

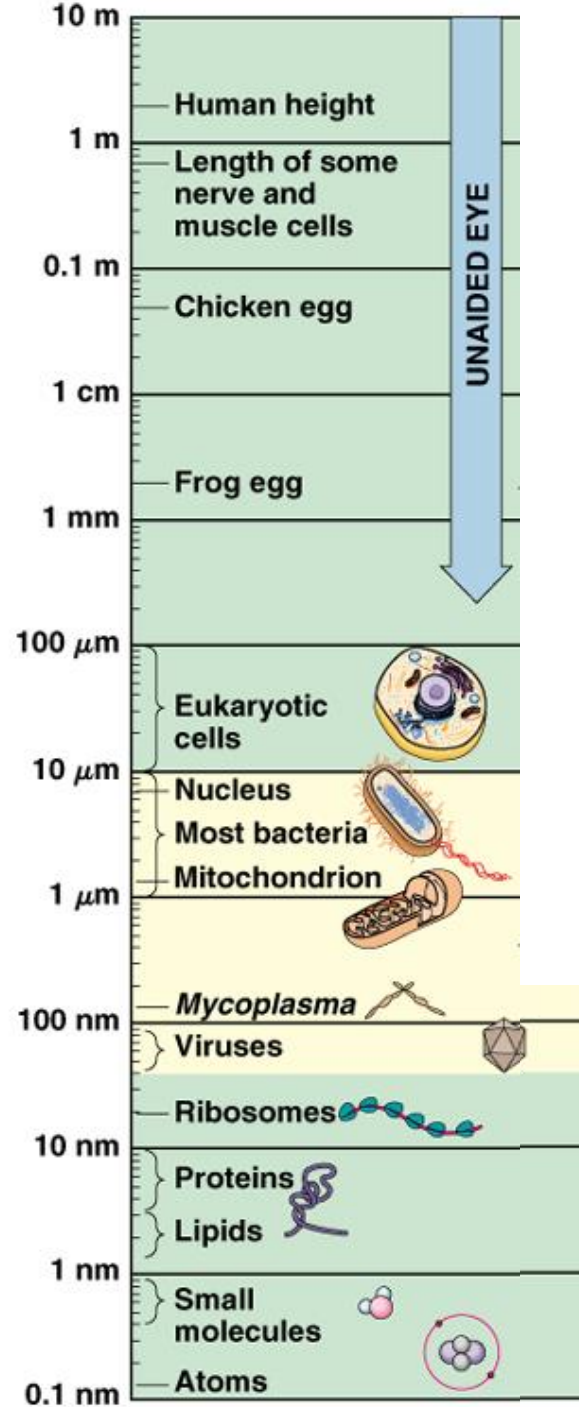
# La cryo-microscopie électronique en biologie structurale

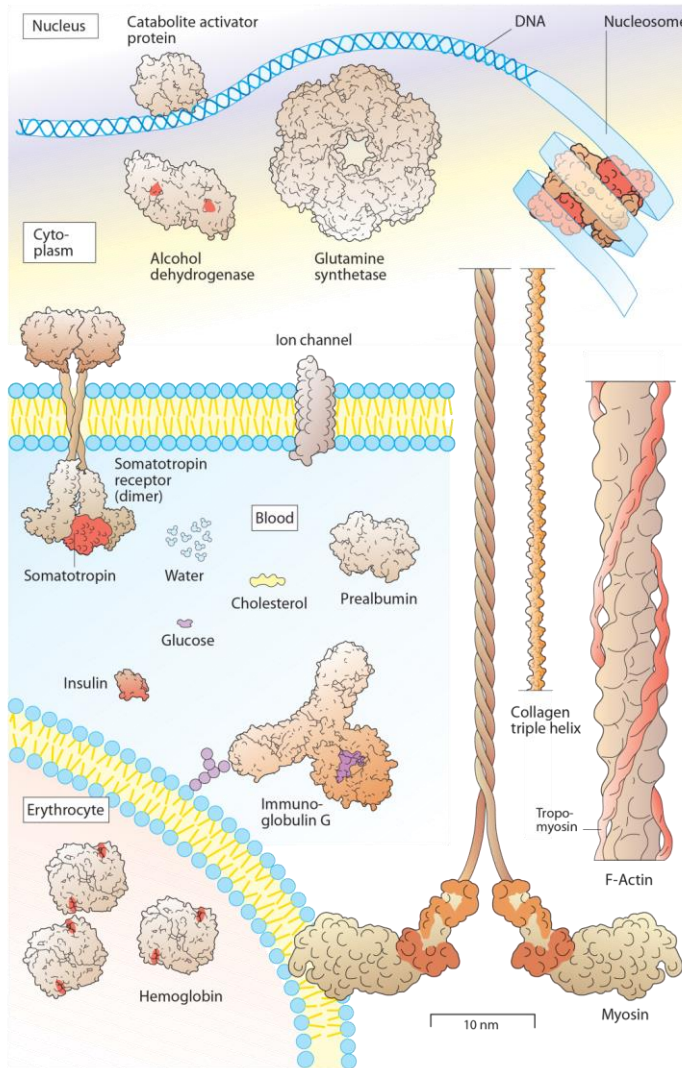
Guy Schoehn  
[guy.schoehn@ibs.fr](mailto:guy.schoehn@ibs.fr)



67<sup>ème</sup> congrès national des professeurs de physique et de chimie  
30 octobre 2019

# Les différentes échelles de résolution





Les protéines sont présentes à toutes les étapes de la vie de la cellule :

- Mouvements cellulaires
- Rôle structural et mécanique
- Défense de l'organisme
- Rôle informatif
- Contrôle et régulation
- Etc...

# La microscopie

- Grec : « mikros », qui signifie petit ; « skopein », qui signifie observer.
- Regarder des objets de plus en plus petits
- Sciences du vivant : structure de cellules, de bactéries, de virus et de complexes macromoléculaires.
- Sciences de la matière : inhomogénéités et imperfections dans les métaux, batteries, cellules photo-voltaïques et céramiques...
- Géologie : roches, minéraux, etc...

