

# EM Sample Preparation High Pressure Freezing

July 2013

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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

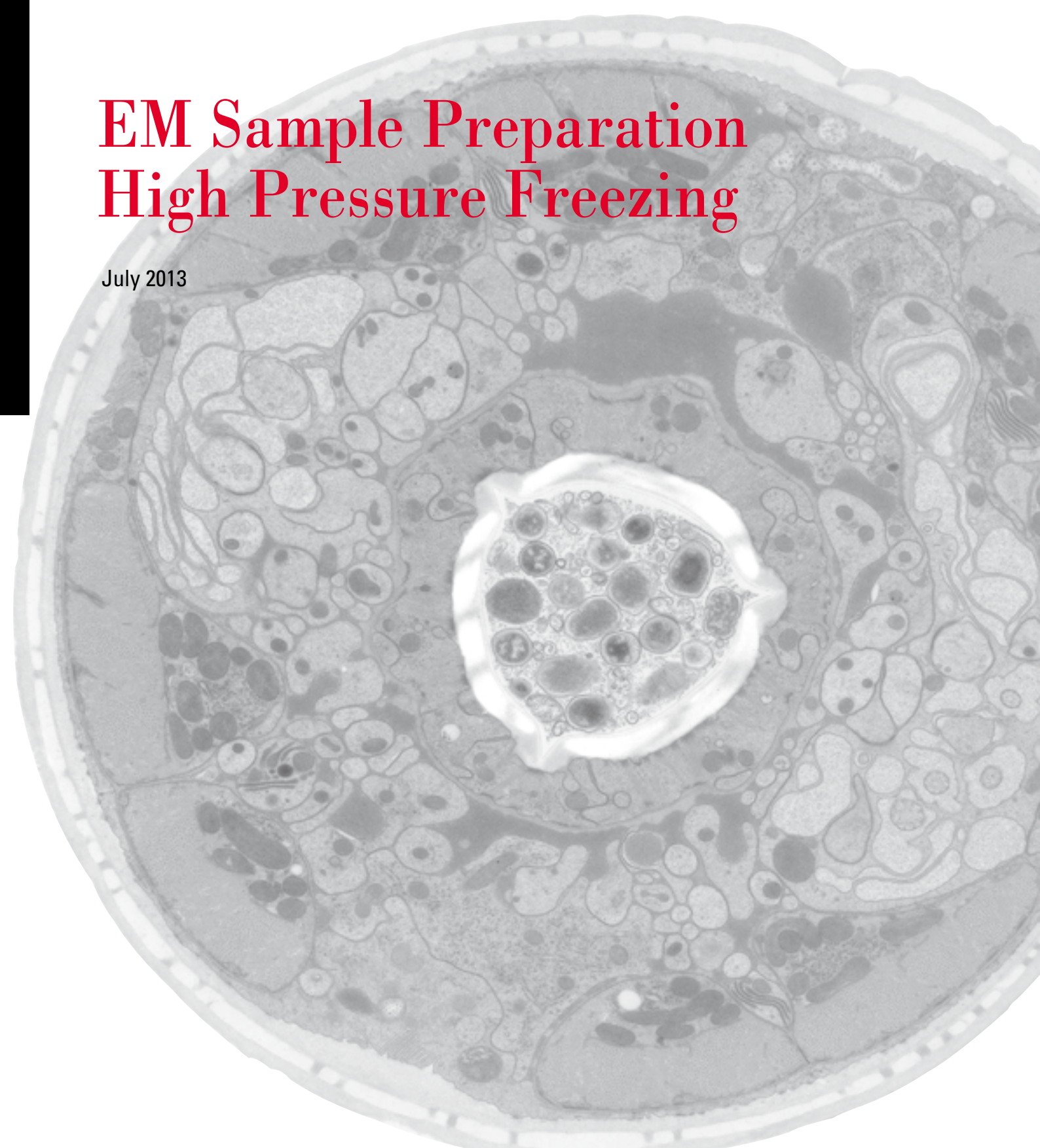
The Leica Microsystems Industry Division's focus is to support customers' pursuit of the highest quality end result. Leica Microsystems provide the best and most innovative imaging systems to see, measure, and analyze the microstructures in routine and research industrial applications, materials science,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

#### BIOSYSTEMS DIVISION

The Leica Microsystems Biosystems Division brings histopathology labs and researchers the highest-quality, most comprehensive product range. From patient to pathologist, the range includes the ideal product for each histology step and high-productivity workflow solutions for the entire lab. With complete histology systems featuring innovative automation and Novocastra™ reagents, Leica Microsystems creates better patient care through rapid turnaround, diagnostic confidence, and close customer collaboration.

#### 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.






## High Pressure Freezing

Water is the most abundant cellular constituent and therefore important for preserving cellular ultra-structure. Currently the only way to fix cellular constituents without introducing significant structural alterations is by cryo-fixation. There are currently two common methods employed; plunge freezing and high pressure freezing.

Cryo-fixation has two distinct advantages over chemical fixation. It is achieved within milliseconds and it ensures simultaneous immobilization of all macromolecular components. Many protein networks are very labile and fall apart with the slightest osmotic or temperature change and these unwanted effects are minimized during cryo-fixation. These techniques allow the study of biological samples with improved ultra-structural preservation, and can facilitate the study of dynamic processes.

Currently, the only method to vitrify thicker samples (up to 200  $\mu\text{m}$ ) is by HPF.

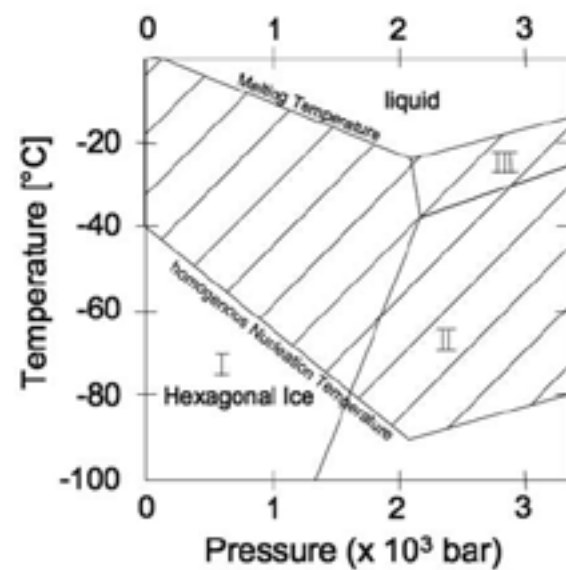
Successful cryo-fixation (vitrification) is the transformation of water from a liquid to an amorphous state without inducing the nucleation of ice crystals. The nucleation of ice crystals is temperature- and pressure- dependent (see diagram below). Crystallisation also depends on the cooling rates as freezing is a time dependant process. The cooling rates depend on the thermal properties of water, the sample thickness and the heat extraction flow at the surface of the specimen.



Mouse embryonic fibroblast  
grown on sapphire disc.  
Paul Verkade, University of Bristol, UK

## High Pressure Freezing continued

The idea of freezing biological samples under pressure was first introduced by Moor and Riehle (Moor and Riehle, 1968) The HPF device became commercially available in the 1985. All HPF machines available currently, despite having different design, deliver synchronized pressurization and cooling of the sample within 20 ms in a highly reproducible manner.



The diagram (Kanno et al. 1975) shows the states of water depending on pressure and temperature. At a pressure of 2045 bar the melting point of water is lowered to 251K and the temperature for homogenous nucleation is reduced to 181K.

Kanno H, Speedy RJ, Angell CA (1975) Supercooling of water to  $-92^{\circ}\text{C}$  under pressure. *Science* 189:880–881  
 Moor H, Riehle U. Snap-freezing under high pressure: A new fixation technique for freeze-etching. *Proc Fourth Europ Reg Conf Elect Microsc.* 1968; 2:33-34.

## A New Dimension in High Pressure Freezing

Based on the principle of Self Pressurized Rapid Freezing introduced by Leunissen and Yi (*J. Microsc.* 235: 25–35 [2009]) a new technology has been developed by Leica Microsystems.

It uses the tendency for water inside a sealed specimen carrier to expand upon cooling, thereby generating pressure intrinsically instead of using an external hydraulic system.

This pressure is likely to be the result of crystalline and low density ice formation within the sealed specimen carrier.

To achieve pressure (2010 bar) where the melting point of ice is lowered to 251 K (Kanno et al. 1975, *Science* 189: 880–881 [1975]) 60 % of the water inside the specimen carrier needs to be converted to low density ice.

### THROUGH THE LOOKING GLASS OF PHYSICS

Low density ice formation causes a volume expansion relative to liquid water. Numerical simulations show that freezing along the tube walls proceeds freezing in the center, producing a strongly curved ice front. This effect is prominent when the immersion speed is the highest. The separation between regions containing low density ice and well-frozen or vitrified parts is considered to be best when the ice front is as flat as possible. This can be achieved by alteration of the freezing parameters for each specific case within the Leica EM SPF program interface. During the immersion movement, the ice formation front inside the tube is always above the level of the cryogen surface. The distance of this ice front depends on the immersion speed.



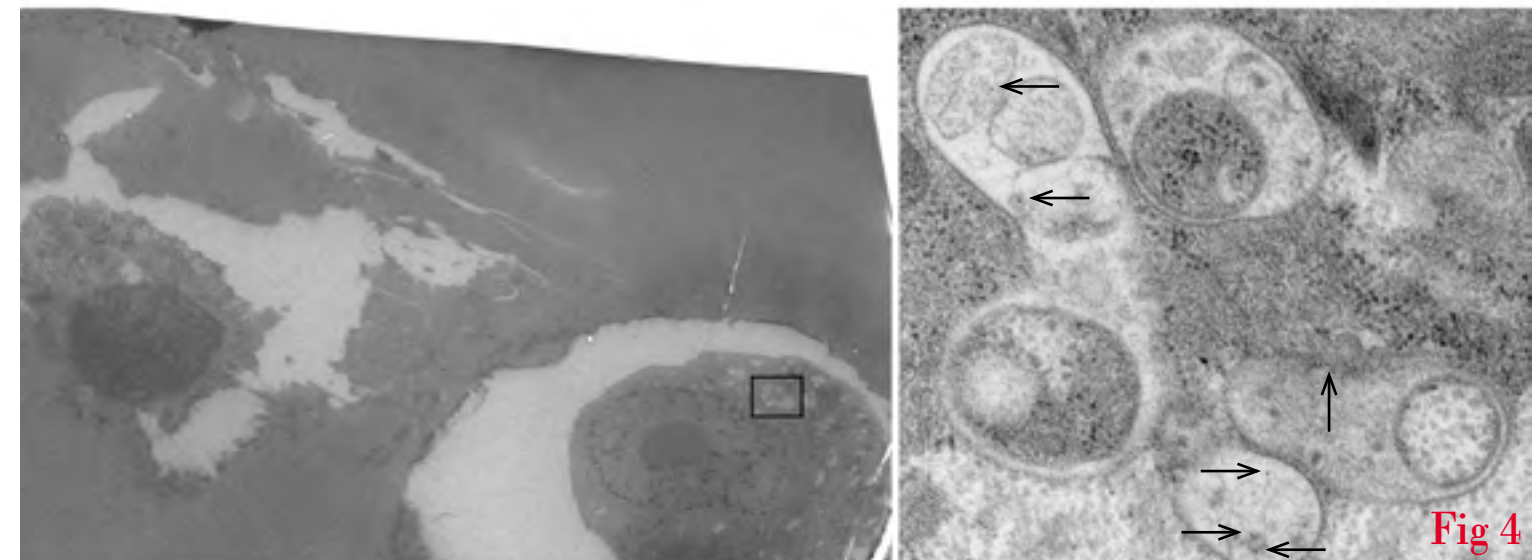
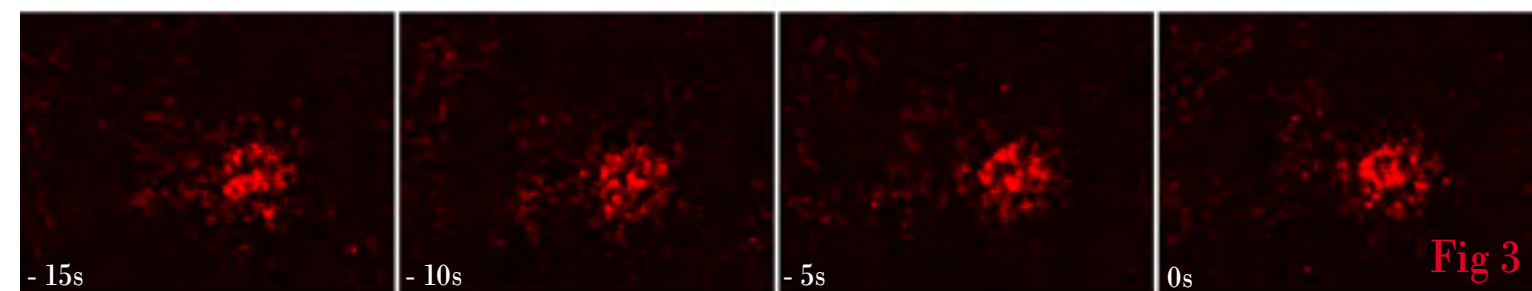
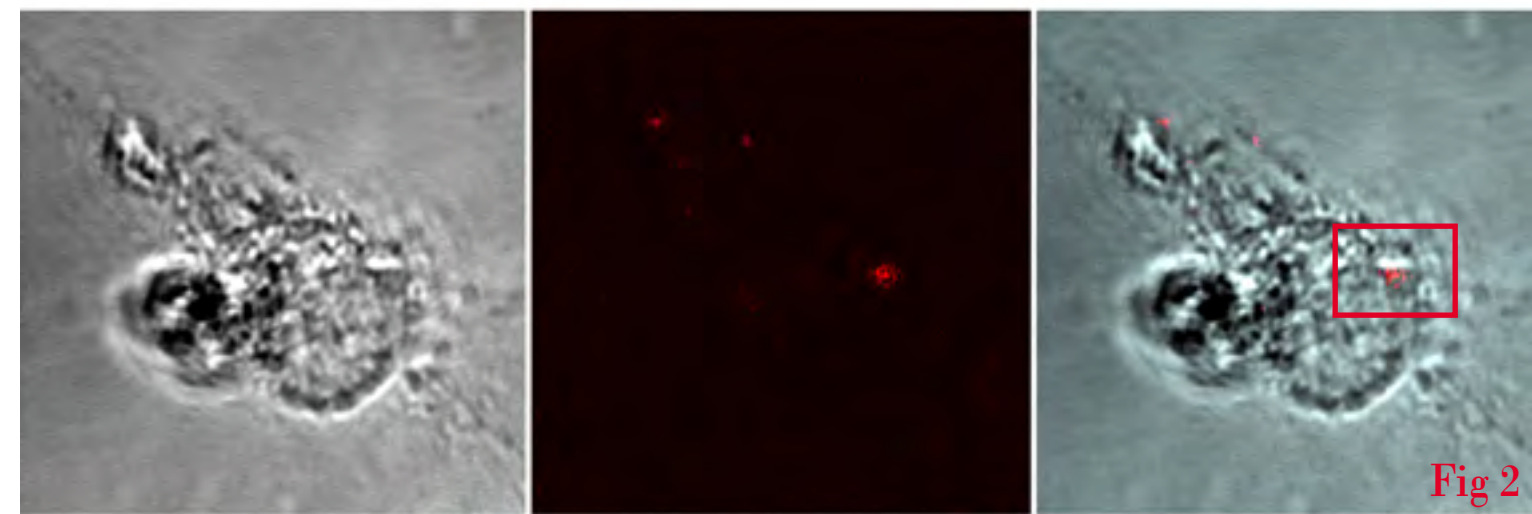
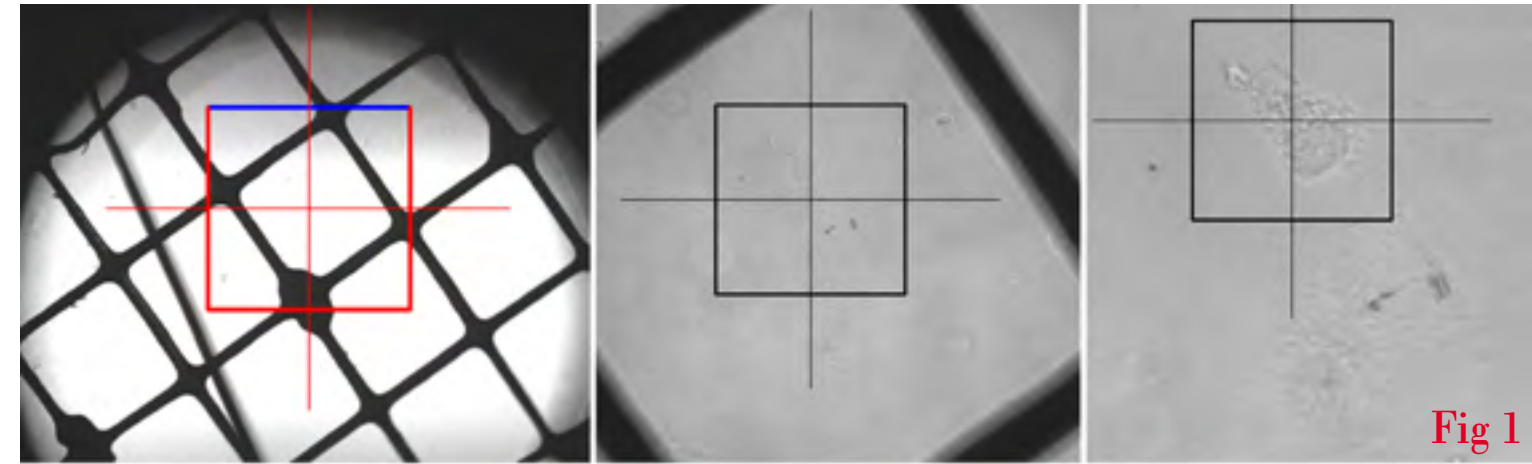
## Application - CLEM

### (Correlative Light Electron Microscopy)

- Fig 1 - **Overview images of PtK2 cells grown on sapphire discs**  
The finder grid located close above the cells for retracing the sample.
- Fig 2 - **DIC and fluorescent images**  
When a cell of interest was located DIC and fluorescent images (internalized EGF-QD655) were made. The overlay (right image) shows the location of the EGF-QD655 containing structures in the cell. The boxed area is the area of interest within the cell.
- Fig 3 - **Fluorescent quantum dots inside the cell of interest**  
The structures containing the fluorescent quantum dots inside the cell of interest were followed live, making a movie sequence with images taken every second. Still images, showing images 5 seconds apart, from this movie sequence are shown. The last image was the last image of the sequence (hence time = 0s). At this moment the rapid loader was transferred from the stage insert into the RTS and the sample was frozen.
- Fig 4 - **Electron micrograph of the cell of interest (left), zoom into structure of interest (right)**  
Electron micrograph of the cell of interest (left). The boxed area is the area that contains the structure of interest and a zoom into the structure of interest is shown on the right. Arrows indicate quantum dots that can be identified inside the structure. Compare the C-shape of this structure with the last image of Figure 3, and a comparable C-shape can be observed. Note that the electron micrograph is from a 70 nm section while the fluorescent image is approximately 1  $\mu\text{m}$  thick.  
Courtesy of: Paul Verkade, University of Bristol, UK

### Related Instruments:

Leica EM PACT2 with Leica EM RTS



## Application - CEMOVIS

The sections are of yeast frozen with a Leica HPM100 in the copper tube system, the cell paste was mixed with a pH 6.5 MES/dextran buffer so that a final MES concentration of 50mM and a dextran concentration of 20% was achieved.

The samples were sectioned on a Leica EM UC7/ EM FC7 with Micromanipulator at -140°C and the section thickness was set to 50nm. The sections were attached to Agar lacey grids.

The sections were imaged using a Tecnai Polara 300KeV (FEI, The Netherlands) microscope fitted with a 4K Gatan CCD camera. The magnification for the sections was 23K, with a defocus of -6µm for the tomogram and -8µm for the projection image, and the diffraction was done with a camera length of 930mm. The image in panel A is an average of the central 10 slices of a reconstruction done with the IMOD package (Kremer et. al., 1996) image processing software from a tomogram collected using the FEI software.

Courtesy of Dr. Jonathan O'Driscoll, Dr. Daniel Kofi Clare and Prof. Helen Saibil, prepared at the Department of Crystallography, Birkbeck, University of London

Related Instruments  
Leica EM HPM100



Fig A

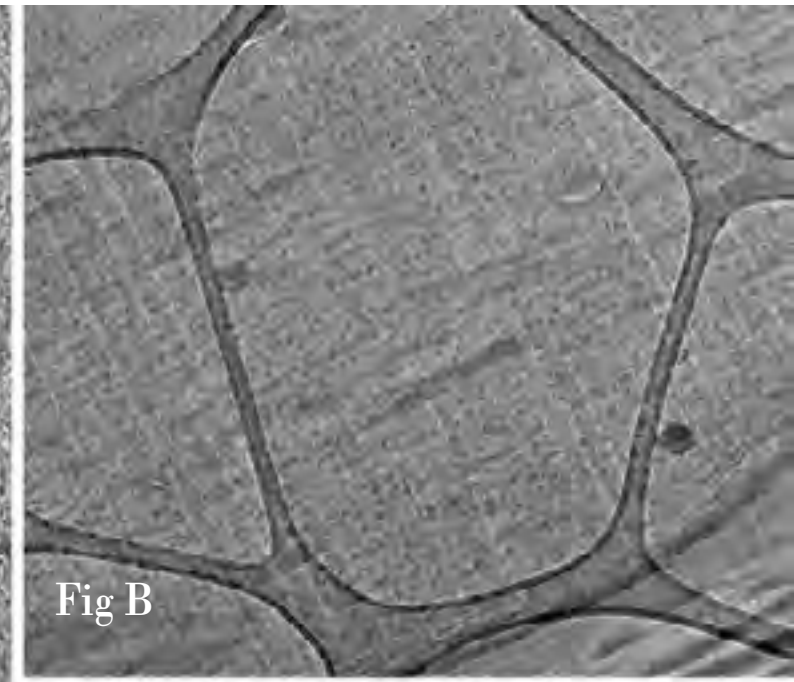


Fig B

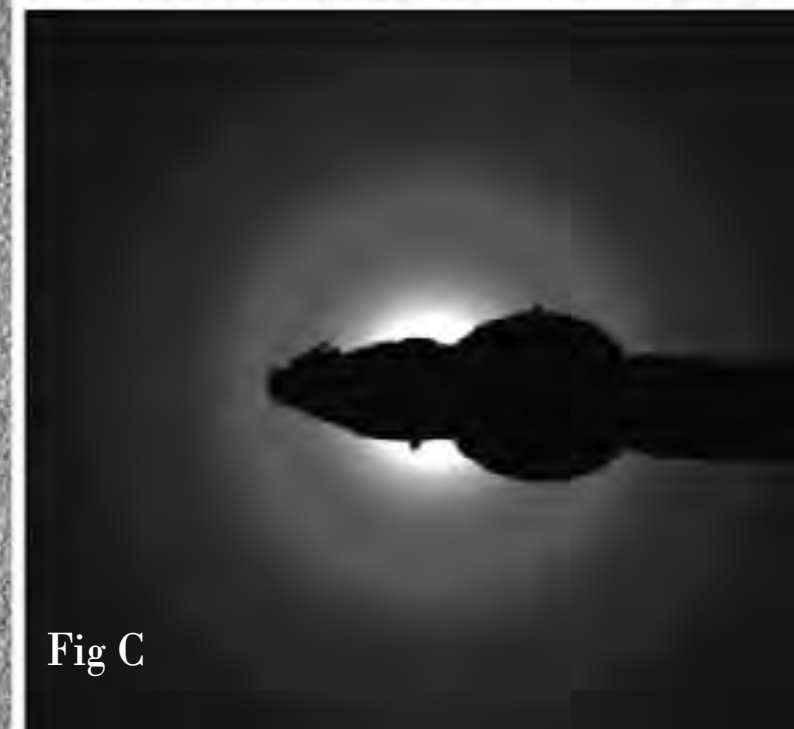


Fig C

A – optical slice from a tomographic reconstruction

B – micrograph of a vitrified yeast cell

C – diffraction pattern image



## Applications - Freeze Fracture and Freeze Substitution

Fig 1 **Pyramidal Cell**

Synaptic connection between a bouton (P-face) and a dendritic spine (E-face) of a pyramidal cell.

Courtesy of Akos Kulik, Institute of Anatomy and Cell Biology, University of Freiburg, Germany.

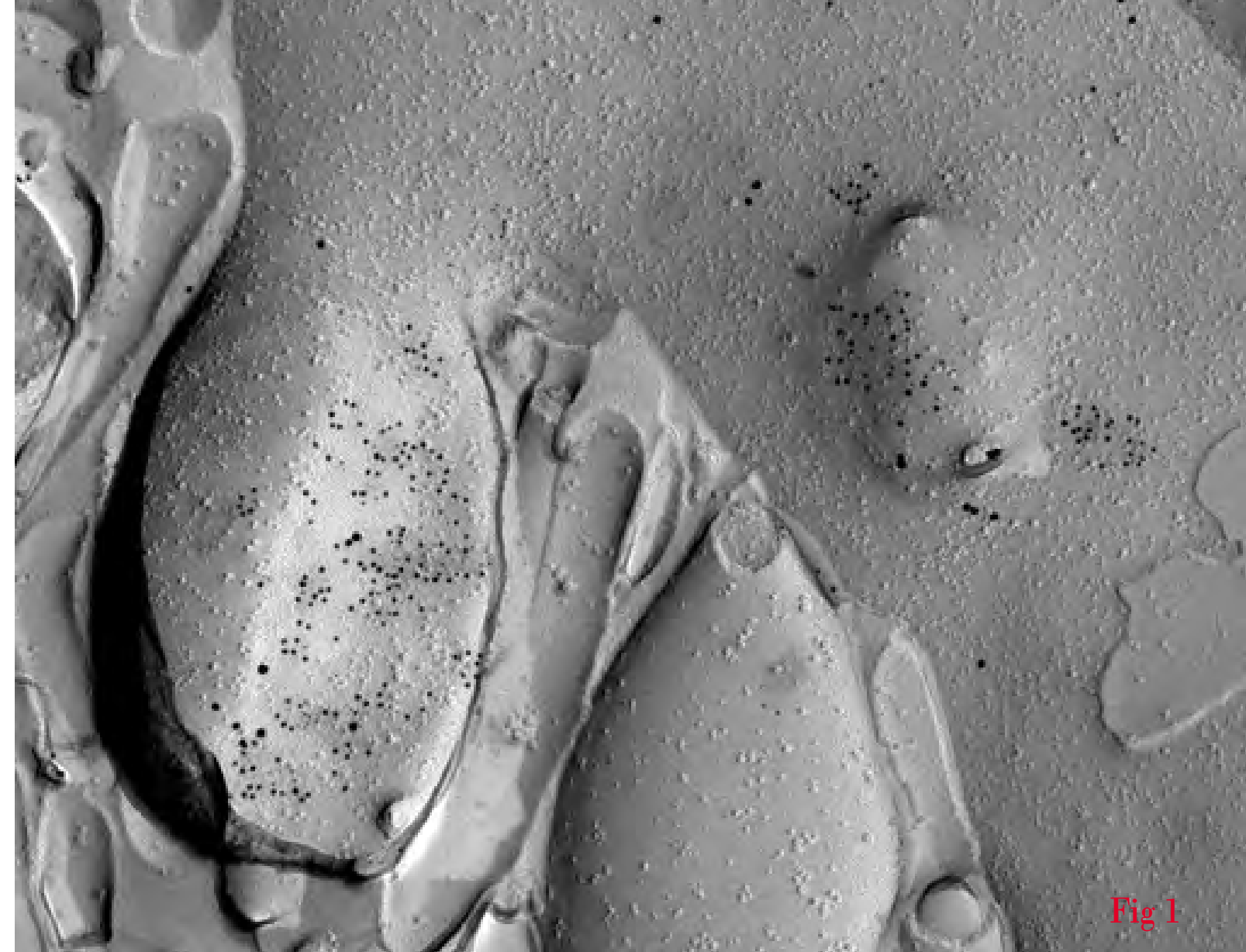


Fig 1

Fig 2 **Higher magnification of inclusion of cell in figure 1**

(C)... Chlamydia pneumoniae cells, (G)... Glycogen granules, (Go)... Golgi, (M)... Mitochondrion, (Mt)... Microtubules, (R)... Ribosomes, (V)... Vesicles.

Scale bar 500 nm.

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

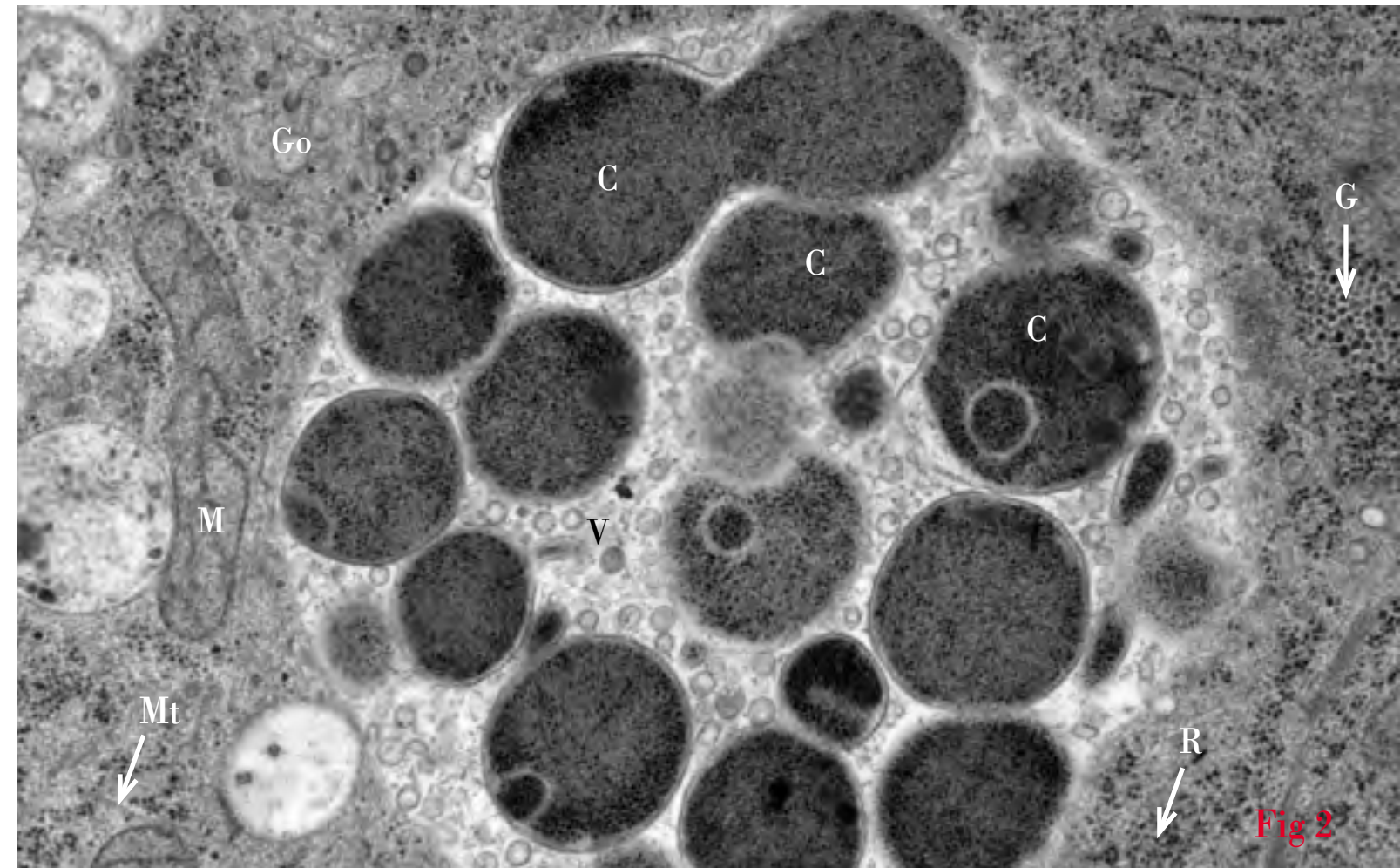


Fig 2

### Related Instruments

Leica EM HPM100



## Applications - SPF

Fig 1 - **CEMOVIS of *Saccharomyces cerevisiae***  
(Courtesy A. Al-Amaudi, DZNE , Bonn)

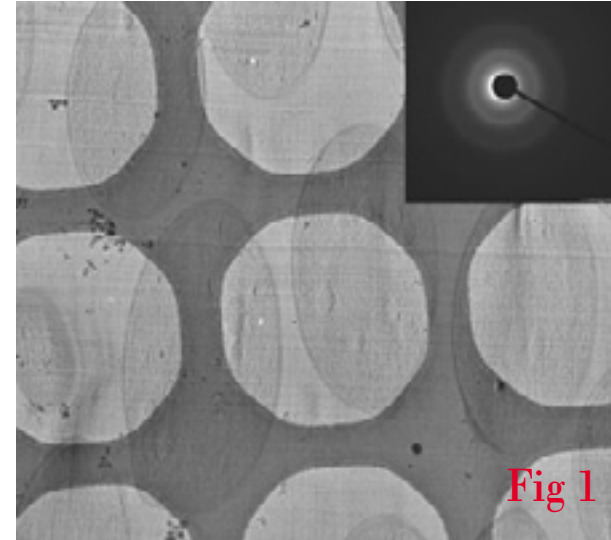


Fig 1

Fig 2 - **Freeze Substitution of *Pseudomonas deceptionensis***  
(Courtesy Dr. Carmen Lopez-Iglesias, Lidia Delgado and Elena Mercade, CC i-University Barcelona, Science Park)

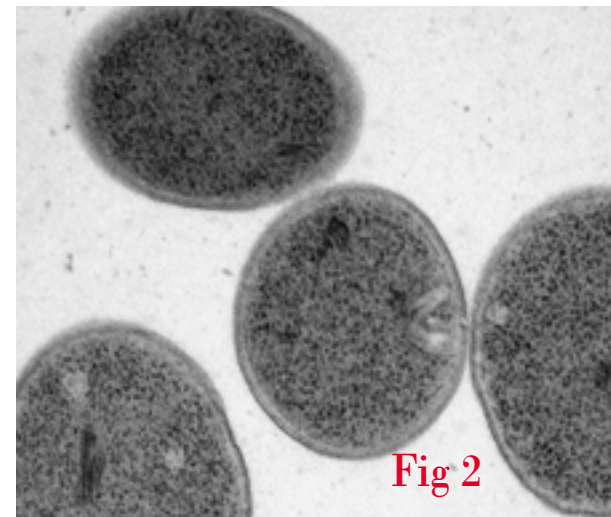


Fig 2

Fig 3 - **Freeze Substitution of *Caenorhabditis elegans***  
(Courtesy of the Delaware Biotechnology Institute Bio-Imaging Center (Shannon Modla, Scott Jacobs, Jeff Caplan and Kirk Czymbek) and University of Pennsylvania (Jessica Tanis))

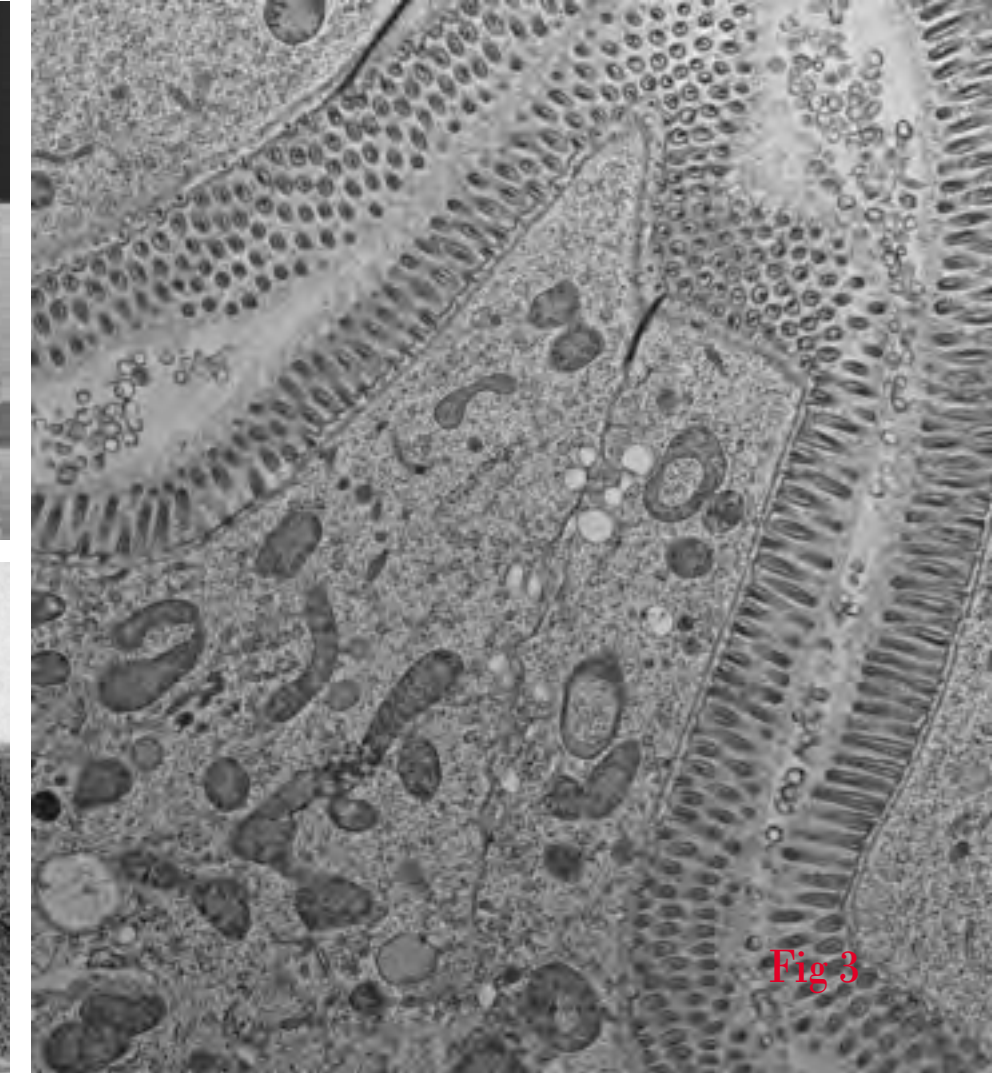


Fig 3

Fig 4 - **Freeze Substitution of *Lingulodinium polyedra***  
(extrusomes) (Courtesy Elena Lindemann, Fraunhofer IG B [Functional Genomics], Stuttgart)

