# *Electron Cryo Tomography and CLEM*

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***CryoEM EMBO-Course 05. September 2019, London***

## *TECHNICAL ASPECTS*

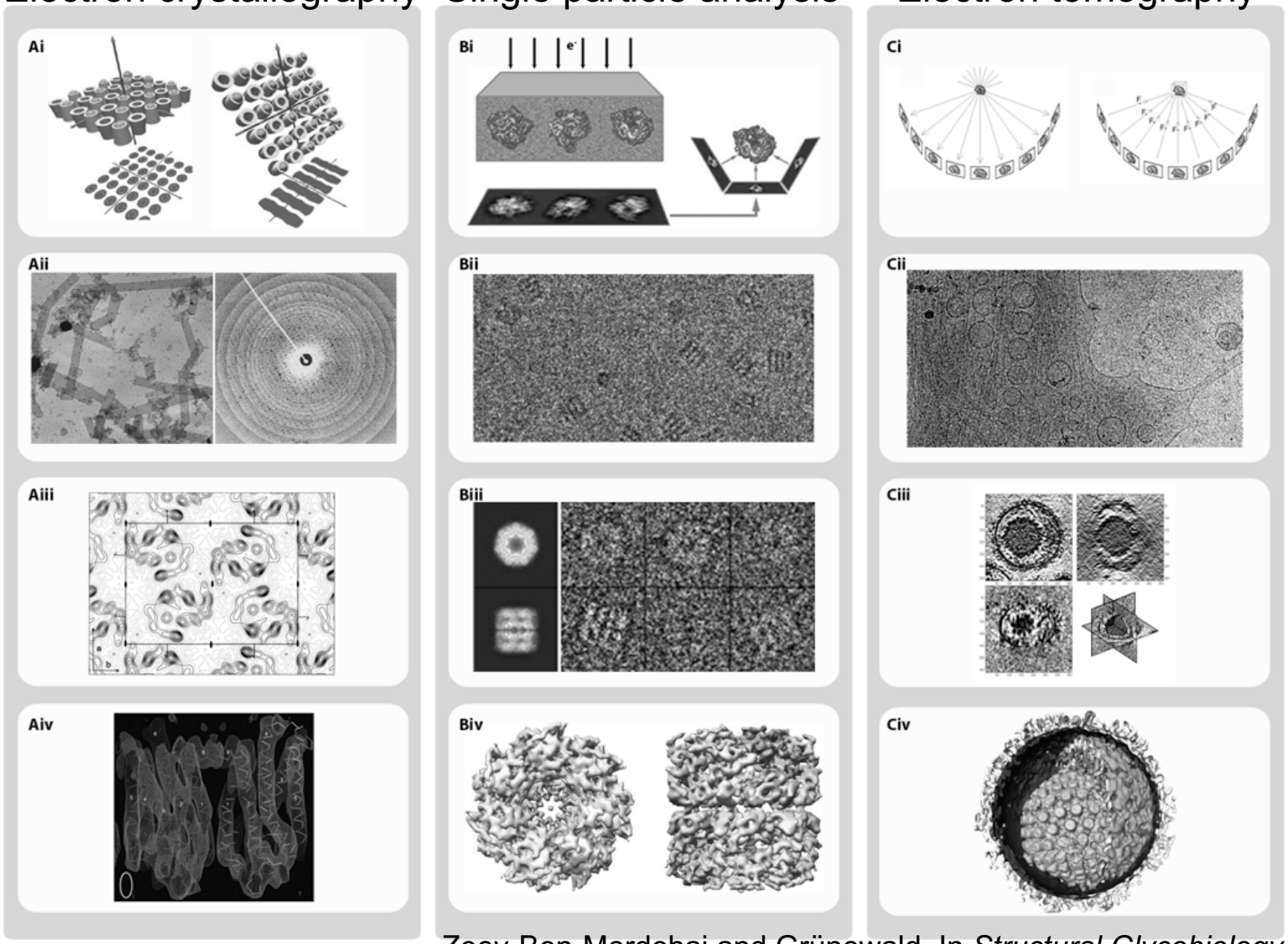
Principle of Electron Tomography
Sample Preparation
Data Acquisition
Alignment
Reconstruction
Data Analysis - Image Processing
Outlook

## *BIOLOGICAL APPLICATIONS and CLEM*

Hybrid Approaches

# *Molecular cryoEM modalities*

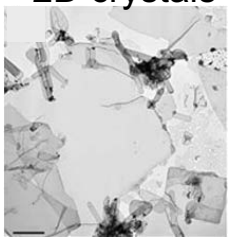
Electron crystallography | Single particle analysis | Electron tomography



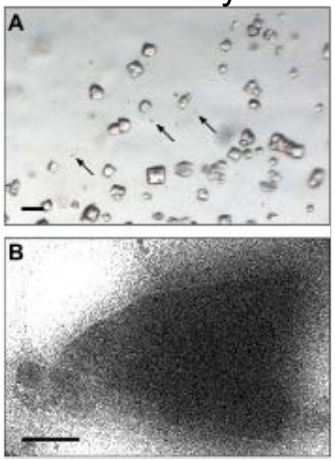

Zeev-Ben-Mordehai and Grünewald. In *Structural Glycobiology*. 2013

- ***Electron crystallography***
  - ***(purified) objects arranged in an ordered lattice, i.e. needs crystals, either 2D or small 3D***
    ***Aquaporin structure at 1.9 Å Gonen, et al. Nature. 2005***
    ***Lysozyme from 3D micro-crystal at 2.9 Å Shi, et al. 2013***

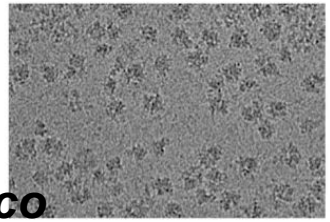
2D crystals

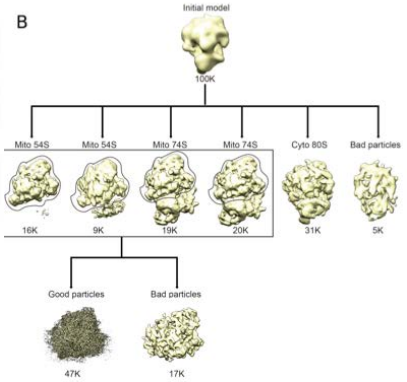


3D micro crystals

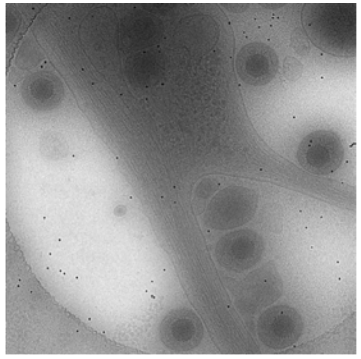



- ***Single particle analysis (SPA)***
  - ***(purified) objects arranged in many different random orientations***
  - ***Particles are computationally aligned - 'in-silico crystallisation'***
    ***Mitochondrial Large Ribosomal Subunit at 3.2 Å Amunts, et al Science 2014***



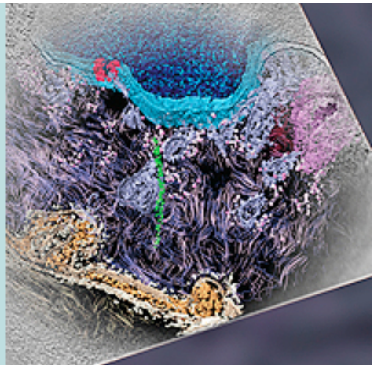

- ***Electron tomography (ET)***
  - ***unique biological objects, i.e. pleomorphic objects with a unique shape without symmetry***
  - ***cells, organelles, subcellular structures, viruses, macromolecules***



EMBL Conference

**Sixth International Congress on Electron Tomography**

**EMBL Heidelberg, Germany**
Thursday 5 May - Sunday 8 May 2011



http://www.embl.de/training/events/2011/TOM11-01/

ZING CONFERENCES

**7th International Electron Tomography Conference November 2014, Cancun**
www.zingconferences.com/7th-international-electron-tomography-conference-2014/
*'Welcome to the vast uncharted lands of electron tomography!'*

Grant Jensen, session chair, 3DEM Gordon Conference June 2015

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**8th International Conference on Electron Tomography, September 2018, Les Diablerets:** www.colorado.edu/symposium/etm2018/

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**Next:**
**9th International Conference on Electron Tomography,** September 13-16 2020 Egmond aan Zee, The Netherlands. https://tomo2020.org
(Organizers: Ariane Briegel / Friedrich Förster)

# *Modalities of biomolecular cryoEM*

Electron crystallography

Single particle analysis

**Electron tomography (ET)**

Ideal for **pleomorphic objects**

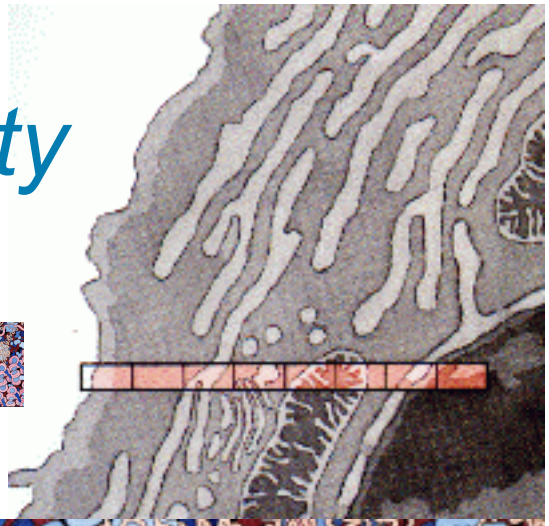
***Note***: cryoET is just a fraction of field; much larger community of ET of plastic embedded specimens

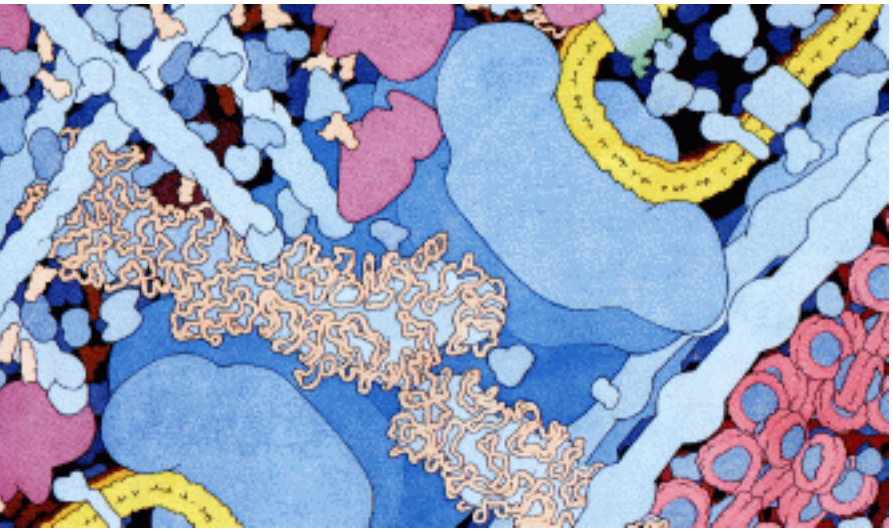
**Cellular tomography:**

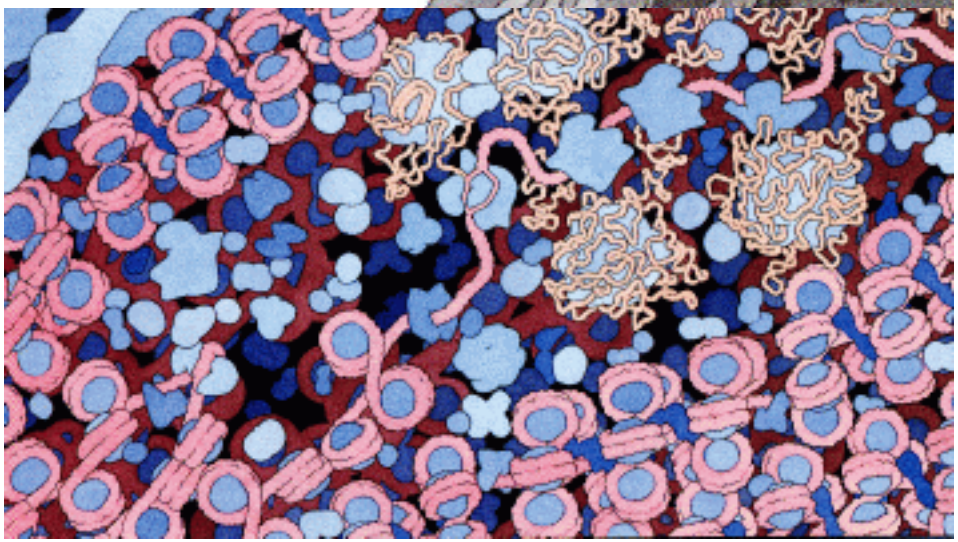
Concept of cell as a *giant supramolecular assembly*

**Macromolecules in their native cellular context!**

# *An artists view of the structure – function complexity*







David S. Goodsel

Rendered in watercolor on Arches paper.

Published in Moran, L.A. and Scrimgeour K.G. (1994) "Biochemistry"
Neil Patterson Publishers /Prentis Hall, North Carolina.



http://mgl.scripps.edu/people/goodsell

# *CryoET - not a new concept*

**Electron Microscopy of Unstained Biological Material: The Polytropic Montage**

Abstract. *With use of an electronic picture-scanning device and a digital computer, electron micrographs taken of a specimen along several different directions can be superimposed to form a montage that is more informative than the component images. Preliminary results indicate that one may thus study unstained, unshadowed biological material at high resolution.*

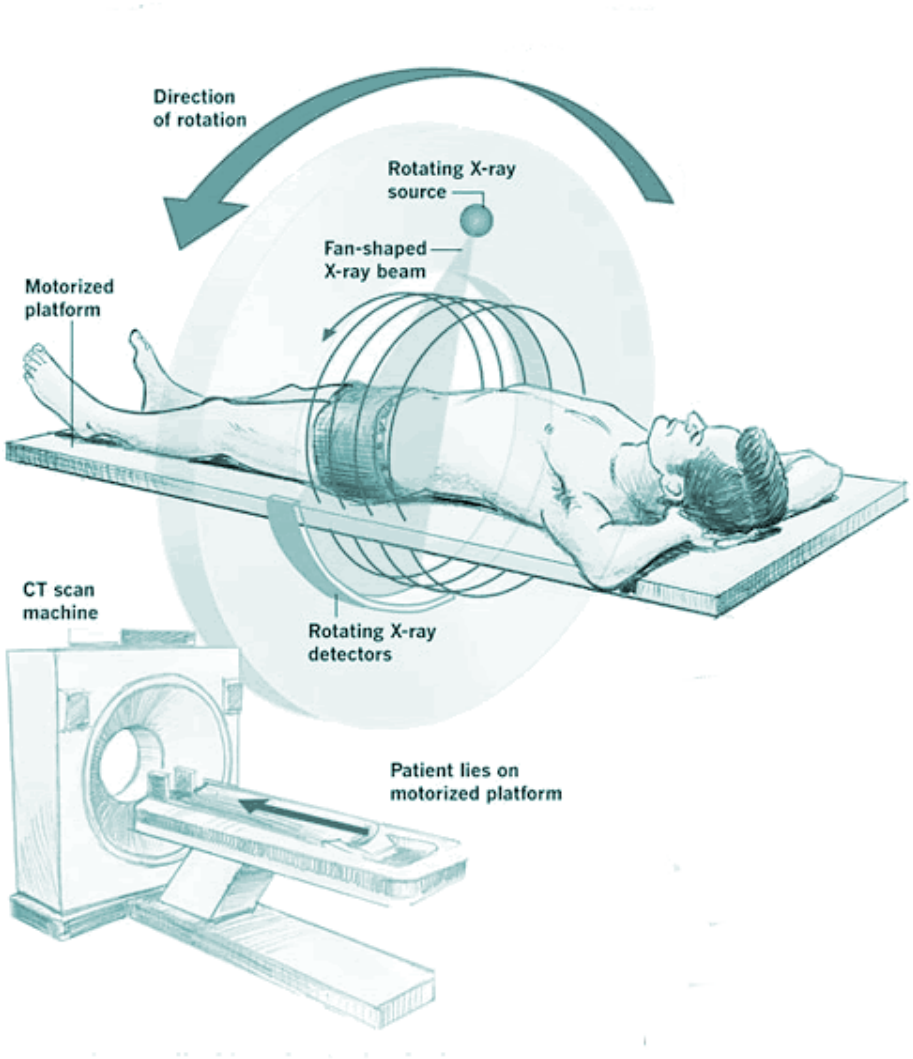
R.G. Hart, (1968) *Science* 159: 1464-1467.

**The dose fractionation theorem**
**Hegerl and Hoppe 1976**

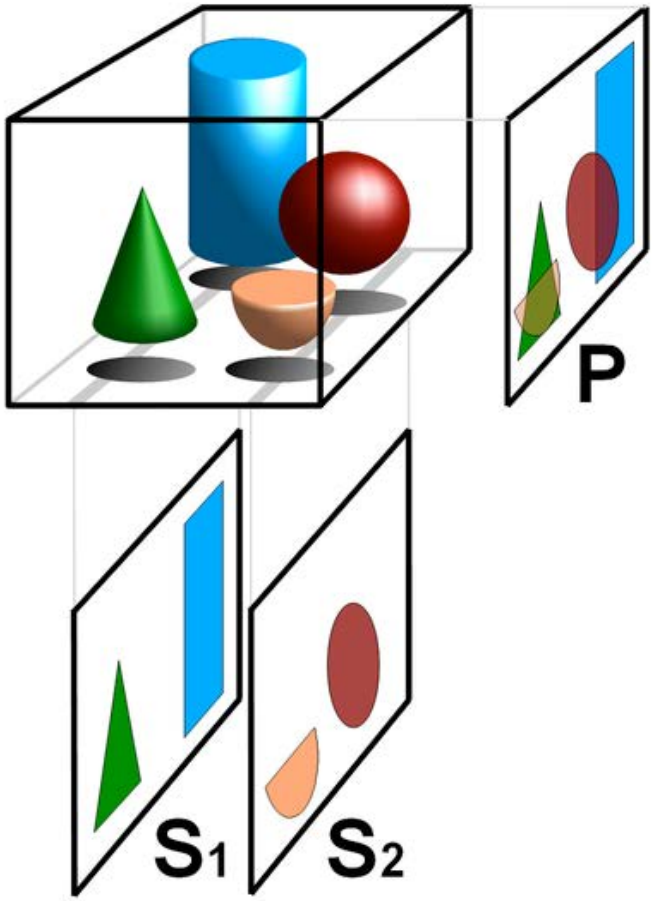
more information can be obtained from a tomogram than from a single projection by using the same electron dose

# *Tomography*

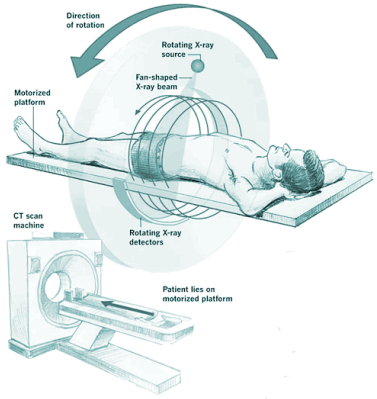
- ***collecting 2D projections from different directions***
- 
- ***recombine into 3D volume***
- ***analysing slices of the volume***

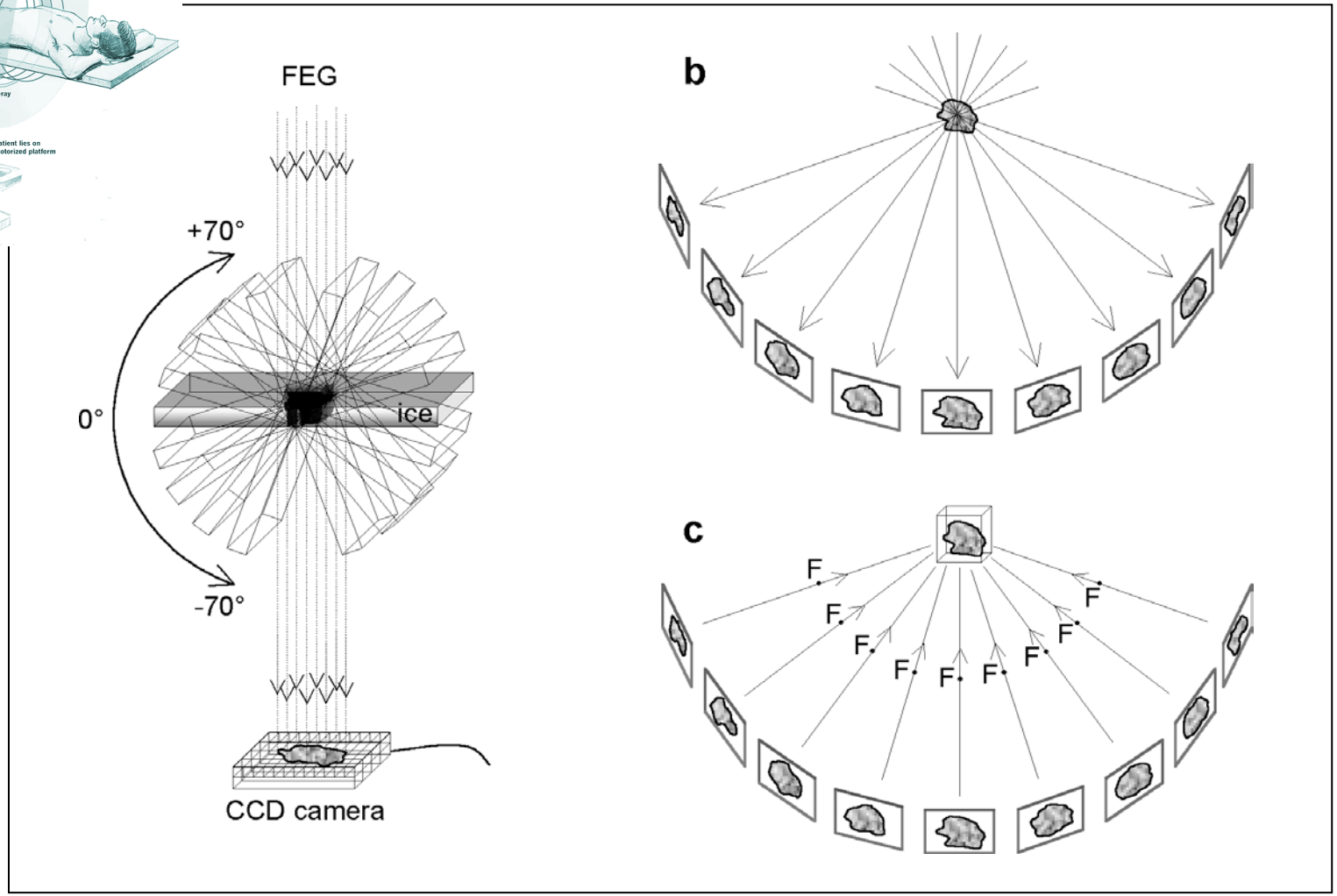


# *Difference between projection and slice*

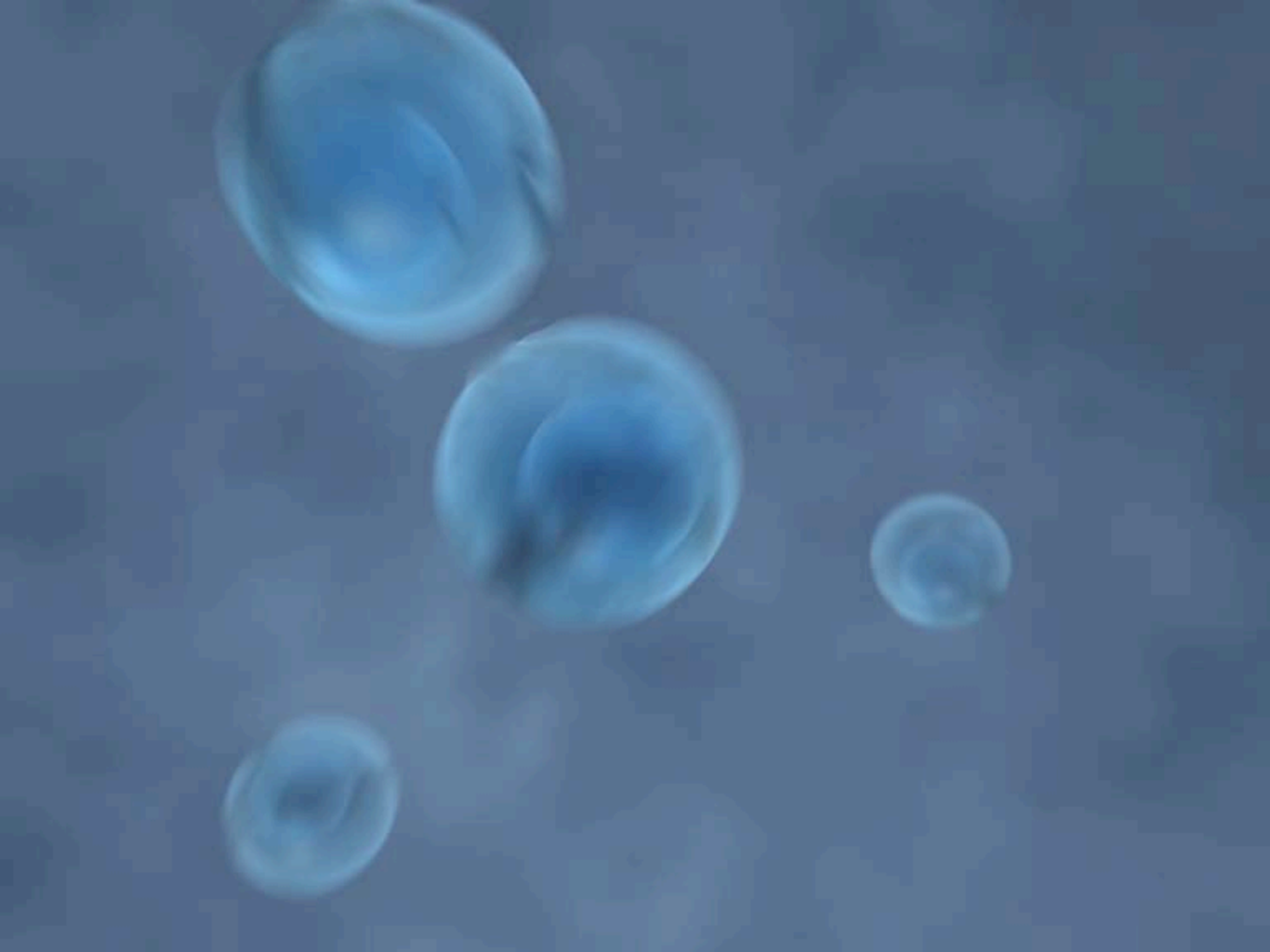


# *Principle of cryo-ET*

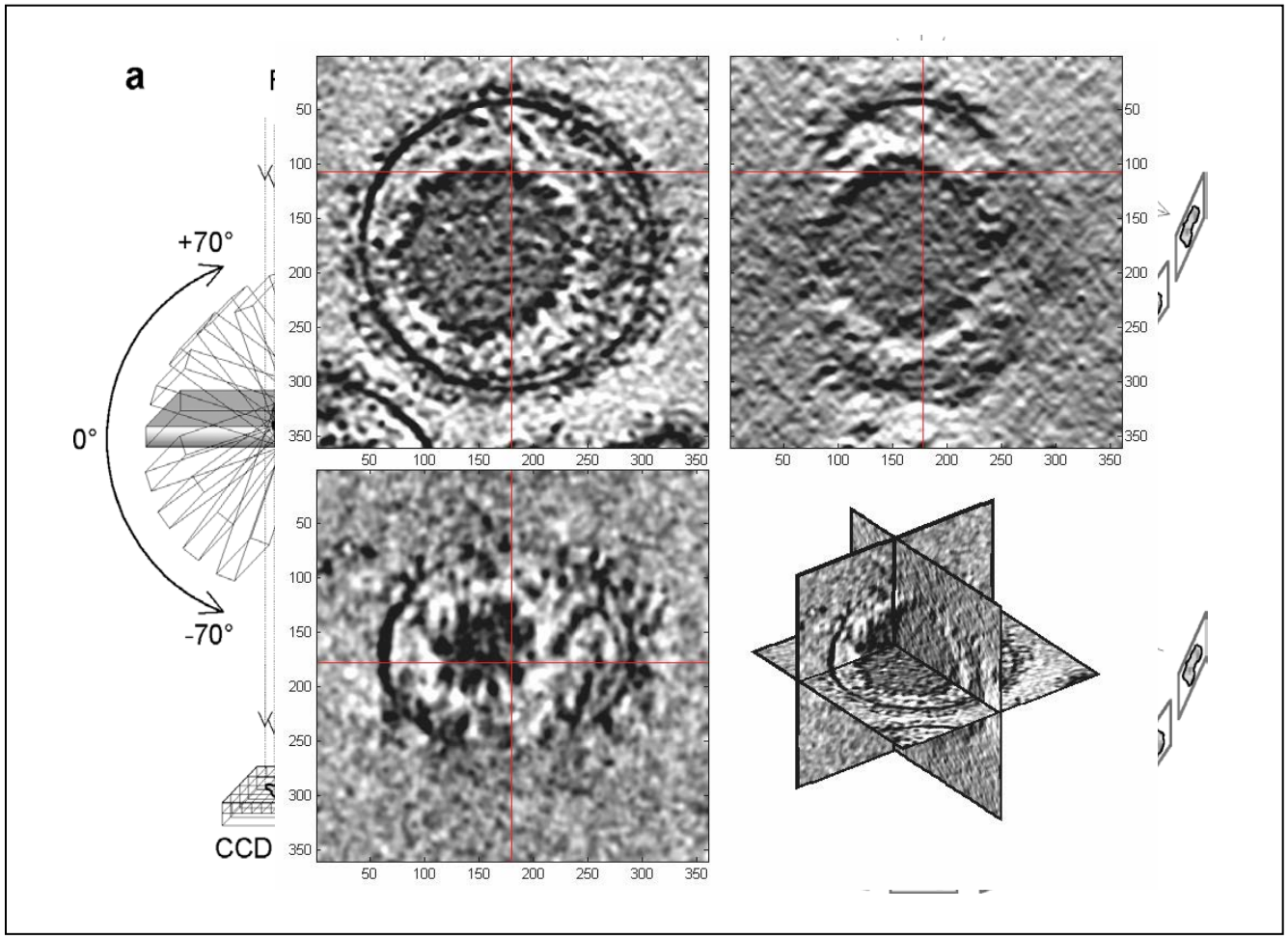


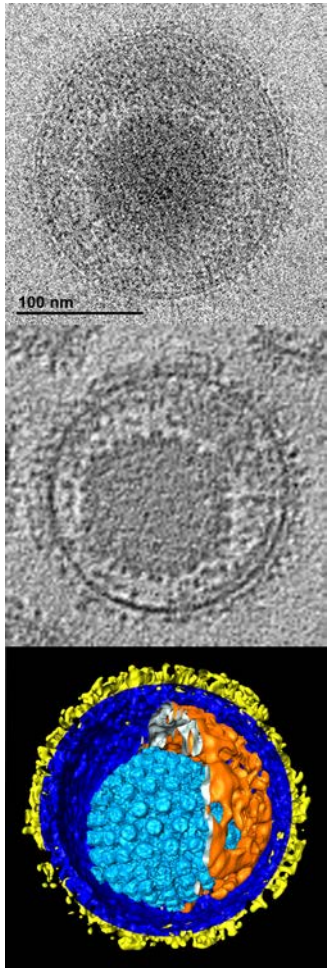




Grünewald *et al.* (2003) *Biophys. Chem.* 100: 577-591.



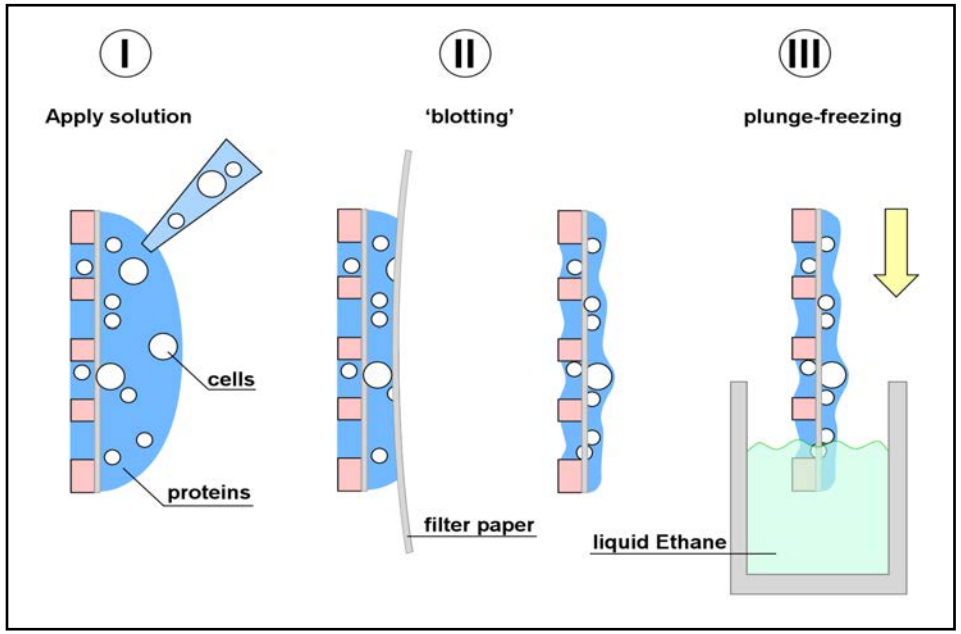
# *Electron cryo tomography*

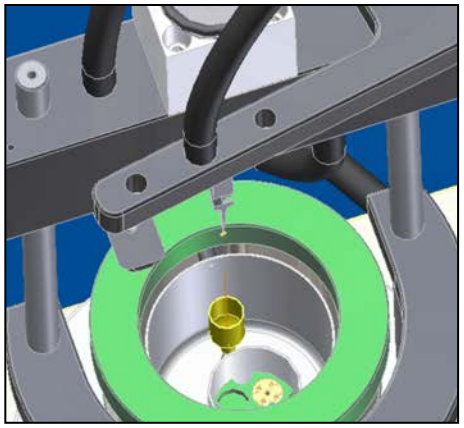


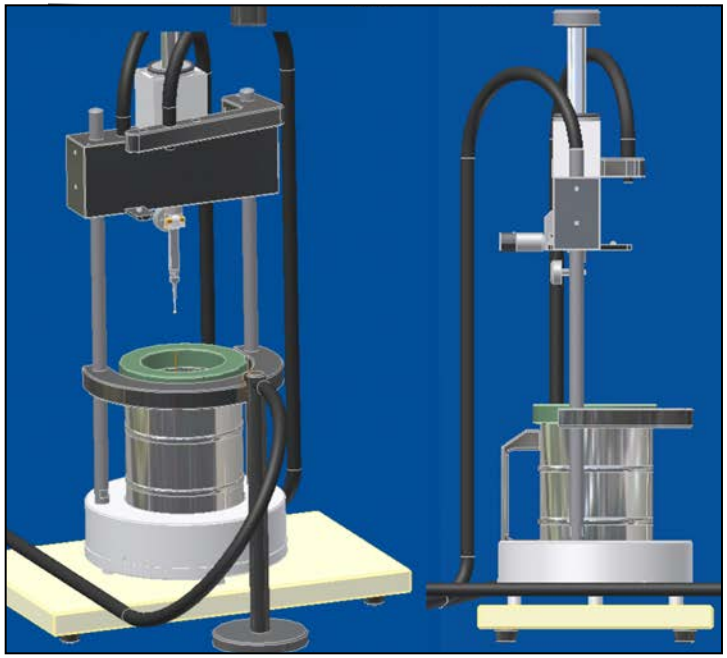




Resolution is anisotropic!

