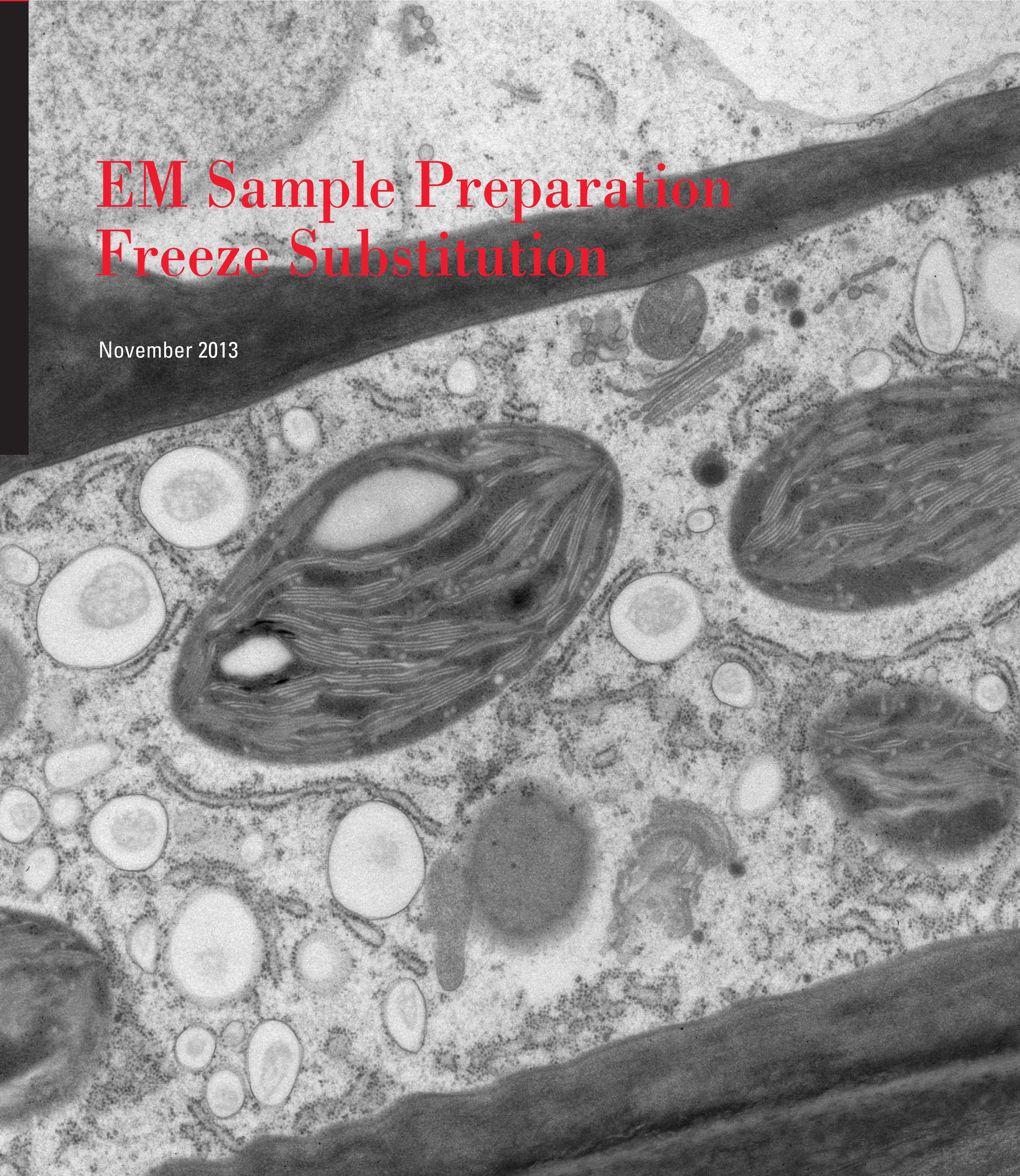
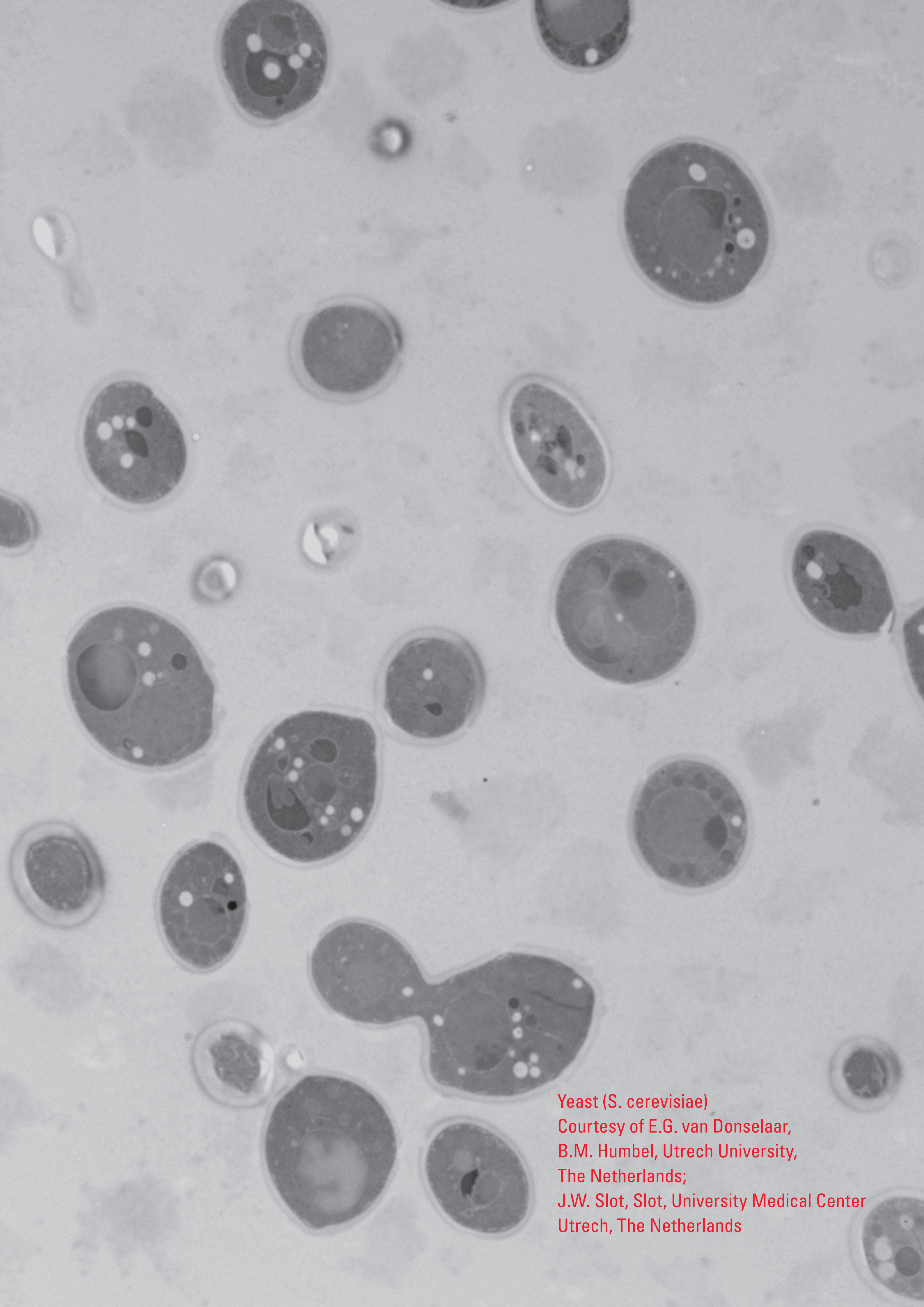
Living up to Life

