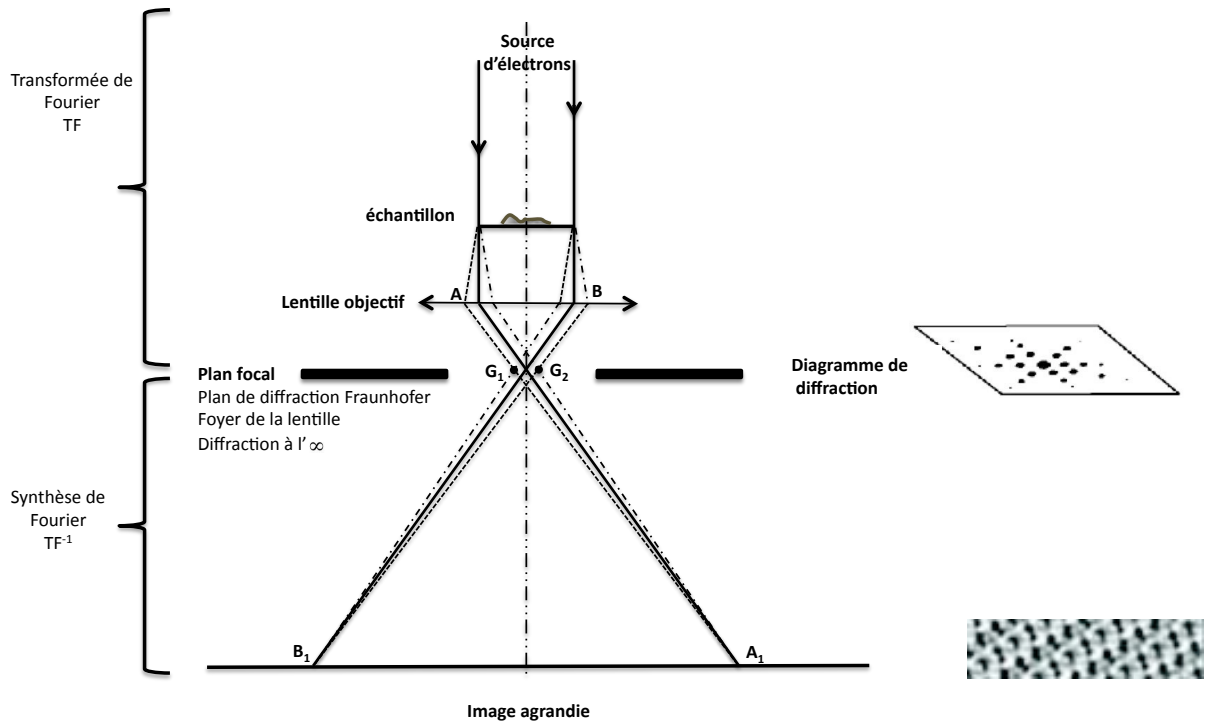
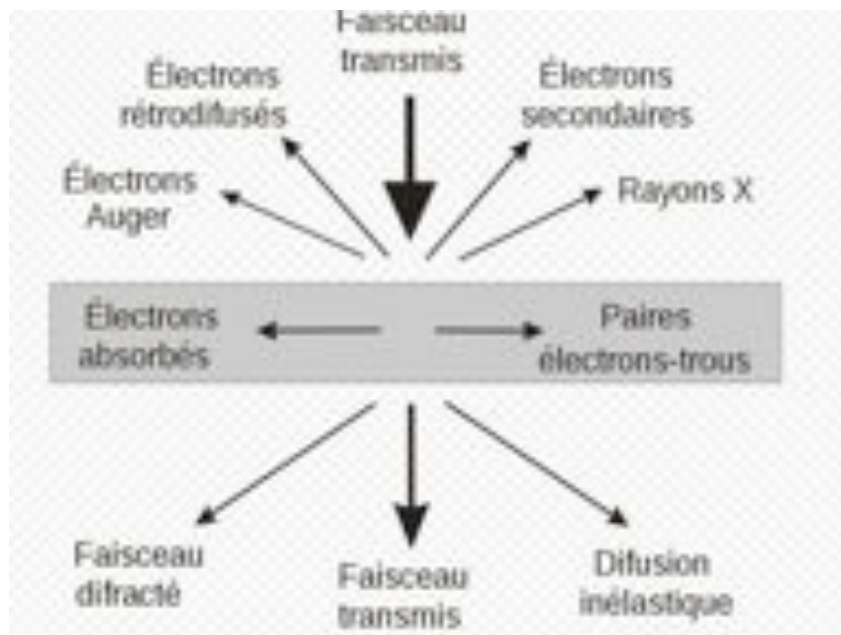


Formation de l'image, Théorie d'Abbe (1873)



Les différents modes d'observation

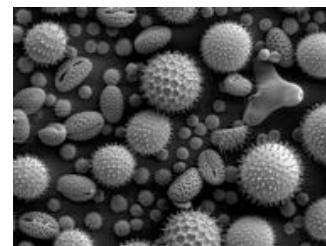
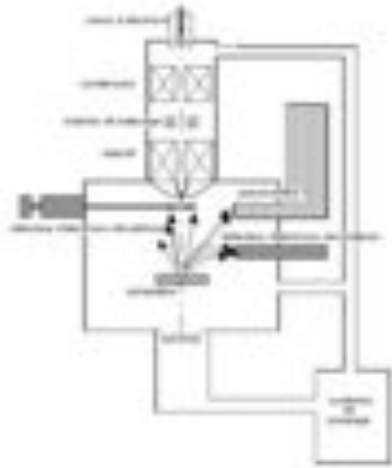


Microscopie électronique à balayage (MEB)

Scanning electron microscopy (SEM)

Electron secondaires et primaires rétrodiffusés

- Spécimen très épais
- Topographie 3D
- Résolution faible
- Grande profondeur de champ
- Tensions d'accélération faibles

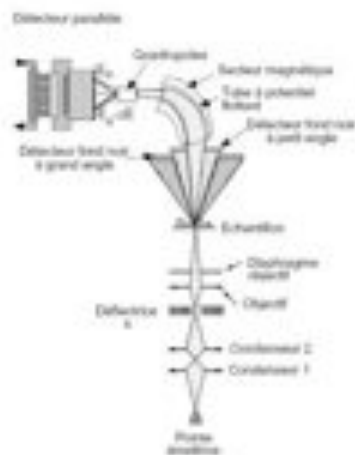


Les électrons secondaires apportent de l'information sur la topographie
Les électrons rétro-diffusés apportent le contraste de l'image

La microscopie électronique analytique

La microscopie électronique à balayage en perte d'énergie

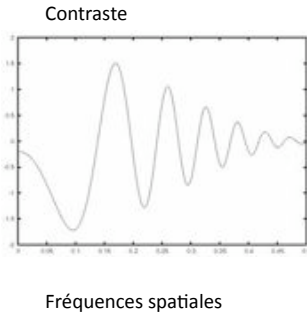
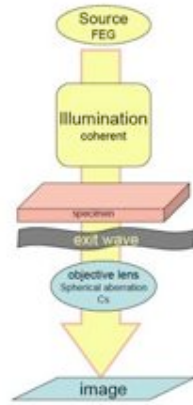
STEM-EELS



La fonction de transfert de contraste

Finalement, l'image représente une projection de la structure de l'objet modulé par un terme dépendant des caractéristiques optiques du microscope. Ce terme, appelé la fonction de transfert de contraste, décrit les effets de la qualité du contraste provenant à la fois d'effets d'amplitudes et d'effets de phases mais aussi la mise au point, l'aberration de sphéricité, la cohérence du faisceau électronique etc...