# *Sample preparation: 1. Suspensions*





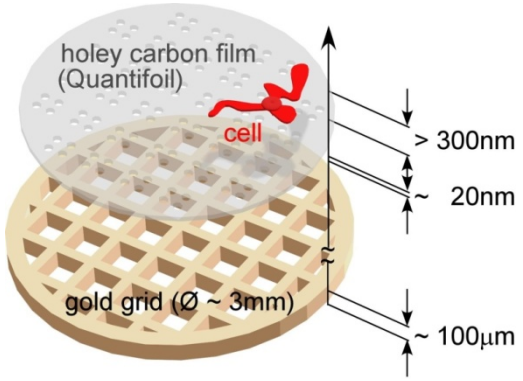
- Vitrification of biological objects
- densities relate directly to biological material (protein, lipid, nucleic acid …)
- Plunge freezing (or alternative routes) of suspension samples
- **Tomography samples more versatile**:
  - Purified larger complexes
  - Subcellular fractions, viruses, bacteria …
  - *In vitro* reconstituted assays
- **Add fiducial markers!**

# *Fiducial markers*

- **Rational**:
  - addition of easily recognisable features into samples
  - aiding the alignment of the tilt series before reconstruction
- Typically small **gold nanoparticles**:
  - electron dense
  - coated with protein shell (BSA, ProteinA ….)
    otherwise particles will often stick to sample!
  - size (10 nm, 5 nm… )
    depends on pixel size used, accuracy vs. easy visibility …
- Ways of **application**:
  - Mix with sample, just prior to plunging (on grid; in tube …)
  - apply to support film first ….
  - combinations …

# *Sample preparation: 2. Cellular cryoET*

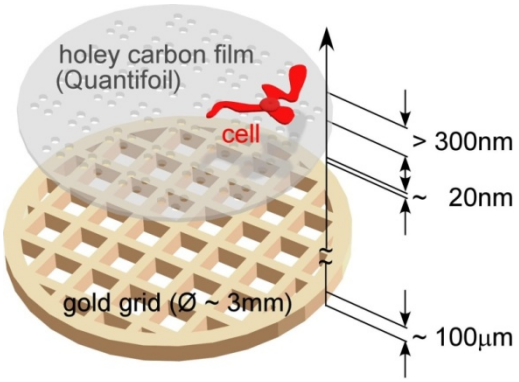
- growing cells directly on support of EM grid
- use gold grids (Au is inert, while is Cu is toxic)
- more fragile grids → handling considerations:
  - good pair of tweezers, check!
  - minimize handling steps!




**<u>Steps:</u>**

- glow discharge
- coating of grids (fibronectin, poly-lysine …; fiducial markers?)
- washing / incubation with medium (in low rim petri dish or ibidi slide …)
- Seeds cells, grow in low density (application dependent)
- freeze (plunge freezing)
- transfer to electron microscope
- *<u>optional:</u>* mount in autogrids, fluorescence observation … sample thinning …

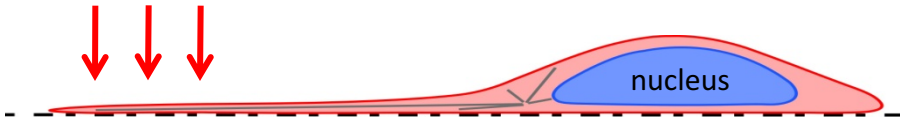
# Sample preparation: 2. Cellular cryoET




- dose fractionation theorem: very low signal to noise
- beam penetration limit under low dose conditions: ~1µm

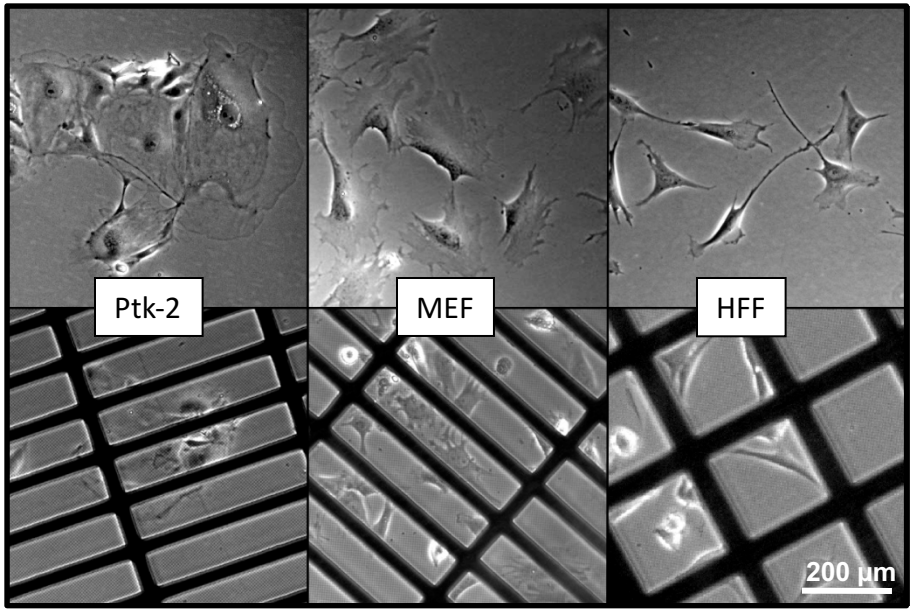
  keep your specimen thin! (200-500nm)
- Crowther criterion: d ~ π D/N

  Crowther *et al.* (1970) *Proc. Roy. Soc. London* A 317: 319-340.

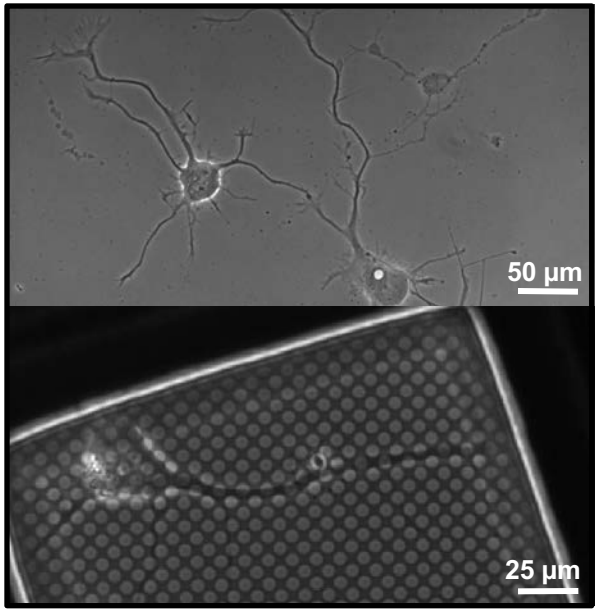



adherent cell on support film

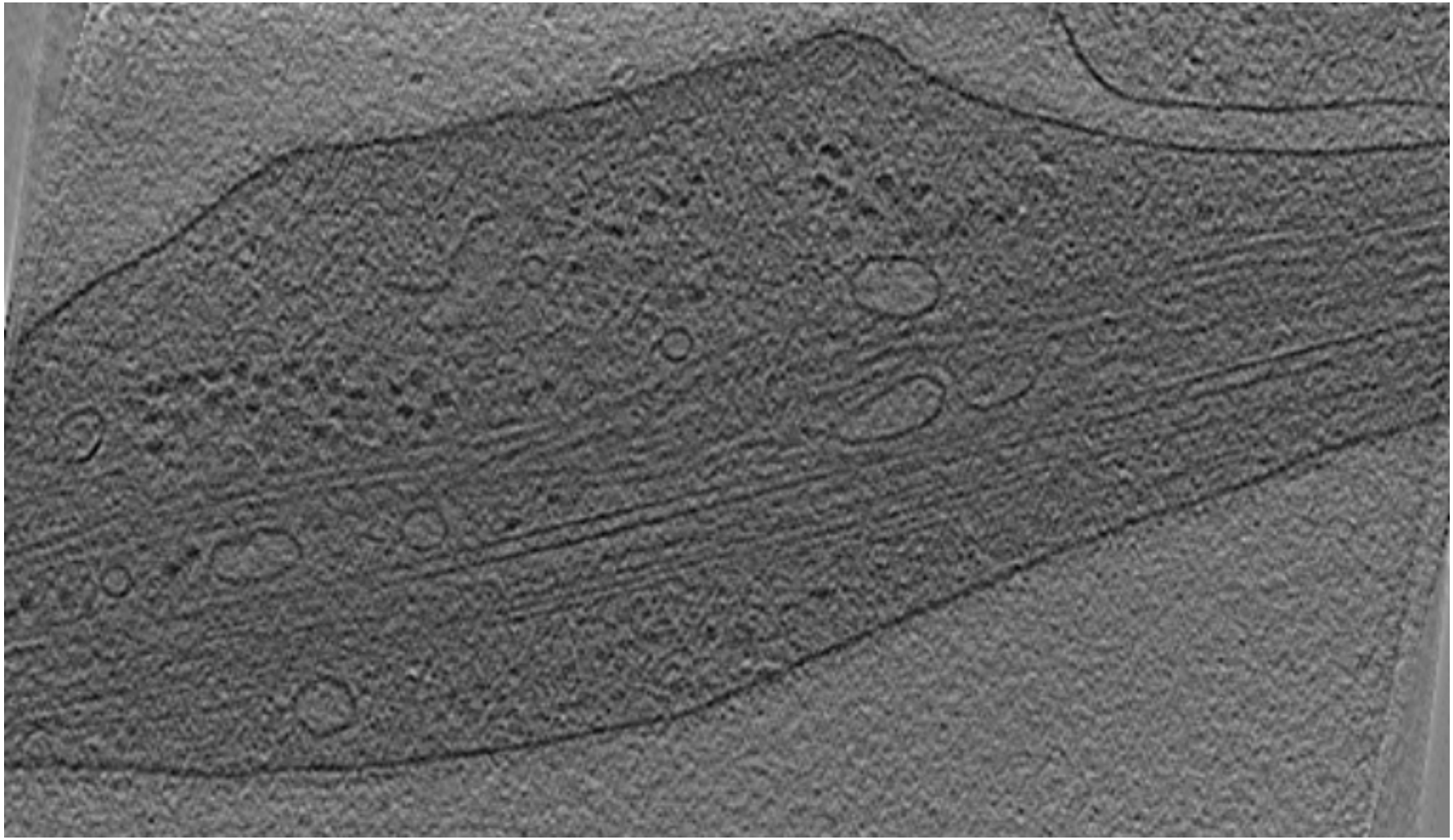
Non-neuronal cell lines



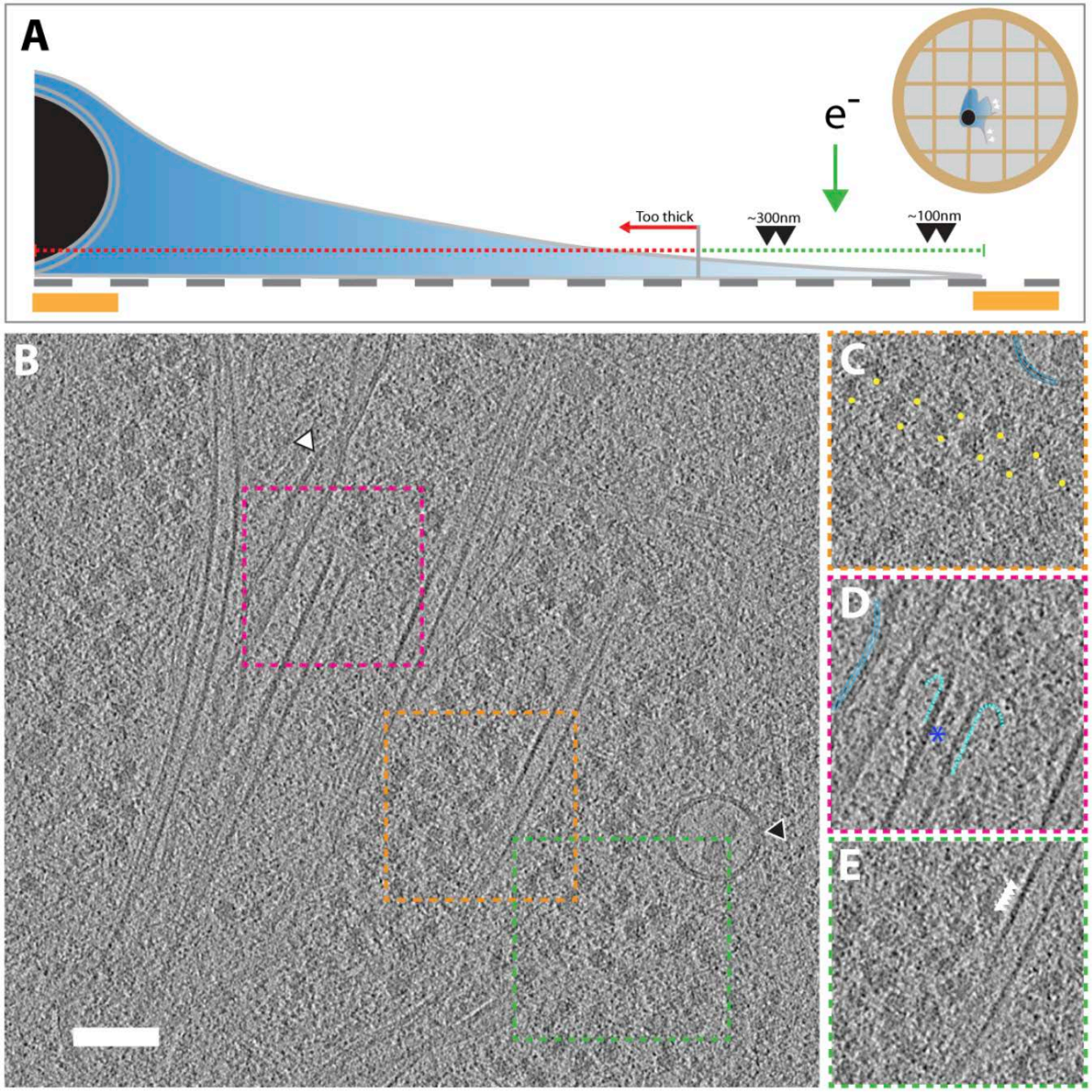

Hippocampal neurons



# *Cellular Cryo-ET: whole mount, thin area*

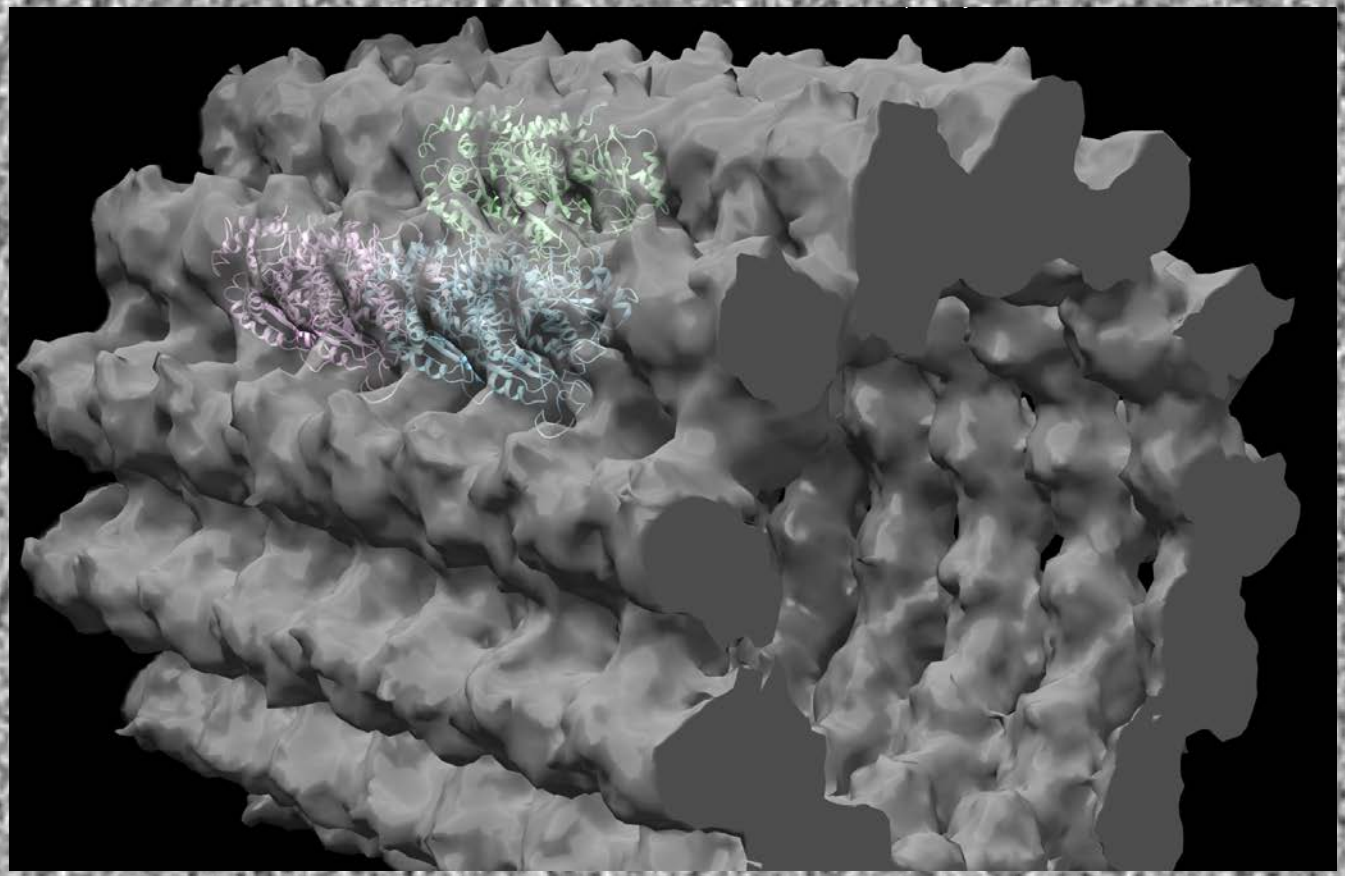


# *Cellular electron cryo tomography: complexes in situ*



Grange *et al.* (2016) *J. Struct. Biol*. doi: 10.1016/j.jsb.2016.06.024.

Michael Grange



# Macromolecular machines *in situ*: CELLULAR ELECTRON CRYO TOMOGRAPHY

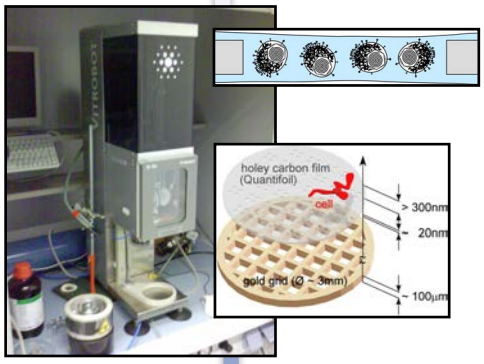
100nm

# Specimen thinning

**Cryo-immobilization:**

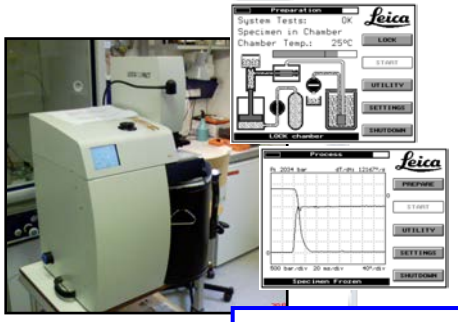
**sample**

**Plunge-freezing (< 5 µm)**

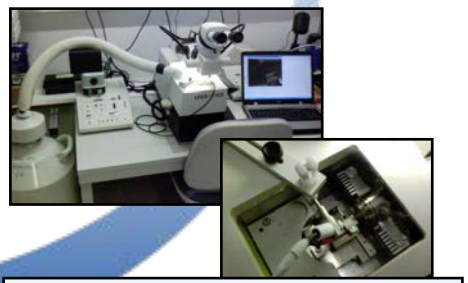



**direct**

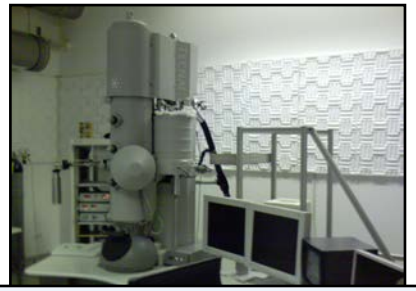
**High pressure-freezing (200-300 µm)**



**CEMOVIS**



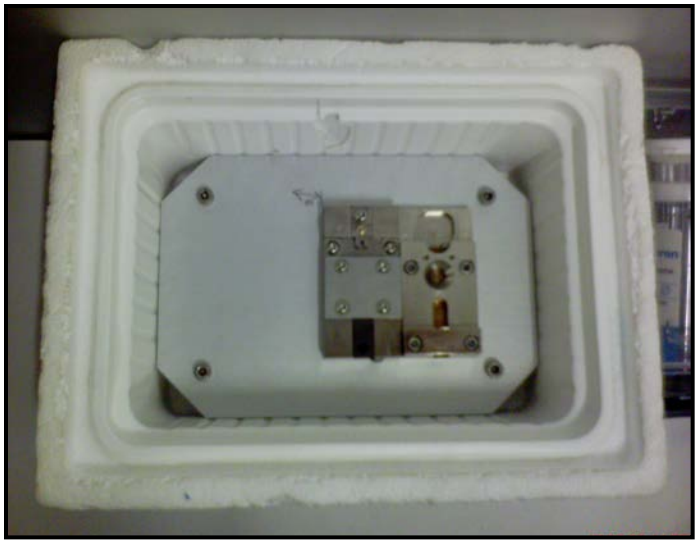
**Cryo-sectioning (< 138 K)**

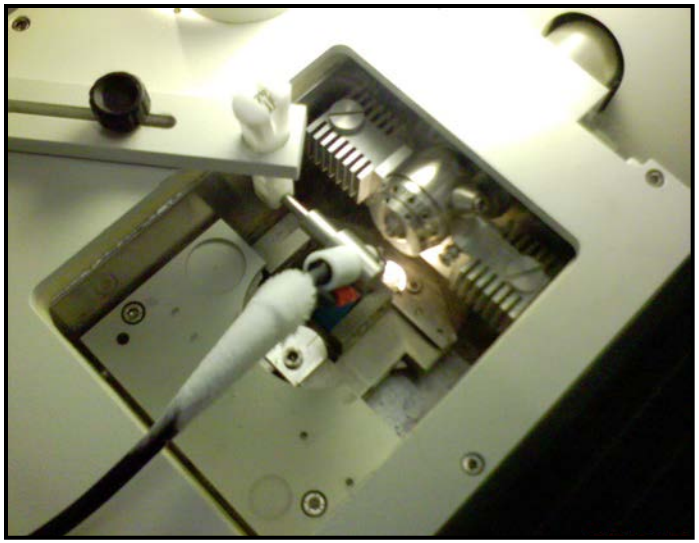


**CryoEM / Tomography**

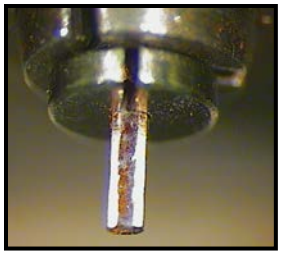
**sample thickness limit** for cryoET: **~ 1 µm** *(Lučić et al. (2005) Ann. Rev. Biochem. 74: 833-865)*

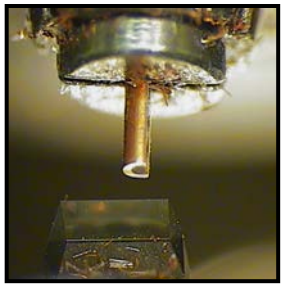
# *High-pressure freezing and cryo-sectioning*

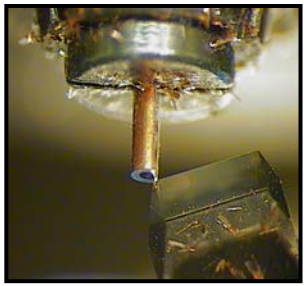




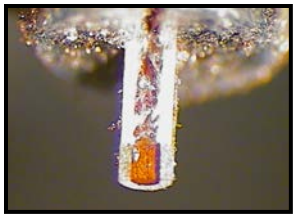
Leica EMPACT (copper tube freezing), tube slicing (‘banana splitter’) and transfer to Leica FCS cryo-chamber, …

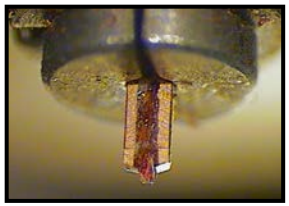


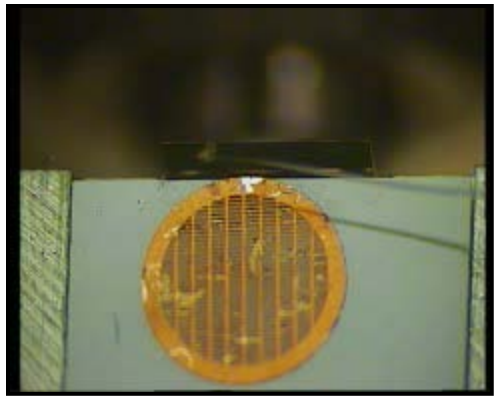




… **targeted** trimming of the sliced copper tube …



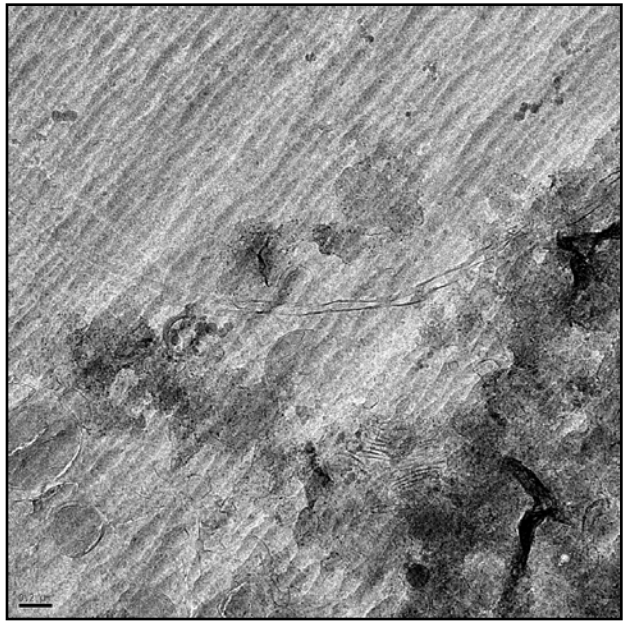




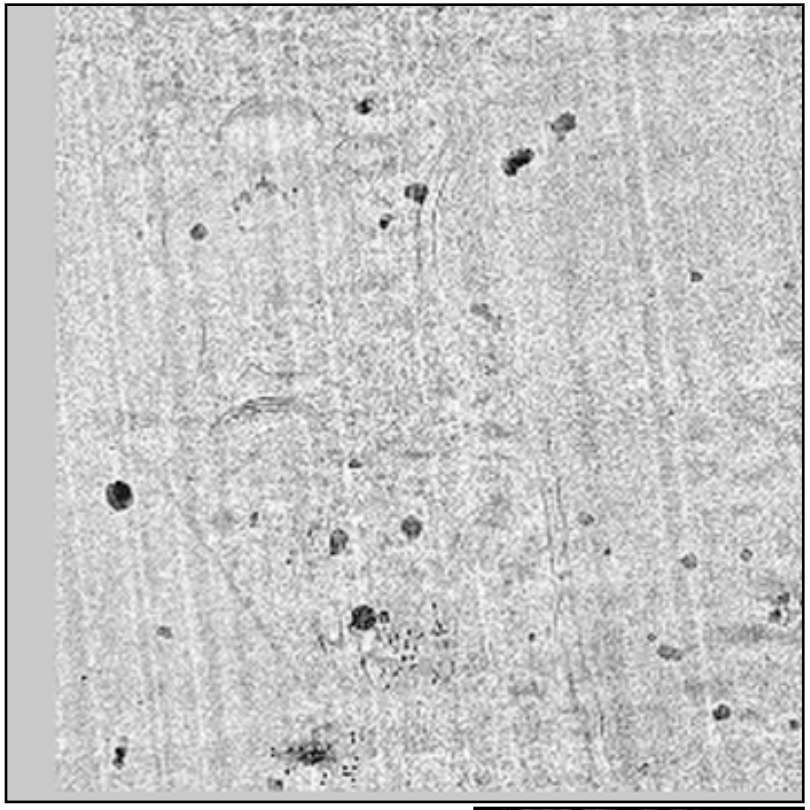
… and cutting.

**CEMOVIS is art!**

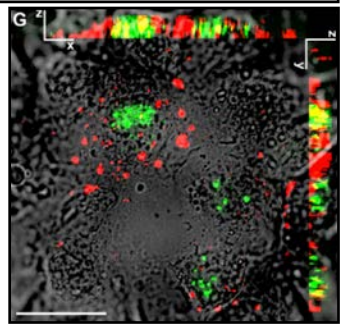
# CEMOVIS: marker problem



**Gold marker deposition** according to *Gruska et al. (2008), J. Struct. Biol. 161: 384-392.*

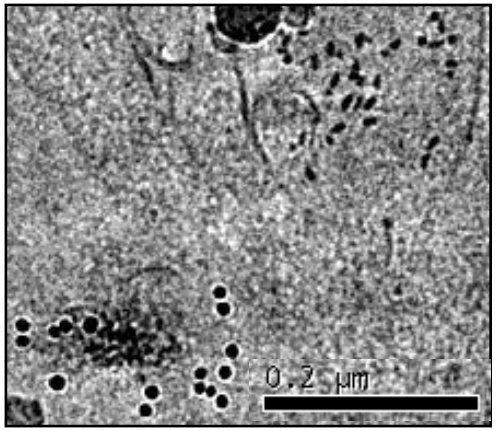


Adherent **Vero cell, 30 nm** nominal section feed, Tecnai F20, -60° -> 60°, Saxton scheme, 1/cos



*Hagen & Grünewald (2008), J. Microsc. 230: 288-296.*

**Quantum dots** (655 nm) and **BSA-protected 13 nm gold beads** taken up endocytotically by a Vero cell

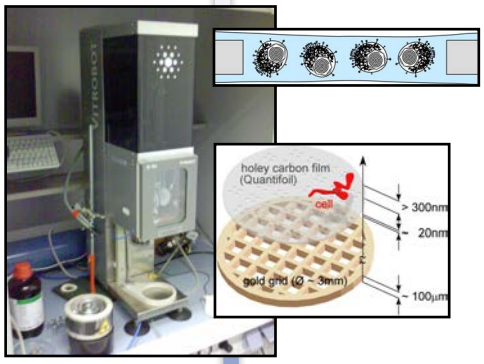


# *Specimen thinning*

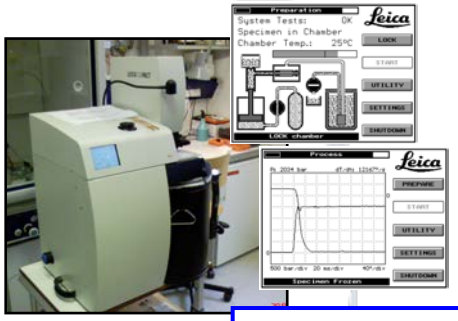
**sample**

**Cryo-immobilization:**

**Plunge-freezing (< 5 µm)**



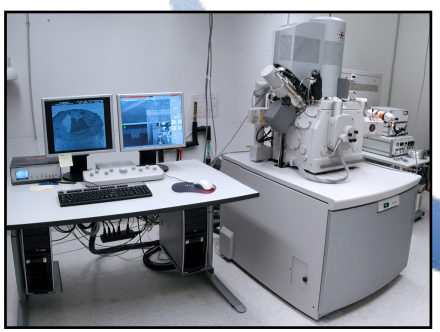

**High pressure-freezing (200-300 µm)**



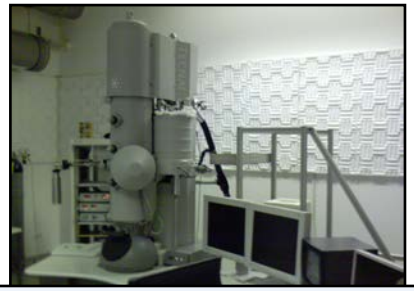
**CEMOVIS**

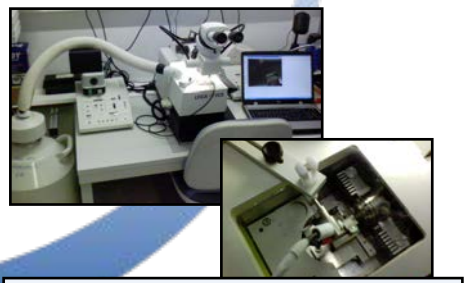
**Focused-ion-beam (FIB) milling**

*Marko et al. (2007) Nat. Meth. 4: 215-217.*
*Rigort et al. (2010) JSB **172**: 169-173.*



**direct**



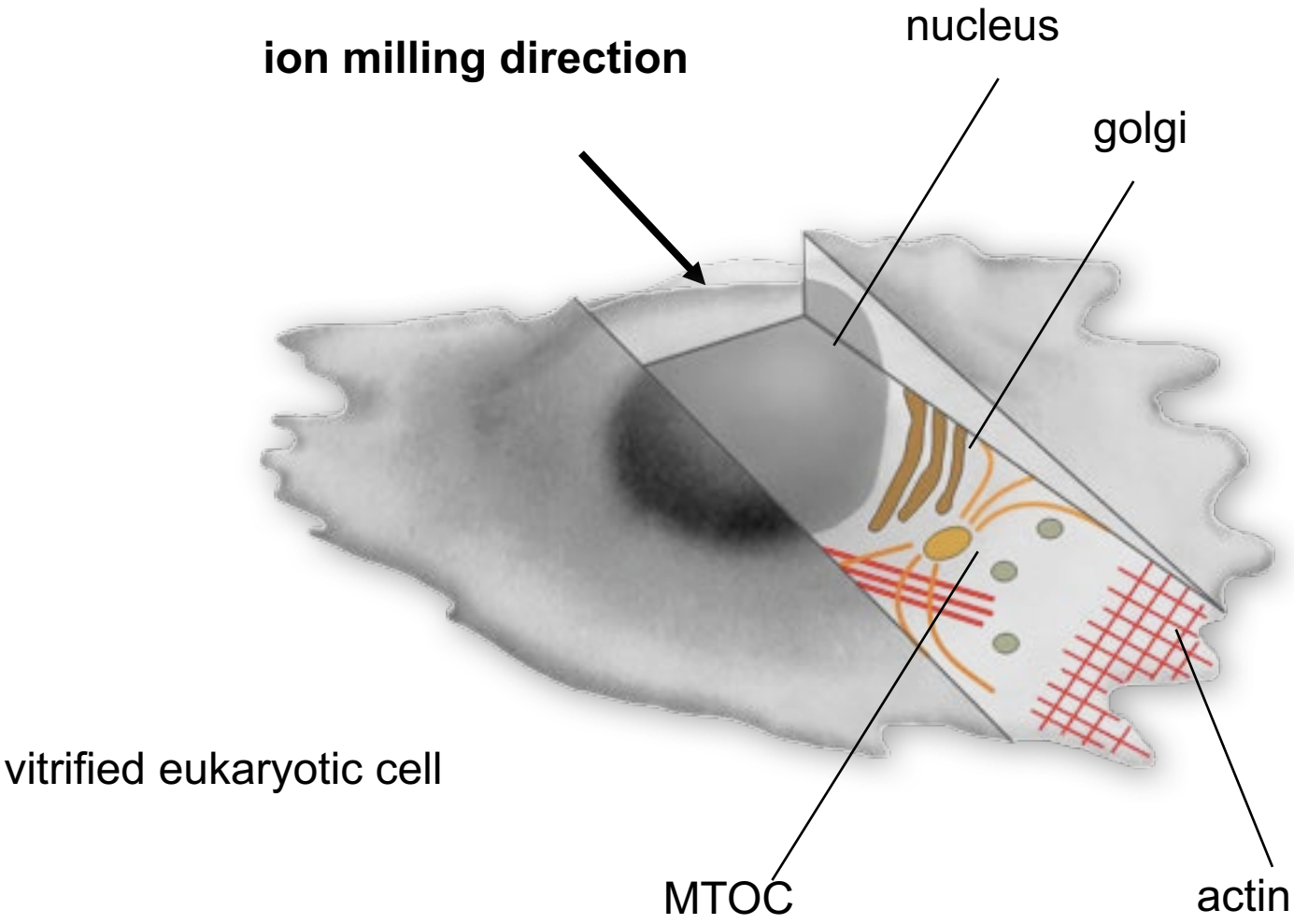


**Cryo-sectioning (< 138 K)**

**CryoEM / Tomography**

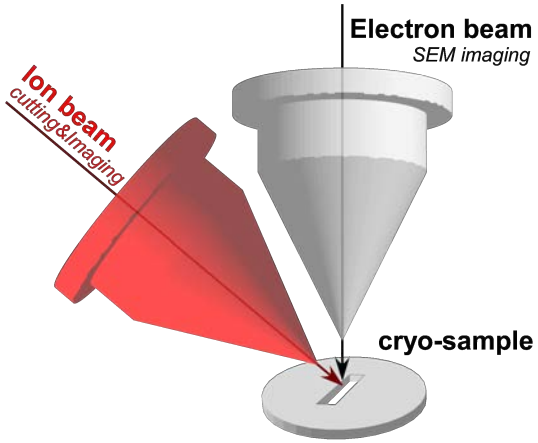
**sample thickness limit** for cryoET: **~ 1 µm** *(Lučić et al. (2005) Ann. Rev. Biochem. 74: 833-865)*

