Challenges in Cellular Structure Determination

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EMBL

Challenges in Cellular Structure Determination

Syllabus from Bridget/Ron/Clint.

Including results/ideas from many of you.

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EMBL

In situ or in vitro?

Complex systems
Components in situ

Simple systems
Purified components in vitro

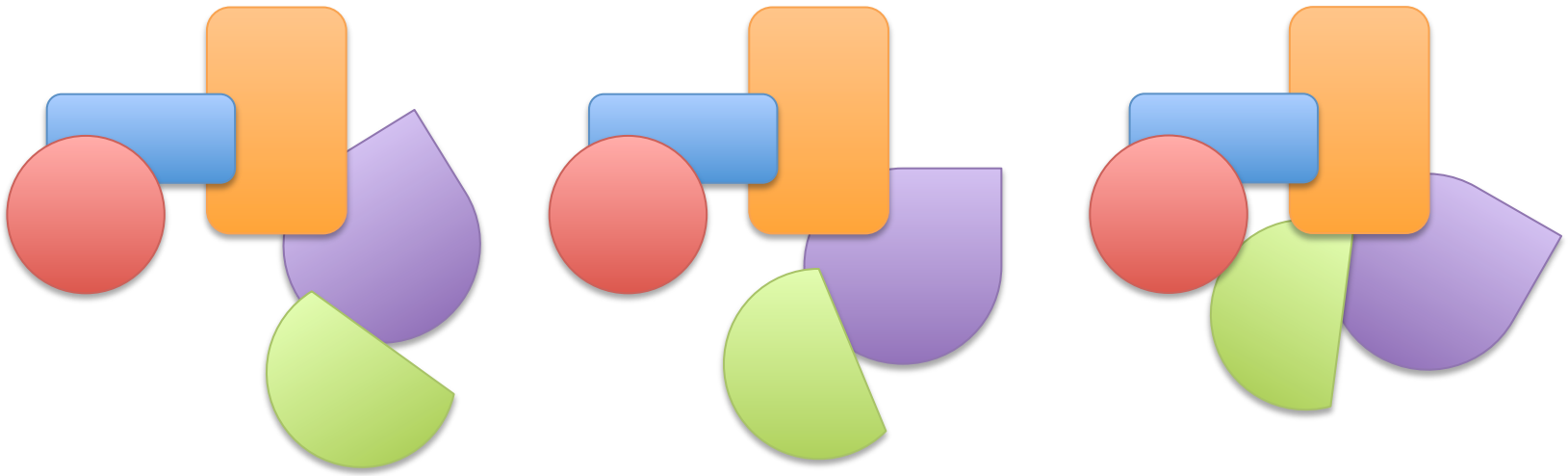
Low-resolution “blobs”

High-resolution detail

Challenges in Cellular Structure Determination

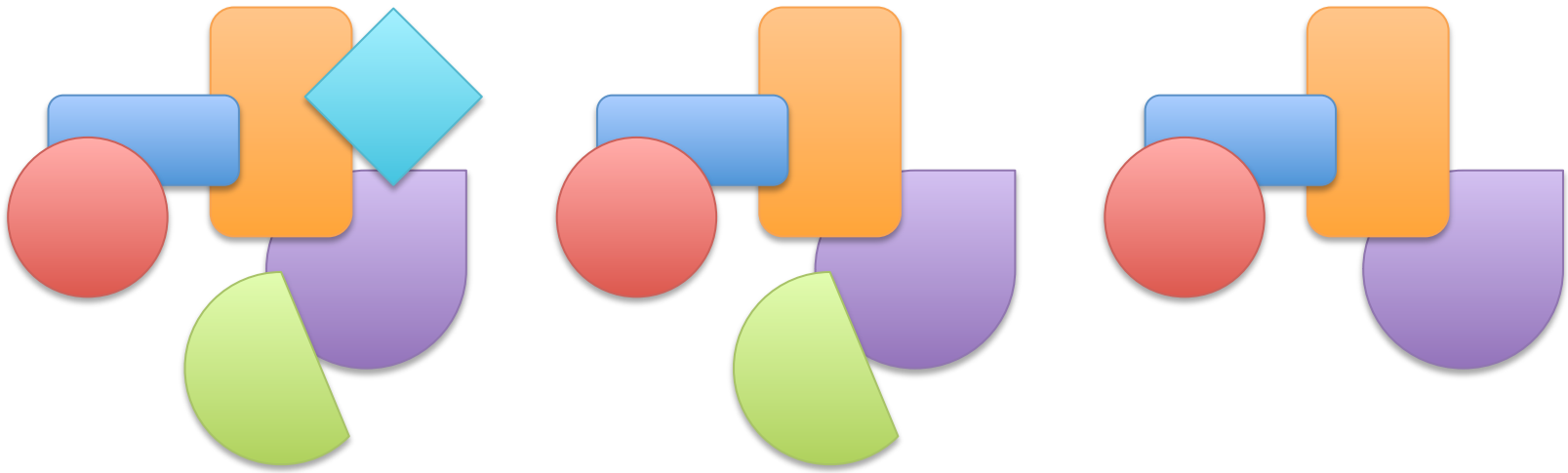
1. Why do it?

Multidimensional structures



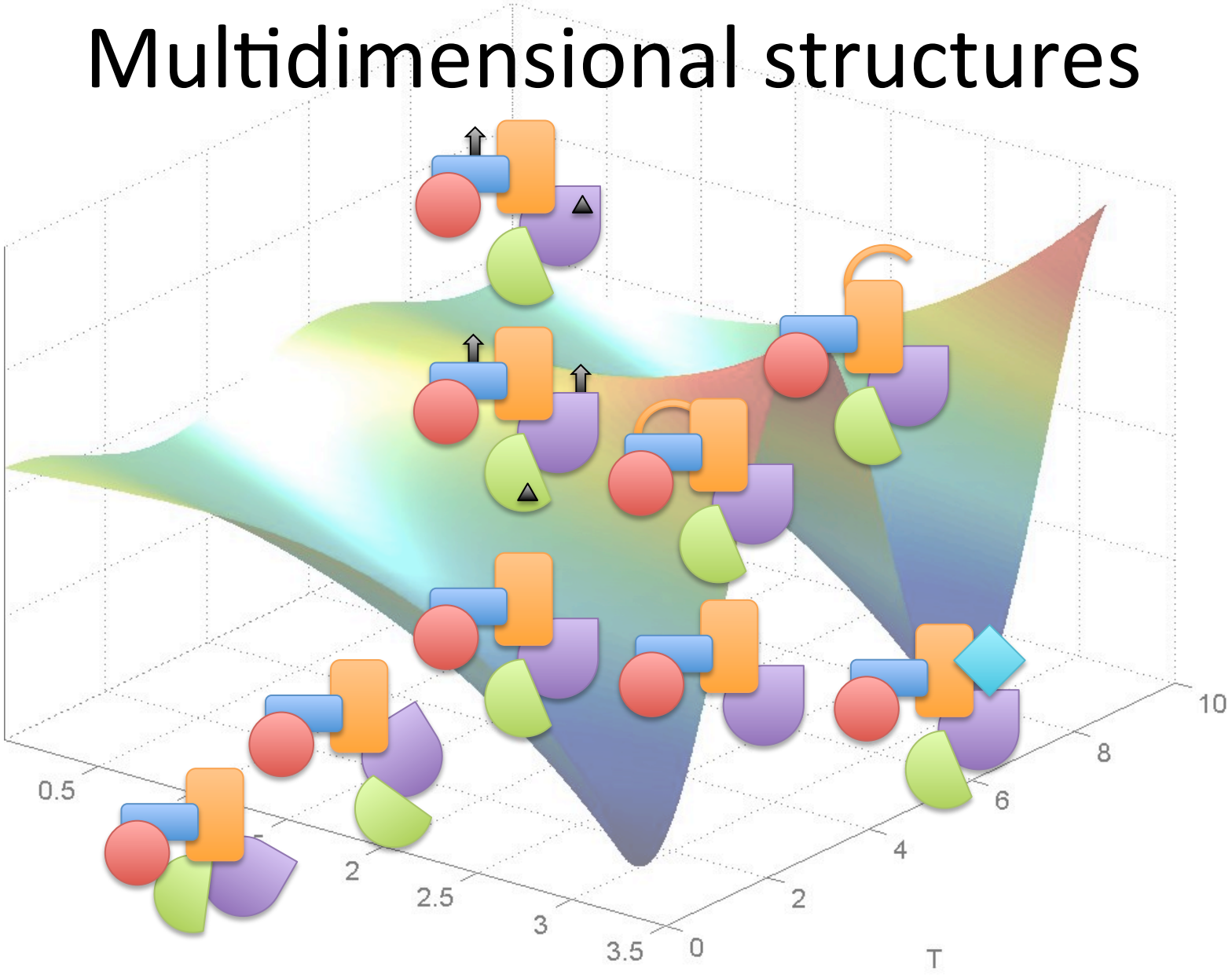
Complex may flex between many different conformations

Multidimensional structures



Complex may have many different compositions, depending on which sub-components are bound

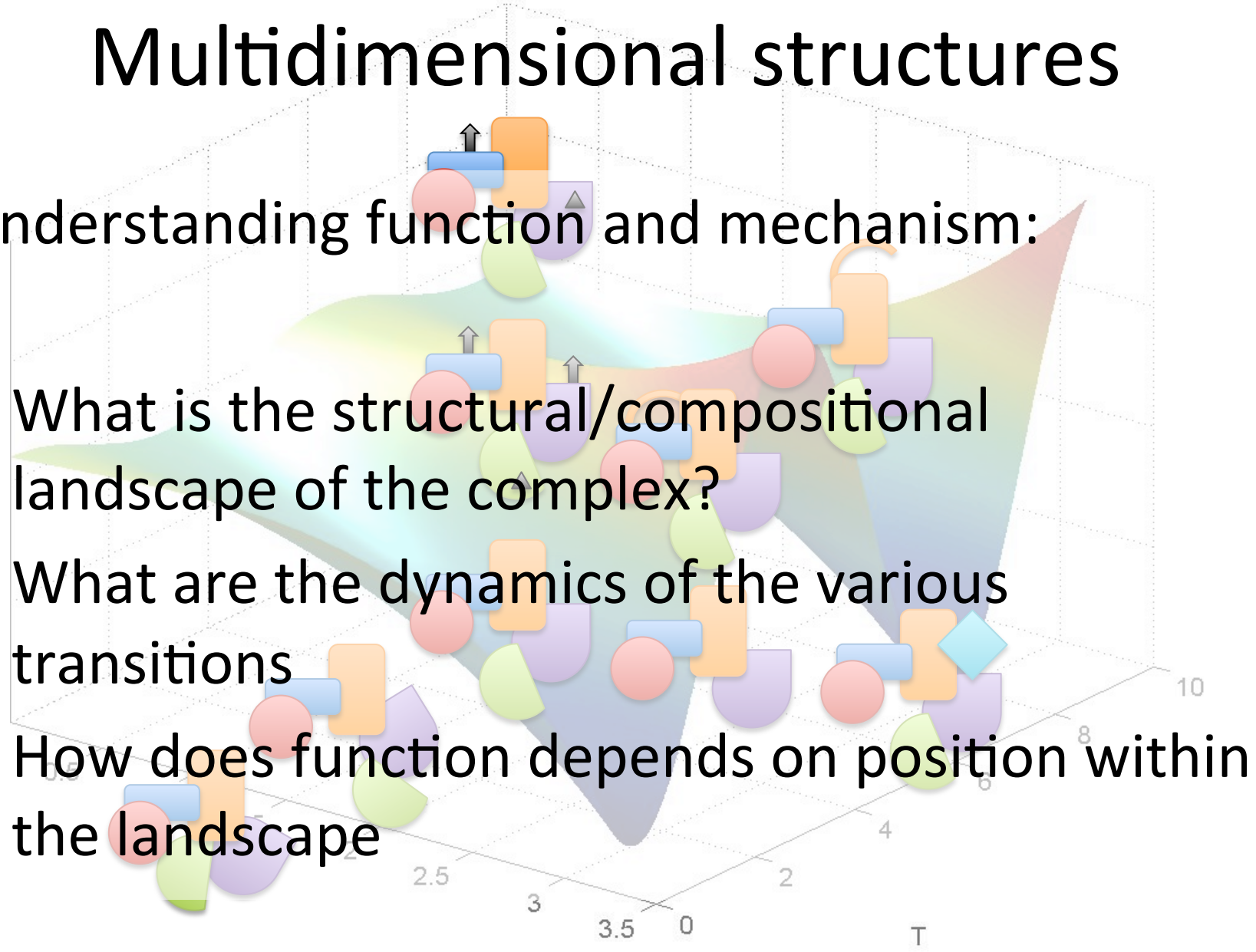
Multidimensional structures



Multidimensional structures

Understanding function and mechanism:

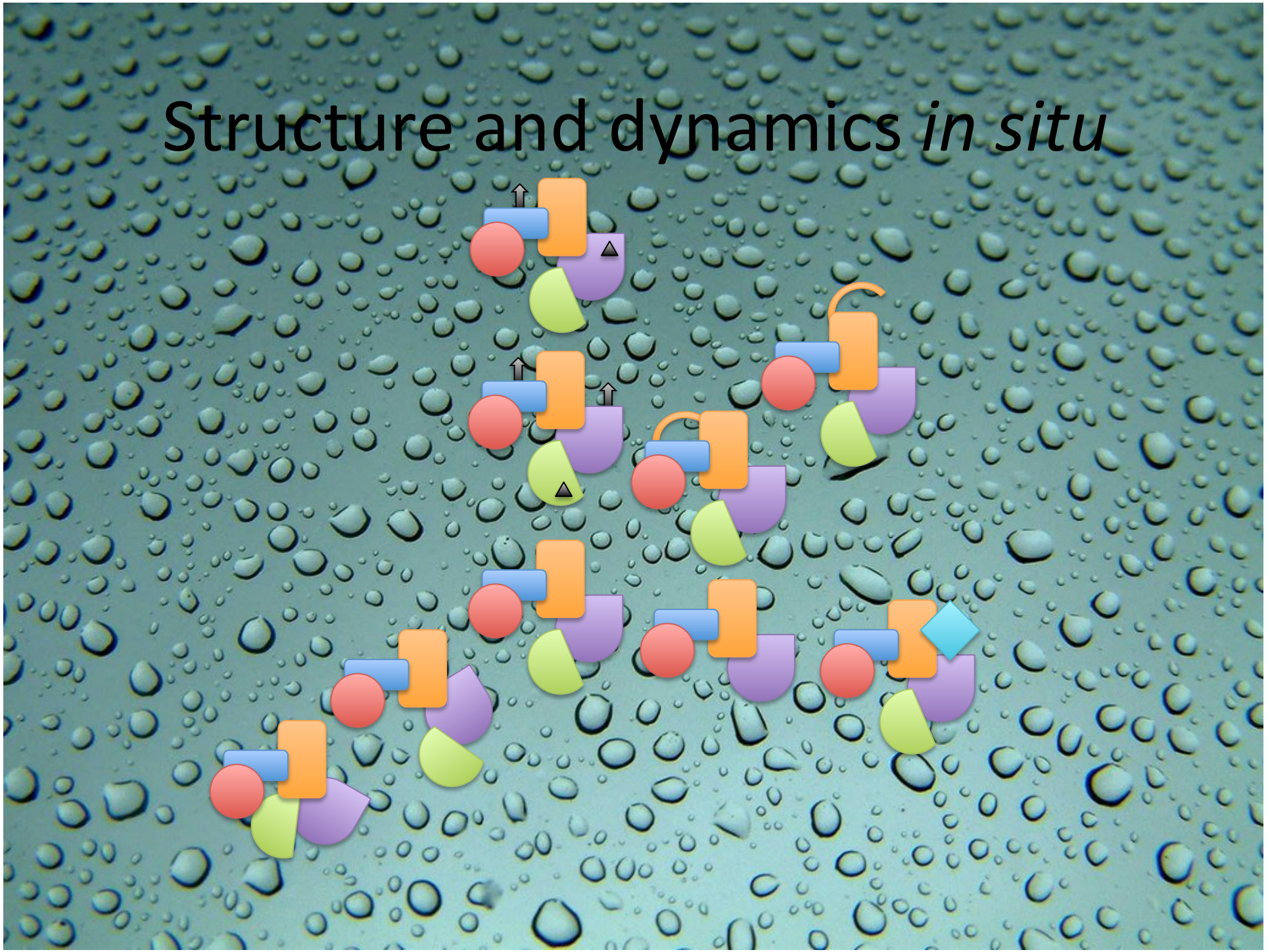
- What is the structural/compositional landscape of the complex?
- What are the dynamics of the various transitions
- How does function depends on position within the landscape



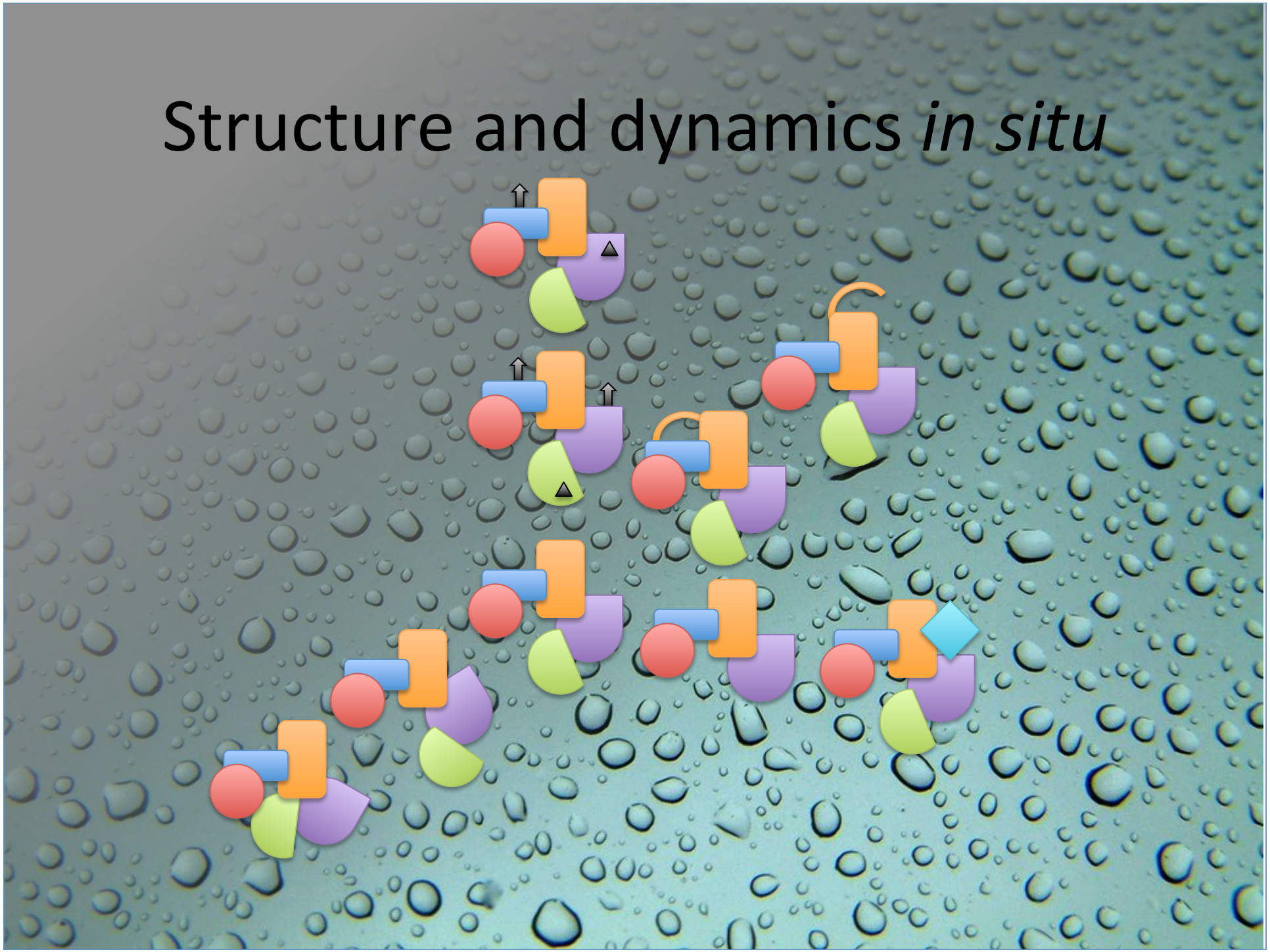
Multidimensional structure

Many of these questions can be addressed by single particle approaches, combined with functional/dynamic assays. But…