En cryo le contraste d'amplitude n'est que de 7% donc on le néglige pour se concentrer sur la fonction de contraste de phase qui se présente sous la forme :



$$H(k) = 2 \cdot [(1-W) \cdot \sin\gamma - W \cdot \cos\gamma]$$

Avec:

k = fréquence spatiale (espace réciproque 1/distance)

$\gamma = 2\pi \cdot (0,25 \cdot k^4 \cdot Cs \cdot \lambda^3 - 0,5 \cdot \lambda \cdot \Delta z \cdot k^2)$

W = pourcentage du contraste d'amplitude

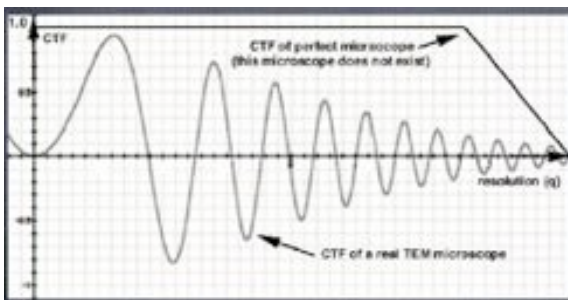
λ = longueur d'onde des électrons

Cs = Constante d'aberration de sphéricité de la lentille

Δz = sous-focalisation durant la prise de vue

Contrast transfer function

The image formation in electron microscopy can be described by the action of the contrast transfer function (CTF). This CTF is independent of any particular specimen, it is only dependent of the optical characteristics of the EM used.



The shape of the CTF:

CTF oscillates as we go from low to high spatial frequencies.

There are "passbands" where it is NOT equal to zero (good "transmittance") and there are "gaps" where it IS equal (or very close to) zero (no "transmittance").

When it is negative, positive phase contrast occurs, meaning that atoms will appear dark on a bright background.

When it is positive, negative phase contrast occurs, meaning that atoms will appear bright on a dark background.

When it is equal to zero, there is no contrast (information transfer) for this spatial frequency. The exact locations of the zero crossings (where no contrast is transferred, and information is lost) depends on the defocus.

The shape of the CTF depends on several parameters:

- defocus [A] - which describes the deviation in the focus of the objective lens from the "Gaussian focus."
- spherical aberration coefficient [mm] - which describes the spherical aberration of the wave front in the objective lens.
- source size [1/A] - which describes the illumination divergence, expressed as a size in the back focal plane (hence a quantity in reciprocal space).
- defocus spread - which describes the spread of defocus due to the spread of electron energies or to the fluctuation of lens current.

The only parameter being varied in the experiment is the defocus. Depending on the defocus setting, different features of the object appear enhanced or suppressed in the image

Contrast transfer function

Dans un microscope électronique, la FTC caractérise la proportion et la qualité du contraste provenant à la fois d'effets d'amplitudes et d'effets de phases. Elle se présente sous la forme :

$$H(k) = 2 \cdot [(1-W) \cdot \sin\gamma - W \cdot \cos\gamma]$$

Avec:

k = fréquence spatiale (espace réciproque 1/distance)

$$\gamma = 2\pi \cdot (0,25 \cdot k^4 \cdot Cs \cdot \lambda^3 - 0,5 \cdot \lambda \cdot \Delta z \cdot k^2)$$

W = pourcentage du contraste d'amplitude

λ = longueur d'onde des électrons

Cs = Constante d'aberration de sphéricité de la lentille

Δz = sous-focalisation durant la prise de vue

Phase contrast transfer function

Phase factors: -Spherical aberration

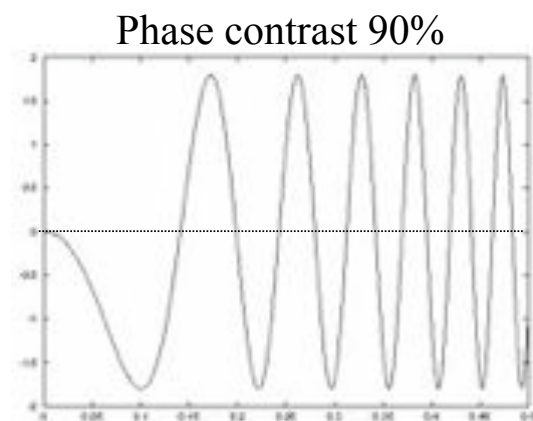
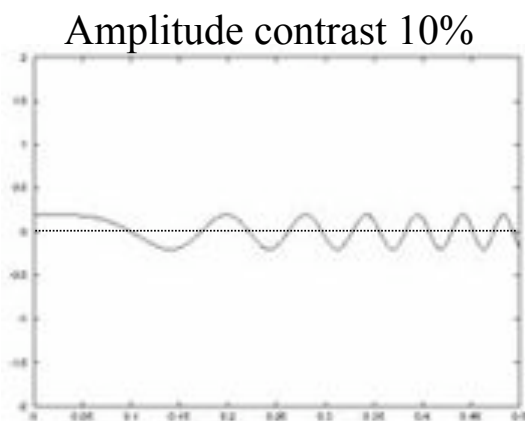
-Defocus value

Amplitude contrast transfer function

Amplitude factors: -(objective) apertures

-Spatial coherence envelope (non-parallel, convergent beam)

-Temporal coherence envelope (non monochromatic beam, instabilities of the gun and lenses).

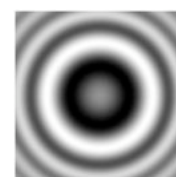
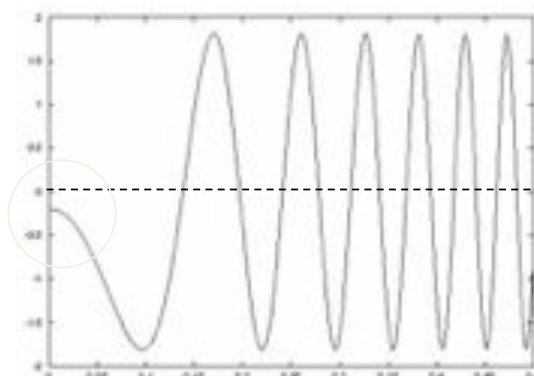


$$2 \cdot W \cdot \cos\gamma$$

$$2 \cdot (1-W) \cdot \sin\gamma$$

CTF total

W = 10 %,
Cs = 1 mm,
 $\lambda = 0,025 \text{ \AA}$
 $\Delta z = -3,5 \text{ \mu m}$



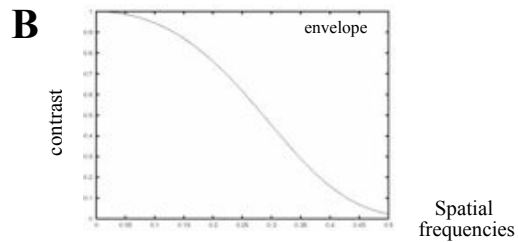
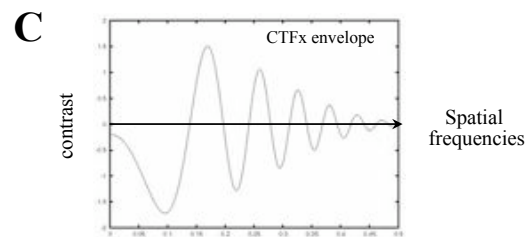
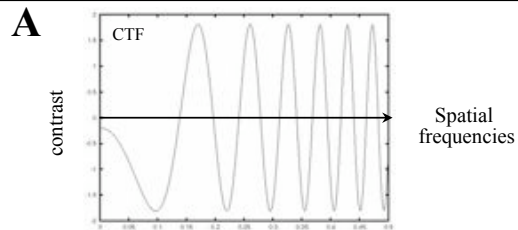
Do not forget
the envelope

Envelope function from the CTF :

Determine the maximum of the transmitted spatial frequencies (limit of the information *i.e.* the highest resolution attainable with the microscope)

$$E(u) = E_s(u)E_c(u)E_d(u)E_v(u)E_D(u)$$

$E_s(u)$: instability of the source $E_v(u)$: spécimen vibration
 $E_c(u)$: Chromatic aberration $E_D(u)$: detector
 $E_d(u)$: Specimen drift

