Third annual Cryo-EM Course at LBMS, BNL (June 20-23rd, 2023)

CryoET sample preparation tutorial & demonstration

Jianfeng Lin

Yale CryoEM Resource

6-22-2023

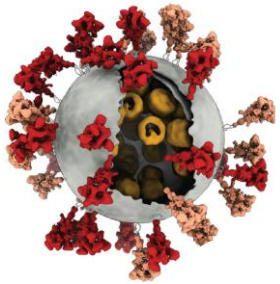
Main contents

1. Specimens accessible by cryoET

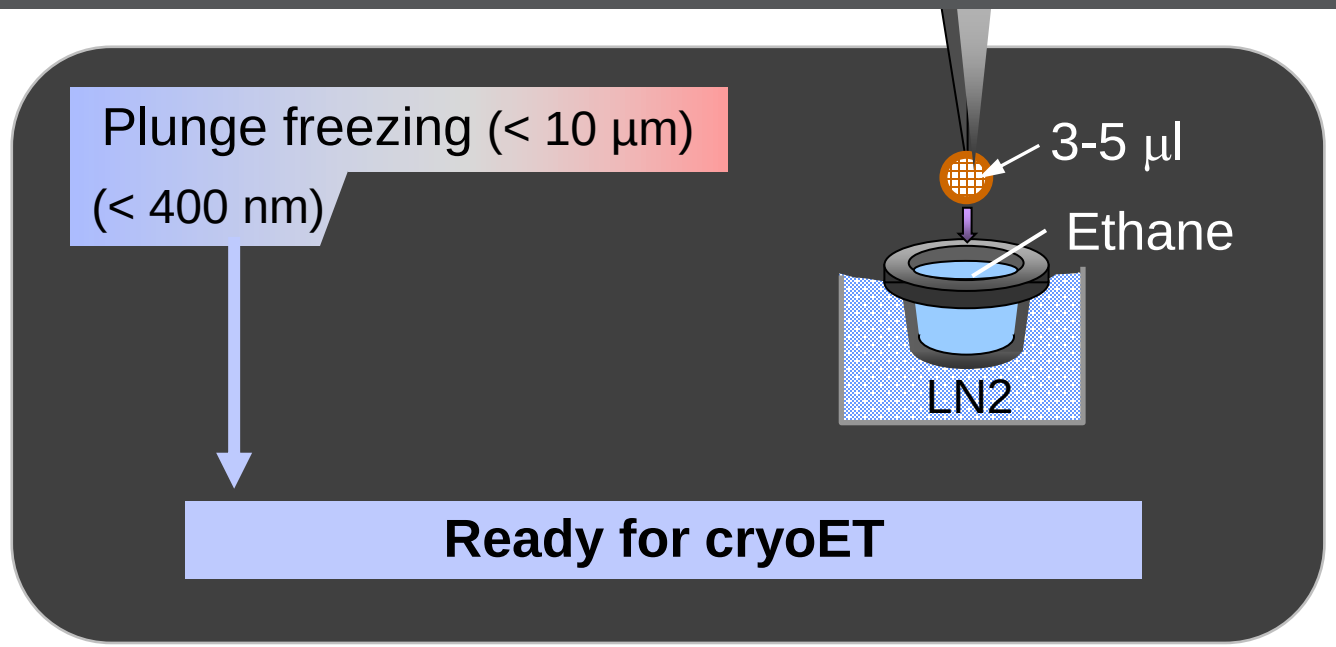
2. Five considerations for cryoET sample preparation by plunge freezing

3. Tutorial of major steps of cryo lamella preparation

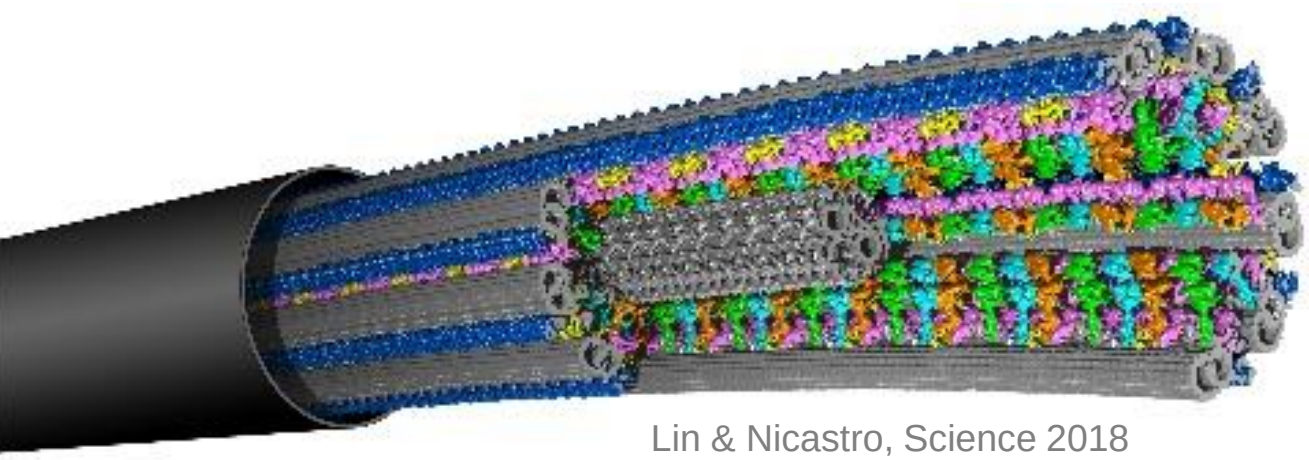
1. Specimens accessible by cryoET



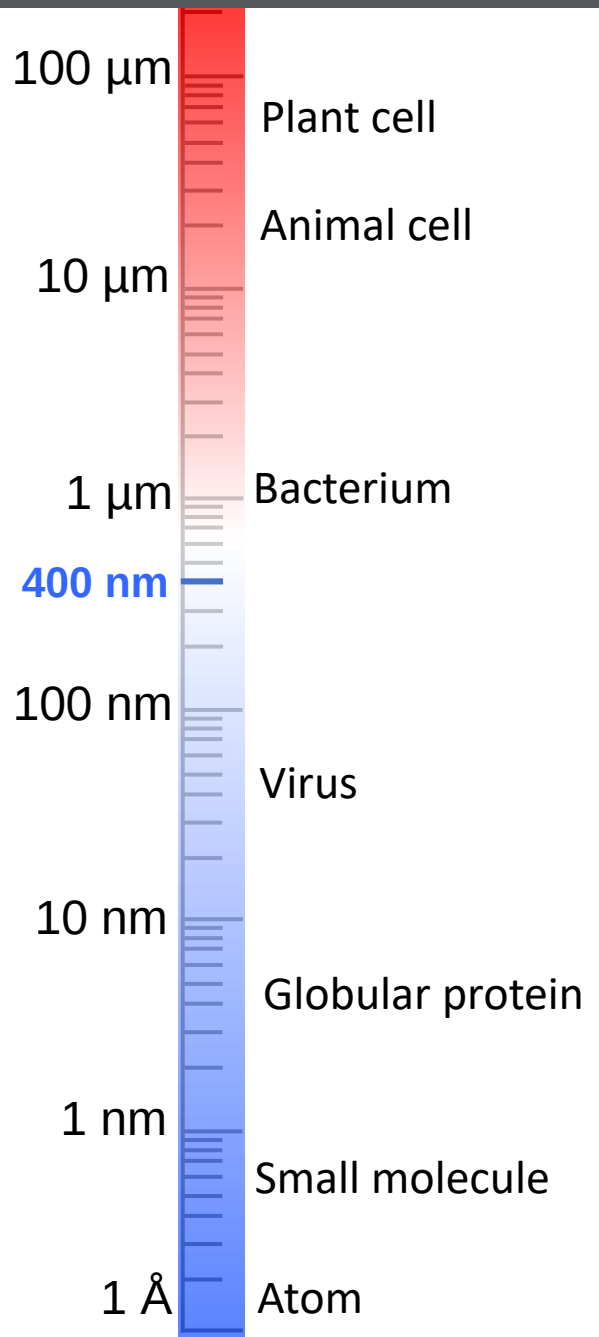
Yao et al., Cell 2020



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

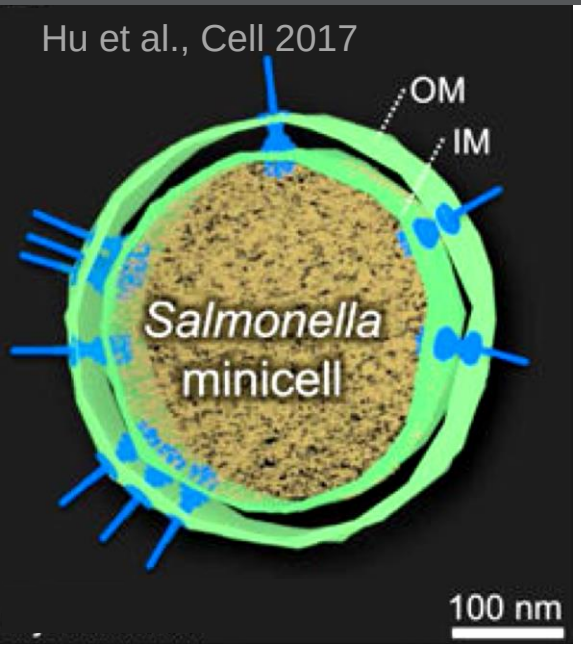


Lin & Nicastro, Science 2018

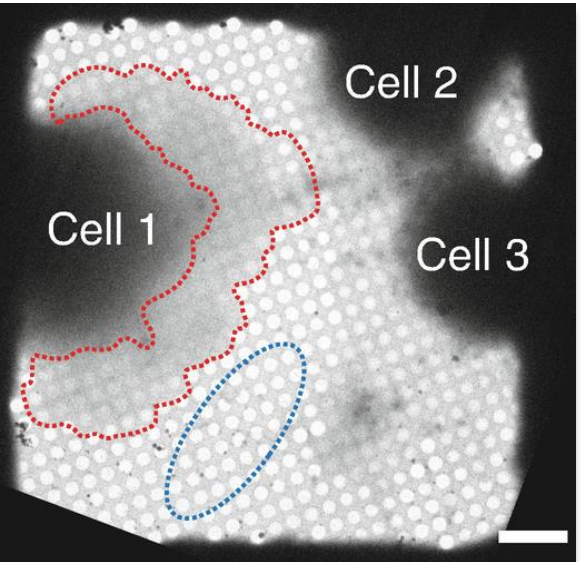


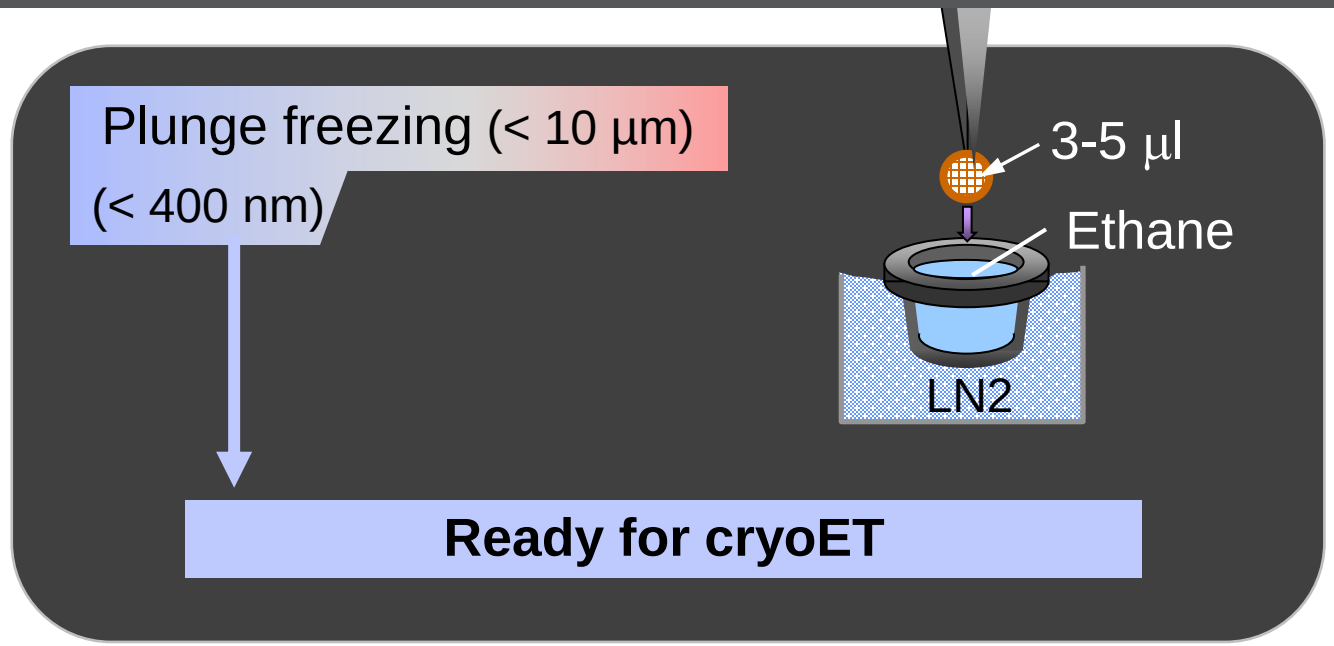
1. Specimens accessible by cryoET

Hu et al., Cell 2017

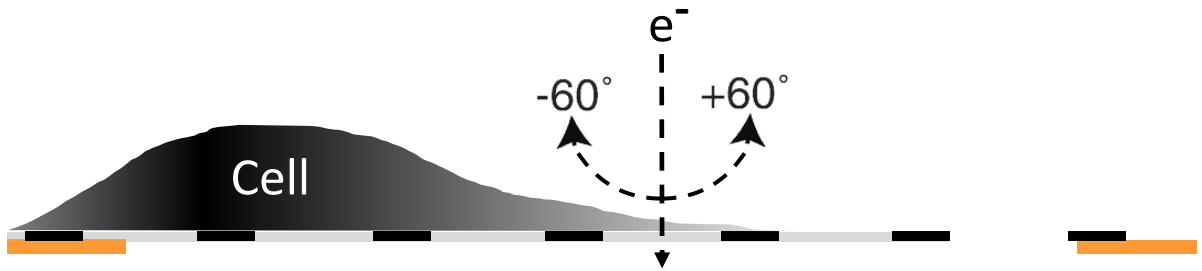


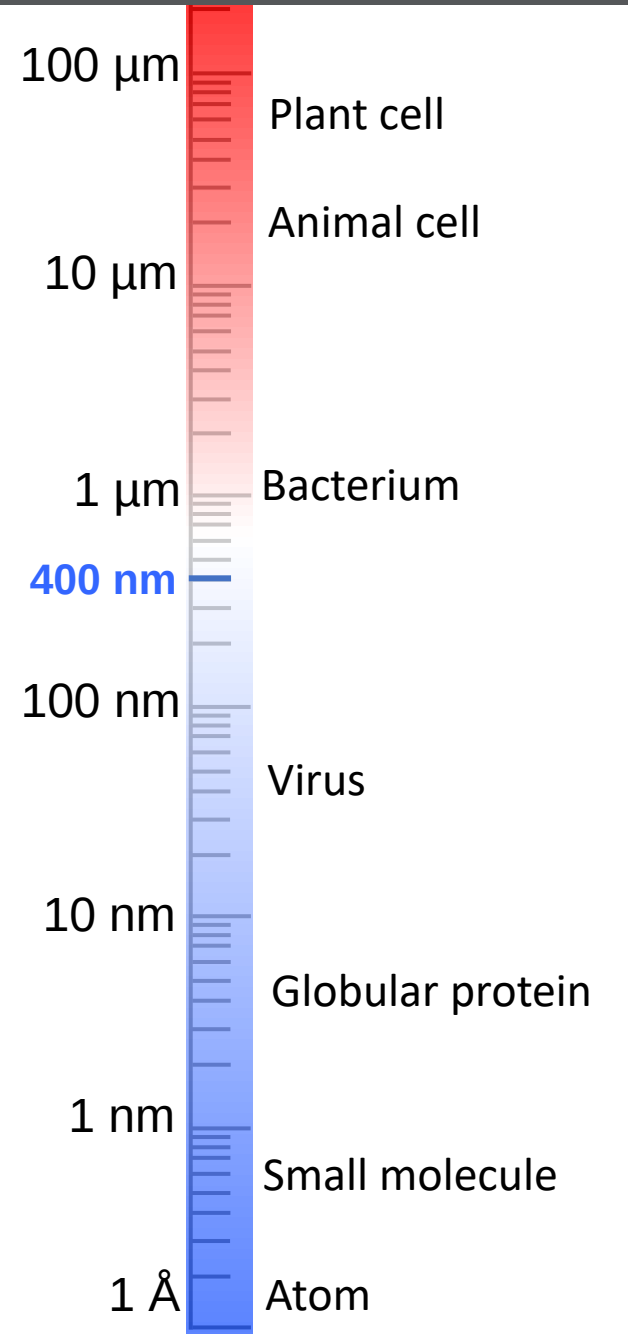
Serwas & Davies.
Methods Mol Biol. 2021



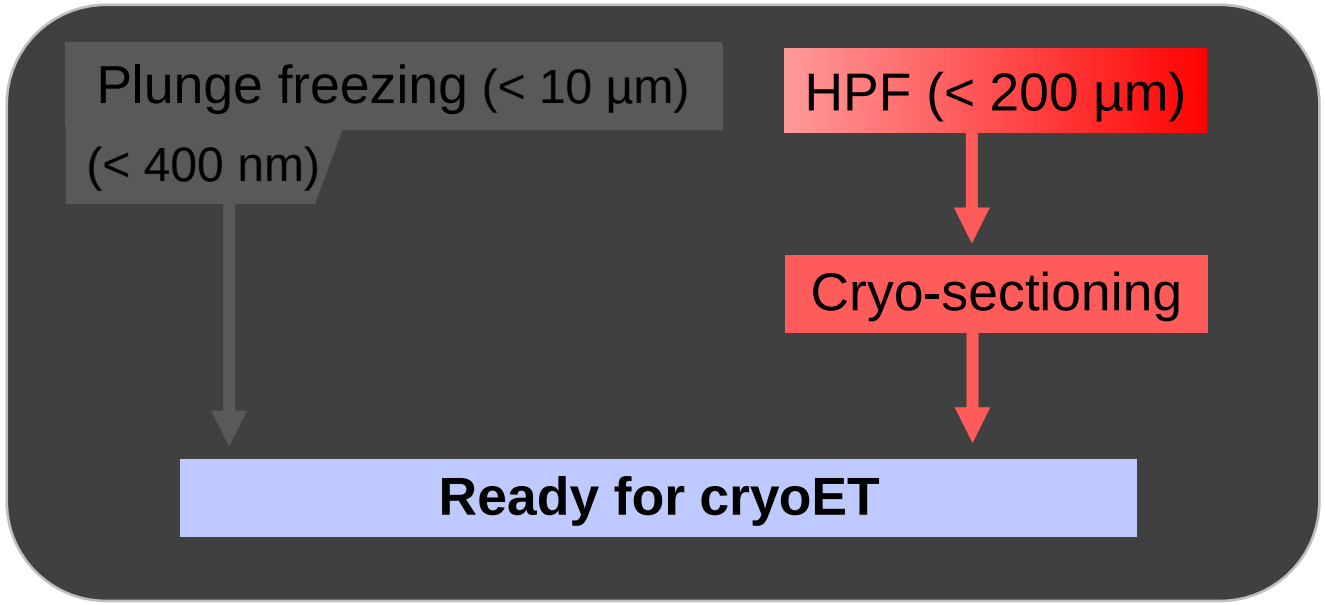


- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells

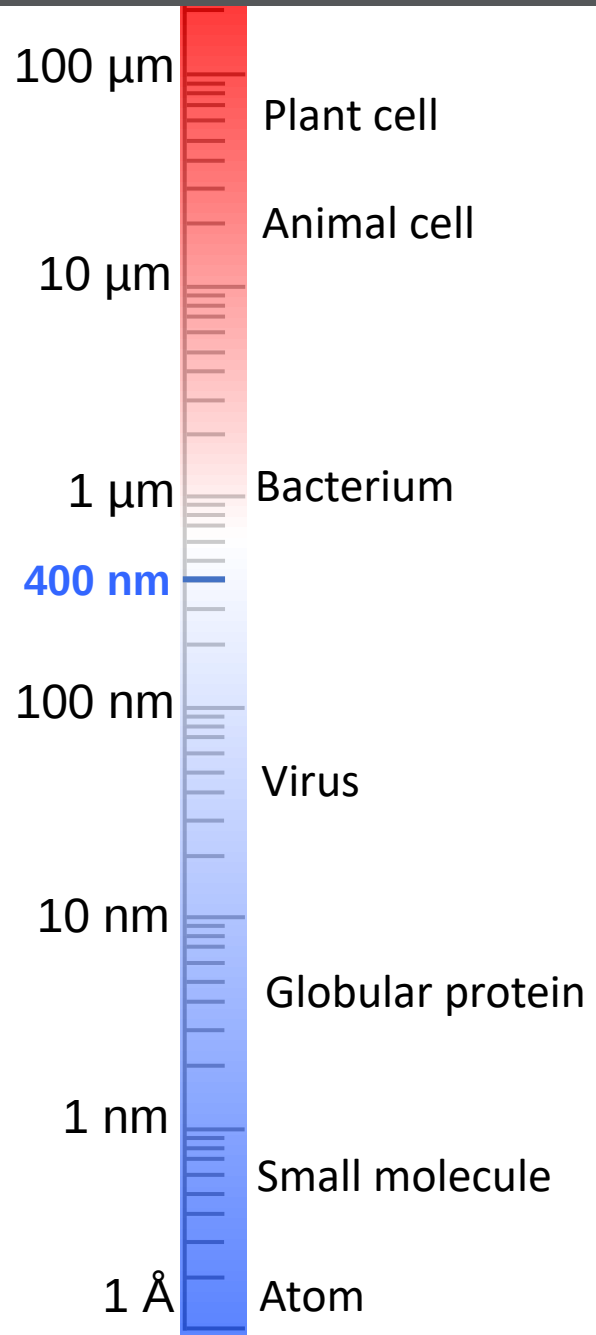




1. Specimens accessible by cryoET

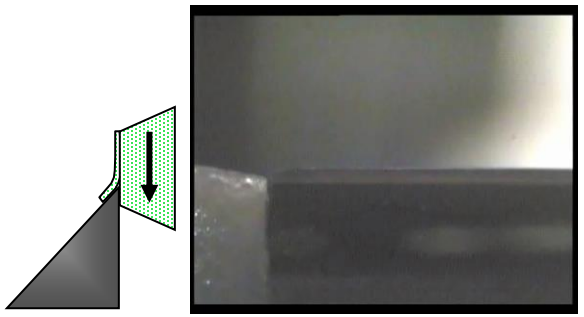


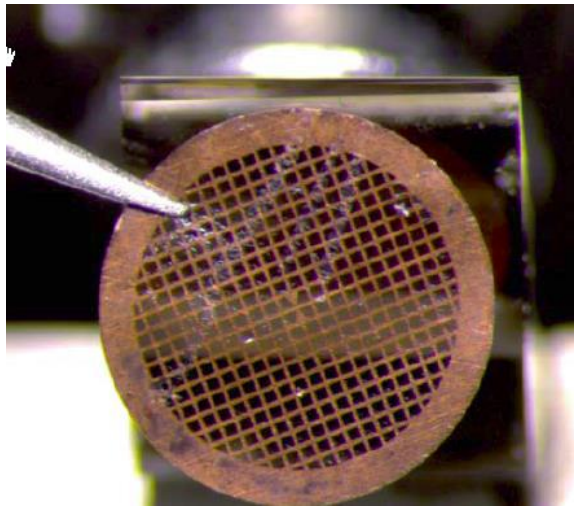
- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells
- Cryo-sections



1. Specimens accessible by cryoET

CEMOVIS





Adapted from Thermo Fisher Scientific (TFS)

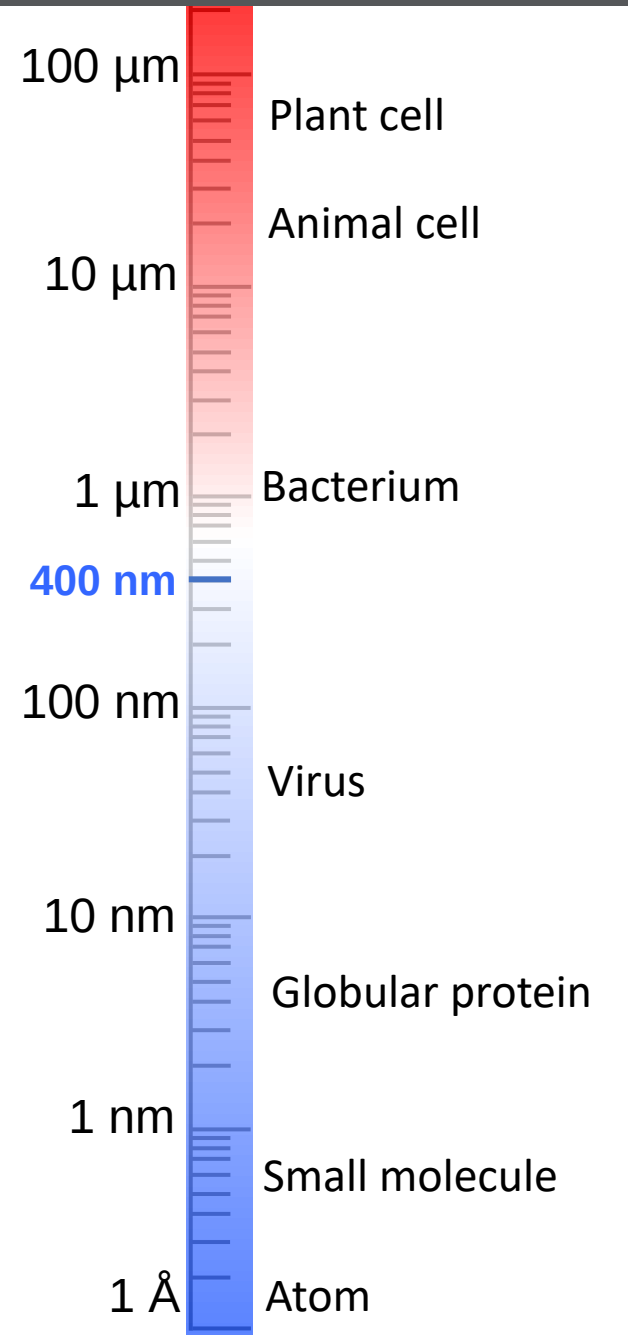
Plunge freezing (< 10 µm)
(< 400 nm)

HPF (< 200 µm)

Cryo-sectioning

Ready for 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

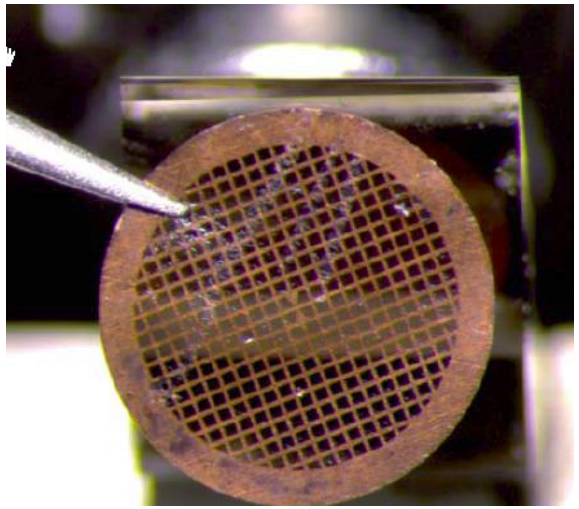
Plunge freezing (< 10 µm)

(< 400 nm)

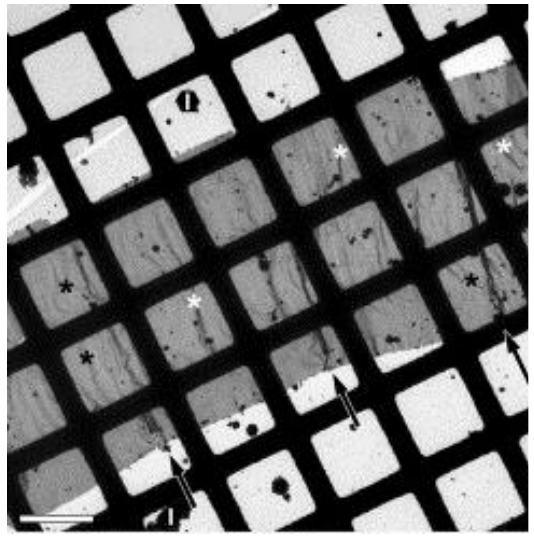
HPF (< 200 µm)

