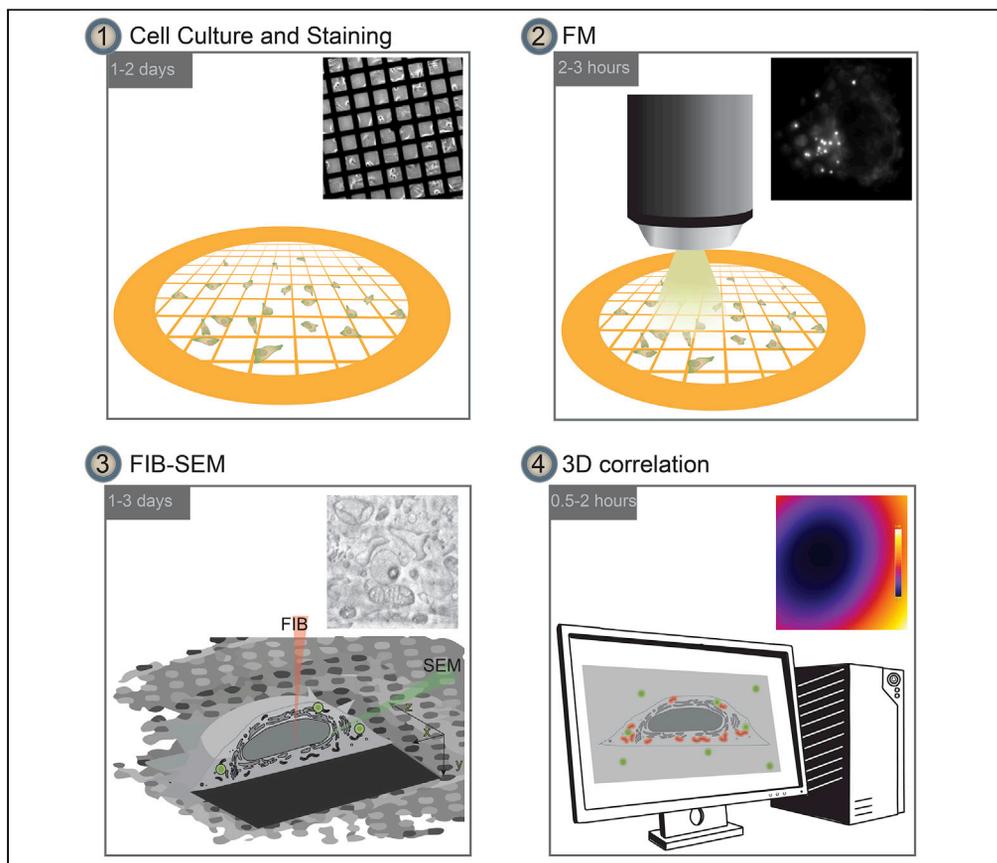


Protocol

Sample preparation and image registration for correlative cryo-FM and cryo-FIB-SEM of plunge-frozen mammalian cells



We recently demonstrated how lipid droplets can serve as *in situ* fiducials for correlating cryo-fluorescence microscopy (cryo-FM) and cryo-focused ion beam scanning electron microscopy (cryo-FIB-SEM) datasets of mammalian cells grown on grids. Here we describe a step-by-step protocol for correlative cryo-FM and cryo-FIB-SEM, starting from sample preparation of C2C12 cell line, followed by imaging with cryo-FM and cryo-FIB-SEM. Finally, we detail how to perform the 3D-correlation with sub-micron accuracy.

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Highlights

Detailed protocol for correlative cryo-CLEM

Step-by-step guide for cryo-FM and cryo-FIB-SEM of cells grown on EM grids

3D image registration pipeline and an example dataset for 3D-CLEM

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Protocol

Sample preparation and image registration for correlative cryo-FM and cryo-FIB-SEM of plunge-frozen mammalian cells

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SUMMARY

We recently demonstrated how lipid droplets can serve as *in situ* fiducials for correlating cryo-fluorescence microscopy (cryo-FM) and cryo-focused ion beam scanning electron microscopy (cryo-FIB-SEM) datasets of mammalian cells grown on grids. Here we describe a step-by-step protocol for correlative cryo-FM and cryo-FIB-SEM, starting from sample preparation of C2C12 cell line, followed by imaging with cryo-FM and cryo-FIB-SEM. Finally, we detail how to perform the 3D-correlation with sub-micron accuracy.

For complete details on the use and execution of this profile, please refer to Scher et al. (2021).

BEFORE YOU BEGIN

Read the materials data safety sheets before working on this protocol, order all the materials needed for the protocol, and ensure you have access to the instrumentation needed. Use safety equipment when working with liquid nitrogen and liquid ethane. The protocol below was utilized in a recent publication demonstrating the use of lipid droplets (LDs) as *in situ* fiducial markers for 3D correlative cryo light and electron microscopy (cryo-CLEM) (Scher et al., 2021). The following protocol was optimized for work with mammalian C2C12 cells and 200 mesh Au Quantifoil R3.5/1 electron microscopy (EM) grids.

Maintaining C2C12 cell culture

C2C12 cells were handled according to the ATCC handling information (<https://www.atcc.org/products/crl-1772>).

Hardware and software setup for the 3D correlation

⌚ Timing: 0.5–3 h

1. Ensure you have the necessary processing capacity for 3D data registration. Sufficient RAM and hard disk allocation will ensure expediency, given the large amount of imaging data involved (circa 1Gb for each 3D volume). The minimal requirements are 16GB of RAM (ideally 32GB ≤) and several GB of additional free storage space for paging in case Icy runs out of RAM, as well as a dedicated GPU. If you use the example dataset provided, 4GB of RAM will be sufficient.



2. Ensure you have the necessary software in place:
 - a. Download Icy from <http://icy.bioimageanalysis.org/download/> and install it according to the instructions on the site. The version used in this protocol is 2.1.0.1.

Note: Many plugins, including Spot Detector, are installed by default.

- b. Configure Icy to use the maximum available RAM. Depending on your system and the available memory on your computer, the memory settings may be automatically set up during the first run. These settings can be changed in the 'Preferences' panel ('Max Memory' option in the 'General' panel in the 'Preferences' panel).

△ CRITICAL: Icy requires Java in order to work. Download the correct version for your operating system from the link given on the Icy FAQ webpage: <http://icy.bioimageanalysis.org/faq/>. Java version 1.8 is advised, and 64-bits is mandatory; use the provided links on the Icy website.

Note: After launching Icy, the correct version of the Java machine will be visible in the 'Output' panel. For Java Oracle Java (TM) SE Runtime Environment 1.8.0_261-b12 (64 bit), make sure the VTK 8.0.1 library loads successfully. If a message notifying of the library's successful uploading does not appear, check the Icy FAQ, in particular, <https://forum.image.sc/t/vtk-not-loading-in-icy-version-2-1-x/47248/2> (permanent link).

- c. Download the ec-CLEMv2 plugin (not the ec-CLEM plugin). We used version 2.2.0b in this protocol.
 - i. In Icy, go to 'Preferences' in the 'Plugins' panel and ensure that 'Allow beta version' is selected. If not, check it, click 'Apply' and then 'OK.'
 - ii. Type 'ec-clemv2' in the search bar; it should appear as an online plugin. Select this plugin; this will prompt its automatic download.
 - iii. Close Icy for now.
3. To get familiar with Icy, use the online resource from Train others & yourself - Icy – Open-Source Image Processing Software (bioimageanalysis.org)

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene)	Thermo Fisher Scientific	Cat#D3922
DMSO, anhydrous	Biotium	Cat#90082
DMEM, high glucose	Thermo Fisher Scientific	Cat#41965039
HEPES 1M	Biological Industries	Cat#03-025-1B
Sodium Pyruvate 100mM	Biological Industries	Cat#03-042-1B
Penicillin-streptomycin solution	Biological Industries	Cat#03-031-1B
FBS	Thermo Fisher Scientific	Cat#12657-029
DPBS	Biological Industries	Cat#02-020-1A
DPBS without calcium and magnesium	Biological Industries	Cat#02-023-1A
Trypsin EDTA solution B	Biological Industries	Cat#02-052-1B
Experimental models: Cell lines		
Mouse: C2C12 cell line	ATCC	Cat#CRL-1772
Deposited data		
BODIPY signal cryo-FM 3D dataset <i>source_c1.tif</i>	(Scher et al., 2021)	https://doi.org/10.34933/wis.000399
Cryo-FIB-SEM dataset, binned ' <i>target_fibsem.tif</i> '	(Scher et al., 2021)	https://doi.org/10.34933/wis.000399

