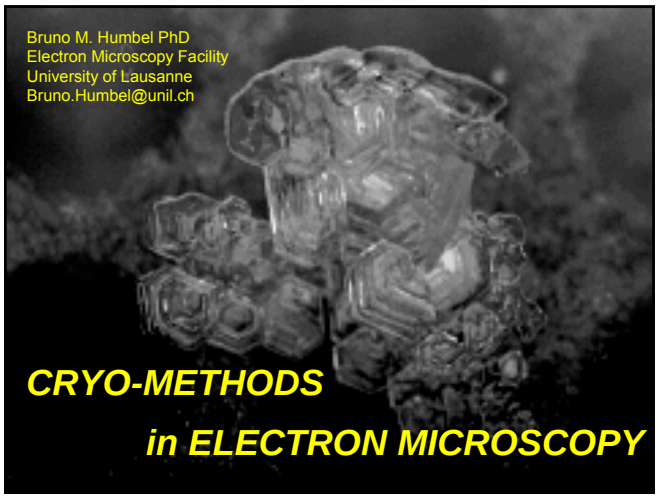
Bruno M. Humbel PhD
Electron Microscopy Facility
University of Lausanne
Bruno.Humbel@unil.ch



CRYO-METHODS in ELECTRON MICROSCOPY

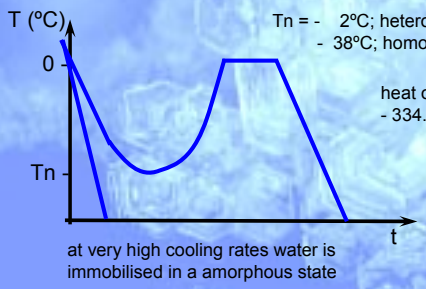
Kutchan
Hokkaido
Japan

CRYO-FIXATION

Solidification of a Biological Specimen by Cooling with the Aim of minimal Displacement of its Components

In: *Cryotechniques in Biological Electron Microscopy,* Steinbrecht RA, Zierold K (eds), Springer-Verlag, Heidelberg, 1987

Methods of Cryo-Fixation



Tn = - 2°C; heterogeneous nucleation
- 38°C; homogeneous nucleation

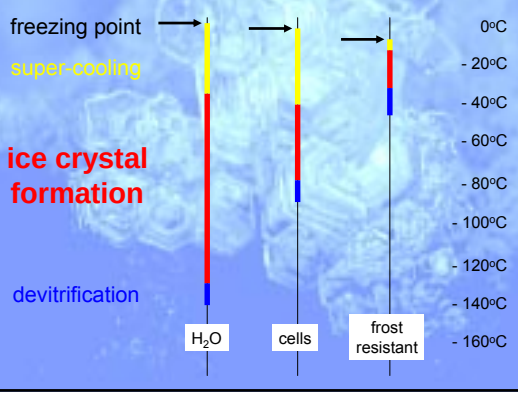
heat of crystallisation:
- 334.9 kJ/kg (- 80 kcal/kg)

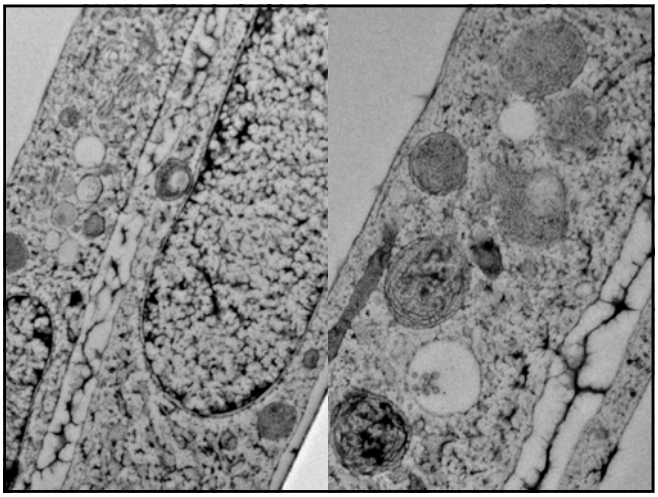
at very high cooling rates water is immobilised in a amorphous state

→ **amorphous, vitrified water**

Robards AW, Sleytr UB, Low Temperature Methods in Biological Electron Microscopy. In: *Practical Methods in Electron Microscopy,* Glauert AM (ed), vol 10, Elsevier, Amsterdam, 1985.

Freezing





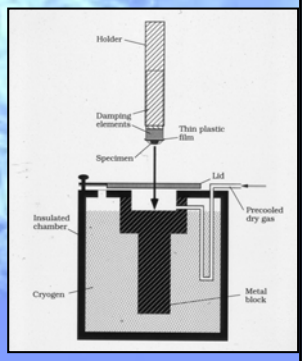
Methods of Cryo-Fixation

- Slamming

  flat specimen,
  one-sided cooling

  high thermal conductivity

  TISSUE (single cells)



Dr. H. Hohenberg, Heinrich-Pette-Institute, Hamburg

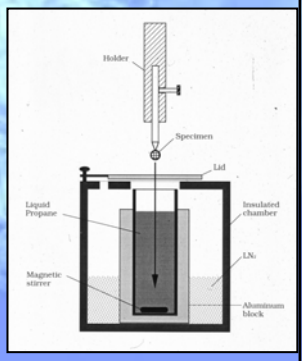
Methods of Cryo-Fixation

- Plunging

  flat specimen; (one) or
  two-sided cooling

  good contact with cryogen,
  high convection

  TISSUE and
  SINGLE CELLS



Dr. H. Hohenberg, Heinrich-Pette-Institute, Hamburg

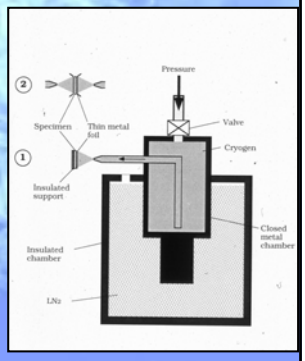
Methods of Cryo-Fixation

- Propane-Jet

  flat specimen; one
  or two-sided cooling

  good contact with cryogen,
  high convection

  SINGLE CELLS
  (TISSUE)



Dr. H. Hohenberg, Heinrich-Pette-Institute, Hamburg

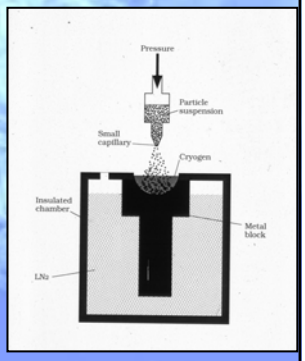
Methods of Cryo-Fixation

- Spray-Freezing

  sphere

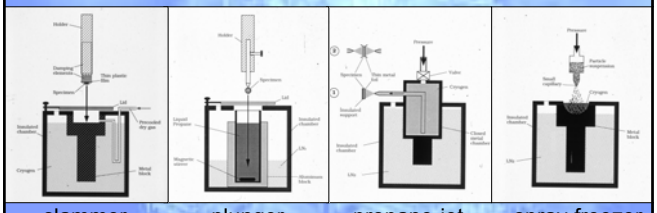
  good contact with cryogen,
  high convection

  SINGLE CELLS



Dr. H. Hohenberg, Heinrich-Pette-Institute, Hamburg

Methods of Cryo-Fixation



slammer plunger propane-jet spray-freezer

maximum depth of cryo-fixation
10 - 20 µm

Methods of Cryo-Fixation

Change the physical properties of water

- Cryo-protectant

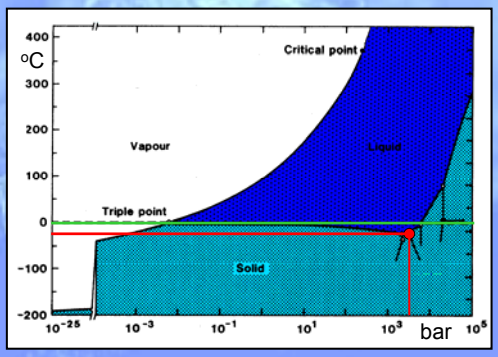
Methods of Cryo-Fxation

Change the physical properties of water

- Cryo-protectant
- High-Pressure

Methods of Cryo-Fixation: HPF

2045 bar
-21°C

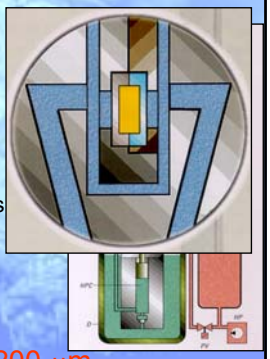


Robards AW, Sleytr UB, Low Temperature Methods in Biological Electron Microscopy. In: *Practical Methods in Electron Microscopy*, Glauert AM (ed), vol 10, Elsevier, Amsterdam, 1985.