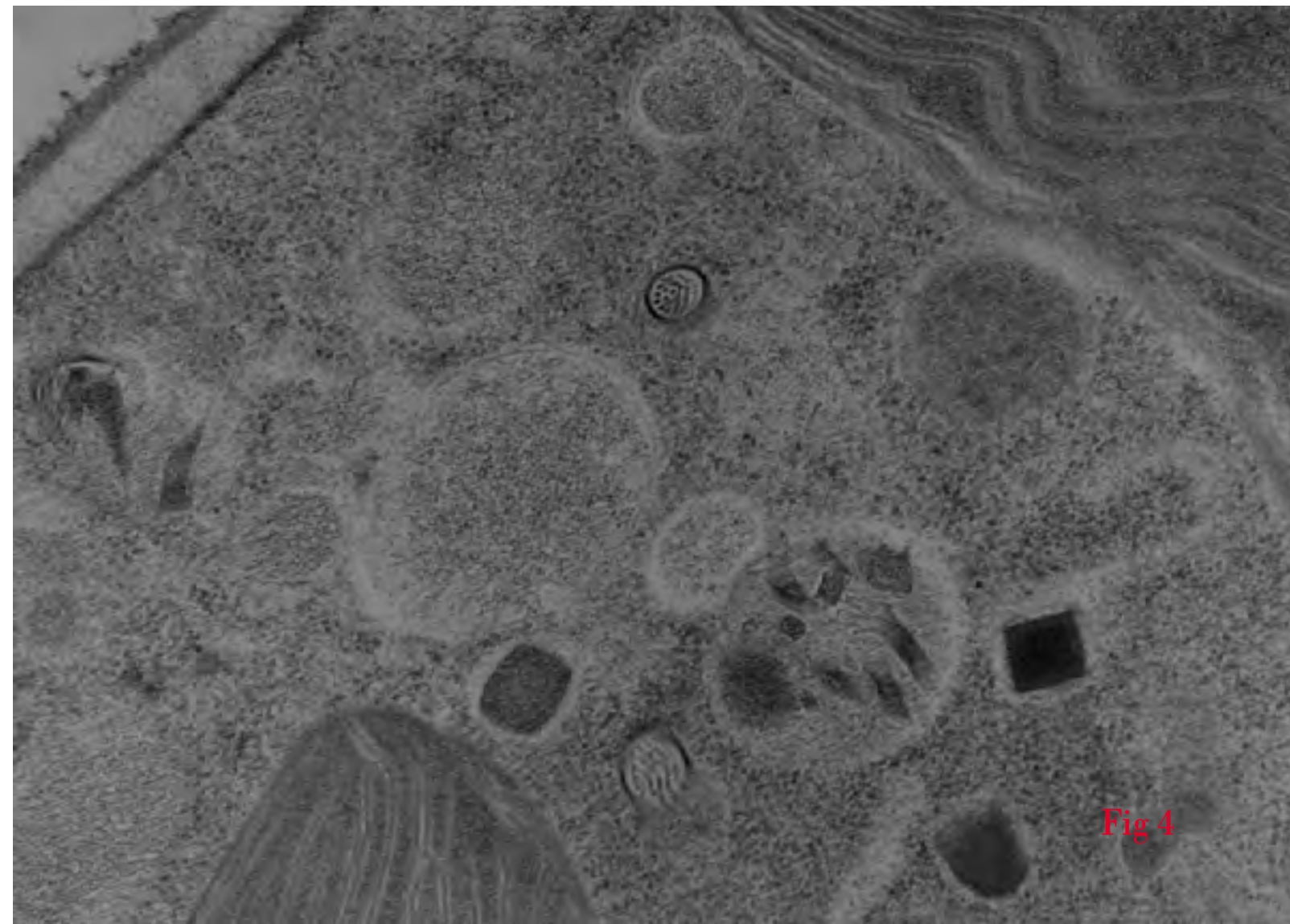


Fig 4

Related Instruments: Leica EM SPF

## 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.

## Leica EM HPM100



High pressure freezing is by far the most significant 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 vitrified up to a thickness of 200  $\mu\text{m}$ , 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.



## Leica EM SPF High Pressure Freezer



The Leica EM SPF works with U-tubes as a specimen carrier. The aim is to keep the low density ice located predominantly in the leg areas, whereas the arc area of the U-tube freezes last and while pressurized.

This approach for cryo-fixation allows freezing of biological specimens in their native environment without any specific preparation or addition of cryo-protectants, which can alter the initial physiological balance of the sample. Almost any type of cells, free-living bacteria, yeast cells, unicellular organisms etc., can be cryo-immobilized directly after being isolated from their natural habitat.

The Leica EM SPF is a unique entry-level product offering an alternative cryo-fixation method

www.leica-microsystems.com

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MICROSYSTEMS

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