

CryoEM Course at LBMS, BNL (June 11-14th, 2024)

CryoET sample preparation tutorial & demonstration

Jianfeng Lin

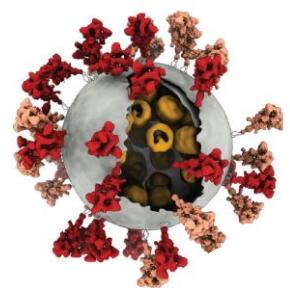
Yale CryoEM Resource

6-13-2024

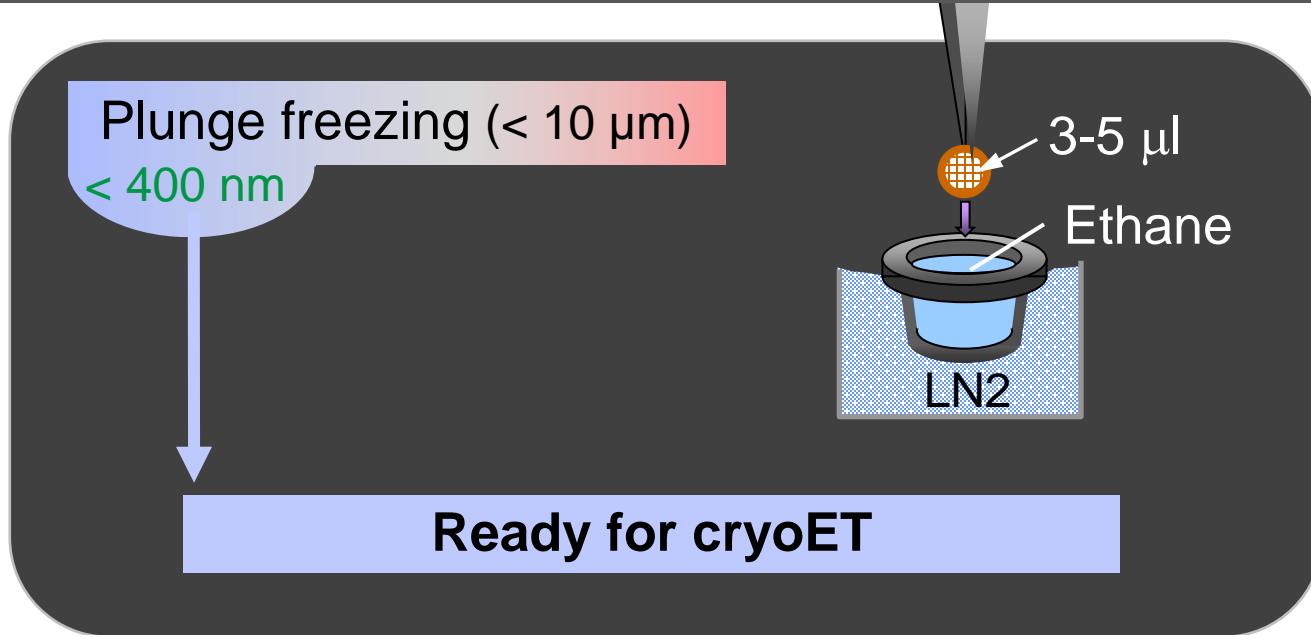
Main contents

1. Specimens accessible by cryoET
2. Five considerations for cryoET sample preparation
3. Tutorial of major steps of cryo lamella preparation

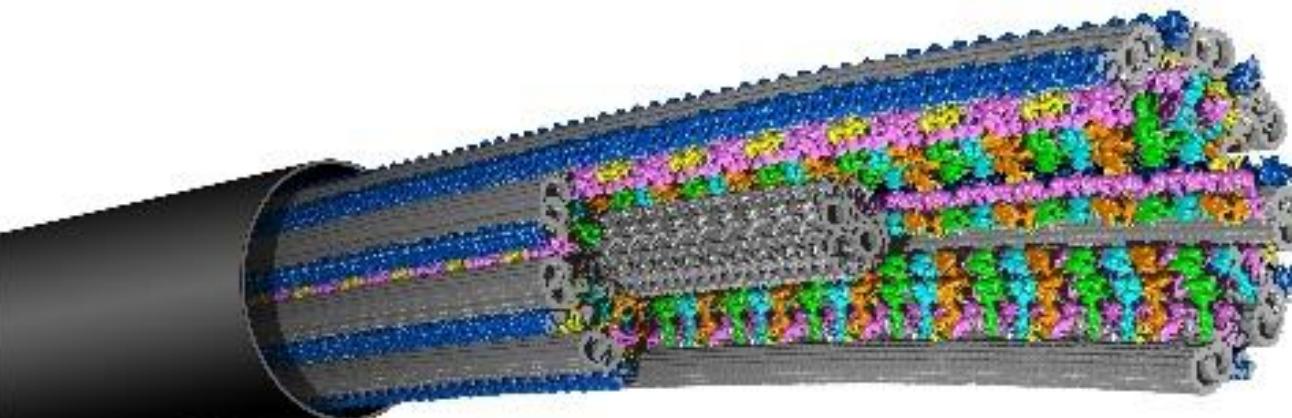
1. Specimens accessible by cryoET



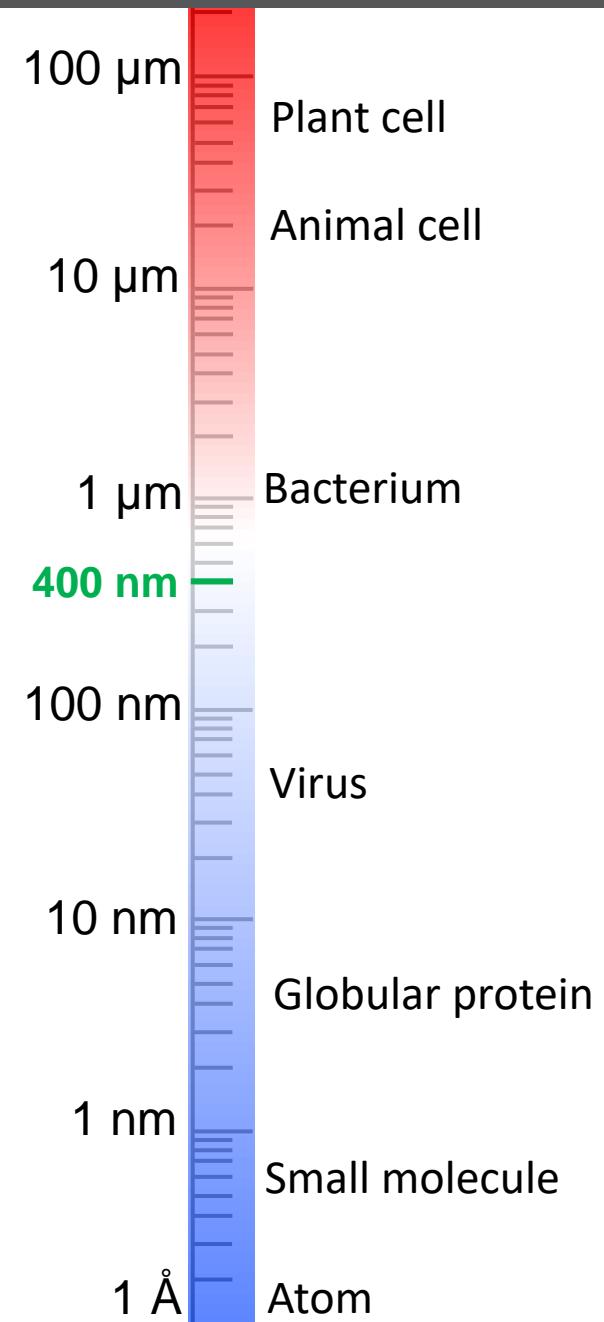
Yao et al., Cell 2020



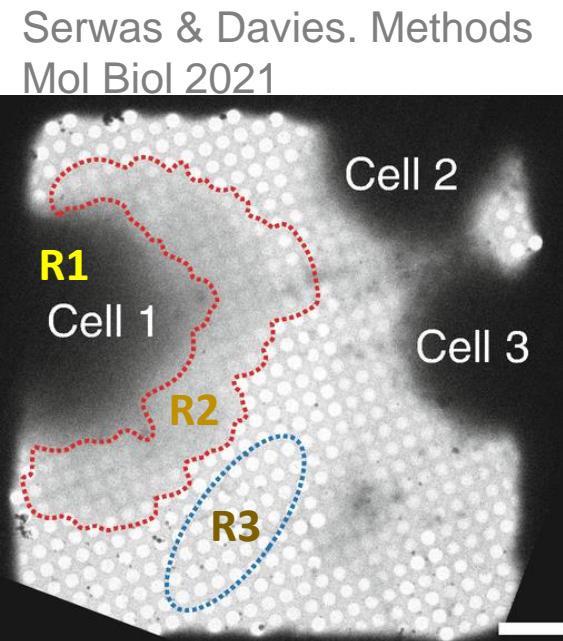
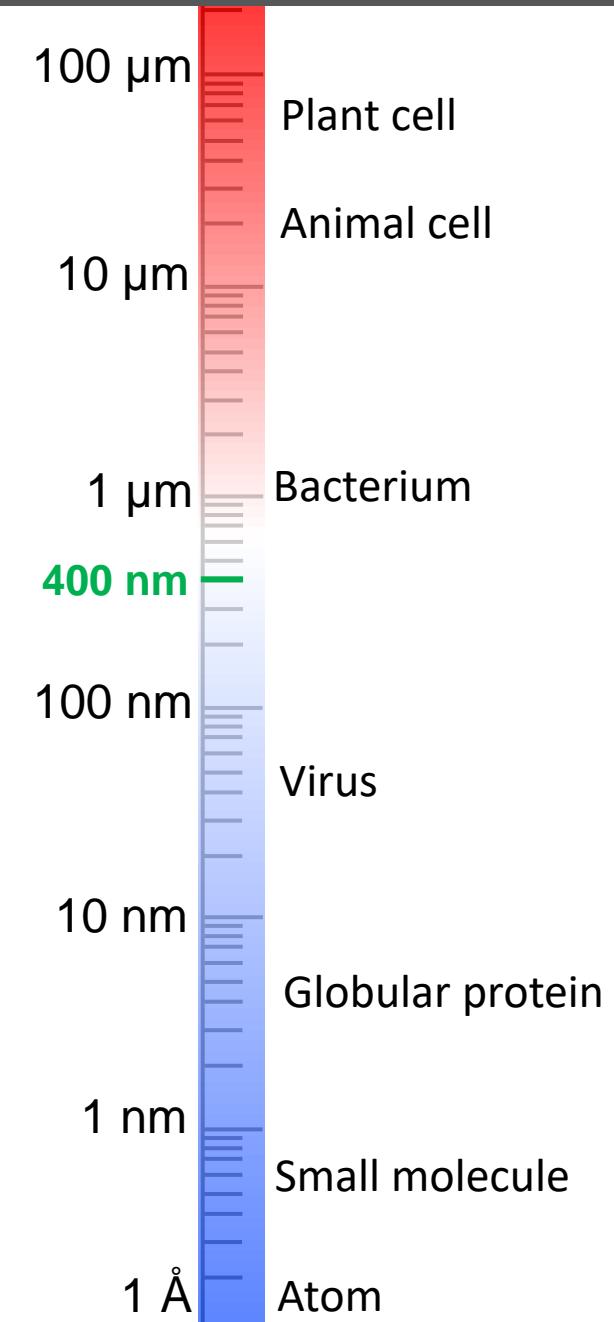
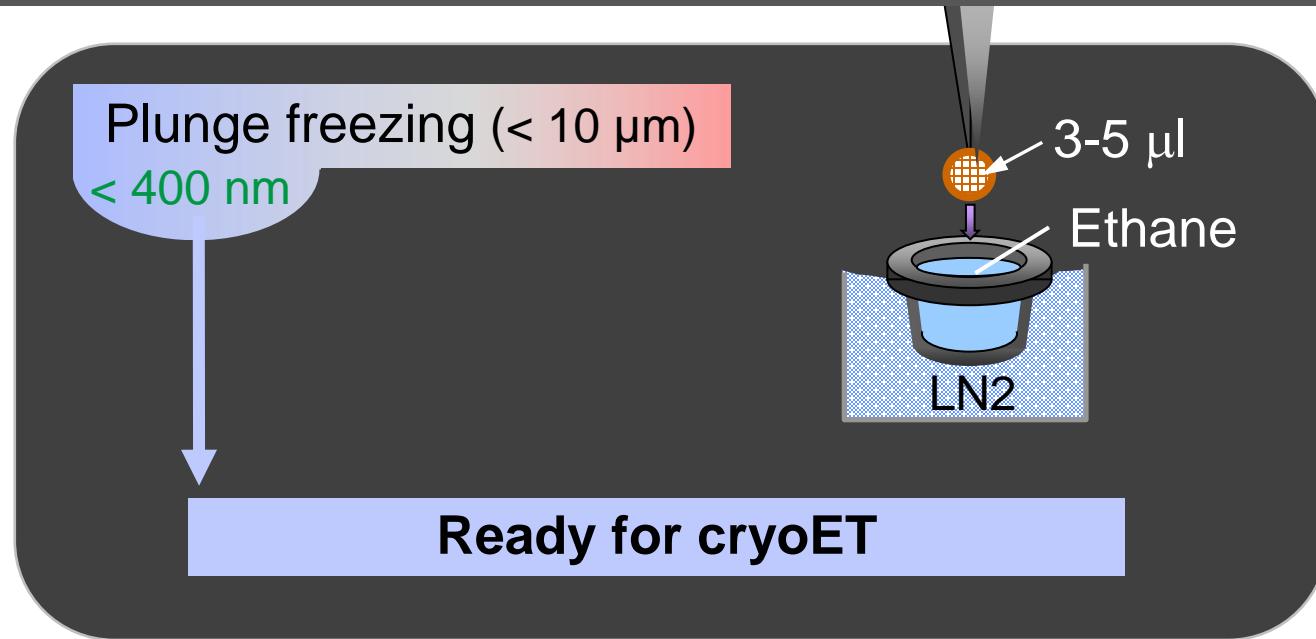
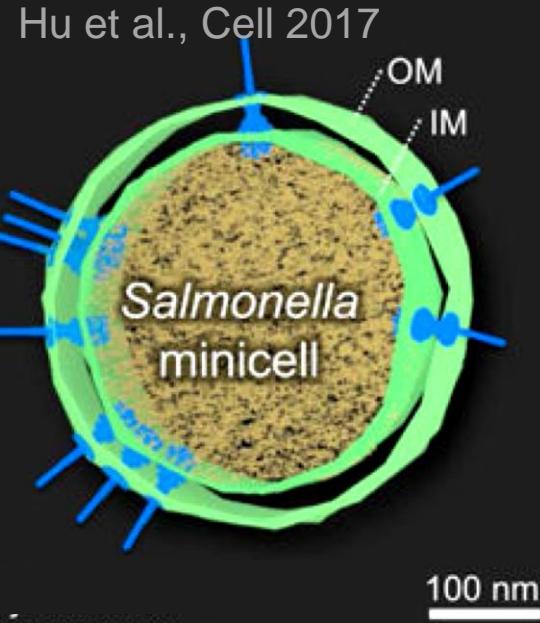
- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme



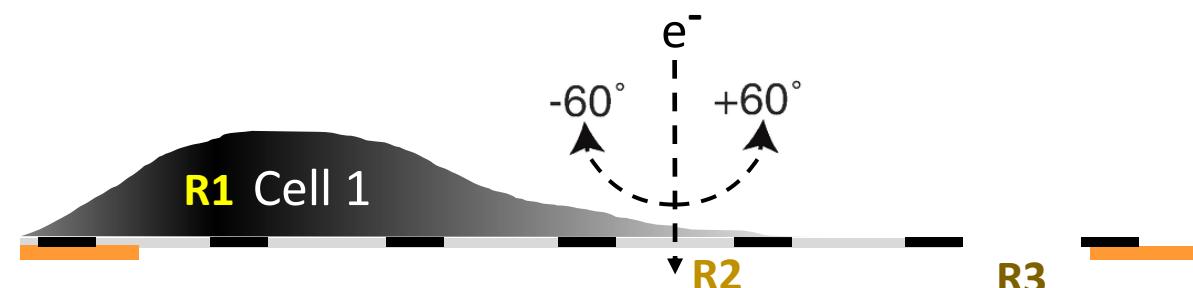
Lin & Nicastro, Science 2018



1. Specimens accessible by cryoET



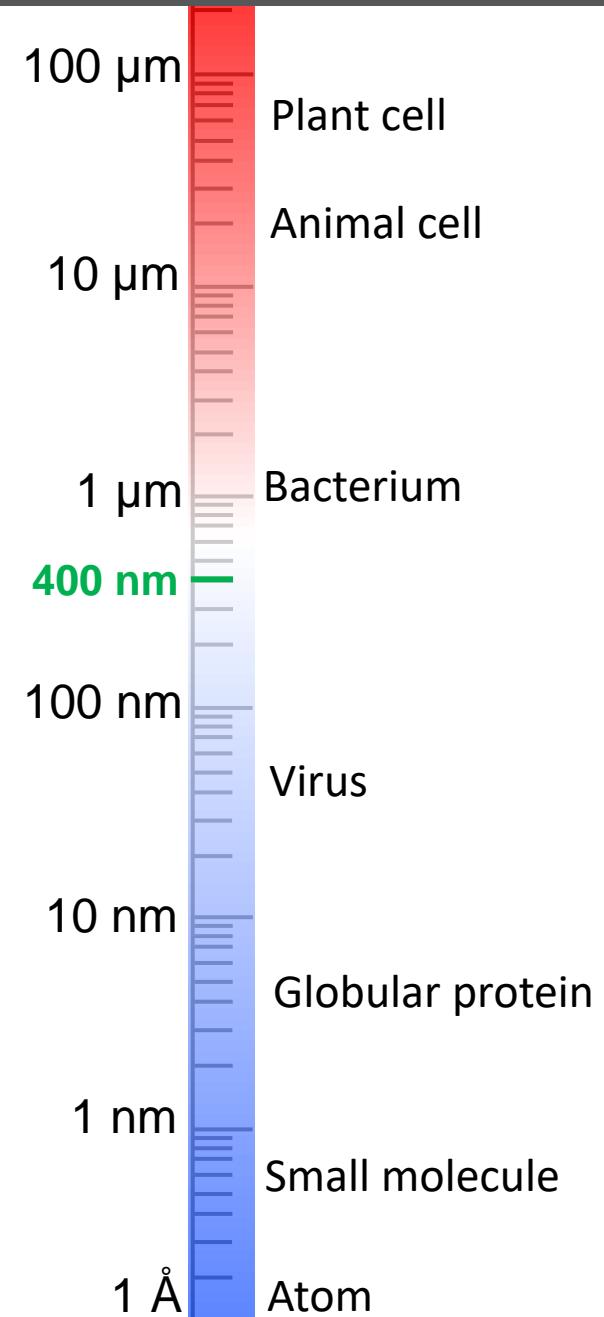
- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells



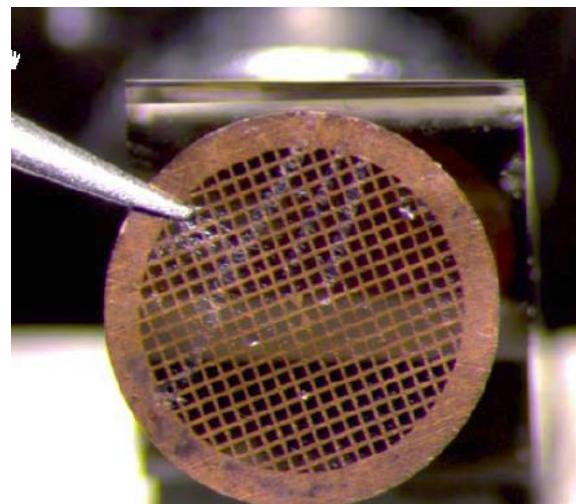
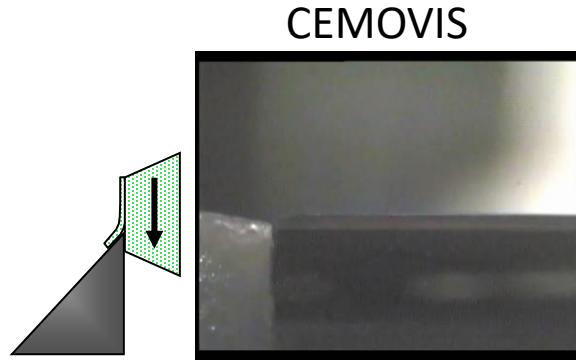
1. Specimens accessible by cryoET



- 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

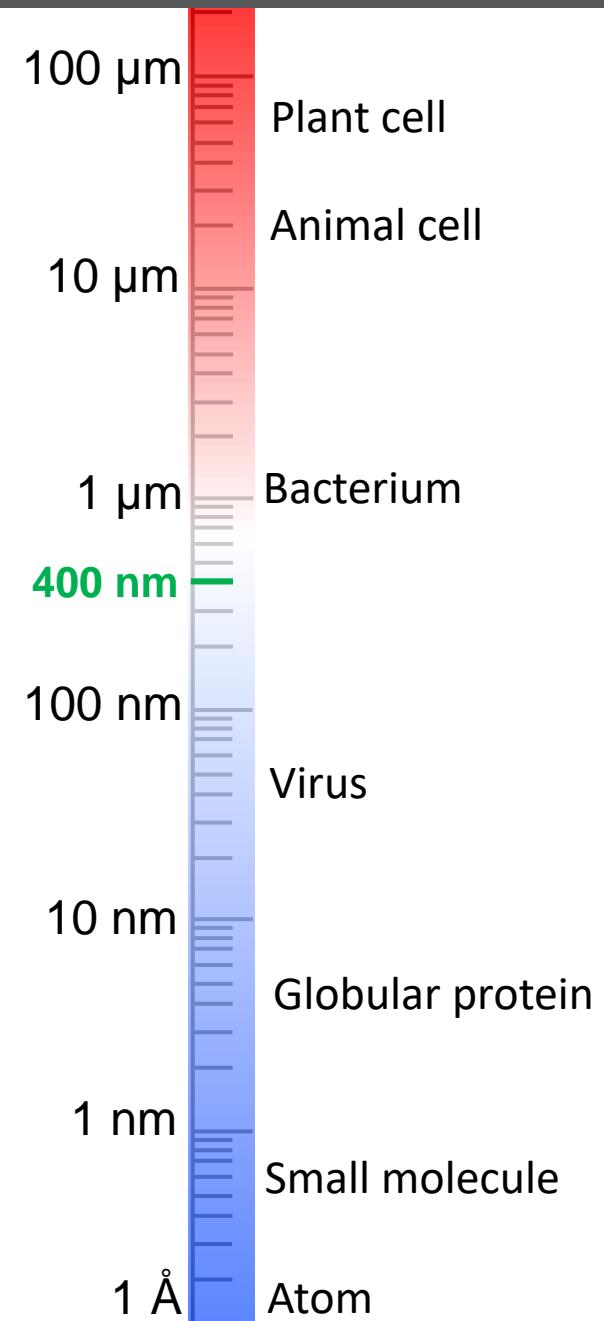


1. Specimens accessible by cryoET

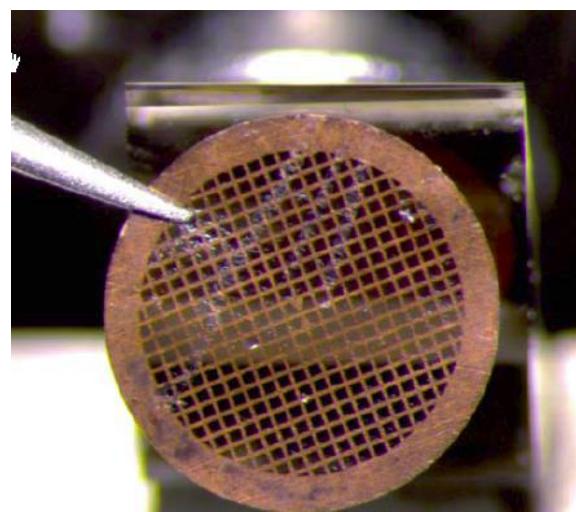
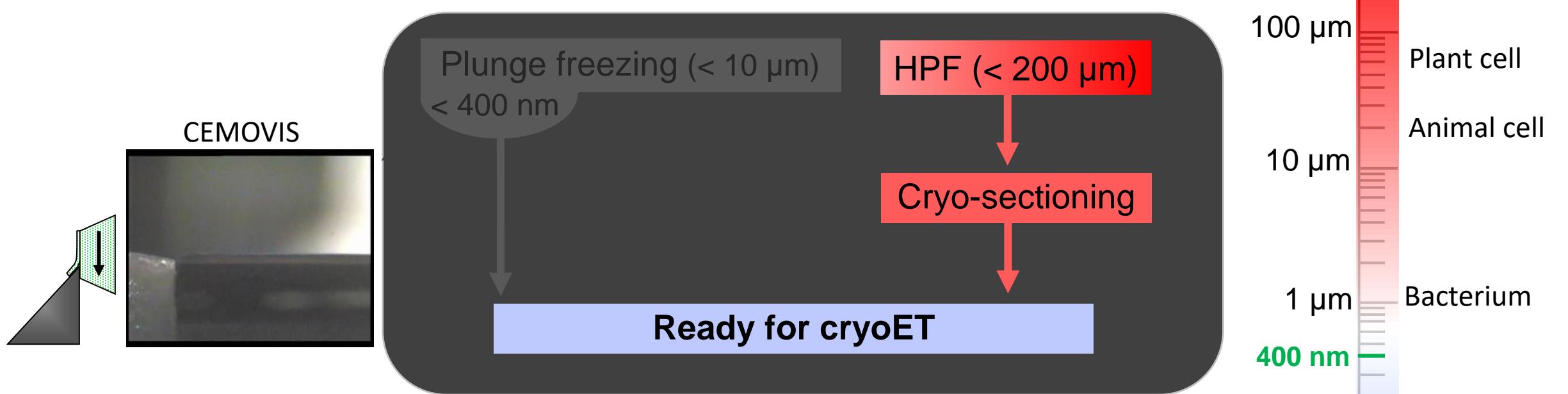


- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells
- **Cryo-sections**

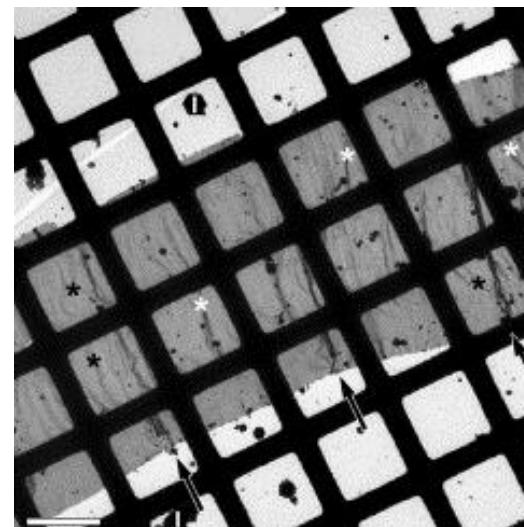
Adapted from Thermo Fisher Scientific (TFS)



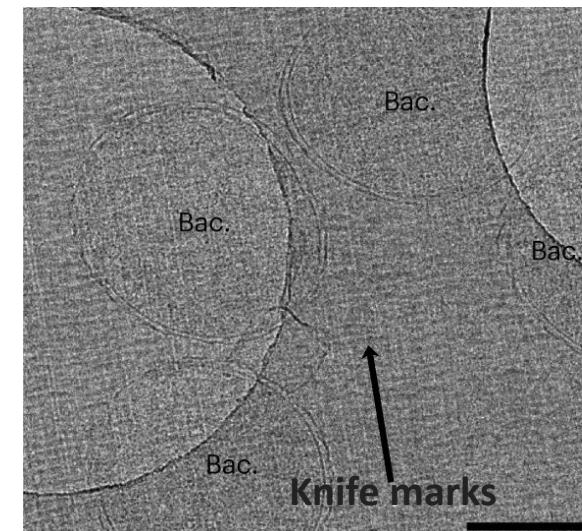
1. Specimens accessible by cryoET



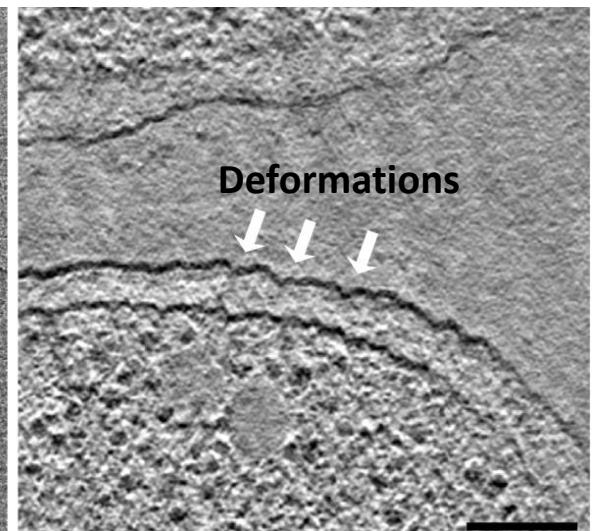
Adapted from TFS



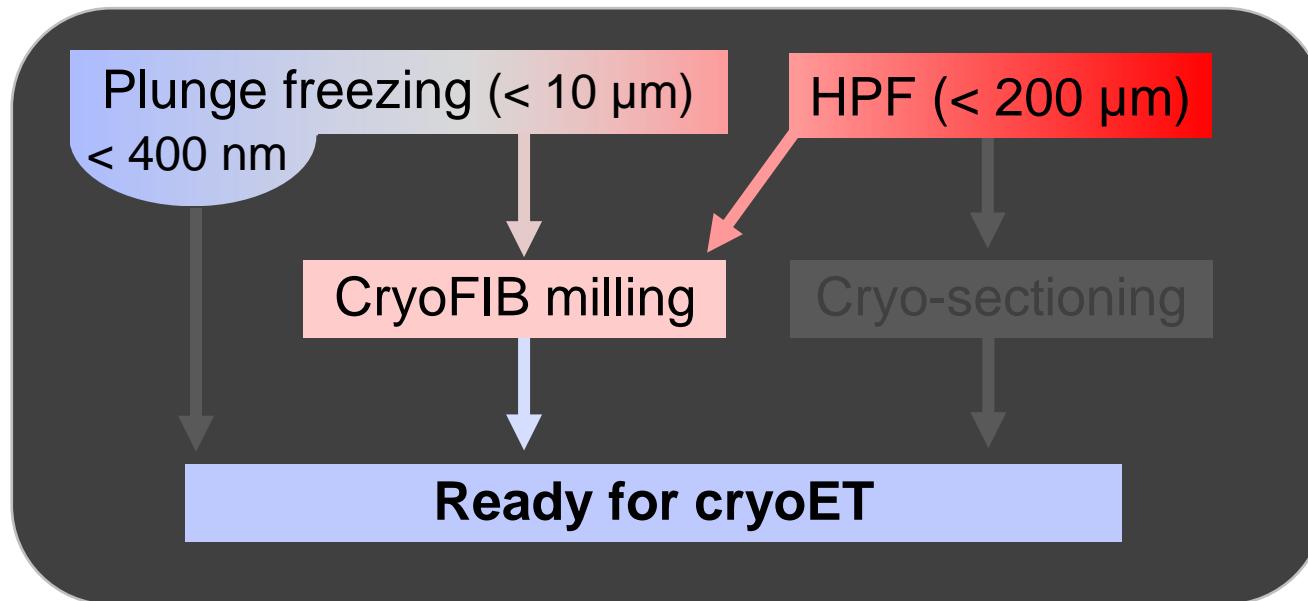
Al-Amoudi et al., J Struct Biol 2005



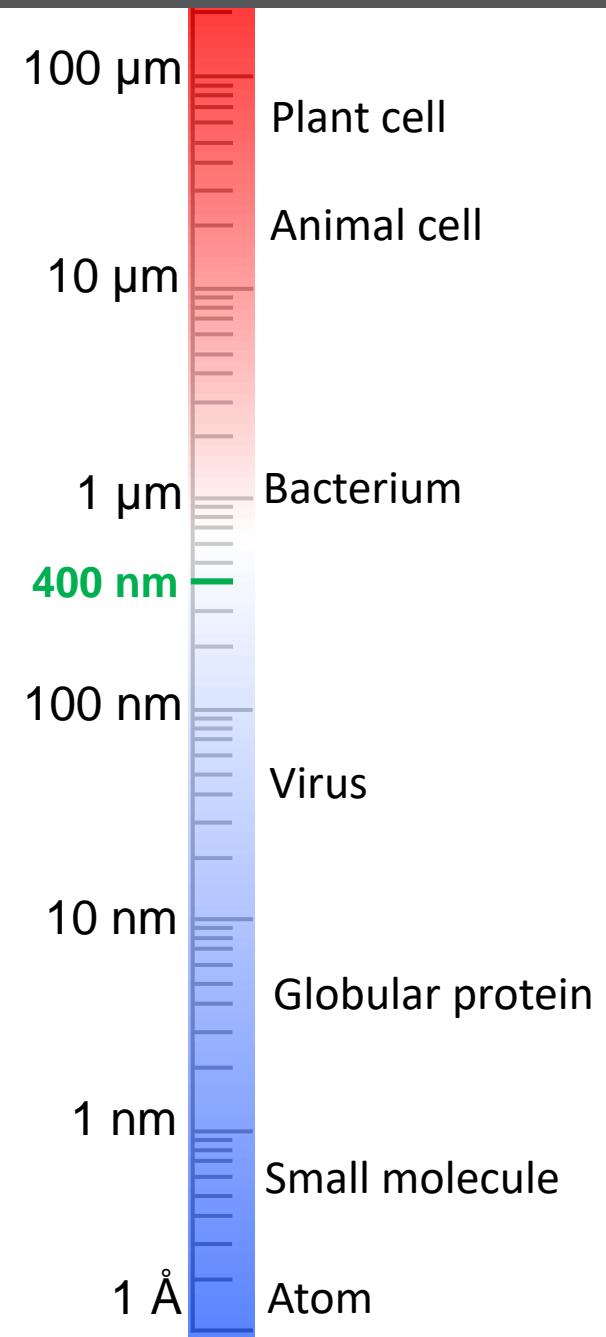
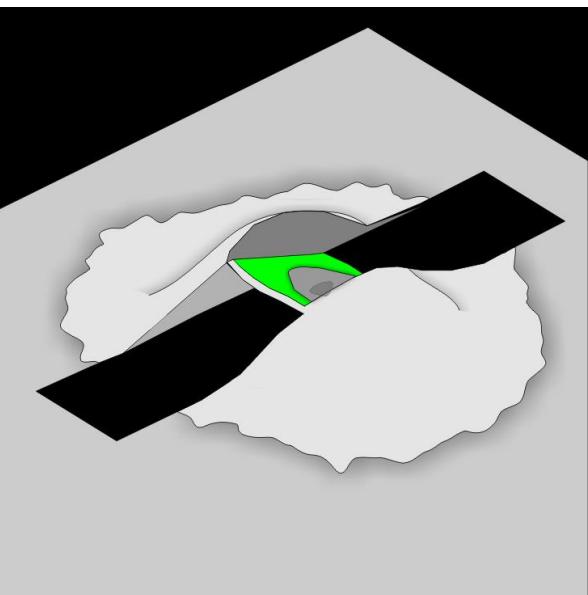
Berger et al., Nature Methods 2023