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
eC-CLEMv2 (v.2.2.0.0b)	(Paul-Gilloteaux et al., 2017)	ec-CLEMv2 - Icy – Open Source Image Processing Software (bioimageanalysis.org)
Spot Detector (Version 1.8.0.0)	(Olivo-Marin, 2002)	Spot Detector - Icy – Open Source Image Processing Software (bioimageanalysis.org)
ICY v2.2.0	(De Chaumont et al., 2012)	http://icy.bioimageanalysis.org/ RRID:SCR_010587
Adobe Illustrator	Adobe	http://www.adobe.com/products/illustrator.html RRID:SCR_010279
Amira (v2019.3)*	Thermo Fisher Scientific	http://www.fei.com/software/amira-3d-for-life-sciences/ RRID:SCR_007353
Fiji*	(Schindelin et al., 2012)	https://fiji.sc/ RRID:SCR_002285
Python 3.7*	Python software foundation	http://www.python.org/ RRID:SCR_008394
PyCharm – community version*	JetBrains	https://www.jetbrains.com/pycharm/ RRID:SCR_018221
Image processing python script*	(Spehner et al., 2020)	https://doi.org/10.1016/j.jsb.2020.107528
Other		
Quantifoil R 3.5/1 200 Au mesh	Quantifoil Micro Tools GmbH	EMS Cat#Q250AR-35
C-Clip Ring	Thermo Fisher Scientific	Cat#1036173
C-Clips	Thermo Fisher Scientific	Cat#1036171
EMS Swiss Line, standard Tweezer Style 5	Electron Microscopy Sciences	Cat#7800-5
35mm Glass bottom dish with 14mm micro-well #1.5 cover glass	Cellvis	Cat#D35-14-1.5-N
Plasma cleaner**	PIE Scientific	https://piescientific.com/tergeo_plasma_cleaner/
Automatic plunge freezer**	Leica Microsystems GmbH	EM-GP
Grid storage system	Subangstrom	Cat#GSS02
Cryo-fluorescence microscope**	Leica Microsystems GmbH	CryoCLEM
Cryo-coating system**	Leica Microsystems GmbH (Baltek)	EM-BAF060
Focused ion beam scanning electron microscope**	Carl Zeiss GmbH	Crossbeam 550 Ion Sculptor
Automatic liquid nitrogen filling system	Norhof LN2 microdosing systems	Cat#905
Cryo-sample holder (Modified)***	Leica Microsystems GmbH	Cryo-specimen holder #9 - blank holder
Vacuum cryo-transfer system**	Leica Microsystems GmbH	EM-VCT100
Vacuum cryo-manipulation system**	Leica Microsystems GmbH	EM-VCMS500

*The following softwares were used for image processing and analysis in the original study. This protocol does not describe them.

****Alternatives:** These instruments can be substituted with others that are capable of the same functions used in this protocol.

*****Optional:** To prevent sample exposure to ice contamination during the transfer, we modified a standard Leica cryo-holder by adding a pre-tilt and a copper lid to it.

MATERIALS AND EQUIPMENT

Recipes

C2C12 growth media (GM)		
Reagent	Final concentration	Amount
Fetal bovine serum	10%	50 mL
HEPES (1M)	2%	10 mL
Sodium pyruvate (0.1M)	1%	5 mL
Penicillin-Streptomycin (Pen: 10,000 units/mL, Strep: 10 mg/mL)	1%	5 mL
Dulbecco's Modified Eagle Media, high glucose	n/a	450 mL
Total	n/a	500 mL

Store at 4°C when not used. Open bottle only under biosafety cabinet.

BODIPY stock solution

Reagent	Final concentration	Amount
BODIPY (10 mg)	1 mg/mL	10 mg
DMSO anhydrous	n/a	10 mL
Total	n/a	10 mL

Keep in aliquots at -20°C; can be stored for a very long time as long as it does not go through cycles of thawing and freezing.

BODIPY staining solution

Reagent	Final concentration	Amount
BODIPY (1 mg/mL in DMSO)	1 µg/mL	4 µL
Dulbecco's Phosphate Buffered Saline	n/a	4 mL
Total	n/a	4 mL

Make fresh in a biosafety cabinet right before use, use PBS at 37°C and keep in 37°C until used.

Alternatives: Other neutral lipid dyes with different wavelengths can be used in the same manner, such as HCS LipidTOX or Nile Red (Greenspan et al., 1985).

STEP-BY-STEP METHOD DETAILS

Sample preparation – seeding cells

⌚ Timing: ~12–24 h

This part of the protocol addresses cell seeding, from grid preparation until the cells are ready for staining and plunge-freezing.

1. Glow discharge EM grids:

Note: While working with cells, use Au mesh grids.

- Place a glass slide inside a petri dish (100 mm round dish).
- With fine tweezers, gently place the desired number of grids, carbon layer facing up, on the edge of the glass slide so that $\frac{1}{4}$ of the grid is not touching the slide.
- Place the petri dish with the grids inside a glow discharge instrument; remove the cover of the dish.
- Use plasma cleaning to make the grid's surface hydrophilic: with air (15 sccm), radio frequency (RF) power 18W, duration 2min, pulse ratio 50/255.
- Take the dish out of the plasma cleaner and transfer it to a biosafety cabinet using proper aseptic techniques for working in a class II biosafety cabinet.

2. Prepare the dish for seeding:

Note: From this moment on, all the steps are performed in a biosafety cabinet.

- Inside the biosafety cabinet, prepare 2 glass-bottomed 35 mm imaging dishes. The volumes used in this protocol were optimized for the 14 mm glass-bottom dishes.
- Place 350 µL of GM inside each glass-bottomed well (only inside the well).
- Gently transfer up to 4 grids to each dish, carbon layer facing up. The grids should sink to the bottom. If not, gently poke the grid edge with a 10 µL tip until they break the surface tension and sink.

Optional: Alternately, carefully insert the grids on the edge using fine tweezers. This will require a better handling on the grids and will likely break less of the carbon film.

- d. Check the integrity of the holey carbon layer. Do not use grids in which more than ~75% of the grid squares have torn carbon.
- e. Keep at 37°C, 5% CO₂ until seeding.

Note: there is probably no upper limit for how long grids can be kept under these conditions.

Optional: For easier handling, put the 35 mm dishes inside a 100 mm plastic petri dish.

3. Seeding cells:

Note: All the solutions used in the following part are pre-warmed to 37°C.

- a. Wash the cells twice with Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium.
- b. Add 2 mL of trypsin solution to the cells and incubate for 5 min at 37°C, 5% CO₂.
- c. Check under the microscope that the cells detach; they will be round and move upon the gentle shaking of the dish. If most of the cells are still attached, incubate for an additional minute. If the cells do not detach, consider opening a new batch of trypsin.
- d. Add 8 mL of growth medium (GM) to the dish, tilt it to one side and wash it several times with the same solution to aspirate as many cells as possible. Transfer the cells to a 15 mL conical tube.
- e. Spin at 180×g for 5 min at room temperature.

Note: In this protocol, room temperature is within the range of 21°C–25°C, and we refer to this range throughout the protocol.

- f. Discard the supernatant and resuspend the cell pellet in 1.5–2 mL of GM.
- g. Remove 200 µL of GM from each of the dishes prepared in step 2. e. and add 3×10⁴ cells in 200 µL of GM only to the glass-bottomed area.

Note: The number of cells depends on the type of cell and grid used. The numbers here are optimized for C2C12 on a 200 mesh Au, Quantifoil R3.5/1 grid.

- h. Shake the dish gently to spread the cells evenly on the glass bottom.
- i. Incubate at 37°C, 5% CO₂ for 2–4 h.
- j. Check under the microscope that the cells have attached to the grids before gently adding 2 mL of growth media to each dish (Figure 1).

▮▮ **Pause point:** Incubate the cells for an additional 8–18 h at 37°C, 5% CO₂ before proceeding.

Sample preparation – staining

⌚ **Timing:** 30–45 min

To preserve the staining in the frozen cells, stain the sample right before the plunge-freezing. Although we describe staining only with BODIPY 493/503, additional staining, such as Hoechst or MitoTracker, can be applied simultaneously (Figure 2). Generally, every chemical dye can be used if it does not interfere with the other dyes. Skip the staining steps if the cells are genetically expressing all the desired fluorescent markers.

4. Wash cells 2–3 times with warm DPBS.
5. Incubate the cells with a warm staining solution of 1 µg/mL BODIPY 493/503 in DPBS for 20 min at 37°C, 5% CO₂.

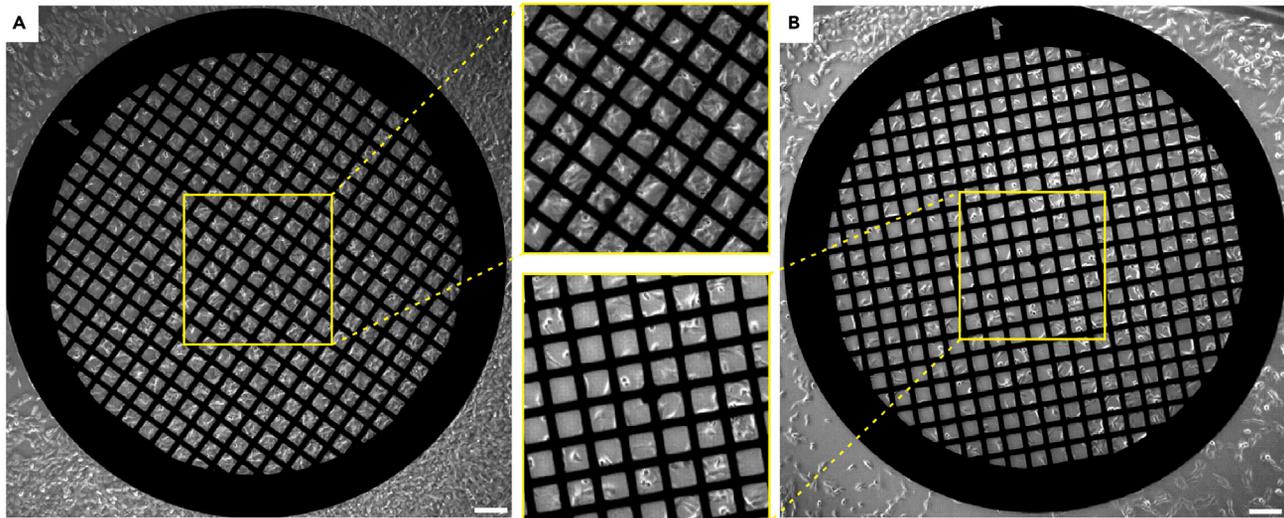


Figure 1. C2C12 cells on EM grids. Cells after 8–18 h of incubation

(A) When the cell concentration is too high, many cells are placed in every grid square. An overcrowded grid compromises the targeting of specific cells later on in the workflow.
(B) An optimal cell concentration is one that gives a good compromise between the number of cells and the ability to target a specific cell easily. Scale: 200 μm

Note: In our hands, we observed a decrease in signal intensity after a couple of hours. This is probably due to cellular processes. Therefore, it is important that plunge freezing will be done within an hour from BODIPY 493/503 wash to retain staining of lipid droplets.