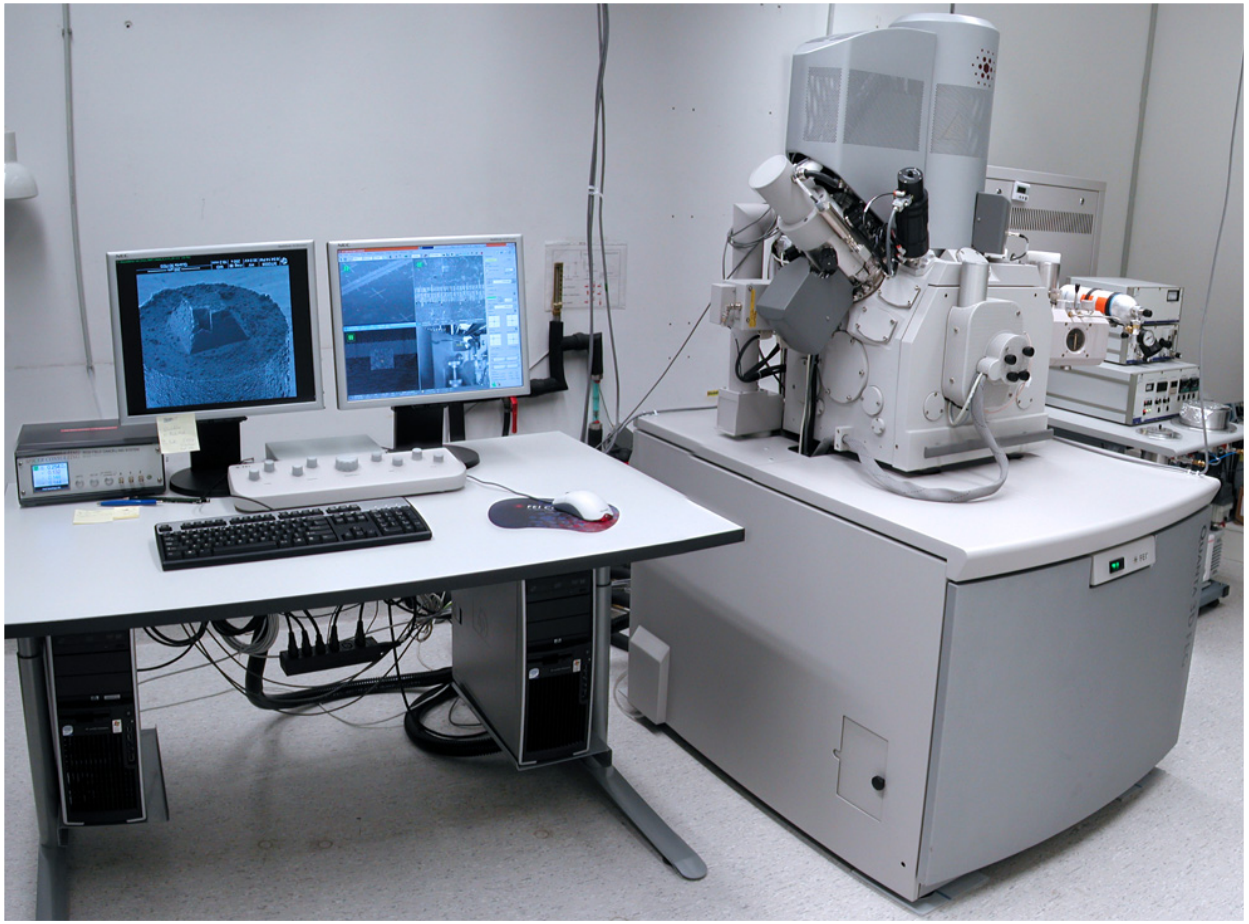
# *Milling rationale*




*(slide courtesy of  Jürgen Plitzko)*

# *Focused Ion Beam – FIB*



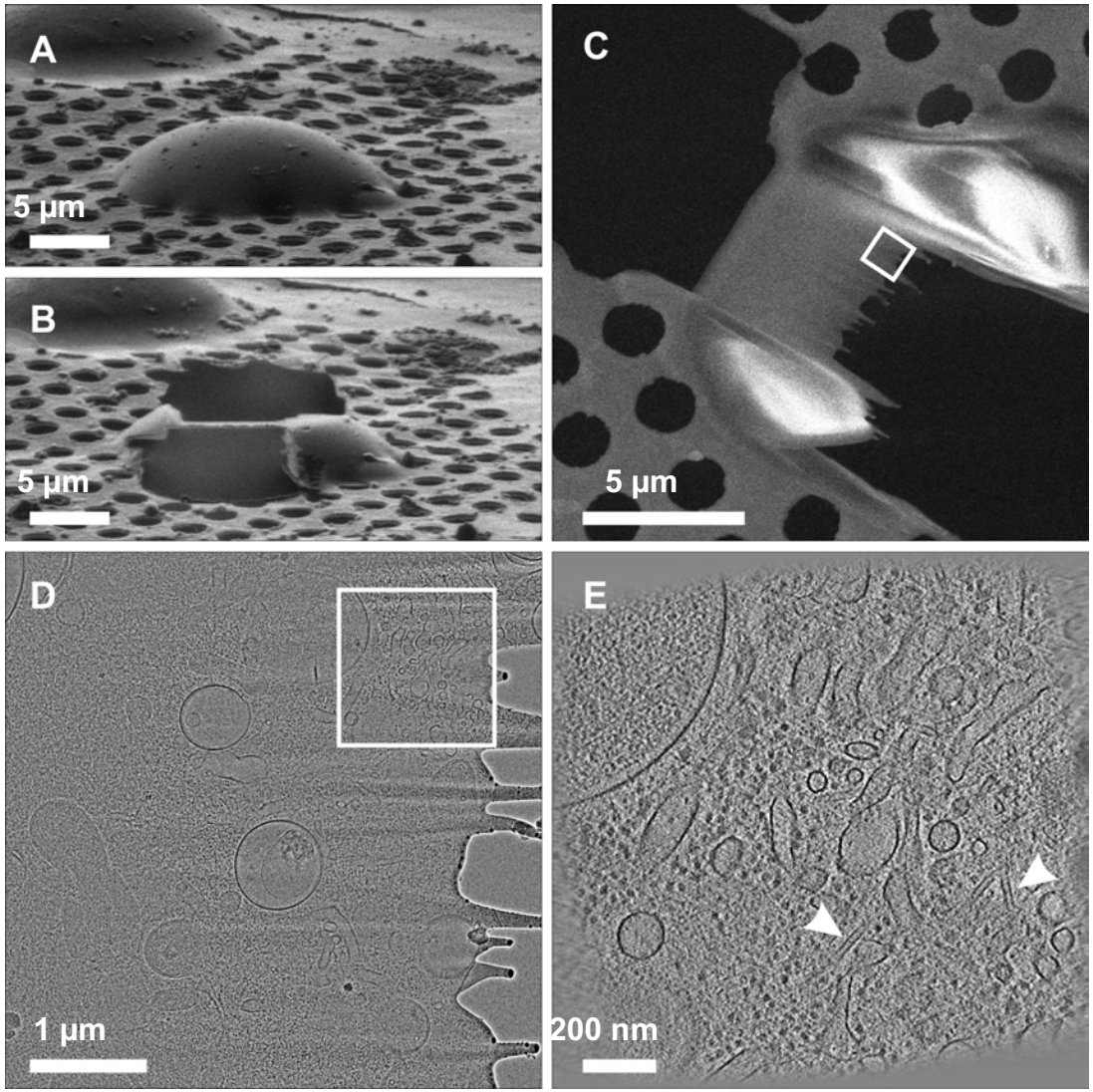



*FEI Quanta 3D FEG dual beam FIB/SEM instrument as installed at the MPIB*

# *Cryo-ET of FIB milled specimens*

FIB milling
(**A** before; **B** after)

TEM projection
(high-pass filtered)




thinned region
(Cryo-SEM; top view)

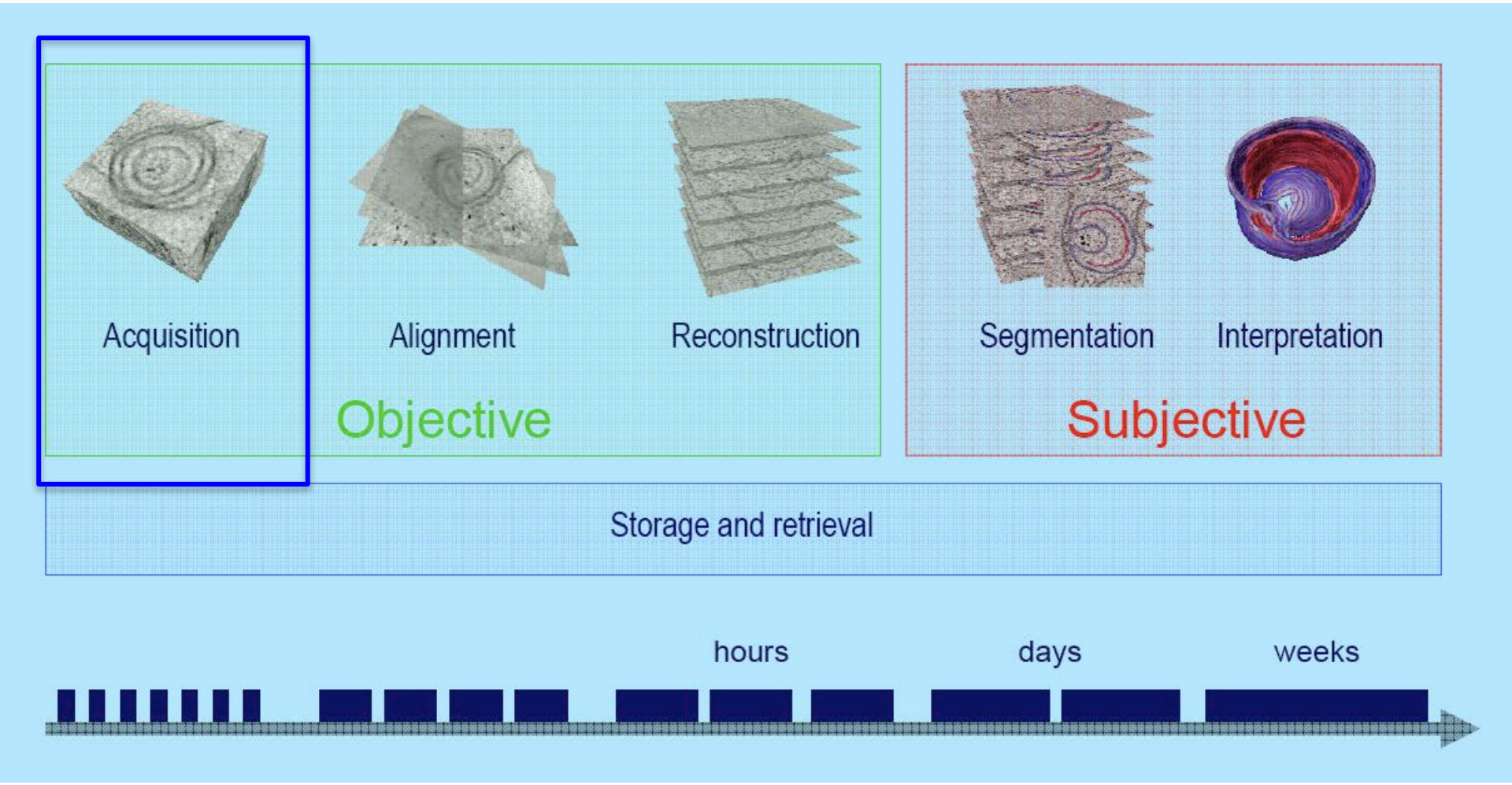
Specimen transfer to TEM

slice from 3D-reconstruction

Rigort *et al.* (2010) *JSB* 172: 169-173.

**→ cf. dedicated talk by Julia Mahalid tomorrow!**

# Tomography workflow

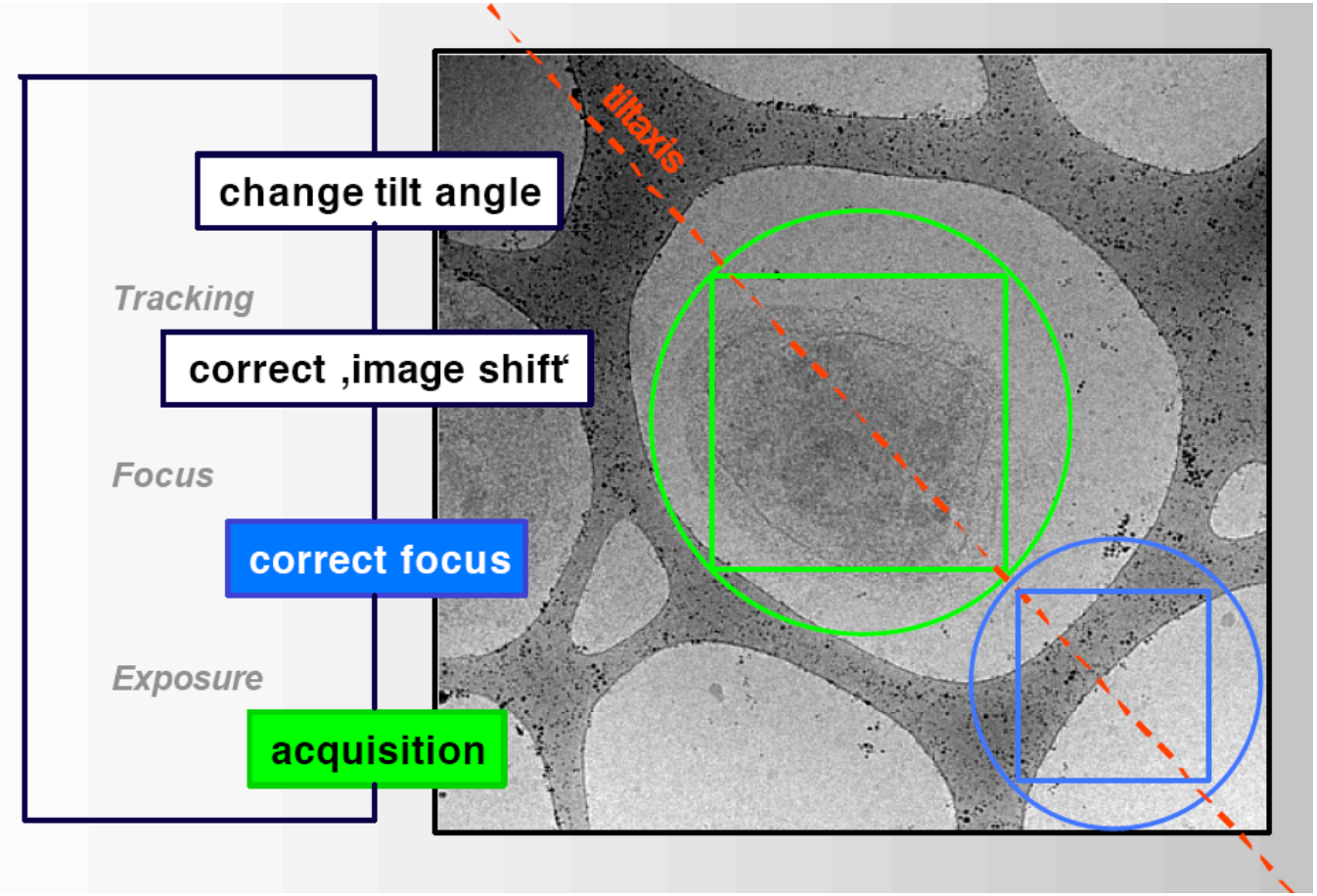



(courtesy of Bram Koster)

# *Acquisition software*

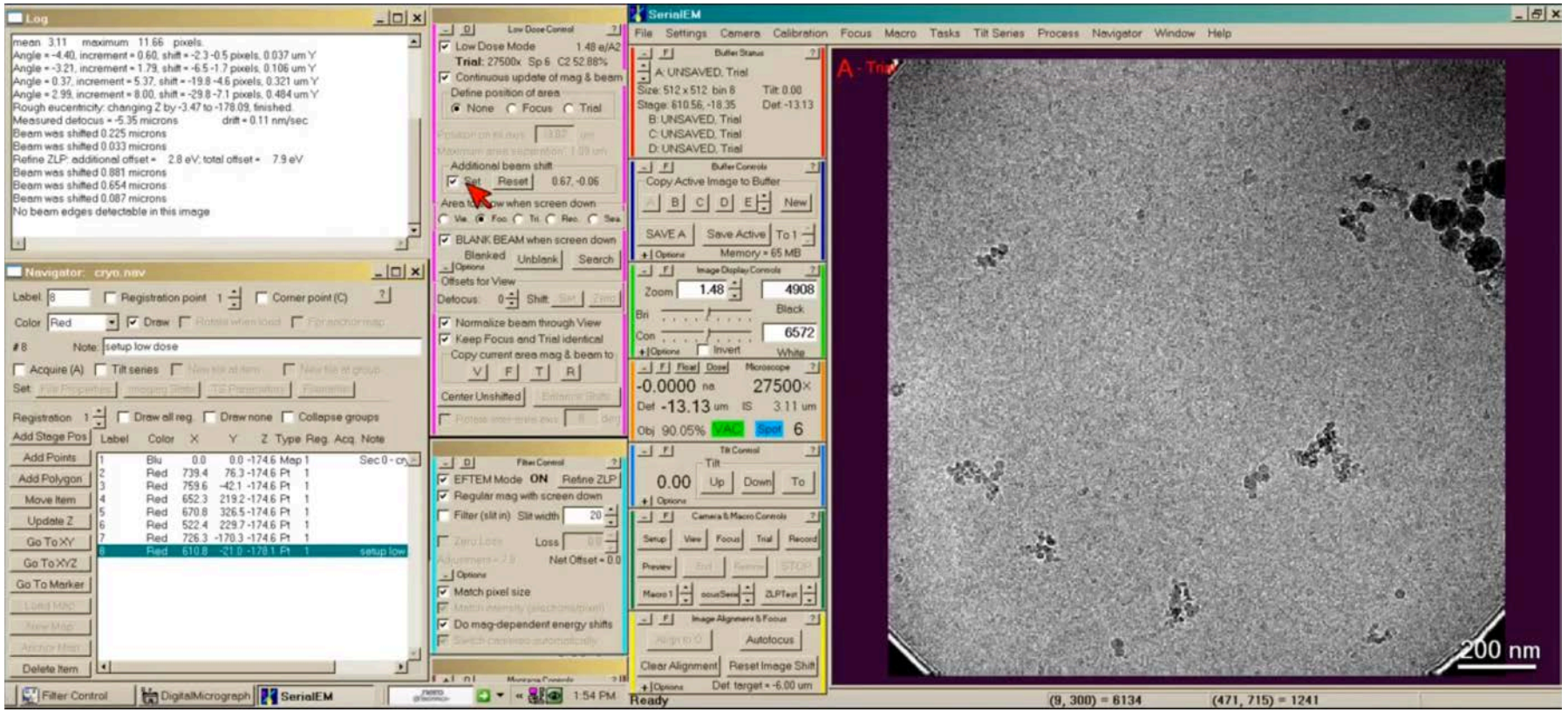
- 1990: autofocus routines (Bram Koster/David Agard)
- 1991-96: first ‘automated’ data acquisition software (MPI, Martinsried: Dierksen *et al.* , Rudo Grimm *et al.*)
- 199?: EM Menue (Hans Tietz)
- 2001: Open Tomography (Bram Koster)
- 2002: FEI Tomography
- 2003: UCSF Tomo, **SerialEM**
- 2004: Jeol and Gatan implementations

# Data acquisition steps




- good alignments / calibrations
  (image shifts, stage shifts, rotational centre, tilt axis, dose … )
- stage pre-calibration
- predictive tracking implementations

# Serial EM




- Academic software by David Mastronarde (Boulder) (+ support by G. Resch)
  http://bio3d.colorado.edu/SerialEM/

- Range of tutorial videos on YouTube by Cindy Schwartz and others (BL3DEMC)

# Serial EM scripts as extension

## The SerialEM Script Repository

SCRIPTS BY CATEGORY  SCRIPTS BY AUTHOR  LINKS  LOGIN/REGISTER

### Scripts by Category

Add Script

#### Calibrations

- Find Tilt Axis Offset (Guenter Resch)
- TuneScope (Wim Hagen)
- Titan IlluminatedAreaLimits (Guenter Resch)

#### High-Level Functions

- Center Beam (Juha Huiskonen)
- MapGrids (Wim Hagen)
- Refine Eucentricity with Threshold (Guenter Resch)
- Center Detail (Juha Huiskonen)
- Focus Pair (Juha Huiskonen)
- Restore Microscope State (Guenter Resch)
- Wait For Drift (Juha Huiskonen)
- Save Microscope State (Guenter Resch)

#### Tomography

- Dose-symmetric tomography (Wim Hagen)
- Fast-Incremental Single-Exposure tomography (Fabian Eisenstein)
- Find Tilt Range (Martin Schorb)
- Quick Tomography (Martin Schorb)
- Grouped dose-symmetric tomography (Wim Hagen)
- Pre-calibrations for FISE tomography (Fabian Eisenstein)

#### Single Particle

- SPAK2aligntomap (Wim Hagen)
- Tilt Pair (Juha Huiskonen)
- Auto Acquire on Holey Carbon (Juha Huiskonen)
- SPA multiple images Test Radius (Wim Hagen)
- test hole centering (Wim Hagen)
- LD (Henning Stahlberg)
- Manual Acquisition (Juha Huiskonen)
- SPA multiple images (Wim Hagen)
- Parameters (Henning Stahlberg)

#### Other Data Acquisition

- Serial Section Maps (Martin Schorb)
- Prepare gridsquare (set eucentric height) (Jason de la Cruz)
- Continuous image acquisition (Jason de la Cruz)
- CRmov (Jason de la Cruz)
- Check grid (Jason de la Cruz)
- Random Grid Sampler (Guenter Resch)

#### Function Libraries

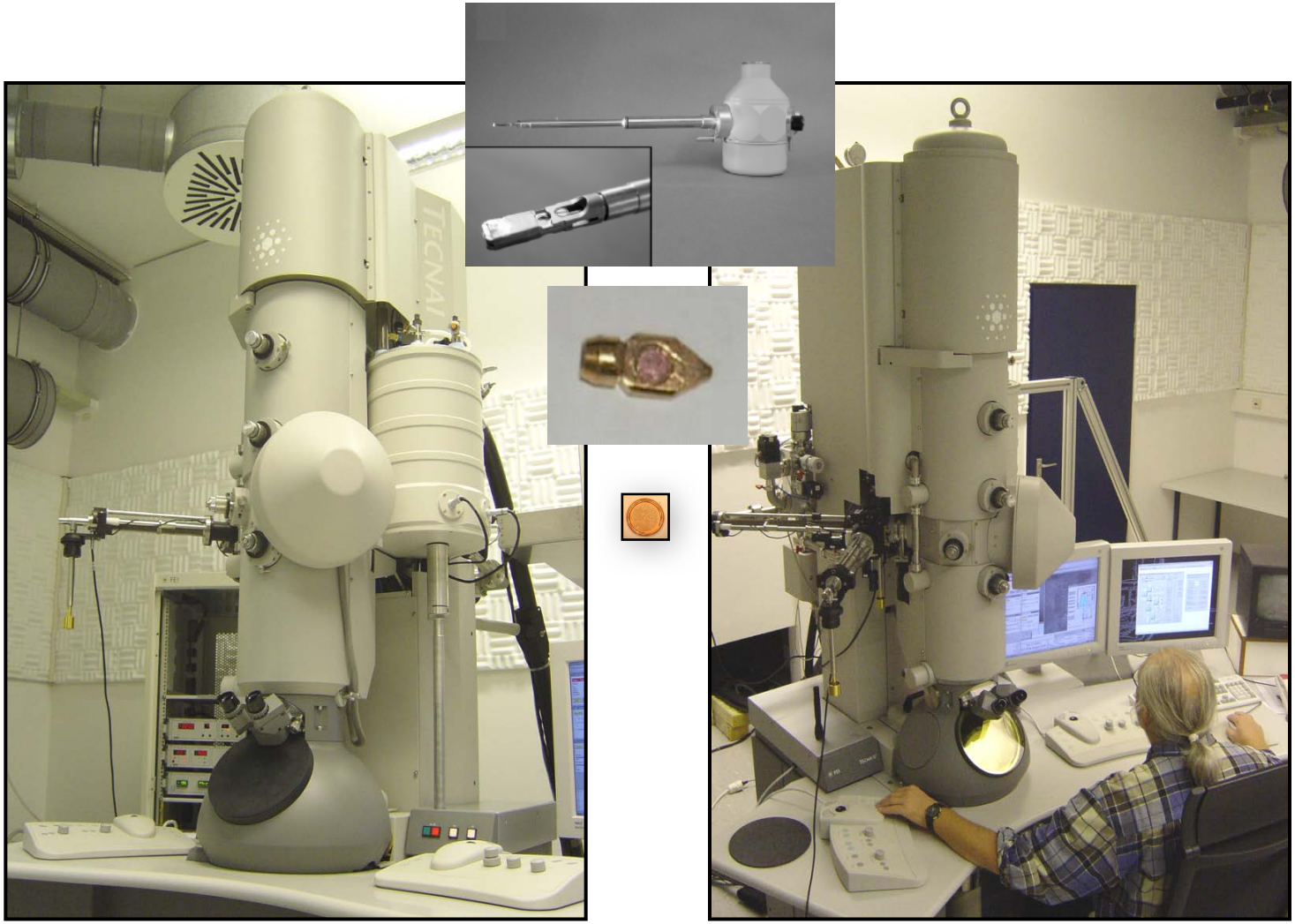
- CINACronJob (Henning Stahlberg)
- WaitForRefilling (Chen Xu)
- CINAAutoFocus (Henning Stahlberg)

#### Testing

- Set intensity value (condenser lens) (Jason de la Cruz)
- K2 alignment timing test (David Mastronarde)
- Probe intensity value (condenser lens) (Jason de la Cruz)
- Beam Intensity Calibration Test (Guenter Resch)

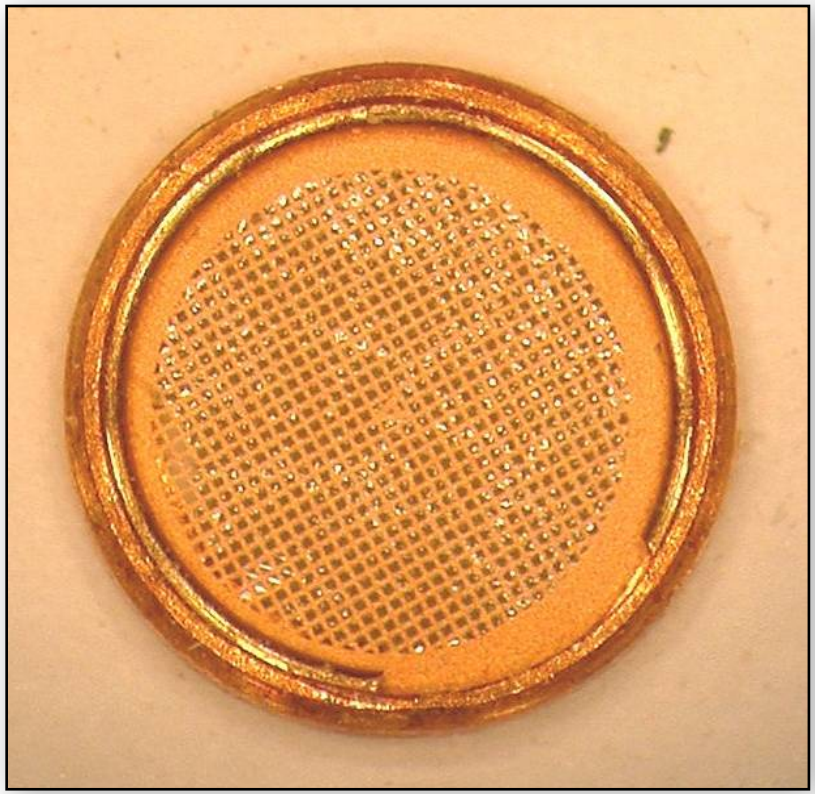
https://serialemscripts.nexperion.net

# Automation - stage stability

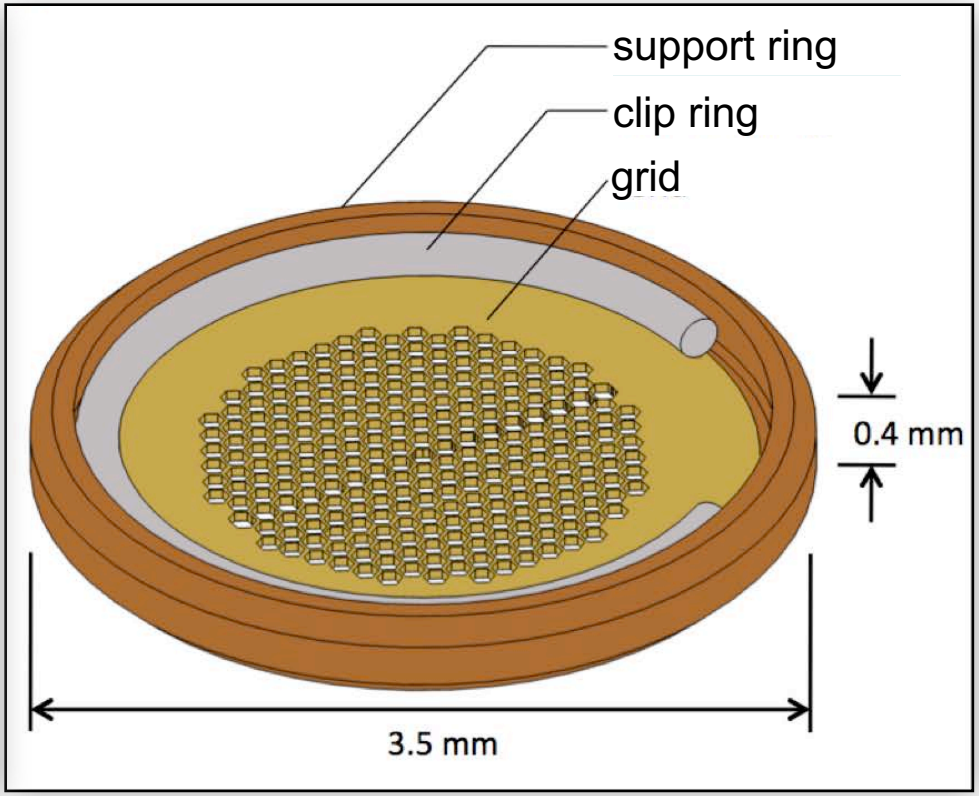


*2001: Introduction of FEI 'Polara': Cartridges*

# *Autogrid: Optimizing multiple transfers*



‘Smallest’ possible TEM holder




Suitable for robotic sample/grid handling

*(used in FEI Titan ‘Krios’)*

# *Challenge in electron tomography*

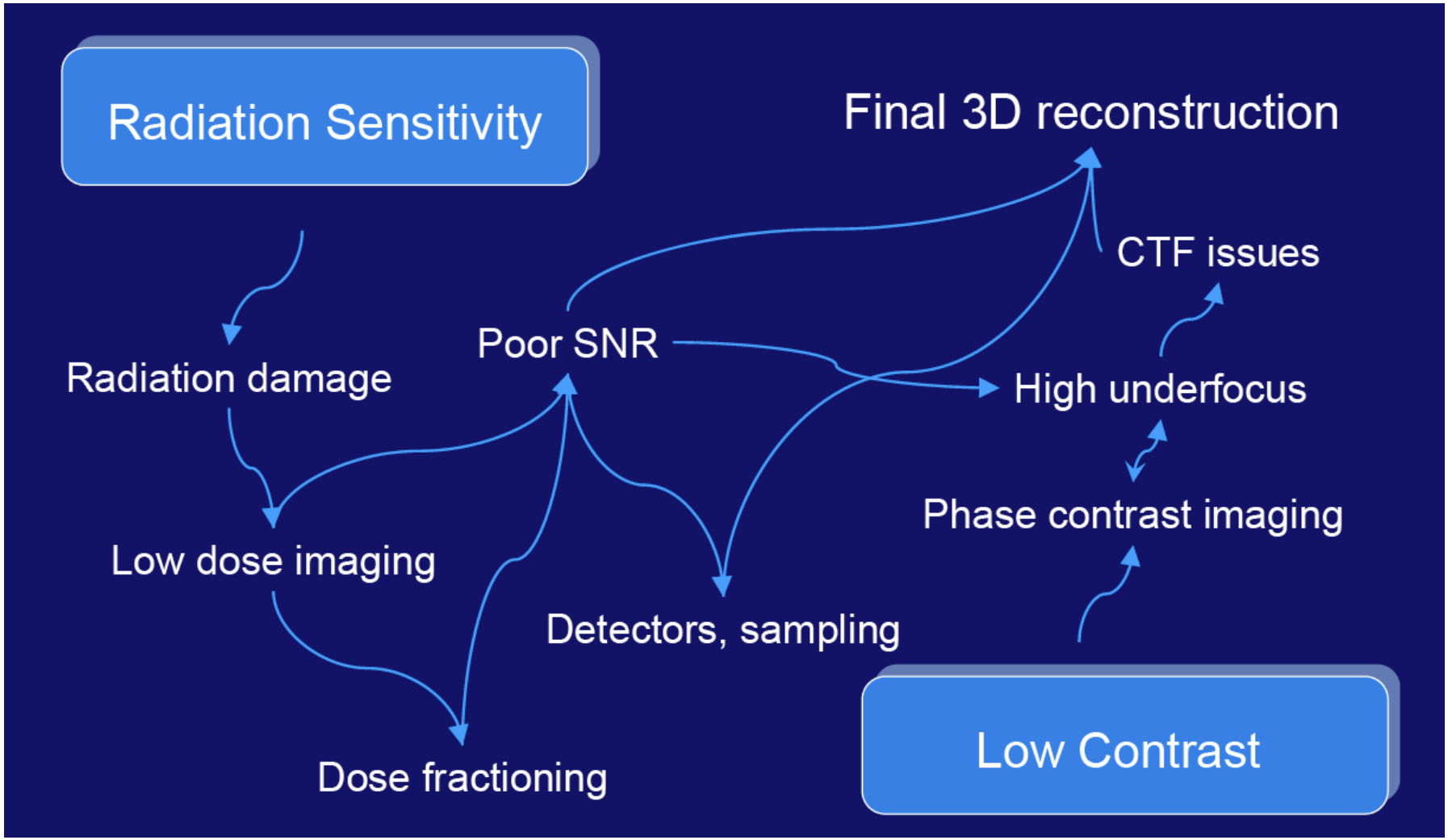
- d ~ π D/N (Crowther)
- reconcile two conflicting requirements :
  - ***maximizing*** the number of projections

    vs.

