

The 5th CryoEM Course at LBMS, BNL

***In situ* sample preparation tutorial & demonstration**

Jianfeng Lin

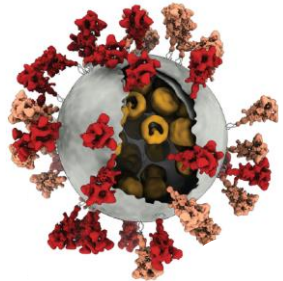
Yale CryoEM Resource

6-5-2025

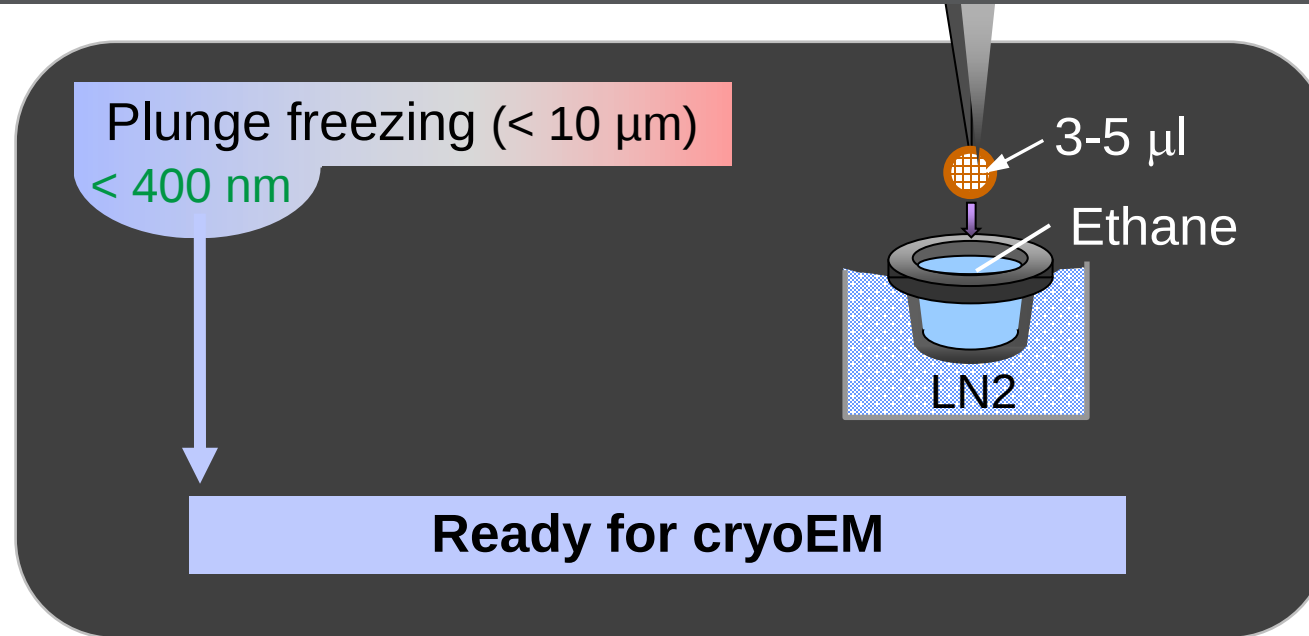
Main contents

1. *In situ* specimens accessible by cryoEM
2. Five considerations for *in situ* sample preparation
3. Tutorial of major steps of cryo lamella preparation

1. *In situ* specimens accessible by cryoEM



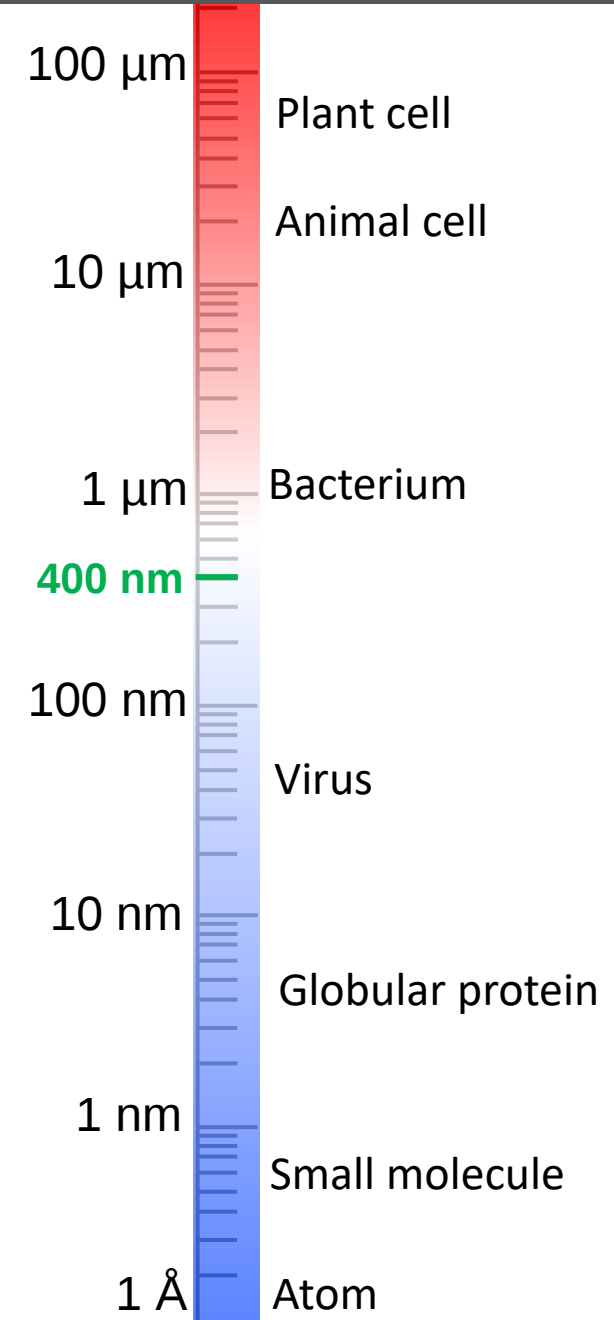
Yao et al., Cell 2020



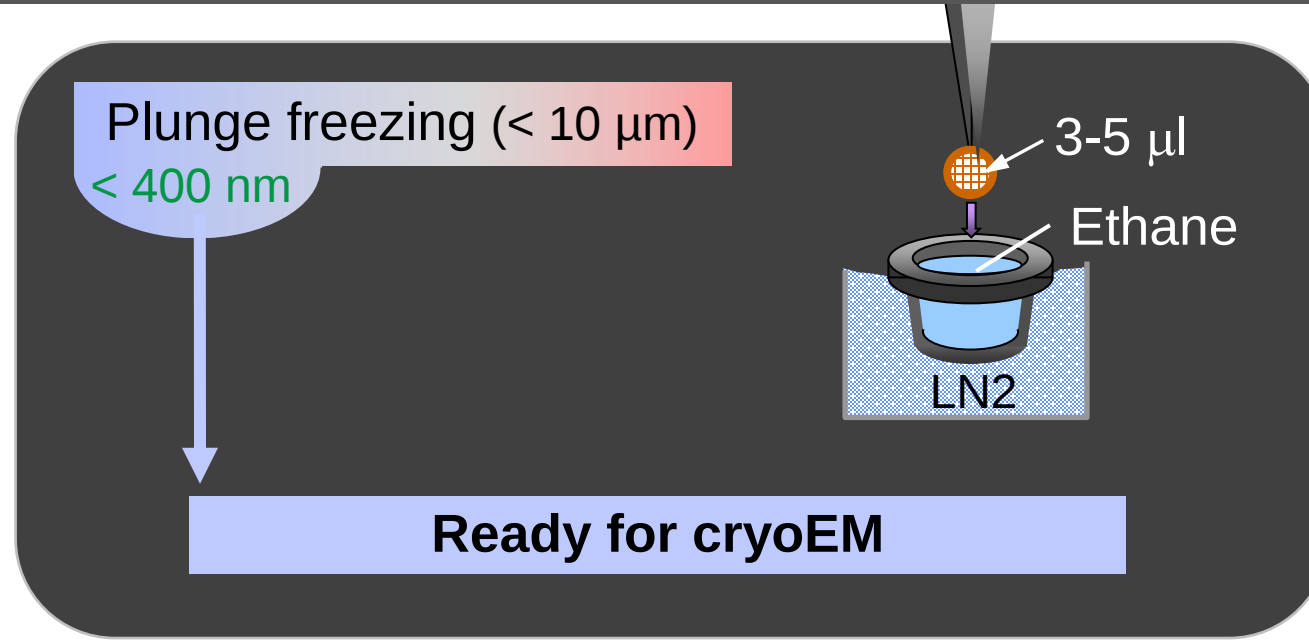
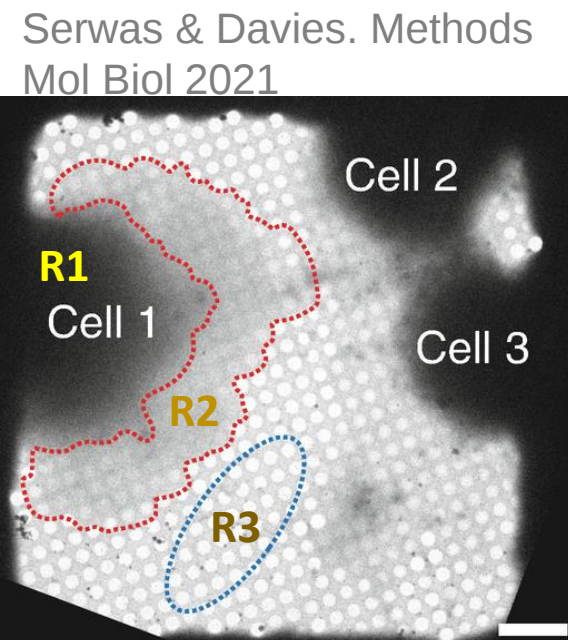
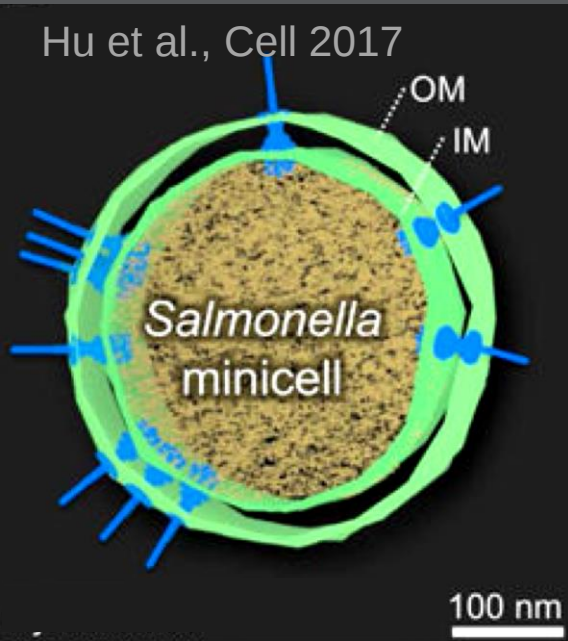
- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme



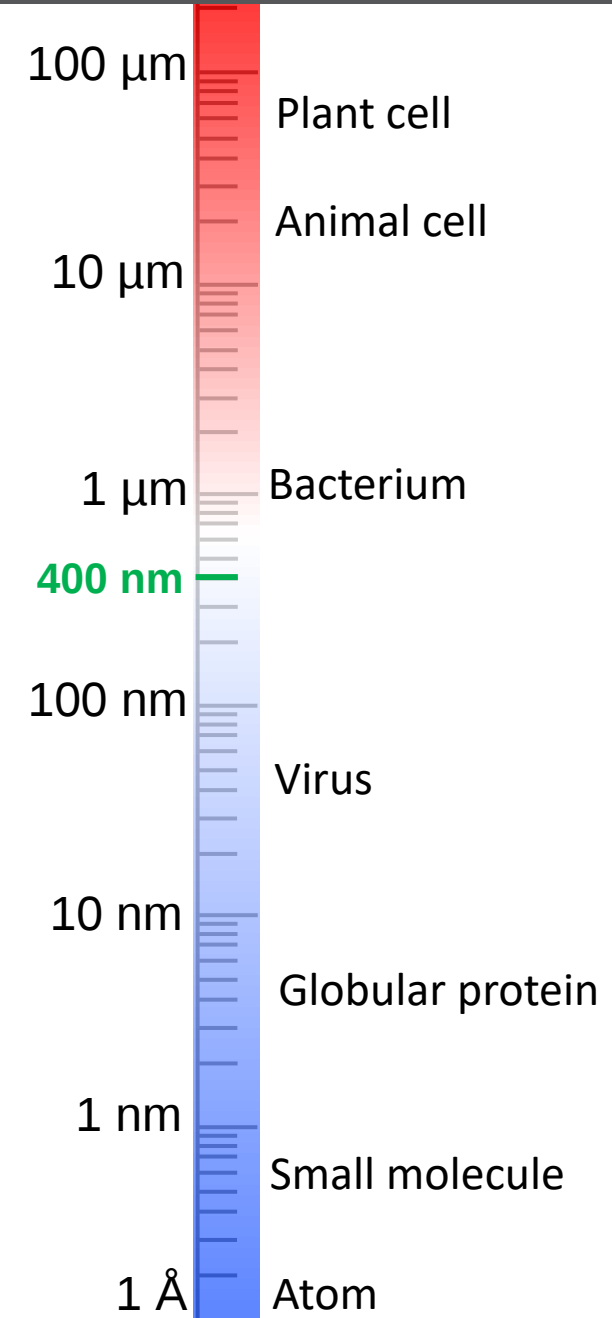
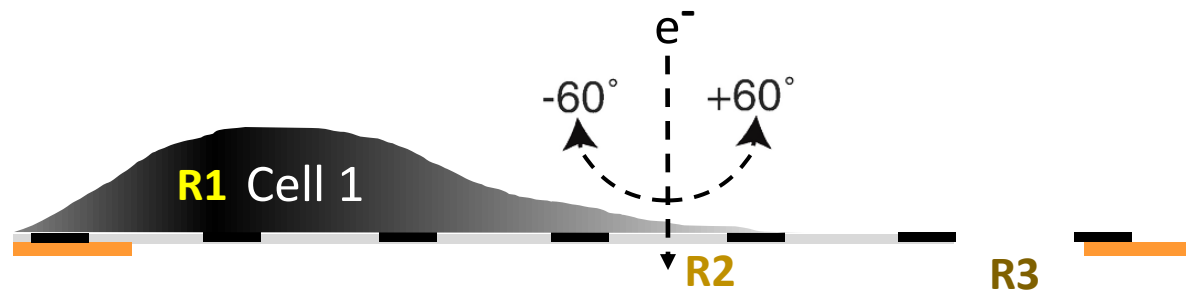
Lin & Nicastro, Science 2018



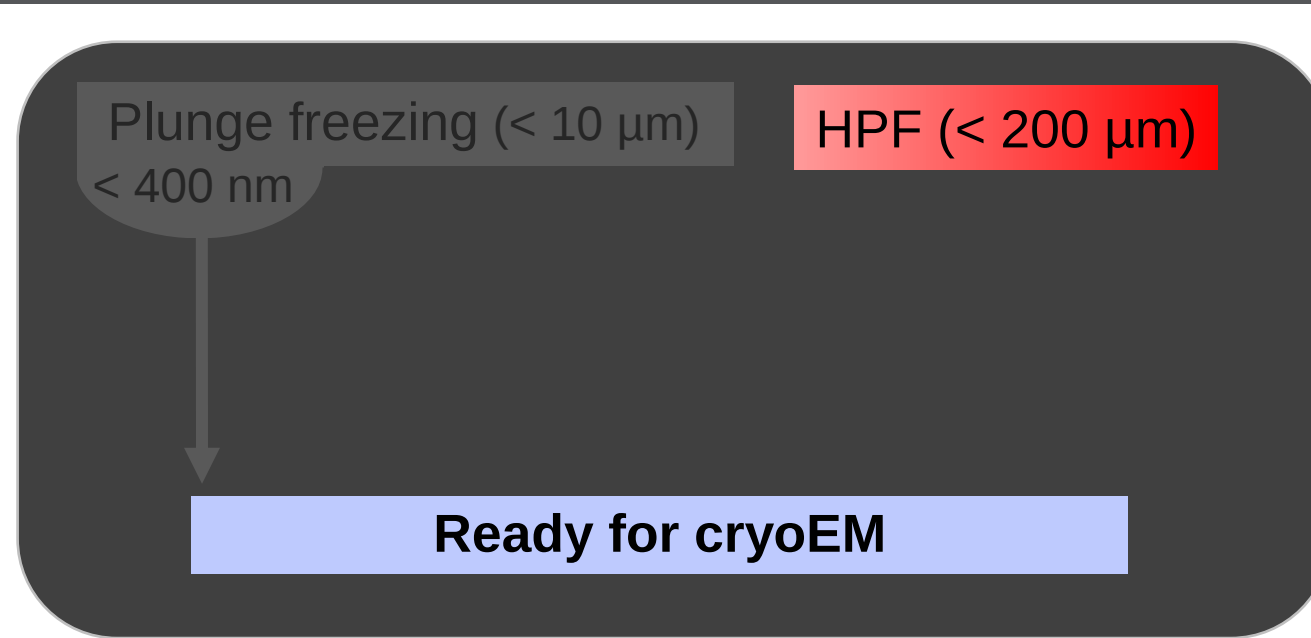
1. *In situ* specimens accessible by cryoEM



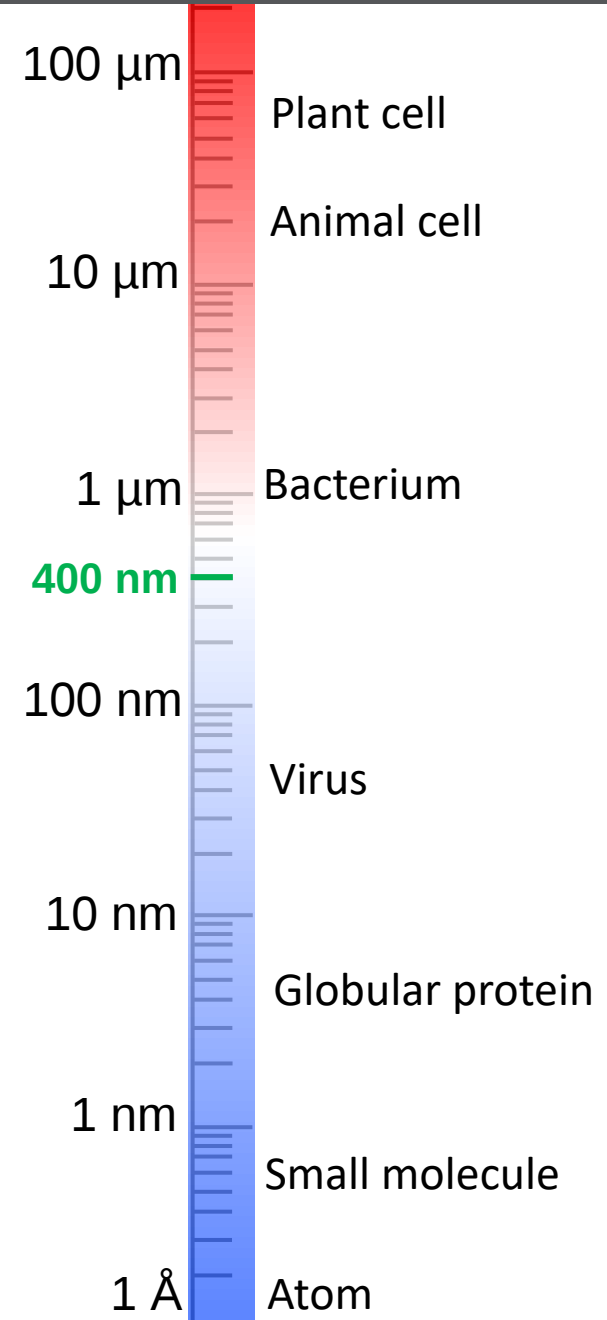
- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells



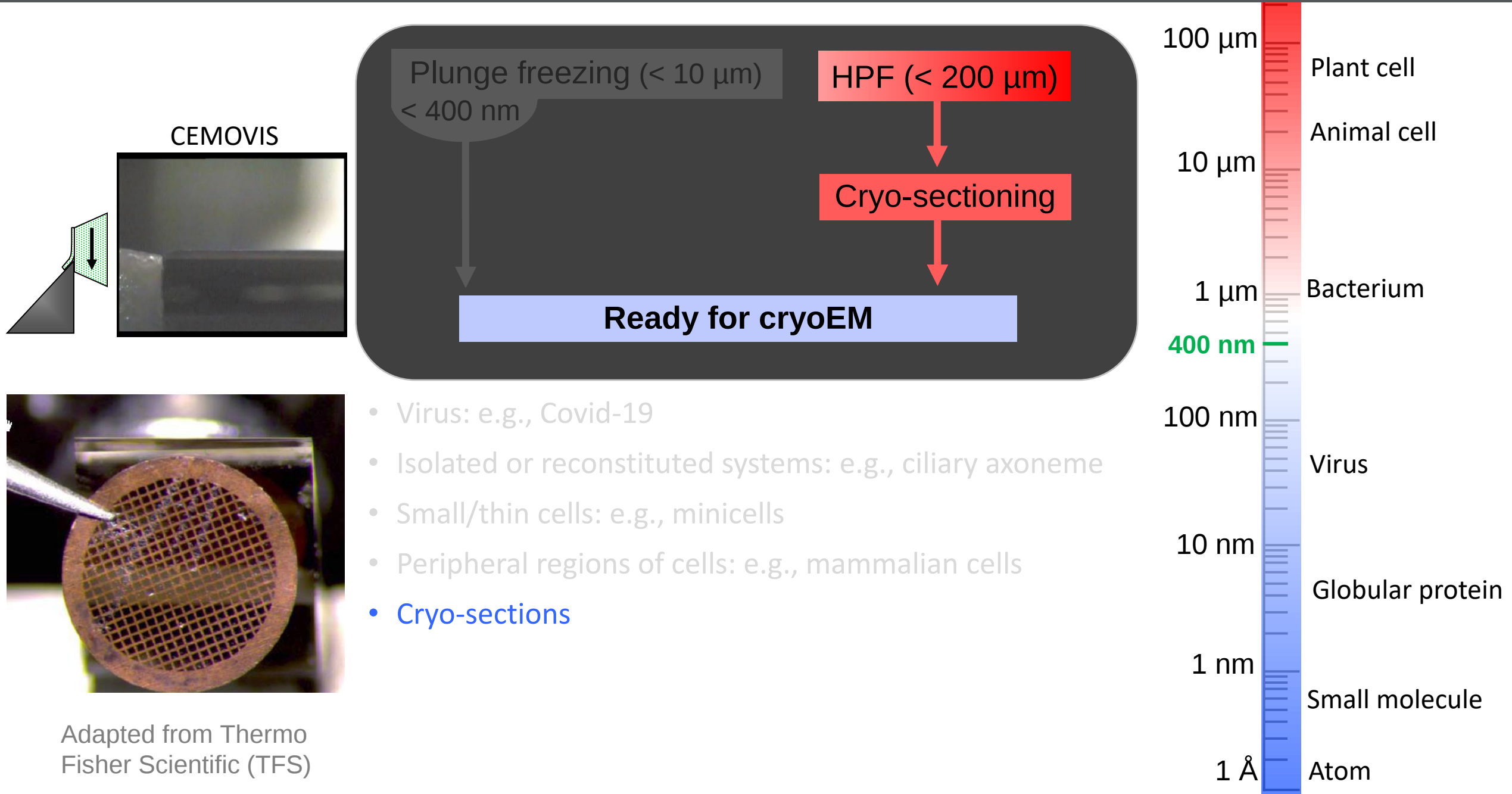
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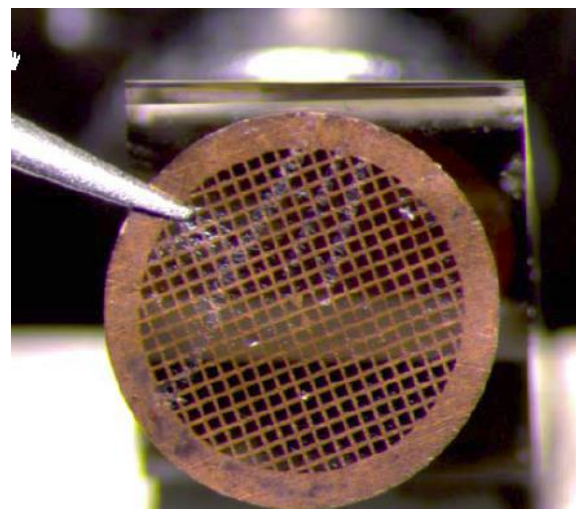
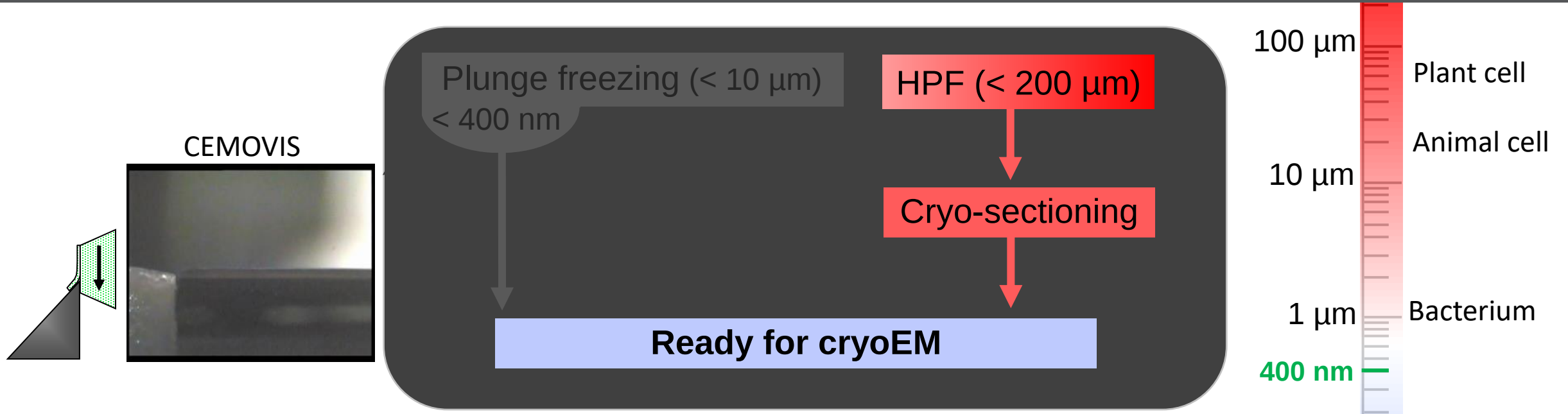
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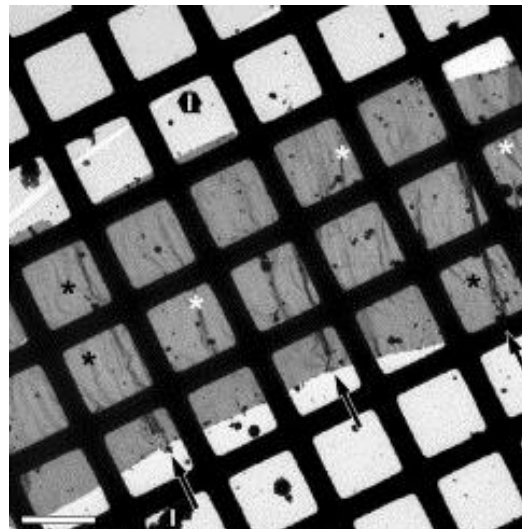
1. *In situ* specimens accessible by cryoEM



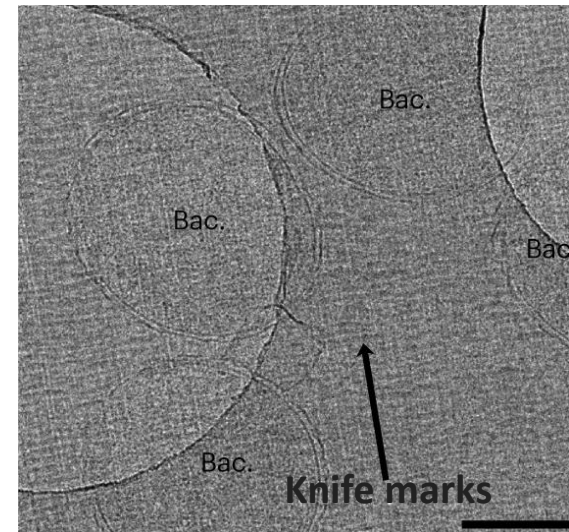
1. *In situ* specimens accessible by cryoEM



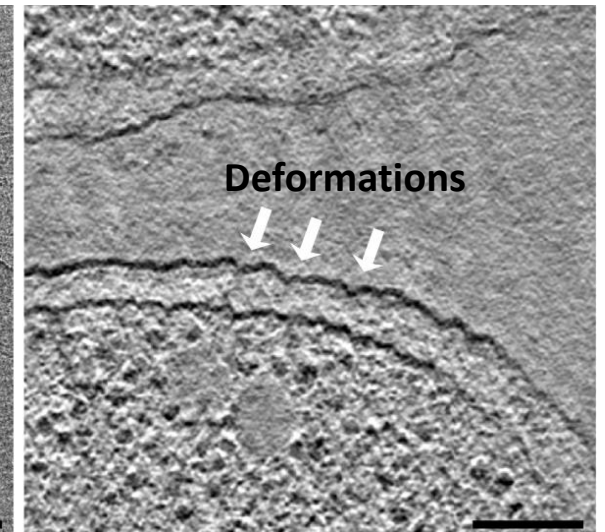
Adapted from TFS



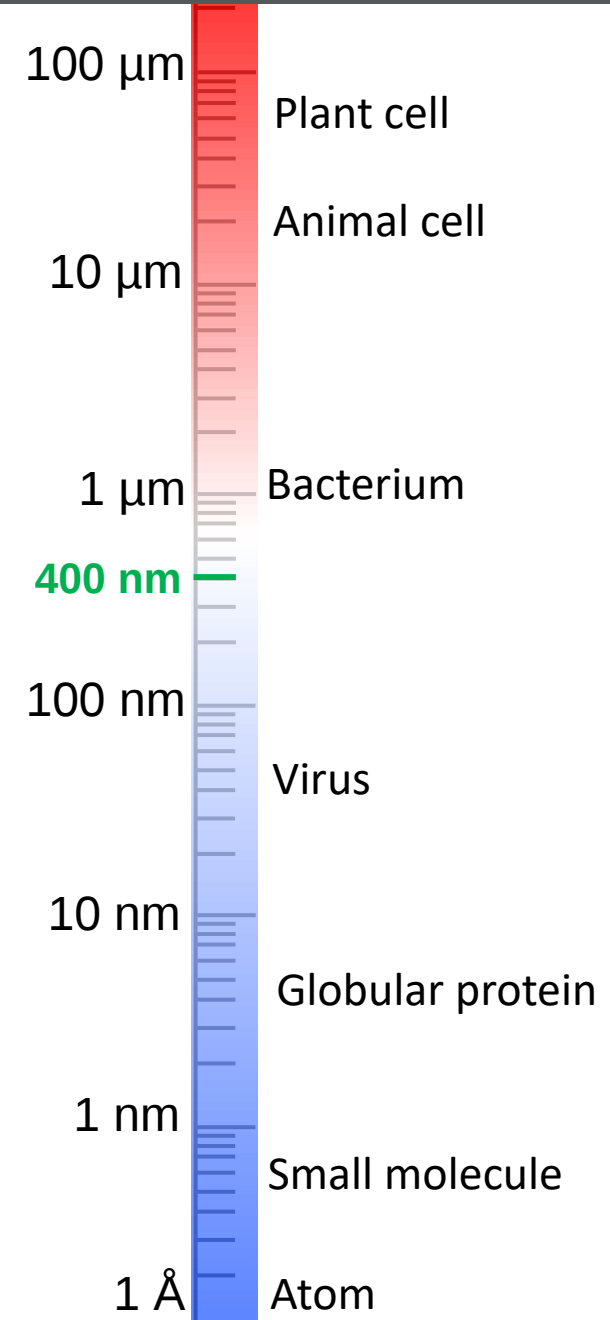
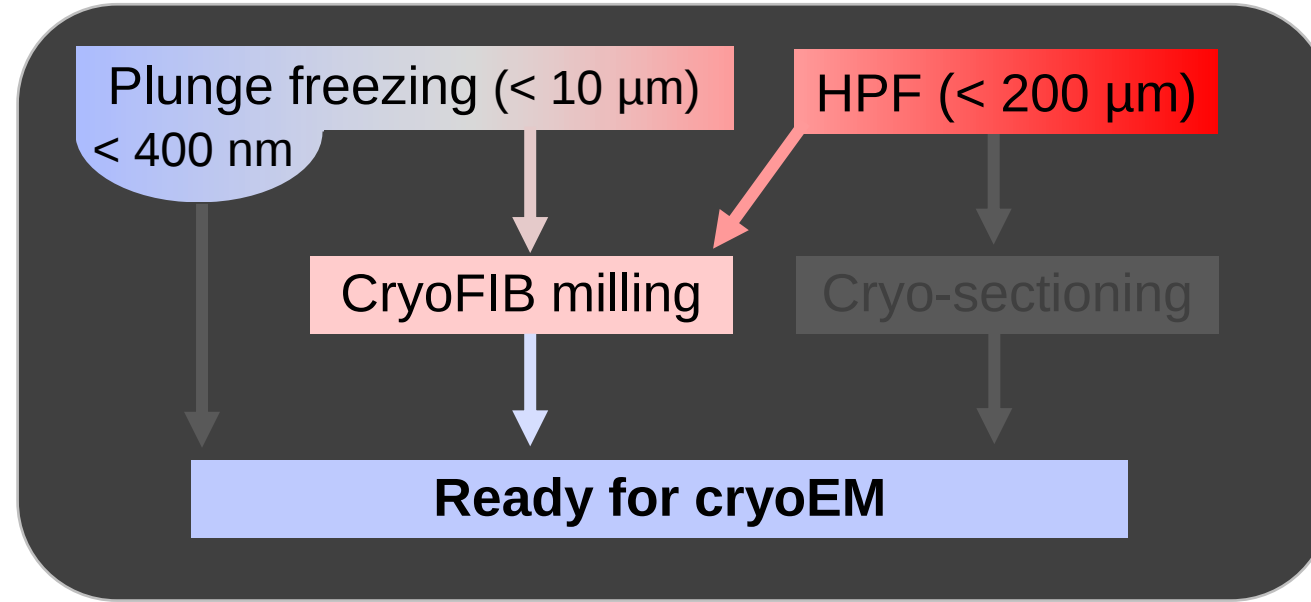
Al-Amoudi et al., J Struct Biol 2005



