„Biological electron microscopy"

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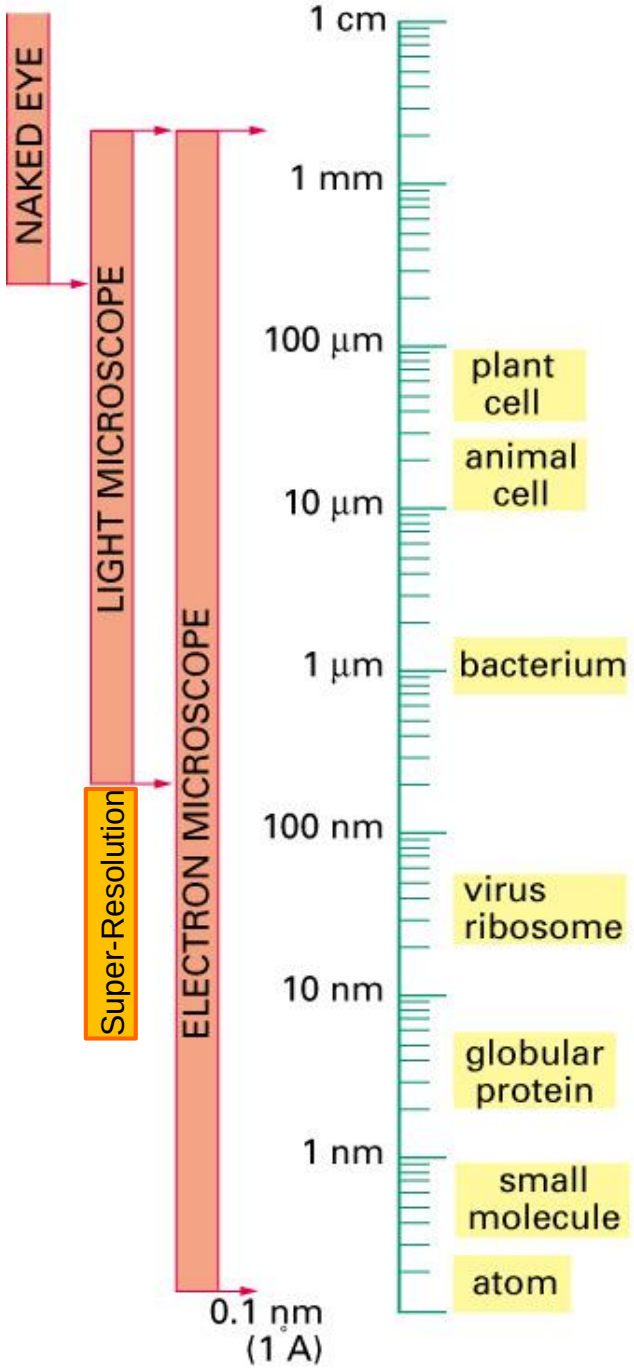
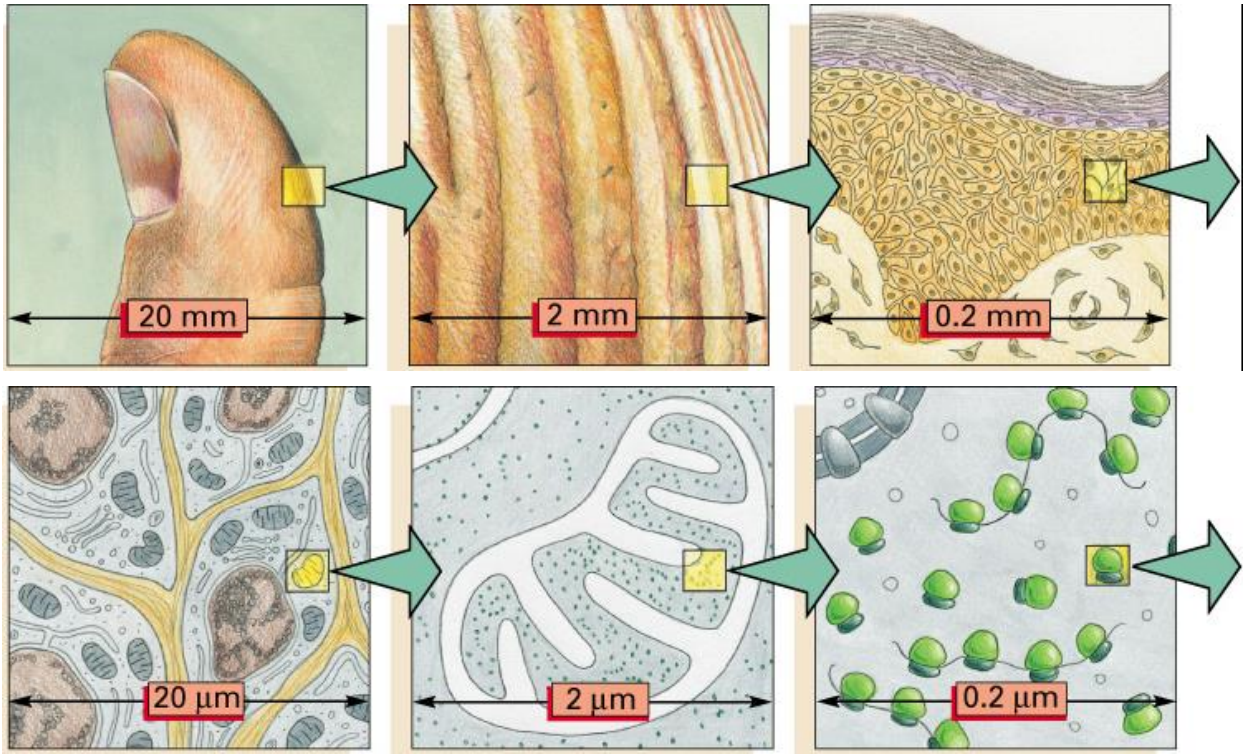
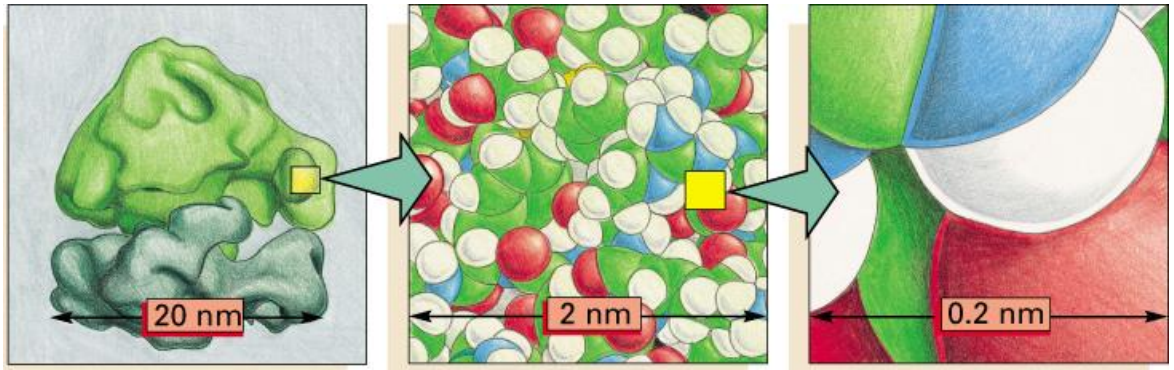
- Introduction: Scanning electron microscopy (SEM)
  Transmission electron microscopy (TEM)
- Analysis of cells and tissues by TEM
- Chemical fixation – cryofixation
- Immunoelectron microscopy
- Correlative microscopy
- 3D electron microscopy
- Analytical microscopy

Microscopy





Light microscope (LM) TEM →



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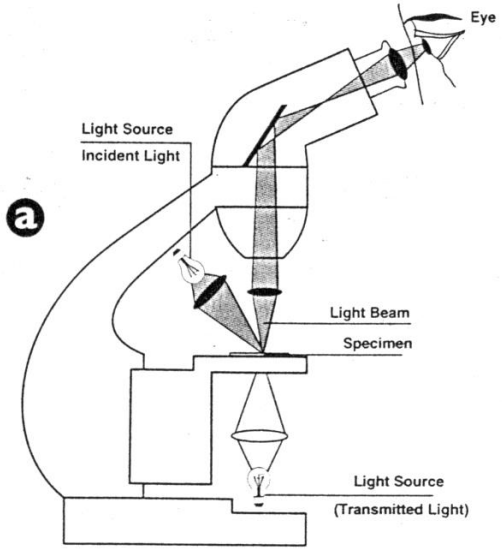
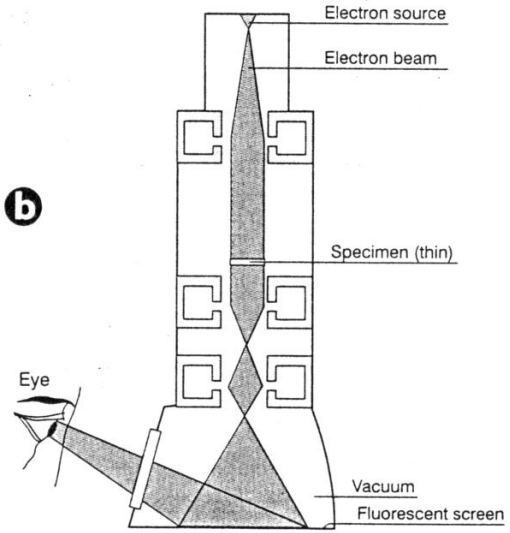
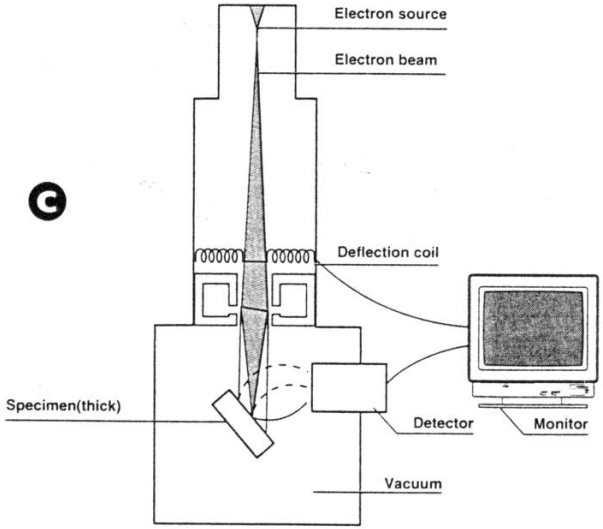
Microscopes

electron microscopes







Light microscope (LM)

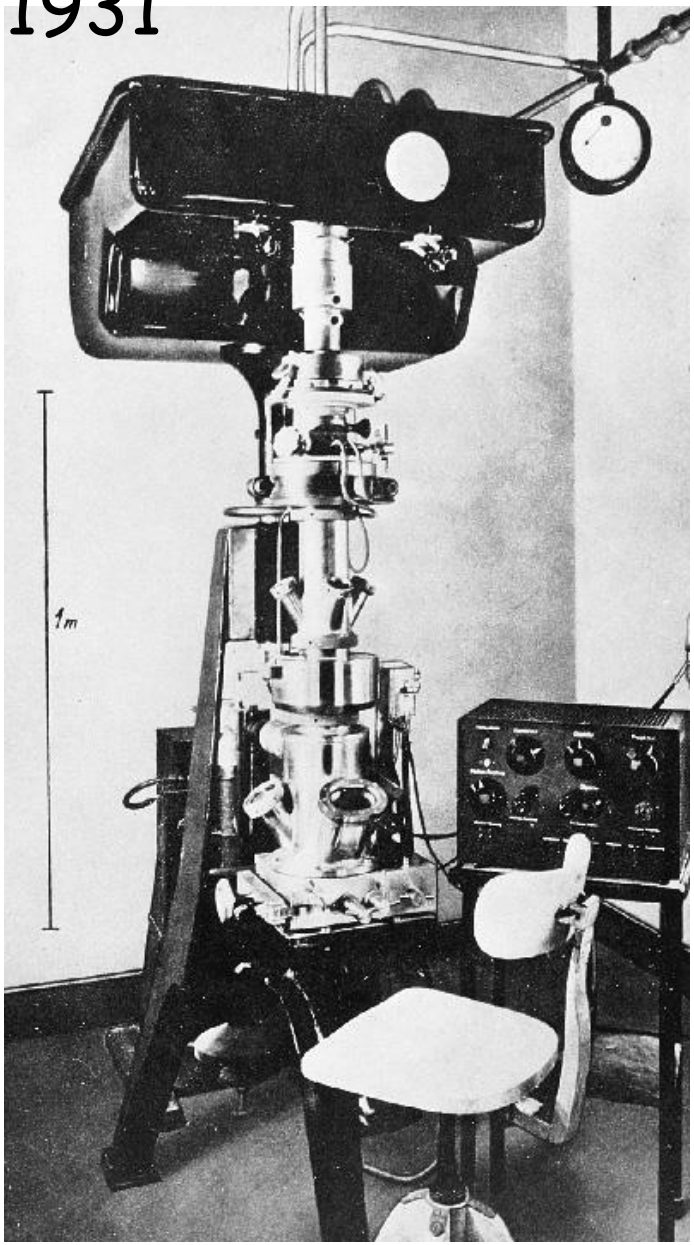
Transmission electron microscope (TEM)

Scanning electron microscope (SEM)

Plattner/Zingsheim 1987

Resolution:	0.2 µm (standard)	0.2 nm	
Wave length:	400 – 800 nm	0.002 nm (300 kV)	

The beginning of new era in microscopy and cell biology: First TEMs 1931

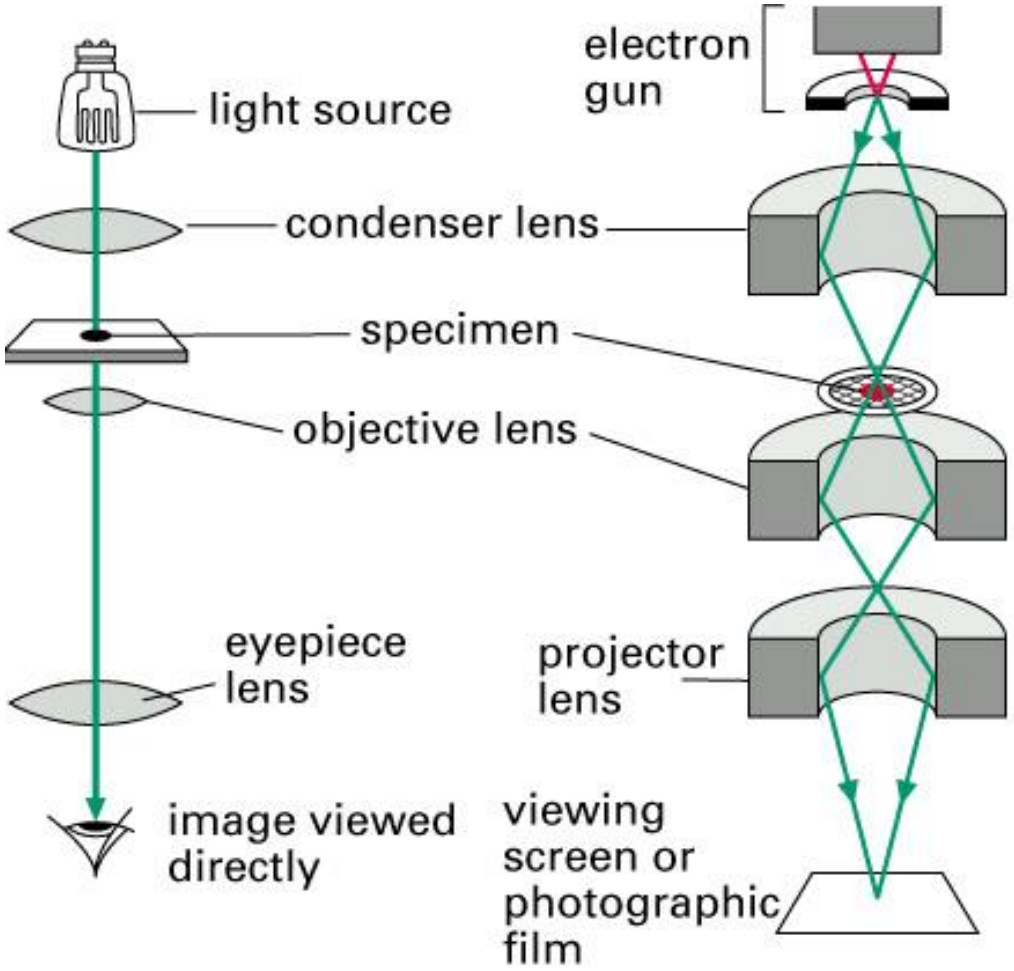


Max Knoll & **Ernst Ruska**
1931.
Ernst Ruska 1986 Nobel Price
for Physics.

Light versus transmission electron microscope

Light microscope (LM)

Transmission electron microscope (TEM)



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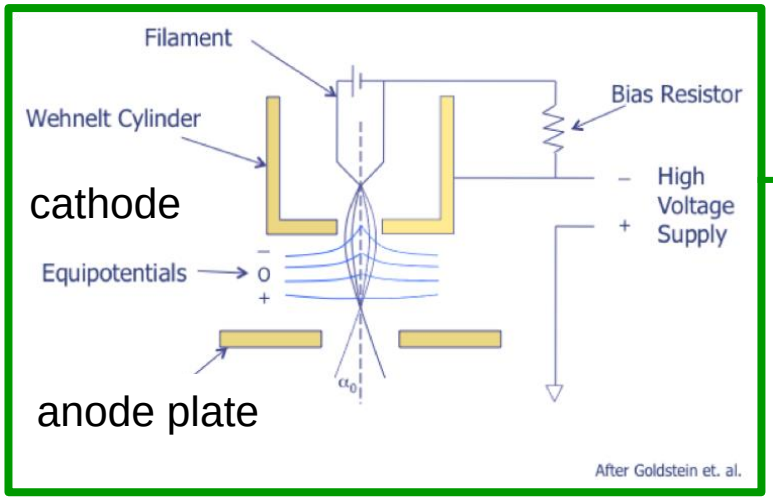
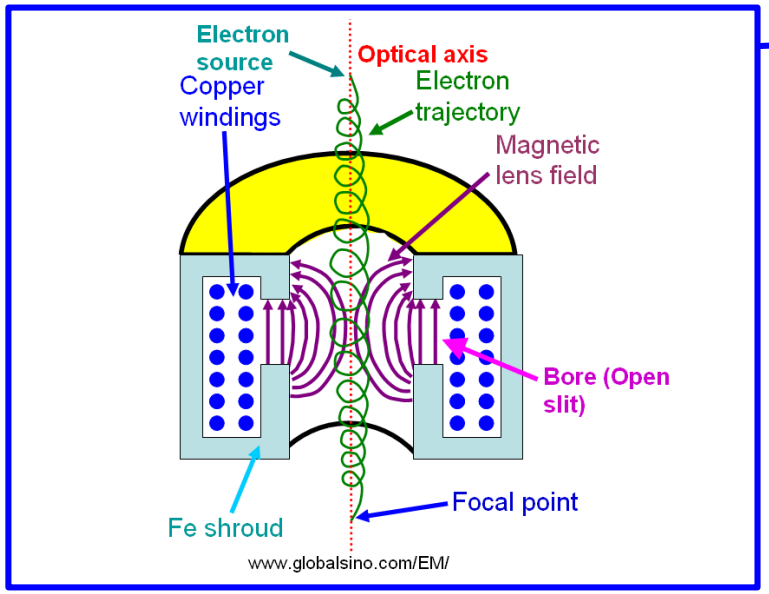
Transmission electron microscope

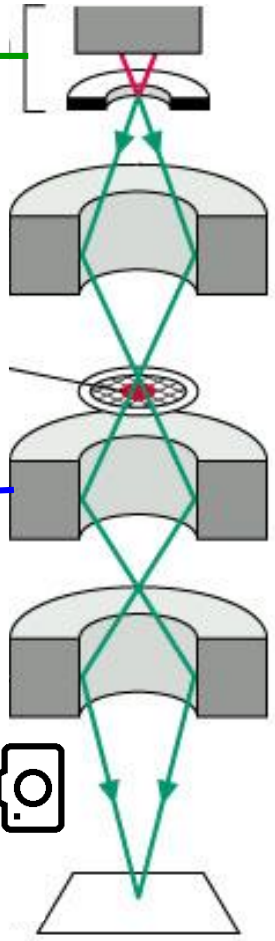
Electron gun & Wehnelt cylinder



10 -100 - 1000 kV

Electromagnetic lens





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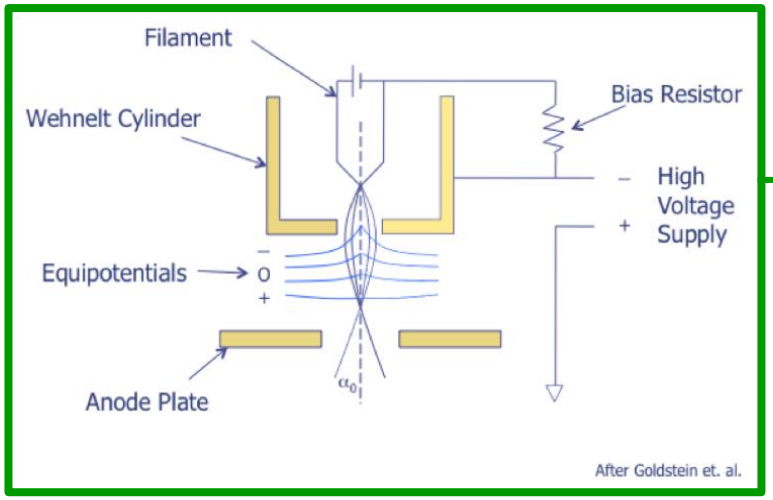
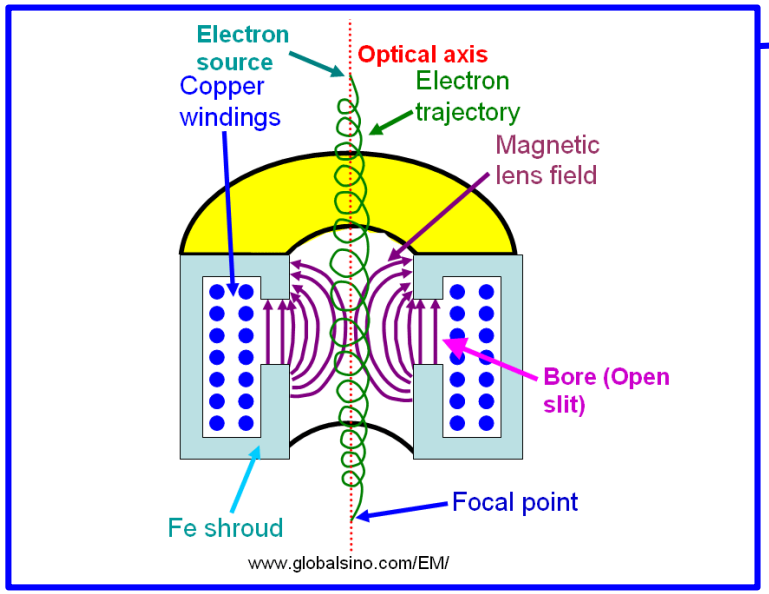
CCD cameras

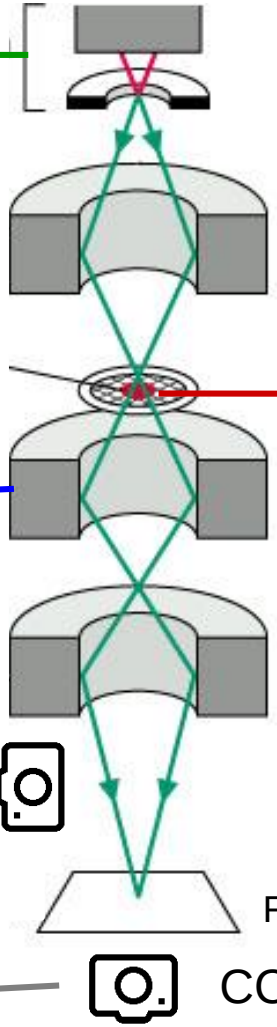
Transmission electron microscope

Electron gun & Wehnelt cylinder

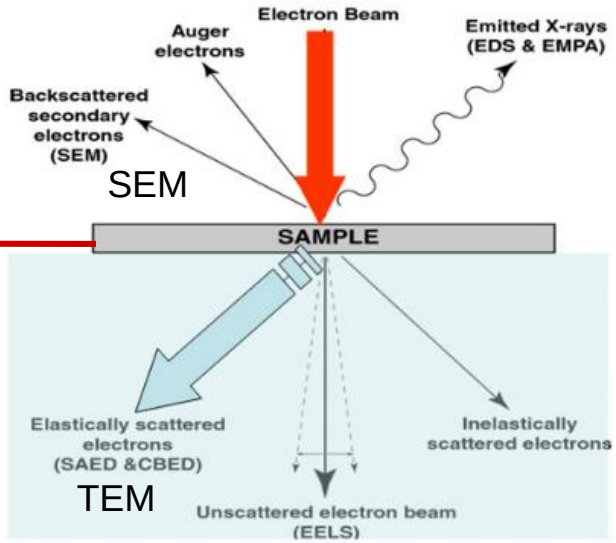


Electromagnetic lens





Electron beam speciamen Interaction

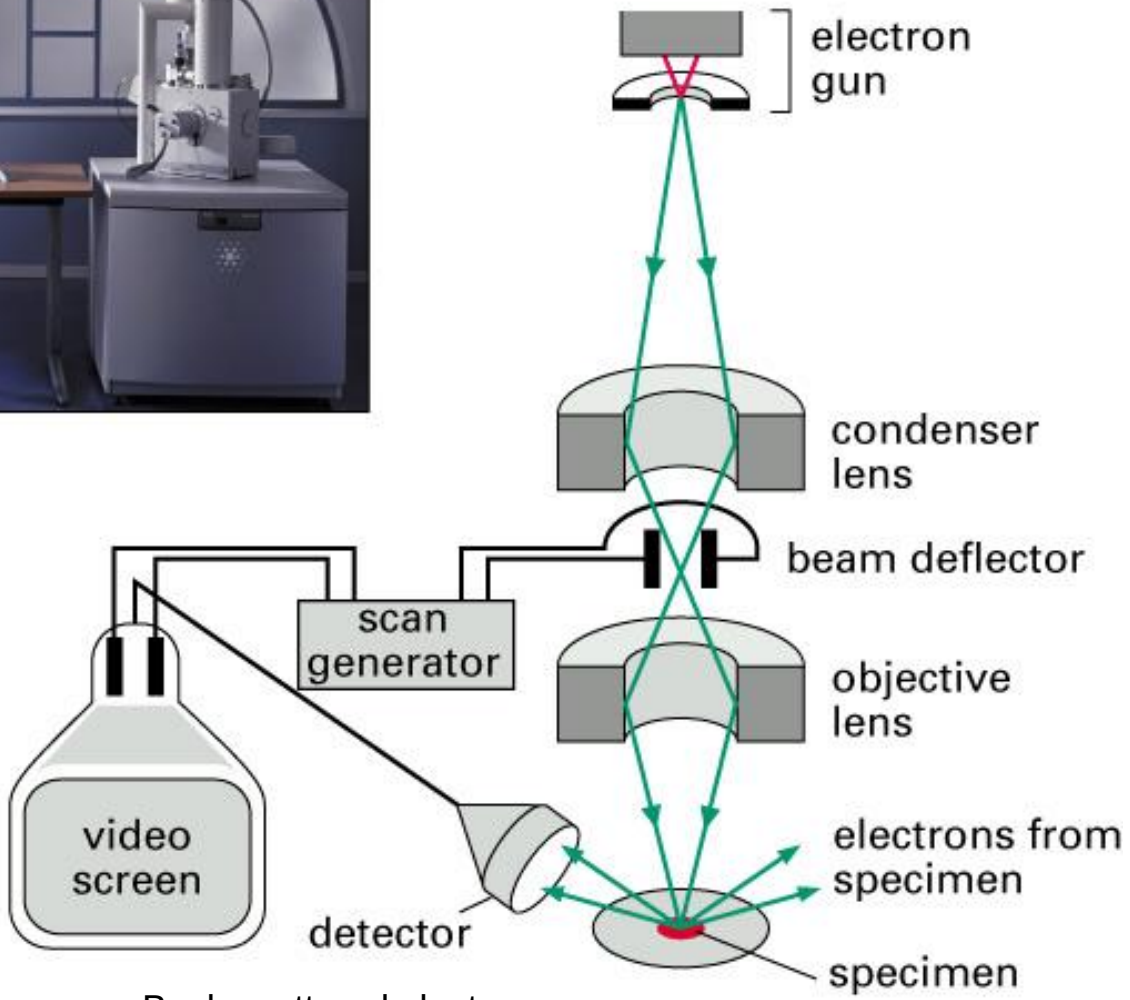


Elastic scattered electrons

Fluorescent screen

CCD cameras

Scanning Electron Microscope (SEM)



