

*Third annual Cryo-EM Course at LBMS, BNL (June 20-23<sup>rd</sup>, 2023)*

# **CryoET sample preparation tutorial & demonstration**

**Jianfeng Lin**

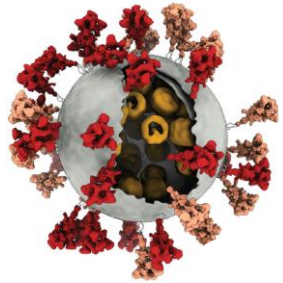
Yale CryoEM Resource

6-22-2023

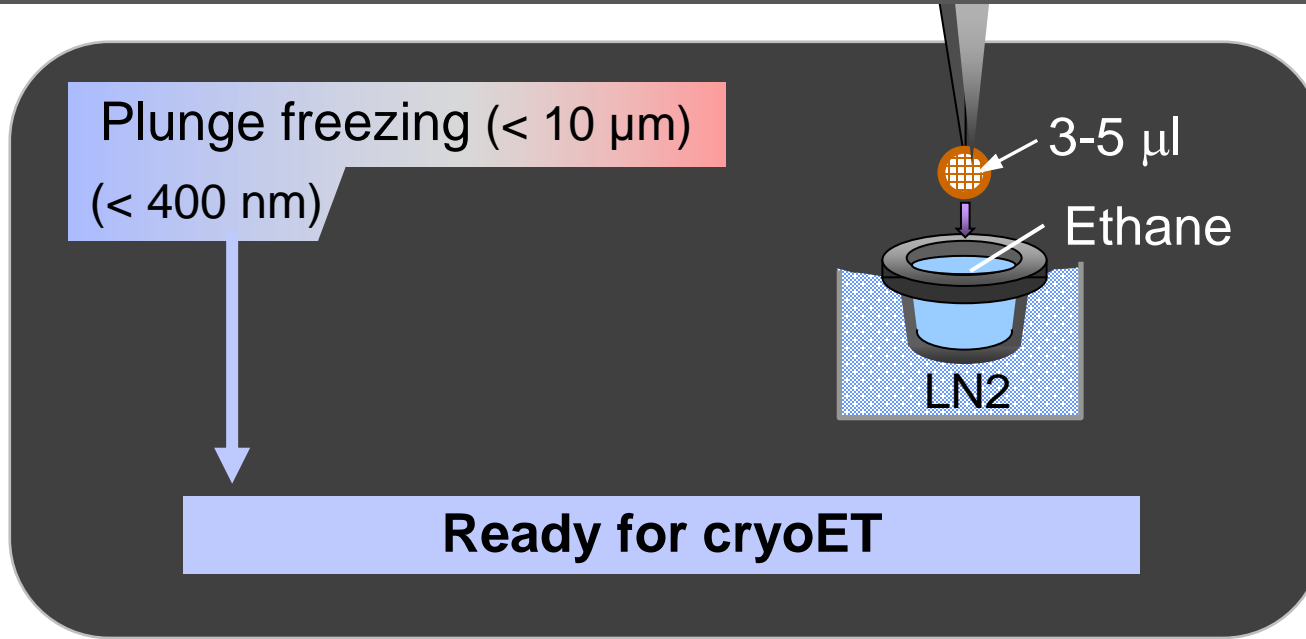
# Main contents

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

# 1. Specimens accessible by cryoET



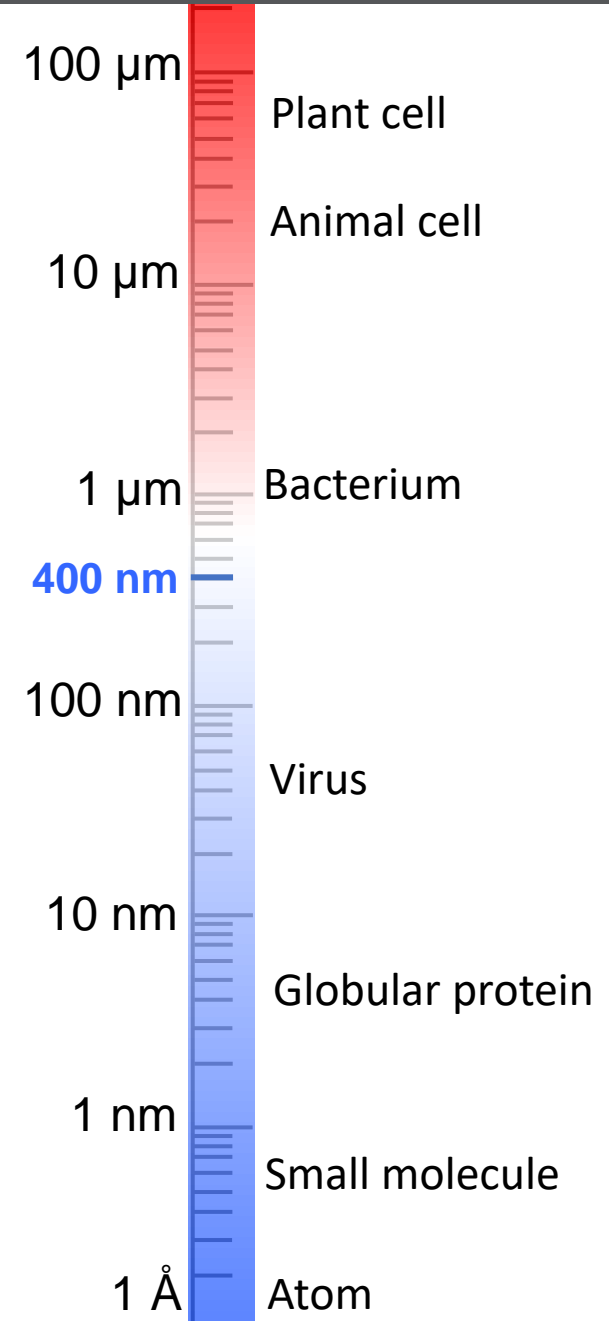
Yao et al., Cell 2020



- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., cilia & flagella

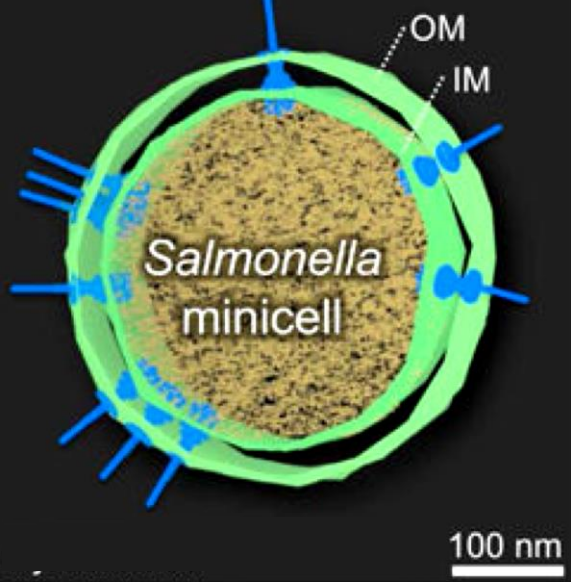


Lin & Nicastro, Science 2018

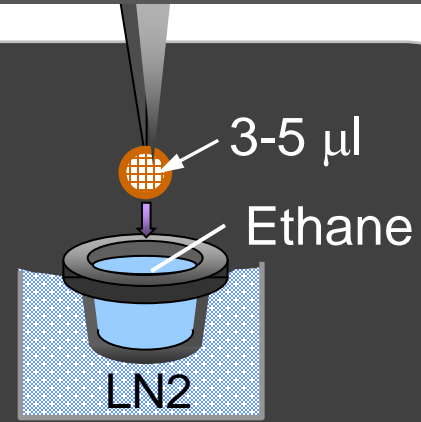


# 1. Specimens accessible by cryoET

Hu et al., Cell 2017



Plunge freezing ( $< 10 \mu\text{m}$ )  
( $< 400 \text{ nm}$ )



Ready for cryoET

100  $\mu\text{m}$

Plant cell

10  $\mu\text{m}$

Animal cell

1  $\mu\text{m}$

Bacterium

400 nm

100 nm

Virus

10 nm

Globular protein

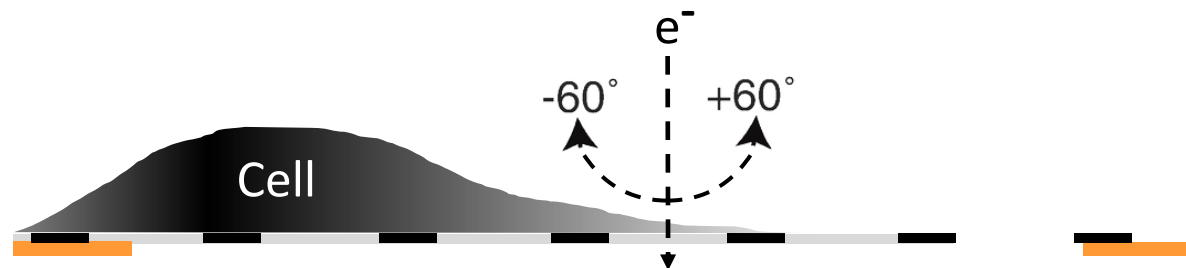
1 nm

Small molecule

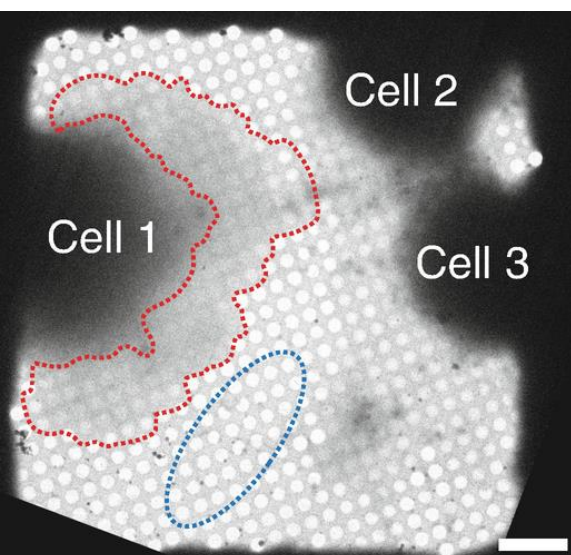
1  $\text{\AA}$

Atom

- 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

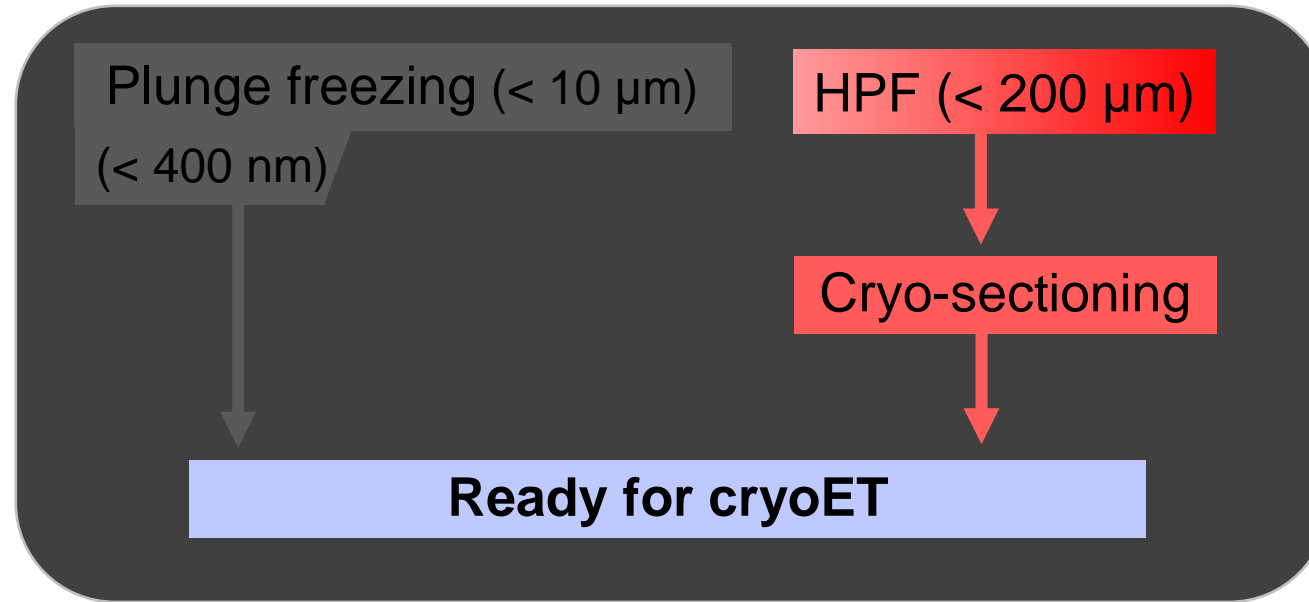


Serwas & Davies.  
Methods Mol Biol. 2021

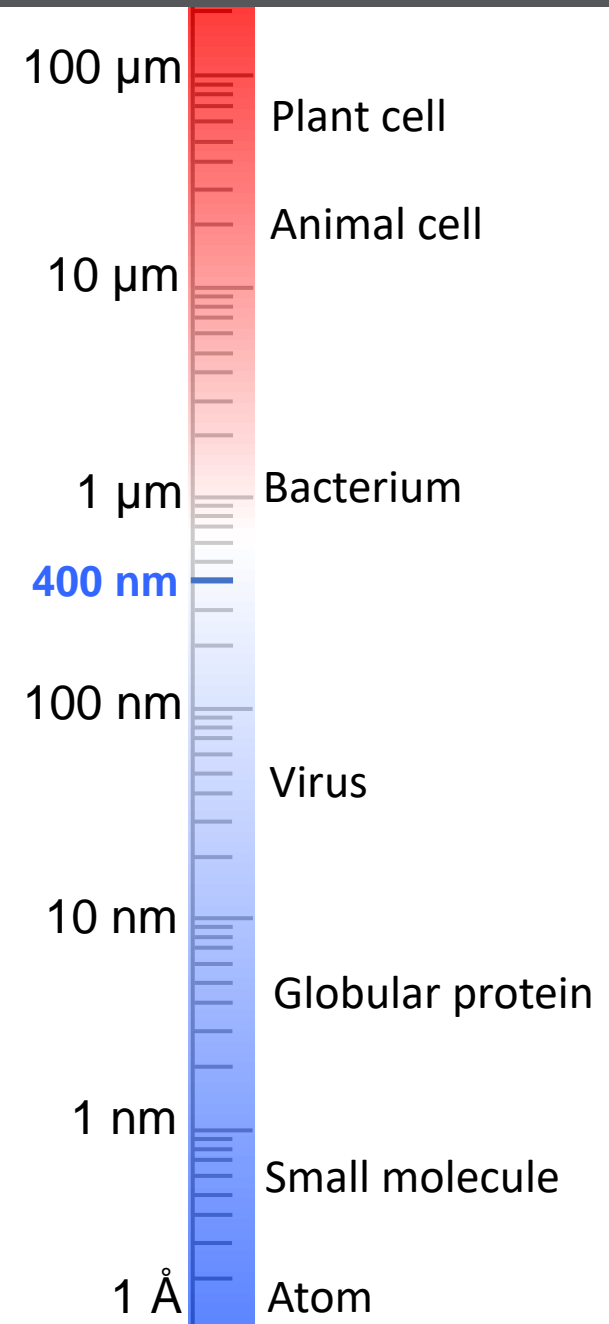




# 1. Specimens accessible by cryoET



- 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**



# 1. Specimens accessible by cryoET

CEMOVIS



Plunge freezing ( $< 10 \mu\text{m}$ )  
( $< 400 \text{ nm}$ )

HPF ( $< 200 \mu\text{m}$ )

Cryo-sectioning

Ready for cryoET

100  $\mu\text{m}$

Plant cell

10  $\mu\text{m}$

Animal cell

1  $\mu\text{m}$

Bacterium

400 nm

100 nm

Virus

10 nm

Globular protein

1 nm

Small molecule

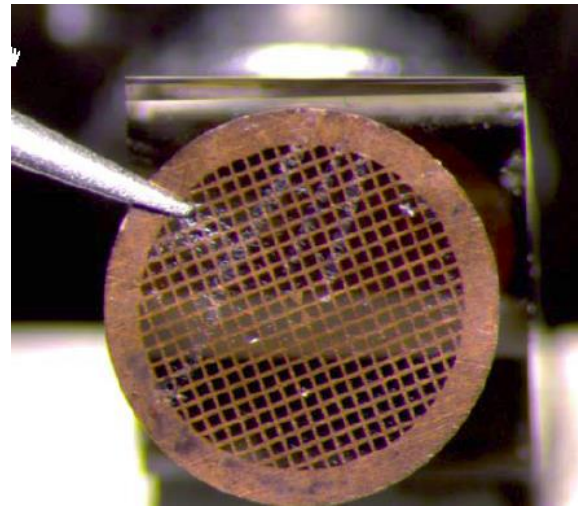
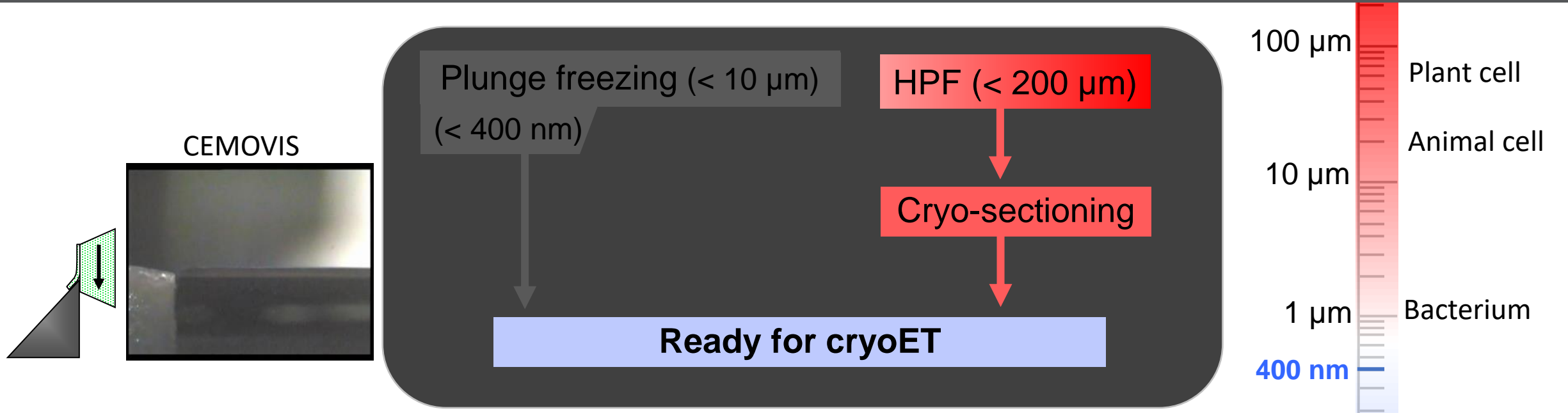
1  $\text{\AA}$

Atom

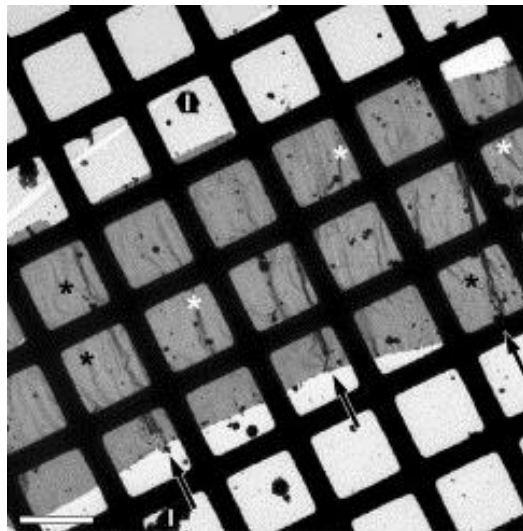
- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells
- **Cryo-sections**

Adapted from Thermo  
Fisher Scientific (TFS)

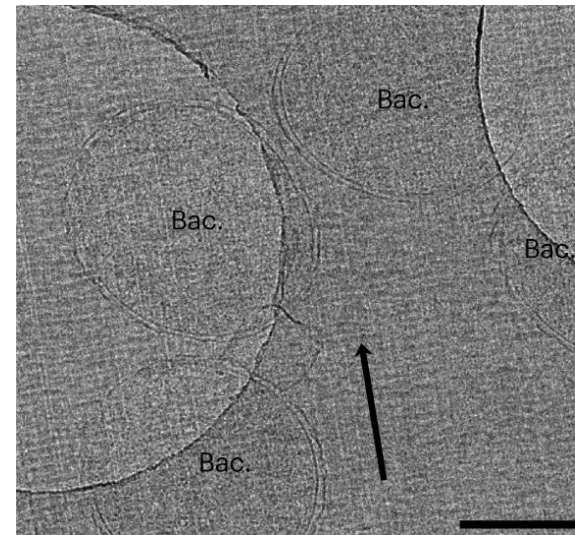
# 1. Specimens accessible by cryoET



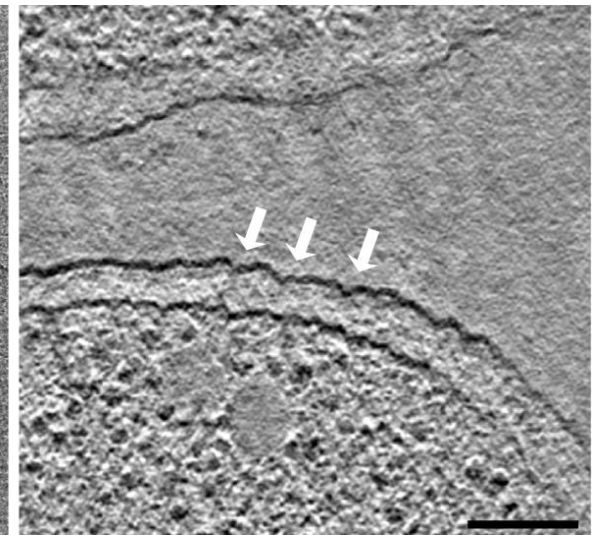
Adapted from TFS



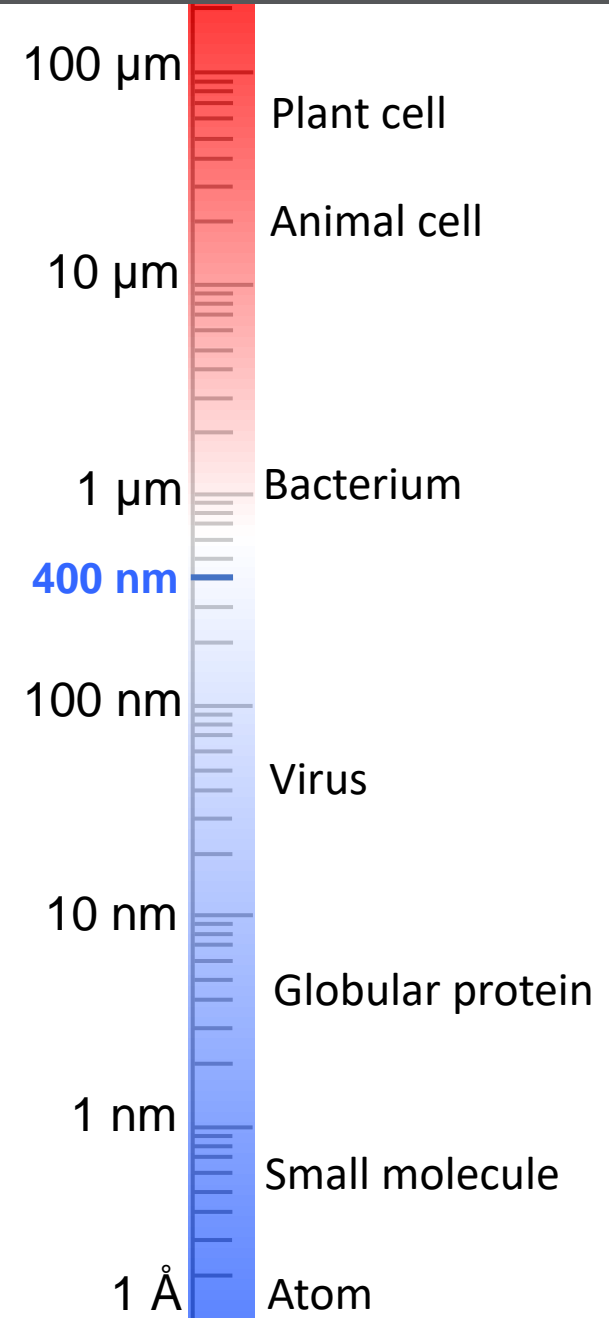
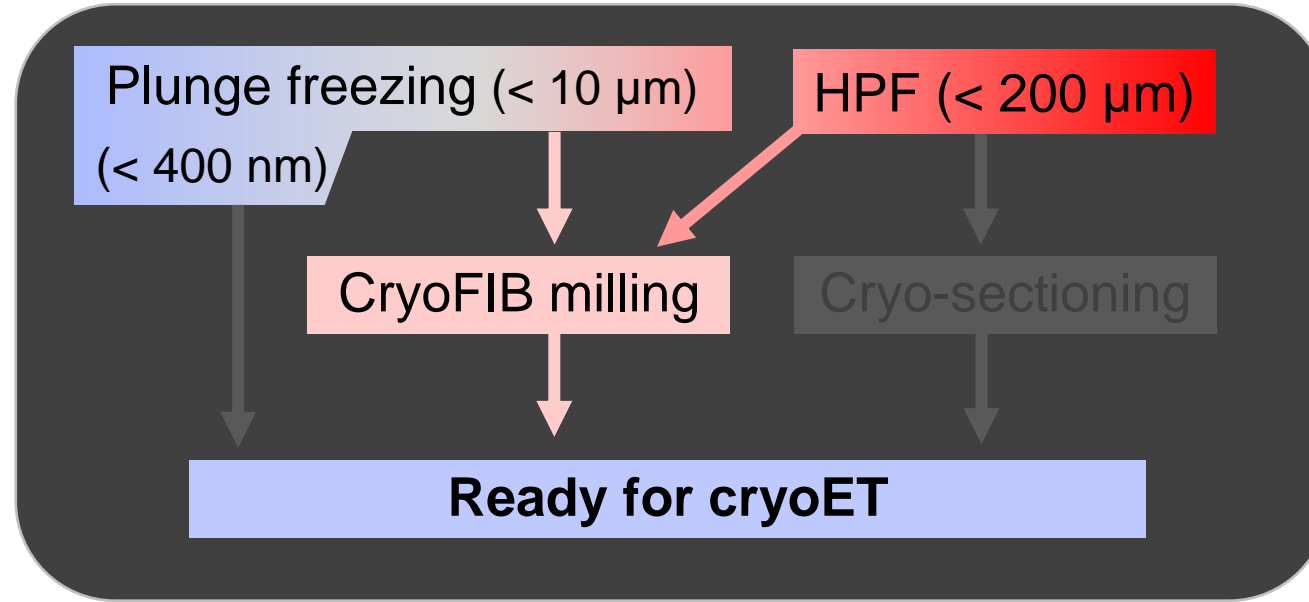
Al-Amoudi et al., J Struct Biol. 2005