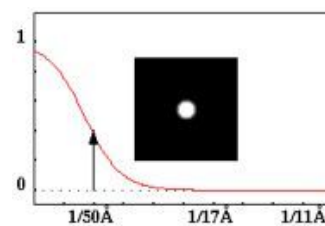


Image



Pixel size: 5.5 Å

Low-pass filter

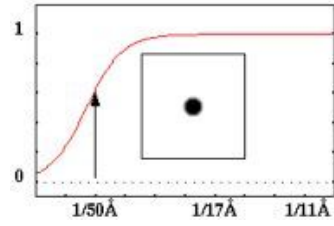


Image



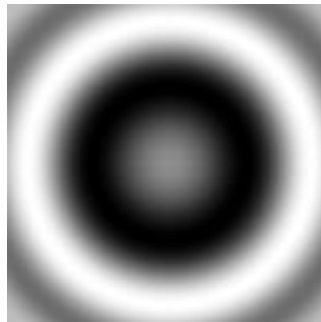
Pixel size 5.5 Å

High pass-filter



object

X



CTF defocus 1

=



Image altered by CTF

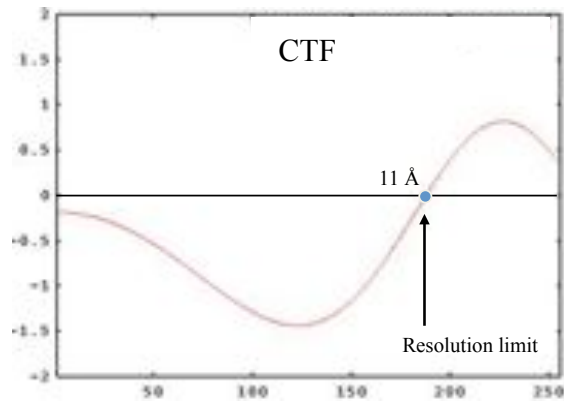
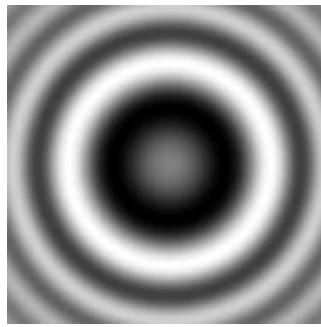


Image altered by CTF and contrast inverted



object

X



CTF defocus 2

=



Image altered by CTF

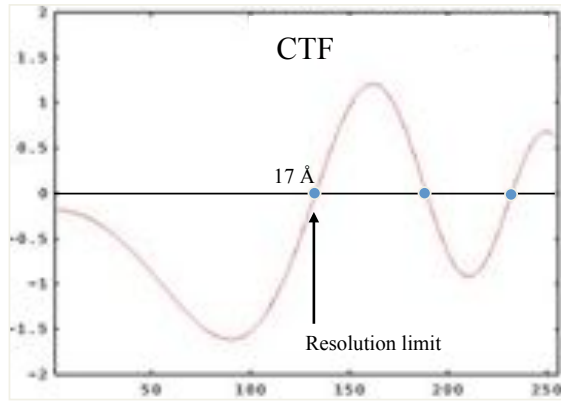
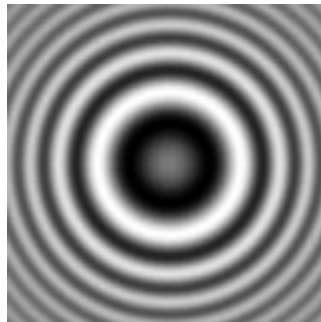


Image altered by CTF and contrast inverted



object

X



CTF defocus 3

=



Image altered by CTF

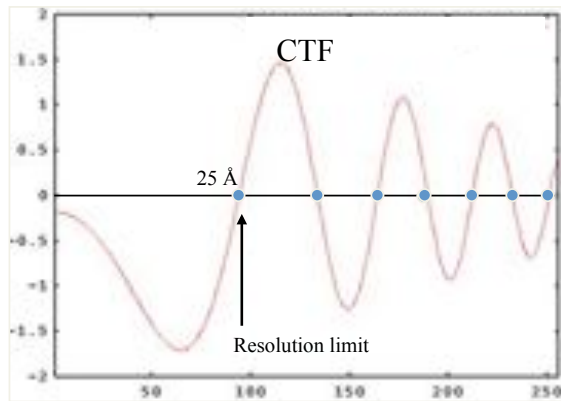
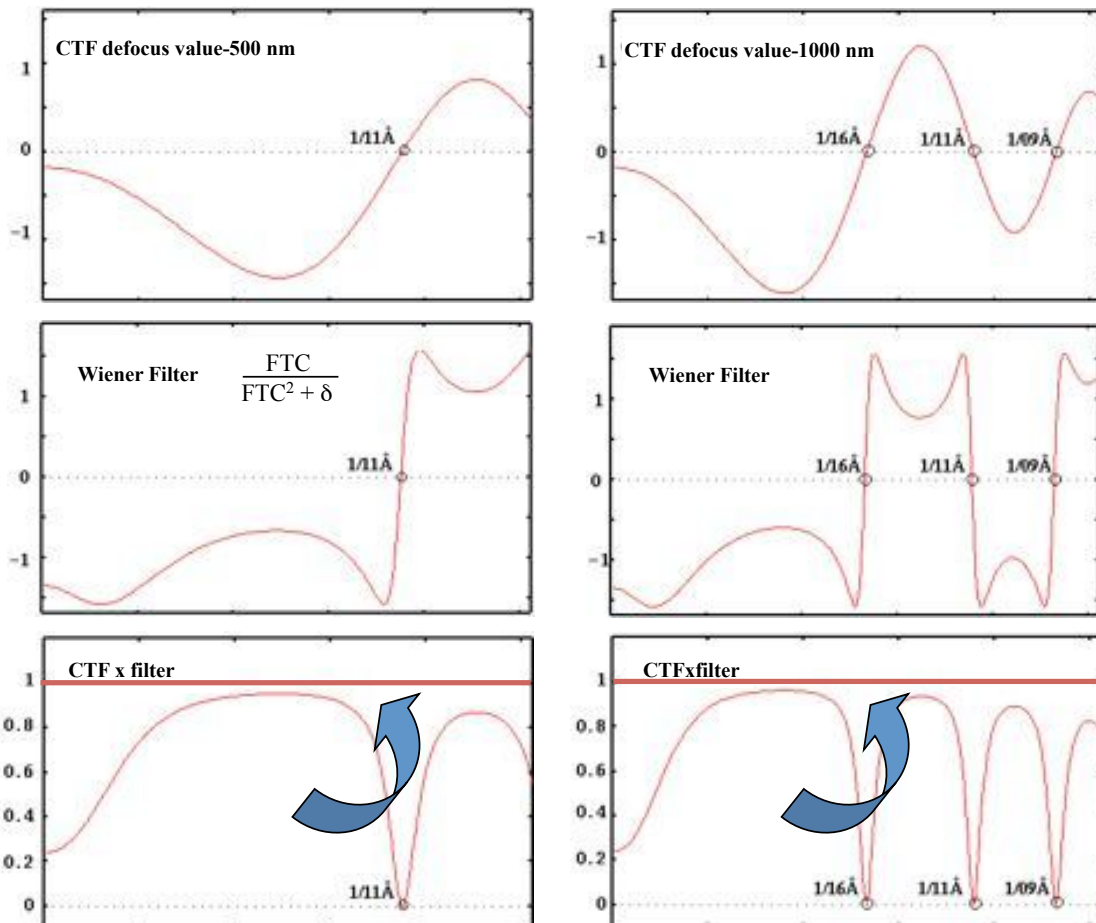
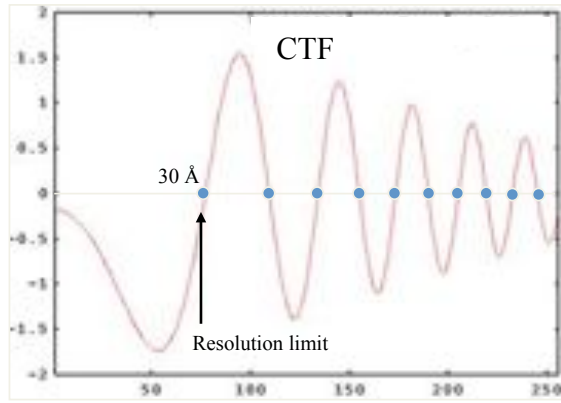
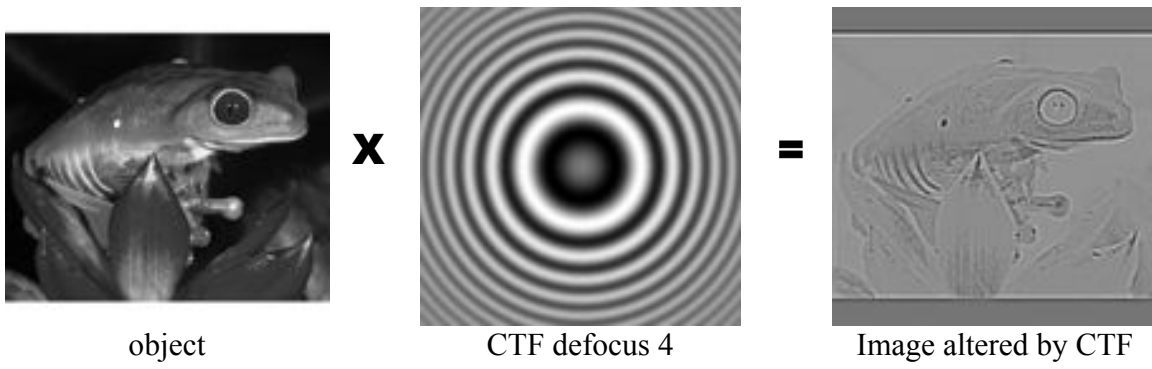
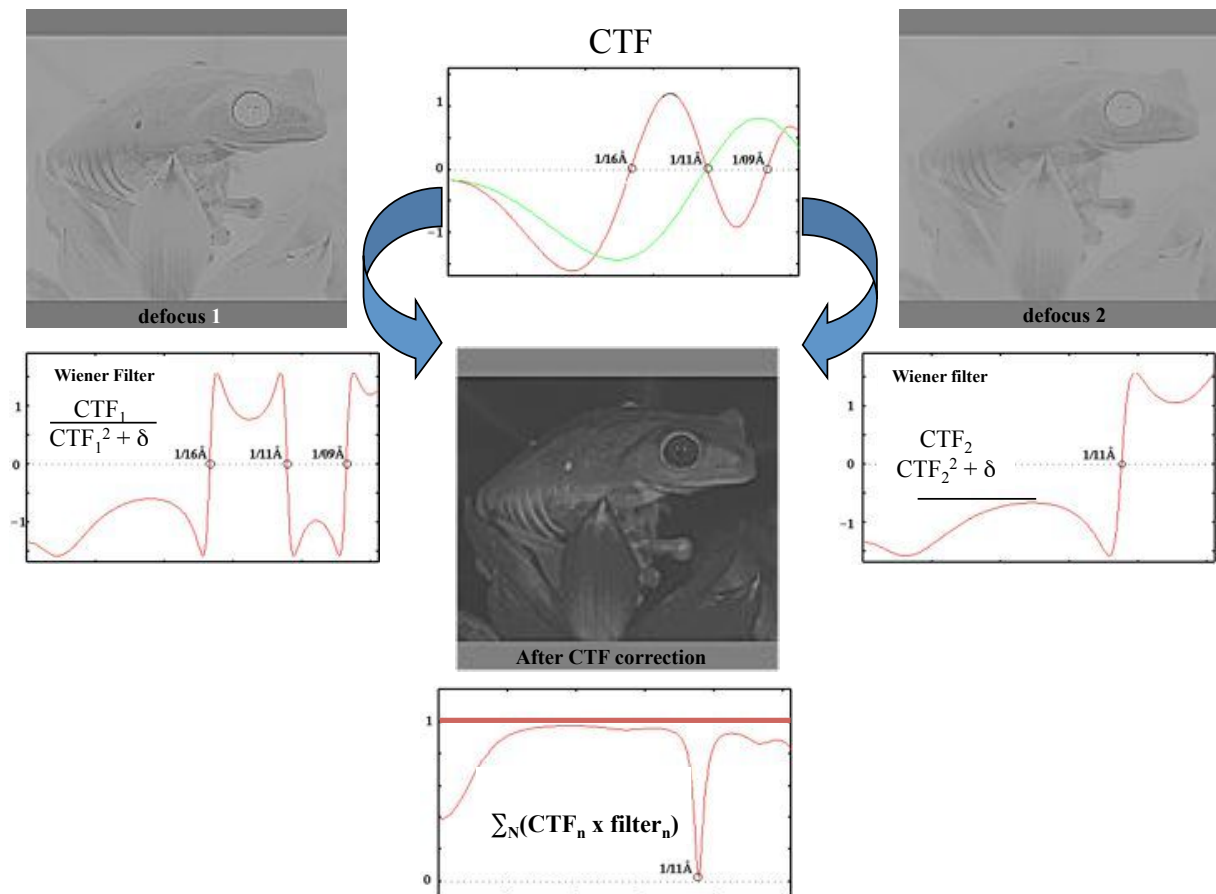


