Living up to Life

EM Sample Preparation

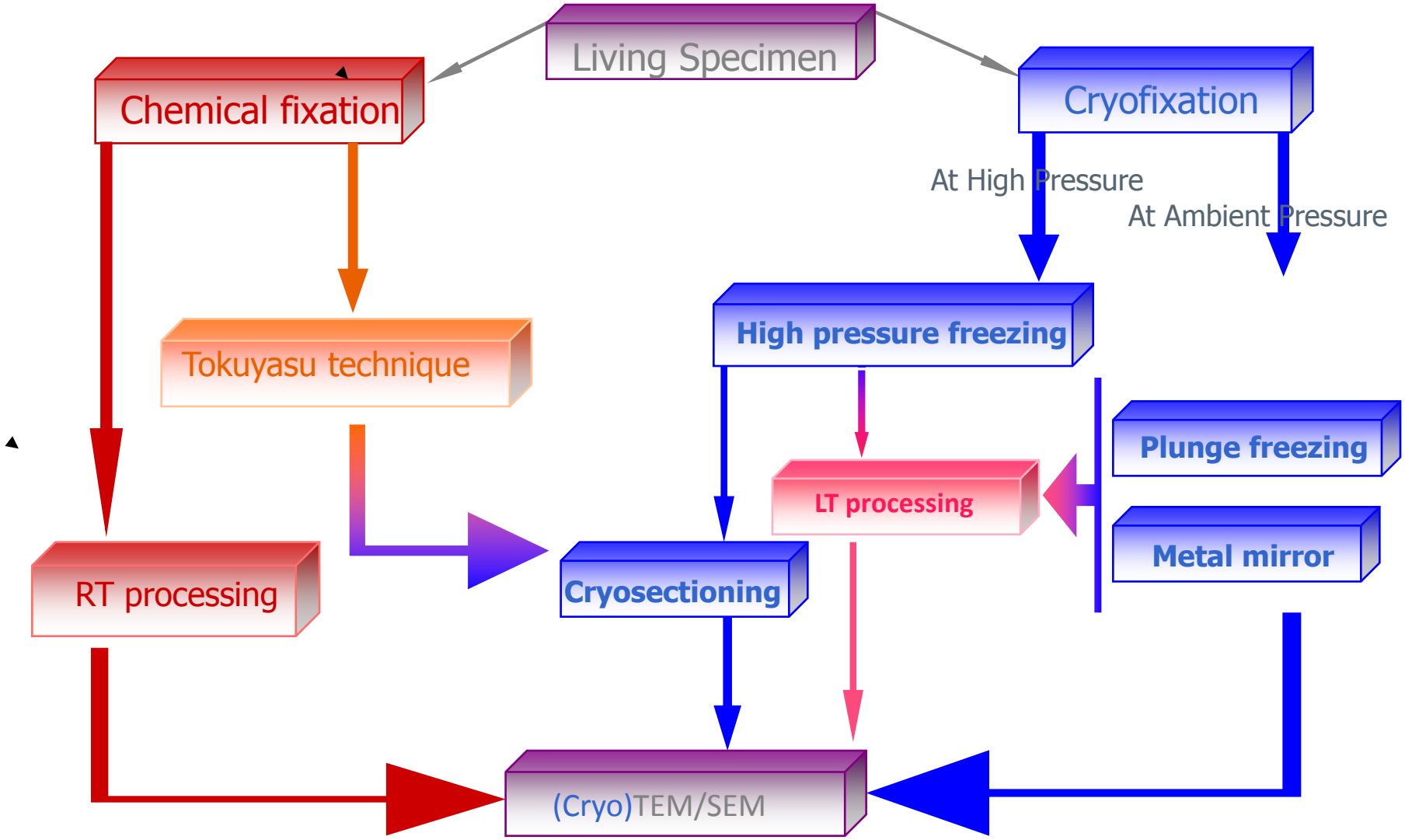
Techniques and pathways

By Leica Nanotechnology

Santiago Sevillano

SOME TECHNIQUES….

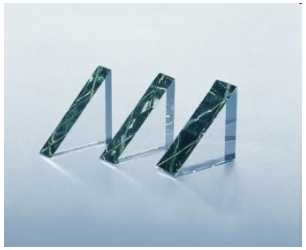
ULTRAMICROTOMY / CRYO-ULTRAMICROTOMY **LEICA UC6-FC6**	TRIMMING/ KNIFE-MAKER **LEICA KMR2**	CRITICAL POINT DRYING **IN BRAGA?**
CONTRASTING /LABELLING	PROCESSING/ DEHIDRATATION CHEMICAL FIXATION RESIN EMBEDING	COATING **HIGH VACUUM EVAPORATOR POLARON CRESSINGTON SPUTTER**
FREEZE ETCHING / FREEZE FRACTURE	CRYO-TRANSFER	FREEZE SUBSTITUTION
CRYO-FIXATION HIGH PRESSURE FREEZING	CRYO-FIXATION BARE GRID TECHNIQUE PIN INMERSION	CRYO-FIXATION SELF PRESSURE FREEZING
TARGET PREPARTION (CUTTING, MILLING, SAWING, POLISHING, GRINDING) **IN MINHO?**	ION BEAM CUTTING	ION MILLING SYSTEM

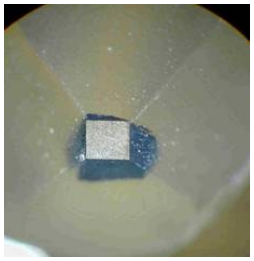

Living Specimen
Chemical fixation
Cryofixation
At High Pressure
At Ambient Pressure
Tokuyasu technique
High pressure freezing
Plunge freezing
LT processing
Metal mirror
RT processing
Cryosectioning
(Cryo)TEM/SEM