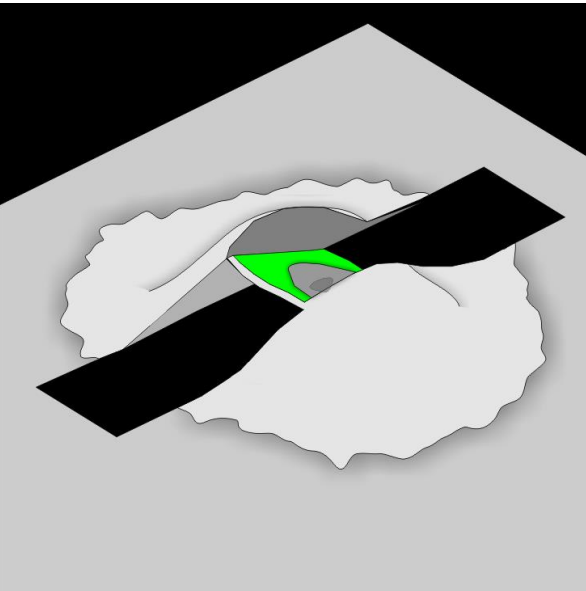
Berger et al., Nature Methods 2023



1. *In situ* specimens accessible by cryoEM



- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells
- Cryo-sections
- Cryo-lamellae



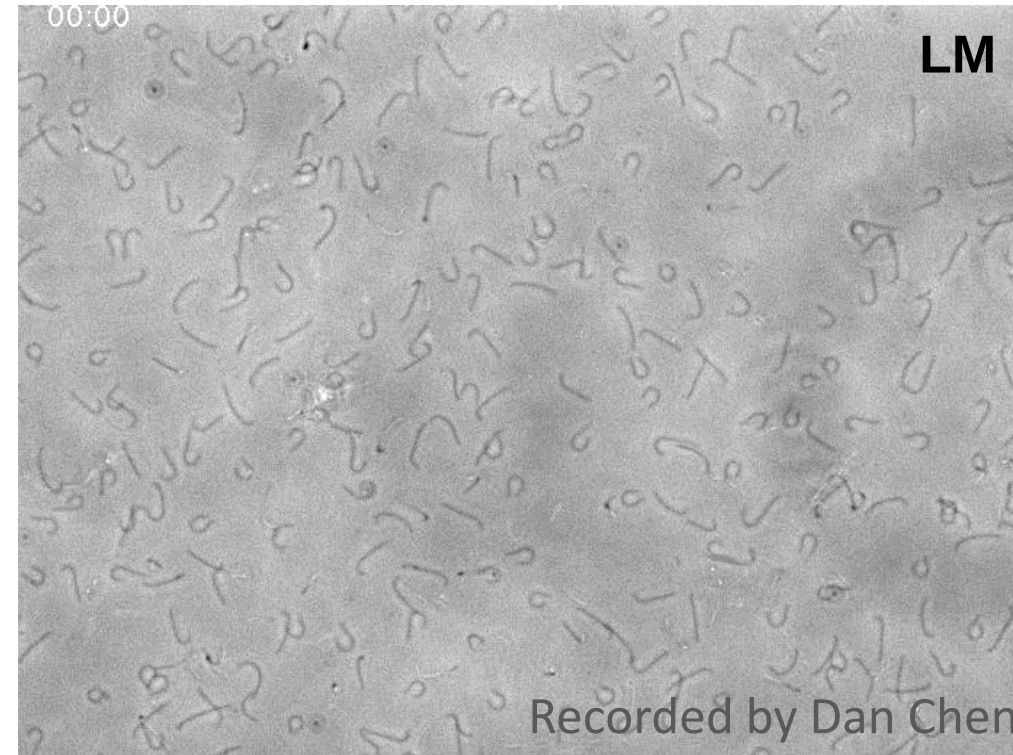
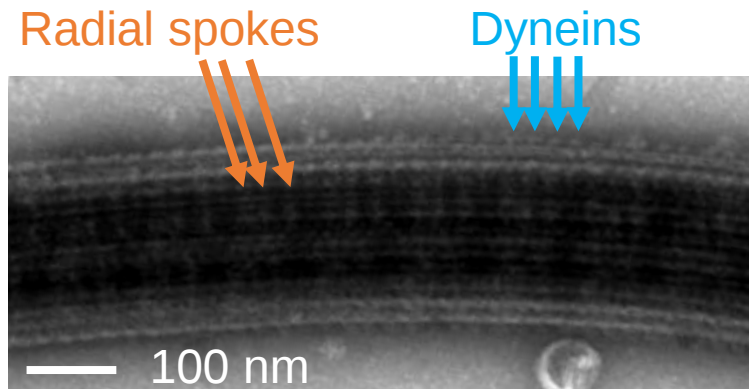
Main contents

1. *In situ* specimens accessible by cryoEM
2. Five considerations for *in situ* sample preparation
3. Tutorial of major steps of cryo lamella preparation

2. Five considerations for *in situ* sample preparation

2.1 Validation of the sample quality e.g., Negative stain EM & Reactivation of flagellar axoneme

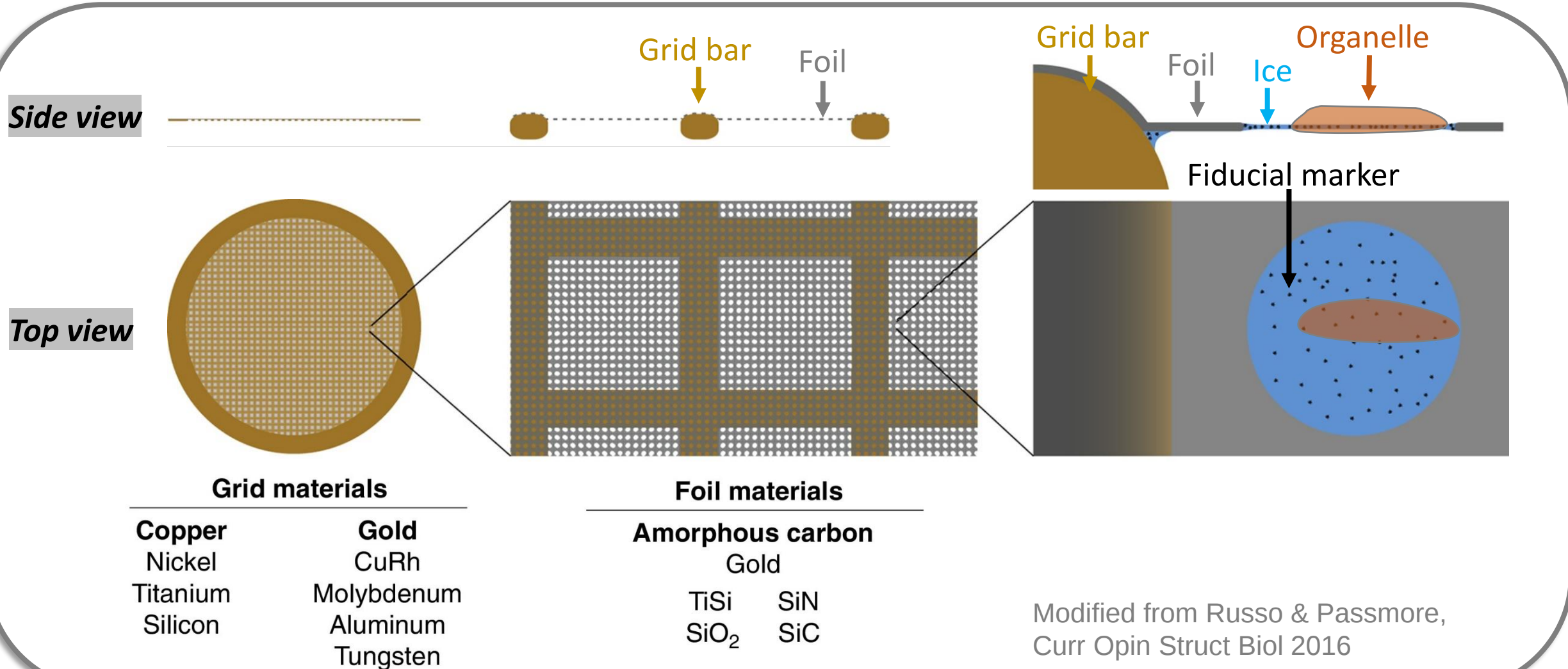
Negative stain EM



2. Five considerations for *in situ* sample preparation

2.1 Validation of the sample quality

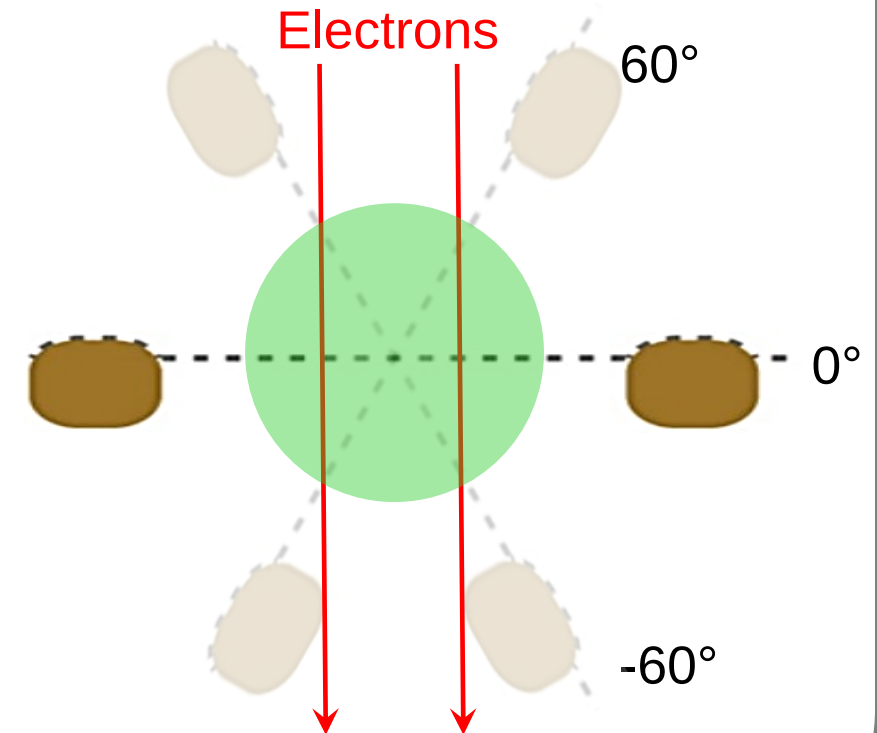
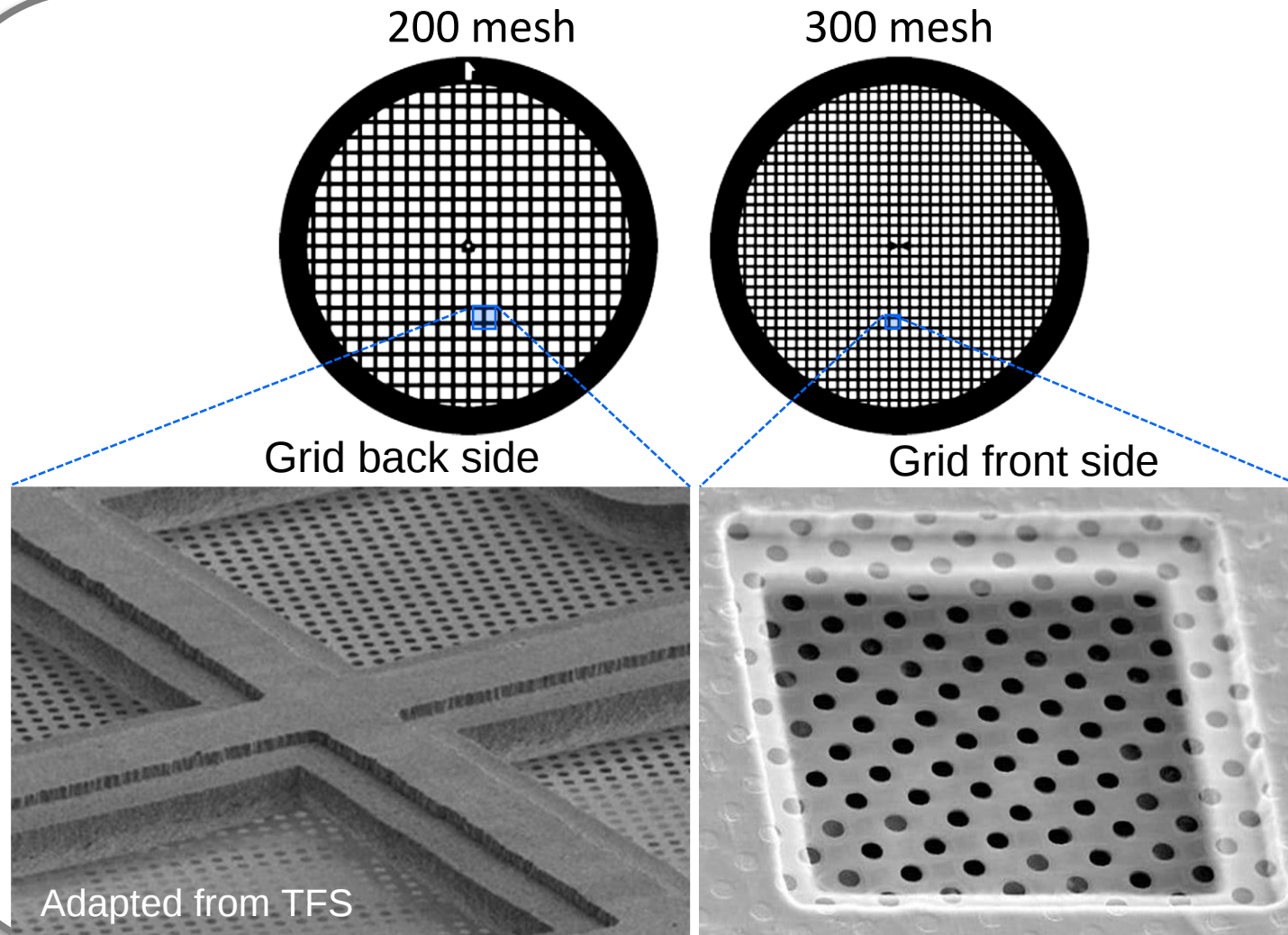
2.2 EM grid => Grid / Foil materials



2. Five considerations for *in situ* sample preparation

2.1 Validation of the sample quality

2.2 EM grid => Mesh / Hole size

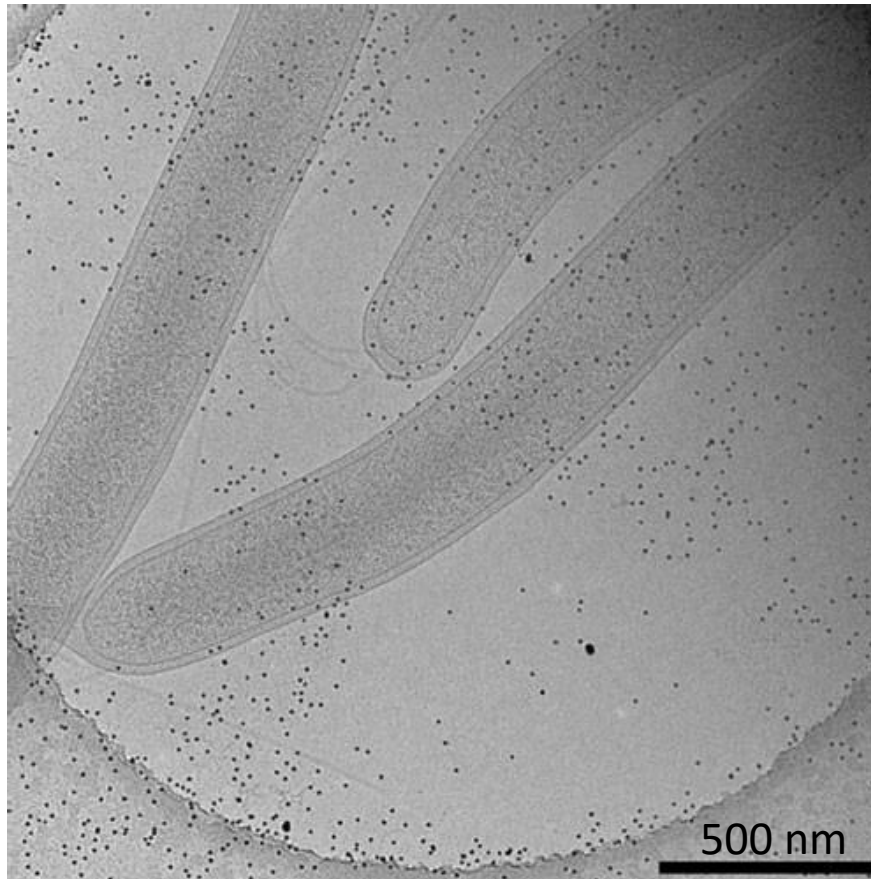


2. Five considerations for *in situ* sample preparation

2.1 Validation of the sample quality

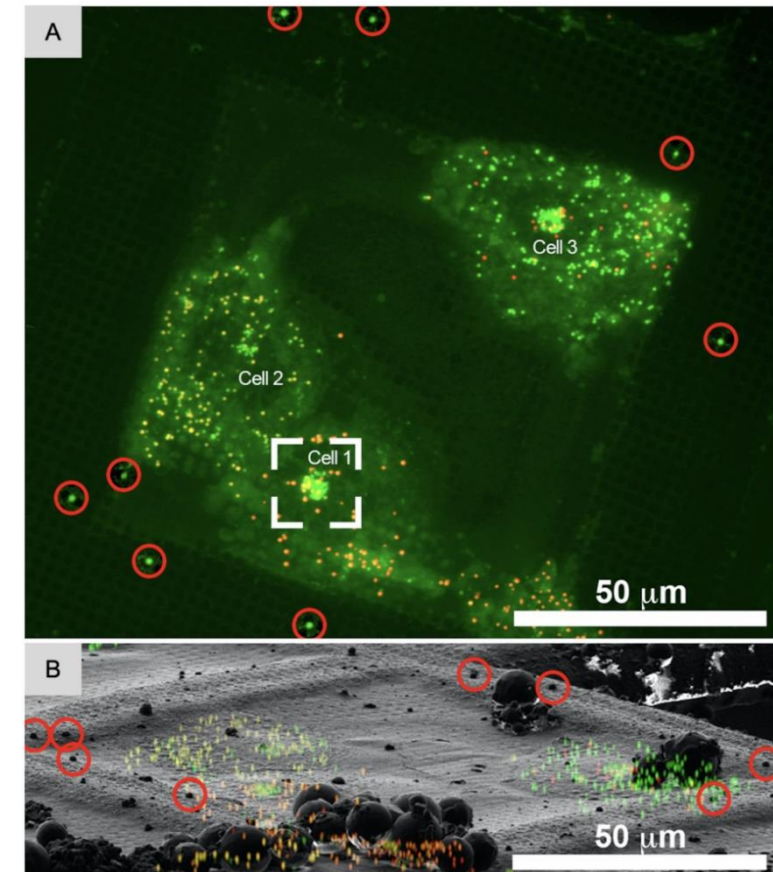
2.2 EM grid

2.3 Fiducial markers e.g., 10-nm BSA-treated colloidal gold for tilt series alignments



Iancu et al., Nature Protocols 2006

e.g., 1- μ m Magnetic beads for FLM and SEM/FIB microscopy.



Arnold et al., Biophysical Journal 2016

2. Five considerations for *in situ* sample preparation

2.1 Validation of the sample quality

2.2 EM grid

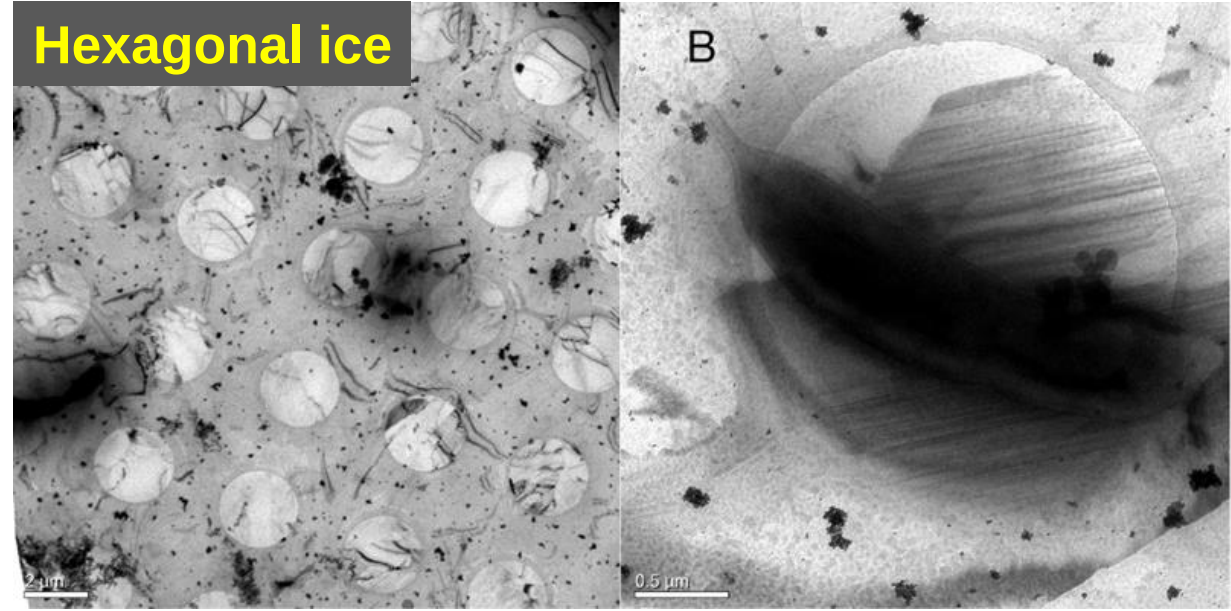
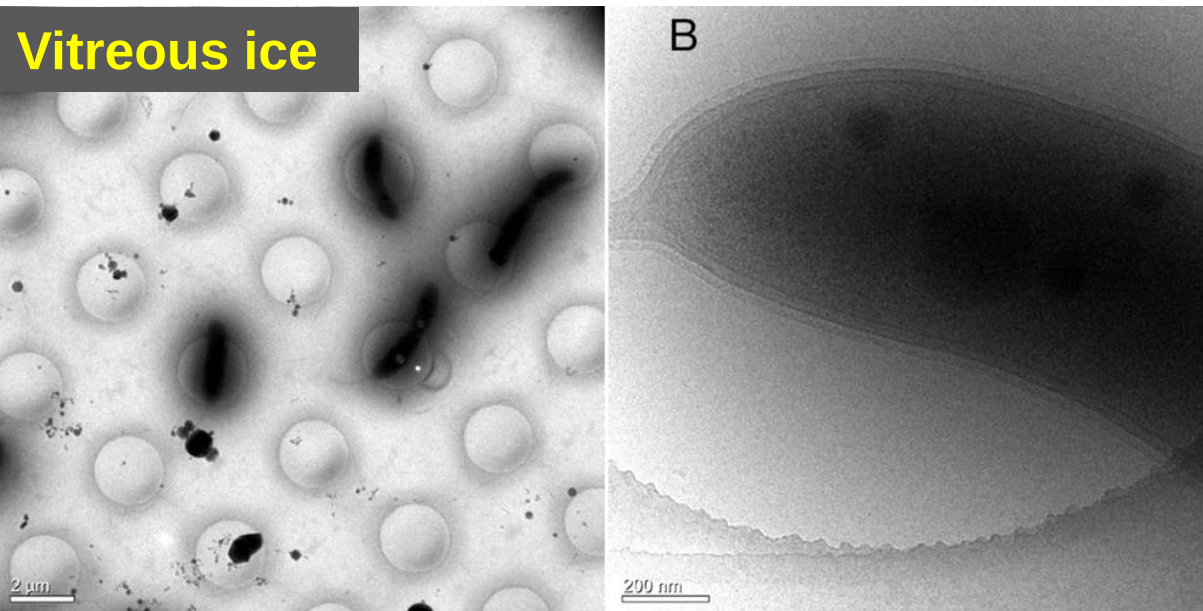
2.3 Fiducial markers

2.4 Cryogen e.g., LE or 37% LE-63% LP mixture

$$T_{LE} = -182.8\text{ °C} \sim -88.6\text{ °C}$$

$$T_{LP} = -189.7\text{ °C} \sim -42.2\text{ °C}$$

$$T_{LN2} = -210\text{ °C} \sim -195.8\text{ °C}$$



2. Five considerations for *in situ* sample preparation

2.1 Validation of the sample quality

2.2 EM grid

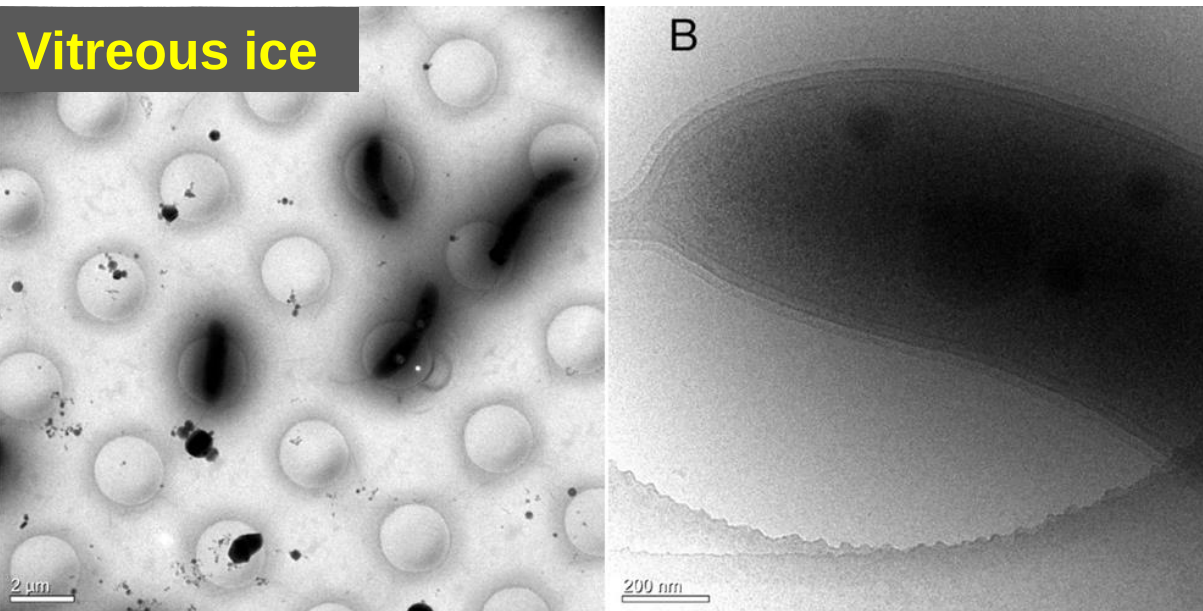
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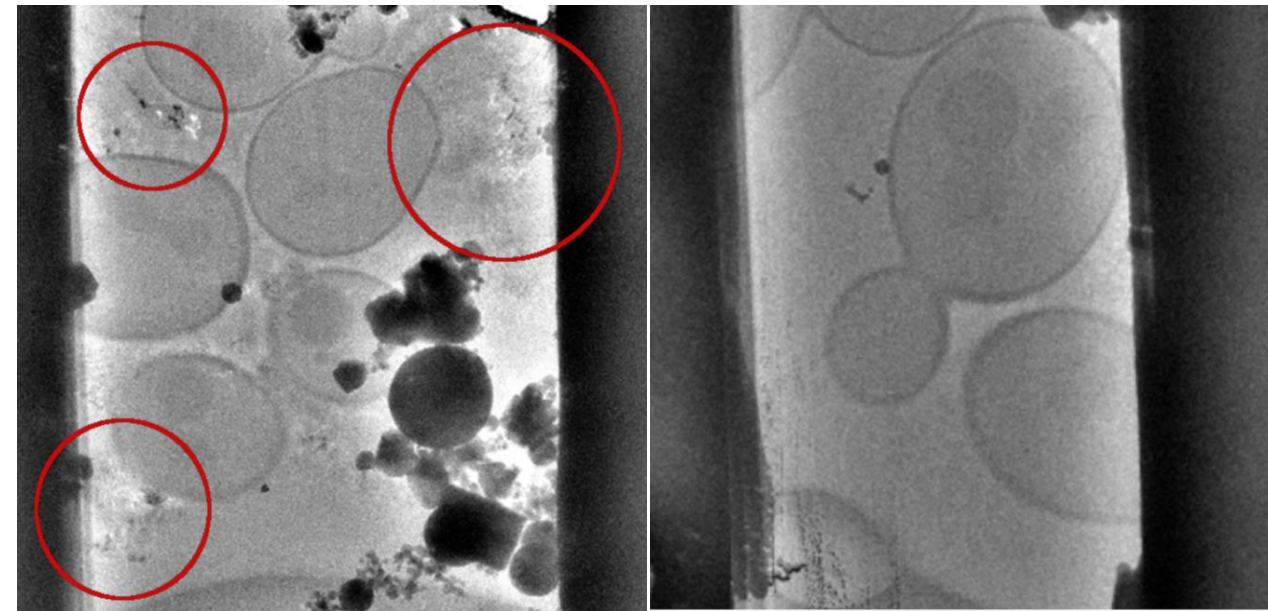
$$T_{LN2} = -210\text{ °C} \sim -195.8\text{ °C}$$



