From Eye to Insight

The 1st Cryo Electron Microscopy and 3D Image Processing of Macromolecular Assemblies and Cellular Tomography (CEM3DIP) 2016

EM CRYO PREPARATION WORKFLOWS
Update on the Latest Instrumentation
Why Cryo?

- Low Contrast in EM
- Critical Temperature -140°C
- Sensitive to Electron Beam
- Contamination (ice)

+ Low Artefact Formation During Fixation
+ Most Native Fixation and Analysis Method
+ Fastest Fixation
+ Frozen Hydrated Samples

Toxoplasma gondii
The “No-Ice Challenge”

Connectivity and Cryo-Preparation Workflows

1. Cryo-CLEM
2. Cryo Fixation by High Pressure Freezing
3. Freeze Fracture, Freeze Etching and Coating
4. Cryo-Transfer
5. Cryo Ultramicrotomy

Cryo Fixation by High Pressure Freezing
Cryo Transfer
Cryo-Transfer
Cryo EM
Cryo Fixation by High Pressure Freezing
Cryo-Transfer
Cryo-Transfer
Correlative Light and Electron Microscopy (CLEM) combines fluorescence light microscopy (FLM) and electron microscopy (EM) imaging of the same sample.

A method which allows FLM rapid screening of large areas and fast determination of regions of interest in EM. This reduces the user interaction time on the EM significantly.

Provides deeper understanding of analyzed sample by overlaying complementary information such as high resolution LM localization and EM ultrastructural context data.
Without FLM prior the EM the user might have to check all grid squares to find adequate target structures.

With FLM prior the EM the user knows which grid squares contains promising target structures.
Cryo CLEM System

LN2-Pump Controller

Microscope Controller

Leica DM6 FS Microscope

PC-System + LAS Widefield Software

STP6000 SmartTouch Panel

5l Dewar

EL6000 external light source

cryo Stage

Manual Stage

DFC Cameras (DFC365FX)

3x Mag. Changer

50x Cryo Obj.

cryo Transfer System
Cryo CLEM System

Plunge frozen or HPF/UM sample

Cryo transfer system with cartridge loading station

Cryotransfer system docked to cryo stage

Cartridge loading station with empty cartridge

Cartridge with 3mm grid

Leica cryo stage with cover and cryo CLEM objective.
Temp. range –195 °C to +60 °C
Cryo CLEM System - Objective

Leica HCX PL APO 50x / 0,90 CLEM objective

- Apochromatically corrected
- Thermally decoupled lens
- NA 0.9
- Low working distance of 0.28 mm
- Localization of ~50nm
Workflow Solution

Leica LAS X Widefield Images used for correlation of LM marked structures in EM

High Pressure Freezing, Cryo-Ultramicrotomy

or

Grid Plunging

Cryo-Transfer System

Leica Cryo Light Microscopy

Cryo CLEM

Image Analysis

Cryo-TEM

Transfer to cryoTEM
A NEW ERA IN HIGH PRESSURE FREEZING
A High Pressure Freezer arrests aqueous samples in their native state to deliver the best possible sample preservation.
Leica EM ICE is a platform for game-changing discoveries.
LEICA EM ICE LIGHT STIMULATION
New possibilities for researchers in life science and industry
WHY LIGHT STIMULATION?

Utilizing light to understand the complex process of neurotransmission
The synchronization of light stimulation and high pressure freezing allows the visualization of highly dynamic process at a nanometer resolution and millisecond precision.

Nature. 2013 Dec 12; 504(7479):242-7
Variety of Light Spectra

- Five modules, five wavelengths automatically recognized by the instrument software

Program Your Experiment

- Precise correlation between the light impulse and the time of freezing
- One minute recovery time between freezing cycles
- One second from fresh-to-frozen
- Only 20 minutes cool-down time
- Only 30 L daily consumption of LN2
FOCUS ON YOUR SAMPLE
One move, fully automated loading

Closing the cover triggers the perfect alignment of cartridge assembly followed by the freezing process
SAMPLE STORAGE DEWAR

- Three separate positions
- Nine consecutive freezing cycles
- Programmed rotation
Apply light stimulation to any light sensitive compounds

Sunscreen lotion frozen after millisecond UV light stimulation.
3. Cryo-preparation – Freeze Fracture

- Cryo Fixation
- High Pressure Freezing
- Cryo-Transfer
- Cryo SEM
- Freeze Fracture, Freeze Etching and Coating
A REVOLUTION IN FREEZE FRACTURE
READY TO OPERATE

- Load locks for sample, microtome, e-beams
- New cooling, shielding, microtome, e-beams
- High vacuum 10-8mbar
- Connection to VCT500
PRECISE AND CLEAN CUTTING
Exchangeable and 3 axis movable knife
FREEZE FRACTURE

Freeze Fracture breaks frozen specimens to reveal internal structures

Euglina gracilis, courtesy of Andres Käch, Center for Microscopy and Image Analysis, University of Zurich, Switzerland
FREEZE ETCHING

Freeze Etching is the sublimation of surface ice under vacuum to reveal details of the fractured face that were originally hidden.

