High pressure freezing

Introduction

Technique

Freeze-substitution

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Introduction

Plunge freezing:

- Only suspensions (< 1 µm) or thin tissues containing anti-freeze (anti-freeze -> osmotic effects!)

Slam freezing:

- Suspensions and thin tissues (few µm, only front well frozen ca. 1 µm)

Propane jet freezing (JFD):

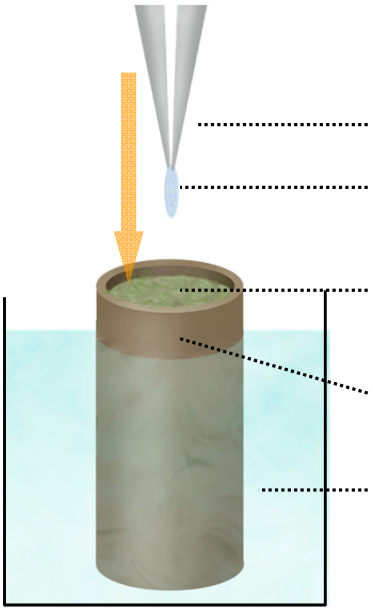
- Adequate freezing of suspensions not thicker than 15 µm
- Thicker specimen require anti-freeze

High pressure freezing (HPM)

- Freezing under high pressure (2100 bar)
- Adequate freezing of samples up to 200 µm thickness without anti-freeze

Introduction: Freezing at ambient pressure

Plunge freezing



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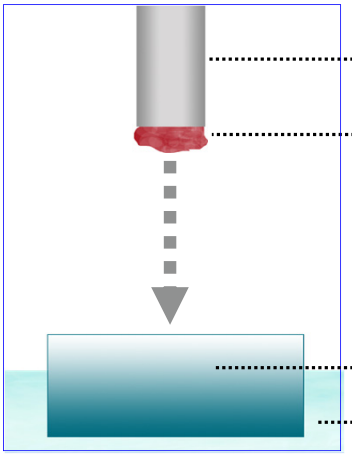
Introduction: Freezing at ambient pressure

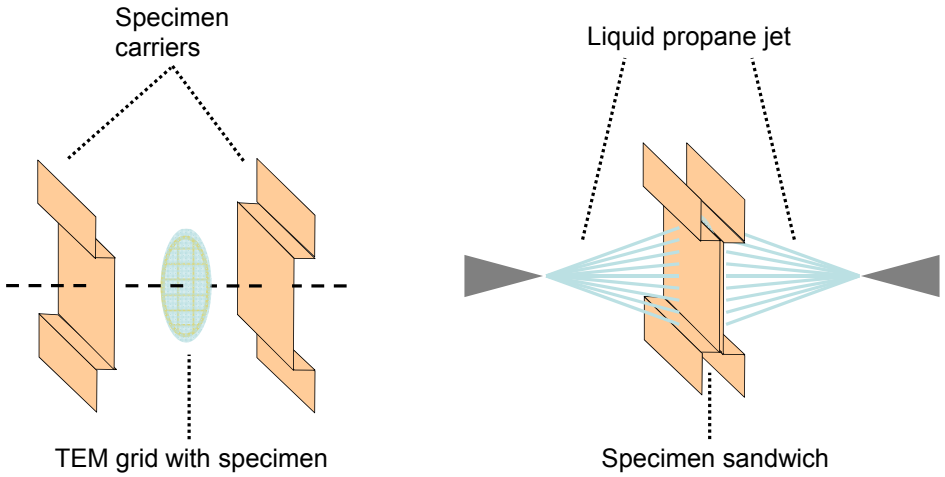
Slam freezing



- Suspensions and thin tissues up to a few µm, only front well frozen ca. 1 µm

Introduction: Freezing at ambient pressure

Propane jet freezing



- Adequate freezing of suspensions up to 15 µm
- Thicker specimen require anti-freeze

Introduction: Freezing under high pressure

Only way to freeze samples thicker than 15 µm

- Freezing under high pressure (2100 bar)
- Changing the physical properties of the specimen!
- Adequate freezing of samples up to 200 µm thickness without anti-freeze

Introduction

Plunge/slam freezer

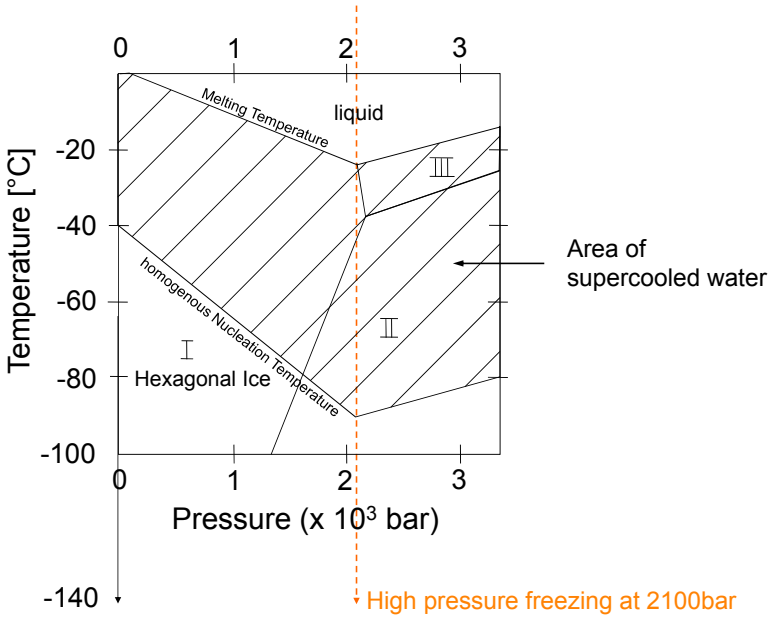
Propane jet freezer

High-pressure freezer

Relative sizes

HPF technique

The fundamentals - Phase diagram of water



Redrawn from Kanno H, (1975) supercooling of water to -92°C under pressure Science 189: 880-881

Basic demands

- Pressure built-up must be as rapide as possible (Dissociation constants)
- Immediately after reaching 2100 bar, cooling should start and run as fast as possible