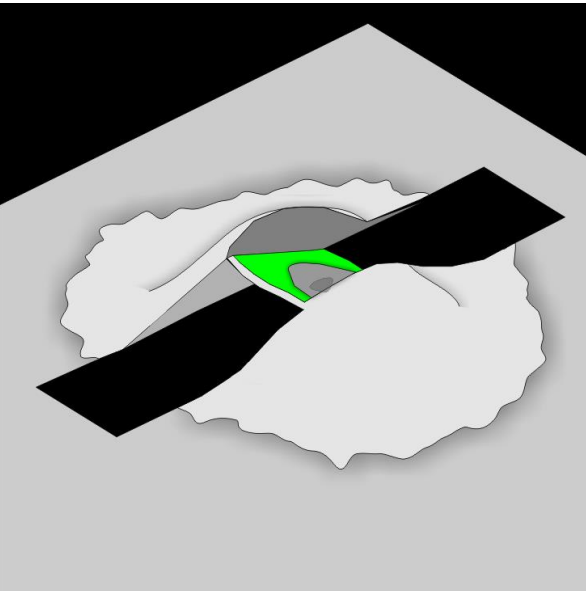
Casper Berger et al., Nature Methods. 2023



# 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

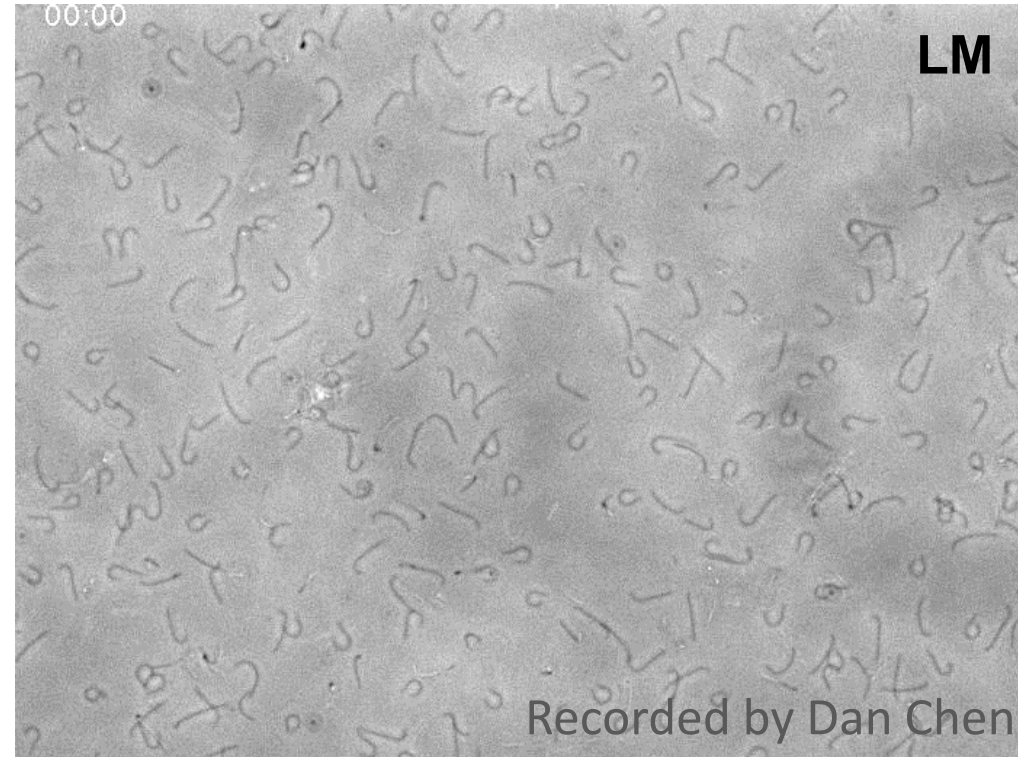
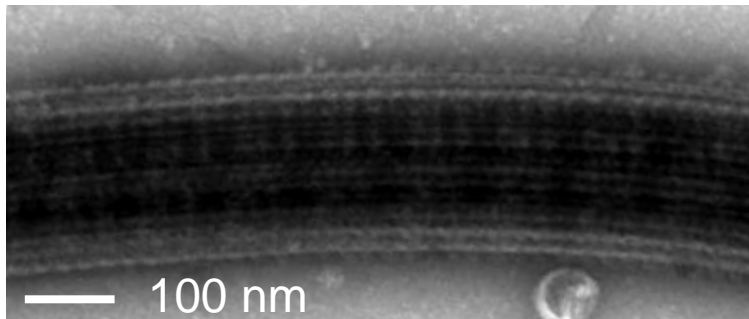
1. Specimens accessible by cryoET
- 2. Five considerations for cryoET sample preparation by plunge freezing**
3. Tutorial of major steps of cryo lamella preparation



## 2. Five considerations for cryoET sample preparation

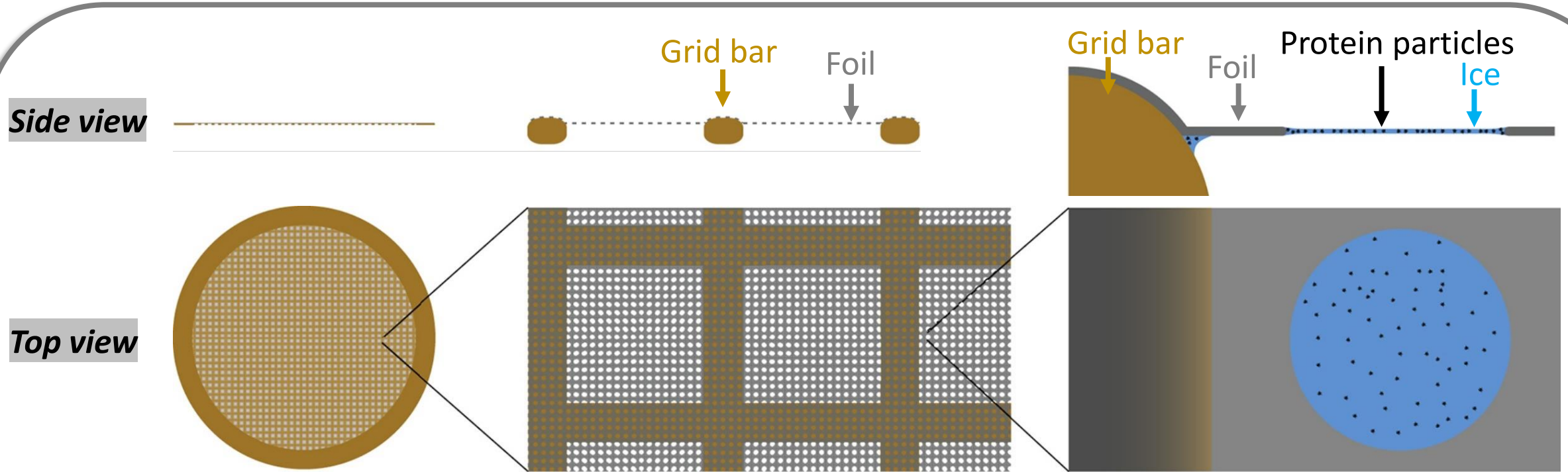
- Validation of the sample quality e.g., Negative stain EM & Reactivation of flagellar axoneme

**Negative staining EM**



## 2. Five considerations for cryoET sample preparation

- Validation of the sample quality
- **EM grid => Grid / Foil materials**



### Grid materials

Copper  
Nickel  
Titanium  
Silicon

Gold  
CuRh  
Molybdenum  
Aluminum  
Tungsten

### Foil materials

Amorphous carbon

Gold  
TiSi    SiN  
SiO<sub>2</sub>    SiC

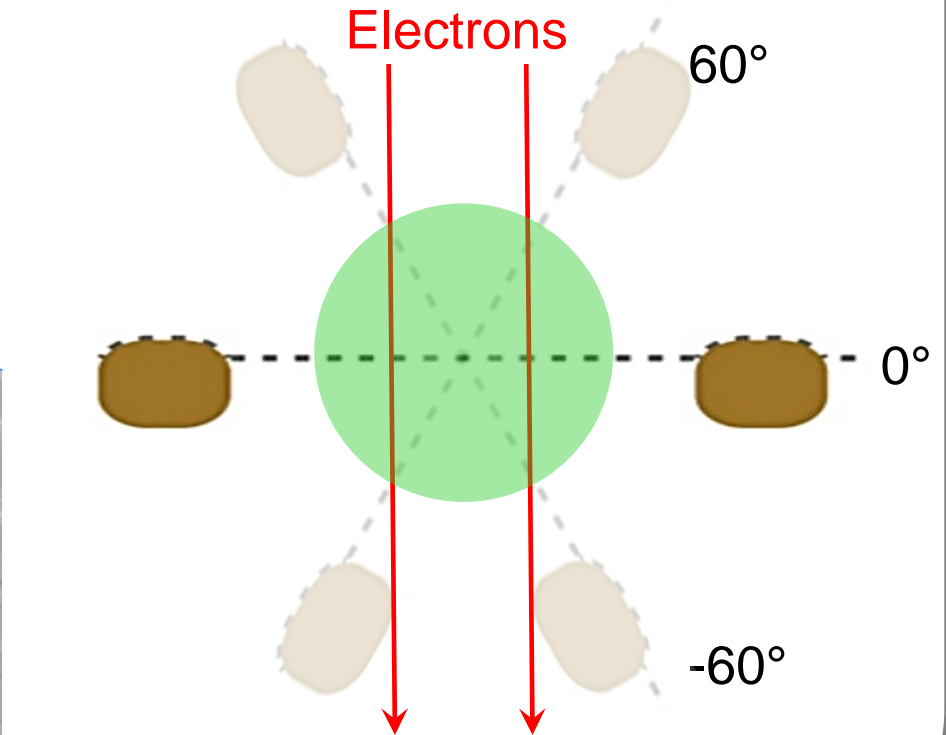
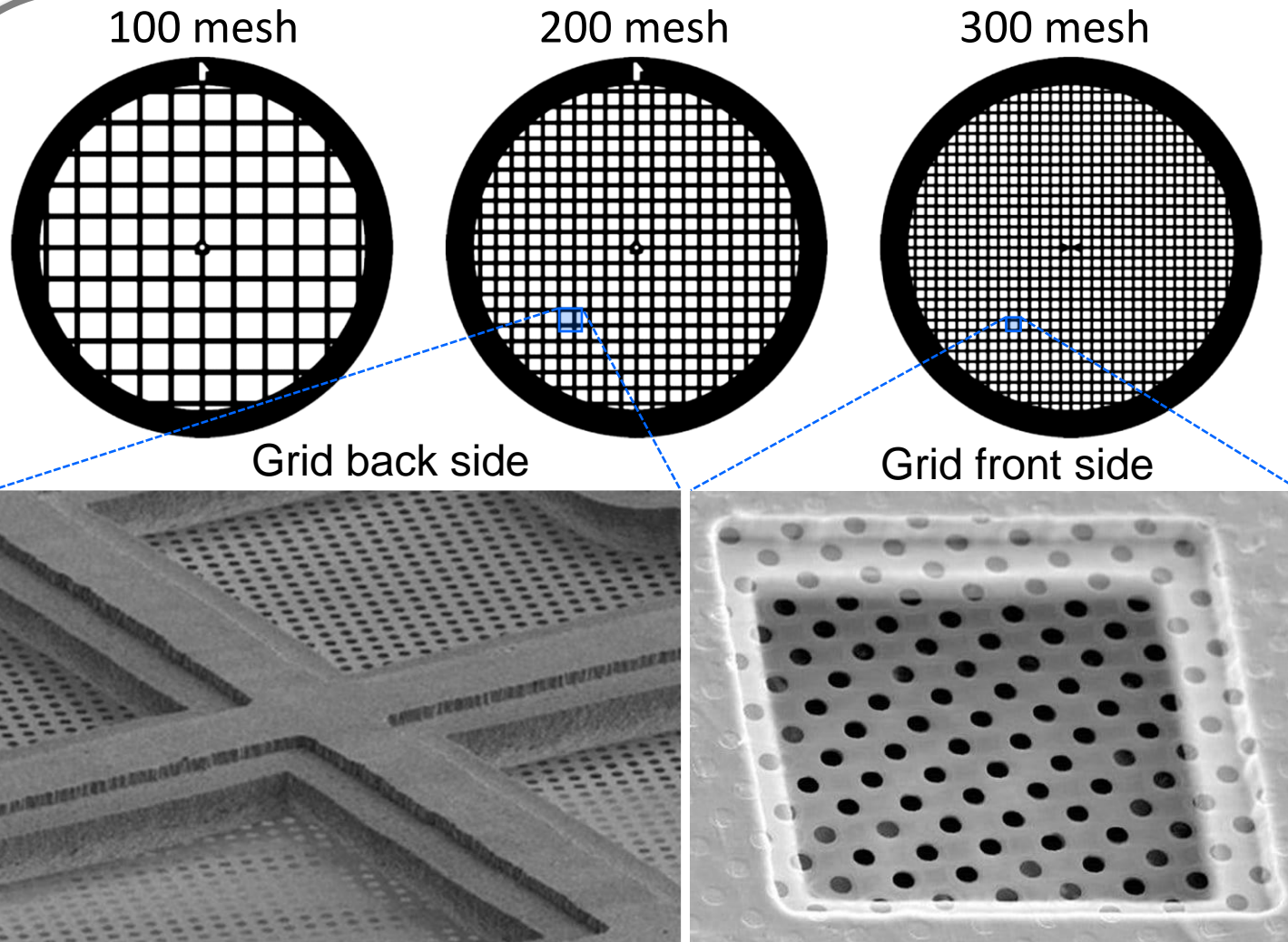
Russo & Passmore, Curr Opin Struct Biol, 2016.



## 2. Five considerations for cryoET sample preparation

➤ Validation of the sample quality

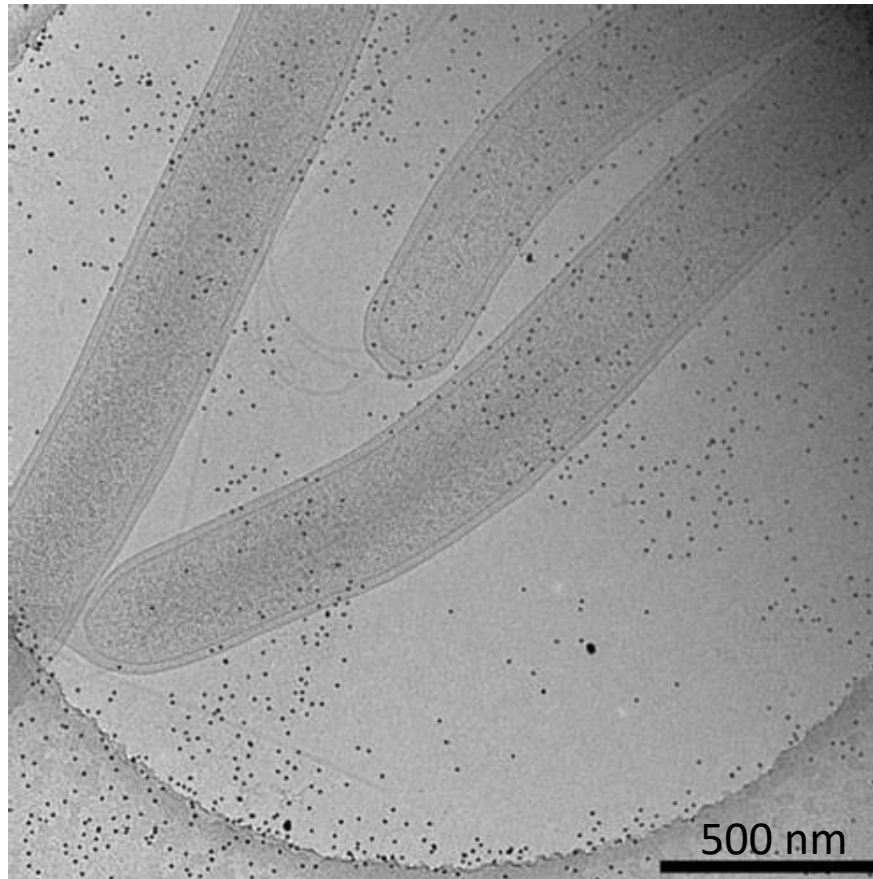
➤ EM grid => Mesh / hole size



Adapted from TFS

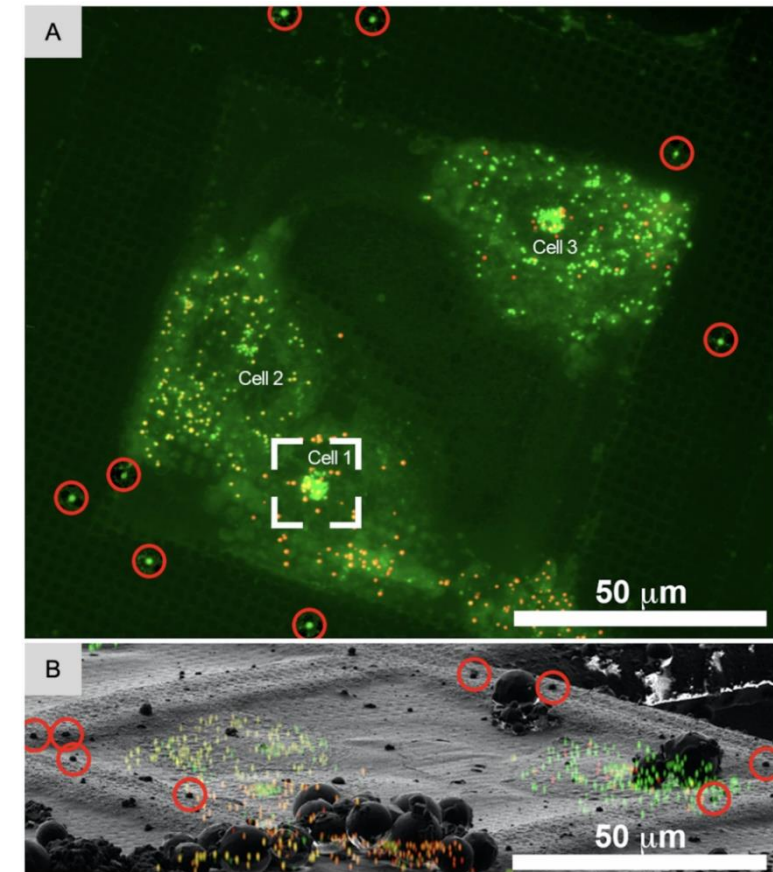
## 2. Five considerations for cryoET sample preparation

- Validation of the sample quality
- EM grid
- **Fiducial markers** e.g., 10-nm BSA-treated colloidal gold for tilt series alignments



Iancu et al., Nature Protocols 2006

- e.g., 1- $\mu\text{m}$  Magnetic beads for FLM and SEM/FIB microscopy.



Arnold et al., Biophysical Journal 2016

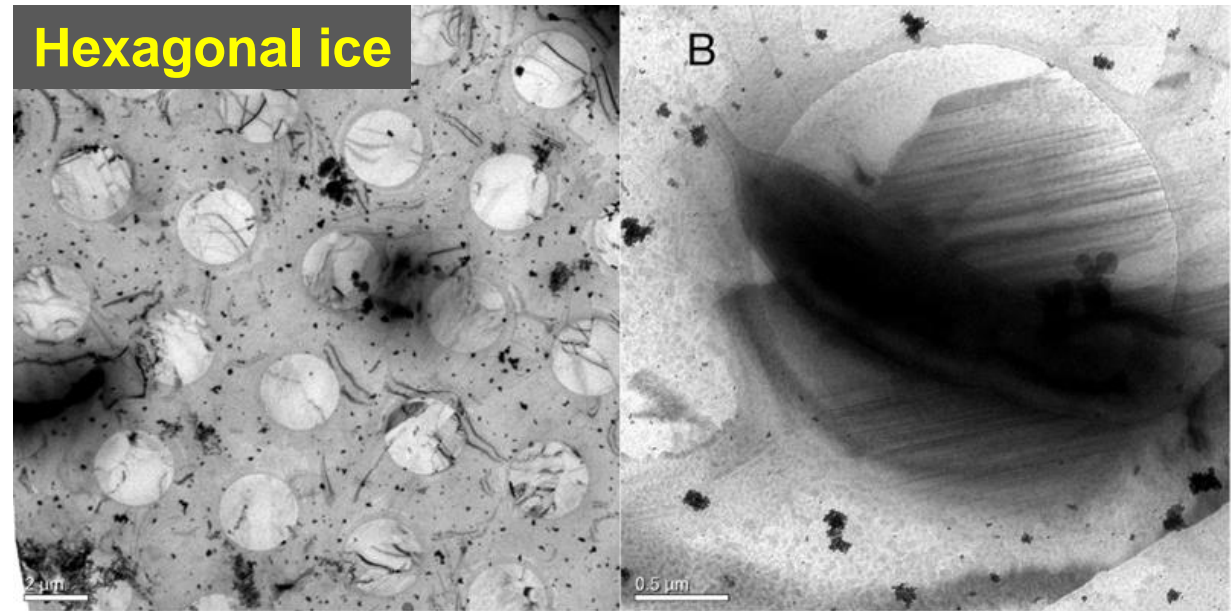
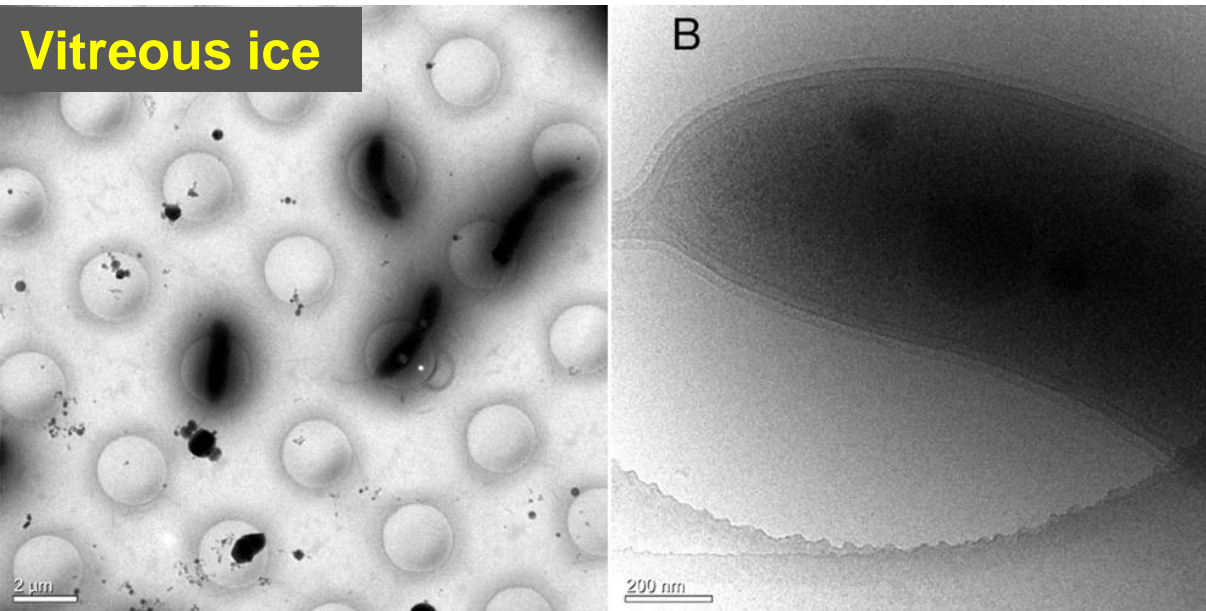
## 2. Five considerations for cryoET sample preparation

- Validation of the sample quality
- EM grid
- Fiducial markers
- **Cryogen** e.g., LE or 37% LE-63% LP mixture

