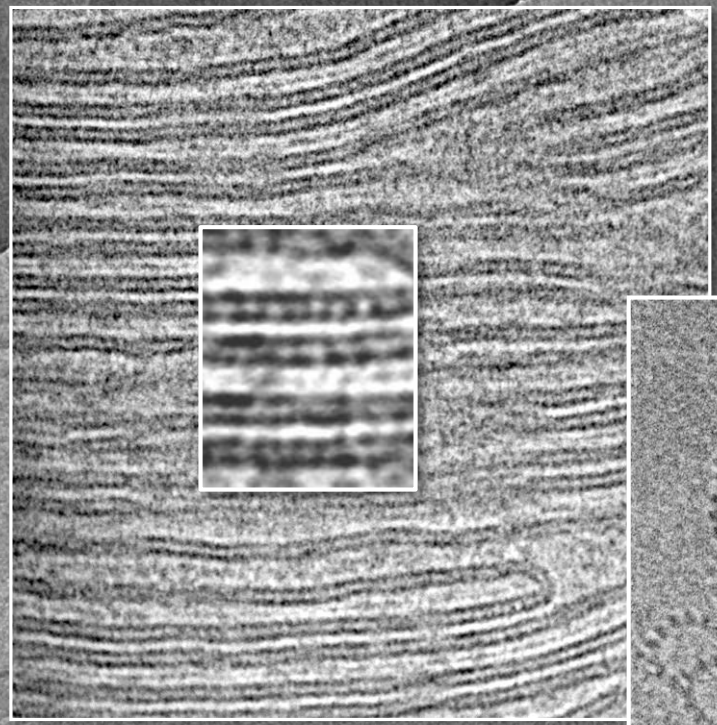
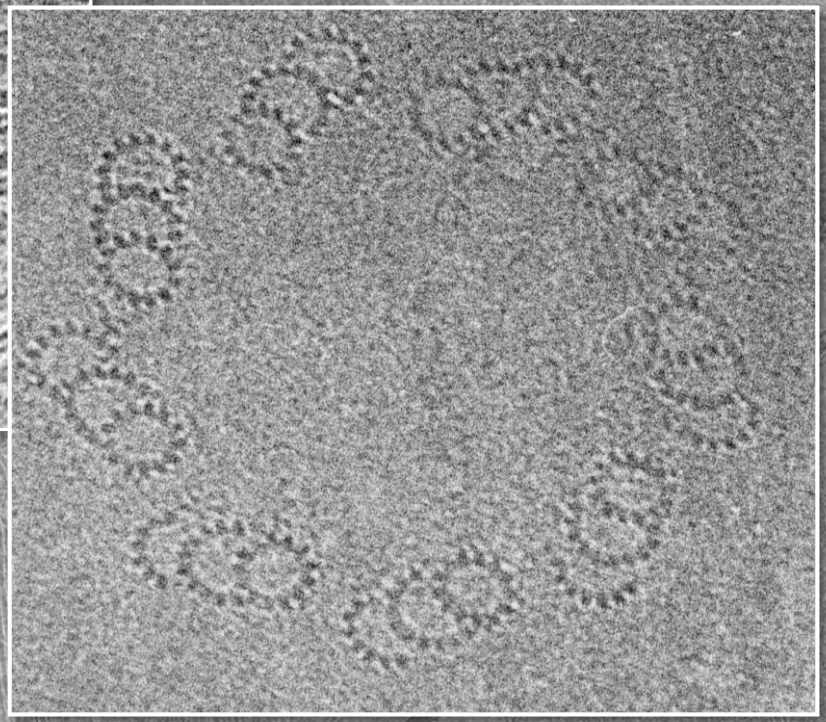
Structural biology *in situ*

Cryo-Electron Microscopy of the Cell





Amélie Leforestier, Laboratoire de Physique des Solides, UPS, Orsay

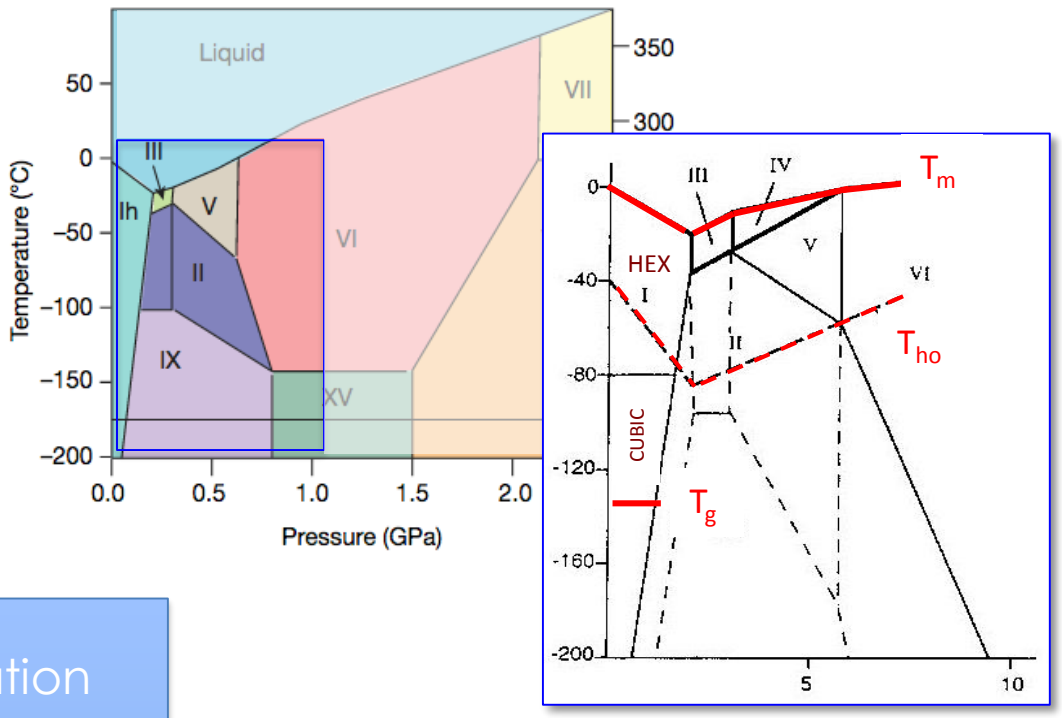
Outline

SAMPLE PREPARATION
- vitrification of massive hydrated specimens
- thinning specimens(electron lucent)

IMAGING
- a new cellular landscape
- 3D structures of unique objects: cryo-tomography
- targetting molecules of interest: cryoCLEM

OTHER CRYO-IMAGING METHODS
- cryoSTEM
- cryo X ray microscopy

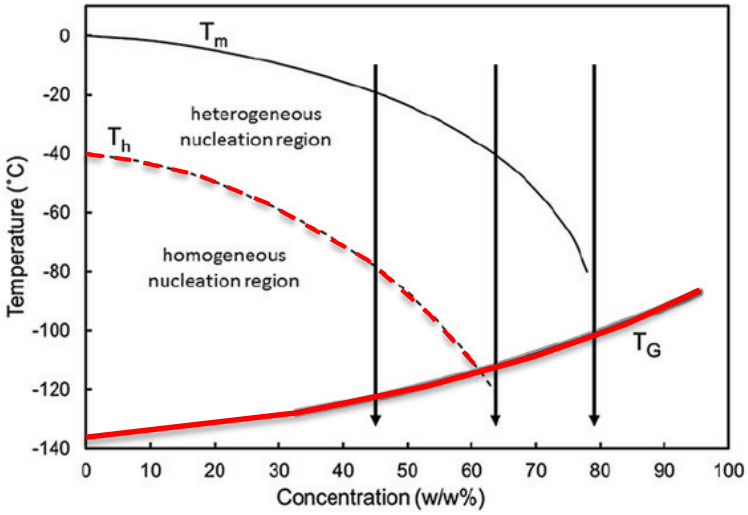
Vitrification



Cooling rate > crystallisation

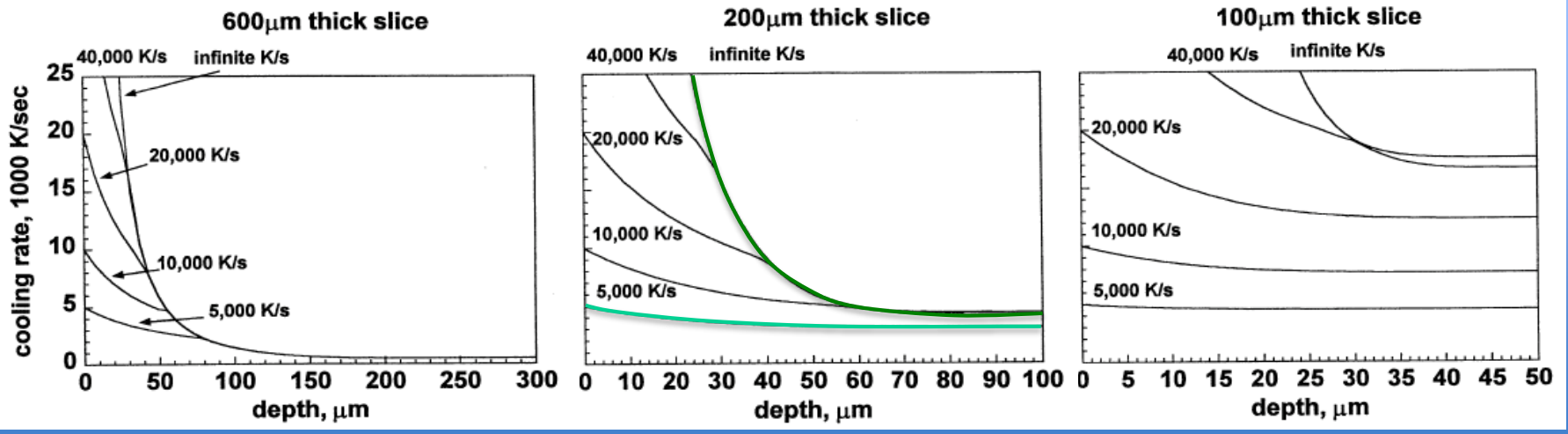
$\Delta T = T_{ho} - T_g$

	100 % water	70% water
1 bar	10^5-10^6 Ks^{-1}	10^4 Ks^{-1}
2045 bars	10^4 Ks^{-1}	10^2-10^3 Ks^{-1}



Dubochet et al, 1988; Studer et al, 2001 ; Vanhecke et al, 2007

Water & biological specimens: poor thermal conductors

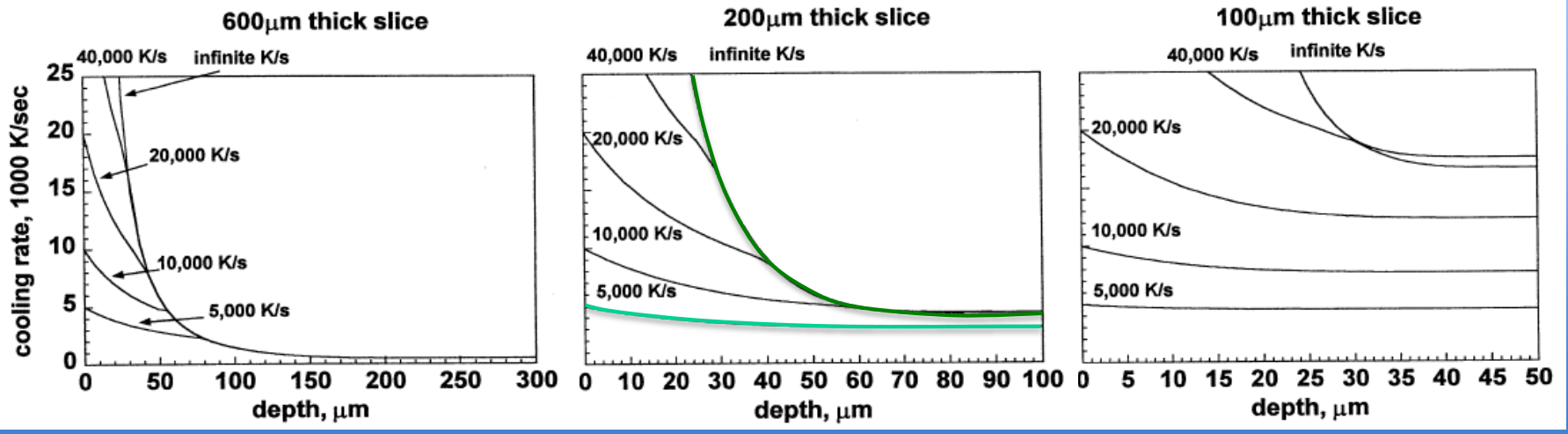


Studer et al, 1995

	100 % water	70% water
1 bar	10^5-10^6 Ks^{-1}	>10^4 Ks^{-1}
2045 bars	10^4 Ks^{-1}	>10^2 -10^3 Ks^{-1}

+ nucleation & growth of ice crystals in deep zones = exothermic process, which in turn slow down cooling rate
(Escaig, 1982)

Water & biological specimens: poor thermal conductors



Studer et al, 1995

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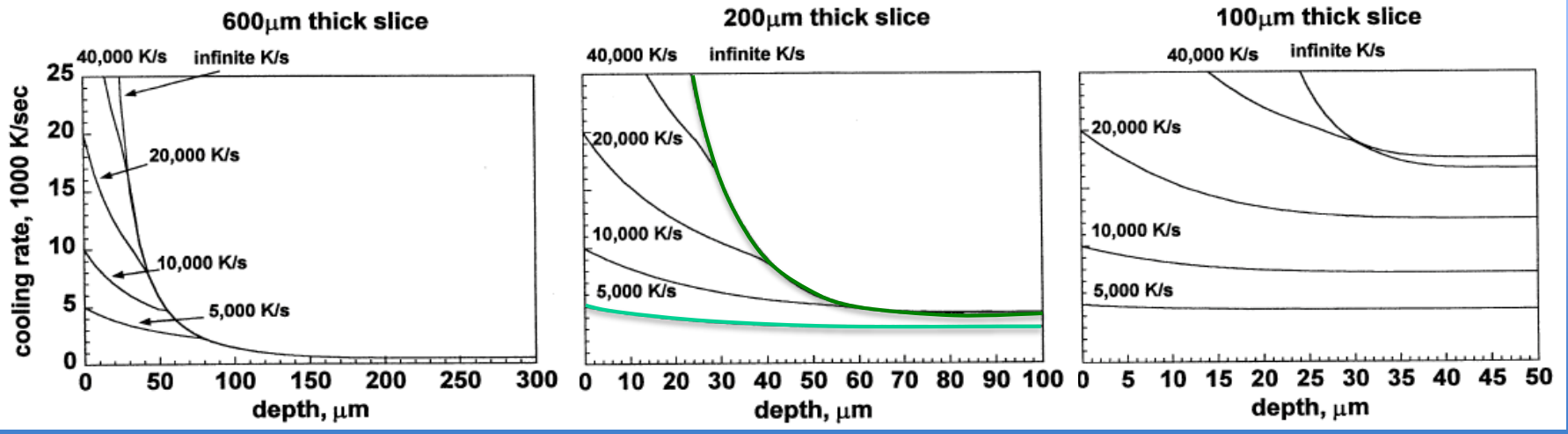
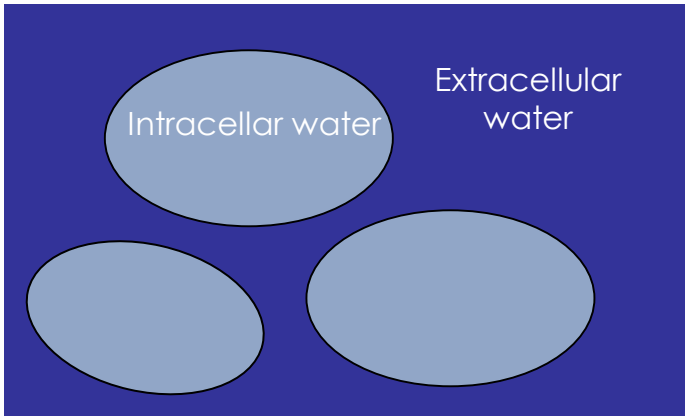
1 bar	10^5-10^6 Ks^{-1}	>10^4 Ks^{-1}
2045 bars	10^4 Ks^{-1}	>10^2-10^3 Ks^{-1}

No pure water bulk vitrification
Water content ≥ 80 %

Water & biological specimens: poor thermal conductors



Studer et al, 1995



Addition of cryo-protectants

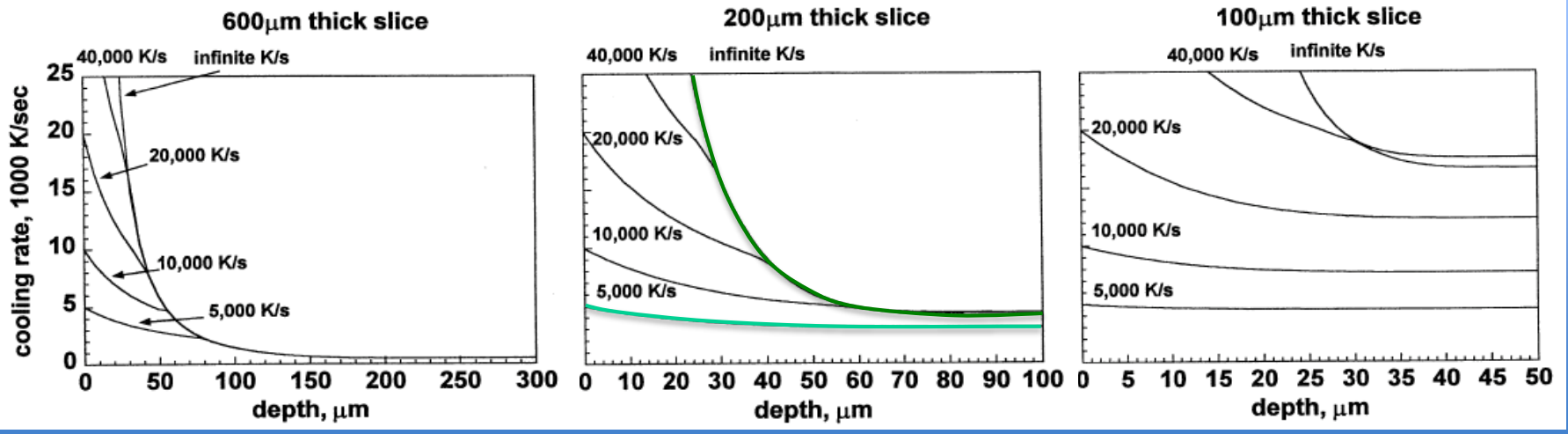
glycerol
glucose
threhalose

toxicity
sectionning
imaging

dextran
PEG
BSA

osmotic stress

Water & biological specimens: poor thermal conductors



Studer et al, 1995

	100 % water	70% water
1 bar	10^5-10^6 Ks^{-1}	>10^4 Ks^{-1}
2045 bars	10^4 Ks^{-1}	>10^2-10^3 Ks^{-1}

@ 1 bar

Plunge Freezing (ethane)

1-3 μm

Jet freezing ? (ethane)

Slam Freezing (helium)

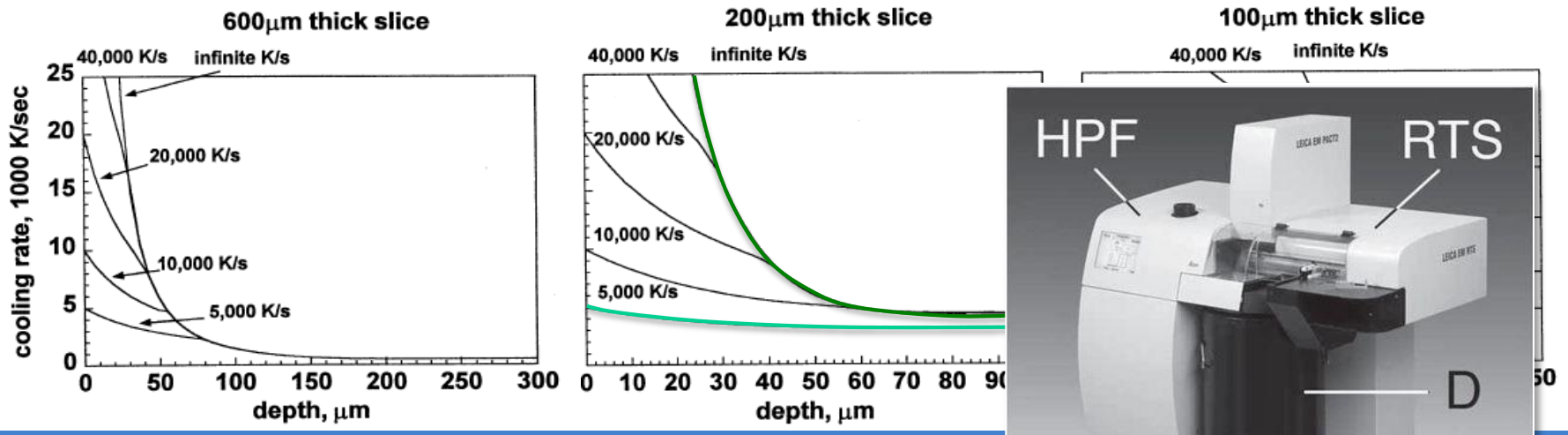
10-20 μm

@ 2045 bar

High Pressure Freezing (HPF)

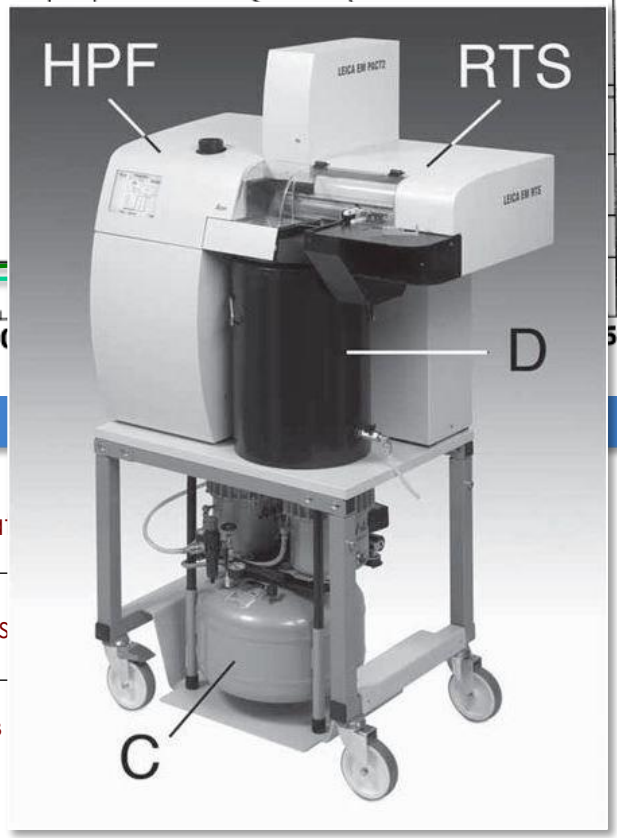
100-200 μm

Water & biological specimens: poor thermal conductors



Studer et al, 1995

	100 % water	70% wa
1 bar	10^5-10^6 Ks^{-1}	>10^4 Ks
2045 bars	10^4 Ks^{-1}	>10^2-10^3