EXAMPLE: Workflow of Room Temperature Specimen Preparation for TEM

Fast

AMW

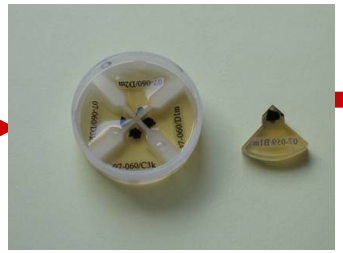




AC20

TP

Standard



KMR3

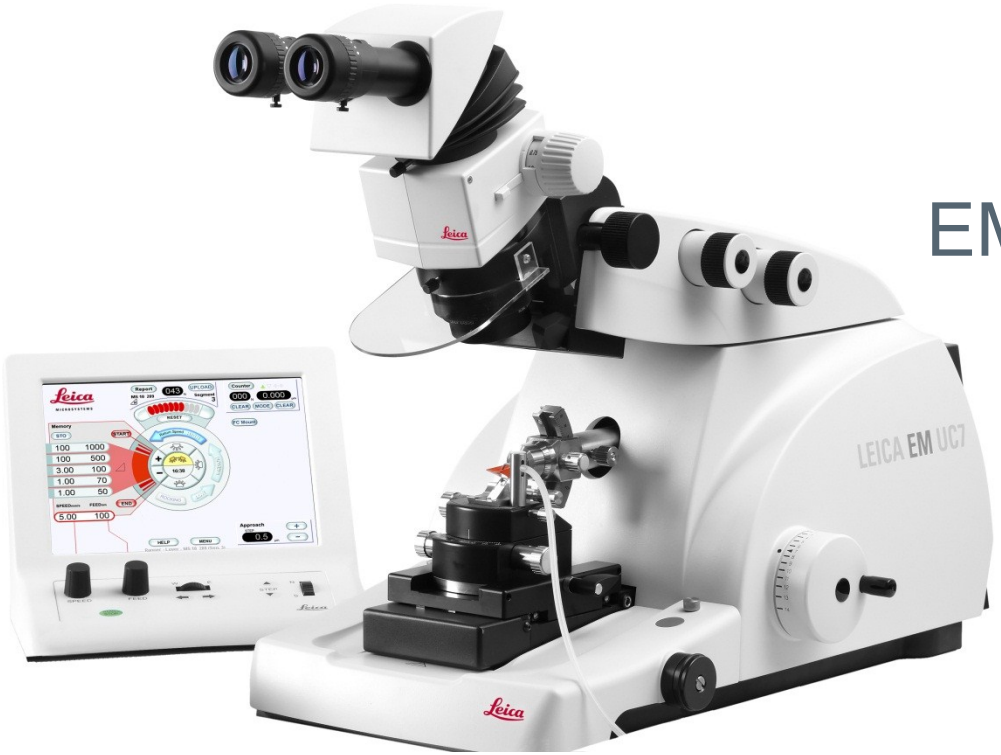
Trim2

UC7

IGL

Tissue Processing	Knifemaking	Trimming	Ultrathin sectioning	Contrasting or Immunogold labelling

EM UC7:Ultramicrotome



EM KMR3 : Knifemaker

The Leica Ultracut EM UC7

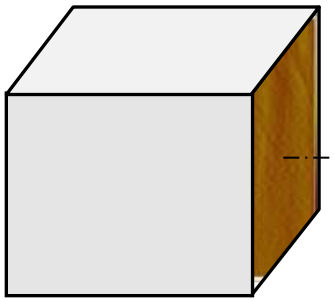
- is used for ultrathin sectioning for TEM
- Surface planing of AFM and **SEM samples**. Industrial material manufacturers and research (polymer, rubber, and materials), as well as cosmetic samples.
- Semithin sections for LM and FT-IR
- 3D reconstruction (tomography, serial sectioning)

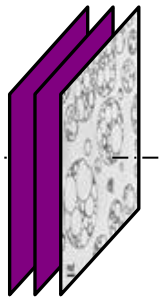
Leica EM UC7 application

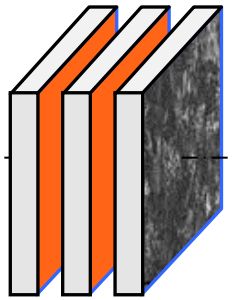
Sample surface:
SEM, AFM, SIMS

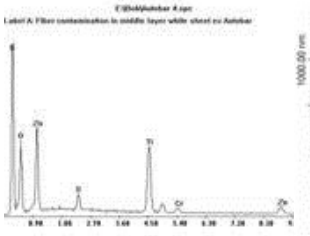
Ultra thin section:
(< 200nm) TEM

Thin section:
(0.2-15µm) LM, FT-IR

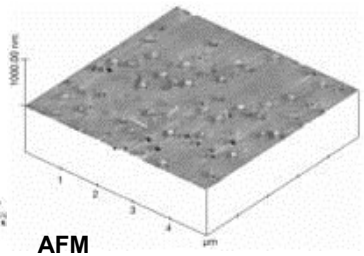




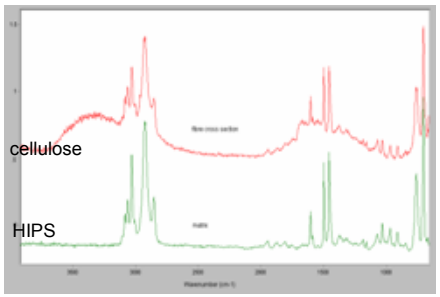




EDX



AFM



FT-IR

Trimming and /or Targeting

TRIM2

Why using a trimming tool?

1)Save time and money
2)Save diamond/glass knives

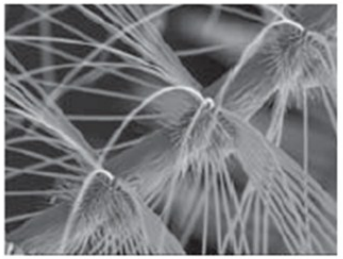
Synergies - Room temperature SEM workflow

Tissue Processing – Leica EM TP ❯ Critical Point Drying – Leica EM CPD300 ❯ Coating – Leica EM ACE200 and ACE600 ❯ Imaging and analysis

Automated tissue processing for fixation and dehydration.

Automated critical point drying for extremely well perserved sample structures.

Automated coating for reproducible, thin and conductive layers.



Antenna of male mosquito (Gold sputter coating), SEM.

H_2O concentration

Liquid CO_2 concentration

Ethanol, Acetone concentration

Carbon, Gold, Platinum...

Material Research Samples

Tissue Processing

TP

AMW

- **WHY ? SAFETY REASONS !!!**

1) **Automatic processing of tissue:**

   Minimizes contact with hazardous reagents (eg. Uranyl, Osmiun or Glutaraldehyde during the CHEMICAL FIXATION).

