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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 107 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 107 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).