mosaicity?"
This possibility is mentioned in the Discussion (cf. answer to Reviewer #1).

- "It is indicated that the concentration needs to be adjusted to 10^7 crystals/ml, but how do you estimate the amount of crystals? This procedure is not clear."
The text has been updated to clarify this point in point 2a.9) (line 296):

> *"Estimate the concentration of crystals by using a haemocytometer as described by the manufacturer for cells. Count crystals as you would be counting cells."*

and 3.2a.1) (line 451):

> *"Based on the crystal concentration calculated in step 2a.9), adjust the concentration to 10^7 crystals/ml by diluting the sample (…)".*

- "Is the incubation time relevant to work "in cellulo" or for extraction?"
This has now been specified in step 3.1.4) with suggestions on incubation times to be tested as a first screen.

- "Is mesh-size relevant? In reference [2] it was used 800 µm."
The following text was added to step 3.2a.3):

> *"We recommend using 700 µm diameter meshes with holes of 25 µm as a starting point. The area of the mesh is not critical but a larger area provides more crystals per mesh and will lead to slower evaporation during preparation. The size of holes may need to be optimized to match the size of the crystals. Indexed meshes are available to facilitate a methodical grid scan for systematic studies."*

- "When the protocol is developed "in cellulo", how many crystals are obtained in each cell?"
This information has been included in Table 1. Also, the text now states:

> *"Note: The proportion of cells containing crystals will be different in each case and can even vary between preparations but in general one should not expect to find crystals in all cells. Similarly, the number of crystals per cell is also variable (see Table 1), and more than one crystal may be found in one cell. These factors need to be optimized where possible by adjusting the multiplicity of infection, altering the length of expression and varying the protein construct. On one hand, conditions of protein expression and cell growth that maximize the number of crystal-containing cells will improve productivity (e.g. higher multiplicity of infection and late harvesting of cell culture). On the other hand, conditions where cells contain a single crystal facilitate data collection (e.g. low multiplicity of infection). Given the enrichment brought by flow sorting described in step 2b, we recommend aiming for larger crystals (e.g. a single crystal per cell) even if the proportion of crystal-containing cells is low."*

- Do you need to orient the cell somehow to get single crystals under diffraction condition? This protocol is not completely clear.
A note after step 4.4) has been added to include this information:

> *"(…)If the collimated microbeam has approximately the same size as the cells (~10µm), all crystals contained in a cell will be illuminated simultaneously. For cells containing multiple microcrystals, this leads to the observation of multiple diffraction patterns, which can be difficult to process. However, diffraction from one of the crystals often tends to dominate the diffraction pattern and is preferentially indexed during data processing. For beamlines with an X-ray beam*

significantly smaller that the cell, rastering at very low X-ray flux (>95% attenuation) should be used to center on a single crystal before data collection."

- If full data sets can be collected at RT, why use cryo-preservation methods? It is clear that radiation damage can be reduced at 100 K but also manipulation (cryo-protection, etc.) can damage crystals affecting the final data quality.
While high quality data sets can be collected without the addition of cryoprotectant, they are still analysed at 100 K. We have clarified the text to avoid confusion by adding a sentence in step 3.2b.5):

"This only applies to the incubation of the cells with cryoprotectant solution; the sample should still be analyzed at 100K to minimize the effects of radiation damage on the crystals."

We now also mention this point in the Discussion:

"The in cellulo protocol also bypasses the requirement for incubation in cryoprotectant solution, while still allowing data collection at cryogenic temperature."

- Other JoVE article relevant for this work: Ishchenko et al. J. Vis. Exp. (115), e54463, doi:10.3791/54463 (2016).
This article is now cited in the Discussion.

Reviewer #3:
We have rewrote the protocol to present an in depth description of the identification and manipulation of microcrystals and crystal-containing cells for X-ray diffraction experiments. Some aspects will be complemented by the video protocol that aims to provide a visual guidance to microcrystal identification and manipulation.