  - ***minimizing*** the cumulative electron dose (dose fractionation)

- compensate for mechanical imperfections

# *Issues in detail …*




*(from Montserrat Bárcena)*

# *Considerations:*
# ***Choice of data acquisition parameters***

- ***Microscope***

  (accelerating voltage, stage stability, energy filtering, detector …)
- ***Magnification*** (Pixel size, binning …, → fiducial marker density)
- ***Defocus***
- ***Total dose***
- ***Tilt range, increments and scheme***

***…***

**→ dependent on specific aim of study**

# Data acquisition schemes

Journal of Structural Biology 197 (2017) 191–198

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi


## Implementation of a cryo-electron tomography tilt-scheme optimized for high resolution subtomogram averaging

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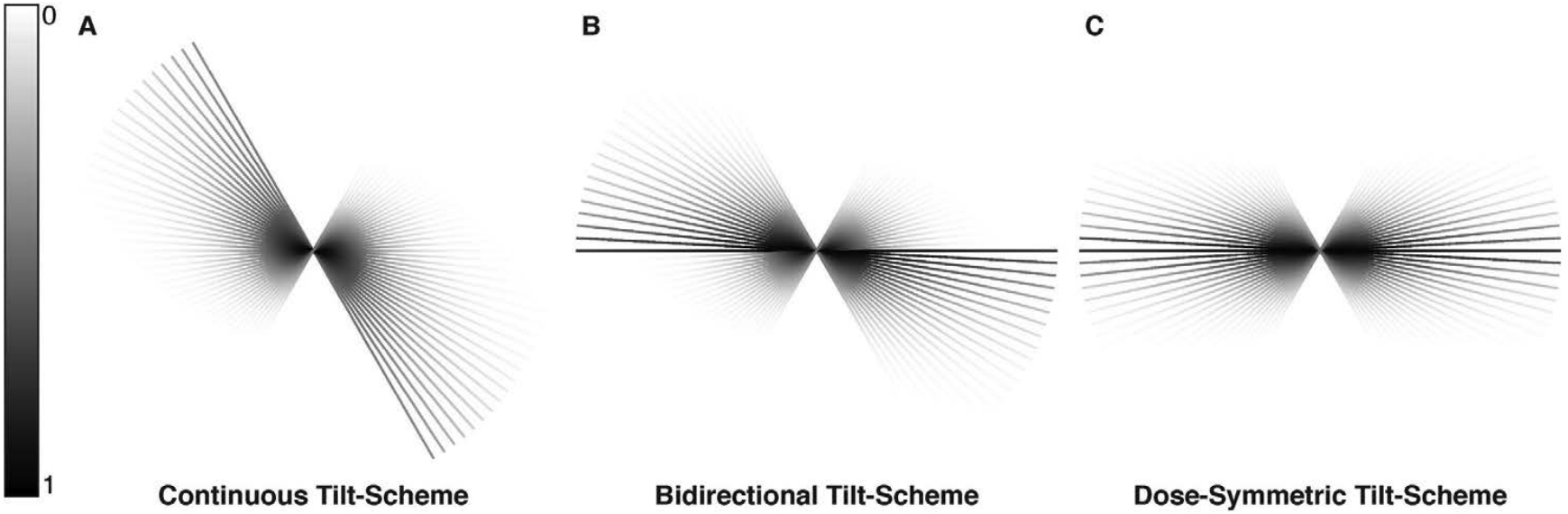
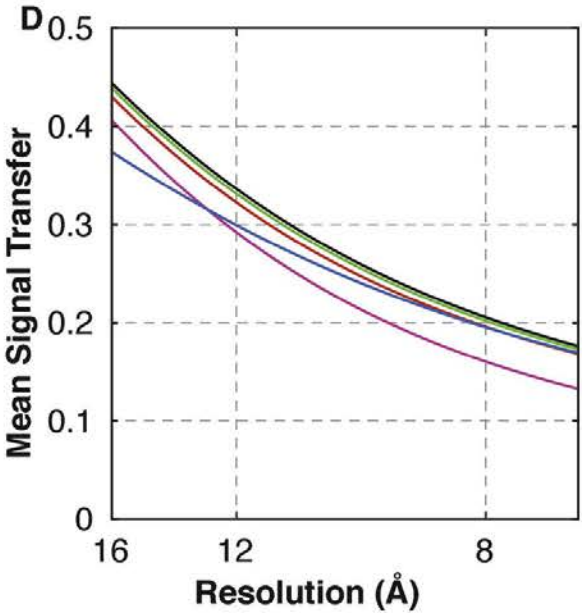
ABSTRACT

Cryo-electron tomography (cryoET) allows 3D structural information to be obtained from cells and other biological samples in their close-to-native state. In combination with subtomogram averaging, detailed structures of repeating features can be resolved. CryoET data is collected as a series of images of the sample from different tilt angles; this is performed by physically rotating the sample in the microscope between each image. The angles at which the images are collected, and the order in which they are collected, together are called the tilt-scheme. Here we describe a "dose-symmetric tilt-scheme" that begins at low tilt and then alternates between increasingly positive and negative tilts. This tilt-scheme maximizes the amount of high-resolution information maintained in the tomogram for subsequent subtomogram averaging, and may also be advantageous for other applications. We describe implementation of the tilt-scheme in combination with further data-collection refinements including setting thresholds on acceptable drift and improving focus accuracy. Requirements for microscope set-up are introduced, and a macro is provided which automates the application of the tilt-scheme within SerialEM.

# Data acquisition schemes



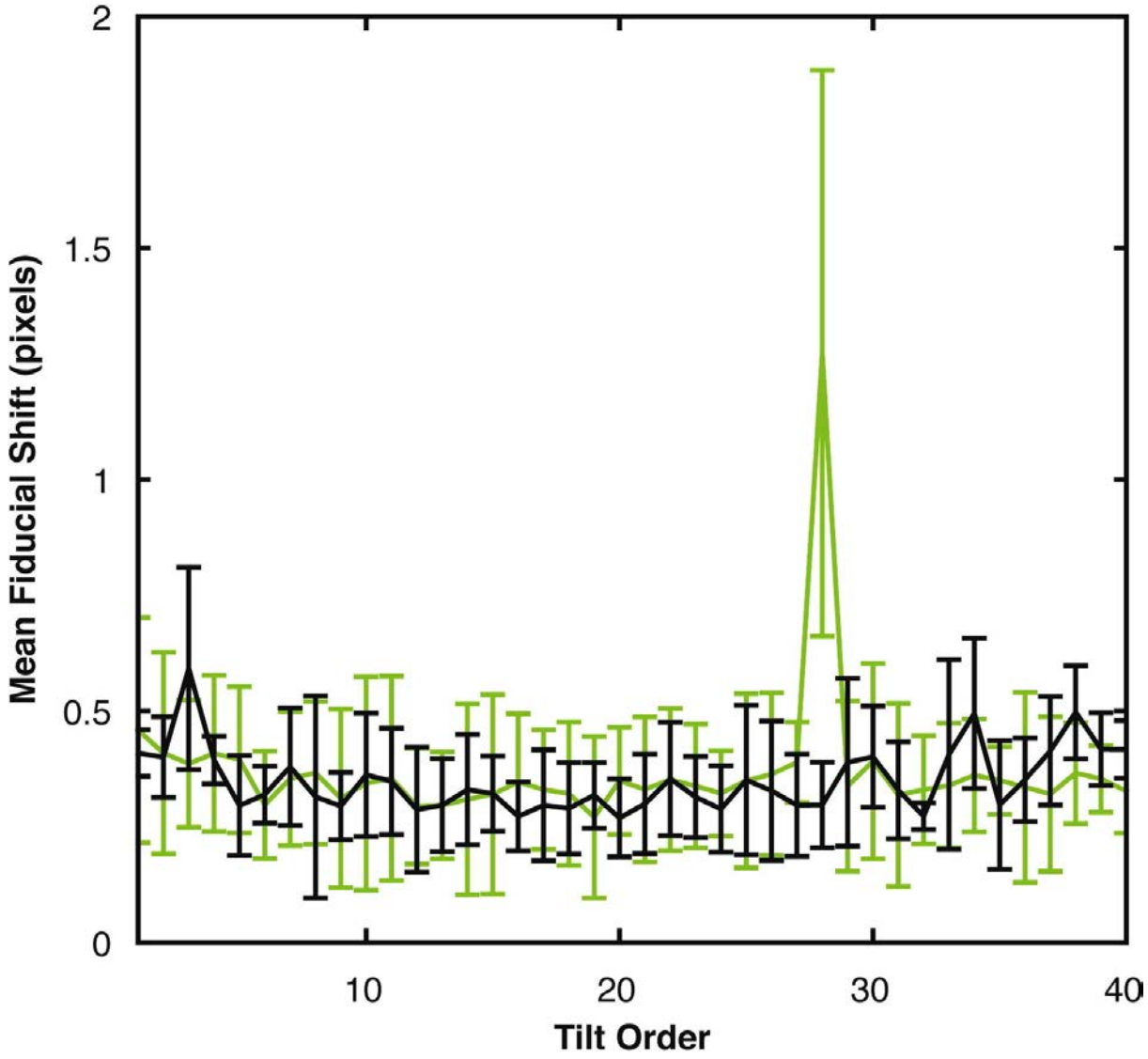




Continuous
bidirectional starting at 0°
bidirectional starting at −21°
bidirectional starting at −21° for case that the second branch is deleted before averaging to reduce impact of the jump-at-start problem
**dose-symmetric (black).**

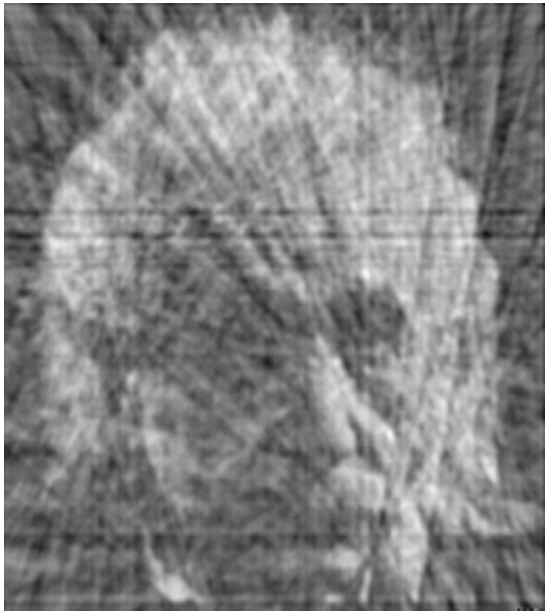
Mean signal transfers are calculated as the mean of the signal transfer for all tilts within the tomogram.

Hagen *et al.* (2017) JSB 197-191-198: 833-865.

# *Data acquisition schemes*

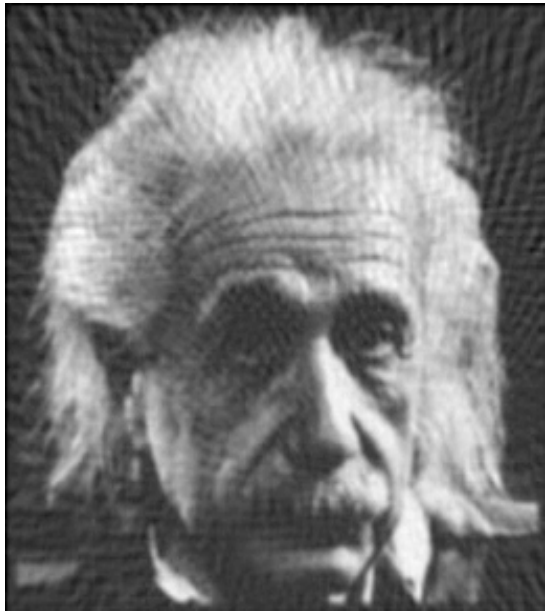


# *The effect of sampling*



18 Proj. / 10 deg.

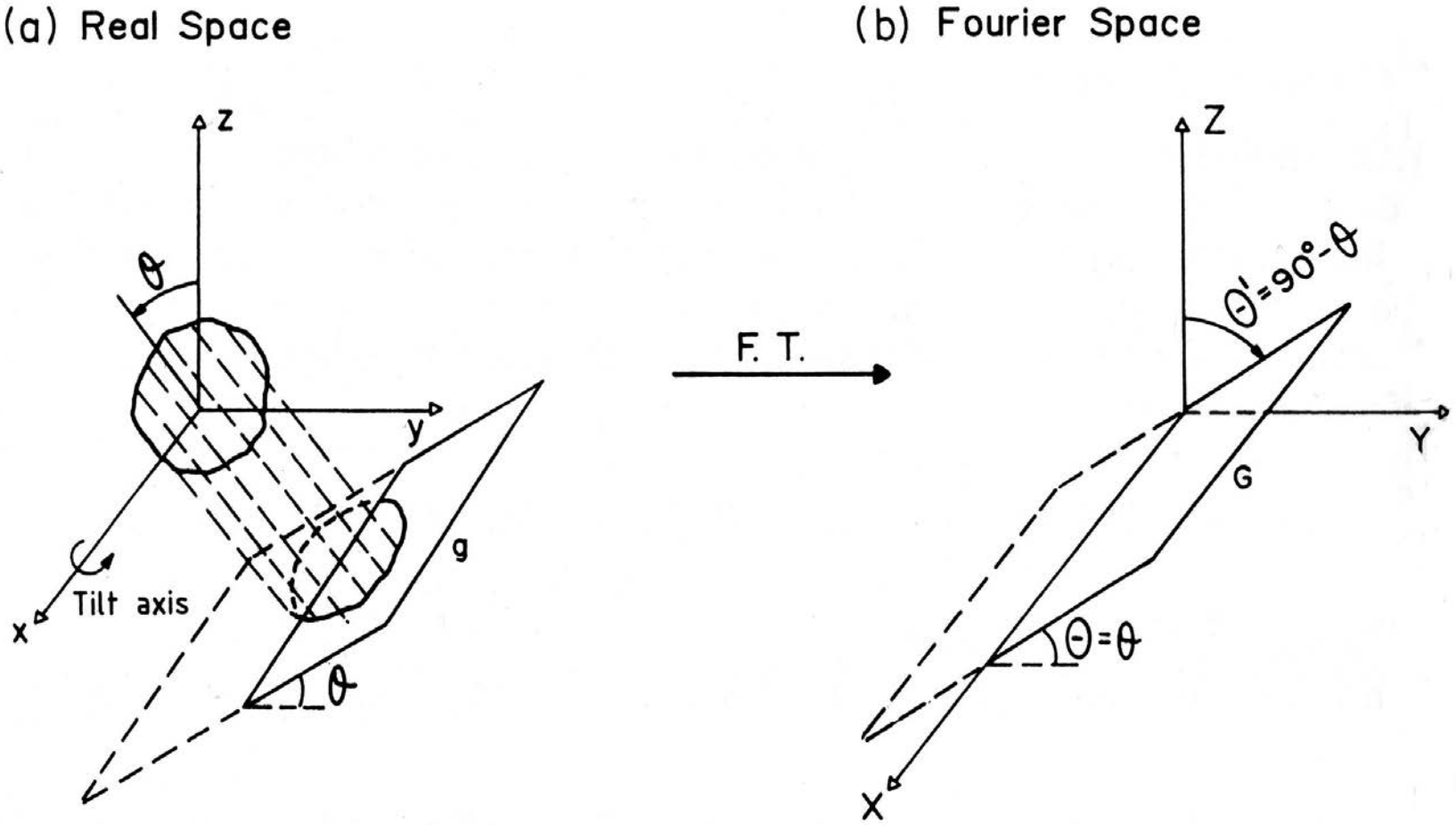
36 Proj. / 5 deg.



72 Proj. / 2.5 deg.

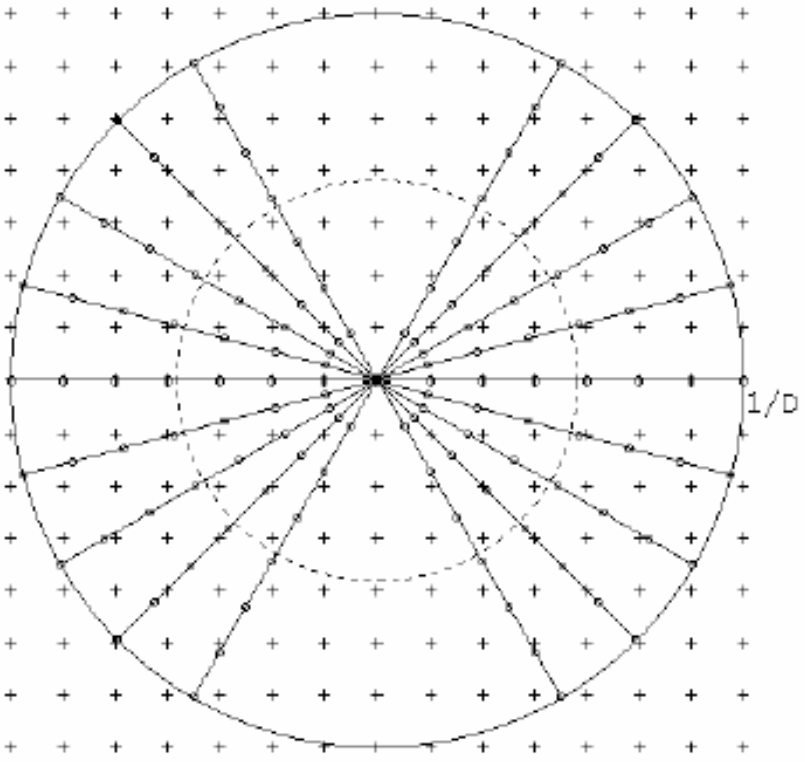
# *The Central Section Theorem*

The 2-D Fourier transform of a projection image is a central plane through the 3D Fourier transform of the object



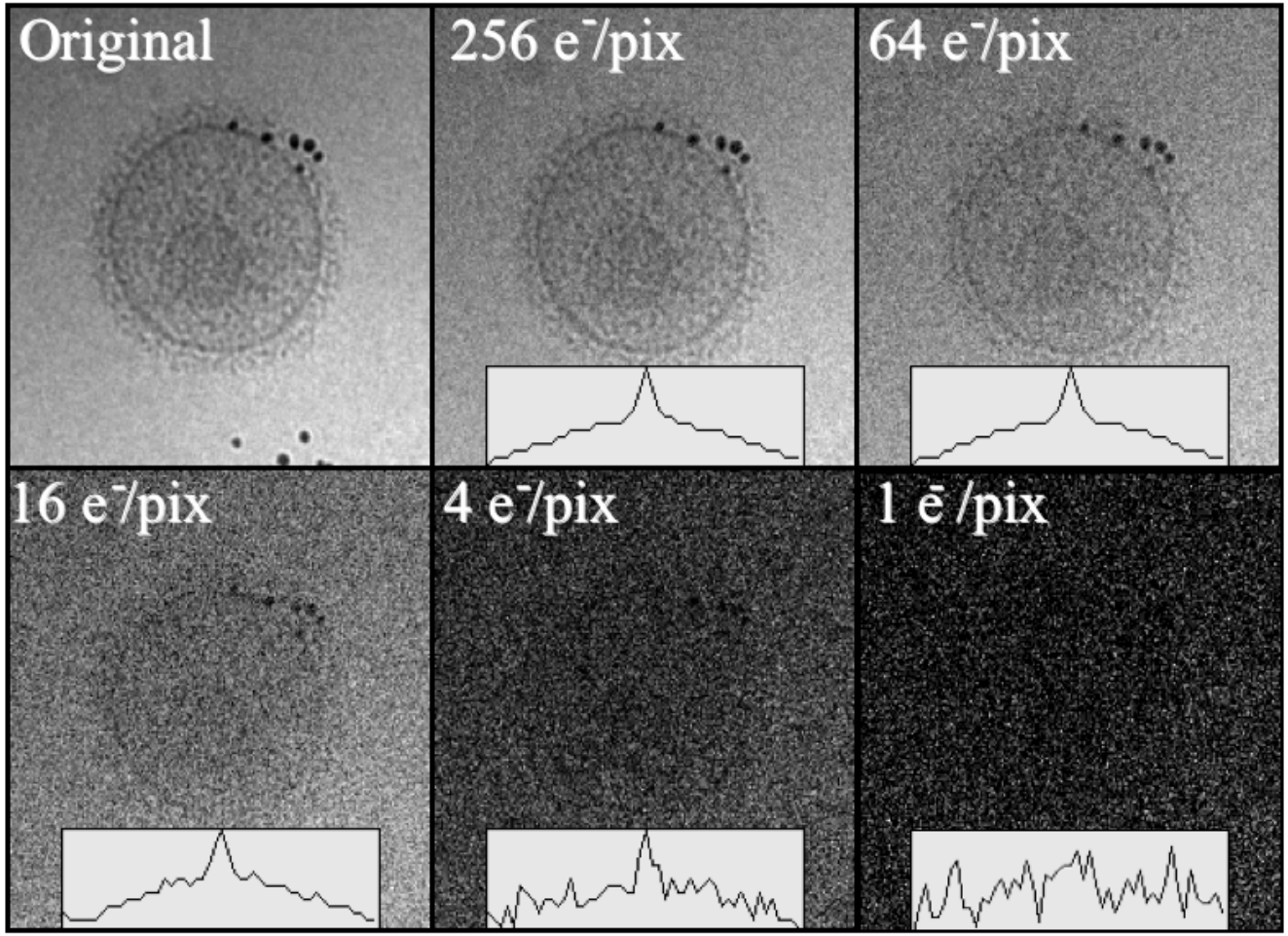

(From Carazo, 1992)

# *Sampling, resolution*




Crowther criterion: R~π*D/N
Anisotropic resolution!
oversampling vs. undersampling

# *Electron dose and signal/noise ratio*



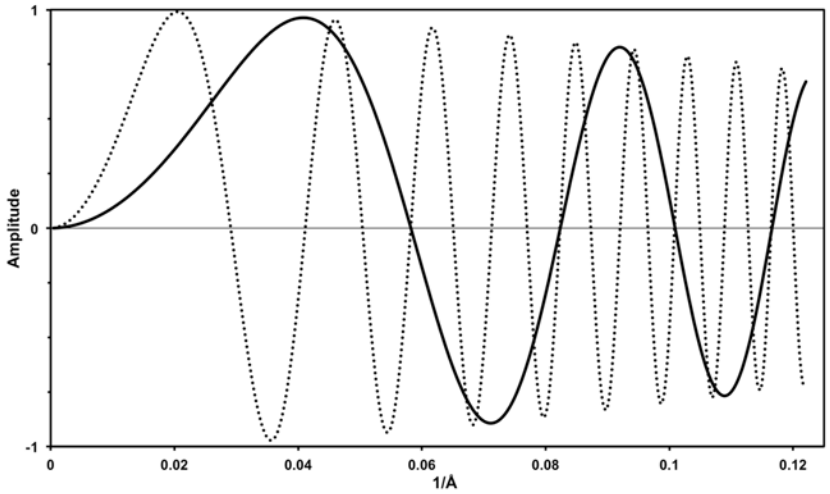

- total dose of 40-100 $e^-/Å^2$ for 60-140 projection images

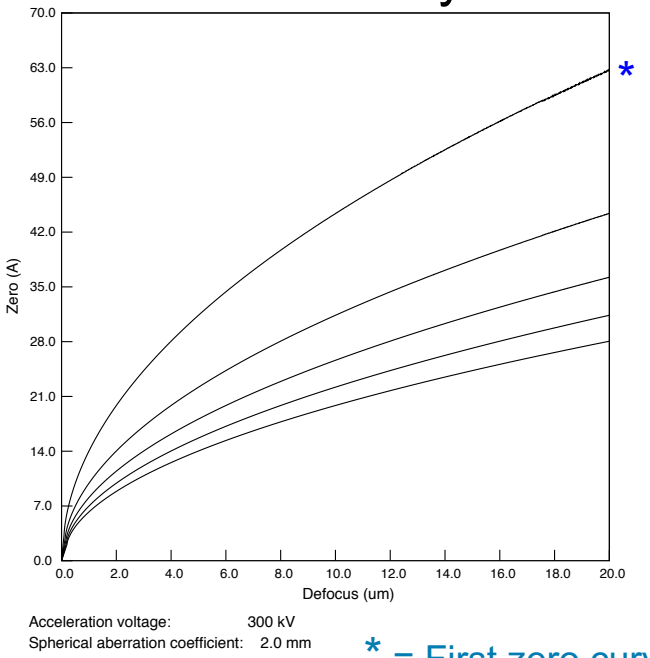
# *CTF issues*

- **create phase contract by under-focussing**

– Without CTF correction, the interpretable resolution is limited to the first zero of the CTF

– Before any correction, the CTF needs to be determined accurately




* = First zero curve

- **CTF determination / correction**

– SPA: FT periodogramms of overlapping patches

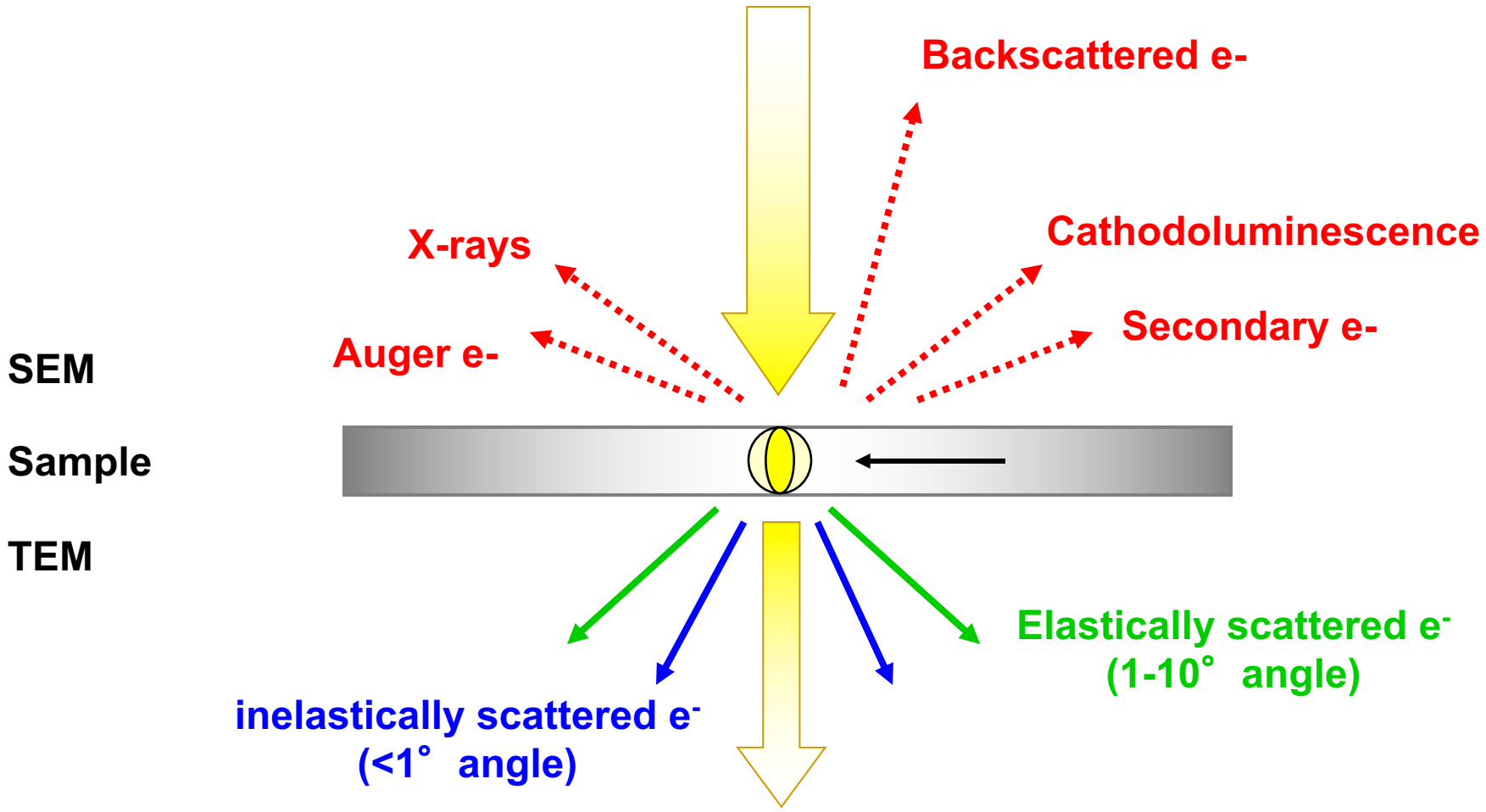
**– ET: lower signal per image + tilted images**

**– stripe defocus determination, correction**

**(problem: power in image stripes typically not sufficient)**

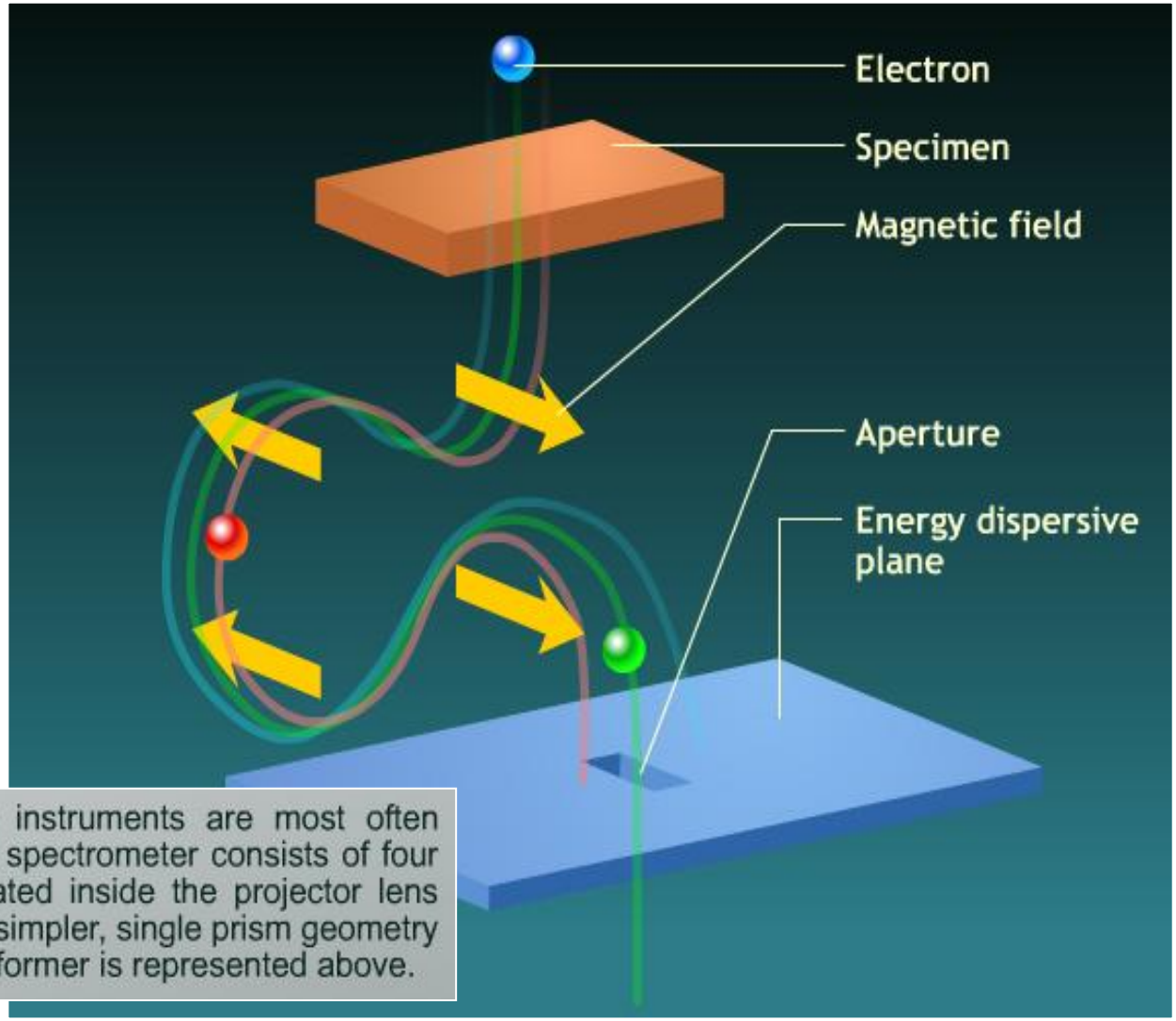
**- NovaCTF….** Implementations **(cf talk of Dan Clare yesterday)**

# *Electron sample interactions*



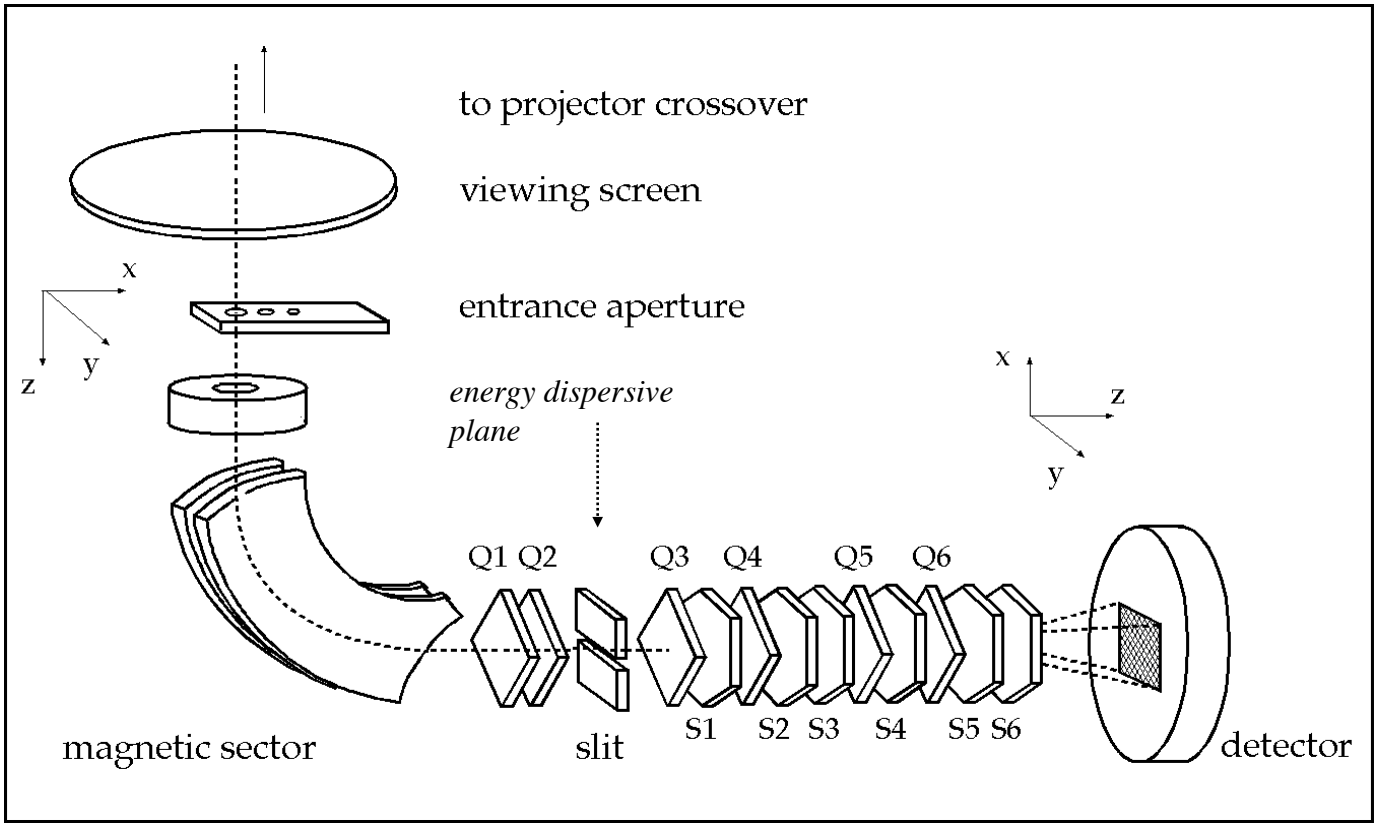

… mean free path for electrons depending on acceleration voltage and sample material, consider this relative to sample thickness …

# *In-column energy filtering*




Two types of energy-filtering instruments are most often used: In-column filters, whose spectrometer consists of four magnetic prisms and are located inside the projector lens and post-column filters, with a simpler, single prism geometry based on multiple lenses. The former is represented above.