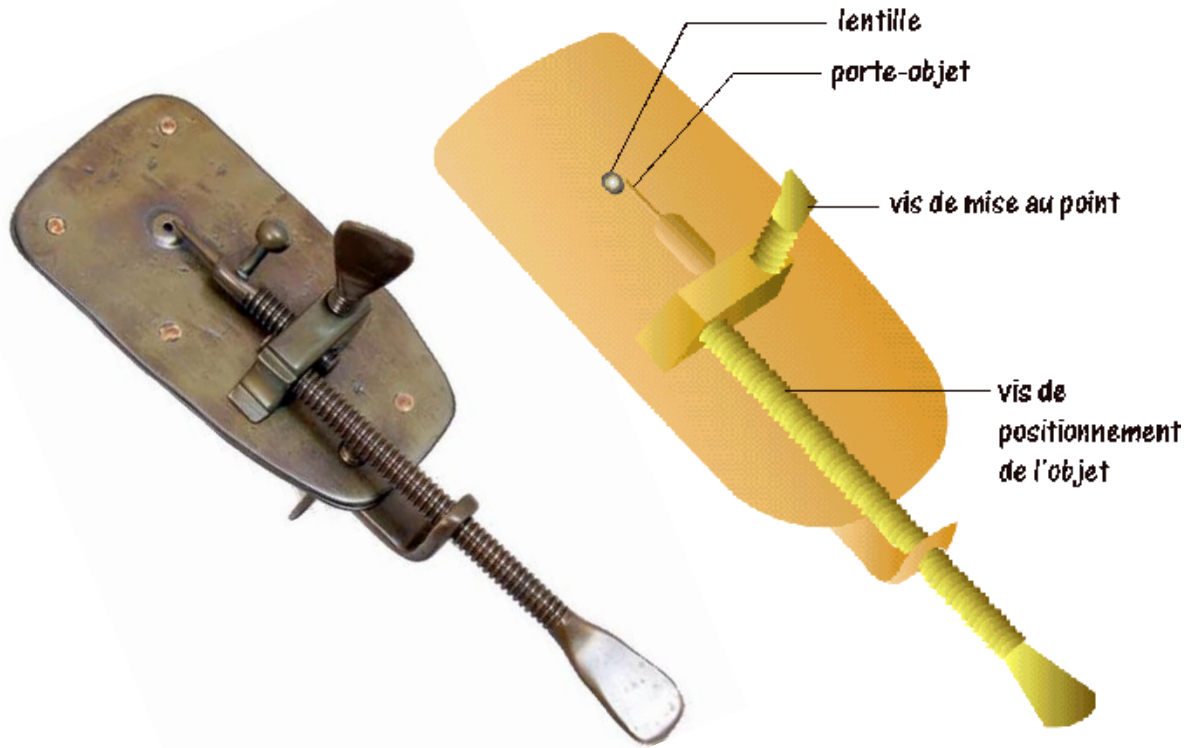
# Invention des microscopes

Les différents types de microscopes



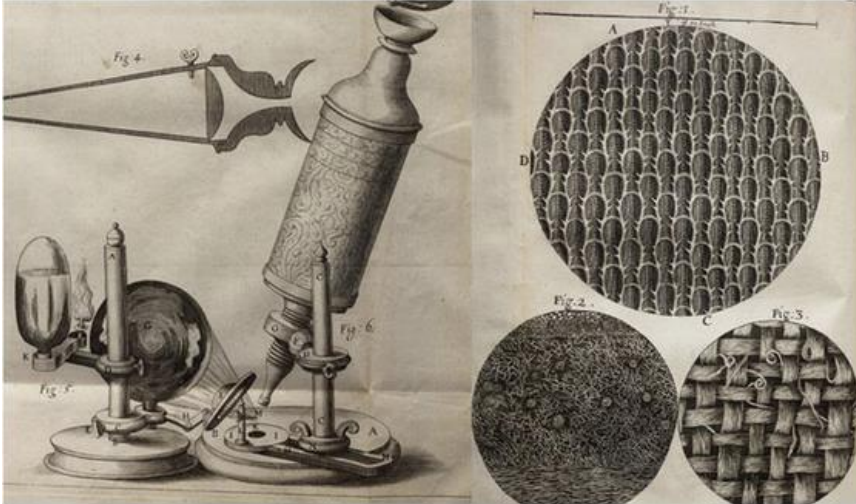
microscope optique  
d'Antoni van Leeuwenhoek  
(1632-1723)

Résolution de 1,5 microns

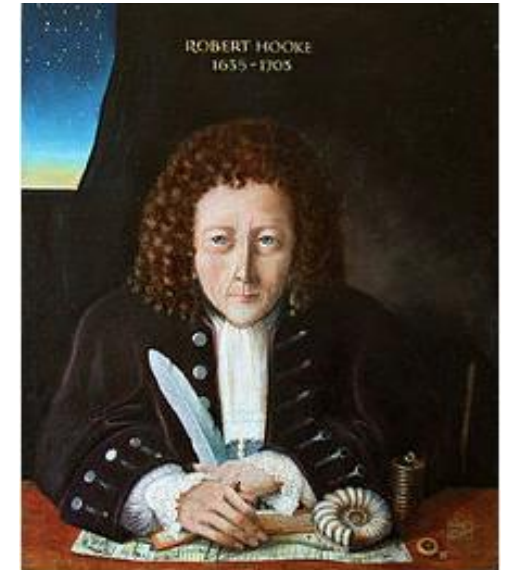
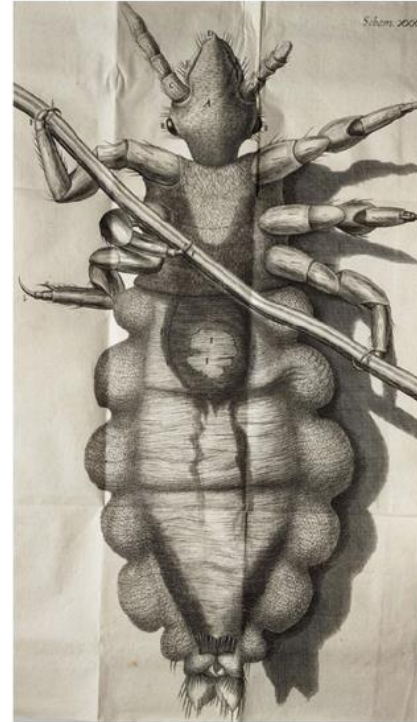


# Invention des microscopes

Les différents types de microscopes



Robert Hooke (1635-1703)



# Invention des microscopes

Les différents types de microscopes

Au 17<sup>ème</sup> siècle, les microscopes évoluent  
mais le principe reste le même.



La résolution limite en MO est donc 0,2  $\mu\text{m}$ .



## ➤ Les limites:

En grandissement 1000x

En pouvoir de résolution : formule d'Abbé:

Soit  $d \geq 0,61 \lambda / ON$

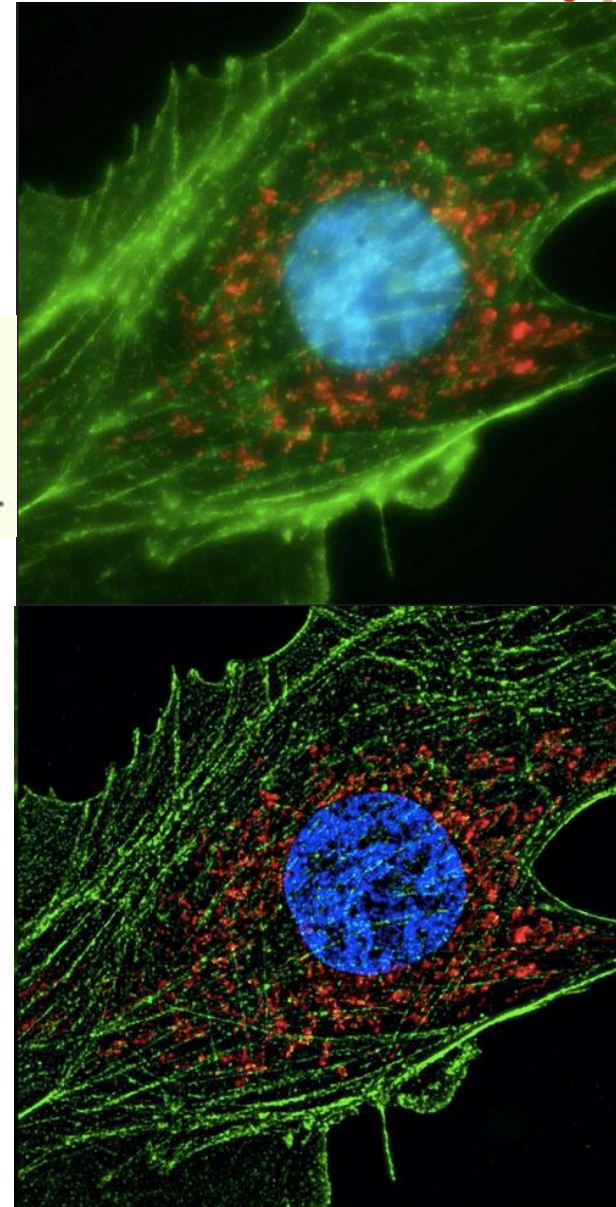
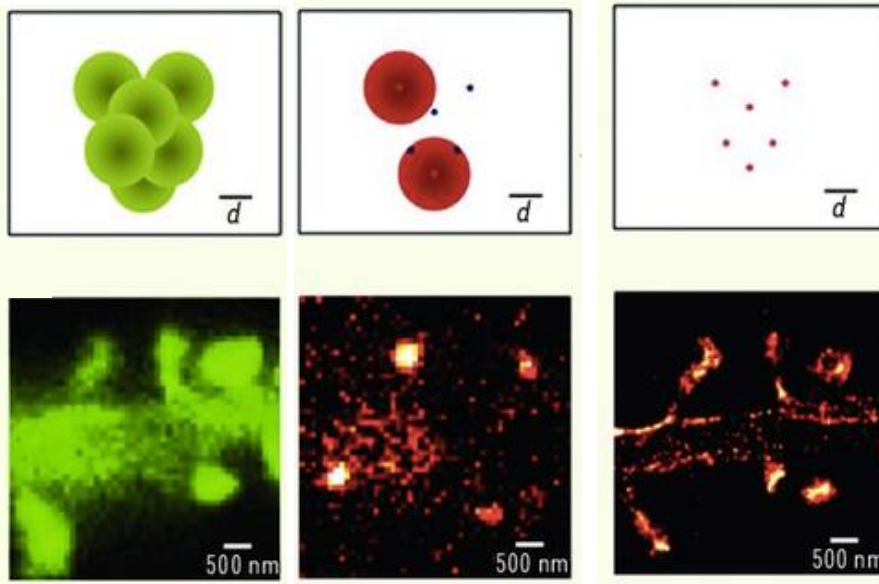
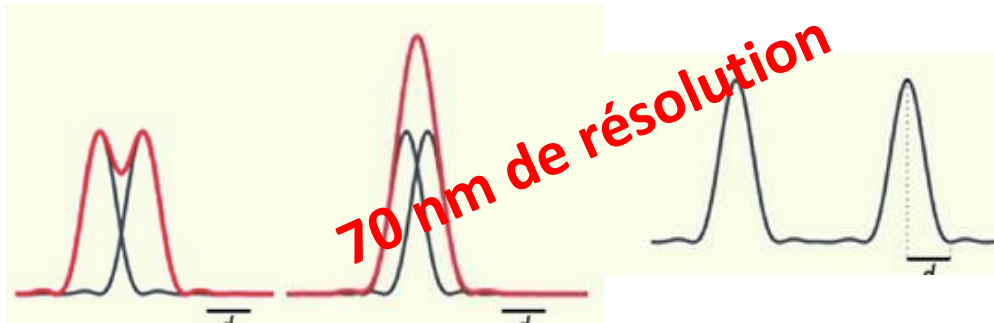
– d = distance