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

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

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





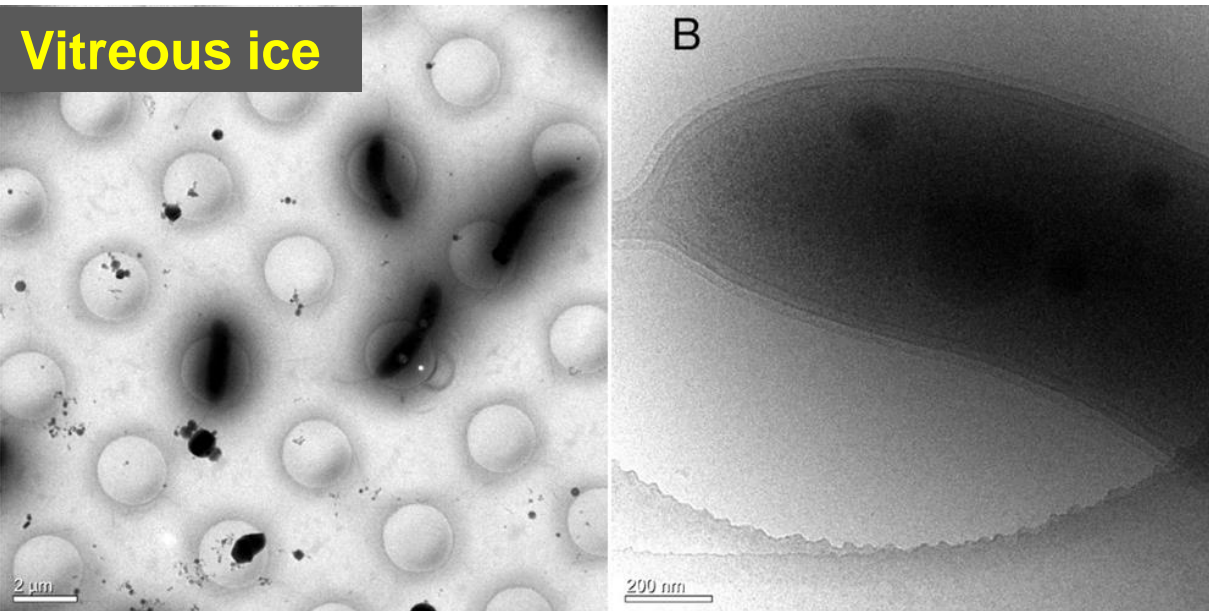
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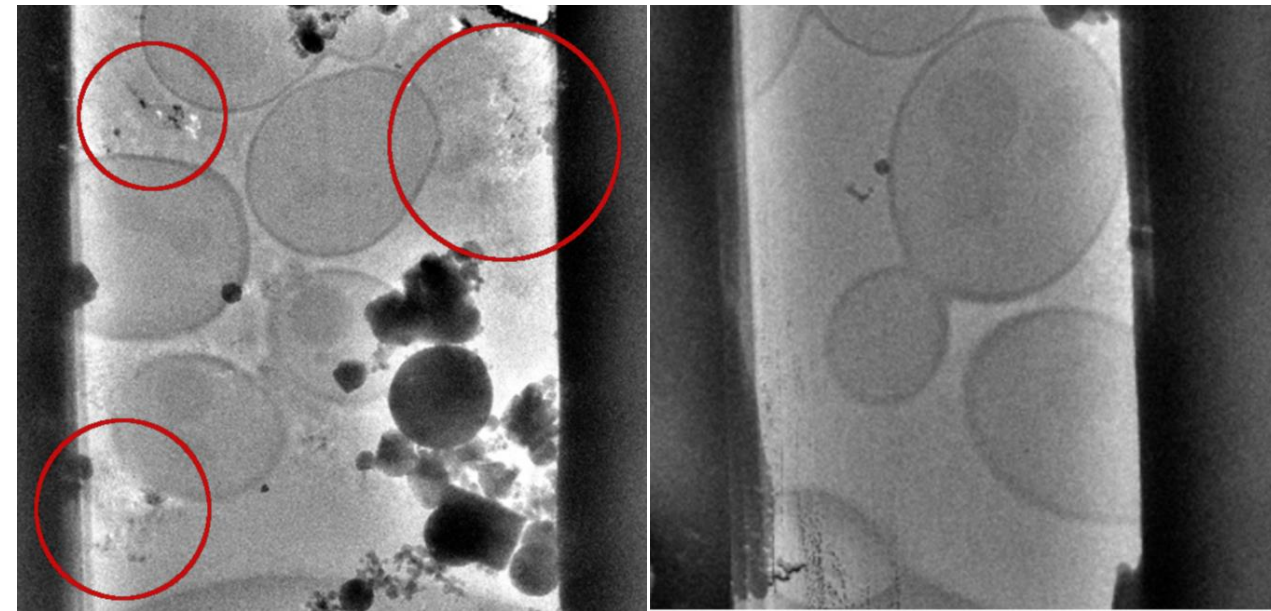
$$T_{LP} = -189.7 \text{ }^{\circ}\text{C} \sim -42.2 \text{ }^{\circ}\text{C}$$

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



Without glycerol

5% glycerol



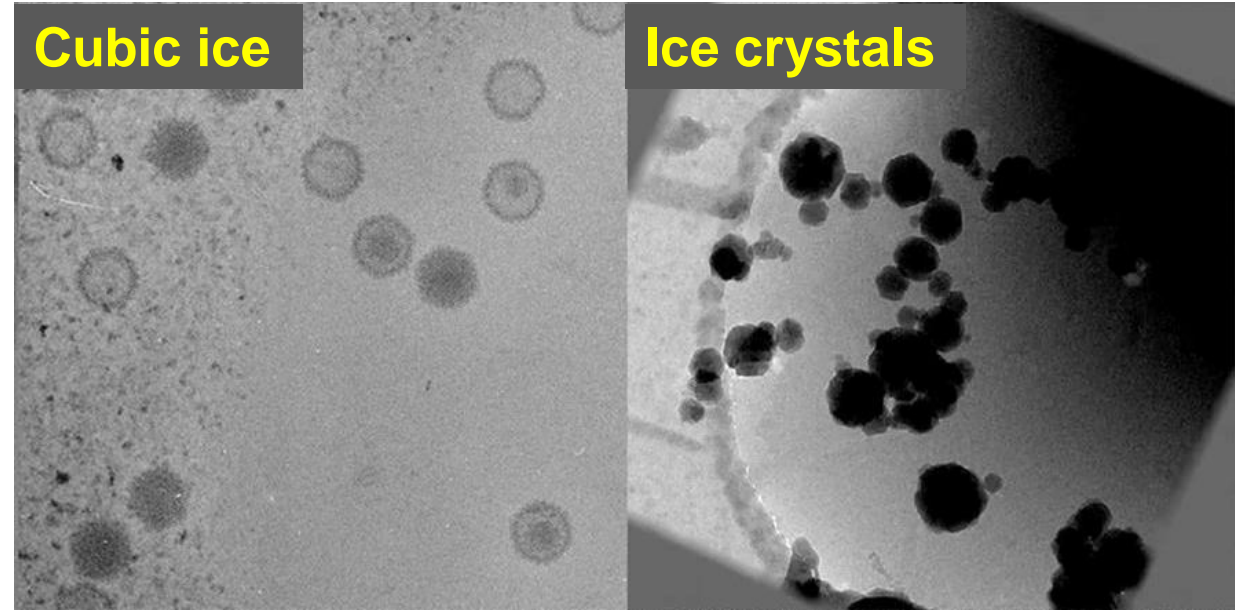
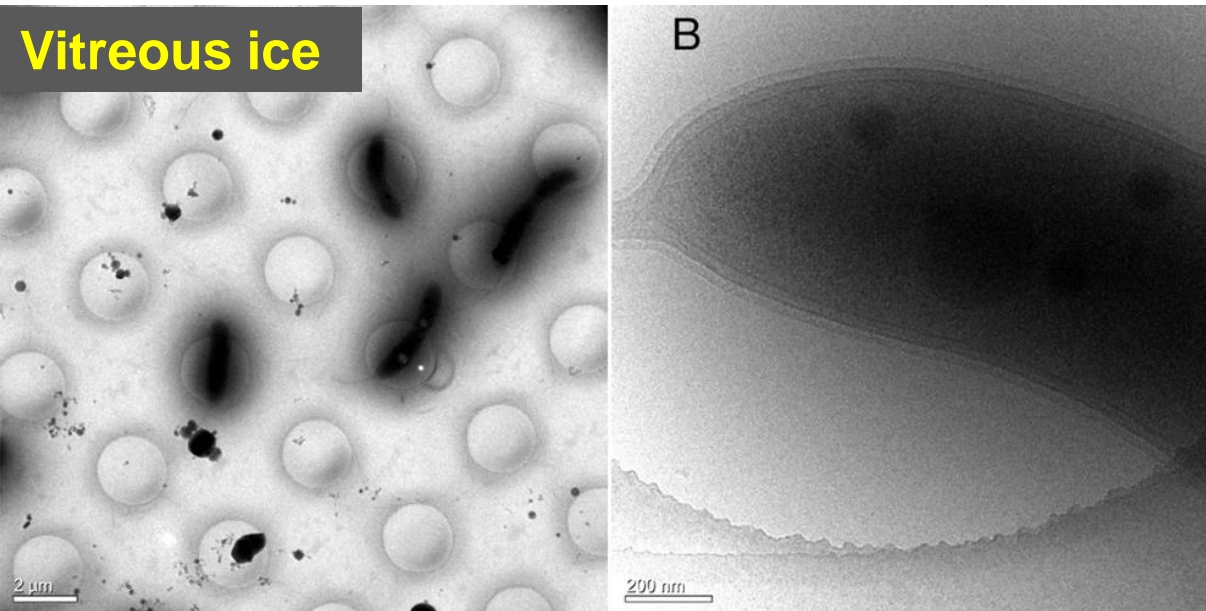
## 2. Five considerations for cryoET sample preparation

- Validation of the sample quality
- EM grid
- Fiducial markers
- **Cryogen** e.g., LE or 37% LE-63% LP mixture

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

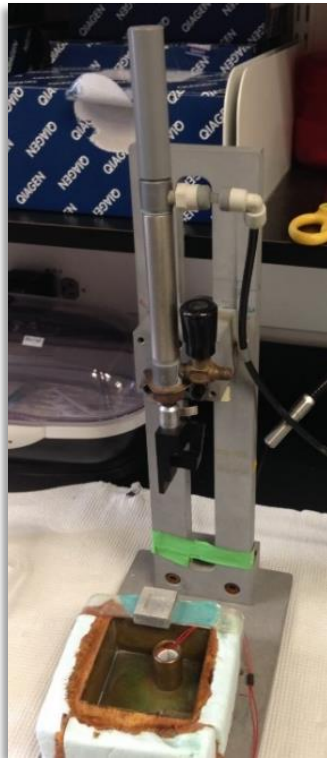
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$$T_{LN2} = -210 \text{ }^{\circ}\text{C} \sim -195.8 \text{ }^{\circ}\text{C}$$



## 2. Five considerations for cryoET sample preparation

- Validation of the sample quality
- EM grid
- Fiducial markers
- Cryogen
- **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 by plunge freezing
3. Tutorial of major steps of cryo lamella preparation



# Examples of cryoFIB milling instruments

## Arctis

(Thermo Fisher Scientific)



## Scios, Aquiclos 1/2

(Thermo Fisher Scientific)



## Crossbeam (ZEISS)



# Main contents

1. Specimens accessible by cryoET
2. Five considerations for cryoET sample preparation by plunge freezing
3. Tutorial of major steps of cryo lamella preparation (with Vitrobot & Aquilos 2)



# 3.1 Get 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)

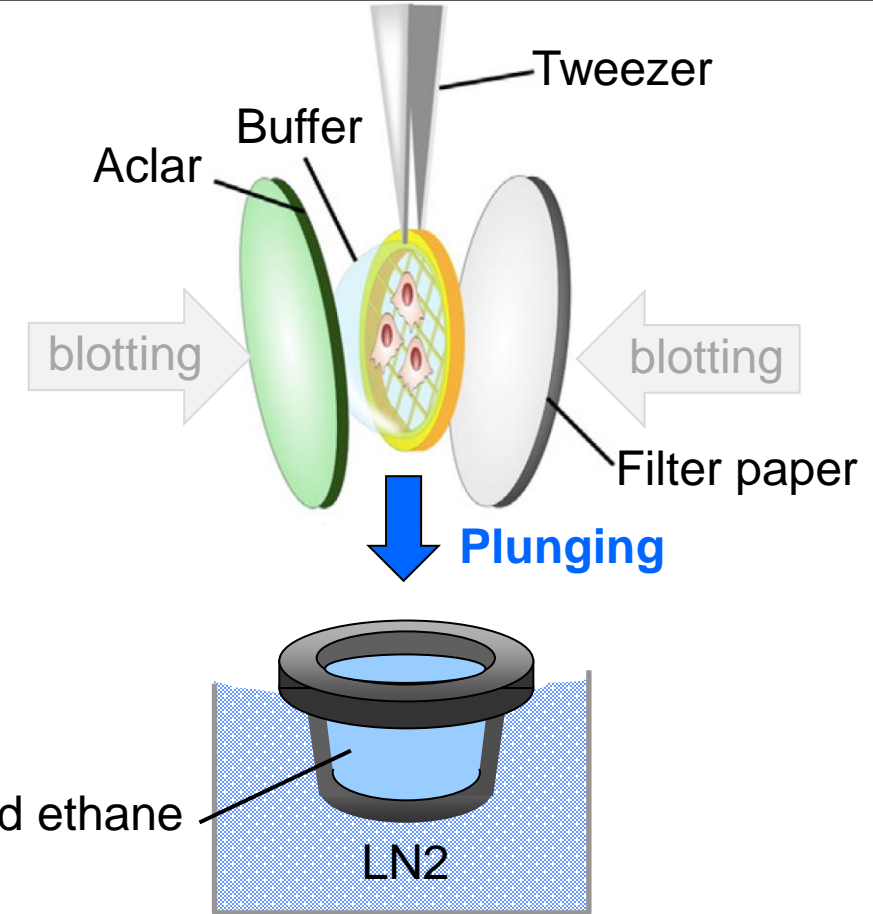
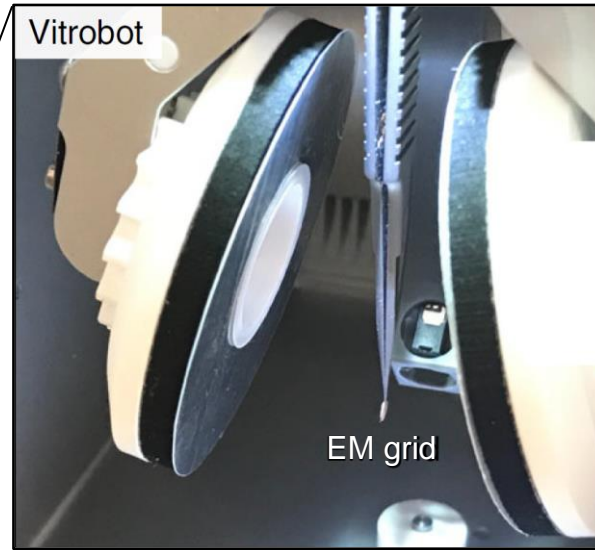
Target confirmation

#### Pt sputter (Optional)

Lamella conductivity

T

### CryoET



## 3.2 Transfer the grids to Aquilos 2

**Vitrification**

↓ **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**

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

# Aquilos 2 & software used in this tutorial

## In Support PC



Flow DDE



FlowView

## In Aquilos 2 PC



Microscope Control v32.1



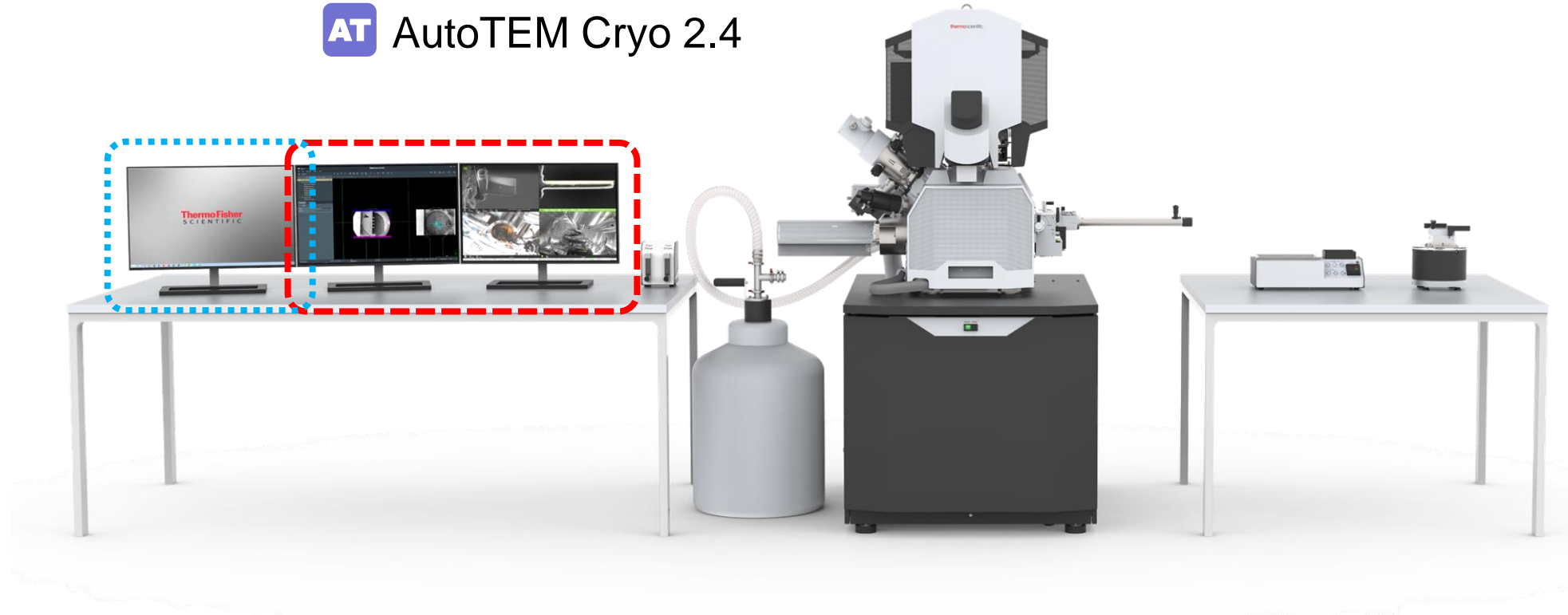
Maps 3.22



Fluorescence Microscope Control 1.2.0



AutoTEM Cryo 2.4





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

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)

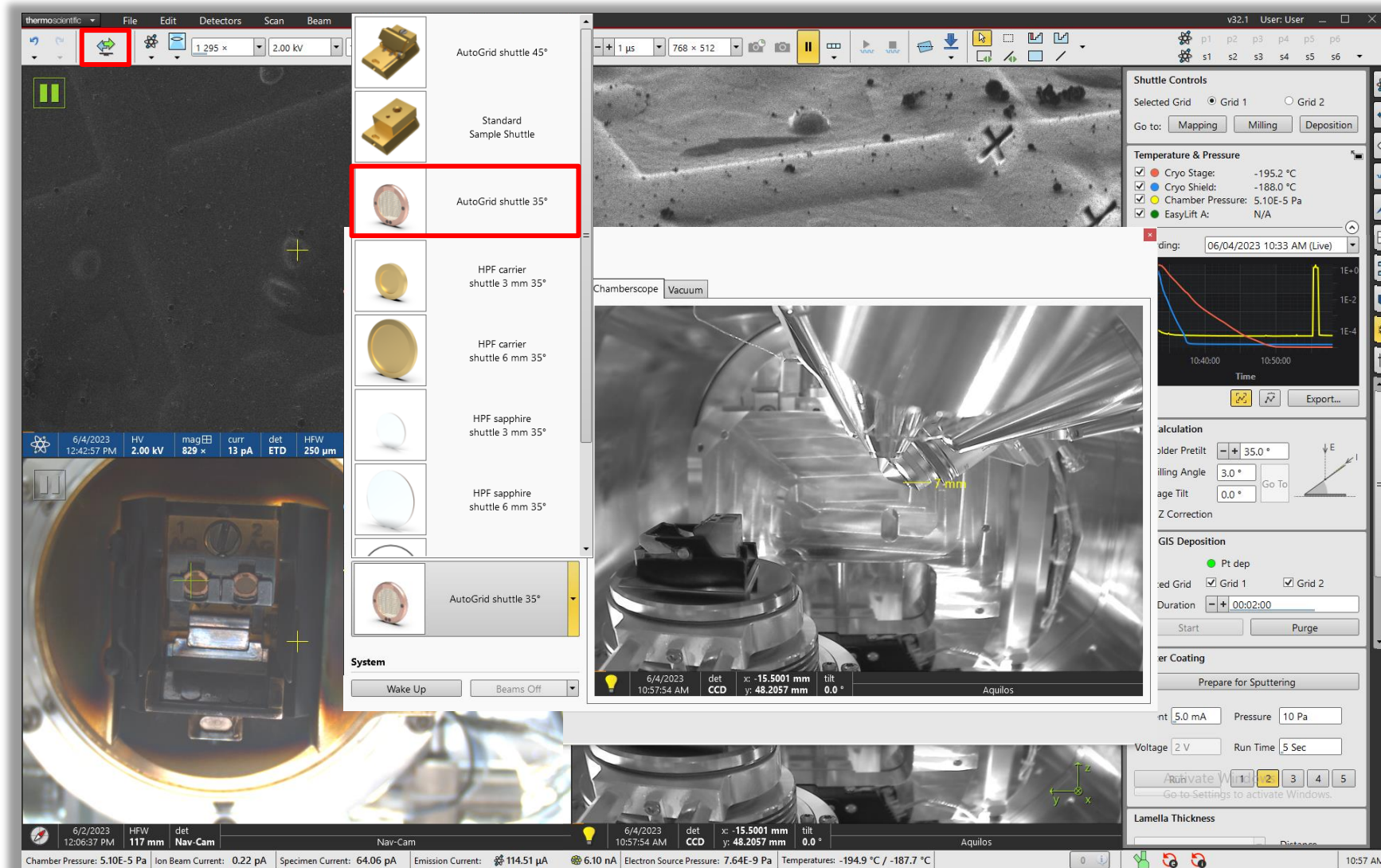
Target confirmation

**Pt sputter** (Optional)

Lamella conductivity

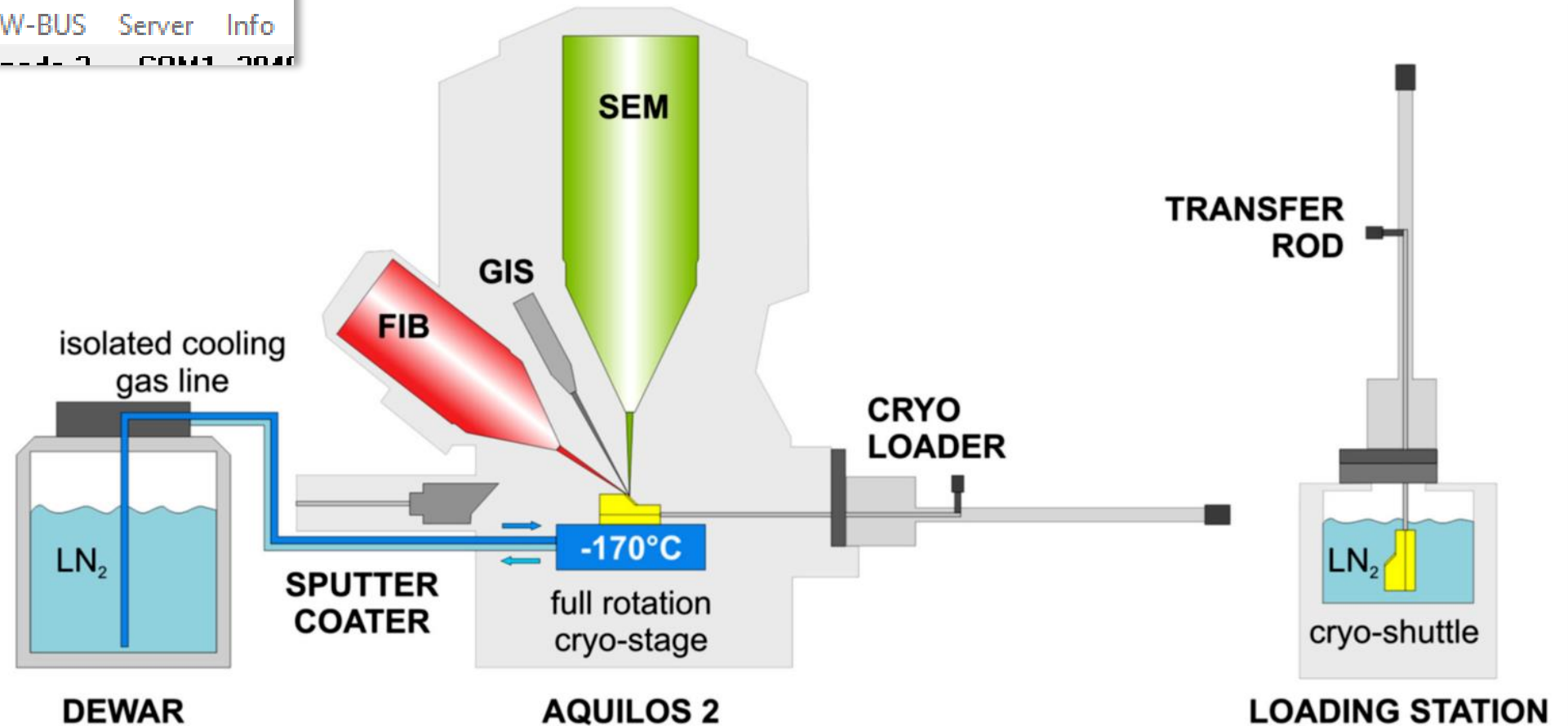
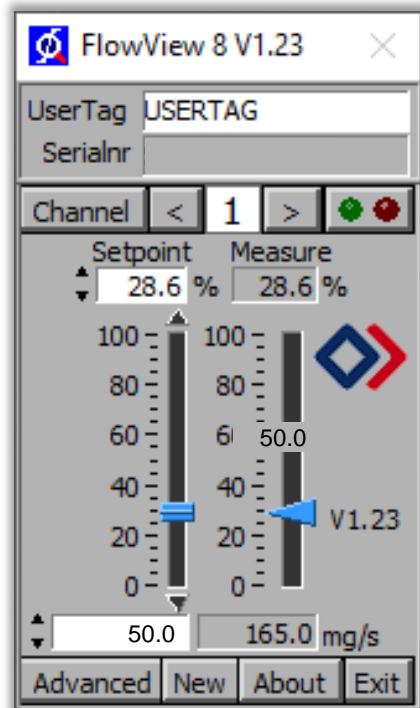
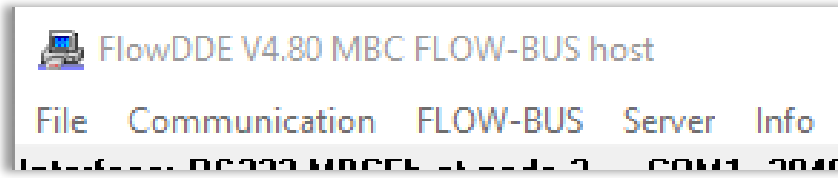


**CryoET**



### 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)**

Kuba et al., J Microsc. 2021

The diagram illustrates the setup for cryo-SEM. It features a central SEM column with a GIS (Gas Inlet System) and a FIB (Focused Ion Beam) source. The sample is mounted on a full rotation cryo-stage maintained at -170°C. This stage is connected to a SPUTTER COATER and a DEWAR containing LN<sub>2</sub> (liquid nitrogen) via an isolated cooling gas line. A CRYO LOADER is positioned to the right of the cryo-stage. The entire system is housed within the AQUILOS 2 microscope.

thermoscientific File Edit Detectors Scan Beam Patterning Stage Tools View Help

116 x 30.00 kv 30 pA 200 ns 768 x 512

**Vacuum**

- High Vacuum
- Sputter Vacuum

Purge

Number of Cycles:

Chamber Pressure: 1.77E-4 Pa

**System**

Wake Up Sleep

**Column**

Beam On Beam Current:

High Voltage:

**Magnification**

Couple Magnifications

Magnification:

**Beam**

Stigmator Beam Shift

**Beam Deceleration**

On Stage Bias:

**Scan Rotation**

Scan Rotation:

**Detectors**

Contrast:

Brightness:

5/31/2021 7:54:37 PM HFW 116 mm det Nav-Cam Nav-Cam

6/12/2021 2:44:48 PM det CCD x: 3.3083 mm tilt 18.0 ° y: 3.4435 mm Aquilos

Chamber Pressure: 1.79E-4 Pa Ion Beam Current: -0.02 pA Specimen Current: 0 pA Emission Current: 114.01 μA 6.10 nA Electron Source Pressure: 1.02E-8 Pa Temp 0

2:44 PM

## 3.2.1 Prepare the system **\_Purge the system**

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

Kuba et al., J Microsc. 2021

The diagram illustrates the cryogenic system for the SEM. It includes a DEWAR containing LN<sub>2</sub> connected to an isolated cooling gas line. This line leads to a SPUTTER COATER, which is connected to a full rotation cryo-stage maintained at -170°C. The cryo-stage is equipped with a FIB (Focused Ion Beam) and a GIS (Gas Inlet System). The SEM (Scanning Electron Microscope) is positioned above the cryo-stage. A CRYO LOADER is also shown. The system is controlled via the xT Microscope Control software.

