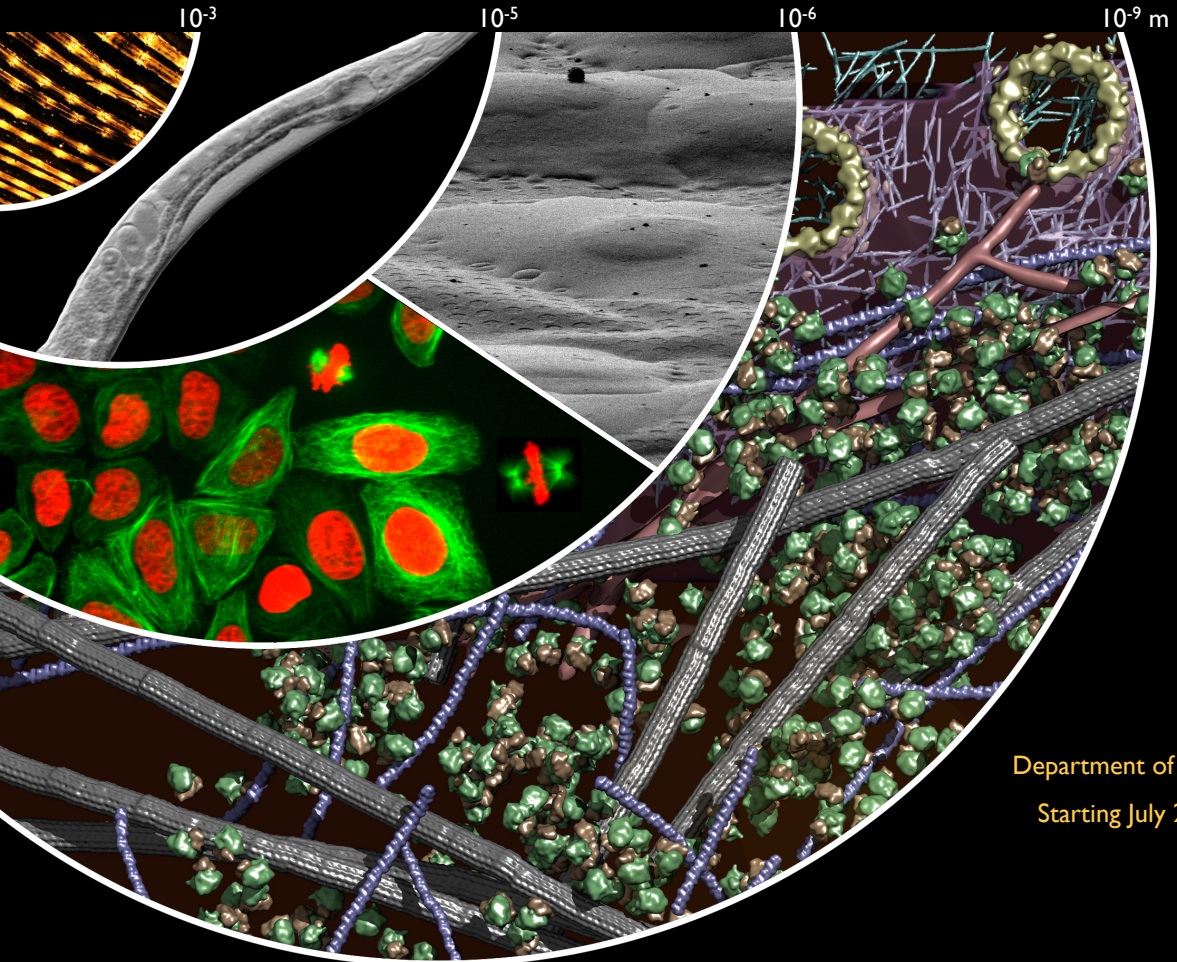
Use proven methods, avoid shortcuts.

Challenges and Opportunities:
Cellular Cryo-ET



Julia Mahamid

Department of Molecular Structural Biology, MPI of Biochemistry, Martinsried

Starting July 2017: Structural & Computational Biology, EMBL, Heidelberg

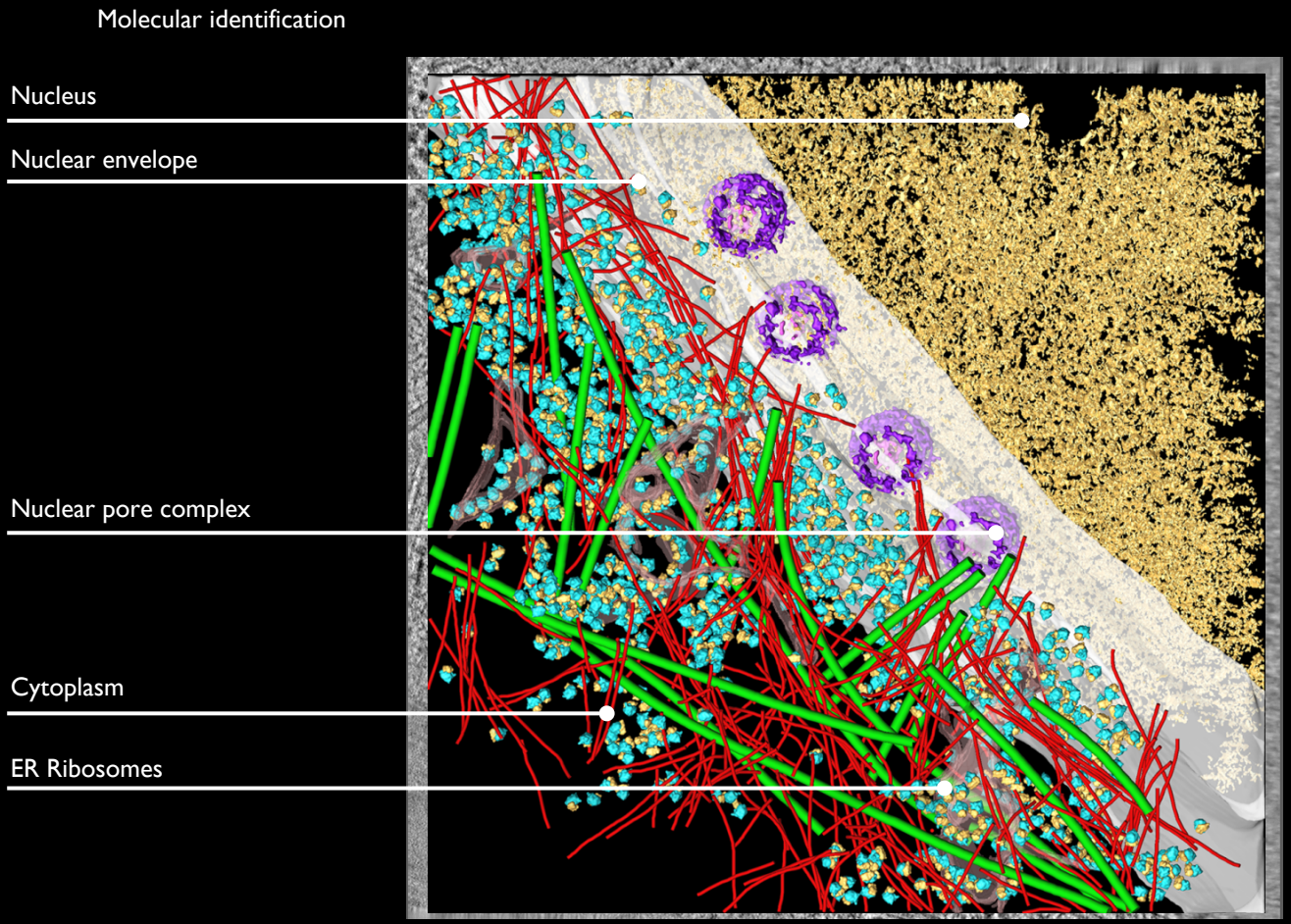
Challenges and Opportunities: Cellular Cryo-ET

- What is next for these challenging methods?
- How hard will it be to do accurate 3D localization for site-specific preparations with cryo-FIB and navigation of tomography data acquisition?
- Will super-resolution cryo-LM become a reality?
- Will high-pressure freezing and FIB lift-out become routine for bulk specimens?
- How will we solve the segmentation problem? Will deep learning methods help with this or are they over hyped?

Challenges and Opportunities: Cellular Cryo-ET

- What is next for these challenging methods?
- How hard will it be to do accurate 3D localization for site-specific preparations with cryo-FIB and navigation of tomography data acquisition?
- Will super-resolution cryo-LM become a reality?
- Will high-pressure freezing and FIB lift-out become routine for bulk specimens?
- How will we solve the segmentation problem? Will deep learning methods help with this or are they over hyped?