$ON = n \sin(\theta)$

–  $\lambda$  = longueur d'onde de la lumière utilisée

– ON = Ouverture Numérique de l'objectif

# Microscopie photonique super résolution

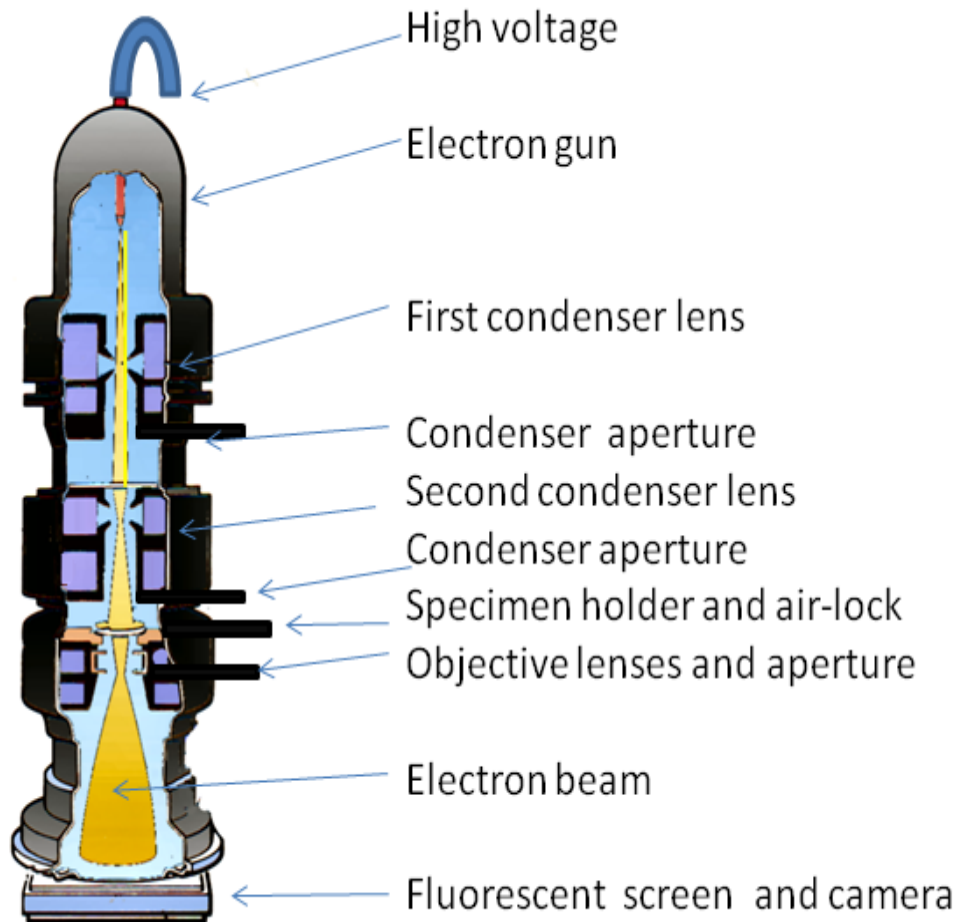


2014, Prix [Nobel](#) de Chimie 2014: [Eric Betzig](#), [William E. Moerner](#) and [Stefan Hell](#)

# Comment aller plus loin?

- En 1920 : les électrons accélérés dans le vide se comportent de manière identique à la lumière
- Electron** → les longueurs d'onde peuvent être très faibles:  $0.037 \text{ \AA}$  à une accélération de 100 kV
- Les champs électriques et magnétiques** modifient la trajectoire des électrons
- En 1931 Ernst Ruska**, Berlin, a combiné ces caractéristiques pour construire le premier microscope électronique à transmission (Prix Nobel de Physique en 1986)
- Le premier microscope électronique** utilisait 2 lentilles magnétiques (trois ans après) pour **une résolution de 100 nm**, deux fois mieux qu'un microscope photonique.

# Le microscope électronique à transmission (TEM)

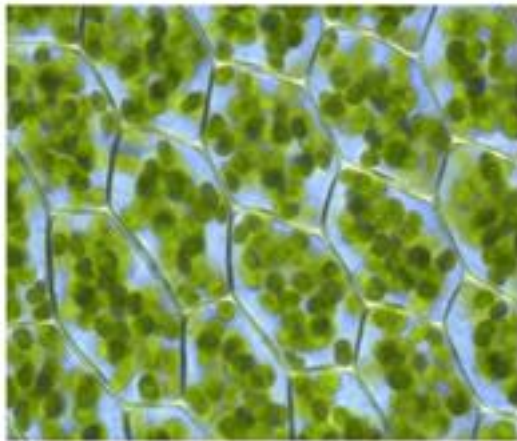
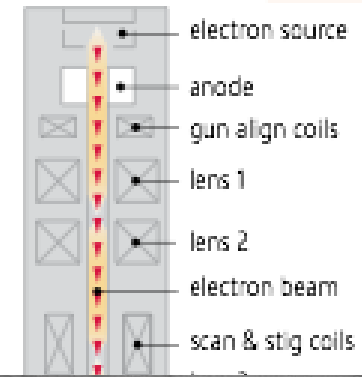
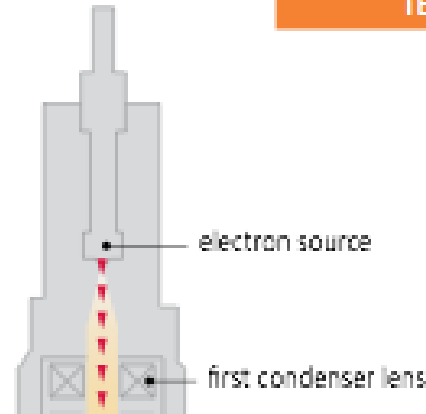


