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













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1. Specimens accessible by cryoET

2. Four considerations for cryoET sample preparation

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

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



> Validation of the sample quality.





Russo & Passmore, Curr Opin Struct Biol, 2016.



> Validation of the sample quality.

➢ Grid selection. ⇒ Mesh





> Validation of the sample quality.

≻ Grid selection.

Fiducial marker. e.g., BSA-treated colloidal gold



lancu et al., Nature Protocols 2006

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

T_{LE}= -182.8 °C ~-88.6 °C T_{LP}= -189.7 °C ~ -42.2 °C T_{LN2}=-210 °C ~-195.8 °C



Tivol et al., Microsc Microanal. 2008

- ➤ Validation of the sample quality.
- ≻ Grid selection.
- ≻ Fiducial marker.
- Vitrification. e.g., LE or 37% LE-63% LP mixture
 Without glycerol
 5% glycerol

T_{LE}= -182.8 °C ~-88.6 °C T_{LP}= -189.7 °C ~ -42.2 °C T_{LN2}=-210 °C ~-195.8 °C



Moravcová et al., J Vis Exp. 2021

Tivol et al., Microsc Microanal. 2008

- ➤ Validation of the sample quality.
- ≻ Grid selection.
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Bhella D, Biophysical Reviews. 2019

Tivol et al., Microsc Microanal. 2008

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3. Workflow of cryo lamellae preparation

Vitrification **Plunge-freezing** Cryo-FM Cryo-FIB **Pt sputter** Sample conductivity Pt GIS **Protective coating** Lamella milling Parallel & pre-wedge milling geometries Pt sputter Lamella conductivity **Cryo-electron** tomography Schaffer et al., JSB 2017



nature

protocols

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 ^{옷 1} 쯔, Julia Mahamid ¹, Benjamin D. Engel, Tim Laugks, Wolfgang Baumeister, Jürgen M. Plitzko 옷 ⊠

PROTOCOL

https://doi.org/10.1038/s41596-020-0320->

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 Schampers², Digvijay Singh¹, Hans Persoon², Miroslava Schaffer³, Peter Fruhstorfer^{2,5}, Jürgen Plitzko¹³ and Elizabeth Villa¹¹[∞]

T Cryo-FM Cryo-FIB Pt sputter Sample conductivity Pt GIS Protective coating

Vitrification

Plunge-freezing

Parallel & pre-wedge milling geometries

Pt sputter Lamella conductivity

Т

Cryo-electron tomography Schaffer et al., JSB 2017







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

Vitrification **Plunge-freezing** Cryo-FM Т **Cryo-FIB Pt sputter** Sample conductivity Pt GIS **Protective coating** Lamella milling Parallel & pre-wedge milling geometries Pt sputter Lamella conductivity **Cryo-electron**

tomography Schaffer et al., JSB 2017





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

Vitrification **Plunge-freezing** Cryo-FM Т **Cryo-FIB** Pt sputter Sample conductivity Pt GIS **Protective coating** Lamella milling Parallel & pre-wedge milling geometries Pt sputter Lamella conductivity **Cryo-electron** tomography Schaffer et al., JSB 2017





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

Schaffer et al., JSB 2017



Lam & Villa, Methods Mol Biol. 2021

3.2 Transfer the grids_clip the grid for cryo-FIB milling



Cryo-FIB Pt sputter Sample conductivity

Pt GIS Protective coating

Lamella milling Parallel & pre-wedge milling geometries

Pt sputter Lamella conductivity

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



Schaffer et al., JSB 2017

Orientating the lamellae

Cryo-FIB Pt sputter Sample conductivity

Vitrification

Plunge-freezing

Т

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

Cryo-electron tomography Schaffer et al., JSB 2017





LEICA Cryo FM

Adapted from TFS

3.3 (Optional) Cryo-fluorescence correlative microscopy localizes target

Vitrification Plunge-freezing







Schaffer et al., JSB 2017

Kuba et al., J Microsc. 2021

Adapted from TFS

No.