Open system

- LN_2 at 2100bar used for pressure build-up AND cooling

HPM 010

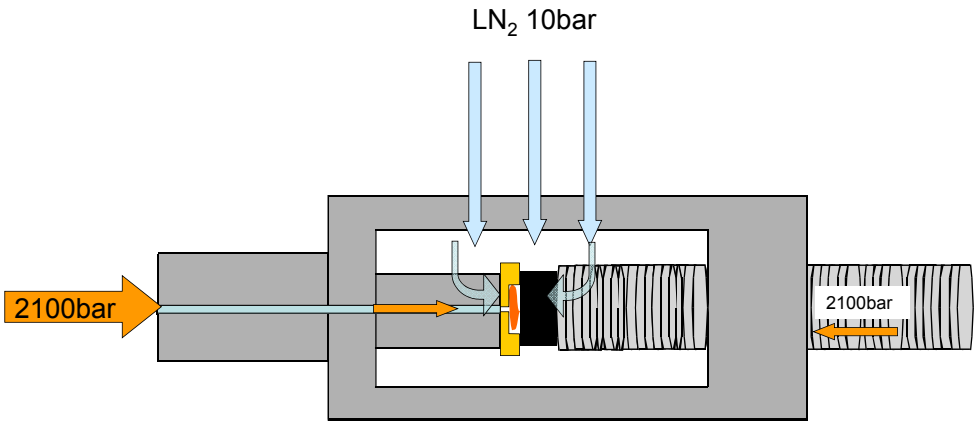
Compact 1

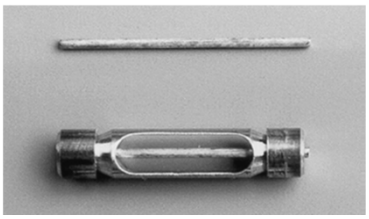
HPM 100

Closed system

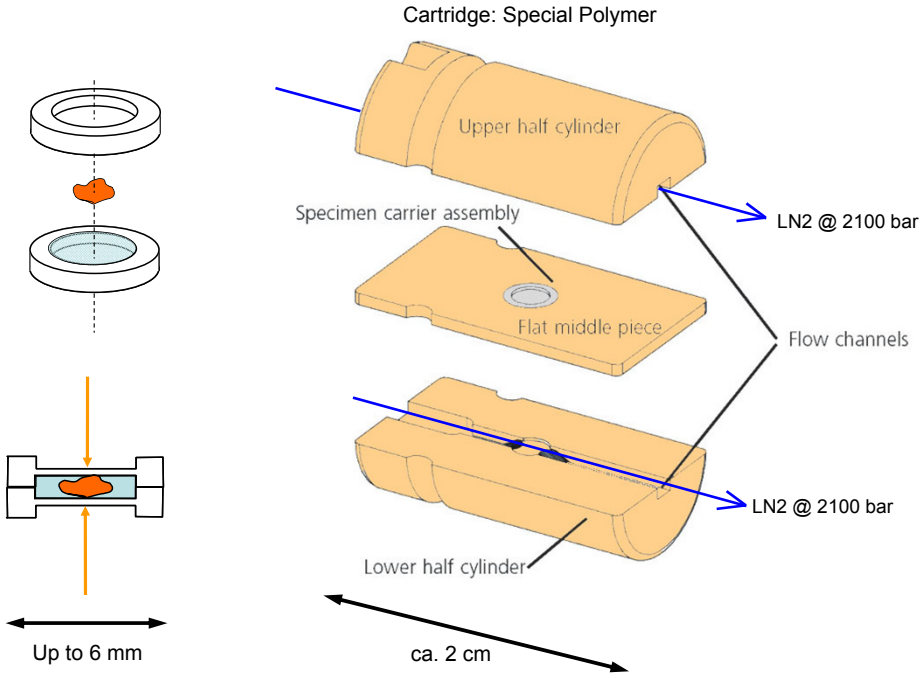
- Pressure build-up and cooling separated

Empact II





The implementation – Open system HPM100



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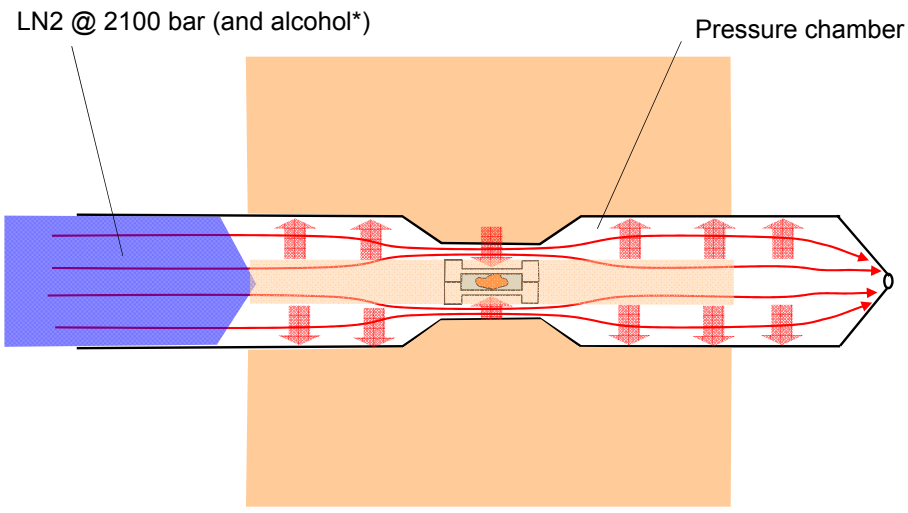

*used for sychronization of presssure build-up and cooling

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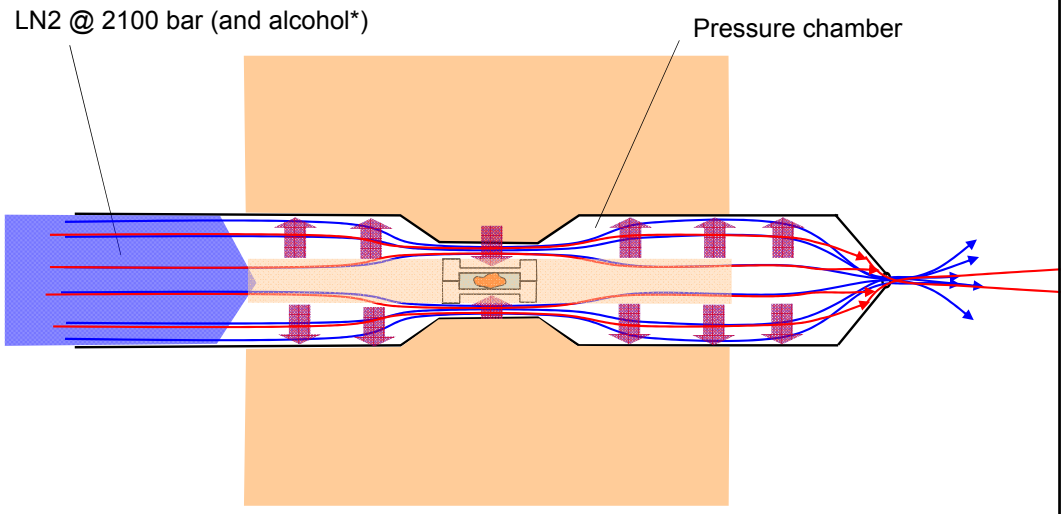

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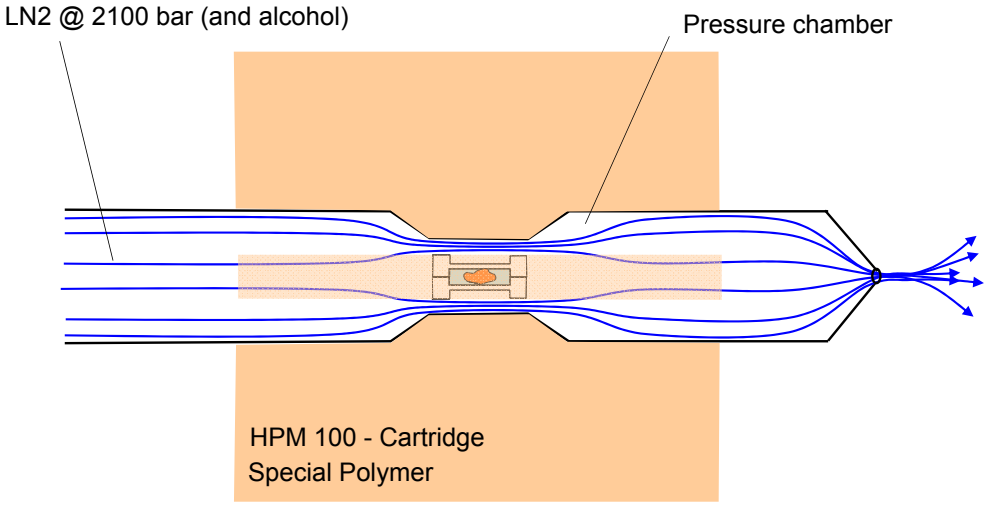


The implementation – Open system HPM100

Advantages:

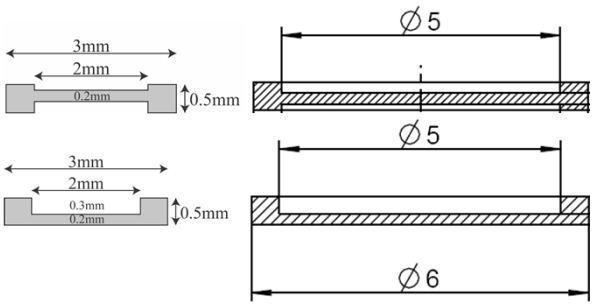
- Pressure everywhere the same in pressure chamber:
  Very thin-walled carriers can be used – “no limit”
- Cooling primarily performed via thin membranes
- In HPM 100: Cooling of specimen only (cartridge is insulator)
- In HPM 100: Cartridge can be adapted to specimen geometry