- "How to find out if the cells actually contain crystals worth purifying."
We have added examples of what in vivo crystals may look like in Fig. 2 and table 1. We provide references for more examples in Table 1 and Ref. 21 for a review. However, like classical crystallization, the only definitive proof that crystals worth purifying are present is by obtaining X-ray diffraction and their aspect may not correlate with their usefulness for diffraction experiments.

-"How many crystals would I expect to see in a cell? Where would I expect to see them?".

In theory, all scenarios can occur for *in vivo* crystallization and many have already been described in the literature. Crystals have been observed in the ER, nucleus, cytoplasm and other sub-cellular compartments so that it is hard to predict where a new target might crystallize unless it is addressed to a particular compartment. The number of crystals per cell is now discussed in section 1 and to guide the reader we present examples in Fig. 2. (see also answer to Reviewer #2).

- How can one tell if PBS is the appropriate buffer system for any crystals?
PBS appears as a good starting point as a standard isotonic buffer with the aim of mimicking the cellular environment where the in vivo crystals grew. It has been used for most *in vivo* crystals grown in cell culture to date (Coulibaly et al. 2007, *Nature*; Koopmann et al. 2012, *Nature Methods*; Baskaran et al. 2015, *Nature Comm*). DMEM medium has also been used for *in vivo* crystals grown in mammalian cell cultures (Tsutsui et al., 2015, *Mol Cell*). If crystals visibly suffer from their transfer in these buffers or present weak or poor diffraction this parameter should be investigated as a potential source of degradation. This is now specified in a note at section 2a.3).

- How often do in-situ grown crystals dissolve or crack during harvesting?
Most naturally occurring protein crystals are very robust and we have not observed damage for any in vivo crystals produced by insect viruses. However there are reports of highly dynamic crystallization processes in vivo and more fragile crystals. This point is mentioned in the revised manuscript as described in more details in response to the main question of Reviewer #1.

- How do you know (what are you looking for) that ethylene glycol at 50% might not be the appropriate cryoprotectant?
The optimization of the cryoprotecting solution follows the same approach as classical crystallography. We have added a reference containing this information to guide the reader. Step 3.2a.5) now reads:

> *"Add cryoprotectant: pipet 0.5 µl of a solution of 50% ethylene glycol in water onto the mesh (the composition of the cryobuffer is to be adapted to the sample; suggestions for optimization of cryoprotectant can be found in Armour et al. 2013, JoVE)"*

- On line 246, "For other types of crystals the incubation period and concentration of heavy atoms should be determined experimentally". Well yes, but how can I tell it didn't work (do I have to go all the way to the diffraction experiment?)
Binding of heavy atoms to in vivo crystals is difficult to monitor because they may be lost during purification of the crystals and biophysical analysis. Moreover this approach would only confirm binding and not the usefulness of the crystals after treatment (i.e. diffraction power; isomorphism; phasing power). Thus, we concur with the reviewer that the only way to determine whether heavy atom soaks have been successful is by going all the way to the diffraction experiment and processing the data to detect if useful phasing signal is present. As suggested by both Reviewers #2 and #3 we refrain to provide detailed protocols and methods for structure determination steps, which are beyond the scope of this protocol.

This has now been clarified in step 3.1.4):

> *"(…) the incubation period and concentration of heavy atoms should be determined experimentally. Too short incubation times will not allow the incorporation of proper amounts of the heavy atom, while long incubation times can affect the integrity of the crystals, hence lowering their diffraction quality or even dissolving them. As a starting point, try 3 or 4 different incubation times for each heavy atom solution (e.g. 1 min, 1h, overnight, 3 days). Successful derivatization is assessed by analysis of diffraction data as briefly described in section 5 and references therein".*

- There seems to be an inconsistency between having to heavy atom soak for 3 days (line 244) and not storing crystals or cells for more than 3 days (line 204).
We have removed the specific time in section 2b.6). Although crystal-containing cells should be processed as soon as possible, in the case of polyhedra (and possibly other in vivo crystals), the incorporation of heavy atoms into the polyhedra crystal lattice requires very long incubation times to be effective because of the extremely low solvent content.

- Where does one irradiate a cell? Just point a microbeam somewhere and hope to see diffraction?
We have added details describing this process and the video will clarify this step (cf. answer to Reviewer #2).