1. Specimens accessible by cryoET



- 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**

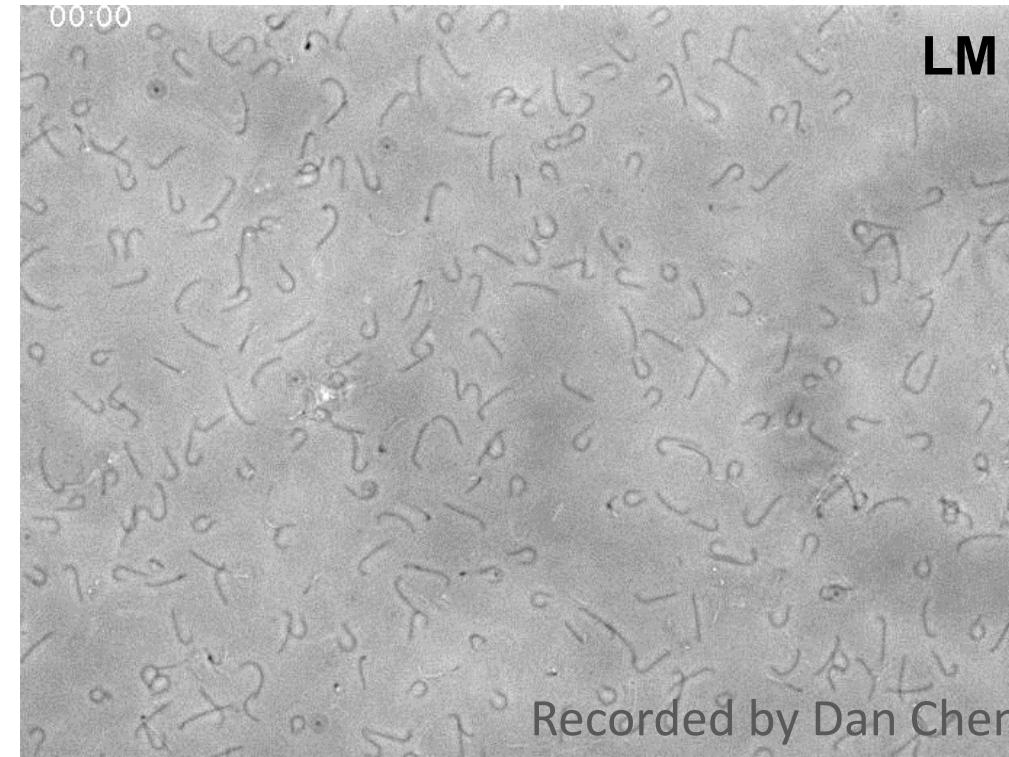
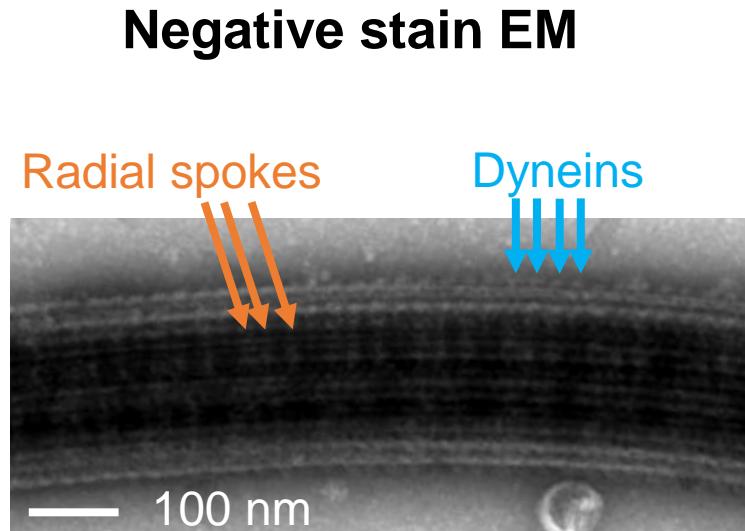


Main contents

1. Specimens accessible by cryoET
2. Five considerations for cryoET sample preparation
3. Tutorial of major steps of cryo lamella preparation

2. Five considerations for cryoET sample preparation

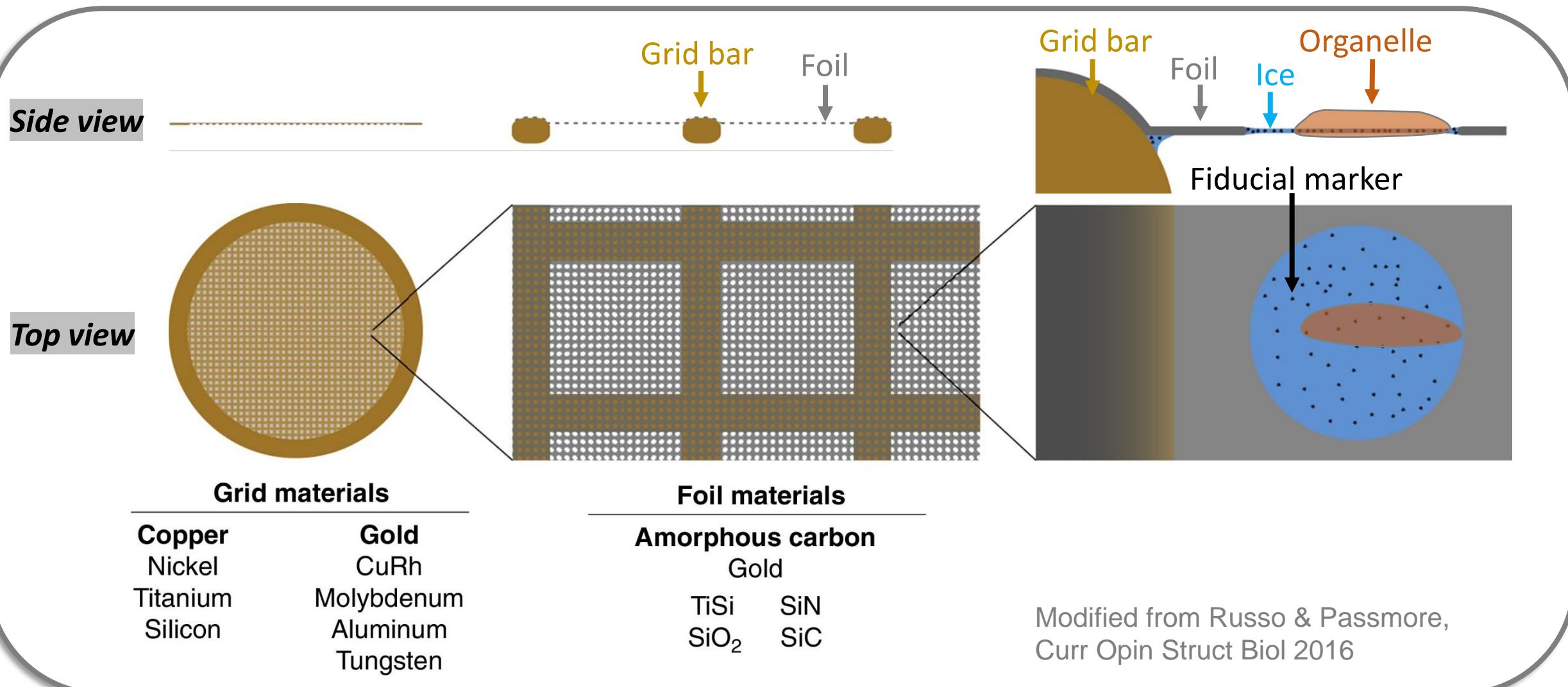
2.1 Validation of the sample quality e.g., Negative stain EM & Reactivation of flagellar axoneme



2. Five considerations for cryoET sample preparation

2.1 Validation of the sample quality

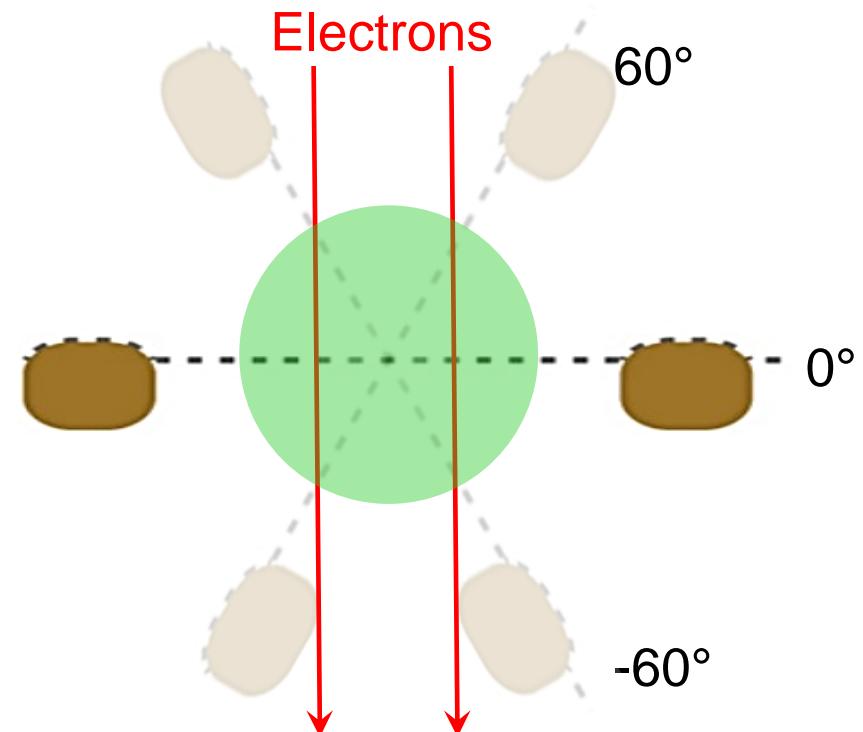
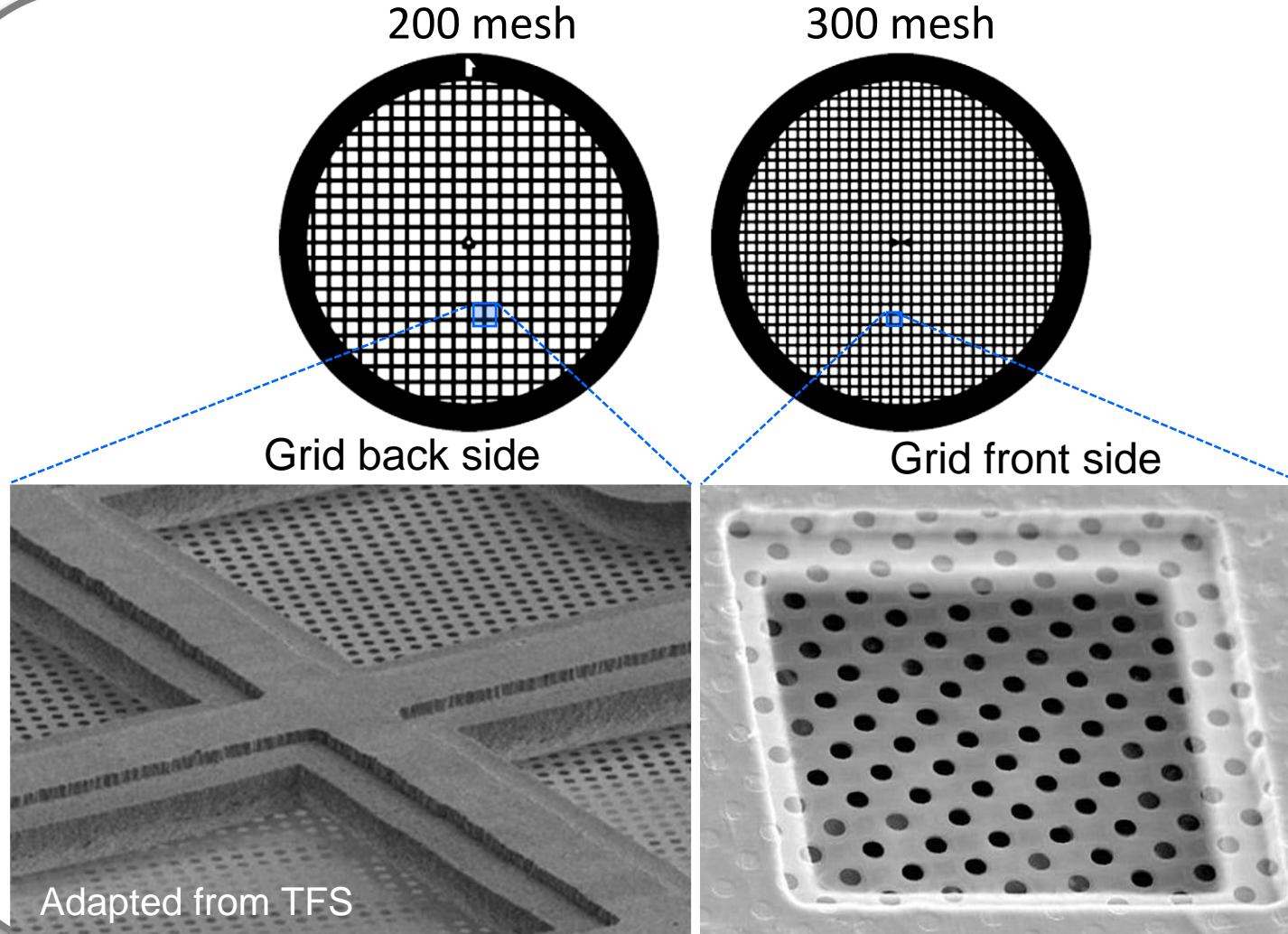
2.2 EM grid => Grid / Foil materials



2. Five considerations for cryoET sample preparation

2.1 Validation of the sample quality

2.2 EM grid => Mesh / Hole size

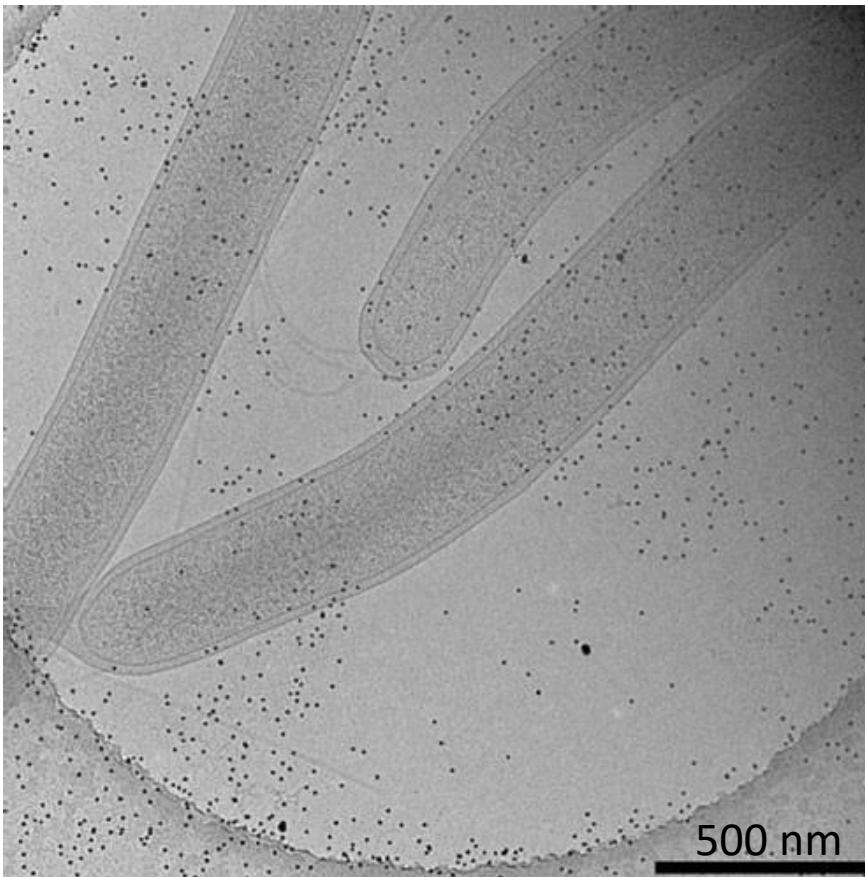


2. Five considerations for cryoET sample preparation

2.1 Validation of the sample quality

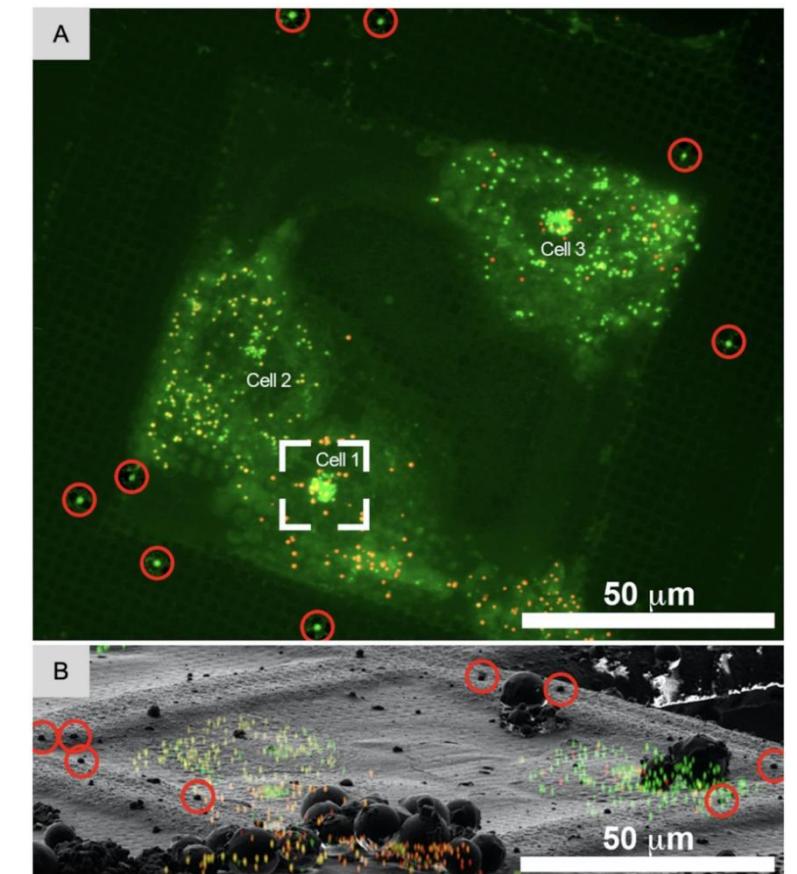
2.2 EM grid

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 cryoET sample preparation

2.1 Validation of the sample quality

2.2 EM grid

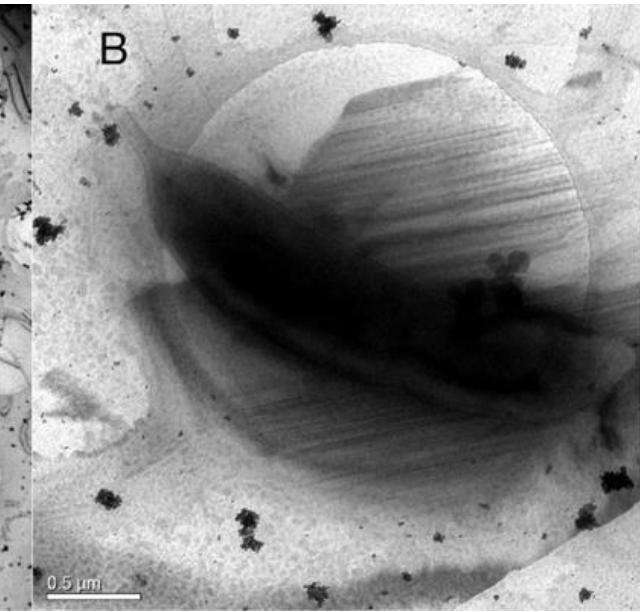
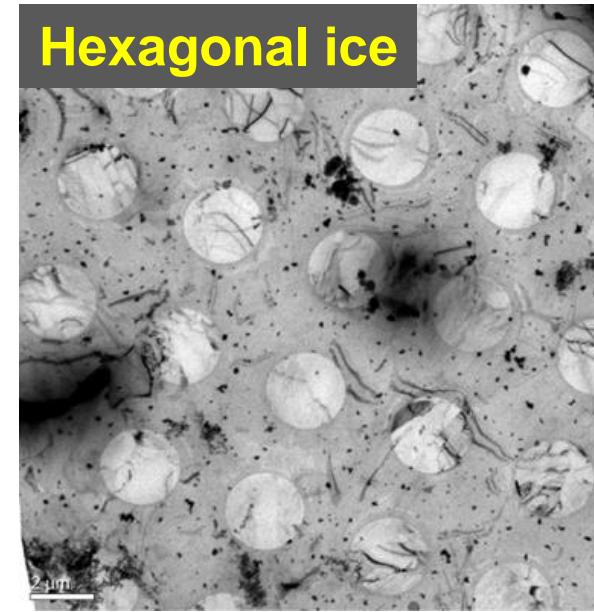
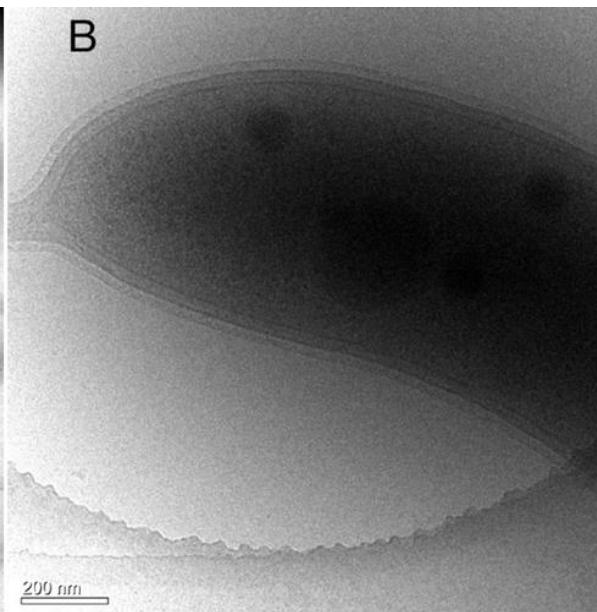
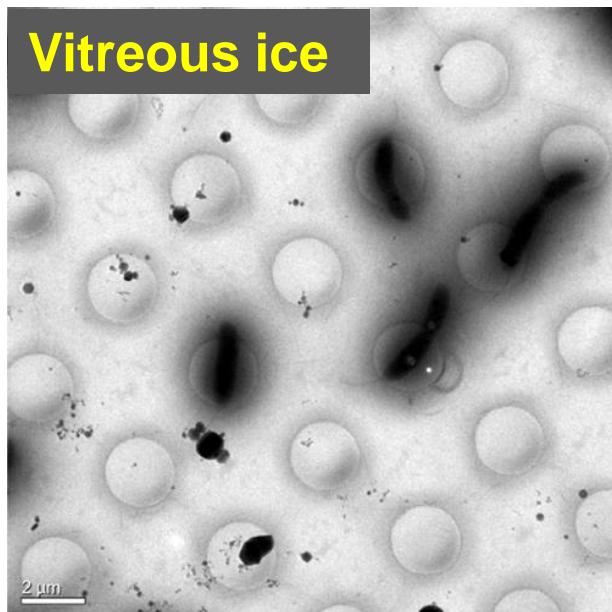
2.3 Fiducial markers

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

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

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

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



2. Five considerations for cryoET sample preparation

2.1 Validation of the sample quality

2.2 EM grid

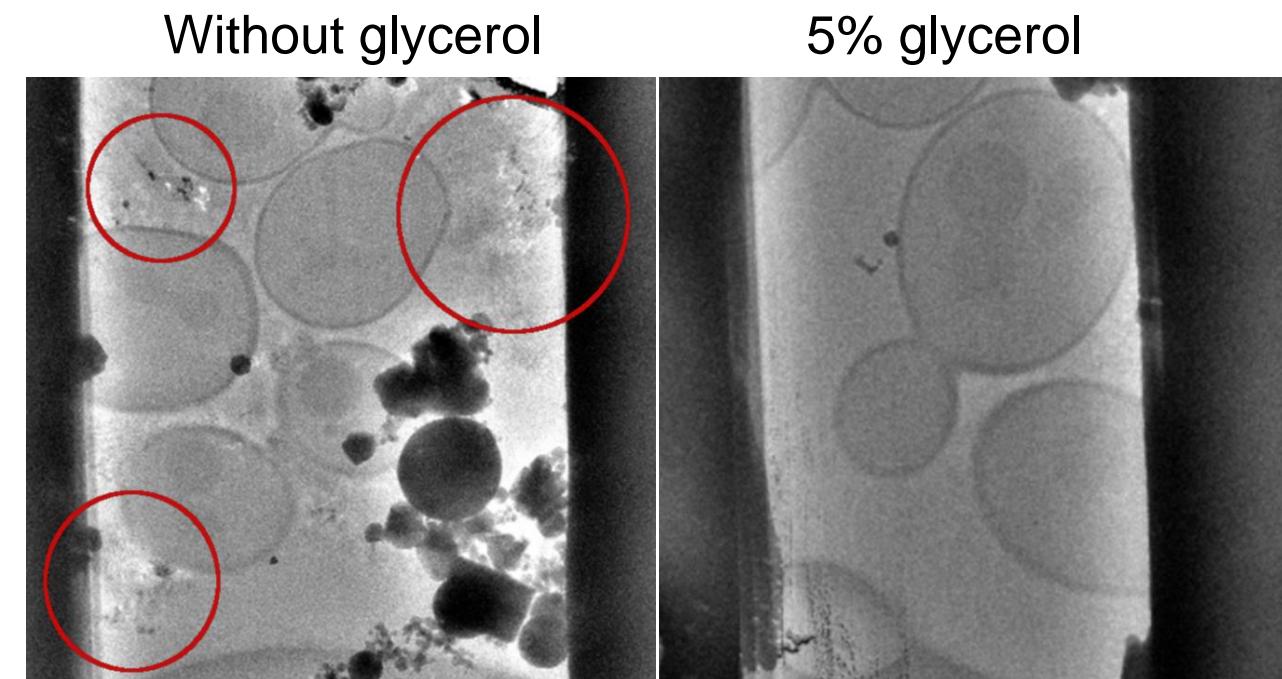
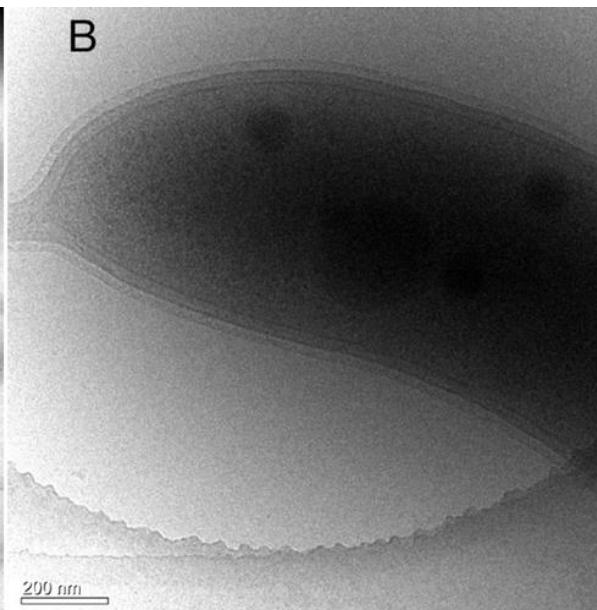
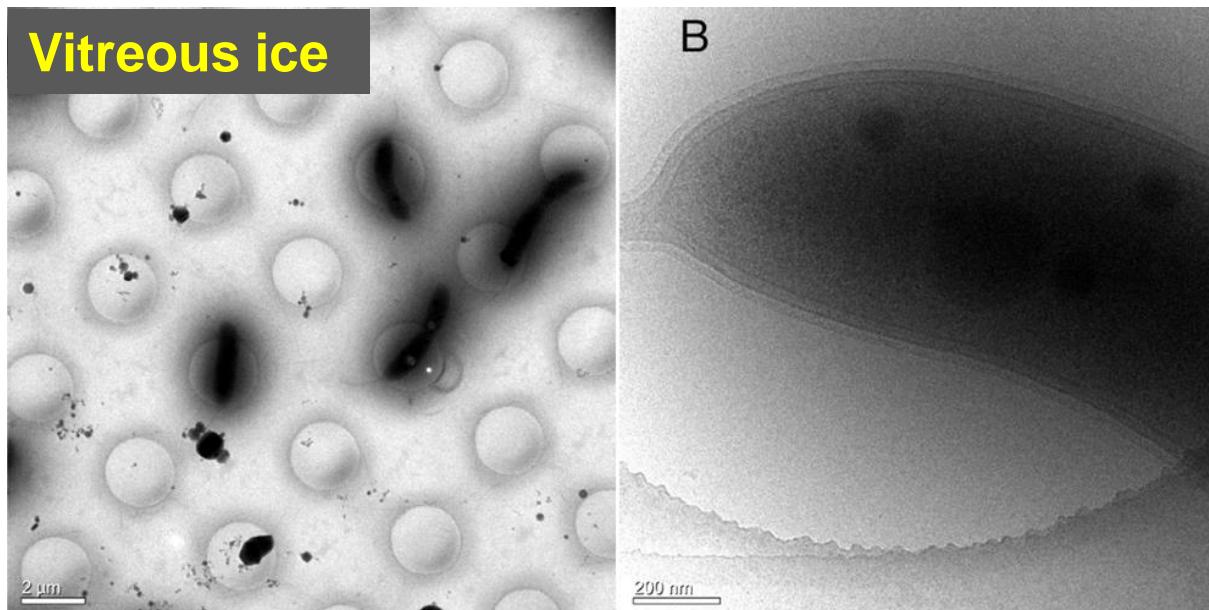
2.3 Fiducial markers

