Dr. Philipp S. Erdmann
Group Leader
Fondazione Human Technopole
philipp.erdmann@fht.org

21/07/21

To: The Editor
Journal of Visualized Experiments (JOVE)

Submission of a Revised Manuscript

Please find attached our revised manuscript entitled “Sample Preparation by 3D-Correlative Focused Ion Beam Milling for High-Resolution Cryo-Electron Tomography” and our point to point response to the reviewers’ comments. We would like to thank both reviewers and the editor for the constructive feedback and hope that our protocol is now suitable for filming and publication in JOVE.

Sincerely yours,

Philipp S. Erdmann
Research Group Leader in Structural Biology
Fondazione Human Technopole

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the manuscript entitled ‚Sample Preparation by 3D-Correlative Focused Ion Beam Milling for High-Resolution cryo-Electron Tomography', Bieber et al. provide a protocol for correlative light and electron microscopy of focused ion beam milled cells after plunge freezing. This protocol is based on the pioneering work by Arnold et al. (2016) and was recently applied by Wilfling et al. (2020). Such a protocol is very welcomed and it will facilitate the application of this complex but important cryo-CLEM workflow in other labs of the cryo-EM community. However, as it is written now, the protocol lacks many important details.

Concerns:

The authors present the workflow in a generalized way, although it is very likely that the workflow was performed using Aquilos dual-beam microscope (Thermo Fisher Scientific) and a cryo-confocal microscope (Leica). This generalization leads to a lack of necessary details that are required to reproduce the presented workflow. It would be more valuable for the reader if the protocol would include details concerning the systems that were used.

Since there's only a "limited" selection of equipment available at our institute, these experiments were of course not performed and tested on all the FIB/SEMs and cryo-FLM setups that exist. We've added a statement commenting on this in the introduction. Nonetheless, we have sampled quite a significant space from Quanta 3D FEG to Scios, and Aquilos1/2 on the FIB/SEM side, and Corrsight, Leica Thunder, and Leica SP8 cryo as cryo-FLMs, which here serve as a basis for our generalized workflow. Individual settings for these instruments are now provided (see updated Materials xsl). However, the workflow doesn't change for any of these systems and hence we prefer to keeping the general tone. This might also help reduce the impression, that only the latest high-end equipment can be used for this workflow. In fact, the "Representative Results" we chose to show were prepared using the oldest machines in our list: Quanta3D FEG and Corrsight.

In addition, the authors should add a section on the compatibility of this protocol with different systems such as Zeiss cross-beams, Linkam cryo-LM, integrated cryo-LM-FIB-SEM, etc.

Unfortunately, places with access to FIB/SEM instruments other than the TFS brand are rare - even more so are the TEM alternatives and we haven't been able to test the

workflow there. However, we hope that the provided settings will allow experienced users to successfully transfer our workflow to other microscopes.

A discussion on integrated FLM solutions is present in the discussion section. However, most of these systems (iFLM, METEOR, PIE-Scope) have either not been delivered (yet), or are at a late prototype stage. Accordingly, significant changes (hardware/software) are to be expected. Due to this, we haven't added a detailed protocol on their use, yet. That said, we've tested and validated the approach on a prototype of Delmic's METEOR with great success.

In the following you can find a list of specific points which need to be addressed:
1) Line 42: "While it is possible to combine FLM and cryo-ET data only after TEM acquisition" Please also provide the reference of the detailed description of the mentioned method: doi.org/10.1016/bs.mcb.2020.12.009

In addition to the original publication, we now also direct the reader to the protocol.

2) Line 71: "Note: The structure of interest should be present in the majority of your cells, which will significantly increase the throughput of the correlative approach." This contradicts the statement in the abstract: Line 27-29: "Using this technique, rare cellular events and structures can be targeted with high accuracy and visualized at molecular resolution using cryo-transmission electron microscopy (cryo-TEM)".

We now explain this in more detail: while 3D-targeted FIB milling is in principle so selective that a single POI-positive cell/grid could be targeted, this would not be very productive as each time after finishing one lamella grids would have to be unloaded and a new sample placed in the FIB/SEM.

Since there is no publication showing that rare cellular events and structures can be targeted with high accuracy on lamella yet, the statement in the abstract should be revised.

Both Arnold et al. and Wilffiling et al. in fact show localization, targeting, and milling of structures ~ 500 nm in dimension on lamellas. In Wilfling et al., we used the approach on two different target structures: END compartments and autophagic bodies. For the END, 1-2 structures are present per cell (~rare). It is true of course that there is (among other limitations) a size limit. A detailed discussion on the limitations (and how they are related) is now present in the discussion section.

