Specimen preparation for SEM

Jana Nebesářová
Biology Centre CAS, České Budějovice
Faculty of Science, Charles University in Prague
nebesaro@natur.cuni.cz
The current HR SEM offers a number of applications in biology and biomedicine. Sample preparation depends on the purpose of the observation.
Specimen criteria for the EM examination

- Removing of water or other volatile components from the specimen:
  - totally – HV SEM (dry specimen)
  - partly – LV SEM (70%)
  - without – ESEM (wet)
- Ability to remain unchanged under high vacuum conditions
- Stability when exposed to electron beam
- Sufficient production of detected signals
- Stopping the changes associated with removing and processing of the sample
- Appropriate size for the SEM
Standard procedure of specimen preparation at RT

- Fixation
- Dehydration
- Drying
- Coating
- Examination in SEM
Chemical fixation

- To preserve cell and tissue organization as near as possible to the native state.
- To arrest living process in cells.
- To protect the sample against all damages in following preparation steps leading to minimal deterioration of fine structure.
- To arrest autolysis and bacterial decay.

*Plant sample sizing*
Glutaraldehyde \( \text{OHC-} \text{(CH}_2\text{)}_3\text{-CHO} \)

- Aliphatic dialdehyde which forms colourless crystals that are highly soluble in water, ethanol and most organic solvents.
- In aqueous solutions, GA polymerizes into polymer chains with variable size.
- GA fixative solutions must contain monomer and low polymers with small molecules to penetrate cell membranes fast enough.
- GA (EM grade, 8-25 %) diluted in solutions (1-4%) are used for fixation, which are relatively stable with a pungent odour.
- GA is able to cross-link proteins rapidly, effectively and irreversibly and forms large, three-dimensional network throughout the cytoplasm in tenths of second to minutes.
- GA in solution is uncharged and can thus rapidly cross all biological membranes.

Introduced by Sabatini et al, 1962
Glutaraldehyde

- GA preserves mainly **proteins**, because it can react with several functional groups of proteins, such as amine, thiol, phenol and imidazol.
- GA cross linking preserves 3D structure of proteins
- It preserve also other macromolecules associated with proteins (lipoproteins, histoproteins associated with DNA). GA cross-linking reactions are horribly complex leading to the formation of broad spectrum of conjugates.
- **Glycogen** may be preserved but the majority of carbohydrates will be extracted in the next preparation steps.
- Most **lipids** do not react with GA, with exception of phospholipids containing primary amines.
- Many **enzymes** remain active after aldehyde treatment. Molecules that are not immobilized may be relocated resulting in a false location or negative results in immunolabelling.
Glutaraldehyde

- The reaction is influenced by a ratio of GA to free amines (2:1), too high concentration of GA can inhibit the formation of the rapid cross-links.
- The reaction with amines is accompanied by a significant release of protons and ensuing drop in pH – buffered fixation solution.
- The fixation process consumes oxygen - adding azide (inhibit respiration) or hydrogen peroxide hepl cross-linking.

Work with GA carefully! Avoid the contact with the skin and eyes, prolonged breathing. Repeated exposure to GA may cause contact dermatitis. A well-ventilated hood, gloves and protective clothing are necessary!
Formaldehyde  

At room temperature FA is a colorless gas highly soluble in water. Both liquid and gas polymerize spontaneously.

FA is commercially available as a concentrated aqueous solution (Formalin) or in a polymerized state as a dehydrated powder – paraformaldehyde (PFA).

FA molecule is smaller than GA, thus rapidly penetrates into sample (5 times faster than GA).

Karnovsky’s fixative – the combination of FA and GA (recommended for larger size samples with a poor penetration).

Substances such as carbohydrates, lipids and nucleic acids are trapped in a matrix of insolubilized and cross-linked protein molecules but they are not chemically changed by formaldehyde.

FA links weakly proteins, therefore it is a preferred fixative for immunolabeling techniques.
Osmium tetroxide

- The molecule is symmetrical and contains four double bonded oxygen atoms.
- Osmium can exist in nine oxidative states, five of which are quite stable.
- Osmium tetroxide is soluble in both polar (aqueous) and non-polar media. Thus can fix both hydrophobic (e.g. membrane phospholipids) and hydrophilic domains in cells.
- It is highly volatile, it can be used also as a vapor fixative.
- Os is electron opaque, works as a stain, as well as fixative.
- Osmium tetroxide also act as a mordant, it enhances lead staining.
- It causes rapid permeabilization of membranes with cessation of cytoplasmic movement within second to minutes.
- It is the most slowly penetrating fixative and has no cross-linking capabilities.
- It reacts with ethanol to form black precipitates:
  \[ \text{OsO}_4 + C_2H_5OH = \text{OsO}_2 + 2 \text{CH}_3\text{CHO} + 2 \text{H}_2\text{O} \]
Osmium tetroxide