Image altered by CTF and contrast inverted





CTF correction

In CTF correction, we attempt to retrieve the undistorted object from the image. This attempt is compromised by the presence of noise; i.e., recovery of the object is never ideal.

Since the CTF always has zero crossings, part of the information about the object is lost.

This is why we make use of *several images obtained at different defocus settings, hoping that the resulting CTFs $H_n(k)$ jointly (after appropriate weighting) cover the whole Fourier space without gap.*

We also apply a filter: Wiener filter in order to retrieve some of the information.

Préservation de la structure

- Avant l'acquisition d'images l'échantillon biologique doit être protégé contre la radiation et le vide poussé générés par le microscope électronique
 - les électrons interagissant avec la matière organique non-protégée détruisent irrémédiablement sa structure



Préservation de la structure en MET

- **Specimens fins (0.5 μm), particules isolées et cristaux 2D haute résolution**
 - Coloration négative
 - Cryo-microscopie électronique
- **Specimen épais: cellules et organismes**
 - Tomographie électronique cellulaire
 - CEMOVIS (cryo electron microscopy of vitreous sections) pour échantillons très épais.

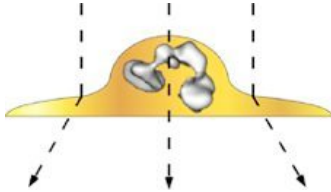
Preparation of the specimen

Specimen needs to be protected against:

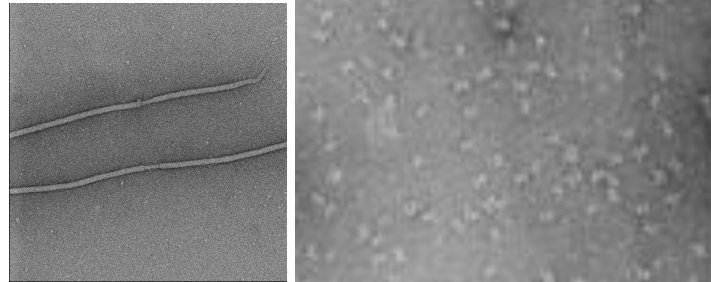
- High vacuum
- Severe irradiation from the electron beam

Biological specimen is made of light atoms (poor contrast)

Negative staining



When stain (heavy metal salts) is added to a sample, the stain surrounds the sample but is excluded from the volume occupied by the sample; hence the use of the term 'negative stain'



Benefits

- Very high contrast
- Radiation damage limited
- Sample easy to prepare

Drawbacks:

- Slight distortion due to drying process
- Resolution limited to 20Å

Protocole Coloration négative simple couche



- Les grilles de cuivre sont préparées au préalable et recouverte d'un film de carbone (film continu ou film à trous),
- Une meilleure répartition des particules est obtenue en soumettant les grilles avant adsorption à un effluvage (sous un vide peu poussé, l'effluvage sera obtenu par une décharge électrique qui ionisera les molécules d'air résiduelles, la surface des grilles sera alors hydrophile).
- Addition de 3 μ l de la solution contenant la macromolécule à étudier
- Addition d'une solution d'acétate d'uranyle 2% à la goutte contenant la macromolécule à étudier, puis l'excès de liquide est éliminé à l'aide d'un papier filtre et la grille est séchée.

