***Read through and understand this document before contacting* eBIC staff**

Guidance for cryoFIB proposals

Find the latest document here:
https://www.diamond.ac.uk/Instruments/Biological-Cryo-Imaging/eBIC/Current_calls.html

Who should apply

Researchers who want to examine structures within the interior of cells at high resolution using tomography or diffraction of crystals in a cryogenic TEM.

Important points

- Only automated, on-grid thinning is performed
- TEM grid atlases are required for a rapid proposal
- The number of lamellae prepared will vary depending on the size, density and location of milling sites as well as ice thickness
- For CLEM experiments, inform the LC at least three weeks in advance
- Instrument cooling starts at the beginning of the session. Onsite LC support ends at 5 pm.
- Samples entering eBIC must be biosafety level 2 or lower *and* be deactivated by 70% ethanol

How to apply

Proposals are made through the user administration system (https://uas.diamond.ac.uk).

Application and experiment information:

- For rapid access:
  - You must be ready to carry out your session ***before*** you submit your proposal
  - You will need at least two good grids per session
  - Proposals are reviewed weekly by eBIC staff and scheduled for the next available slot
- For BAG proposals:
  - Sessions can be scheduled any time within the AP you apply for
- For either rapid or BAG routes, grids can be supplied over multiple weeks. Grids will be clipped into an AutoGrid ring. A maximum of two grids are loaded for a 48-hour session.
- Investigators can attend remotely
- Lamellae can be stored at eBIC
- Lamellae can be examined on an eBIC Krios using BAG time or a rapid session or at another institution

In the UAS:

- For uploading the Science Case there is a two A4 page and a 2 MB limit. Links to file hosting services can be within this document.
- Under Instruments, select “Aquilos”
- Choose 4 shifts *i.e.* 32 hours

What makes a good submission

Showing frozen grids and demonstrating grid quality in your application is essential. Screen your grids in a TEM and show the TEM atlas.

Good grids (Figure 1) will have:

- Thin vitreous ice – observe transmission around the milling sites
- Enough milling sites close to the centre of grid squares
- An intact support film

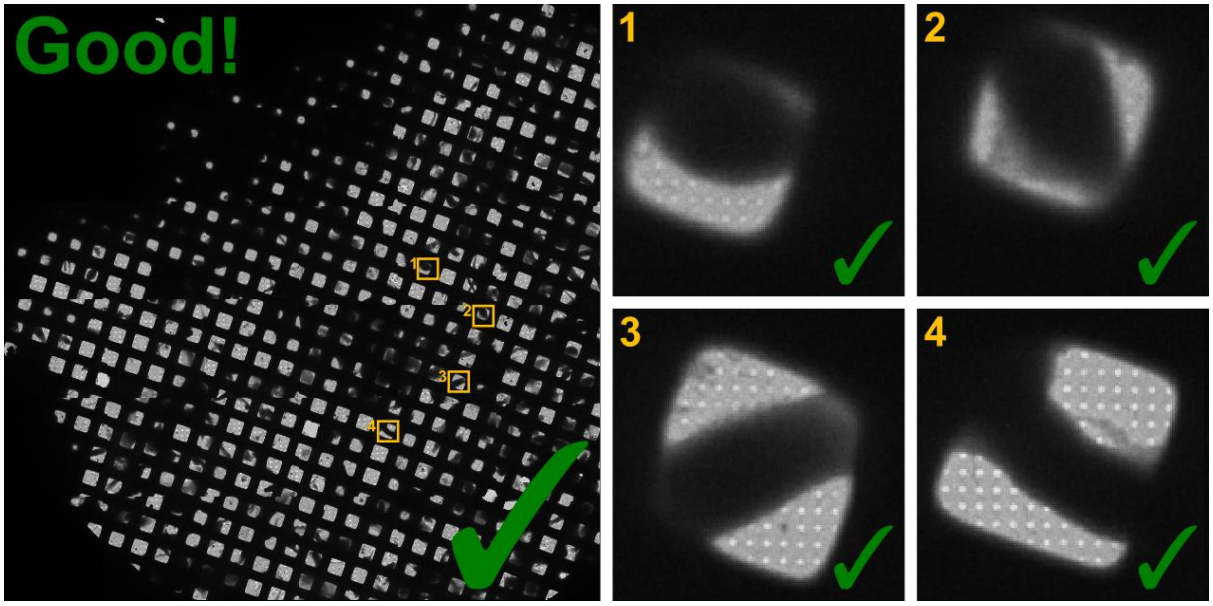


Figure 1 A TEM atlas of a grid suitable for cryoFIB milling.