# Les différents types de microscopes

light

TEM

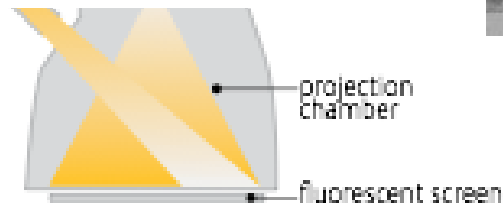
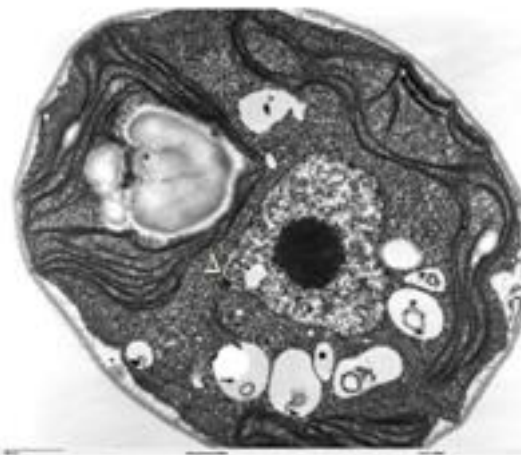
SEM



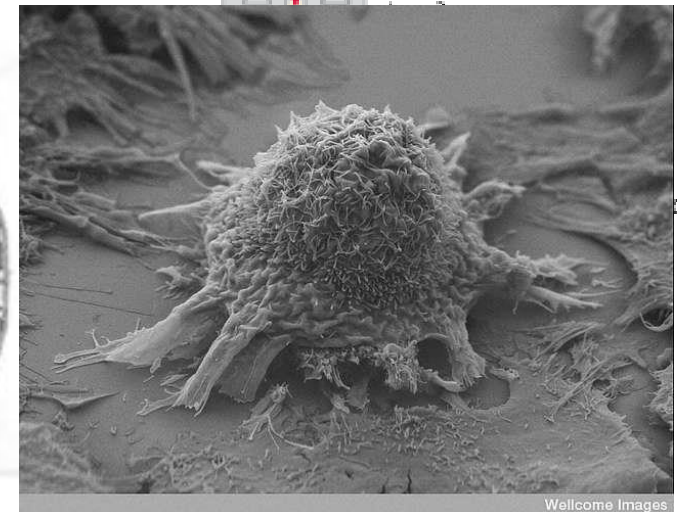
condenser aperture

object aperture

select area aperture

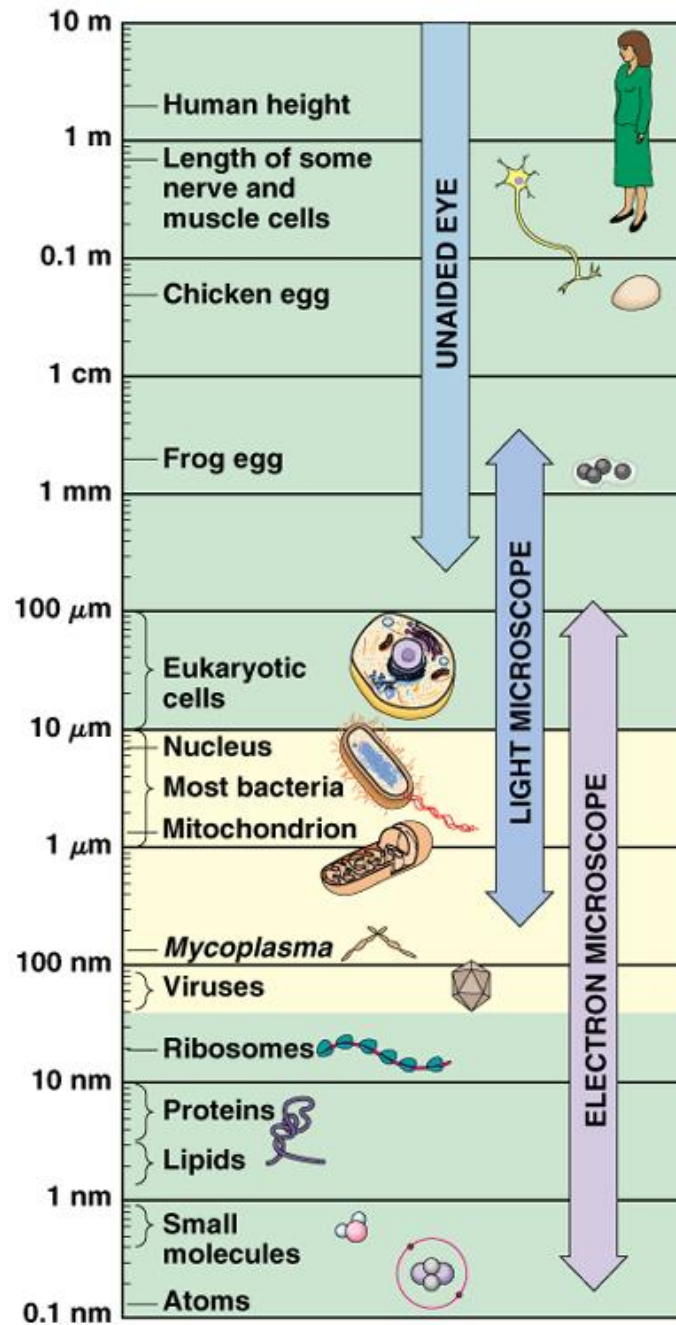


Light source



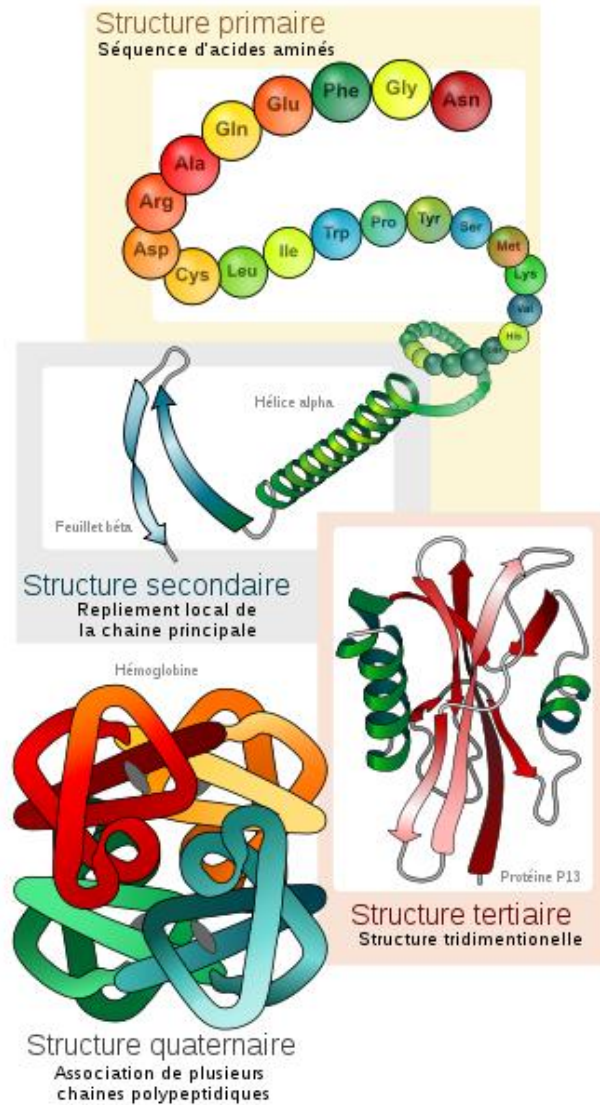
roughing line

# Les différentes échelles de résolution



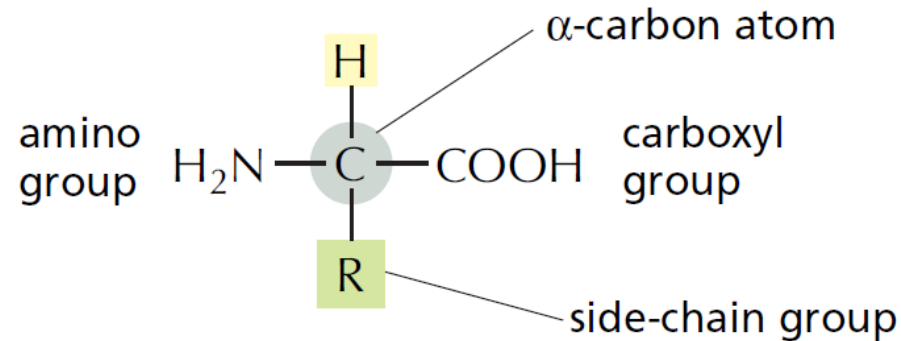
©Addison Wesley Longman, Inc.

# La biologie structurale



➤ **Primaire** : Séquence en acides aminés

# La structure primaire



## Glossaire

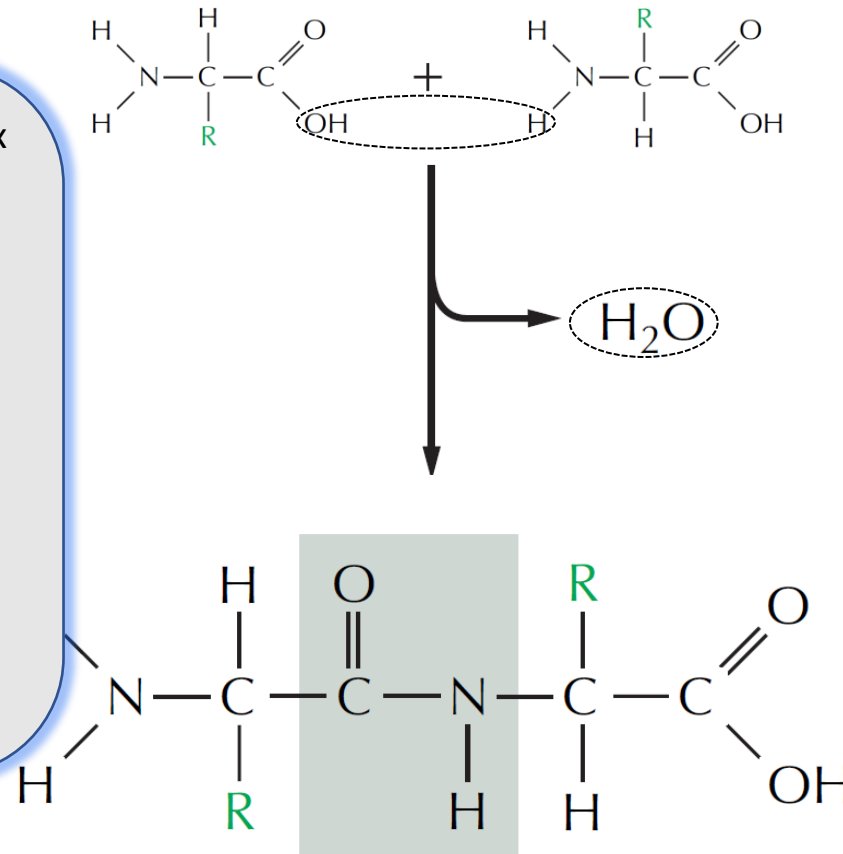
- Un acide aminé est constitué de trois fonctions chimiques :