SEM

FIB

GIS

CRYO LOADER

DEWAR

LN<sub>2</sub>

isolated cooling gas line

SPUTTER COATER

-170°C

full rotation cryo-stage

DEWAR

AQUILOS 2

thermoscientific

File Edit Detectors Scan Beam Patterning Stage Tools View Help

116 x 30.00 kV 30 pA 200 ns 768 x 512

Shuttle Controls

Selected Grid  Grid 1  Grid 2

Go to: Mapping Milling Deposition

Temperature & Pressure

Cryo Stage: 27.4 °C

Cryo Shield: 25.4 °C

Chamber Pressure: 1.79E-4 Pa

Recording: 06/03/2021 11:00 AM

Time

Tilt Calculation

Holder Pretilt: 35.0 °

Milling Angle: 10.2 °

Stage Tilt: 7.2 °

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

5/31/2021 7:54:37 PM HFW 116 mm det Nav-Cam Nav-Cam

6/12/2021 2:44:48 PM det CCD x: 3.3083 mm tilt 18.0 ° y: 3.4435 mm

Chamber Pressure: 1.79E-4 Pa Ion Beam Current: -0.02 pA Specimen Current: 0 pA Emission Current: 114.01 μA 6.10 nA Electron Source Pressure: 1.02E-8 Pa Temp 0

2:44 PM

# 3.2.1 Prepare the system **Cool down the system**

The screenshot displays the xT Microscope Control software interface. At the top, a menu bar includes File, Edit, Detectors, Scan, Beam, Patterning, Stage, Tools, View, and Help. The main window features a schematic diagram of the SEM system with the following components labeled: DEWAR (containing LN<sub>2</sub>), SPUTTER COATER, FIB (Focused Ion Beam), GIS (Gas Inlet System), SEM (Scanning Electron Microscope), CRYO LOADER, and a full rotation cryo-stage maintained at -170°C. The schematic is attributed to Kuba et al., J Microsc. 2021. On the left, the FlowView 8 V1.23 window shows a control panel with a UserTag of USERTAG, a Serial number field, and two vertical sliders. The top slider is set to 28.6% (Setpoint and Measure) and the bottom slider is set to 165.0 mg/s (Setpoint and Measure). On the right, the Shuttle Controls panel is highlighted with a red box, showing Temperature & Pressure settings: Cryo Stage at 27.4 °C, Cryo Shield at 25.4 °C, and Chamber Pressure at 1.79E-4 Pa. Below these settings is a graph showing temperature and pressure over time from 11:20:00 to 12:20:00. The bottom status bar provides real-time data: Chamber Pressure: 1.79E-4 Pa, Ion Beam Current: -0.02 pA, Specimen Current: 0 pA, Emission Current: 114.01 μA, 6.10 nA, Electron Source Pressure: 1.02E-8 Pa, Temp: 0 °C, and a timestamp of 2:44 PM.

FlowView 8 V1.23

UserTag USERTAG

Serialnr

Channel < 1 >

Setpoint Measure

28.6 % 28.6 %

100 100

80 80

60 60

40 40

20 20

0 0

165.0 165.0 mg/s

Advanced New About Exit

Varies by instruments



## 3.2.2 Prepare the grids **\_Clip the grid**

**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)

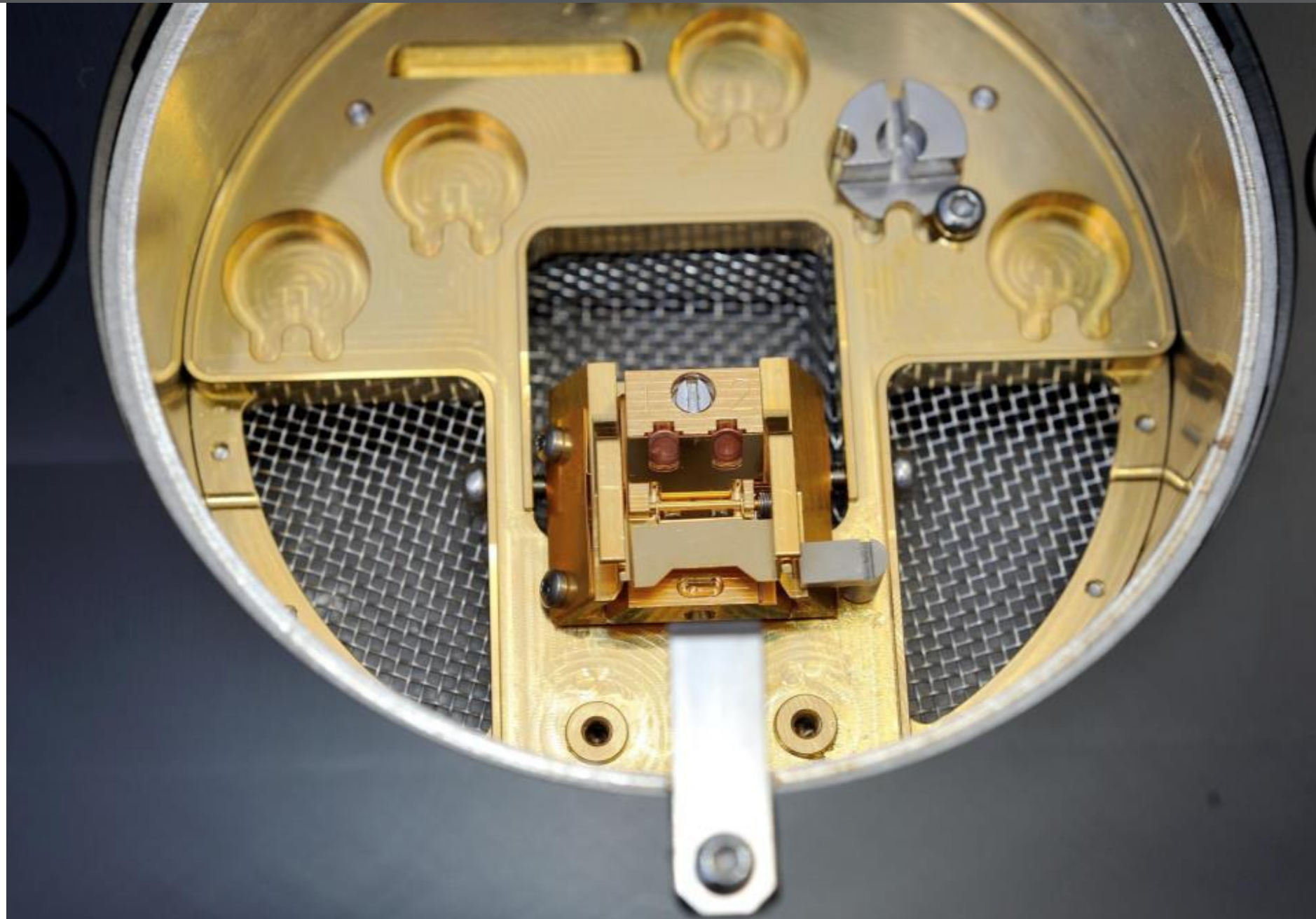
Target confirmation

**Pt sputter** (Optional)

Lamella conductivity



**CryoET**



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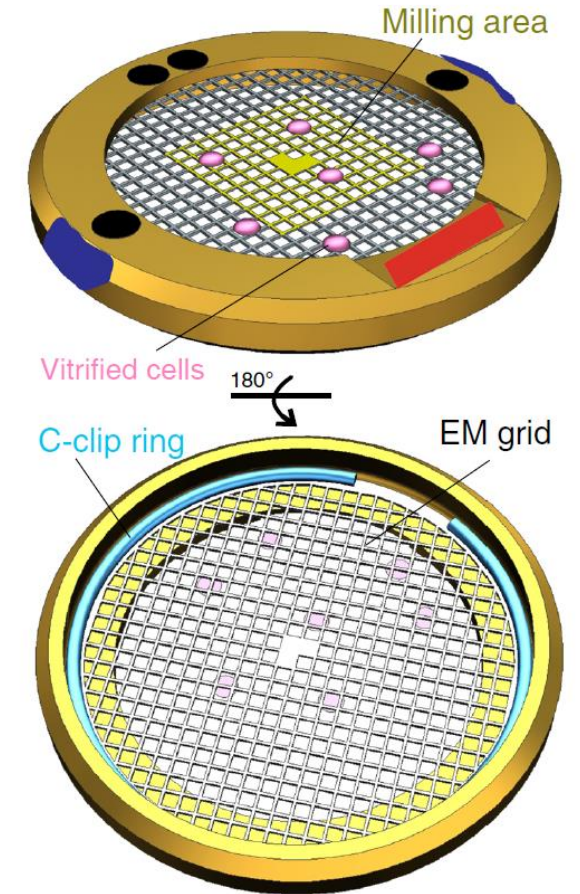
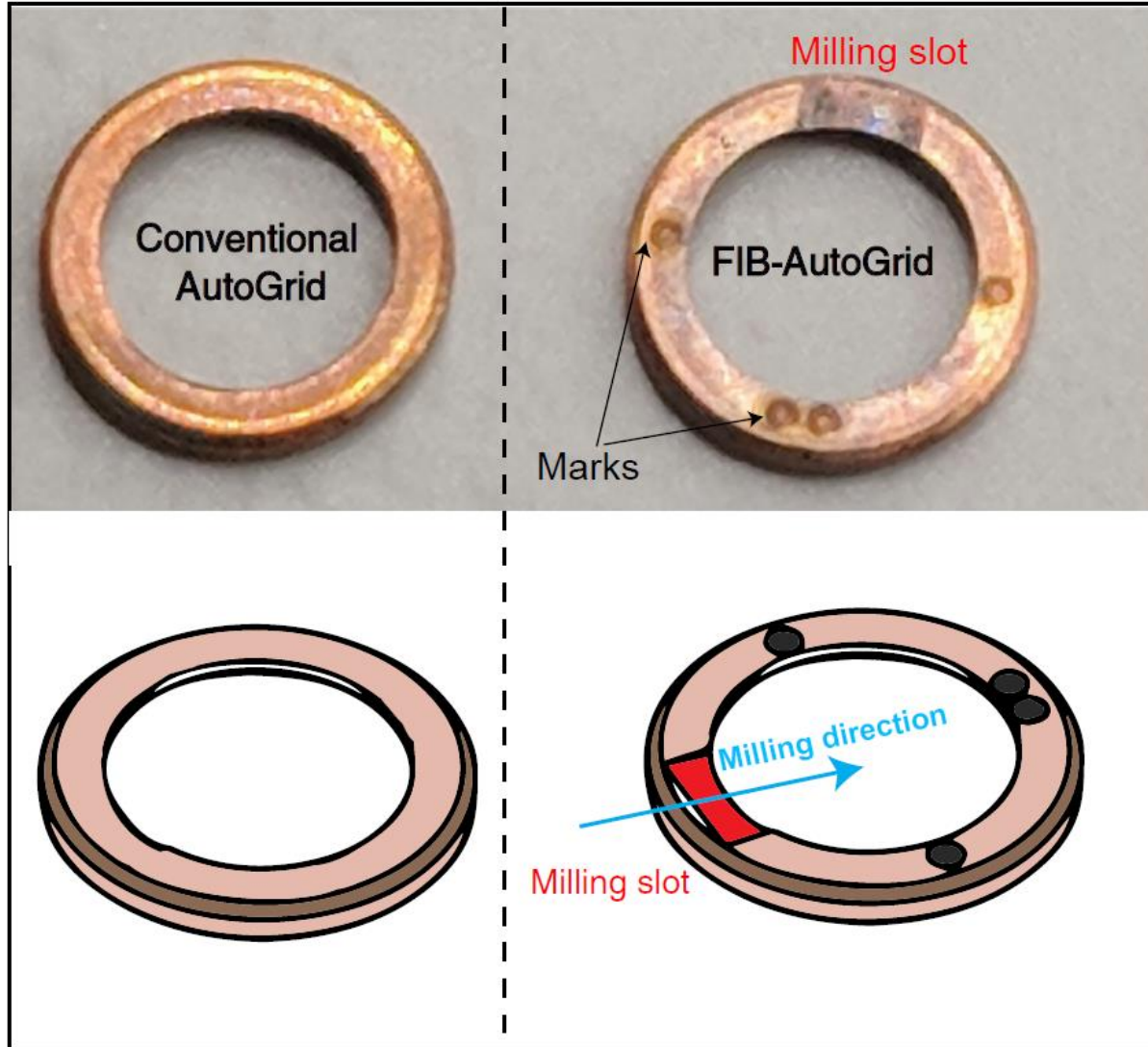
Target confirmation

**Pt sputter** (Optional)

Lamella conductivity



**CryoET**



Wagner, et al., Nature Protocols, 2020



### 3.2.2 Prepare the grids **\_Mark the grid rim for future lamellae orientation**

**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)

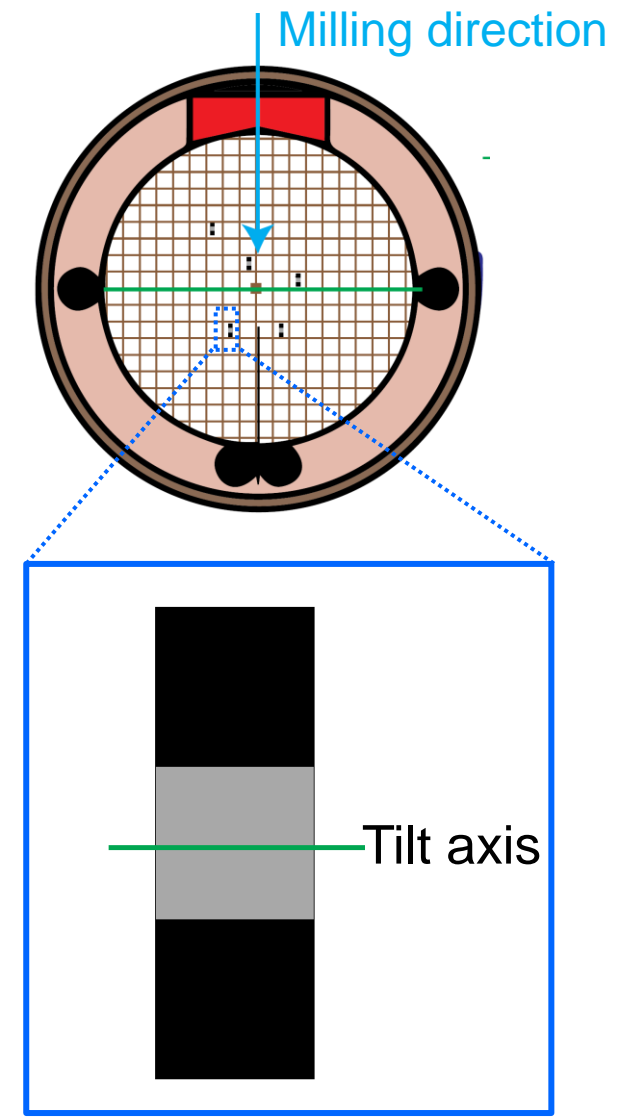
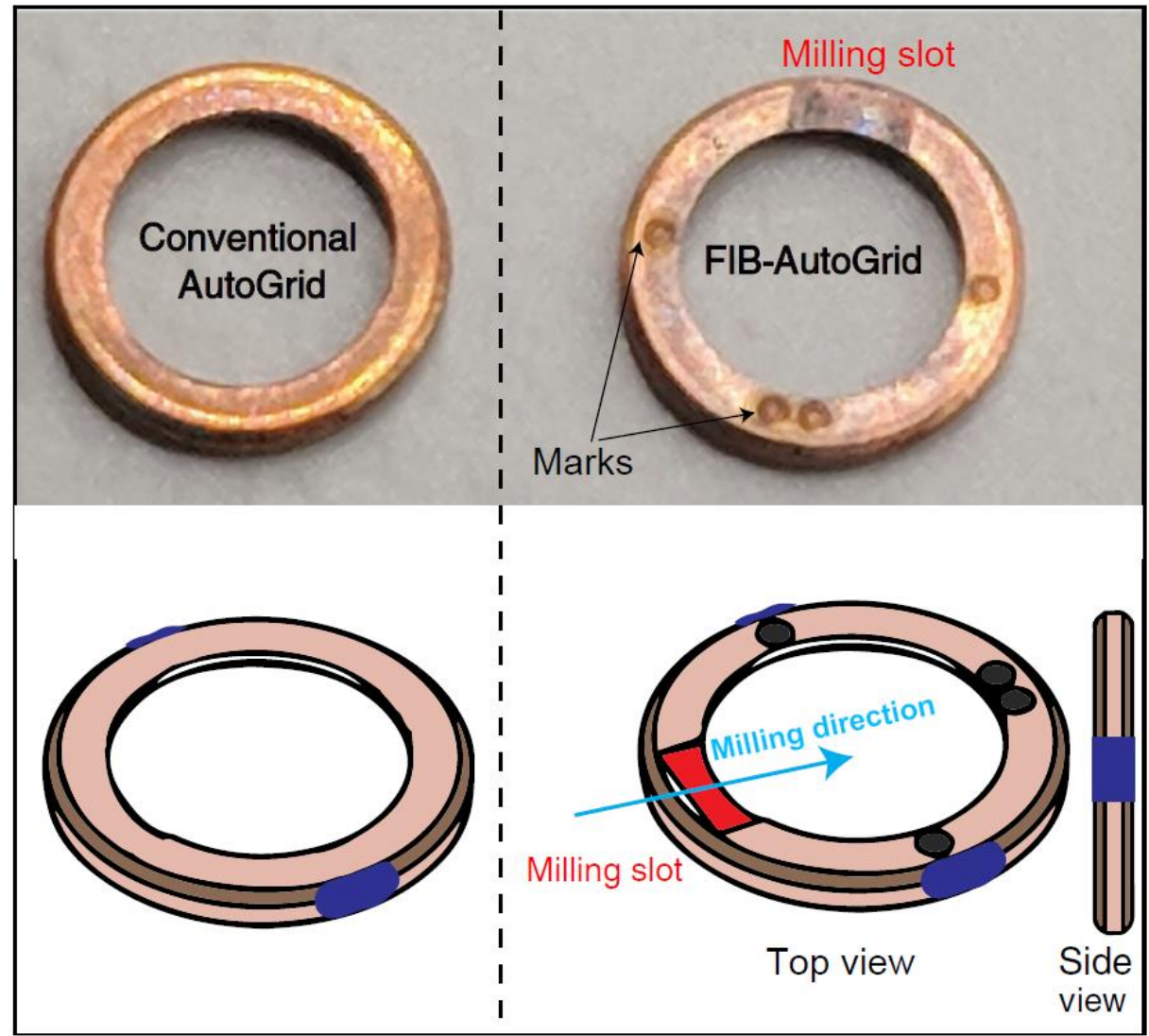
Target confirmation

**Pt sputter** (Optional)

Lamella conductivity



**CryoET**



Wagner, et al., Nature Protocols, 2020

# Orientating the lamellae

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)

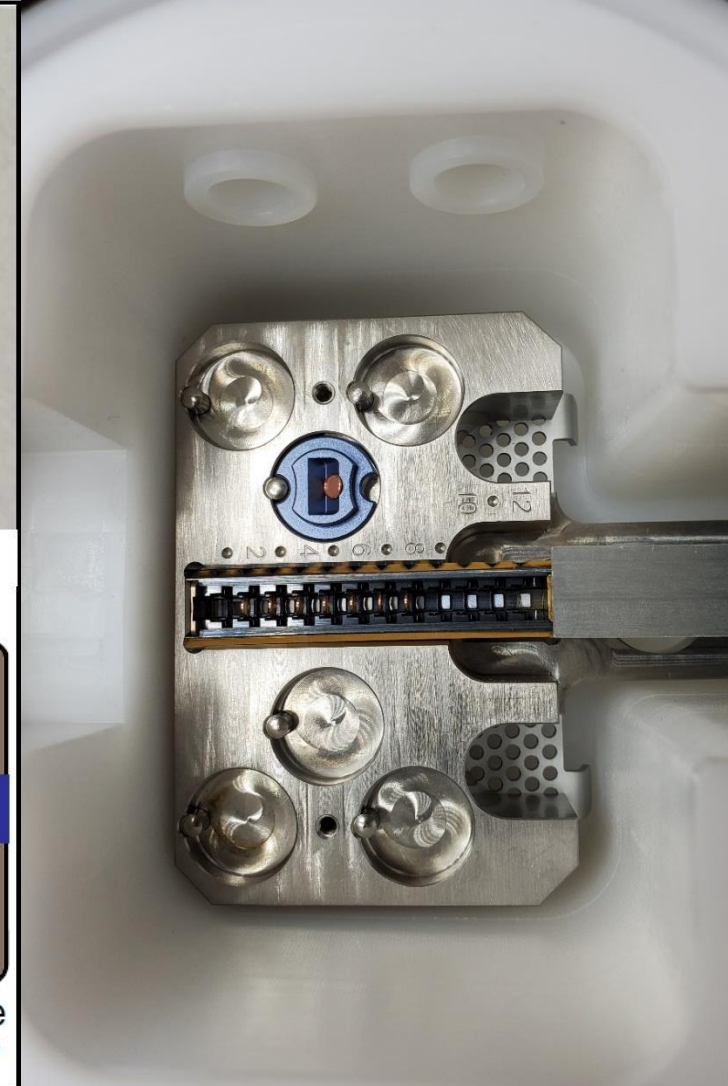
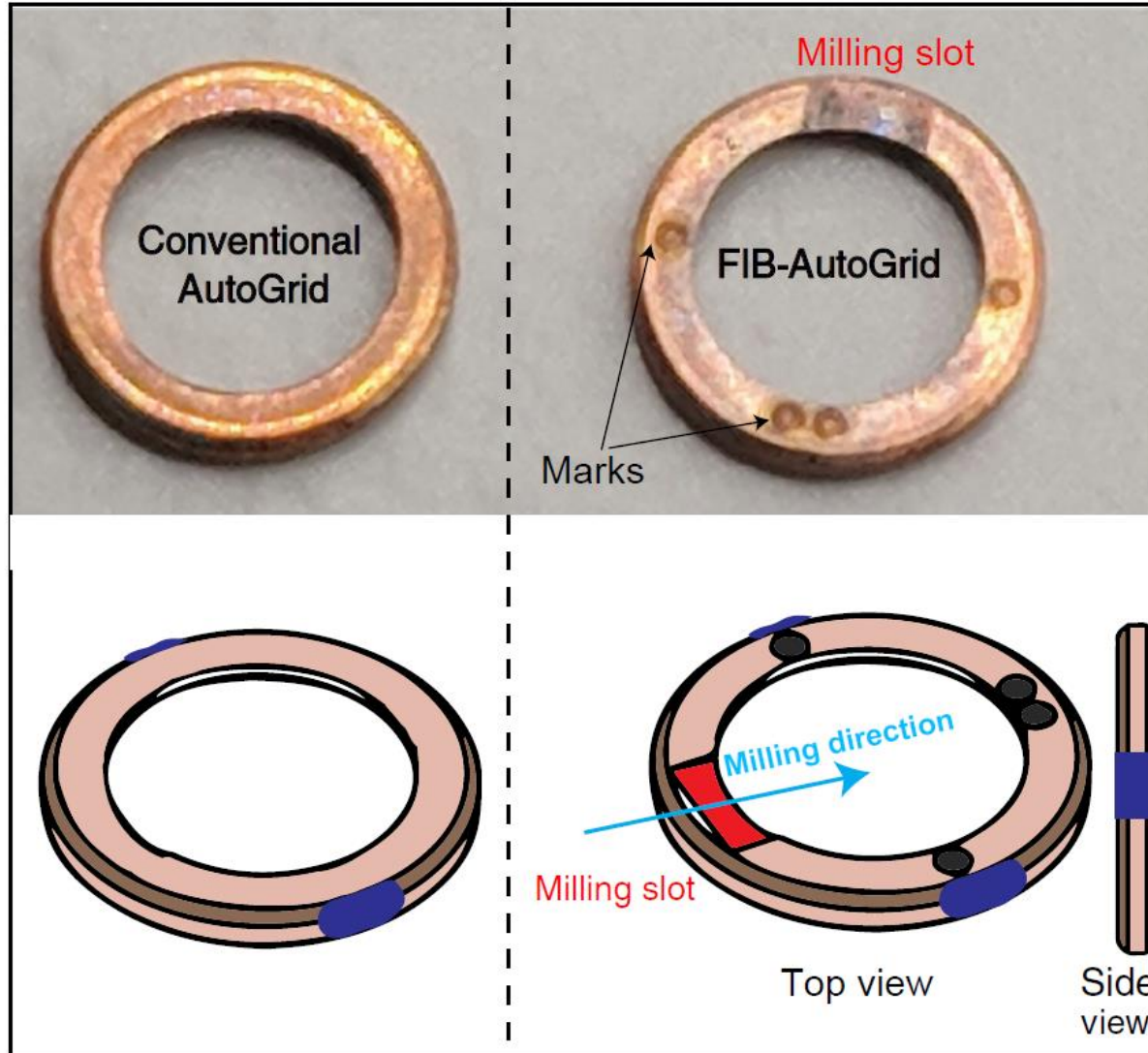
Target confirmation

**Pt sputter** (Optional)

Lamella conductivity



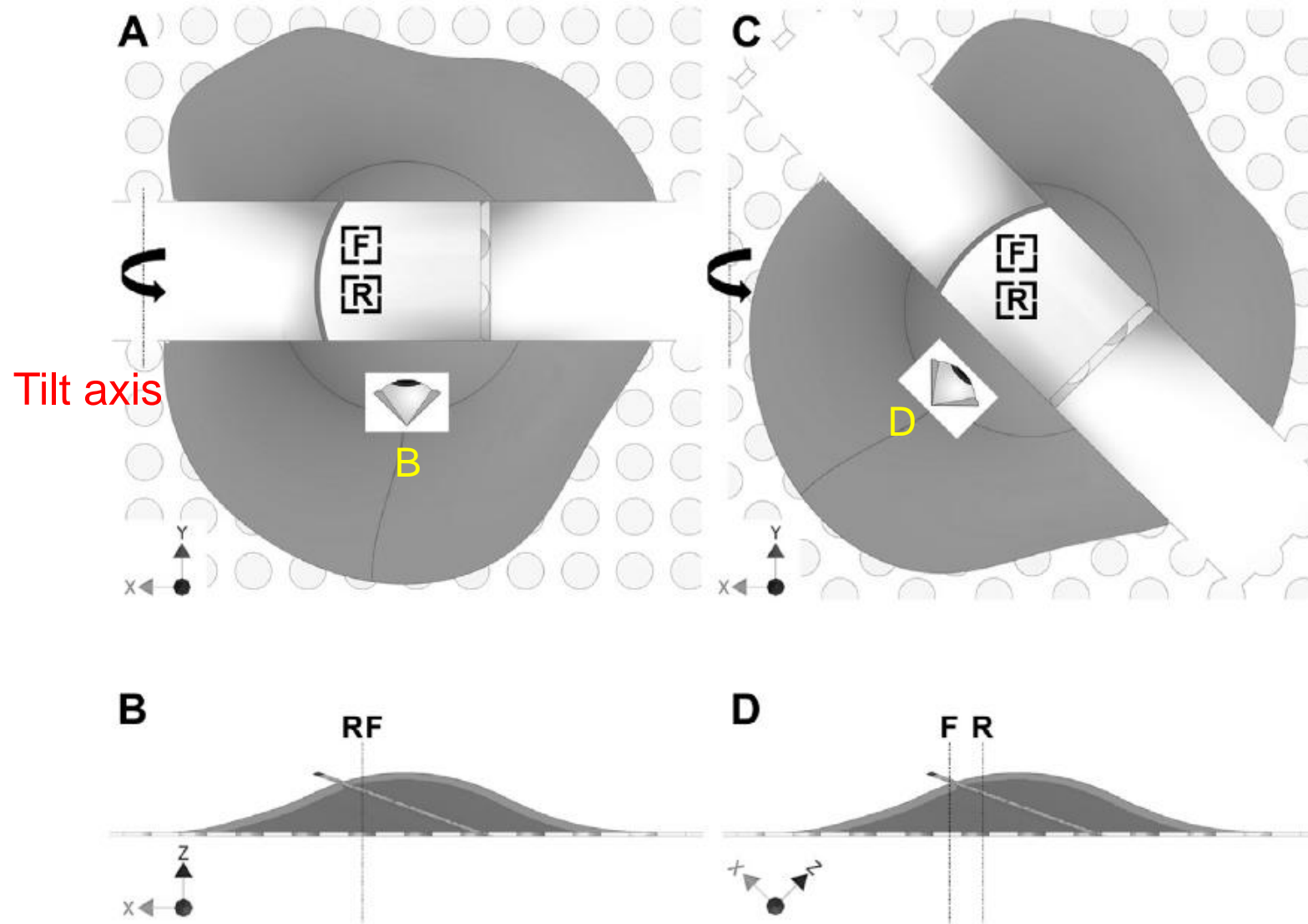
**CryoET**



Wagner, et al., Nature Protocols, 2020



# Poor orientation leads to inaccurate focusing & occlusion at high tilt angles



### 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

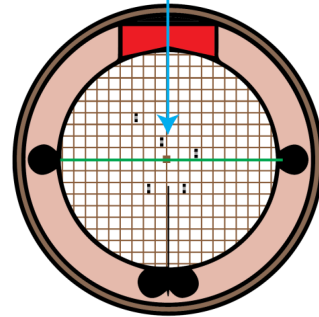
**Pt sputter** (Optional)

Lamella conductivity

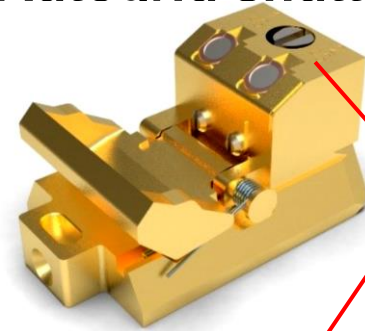


**CryoET**

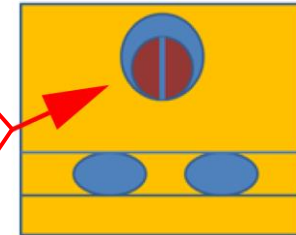
Milling direction



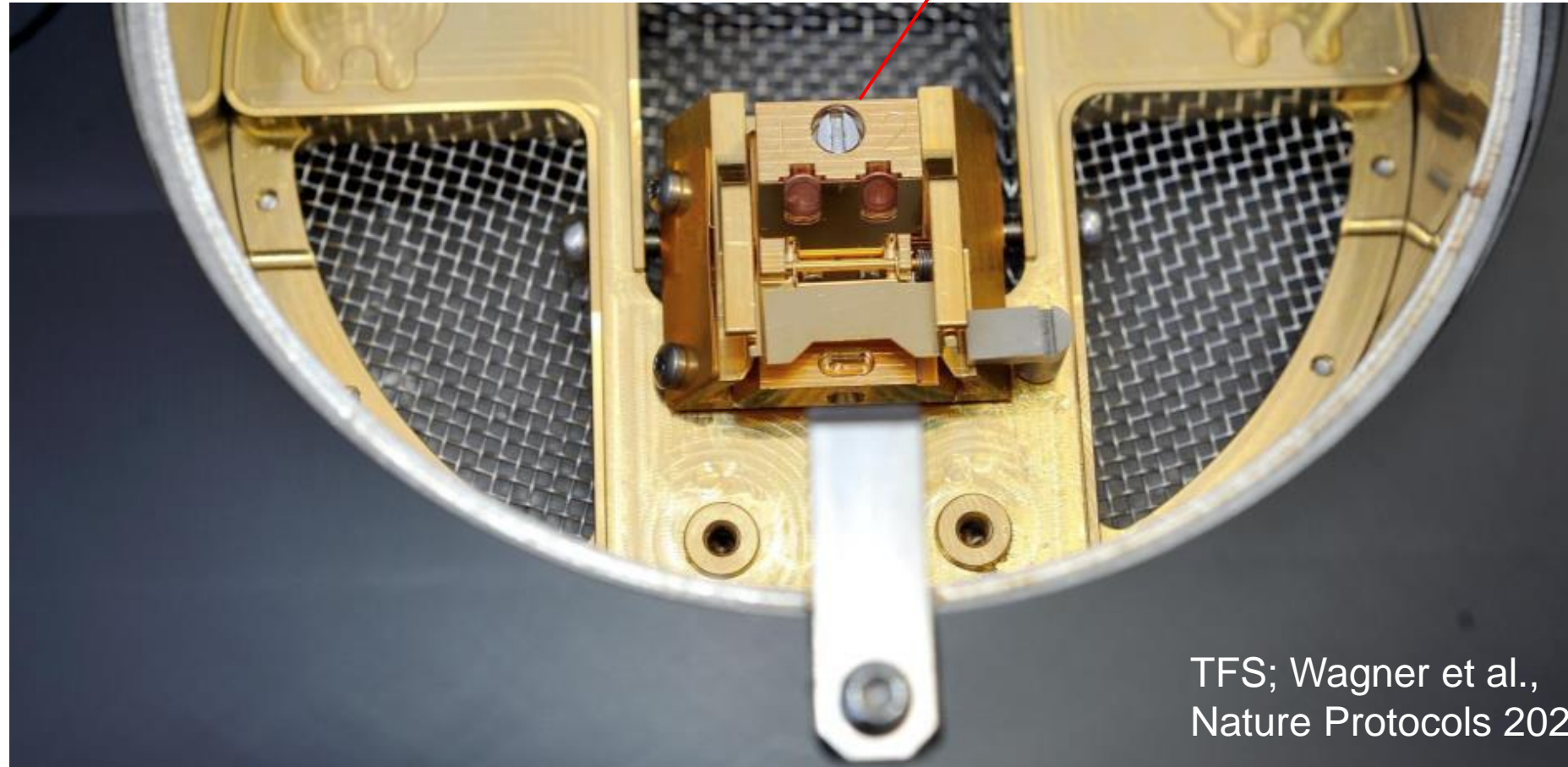
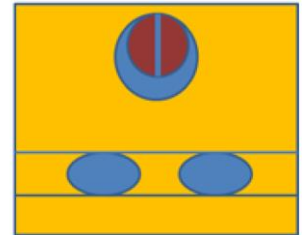
**Autogrid shuttle**



Open



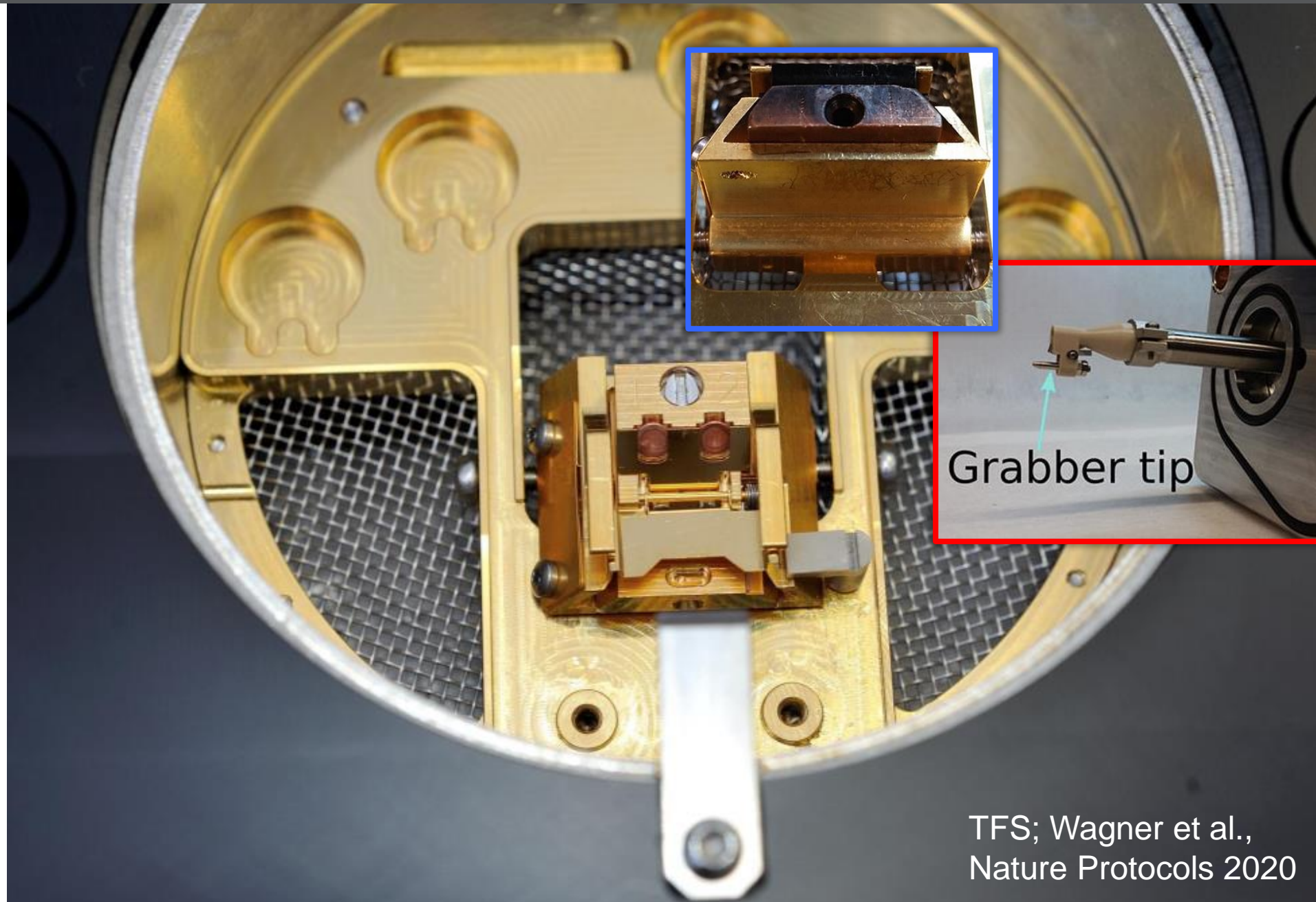
Closed



TFS; Wagner et al.,  
Nature Protocols 2020



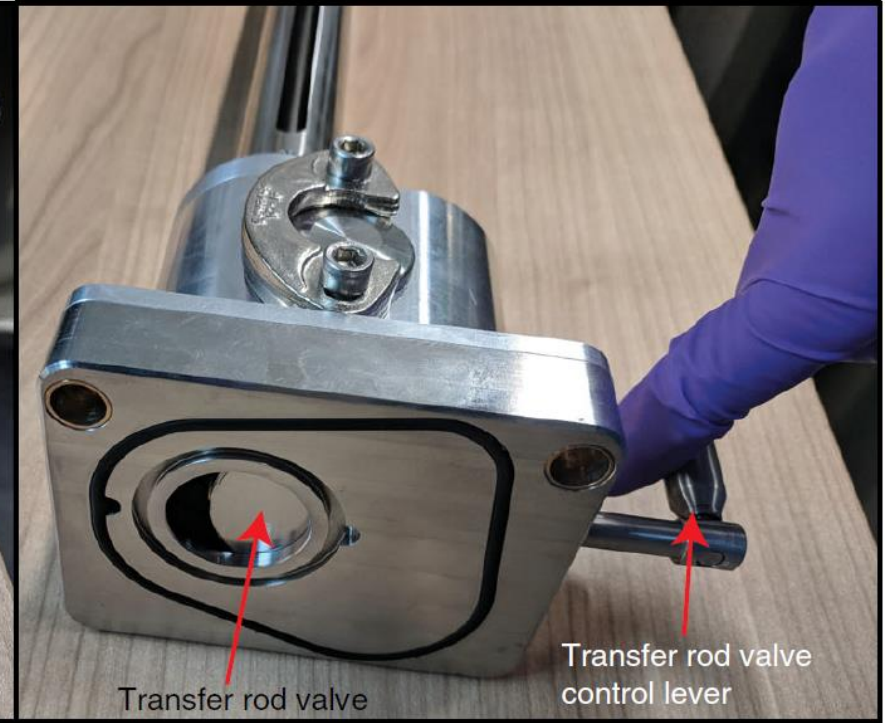
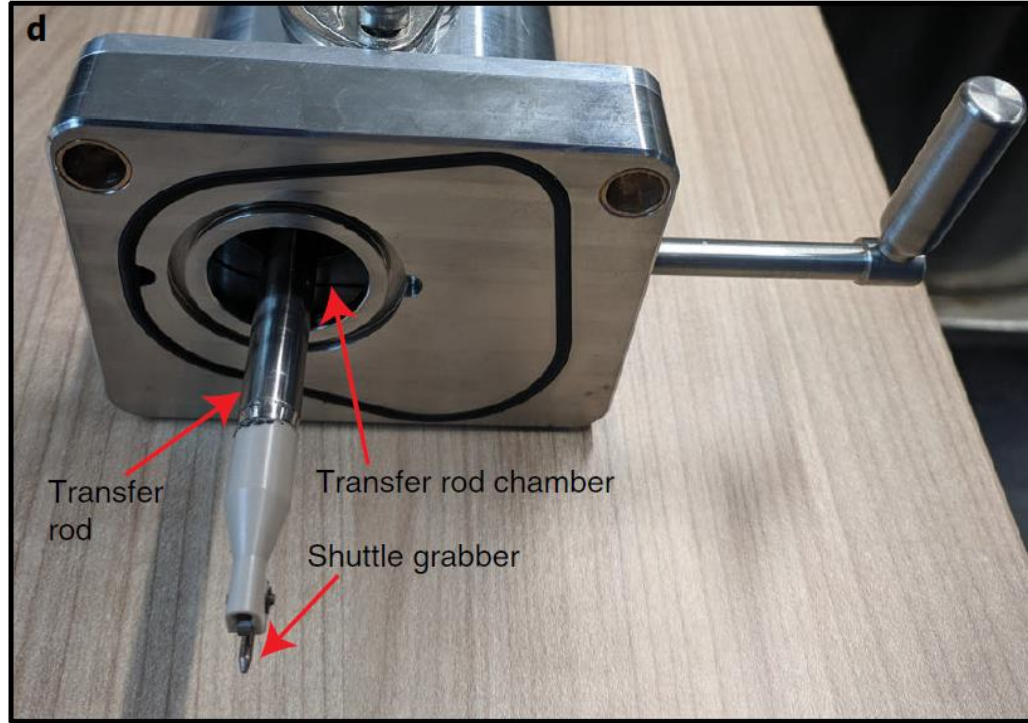
### 3.2.3 Transfer the grids Load shuttle to Transfer rod



TFS; Wagner et al.,  
Nature Protocols 2020



### 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

(Video from TFS)





# 3.3 Sample screening **\_Setup E-beam**

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. The main window is divided into four panels. The top-left panel shows a circular grid of sample sites. The top-right panel shows a high-magnification SEM image of a lamella. The bottom-left panel shows a close-up of the sample holder. The bottom-right panel shows a 3D schematic of the microscope column. The interface includes a menu bar (File, Edit, Detectors, Scan, Beam, Patterning, Stage, Tools, View, Help) and a toolbar. The right-hand control panel contains various parameters and controls, including Vacuum (High Vacuum, Sputter Vacuum), System (Wake Up, Beams Off), Column (Beam On, Beam Current, High Voltage), Magnification (Magnification, Couple Magnifications), Beam (Stigmator, Beam Shift), Beam Deceleration (On, Stage Bias), Scan Rotation (Scan Rotation), and Detectors (Contrast, Brightness). A red box highlights the 'Beam On' button in the Column section. The status bar at the bottom shows various parameters such as Chamber Pressure, Ion Beam Current, Specimen Current, Emission Current, Electron Source Pressure, and Temperatures.



# 3.3 Sample screening **Quickly check the grids**

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. The main window is divided into four quadrants showing different stages of sample preparation and analysis. The top-left quadrant shows a grid overview with a 500µm scale bar. The top-right quadrant shows a milling view with a 20µm scale bar. The bottom-left quadrant shows a sputter view with a 117mm scale bar. The bottom-right quadrant shows a GIS view with a 7mm scale bar. The right sidebar contains several control panels: Shuttle Controls (Selected Grid: Grid 1, Go to: Mapping, Milling, Deposition), Temperature & Pressure (Cryo Stage: -195.2 °C, Cryo Shield: -188.0 °C, Chamber Pressure: 5.10E-5 Pa, EasyLift A: N/A), Tilt Calculation (Holder Pretilt: 35.0°, Milling Angle: 26.2°, Stage Tilt: 23.2°), Cryo GIS Deposition (Gas: Pt dep, Selected Grid: Grid 1, Grid 2, Flow Duration: 00:02:00), Sputter Coating (Current: 30.0 mA, Pressure: 10 Pa, Voltage: 2 V, Run Time: 15 Sec), and Lamella Thickness. The bottom status bar shows various parameters: Chamber Pressure: 5.10E-5 Pa, Ion Beam Current: 0.22 pA, Specimen Current: 64.06 pA, Emission Current: 114.51 µA, 1.97 µA, Electron Source Pressure: 9.36E-9 Pa, Temperatures: -195.2 °C / -188.0 °C.



# 3.3 Sample screening **\_Link Z to FWD**

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. The main window is divided into four quadrants showing different views of the sample and the microscope. The top-left quadrant shows a low-magnification SEM image of a sample with a yellow crosshair. The top-right quadrant shows a higher magnification SEM image of the same sample. The bottom-left quadrant shows a close-up of the sample stage with a yellow crosshair. The bottom-right quadrant shows a close-up of the electron gun and column with a yellow crosshair and a 7 mm scale bar. The interface includes a menu bar at the top with options like File, Edit, Detectors, Scan, Beam, Patterning, Stage, Tools, View, and Help. A toolbar below the menu bar contains various icons for navigation and control. On the right side, there is a 'Stage' control panel with fields for X, Y, Z, T, and R coordinates. The Z coordinate is highlighted with a red box and shows a value of 22.4671 mm. Below the stage control panel is a 'Last Position' section with buttons for 'Add', 'Update', 'Remove', and 'Remove All'. There is also a 'Touch Alarm Enabled' checkbox and a small video window showing the microscope's internal view. At the bottom, there is a status bar with various parameters such as Chamber Pressure, Ion Beam Current, Specimen Current, Emission Current, Electron Source Pressure, and Temperature.

# 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)

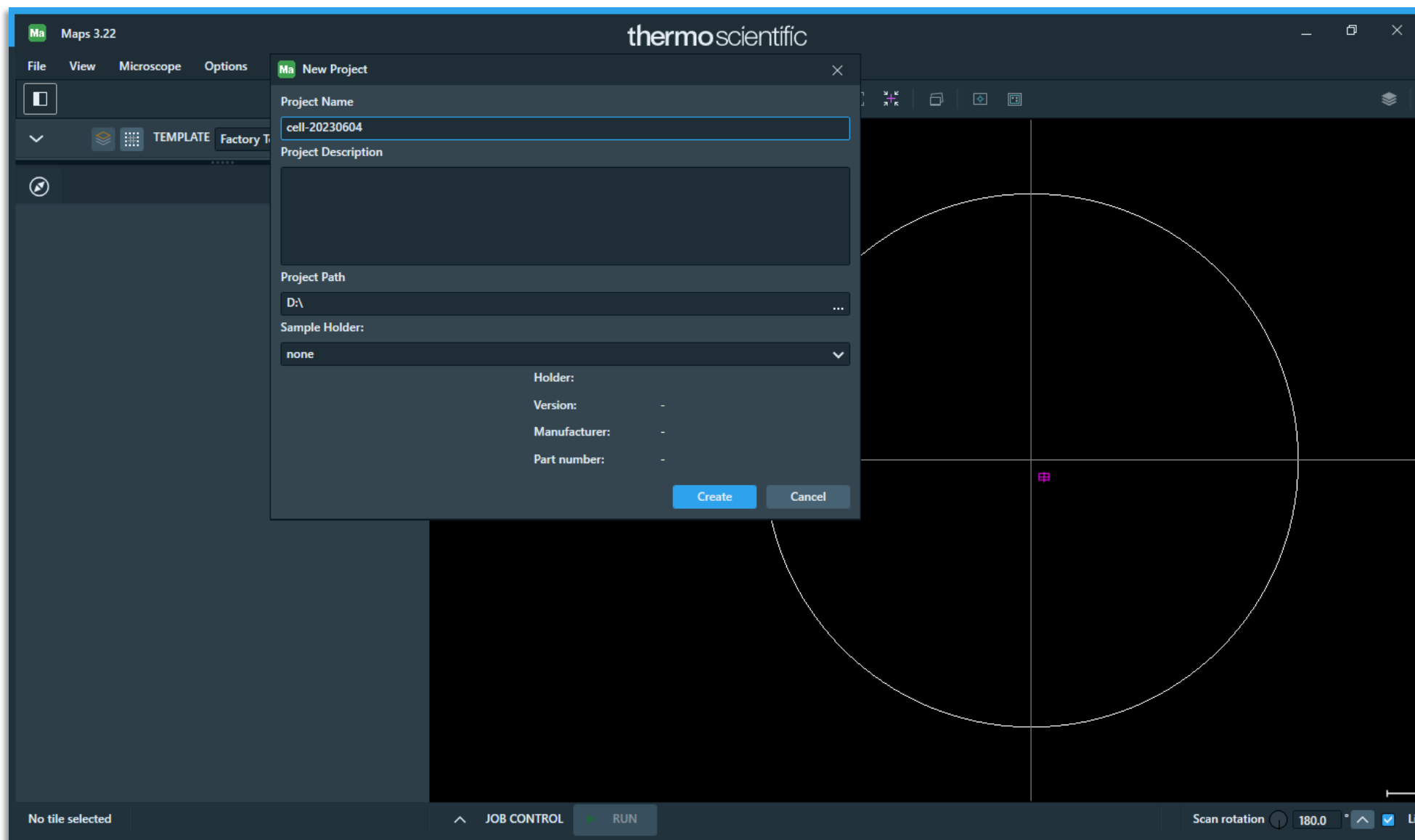
Target confirmation

**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

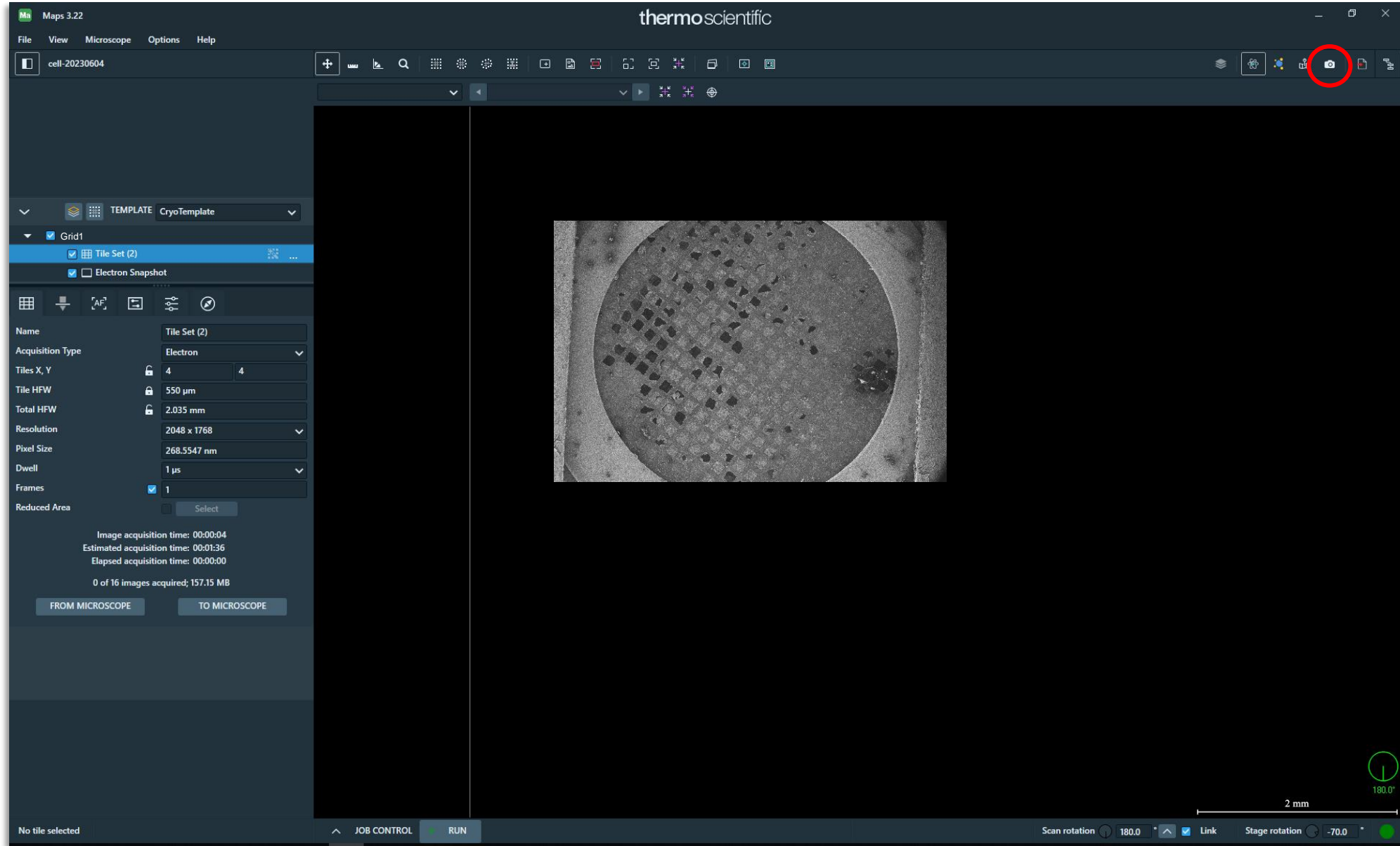
**Pt sputter** (Optional)

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

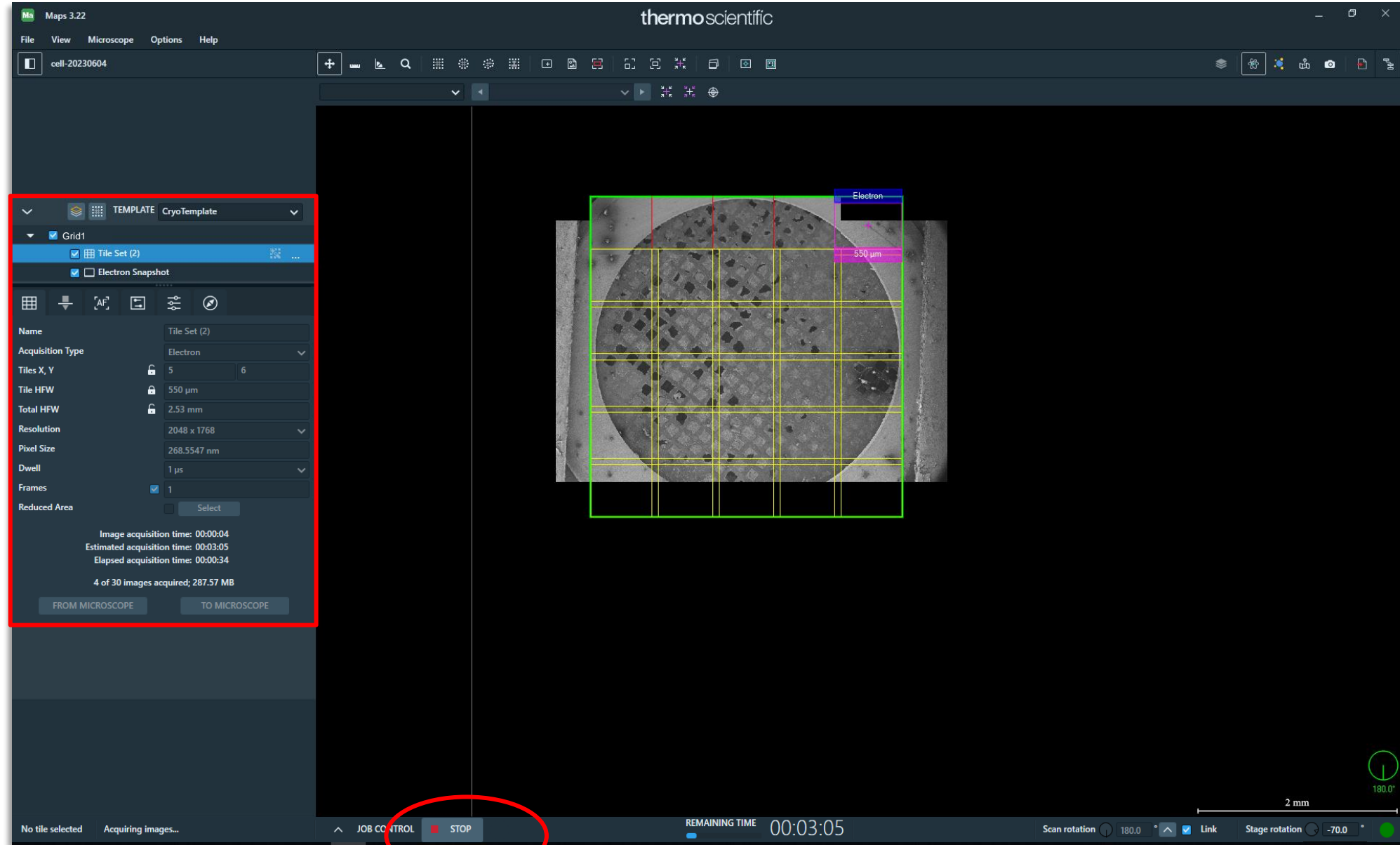
**Pt sputter** (Optional)

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

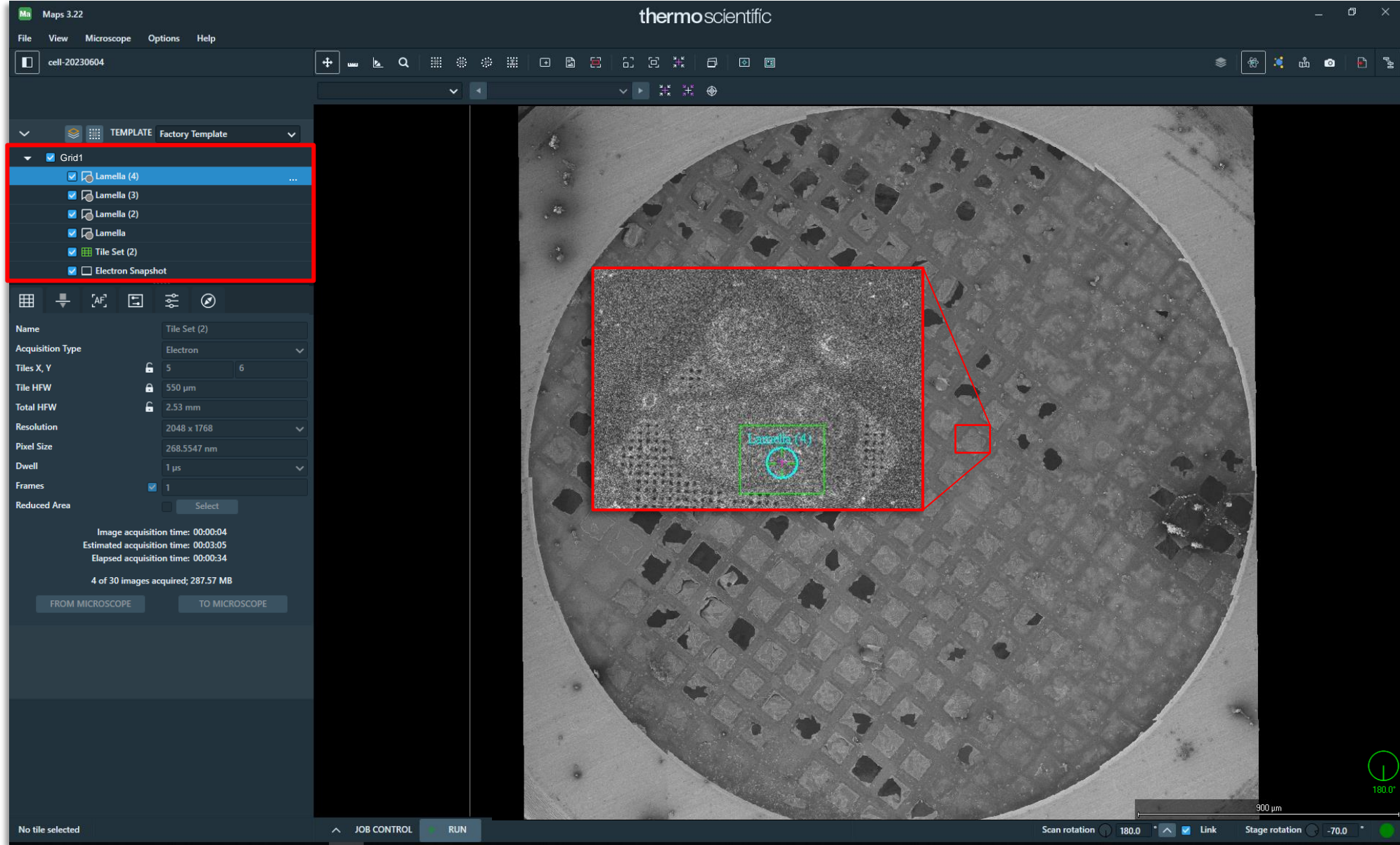
**Pt sputter** (Optional)  
Sample conductivity

**Lamella milling**  
Preparation, Milling,  
& thinning

**iFLM** (Optional)  
Target confirmation

**Pt sputter** (Optional)  
Lamella conductivity

↓ T  
**CryoET**



# 3.4 CryoFLM for 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)**

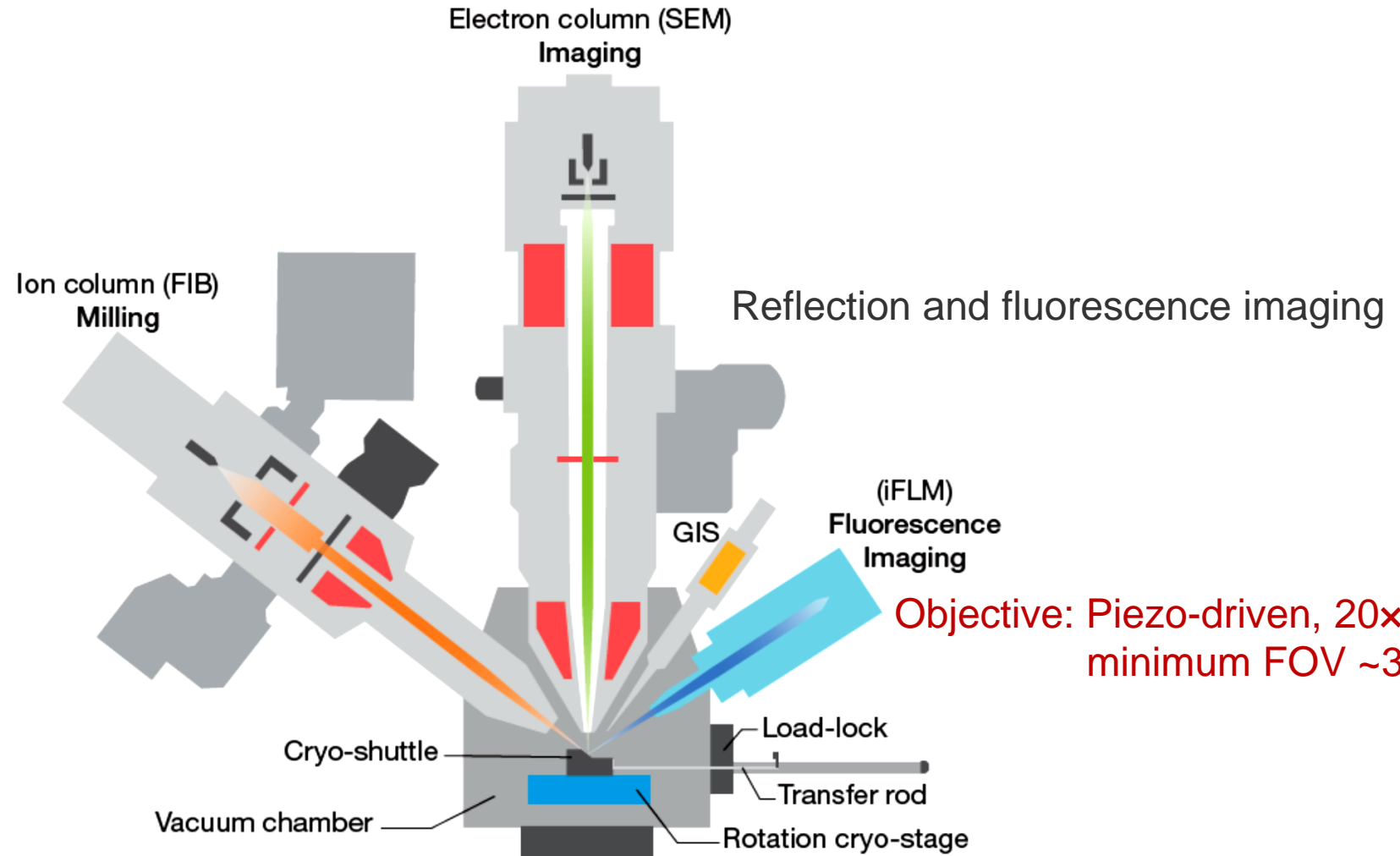
Target confirmation

**Pt sputter (Optional)**

Lamella conductivity



**CryoET**



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

# 3.4 CryoFLM \_Focus with Objective

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

The screenshot displays the 'Fluorescence Microscope Control 1.2.0' software interface. The 'Stage & Objective Control' panel is highlighted with a red box and contains the following controls:

- Buttons for SEM, LM, LM, and FIB.
- Current Relative Position (µm): 0.00
- Saved Focus Position (µm): 2509.62
- Buttons: Set Focus Position, Go to Focus Position
- Step Size (µm): 1
- Buttons: Retract (-), Advance (+)
- Reset Histogram Adjustment, Show Crosshair checkbox
- Objective inserted:
- Live View:
- Light Configuration: Fluorescence (selected), Reflection
- Excitation: 385nm, Binning: 1, Intensity: 5.0 [%], Exposure: 50.000 [ms]
- Snapshot button
- Binning, Gain, Gamma sliders
- Excitation/Emission/Intensity/Exposure table:

Excitation	Emission	Intensity	Exposure
<input type="checkbox"/> 625nm	<input type="checkbox"/>	10.0 [%]	10.000 [ms]
<input type="checkbox"/> 565nm	<input type="checkbox"/>	15.0 [%]	100.000 [ms]
<input type="checkbox"/> 470nm	<input type="checkbox"/>	20.0 [%]	50.000 [ms]
<input checked="" type="checkbox"/> 385nm	<input checked="" type="checkbox"/>	5.0 [%]	50.000 [ms]
- Create Stack section with Save Raw Copy, Pos. Start/End (µm), Number of Slices, and Slice Distance (µm) fields.
- Start Acquisition button

The 'Live View' panel shows a grayscale image of a sample with a 7 µm scale bar and scan rotation information: Scan Rotation E: 180.0°, Scan Rotation I: 180.0°. The bottom status bar shows: 6/4/2023 2:06:11 PM, det CCD, x: 43.5855 mm, tilt 17.0°, y: -6.1440 mm.

The 'Shuttle Controls' panel includes 'Selected Grid' (Grid 1 selected), 'Go to' buttons for Mapping, Milling, and Deposition, and a red circle highlighting the 'Mapping' button.



The top status bar displays the following parameters: 6/4/2023 2:01:02 PM, HV 30.00 kV, curr 10 pA, det ETD, mode SE, HFW 592 µm, WD 19.1 mm, rotation -70°. A 100 µm scale bar and 'Aquilos' label are also present.

The bottom status bar shows: 6/4/2023 2:06:11 PM, det CCD, x: 43.5855 mm, tilt 17.0°, y: -6.1440 mm. An inset image shows a histogram of the fluorescence data with a peak at approximately 4000 units.



# 3.4 CryoFLM **\_Setting up imaging parameters** ( 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

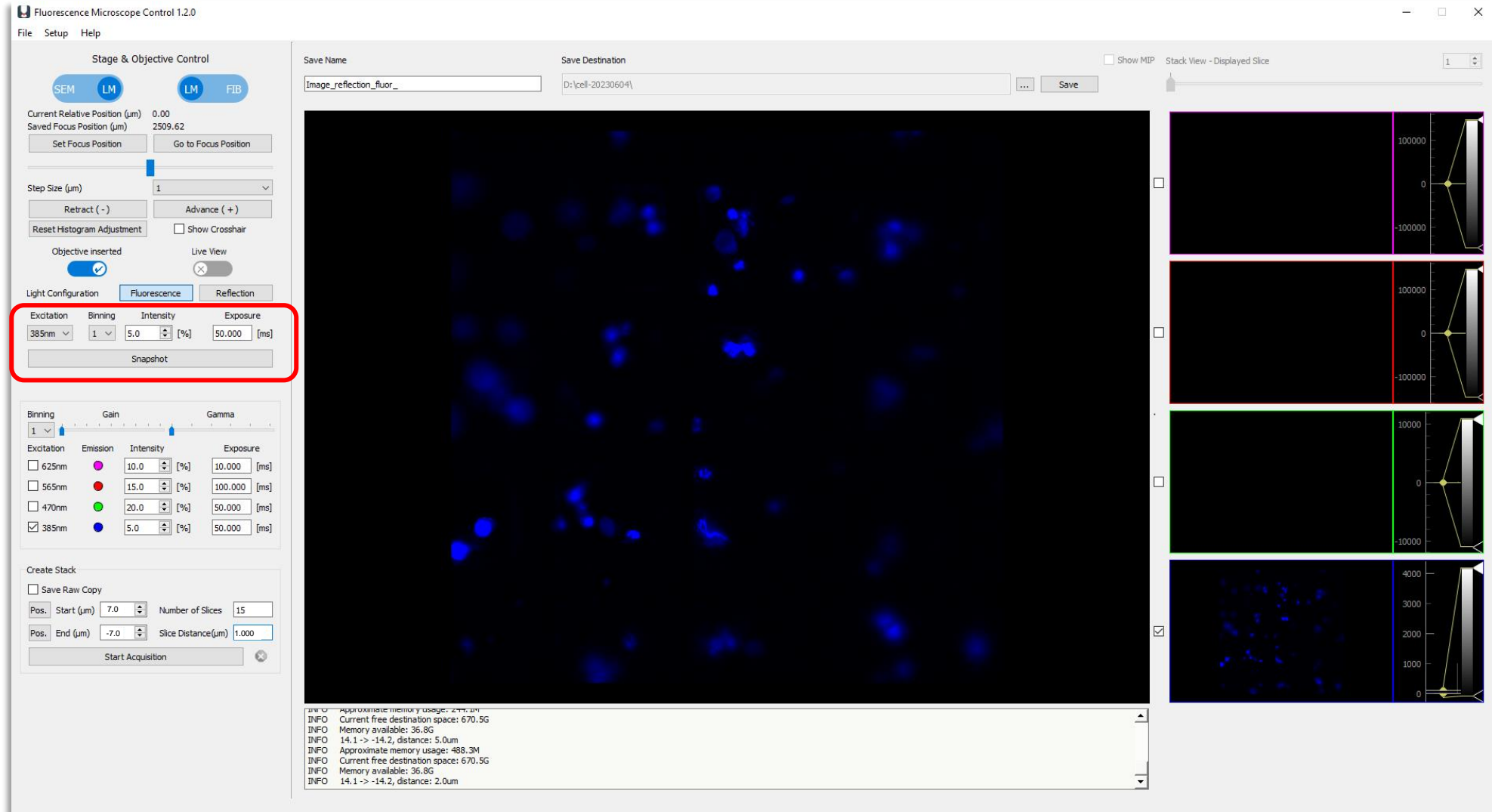
**iFLM (Optional)**

Target confirmation

**Pt sputter (Optional)**

Lamella conductivity

↓ T  
**CryoET**



Fluorescence Microscope Control 1.2.0

File Setup Help

Stage & Objective Control

SEM LM LM FIB

Current Relative Position (µm) 0.00  
Saved Focus Position (µm) 2509.62

Set Focus Position Go to Focus Position

Step Size (µm) 1

Retract (-) Advance (+)

Reset Histogram Adjustment Show Crosshair

Objective inserted Live View

Light Configuration Fluorescence Reflection

Excitation Binning Intensity Exposure

385nm 1 5.0 [%] 50.000 [ms]

Snapshot

Binning Gain Gamma

Excitation Emission Intensity Exposure

625nm 10.0 [%] 10.000 [ms]

565nm 15.0 [%] 100.000 [ms]

470nm 20.0 [%] 50.000 [ms]

385nm 5.0 [%] 50.000 [ms]

Create Stack

Save Raw Copy

Pos. Start (µm) 7.0 Number of Slices 15

Pos. End (µm) -7.0 Slice Distance (µm) 1.000

Start Acquisition

Image\_reflection\_fluor\_ D:\cell-20230604\ Save

Show MIP Stack View - Displayed Slice 1

Approximate memory usage: 244.4M  
INFO Current free destination space: 670.5G  
INFO Memory available: 36.8G  
INFO 14.1 -> -14.2, distance: 5.0um  
INFO Approximate memory usage: 488.3M  
INFO Current free destination space: 670.5G  
INFO Memory available: 36.8G  
INFO 14.1 -> -14.2, distance: 2.0um



# 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

Fluorescence Microscope Control 1.2.0

File Setup Help

Stage & Objective Control

SEM LM LM FIB

Current Relative Position (µm) 0.00  
Saved Focus Position (µm) 2509.62

Set Focus Position Go to Focus Position

Step Size (µm) 1

Retract (-) Advance (+)

Reset Histogram Adjustment Show Crosshair

Objective inserted Live View

Light Configuration Fluorescence Reflection

Excitation	Binning	Intensity	Exposure
385nm	1	5.0 [%]	50.000 [ms]

Snapshot

Binning Gain Gamma

Excitation	Emission	Intensity	Exposure
<input type="checkbox"/> 625nm		10.0 [%]	10.000 [ms]
<input type="checkbox"/> 565nm		15.0 [%]	100.000 [ms]
<input type="checkbox"/> 470nm		20.0 [%]	50.000 [ms]
<input checked="" type="checkbox"/> 385nm		5.0 [%]	50.000 [ms]

Create Stack

Save Raw Copy

Pos. Start (µm) 7.0 Number of Slices 15

Pos. End (µm) -7.0 Slice Distance (µm) 1.000

Start Acquisition

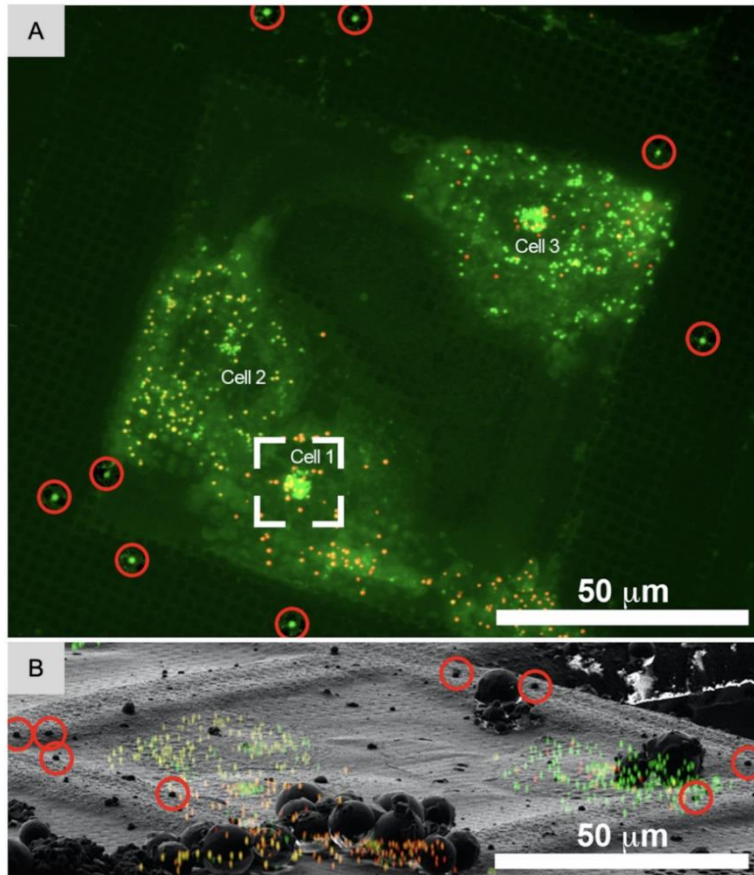
Save Name: Image\_reflection\_fluor\_ Save Destination: D:\cell-20230604\ Save

Show MIP Stack View - Displayed Slice 1

Approximate memory usage: 244.4M  
INFO Current free destination space: 670.5G  
INFO Memory available: 36.8G  
INFO 14.1 -> -14.2, distance: 5.0um  
INFO Approximate memory usage: 488.3M  
INFO Current free destination space: 670.5G  
INFO Memory available: 36.8G  
INFO 14.1 -> -14.2, distance: 2.0um