- Interact directly with unsaturated lipids oxidizing double bonds, leading to the formation of monoesters, diesters and dimeric monoesters.
- Specimens generally turn black after osmification.
- It causes hardening of tissues.
- Osmium tetroxide can also react with some proteins and lipoproteins complexes. Prolonged fixation results in the progressive denaturation of proteins.
- As a strong oxidant it damages the majority of antigents.
- Limited penetration into tissue (200 µm)

Be extremely careful in handling OsO₄. It is dangerous to eyes, respiratory and alimentary membranes. Used or excess of OsO₄ must be stored in sealed glass containers. Avoid the direct contact by wearing plastic gloves and working in well-ventilated hood.
Fixation solution

- Glutaraldehyde, formaldehyde: 1-4%
- Osmium tetroxide: 1-4%
- Buffers - phosphate, cacodylate, HEPES…
- Other substances improving fixation like hydrogen peroxide, tannic acid etc.
- Isotonic or slightly hypertonic solution
Dehydration

• To replace water from samples by organic solvents (ethanol, acetone, propylene oxide)
• Caused shrinkage, extraction of various cellular components, changes in shape and size of sample
• Usually performed using a solution series with increasing concentration of organic solvent

Dehydrants are hygroscopic, they are able to absorb water from the air. It is important to keep dehydrants sealed!
Drying

Critical point drying method - phase change from the liquid to the dry gas without the effects of surface tension on a specimen

<table>
<thead>
<tr>
<th>Substance</th>
<th>Temp. °C</th>
<th>P.S.I</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYDROGEN</td>
<td>-234.5</td>
<td>294</td>
</tr>
<tr>
<td>OXYGEN</td>
<td>-118</td>
<td>735</td>
</tr>
<tr>
<td>NITROGEN</td>
<td>146</td>
<td>485</td>
</tr>
<tr>
<td>CARBON DIOXIDE</td>
<td>+31.1</td>
<td>1072</td>
</tr>
<tr>
<td>CARBON MONOXIDE</td>
<td>+141.1</td>
<td>528</td>
</tr>
<tr>
<td>WATER</td>
<td>+374</td>
<td>3212</td>
</tr>
</tbody>
</table>

Surface topography
The ethanol/aceton is replaced by liquid carbon dioxide, then the CO$_2$ is brought to its critical point and converted to the gaseous phase without crossing the interfaces between liquid and gaseous avoiding the damaging effects.
Specimen Coating

Charging – a material cannot conduct the beam energy imparted to it.

Contamination – the interaction of the electron beam with residual gases and hydrocarbons on the specimen surface.
Specimen mounting

• The dry specimen is mounted on a metal stub using a sticky carbon/copper disc/tape which increases conductivity. Silver-containing glue can additionally be applied for even more conductivity.

• Dry biological samples are not conductive. To prevent charge build-up on specimen surface, it is coated with a conductive material. The metal is applied in a controlled manner in special devices. It is critical that the coating is thick enough to prevent charging (typically around 10 nm) but not thick enough to obscure specimen surface details.
Specimen Coating

- increasing of the conductivity of dry specimen
- reduction of the charging effect
- reduction of thermal damage
- improvement of SE and BSE emission

<table>
<thead>
<tr>
<th>Element</th>
<th>Z</th>
<th>Thermal conductivity at RT (W/cm/K)</th>
<th>Resistivity at RT (W.m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>6</td>
<td>1.29</td>
<td>3.5x10^-5</td>
</tr>
<tr>
<td>Aluminium</td>
<td>13</td>
<td>2.37</td>
<td>2.82x10^-8</td>
</tr>
<tr>
<td>Palladium</td>
<td>46</td>
<td>0.72</td>
<td>1.1x10^-7</td>
</tr>
<tr>
<td>Silver</td>
<td>47</td>
<td>4.29</td>
<td>1.60x10^-8</td>
</tr>
<tr>
<td>Platinum</td>
<td>78</td>
<td>0.72</td>
<td>1x10^-7</td>
</tr>
<tr>
<td>Gold</td>
<td>79</td>
<td>3.17</td>
<td>2.44x10^-8</td>
</tr>
</tbody>
</table>

Surface topography
Specimen coating

The most important parameters – film thickness, homogeneity and granularity. The film thickness depends – deposition rate, sputtered material, the distance between the metal target and sample position.