Leica
MICROSYSTEMS



EM Sample Preparation
Freeze Substitution

November 2013



Yeast (S. cerevisiae)
Courtesy of E.G. van Donselaar,
B.M. Humbel, Utrech University,
The Netherlands;
J.W. Slot, Slot, University Medical Center
Utrech, The Netherlands

Freeze Substitution

Freeze-substitution is a process of dehydration, performed at temperatures low enough to avoid the formation of ice crystals and to circumvent the damaging effects observed after ambient-temperature dehydration. During freeze substitution the "frozen" water is dissolved by an organic solvent, which usually also contains chemical fixatives (Steinbrecht and Müller 1987). Freeze-substitution links instant physical immobilization of the cell constituents (cryo-fixation) and resin embedding. Once substitution is complete, samples are gradually warmed-up and processed further as for conventionally prepared samples. Successful cryo-fixation followed by FS shows superior preservation of fine structure compared to chemical fixation techniques (Müller 1992). Aggregation of macromolecules in organic solvents and changes of the hydration shell surrounding the biological molecules can occur even at very low temperatures, but it is reasonable to assume that FS at temperatures below a specific threshold preserves the hydration shell (Hobot et al. 1985; Kellenberger 1991).
This technique also gives the possibility of examining thick (200–300 nm sections) samples by ET, so that relatively large cellular volumes can be studied in 3D. This approach is very beneficial for an understanding of the complex relation between different cellular organelles and randomly occurring events.

Hobot JA, Villiger W, Escaig J, Maeder M, Ryter A, Kellenberger E (1985) The shape and fine structure of the nucleoid observed on sections of ultra rapid frozen and cryosubstituted bacteria. J Bacteriol 162:960–971

Kellenberger E (1991) The potential of cryofixation and freeze substitution: observations and theoretical considerations. J Microsc 163:183–203

Müller M (1992) The integrating power of cryofixation-based electron microscopy in biology. Acta Microsc 1:37–44

Steinbrecht RA, Müller M (1987) Freeze-substitution and freeze-drying. Cryotechniques in biological electron microscopy. Springer, Berlin, pp 149–172

Electron Microscopy of high pressure frozen and freeze substituted Arabidopsis thaliana root tips cells

COURTESY

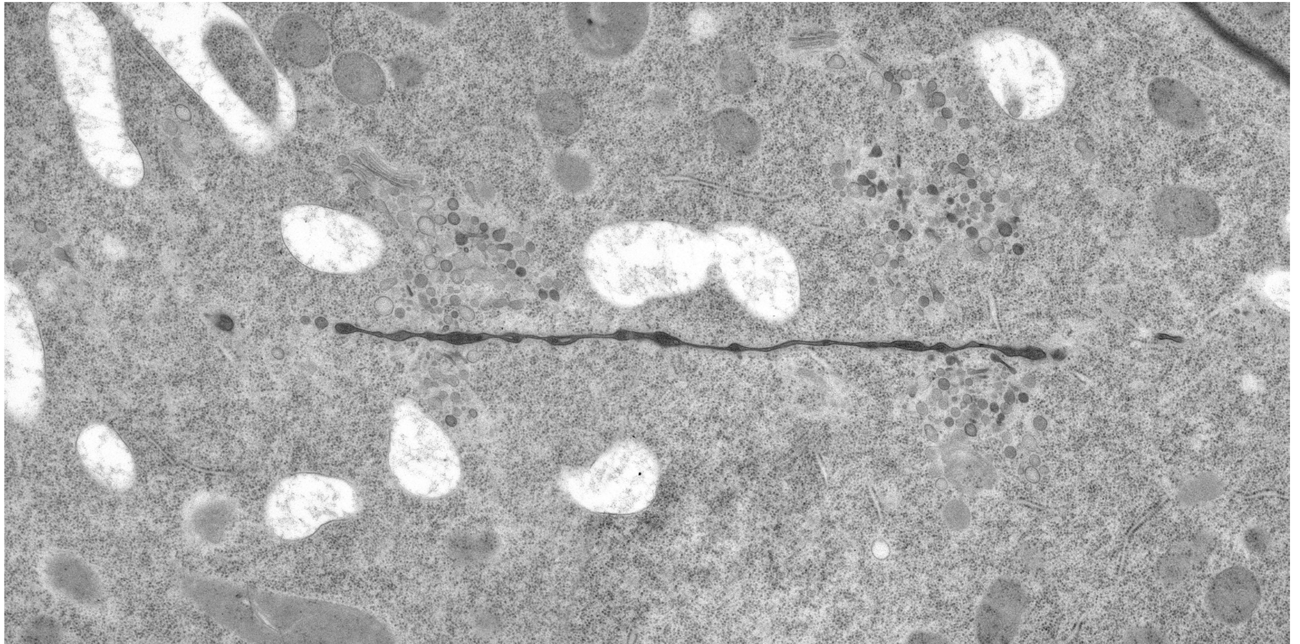
Riet De Rycke
resp. DMBR-PSB Transmission Electron Microscopy - Core facility
VIB1 - Department for Molecular Biomedical Research
VIB2 - Plant Systems Biology
9000 Gent