Back scattered electrons

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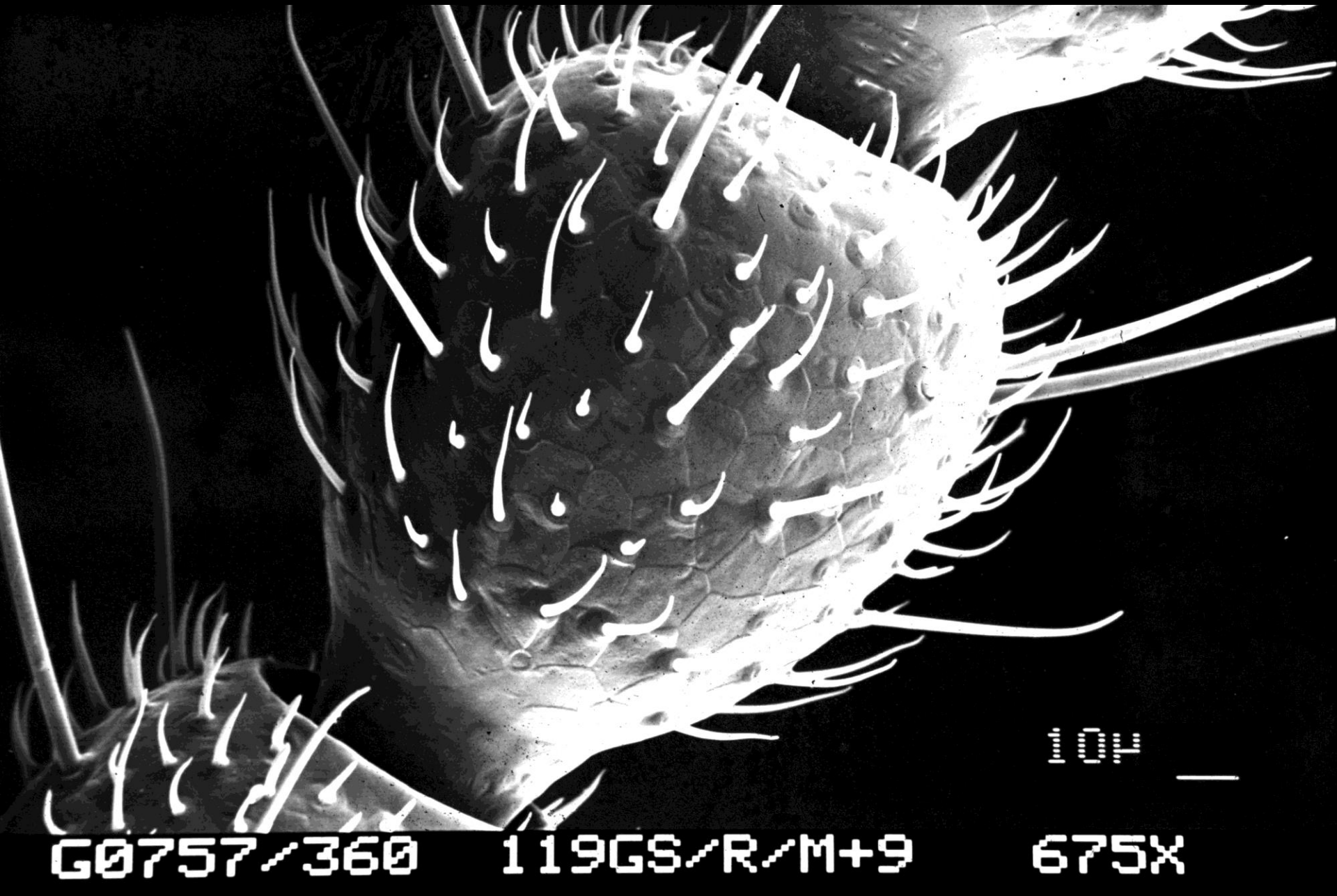
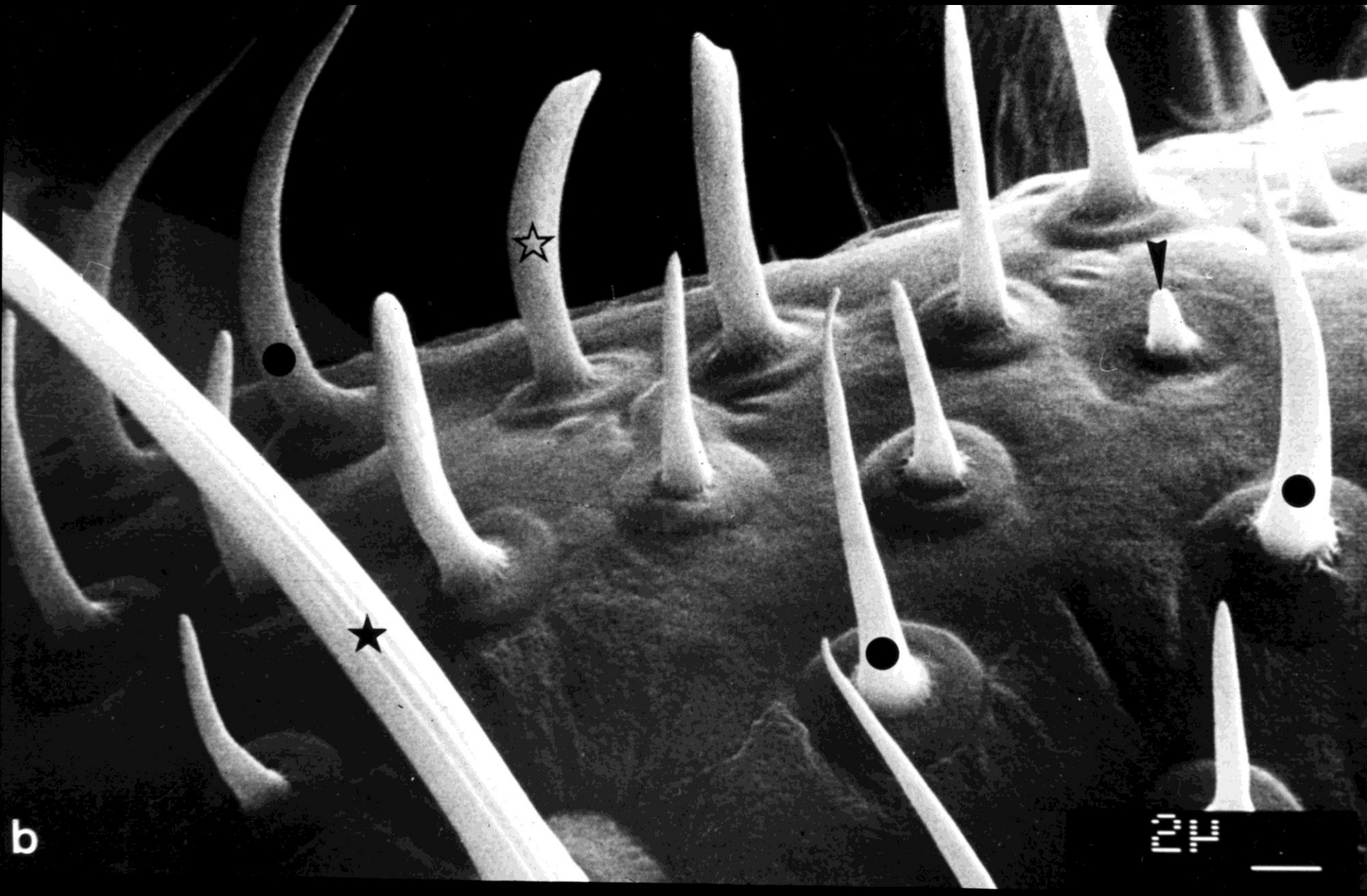
Schedorhinotermis lamanianus (Isoptera)

Ivory Coast

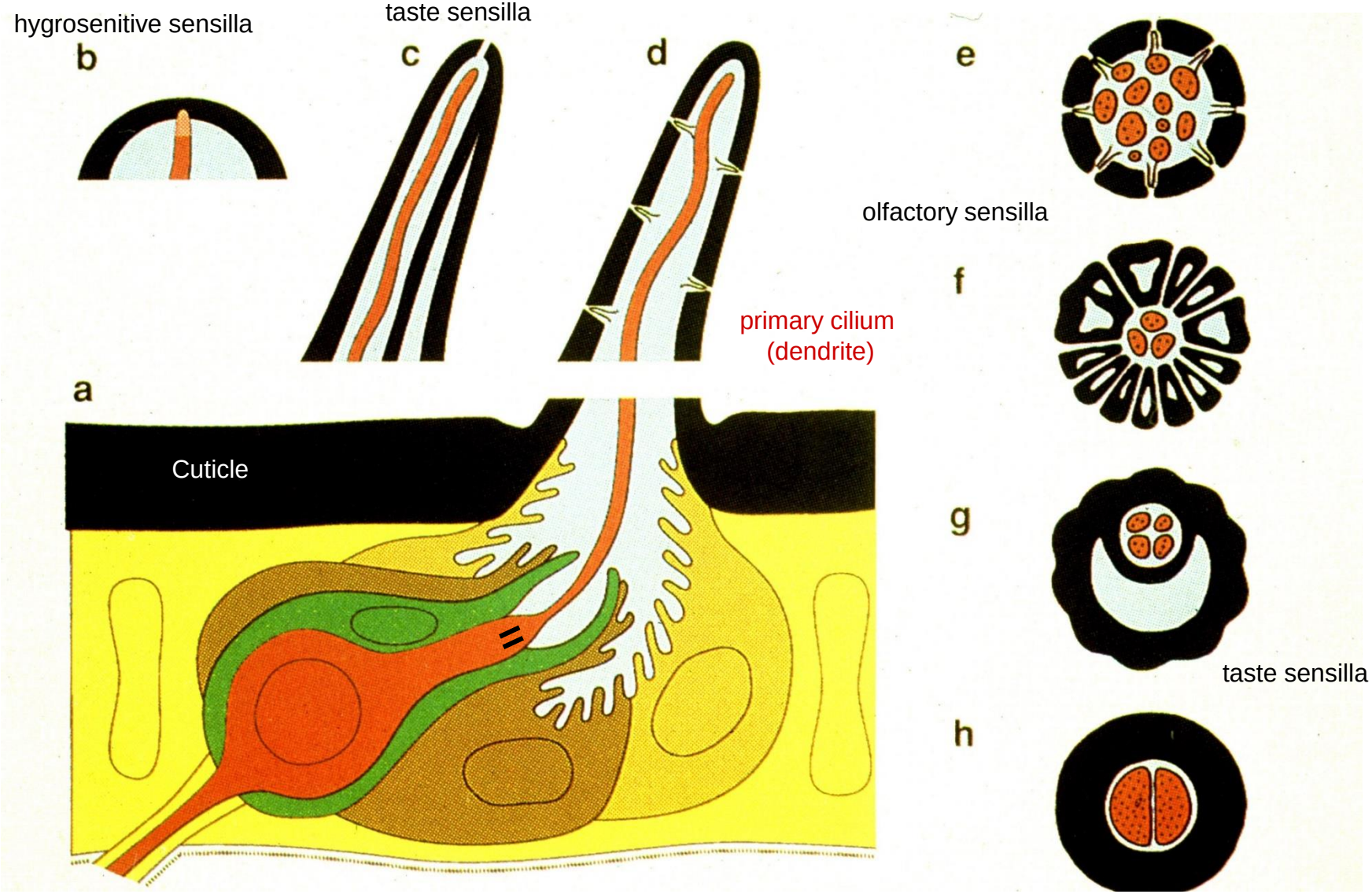
9th flagellar segment of *S. lamanianus* (l. soldier)



Cuticular hair sensilla of *S. lamanianus*



Cuticular hair sensilla



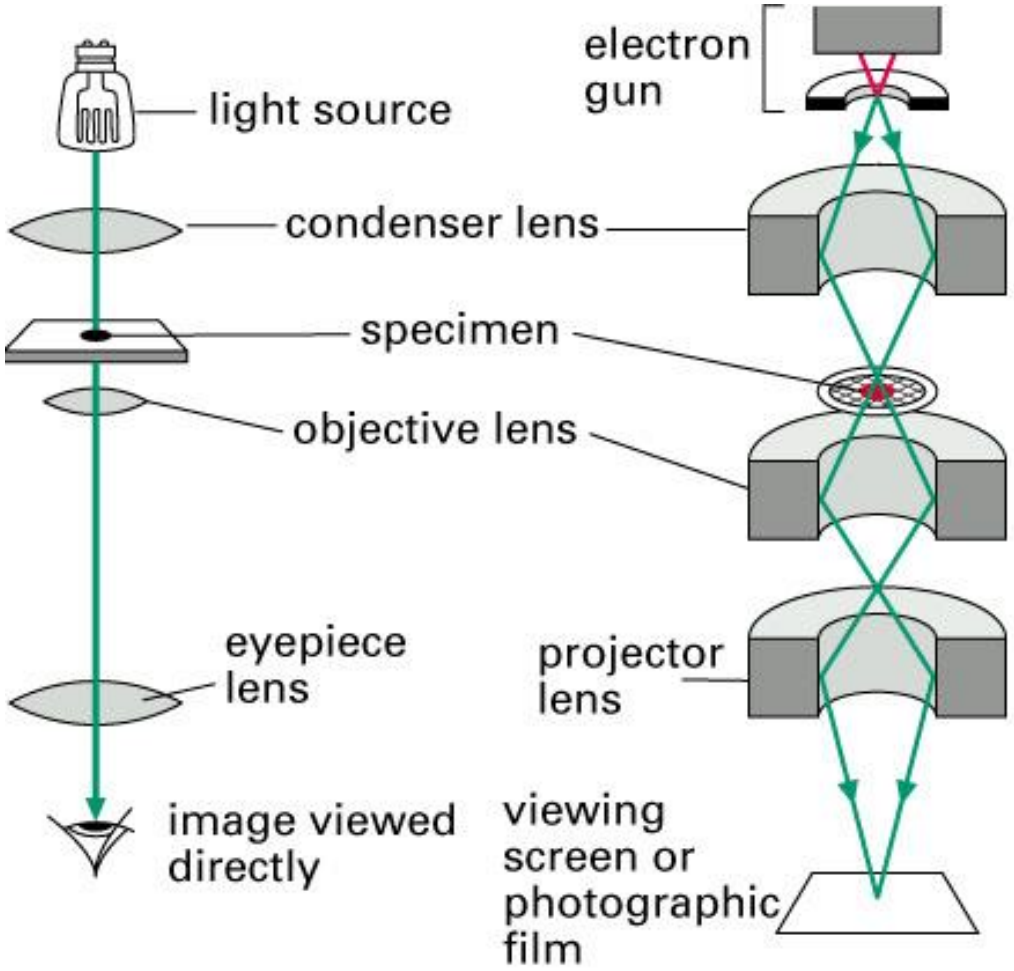
Sensory cell/neuron with primary cilium (dendrite)

Steinbrecht 1980

Light versus transmission electron microscope

Light microscope (LM)

Transmission electron microscope (TEM)



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Molecular Biology of the Cell, 4th Edition.

Major aims in biological electron microscopy

- **Resourcing the basis for the correlation of structure and function in biologic species.**

- Preservation of cellular composition as closely as possible to the living state.

- Subcellular localization of
  - macromolecules/antigenes
    (cytochemistry, immunoelectron microscopy)
  - molecules and ions
    (analytical electron microscopy)

Major problems in biological electron microscopy

- Tissue dissection – preparation
- Samples have to resist high vacuum (10^{-4}-10^{-7} Pa) and strong electron beam.
- Water has to removed or bound.
- Subcellular analysis of tissues and cells requires ultrathin sections.

  … or cryosectioning

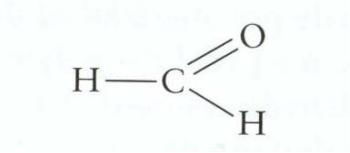
  – Embedding in epoxid or metacrylate resins
  – Dehydration with organic solvents

→ **chemical or physical (cryo)fixation**

Chemical fixation

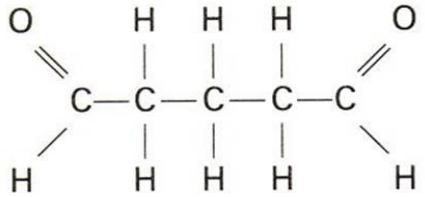
1. Fixatives

• Aldehydes – fixate proteins



a. Paraformaldehyd (PFA) polymer of formaldehyd

- weak fixation – water soluble
- small molecule → quick penetration
- often not suitable alone as fixative in biological electron microscopy.

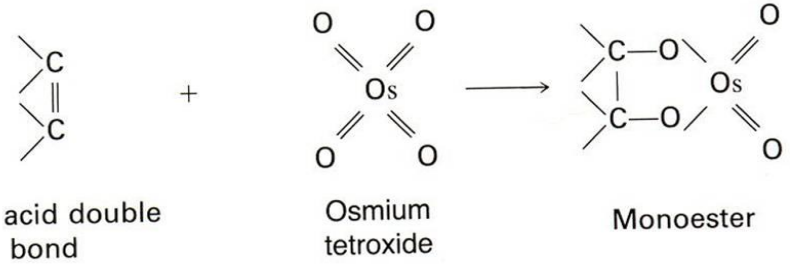


b. Glutaraldehyd (GA)

- irreversible fixation – two binding sites (suitable for cross-linking of protein)
- ~ large molecule → slow penetration

• Osmium tetroxid (OsO_4) fixate lipids

- reacts with double bounds of non saturated fatty acids:



All fixatives are highly toxic !!!!

….- dehydration – "plastic" embedding

2. Dehydration

Water soluble organic solution – **acetone** – manly **ethanol** (EM grade) – graduate increasing concentrations towards 100%

Intermedium Propylenoxid is mixable with ethanol and epoxid resins.

3. Embedding in resins:

e.g. **Epoxid resins** (e.g. Araldite, Epon, Dorcopan)
conventional TEM analysis
heat polymerization (~ 65°C)

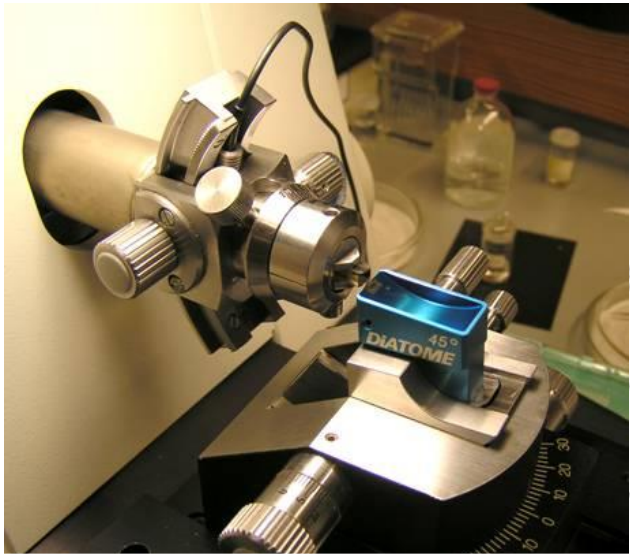
Metacrylate resins (e.g. LR-white, LR-gold, Lowicryls)
ImmunoTEM (slightly hydrophilic)
heat polymerization (~ 60°C)
UV polymerization in the cold (4°C - -70°C) !

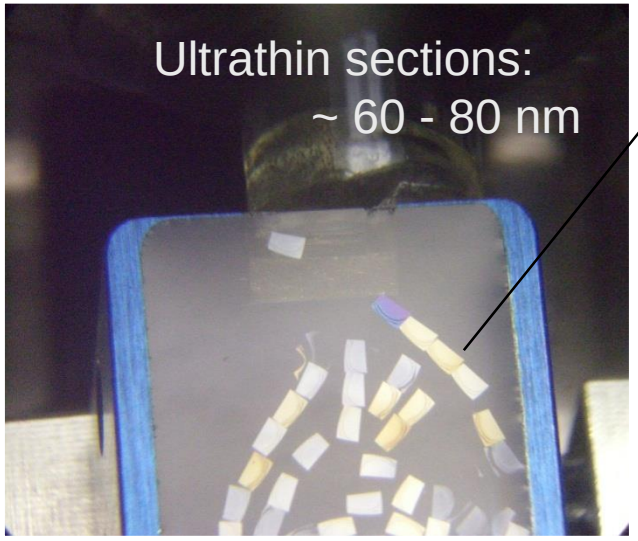
5. Ultrathin sectioning & counter staining:

on grid staining with lead citrate & uranyl acetate – better contrast

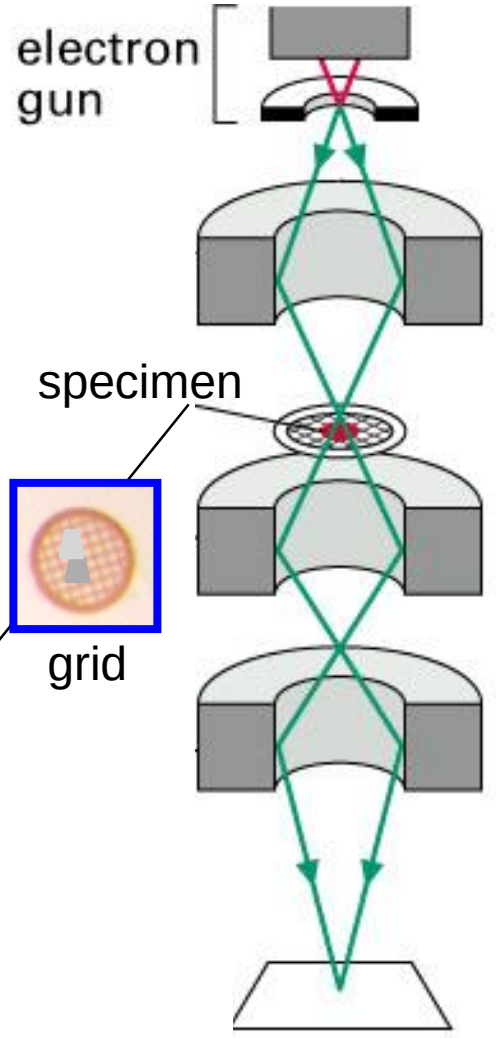
Transmission electron microscope

Ultramicrotomy





Transmission electron microscope (TEM)



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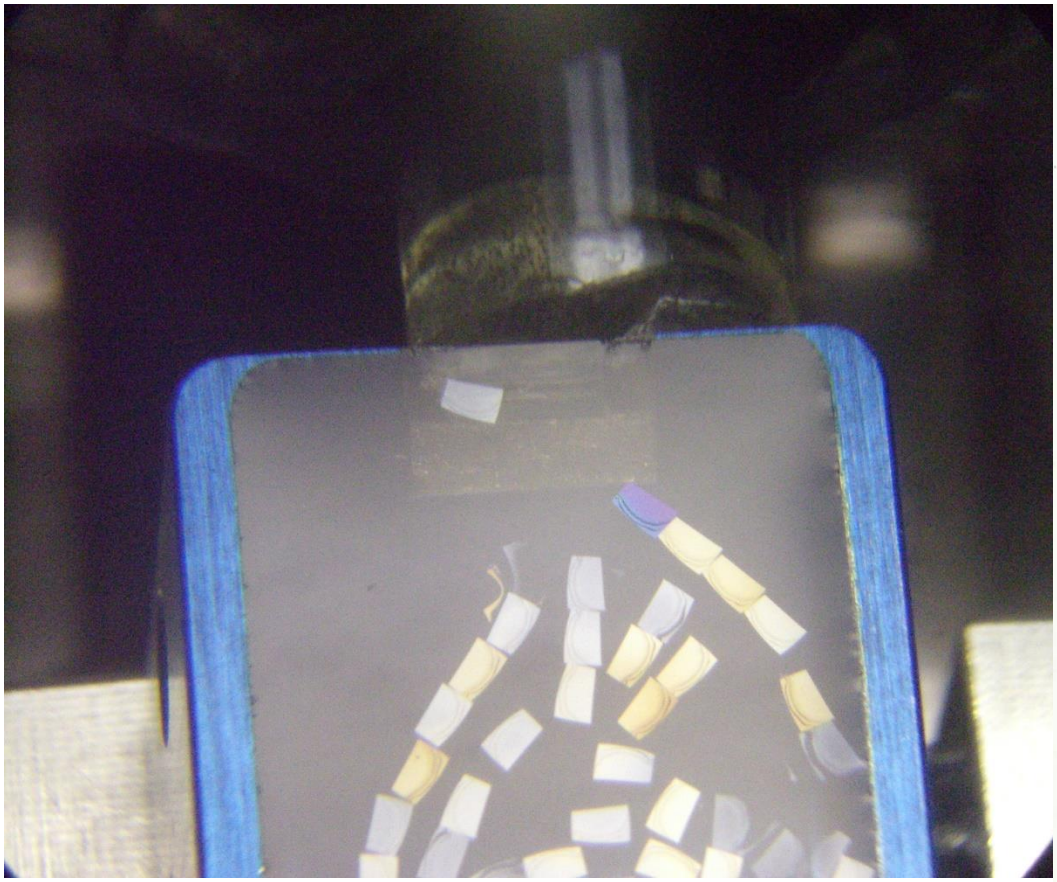
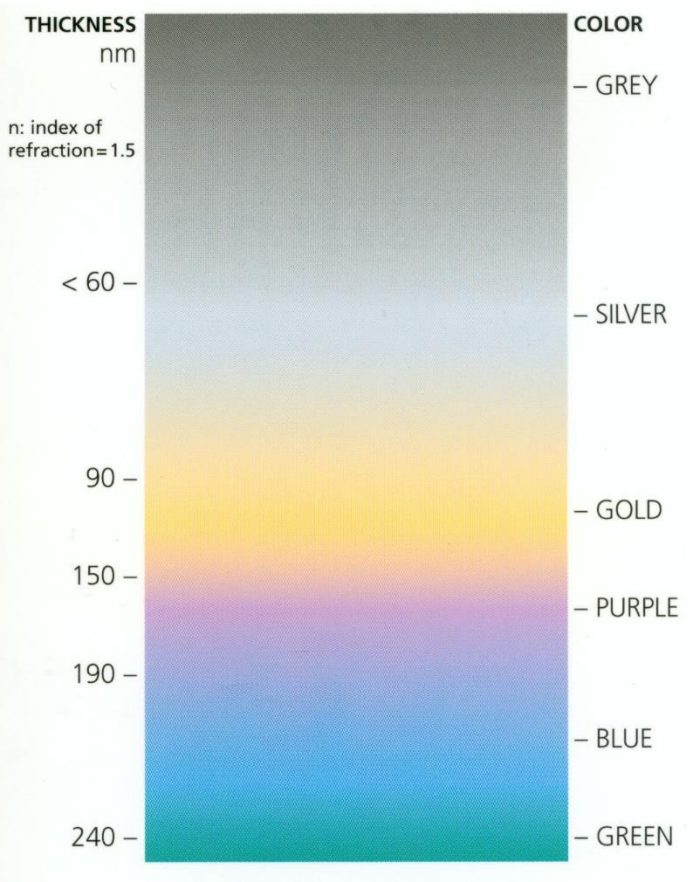
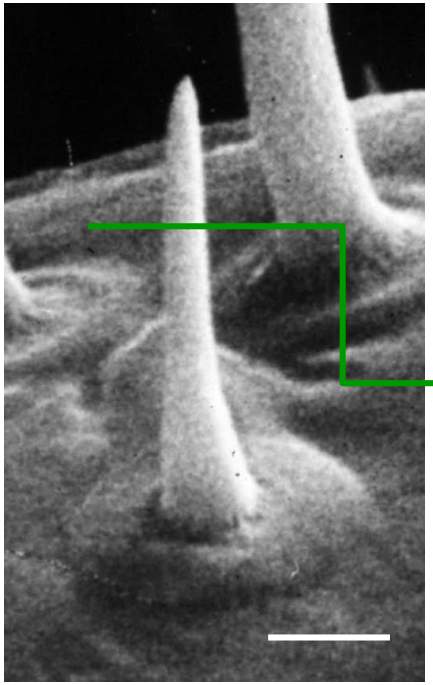
Ultramicrotomy - sectioning



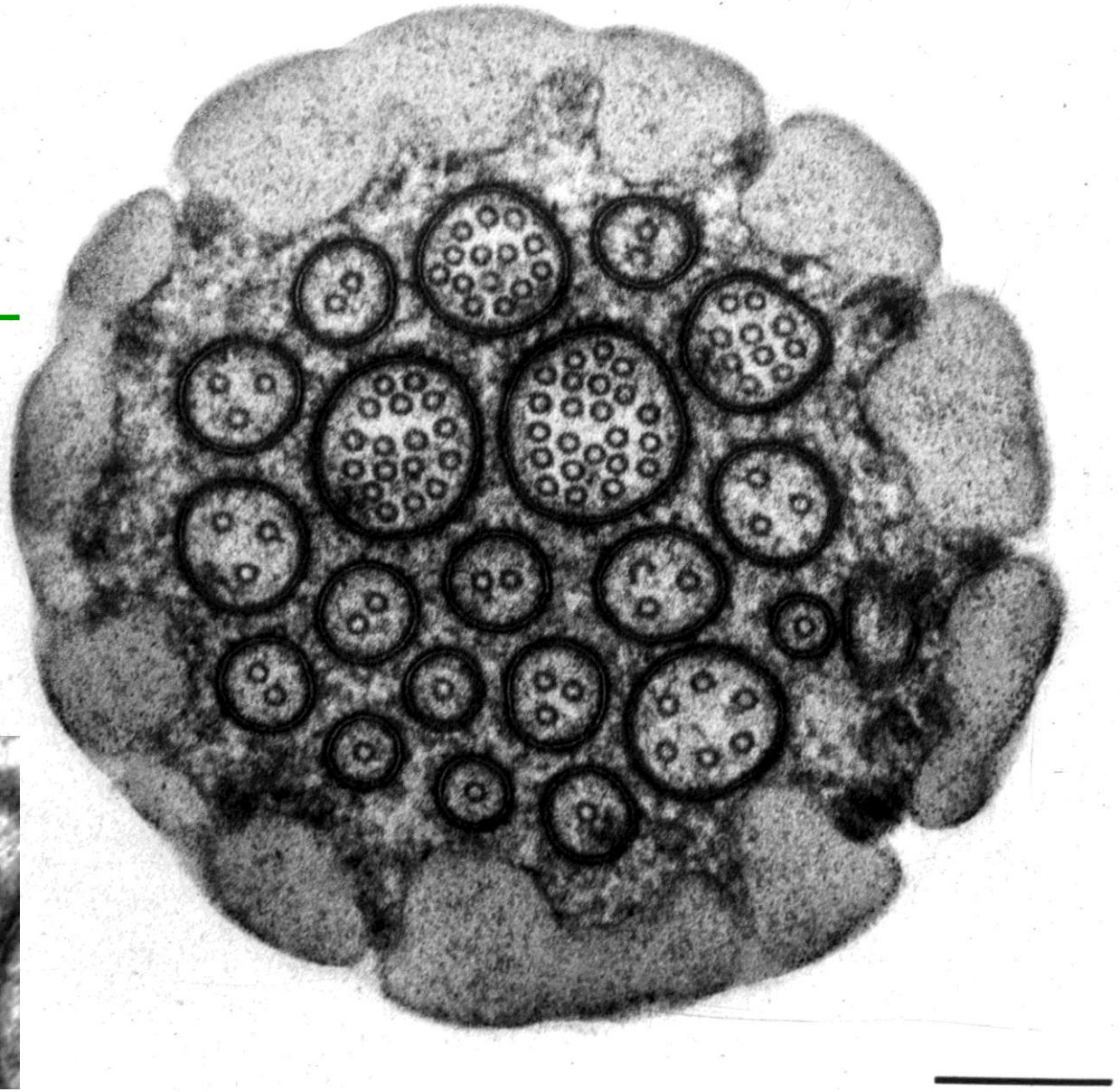


- Ultrathin sections on the surface of the water vat of a diamond knife.
- Interference colors indicate thickness of sections.
- Fishing of sections and transfer to a grid

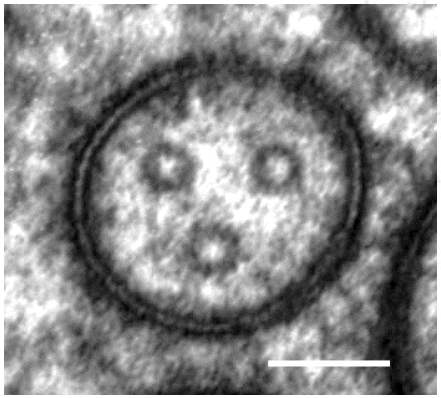
SEM



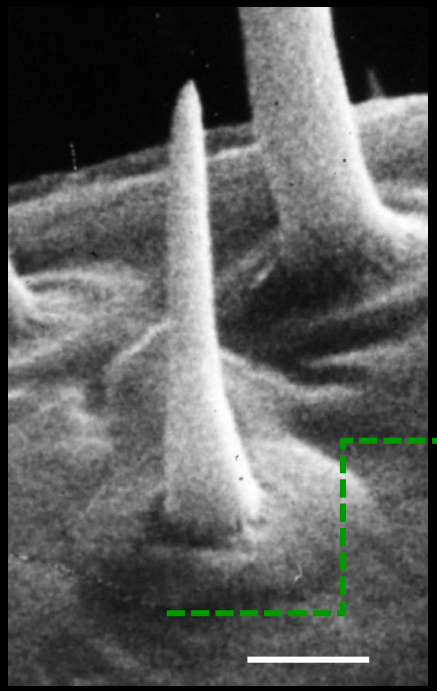
TEM



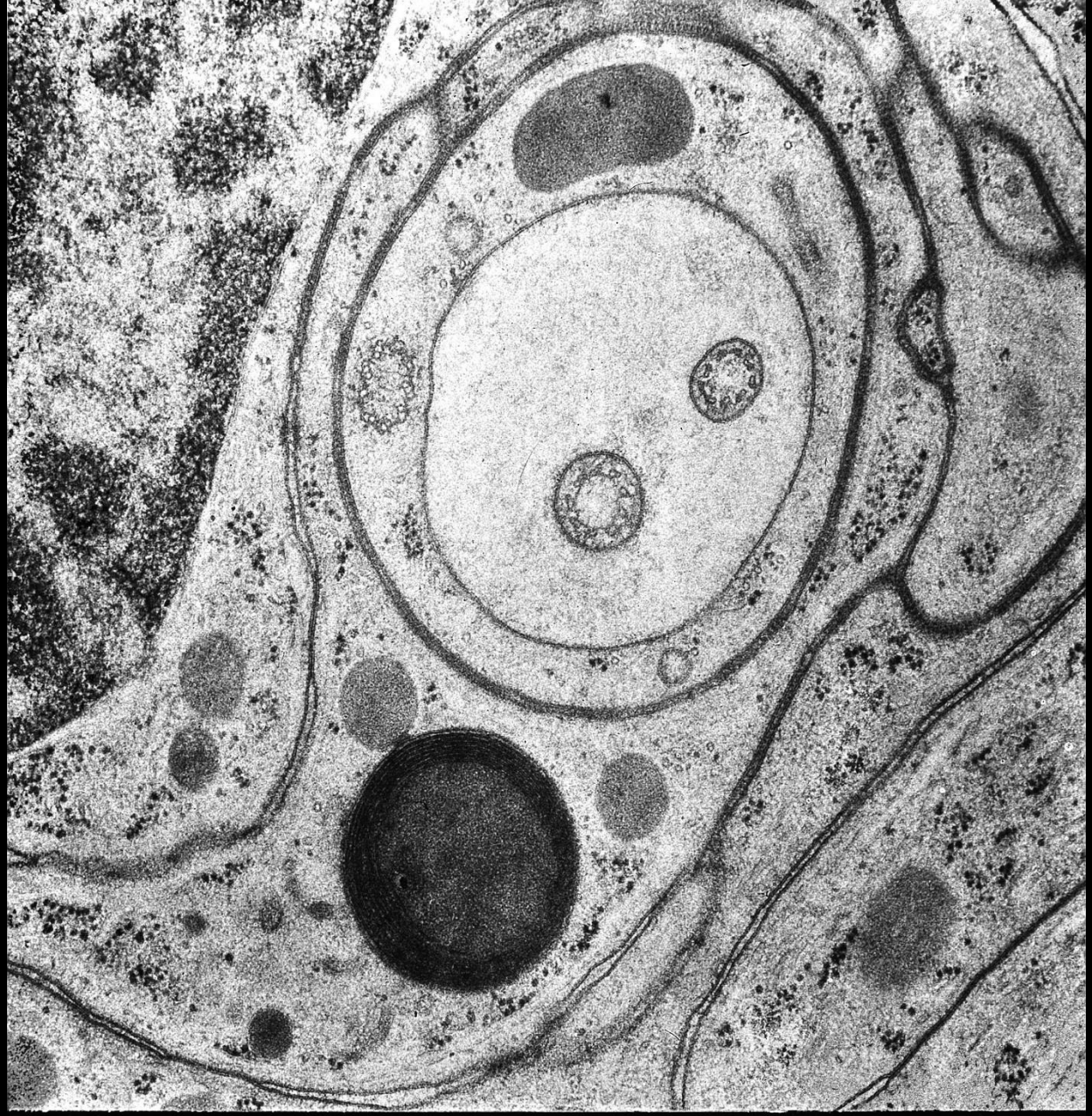
zoom



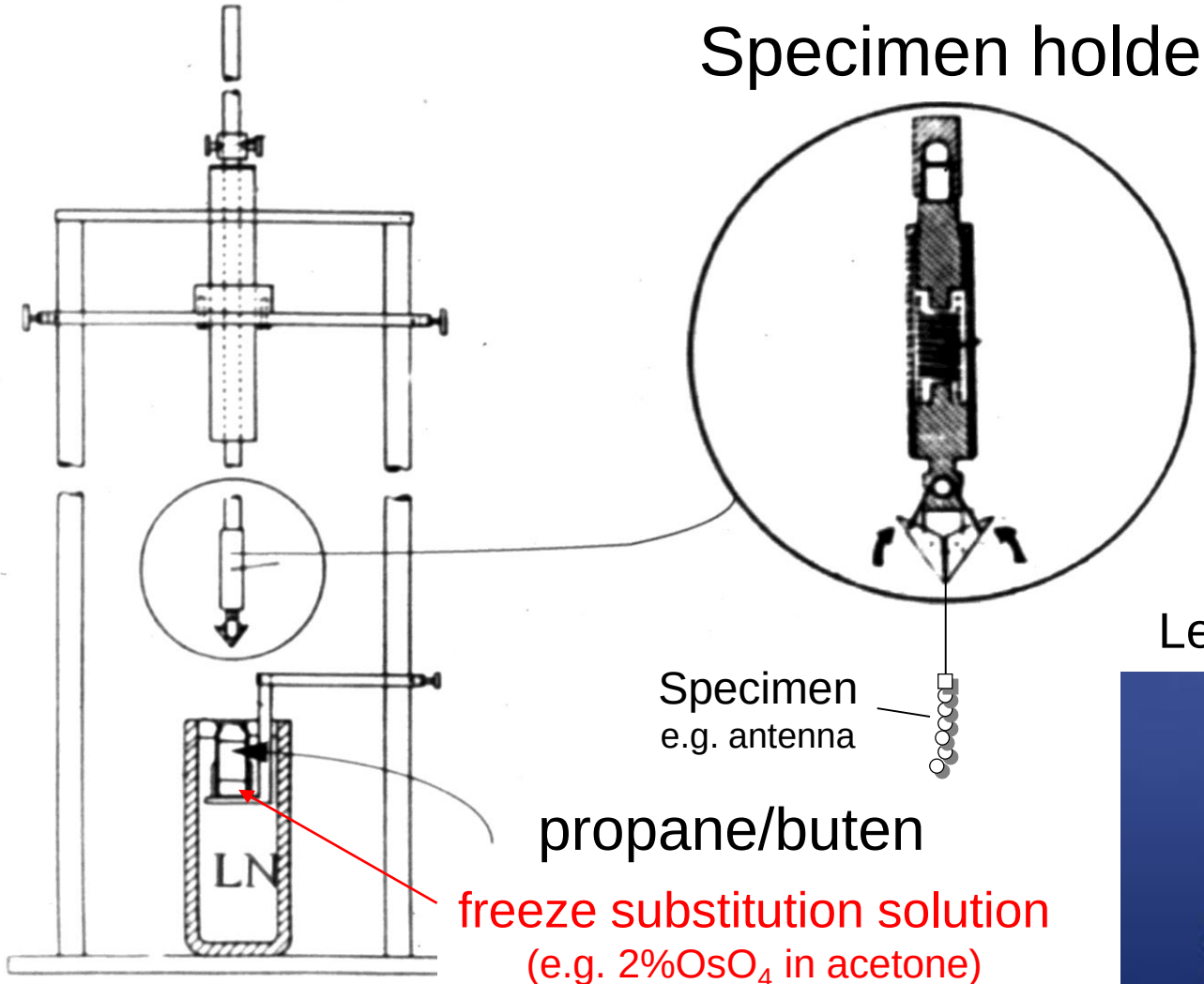
SEM



TEM



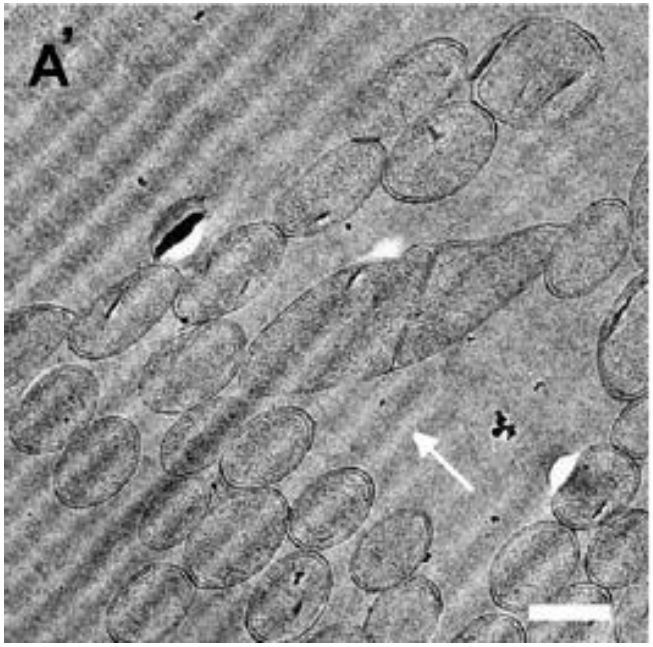
Plunge freezing at ambient pressure

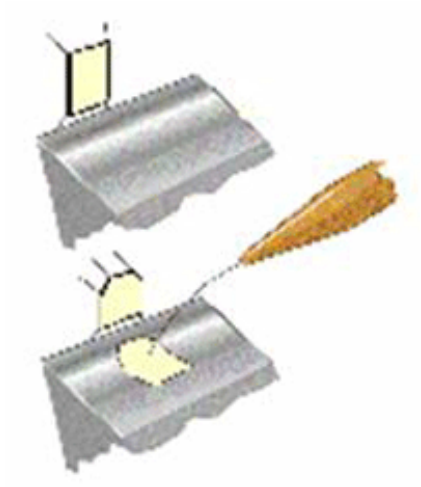


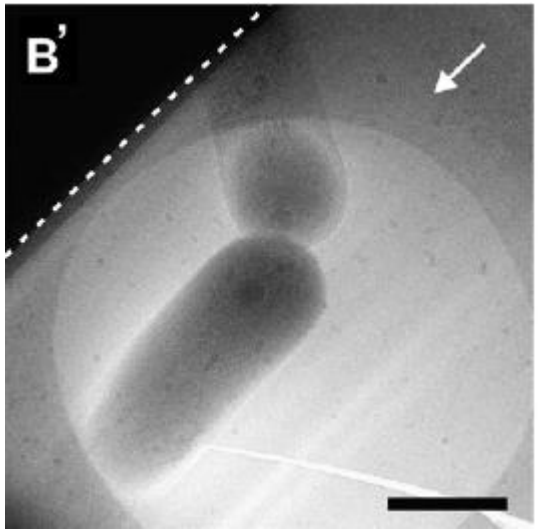
Leica slam freezer

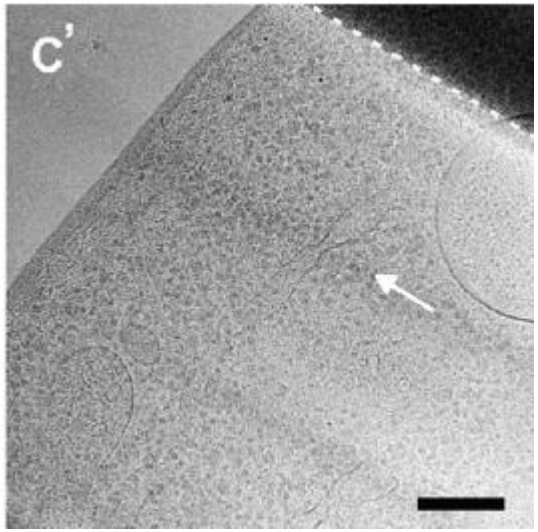
Cooling rates: 10^3 °C/sec

Ultramicrotomy – cryo sectioning









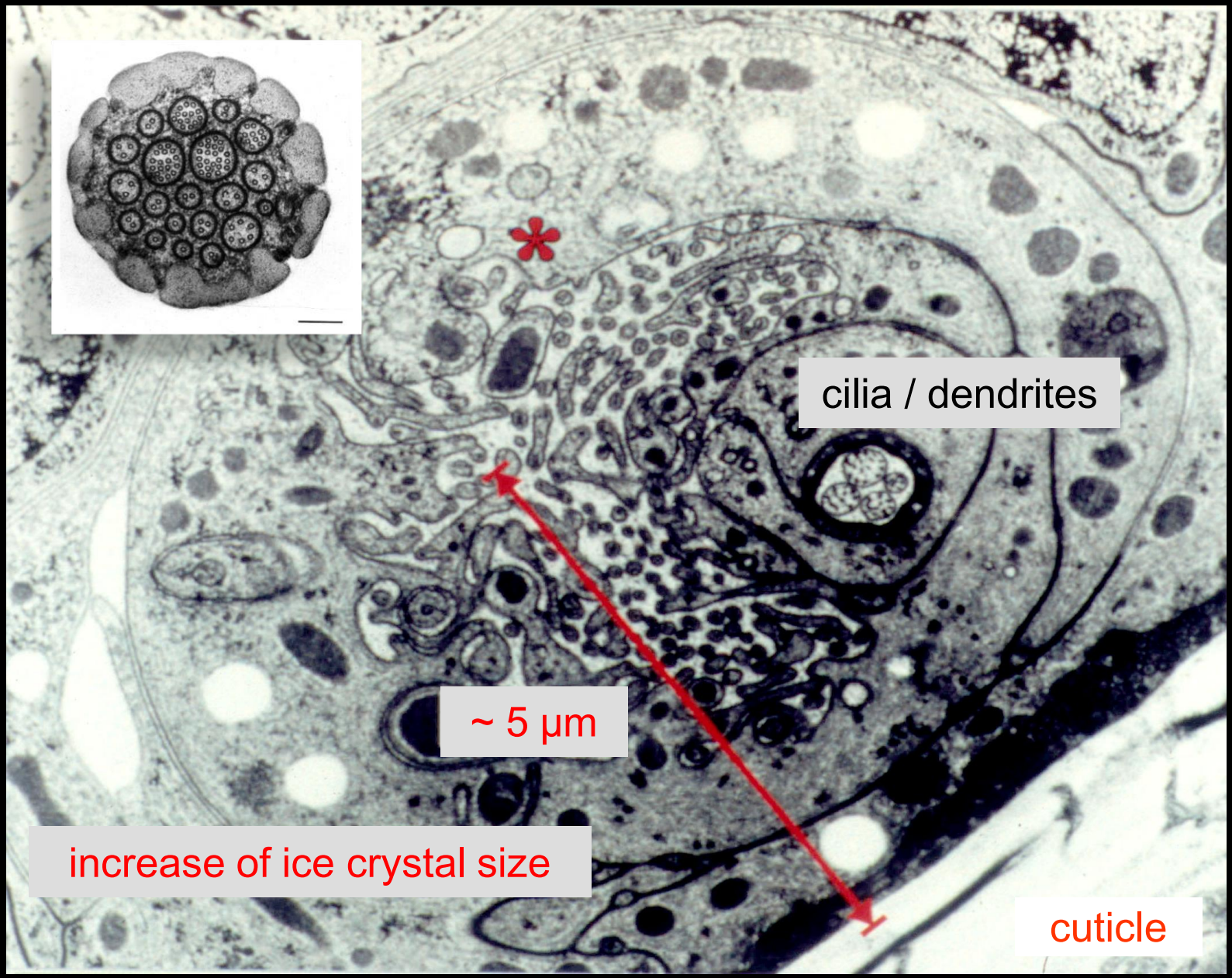
Freeze substituion: systems & standard protocol

Balzers FSU10

Leica EM AFS2

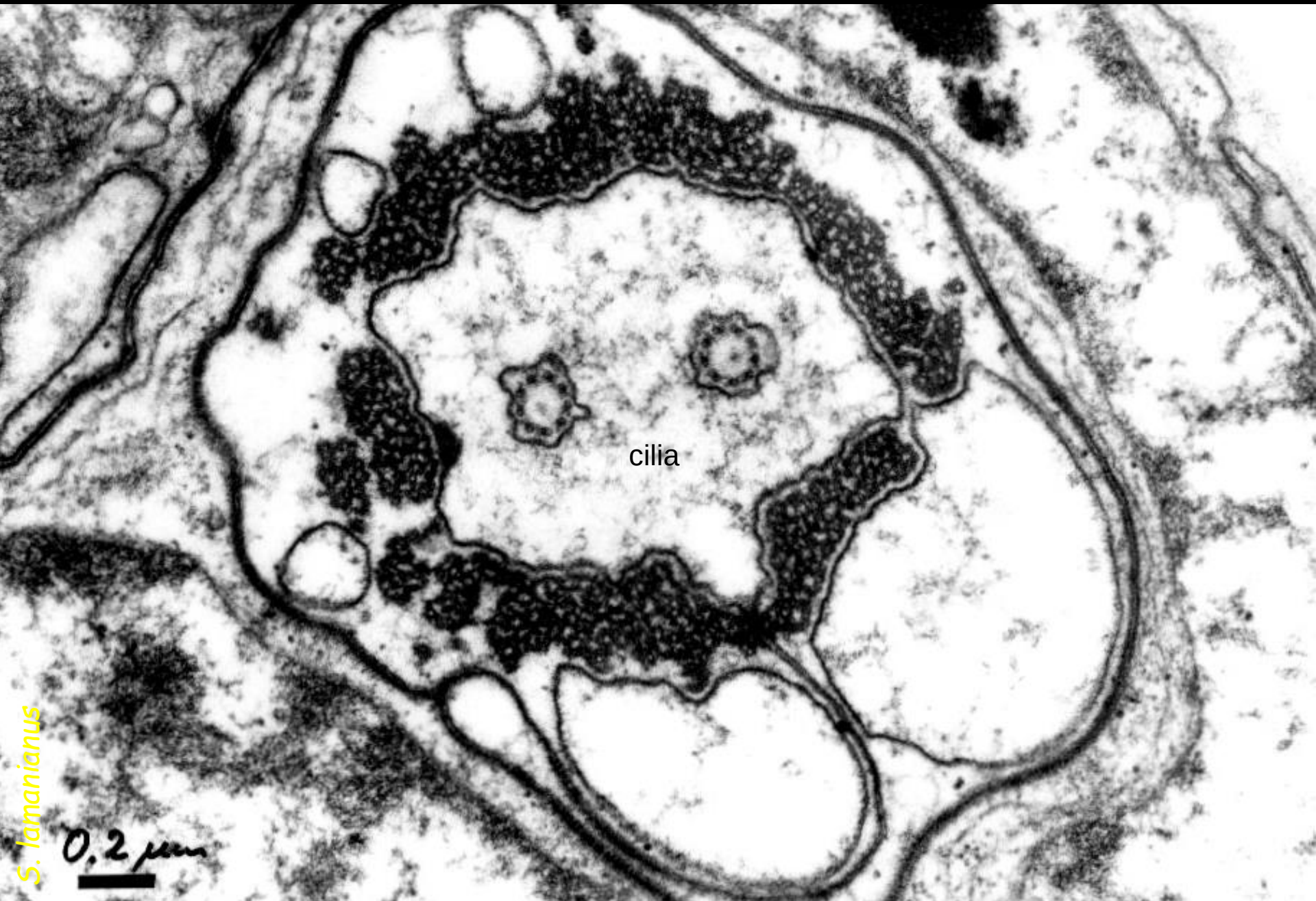
Step	Reagent	Time [h]	Temp. [°C]	Slope [°Ch^{-1}]
1	Acetone containing 2% OsO_4	26	-90	
2		15		+2
3		8	-60	
4		15		+2
5		8	-30	
6	Acetone 100%	0.5	-30	
7	Acetone 100%	0.5	-30	
8	Epon:acetone 1:2	3	+4	
9	Epon:acetone 2:1	ON	RT	
10	pure Epon	ON	+20	
11	pure Epon + 1.5% BDMA			
12	Polymerisation	72	+60	

Plunge frozen hair sensillum

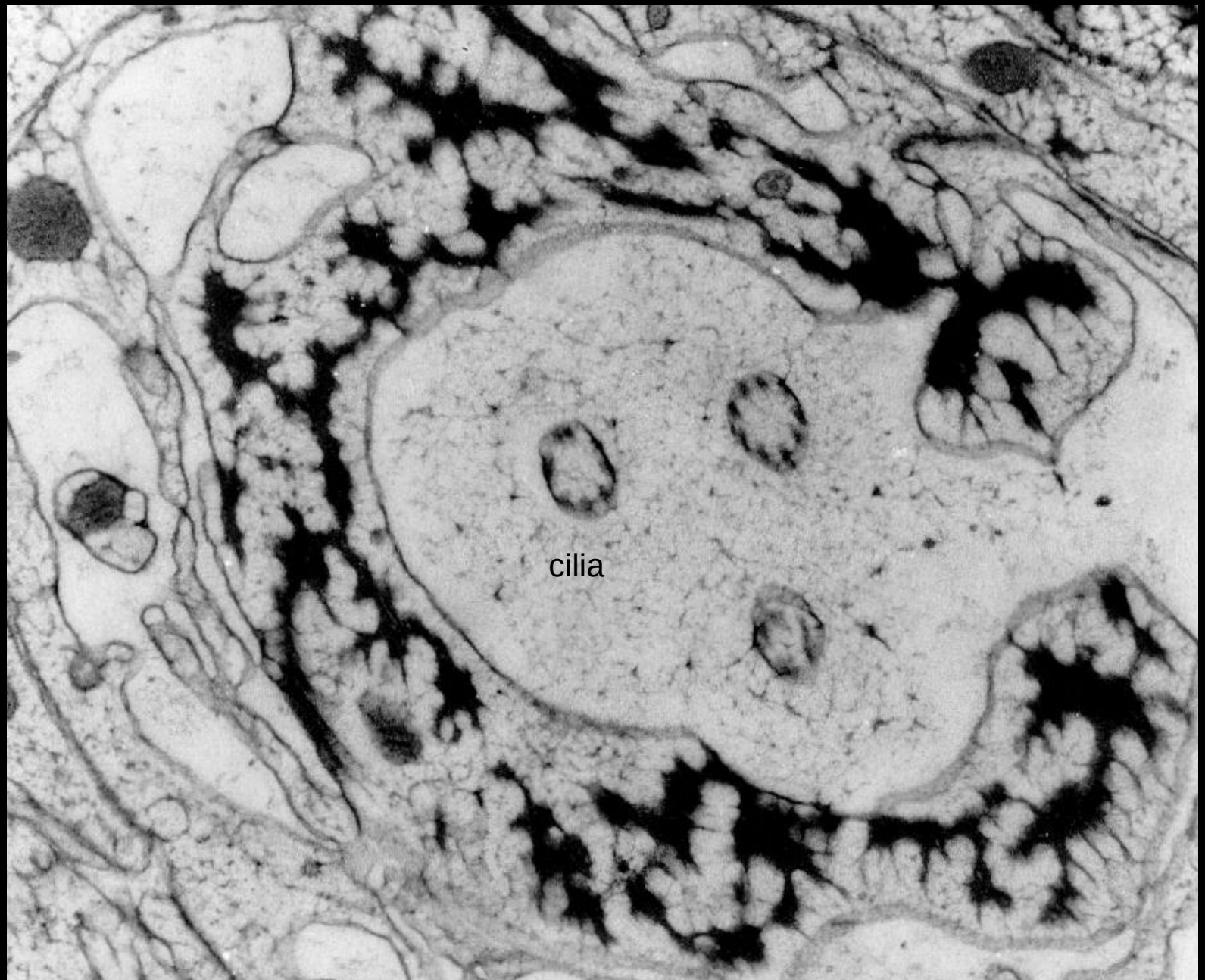


S. lamanianus

Chemical fixed scolopidium



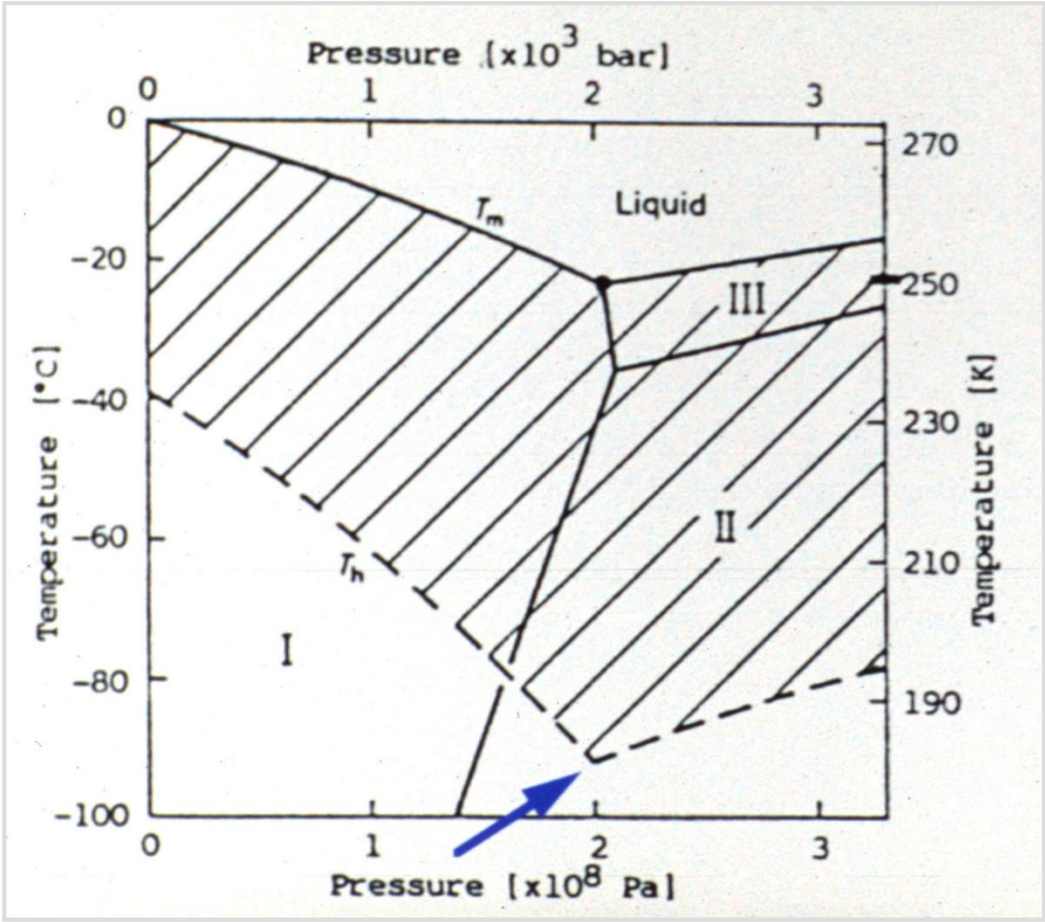
Plunge frozen scolopidum



S. lamanianus

Principle of high pressure freezing I

Pressure/temperature phase diagram of water



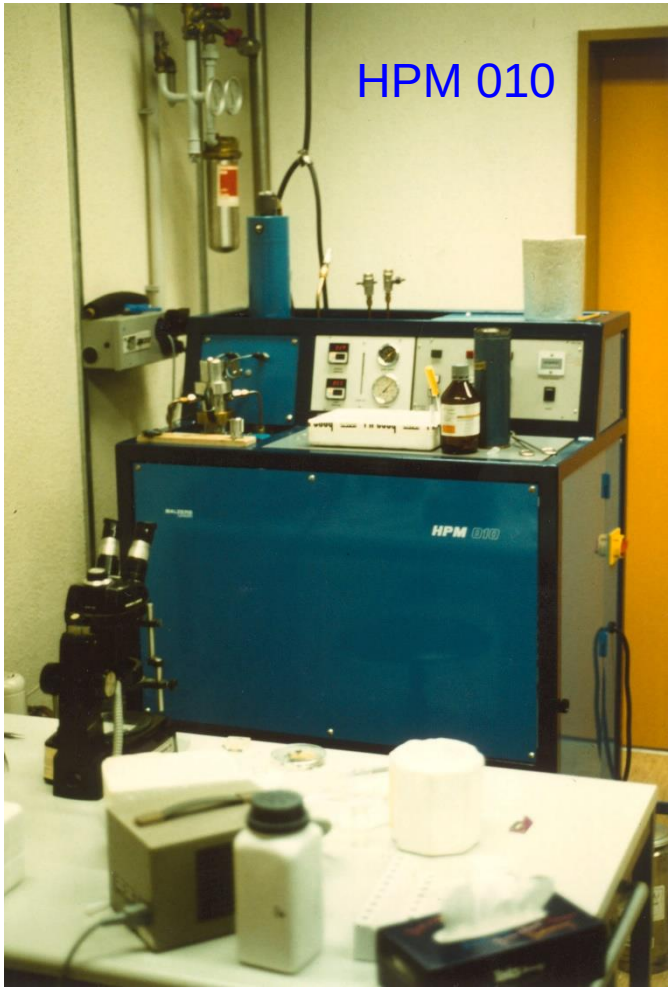
T_m melting temperature

T_h homogeneous nucleation

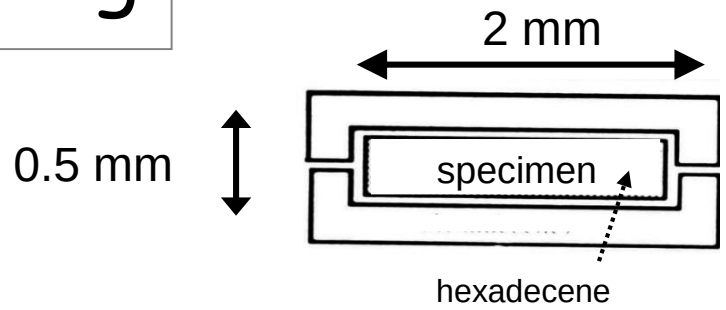
Super cooling point of water at 2100 bar

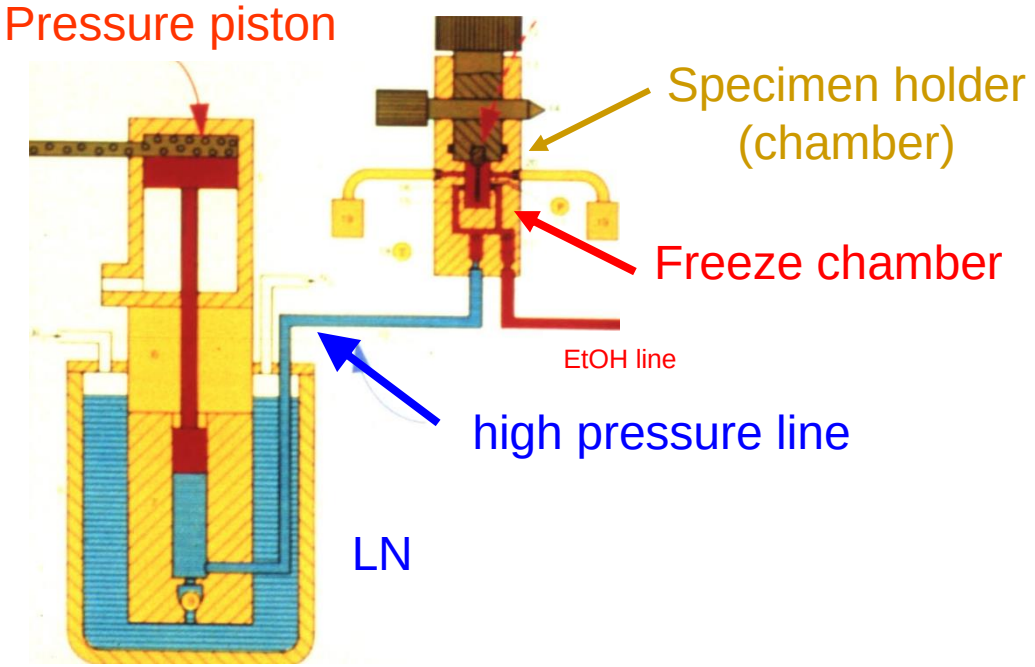
I, II, III different stable ice polymorphs

High pressure freezing

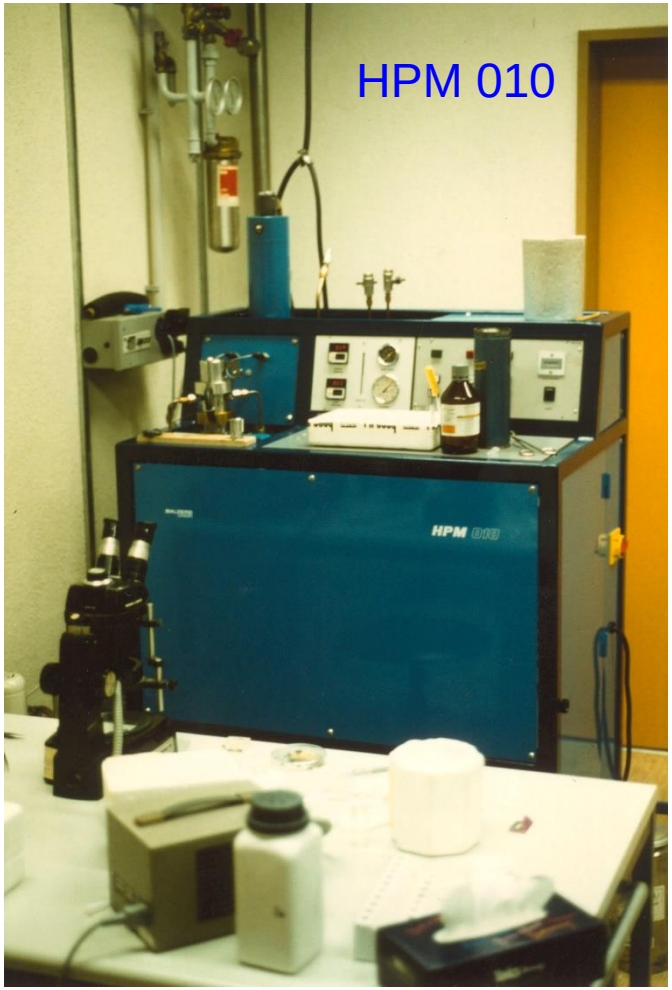


Specimen chamber

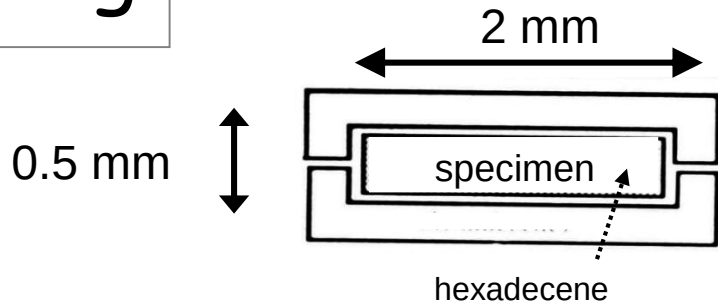




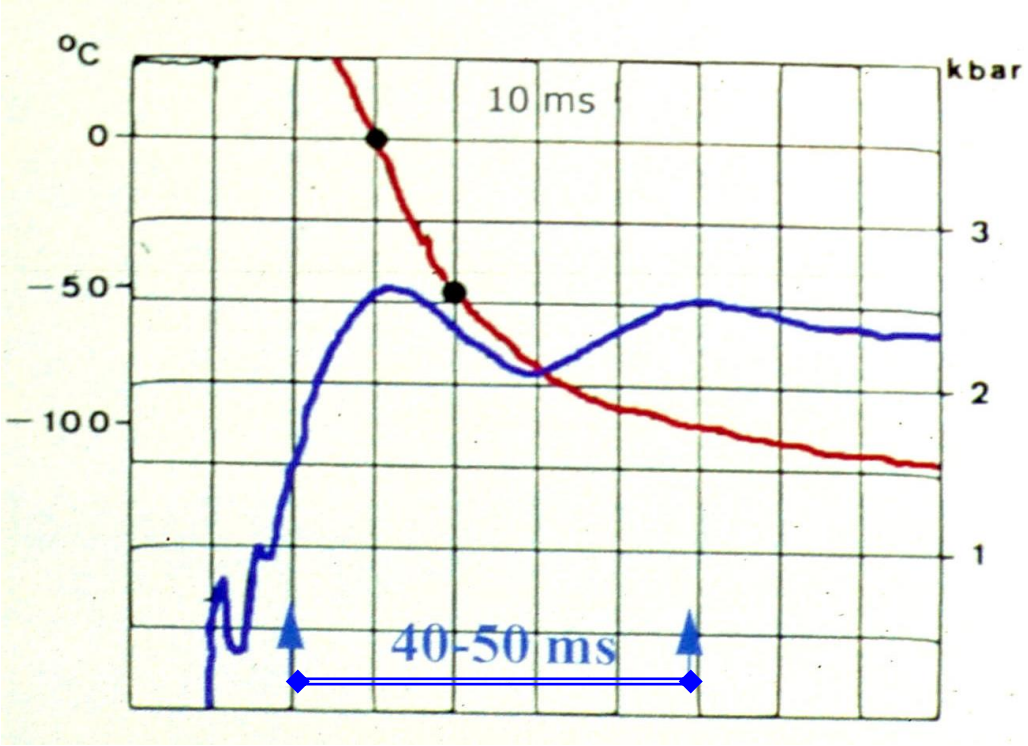
High pressure freezing



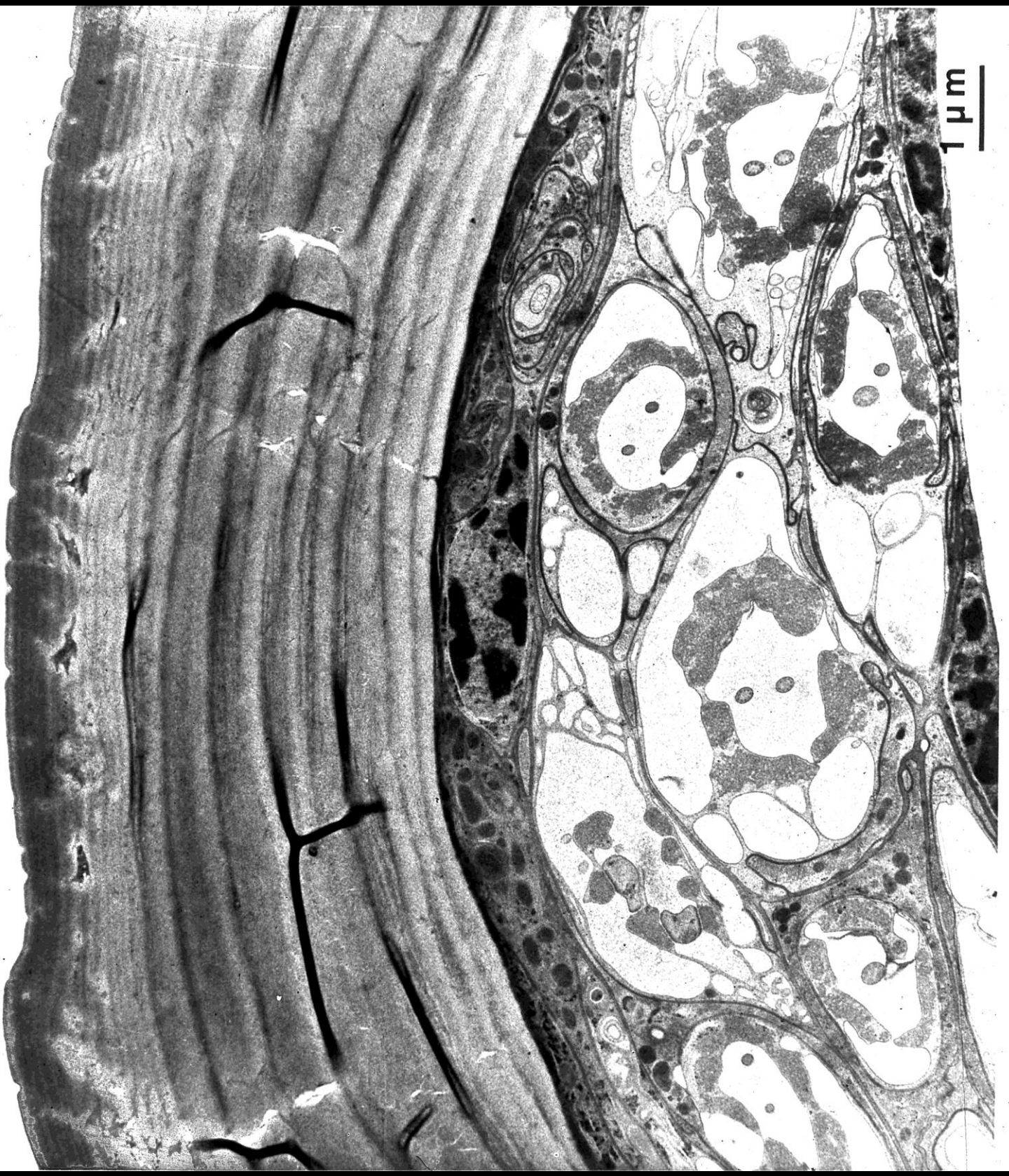
Specimen chamber

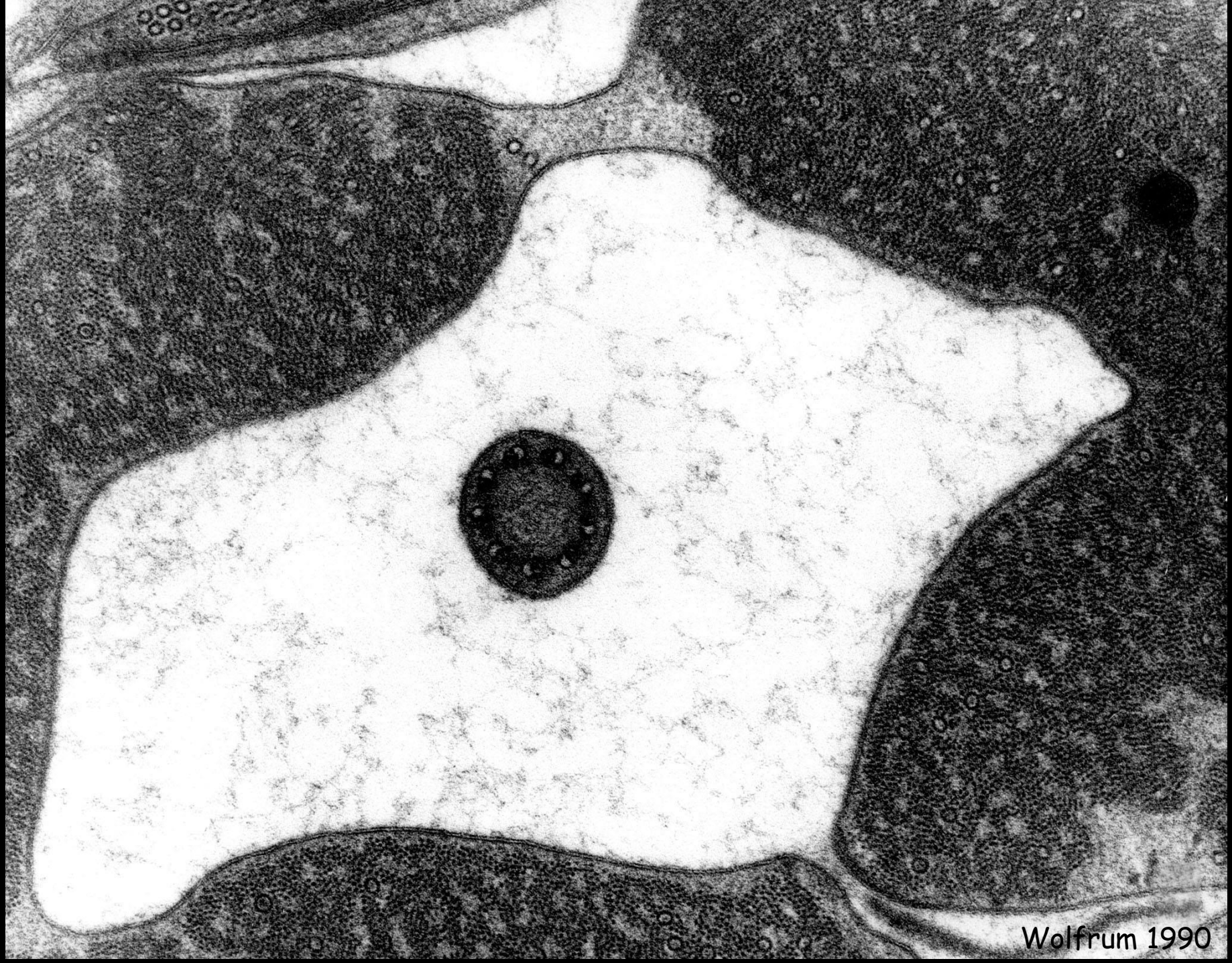


Time courses of temperature and pressure in the specimen chamber



High pressure frozen pecellus of *S. lamanianus*




Wolfrum 1990

Summary cryotechniques

- Immobilizing of native structures are restricted to cryotechniques.
- Rapid freezing methods are ideal for the fixation of thin layers and are necessary for freezing of fast/dynamic processes.
- Cryofixation of thick samples requires high pressure freezing.
  - high hydrostatic pressure may affect molecular structures
  - in particularly the cytoskeleton.

In any way, critical discussion of the results obtained is necessary !

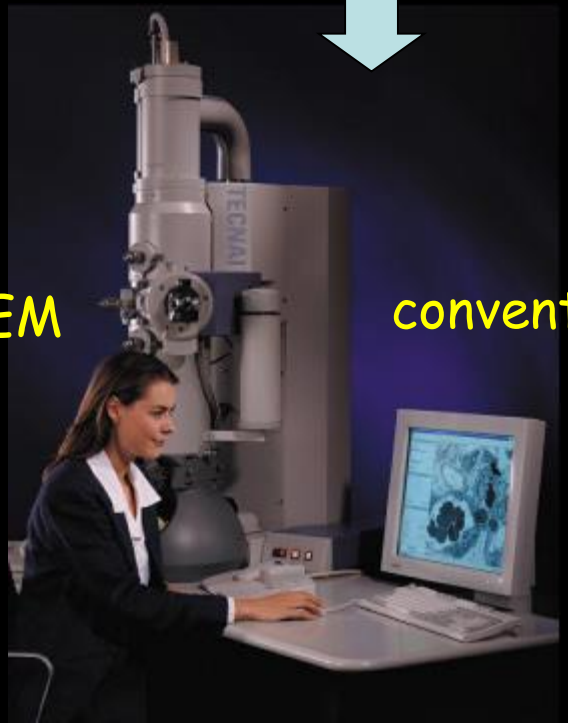
Cryofixation

Freeze substitution

Embedding &
Ultramicrotomy

Cryoultramicrotomy

CryoTEM



conventional TEM

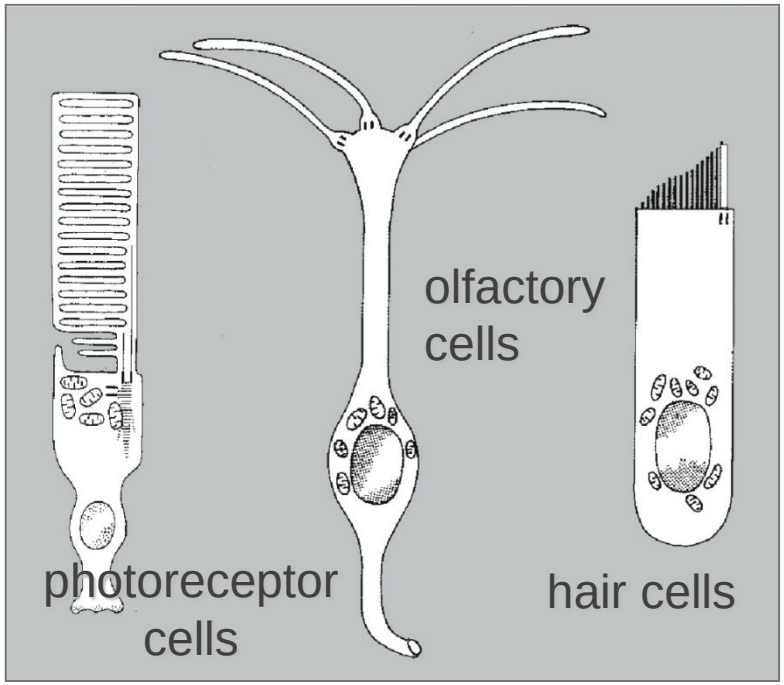
analysis

Molecular cell biology

Wolfrum Lab, JGU Mainz

Institute of Molecular Physiology (IMP), JGU Mainz, Germany

The molecular function of ciliated sensory cells



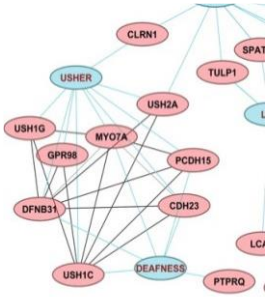
… and of primary sensory cilia.

Protein network team

Uwe Wolfrum

Identification & characterization of novel molecules related to primary sensory cilia.

Protein networks related to the Usher syndrome & other retinal ciliopathies.

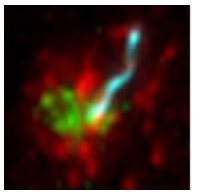


Protein function: transport, endocytosis, splicing … in health and disease.

Therapy team

Kerstin Nagel-Wolfrum

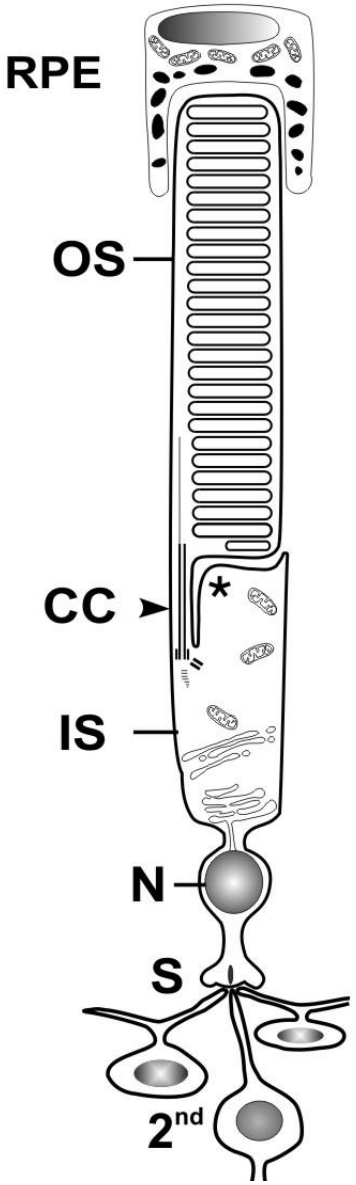
Pathomechanisms in senso-neuronal degenerations.

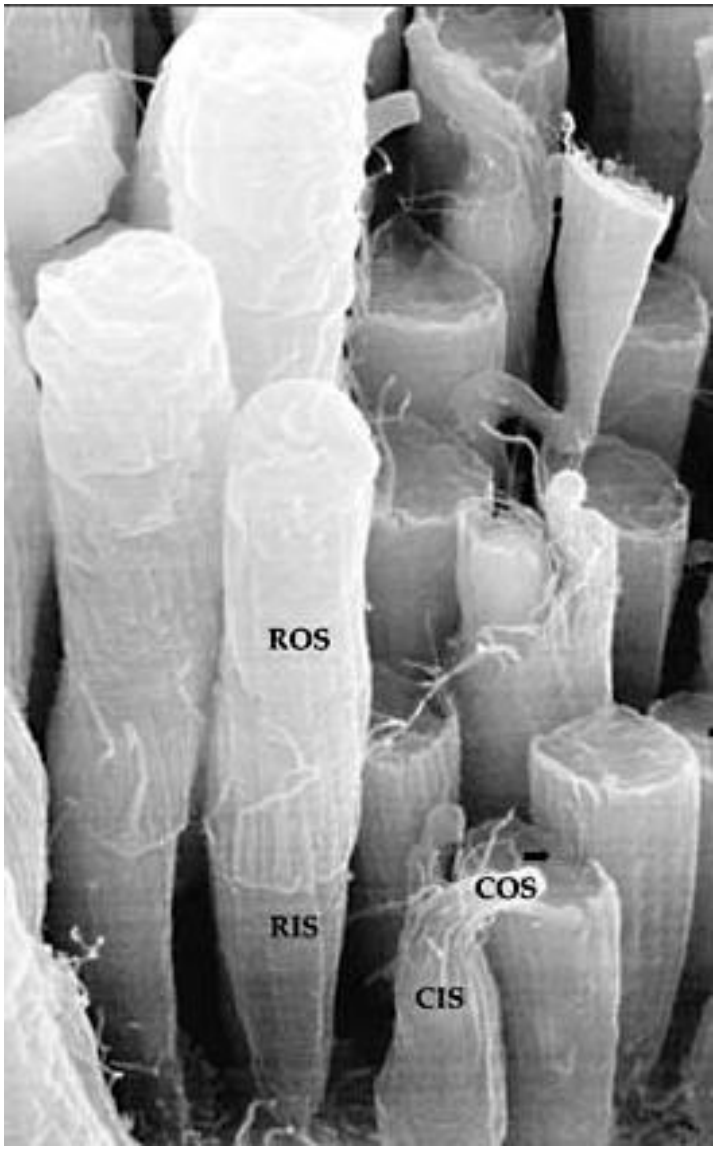


Evaluation of therapeutic strategies:

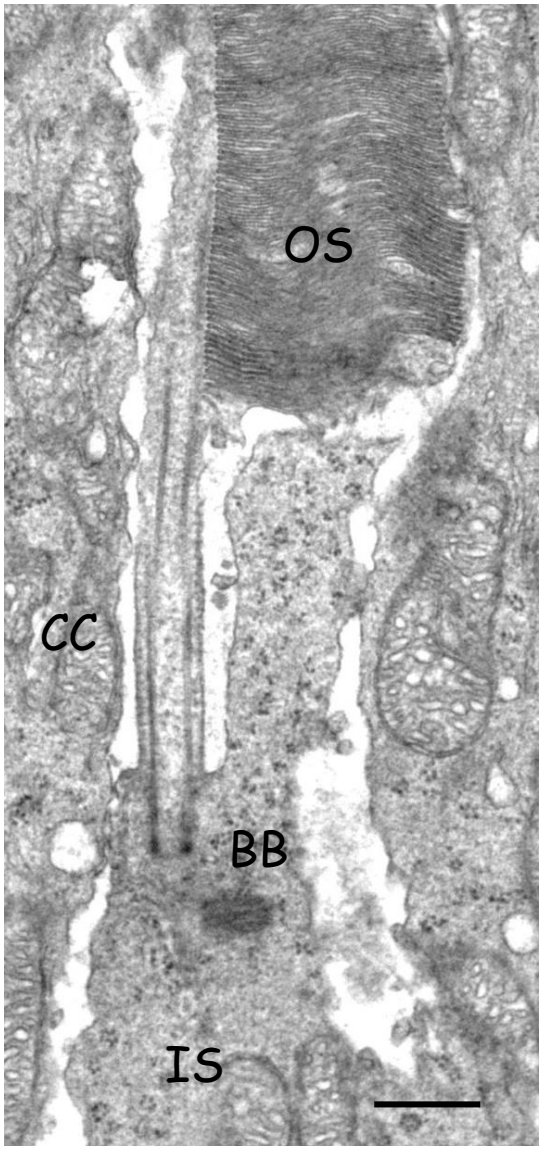
Gene based therapies

Electron microscopy of retinal photoreceptors



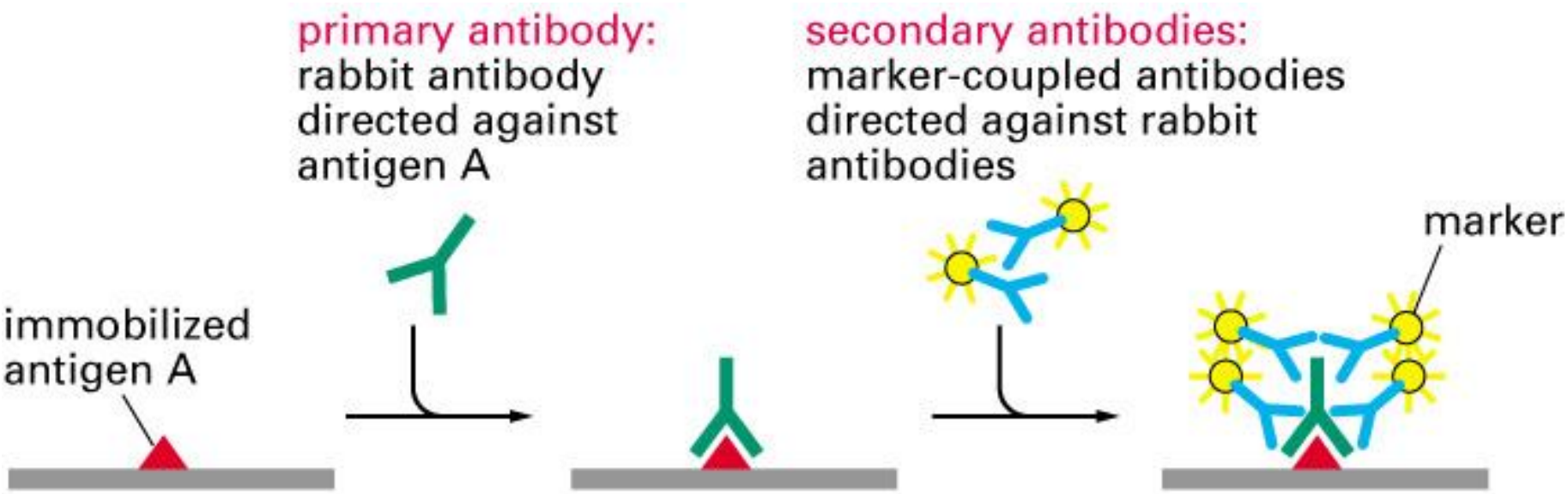


SEM



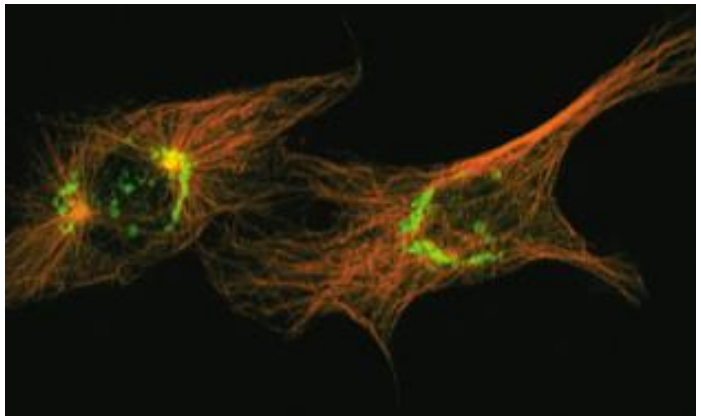
TEM

Indirect immunocyto/histochemistry

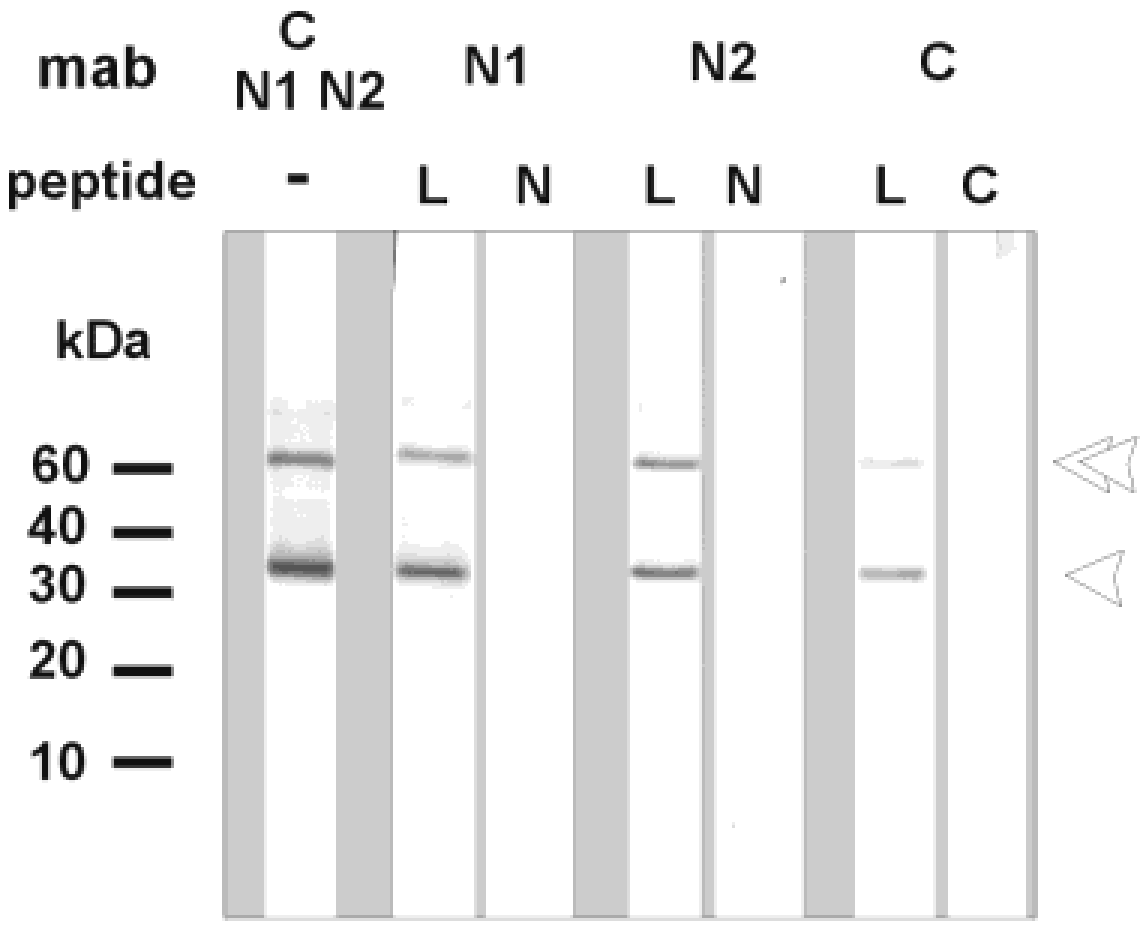


Indirect Immunofluorescence

1st ab: anti-tubulin,
2nd ab: anti-mouse-rhodamin
(microtubules)
1st anti-GM130;
2nd ab anti-rabbit-FITC
(Golgi apparatus)



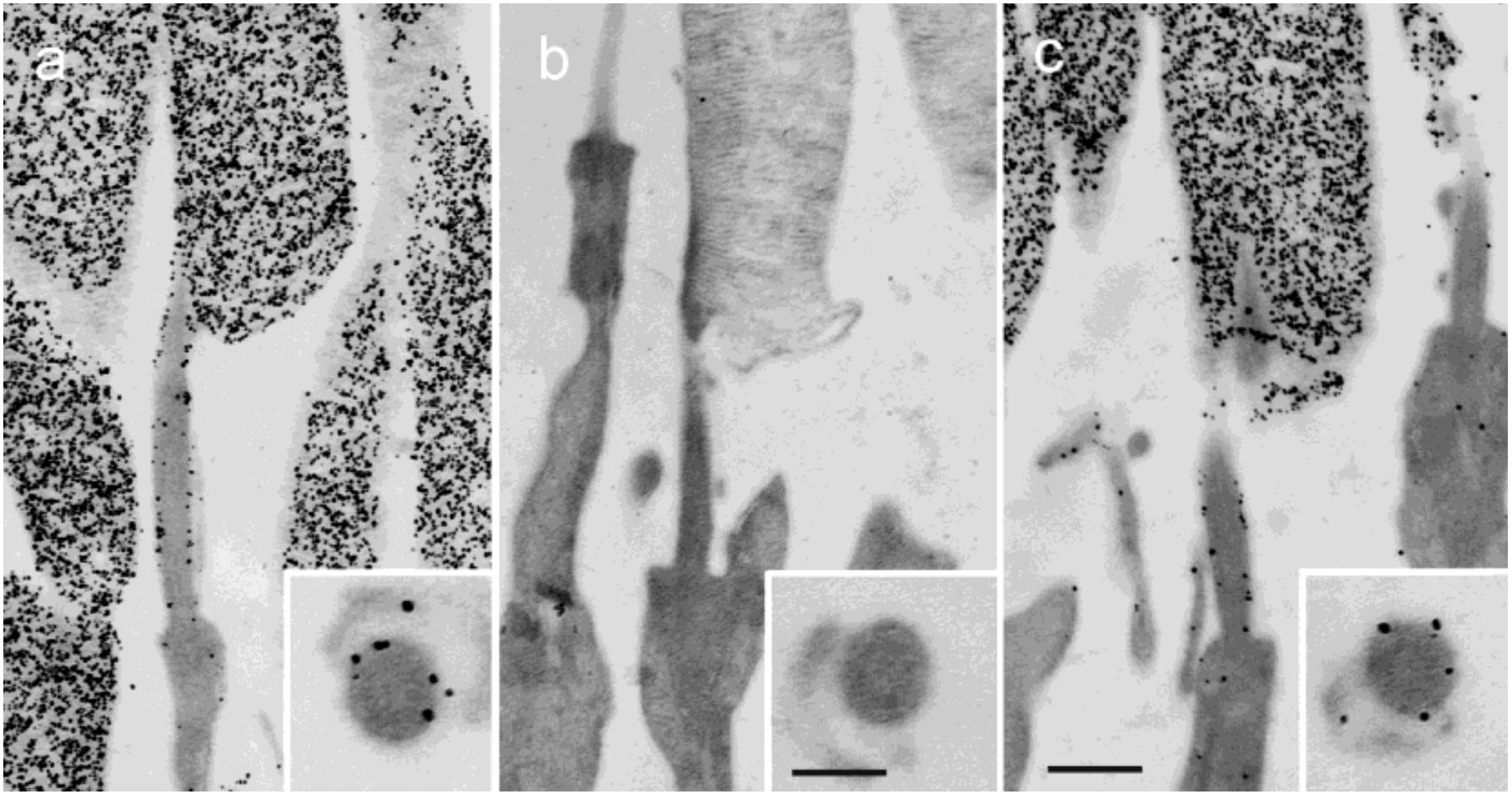
Specificity tests of monoclonal antibodies opsin



Wolfrum & Schmitt (2000) Cell Motility & Cytoskeleton

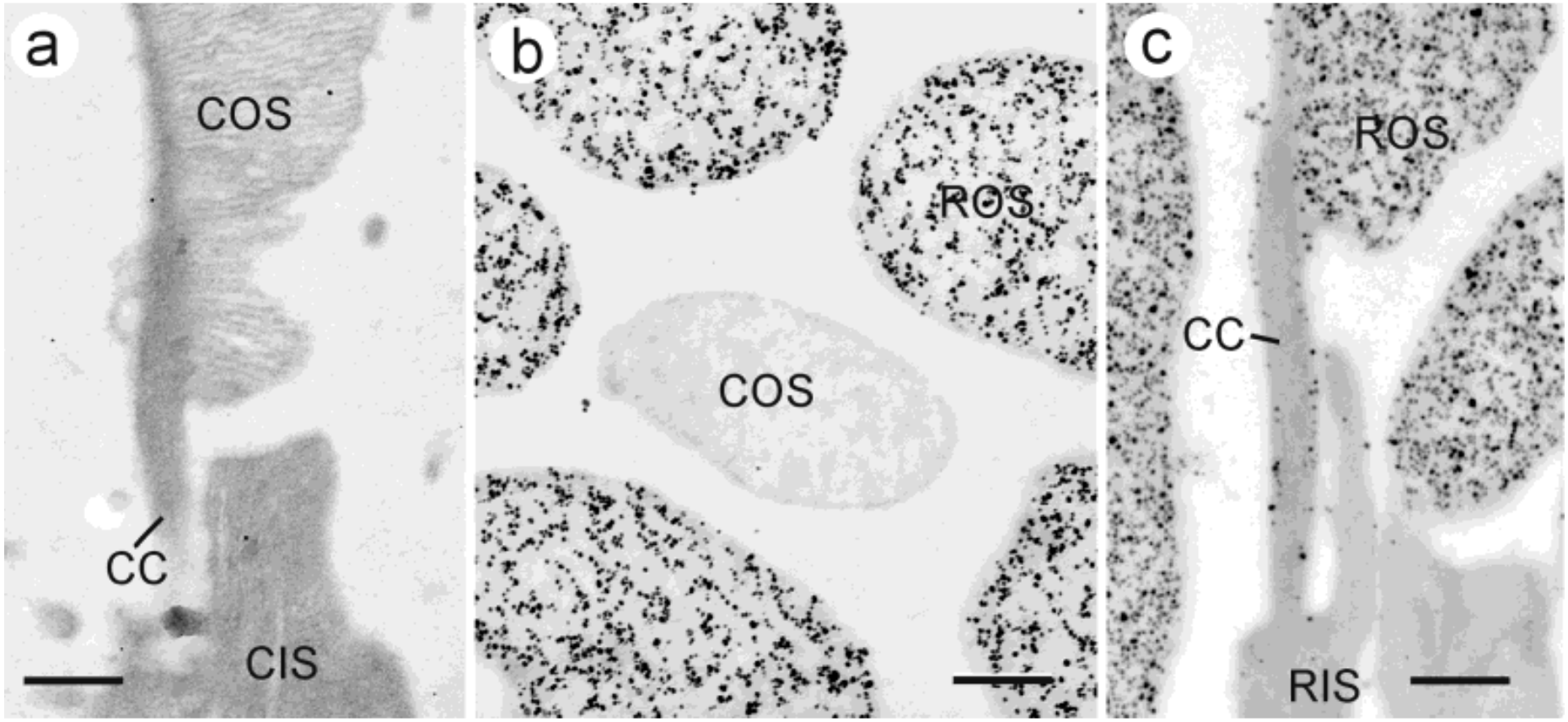
Specificity tests of monoclonal antibodies against rhodopsin

N2 | N2 & N2 peptide | N2 & L peptide



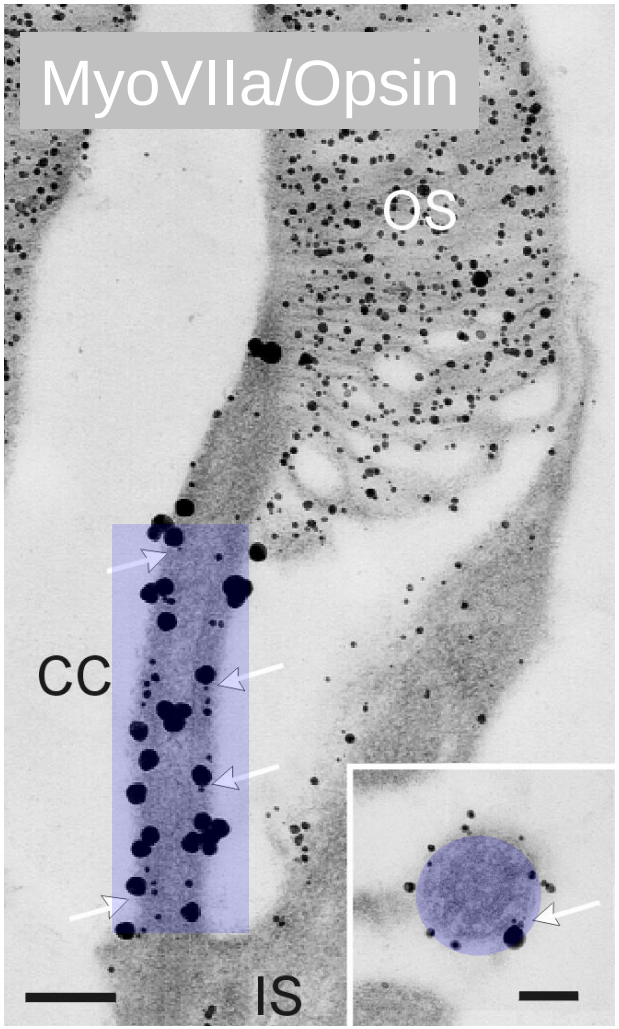
Wolfrum & Schmitt (2000) Cell Motility & Cytoskeleton

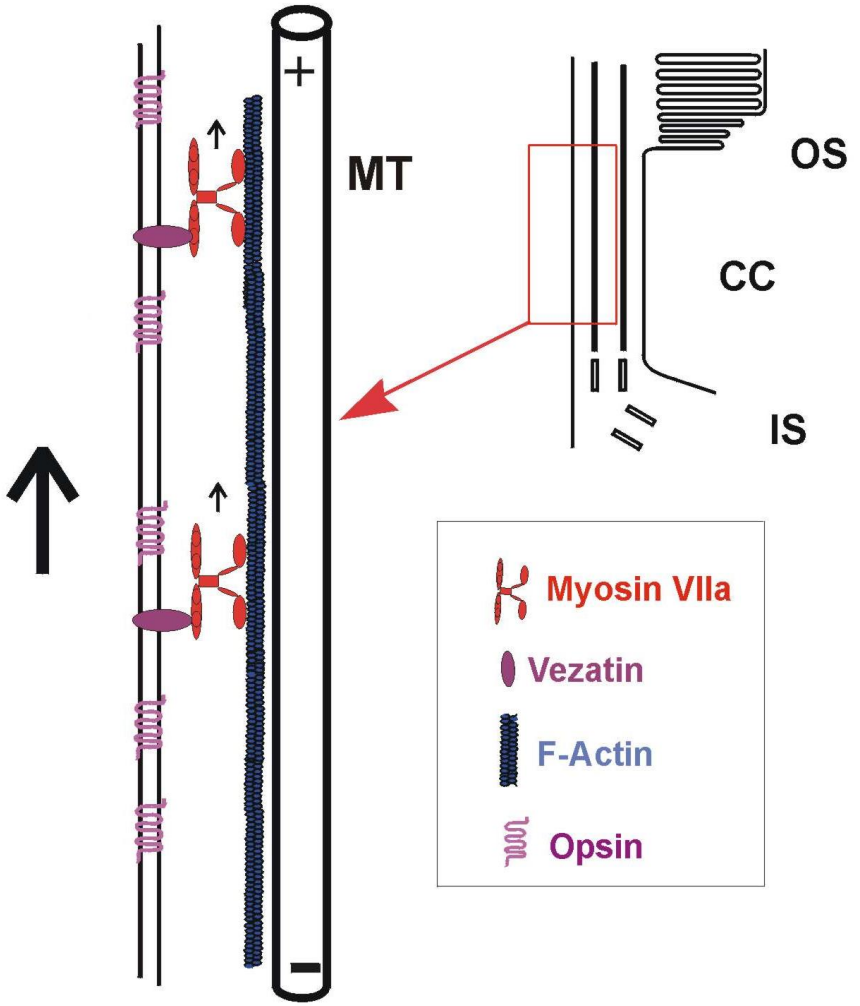
Loclization of rhodopsin in photoreceptor cells



Wolfrum & Schmitt (2000) Cell Motility & Cytoskeleton

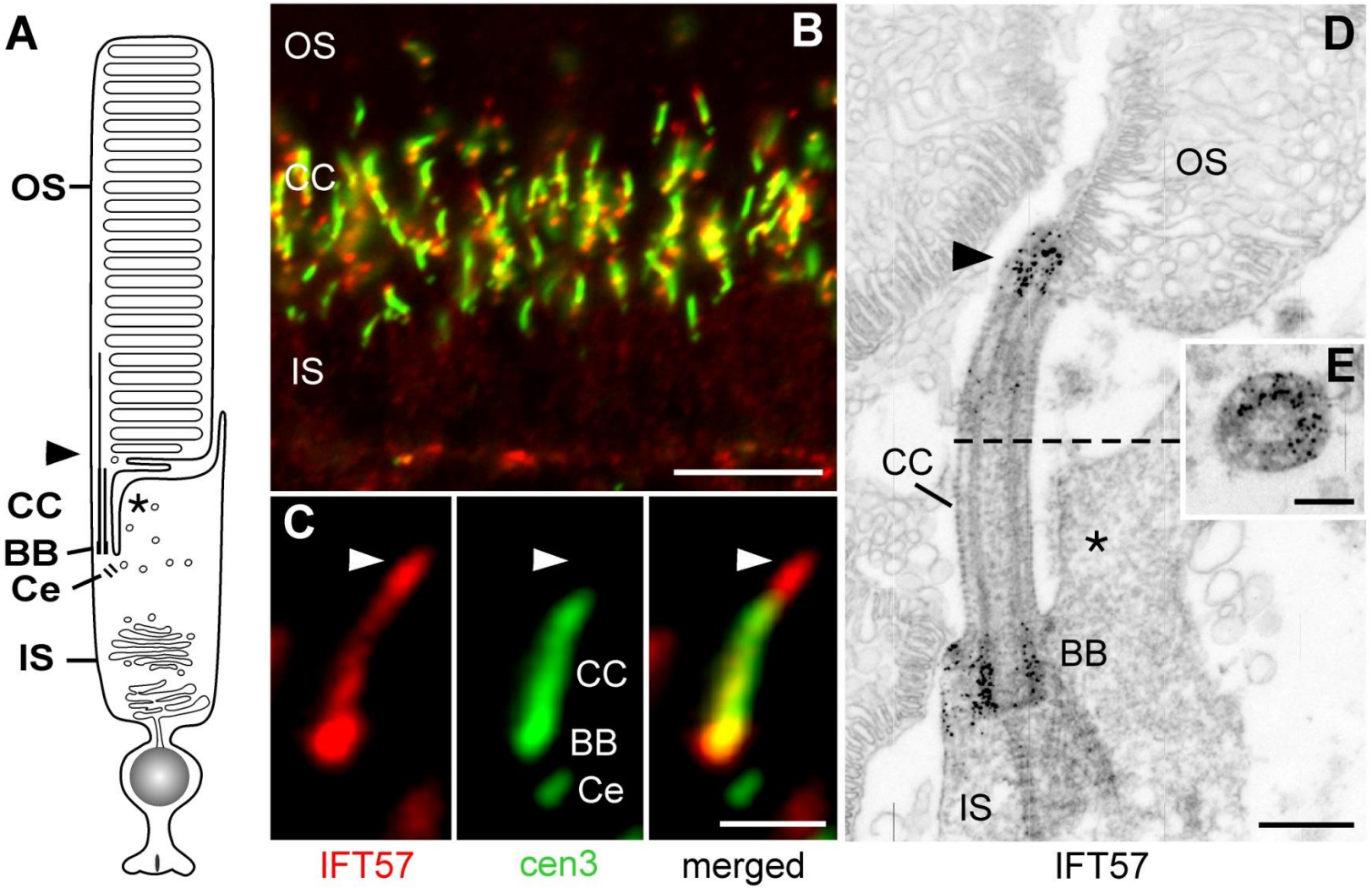
Myosin VIIa participates in rhodopsin transport across the connecting cilium





Liu et al. 1995, 1999, Wolfrum et al. 1998, Wolfrum & Schmitt, 2000

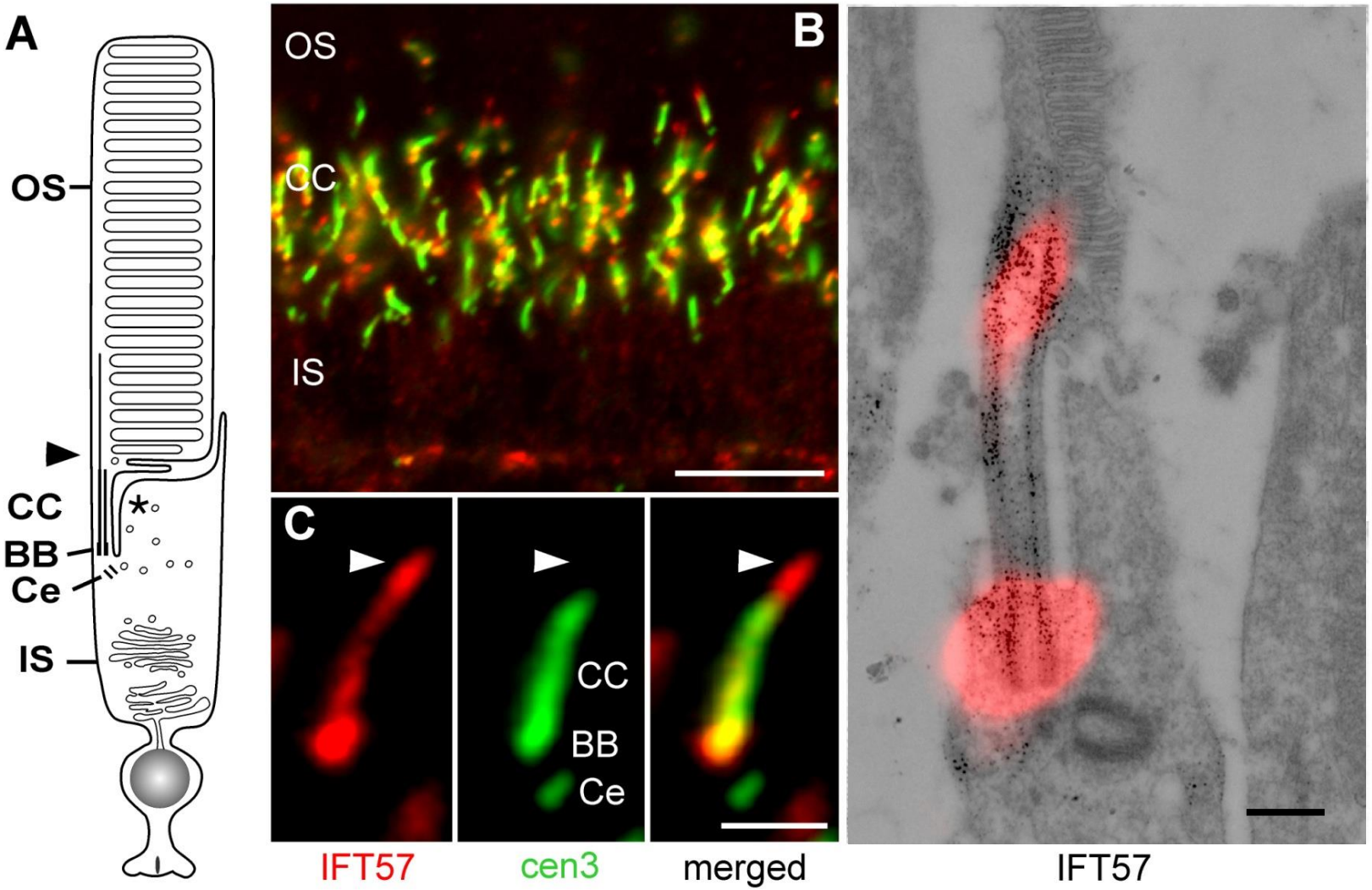
Combined immunofluorescence and immunoelectron microscopic analysis