2) **Closed processing chamber with fume extraction system**

Critical Point Drying

CPD300

Cells

Insects

Tissues

Wafers

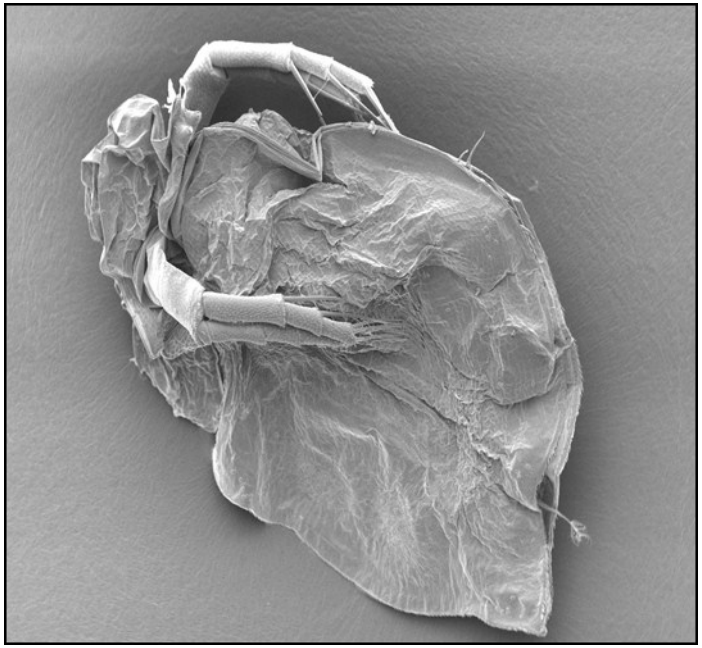
Etc…

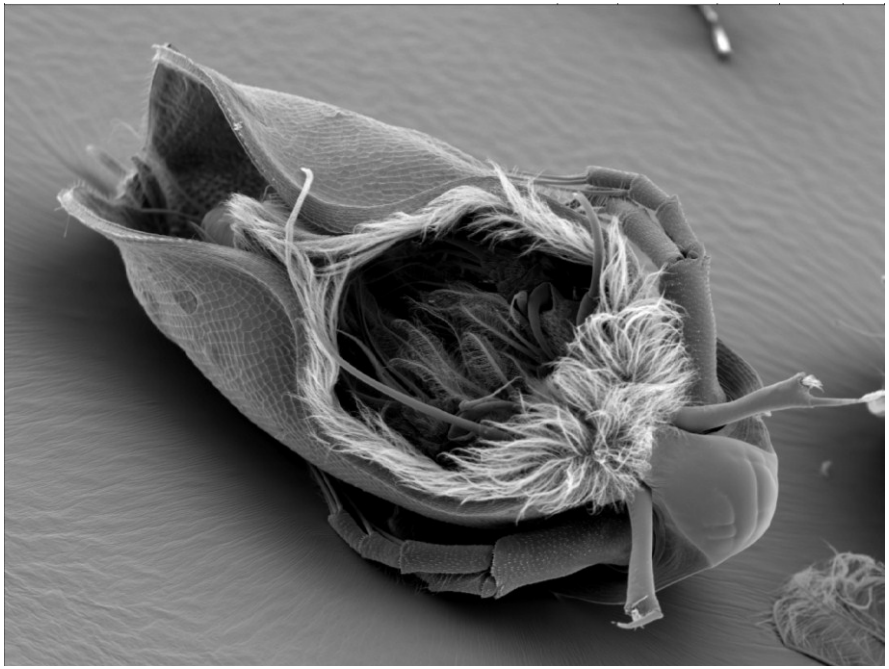
Why ?

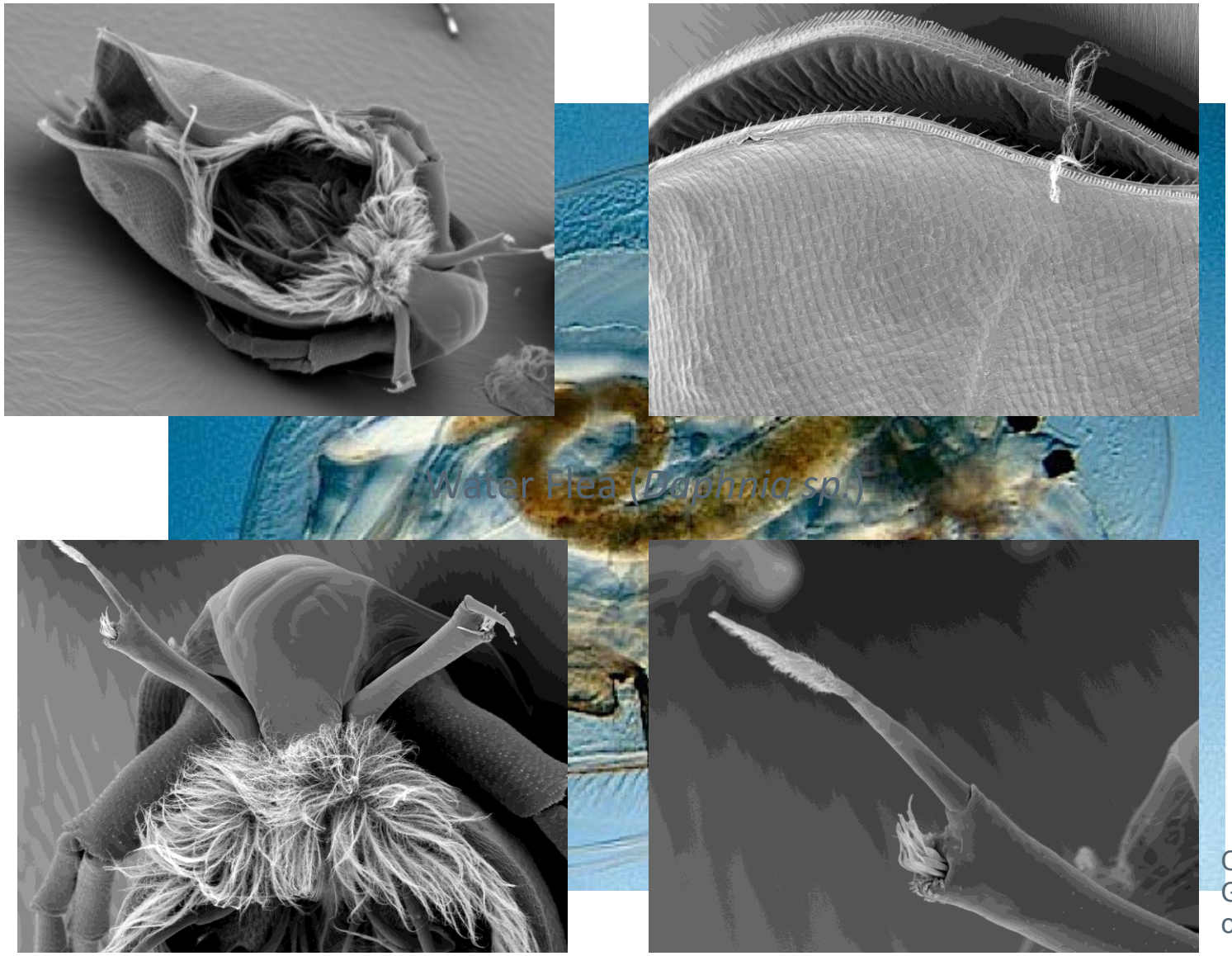
Surface Tension of Water Damages Sample, but not CO2