Propriétés de la coloration négative

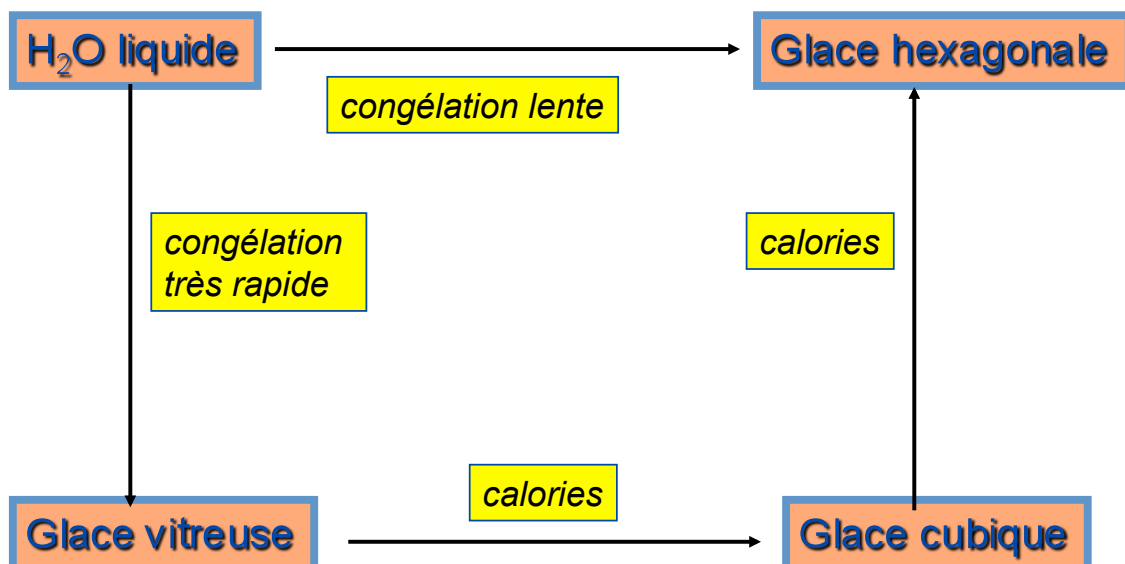
- Bon contraste
- Contraste d'amplitude (lié à la perte d'électrons au passage du faisceau dans l'échantillon et les diaphragmes des lentilles à cause de la diffusion (élastique) augmentée d'électrons par les atomes lourds)
- Les images ne montrent pas fidèlement les structures internes du matériel biologique mais seulement les surfaces moléculaires accessibles au colorant
 - Utile pour les études structurales à basse résolution, 20Å au mieux (pour calculer un modèle initial 3D qui sera raffiné par la suite en utilisant les images de haute résolution *i.e.*, les images de la cryo-MET à faible dose d'électrons et sans colorant)
- Il peut y avoir des artefacts dus à l'utilisation d'un colorant: accumulation du colorant, légère déformation du spécimen.

Cryo-microscopie électronique

(Préservation du spécimen dans des conditions proches des conditions physiologiques)

La meilleure des solutions pour la préparation des échantillons !!!

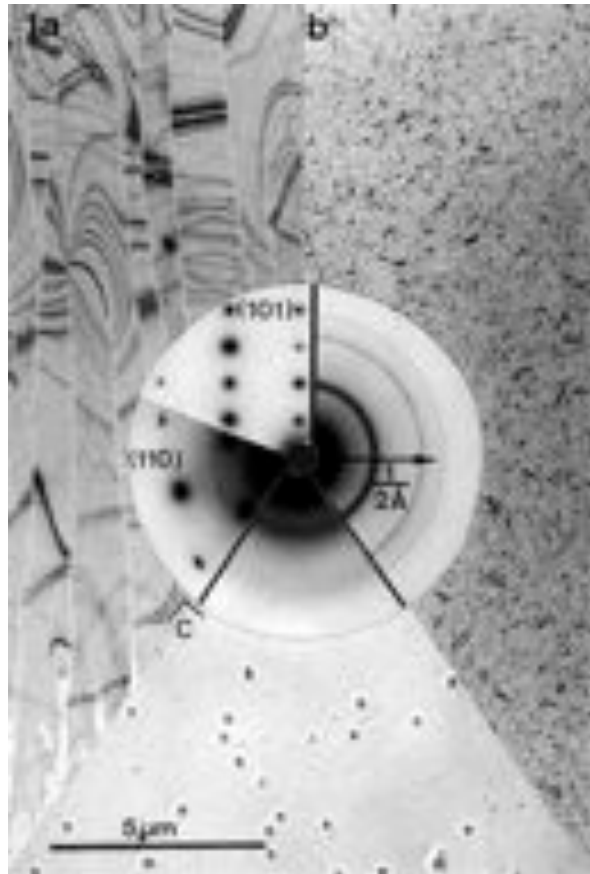
Vitrification : La clé est la congélation rapide



Trois états de l'eau glace

- a) hexagonale
- b) cubique
- c) vitreuse

Dubochet *et al.*, (1982)



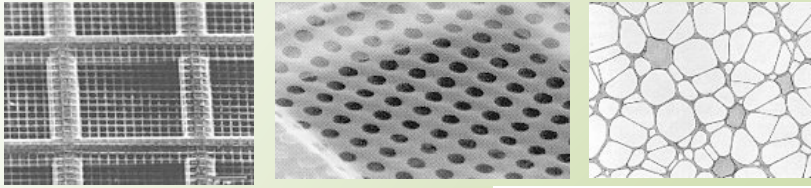
Pourquoi la congélation rapide ???

- **Pour produire la glace vitreuse en évitant la formation des cristaux**
 - Spécimens minces (<500 nm; e.g., organelles et protéines isolées et petites cellules)
 - “Plunge-freezing” où le spécimen est rapidement plongé dans éthane liquide (-180°C), ainsi la température chute avec la vitesse de 10^4 °C/s.
 - Echantillon congelé-hydraté obtenu par “plunge-freezing” est maintenu à la température d’azote liquide ou de hélium liquide pendant la prise d’images

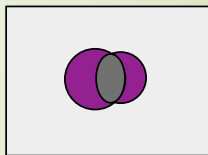
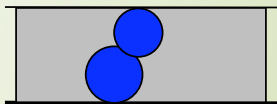
La cryo-MET sur spécimen congelé-hydraté

Protocole de préparation des échantillons

Supports d'observation

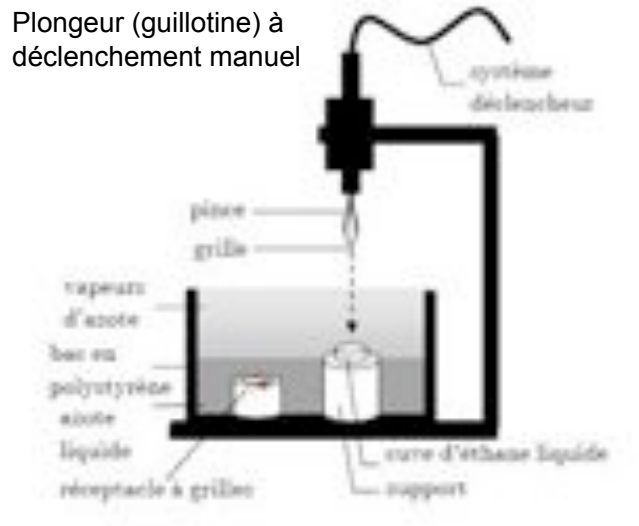


Une couche de glace vitreuse se forme dans les trous du carbone



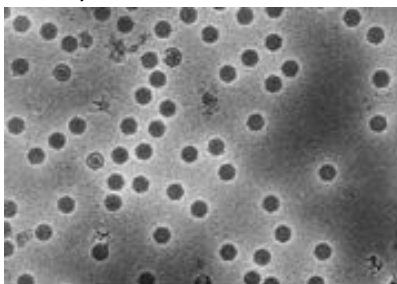
Condition physiologique
Contraste de phase

Plongeur (guillotine) à déclenchement manuel

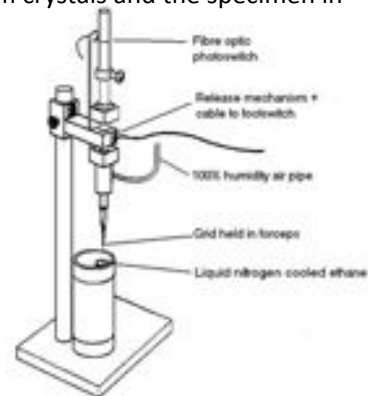


Frozen-hydrated specimen -Cryo Electron Microscopy

The specimen is frozen, vitrified at liquid nitrogen temperature using liquid ethane. The process is very fast so the water does not have time to form crystals and the specimen is frozen-hydrated



Electron micrograph of adenovirus embedded in rapidly frozen vitreous ice. This picture was taken by Dubochet and colleagues (Adrian et al., (1984)) and appeared on the cover of the issue of Nature.



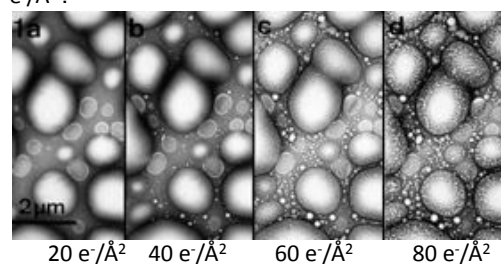
Severe irradiation damage: a dose higher than 20 electrons/Å² causes severe irradiation damage. We need to work at low dose of electrons, typically 15 e⁻/Å²:

Very low contrast:

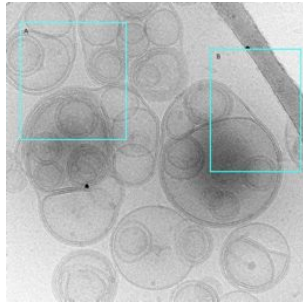
Reduction of noise by averaging



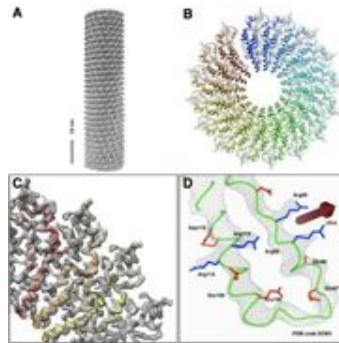
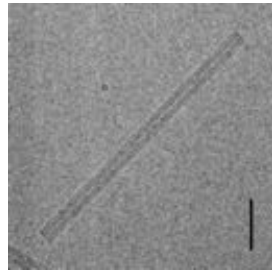
2 5 10 25 200 images



20 e⁻/Å² 40 e⁻/Å² 60 e⁻/Å² 80 e⁻/Å²



Vesicles and lipid bilayer

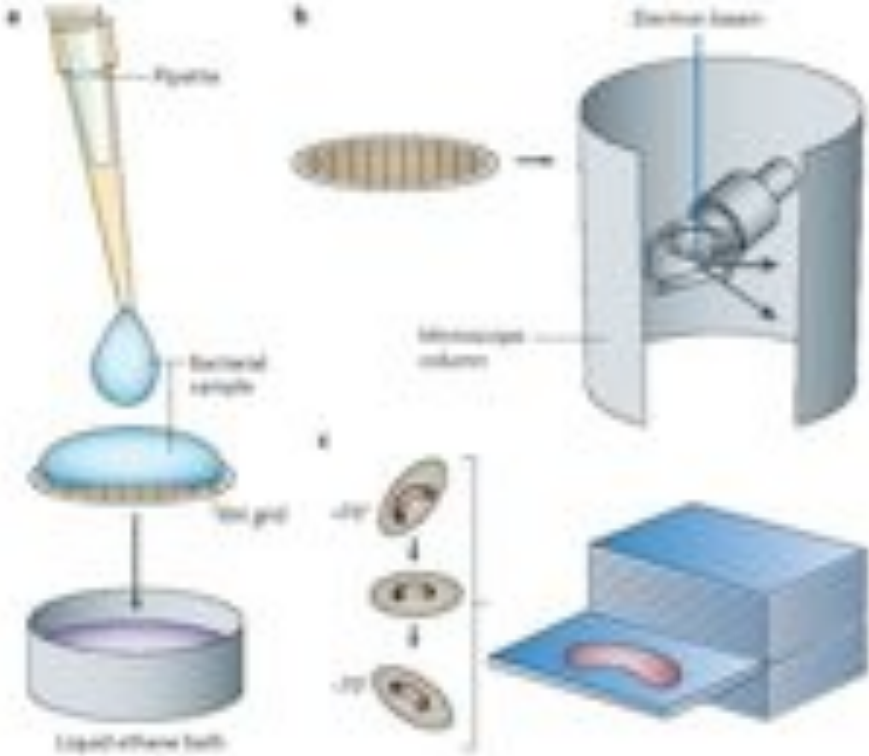


Tobacco Mosaic Virus Sachse et al., 2007.
J. Mol. Biol. 371:812-35

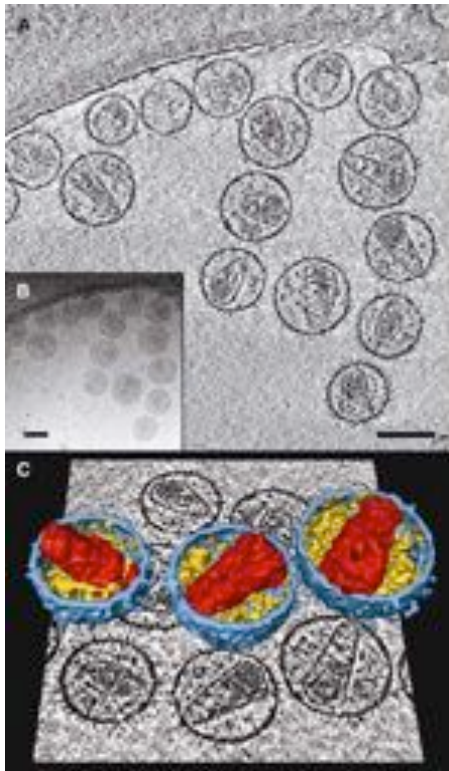
Echantillons épais:

TOMOGRAPHY CELLULAIRE CEMOVIS

Tomographie cellulaire



Electron tomography – Examples (1)



Electron tomography can reveal heterogeneity of complex assemblies.

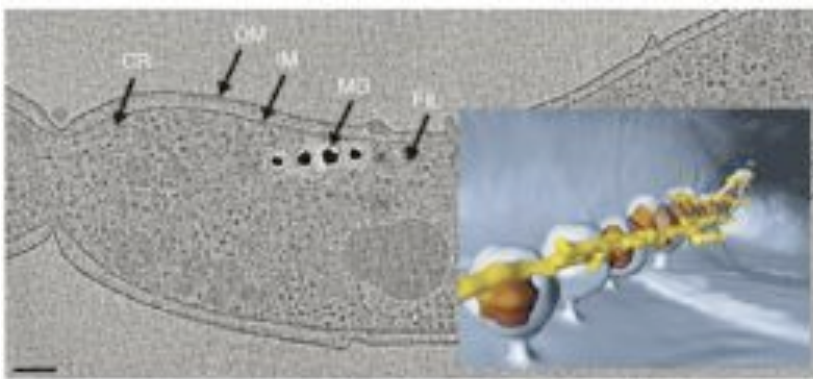
Use of cryo-ET to reveal the heterogeneity of the core of HIV-1 virions in size and shape.

A) Slice through the computed tomogram. HIV-1 particles were purified, inactivated, mixed with 10 nm colloidal gold and vitrified. The virions have an approximately spherical shape, with diameters between 106 and 183 nm.

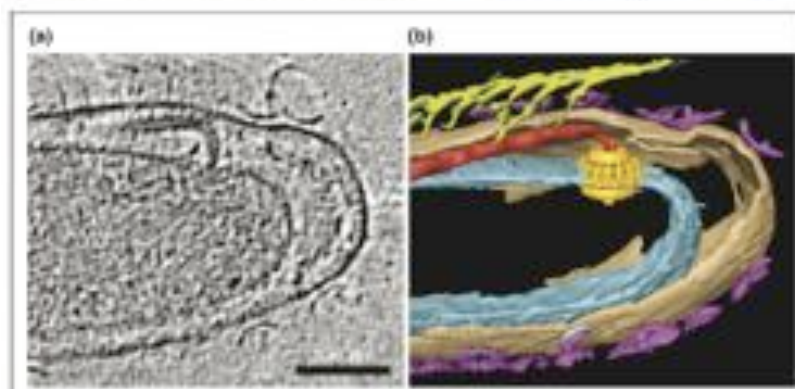
B) Projection image of the same area with visible gold fiducial markers.