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

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

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

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



Tivol et al., Microsc Microanal 2008

Moravcová et al., J Vis Exp 2021

2. Five considerations for cryoET sample preparation

2.1 Validation of the sample quality

2.2 EM grid

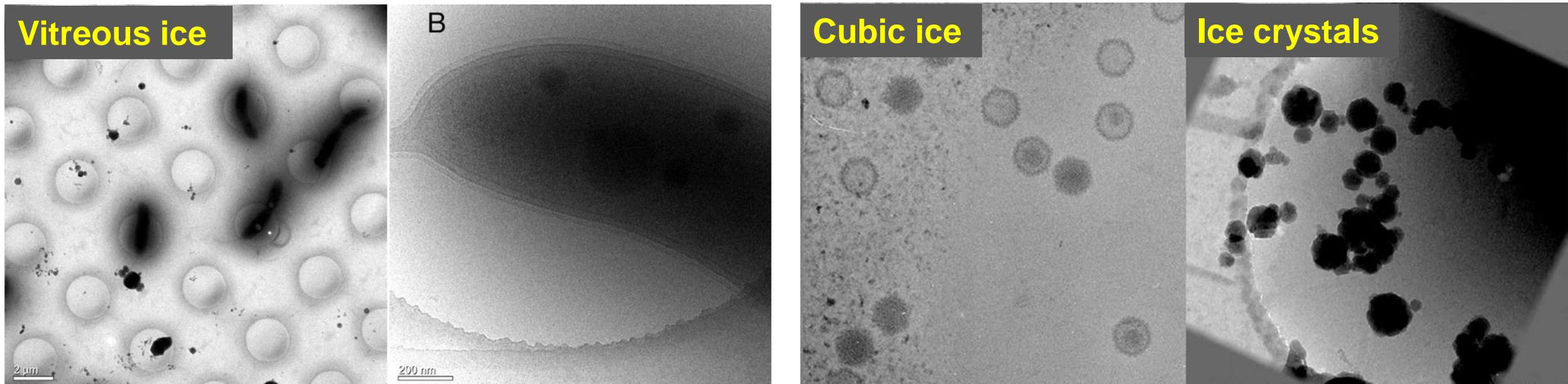
2.3 Fiducial markers

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

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

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

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



Tivol et al., Microsc Microanal 2008

Bhella D, Biophysical Reviews 2019

2. Five considerations for cryoET sample preparation

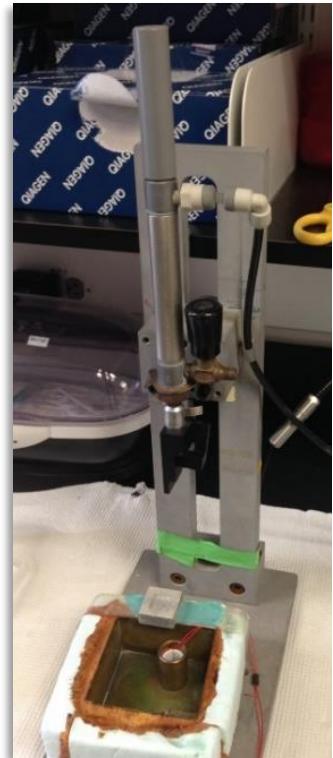
2.1 Validation of the sample quality

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. Specimens accessible by cryoET
2. Five considerations for cryoET sample preparation
3. Tutorial of major steps of cryo lamella preparation

Examples of cryoFIB milling instruments

Crossbeam (ZEISS)

Arctis

(Thermo Fisher Scientific)



Scios, Aquiclos 1/2
(Thermo Fisher Scientific)



Main contents

1. Specimens accessible by cryoET
2. Five considerations for cryoET 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)

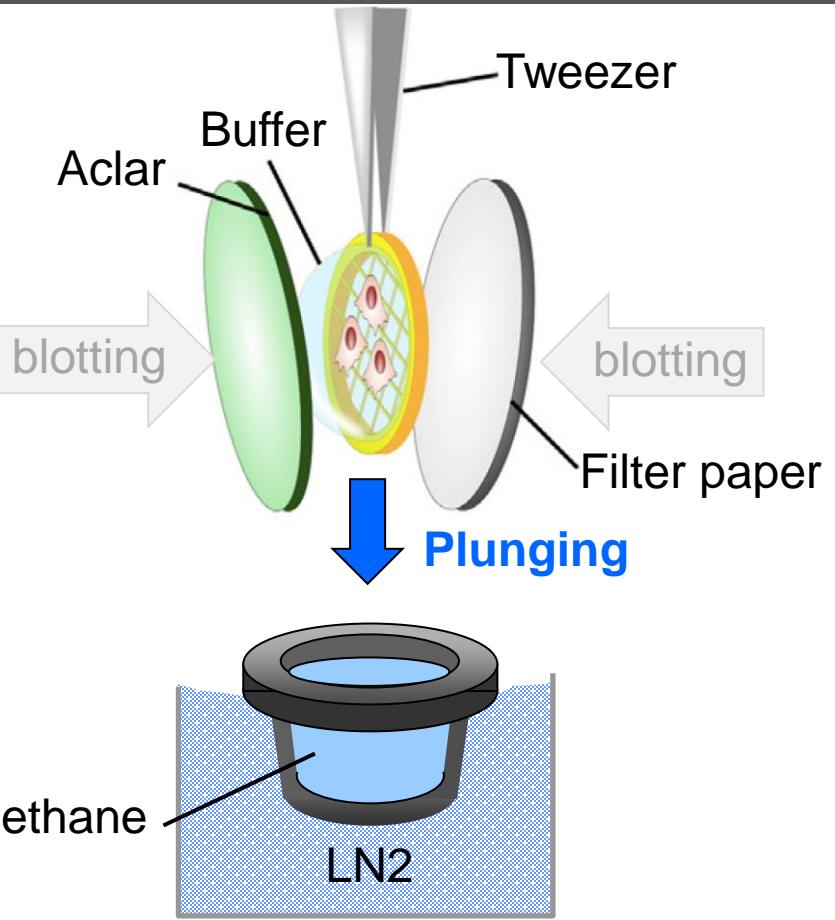
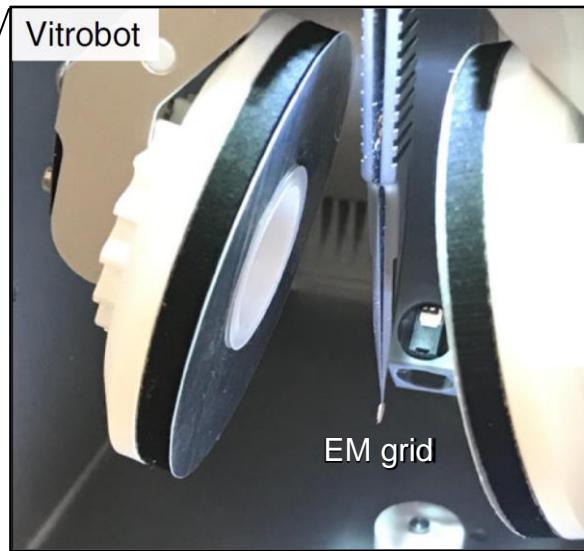
Target confirmation

Pt sputter (Optional)

Lamella conductivity

T

CryoET



Schaffer et al., JSB 2017; Medeiros et al., Curr Opin Microbiol 2018; Wagner et al., Nature Protocols 2020

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

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



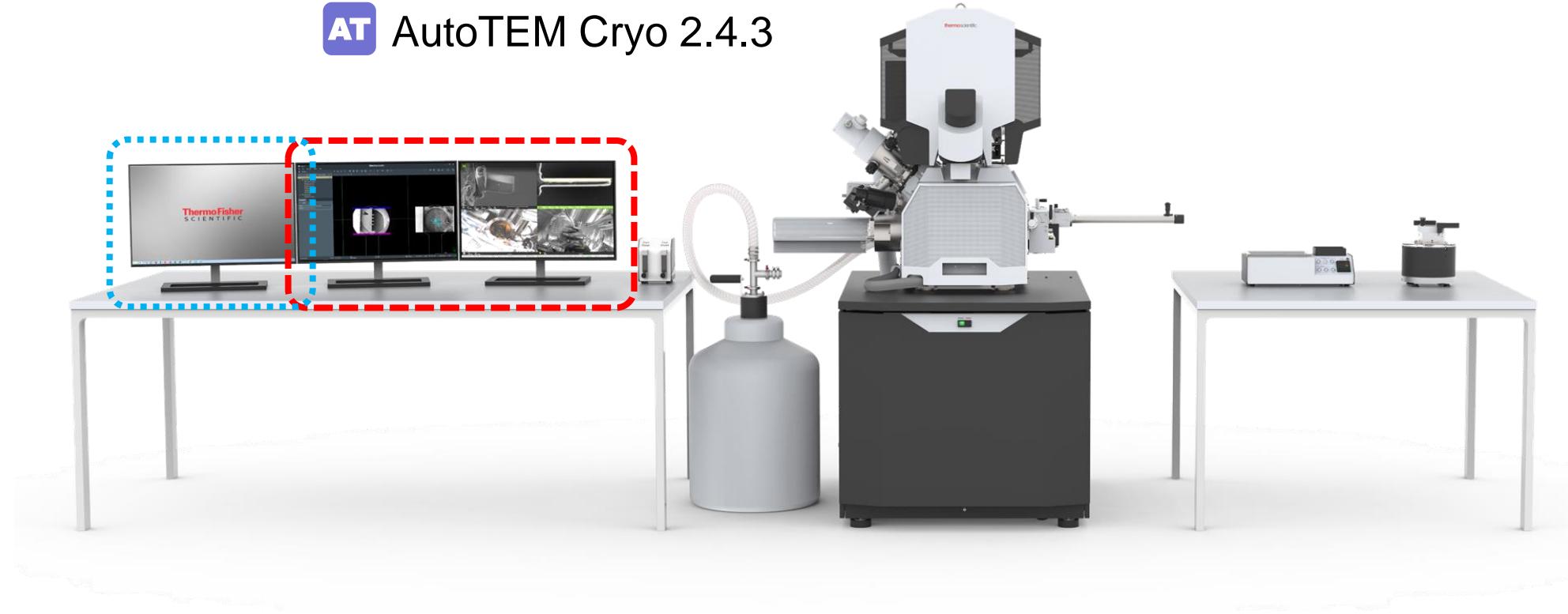
Aquilos 2 & Software used in this tutorial

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

( 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

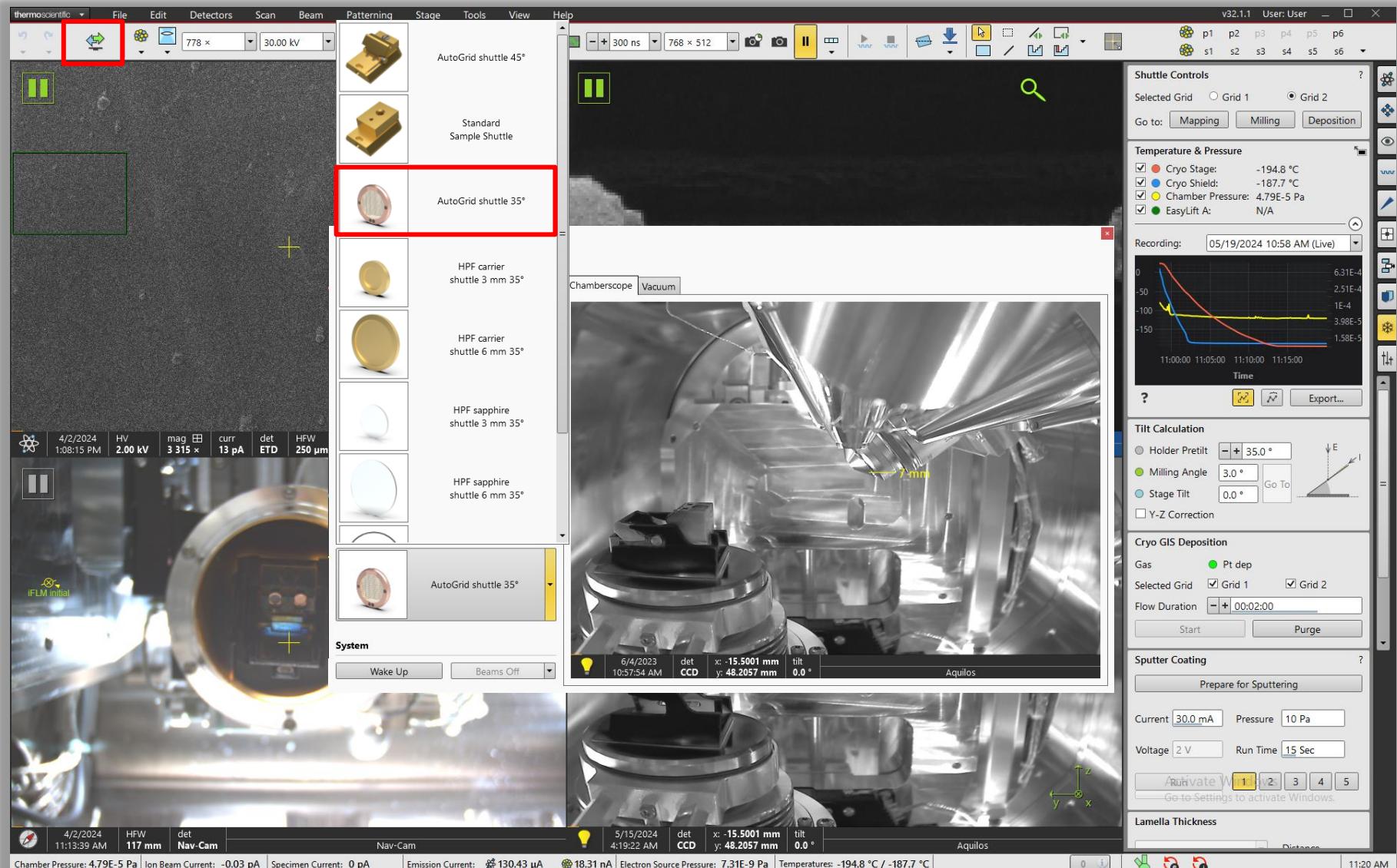
Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

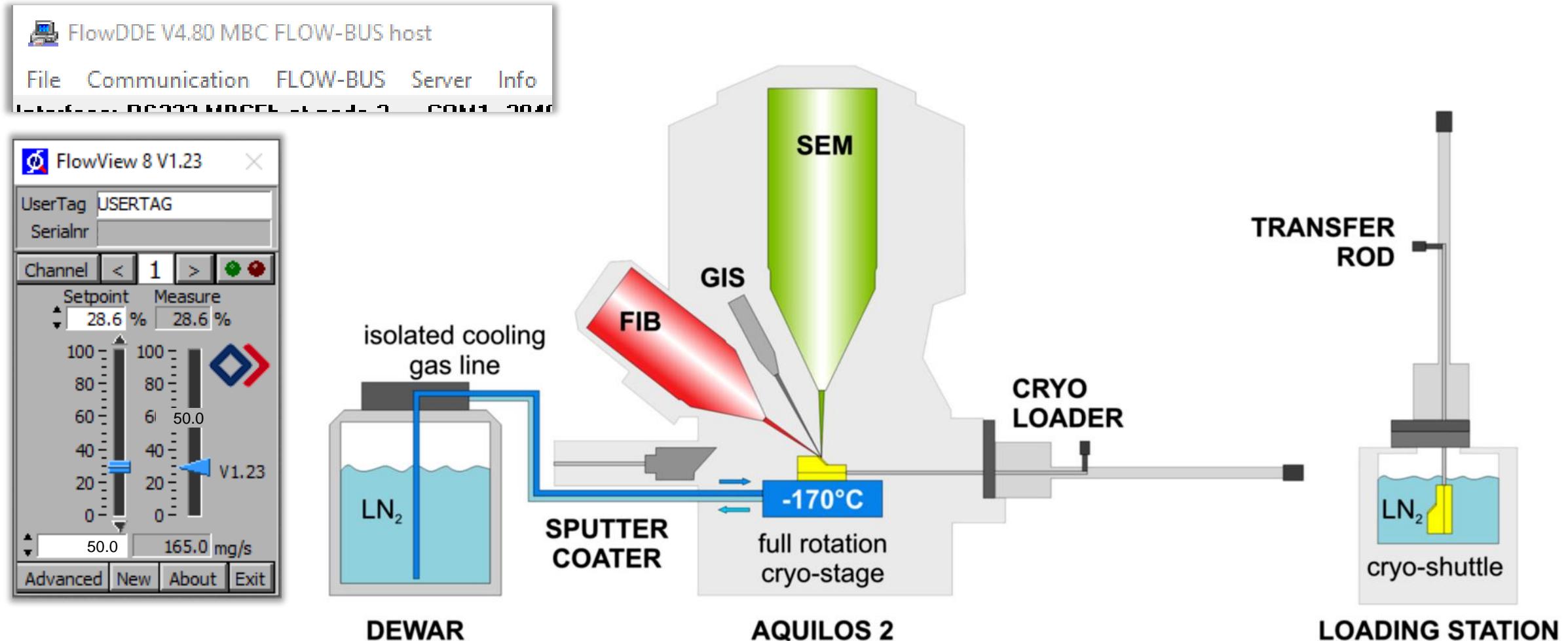
Pt sputter (Optional)

Lamella conductivity



3.2.1 Prepare the system _Purge the system

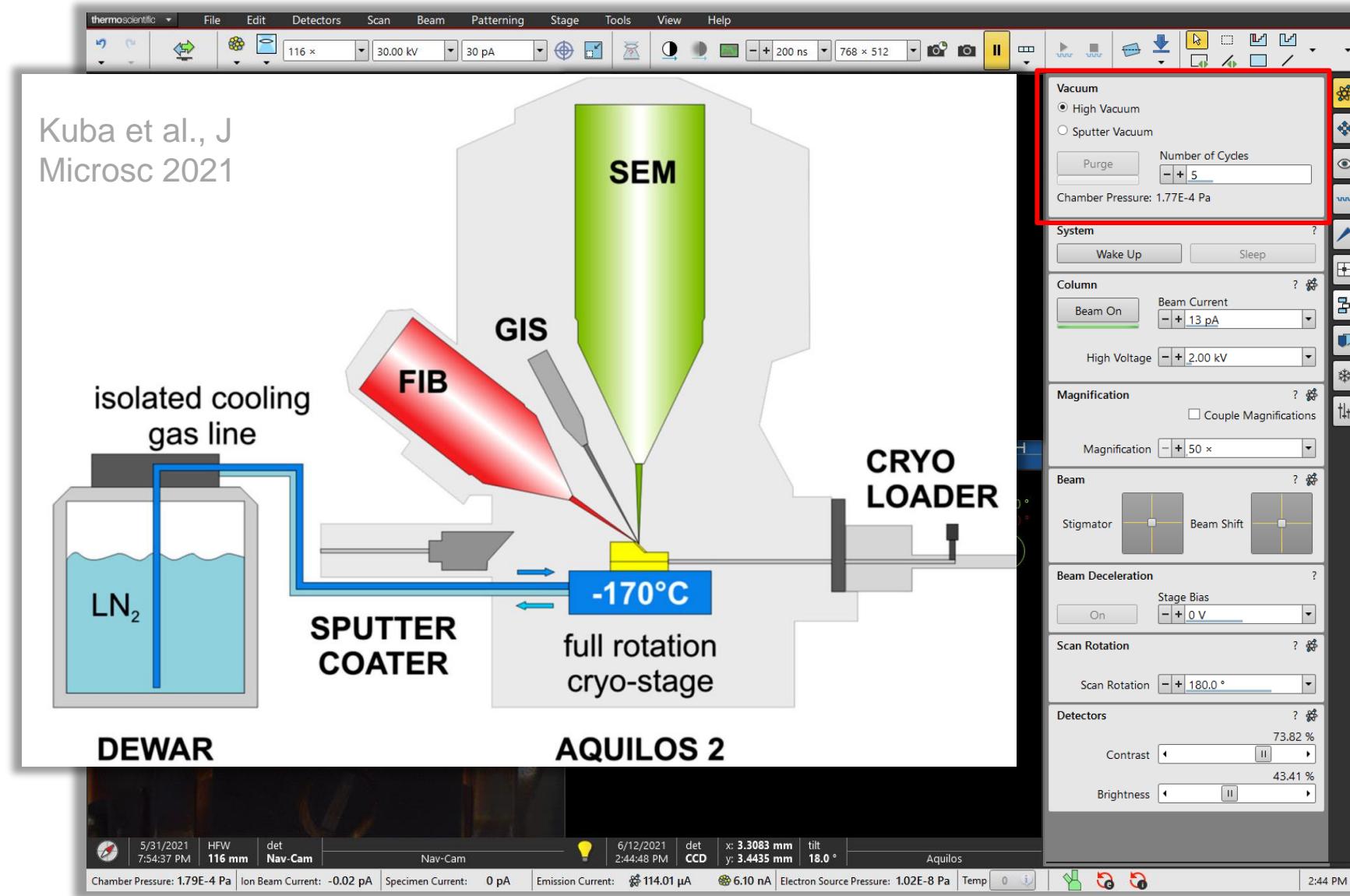
- Cooling-gas line, Loading station (>0.5h)



3.2.1 Prepare the system _Purge the system

( Microscope Control)

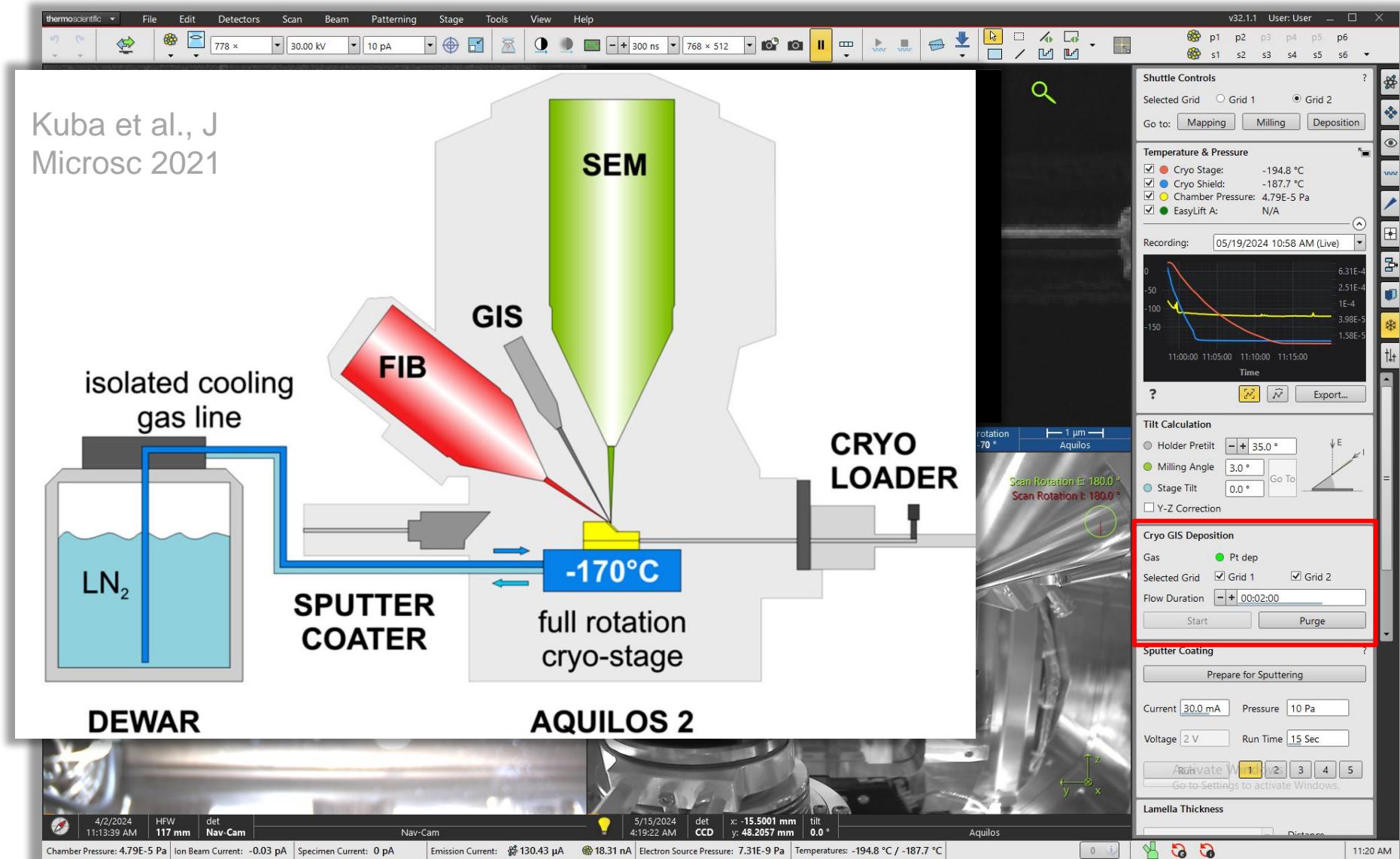
- Cooling-gas line, Loading station, Argon line (5 cycles)



3.2.1 Prepare the system _Purge the system

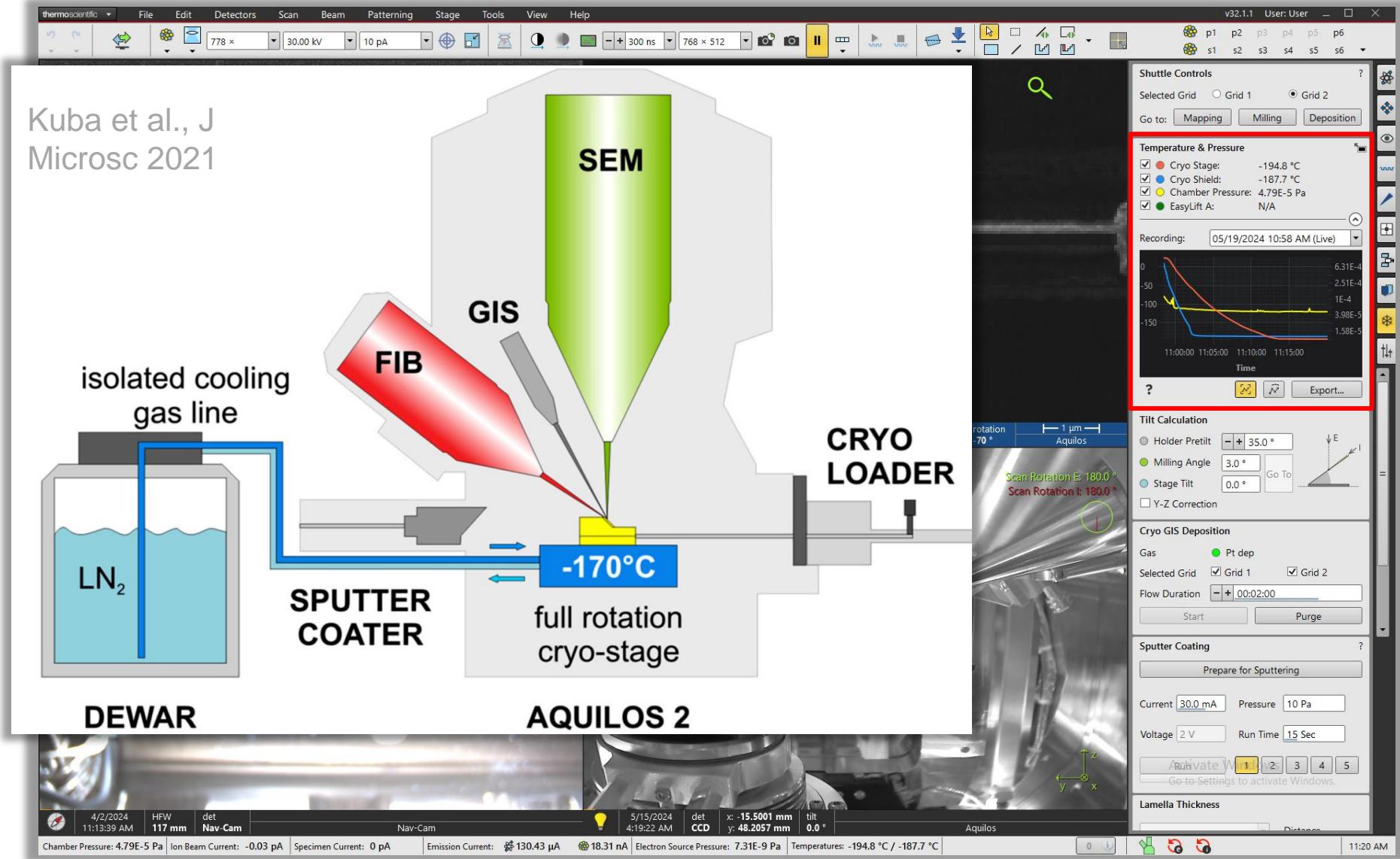
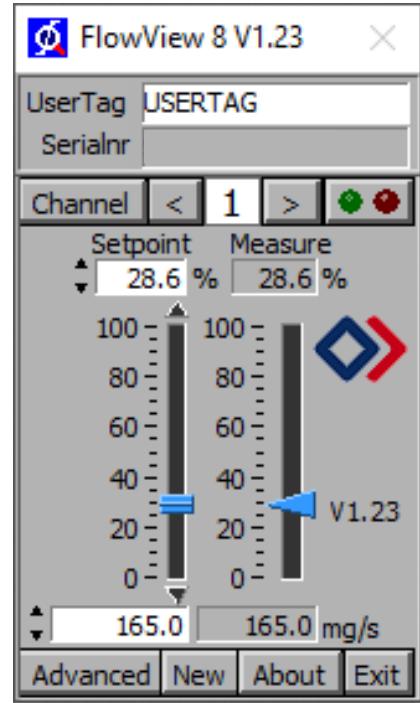
( Microscope Control)

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



3.2.1 Prepare the system _Cool down the system

( Microscope Control)



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)

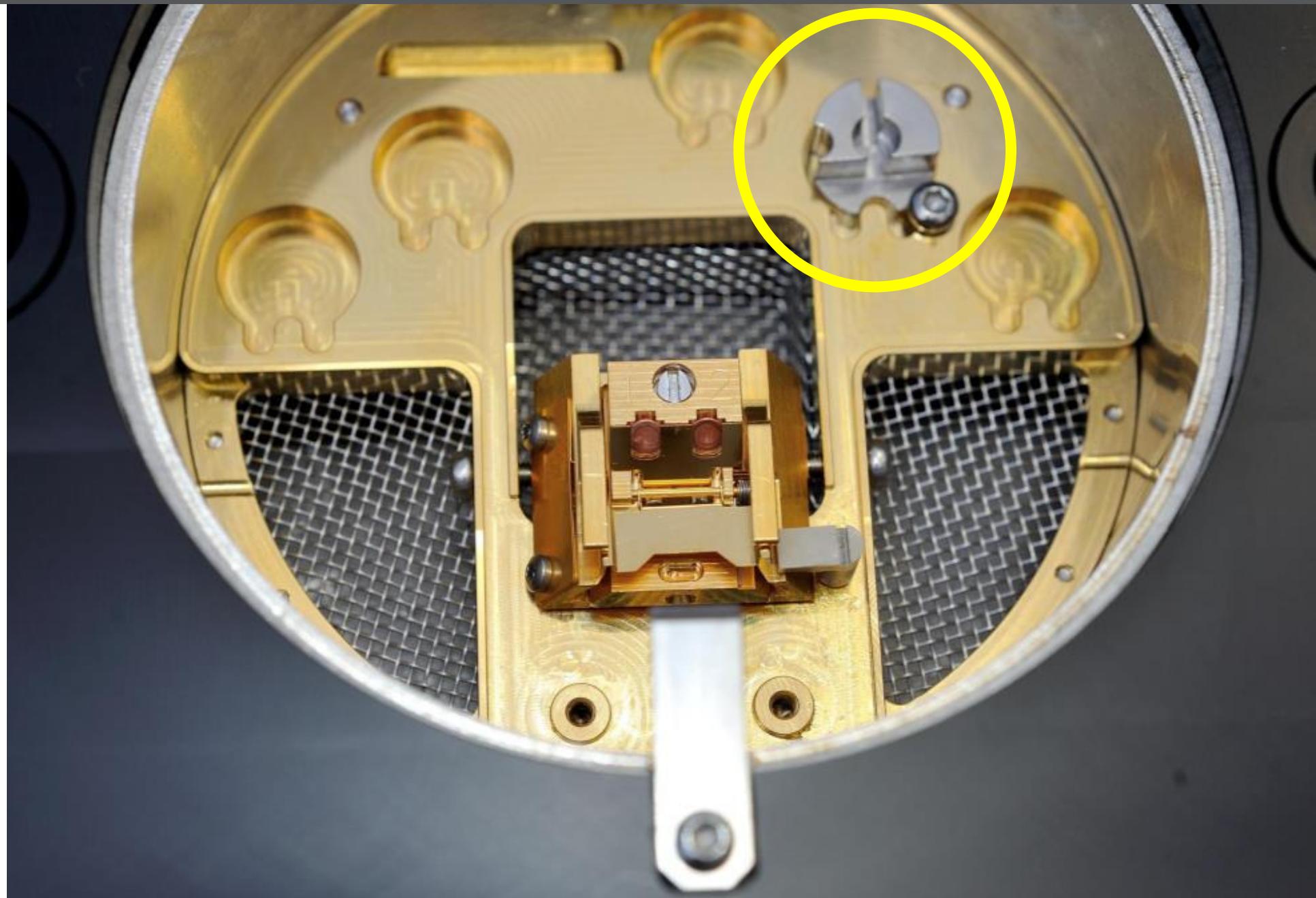
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.2.2 Prepare the grids Clip the grids

Vitrification



CryoFIB

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)

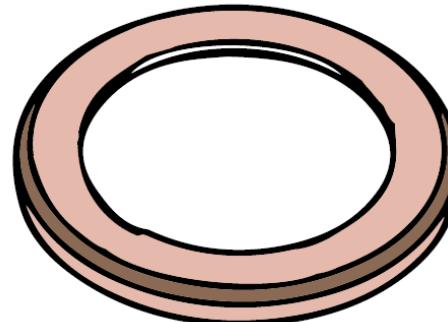
Target confirmation

Pt sputter (Optional)