The measurement of film thickness:
- Quartz crystal thickness monitor
- Contaminations: The cleanness of vacuum chamber
- The film granularity: Selection of coating Metals.
  - Au, Pt, Pd for HR
  - W, Ir, Ch for ultra HR

Sputter coater
Vacuum evaporator

BalTec SCD050
JEOL JEE4C
Special methods

- Fixation in OsO$_4$ vapors:
  - the sample is enclosed in a sealed container together with the crystal of OsO$_4$ and placed in the freezer for 2-3 weeks. Fixation with OsO$_4$ vapors strengthens the sample surface, which then gradually dries without any manipulation and deformation.
- Suitable for gentle samples
Special methods

- Dehydration with t-Butyl Alcohol:
- Ethanol is used as a dehydration agent. A sample in 100% ethanol is transferred to 100% t-BA at a temperature above 25°C.
- Then the sample is placed in the refrigerator (6 °C). Amorphous solid tBA is sublimated using a rotary vacuum pump.
- Suitable for gentle samples
Artifacts

• Sample compression or shape changes caused by dehydration and high pressure during CPD
• Insufficient or irregular thickness of conductive layers can cause charging
• Microwave irradiation can be used in each step of specimen preparation and improves the sample preservation
Cryo methods

Advantages:
• structure preservation close to native state
• no fixation, no dehydration, no coating
• low accelerating voltage (>5 kV)
• observation of time depending processes in cells

Disadvantages:
• expensive equipment (cryo-attachment)
• necessity to store the sample in liquid nitrogen
Cryo fixation

- Cryo-fixation - based on vitrification of samples without ice crystal formation during cooling (amorphous ice)
- High pressure freezing (HPF) – the best methods of vitrification of bulk samples containing water (biological samples, hydrogels etc.)
Cryo - fixation

Cooling speed

$> 10^5 \text{ K/s}$

Freezing and vitrification

Vitrification (from Latin vitreum, "glass" via French vitrifier) is the transformation of a substance into a glass.

crystalline ice
- lower density than water in liquid state

vitreous = amorphous = glassy ice
- density of liquid water and vitreous ice is about the same
- no segregation of solutes and solvents
Vitrification and amorphous ice

- Brügeller P and Mayer E. 1980 - vitrification of water

Water becomes solid while remaining in amorphous state.
Cooling rate must be high enough that the crystals do not have time to form
Cryo - SEM

- Direct observation of frozen specimens in FESEM equipped with cryo-attachment

1. Cryofixation with slushy nitrogen
2. Transfer to preparation chamber under vacuum
3. Treatment of sample: Fracturing, etching, coating
4. Transfer to the cold stage of SEM and the examination
Ice sublimation

Cryo methods

Sublimation (freeze etching)
T~160K to 180K
(-110°C to -90°C)

Examination:
T < 140K (-130°C)
Sublimation rate <0.001 nm/s
<4 nm/h
Ice sublimation of ice cream

Stokes D.J. et al., Journal of Microscopy, 213, 2004, 198-204
Freeze fracturing

The technique used to look at inner ultrastructure of a frozen sample
Characterization of yeast biofilm

C. parapsilosis and C. albicans

Cryo methods

- plunging into LN$_2$
- plunging into liquid propane
- high pressure freezing

K. Hrubanova et al.: Micron, 110, 2018
Cryo methods

Freeze drying

Freeze substitution

- The combination of chemical methods with cryo methods
- The ice is replaced at low temperature by anhydrous solvent
- The organic solvent must be liquid at low temperature and dissolve additives such as osmium tetroxide or GA
- Reduced shrinkage and shape changes
- Resulting in dry samples

Living biological specimen

Cryofixation (HPF)

Freeze substitution

Drying (CPD)

HPF, plunge freezing

Aceton with GA, OsO₄

FS unit Leica
Temperature range: -90°C - 20 °C
Application: Characterization of yeast biofilm

*Candida parapsilosis* grown on the glass substrate and fixed by plunging in nitrogen slush

V. Krzyzanek, K. Hurbanová, J. Nebesářová, F. Růžička: Novel technique in cryo-SEM freeze fracturing demonstrated on microbial film. Submitted
Application: Characterization of yeast biofilm

- Freeze fracture of *Candida parapsilosis* grown on sapphire discs and fixed by plunging in liquid ethan/propan
Application: Characterization of yeast biofilm

Cryo methods

Freeze fracture of Candida parapsilosis grown on a sapphire discs (1.4 mm) and fixed by HPF. HPF Leica EM Pact II.
Penicillium

Fixation: 1% OsO₄
Dehydration: aceton
Drying: CPD
Coating: Au
Penicillium

Fixation: OsO₄ vapors at freezer temperature
Drying: freeze drying
Coating: Au
Penicillium

Fixation: Cryo-fixation
Freeze substitution: aceton with 1%OsO₄
Dehydration: aceton
Drying: CPD, Coating: Au
Penicillium

Cryo-SEM
Cryo-fixation
Sublimation at -95°C
Coating: Pt
Freeze drying and cryo SEM

- Suitable filter for water removing

Undescribed species of Microsporidia from microcrustacea (Vavra J. 2013)
Comparison of sample size prepared by different methods

Size distribution of sturgeon sperm depending on the method of preparation:
A/ CPD drying
B/ t-butyl alcohol
C/ ESEM
D/ cryo-SEM

Pšenička et al.: Micron, 41(5), 2010
Thanks for your attention!

Cryo-SEM:
Larva of Chymomyza Costata (Drosophilidae)