C) Three-dimensional structure of three virions segmented from the tomogram shown above a central slice through it. Viral membrane, blue; density between the membrane and the core, yellow; viral capsid, red. The core was revealed by removing computationally half of the blue and yellow densities. The scale bars are 100 nm. (Briggs *et al* (2006). *Structure* 14:15-20)

Exemples

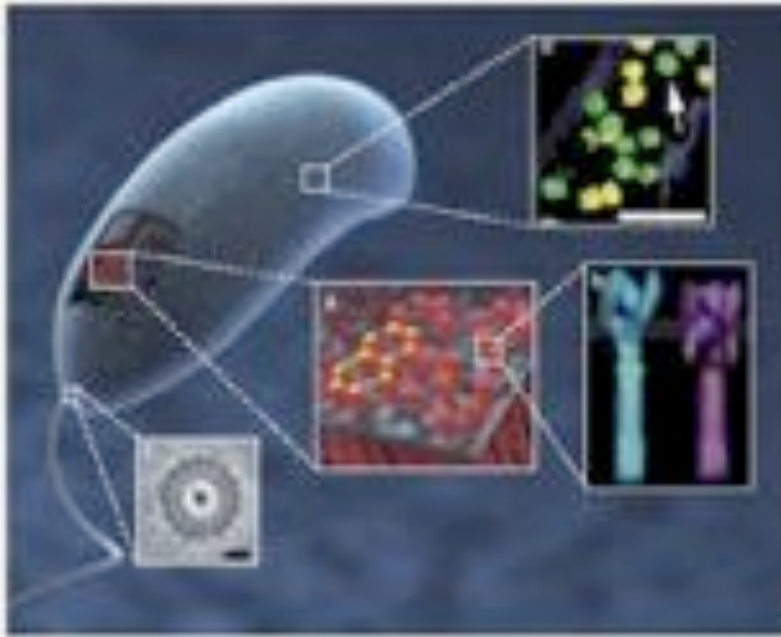


Cryo-tomographie
d'une bacterie magnetotactique



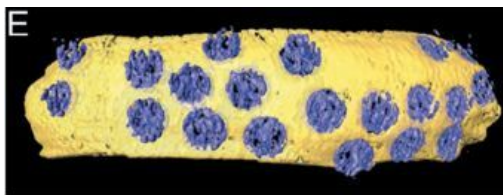
Détermination
d'un moteur flagellaire

Exemples (2)



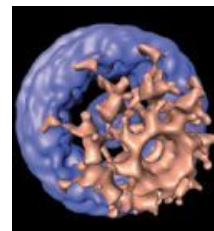
Electron tomography – Examples (3)

Combination of single-particle and electron tomography techniques



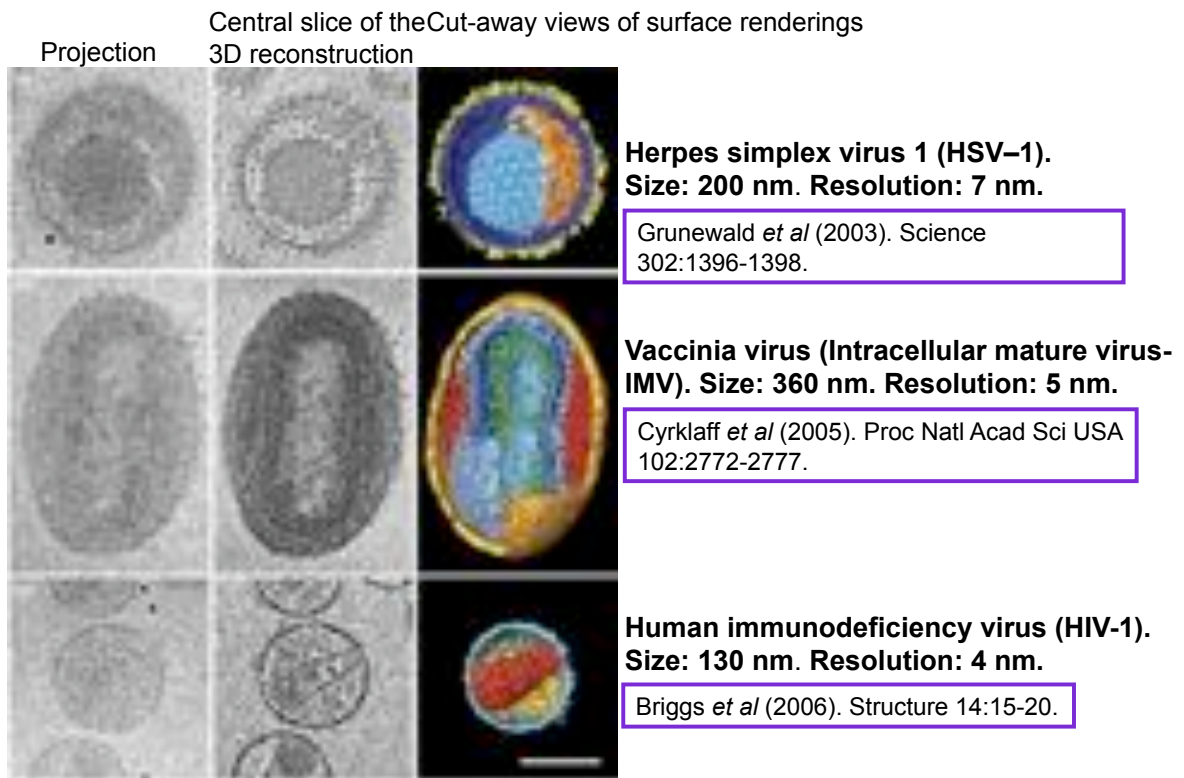
Nuclear envelope with nuclear pore complexes at 8-9 nm resolution.

Beck *et al* (2004). *Science* 306, 1387-1390.

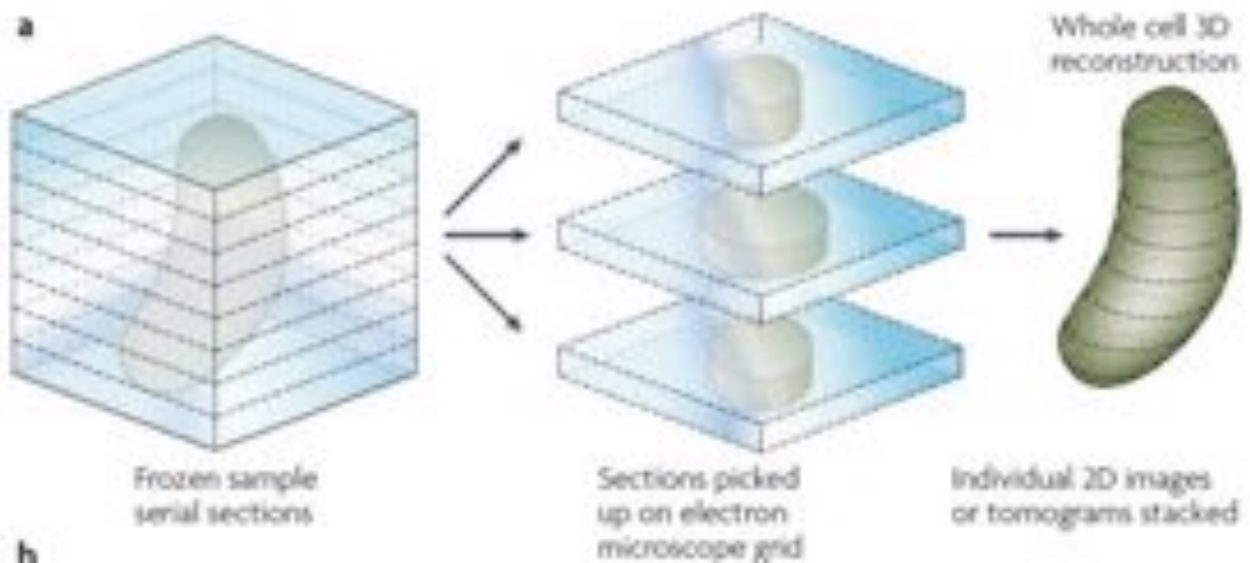


When the sample contains many identical copies of the same structure, particle volumes extracted from tomograms can be averaged or traditional single-particle analysis strategies (classification and averaging) can be used to average projection images from identical particles and, thus, improve the signal-to-noise ratio and the resolution of the reconstructed volume.