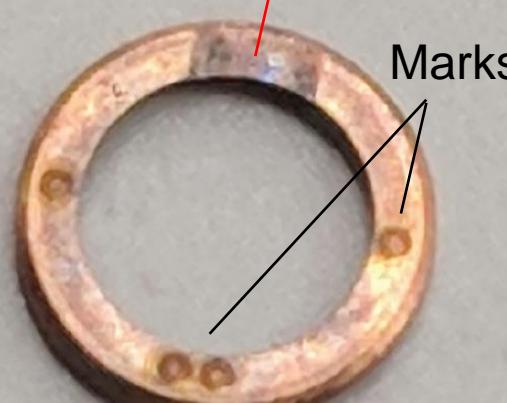
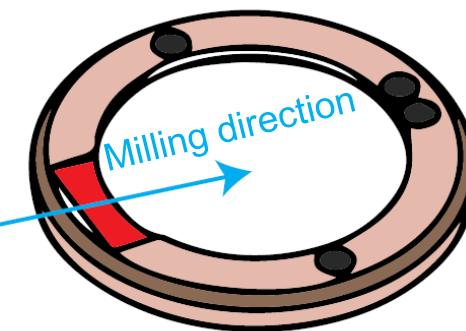
Lamella conductivity



Conventional AutoGrid



FIB-AutoGrid

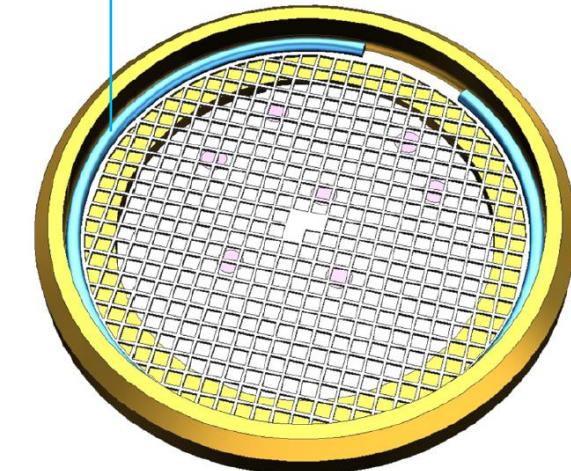


Milling area

EM grid

Vitrified cells

C-clip 180°



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)

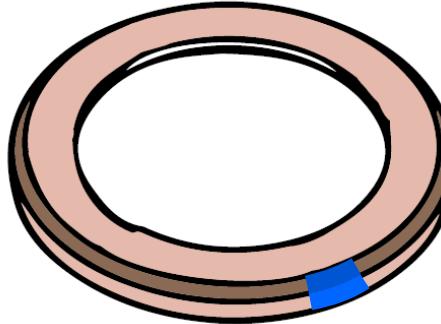
Target confirmation

Pt sputter (Optional)

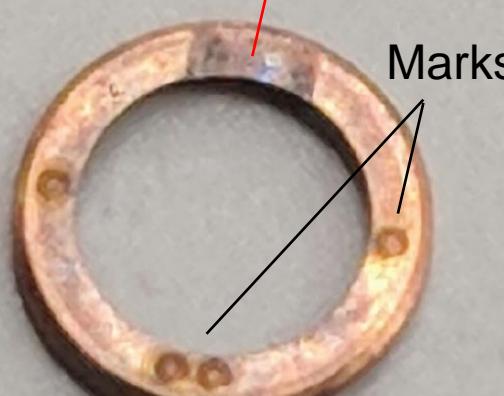
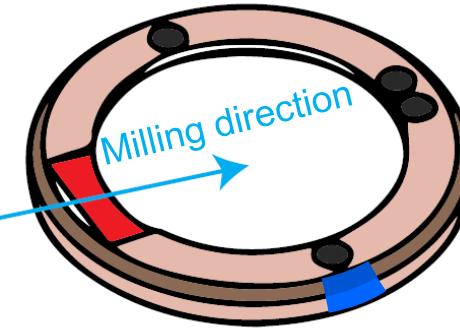
Lamella conductivity



Conventional AutoGrid

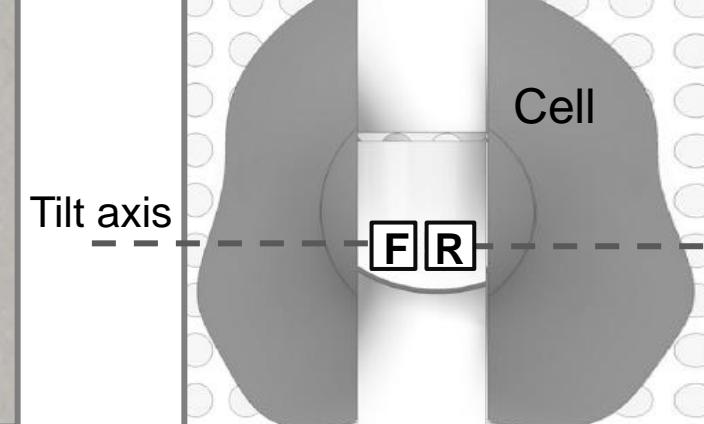
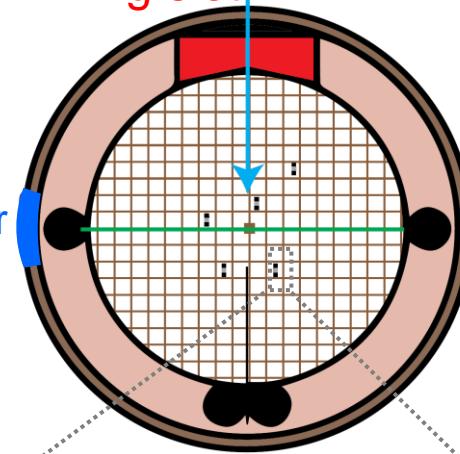


FIB-AutoGrid



Milling direction

Milling slot



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

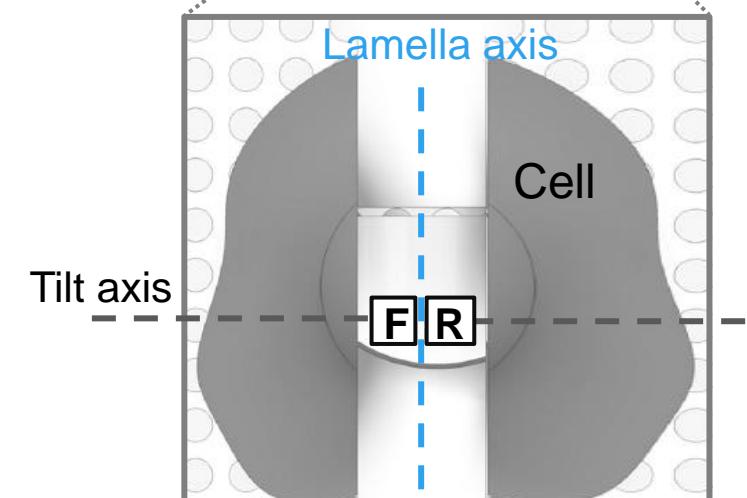
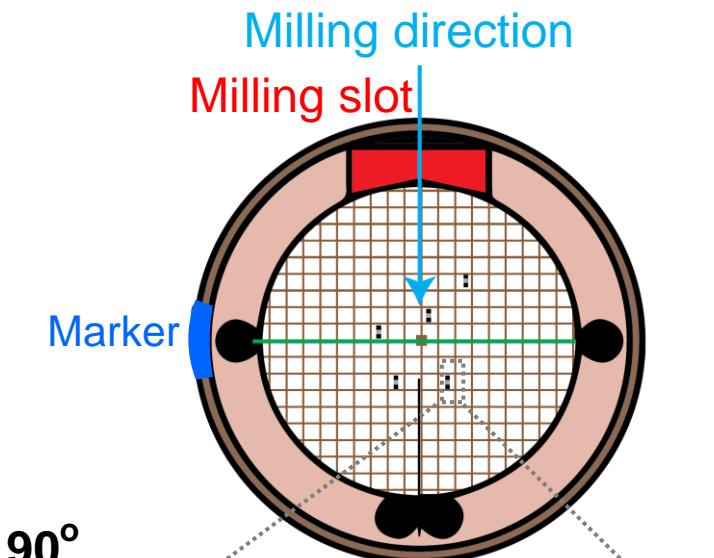
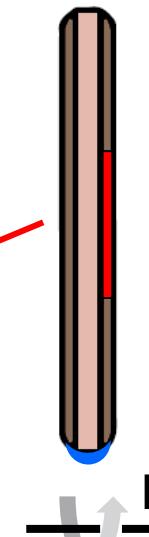
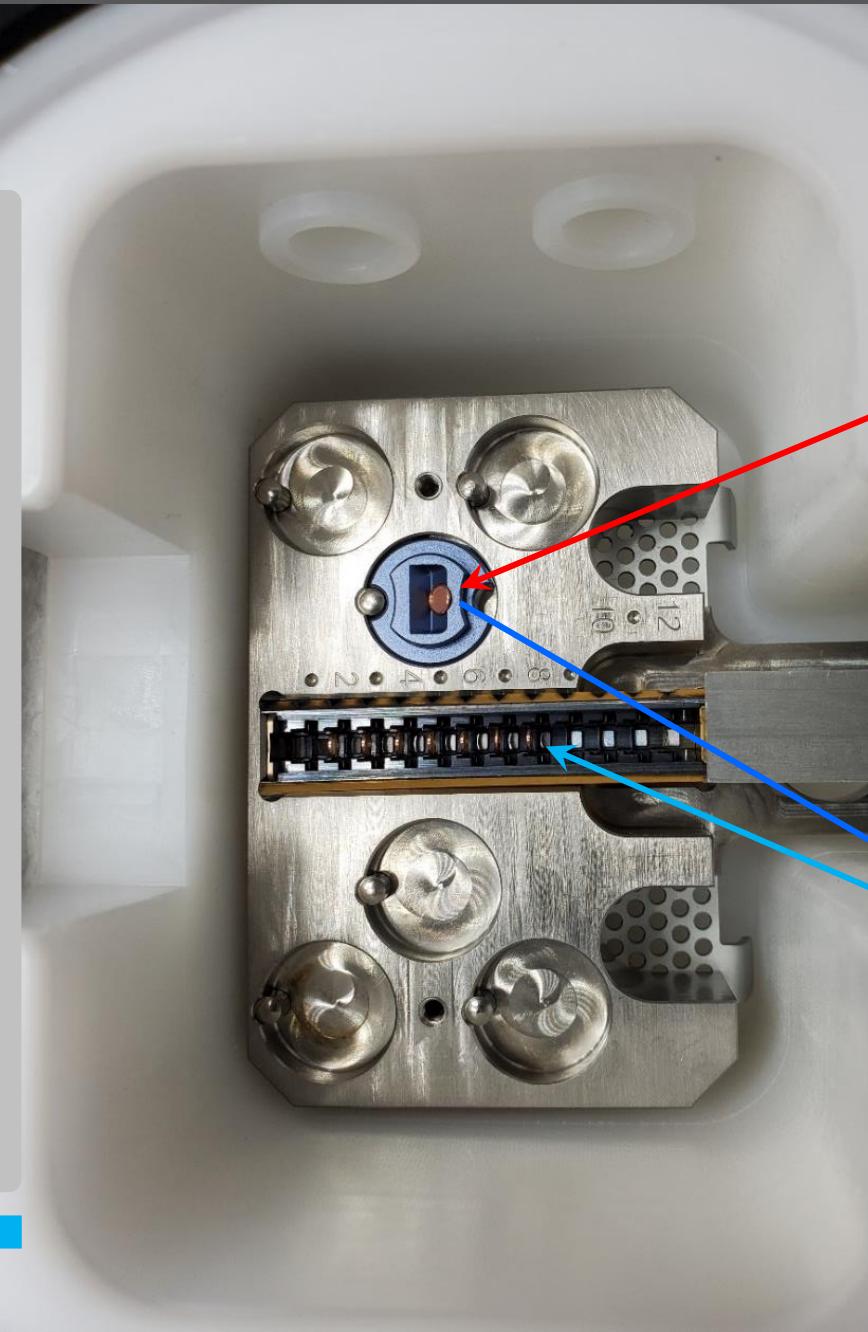
Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

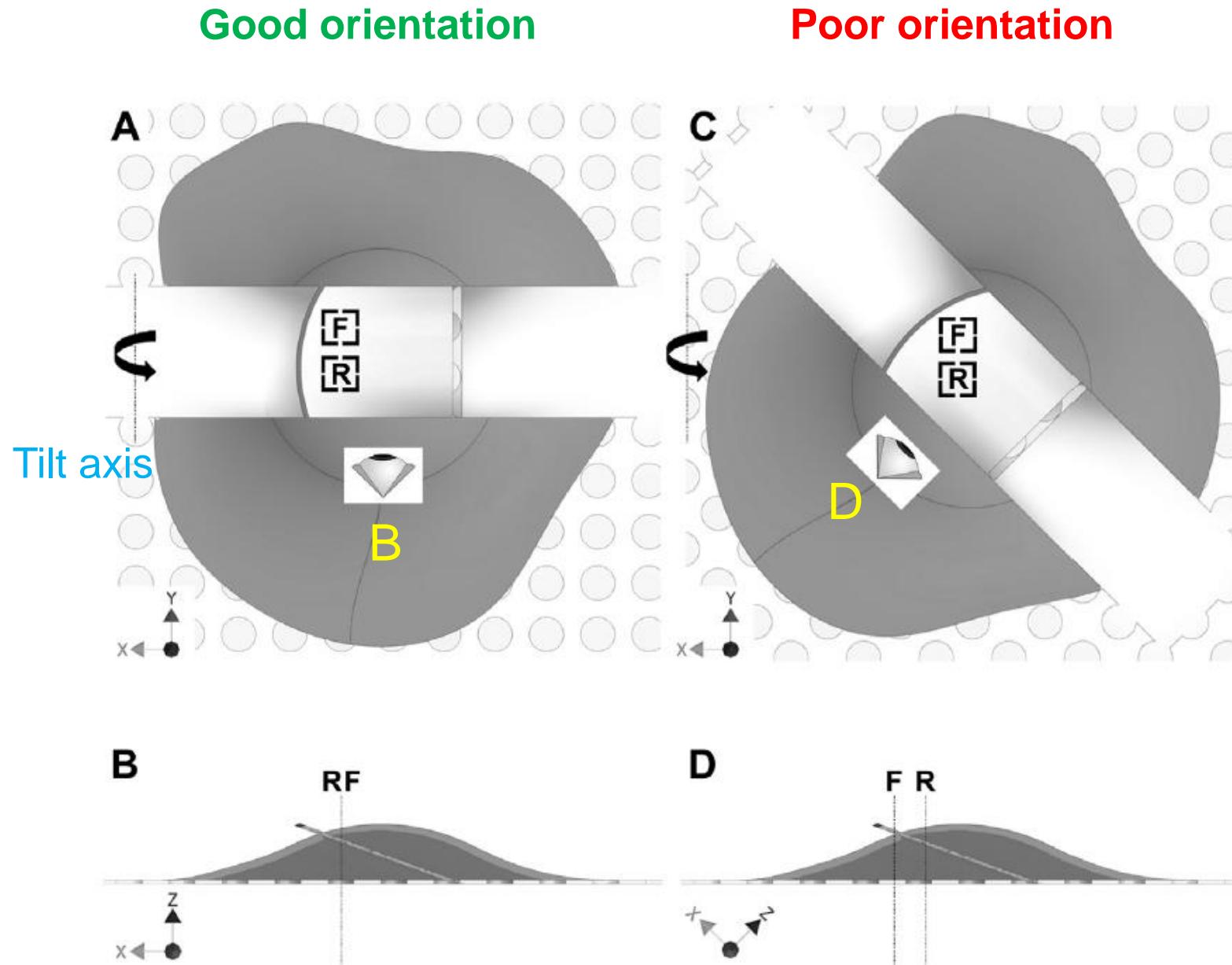
Pt sputter (Optional)

Lamella conductivity



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

Poor orientation causes occlusion at high tilt angles & inaccurate focusing



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)

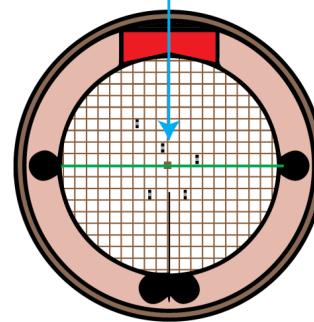
Target confirmation

Pt sputter (Optional)

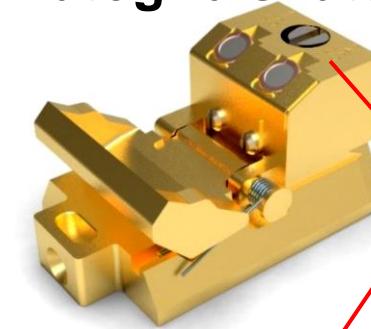
Lamella conductivity



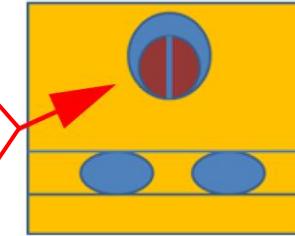
Milling direction



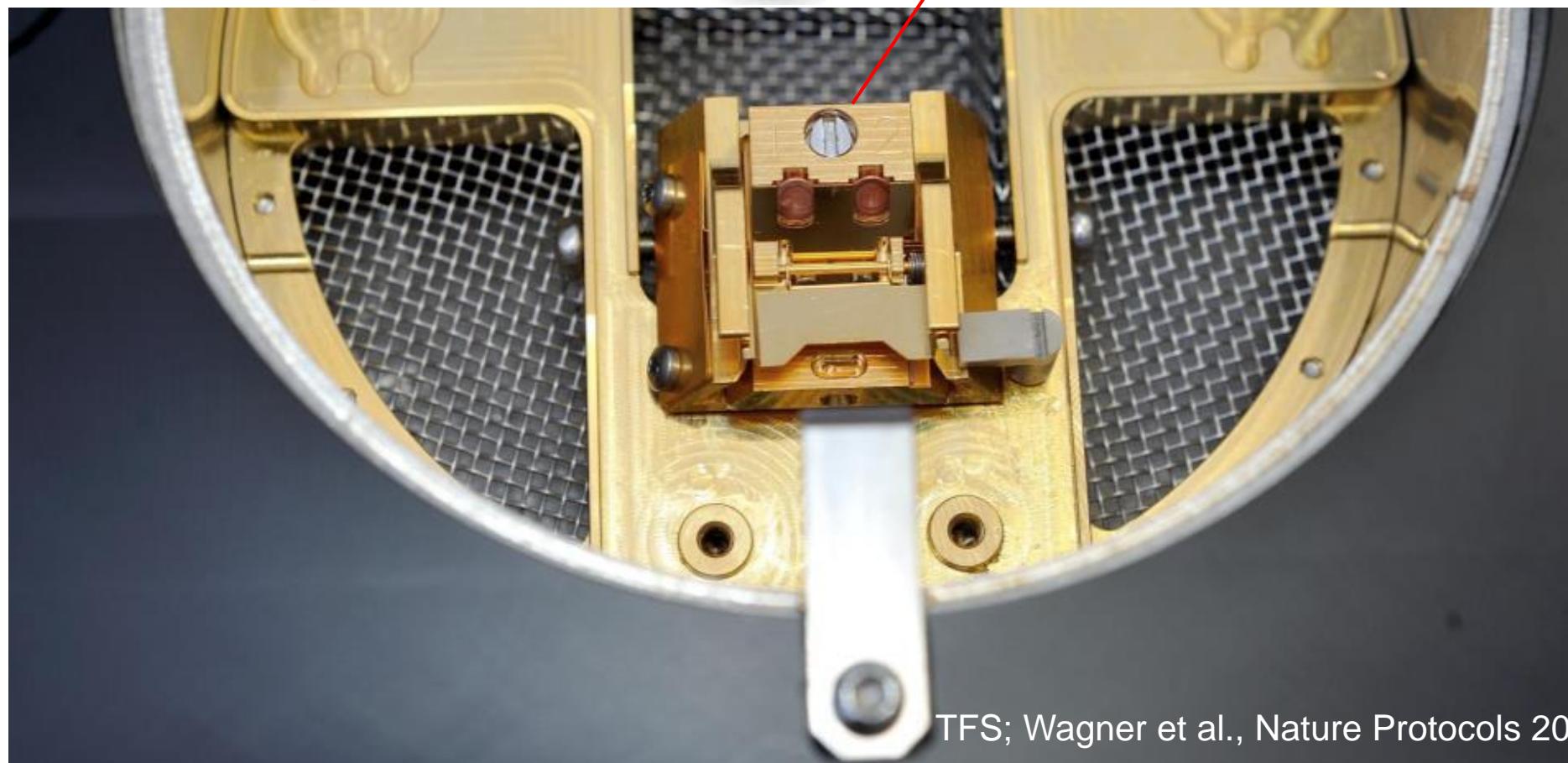
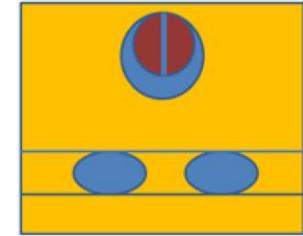
Autogrid shuttle



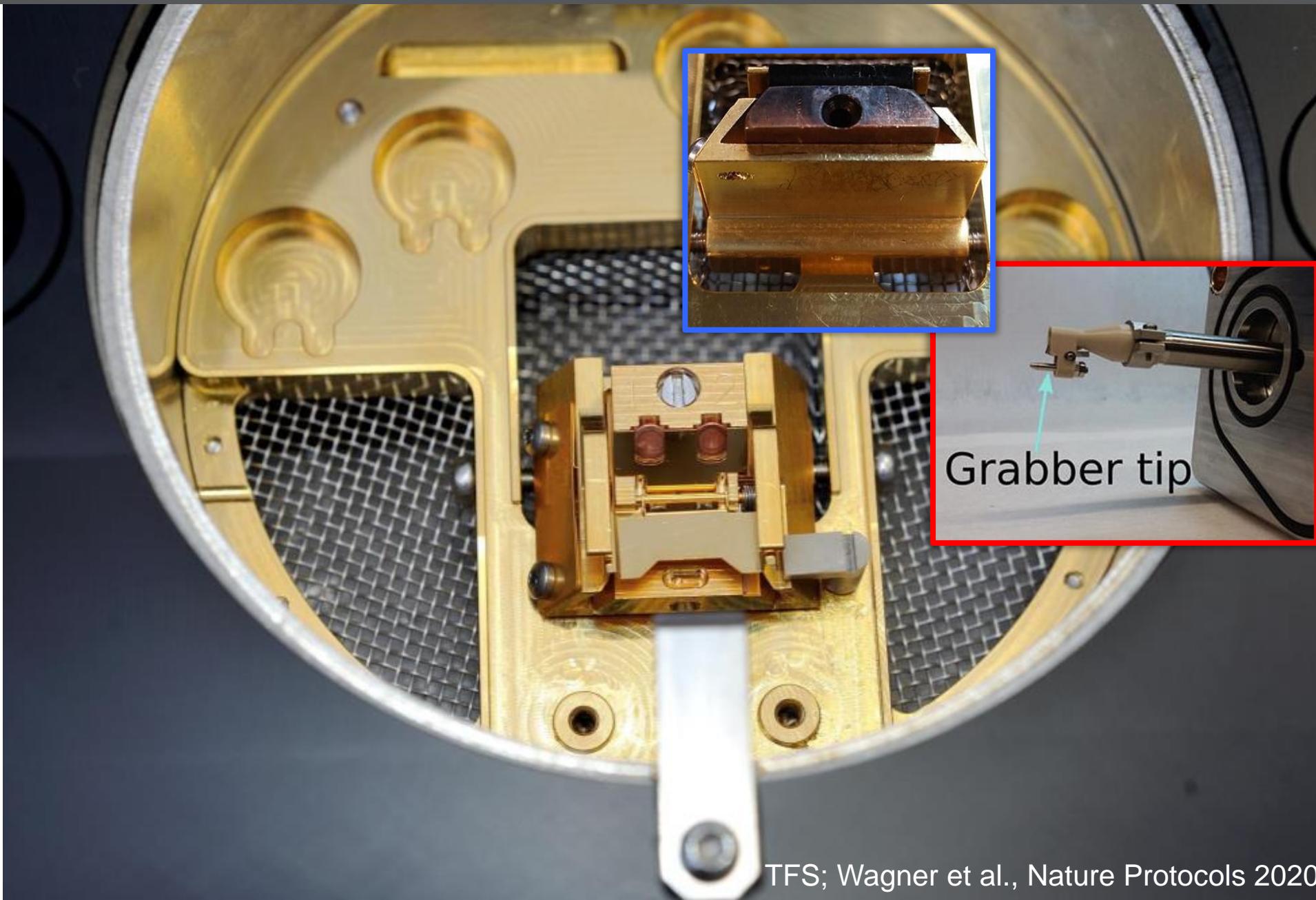
Open



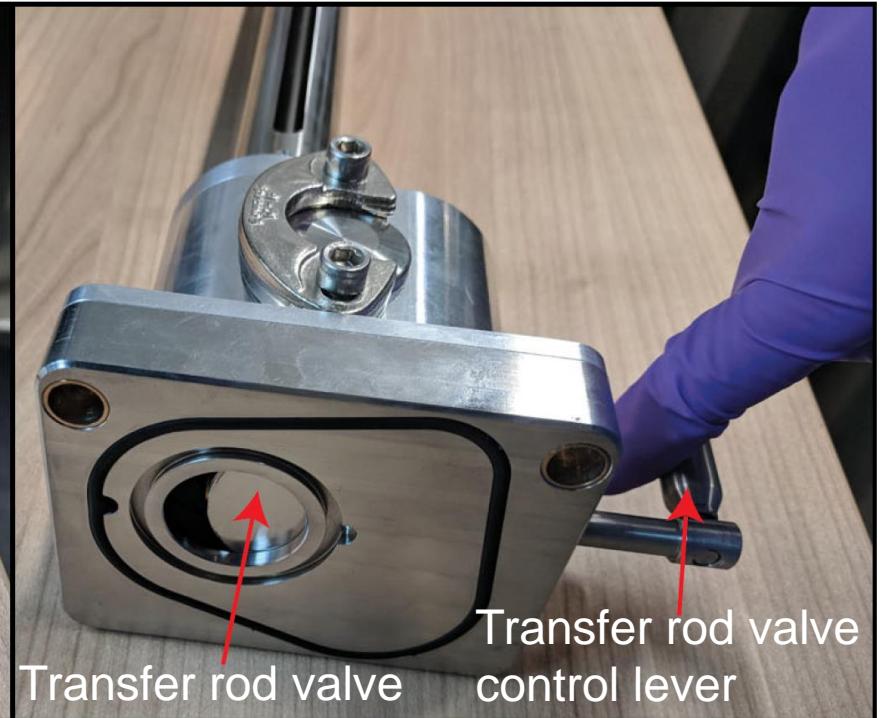
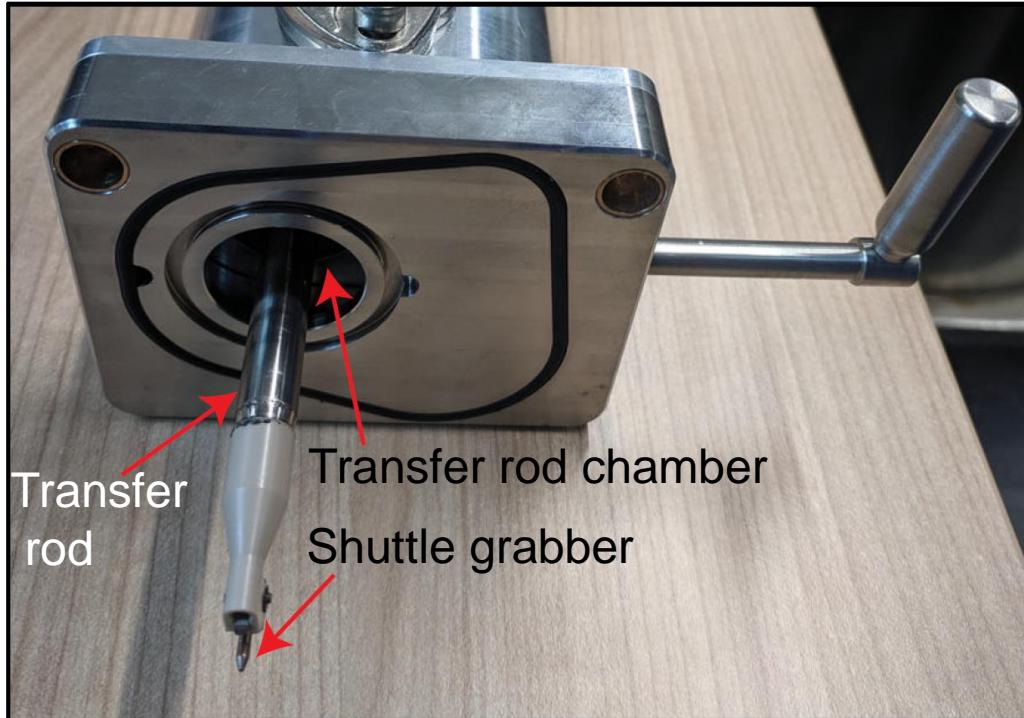
Closed



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

(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)

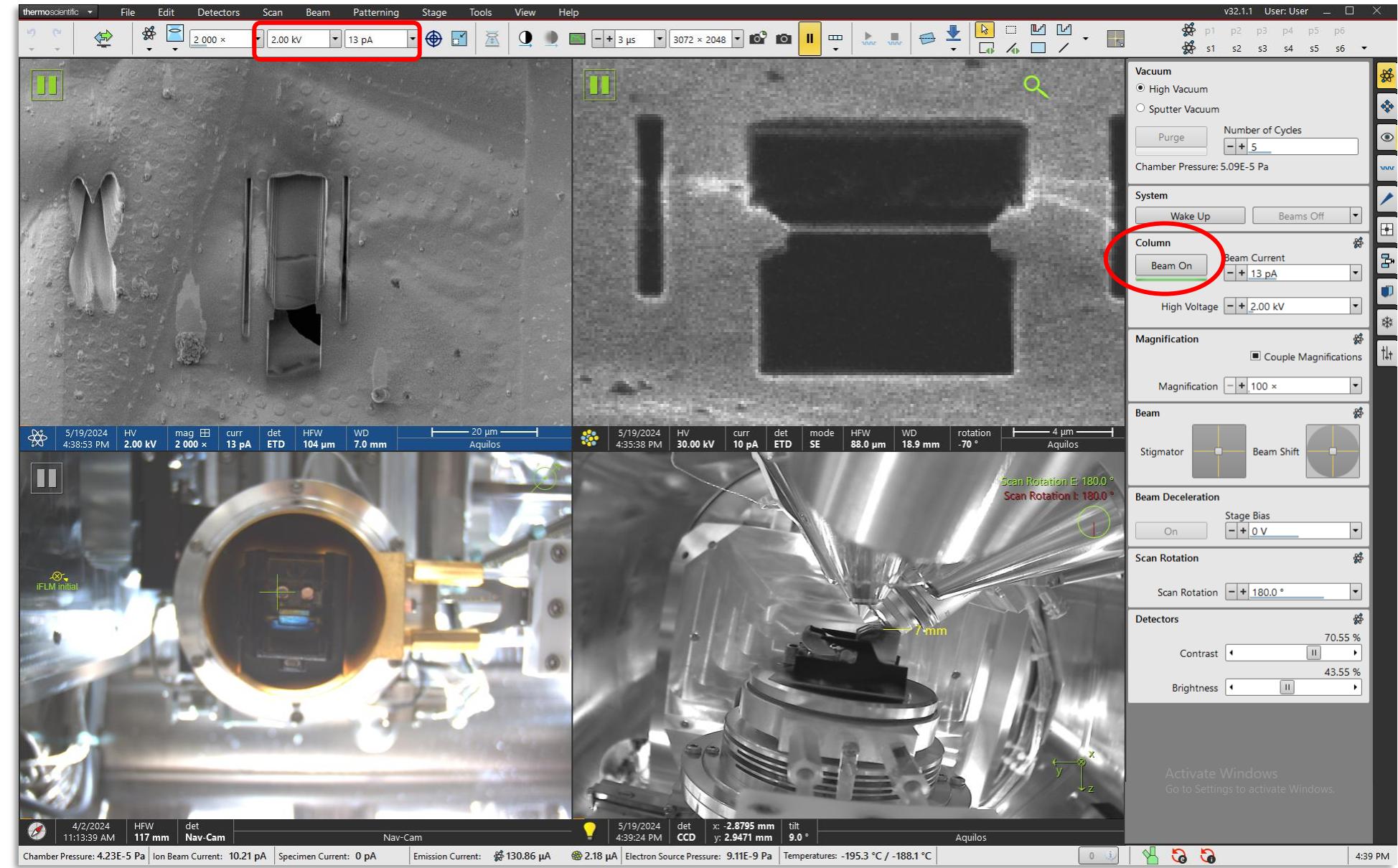
Target confirmation

Pt sputter (Optional)

Lamella conductivity

T

CryoET



3.3 Sample screening _Link Z to FWD

(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)