@ 1 bar

@ 2045 bar

Plunge Freezing (ethane)

Slam Freezing (helium)

High Pressure Freezing (HPF)

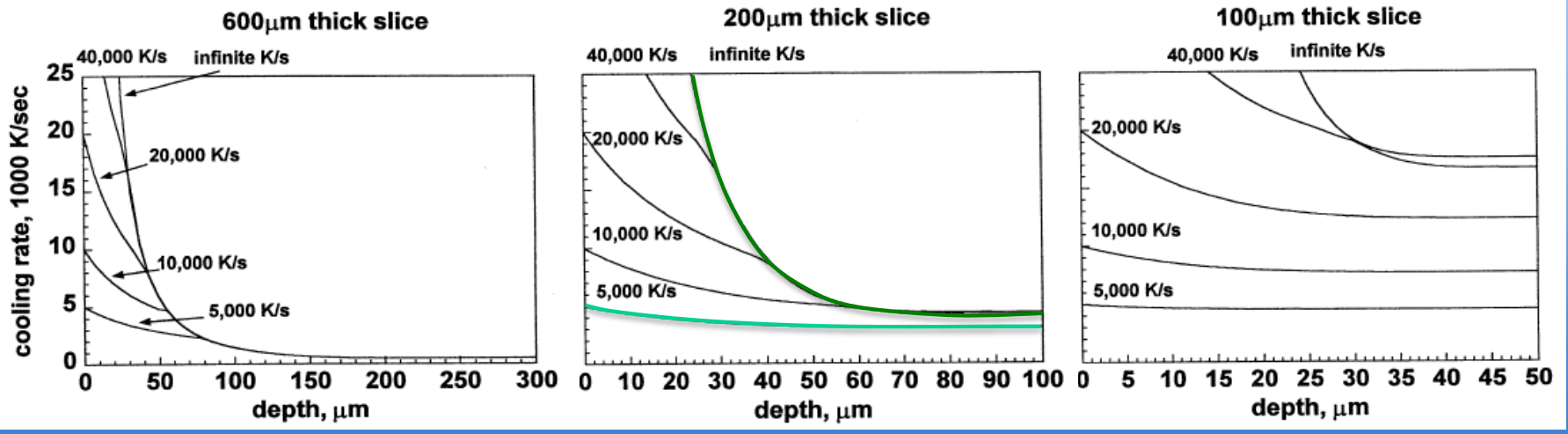
1-3 μm

Jet freezing ? (ethane)

10-20 μm

100-200 μm

Water & biological specimens: poor thermal conductors



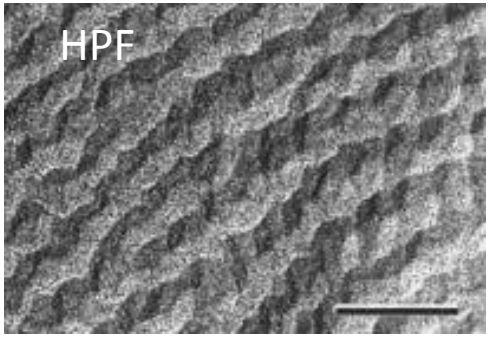
Studer et al, 1995

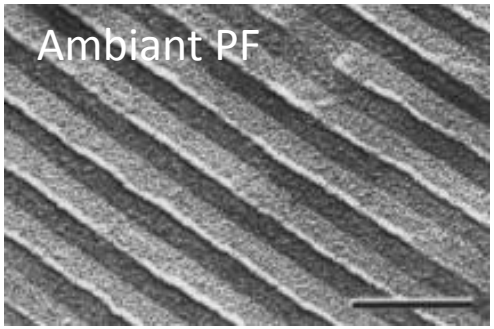
cell extensions, bacteria, picoeukaryotes	isolated cells, bacteria, peripheral tissues	cells, tissues, organs, organisms
@ 1 bar		@ 2045 bar
Plunge Freezing (ethane)	Slam Freezing (helium)	High Pressure Freezing (HPF)
1-3 μm	10-20 μm	100-200 μm

Problems ?

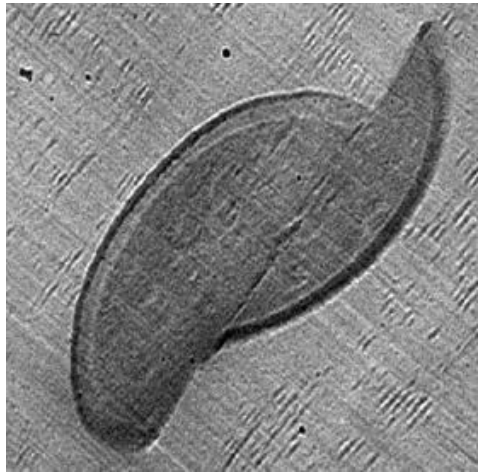
Phase transitions (DNA, lipids)	Cell techtonics	High density amorphous water *vs* LDA

Leforestier *et al*, 1992; Semmler et al, 1995

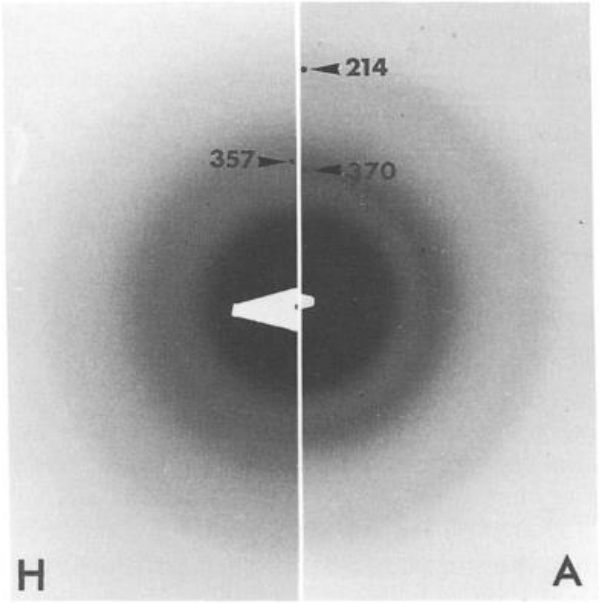




Dubochet et al, 2007



Richter, 1994



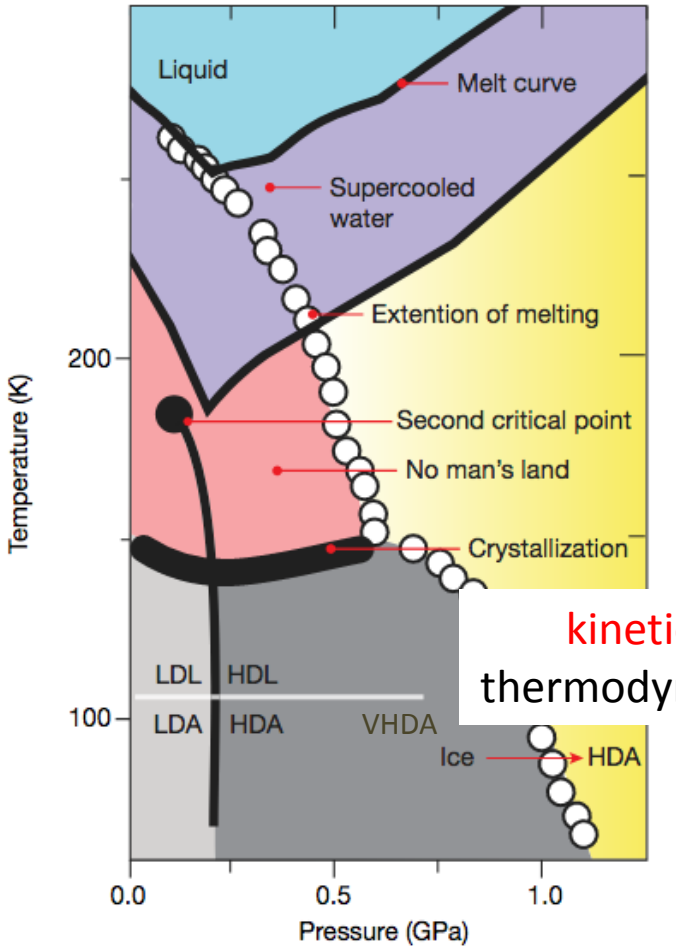
0.94 g/cm^3 1.15 g/cm^3

imaging

Vitreous does not (always) mean the native state is preserved

Problems, … and perspectives ?

Voyage through water poly(a)morphs ?



Tulk et al, Nature 2019

kinetics

thermodynamics

✓ Transitions upon pressure changes
✓ Transitions upon temperature changes !
✓ Transitions upon beam irradiation

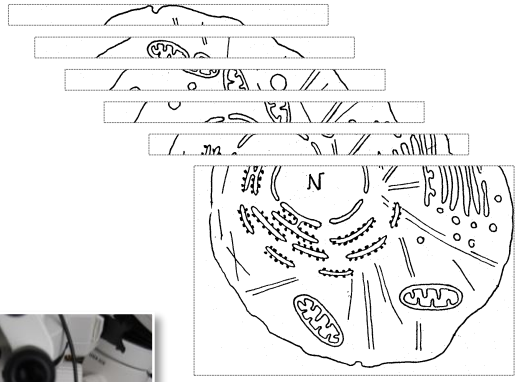
Check the state of water of your sample !

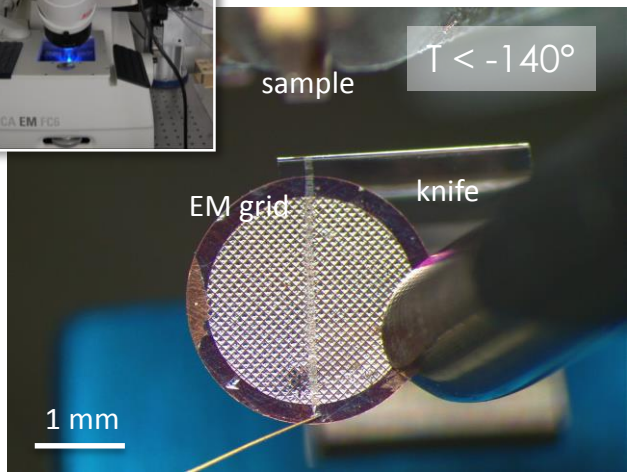
Pure water vitrification ?
Large volumes ?

Obtaining a thin (electron lucent) specimen

Cryo-sectioning

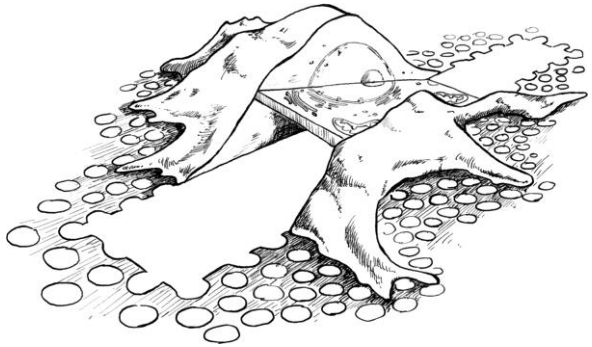
Cryo-Electron Microscopy Of Vitreous Sections



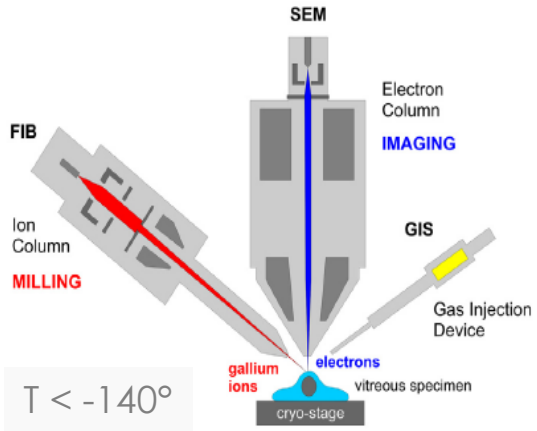


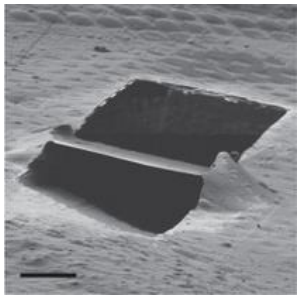
vitrified cells

Cryo-FIB milling



Villa *et al, Curr. Opin. Struct. Biol*, 2013

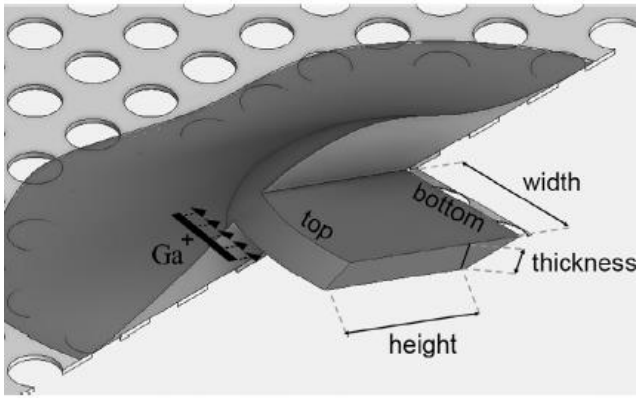




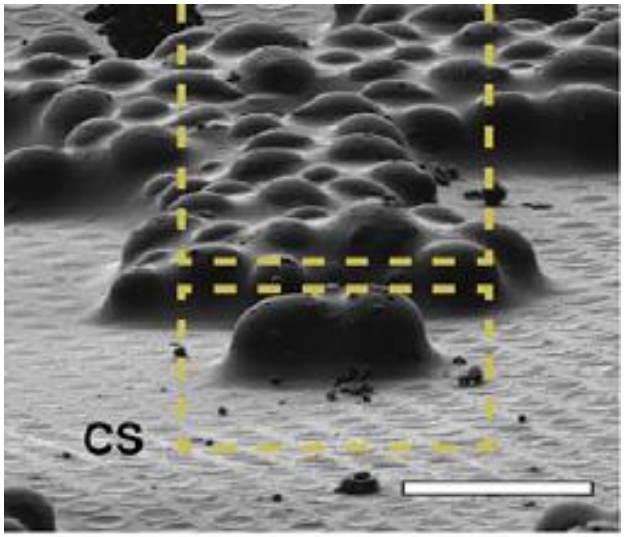
Cryo-FIB milling

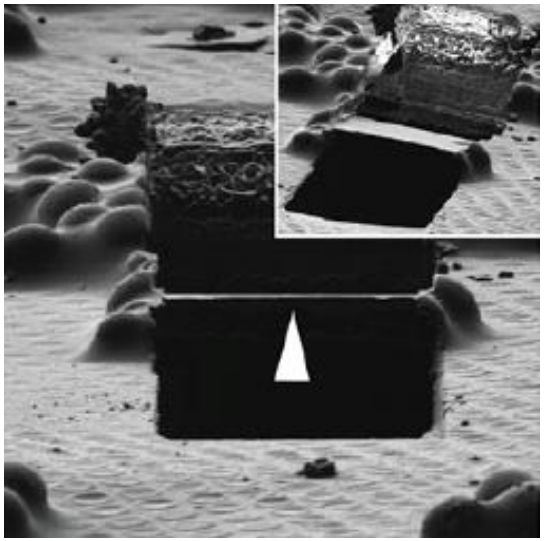
I. Plitsko (Villa et al, 2013)

Cells deposited/cultured on grids



Schaffer et al (2017) JSB, 197



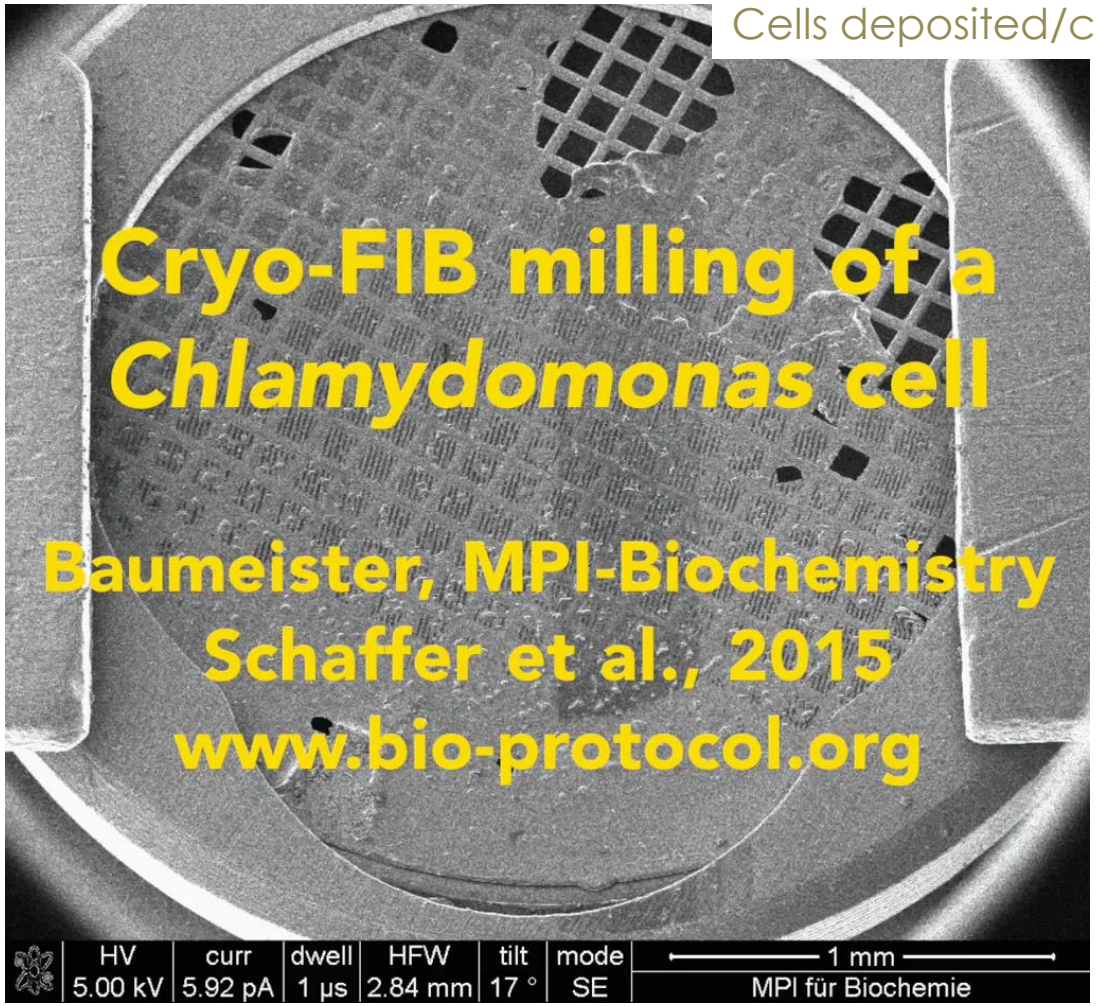


lamella thickness
≥ 100-150 nm

Cryo-FIB milli

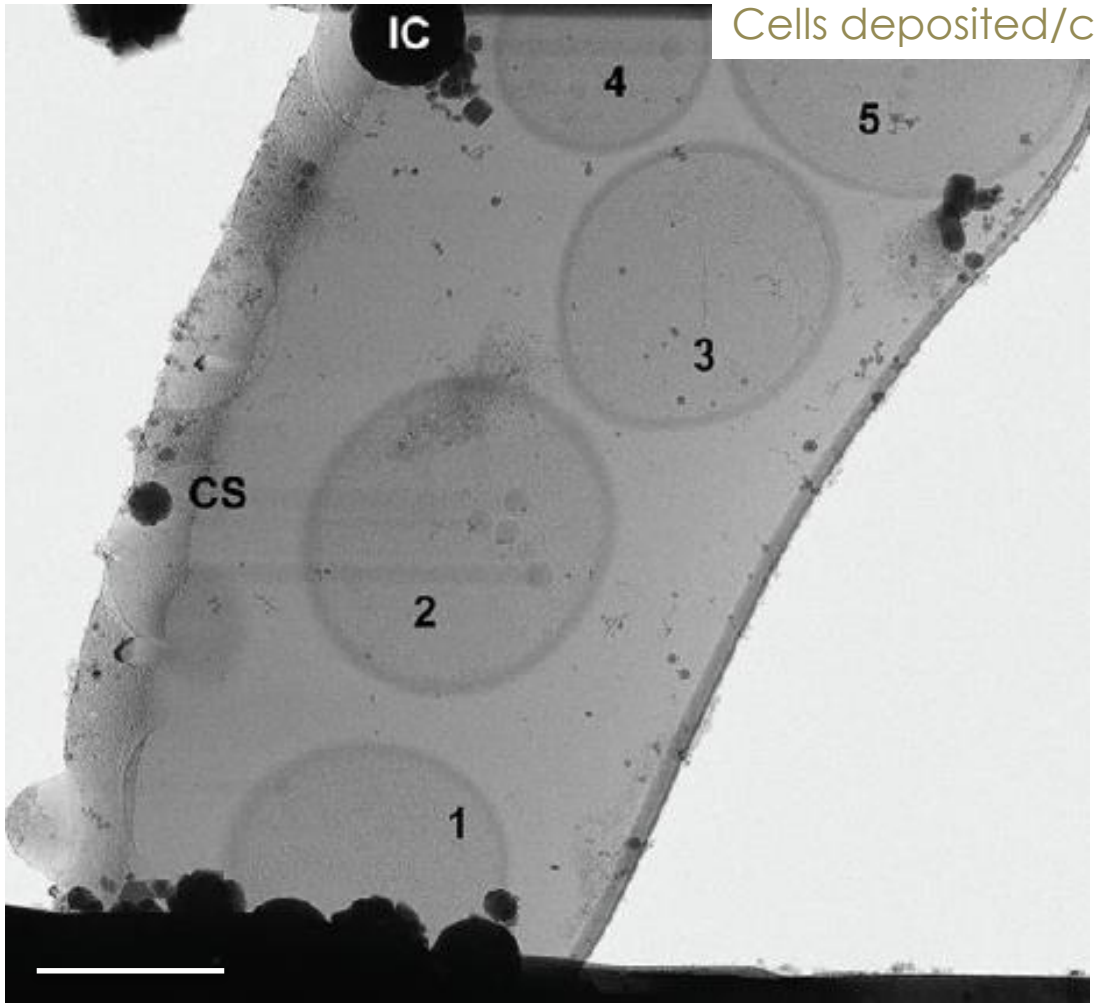
I. Plitsko (Villa et al, 2013)