Tivol et al., Microsc Microanal 2008

Without glycerol

5% glycerol



Moravcová et al., J Vis Exp 2021

2. Five considerations for *in situ* sample preparation

2.1 Validation of the sample quality

2.2 EM grid

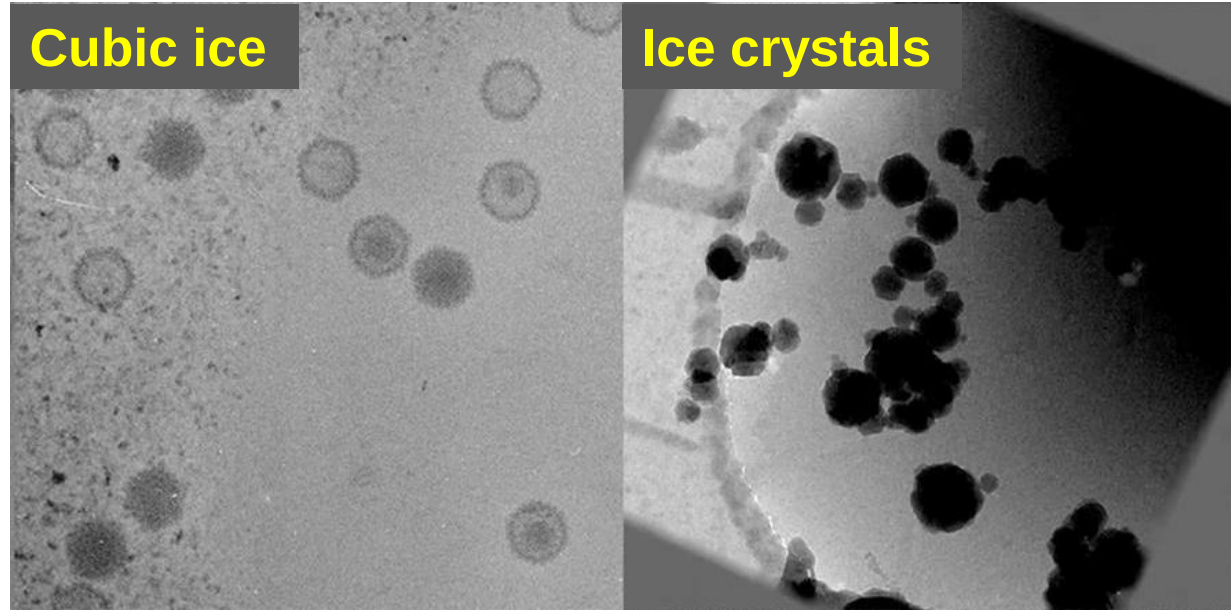
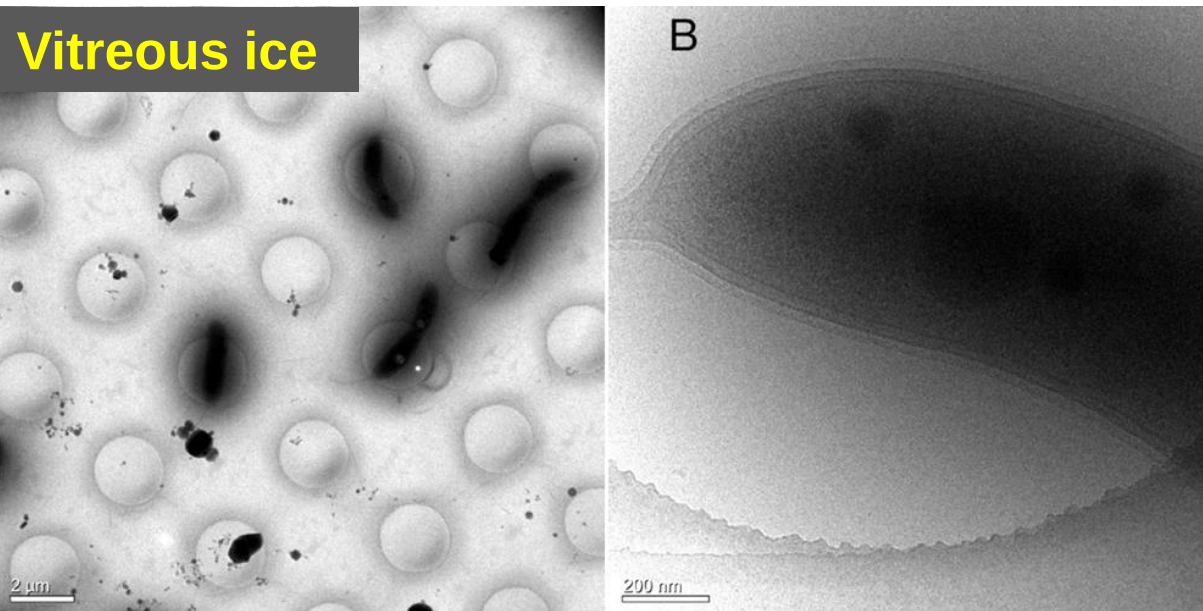
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2. Five considerations for *in situ* sample preparation

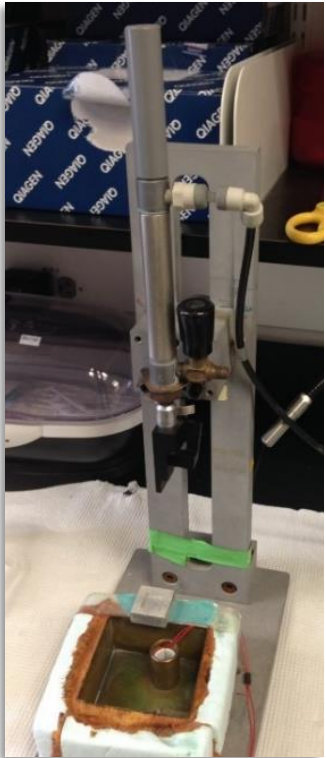
2.1 Validation of the sample quality

2.2 EM grid

2.3 Fiducial markers

2.4 Cryogen

2.5 Plunger



Homemade



EMS-002 (EMS)



EM GP2 (Leica)



Vitrobot Mark IV (TFS)



Cryoplunge™3 (Gatan)

Main contents

1. *In situ* specimens accessible by cryoEM
2. Five considerations for *in situ* sample preparation
3. Tutorial of major steps of cryo lamella preparation

Examples of cryoFIB milling instruments

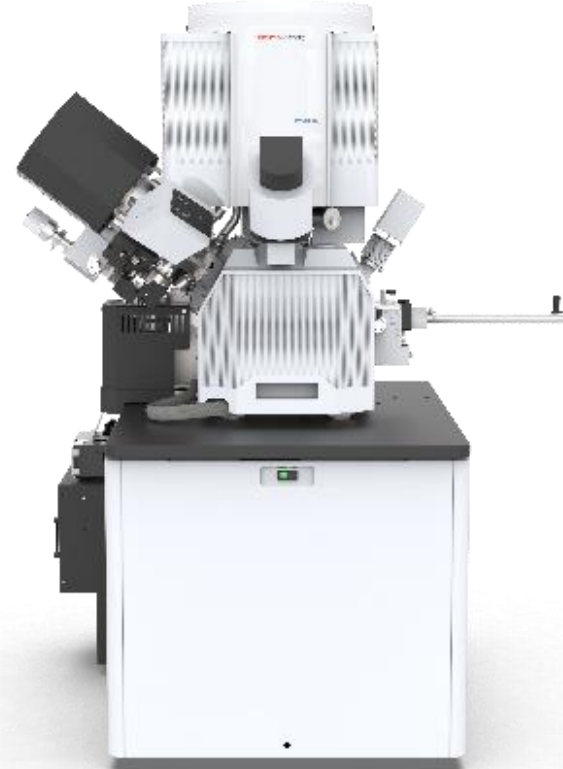
Crossbeam
(ZEISS)



Scios, Aquilos 1/2
(Thermo Fisher Scientific)



Hydra Bio Plasma-FIB
(Thermo Fisher Scientific)



Arctis Cryo-Plasma-FIB
(Thermo Fisher Scientific)



Main contents

1. *In situ* specimens accessible by cryoEM
2. Five considerations for *in situ* sample preparation
3. Tutorial of major steps of cryo lamella preparation (with Vitrobot & Aquilos 2)



3.1 Prepare frozen-hydrated cells on an EM grid

Vitrification

T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

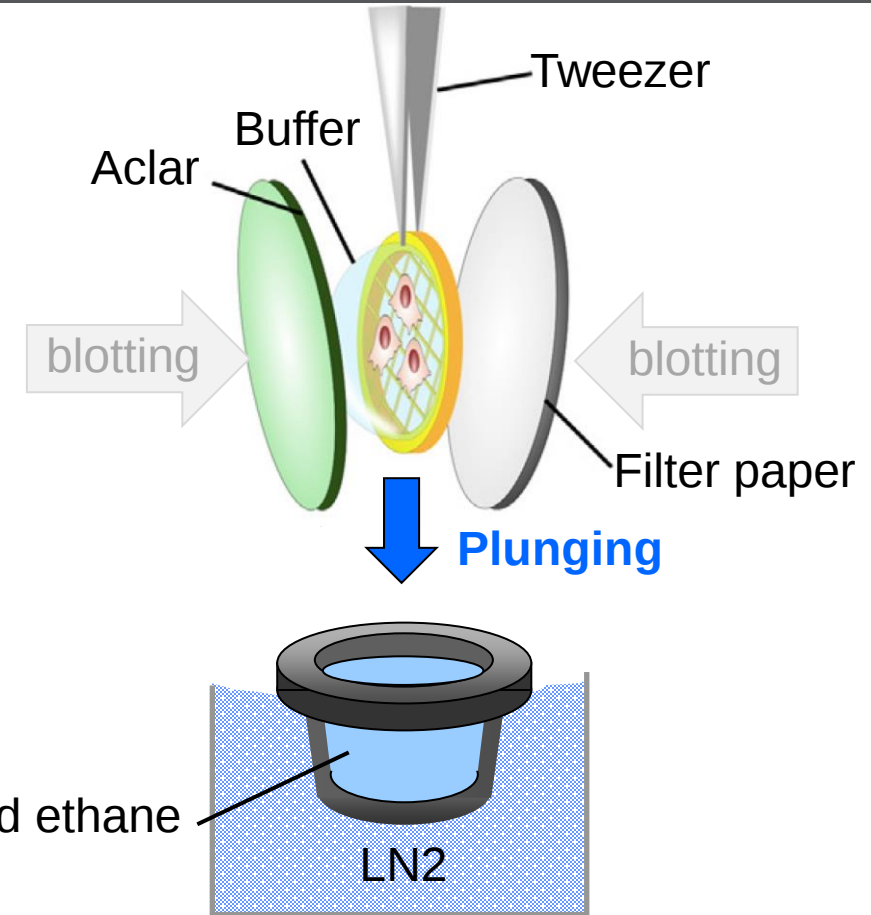
Target confirmation

Pt sputter (Optional)

Lamella conductivity

T

CryoET



3.2 Transfer the grids to Aquilos 2

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET

1. Prepare the Aquilos 2.
2. Prepare the grids.
3. Transfer the grids to the Aquilos 2.

Aquilos 2 & Software used in this tutorial

In Support PC



Flow DDE



FlowView

In Aquilos 2 PC



Microscope Control v32.1.1



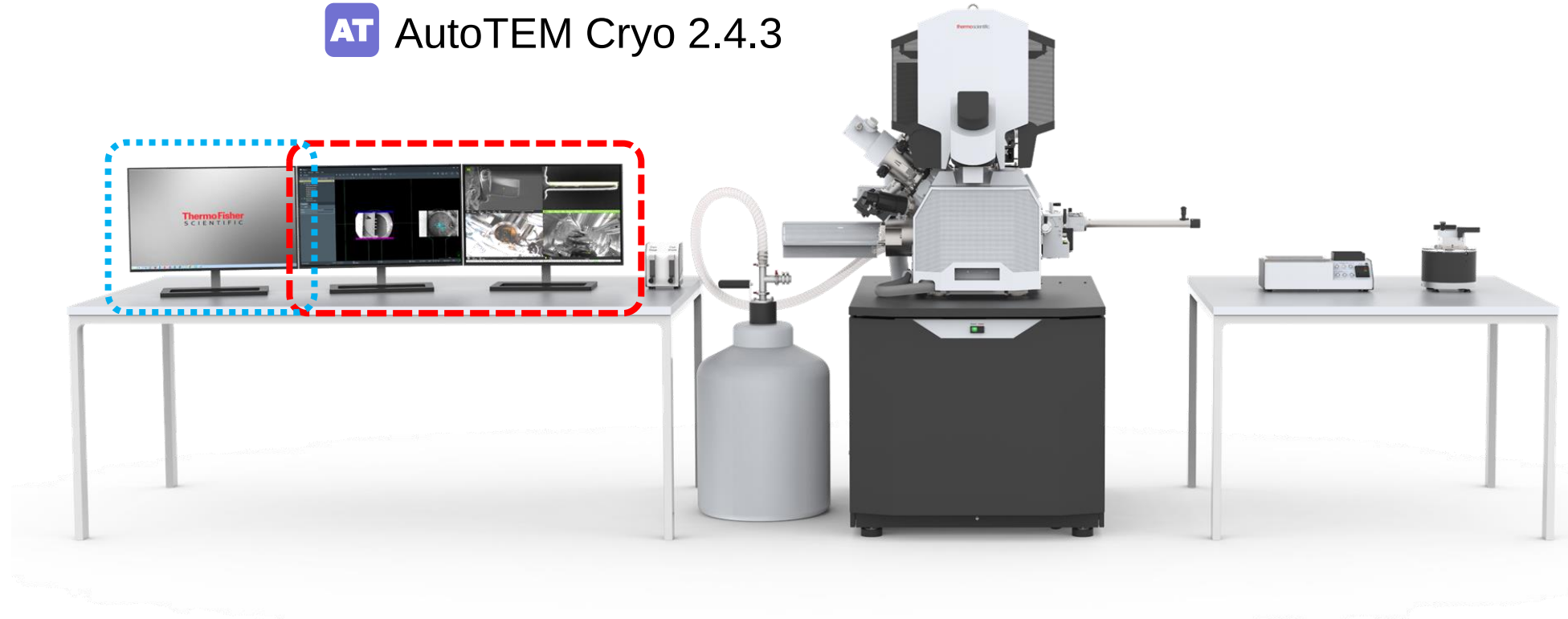
Maps 3.22



Fluorescence Microscope Control 1.3.0



AutoTEM Cryo 2.4.3



3.2.1 Prepare the system **Select the Shuttle type**

(**xT** Microscope Control)

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

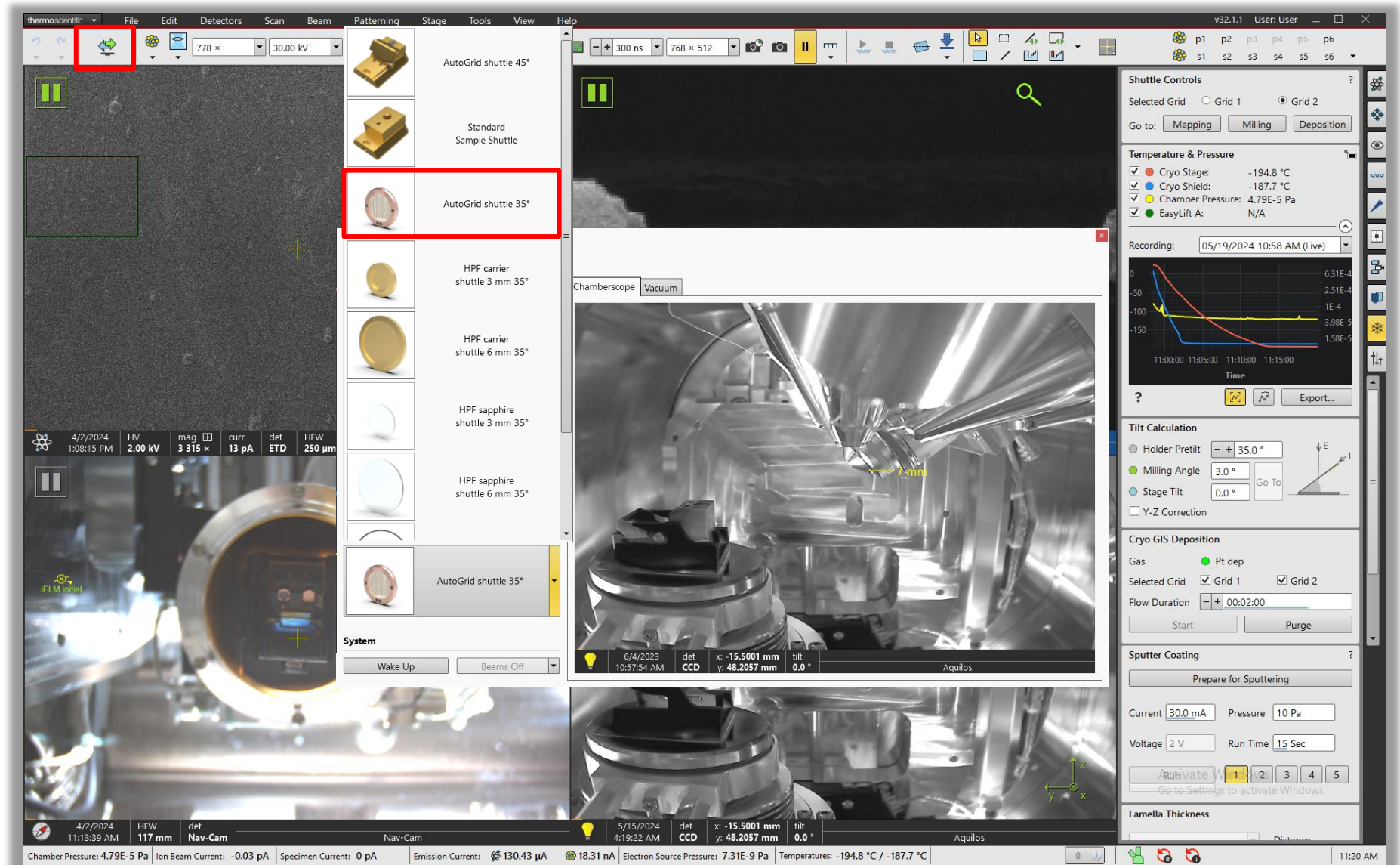
Target confirmation

Pt sputter (Optional)

Lamella conductivity

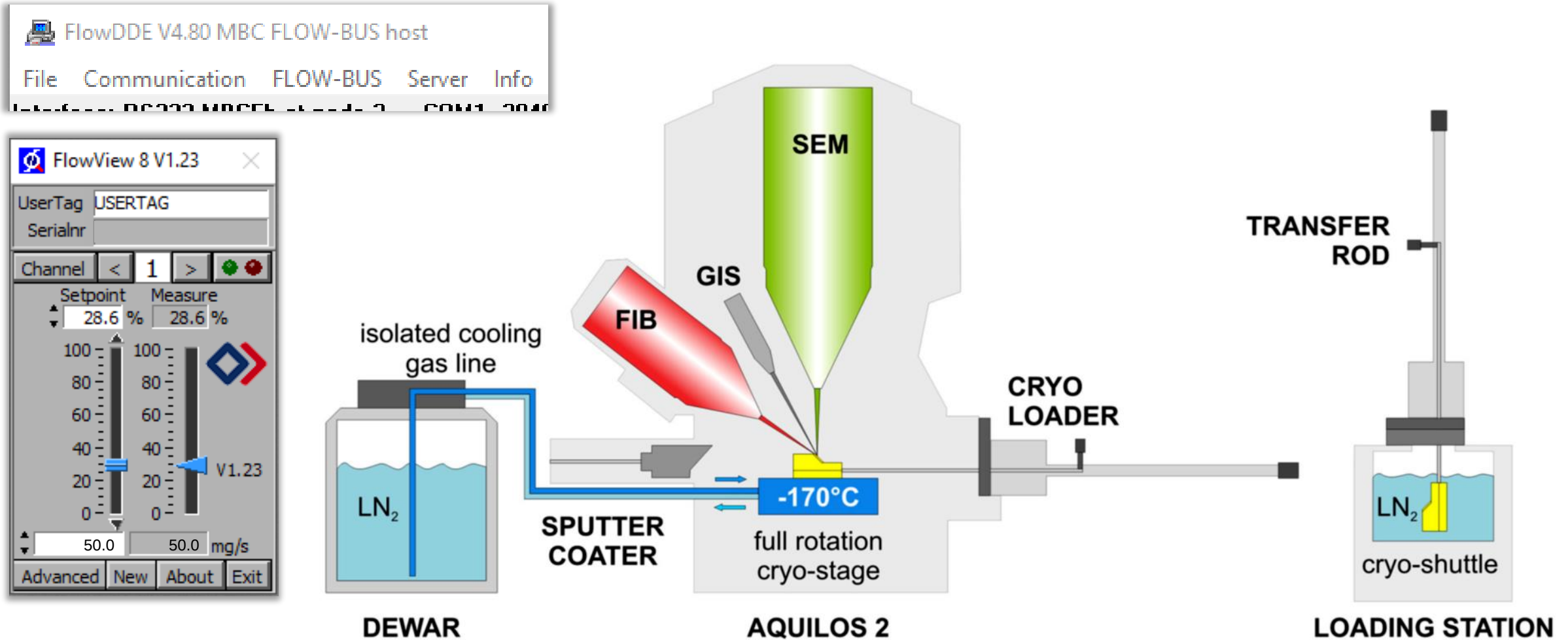


CryoET



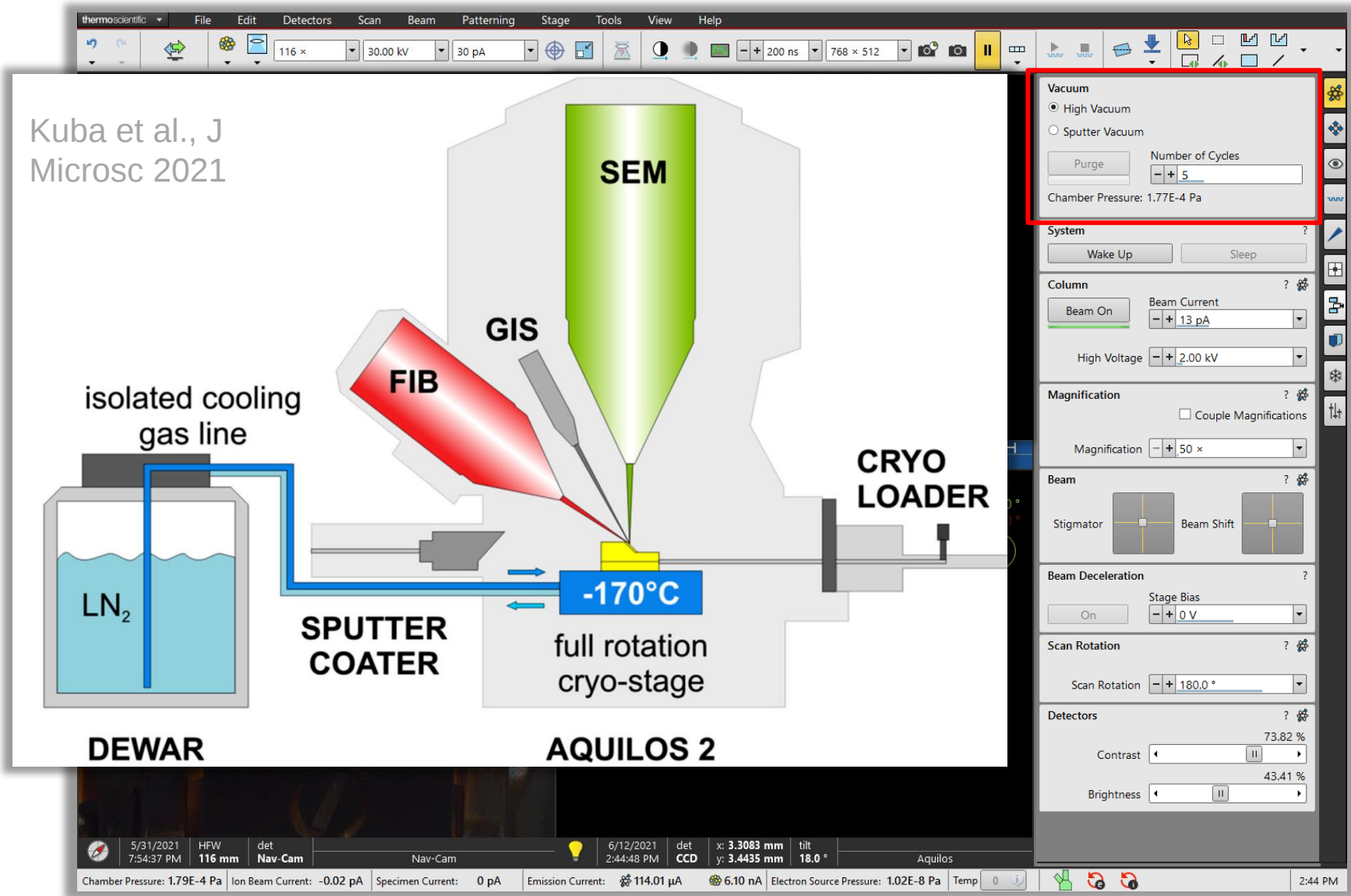
3.2.1 Prepare the system **_Purge the system**

- Cooling-gas line, Loading station (>0.5h)



3.2.1 Prepare the system **_Purge the system**

- Cooling-gas line, Loading station, Argon line (5 cycles)



3.2.1 Prepare the system **_Purge the system**

(**xT** Microscope Control)

- Cooling-gas line, Loading station, Argon line, **GIS (2 mins)**.

Kuba et al., J Microsc 2021

SEM

GIS

FIB

CRYO LOADER

isolate cooling gas line

LN₂

DEWAR

SPUTTER COATER

-170°C

full rotation cryo-stage

AQUILOS 2

thermoscientific

File Edit Detectors Scan Beam Patterning Stage Tools View Help

778 x 30.00 kV 10 pA 300 ns 768 x 512

v32.1.1 User: User

Shuttle Controls

Selected Grid ☐ Grid 1 ☒ Grid 2

Go to: Mapping Milling Deposition

Temperature & Pressure

- ☒ Cryo Stage: -194.8 °C
- ☒ Cryo Shield: -187.7 °C
- ☒ Chamber Pressure: 4.79E-5 Pa
- ☒ EasyLift A: N/A

Recording: 05/19/2024 10:58 AM (Live)

Tilt Calculation

- ☐ Holder Pretilt:
- ☐ Milling Angle:
- ☐ Stage Tilt:

☐ Y-Z Correction

Cryo GIS Deposition

Gas ☒ Pt dep

Selected Grid ☒ Grid 1 ☒ Grid 2

Flow Duration

Start Purge

Sputter Coating

Prepare for Sputtering

Current Pressure

Voltage Run Time

Activate Windows

Go to Settings to activate Windows.

Lamella Thickness

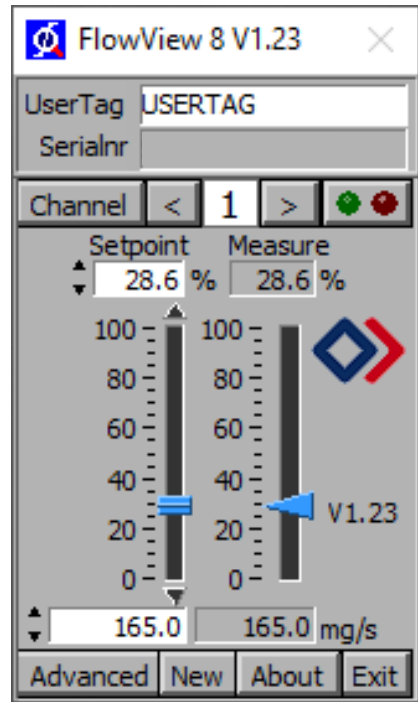
4/2/2024 11:13:39 AM HFW 117 mm det Nav-Cam

5/15/2024 4:19:22 AM det CCD x: -15.5001 mm y: 48.2057 mm tilt 0.0°

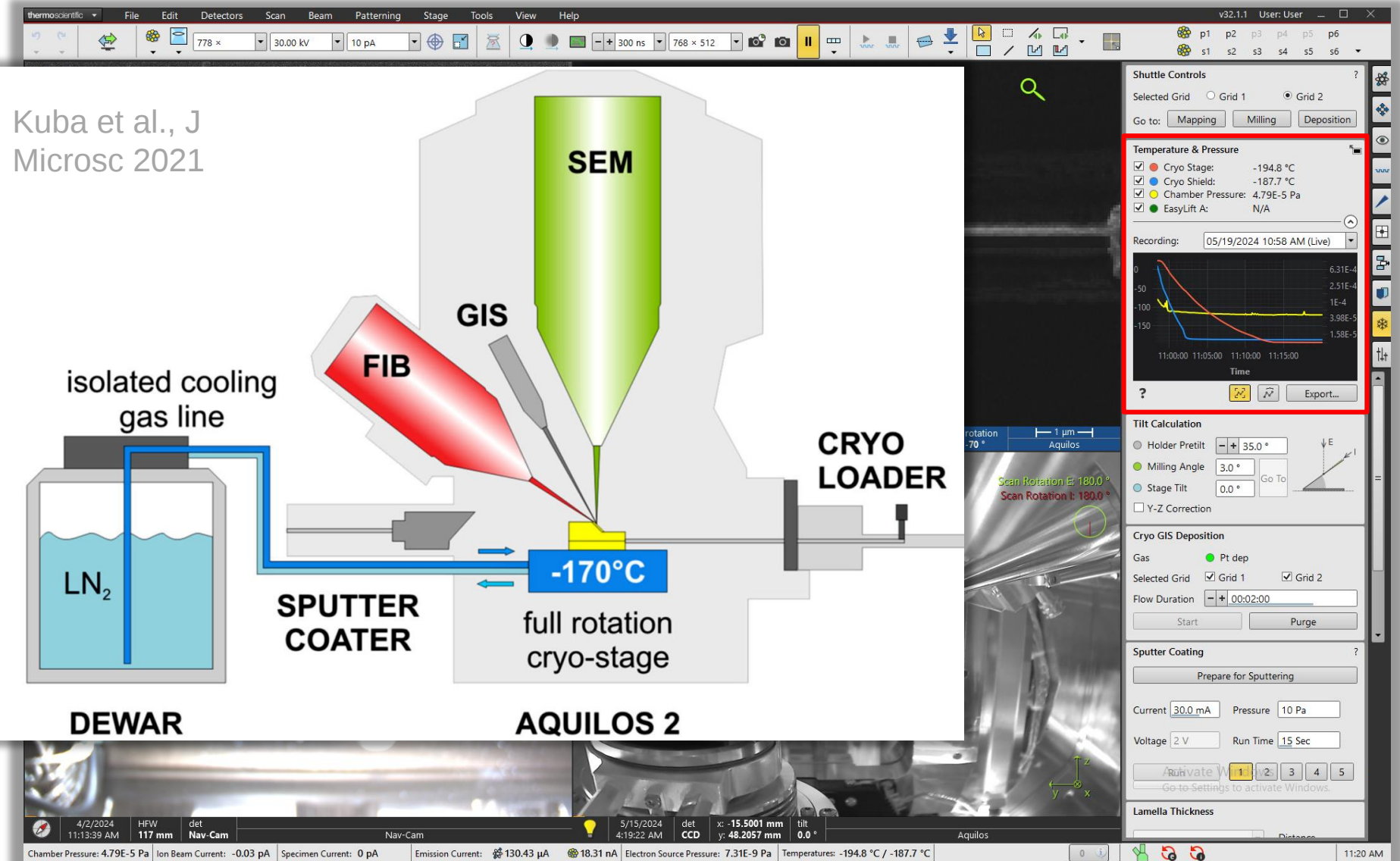
Chamber Pressure: 4.79E-5 Pa Ion Beam Current: -0.03 pA Specimen Current: 0 pA Emission Current: 130.43 μA 18.31 nA Electron Source Pressure: 7.31E-9 Pa Temperatures: -194.8 °C / -187.7 °C

11:20 AM

3.2.1 Prepare the system _Cool down the system



Varies by instruments



3.2.2 Prepare the grids **_Clip the grids**

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

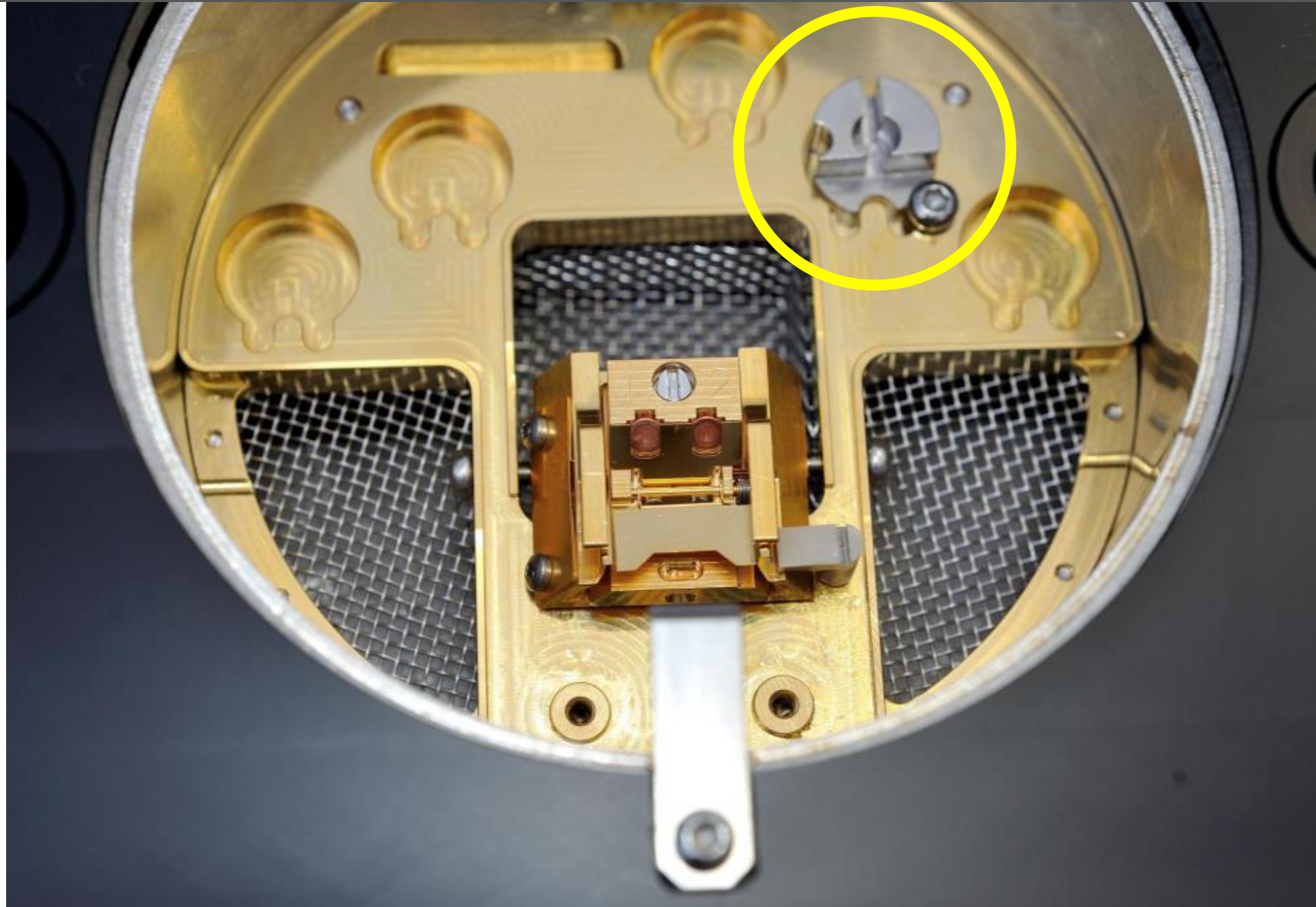
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.2.2 Prepare the grids **_Clip the grids**