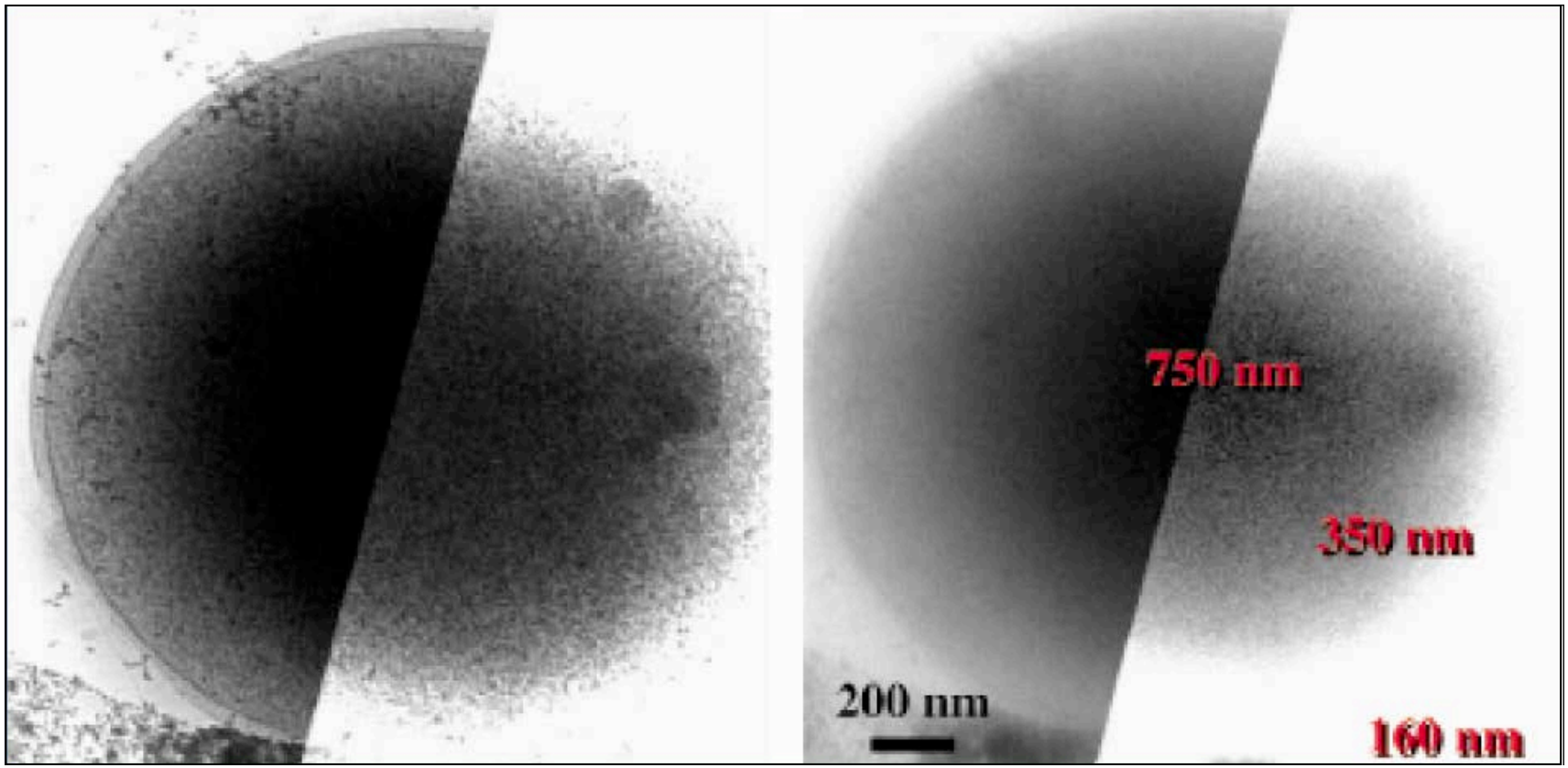
# Post-column energy filter



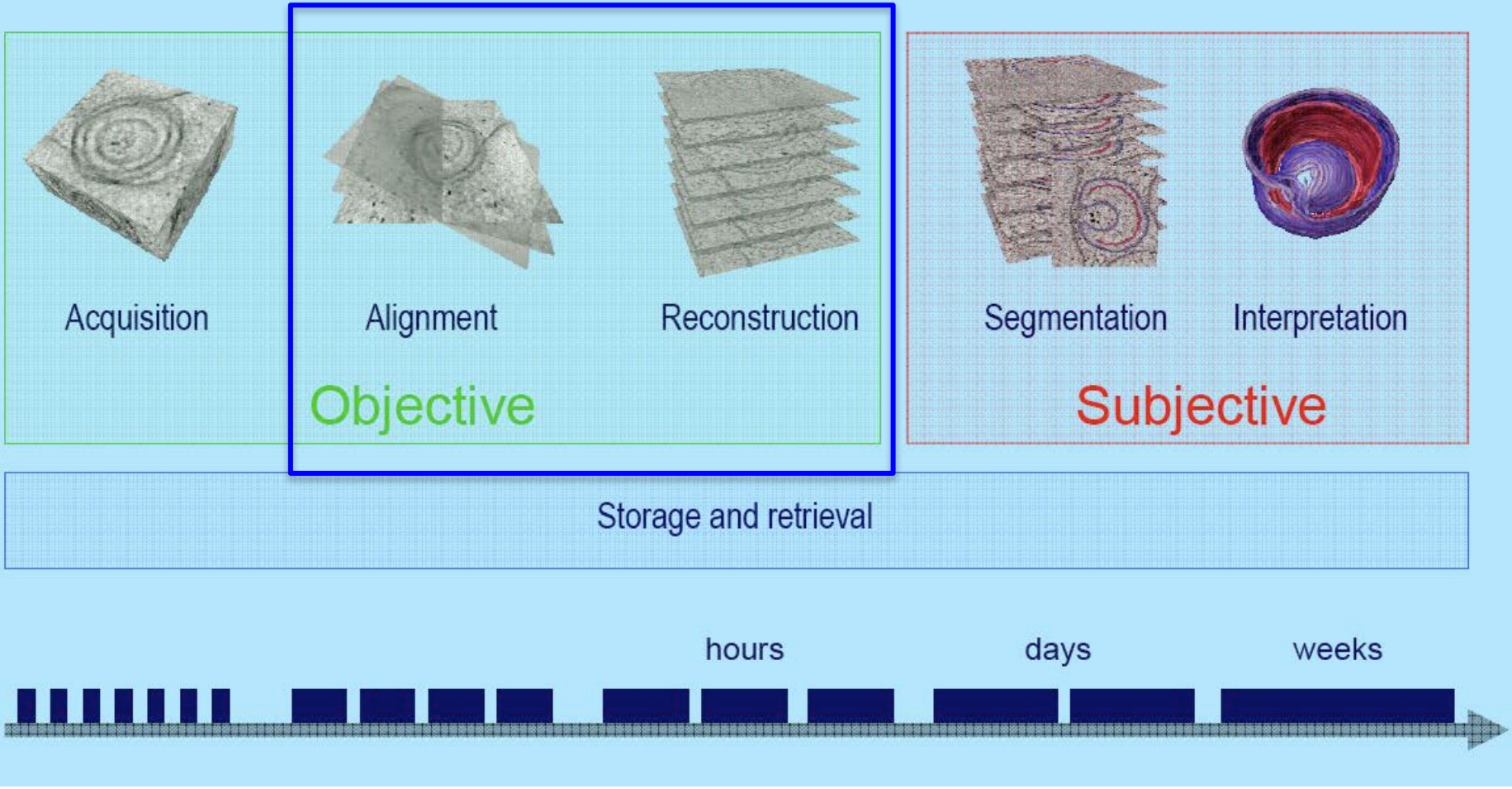

Gatan Imaging Filter (GIF)

# *Zero-loss filtering*

‘filtered’ ‘unfiltered’



# Tomography workflow




(courtesy of Bram Koster)

# *Reconstruction*

from acquired tilt series (projection image)

1. Remove X-rays
2. Alignment

*i.e.* registering projection images to each other

3. Reconstruction of 3D volume, the **tomogram**

Dedicated software,

most popular: **IMOD** (bio3d.colorado.edu/imod/)

Other: EM, BSOFT, FEI Inspect 3D …

# *Tilt series alignment*

Method A: Alignment by cross correlation
- only for thin specimen
- requires stretching of the projection images perpendicular to the tilt axis

Method B: Use of marker points (colloidal gold)

$\mathbf{r}_i = (x_i, y_i, z_i)$ coords of marker point $i$

$\mathbf{u}_{ik} = (u_{ik}, v_{ik})$ projection of marker point $i$ in tilt $k$

$\mathbf{a}_k = (a_k, b_k)$ displacement of tilt $k$

$\mathbf{u}_{ik} = \mathbf{M}_k \mathbf{r}_i + \mathbf{a}_k$ Rotation and Projection

Use marker point $j$ as a reference and solve

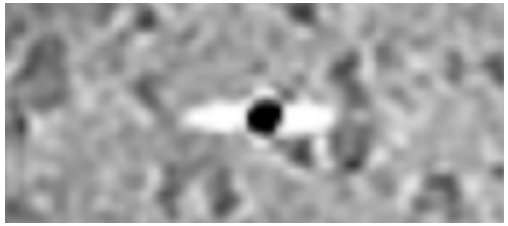
$$\sum_i \sum_k |\mathbf{u}_{ik} - \mathbf{u}_{jk} - \mathbf{M}_k(\mathbf{r}_{ik} - \mathbf{r}_{jk})|_2 \Rightarrow \min$$

for the difference vectors $\mathbf{r}_{ik} - \mathbf{r}_{jk}$

# *Quality of tilt series alignment*

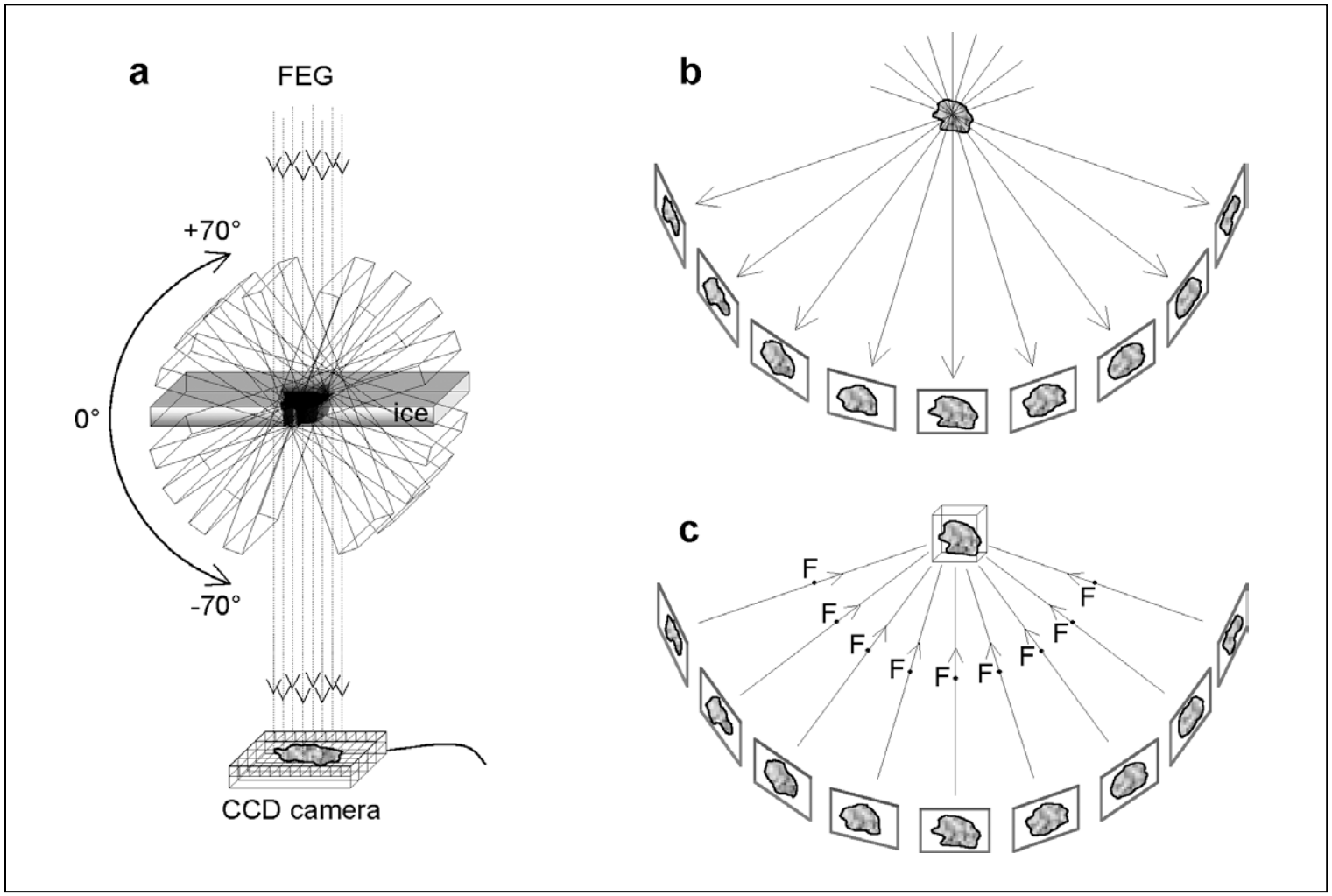
- only partial compensation for any problems in acquisition!

- **pixel error** from marker alignment
- **visual** (looking at reconstruction):
  drifting appearance?
  Straight or banana-shape ‘shadow’ around gold markers?



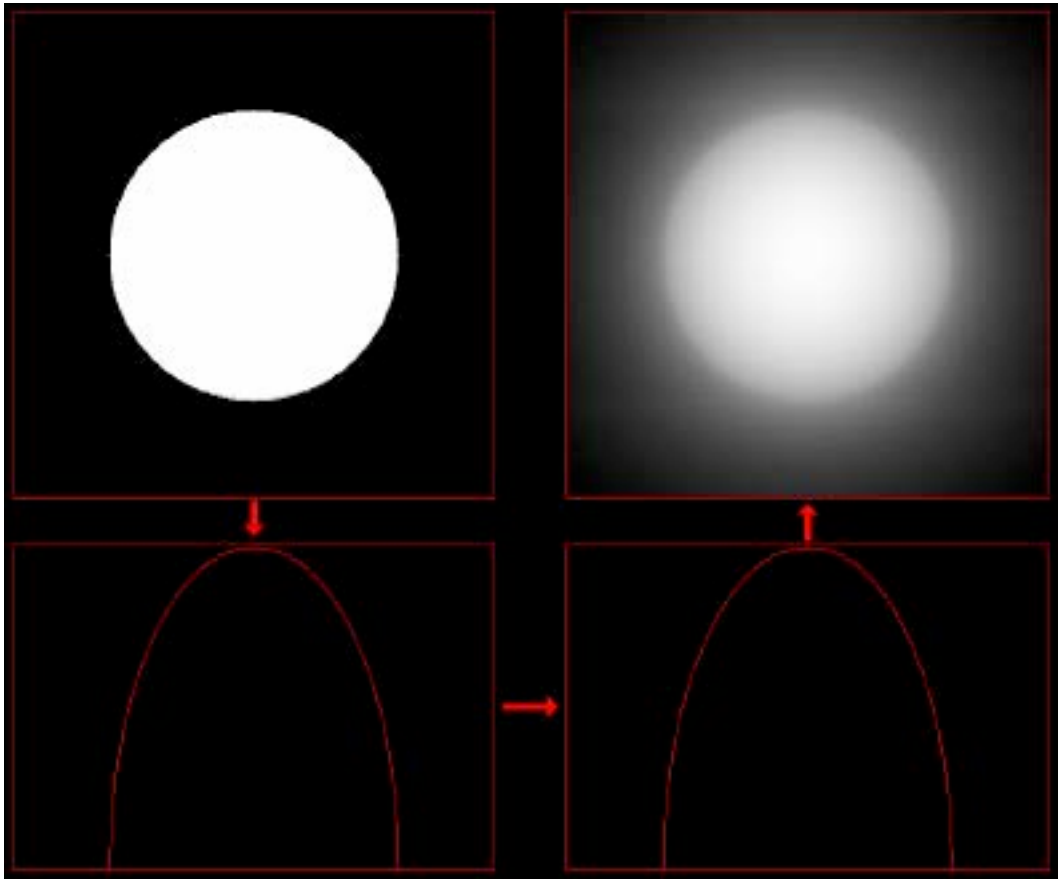
- **global versus local alignment!**

# *Reconstruction by backprojection*

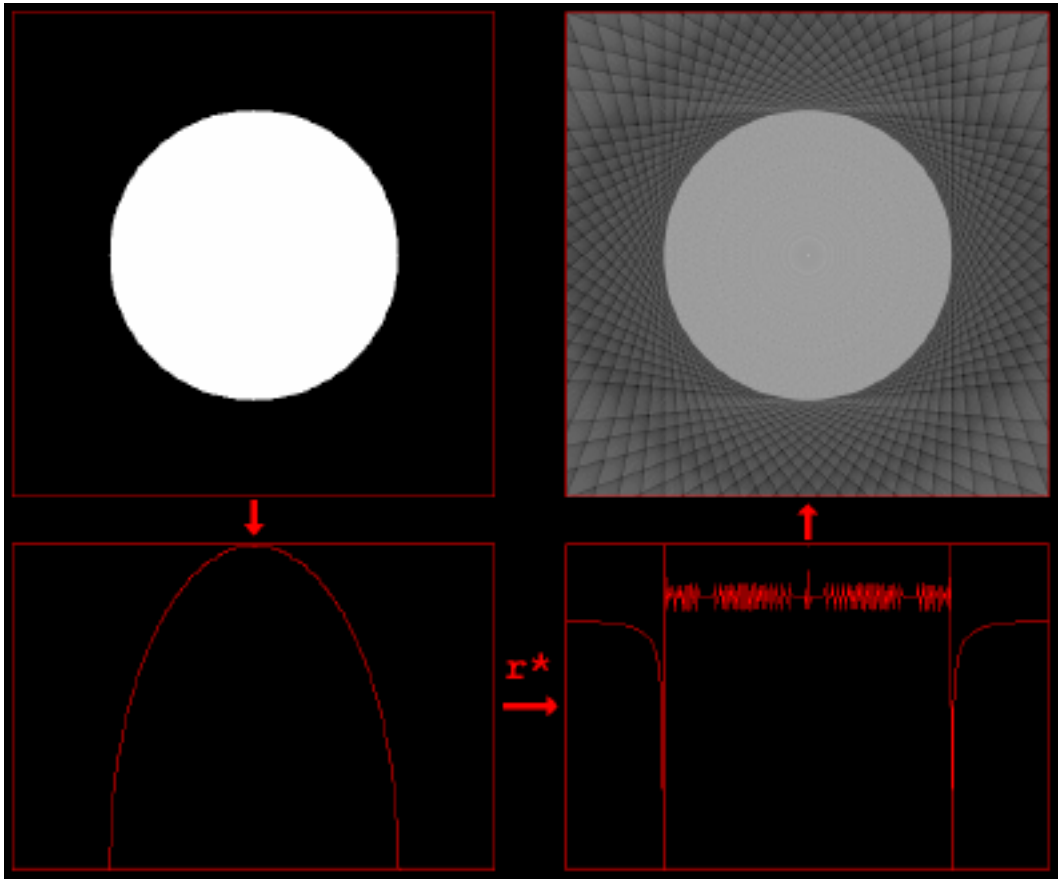



Grünewald *et al.* (2003) *Biophys. Chem.* 100: 577-591.

# *Reconstruction (Backprojection)*



# Reconstruction (Weighted BP)



# *Algebraic reconstruction methods*

- ART
- SIRT

  various implementations

  iterative process

  memory hungry, best done on GPU

→ be sure to understand all the parameters … filtering!
  (refer to *e.g.* IMOD description)

# *Resolution Determination in Tomography*

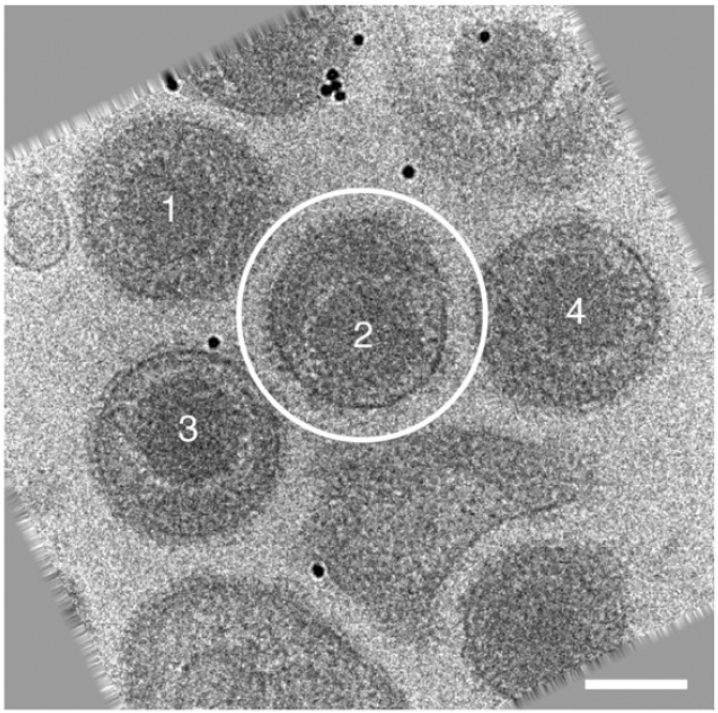
- **Anisotropic** resolution
- criteria used in SPA not directly applicable (uniqueness of tomographic volume)
- Crowther criterion: **R= π * D/N**

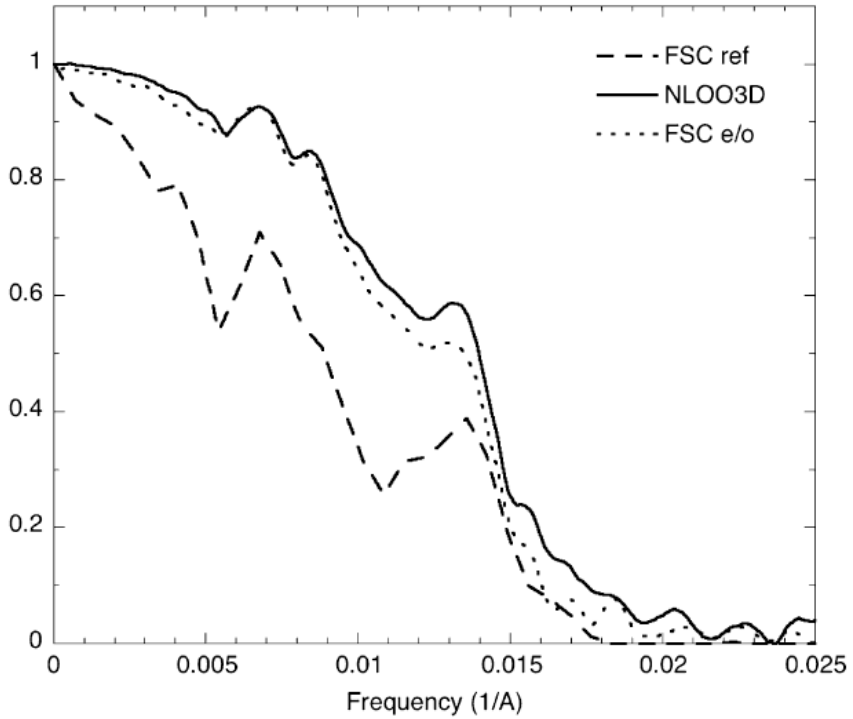
but in practice; structural preservation or noise limiting!

- cross-validation approach:
  - **$FSC_{e/o}$** tomograms from even vs. odd projections
  - **NLOO** (noise compensated leaf one out):

Fourier Ring Correlation comparison between an original projection and the corresponding re-projection of the tomogram calculated from all the other projections, taking into account the differing noise statistics.

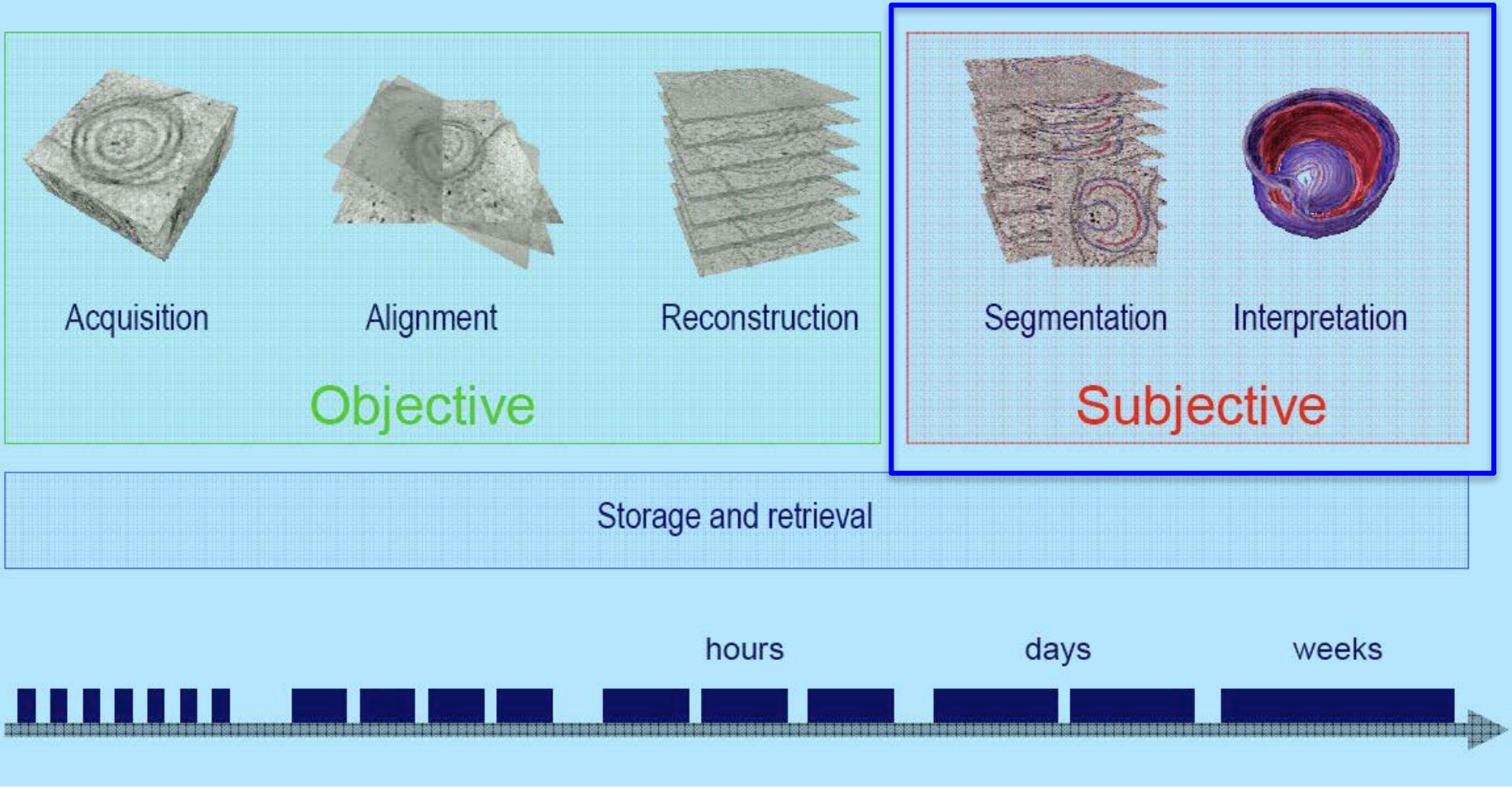
# Resolution Determination in Tomography




Cardone, G. *et al.* (2005) *JSB* 151:117-129.

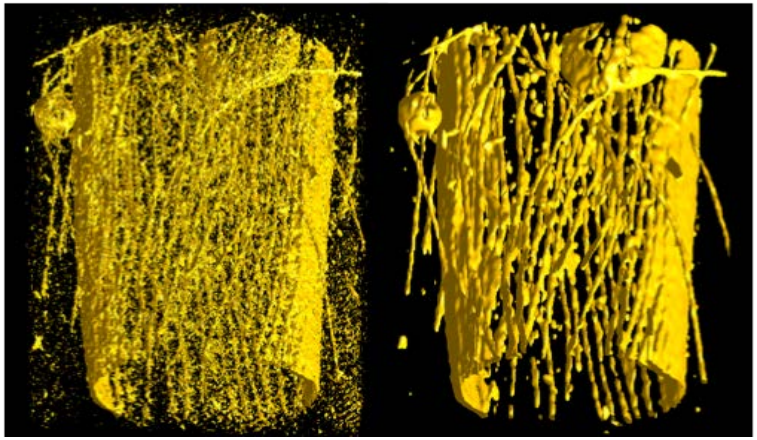
# Tomography workflow




(courtesy of Bram Koster)

# *Data Mining, Visualisation*

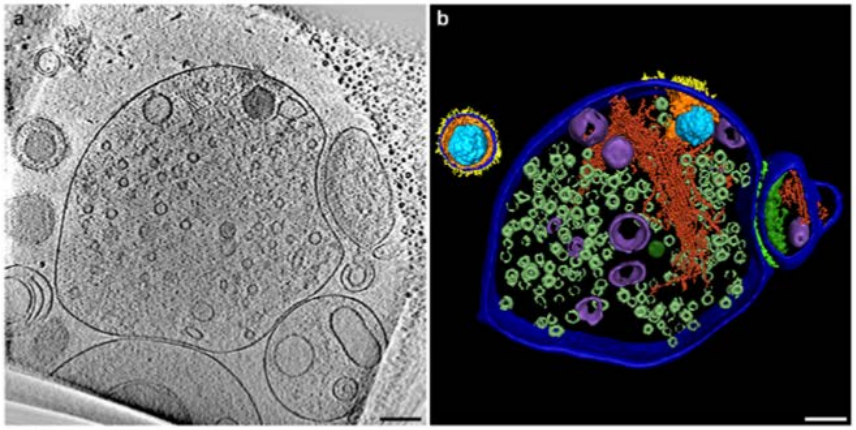
*Denoising by nonlinear anisotropic diffusion:*
*Reconstruction of a DMPC vesicle with actin filaments*
*Grimm et al. (1997) Biophys. J. 72: 482-489.*



- **Denoising**
  - Linear Filters (Real and Fourier Space)
  - Nonlinear Filters (Median and NAD)

- **Segmentation**
  - Manual, **subjective!**
  - Binary Operations / Morphological Operations
  - More Sophisticated Algorithms

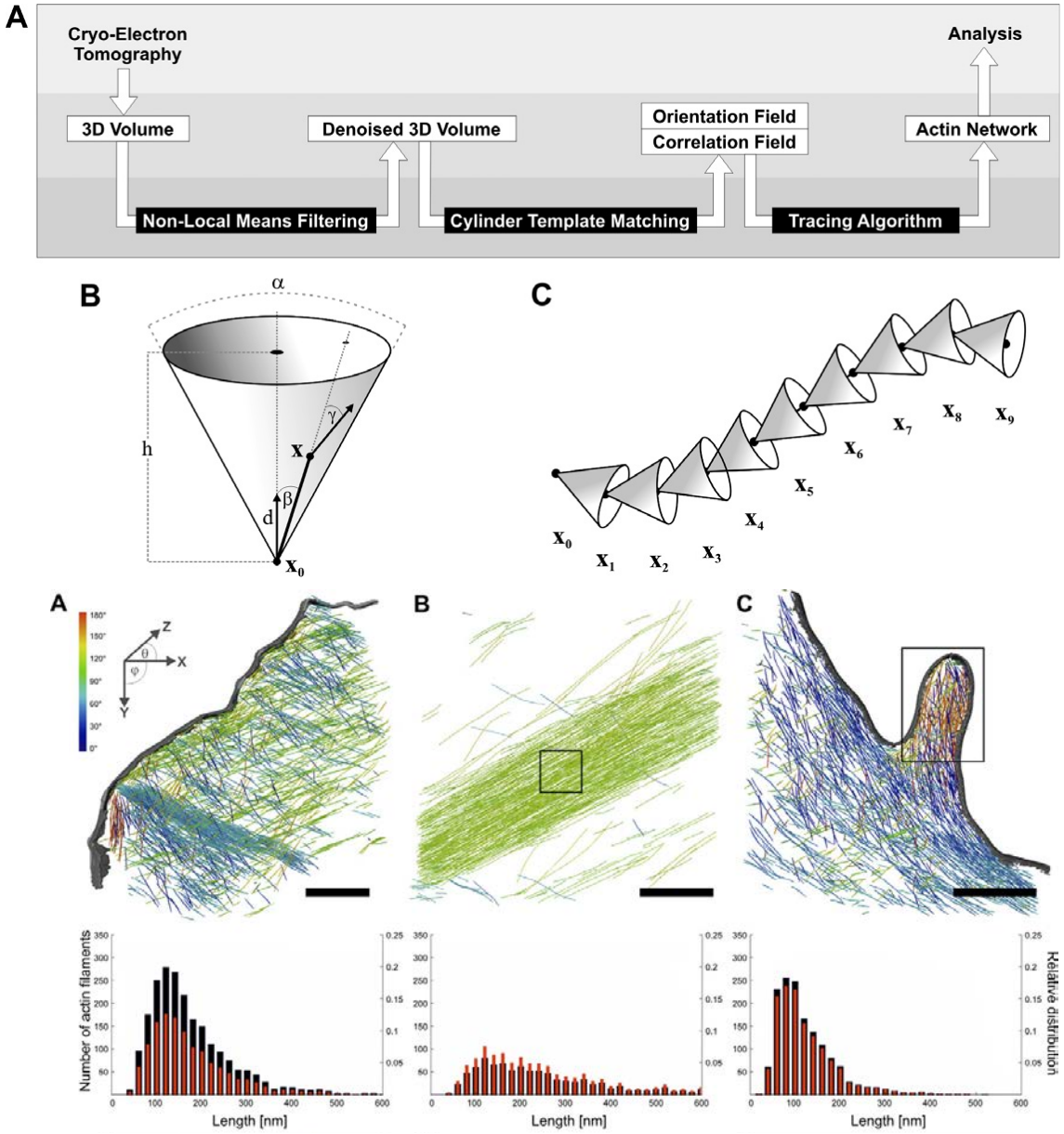
*Segmentation: Entry into synaptosomes*
*Maurer et al. (2008) PNAS 105: 10559-10564.*

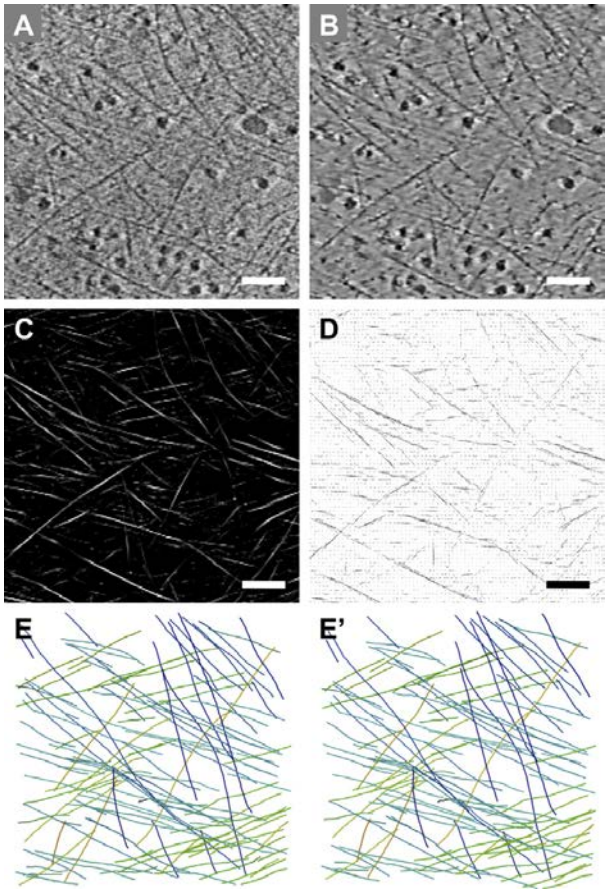



- **Motif Search**

  **- Template matching**

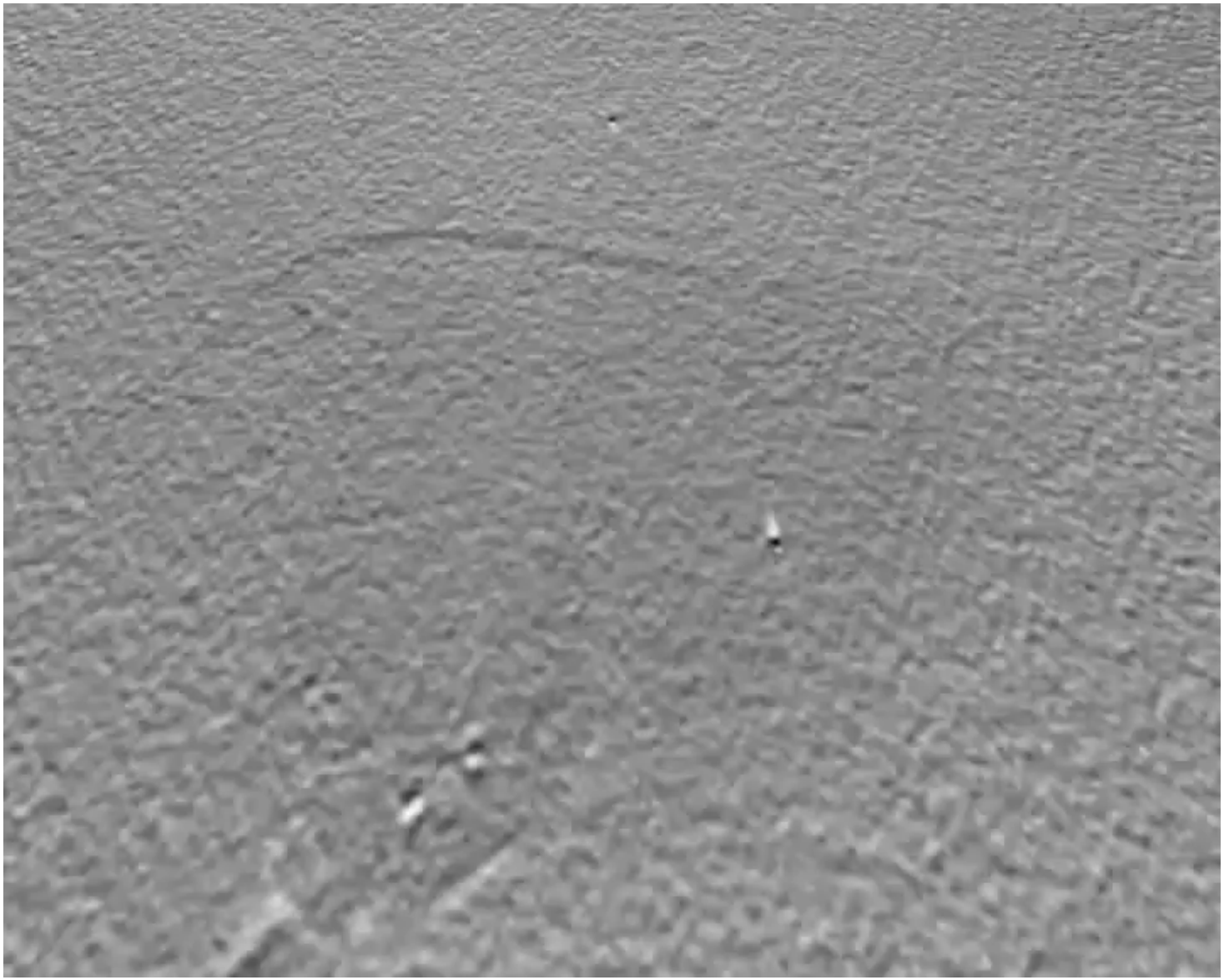
# Automated segmentation




Rigort *et al.* (2012) *JSB* **177**: 135–144.