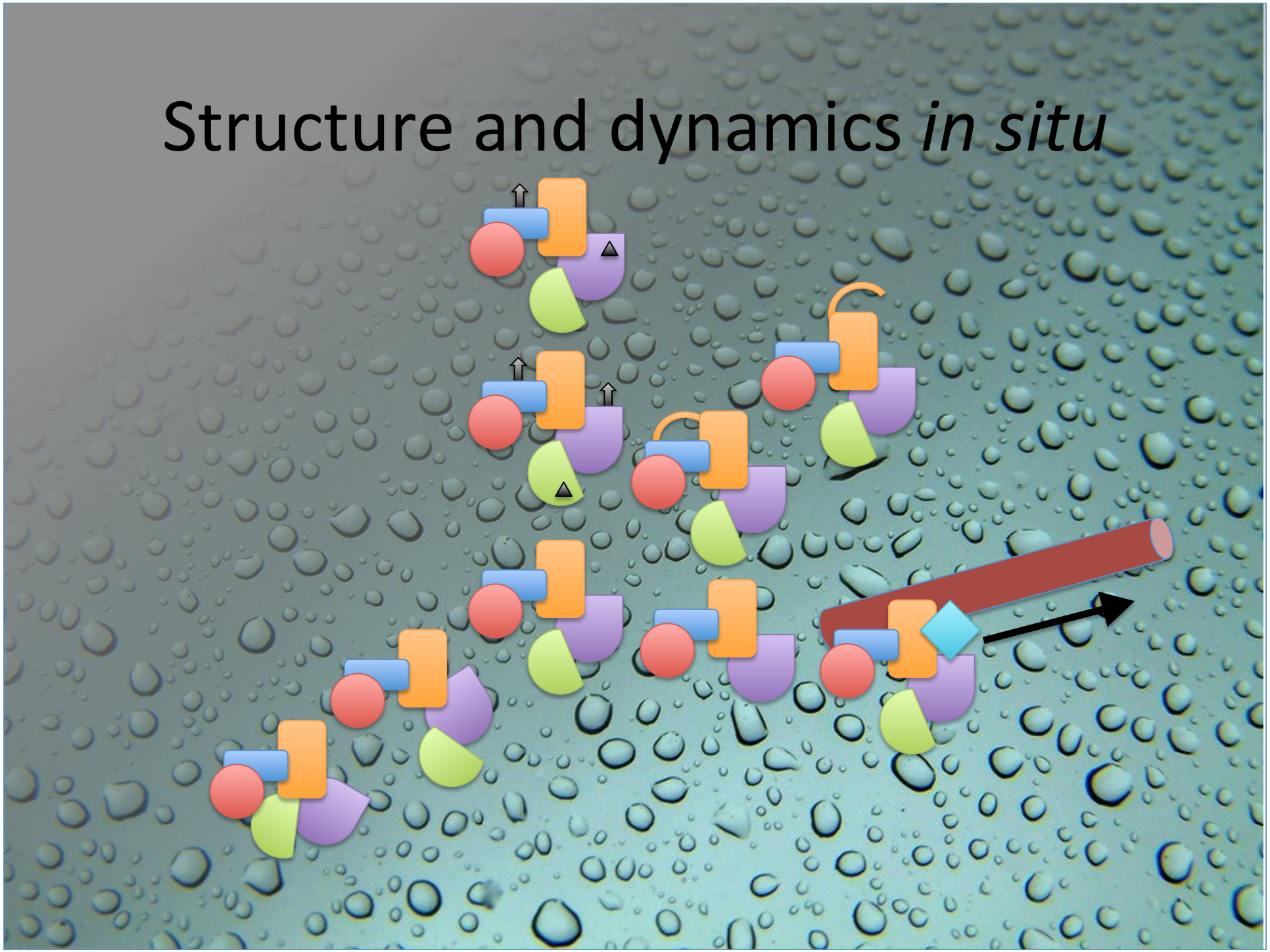
Structure and dynamics *in situ*



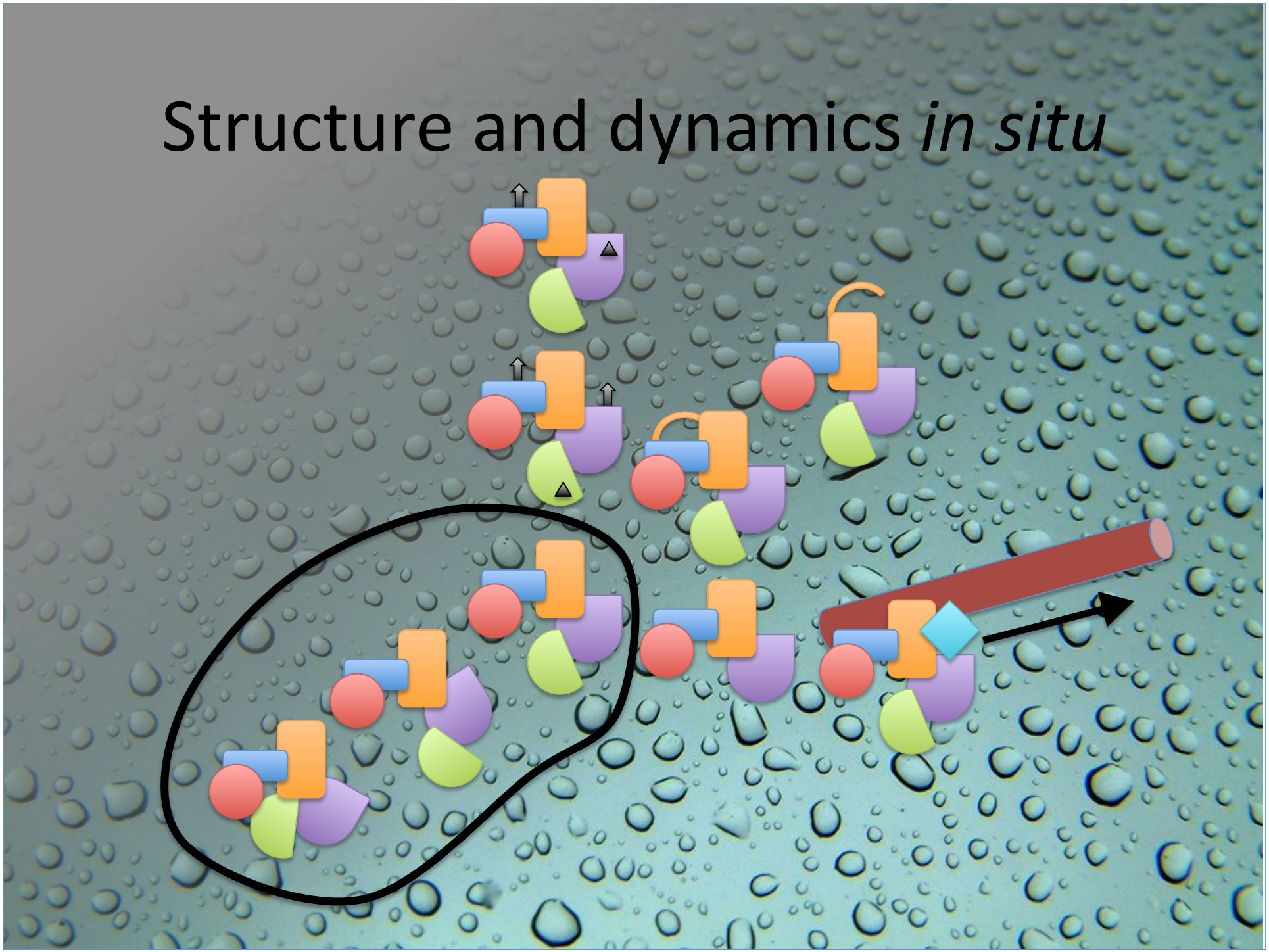
Structure and dynamics *in situ*



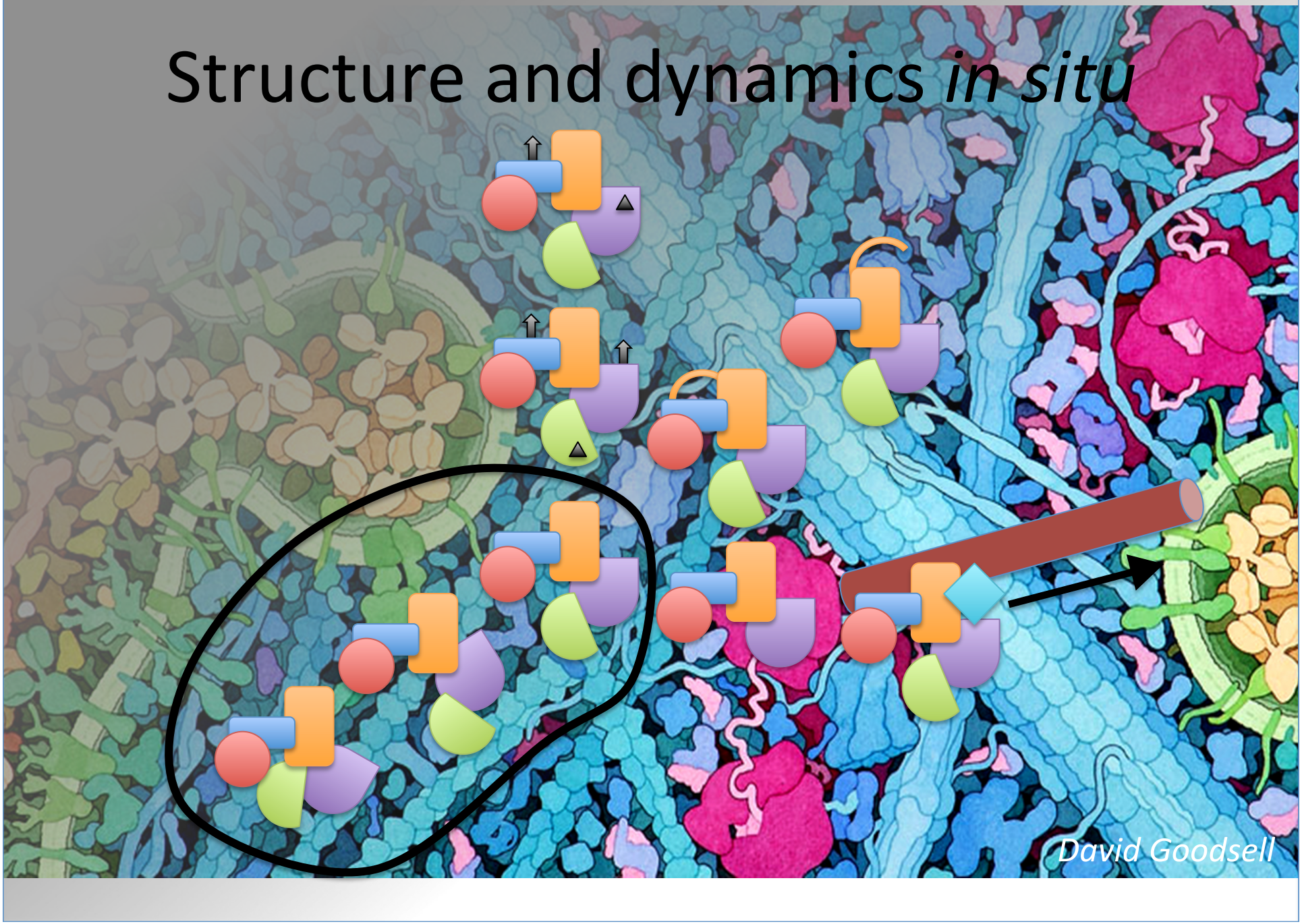
Structure and dynamics *in situ*



Structure and dynamics *in situ*



Structure and dynamics *in situ*



Structure and dynamics *in situ*

- The composition, conformation and dynamics of the complex may be different depending on the cellular environment

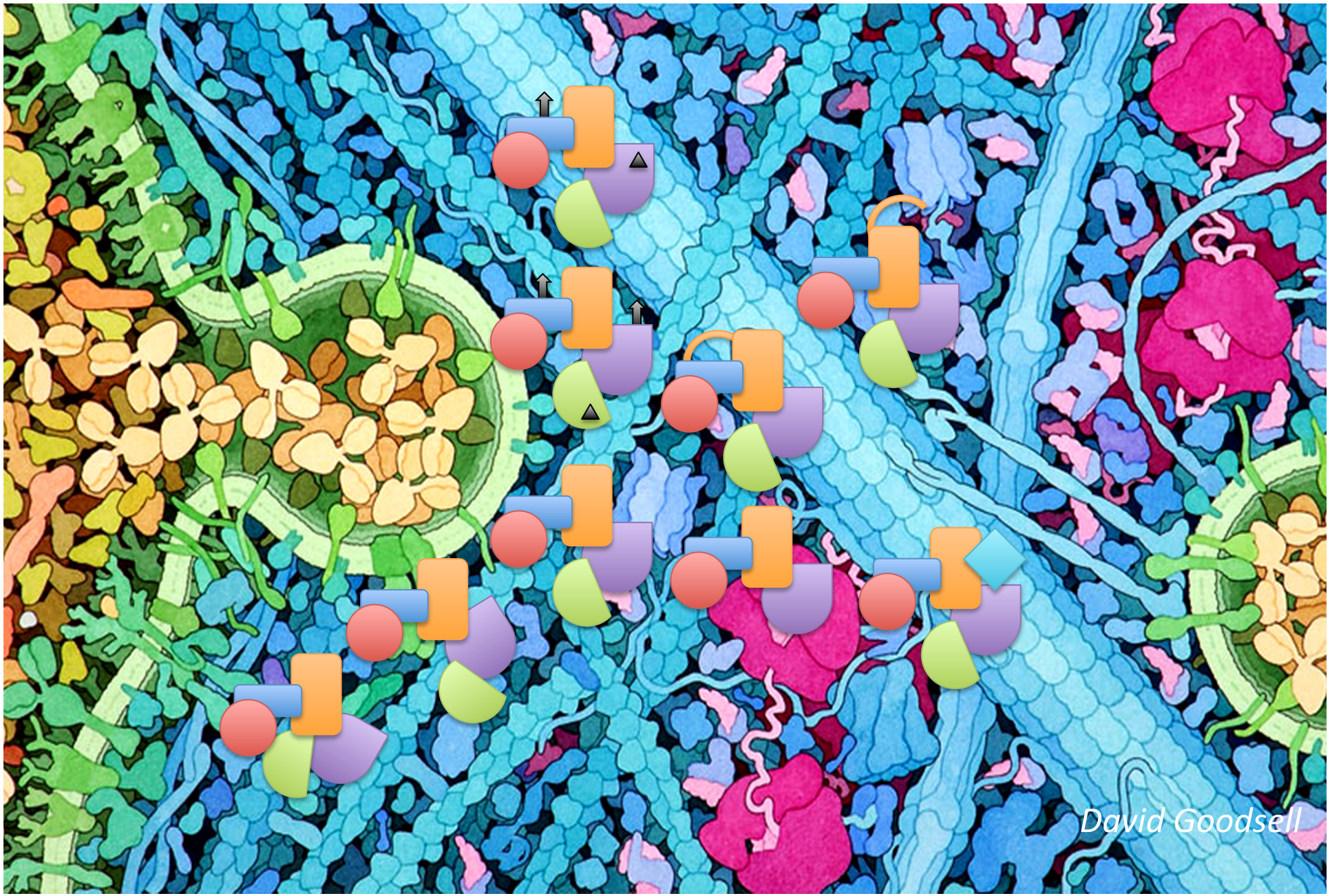
- Ideally we would determine structure within the cell

Structure and dynamics *in situ*

- For many protein complexes the cellular context is inseparable from function

- eg nuclear pore, endocytosis, electron transport chain

Investigate structure and dynamics in the cell

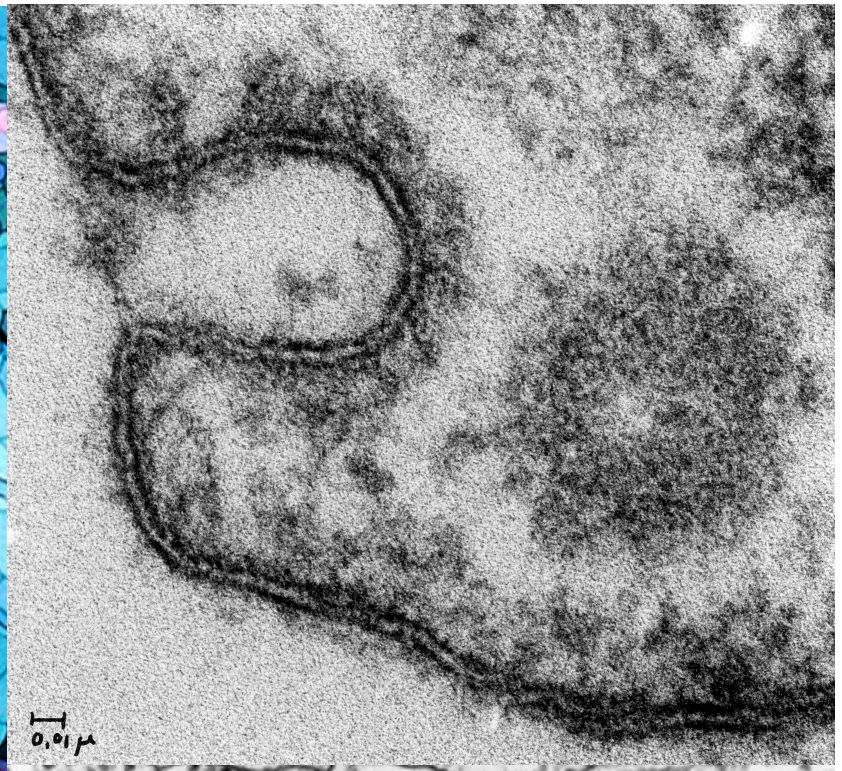


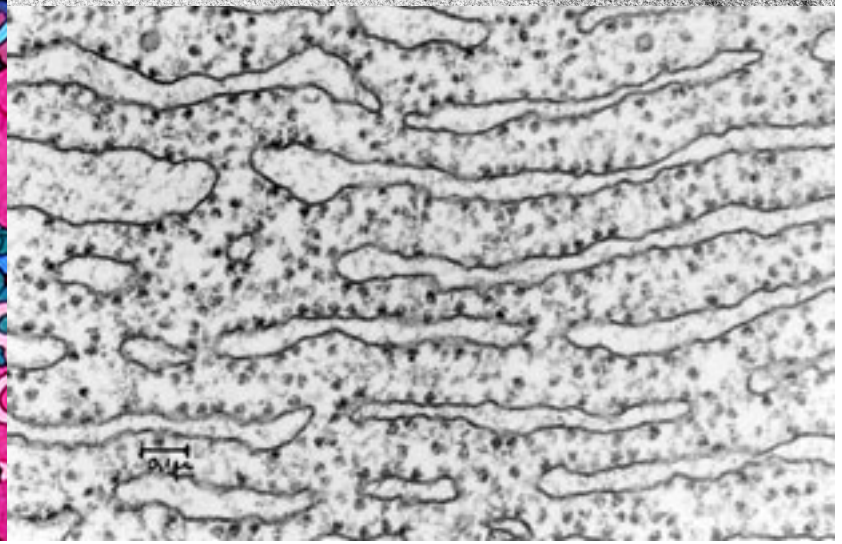
Investigate structure and dynamics in the cell

Challenges in Cellular Structure Determination

2. A brief intro to how it is done today

Cellular ultrastructure determination. 1960's Palade





How it is done today

Sample preparation

Data collection

Image processing

How it is done today

Sample preparation

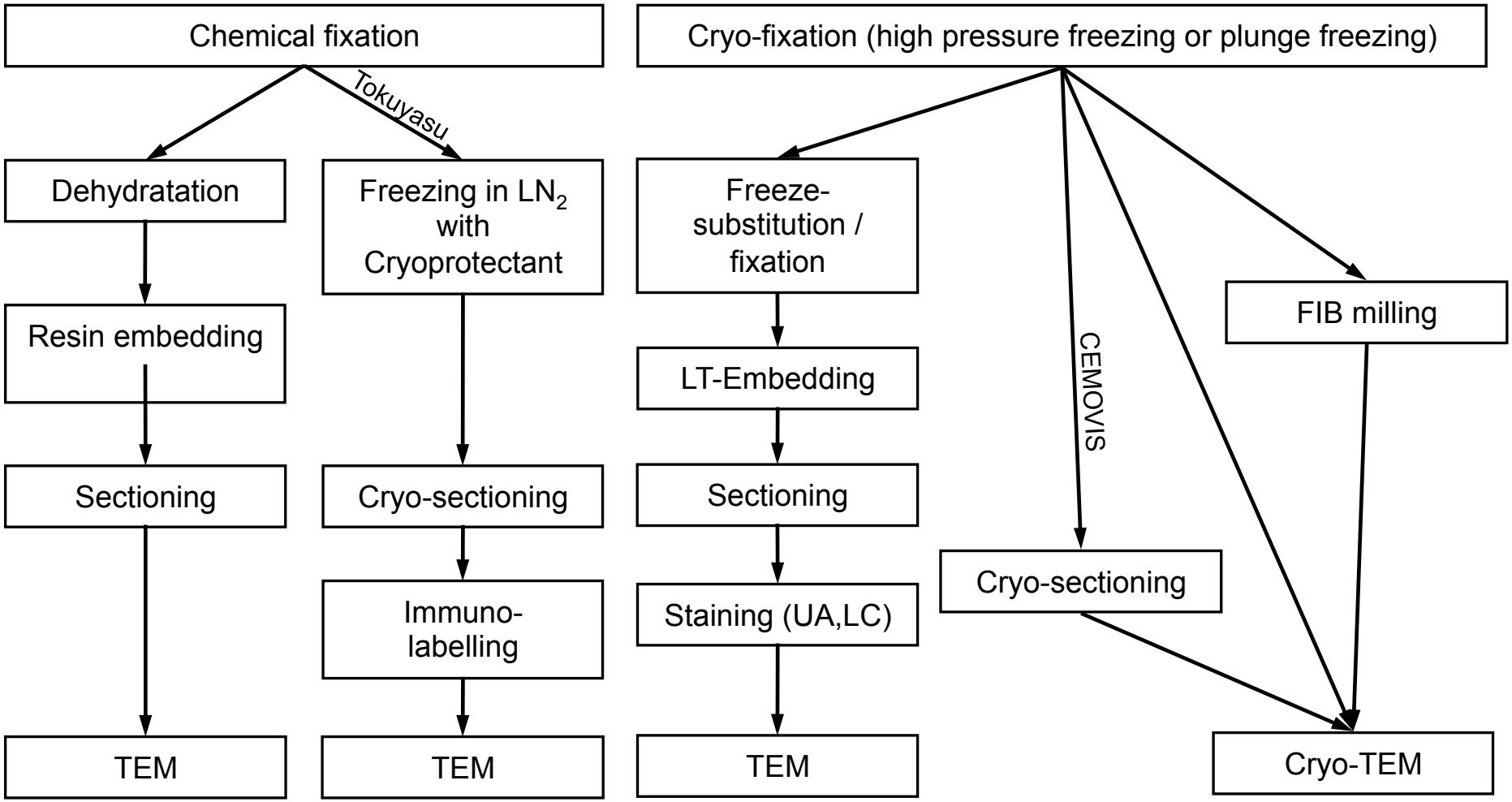
Data collection

Image processing

Sample Requirements…

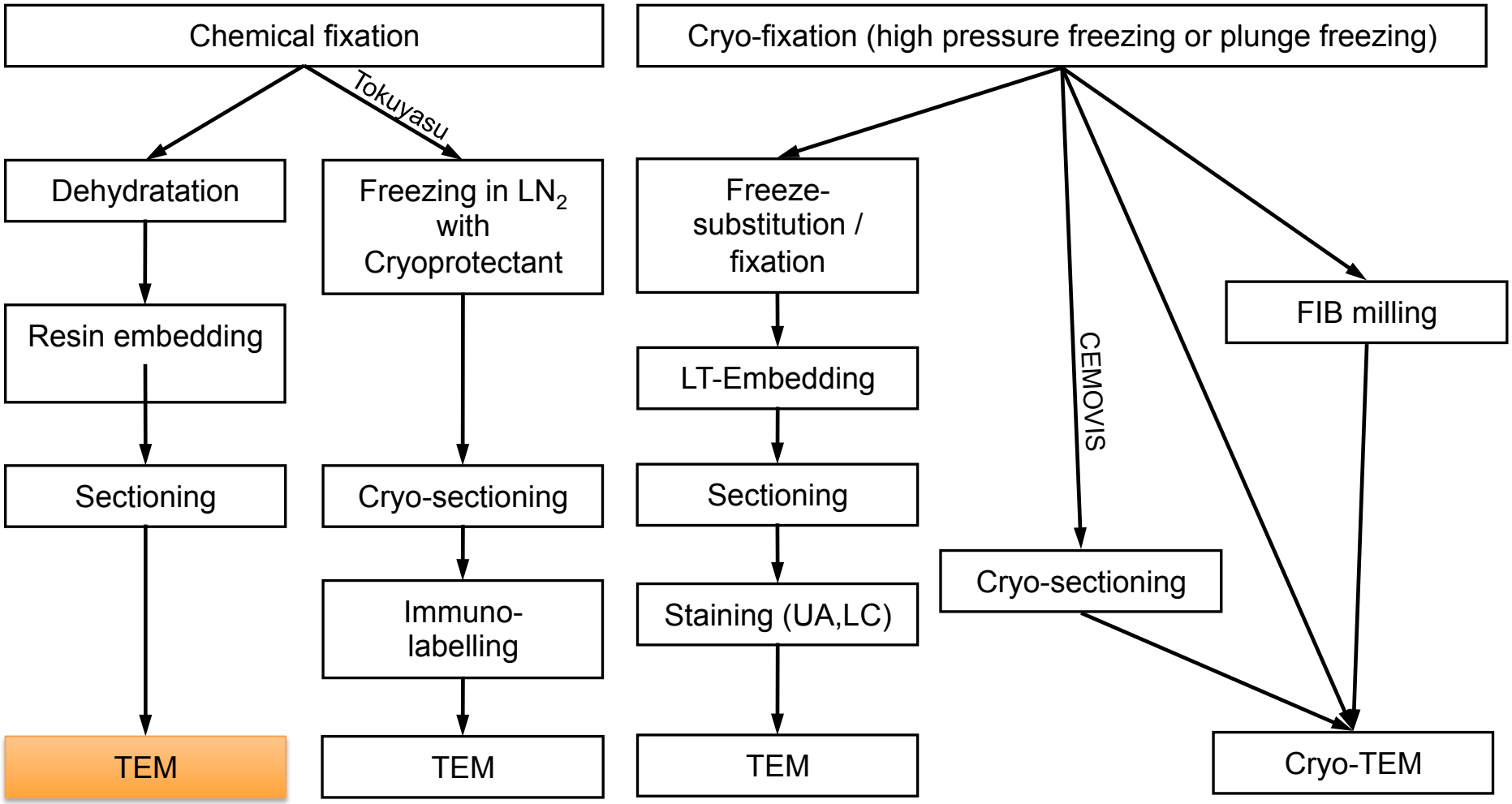
- Sample must preserve structure
- Sample must be thin
- Sample must give contrast
- Sample must be stable in vacuum