Electrons

Vitreous cells

Gallium ions

Adapted from TFS

10.0






3.4 Cryo-FIB milling with DualBeam system



3.4 Cryo-FIB milling with DualBeam system



3.4.1 Eucentric height & tilt calculation



Wagner et al., Nature Protocols 2020

3.4.1 Eucentric height & tilt calculation



Wagner et al., Nature Protocols 2020

3.4.1 Eucentric height & tilt calculation



Wagner et al., Nature Protocols 2020

3.4.2 Stepwise milling procedure



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

Regular milling



Schaffer et al., JSB 2017

Regular milling





Schaffer et al., JSB 2017

Regular milling



Wedge pre-milling



Schaffer et al., JSB 2017

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

AutoTEM Cryo thermoscientific – –									
File Project Site ?						Projects	Templates	Jianf	eng_20220604-test ×
: TEMPLATE LIST		💥 Preparatio	n action ~		702 Milling	action		🐣 Thinning	action 🗸
Search templates	Ion HFW Oversize	30.0 µm			8.5 μm	× 3.0 µm	Final Thickness	200.0 nm	
<i>70</i> Ľ	Sample Pretilt	45.0 °		Correction Factor	0.50		Enable Windows		
Yeast default template	Milling angle	Not Available		V DELAY			V DELAY		
Спуо	EUCENTRIC TILT			✓ ELECTRON REFER	RENCE DEFINITION		POLISHING 1		
Yeast-Practice1 X Cryo	Maximal tilt step	10.0 °		✓ REFERENCE DEFII	NITION		Pattern Offset	150.0 nm	
	Preparation HFW	250.0 μm		✓ STRESS RELIEF CU			Overtilt	0 °	
	Resolution (guided)	1536 x 1024 🗸		✓ REFERENCE REDE	FINITION 1		Depth Correction	160.0 %	
	Electron ACB			A ROUGH MILLING			High Voltage	30 kV 🗸	
	Ion ACB			Pattern Offset	1.0 μm		Milling Current	50 pA 🗸 🗸	
	Electron AutoFocus			Front Pattern Height	3.6 μm		DCM Rescan Interval	30 s	
	Ion AutoFocus			Rear Pattern Height	5.6 μm		POLISHING 1 - I	ELECTRON IMAGE	
	MILLING ANGLE			Depth Correction	75 %		Decolution	1526 - 1024	2
	Target Milling Angle	10.0 °		Front Width Overlap	1.5 μm	1.5 μm	ACR	1536 X 1024 Y	× 245
	Clearance Angle	2.0 °		Rear Width Overlap	1.0 μm	1.0 μm	Auto Focus		
	Enforce target	•		Milling Current	0.50 nA 🗸 🗸		Imaging HFW	70.0 μm	
				Pattern Type	Rectangle 🗸 🗸		Notification		
				DCM Rescan Interval	120 s				
	Ion HFW Oversize	120 %		Show Graphics			POLISHING 2		
	Resolution	1536 x 1024 🗸 🗸	@ 500 ns 🗸 🗸	ROUGH MILLING	- ELECTRON IMAGE	\bigcirc	Pattern Offset	0 μm	
	ACB			Resolution	1536 x 1024 🗸 🗸	@ 3 us 🗸	Overtilt	0 °	
	Auto Focus			ACB	✓		Depth Correction	160.0 %	
	A LAMELLA PLACEMENT						High Voltage	30 kV 🗸	
	Ion HFW Oversize	120 %		Imaging HFW	70.0 μm		Milling Current	30 pA 🗸	
				Notification			DCM Rescan Interval	10 s	
				✓ REFERENCE REDE	FINITION 2		POLISHING 2 - I	ELECTRON IMAGE	
				V MEDIUM MILLING			Resolution	1536 x 1024 🗸 @	3 µз 🗸
				V MEDIUM MILLING - ELECTRON IMAGE			АСВ		
				V FINE MILLING			Auto Focus		
				V FINE MILLING - ELECTRON IMAGE			Imaging HFW	70.0 μm	
				✓ FINER MILLING			Notification		
2.2.0 (core: 10.0.3)									
No tile selected		∧ JOB CONTROL	⊳ RUN					Scan rotation 🕧 180.0 ° 🔨 🔽	Link Stage rotation

