


RMS

SEM Specimen Preparation for Tissues and Biomaterials

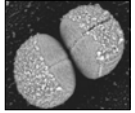


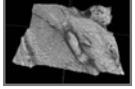
Iolo ap Gwynn
The University of Wales Bioimaging Laboratory
Aberystwyth
Wales
iag@aber.ac.uk



RMS/ESB Workshop Sorrento 2005

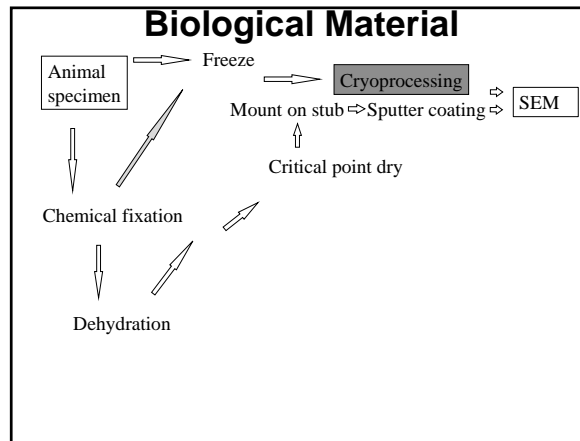
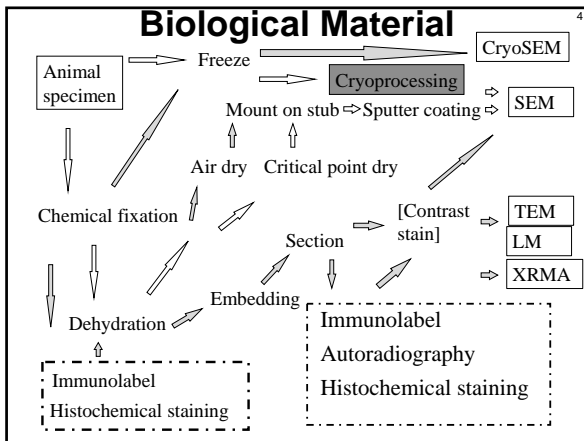
SEM Imaging

- Information required ?
 - Signal types
 - Resolution ?
- Specimen preparation
 - Preservation ?
 - Dehydration ?
 - Coating
- Microscope settings
- Interpretation
 - Analysis

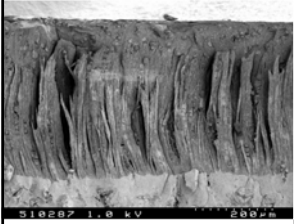





Specimen types (general)

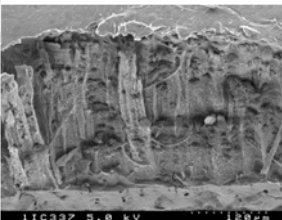
- Biomaterial
 - Surfaces
 - Particles
 - Matrices
- Biological material
 - Cells
 - Tissues
- Combined Biological/Material
 - Interfaces



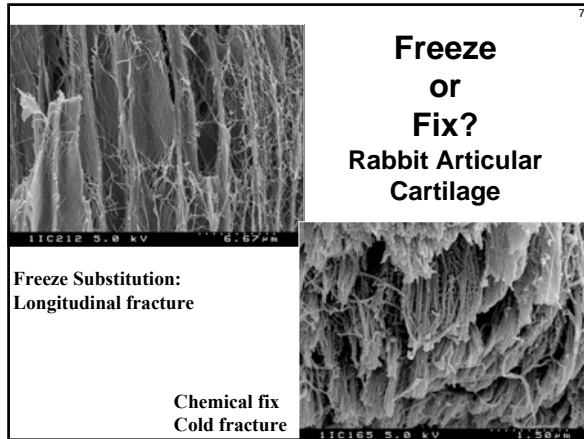
Freeze or Fix? Rabbit Articular Cartilage



Freeze fracture



Chemical fix



- 8
- ### Specimen Preparation Procedures
- What do think you need to know?
 - Search literature for methods
 - Discuss with microscopist(s) !
 - Choose possible approach(es)
 - Experiment
 - Choose final approach(es)
 - Interpret results

- 9
- ### Why Not Freeze Always?
- Possible artefact formation
 - Rapid freezing not possible
 - Comparison to published work
 - Not always correct!
 - Access to fresh tissue not possible
 -etc.