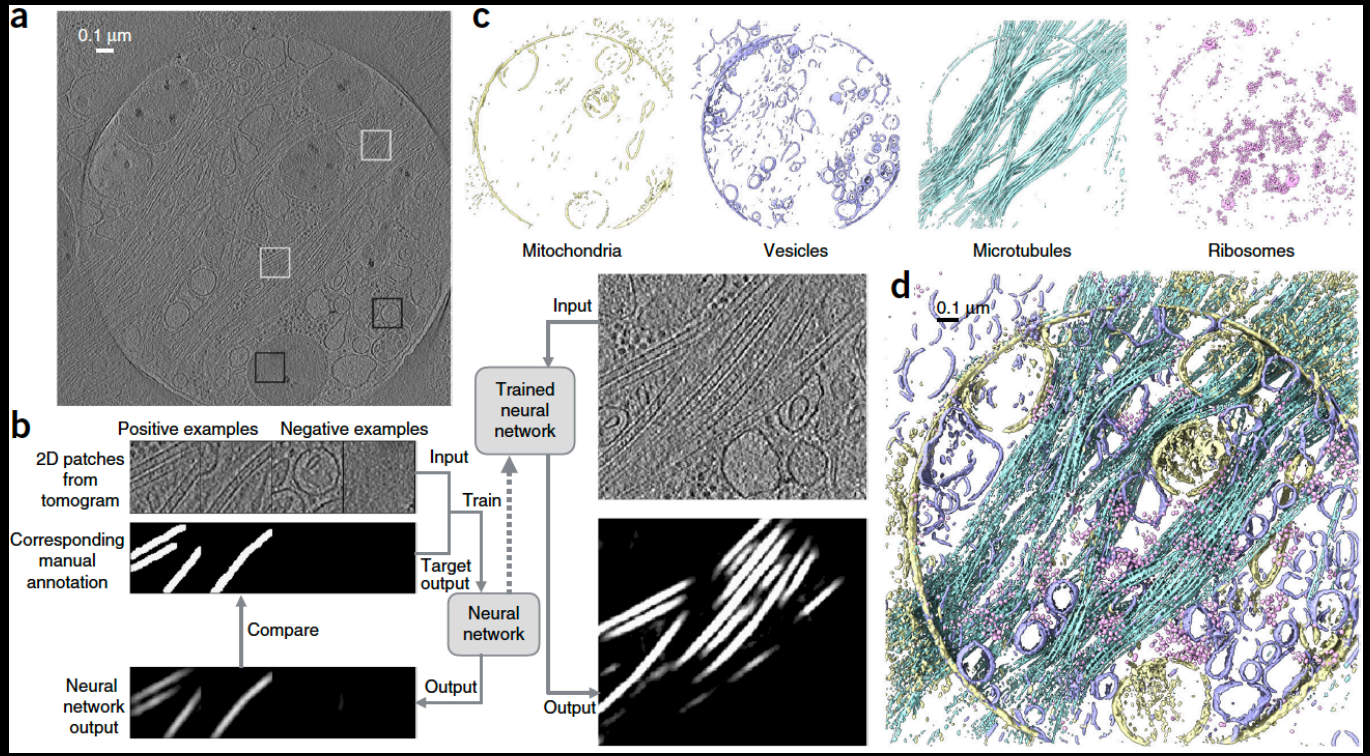
How will we solve the segmentation problem?



Mahamid et al. *Science* 2016

How will we solve the segmentation problem? Will deep learning methods help or are they over hyped?

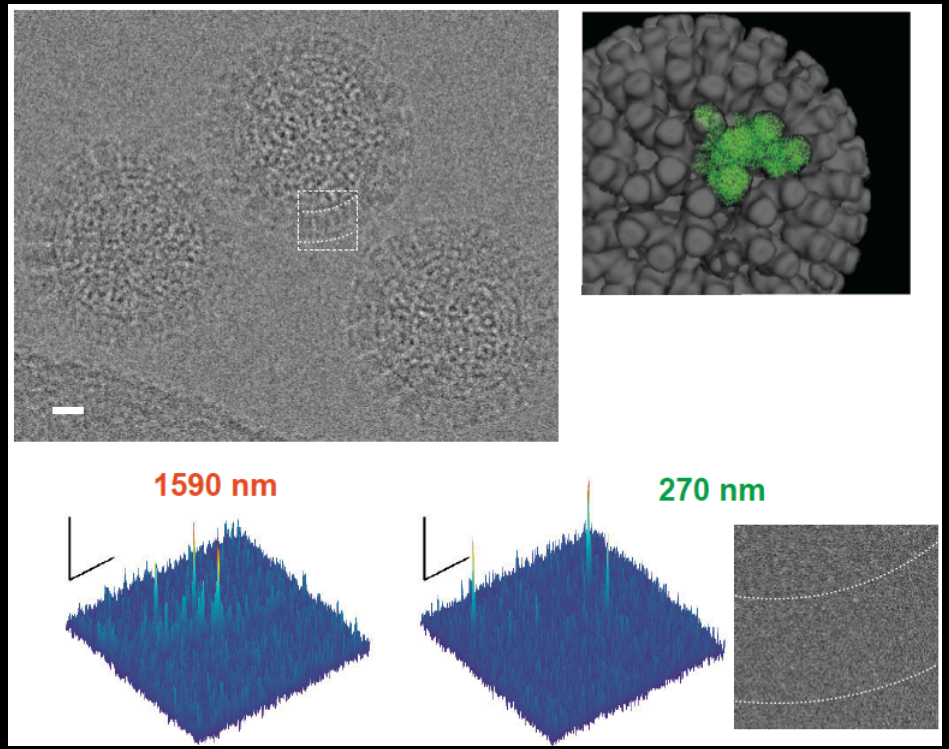
Automated annotation of cellular cryo-electron tomograms



Chen, Dai, Sun, Jonasch, He, Schmid, Chiu & Ludtke. *Nature Methods* 2017

How will we solve the segmentation problem? Will deep learning methods help or are they over hyped?

Single-protein detection in crowded molecular environments in cryo-EM images



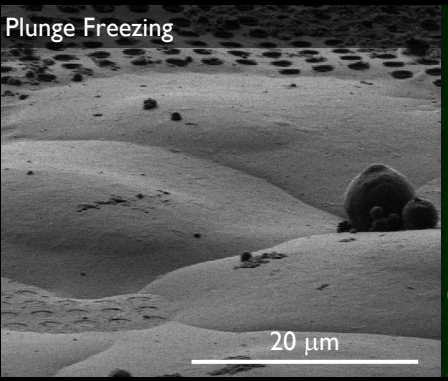
Rickgauer, Grigorieff, Denk. *eLife* 2017

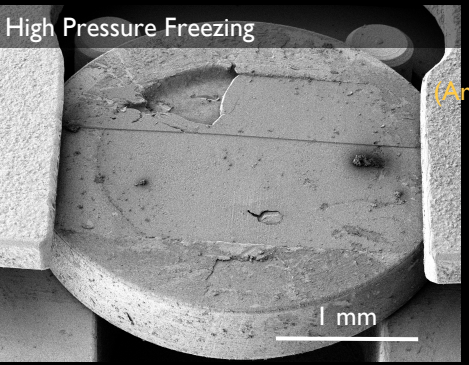
What is next for these challenging methods?

Cellular Cryo-Electron Tomography:
Sample Preparation Magic

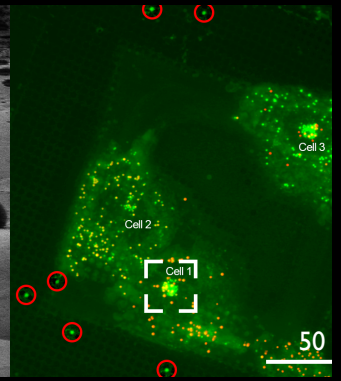
Vitrification
Structural Preservation

-180° C



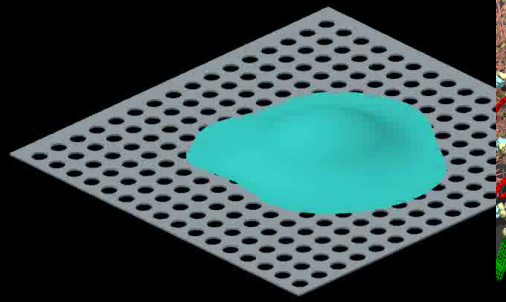


Cryo-Correlative
Fluorescence Microscopy:
3D Confocal Imaging



3D Correlative Microscopy
Targeted Cryo-FIB
(Arnold, Mahamid et al. Biophys J 2016)

Cryo-Focused
Ion Beam (FIB):
Site Specific Thinning

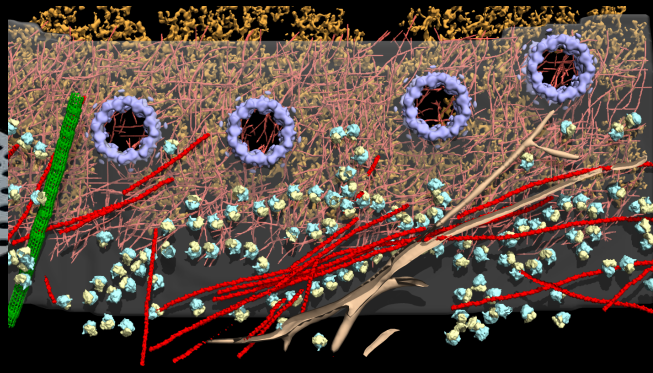


Optimization & Reproducibility
(Schaffer, Mahamid et al. JSB 2017)

Conductivity
(Mahamid et al. Science 2016)

Multicellular Organisms & Tissues
(Mahamid, Schampers et al. JSB 2015)

Cryo-Electron
Tomography:
3D Volume Imaging



Zero-loss energy filter
K2 direct detector
Volta phase plate
(Mahamid et al. Science 2016)

Animation by Tim Laugks , MPI of Biochemistry, Martinsried

What is next for these challenging methods?

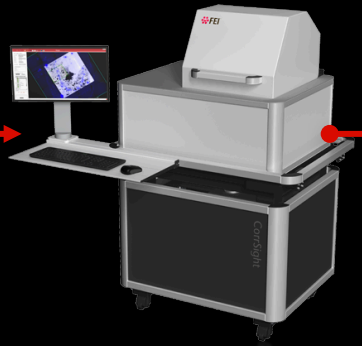
Vitrification
Plunge freezing

-180° C

Incomplete vitrification even in mammalian cell cultures

Forces from blotting lead to structural deformation

3D Cryo-Correlative
Fluorescence
Microscopy



Not commercially available

Done at atmospheric pressure

Low resolution

Cryo-Focused
Ion Beam

Systems are frequently home-made or heavily modified

Stages and imaging instability

In chamber condensation

Precise control over thickness

Conductivity

Extremely time consuming

Cryo-Electron
Tomography

Zero-loss Energy filter

K2 Direct detector

Volta phase plate

Automated acquisition is tricky

Routine pipelines for dose weighting and 3D CTF

Tilt series alignment (no fiducials)

Objective localization of structures of interest

3D averaging with particle polishing (at the level of the tilt series)

Sputter power &
control unit

Pt sputter

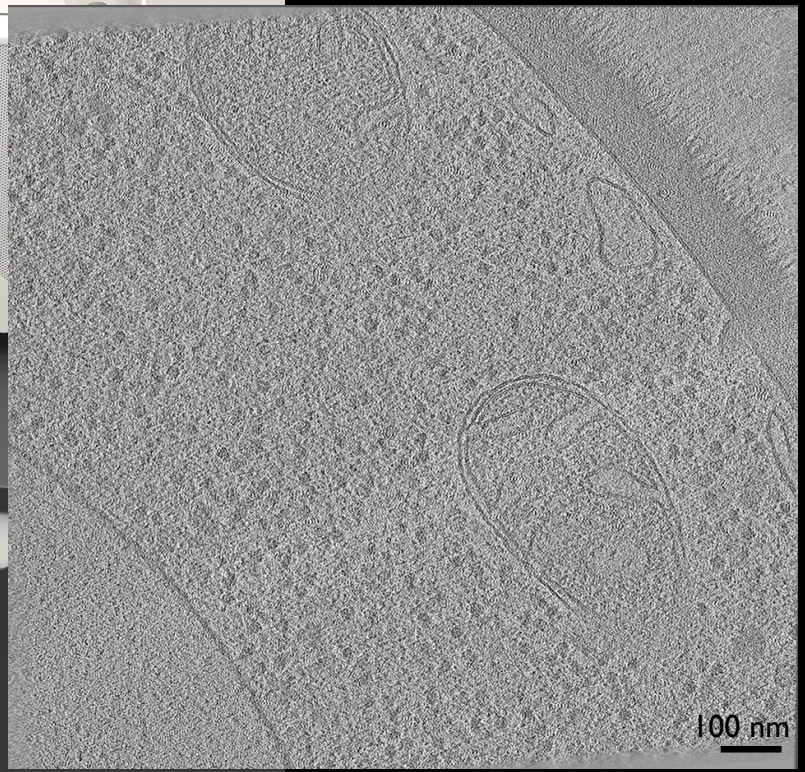
Airlock

Chamber pressure ~ 3

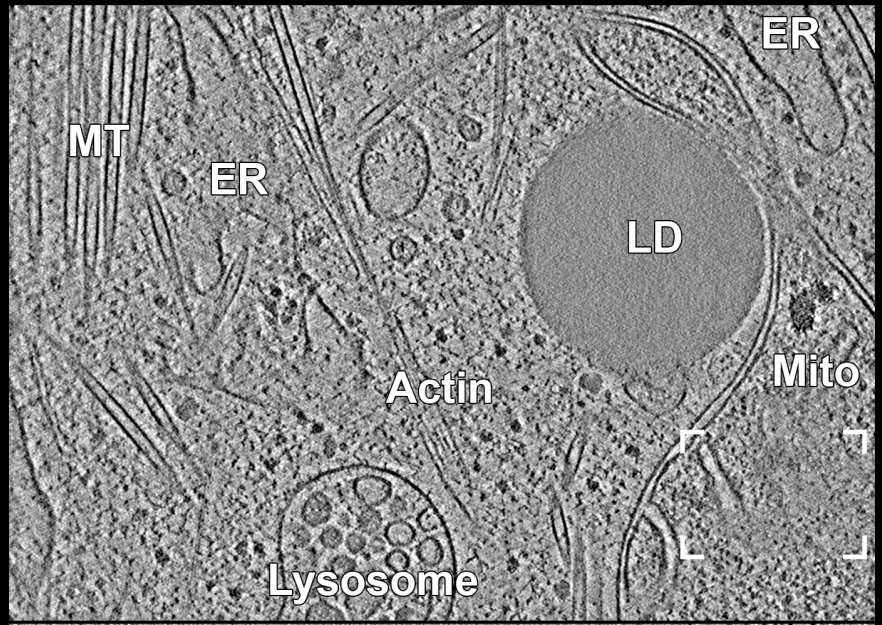
Would automation & high throughput become possible?

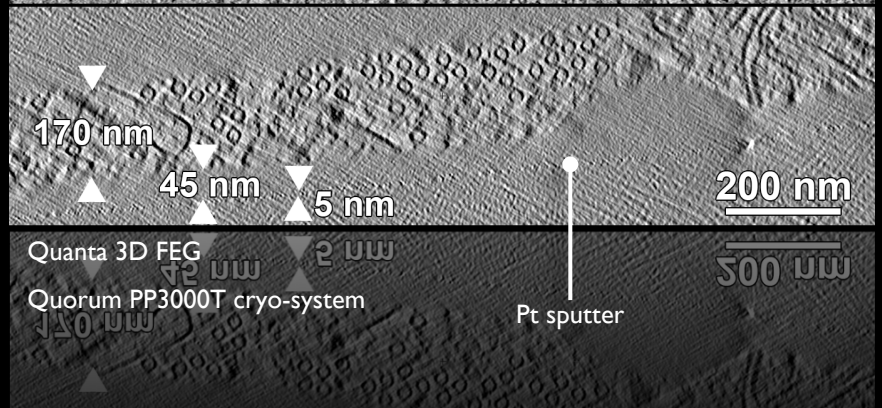
Stable stage & temperature
Better vacuum

Still need more characterization

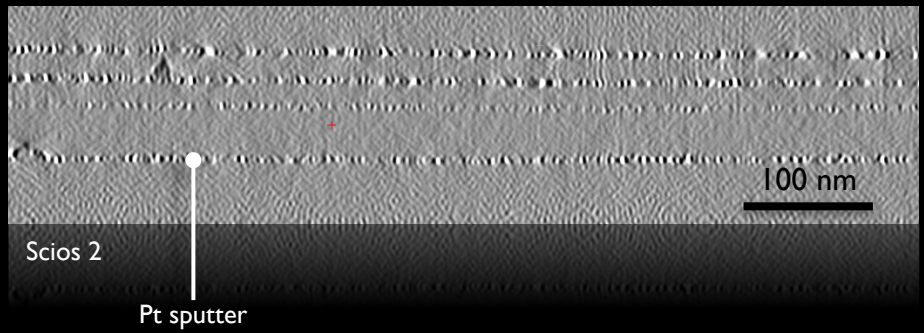


Cryo-FIB Lamella: Condensation Rates



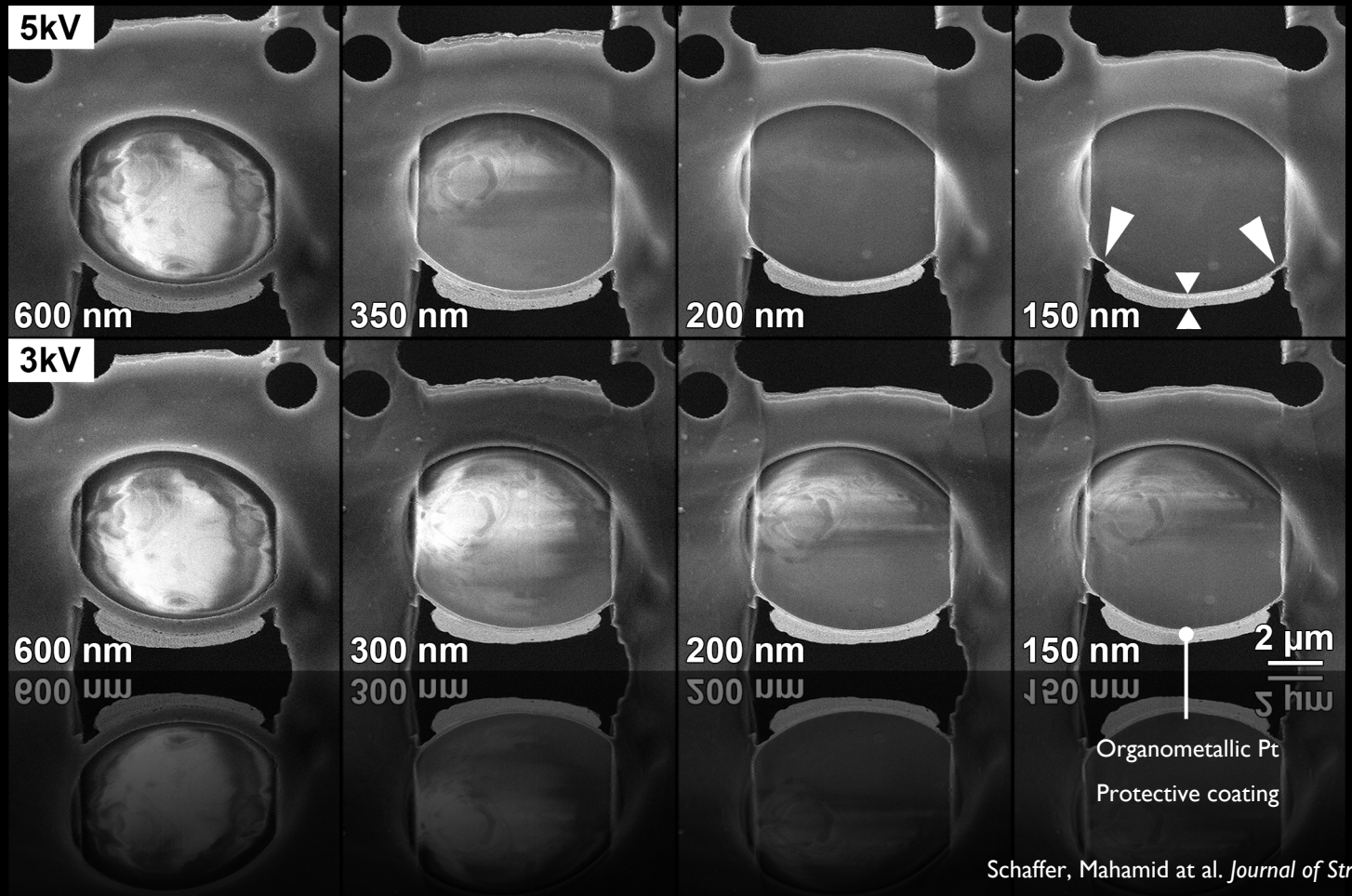


Condensation rates: 1-3 nm/min



Schaffer, Mahamid at al. *Journal of Structural Biology* 2017

Cryo-FIB Lamella: Thickness Calibration



Schaffer, Mahamid at al. *Journal of Structural Biology* 2017

How hard will it be to do accurate 3D localization for site-specific preparations with cryo-FIB and navigation of tomography data acquisition?

- High precision is needed to locate things
- High/Super- resolution is needed to separate signals that are close

3D Correlative Cryo-FLM & EM:
Aimed at Targeted FIB Milling

Bellow de-vitrification temperature (-135° C)

Thermal and mechanical stability

Avoid frost

Optical z-sectioning: spinning disk confocal microcopy

Adequate sensitivity

Appropriate fiducial markers: overcoming the resolution limit

Schorb and Briggs. *Ultramicroscopy* 2014

Schellenberger…Grünewald. *Ultramicroscopy* 2014

Computing coordinate transformation

From 3D LM stacks to 2D topographic images

Arnold, Mahamid et al. *Biophysical Journal* 2016

3D Correlative Cryo-FLM & EM:
Aimed at Targeted FIB Milling

Bellow de-vitrification temperature (-135° C)

Thermal and mechanical stability

Avoid frost

Optical z-sectioning: spinning disk confocal microcopy

Adequate sensitivity

Appropriate fiducial markers: overcoming the resolution limit

Schorb and Briggs. *Ultramicroscopy* 2014

Schellenberger…Grünewald. *Ultramicroscopy* 2014

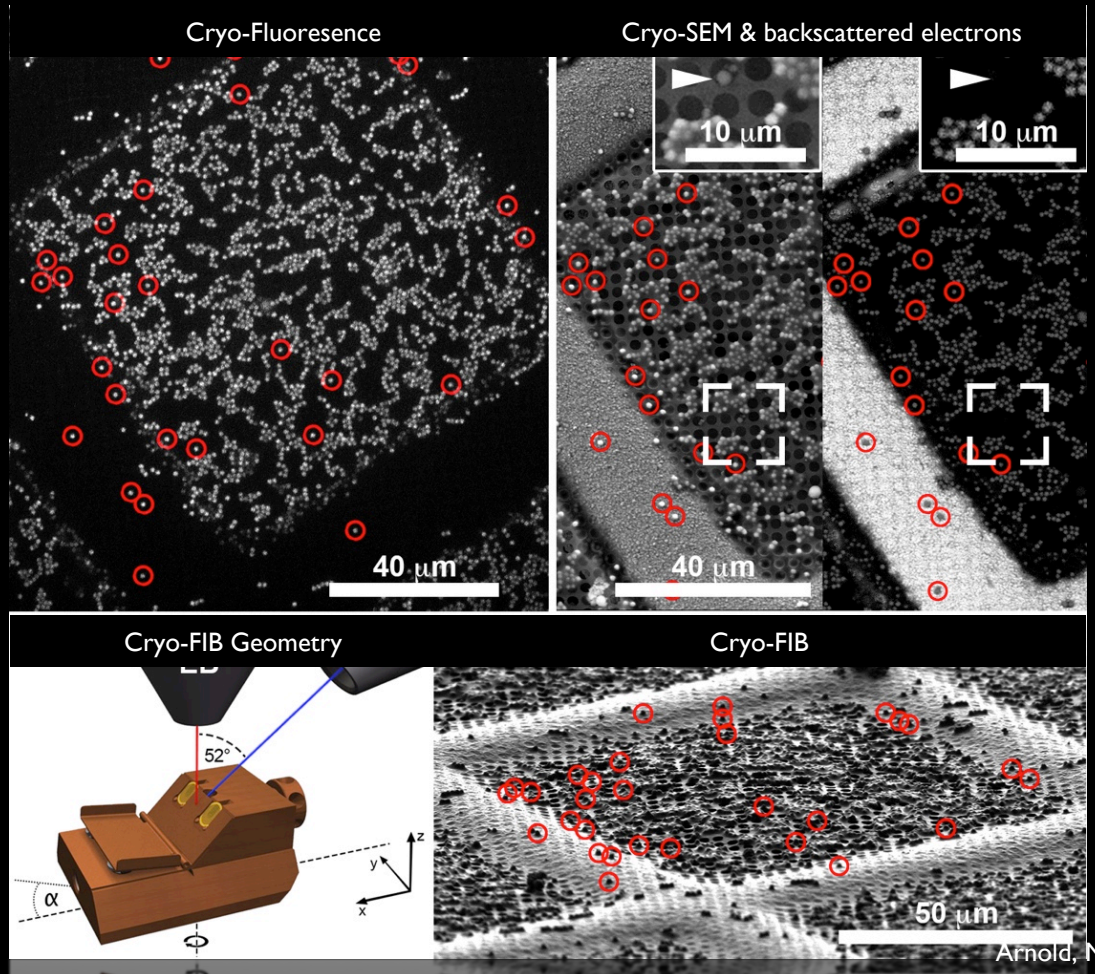
Computing coordinate transformation

From 3D LM stacks to 2D topographic images

Reliably recall positions in FIB/SEM, avoid (stage/image) drift during milling

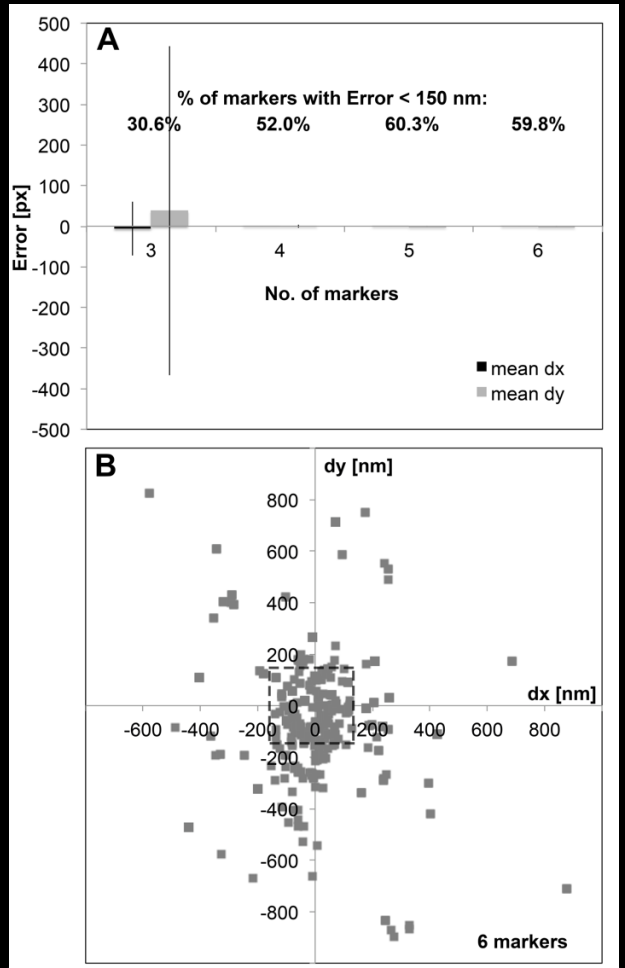
Arnold, Mahamid et al. *Biophysical Journal* 2016

3D Correlative Cryo-FLM & EM:
Fiducial Markers



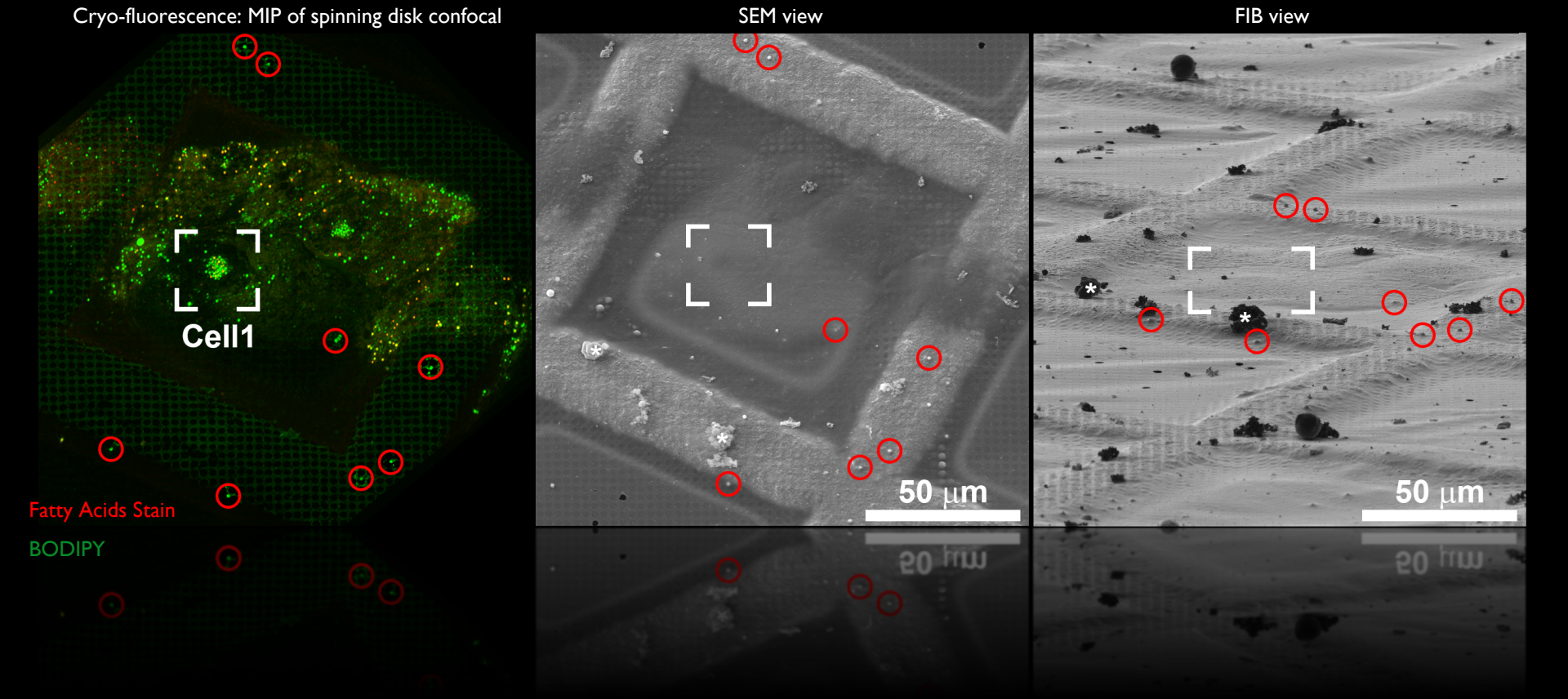
Arnold, Mahamid et al. *Biophysical Journal* 2016

3D Correlative Cryo-FLM & EM:
Correlation Accuracy



Arnold, Mahamid et al. *Biophysical Journal* 2016

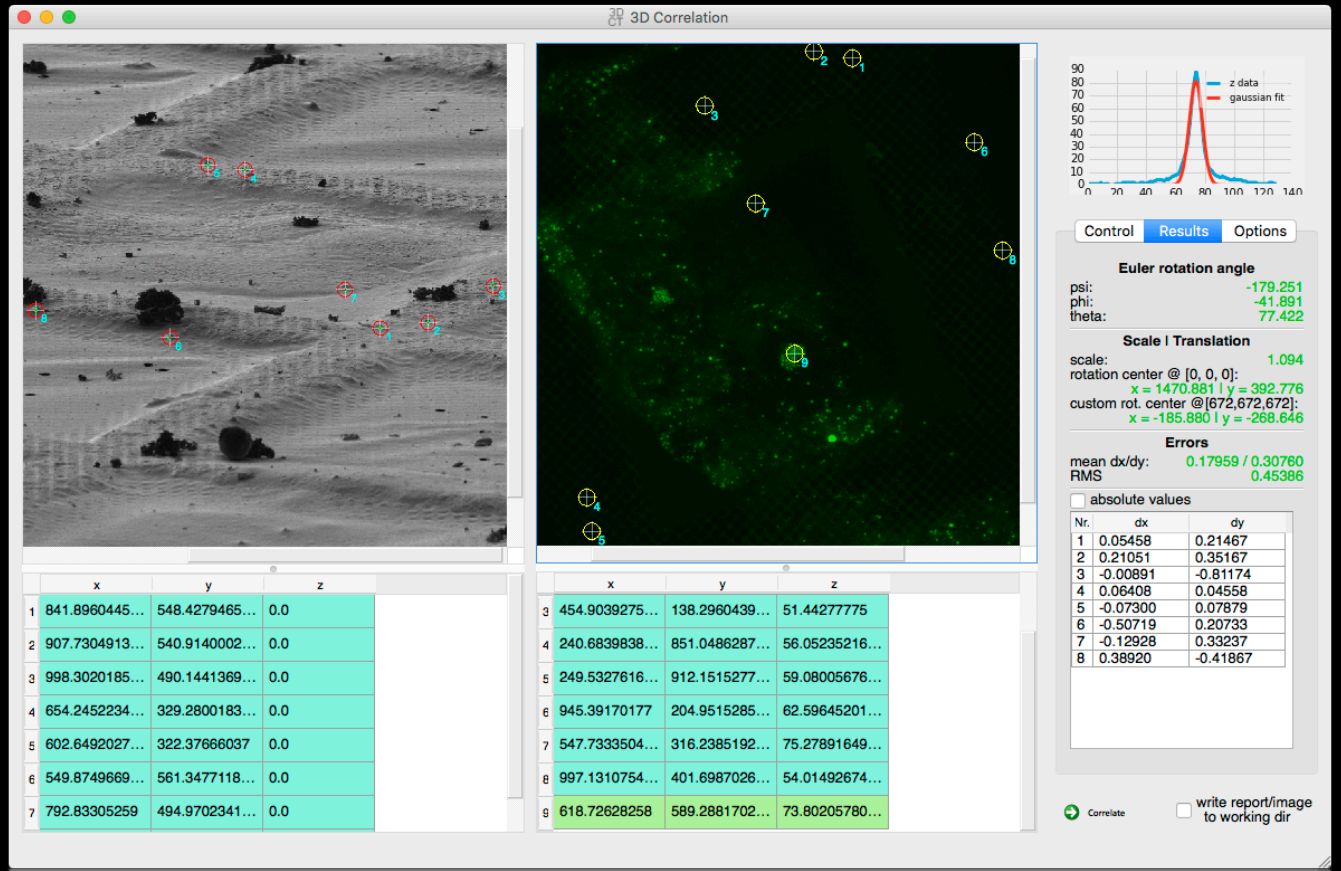
3D Correlative Cryo-FLM & EM:
Targeted FIB Milling in Cellular Samples



https://3dct.semper.space/

Mahamid, Arnold & Plitzko. *Microscopy and Microanalysis* 2017

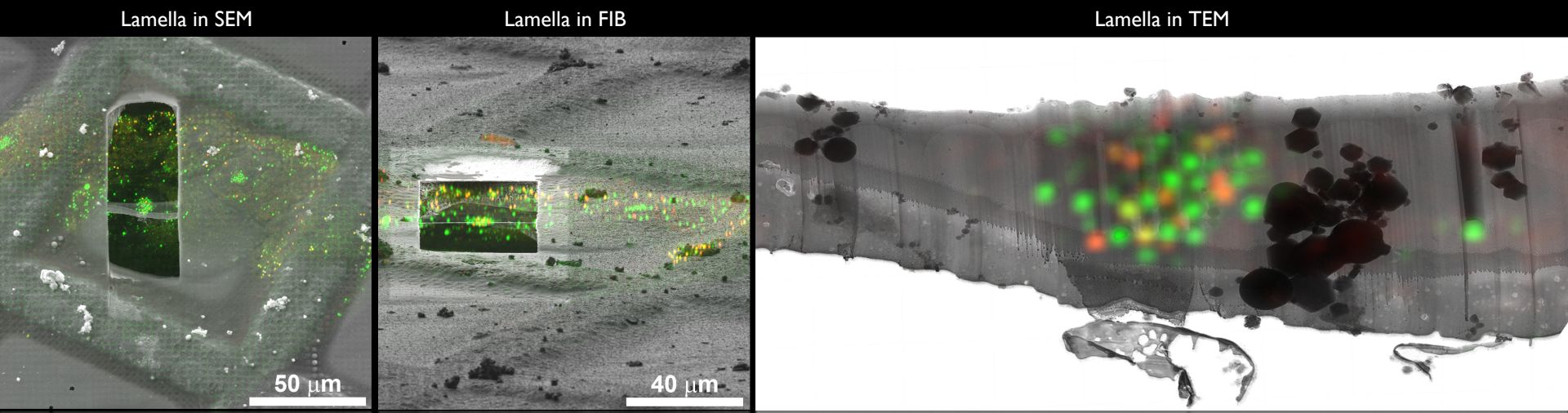
3D Correlative Cryo-FLM & EM:
Online Targeted FIB Milling



https://3dct.semper.space/

Mahamid, Arnold & Plitzko. *Microscopy and Microanalysis* 2017

3D Correlative Cryo-FLM & EM:
Targeted FIB Milling and TEM Navigation



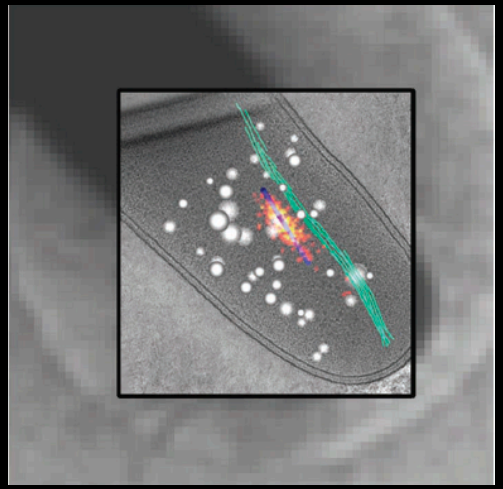
https://3dct.semper.space/

Mahamid, Arnold & Plitzko. *Microscopy and Microanalysis* 2017

Will super-resolution cryo-LM become a reality?

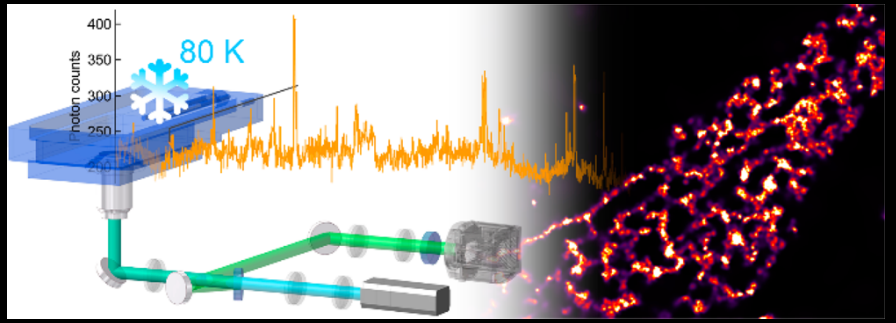
Cryo-PALM (photoactivated localization microscopy)

PA-GFP; NA of 0.7; 5% Ficoll PM 70 as a cryoprotectant



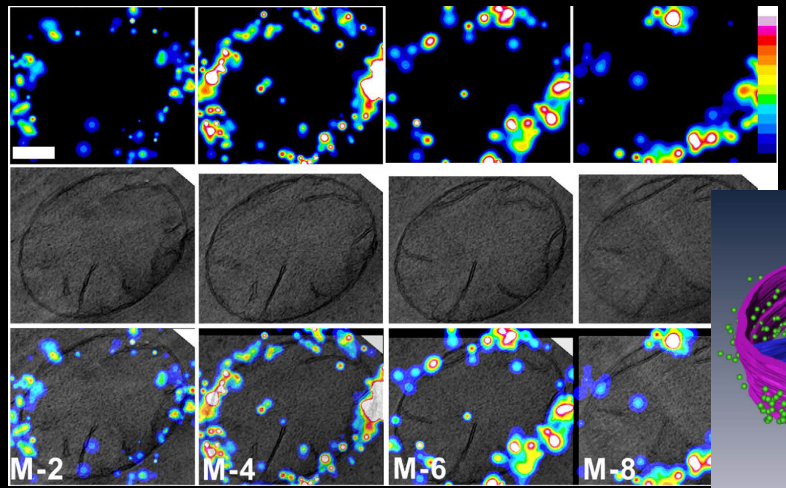
Chang, Chen, Tocheva, Treuner-Lange, Löbach, Søgaard-Andersen & Jensen. *Nature Methods* 2014

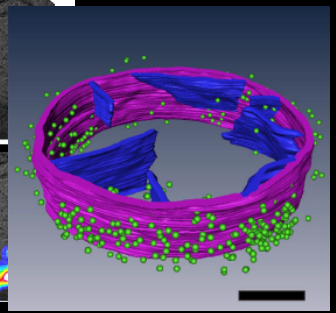
Cryo-single molecule localization microscopy; mVenus; NA of 0.75



Kaufmann, Schellenberger, Seiradake, Dobbie, Jones, Davis, Hagen, Grünewald. Nano Letters 2014

Cryo-PALM; Dronpa; NA of 0.8; cryo-sections



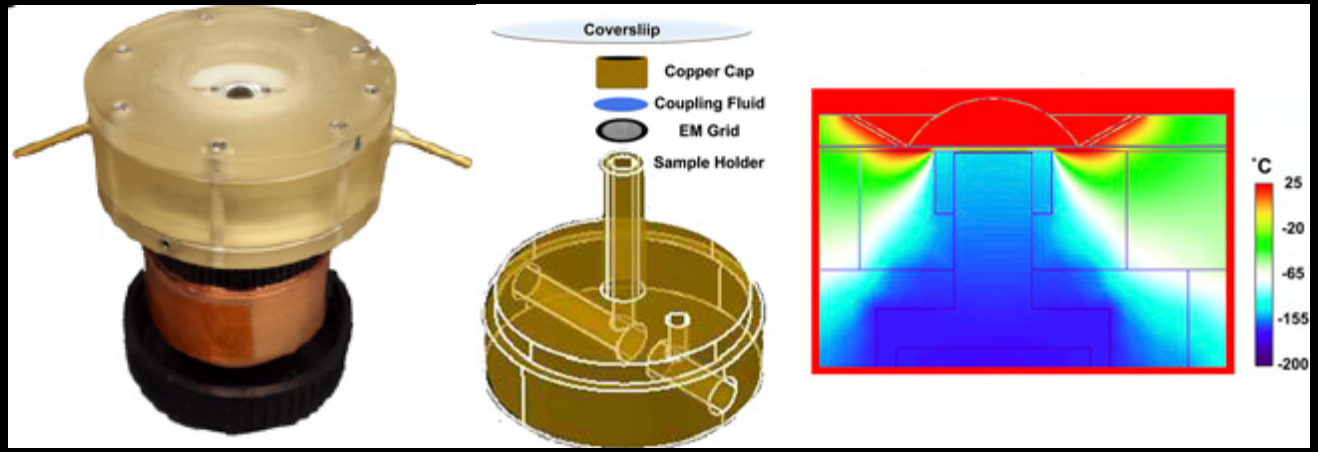


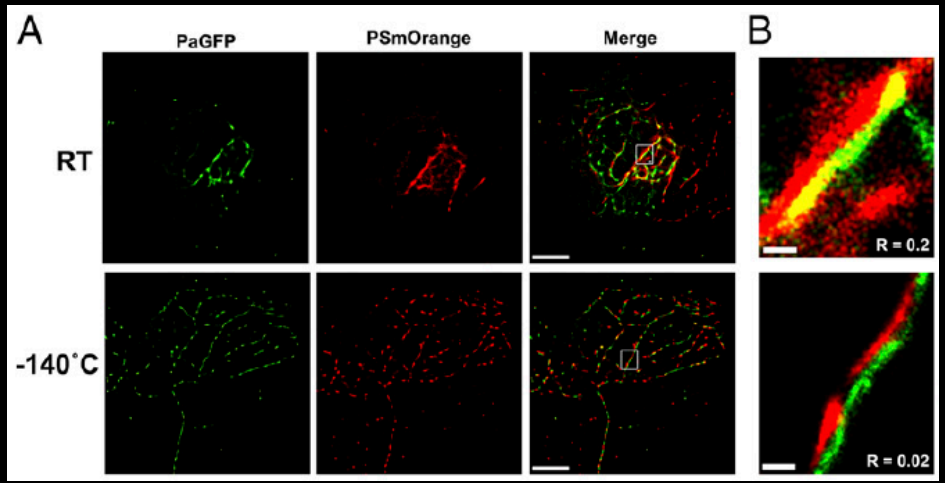
Liu, Xue, Zhao, Chen, Fan, Gu, Zhang, Zhang, Sun, Huang, Ding, Sun, Ji & Xu. *Scientific Reports* 2015

Wolff, Hagen, Grunewald, Kaufmann. *Biol. Cell* 2016

Will super-resolution cryo-LM become a reality?

Cryo-PALM; PaGFP and PSmOrange; organic solvent immersion; NA of 1.2;





Nahmani, Lanahan, DeRosier, Turrigiano. *PNAS* 2017

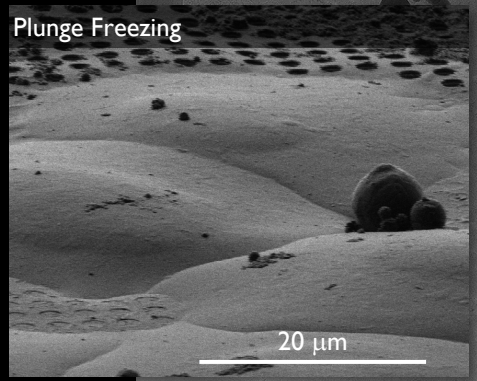
Reiner Kaufmann, Hamburg

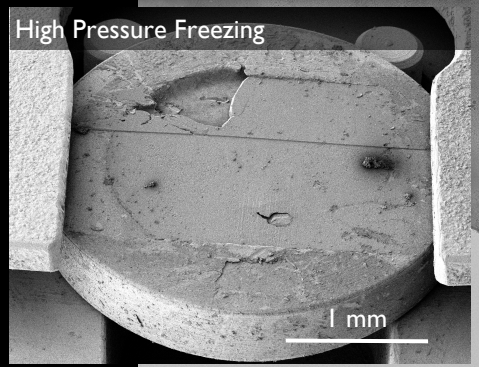
David Hoffman, Harald Hess, Eric Betzig, Janelia

Will high-pressure freezing and FIB lift-out become routine for bulk specimens?

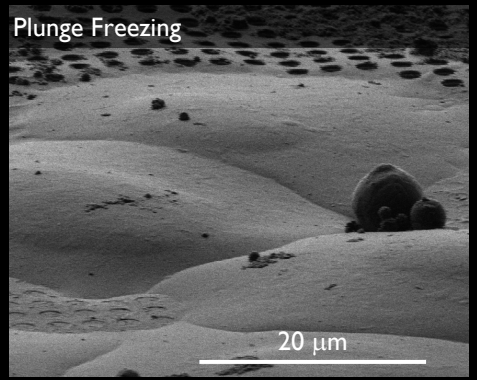
- The ultimate objective is to freeze the specimen so rapidly (at 10^4 to 10^6 K/s) that ice crystals are unable to form – limited to a few micrometers thickness
- At a pressure of 2000 bar the freezing point of water drops to -22° C; one achieves a depth of vitrification of ~200 μm

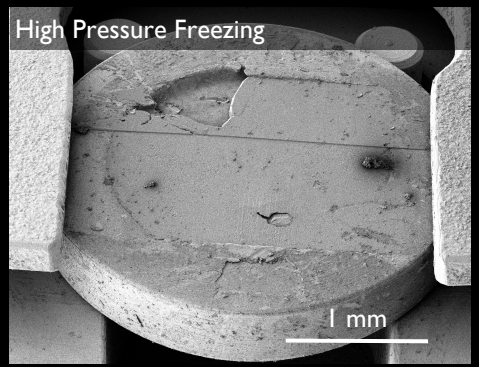
McDonald, Schwarz, Müller-Reichert, Webb, Buser, Morphew. Methods Cell Biol. 2010

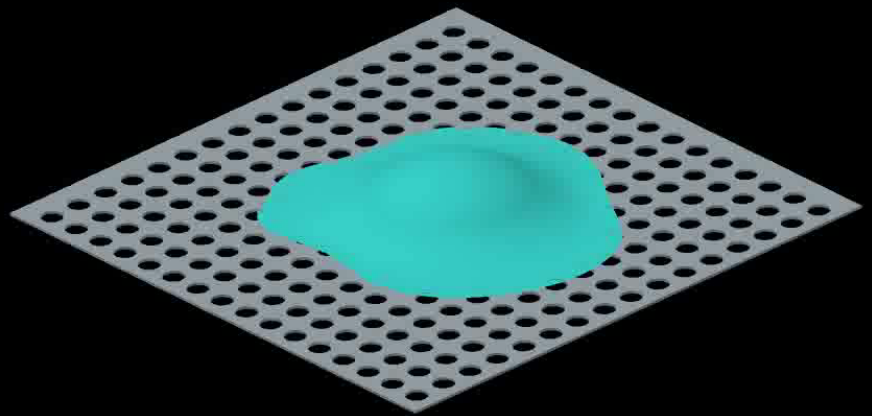




2 μm



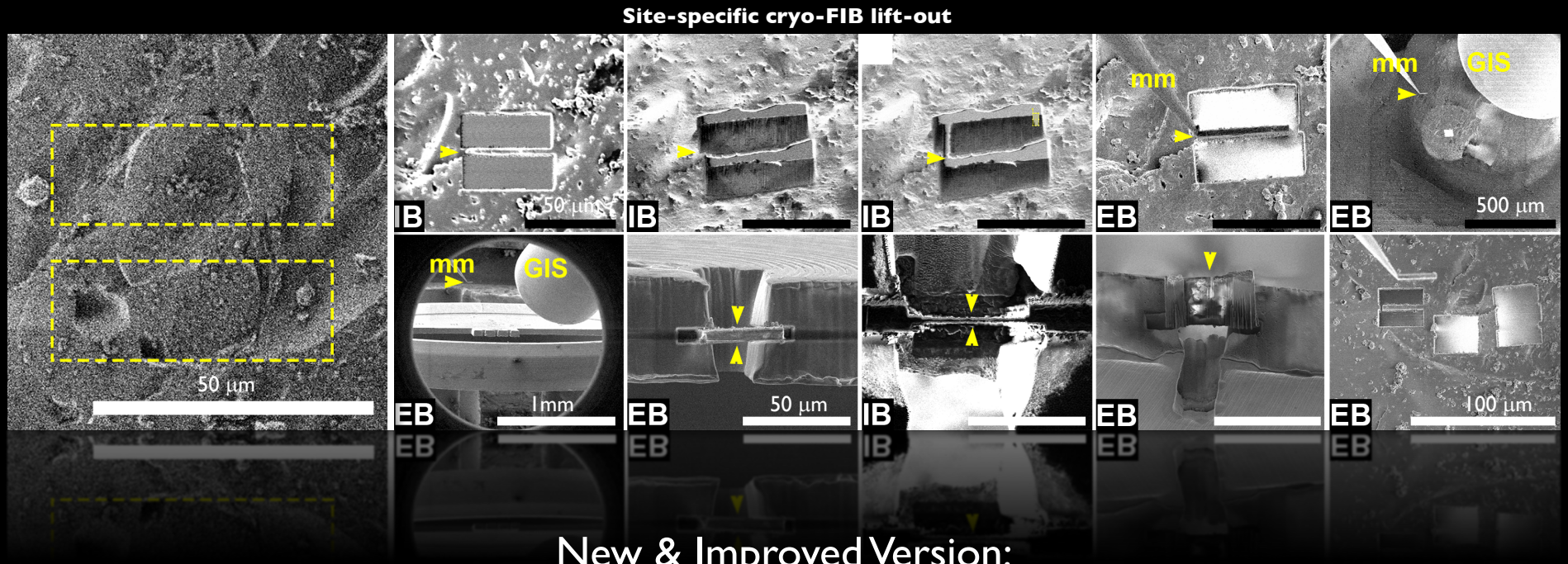




Rigort, Baeuerlein, Villa, Eibauer, Laugks, Baumeister & Plitzko, *PNAS 2012*

Animation by Tim Laugks , MPI of Biochemistry, Martinsried

Cryo-FIB Lift-out: 'Biopsies' on the Micron Scale



New & Improved Version:

Miroslava Schaffer, In Collaboration with Kleindiek

Mahamid, Schampers et al. *Journal of Structural Biology*, 2015

Will high-pressure freezing and FIB lift-out become routine for bulk specimens?

The real question: is it going to happen anytime soon?

Bulk specimen polishing (automated cryo-ultramicrotomy/plasma)

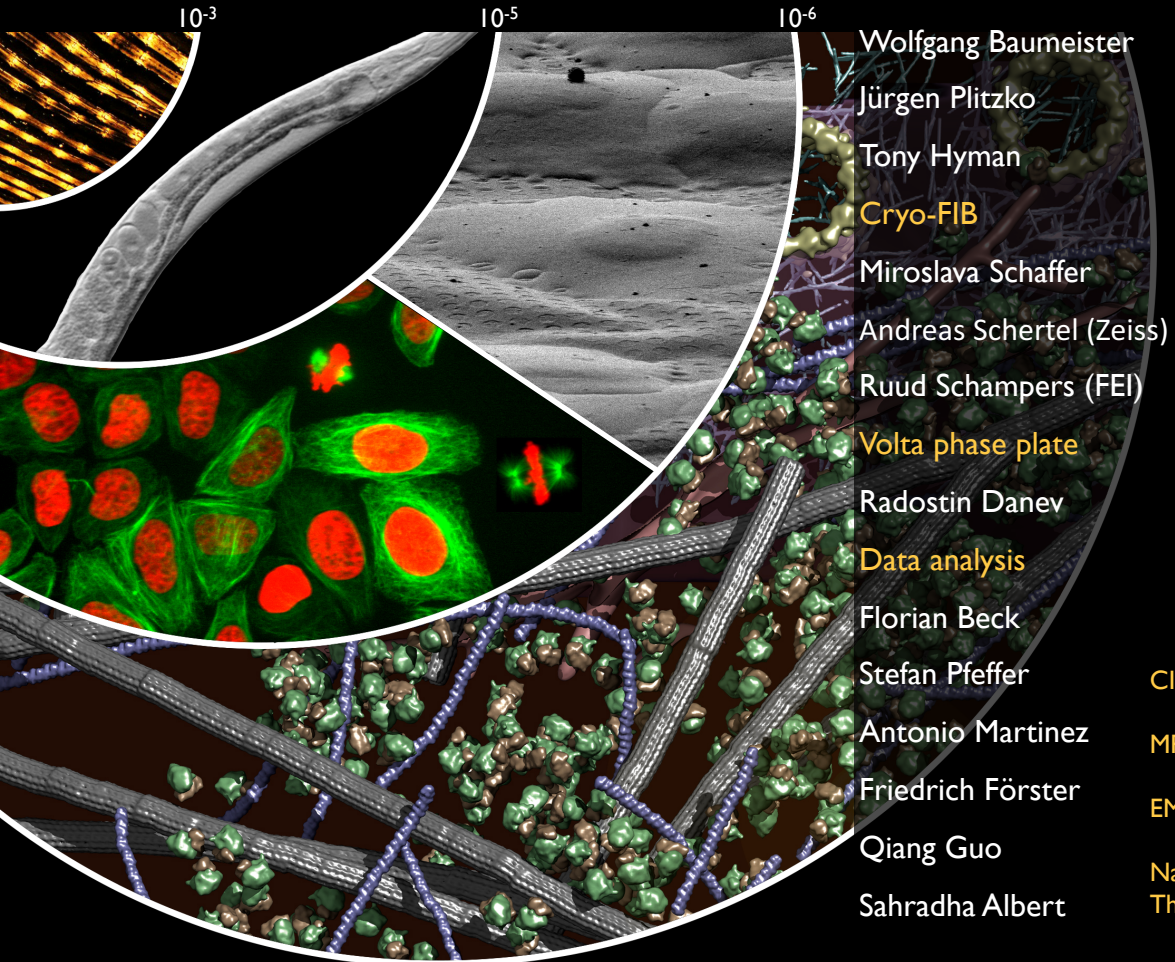
Precise correlation approaches

Faster milling sources (initial preparation of trenches)

Fabrication/micro-printing of carriers

Reliable attachment of lamellae to carrier

Challenges and Opportunities:
Cellular Cryo-ET



Wolfgang Baumeister

Jürgen Plitzko

Tony Hyman

Cryo-FIB

Miroslava Schaffer

Andreas Schertel (Zeiss)

Ruud Schampers (FEI)

Volta phase plate

Radostin Danev

Data analysis

Florian Beck

Stefan Pfeffer

Antonio Martinez

Friedrich Förster

Qiang Guo

Sahradha Albert

Retina project

Matthias Pöge

Krzysztof Palczewski

Sanae Sakami

Ning Zhang

MPIB Animal facility

3D Correlation

Jan Arnold

Alex de Marco

Vladan Lucic

Tobias Mayer

Tim Laugks

CIFAR

MPIB Junior Research Award

EMBO & HFSP Postdoctoral Fellowships

National Postdoctoral Award for Women in Science – The Weizmann Institute of Science