The implementation – Open system HPM100

Large specimen carriers: 6 mm

Aluminium specimen carriers and Sapphire discs

Area for specimen	3.1 mm^2	19.6 mm^2
Volume 0.2 mm cavity	0.6 mm^3	3.9 mm^3



Basic rules

Reaching good freezing quality with HPF

- Smallest (thinnest, <200 µm) possible aqueous specimen surrounded by the thinnest possible metal support.
- Optimized transfer of the cold to the aqueous sample by 1-Hexadecene, other paraffin oils or extracellular cryoprotectants such as Polyvinylpyrrolidone, Hydroxyethyl starch, Dextrane, BSA.
  -> Removal of air (twisted carriers)!
- During freezing, the specimen sandwich must be firmly kept in place by the sample holder
- Always use “fresh specimen”

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Reaching good freezing quality with HPF

Hexadecene

- Low surface tension
- No osmotic activity
- Does not mix with water
- (is not an antifreeze)

Dextrane, BSA

- Use in buffer or culture medium (not in distilled water)
- Attention: These solutions dry fast
- Better cutting properties than hexadecene (cryo-ultramicrotomy)

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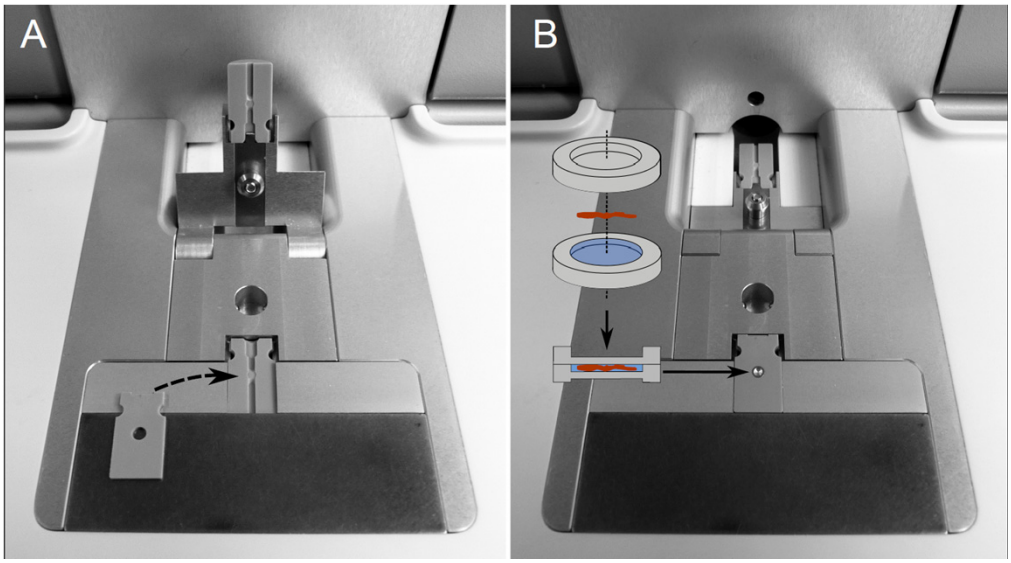
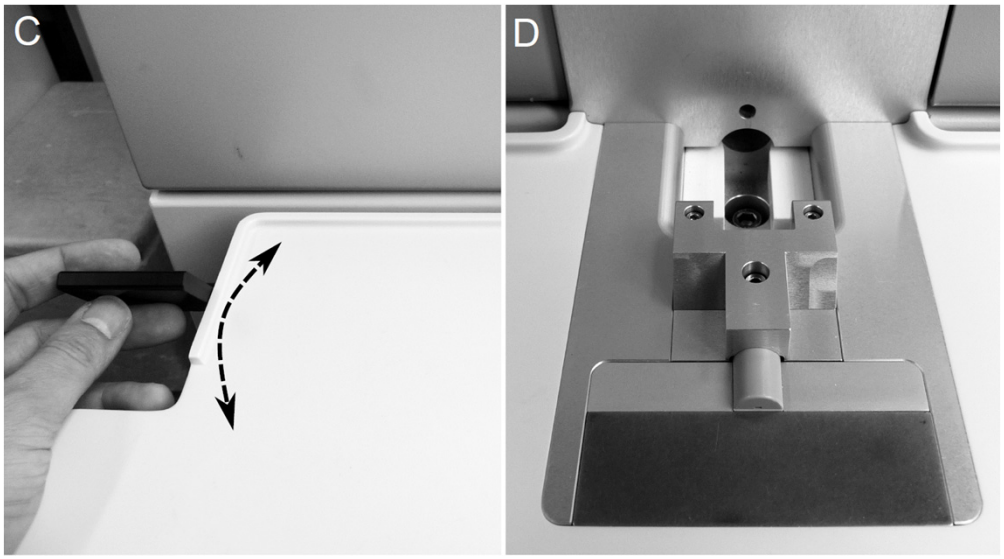
Specimen preparation and carriers

Specimen preparation HPM 100

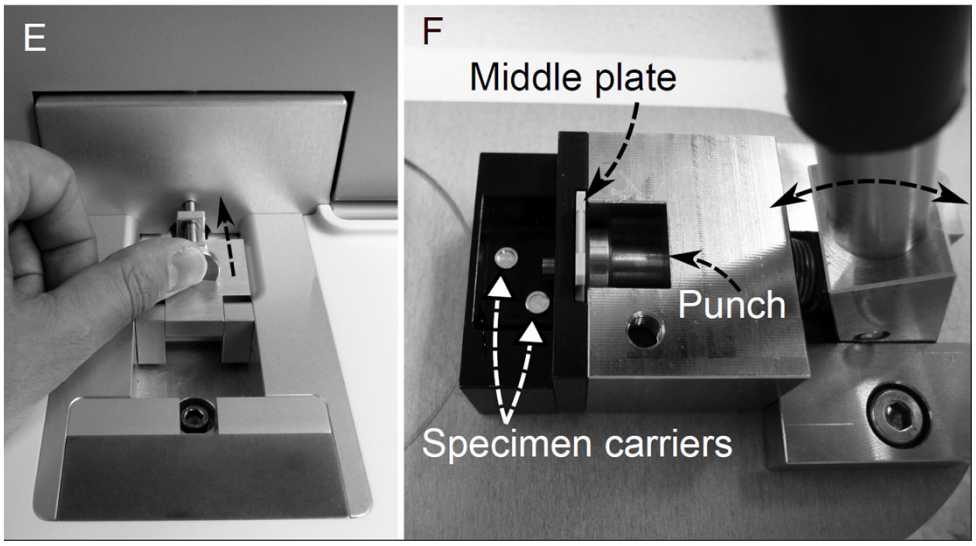
Specimen preparation HPM 100



Specimen preparation HPM 100



Specimen preparation HPM 100



Specimen preparation HPM 100

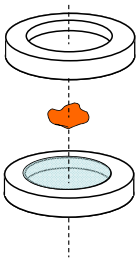
Carriers are nice…but how do we get a native sample into them

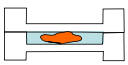
Suspensions

Adherent cell cultures

Tissue of animals (and plants)

Technical/chemical systems
> freeze-fracturing

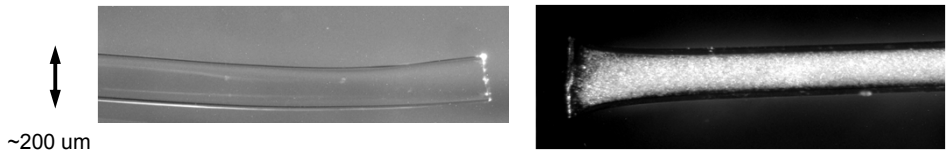




Sample preparation – Suspensions

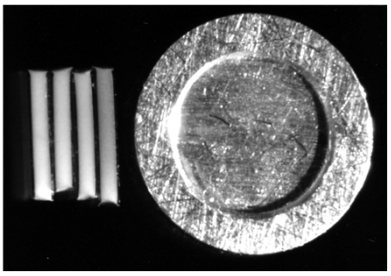
Cellulose capillary tubes

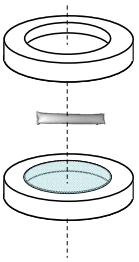
Filled by capillary forces or with pipette