Cellular Sample preparation methods

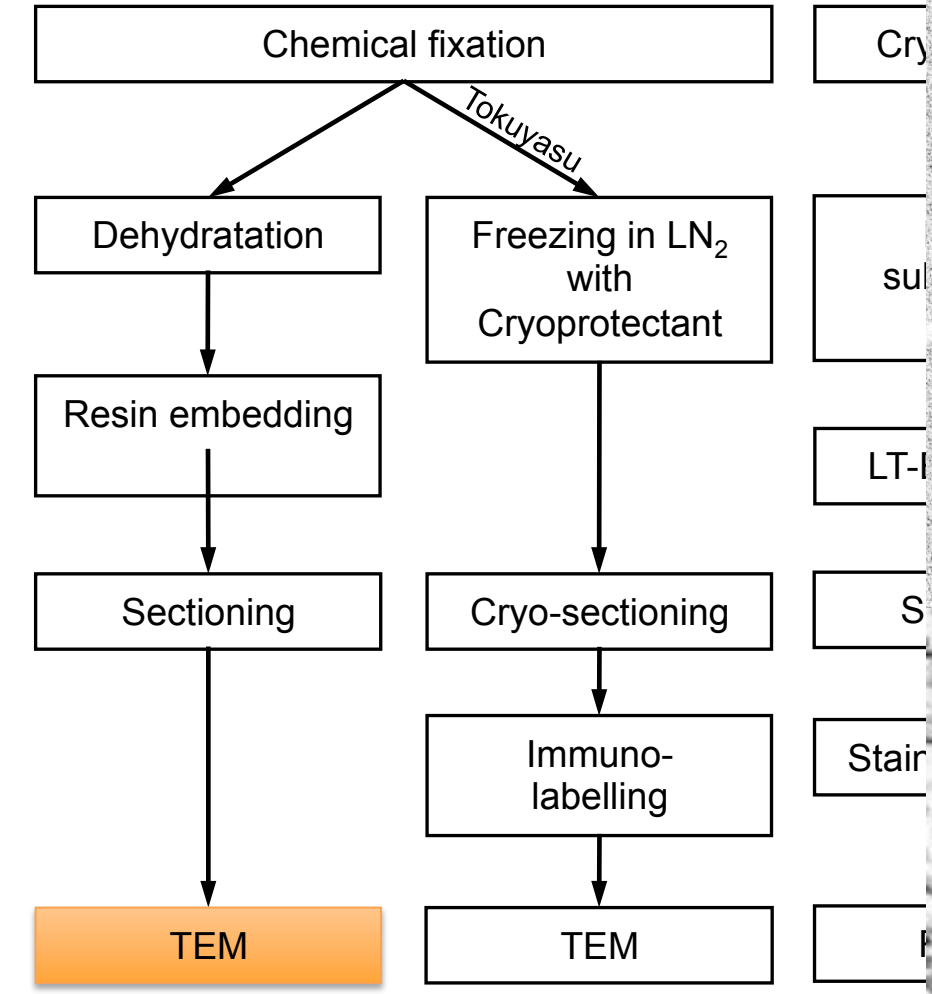


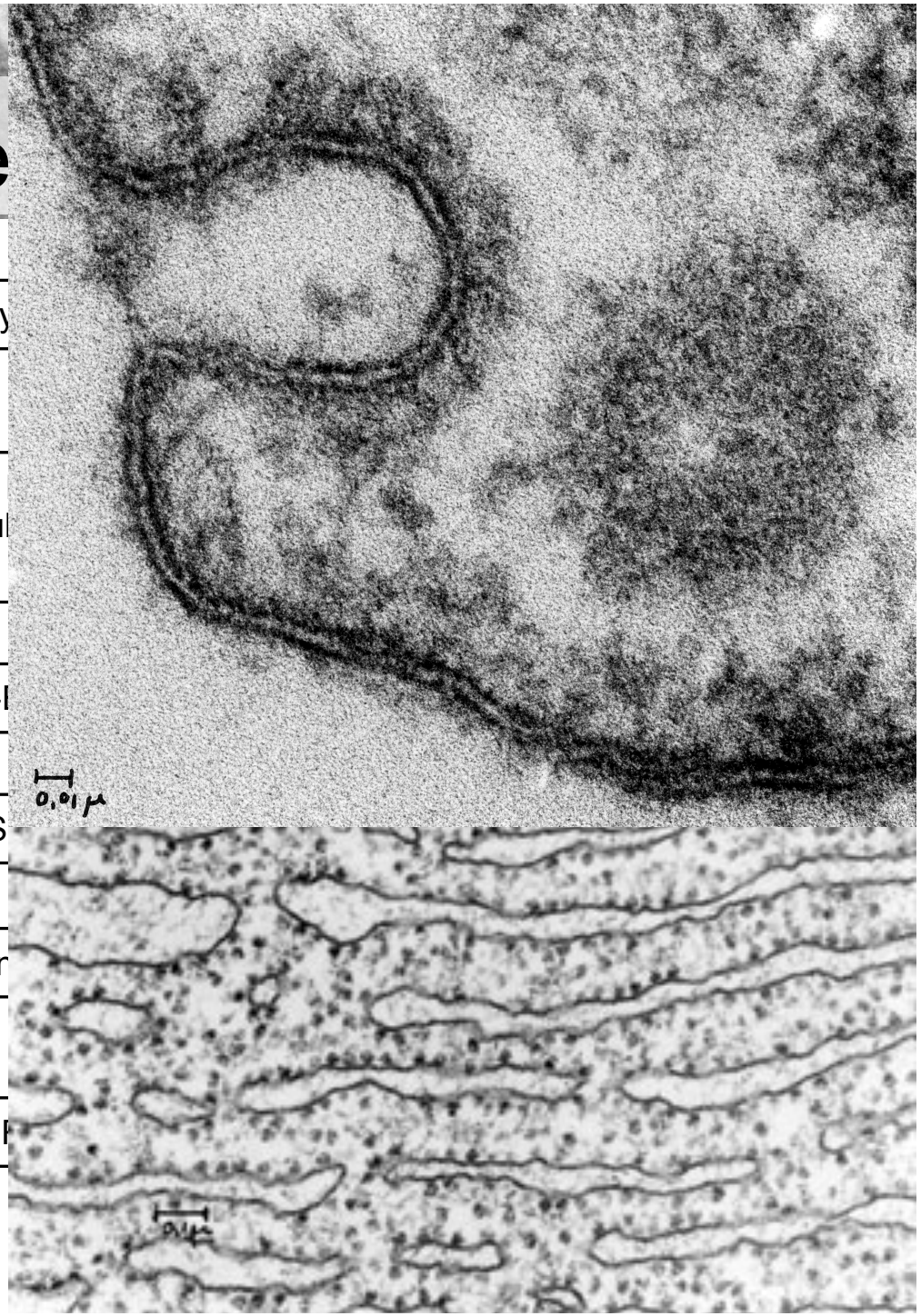
Claude Antony

Cellular Sample preparation methods

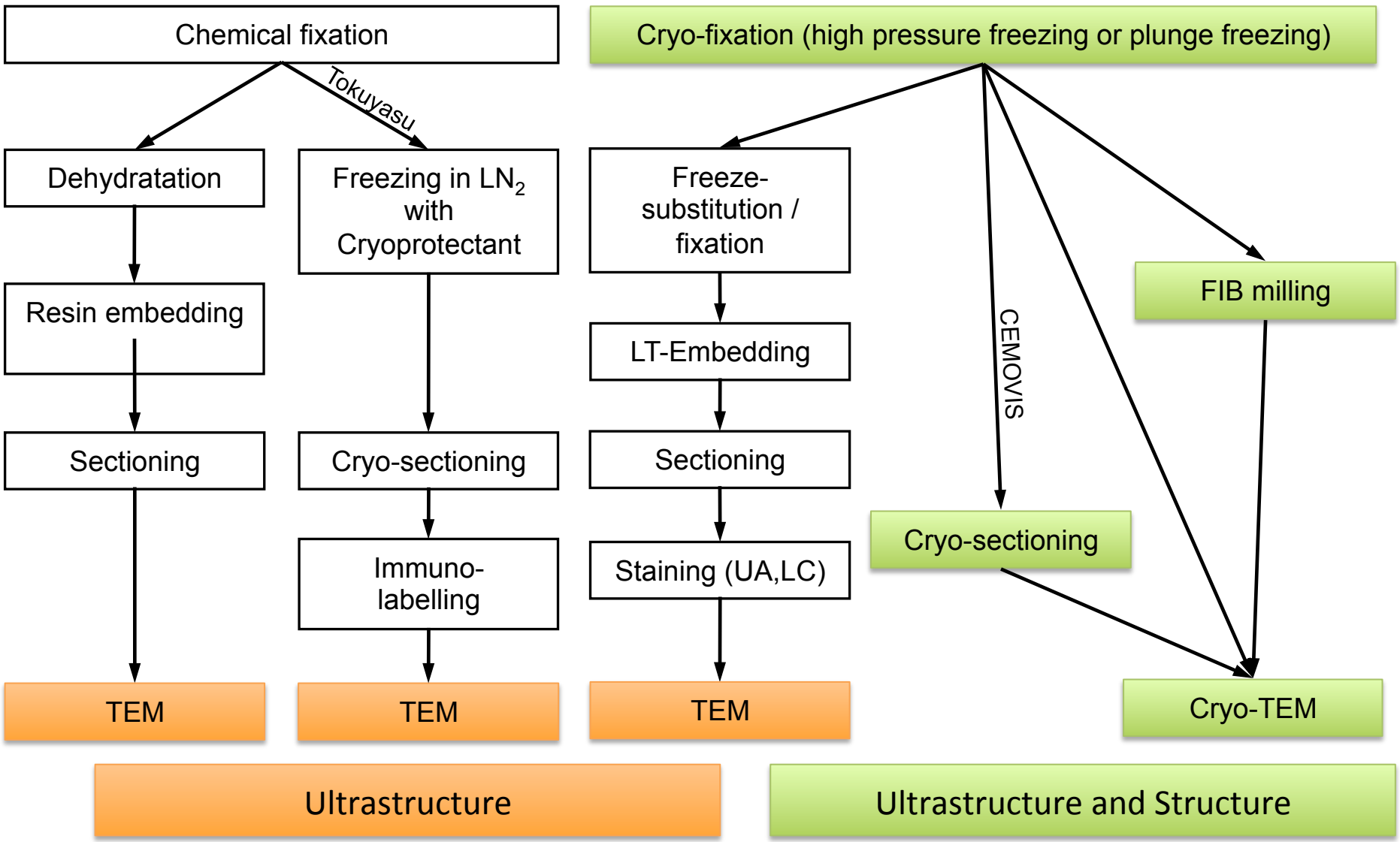


Cellular Sample pre

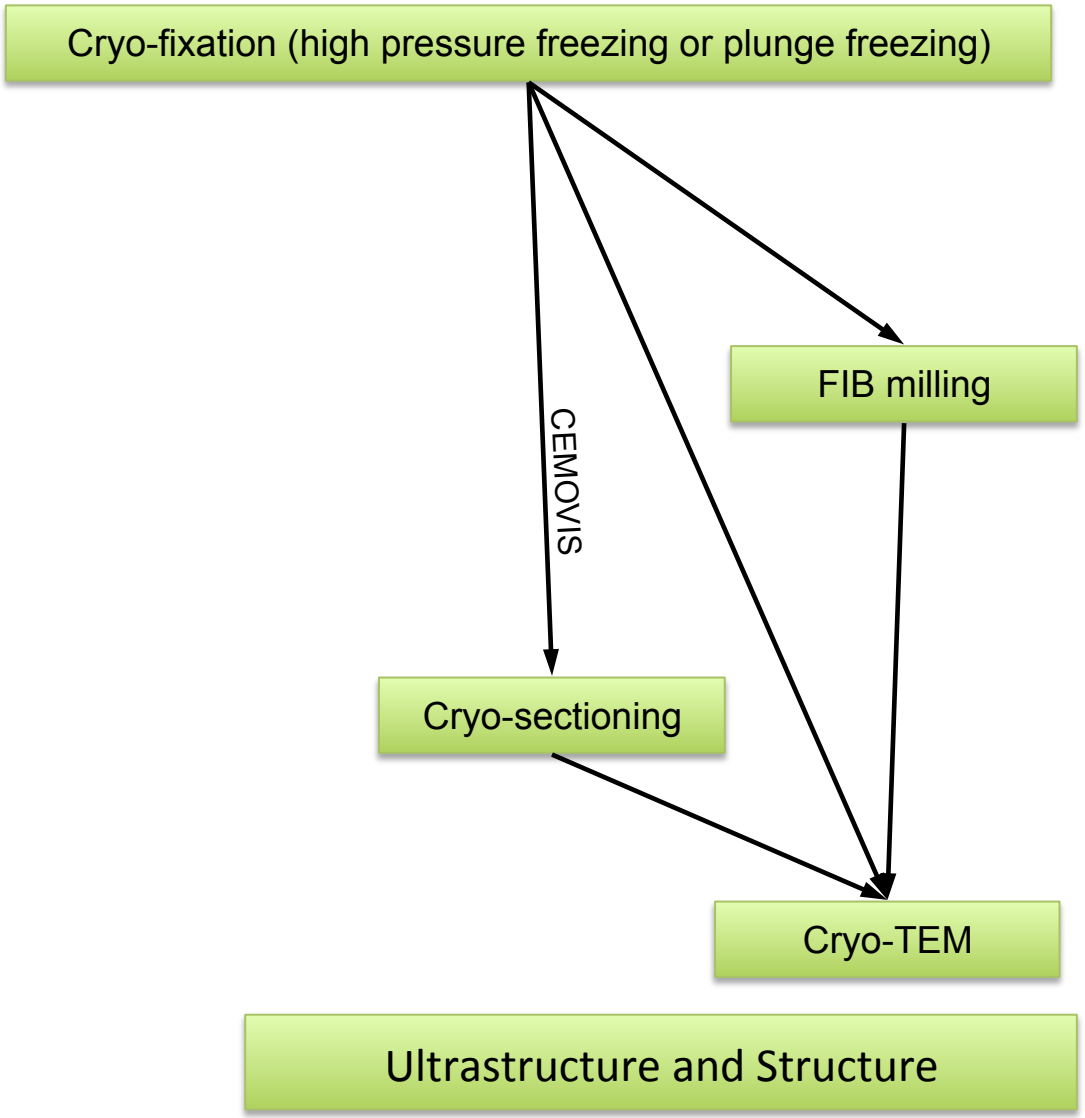




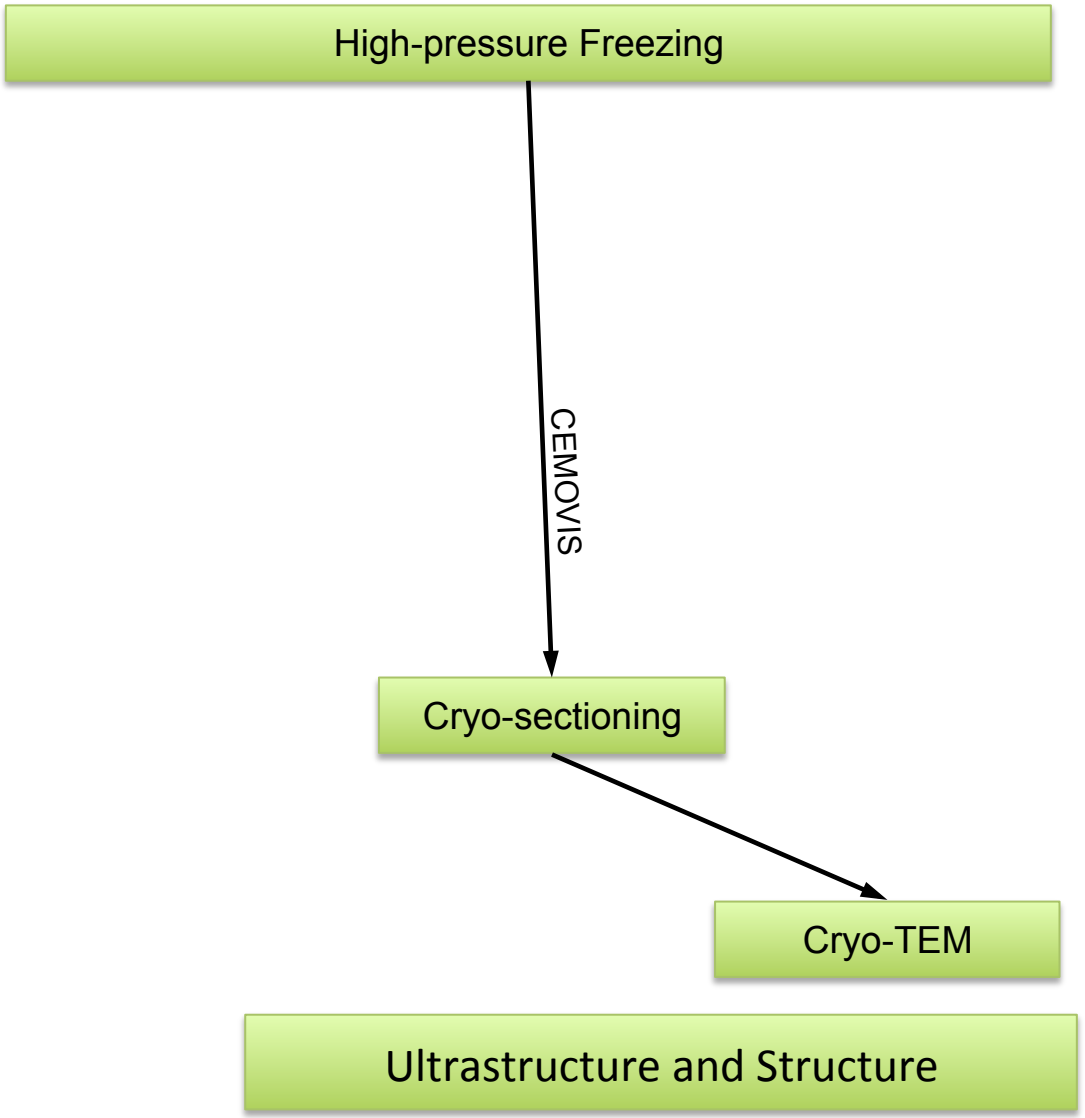
Ultrastructure or Structure?

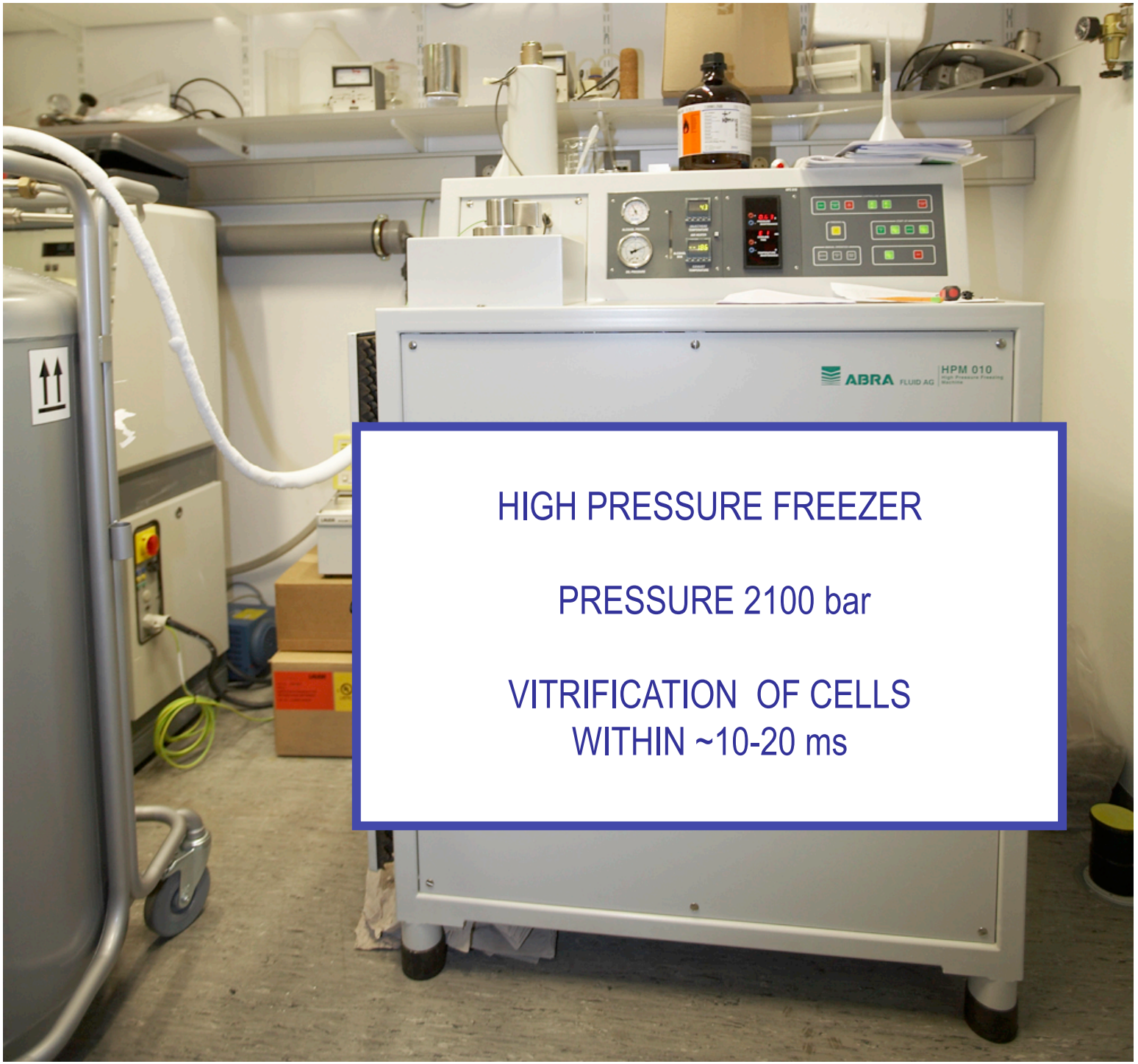


Structure


Cryo-fixation (high pressure freezing or plunge freezing)
FIB milling
CEMOVIS
Cryo-sectioning
Cryo-TEM
Ultrastructure and Structure

Structure


High-pressure Freezing
CEMOVIS
Cryo-sectioning
Cryo-TEM
Ultrastructure and Structure


ABRA FLUID AG
HPM 010
HIGH PRESSURE FREEZER
PRESSURE 2100 bar
VITRIFICATION OF CELLS
WITHIN ~10-20 ms


Leica
LEICA EM FC6
Leica
SPEED
FEED
STEP
Leica


27
30
33

CEMOVIS

High pressure freezing (HPF)

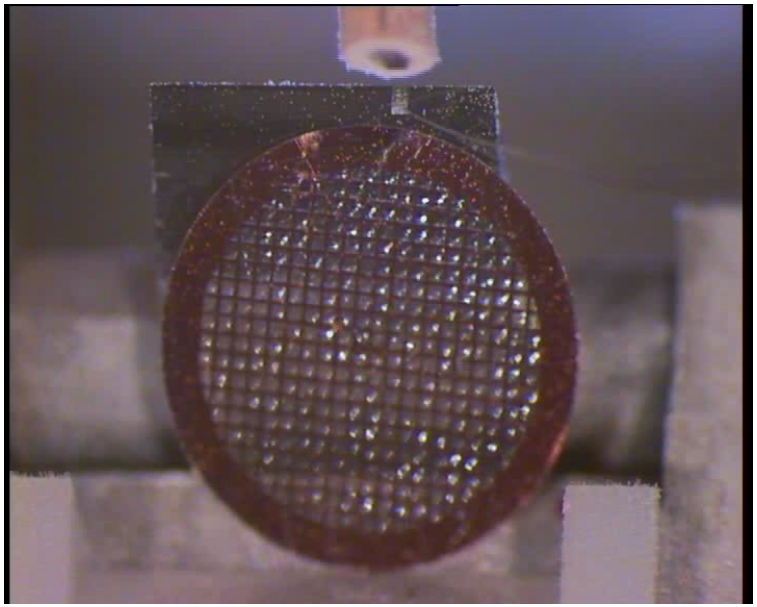
→ vitreous ice (no crystals)

- 2050 bar the freezing point of pure water drops to -22ºC
- Freezing rate 500ºC/s

HPF device

Cryo-sectioning

- 50-200 nm sections are cut at -(140-160ºC)