Cells deposited/cultured on grids



Cryo-FIB milli

J. Plitsko (Villa et al, 2013)

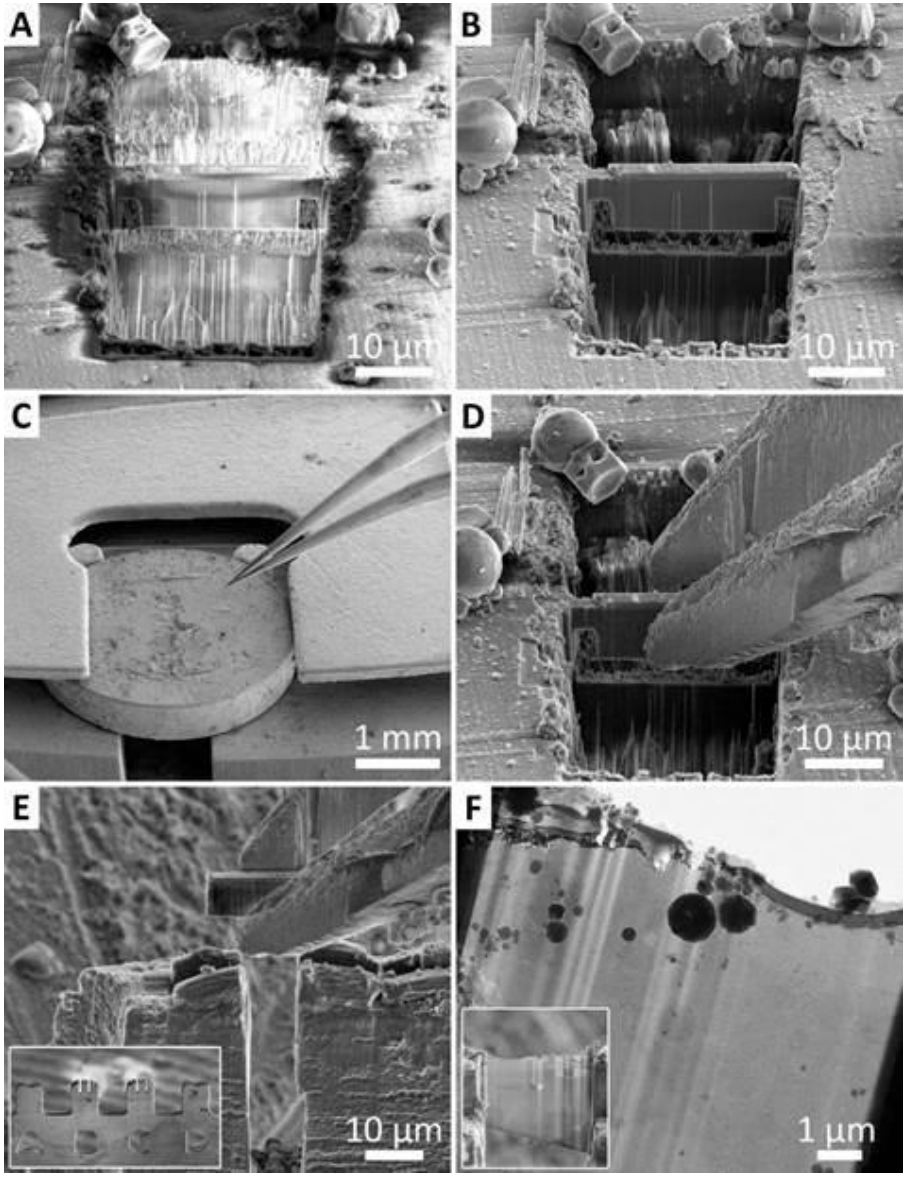


Cells deposited/cultured on grids

Cryo-FIB milling

Multicellular tissues & organisms: « lift out »

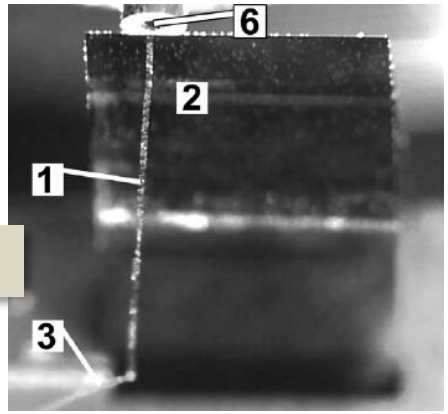
Mahamid et al (2015) J. Struct. Biol. 192, 262-269
Hsieh et a (2014) J. Struct. Biol, 185, 32-41, 2014
Harapin et al (2015) Nature methods, 12, 634-636.

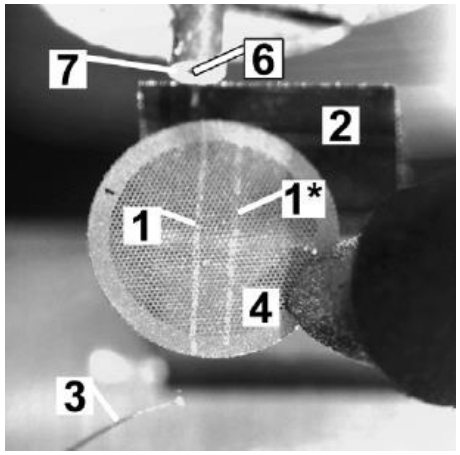


CEMOVIS

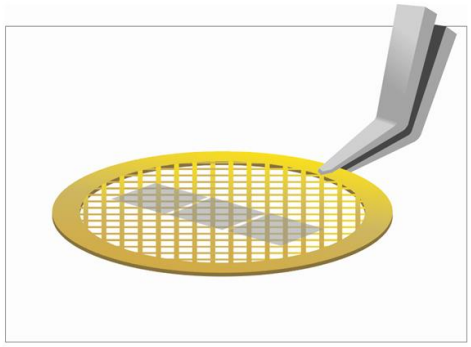
https://www.youtube.com/watch?v=d3tHAWde1GQ

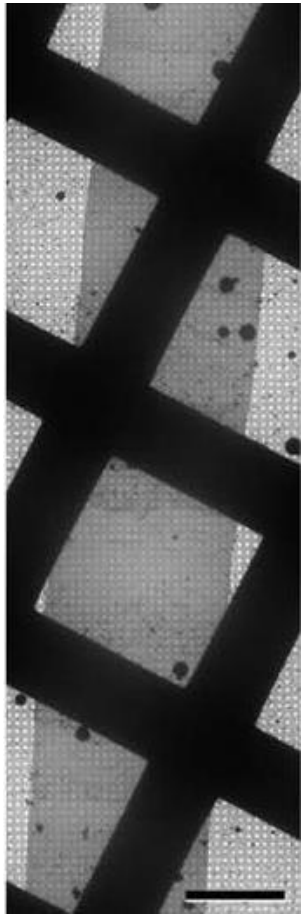
Bouchet-Marquis et al (2007) Biol. Cell, 99, 45

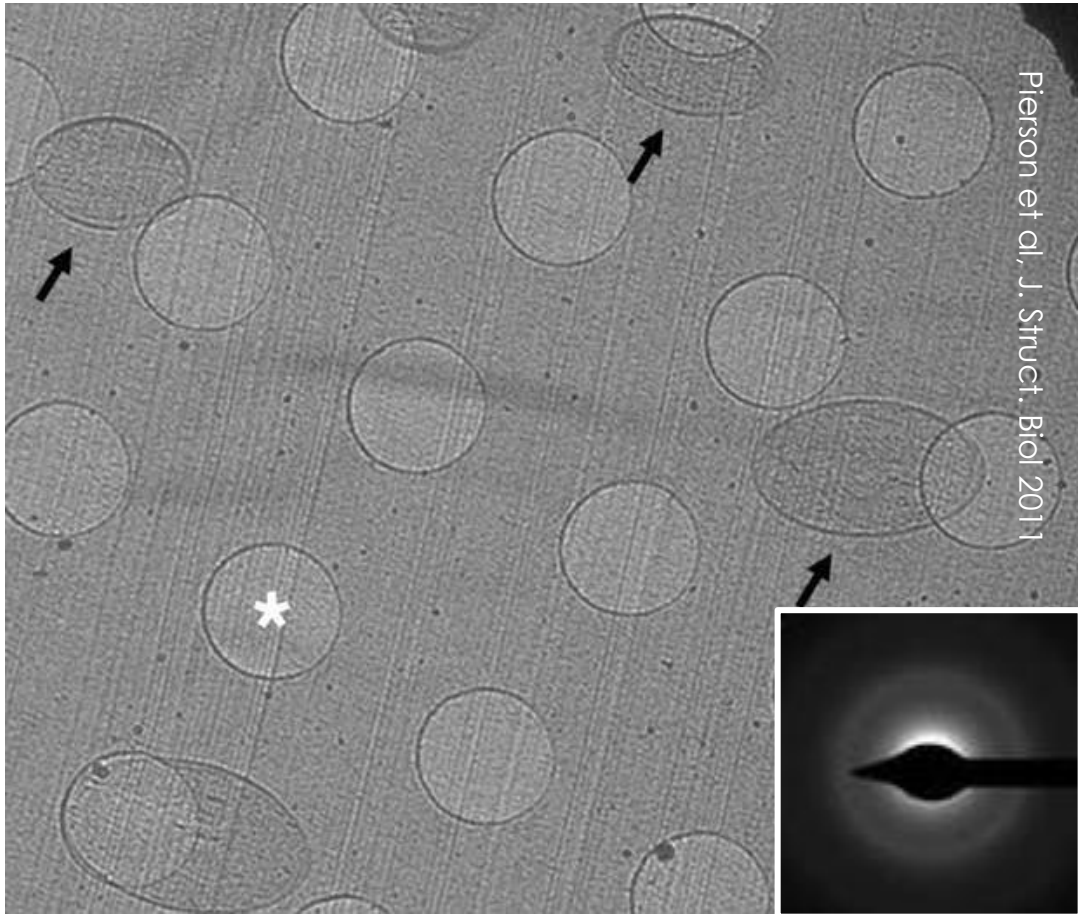




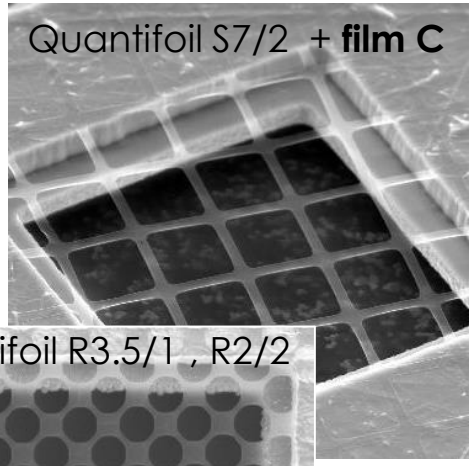
D. Studer et al (2014) J. Struct. Biol. 185, 125

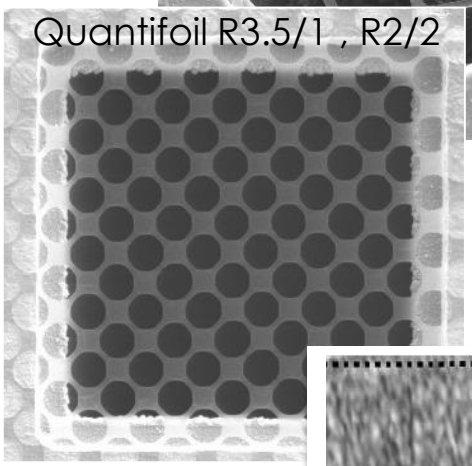




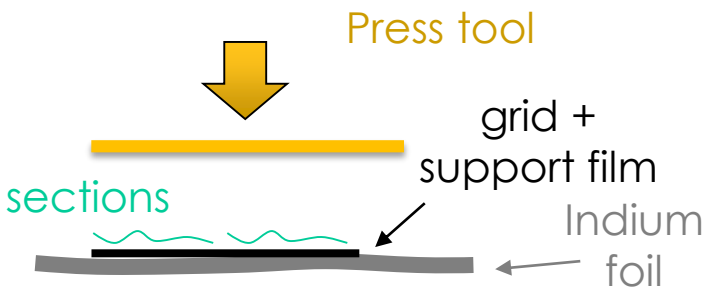


CEMOVIS: adhesion, flatness

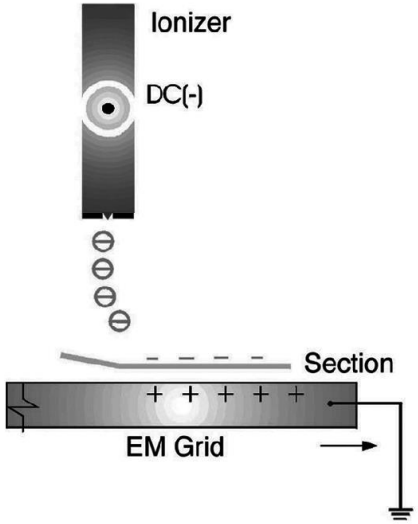


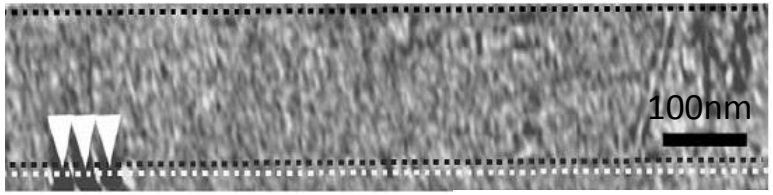


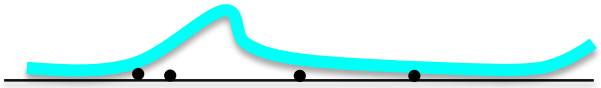
Mechanical presss

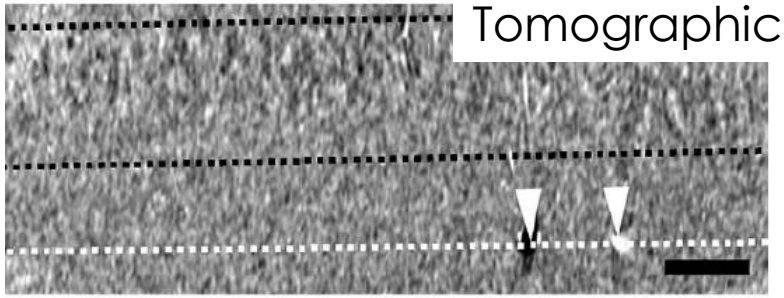


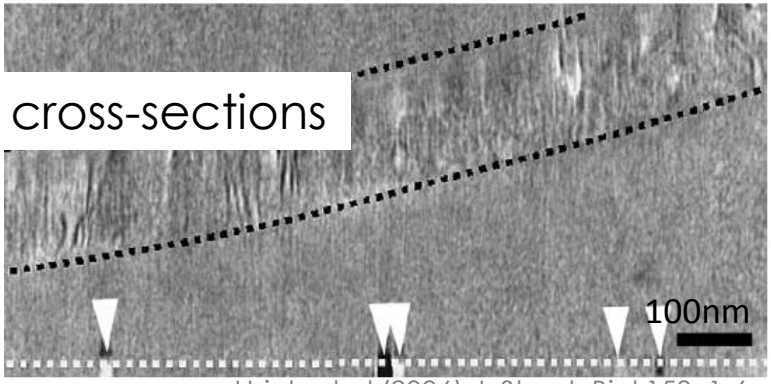
Electrostatic press











Tomographic cross-sections

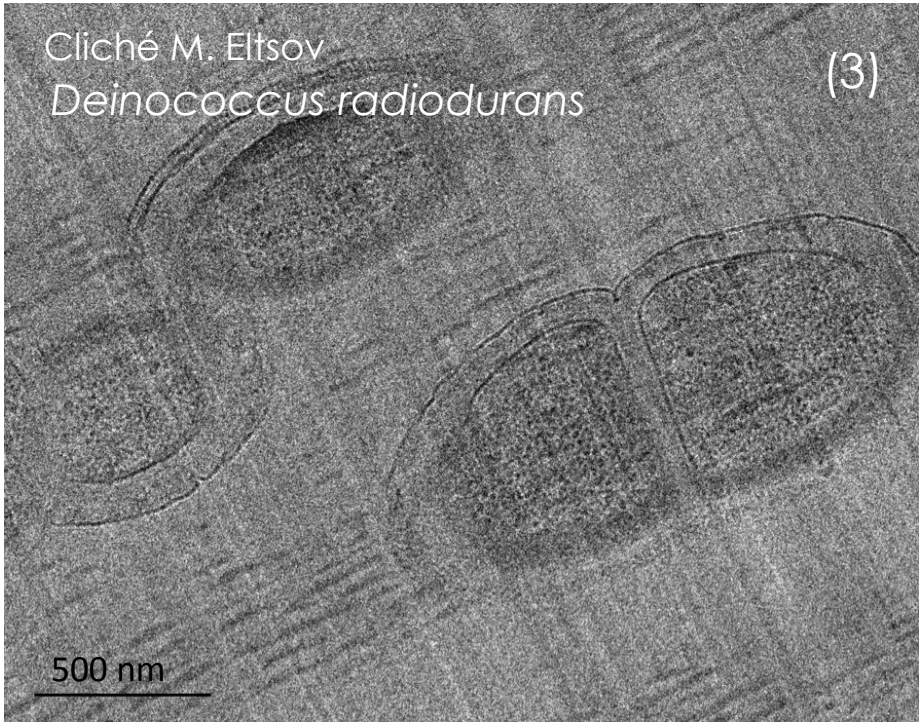
Hsieh et al (2006) J. Struct. Biol 153, 1-6

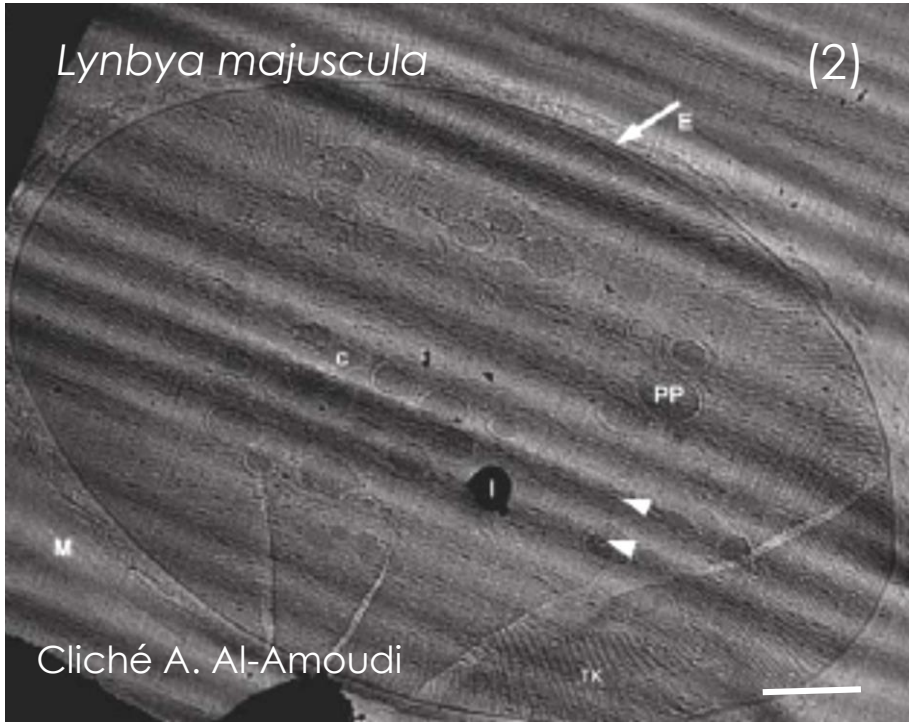
CEMOVIS: Problems & artefacts

- knife marks (1)
- chatter (2)
- crevasses (3)

nature specimen
relative orientation molecules/section
thickness

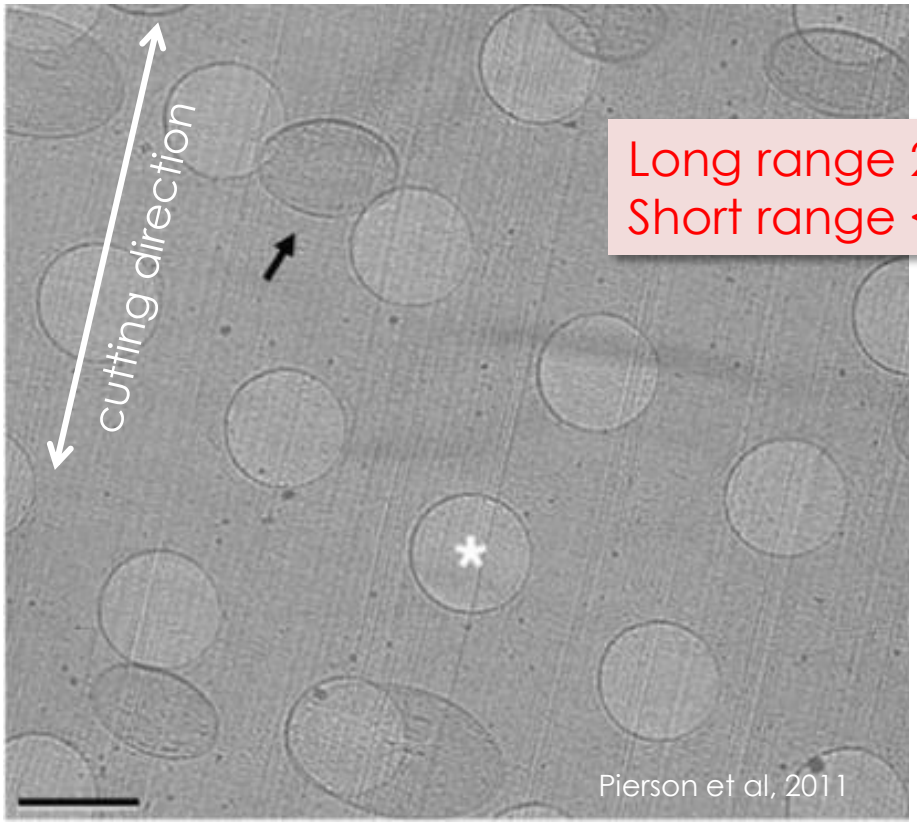
- compression





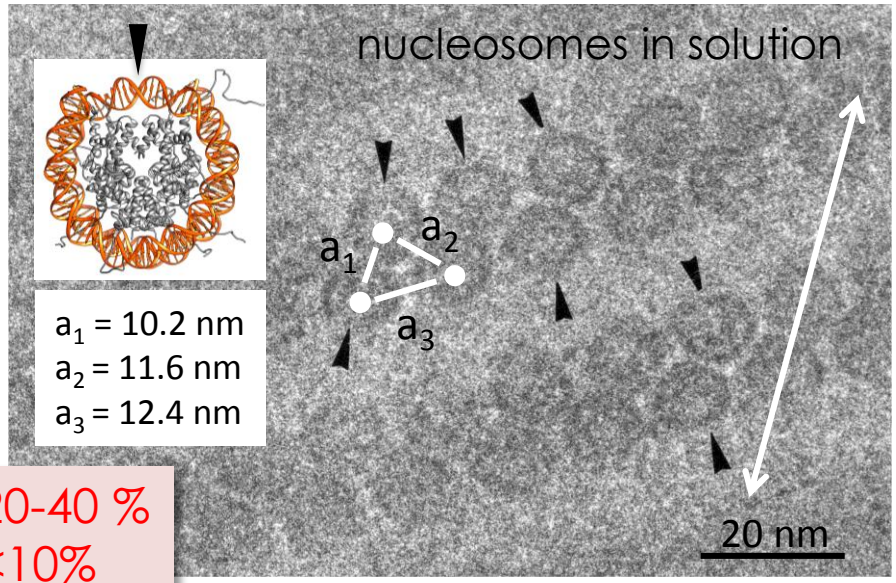
CEMOVIS: Compression ?