~200 um

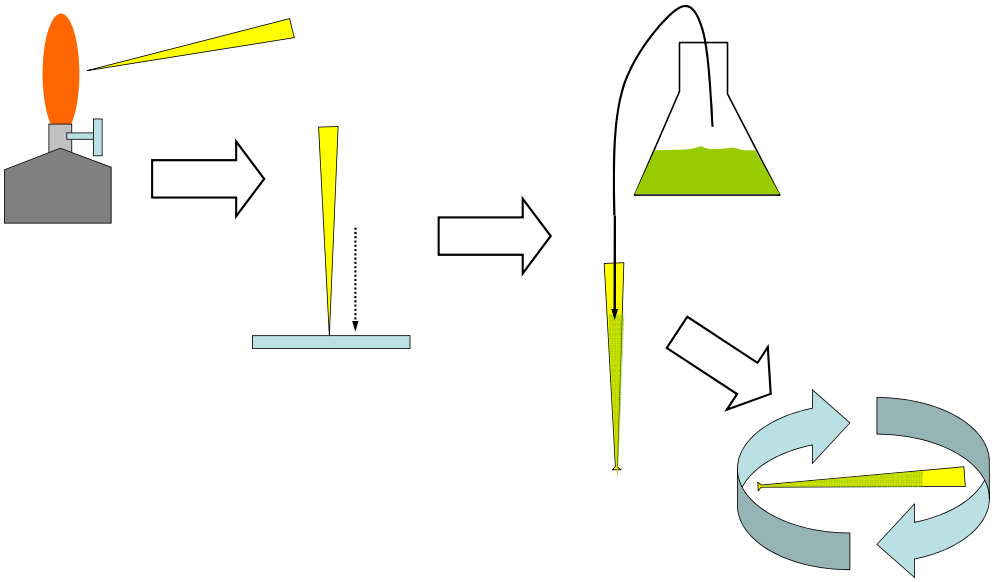
Tubes are cut into pieces fitting into carrier using scalpel





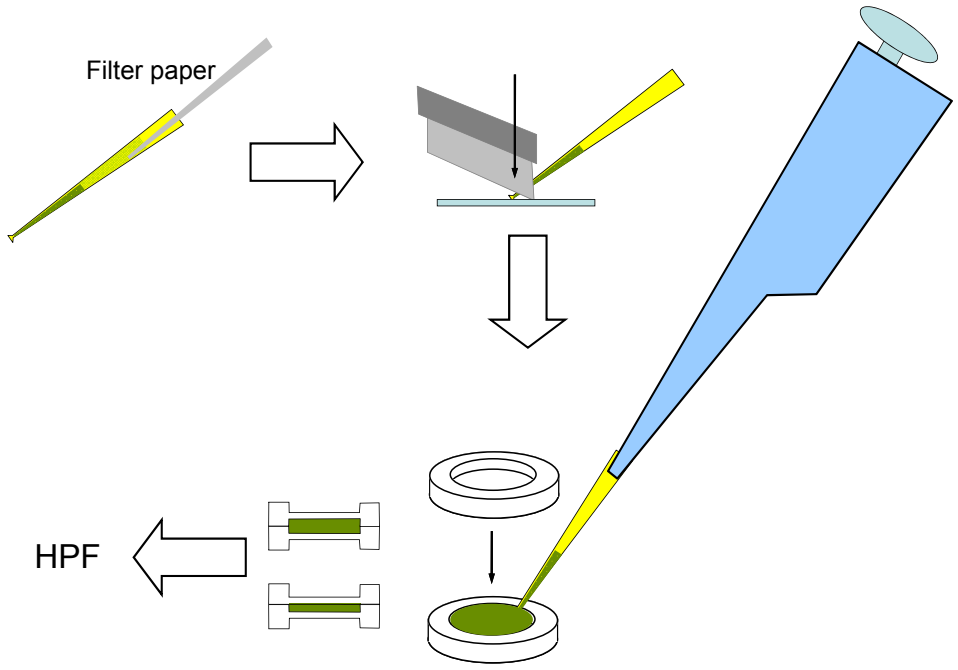
Sample preparation – Suspensions

Centrifugation of suspensions in closed pipette tips (A. McDowall)



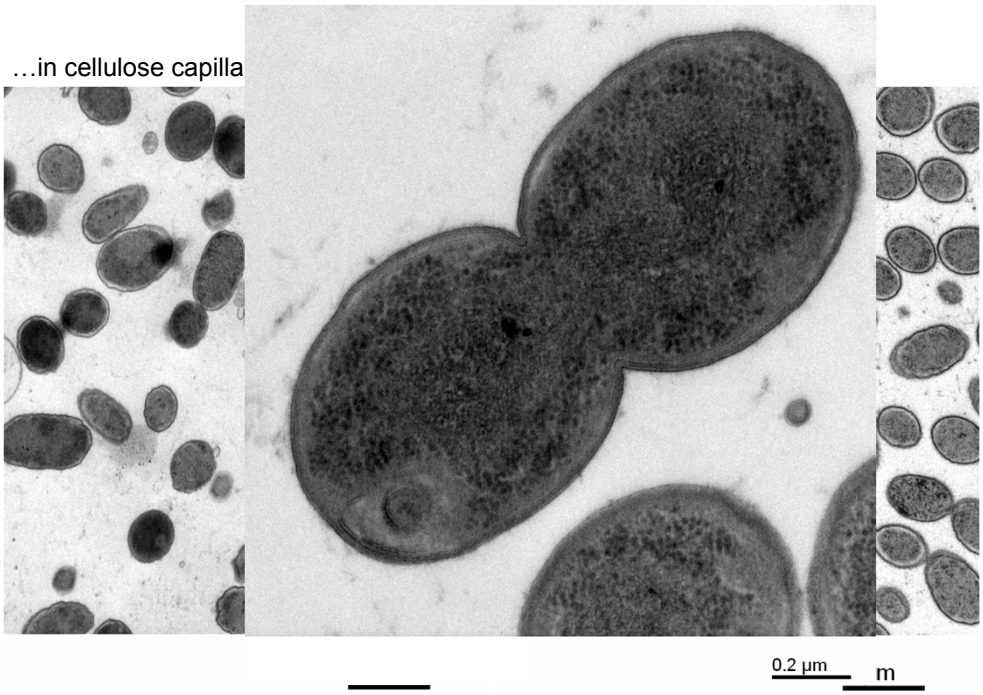
Centrifugation (as well possible in Eppendorf tube)

Sample preparation – Suspensions



Sample preparation – Suspensions

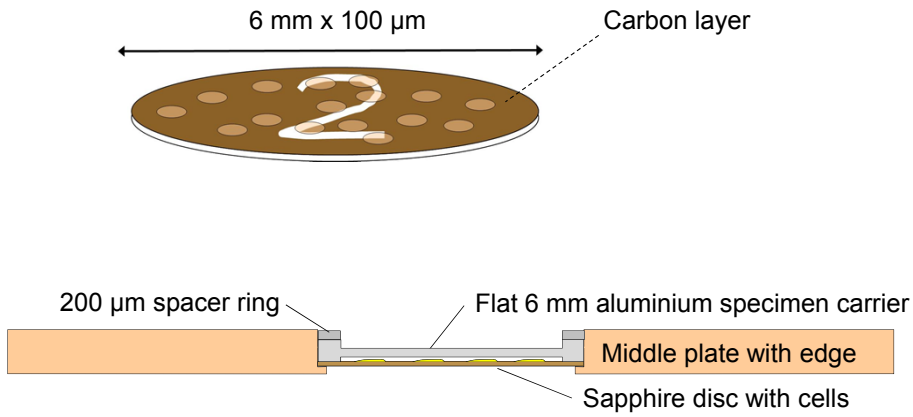
…in cellulose capilla



Srinivas, N., P. Jetter, et al. (2010). Peptidomimetic Antibiotics Target Outer-Membrane Biogenesis in Pseudomonas aeruginosa." Science 327(5968): 1010-3