Vitrification



CryoFIB

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Atlas & lamella sites

iFLM (Optional)

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Sample conductivity

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Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

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Target confirmation

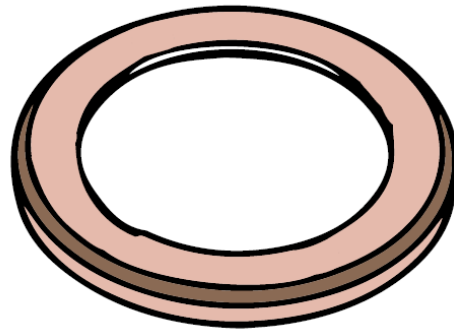
Pt sputter (Optional)

Lamella conductivity

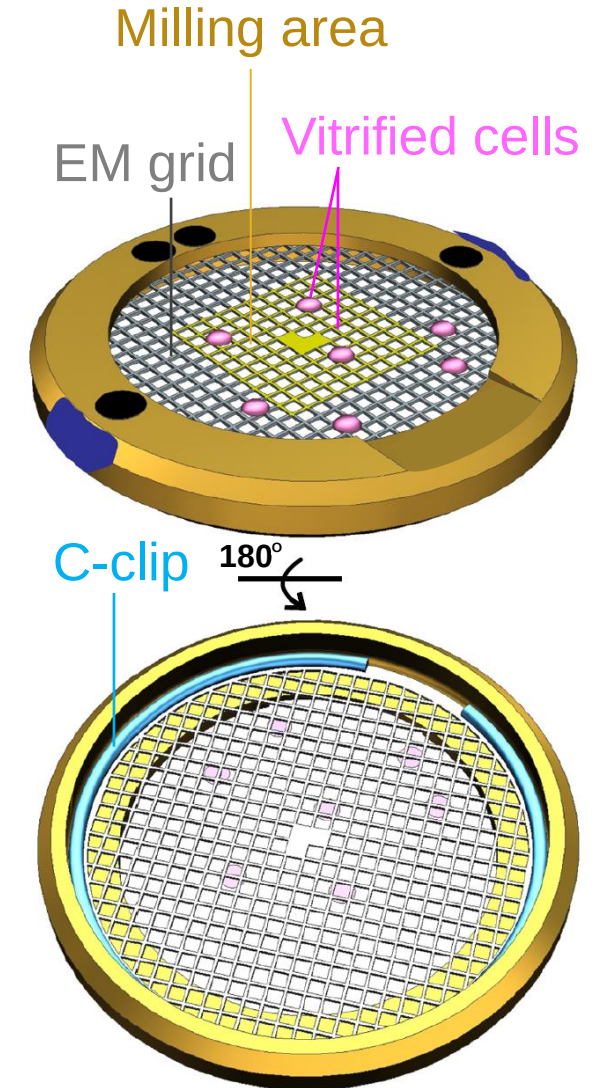
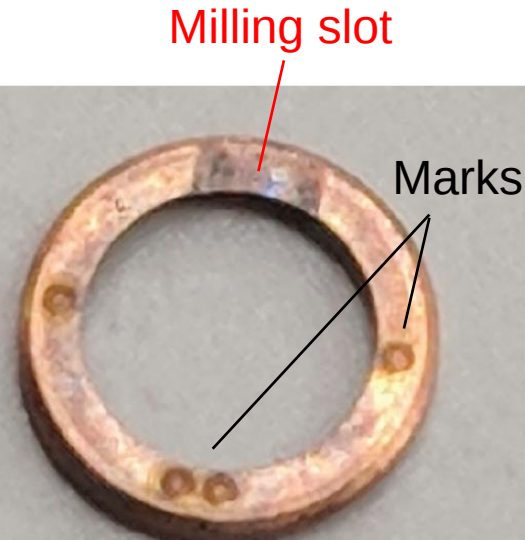
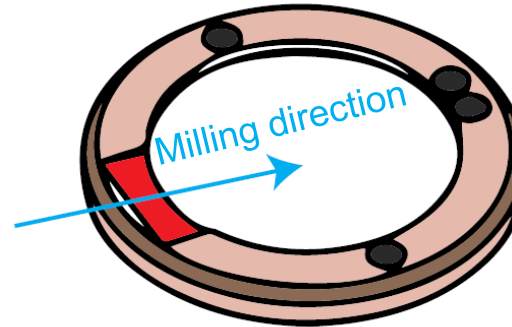


CryoET

Conventional AutoGrid



FIB-AutoGrid



Adapted from Wagner, et al., Nature Protocols 2020

Mark the AutoGrid rim to facilitate orienting the lamellae during grid loading

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

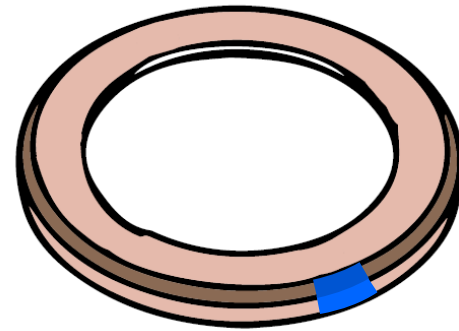
Pt sputter (Optional)

Lamella conductivity

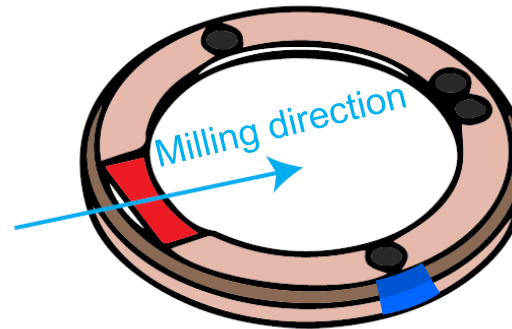


CryoET

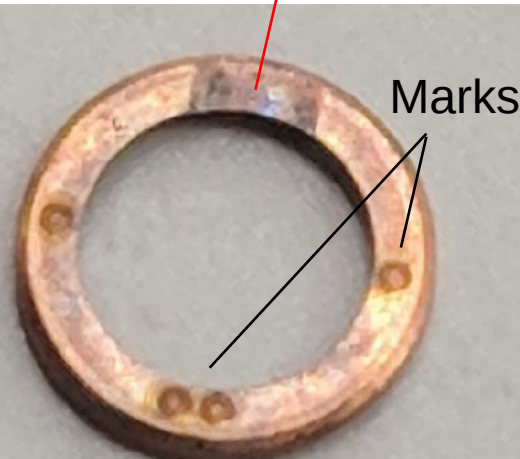
Conventional AutoGrid



FIB-AutoGrid



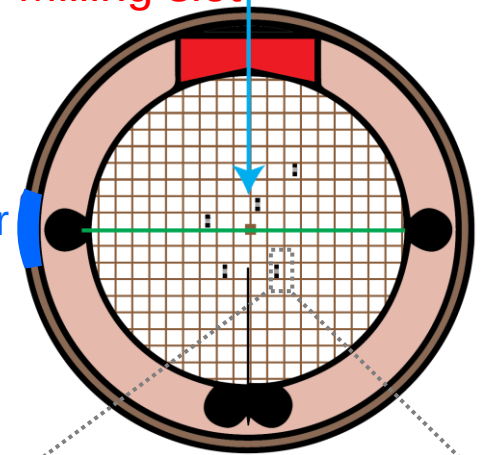
Milling slot



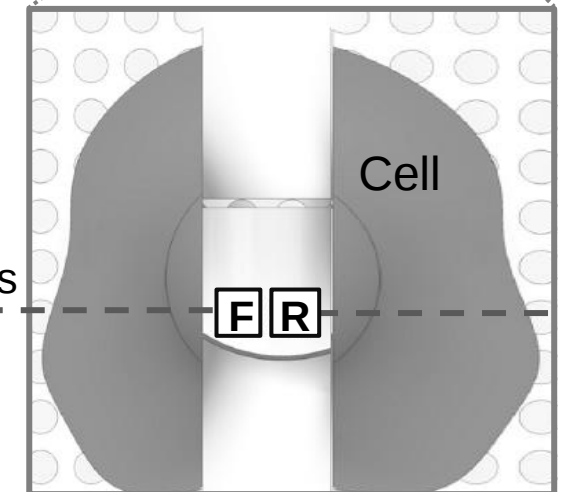
Marks

Milling direction
Milling slot

Marker



Tilt axis



Cell

F R

Adapted from Wagner, et al., Nature Protocols 2020; Schaffer et al., JSB 2017

Mark the AutoGrid rim to facilitate orienting the lamellae during grid loading

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

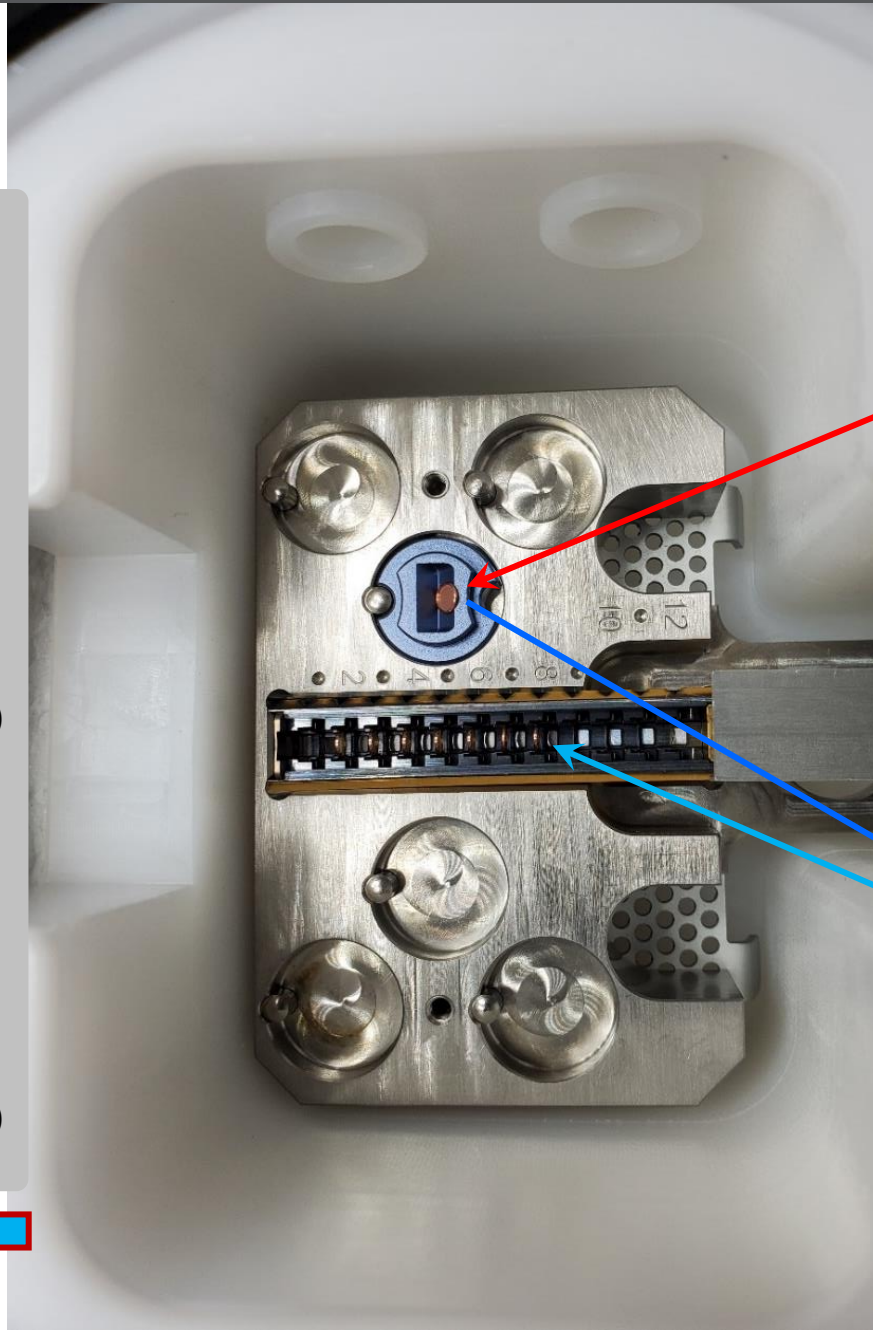
Target confirmation

Pt sputter (Optional)

Lamella conductivity



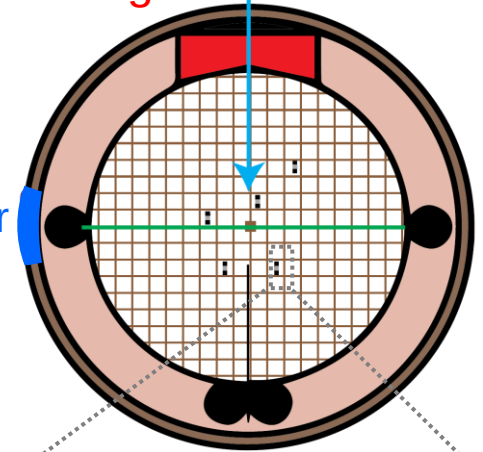
CryoET



Rotate 90°

Milling direction
Milling slot

Marker

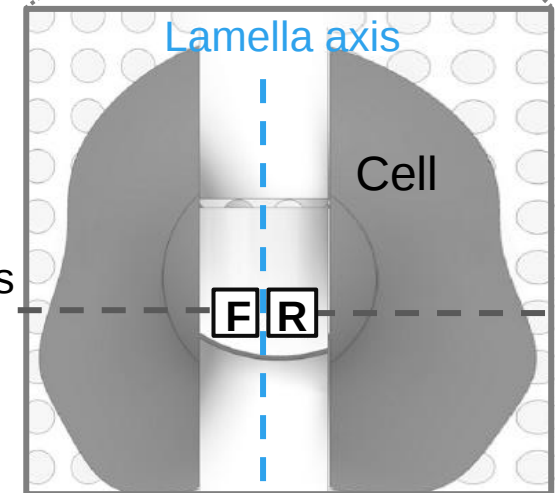


Lamella axis

Cell

Tilt axis

FIR

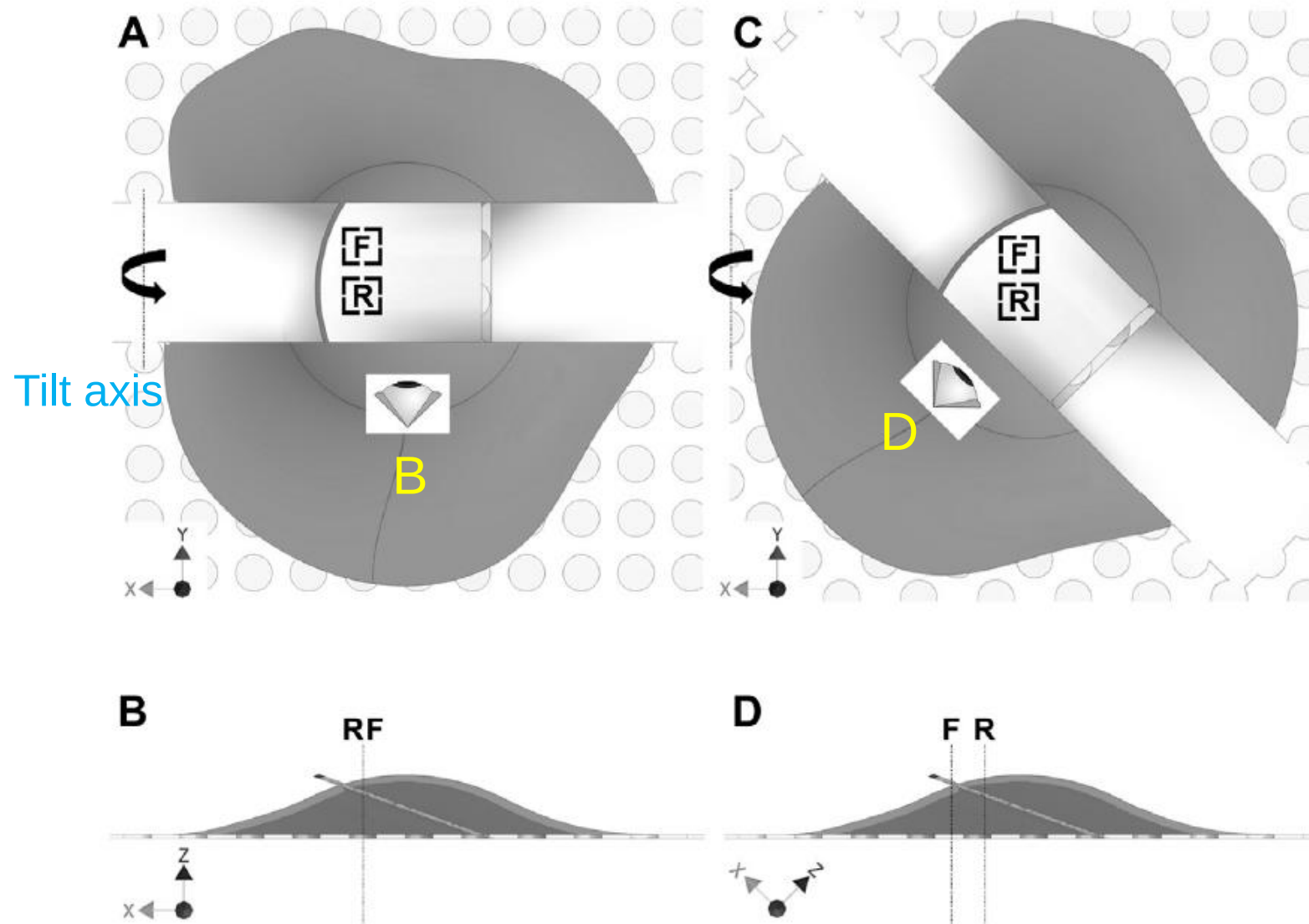


Adapted from Wagner, et al., Nature Protocols 2020;
Schaffer et al., JSB 2017

Poor orientation causes occlusion at high tilt angles & inaccurate focusing

Good orientation

Poor orientation



3.2.3 Transfer the grids **_Load grids to Autogrid shuttle**

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

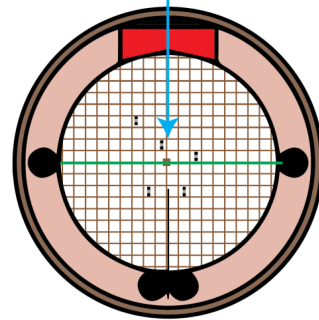
Pt sputter (Optional)

Lamella conductivity

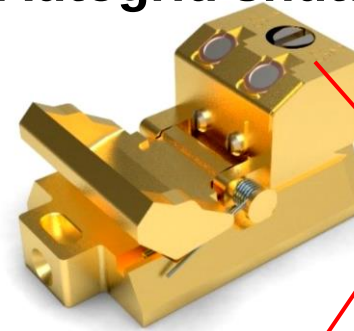


CryoET

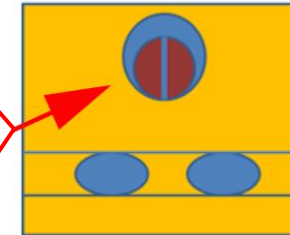
Milling direction



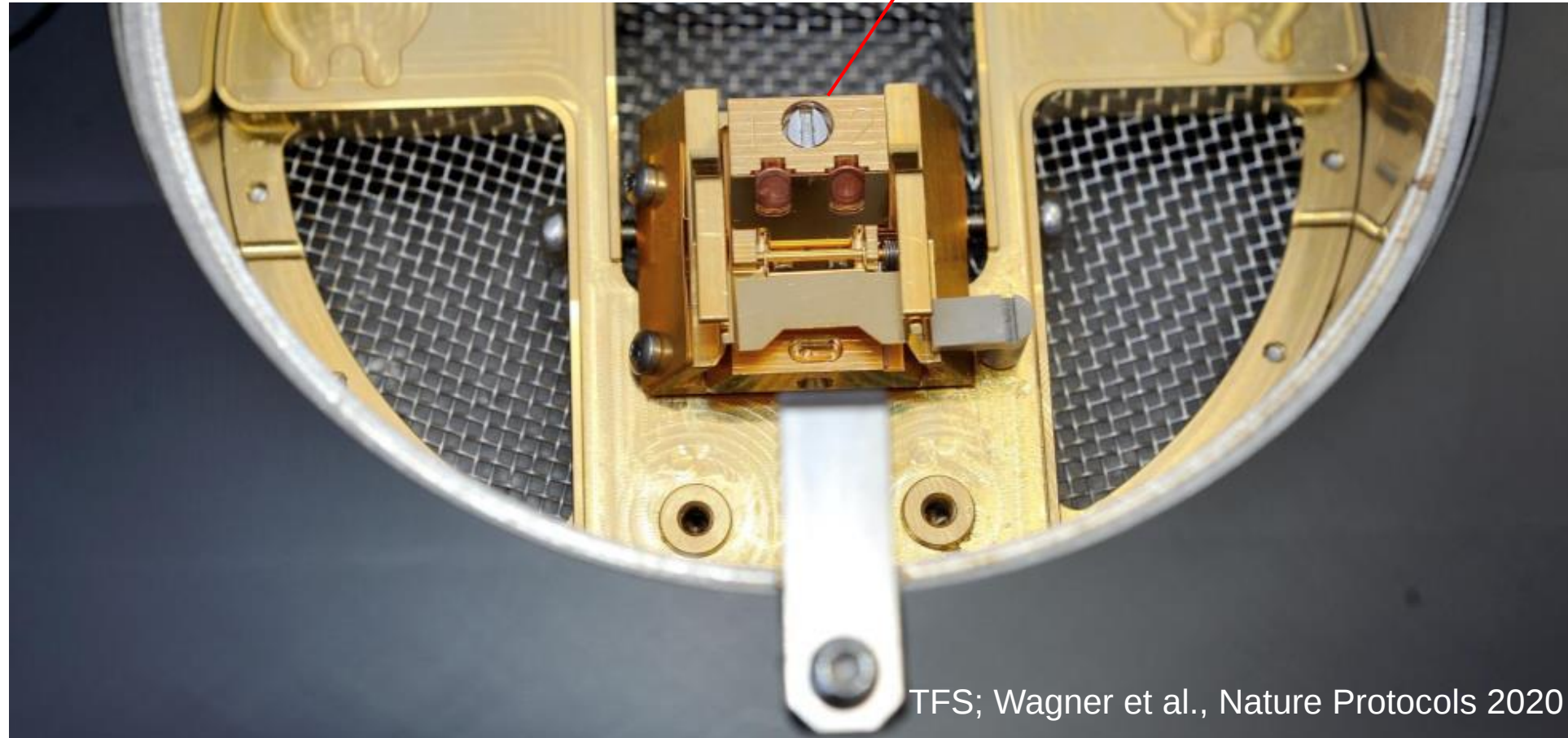
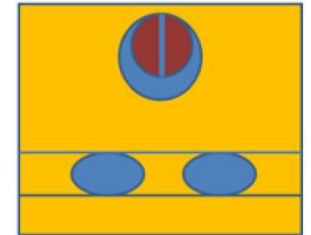
Autogrid shuttle



Open

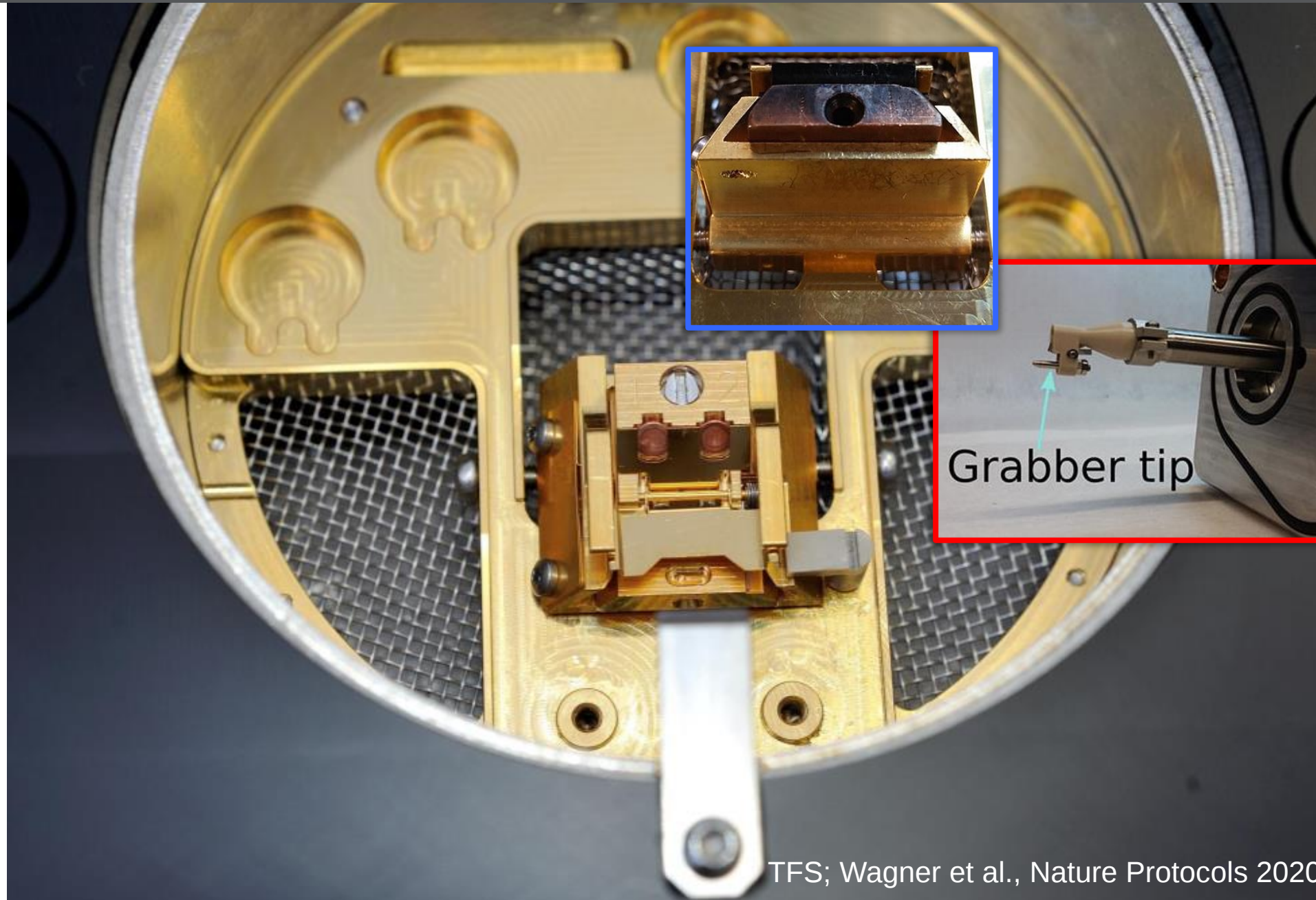


Closed

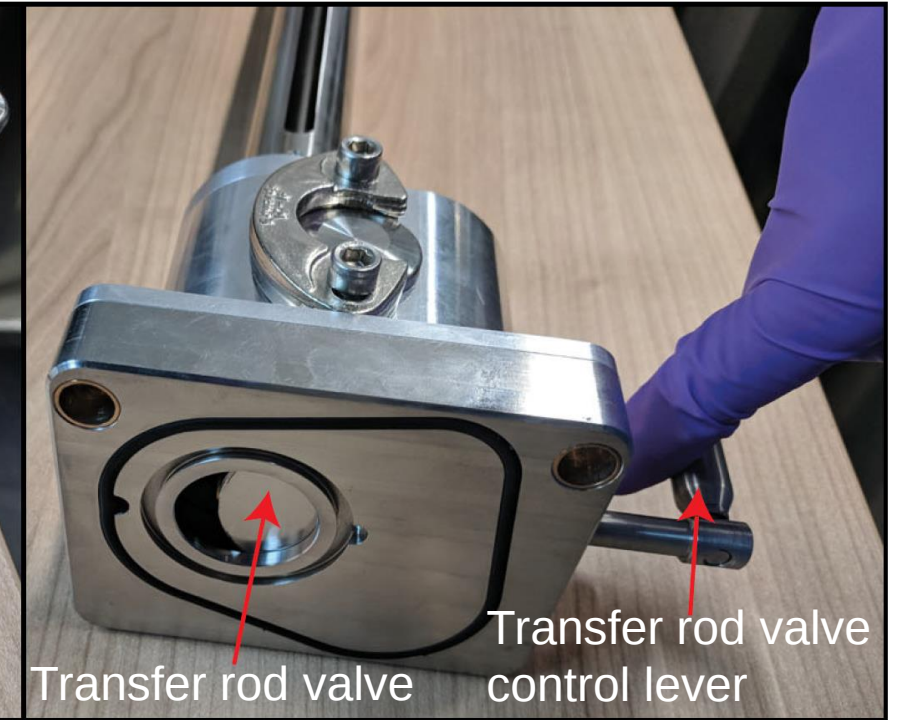
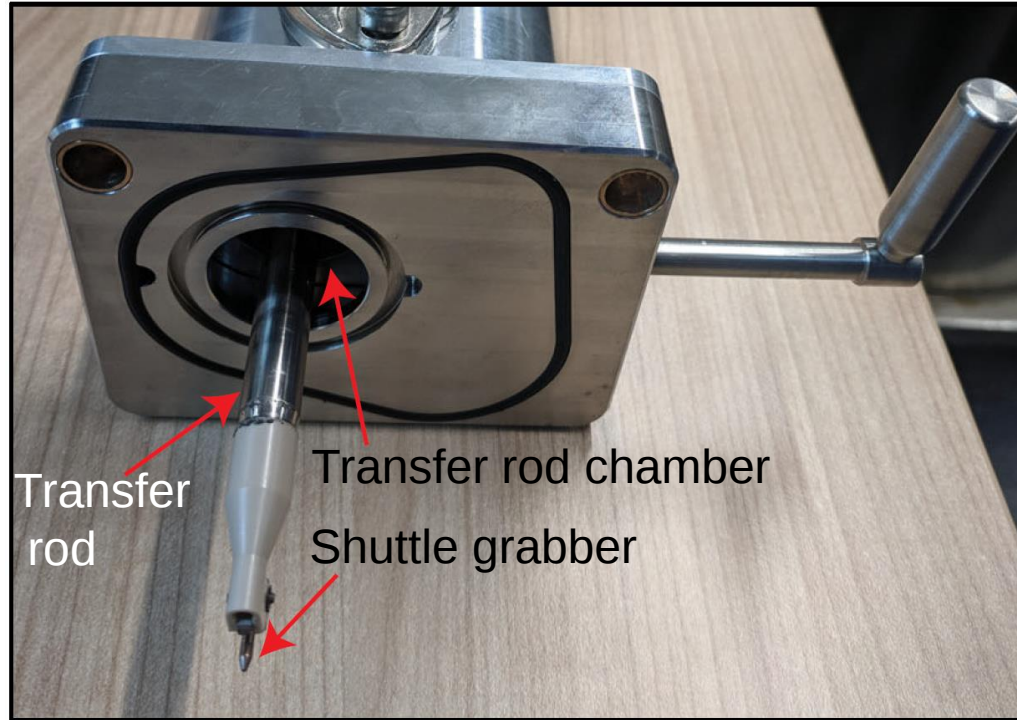


TFS; Wagner et al., Nature Protocols 2020

3.2.3 Transfer the grids Load shuttle to Transfer rod



3.2.3 Transfer the grids **_Load shuttle to Transfer rod**



TFS; Lam & Villa, Methods Mol Biol 2021; Wagner et al., Nature Protocols 2020

3.2.3 Transfer the grids to the Aquilos 2

(Video from TFS)



3.3 Sample screening _Setup E-beam

(**xT** Microscope Control)