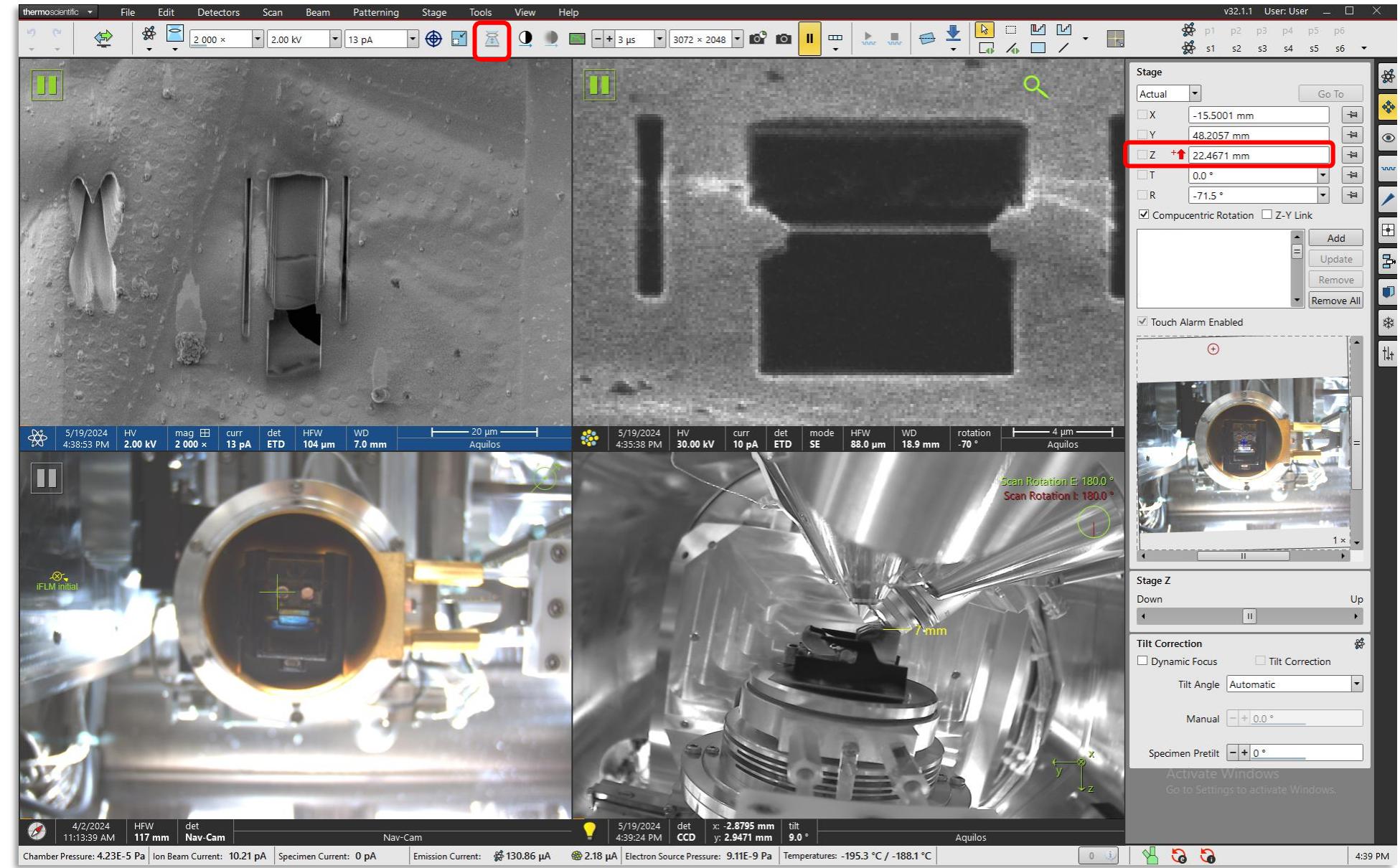
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.3 Sample screening _Quickly check the grids

(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)

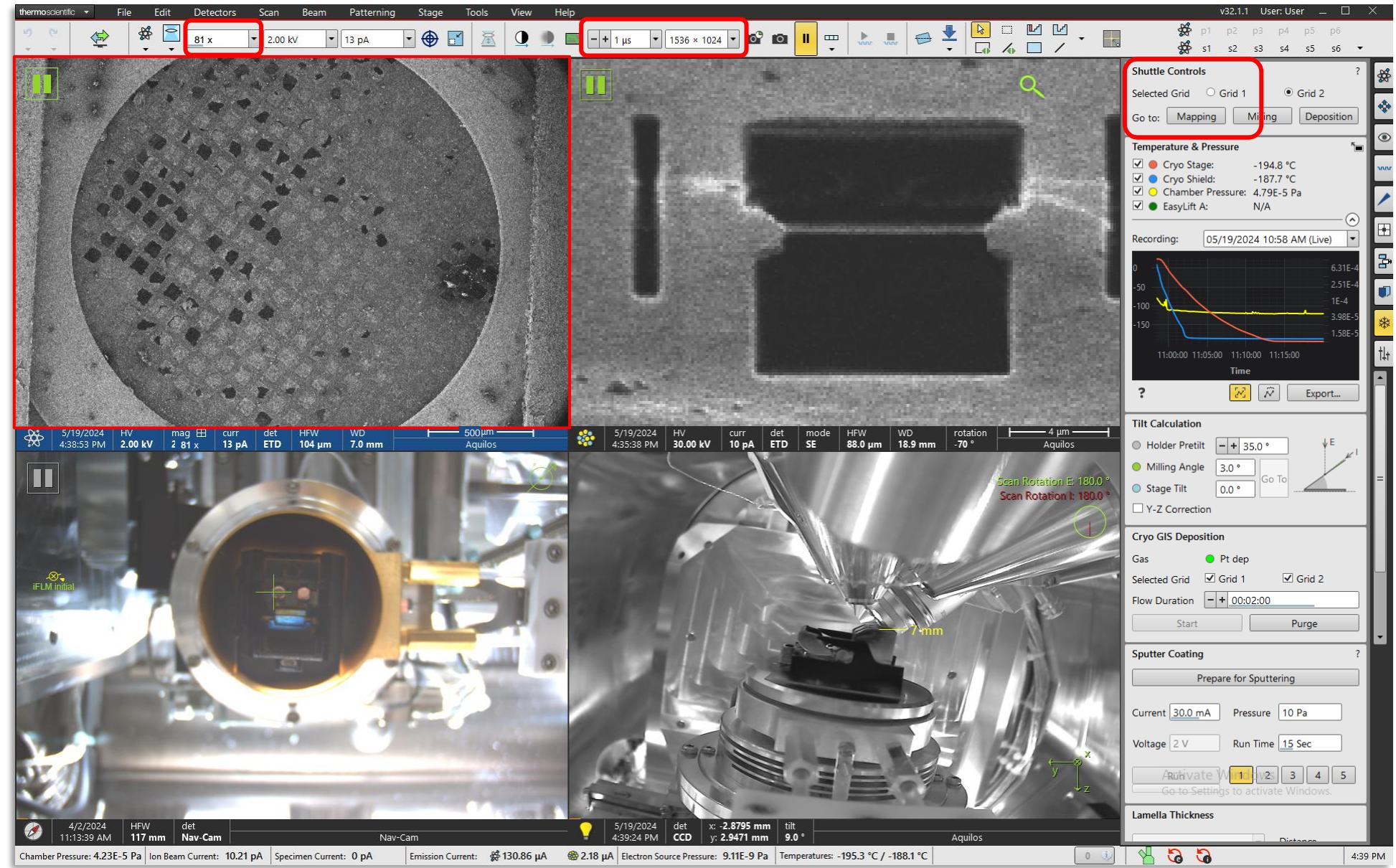
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.3 Sample screening _Create a Maps Project

(Ma) Maps

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)

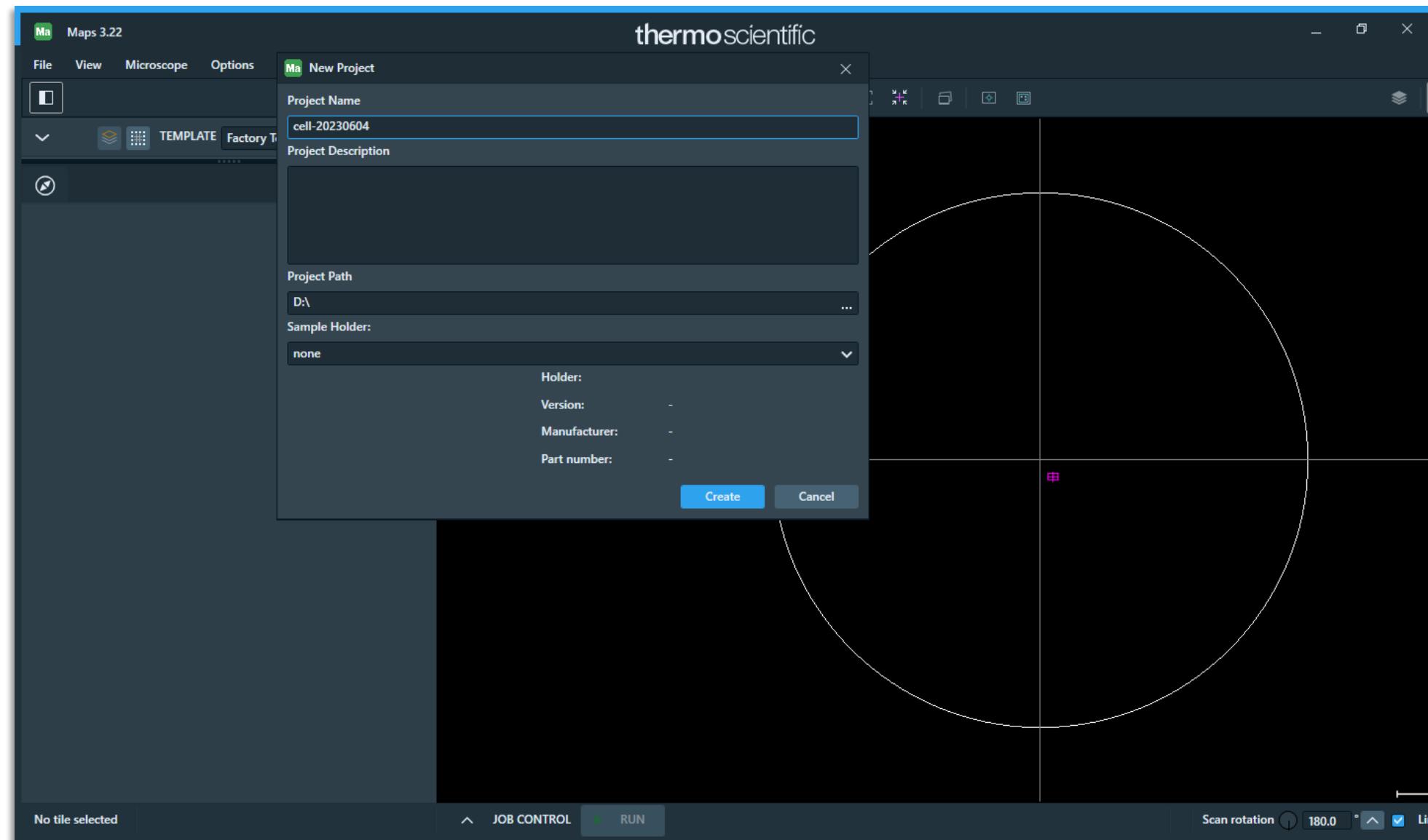
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.3 Sample screening _Take a snapshot of the grid

(Ma Maps)

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)

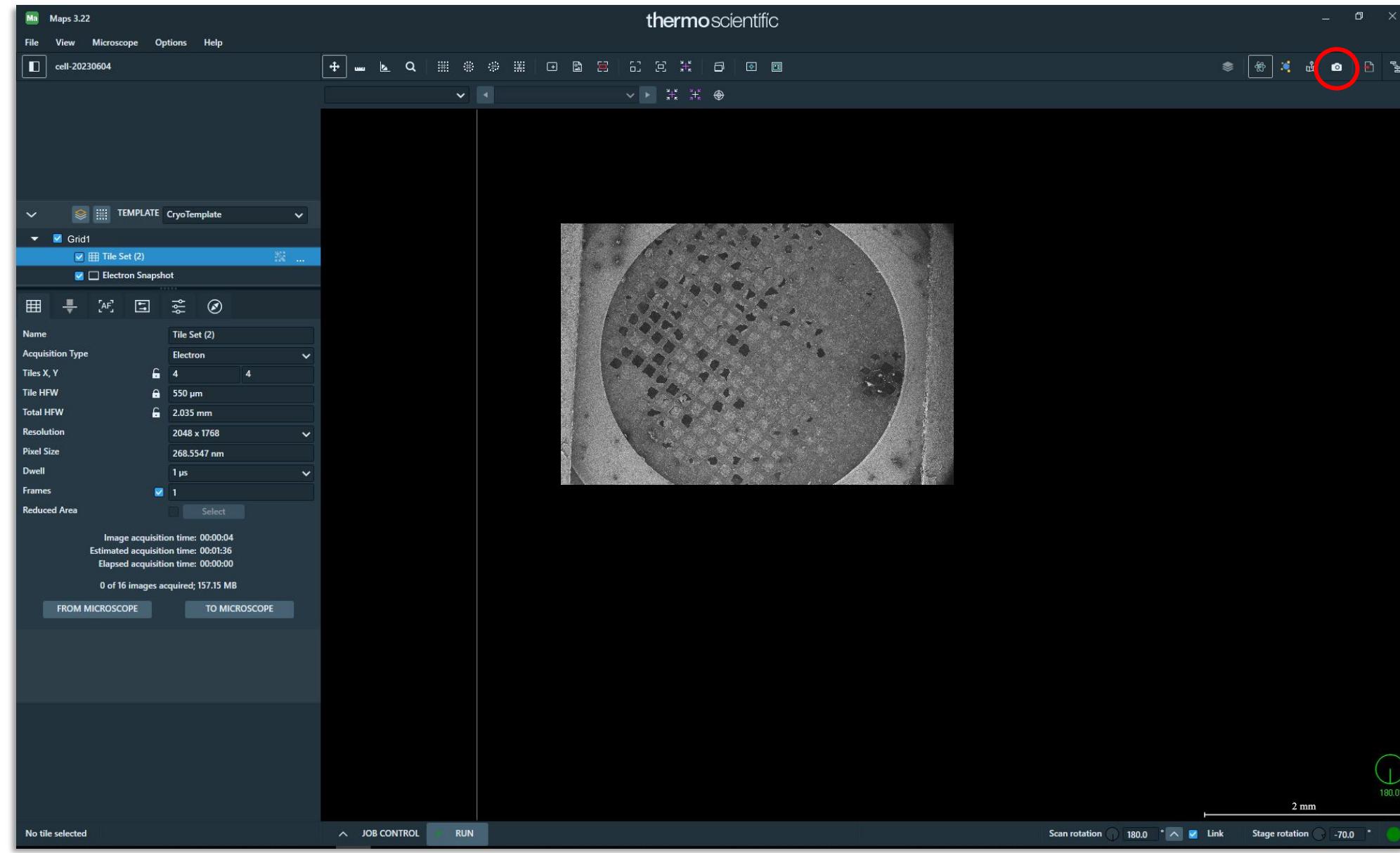
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.3 Sample screening _Set up Tile Set & Run atlas acquisition

(Ma Maps)

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)

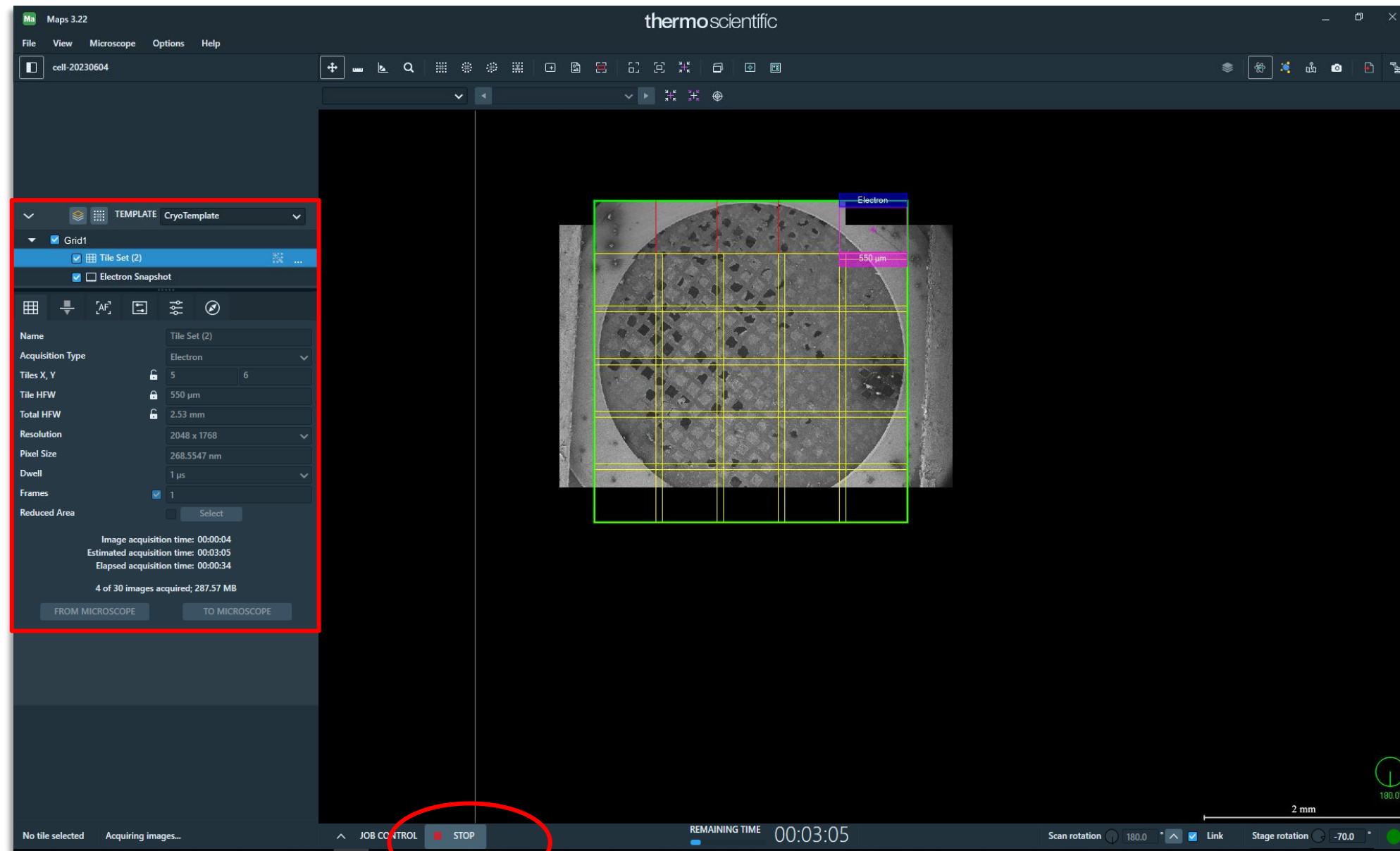
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.3 Sample screening _Add candidate lamella sites

(Ma Maps)

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)

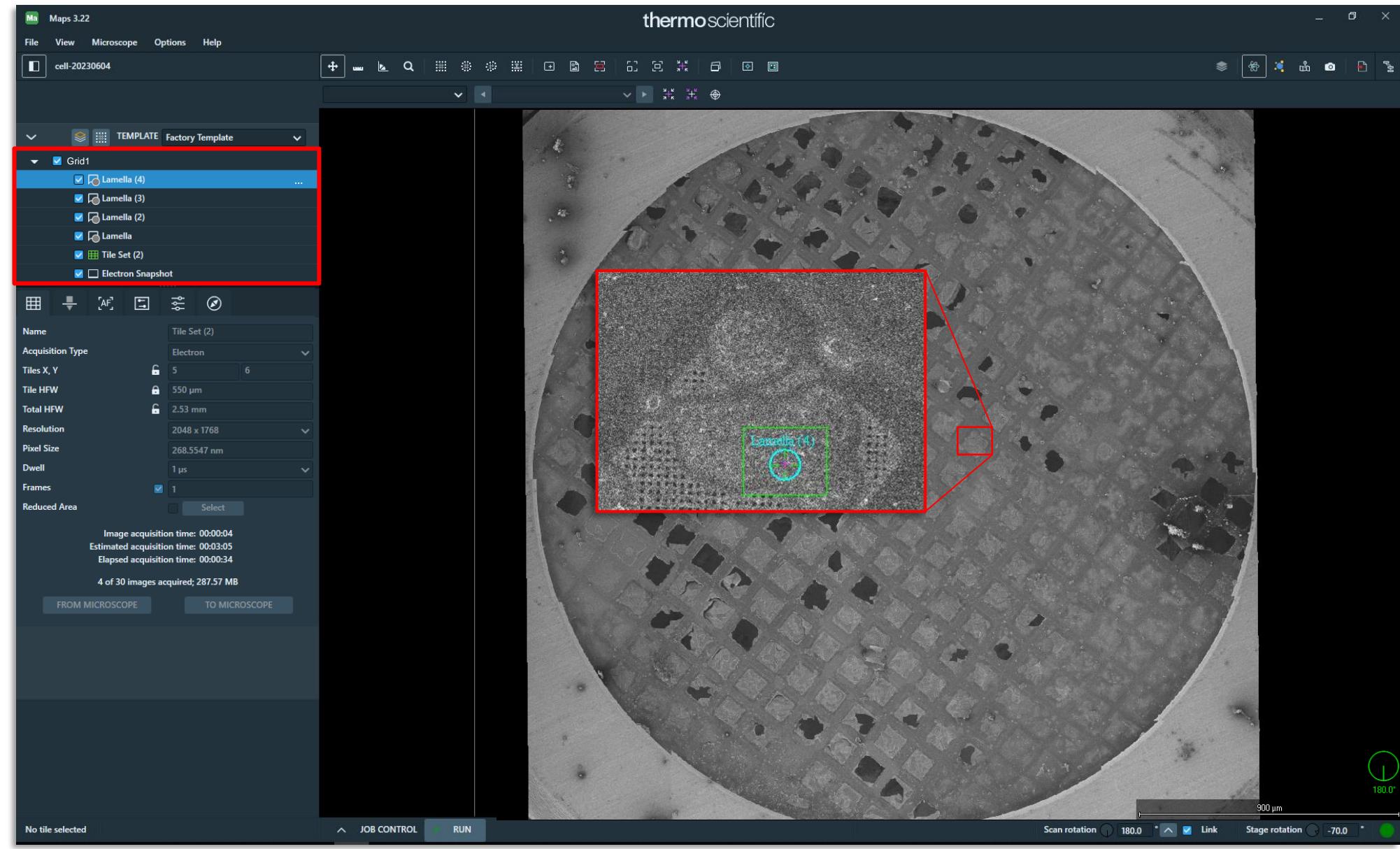
Target confirmation

Pt sputter (Optional)

Lamella conductivity



CryoET



3.4 CryoFLM for tart selection

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

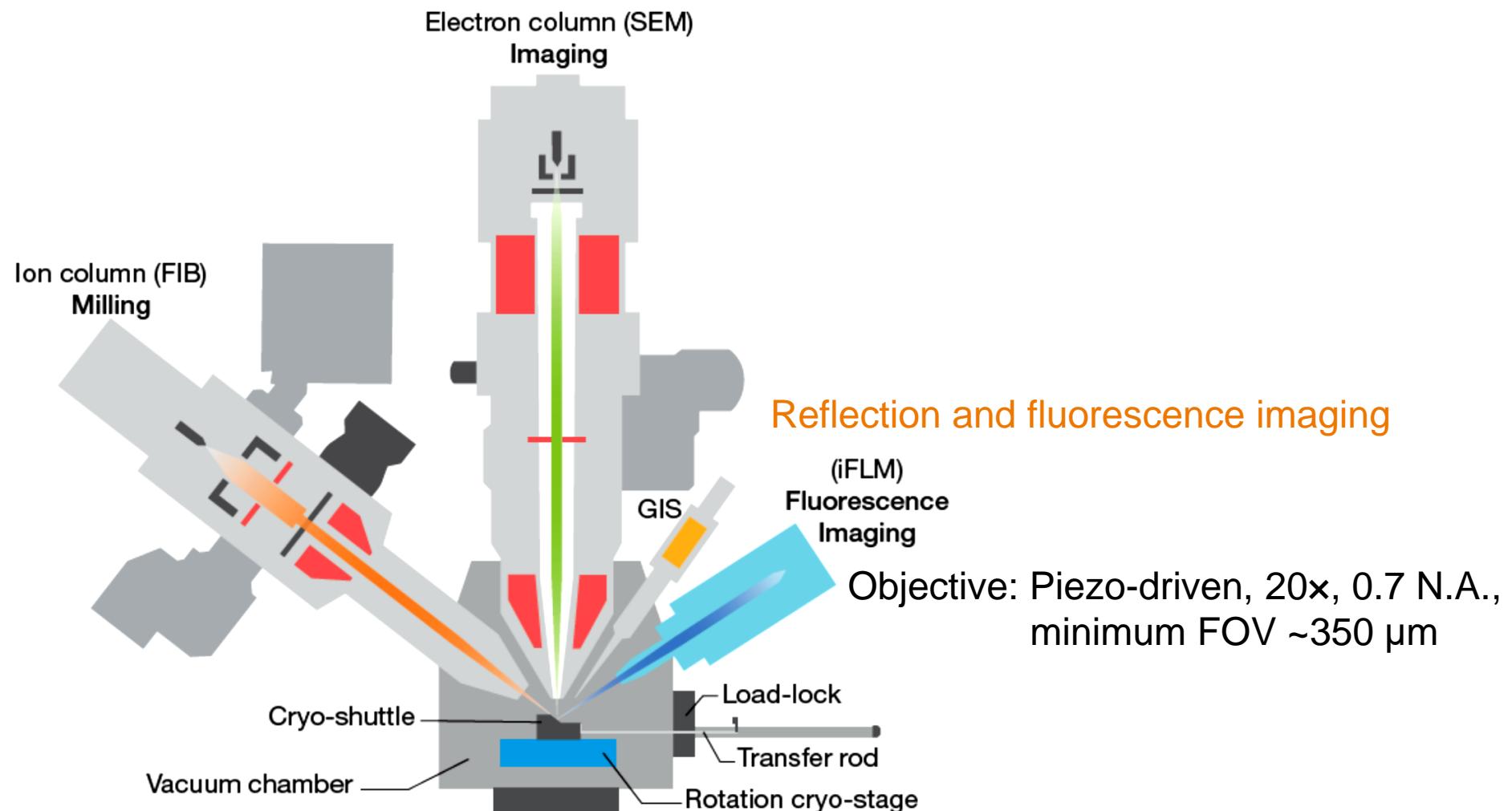
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T
CryoET



Reflection and fluorescence imaging

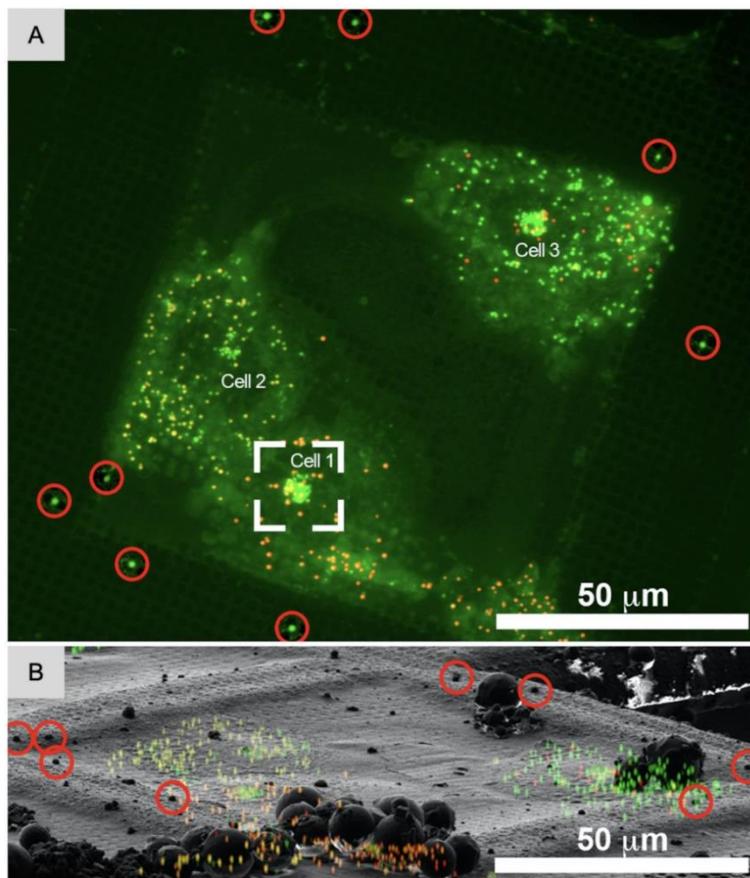
(iFLM)
Fluorescence
Imaging

Objective: Piezo-driven, 20x, 0.7 N.A.,
minimum FOV ~350 μm

Adapted from TFS

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

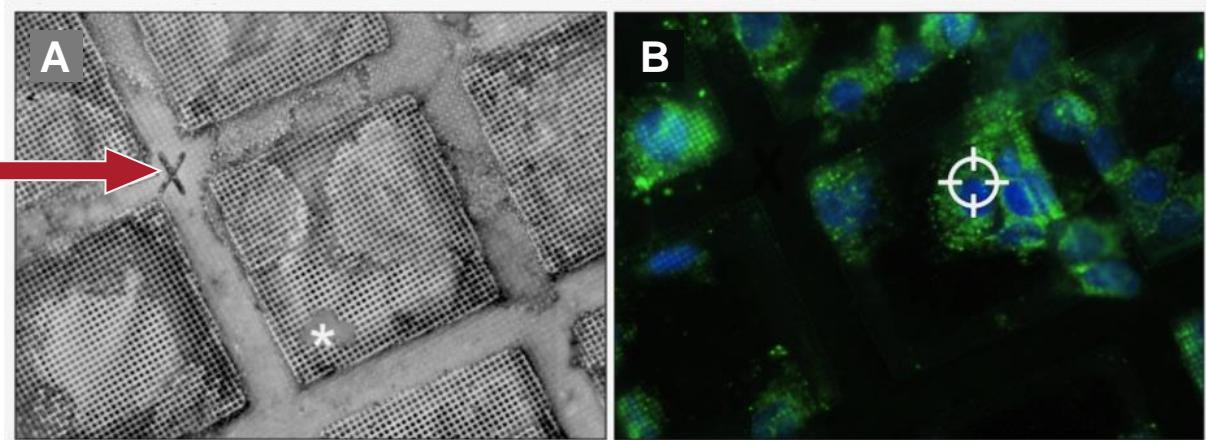
1- μ m Magnetic beads



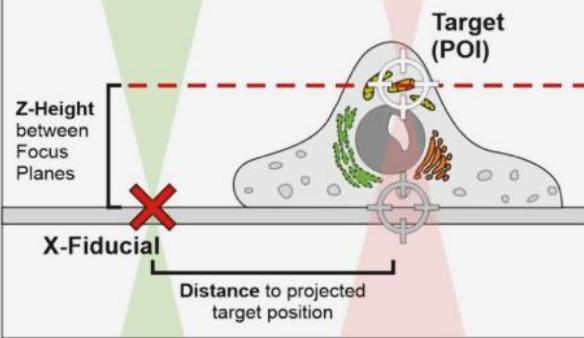
Arnold et al., Biophys J 2016

X-Fiducial

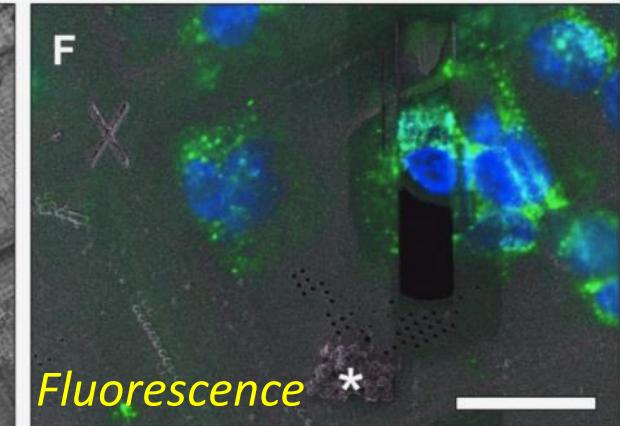
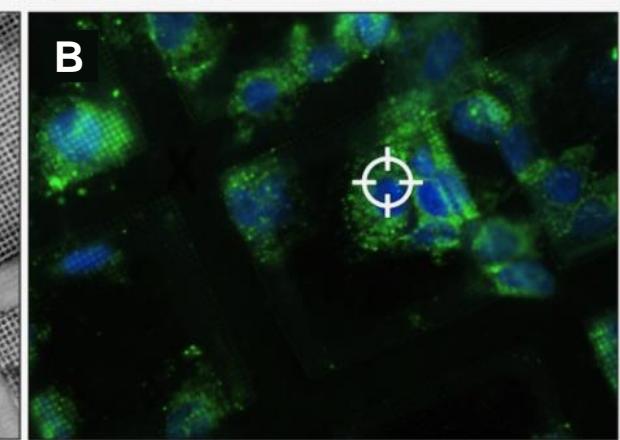
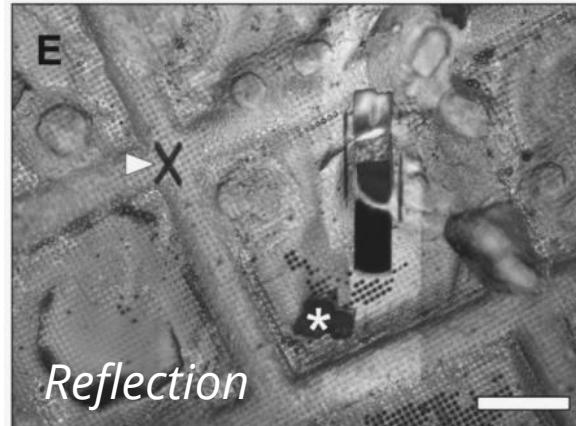
Before
milling