  1. Une fonction **amine** ( $\text{NH}_2$ )
  2. Une fonction **acide carboxylique** ( $\text{COOH}$ )
  3. Une chaîne latérale R qui diffère en fonction de l'acide aminé
- L'ensemble s'articulant sur un carbone central : le **carbone alpha**

# La structure primaire

- Les acides aminés se lient les uns aux autres grâce à la formation d'une liaison **covalente** :

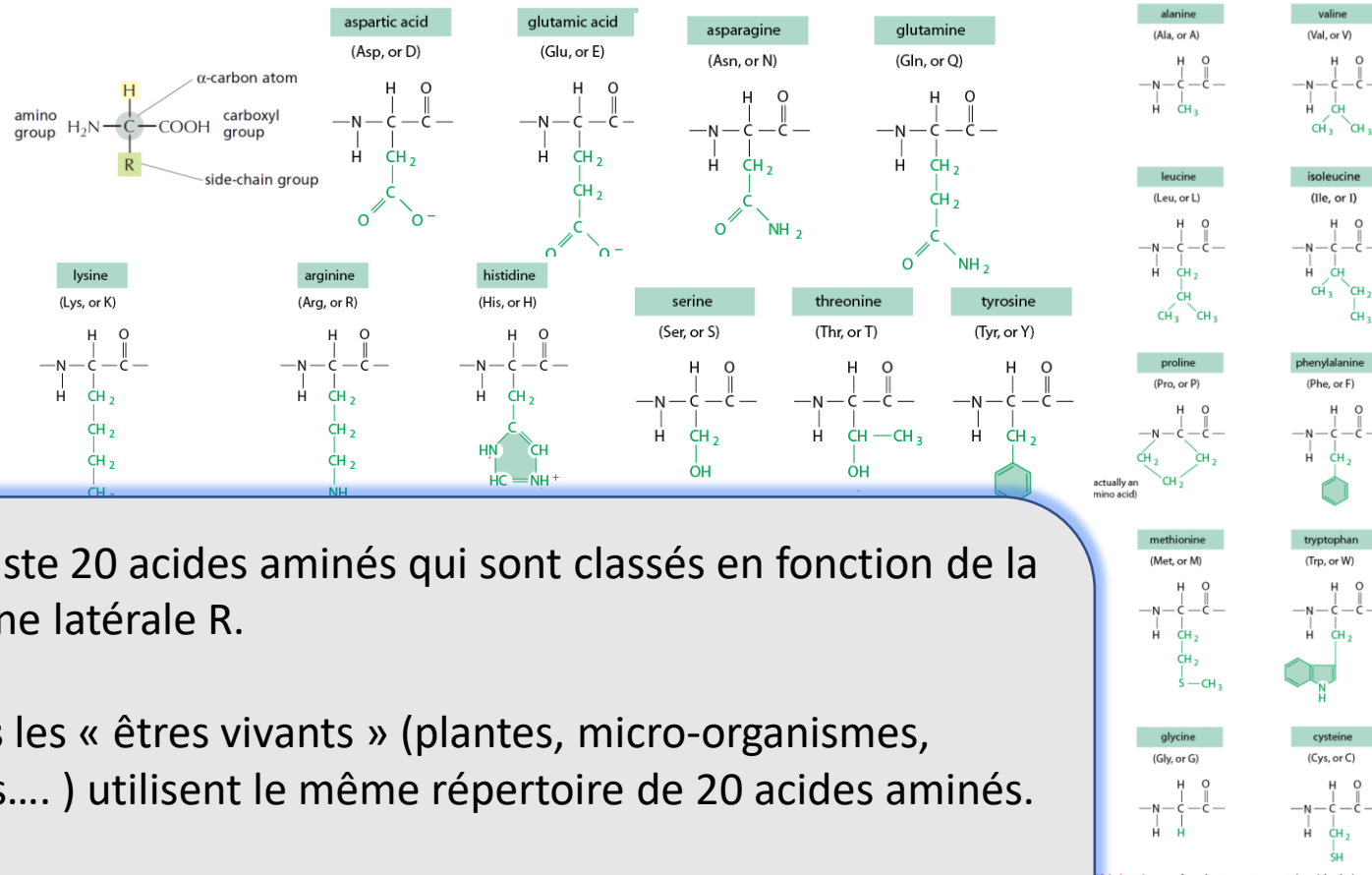
**La liaison peptidique.**

- La liaison est le résultat de la réaction entre **la fonction acide carboxylique COOH du premier acide aminé** et **la fonction amine NH<sub>2</sub> du deuxième**, avec comme produit secondaire une molécule d'eau H<sub>2</sub>O.