Vitrification

↓ T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

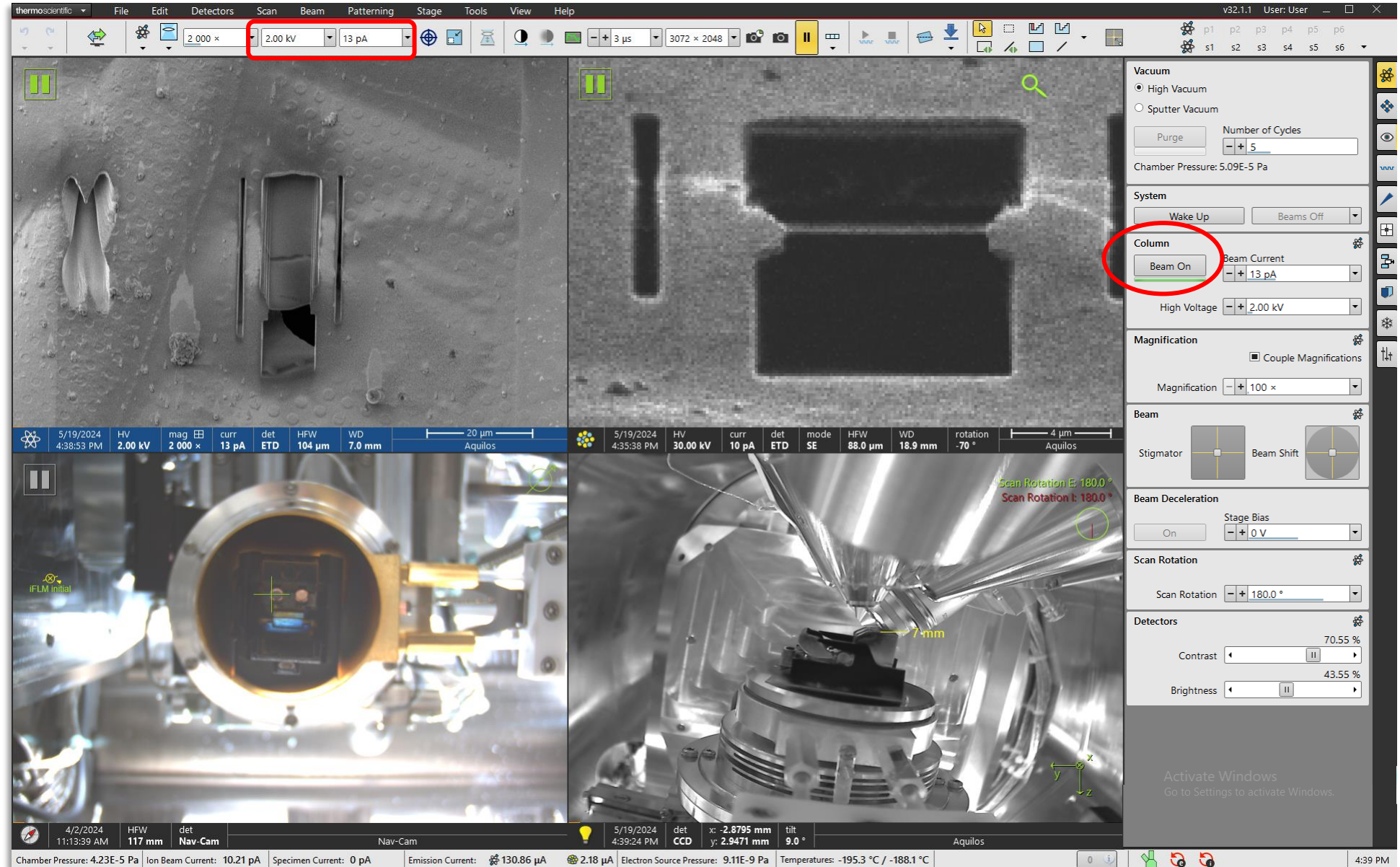
Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T

CryoET



3.3 Sample screening Link Z to FWD

(**xT** Microscope Control)

Vitrification

↓ **T**
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

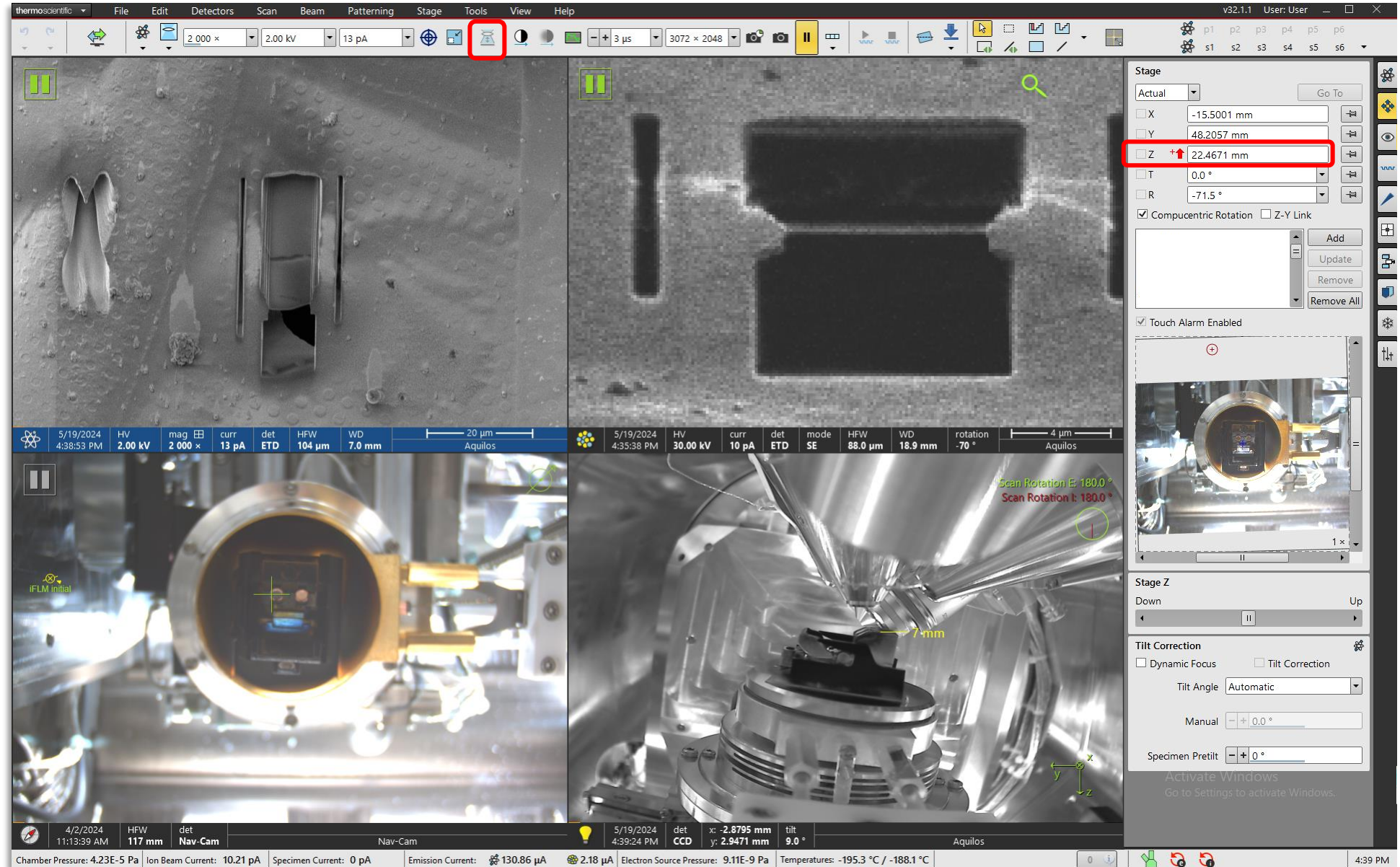
iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ **T**
CryoET



3.3 Sample screening **Quickly check the grids**

(**xT** Microscope Control)

Vitrification

↓ T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T

CryoET

The screenshot displays the xT Microscope Control software interface, which is used for managing the microscope's operations. The interface is divided into several sections:

- Top Panel:** Contains a menu bar (File, Edit, Detectors, Scan, Beam, Patterning, Stage, Tools, View, Help) and a toolbar with various icons for navigation and control. The main display area is split into four quadrants, each showing a different view of the sample or the instrument.
- Left Quadrant:** Shows a high-magnification view of a grid, with a red box highlighting the magnification settings (81 x, 2.00 kV, 13 pA).
- Right Quadrant:** Shows a lower-magnification view of the sample, with a red box highlighting the magnification settings (1536 x 1024).
- Bottom-Left Quadrant:** Shows a view of the iFLM (Ion Focused Low Magnification) system, with a red box highlighting the magnification settings (2.81 x, 13 pA).
- Bottom-Right Quadrant:** Shows a view of the Pt sputter system, with a red box highlighting the magnification settings (10 pA, 30.00 kV).

The right-hand sidebar contains several control panels:

- Shuttle Controls:** Includes buttons for Mapping, Milling, and Deposition, and a section for Selected Grid (Grid 1, Grid 2).
- Temperature & Pressure:** Displays real-time data for Cryo Stage, Cryo Shield, Chamber Pressure, and EasyLift A.
- Recording:** Shows a graph of the recording process over time, with a red box highlighting the recording status (05/19/2024 10:58 AM (Live)).
- Tilt Calculation:** Includes a section for Holder Pretilt, Milling Angle, and Stage Tilt, with a red box highlighting the tilt settings (3.0°, 0.0°).
- Cryo GIS Deposition:** Includes a section for Gas (Pt dep), Selected Grid (Grid 1, Grid 2), Flow Duration, and buttons for Start and Purge.
- Sputter Coating:** Includes a section for Current (30.0 mA), Pressure (10 Pa), Voltage (2 V), and Run Time (15 Sec), with a red box highlighting the sputter settings.
- Lamella Thickness:** Includes a section for Lamella Thickness and a red box highlighting the thickness settings.

The status bar at the bottom of the interface displays various parameters, including Chamber Pressure, Ion Beam Current, Specimen Current, Emission Current, Electron Source Pressure, and Temperatures.

3.3 Sample screening **_Create a Maps Project**

Vitrification

↓ T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

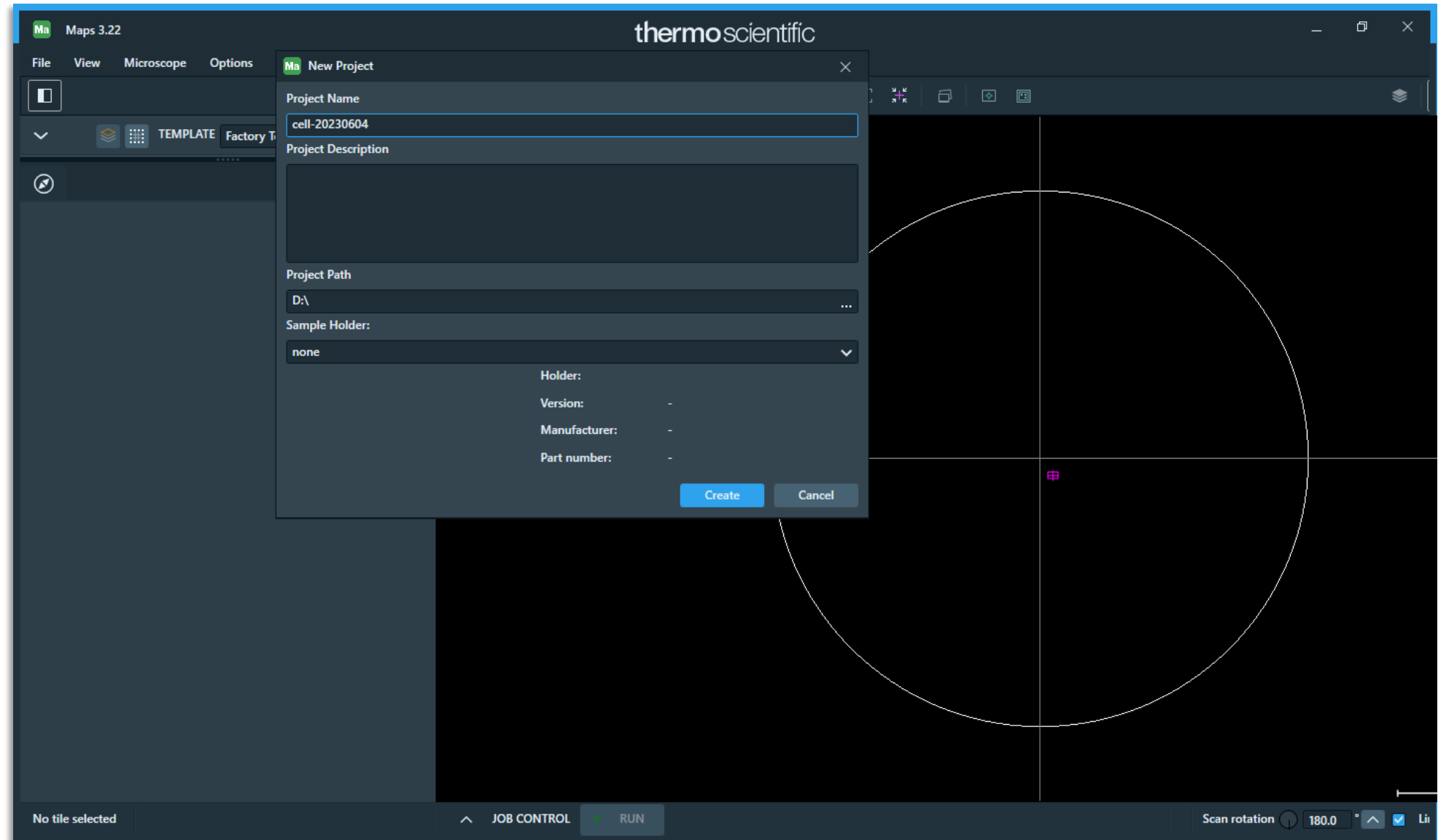
Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T

CryoET



3.3 Sample screening **_Take a snapshot of the grid**

Vitrification

↓ T
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

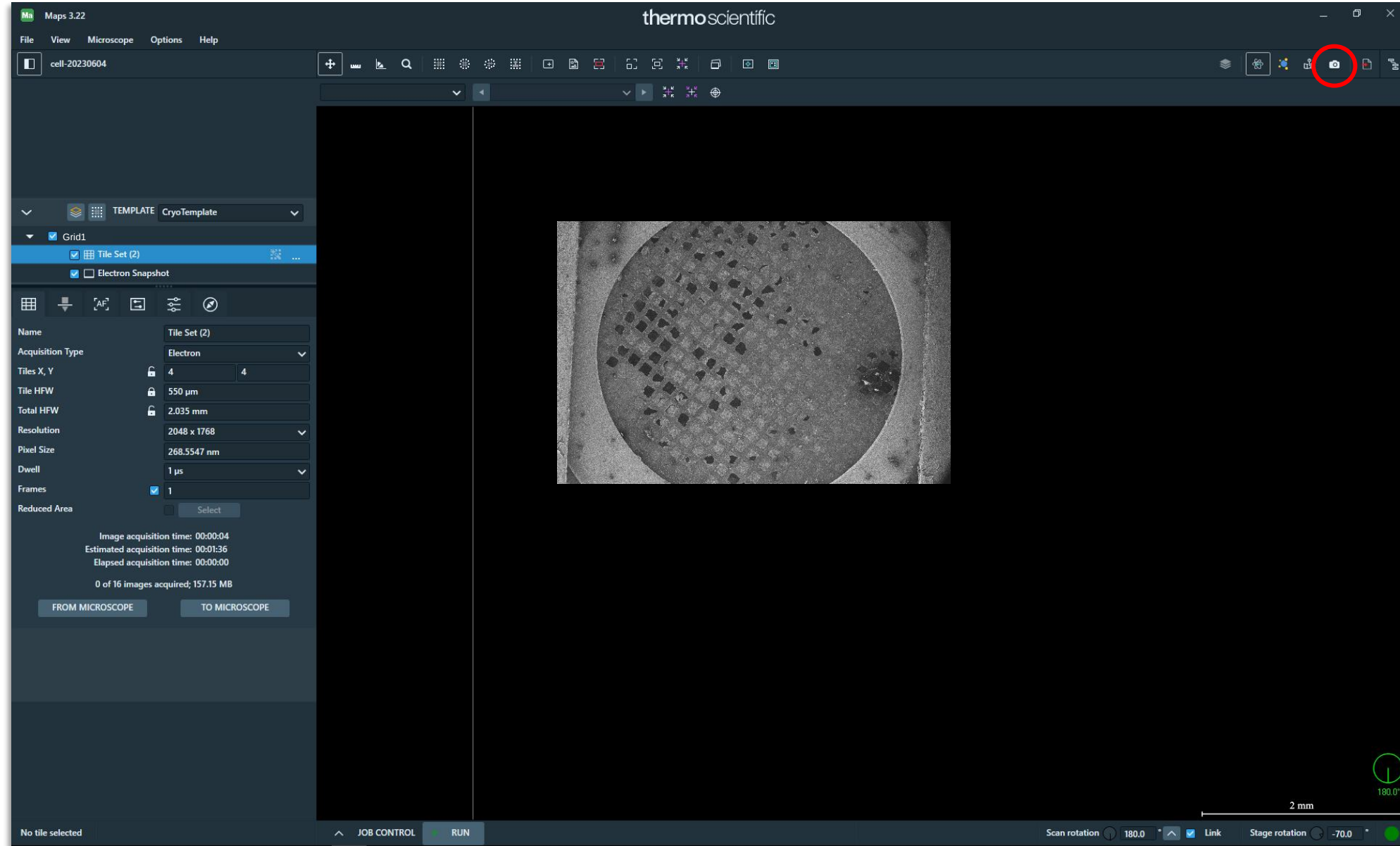
iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T
CryoET



3.3 Sample screening **_Set up Tile Set & Run atlas acquisition**

Vitrification

↓ T
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

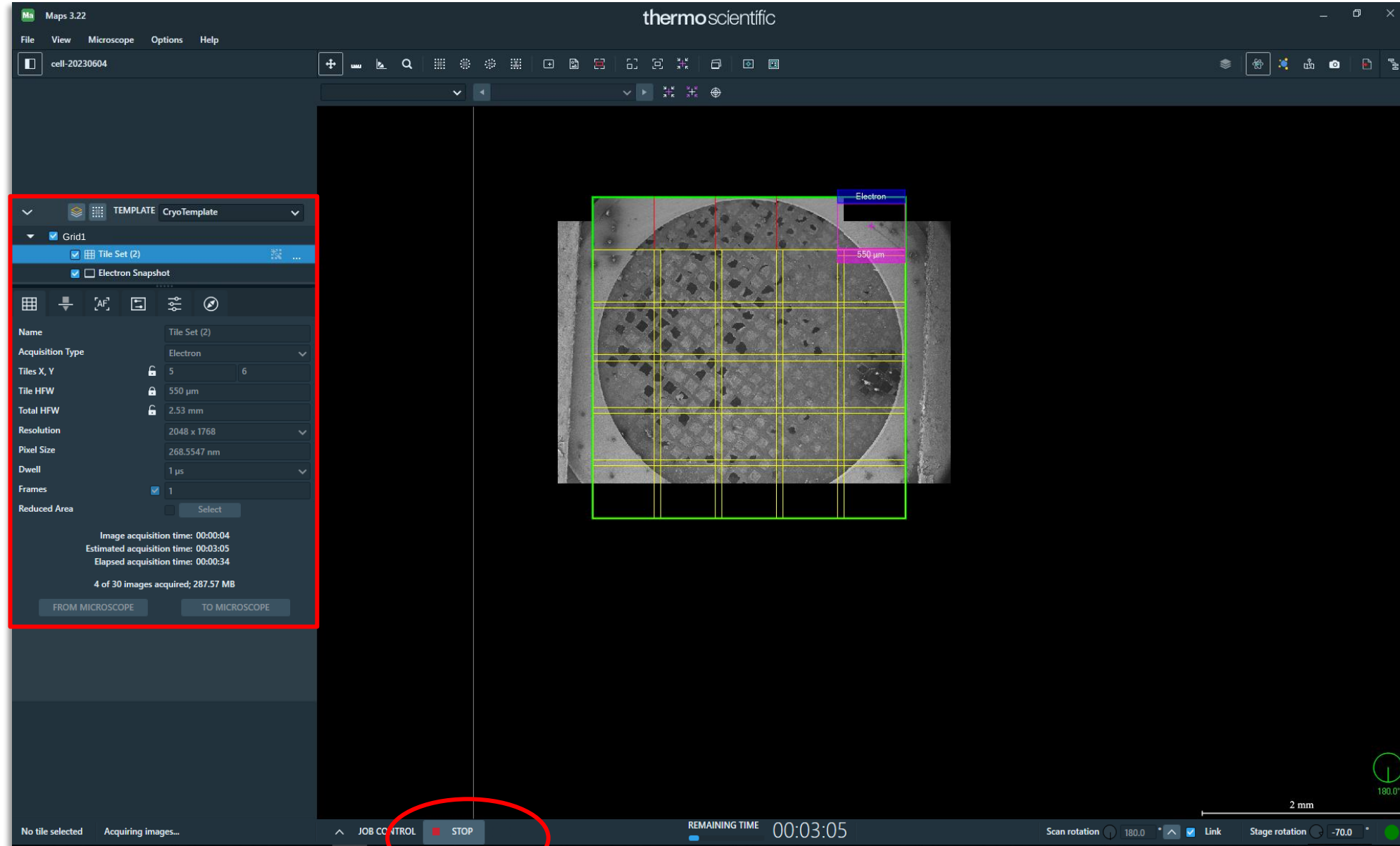
iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T
CryoET



3.3 Sample screening **_Add candidate lamella sites**

Vitrification

↓ T
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

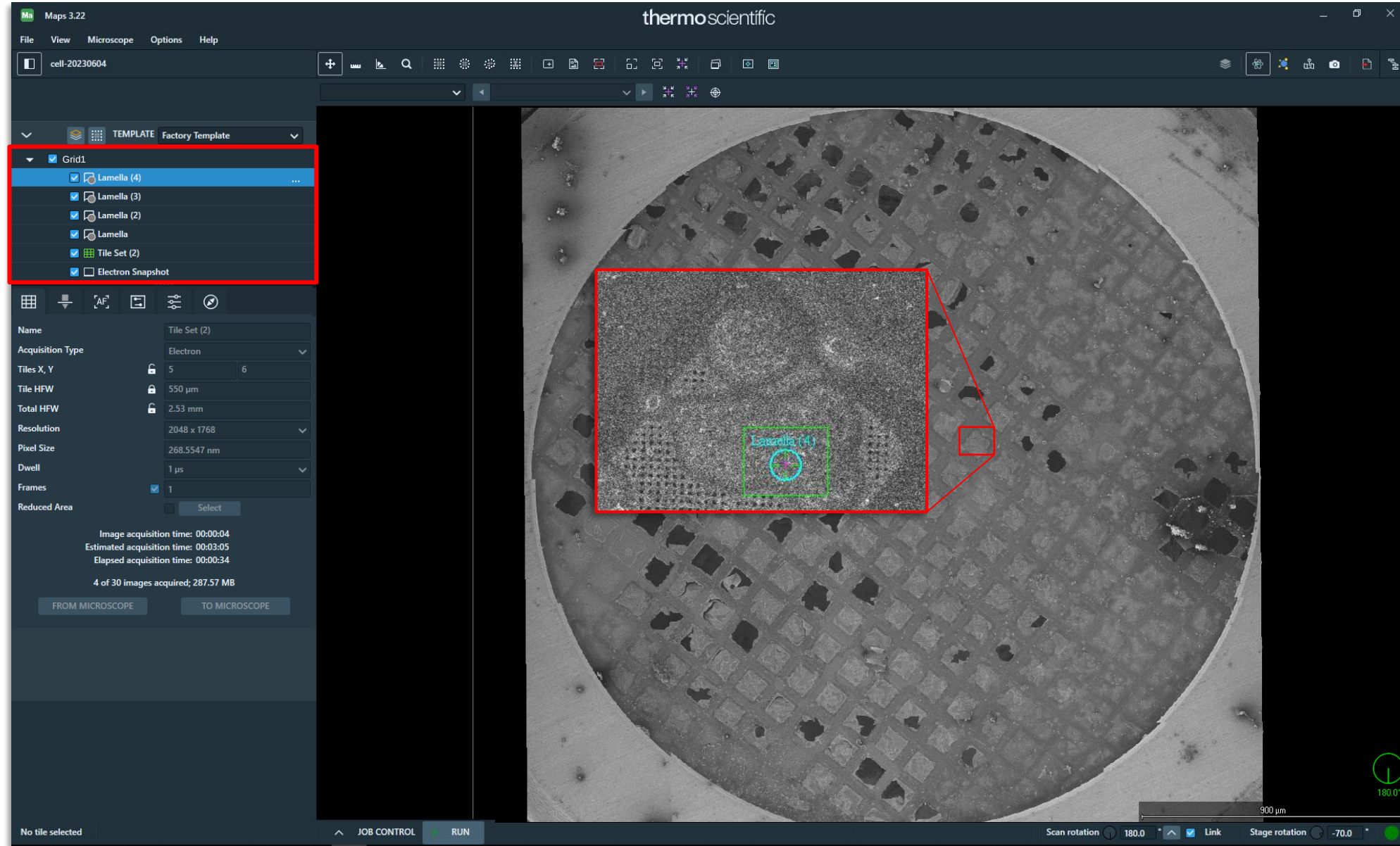
iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T
CryoET



3.4 CryoFLM for target selection

Vitrification

↓ **T**

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

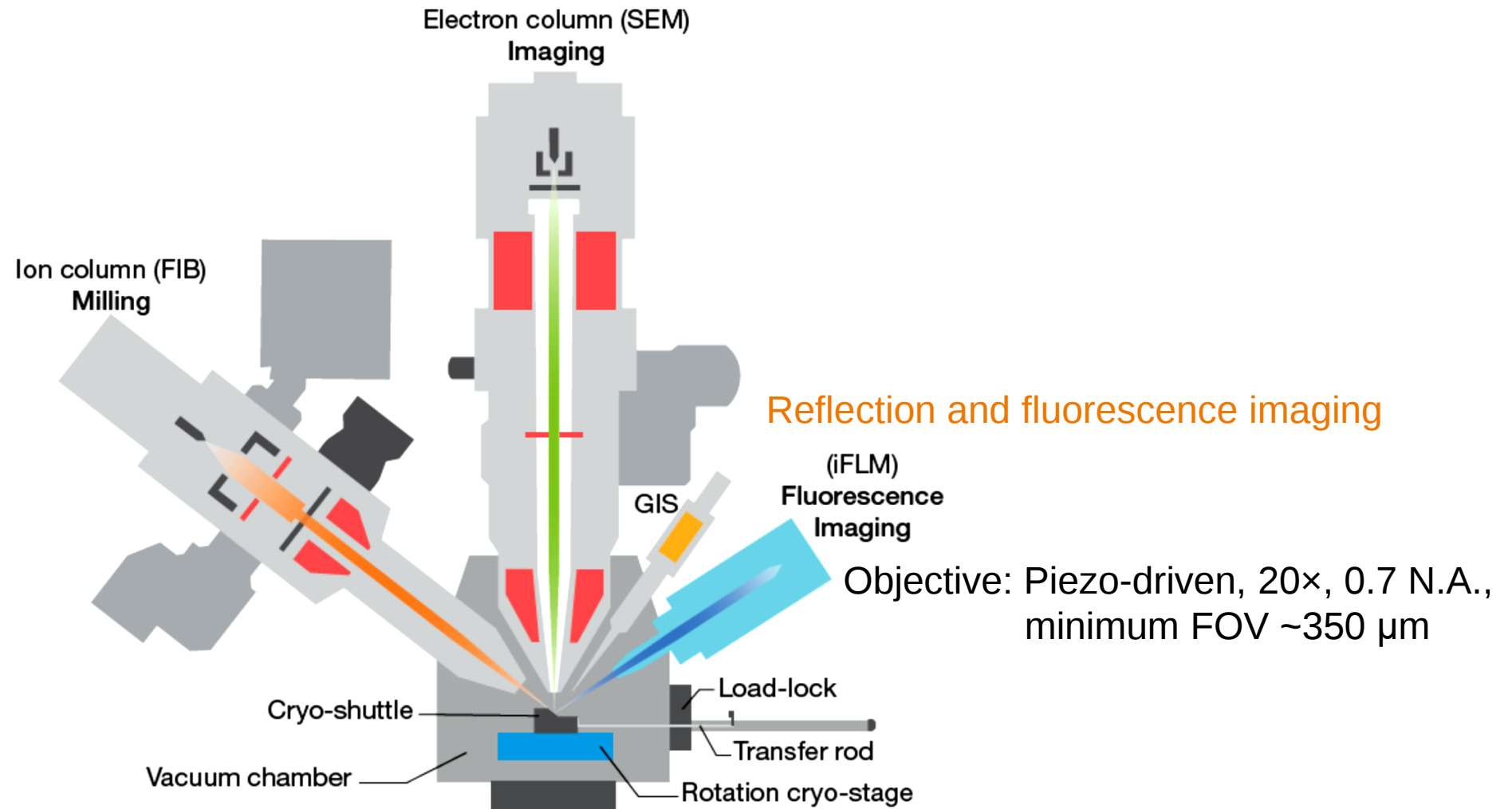
Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ **T**

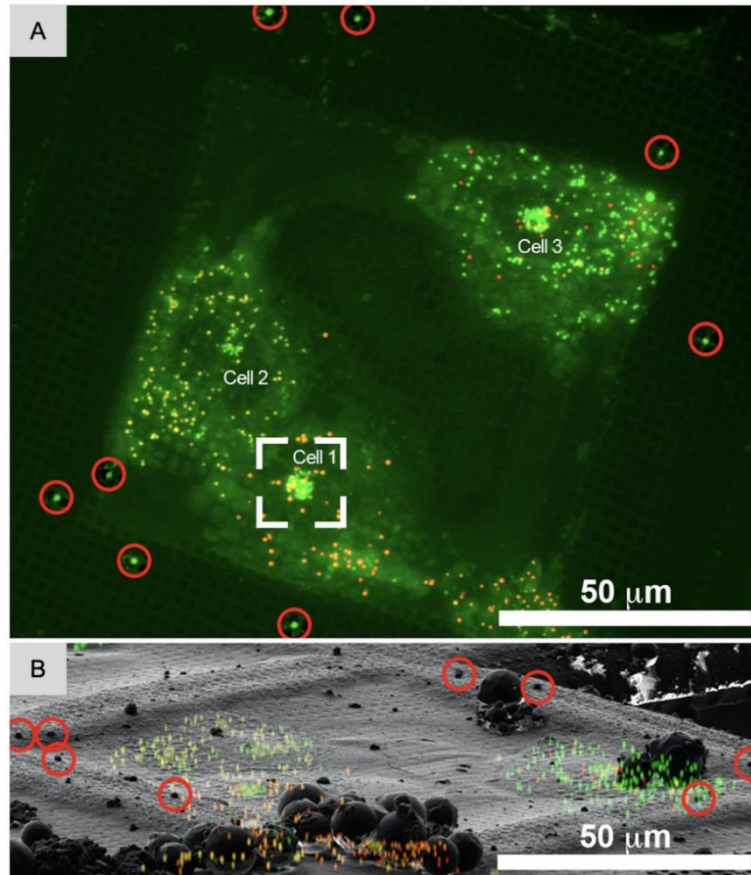
CryoET



Adapted from TFS

Fiducial markers & X-Fiducials for accurate FLM-SEM alignment and target positioning

