Cryogenic sample prep for electron microscopy: considerations and techniques

Reiner Bleher
Cryogenic vs. conventional processing

- High-pressure freezing
- Plunge freezing
- Chemical fixation, dehydration, resin embedment or CPD

permeability changes, redistribution/loss of diffusible ions and small molecules and extraction of lipids

shrinkage/distortion/collapse
Cryofixation without ice crystal formation

### Realizable cooling rates

**I** Range of vitrified pure water

**II** Range of vitrified animal cells and tissues

**III** Range of specimens vitrified with high pressure

### Which cryogens are suitable?

<table>
<thead>
<tr>
<th>Cryogen</th>
<th>Melting Pt. [°C]</th>
<th>Boiling Pt [°C]</th>
<th>Freezing Rate [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freon 13 (CCIF3)</td>
<td>-181</td>
<td>-81</td>
<td>98000</td>
</tr>
<tr>
<td>Propane</td>
<td>-188</td>
<td>-42</td>
<td>98000</td>
</tr>
<tr>
<td>Ethane</td>
<td>-183</td>
<td>-89</td>
<td>97000</td>
</tr>
<tr>
<td>Isopentane</td>
<td>-160</td>
<td>28</td>
<td>45000</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>-209</td>
<td>-196</td>
<td>16000</td>
</tr>
</tbody>
</table>

Cryofixation

<table>
<thead>
<tr>
<th>Device</th>
<th>Freezing depth (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plunge Freezer</td>
<td>≤ 10</td>
</tr>
<tr>
<td>Spray Freezer</td>
<td>≤ 15</td>
</tr>
<tr>
<td>Slam Freezer</td>
<td>≤ 15</td>
</tr>
<tr>
<td>Propane Jet</td>
<td>≤ 40</td>
</tr>
<tr>
<td>High-Pressure Freezer</td>
<td>≤ 500</td>
</tr>
</tbody>
</table>

Basic cryogenic workflows

Plunge freezing

- Cryo-EM (TEM, STEM, SEM)

Plunge freezing variant:

Flash freezing
- Requires the use of cryoprotectants (Sucrose, Glycerol, OCT)
- Used for:
  - immuno-EM (Tokuyasu method)
  - immunohistochemistry

High-pressure freezing

Freeze drying → Coating → SEM

Freeze fracture/etching/coating → Cryo-SEM

Freeze substitution → Resin embedment → Ultra microtomy → EM (TEM, STEM, SEM)

Cryo ultra-microtomy or cryo planing → Cryo-EM (TEM, STEM, SEM)
Plunge freezing
Plunge freezing


<table>
<thead>
<tr>
<th>Samples</th>
<th>Blot time / drain time (s)</th>
<th>Blot total</th>
<th>Blot offset (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large protein complexes</td>
<td>3 - 4</td>
<td>1 - 2</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Viruses</td>
<td>2 - 3 + drain time 0.5 - 1</td>
<td>1 - 2</td>
<td>2</td>
</tr>
<tr>
<td>Organelles</td>
<td>1 - 2</td>
<td>1 - 2</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Cells</td>
<td>1 - 2 + drain time 1</td>
<td>1 - 2</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Gels (low viscosity)</td>
<td>5 - 6 + drain time 1</td>
<td>1 - 2</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Gels (high viscosity)</td>
<td>5 - 6 + drain time 1</td>
<td>2 - 3</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Liquid emulsions</td>
<td>2 - 3</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
Plunge freezing

Mark III

Mark IV

NUANCE
Northwestern University Atomic and Nanoscale Characterization Experimental Center

EXPLORING INNER SPACE
Plunge freezing

4 stages:

1. Sample optimization
2. Sample carriers and treatments
3. Deposition
4. Vitrification

Each of these stages is subdivided into options users are faced with in the workflow.

Plunge freezing

C-flat™

UltrAuFoil™ Holey Gold Films

Thickness of Gold Foil about 500 Å
Structure of Gold Foil
regular square array of micrometer-sized circular holes
Plunge freezing - artifacts

Evaporation rates at different temperatures and humidities

Osmotic collapse of spherical liposomes into “vaselike” structures

Ice contamination as a result of a high partial water vapor pressure in the microscope column.

Plunge freezing – new developments

Noble, Alex J., Hui Wei, Venkata P. Dandey, Zhening Zhang, Yong Zi Tan, Clinton S. Potter, and Bridget Carragher. "Reducing effects of particle adsorption to the air–water interface in cryo-EM." Nature

Plunge freezing – new developments

**Basic cryogenic workflows**

**Plunge freezing**

- Cryo-EM (TEM, STEM, SEM)

Plunge freezing variant:

- **Flash freezing**
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**High-pressure freezing**

- Freeze drying
- Freeze fracture/etching/coating
- Freeze substitution
- Cryo ultra-microtomy or cryo planing
- Cryo-SEM
- Resin embedment
- Ultra microtomy
- EM (TEM, STEM, SEM)
When water freezes, its volume increases
(Chatelier’s Principle)

High pressure (~2050 bar):
1. inhibits volume expansion
   and
2. reduces the critical freeze rate to a range between 100 and 500 °/s

How?
1) Lowering of the freezing point
2) Lowering the supercooling temp. limit
3) Reduction in the rate of ice crystal nucleation
4) Slowing the growth of ice crystals

<table>
<thead>
<tr>
<th></th>
<th>melting point</th>
<th>devitrification temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pure water</td>
<td>-0.15°C (273 K)</td>
<td>-133.15°C (140 K)</td>
</tr>
<tr>
<td>physiologically active cells and tissues</td>
<td>-2.15°C (271 K)</td>
<td>-80.15°C (193 K)</td>
</tr>
<tr>
<td>frost-hardy cells with reduced water content</td>
<td>-13.15°C (260 K)</td>
<td>-43.15°C (230 K)</td>
</tr>
</tbody>
</table>

Freezing damage
A brain slice (ms) of 300 µm thickness was frozen in liquid nitrogen
High-Pressure Freezing

HPM100

HPM100: temperature and pressure dynamics during a freeze.
High-pressure freezing
Freeze substitution involves replacing the frozen water of the cell with an organic solvent at low temperature, thus avoiding the damaging effects of dehydration that occur at ambient temperature (Steinbrecht and Müller, 1987).

**FS is fully automated with the Leica AFS2**

- FS medium: UA, OsO₄ or GA in acetone
- From ~90°C to RT in several days
- Steps with holding and ramping up (e.g., 5°C per hour) temperature
- Ending at RT, then rinsing with acetone and resin embedment

or (mainly for immuno-EM or fluorescent samples)

- Ending at low temp. then automated rinses and resin infiltration at low temp., usually -50, -40, or -20 °C, depending on resin used (e.g., GMA, LR Gold, K4M, HM20)
- Polymerization with UV light at low temperature


Cryosections of high-pressure frozen E. coli ΔcusR on silicon nitride windows

Window #1 with sections

Window #2 with sections
Thanks for your Attention!

Q & A

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