3D Solutions in Transmission Electron (Cryo) -Microscopy in Biology

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Abstract. One of the main problems in transmission electron microscopy in the biological field is the tri-dimensionality. This article explains the technical procedures and requirements to prepare biological specimens preserving them closest to their native state to perform 3D reconstruction of the macromolecular complexes and cellular structures in their natural environment.

1. Introduction

Transmission electron microscopic (TEM) studies of biological samples are limited by four general factors:

- Electrons need high vacuum to form a beam able to traverse the sample and to form an image from it.
- Electrons heat the sample.
- The penetration power of electrons is very low.
- The image is a 2D projection of the specimen.

Therefore, the followings points must be considered:

- Firstly, the specimens cannot be alive, they must be immobilized or fixed. The high water content of cells (around 80%) needs to be removed or, preferably, stabilized by freezing [1].
- Secondly, the samples have to be dried, embedded in a resin resistant to heating or the electron microscope (EM) must be able to work under particular conditions ("low dose" of electrons) without damaging the sample [2].
- Thirdly, the thickness of the sample has to be between 50 and 400 nm, depending on the voltage of the EM.
- Finally, the viewing of 3D images from the specimen requires certain strategies in working with the EM and special software, called generally: "single particle analysis/reconstruction" and "electron tomography" [3].

3D solutions in EM refer to technical procedures that bring the electron microscopic study closer to the native "hydrated" and "three-dimensional" state of biological specimens. These technical procedures are summarized as follows (see Table 1):

- *CRYO-IMMOBILIZATION:* The optimal way to immobilize or fix biological specimens in a close-to-native state, using physical procedures based on freezing. It is the recommendable first step in any 3D procedure. The goal is to get "vitreous" or amorphous ice from the cell water after ultrarapid freezing, without destroying cell structures and preserving their *in vivo* rapidly changing interactions. Vitrification allows spatial and temporal resolution of cellular events.
- *VITREOUS SECTIONING & FREEZE-SUBSTITUTION*: Two procedures to prepare bulk specimens for three-dimensional reconstruction. Cryo-sectioning of vitreous samples allows thin sections of cells or tissues at low temperatures to be taken without loss of their vitreous state. Freeze-substitution consists of the substitution of the vitreous ice by an organic solvent followed by embedding in a resin which after polymerization leads to thin sections of the specimens
- *FREEZE-DRYING & FREEZE-FRACTURE*: Two different procedures to prepare bulk specimens for three-dimensional view but not for 3D-reconstruction. Freeze-drying allows to dry vitreous samples keeping all the nano-features immobilized by vitrification. Freeze-fracture opens lipid bilayers showing intra-membrane proteins. In both cases, a replica of the rough surface obtained by drying or fracture of the vitreous sample, is performed by shadowing with platinum (or tantalum) and carbon.
- *ROOM TEMPERATURE 3D ELECTRON MICROSCOPY*: Possibilities for 3D imaging of molecular or cell structures at room temperature. The procedures for the preparation of the samples for room temperature 3D-reconstruction and tomography are: i) negative staining (for small isolated particles) and ii) vitrification followed by freeze-substitution and resin embedding allowing sectioning at room temperature (for bulk specimens)
- *3D CRYO-ELECTRON MICROSCOPY*: The same three-dimensional reconstruction strategies mentioned above, but working under cryo-conditions (keeping the sample at a temperature below -170ºC and working with a "low dose" of electrons). The procedures for

the preparation of the samples for 3D Cryo-EM are: i) vitrification (for small isolated particles) and ii) vitrification followed by cryosectioning (for bulk specimens)

- *COMPUTATIONAL METHODS FOR 3D-RECONSTRUCTION*: After image acquisition, different software are used for data alignment, reconstruction and viewing. The reconstruction can be considered as the inversion of the imaging process. The projections obtained from the 3D-specimen at different angles or orientations are deprojected into the reconstructed volume.

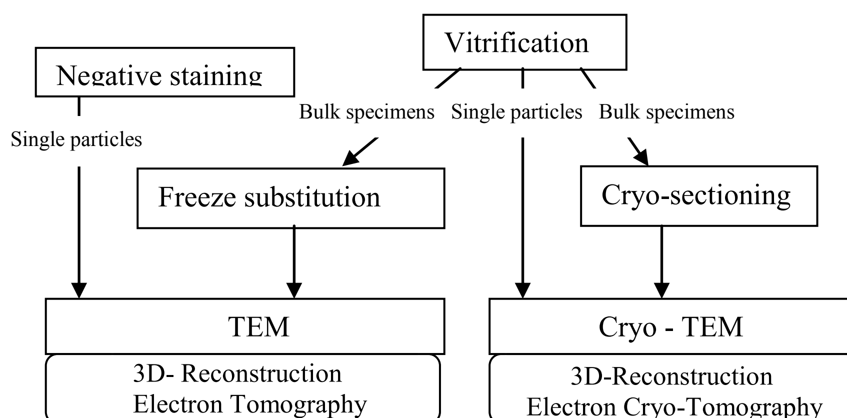

Table 1. Technical procedures for 3D solutions in EM

2. Methodology

2.1. Cryo-immobilization

The first step in the 3D reconstruction of cells or molecules in EM is the preservation of the biological specimens in a close-to-native state.

In the EM, samples have to be observed under high vacuum and the electron beam leads to the destruction of native biological structure. Furthermore, the electron beam cannot usually penetrate an entire cell, so cells and tissues have to be cut into thin sections (50-400 nm in thickness depending on the voltage of the EM used) for observation. A conventional and classic way to solve these problems involves chemical fixation of the specimens, followed by dehydration or drying by various methods. Usually, chemical fixation is achieved by aldehydes such as glutaraldehyde and paraformaldehyde in a buffer. These mixtures fix the structures by selective cross-linking between certain amino acid residues of proteins. The rest of the macromolecules, small molecules and ions are not immobilized by this method. Thus, they are easily removed or re-distributed during the dehydration or drying. Another important drawback of conventional chemical methods is the time needed for the fixation. The chemicals penetrate the sample by diffusion. For thin samples, less than a few micrometers in the shortest dimension, diffusion is fast enough to give rapid fixation, but for larger pieces of tissue or organisms with natural diffusion barriers, such as cell walls, exoskeletons or special layers of embryos, diffusion throughout all cells is very slow. During this time, up to hours in some cases, the cell structure outside its natural environment is slowly destroyed.

In contrast to these conventional methods, ultrarapid freezing immobilizes all molecules in a cell within milliseconds. It is also called "quick freezing" "cryo-immobilization" or "cryo-fixation". Ultrarapid freezing is highly desirable, because it allows the instantaneous fixation of all molecules in their current position. After this instantaneous immobilization it is very important to maintain this situation until the observation or image acquisition in the EM, in order to have a true snapshot of the cell at the moment of freezing [4].

The next optional steps are explained in the "*Cryosectioning and Freeze-substitution"* and *"Cryo-Electron Microscopy"*.

There are several methods of freezing cells, but they all have the same goal of removing heat from the sample so quickly that water molecules form an amorphous or vitreous non-crystalline ice. Thus, the optimal cryofixation is "vitrification". Such samples are called: frozen-hydrated or vitreous samples.

The methods to cryo-immobilize cells and molecules are:

- Plunge freezing
- Impact freezing (or slam- freezing)
- High pressure freezing
- Self-pressurized rapid freezing

For freezing suspensions or molecules or even small cells, plunging into a cooled cryogen, such as ethane or propane, is a common method. Another method, faster than plunging in its heat transfer, is slamming cells or tissues against a cooled metal block. For both impact- and plunge-freezing, it is possible to achieve up to 2-20 microns of good freezing depth. Beyond that, ice crystals form and destroy the tissue. However, most cells and tissues are much larger than a few microns, so we need another method to freeze them without ice crystal damage. The best method in these cases is High-Pressure Freezing (HPF), which freezes samples with liquid nitrogen under high-pressure conditions. Using HPF, relatively large volumes (200 microns and more) can be frozen without ice crystal damage.

2.1.1. Plunge freezing

Plunging samples into a cooled cryogen is a common method for freezing suspensions (viruses, liposomes, bicelles, micelles...), molecular assemblies (proteins, nucleic acids...), isolated organelles and cell structures and small cells (bacteria, parasites...) [5].

Two types of gas can be used: ethane and propane. Both of them are gases at room temperature and become liquids at low temperature: Propane is liquid between - 42ºC and -188ºC; and ethane, between -88ºC and -172ºC. They are transferred from a gas bottle to a container cooled by liquid nitrogen at -196ºC and they liquefy instantaneously. They are used because of their low temperature in liquid state, which allows plunging for freezing, and for their high thermal conductivity, which allows a quick transfer of heat from the sample to the liquid. They are kept in liquid nitrogen during the freezing process to ensure that they remain liquid and at the lowest temperature.

The samples must be immersed at high speed into the liquid coolant to optimize the heat exchange required to vitrify the specimen. Usually, acceleration 3 times greater than the gravitational force is required. Under these conditions, high freezing velocity, around 10^4 ºC/sec, is attained.

Figure 1. Plunge freezing equipment: Vitrobot Mark III (FEI Company) atthe CCiTUB Cryo-EM laboratory.

2.1.2. Impact freezing

Slamming or impact-freezing or "metal-mirror" is a process of rapidly projecting cells or tissue onto a cooled metal block. The copper block is cooled by liquid nitrogen or liquid helium from a Dewar flask. Some instruments have the copper block placed in a vacuum chamber in order to avoid any moisture contamination on the block surface. Good structural preservation, without ice crystal damage, goes from 10 to 30 microns in depth.

Impact-freezing is used to cryofix cells and tissues for further freeze-substitution or freeze-drying, or even for cryo-sectioning and cryo-EM [6].

The main advantage of this method is the possibility of vitrifying surface regions of quite large samples, nearly as large as the cooling block (between 1 and 3 cm in diameter, depending on the type of instrument). Another advantage, compared with plunge-freezing, is the high thermal conductivity of copper, much higher than organic liquids. The same amount of heat is transferred 10,000 times faster through copper than through liquid cryogen. This is why it is used for samples larger than "single particles" (seen in plunge-freezing).

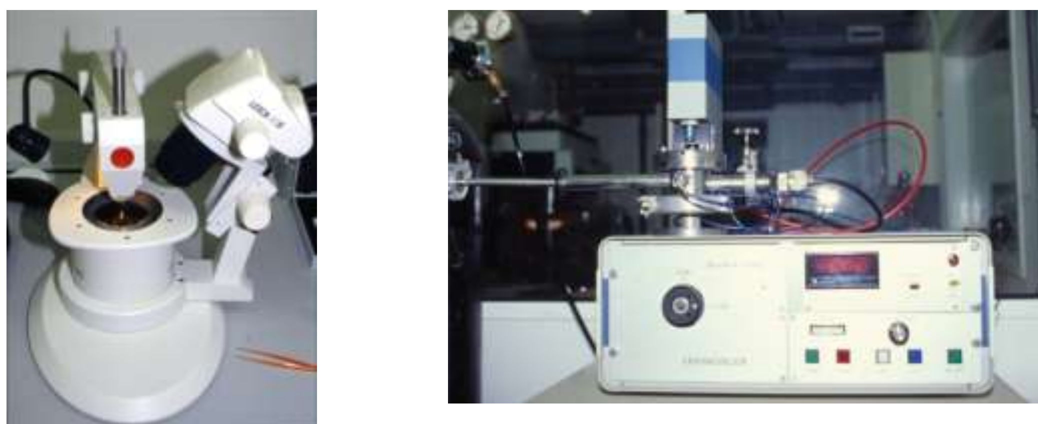

Figure 2. Impact freezing equipments: Leica EM-CPC cooled by liquid nitrogen and Leica Cryoblock, cooled by liquid nitrogen or liquid helium. Both at the CCiT Cryo-EM Laboratory.