Sedmak & Wolfrum, J Cell Biol, 2010

Sedmak et al., Methods in Cell Biol, 2009

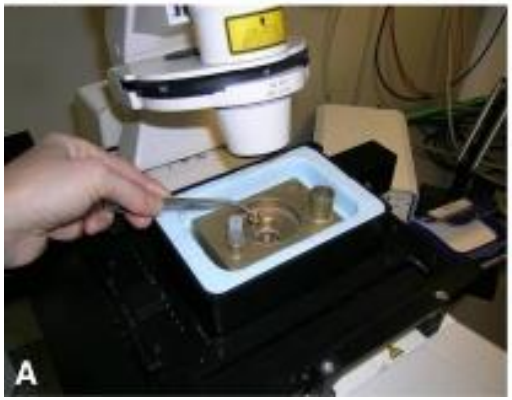
Prospect correlative microscopy: Transfer from light to electron microscopic analysis

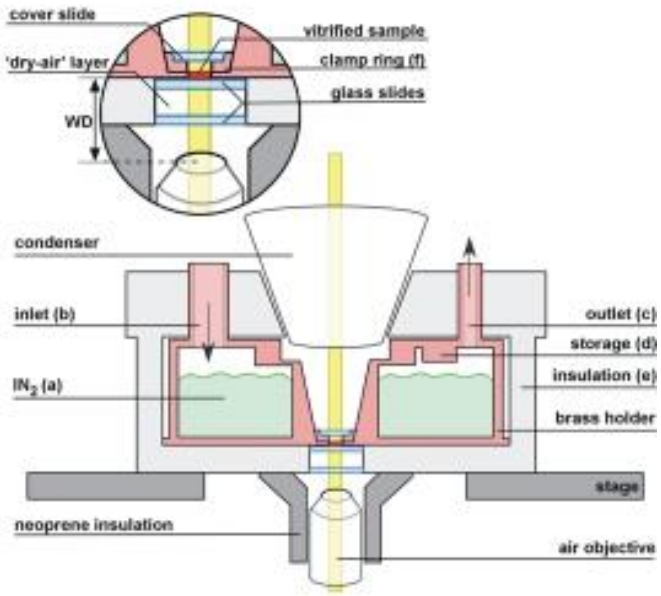


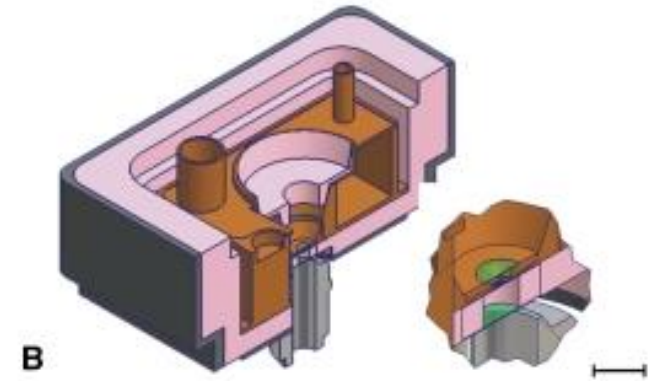
Sedmak & Wolfrum, J Cell Biol, 2010

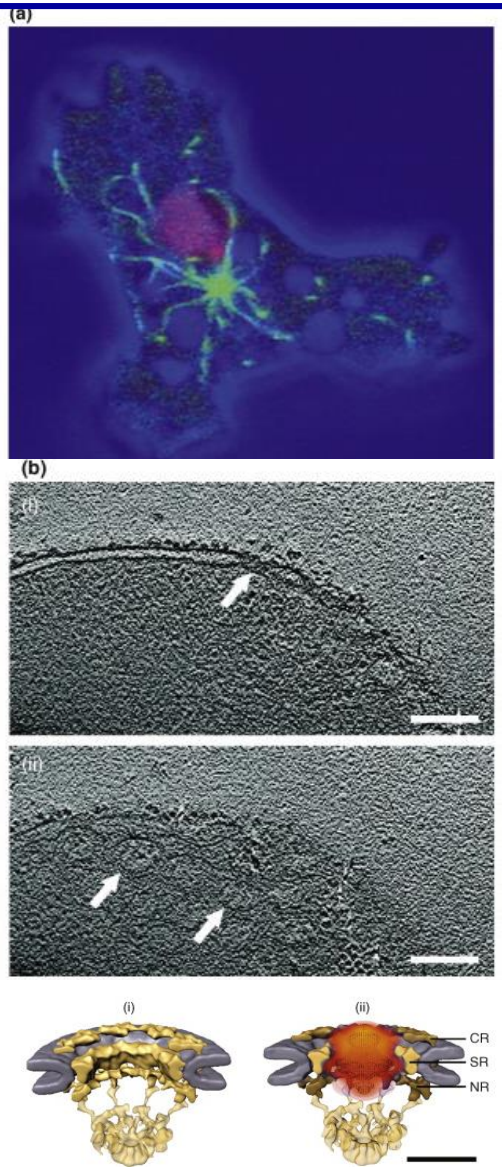
Sedmak et al., Methods in Cell Biol, 2009

Prospect correlative microscopy: Transfer from light to electron microscopic analysis



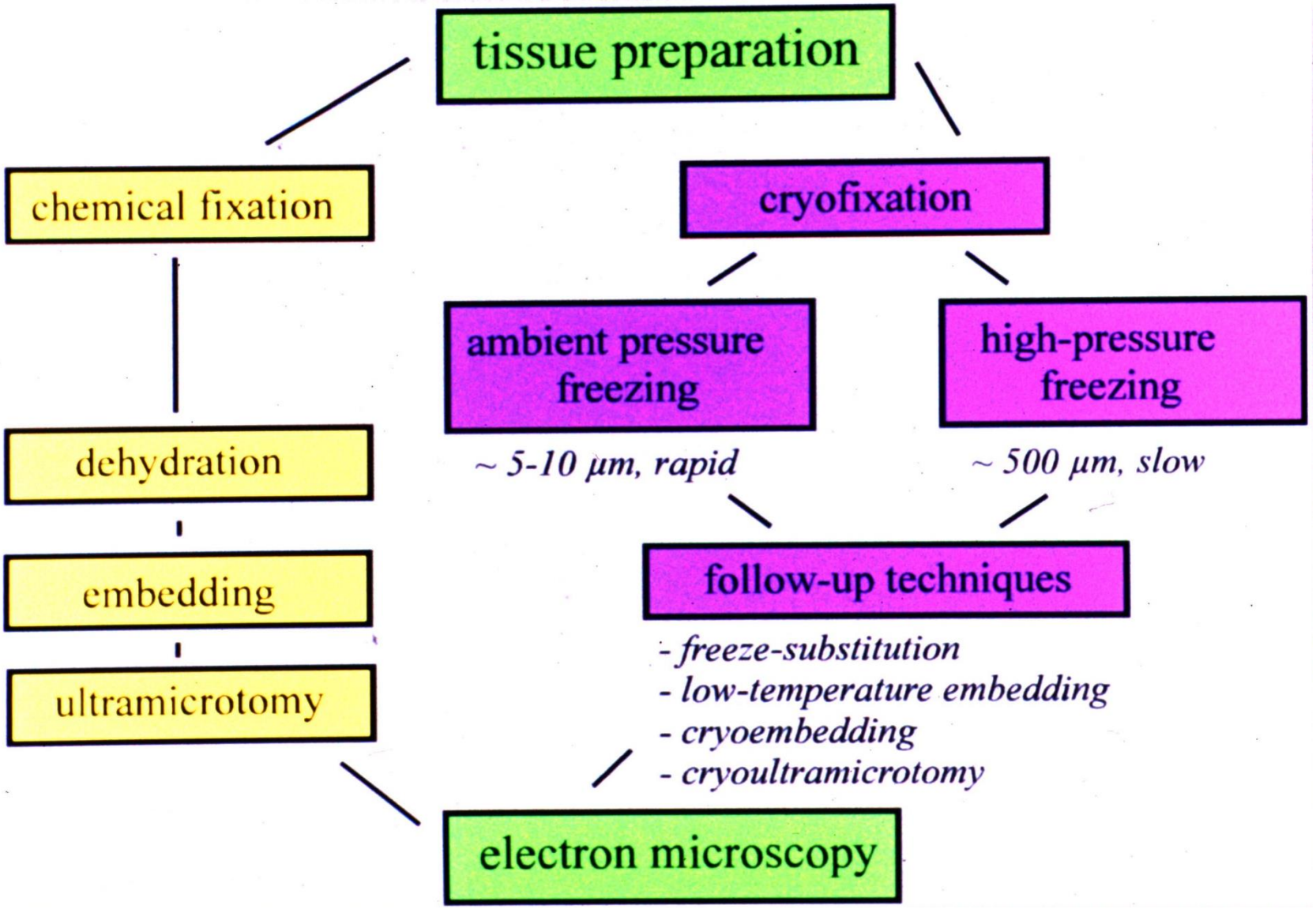






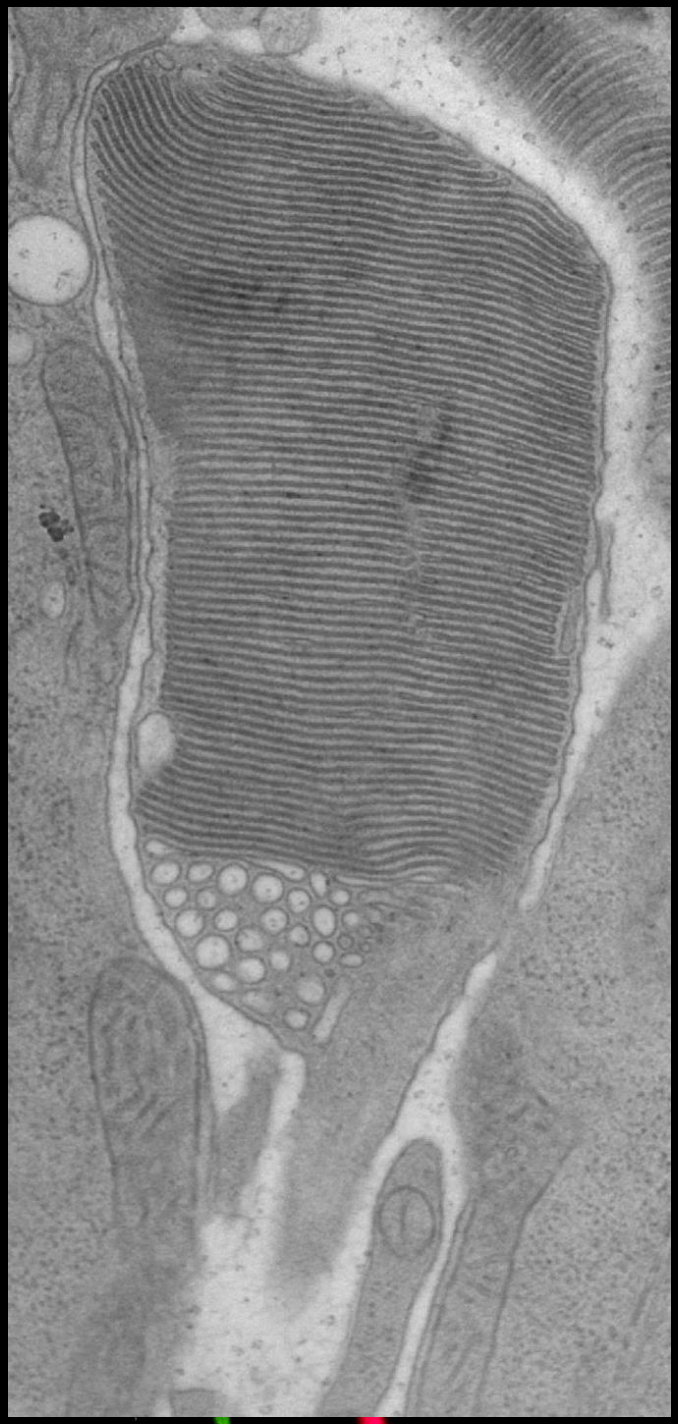
Borrowed from W. Baumeister lab, MPI Munich

Techniques in biological electron microscopy

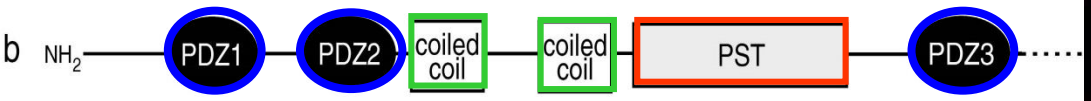


Wolfrum 1997

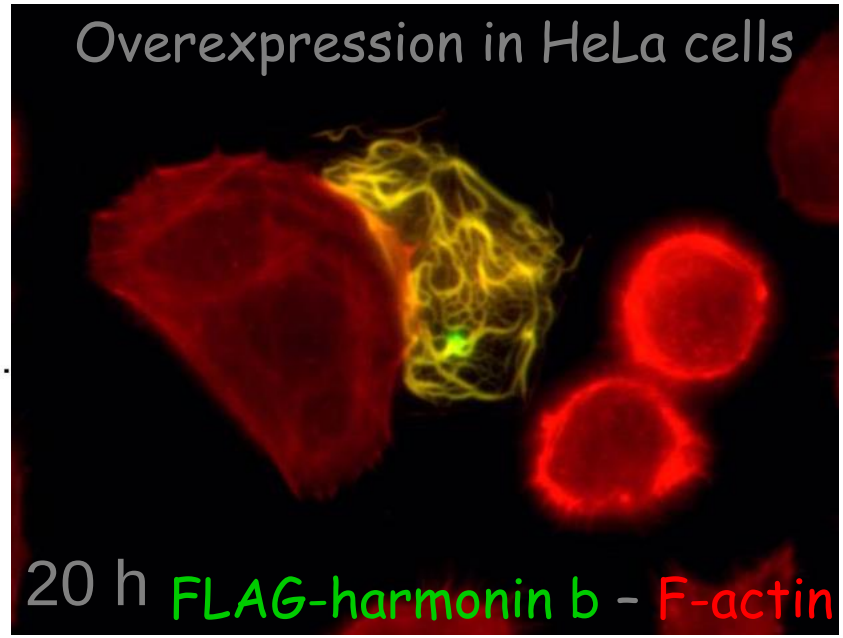
Thank you!



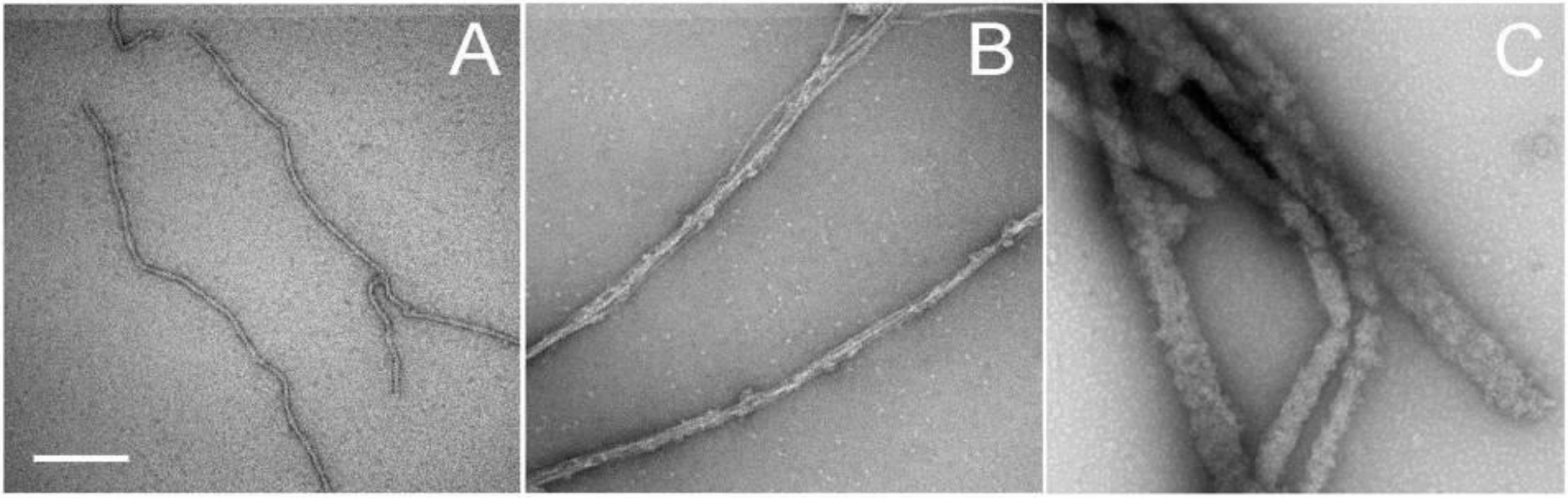
Harmonin b bundles actin filaments



harmonin (slicing variant) isoform b



Negative staining of actin filaments



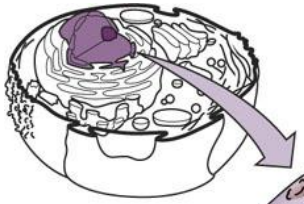
F-actin & GST

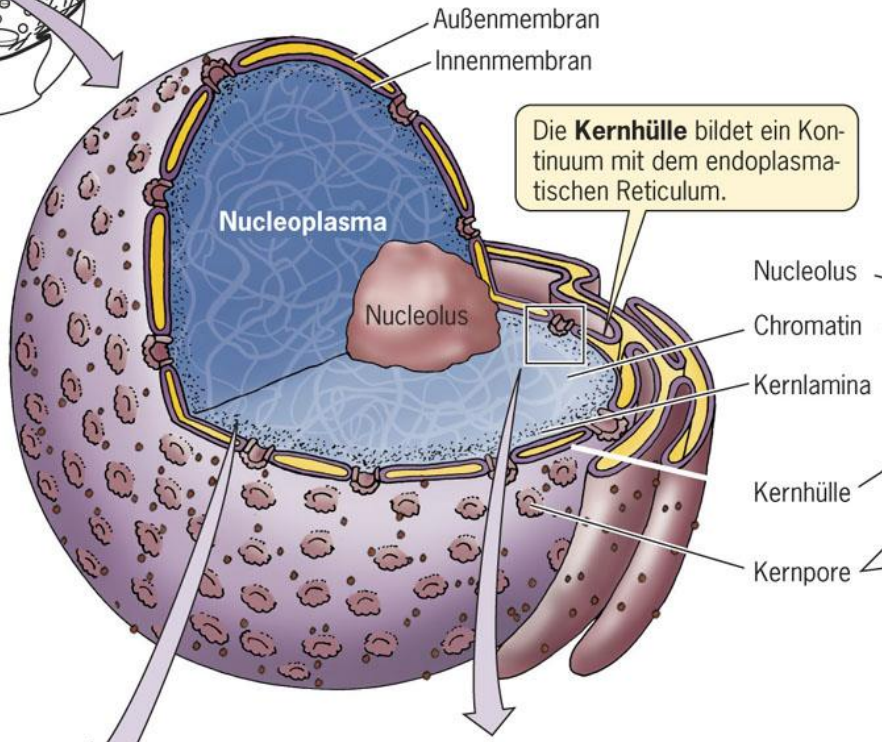
F-actin & GST-harmonin b (s)

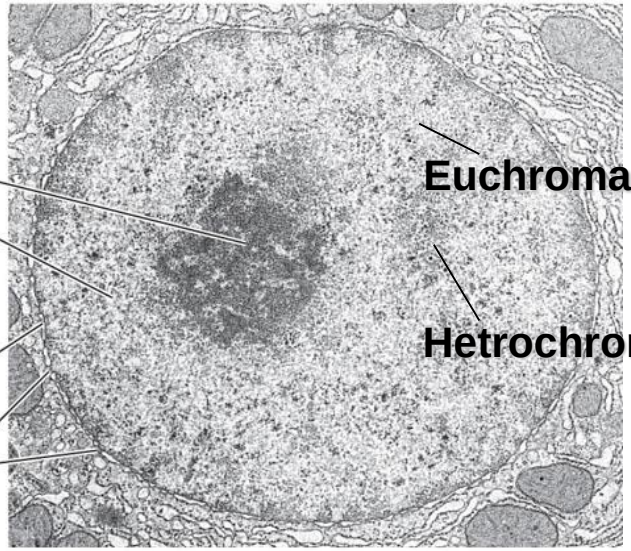
F-actin & GST-harmonin b (l)

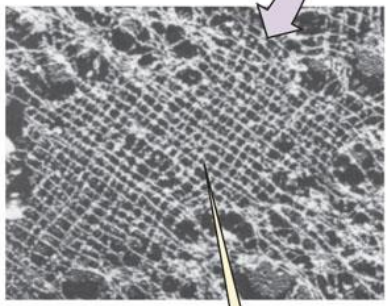
Boeda et al. 2002 EMBO J

Der Zellkern

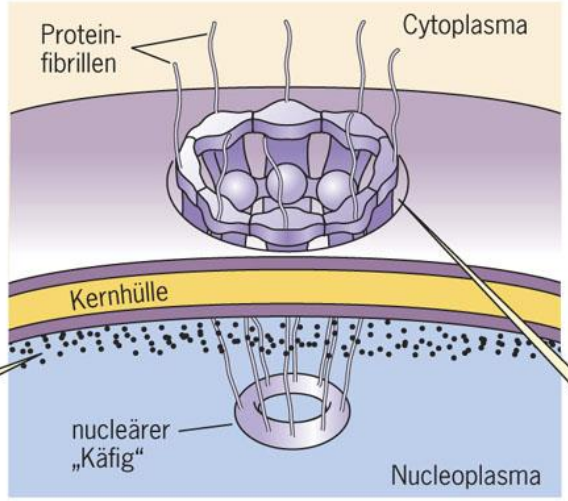


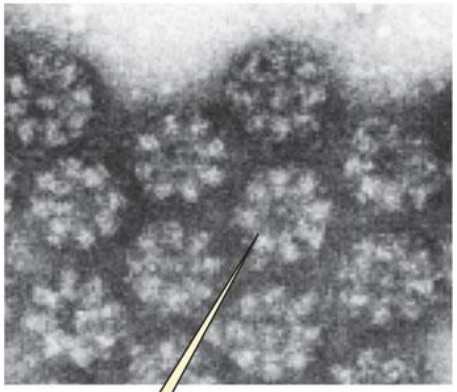






Die **Kernlamina** – ein Netzwerk aus Proteinfilamenten, das sich unmittelbar an der Innenseite der Kernhülle befindet und an diese angeheftet ist – steht in Wechselwirkung mit dem Chromatin und stützt die Kernhülle.



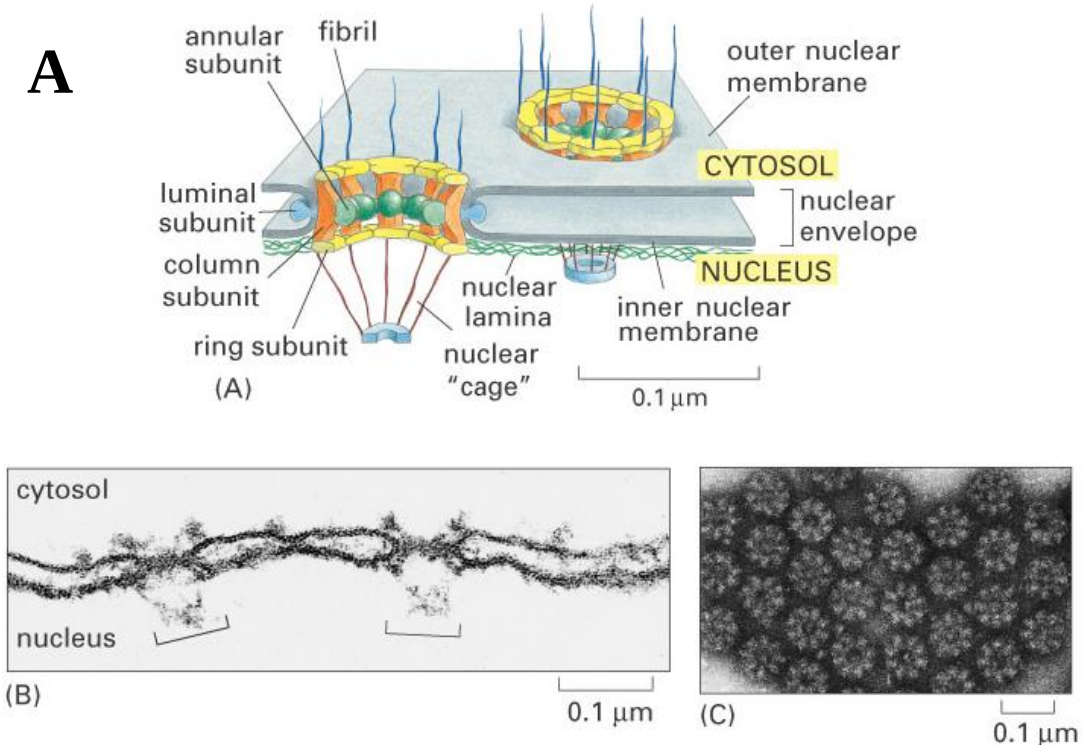


Jede **Kernpore** ist von einem Achteck aus Proteinkomplexen umgeben; Proteinfibrillen auf der Kerninnenseite bilden eine käfigähnliche Struktur.

- Lamine - Intermediär Filamente - Cytoskelett

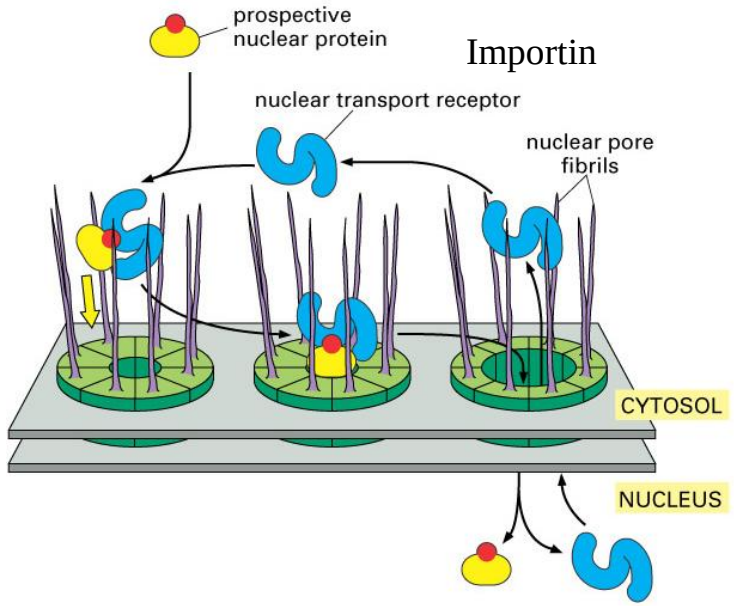
Der Kernporenkomplex

A



Die Kernporen bestehen aus einem Multiproteinkomplex.