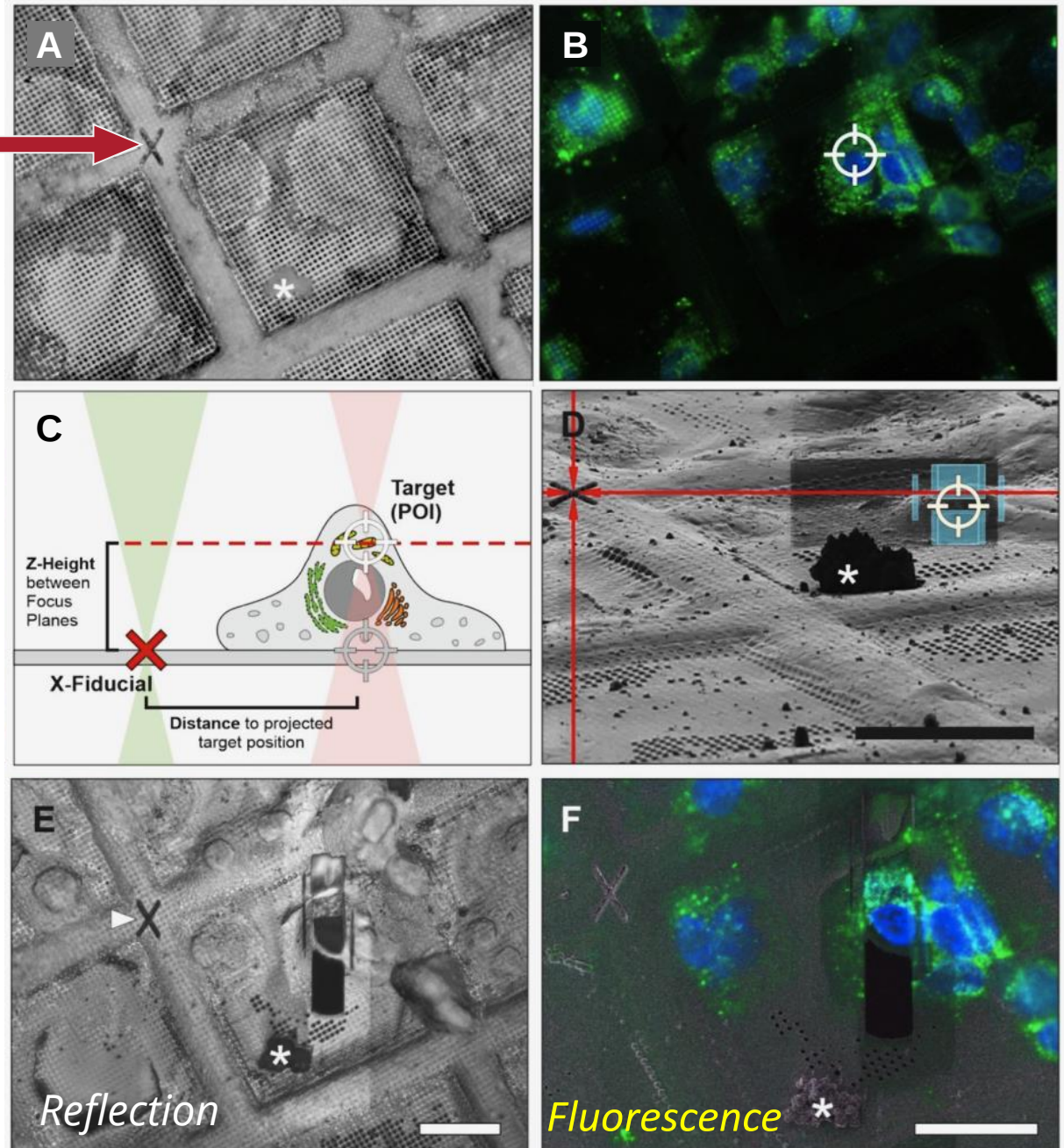
1- μm Magnetic beads



Arnold et al., Biophys J 2016

X-Fiducial

Before
milling



After
milling

Adapted from <https://cryoem101.org/chapter-2-et/>

3.4 CryoFLM _Focus with Objective

( Fluorescence Microscope Control)

Vitrification

↓ T
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

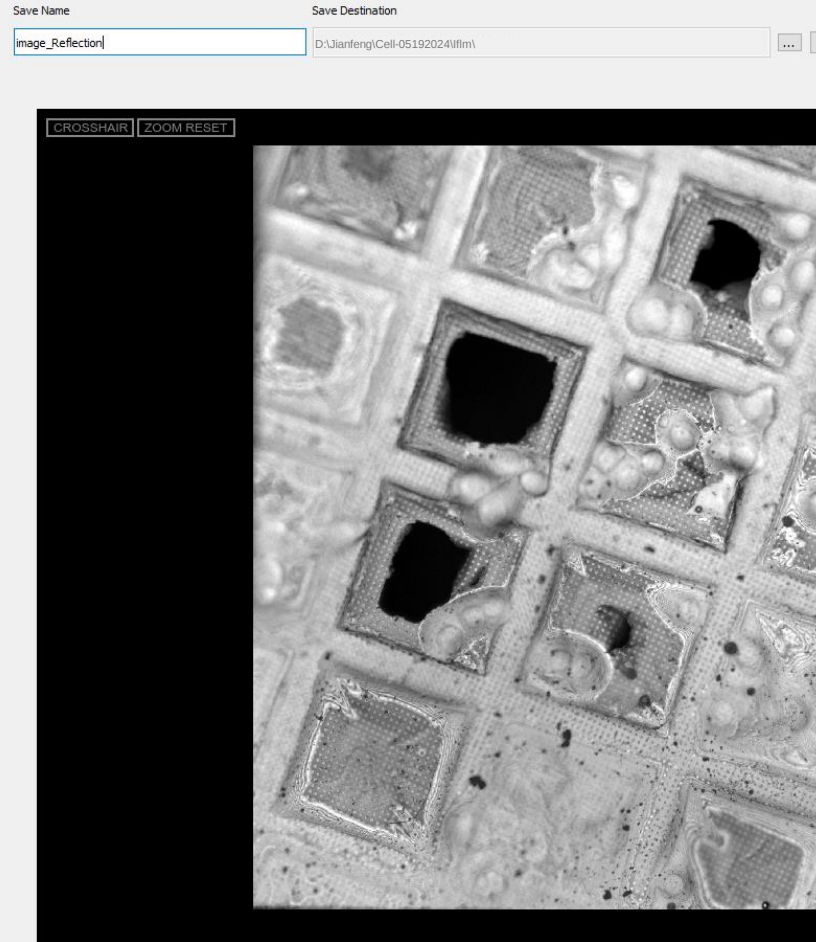
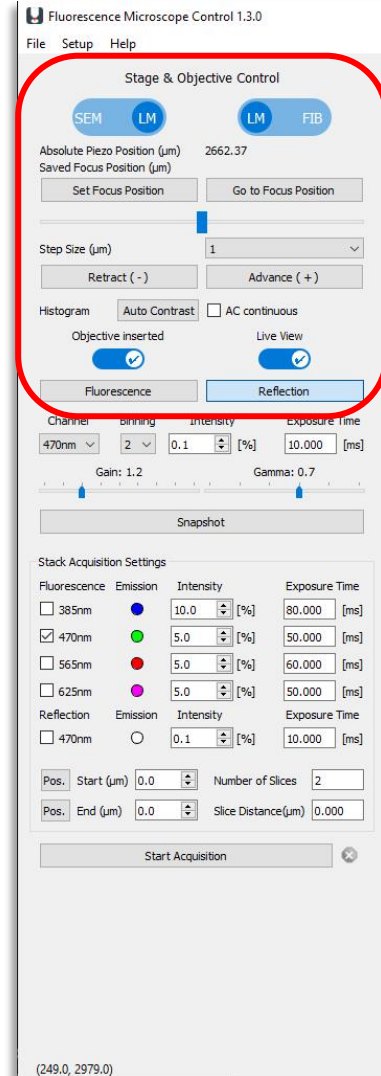
iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T
CryoET



3.4 CryoFLM _Setting up imaging parameters

( Fluorescence Microscope Control)

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

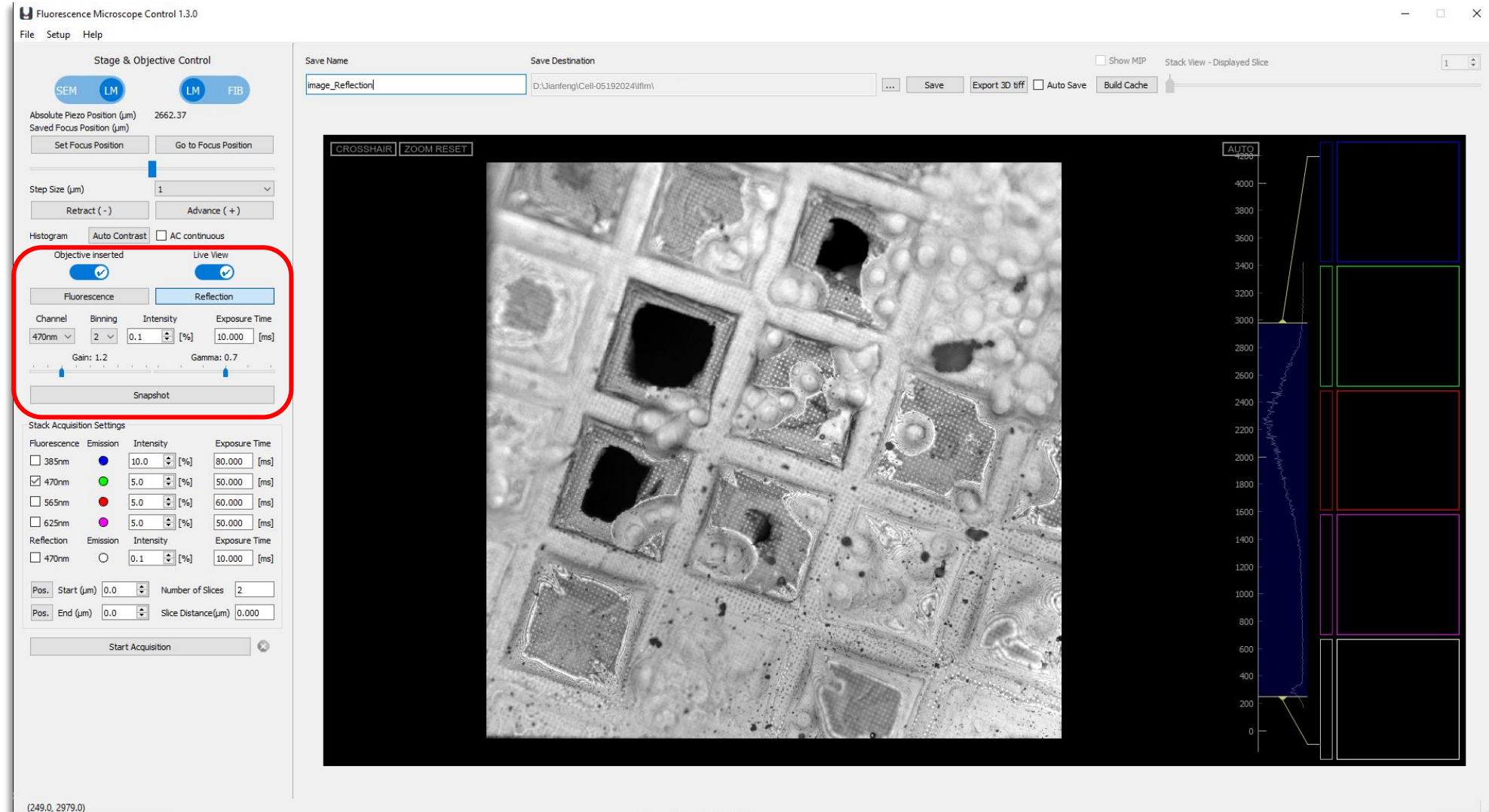
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.4 CryoFLM _Setting up imaging parameters

( Fluorescence Microscope Control)

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

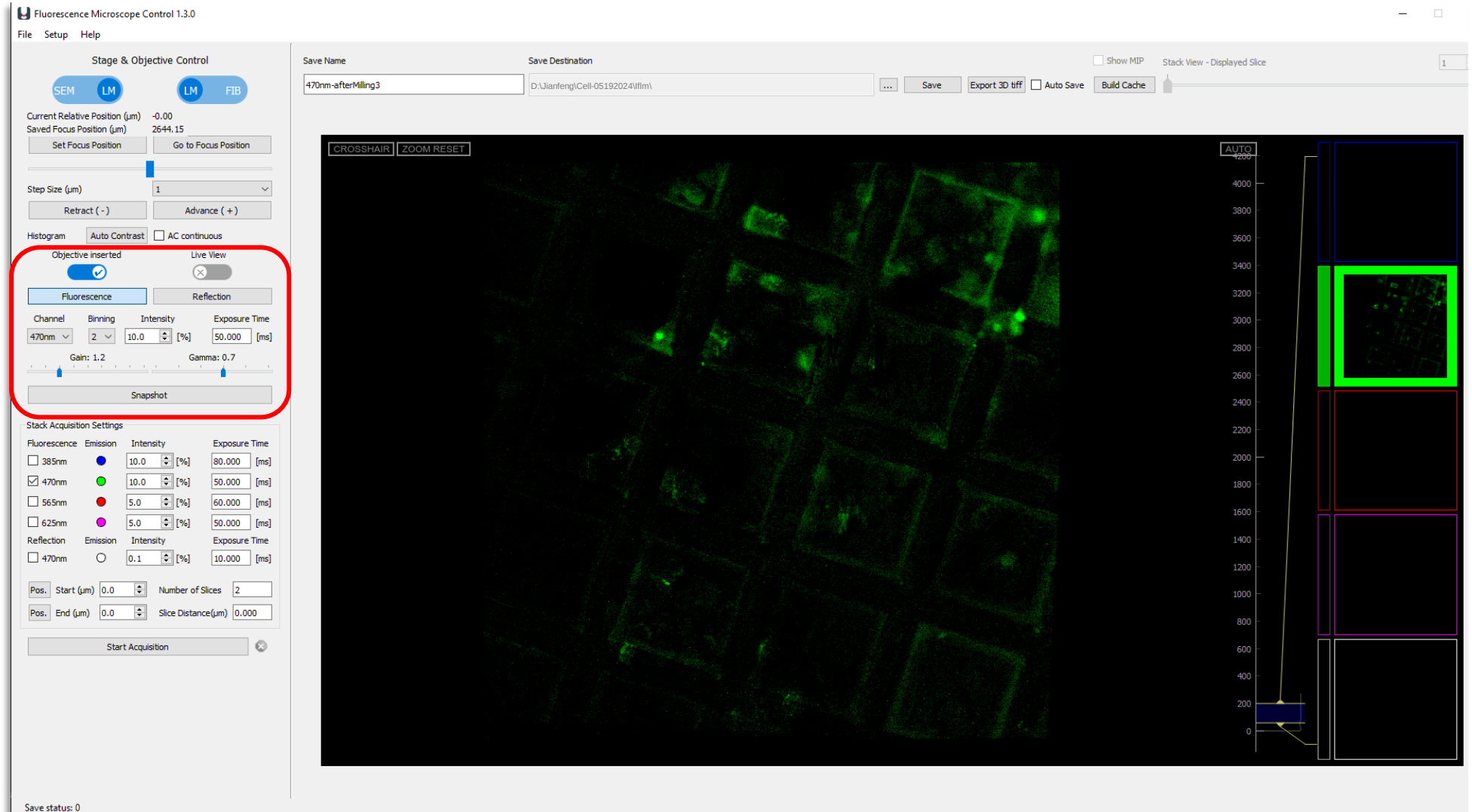
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.4 CryoFLM _Setting up Z-stack

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

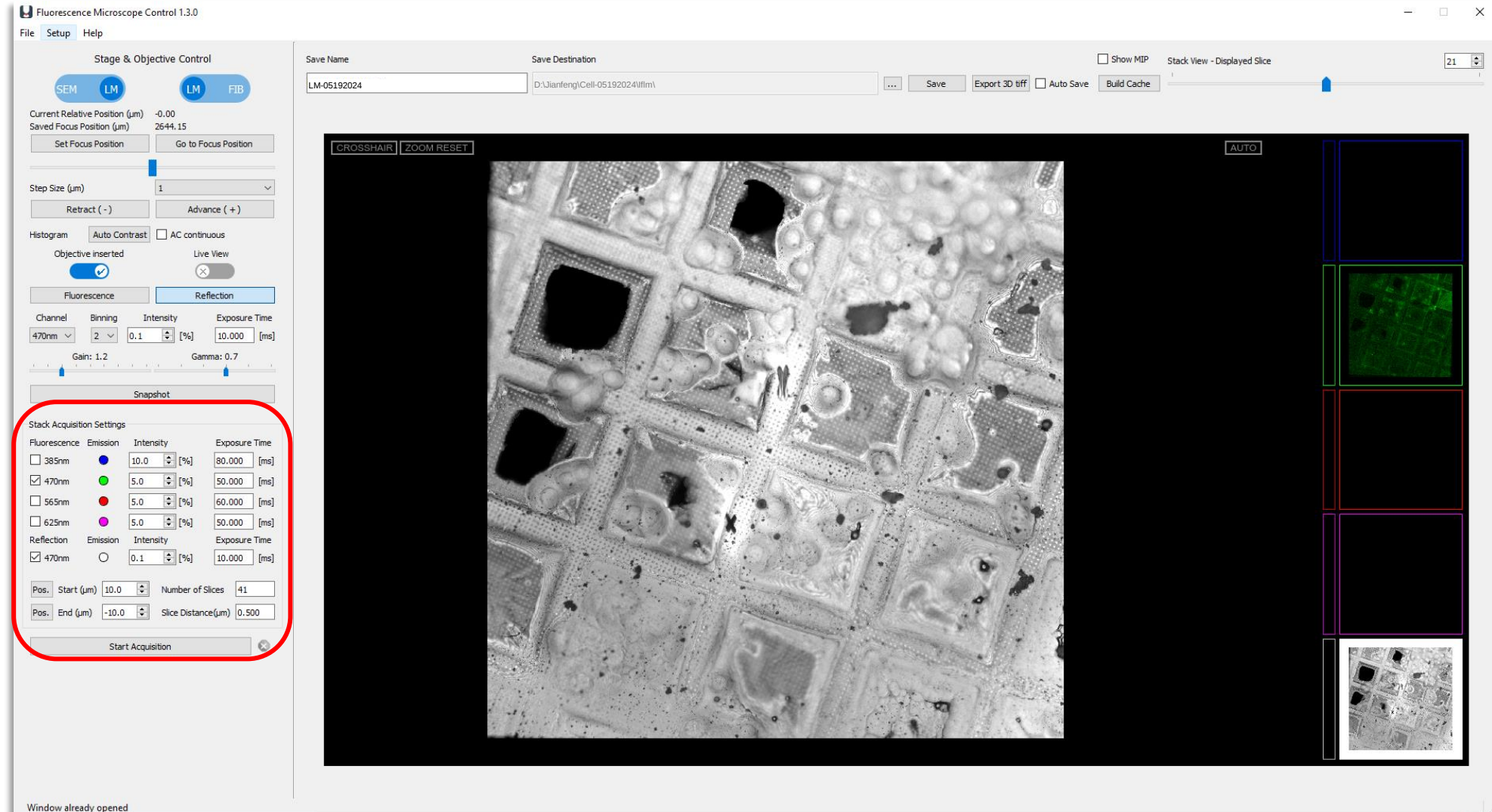
Target confirmation

Pt sputter (Optional)

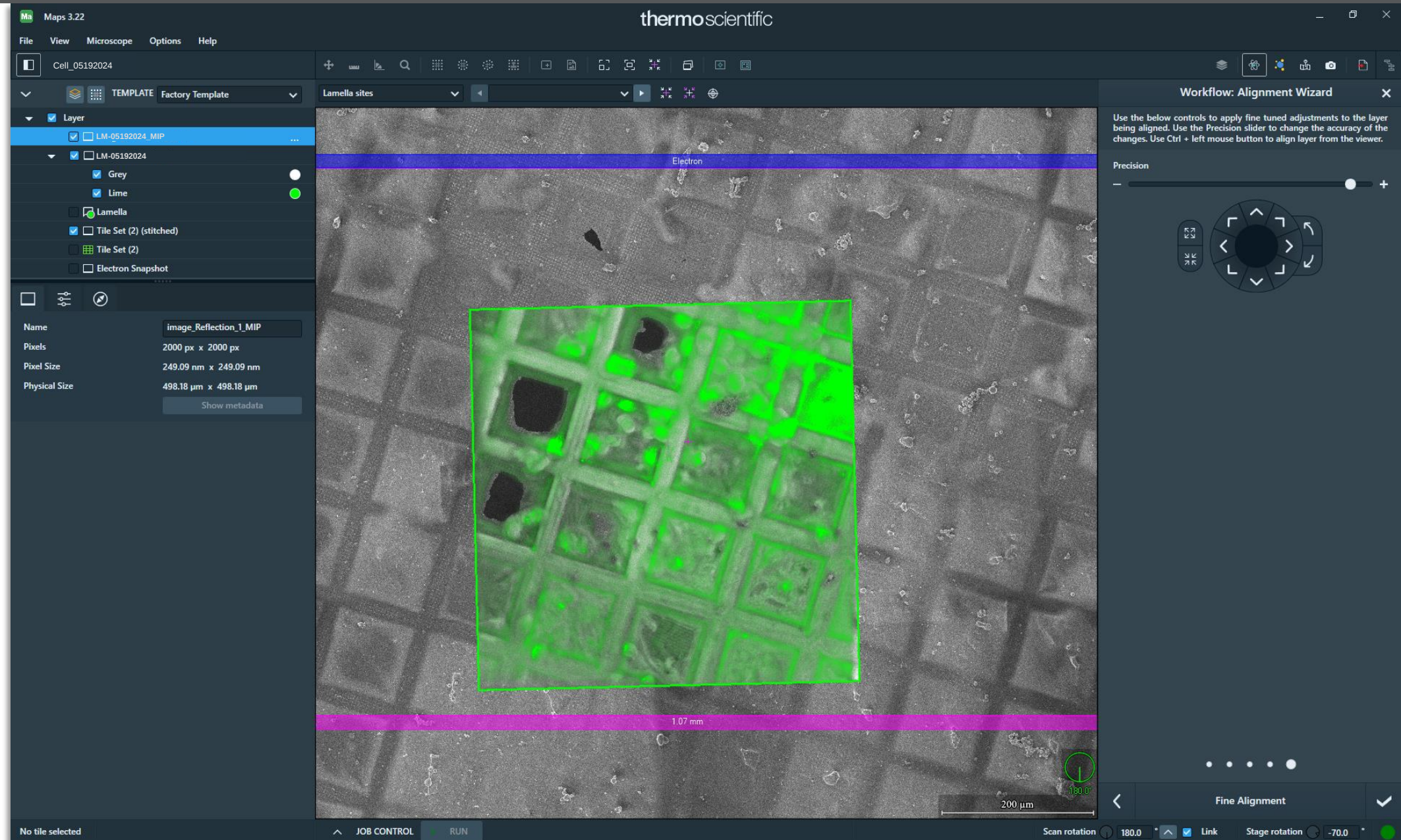
Lamella conductivity



CryoET



3.4 CryoFLM _FLM-SEM alignment by Fine Alignment



3.4 CryoFLM _FLM-SEM alignment by Multi-Point Alignment

Ma Maps 3.22

thermoscientific

File View Microscope Options Help

cell-20230604

Lamella sites

Zoom
Semi-log
Invert
Auto

TEMPLATE CryoTemplate

grid2

- Image_300nm_fluor_green_stack_15_MIP
- Image_300nm_fluor_green_stack_15
- Image_300nm_fluor_blue_stack_15_MIP
- Image_300nm_fluor_blue_stack_15
- Electron Snapshot (5)
- Lamella (7)
- Electron Snapshot (3)

Name: Electron Snapshot (5)
Pixels: 6144 px x 4096 px
Pixel Size: 96.35 nm x 96.35 nm
Physical Size: 592 μm x 394.67 μm
Show metadata

Workflow: Alignment Wizard

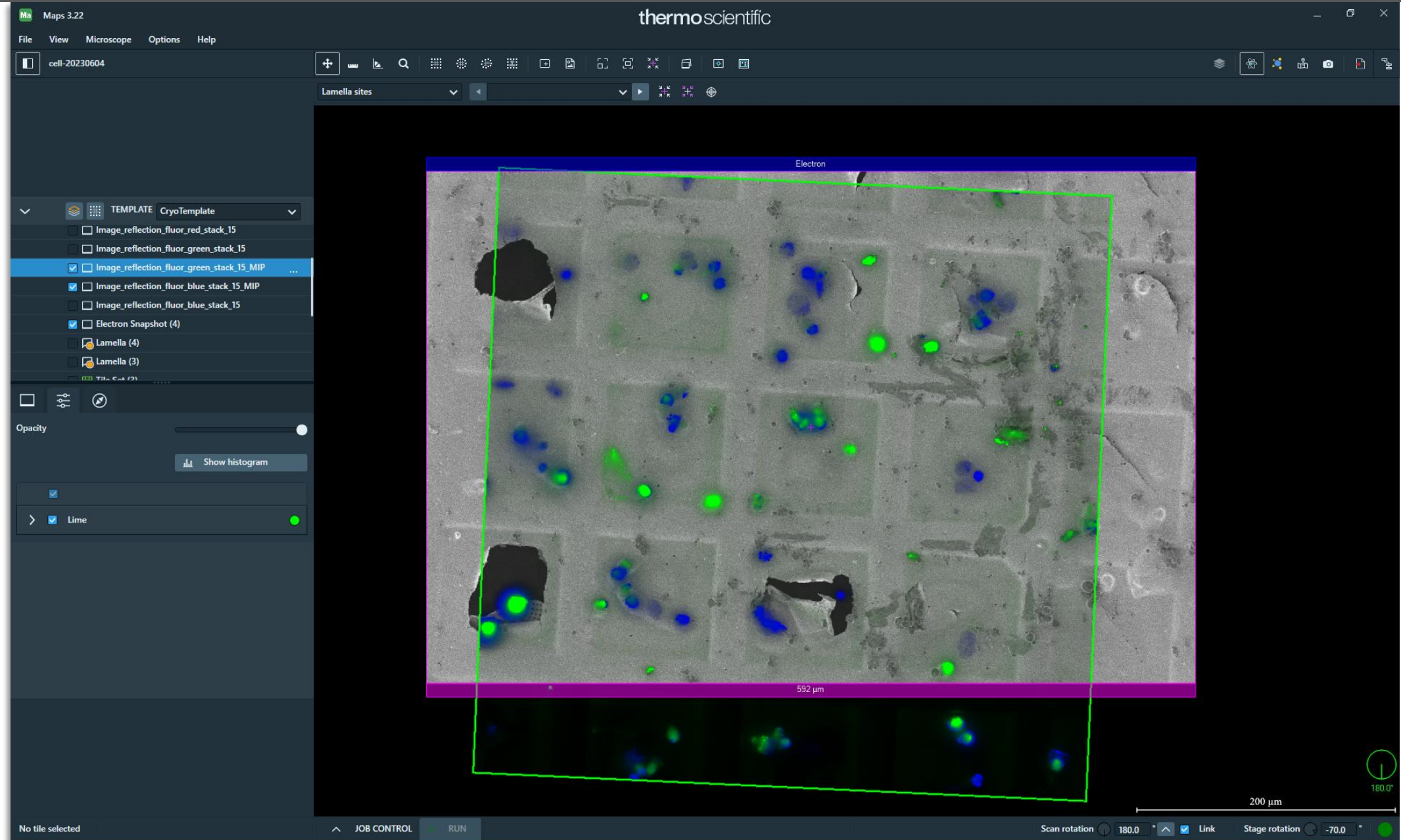
The viewer below displays the data that will be moved. Select distinct alignment points by right mouse click and then choose 'Place Point'. Next find the same features in the viewer to the left and place the points in the same way. Use the 'Snapshot' feature to acquire more images if needed.

Points: 1 2 3 Link Rotation
Stage Axes: $\leftrightarrow$ $\updownarrow$ $\otimes$
Mirror Image: $\leftrightarrow$ $\updownarrow$

Multi-Point Alignment

Scan rotation: 180.0° Link Stage rotation: -70.0°

3.4 CryoFLM _FLM-SEM alignment by Multi-Point Alignment



3.4 CryoFLM _Define Fiducial & ROI markers for each lamella

(Ma Maps)

Ma Maps 3.22

File View Microscope Options Help

Cell_05192024

TEMPLATE Factory Template

Layer

Lamella (3)

ROI

Fiducial (2)

Lamella (2)

ROI (2)

Fiducial

LM-05192024_MIP

LM-05192024

Grey

Lime

Lamella

Tile Set (2) (stitched)

Tile Set (2)

Electron Snapshot

image_reflection_1

Depth

0 μm 20 μm

Plane

17 of 41 (8 μm)

Pixels

2000 px x 2000 px

Pixel Size

249.09 nm x 249.09 nm

Physical Size

498.18 μm x 498.18 μm

Show metadata

No tile selected

thermoscientific

Lime

Zoom

Semi-log

Invert

Auto

Lamella sites

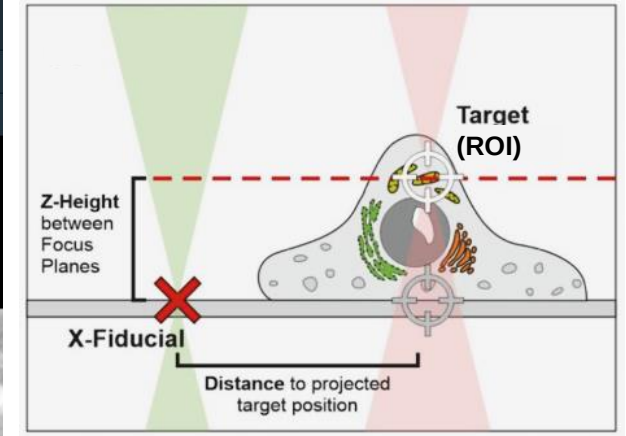
Fiducial

Lamella

100 μm

180.0°

Stage rotation -70.0°



Adapted from
<https://cryoem101.org/chapter-2-et/>

3.4 CryoFLM _Define Fiducial & ROI markers for each lamella

(Ma Maps)

thermoscientific

Maps 3.22

File View Microscope Options Help

Cell_05192024

TEMPLATE Factory Template

Layer

- Lamella (3)
 - ROI
 - Fiducial (2)
- Lamella (2)
 - ROI (2)
 - Fiducial
- LM-05192024_MIP
- LM-05192024
 - Grey
 - Lime
- Lamella
- Tile Set (2) (stitched)
- Tile Set (2)
- Electron Snapshot

image_reflection_1

Depth: 0 μm to 20 μm

Plane: 21 of 41 (10 μm)

Pixels: 2000 px x 2000 px

Pixel Size: 249.09 nm x 249.09 nm

Physical Size: 498.18 μm x 498.18 μm

Show metadata

Lamella sites

Zoom

Semi-log

Invert

Auto

Fiducial

Fiducial (2)

Lamella (2)

Lamella (3)

ROI

ROI

100 μm

180.0°

180.0°

Stage rotation: -70.0°

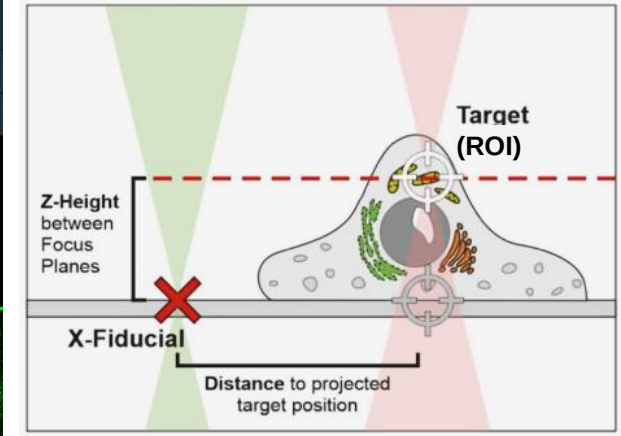
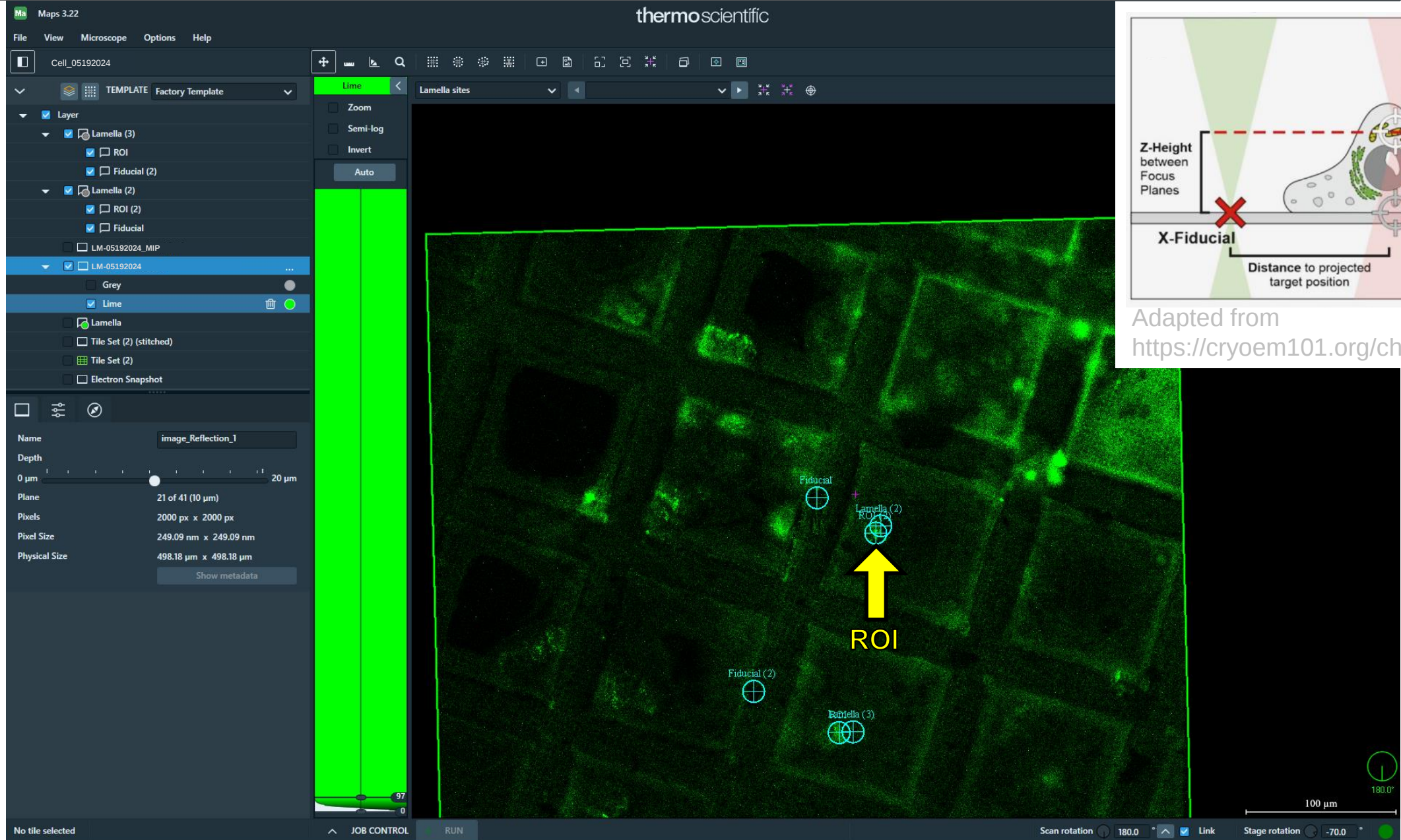
Scan rotation: 180.0°