6. Wash out the staining solution thrice with warm GM and transfer the cells to an incubator.

Note: It is recommended to keep cells in optimal incubation conditions (37°C, 5% CO₂) and so, we recommend having an incubator in proximity to the plunger.

Sample preparation – plunge freezing and clipping

⌚ Timing: 2 h

Plunge freezing is a cryo-immobilization approach in which the sample is rapidly plunged into a liquid cooled to cryogenic temperature. The rapid freezing prevents ice crystal formation and is essential for the sample preservation (Dubochet et al., 1988). Our workflow uses clipped grids for easier grid handling, which increases the robustness of the workflow, however, this is not a requirement and unclipped grids work as well.

Note: The workflow presented here uses a Leica EM-GP plunger (Leica Microsystems, Wetzlar, Germany); modifications may be required for other plungers. The following link has a short video tutorial demonstrating the plunge freezing process in Leica EM-GP: https://www.youtube.com/watch?v=Cyu9IggIqKg&ab_channel=LeicaMicrosystems. Always work in accordance with the manufacturer’s guidelines.

7. Check the integrity of the plunging tweezers, as bent tweezers might damage the grids upon plunging.
8. Turn on the plunger and fill the humidity bath with deionized H₂O.
9. Replace the blotting paper in the plunger and set the plunger to the following parameters:

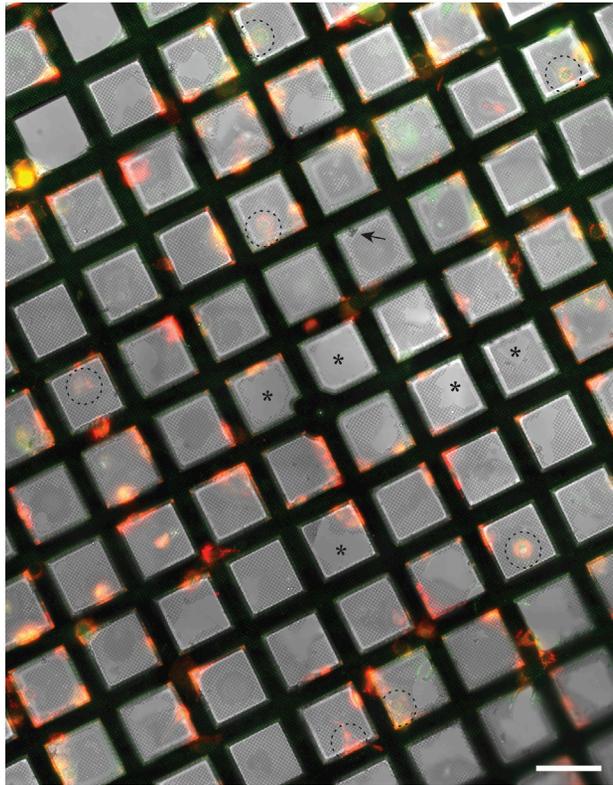


Figure 2. Plunge-frozen grid under cryo-FM

An EM grid of C2C12 cells stained with BODIPY493/503, to label lipid droplets (green), and MitoTracker DeepRed, to label mitochondria (red). Potential cells for cryo-FIB-SEM image acquisition are cells that are positioned close to the middle of the grid square (black, dashed circles). During the sample preparation steps, part of the carbon layer on top of the grid tears (black asterisks) and ice particles attach to the sample (black arrow), which may block the cells below them. Both tears and large ice particles can help with sample re-orientation during the cryo-FIB-SEM session. Scale: 100 μm .

- a. Humidity: 90%.
- b. Temperature: 37°C.
- c. 2.5 s back blotting.

Note: The calibration of the plunger changes between different instruments of the same type. Therefore, in the beginning of each session we perform a mock test with a wet grid to determine the blot arm position parameter. Check that the blot arm touches the grid pushing it slightly forward. A wet blotting mark appears on the blotting paper after the blotting.

Note: The blotting time is calibrated according to the cell density and the grids used in [Scher et al. \(2021\)](#). If using other cell types or grids, the protocol may need to be adjusted. Different cells may also require a different level of humidity and temperature.

- d. Automatic plunging: on. This mode immediately plunges the grid after blotting.
10. Cool the system by pouring dry liquid N_2 into the reservoir. When the vigorous bubbling stops, fill the rest of the reservoir and the grid box place.
 11. Place a grid box in the designated place next to the ethane cup in the plunger.
 12. Fill the liquid ethane cup to its brim with ethane.

Note: To prevent waste of gaseous ethane, stop filling ethane if the temperature indicator rises above -160°C , and continue once the temperature returns to -178°C .

13. Pick one grid using the plunger's tweezers. When the tweezers are mounted, the cells should face away from the blotting paper.
14. Move the tweezers to the loading position, press blot and check that a blotting mark remains on the blotting paper ([problem 1](#)).
15. Press 'transfer' and gently detach the tweezers with one hand while holding the lower part of the tweezers with the other to keep the grid from hitting the cup.

Note: To reduce the contamination hazard from breathing, wear a face shield while handling the frozen samples.

16. Rapidly transfer the grid to the box.
17. Once all the grids are plunged, store the box in liquid N_2 and dry the plunger by starting the plunger's bake-out function.

△ CRITICAL: From this moment on, every tool that touches the frozen grids needs to be cooled to liquid N_2 temperature before handling of the grids.

Note: The grids can be clipped at a different time. However, we recommend clipping immediately after the plunge-freezing.

Optional: Clipping. Briefly, clipping is done by fixing a c-shaped clip into a c-clip ring with an EM grid in it under liquid N_2 and using a special clipping tool and station. To account for the short working distance (WD) of the cryo-objective in the cryo-FM, it is important that the cells face away from the c-clip while clipping. For a detailed clipping tutorial, go to the Unit 2 Sample Preparation lecture of the Cryo-EM Course by Caltech (course website: <http://cryo-em-course.caltech.edu/>).

⏸ Pause point: Store the grids in a liquid N_2 storage system until imaging.

Cryo-fluorescence microscopy

⌚ Timing: 2–3 h per grid

Leica cryo-CLEM FM transfer shuttle can accommodate clipped grids. For older cryo-CLEM models and workflow, including video demonstrations, see [Schorb et al. \(2017\)](#).

18. Turn on the operating computer and all the microscope's components.

Note: To prevent communication errors, wait until the computer finishes booting before turning the camera on.

19. To reduce thermal fluctuations, pre-cool the objective:
 - a. Lower the cryo-objective $\frac{3}{4}$ of its range.
 - b. Insert the pump into the liquid N_2 Dewar slowly. After the bubbling of liquid N_2 relaxes, start cooling.
 - c. Allow the system 45 min to stabilize.
20. While the microscope is cooling:
 - a. Turn on the LASX software and allow stage initialization.
 - b. Open Leica's MatrixScreener HCSA (High Content Screening Automatization) software and load the CLEM module ([Schorb et al., 2017](#)).

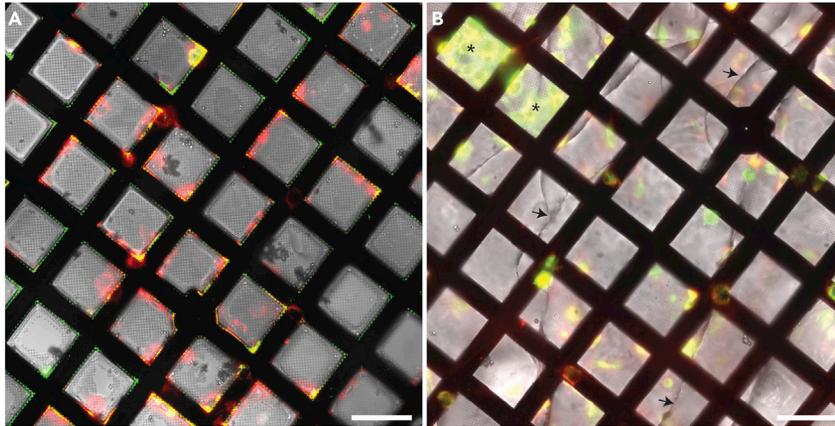


Figure 3. Thick ice under cryo-FM

(A and B) EM grids of C2C12 cells stained with BODIPY493/503, to label lipid droplets (green), and MitoTracker DeepRed, to label mitochondria (red), in an overall good condition (A) and an overall bad condition with respect to ice quality (B). In (B), fluorescent signal is hazy (black asterisks), and cracks are apparent in the ice (black arrows). Scale: 100 μm .