Electron tomography – Examples (2)



Tomographie (2) Méthode CEMOVIS



Congélation rapide d'échantillons épais

- Pour produire la glace vitreuse en évitant la formation des cristaux
 - Spécimens épais (> 500 nm; e.g. cellules, tissus et petite organismes)
 - Difficiles à vitrifier car la chaleur ne peut pas quitter le milieu du spécimen assez rapidement
 - Congélation sous haute (supérieure ou égale à 2000 atmosphère) pression (des spécimens jusqu'à $500\ \mu\text{m}$ peuvent être vitrifiés sans formation de cristaux)
 - Augmentation rapide de la pression suivie immédiatement de la congélation rapide pour éviter les dégâts potentiels de la haute pression

High-pressure freezing cemovis

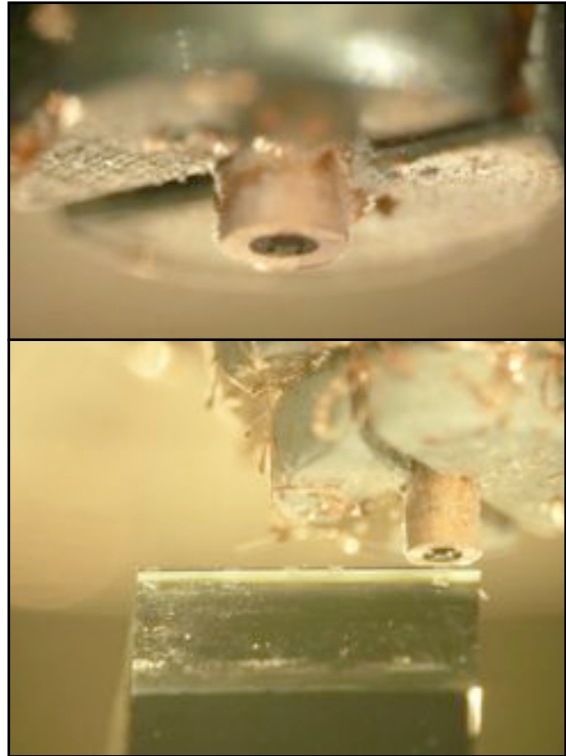


- Vitrification of samples at high pressure (2000 bar) in few ms
- Two types of specimen holders
 - Tube system (0.35mm)
 - Good for cell suspensions, bacteria, small organisms
 - Flat system (1.5mm, 0.2mm)
 - Good for larger organisms, tissue samples, bacteria



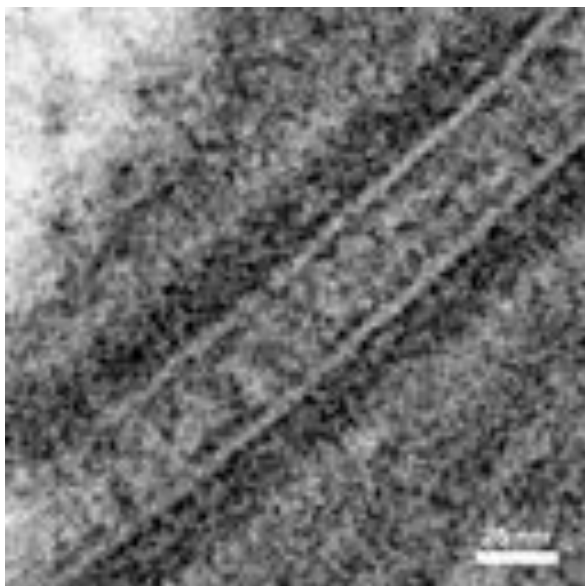
Sectioning cemovis

- Sample trimmed to pyramid shape
- Thin (>50nm) or semi-thin (<50nm) sections cut from pyramid
- Transfer ribbons to grid by eyelash

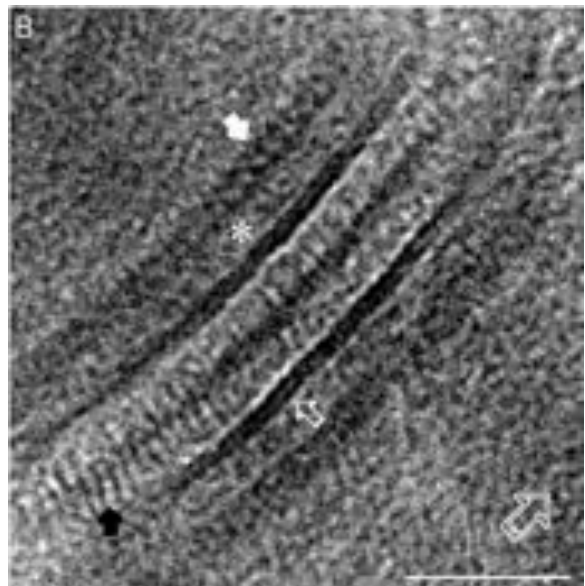


Skin cell desmosomes

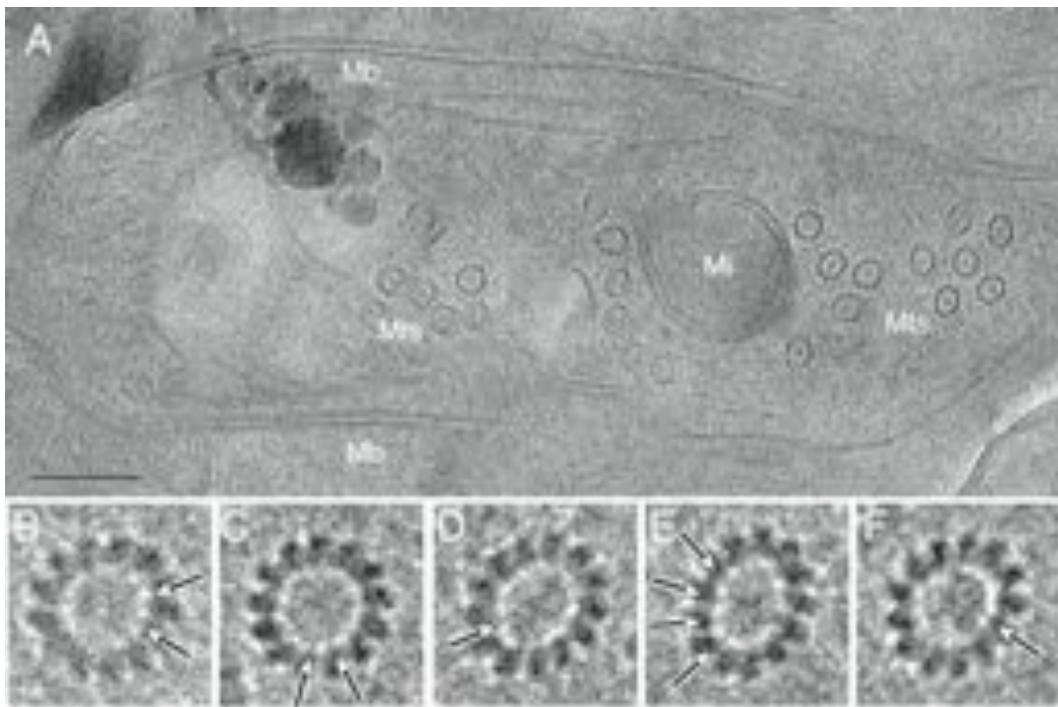
Resin section



Cryo section



Microtubules rat brain tissue



Garvalov BK *et al*, J Cell Biol. 2006
11;174(6):759-65