You should avoid water in the EM column

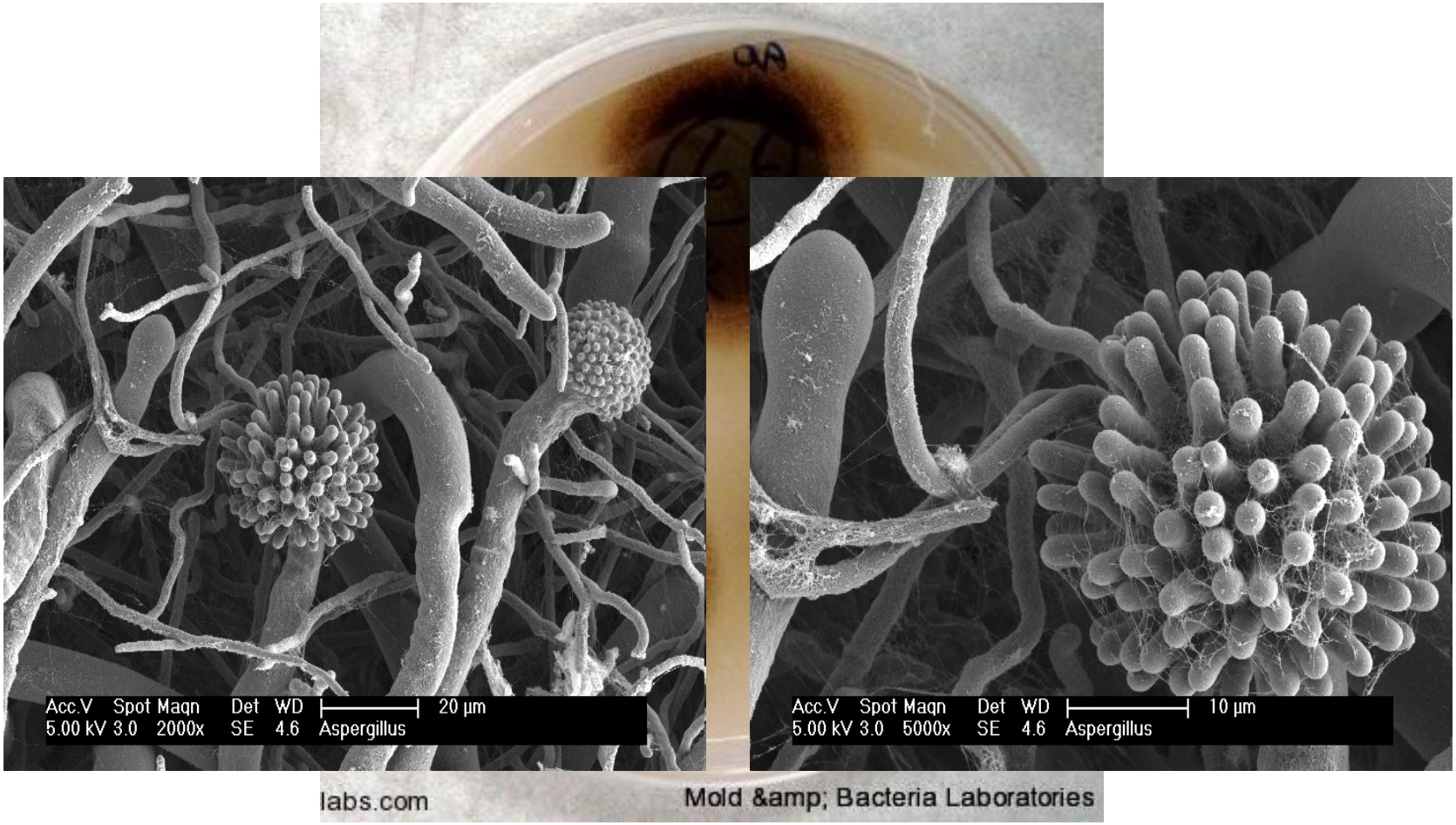
And the result is….







Courtesy of D. Gruber, University of Vienna, Austria



W. Müller, University of Utrecht, Netherlands

Coating

ACE200

ACE600

Cryo-ACE600

At SEMAT:

Evaporator Polaron

Sputter Cressington

Why do we coat ?

SEM

- **Reduce charging, inhibits charging**
- **Improves the secondary electron signal**
- **Higher electron yield from specimen surface**
- **Prevent specimen thermal damage**

TEM

- **Support films for TEM-grids (carbon on formvar)**
- **Generate contrast on thin samples (low-angle shadowing)**
- **Freeze-fracture replica technique**

Or, to produce conductive layers in Micro-electronics research.

Different Coatings

- **WHY METAL SPUTTERING COATING?**
- Chemically inert, does not react with specimens or acids
- Stable in the electron beam
- Transparent to the electron beam (does not create contrast!)
- Conductive - reduces charging

- **WHY CARBON COATING?**
- Reduce charging
- Localize secondary electron (SE) and back-scattered electron (BSE) signal to the surface in SEM
- Reduce heating of non-conductive specimens to increase potential exposure time to the electron beam
- Reveal topography with shadowing (TEM or SEM)

Other techniques in your coater:

- **Sputtering metals** like gold, gold/palladium, silver, platinum etc… and additionally to LowVac, HigVac sputtering for materials like Aluminum, Chromium, Iridium, Molybdenum, Titanium Tungsten etc.

- **Conductive carbon films** for X-ray analyses, grids and backing of collodion and formvar films for biological EM via carbon thread /rod evaporation

- **Thermal resistance evaporation of metal and carbon.**

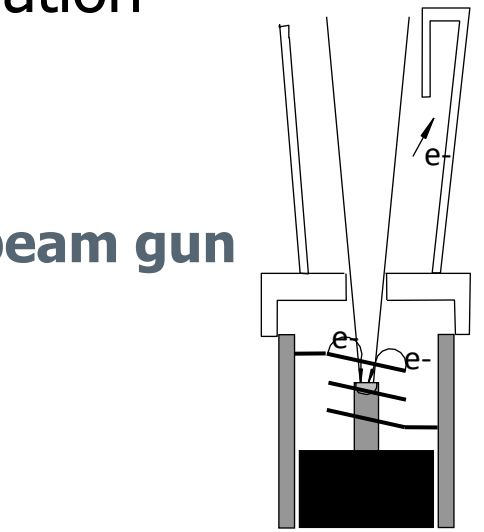
- **e-beam evaporation**. The finest layers, either carbon or metals.

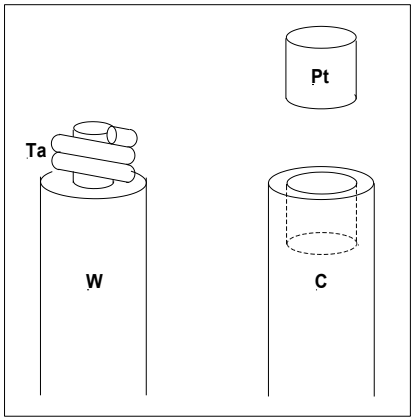
- **Freeze drying, freeze etching, in combination with a VCT100**: Freeze fracturing **double replica**, cryo-transfer.

Metal coating: Electron beam evaporation

Evaporation of metals using the electron beam gun

- For materials with high melting point
- For very fine grained layers (W, Ta/W, Cr, Pt/C)
- Shadowing
- Homogeneous layers with DARS (Double Axis Rotary Shadowing)
- **Low heat transfer to specimen**
- **No charged particles to your sample**





Coating quality depends on:

- Coating technique
- **Layer thickness**
- **Coating material**
- **Vacuum conditions**
- Specimen temperature
- The specimen itself (“decoration effects”)
- Effects after coating

Coating quality - Specimen temperature
WHY CRYO-COATING ???

- Reorganization of the coating material depends on the surface temperature.
- Colder temperature reduces mobility of the coating material causing:
  - Smaller distance between grains
  - Finer structure

Synergies - Cryo SEM workflow

High Pressure Freezing –
Leica EM HPM100, Leica EM PACT2

Superior cryo fixation to observe aqueous biological and industrial samples near to native state.

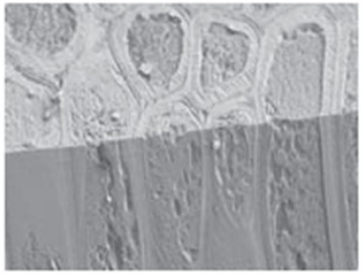
Cryo Ultramicrotomy –
Leica EM UC7 with EM FC7

High quality ultrathin sectioning/planing for light, electron, and atomic force microscopy examination.

Coating –
Leica EM ACE600 cryo outfit

High vacuum coating in conjunction Leica EM VCT100 (vacuum cryo transfer) system for the finest metal and carbon layers.

Imaging and analysis



Mouse kidney (Platinum cryo-sputter coating), Cryo SEM.

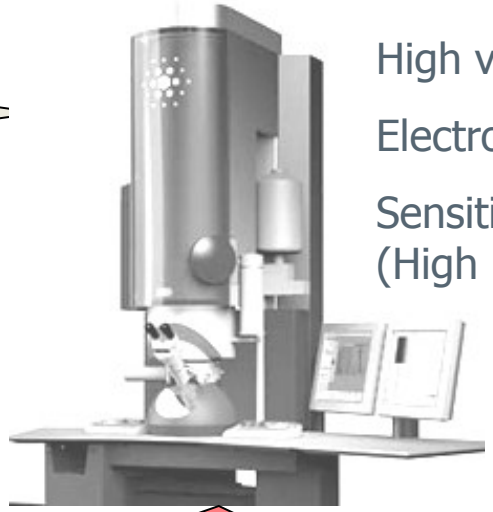
Biology

Soft and "Large"

Aqueous/hydrated

Light elements
(C, O, H, N, S, P etc.)

Electron microscope



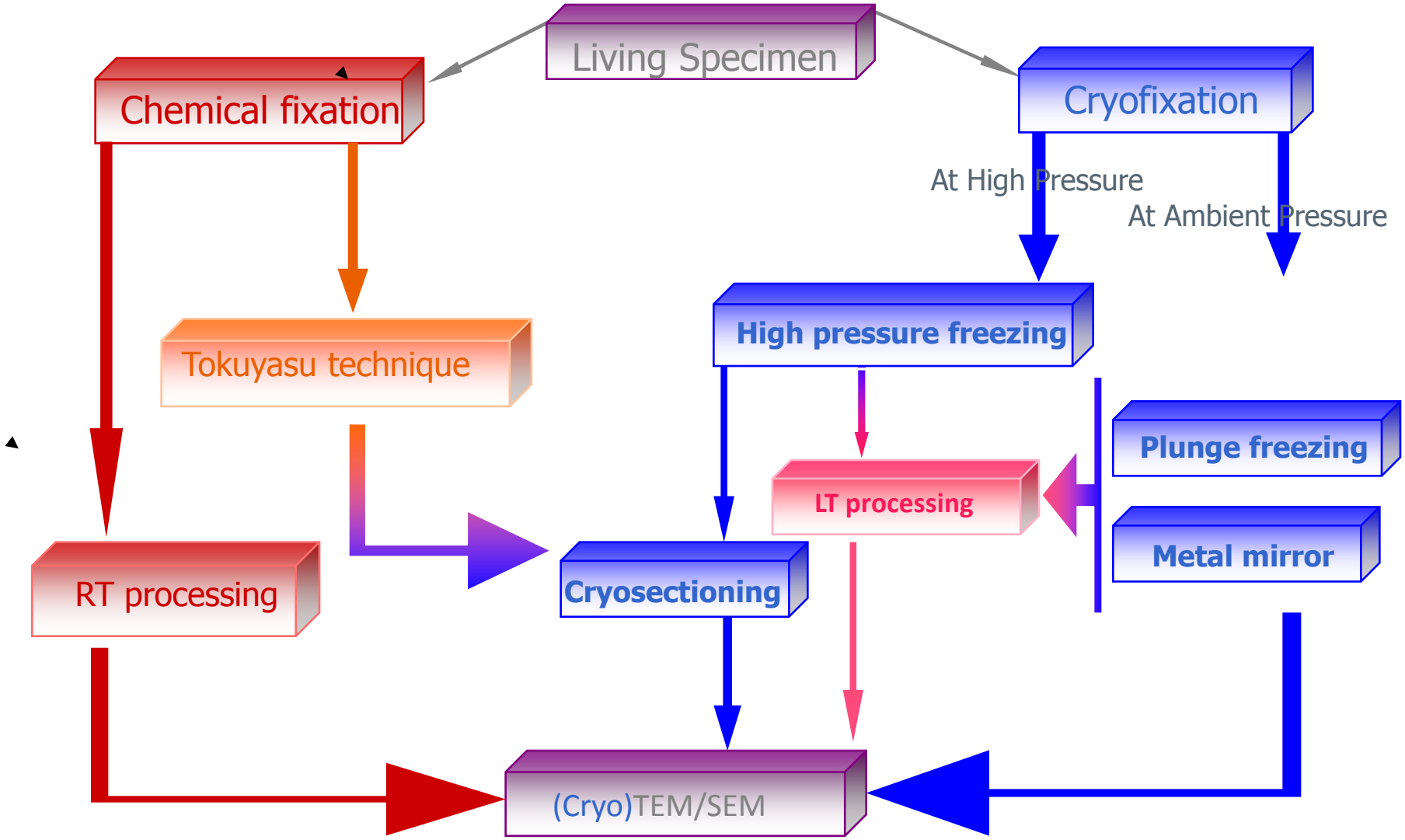
High vacuum

Electron beam

Sensitive to vibration
(High magnifications)

Any treatment induces changes in the specimen

Contrast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Cryosectioning
(Cryo)TEM/SEM

Why cryofixation?

- The cellular constituent is rapidly immobilized
- Cryofixation is a physical fixation of all cellular components simultaneously
- Enzymes and antigens are not denatured (antigenecity)
- Cells retained in their 'native' state
- The physical properties of a frozen sample allow cryo-sectioning without any additional support by embedding medium (CEMOVIS).

Why High Pressure Freezing?

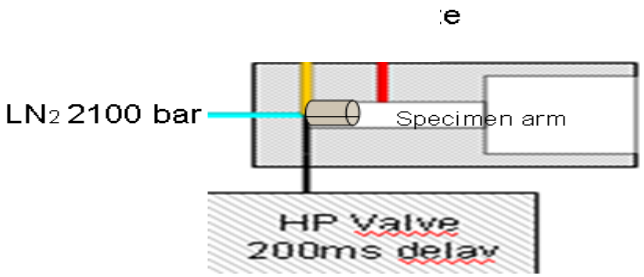
- No changes in the physical equilibrium because of fast and precise correlation between pressure and temperature
- No cryo-protection needed, so no alteration of the cellular processes
- Vitrification of sample thickness up to 200 µm

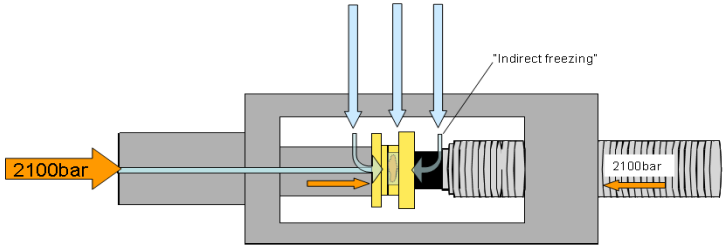
HPM100

High pressure freezing

PACT2

LN_2 10bar





- LN2 at 2100bar used for pressure build-up AND cooling
- Alcohol is synchronizing pressure build-up and cooling
- Pressure is the same everywhere

- Pressure build-up and cooling separated
- LN2 at 10 bar used for cooling only
- Pressure transferred only to specimen (2100bar) by filler

Cryo-fixation
Plunge Freezing and Metal Mirror

In biological research, virology, protein crystallography, pharmaceutical labs, cosmetic companies and paint research.