Methods of Cryo-Fixation: HPF

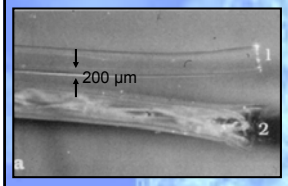
- High-Pressure-Freezing
  - flat specimen, two-side cooling
  - high convection
  - low freezing rate
  - changes the physical properties of water

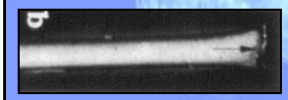
TISSUE and SINGLE CELLS

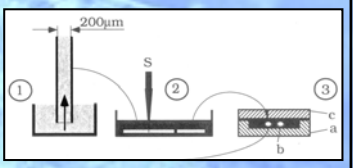


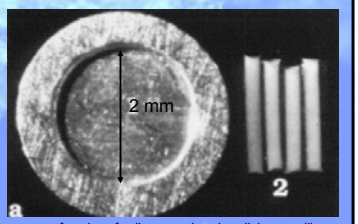
~ 10 times better: 100 - 200 μm

HPF: Specimen Preparation



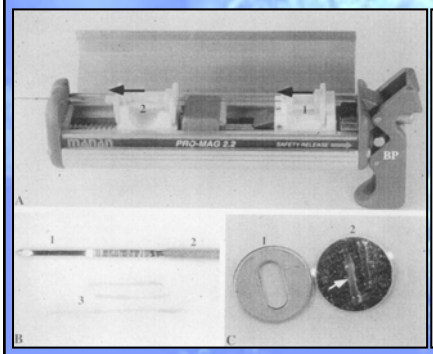


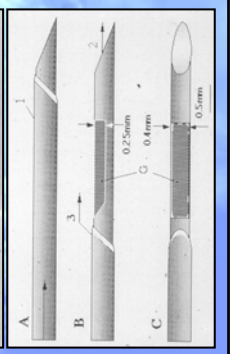




Hohenberg H, Mannweiler K, Müller M. 1994. High-pressure freezing of cell suspensions in cellulose capillary tubes. J Microsc 175, 34-43.

HPF: Specimen Preparation





Hohenberg H, Tobler M, Müller M. 1996. High-pressure freezing of tissue obtained by fine-needle biopsy. J Microsc, 183, 133-139.

5 Machines on the Market *2 principles*

- HPM 010
- Compact 2
- EMPact 2
- HPM 100
- EM SPF

The cryogen is also the pressurising medium

Müller M, Moor H. 1984. Cryofixation of thick specimens by high pressure freezing. In *Science of Biological Specimen Preparation 1983*, Revel JP, Barnard T, Haggis GH. (eds). SEM Inc.: AMF O'Hare; 131-138.

Separated cryogen and pressurising system

Riehle U. 1968. *Über die Vitrifizierung verdünnter wässriger Lösungen*. Federal Institute of Technology (ETH).

Studer D, Graber W, Al-Amoudi A, Eggli P. 2001. A new approach for cryofixation by high-pressure freezing. *J Microsc*, **203**, 285.

Leunissen JLM, Yi H. 2009. Self-pressurized rapid freezing (SPRF): a novel cryofixation method for specimen preparation in electron microscopy. *J Microsc*, **235**, 25-35.

5 Machines on the Market *2 principles*

- HPM 010
- Compact 2
- EMPact 2
- HPM 100
- EM SPF

The cryogen is also the pressurising medium

Müller M, Moor H. 1984. Cryofixation of thick specimens by high pressure freezing. In *Science of Biological Specimen Preparation 1983*, Revel JP, Barnard T, Haggis GH. (eds). SEM Inc.: AMF O'Hare; 131-138.

Separated cryogen and pressurising system

Riehle U. 1968. *Über die Vitrifizierung verdünnter wässriger Lösungen*. Federal Institute of Technology (ETH).

Studer D, Graber W, Al-Amoudi A, Eggli P. 2001. A new approach for cryofixation by high-pressure freezing. *J Microsc*, **203**, 285.

Leunissen JLM, Yi H. 2009. Self-pressurized rapid freezing (SPRF): a novel cryofixation method for specimen preparation in electron microscopy. *J Microsc*, **235**, 25-35.

5 Machines on the Market *2 principles*

- HPM 010 most publications !
- Compact 2
- EMPact 2
- HPM 100
- EM SPF

The cryogen is also the pressurising medium

Müller M, Moor H. 1984. Cryofixation of thick specimens by high pressure freezing. In *Science of Biological Specimen Preparation 1983*, Revel JP, Barnard T, Haggis GH. (eds). SEM Inc.: AMF O'Hare; 131-138.

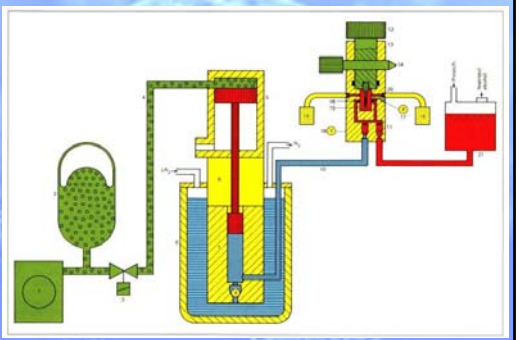
Separated cryogen and pressurising system

Riehle U. 1968. *Über die Vitrifizierung verdünnter wässriger Lösungen*. Federal Institute of Technology (ETH).

Studer D, Graber W, Al-Amoudi A, Eggli P. 2001. A new approach for cryofixation by high-pressure freezing. *J Microsc*, **203**, 285.

Leunissen JLM, Yi H. 2009. Self-pressurized rapid freezing (SPRF): a novel cryofixation method for specimen preparation in electron microscopy. *J Microsc*, **235**, 25-35.

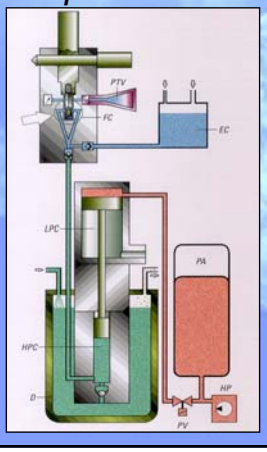
Bal-Tec HPM 010 *now Boeckeler RMC and ABRA*



http://www.high-pressure-freezing-machine-hpm-010.com/high-pressure-freezer.html
http://www.rmcproducts.com/cms/index.cfm/path/17933/25999/24156/24167/

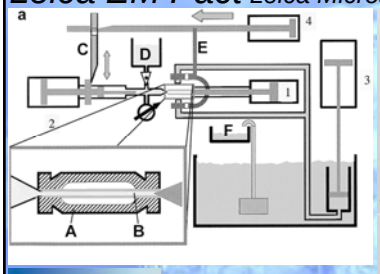
Müller M, Moor H. 1984. Cryofixation of thick specimens by high pressure freezing. In *Science of Biological Specimen Preparation 1983*, Revel JP, Barnard T, Haggis GH. (eds). SEM Inc.: AMF O'Hare; 131-138.

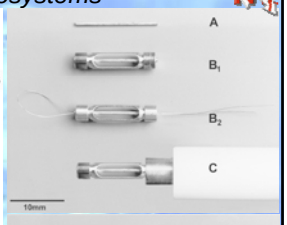
Compact 2 *Wohlwend Engineering*

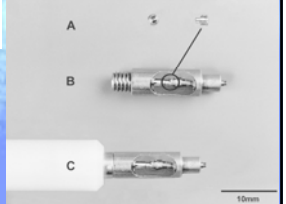


Engineering Office M. Wohlwend GmbH
Bifig 14
9466 Sennwald
Switzerland
phone: 0041 81 7571924
Facsimile: 0041 81 7572243
e-mail: martin-wohlwend@bluewin.ch

Leica EM Pact *Leica Microsystems*

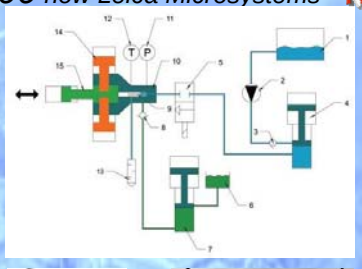


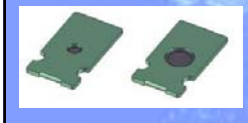




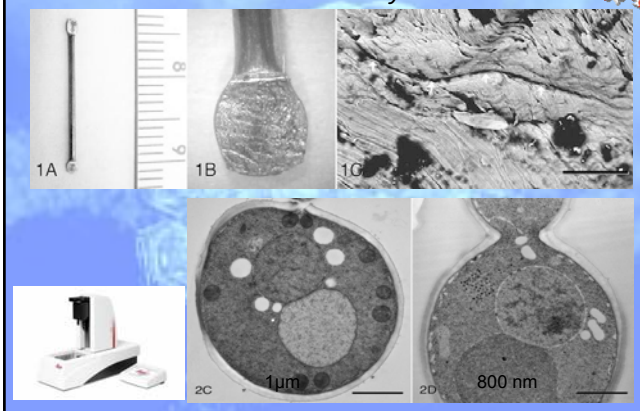
Studer D, Graber W, Al-Amoudi A, Eggli P. 2001. A new approach for cryofixation by high-pressure freezing. *J Microsc*, **203**, 285.

Bal-Tec HPM 100 *now Leica Microsystems*





Leica EM SPF Leica Microsystems



Leunissen JLM, Yi H. 2009. Self-pressurized rapid freezing (SPRF): a novel cryofixation method for specimen preparation in electron microscopy. *J Microsc*, **235**, 25-35.

Chemical Fixation

Neanderthals Courtesy of Prof Dr Arie J Verkleij

Why Cryo ?

