"Beauty and benefit of Cryo-LM & Cryo-EM: our achievements with method development"

Disclosure:
Co-founder of CryoSol-World - licensed some of our discoveries
Jacques Dubochet, Joachim Frank and Richard Henderson win the 2017 Nobel prize in Chemistry for developing cryo-electron microscopy

First cryoEM-holder 1980 in Maastricht

Alasdair McDowall

Thermo Fisher Scientific, Eindhoven; Krios wins the 2018 Gold Edison Award

Werner Hax Knighted by the Dutch King 2019

Dominique Hubert, Raymond Wagner, Ben Bormans, Uwe Luecken, Raymond Schrijver, Marc Storms, Rob Fastenau and many others at FEI
Specimens prepared in an uncontrolled laboratory atmosphere are subject to evaporation and heat transfer, which may introduce artifacts caused by concentration, pH, ionic strength, and temperature changes.
Pico-liter volumes: prevent evaporation!
Climate chamber not sufficient → dew point control
The pioneers of the VitroJet
Raimond Ravelli, Rene Henderikx, Frank Nijpels, Sanne Thewissem, Pascal Huysmans, Bart Beulen, Carmen Lopez-Eglezias & Peter J. Peters

Interested in working with us?  
[Email Address]  
Maastricht MultiModal Molecular Imaging Institute
A. Blotting

B. Droplet-based

C. Scribing
Automated Handling
Integrated Glow Discharger
Sample Deposition
Dewpoint Control

-1.2°C

80nm
Pin Print Model
Jet Vitrification
Cryo-EM
Cryo-EM structures from sub-nl volumes using pin-printing and jet vitrification


The increasing demand for cryo-electron microscopy (cryo-EM) reveals drawbacks in current sample preparation protocols, such as sample waste and lack of reproducibility. Here, we present several technical developments that provide efficient sample preparation for cryo-EM studies. Pin printing substantially reduces sample waste by depositing only a sub-nanoliter volume of sample on the carrier surface. Sample evaporation is mitigated by dewpoint control feedback loops. The deposited sample is vitrified by jets of cryogen followed by submersion into a cryogen bath. Because the cryogen jets cool the sample from the center, pre-mounted autogrids can be used and loaded directly into automated cryo-EMs. We integrated these steps into a single device, named VitroJet. The device’s performance was validated by resolving four standard proteins (apo-ferritin, GroEL, worm hemoglobin, beta-galactosidase) to ~3 Å resolution using a 200-kV electron microscope. The VitroJet offers a promising solution for improved automated sample preparation in cryo-EM studies.
Benefits

- Minimize operator variability
- Prevent grid damage
- Reduce contamination
- Decrease sample usage
- Visual quality inspection
Founded March 2018
A leading developer of sample preparation technology for cryo-EM From ‘high content’-technology to ‘high throughput’-solution
Demcon invests in CryoSol technology

Erik-Jan de Hoon

Dennis Schipper
Director
### Timeline

<table>
<thead>
<tr>
<th>Year</th>
<th>2015</th>
<th>2016</th>
<th>2017</th>
<th>2018</th>
<th>2019</th>
<th>2020</th>
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<td>Q4</td>
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<td>Q3</td>
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</table>

**Module testing**

**Prototype**

**Functional Model**

**Comm. Concept**

**Alpha**

**Beta**

**Production**
Calibration Velocity

Jet velocity vs pressure
LIFE IN THE CELL

How to study it?
Producing sections
- Mastering cutting artefacts (cont.)
- Understanding knife marks: test of the cut/fracture hypothesis
- Test of Al-Anowid model
- Test of deformation equation: effect of knife angle; control of friction
- Understanding gliding on knife surface
- Glass knives, knives by other producers
- Oscillating knife
- Floating on a liquid surface, NH₃, alcohol
- Computer simulation of the cutting process cf:
  http://www.rei.rutgers.edu/~oze/phaseash01.htm

Imaging and CET
- Optimal imaging condition (defocus, coherence)
- Evaluate the advantage of energy loss filter
- Evaluate cryo-protection at very low temperature
- Reducing drift (attachment to the support; coating of the section)
- Edicial marks
- Test specimen: TPP II (6.6MD); T4; protein and DNA crystal

Technical developments
- Vitrification
  - Improved biopsy for HP freezer
- Cutting
  - Improved knife
  - Oscillating knife
  - Micro-manipulator in the microtome
  - Determination of the hardness in the cm
  - AFM in the microtome
  - Biopsy device