Sample preparation – Adherent cell cultures

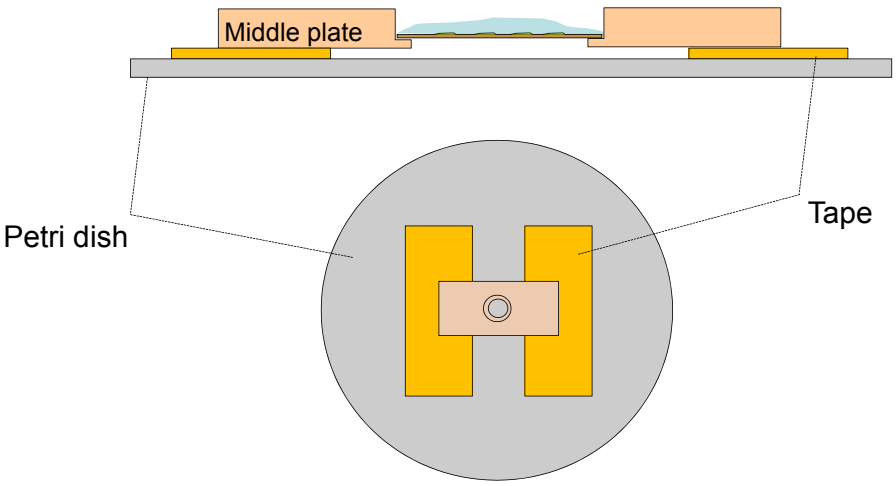
Specimen carriers for cell monolayers on Sapphire discs



Sample preparation – Adherent cell cultures

NOTE:

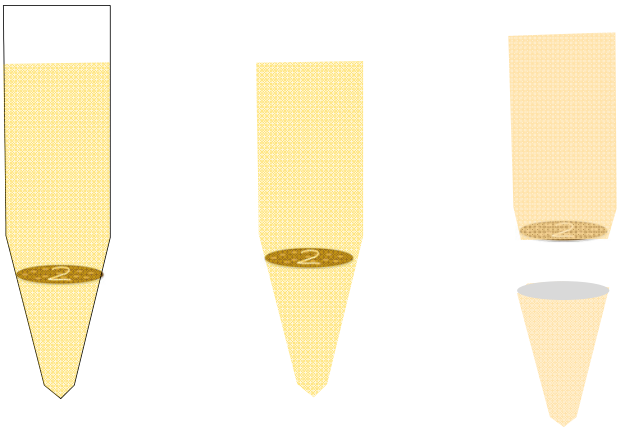
- Capillary forces need to be taken into account for loading



Sample preparation – Adherent cell cultures

Sapphire discs with cell cultures

- Polymerization in 1.5 ml Eppendorf tubes optimal
- Direct access to whole area



Sample preparation – Adherent cell cultures

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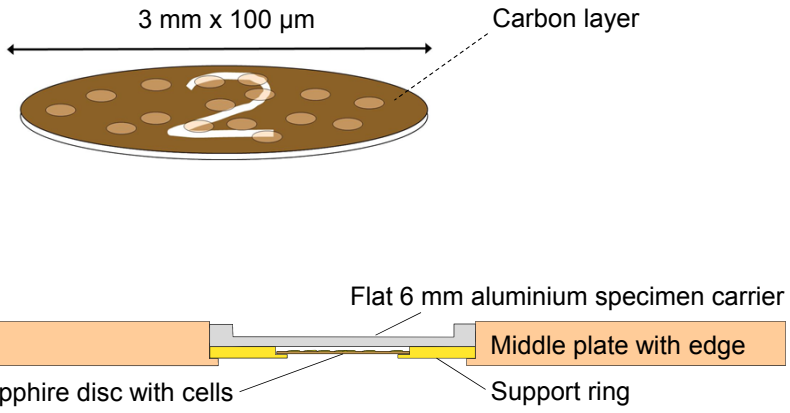
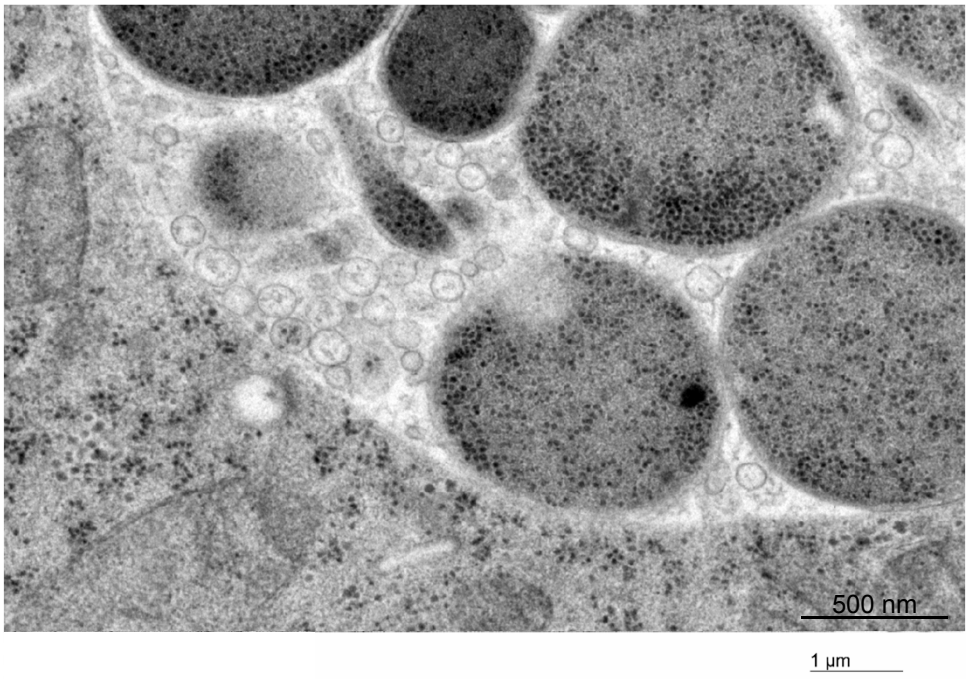


Sample preparation – Adherent cell cultures

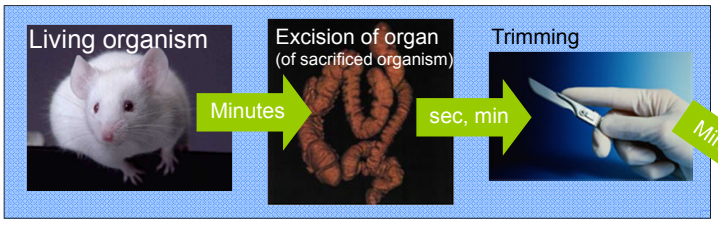
Hep-2 cells infected with Chlamydia pneumonia



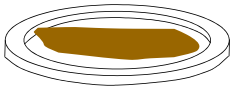
Sample preparation – Mammal tissues

Excision and trimming must be as quick as possible
to preserve the native state of the tissue before fixation (thickness < 200 um)

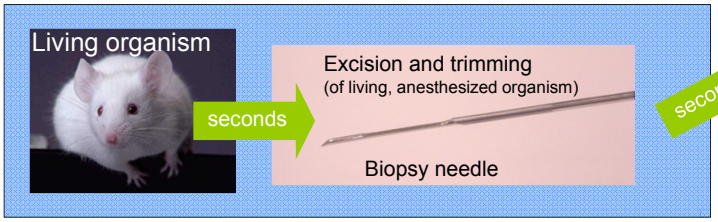
Manual dissection



High-pressure freezing:
Seconds/minutes
(ev. oxygenation required)