C



After
milling



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

3.4 CryoFLM _Focus with Objective



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

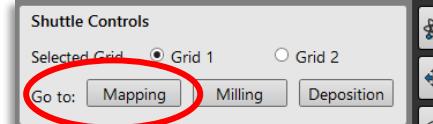
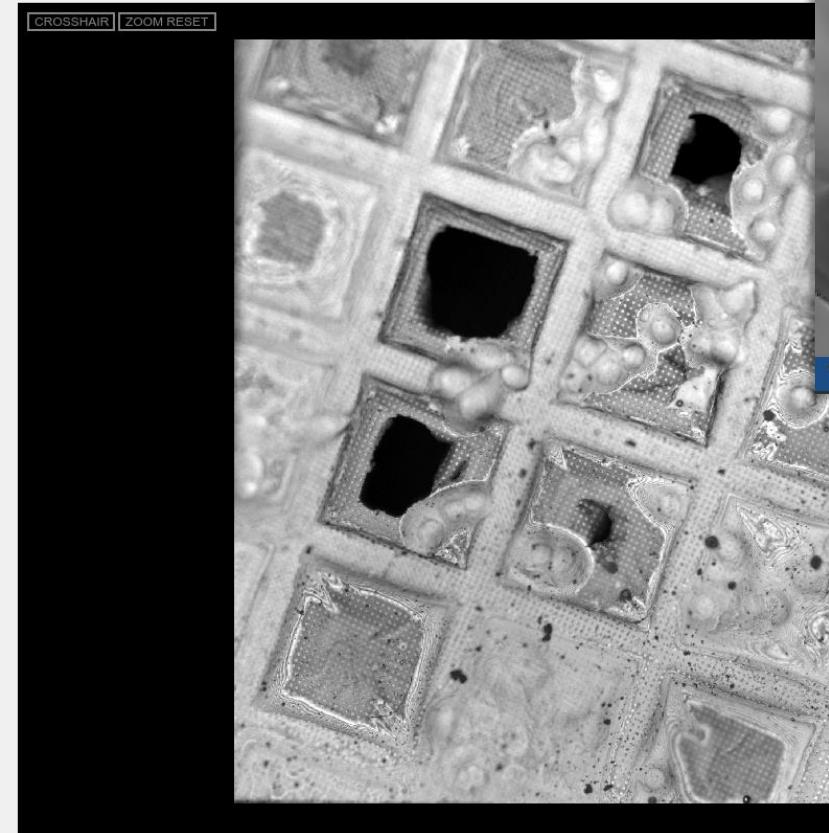
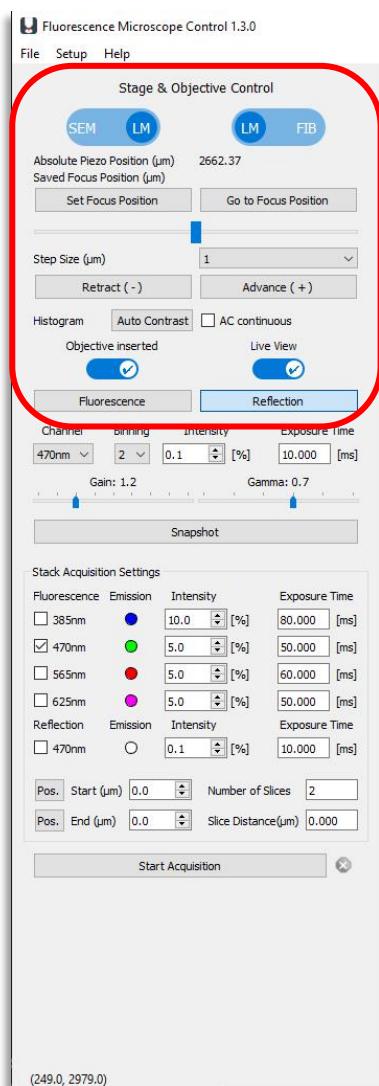
iFLM (Optional)
Target confirmation

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

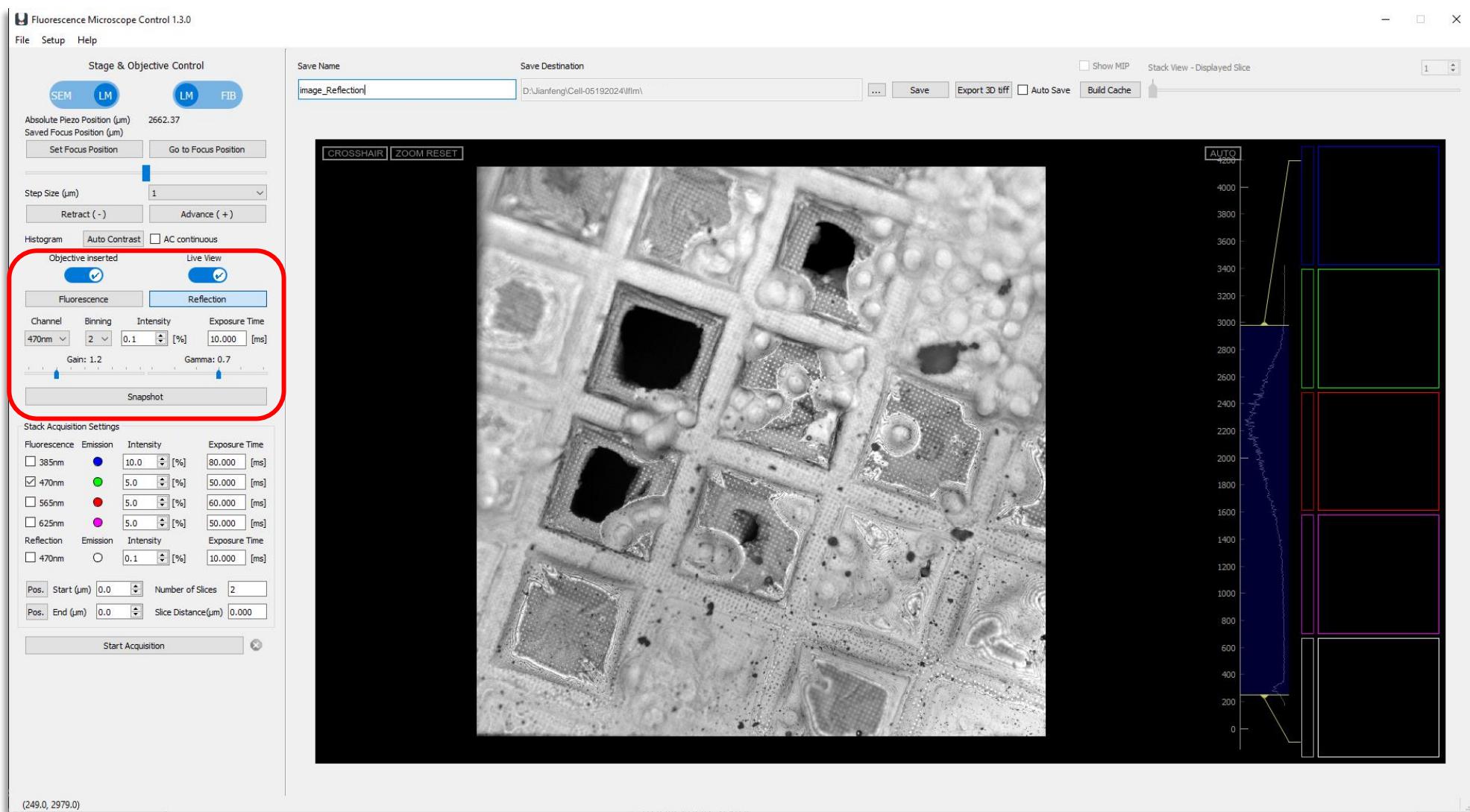
iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ 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

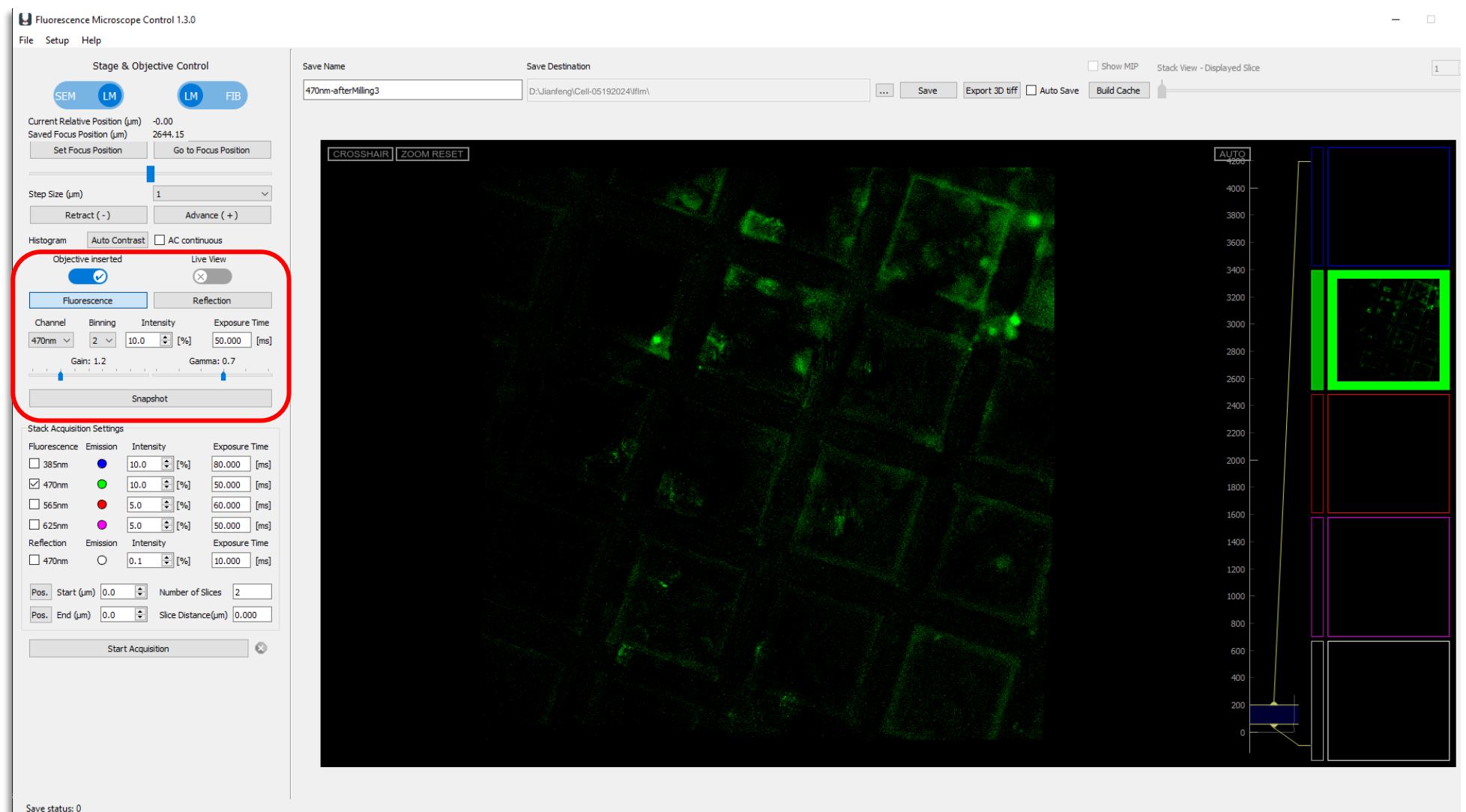
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)

Lamella conductivity

↓ T
CryoET



3.4 CryoFLM _Setting up Z-stack



Fluorescence Microscope Control)

Vitrification



CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

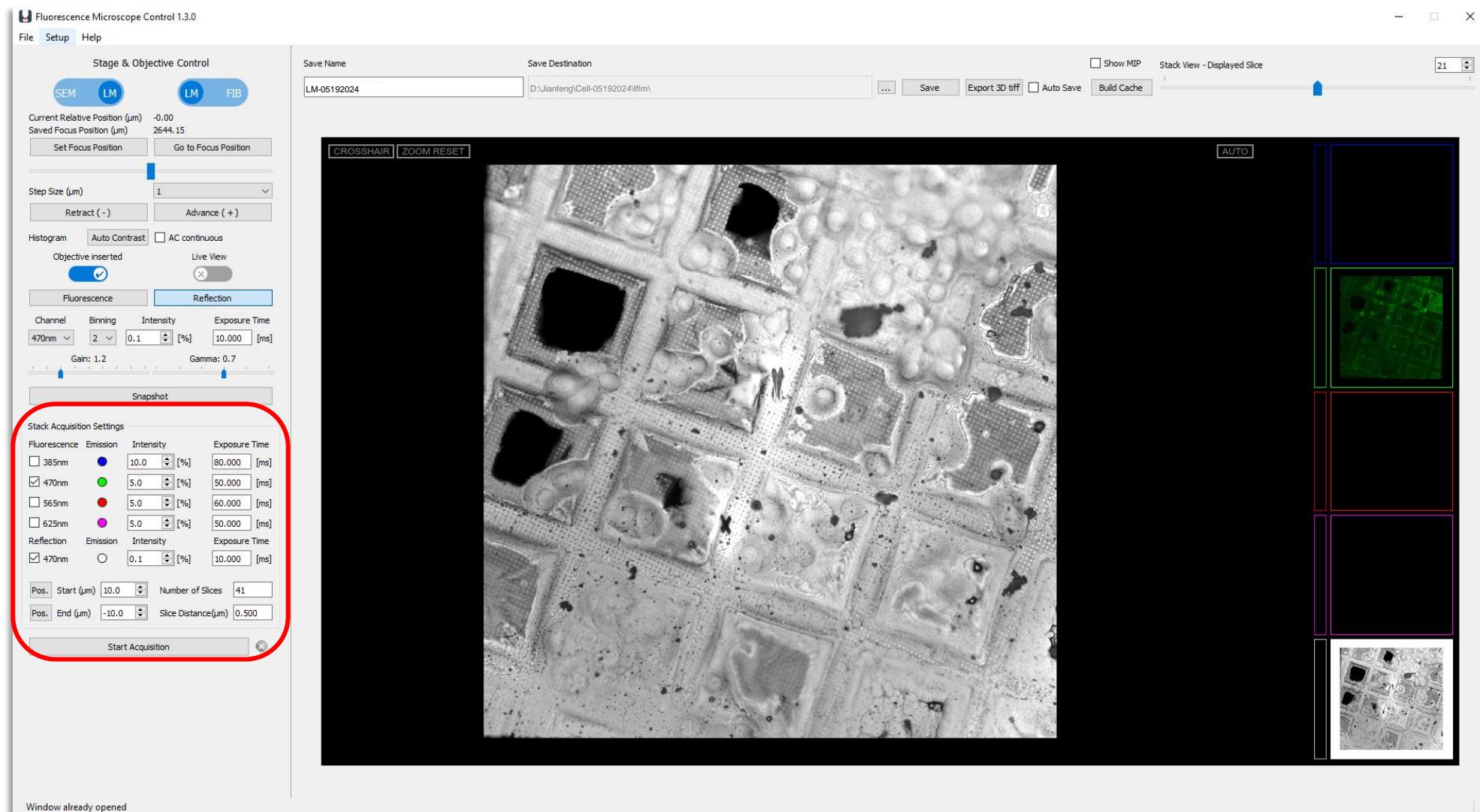
Pt sputter (Optional)
Sample conductivity

Lamella milling
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

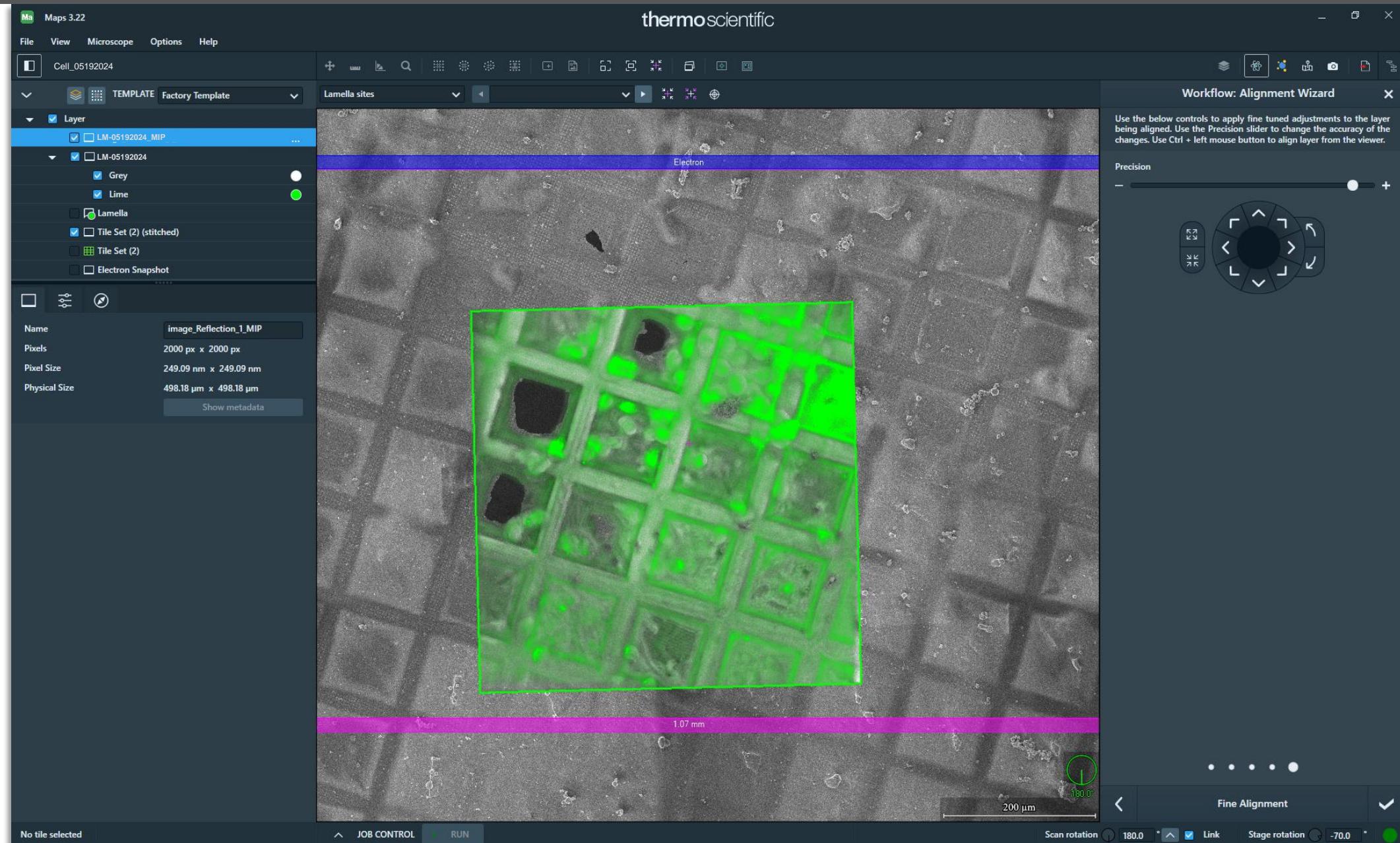
Pt sputter (Optional)
Lamella conductivity

↓
T
CryoET



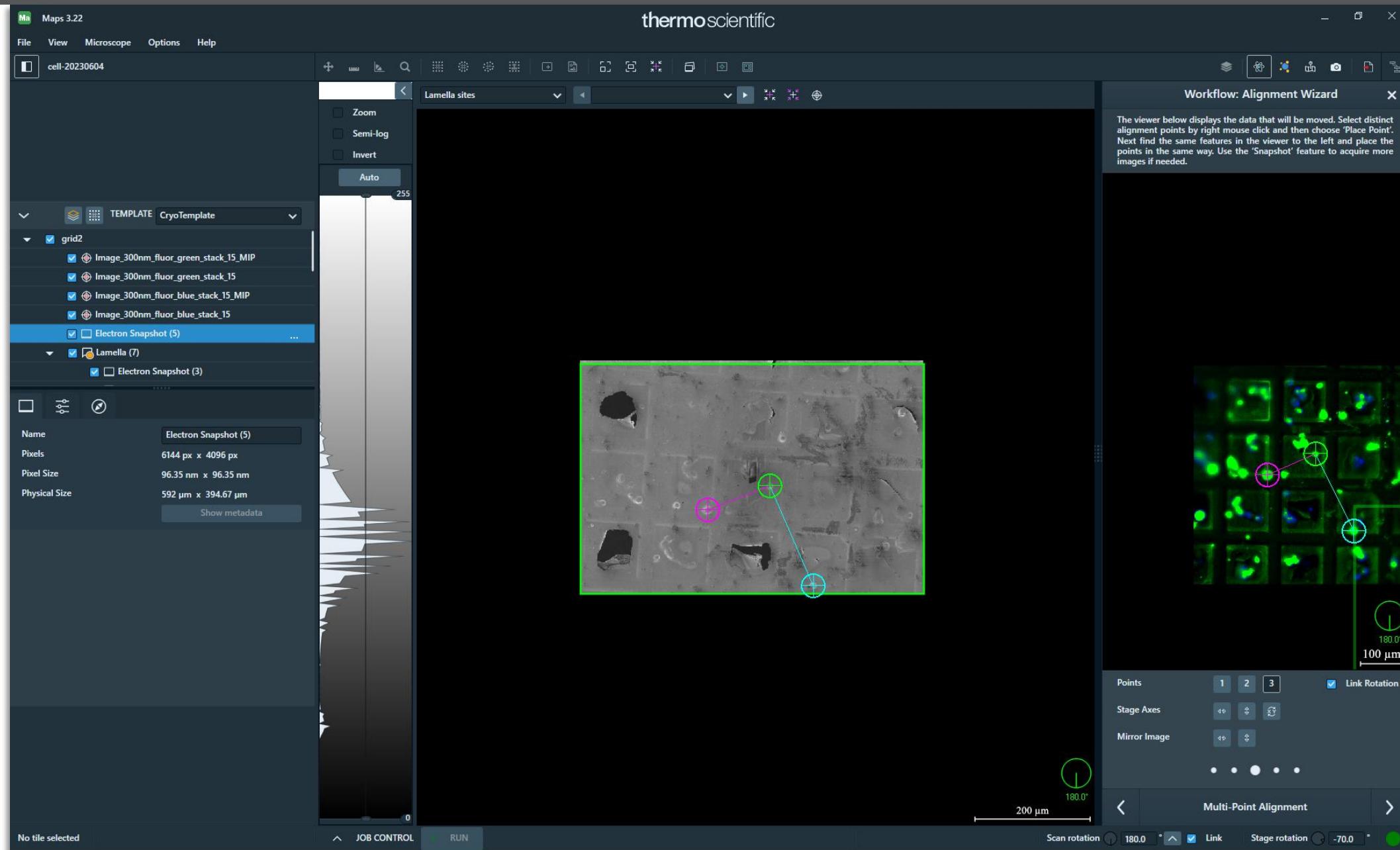
3.4 CryoFLM _FLM-SEM alignment by Fine Alignment

(Ma Maps)



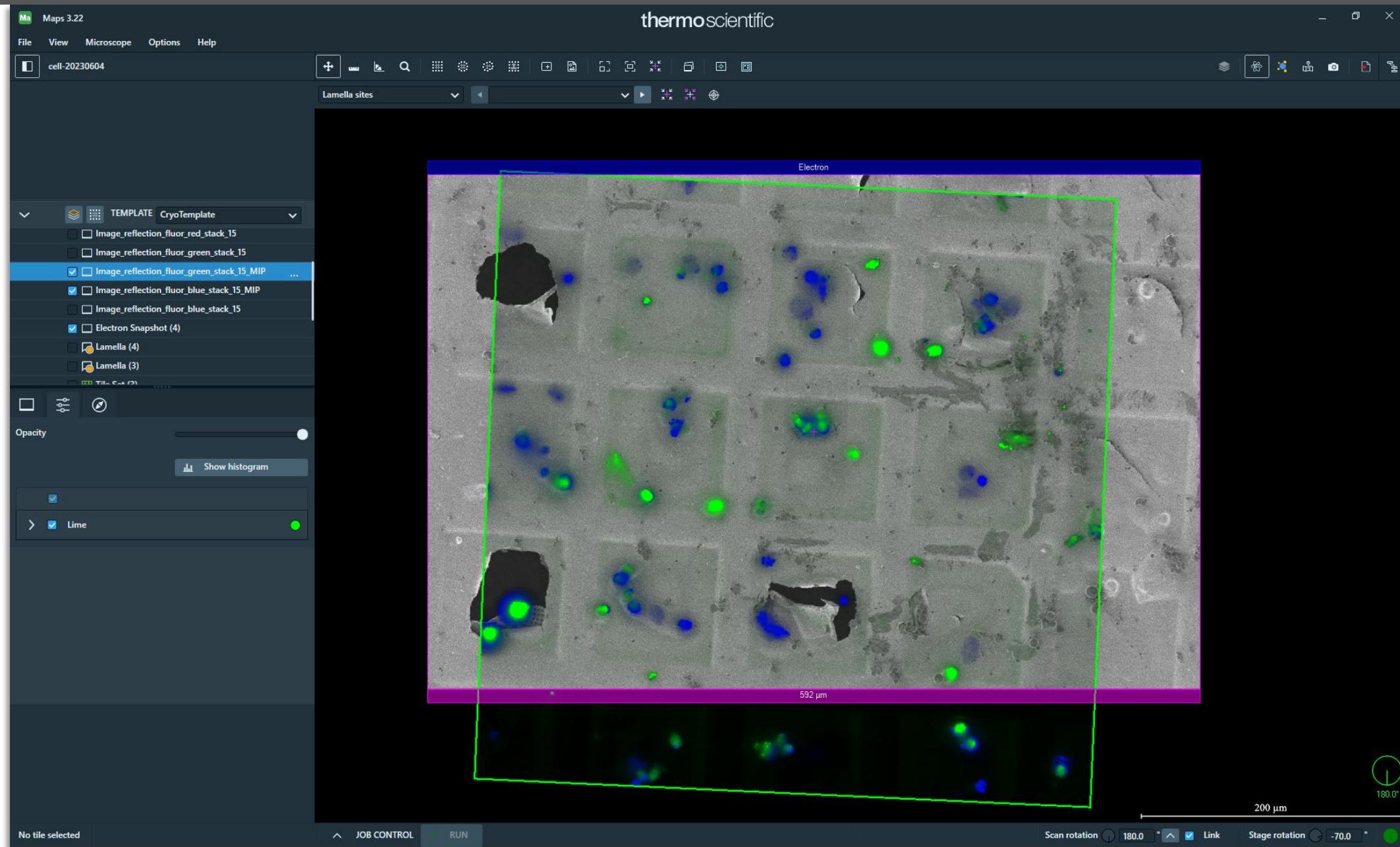
3.4 CryoFLM _FLM-SEM alignment by Multi-Point Alignment

(Ma) Maps



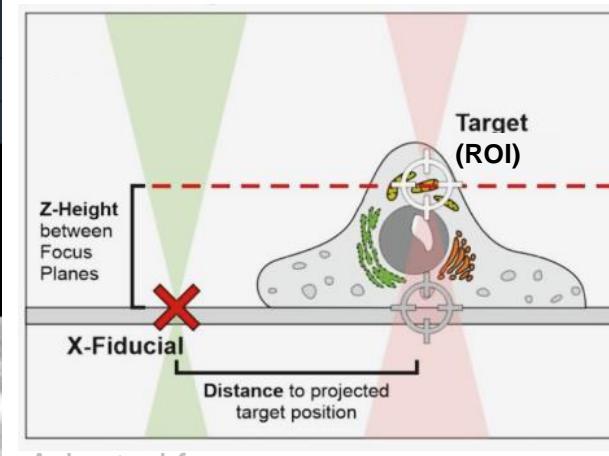
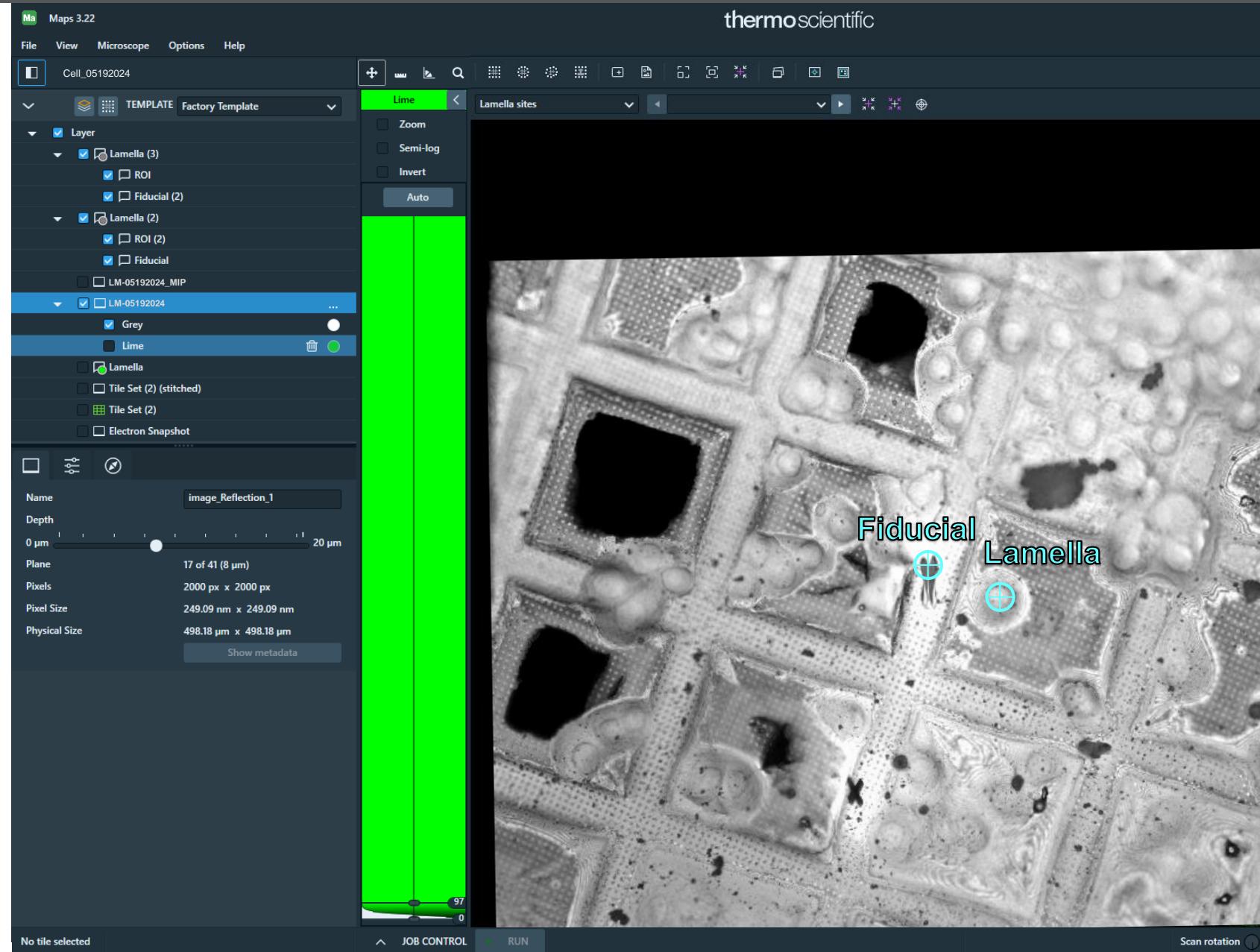
3.4 CryoFLM _FLM-SEM alignment by Multi-Point Alignment

(Maps)