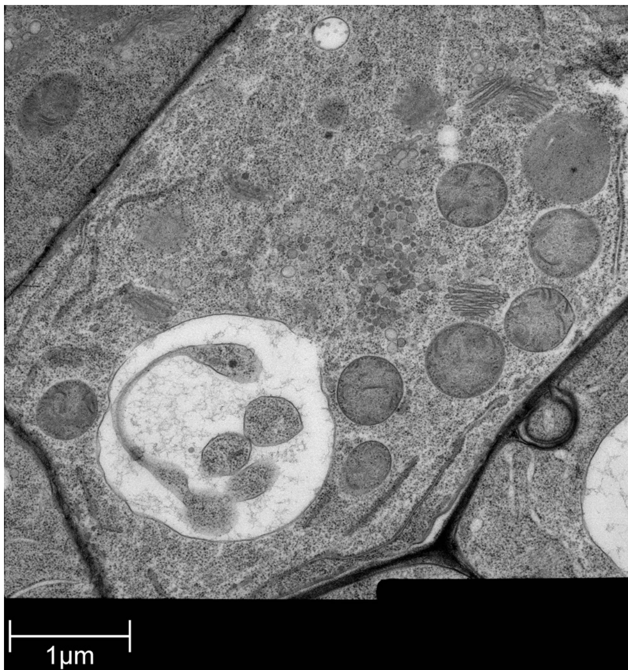
PROTOCOL HPF - AFS FOR MORPHOLOGICAL ANALYSIS:

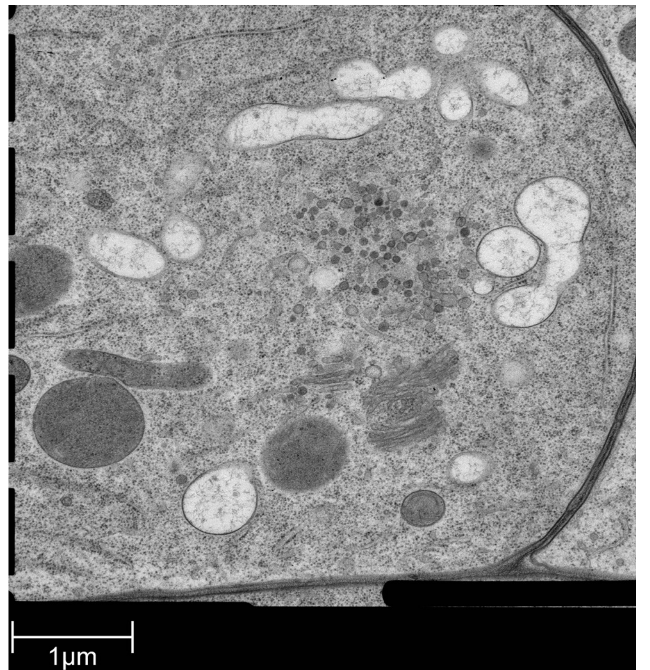
Arabidopsis thaliana roots (mutant PIN1pro:PIN1-GFP;bex5-1) were excised, immersed in 20 % (w/v) BSA and frozen immediately in a high-pressure freezer (EM PACT; Leica Microsystems, Vienna, Austria).
Freeze substitution was carried out using a Leica EM AFS2 (Leica Microsystems) in dry acetone containing 1 % ddH2O, 1 % OsO4 and 0.5 % glutaraldehyde over a 4-days period as follows:
-90 °C for 24 hours, 2 °C per hour increase for 15 hours, -60 °C for 24 hours, 2 °C per hour increase for 15 hours, and -30 °C for 24 hours. Samples were then washed 3 times in pure acetone between
-30 °C and 0 °C and slowly warmed up to 4 °C, infiltrated stepwise over 3 days at 4 °C in Spurr's resin and embedded in capsules. The polymerization was performed at 70 °C for 16 h.
Ultrathin sections were made using an ultramicrotome (Leica EM UC6) and post-stained in in a Leica EM AC20 for 40 min in uranyl acetate at 20 °C and for 10 min in lead citrate at 20 °C. Grids were viewed with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV using Image Plate Technology from Ditabis (Pforzheim, Germany).