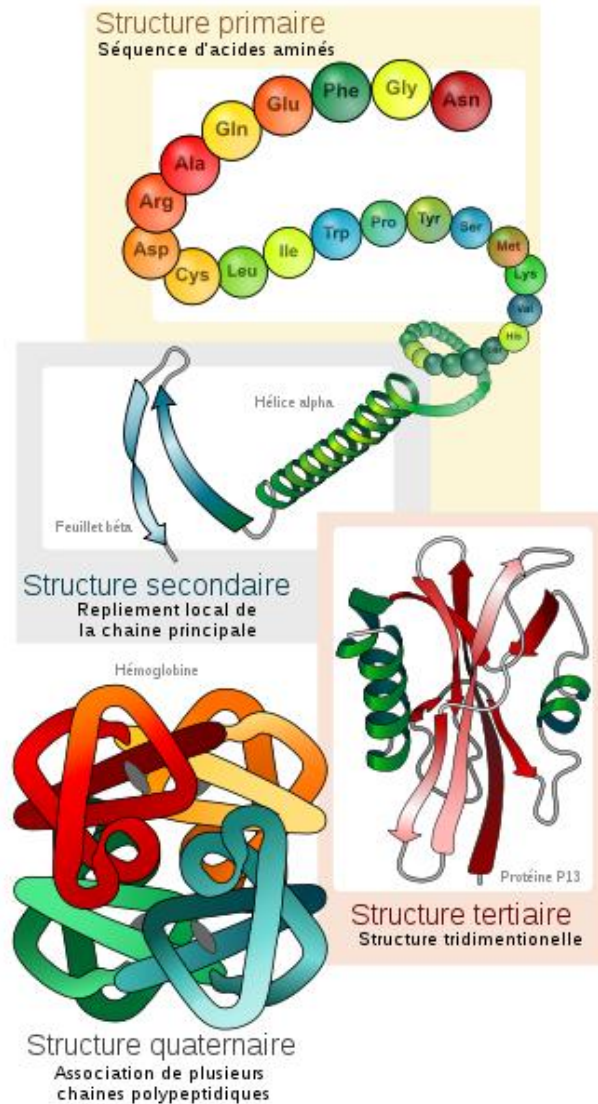


# La structure primaire

## 20 acides aminés différents



- Il existe 20 acides aminés qui sont classés en fonction de la chaîne latérale R.
- **Tous** les « êtres vivants » (plantes, micro-organismes, virus.... ) utilisent le même répertoire de 20 acides aminés.
- C'est le radical R qui va marquer la différence entre les acides aminés et par conséquent leurs propriétés physiques et chimiques.

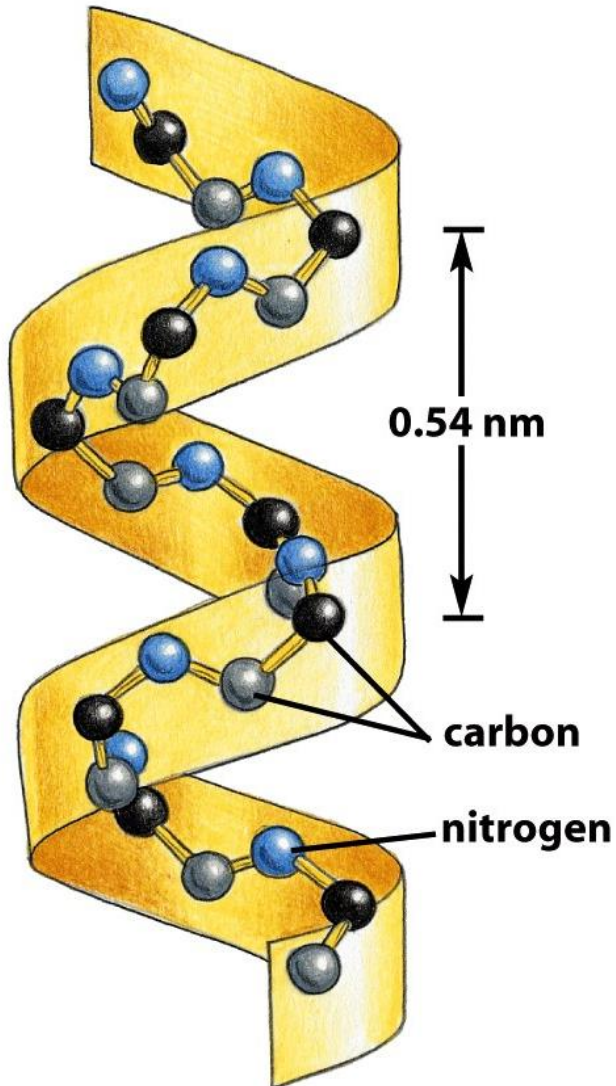


➤ **Primaire** : Séquence en acides aminés

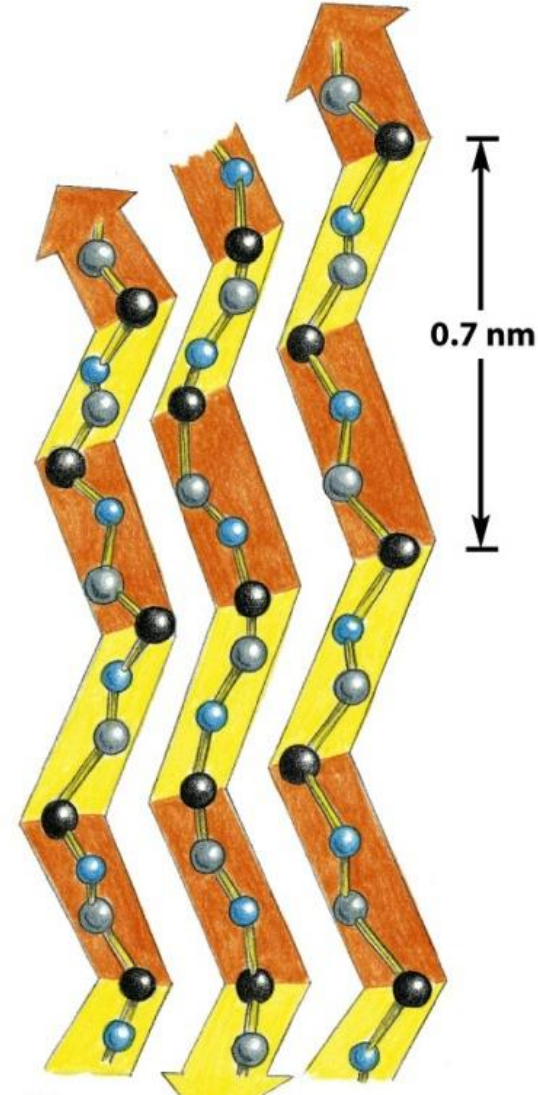
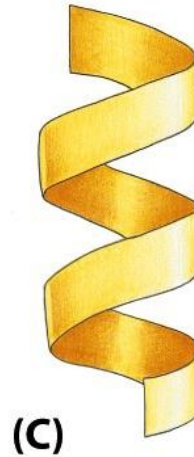
➤ **Secondaire** : Repliement **local** et **régulier** des acides aminés.