As the authors point out later in the manuscript the major limitations of the correlation are the Z resolution and the diffraction-limited resolution of FLM (Lines 399-401). One should point out that with the current setup a rare event can be targeted but it has to be a fairly large structure.

This is pointed out in the discussion section.

3) Line 76: "We significantly increased our success rate using more rigid SiO2 films." Please provide data that would prove the significance. In our experience, we have not found much of a difference between carbon and SiO2 but we do not have data to show it. It would be very useftul to the community if this was systematically tested and shown.

We agree that a systematic test would be preferable, but this would be outside the scope of this protocol. Our statement is more “qualitative”, summarizing the general experience in our department (several publications pending). Also, we want to point out alternatives for Au/C and Cu/C grids to (frustrated) FIB 3D correlation newcomers. Our suggestion is also in line with what has been reported for SPA (stability) and recently by Mahamid et al. in their SerialFIB pre-print, which suggest Ti/SiO_2 to be even better. Of course, the verdict is still out whether mechanical stability or matched thermal expansion coefficients (or both) is the determining factor for successful 3D FIB correlation. We’ve added an appropriate “disclaimer” to our statement.

4) Line 85: Which type of grids do you recommend for yeast, bacteria, adherent cells?

We’ve added a list of recommended grids.

5) Line 94: Note. Please provide the exact protocol on poly-L-lysin/concanavalin coating.

We’ve added the respective protocols.

6) Line 106: 4 Hela cells per square (200 Mesh) are too many as they would occupy the entire square and likely be poorly vitrified. 1-2 adherent cells are more desirable.

The text has been changed as suggested. While it is true that 1 cell/square would be ideal, depending on where lamellas are cut, we have observed vitreous HeLa calls at up to 4/5 cells/grid. We now also point out the potential use of glycerol as protective agent.

7) Line 107: Please provide more details on grid patterning and add a reference.

We've added a short explanation why grid pattering is useful and now provide the (missing) reference. For a detailed protocol, please refer to said publications as it is outside the scope of this protocol and also not required for successful 3D-correlative FIB milling.

8) Line 109: Please provide more details on how to remove grids carefully from the dish. This step is challenging and bent grids will negatively influence the outcome. 3D printed holders or the addition of the PDMS layer can be used to facilitate grid recovery from tissue culture plasticware.

We have tested the 3D printed holders with little success in our lab. Accordingly, we cannot recommend it. The PDMS layer is a great suggestion and we've added it (including the reference from above).

9) Line 114: "Fiducials should be chosen both in size and brightness to be compatible". Please specify which beads (company and material numbers) are recommended.

According to JOVE guidelines, brand names etc. should not be present in the main text. We provide bead vendors (1 µm) in the materials xls file. Concentrations are provided in the main text.

10) Line 117-119: Statement: "…do not use beads too bright…" Please be more specific.

We've added a respective comment.

11) Line 137: Please specify how to judge optimal blotting time.

As requested, a comment has been added.

12) Line 145: Autogrids without cutouts are suboptimal and should not be recommended here.

We agree that this might be confusing to newcomers and we now only suggest cutout-autogrids. However, we still successfully use regular autogrids on occasion ...

13) Lines 143-169: Section 2 "Cryo-Fluorescence Light Microscopy" is missing many important details. Please provide settings for Z-stack sampling, NA, and magnification of the objective, if confocal or wide-field imaging was used. Please provide the X-Y, Z-

theoretical Nyquist resolution. This is very important for others to know to judge whether their system is suitable for the protocol.

Details have been added to the text and the “Settings” tab of the Materials .xls

14) Lines 174-222; Section 3 "Focused Ion Beam Milling" is again missing many details. This section is most crucial for the complete workflow and needs to be completely revised. Please provide a separate section with a detailed description of how the correlation and transfer of positions are done. Describe how to use 3DCT software using detailed figures describing step by step how this is performed. While this may be captured on the JoVE video later, there should be also a detailed written protocol available. The 3DCT seems to be a major advancement since Arnold et al. publication, yet it is not well explained here and many reader, buts may not be aware of it.

We have added a more detailed explanation to our text and also point out the online tutorial for 3DCT. The figures have been updated and improved as well.

15) Line 203: Note. The fact that fine milling must be done immediately after rough milling is limiting the number of lamellae that can be prepared due to ice deposition on polished lamellae. How many lamellae are recommended per session?