Cryo-sectioning

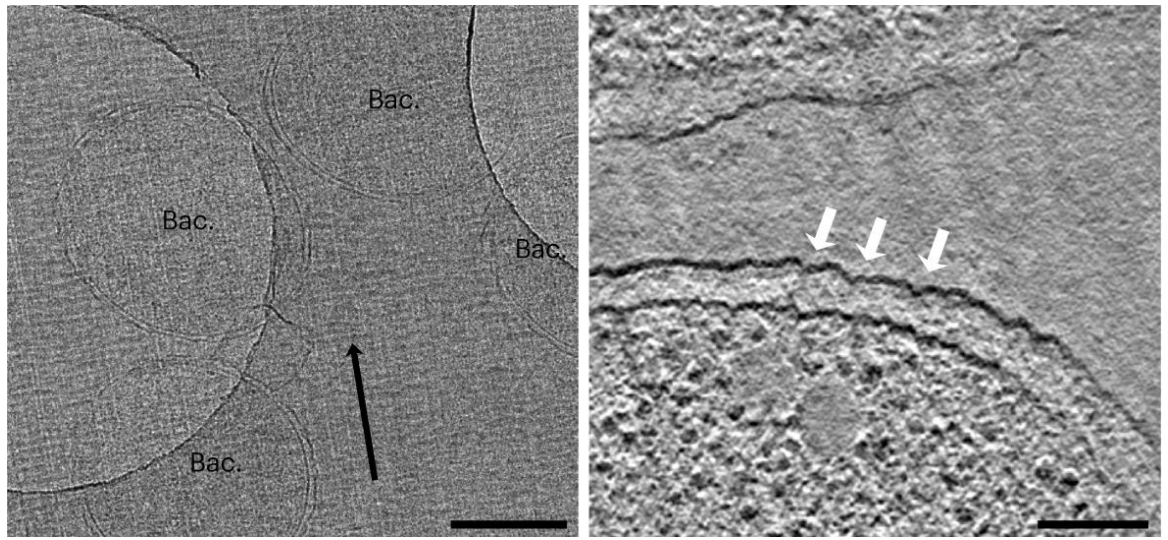
Ready for cryoET



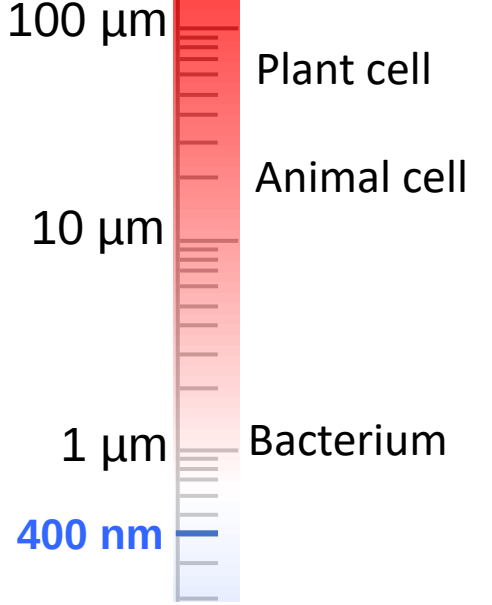
Adapted from TFS



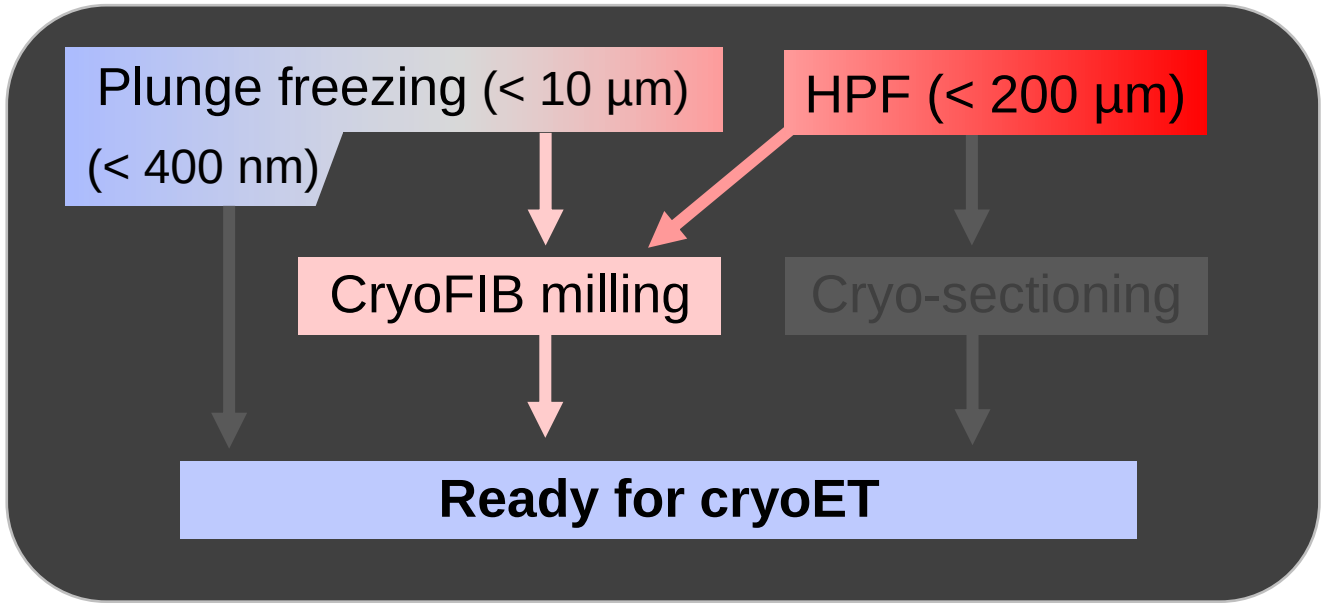
Al-Amoudi et al., J Struct Biol. 2005

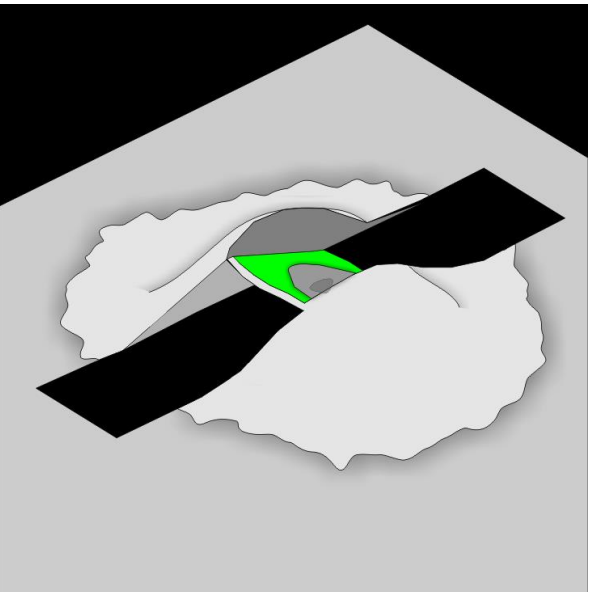


Casper Berger et al., Nature Methods. 2023

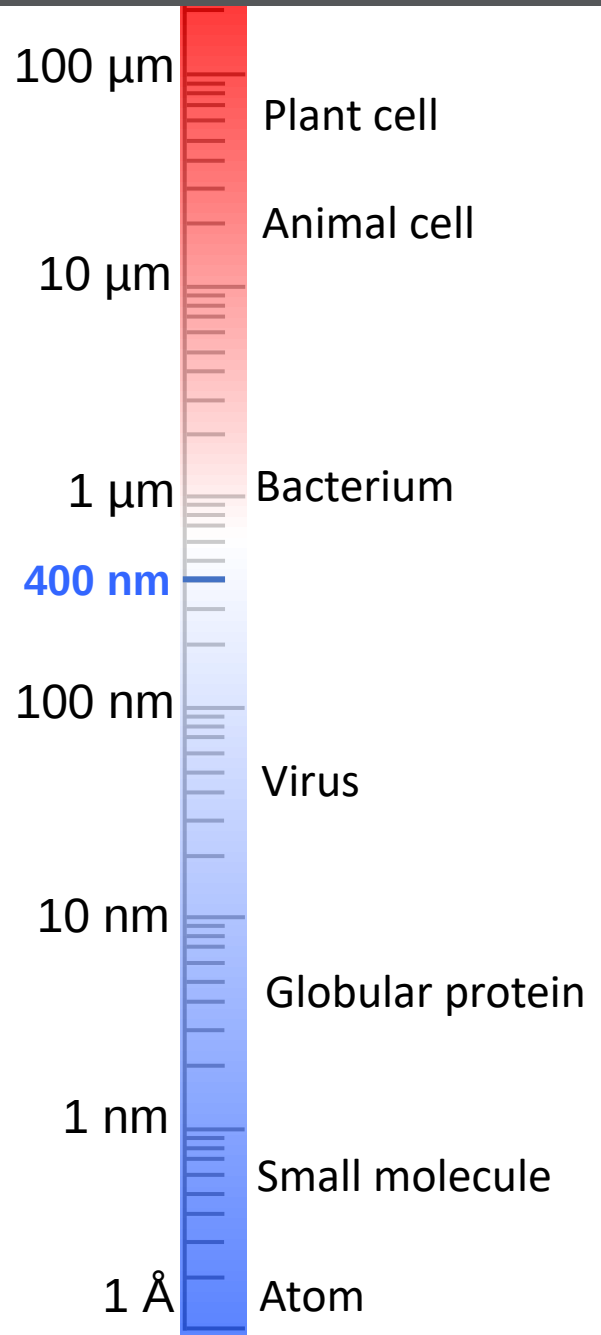


1. Specimens accessible by cryoET





- 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

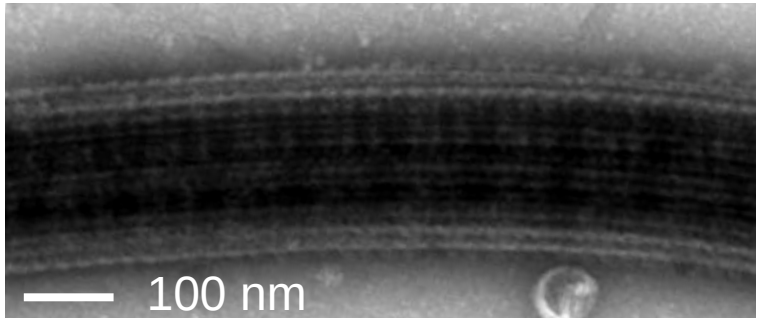
2. Five considerations for cryoET sample preparation by plunge freezing

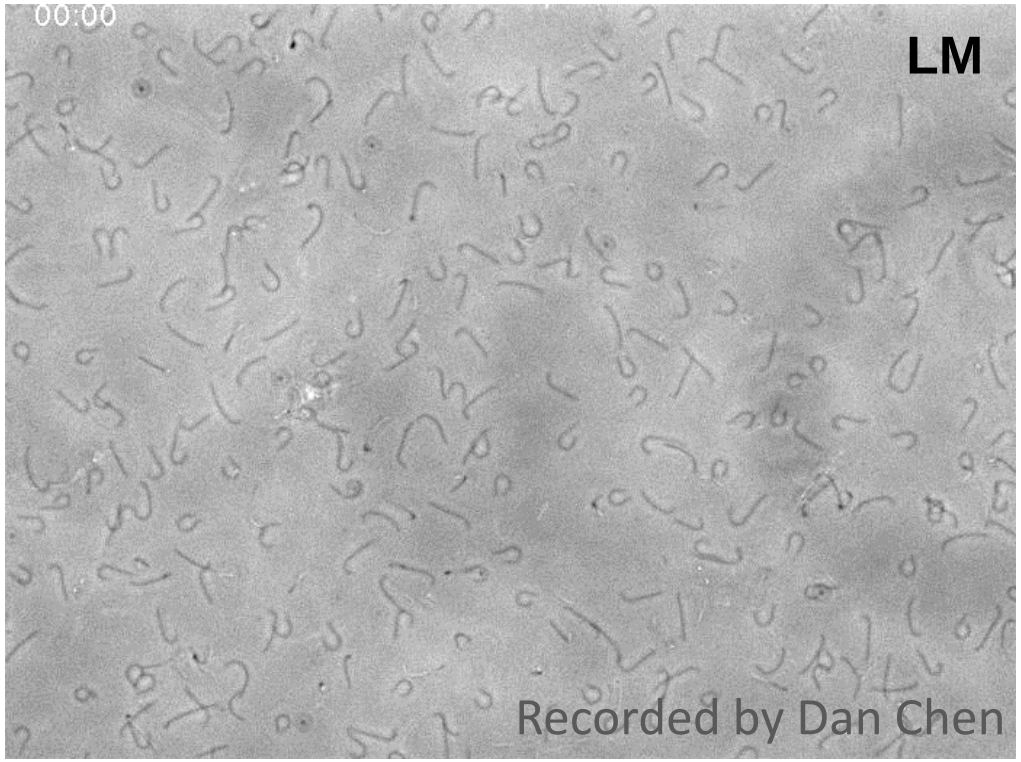
3. Tutorial of major steps of cryo lamella preparation

2. Five considerations for cryoET sample preparation

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

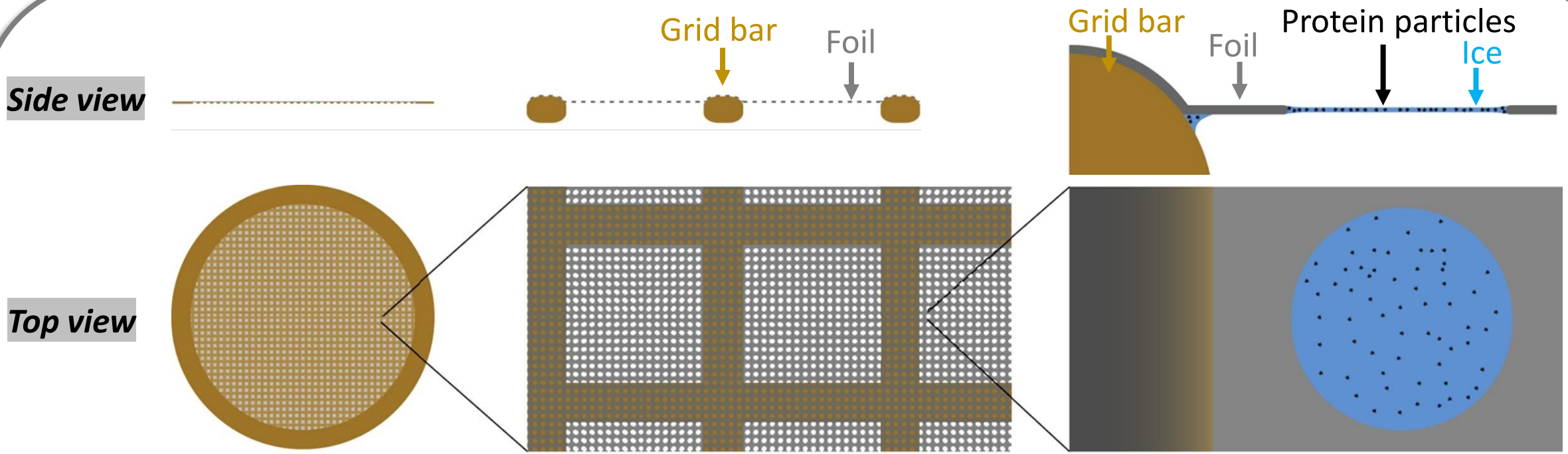
Negative staining EM





2. Five considerations for cryoET sample preparation

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



Grid materials

Copper	**Gold**
Nickel	CuRh
Titanium	Molybdenum
Silicon	Aluminum
	Tungsten

Foil materials

Amorphous carbon

Gold

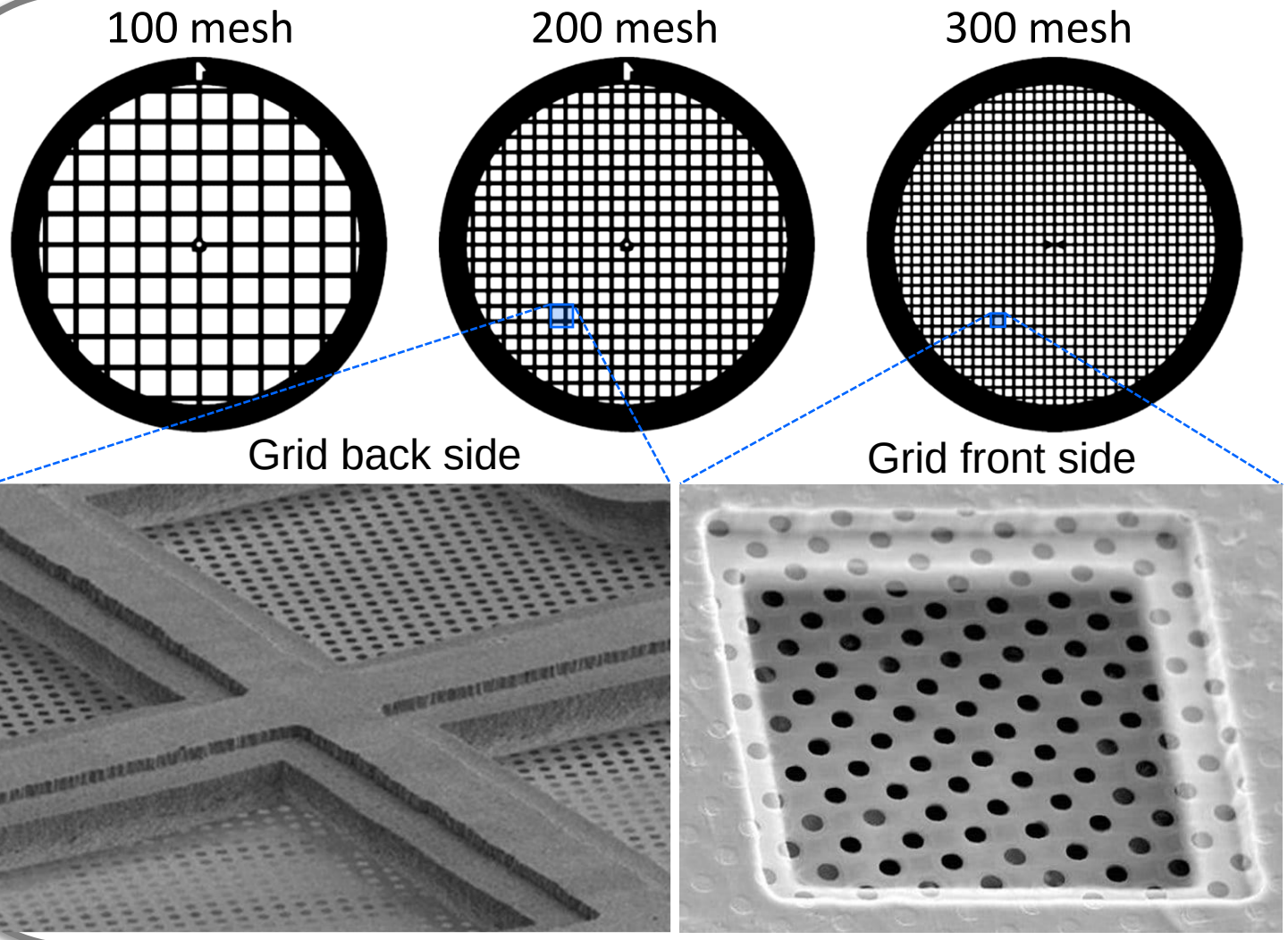
TiSi SiN

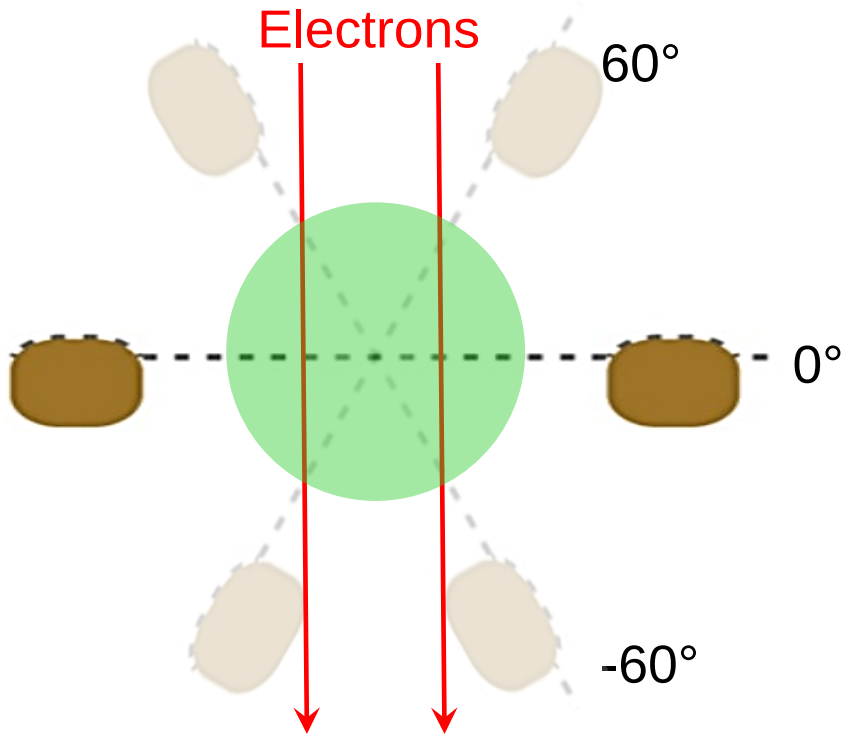
SiO_2 SiC

Russo & Passmore, Curr Opin Struct Biol, 2016.

2. Five considerations for cryoET sample preparation

- Validation of the sample quality
- EM grid => Mesh / hole size



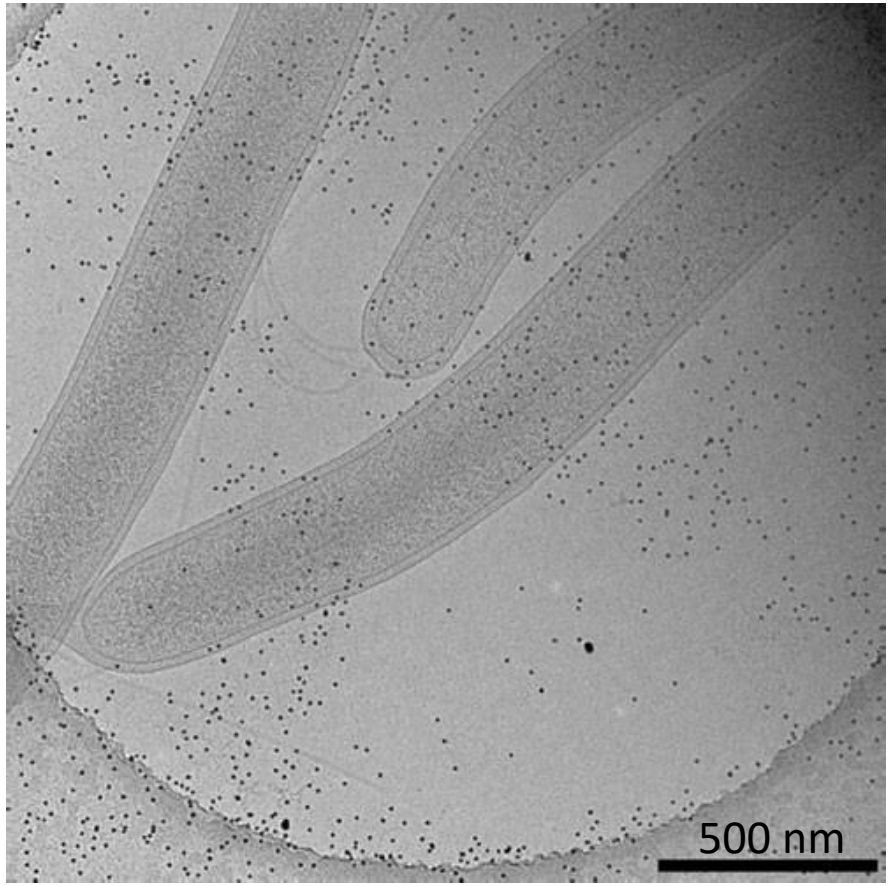


Adapted from TFS

2. Five considerations for cryoET sample preparation

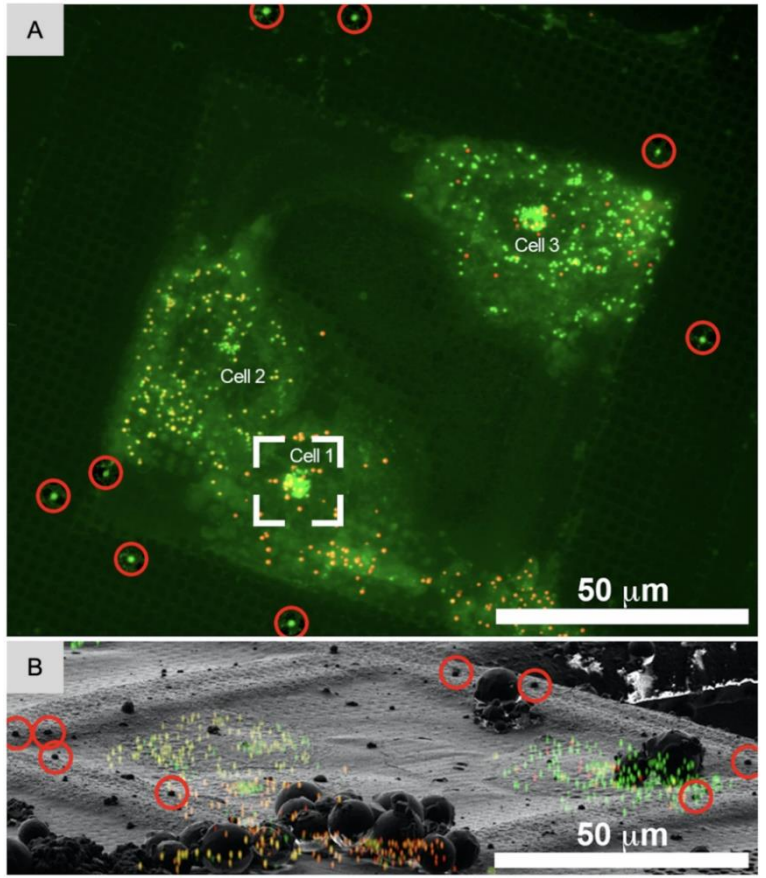
- Validation of the sample quality
- EM grid
- Fiducial markers

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



Iancu et al., Nature Protocols 2006

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



Arnold et al., Biophysical Journal 2016

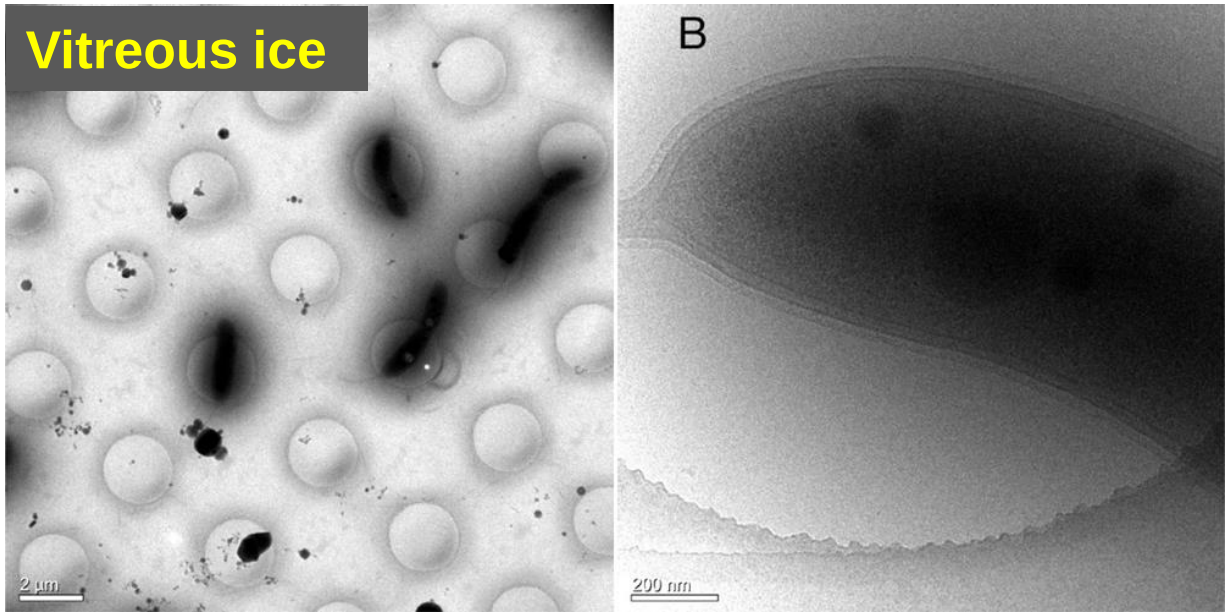
2. Five considerations for cryoET sample preparation

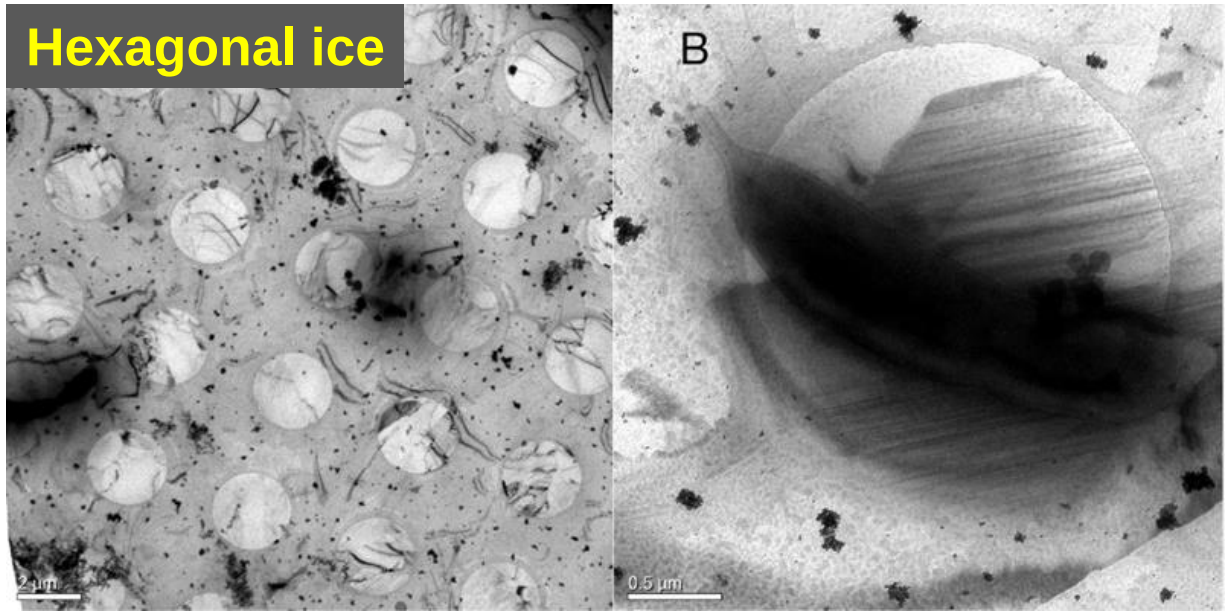
- Validation of the sample quality
- EM grid
- Fiducial markers
- Cryogen e.g., LE or 37% LE-63% LP mixture

T_{LE}= -182.8 ℃ ~-88.6 ℃

T_{LP}= -189.7 ℃ ~ -42.2 ℃

T_{LN2}=-210 ℃ ~-195.8 ℃





Tivol et al., Microsc Microanal. 2008

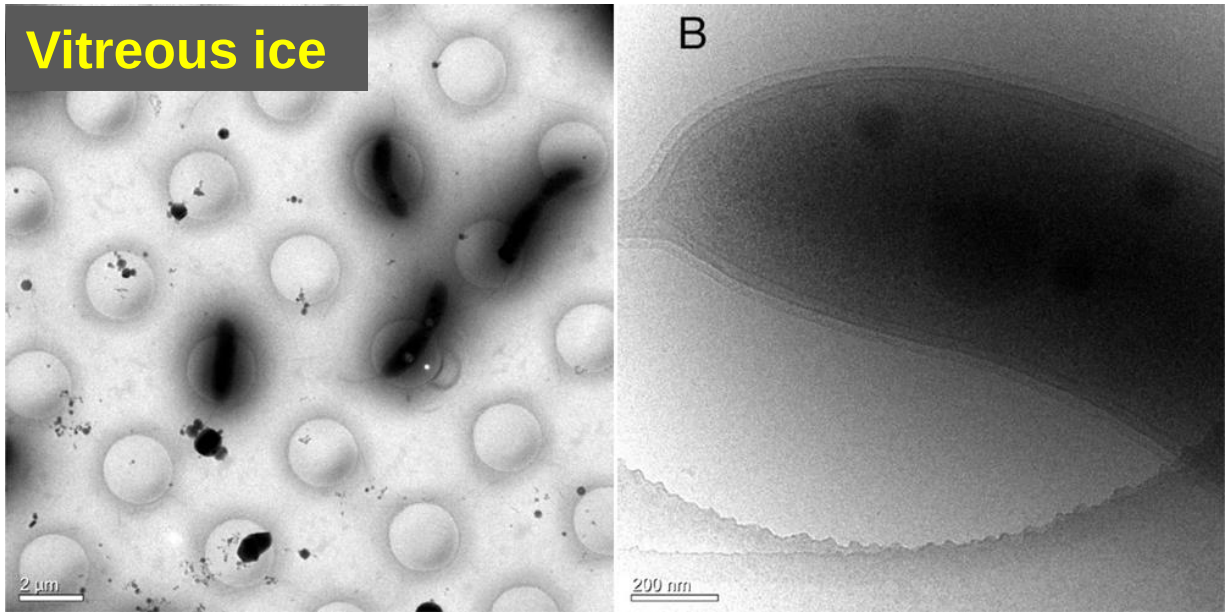
2. Five considerations for cryoET sample preparation

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

T_{LE}= -182.8 ℃ ~-88.6 ℃

T_{LP}= -189.7 ℃ ~ -42.2 ℃

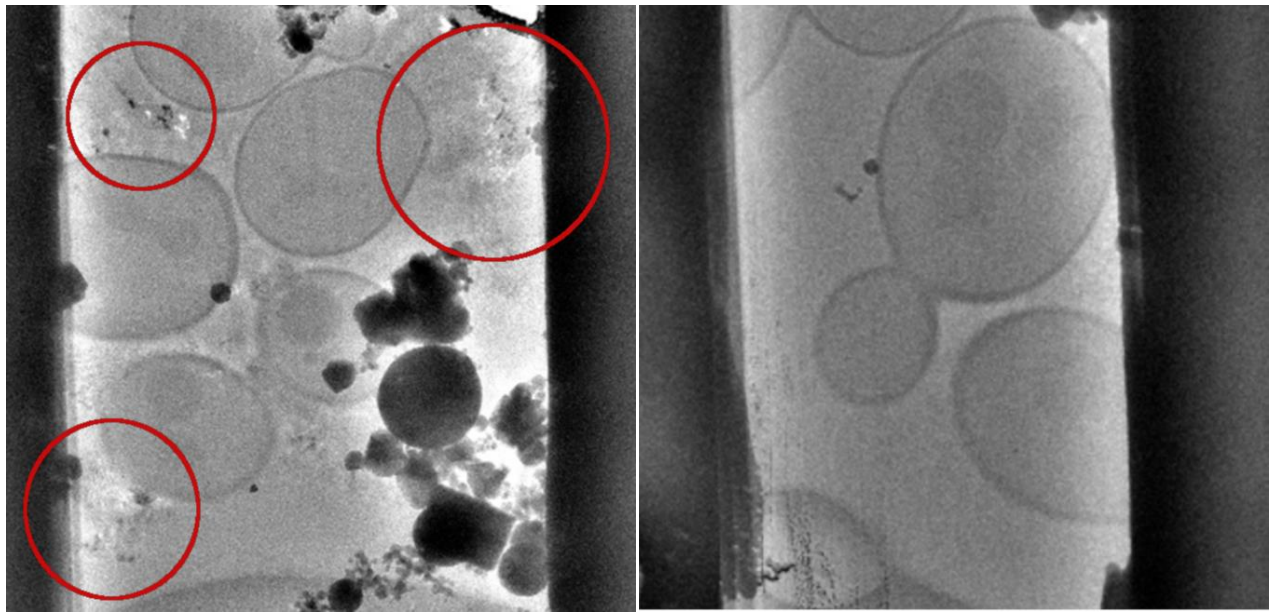
T_{LN2}=-210 ℃ ~-195.8 ℃



Tivol et al., Microsc Microanal. 2008

Without glycerol

5% glycerol



Moravcová et al., J Vis Exp. 2021

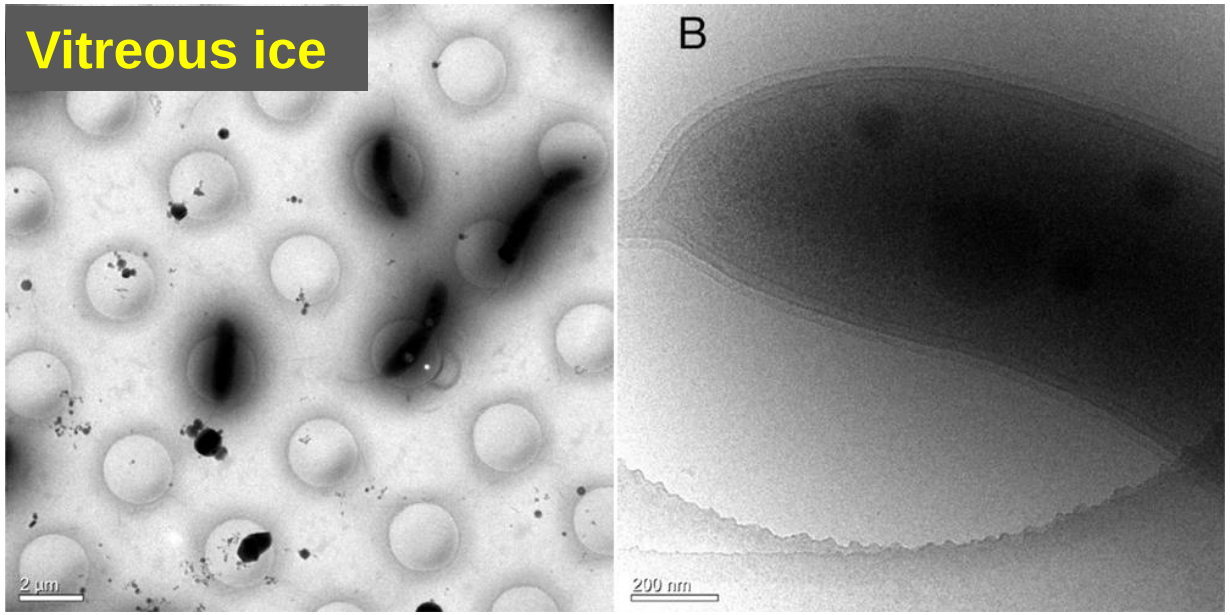
2. Five considerations for cryoET sample preparation

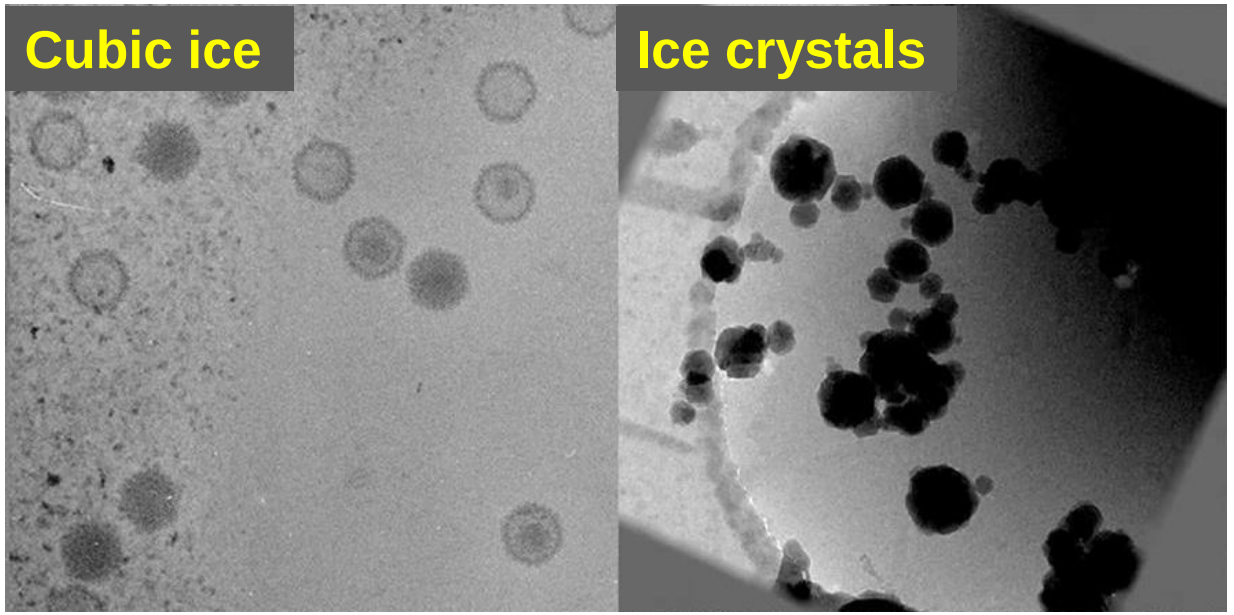
- Validation of the sample quality
- EM grid
- Fiducial markers
- Cryogen e.g., LE or 37% LE-63% LP mixture

T_{LE}= -182.8 ℃ ~-88.6 ℃

T_{LP}= -189.7 ℃ ~ -42.2 ℃

T_{LN2}=-210 ℃ ~-195.8 ℃



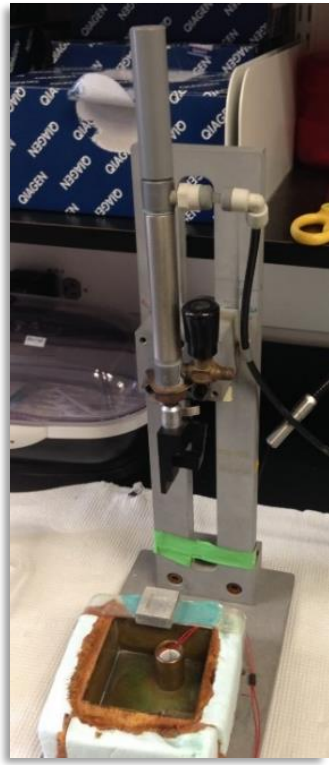


Tivol et al., Microsc Microanal. 2008

Bhella D, Biophysical Reviews. 2019

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)

Crossbeam (ZEISS)

Scios, Aquiclos 1/2
(Thermo Fisher Scientific)

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

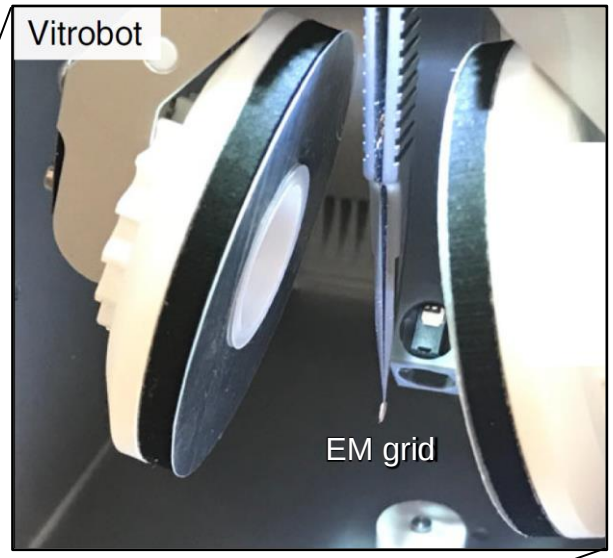
Lamella milling
Preparation, Milling, & thinning

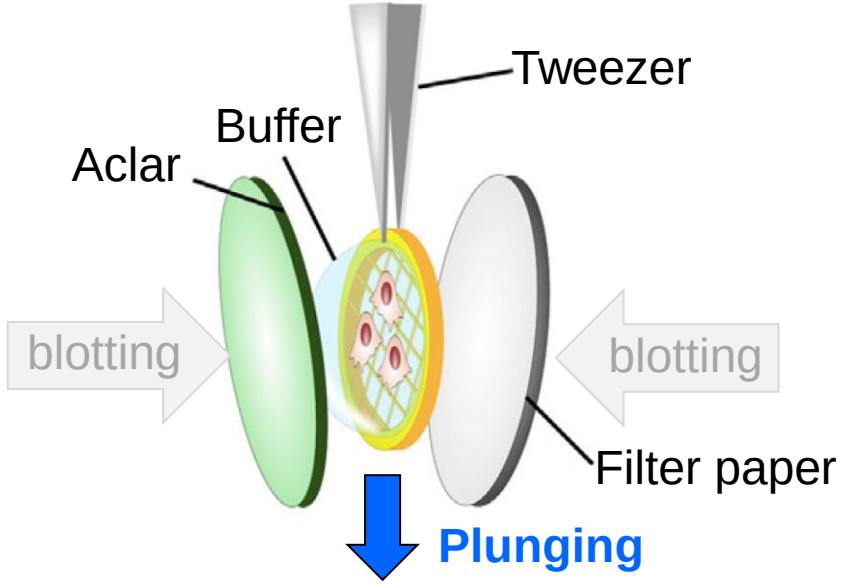
iFLM (Optional)
Target confirmation

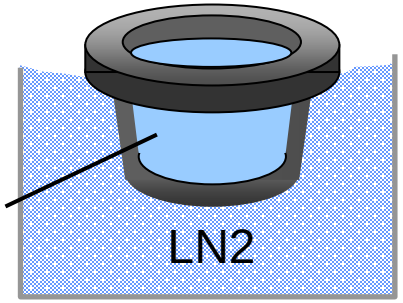
Pt sputter (Optional)
Lamella conductivity

↓ **T**

CryoET

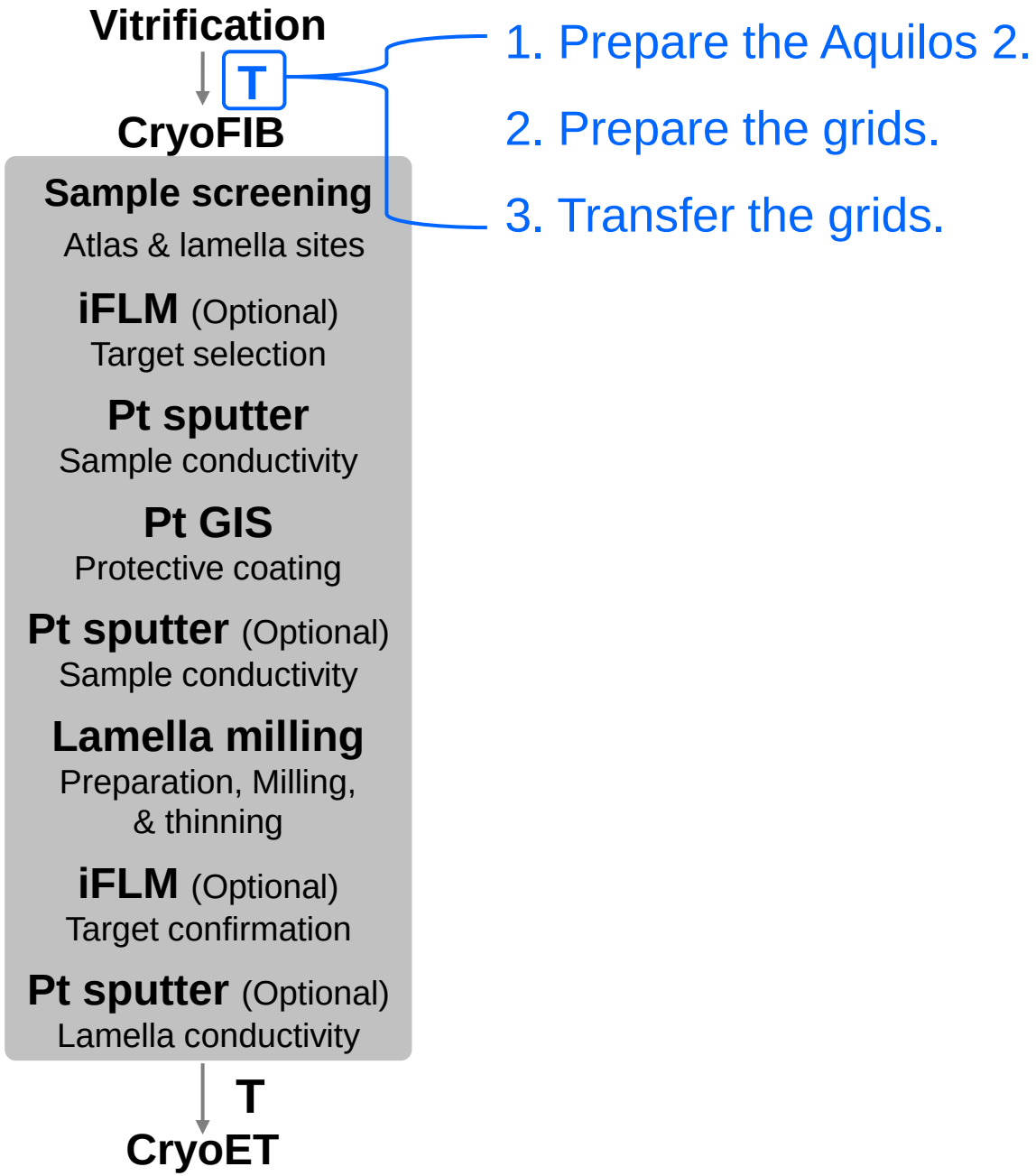






Schaffer et al., JSB 2017; Medeiros et al., Curr Opin Microbiol. 2018; Wagner, et al., Nature Protocols, 2020

3.2 Transfer the grids to Aquilos 2



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

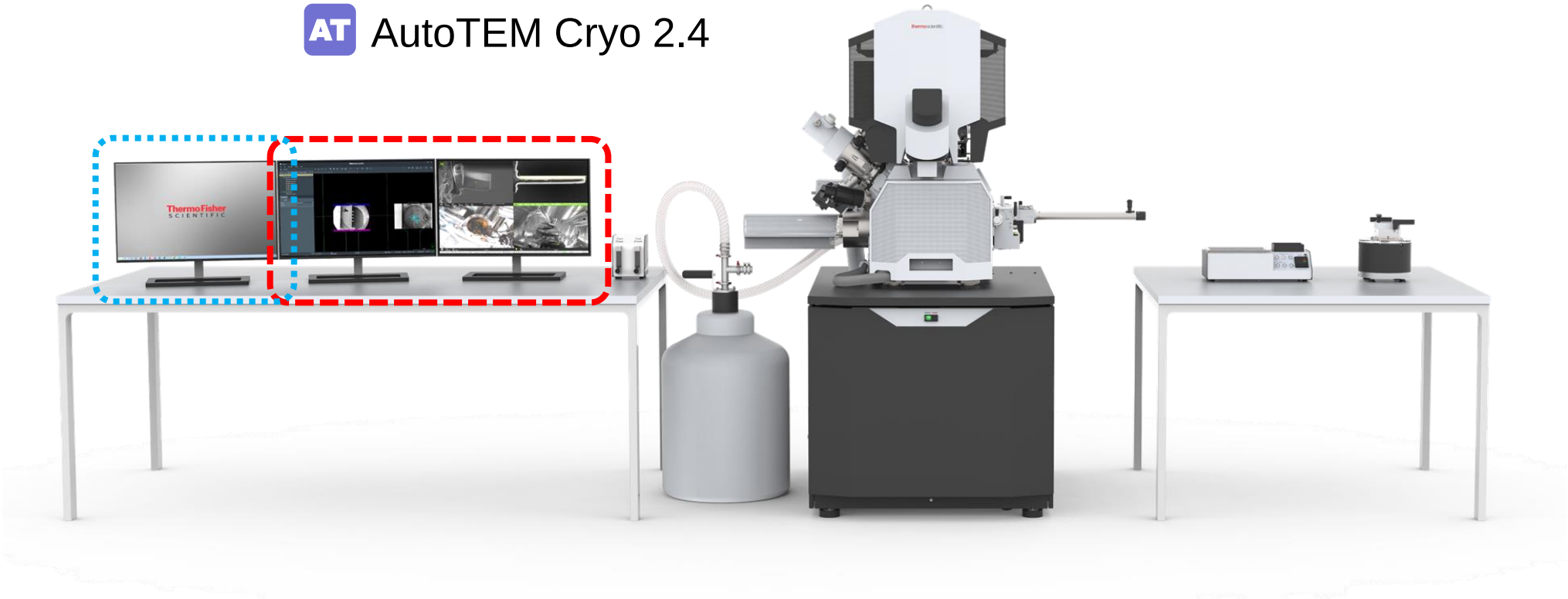
Aquilos 2 & software used in this tutorial

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

(xT Microscope Control)

Vitrification

↓ T

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

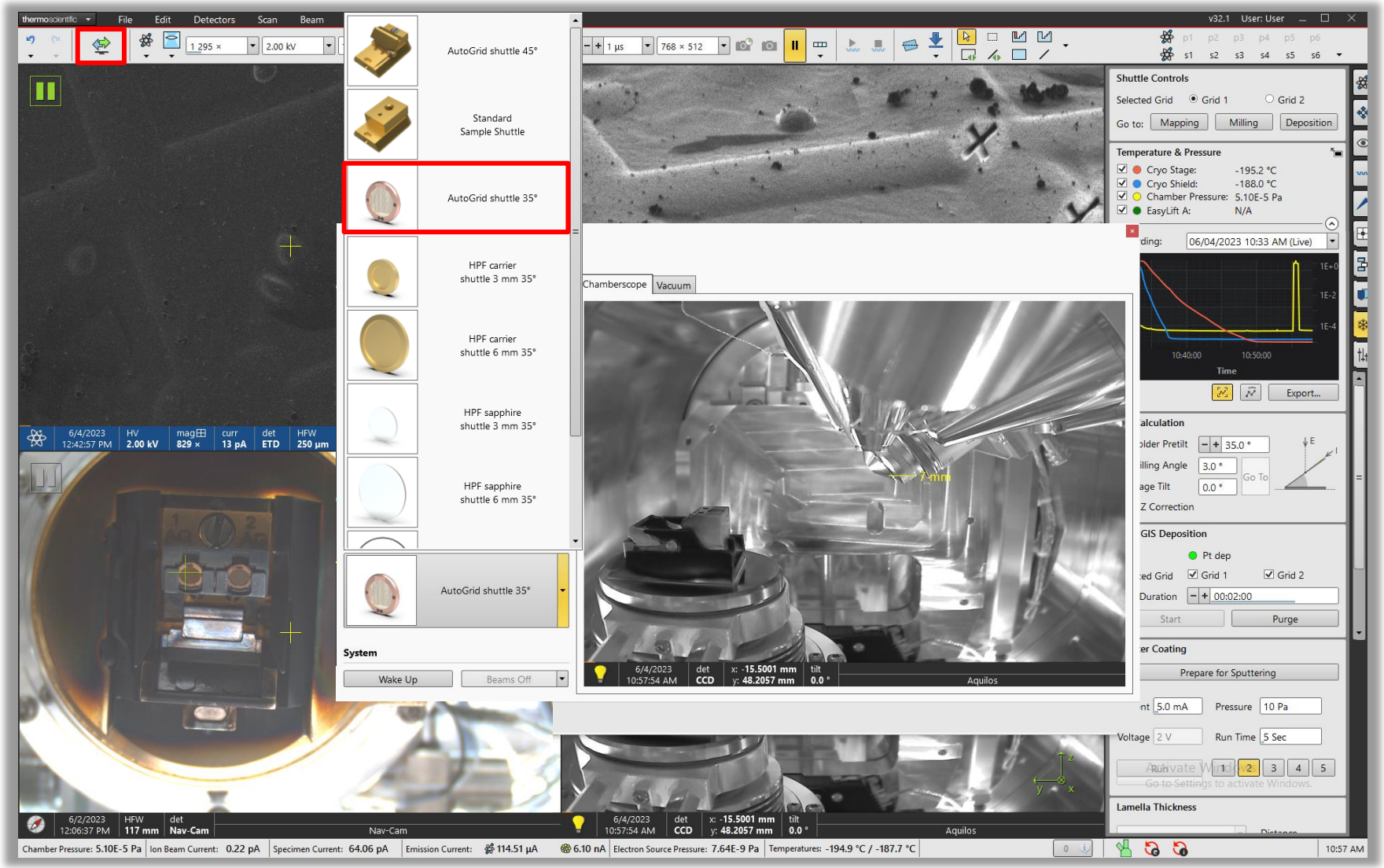
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

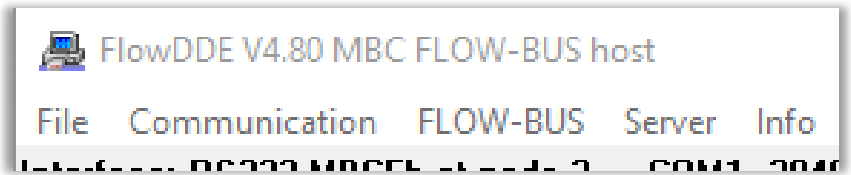
↓ T

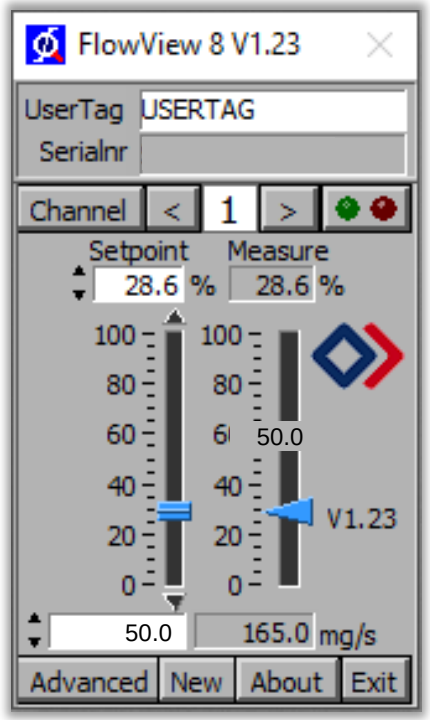
CryoET

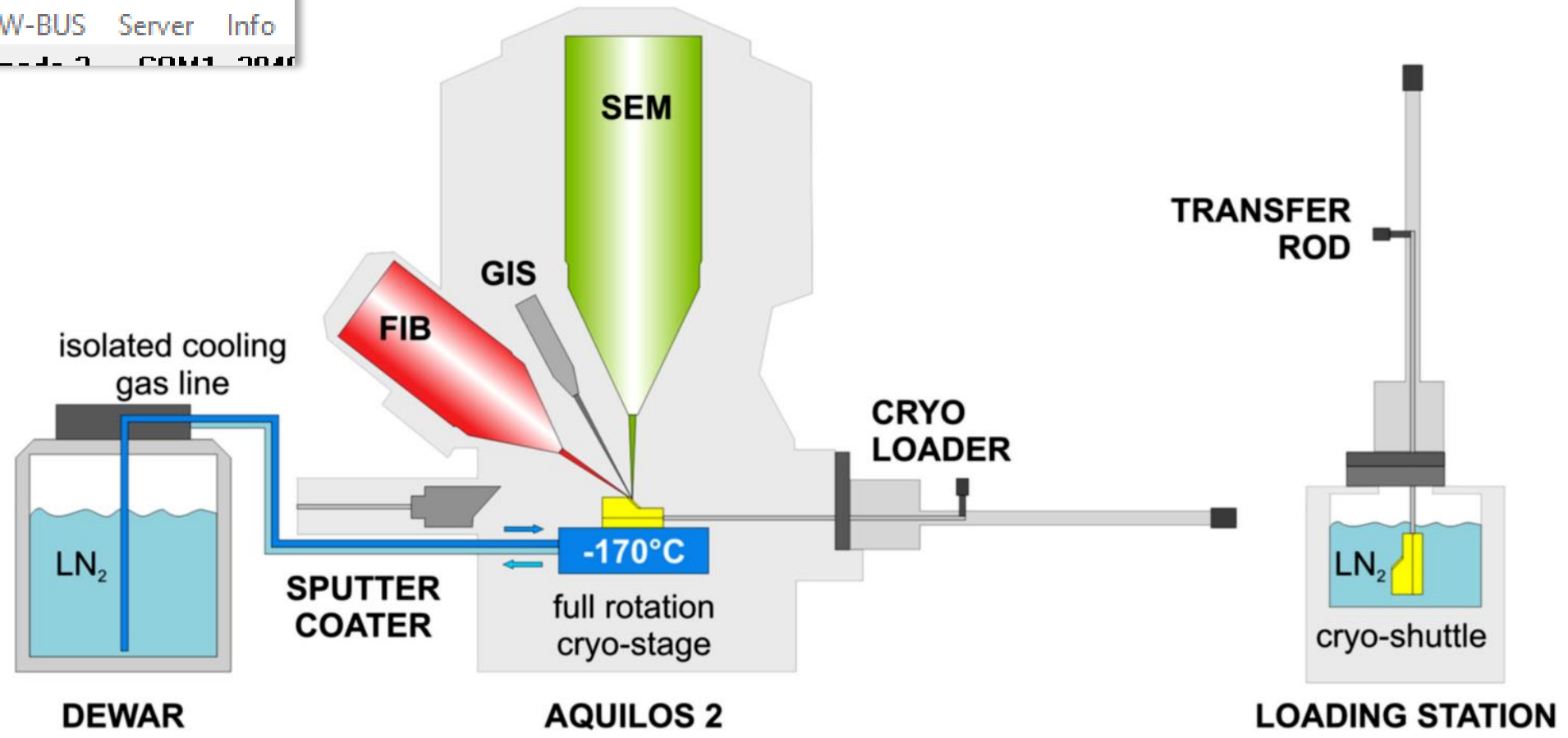


3.2.1 Prepare the system _Purge the system

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





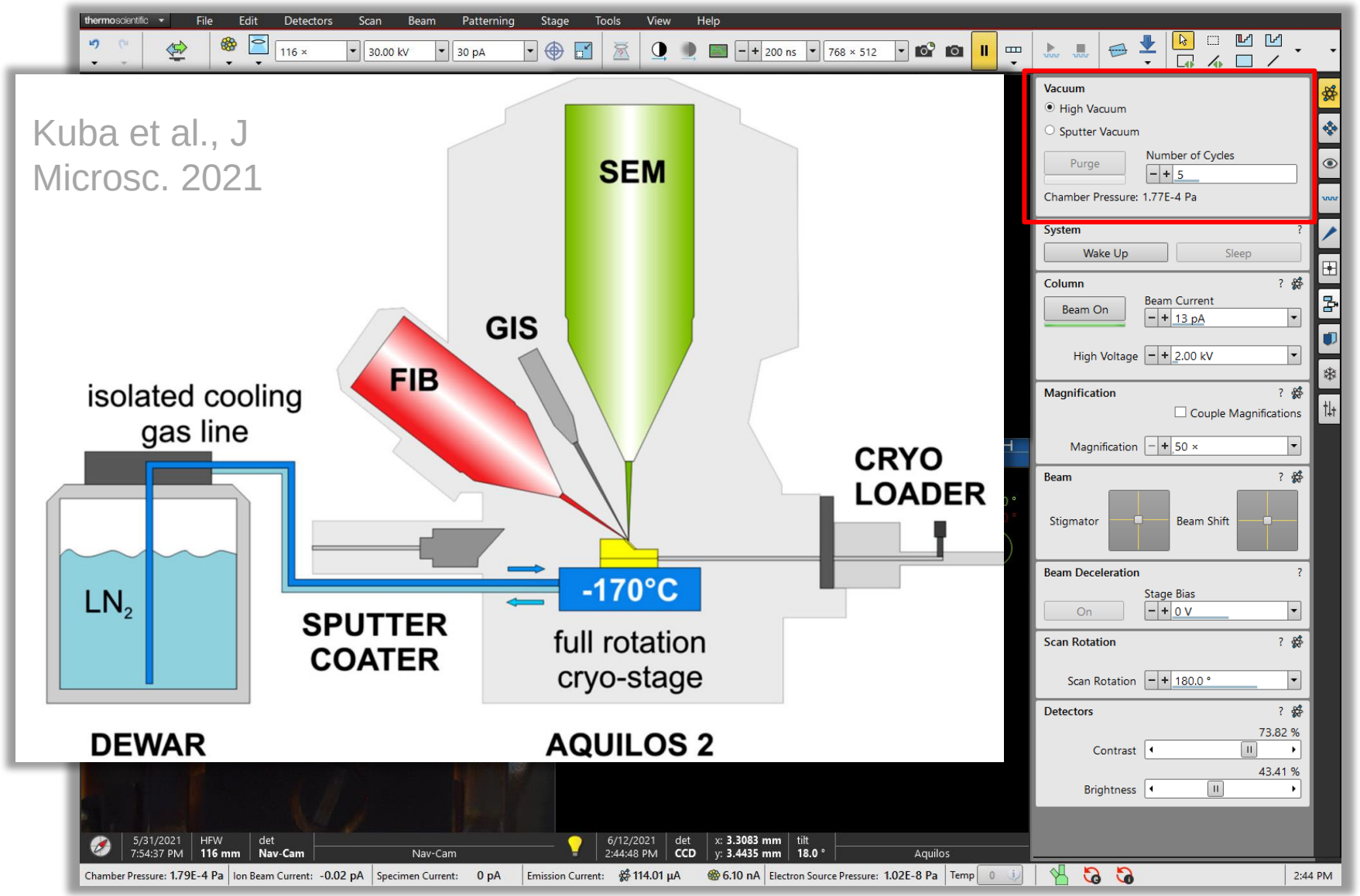


Kuba et al., J Microsc. 2021

3.2.1 Prepare the system _Purge the system

(xT Microscope Control)

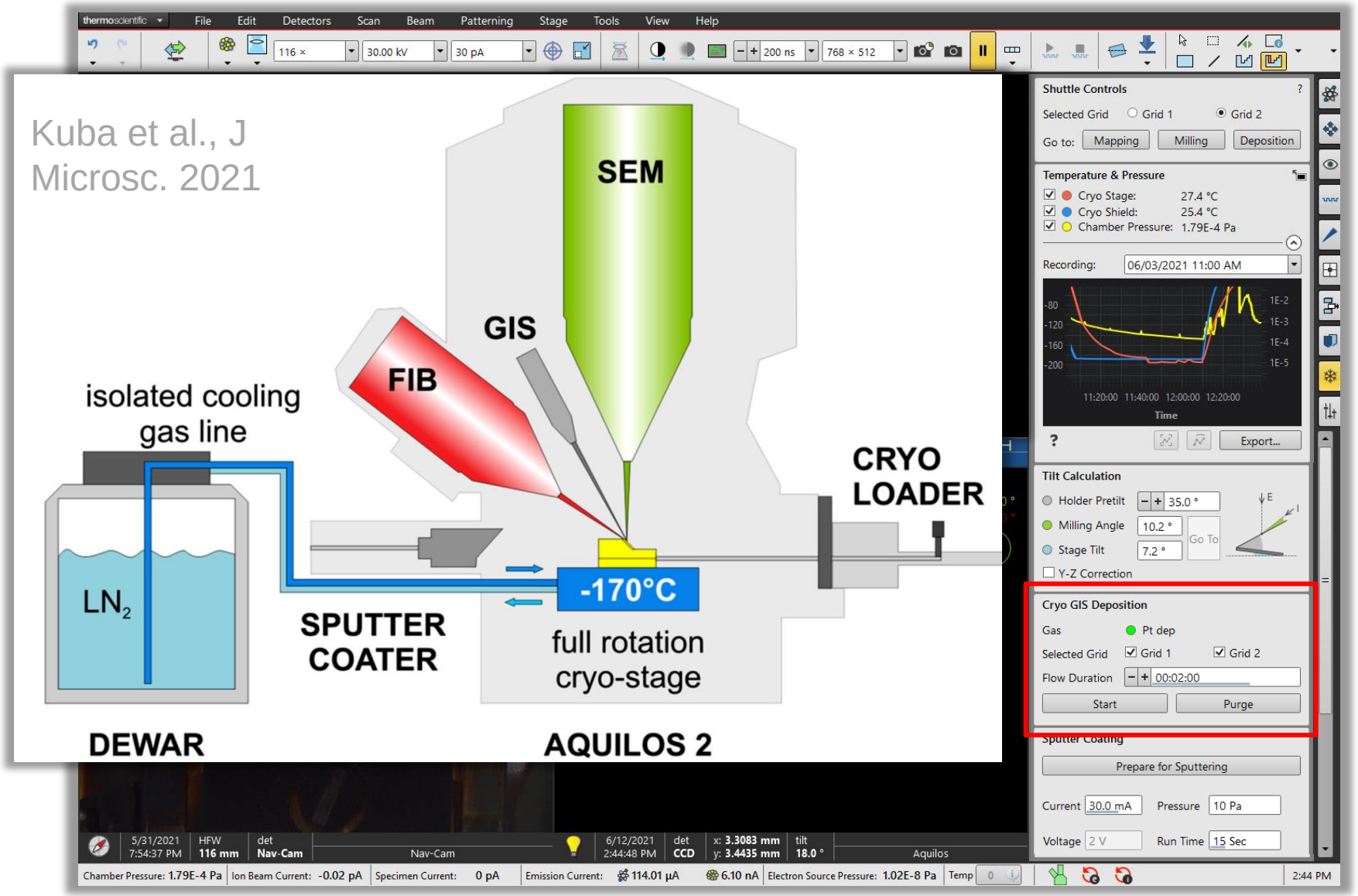
- Cooling gas line, Loading station, Argon line (5 cycles)



3.2.1 Prepare the system _Purge the system

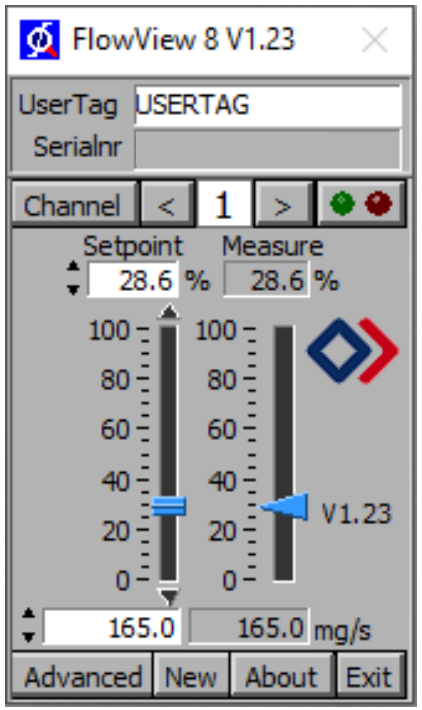
(xT Microscope Control)