# Les structures secondaires

## L'hélice $\alpha$ et le feuillet $\beta$



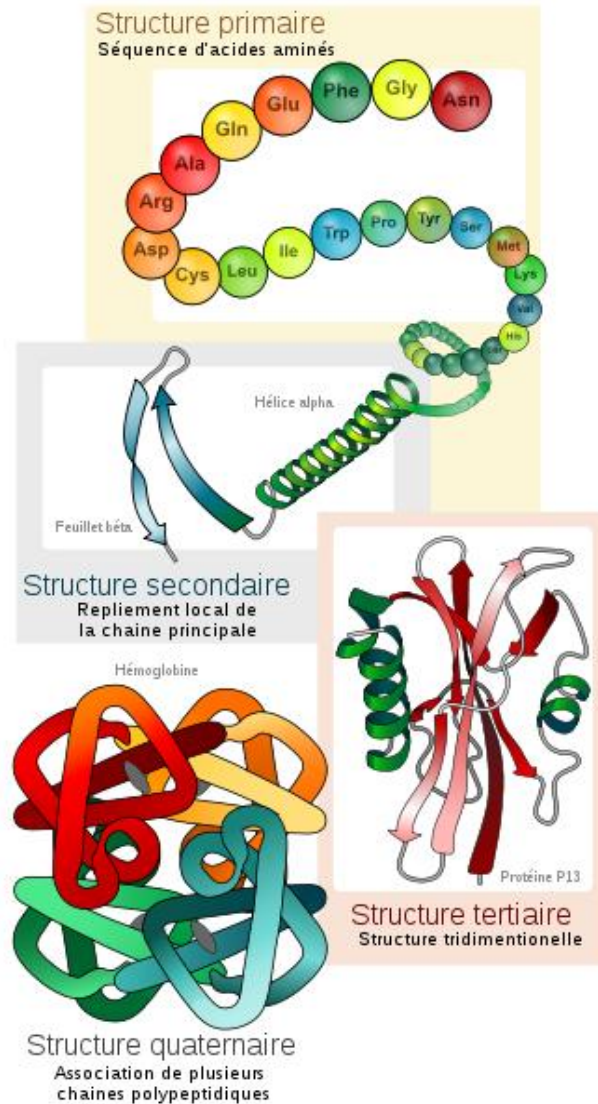
$\alpha$  helix



$\beta$  sheet



# La biologie structurale



➤ **Primaire** : Séquence en acides aminés

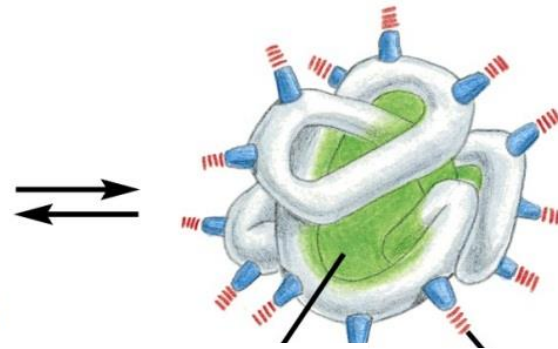
➤ **Secondaire** : Repliement **local et régulier** des acides aminés.

➤ **Tertiaire** : Arrangement tridimensionnel des éléments de structures secondaires.

# Les structures tertiaires

Ce repliement va suivre deux grandes règles :

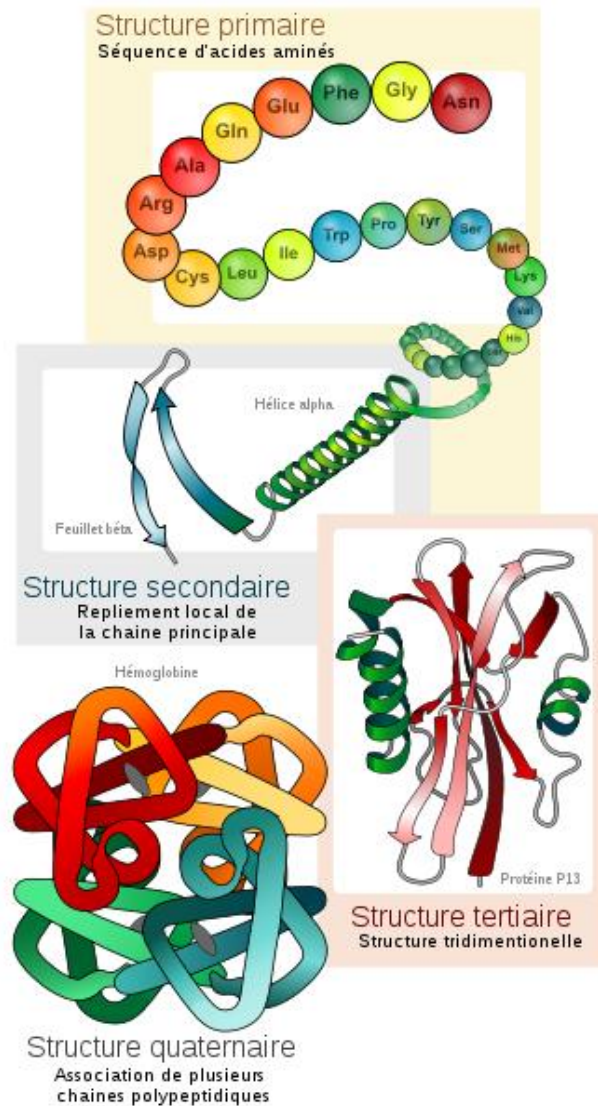
- Les parties apolaires ou hydrophobes vont être éloignées de l'eau, pour former le **cœur hydrophobe**.
- Les parties hydrophiles vont être exposées au solvant, permettant la formation des liaisons avec l'eau.



Cœur  
hydrophobe  
de la protéine

Chaînes  
polaires en  
contact avec le  
milieu aqueux

**Ce repliement dans l'espace va rapprocher des régions qui peuvent être très éloignées dans la séquence primaire.**



➤ **Primaire** : Séquence en acides aminés

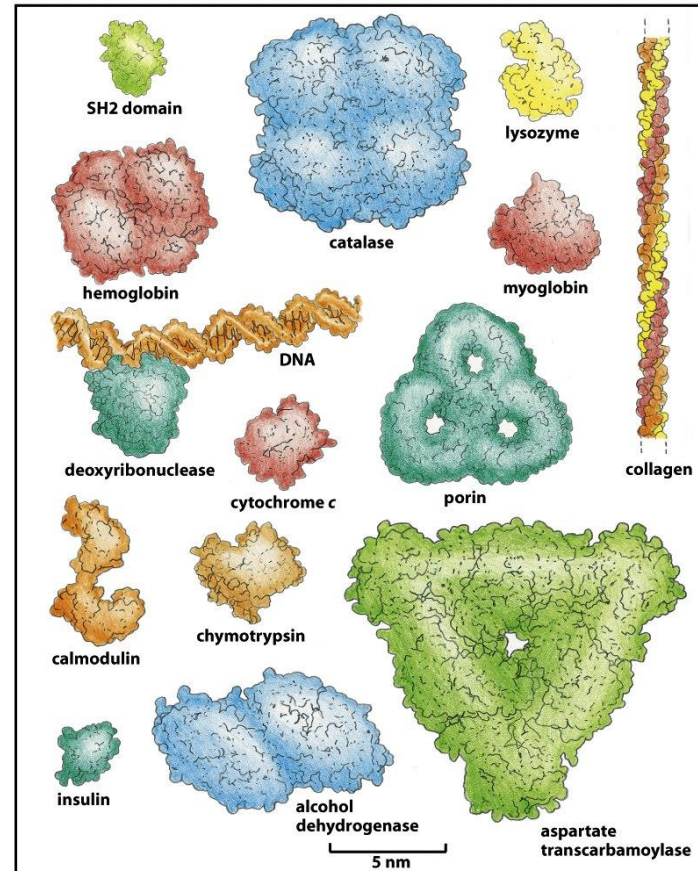
➤ **Secondaire** : Repliement **local et régulier** des acides aminés.