- **Process Automation**
  - Automatic blotting with optional sensor control
  - Controllable ethane container temperature

Reproducibility with high sample quality

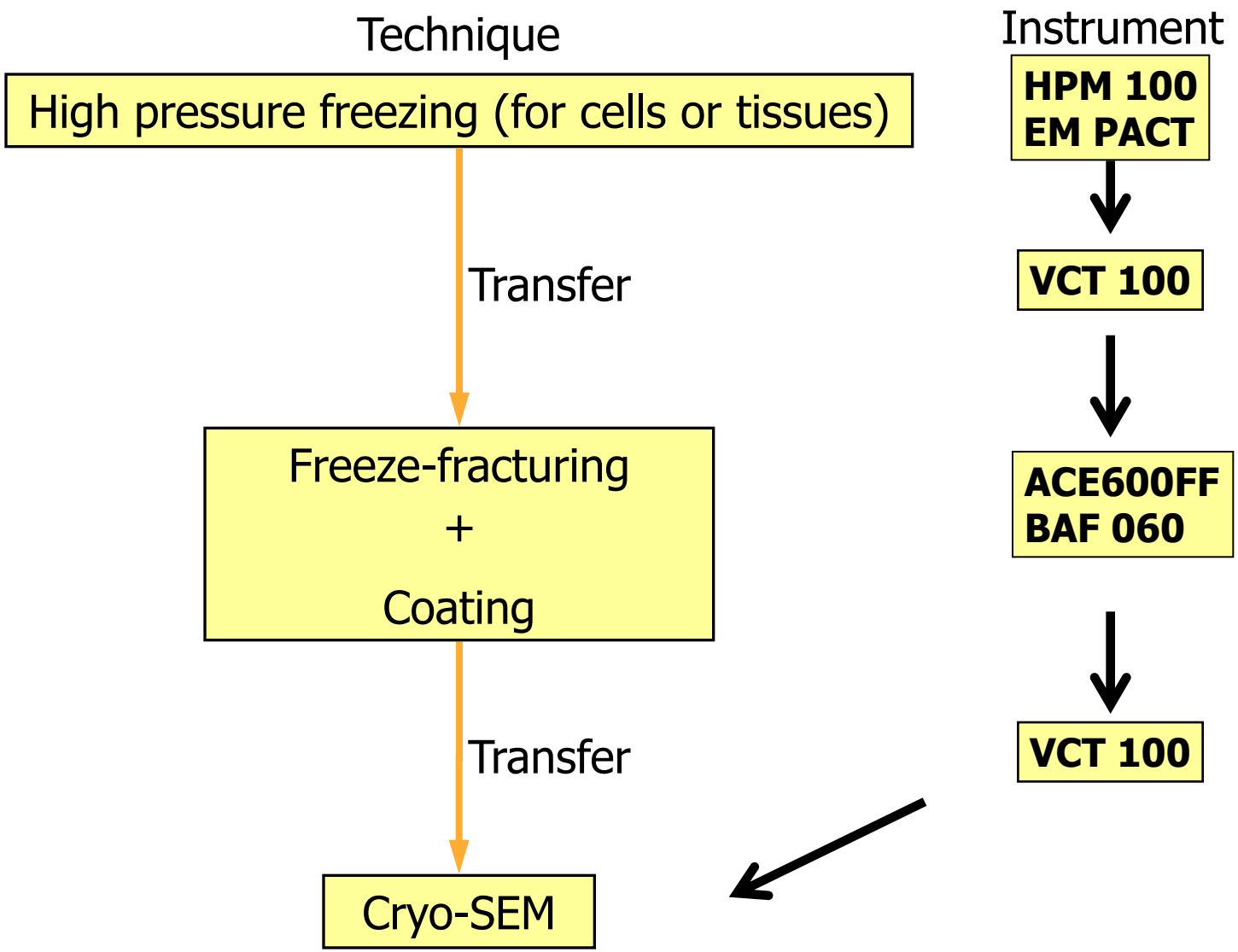
Leica EM FC7

- Three different cryo-condition modes:
  - standard mode
  - high gas-flow (to reduce ice contaminations)
  - wet sectioning (for polymer sectioning at wet condition using DMSO), wide range of different temperature settings of knife / specimen and gas (-40°C to -160°C)

SPECIMEN MOUNTING AND TRANSFER
Why? How could I transfer my sample?

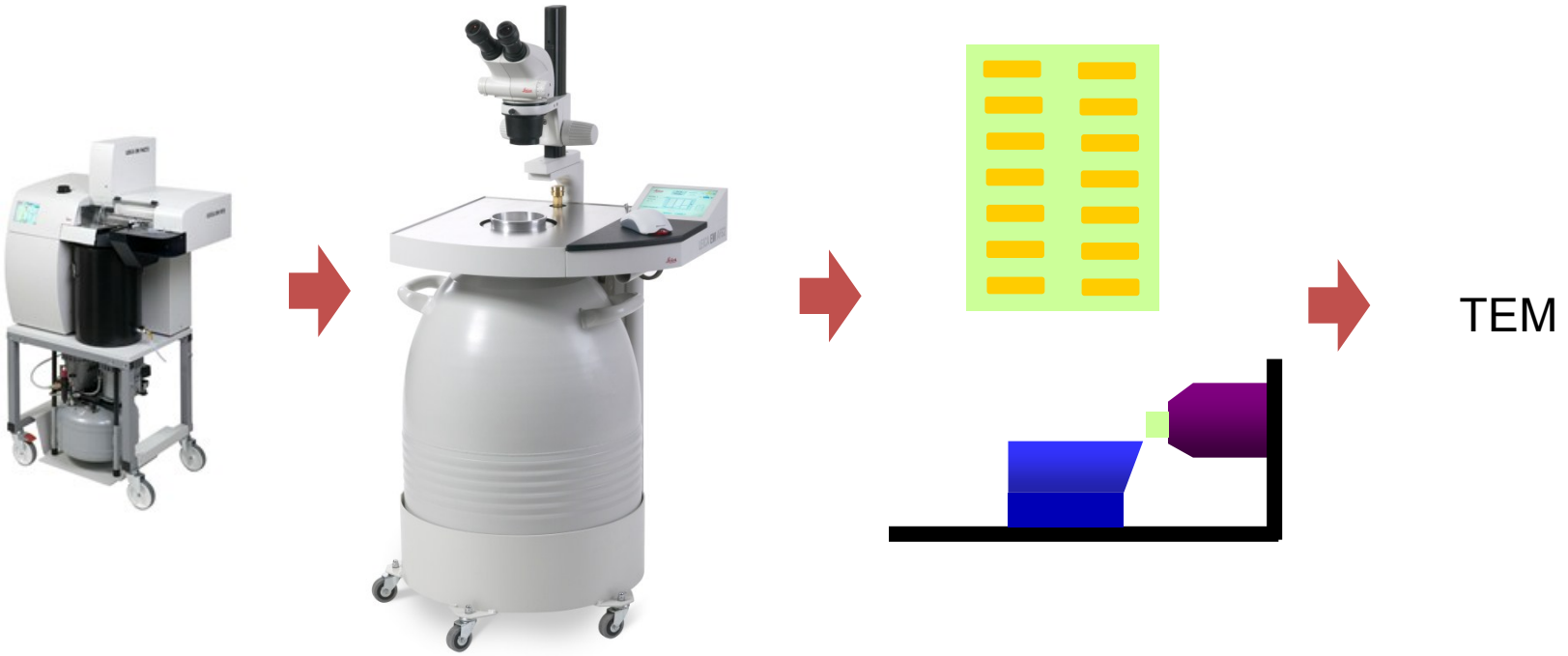
SHUTTLE

LOADING STATION


Technique
Instrument
High pressure freezing (for cells or tissues)
HPM 100
EM PACT
Transfer
VCT 100
Freeze-fracturing
+
Coating
ACE600FF
BAF 060
Transfer
VCT 100
Cryo-SEM

VCT 100 - HIGH VACUUM CRYO TRANSFER TO SEM

Pathway with Freeze Substitution



Dehydration of a cryofixed specimen by exchange of ice with an organic solvent

Freeze substitution is a link between cryofixation and room temperature TEM observation.