- c. Fill a dry Dewar with liquid N_2 to cool the cryo-CLEM shuttle.
 - d. Bring the grid box from the liquid N_2 storage.
 - e. Put the sample holder on the sample holder loading station.
 - f. In the last 5–10 min of stabilization, while wearing a face shield, cool the shuttle and keep it covered.
21. Loading the sample:
- a. Transfer the grid box to the designated place in the shuttle and mount one grid, with its cells facing up, to the sample holder.
 - b. Grab the sample holder with the transfer rod, retract the rod, and move away the sample holder loading station.
 - c. Cover the shuttle and attach it to the microscope.
 - d. Raise the objective slightly to avoid damage during the transfer.
 - e. Open the valves of the microscope and the shuttle, transfer the sample, retract the rod, close the valves, and detach the shuttle.
 - f. Lower the objective to its final position.
- Note:** For a detailed tutorial of the cryoCLEM protocol, check Appendix A. of Schorb et al., in particular the Methods video S1 (Schorb et al., 2017).
22. Find focus using the brightfield.
 23. To observe the grid's condition, apply a spiral scan with brightfield and GFP. This step will allow you to determine the overall condition of the grid, including the quality of the ice (Figure 3). If the grid is badly bent or the ice is too thick, do not use this grid. Replace it with another grid and refer to [problem 1](#).
 24. Mapping the grid:
 - a. Define a region in the middle of the grid, excluding the grid squares close to the c-clip ring. The view of the cells close to the c-clip might be obscured once the sample is tilted in the FIB-SEM.
 - b. Acquire maps in all relevant channels. The step size is 1–2 μm over a range of 50–100 μm .
 - c. Go over the generated mosaic and mark cells of interest with an auto-focus point indicator.
 - d. To obtain a map for re-orientation in the FIB-SEM, save a snapshot of the mosaic with all the focus points.
 25. Acquire a single tile stack for every cell of interest using a step size of 0.15–0.25 μm over 10 μm .

Note: Change the range according to your cell's height.

Note: While 0.15 μm step size is beyond the z resolution of the instrument, we wanted to have the option to apply deconvolution to the stacks.

26. Unloading the sample:
 - a. Cool the shuttle and attach it to the microscope's stage.
 - b. Raise the objective slightly to avoid damage during transfer.
 - c. Open the valves of the microscope and the shuttle, grab the sample, retract the rod, close the valves, and detach the shuttle.
 - d. Store the grid in its box.
27. If another grid is to be imaged, repeat steps 21–26. Otherwise, proceed to next step.

Optional: Bake out:

- a. Raise the objective to the maximal height.
- b. Heat the microscope stage from the pump controller.
- c. Turn off all the components but the pump.
- d. After the temperature reaches 60°C and the stage is dry, turn off the pump and take it out of the liquid N₂.

▮▮ Pause point: The grids are stored until FIB-SEM imaging.

Cryo-FIB-SEM

⌚ **Timing:** 1–3 days

Our Crossbeam 550 (Carl Zeiss, Oberkochen, Germany) cryo-FIB-SEM workflow uses a sample transfer system by Leica (Leica Microsystems, Wetzlar, Germany), which includes a cryo-stage, Vacuum Cryo-Transfer shuttle (VCT), and a modified Vacuum Cryo-Manipulation system (VCM). The modification of EM-VCM was made to reduce ice contamination to the sample while exposed during mounting. The instrument loading chamber was enclosed in a thick nylon sheet to form an improvised glove box around it. To dry the air inside the enclosed space, dry gaseous N₂ flows into the chamber at ~ 1.5 psi (Figures 4B–4D, Methods video S1). The steps related to the modified version of the VCM will be marked as **optional**.

Optional: We recommend performing a mock experiment with the instrumentation before putting a sample in the cryo-FIB. This step will ensure that minimal problems are encountered during the actual experiment.

28. Microscope preparation:
 - a. Install the cryo-stage in the chamber and pump down the system. We recommend doing this a night before the session so the system will have time to stabilize.
 - b. Initialize the stage and move it to the cryo-transfer position.
 - c. Cool down the microscope with liquid N₂; the working temperature should be lower than -140°C.

Note: Start cooling the microscope once the vacuum is lower than 5×10^{-6} mbar.

29. Loading sample:
 - a. Place a cryo-sample holder inside the VCM (Figure 4A, Methods video S1).
 - b. Turn on Leica's VCM.

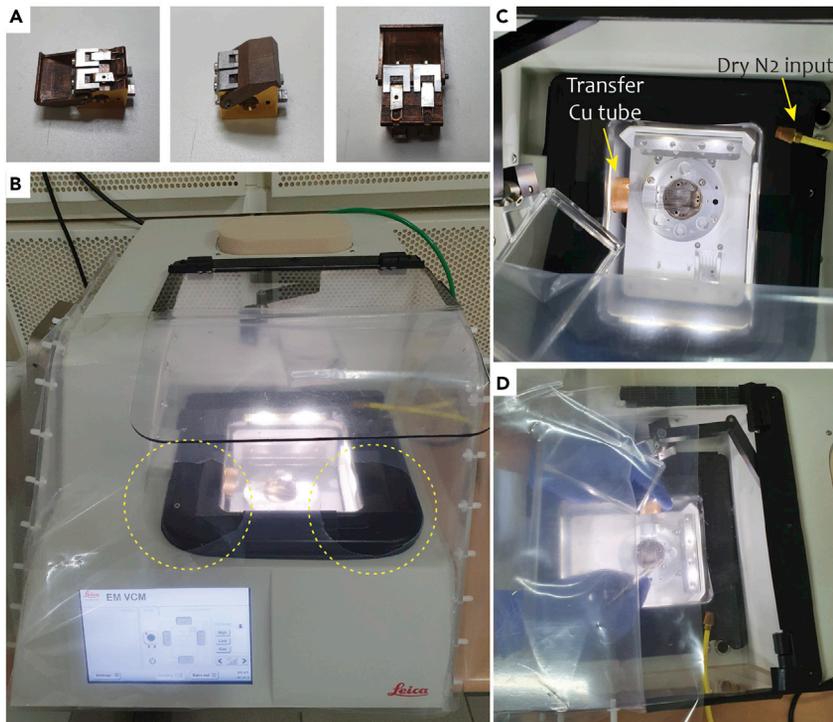


Figure 4. Modified cryo-sample holder and vacuum cryo-manipulation (VCM) setups

(A) A cryo-holder designed based on Leica's sample holder, shown from three different angles. The stage holder has a pre-tilt of 25° and a copper lid to protect the sample during transfer. In the right image, a clipped grid is placed in the left slot.

(B) A modified version of a Leica EM-VCM. The instrument is sealed with two clear plastic sheets, with a two-layered plastic tarp located between them. The lower layer of the tarp has holes for the entrance of hands (yellow circles).

(C) Top view of the cryo-manipulation chamber. To keep a dry environment with as little vapor contamination as possible, dry N₂ is inserted into the chamber (~1.5 psi). An additional Cu tube is installed in the transfer inlet gate as a cryo-shield over a gap between the inlet gate and the VCM cold bath cage.

(D) A demonstration of the work inside the VCM through the glove box. The upper layer of the tarp rests on the forearms of the user, ensuring a closed environment. Throughout the work process, a constant flow of dry N₂ and a low-humidity environment (< 3% H₂O) is maintained.

Optional: To work in a drier environment, we recommend warming the VCM chamber to 50°C–70°C for one hour, and to purge the enclosed space with dry N₂ for 5–10 min before cooling it down.

- c. Fill the reservoir with dry liquid N₂.
- d. Start pumping liquid N₂ to the chamber using the 'low fill' mode.
- e. Pump a VCT and keep it pumped in standby. We pump the VCT while it is connected to a BAF060 (Bal-Tec, Liechtenstein) instrument, but it can be connected to any other VCT shuttle compatible port.
- f. When the VCM chamber is ready, place the grid box inside the chamber.
- g. Open the box and transfer grids onto the sample holder, cells facing up.

Note: To increase the success rate, we recommend loading two grids at once, in case one sample was damaged or the regions of interest are not easy to relocate.

- h. Cover the sample holder with its lid to prevent sample contamination.
- i. Cool the pumped VCT and attach it to the VCM.
- j. Grab the sample holder with the VCT rod and rapidly retract it to the VCT chamber.

- k. Detach the VCT and attach it to the Crossbeam.

Optional: To reduce unwanted charging effects and improve the image quality, coat the sample surface with a 5–10 nm conductive layer of Pt or carbon prior to loading it into the FIB-SEM. We use a BAF060 instrument for this purpose.

- l. Transfer the sample holder to the chamber. Make sure it slides into the cryo-stage before retracting it a little to slightly turn it and remove the lid from the sample.
- m. Detach the VCT from the crossbeam.

Optional: Close the dry N₂ valve to stop the flow to the VCM.

30. Cryo-Serial Surface Imaging (SSI):

- a. Turn on both the FIB and the SEM columns.
- b. Move the sample to the SEM observation position and adjust the working distance (WD) to the FIB-SEM coincidence point (5 mm). Choose an object in the central area of the grid square for this adjustment.
- c. Take a wide field of view image of the entire grid (~100X magnification) at high resolution (at least 3027x2304 pixels). This allows future digital zoom-in on regions of interest (ROIs).