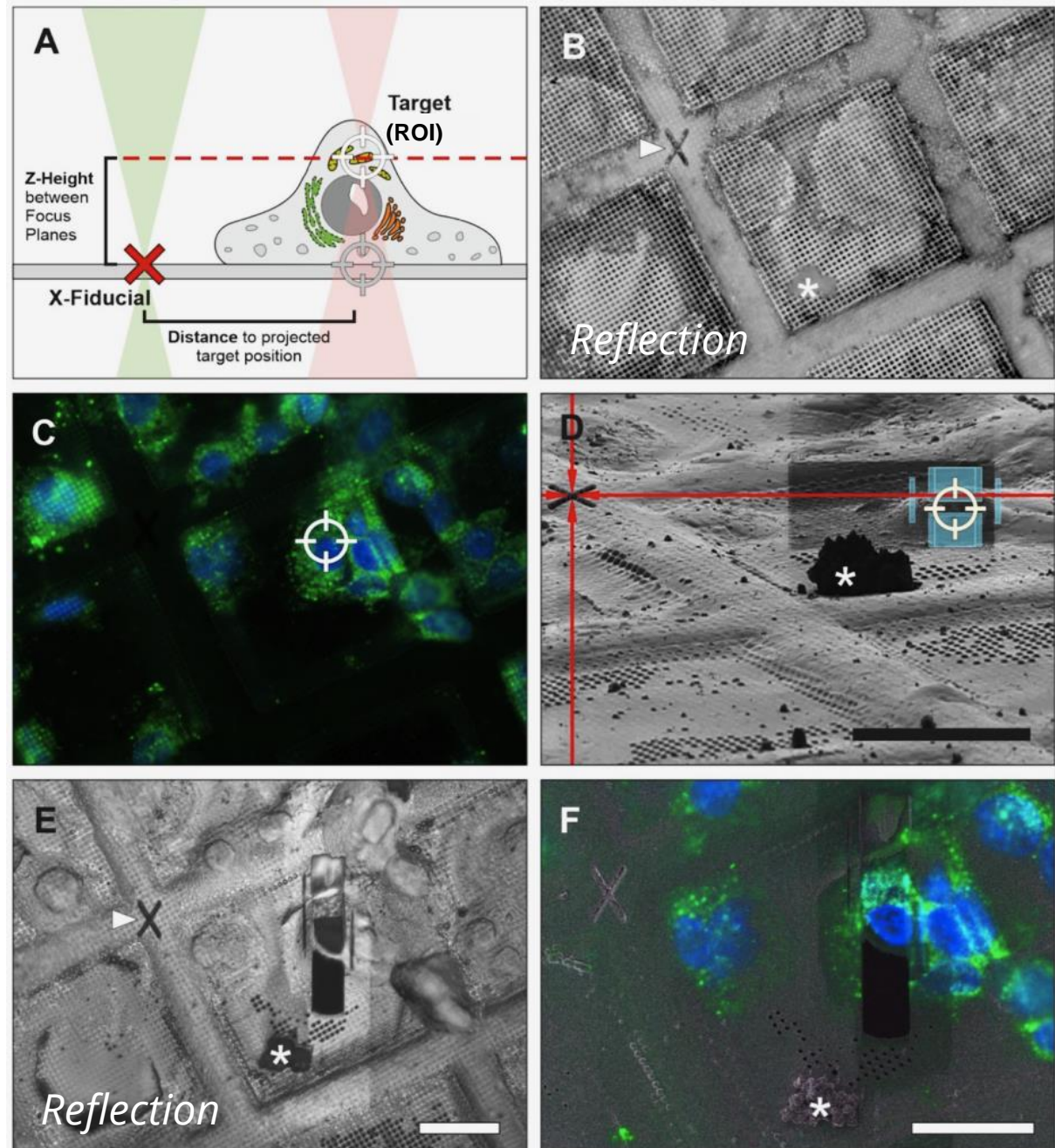
# Fiducial markers & X-Fiducials for accurate FLM-SEM alignment and target positioning

## 1- $\mu\text{m}$ Magnetic beads



Arnold et al., Biophysical Journal 2016

## X-Fiducials



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

# 3.4 CryoFLM \_FLM-SEM alignment

The screenshot displays the Ma Maps 3.22 software interface. The main window shows a grid of images with three alignment points marked: a pink circle, a green circle, and a blue circle. A scale bar at the bottom indicates 200 μm. The interface includes a menu bar (File, View, Microscope, Options, Help), a toolbar, and a sidebar with a file tree. The file tree shows a project named 'cell-20230604' with a 'Lamella sites' folder. The 'Electron Snapshot (5)' is selected. The 'Workflow: Alignment Wizard' panel on the right provides instructions: '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.' The wizard panel also includes controls for 'Points' (1, 2, 3), 'Link Rotation' (checked), 'Stage Axes', and 'Mirror Image'. At the bottom, the 'Multi-Point Alignment' section shows 'Scan rotation' at 180.0° and 'Stage rotation' at -70.0°.

thermoscientific

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:

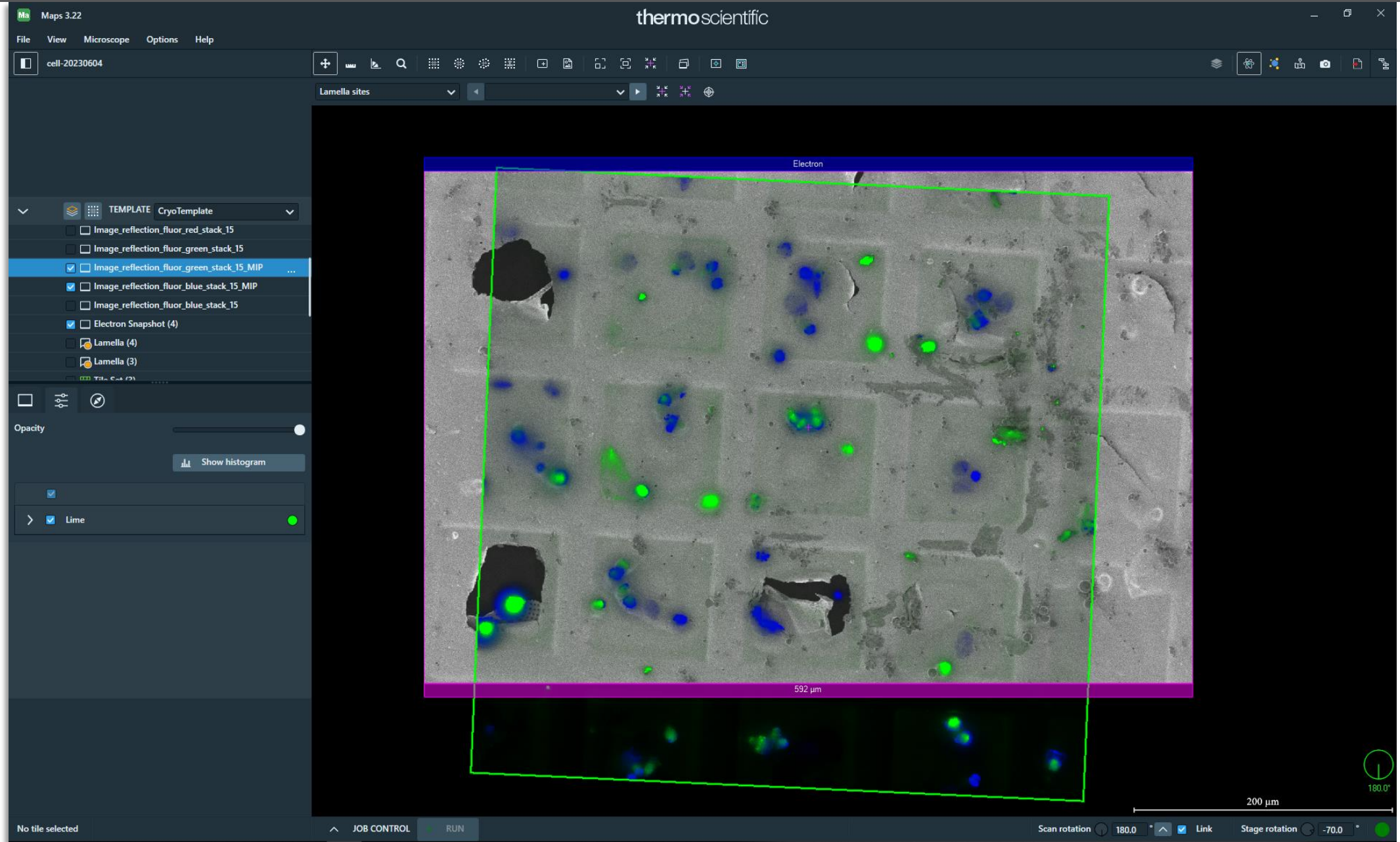
Mirror Image:

Multi-Point Alignment

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



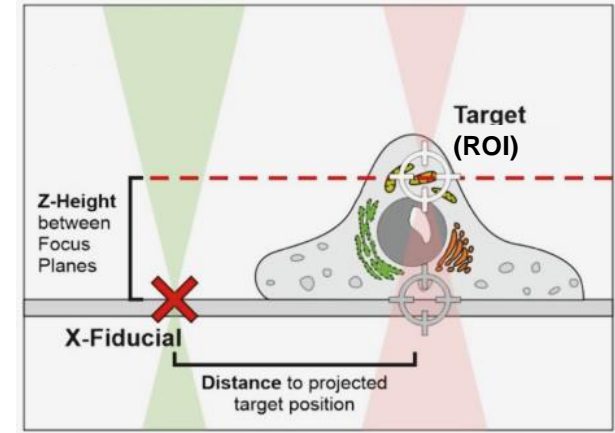
# 3.4 CryoFLM \_FLM-SEM alignment





# 3.4 CryoFLM \_Define ROI and Fiducial for each lamella

The screenshot shows the Ma Maps 3.22 software interface. The main window displays a cryo-EM image of a lamella with a green rectangular ROI and several blue fiducial markers. The left sidebar shows a template list with 'Image\_reflection\_fluor\_blue\_stack\_15' selected. The bottom status bar indicates 'JOB CONTROL' and 'RUN' buttons, along with scan rotation (180.0) and stage rotation (-70.0) settings.



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

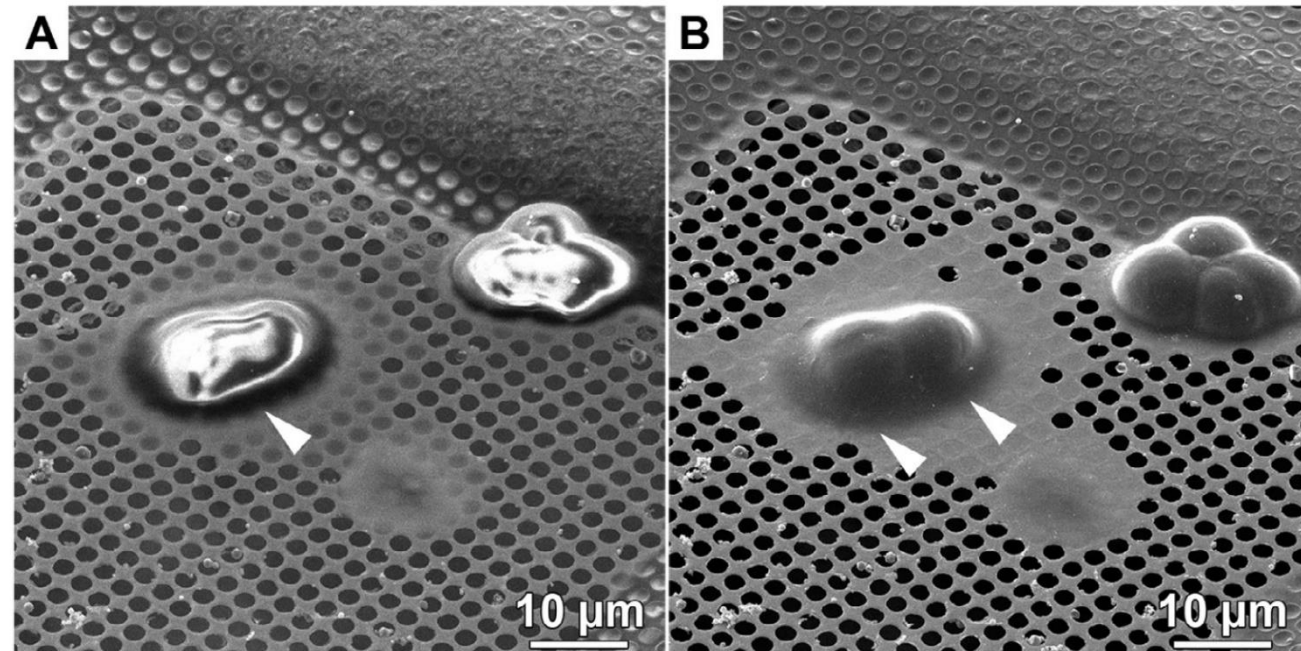
**Pt sputter** (Optional)

Lamella conductivity



**CryoET**

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





# 3.5 Pt sputter

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

FlowView 8 V1.23

UserTag USERTAG

Serialnr

Channel < 1 >

Setpoint Measure

28.6 % 28.6 %

100 80 60 40 20 0

100 80 60 40 20 0

V1.23

200.0 200.0 mg/s

Advance New About Exit

Increase to 200 mg/s  
(varies by instruments)

FEI File Edit Detectors Scan Beam Patterning Stage Tools View Help

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

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

Time

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: 00:01:30

Start Purge

Sputter Coating

Prepare for Sputtering

Current: 30.0 mA Pressure: 10 Pa

Voltage: 2 V Run Time: 15 Sec

Run 1 2 3 4 5

Activate Windows

Go to Settings to activate Windows.

Chamber Pressure: 14 Pa Ion Beam Current: -0.04 pA Specimen Current: 2.04 pA Emission Current: 113.61 μA 2.13 μA Electron Source Pressure: 1.87E-8 Pa

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

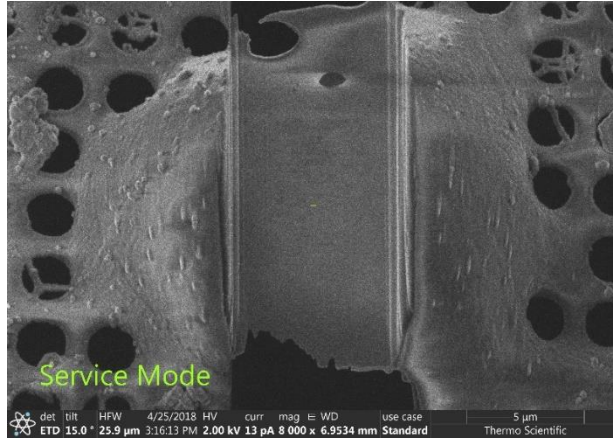
**Pt sputter** (Optional)

Lamella conductivity

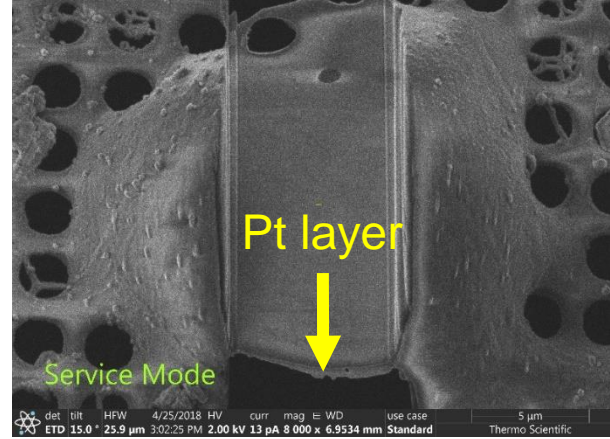


T

CryoET



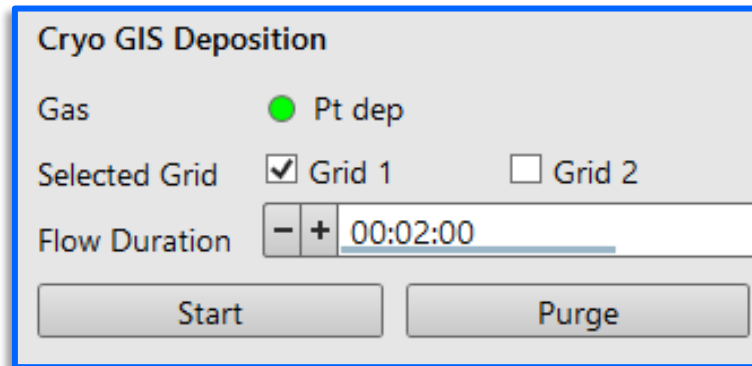
**Without Pt GIS coating**



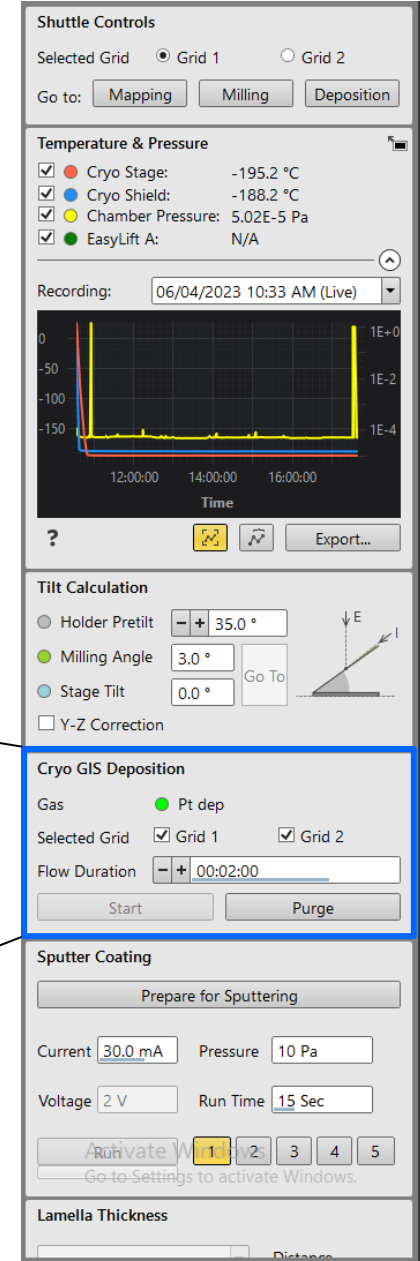
**With Pt GIS coating**

↑  
Milling  
direction

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

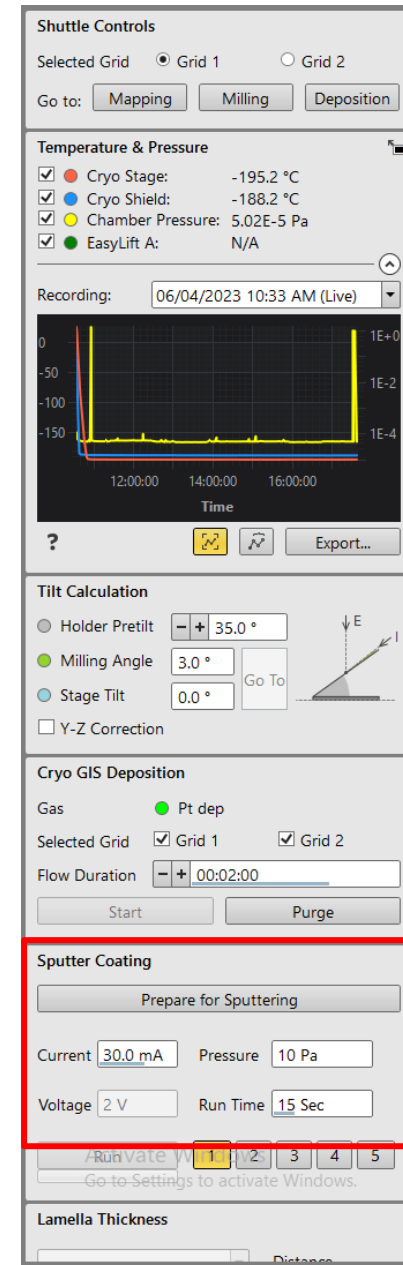
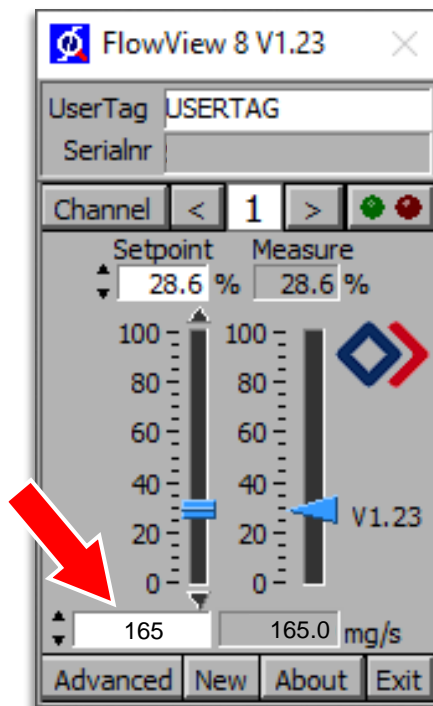
**Pt sputter** (Optional)

Lamella conductivity



**CryoET**

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



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

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**

The screenshot displays the AutoTEM Cryo AT software interface, which is organized into three main vertical panels: Preparation, Milling, and Thinning. The Preparation panel includes sections for EUCENTRIC TILT, ARTIFICIAL FEATURES, MILLING ANGLE, IMAGE ACQUISITION, and LAMELLA PLACEMENT. The Milling panel includes sections for DELAY, REFERENCE DEFINITION, STRESS RELIEF CUTS, and ROUGH MILLING. The Thinning panel includes sections for DELAY, POLISHING 1, and POLISHING 2. The interface is dark-themed and includes a top navigation bar with 'File', 'Project', and 'Site' menus, and a bottom status bar showing the version '2.4.2 (core 10.0.6.47)'.

# 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

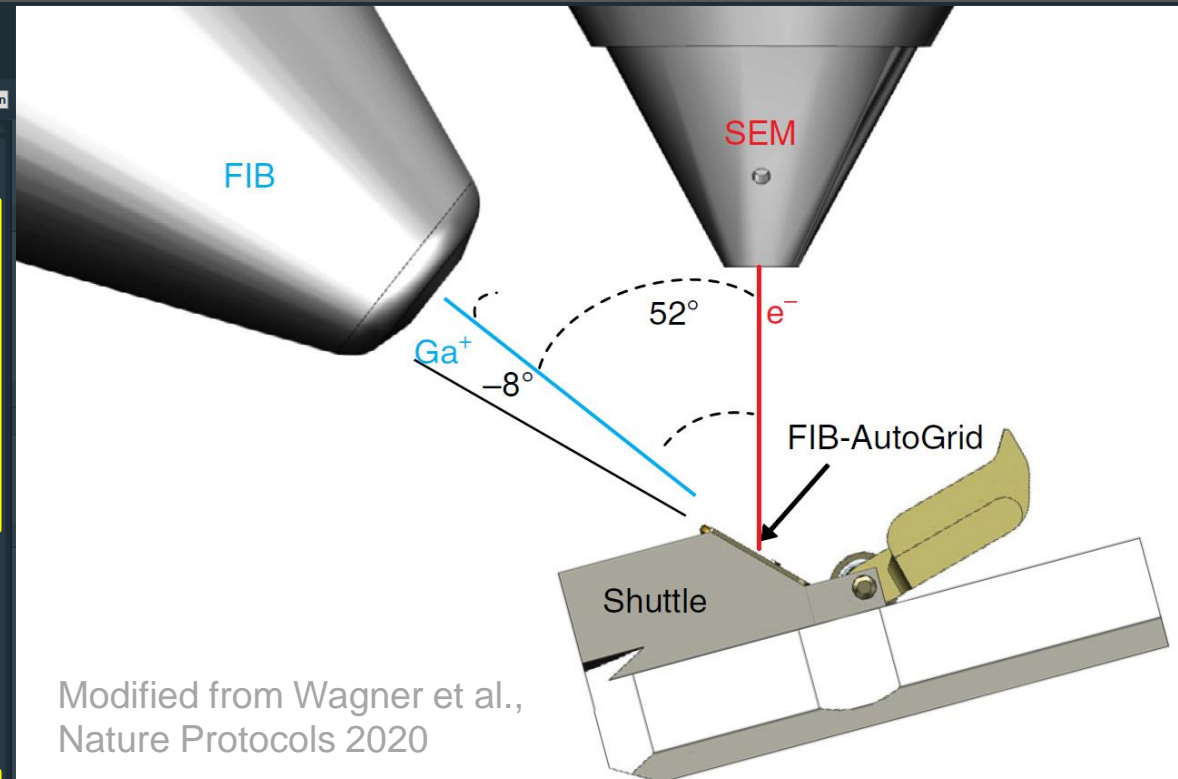
**Pt sputter** (Optional)

Lamella conductivity



CryoET

The screenshot shows the 'Preparation' settings in the AutoTEM Cryo software. The 'EUCENTRIC TILT' section is highlighted with a yellow box and includes: Maximal Tilt Step (10.0 °), Preparation HFW (250.0 μm), Resolution (guided) (1536 x 1024), and several checked options: Electron ACB, Ion ACB, Electron AutoFocus, Ion AutoFocus, Precise Coincidence, and Rough Centering. The 'MILLING ANGLE' section is also highlighted with a yellow box and includes: Target Milling Angle (9.0 °), Clearance Angle (2.0 °), Enforce Target (unchecked), and HFW (160.0 μm). Other sections visible include '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) and 'IMAGE ACQUISITION' (Ion HFW Oversize: 120 %, Resolution: 1536 x 1024 @ 4 μs, Enable ACB and Auto Focus checked). The 'LAMELLA PLACEMENT' section shows Ion HFW Oversize: 120 %.



Modified from Wagner et al., Nature Protocols 2020

The screenshot shows the 'ROUGH MILLING' and 'POLISHING 2' settings in the AutoTEM Cryo software. The 'ROUGH MILLING' section includes: 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), and DCM Rescan Interval (120 s). The 'POLISHING 2' section includes: Enable Auto Focus (unchecked), HFW (70.0 μm), Add lamella to HFW (checked), Notification (unchecked), High Voltage (2 kV), Beam Current (13 pA), Pattern Offset (0 μm), Overtilt (0 °), Depth Correction (160.0 %), High Voltage (30 kV), and Milling Current (30 pA).