Serving and CET
- Laser software (Test of COMET)
Vitreous cryo-sections: cryo-electron tomography, subvolume averaging & template matching

P. J. Peters
The Cryo Tomography Workflow: Enabling *In Situ* Structural Biology

1. **Vitrification**
   - Cell culture
   - Fluorescence imaging
   - Identification

2. **Cryo-correlative microscopy**
   - Cryo-correlative microscopy

3. **Cryo-focused ion beam milling**
   - Ion beam
   - Thinning

4. **Cryo-electron tomography**
   - Electron beam
   - 3D acquisition

5. **Structural analysis**
Sample Preparation

Cell → Carbon foil → Electron microscopy grid (~20 nm) → Vitrified cells on grid (gold mesh, Ø 3 mm) → FEI Autogrid → Cryo-LM

~2-5 μm, ~100 μm
“The lamella exhibits transparent amorphous ice at the edges of the cell.

However, the center of the cell clearly undergoes incomplete 
vitrification due to the low heat transfer capacity of biological material.

We thus avoided data acquisition deep inside the nuclear volume
and choose to target areas at the front of the lamella for cryo-ET data collection.”
Greatly improved Cell Vitrification

- Healthy cells – prevent drying before freezing.
- Vitreous ice after freezing.
- No ice on back of the grid.
- No cracks.
- Cells in the center area of the grid and in the center of the grid square.
- Marks for lamella orientation.
FIB-lamellae fabrication
intracellular infection of primary immune cells with *Yersinia enterocolitica*

Rapid freezing + focused ion-beam lamella preparation

Cryo-electron tomography of intracellular bacteria in cryo-FIB lamellae

Injectisomes contact and deform host membranes

Subtomogram averaging structure of entire injectisome
AutoGrid jet vitrification

- Time of flight: from application to vitrification
- Ramp down to jet position
- Timing of the jet valve
- Length of the jet
- Temperature of the jet
- Synchrony of the jets
- Position of the grid between the jet
- Diameter of the jet
- Velocity of the jet
- Graphene grids work well

Movie: 17,000 frames / second
Cooling rate
Vitrobot plunge 40.000 Kelvin/sec normal grids
Ethane jet 3.150.000 Kelvin/sec with AutoGrids
How can nanobiology contribute to have a happy, healthy sexual life | Peter Peters | TEDxMaastricht
M4I Nanoscopy:
Raimond Ravelli
Carmen López Iglesias
Kevin Knoops
Navya Premaraj
Casper Berger
Ye Gao
Axel Siroy
Hans Duimel
Abril Gijsbers
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Interested in working with us?
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Disclosure: CryoSol has licensed some of our discoveries.
<table>
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<tr>
<th>Blotting</th>
<th>Deposition Technique</th>
<th>Licence</th>
<th>Droplet Based</th>
<th>Scribing Based</th>
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</thead>
<tbody>
<tr>
<td>Pipetting</td>
<td>Manual plunger, Vitrobot, EM GP, Cryoplunge 3</td>
<td>Back-It-Up, Shake-it-off</td>
<td>De Marco group, TED (Muench group), Frank group, Trinick group, Spotron, Chameleon</td>
<td>Vitrotek</td>
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<tr>
<td>Ultrasonic Spray</td>
<td>High frequency droplet generation with through-grid wicking</td>
<td>High frequency droplet generation with self-wicking grids</td>
<td>Surface acoustic waves through microfluidic device</td>
<td>Gas sheath around nozzle using optional high voltage to steer droplets</td>
</tr>
<tr>
<td>Gas Pressurized Spray</td>
<td>High potential difference between nozzle and grid</td>
<td>High potential difference between nozzle and grid</td>
<td>Droplets formed by piezoelectrically dispensed onto self-wicking grids</td>
<td>Dip pen deposition while maintaining dewpoint</td>
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<tr>
<td>Electrostatic Spray</td>
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<td>Capillary deposition with controlled evaporation or re-aspiration</td>
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<tr>
<td>Inkjet</td>
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<tr>
<td>Pin Printing</td>
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<tr>
<td>Capillary writing</td>
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</table>