Why Freeze Substitution?

- Enables sectioning at room temperature
- Avoids artifacts produced by conventional room temperature procedures
- Preserves **antigenicity**

High Pressure Freezing

(I

Freeze substitution
LT embedding

TEM

Contrasting

(EM AC20)

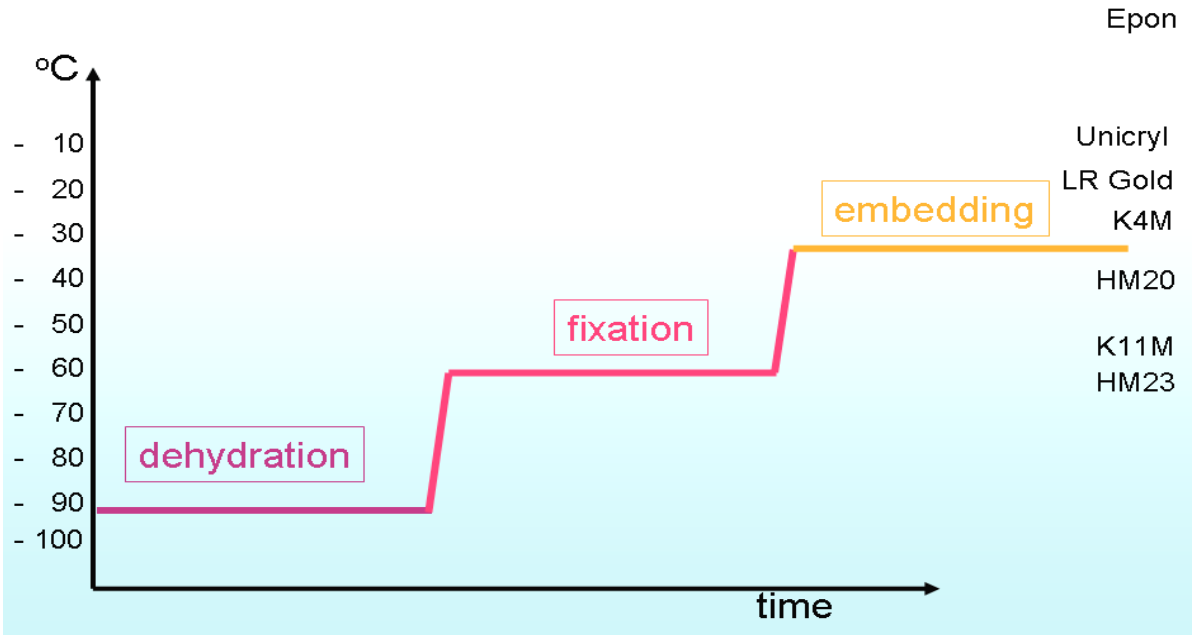
Sectioning

Trimming

(EM TRIM2)

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)

FSP

Freeze **S**ubstitution **P**rocessor
for EM AFS2

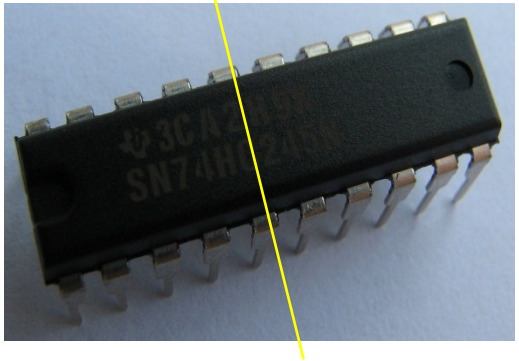
- Automatic reagent handling
- Special embedding moulds for
  - FS of high pressure frozen samples
  - PLT samples

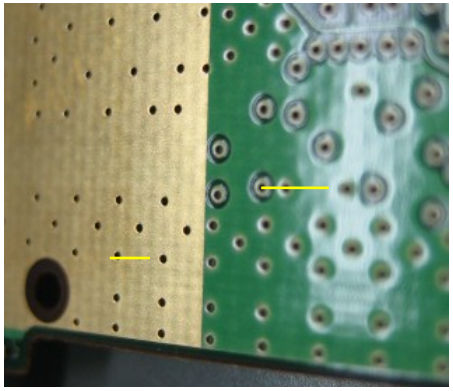
“Load and Leave”

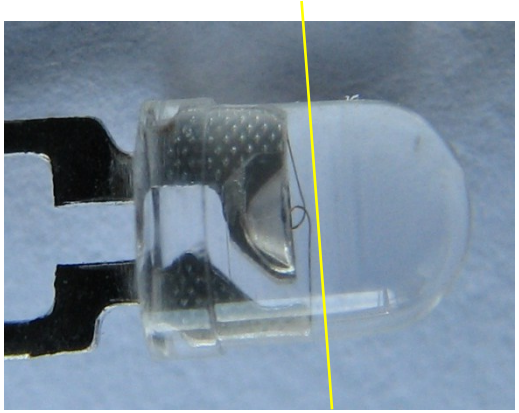
MATERIAL SCIENCES

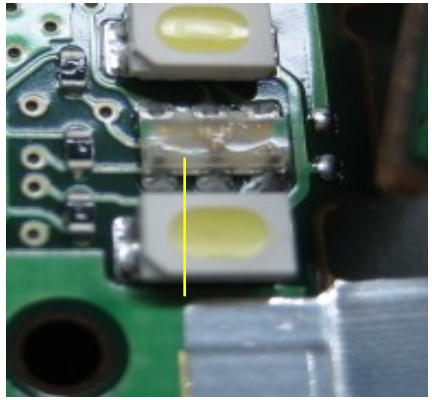
EM TXP

Target Surfacing Device for SEM, LM, TEM









- For site specific cross sections (target preparation)
- Surfacing of small samples
- Pre-preparation prior to ion beam polishing (SEM)
- Pre-preparation prior to ion beam thinning (TEM)
- Pre-preparation prior to ion beam slope cutting (SEM)
- Pre-preparation priot to ultramicrotomy (SEM, TEM) **TRIMMING**

➢ Microelectronic market
➢ Watch industry
➢ Material reseach (Nano technoloy)