➤ **Tertiaire** : Arrangement tridimensionnel des éléments de structures secondaires.

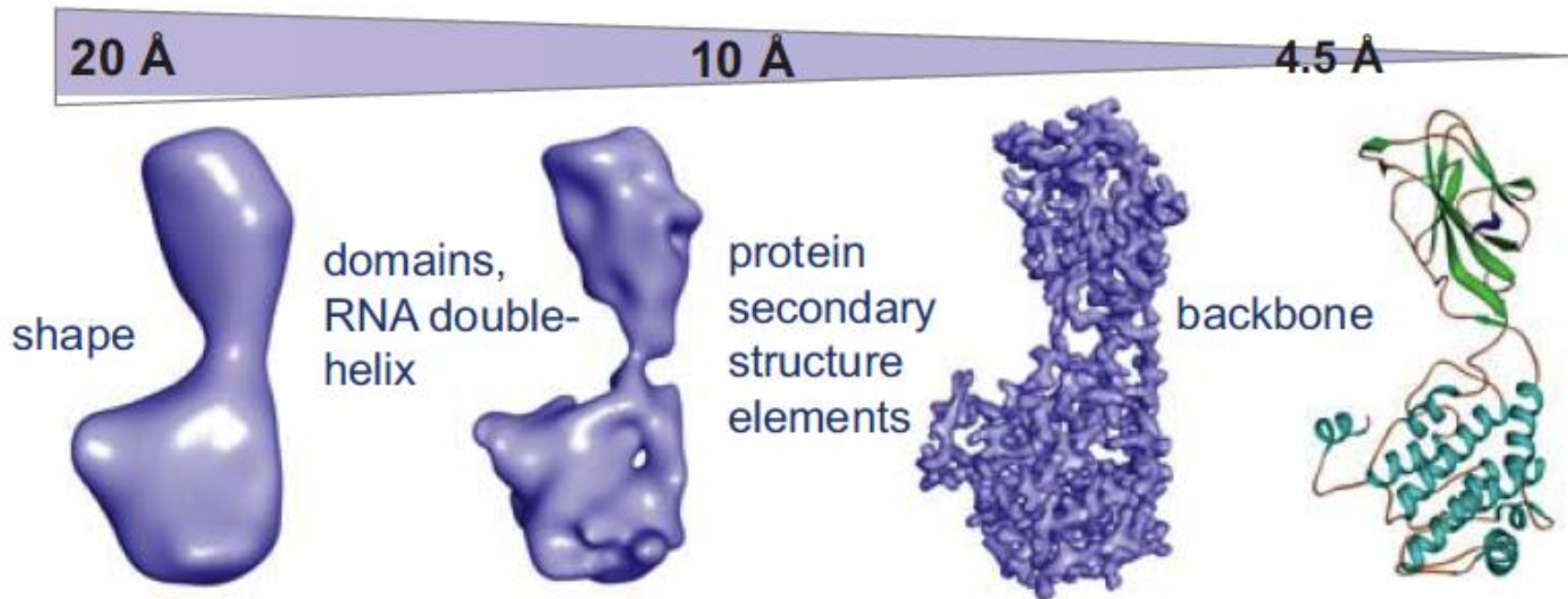
➤ **Quaternaire** : Association stable de plusieurs protéines sous forme d'un complexe.

# Les structures quaternaires

- Il y a autant de structures quaternaires que de protéines, même si certaines formes générales reviennent (globulaires, en filaments...).



# Les structures en fonction de la résolution



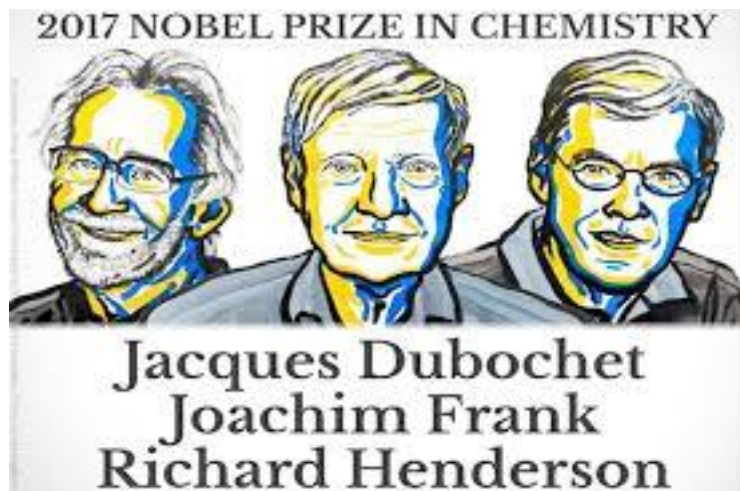
PERSPECTIVE | BIOCHEMISTRY

# The Resolution Revolution

Werner Kühlbrandt

+ See all authors and affiliations

Science 28 Mar 2014:  
Vol. 343, Issue 6178, pp. 1443-1444  
DOI: 10.1126/science.1251652



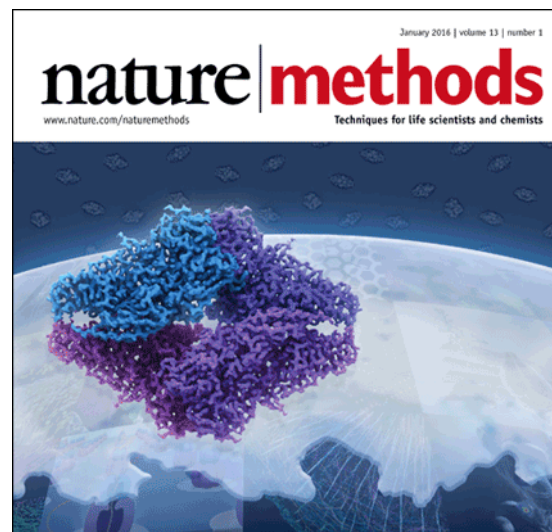
NATURE | NEWS FEATURE

## The revolution will not be crystallized: a new method sweeps through structural biology

Move over X-ray crystallography. Cryo-electron microscopy is kicking up a storm by revealing the hidden machinery of the cell.

Ewen Callaway

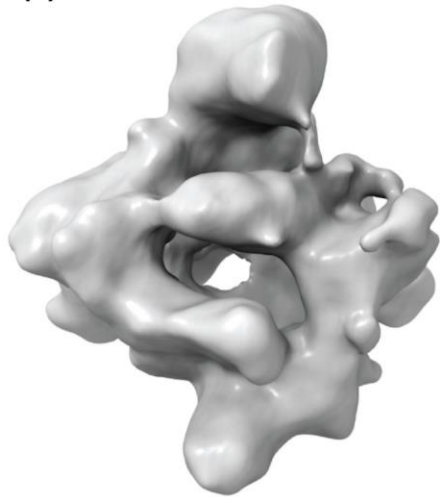
09 September 2015



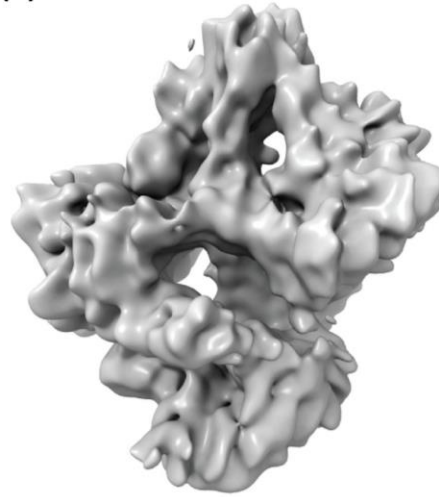
## