Second annual Cryo-EM Course at LBMS, BNL (June 14-17th, 2022)

Get on the ready line of tilting

Jianfeng Lin

The Yale CryoEM Resource, Yale University

6-16-2022

Main contents

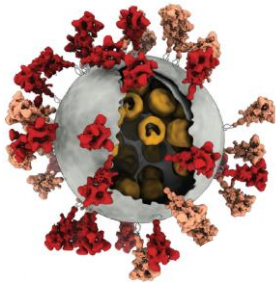
1. Specimens accessible by cryoET

2. Four considerations for cryoET sample preparation

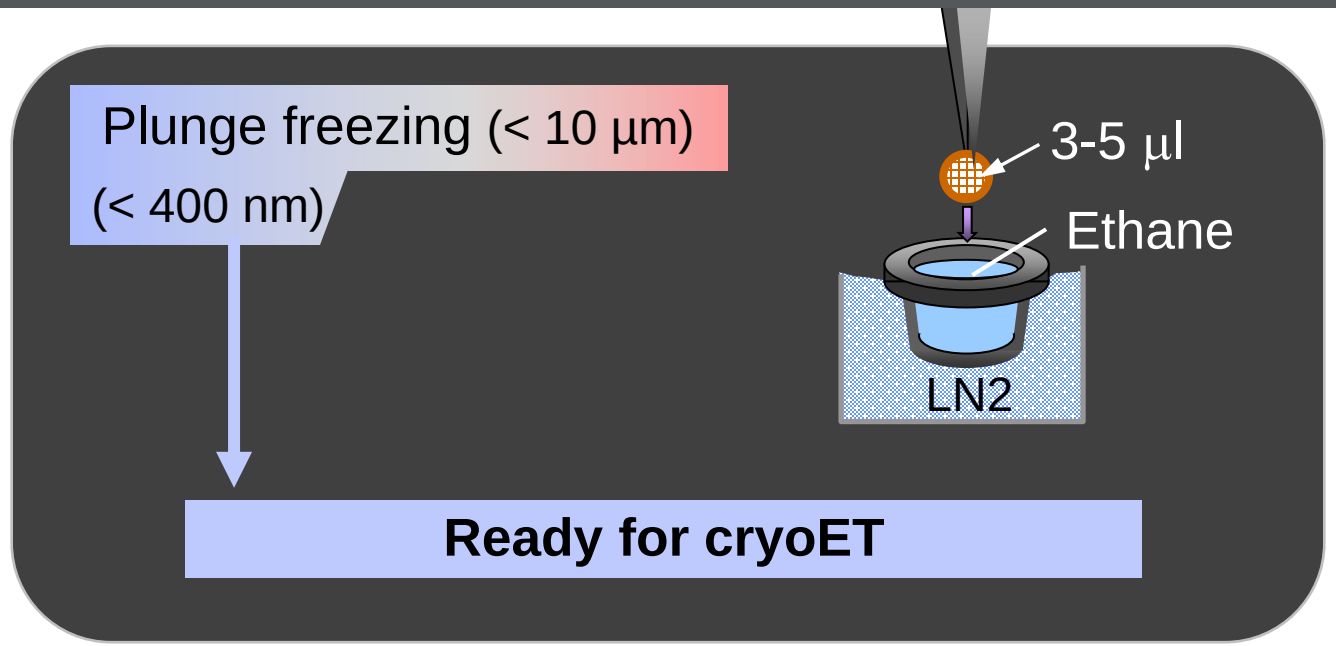
3. Workflow of cryo lamellae preparation

4. Tutorial of major steps of cryo lamella preparation with Aquilos 2

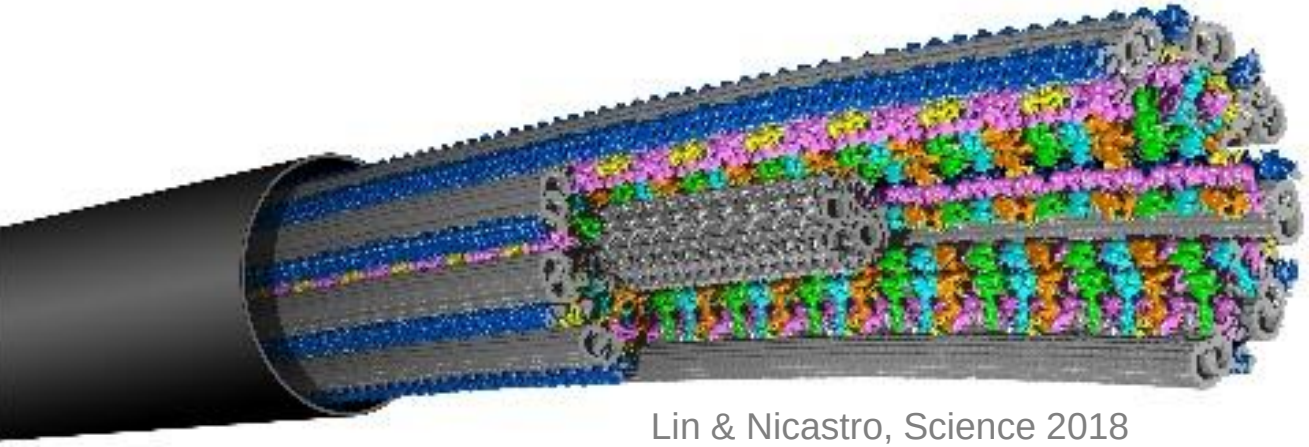
1. Specimens accessible by cryoET



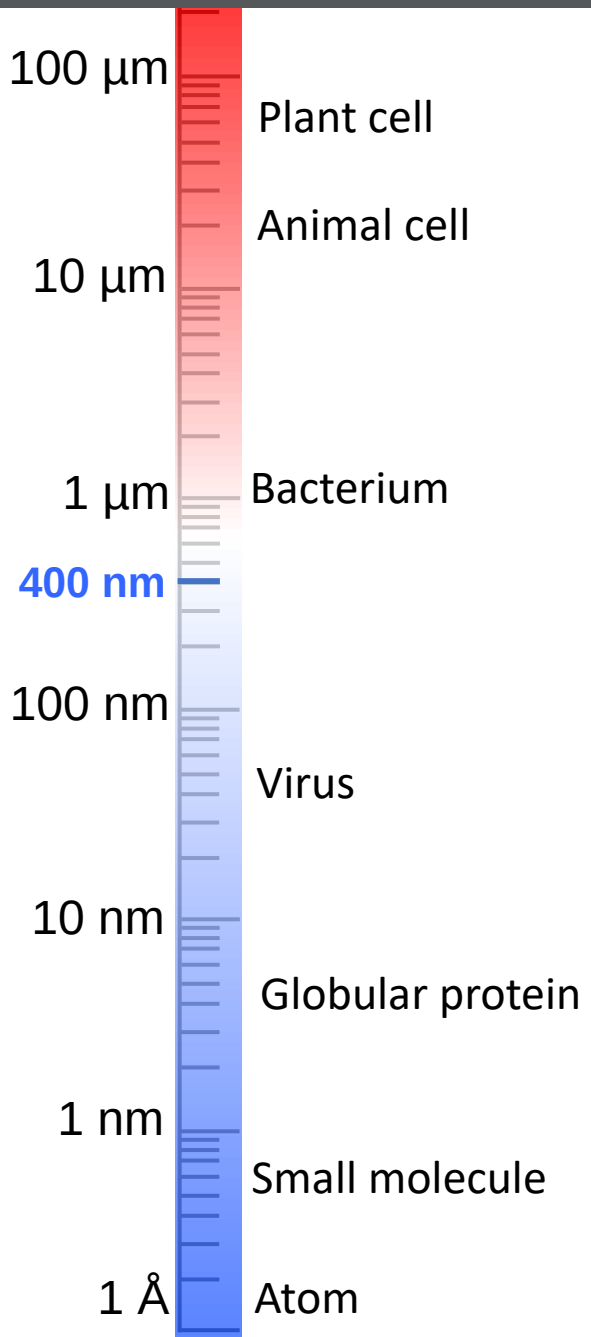
Yao et al., Cell 2020



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

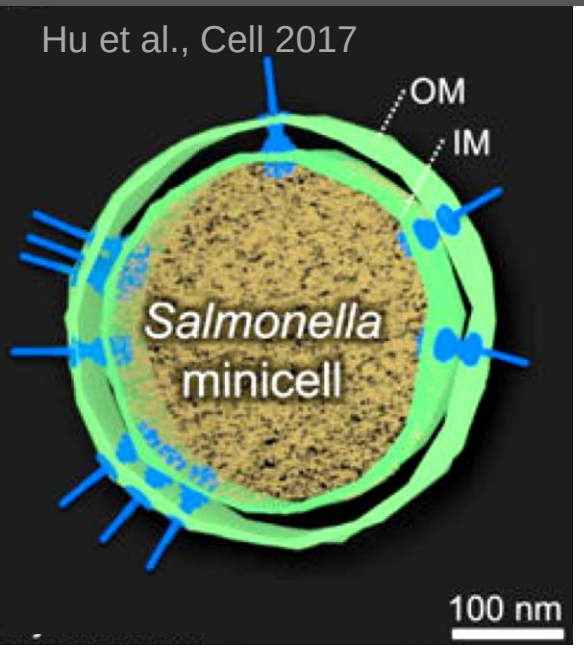


Lin & Nicastro, Science 2018

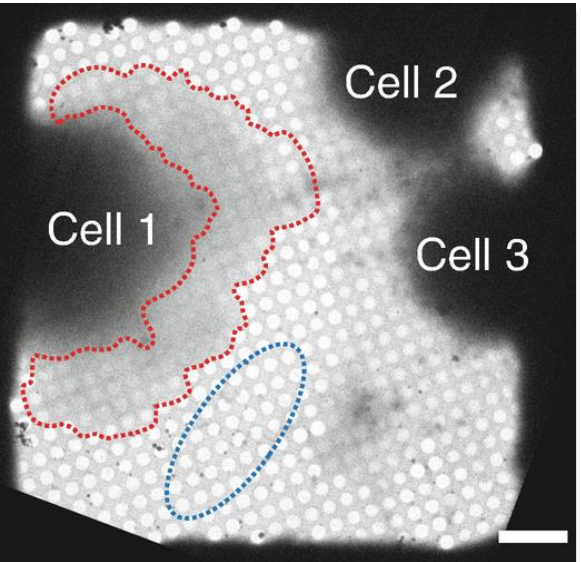


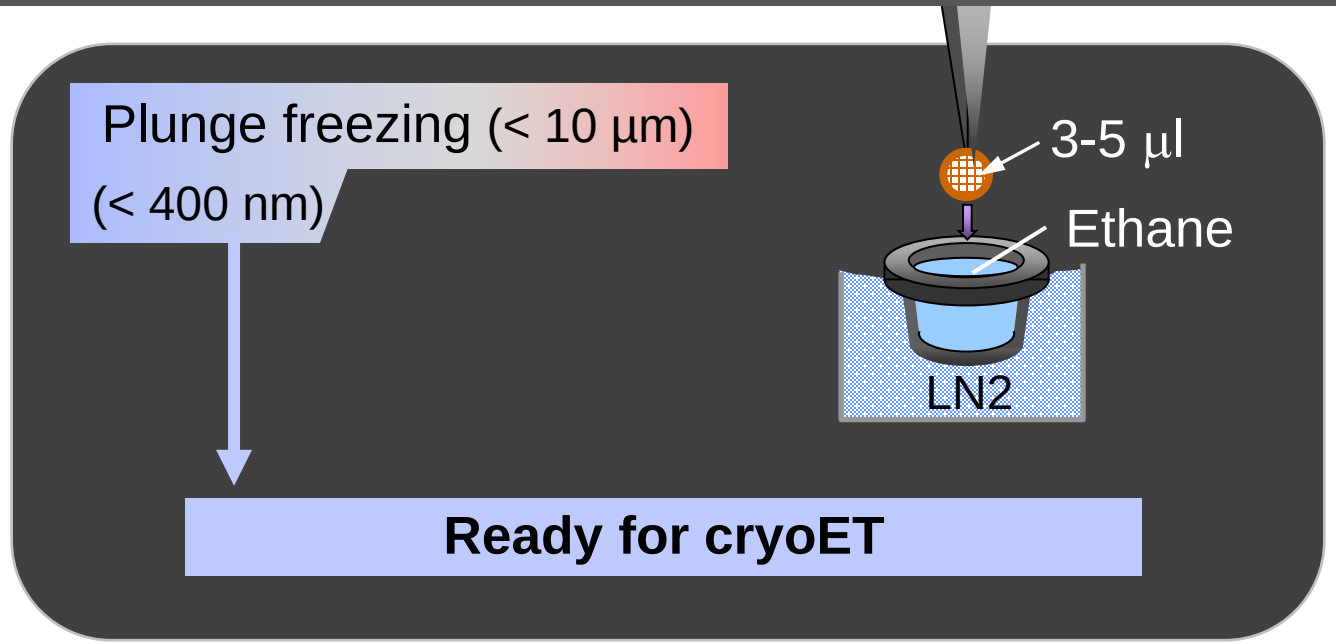
1. Specimens accessible by cryoET

Hu et al., Cell 2017

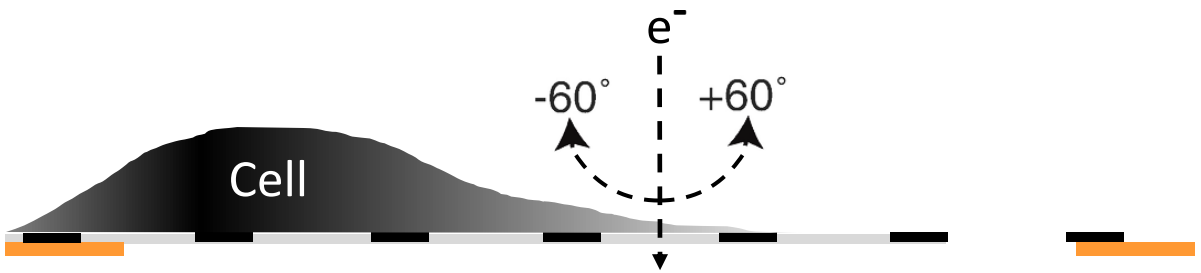


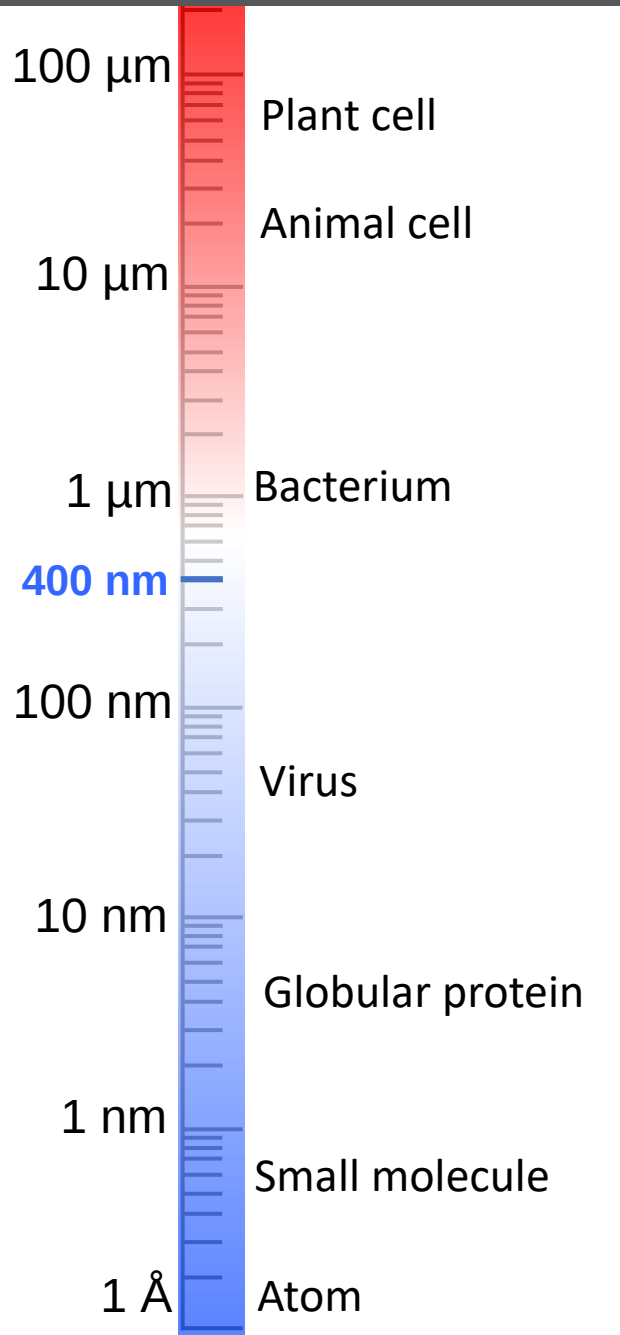
Serwas & Davies.
Methods Mol Biol. 2021



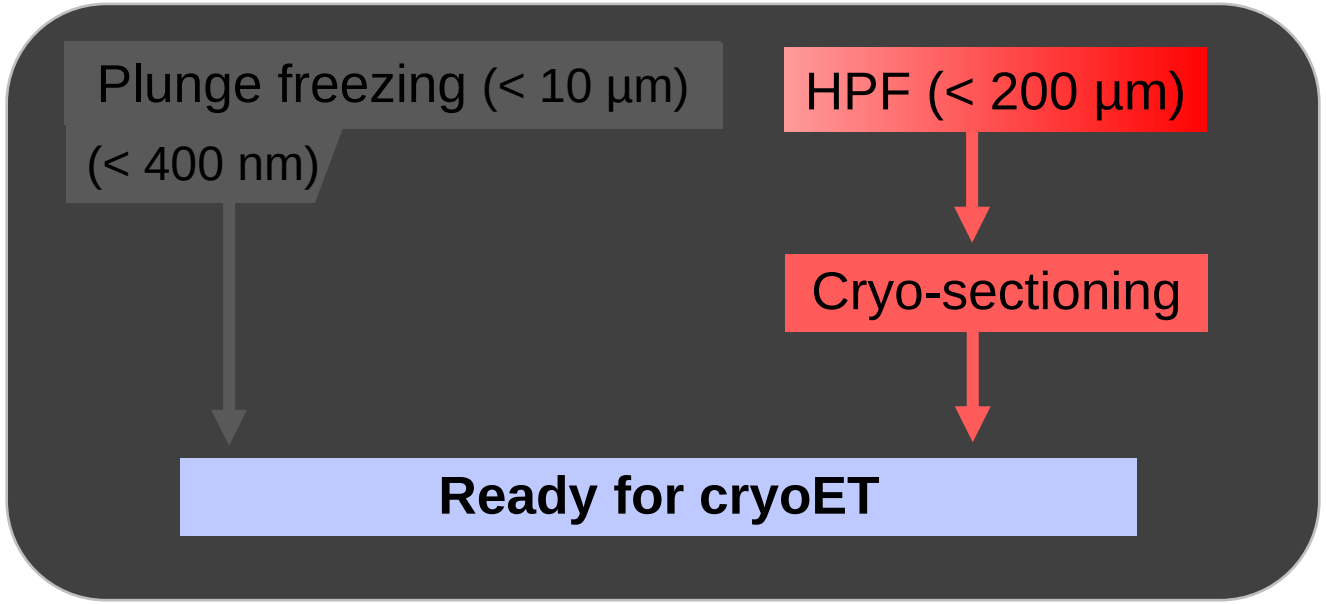


- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells

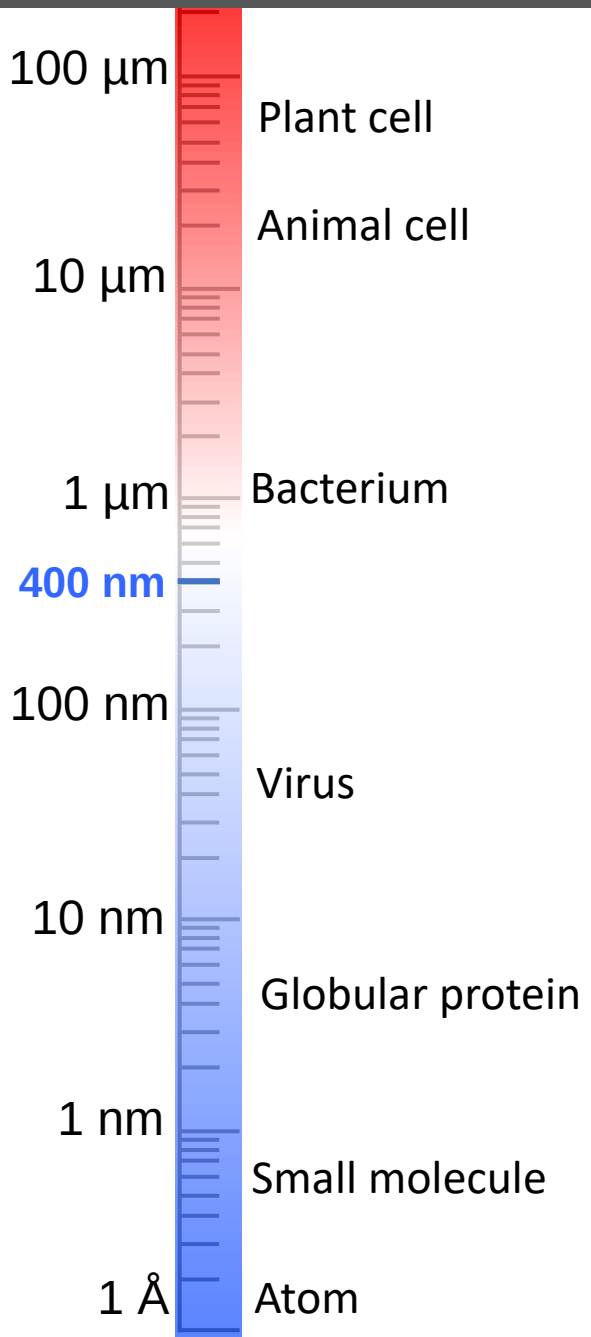




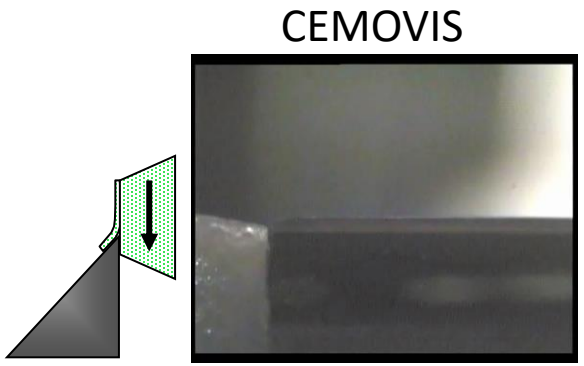
1. Specimens accessible by cryoET

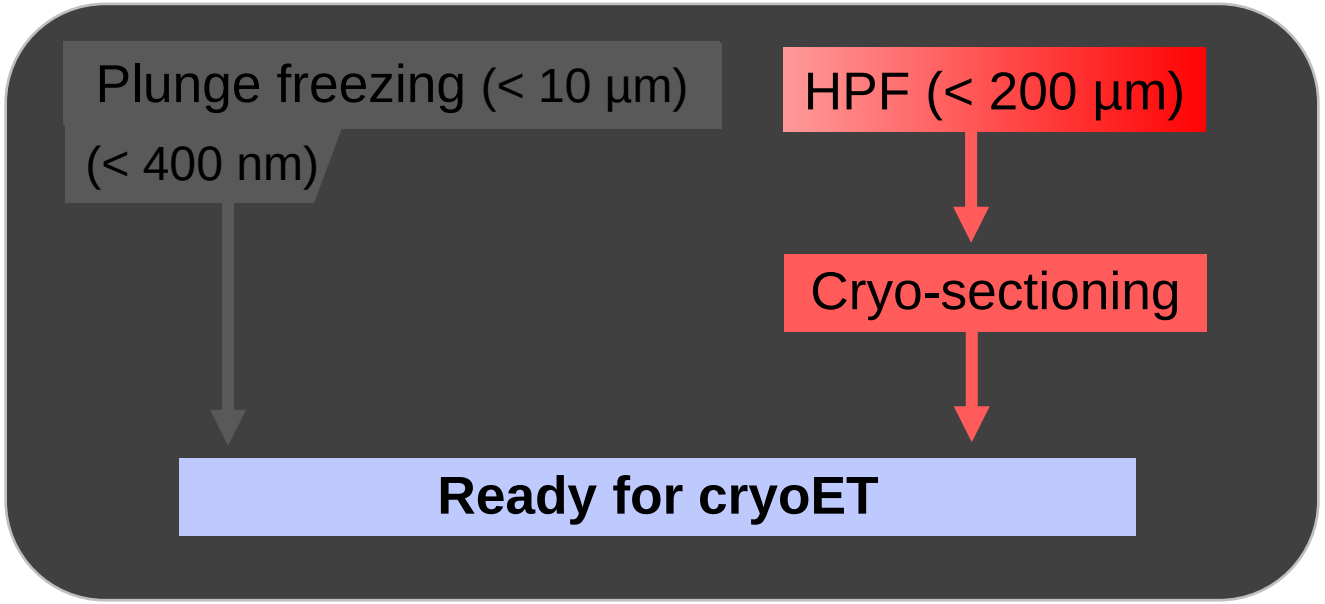


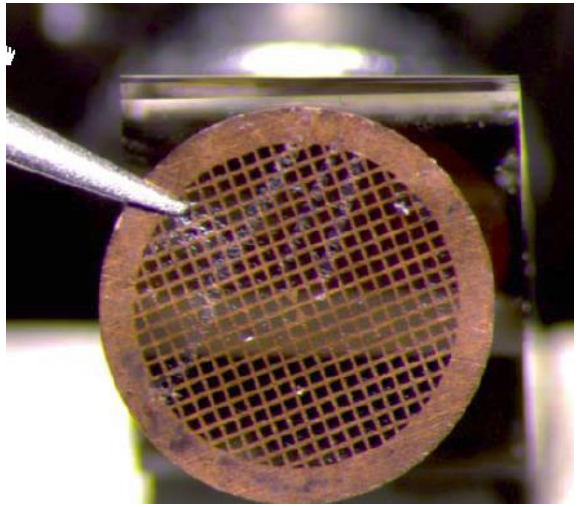
- Virus: e.g., Covid-19
- Isolated or reconstituted systems: e.g., ciliary axoneme
- Small/thin cells: e.g., minicells
- Peripheral regions of cells: e.g., mammalian cells
- Cryo-sections



1. Specimens accessible by cryoET

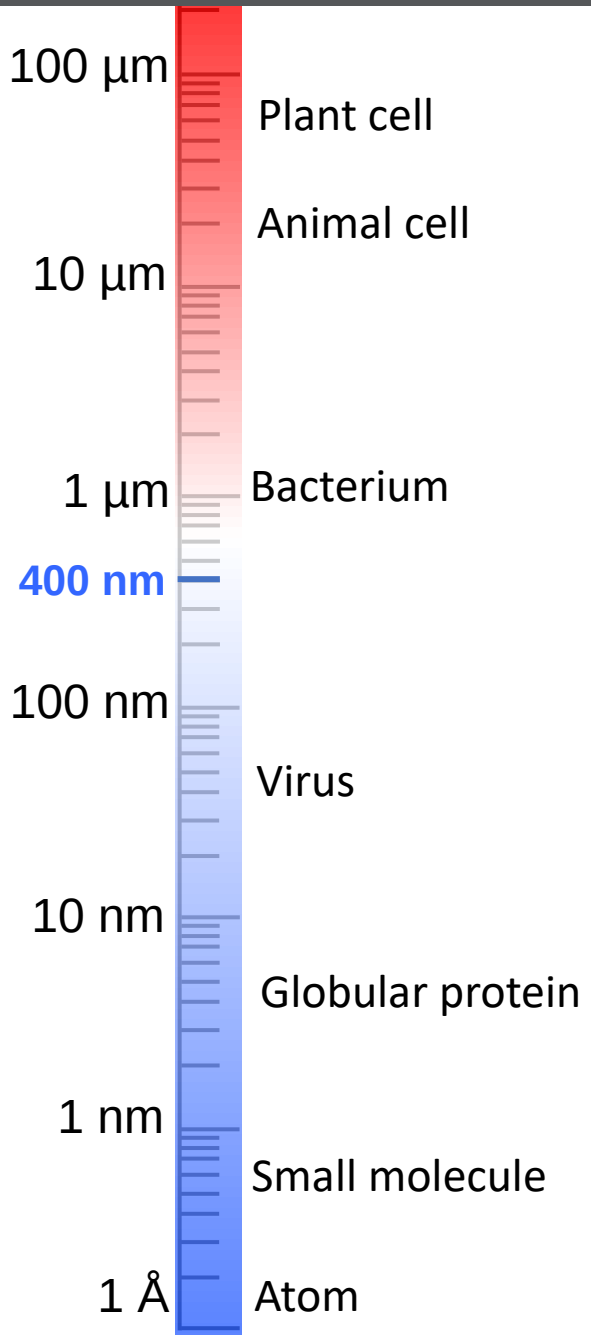






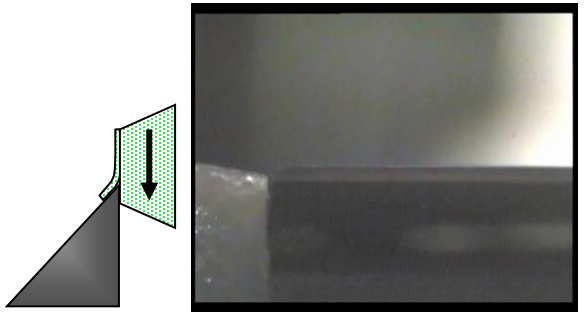
Adapted from Thermo Fisher Scientific (TFS)

- 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



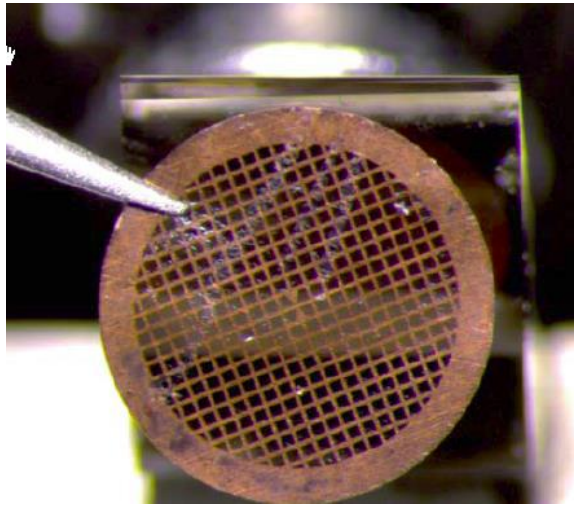
Plunge freezing (< 10 µm)

(< 400 nm)

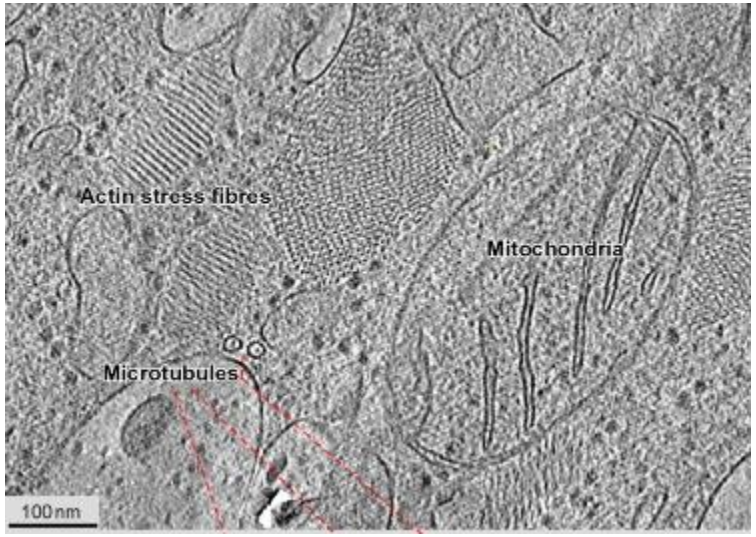
HPF (< 200 µm)

Cryo-sectioning

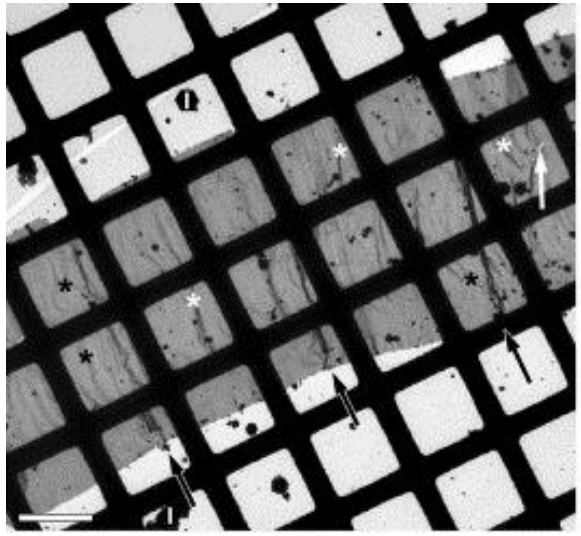
Ready for cryoET



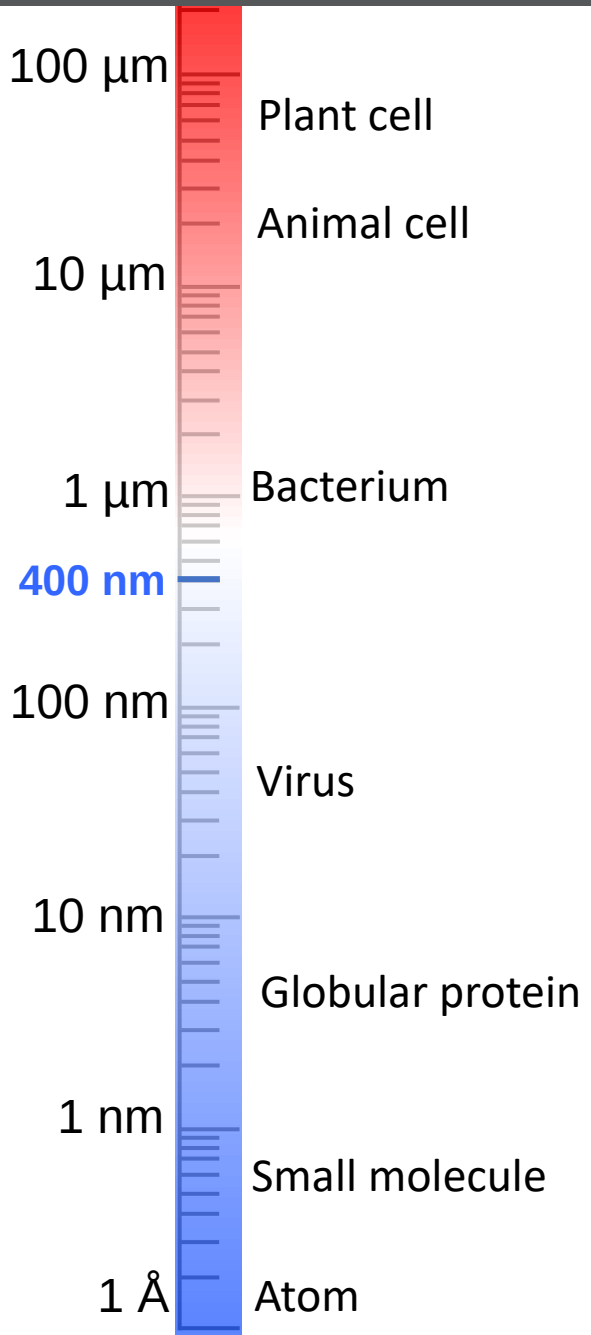
Adapted from TFS



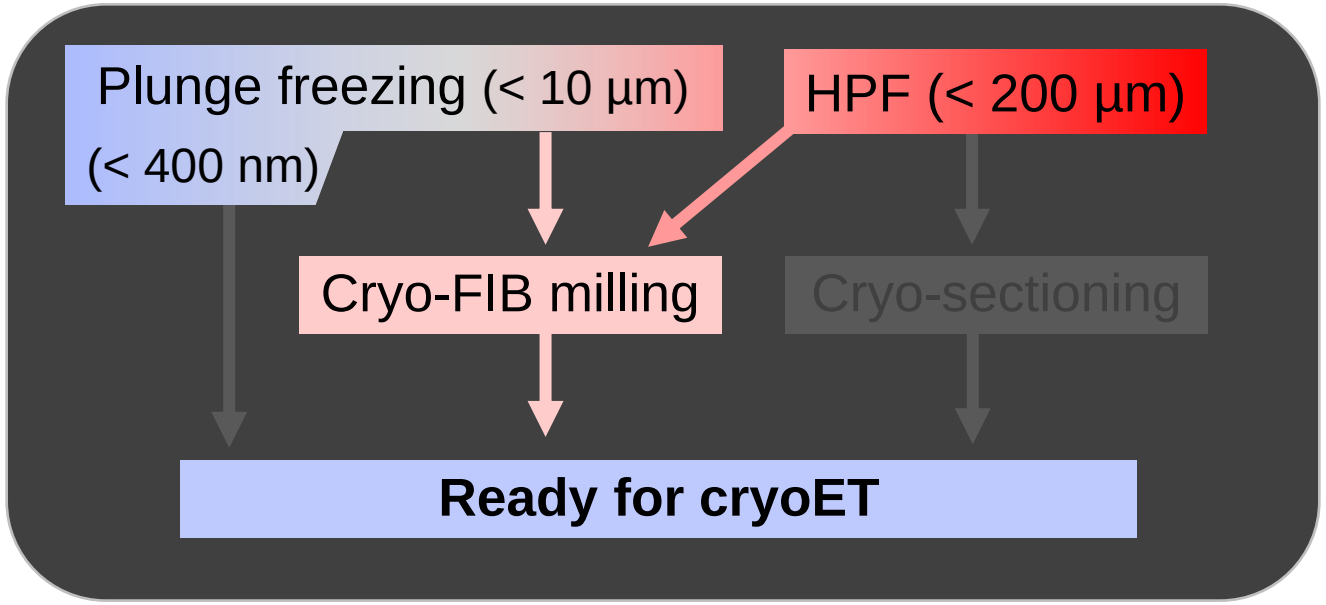
Hoenger & Bouchet-Marquis. Adv Protein Chem Struct Biol. 2011

