From Eye to Insight

High Pressure Freezing Applications
Why cryofixation?

- Cryo-fixation is a physical process = instantaneous and simultaneous immobilisation of all cellular components
- Cells retained in their ‘native’ state
- Enzymes and antigens are not denatured
- The physical properties of a frozen sample allow cryo-sectioning
Ice and cellular structure
Phase diagram of Water

Liquid water is easily supercooled, but difficult to gasify

In glassy state the viscosity is extremely high and matter is metastably solid

http://www.lsbu.ac.uk/water/phase.html
Why High Pressure Freezing?

- No changes in the physical equilibrium (precise correlation between pressure and temperature)
- No cryo-protection needed (no alteration of the cellular processes)
- Vitrification of sample thickness up to 200 µm
- Not all samples are the same: proteins/lipids/sugars
Cooling rate

- Water has a very poor heat conductivity

Thicker specimen = lower cooling rate
Ice crystal formation = Segregation

Sample thickness (µm):
- 200 µm
- 600 µm
Cooling rate

vitrified sample

ice crystals-segregation

Sample thickness (um)

200 µm

600 µm

° C/s

24 September 2015
Biology

Soft and “Large”
Aqueous/hydrated
Light elements (C, O, H, N, S, P etc.)

Extracting the area of interest

Matching the size (Volume & shape)

Timing is everything

High Pressure Freezer
Cooling rates
High pressure
Mechanical damage - Cutting

Biological mater is extensible complex material

Elasticity curves

- Very low shear forces preventing local stress increase that could evoke failure
- Difficult to tear (skin vs aluminum sheet)
- Immense variety of combinations leads to complex mechanical behavior

Cutting should be with a sharpest possible blade and with high acceleration and oscillation
General rules

- Filling the carriers

- Choosing the right filler
  - Physiologically compatible
  - Should not alter or compromise the sample
  - Effective in heat transfer

- Timing is everything
Freeze substitution

Cryosectioning

High pressure freezing

Step 1

Step 2

Freeze substitution

Cryosectioning

Freeze fracture

LT embedding

RT sectioning & contrasting

Freeze drying

Replica

(Cryo)TEM/SEM
Golden delicious apple leaf, high pressure frozen

Freeze-substitution  Freeze-fracturing  Cryosectioning

Elektronenmikroskopie ETH Zürich
CEMOVIS
Cryo-Electro Microscopy Of Vitreous Sections

High Pressure Freezing

CryoTEM /CryoET

Cryosectioning under controlled environment

(EM UC7+ FC7)

(EM ICE)
CEMOVIS

- Water is present as immobilized liquid
- Faithful representation of the ‘native’ state
- Absence of any stain
- The image is a fair representation of the density of the material

- In practice, there are a number of difficulties
Cryosectioning

Production of the ribbon

Courtesy Robert Ranner

http://www.youtube.com/user/LeicaMicrosystems?feature=mhum#g/c/6089D886E4DF19B1
New development

FC7 vertical holder for EM Pact and HPM 100 specimen carriers
Cellular context at molecular level

Courtesy J. Pierson and P. Peters
Freeze fracturing

High Pressure Freezing
(EM BAF900)

Cryo SEM/TEM
(EM VCM + VCT500)

High vacuum cryo transfer
(EM VCM + VCT500)

Freeze Fracturing/Etching
(EM ICE)

Freeze fracturing
Freeze fracturing

- Fracturing of adequately frozen biological samples
- High vacuum conditions allow sublimation at low temperature and deposition of platinum–carbon

Frozen hydrated biological specimen

(Cryo)SEM
Cryo SEM after Freeze-fracture

Mouse intestine

Mouse brain

Electron Microscopy Center Zurich (EMEZ)
Freeze fracturing

- The biological material is removed from the replica
- The replica are mounted on grids and examined in the TEM

Sodium hypochlorite/ Chromic acid
Replica

myelinated axons

synaptic connection PSD95 (15nm), KCTD12 (10nm), and GABA(B1) (5nm)

Courtesy Akos Kulik, (University of Freiburg)
Freeze-Substitution

Freeze substitution

High Pressure Freezing

TEM/ET

Contrasting

Sectioning

Trimming
Freeze-Substitution

- Dehydration at low temperature
- Fixatives are uniformly distributed
- Immunocytochemistry

Bacteria

Plant cells

Eukaryotic cells

C. Lopez-Iglesias

Riet de Rycke

Bram Koster
Endosomes and lysosomes in HepG2 cells

Murk et. al., 2003
Tomographic reconstruction of early endosomes

Murk et. al., 2003
Freeze Substitution

- Minimized aggregation and redistribution of diffusible elements
- Fixatives are uniformly distributed throughout the sample

*Cryotechniques in Biological Electron Microscopy, 1987, Steinbrecht RA, Zierold K*
Solvents capacity to dissolve water

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Melting point</th>
<th>% (v) Water dissolved at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>183 K</td>
</tr>
<tr>
<td>Methanol</td>
<td>179.1, -93.9</td>
<td>29</td>
</tr>
<tr>
<td>Acetone</td>
<td>177.6, -94.4</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Diethylether</td>
<td>156.8, -116.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Ethanol</td>
<td>155.7, -117.3</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> 0.14% at 213 K.

Data on water capacity of acetone from Van Harreveld et al. (1965), of diethylether from Humbel (1984), of methanol and ethanol from J.-D. Acetarin and E. Carlemalm (unpubl.).
Cartilage collagen fibrils in high-pressure frozen samples

acetone

ethanol

methanol

D. STUDER, M. CHIQUET, AND E. B. HUNZIKER
2%OsO4-0.5%GA-10%H2O
The choice of resin

Conventional Os-2%-Epon

0.1%UA- HM20
CLEM  Correlative Light and Electron Microscopy

Live cell imaging to EM visualization in 7 seconds

- High pressure freezing
- Freeze substitution
- LT embedding
- RT sectioning & contrasting
- TEM

Light Microscopy

Live cell video microscopy
LM micrographs Hela- GFP tubulin and H2B cherry

20 nm carbon layer

Courtesy Andres Kaech
Processing the 6mm sapphires

Sample ready for trimming/sectioning

Polymerized block
Correlation between the LM of a live cell and a TEM section. (Hela cell, MLN64-gfp)

courtesy of Y. Schwab
The GFP signal is localized at the lysosome periphery.

courtesy of Y. Schwab
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