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

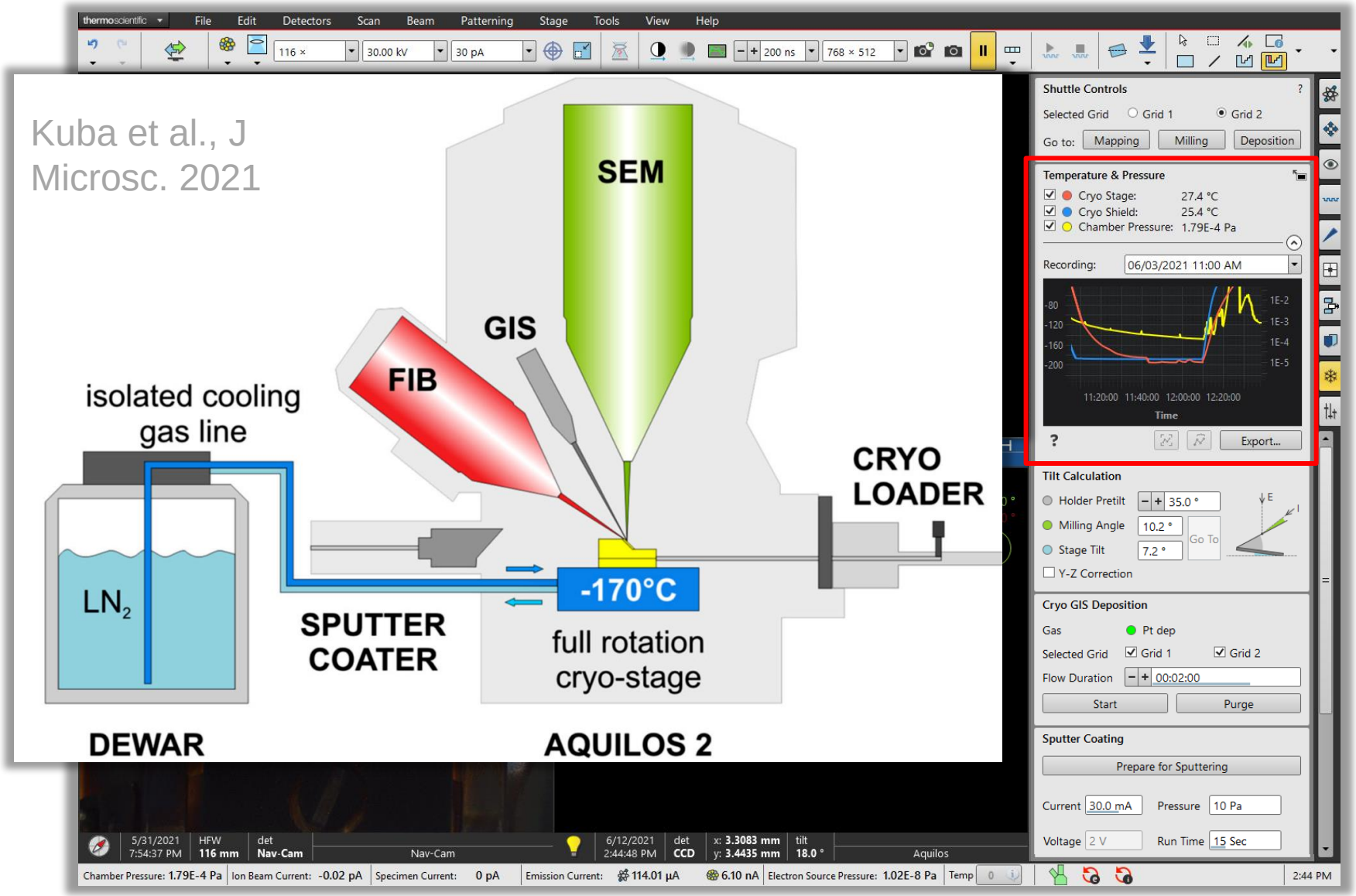


3.2.1 Prepare the system _Cool down the system

(xT Microscope Control)



Varies by instruments



3.2.2 Prepare the grids _Clip 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

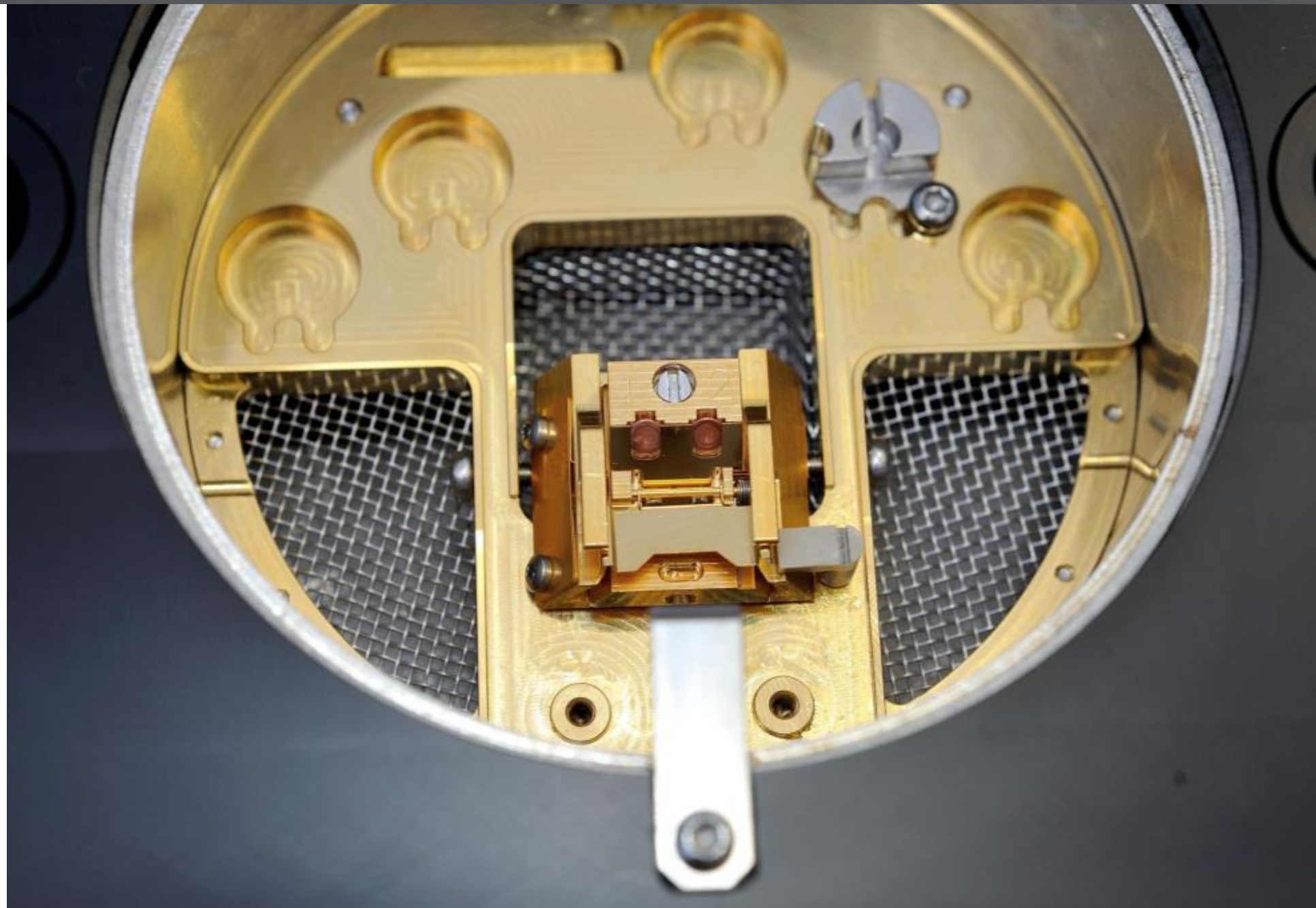
Lamella milling
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.2.2 Prepare the grids _Clip 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

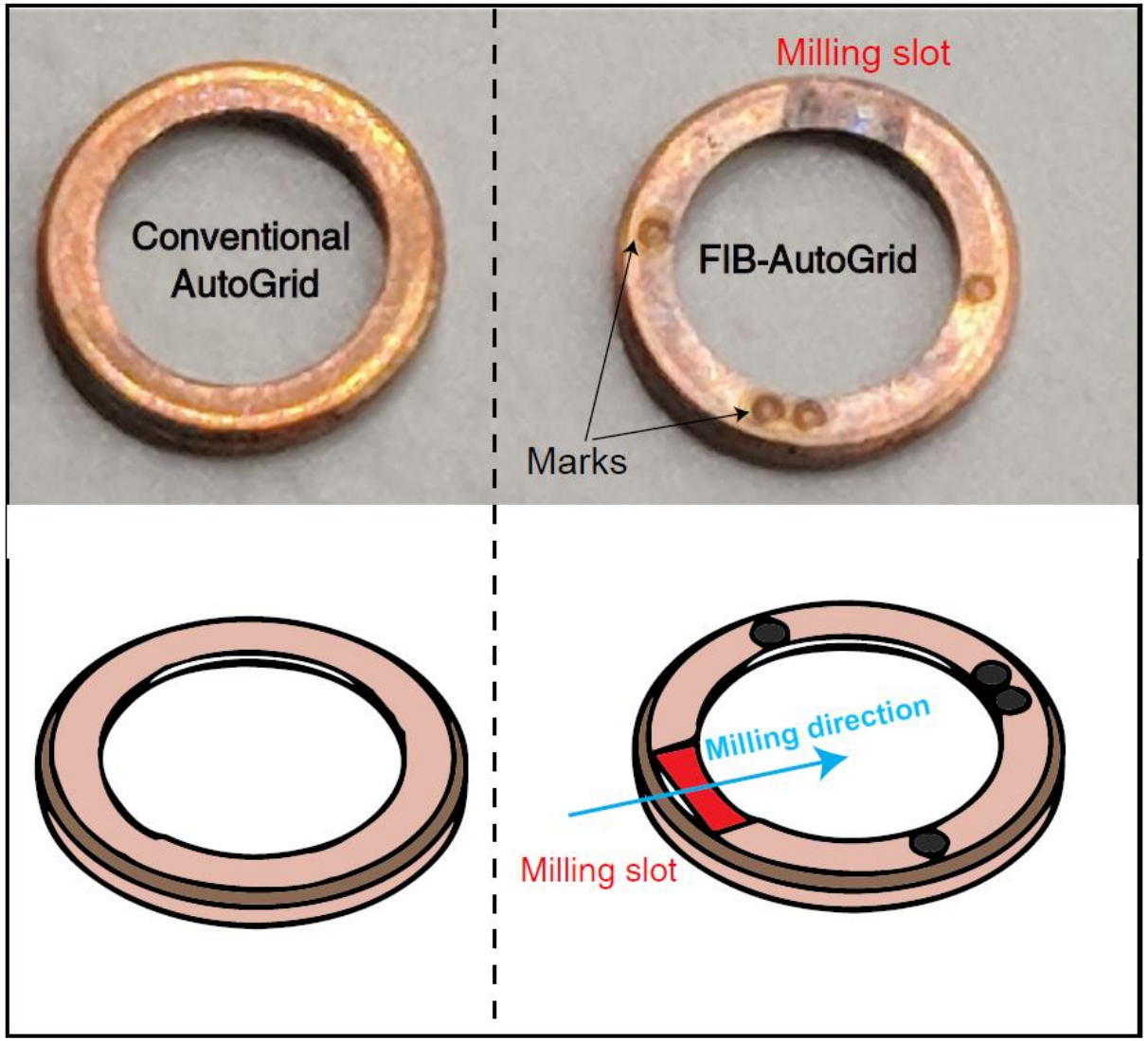
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

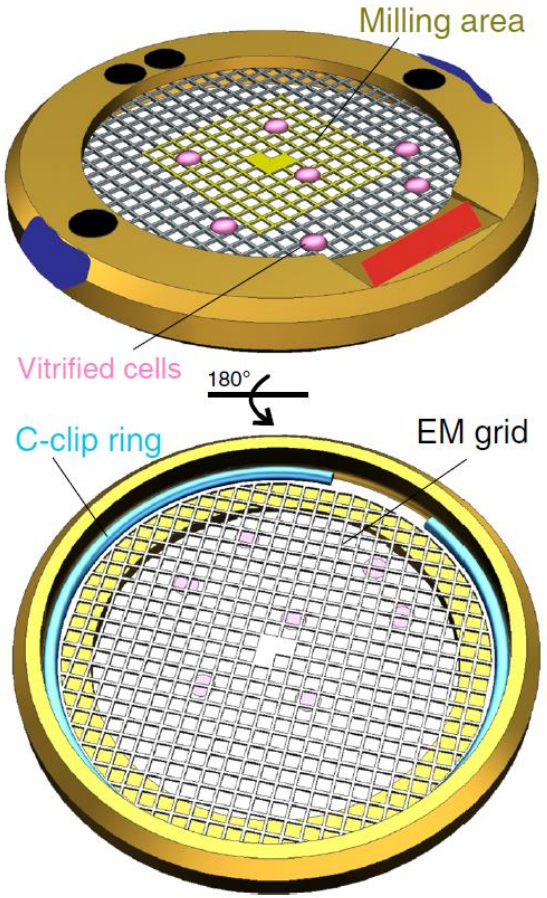
Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



Wagner, et al., Nature Protocols, 2020



3.2.2 Prepare the grids _Mark the grid rim for future lamellae orientation

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

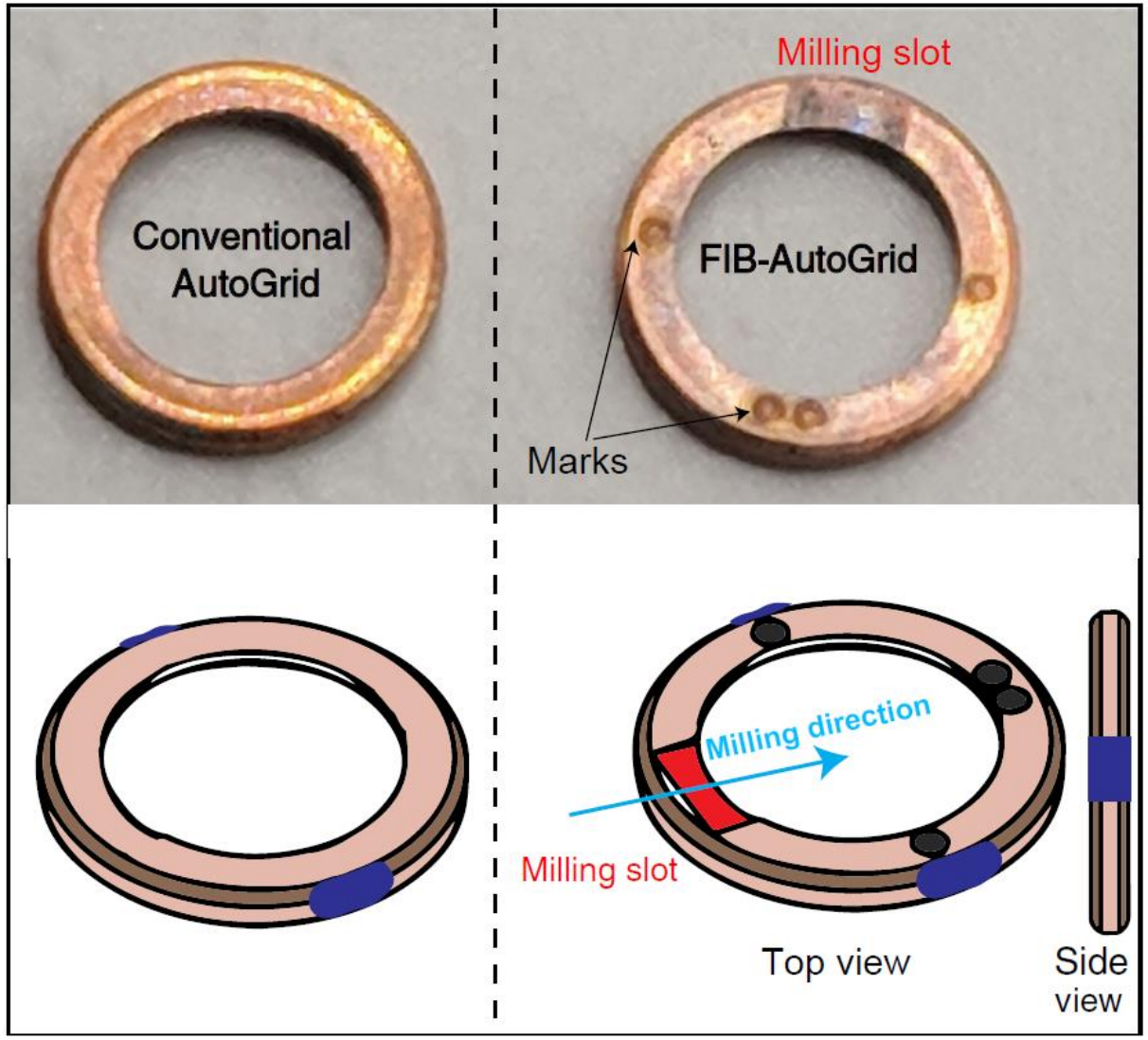
Lamella milling
Preparation, Milling, & thinning

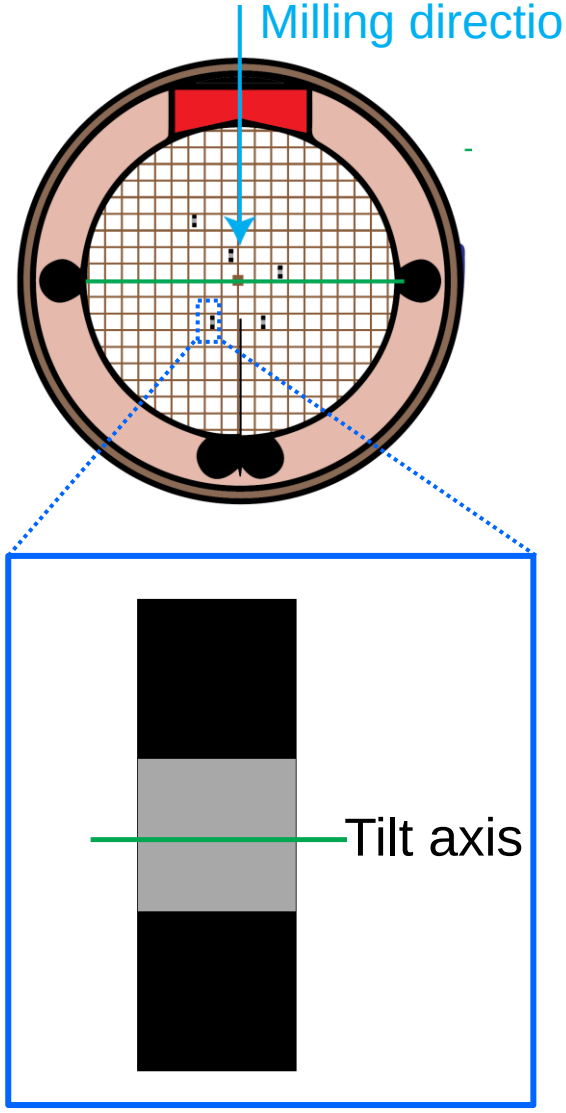
iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ **T**

CryoET





Wagner, et al., Nature Protocols, 2020

Orientating the lamellae

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

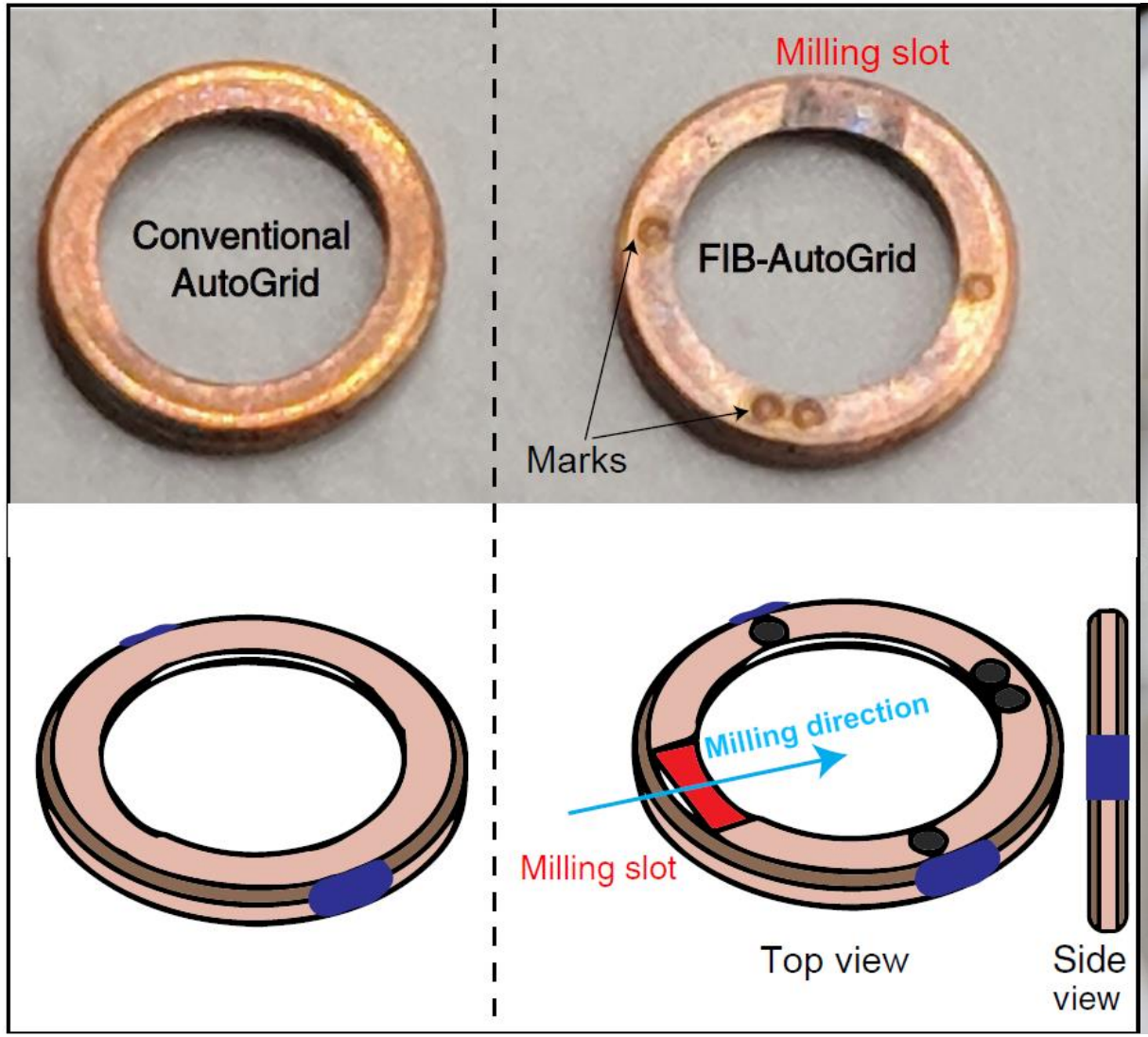
Lamella milling
Preparation, Milling, & thinning

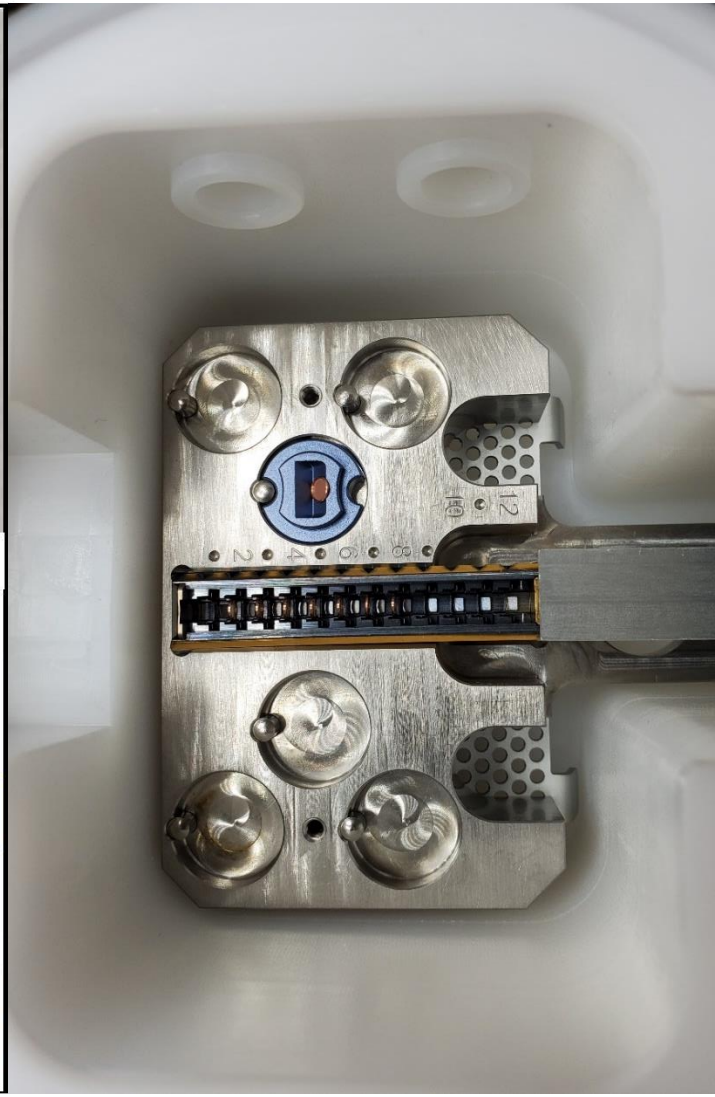
iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ **T** ←

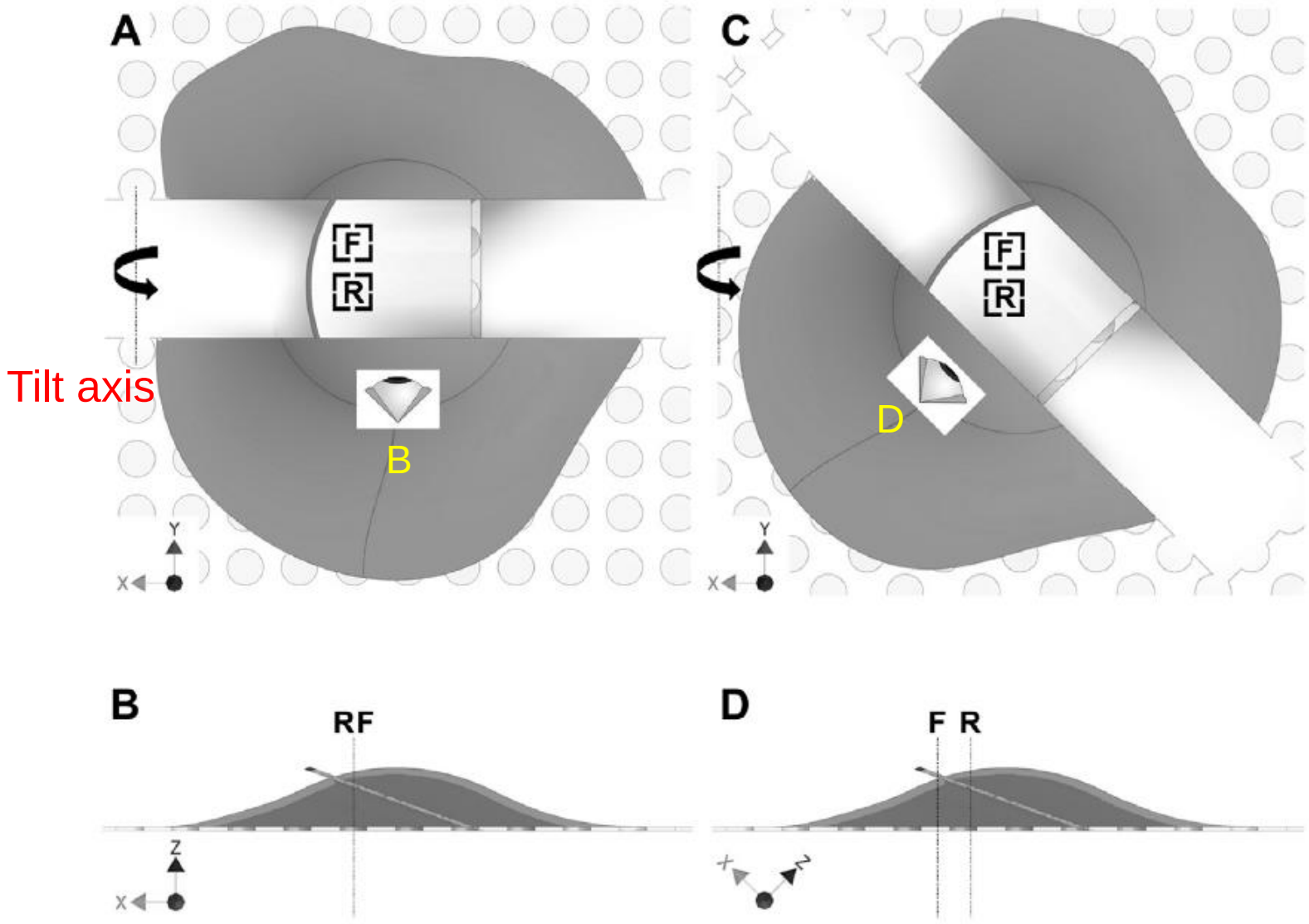
CryoET





Wagner, et al., Nature Protocols, 2020

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



Schaffer et al., JSB 2017

3.2.3 Transfer the grids _Load grids to Autogrid shuttle

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

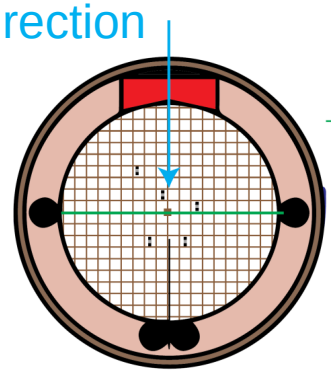
iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

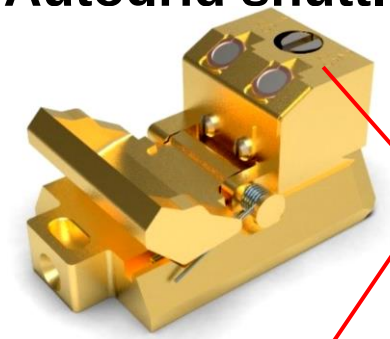
↓ **T**

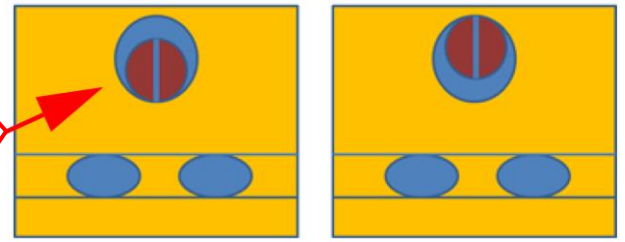
CryoET

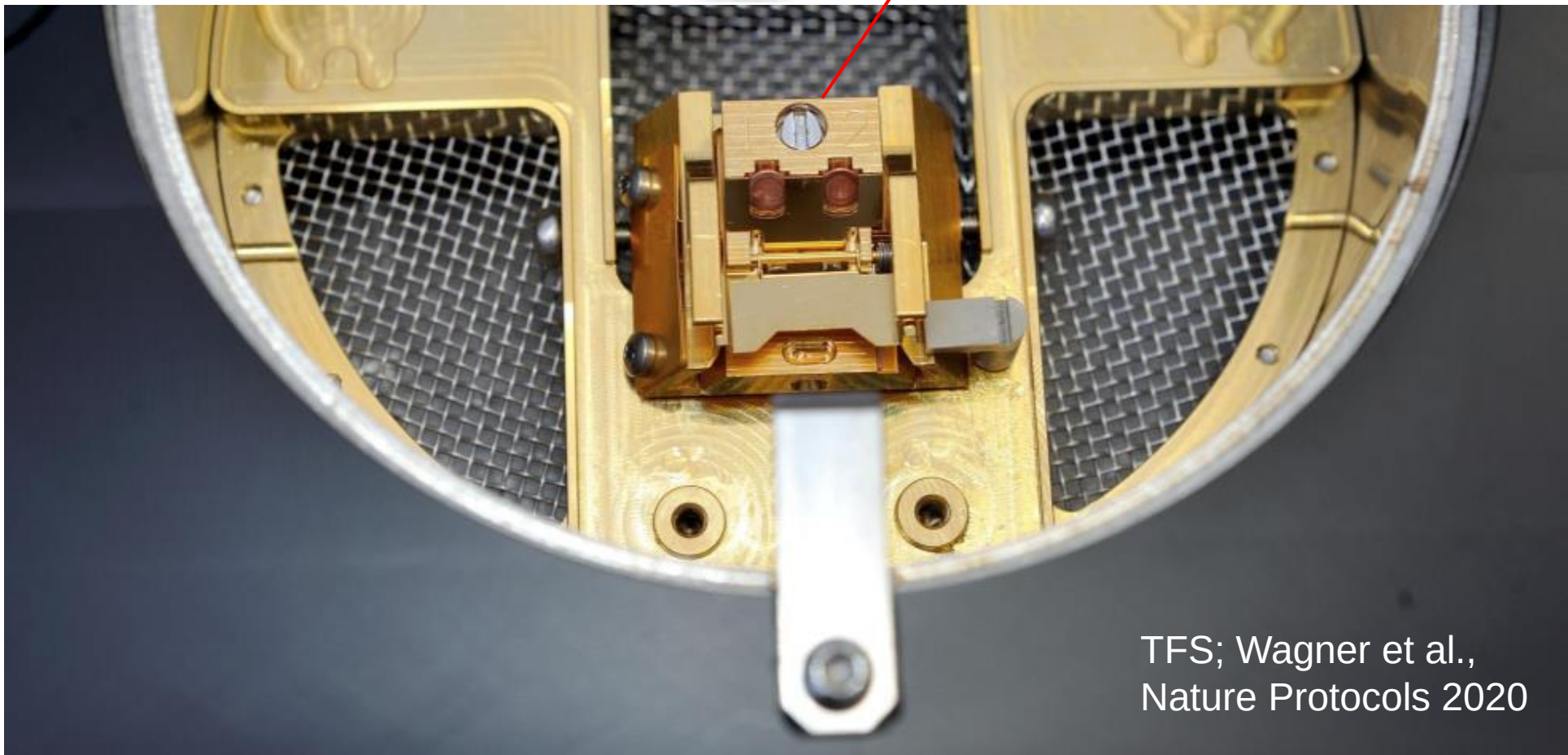
Milling direction



Autogrid shuttle

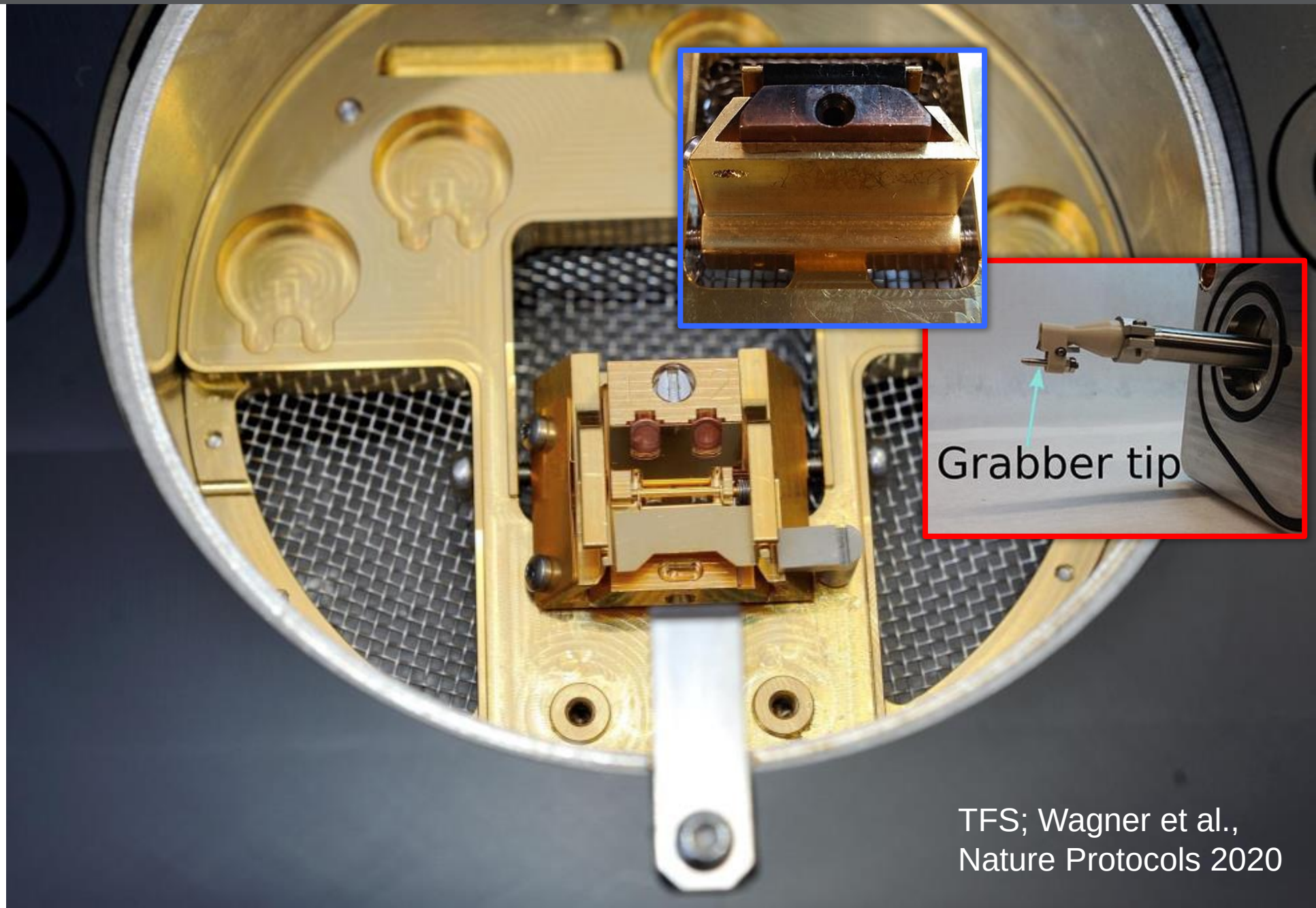






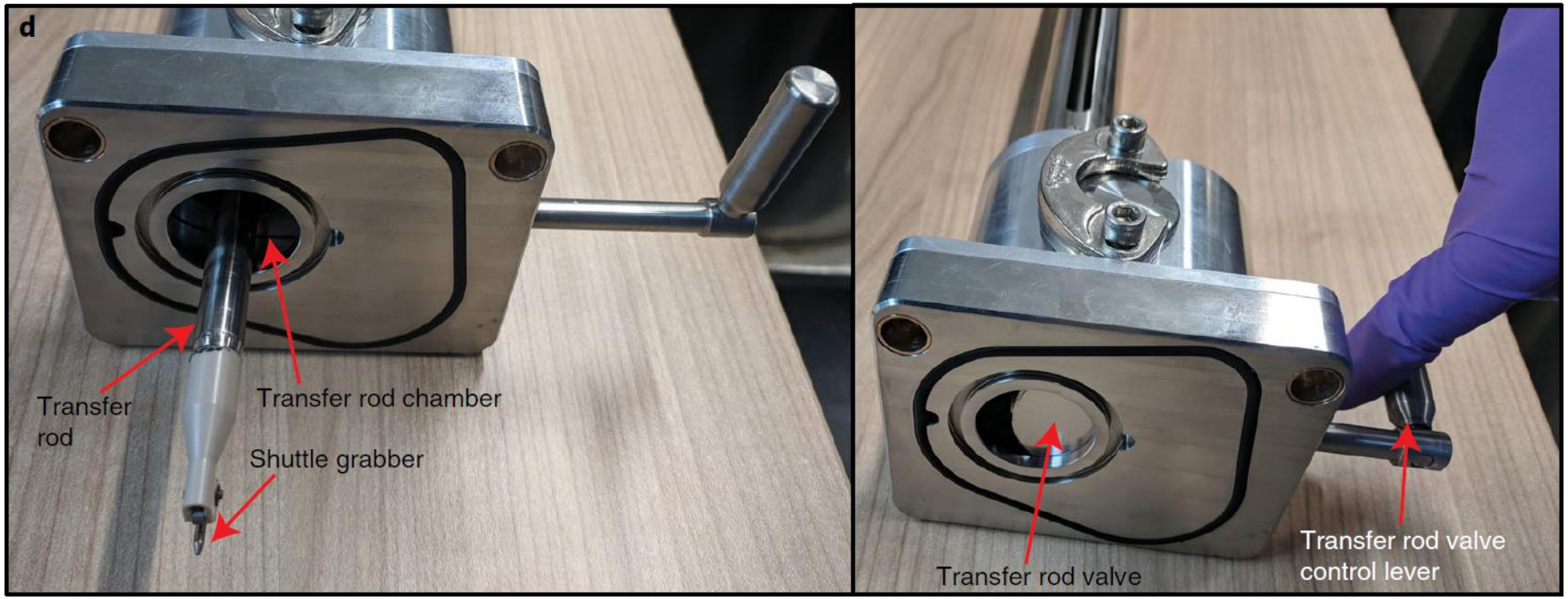
TFS; Wagner et al.,
Nature Protocols 2020

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

(xT Microscope Control)

Vitrification

↓ T

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

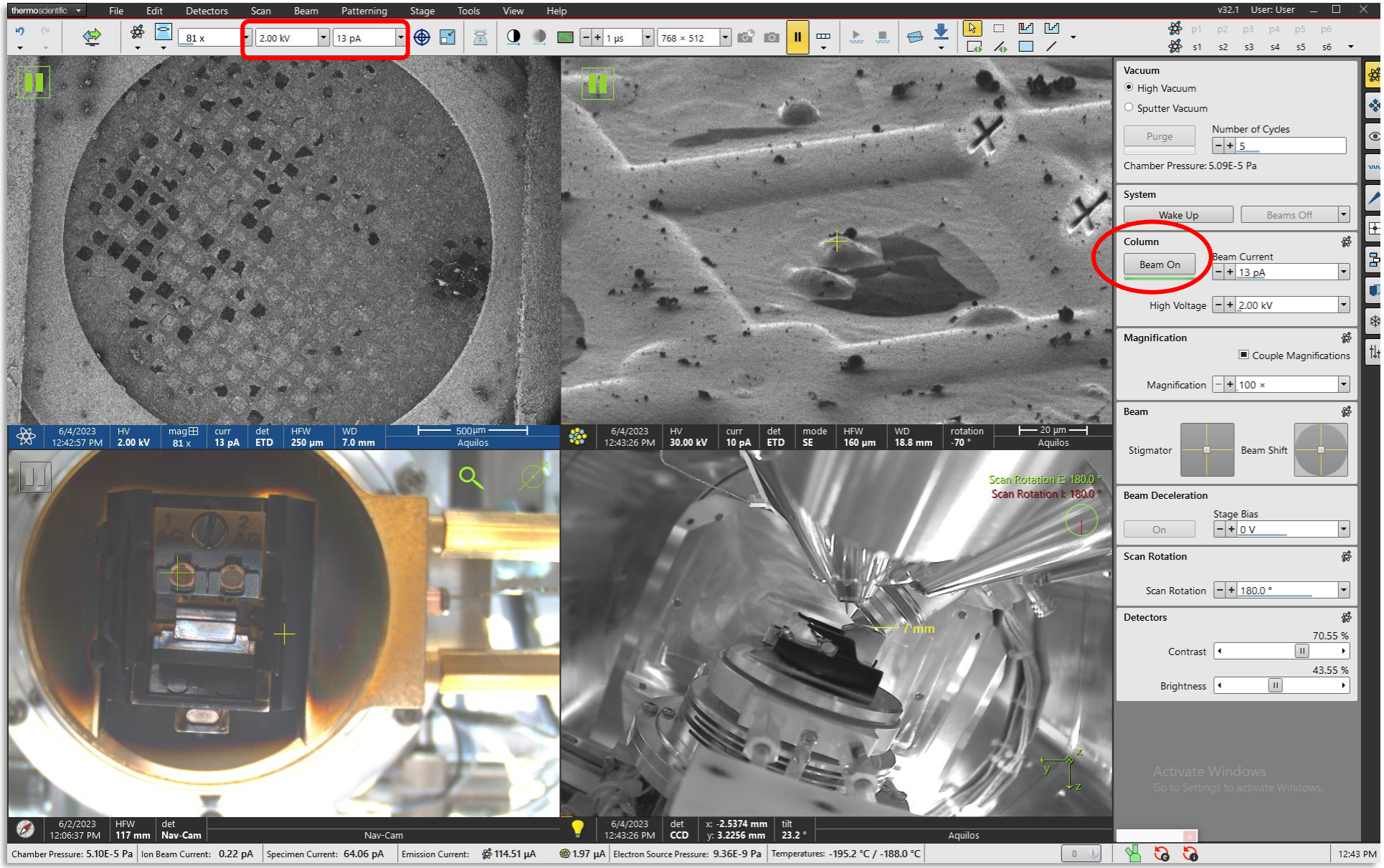
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.3 Sample screening _Quickly check the grids

(xT Microscope Control)

Vitrification

↓ T

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

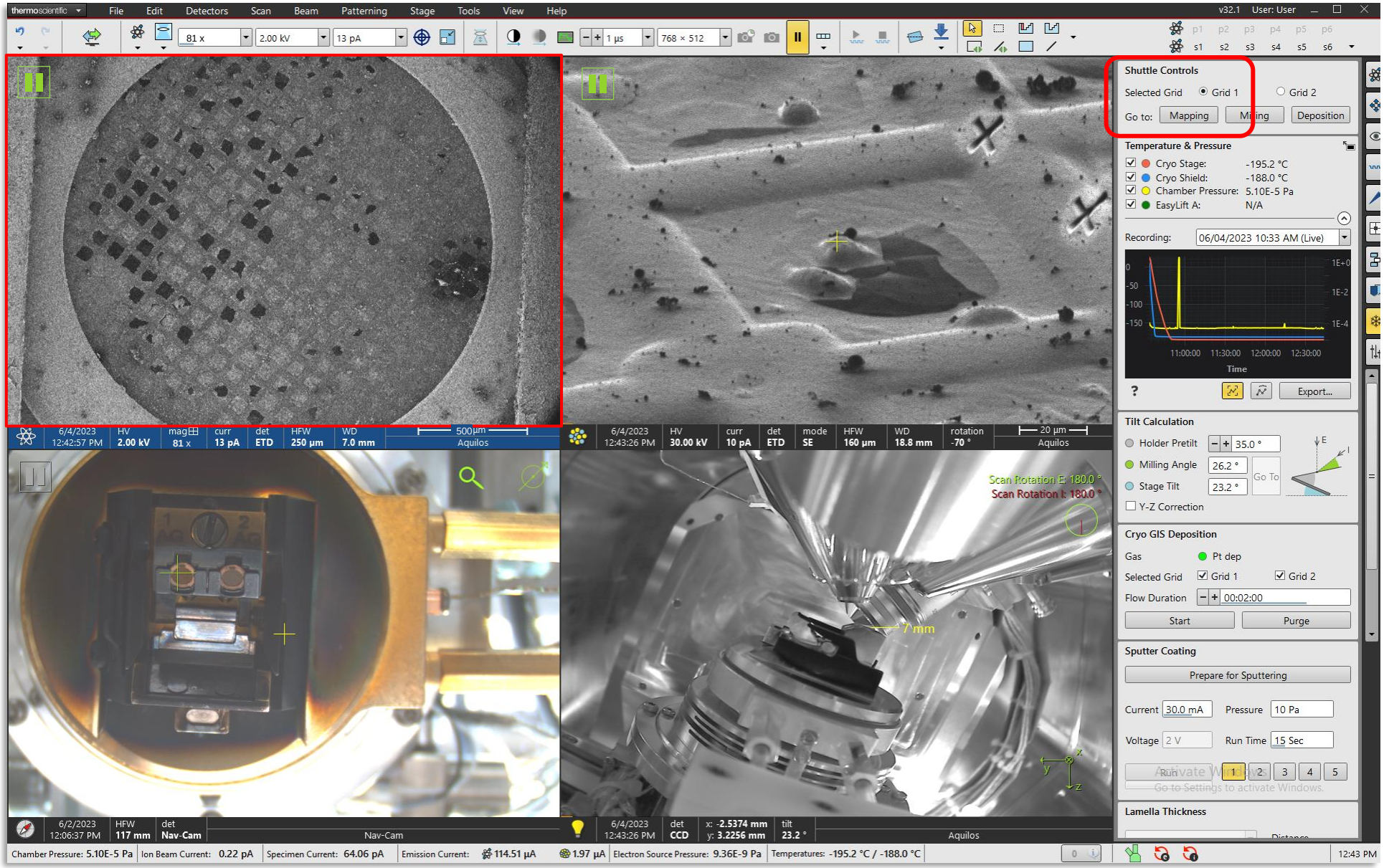
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.3 Sample screening _Link Z to FWD

(xT Microscope Control)

Vitrification

↓ T

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

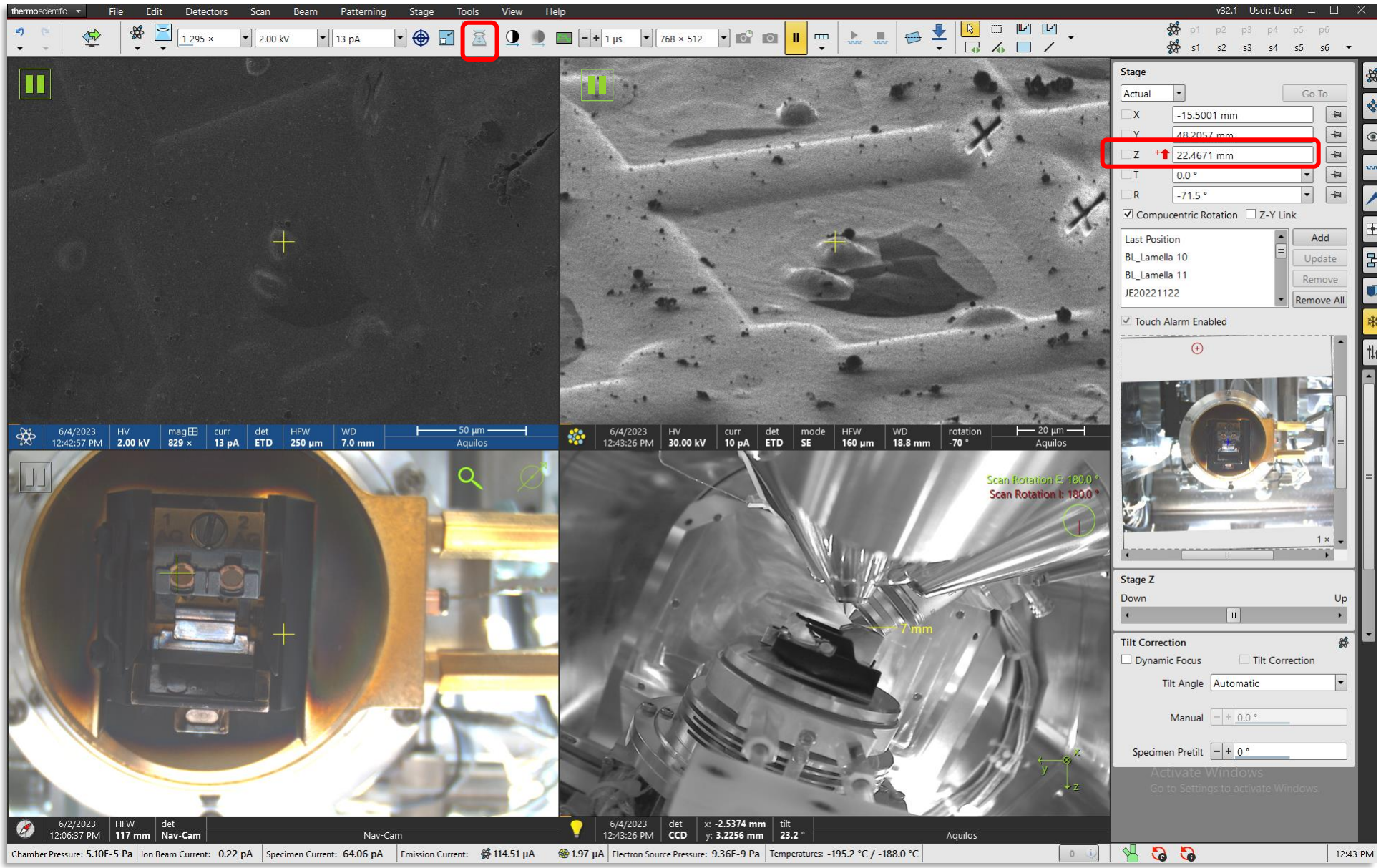
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.3 Sample screening _Create a Maps Project

(Maps)

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

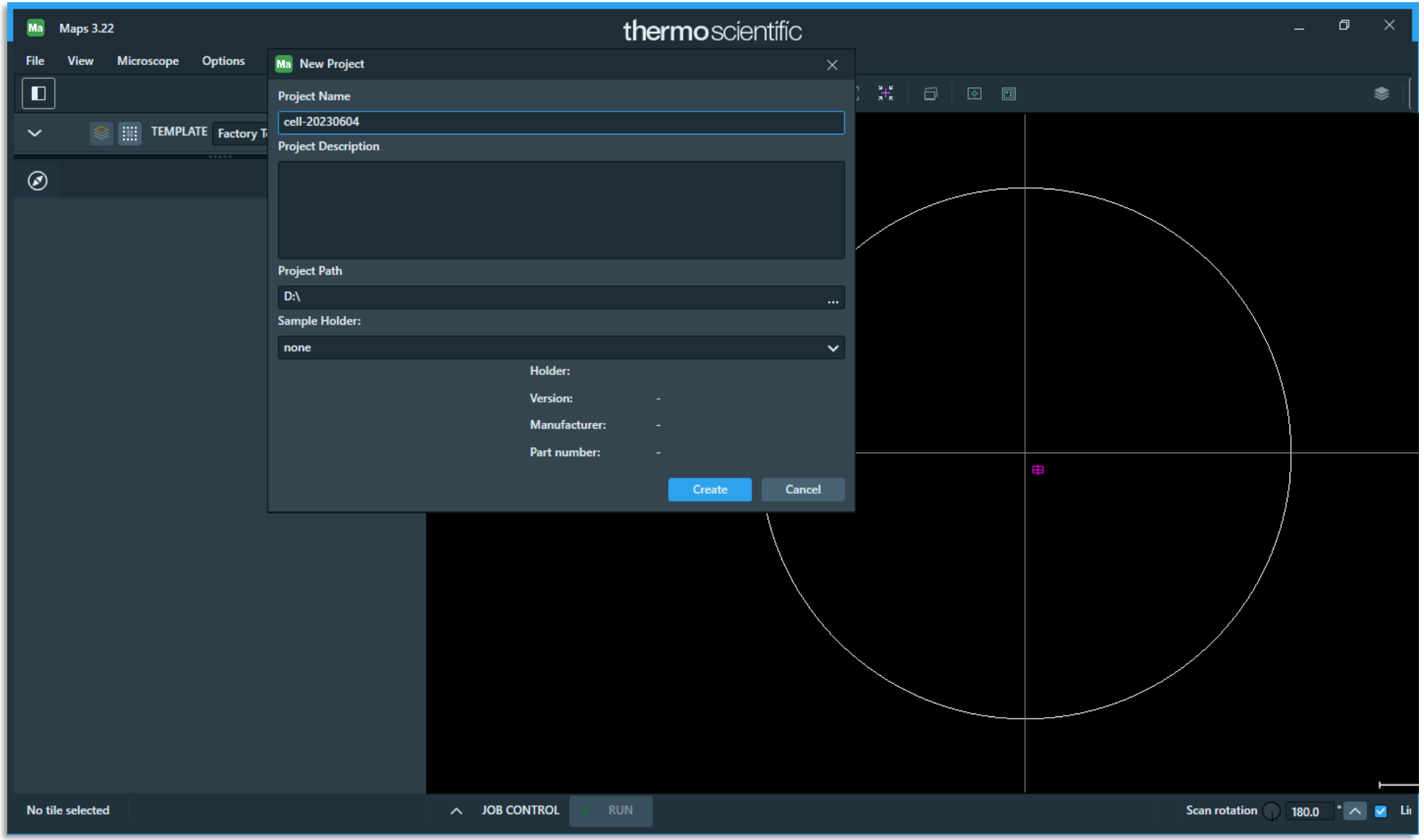
Lamella milling
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.3 Sample screening _Take a snapshoot of the grid

(Maps)

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

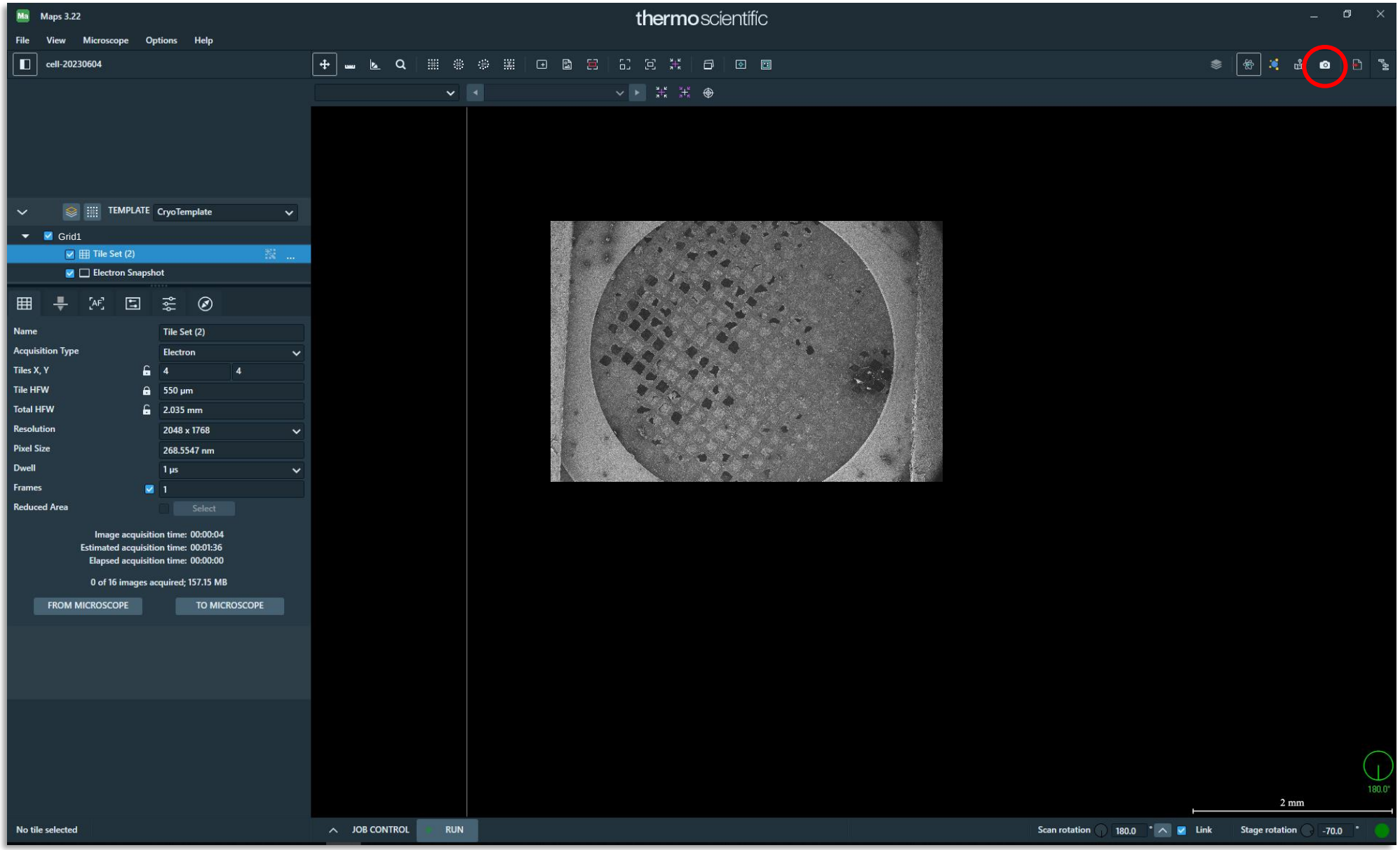
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.3 Sample screening _Set up Tile Set & Run atlas acquisition

(Maps)

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

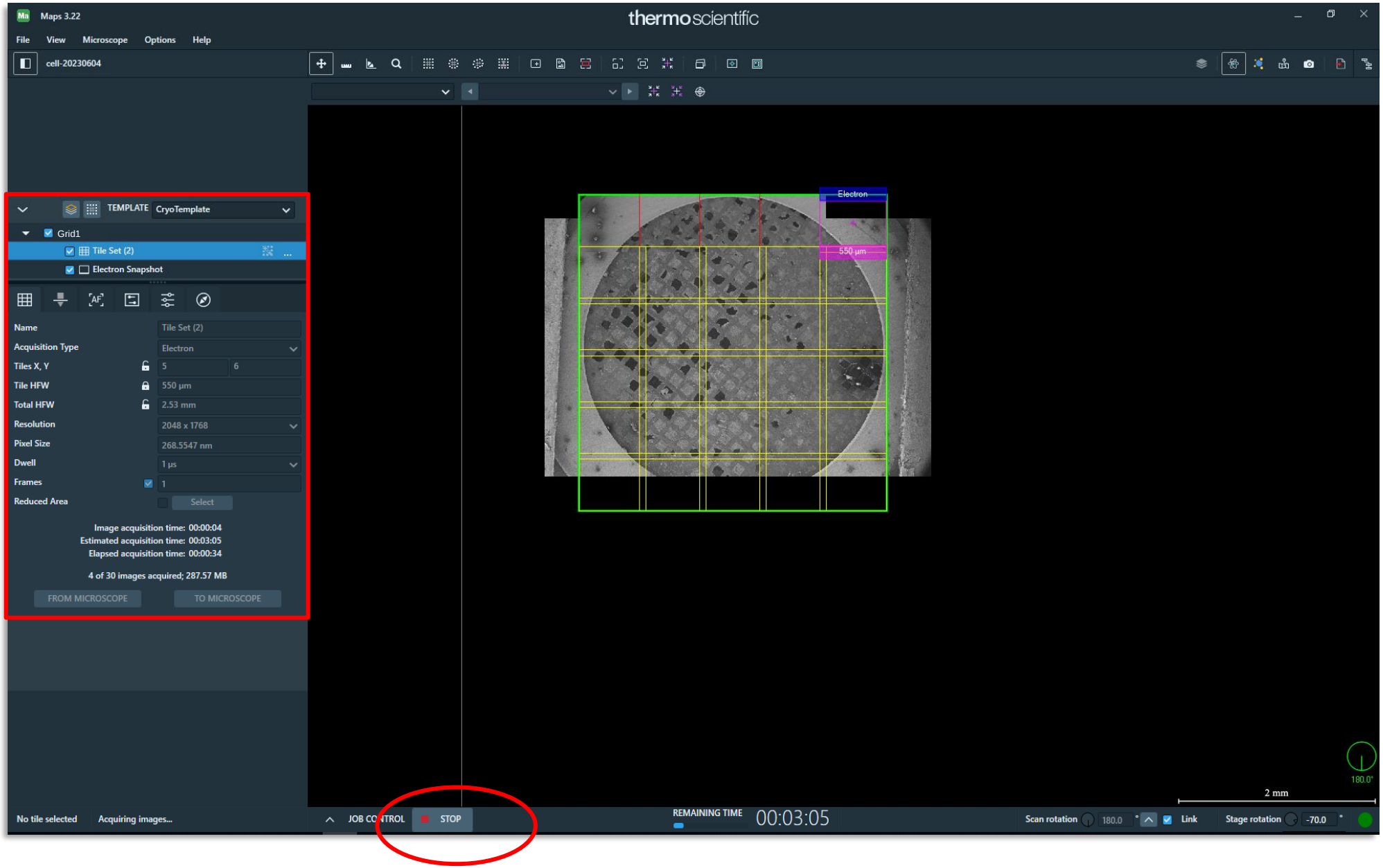
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.3 Sample screening _Add candidate lamella sites

(Ma Maps)

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

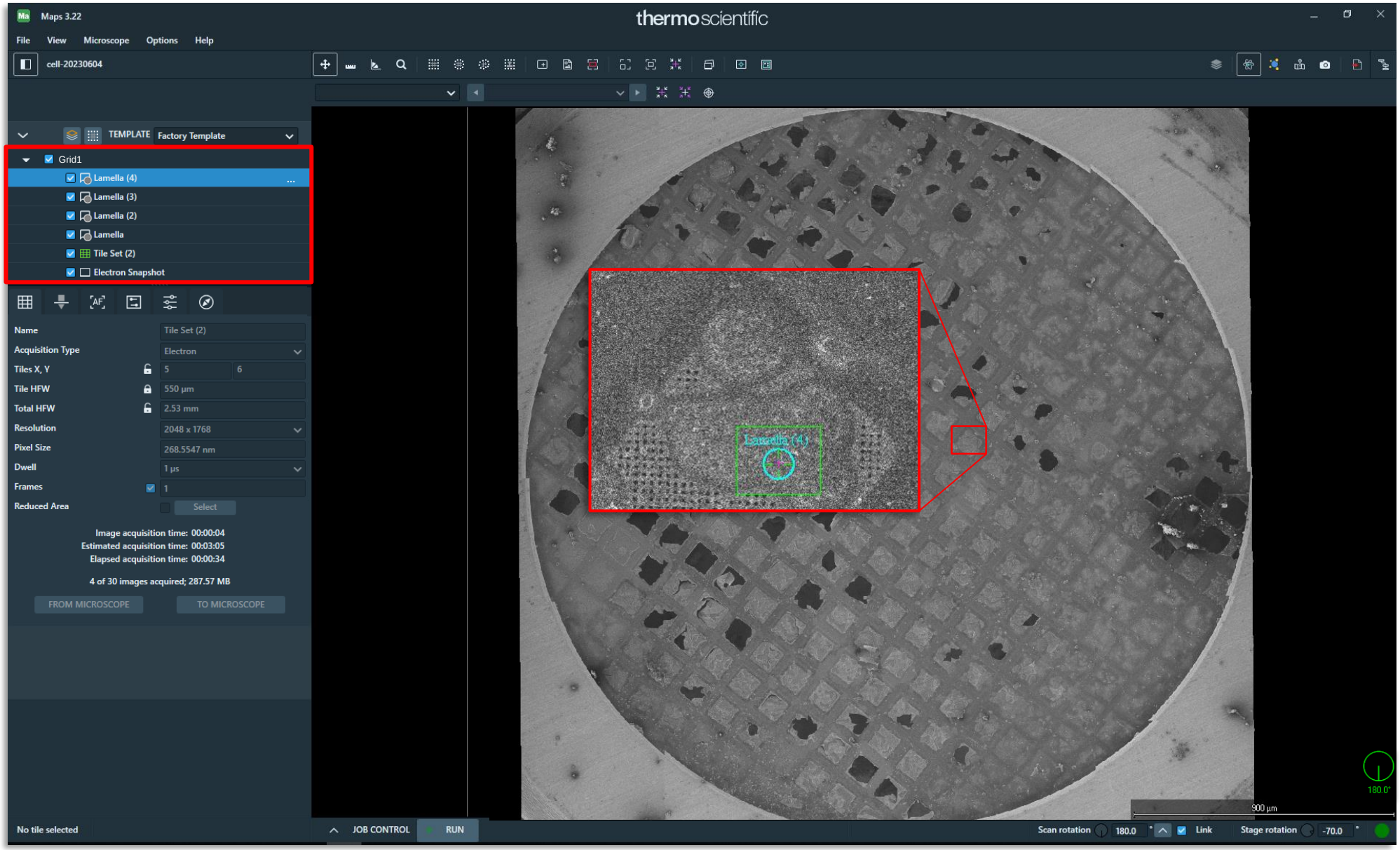
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.4 CryoFLM for tart selection

Vitrification

↓ **T**

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

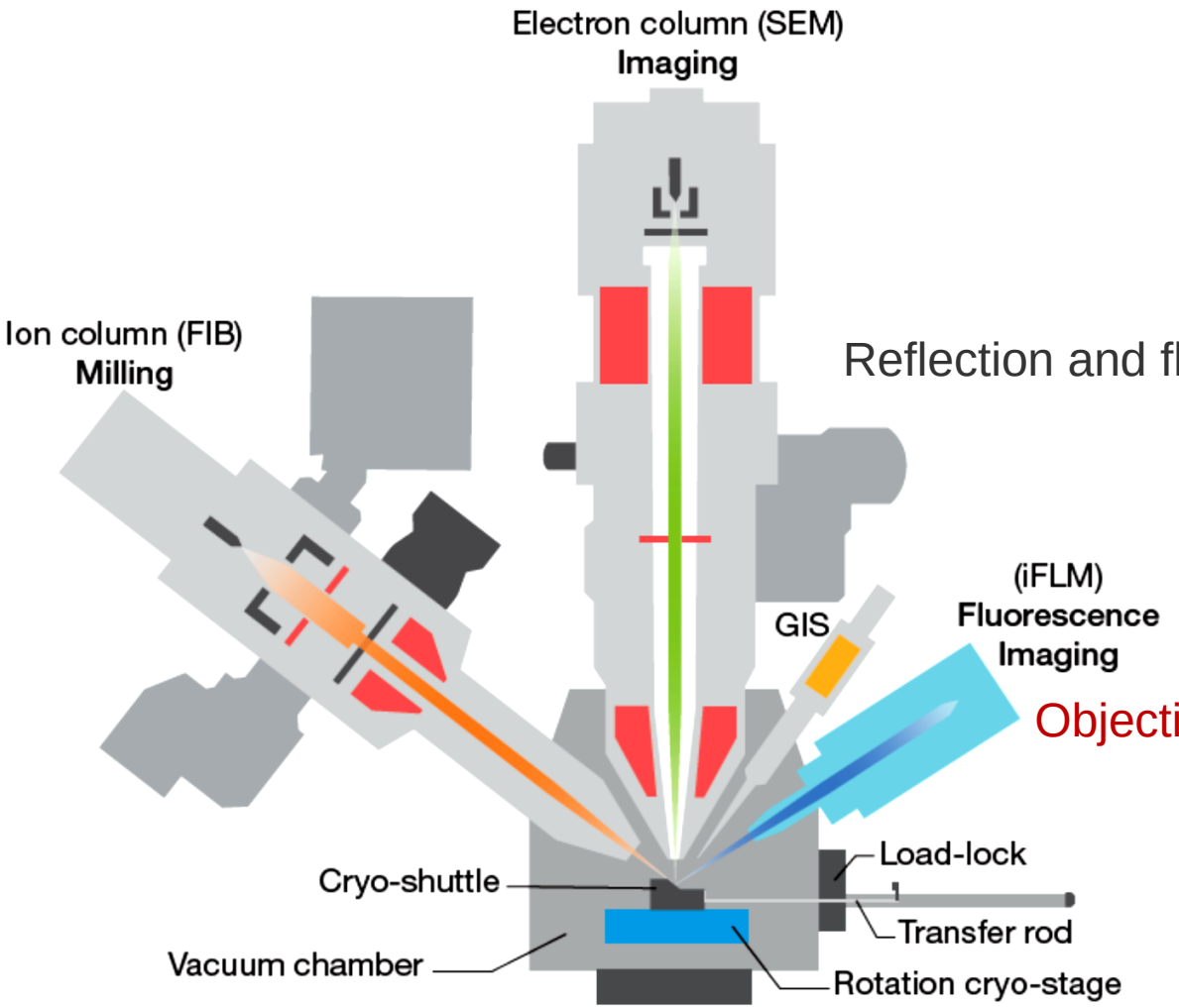
Lamella milling
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ **T**

CryoET



Reflection and fluorescence imaging

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

Adapted from TFS

3.4 CryoFLM _Focus with Objective

(Fluorescence Microscope Control)

Vitrification

↓ T

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

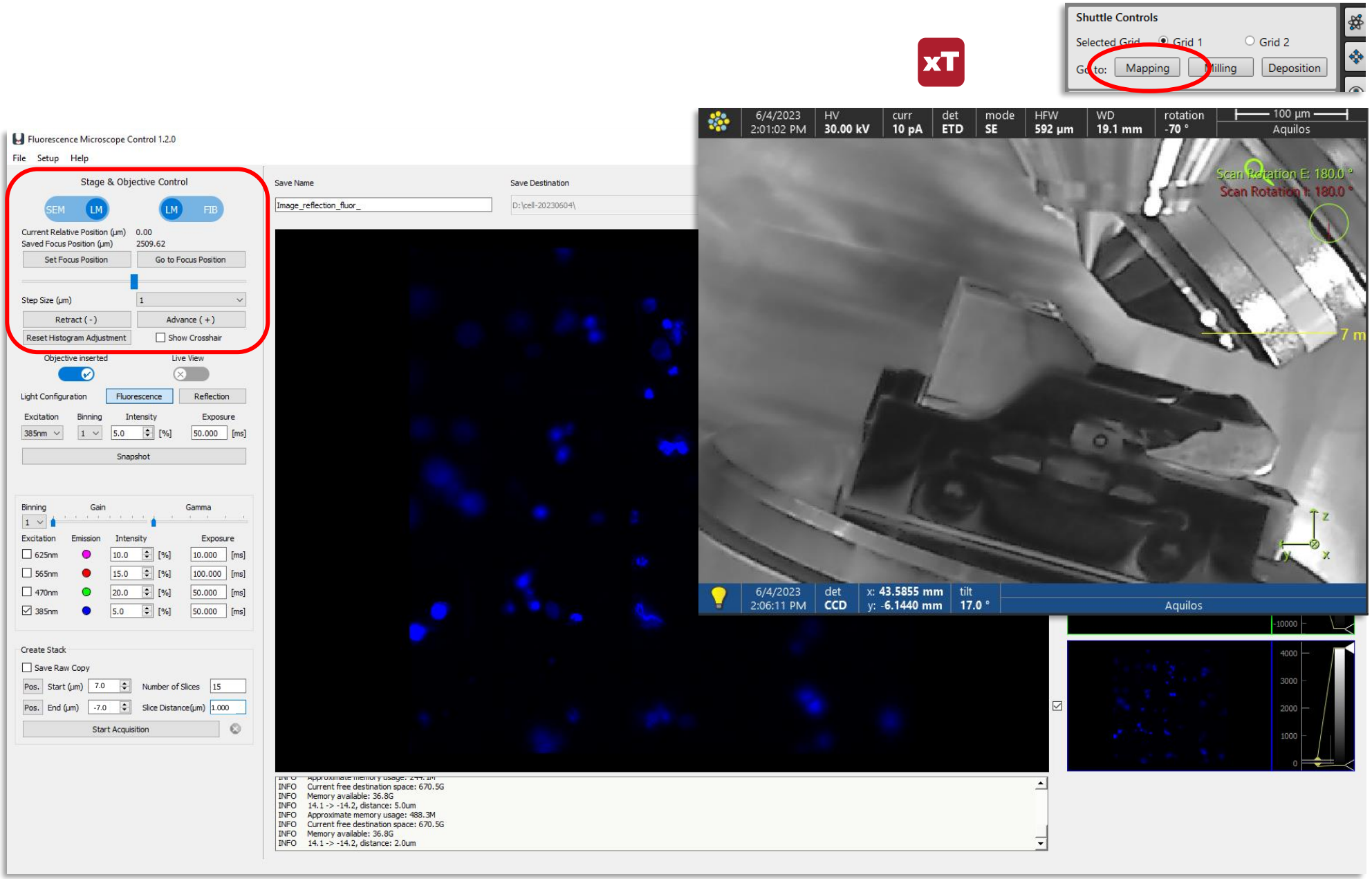
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.4 CryoFLM _Setting up imagining 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

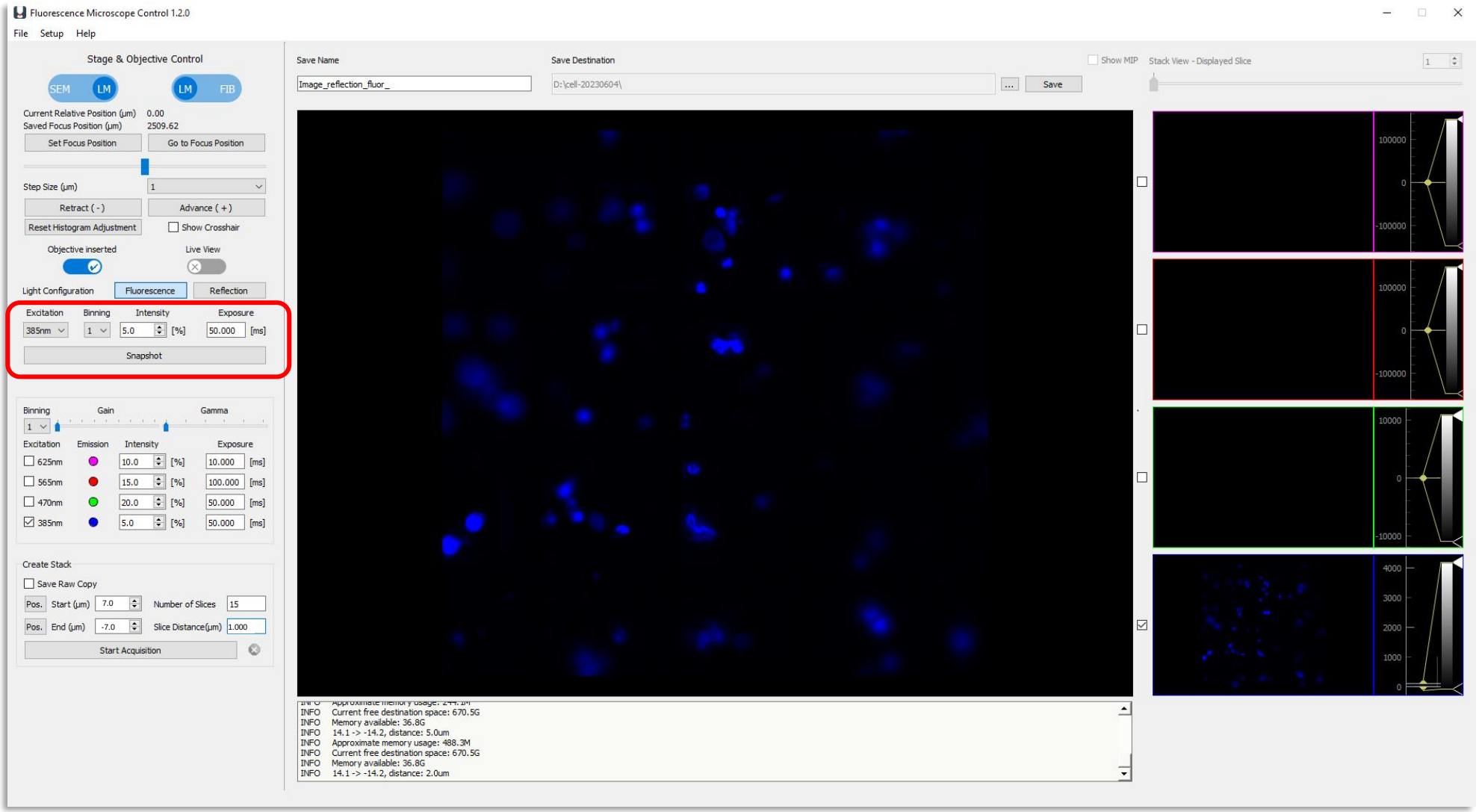
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.4 CryoFLM _Setting up Z-stack

(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

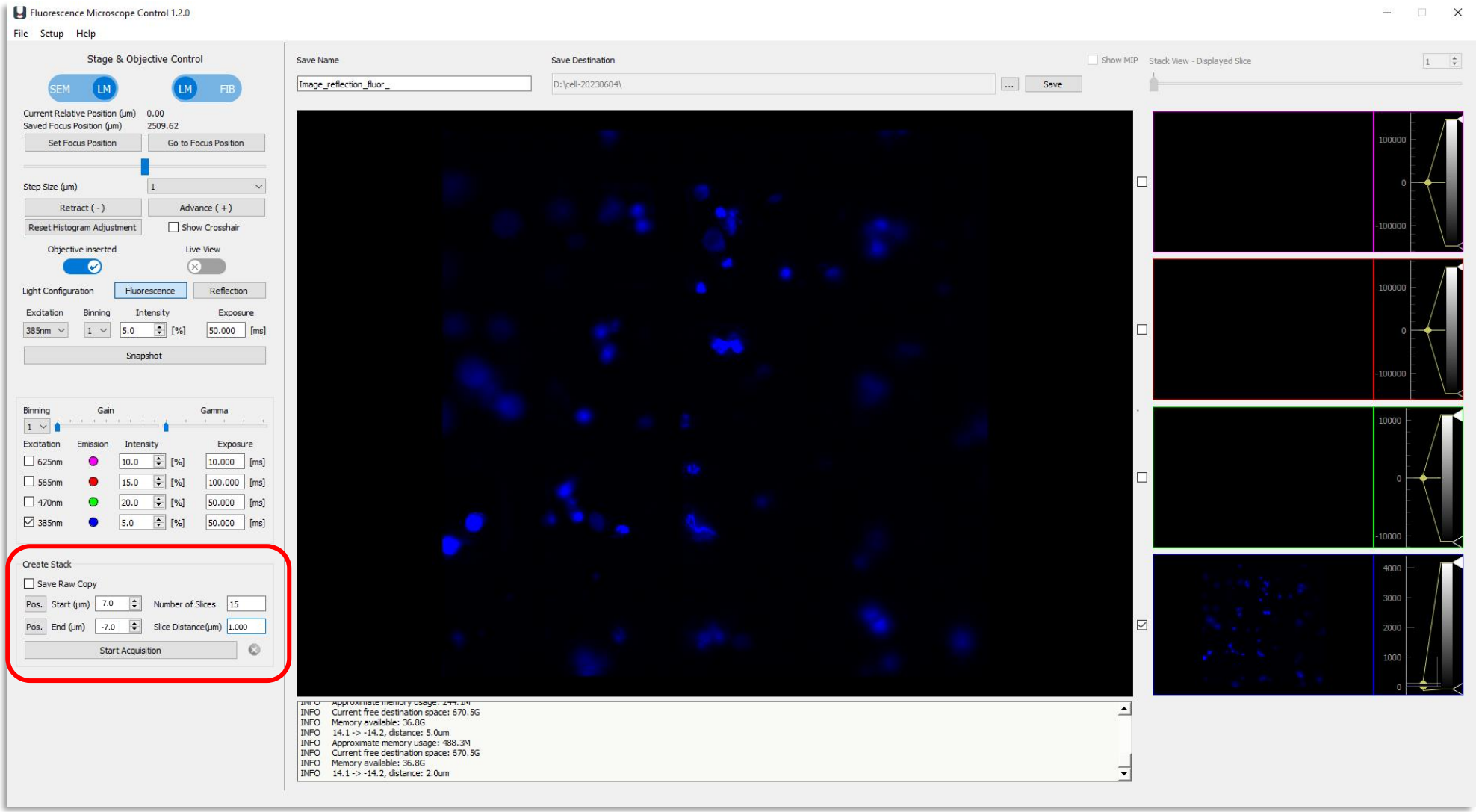
Lamella milling
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

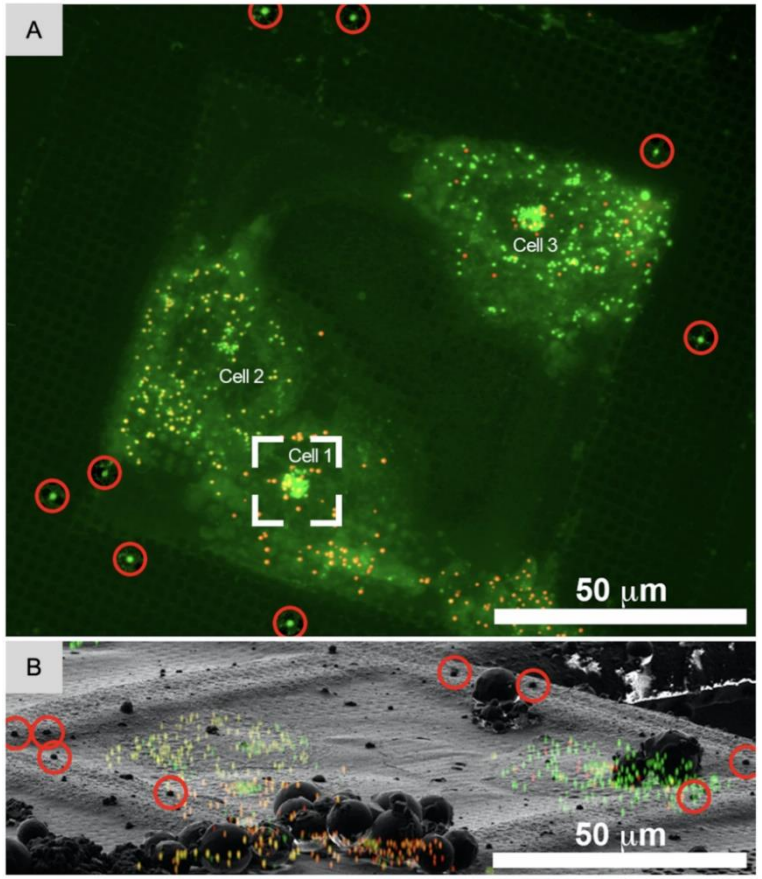
CryoET



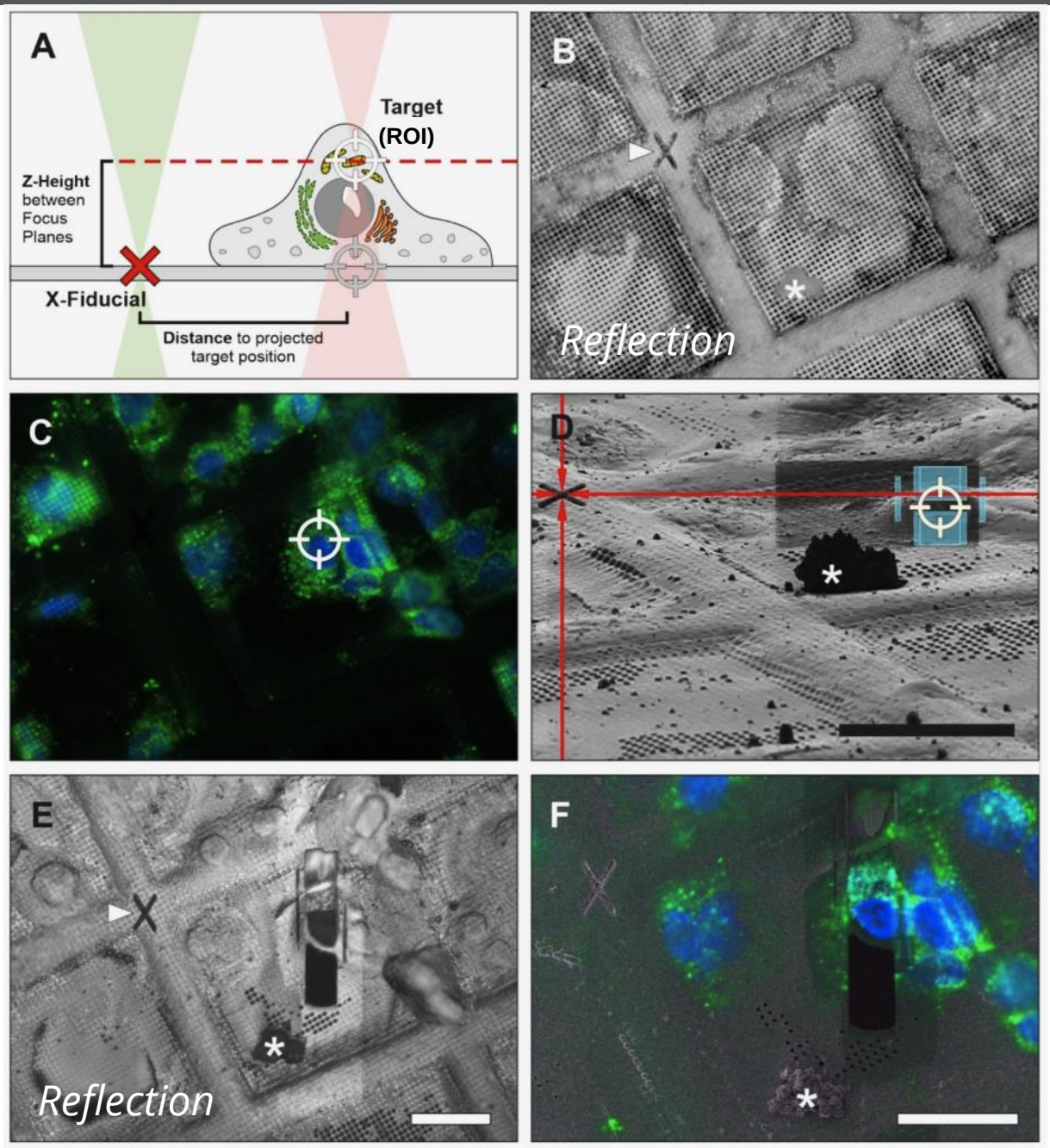
Fiducial markers & X-Fiducials for accurate FLM-SEM alignment and target positioning

X-Fiducials

1-µm Magnetic beads



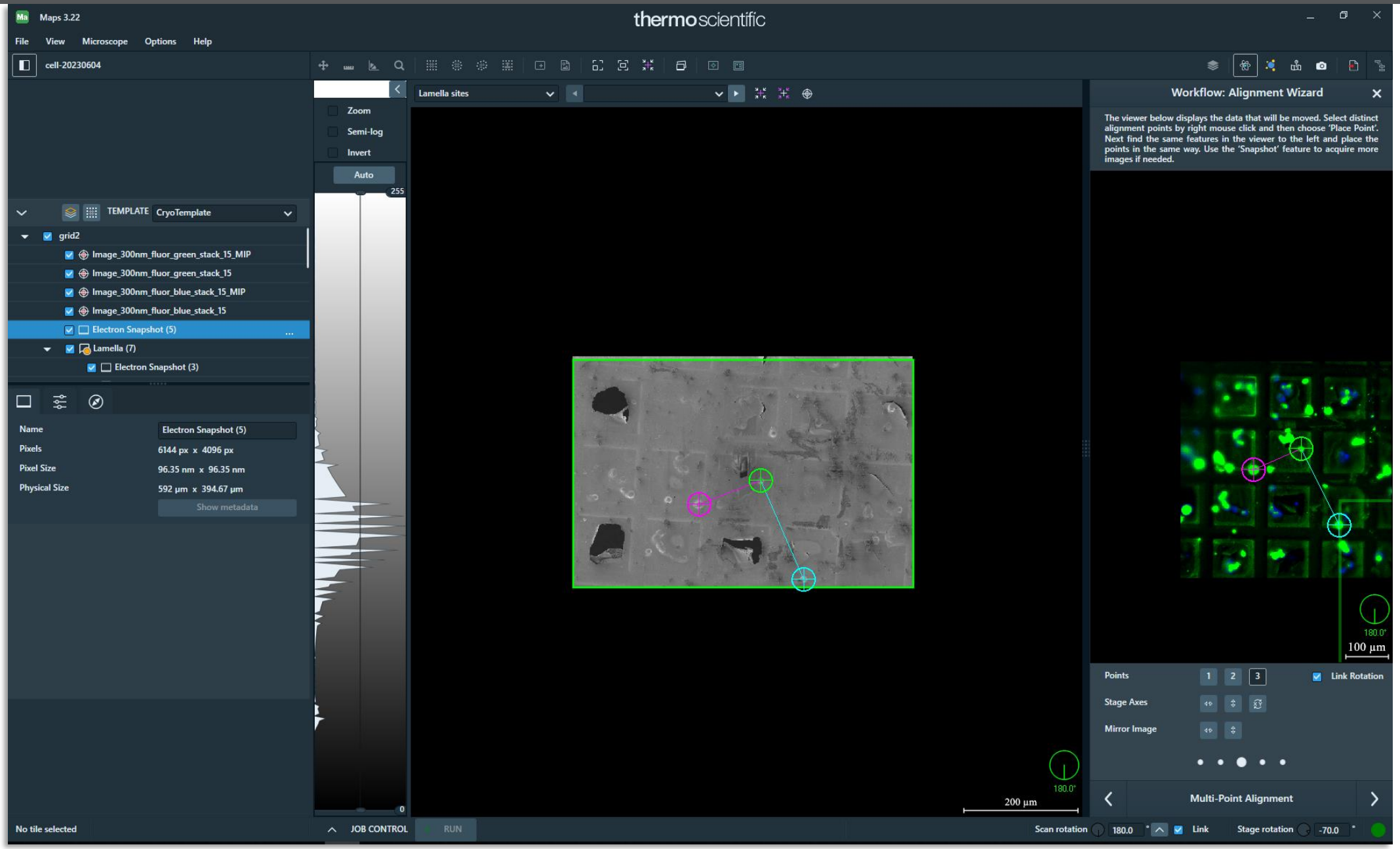
Arnold et al., Biophysical Journal 2016



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

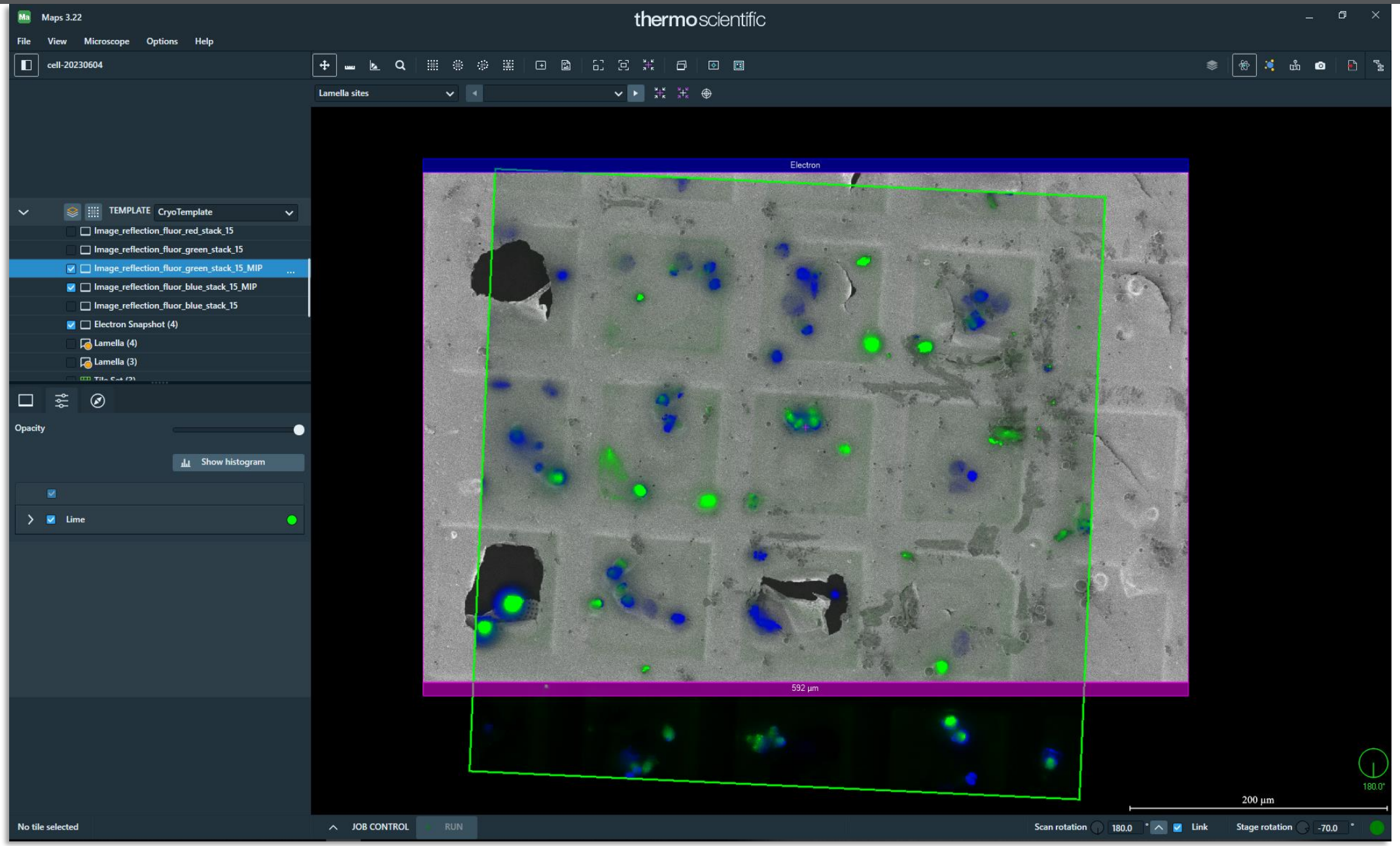
3.4 CryoFLM _FLM-SEM alignment

(Maps)



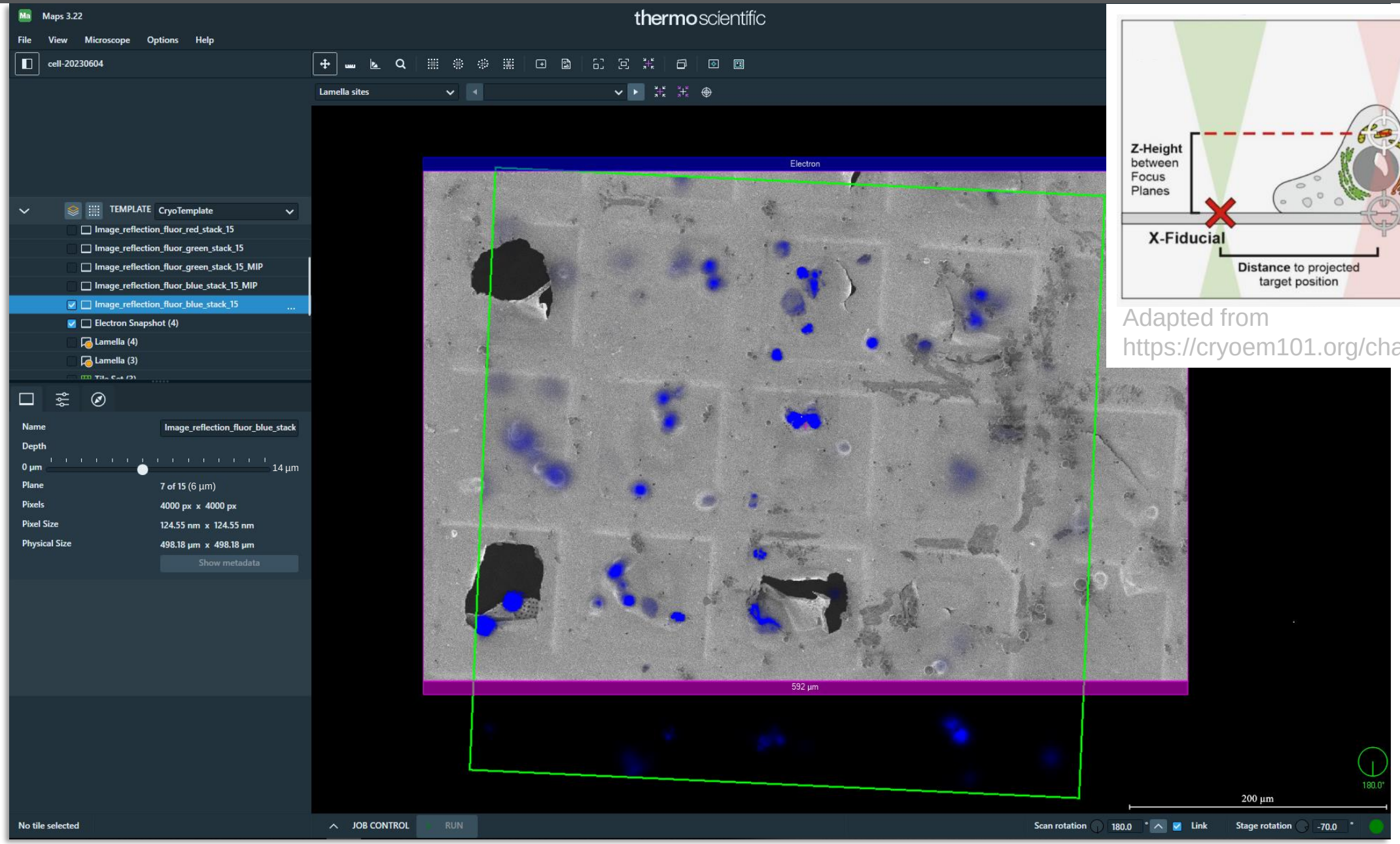
3.4 CryoFLM _FLM-SEM alignment

(Maps)



3.4 CryoFLM _Define ROI and Fiducial for each lamella

(Maps)



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

3.5 Pt sputter

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

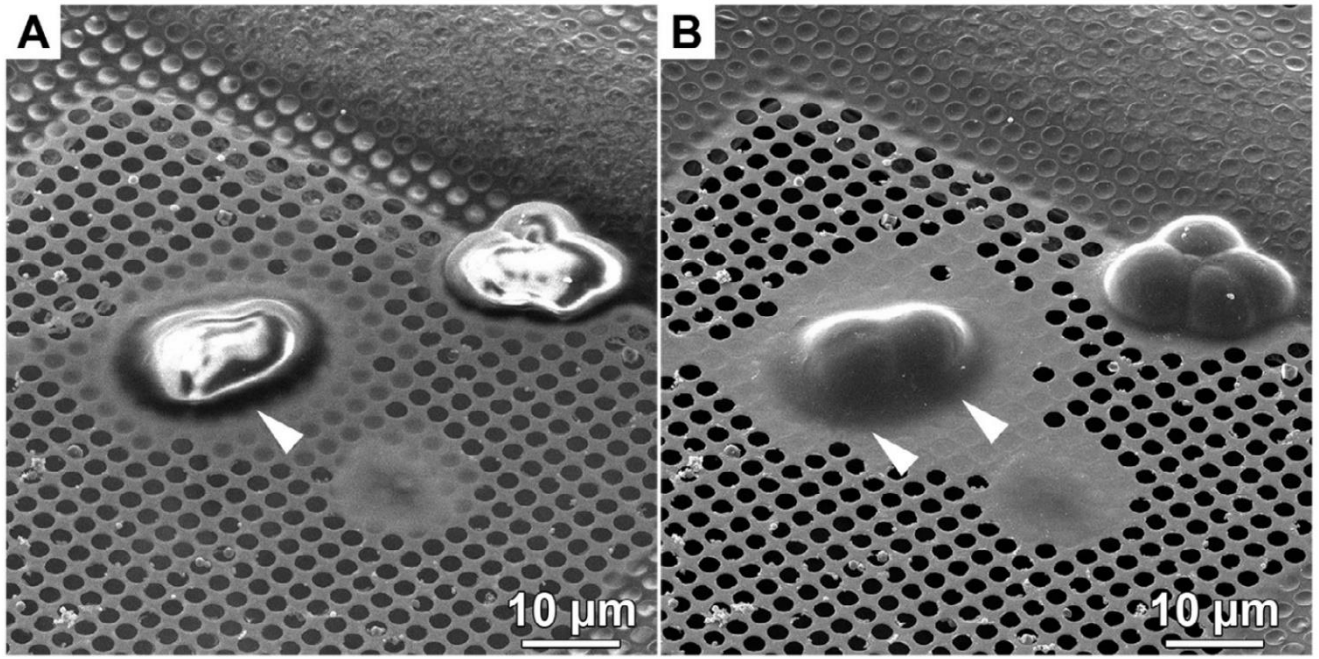
iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ **T**

CryoET

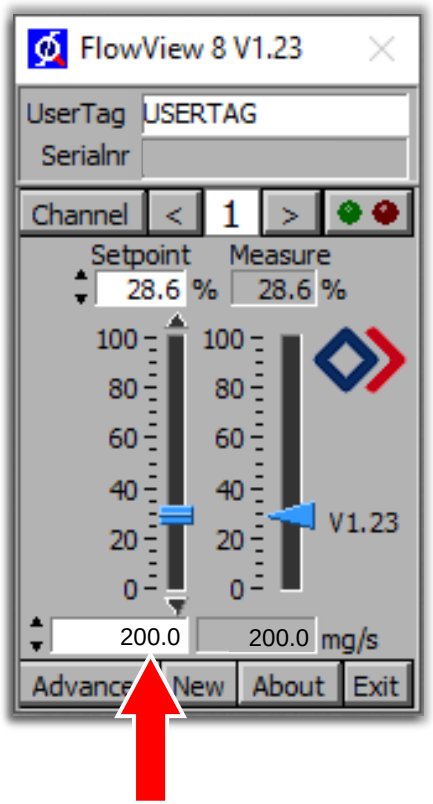
(Optional) Inorganic Pt minimizes charging, ensure targeting and precise milling.



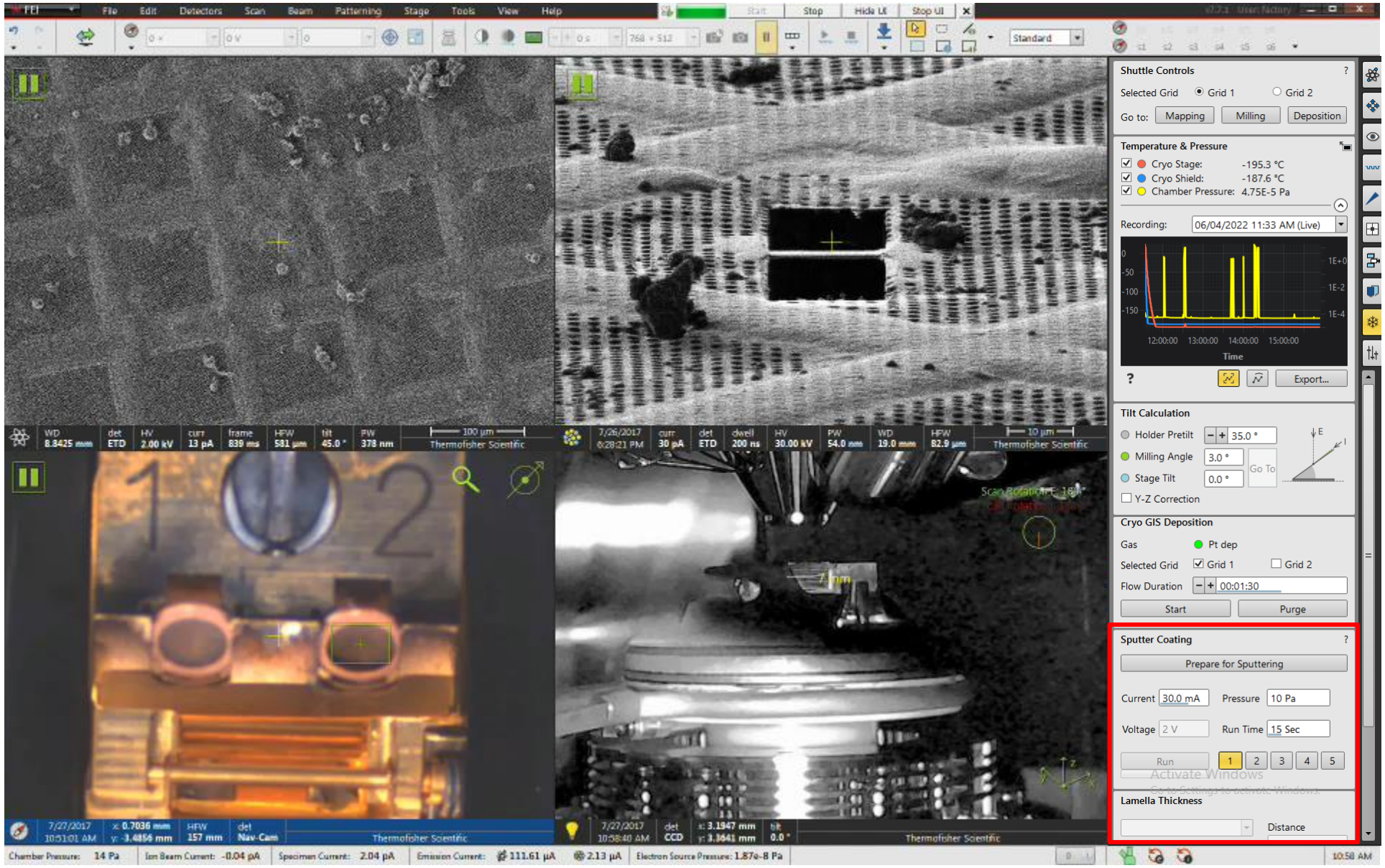
Schaffer et al., JSB 2017

3.5 Pt sputter

(Video from TFS; xT Microscope Control)



Increase to 200 mg/s
(varies by instruments)



3.5 Pt GIS

(xT Microscope Control)

Vitrification

↓ T

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

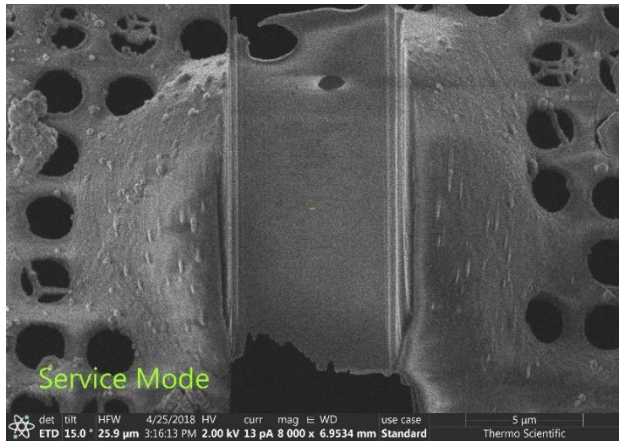
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

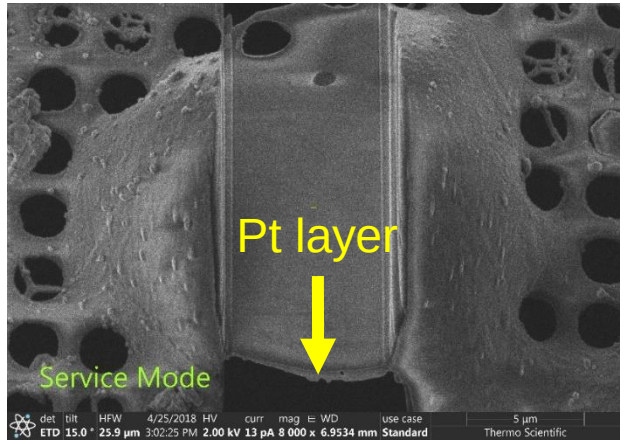
Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



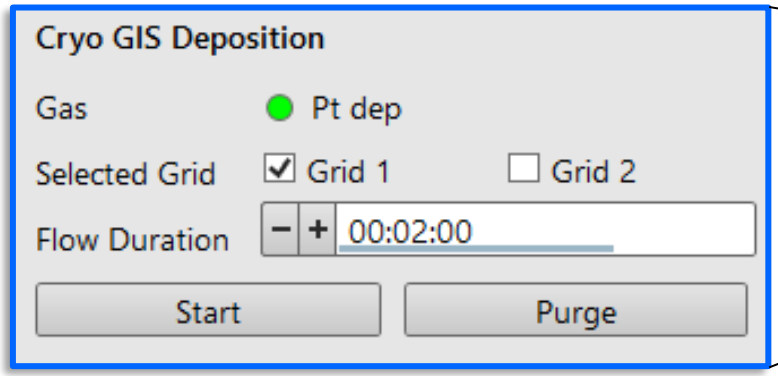
Without Pt GIS coating



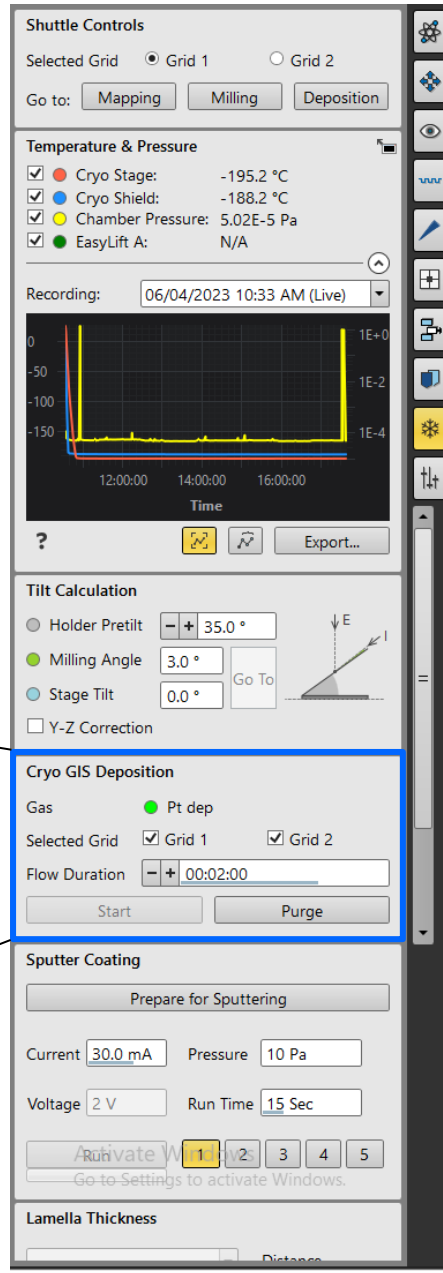
With Pt GIS coating

Milling direction

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



Duration varies by samples / instruments



3.6 Pt sputter (Optional)

(xT Microscope Control)

Vitrification

↓ **T**

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

Lamella milling
Preparation, Milling, & thinning

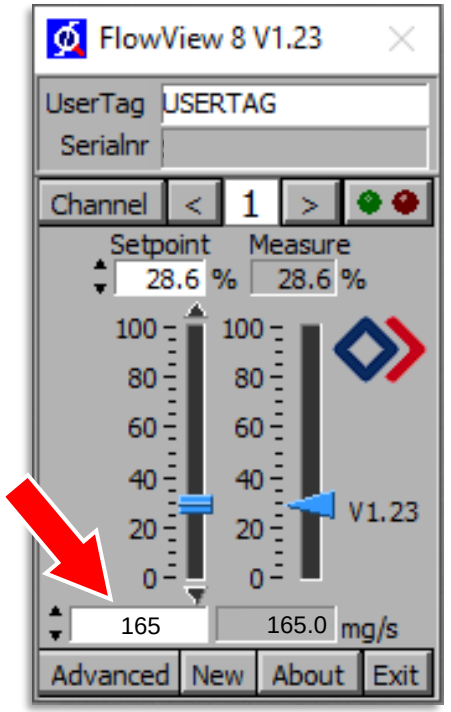
iFLM (Optional)
Target confirmation

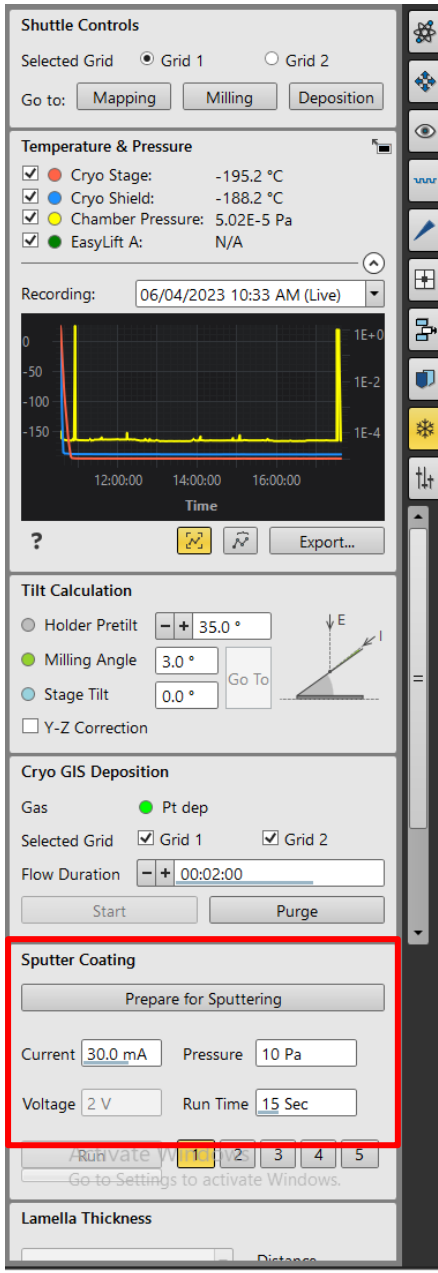
Pt sputter (Optional)
Lamella conductivity

↓ **T**

CryoET

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





3.7 Automated lamella milling using AutoTEM Cryo AT

Vitrification

↓ T

CryoFIB

Sample screening
Atlas & lamella sites

iFLM (Optional)
Target selection

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Pt sputter (Optional)
Sample conductivity

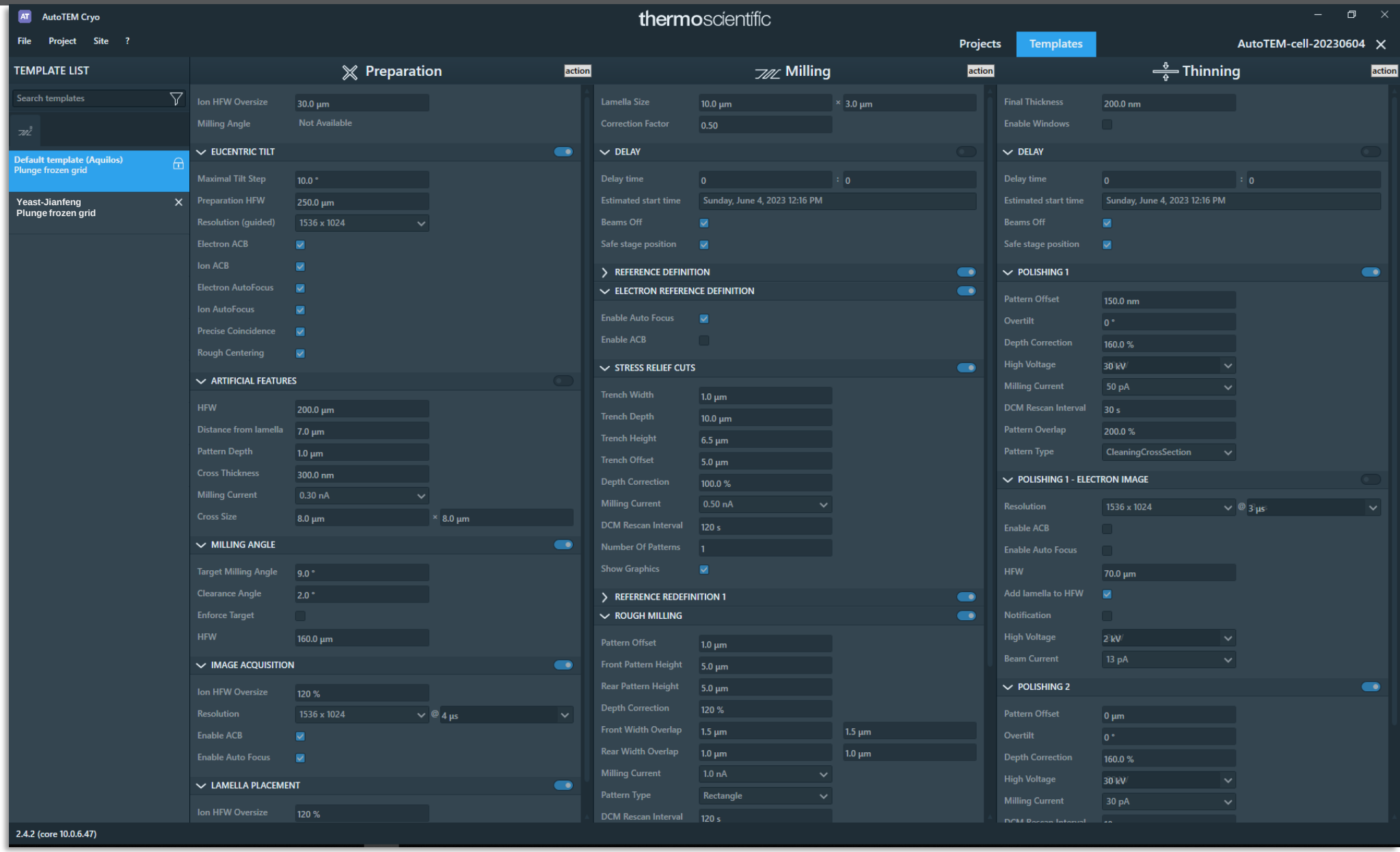
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



Eucentric height & tilt calculation

(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

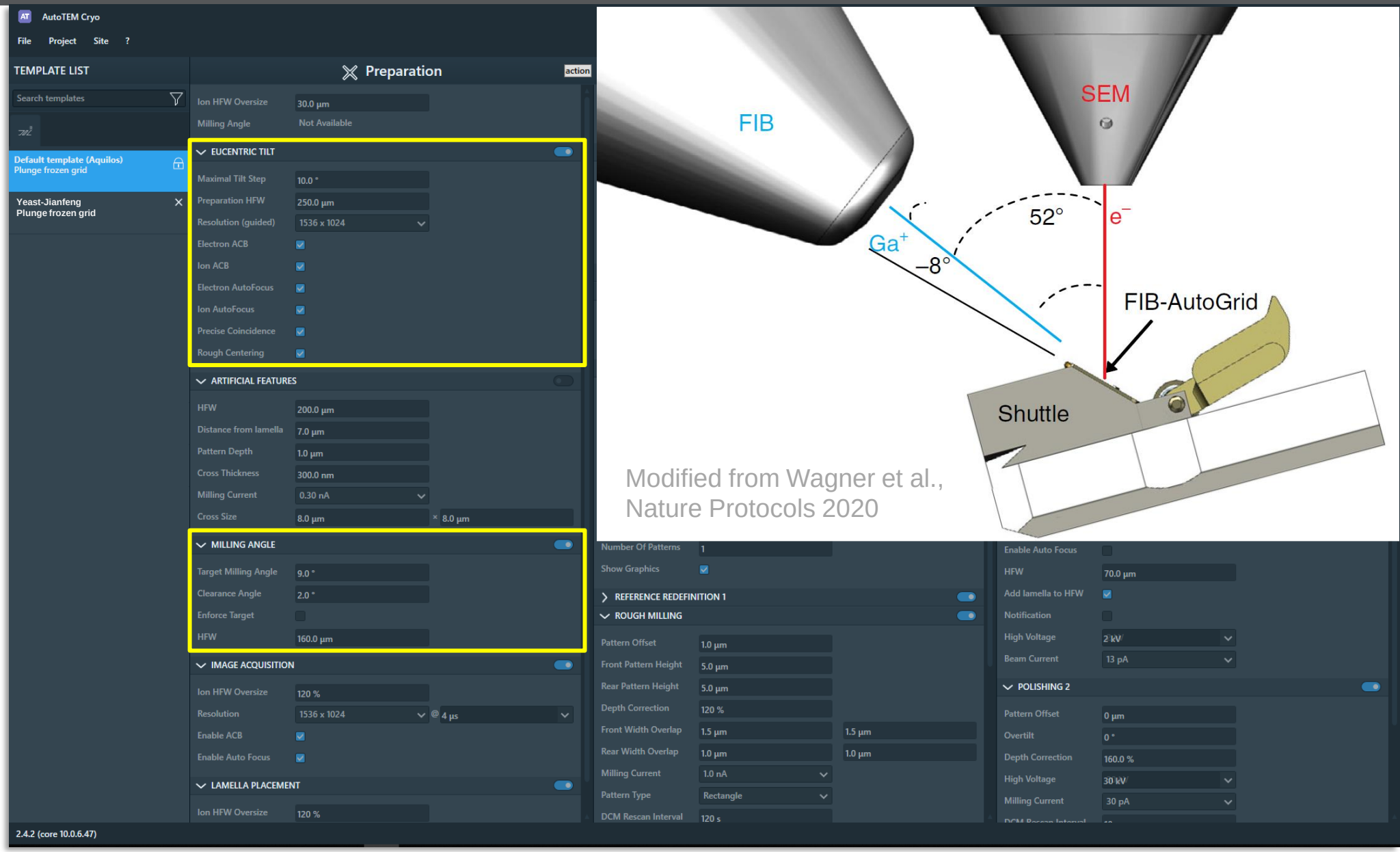
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



Artificial Features (Optional)

(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

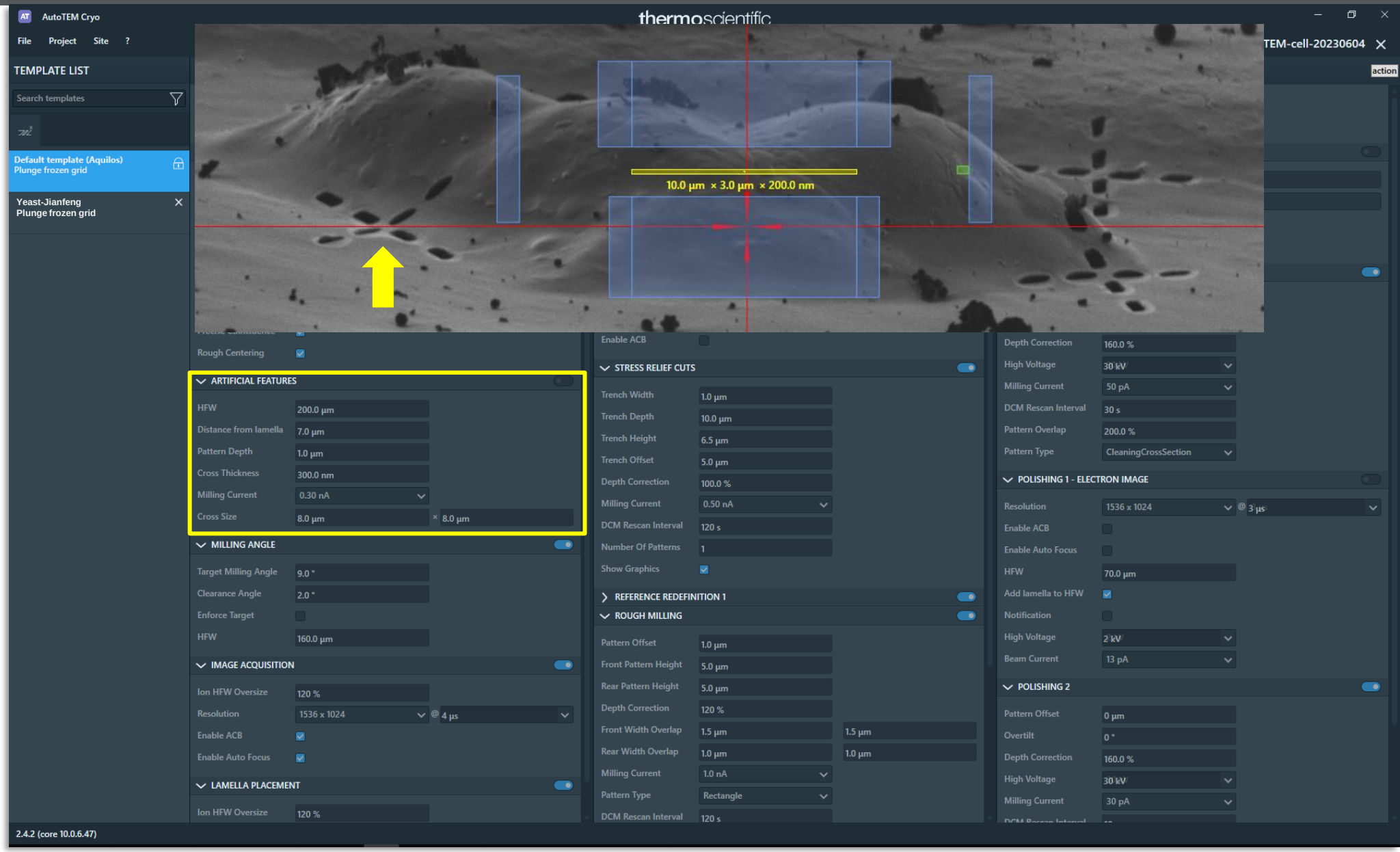
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



Micro-expansion joints

(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

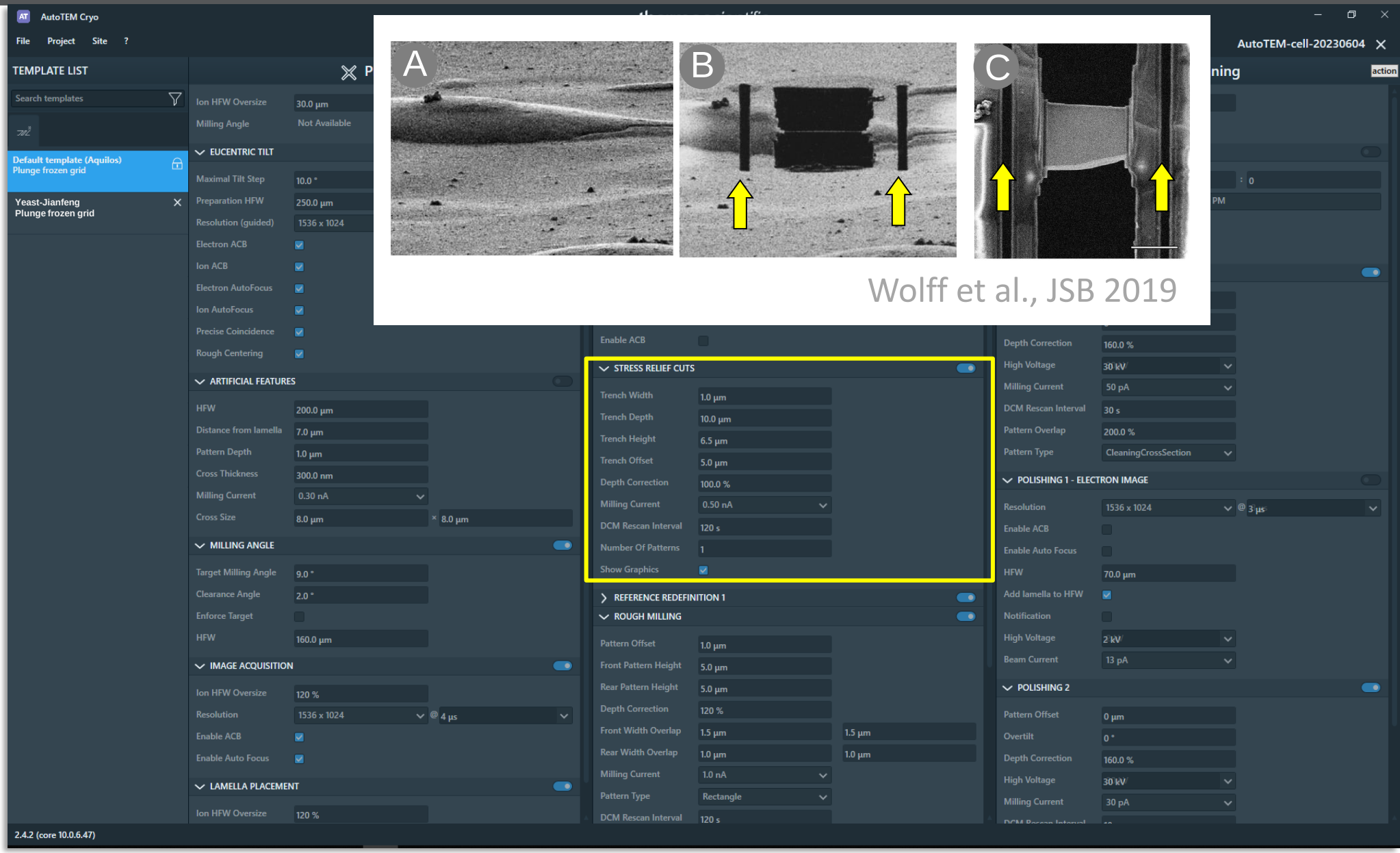
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



Wolff et al., JSB 2019

Stepwise milling procedure

(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

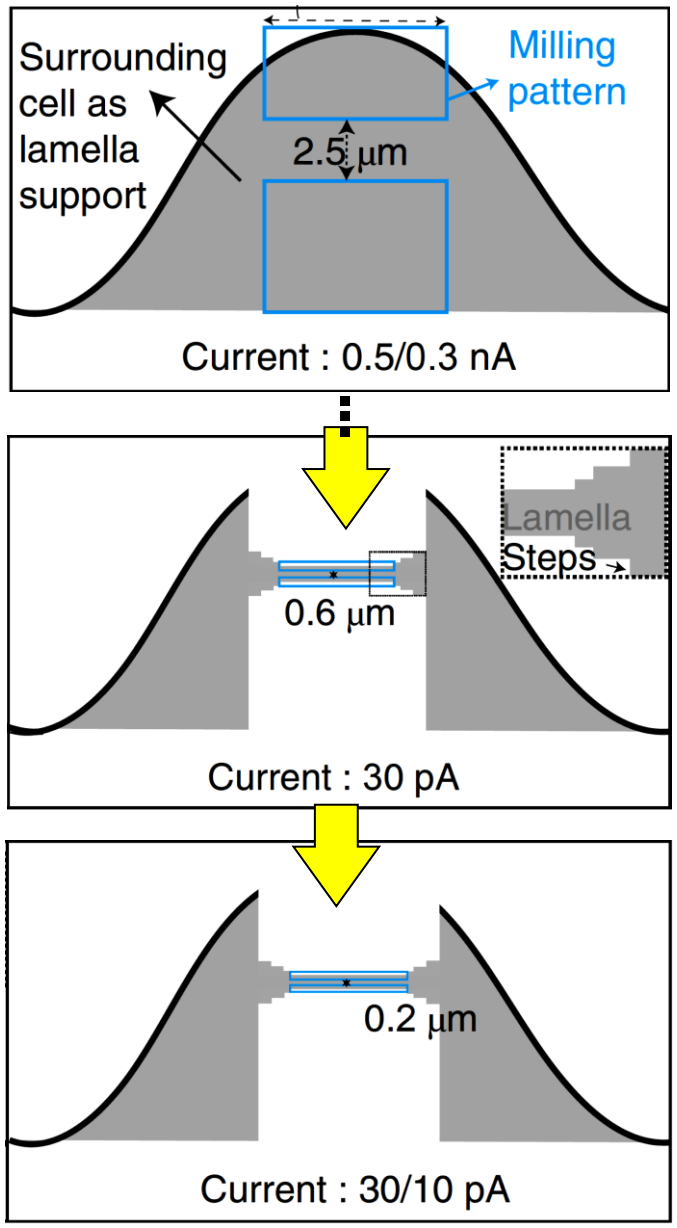
Lamella milling
Preparation, Milling, & thinning