- 10
- ### Chemical Fixation
- The composition of a fixative
 - Fixing agent(s)
 - Vehicle (buffer, ions etc.)
 - Fixation conditions
 - Time; Temperature; pH
 - Dehydration
 - Drying methods

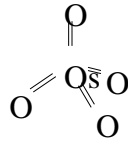
- 11
- ### The Fixing Agent
- Macromolecule cross-linker
 - Kill cells
 - Possible provider of contrast
 - Will create artefacts
 - Several often used together

- 12
- ### The Aldehydes
- Glutaraldehyde
 - Popular since 1960s – slow penetrating
 - Needs oxygen
 - Formaldehyde
 - Used in combination with glutaraldehyde
 - Faster penetration BUT unstable
 - Potentially least disruptive
 - Acrolein (acrylic aldehyde)
 - Highly reactive and fast penetration
 - All crosslink proteins
 - All remove basic groups
- $$\begin{array}{c} \text{H}_2 \\ | \\ \text{H}_2\text{C} - \text{C} - \text{CH}_2 \\ | \quad | \\ \text{CHO} \quad \text{CHO} \end{array}$$

$$\begin{array}{c} \text{O} \\ || \\ \text{H} - \text{C} - \text{H} \end{array}$$

$$\begin{array}{c} \text{O} \\ || \\ \text{H} - \text{C} = \text{C} - \text{H} \\ | \quad | \\ \text{H} \quad \text{H} \end{array}$$

Osmium Tetroxide



- Cross linker mainly of unsaturated lipids, some proteins & phenolic compounds
- Main use in secondary fixatives
- Causes elastic electron scattering (BSE)
- Can solubilise some proteins

13

Effects of Fixing Agents

- Main reaction = proteins
 - Some with lipids (fats)
 - Rarely with carbohydrates
- Reduction in pH (Buffer)
 - Cell death = acidification
- Acidification
 - Solubilisation/extraction (cations)
 - Artefacts

14

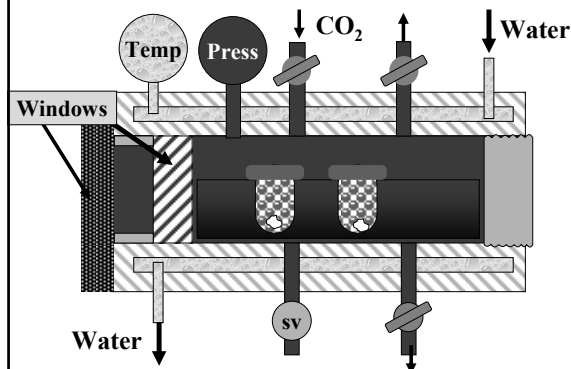
Specimen Dehydration

2 Major Steps:

- 1. Water replaced by organic solvents:
 - Ethanol or acetone (time & temp.)
- 2. Remove organic fluids:
 - by Critical Point Drying (CPD)
 - By 'air drying'
 - tissue distortion
 - by sublimation (freeze drying)

15

Critical Point Drying

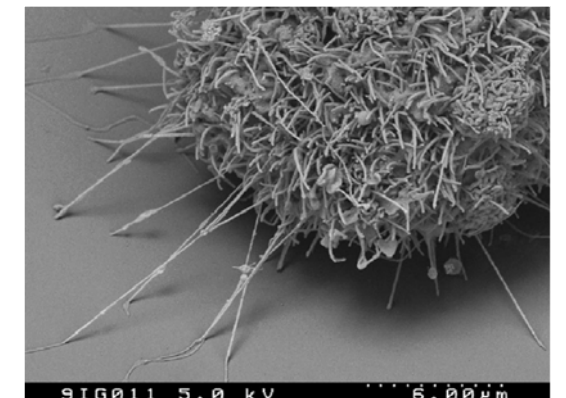


16

Critical Point Drying

- Normal shrinkage 10-15%.
 - Embryonic tissue can shrink by >60%.
 - Shrinkage is spatially unequal.
- CPD causes selective solubilisation
 - supercritical CO₂ used to decaffeinate coffee
- With careful use it can give good results

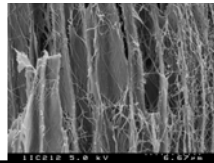
17



18

Cryotechniques: Why ?