There is no definitive answer for that. It appears that all (TFS) FIB/SEM systems show slightly different recontamination rates (< 50 nm/h is “within specs”). We now try to explain this concept in more detail to allow users to make an educated decision on the exact number of lamellas that can be cut. But this needs to be up to the user and how fast they are ...

16) Line 205: "…or by using scripting…" The transfer of the milling position is the most crucial step for the precision of the targeted milling. A "manual" transfer of the position is certainly not precise enough to target any individual cellular structures. Please provide the code of the mentioned script and describe in detail the exact procedure.

Manual transfer in fact is precise enough (given precise enough measurements) and was used for the “Representative Results” here. However, automated transfer is of course preferable. We now also cite SerialFIB, which contains functionality to transfer positions from 3DCT to the FIB.

17) Line 206: Please add the missing reference for stress relief cuts (doi.org/10.1016/j.jsb.2019.09.006).

The reference has been added.

18) Line 213: "…generally thought to reduce data quality…". Please provide a reference for this statement.

We explain this now in more detail. Thicker samples correspond to more electron scattering events. A sample should therefore be thin and only consist of the material of interest, i.e. the cellular lamella. Recontamination adds unwanted, amorphous ice to both sides of the lamella. For an Aquilos1 for example, up to 50 nm/h is still considered “within specs”! This unwanted material of course will cause unwanted scattering events and hence deteriorate the signal.

19) Line 219; Section 3.6. Please provide more details on the evaluation of the accuracy of milling. What should one do in case the lamella is bent or has moved and how can one recognize that this has not caused that the FM signal is not anymore preciously corresponding to the initial coordinates?

We’ve added a comment, that users should regularly check for such movements. If it becomes too excessive (>300 nm), the site should be discarded as the correlation will be out of registration. A potential solution, i.e. integrated FLM setups, is also pointed out.

20) Lines 226-241; Section 4 "Correlative TEM": Please provide more details on how this step is performed. Especially Step 3.4 is essential for successful targeting and is not described well with essential information missing. Please add detailed step-by-step instructions on how this correlation is performed. Is the correlation done in SerialEM, in MAPS, or another software? A figure describing this step is necessary, as it is together with step 3.3 the most crucial step for the correlation.

Details have been added and are now supported with a dedicated figure.

21) Line 244: "While we have successfully applied this protocol to a variety of different samples" Please provide all references for the different samples on which the workflow was applied.

Our protocol in fact summarizes our experience on three different published target structures: lipid droplets (Arnold et al.), the END, and autophagic bodies (containing END) removed by autophagy (Wilfling et al.). There are several more pending for publication, including autophagy intermediates in yeast and mammalian cells, LLPS compartments involved in B-cell priming, and centrosomes just to name a few.

Obviously, they cannot be revealed at this point. The statement has been adjusted to account for that.
As a community service, however, we would like to make our experience available to other labs now.

22) Line 245-246: "…we here provide a walkthrough of the pipeline that was used in the discovery of the Ede1-dependent endocytic protein deposit (END) in S. cerevisiae." Please add the missing reference to the publication on which your representative results are based: Wilfling et al., 2020, doi.org/10.1016/j.molcel.2020.10.030

The reference has been added.

23) Line 255: "FLM stack acquisition" Please provide details on the used microscopy system.

Details have been added to the resource file.

24) Lines 269-270: "Since the correlation was found to be accurate.." Please describe how accuracy was determined and which accuracy exactly was measured. This is essential to evaluate the precision of the here described targeted milling.

The details on how accuracy can be judged have been provided in the protocol and discussion section.

25) Line 288: "Overall, a correlation success of ~75% was achieved" Please describe how the correlation success was defined and quantified. Please provide all correlation examples for this analysis.

Correlation success is defined as correlated lamellas that survived transfer to the TEM and sowed the END compartment. It is hardly possible to show all the positions that have been processed within the scope of this (video) protocol. We've added the total numbers (12 milled lamellas, 9 END structures found) to the statement.

26) Line 340, Figure 8: Please provide an actual correlated image of the FLM and TEM image in addition to the red cross which only indicates where the fluorescent signal is supposed to be.

The classical CLEM view has been added (Fig. 7B).

27) Line 356: "In this regard, testing different denoising or deconvolution techniques on the FLM data is also advised, as it may considerably improve the localization of fiducials and cellular signals.". Please provide references for denoising and deconvolution techniques suitable for cryo samples.