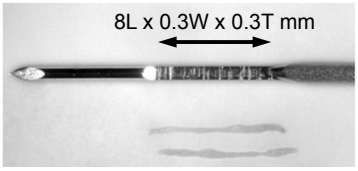
Biopsy gun

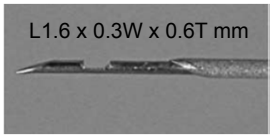


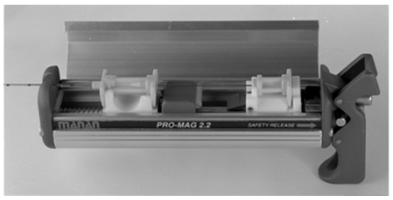
Sample preparation – Mammal tissues

Limitations of biospsy/manual dissection:

- Biopsy from specific site of organ difficult
- Orientation of tissue during/after freezing difficult
- Only one biopsy possible: First biopsy leads to trauma of organ
- Commercially available needles have too large indentation







Sample preparation – Mammal tissues

Compromise, alternatives, workaround:

- Perfusion fixation (FA/GA) prior to HPF
  - » Time for excision of specific region and trimming
  - » Addition of cryo-protectants possible during/after perfusion
- Native tissue cultures
  - » Brain slice culture
  - » 3D cell cultures

Sosinsky, G. E., J. Crum, et al. (2008). "The combination of chemical fixation procedures with high pressure freezing and freeze substitution preserves highly labile tissue ultrastructure for electron tomography applications." J Struct Biol 161(3): 359-71.

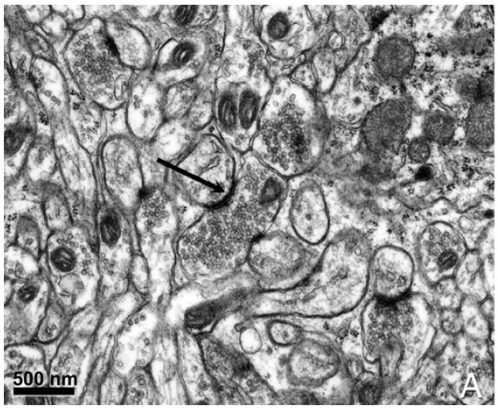
Mobius, W. (2009). "Cryopreparation of biological specimens for immunoelectron microscopy." Ann Anat.

Muhlfeld, C. (2010). "High-pressure freezing, chemical fixation and freeze-substitution for immuno-electron microscopy." Methods Mol Biol 611: 87-101.

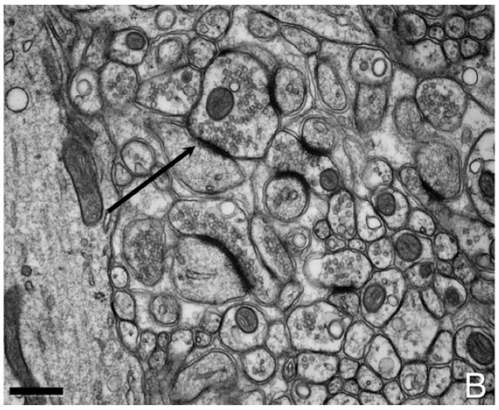
Sample preparation – Mammal tissues

- Perfusion fixation (FA/GA) prior to HPF

Perfusion fixation



Perfusion fixation - HPF



(2% of paraformaldehyde/2.5% of glutaraldehyde in 0.15 M cacodylate buffer)

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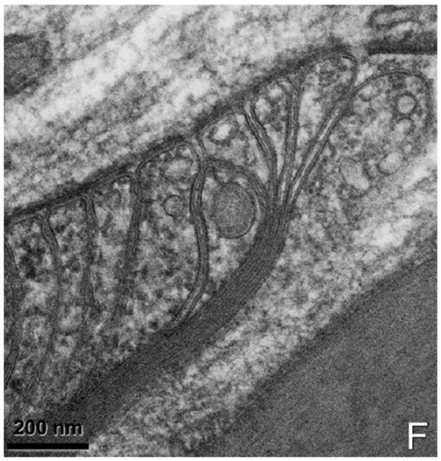

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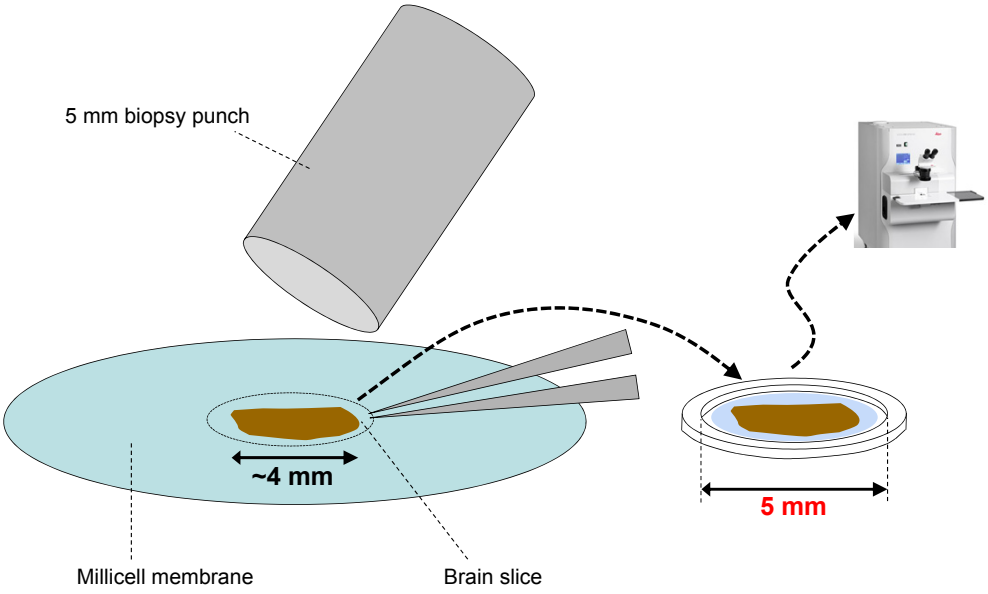
Sample preparation – Mammal tissues

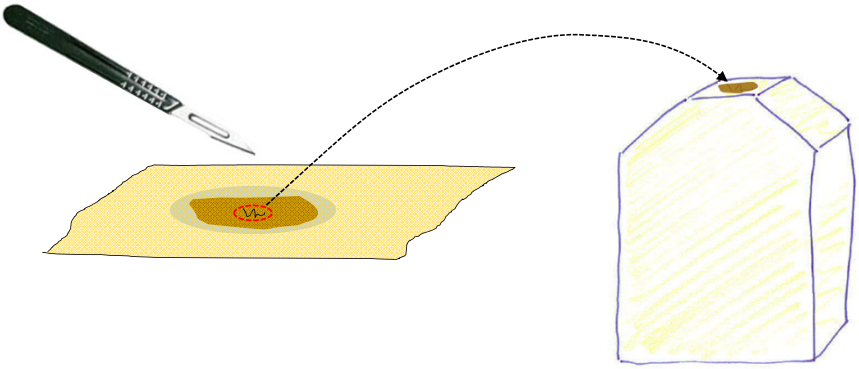
Brain slice cultures grown on Millicell membranes