- Drawbacks of chemical fixation
- Only method suitable
- Arrest metabolic or contractile processes
- Immunocytochemistry



19

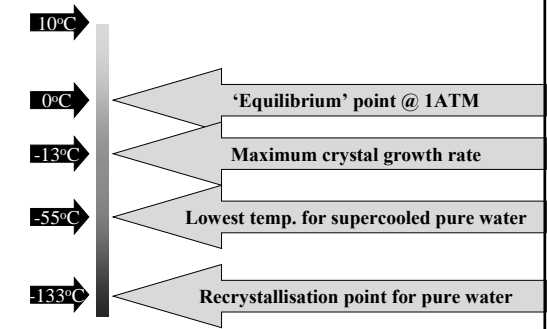
Cooling Pure Water Below 0°C @ 1ATM

Crystal melts

Latent heat of fusion

20

Cooling Pure Water



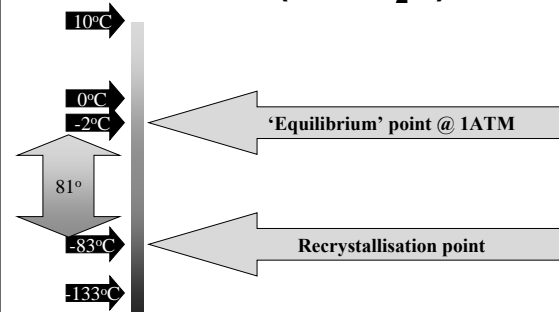
21

Avoid Crystallisation

- Rapid removal of heat
 - latent heat of fusion removed faster than it is released
- Virtually impossible with pure water
- Possible in biological tissue
 - Cryo-protectants (e.g. glycerol, methanol)
 - Rapid freezing

22

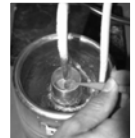
Cooling Biological Material (80% H₂O)



23

Cooling Methods

- Liquid Nitrogen
 - Leidenfrost effect
- Nitrogen Slush
- Intermediate liquid
 - Propane, Freons, Iso-pentane
 - Immersion or spraying
- ‘Slam’ freezing
- High Pressure
 - Special apparatus



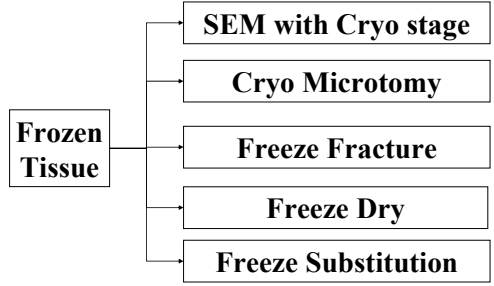
24

Conclusions

- Optimising freezing is possible
- Smaller samples are easier
- Many approaches possible
- Experimentation necessary
- Artefacts can form
- Care with cryogens

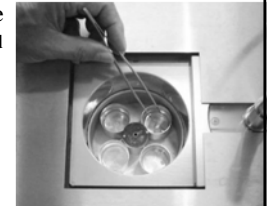


After Freezing



Freeze Substitution

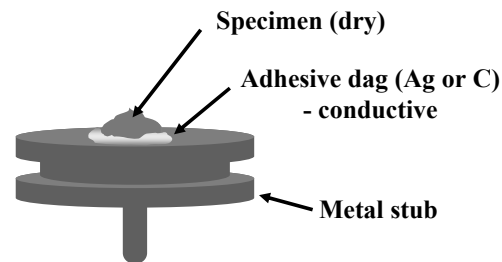
- Keep specimens @ < -90°C
- Place in organic solvent for several days
 - Changes of solvent
 - Staining / crosslinking agents
 - Agitate container
- Bring to room temperature
 - Critical Point Dry or Embed



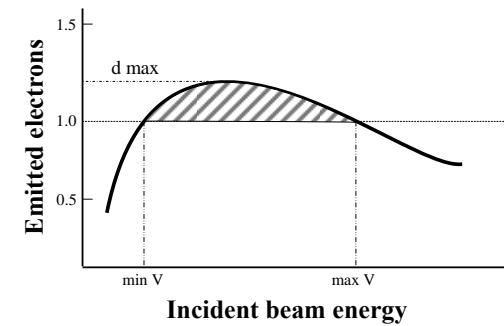
Conclusions - Freezing

- Can be better than fixation
- Artefacts are formed
- Care required with interpretation
- Cryogens can be dangerous
- Only choice for some tissues

Specimen Mounting

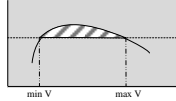


Specimen Coating? Use low kV?