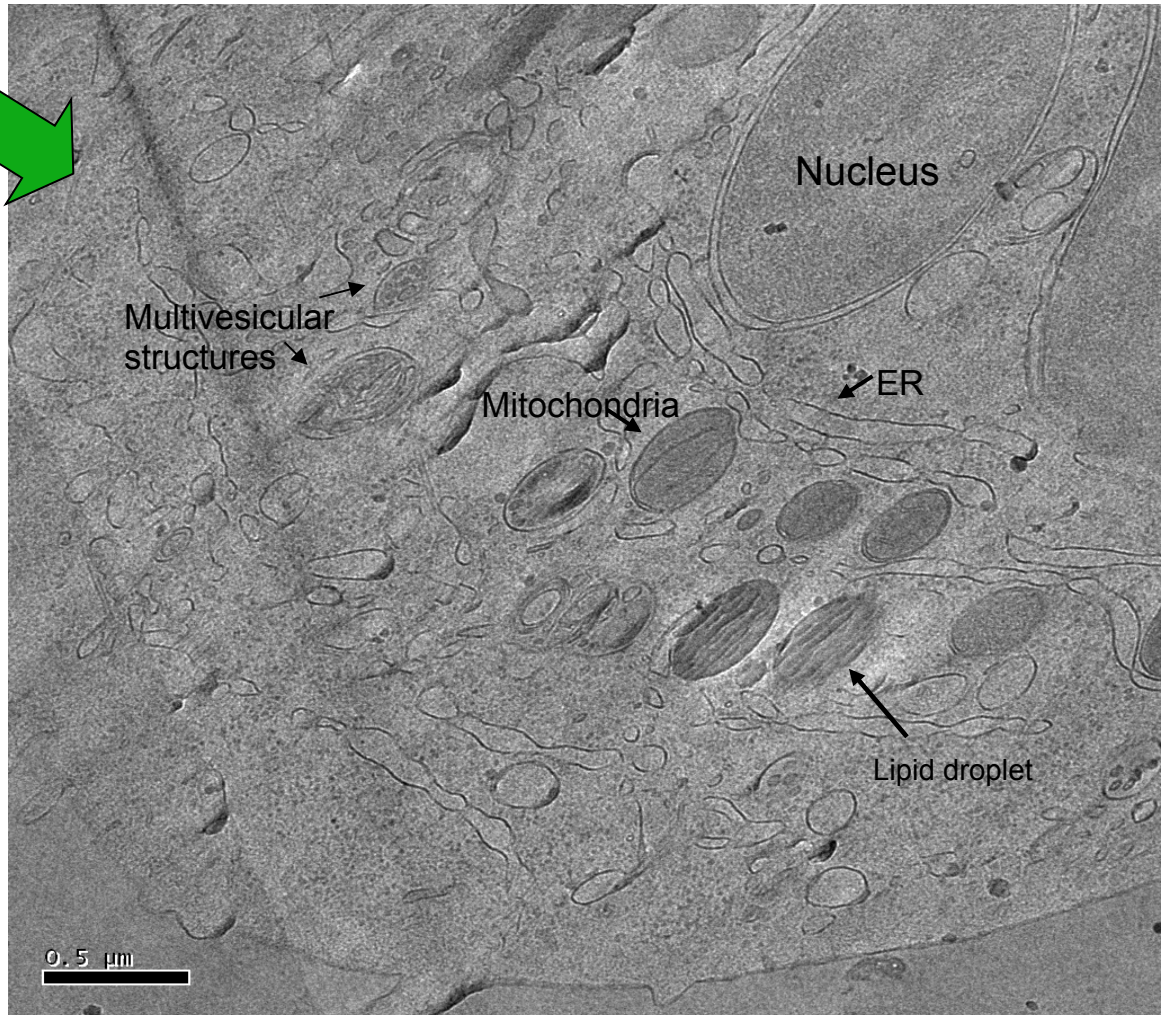


picture by Peter Peters

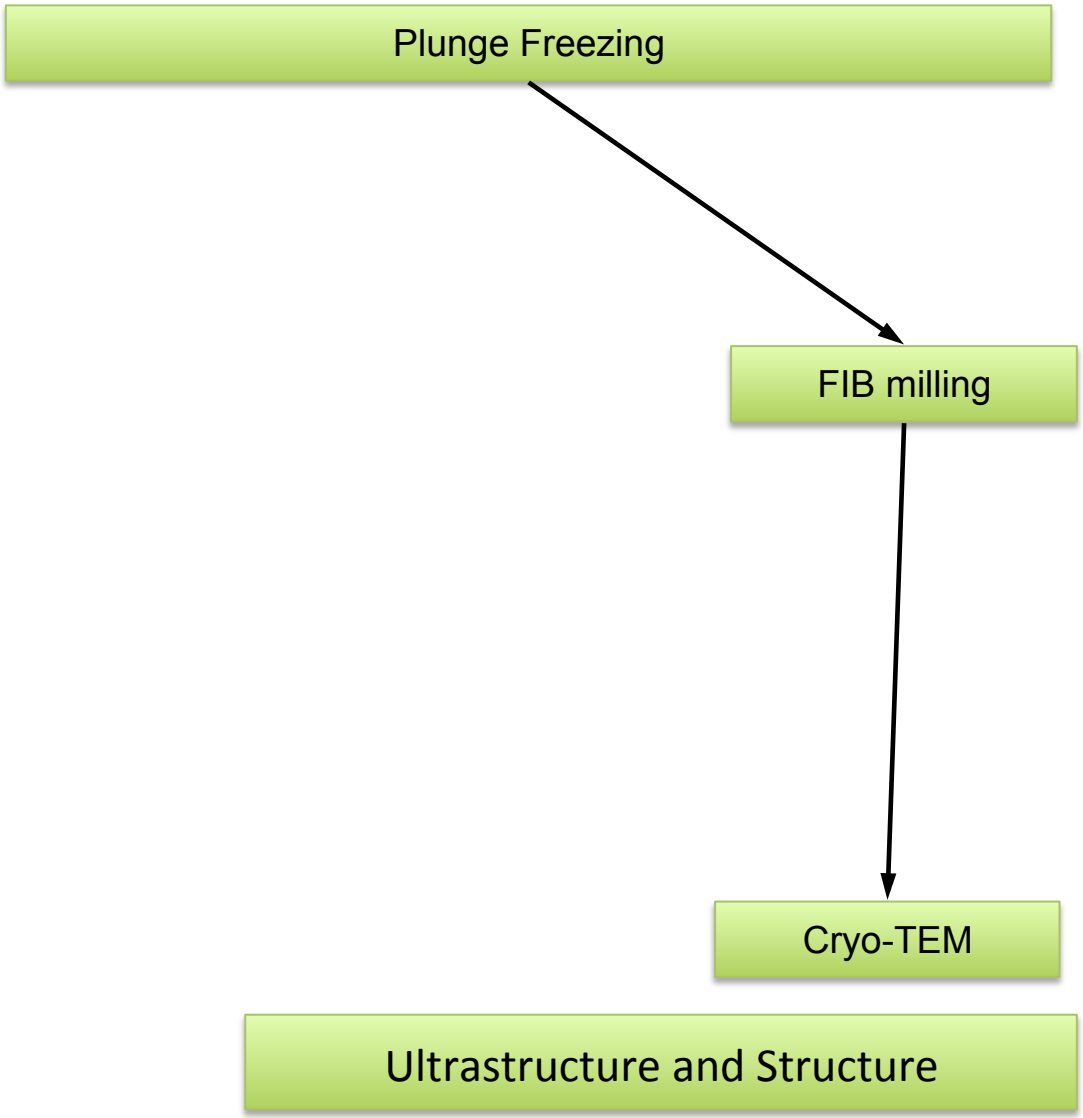
CEMOVIS

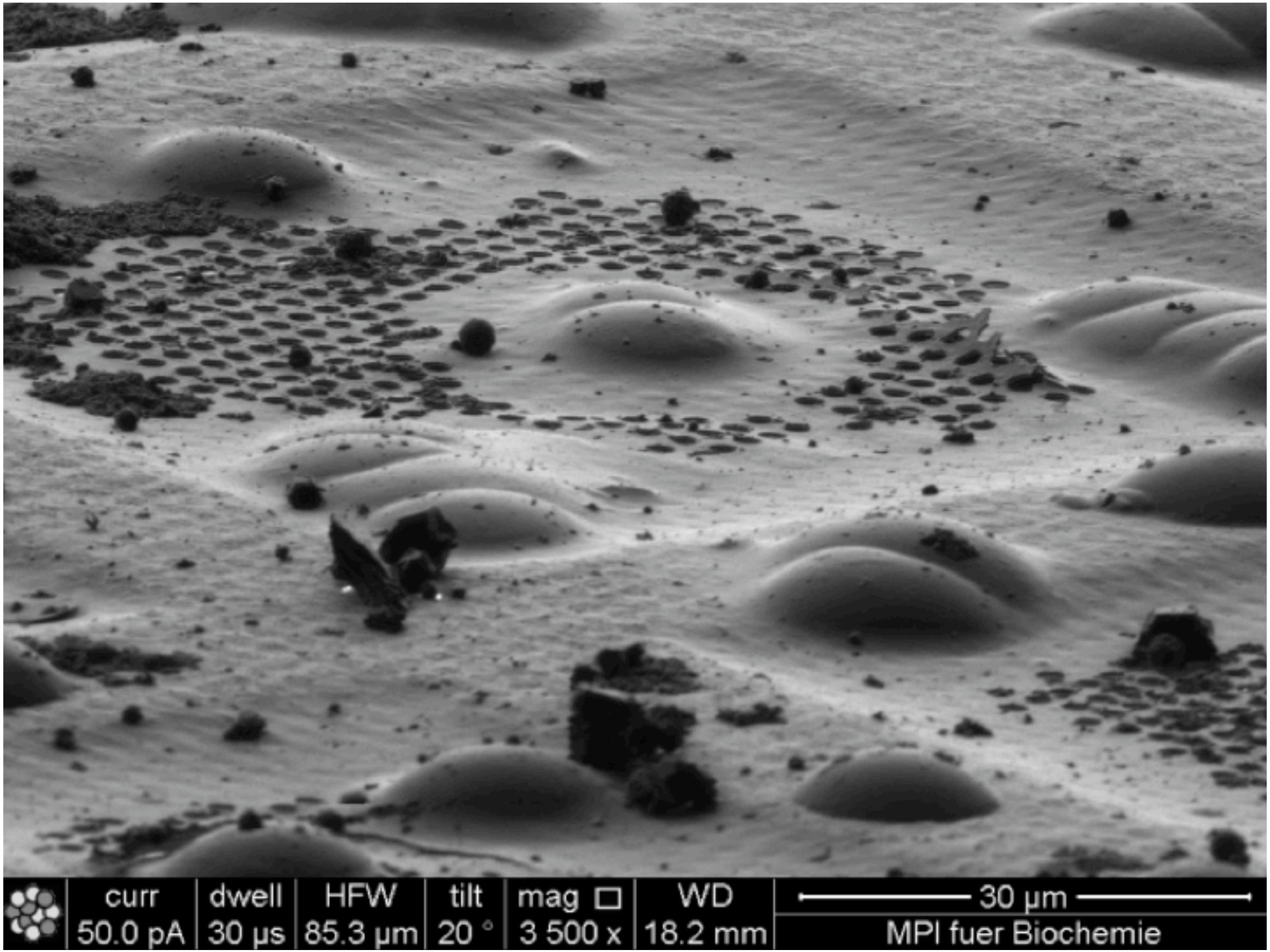
HPF → CEMOVIS

no chemical fixation
no dehydration
no contrasting

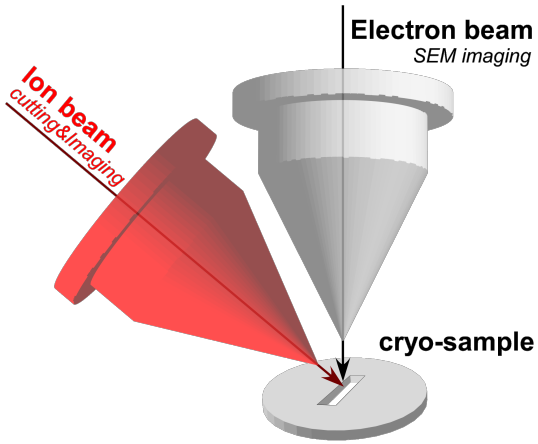


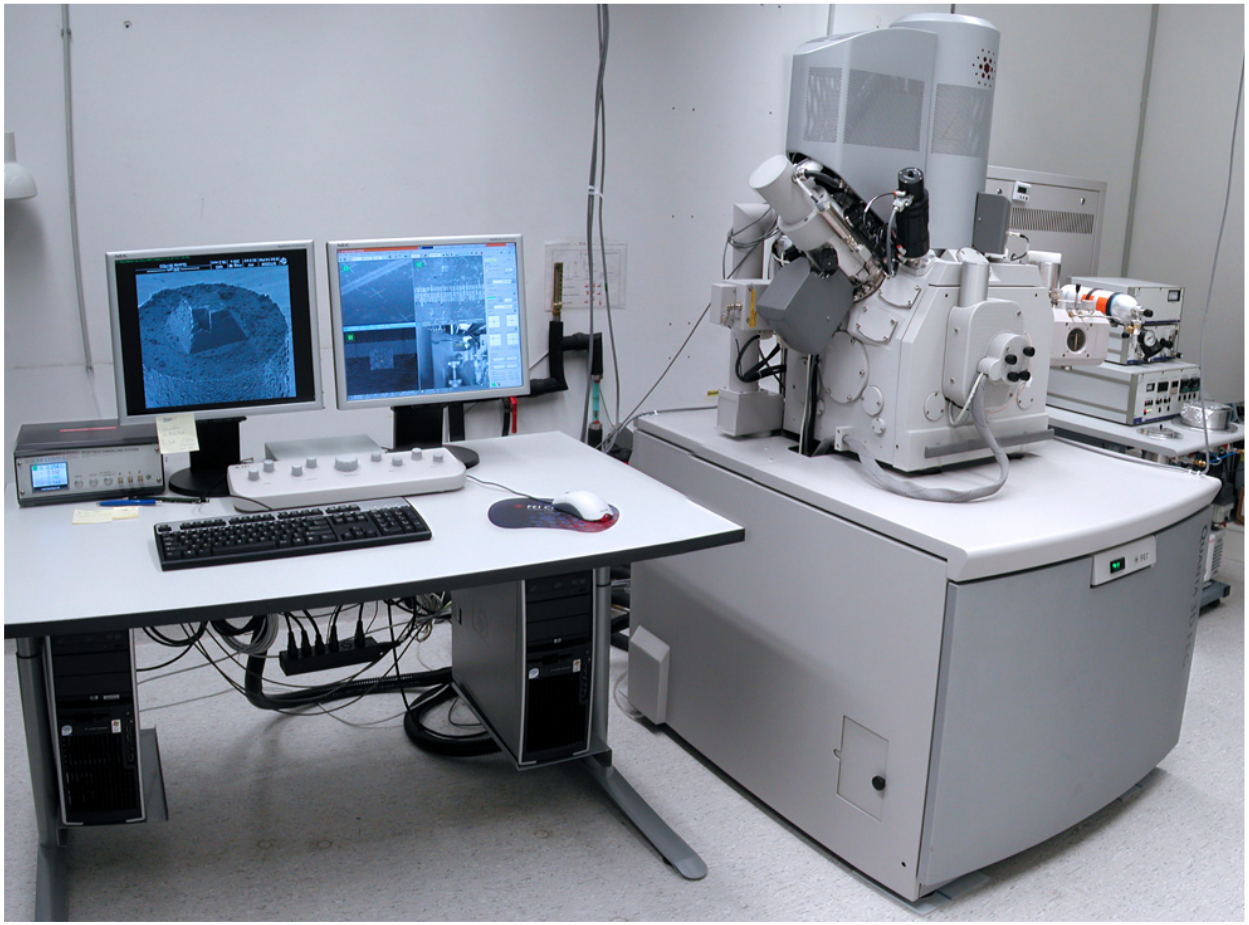
Structure


Plunge Freezing
FIB milling
Cryo-TEM
Ultrastructure and Structure


curr
50.0 pA
dwell
30 µs
HFW
85.3 µm
tilt
20 °
mag
3 500 x
WD
18.2 mm
30 µm
MPI fuer Biochemie

Focused Ion Beam – FIB



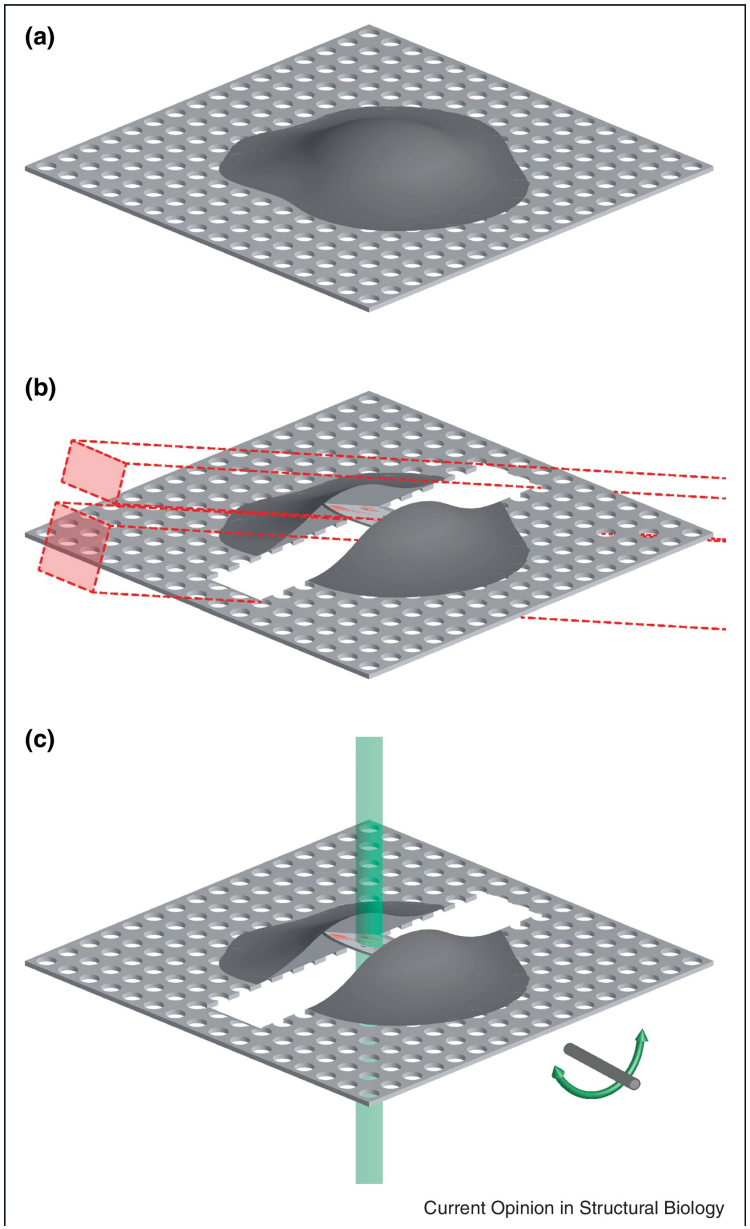


Bundesministerium
für Bildung
und Forschung

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

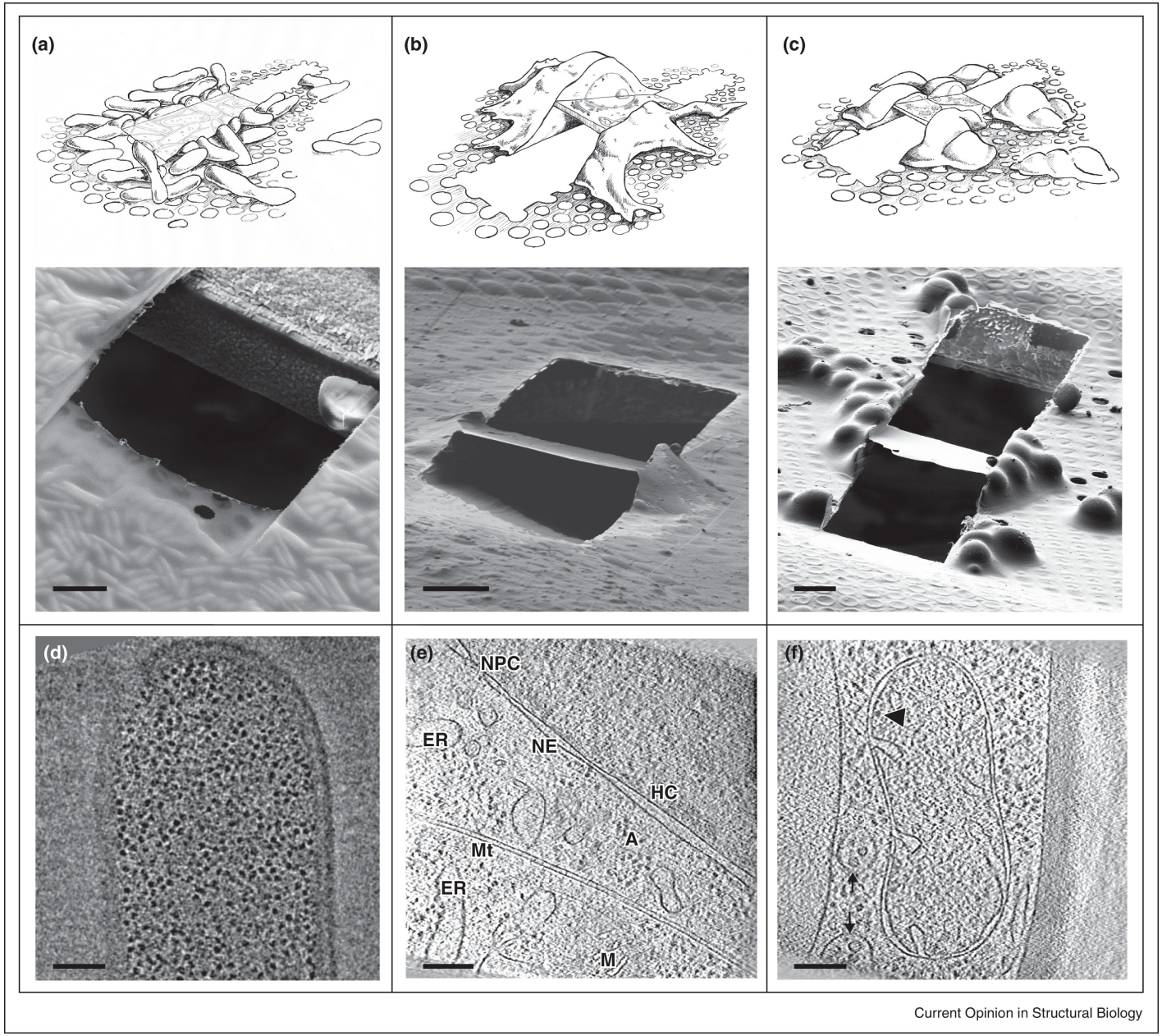
Juergen Plitzko

Focused ion beam milling



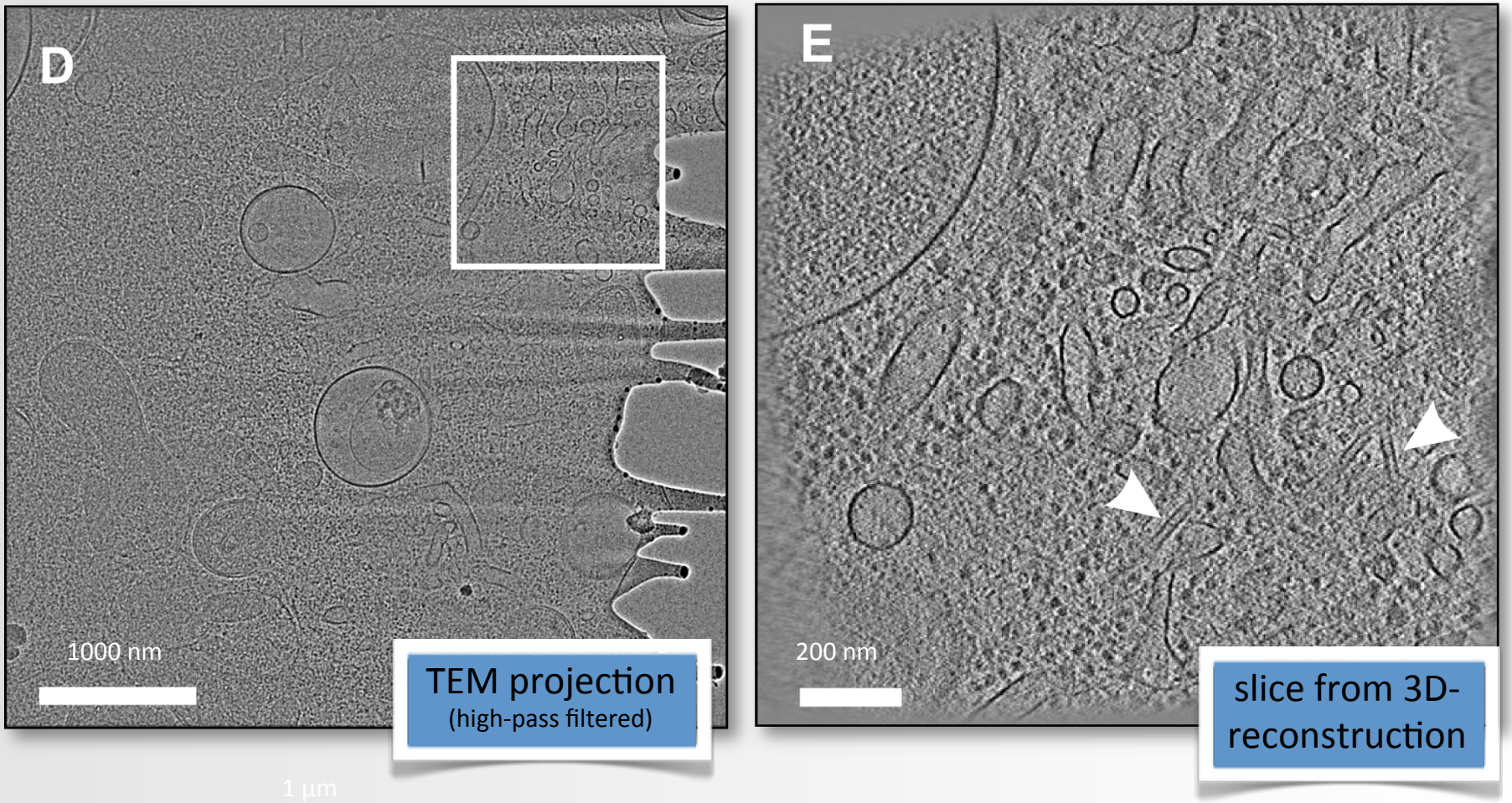
Villa et al. 2013

Focused ion beam milling



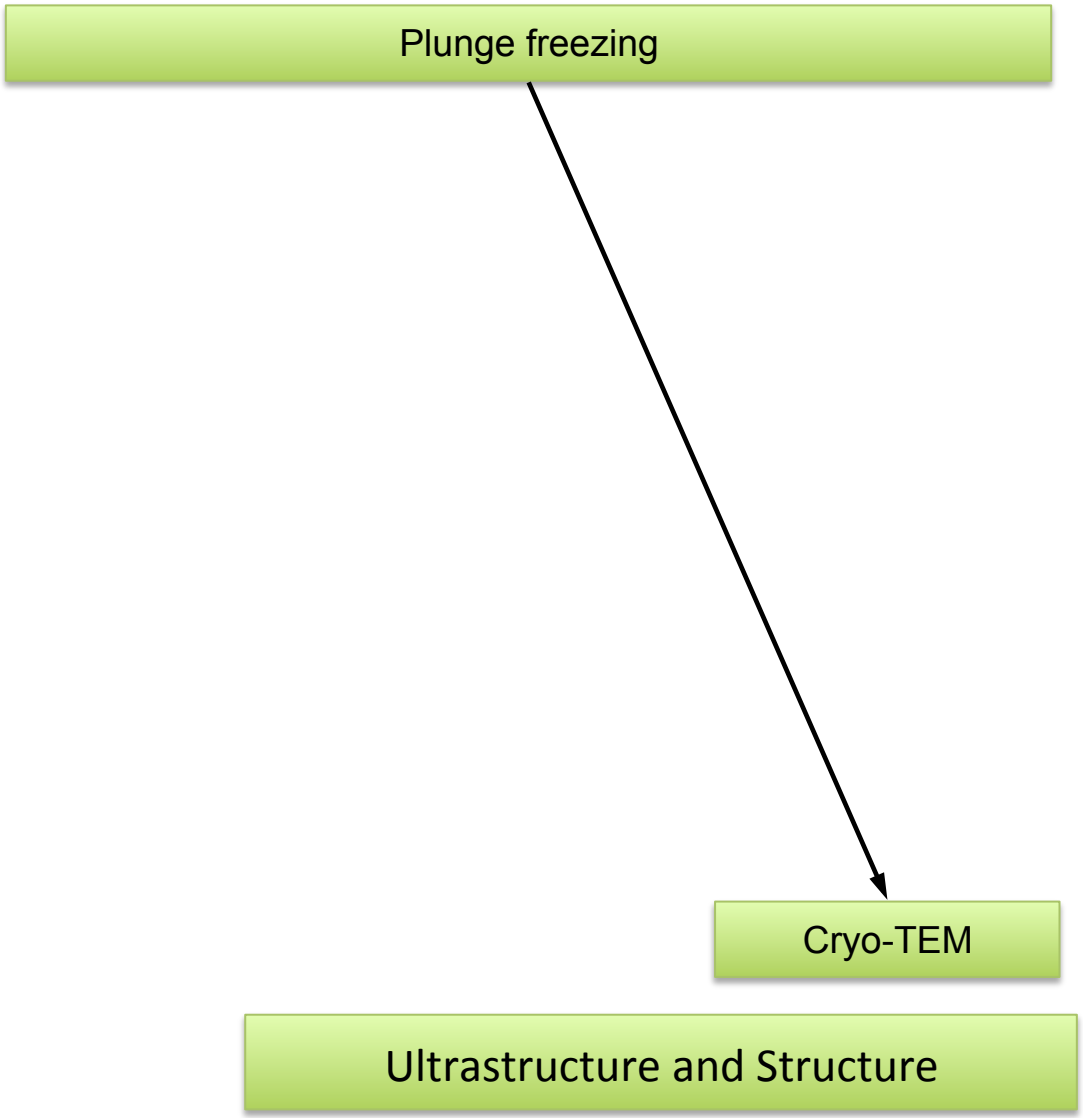
Villa et al. 2013

Cryo-ET of FIB milled specimens



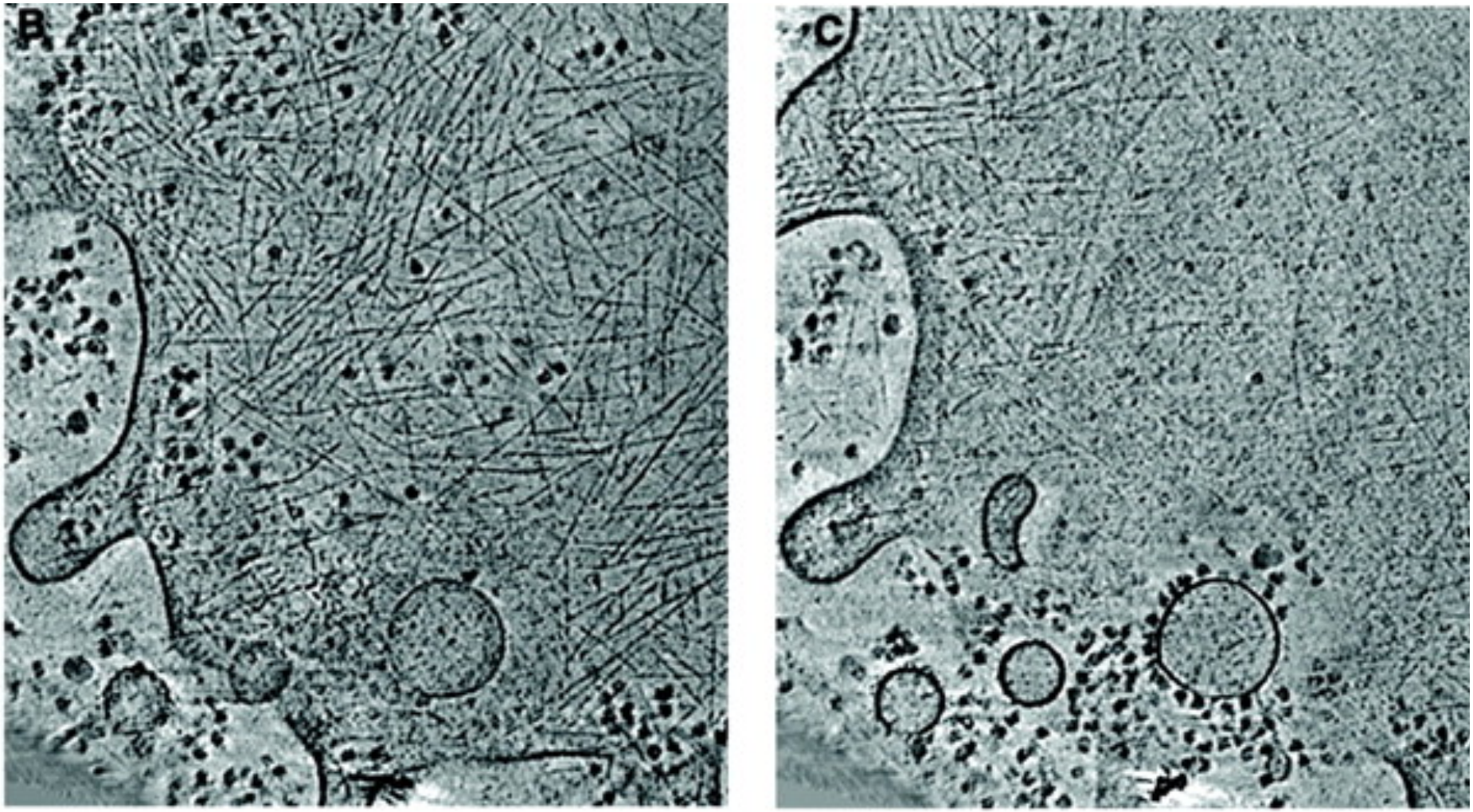
Juergen Plitzko

Structure

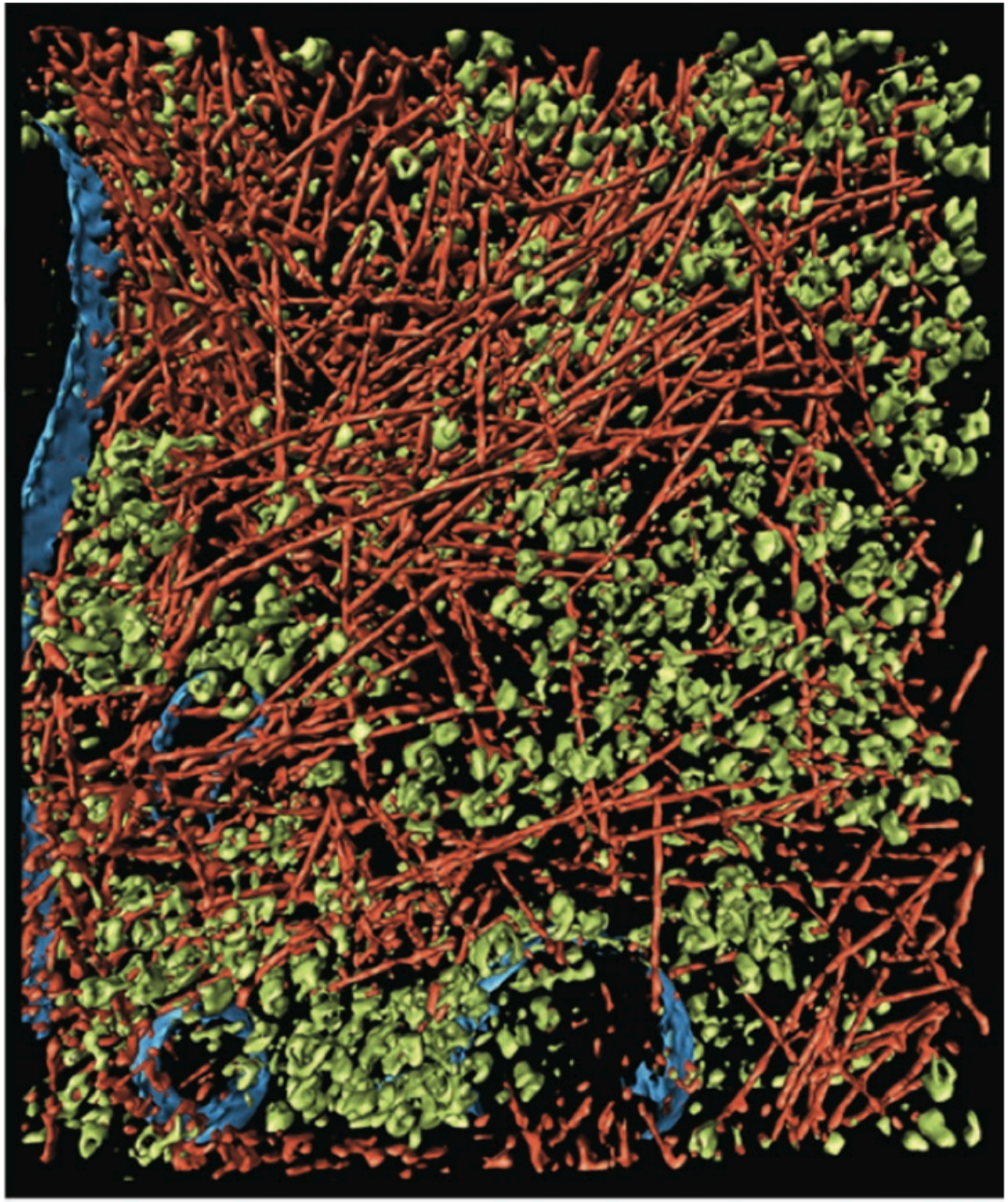


Cryo-electron tomography

Medalia et al. Science 2002



Medalia et al. 2002



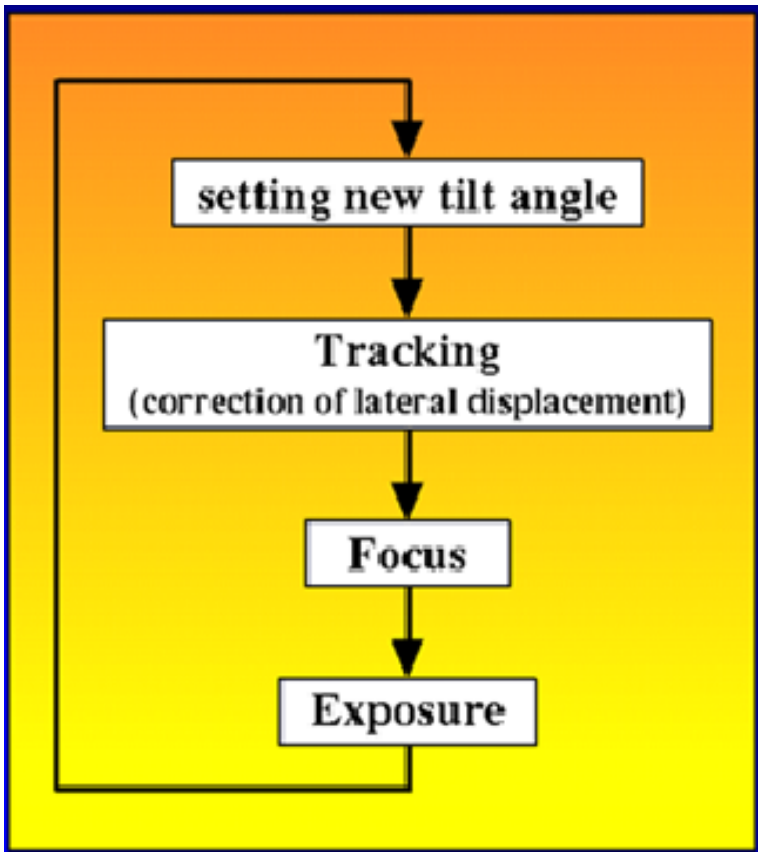
How it is done today

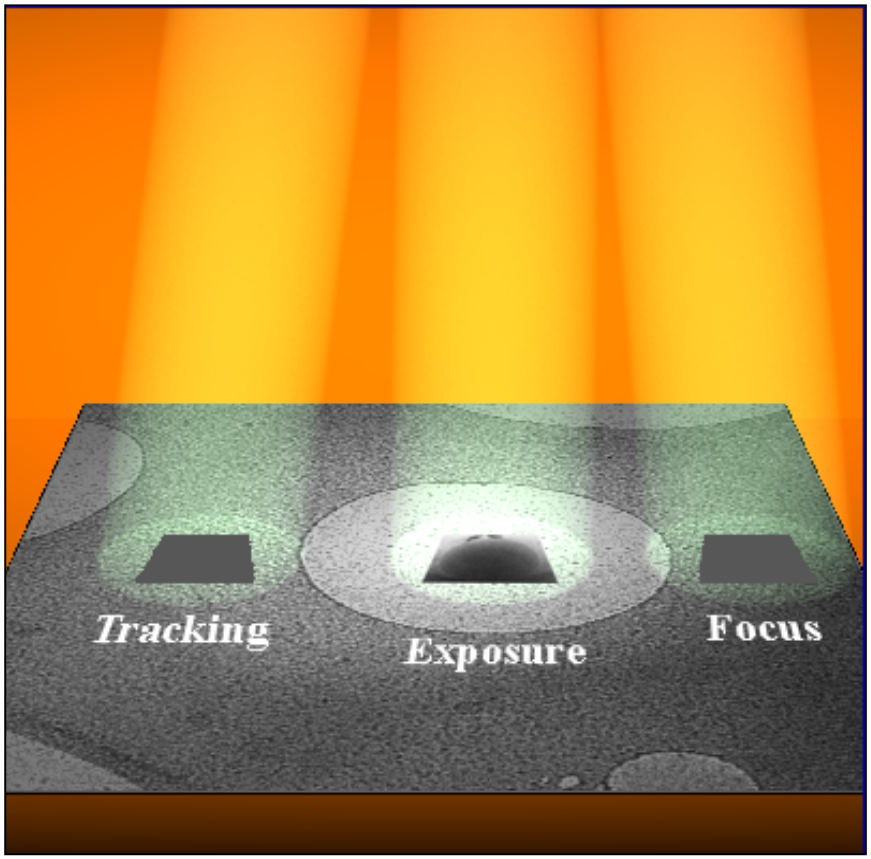
Sample preparation

Data collection

Image processing

Automated Electron Tomography





Thicker samples means benefits from use of an energy filter and 300kV

How it is done today

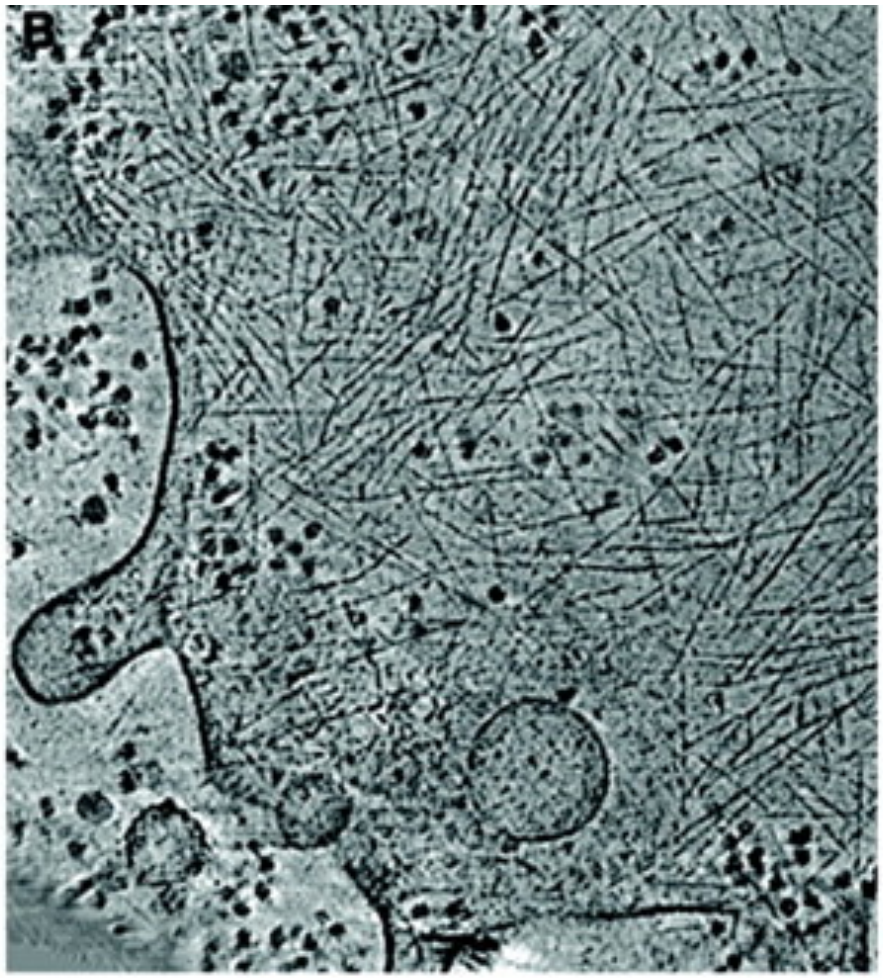
Sample preparation

Data collection

Image processing

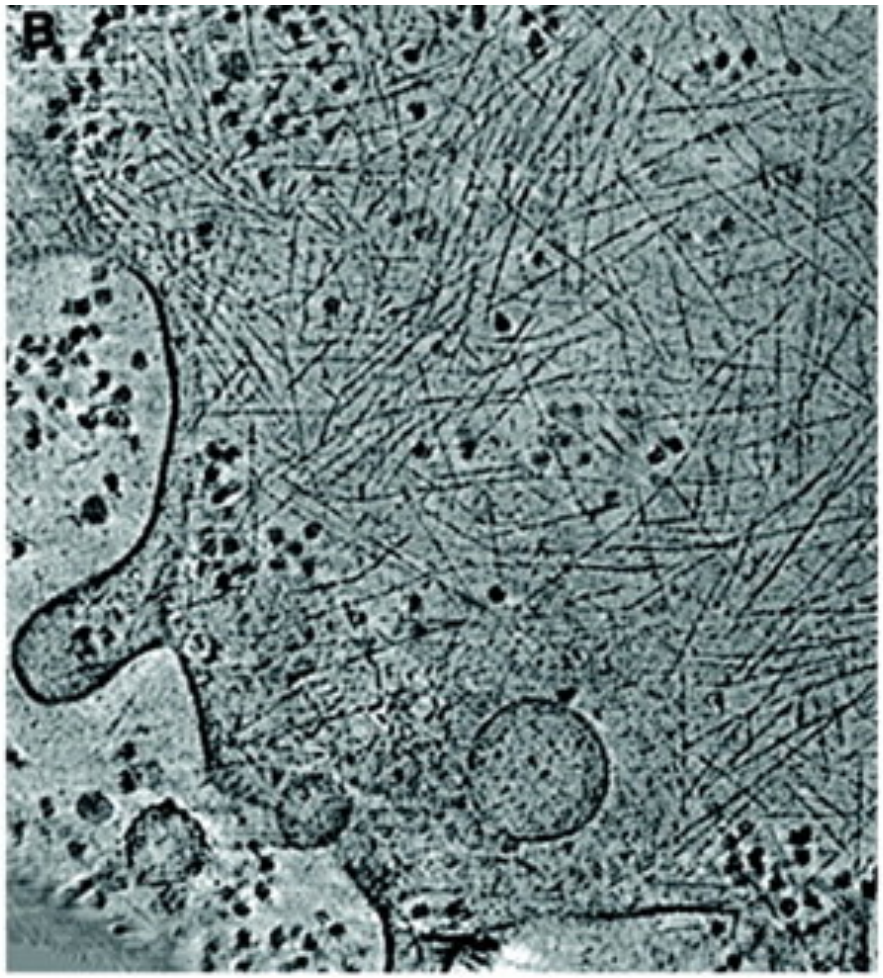
Analysing the images

- Direct functional insights
- Influences how we think about biological problems, how we develop hypotheses, how we design experiments.

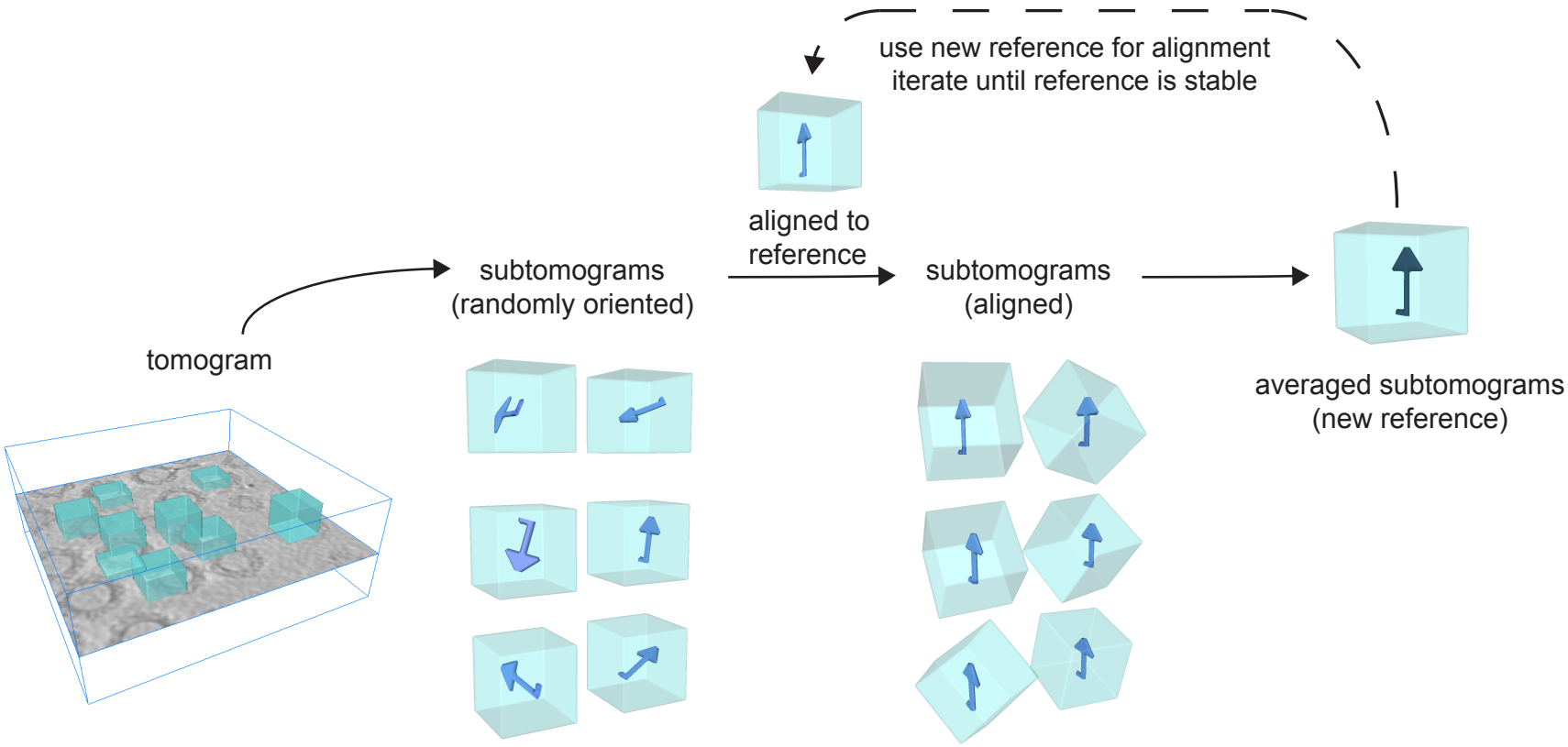


Analysing the images - Stereology

- Should ideally be analysed quantitatively.
- There are robust and powerful approaches for extracting quantitative data from such images, used within cellular EM community. Systematic uniform random sampling. Stereology.



From ultrastructure to structure
Subtomogram averaging

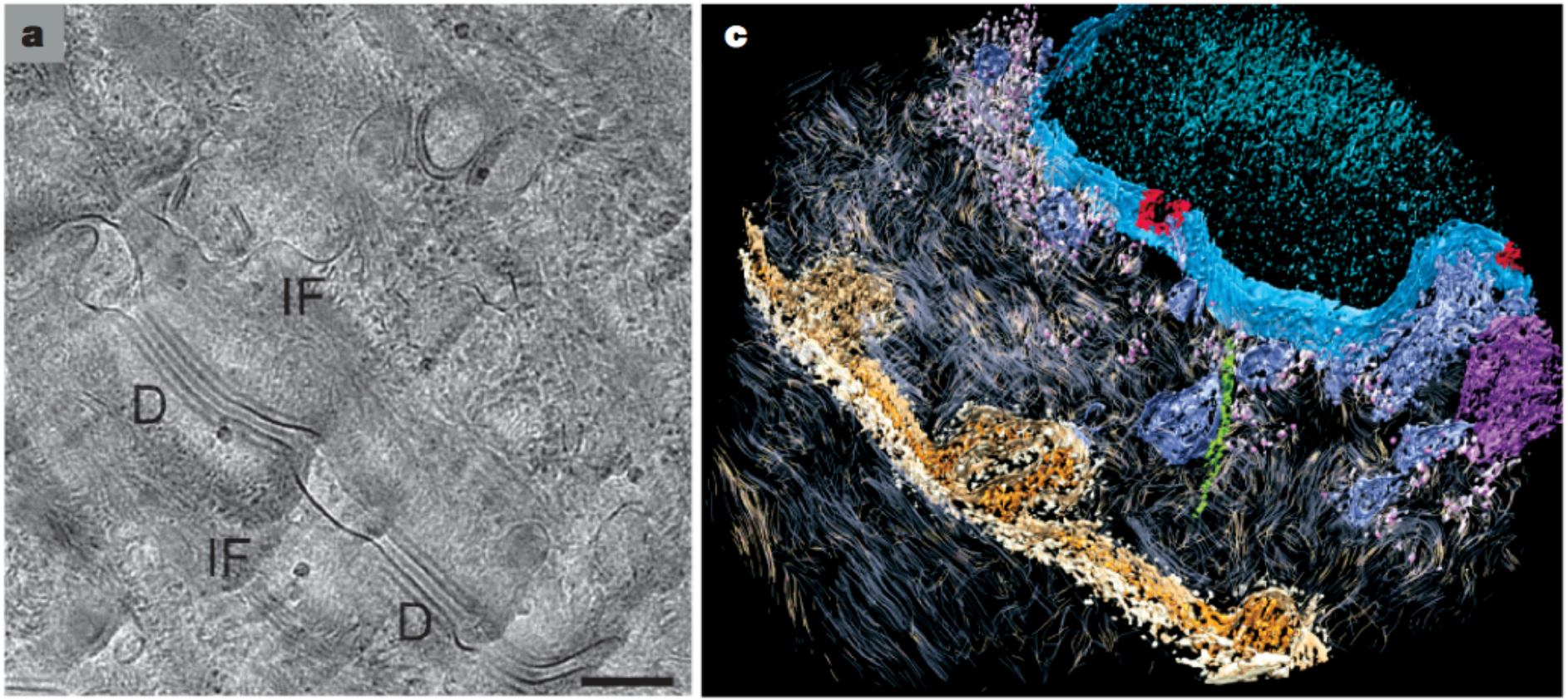


Briggs 2013, *Curr. Opin. Struct. Biol.*

Challenges in Cellular Structure Determination

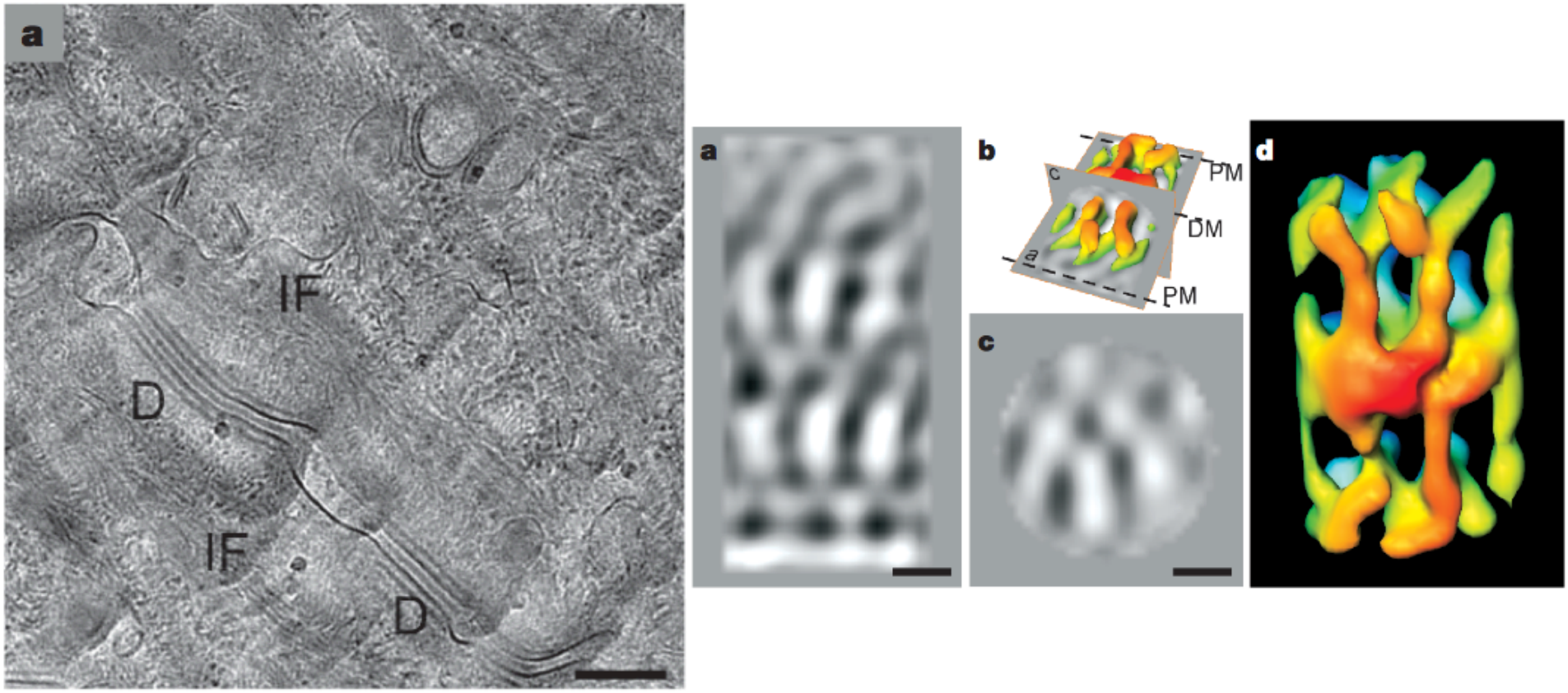
3. Some examples from the literature

Desmosomes (skin)



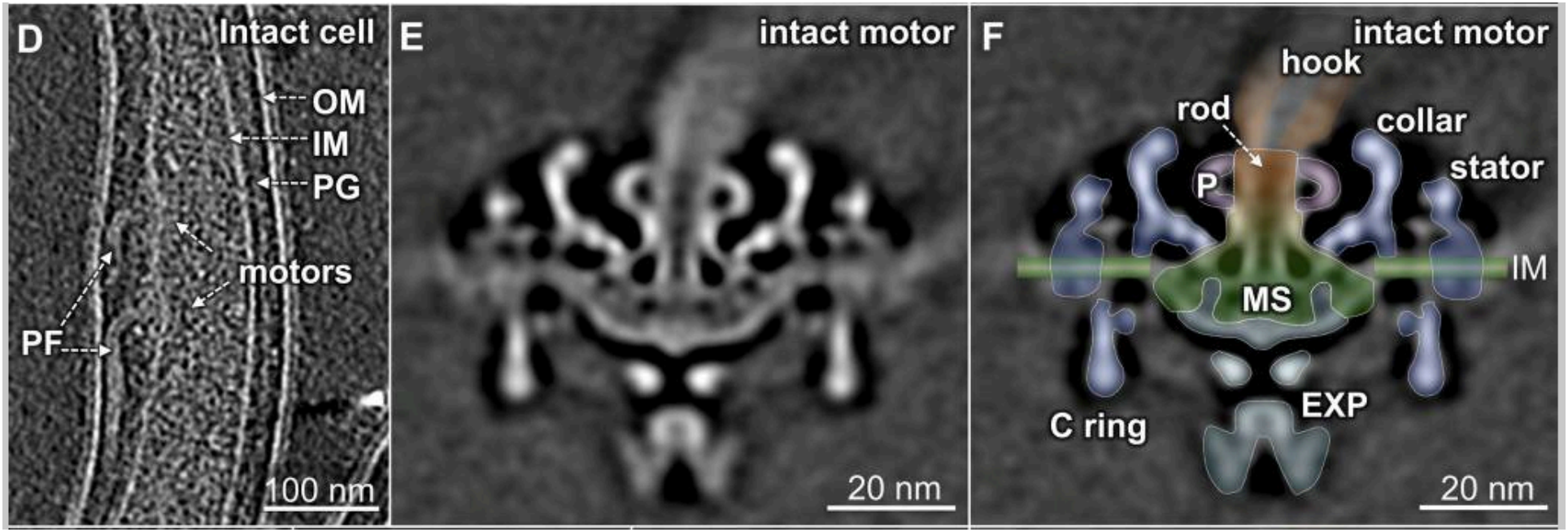
Al Ahmoudi … Frangakis. Nature, 2011

Desmosomes (skin)



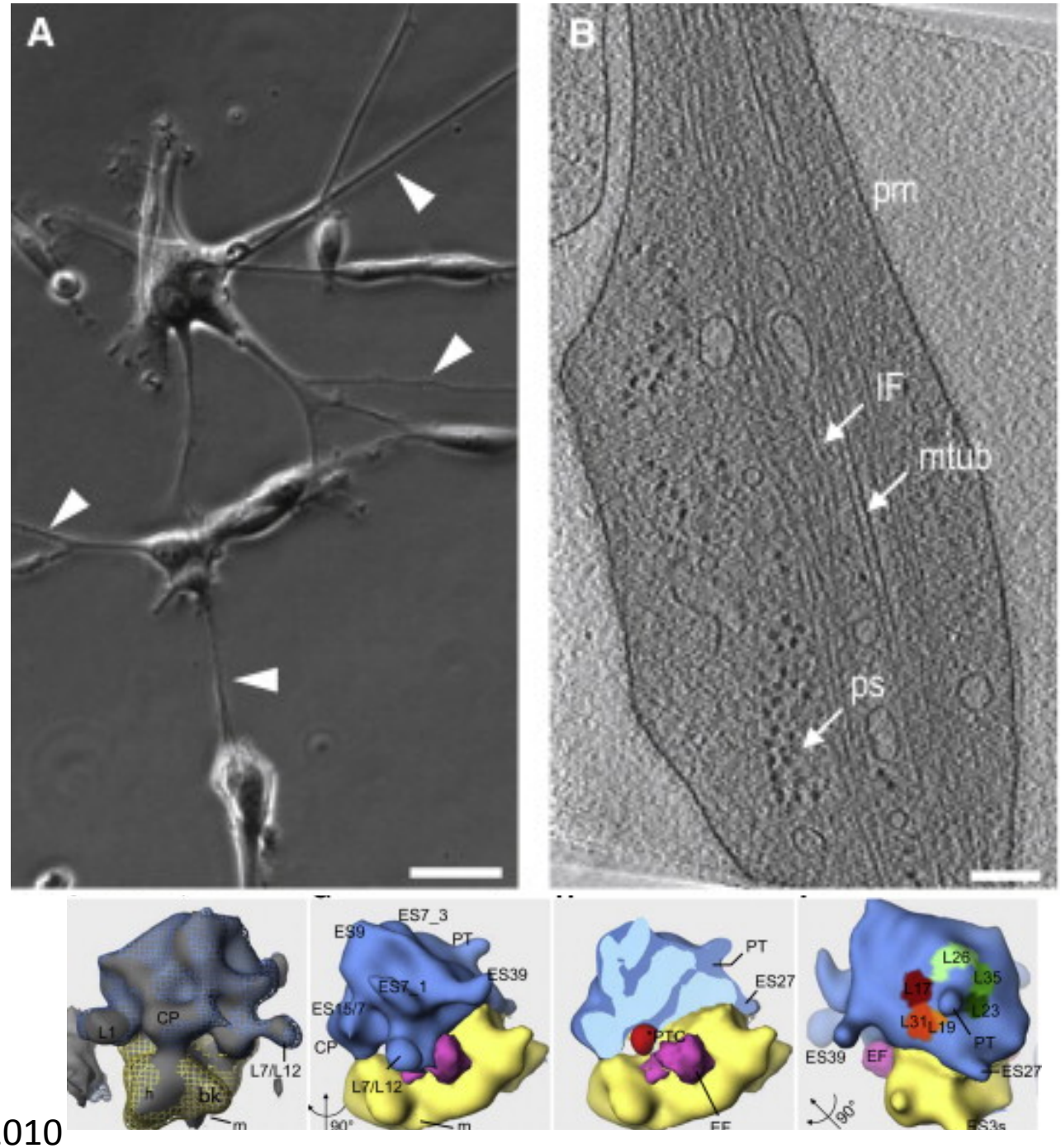
Al Ahmoudi … Fragakis. Nature, 2011

Flagellar motors



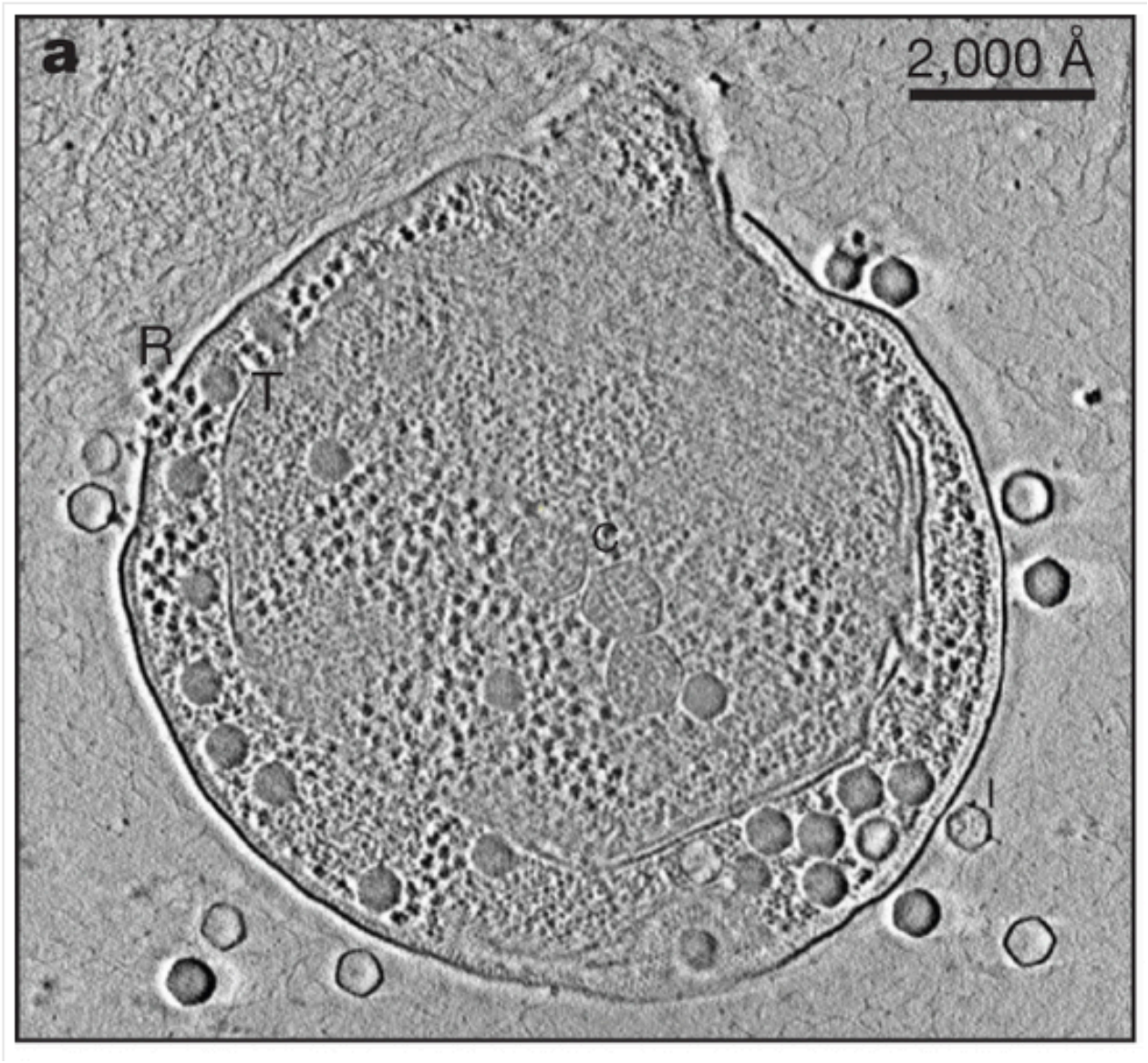
Zhao … Liu, PNAS 2013

Ribosomes in intact cells

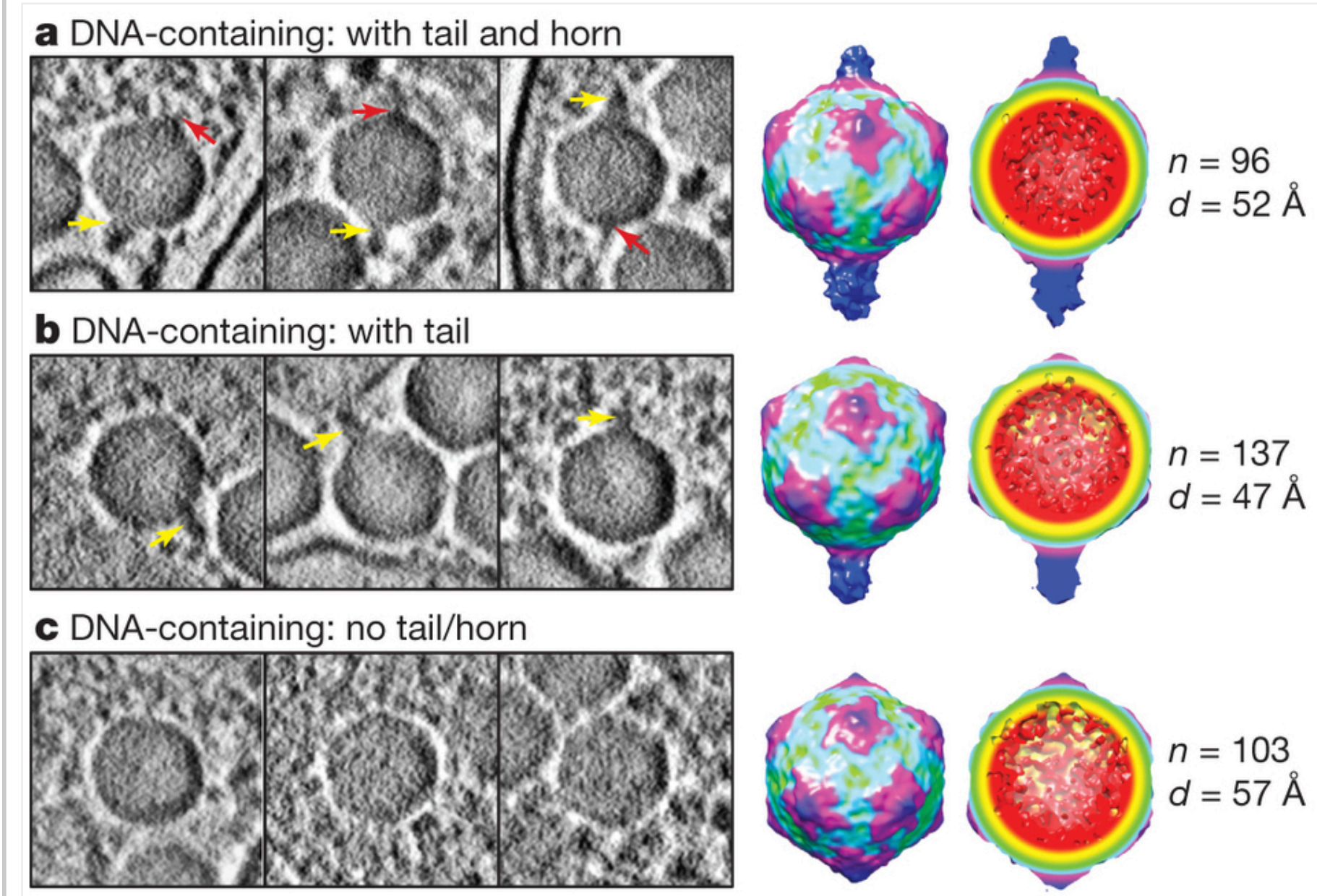


Brandt … Grünewald. Mol.Cell 2010

Phages (with Zernike Phase Plate)



Dai … Chiu, Nature 2013



Dai … Chiu, Nature 2013

There are not many examples in cells.

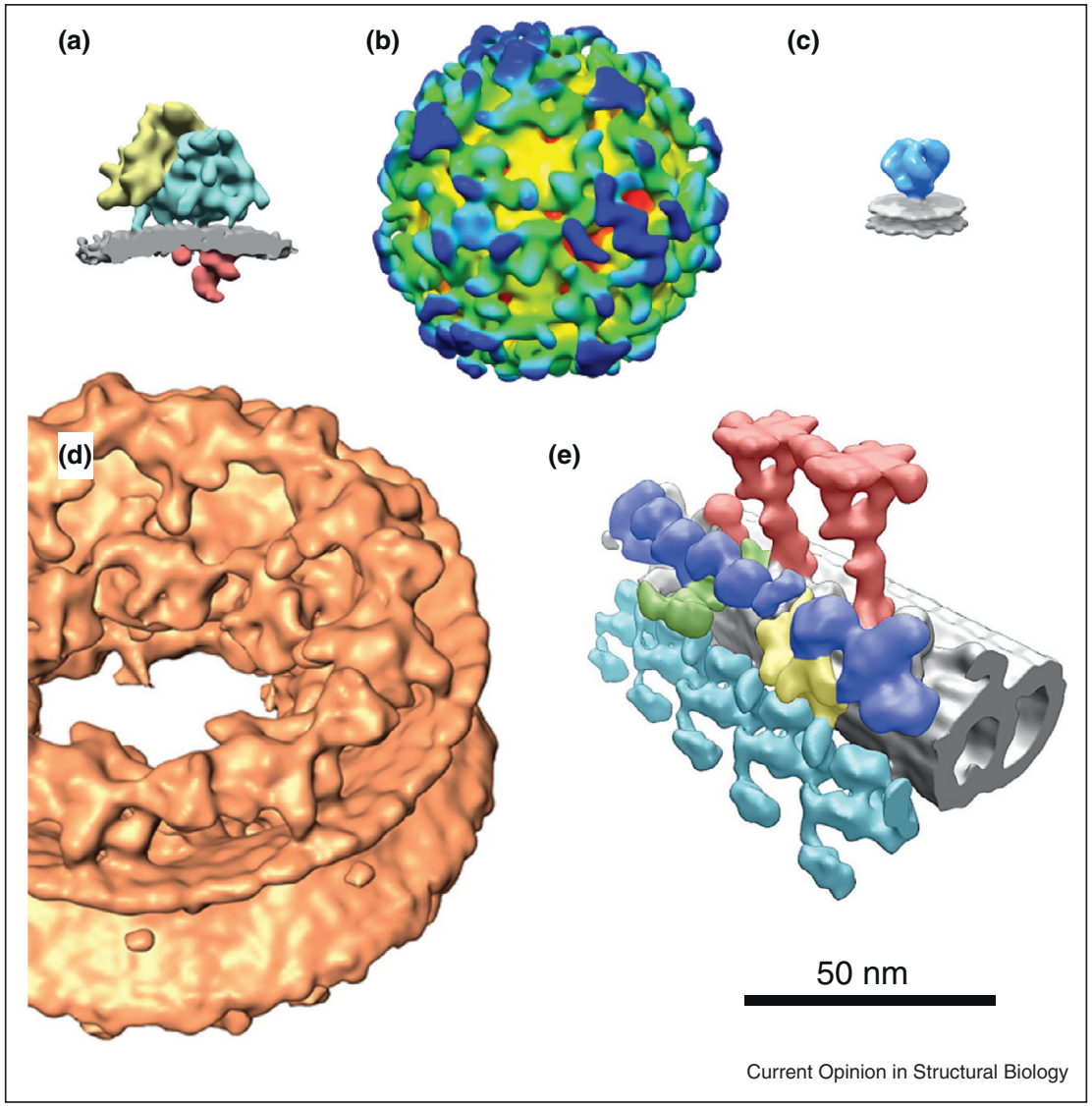
More examples in “intermediate systems”:

lysates
organelles
in vitro reconstituted systems
pleiomorphic viruses

Figure 2 Briggs 2013, *Curr. Opin. Struct. Biol.*

Förster lab
Ribosomes

Medalia lab
Nuclear Pore



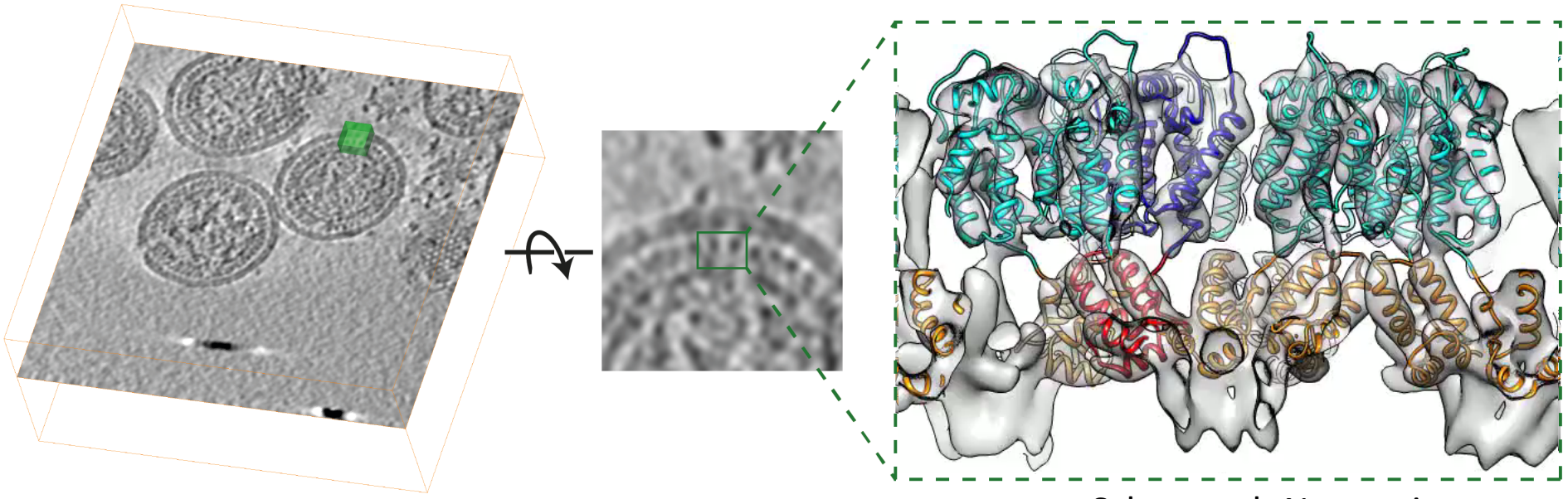
Subramaniam lab
HIV-1 glycoprotein

Briggs lab
COPI vesicles

Ishikawa lab
Flagellum

Examples of recent structures solved by subtomogram averaging, shown approximately to scale. **(a)** Ribosomes on the ER membrane [46••]. **(b)** COPI coated vesicles [30••]. **(c)** The glycoprotein spike of HIV [11]. **(d)** The human nuclear pore [23]. **(e)** A microtubule doublet from a Chlamydomonas flagellum [36]. Panels were adapted from the original references. Panel e © 2011 Rockefeller University Press. Originally published in Journal of Cell Biology. 195:673–687. http://dx.doi.org/10.1083/jcb.201106125.

Structure of the immature HIV-1 capsid in intact virus particles at 8.8 Å resolution.
Cryo-electron tomography and subtomogram averaging



Schur et al. *Nature*, in press

Typical resolutions have been around 2nm (with CCD cameras).
Resolutions of 8 Å have been obtained using both CCD cameras and DDs.
Further improvements should come soon from DD equipped labs.