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

Vitrification **Plunge-freezing** Т Cryo-FIB **Pt sputter** > (Optional) minimize charging; ensure targeting and precise milling. Sample conductivity > Protect the sample surface during milling; reduces curtaining and the Pt GIS redeposition of milled material. **Protective coating** Lamella milling > Rough milling, thinning and cleaning for thin lamella. Parallel & pre-wedge milling geometries (Optional) minimize charging; ensure low beam-induced movement and Pt sputter Lamella conductivity using of VPP. Т **Cryo-electron** tomography Schaffer et al., JSB 2017

3.4 Cryo-FIB mining with DualBeam system



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

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In Aquilos 2 PC: Microscope Control v20.1.1



In Aquilos 2 PC: T M > Create grid atlas > Align LM & SEM (optional)

Maps 3.19 > Add lamella sites

Eucentric height & tilt calculation (optional)



In Aquilos 2 PC: The wedge pre-milling technique > Batch & Stepwise lamella milling



In Aquilos 2 PC: 🏧 🔤 🏧

In Support PC

FlowDDE V4.80 MBC FLOW-BUS host

File Communication FLOW-BUS Server Info



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



Kuba et al., J Microsc. 2021

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



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



> Cool down the system.



> Cool down the system.



Jakub Kuba at Brno; TFS

4.2 Clip grids



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



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

хT



4.4 Acquire grid atlas_ setup E-beam imagining conditions

хT



4.4 Acquire grid atlas _ take a snapshoot of the grid

Maps

Ma Maps 3.19	thermo scientific	_ Ø ×
File View Microscope Options Help		
Jianfeng_20220604-test		🃚 🕷 🤾 🔐 🙆 🚡
✓ S III TEMPLATE CryoTemplate ✓		
👻 🔽 Layer		
☑ Ⅲ Tile Set (2) 🎇		
Electron Snapshot		
⊞ – [≁] ⊑ 幸 ⊘		
Name Tile Set (2)		
Acquisition Type Electron V		
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Tile HFW		
Resolution 20.49 + 1759		
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Dwell 1µs v	a start in the second	
Frames 🗾 1		
Reduced Area Select		
Image acquisition time: 00:03:03 Elapsed acquisition time: 00:03:03 Elapsed acquisition time: 00:00:34 4 of 30 images acquired; 287.37 MB FROM MICROSCOPE TO MICROSCOPE		2mm
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🔳 🔎 📑 💄 🥵 🚾 🔤		2:12 PM 6/4/2022

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

thermo scientific Maps 3.19 Microscope Options Help File View Jianfeng_20220604-test ✓ ► ³⁺^k ³⁺^k ⊕ TEMPLATE CryoTemplate 👻 🗹 Layer V III Tile Set (2) 🗹 🔲 Electron Snapshot -\$**₩** Ħ Electron Tiles X. Y 6 5 Tile HFW 🔒 550 μm Total HFW 🔒 2.53 mm Resolution Pixel Size Dwell Frames 2 1 Reduced Area Image acquisition time: 00:00:04 Estimated acquisition time: 00:03:05 Elapsed acquisition time: 00:00:34 4 of 30 images acquired; 287.57 MB REMAINING TIME 00:03:05 No tile selected A JOB CONTROL Scan rotation 🕥 180.0 ° 🔨 🔽 Link Stage rotation Acquiring images... 2:12 PM · 오 븀 💄 🚅 хT Ma 6/4/2022

Maps
4.4 Acquire grid atlas

Maps



(Video from TFS)

4.5 Pt sputter





4.6 Targeting cells_eucentric Position

6/12/2021 det x: -0.0001 mm tilt 1:55:49 PM CCD y: 0.0000 mm 0.0 °



Aquilos

хT



4.6 Targeting cells_eucentric Position



Maps

хT



4.7 Pt GIS

хT



Duration varies by samples / instruments

	Cryo GIS Deposition				
/	Gas	😑 Pt dep			
	Selected Grid	Grid 1	Grid 2		
	Flow Duration	- + 00:01:30			
	Start		Purge		

5 FlowView 8 V1.23 UserTag USERTAG Serialnr Channel >Measure Setpoint 28.6 % 28.6 % 100 -100 80 E 80 E 60 E 60 E 40 - **40** E V1.23 20 E 20 -0 0 -165.0 mg/s 165 Ŧ Advanced New About Exit

Then, decrease the N2 to

165 (varies by instruments)

Vitrification	AutoTEM Cryo thermoscientific – –				
Vicinication			action Y 700 Million	action ~	Jianteng_20220604-test ×
Plunge-freezing	Search templates	Ion HFW Oversize 30.0 µm	Size 8.5 µm × 3.0 µm	Final Thickness	200.0 nm
Τ	7/1/L Yeast default template Cryo Yeast-Practice1 Cryo	Sample Pretiit 45.0 ° Milling angle Not Available CUCENTRIC TILT Maximal tilt step 10.0 °	Correction Factor O.SU O DELAY ELECTRON REFERENCE DEFINITION REFERENCE DEFINITION	DELAY POLISHING 1 Pattern Offset	150.0 nm
Cryo-FIB		Preparation HFW 250.0 μm Resolution (guided) 1536 x 1024 Electron ACB	 STRESS RELIEF CUTS REFERENCE REDEFINITION 1 ROUGH MILLING 	Overtilt Depth Correction High Voltage	0 ° 160.0 % 30 kV ~
Pt sputter Sample conductivity		Ion ACB Electron AutoFocus Ion AutoFocus	Pattern Offset 1.0 μm Front Pattern Height 3.6 μm Rear Pattern Height 5.6 μm	Milling Current DCM Rescan Interval	50 pA ~ 30 s
Pt GIS Protective coating		MILLING ANGLE Target Milling Angle Clearance Angle 2.0 ° Enforce target	O Depth Correction 75 % Front Width Overlap 1.5 μm 1.5 μm Rear Width Overlap 1.0 μm 1.0 μm Milling Current 0.50 nA ✓	ACB Auto Focus Imaging HFW	1536 x 1024 Υ @ 3 μs Υ
Lamella milling Parallel & pre-wedge milling geometries		IMAGE ACQUISITION ION HFW Oversize Resolution 1536 x 1024 © 500 ns ACB Auto Focus	CCM Rescan Interval 120 s Show Graphics ♥ COUGH MILLING - ELECTRON IMAGE Resolution 1536 x 1024 ♥ @ 3 µs	POLISHING 2 Polishing 2 Pattern Offset Overtilt Partle Correction	Ο μm 0 ° 160.0 %
Pt sputter Lamella conductivity		Ion HFW Oversize 120 %	ACB ✓ Auto Focus Imaging HFW 70.0 µm Notification	High Voltage Milling Current DCM Rescan Interval	30 kV 30 pA 10 s
Τ			 REFERENCE REDEFINITION 2 MEDIUM MILLING MEDIUM MILLING - ELECTRON IMAGE 	POLISHING 2 - E Resolution ACB Auto Focus	LECTRON IMAGE Ο
Cryo-electron tomography	2.2.0 (core: 10.0.3)		 FINE MILLING FINE MILLING - ELECTRON IMAGE FINER MILLING 	Imaging HFW Notification	70.0 μm
Schaffer et al., JSB 2017	No tile selected	A JOB CONTROL RUN			ican rotation 🎧 180.0 ° 🦳 🗹 Link Stage rotation 🕞









Vitrification Plunge-freezing

Takes ~30 minutes

Cryo-FIB Pt sputter

Sample conductivity

Pt GIS Protective coating

Lamella milling Parallel & pre-wedge milling geometries

Pt sputter Lamella conductivity

Cryo-electron tomography Schaffer et al., JSB 2017

E-beam micrograph



I-beam micrograph



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

Vitrification Plunge-freezing

Cryo-FIB Pt sputter Sample conductivity

Pt GIS Protective coating

Lamella milling Parallel & pre-wedge milling geometries

Pt sputter Lamella conductivity

Cryo-electron tomography Schaffer et al., JSB 2017



4.9 Pt sputter (optional)







4.10 Unloading grids

(Video from TFS)

T Cryo-FIB Pt sputter Sample conductivity

Vitrification

Plunge-freezing

Pt GIS Protective coating

Lamella milling Parallel & pre-wedge milling geometries

Pt sputter Lamella conductivity

Т

Cryo-electron tomography Schaffer et al., JSB 2017



Thank you!

Comments & Questions?

Jianfeng.lin@yale.edu