Note: The typical SEM settings for imaging of unstained cryo-cross sections are 1.6–2.3 kV, 25–40 pA. To avoid damaging the cross-section during scanning, keep the electron dose as low as possible, while considering signal-to-noise ratio. This is done by reducing beam current and pixel dwell time. The origin of contrast mechanism is in changes of secondary electrons (SE) yield, which are influenced by local surface potential ([Grabenbauer et al., 2013](#); [Vidavsky et al., 2016](#)). Fine alternations in accelerating voltage need to be made to optimize the visualization, and change from sample to sample, therefore a range is given.

- d. Upload an FM map on a support computer and align it to the same orientation of the SEM map by flipping / rotation, using an image-processing editor (we used Fiji standard image transform functions ([Schindelin et al., 2012](#))). Every landmark can aid in retracing an ROI: central mark, finder grid labeling, broken grid squares etc.
- e. Navigate the stage to ROIs, inspect them with a higher magnification of the SEM.

Optional: Upload the transformed FM image to the 'Image Navigation' folder of the Smart-SEM platform. Three-point registration will allow you to navigate the stage by clicking on the FM map positions.

- f. Adjust the height of the stage to the WD of the coincidence point for each ROI.
- g. Run SmartFIB software to operate the FIB.
- h. Inspect the ROI with the FIB. The ion beam is destructive to the specimen; therefore, it is recommended to use low ion beam currents for imaging. Typical parameters for imaging are 30 kV, 10–50 pA. Adjust the stage tilt to make the ROI clearly visible at the FIB field-of-view. Correct the WD if needed.

Note: There are two possible setups for SSI: Normal-to-FIB and Low Angle. The Normal-to-FIB orientation positions the FIB perpendicular to the surface and minimizes the ion beam damage on the surface. However, when used on plunge-frozen cells on grids, this limits the exposed area on the cross-section. The Low Angle orientation has a smaller tilt angle and is usually used for lamella preparation. It exposes a larger area on the cross-section at the expense of the beam causing greater damage to the edges of the cross-section. We prefer to work as close as possible to the Normal-to-FIB orientation.

- i. Perform Pt-organometallic layer deposition to protect the sample surface from damage during milling:

Note: The deposition in this protocol is neither electron beam nor ion beam induced. The precursor condensates on the colder surface of the specimen.

- i. Make sure that the Pt precursor is at room temperature.
- ii. Increase the WD to 7–8 mm.
- iii. Open and close the nozzle of the gas-injection system (GIS) for 3 cycles of 30 s each.

Note: We use a multi-port GIS, but the steps are similar with a uni-port GIS.

- j. Move back to the working position and perform a milling test.

△ CRITICAL: Adjusting the FIB-SEM settings for milling and imaging are performed on another, unimportant, cell.

- k. Adjust the ion dose factor and dwell time to suit the sample's particular properties. For the mammalian cells used in our study, we used an ion dose of (160–200 mC/cm²). The typical milling current range is 50–300 pA for a 10–30 nm slice thickness.

Note: The dose factor, milling current, and slice thickness change the milling duration and affect the acquisition duration. To cover large volumes in several hours, we suggest increasing the slice's thickness according to the object of interest's dimensions and milling currents, when possible.

- l. Cut a cross-section with a reasonable exposed area, showing at least 2 μm of the cell in height for SEM imaging adjustment. It is important to have some features on the cross-section to focus on, even milling artifacts can help at first, before image adjustment on a cellular object.
- m. Optimize the SEM parameters to obtain an acceptable image quality. Our acquisition parameters were as follows: acceleration voltage 1.6 kV, current 40 pA, pixel dwell time 12–22 μs, pixel size 10 nm, line average N=140. Detection: InLens SE and external SE2 were mixed at a ratio of 0.6–0.7 to reduce charging artifacts and optimize the signal to noise ratio ([problem 2](#)).

Note: To prevent electron beam damage, we keep the scan speed as fast as possible while the image quality is not greatly compromised. This, in turn, helps shorten the session's overall duration. SEM imaging parameters may vary for different cells.

△ CRITICAL: Always work with the tilt correction feature on –36° when imaging a cross-section. The FIB and SEM alignment always sets a cross-section at a 36° angle to the SEM.

Optional: Some samples may require back-scattered electron (BSE) detection in addition to SE detection. Atomic number-based contrast may add information which is not available with SE detection. This is the reason why heavy metal staining in room temperature FIB-SEM imaging generates contrast from a smooth cross-section. Despite the fact no heavy metals are introduced in cryo-FIB-SEM imaging, BSE detection is helpful when there are inherent heavy atoms in the samples, such as in biominerals. In such cases, we recommend using the dual channel feature in SmartSEM and collecting images using the energy-selective back scatter (EsB) detector in parallel.

Optional: Setting the dynamic focus and scan rotation: In some cases, the focal point in different heights along a cross-section might be slightly different. To adjust the focal point using dynamic focus, first adjust the focus in the middle of the field of view. Then, use 'reduced scanning area' in the upper right part of the image and change the dynamic focus value to

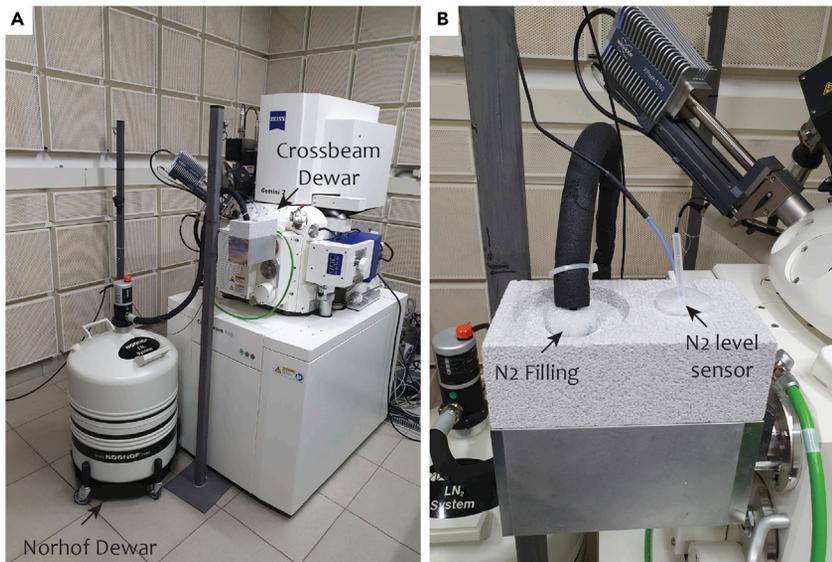


Figure 5. Liquid nitrogen refilling for long unsupervised acquisition.

(A) For long acquisitions, the microscope Dewar is connected to a microdosing system that refills liquid N₂ every two hours. (B) Close-up of the connection between the microdosing Dewar and the microscope Dewar. The N₂ is replenished every two hours and the filling stops once the level of liquid reaches the sensor.

obtain a focused image. Check if the image is in focus on the bottom left part as well and re-adjust iteratively. Use the scan rotation feature to align the base of the cell with the x-axis.

- n. Move to the desired ROI and take a FIB image with a low current (20–50 pA).
- o. On the FIB image: Place a milling pattern on the ROI. Select or create a recipe for serial sectioning with the adjusted milling parameters.
- p. Create a folder for data collection, link the path to the recipe.
- q. Start the serial milling. If necessary, correct the SEM image quality on the exposed section's face.
- r. Adjust a reduced scanning area to minimize the scanning time per frame.
- s. Follow the SSI process for the first 1–2 h and, if the session is stable, continue for long-term acquisition. This time is required for the system to reach a steady state, balancing milling and imaging iterations. If the cross-section drifts, refer to Problems 2–3.

Note: Other instruments may require shorter or longer stabilization times.

- t. Long-term acquisition requires an automatic liquid N₂ supply for the periodic refilling of the cryo-FIB-SEM Dewar, which normally needs to be refilled every 2 h. We use Norhof #905 (NORHOF LN₂ MICRODOSING SYSTEM, The Netherlands) with a volume of 50 liters in the one-sensor controllable mode. The microdoser pumps liquid N₂ into the microscope's Dewar once every two hours, until it reaches the sensor's level. The 50-liter container can supply liquid N₂ for 2–3 days of continual work (Figure 5).

▮▮▮ **Pause point:** After the data collection is completed, transfer the data to a third-party computer for further processing. For detailed image processing pipelines, see [Spehner et al. \(2020\)](#). Take out the grids, passing through the VCM if they are to be kept, and store until future use.

3D correlation: general housekeeping

⌚ Timing: 0.5–3 h

Table 1. List of deposited files

File name	Image type	What is it?	Instrument /acquisition software	Image data properties			
				File extension	Data units	Pixel size (μm)	Field of view dimensions (pixels)
<i>source_c1</i>	Uni-channel FM stack	Cryo-FM z-series in GFP filter (ex430-490/em500-550)	Leica cryo-CLEM/LASX	.tif	8-bit integer	0.129 × 0.129 × 0.15	584 × 584 × 71
<i>target_fibsem</i>	Binned tomogram	Cryo-FIB-SEM serial section imaging	Zeiss Crossbeam 550/SmartSEM and SmartFIB	.tif	8-bit integer	0.040 × 0.040 × 0.040	884 × 892 × 550

For this protocol, we have uploaded a dataset for correlation (DOI: <https://doi.org/10.34933/wis.000399>); the details of the dataset are presented in Table 1.