Preservation in time and space

Mammoth Siberia
Courtesy of Prof Dr Arie J Verkleij

Why Cryo-Fixation

- Reduced Fixation Artifacts
  - membrane blisters
  - mesosomes
  - nuclear equivalent

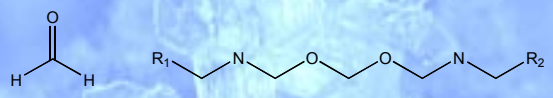
Chemical Fixation

to protect the ultrastructure from collapse and loss of cellular components

- cross-linking of proteins: aldehydes
- cross-linking of lipids: uranyl, osmium

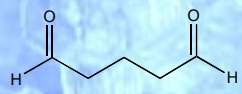
distortion of proteins
proteolysis
masking of epitopes

Formaldehyde



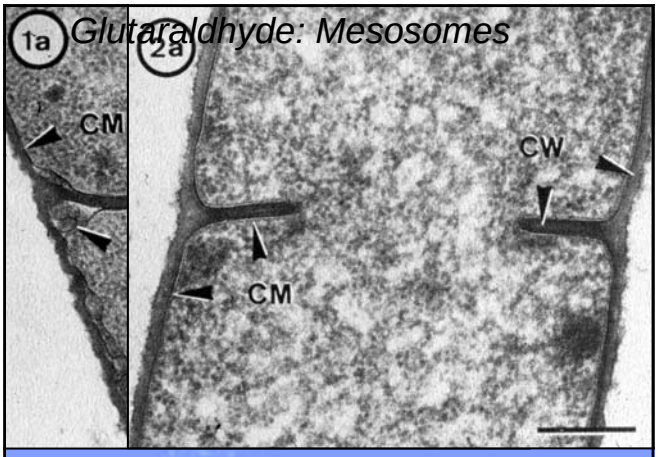
- reacts primarily with $-NH_2$ groups
- and C=C groups
- cross-links proteins, unsaturated fatty acids
- fast penetration
- dissociates easily at concentrations < 4%
- little influence on antigenicity

Glutaraldhyde



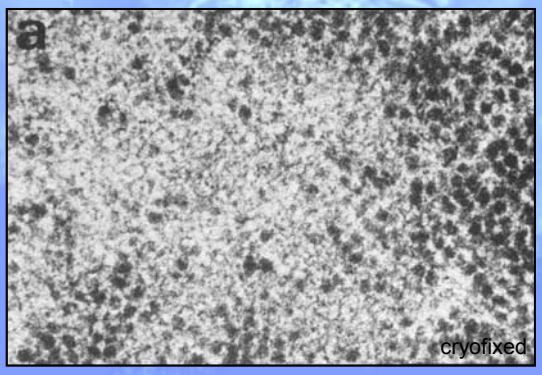
- reacts primarily with $-NH_2$ groups
- cross-links proteins
- slow penetration
- cross-linking is irreversible
- cave: pH drop => strong buffers
- great influence on antigenicity

Glutaraldhyde: Mesosomes



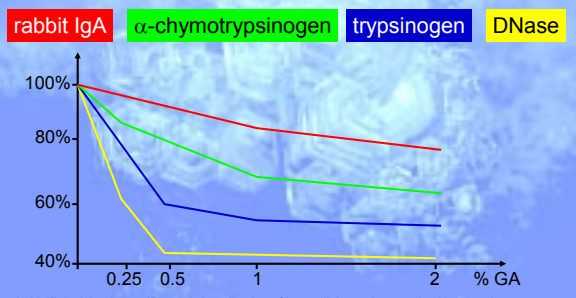
Ebersold HR, Cordier JL, Lüthy P. 1981. Bacterial mesosomes: method dependent artifacts. *Arch Microbiol* **130**,19-22.

Glutaraldehyde



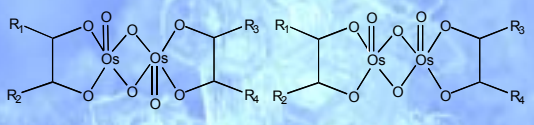
Hobot JA, Villiger W, Escaig J, Maeder M, Ryter A, Kellenberger E. 1985. Shape and fine structure of nucleoids observed on sections of ultrarapidly frozen and cryosubstituted bacteria. *J Bacteriol* **162**, 960 - 971.

GA/ antigenicity: concentration



Krähenbuhl JP and Jamieson JD. 1973. Localization of intracellular antigens using immunoelectron microscopy. *In* Electron Microscopy and Cytochemistry. North-Holland Publ. Com., Amsterdam. 181-192.

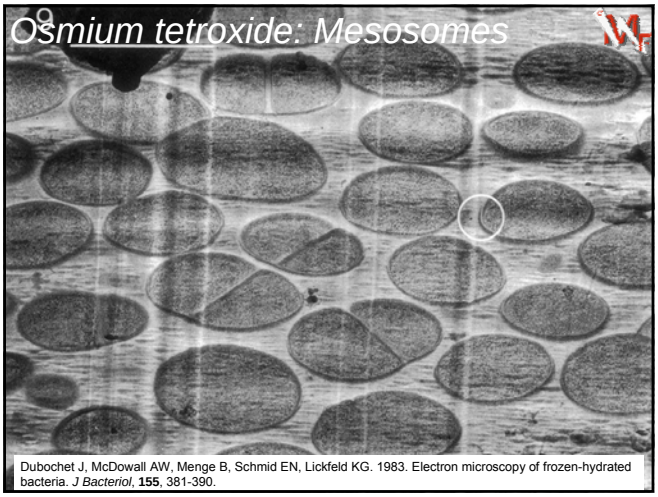
Osmium Tetroxide



- reacts C=C groups
- cross-links proteins, unsaturated fatty acids
- proteolytic
- fast penetration
- great influence on antigenicity

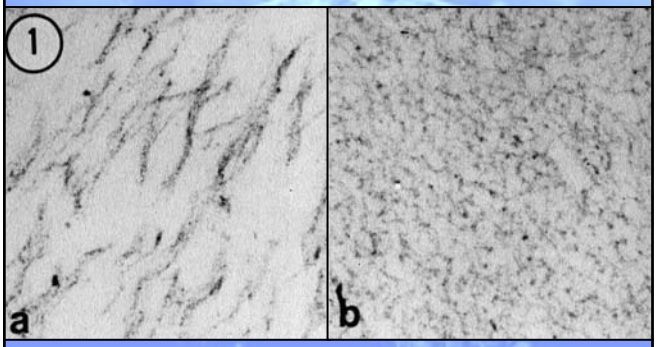
Behrman EJ. 1983. The chemistry of osmium tetroxide fixation. *In* The Science of Biological Specimen Preparation 1983. Revel JP Barnard T and Haggis GH, eds. SEM Inc., AMF O'Hare, IL 60666. 1-5.

Osmium tetroxide: Mesosomes



Dubochet J, McDowall AW, Menge B, Schmid EN, Lickfeld KG. 1983. Electron microscopy of frozen-hydrated bacteria. *J Bacteriol*, **155**, 381-390.

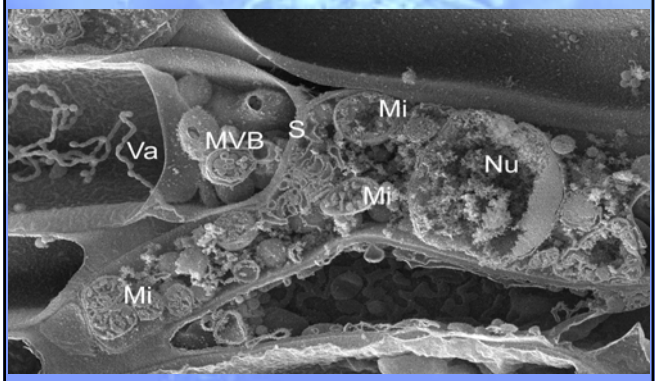
Osmium Tetroxide: 'Protease'



30' 100 mM GA rt; 10' 4 mM OsO_4 2°C 30' 100 mM GA rt; 60' 40 mM OsO_4 rt

Maupin P, Pollard TD. 1983. Improved preservation and staining of HeLa cell actin filaments, clatrin-coated membranes, and other cytoplasmic structures by tannic acid-glutaraldehyde-saponin fixation. J Cell Biol, **96**, 51-62.

Osmium Tetroxide

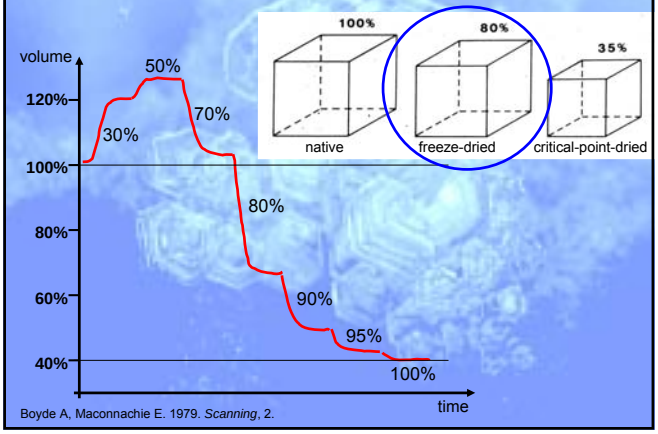


Dr. WH Müller, Utrecht University, Utrecht, The Netherlands

Why Cryo-Fixation

- Reduced Fixation Artifacts
  - membrane blisters
  - mesosomes
  - nuclear equivalent
- Reduced Shrinkage

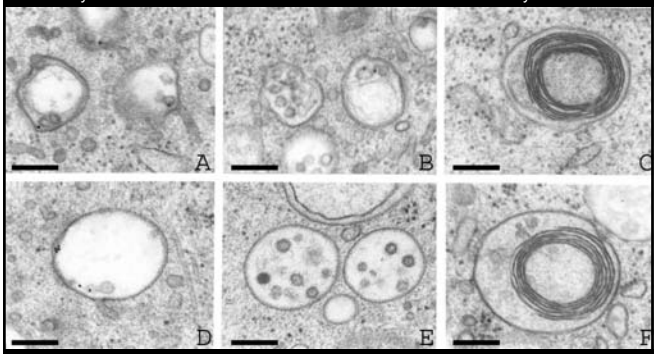
Shrinkage



Boyde A, Maconnachie E. 1979. *Scanning*, 2.

Chemical vs Cryo-Fixation

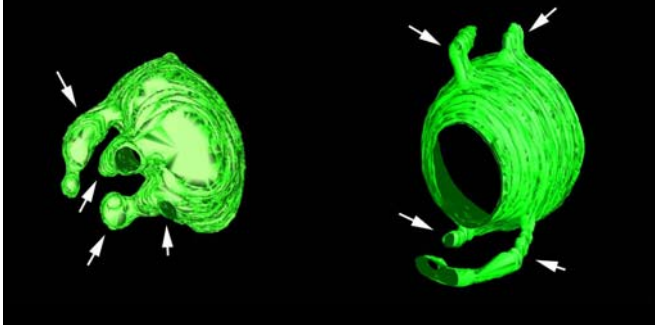
35% early endosome 30% late endosome 9% lysosome



Murk JLAN, Posthuma G, Koster AJ, Geuze HJ, Verkleij AJ, Kleijmeer MJ, Humbel BM. 2003. Influence of aldehyde fixation on the morphology of endosomes and lysosomes. J Microsc, **212**, 81-90.

Chemical vs Cryo-Fixation

chemical fixation cryo-fixation



Murk JLAN, Posthuma G, Koster AJ, Geuze HJ, Verkleij AJ, Kleijmeer MJ, Humbel BM. 2003. Influence of aldehyde fixation on the morphology of endosomes and lysosomes. J Microsc, **212**, 81-90.

Why Cryo-Fixation

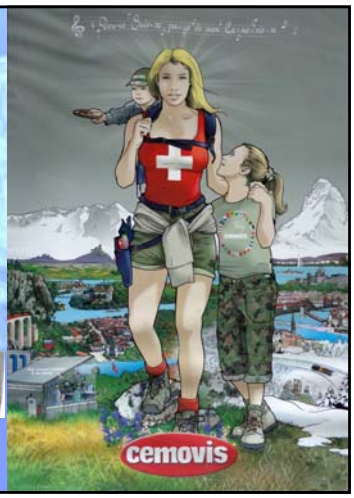
- Reduced Fixation Artifacts
  - membrane blisters
  - mesosomes
  - nuclear equivalent
- Reduced Shrinkage
- Reduced Extraction of Cellular Components
  - lipids
  - proteins
  - proteoglycans

Reduced Extraction

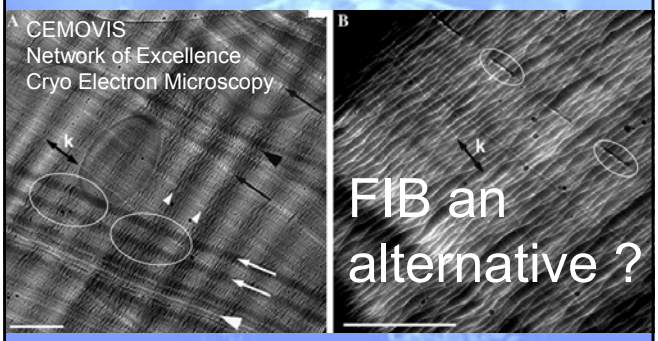
- Lipids
  - acetone: 5%[3]
  - methanol: 15% – 45%[3]
  - methanol at -70°C: 9%[2]
  - methanol at -30°C: 15%[2]
  - methanol + UA at -70°C: 2%[2]
  - methanol + UA at -30°C: 4%[2]
- Proteins
  - 2.2 %[1]
- Proteoglycans
  - 0.25%[1]

[1]Hunziker EB, Herrmann W. 1987. In situ localization of cartilage extracellular matrix components by immunoelectron microscopy after cryotechnical tissue processing. *J Histochem Cytochem*, **35**, 647-655.
[2]Humbel BM, Schwarz H. 1989. Freeze-substitution for immunochemistry. In *Immuno-Gold Labeling in Cell Biology*, Verkleij AJ, Leunissen JLM. (eds). CRC Press: Boca Raton; 115-134.
[3]Weibull C, Villiger W, Carlemalm E. 1984. Extraction of lipids during freeze-substitution of Acholeplasma laidlawii-cells for electron microscopy. *J Microsc*, **134**, 213-216.

Cryo-Electron Microscopy of Vitrous Sections

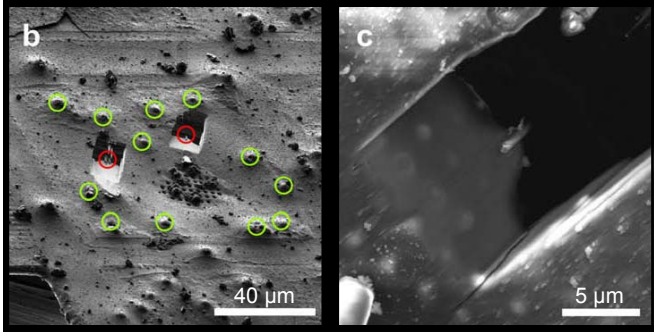


Cryo-Electron Tomography



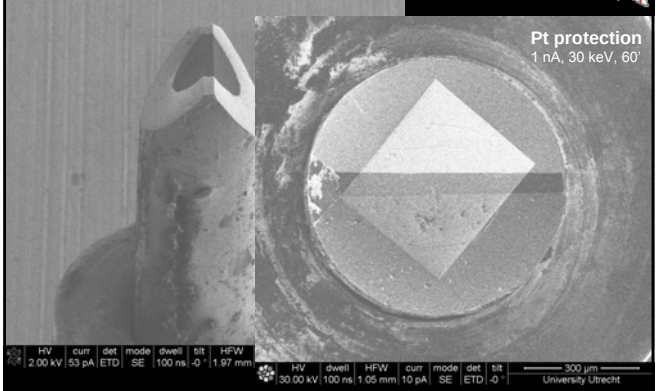
Al-Amoudi A, Studer D, Dubochet J. Cutting artefacts and cutting process in vitreous sections for cryo-electron microscopy, *J. Struct. Biol.*, 150, 109, 2005.

FIB Thinning



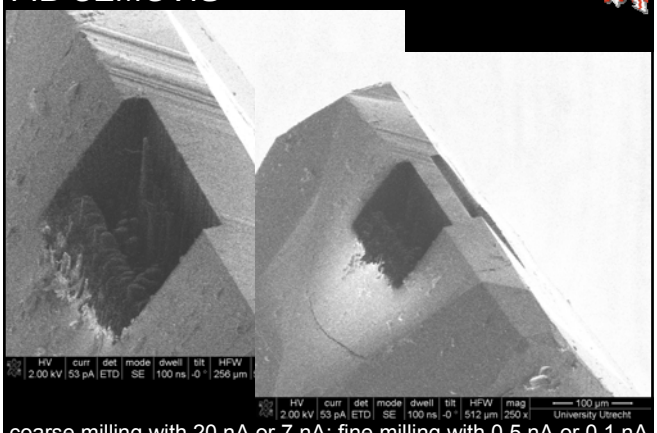
Rigort A, Bäuerlein FJB, Leis A, Gruska M, Hoffmann C, Laugks T, Böhm U, Eibauer M, Gnaegi H, Baumeister W, Plitzko JM. 2010. Micromachining tools and correlative approaches for cellular cryo-electron tomography. *J Struct Biol*, in press.

FIB CEMOVIS



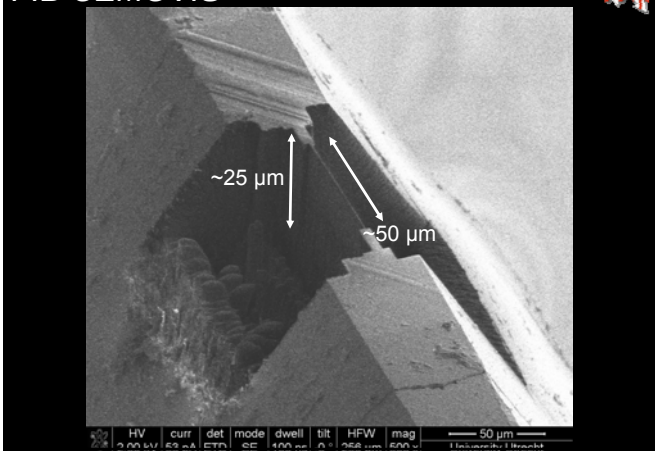
Hayles MF, de Winter DAM, Schneijdenberg CTWM, Meeldijk JD, Luecken U, Persoon H, de Water J, de Jong F, Humbel BM, Verkleij AJ. 2010. The making of frozen-hydrated, vitreous lamellas from cells for cryo-electron microscopy. *J Struct Biol*, **172**, 180-190.

FIB CEMOVIS

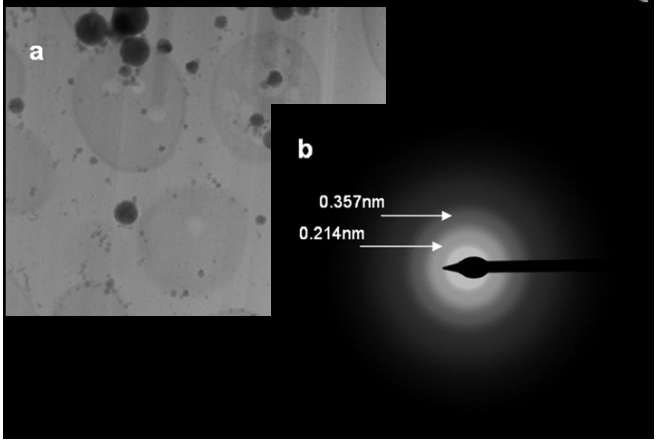


coarse milling with 20 nA or 7 nA; fine milling with 0.5 nA or 0.1 nA

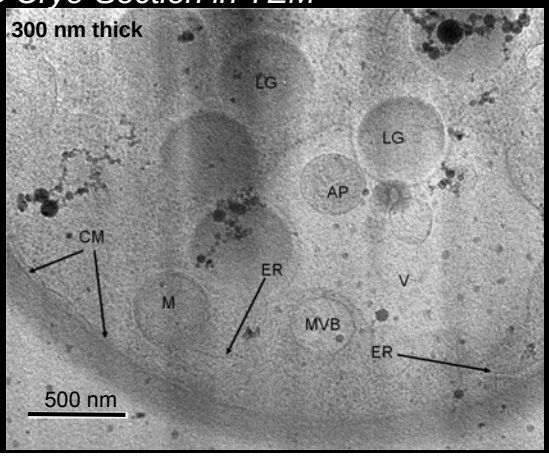
FIB CEMOVIS



FIB Cryo-Section in TEM



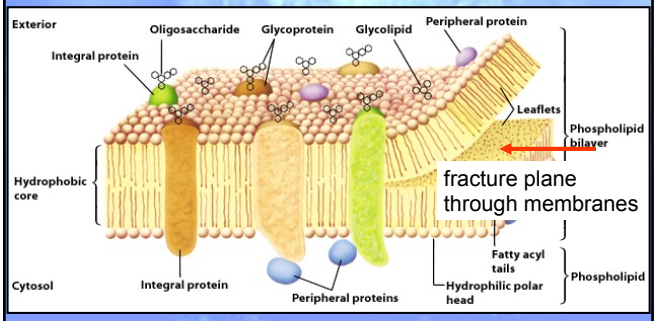
FIB Cryo-Section in TEM



Freeze-Fracture
Freeze-Etching

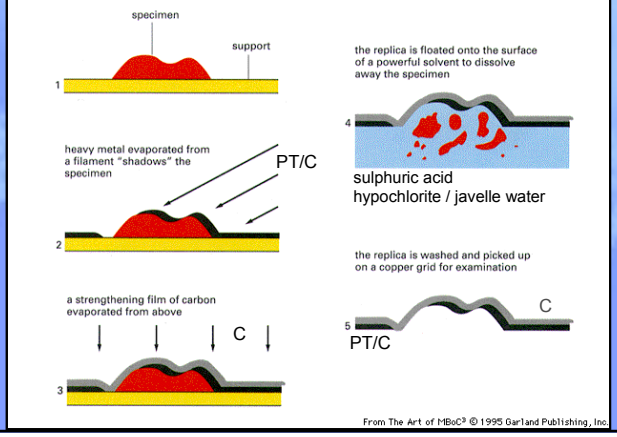
Freeze-Fracturing

Friederich Kopp, ETHZ & Daniel Branton, Harvard

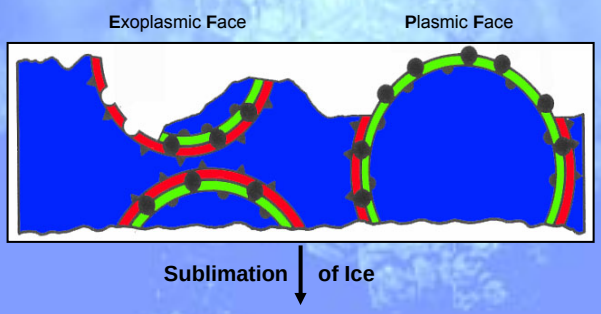


Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell JE, 2000, Molecular Cell Biology WH Freeman & Co, New York.

Freeze-Fracturing

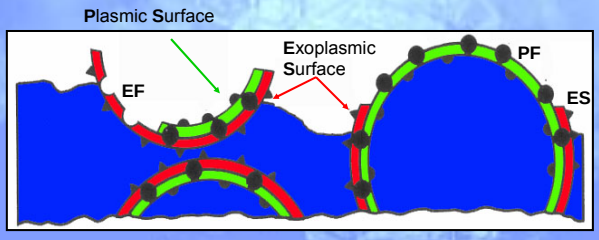


Freeze-Fracturing

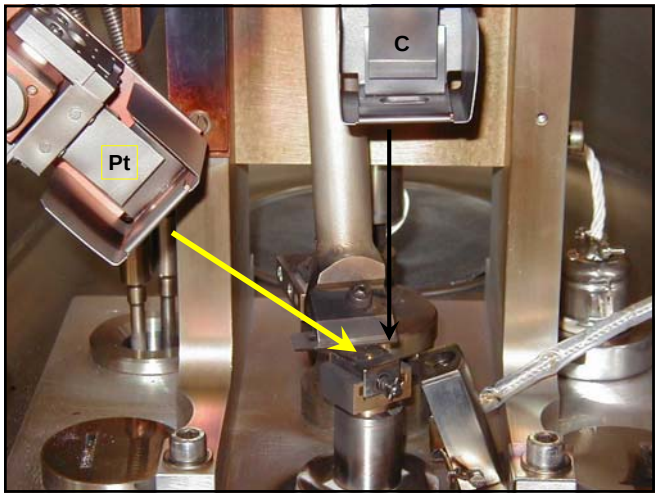


Flegler SL, Heckman JW, Klomparens KL, 1993, Scanning and Transmission Electron Microscopy, WH Freeman & Co, New York.

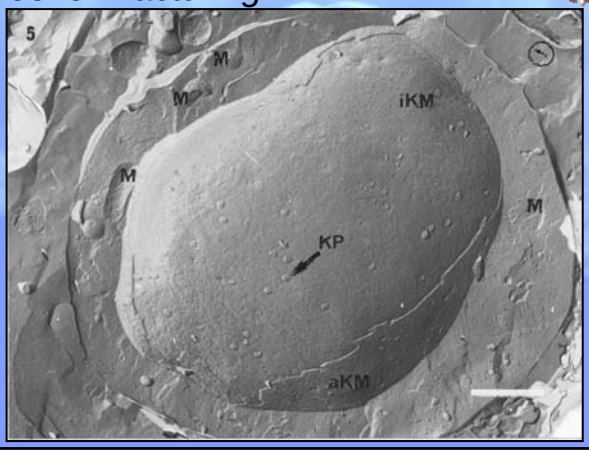
Freeze-Etching



Flegler SL, Heckman JW, Klomparens KL, 1993, Scanning and Transmission Electron Microscopy, WH Freeman & Co, New York.



Freeze-Fracturing

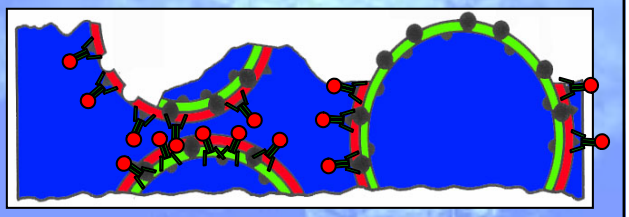


Freeze-Fracturing

Nettle
frozen
Pt coated
cryo-SEM

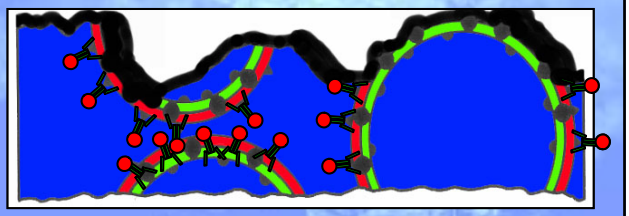
Antonio Mucciolo
Willy Blanchard
EMF
Dr Edward Farmer
DBMV
Lausanne

Label-Fracturing



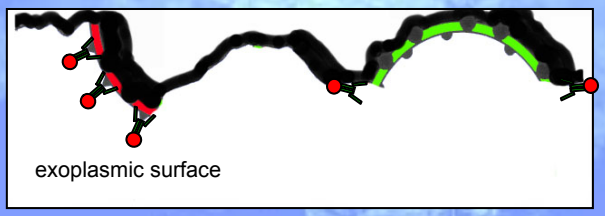
Flegler SL, Heckman JW, Klomparens KL, 1993, Scanning and Transmission Electron Microscopy, WH Freeman & Co, New York.

Label-Fracturing



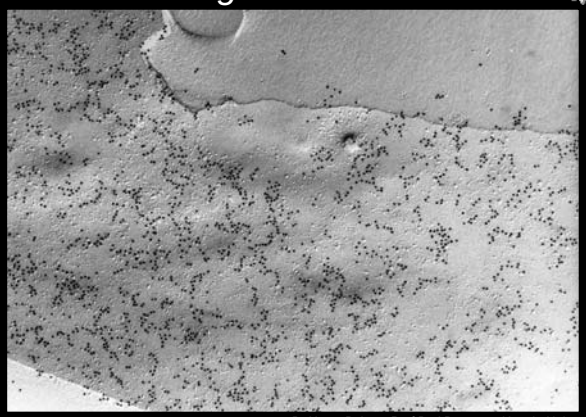
Flegler SL, Heckman JW, Klomparens KL, 1993, Scanning and Transmission Electron Microscopy, WH Freeman & Co, New York.

Label-Fracturing



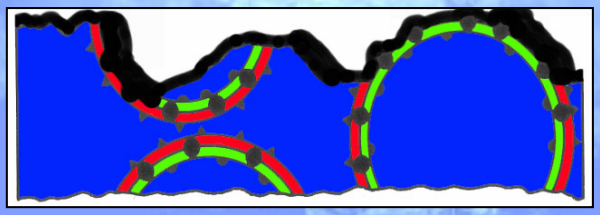
Flegler SL, Heckman JW, Klomparens KL, 1993, Scanning and Transmission Electron Microscopy, WH Freeman & Co, New York.

Label-Fracturing



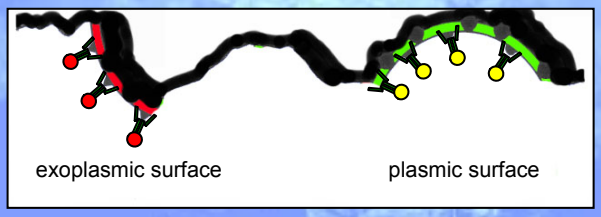
Dr N van Belzen, Prof Dr J Boonstra, Utrecht University

Fracture-Labelling



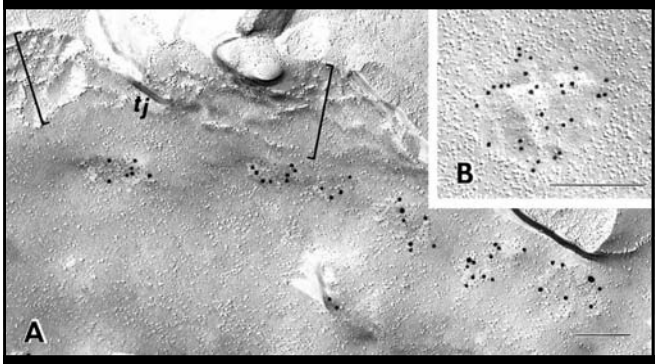
Flegler SL, Heckman JW, Klomparens KL, 1993, Scanning and Transmission Electron Microscopy, WH Freeman & Co, New York.

Fracture-Labelling



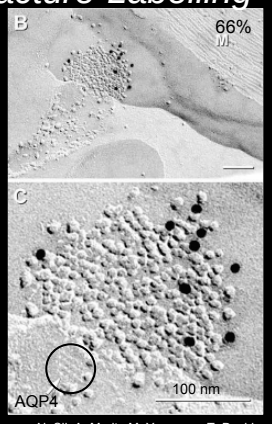
Fujimoto K. 1995. Freeze-fracture replica electron microscopy combined with SDS digestion for cytochemical labeling of integral membrane proteins. *J Cell Sci* **108**, 3443-3449.

Fracture-Labelling



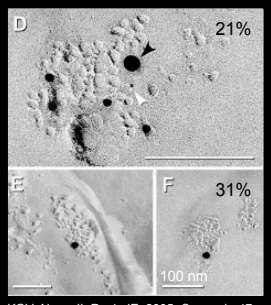
Fujimoto K. 1995. Freeze-fracture replica electron microscopy combined with SDS digestion for cytochemical labeling of integral membrane proteins. *J Cell Sci* **108**, 3443-3449.

Fracture-Labelling



Gap junction outer surface of myelin
- connexin-47
- connexin-32

AQP4 aquaporin-4



Kamasawa N, Sik A, Morita M, Yasumura T, Davidson KGV, Nagy JI, Rash JE. 2005. Connexin-47 and Connexin-32 in gap junctions..... *Neuroscience*, **136**, 65-86.

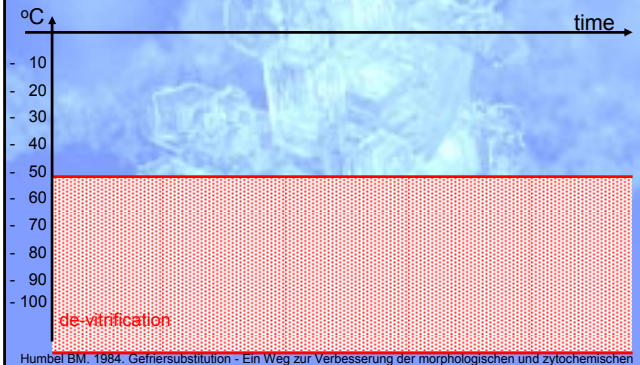
Freeze-Substitution

FREEZE-SUBSTITUTION

Dehydration of a Cryo-fixed Specimen by Exchange of Ice against an Organic Solvent

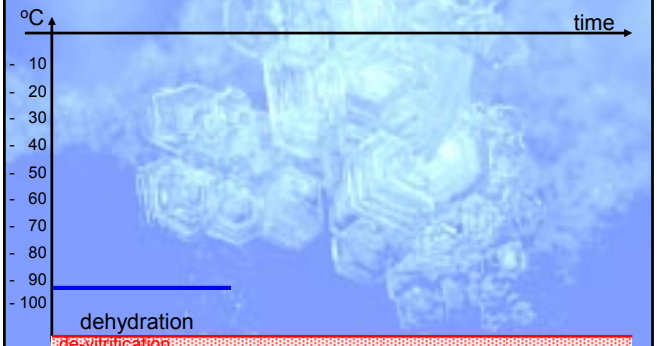
In: *Cryotechniques in Biological Electron Microscopy,* Steinbrecht RA, Zierold K (eds), Springer-Verlag, Heidelberg, 1987

Freeze-Substitution



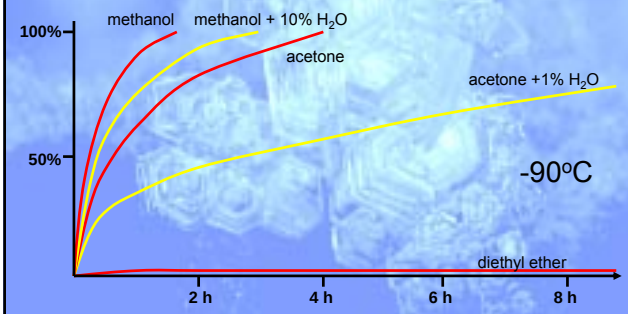
Humbel BM. 1984. Gefriersubstitution - Ein Weg zur Verbesserung der morphologischen und zytochemischen Untersuchungen biologischer Proben im Elektronenmikroskop, ETH, Zürich, Switzerland. ETH nr. 7609

Freeze-Substitution



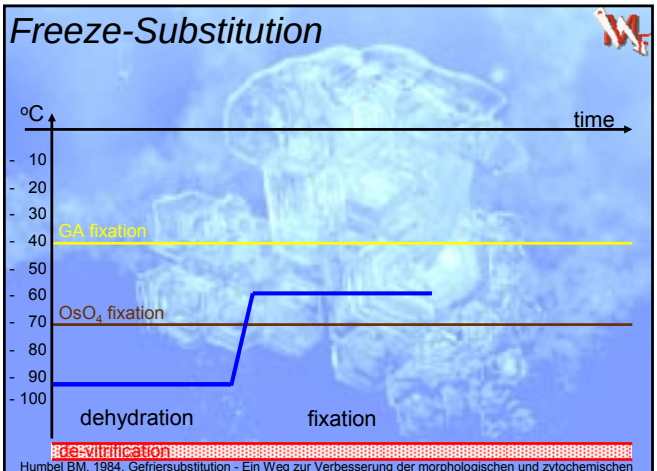
Humbel BM. 1984. Gefriersubstitution - Ein Weg zur Verbesserung der morphologischen und zytochemischen Untersuchungen biologischer Proben im Elektronenmikroskop, ETH, Zürich, Switzerland. ETH nr. 7609

Substitution Capacity of Solvents



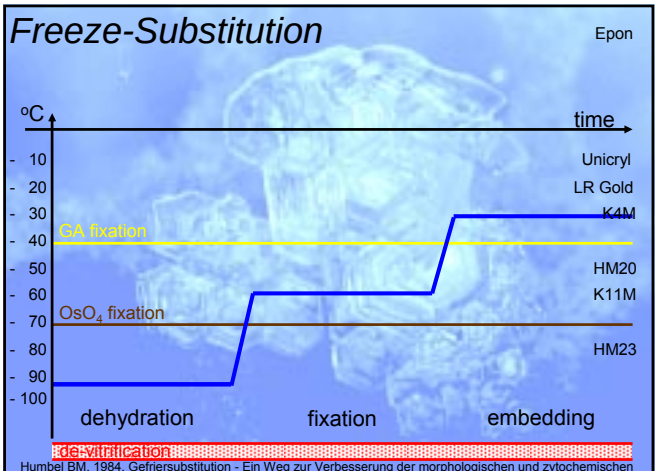
Humbel B, Marti T, Müller M. 1983. Improved structural preservation by combining freeze substitution and low temperature embedding. In *Beitr Elektronenmikroskop Direktabb Oberfl*, Pfefferkorn G. (ed): Antwerpen; 585-594.