2.1.3. High-pressure freezing

High pressure freezing freezes samples with liquid nitrogen under high pressure conditions. Using HPF, relatively large volumes (200 microns and more) can be frozen without ice crystal damage [7] [8]. This is possible because at high pressure (about 2,000 atmospheres or bar) ice crystal nucleation and growth are slowed down (homogeneous nucleation of water molecules to form crystals starts at -40ºC at ambient pressure and drops to -90ºC at 2,000 bar; and water is 1,500 times more viscous than at ambient pressure, reducing the ice crystal growth rate). High pressure lowers the freezing point of water (at 2,000 bar the freezing point is at a minimum of -22ºC) [9].

When freezing at ambient pressure, it takes a very high freezing rate to achieve good preservation, between 10^5 and 10^6 ºC/sec. In very viscous water under high pressure the cooling rate can be much lower, in the range of 10,000 to 30,000ºC/sec.

In HPF machines the pressure increase is synchronized with the temperature decrease. Pressure can be harmful when applied for too long times (>100 ms) to a biological sample. Therefore, pressure and cold must be simultaneously applied to the sample to achieve vitreous ice. The only problem in freezing a sample with HPF is the gaseous compartments, because these are compressed and their collapse may distort the sample. However, the aqueous phase is almost incompressible.

2.1.4. Self-pressurized rapid freezing (SPRF)

This new rapid-freezing method uses the increase of volume of water when it freezes naturally. The sample, preferably in an aqueous medium or buffer, is located into a thin-copper tube, which is projected into liquid ethane or propane in two speed steps. Thus, the cooling rate is slow at the ends of the copper tube and very fast in the middle of it. Therefore, the increasing of water volume at the

ends of the copper tube by ice crystals formation pressurizes the middle of the tube at the same time that freezing takes place in this part.

SPRF is a totally new technology described by Leunissen et al [10] recently.

Figure 3. High-pressure freezing Leica EMPact machine at the CCiT Cryo-EM Laboratory.

2.2. Cryo-sectioning of vitreous samples & Freeze substitution

2.2.1. Cryo-sectioning of vitreous samples

Cryo-sectioning of vitreous samples allows thin sections of cells or tissues at low temperatures to be taken without loss of their vitreous state. Most cells are too big to be imaged by EM, so microtomy is needed to make samples thin enough to be viewed under the electron beam. Even if the cells are small enough to be imaged by an EM of a certain voltage, it has been tested that the resolution is higher after sectioning.

Cryo-tomography of vitreous sections allows 3D reconstruction of macromolecular complexes within the natural cell environment [11].

The necessary instrumentation for vitreous sectioning is a cryo-ultramicrotome in an atmosphere totally free of moisture. One option is an anti-contamination glove box covering the cryo-ultramicrotome where it is possible to reach 0% of relative humidity (Fig. 4).

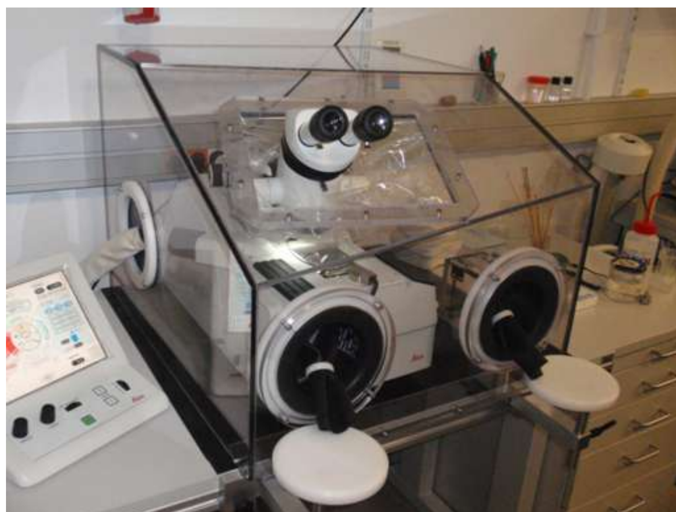

Figure 4. Cryo-ultramicrotome Leica UC6/FC6 with an anti-contamination glove box based on the prototype of Peters Lab. [12], at the CCiTUB Cryo-EM Laboratory.

2.2.2. Freeze substitution

Freeze substitution consists of the substitution of the vitreous ice by an organic solvent followed by embedding in a resin which after polymerization allows to take thin sections of the specimens. The samples are cryo-immobilized to get vitrification of the cell water and the amorphous ice is dissolved by the solvent at about -90ºC during freeze substitution. The samples are put in a medium composed by acetone, methanol or ethanol containing chemical fixatives such as uranyl acetate, osmium tetroxide, or glutaraldehyde, in different mixtures and different concentrations, depending on the type of sample and on the technique to be carried out later [13]. The samples are kept in the chosen medium at about -90ºC from several hours to 3 days. The freeze substitution time is dependent on the specimen, e.g., for culture cells we use 24 hours or less and for yeast or plant cells, 72 hours. After substitution the temperature is raised to the appropriated embedding temperature depending on the resin used. Epoxy resins are used for structural and tomographic studies without molecular labels and they polymerised at room temperature. Acrylic resins are used for immunolabeling, *in situ* hybridization and also for tomographic studies searching for their higher transparency [14].

Freeze substitution protocol for tomography
0.5% uranyl acetate in acetone

Step	Time	Temperature	Slope
T1	24 h	- 90˚C	
S1	2,5 h	until -40ºC	20˚C/h
T2	2h	- 40˚C	

Followed by Lowicryl HM 23 embedding at -40ºC.

Figure 5. Leica EMAFS (Automatic Freeze Substitution System) at the CCiTUB Cryo-EM Laboratory

2.3. Freeze drying and freeze fracture

2.3.1. Freeze drying

This term refers to the removal of ice from the vitrified sample by vacuum sublimation, before a heavy metal-carbon replica of the tri-dimensional surface is made. The replica is obtained by oblique and rotary deposition of a heavy metal, usually platinum, followed by coating with a strengthening layer of electron-translucent carbon. The angle of platinum deposition is 45º in a standard way, but it is changed depending on the height of the structure to be shadowed. For example, for caveolae, 23º are used and for DNA, around 8º. Then, the biological material is removed and the cleaned replica is mounted on a grid for examination in the TEM [6] [15].

2.3.2. Freeze fracture

The freeze-fracture technique involves physically fracturing a suitably frozen sample, making a metal-carbon replica of the frozen fractured surface under vacuum. The replica is made by oblique and unidirectional deposition of a heavy metal followed by carbon as it was explained before. The difference with the freeze-drying process is the uni-directionally of the shadowing [15].

Improved resolution in all replication techniques can be achieved using a mixture of tungsten/tantalum instead of platinum, but this metal mixture is more difficult to use reproducibililty.

In frozen cells the preferably planes of fracture are the interior of lipid bilayers of cell membranes [16]. For this reason, freeze-fracture is very useful to study intra-membrane proteins.

Figure 6. Freeze-etching system BAF 060 from BalTec at the CCiTUB CryoEM Laboratory

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2.4. Room temperature 3D electron microscopy

This subsection refers to the possibilities for 3D imaging of molecular or cell structures at room temperature. In general, the term 3D reconstruction is used when images are acquired in the EM in only one direction of the electron beam. One case is the reconstruction from the different orientations of a single particle on a grid. Single particles are isolated macromolecules, molecular assemblies, small organelles, small cellular structures or small organisms not needing sectioning. Another case is the reconstruction of a cell structure or organism from images obtained from serial sections.

The term electron tomography is commonly used when images from a specimen in the EM are recorded in as many directions of the electron beam as possible. This is achieved by tilting the holder supporting the grid containing the specimen. The sample can be an isolated specimen not needing sectioning or a structure inside a section. In practice, we tilt the grid around the a tilt axis of the goniometer, typically over the range from -70º to +70º, recording images at regular tilt intervals of 1º for example. These tilted images form a tilt series and are processed by software. Then, the acquired images are aligned with each other using suitable software, and from them a three-dimensional volume consisting of a stack of parallel images is reconstructed. The next step is the visualization or modelling of the 3D-structure. Summarizing, acquisition, alignment/reconstruction and modelling are the three steps in tomography.

There are two ways of preparing samples for EM 3D studies at room temperature: negative staining and freeze substitution. Negative staining is used for single particle analysis and reconstruction. It is a very simple technique based on the use of salts containing heavy metals such as uranyl acetate or formiate, ammonium molibdate or phosphotungstic acid. They provide an electrodense background to the grids and the single particles protrude from it. As a result, the image presents a negative contrast, the particles become clear over a dark background. On the other hand, freeze substitution is used, as it was mentioned before, to enable thin sectioning of bulk cells or specimens [17].

2.5. 3D Electron Cryo-Microscopy

This subsection refers to the possibilities for 3D imaging of molecular or cell structures in cryo-conditions. Single particle analysis of vitrified samples and electron cryo-tomography of vitreous specimens or sections differs from room temperature 3D EM in the temperature used to observe and image the specimen and enables a three-dimensional visualization of the macromolecular complexes, cells or tissues in a quasi-native environment [18] [19]. The temperature of the vitrified

sample during the cryo-work should be below -170ºC and the specimens aren't chemically fixed and stained with heavy metals. Although the radiation sensitivity of vitrified samples and their low contrast pose problems for information imaging, methods used now like low- dose of electrons imaging and high resolution CCD cameras make more efficient the cryo-EM work.

The procedures for sample preparation for 3D Cryo-EM are: vitrification for small isolated particles and vitrification followed by cryo-sectioning for bulk specimens.

Figure 7. Tecnai Spirit electron microscope, equipped for cryo and tomography at the CCiT Cryo-EM Laboratory.

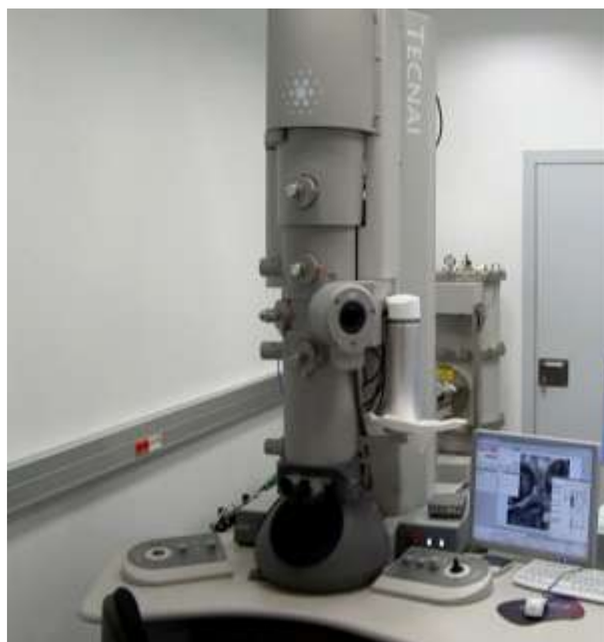
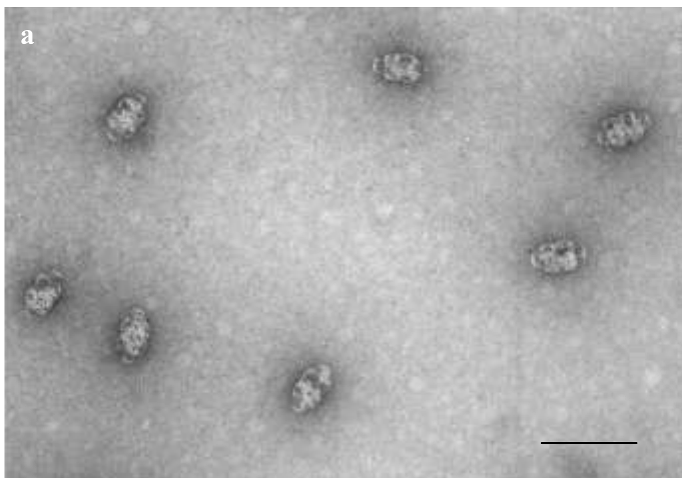
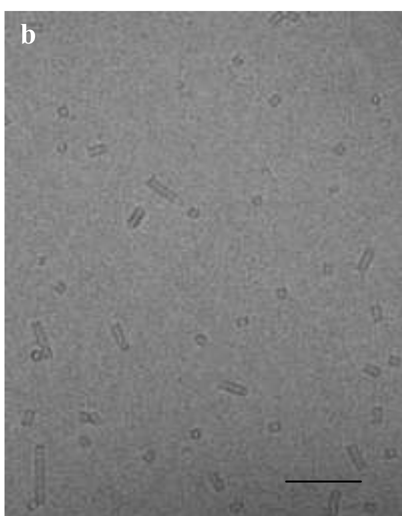


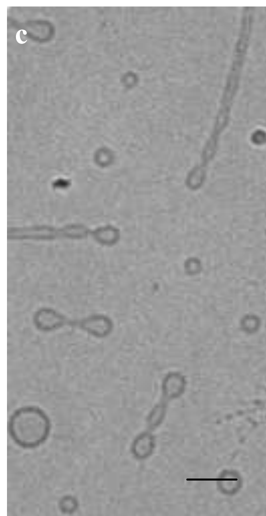
Figure 8. Tecnai F20 200 kV and field-emission gun (FEG) electron microscope. It is equipped for cryo and tomographic work with an anti-contamination device, the cryo-box, a low dose imaging mode, a 4k x 4k CCD Eagle camera and the FEI tomography software package. At the CCiT Cryo-EM Laboratory

3. Examples of applications

In the next plates, different examples of the describedr procedures are showed. They are focused mainly onthe optimal preparation methods to do three-dimensional studies in biological structures.







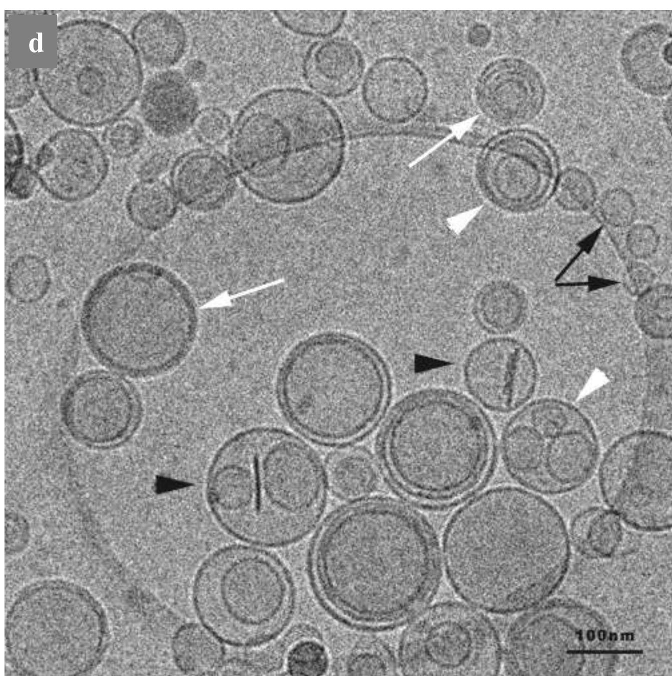


Figure 9. Single particles

a. Vaults imaged after negative staining with 2% uranyl acetate in water.
This image belongs to an study of these ribonucleoprotein particles [20]. It shows the high resolution that can be achieved with basic techniques such as negative staining to perform 3D-studies.

b. Hemocyanin protein . A solution of this protein was vitrified by plunge freezing in ethane using the Vitrobot. 3 µl of the solution were deposited on a holey carbon copper grid, kept during 30 seconds in a 100% humidity chamber and blotting with filter papers before plunging in the liquid ethane cooled by liquid nitrogen.
This is a second way to prepare single particles to perform 3D-studies. In this case the observation and imaging were made in an electron cryo-microscope.

c. Vesicles isolated from the extracellular matrix in a bacterial colony. The vesicles were vitrified by plunging in ethane and directly transferred into liquid nitrogen to the electron cryo-microscope and imaged at -180ºC.

d. Bicosomes: liposomes containing bicelles.This image was taken at -180ºC in the F20 cryo-microscope after vitrification in liquid ethane. Bicellar systems are mixtures of aliphatic long chain and short-chain phospholipids. Their morphology depends on the composition, temperature, hydration and the long/short chain phospholipid molar ratio. These systems may form spherical micelles, discoidal bilayers, rod-like micelles and perforated bilayers. Freeze-fracture EM and cryo-TEM are the most suitable techniques to study these systems [21].