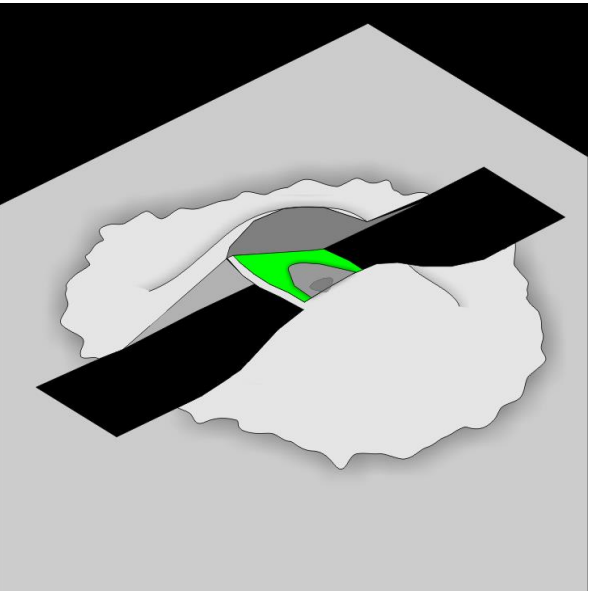


Al-Amoudi et al., J Struct Biol. 2005

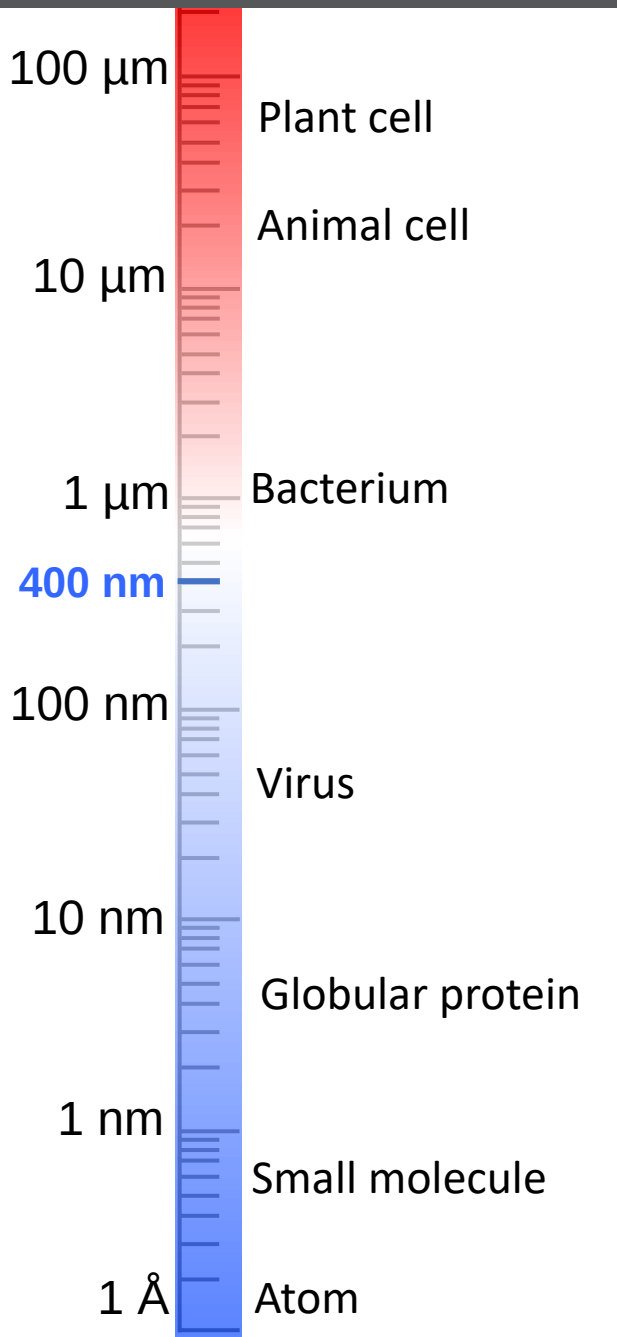


1. Specimens accessible by cryoET





- 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

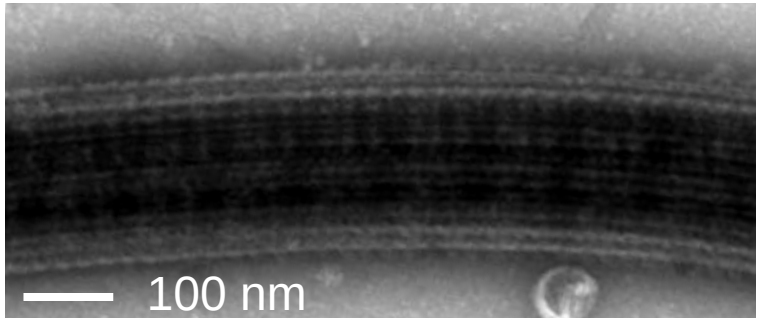


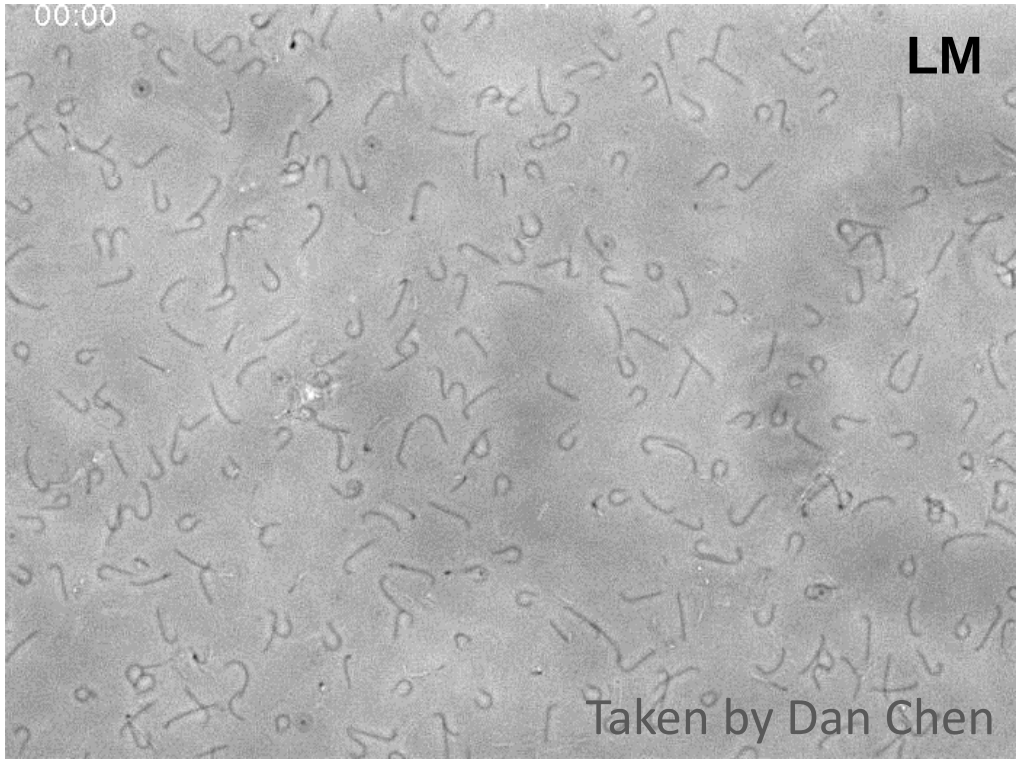
Main contents

2. Four considerations for cryo ET sample preparation

- Validation of the sample quality. e.g., ciliary axoneme

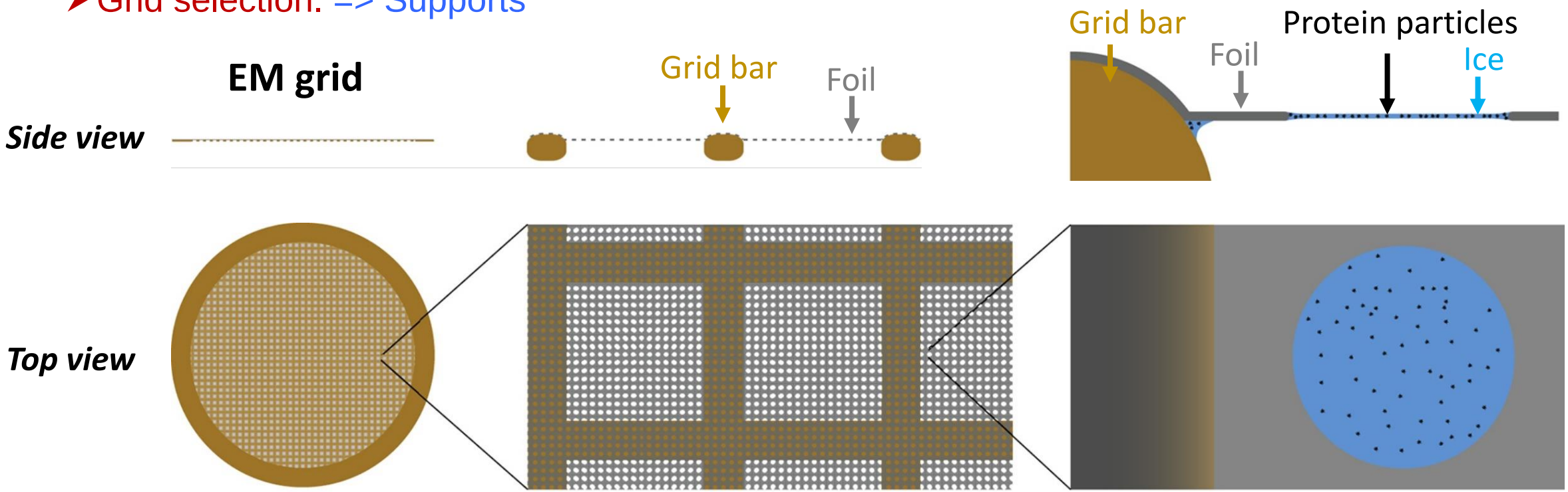
Negative staining EM





2. Four considerations for cryo ET sample preparation

- Validation of the sample quality.
- Grid selection. => Supports



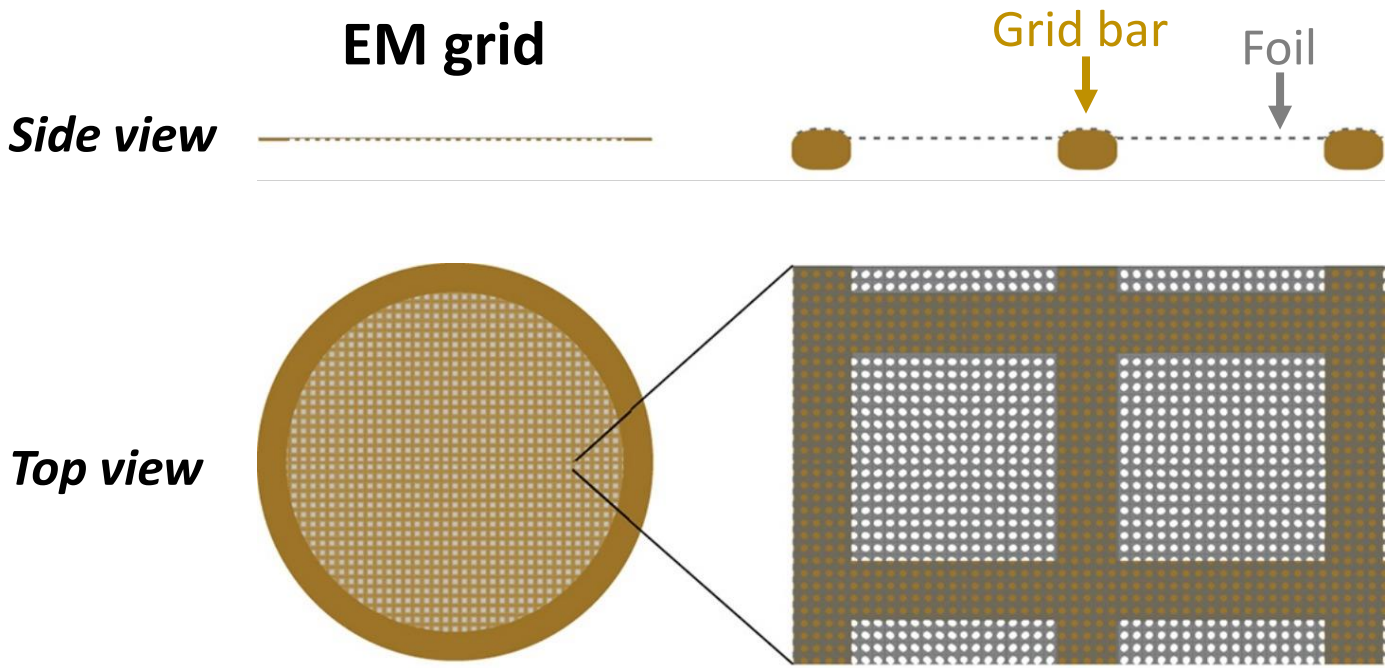
Grid materials	
Copper	**Gold**
Nickel	CuRh
Titanium	Molybdenum
Silicon	Aluminum
	Tungsten

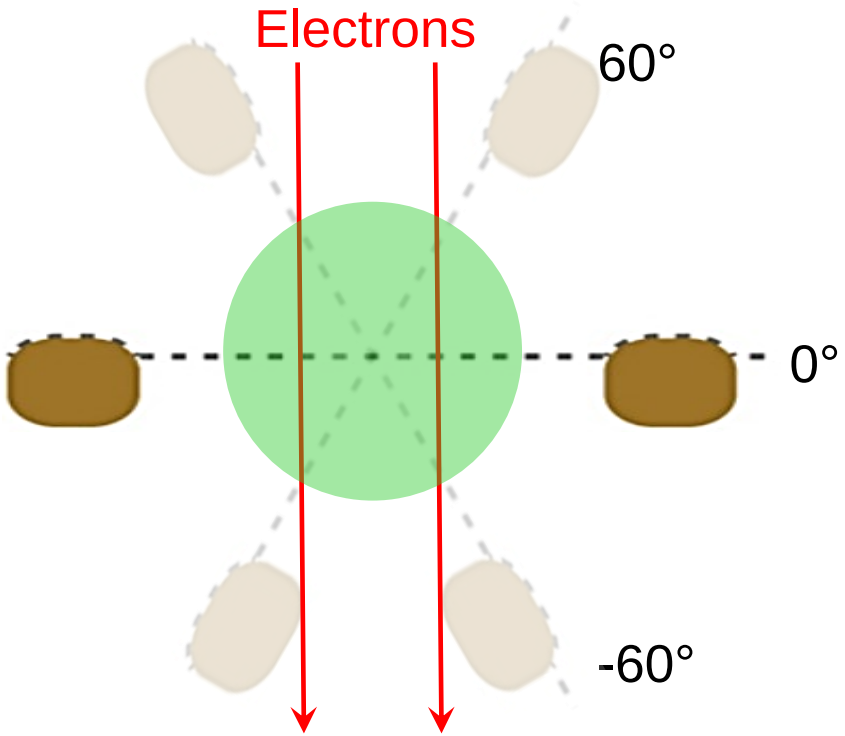
Foil materials	
Amorphous carbon	
Gold	
TiSi	SiN
SiO_2	SiC

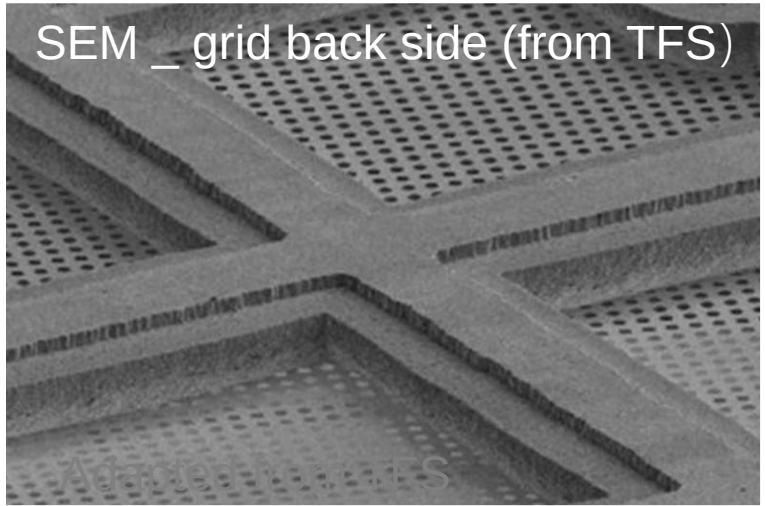
Russo & Passmore, Curr Opin Struct Biol, 2016.

2. Four considerations for cryo ET sample preparation

- Validation of the sample quality.
- Grid selection. => Mesh



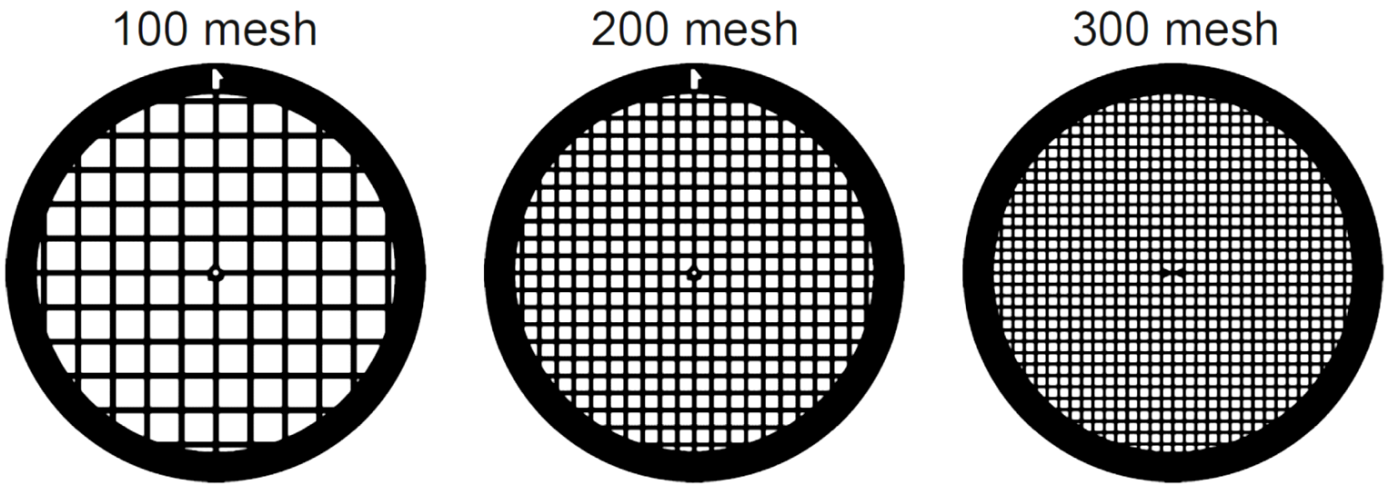


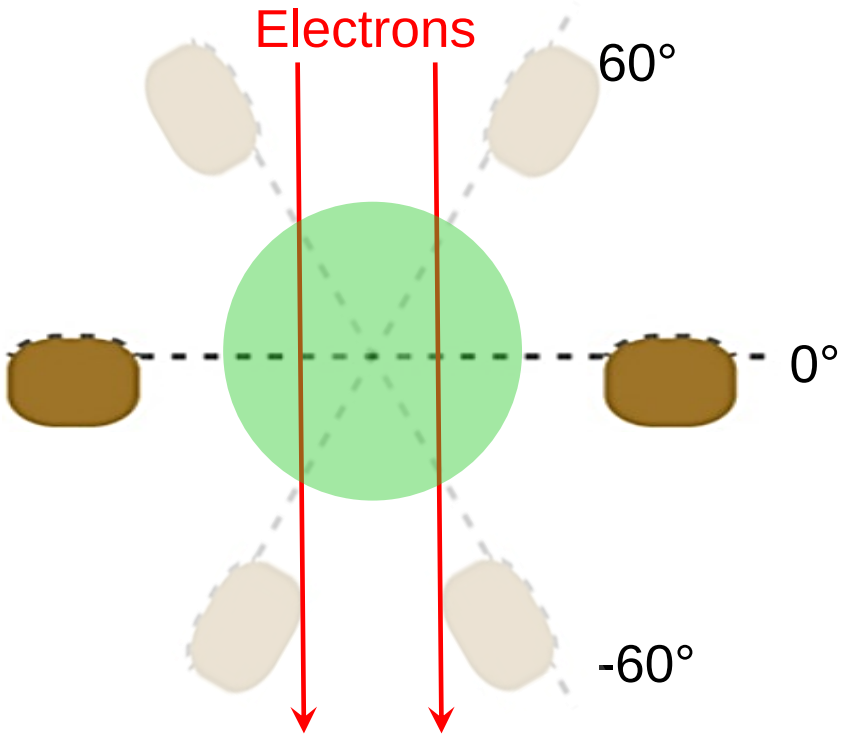


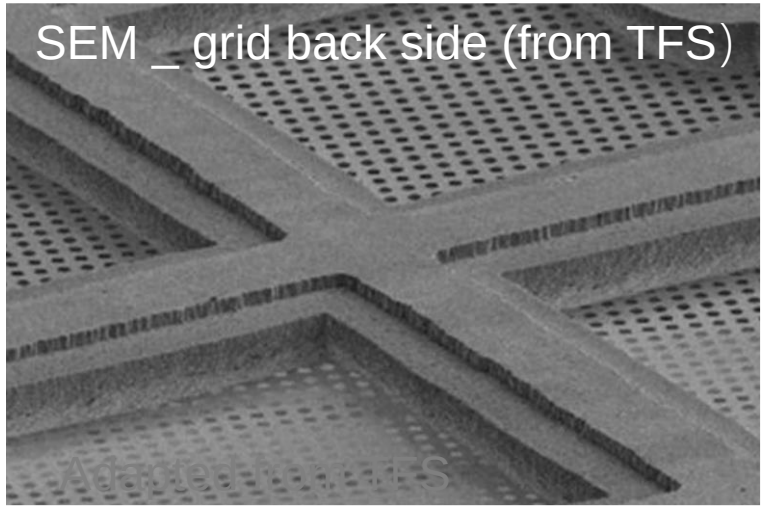
Russo & Passmore, Curr Opin Struct Biol, 2016.

2. Four considerations for cryo ET sample preparation

- Validation of the sample quality.
- Grid selection. => Mesh

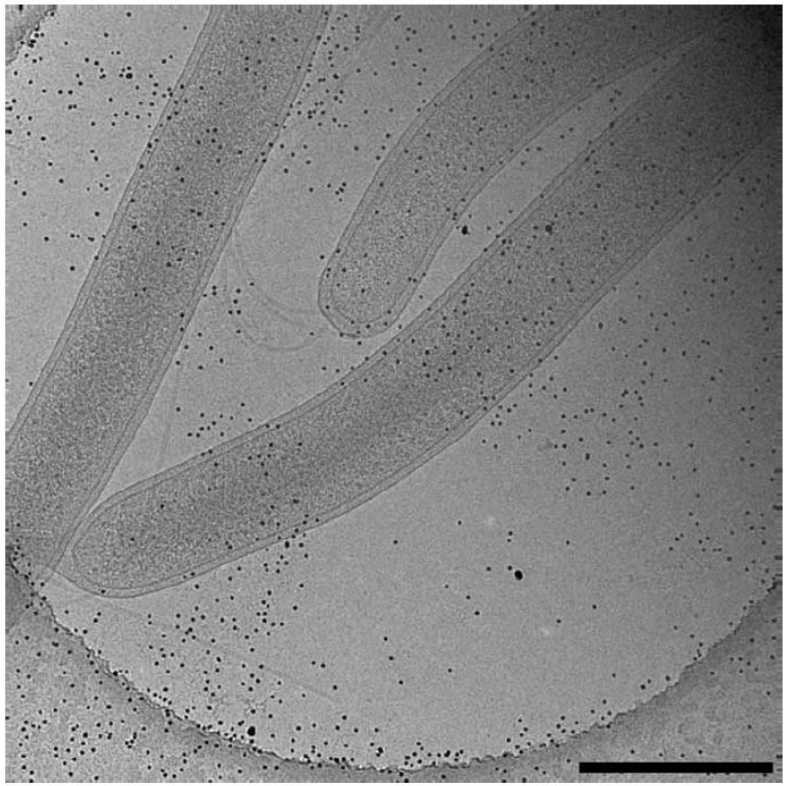






2. Four considerations for cryo ET sample preparation

- Validation of the sample quality.
- Grid selection.
- Fiducial marker. e.g., BSA-treated colloidal gold



Iancu et al., Nature Protocols 2006

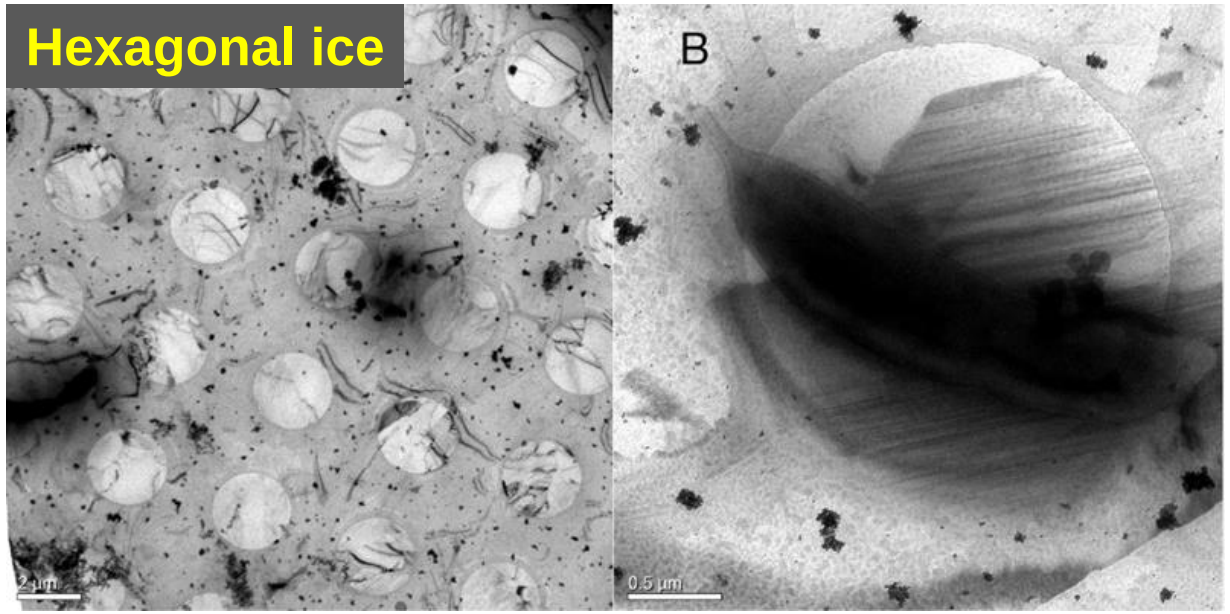
2. Four considerations for cryo ET sample preparation

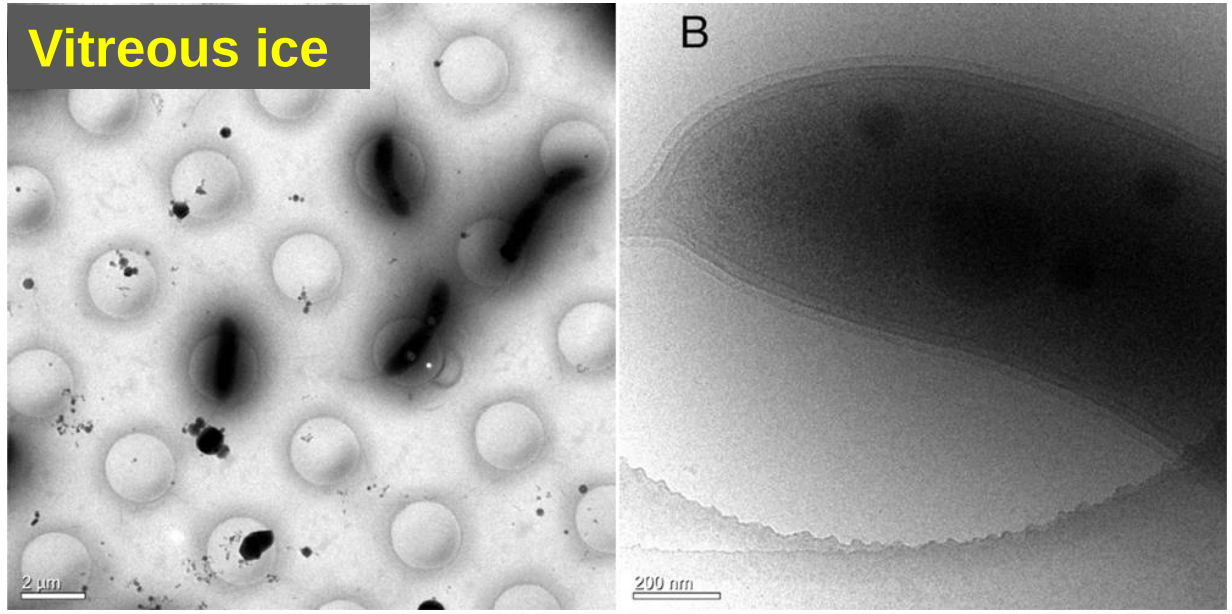
- Validation of the sample quality.
- Grid selection.
- Fiducial marker.
- Vitrification. e.g., LE or 37% LE-63% LP mixture

T_{LE}= -182.8 ℃ ~-88.6 ℃

T_{LP}= -189.7 ℃ ~ -42.2 ℃

T_{LN2}=-210 ℃ ~-195.8 ℃





Tivol et al., Microsc Microanal. 2008

2. Four considerations for cryo ET sample preparation

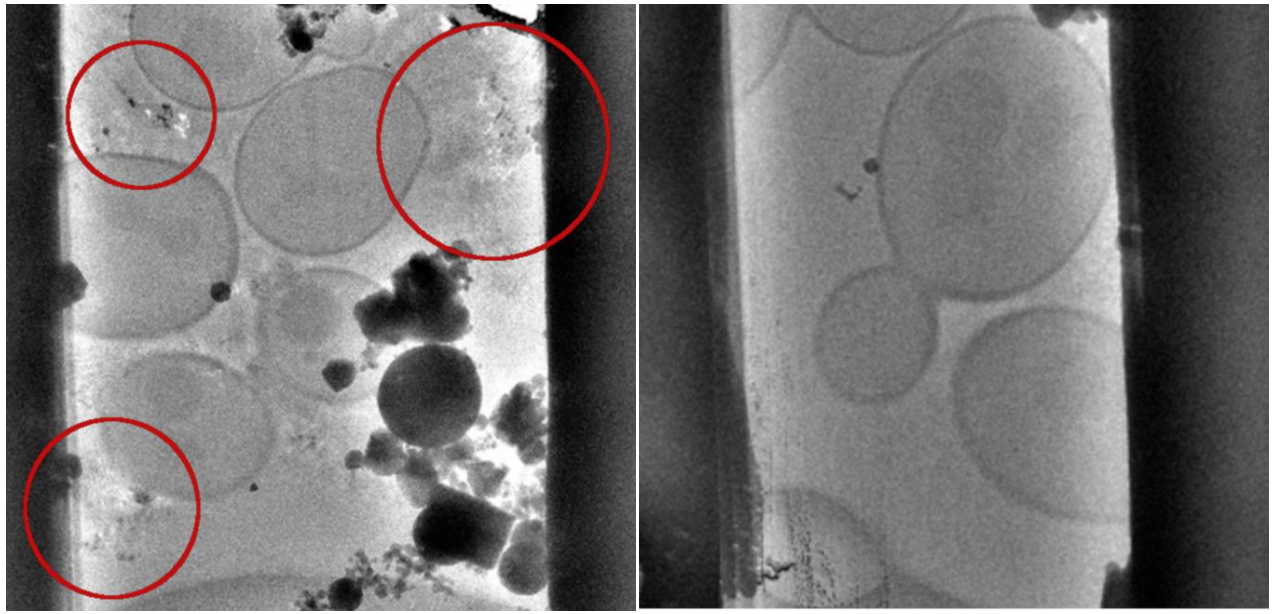
- Validation of the sample quality.
- Grid selection.
- Fiducial marker.
- Vitrification. e.g., LE or 37% LE-63% LP mixture

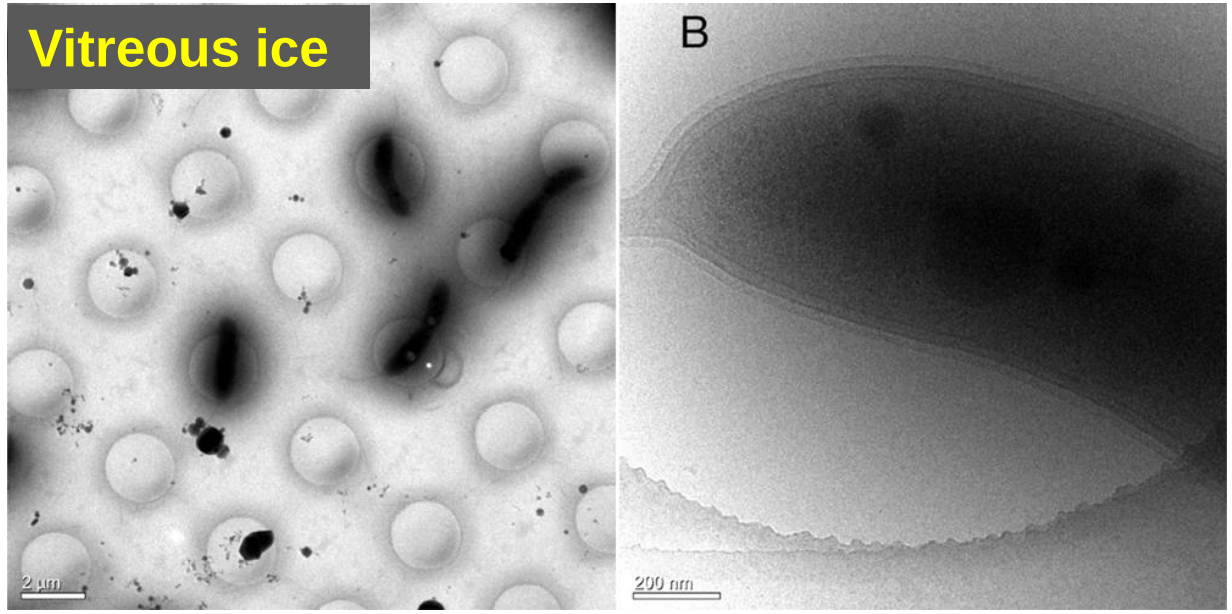
T_{LE}= -182.8 ℃ ~-88.6 ℃

T_{LP}= -189.7 ℃ ~ -42.2 ℃

T_{LN2}=-210 ℃ ~-195.8 ℃

Without glycerol 5% glycerol





Moravcová et al., J Vis Exp. 2021

Tivol et al., Microsc Microanal. 2008

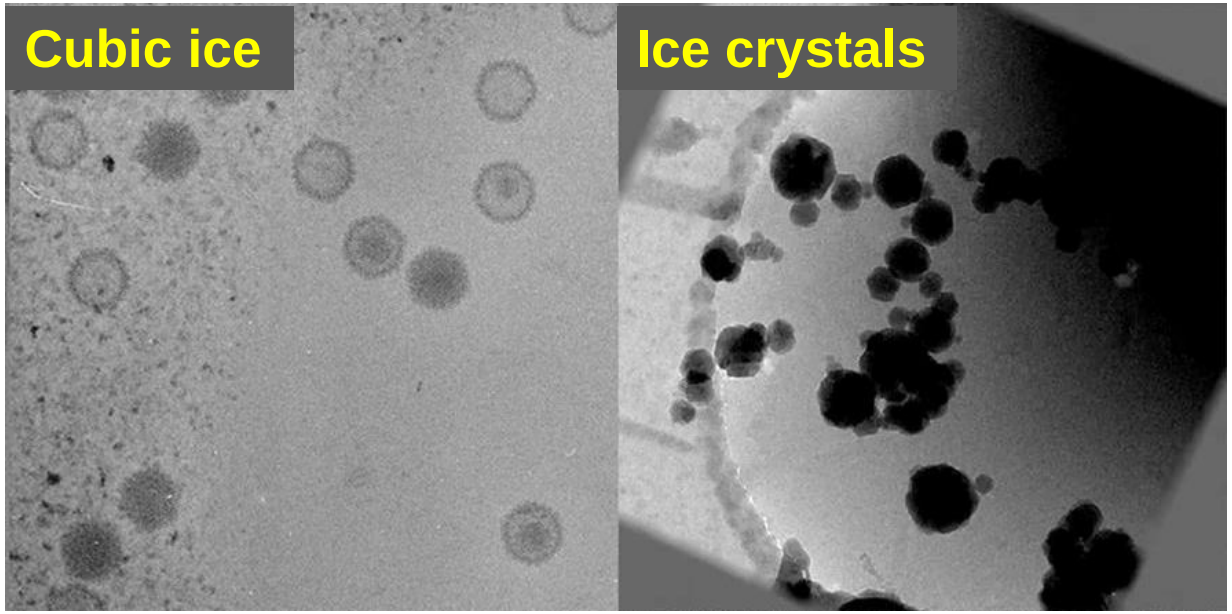
2. Four considerations for cryo ET sample preparation

- Validation of the sample quality.
- Grid selection.
- Fiducial marker.
- Vitrification. e.g., LE or 37% LE-63% LP mixture

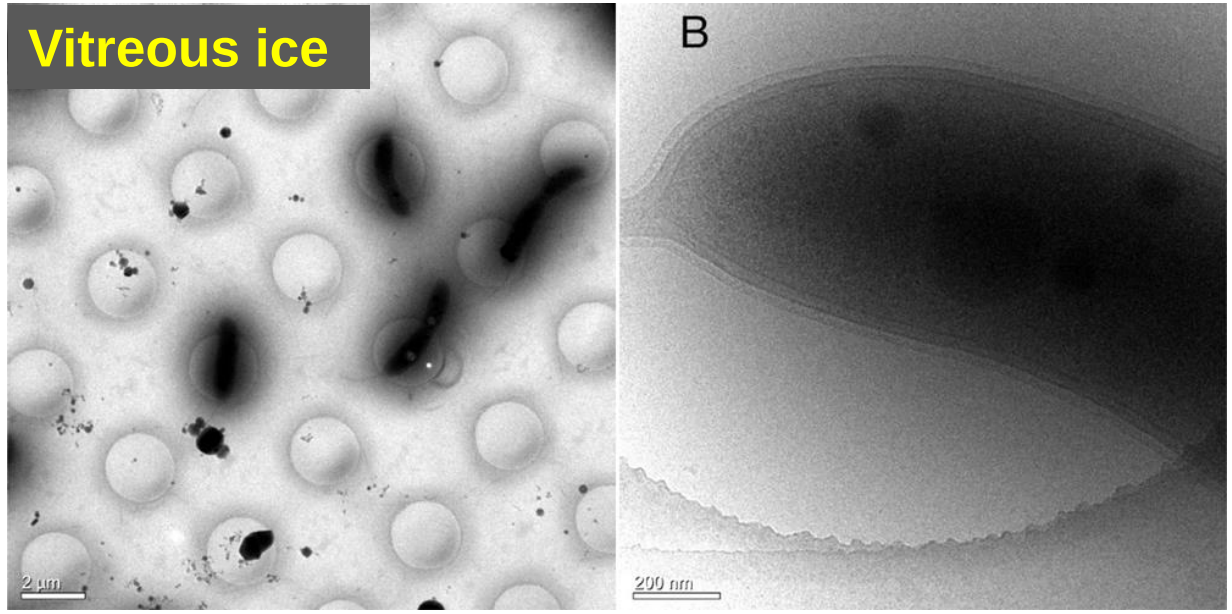
T_{LE}= -182.8 ℃ ~-88.6 ℃

T_{LP}= -189.7 ℃ ~ -42.2 ℃

T_{LN2}=-210 ℃ ~-195.8 ℃



Bhella D, Biophysical Reviews. 2019



Tivol et al., Microsc Microanal. 2008

Main contents

3. Workflow of cryo lamellae preparation

Vitrification
Plunge-freezing

T — Cryo-FM

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017

ELSEVIER

Journal of Structural Biology
Volume 197, Issue 2, February 2017, Pages 73-82

Optimized cryo-focused ion beam sample preparation aimed at in situ structural studies of membrane proteins

Miroslava Schaffer [1], Julia Mahamid [1], Benjamin D. Engel, Tim Laugks, Wolfgang Baumeister, Jürgen M. Plitzko

nature protocols

PROTOCOL
https://doi.org/10.1038/s41596-020-0320-x

Check for updates

Preparing samples from whole cells using focused-ion-beam milling for cryo-electron tomography

Felix R. Wagner[1,4,6], Reika Watanabe[1,6], Ruud Schampers[2], Digvijay Singh[1], Hans Persoon[2], Miroslava Schaffer[3], Peter Fruhstorfer[2,5], Jürgen Plitzko[3] and Elizabeth Villa[1]

3.1 Get frozen-hydrated cells on an EM grid

Vitrification
Plunge-freezing

T — Cryo-FM

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

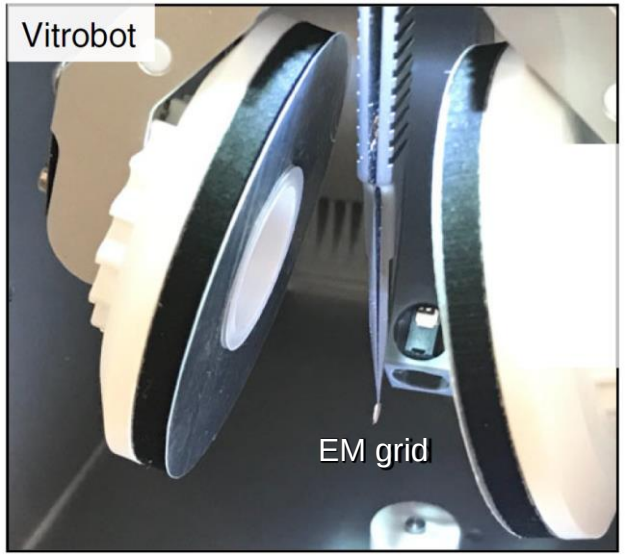
Lamella milling
Parallel & pre-wedge milling geometries