<table>
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<tr>
<th>Devices</th>
<th>Methodology</th>
<th>Sample carrier compatibility</th>
<th>Stock volume</th>
<th>Volume per grid</th>
<th>Dewpoint control</th>
<th>Layer inspection</th>
<th>Time deposition to vitrification</th>
<th>Grid coverage</th>
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</thead>
<tbody>
<tr>
<td>Wicking of liquid through filter paper(s)</td>
<td>High frequency droplet generation with through-grid wicking</td>
<td>All</td>
<td>0.5 - 1 µL</td>
<td>3 - 5 µL</td>
<td>No</td>
<td>No</td>
<td>± 130 ms</td>
<td>100%</td>
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<tr>
<td>High frequency droplet generation with self-wicking grids</td>
<td>High frequency droplet generation with self-wicking grids</td>
<td>All</td>
<td>0.5 - 1 µL</td>
<td>3 - 5 µL</td>
<td>No</td>
<td>No</td>
<td>&lt; 100 ms</td>
<td>25 - 35%</td>
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<tr>
<td>Surface acoustic waves through microfluidic device</td>
<td>Surface acoustic waves through microfluidic device</td>
<td>All</td>
<td>0.05-5 µL</td>
<td>1.5 - 2 mL</td>
<td>No</td>
<td>No</td>
<td>10 - 1000 ms</td>
<td>5 - 10%</td>
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<tr>
<td>Gas sheath around nozzle using optional high voltage to steer droplets</td>
<td>Gas sheath around nozzle using optional high voltage to steer droplets</td>
<td>All</td>
<td>33 µL</td>
<td>4 mL</td>
<td>No</td>
<td>No</td>
<td>2 - 200 ms</td>
<td>1 - 5%</td>
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<td>Gas sheath around nozzle</td>
<td>Gas sheath around nozzle</td>
<td>All</td>
<td>&gt; 30 µL</td>
<td>9 µL</td>
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<td>No</td>
<td>10 - 1000 ms</td>
<td>5 - 10%</td>
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<td>High potential difference between nozzle and grid</td>
<td>High potential difference between nozzle and grid</td>
<td>All</td>
<td>5 - 10 µL</td>
<td>1 - 2 mL</td>
<td>No</td>
<td>No</td>
<td>&gt; 1 s</td>
<td>5 - 10%</td>
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<tr>
<td>Droplets formed by piezoelectrically dispensed onto self-wicking grids</td>
<td>Droplets formed by piezoelectrically dispensed onto self-wicking grids</td>
<td>All</td>
<td>3 - 5 µL</td>
<td>2 - 16 mL</td>
<td>No</td>
<td>No</td>
<td>50 - 2500 ms</td>
<td>10 - 15%</td>
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<td>Dip pen deposition while maintaining dewpoint</td>
<td>Dip pen deposition while maintaining dewpoint</td>
<td>All</td>
<td>0.5 µL</td>
<td>1 mL</td>
<td>Yes</td>
<td>Yes</td>
<td>1 - 5 s</td>
<td>15 - 25%</td>
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<td>Capillary deposition with controlled evaporation or re-aspiration</td>
<td>Capillary deposition with controlled evaporation or re-aspiration</td>
<td>All</td>
<td>15 - 25 nL</td>
<td>0.1 nL</td>
<td>Yes</td>
<td>Yes</td>
<td>1 - 3 s</td>
<td>10 - 20%</td>
</tr>
</tbody>
</table>
Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein.

Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D.
PMID: 32155444 Free PMC Article

Structure of mouse coronavirus spike protein complexed with receptor reveals mechanism for viral entry.

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Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2.

Yan R, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q.
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Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation.

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Stabilized coronavirus spikes are resistant to conformational changes induced by receptor recognition or proteolysis.

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Cryo-EM structure of the SARS coronavirus spike glycoprotein in complex with its host cell receptor ACE2.

Song W, Gui M, Wang X, Xiang Y.
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Cryo-EM structures of MERS-CoV and SARS-CoV spike glycoproteins reveal the dynamic receptor binding domains.

Nat Commun. 2017 Apr 10;8:15092. doi: 10.1038/ncomms15092.
PMID: 28303837 Free PMC Article

Cryo-electron microscopy structures of the SARS-CoV spike glycoprotein reveal a prerequisite conformational state for receptor binding.

PMID: 28008926 Free PMC Article

Pre-fusion structure of a human coronavirus spike protein.

PMID: 26935699 Free PMC Article

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Hans Clevers Lab

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Wim Voorhout
Disclosure: Shareholder of CryoSol
2e alternatief voor capillair vulling