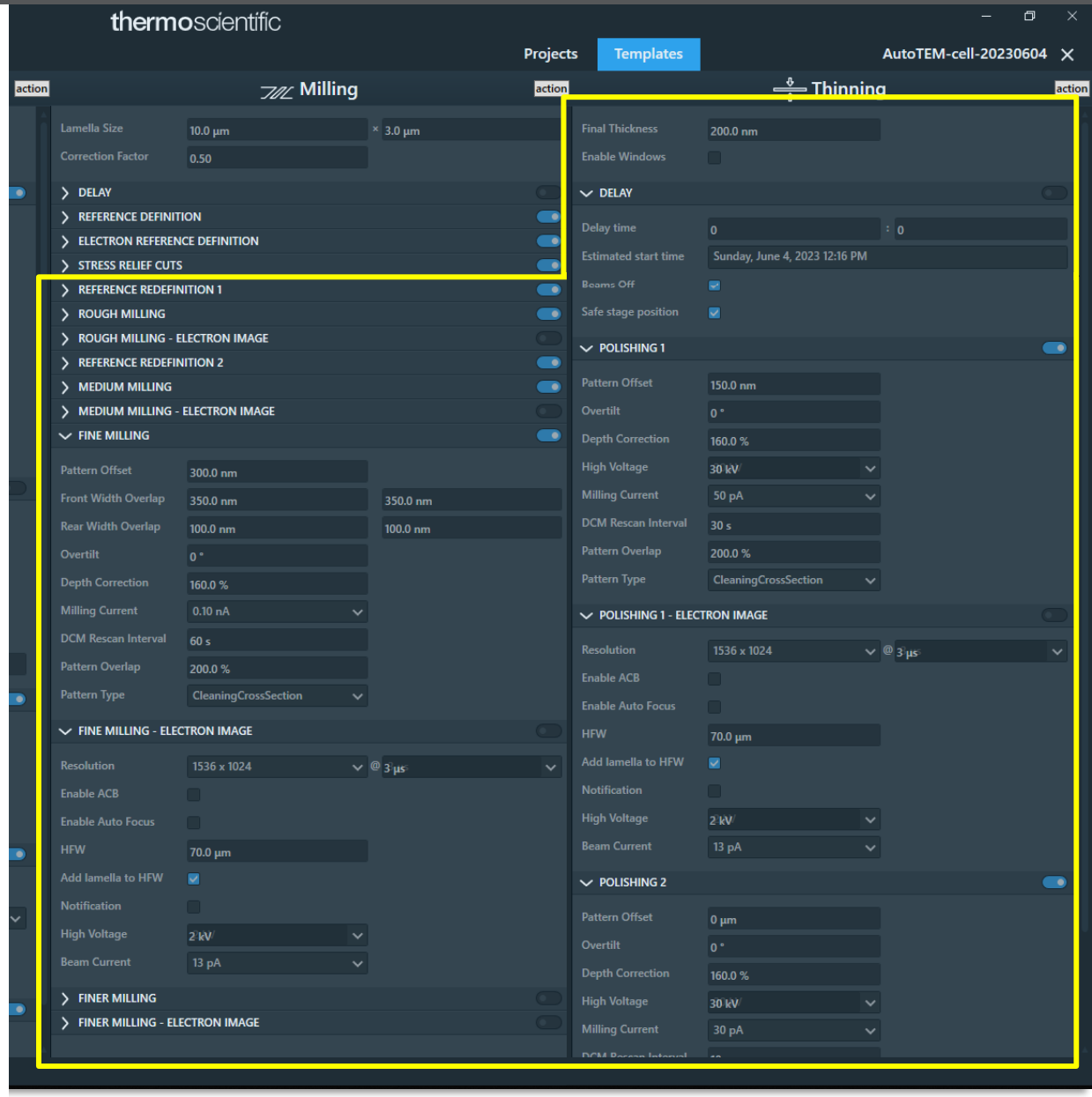
iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET

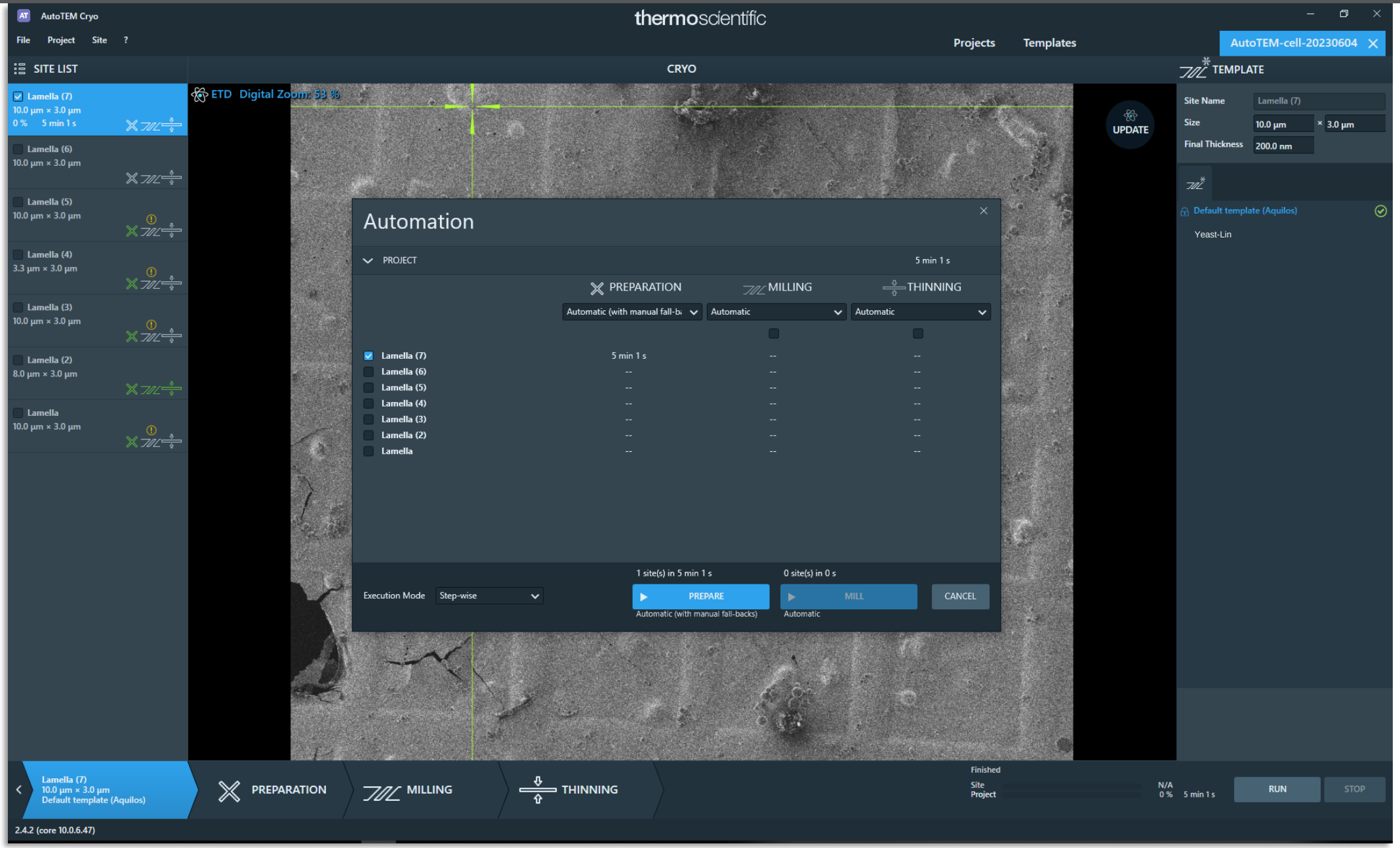




Wagner et al., Nature Protocols 2020

3.7.1 Automated lamella milling _Preparation

(AutoTEM Cryo)

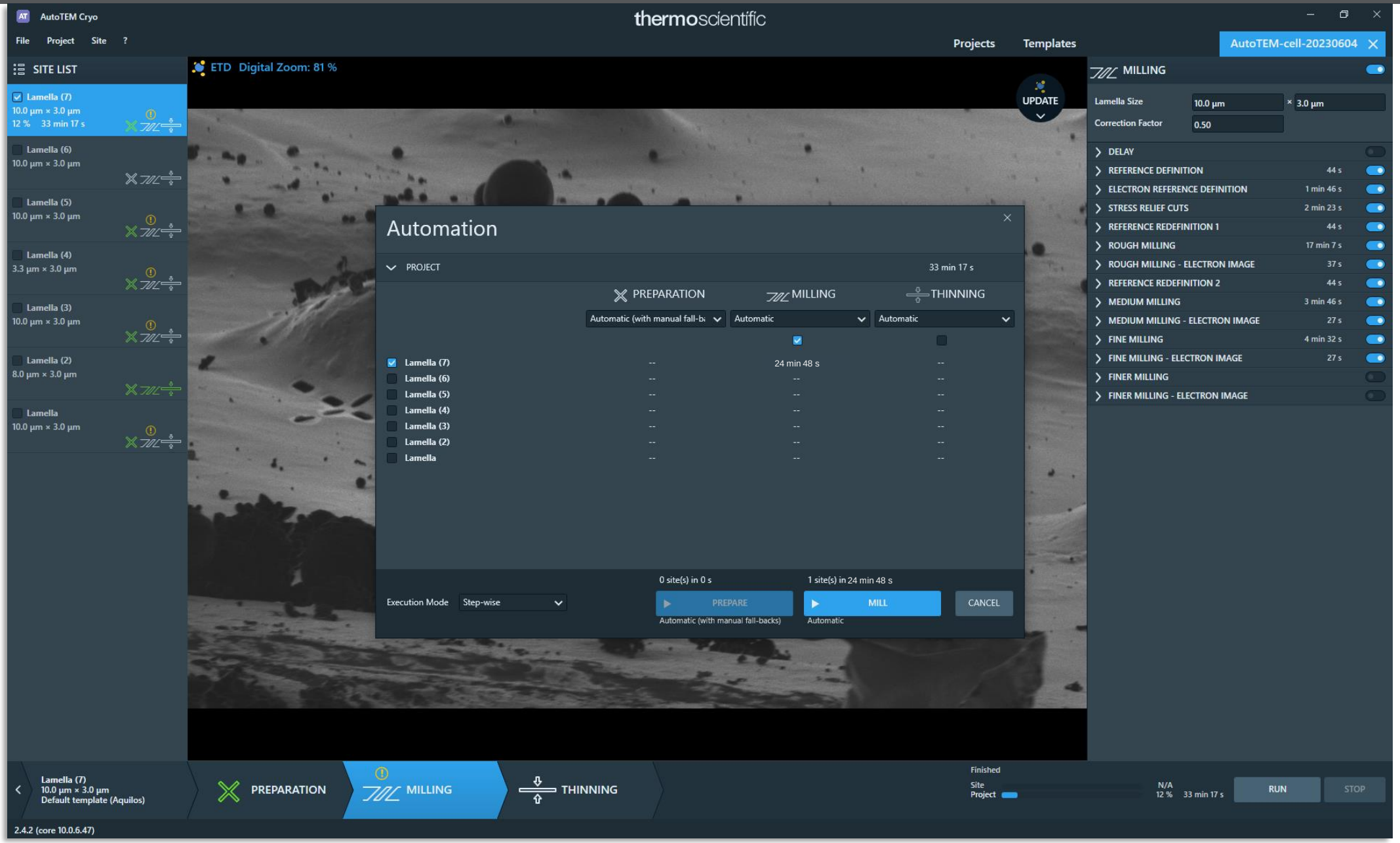


3.7.1 Automated lamella milling _Preparation

(AT AutoTEM Cryo)

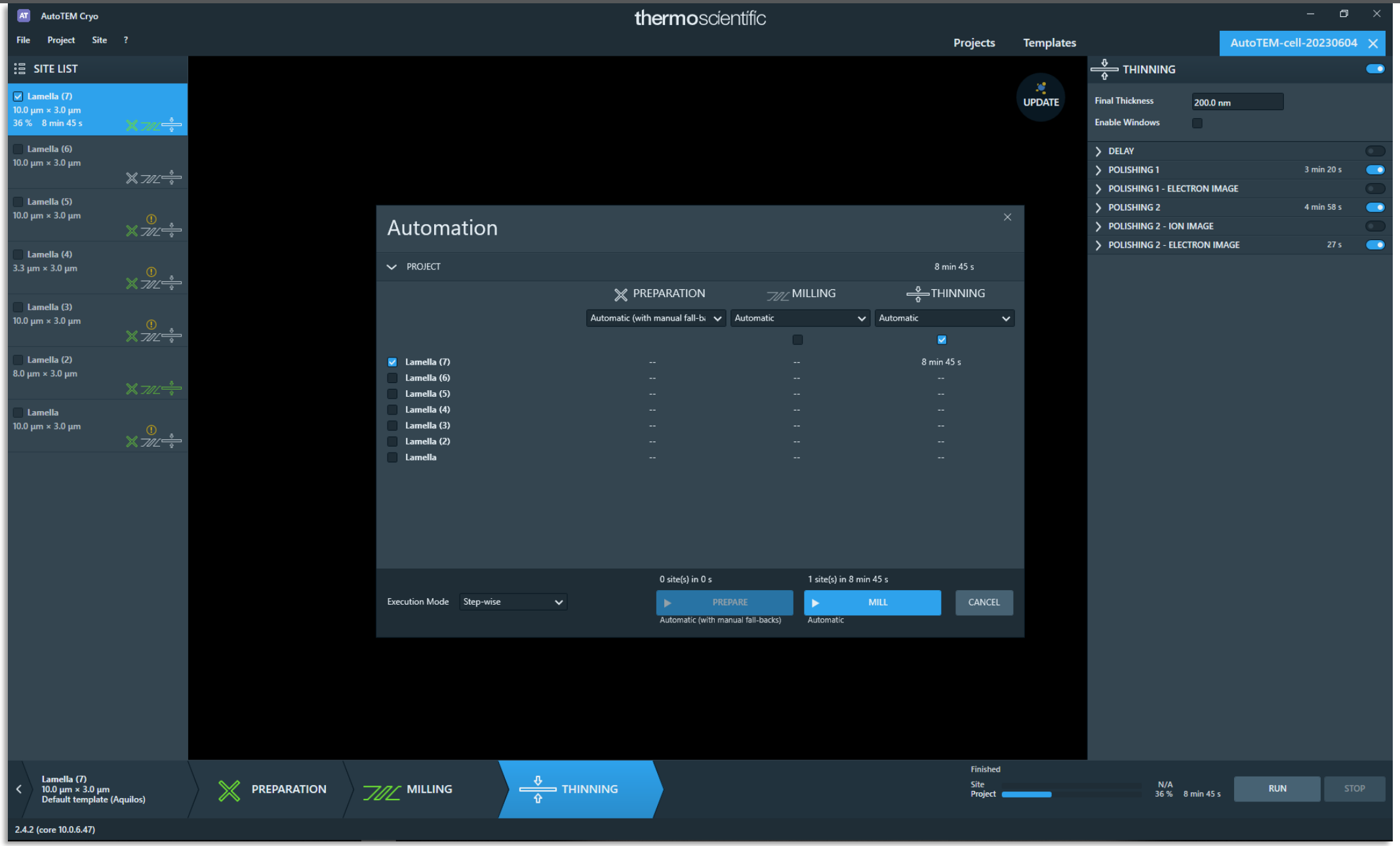
3.7.2 Automated lamella milling _Milling

(AutoTEM Cryo)



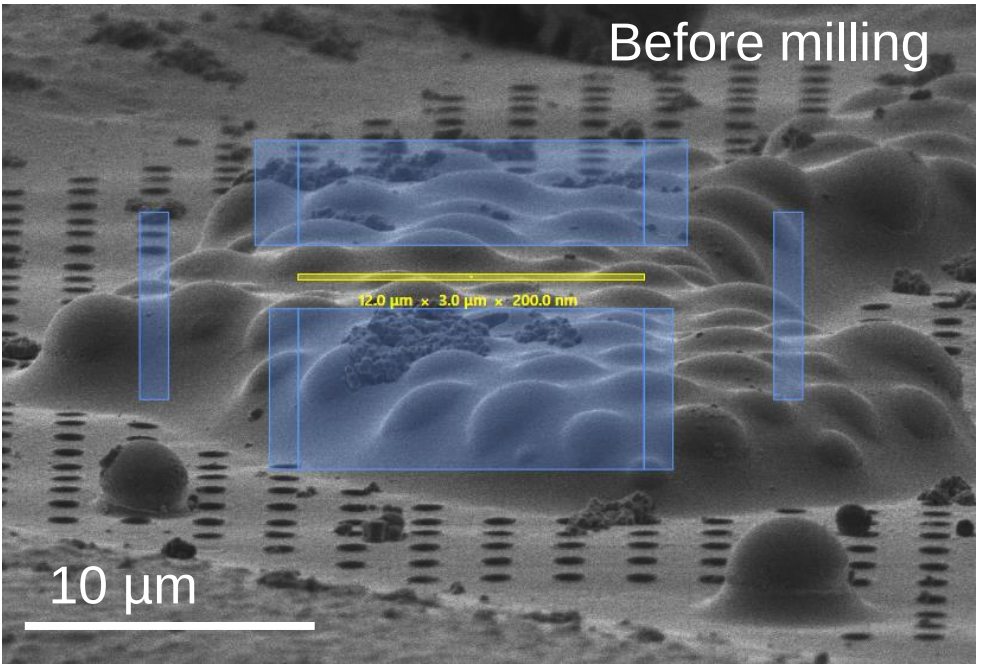
3.7.3 Automated lamella milling _Thinning

(AutoTEM Cryo)

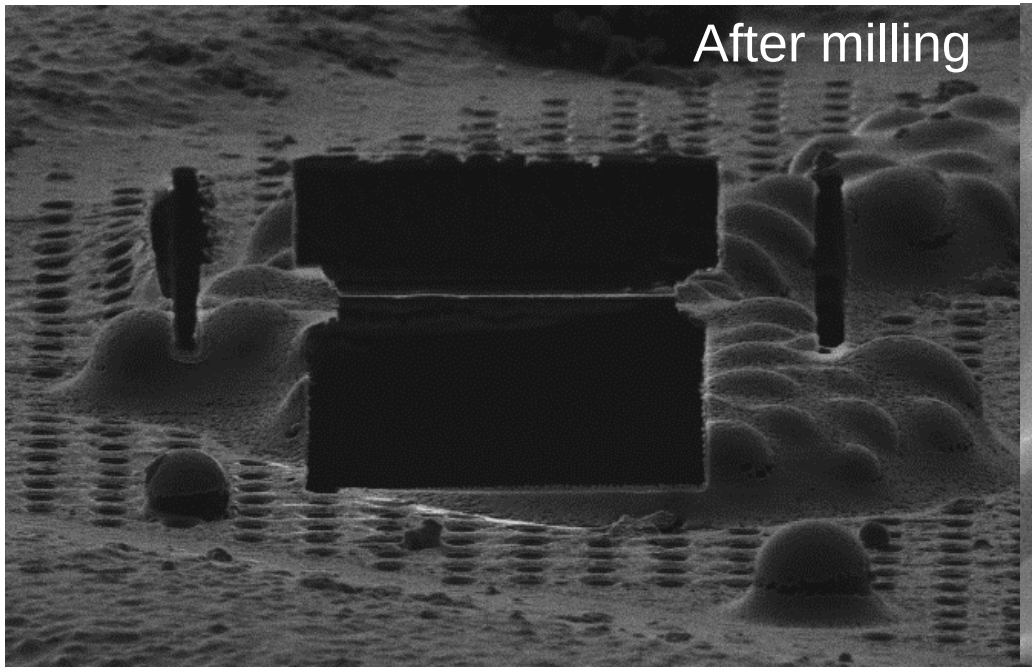


3.7.3 Automated lamella milling _Obtained cryo-lamella #1 (xT Microscope Control)

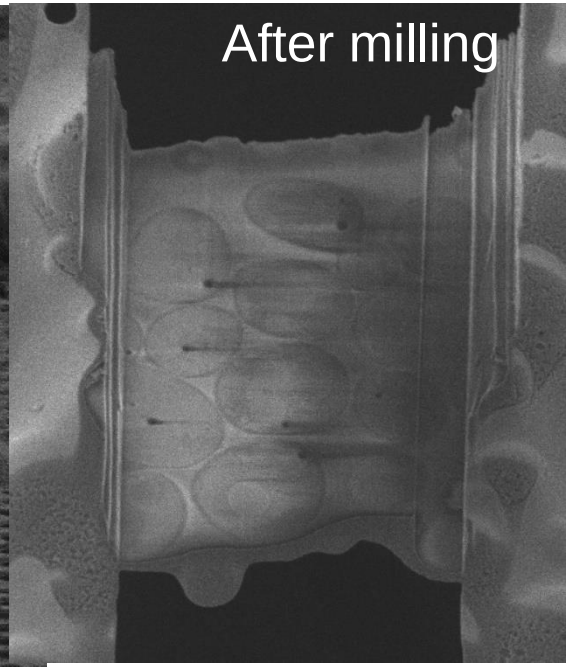
I-beam



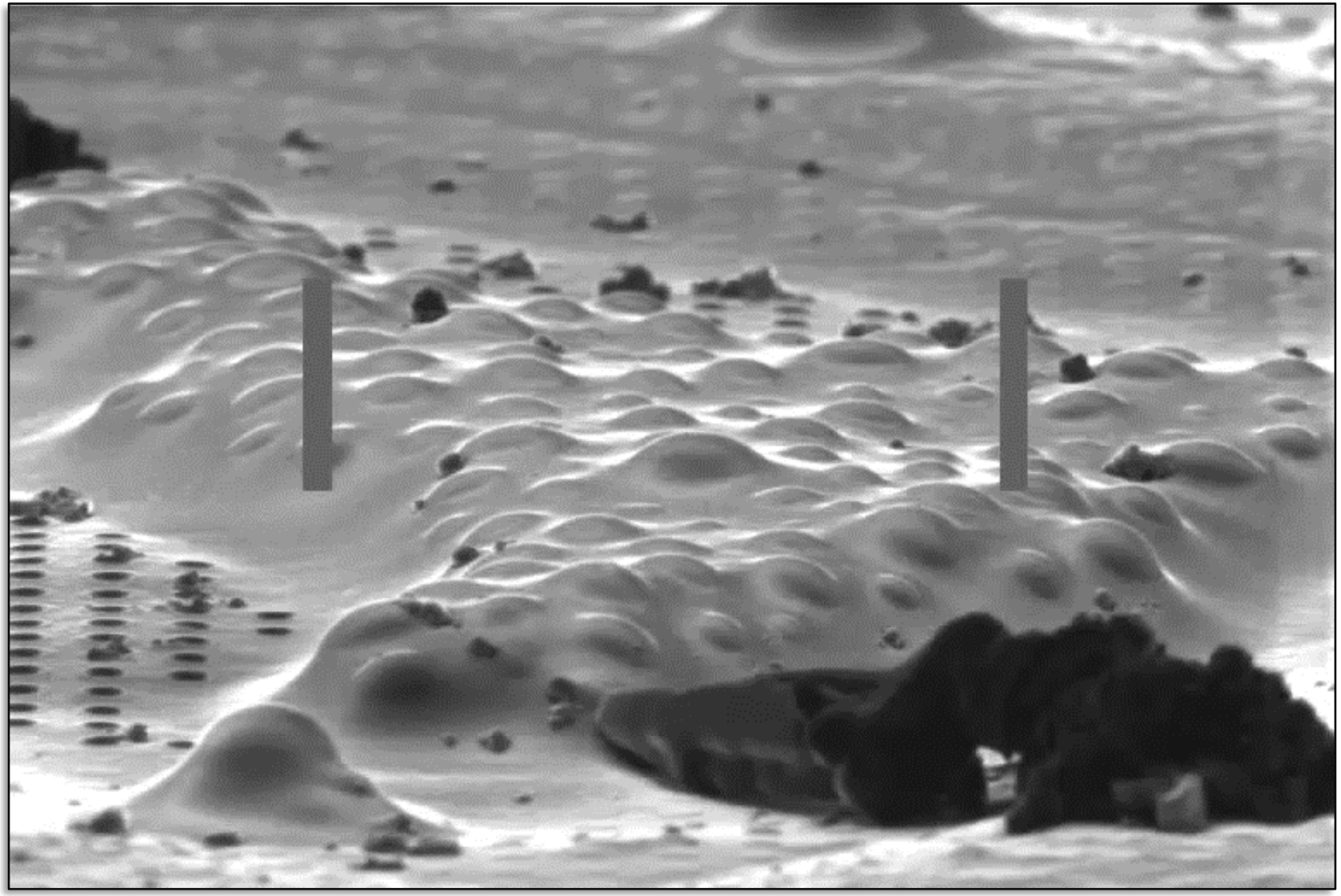
I-beam



E-beam



3.7.3 Automated lamella milling _Video of cryo-lamella #2 (30x accelerated video)



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

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

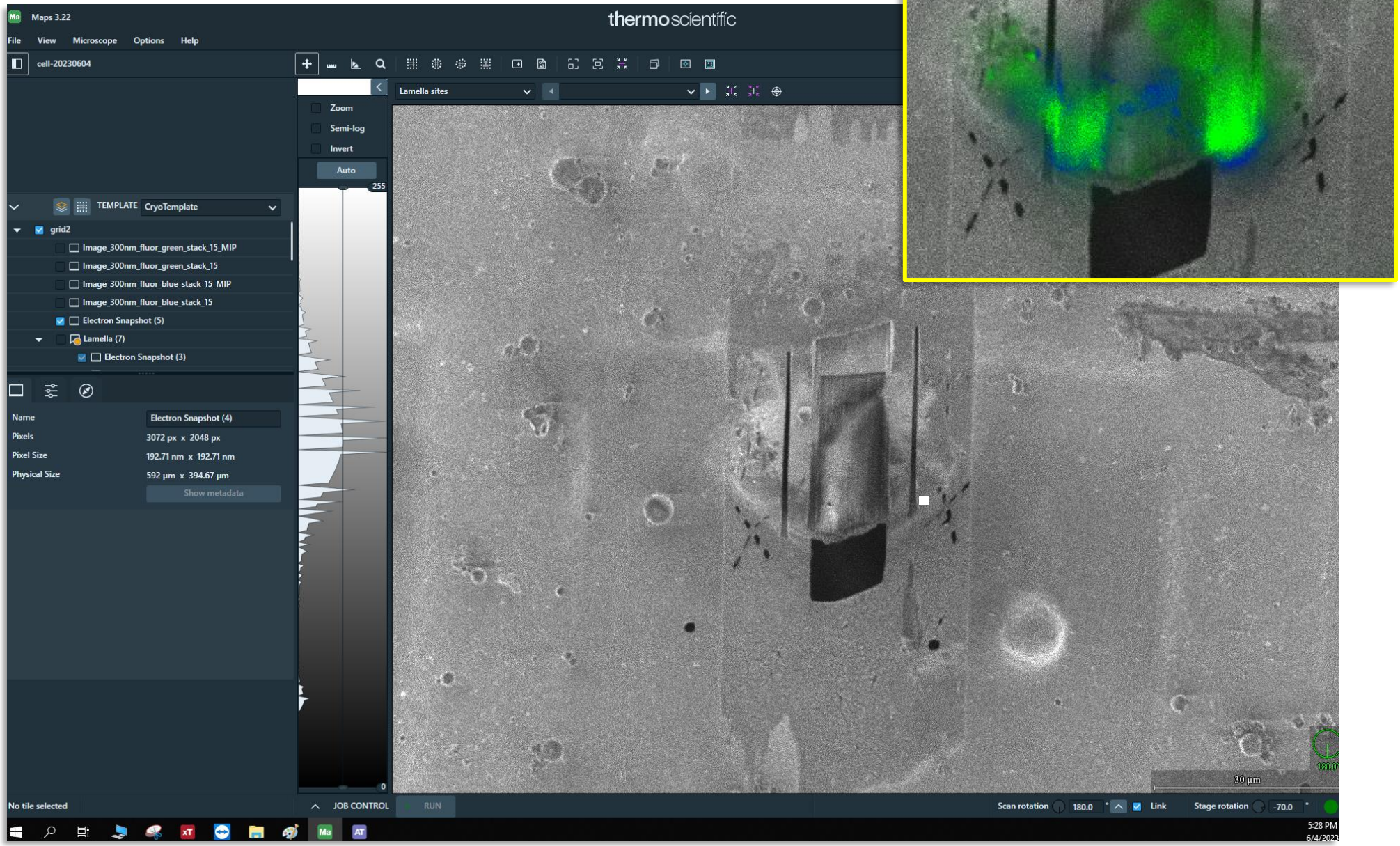
Lamella milling
Preparation, Milling,
& thinning

iFLM (Optional)
Target confirmation

Pt sputter (Optional)
Lamella conductivity

↓ T

CryoET



3.9 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

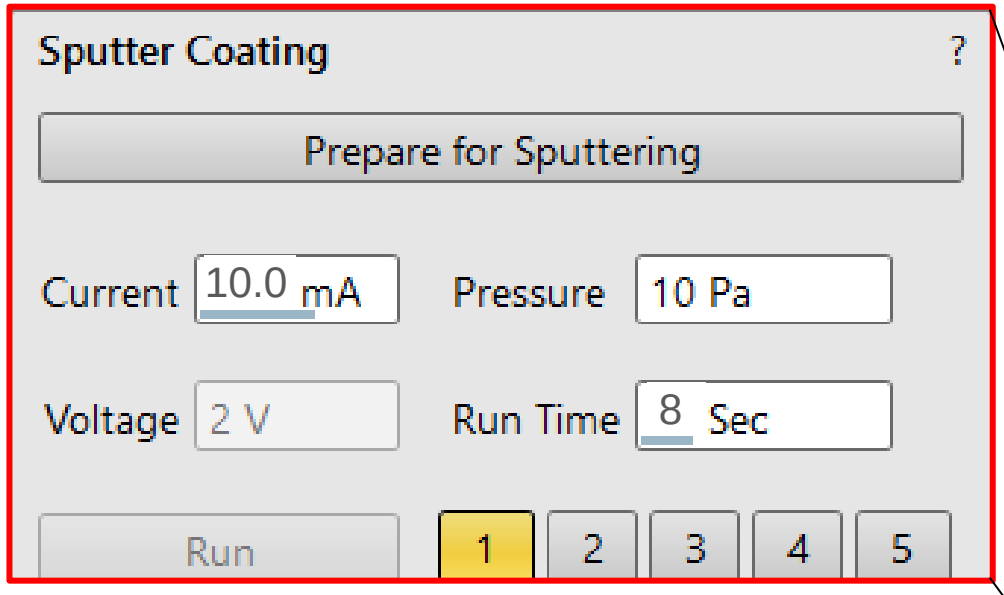
Lamella milling
Preparation, Milling, & thinning

iFLM (Optional)
Target confirmation

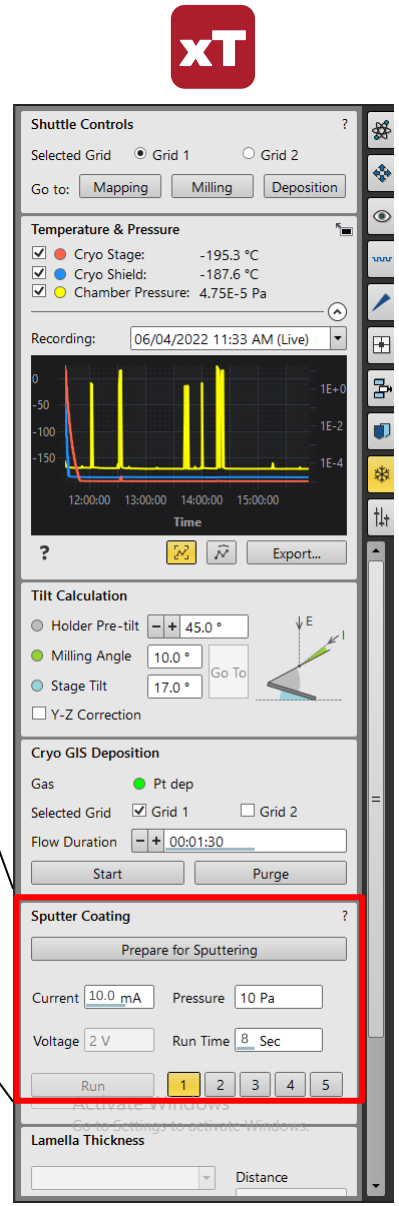
Pt sputter (Optional)
Lamella conductivity

↓ **T**

CryoET



(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

↓ T

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

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 the lamellae #1&2

(Video from TFS)

Thank you!

Comments & Questions?

Jianfeng.lin@yale.edu