References for algorithms that can be used for deconvolution are provided (e.g. Huigens). But since there's nothing special about cryo-FLM data, any algorithm that works at RT should also be applicable to cryo-conditions. We now also cite Florian Jug's N2V.

28) Lines 392-401, Section 3 "Limitations of the method": Please make clear that this approach is sensitive to lamellae movement during milling in respect to the fiducial markers. In addition, it should be mentioned that fine milling should be performed immediately after the rough milling of each lamella. In such a case, the throughput is limited to few lamellae or it leads to increased ice contamination which is detrimental for subsequent cryo-ET.

We've added more discussion and suggestions regarding this topic in both the protocol and the discussion section. We also do recommend the site-wise approach. This however still allows experienced users to finish ~4-6 lamellas in a time-frame compatible with the recontamination rates of the FIB/SEMs.

Reviewer #2:

Manuscript Summary:

This manuscript provides a step-by-step protocol of a cryo-correlative microscopy workflow for 3D localisation of fluorescent signals in cells that are thinned by cryo-FIB milling and imaged by cryo-ET. Such a detailed description comes very timely as these methods are gaining popularity while they still require significant expert know-how. While for cryo-FIB milling and cryo-ET of cells, a number of good methodological papers are available, the practical aspects of how to use 3D correlative microscopy to prepare lamellae in a targeted fashion by precisely locating fluorescent signals during the milling process have been missing. The authors use the example of aberrant yeast endocytic sites which they and others have previously described to be autophagy-precursors and liquid-liquid phase separation events (Wilfling et al Mol Cell 2020, Kozak & Kaksonen, bioRxiv 2019).

While I support the publication of such a protocol article, I think the authors could improve the manuscript in multiple ways to make it a more helpful resource. Here's my suggestions for improvements, as well as some needed clarifications.

Major Concerns:
- As the correlative part is the central advance presented in this paper, the correlation procedures used should be most extensively explained. The descriptions of the correlation procedures (performed at multiple steps) do not contain sufficient details to be useful. This relates to all steps at which correlations between image modalities are done. It would also be good to explain whether and why certain software or algorithms are better for 2D or 3D correlations. Further example, in point 3.3 of the protocol and legend to Fig 4: what is 3DCT? If this is the preferred software, then it may be helpful to use it as a paradigm to describe the procedures in more detail.

As suggested, we now discuss the 3D correlation toolbox (3DCT; Arnold et al.) in more detail and point out an online walkthrough of its features and use.

- line 61- 62: The statement 'Ensure that sites of interest can be localized successfully with a good signal to noise ratio at room temperature in a fluorescence microscope' is very vague. What is a good signal to noise ratio? Can the authors provide some estimate? And what configuration of a fluorescence microscope is meant?

We now provide details on the microscopes and their settings (see Materials.xls). There is no estimate on the exact SNR that can be useful. We've changed the statement to rather alert the reader of the (obvious) fact that the signal of interest needs to stand out above background (in cryo!).

- line 67: In what respect did mVenus perform better than EGFP? This is again a vague and therefore not helpful statement.i

The hypsochromic shift is now discussed and why it may be useful for standard filter combinations. mVenus is also ~1.6x brighter than EGFP (ref. 25 in text) and hence leads to higher photon count and ultimately higher localization accuracy. [This note has been moved to discussion section]

- line 68-69: I do not think the sentence regarding trade-off between wavelength and localisation accuracy with reference to Abbe's diffraction limit makes sense. For the localisation precision of point sources (as are fiducials and the majority of signals dealt with here), Abbe's law is not limiting. The emission wavelength of the fluorophore can almost certainly be neglected for the localisation accuracy in CLEM. However, what does matter is the numerical aperture of the objective, because the number of collected photons (and hence the sensitivity) depends on it.

This conceptual issue seems to also be reflected in lines 117 -118: The localisation limits of conventional FLMs are at least one magnitude smaller than the size of beads of 0.5-1 µm, so I don't understand this statement. Beads of diffraction-limited size (like 50 nm or 100 nm) can be localised equally well - it is a matter of detectability and number of emitted and detected photons, not size.

It is true that the Abbe diffraction limit is not (the only) limiting factor here. This has been corrected and is now discussed in more detail. [This note has been moved to discussion section]

- line 88-89 and lien 355: What is an 'optimal' dilution of fiducial markers? Also, fiducial markers are mentioned early on in the protocol, without really explaining their purpose, which would make it easier to assess what is meant by optimal.