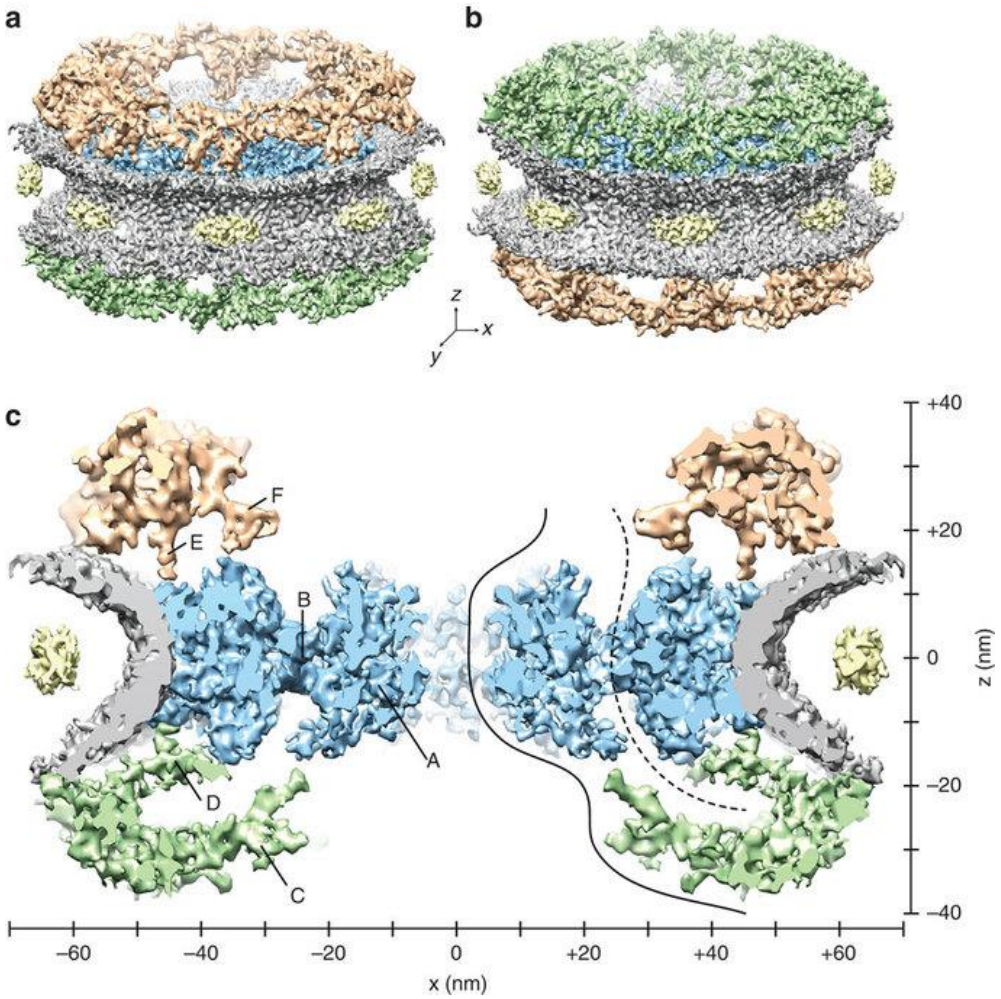
B

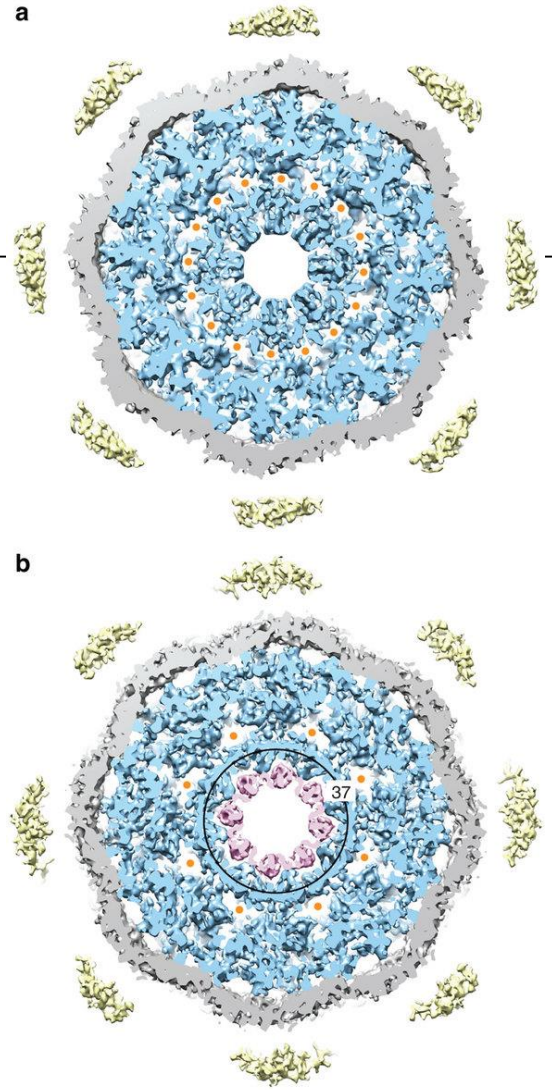


Transport durch Kernporenkomplex ist **selektiv** und **aktiv:**
Moleküle besitzen:
Kern-Lokalisations-Sequenz (**NLS**) bzw.
Kern-Export-Sequenz (**NES**), als Erkennun
für: Importinen bzw.
Exportinen

Alberts, Lehrbuch der Molekularen Zellbiologie

Der Kernporenkomplex - TEM Tomographie





Eibauer et al. 2015 Nat. Com.

Nobelpreis in Chemie 2017

Jacques Dubochet
Univ. of Lausanne

Joachim Frank
Columbia University

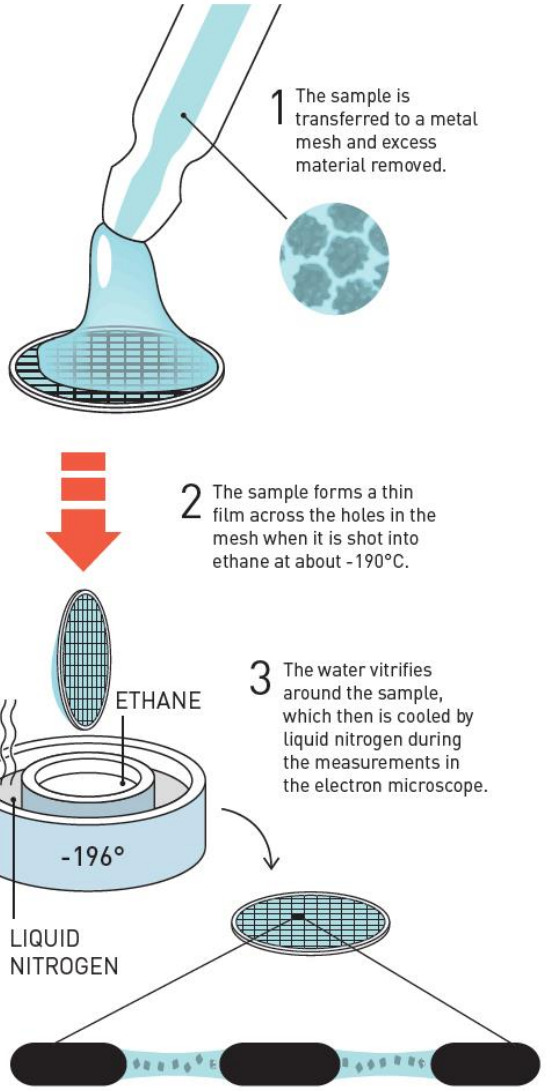
Richard Henderson
MRC Cambridge

.

"….. developing cryo-electron microscopy (cryo-EM), a method for imaging biomolecules at an atomic resolution."

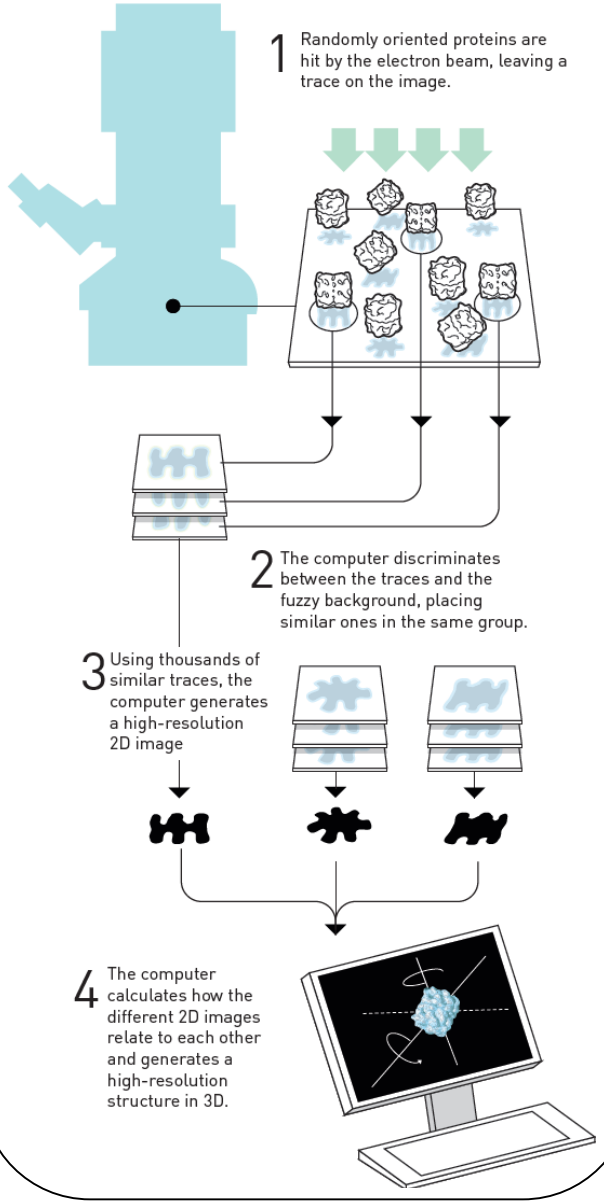
Jacques Dubochet

Cryo-Fixation



Joachim Frank

Image processing



Richard Henderson

EM analysis

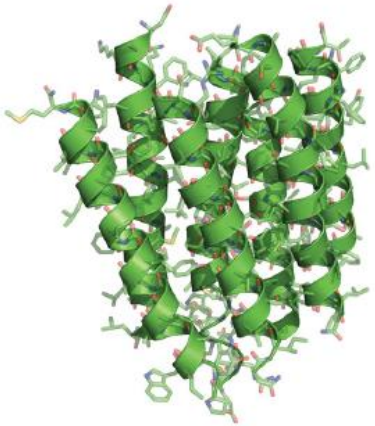
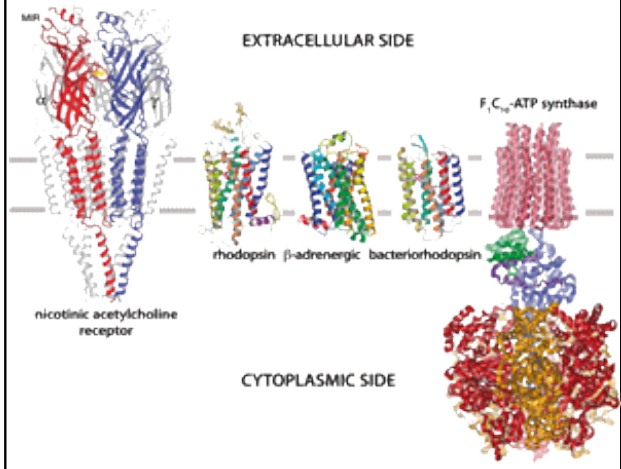
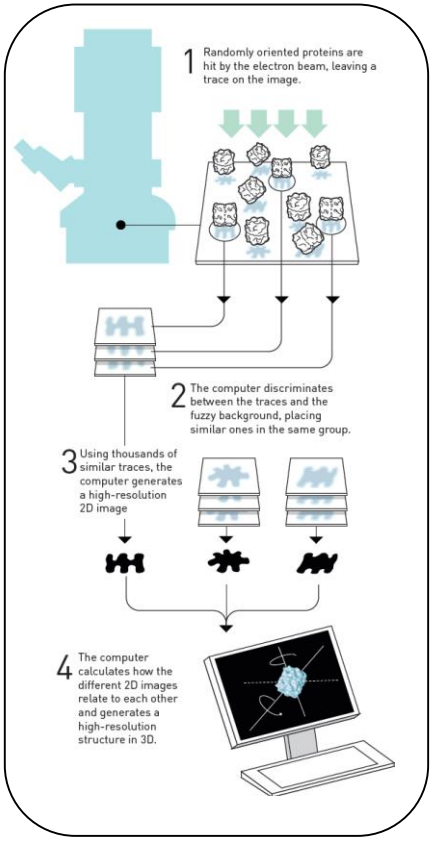
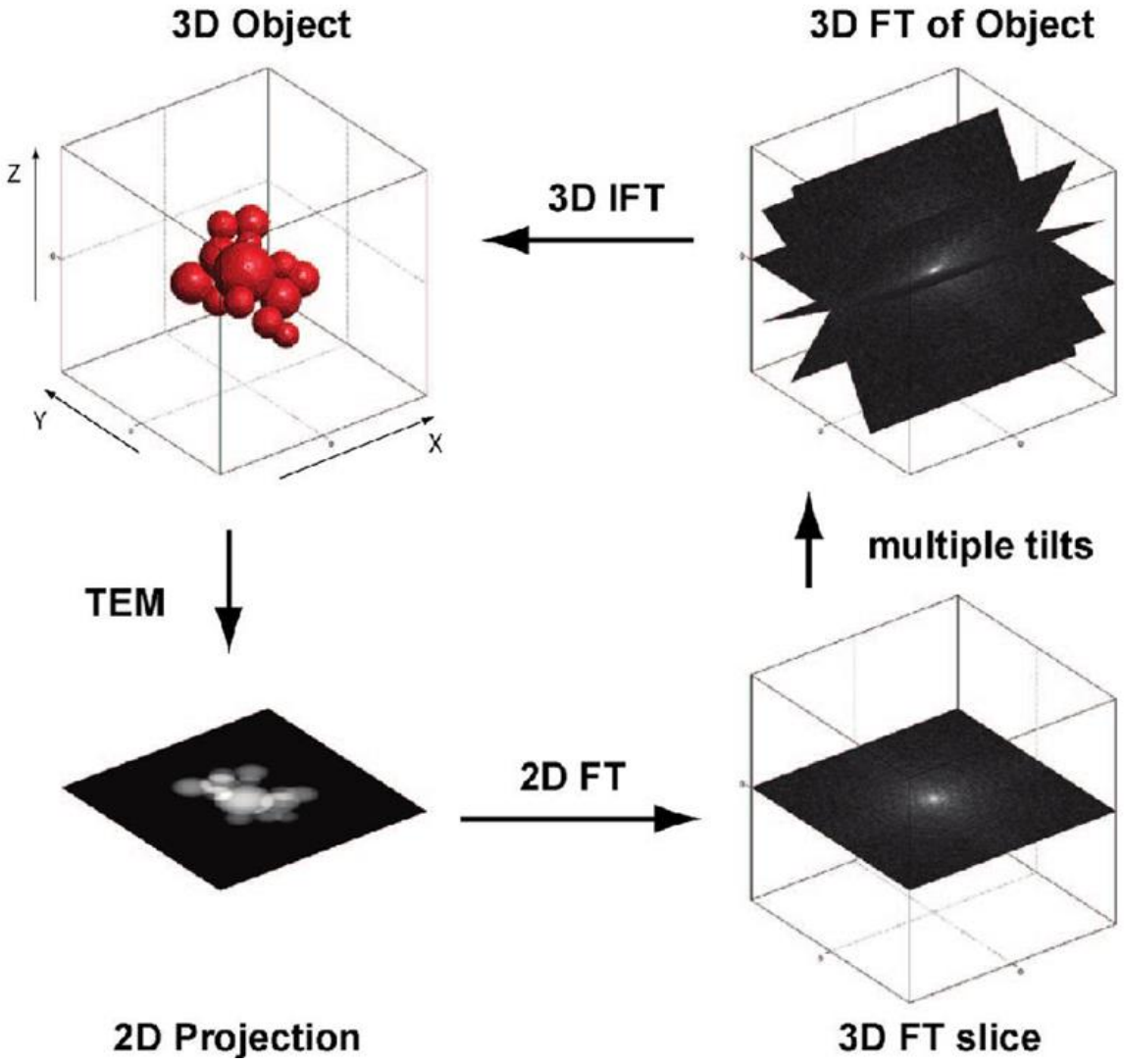


Figure 3. In 1990, Henderson presented a bacteriorhodopsin structure at atomic resolution.

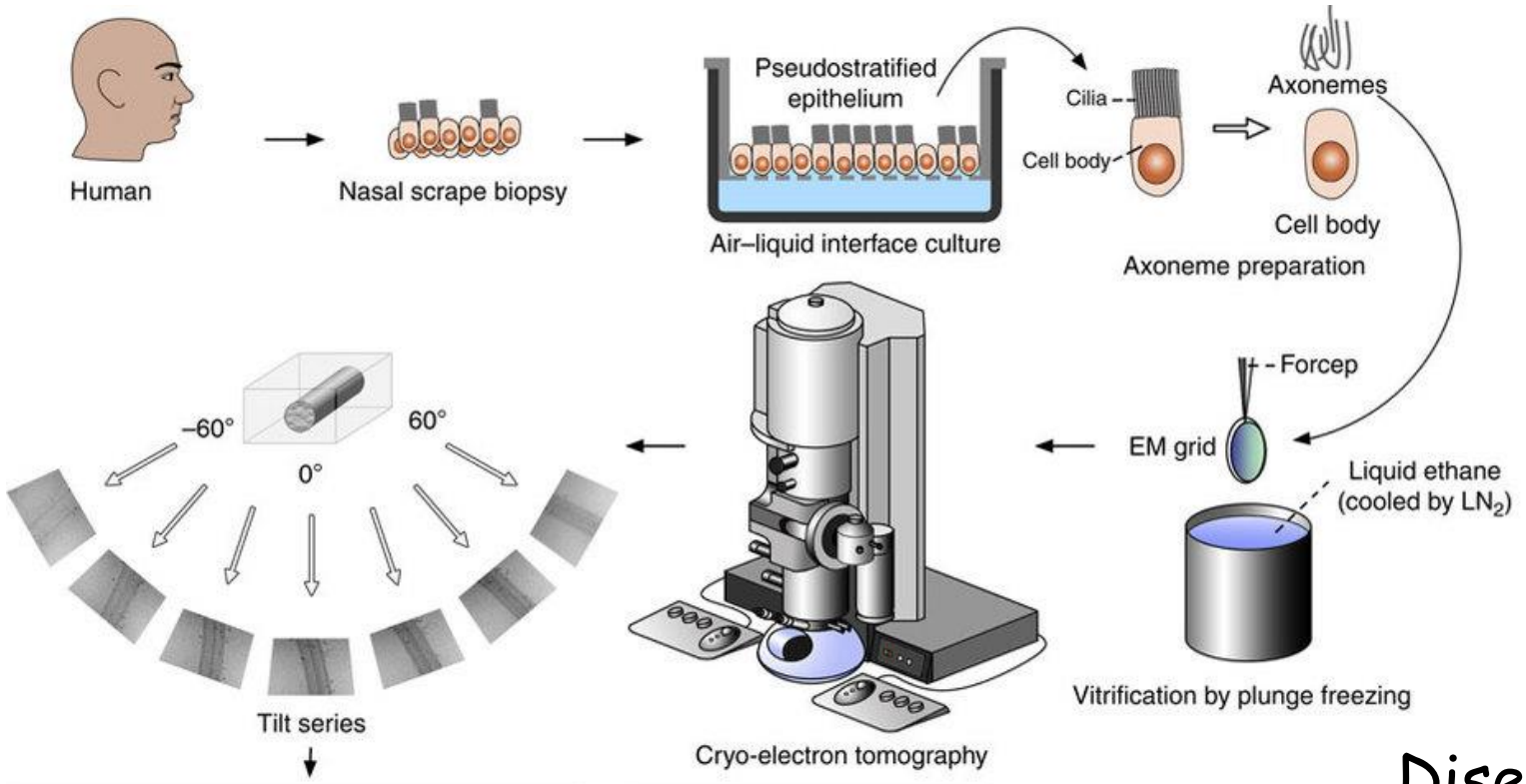


Elektronenmikroskopische -Tomographie

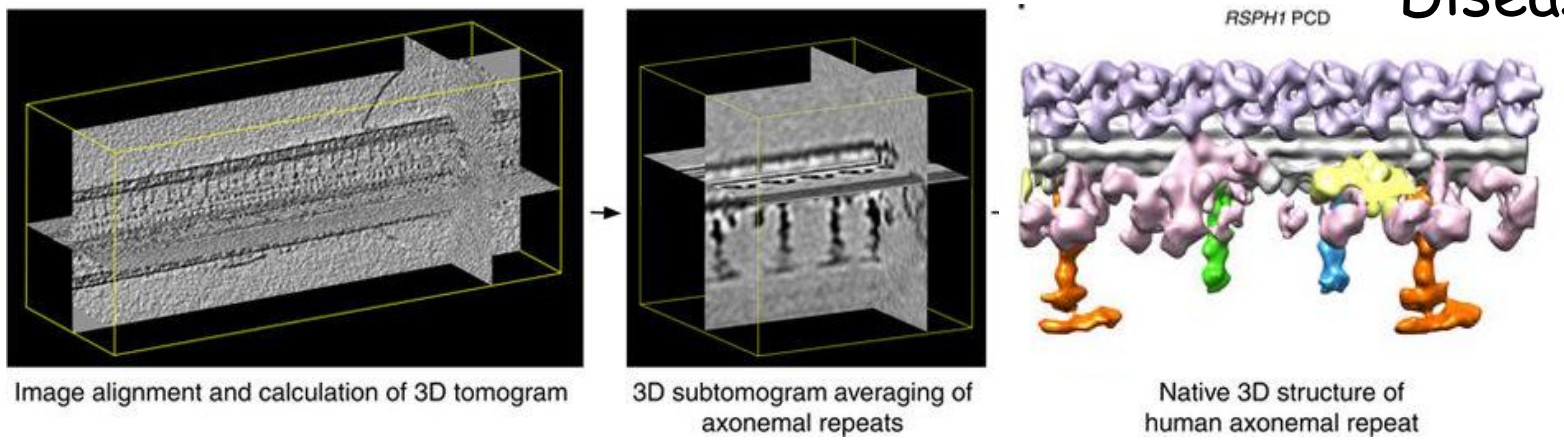




Cryo-EM-tomography of human nasal cilia



Disease



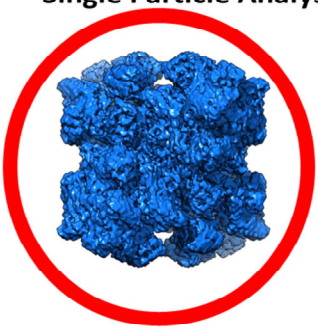
Li et al. (2014) Nat. Com.

3D and Analytical Electron Microscopy

Frank Depoix

3D Electron Microscopy

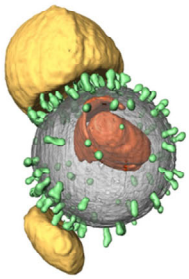
Single Particle Analysis: proteins (> 100 kDa)
molecular complexes



Nobel Price Laureates
2017 (Chemistry)

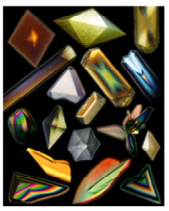
Jacques Dubochet | Joachim Frank | Richard Henderson

Electron Tomography: molecular complexes
cellular compartements
cells
tissues



2

Röntgenkristallographie

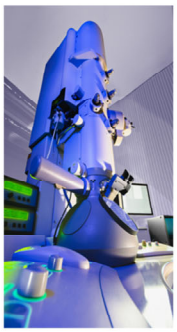


- hohe Auflösung (bis zu 1,5 Å)
- Kristallbildung notwendig
- kleine bis mittelgroße Proteine (max. 1,5 mDa)

NMR-Spektroskopie

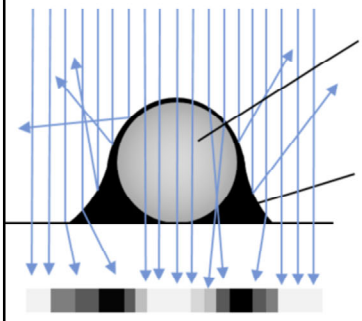
- hohe Auflösung (bis zu 3 Å)
- Protein liegt in Puffer vor
- hohe Proteinkonzentration erforderlich
- nur für kleine Proteine (max. 100 kDa)

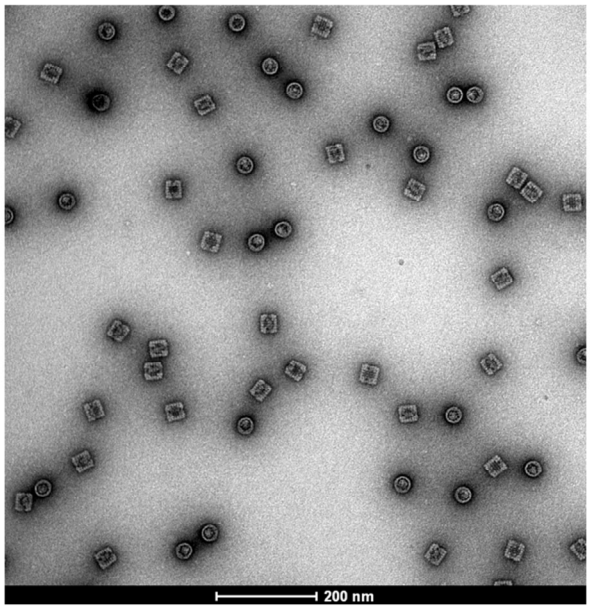
Transmissionselektronenmikroskopie

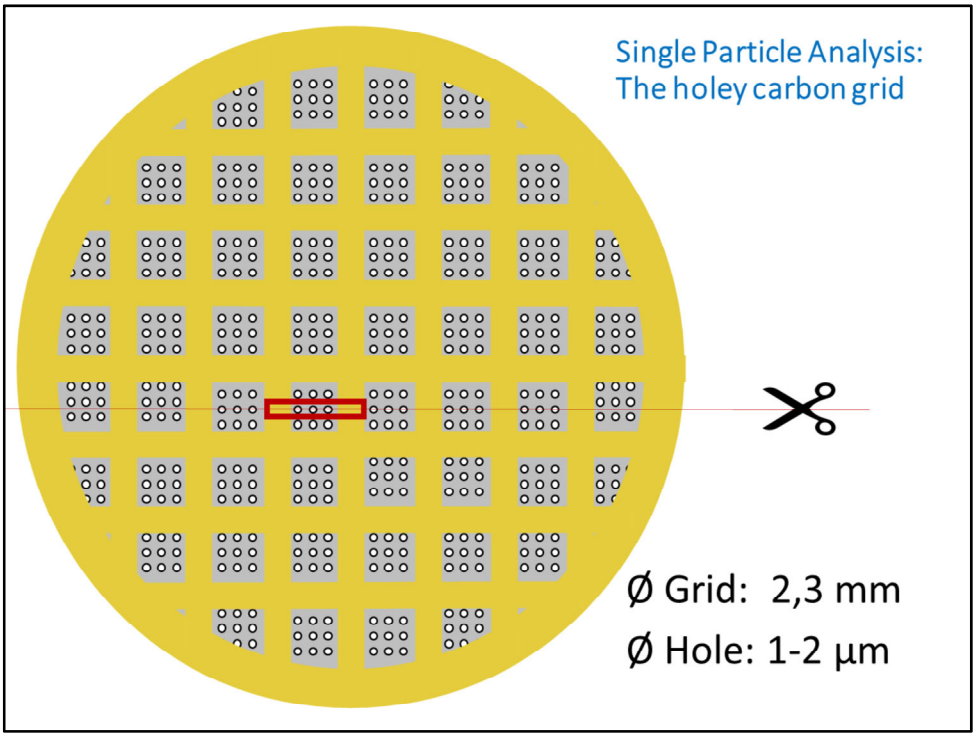


- hohe Auflösung (bis zu 2 Å)
- Proben in physiologischem Medium
- mittelgroße bis große Proteine (min. 200 kDa)

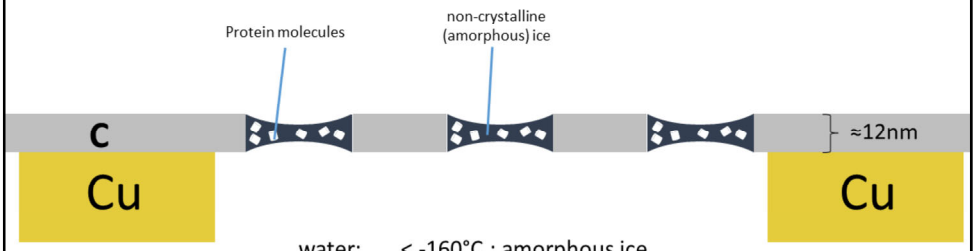
Negative staining shows proteins and particles quick and easy