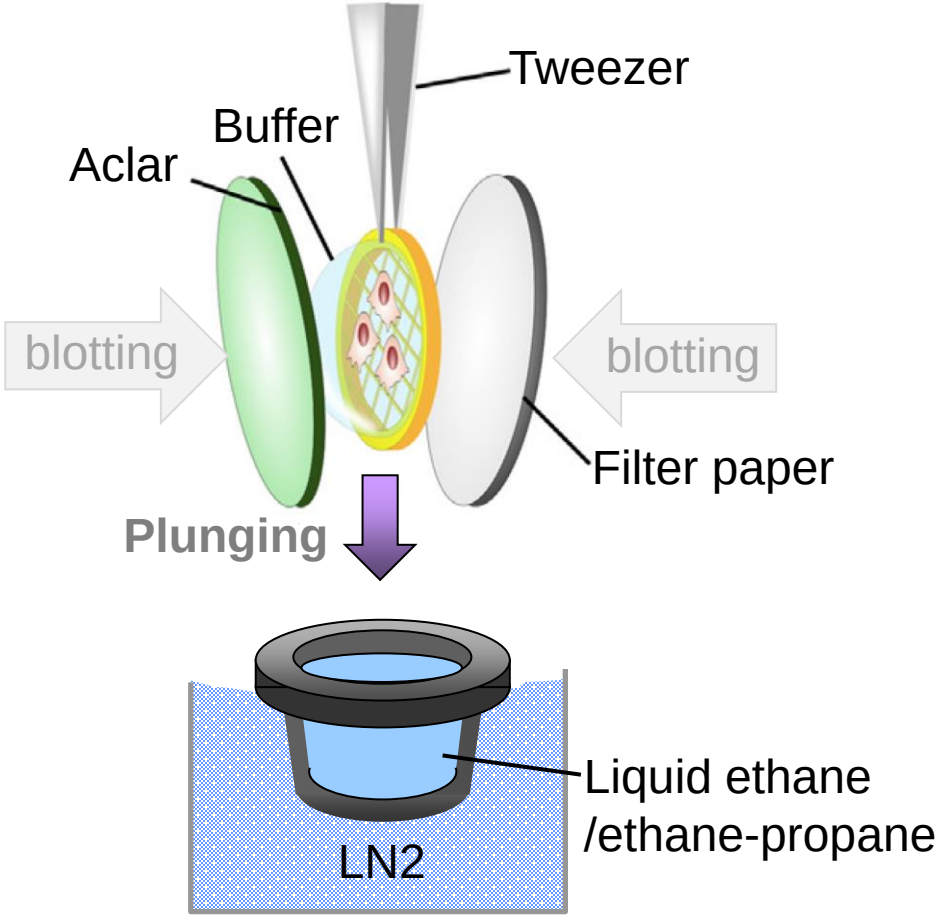
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017





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

3.1 Get frozen-hydrated cells on an EM grid

Vitrification
Plunge-freezing

T — Cryo-FM

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

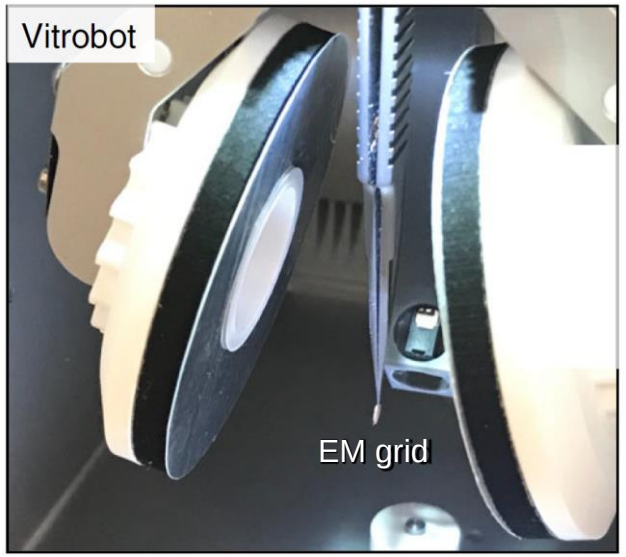
Lamella milling
Parallel & pre-wedge milling geometries

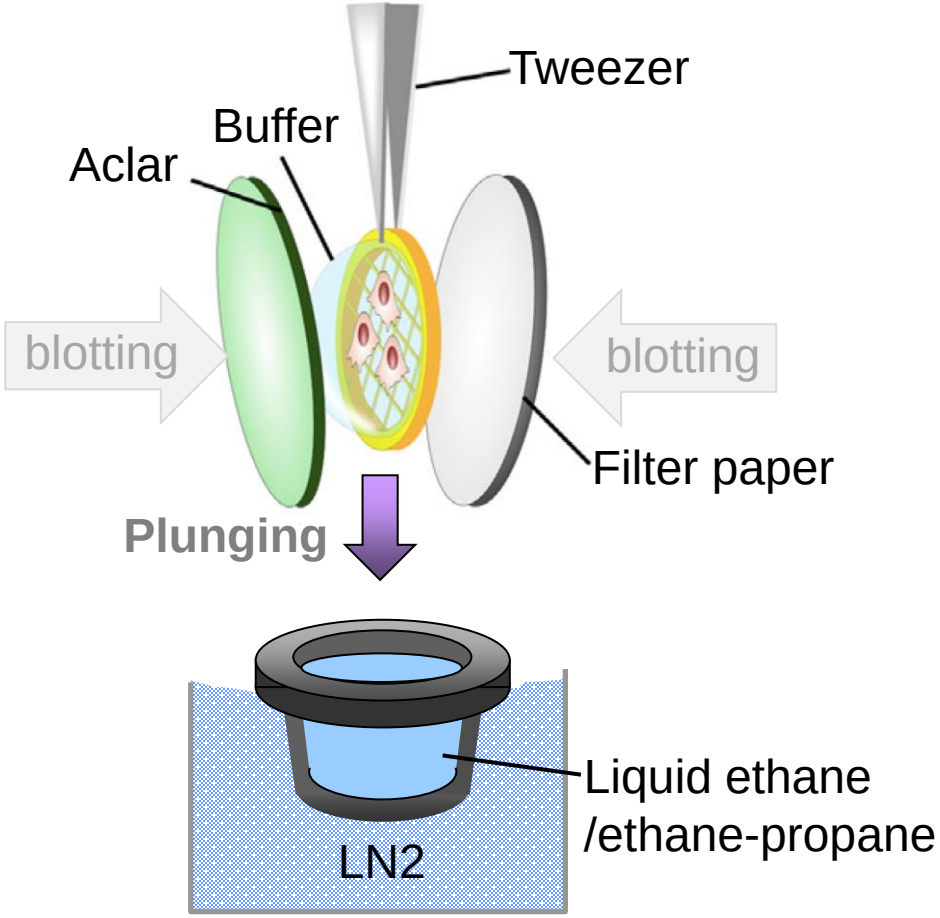
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017





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

3.1 Get frozen-hydrated cells on an EM grid

Vitrification
Plunge-freezing

T — Cryo-FM

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

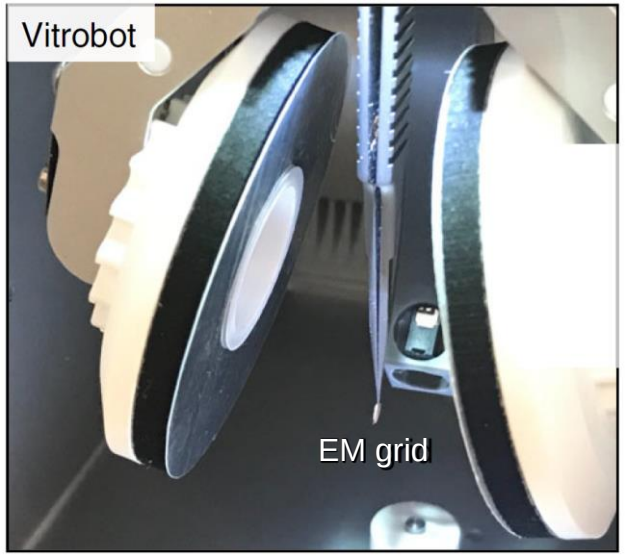
Lamella milling
Parallel & pre-wedge milling geometries

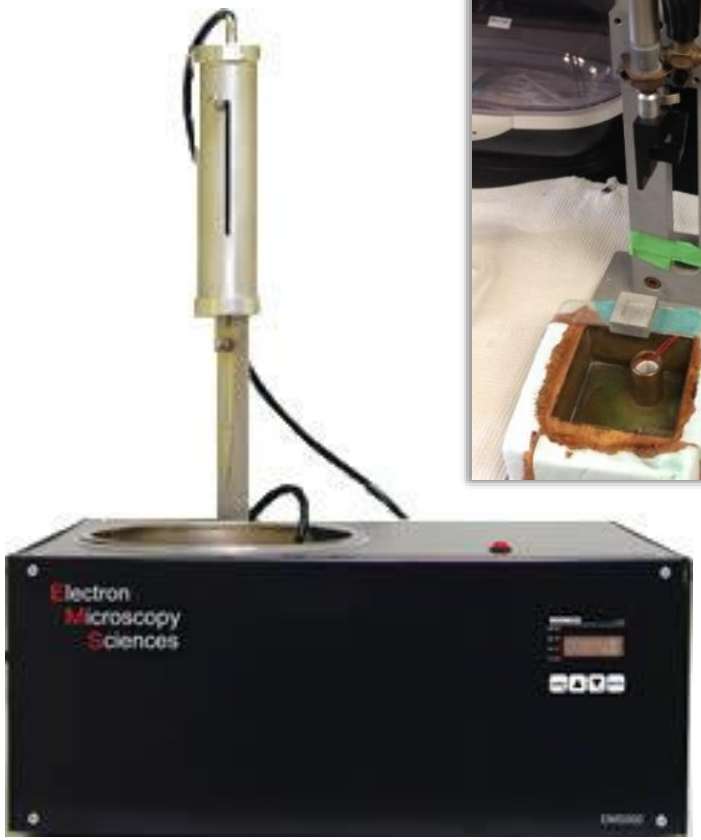
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017





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

3.1 Get frozen-hydrated cells on an EM grid

Vitrification
Plunge-freezing

T — Cryo-FM

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

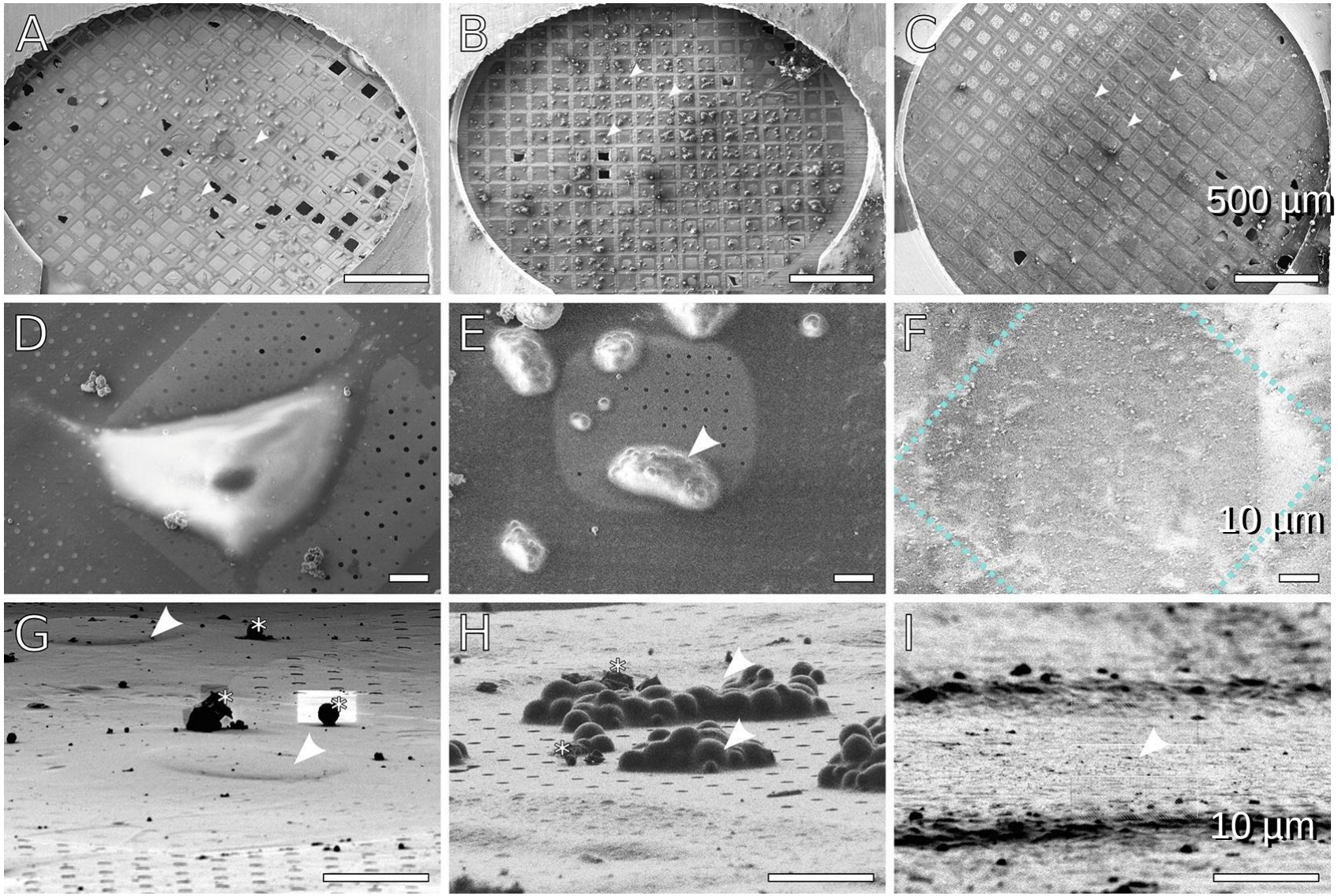
Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Mammalian | Yeast | Bacterial



Schaffer et al., JSB 2017

Lam & Villa, Methods Mol Biol. 2021

3.2 Transfer the grids_clip the grid for cryo-FIB milling

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

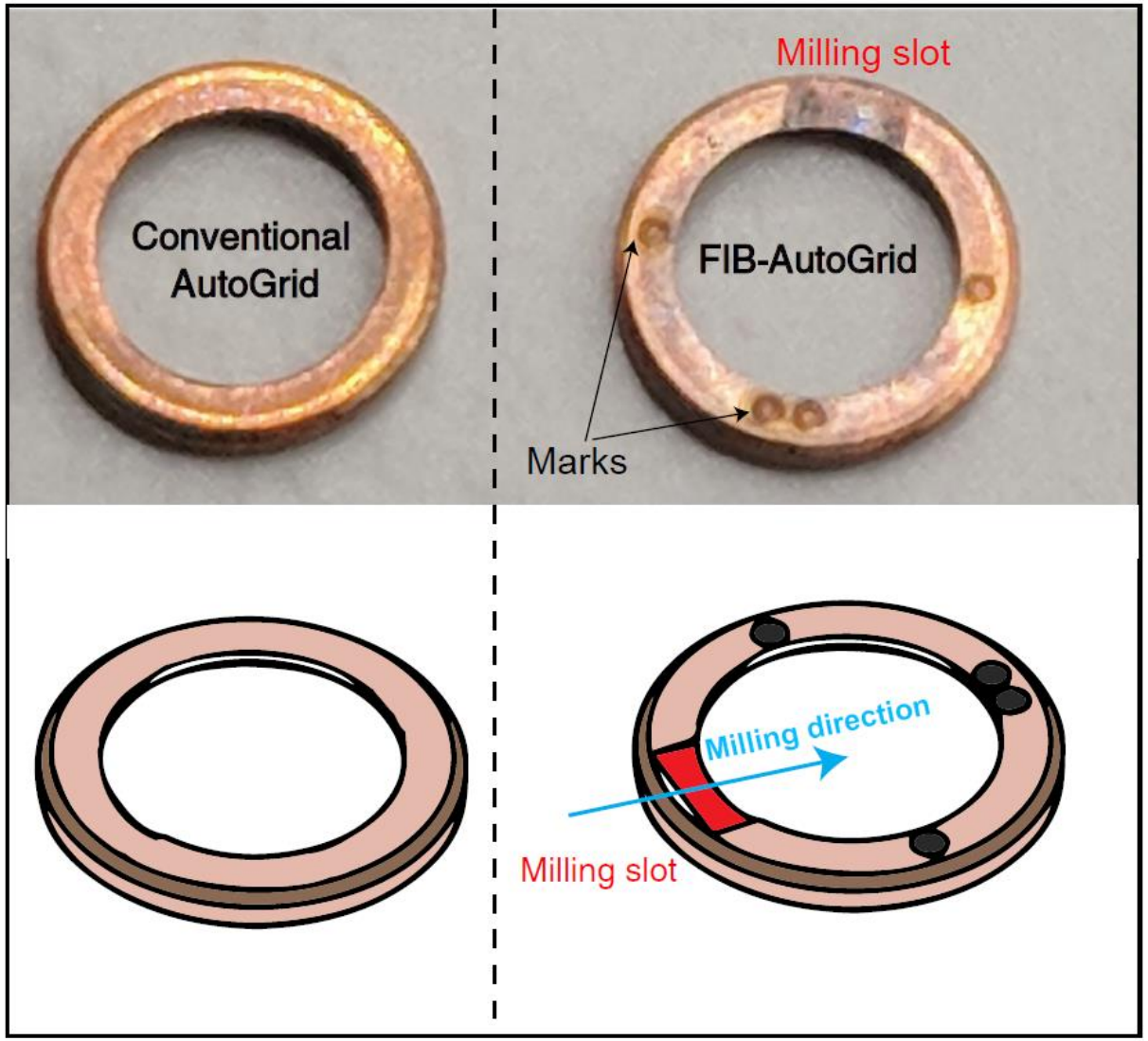
Lamella milling
Parallel & pre-wedge milling geometries

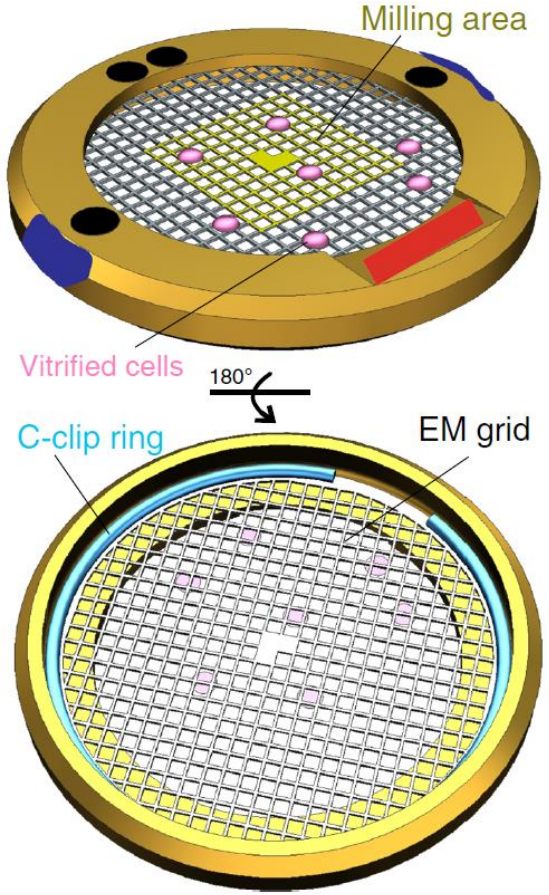
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

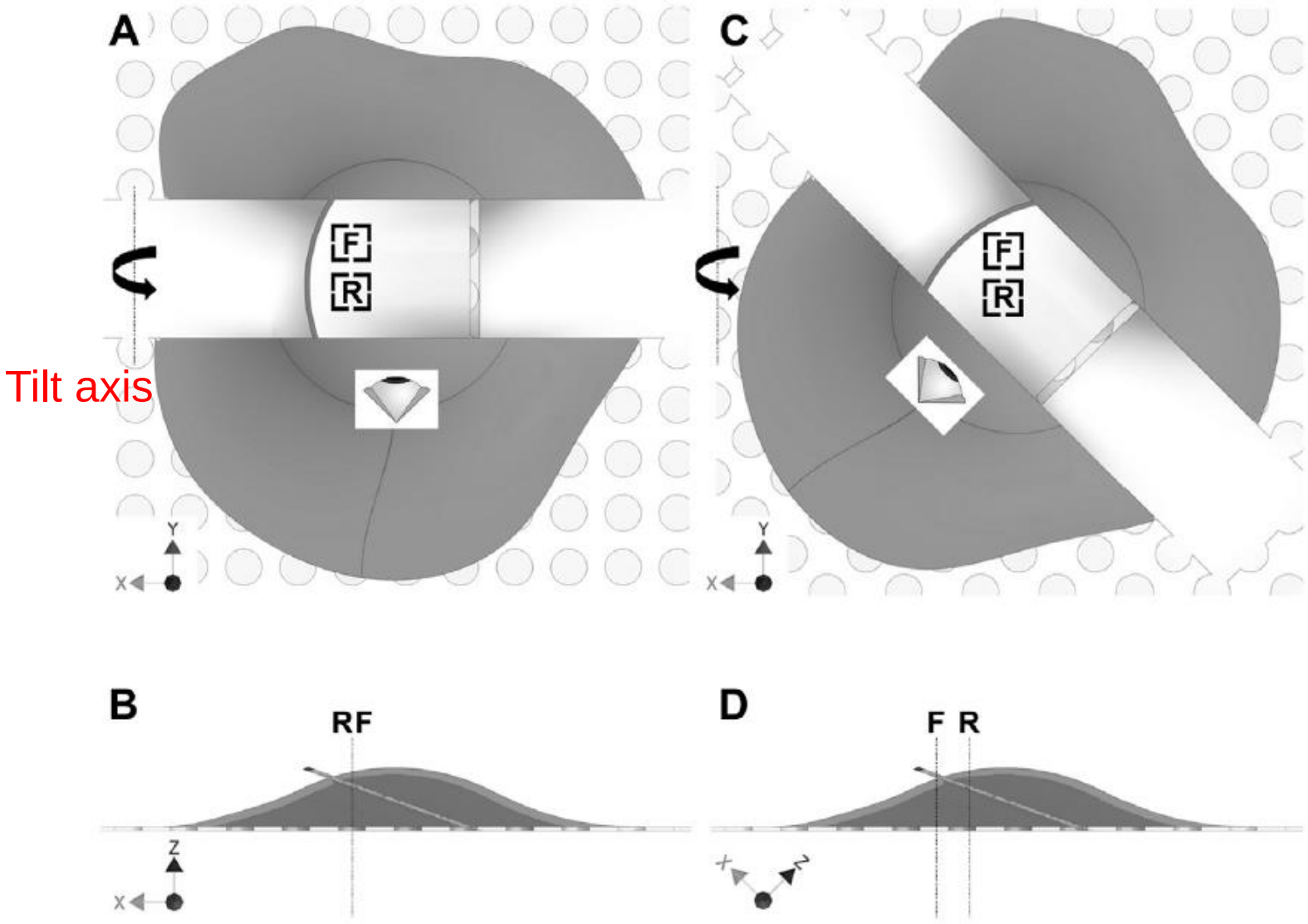
Schaffer et al., JSB 2017





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

Poor orientation causes inaccurate focus and occlusion at high tilt angles



Schaffer et al., JSB 2017

Mark the grid rim for later orientating the lamellae

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

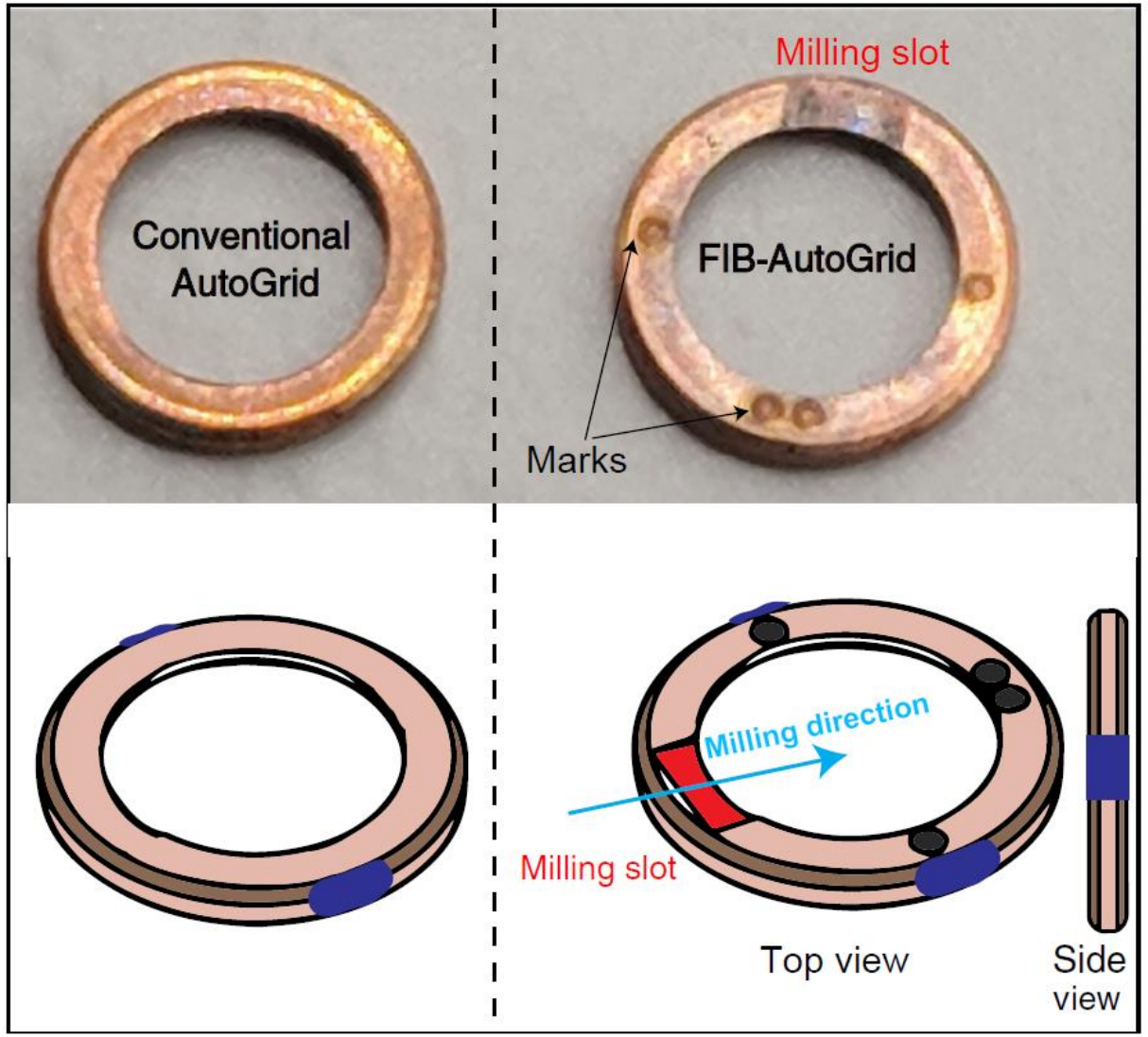
Lamella milling
Parallel & pre-wedge milling geometries

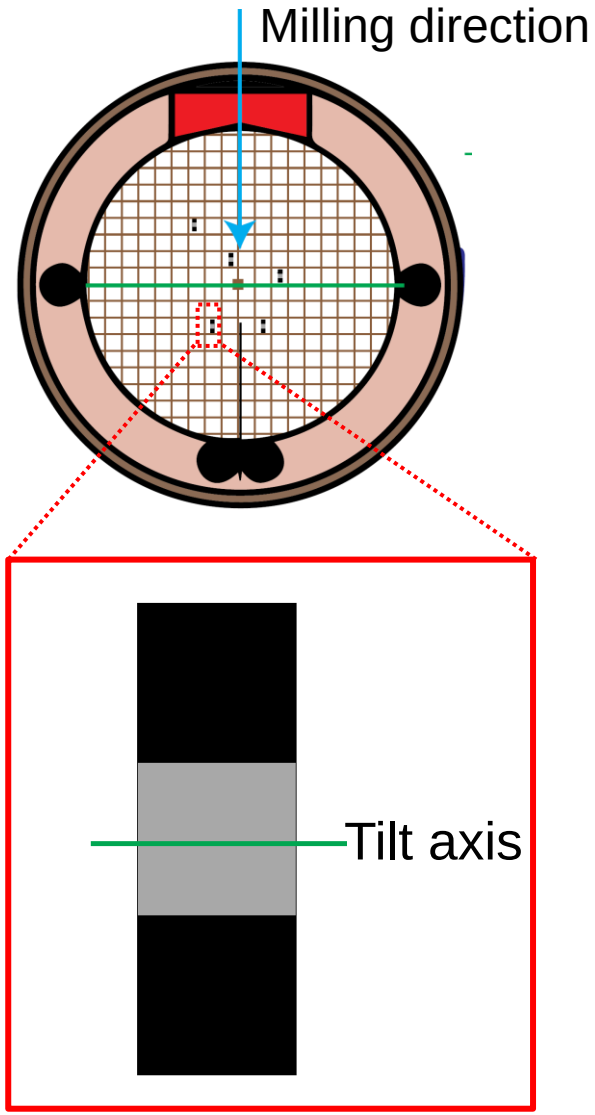
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017





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

Orientating the lamellae

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

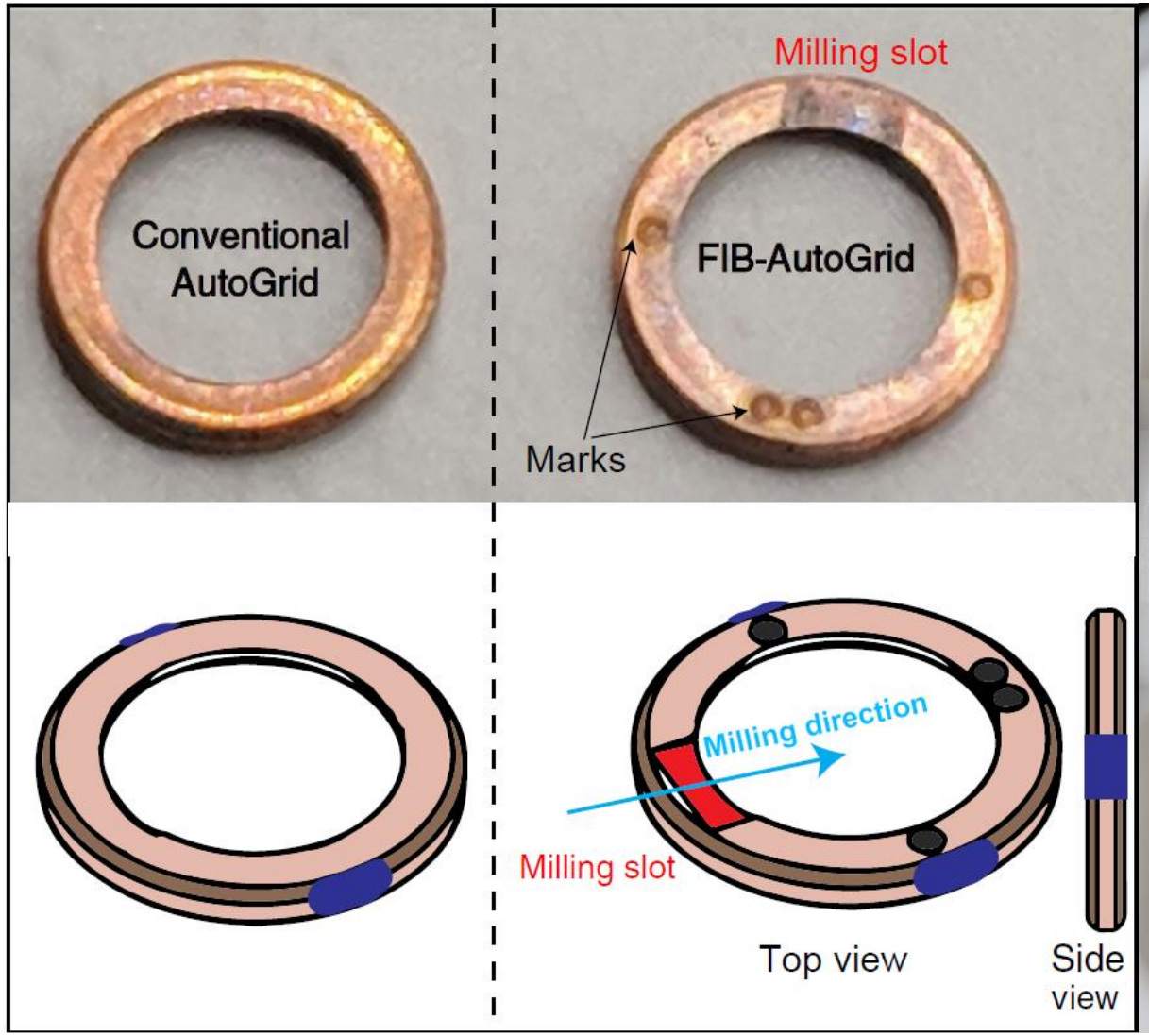
Lamella milling
Parallel & pre-wedge milling geometries

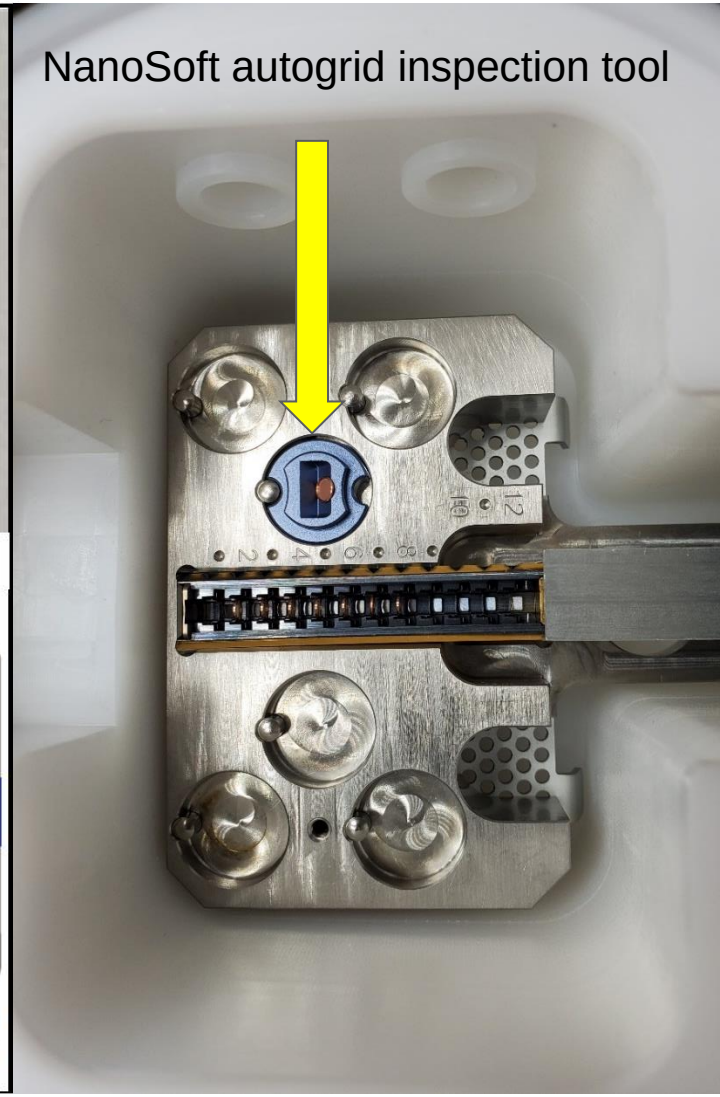
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017





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

3.3 (Optional) Cryo-fluorescence correlative microscopy localizes target

Vitrification
Plunge-freezing

T — **Cryo-FM**

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

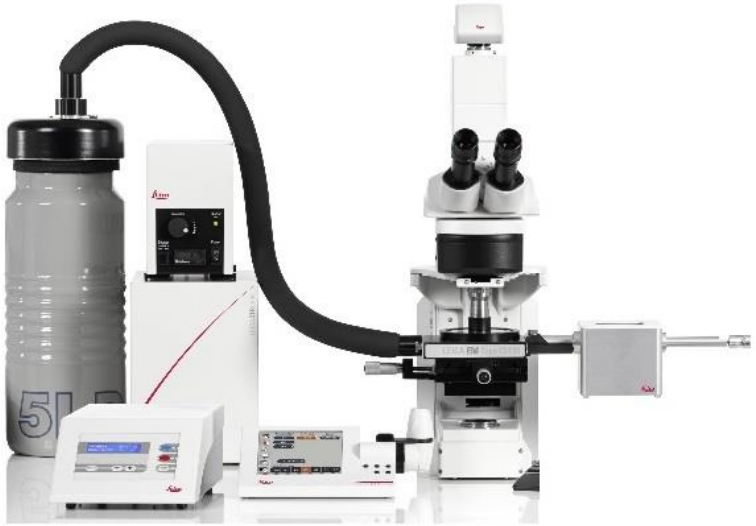
Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

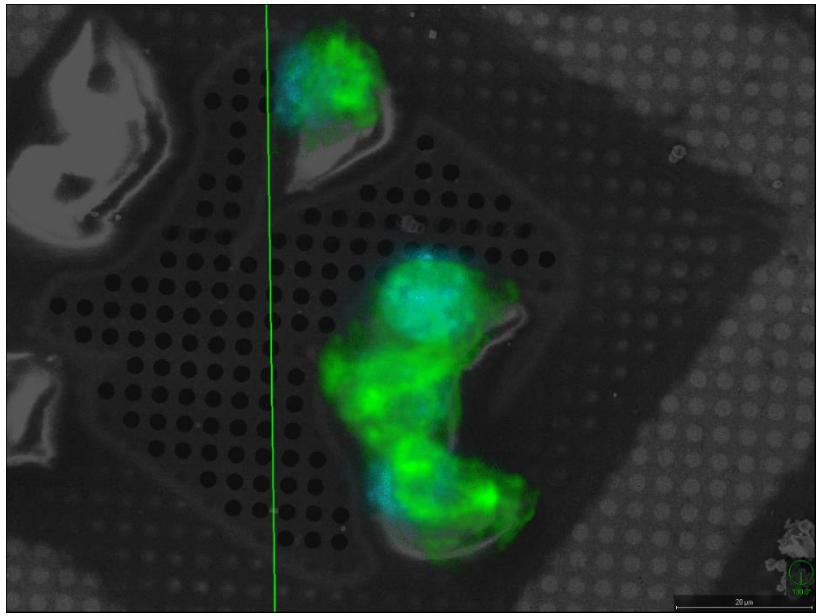
T

Cryo-electron tomography

Schaffer et al., JSB 2017

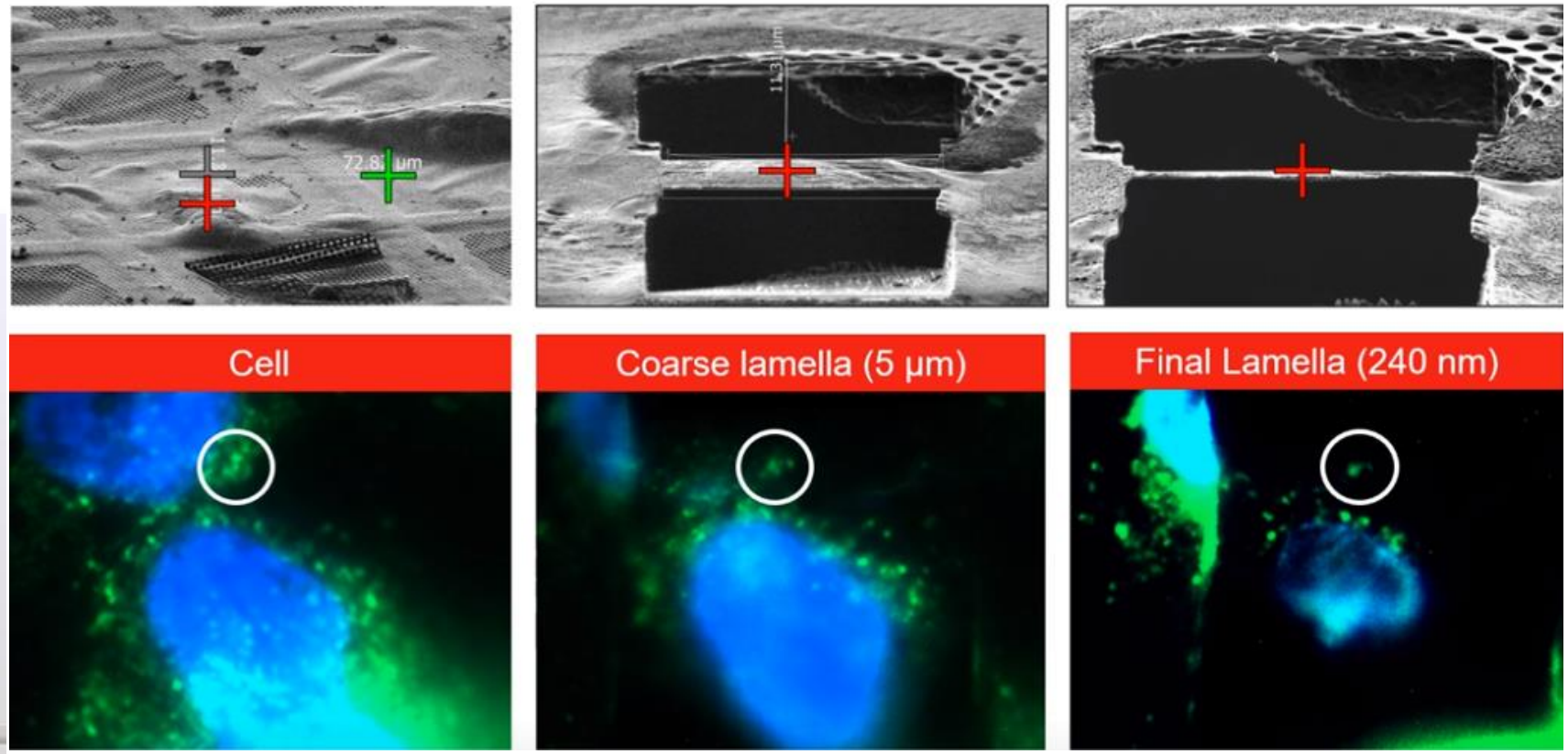


LEICA Cryo FM



Adapted from TFS

3.3 (Optional) Cryo-fluorescence correlative microscopy localizes target



Adapted from TFS

3.4 Cryo-FIB milling with DualBeam system

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

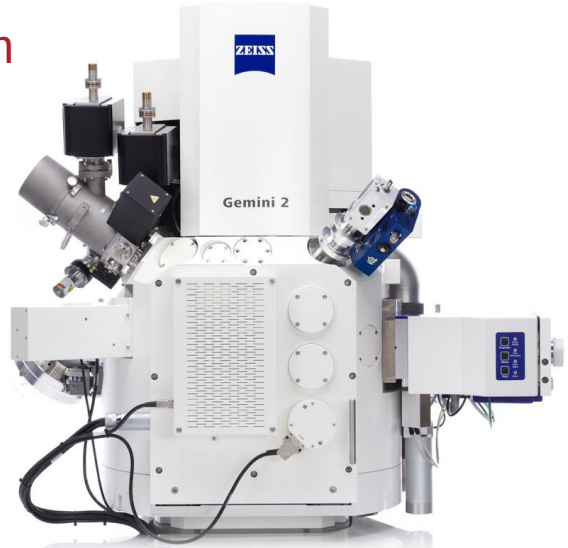
Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography

ZEISS Crossbeam



Thermo Fisher Scientific Aquiclos 1/2

Schaffer et al., JSB 2017

3.4 Cryo-FIB milling with DualBeam system

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

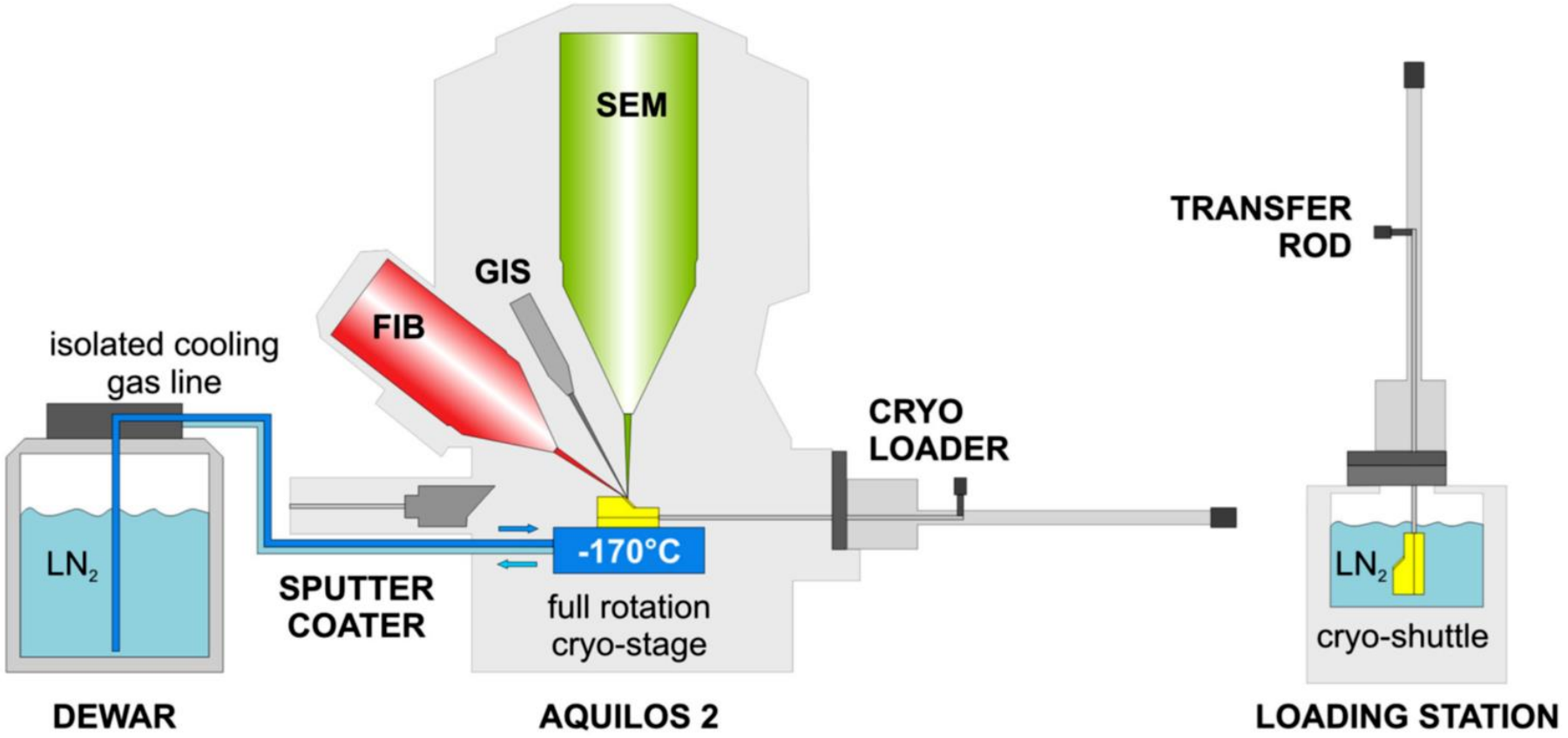
Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

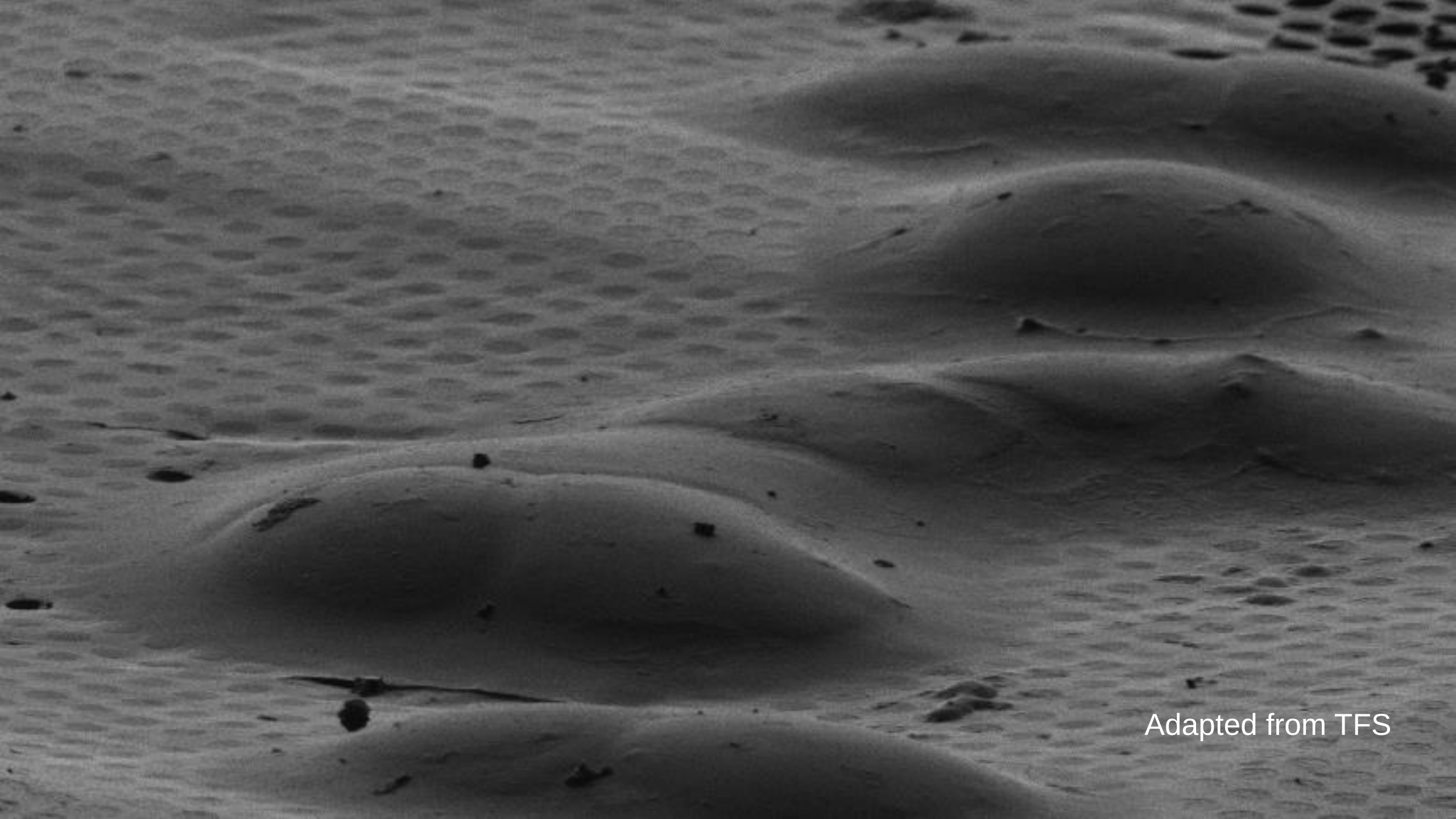
T

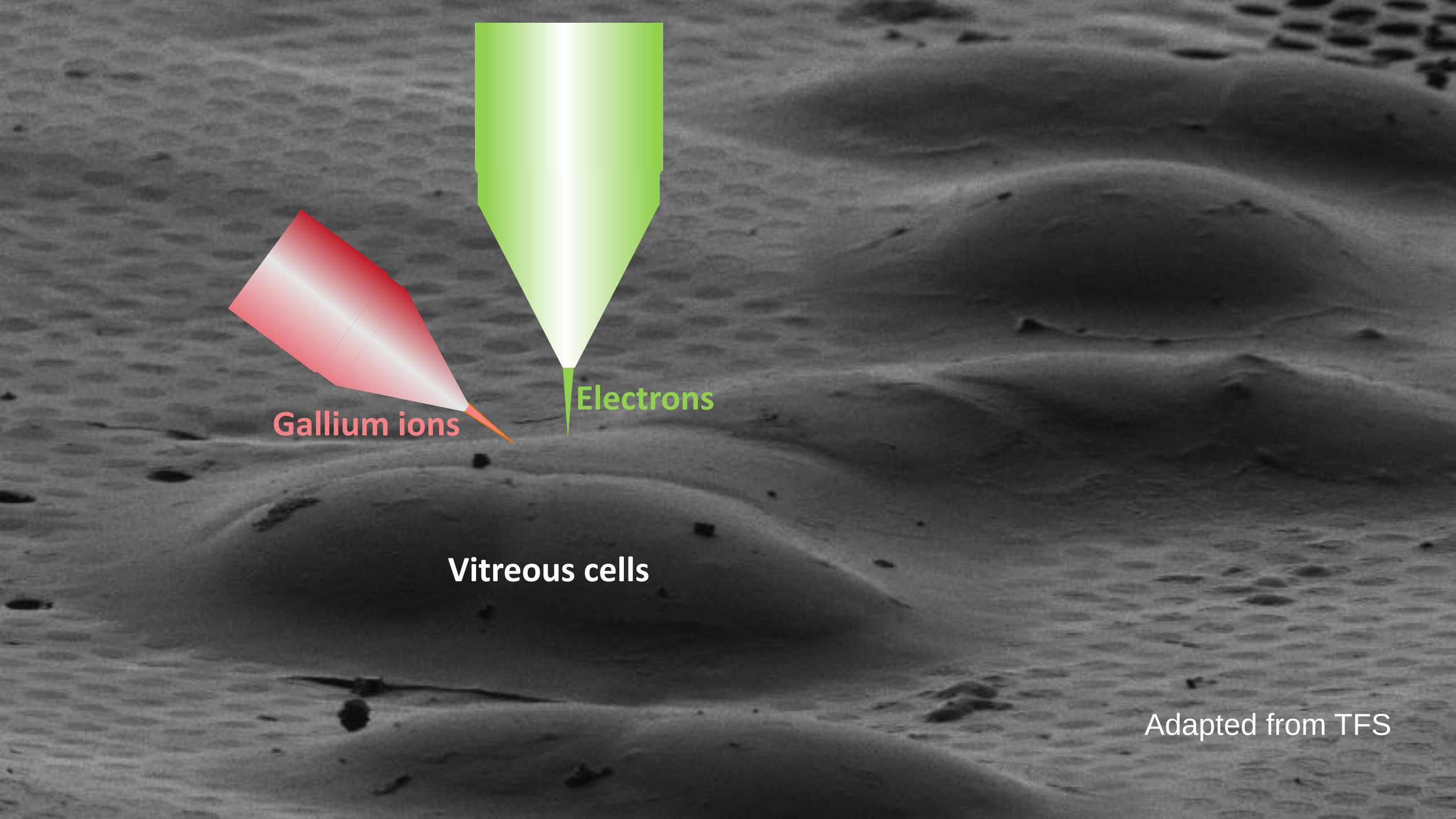
Cryo-electron tomography

Schaffer et al., JSB 2017



Kuba et al., J Microsc. 2021


Adapted from TFS


Electrons
Gallium ions
Vitreous cells
Adapted from TFS

3.4 Cryo-FIB milling with DualBeam system

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

> (Optional) minimize charging; ensure targeting and precise milling.

Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

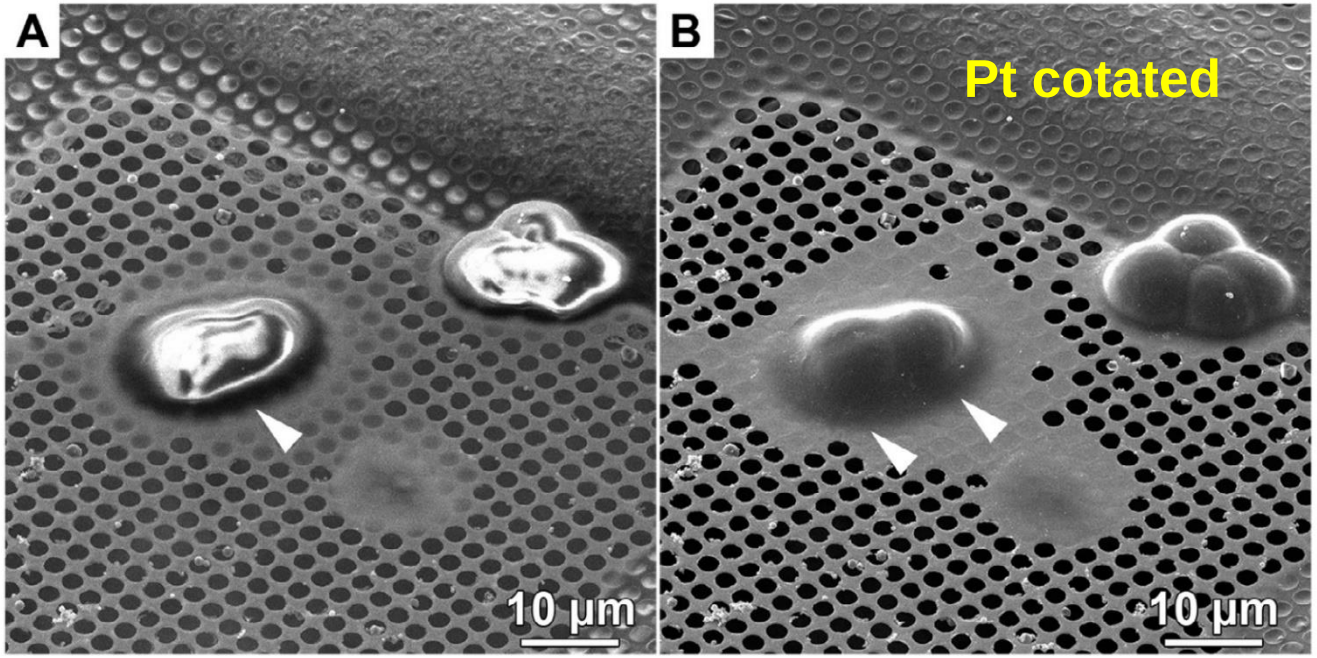
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017

Inorganic Pt



3.4 Cryo-FIB milling with DualBeam system

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

> (Optional) minimize charging; ensure targeting and precise milling.

Pt GIS
Protective coating

> Protect the sample surface during milling

Lamella milling
Parallel & pre-wedge milling geometries

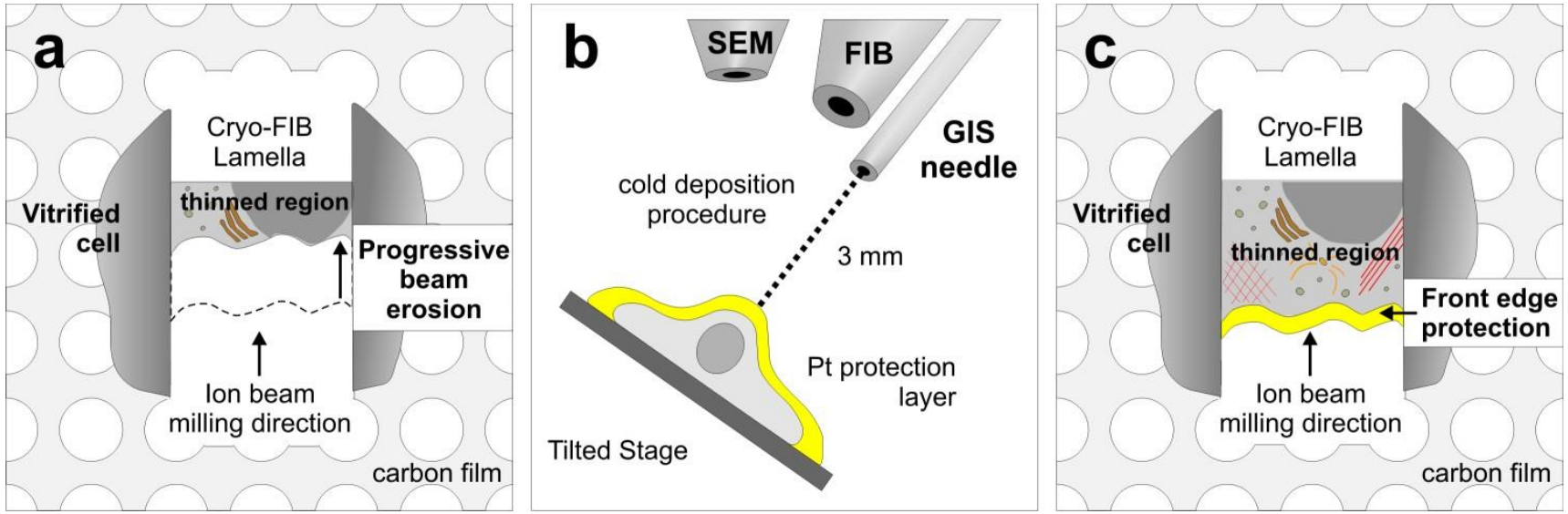
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017

Organometallic Pt



Rigort & Plitzko. Arch Biochem Biophys 2015

3.4 Cryo-FIB milling with DualBeam system

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

> (Optional) minimize charging; ensure targeting and precise milling.

Pt GIS
Protective coating

> Protect the sample surface during milling

Lamella milling
Parallel & pre-wedge milling geometries

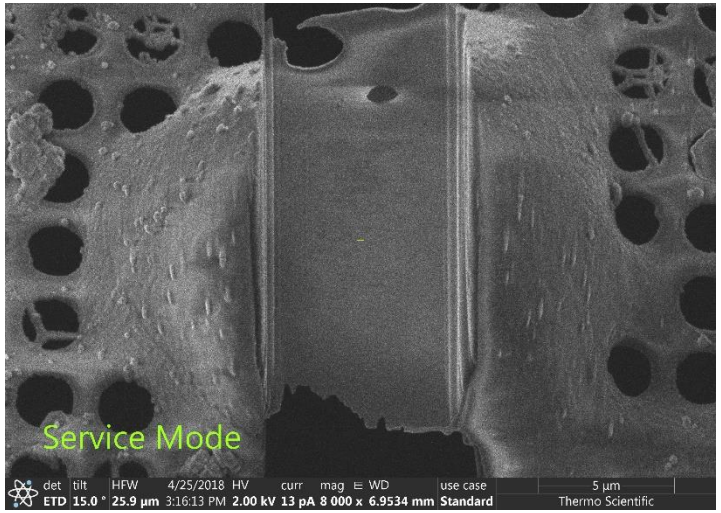
Pt sputter
Lamella conductivity

T

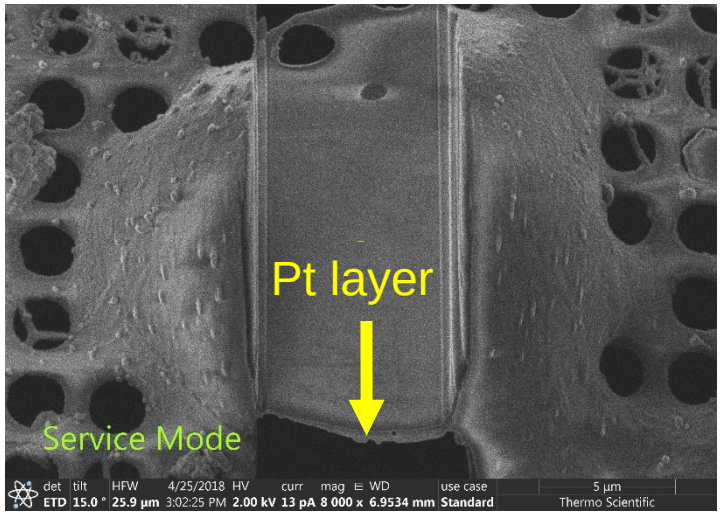
Cryo-electron tomography

Schaffer et al., JSB 2017

Organometallic Pt



Without Pt GIS coating



With Pt GIS coating

Milling direction

3.4 Cryo-FIB milling with DualBeam system

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

> (Optional) minimize charging; ensure targeting and precise milling.

Pt GIS
Protective coating

> Protect the sample surface during milling; reduces curtaining and the redeposition of milled material.

Lamella milling
Parallel & pre-wedge milling geometries

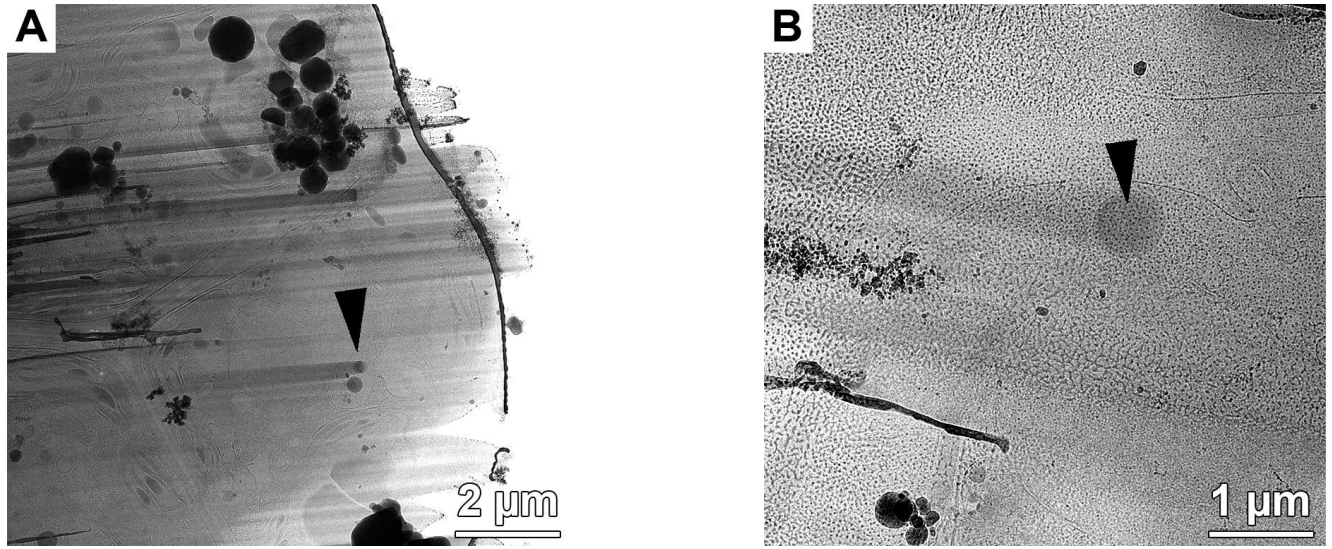
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

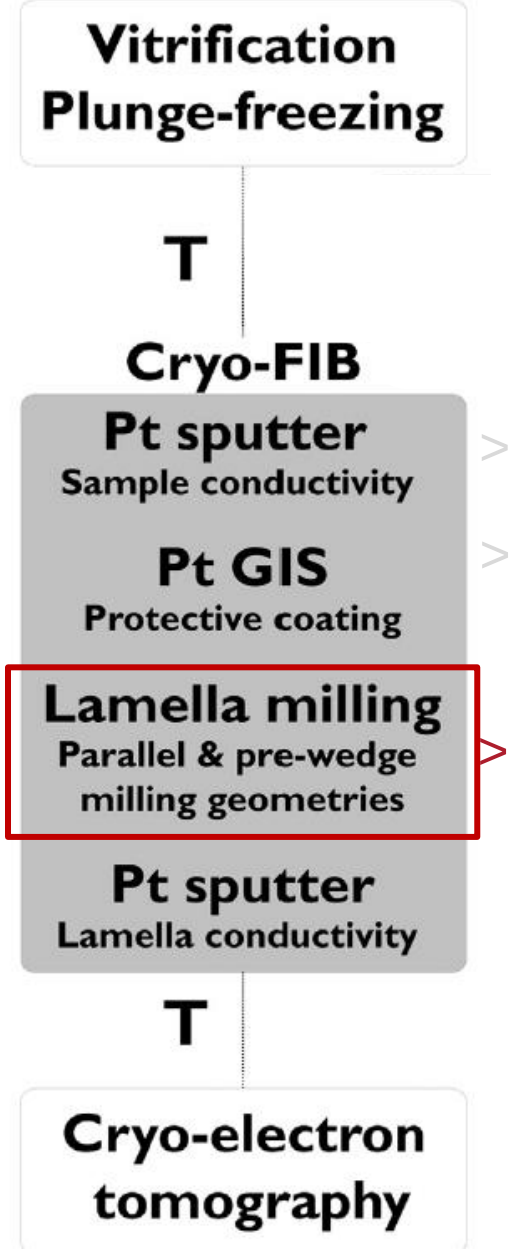
Schaffer et al., JSB 2017

Organometallic Pt



TEM

3.4 Cryo-FIB milling with DualBeam system



Schaffer et al., JSB 2017

> (Optional) minimize charging; ensure targeting and precise milling.

> Protect the sample surface during milling; reduces curtaining and the redeposition of milled material.

> Rough milling, thinning and cleaning for thin lamella.