# *Template matching*

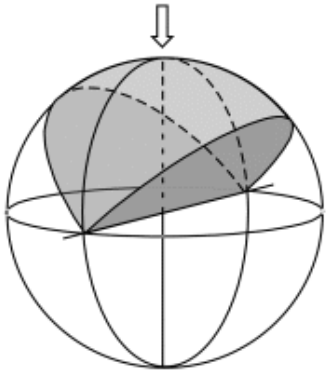


# *Directions for improvement*

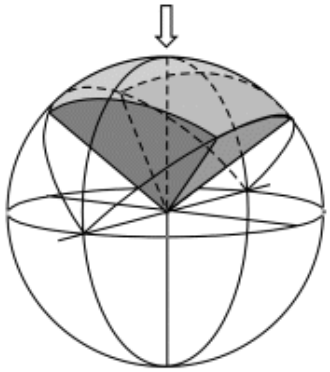
| | |
|---|---|
| ***Dual axis tilting*** | **reduction of missing data** |
| ***Better detectors*** | **direct ‘single’ electron detection** |
| ***Contrast enhancement*** | **phase plates** |
| ***Improved reconstructions*** | **incorp. of boundary conditions** |
| *Better electron optics* | aberration correction? |

# *Single versus dual-axis tilting*

***missing wedge***

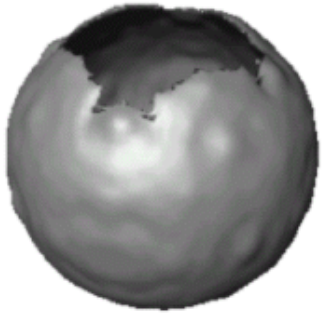


***missing pyramid***



***Fourier space***

- limited tilt range (here ±60° , max ±75° )
- full angular range is not accessible



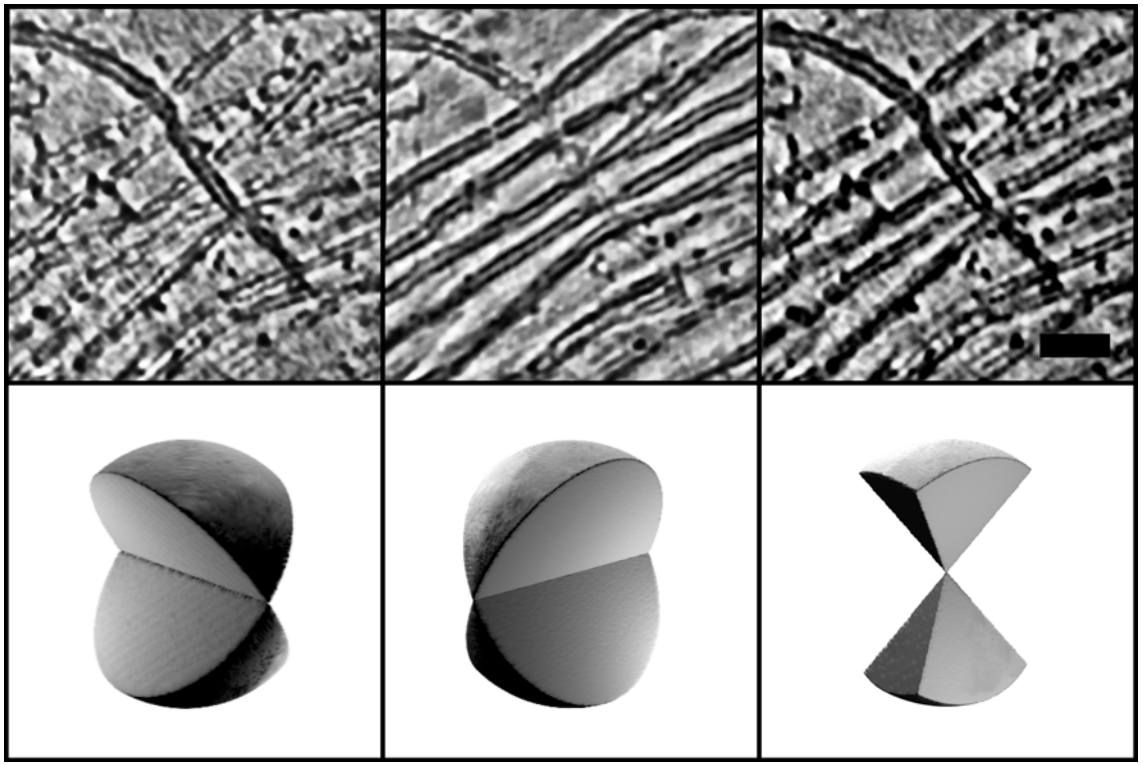
***Real space***

- artifacts from missing information during reconstruction:
  - elongation of object structures along z
  - blurring of the object boundaries

*ideal thin-walled vesicle*

***… gain in information by dual axis tilting, but total dose limit***

# *Single vs. dual-axis tilting*



# *Better Detectors*

**Historically: Indirect detection** (creation of photons first)

- CCD cameras (charged coupled devices)
  - Fibre coupled CCDs
  - Lens coupled CCDs
  - Deceleration

**Direct detection (of electrons)**

- Pixel detectors
  - Active Pixel Sensors (APS)
  - CMOS based detectors

***Detector developments:* Most relevant, higher sensitivity and higher resolution in cryo-ET studies!**

# *Direct detection 'revolution': DD (Gatan K2) vs. Film vs. CCD*

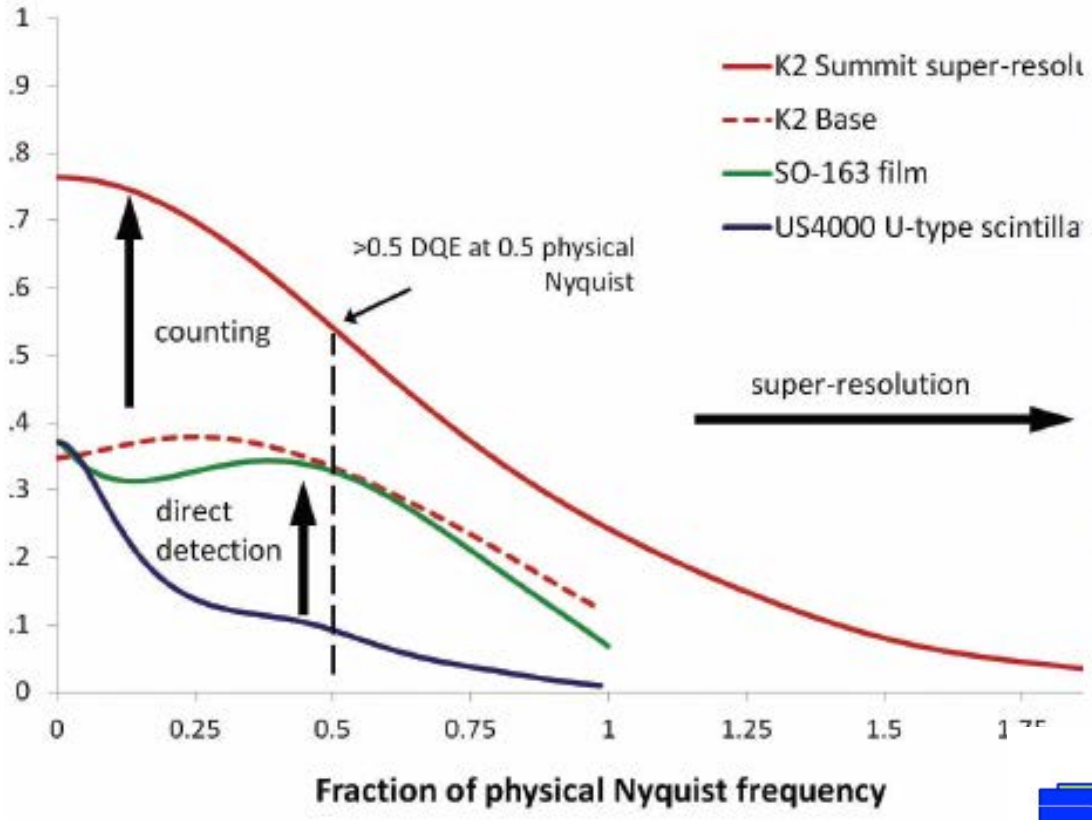
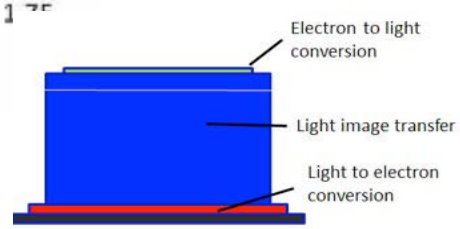
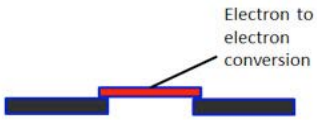



**FIGURE 2**
Detective quantum efficiency (DQE) is the critical metric for evaluating high-performance detectors, such as those used in structural biology EM. This plot shows the relative performance of four common detectors used in structural biology: film, a scintillator-based CCD and two types of direct detectors. The CCD camera does not fully match the performance of film. The charge integrating direct detector, offers a large performance increase over CCDs and gives a slight improvement over film. Whereas the detector with counting and super-resolution offers a substantial increase in performance over all other detector technology.




Detective Quantum Efficiency = $SN_{out}^2/SN_{in}^2$.

Nyquist frequency → Nyquist/Shannon sampling theorum *i.e.* double the pixel size.
Ability to do electron counting!

# *Hybrid approaches (1)*

## Combination with single particle techniques

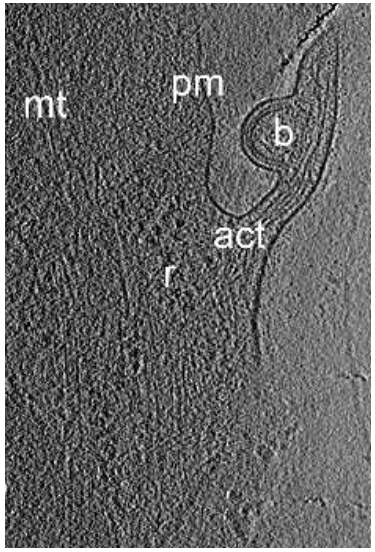
1) Starting models for SPA

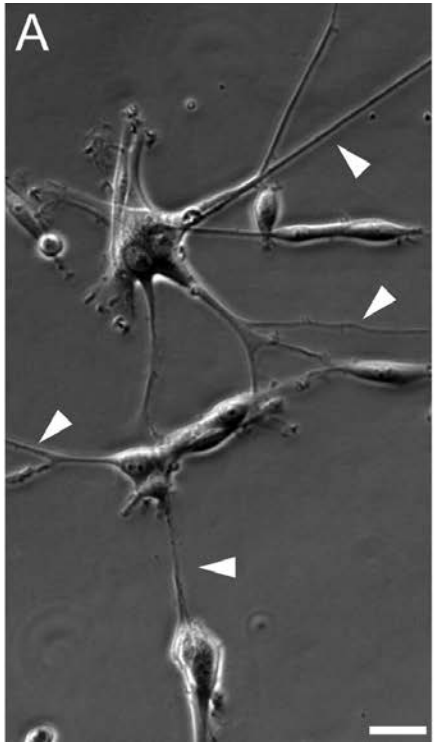
2) Sub-volume / sub-tomogramm averaging:
Single particle type structures inside pleomorphic samples (*e.g.* ribosomes inside cells …)
→ local improvement of resolution!

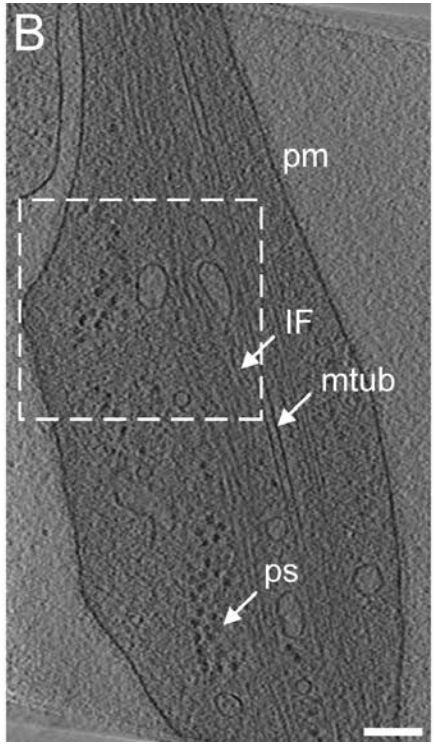
- Particle detection, extraction
- Orientation finding (missing wedge compensation)
- Averaging
- Iterative refinement

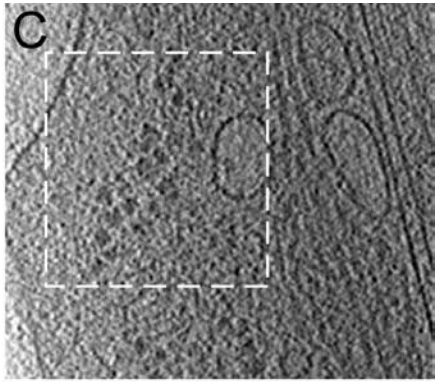
Future:
improved **classification**
(maximum likelihood, take care of missing wedge)

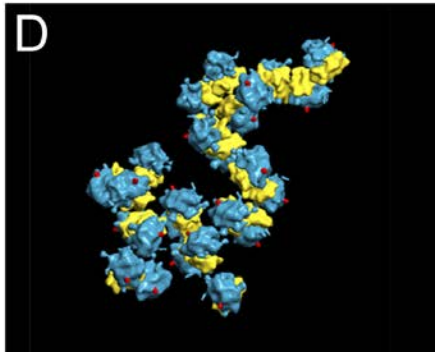
# *Eukaryotic polysome structure in situ*

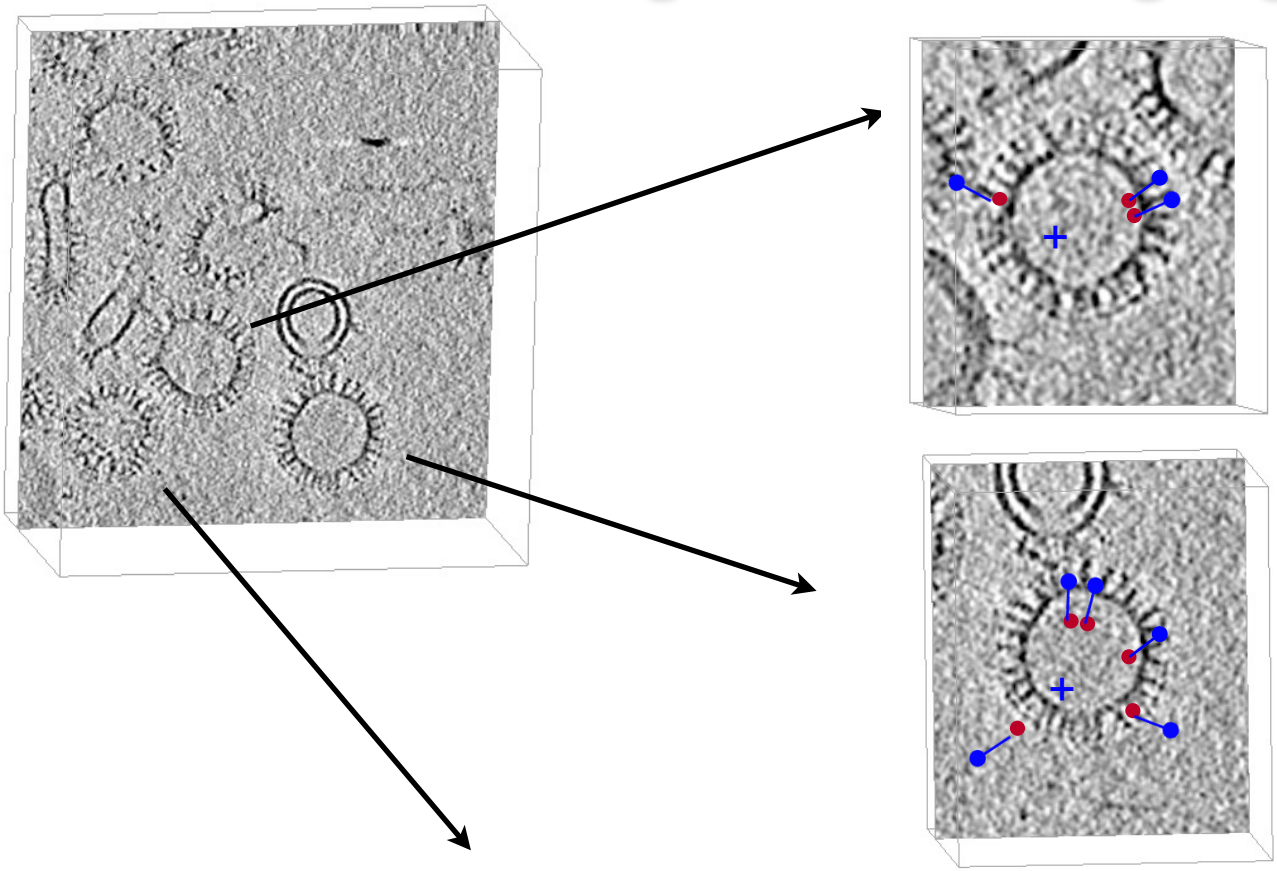




Carlson *et al.*, *PLoS Pathogens 6(11): e1001173*.

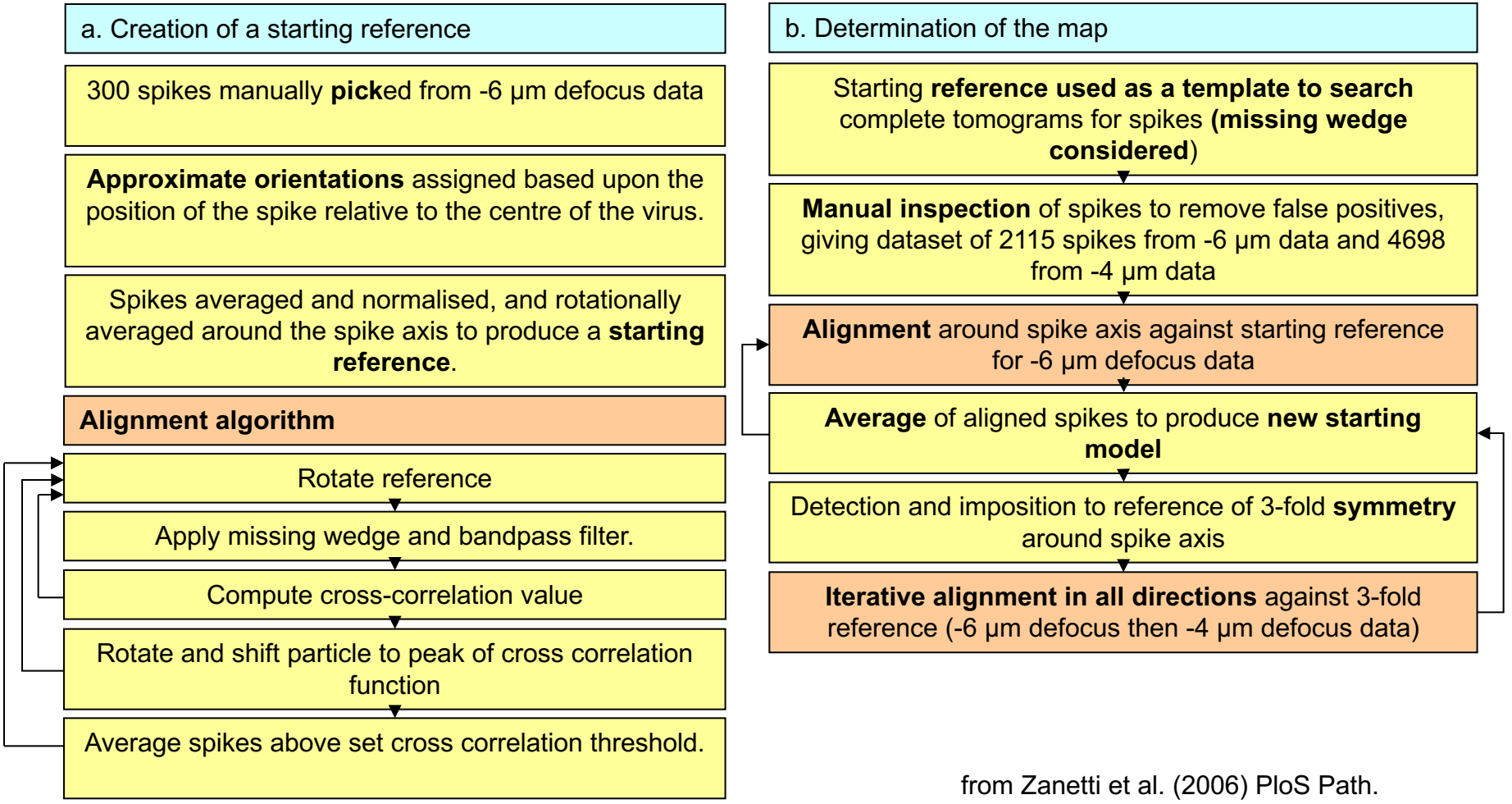
Brandt, Carlson *et al.*, (2010) *Mol. Cell* 39: 560-569.

# *Sub-tomogram averaging*



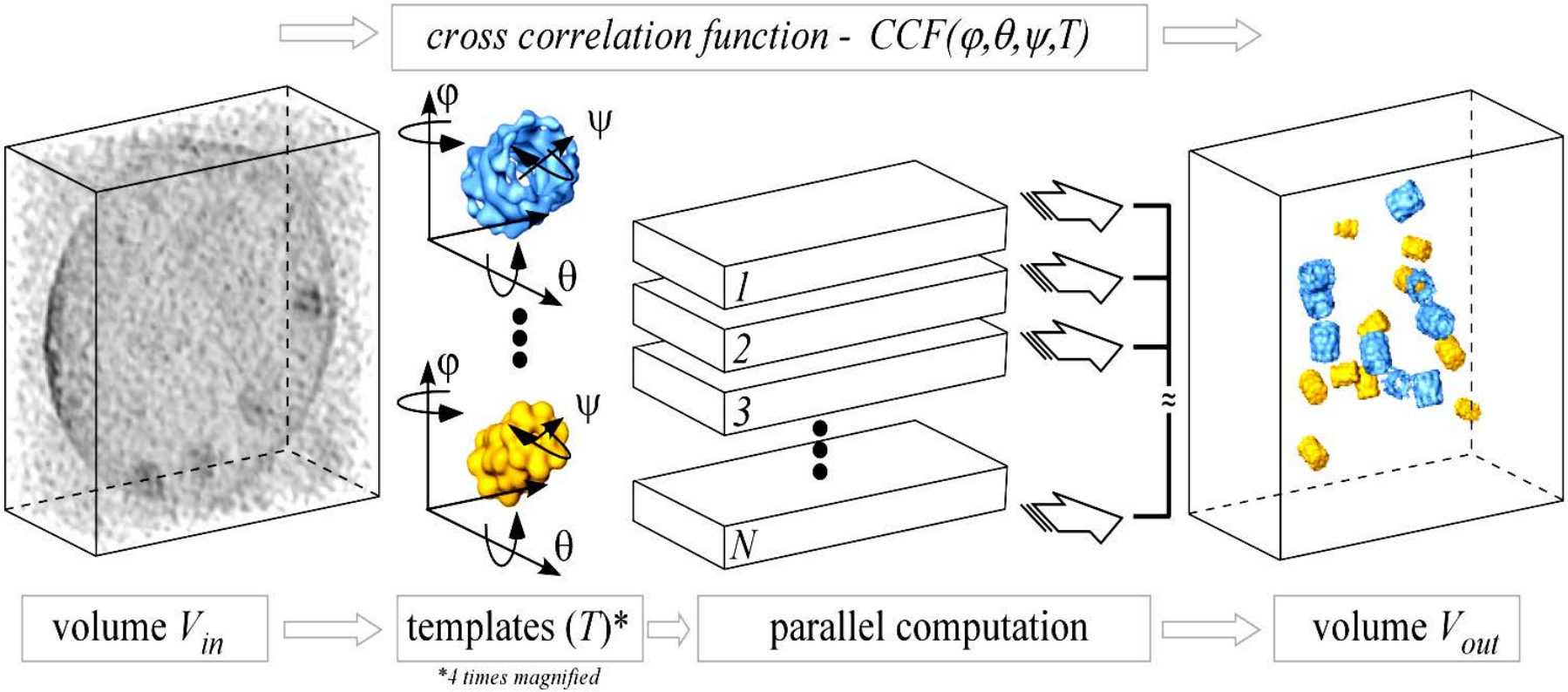
1) initial model calculated by averaging all particles, then iterative averaging:
2) View vector refinement
3) Angle around the spike's long axis refined
3) Symmetry detection and subsequent application
3) refinement of emerging angle

# *Alignment procedure*



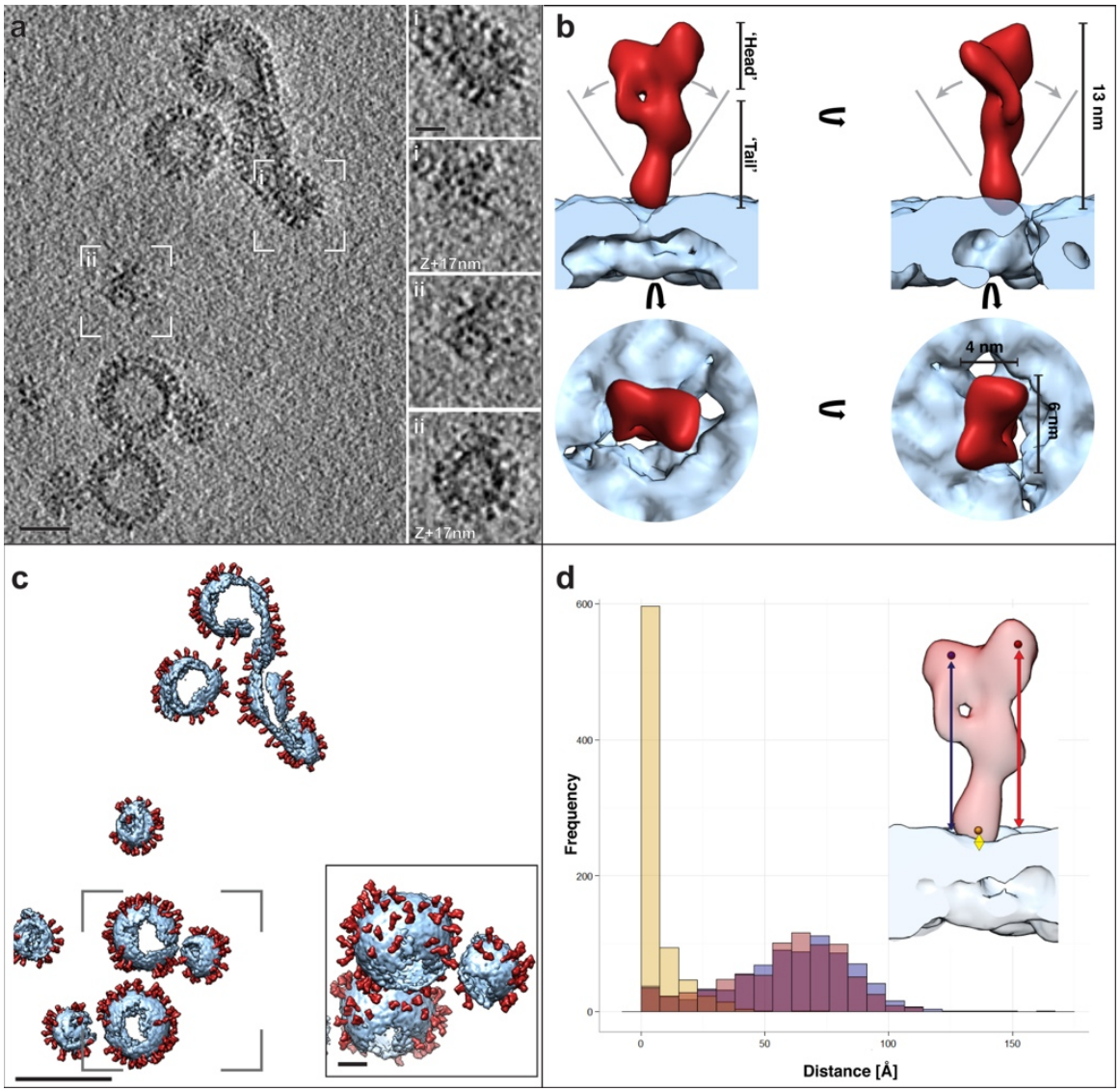

from Zanetti et al. (2006) PloS Path.

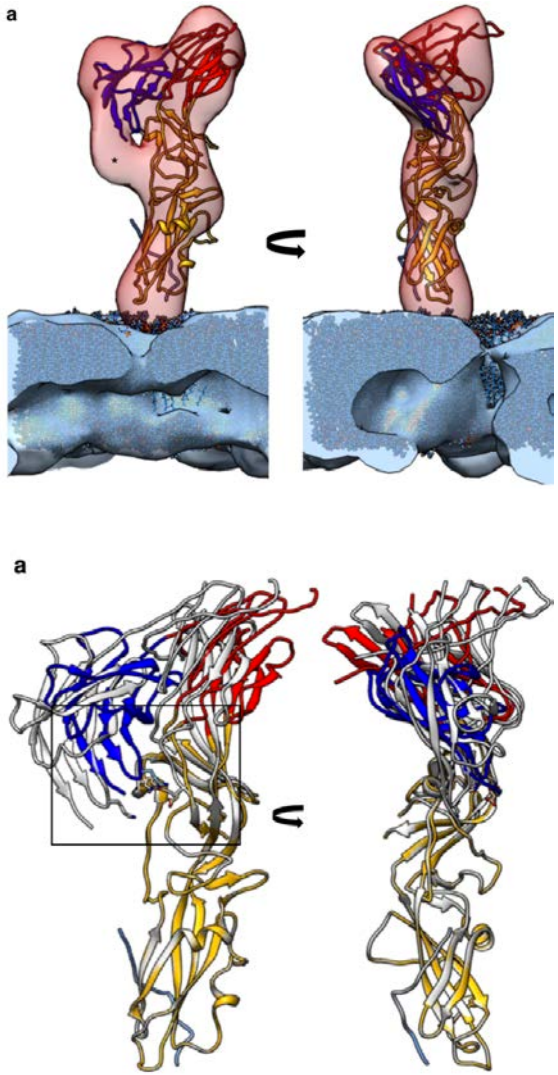
# Detection and identification strategy




A.S. Frangakis, J. Böhm, F. Förster, S. Nickell, D. Nicastro, D. Typke, R. Hegerl, W. Baumeister:
PNAS 99 (2002) 14153-14158.

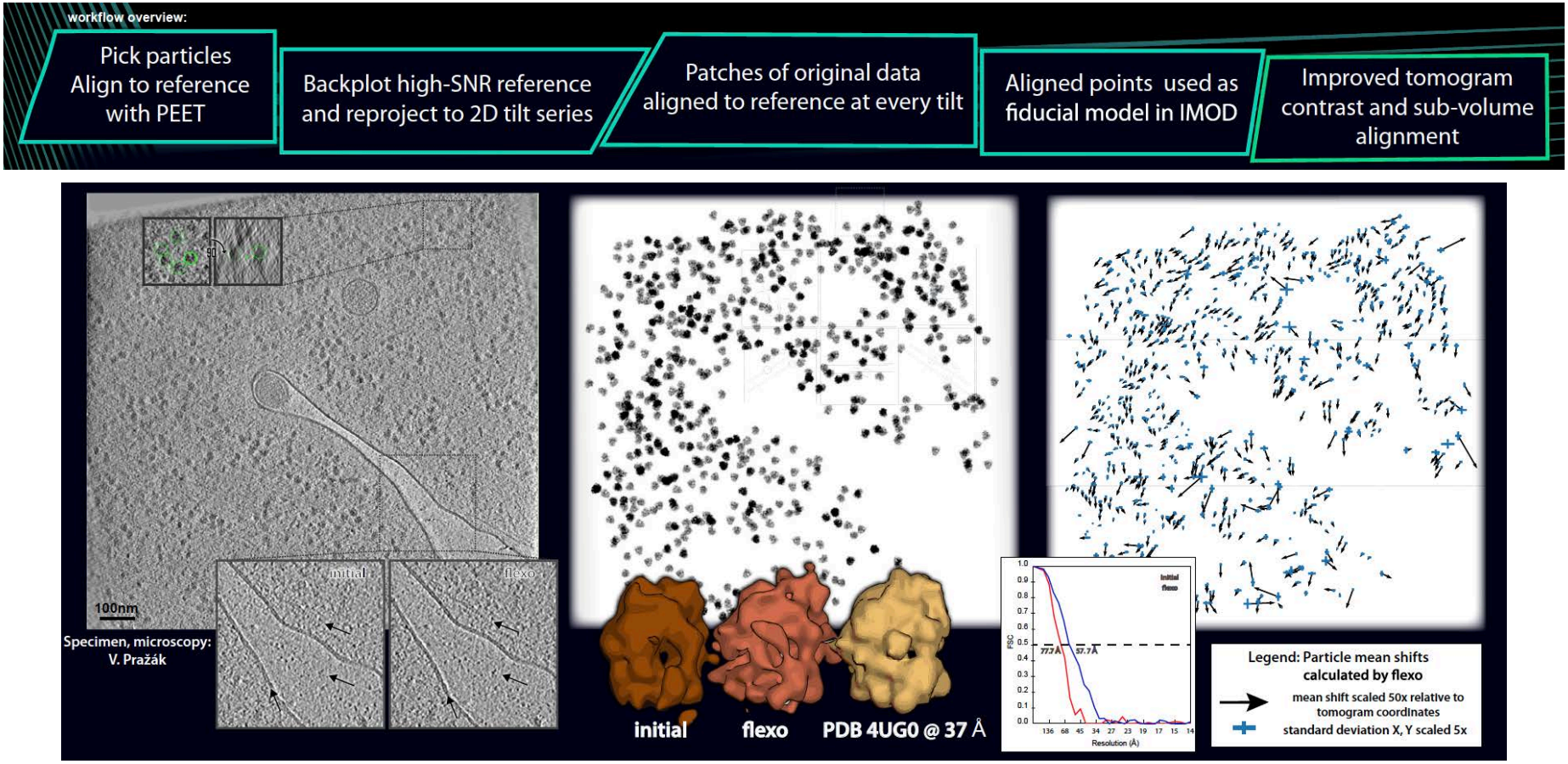
# Sub-tomogram averaging




Zeev-Ben-Mordehai, et al. *Nat Comm.* 2014. 5:3912.

# *FLEXO – unwarping your tomograms (Vojta Pražák)*




similar approaches in EM-Clarity, Tigress etc.

# *Hybrid approaches (2)*

## Correlation with fluorescence microscopy

- Integrating the information (native environment)
- guided approach to target events of interest

## CLEM:

Correlative Light and Electron Microscopy

# Categories of correlative / combined imaging

1. **“Combined Imaging”:**

   Images of different specimens of the same type obtained on different types of instruments.

   Example: Examination of a particular type of cell by both x-ray and electron microscopy under circumstances where the specimen is destroyed by radiation damage after each examination.

2. **Correlative imaging, mode A:**

   Same specimen examined on different instruments.

3. **Correlative imaging, mode B:**

   Same specimen examined in a combined instrument.

# Categories of correlative / combined imaging

1. **"Combined Imaging":**

   Images of different specimens of the same type obtained on different types of instruments.