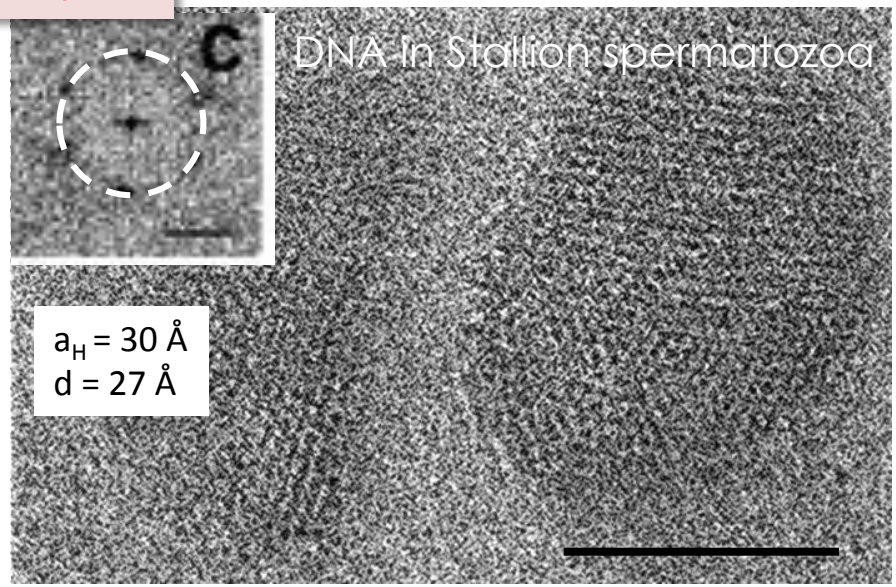
- nature of the specimen
- width of the specimen
- thickness
- scale



Long range 20-40 %
Short range <10%

Intermolecular spacing

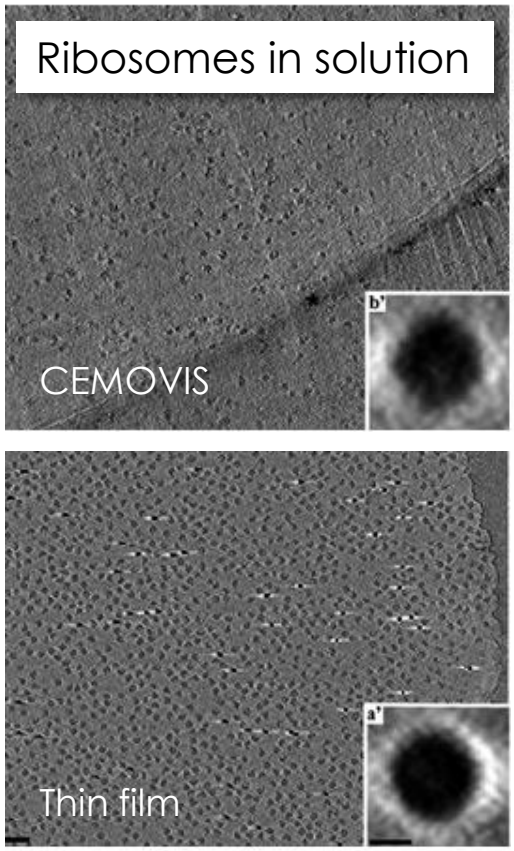




N. Sartori et al (2001) J. Struct. Biol. 134, 76-81

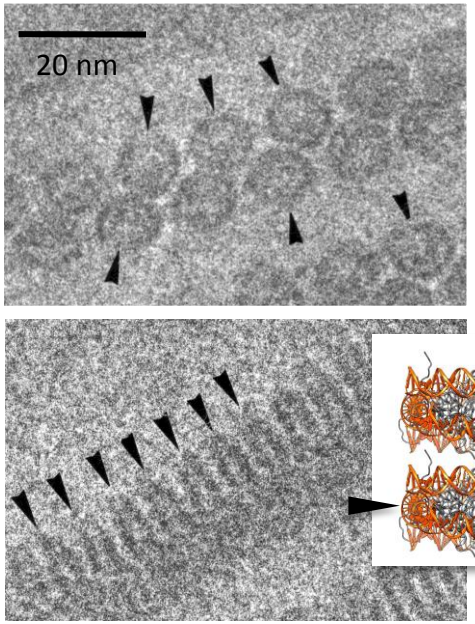
CEMOVIS: compression ?

Ribosomes in solution

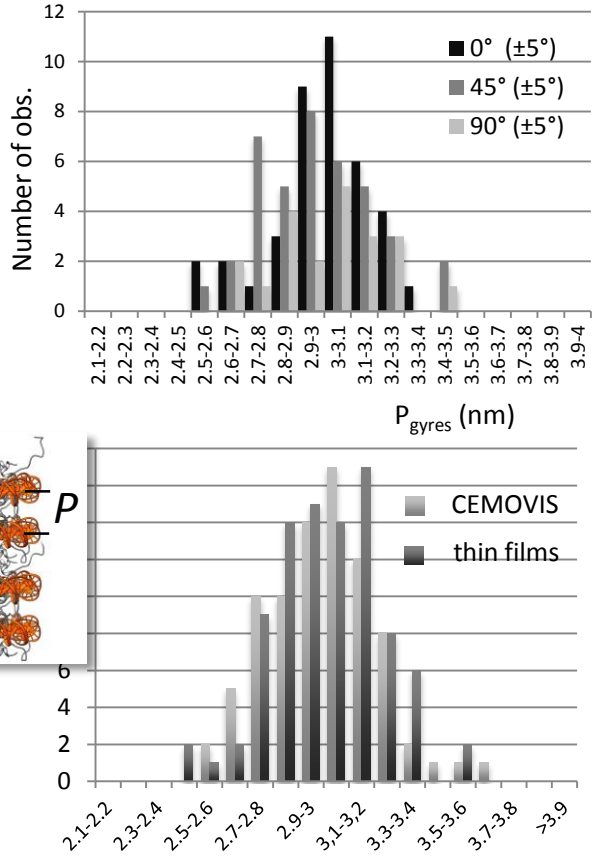


Pierson et al, JSB, 2011

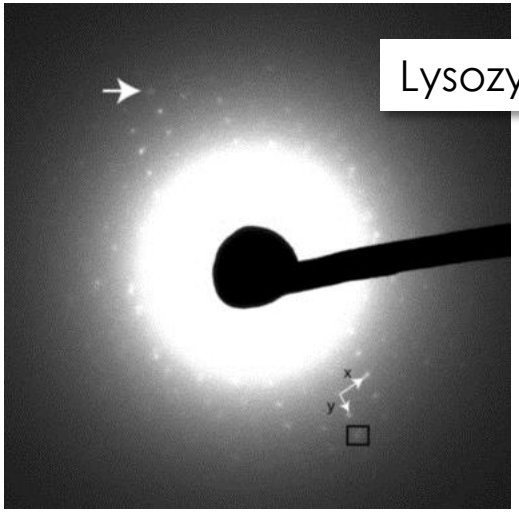
Nucleosomes



Leforestier et al, Biophys. J., 2001
Eltsov et al, NAR, 2018



Lysozyme crystals

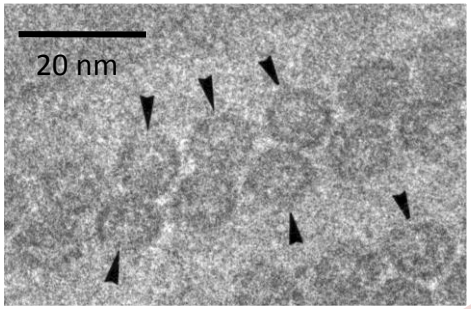


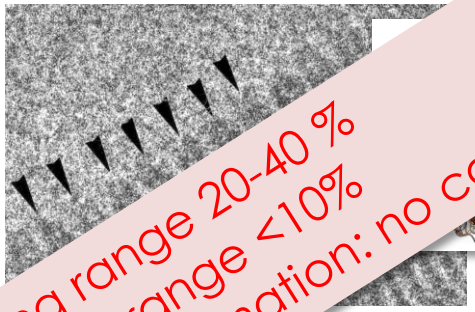
high resolution information 7.9 Å

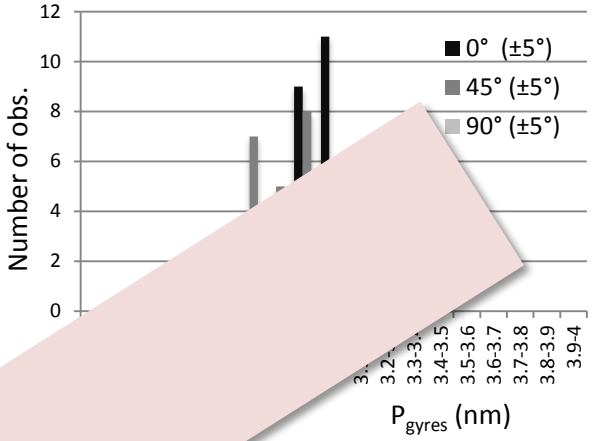
Sader et al, Ultramicroscopy 2009

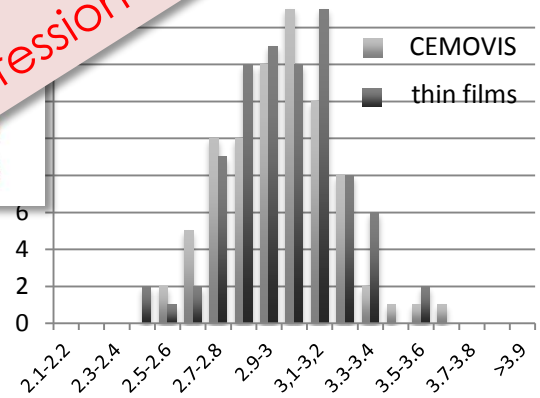
CEMOVIS: compression ?

Nucleosomes



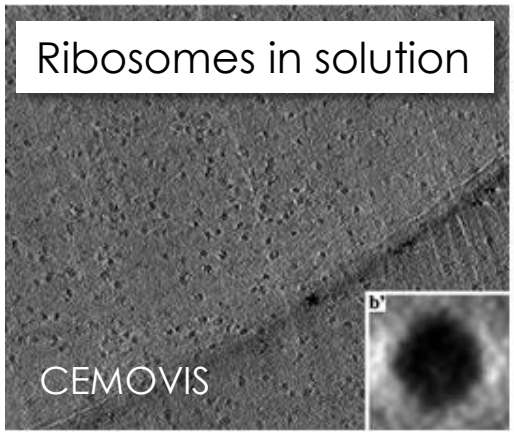


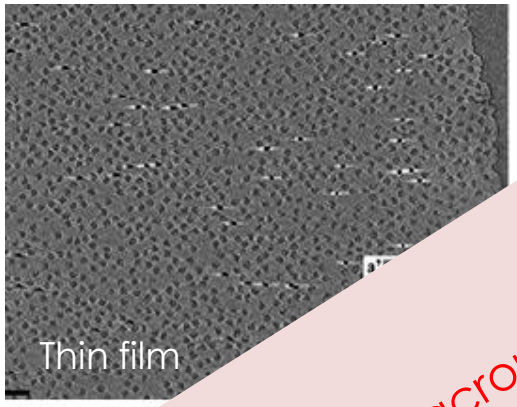




ophys. J., 2001
R, 2018

Ribosomes in solution

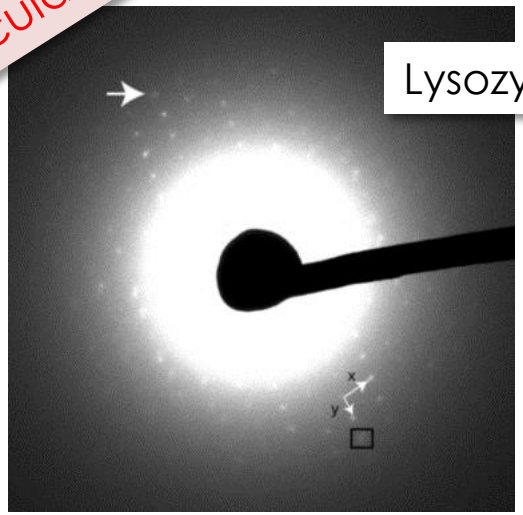




Pierson

Long range 20-40 %
Short range <10%
Macromolecular conformation: no compression

Lysozyme crystals



high resolution information 7.9 Å

Sader et al, Ultramicroscopy 2009

Pros & cons

Cryo-sectioning *versus* cryo-FIB milling ?

Technically demanding	YES	NO (?) / YES for tissue
Surface artifacts	YES crevasses (e ≥ 75nm)	(YES)
Bulk artifacts	YES compression	NO
Serial sections	YES	NO
Surface of observation	50 x 150 μm	10 x 20 μm
Sample thickness	30 -300 (3000) nm	>150 nm
Imaging problems	YES flatness adhesion section-support	(YES) orientation for tomography

Pros & cons

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vibrating knife (Diatome)

- new support films (graphene ?)

CEMOVIS: A new cellular landscape

paramylon

mitochondrion

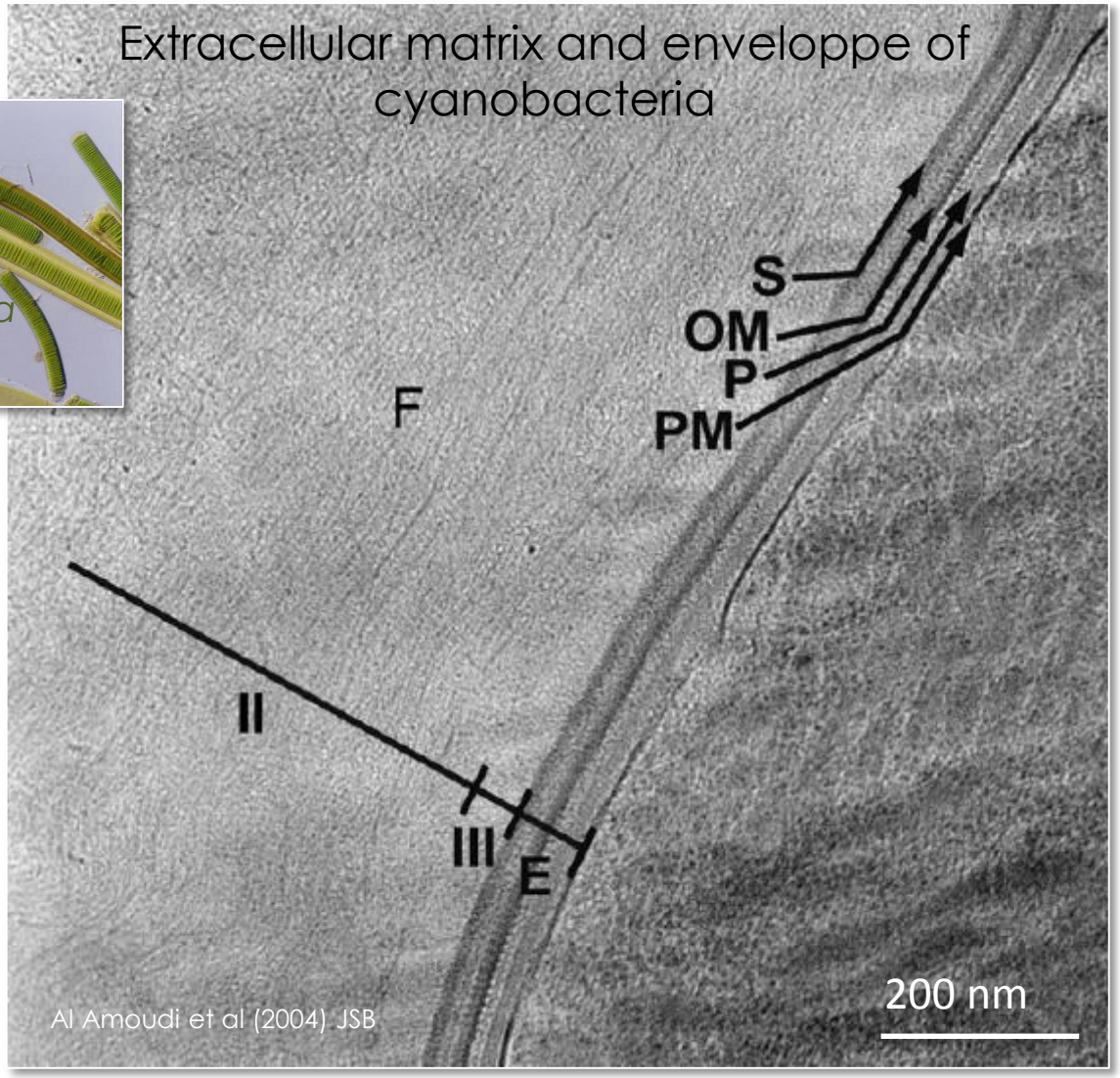
chromosomes

nucleus

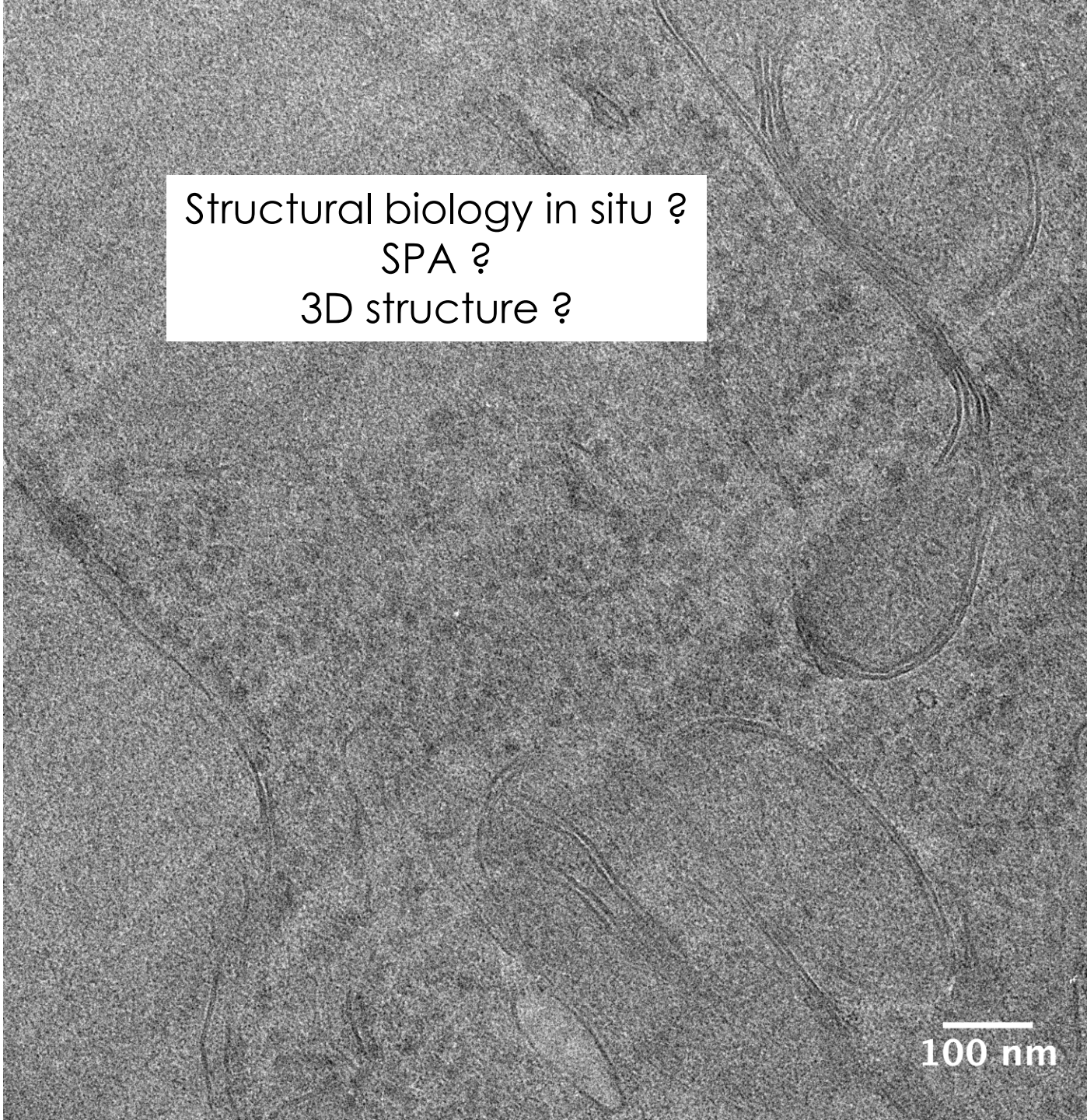
chloroplast

1 μm

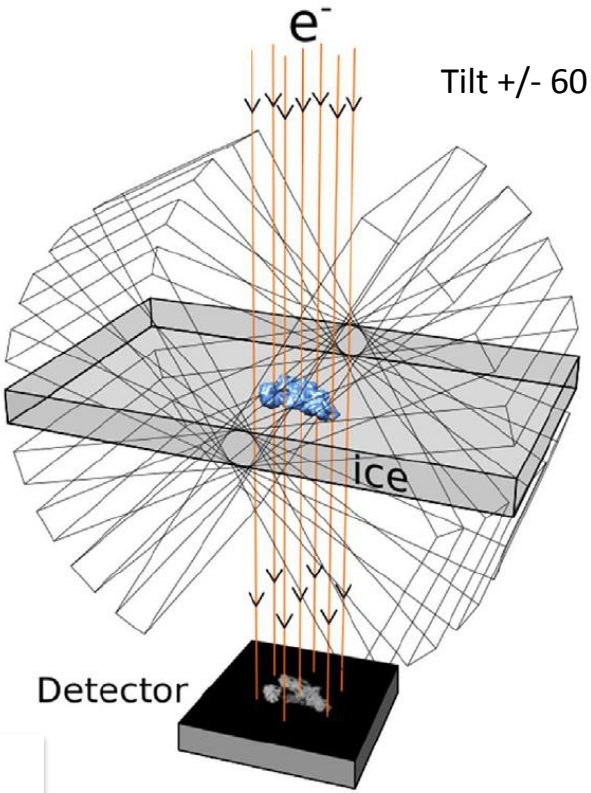
A new cellular landscape



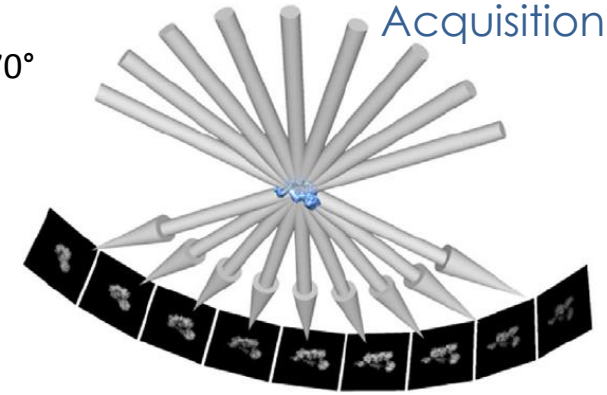
Imaging

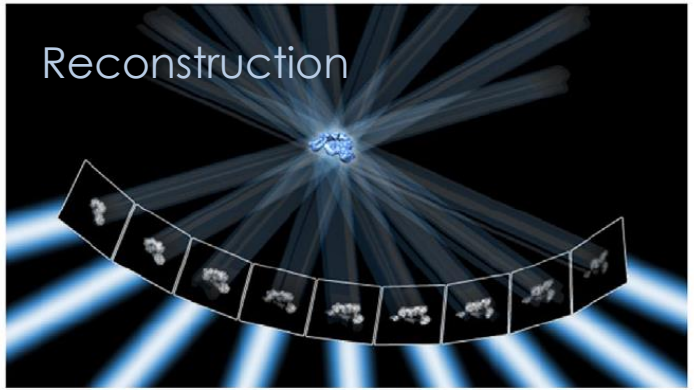


3D structure by cryo-electron tomography



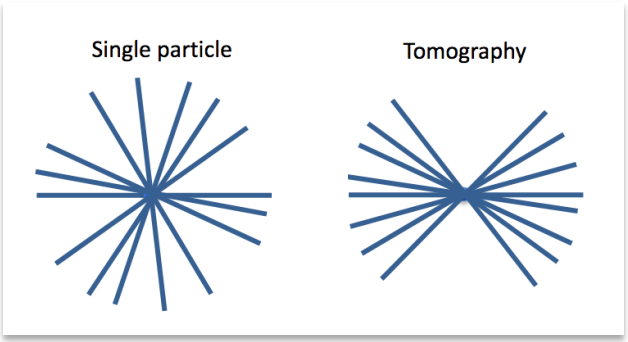
Tilt +/- 60 to 70°





✓ Total electron dose

✓ Missing information



✓ CTF-correction (2D, 3D)