Freeze-Substitution



Humbel BM. 1984. Gefriersubstitution - Ein Weg zur Verbesserung der morphologischen und zytochemischen Untersuchungen biologischer Proben im Elektronenmikroskop, ETH, Zürich, Switzerland. ETH nr. 7609

Freeze-Substitution



Humbel BM. 1984. Gefriersubstitution - Ein Weg zur Verbesserung der morphologischen und zytochemischen Untersuchungen biologischer Proben im Elektronenmikroskop, ETH, Zürich, Switzerland. ETH nr. 7609

Table 1
POTENTIONAL SUBSTITUTION MEDIA IN RELATION TO WATER

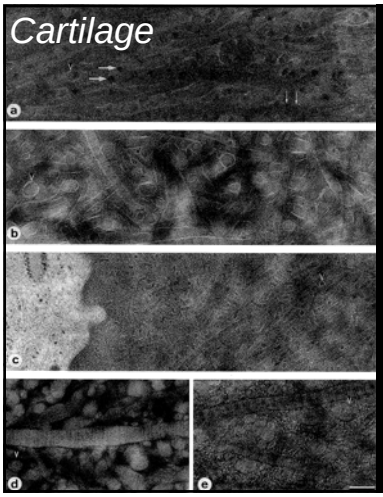
Solvent	Mol. wt. (g/mol)	mp K	mp °C	bp K	bp °C	ε	K	°C
Propane	44.09	83.1	−189.9	228.5	−44.5	1.61	273	0
Diethyl ether	74.12	156.8	−116.2	307.6	34.6	4.34	293	20
						10.4	157	−116
Tetrahydrofuran	72.12	164.	−109.	340.	67.	8.2	293	20
						8.9	274	1
Acetone	58.08	177.6	−95.4	329.0	56.0	20.7	298	25
Ethanol	46.07	155.7	−117.3	351.5	78.5	24.3	298	25
						41.0	213	−60
Methanol	32.04	175.2	−97.8	337.9	64.9	32.6	298	25
						40.0	253	−20
						54.0	193	−80
Acrolein	56.06	185.3	−87.7	326.0	53.0			
Dimethylformamide	37.09	212.5	−60.5	422/9	149/56	36.7	298	25
Water	18.02	273.0	0.0	373.0	100.0	78.36	298	25
						80.18	293	20
						87.9	273	0

Note: Mol. wt., molecular weight; mp, melting point; bp, boiling point; ε, dielectric constant.

Data taken from: *Handbook of Chemistry and Physics, 60th ed.*, Weast, R. C., Ed., CRC Press, Boca Raton, FL, 1979.

Humbel BM, Schwarz H. 1989. Freeze-substitution for immunochemistry. In *Immuno-Gold Labeling in Cell Biology*, Verkleij AJ, Leunissen JLM. (eds). CRC Press: Boca Raton; 115-134.

Cartilage



CEMOVIS (a)
67-nm periodicity of collagen is faintly but clearly visible

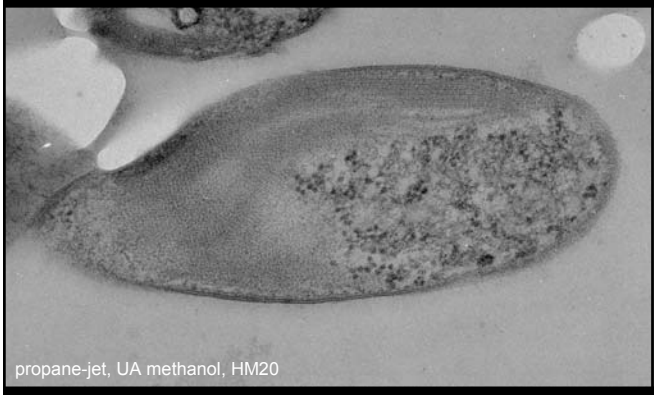
Acetone (b,c)
halo separates collagen fibrils are thicker in ECM
67-nm periodicity of collagen is clearly visible

Studer D, Chiquet M, Hunziker EB. 1996. Evidence for a distinct water-rich layer surrounding collagen fibrils in articular cartilage extracellular matrix. *J Struct Biol*, **117**, 81-85.

Ethanol (d)
67-nm periodicity faintly visible no halo

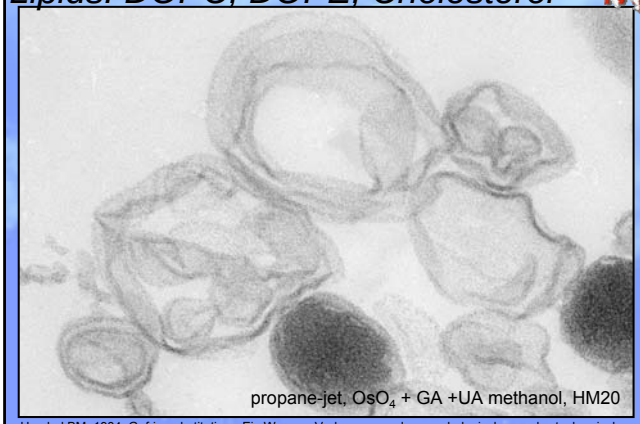
Methanol (e)
67-nm periodicity less distinct

Rhodopseudomonas viridis



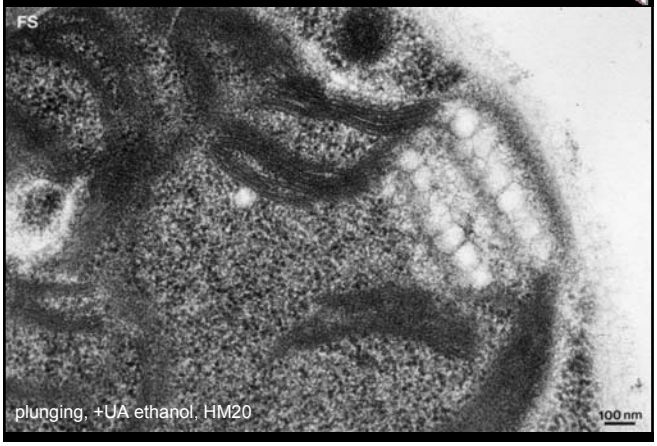
Humbel BM. 1984. Gefriersubstitution - Ein Weg zur Verbesserung der morphologischen und zytochemischen Untersuchungen biologischer Proben im Elektronenmikroskop, ETH, Zürich, Switzerland. ETH nr. 7609

Lipids: DOPC, DOPE, Cholesterol

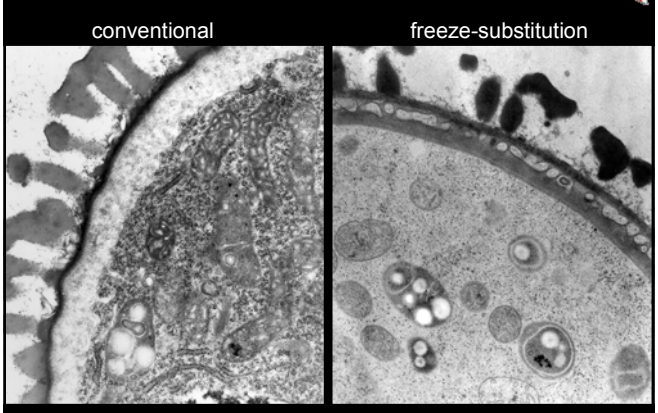


Humbel BM. 1984. Gefriersubstitution - Ein Weg zur Verbesserung der morphologischen und zytochemischen Untersuchungen biologischer Proben im Elektronenmikroskop, ETH, Zürich, Switzerland. ETH nr. 7609

Clamydomonas reinhardtii

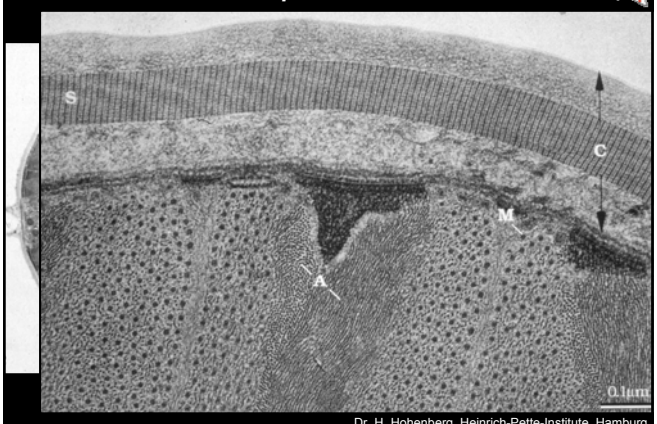


Arabidopsis thaliana: Pollen

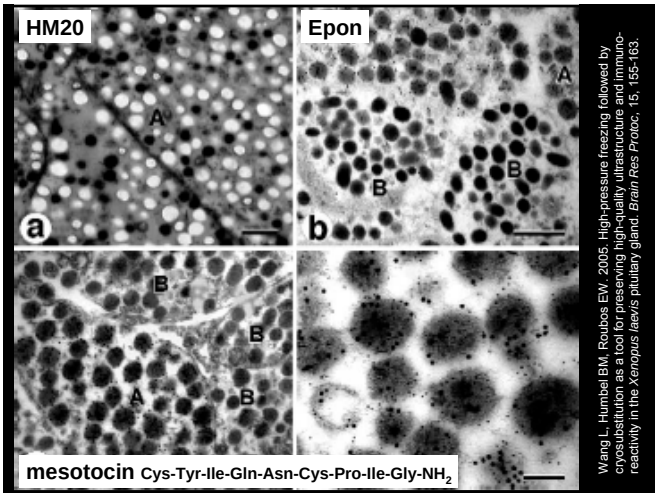


Collaboration: Dr. A. Omedilla, University of Granada, Granada

Heterorhabditis sp.