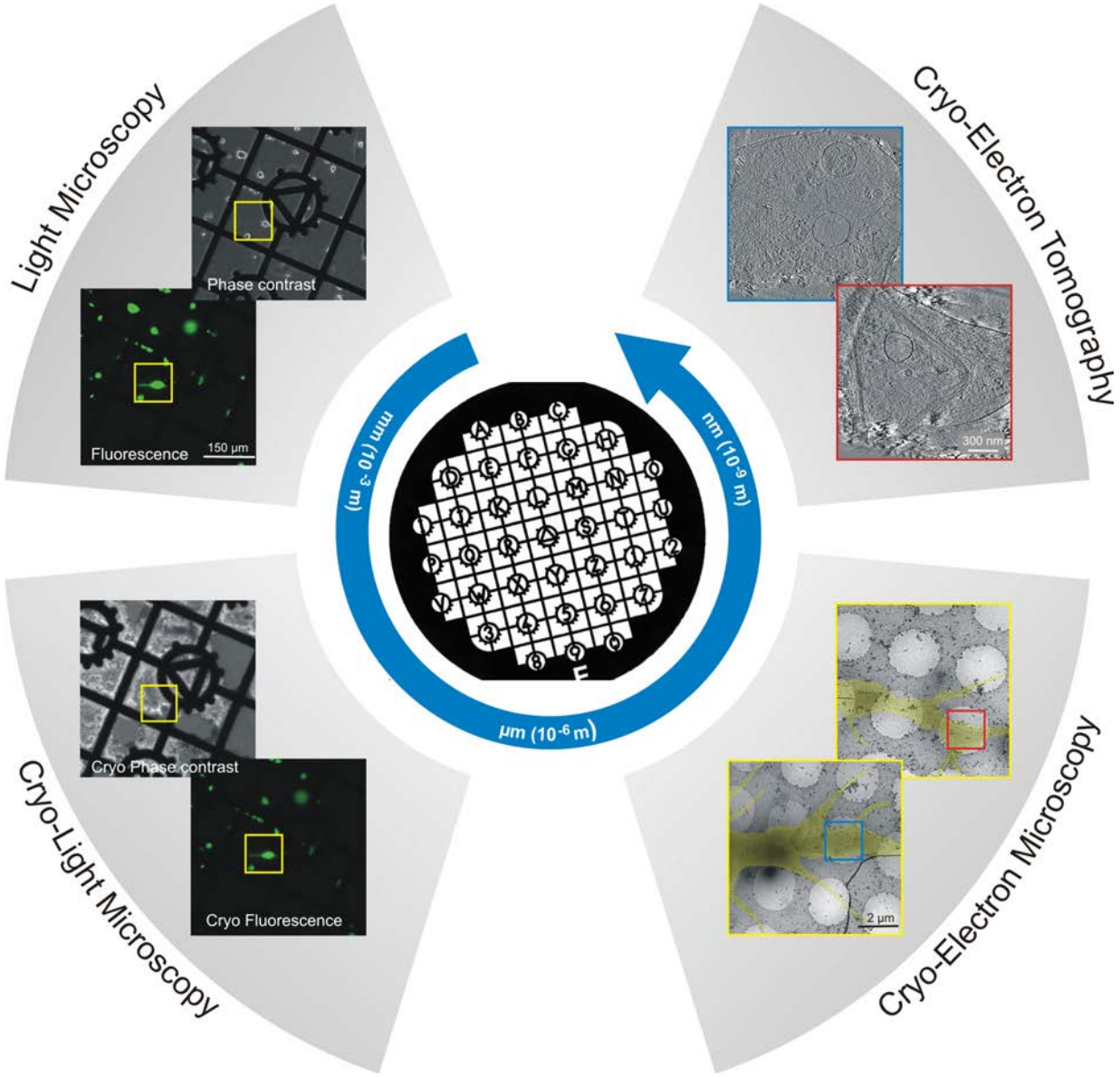
2. **Correlative imaging, mode A:**

   Same specimen examined on different instruments. Specimen preserved for subsequent examination (perhaps after additional specimen treatment). Correlation of images at more detailed level. Need for high quality cross technique fiducial markers. Physically distance of instruments possible. Transfer issues.

   Super resolution light microscopy and cryo-electron tomography.

3 **Correlative imaging, mode B:**

   **Same specimen examined in a combined instrument.**

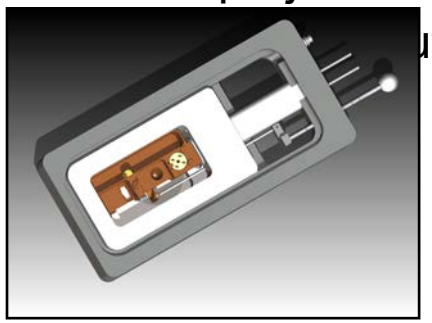


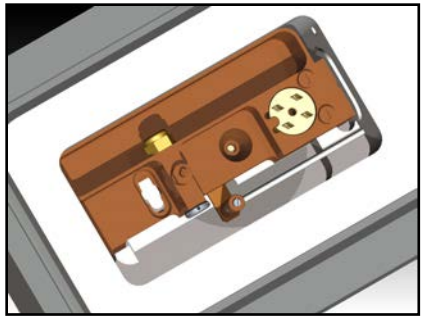

Sartori *et al.* (2008) *JSB*

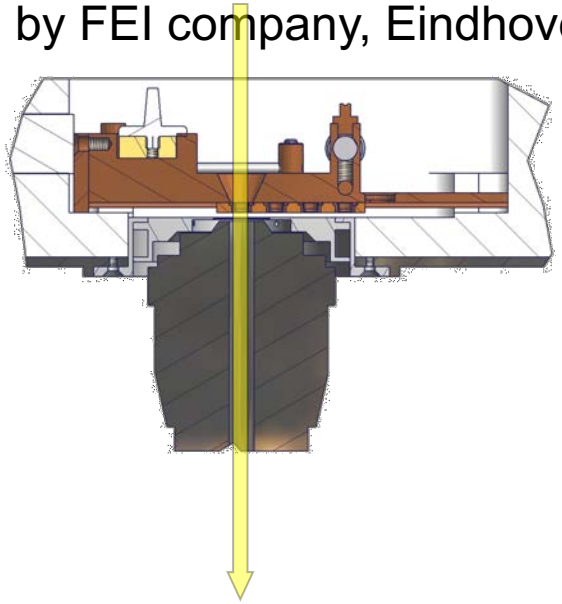
# *Cryo-stage² for LM/FM*

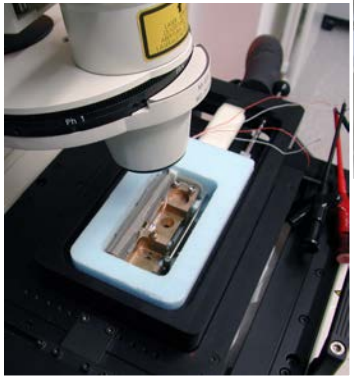
## Characteristics

- Made for inverted LM/FM microscopes – cryo-box for motorized stage
- Automated liquid nitrogen refill for extended screening
- Multiple grid loading (up to 4 EM grids)
- Use of long working distance (LWD) 63x objective (W.D. 2.1 mm, N.A. 0.75)
- MPIB project since 2004, partly in 6th FP Network of Excellence ‘3DEM’
  ution by FEI company, Eindhoven, NL

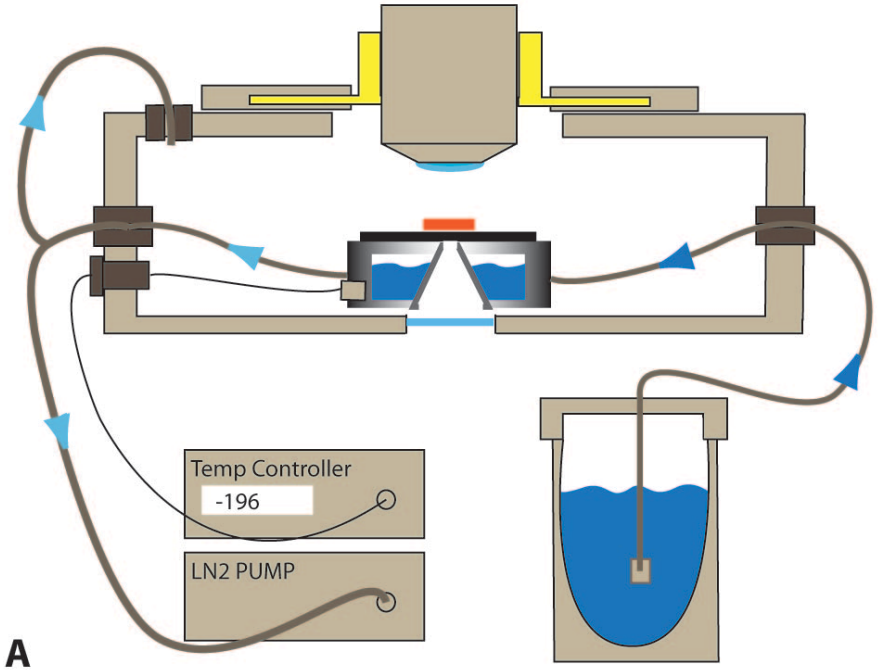




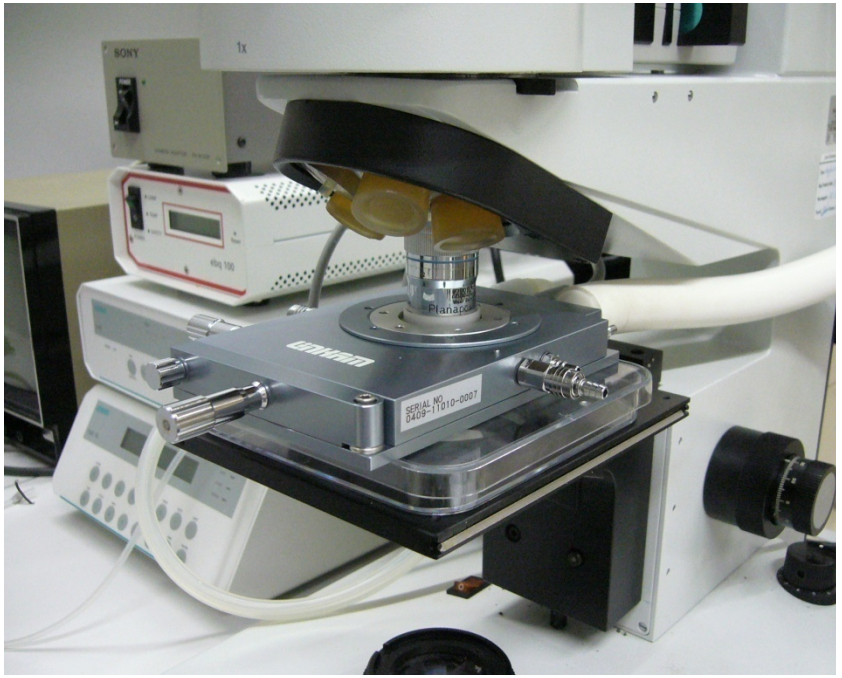




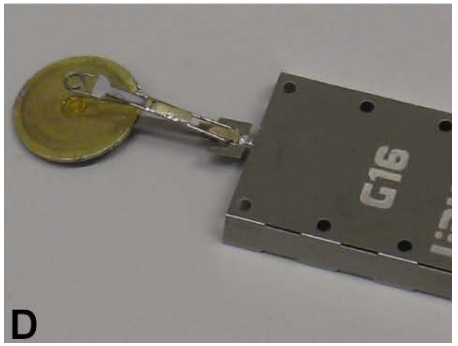
# Cryo stage for correlative microscopy



A



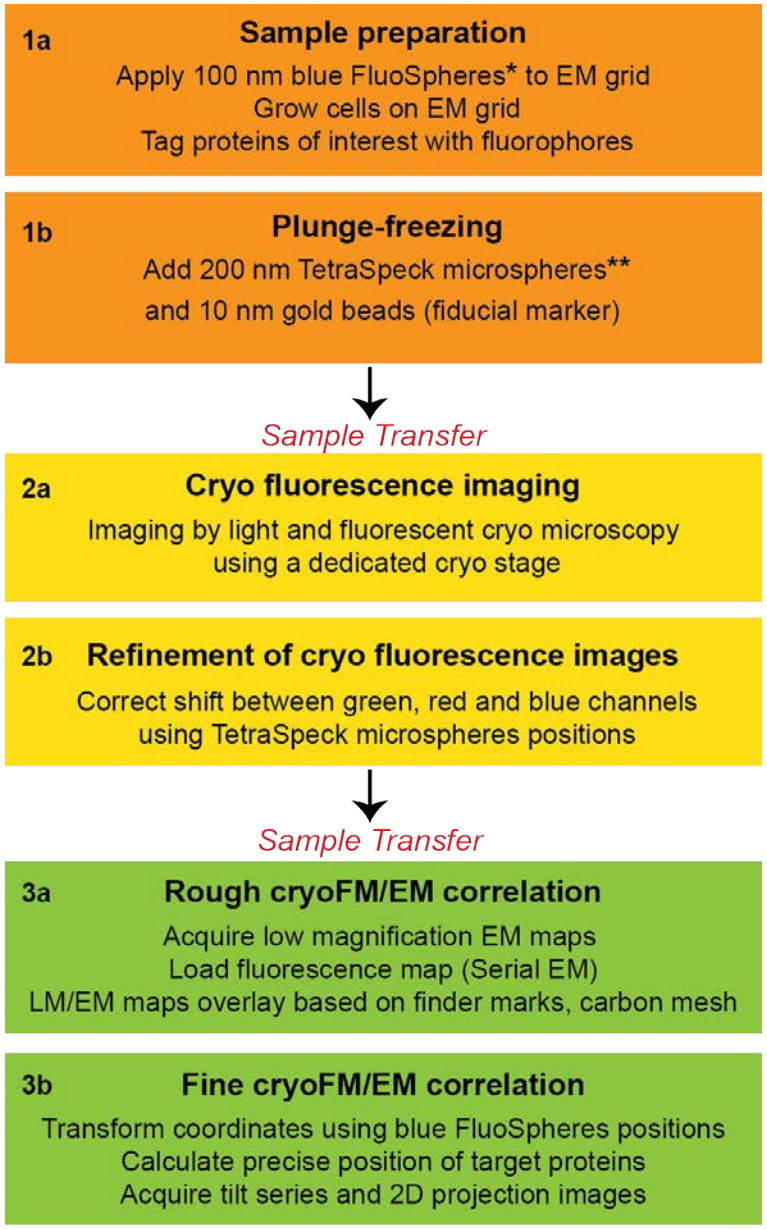
LINKAM freezing stage mounted on a light microscope.
Resolution of 0.4 um.
Workdistance of 4.7 mm
A 100x Leica lens with an NA of 0.75
After LM, the specimen is transferred to a cryoTEM.
Evaluated grid temperature and ice-contamination during transfer



D

Van Driel *et al.* Freezing stage for correlative microscopy. Eur. J. Cell Biology (2009).

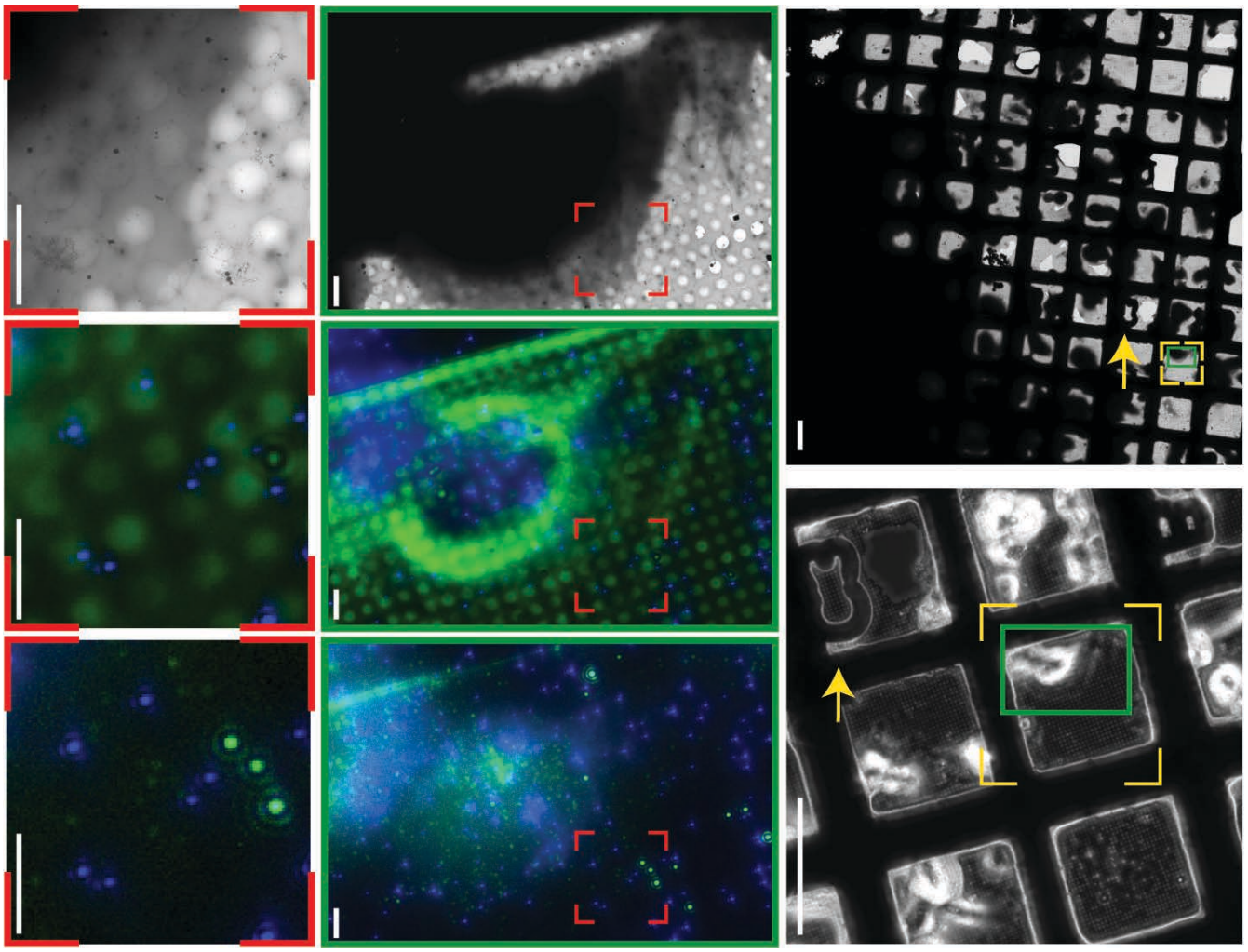
# Correlative imaging workflow




* Markers for correlation between Electron Microscopy (EM) and Fluorescence Microscopy (FM). Can also be appied in step 1b.

** Markers for correction of FM multi channels shifts

# CryoLM and cryoEM correlation from low to high magnification

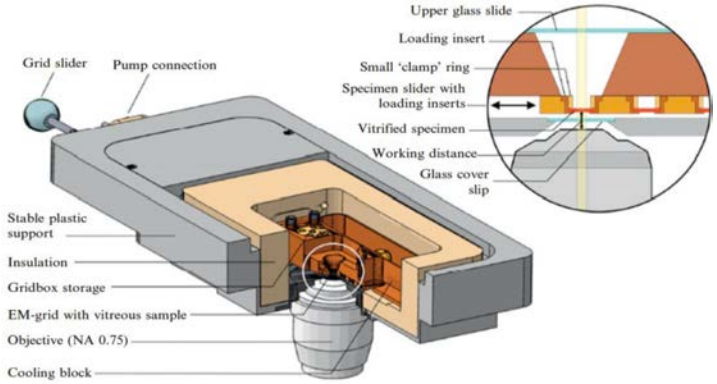


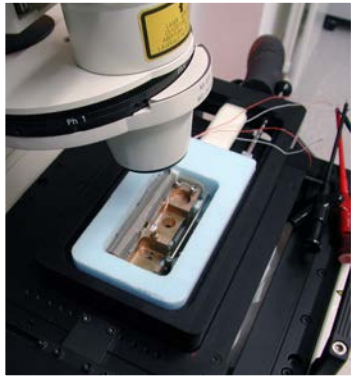
Schellenberger, Kaufmann *et al.* (2014) *Ultramicroscopy*

# Multichannel alignment

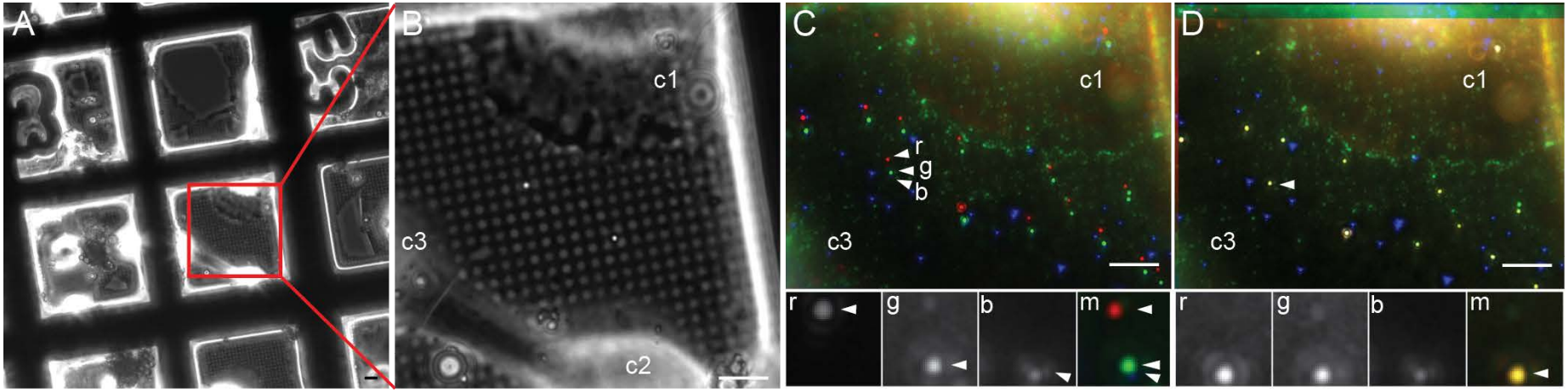
Pascale Schellenberger

Rainer Kaufmann



Based on Cryostage$^2$ (MPI Biochemistry Martinsried)




Schellenberger, Kaufmann *et al.* (2014) *Ultramicroscopy 143, 41−51.*

# High-precision correlative fluorescence and electron cryo microscopy

Pascale Schellenberger

Christoph Hagen

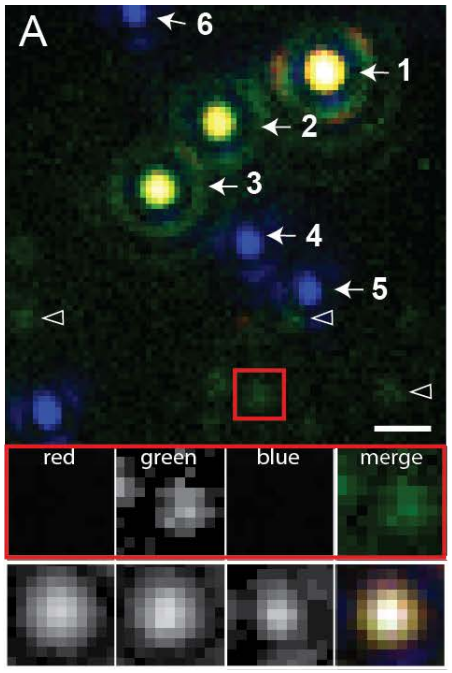
Rainer Kaufmann

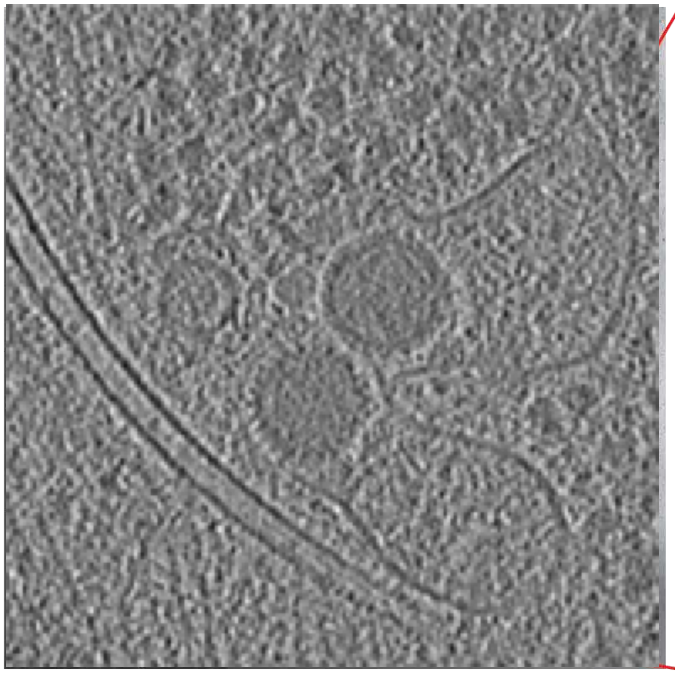
## Calculating precision of correlation $\sigma^T$

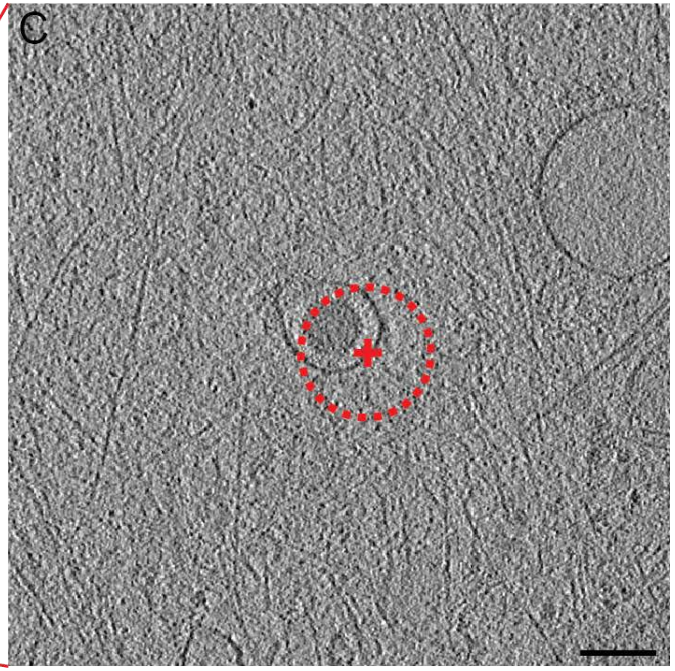
For each event of interest, the correlation accuracy is calculated as:

$$\sigma^T = \sqrt{\left((\sigma^A)^2 + (\sigma^C)^2 + (\sigma^E)^2\right)}$$

- $\sigma^T$: *Total correlation precision* ~ 70 nm
- $\sigma^A$: *precision of cryoFM channel Alignment (200 nmTetraSpeck)* ~ 20 nm
- $\sigma^C$: *precision of Coordinate transformation acc. to [2] (100 nm FluoSpheres)* ~ 30 nm
- $\sigma^E$: *precision of locating Event of interest* ~ 60 nm

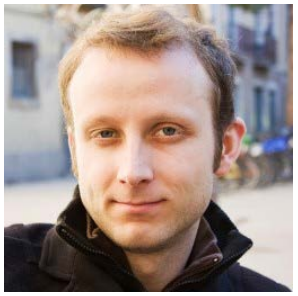


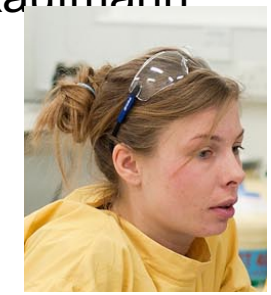



Schellenberger, Kaufmann *et al.* (2014) *Ultramicroscopy 143, 41–51. (cf. also Schorb et al. (Briggs lab)*

# *Cool next thing: super-resolution cryoFM*

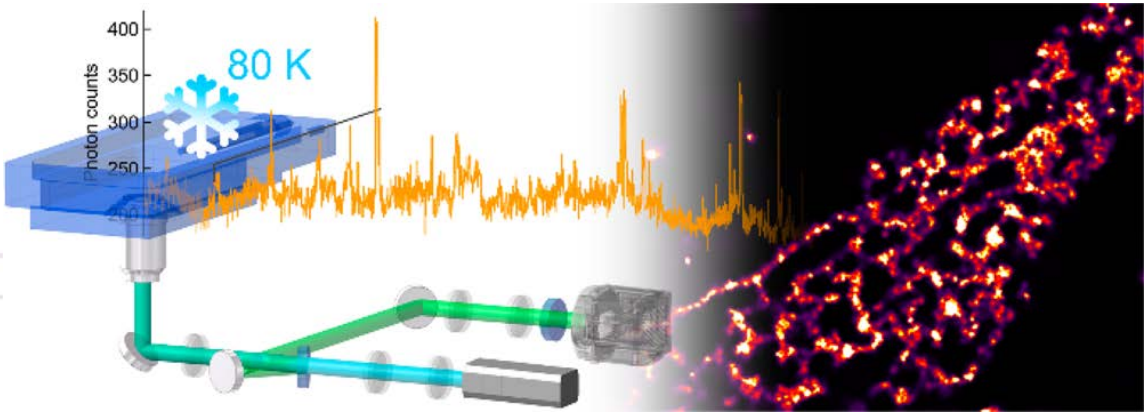


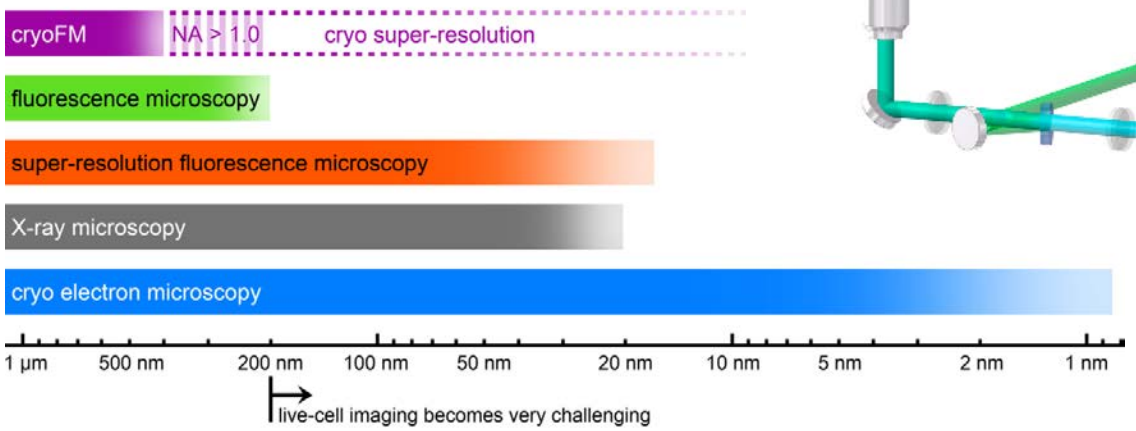
Rainer Kaufmann



Pascale Schellenberger

- **vitrification** preserves specimen at level of resolution analysed
- '**blinking**': reversible photo bleaching of **standard fluorescent proteins** at ~80K suitable for **single molecule localization microscopy**
- **cryo stage stability** for stochastic single molecule super-resolution
- average single molecule localization accuracy ~ 40 nm
- **resolution of ~ 125 nm** (*i.e.* 3-5x improvement)
- using a LWD objective, *i.e.* non-dipping lens!



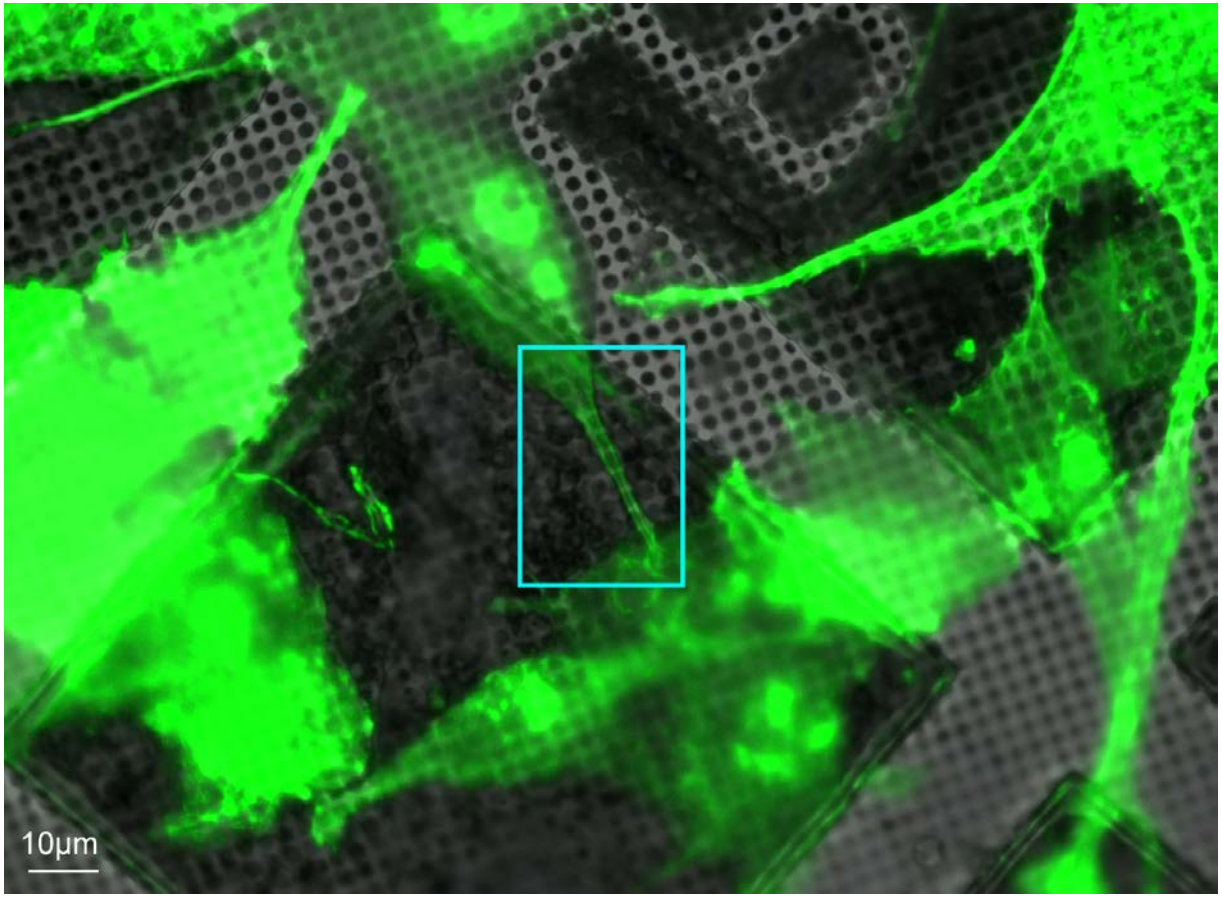

Kaufmann *et al.* (2014) *NanoLetters*

Kaufmann *et al.* (2014) *Curr Rev Chem Biol*.