Low kV Secondary Electron Emission

	min V	max V	d max
Al	300	300	1.0
Cu	200	1500	1.3
C (diamond)	>5000		2.8
Glass	300-450		2-3
Au	150	>2000	1.4
C (graphite)	300	700	1.0
Fe	120	1,400	1.3
Ag	200	>2,000	1.5

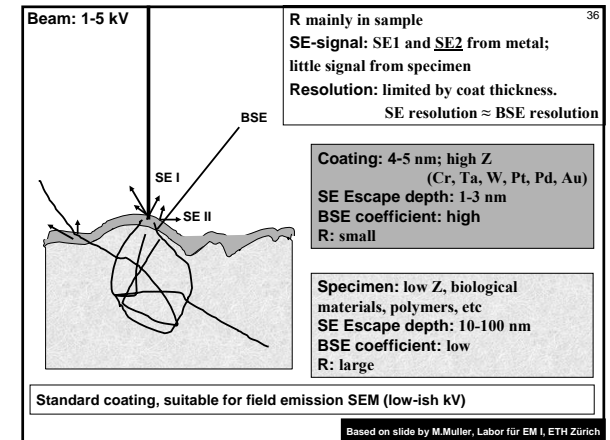
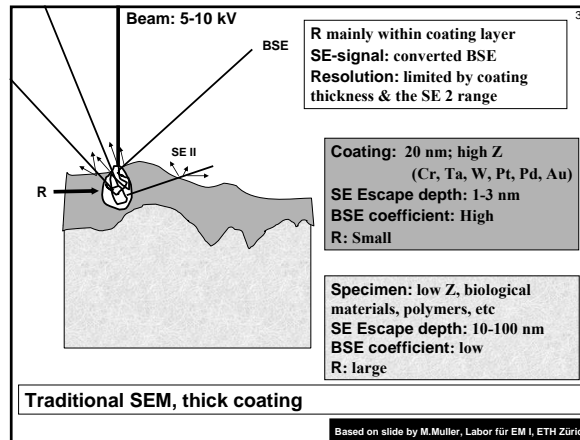
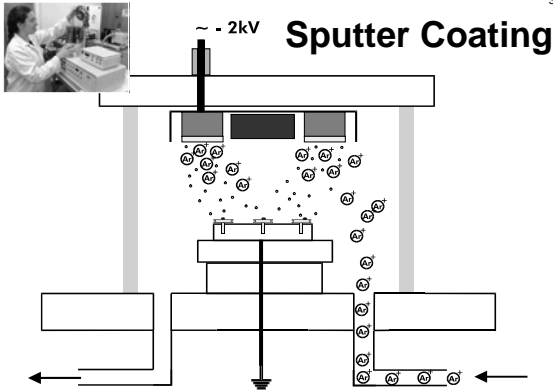


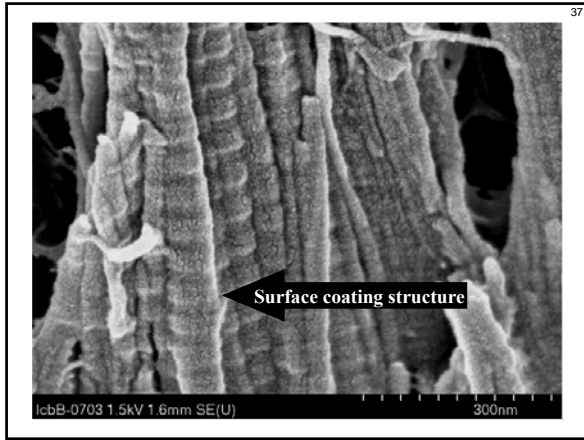
Sample Coating

- Almost all biological samples
- Oxidising metals
- Polymers or ceramics

Sample Coating (Sputtering)

- Removes or reduces electric charge
 - SE very sensitive to specimen charge
- Large number of SE (e.g. Au, Pt, Pd)
- Distributes effects of heating
- Deposited as granules (hi res problem)
- May interfere with X-ray and BSE emission