When we now introduce fiducial markers, we also provide more details on their function and optimal properties.

- line 107: Reference(s) for grid patterning protocols should be added.

We now cite the grid patterning protocol.

- line 111: Why are metal oxide grids mentioned? What would be their purpose? This mention comes out of the blue and it's not clear why.

This was indeed too vague and due to JOVE's instructions to not provide brand names in the main text. This has now been corrected and should be clear now.

- line 131: In fact to minimise contaminations it would be better to perform steps in the gas phase a few centimetres above the liquid phase of lN2, not in the liquid phase.

This seems to be a matter for debate and maybe "culture" of lab. To our knowledge, there is no literature showing that one way is significantly better than the other. In our hands, working in the liquid has worked well so far - at least for beginners. It may be true, however, that with experience (and steady hands) working in the gas phase is better.

- line 160-161, and Figure legend 3: Although convolution is mentioned in the text and shown in Fig 3, an explanation of what is actually done and why it would be beneficial for the CLEM procedure is missing. In particular the panels with graphs and analysis shown in Fig 3 are not explained at all in the legend. Also, the legend

does not say whether the red signals in Fig 3 are fiducials or signals of interest.

Deconvolution and its benefits are now better discussed. Fig. 3 and its legend have been updated.

- line 165-166: Why check for bleed through? In some cases (fiducial markers) bleed through is desirable because it allows to align channels to each other and correct for chromatic aberration.

This has been updated. We now describe why too much bleed-through would be problematic. We've added a comment regarding chromatic aberration.

- line 199-200, line 268 and line 364: accuracy of correlation is mentioned in several instances. However, the authors give no estimate for the accuracy in their example / proof of principle. Also, how dependent is the accuracy of locating the position of the lamella in z on the z-distribution of the fiducials? It would be good to get some estimates or ideas on that, or even better some actual measurements.

We've added a statement on the z-distribution of fiducial beads and that it is indeed beneficial to have a nice z-distribution. A detailed description on the accuracy of the method is available in Arnold. et al. However, we here provide some practical (rough) guidelines on the RMSE values and how to judge (e.g. base on fiducial beads not used for registration) if the correlation is trustworthy or not.

- line 217: Is bending / deformation really such a problem for correlation? If lamellae are bent by dimensions that are similar to correlation accuracy (which I guess is in the hundreds nm range), then they are too deteriorated for high-quality cryo-ET anyway. Deformations that are harmless to cryo-ET are likely to be neglectable for correlation. However, as the authors do not provide an estimate for the correlation accuracy in z, it remains difficult to assess.

We now mention the accuracy on the IB image also derived in Arnold et al., which is ~ 300 nm. This is in fact a combination of x,y, and z due to the tilted IB image with respect to the FLM stack. It is certainly true that lamellas that exceed this value should be discarded because they are too deteriorated. We now instruct the users to regularly check for such movement/bending and to discard the site if bending > 300 nm is detected.

- It may make more sense to swap the order of Fig 4 and 5.

The order of the figures has been revised.

- legend Fig 5: At this stage of the pipeline, the grid is already in the FIB microscope. So how can the title be 'finding suitable grid squares for FLM stack acquisition'? The figure shows steps after FLM.

The legend has been updated.

- legend Fig 8, line 340-341: 'can readily be correlated': How exactly? It may be obvious to the authors but to the reader, this is not helpful.

The details are now provided in the text.

- line 397: The use of stained lipid droplets as fiducials is mentioned. The relevant paper should be cited (Scher N. et al, bioRxiv 2021)

The citation has been added.

Minor Concerns:

- The summary says 'Proteins of interest (POIs) are fluorescently tagged, their 3D position …' the manuscript however does not describe fluorescent tagging of proteins. This should be modified.

This has been corrected.

- In the abstract, the phrasing doesn't make clear that FIB milling is the solution to thinning samples and thus making them amenable to cryo-ET.

This has been adjusted.

- line 74: 'stable are handled' should probably read 'stable and handled'

This has been fixed.

- line 77: By grid hole size, do the authors mean 'grid square size' or (carbon or SiO2) 'film hole size'?

This has been fixed.

- line 116: 'channel the biological' should probably read 'channel than the biological'

This has been fixed.

- line 179: What does 'as required by the system' mean?

What we meant to say was “depending on which TEM is available”. We’ve rephrased the sentence.