Challenges in Cellular Structure Determination

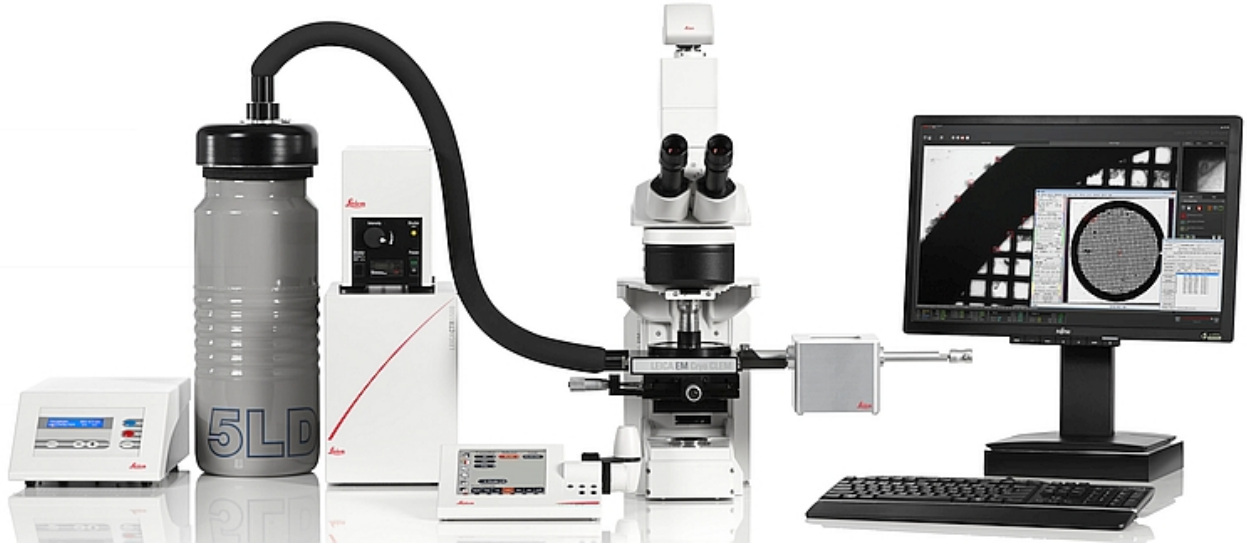
4. Correlative methods and labelling

Cryo-CLEM

- If you are not sure what you are looking for
- If what you are looking for is hard to find/see
- If what you are looking for is rare
- If you need to catch a dynamic state

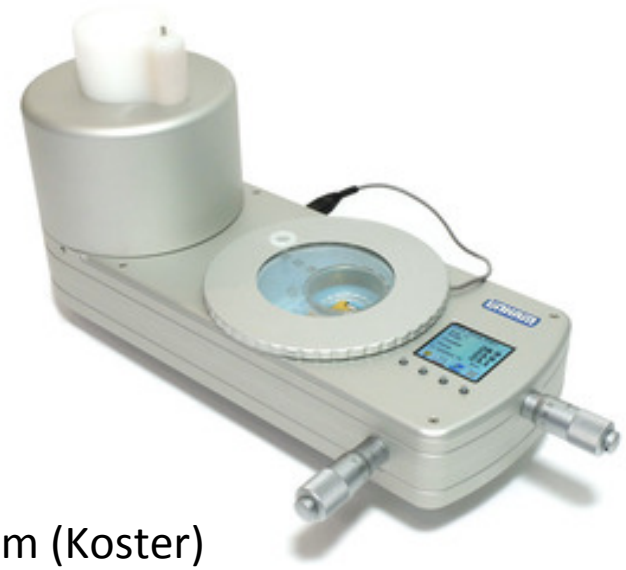
Cryo-CLEM

Some examples
(There are others)



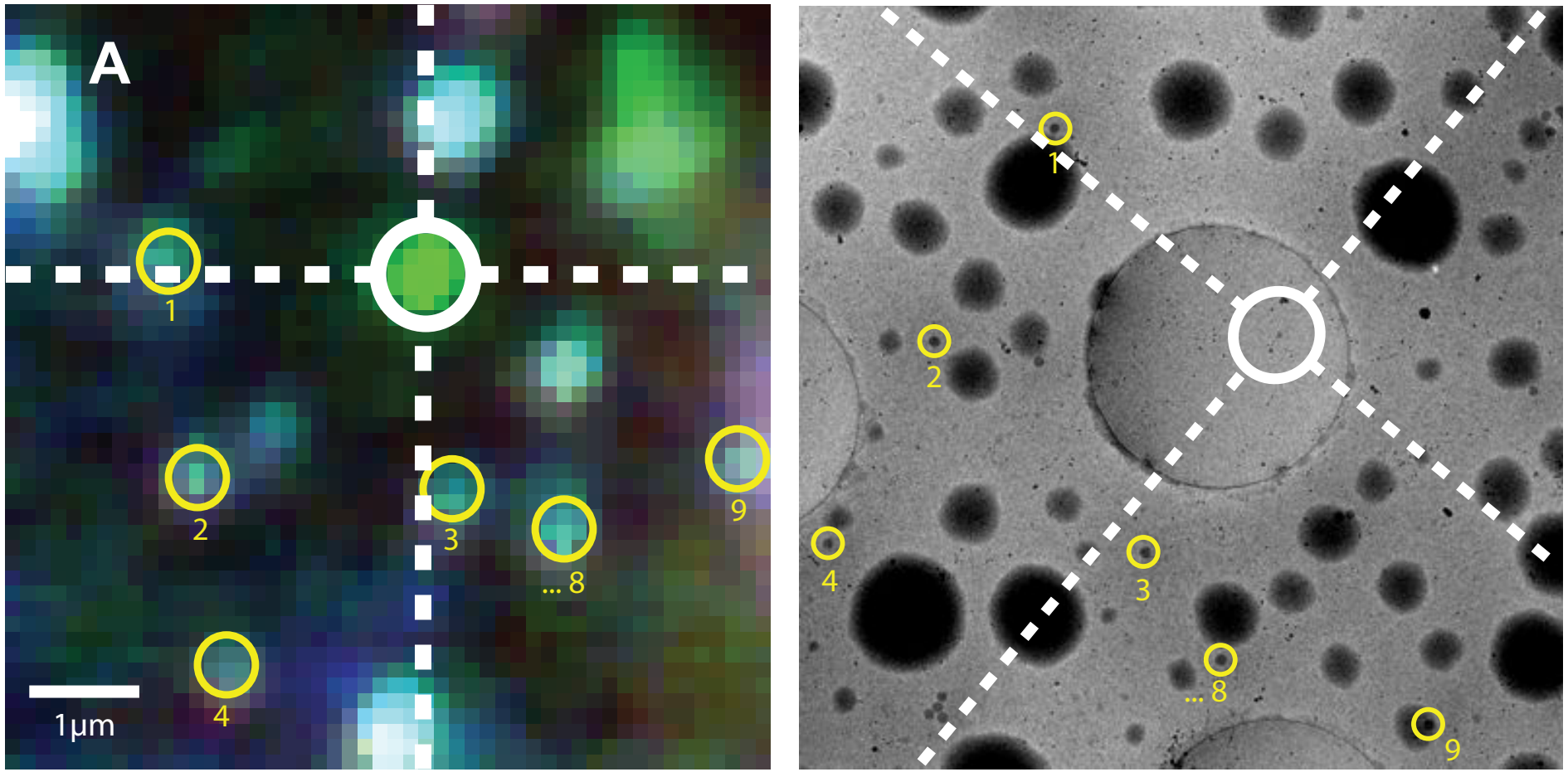
Leica (Briggs)

FEI (Baumeister)



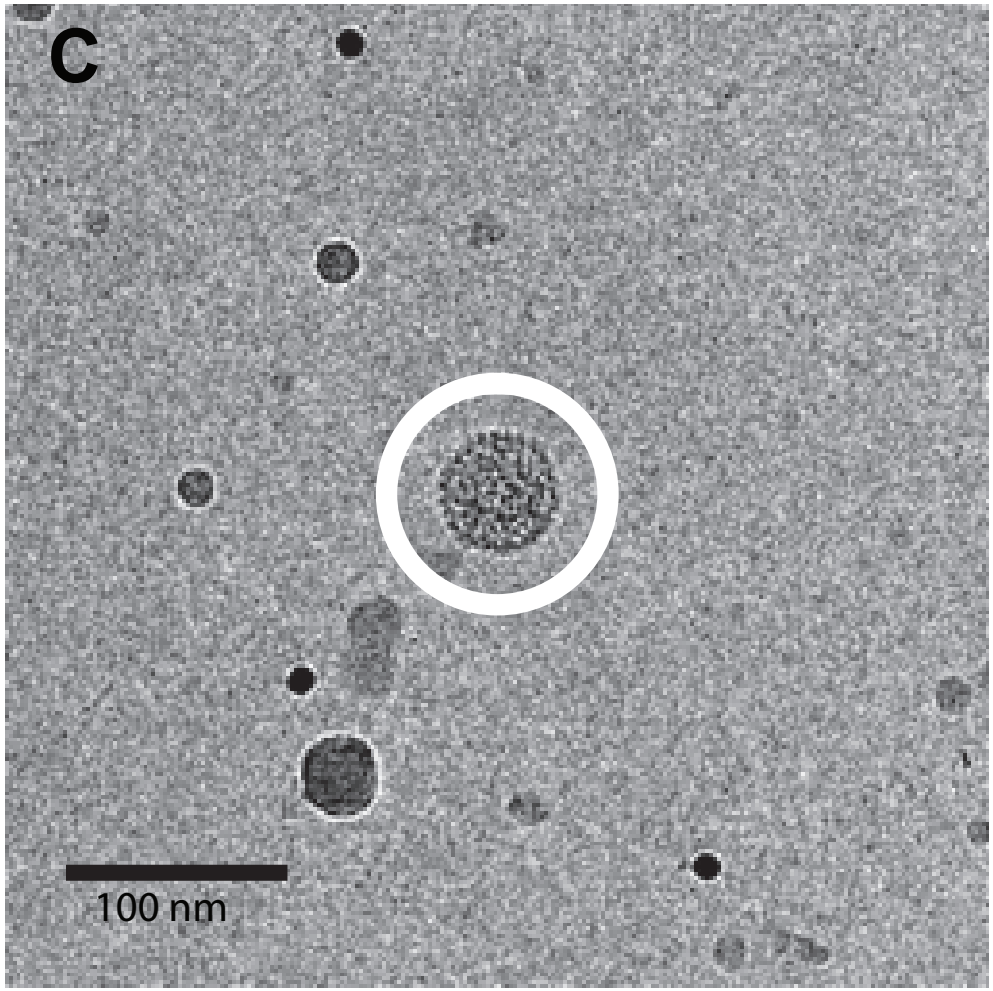
Linkam (Koster)

Fiducial based correlation



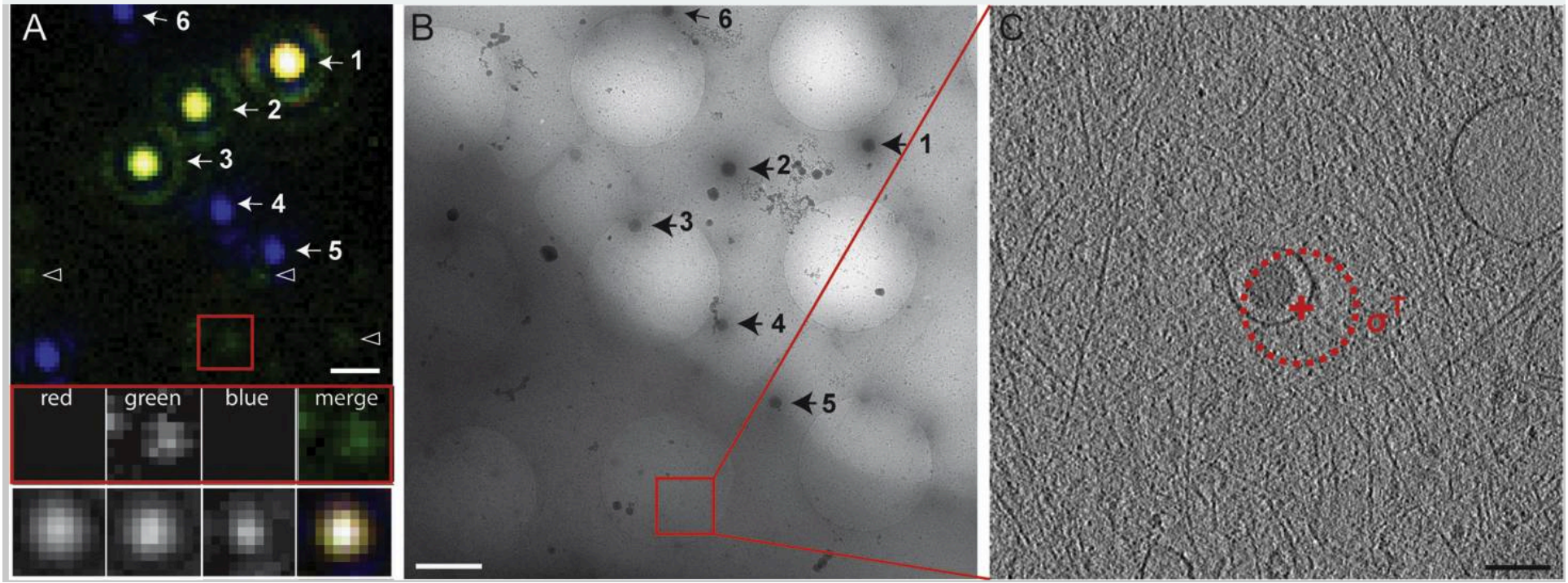
Schorb and Briggs 2014, *Ultramicroscopy*

P22 bacteriophage (circle radius 50 nm)



Schorb and Briggs 2014, *Ultramicroscopy*

Adenovirus



Schellenberger… Grünewald, 2014 *Ultramicroscopy*

Cryo-CLEM

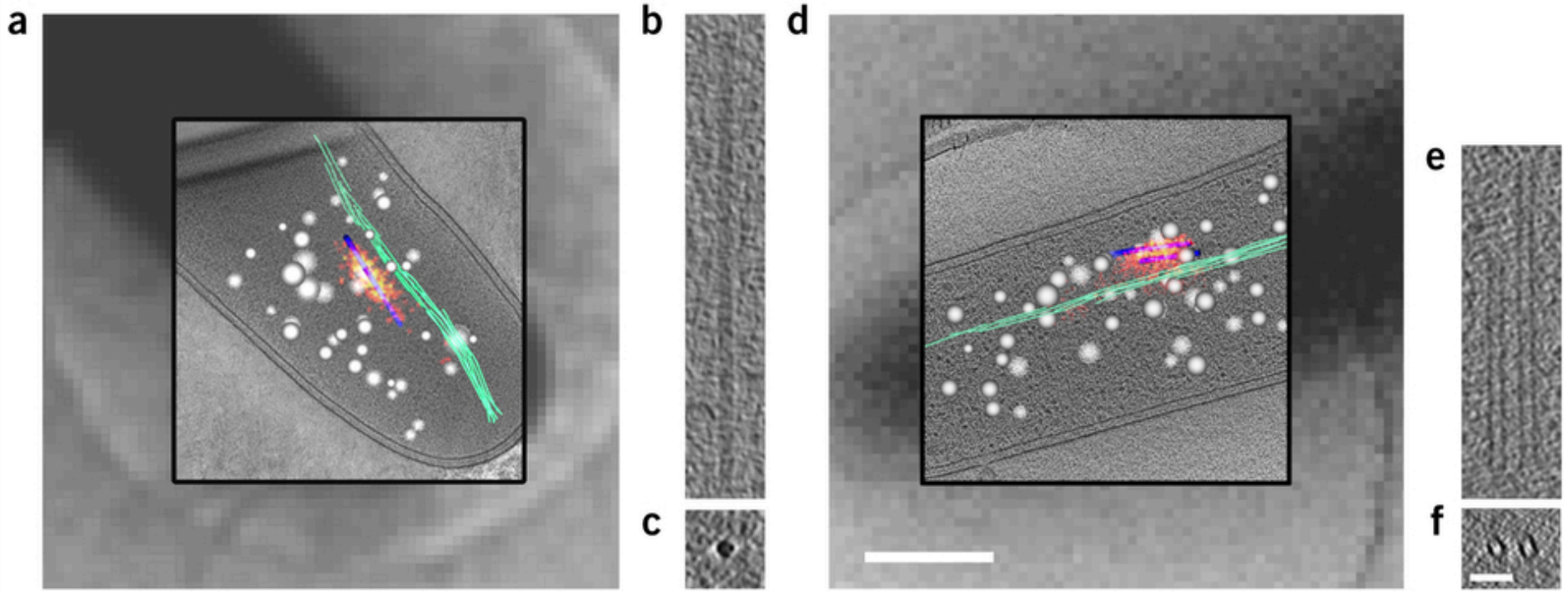
- High precision is needed to locate things
- High (super-) resolution is needed to separate signals that are close together

Correlated cryogenic photoactivated localization microscopy and cryo-electron tomography

Yi-Wei Chang, Songye Chen, Elitza I Tocheva, Anke Treuner-Lange, Stephanie Löbach, Lotte Søgaard-Andersen & Grant J Jensen

Nature Methods **11**, 737–739 (2014) | doi:10.1038/nmeth.2961

Received 06 February 2014 | Accepted 10 April 2014 | Published online 11 May 2014



(**a**,**d**) Low-resolution EM images (grayscale background), cryo-PALM images (red and yellow foreground), slices from high-resolution three-dimensional cryo-tomograms (grayscale foreground), and segmentations of cellular structures (blue, tubular structures; green, filament bundles; white, spherical granules) superposed. The cryo-PALM images reveal VipA–PA-GFP localization (red, low precision; yellow, high precision), identifying the tubular structures as T6SSs. (**b**,**e**) Tomographic slices through the tubular structures (blue) in **a**,**d** showing extended and contracted T6SS sheaths, respectively. (**c**,**f**) Cross-sectional views of **b** and **e**, respectively. Scale bars, 400 nm (**d**; applies to **a**,**d**) and 50 nm (**f**; applies to **b**,**c**,**e**,**f**).

Cryo-CLEM

- Cryo-stages and transfer systems are now becoming robust and user-friendly.

- Continuous improvement of optics and usability.

Cryo-CLEM

- can your feature of interest be labelled?
- does the labelling affect the function?

- Typically there is a need for supporting FM data.

Challenges in Cellular Structure Determination

5. A general strategy

A general strategy

- Prepare the sample
- Identify the features of interest
- Image them by cryo-electron tomography
- Perform image processing to solve the structure

Challenges in Cellular Structure Determination

6. What are the challenges?

The Challenges

- Sample preparation
- Identifying the features of interest
- Finding enough of them
- Identifying optimal conditions for imaging
- Processing the data properly
- Dealing with heterogeneity

The Challenges

- Sample preparation
- Identifying the features of interest
- Finding enough of them
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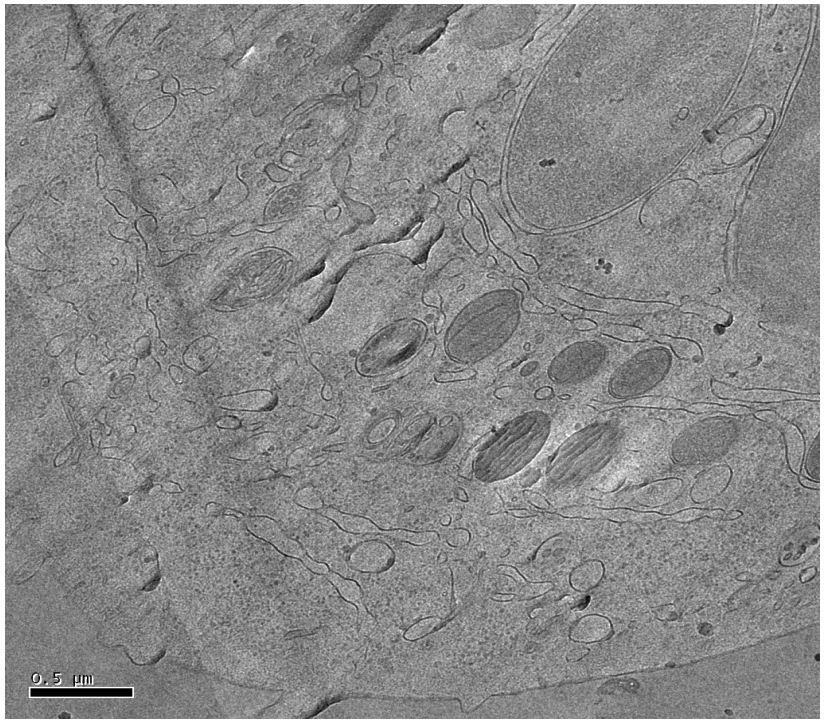
- Dealing with heterogeneity

Ideal sample preparation

- Very large imaging area on the grid, so we can image many copies of rare/disperse objects
- Artefact free
- Robust and cheap

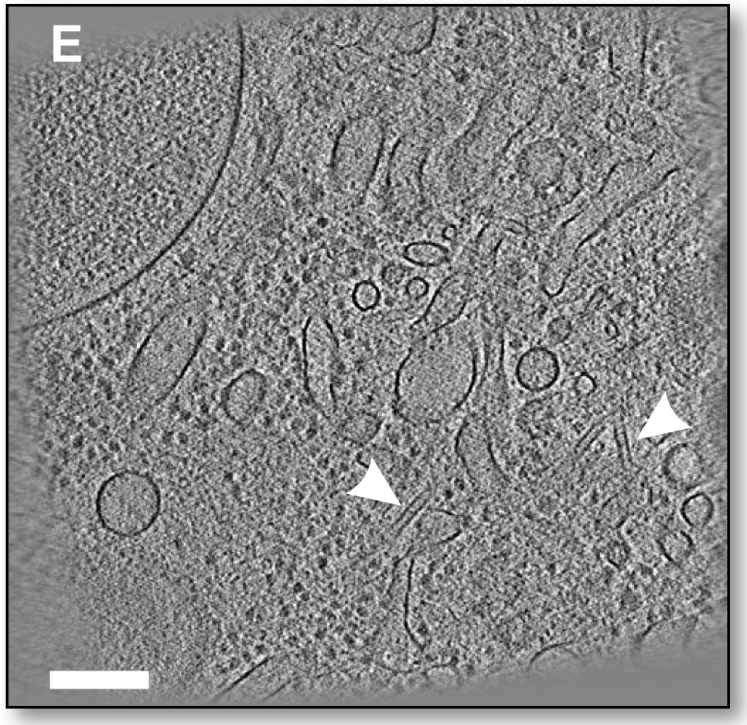
Sample preparation

- CEMOVIS allows large areas to be generated
- Suffers from compression artefacts
- Is robust only in a few labs



Sample preparation

- FIBSEM does not allow rapid generation of large areas
- Seems to be relatively artefact free
- Is robust only in a few labs
- Is not cheap



The Challenges

- Sample preparation
- Identifying the features of interest
- Finding enough of them
- Identifying optimal conditions for imaging
- Processing the data properly
- Dealing with heterogeneity

Ideal methods to find the features of interest.

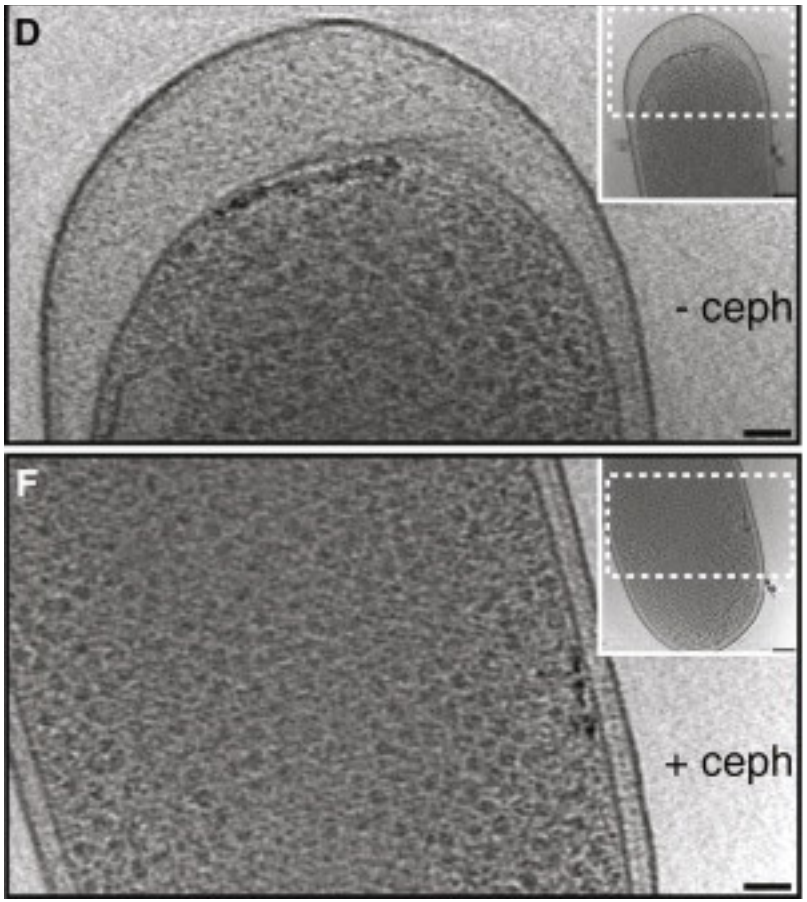
- **Option 1: An EM label**

- A label visible directly in EM in cryo samples
- Visible at single molecule level
- Visible in low mag grid scans.
- Genetically encodable

Ideal methods to find the features of interest.

- **Option 1: An EM label**

- Ferritin
- Metallothionine
- Others?



Wang … Löwe. Structure 2011

Ideal methods to find the features of interest.

- **Option 2: Correlative cryo-FM/EM**

- Single molecule sensitivity cryo-FM
- Super-resolution FM
- Multicolour imaging
- High-precision correlation

- Robust and rapid cryo-FM

Methods to find the features of interest.

- Option 2 may be only a few years away, but there are challenges to overcome.

- Dye/Fluorescent protein behaviour
- Immersion objectives
- Engineering challenges

The Challenges

- Sample preparation
- Identifying the features of interest
- Finding enough of them
- Identifying optimal conditions for imaging
- Processing the data properly
- Dealing with heterogeneity

Finding enough copies of the object of interest

- **How many do we need?**

- If minimal structural heterogeneity in sample of 150nm thick:

Sub-nm with 30000 copies on CCD camera (Schur et al 2013).

(small test dataset collected in Martinsried on K2 gave 12Å with 1500 copies). Others may have more current data?

Finding enough copies of the object of interest

- **How many do we need?**

- If minimal structural heterogeneity in sample of 150nm thick:

Sub-nm with 30000 copies on CCD camera (Schur et al 2013).