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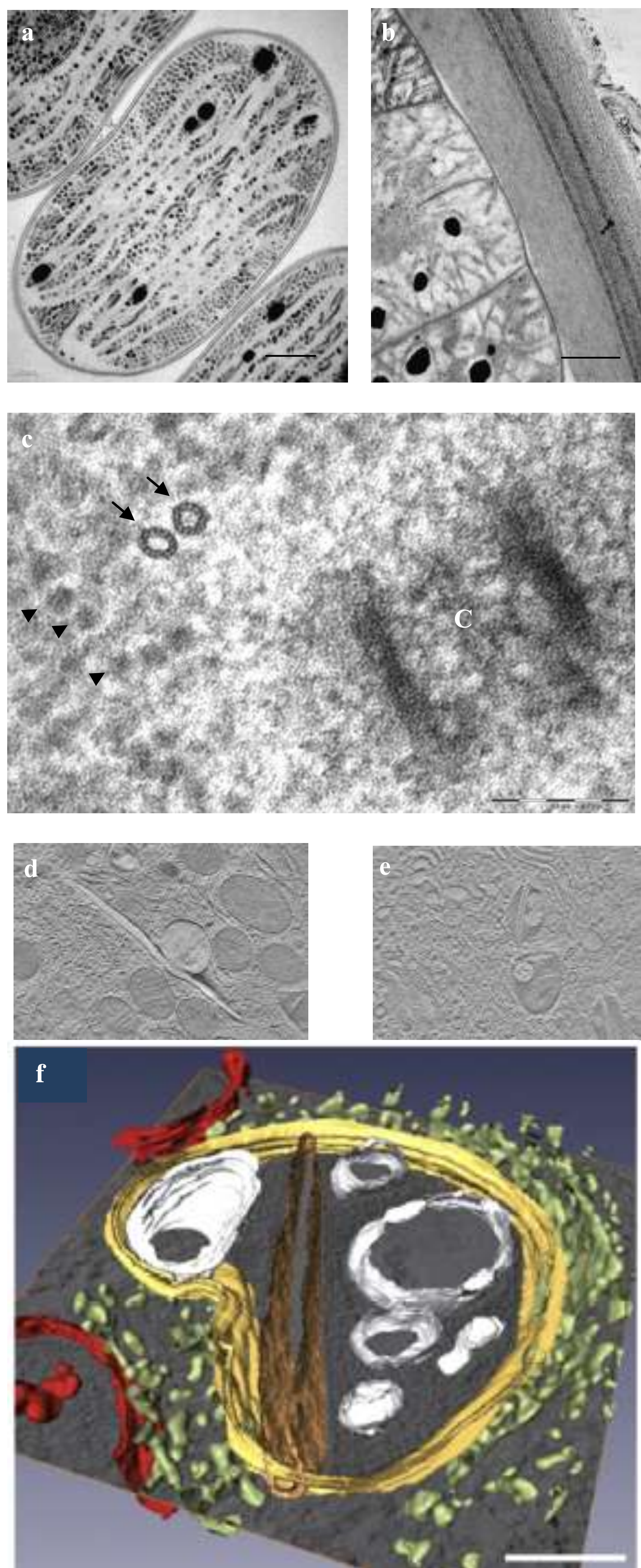


Figure 10. Bulk specimens

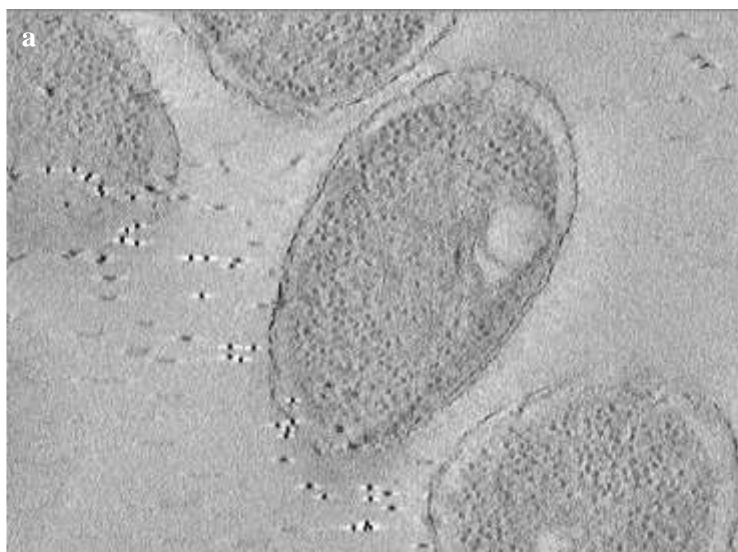
Freeze substitution:

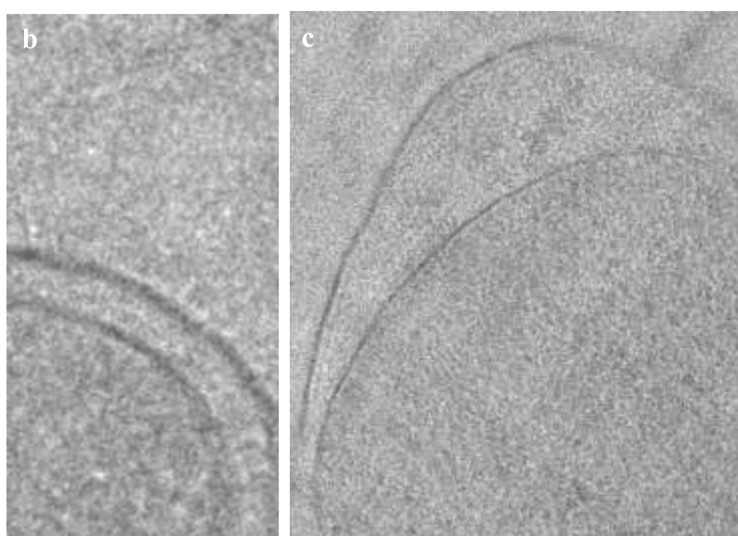
a & b. Cianobacteria. High pressure freezing without any cryoprotectant and filler. Once frozen, they were freeze-substituted in 2% osmium tetroxide and 0.1% uranyl acetate in acetone and embedded in Epon resin [see 22].

c. Centrosome area of a neuroblast in the brain of a Drosophila embryos. A centriole (c), two microtubules (arrows) with the typical subunits and the ribosomes (arrowheads) out of the area surrounding the centrioles can be seen. The embryos brain was cryofixed by high pressure freezing using a mixture of yeast paste and dextran like filler. Then, it was freeze-substituted and embedded in Epon.