3.4 CryoFLM _Define Fiducial & ROI markers for each lamella

(Ma) Maps

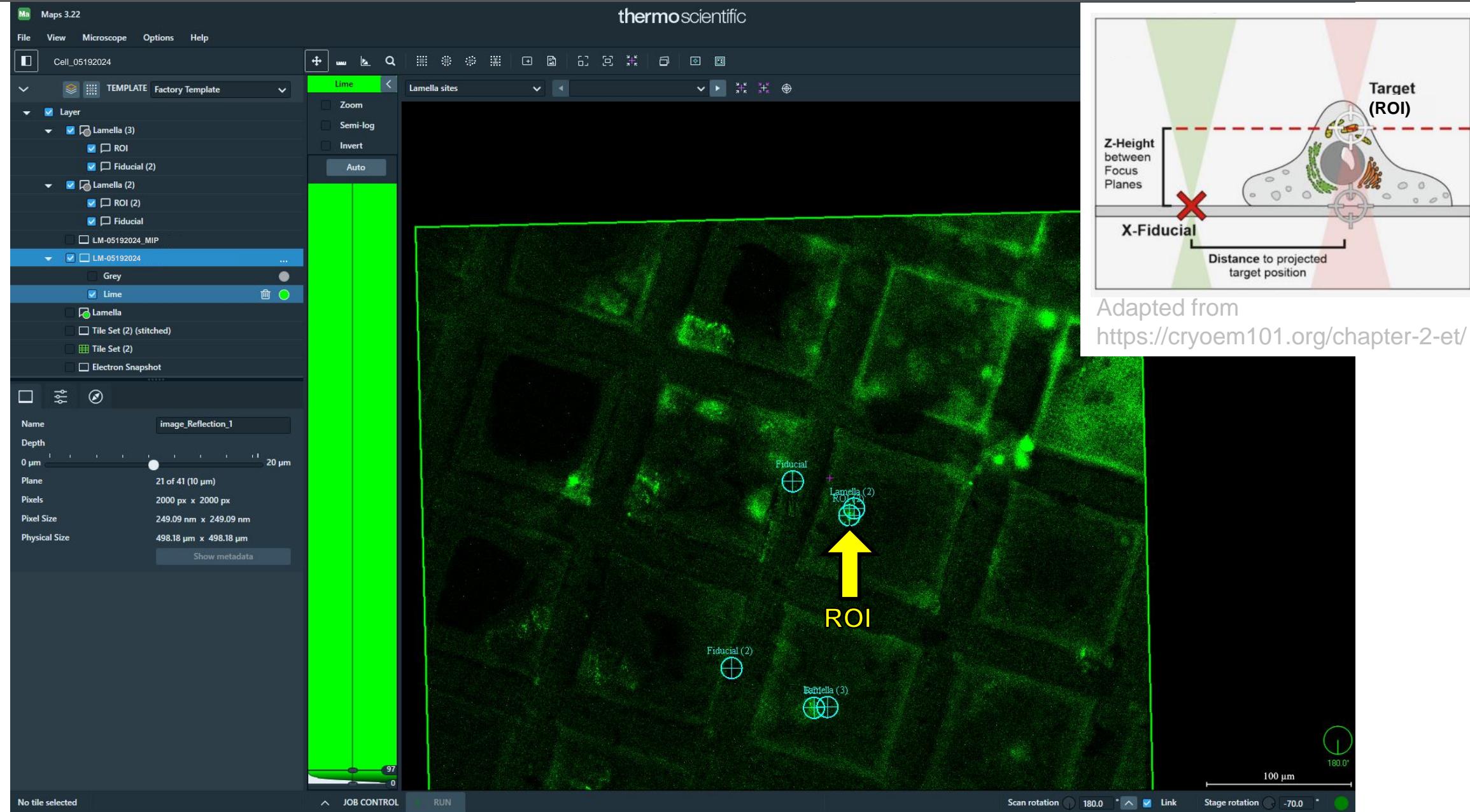


Adapted from
<https://cryoem101.org/chapter-2-et/>



3.4 CryoFLM _Define Fiducial & ROI markers for each lamella

(Ma Maps)



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)

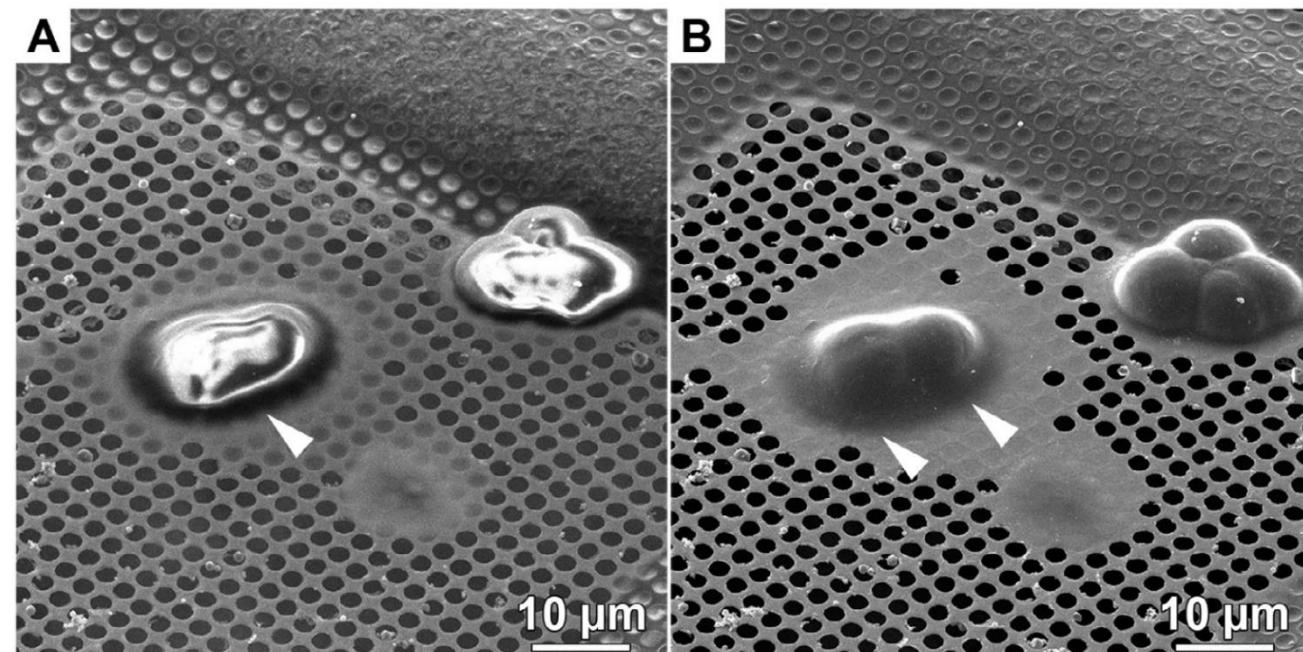
Target confirmation

Pt sputter (Optional)

Lamella conductivity



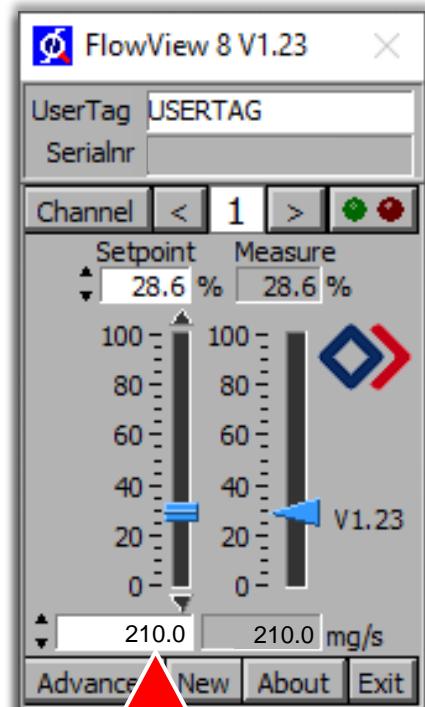
→ (Optional) Inorganic Pt minimizes charging, ensure targeting and precise milling.



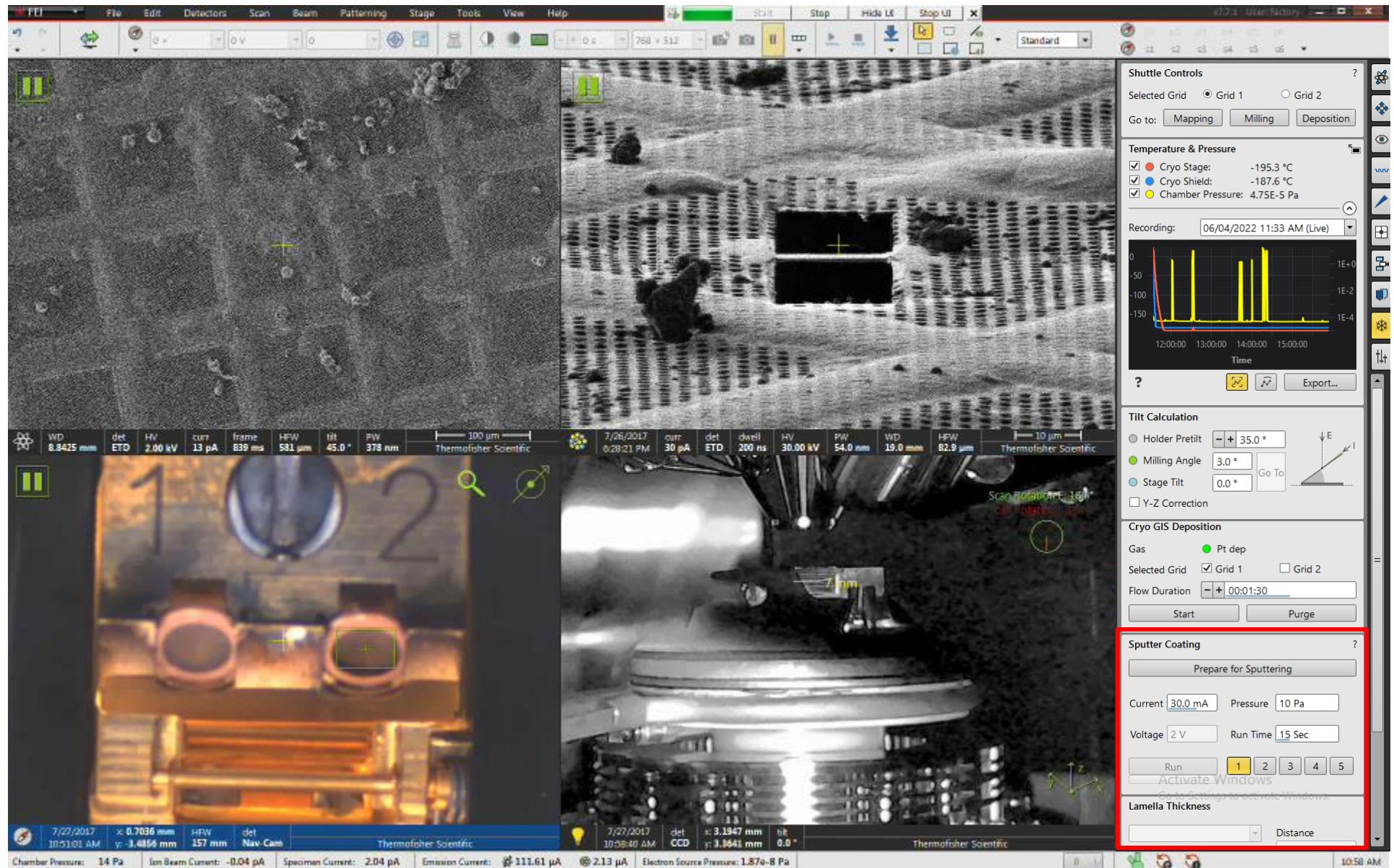
Schaffer et al., JSB 2017

3.5 Pt sputter

(Video from TFS;  Microscope Control)



Increase to 210 mg/s
(varies by instruments)



3.5 Pt GIS

( 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

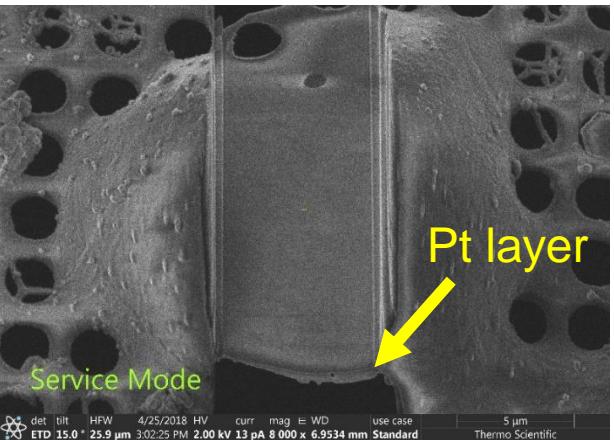
iFLM (Optional)

Target confirmation

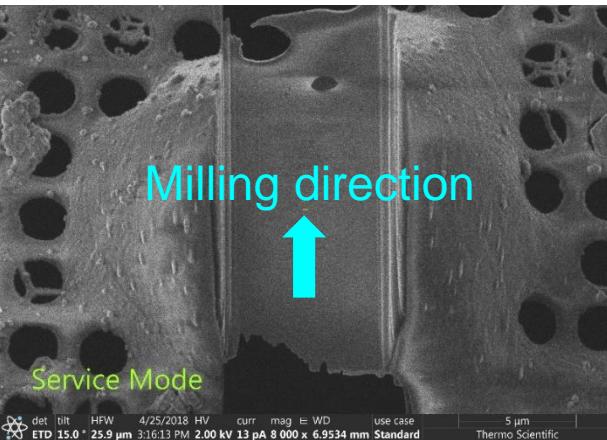
Pt sputter (Optional)

Lamella conductivity

↓
T
CryoET



With an intact Pt layer



With a broken Pt layer

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

Cryo GIS Deposition

Gas Pt dep

Selected Grid Grid 1 Grid 2

Flow Duration - + 00:02:00

Start Purge

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 ° Go To
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

Activate Windows 1 2 3 4 5
Go to Settings to activate Windows.

Lamella Thickness

Distance

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

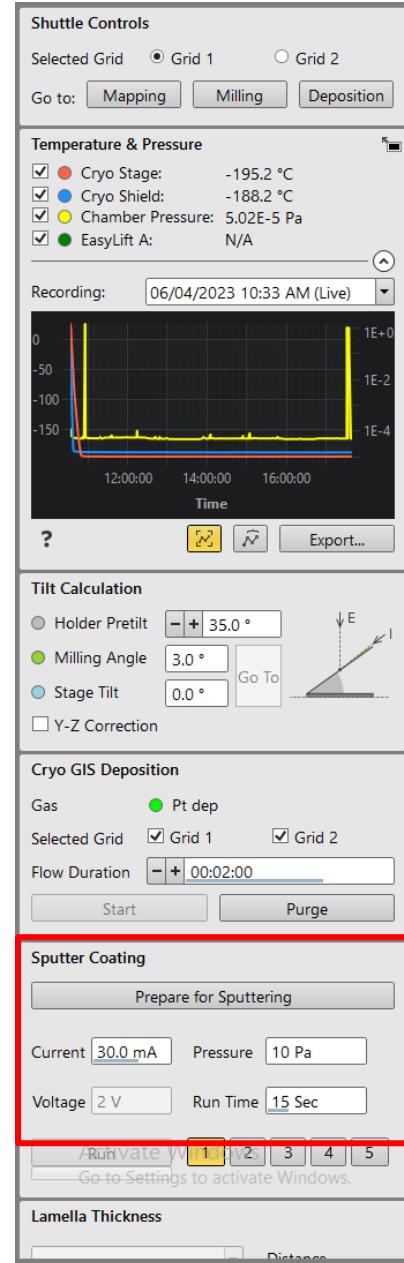
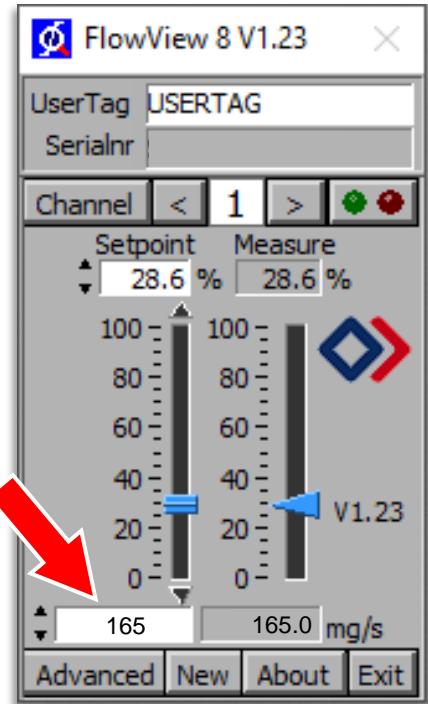
Pt sputter (Optional)

Lamella conductivity



CryoET

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

The screenshot shows the AutoTEM Cryo software interface with the following sections:

- Preparation:** Ion HFW Oversize: 30.0 μm, Milling Angle: Not Available.
- Milling:** Lamella Size: 10.0 μm x 3.0 μm, Correction Factor: 0.50.
- Thinning:** Final Thickness: 200.0 nm, Enable Windows: checked.
- EUCENTRIC TILT:** Maximal Tilt Step: 10.0°, Preparation HFW: 250.0 μm, Resolution (guided): 1536 x 1024.
- ARTIFICIAL FEATURES:** HFW: 200.0 μm, Distance from lamella: 7.0 μm, Pattern Depth: 1.0 μm, Cross Thickness: 300.0 nm, Milling Current: 0.30 nA, Cross Size: 8.0 μm x 8.0 μm.
- MILLING ANGLE:** Target Milling Angle: 9.0°, Clearance Angle: 2.0°, Enforce Target: checked, HFW: 160.0 μm.
- IMAGE ACQUISITION:** Ion HFW Oversize: 120 %, Resolution: 1536 x 1024 @ 4 μs, Enable ACB: checked, Enable Auto Focus: checked.
- LAMELLA PLACEMENT:** Ion HFW Oversize: 120 %.
- REFERENCE DEFINITION:** Electron Reference Definition: checked.
- STRESS RELIEF CUTS:** Trench Width: 1.0 μm, Trench Depth: 10.0 μm, Trench Height: 6.5 μm, Trench Offset: 5.0 μm, Depth Correction: 100.0 %, Milling Current: 0.50 nA, DCM Rescan Interval: 120 s, Number Of Patterns: 1, Show Graphics: checked.
- REFERENCE REDEFINITION 1:** checked.
- ROUGH MILLING:** Pattern Offset: 1.0 μm, Front Pattern Height: 5.0 μm, Rear Pattern Height: 5.0 μm, Depth Correction: 120 %, Front Width Overlap: 1.5 μm, Rear Width Overlap: 1.0 μm, Milling Current: 1.0 nA, Pattern Type: Rectangle, DCM Rescan Interval: 120 s.
- POLISHING 1 - ELECTRON IMAGE:** Resolution: 1536 x 1024 @ 3 μs, Enable ACB: checked, Enable Auto Focus: checked, HFW: 70.0 μm, Add lamella to HFW: checked, Notification: checked, High Voltage: 2 kV, Beam Current: 13 pA.
- POLISHING 1:** Pattern Offset: 150.0 nm, Overtilt: 0°, Depth Correction: 160.0 %, High Voltage: 30 kV, Milling Current: 50 pA, DCM Rescan Interval: 30 s, Pattern Overlay: 200.0 %, Pattern Type: CleaningCrossSection.
- POLISHING 2:** Pattern Offset: 0 μm, Overtilt: 0°, Depth Correction: 160.0 %, High Voltage: 30 kV, Milling Current: 30 pA, DCM Rescan Interval: 30 s.

2.4.3 (core 10.0.7.70)

Eucentric height & tilt calculation

(AT) AutoTEM Cryo)

Vitrification
↓ T
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

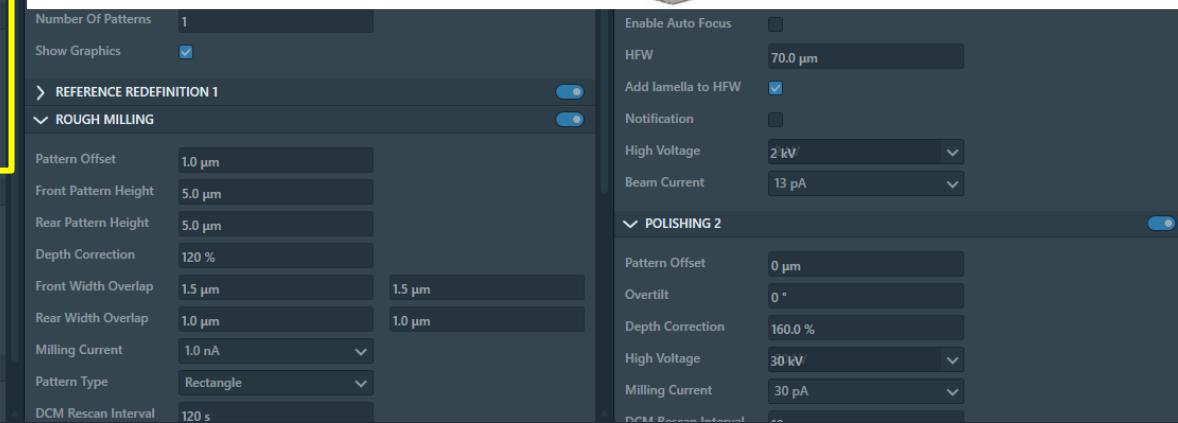
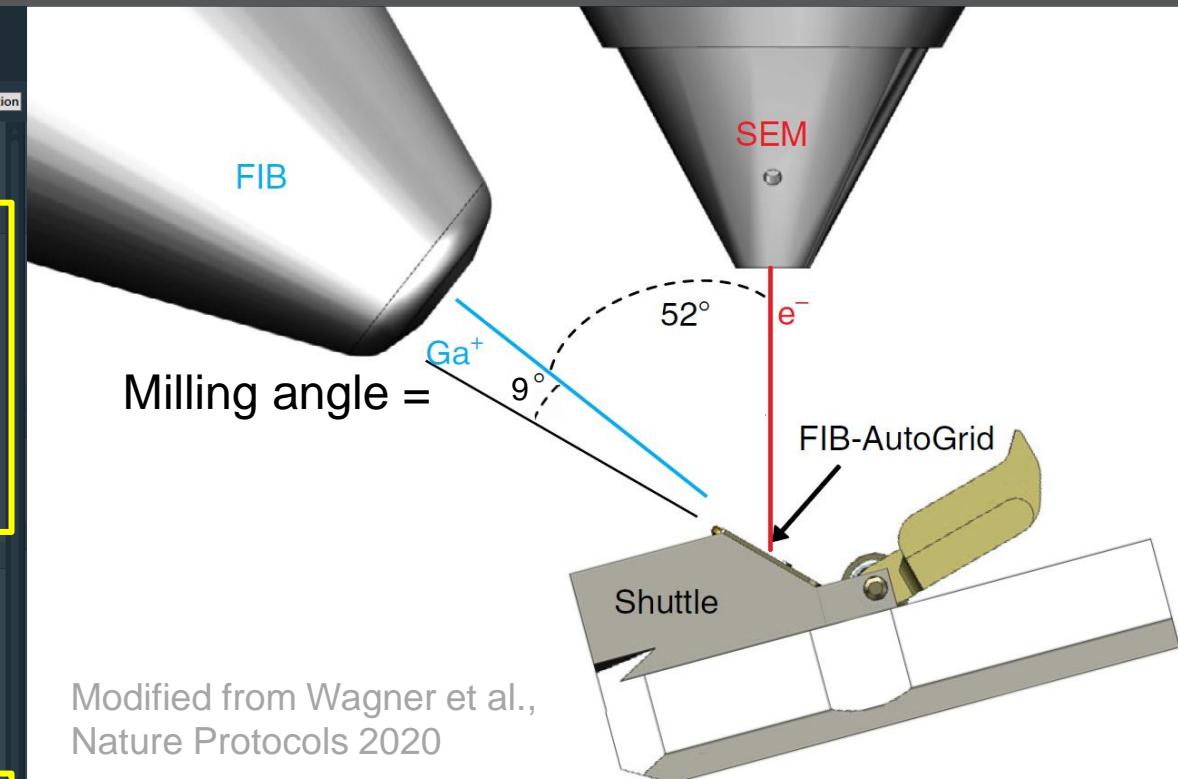
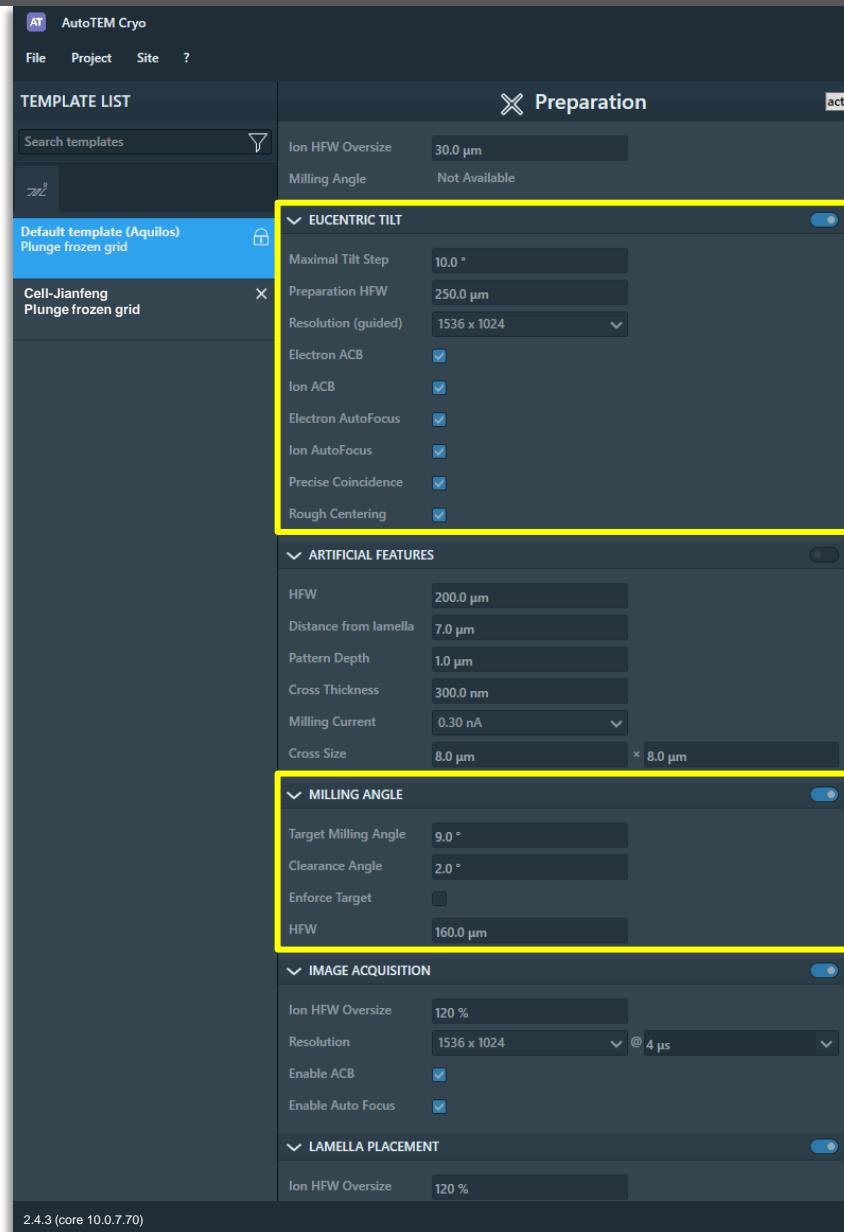
Pt sputter (Optional)
Sample conductivity

Lamella milling
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T
CryoET



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

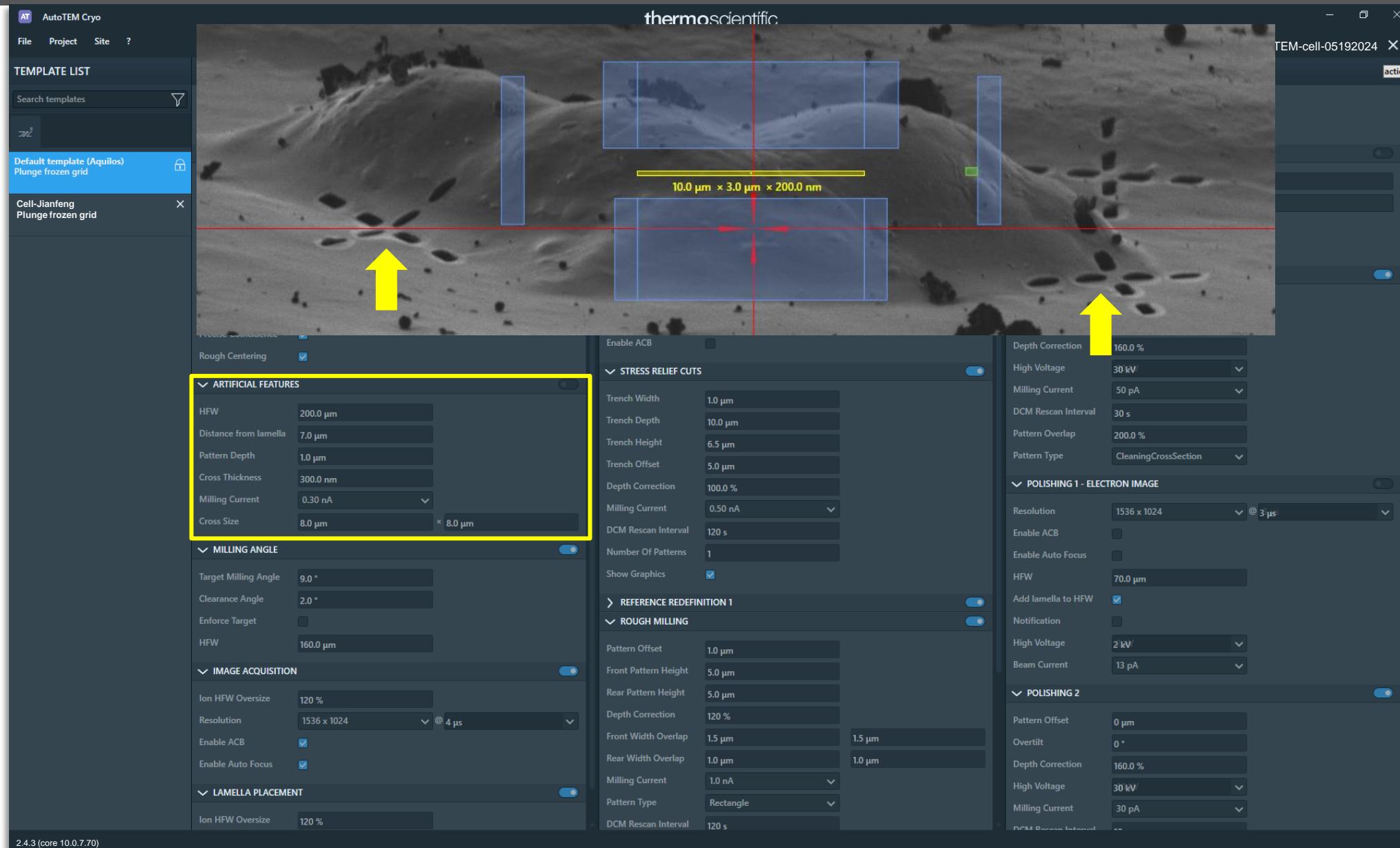
Pt sputter (Optional)
Sample conductivity

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

AutoTEM-cell-05192024

action

AutoTEM Cryo

File Project Site ?