# Artificial Features (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



CryoET

The screenshot displays the AutoTEM Cryo software interface. At the top, a TEM image shows a sample with a yellow arrow pointing to a specific feature. A yellow box highlights the 'ARTIFICIAL FEATURES' panel in the control interface, which includes the following settings:

- HFW: 200.0  $\mu\text{m}$
- Distance from lamella: 7.0  $\mu\text{m}$
- Pattern Depth: 1.0  $\mu\text{m}$
- Cross Thickness: 300.0 nm
- Milling Current: 0.30 nA
- Cross Size: 8.0  $\mu\text{m}$   $\times$  8.0  $\mu\text{m}$

Other visible panels include:

- STRESS RELIEF CUTS:** Trench Width (1.0  $\mu\text{m}$ ), Trench Depth (10.0  $\mu\text{m}$ ), Trench Height (6.5  $\mu\text{m}$ ), Trench Offset (5.0  $\mu\text{m}$ ), Depth Correction (100.0 %), Milling Current (0.50 nA), DCM Rescan Interval (120 s), Number Of Patterns (1).
- ROUGH MILLING:** Pattern Offset (1.0  $\mu\text{m}$ ), Front Pattern Height (5.0  $\mu\text{m}$ ), Rear Pattern Height (5.0  $\mu\text{m}$ ), Depth Correction (120 %), Front Width Overlap (1.5  $\mu\text{m}$ ), Rear Width Overlap (1.0  $\mu\text{m}$ ), Milling Current (1.0 nA), Pattern Type (Rectangle), DCM Rescan Interval (120 s).
- POLISHING 1 - ELECTRON IMAGE:** Resolution (1536 x 1024 @ 3  $\mu\text{s}$ ), HFW (70.0  $\mu\text{m}$ ), Add lamella to HFW (checked), High Voltage (2 kV), Beam Current (13 pA).
- POLISHING 2:** Pattern Offset (0  $\mu\text{m}$ ), Overtilt (0  $^\circ$ ), Depth Correction (160.0 %), High Voltage (30 kV), Milling Current (30 pA).

The interface also shows a 'TEMPLATE LIST' on the left with 'Default template (Aquilos) Plunge frozen grid' selected, and a 'Yeast-Jianfeng Plunge frozen grid' option. The bottom left corner indicates the software version: 2.4.2 (core 10.0.6.47).



# 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. On the left, a 'TEMPLATE LIST' shows 'Default template (Aquilos) Plunge frozen grid' selected. The main panel shows various settings for 'EUCENTRIC TILT', 'ARTIFICIAL FEATURES', 'MILLING ANGLE', 'IMAGE ACQUISITION', and 'LAMELLA PLACEMENT'. A yellow box highlights the 'STRESS RELIEF CUTS' section, which includes parameters like 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), and Number Of Patterns (1). To the right, three TEM images (A, B, C) show micro-expansion joints. Image A shows a lamella with a joint. Image B shows a lamella with two vertical bars and two yellow arrows pointing to the joints. Image C shows a close-up of a joint with two yellow arrows pointing to the expansion points. The text 'Wolff et al., JSB 2019' is visible below the images. The bottom right of the interface shows 'POLISHING 1 - ELECTRON IMAGE' settings, including Resolution (1536 x 1024), High Voltage (2 kV), and Beam Current (13 pA).

# 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)

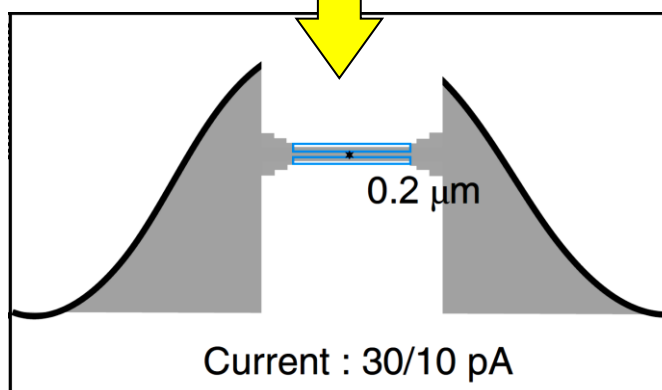
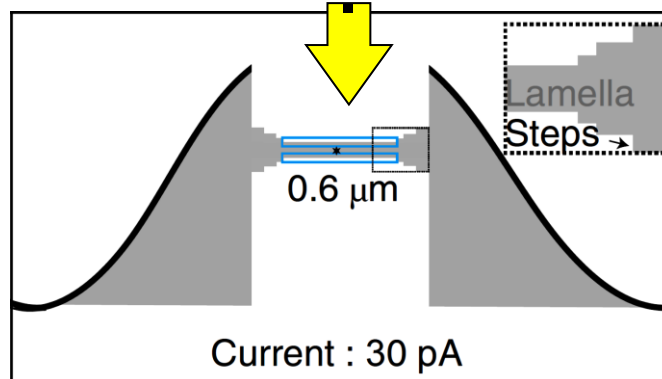
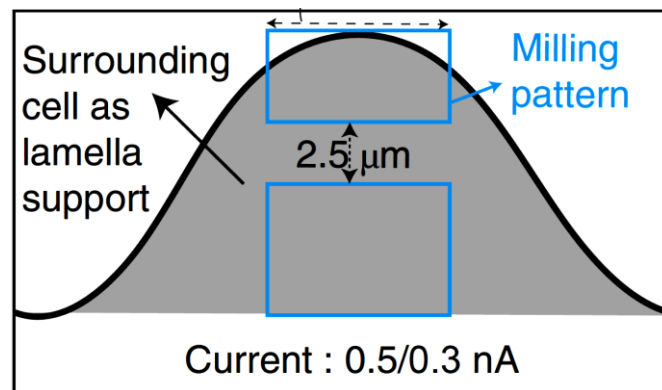
Target confirmation

**Pt sputter** (Optional)

Lamella conductivity

↓ T

**CryoET**



thermoscientific

Projects Templates AutoTEM-cell-20230604

action Milling action Thinning action

Lamella Size 10.0 μm × 3.0 μm

Correction Factor 0.50

Final Thickness 200.0 nm

Enable Windows

DELAY

Delay time 0 : 0

Estimated start time Sunday, June 4, 2023 12:16 PM

Beams Off

Safe stage position

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 Overlap 200.0 %

Pattern Type CleaningCrossSection

POLISHING 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

POLISHING 2

Pattern Offset 0 μm

Overtilt 0°

Depth Correction 160.0 %

High Voltage 30 kV

Milling Current 30 pA

DCM Rescan Interval ...

# 3.7.1 Automated lamella milling Preparation

**Automation**

PROJECT 5 min 1 s

PREPARATION MILLING THINNING

Automatic (with manual fall-backs) Automatic Automatic

Lamella	Time	Milling	Thinning
<input checked="" type="checkbox"/> Lamella (7)	5 min 1 s	--	--
<input type="checkbox"/> Lamella (6)	--	--	--
<input type="checkbox"/> Lamella (5)	--	--	--
<input type="checkbox"/> Lamella (4)	--	--	--
<input type="checkbox"/> Lamella (3)	--	--	--
<input type="checkbox"/> Lamella (2)	--	--	--
<input type="checkbox"/> Lamella	--	--	--

Execution Mode: Step-wise

1 site(s) in 5 min 1 s      0 site(s) in 0 s

**PREPARE** (Automatic (with manual fall-backs))    **MILL** (Automatic)    CANCEL

Finished Site Project N/A 0% 5 min 1 s **RUN** **STOP**

2.4.2 (core 10.0.6.47)



# 3.7.1 Automated lamella milling **\_Preparation**

The screenshot shows the AutoTEM Cryo software interface. On the left is a 'SITE LIST' with seven entries for 'Lamella' (1-7) with dimensions and status. The main window shows a cryo-ET image with a target ROI highlighted in blue. A yellow box highlights the 'Lamella Reposition' button in the 'PREPARATION' settings panel. The settings include: Ion HFW Oversize (30.0 μm), Milling Angle (10.2 °), CORRELATION (checked), Stack Offset (2.00 μm), Fiducial position [X,Y] (3.568233 mm, 3.870685 mm), ROI position [X,Y] (3.558625 mm, 3.867597 mm), and Show Graphics (checked). A diagram labeled 'A' illustrates the geometry of the electron beam, target ROI, and X-fiducial position. The diagram shows a green electron beam cone on the left and a red electron beam cone on the right. A target ROI is shown in the center, with a dashed red line indicating the Z-Height between Focus Planes. An X-Fiducial is marked with a red 'X' on the left. The distance to the projected target position is indicated by a bracket. The diagram is adapted from <https://cryoem101.org/chapter-2-et/>. At the bottom, there is a progress bar for 'Lamella (7)' and buttons for 'PREPARATION', 'MILLING', and 'THINNING'. The status bar shows '2.4.2 (core 10.0.6.47)' and 'Lamella (7) Preparation execution'.

# 3.7.2 Automated lamella milling **\_Milling**

**Automation**

PROJECT 33 min 17 s

	PREPARATION	MILLING	THINNING
<input checked="" type="checkbox"/> Lamella (7)	Automatic (with manual fall-backs)	Automatic	Automatic
<input type="checkbox"/> Lamella (6)	--	24 min 48 s	--
<input type="checkbox"/> Lamella (5)	--	--	--
<input type="checkbox"/> Lamella (4)	--	--	--
<input type="checkbox"/> Lamella (3)	--	--	--
<input type="checkbox"/> Lamella (2)	--	--	--
<input type="checkbox"/> Lamella	--	--	--

Execution Mode: Step-wise

0 site(s) in 0 s      1 site(s) in 24 min 48 s

**PREPARE** (Automatic (with manual fall-backs))      **MILL** (Automatic)      CANCEL

**MILLING** Panel:

- Lamella Size: 10.0 μm × 3.0 μm
- Correction Factor: 0.50
- DELAY: OFF
- REFERENCE DEFINITION: 44 s ON
- ELECTRON REFERENCE DEFINITION: 1 min 46 s ON
- STRESS RELIEF CUTS: 2 min 23 s ON
- REFERENCE REDEFINITION 1: 44 s ON
- ROUGH MILLING: 17 min 7 s ON
- ROUGH MILLING - ELECTRON IMAGE: 37 s ON
- REFERENCE REDEFINITION 2: 44 s ON
- MEDIUM MILLING: 3 min 46 s ON
- MEDIUM MILLING - ELECTRON IMAGE: 27 s ON
- FINE MILLING: 4 min 32 s ON
- FINE MILLING - ELECTRON IMAGE: 27 s ON
- FINER MILLING: OFF
- FINER MILLING - ELECTRON IMAGE: OFF

Bottom Status Bar:

Lamella (7) 10.0 μm × 3.0 μm Default template (Aquilos)      PREPARATION      **MILLING**      THINNING      Finished Site Project      N/A 12 % 33 min 17 s      RUN      STOP

2.4.2 (core 10.0.6.47)

# 3.7.3 Automated lamella milling **\_Thinning**

**Automation**

PROJECT 8 min 45 s

	PREPARATION	MILLING	THINNING
<input checked="" type="checkbox"/> Lamella (7)	Automatic (with manual fall-bi)	Automatic	Automatic 8 min 45 s
<input type="checkbox"/> Lamella (6)			
<input type="checkbox"/> Lamella (5)			
<input type="checkbox"/> Lamella (4)			
<input type="checkbox"/> Lamella (3)			
<input type="checkbox"/> Lamella (2)			
<input type="checkbox"/> Lamella			

Execution Mode: Step-wise

0 site(s) in 0 s      1 site(s) in 8 min 45 s

**PREPARE** (Automatic (with manual fall-backs))      **MILL** (Automatic)      CANCEL

**THINNING**

Final Thickness: 200.0 nm

Enable Windows:

DELAY:

POLISHING 1: 3 min 20 s

POLISHING 1 - ELECTRON IMAGE:

POLISHING 2: 4 min 58 s

POLISHING 2 - ION IMAGE:

POLISHING 2 - ELECTRON IMAGE: 27 s

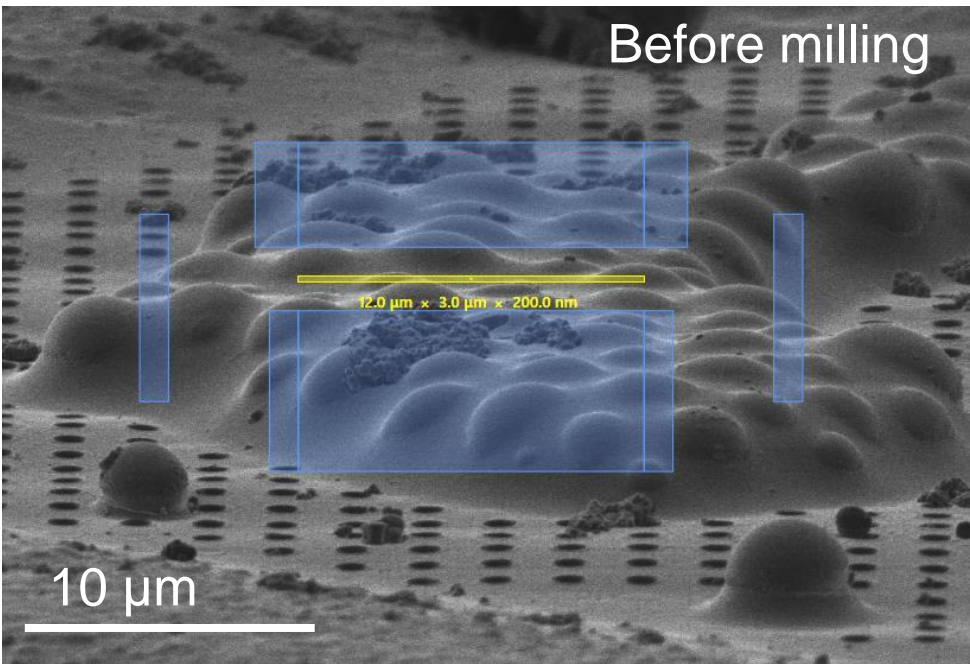
Finished Site Project: N/A 36 % 8 min 45 s

**RUN**      **STOP**

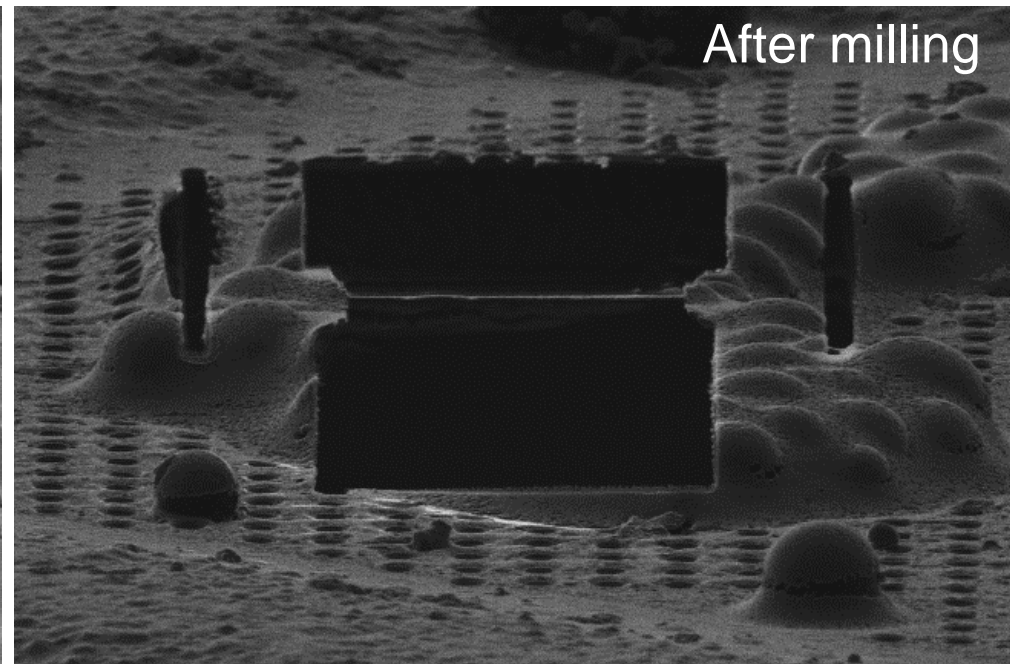
2.4.2 (core 10.0.6.47)



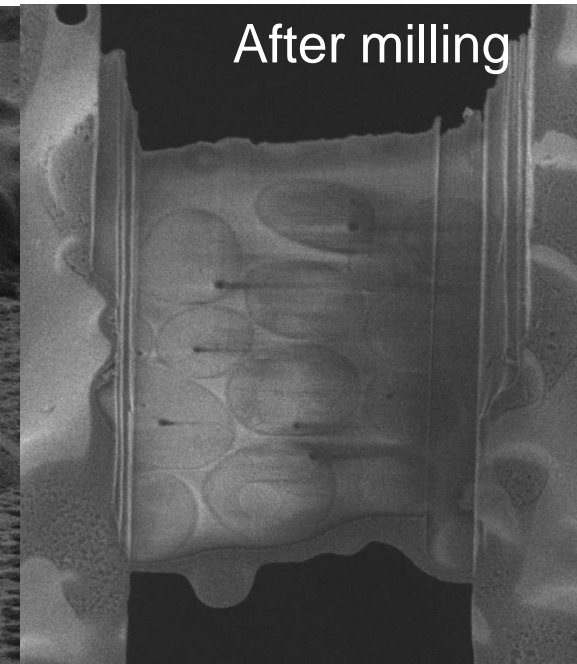
I-beam

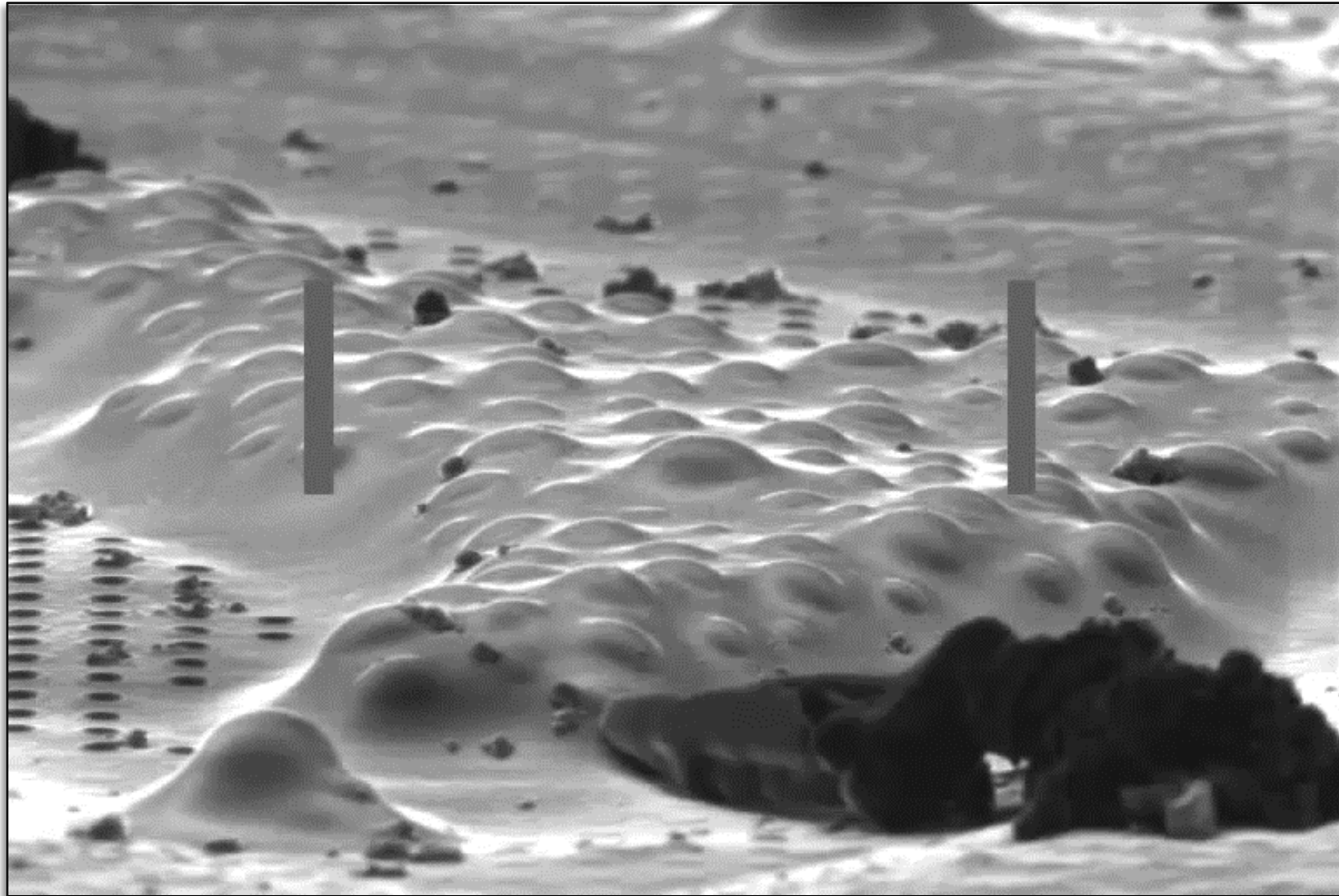


I-beam



E-beam







# 3.8 CryoFLM target confirmation **\_e.g., cryo-lamella #7**

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)

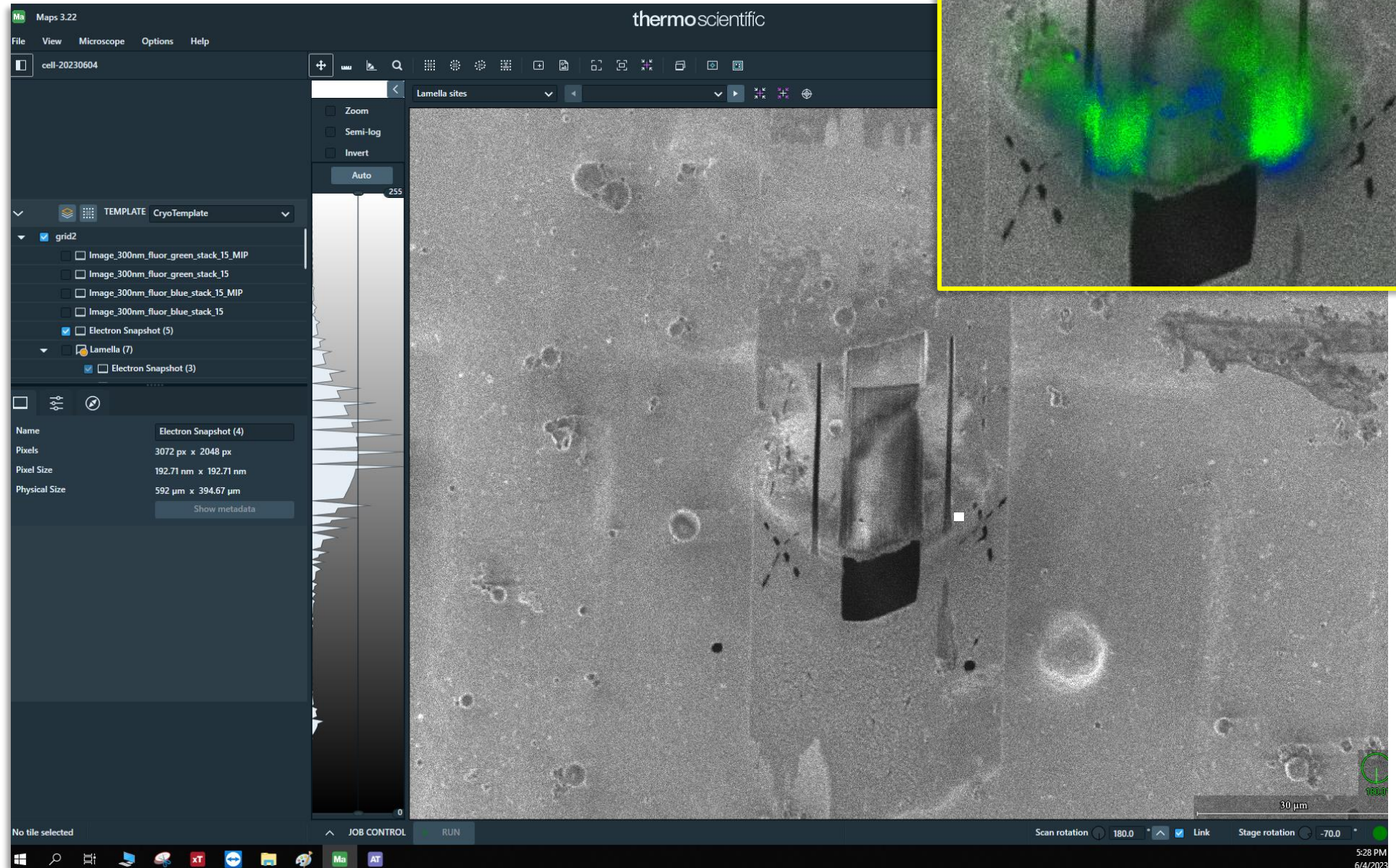
Target confirmation

**Pt sputter** (Optional)

Lamella conductivity



**CryoET**





# 3.9 Pt sputter (optional)

Vitrification



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

### Sputter Coating

Prepare for Sputtering

Current  Pressure

Voltage  Run Time

Run

The screenshot shows the xT software interface. The top panel is 'Shuttle Controls' with options for 'Mapping', 'Milling', and 'Deposition'. Below it is 'Temperature & Pressure' with checkboxes for 'Cryo Stage' (-195.3 °C), 'Cryo Shield' (-187.6 °C), and 'Chamber Pressure' (4.75E-5 Pa). A 'Recording' section shows a date and time. A graph displays data over time. The 'Tilt Calculation' section includes 'Holder Pre-tilt' (45.0 °), 'Milling Angle' (10.0 °), and 'Stage Tilt' (17.0 °). The 'Cryo GIS Deposition' section has 'Gas' set to 'Pt dep', 'Selected Grid' as 'Grid 1', and 'Flow Duration' of 00:01:30. The bottom panel is 'Sputter Coating', which is highlighted with a red box and matches the parameters shown in the inset image above.

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

# 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

## 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

## 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

## 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 the lamellae #1&2

If CLEM is unnecessary



(Video from TFS)

# Thank you!

**Comments & Questions?**

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