d. & e. Computational tomographic slides (~3 nm) of the tomogram obtained from 200 nm sections of high-pressure frozen, freeze-substituted with 0.5% uranyl acetate in acetone and Lowicryl HM23 embedded Rubella infected cells. To stabilize the sections under the electron beam they were collected on holey carbon Quantifoil® grids. Rubella virusbuilds a factory around modified lysosomes, known as "cytopathic vacuoles" or "CPVs", by recruitment of mitochondria, rough endoplasmic reticulum elements and Golgi stacks [23].

f. 3D model after segmentation and visualization of the CPV of a Rubella replicon transfected cell. Single axis tilt series were obtained with angle ranges between -60 and +60º and a 2º angular increment. Color code is as follows: CPV,yelow; straight sheet, brown; RER, green; mitochondria, red; nucleus, pink; vesicles and vacuoles, white; cytoplasm, grey [23].





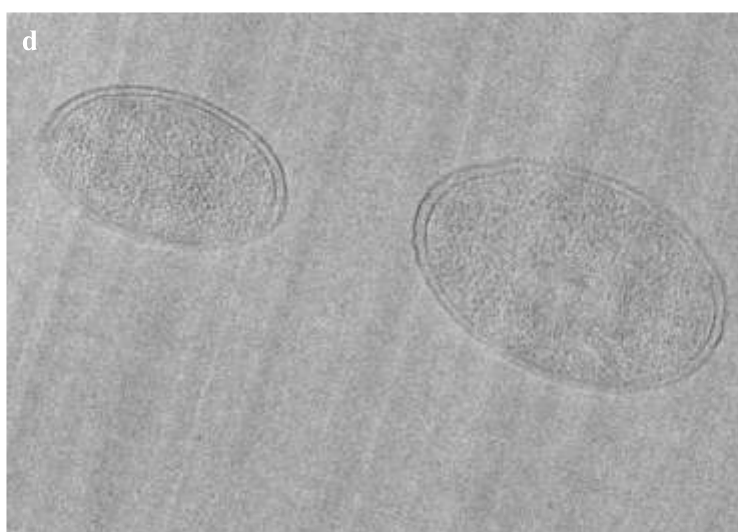


Figure 11. Bulk specimens

a.Antarctic bacteria *Pseudomonas deceptionensis* .

Computational tomographic slides (~3nm) of the tomogram obtained from 200 nm sections of high pressure frozen, freeze-substituted with 2% osmium tetroxide /0.1% uranyl acetate and Epon embeddedAntarctic bacteria.
Gold particles used as fiducial markers for tomography can be seen in the image with a shadow after tilting.

Cryosectioning of vitreous samples:

b, c & d. *Ps. deceptionensis.*
Cryosections of vitreous samples observed in an electron cryo-micoscope (CEMOVIS: Cryo-electron microscopy of vitreous sections).
Bacteria colonies were mixed with dextran at a final concentration of 20%-30% and frozen by high pressure freezing into small copper tubes. The tubes were sectioned at -150ºC in a cryo-ultramicrotome equipped with an anti-contamination glove box. Cryosections of 50 nm were collected on holey carbon Quantifoil grids.
Details of the bacteria envelope and extracellular area is shown in b, periplasmic space in c and a panoramic view of this kind of sections in d.
CEMOVIS allows the study of the macromolecular structures inside the cell (*in situ)* in the closet to the native state conditions.

The final goal of this technique in development known as CETOVIS (cryo-electron tomography of vitreous sections) is to have the 3D-information of the cellular nano-machines in their natural environment.