Link

JOB CONTROL

RUN

No tile selected



Adapted from
<https://cryoem101.org/chapter-2-et/>

3.5 Pt sputter

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

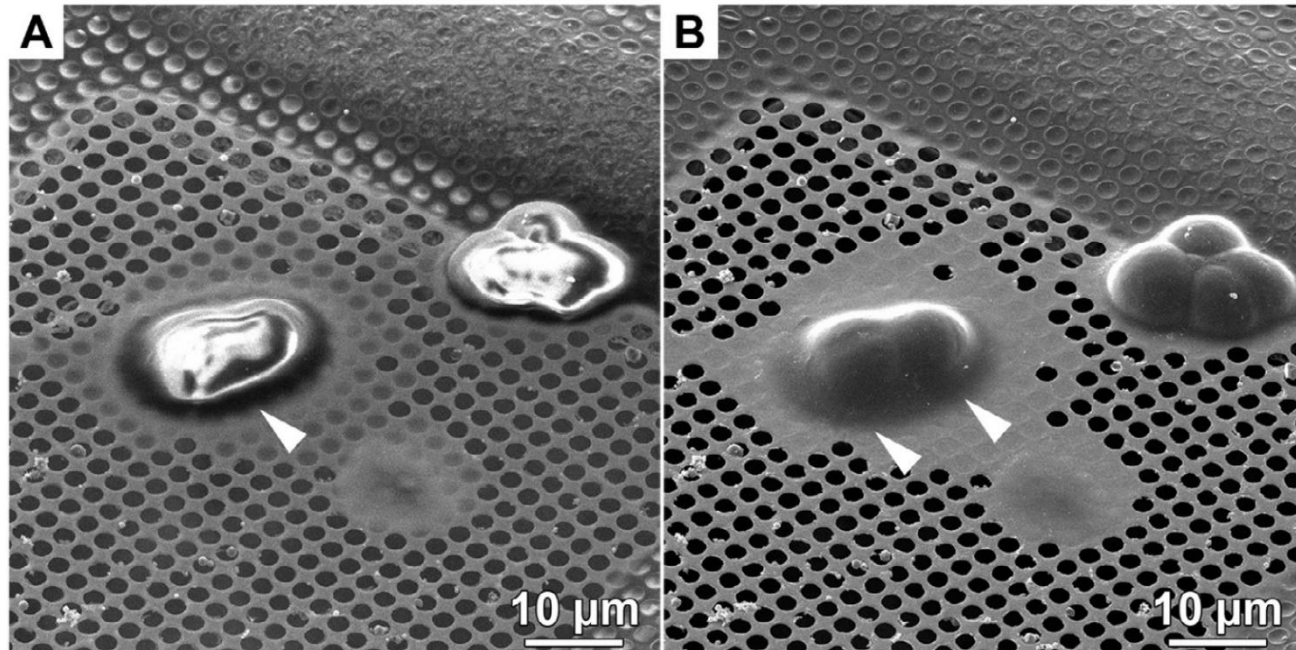
Pt sputter (Optional)

Lamella conductivity



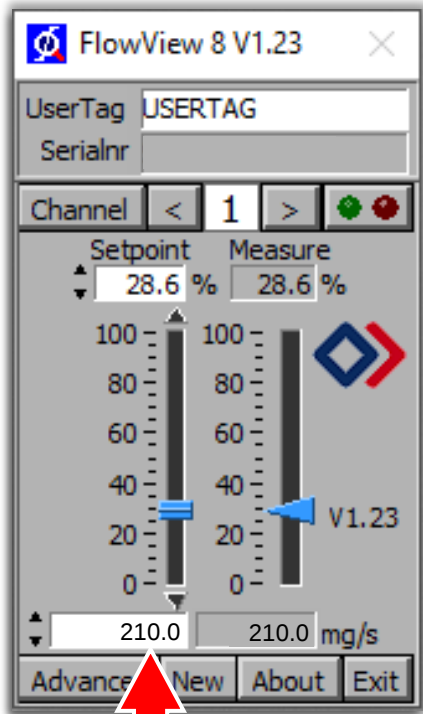
CryoET

(Optional) Inorganic Pt minimizes charging, ensure targeting and precise milling.

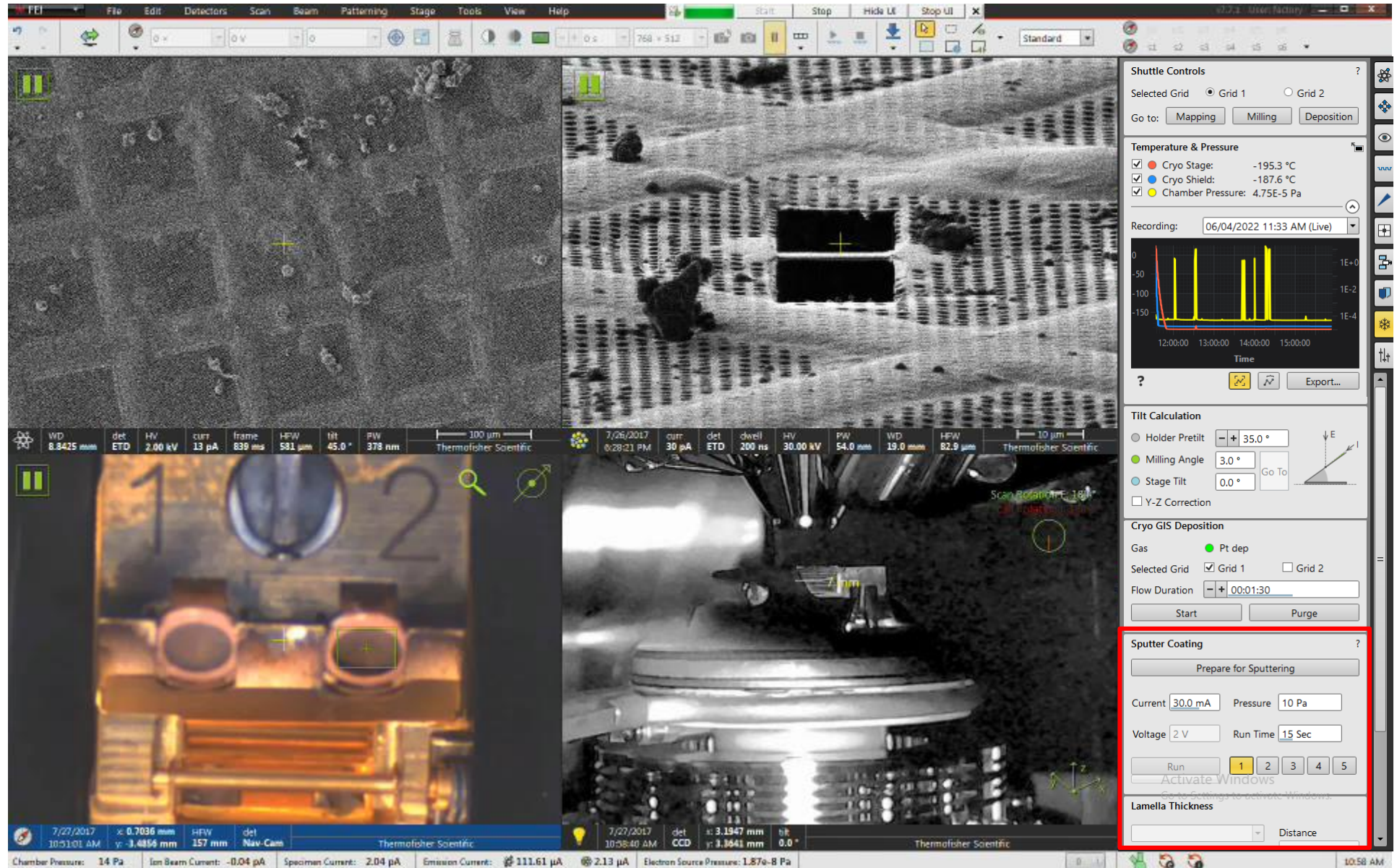


3.5 Pt sputter

(Video from TFS; **xT** Microscope Control)



Increase to 210 mg/s
(varies by instruments)



3.5 Pt GIS

Vitrification

↓ T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

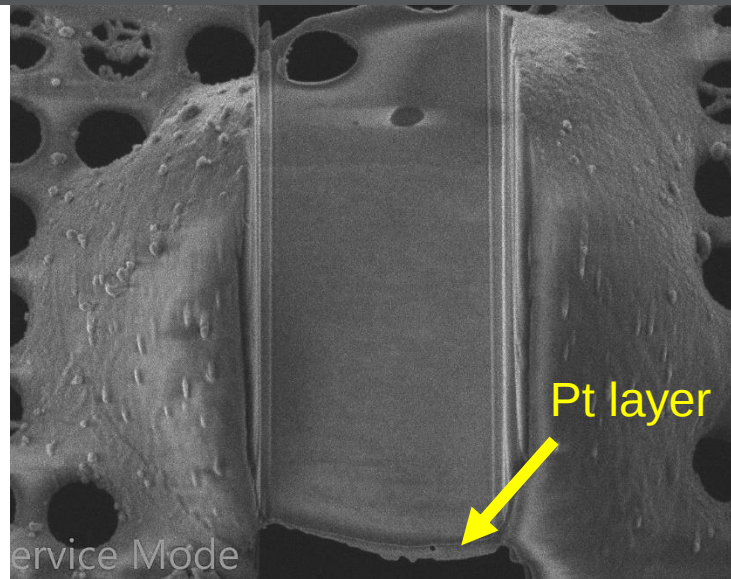
Target confirmation

Pt sputter (Optional)

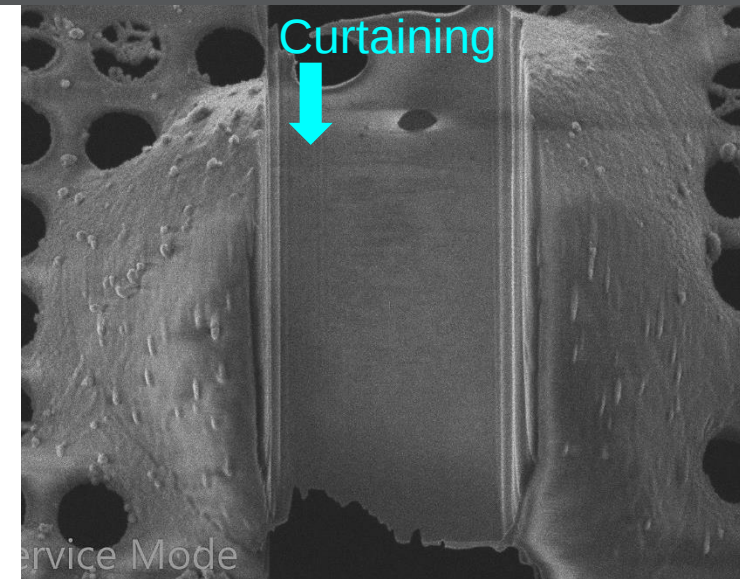
Lamella conductivity

↓ T

CryoET

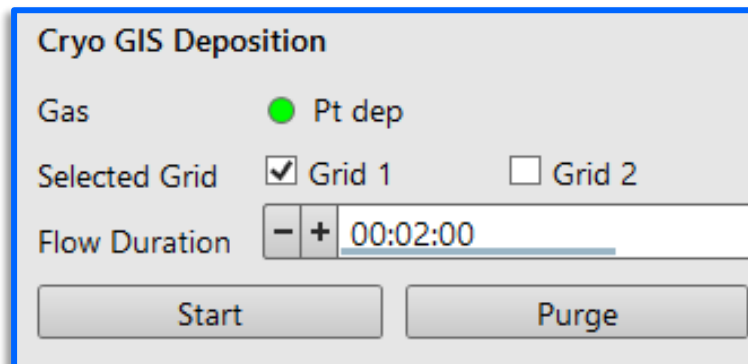


With an intact Pt layer

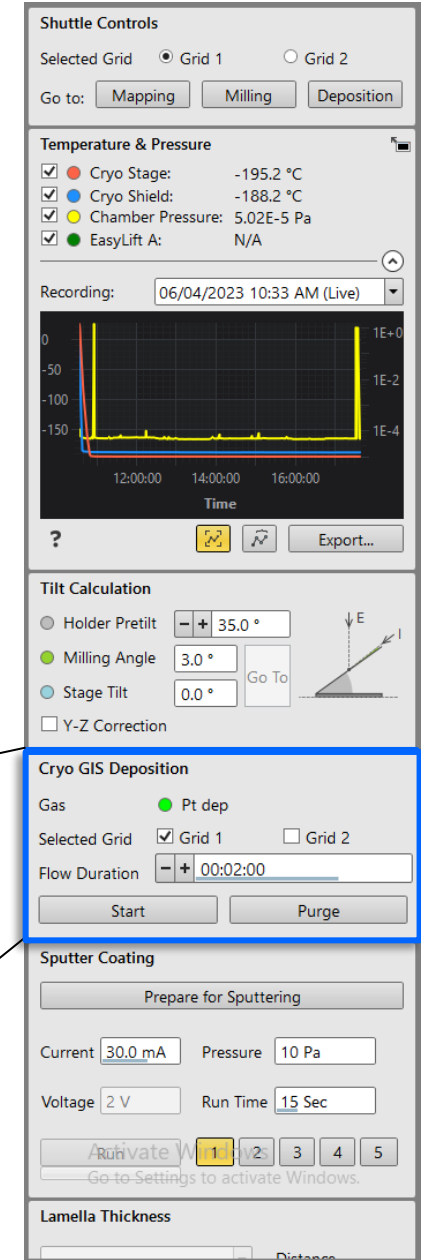


With a broken Pt layer

Organometallic Pt protects sample surface during milling,
minimize curtaining and redeposition.



Duration varies by samples / instruments



3.6 Pt sputter (Optional)

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)
Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET

(Optional) Inorganic Pt minimizes charging, ensure targeting and precise milling.

Shuttle Controls

Selected Grid: ☒ Grid 1 ☐ Grid 2

Go to: Mapping Milling Deposition

Temperature & Pressure

- ☒ Cryo Stage: -195.2 °C
- ☒ Cryo Shield: -188.2 °C
- ☒ Chamber Pressure: 5.02E-5 Pa
- ☒ EasyLift A: N/A

Recording: 06/04/2023 10:33 AM (Live)

Tilt Calculation

- ☐ Holder Pretilt: 35.0 °
- ☒ Milling Angle: 3.0 °
- ☐ Stage Tilt: 0.0 °
- ☐ Y-Z Correction

Cryo GIS Deposition

Gas: ☒ Pt dep

Selected Grid: ☒ Grid 1 ☒ Grid 2

Flow Duration: 00:02:00

Start Purge

Sputter Coating

Prepare for Sputtering

Current: 30.0 mA Pressure: 10 Pa

Voltage: 2 V Run Time: 15 Sec

Lamella Thickness

3.6 Pt sputter (Optional)

Vitrification

↓ **T**
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)
Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

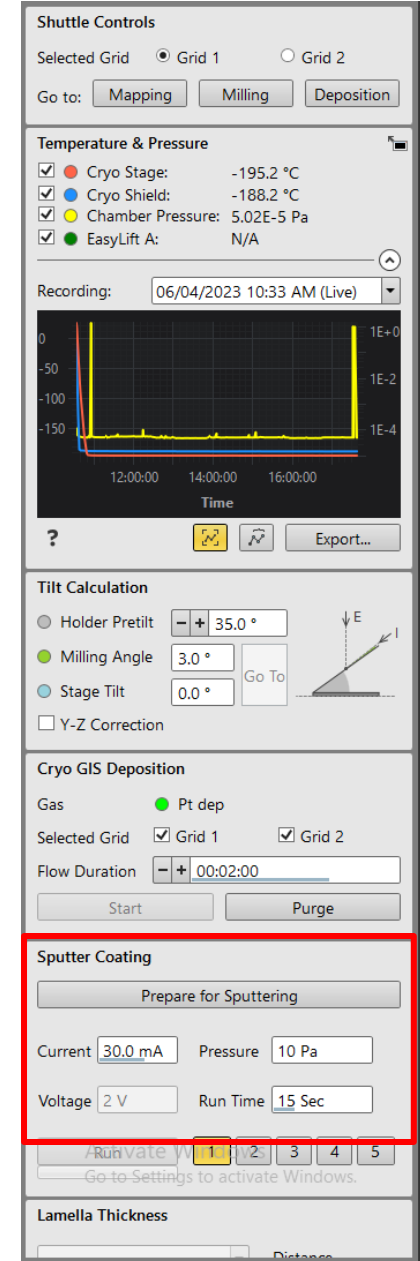
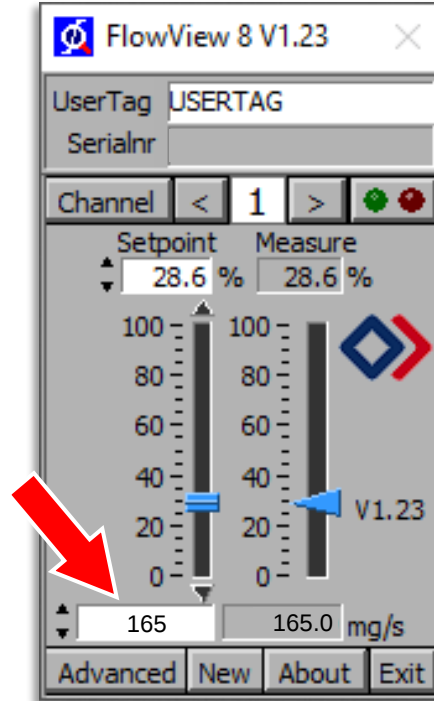
Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ **T**
CryoET

Upon completion,
reduce to 165 mg/s.
(varies by instruments)



3.7 Automated lamella milling using **AutoTEM Cryo** AT

Vitrification

↓ T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

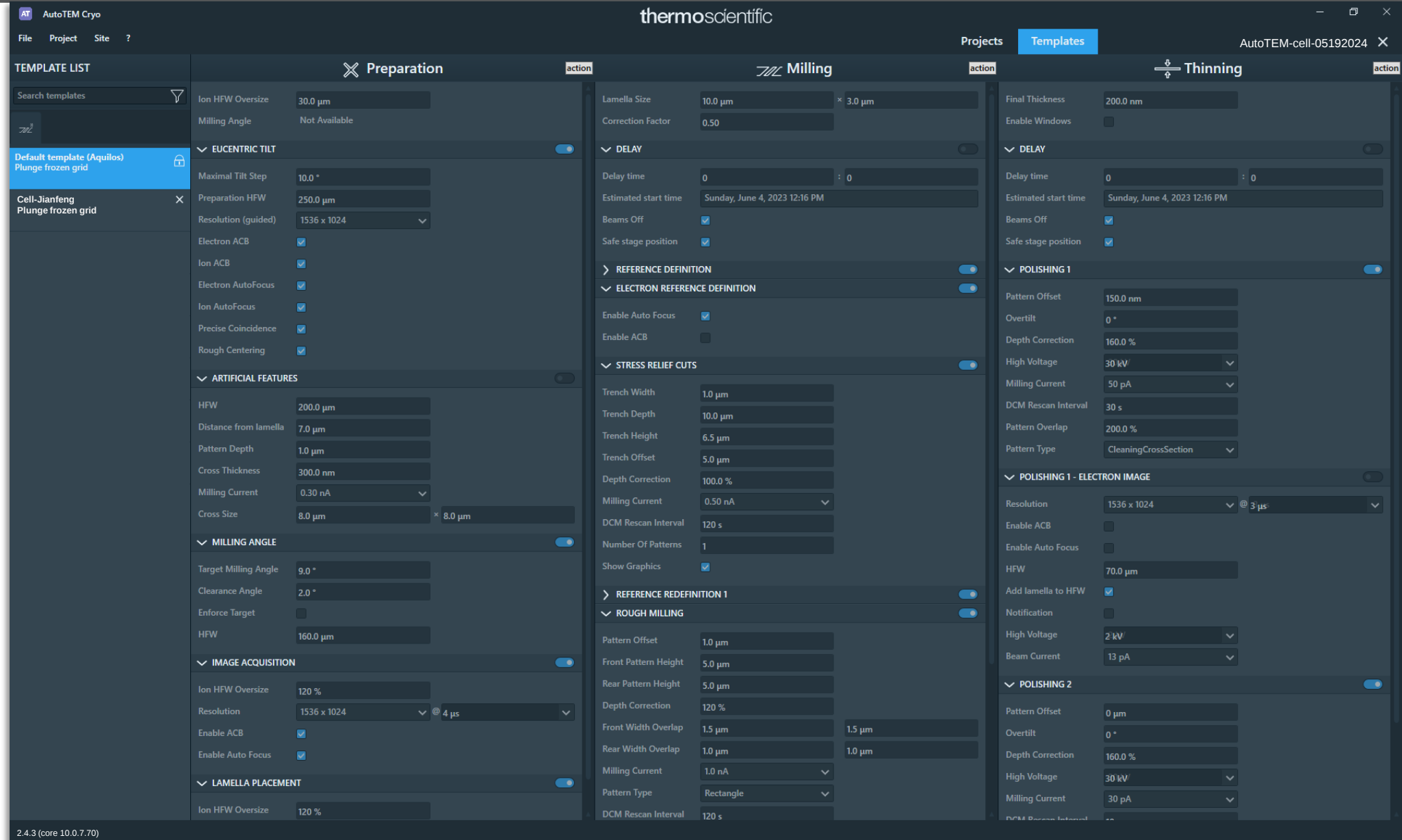
Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T

CryoET



Eucentric height & tilt calculation

Vitrification

↓ T
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

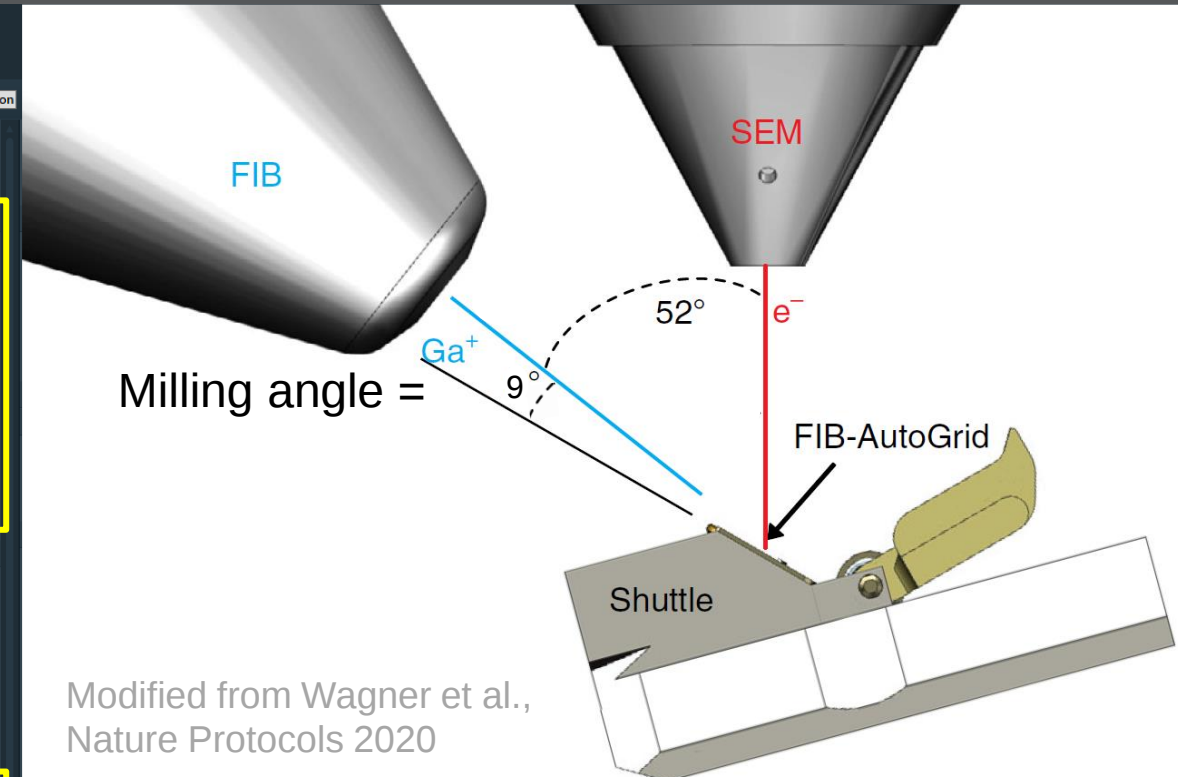
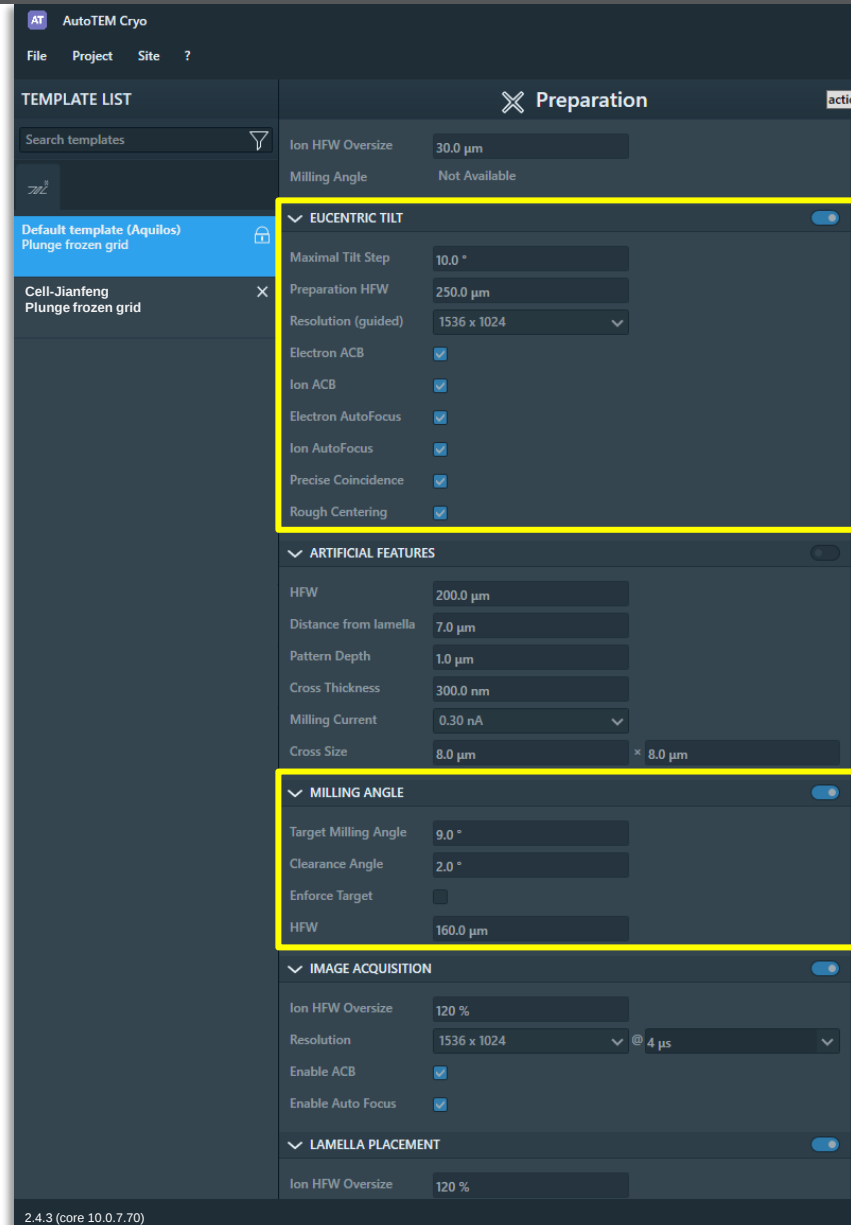
iFLM (Optional)

Target confirmation

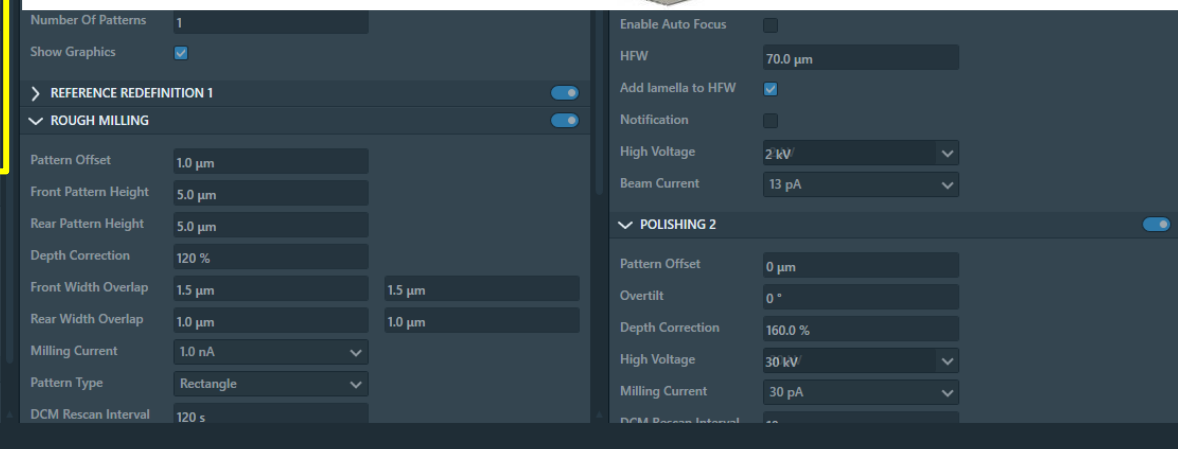
Pt sputter (Optional)

Lamella conductivity

↓ T
CryoET



Modified from Wagner et al.,
Nature Protocols 2020



Artificial Features (Optional)

(**AT** AutoTEM Cryo)

Vitrification

↓ **T**
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

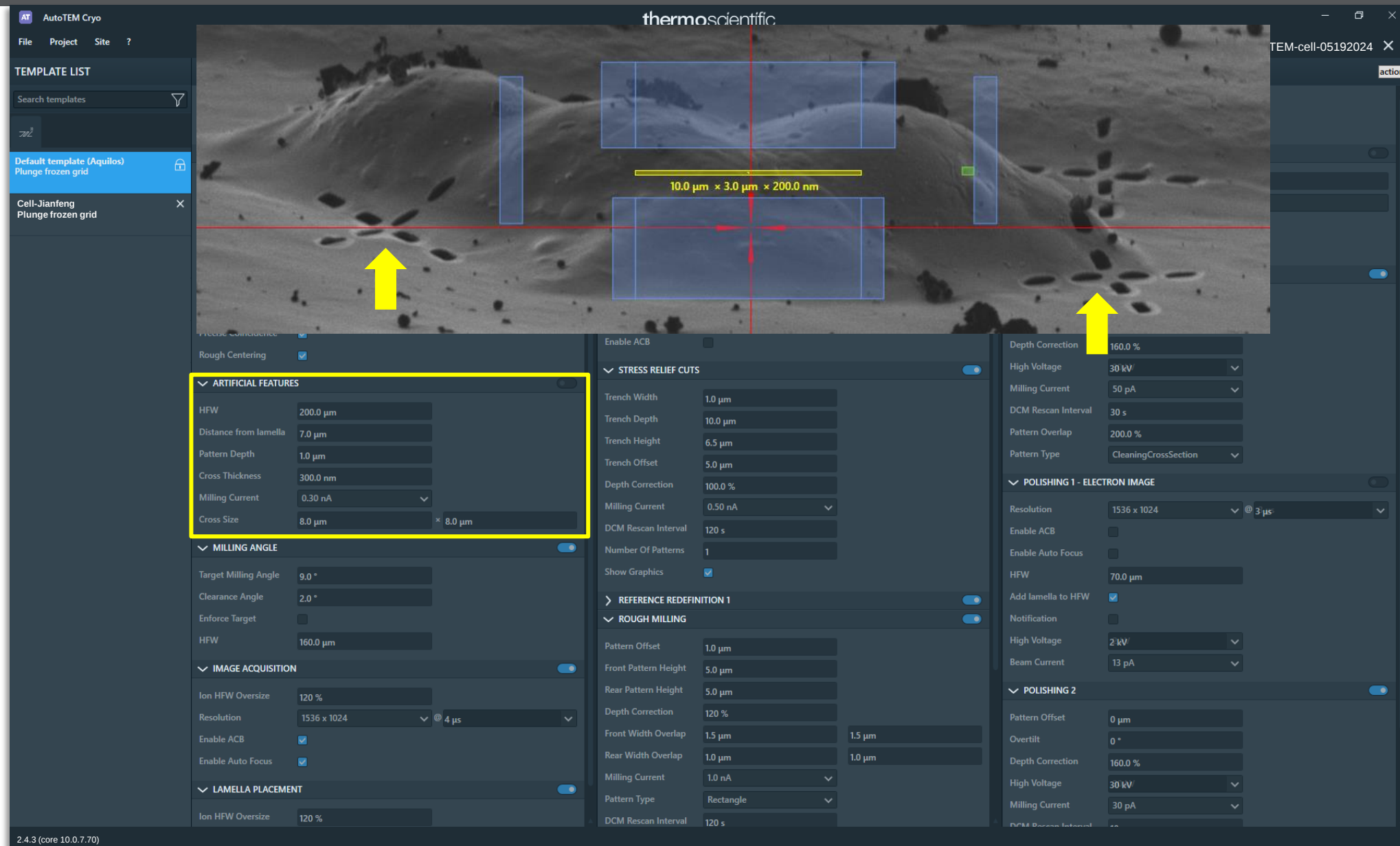
iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ **T**
CryoET



Micro-expansion joints

(**AT** AutoTEM Cryo)

Vitrification



T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity



T

CryoET

The screenshot displays the AutoTEM Cryo software interface, which is used for controlling the electron microscope and the milling process. The interface is divided into several panels:

- TEMPLATE LIST:** Shows a list of templates, including "Default template (Aquilos) Plunge frozen grid" and "Cell-Jianfeng Plunge frozen grid".
- PARAMETERS:** A large section on the right containing various settings for the milling process, such as "Ion HFW Oversize", "Milling Angle", "EUCENTRIC TILT", "ARTIFICIAL FEATURES", "MILLING ANGLE", "IMAGE ACQUISITION", and "LAMELLA PLACEMENT".
- STRESS RELIEF CUTS:** A panel highlighted with a yellow box, showing settings for "Trench Width", "Trench Depth", "Trench Height", "Trench Offset", "Depth Correction", "Milling Current", "DCM Rescan Interval", and "Number Of Patterns".
- REFERENCE REDEFINITION 1:** A panel showing settings for "Pattern Offset", "Front Pattern Height", "Rear Pattern Height", "Depth Correction", "Front Width Overlap", "Rear Width Overlap", "Milling Current", "Pattern Type", and "DCM Rescan Interval".
- ROUGH MILLING:** A panel showing settings for "Pattern Offset", "Front Pattern Height", "Rear Pattern Height", "Depth Correction", "Front Width Overlap", "Rear Width Overlap", "Milling Current", "Pattern Type", and "DCM Rescan Interval".
- POLISHING 1 - ELECTRON IMAGE:** A panel showing settings for "Resolution", "Enable ACB", "Enable Auto Focus", "HFW", "Add lamella to HFW", "Notification", "High Voltage", and "Beam Current".
- POLISHING 2:** A panel showing settings for "Pattern Offset", "Overtilt", "Depth Correction", "High Voltage", "Milling Current", and "DCM Rescan Interval".