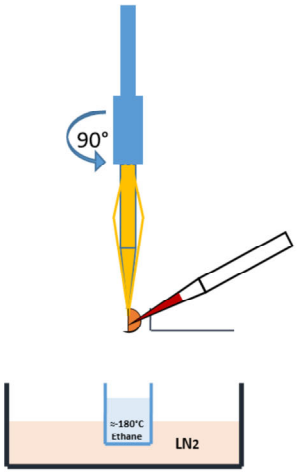

Single Particle Analysis:
The holey carbon grid
Ø Grid: 2,3 mm
Ø Hole: 1-2 µm

Single Particle Analysis: The holey carbon grid

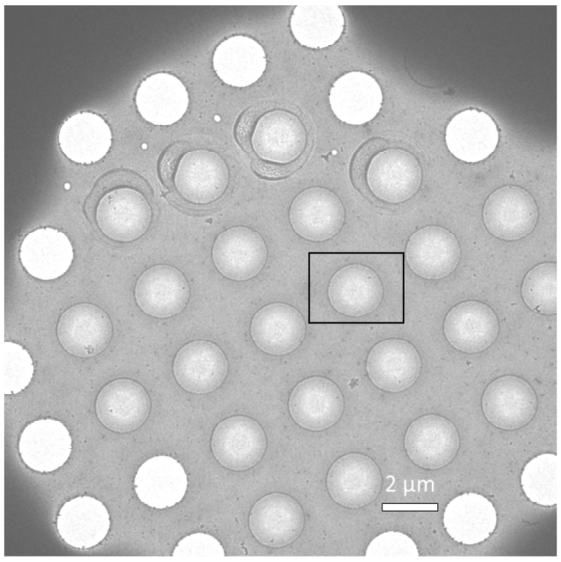


water: < -160°C : amorphous ice
> -160°C : cystalline ice

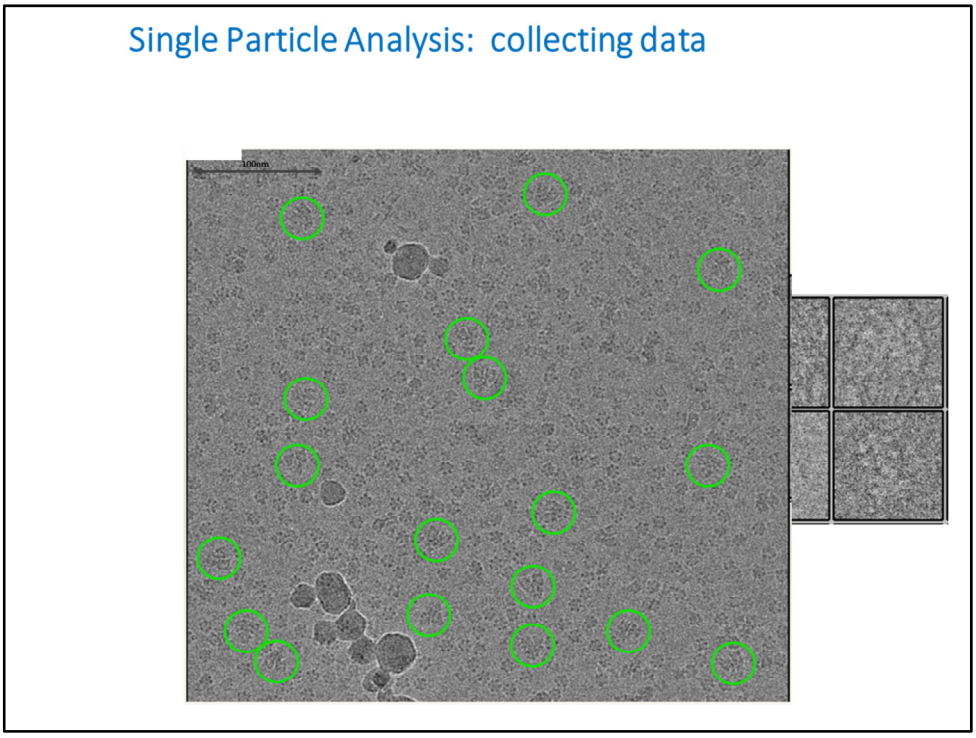
Single Particle Analysis: Cryo vitrification



Single Particle Analysis: in the TEM



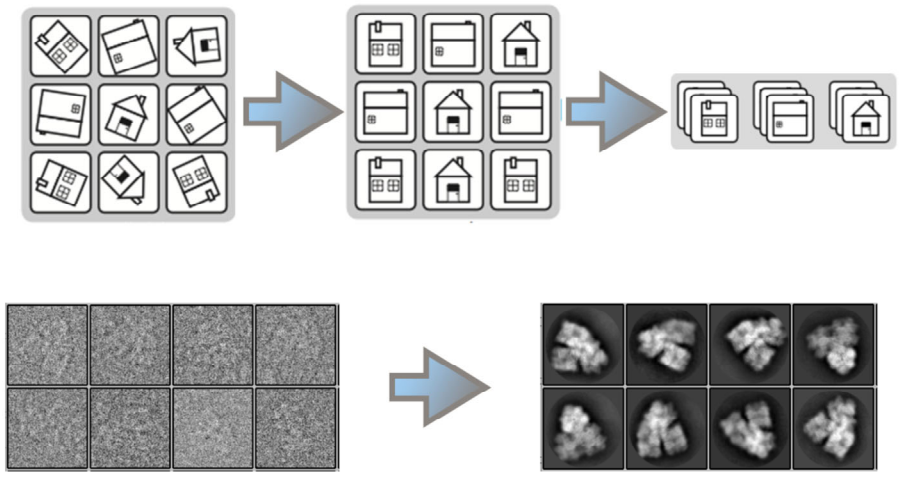
Mag: 1000x



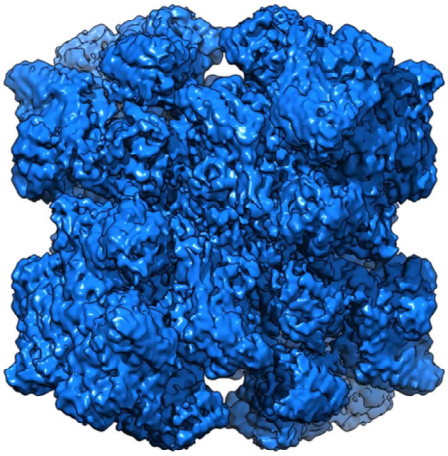
- nach der Präparation wird das Grid mit der Proteinprobe im EM untersucht und mit der Aufnahme der EM-Bilder gestartet
- Aufgrund der neuen Kamerasysteme ist es inzwischen kein Problem mehr die Proteinstrukturen auf den Kryo-Aufnahmen zu identifizieren.
- Ist die Bildakuise abgeschlossen startet man mit der EInzelpartikelanalyse
- Partikel werden einzeln selektiert und als Partikeldatensatz gespeichert.
- anschließend folg die digitale Bildbearbeitung der Einzelpartikel

Single Particle Analysis: computing the data

2D-Classification



Single Particle Analysis: an example
Calianassa truncata hemocyanin

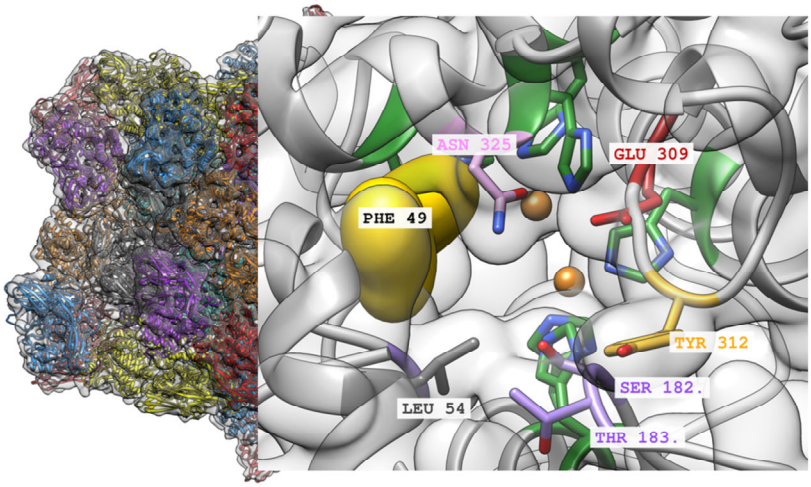


22nm
1,8 MDa

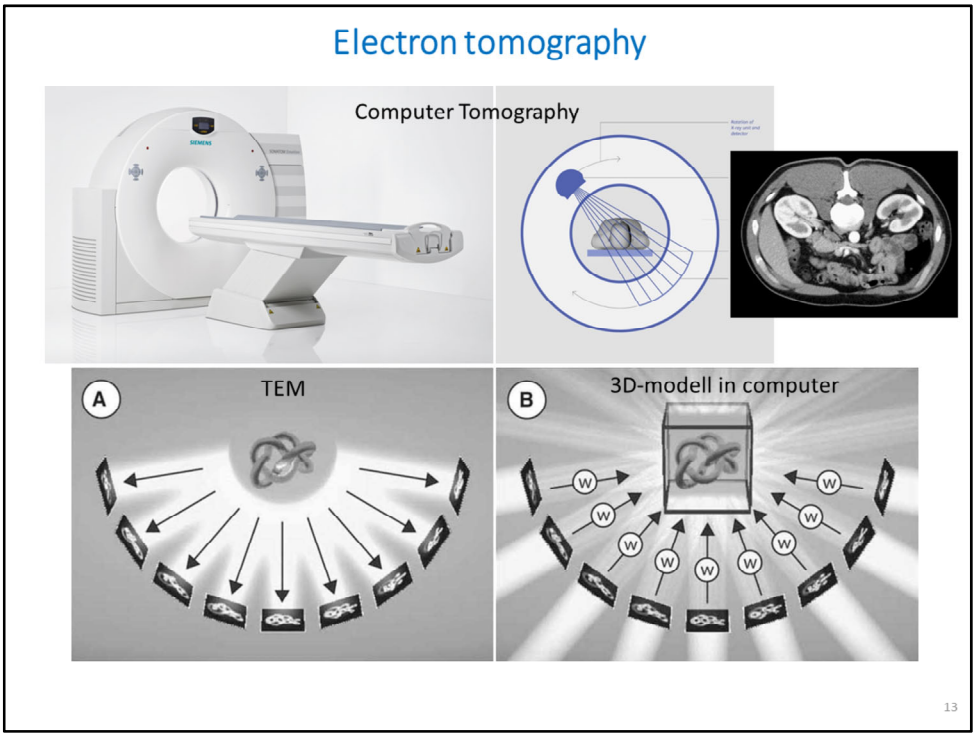
Resolution: 4,9Å (=0,49nm)

Martin Haugwitz 2017

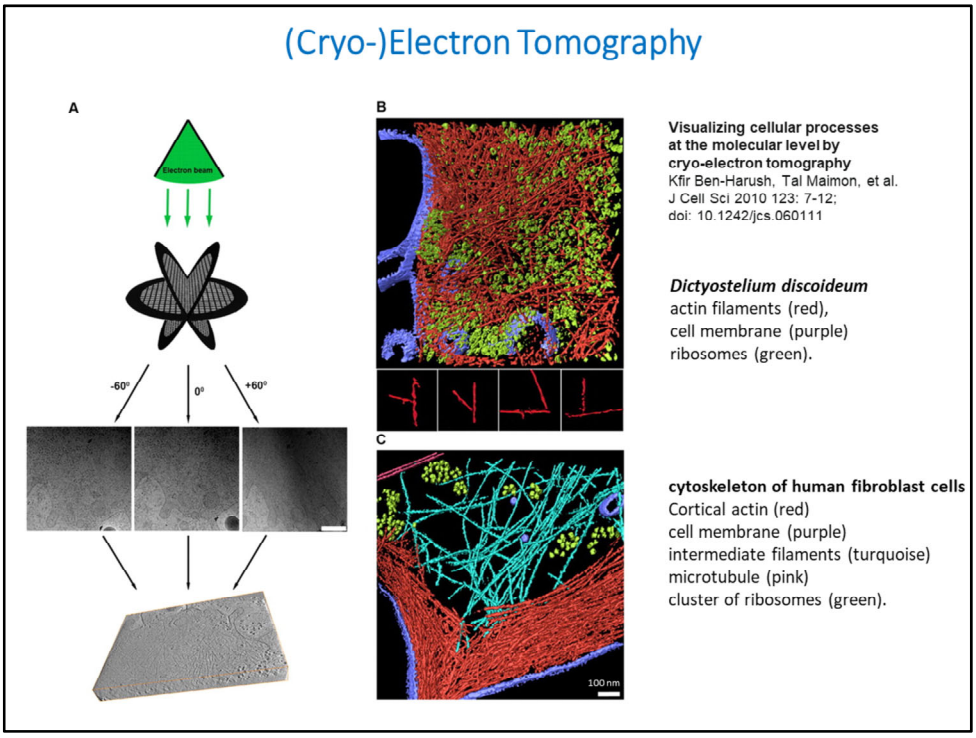
Single Particle Analysis: an example
Calianassa *truncata* hemocyanin



Martin Haugwitz 2017



http://ki.se/en/research/core-facility-for-electron-tomography-0

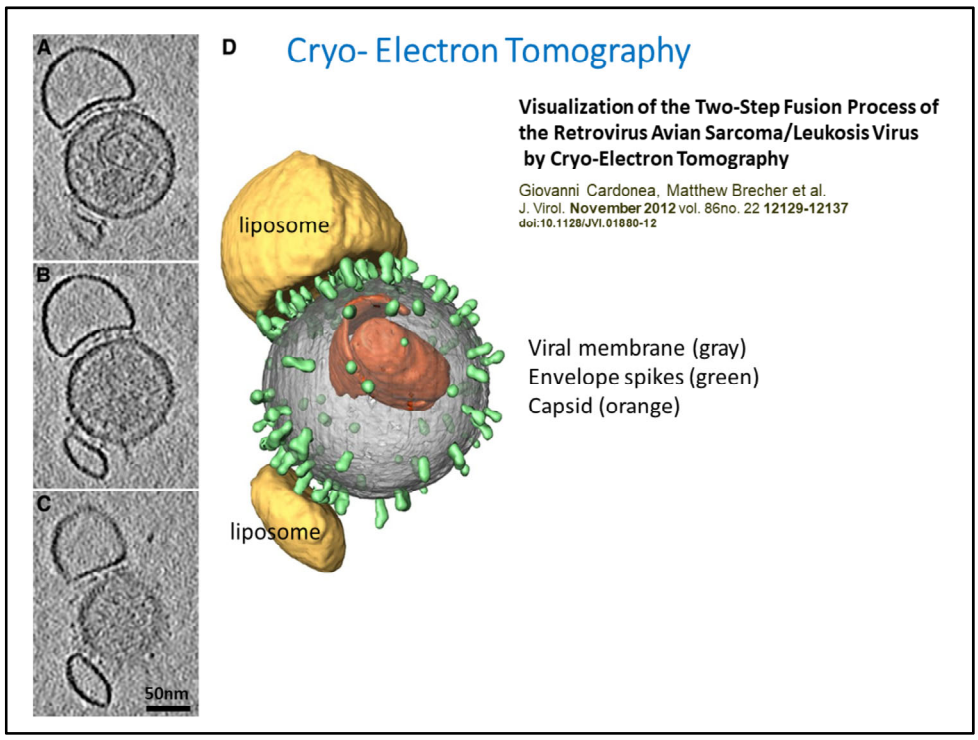


http://jcs.biologists.org/content/123/1/7

Visualizing cellular processes at the molecular level by cryo-electron tomography

Applying cryo-ET to eukaryotic cells. (**A**) 2D projections at different tilt angles for individual 3D objects, such as an intact eukaryotic cell, are recorded by tilting the specimen holder; the projections typically cover ~120°. The holder is tilted incrementally around an axis that is perpendicular to the electron beam. All tilted projections are synthesized into a 3D density map, typically by applying a `weighted back-projection' algorithm (Radermacher, 1992). Shown are projections and a reconstructed volume of a *D. discoideum* cell, adapted from Medalia et al. (Medalia et al., 2002). Scale bar: 300 nm. (**B**) Surface-rendering view of the reconstructed volume shown in A shows the actin filaments (red), cell membrane (purple) and large macromolecular complexes, mostly ribosomes (green). The surface-rendering views were segmented semi-automatically. Colors were chosen subjectively. Branches of actin filaments as found at the cell cortex are shown in the lower panel. (**C**) Surface-rendering view of the reconstructed volume of a human fibroblast cell shows all cytoskeletal elements. Cortical actin (red) is located along the cell membrane (purple), whereas intermediate filaments (turquoise) are localized further into the cell interior; these present a

wider diameter (~10 nm), a different texture and a lower persistence length than actin. In addition, one microtubule (pink) is found in the upper left corner of the tomogram in close proximity to a cluster of ribosomes (green). Scale bar: 100 nm (also for B). (B) Adapted from Medalia et al. (Medalia et al., 2002).

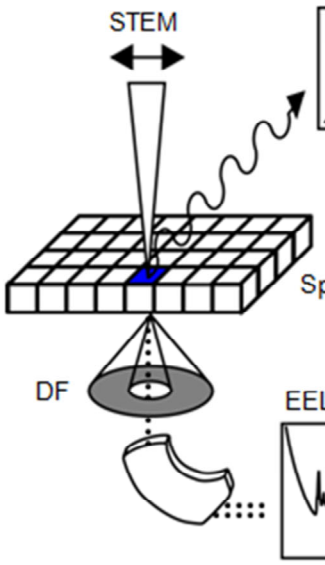


http://jvi.asm.org/content/86/22/12129/F3.expansion.html

Tomographic reconstruction of an ASLV virion interacting with two liposomes.
(A to C) Tomographic slices at different depths. Bar, 50 nm.
(D) Surface rendering with the outer surface of the viral membrane shown in gray, Env spikes in green, liposomes in yellow, and the capsid in orange.

Analytical Electronmicroscopy

Scanning-TEM – Mode (STEM)



X-rays are emitted, when electrons are interacting with the specimen

Energy Dispersive X-ray spectroscopy

Electron Energy Loss Spectroscopy

Electrons can lose energy, when they are interacting with the specimen.

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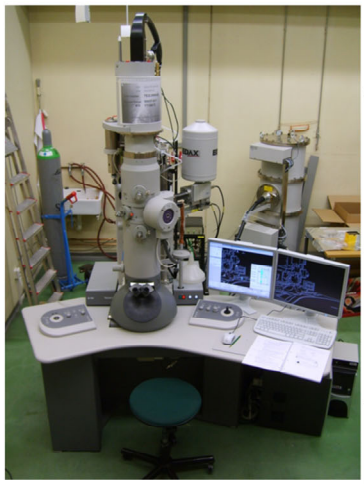
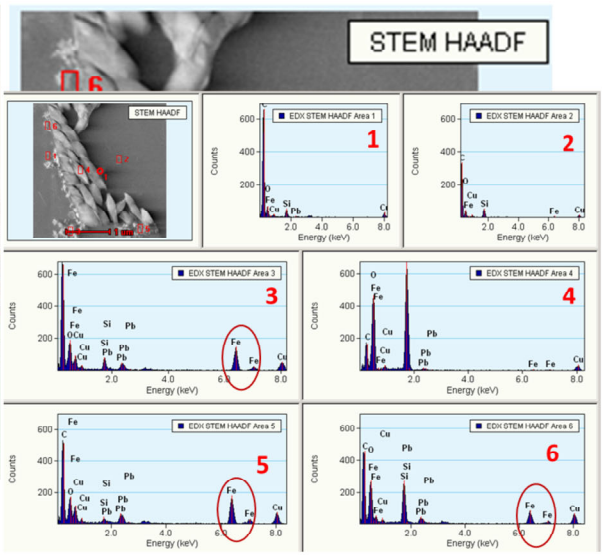
EDX (Energydispersive X-ray spectroscopy)

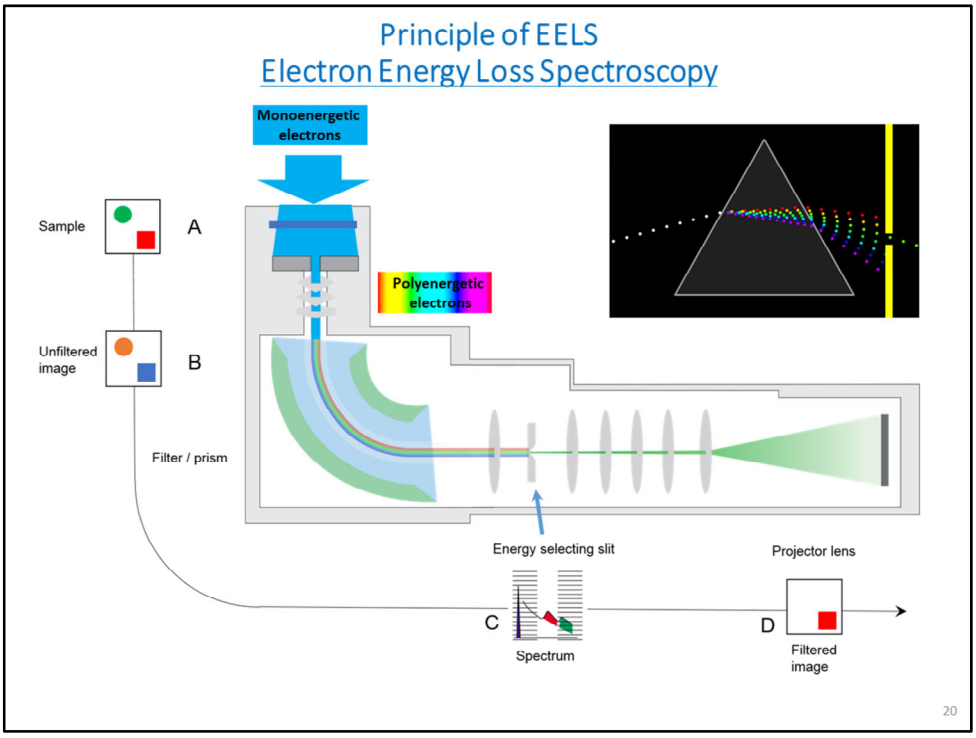
Sycon raphanus

EDX (Energy dispersive X-ray spectroscopy)



EDX on a (S)-TEM





Energy-filtered transmission electron microscopy (EFTEM) is a family of imaging techniques that utilize properties of the energy loss spectrum to increase contrast, remove the effects of chromatic aberration and create unique contrast effects in the image. Key applications include:

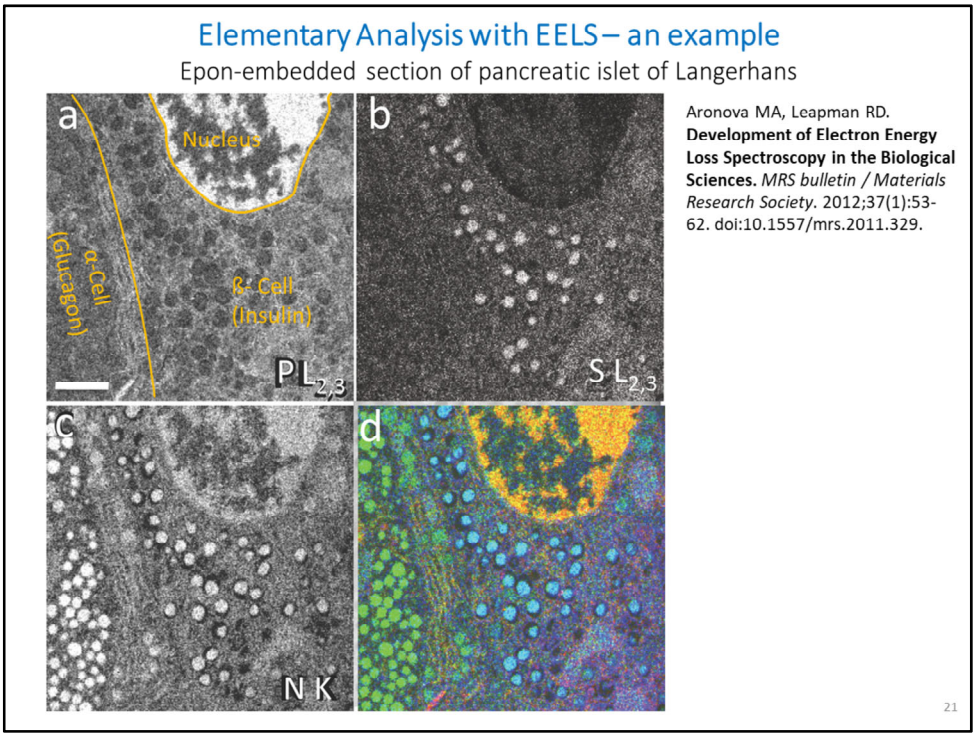
Contrast enhancement – Improves contrast in images and diffraction patterns when it removes inelastically scattered electrons that produce background fog

Including zero-loss filtering, most probable loss imaging, contrast tuning, and pre-carbon imaging

Mapping – Creates elemental/chemical maps at nanometer resolution by forming images with inelastically scattered electrons

Including 2- and 3-window elemental mapping/jump-ratio imaging and chemical mapping – provides fine structure imaging

Analytical – Records and quantifies electron energy loss spectra (and maps) to provide chemical analysis of TEM samples



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3465455/

EFTEM spectrum-imaging of unstained epon-embedded section of pancreatic islet of Langerhans obtained with 5 eV energy loss increments over a 400 eV energy range (from Reference 27). (a) Phosphorus $L_{2,3}$ edge image showing high P concentration in chromatin of cell nucleus. (b) Sulfur $L_{2,3}$ edge image showing high concentration of S in secretory granules of insulin-producing beta cell, but low concentration of S in secretory granules of a neighboring glucagon-producing alpha cell. (c) Nitrogen K edge image showing a strong N signal in granules of both cell types, as well as in the beta cell nucleus. (d) Overlay of the elemental maps with P (red), S (blue), and N (green). (e) Extracted EELS spectrum from beta cell secretory granule illustrating how quantitative analysis can be performed to determine the atomic ratio of sulfur to nitrogen of 0.108:1.

Serial Blockface Imaging

Extracts from Zeiss and Gatan Videos on YouTube :

https://youtu.be/C4-DZilVuAI
https://youtu.be/1ttvyaBlfJc
https://youtu.be/LxZlg1koR6k

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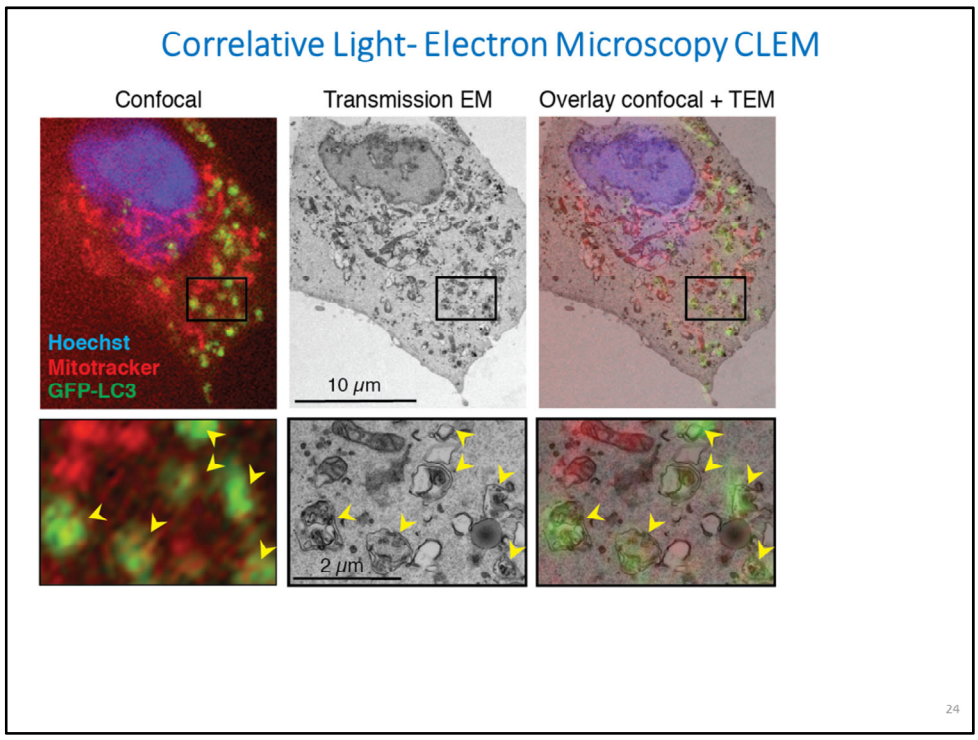
https://www.youtube.com/watch?v=1ttvyaBlfJc

Elektronenmikroskopie-Zentrum Mainz
www.emzm.uni-mainz.de/

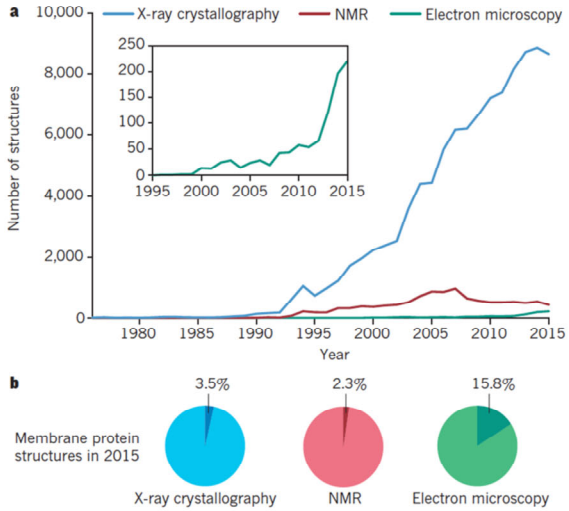
23



Correlative light & electron microscopy of autophagosomes in a wild-type human cell line.
GFP-LC3 marks autophagosomes, and staining the mitochondria with Mitotracker dye improves our ability to register the confocal data with the transmission EM data.

http://www.ucl.ac.uk/lmcb/users/alexander-agrotis

Anzahl publizierter Strukturen der letzten 40 Jahre



Fernandez-Leiro & Scheres, 2016 (*Nature*)