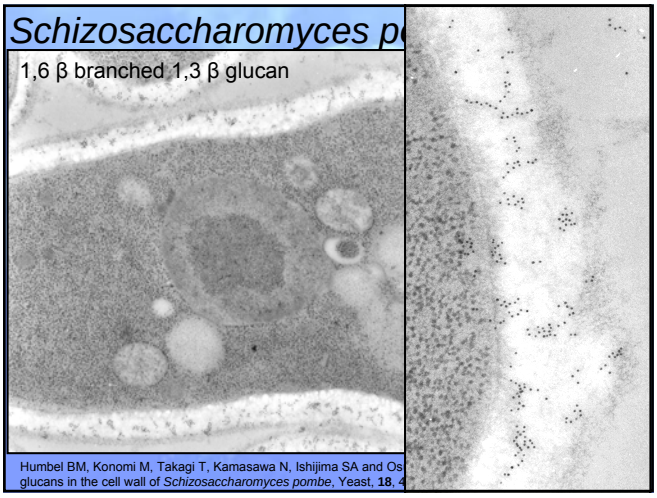
Dr. H. Hohenberg, Heinrich-Pette-Institute, Hamburg



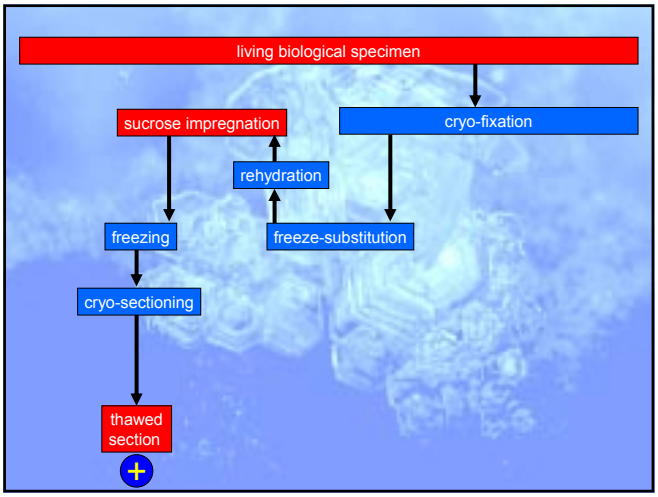
Wang L, Humbel BM, Roubos EW. 2005. High-pressure freezing followed by cryosubstitution as a tool for preserving high-quality ultrastructure and immunoreactivity in the *Xenopus laevis* pituitary gland. *Brain Res Protoc*, 15, 155-163.

Schizosaccharomyces p

1,6 β branched 1,3 β glucan



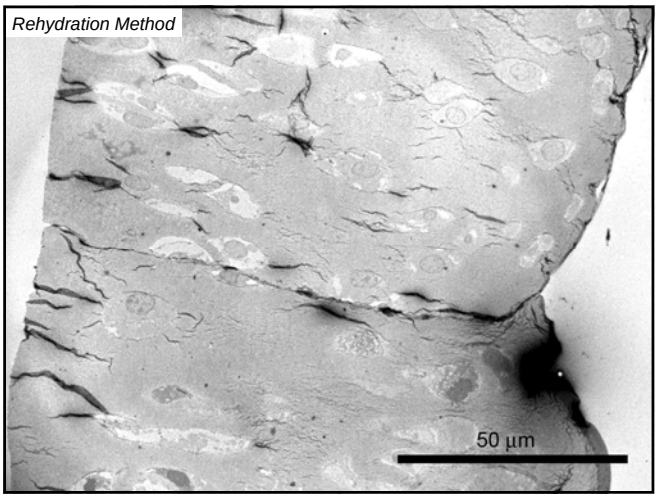
Humbel BM, Konomi M, Takagi T, Kamasawa N, Ishijima SA and Os
glucans in the cell wall of *Schizosaccharomyces pombe*, Yeast, **18**, 4

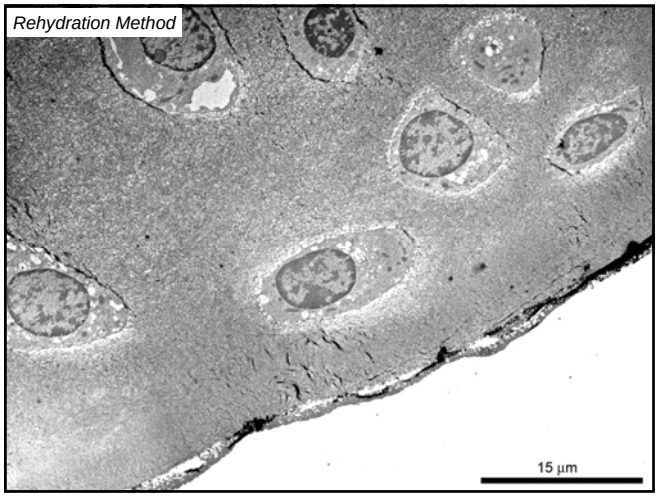


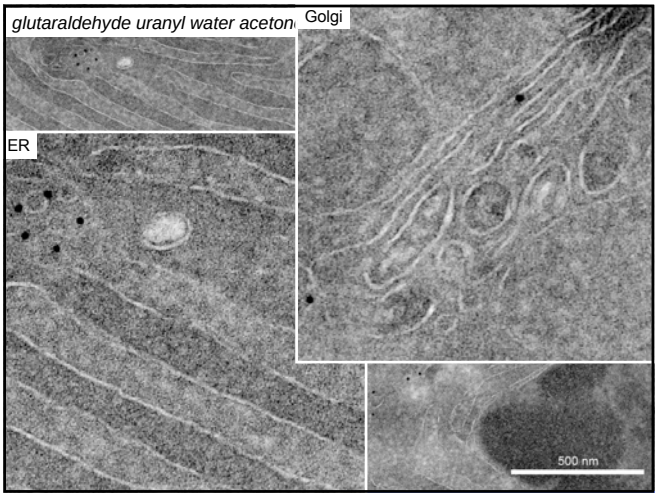
Rehydration Method

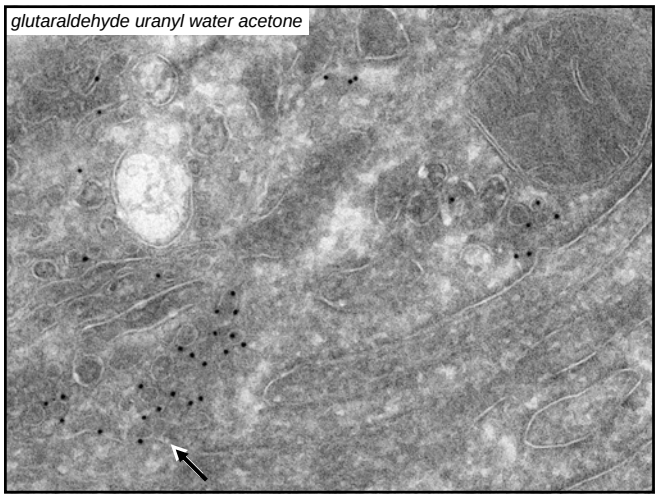
- Cryo-fixation by high-pressure freezing
- freeze-substitution in acetone with or without
  - osmium tetroxide
  - glutaraldhyde
  - uranyl acetate
  - water
- rehydration in water then PHEM buffer
- sucrose – ice embedding
- cryo-sectioning
- methyl cellulose pick-up
- immuno gold labelling

Van Donselaar E, Posthuma G, Zeuschner D, Humbel BM, Slot JW. 2007. Immunogold labeling of cryo-sections from high-pressure frozen cells. *Traffic*, **8**, 471-485.









Conclusion

- Cryo-fixation is an excellent method to preserve the cellular ultrastructure
- Small molecules e.g. ions are not displaced
- Cryo-fixation traps fast cellular processes
- Cryo-fixation is limited to small objects
- Freeze-substitution translates the advantages of cryo-fixation to (thin) resin or Tokuyasu cryo-sections