LEGEND FOR IMAGES:

Ultrastructure of Arabidopsis thaliana primary root cells in PIN1pro::PIN1=GFP;bex5-1 treated for 1h with 50 uM BFA.
For details see Feraru et al., J The Plant Cell, Vol. 24: 1–14, 2012
(http://www.plantcell.org/content/early/2012/07/03/tpc.112.098152)




1µm


1µm

Immuno - Electron Microscopy of high pressure frozen and freeze substituted mouse heart

COURTESY

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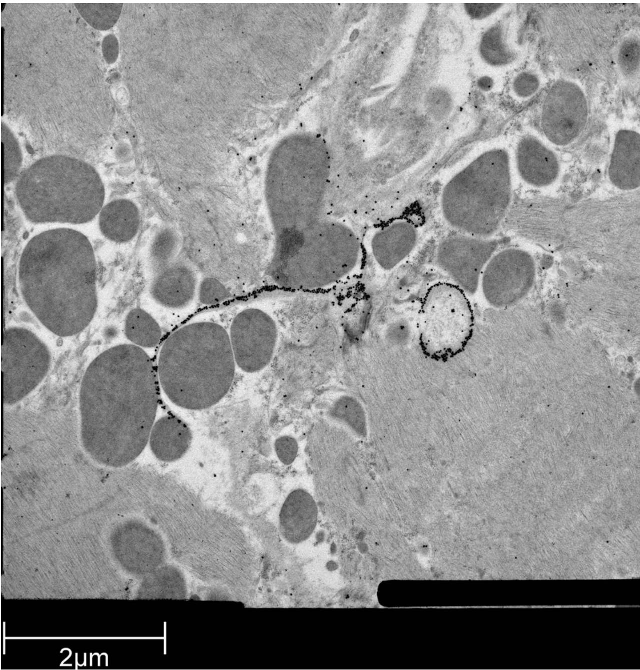
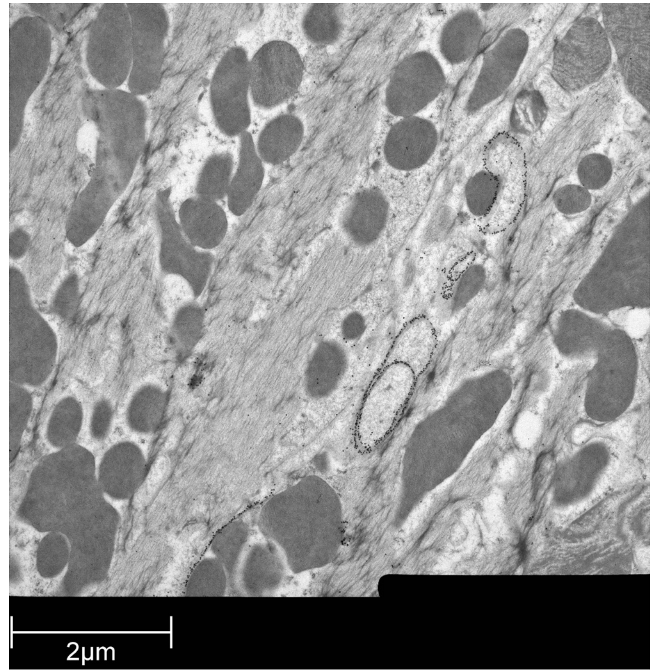
PROTOCOL HPF - AFS MOUSE HEART IEM:

Mouse heart tissue from wild-type (WT) and αT -catenin KO (KO) mice was excised, immersed in 20 % (w/v) BSA and frozen immediately in a high-pressure freezer (EM PACT; Leica Microsystems, Vienna,Austria). Freeze substitution was carried out using a Leica EM AFS (Leica Microsystems) in dry acetone containing 2 % ddH2O, and 0.1 % glutaraldehyde over a 4-days period as follows:
-90 °C for 24 hours, 2 °C per hour increase for 15 hours, -60 °C for 24 hours, 2 °C per hour increase for 15 hours, and -30 °C for 24 hours. Samples were then washed 3 times in pure acetone between -30 °C and 0 °C and slowly warmed up to 4 °C, infiltrated stepwise over 3 days at 4 °C in LR-White and embedded in capsules. The polymerization was performed in Leica EM AFS using UV lamp over 6 days starting at 20 °C and ending at 37 °C.
Ultrathin sections were made using an ultramicrotome (Leica EM UC6) and post-stained in in a Leica EM AC20 for 40 min in uranyl acetate at 20 °C and for 10 min in lead citrate at 20 °C.
Grids were viewed with a JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV using Image Plate Technology from Ditabis (Pforzheim, Germany).
Immunolabeling and label quantification were performed as described previously (Goossens et al., 2007).
The following primary antibodies were used for immuno-EM:
Cx43-polyclonal rabbit (1:50; Sigma) and Desmin polyclonal rabbit (1:50; AbCam).
In the article of Li et al. also other images are shown, processed (with EM Leica EM-Pact and Leica AFS) for spurr's resin and HM20 (with HPM010), but that's no problem, the protocol we explain here is for the shown images.

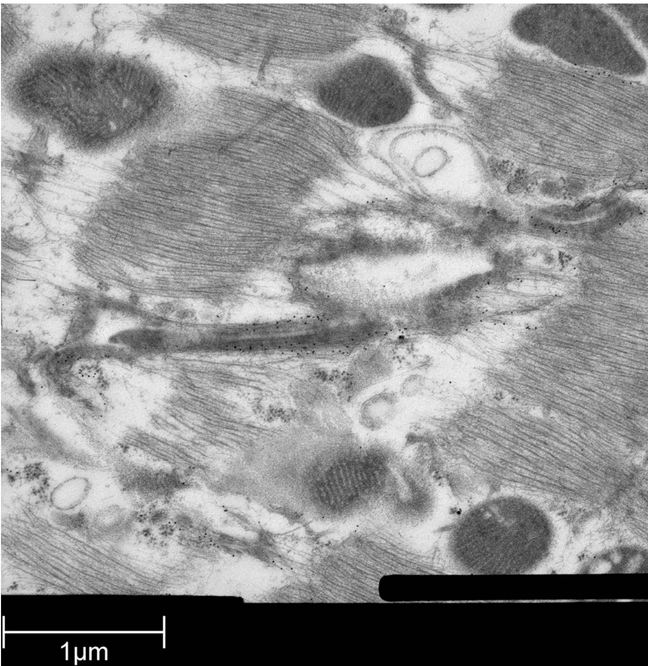
For details see: Li et al., J Cell Sci 125, 1058-1067. , 2012 (http://jcs.biologists.org/content/125/4/1058.long)



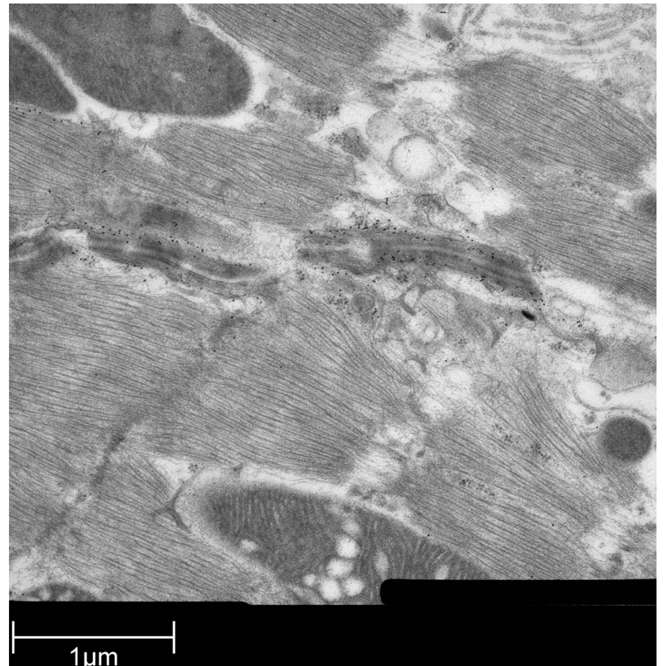
Single immunogold labeling of Cx43 with silver amplification at representative gap junctions of the intercalated disc (ICD) from αT-catenin KO mouse heart.



Single immunogold labeling of Cx43 with silver amplification at representative gap junctions of the intercalated disc (ICD) from WT mouse heart.



Single immunogold labeling of desmin in a desmosome from αT-catenin KO mouse heart.



Single immunogold labeling of desmin in a desmosome from αT-catenin KO mouse heart.

Hep-2 cells infected with Chlamydia pneumoniae

COURTESY

Dr. Andres Kaech
Center for Microscopy and Image Analysis
University of Zurich, Switzerland

PROTOCOL

Hep-2 cells infected with Chlamydia pneumoniae were cultured on carbon-coated 6 mm Sapphire discs. Cells were high-pressure frozen in an EM HPM100 using the 6 mm CLEM middle plate with following setup: Sapphire disc with cells, spacer 200 µm, bare Sapphire disc, 2 spacers 200 µm. Ethanol was used as a synchronization fluid to transfer pressure at room temperature prior to cooling. After freezing, the Sapphire disc was removed from the middle plate in anhydrous acetone at -90 °C and immediately transferred into a 2 ml Eppendorf tube containing 2 % OsO_4 in anhydrous acetone, precooled to -90 °C in an AFS 2 freeze-substitution unit. Samples were substituted for 8 h at -90 °C, 8 h at -60 °C, 8 h at -30 °C, and 1 h at 0 °C with periodic temperature transition gradients of 30 °C/h. Samples were then washed twice with anhydrous acetone at 4 °C, immersed in 33 % Epon/Araldite in anhydrous acetone at 4 °C overnight, followed by 66% Epon/Araldite in anhydrous acetone at 4 °C for 6 h, and finally in 100 % Epon/Araldite at room temperature for 2 h prior to polymerization at 60 °C for at least 24 h in a 1.5 ml Eppendorf tube. Sections were post-stained with uranyl acetate and lead citrate.
NOTE: The use of ethanol as a synchronization fluid during high-pressure freezing is not necessary for cell culture monolayers on Sapphire discs. A simplified sandwich configuration can be used to provide similar results by placing the Sapphire disc in a CLEM middle plate with cells facing up covered with an aluminium specimen carrier dipped in 1-hexadecene with the 100 µm cavity facing the cells.

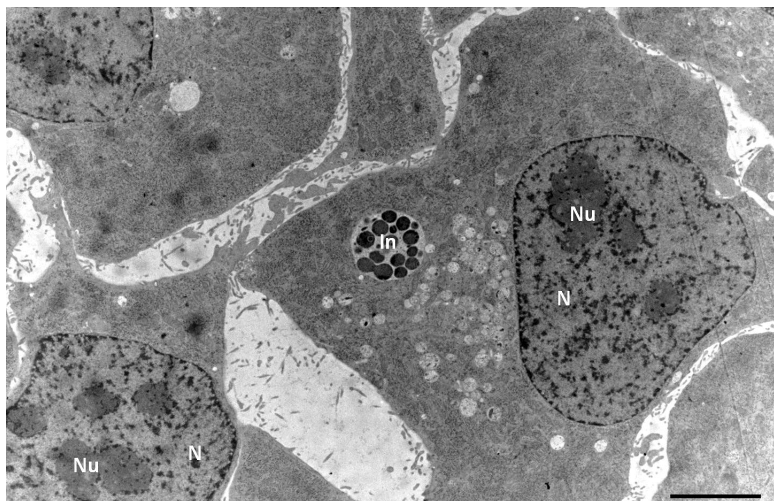


Figure 1. Thin section of Hep-2 cells infected with Chlamydia pneumoniae. Overview. (N)…Nuclei, (Nu)…Nucleoli, (In)…Inclusion. Scale bar 5 µm.

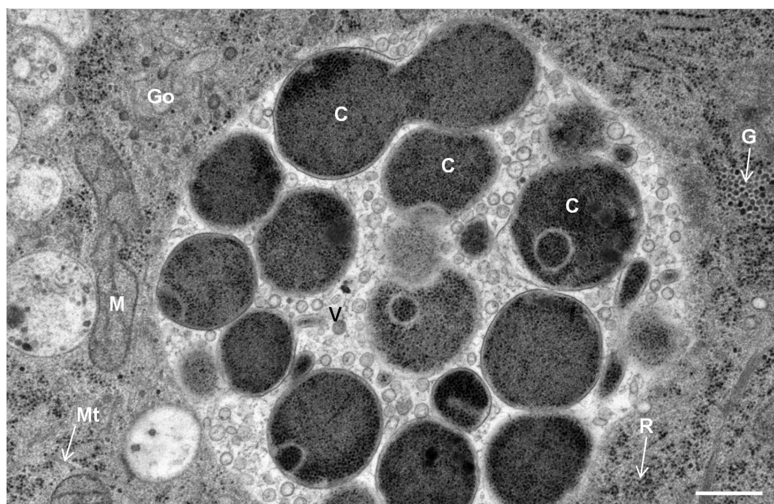


Figure 2. Higher magnification of inclusion of cell in figure 1. (C)…Chlamydia pneumoniae cells, (G)…Glycogen granules, (Go)…Golgi, (M)...Mitochondrium, (Mt)...Microtubules, (R)…Ribosomes, (V)…Vesicles. Scale bar 500 nm.

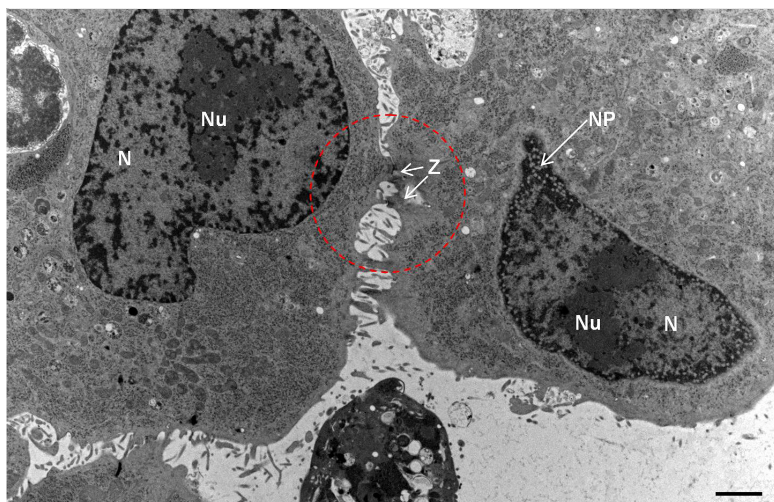


Figure 3. Thin section of Hep-2 cells infected with Chlamydia pneumoniae. Overview. (N)…Nuclei, (Nu)…Nucleoli, (NP)…Nucleopores, (Z)…Zonula adhaerens. Scale bar 2 µm.

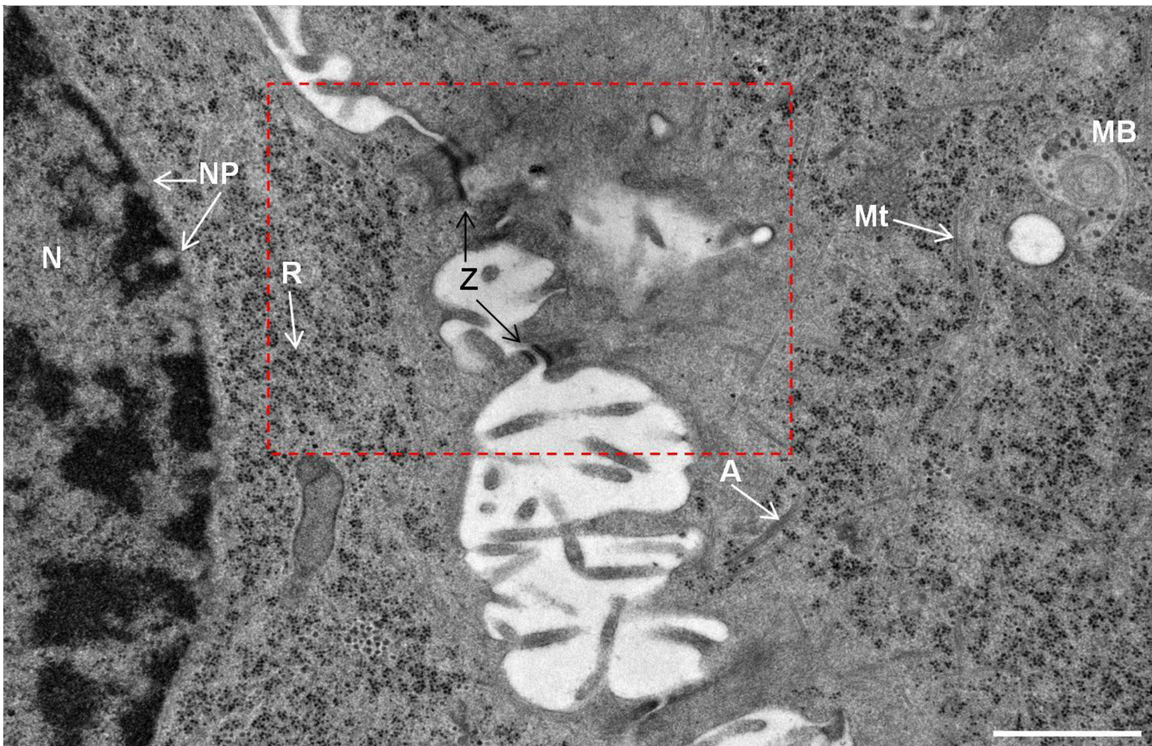


Figure 4. Thin section of Hep-2 cells infected with Chlamydia pneumoniae. Higher magnification of selected area in figure 3. (A)…Actin filaments, (MB)…Mulitvesicular body, (Mt)…Microtubules, (N)…Nucleus, (NP)…Nucleopores, (R)…Ribosomes, (Z)…Zonula adhaerens. Scale bar 1 μm.

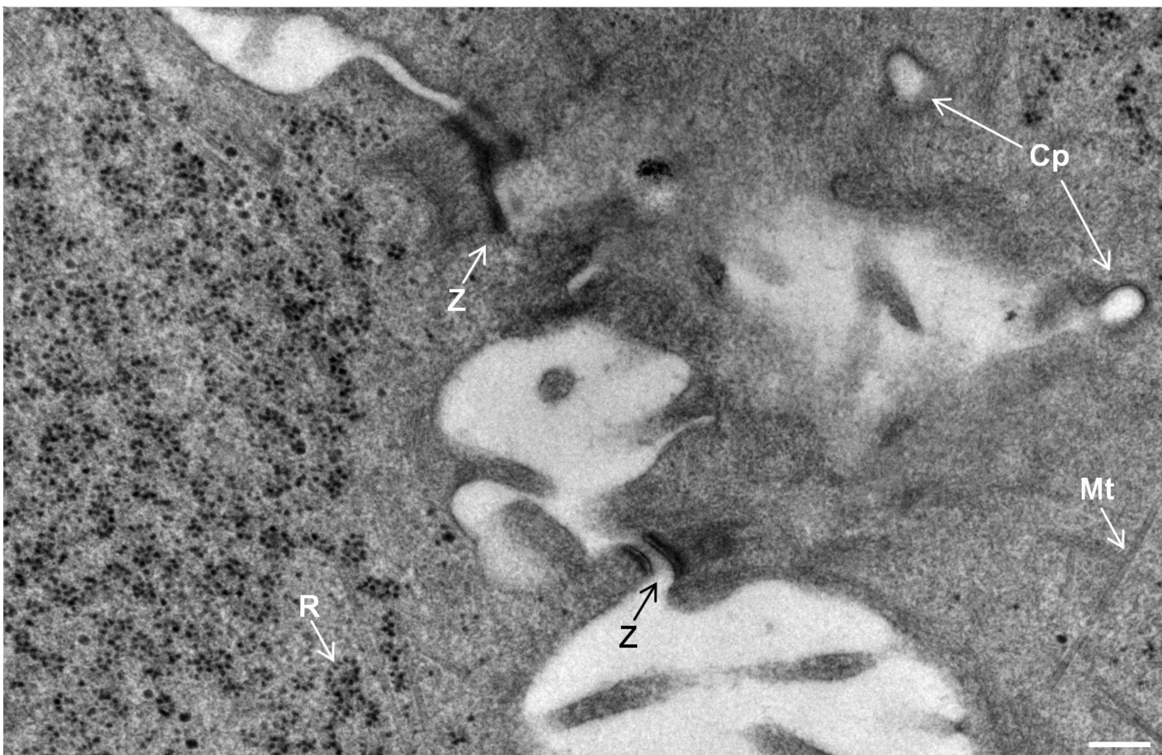


Figure 5. Thin section of Hep-2 cells infected with Chlamydia pneumoniae. Higher magnification of selected area in figure 4. (Cp)…Clathrin coated pit, (Mt)…Microtubules, (R)…Ribosomes, (Z)…Zonula adhaerens. Scale bar 200 nm.

more application images

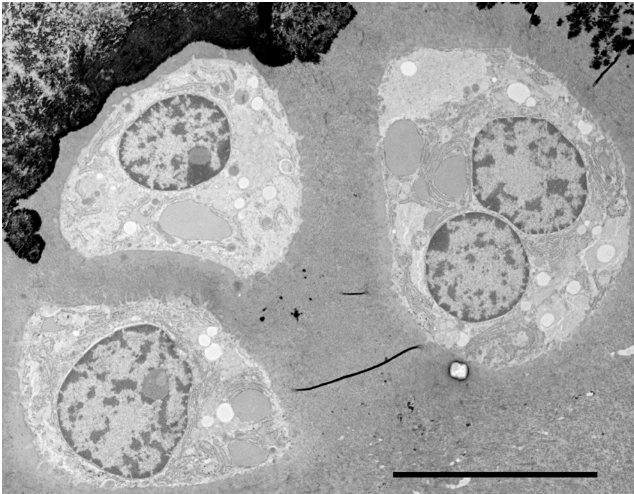


Fig. 1

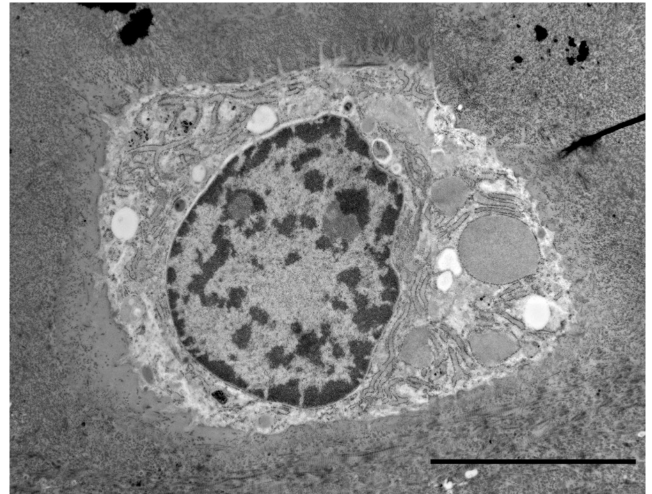


Fig. 2

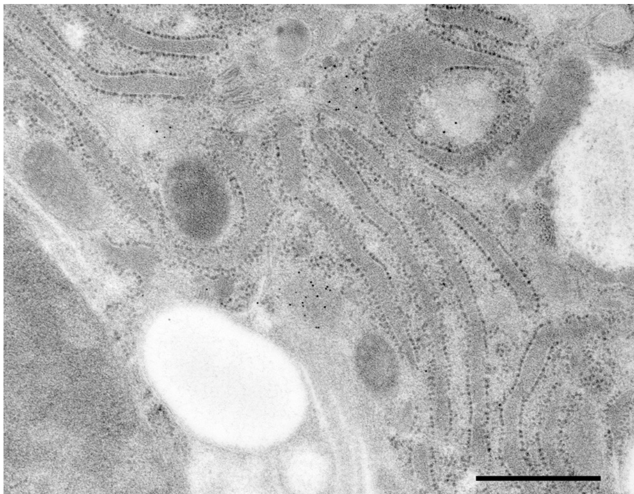


Fig. 3

Figure 1 - 3: Mouse cartilage
Figure 4: Liver HEPG2 cells
Figure 5: Yeast

Courtesy of E.G. van Donselaar,
B.M. Humbel, Utrech University,
The Netherlands;
J.W. Slot, University Medical Center
Utrecht, The Netherlands