3.4.1 Eucentric height & tilt calculation

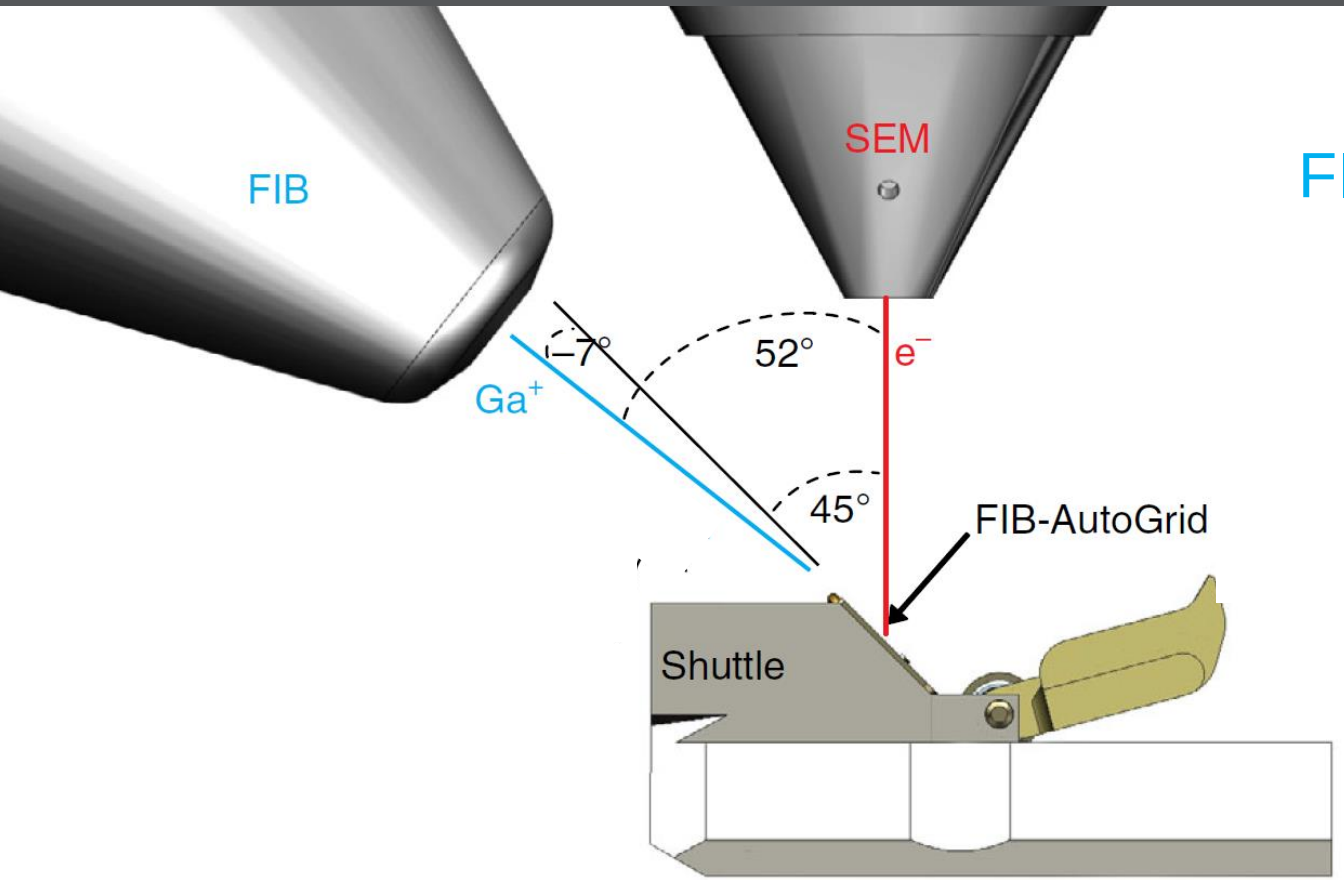
3.4.2 Stepwise milling procedure

3.4.3 Micro-expansion joints technique

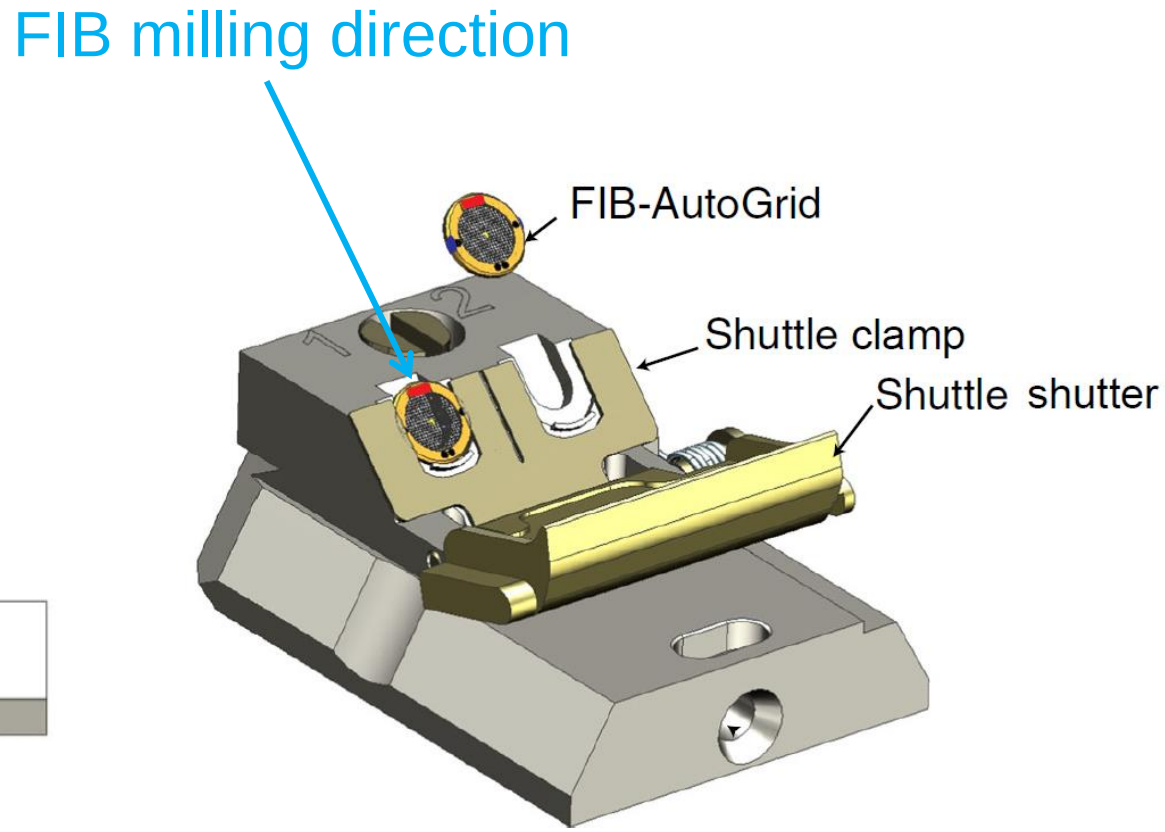
3.4.4 The wedge pre-milling technique

3.4.5 Batch lamella milling

3.4.1 Eucentric height & tilt calculation



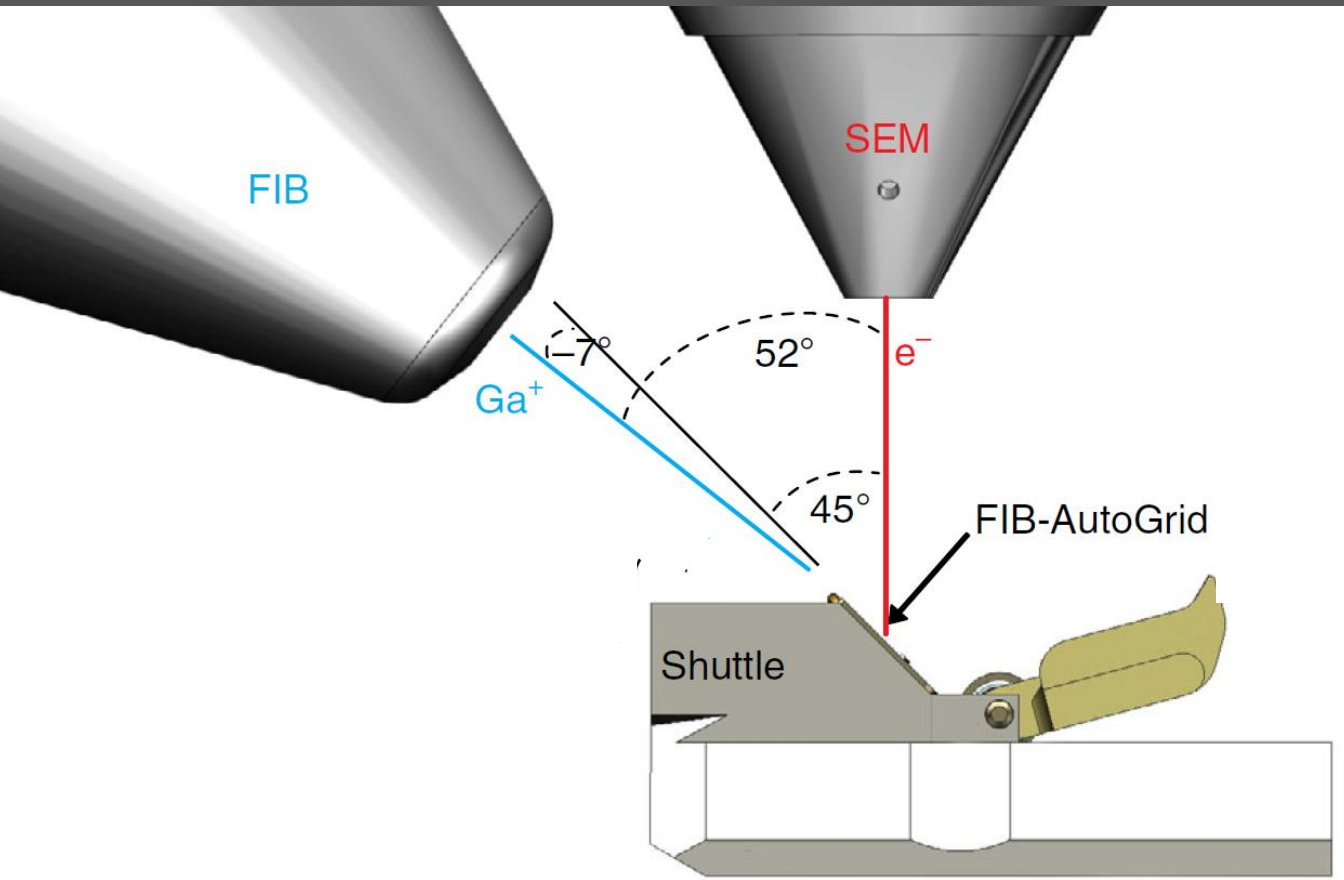
Coincident point of E and I beams

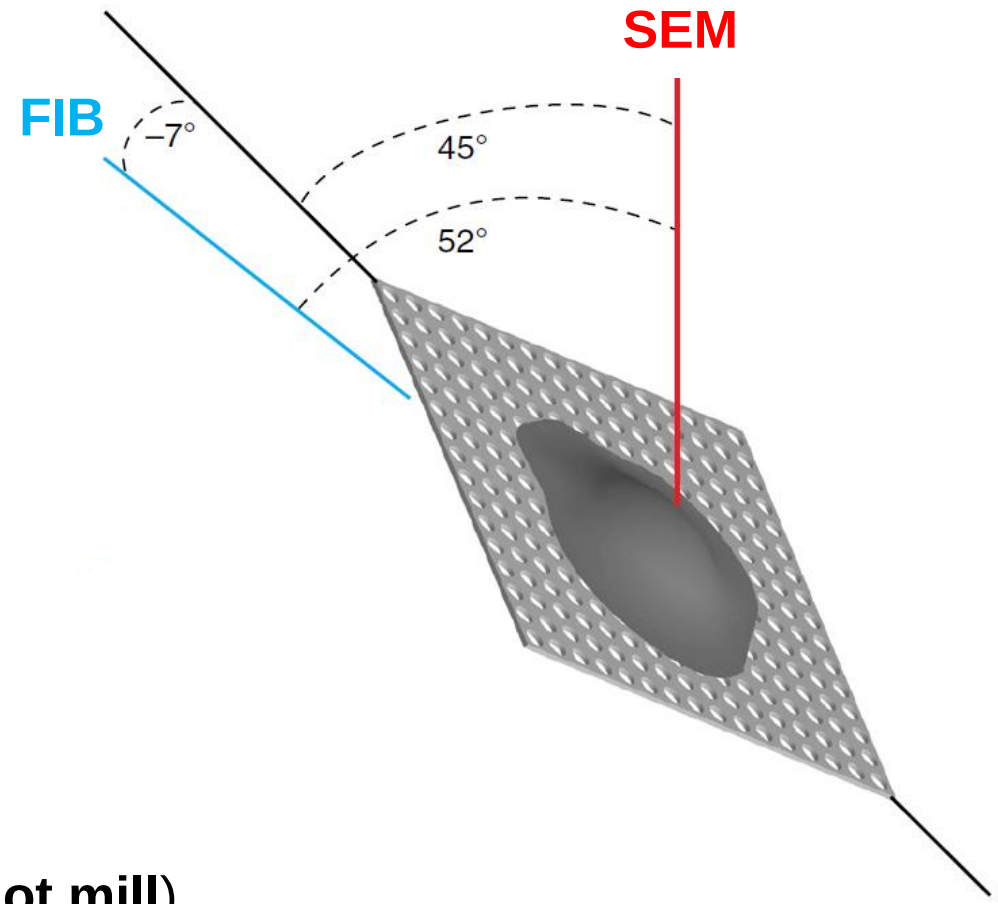


Autogrid shuttle

Wagner et al., Nature Protocols 2020

3.4.1 Eucentric height & tilt calculation



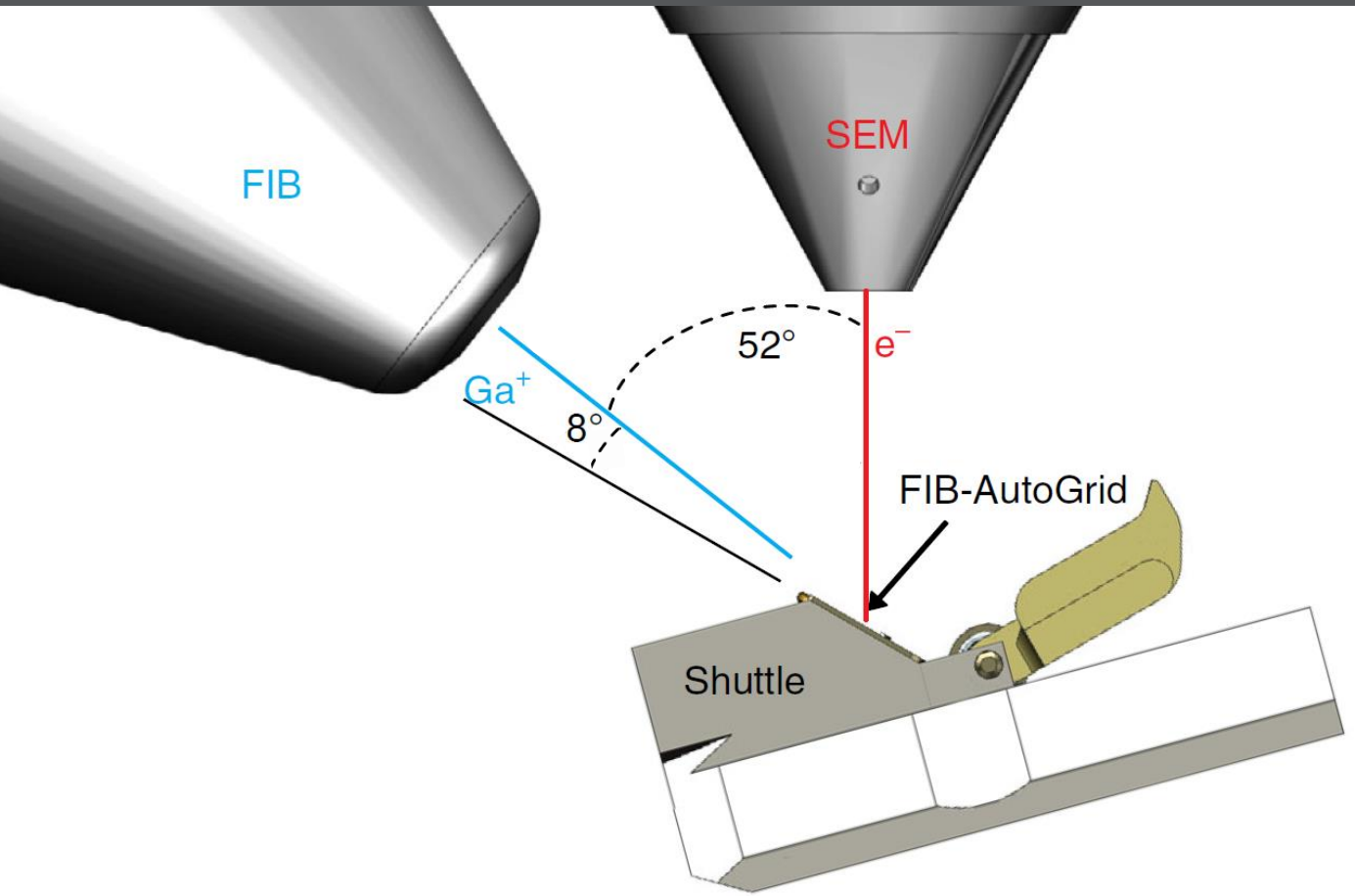


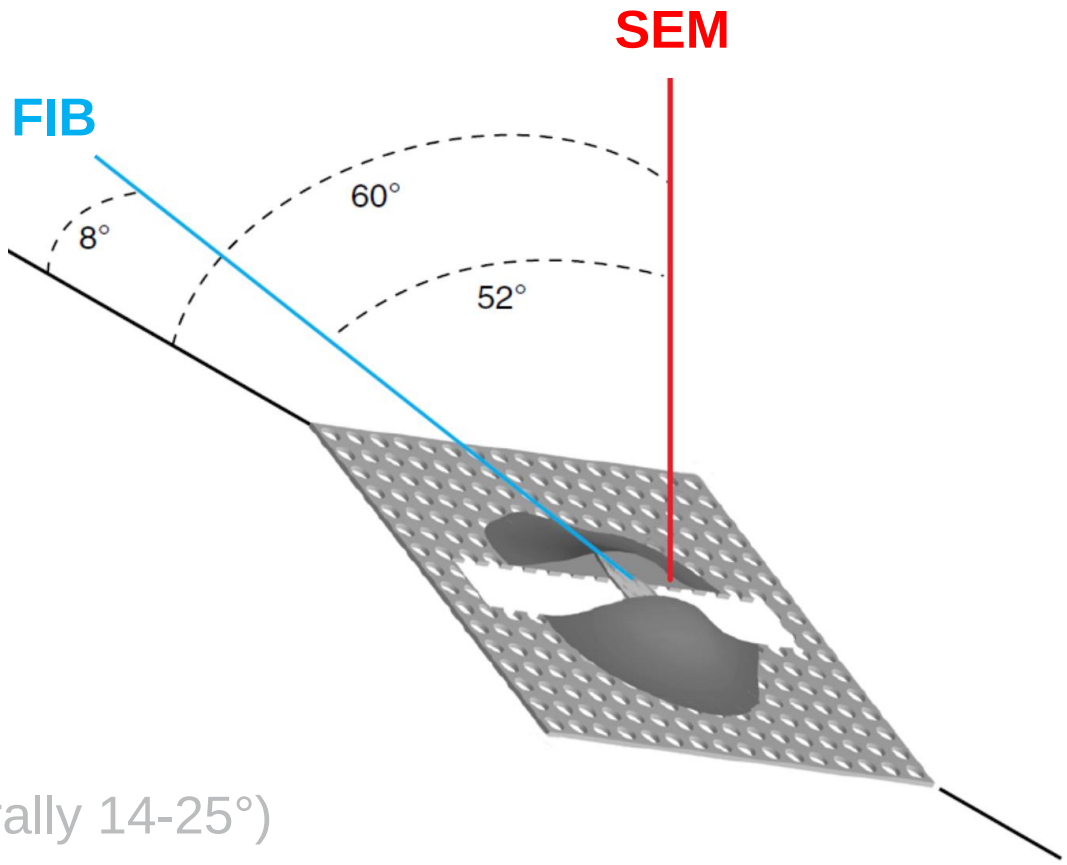
Loading position: Stage tilt angle = 0°

Milling angle = -7° (**can not mill**)

Wagner et al., Nature Protocols 2020

3.4.1 Eucentric height & tilt calculation



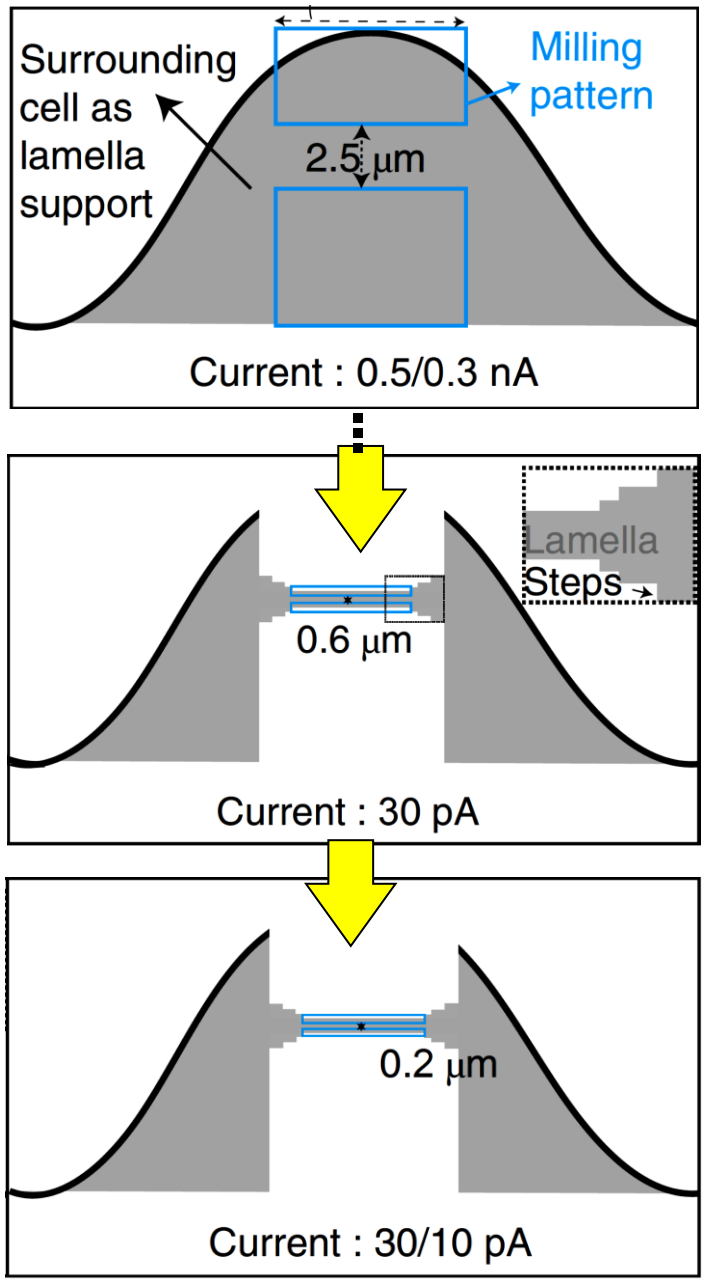


Milling position: Stage tilt angle = 15° (generally 14-25°)

Milling angle = tilt angle -7 ° = 8° (generally 7-18°)

Wagner et al., Nature Protocols 2020

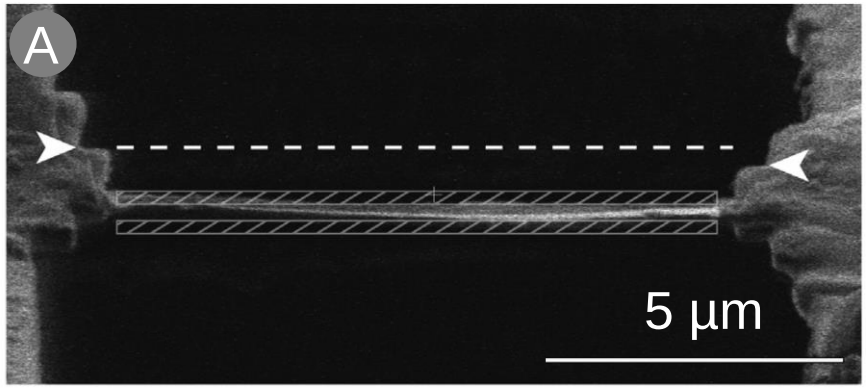
3.4.2 Stepwise milling procedure

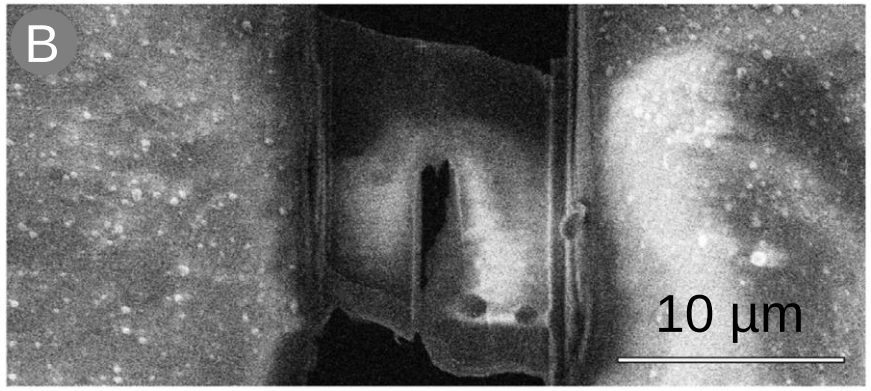


Wagner et al., Nature Protocols 2020

3.4.3 Micro-expansion joints technique

Cryo lamella bulking issue results in broken lamella

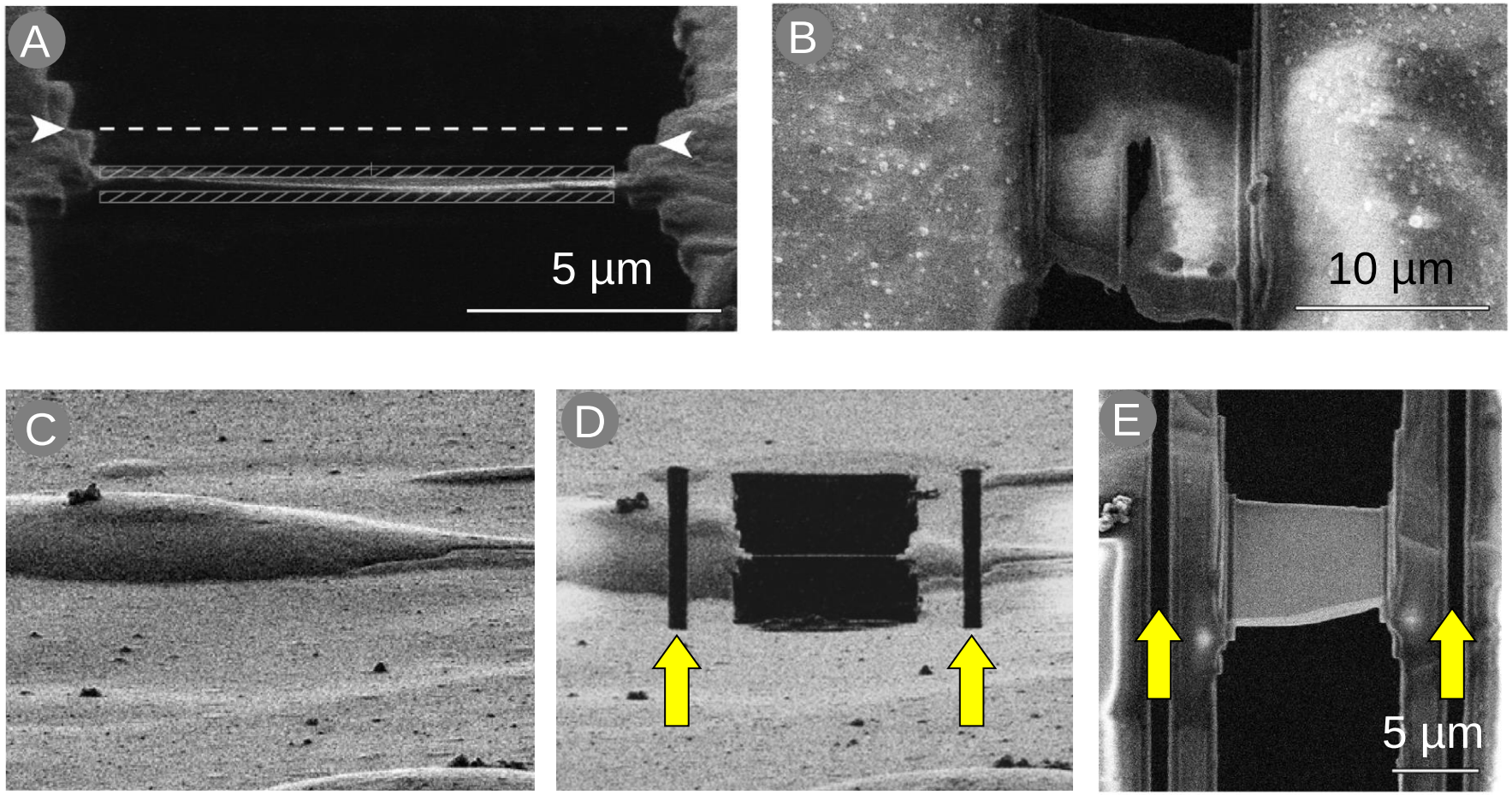




Wolff et al., JSB 2019

3.4.3 Micro-expansion joints technique

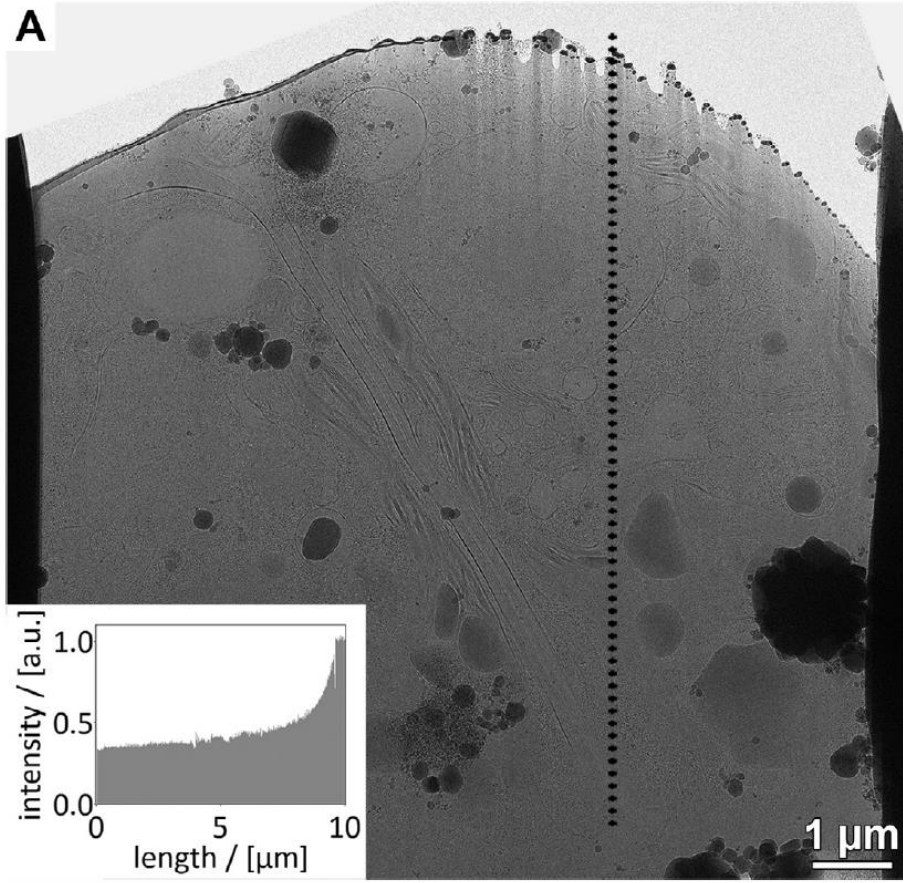
Micro-expansion joints address the cryo lamella bulking issue



Wolff et al., JSB 2019

3.4.4 Get more uniform thickness using the wedge pre-milling technique

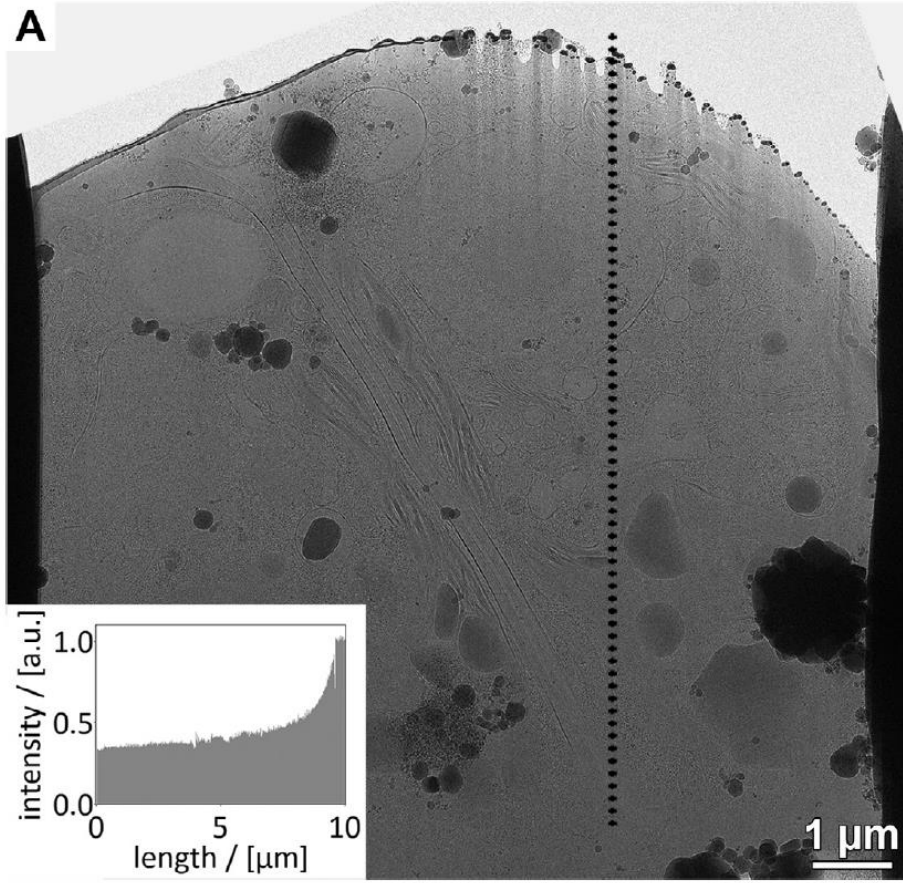
Regular milling

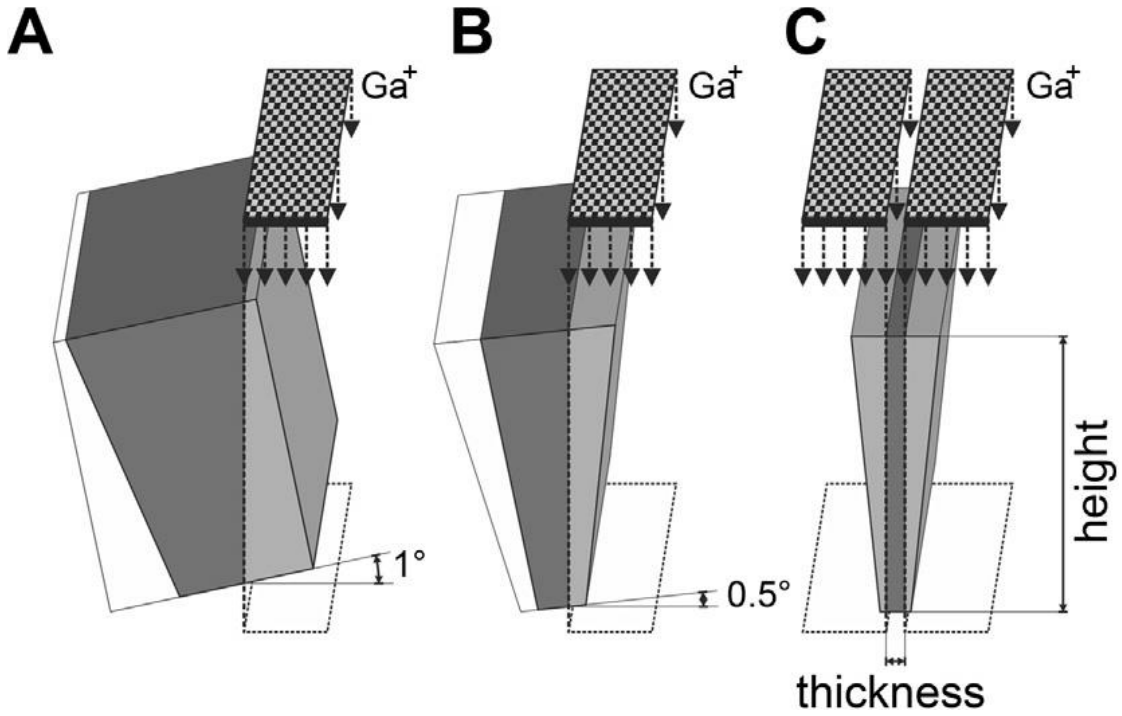


Schaffer et al., JSB 2017

3.4.4 Get more uniform thickness using the wedge pre-milling technique

Regular milling

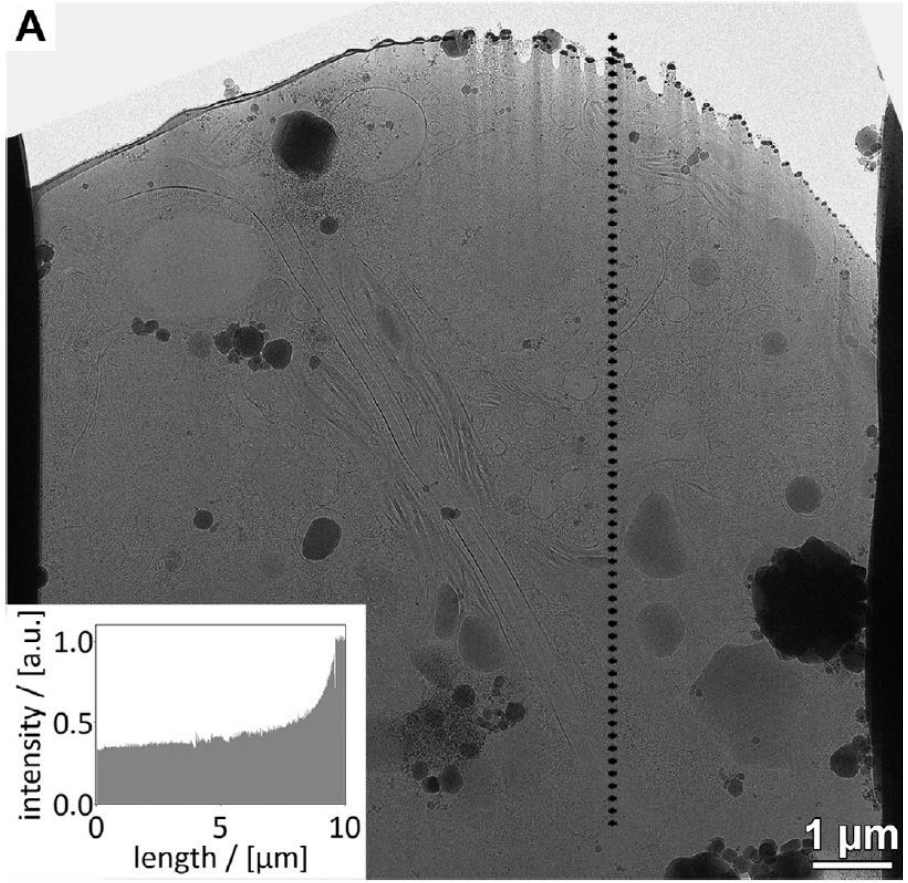




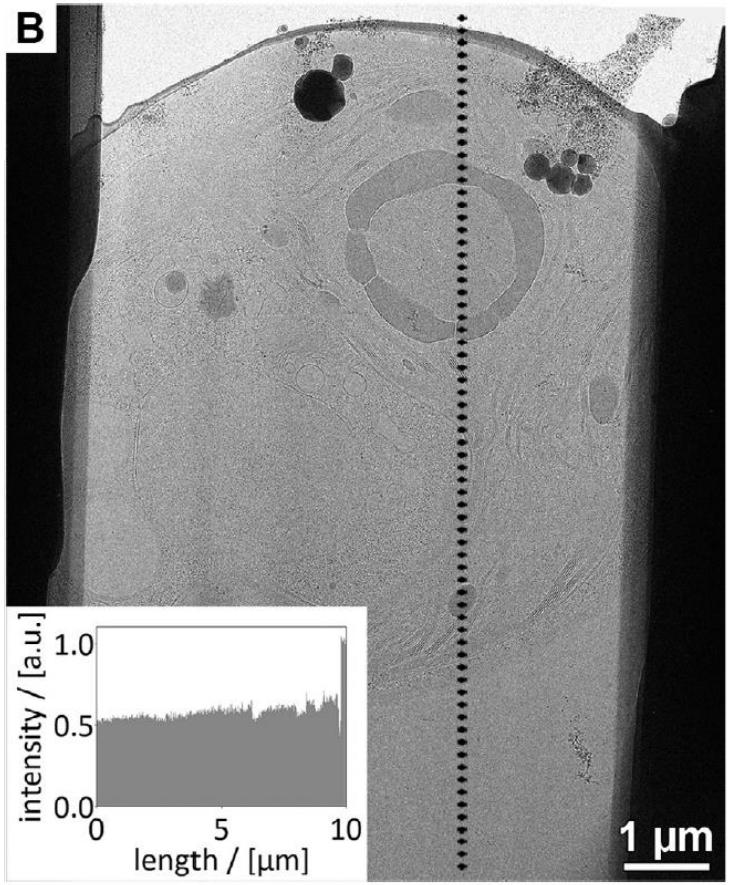
Schaffer et al., JSB 2017

3.4.4 Get more uniform thickness using the wedge pre-milling technique

Regular milling



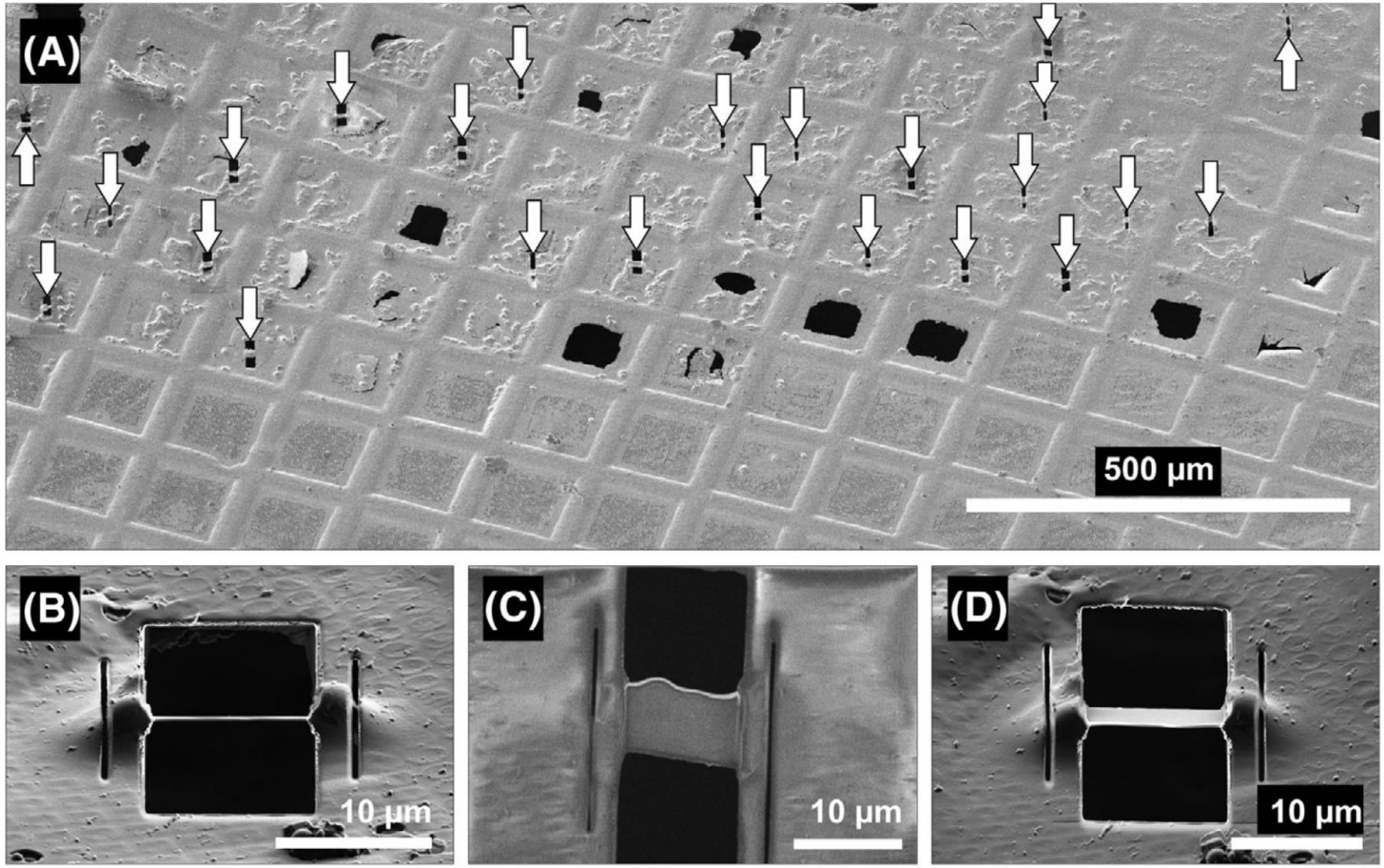
Wedge pre-milling



Schaffer et al., JSB 2017

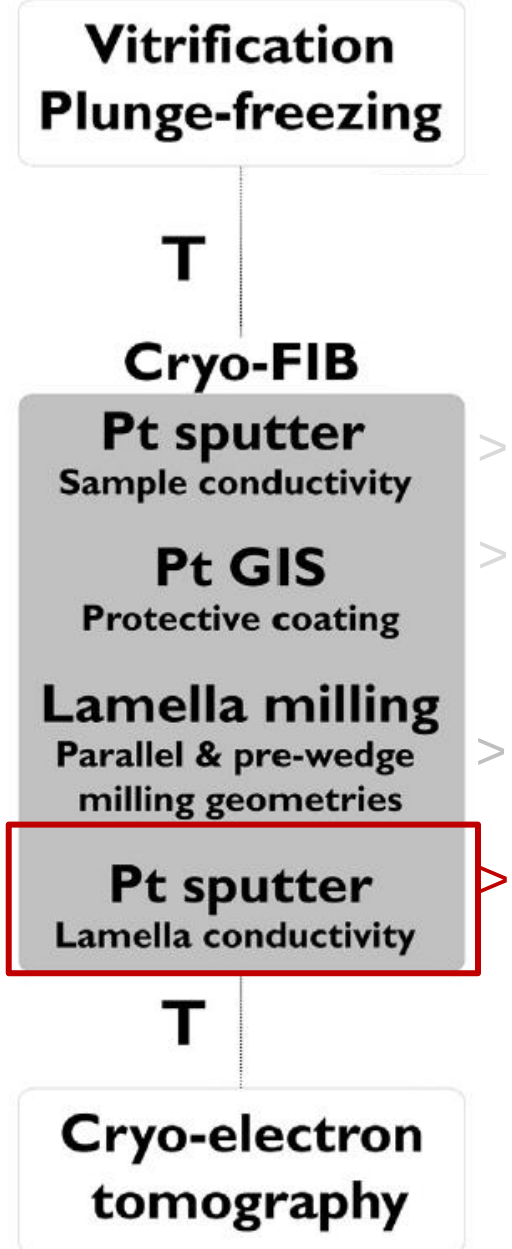
3.4.5 Batch lamella milling_e.g., using AutoTEM Cryo of Aquilos 2

3.4.5 Batch lamella milling using AutoTEM Cryo of Aquilos 2



Kuba et al., J Microsc. 2021

3.4 Cryo-FIB mining with DualBeam system



> (Optional) minimize charging; ensure targeting and precise milling.

> Protect the sample surface during milling; reduces curtaining and the redeposition of milled material.

> Rough milling, thinning and cleaning for thin lamella.

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

Schaffer et al., JSB 2017

3.4 Cryo-FIB mining with DualBeam system

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

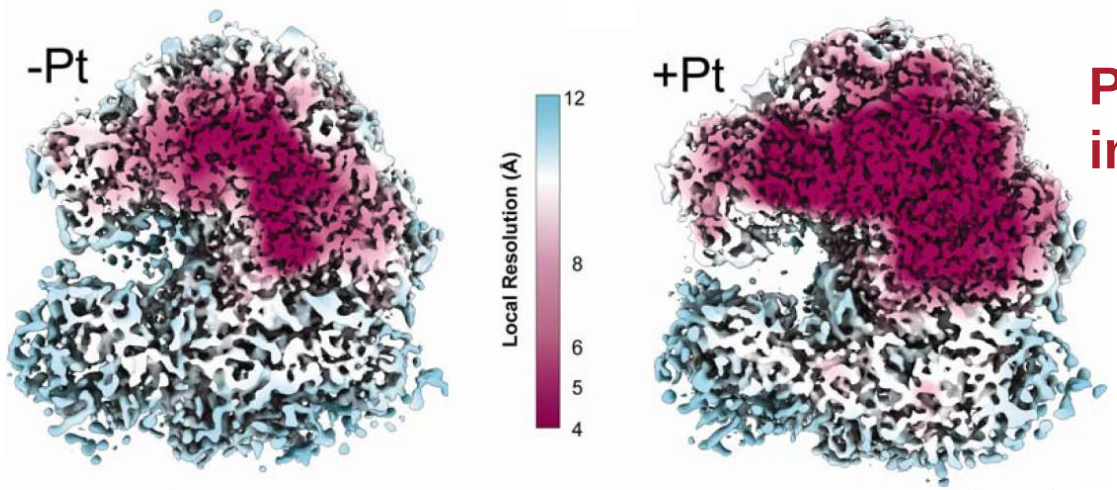
Lamella milling
Parallel & pre-wedge milling geometries

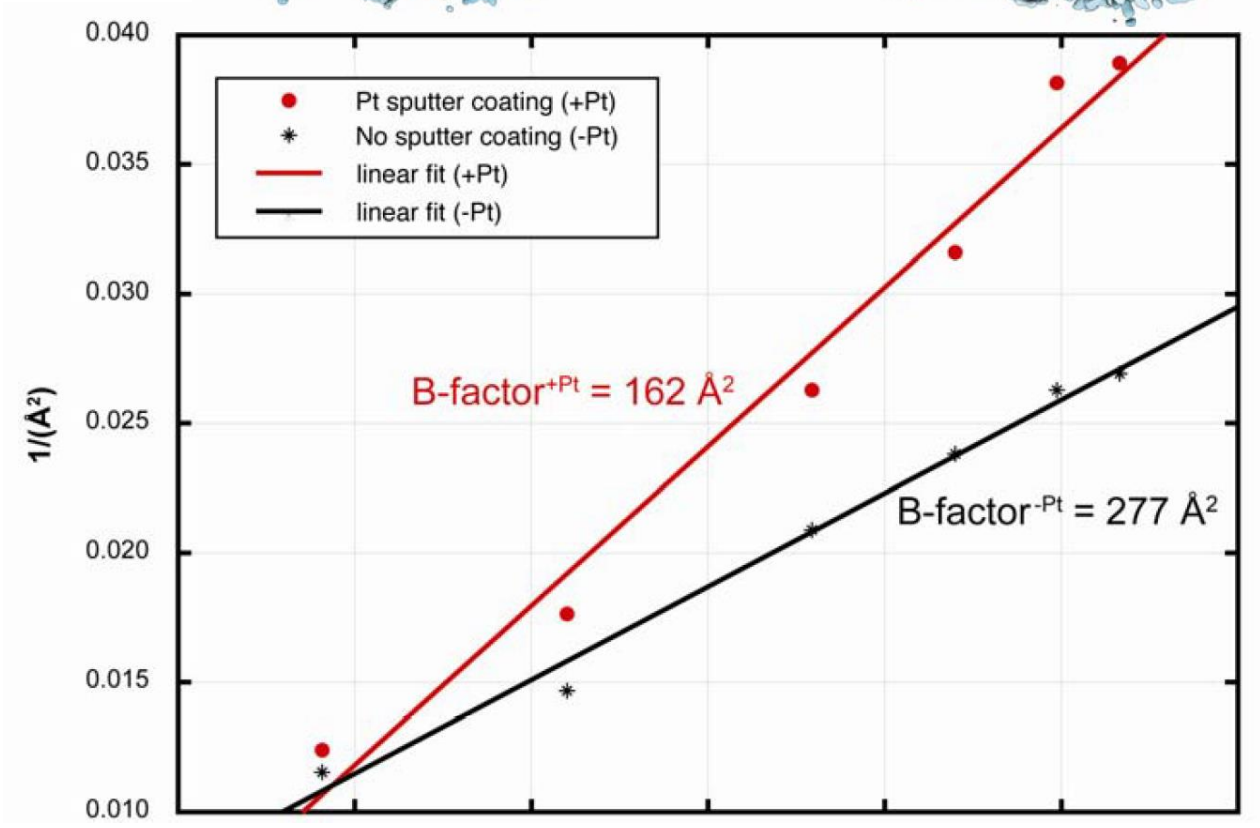
Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017





Pt sputter coating improves B-factors.

Khavnekar et al., bioRxiv. 2022

3.5 Main challenges in cryo lamella preparation

- Precisely **targeting** the structure of interest
- Keeping the lamella **free from contamination**
- Keeping the lamella **Intact**
- Apply **fiducial markers** on the lamella
- Laborous especially for thinning **bulk specimens** (**lift-out technique**)
- **Time consuming**

Main contents

1. Specimens accessible by cryoET

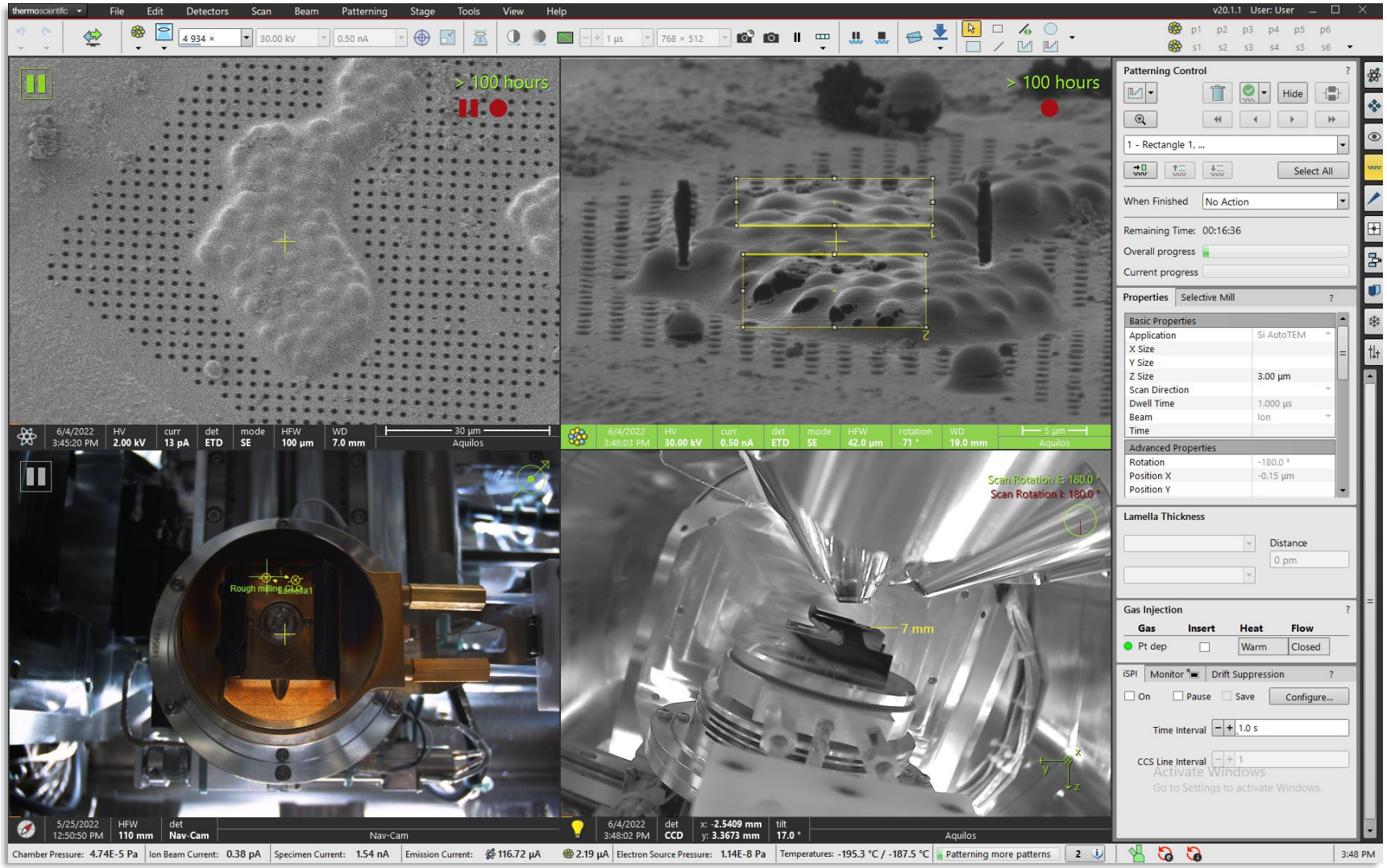
2. Four considerations for cryoET sample preparation

3. Workflow of cryo lamellae preparation

4. Tutorial of major steps of cryo lamella preparation with Aquilos 2

Software packages used in this tutorial

In Aquilos 2 PC: xT Microscope Control v20.1.1

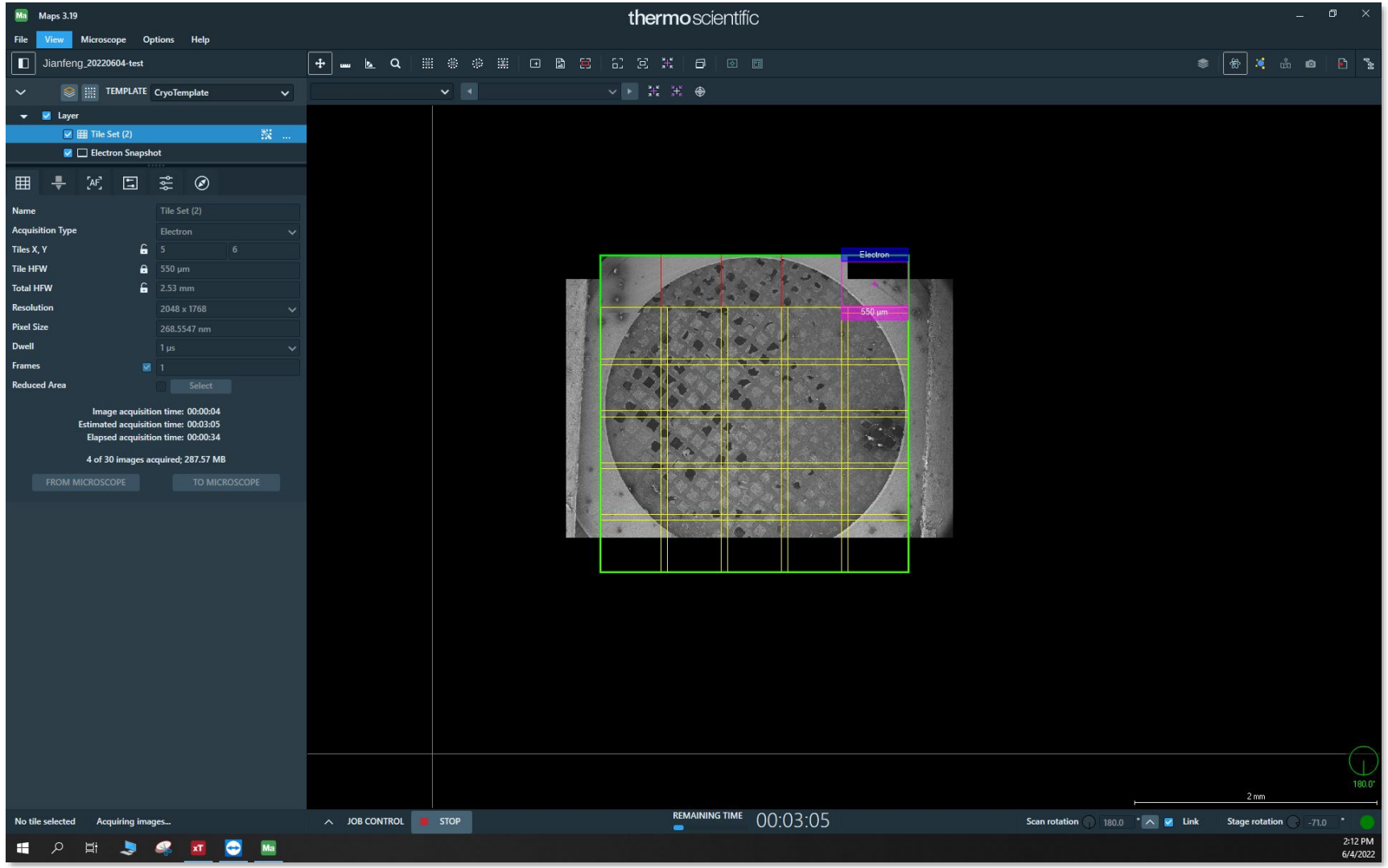


Software packages used in this tutorial

In Aquilos 2 PC: xT Ma

Maps 3.19

- Create grid atlas
- Add lamella sites
- Align LM & SEM（optional）
- Eucentric height & tilt calculation（optional）

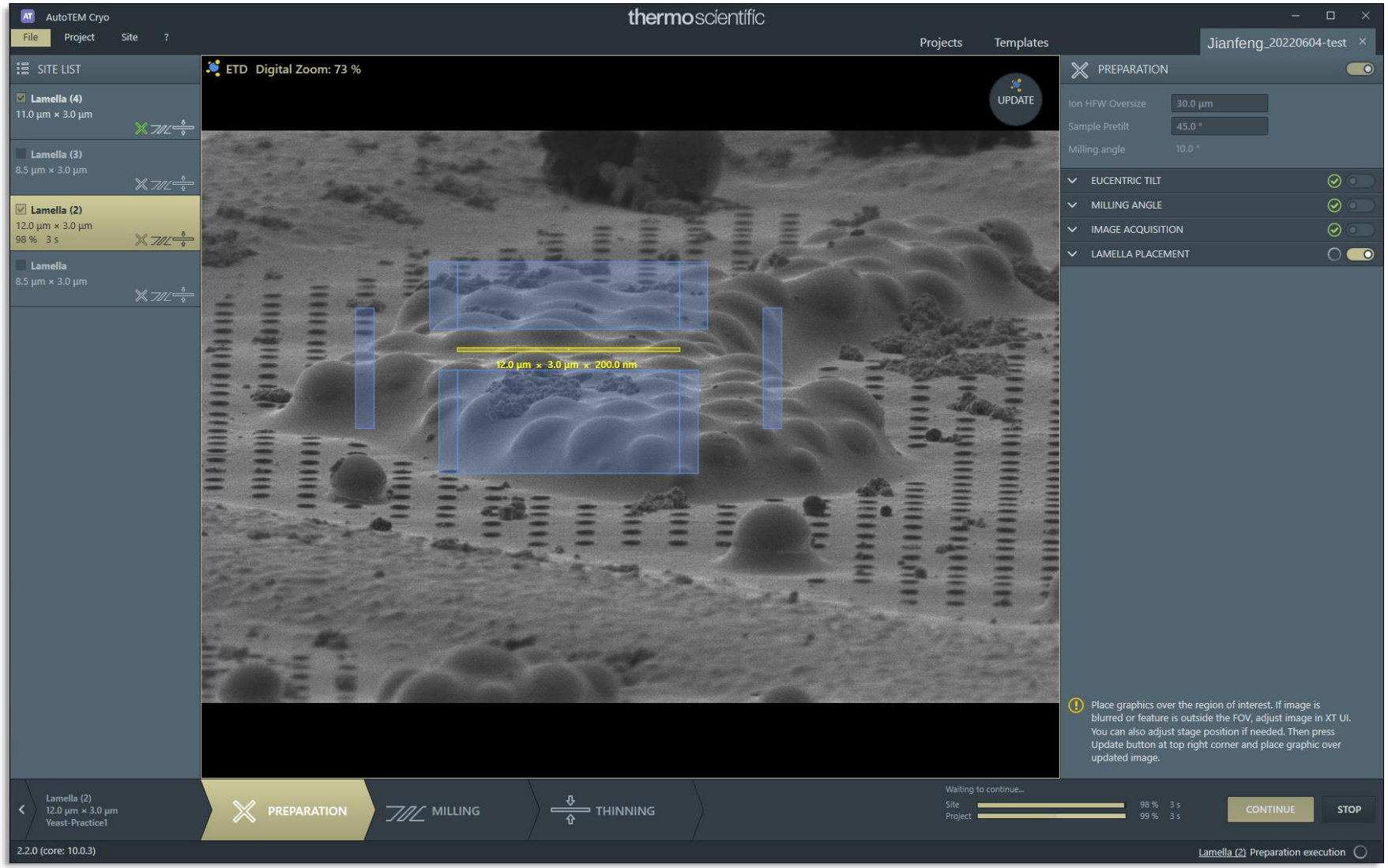


Software packages used in this tutorial

In Aquilos 2 PC: xT Ma AT

AutoTEM Cryo 2.2

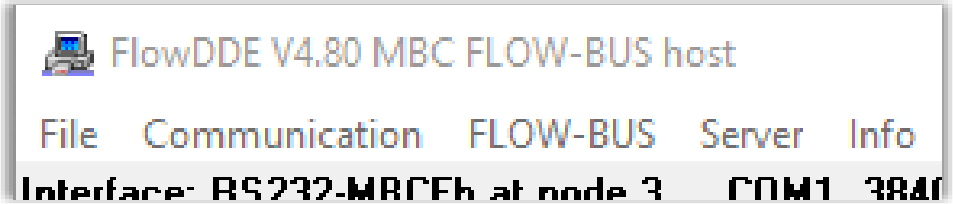
- Eucentric height & tilt calculation（optional）
- Micro-expansion joints
- The wedge pre-milling technique
- Batch & Stepwise lamella milling

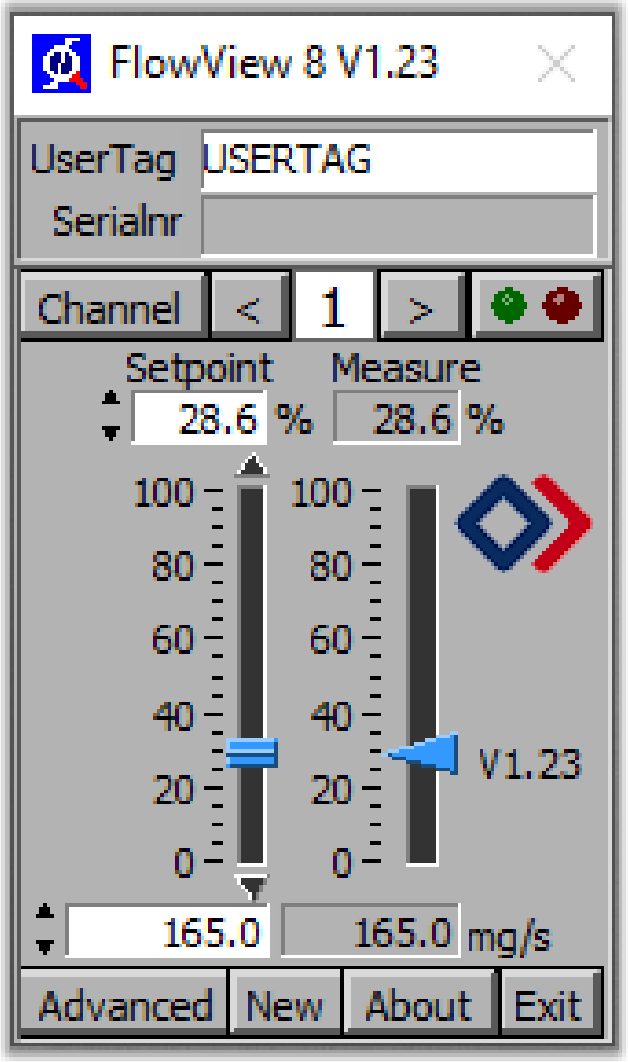


Software packages used in this tutorial

In Aquilos 2 PC: xT Ma AT

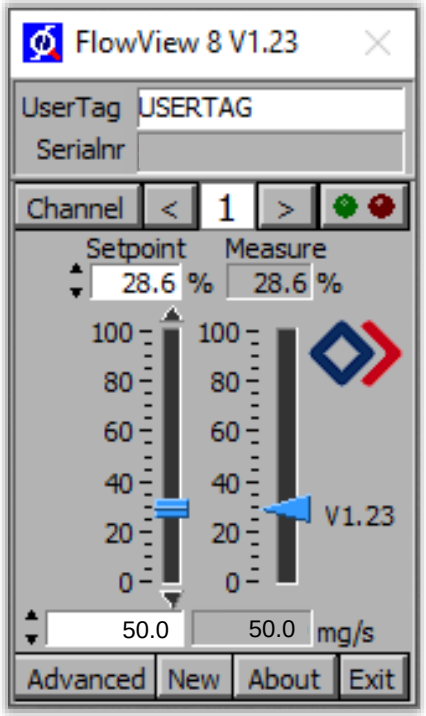
In Support PC

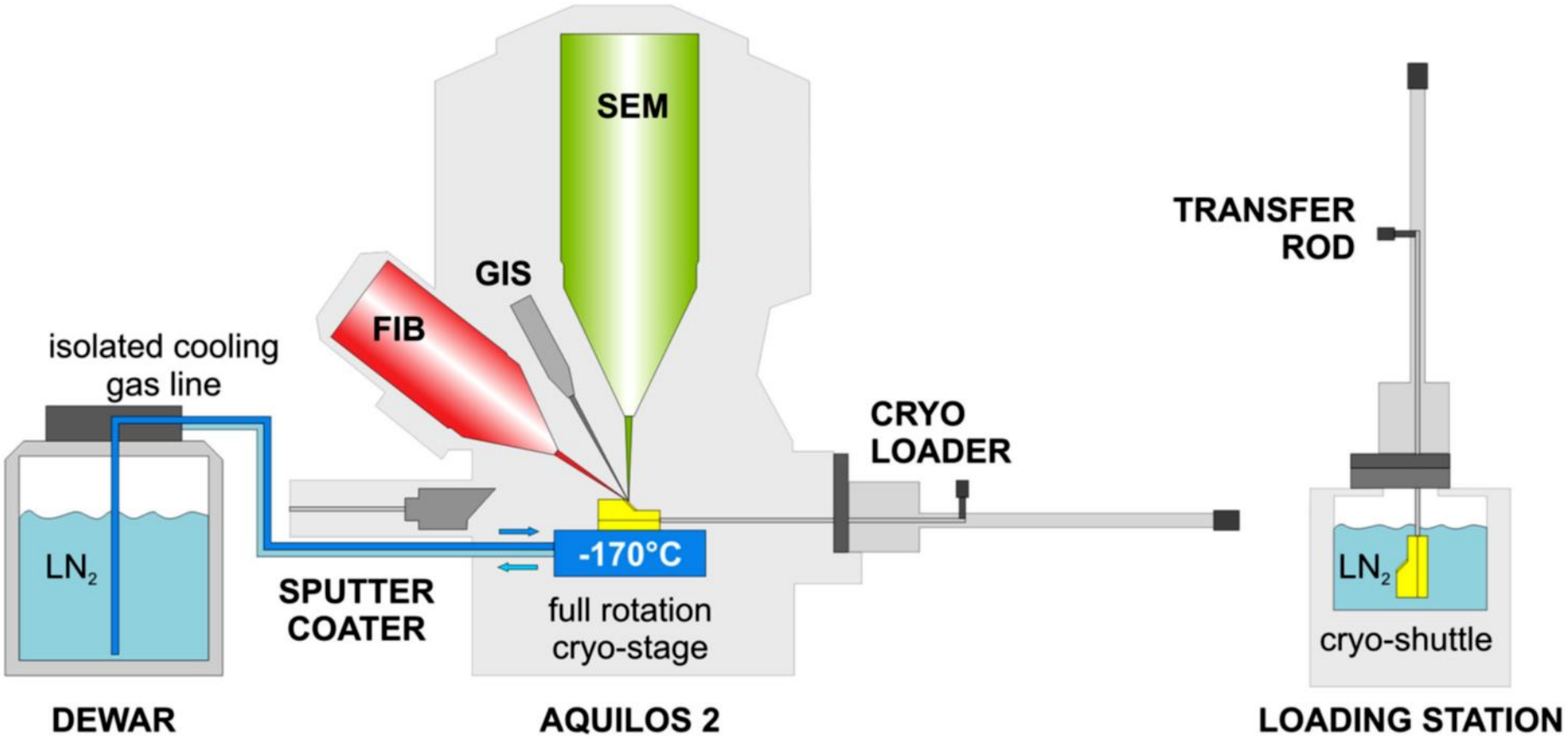




4.1 Preparation the system

➢ **Purge the system:** Cooling gas line, Loading station (>0.5h)

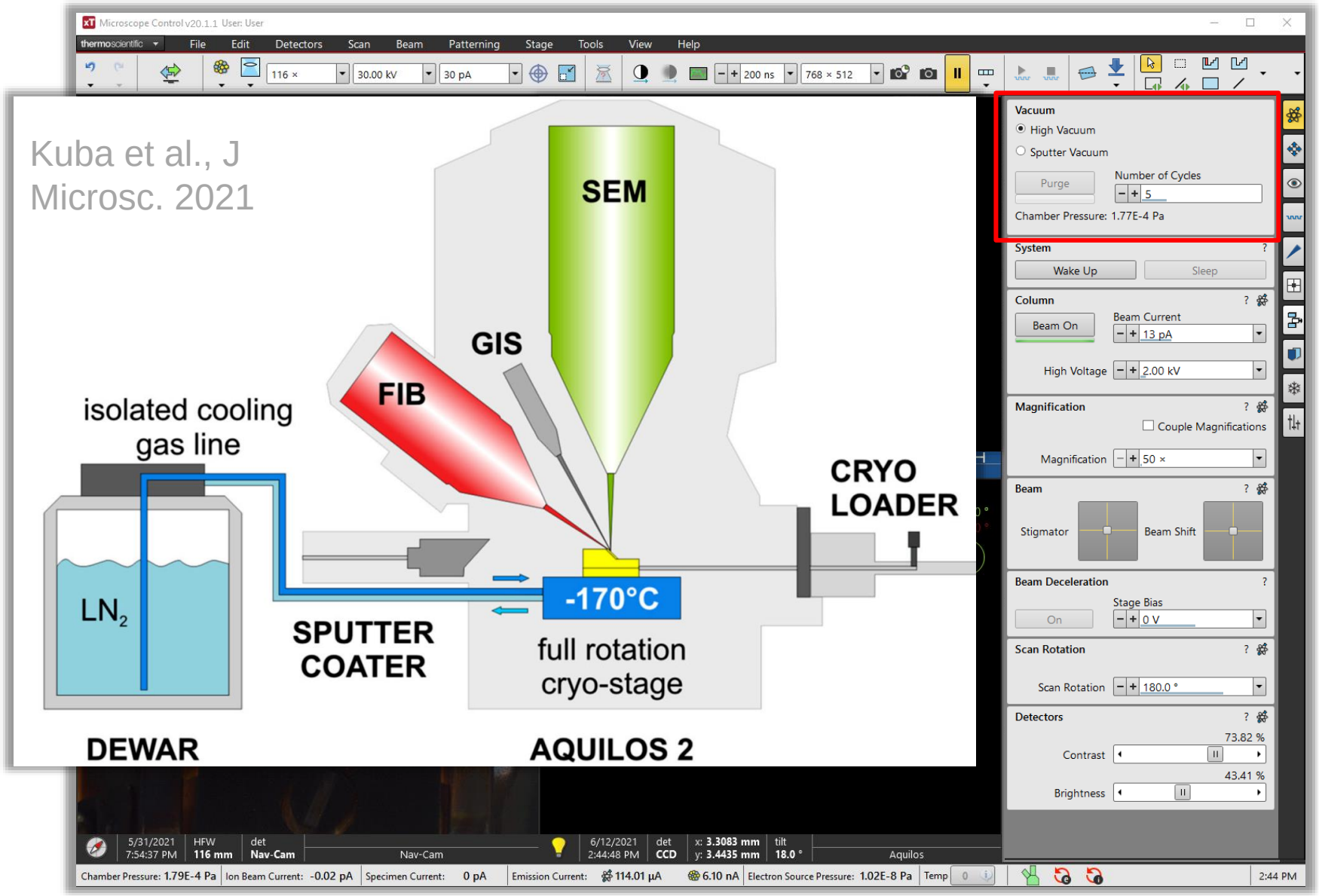




Kuba et al., J Microsc. 2021

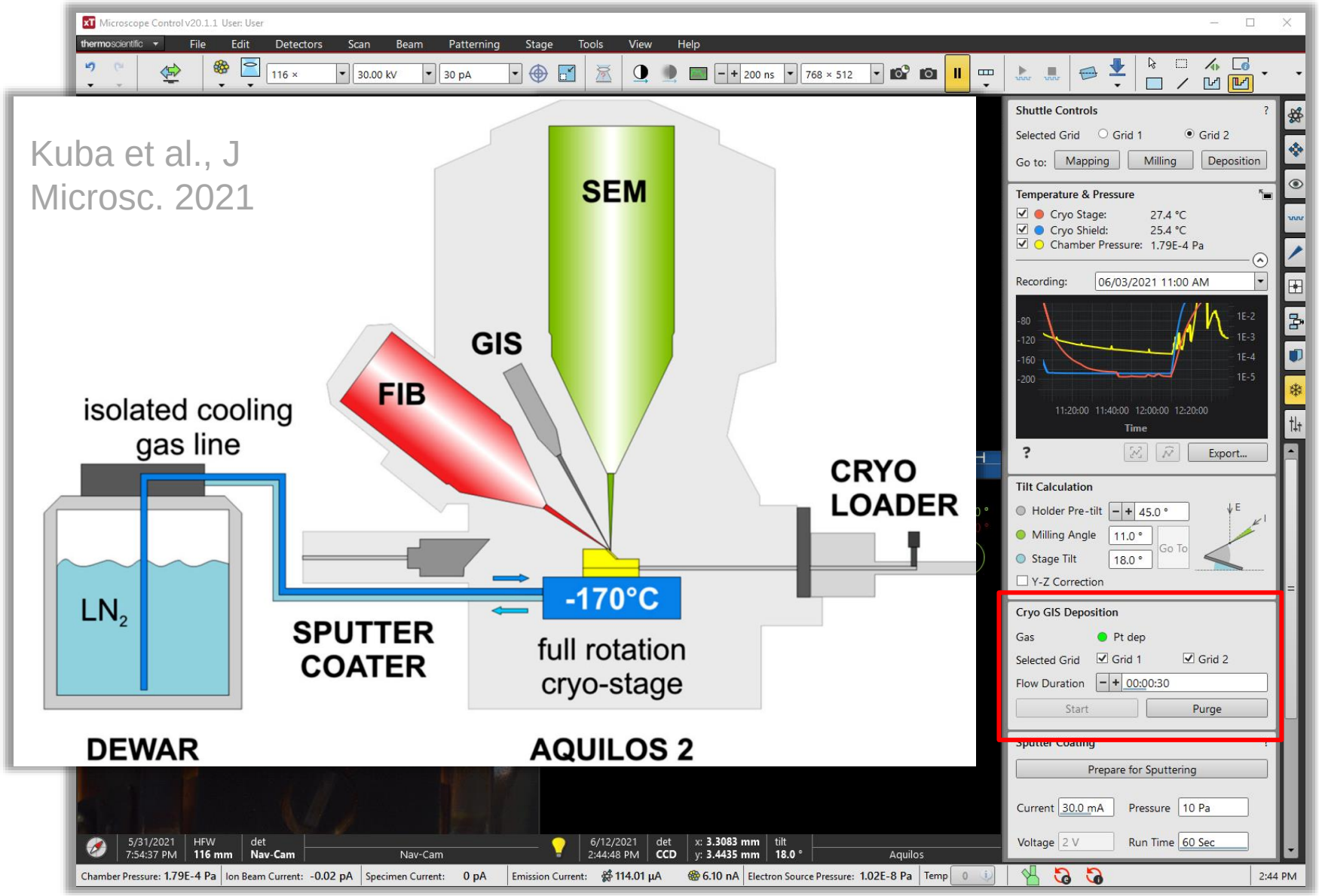
4.1 Preparation the system

- **Purge the system:** Cooling gas line, Loading station, Argon line (5 cycles)



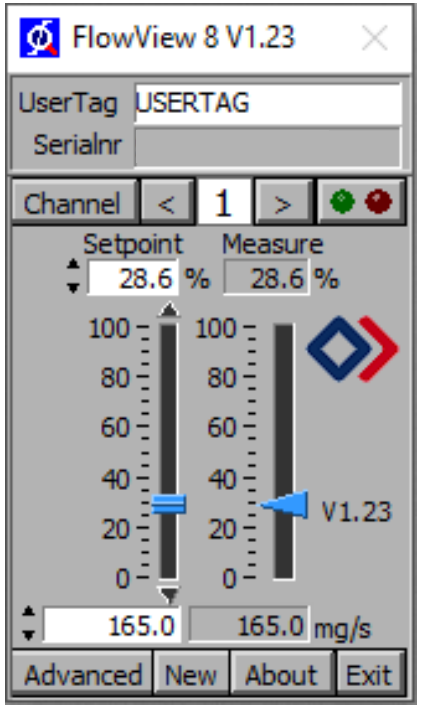
4.1 Preparation the system

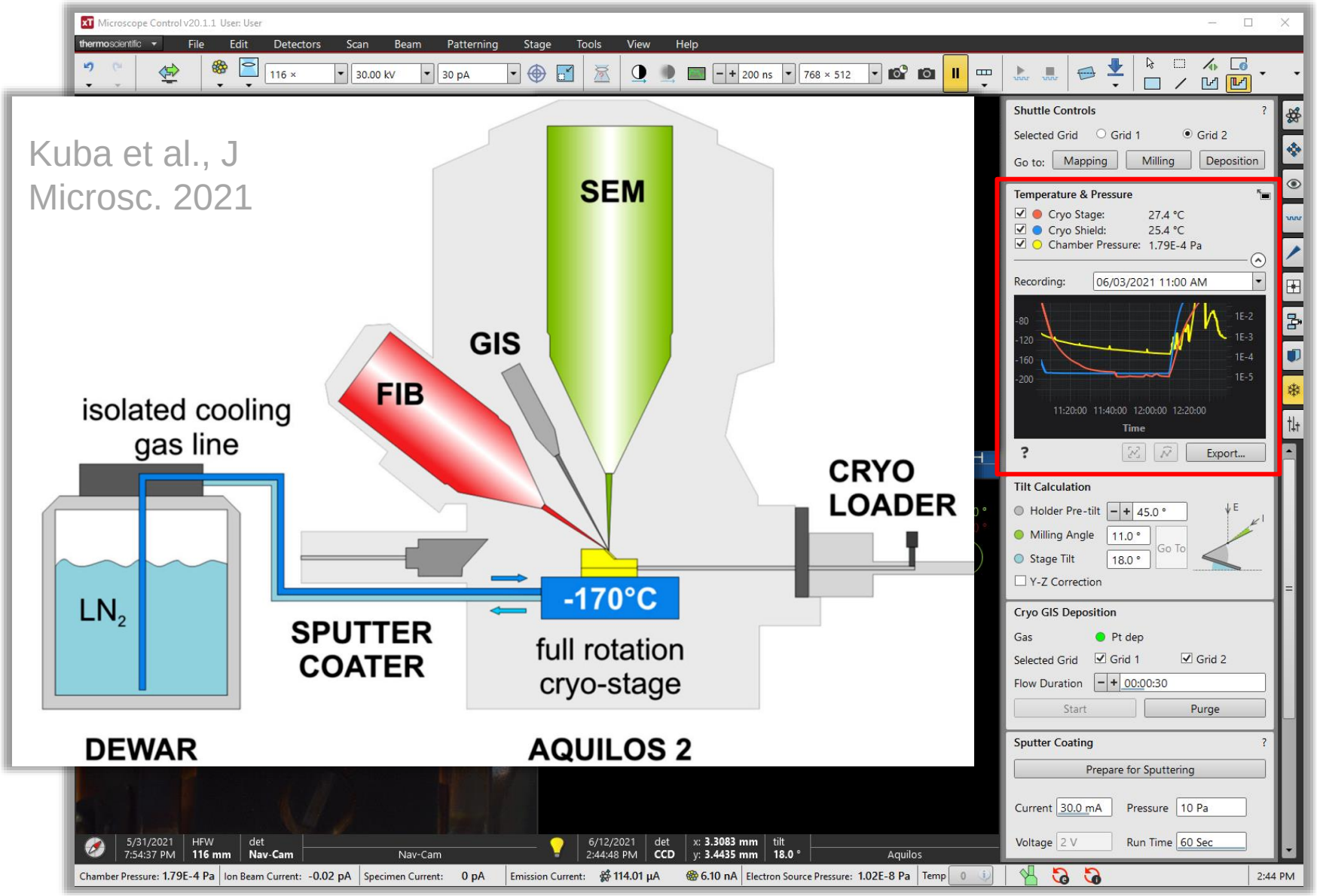
- **Purge the system:** Cooling gas line, Loading station, Argon line, GIS (2 mins).



4.1 Preparation the system

➢ **Cool down the system.**



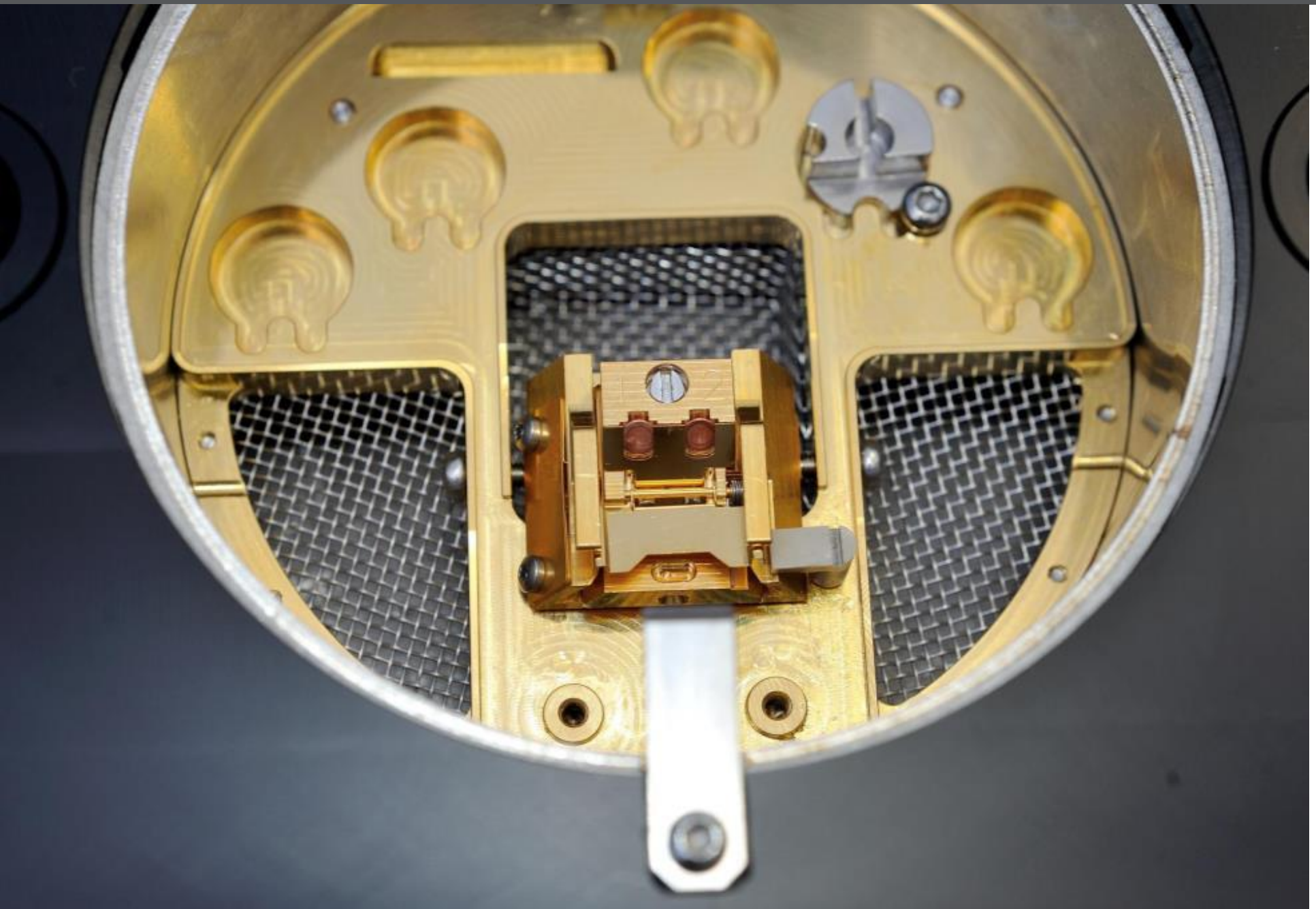


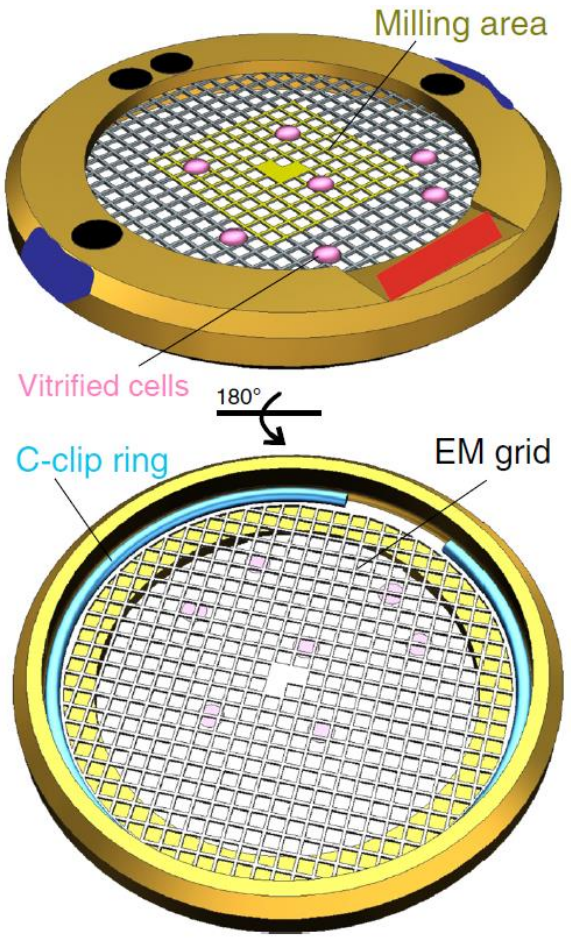
4.1 Preparation the system

- **Cool down the system.**

Jakub Kuba at Brno; TFS

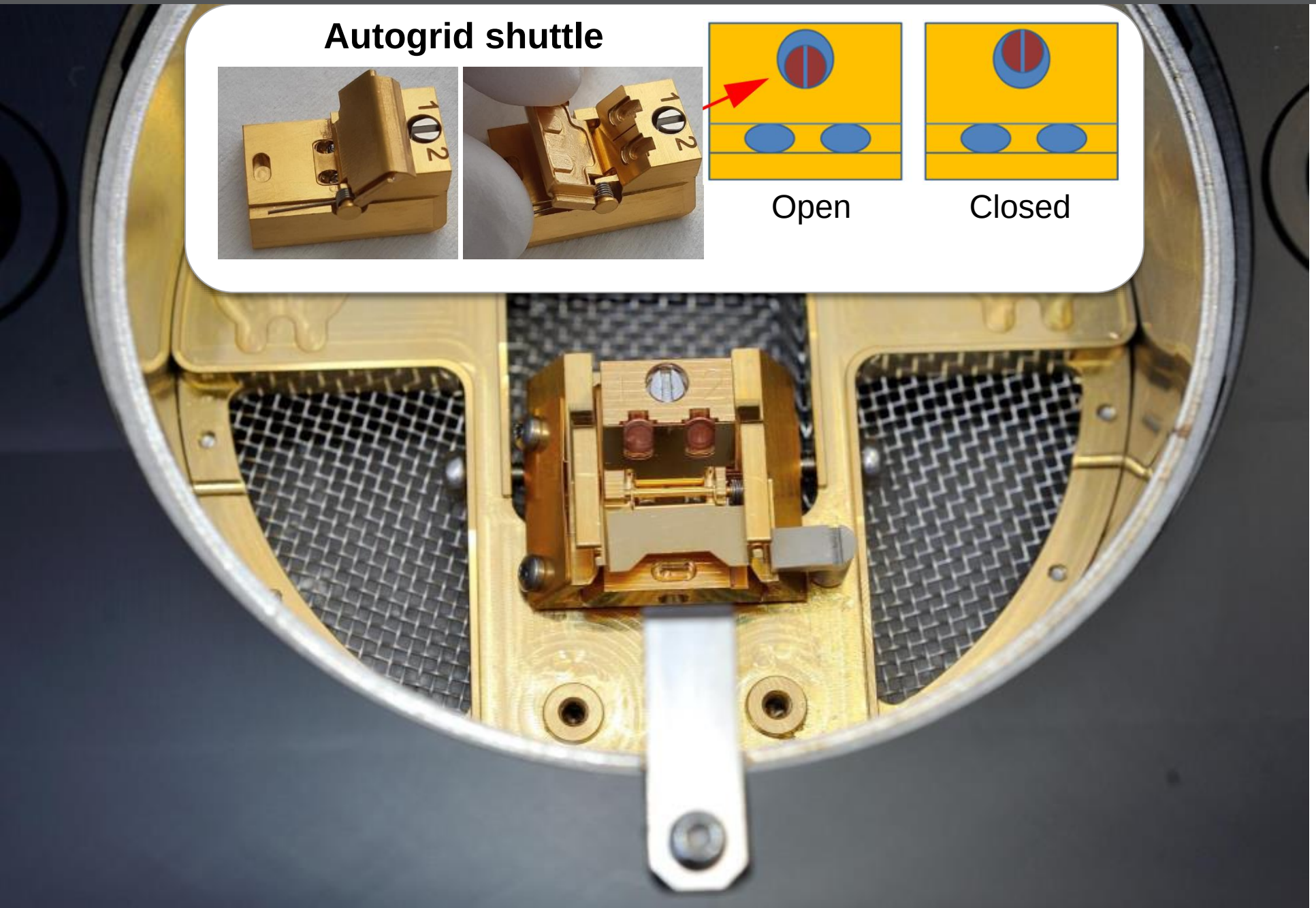
4.2 Clip grids



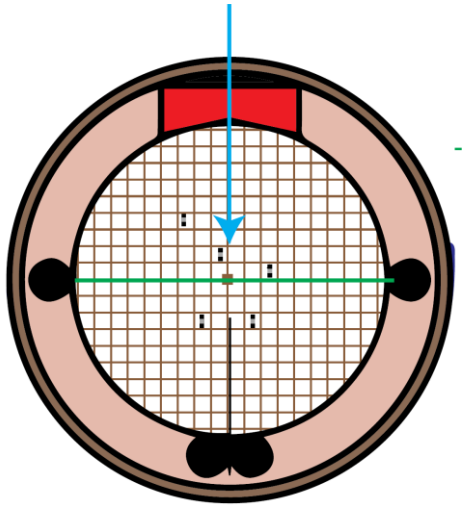


TFS; Wagner et al., Nature Protocols 2020

4.3 Transfer grids to Aquilos 2_Load grids to Autogrid shuttle

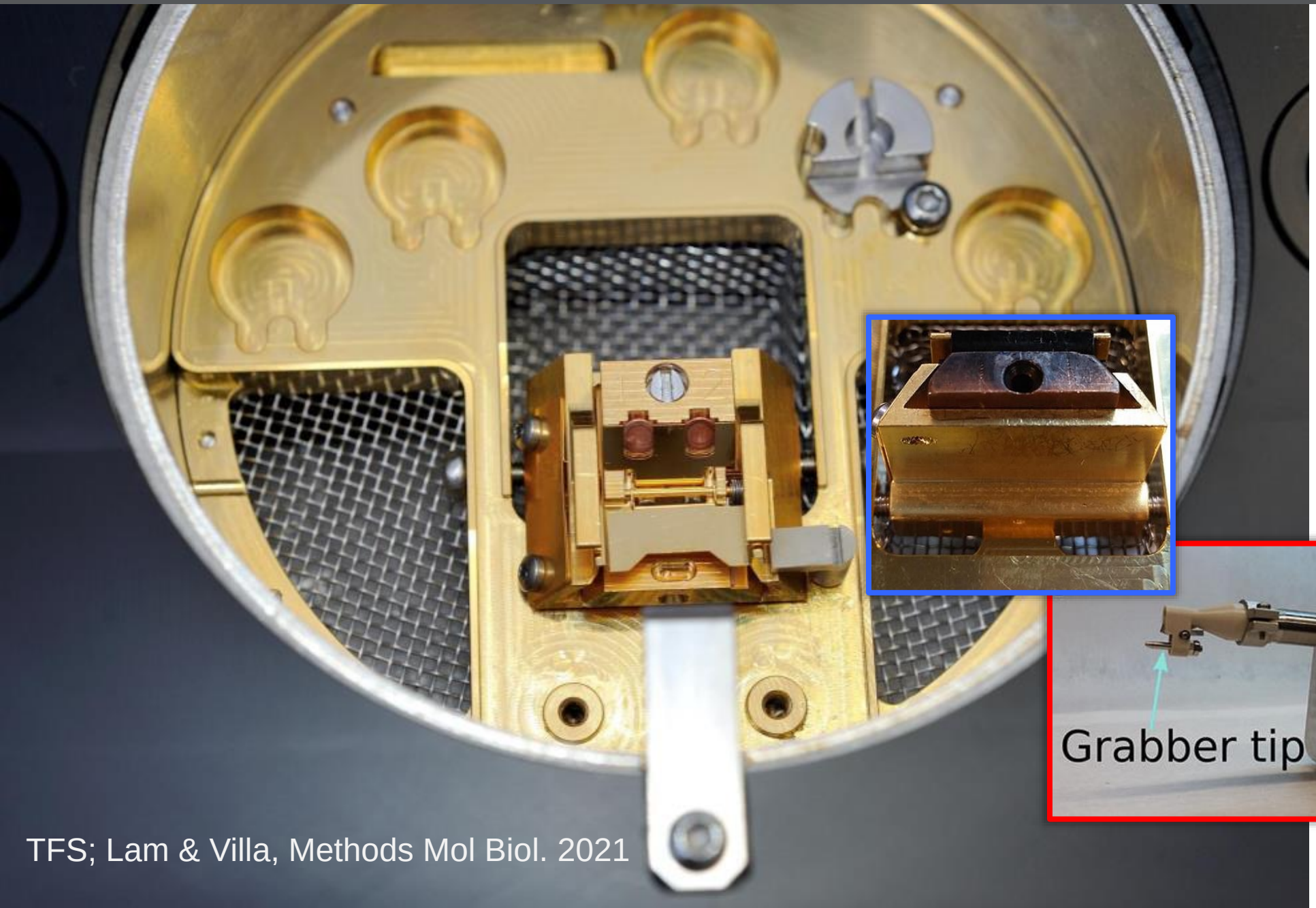


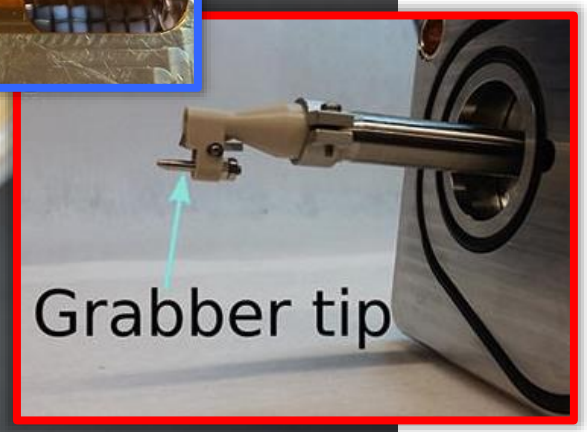
Milling direction



TFS; Wagner et al., Nature Protocols 2020

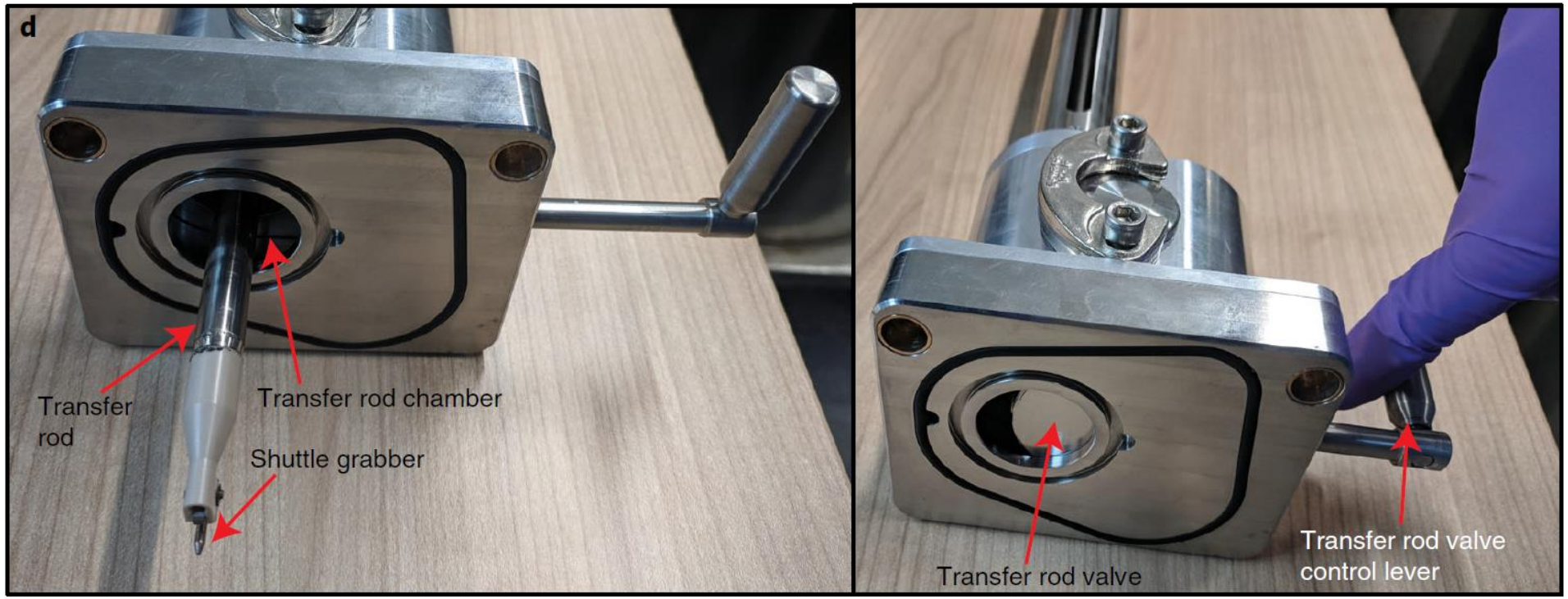
4.3 Transfer grids to Aquilos 2_Load shuttle to Transfer rod

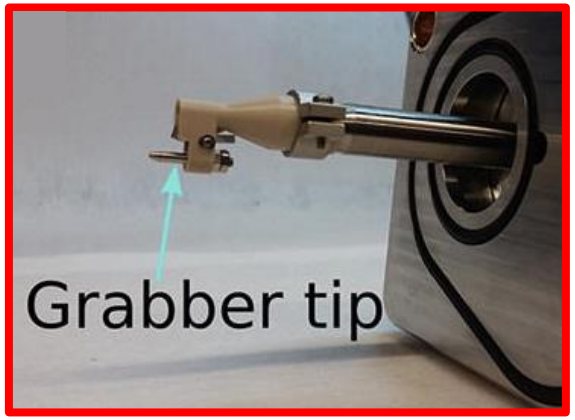




TFS; Lam & Villa, Methods Mol Biol. 2021

4.3 Transfer grids to Aquilos 2_Load shuttle to Transfer rod





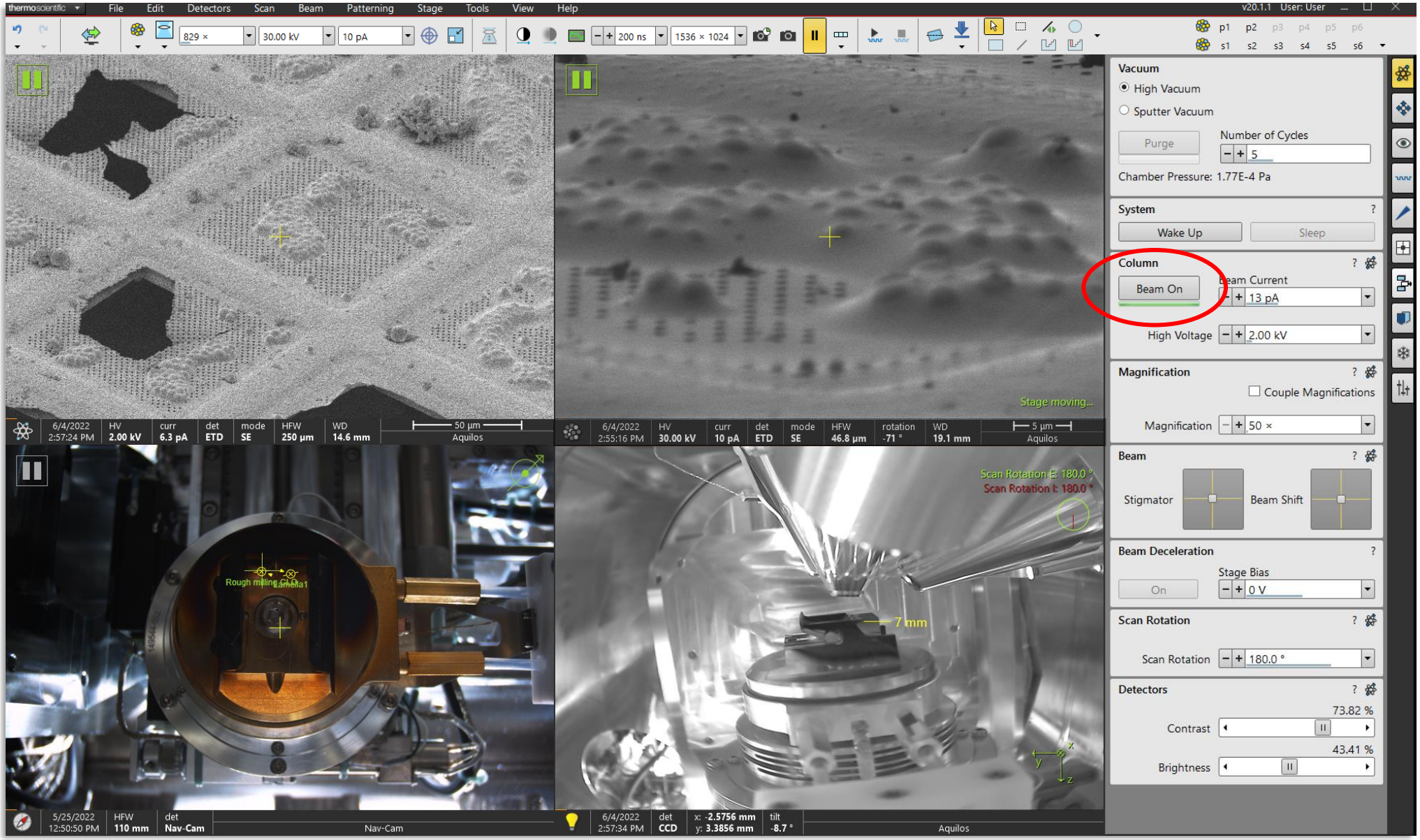
TFS; Lam & Villa, Methods Mol Biol. 2021; Wagner et al., Nature Protocols 2020

4.3 Transfer grids to Aquilos 2

(Video from TFS)

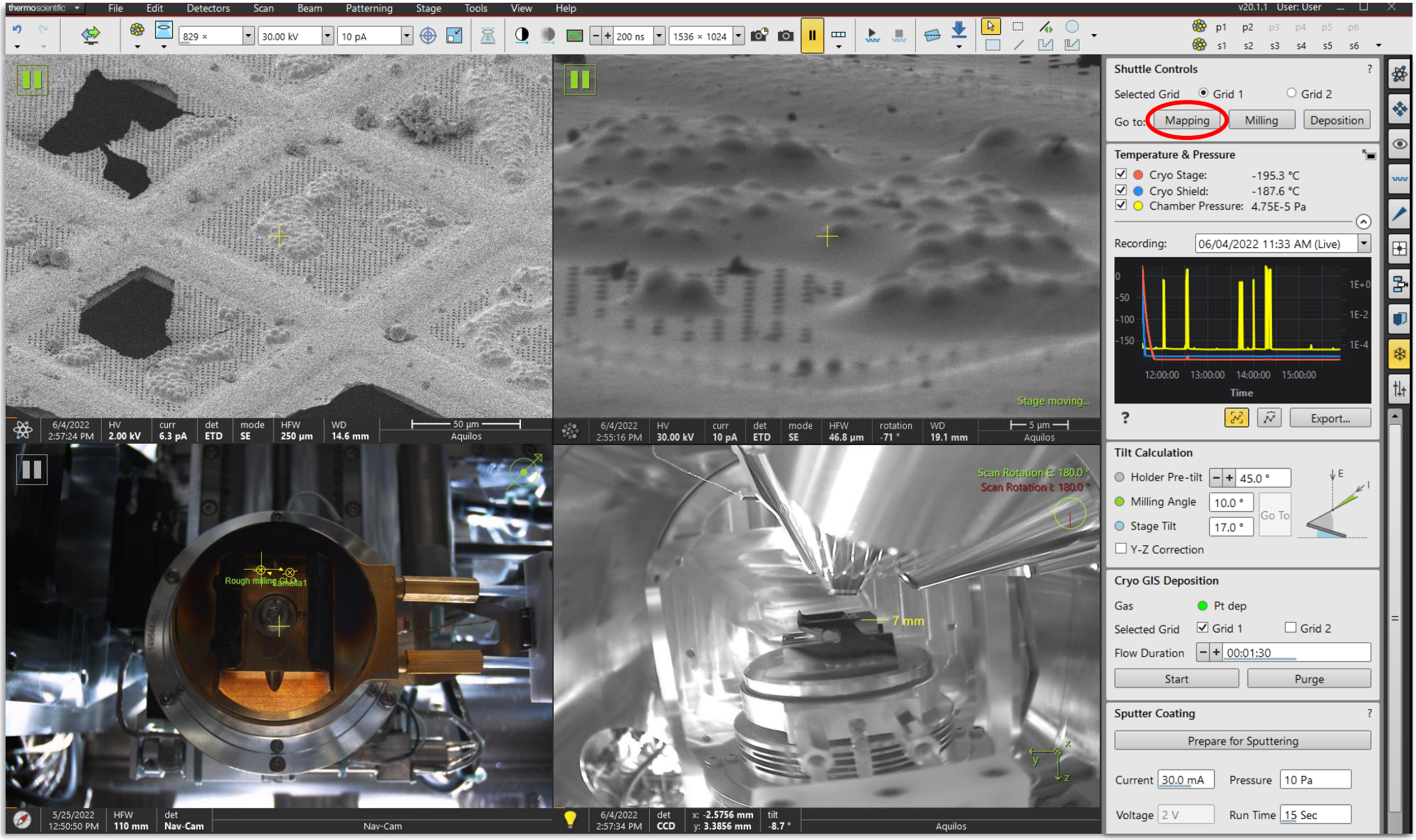
4.4 Acquire grid atlas_ setup E-beam imagining conditions

xT



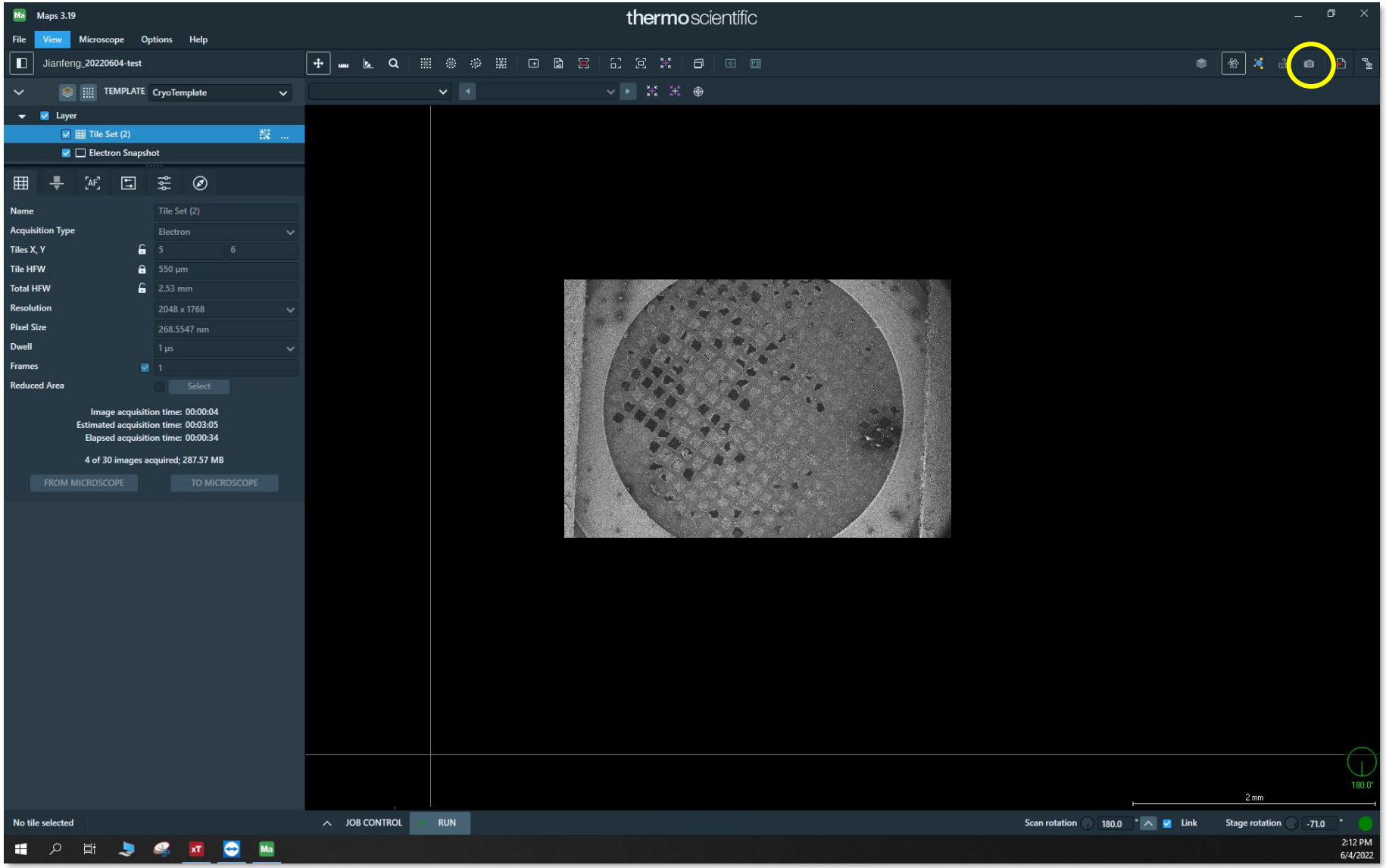
4.4 Acquire grid atlas_ setup E-beam imagining conditions

xT



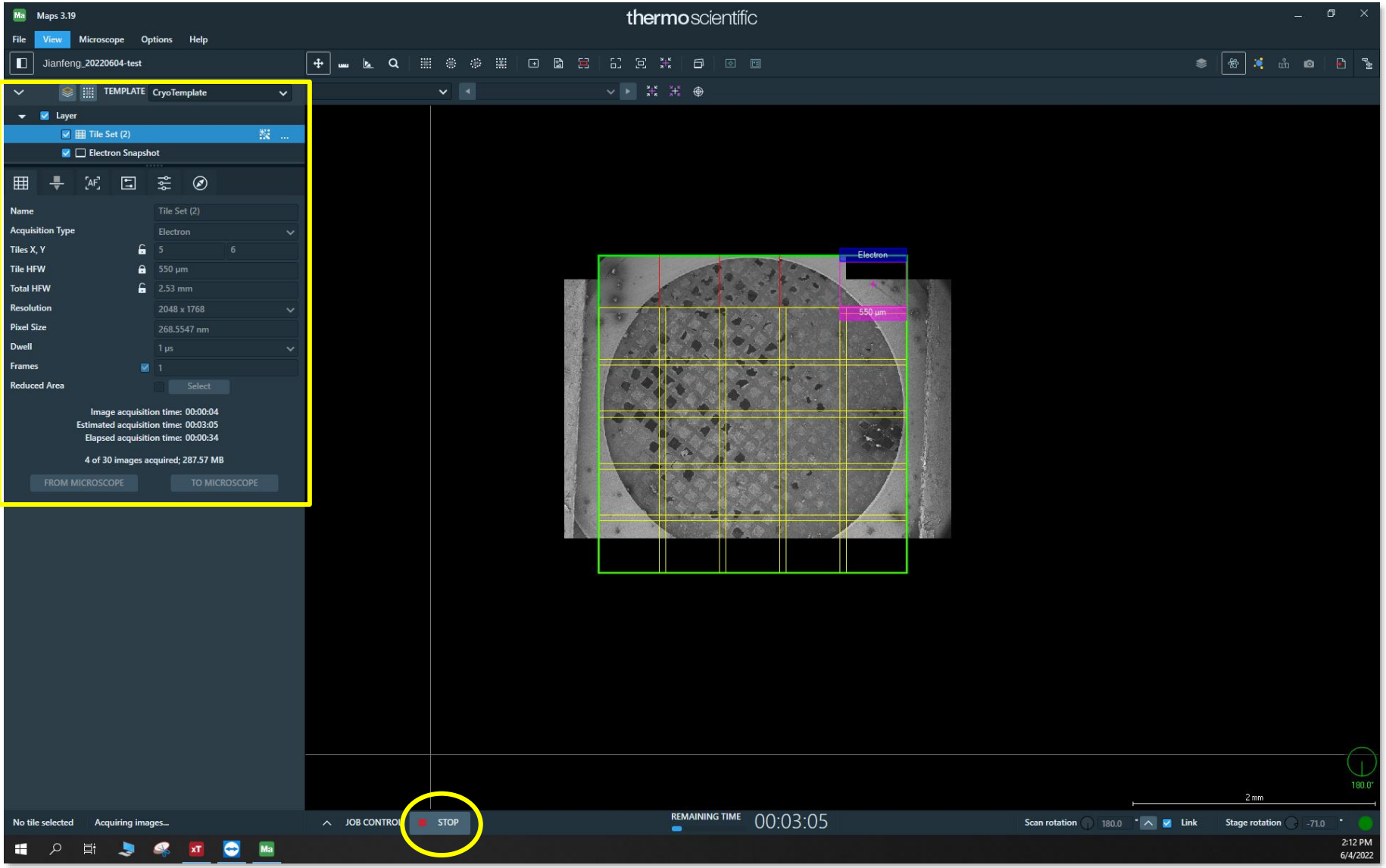
4.4 Acquire grid atlas _ take a snapshoot of the grid

Maps



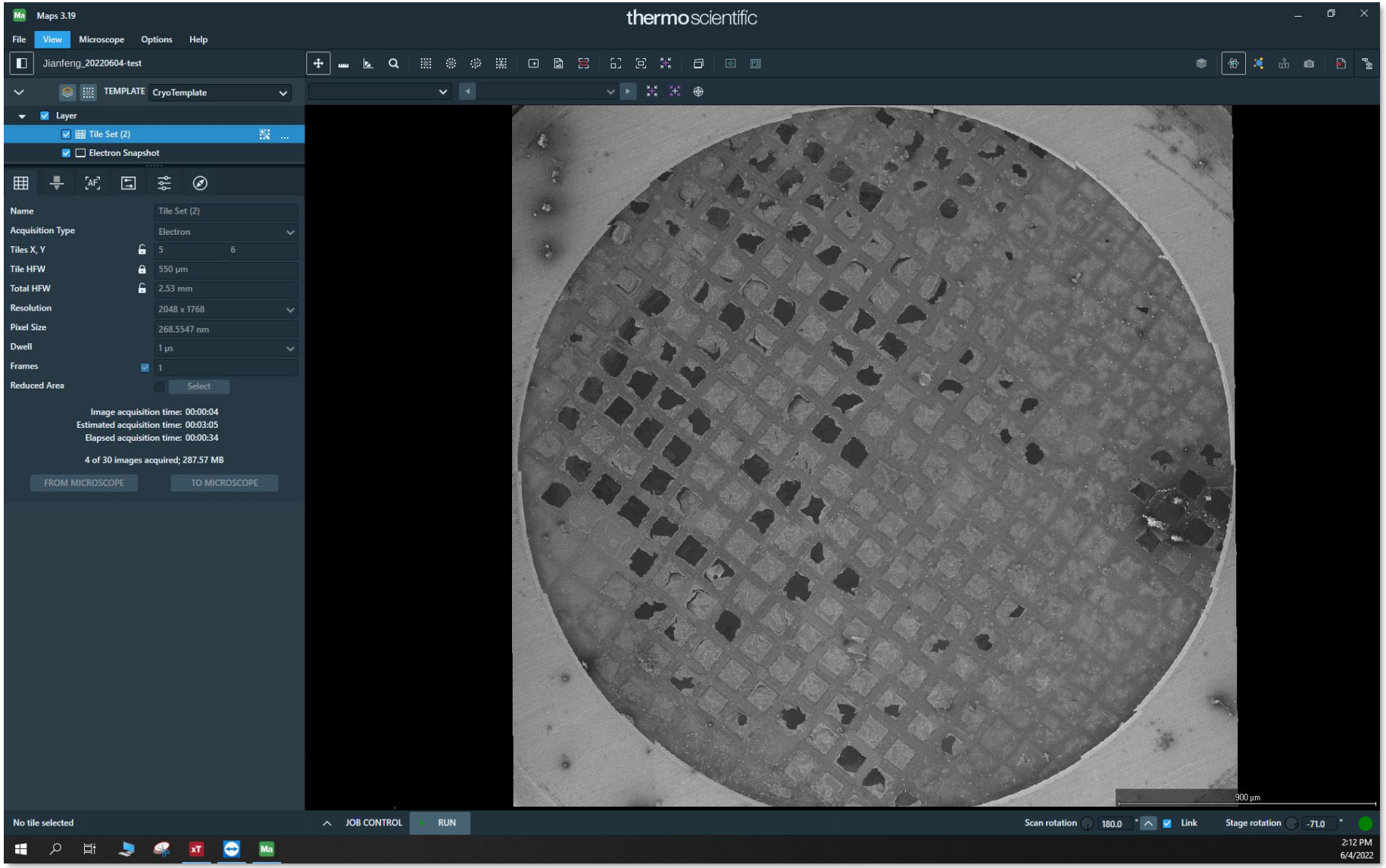
4.4 Acquire grid atlas _ set up Tile Set and Run acquisition

Maps



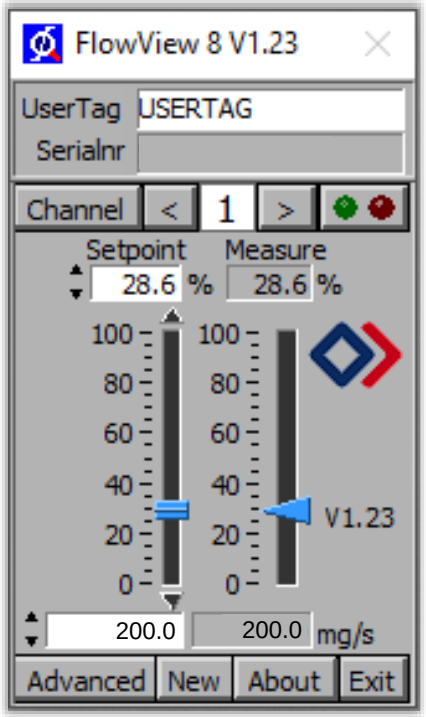
4.4 Acquire grid atlas

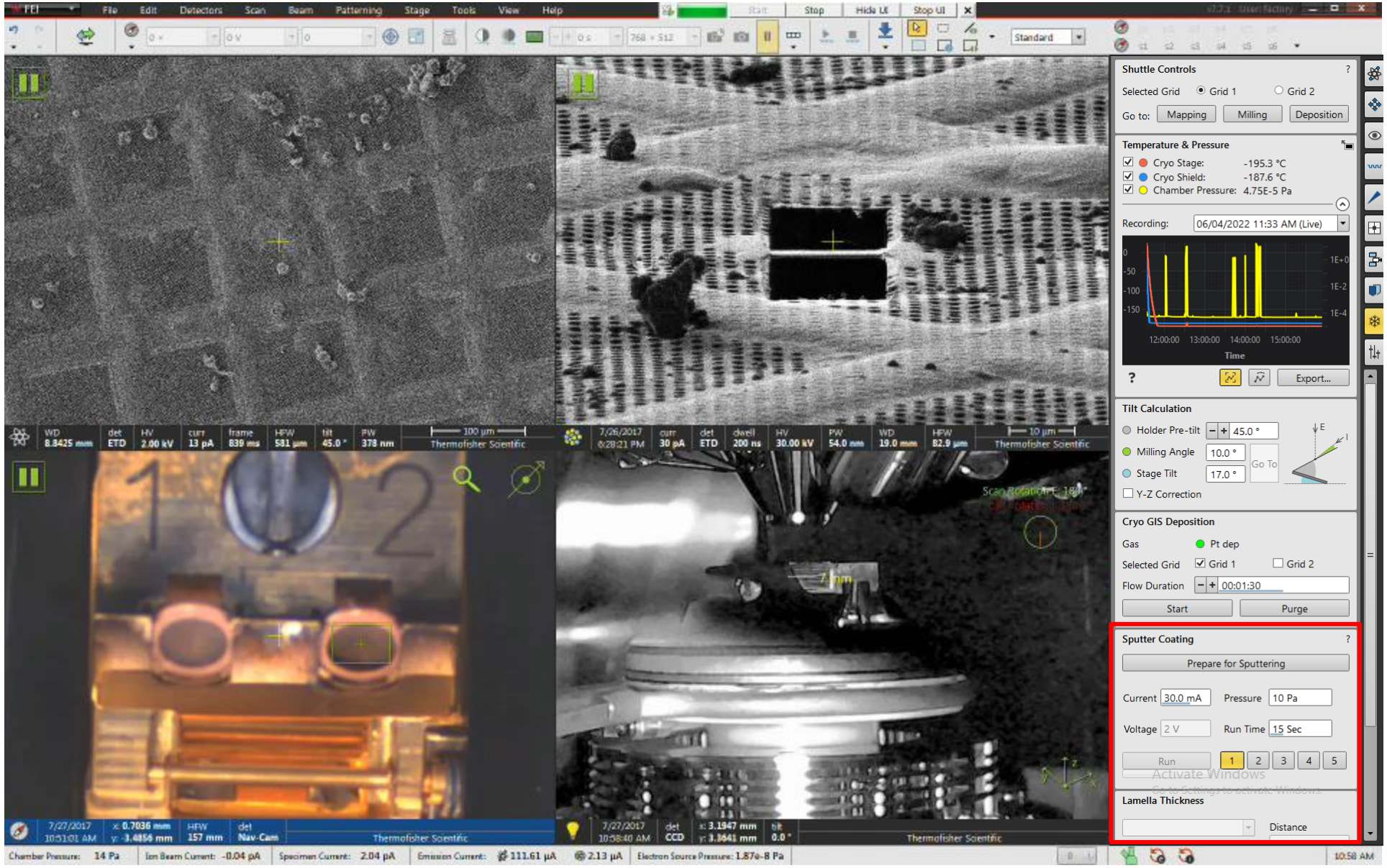
Maps



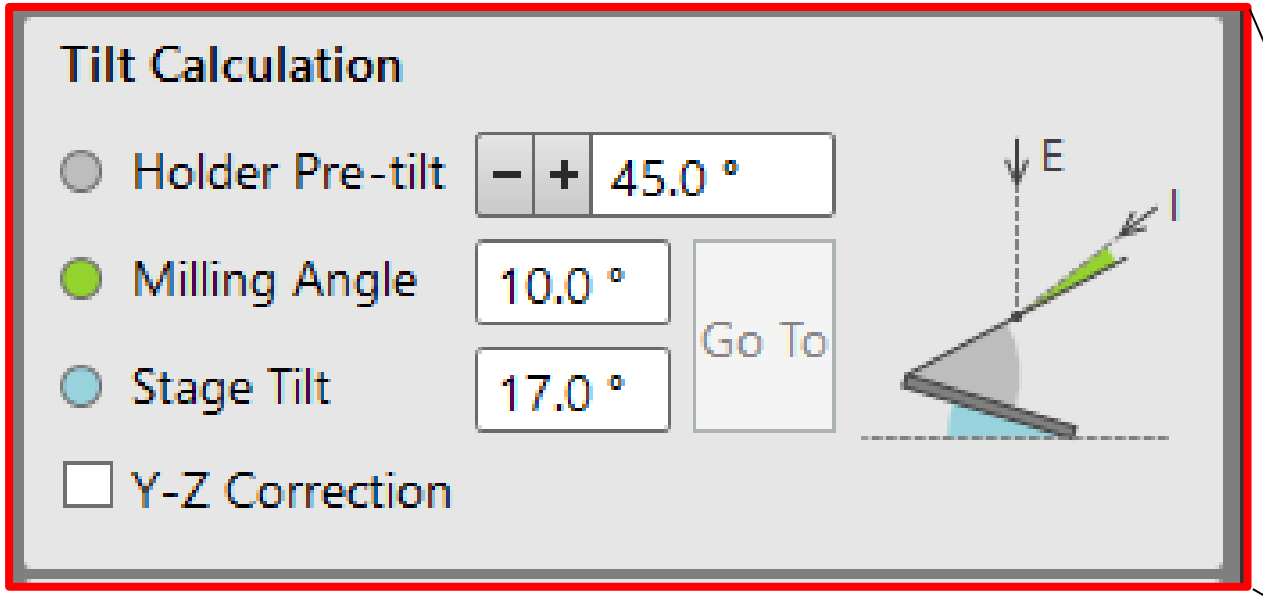
4.5 Pt sputter

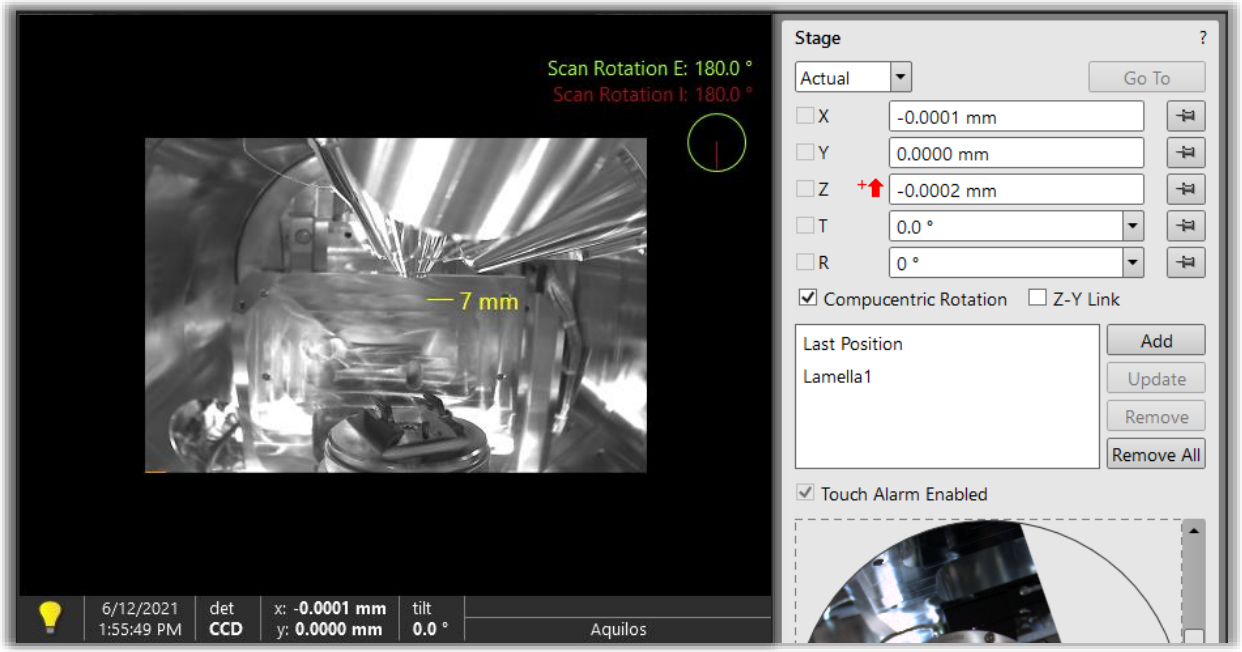
(Video from TFS)