TEMPLE LIST

Search templates

Ion HFW Oversize 30.0 μm

Milling Angle Not Available

EUCENTRIC TILT

Maximal Tilt Step 10.0 °

Preparation HFW 250.0 μm

Resolution (guided) 1536 x 1024

Electron ACB

Ion ACB

Electron AutoFocus

Ion AutoFocus

Precise Coincidence

Rough Centering

ARTIFICIAL FEATURES

HFW 200.0 μm

Distance from lamella 7.0 μm

Pattern Depth 1.0 μm

Cross Thickness 300.0 nm

Milling Current 0.30 nA

Cross Size 8.0 μm x 8.0 μm

MILLING ANGLE

Target Milling Angle 9.0 °

Clearance Angle 2.0 °

Enforce Target

HFW 160.0 μm

IMAGE ACQUISITION

Ion HFW Oversize 120 %

Resolution 1536 x 1024 @ 4 μs

Enable ACB

Enable Auto Focus

LAMELLA PLACEMENT

Ion HFW Oversize 120 %

Enable Auto Focus

STRESS RELIEF CUTS

Enable Auto Focus

Enable ACB

Number Of Patterns 1

Show Graphics

Overtilt 0 °

Depth Correction 160.0 %

High Voltage 30 kV

Milling Current 50 pA

DCM Rescan Interval 30 s

Pattern Overlap 200.0 %

Pattern Type CleaningCrossSection

RESOLUTION 1 - ELECTRON IMAGE

Resolution 1536 x 1024 @ 3 μs

Enable ACB

Enable Auto Focus

HFW 70.0 μm

Add lamella to HFW

Notification

High Voltage 2 kV

Beam Current 13 pA

RESOLUTION 2 - POLISHING

Pattern Offset 0 μm

Overtilt 0 °

Depth Correction 160.0 %

High Voltage 30 kV

Milling Current 30 pA

DCM Rescan Interval 30 s

Wolff et al., JSB 2019

A

B

C

2.4.3 (core 10.0.7.70)

Stepwise milling procedure

(AT) AutoTEM Cryo)

Vitrification
↓ T
CryoFIB

Sample screening

Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

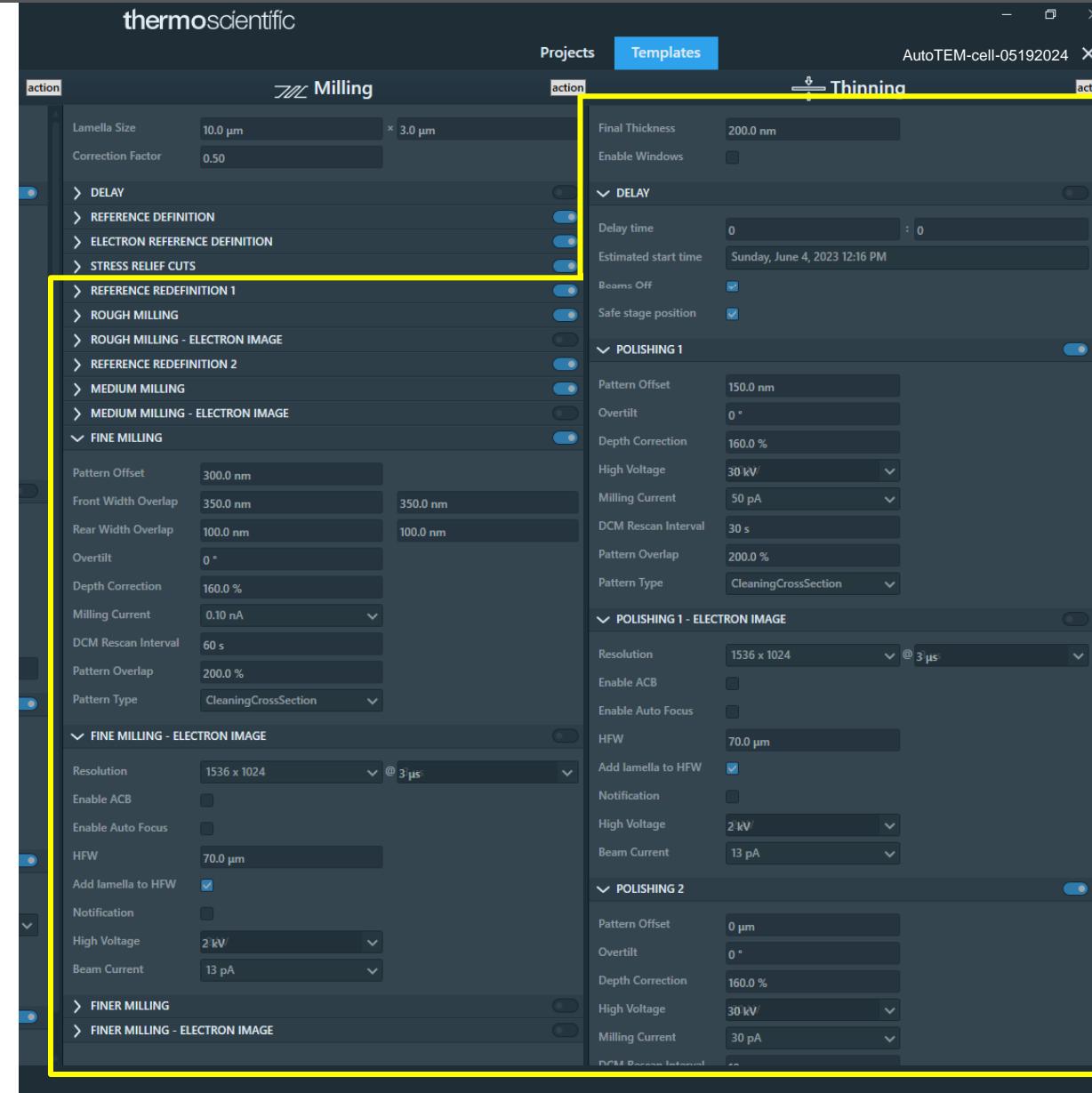
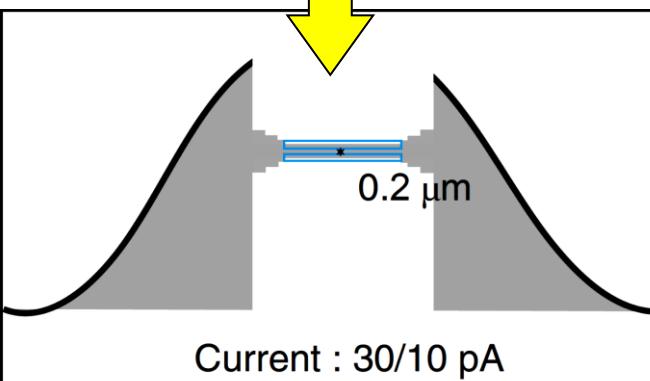
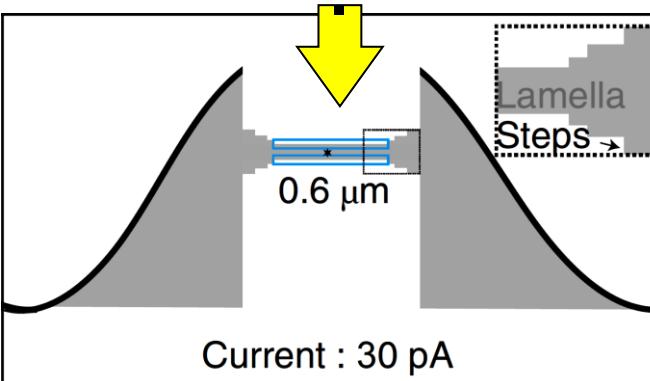
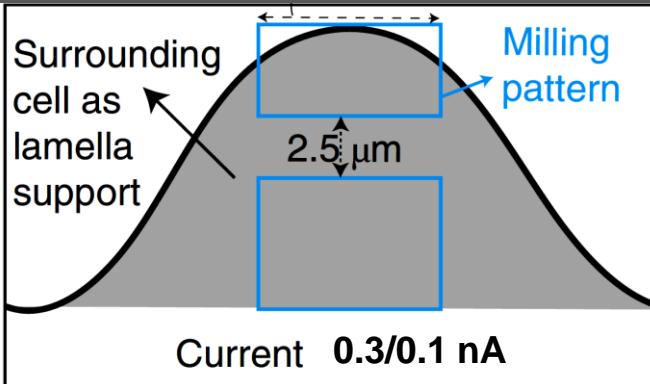
Pt sputter (Optional)
Sample conductivity

Lamella milling
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

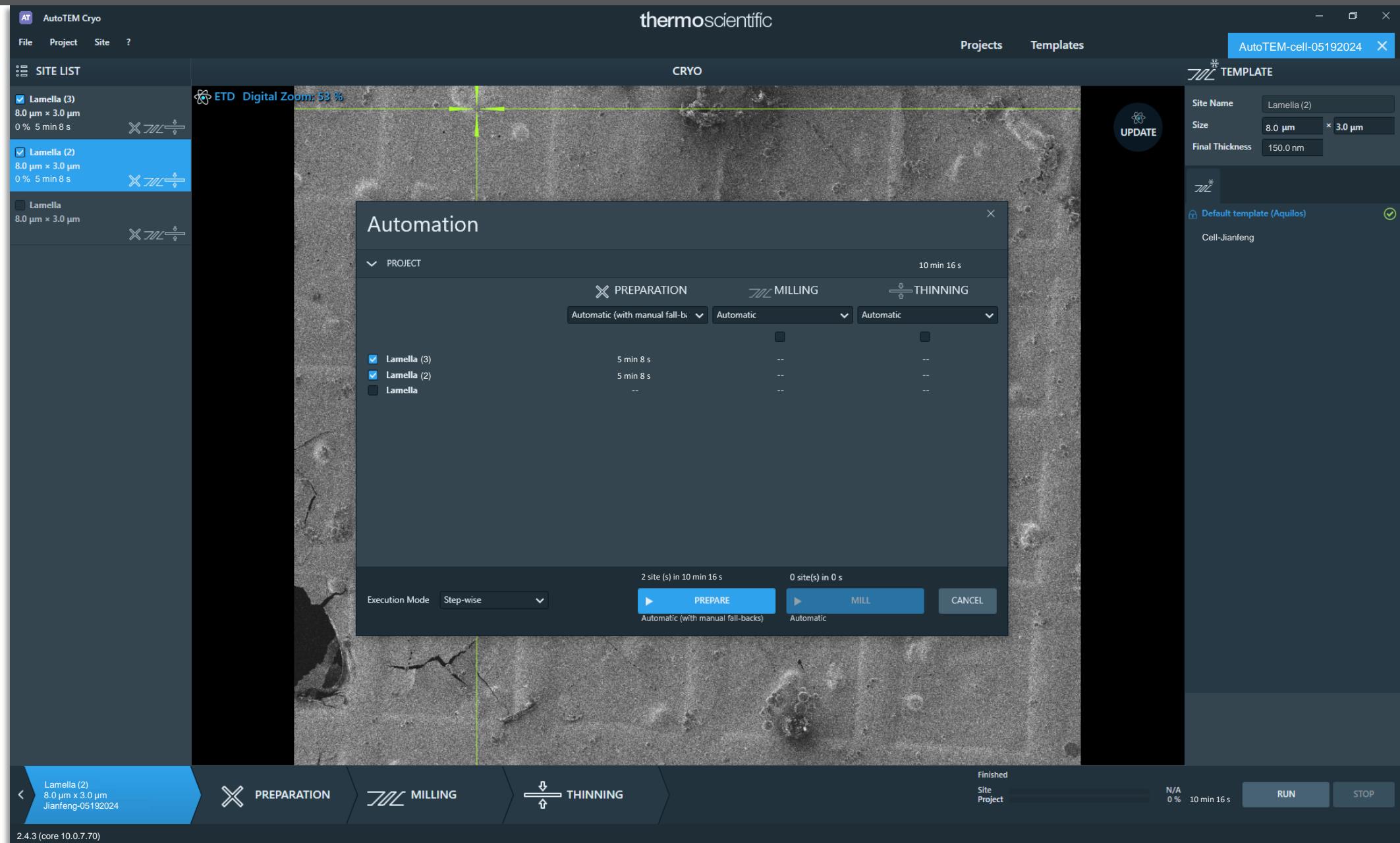
↓ T
CryoET



Modified from Wagner et al., Nature Protocols 2020

3.7.1 Automated lamella milling _Preparation

(AT AutoTEM Cryo)



3.7.1 Automated lamella milling _Preparation

(AT AutoTEM Cryo)

AutoTEM Cryo

thermoscientific

SITE LIST

- Lamella (3)
8.0 μm x 3.0 μm
98 % 3 s
- Lamella (2)
8.0 μm x 3.0 μm
98 % 3 s
- Lamella
8.0 μm x 3.0 μm

ETD Digital Zoom: 81 %

PREPARATION

Ion HFW Oversize: 80.0 μm
Milling Angle: 12.0 °
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**

A

Target (ROI)

Z-Height between Focus Planes

X-Fiducial

Distance to projected target position

Waiting to continue... Site Project 98 % 3 s 98 % 3 s CONTINUE STOP

2.4.3 (core 10.0.7.0) Lamella (2) Preparation execution

3.7.2 Automated lamella milling _Milling

(AT AutoTEM Cryo)

The screenshot displays the AutoTEM Cryo software interface. At the top, the title bar shows "AutoTEM Cryo". The main window features a "thermoscientific" logo and navigation tabs for "File", "Project", "Site", and "?". A "SITE LIST" panel on the left lists three entries: "Lamella (3)" (checked), "Lamella (2)" (checked), and "Lamella" (unchecked). Each entry includes dimensions (8.0 μm x 3.0 μm) and processing time (e.g., 13 % 28 min 16 s). Below the site list is a large "Automation" dialog box. This dialog contains a "PROJECT" section with three steps: PREPARATION, MILLING, and THINNING. Under PREPARATION, "Lamella (3)" and "Lamella (2)" are selected, while "Lamella" is not. The total duration is 58 min 33 s. The "MILLING" step is currently active, indicated by a blue background. The "THINNING" step is shown below it. At the bottom of the dialog are buttons for "Execution Mode" (Step-wise or Automatic), "PREPARE" (disabled), "MILL" (disabled), and "CANCEL". To the right of the Automation dialog is a detailed "MILLING" configuration panel. It specifies "Lamella Size" as 8.0 μm x 3.0 μm and "Correction Factor" as 0.50. The panel lists various sub-processes: 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 (disabled), and FINER MILLING - ELECTRON IMAGE (disabled). The bottom of the screen shows a workflow bar with steps: PREPARATION, MILLING (highlighted in blue), and THINNING. The status bar indicates "Finished Site Project" and "N/A 12 % 58 min 33 s". Buttons for "RUN" and "STOP" are also present.

AutoTEM-cell-05192024

Automation

PROJECT

PREPARATION MILLING THINNING

Lamella (3)
Lamella (2)
Lamella

58 min 33 s

Execution Mode Step-wise

PREPARE MILL CANCEL

MILLING

Lamella Size: 8.0 μm x 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 (disabled)
FINER MILLING - ELECTRON IMAGE (disabled)

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

PREPARATION MILLING THINNING

Finished Site Project

N/A 12 % 58 min 33 s

RUN STOP

2.4.3 (core 10.0.7.0)

3.7.3 Automated lamella milling _Thinning

(AT AutoTEM Cryo)

The screenshot displays the AutoTEM Cryo software interface. At the top, the title bar shows "AutoTEM Cryo" and the version "2.4.3 (core 10.0.7.0)". The main window features a "thermoscientific" logo and navigation tabs for "File", "Project", "Site", and "?".

SITE LIST: On the left, a sidebar titled "SITE LIST" lists three entries under "Lamella":

- Lamella (3): 8.0 µm x 3.0 µm, 45 %, 7 min 56 s
- Lamella (2): 8.0 µm x 3.0 µm, 42 %, 8 min 18 s
- Lamella: 8.0 µm x 3.0 µm

Automation Dialog: A central modal dialog titled "Automation" is open, showing the project setup:

- PROJECT:** 16 min 14 s
- PREPARATION:** Automatic (with manual fall-backs)
- MILLING:** Automatic
- THINNING:** Automatic

The "THINNING" section is expanded, showing settings for "Final Thickness" (150.0 nm) and "Enable Windows". Below these are sections for "DELAY", "POLISHING 1", "POLISHING 1 - ELECTRON IMAGE", "POLISHING 2", "POLISHING 2 - ION IMAGE", and "POLISHING 2 - ELECTRON IMAGE".

Execution Mode: Step-wise

Buttons: PREPARE, MILL, CANCEL

Bottom Navigation: Shows the current step as "PREPARATION" and the next steps as "MILLING" and "THINNING". It also displays progress: "N/A" (43 %), "16 min 14 s", and buttons for "RUN" and "STOP".

3.7.3 Automated lamella milling _Thinning _example-lamella 1

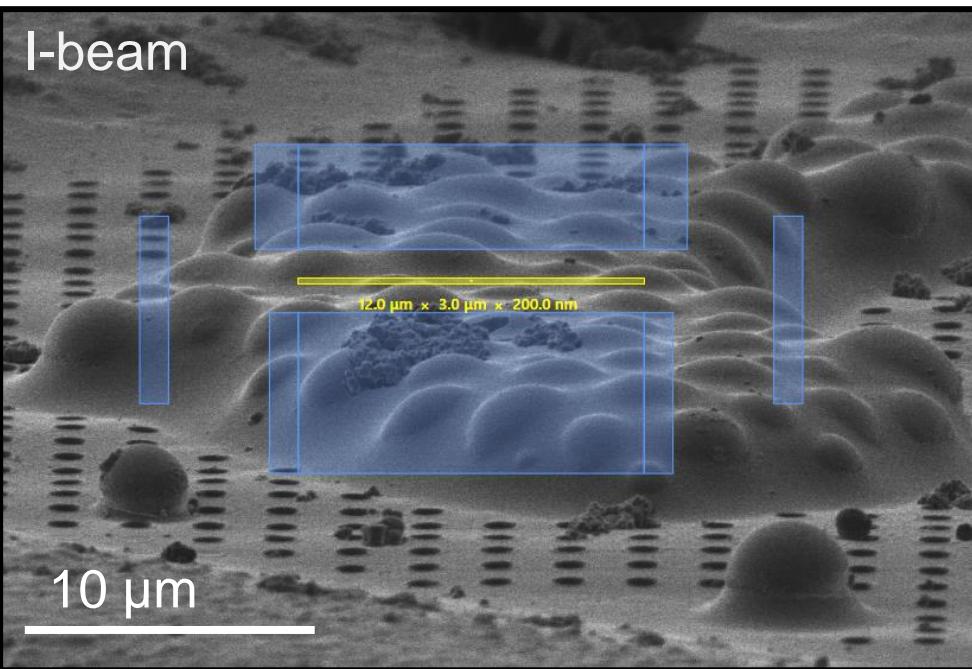
(AT AutoTEM Cryo)



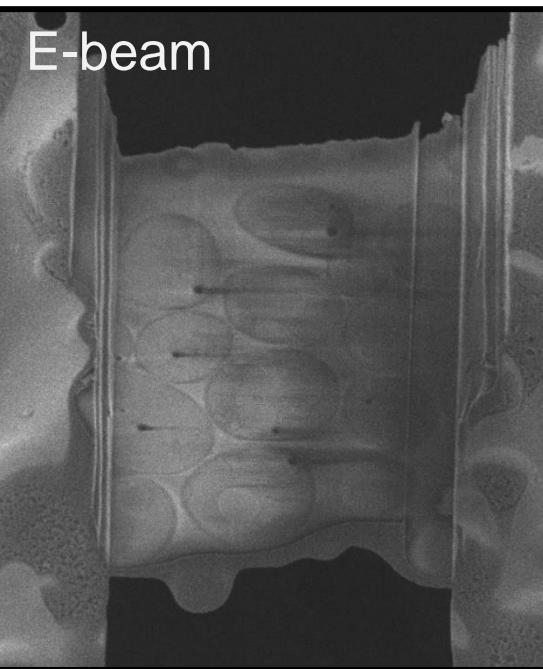
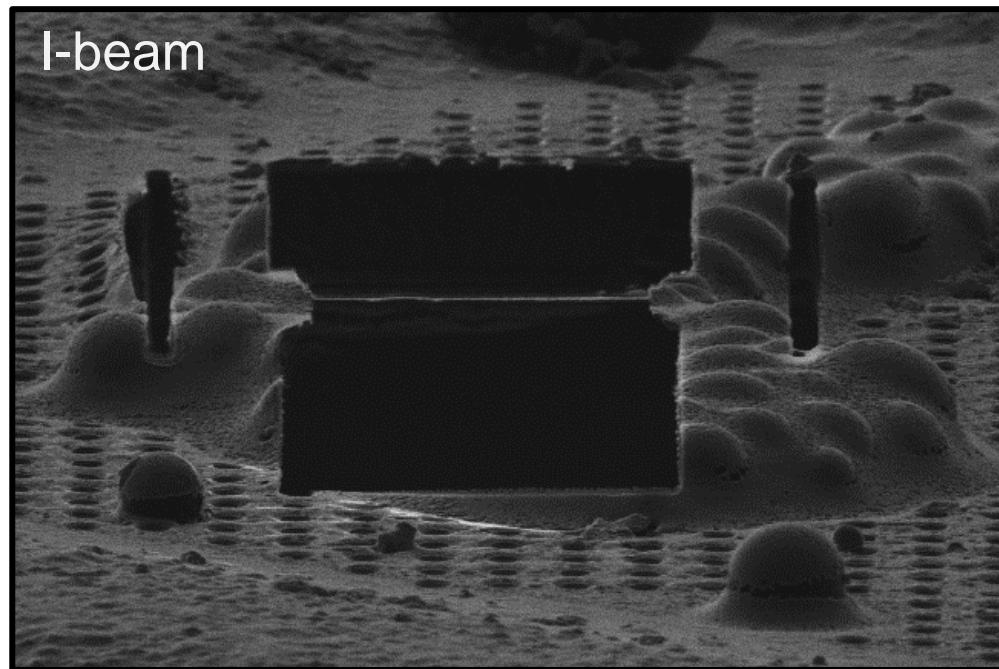
3.7.3 Automated lamella milling _example-lamella 2

( Microscope Control)

Before milling

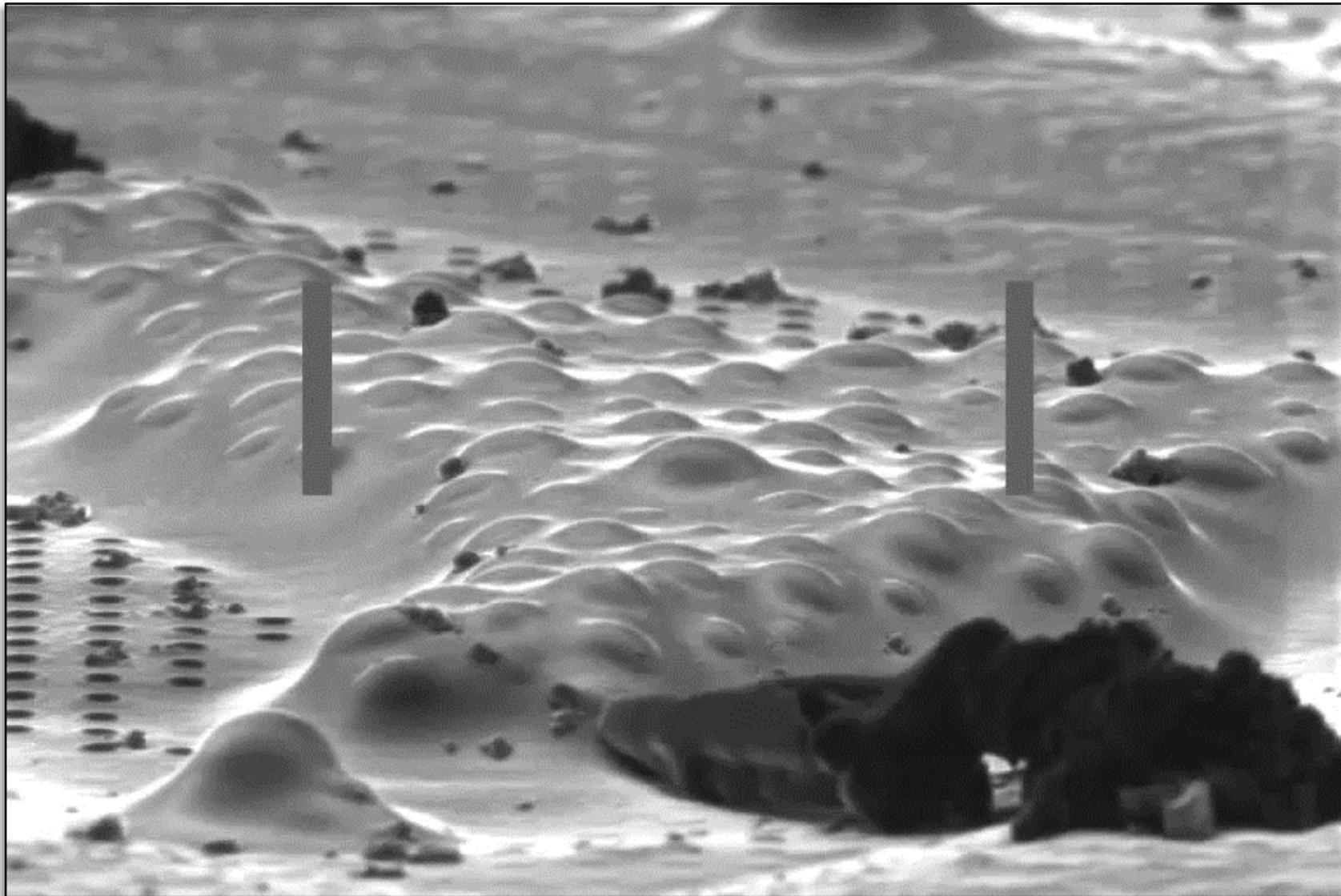


After milling



3.7.3 Automated lamella milling _example-lamella 3

(30x accelerated video)



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)

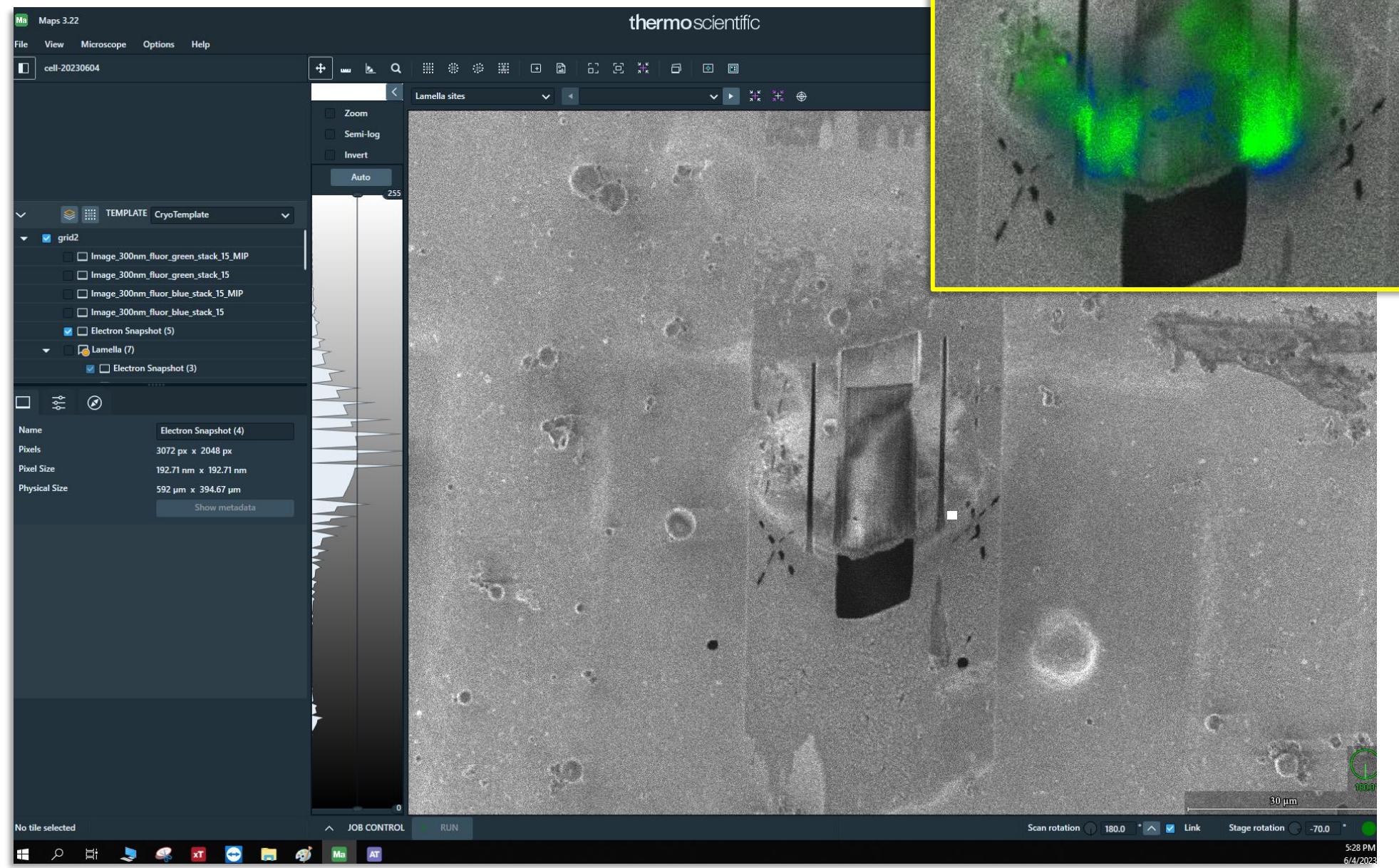
Target confirmation

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

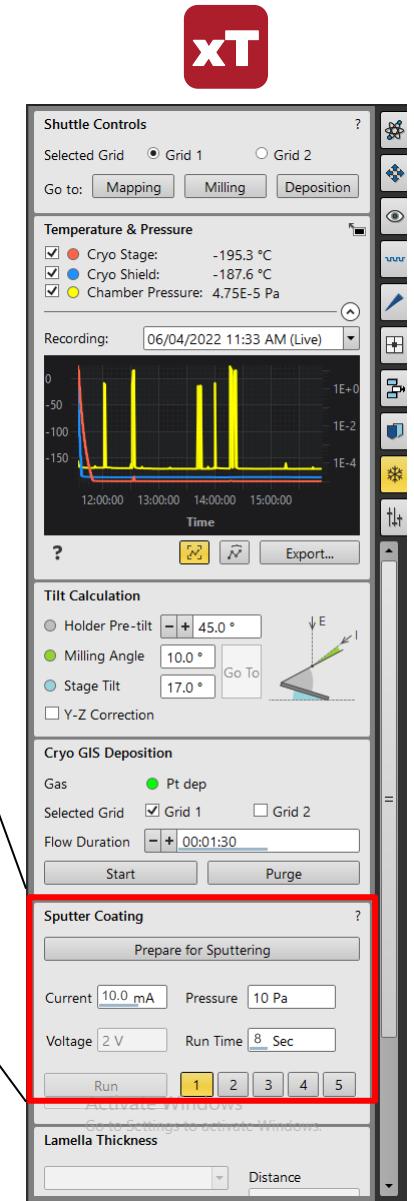
Sputter Coating

Prepare for Sputtering

Current 10.0 mA Pressure 10 Pa

Voltage 2 V Run Time 8 Sec

Run 1 2 3 4 5



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

3.10 Unloading grids

(Video from TFS)

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

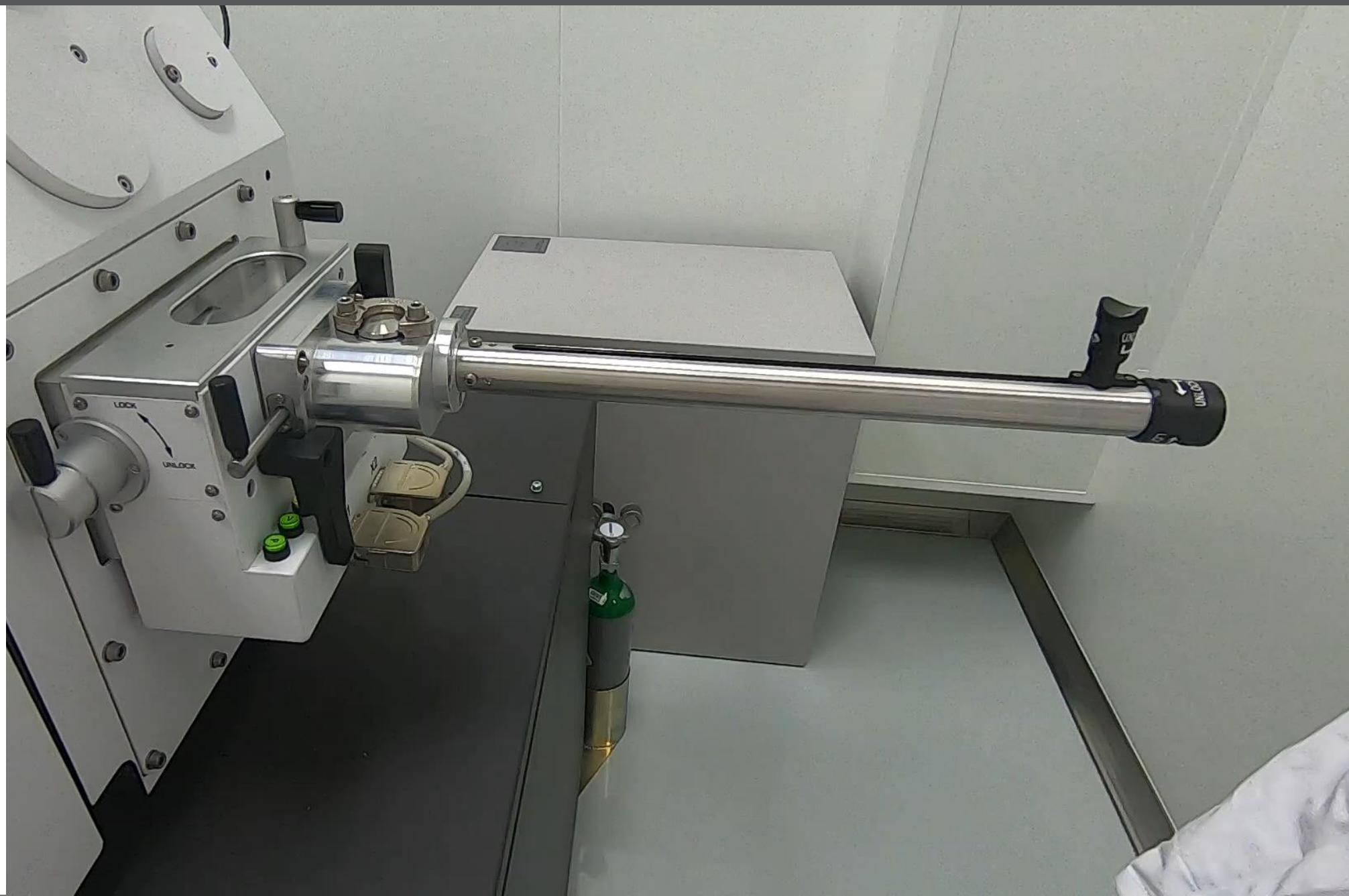
Preparation, Milling,
& thinning

iFLM (Optional)

Target confirmation

Pt sputter (Optional)

Lamella conductivity



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

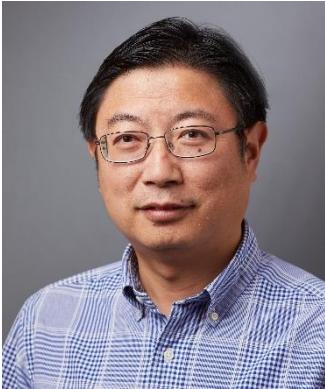
Preparation, Milling,
& thinning

If CLEM is unnecessary
& no charging problems

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

(Video from TFS)

Acknowledgment



Jun Liu
Yale University



Chen Xu
UMass Chan
Medical School



Kangkang Song
UMass Chan
Medical School