*Giardia lamblia,* courtesy of Andres Käch, Center for Microscopy and Image Analysis, University of Zurich, Switzerland
FREEZE FRACTURE & REPLICAS

Frozen hydrated specimen

Carbon
Platinum

Replica

cryo-SEM
Backscattered signal

TEM
Amplitude contrast
THE FUTURE IS CONNECTIVITY

Cryo Fixation
High Pressure Freezing

Cryo-Transfer

Cryo SEM

Cryo-Transfer

Freeze Fracture, Freeze Etching and Coating
WHY VACUUM CRYO TRANSFER?

Contamination-free sample transfer between preparation and analysis systems.

*Euglena gracilis, courtesy of Andres Käch, Center for Microscopy and Image Analysis, University of Zürich, Switzerland*
- All transfer under vacuum
- Contamination-free sample handling
- Improved connectivity

CONNECT WITH LEICA EM VCM
FLEXIBILITY TO CONNECT

- Glovebox
- SEM
- FIB
- Analysis systems, e.g. XPS
- Atomic probe
- Synchrotron
- ....
Connectivity for cryo-preparation

Cryo Fixation, e.g. High Pressure Freezing

Cryo-Transfer

Cryo-CLEM

Freeze Fracture, Freeze Etching and Coating

Cryo Ultramicrotomy

Cryo-Transfer

Cryo Ion Milling
5. Broad Ion Beam Milling Workflow

Sample preparation under controlled environmental conditions
Ion beam slope cutting with Leica EM TIC 3X

- Three ion beams hitting the sample from different directions (reduction of curtaining)
- Fixed sample (better heat transfer)

Features
- Cutting depth >1000µm
- Cutting width > 4000µm
- Cutting speed >150µm/h
Leica EM TIC 3X “Triple ion beam” slope cutting
What About Temperature, or Environmentally Sensitive Samples?
Heat-sensitive polymer fibres with water-soluble portion

Without cooling (~80°C)  With cooling stage (~120°C)
Comparison

Heat-sensitive polymer fibres with water-soluble portion

UC7 with FC7 -140° C

TIC 3X -120° C
Cooling stage result
Environmentally controlled workflow

Sample Freezing

Cryo-loading station with cryo-saw
LN2 sample pre-preparation

EM TIC 3X with cryostage & VCT docking station
(cryo-) BIB milling

(Cryo-)
SEM
FIB

VCT 100 shuttle
(cryo-) vacuum transfer

EM ACE 600 with VCT docking station
(cryo-) coating
Cryo-saw and loading station

Sample size:

- Maximum 10x7x4(thick)mm
- Flat sides (saw-finish)

- Mechanical preparation under LN2 conditions
Preparation Process

- Freeze
- Transfer to cryo-saw preparation station filled with LN2
- Inserted in the VCT holder and pre-prepared with cryo-saw

VCT holder
(sample size max. 10x7x4mm)
Leica EM VCT500 connectivity

Cryogenic sample transfer to and from the broad ion beam etching system, TIC 3X and to (cryo)SEM

- After shaping to size, sample is placed in loading position
- VCT shuttle is attached and the Dewar filled with LN2
- Sample taken on board VCT shuttle, transferred and attached to the EM TIC 3X docking port
- Sample moved into position for ion beam slope cutting under cryo-conditions.
New environmentally controlled workflow

Sample Freezing

Cryo-loading station with cryo-saw
LN2 sample pre-preparation

EM TIC 3X with cryostage & VCT docking station (cryo-) BIB milling

CryoSEM/FIB

VCT 100 shuttle (cryo-) vacuum transfer

EM ACE 600 with VCT docking station (cryo-) coating
Applications

Asparagus; plunged in LN2, pre-prepared with the Cryo-Saw-Loading station, Ion beam cross sectioned with the TIC 3X – VCT at -120°C and 6kV; transferred with the VCT100 and investigated in a “cryo-LM” 20x objective.
Applications

Asparagus; Cryo-TIC preparation; “Cryo-LM”

Asparagus; Cryo-TIC preparation; LM after the sample warmed up
Applications

_Taxus_ sp., plunged in LN2, pre-prepared with the Cryo-Saw-Loading station, ion beam cross sectioned with TIC 3X/VCT at -120C and 6kV; transferred with the VCT100 and investigated in a cryo-FIB SEM

Image courtesy of Harvard University
Connectivity for cryo-preparation

Cryo Fixation, e.g. High Pressure Freezing

Cryo Transfer

Cryo-CLEM

Freeze Fracture, Freeze Etching and Coating

Cryo Ultramicrotomy

Cryo Ion Milling

Cryo Transfer
Future Developments
HPF with Electrical Stimulation
The Concept

- Middle plate includes a circuit board with capacitors to store the electrical energy
The Concept

- Blue LED module used as the trigger for releasing the power and having e-current delivered to the sample
- Software automatically recognising the ES mode. All settings saved in the log-file
Proof of Principle/Functional Model

Initial Sample Test

Successful freezing with the ES set of cartridges in the instrument

Initial Sample Test

Successful stimulation with the pre-ES set outside the instrument
Future Developments

HPF for protein crystallography
Leica Protein Crystallography Solution

Drug discovery development

- Screening for a suitable cryoprotectant
- Disintegration and loss of crystals
- Time consuming

- Standardization
- Reliability
- High throughput

Leica CRYO Stage for CLEM Workflow Solution - TG2 for X-ray diffraction measurements (in-house equipment or a synchrotron)
Thank You