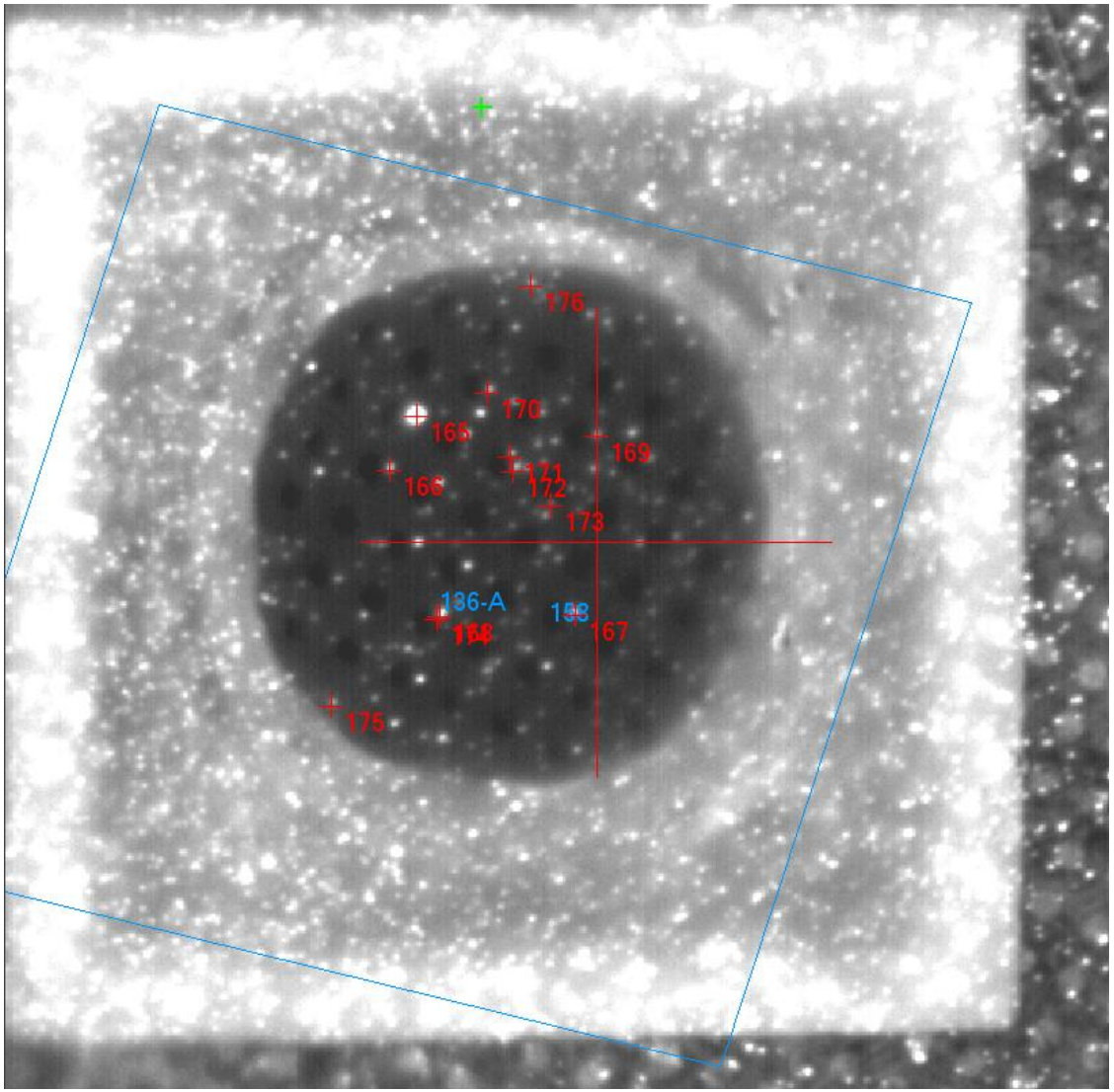
(small test dataset collected in Martinsried on K2 gave 12Å with 1500 copies). Others may have more current data?

Finding enough copies of the object of interest

- Template matching.
- By eye.
- Otherwise CLEM.

Finding enough copies of the object of interest

- Need Highly-automated (correlative) data collection



The Challenges

- Sample preparation
- Identifying the features of interest
- Finding enough of them
- Identifying optimal conditions for imaging
- Processing the data properly
- Dealing with heterogeneity

Data Collection and image processing

Why is the resolution of structures from tomography/subtomogram averaging not as good as for single particle?

Data Collection and image processing

Higher apparent sample thickness (especially at tilt)

Two separate alignment and reconstruction steps

Sample can change during data collection

Higher total electron dose

Smaller datasets (due to more time-consuming data collection)

Difficult in determination of defocus in individual images

Data Collection and image processing

Higher apparent sample thickness (especially at tilt)

Two separate alignment and reconstruction steps

Sample can change during data collection

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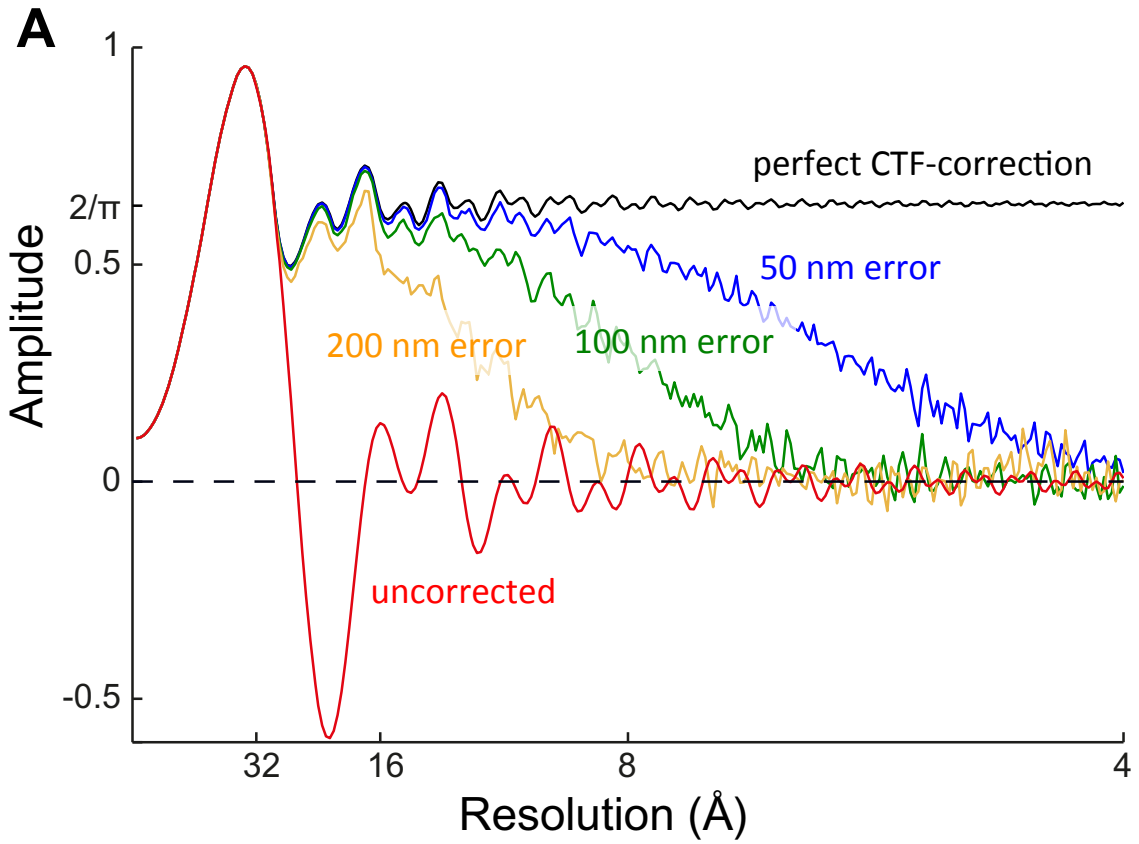
At which resolution will errors in defocus estimation/correction be limiting?



Simulation of CTF of final average from multiple tomograms with mixed defoci

Schur et al. JSB 2013

At which resolution will errors in defocus estimation/correction be limiting?

- Solution 1: stable defocus during data collection. Allows power spectrum averaging and more accurate defocus determination

- Solution 2: buy a quantum K2 and see the Thon rings in individual images – This will make a big difference!

- The need for 3DCTF correction for thick samples?

Data Collection and image processing

Higher apparent sample thickness (especially at tilt)

Two separate alignment and reconstruction steps

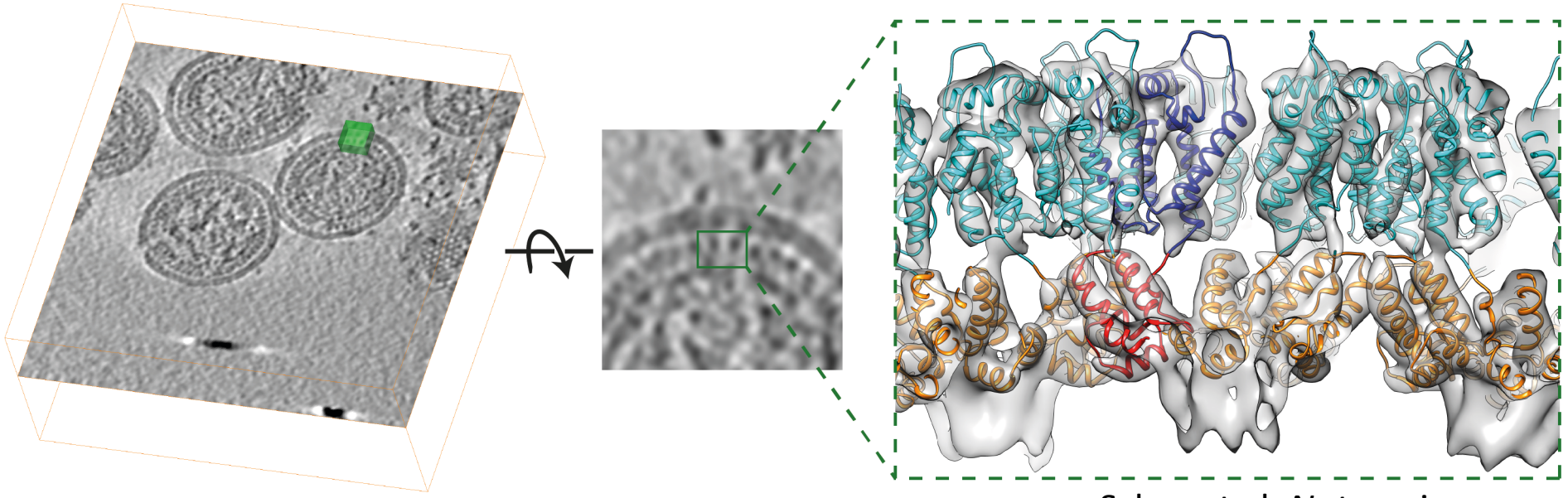
Sample can change during data collection

Higher total electron dose

Smaller datasets (due to more time-consuming data collection)

Difficult in determination of defocus in individual images

Structure of the immature HIV-1 capsid in intact virus particles at 8.8 Å resolution.
Cryo-electron tomography and subtomogram averaging



Schur et al. *Nature*, in press

This structure is from about 200000 unit cells on a CCD camera. Colleagues report at least factor of 10 reduction in dataset size for equivalent resolution when using DD

…so dataset size should not be limiting for such samples.

Data Collection and image processing

Higher apparent sample thickness (especially at tilt)

Two separate alignment and reconstruction steps

Sample can change during data collection

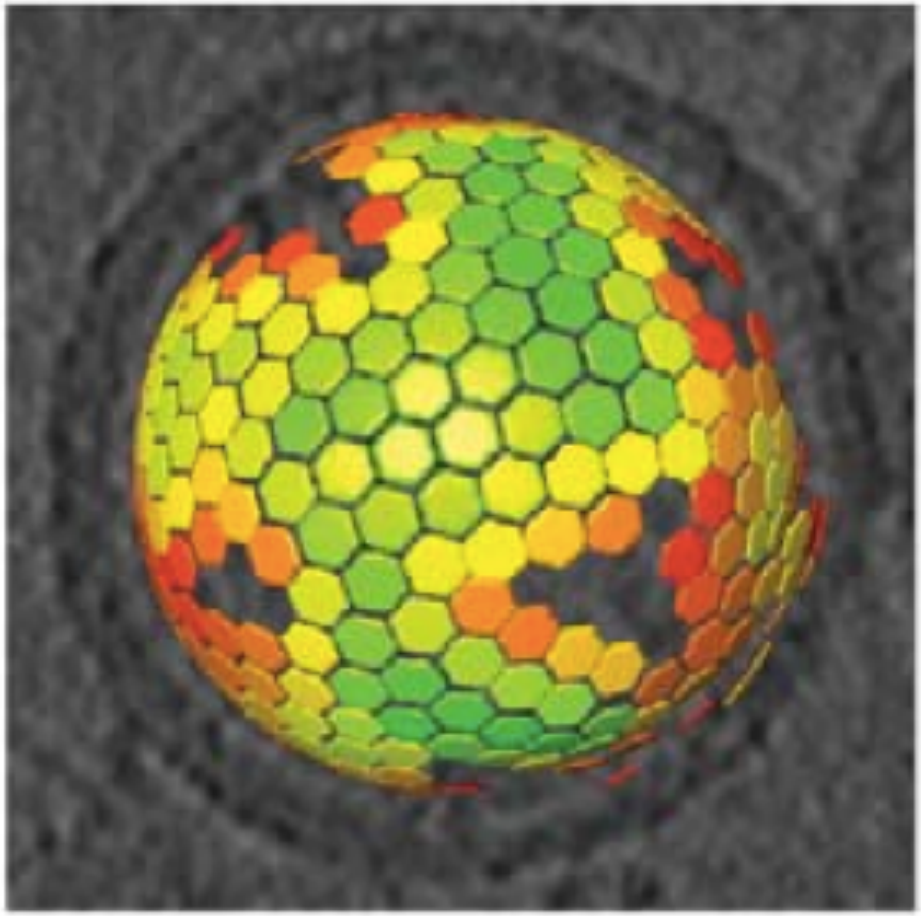
Higher total electron dose

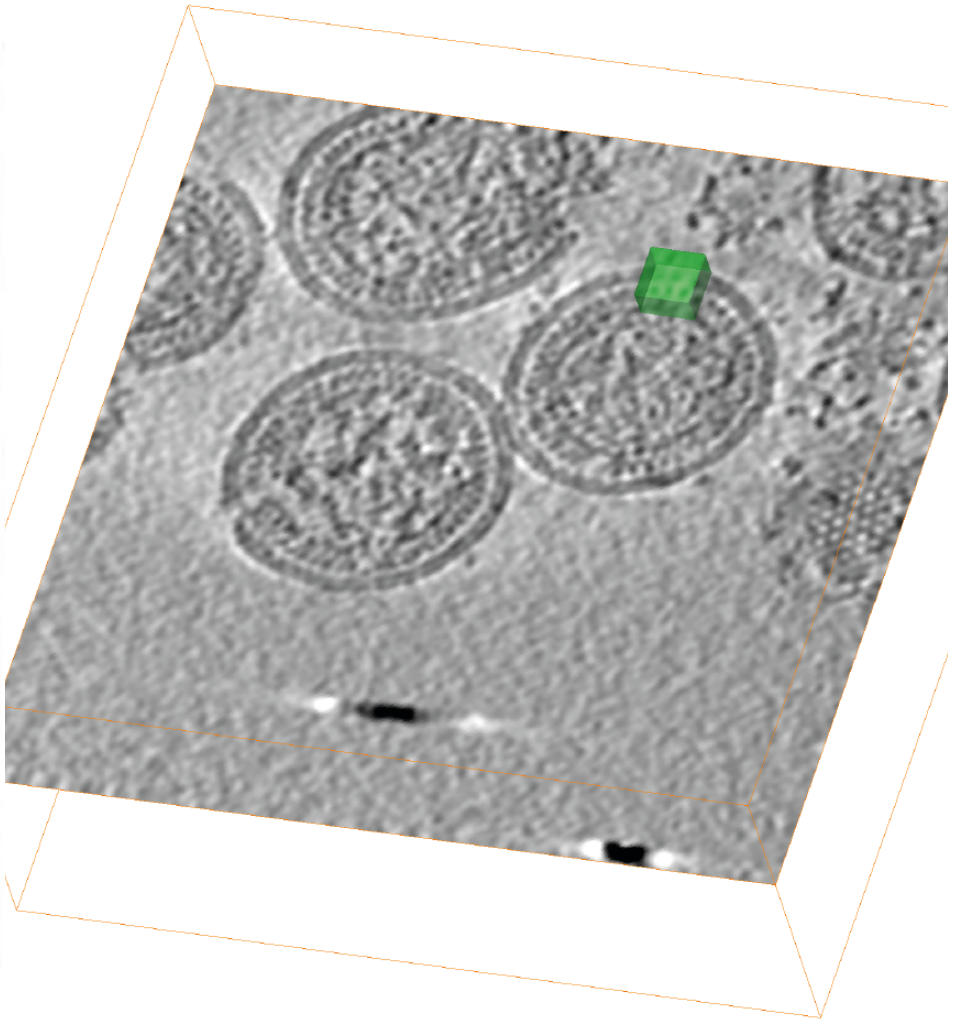
Smaller datasets (due to more time-consuming data collection)

Difficult in determination of defocus in individual images

Data Collection and image processing

Why do we do subtomogram averaging?





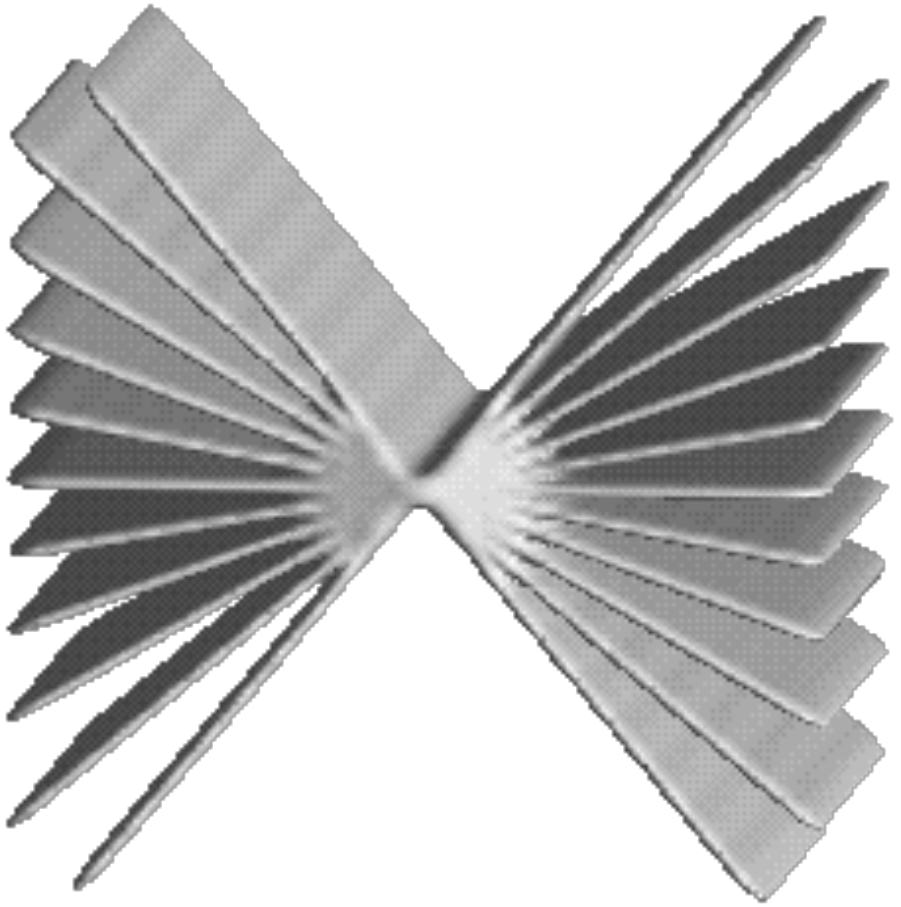
Data Collection and image processing

High-tilt images contain less high-resolution information (thicker sample, and higher accumulated dose)

They are needed to determine alignment

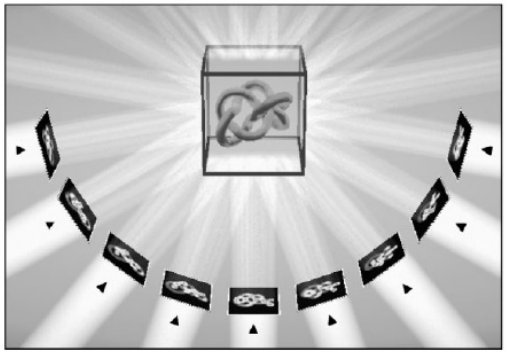
They can be excluded from the final average (or appropriately weighted).

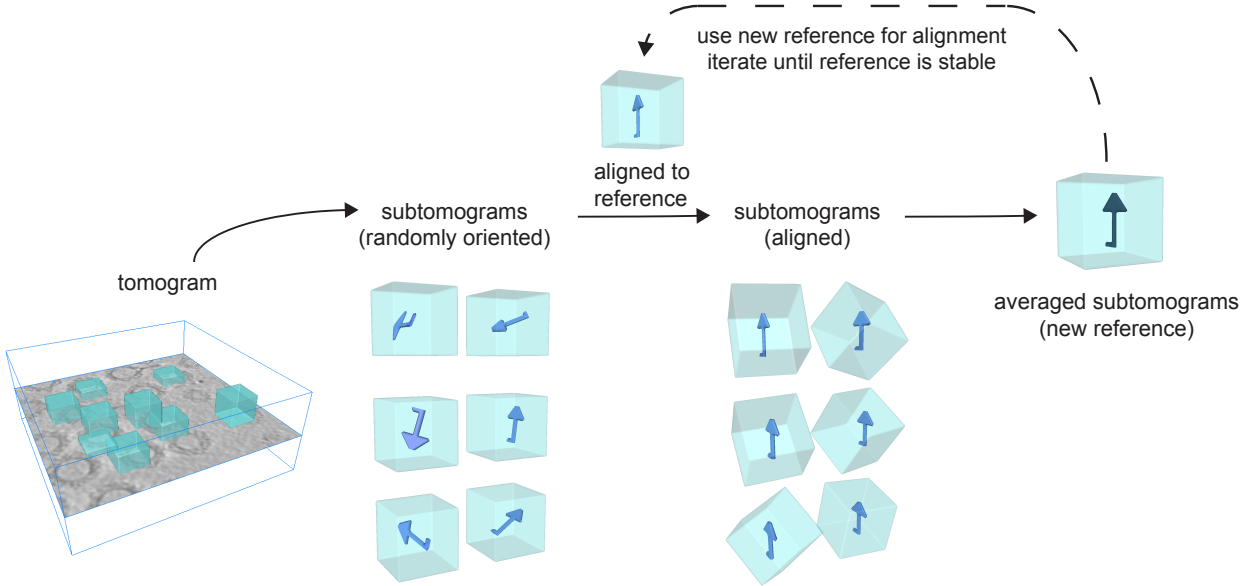
(related to movie processing for single particle)



Data Collection and image processing

A two step process





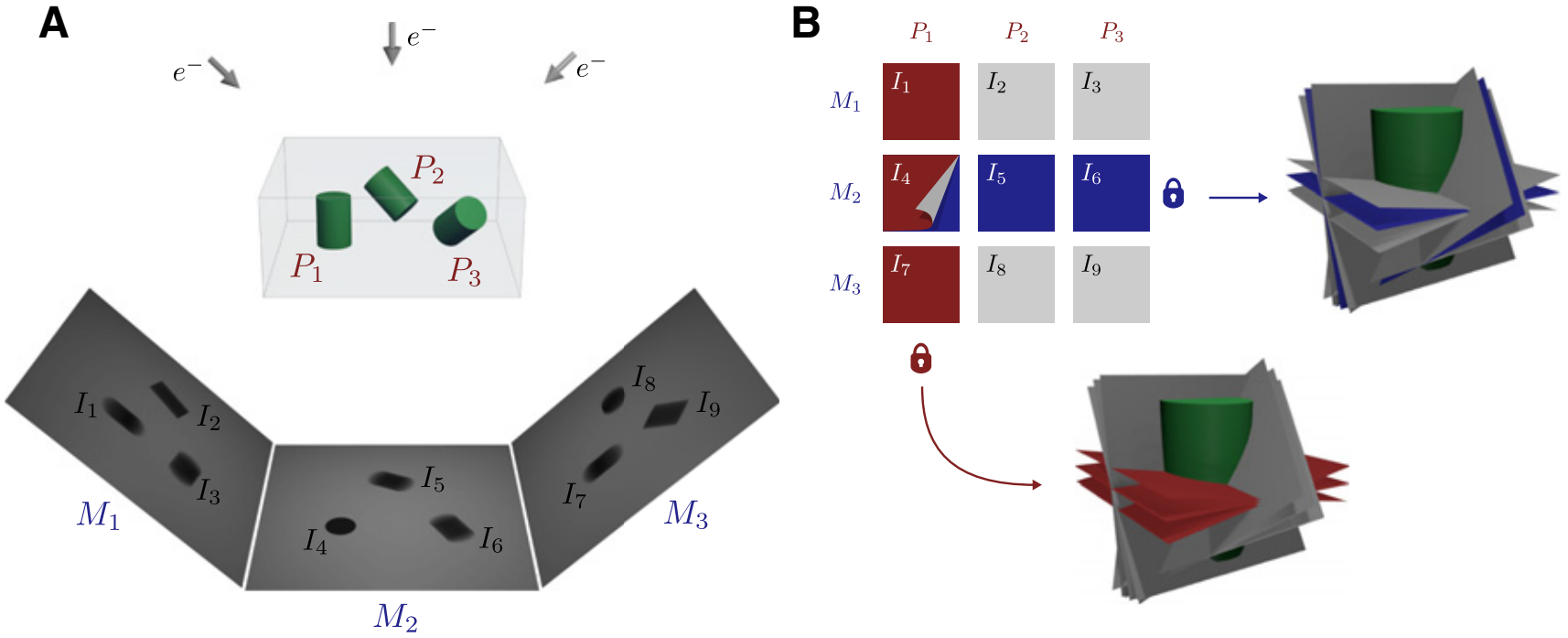
Data Collection and image processing

The final reconstruction can be recalculated from the original tilts to avoid interpolation errors from two reconstruction steps.

But – the alignment of the subtomograms is still dependent on the quality of the alignment of the tomogram.

Can be iterated, or in some cases may be able to do later alignment of individual tilts

“Constrained single-particle tomography”



Bartesaghi et al. 2012

Data Collection and image processing

So we need to

Identifying the optimal data-collection and reconstruction approach.

This will be a hybrid approach, and the optimal approach will depend on both the sample and detector.

It will be hard to overcome the problem of the sample changing during data collection.

3D classification/heterogeneity

Challenges in Cellular Structure Determination

7. The Future

The Future

Improvements in sample preparation

Improvements in targeting and throughput

Development and optimization of hybrid SP/ tomo data processing methods (in ML framework) and understanding how to adapt them to different samples.

Will benefit from technical improvements (detectors, phase plates etc)

Acknowledgements

Organisers (for telling me what to talk about)

My lab for discussions

Colleagues whose work I have shown (or not shown!)

Colleagues whose thoughts and discussions were found in the talk.