Bad grids (Figure 2) will have:

- Ice that is too thick
- Extensive support film damage
- Few milling sites or sites on or adjacent to grid bars

Figure 2 Examples of bad grids determined from TEM atlases

Fluorescent correlation (CLEM) experiments

CLEM can be used to better ensure a subcellular target is within the lamella volume. Identifying sites to prepare lamellae using CLEM can be performed at eBIC. If desired, you must:

- Decide on the CLEM workflow
- If performing 3D correlation, add fluorescent beads onto the grid before freezing
- *Inform eBIC of your requirements at least three weeks beforehand*

Please note:

- Screening in a cryo enabled TEM is still advised
- The quality of the experiment depends on the quality of the sample preparation
- Visualising fluorescent targets does not necessarily mean viable lamellae can be milled there
- The samples must be coated before milling. The coatings obscure the fluorescent signal.
- It is possible, but not routinely advised, to perform post-milling fluorescent imaging directly on the lamellae.

Choosing CLEM workflows

Requesting the correct workflow is vital for success and efficiency. Understand which of the scenarios below best describe your sample and inform eBIC of this at least three weeks in advance.

Scenario 1 – no correlation

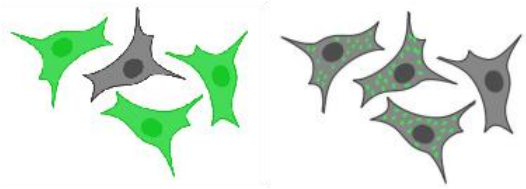
All milling sites contain the fluorescent target *and* it is prevalent within these sites.

In this case:

- No pre-milling fluorescent imaging is needed
- Post-milling fluorescent imaging may be performed at a suitable stage of the preparation

Scenario 2 – 2D correlation

A subset of milling sites contain the fluorescent target, but it is prevalent within these sites.



In this case:

- Pre-milling fluorescent imaging is needed:
    - Fluorescent mapping
- Post-milling fluorescent imaging may be performed at a suitable stage of the preparation

Scenario 3 – 3D correlation

A subset of milling sites contain the target and it is sparse within these sites.

In this case:

- Extensive pre-milling fluorescent imaging is needed:
    - Fluorescent mapping
    - Z-stacks acquired in viable regions
    - Z-stacks will be deconvolved, transformed and correlated to the FIB perspective image
- Post-milling fluorescent imaging is unnecessary

Incorporating fluorescent beads for 3D correlation

Including beads in the grid making process is vital for 3D correlation. This is a process that requires optimisation. The guide below is a starting point – optimisation must follow.

Choose beads that are at least 1 um in diameter. Use either:

- Auto-fluorescent beads – emission dependant on illumination
- Fluorescent beads – specific emission colour but distinct from the target

Before starting

- If beads are supplied in azide, wash with MilliQ water following the manufacturers advice.
- Thoroughly re-suspend the beads in MilliQ water – usually done with a sonicator or vortex mixer

For cells in suspension

- Mix your sample with the beads in a ratio of 9:1 (by volume)
- Using the plunging tweezers, hold a grid horizontally and apply 3-4 uL of the bead mixture to the support film side – incubate for 1 minute
- Plunge freeze using an established protocol

For adherent cells

- Mix your culture media with the beads in a ratio of 9:1 (by volume)
- Using the plunging tweezers, hold a grid horizontally and wick away excess fluid from the grid
- Apply 3-4 uL of the bead mixture to the support film side – incubate for 1 minute
- Plunge freeze using an established protocol