31. Identify the data and metadata required.

Note: Because the images are collected at different time points and on different imaging modalities, it is convenient to have all the files in one directory, which allows one to easily start the different registration steps.

3D correlation of datasets

⌚ Timing: 0.5–2 h

Correlating the binned FIB-SEM dataset and the BODIPY signal involves two main steps. The first is optional automatic registration, which consists of initializing the transformation without identifying the pairs of fiducials (steps 32–35). The second (step 36), which must be done in any case, consists of manual accurate registration by matching fiducials. If you decide to go directly to this manual step, open and check the metadata, as outlined in steps 32 and 33, before jumping to step 36.

Note: The acquired cryo-FIB-SEM dataset was processed as described in Scher et al. (2021) prior to performing the correlation steps described below. Image processing varies based on the data acquired and, therefore, we do not cover this issue in this protocol.

32. Open the FM images, check the metadata, and extract LDs center:

- Launch Icy.
- Drag and drop the fluorescence image stack containing the BODIPY signal. We use *source_c1.tif* in this step. Check the metadata in the 'Sequence Properties' panel (Figure 6A). The stack pixel size should be 0.13 μm in the x and y and 0.15 μm in the z step. Edit the metadata by clicking on the edit button, if needed. Delete any previously added ROI (select all ROIs in the 'ROI' panel).

Note: While 0.15 μm step size is beyond the z resolution of the instrument, we wanted to have the option to apply deconvolution to the stacks.

Note: In the *source_c1.tif* FM stack there are 19 LDs, one of which does not appear in the cryo-FIB-SEM dataset because it was out of the FIB-SEM's field of view.

- Search for the *Spot Detector* plugin in the search bar. Launch it while keeping all the default settings apart from the 'Detector' panel. Select 'Detect bright spots over dark background,' set the spot size to 'Scale 2' (by checking the associated box) and the associated sensitivity to 10 (Figure 6B). In the 'Output' panel, check the box 'Export to ROI' (Figure 6C).
- Click 'Start Detection'; spots should then be displayed as ROIs on *source_c1.tif*.
- Move the z cursor on the stack to parse z and check the segmentation (Figure 6D). Segmented ROIs are represented in green; the red circle indicates the xy position of their

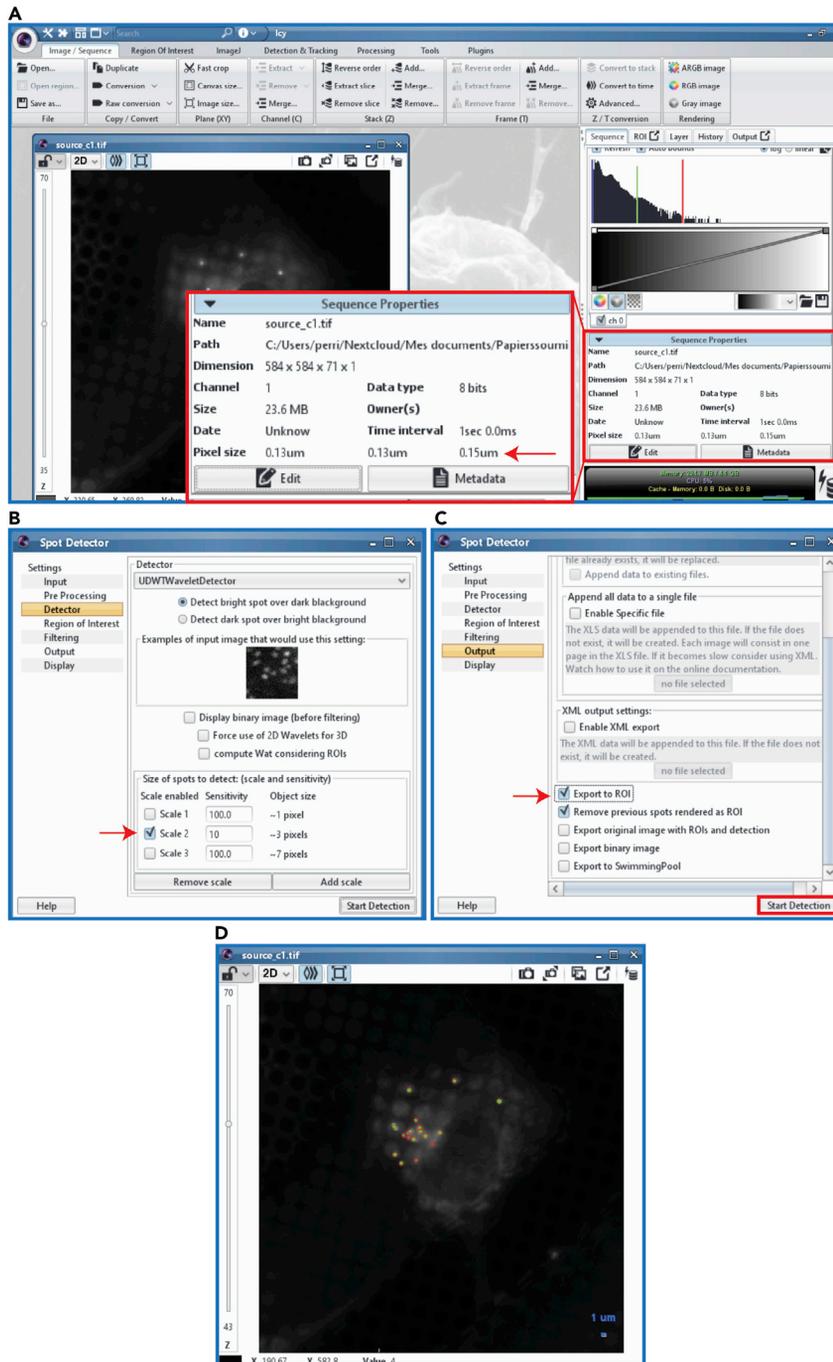


Figure 6. Metadata validation and LDs center extraction

(A) Icy working environment showing the opened FM dataset *source_c1*. The most important step in the correlation procedure is to validate that the metadata in Icy is correct (red box), which involves checking the pixel size (red arrow) and editing, if needed. Without the correct pixel size, the transform is not computed properly.

(B) To find the LDs centers, the Icy Spot Detector plugin is used. Simply search it using the search bar in the upper left corner of the Icy banner (shown in A.). In the 'Detector' panel, set the spot size to 'Scale 2' (red arrow) and check that the associated sensitivity is 10.

(C) Go to the 'Output' panel to export the centers to the ROI list (red arrow). Then, press the 'Start Detection' button (red rectangle). D. The Spot Detector output is presented on the FM stack, which shows the segmented spots (green) and their centers (red circles' centers).