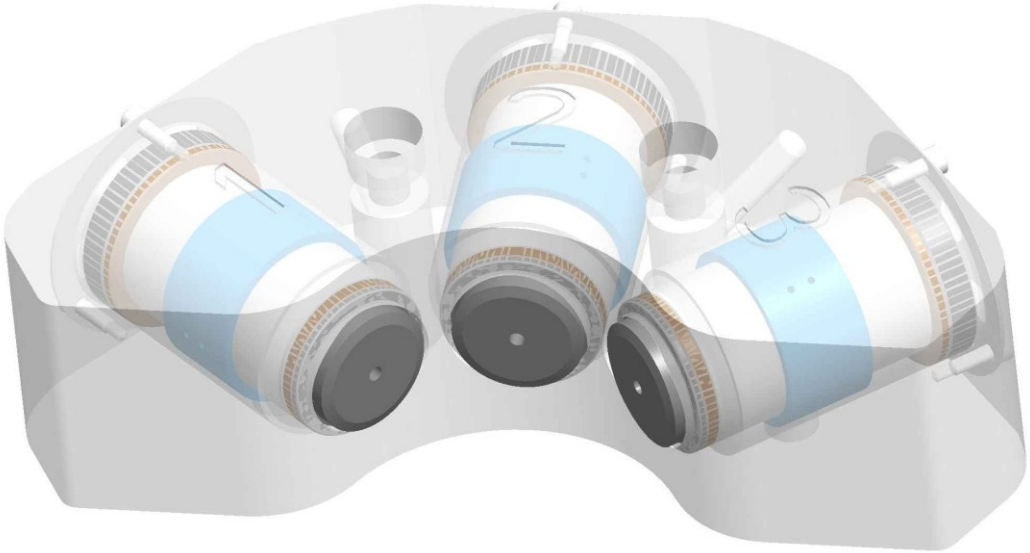


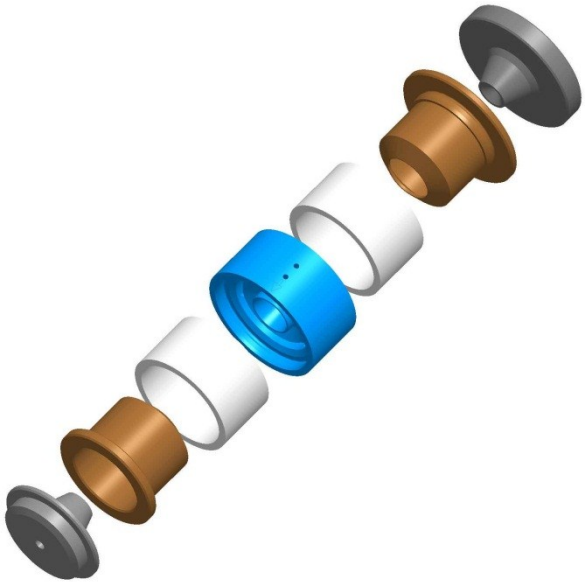

Ion Milling- Slope Cutting FOR SEM

Triple Ion Beam Cutter TIC3X

Used for SEM microstructure analysis
(EDS, WDS, EBSD, CL)
and AFM investigations

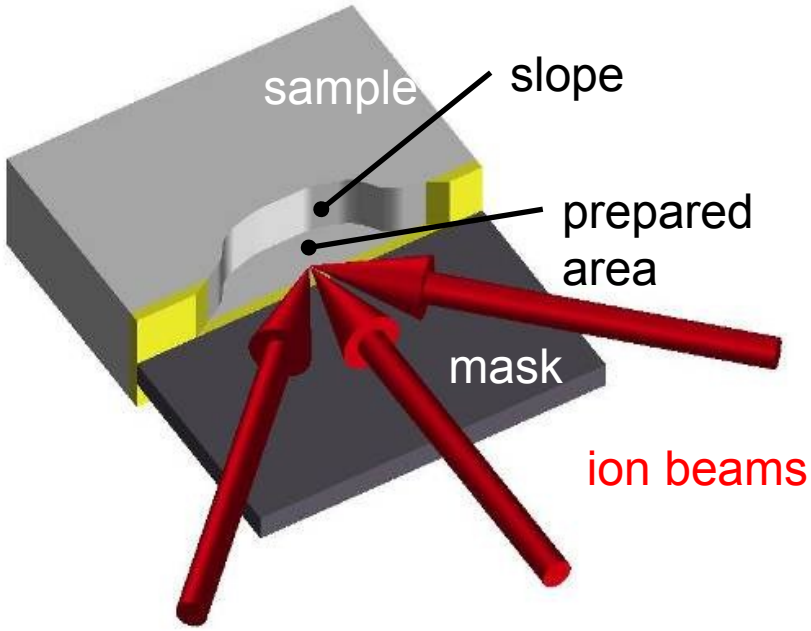
Triple Ion Source





Principle

- Three ion beams hitting the sample from different directions
- Fixed sample

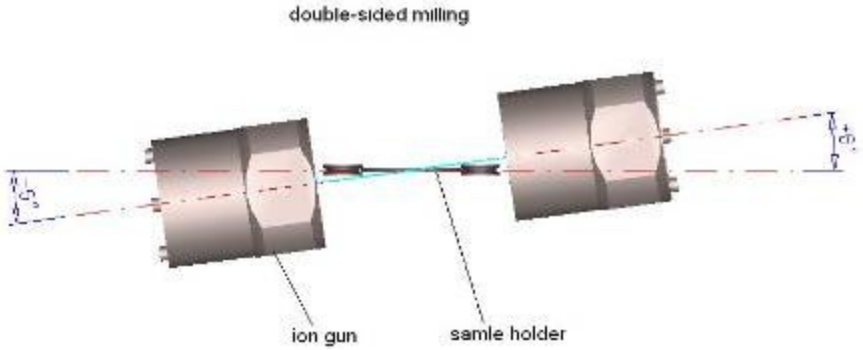


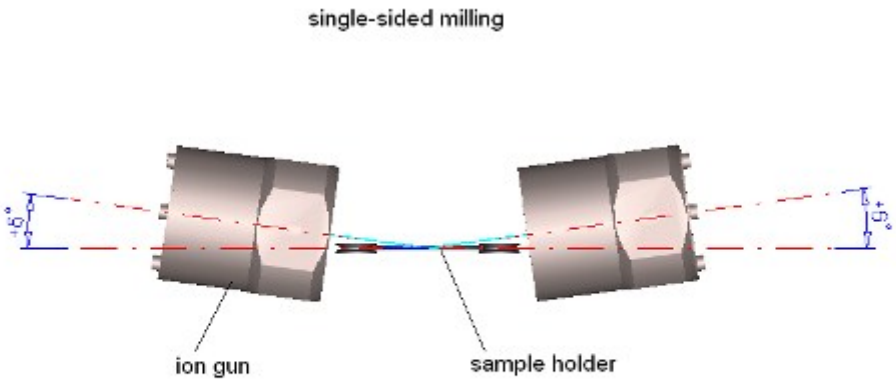
Does not substitute a FIB on SEM, just because the area here is huge.

1x4mm prepared

Ion Milling – Only for TEM

RES 101





Applications of the RES 101

TEM

- Plan view preparation
- Cross-sectional sample preparation
- FIB Cleaning

SEM

- Surface cleaning
- Polishing
- Contrast enhancement
- Ion beam slope cutting (35° and 90°)

LM

Muito obrigado pela vossa atenção!

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