38

Beam - <1 KV

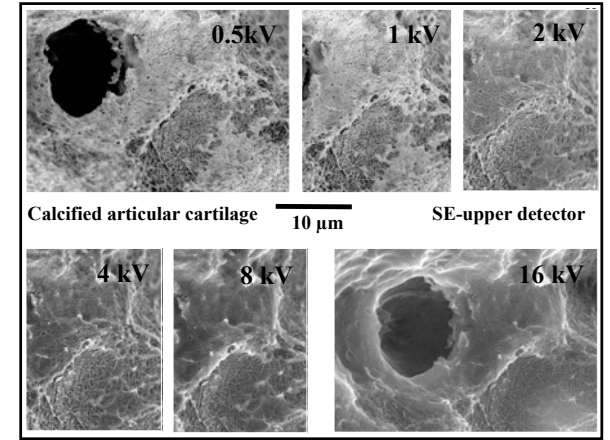
R: mainly in sample
 SE Signal: SE1 + SE2 (small) from metal; little signal from specimen.
 Resolution: limited by coating thickness & diam. of e⁻ beam
 SE produced beneath coating & contained
 Coating discontinuity common

Coating: 1 nm; high Z (Cr, Ta, W)
 SE Escape depth: 1-3 nm
 BSE coefficient: High
 R: Small

Specimen: low Z, biological materials, polymers, etc
 SE Escape depth: 10-100 nm
 BSE coefficient: low
 R: large

High resolution coating for SE1 imaging (FESEM)

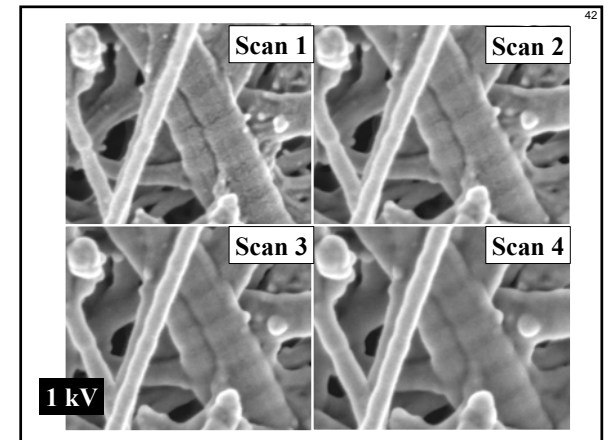
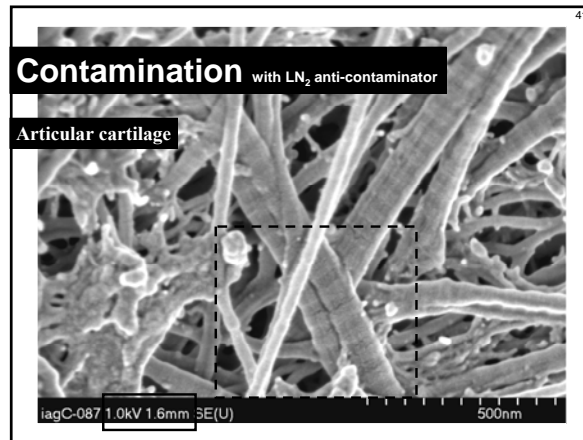
Based on slide by M.Muller, Labor für EM I, ETH Zürich



40

Low Voltage OK
 But ...!

0.3kV 0.5kV 0.7kV 0.9kV 1.0kV 1.2kV 1.5kV



43

Beam
10-30 kV

BSE

R: irrelevant
SE signal: None
BSE signal: Depends on coating
Resolution: High
Beam and BSE penetrate C layer
C layer improves stability & reduces charging

Second coating: 10-60 nm Low Z (C)

Coating: 2 nm; High Z (Pt/Pd)
BSE coefficient: High
R: Small

Specimen: low Z, biological materials, polymers, etc
SE Escape depth: 10-100 nm
BSE coefficient: low
R: large

Sputter Coating for BSE Imaging: Double layer coating

Based on slide by M.Muller, Labor für EM I, ETH Zürich

44

Coating:
Pt/Pd + C

10kV

25wC-0135 10.0kV 8.0mm YAGBSE 200nm

Bacteria & Phage

45

Comparison

1 kV SE
4nm Pt/Pd

3 kV BSE
4nm Pt/Pd

30 kV BSE
4nm Pt/Pd
+ 60nm C

46

Potential SEM Information

- If used incorrectly
 - Very little
 - Waste of time and effort
- If used to its potential
 - Much
 - Dependent upon
 - Specimen preparation
 - Imaging conditions
 - Interpretation and analysis

RMS

SEM Workshop
ap Gwynn & Richards 2005

ESB Sorrento