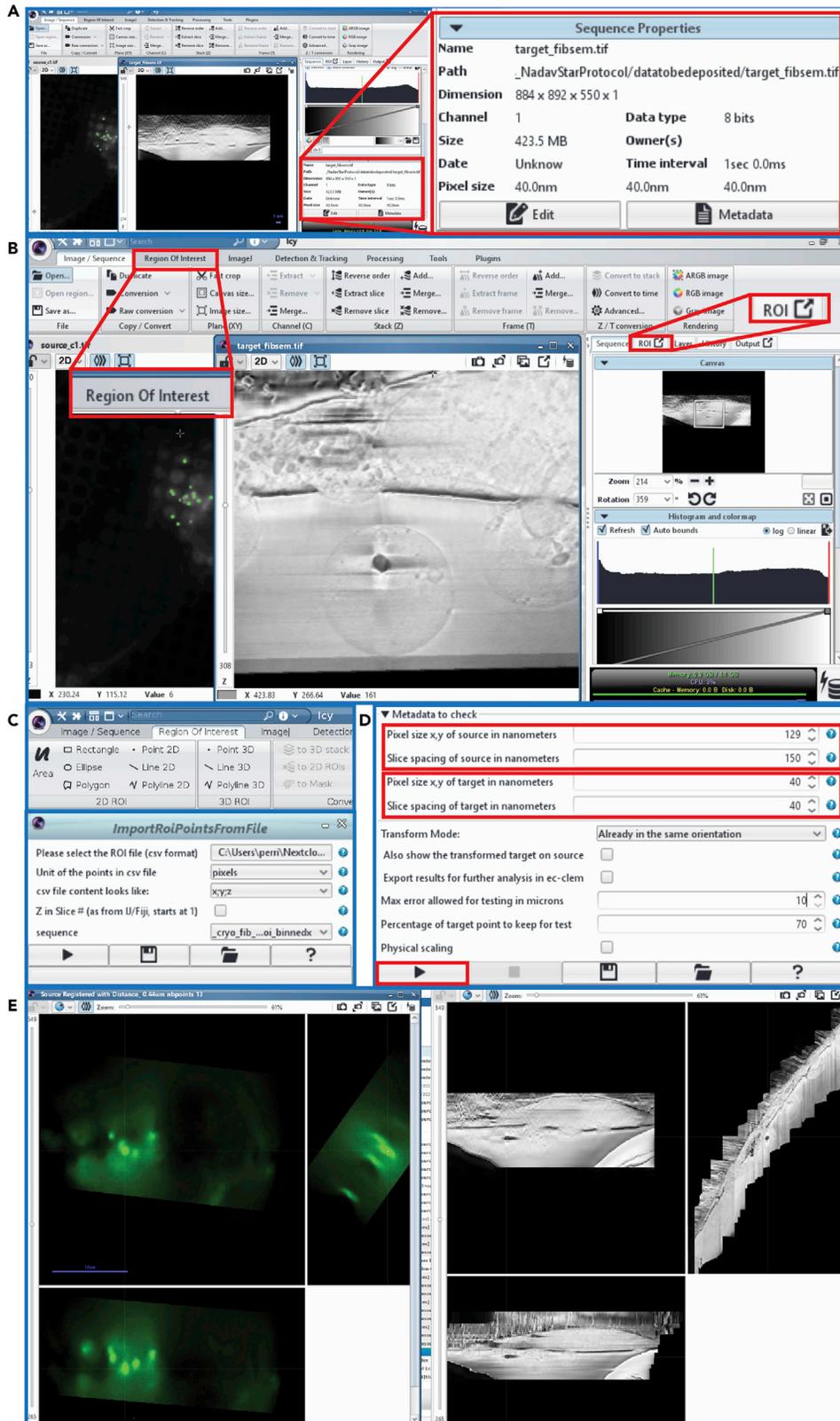


Figure 7. LD picking in FIB-SEM and AutoFinder rough transformation

- (A) When loading the FIB-SEM stack onto Icy, it is critical to validate the entered pixel size dimensions in the 'Sequence Properties' panel (red box).
- (B) LDs can be manually picked using the 'Region of Interest' panel (left red box). Before you begin, make sure to delete any remaining ROIs in the ROI list of the FIB-SEM dataset (right red arrow), zoom in and mark the center of the LDs with a Point 3D object. Make sure to pick the center of the LD in z as well.
- (C) Upper part: the 'ROI' panel; we use Point 3D objects to pick the ROIs. Lower part: In case the centers of LDs were found using different software, such as Amira, import the coordinates from a .csv file, as described in step 33. c.
- (D) After picking the centers of the LDs, the first transformation is done with AutoFinder. The dimensions of both the source (upper red box, FM stack) and the target (lower red box, FIB-SEM stack) need to be checked again; the rest of the parameters are adjusted according to step 34. Press the 'Play' button (bottom left red box) to start the transformation.
- (E) The result is a transformed (rigidly) FM stack that has the same dimensions and orientation as the FIB-SEM data.

centroid, regardless of their z. The centroids disappear when closing the *Spot Detector* plugin. If you are not satisfied with the result, see [problem 4](#).

- f. Close the *Spot Detector* plugin and keep *source_c1.tif* opened.

33. Open the FIB-SEM stack and manually click LDs center of mass:
 - a. Drag and drop the FIB-SEM data, here binned for memory and speed purposes. We use *target_fibsem.tif* in this step. Check the metadata in the 'Sequence' panel ([Figure 7A](#)); the stack dimensions should be 40 nm in x, y, and z. Edit the metadata by clicking on the edit button, if needed. Delete any previously added ROI (select all ROIs in the 'ROI' panel) ([Figure 7A](#) and [7B](#)).
 - b. Use the mouse or the 'Sequence' panel to navigate in the FIB-SEM data and identify the center of LDs ([Figure 7B](#)).
 - c. Select 'Point 3D' from among the 'type of ROI' options in the 'Region of Interest' panel. Click on the approximated center of each LD parsing the z (If you click and no ROI points are appearing, see [problem 5](#)). The provided cryo-FIB-SEM dataset should include 18 LDs but selecting only 15 could be sufficient.

Optional: Alternatively, if you have identified the LDs in another software, such as Amira, you can use `ImportRoiPointsFromFile`. When importing EM ROIs from Excel:

- i. Open the .xls file in Excel and remove the first column and first row. Keep only the columns with the x, y, and z coordinates. Save as a .csv file with comma separation.
- ii. Open `ImportRoiPointsFromFile` in Icy and load your ROIs to the EM data ([Figure 7C](#)).

34. Launch AutoFinder and set the parameters as follows ([Figure 7D](#)):
 - a. Source image: FM with spot-detected ROIs.
 - b. Target image: FIB-SEM with manually picked ROIs.
 - c. Transform mode: Already in the same orientation.
 - d. Max error: 10 μm .
 - e. Percentage of target points to keep: 70%.

The max error and percentage of target points to keep should allow to find a transformation in most cases. Additional explanation and how to change their values are described in [problem 6](#).

35. Press the 'Play' button to initiate the AutoFinder registration ([Figure 7E](#)).

Note: If the initialization provided by this method is not good enough, see [problems 6](#) and [7](#).

36. Compute the 3D image registrations:
 - a. Save the transformed source and give it an explicit name. It is advised to use the `SaveAsTiff-Fast` plugin for Icy. You can now close the *source_c1.tif* stack.

Note: The transformed source image now has the same metadata as the FIB-SEM dataset.

△ **CRITICAL:** For each image opened in Icy, check that the metadata, such as pixel size and image dimensions, are correct. The metadata are shown in the right bar in Icy; edit it if needed. eC-CLEM will not be able to compute the transformation correctly if these details are incorrect.

Note: If the metadata are incorrectly changed in Icy, this can be fixed by deleting the new xml metadata file that was created in the same folder as the image; this should reset the metadata to the original values.

- b. Choose the *Affine* transformation model and the *Isotropic* noise model.
- c. Add fiducial markers to each of the images, dragging the corresponding point to the same place in the other image as it appears. Rotate the field of view of an image if it helps with finding the same area in both images.

Optional: To make it easier to look at the same field of view in both datasets, pick the same numbered lock icon in the top left panel of both stacks. 'Lock 3' keeps the z dimension independent between the datasets, which is convenient for adding fiducial markers.

Optional: z-alignment may be more easily done by observing the orthogonal view of the image stack. In Icy, click on the drop-down arrow in the top left corner of the image and change it from '2D' to 'OrthoViewer.' This may help place the points more accurately in the center of the point spread function of each fluorescent point.

Note: You can zoom in and out using the mouse scroll wheel. The image will zoom to the location of the mouse pointer on the image.

△ **CRITICAL:** When finding locations for fiducial markers, rotate the image using the right mouse button only, as this will not alter the image properties. The images might be flipped with respect to each other. Do not use Icy's 'Flip' tool (or any other image-transformation tool that is not a part of eC-CLEM), as such changes are not tracked by eC-CLEM, which would result in an incorrect final transformation schema. Instead, place 2–3 points and ask eC-CLEM to compute the transform and it will flip the image during the transformation, after which you can easily place more points and re-compute the transform.

△ **CRITICAL:** When picking points in 3D registration in eC-CLEM, make sure to first scroll to the correct slice in both stacks before placing a marker point. The program adds the second marker in the other stack to whichever z slice is displayed, and this cannot be changed.

- d. Click on 'update transform' after placing 3–10 points. The transformation files will be automatically saved in the same folder as the source images.

Note: Three files are saved per computation. The file required for recomputing the 3D transformation is a .xml file with the suffix *transformation_schema*.

- e. Add more points if required and re-compute the transform.
- f. Once you are pleased with the transform, and after result evaluation (see the following section), the same transform can be applied to any additional FM channel acquired for the same dataset by uploading the channel and transforming it with the *transformation_schema* file.

Optional: To avoid a cropping effect and to limit the interpolation of your data, combine the automatic registration and the step 36 registration as a cascaded transform as described in [Vyas et al. \(2021\)](#), by using the EcClemCascadeTransform plugin:

- i. Click 'Add' and select the *transformation_schema* .xml file created by AutoFinder.
- ii. Click 'Add' and select the *transformation_schema* .xml file created by the 3D registration.
- iii. Click 'Choose File Name' and give an explicit name to your transformation schema, such as *original_cryoFM_to_cryoFIBSEM_schema*.
- iv. Press 'Play'.
To use this file, open the original FM data, launch the ApplyTransformation plugin, select the .xml file created in step c., and press 'Run'.
For additional information on cascaded transform, check eC-CLEM documentation and video tutorials over the GitHub page: <https://github.com/anrcrocoval/ec-clem/>.

3D correlation: evaluation of results

The accuracy of the co-registration can be evaluated using eC-CLEM. Two types of error calculations are available: (1) an error map showing the general distribution of the error in the whole image; (2) an error for each of the placed fiducial markers.

37. Generate an error map in eC-CLEM:
 - a. After computing a transform, click 'Compute the whole predicted error map'.
 - b. The error map can be scaled by adjusting the histogram.
 - c. The appearance of the scale bar or color bar can be toggled under the 'layer' panel in the right bar shown in Icy.
 - d. The maximum and minimum values on the color bar show the values of the maximum and minimum error.
38. Install the *histogram* plugin in Icy (search for 'histogram' in Icy) to plot the error map distribution. The width of the plot on the x-axis corresponds to the registration error and the y-axis portrays the number of points with each error value.
39. Measure the error between points used as landmarks by clicking on 'show measured error.'
 - a. Zoom in to the fiducial points to see red lines corresponding to the error.
 - b. Use the 'ruler helper' tool in icy to measure the length of the red line, to obtain the error magnitude.
40. 'Display 3D ellipse,' which represents the 95% likelihood of matching, gives a measurement of the correlation accuracy.

EXPECTED OUTCOMES

Upon completion of the protocol, the two acquired datasets will be co-registered, as demonstrated in [Scher et al. \(2021\)](#), with an error estimation in the range of a few hundred nm. The accuracy of the correlation is dependent on the FM modality and fiducial markers used.