Sample preparation – Mammal tissues

Brain slice cultures grown on Millicell membranes

Flat embedding between Aclar – selection of area easily possble



Sample preparation – Mammal tissues

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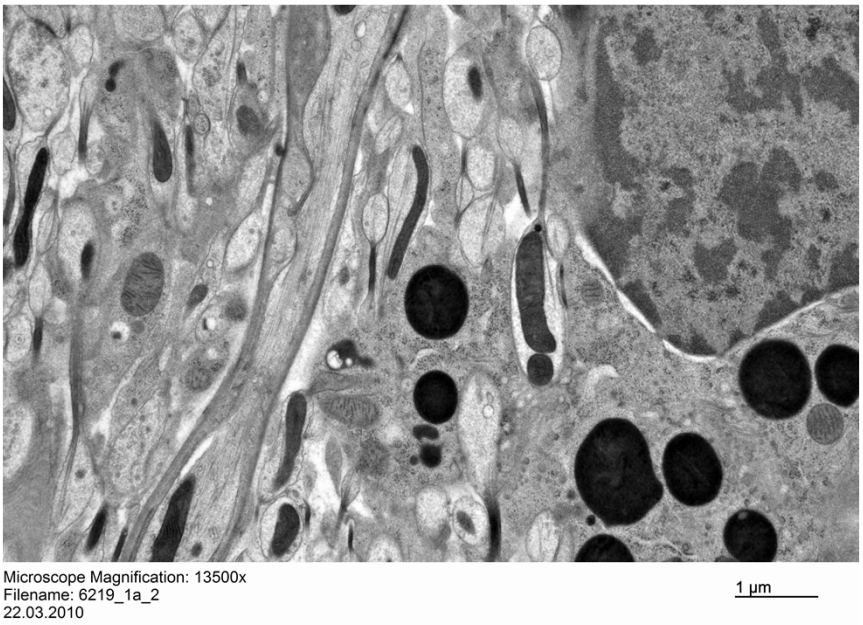

Specimen courtesy of Bettina Sobottka, Neurologische Klinik, University of Zurich

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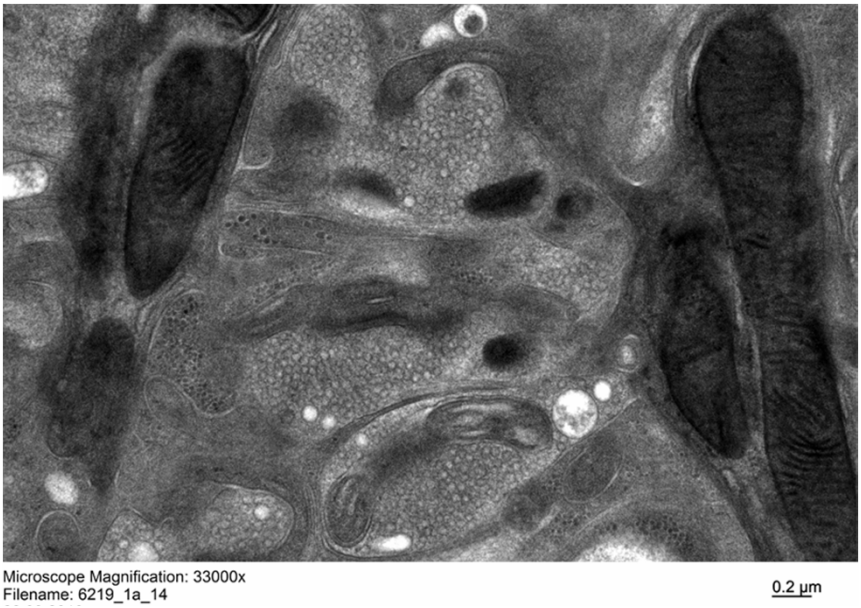

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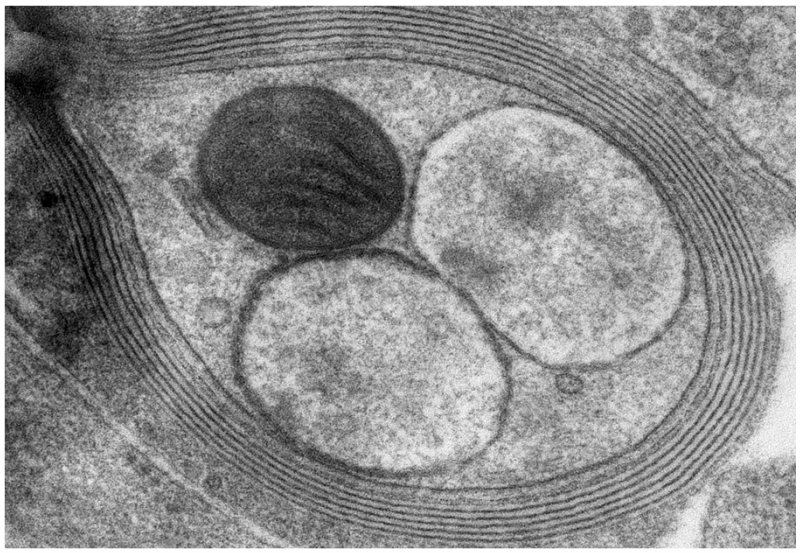


Specimen courtesy of Bettina Sobottka, Neurologische Klinik, University of Zurich