- line 214: 'two-step fashion: first rough, then fine': It is not clear that the authors mean first ALL lamellae are milled roughly, then ALL are milled finely (in contrast to each lamella is milled roughly as well as finely before proceeding to the next lamellae, as they then go on to suggest). This needs to be more clearly phrased.

This has been rephrased as suggested.

- line 229: be more precise what is meant by 'lamella orientation'. It is not clear from this sentence HOW it should be orientated. Perhaps refer to figure, that could help.

This has been updated and is now also illustrated more clearly by Fig. 2.

- line 231: by 'lamella square', do the authors mean 'lamella-containing grid square'?

This has been updated.

- The paragraph introducing Ede1 should cite the relevant literature.

The paper is now cited.

- Line 288: What does 'correlation success of 75%' mean? Does it mean that 75% of targeted spots could be correlated to similar structures found in cryo-ET? Or does it mean that 75% of spots identified by cryo-FM could be located to lamella?

We define as success the number of lamellas that survived milling and loading into the TEM and that contained END structures (9) vs. the total number of lamellas cut (12). This is now explained in the text.

- Figure legend 2: Does the Figure show HeLa cells? It should be specified in each legend what is shown in the figure, especially if it is not yeast cells which are the proof-of-principle in the manuscript.

Even though the protocol is compatible (and has at this point been tested on a variety of cells; several unpublished), we now choose to only show yeast cells as to make the entire protocol more consistent.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We did our best to check for spelling and grammar issues.

2. Please provide an email address for each author.

Email addresses for each author have been added.

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

The summary has been rephrased.

4. Please remove the citations from the Abstract and include them in the Introduction section.

We’ve removed all citations from the abstract.

5. Please ensure that abbreviations are defined at first usage.

Abbreviations should now always be defined upon first use.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. Please sort the Materials Table alphabetically by the name of the material.

Commercial language has been removed. Materials are listed in alphabetical order.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We've updated the protocol as suggested.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol.

Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly, and also one Protocol step cannot have multiple notes. Please consider moving some of the notes about the protocol to the discussion section.

We've updated the protocol as suggested. Where possible, "Notes" have been moved to the discussion section.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We've added more details so that each step is self-explanatory.

10. Please add more details to your protocol steps.

We have added details as also discussed above.

Line 86: what is meant by "any treatment" here? Again, please avoid generic statements in the Protocol step.

As treatments will HIGHLY depend on the biological system targeted by our 3D-correlative FIB approach, a generic term appears to be necessary here. We however now provide examples.

Line 94: Please mention the concentration of the cleaning compounds used and also briefly the cleaning procedure.

We here refer to plasma cleaning (i.e. no reagents). Settings for our instrument are now provided in the Materials xls.

Line 98: Please specify the centrifugation speed and temperature.

Since for some beads the procedure may differ, we have changed this sentence and now refer to the manufacturer's instructions (e.g. dialysis vs. centrifugation).

Step 1.3: Please provide the action steps as discrete steps. Rest can be moved to the Discussion part.

The section has been revised. Note has been moved to the discussion section.

Step 2.4/4.3: Please provide more details if this step needs to be filmed.

2.4 is now 2.3. As deconvolution should be a general enough concept, it doesn't need to specifically be filmed here.
By referencing earlier steps and providing some more detail (including also an improved figure), 4.3 should now be better explained, sufficient for filming.

Line 176: Please mention how to take the note on orientation.

This has been updated and should now be better explained in text and using Fig. 2A

Line 179: What organometallic and conductive layers to be applied? Please specify the amount and concentration.

Details are now provided. Settings are summarized in the Materials xls.

Step 3.2: Please provide more details if this step is to be filmed. Also, please see if the angle can be specified here.

Details are now provided.

Line 193: Please define the term IB.

Ion beam (IB) is now defined in 3.4 upon first use.

11. In the software, please ensure that all button clicks and user inputs are provided throughout.

We now also point out button clicks. Additionally, we reference a walk-through that explains individual buttons in 3DCT and their use.

12. Line 245: Please define Ede-1.

EH domain-containing and endocytosis protein 1 (Ede1) is now defined.

13. Line 388: Please define CLEM.

Correlative light and electron microscopy (CLEM) is now defined (line 59).

14. Please sub-number the Figures as (A), (B), (C), etc., where multiple panel figures are there.

Panel figures are now sub-divided in A), B), C) etc.

15. Figure 3: Please ensure that the description of the xy-axis is clearly visible in the graphical representations.

We've added the necessary labels to the axes.

16. Please remove the DOI numbers from the References

DOIs have been removed.