Three micrographs (A, B, C) are shown at the top, illustrating the process of creating micro-expansion joints. Micrograph A shows a cross-section of a sample. Micrograph B shows a cross-section of a sample with two vertical lines indicating the location of the joints. Micrograph C shows a cross-section of a sample with two vertical lines indicating the location of the joints. The caption below the micrographs reads "Wolff et al., JSB 2019".

Stepwise milling procedure

Vitrification

↓ **T**
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

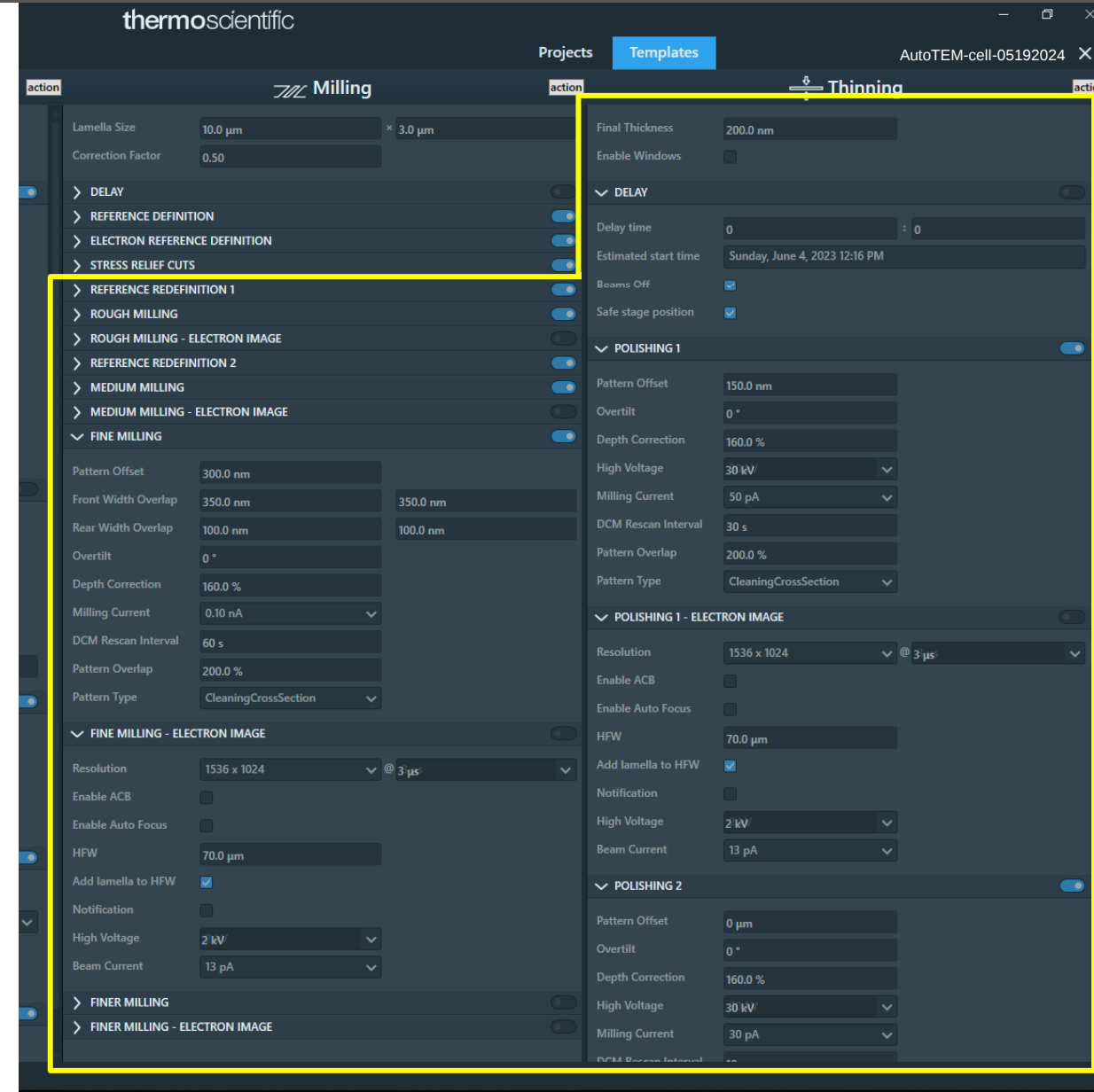
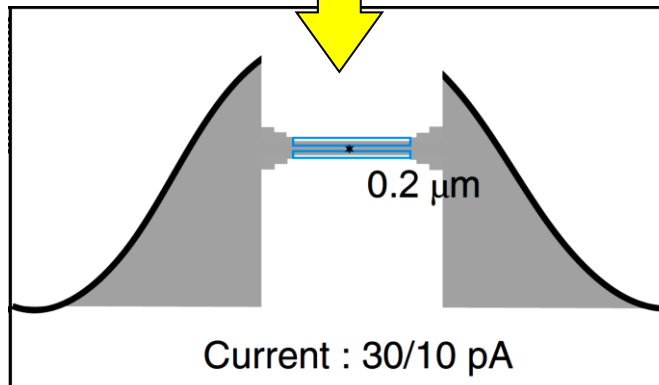
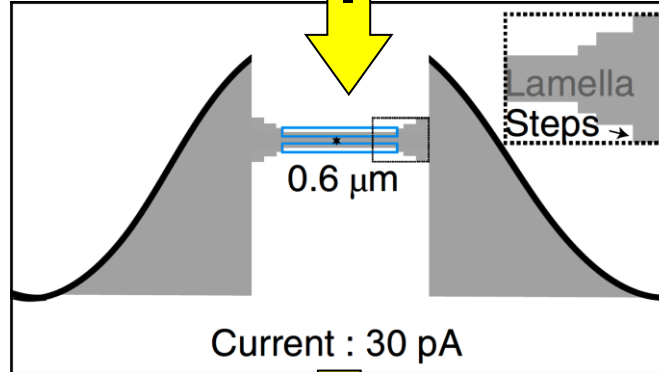
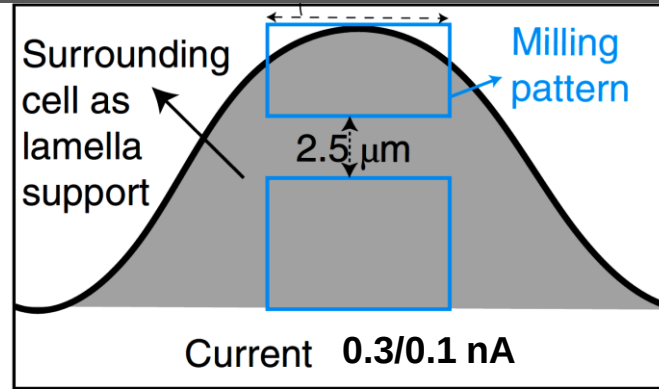
iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ **T**
CryoET



3.7.1 Automated lamella milling

Preparation

(AT AutoTEM Cryo)

AutoTEM Cryo

thermoscientific

Projects Templates

AutoTEM-cell-05192024

SITE LIST

Lamella (3)
8.0 μm × 3.0 μm
0% 5 min 8 s

Lamella (2)
8.0 μm × 3.0 μm
0% 5 min 8 s

Lamella
8.0 μm × 3.0 μm

CRYO

ETD Digital Zoom: 58 %

Automation

PROJECT

10 min 16 s

PREPARATION

MILLING

THINNING

Automatic (with manual fall-back)

Automatic

Automatic

Lamella (3)
5 min 8 s

Lamella (2)
5 min 8 s

Lamella
--

--

--

--

Execution Mode Step-wise

2 site(s) in 10 min 16 s

0 site(s) in 0 s

PREPARE

MILL

CANCEL

Automatic (with manual fall-backs)

Automatic

TEMPLATE

Site Name Lamella (2)

Size 8.0 μm × 3.0 μm

Final Thickness 150.0 nm

Default template (Aquilos)

Cell-Jianfeng

Lamella (2)
8.0 μm × 3.0 μm
Jianfeng-05192024

PREPARATION

MILLING

THINNING

Finished

Site N/A

Project 0 % 10 min 16 s

RUN

STOP

2.4.3 (core 10.0.7.70)

3.7.1 Automated lamella milling _Preparation

(**AT** AutoTEM Cryo)

thermoscientific

AutoTEM Cryo

File Project Site ?

SITE LIST

- Lamella (3)
8.0 μm $\times$ 3.0 μm
98 % 3 s
- Lamella (2)**
8.0 μm $\times$ 3.0 μm
98 % 3 s
- Lamella
8.0 μm $\times$ 3.0 μm

ETD Digital Zoom: 81 %

UPDATE

PREPARATION

Ion HFW Oversize: 80.0 μm

Milling Angle: 12.0 °

CORRELATION

Stack Offset: -2.00 μm

Fiducial position [X,Y]: -2.820553 mm, 3.513866 mm

ROI position [X,Y]: -2.860039 mm, 3.536954 mm

Show Graphics: ☒

Lamella Reposition

EUCENTRIC TILT: ☒

ARTIFICIAL FEATURES: ☐

MILLING ANGLE: ☒

IMAGE ACQUISITION: ☒

LAMELLA PLACEMENT: 2 s ☒

8.0 μm $\times$ 3.0 μm $\times$ 150.0 nm

A

Target (ROI)

Z-Height between Focus Planes

X-Fiducial

Distance to projected target position

Adapted from <https://cryoem101.org/chapter-2-et/>

Waiting to continue...

Site: 98 % 3 s

Project: 98 % 3 s

CONTINUE STOP

Lamella (2) Preparation execution

2.4.3 (core 10.0.7.70)

3.7.2 Automated lamella milling_Milling

(AutoTEM Cryo)

AutoTEM Cryo

FileProjectSite?

SITE LIST

Lamella (3)

8.0 μm × 3.0 μm

13 % 28 min 16 s

Lamella (2)

8.0 μm × 3.0 μm

12 % 30 min 17 s

Lamella

8.0 μm × 3.0 μm

thermoscientific

ProjectsTemplates

AutoTEM-cell-05192024

MILLING

Lamella Size

8.0 μm × 3.0 μm

Correction Factor

0.50

DELAY

REFERENCE DEFINITION

44 s

ELECTRON REFERENCE DEFINITION

1 min 46 s

STRESS RELIEF CUTS

2 min 23 s

REFERENCE REDEFINITION 1

44 s

ROUGH MILLING

14 min 7 s

ROUGH MILLING - ELECTRON IMAGE

37 s

REFERENCE REDEFINITION 2

44 s

MEDIUM MILLING

3 min 46 s

MEDIUM MILLING - ELECTRON IMAGE

27 s

FINE MILLING

4 min 32 s

FINE MILLING - ELECTRON IMAGE

27 s

FINER MILLING

FINER MILLING - ELECTRON IMAGE

Automation

PROJECT

58 min 33 s

PREPARATION

MILLING

THINNING

Automatic (with manual fall-back)

Automatic

Automatic

Lamella (3)

--

28 min 16 s

--

Lamella (2)

--

30 min 17 s

--

Lamella

--

--

--

Execution Mode

Step-wise

0 site(s) in 0 s

2 site (s) in 58 min 33 s

PREPARE

MILL

CANCEL

Automatic (with manual fall-backs)

Automatic

Lamella (2)

8.0 μm × 3.0 μm

Jianfeng-05192024

PREPARATION

MILLING

THINNING

Finished

Site

Project

N/A

12 %

58 min 33 s

RUN

STOP

2.4.3 (core 10.0.7.70)

3.7.3 Automated lamella milling **_Thinning**

(**AT** AutoTEM Cryo)

AutoTEM Cryo

FileProjectSite?

SITE LIST

Lamella (3)

8.0 μm × 3.0 μm

45 % 7 min 56 s

Lamella (2)

8.0 μm × 3.0 μm

42 % 8 min 18 s

Lamella

8.0 μm × 3.0 μm

thermoscientific

ProjectsTemplates

AutoTEM-cell-05192024

THINNING

Final Thickness150.0 nm

Enable Windows

DELAY

POLISHING 13 min 20 s

POLISHING 1 - ELECTRON IMAGE

POLISHING 24 min 58 s

POLISHING 2 - ION IMAGE

POLISHING 2 - ELECTRON IMAGE

Automation

PROJECT16 min 14 s

PREPARATION

MILLING

THINNING

Automatic (with manual fall-bi)

Automatic

Automatic

Lamella (3)

--

--

7 min 56 s

Lamella (2)

--

--

8 min 18 s

Lamella

--

--

--

Execution ModeStep-wise

0 site(s) in 0 s

2 site (s) in 16 min 14 s

PREPARE

MILL

CANCEL

Lamella (2)

8.0 μm × 3.0 μm

Jianfeng-05192024

PREPARATION

MILLING

THINNING

Finished

Site

Project

N/A

43 % 16 min 14 s

RUN

STOP

2.4.3 (core 10.0.7.70)

3.7.3 Automated lamella milling Thinning _example-lamella 1

(**AT** AutoTEM Cryo)

thermoscientific

AutoTEM Cryo

File Project Site ?

SITE LIST

- Lamella (3)
8.0 μm $\times$ 3.0 μm
- Lamella (2)**
8.0 μm $\times$ 3.0 μm
- Lamella
8.0 μm $\times$ 3.0 μm

ETD Digital Zoom: 162 %

THINNING

Final Thickness: 150.0 nm

Enable Windows: ☐

DELAY

POLISHING 1

UPDATE

Lamella (2)
8.0 μm $\times$ 3.0 μm
Jianfeng-05192024

PREPARATION

MILLING

THINNING

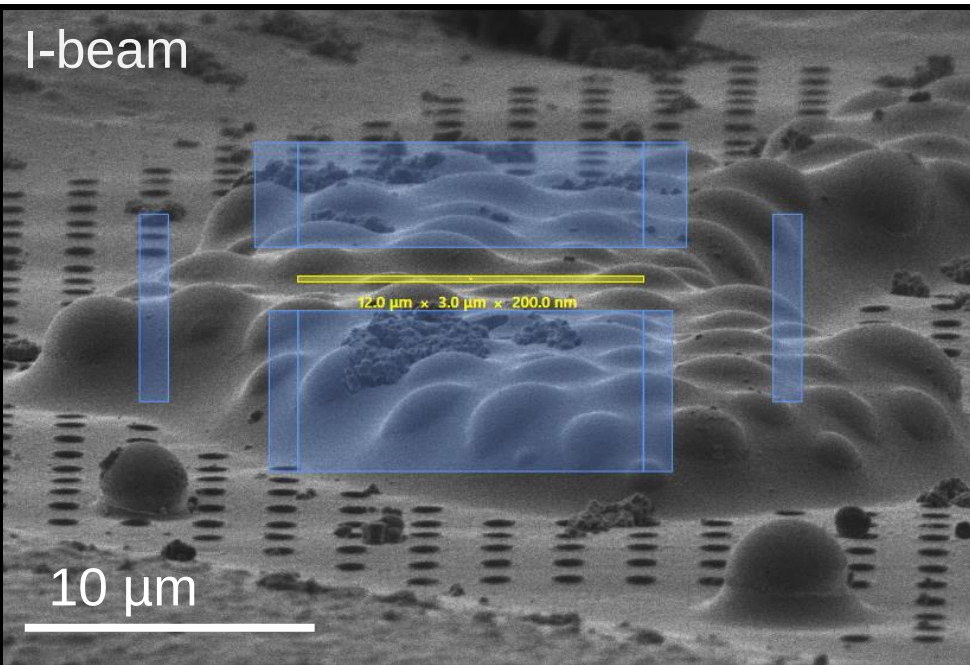
Finished
Site
Project

N/A
0 %

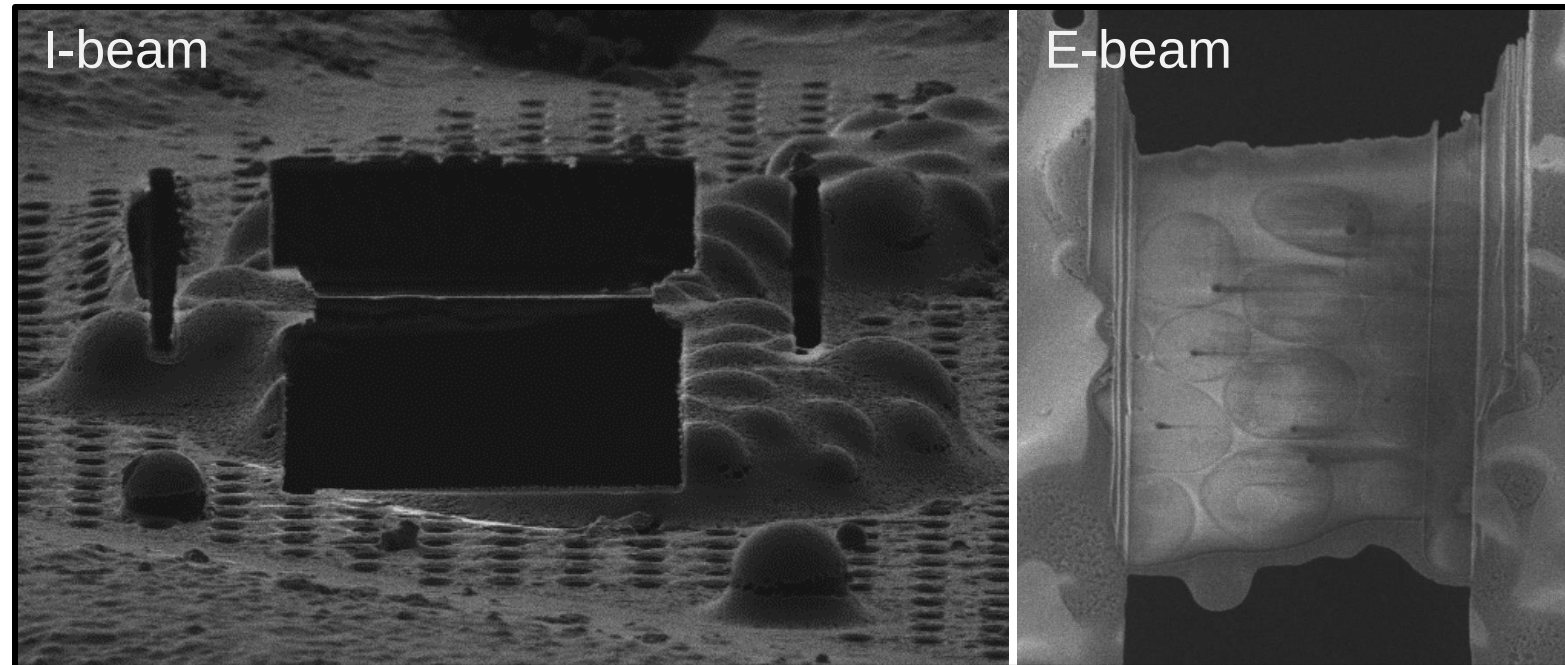
RUN STOP

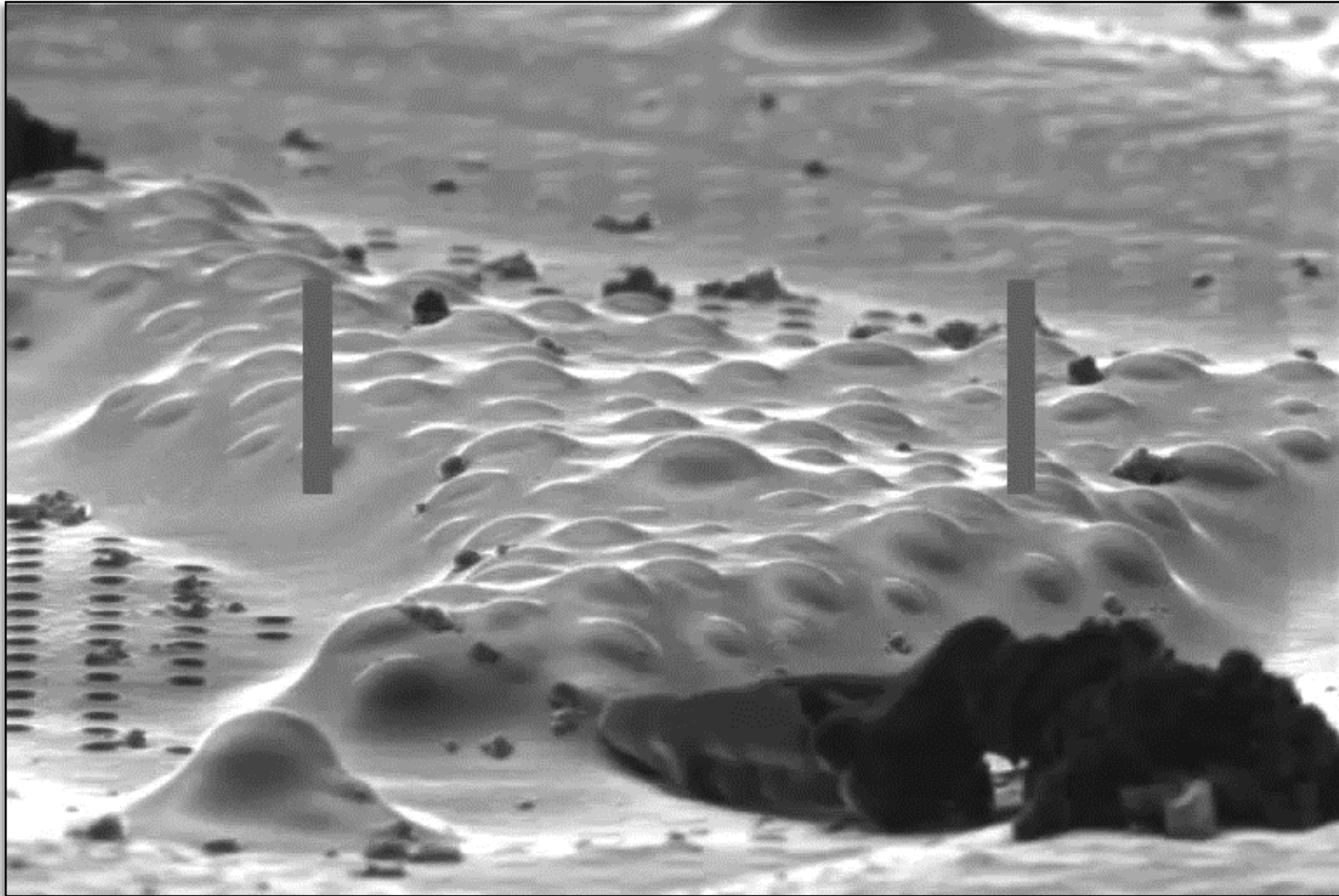
2.4.3 (core 10.0.7.70)

Before milling



After milling





3.8 CryoFLM target confirmation example-lamella 4

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

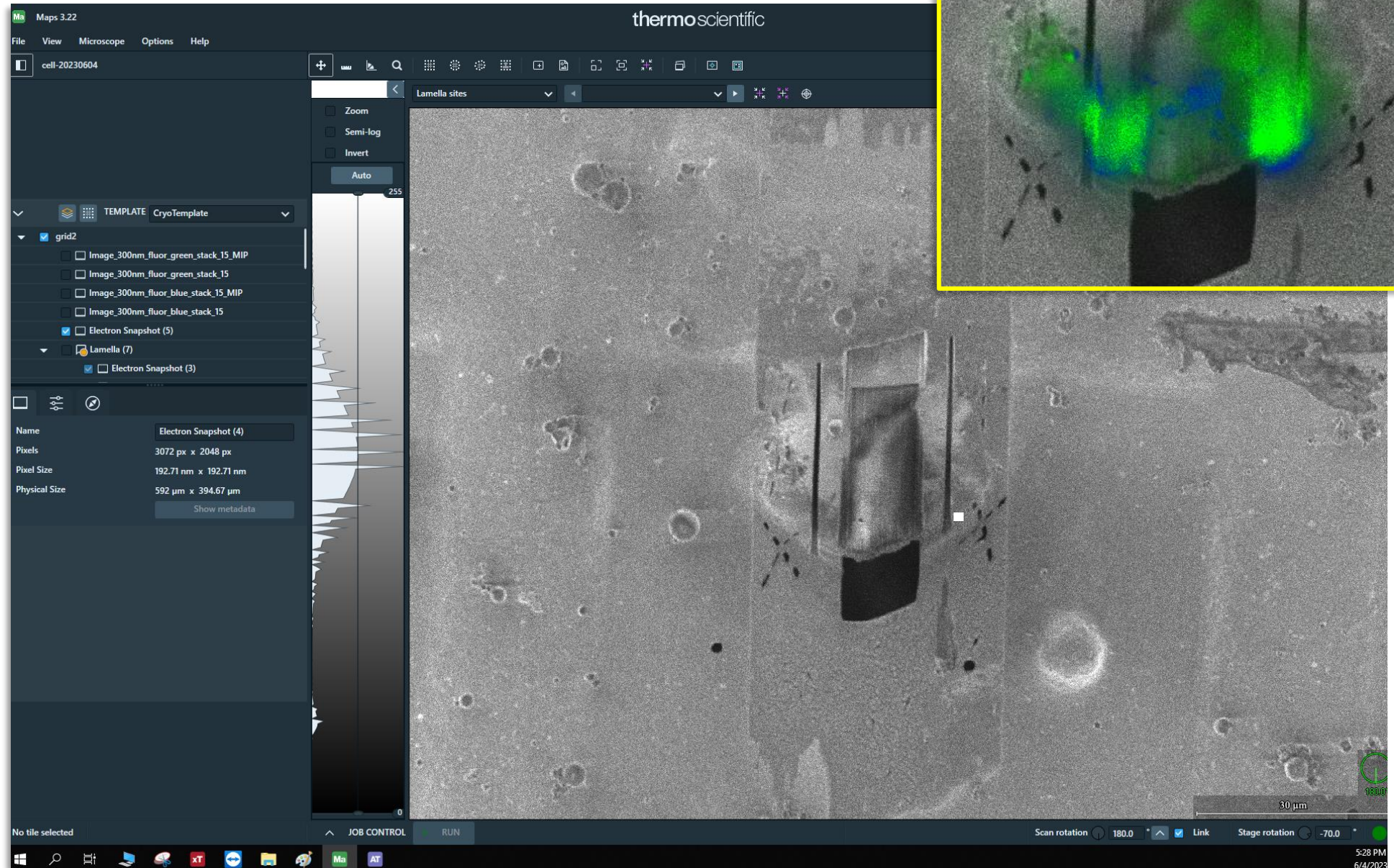
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.9 Pt sputter (optional)

Vitrification

↓ T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T

CryoET

Sputter Coating ?

Prepare for Sputtering

Current mA Pressure Pa

Voltage V Run Time Sec

Run

(Optional) minimize charging; ensure low beam-induced movement and using of VPP.

xT

Shuttle Controls ?

Selected Grid ☒ Grid 1 ☐ Grid 2

Go to: Mapping Milling Deposition

Temperature & Pressure

☒ Cryo Stage: -195.3 °C

☒ Cryo Shield: -187.6 °C

☒ Chamber Pressure: 4.75E-5 Pa

Recording: 06/04/2022 11:33 AM (Live)

0 -50 -100 -150 1E+0 1E-2 1E-4

12:00:00 13:00:00 14:00:00 15:00:00

Time

? Export...

Tilt Calculation

☐ Holder Pre-tilt °

☒ Milling Angle °

☐ Stage Tilt °

Go To

☐ Y-Z Correction

Cryo GIS Deposition

Gas ☒ Pt dep

Selected Grid ☒ Grid 1 ☐ Grid 2

Flow Duration

Start Purge

Sputter Coating ?

Prepare for Sputtering

Current mA Pressure Pa

Voltage V Run Time Sec

Run

Lamella Thickness

Distance

3.10 Unloading grids

(Video from TFS)

Vitrification



T

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



Some common cryoFIB workflow variants

CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)

Target selection

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

e.g., for example-
lamella 4

CryoFIB

Sample screening

Atlas & lamella sites

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

iFLM (Optional)

Target selection

Lamella milling

Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

e.g., for example-
lamella 1

CryoFIB

Sample screening

Atlas & lamella sites

Pt sputter

Sample conductivity

Pt GIS

Protective coating

Pt sputter (Optional)

Sample conductivity

Lamella milling

Preparation, Milling,
& thinning

Pt sputter (Optional)

Lamella conductivity

If CLEM is unnecessary

CryoFIB

Sample screening

Atlas & lamella sites

Pt sputter

Sample conductivity

Pt GIS

Protective coating

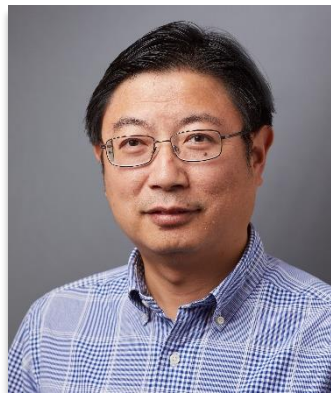
Lamella milling

Preparation, Milling,
& thinning

If CLEM is unnecessary
& no charging problems

e.g., for example-
lamellae 2&3

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