LIMITATIONS

The given protocol should work with any type of cell grown on grids. However, different cells will have a different LD distribution, which might require further optimization of the protocol or finding a different internal fiducial marker altogether. In addition, poor data quality in cryo-FIB-SEM may cause the correlation procedure to be challenging and lower the accuracy. We recommend following imaging protocols such as this one or those of other published works ([Grabenbauer et al., 2013](#); [Scher et al., 2021](#); [Spehner et al., 2020](#)) to find the best imaging parameters suitable for your samples in cryo-FIB-SEM.

TROUBLESHOOTING

Problem 1

The ice on the grids is too thick (Figure 3). Determining if the ice is too thick is made mainly by two observations in the cryo-FM: the appearance of large cracks (in brightfield), and the haziness of the fluorescence signal, both demonstrated in Figure 3.

Potential solution

Ice thickness is determined by the blotting during plunge-freezing protocol. Therefore, make sure that the blotting paper touches the back side of the grid and that a clear liquid mark is apparent on the blotting paper after blotting (step 14). If the ice obtained is still too thick, consider increasing the blotting duration, or change the blot arm position (step 9.c) of the Leica EM-GP (analogous to blotting force in the Vitrobot).

Problem 2

Charging artifacts on the cross-section during cryo-FIB-SEM image acquisition.

Potential solution

As discussed in step 30.c., the accelerating voltage has a strong influence on the SE yield, thus altering the local surface potential. We recommend altering the accelerating voltage, SEM scan settings (scan speed, different noise reduction features; line or frame averaging), and change mix detection ratio; add more weight to the SE2, which is less sensitive to the charging of the cross-section.

Problem 3

The imaging region drifts in the SEM during cryo-FIB-SEM image acquisition. Usually, image drifting is caused by the suboptimal conductance of the specimen, which will cause the cross-section to slowly drift in one direction until it disappears, causing the loss of information. Image drifting can also happen if the geometry used for imaging is different from the recommended for normal-to-FIB orientation. In this case, the cross-section will disappear in an upward direction.

Potential solution

To solve drifting caused by suboptimal conductance, repeat the steps in 30.i. to apply an additional platinum organometallic compound on the surface. If this step does not improve the conductivity, coat the sample with a 5–10 nm-thick conductive layer using either C or Pt (see optional in step 29.k) in a Bal-Tec BAF060 or comparable system (e.g., Leica ACE600/ACE900).

If the drift is caused by the geometry used (see note in step 30.h), check the imaging session occasionally, and once the cross-section is close to the upmost edge of the image, pause the session and shift the SEM along the y-axis to place the cross-section in the center.

Problem 4

Difficulty in segmenting the BODIPY signal automatically in FM.

Potential solution

Make sure the imaging parameters on the microscope are optimized (e.g., images are not over- or under-exposed). Moreover, improving the FM image using image processing may enhance the detected signal. Among the tools that can help are deconvolution, denoising tools, filtering such as an unsharp mask, and local contrast-enhancement tools. If the spot detection still does not work, you can always manually pick LDs.

Problem 5

Clicking on the image does not add new ROI points.

Potential solution

Go to the 'Region of Interest' panel and select again 'Point 3D' (See [Figure 7C](#), upper part).

Problem 6

AutoFinder cannot find a correct solution for correlation.

Potential solution

If incorrect image transformation ([problem 7](#)) does not fix incorrect image transformation, try changing the parameters proposed in steps 34.d. and 34.e. (max error allowed for testing and percentage of target point to keep for test). Those parameters are used in the Random Sample Consensus – Iterative Closest Point (RANSAC-ICP) procedure of AutoFinder in eC-CLEMv2. Briefly, this procedure consists of randomly extracting a subset of points in both images and register them. Then testing what percentage of the full number of points is registered correctly, i.e., the registered point distance is below the maximal error allowed for testing (in this protocol, 10 μm). Because this process is iterative, if the maximum distance value is too restrictive, the first steps of this process will stop too soon. The default parameters proposed by eC-CLEM are 10 times the largest voxel dimension. In this protocol, the z sampling was 0.15 μm , but we expected a larger error due to the resolution in z itself, about 1 μm , so we used 10 μm as maximal error value. AutoFinder will try to find a rigid transformation, however if the transform is not perfectly rigid as in this case, it is advised to insert a higher maximal error value (10 μm).

The percentage of points to be kept depends on the ratio of the number of detections: it represents some minimal number of points that need to be matched and aims to account for outliers, or detections counted twice in one image. For example, 70% of 20 points means that at least 14 points will need to be registered correctly. A higher value for the percentage of target point to keep for test will push the software to look for additional matching, and may give a more accurate result, while increasing the risk to find no transformation at all. A lower value may decrease accuracy and lead to false matching.

Problem 7

Incorrect image transformation.

Potential solution

Usually, incorrect image transformation happens due to incorrect pixel size settings. In this case, reopen the stack and check that the metadata shown in Icy is correct (steps 32.b and 33.a).

The final 3D transformation may be incorrect if the 2D transformations have a large registration error. Check the transformation accuracy in the 2D images beforehand by merging the reference and the target images after each 2D registration. If there are still issues with the image registration, use the sample data-set used in this study (see data and code availability section) to practice the registration steps.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ori Avinoam, ori.avinoam@weizmann.ac.il.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets used to demonstrate the correlation protocol in this study have been deposited to Weizmann Institute of Science Library: <https://doi.org/10.34933/wis.000399>.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101142>.

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AUTHOR CONTRIBUTIONS

N.S. and O.A. conceived and designed the experiments and analyzed the data. N.S. with help from K.R. carried out the experiments. N.S. with the help of P.P.-G. correlated the data. All the authors wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- De Chaumont, F., Dallongeville, S., Chenouard, N., Hervé, N., Pop, S., Provoost, T., Meas-Yedid, V., Pankajakshan, P., Lecomte, T., Le Montagner, Y., et al. (2012). Icy: an open bioimage informatics platform for extended reproducible research. *Nat. Methods* 9, 690–696. <https://doi.org/10.1038/nmeth.2075>.
- Dubochet, J., Adrian, M., Chang, J.-J., Homo, J.-C., Lepault, J., McDowell, A.W., and Schultz, P. (1988). Cryo-electron microscopy of vitrified specimen. *Q. Rev. Biophys.* 21, 129–228.
- Grabenbauer, M., Schertel, A., Ruhwedel, T., Möbius, W., Snaidero, N., Laue, M., and Han, H.-M. (2013). Cryo FIB-SEM: volume imaging of cellular ultrastructure in native frozen specimens. *J. Struct. Biol.* 184, 355–360. <https://doi.org/10.1016/j.jsb.2013.09.024>.
- Greenspan, P., Mayer, E.P., and Fowler, S.D. (1985). Nile red: a selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* 100, 965–973. <https://doi.org/10.1083/jcb.100.3.965>.
- Olivo-Marin, J.C. (2002). Extraction of spots in biological images using multiscale products. *Pattern Recognit* 35, 1989–1996. [https://doi.org/10.1016/S0031-3203\(01\)00127-3](https://doi.org/10.1016/S0031-3203(01)00127-3).
- Paul-Gilloteaux, P., Heiligenstein, X., Belle, M., Domart, M.C., Larjani, B., Collinson, L., Raposo, G., and Salamero, J. (2017). EC-CLEM: flexible multidimensional registration software for correlative microscopies. *Nat. Methods* 14, 102–103. <https://doi.org/10.1038/nmeth.4170>.
- Scher, N., Rechav, K., Paul-Gilloteaux, P., and Avinoam, O. (2021). In situ fiducial markers for 3D correlative cryo-fluorescence and FIB-SEM imaging. *iScience* 24, 102714. <https://doi.org/10.1016/j.isci.2021.102714>.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. <https://doi.org/10.1038/nmeth.2019>.
- Schorb, M., Gaechter, L., Avinoam, O., Sieckmann, F., Clarke, M., Bebeacua, C., Bykov, Y.S., Sonnen, A.F.P., Lihl, R., and Briggs, J.A.G. (2017). New hardware and workflows for semi-automated correlative cryo-fluorescence and cryo-electron microscopy/tomography. *J. Struct. Biol.* 197, 83–93. <https://doi.org/10.1016/j.jsb.2016.06.020>.
- Spehner, D., Steyer, A.M., Bertinetti, L., Orlov, I., Benoit, L., Pernet-Gallay, K., Schertel, A., and Schultz, P. (2020). Cryo-FIB-SEM as a promising tool for localizing proteins in 3D. *J. Struct. Biol.* 211, 107528. <https://doi.org/10.1016/j.jsb.2020.107528>.
- Vidavsky, N., Akiva, A., Kaplan-Ashiri, I., Rechav, K., Addadi, L., Weiner, S., and Schertel, A. (2016). Cryo-FIB-SEM serial milling and block face imaging: large volume structural analysis of biological tissues preserved close to their native state. *J. Struct. Biol.* 196, 487–495. <https://doi.org/10.1016/j.jsb.2016.09.016>.
- Vyas, N., Kunne, S., Fish, T.M., Dobbie, I.M., Harkiolaki, M., and Paul-Gilloteaux, P. (2021). Protocol for image registration of correlative soft X-ray tomography and super-resolution structured illumination microscopy images. *STAR Protoc.* 2, 100529. <https://doi.org/10.1016/j.xpro.2021.100529>.