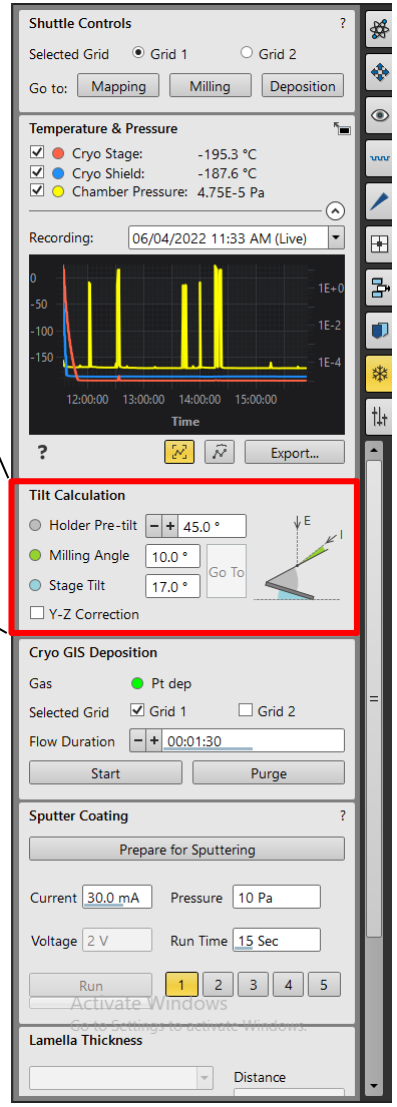


4.6 Targeting cells_eucentric Position

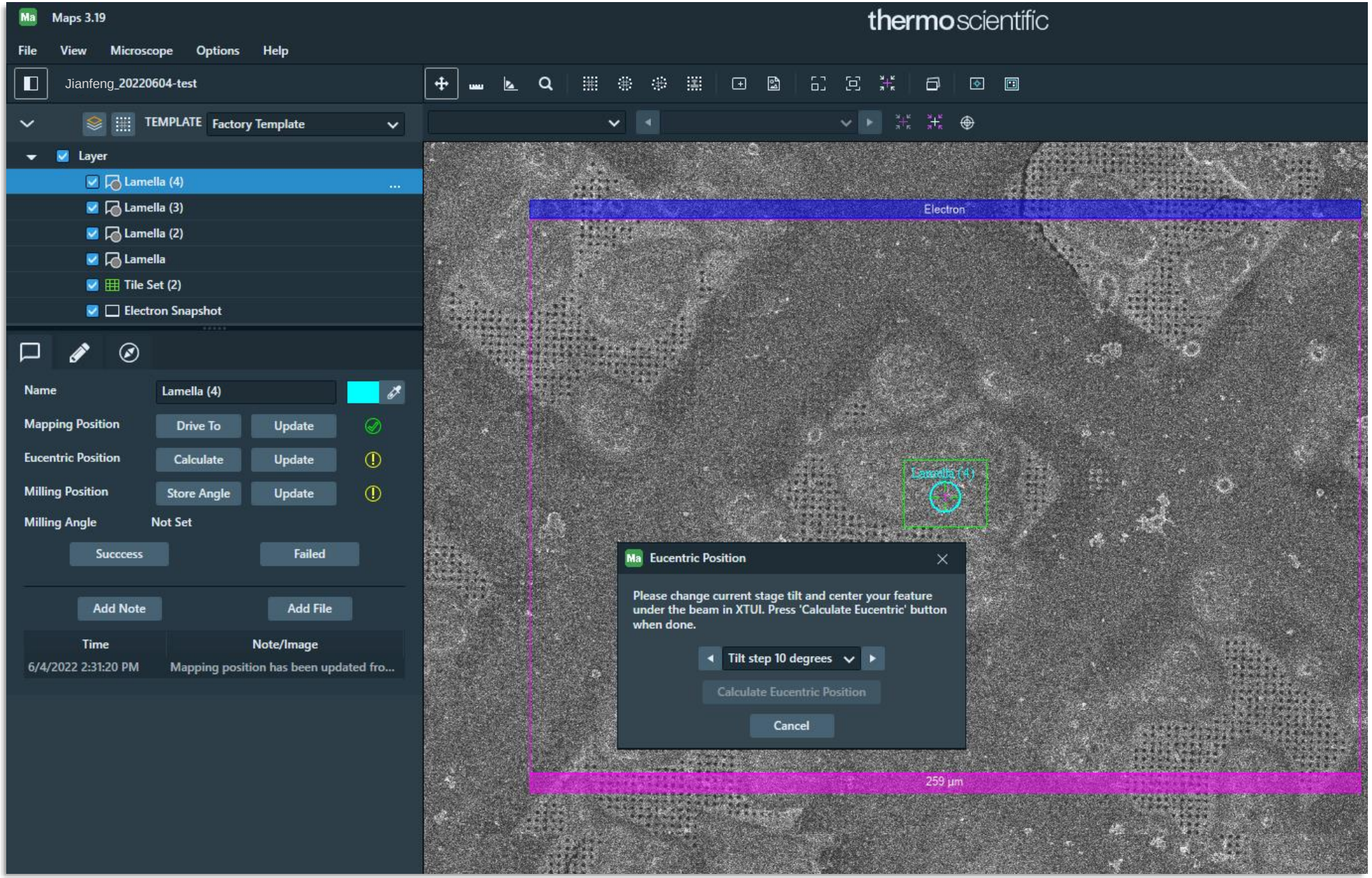




xT



4.6 Targeting cells_eucentric Position

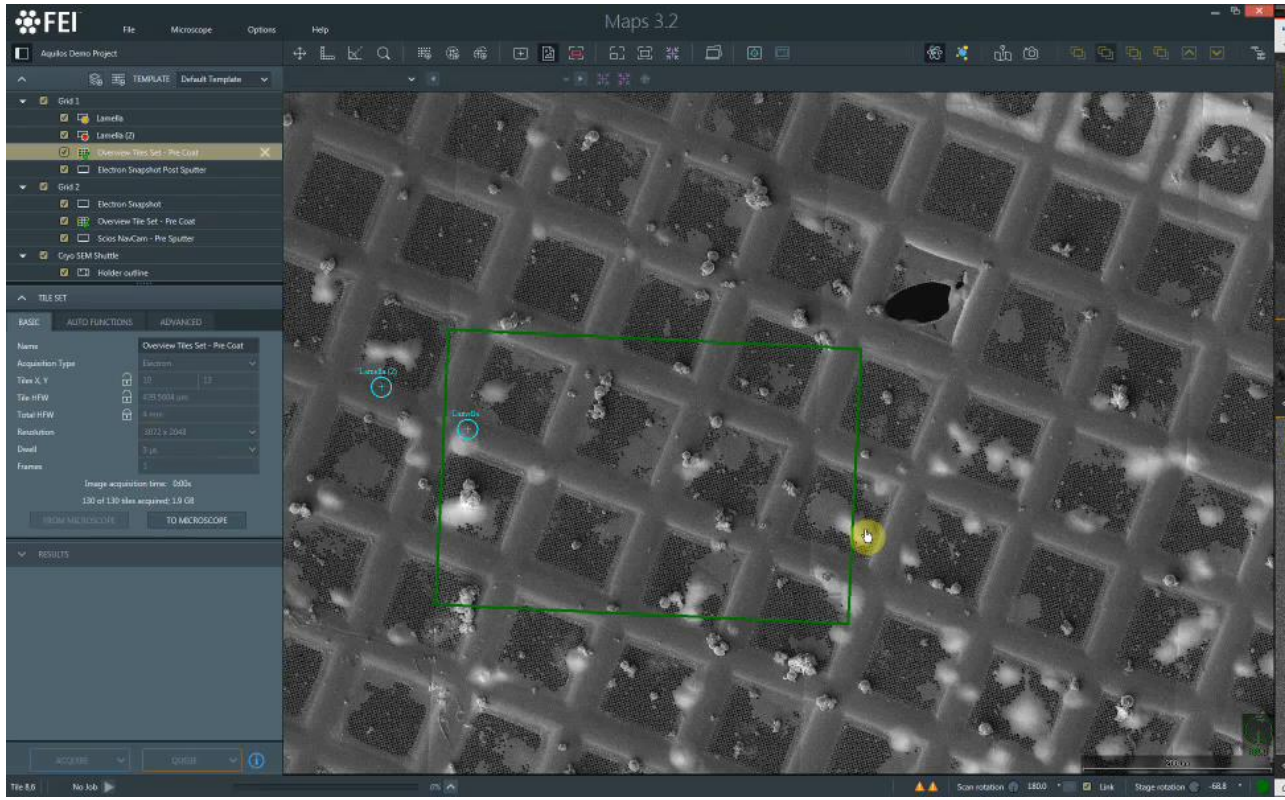


Maps

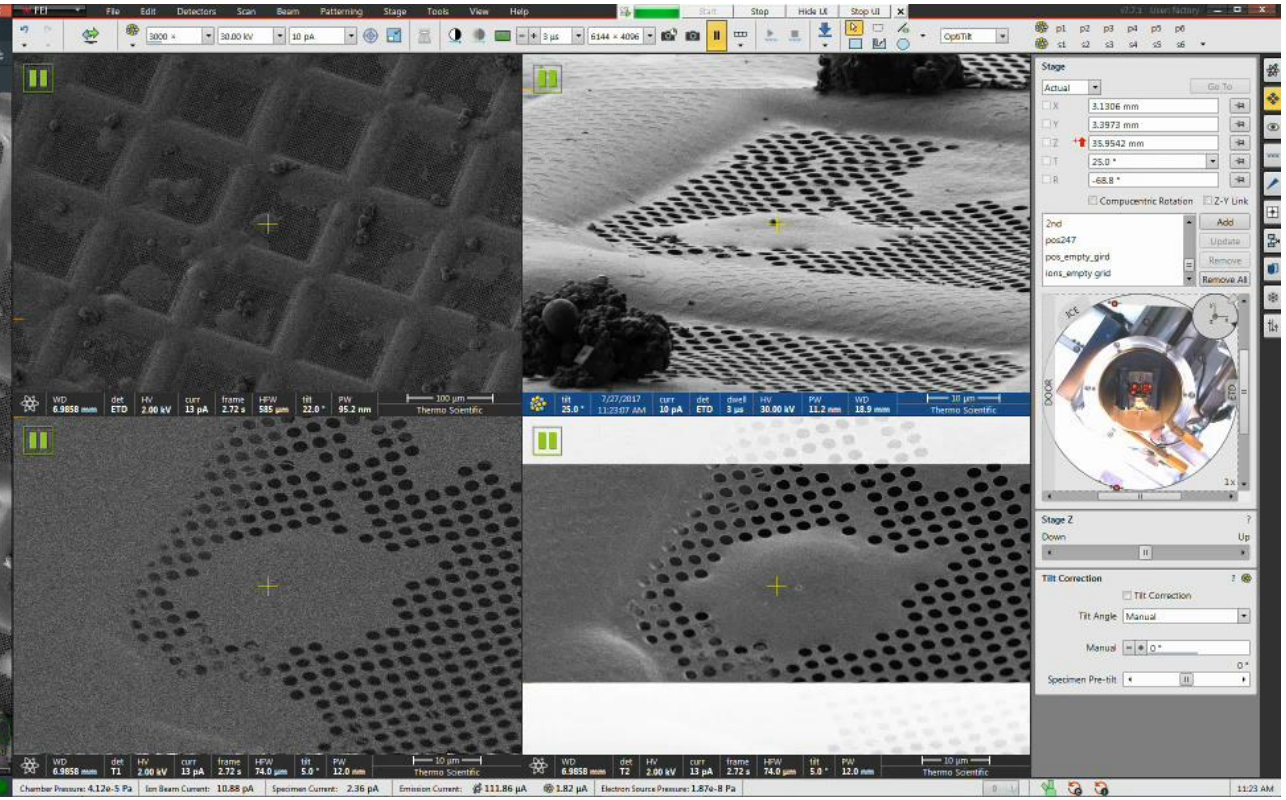
4.6 Targeting cells_by Maps & xT

(Video from TFS)

Maps

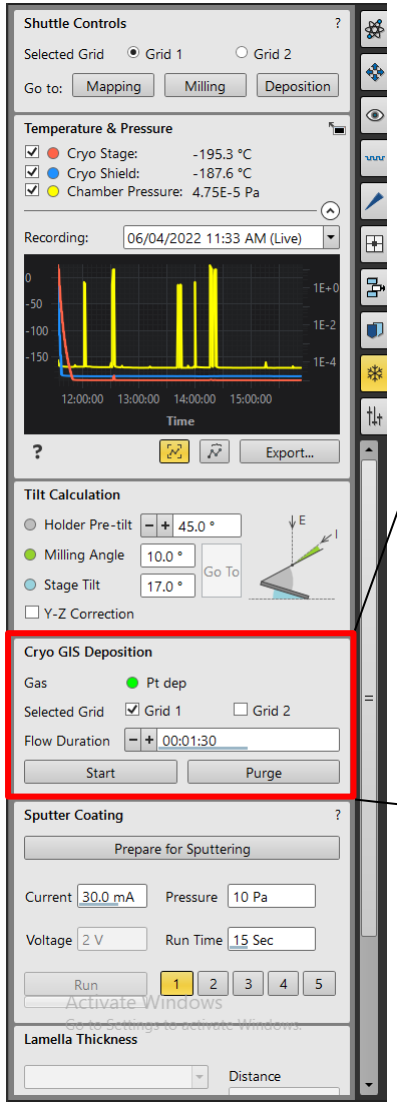


xT

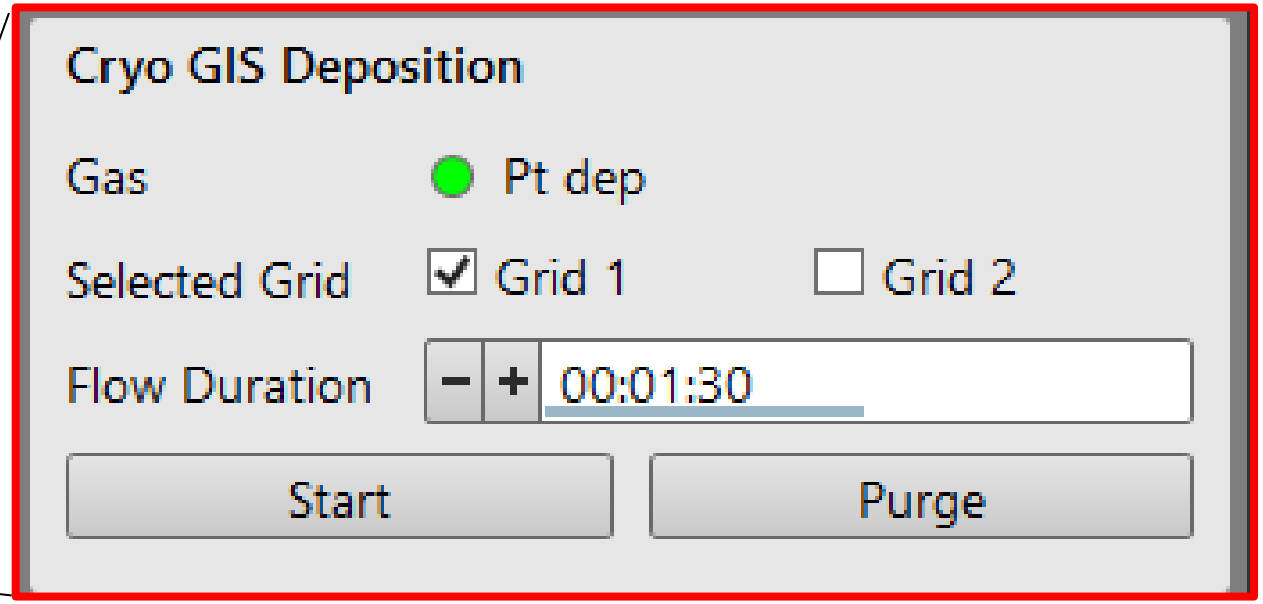


4.7 Pt GIS

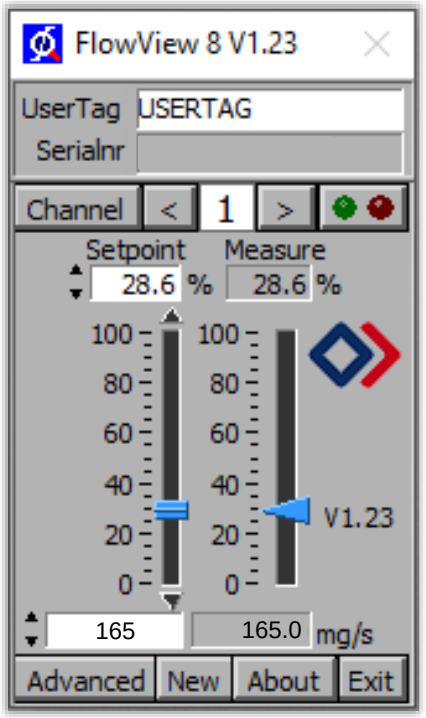
xT



Duration varies by samples / instruments



Then, decrease the N2 to 165 (varies by instruments)



4.8 Automatic milling of the lamellae with AutoTEM Cryo

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017

4.8 Automatic milling of the lamellae with AutoTEM Cryo

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

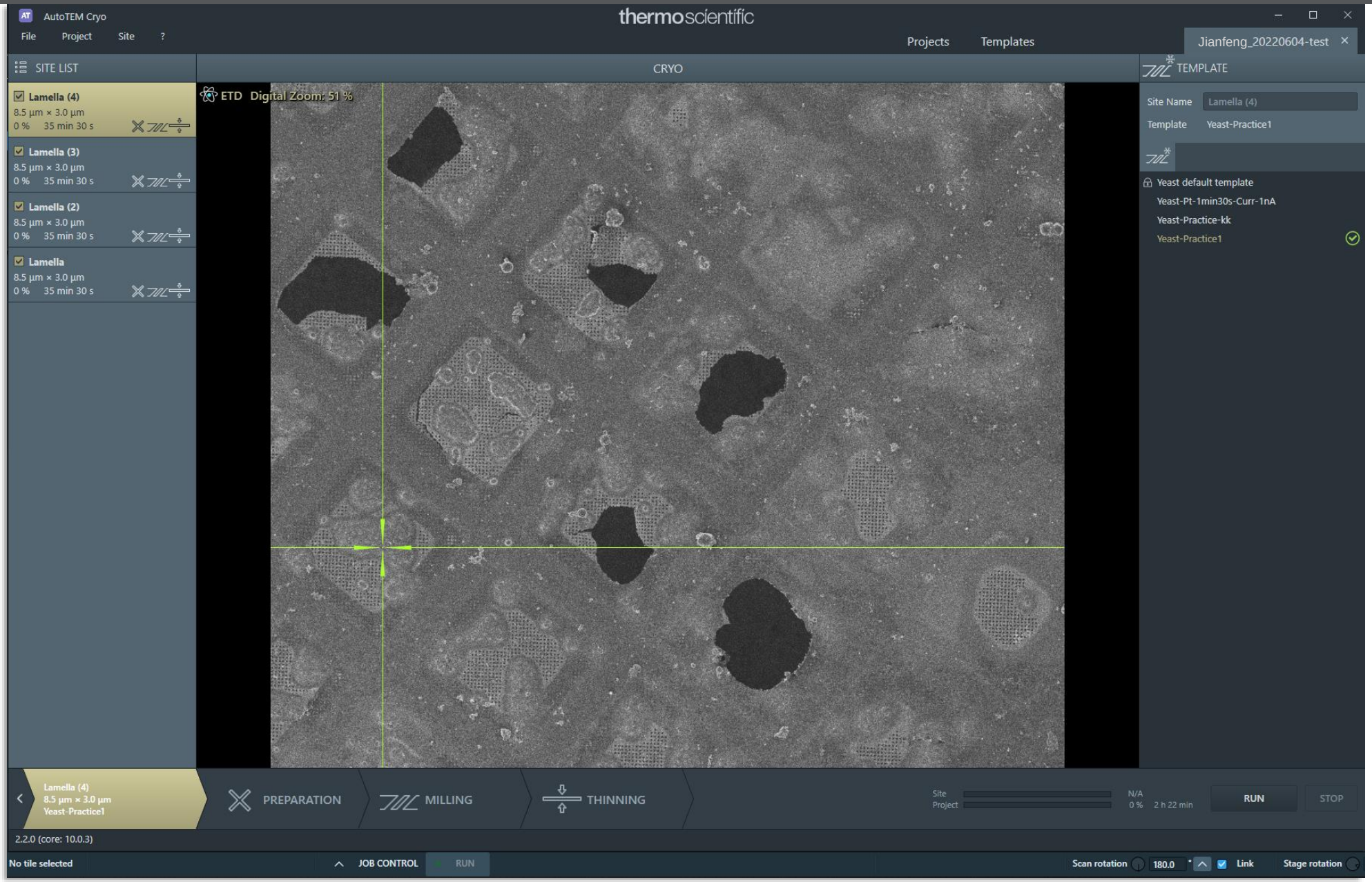
Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography



Schaffer et al., JSB 2017

4.8 Automatic milling of the lamellae with AutoTEM Cryo

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

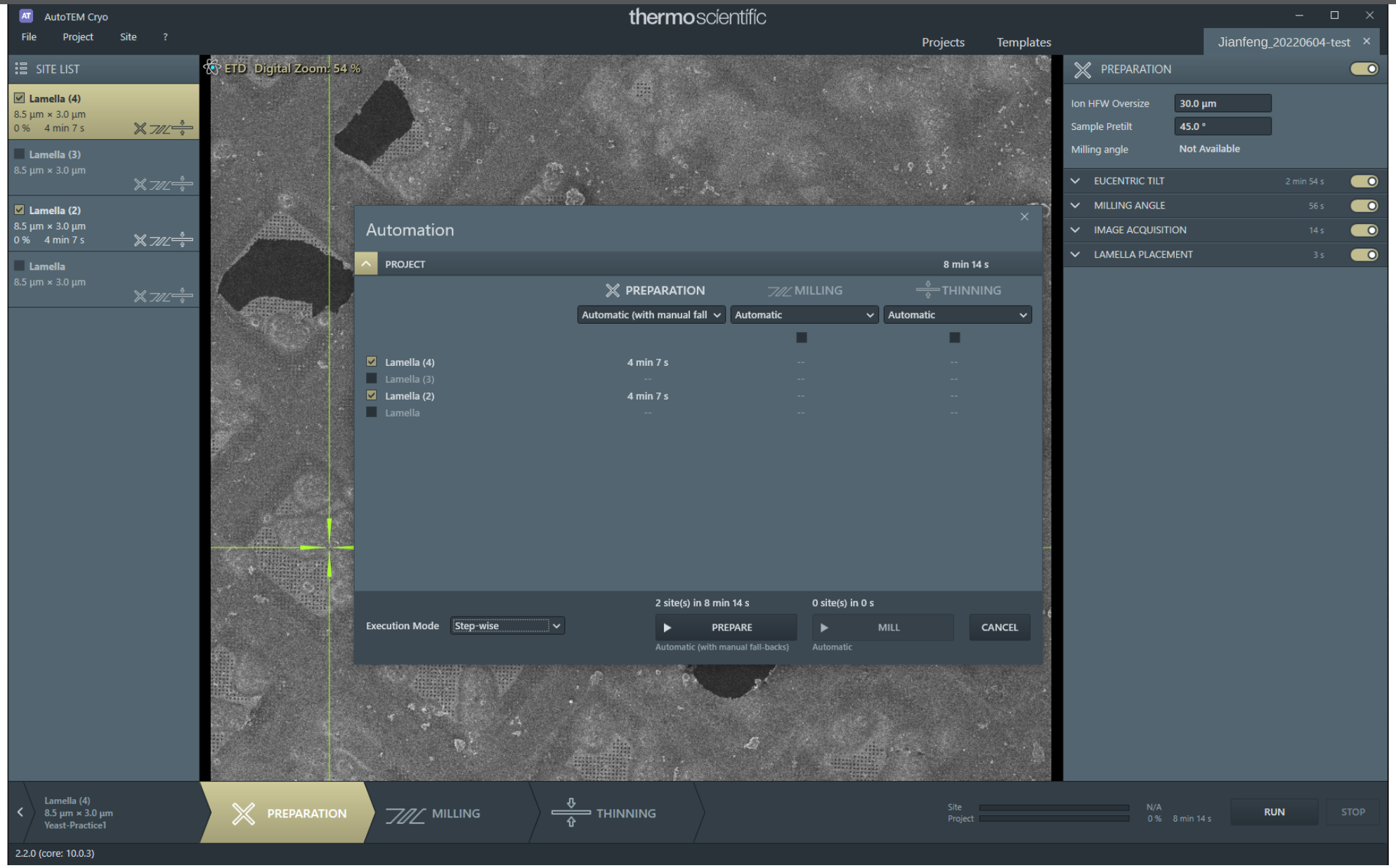
Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography



Schaffer et al., JSB 2017

4.8 Automatic milling of the lamellae with AutoTEM Cryo

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

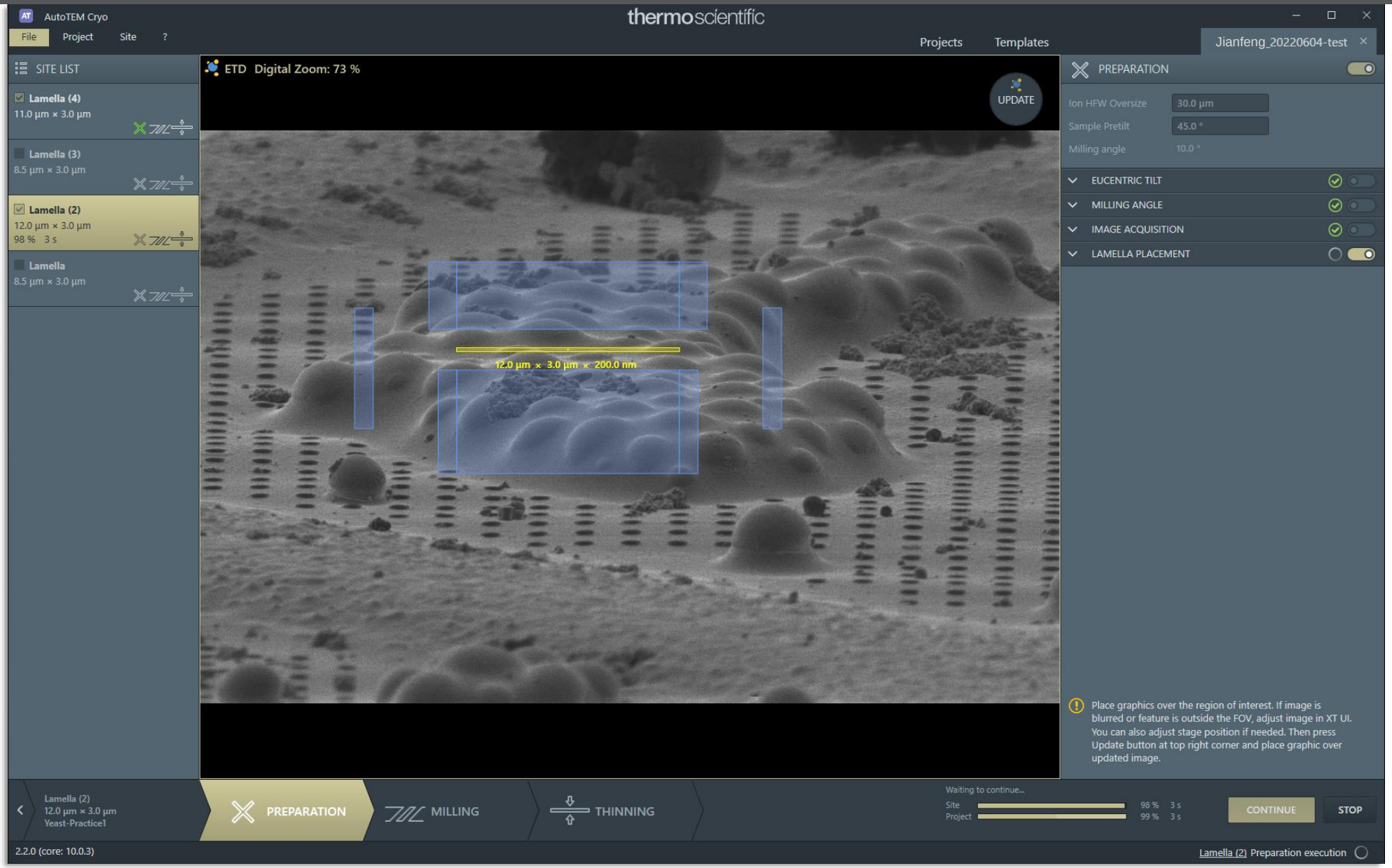
Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography



Schaffer et al., JSB 2017

4.8 Automatic milling of the lamellae with AutoTEM Cryo

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017

4.8 Automatic milling of the lamellae with AutoTEM Cryo

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

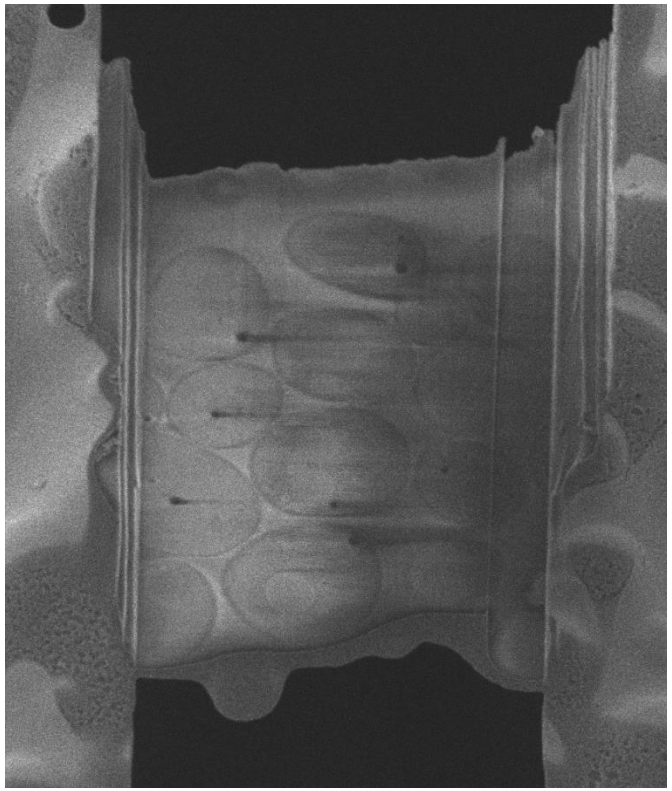
Pt sputter
Lamella conductivity

T

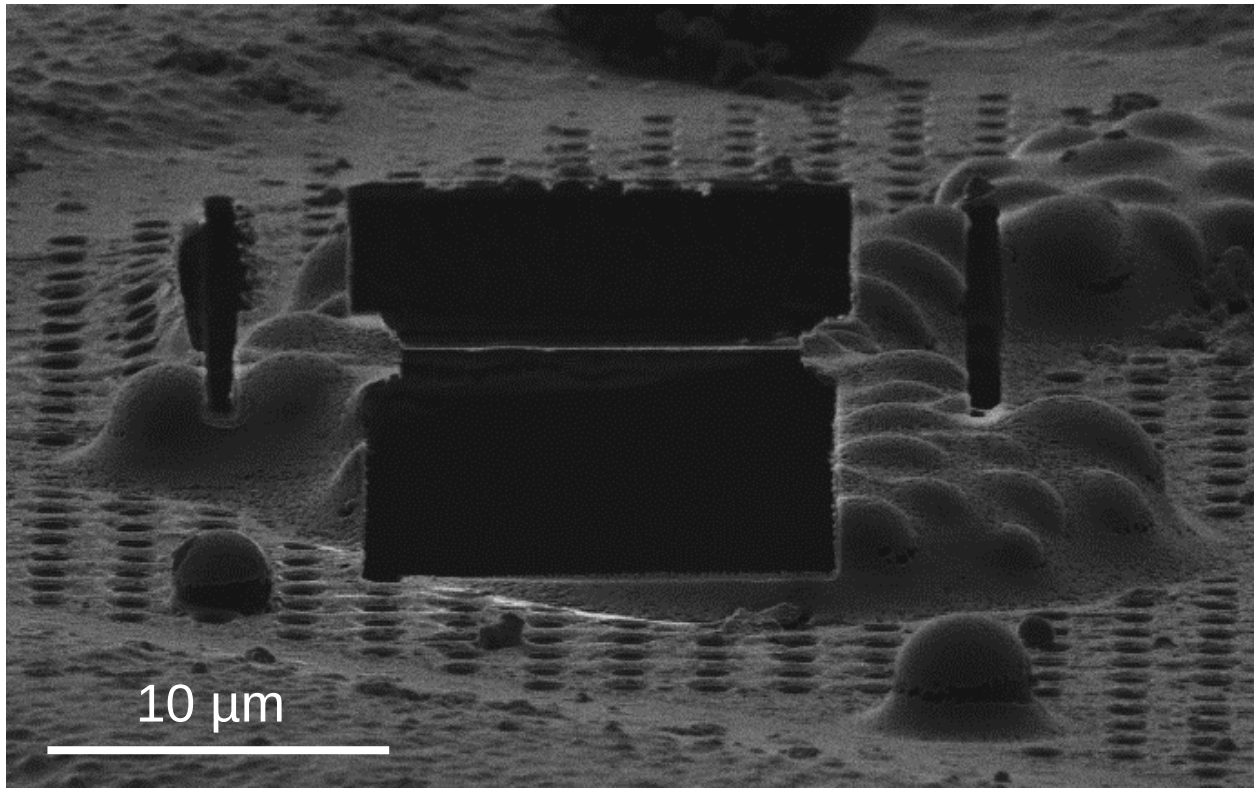
Cryo-electron tomography

Takes ~30 minutes

E-beam micrograph



I-beam micrograph



Schaffer et al., JSB 2017

4.8 Automatic milling of the lamellae with AutoTEM Cryo (30x accelerated video)

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

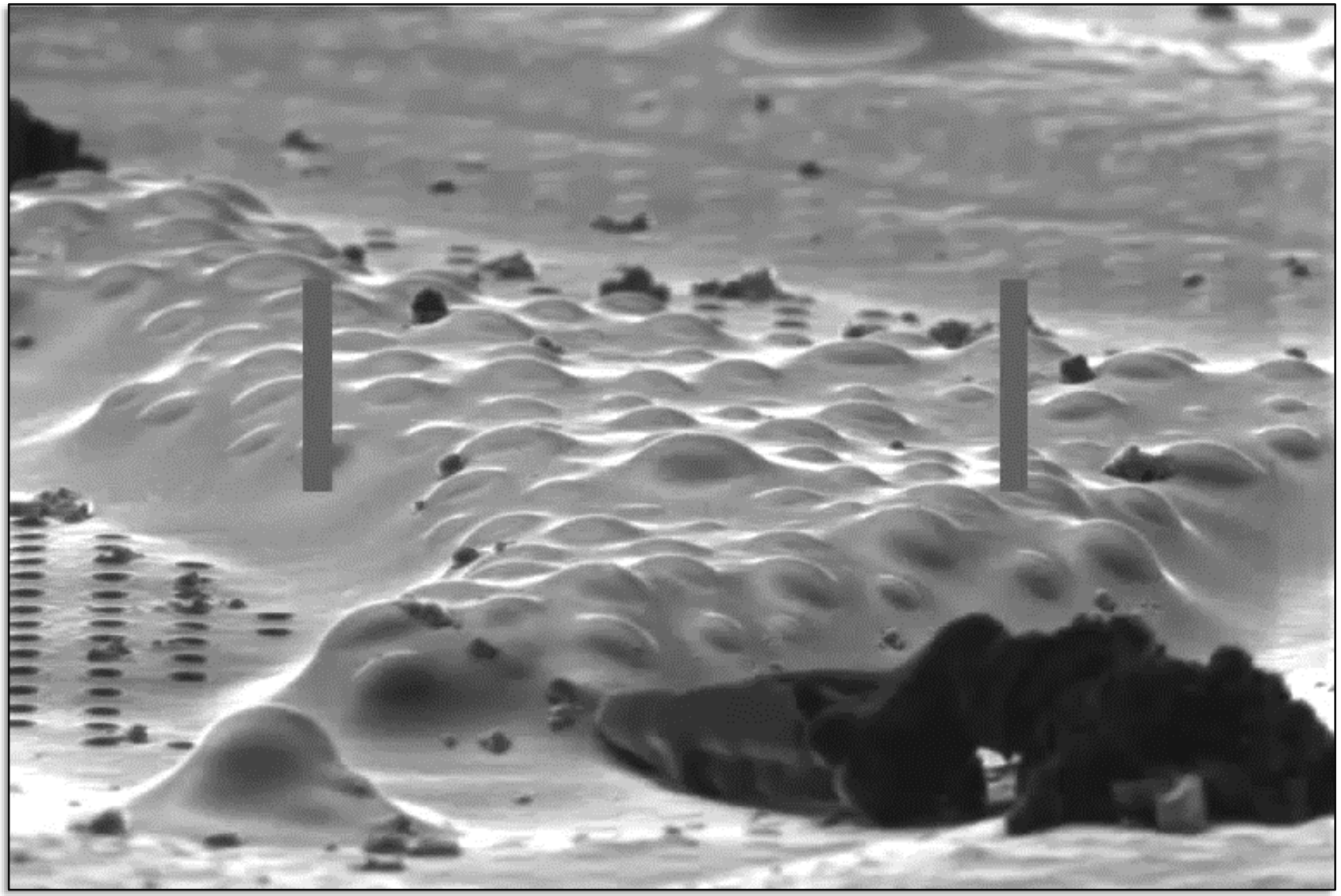
Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography



Schaffer et al., JSB 2017

4.9 Pt sputter (optional)

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

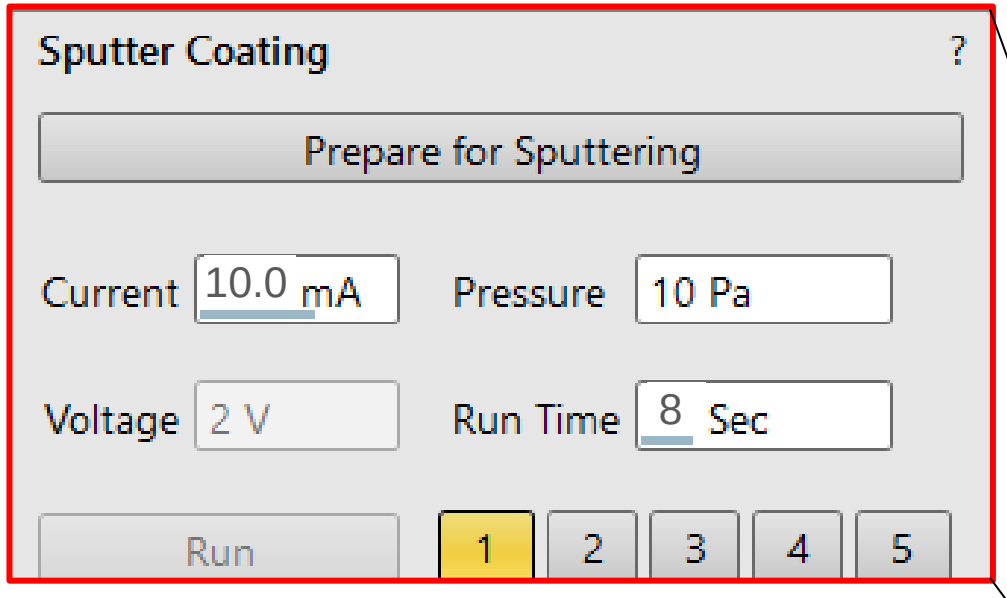
Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

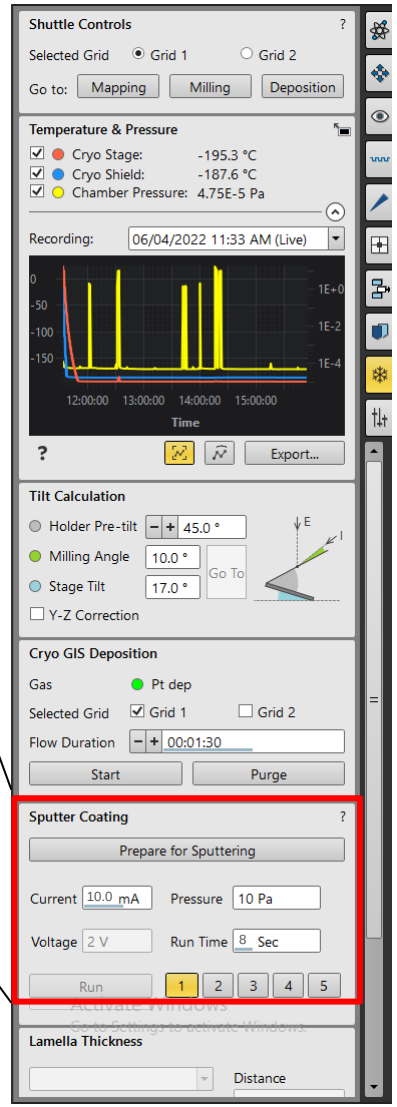
T

Cryo-electron tomography

Schaffer et al., JSB 2017



xT



4.10 Unloading grids

(Video from TFS)

Vitrification
Plunge-freezing

T

Cryo-FIB

Pt sputter
Sample conductivity

Pt GIS
Protective coating

Lamella milling
Parallel & pre-wedge milling geometries

Pt sputter
Lamella conductivity

T

Cryo-electron tomography

Schaffer et al., JSB 2017

Thank you!

Comments & Questions?

Jianfeng.lin@yale.edu