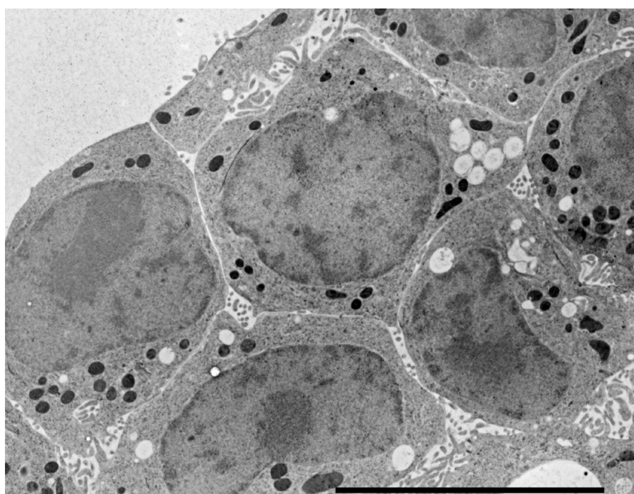


Fig. 4

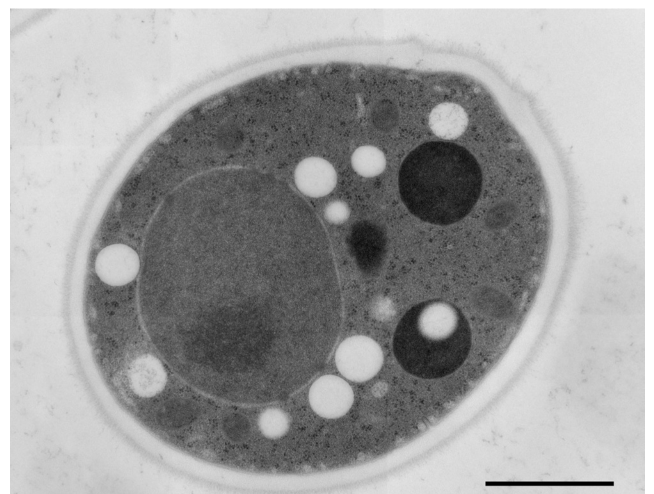


Fig. 5

Leica EM AFS2

Automatic Freeze Substitution System

Freeze Substitution (FS) of specimens in methanol, acetone or any other FS media at low temperatures is THE follow-on procedure to high pressure freezing and other cryo fixation methods.

Progressive Lowering of Temperature (PLT) allows substitution and resin infiltration of chemically fixed specimens.

Finally, the sample is polymerized under UV light in the AFS2 and can be cut and immuno labelled.

Visit the Website: Leica EM AFS2

Publications

Brochure

Science Lab

Leica EM **HPM100**

High pressure freezing is by far the most signifi cant sample preparation method for morphological and immunocytochemical high resolution studies for electron microscopy.

High pressure freezing has made it possible to observe aqueous biological and industrial samples near to native state.

The 2100 bar of high pressure applied to the sample during high pressure freezing using the Leica EM HPM100 suppresses ice crystal formation and growth, while cryo immobilization immediately after pressurization prevents structural damage to the sample.

High pressure frozen samples can be completely vitrifi ed up to a thickness of 200 µm, a 10 to 40-fold increase in the depth of amorphous ice. No conventional freezing method can generate such large, well frozen samples.

The unique 6 mm diameter carrier system of the Leica EM HPM100 allows even more sample area to be frozen, like no other high pressure freezing instrument.

The state-of-the-art design of the Leica EM HPM100 enables express sample handling and easy use with perfect freezing results.

Visit the Website: Leica EM HPM100

Publications

Brochure

Science Lab

Leica EM PACT2

High Pressure Freezer

The Leica EM PACT2 high pressure freezer serves the needs of molecular and cell biologists and all researchers who want an "in vivo" impression of their cellular structures and functions in question – without the artefacts of chemical fixation but with the high resolution information of EM immunocytochemistry, frozen hydrated sections and freeze fracturing.

The Rapid Transfer System EM RTS allows correlative LM/EM experiments, taking a live specimen from a light microscope (e.g. a confocal microscope) to freezing in less than 5 seconds. In the same way, time resolved experiments are possible. Safety and reproducibility for the specimen are increased while operator mistakes are reduced.

Visit the Website: Leica EM PACT2

Publications

Brochure

Science Lab

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Leica
MICROSYSTEMS

The statement by Ernst Leitz in 1907, "With the User, For the User," describes the fruitful collaboration with end users and driving force of innovation at Leica Microsystems. We have developed five brand values to live up to this tradition: Pioneering, High-end Quality, Team Spirit, Dedication to Science, and Continuous Improvement. For us, living up to these values means: Living up to Life.

Leica Microsystems operates globally in four divisions, where we rank with the market leaders.

LIFE SCIENCE DIVISION

The Leica Microsystems Life Science Division supports the imaging needs of the scientific community with advanced innovation and technical expertise for the visualization, measurement, and analysis of microstructures. Our strong focus on understanding scientific applications puts Leica Microsystems' customers at the leading edge of science.

INDUSTRY DIVISION

The Leica Microsystems Industry Division's focus is to support customers' pursuit of the highest quality end result. Leica Microsystems provide the best and most innovative imaging systems to see, measure, and analyze the microstructures in routine and research industrial applications, materials science, quality control, forensic science investigation, and educational applications.

MEDICAL DIVISION

The Leica Microsystems Medical Division's focus is to partner with and support surgeons and their care of patients with the highest-quality, most innovative surgical microscope technology today and into the future.

Image Front page: Arabidopsis thaliana root tips cells
Courtesy of: Riet De Rycke, VIB2 - Plant Systems Biology, Gent, Belgium

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