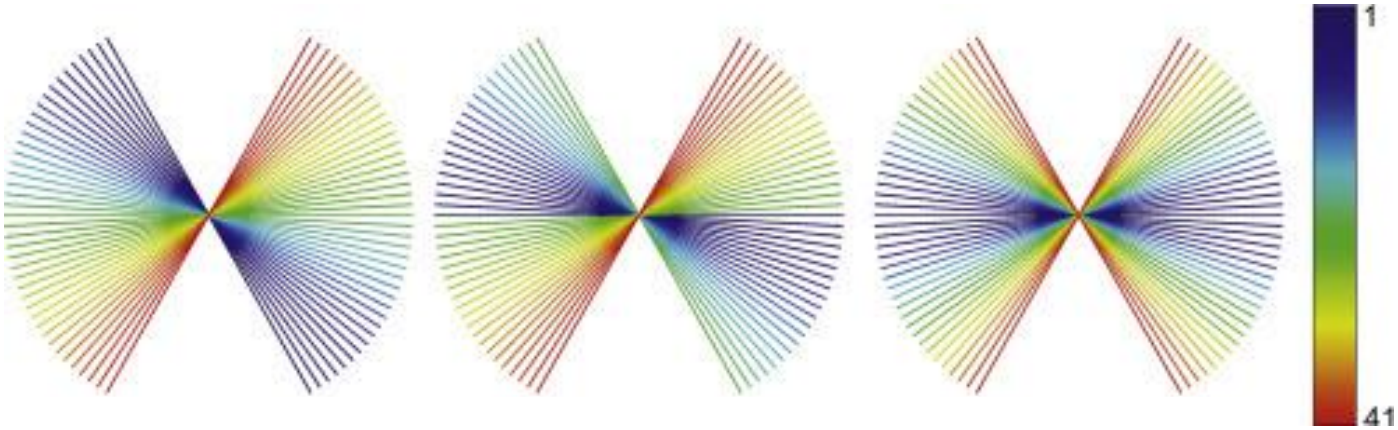
R.I. Koning et al. / Annals of Anatomy 217 (2018) 82–96

Acquisition schemes

Continuous

Bi-directionnal from 0

Dose symmetric



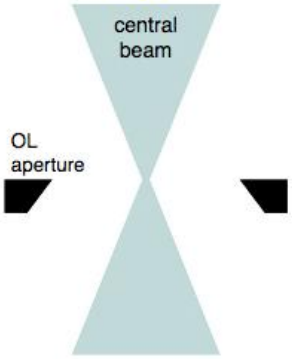
Hagen et al. JSB 2017

« Phase plates »

(d)

Defocus Phase Contrast

DPC



$\sin(\gamma(\mathbf{k}))$

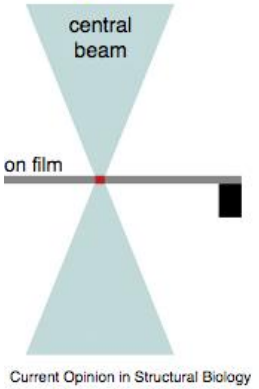
Potentiel Volta
(interaction film-faisceau)

$\cos(\gamma(\mathbf{k}))$

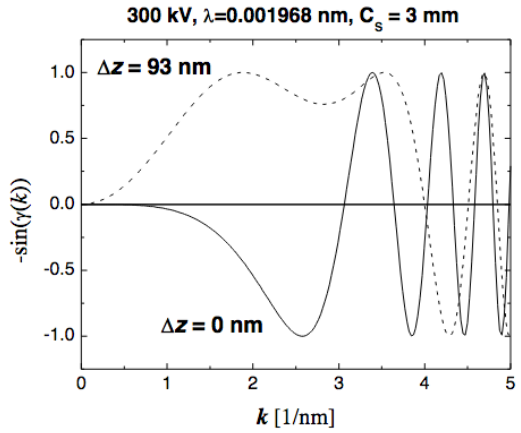
(f)

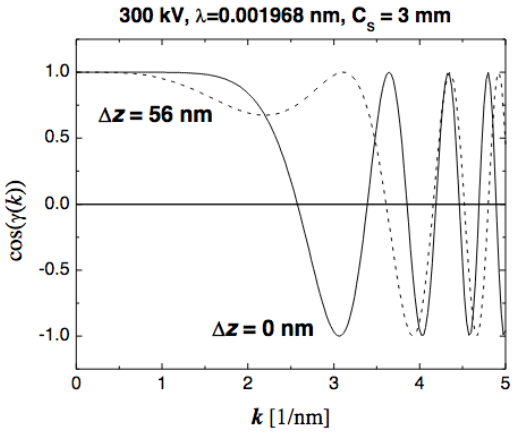
Volta Phase Plate

VPP

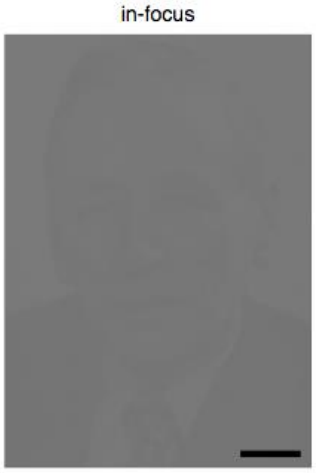


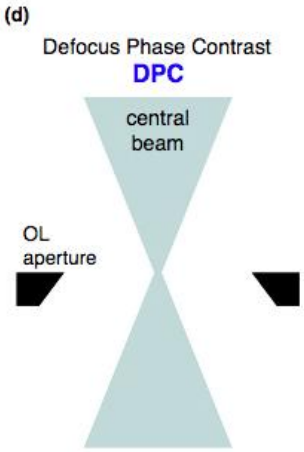
Current Opinion in Structural Biology

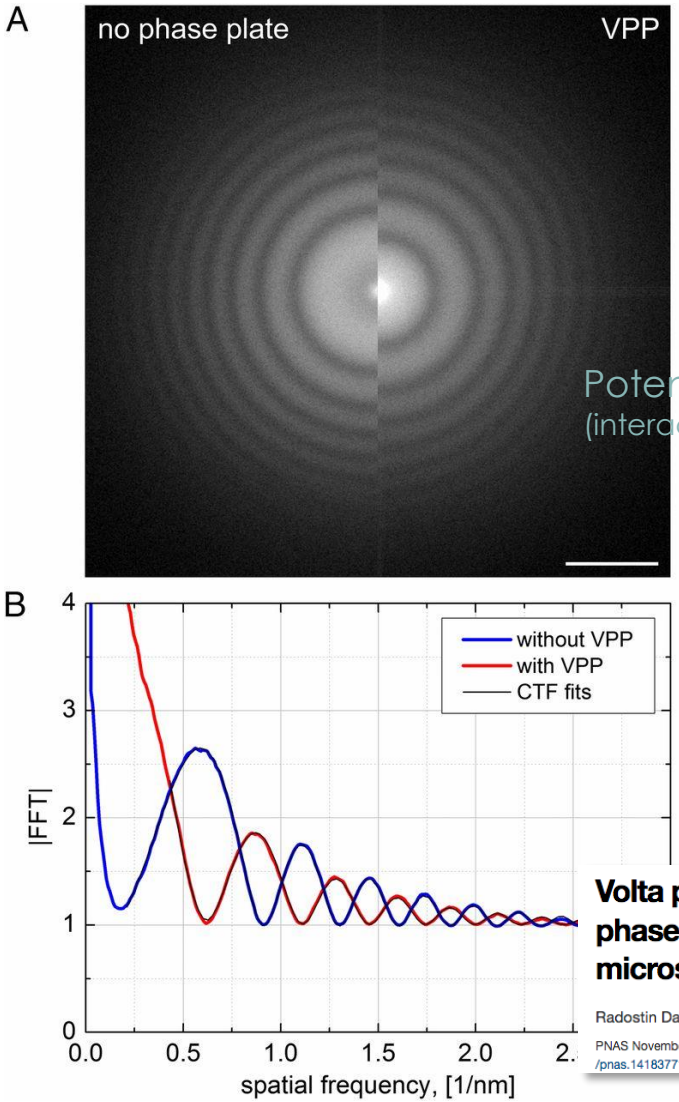




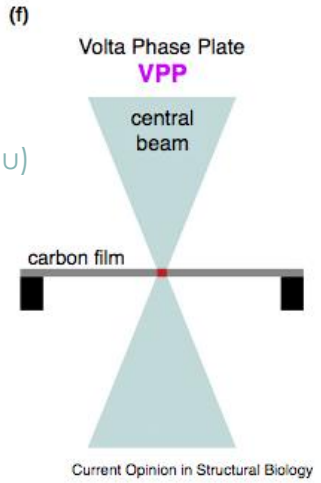
« Phase plates »







Potentiel Volta
(interaction film-faisceau)

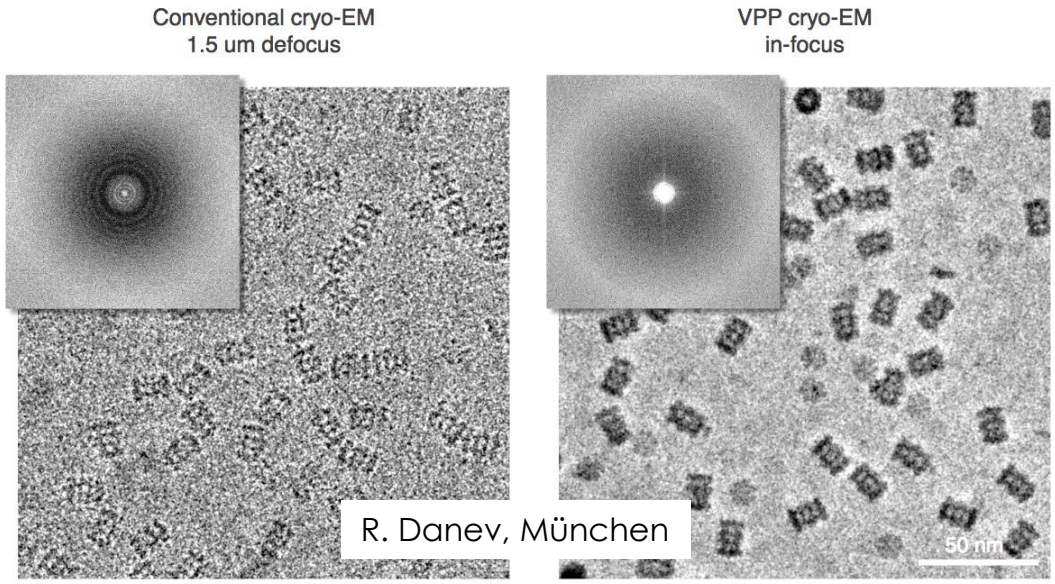


Volta potential phase plate for in-focus phase contrast transmission electron microscopy

Radostin Danev, Bart Buijsse, Maryam Khoshouei, Jürgen M. Plitzko, and Wolfgang Baumeister

PNAS November 4, 2014 111 (44) 15635-15640; published ahead of print October 20, 2014 https://doi.org/10.1073/pnas.1418377111

« Phase plates »



R. Danev, München

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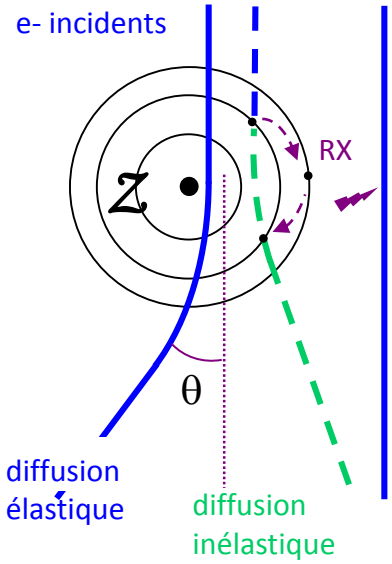
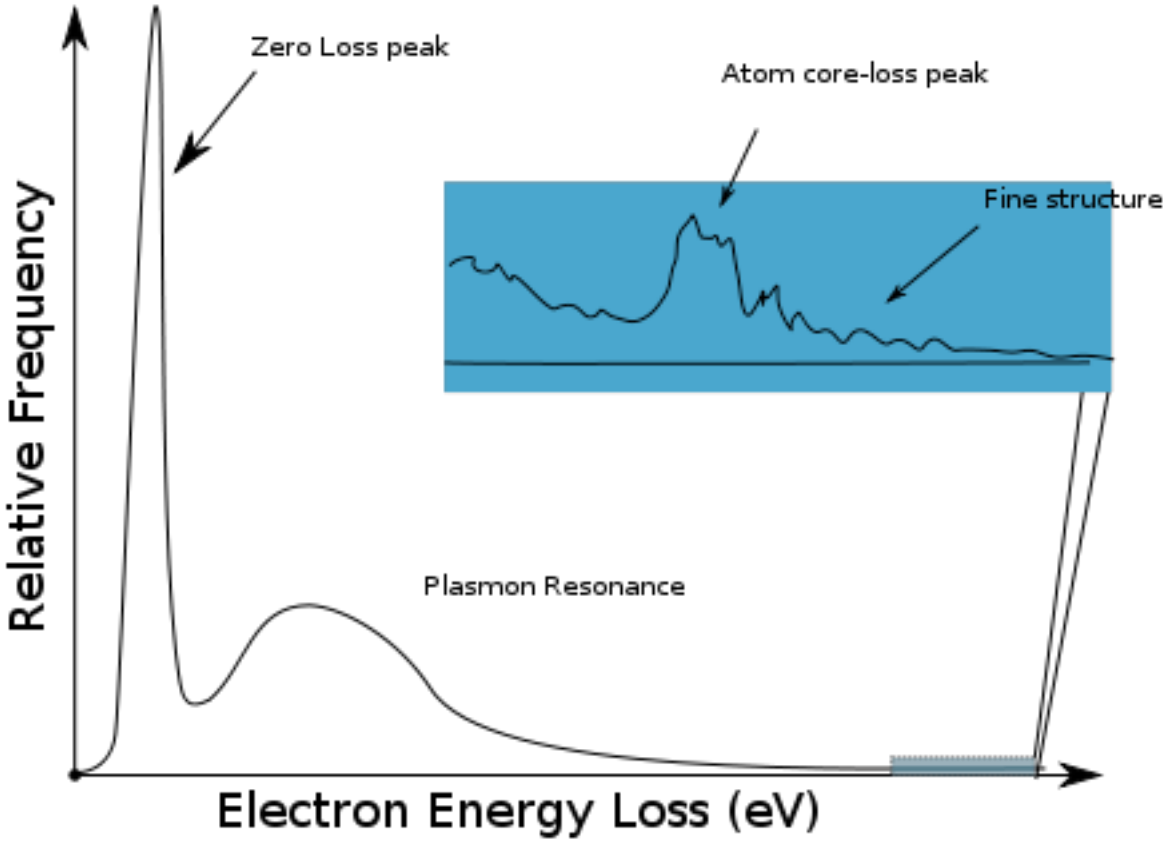
Zero loss filtering