Sample preparation – for cryo-sectioning

Tube system: Copper tubes

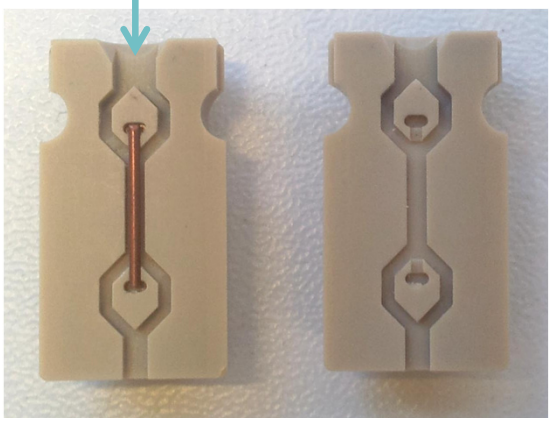
Inner diameter: 350 µm

Outer diameter: 650 µm

LN2@2100 bar

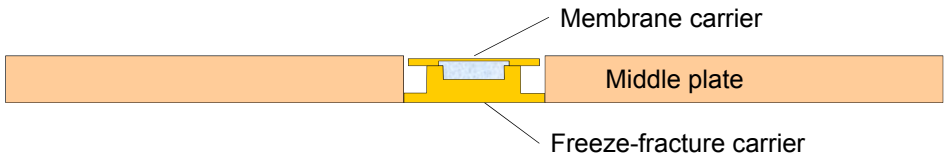
Open end

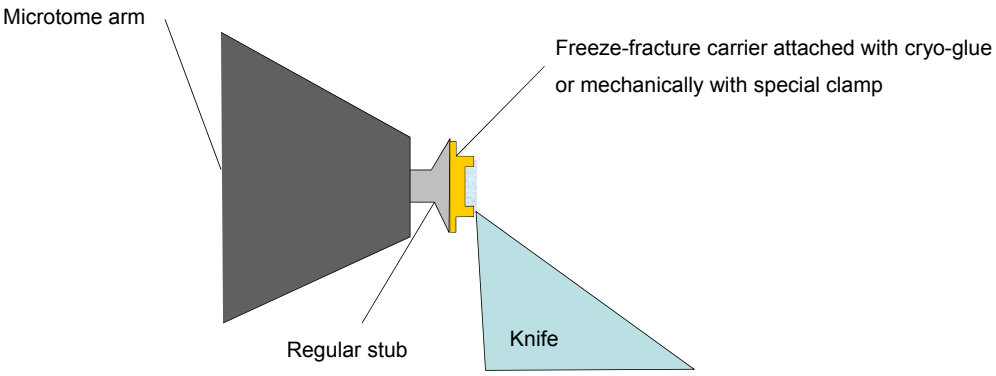
Closed end



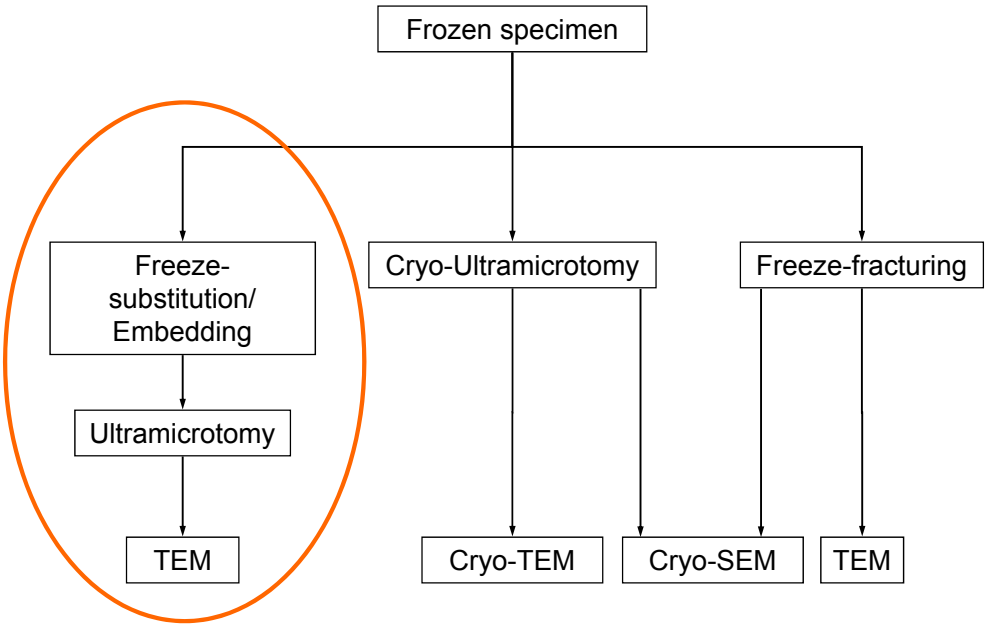
Sample preparation – for cryo-sectioning

"Flat" carrier system:





Post-processing after freezing



Freeze-substitution

Low-temperature dehydration with a solvent!

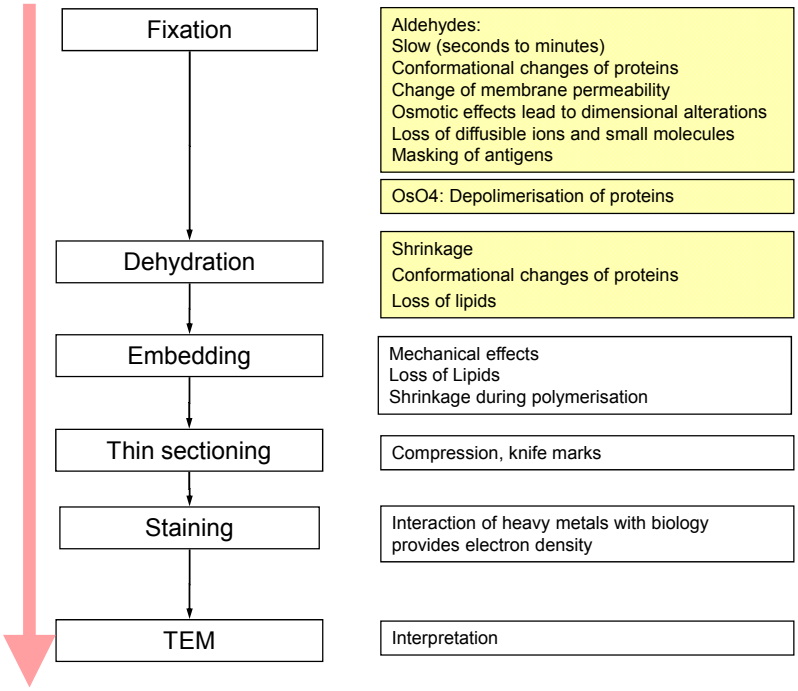
- Starting at the lowest possible temperature
- Simultaneous fixation, if desired (e.g. UAc, OsO4, Epoxy, GA, tannic acid)
- Temperature/time course to temperature for embedding (0° C, RT, -50°C)

Note:

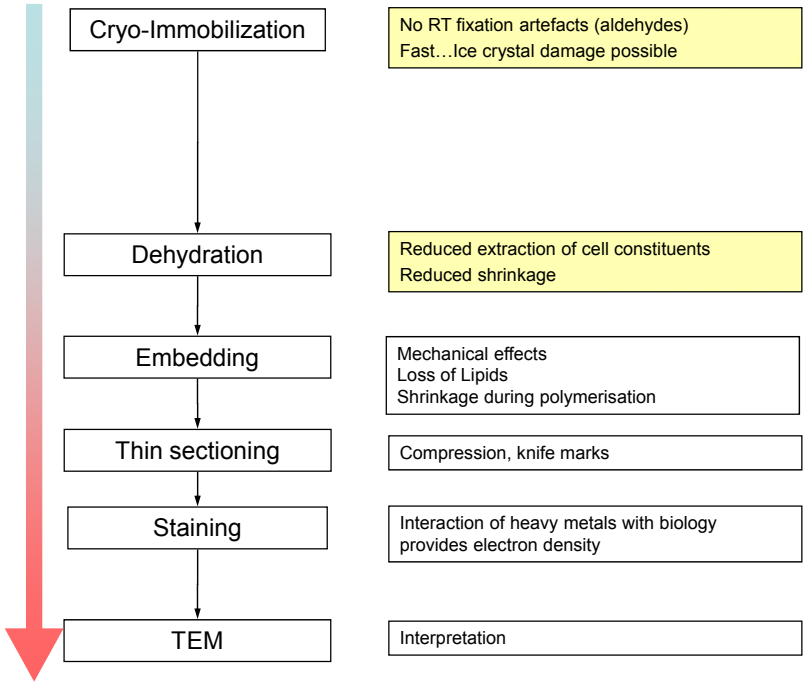
- Acetone at -90°C dissolves only H_2O up to 1 %

Steinbrecht & Müller 1987 in Cryotechniques in Biological Electron Microscopy (Steinbrecht and Zierold), Springer Verlag
Humbel 2009 in Handbook of Cryopreparation Methods for Electron Microscopy, CRC Press
Giddings 2003, Journal of Microscopy

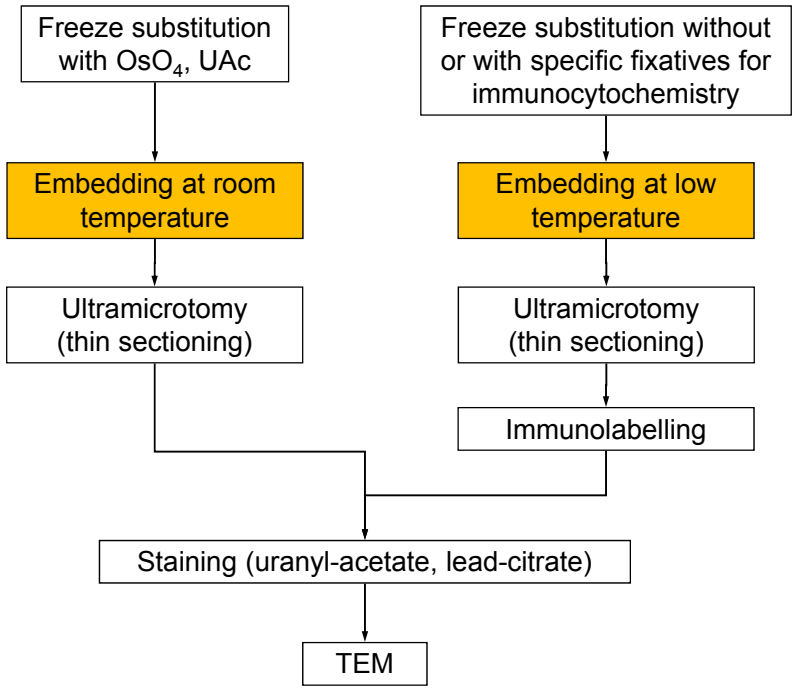
Freeze-substitution



Freeze-substitution



Freeze-substitution



Freeze-substitution