# Making it work correlative:

# *Cryo-SOFI for super-resolution cryo-CLEM*

Cryo **S**uper-resol. **O**ptical **F**luctuation **I**maging: → **Lower laser intensity alternative!**




Dendra2-actin

reflected light + wide-field fluorescence

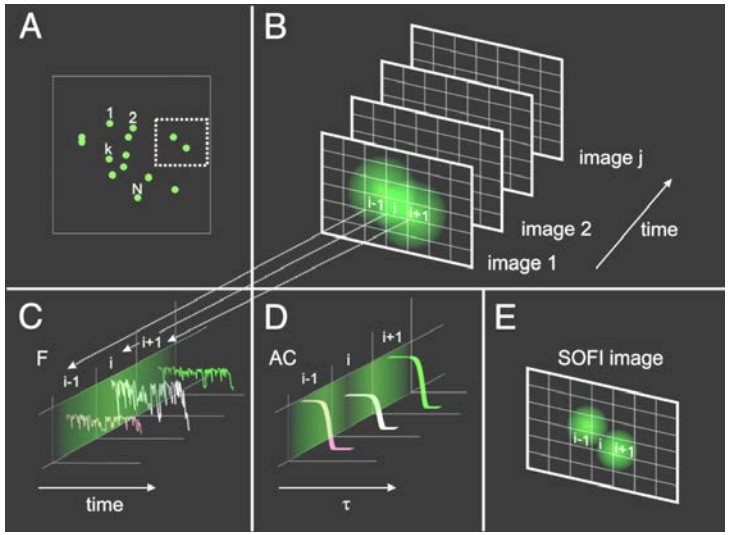
Rainer Kaufmann
Felipe Moser
Vojta Prazak

Leica cryo-CLEM system
based
0.9 NA objective lens

# cryo-SOFI (cryo super-resolution optical fluctuation imaging)

## a low laser intensity alternative for super-resolution cryo-CLEM

SOFI principle:




Dertinger et al. (2009) PNAS

SOFI theory:

$$I_k(t) = \epsilon_k \cdot s_k(t)$$

$$F(\vec{r},t) = \sum_{k=1}^{N} U(\vec{r}-\vec{r}_k) \cdot \epsilon_k \cdot s_k(t)$$

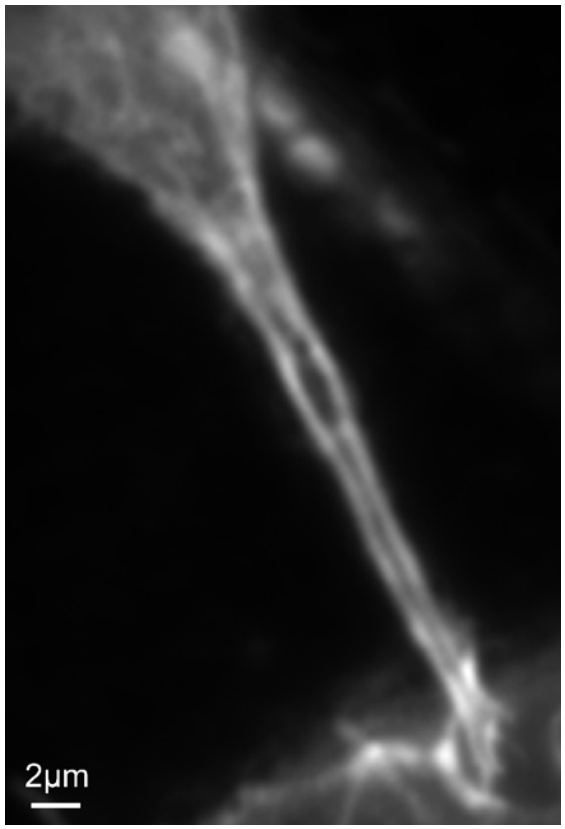
$$\delta F(\vec{r},t) = \sum_{k} U(\vec{r}-\vec{r}_k) \cdot \epsilon_k \cdot \delta s_k(t)$$

$$G_2(\vec{r},\tau_1,\tau_2) = \sum_{k} U^2(\vec{r}-\vec{r}_k) \cdot \epsilon_k^2 \cdot \langle \delta s_k(t+\tau_1) \cdot s_k(t+\tau_2) \rangle_t$$

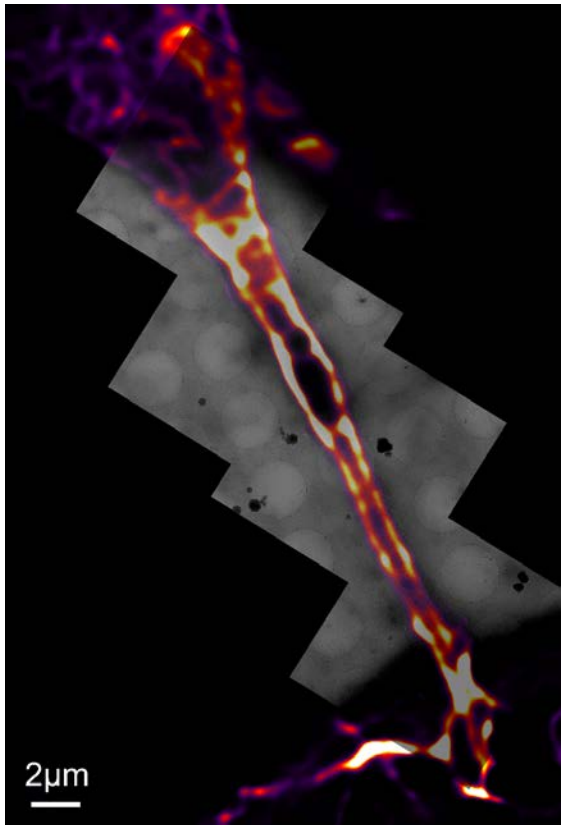
# *First proof: Cryo-SOFI for super-resolution cryo-CLEM*

Dendra2-actin

conventional cryo-FM




cryo-SOFI + low mag montage



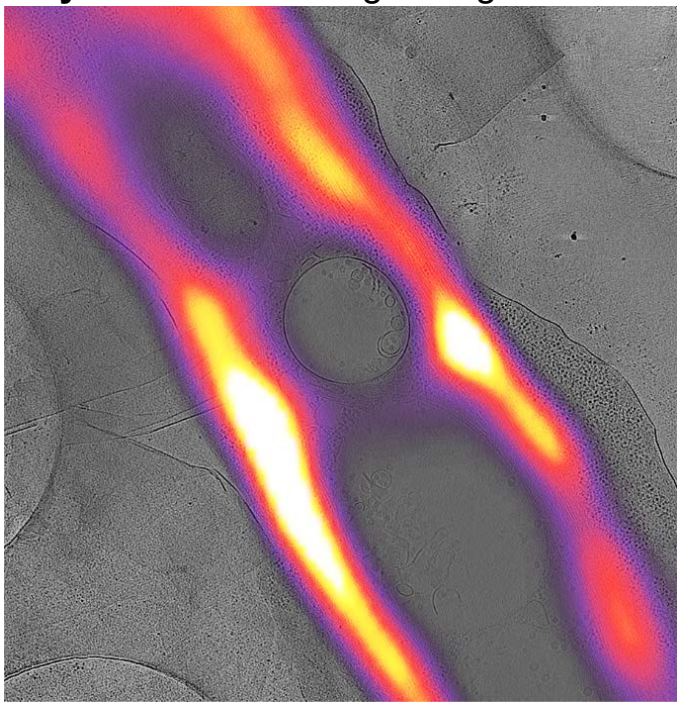

Moser *et al.* (2019) *PNAS* 116(11):4804-4809. doi: 10.1073/pnas.1810690116.

# *Cryo-SOFI for super-resolution cryo-CLEM*

Dendra2-actin

**conv. cryo-FM** + low mag tomogram

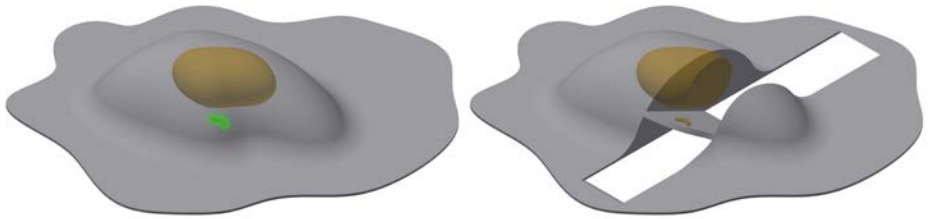
**cryo-SOFI** + low mag tomogram



Moser *et al.* (2019) *PNAS* 116(11):4804-4809. doi: 10.1073/pnas.1810690116.

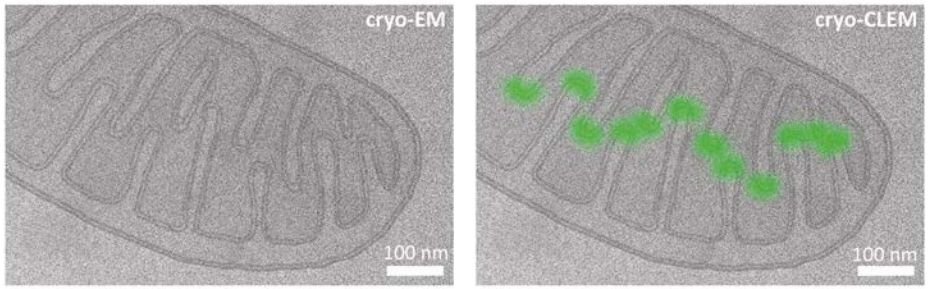
# *Outlook: directions for super-resolution cryoFM*

**Cryo-SIM / Cryo-Airyscan**



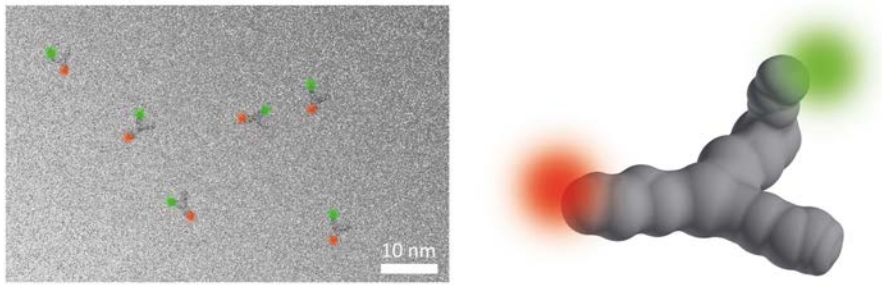
Cryo-FM gives 3D coordinates, e.g. for FIB-milling

**Cryo-STED / Cryo-SMLM**

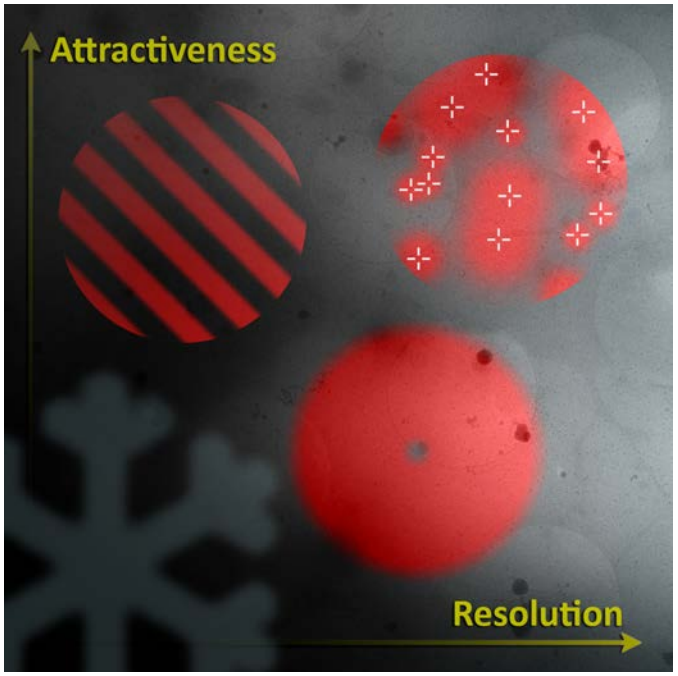



Cryo-FM gives distributions of labelled molecules in structural context of cryo-EM image

**Ultra-high-precision cryo-CLEM**




Cryo-FM gives (3D) orientation of particles, e.g. for particle averaging




Wolf *et al.* (2016) *Biol Cell*

# Categories of correlative / combined imaging

1. **“Combined Imaging”:**

   Images of different specimens of the same type obtained on different types of instruments.

2. **Correlative imaging, mode A:**

   Same specimen examined on different instruments.

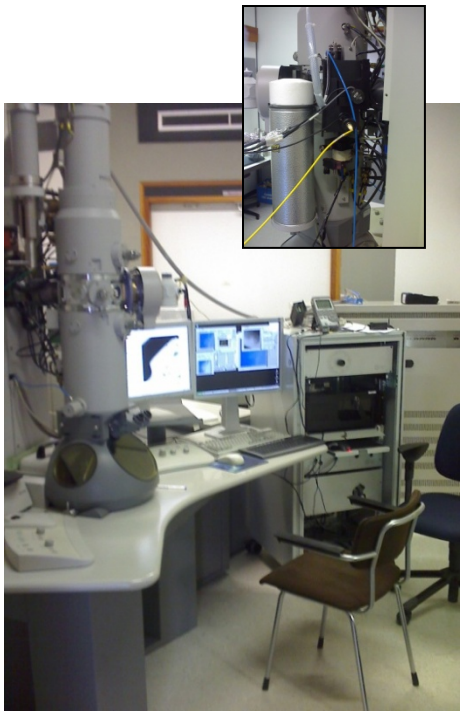
3. **Correlative imaging, mode B:**

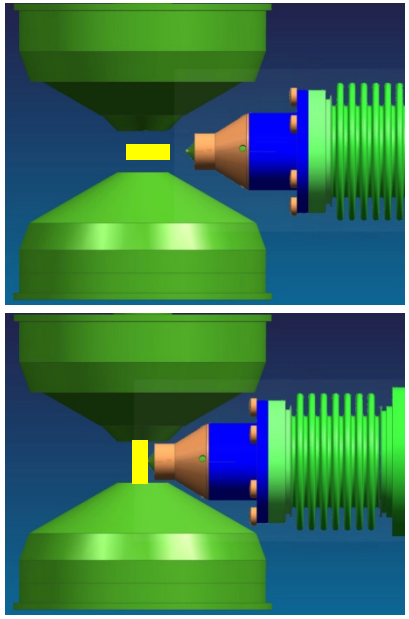
   Same specimen examined in a combined instrument.

   Example: electron or x-ray microscope equipped with a more basic scanning fluorescence microscope, both examining sample at cryo-temperatures. Potential for high-throughput instrument for correlated imaging. No transfer issues, fast change of mode possible.

   → future: inside FIB?

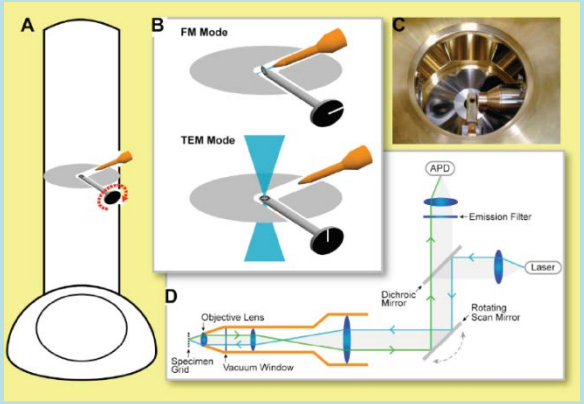
# Correlative microscope developments





FEI Tecnai 120 kV
Twin objective lens

integrated light scanning microscope in a TEM

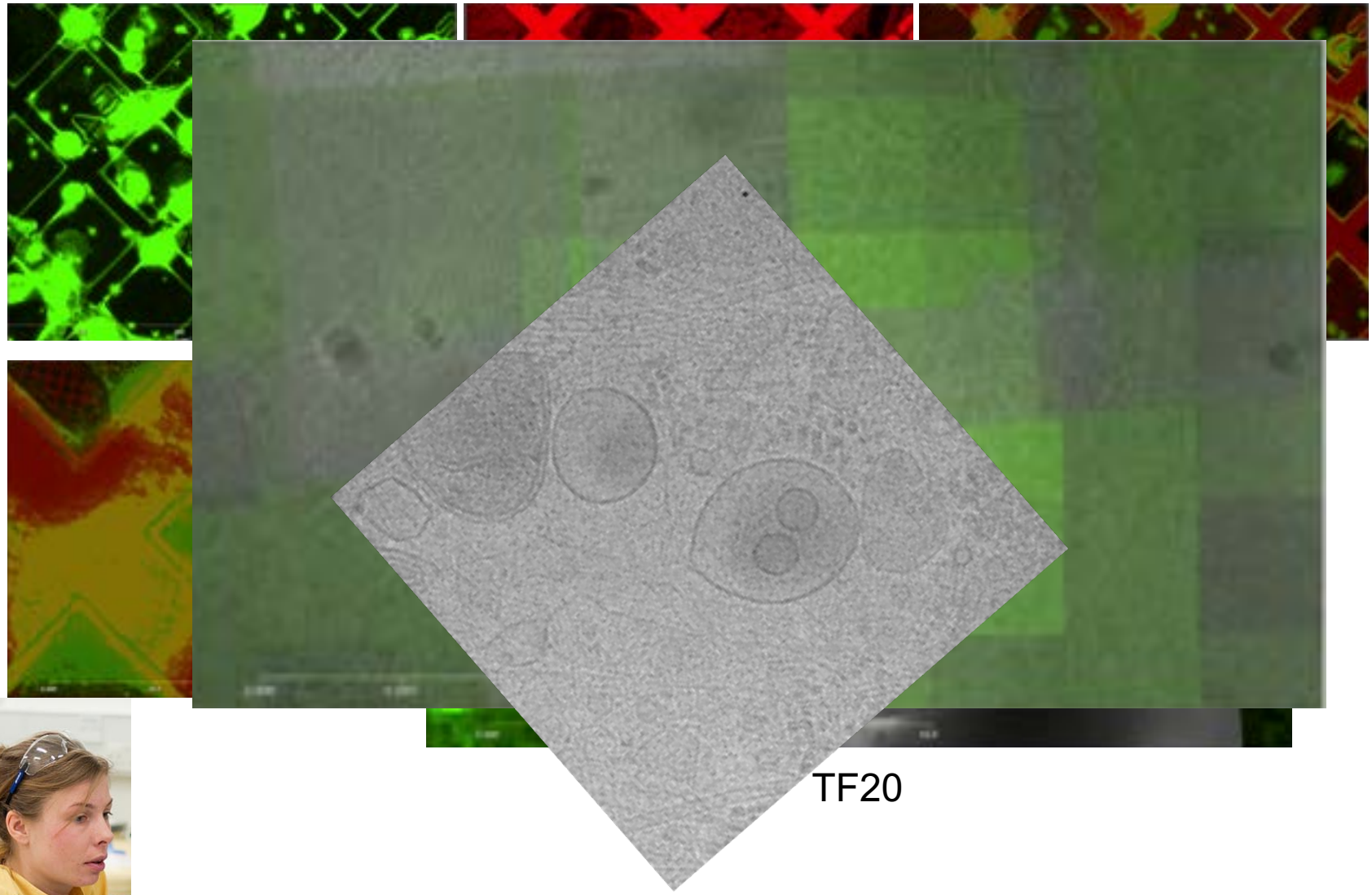



Utrecht University
Sasha Agronskaia
Hans Gerritsen, Arie Verkleij

LUMC
Bram Koster
Linda van Driel, Jack Valentijn

Agronskaia et al (2008) J Struct Biol

# *iCorr – our cellular cryo tests*



TF20



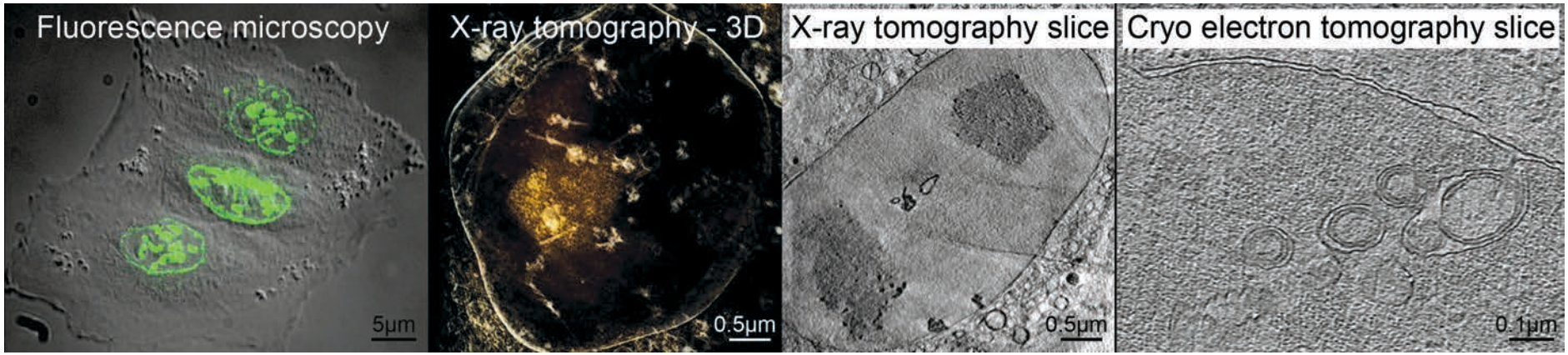
Pascale Schellenberger

with Linda van Driel, Alevtyna Yakushevska, Wim Voorhout (FEI)

# *Hybrid approaches (3)*

## Combination with soft X-ray cryo tomography

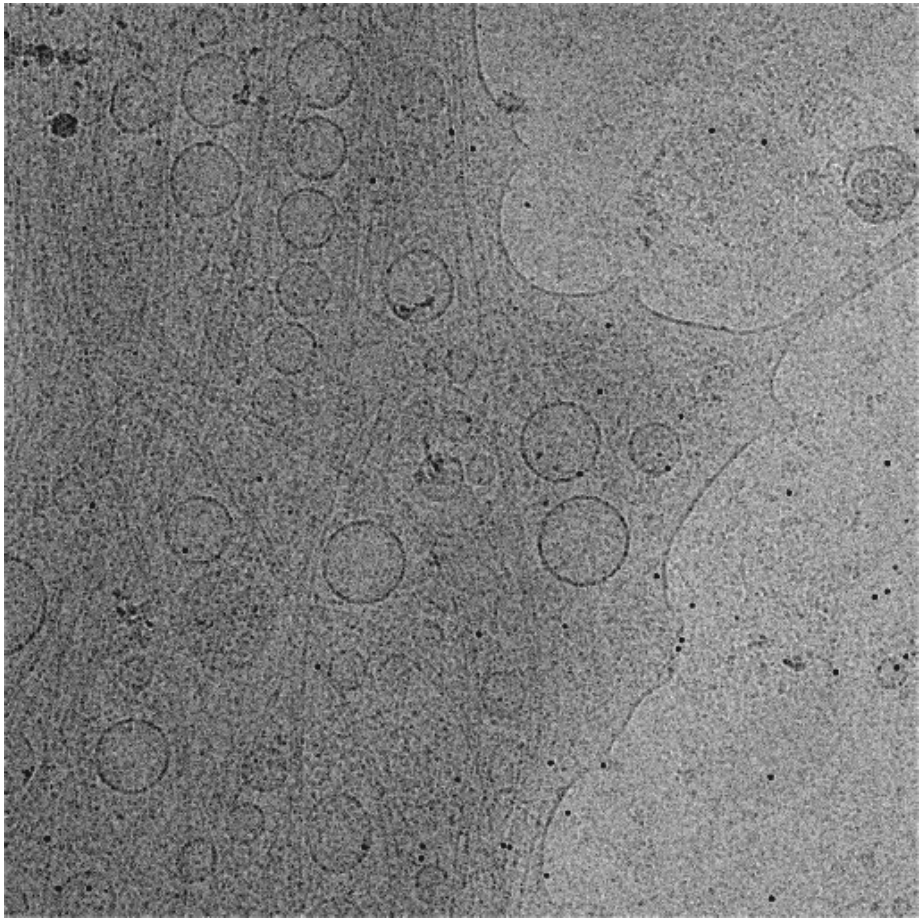
- better penetration power!
- resolution ~25-40 nm, *i.e.* ~10x lower than ET
- larger fields of view
- enabling statistic analyses
- in-scope fluorescence cryo microscopy possible



# ***Summary*** *Cryo Electron Tomography*

- Resolution of raw tomogramms currently ~2-6 nm
- 3-20Å for subtomo (cf. following talk of John Briggs)
- For large structures with unique topologies (organelles or cells) at macromolecular resolution
- Mapping functional interactions of supramolecular highly dynamic assemblies *in situ* in 3D
- Excellent platform for integrative and hybrid approaches (*e.g.* with single particle techniques and fluorescence microscopy; mass spec. …)
- For best performance dedicated investigation systems needed.

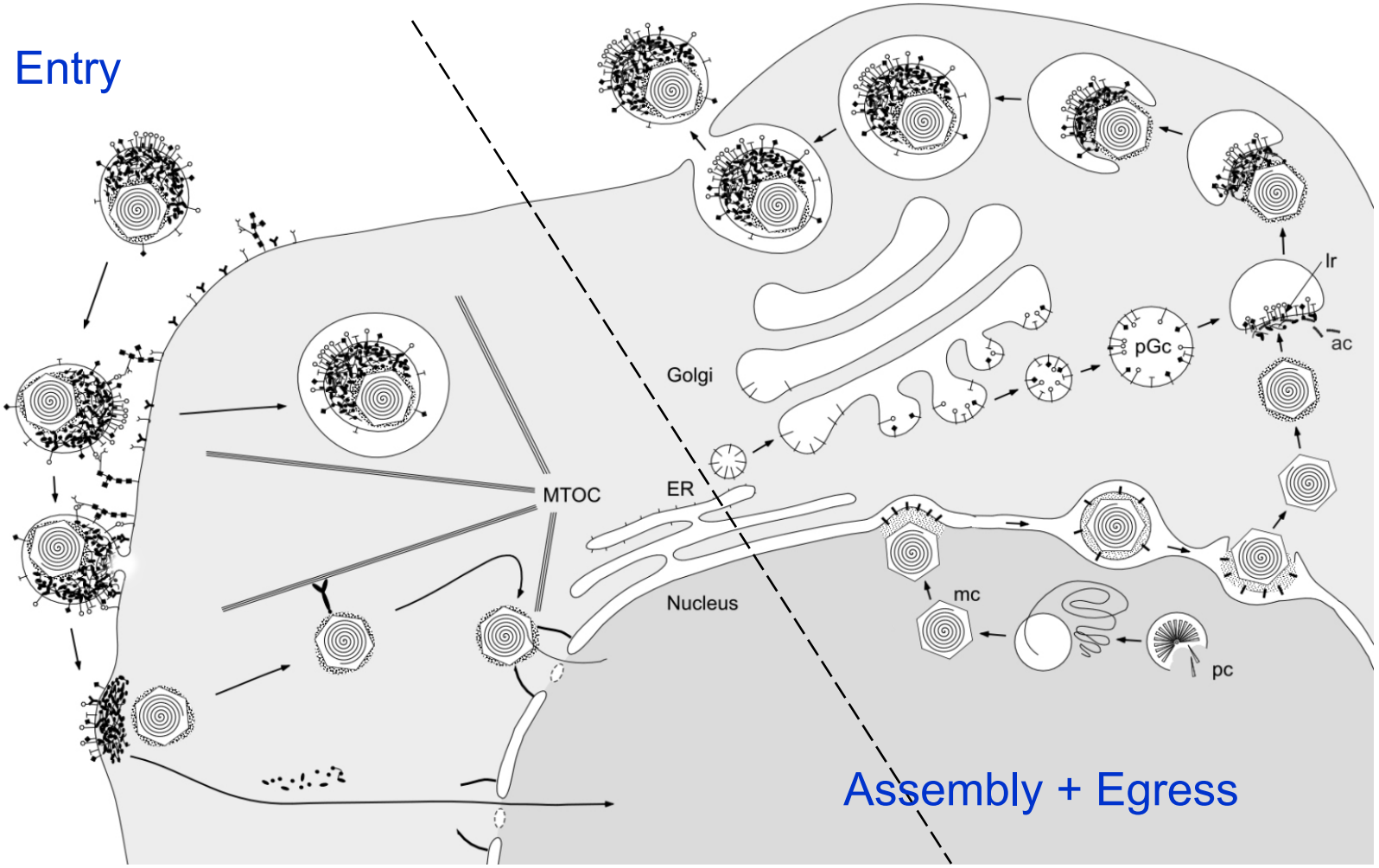
# *Structural cell biology of virus infection by cryoET*



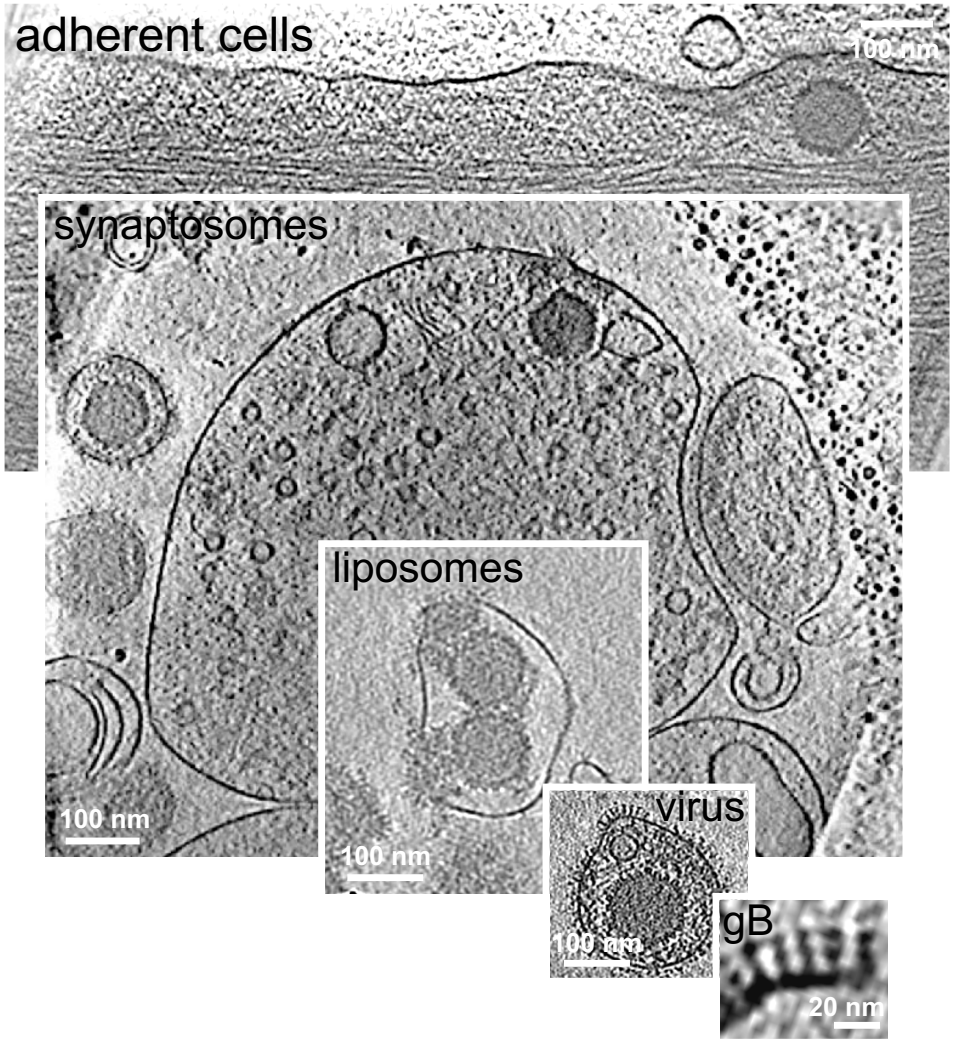
**Problem:**
non-invasive labelling techniques for intact cell lacking

**Our approach:**
virus as probe, structural signature allows recognition inside the crowded cytosol

# *Herpes simplex virus 1 'life cycle'*



# *Dedicated experimental sub-systems to address different levels of complexity*




Complexity & nativeness

interaction with adherent cells

entry into parts of cells

fusion with liposomes

isolated virions

recombinant fusion proteins

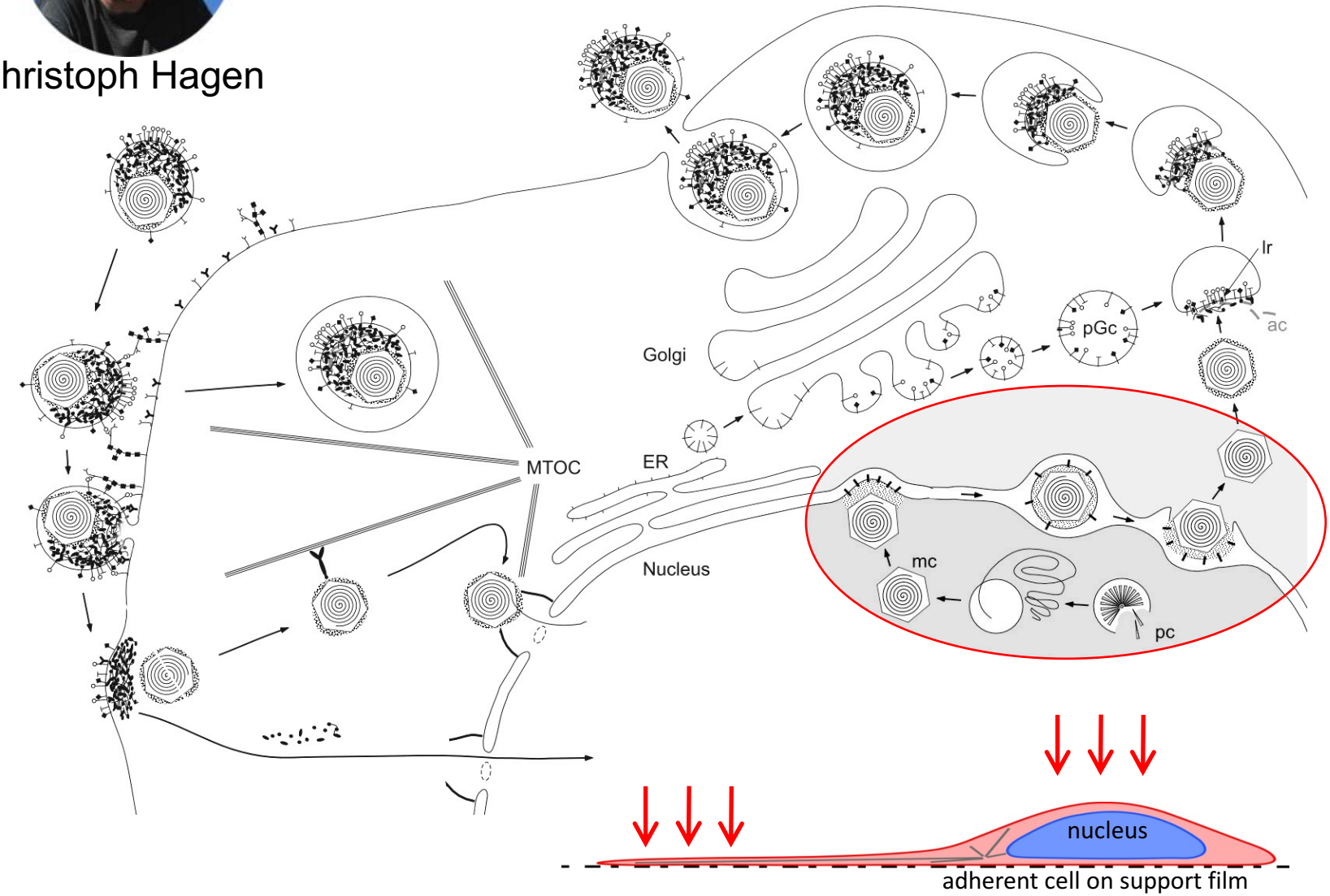
Possible attainable resolution

... towards ‘molecular gymnastics‘ of glycoproteins

Zeev-Ben-Mordehai *et al.* (2014) *Curr. Opp. Virol.* 5: 42–4

Christoph Hagen

# *Nuclear egress of herpesvirus capsids*

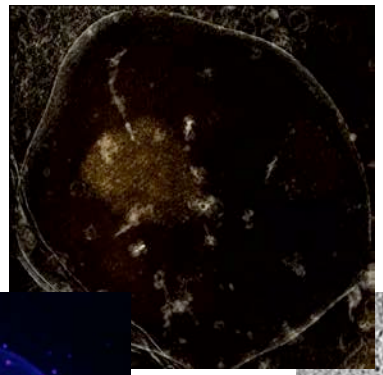


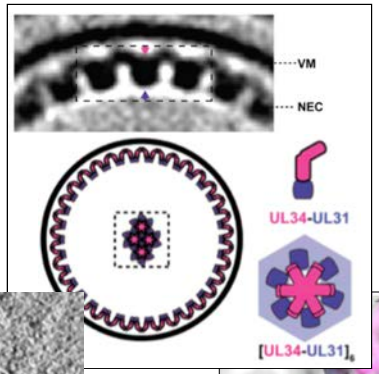
# Increasing biological complexity and integrity

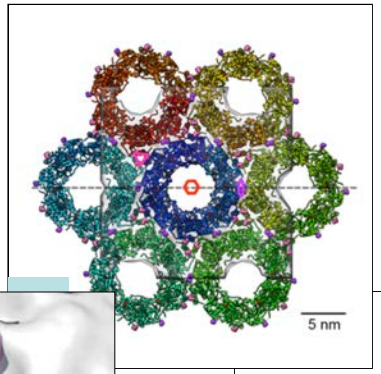
Soft X-ray microscopy

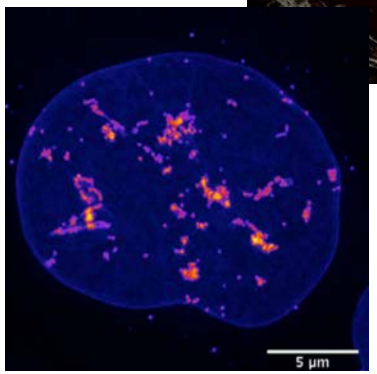
Sub-volume avg./model

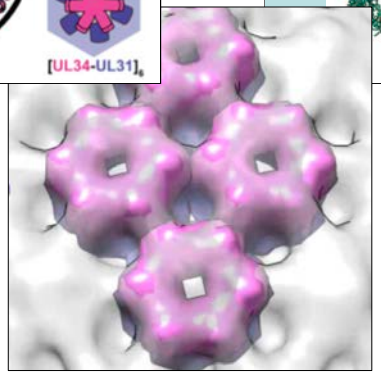
Curved lattice fit

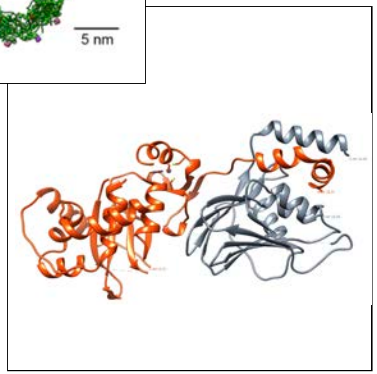






Fluoresc. microsc.
(life-cell SIM, cryoSMLM)

Cellular cryoET
(FIB-SEM prepared)

Fitted SAXS model

Crystallography
$U_L31/34$ heterodimer

Increasing resolution

µm

nm

Å