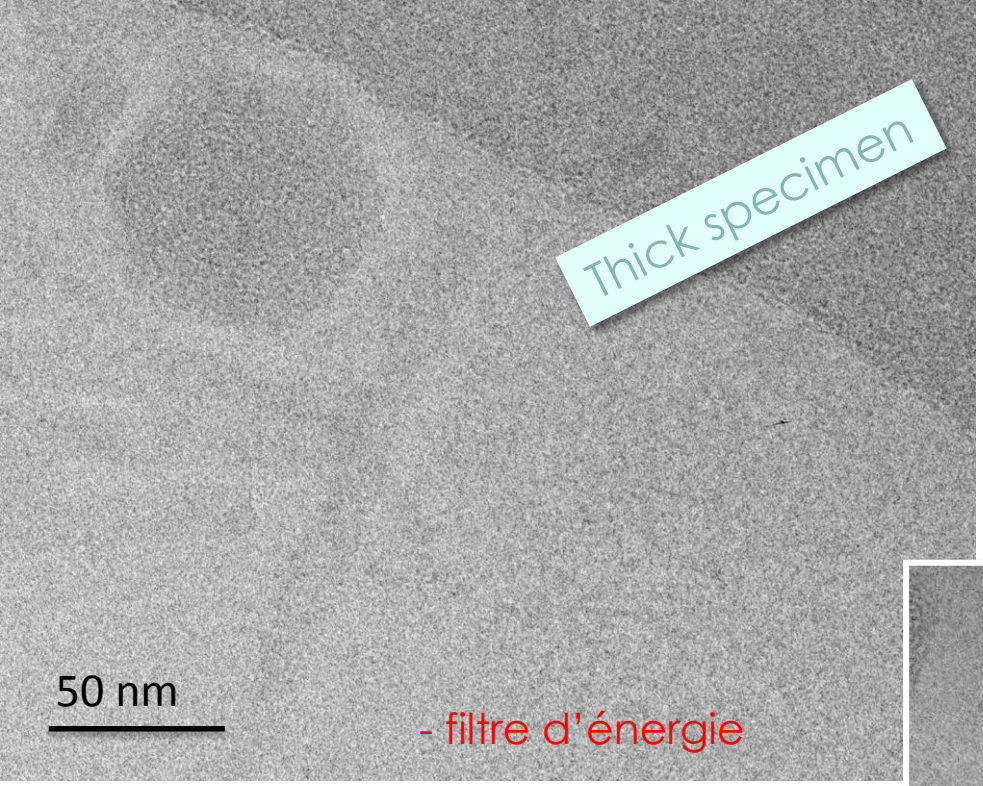


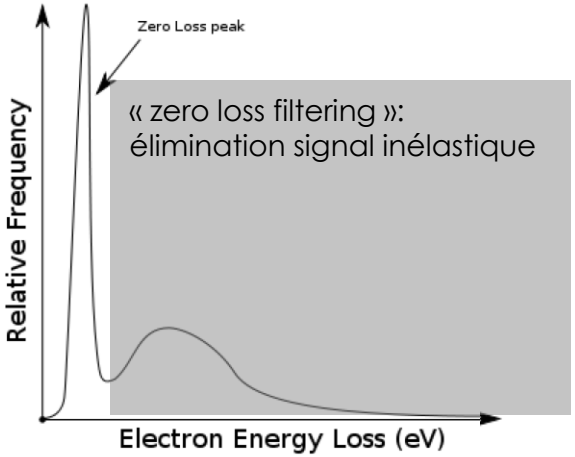


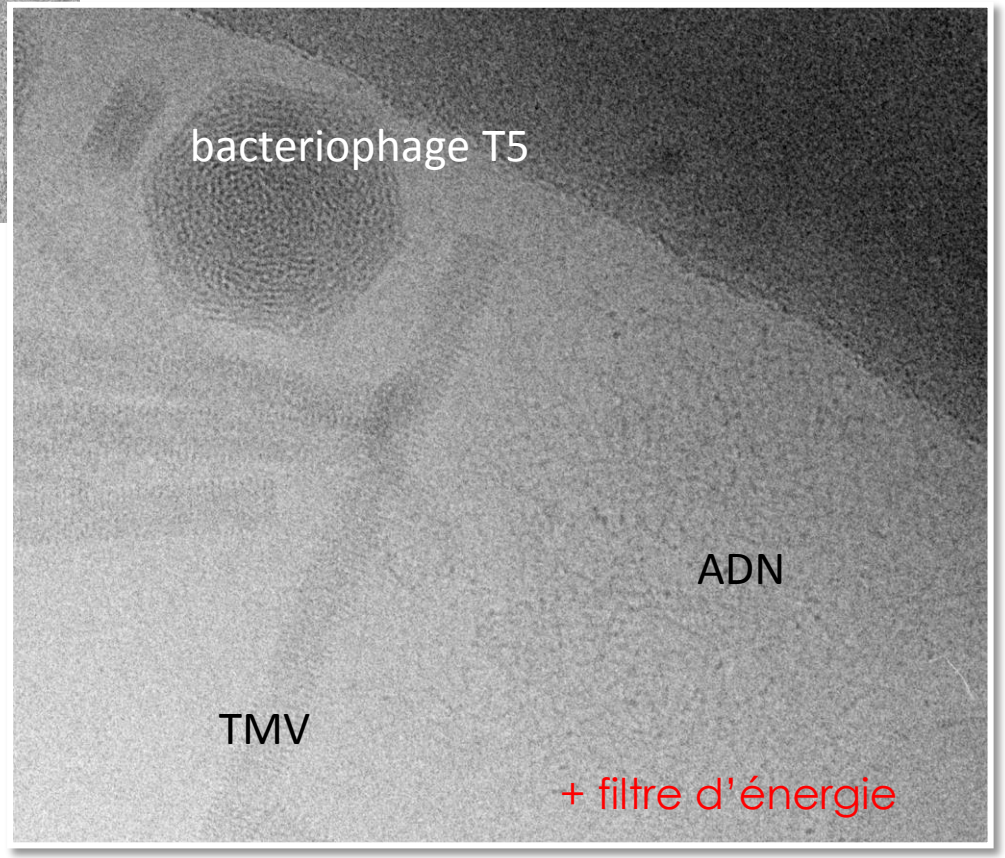
Zero loss filtering

- thick specimen (> 150 nm)
- tomography

JEOL 2200FS



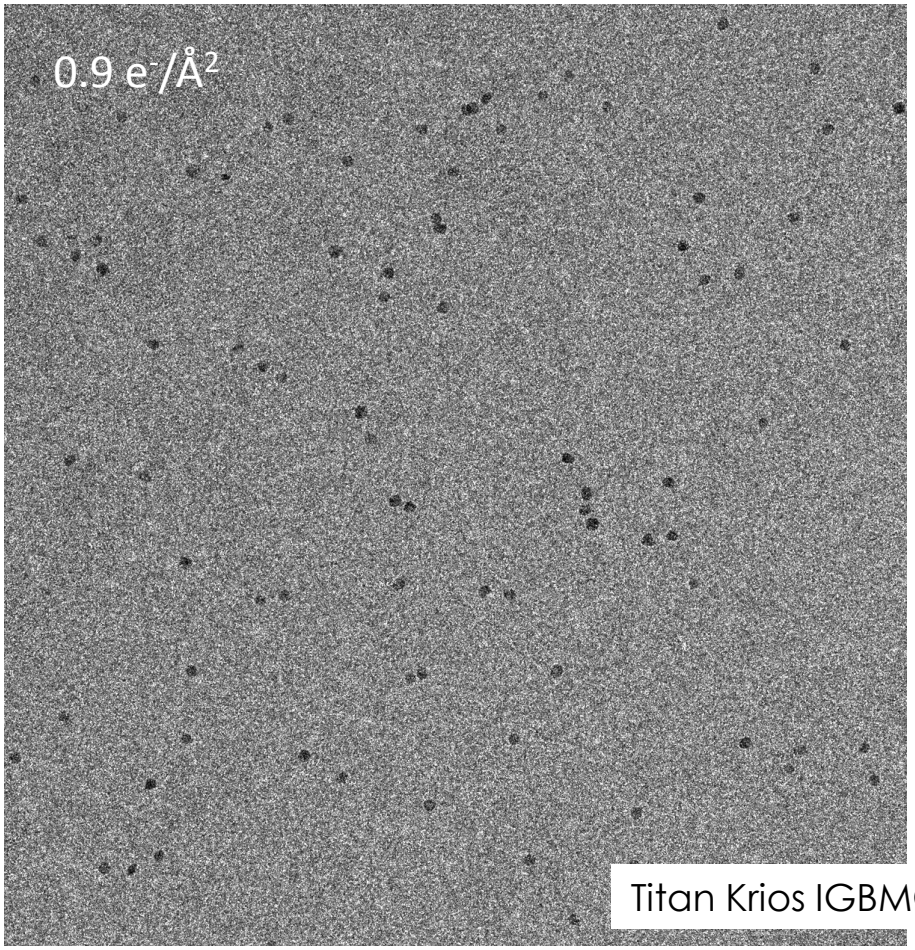


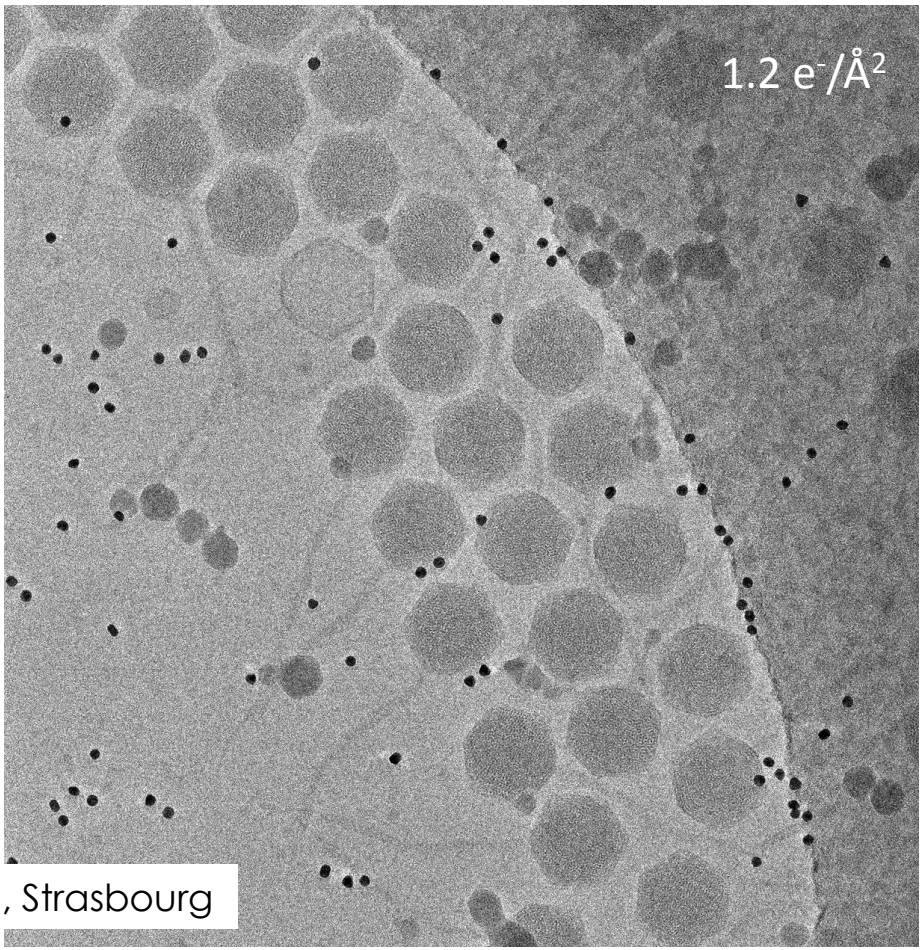


« Volta Phase Plate » + energy filter

Conventional cryoEM
1µm defocus

VPP cryoEM
In-focus + energy filter

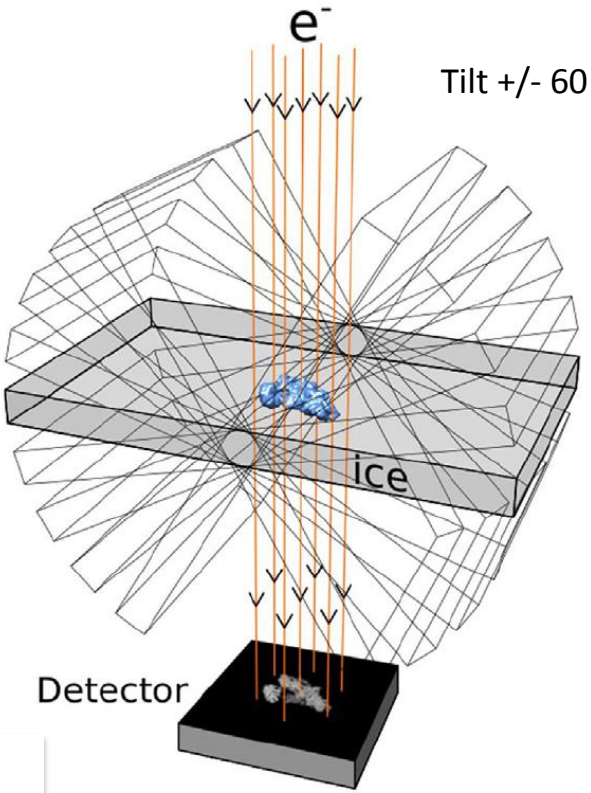




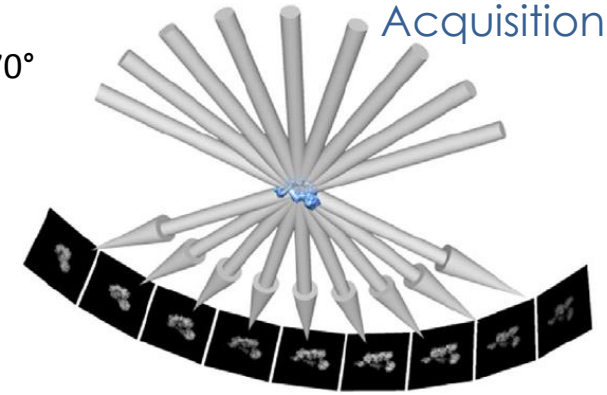
Titan Krios IGBMC, Strasbourg

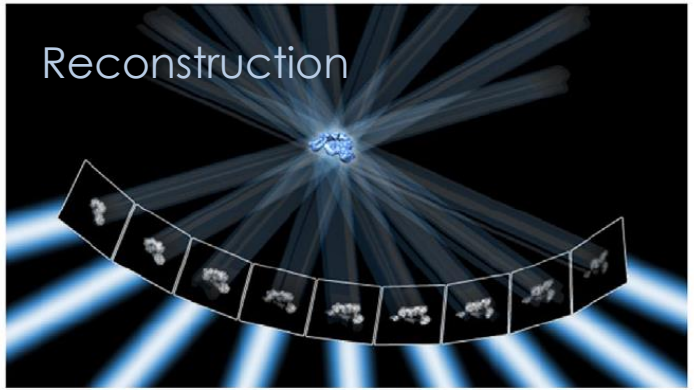
Bacteriophage T5
(80 nm) + NP or (10 nm)

3D structure by cryo-electron tomography



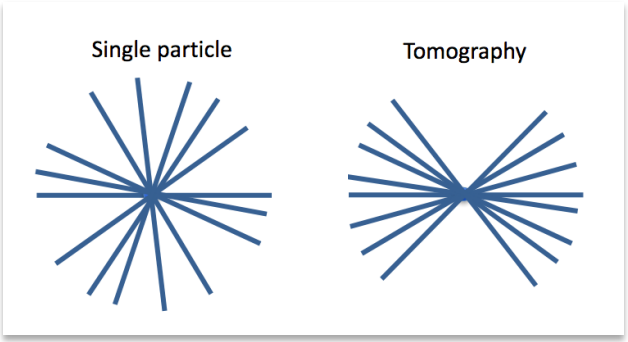
Tilt +/- 60 to 70°





✓ Total electron dose

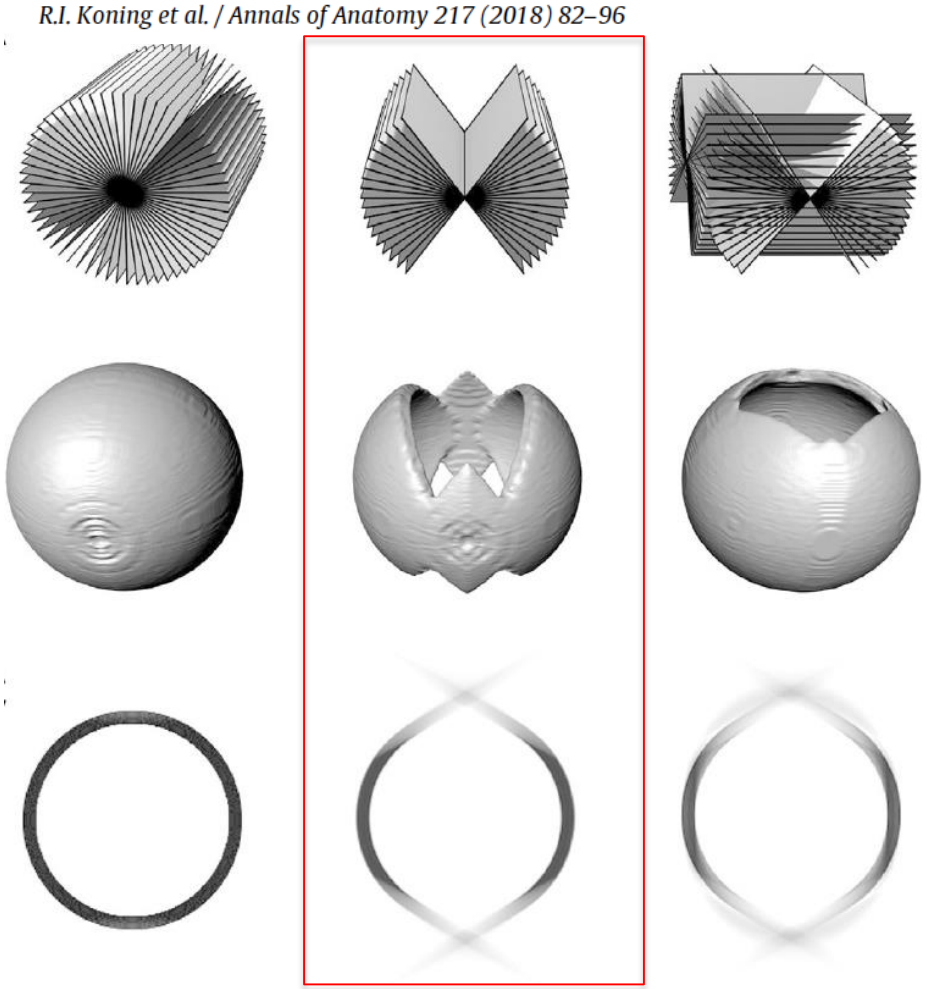
✓ Missing information

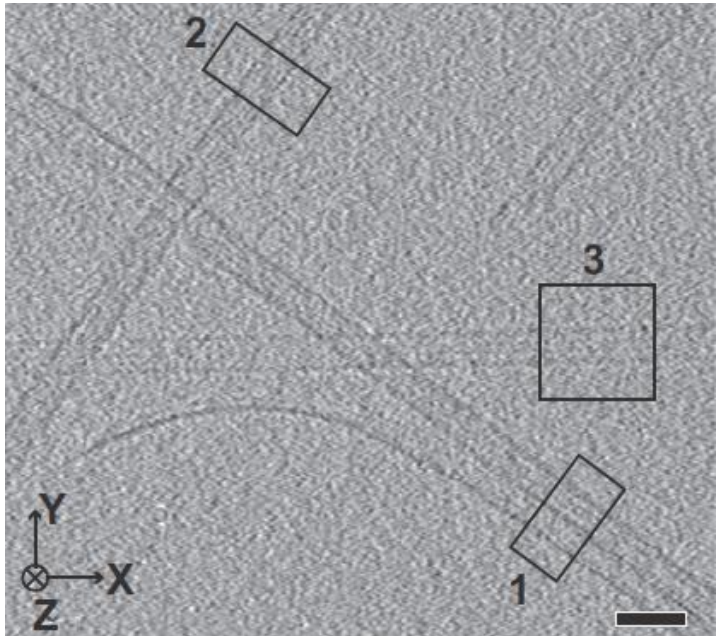


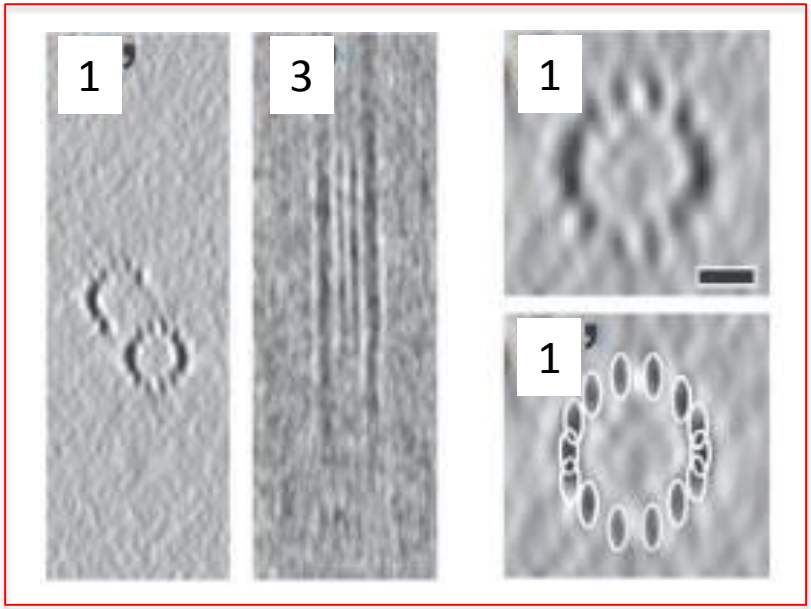
✓ CTF-correction (2D, 3D)

R.I. Koning et al. / Annals of Anatomy 217 (2018) 82–96

« missing wedge », « missing cone »





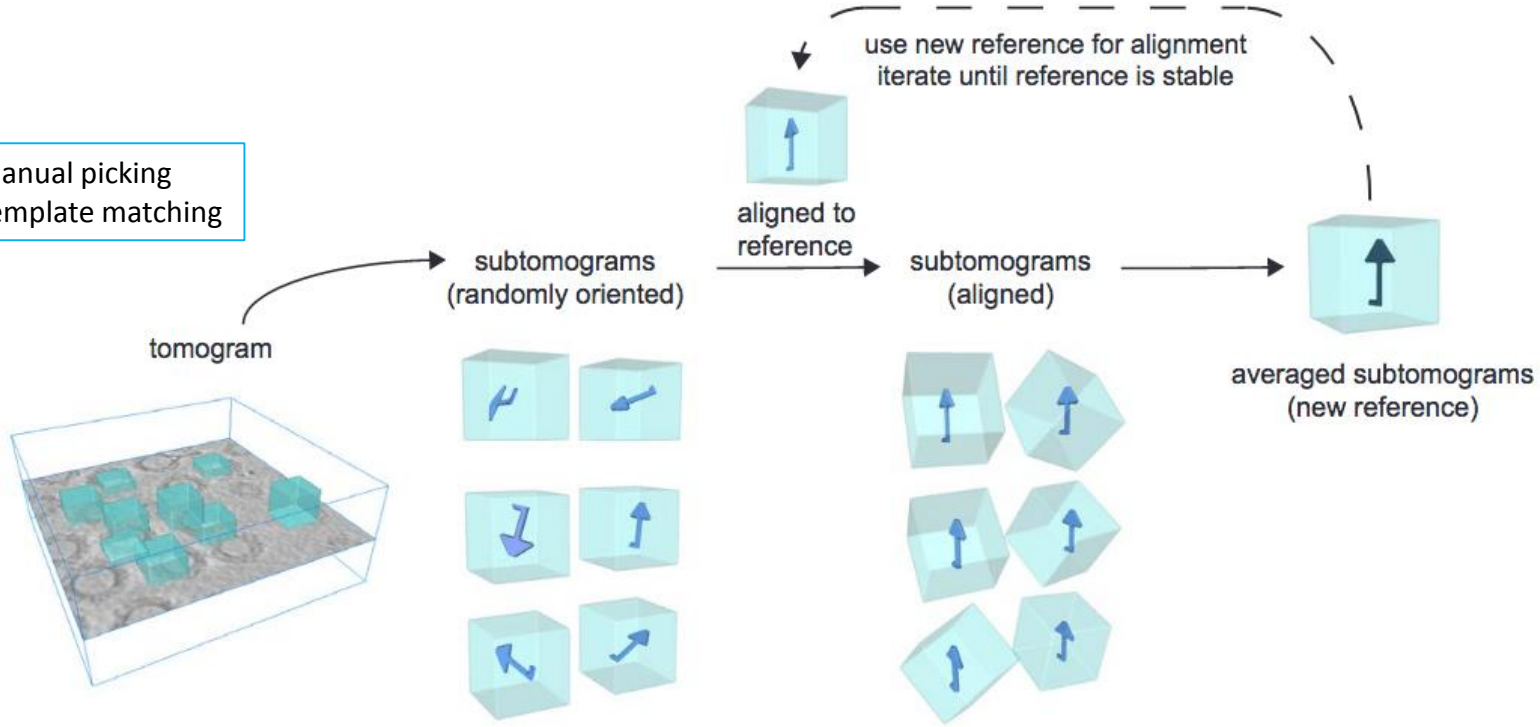


$R_x = R_{2D}$
$R_y = \pi\ D\ /\ N$
$R_z = f(\alpha)\ R_y$

α max tilt angle,
D object diameter,
N ≈ number of images(angles)

Sub-tomogram averaging

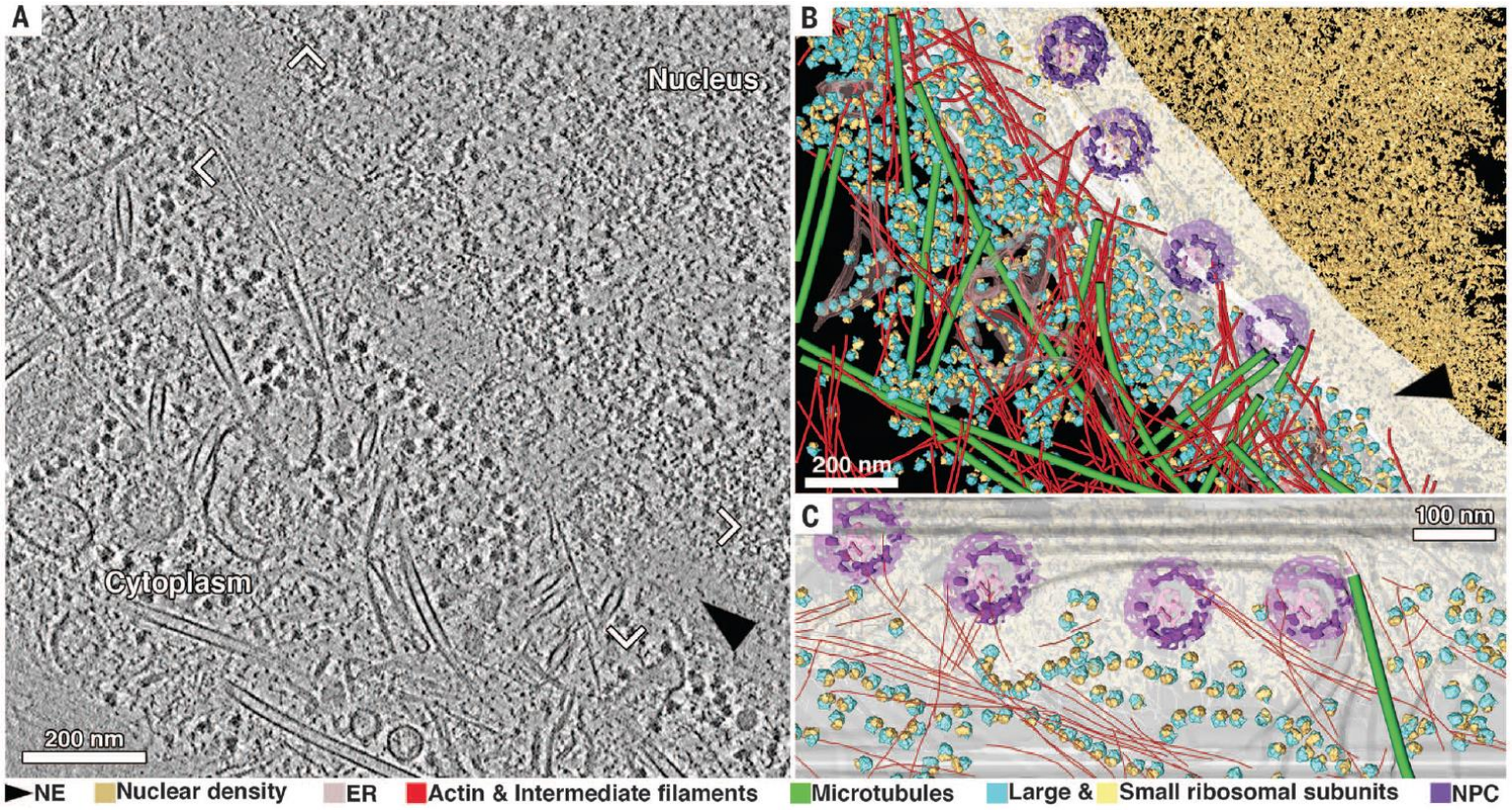
- ✓ Manual picking
- ✓ Template matching



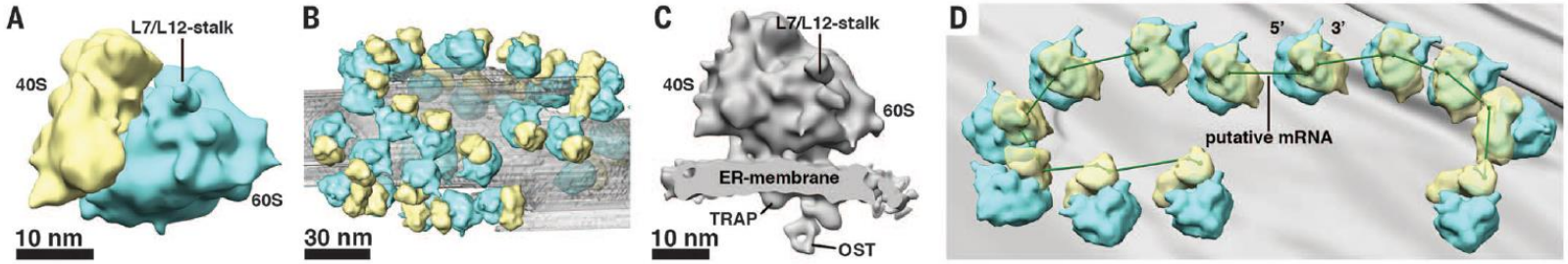
Wan, W., & Briggs, J. A. G. (2016). Cryo-electron tomography and subtomogram averaging. In *Methods in enzymology* (Vol. 579, pp. 329-367). Academic Press

« Molecular sociology »

Nuclear periphery (HeLa cell)



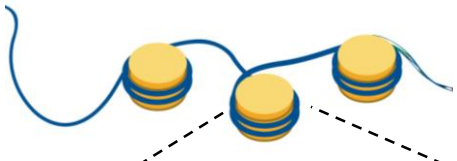
Sub-tomogram averaging



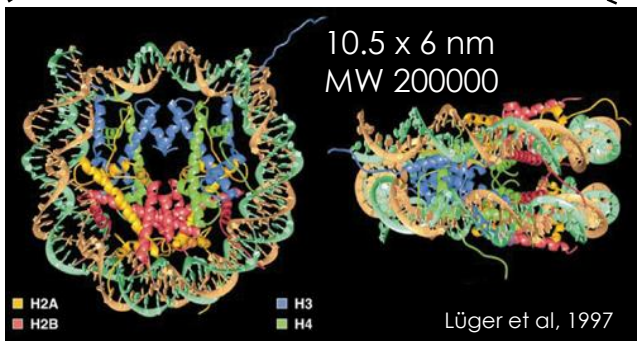
+VPP

Mahamid et al (2016) Science, 351

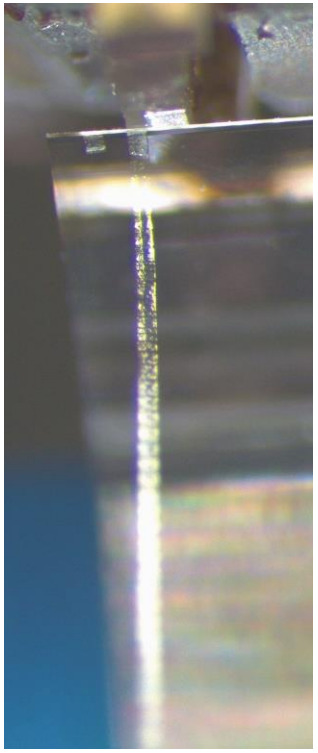
Imaging the nucleosome in its cellular context

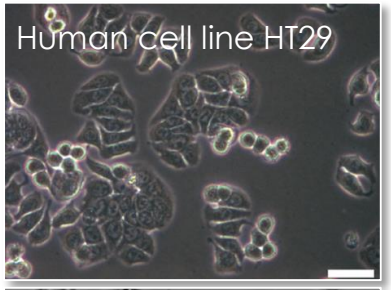


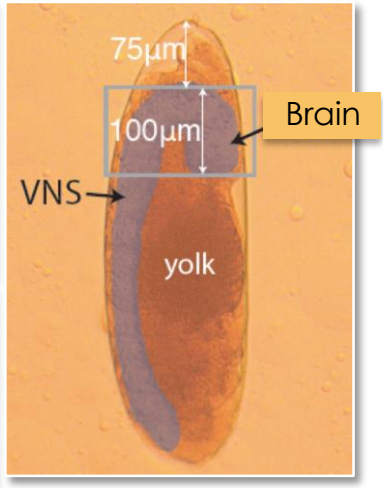
« bead-on-string »
chromatin filament



Mikhail Eltsov Diana Grew
Buchmann Institute, Frankfurt

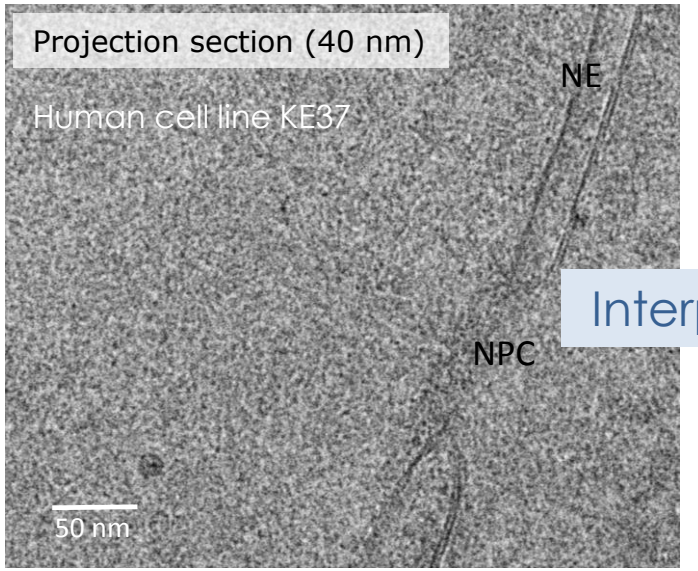


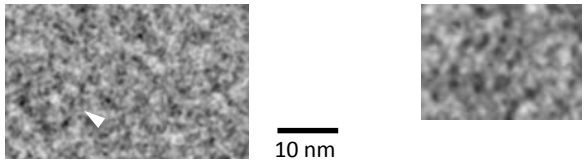




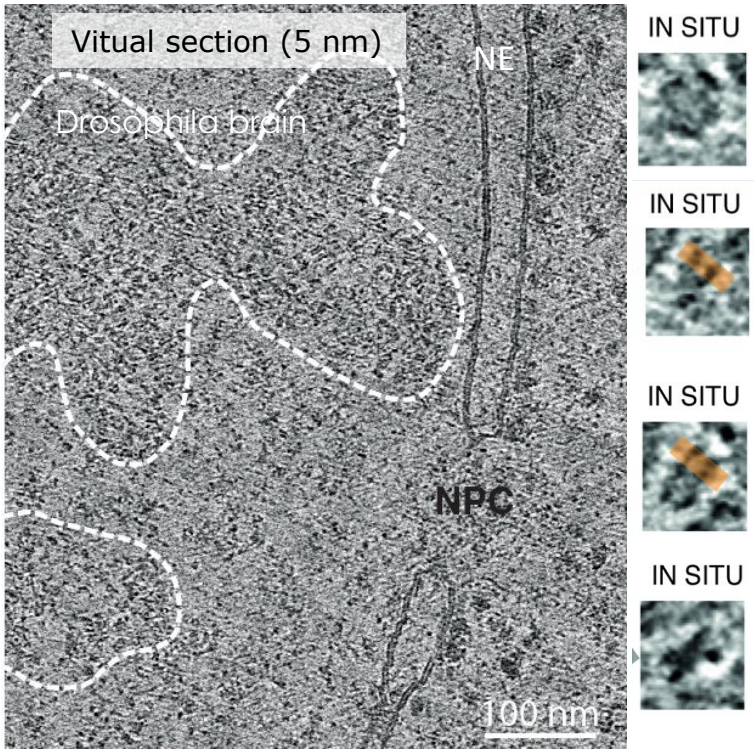
Drosophila embryo

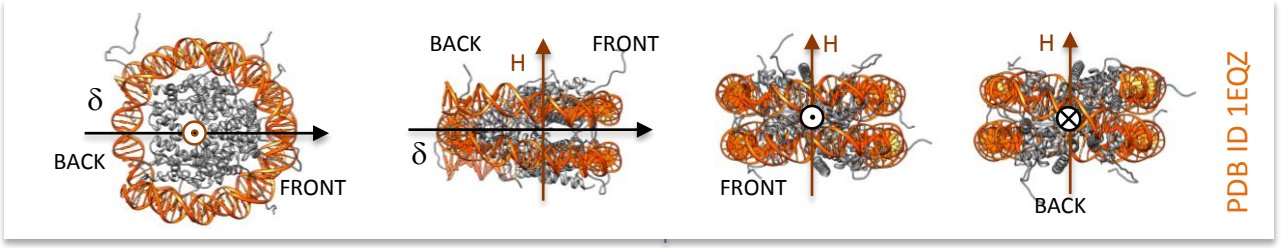
Imaging the nucleosome in its cellular context





Interphase nuclei





2D imaging

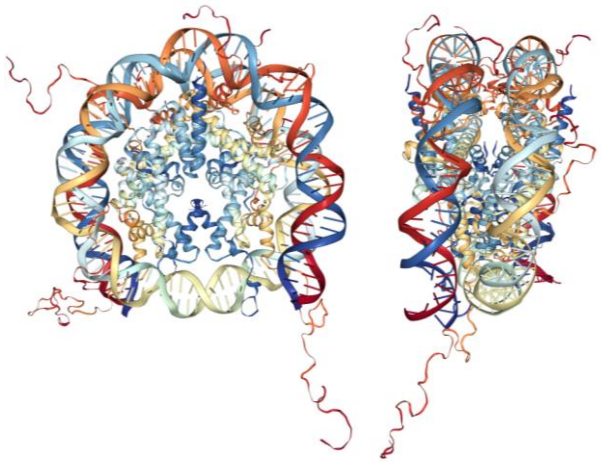
Tomography

Eltsov et al, NAR 2018

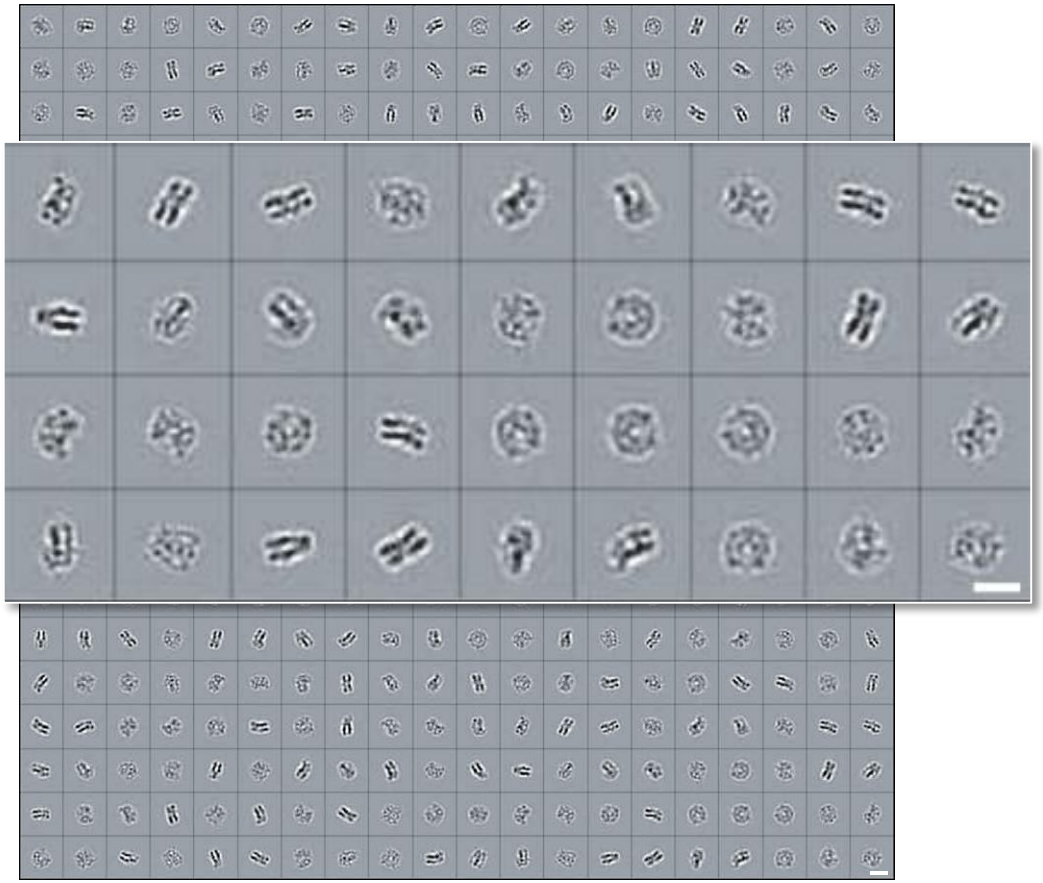
Simulation of cryoEM nucleosomes

Nucleosome canonical structure PDB ID 1EQZ

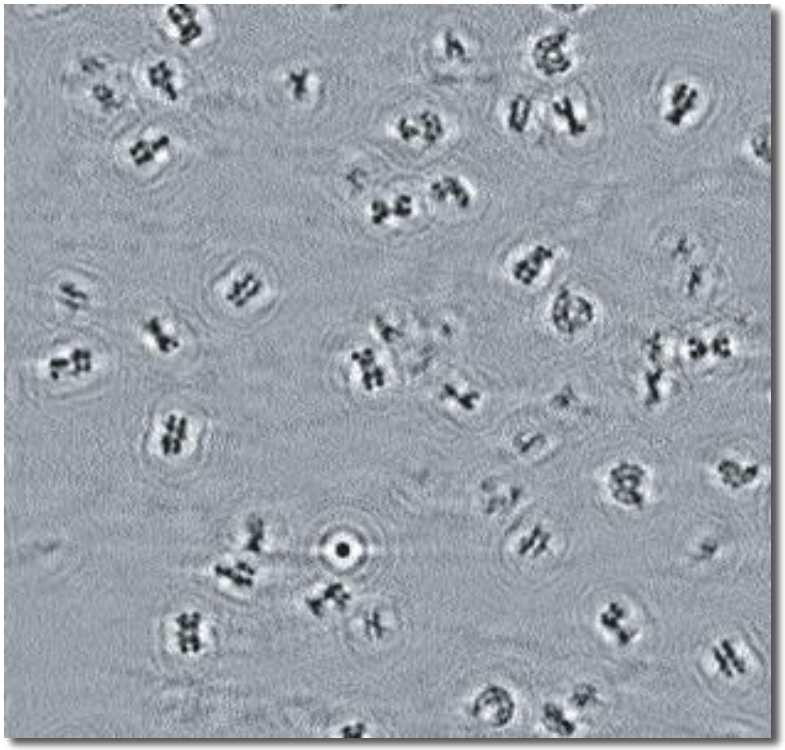
CTF-modulated



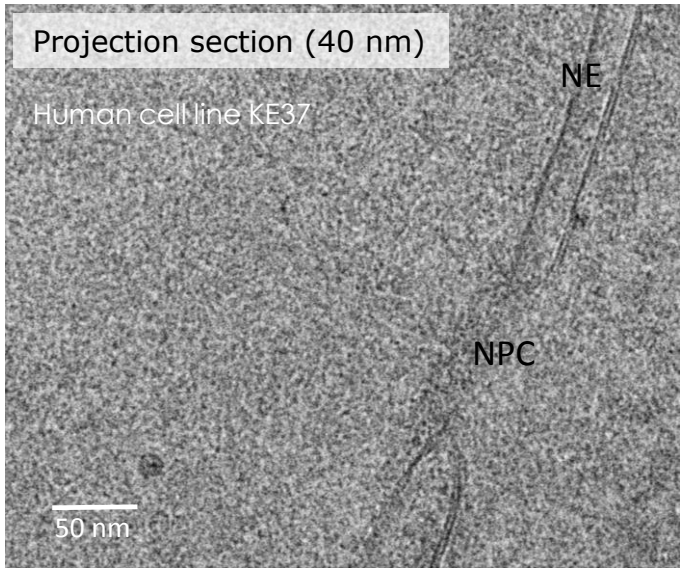
2D projections

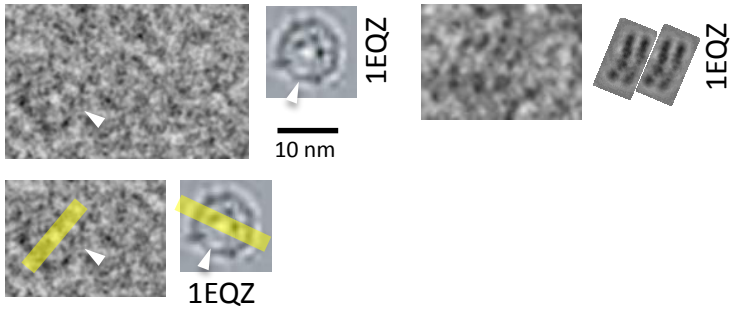


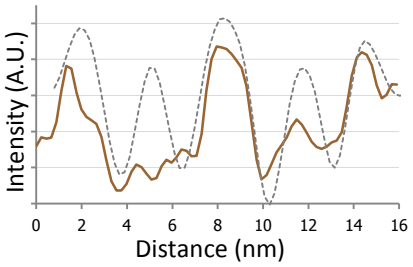
5-nm slices in simulated tomographic reconstructions

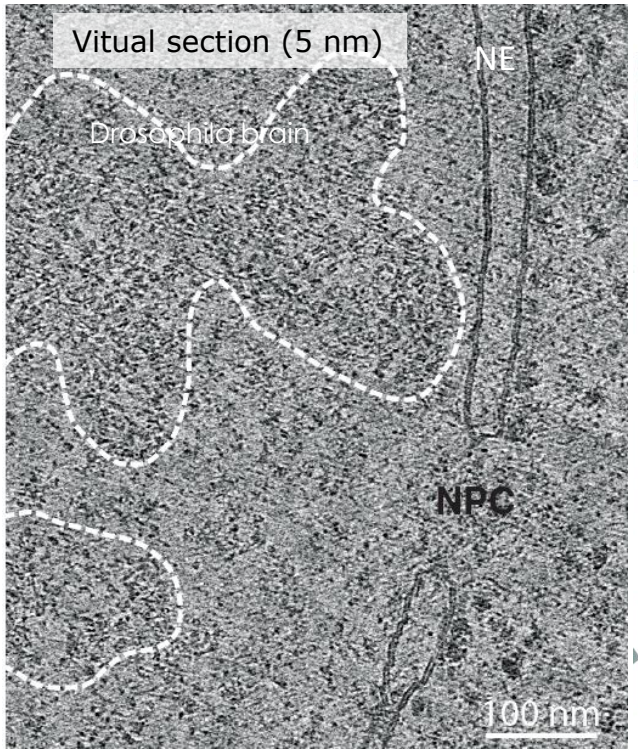


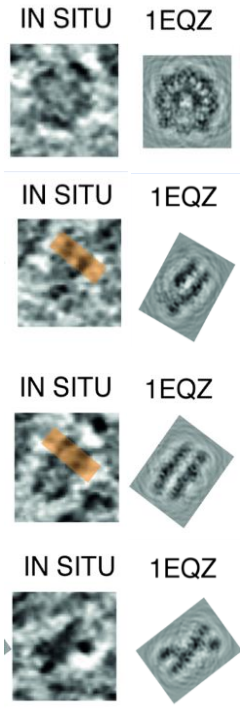
Can we visualise the nucleosome in its cellular context ?

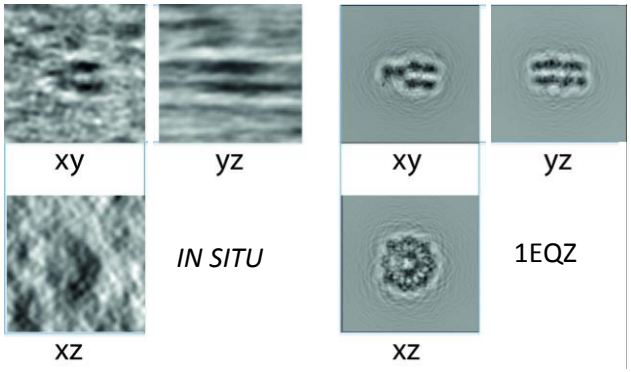












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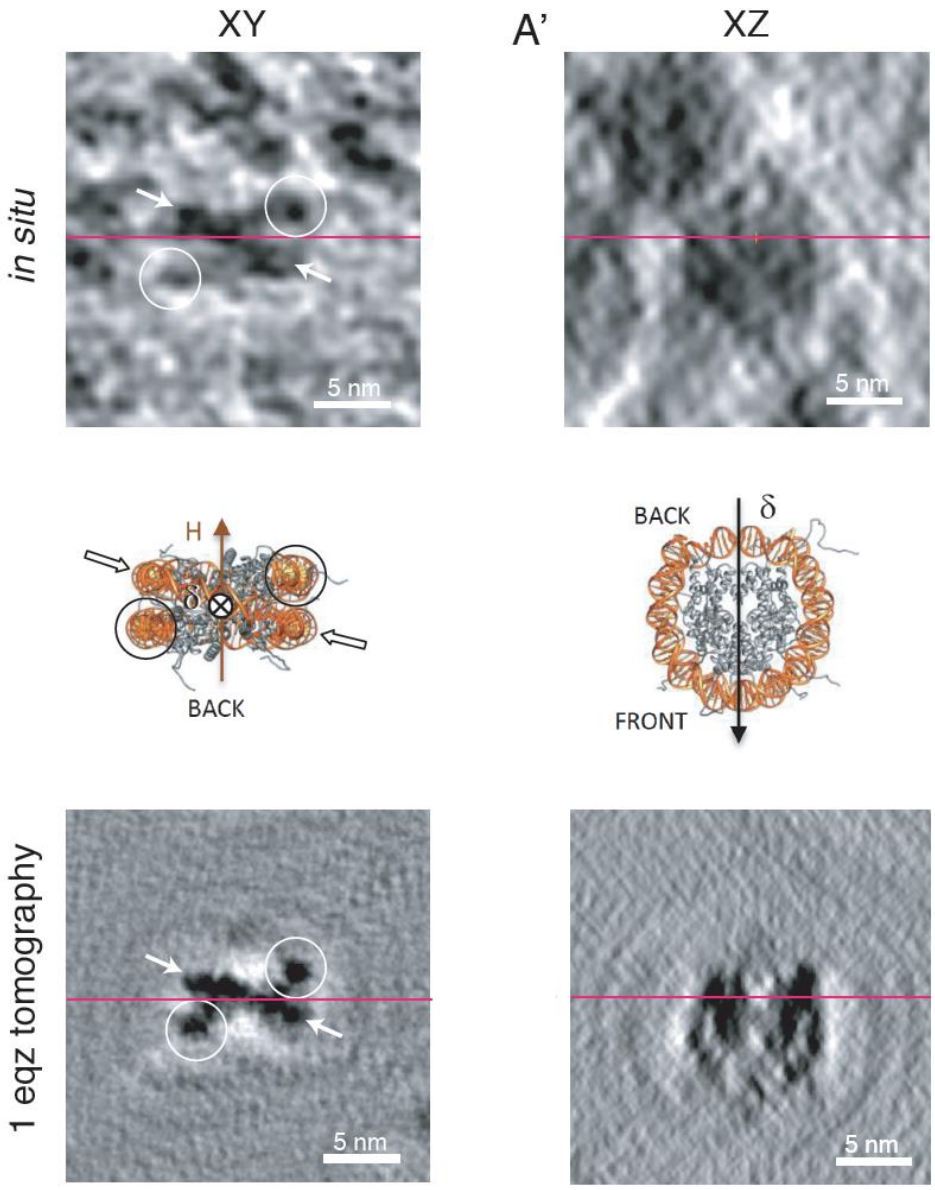
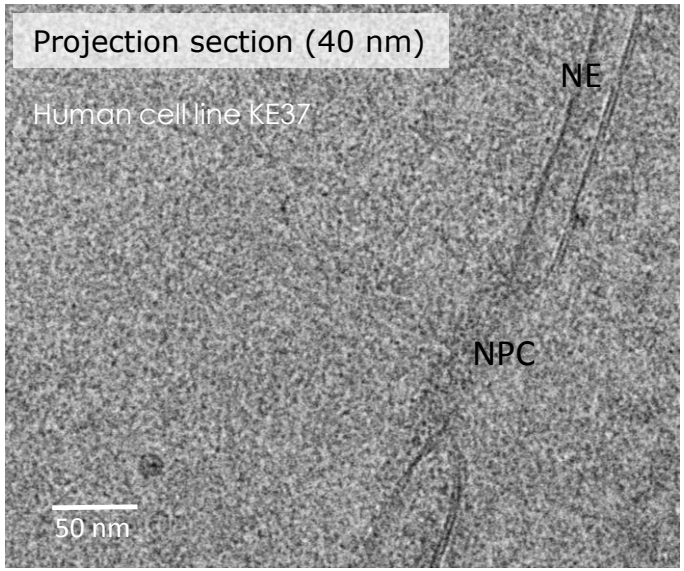
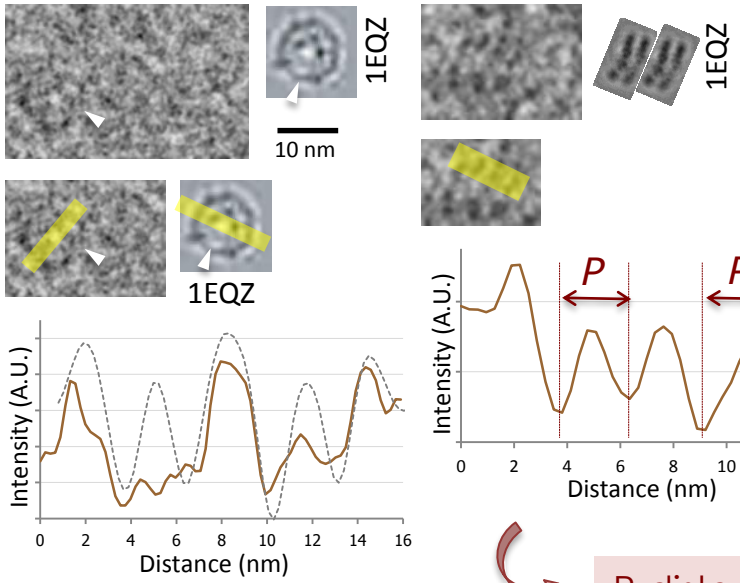
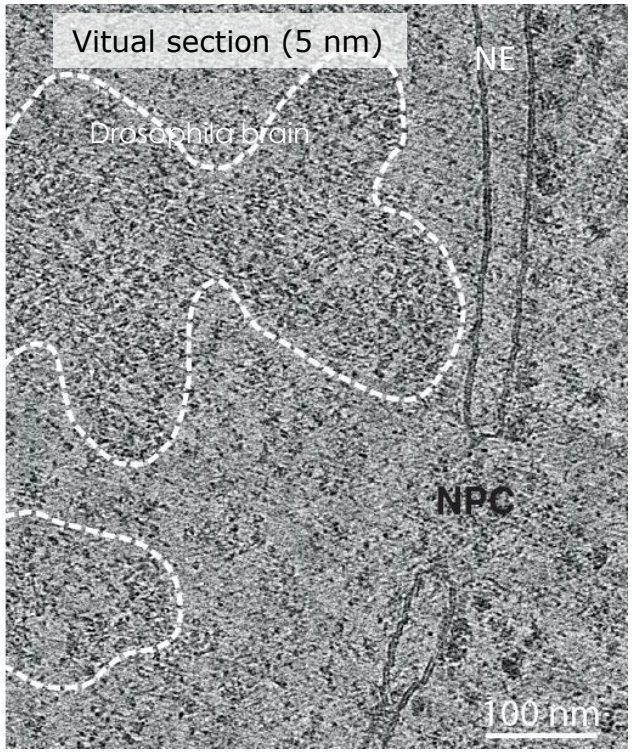
A left-handed DNA superhelix

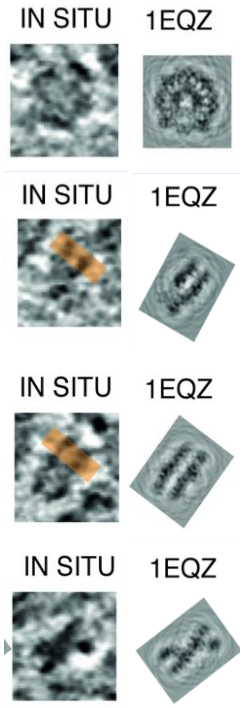


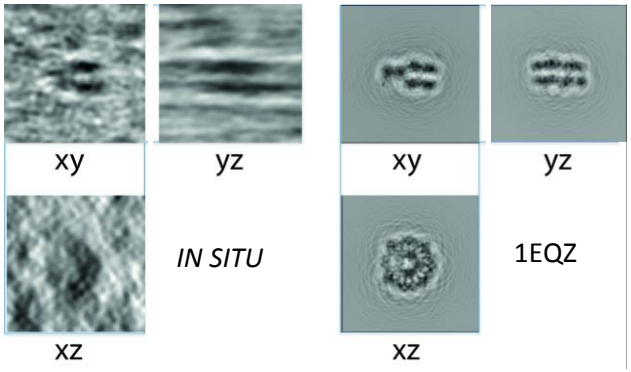
Can we visualise the nucleosome in its cellular context ?









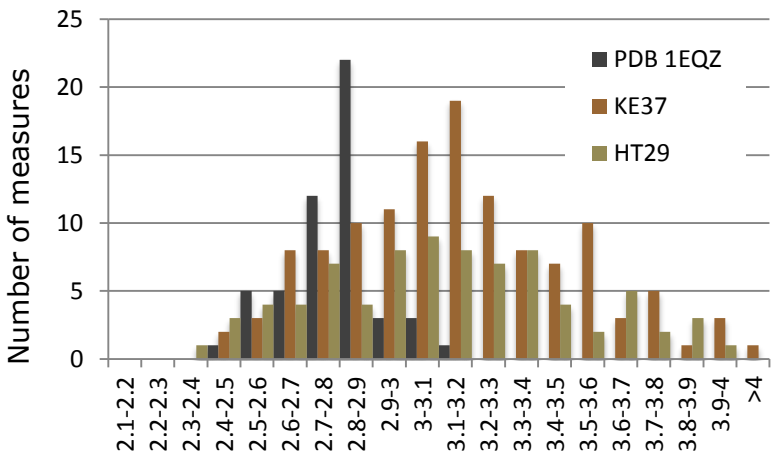


P-distances between DNA gyres

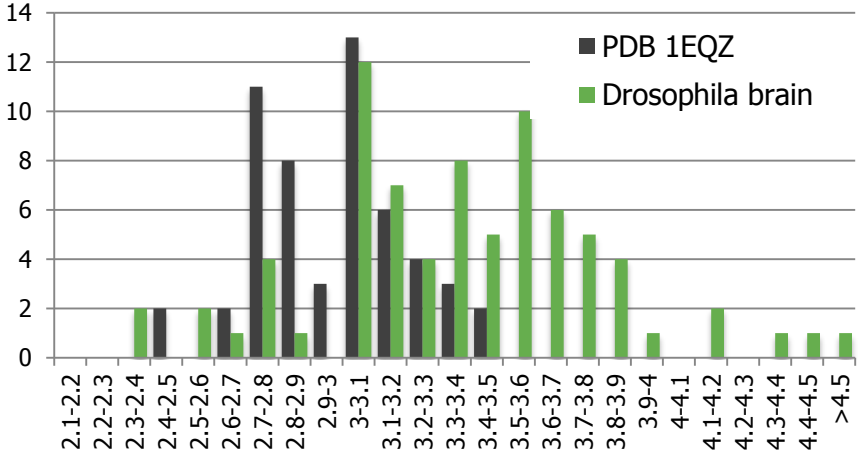
Eltsov et al, NAR 2018

The nucleosome conformation in interphase nucleus is more open than the crystallographic structure.

2D imaging data

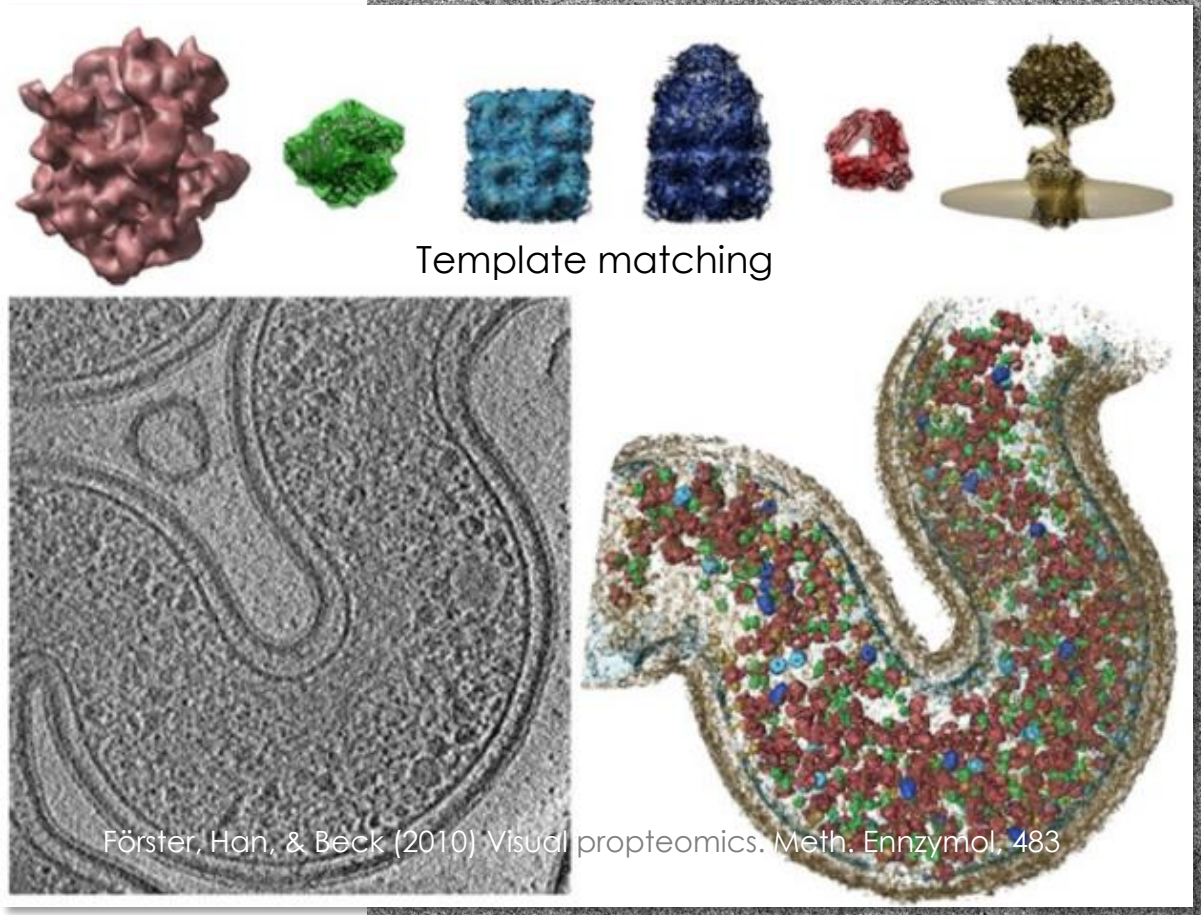


Cryo-tomo data



P-distance between DNA gyres (nm)

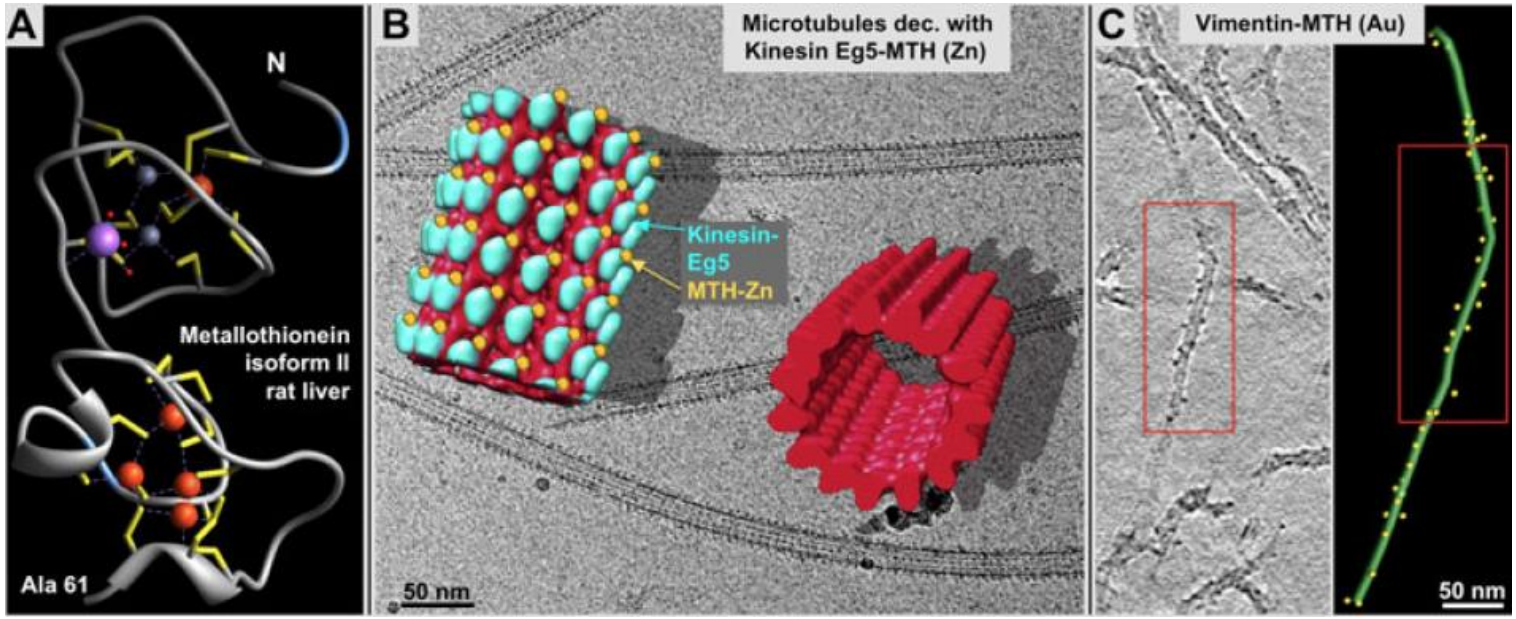
Targetting molecules of interest ?



100 nm

Identification of macromolecules in the cellular (complex) context

Electron dense clonable labels ?



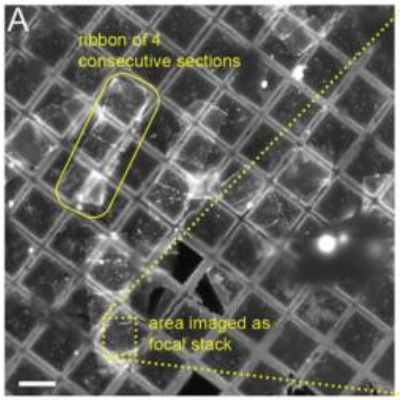
Proof of concept

metallothionein

Mercogliano & De Rosier, 2004
Bouchet Marquis et al, 2012
Hirabayashi et al, 2014

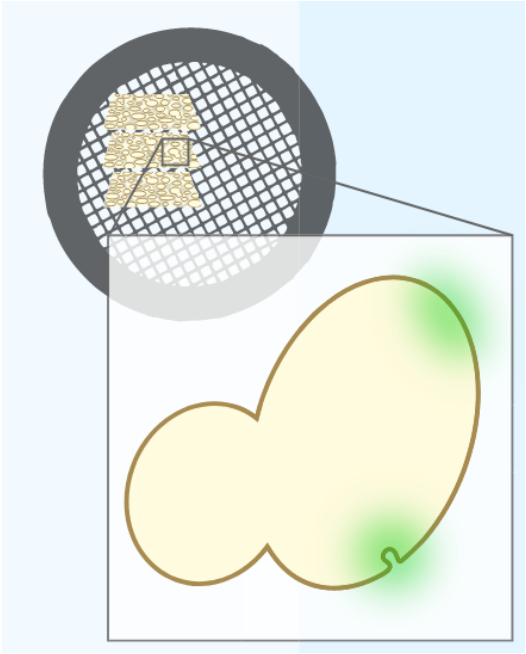
Toxicity
Endogeneous metallothionein

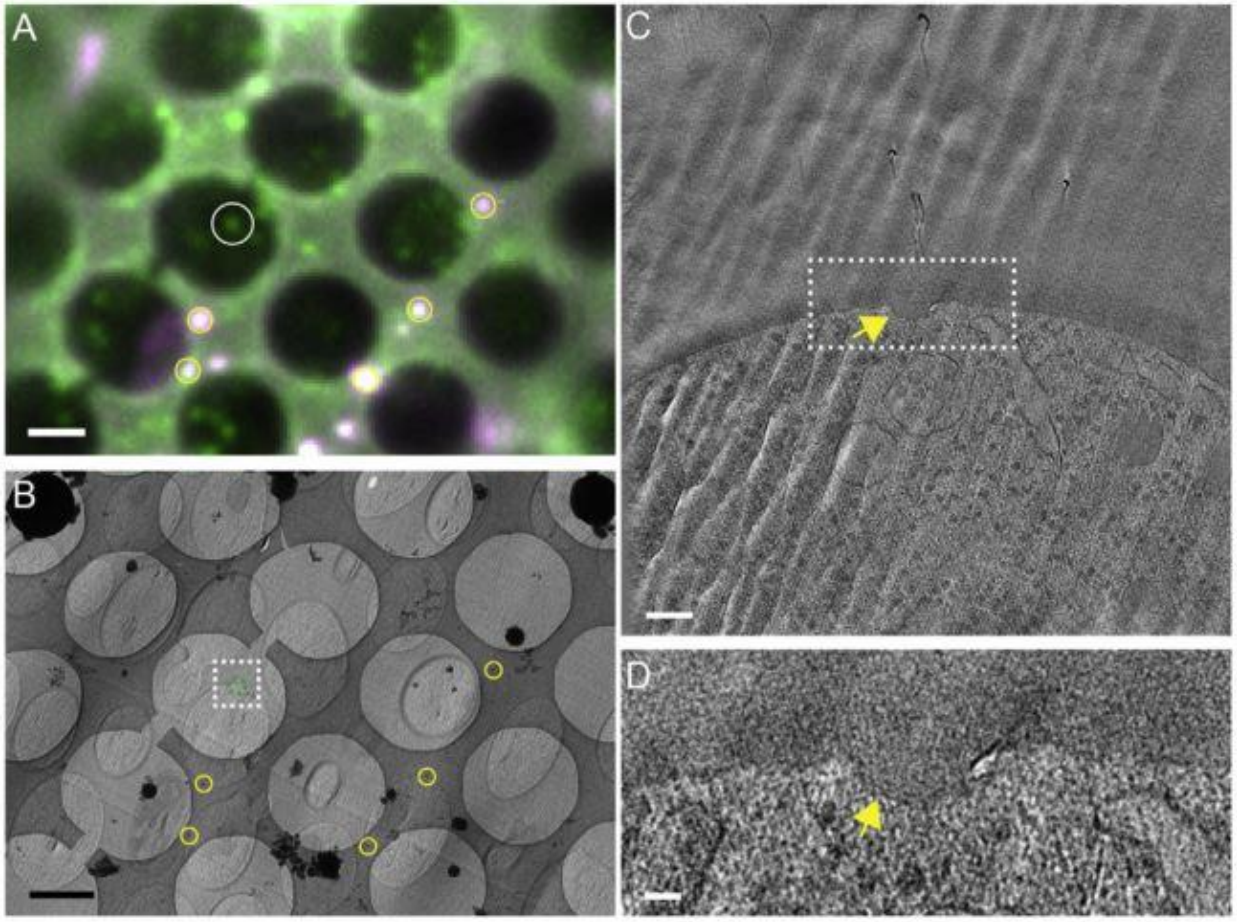
Identification of macromolecules in the cellular (complex) context



Cryo-CLEM

- cryo-fluorescence microscopy
- cryo-EM/ET





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Purified molecules in solution	Molecules in cells
Freezing Imaging CTF correction SPA	Freezing Thinning Imaging: tomography CTF correction Tomogram reconstruction Sub-tomogram averaging

Target/detect molecule of interest

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CRYO-FIB Milling

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CRYO-TOMO

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