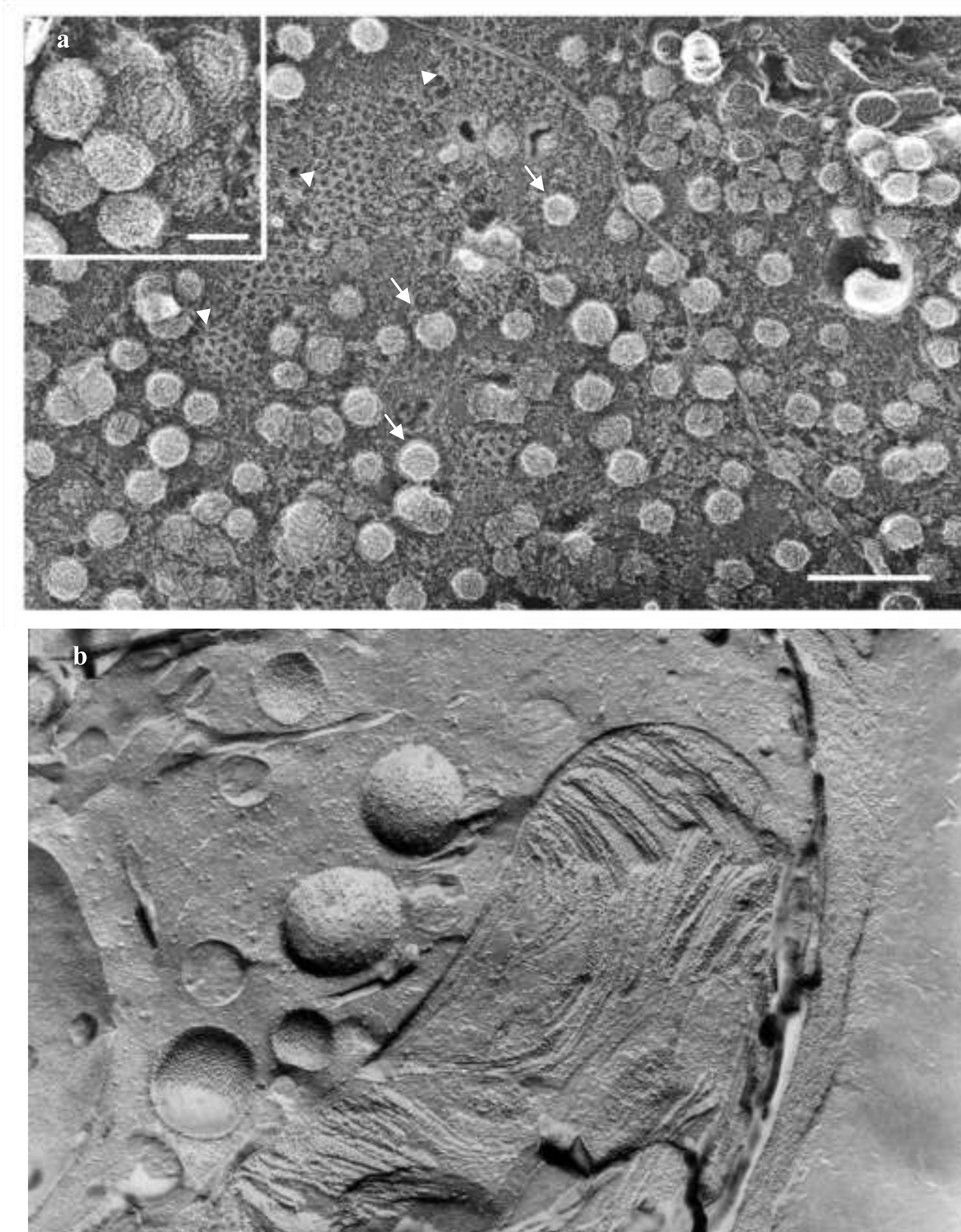


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Figure 12.
a. Plasma membrane lawn of the ventral membrane of a 3T3 adipocyte. Membranes attached to a substrate were frozen by impact freezing and dried at 10^{-7} mbar and -90ºC . The dried surface was shadowed with platinum and carbon in a rotator way in order to make a replica of it. Caveolae (arrows) and clathrin lattices (arrowheads) are revealed [24].
b. Alga frozen by propane immersion which was freeze-fractured at -150ºC and the rough surface obtained was shadowed with platinum at 45º and carbon at 90º in an unidirectional way. Intramembrane proteins can be studied with this technique [13].

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References

[1] [Echlin P. 1992 *Low Temperature Microscopy and Analysis*, Plenum Publishing Corporation, New York

[2] [Steinbrecht R.A, Zierold K. 1987 *Cryotechniques in Biological Electron Microscop,y* Springer-Verlag

[3] Frank J. 2006 *Three-dimensional electron microscopy of macromolecular assemblies: visualization of biological molecules in their native state,* Oxford University Pres

[4] Cavalier A., Spehner D, Humbel B.M. 2008 *Handbook of Cryo-preparation Methods for Electron Microscopy*, CRC Press

[5] Dubochet J., Adrian M., Chang J-J., Homo J-C., Lepault J., McDowall A.W., Schulz P. 1988 *Quarterly Review of Biophysics* **21** 129

[6] Ros-Baro A., López-Iglesias C., Peiro S., Bellido D., Palacin M., Zorzano A., Camps M. 2001
Proc Natl Acad Sci U S A **98** 12050

[7] Galy V., Askjaer P., Franz C., López-Iglesias C., Mattaj I.W. 2006 *Curr Biol.* **16** 1748.

[8] Nebot M., Deroncele V., López-Iglesias C., Bozal N., Guinea J., Mercade E. 2006 *Microb Ecol*. **51** 501

[9] Studer D., Humbel B. M., Chiquet M. 2008 *Histochem. Cell Biol.* **130** 889

[10] Leunissen J.L.M., abd Yi H. 2009 *J. Microsc.* **235** 25

[11] Pierson J., Sani M., Tomova C., Godsave S., Peters P.J. 2009 *Histochem Cell Biol.* **132** 253

[12] Pierson J., Fernández J.J., Bos E., Amini S., Gnaegi H., Vos M., Bel B., Adolfsen F., Carrascosa J.L., Peters P.J. 2010 *J Struct Biol.* **169** 219

[13] Porta D., López-Iglesias C. 1998 *Tissue Cell.* **30** 368

[14] Visa N., Quintana C., López-Iglesias C., Fibla J., Gonzàlez-Duarte R., Santa-Cruz MC. 1993 *Microsc Res Tech*. **24** 453

[15] Severs J., Shotton D.M. 1995 *Rapid freezing, freeze fracture and deep etching*. Wiley & Sons. New York

[16] López O., López-Iglesias C., Cócera M., Walther P., Parra J.L., De La Maza A. 2004 *J Struct Biol*. **146** 302

[17] McIntosh J.R. 2007 *Methods in Cell Biology*. Vol.**79**. Elsevier

[18] Baumeister, W. 2002 *Curr Opin Struct Biol* **12** 679

[19] Al-Amoudi, A., Chang J.J., Leforestier, A., McDowall, A., Salamin, L.M., Norlen, L.P.O., Richter, K., Sartori Blanc, N., Studer, N. and Dubochet, J. 2004 *EMBO J.* **23** 3583

[20] Querol-Audi J., Pérez-Luque R., Fita I., López-Iglesias C., Castón J.R., Carrascosa J.L, Verdaguer N. 2005 *J.Struct. Biol.* **151** 111

[21] Rodríguez G., Soria G., Coll E., Rubio L., Barbosa-Barros L., López-Iglesias C., Planas A.M.., Estelrich J., de la Maza A., López O. 2010 Biophys J. **99** 480

[22] Frias A., Manresa A., de Oliveira E., López-Iglesias C., Mercade E. 2010 *Microb Ecol.* **59** 476

[23] Fontana J., López-Iglesias C., Tzeng W.P., Frey T.K., Fernández J.J., Risco C. 2010 *Virology* **405** 579

[24] González-Muñoz E., López-Iglesias C., Calvo M., Palacín M., Zorzano A., Camps M. 2009 *Endocrinology* **150** 3493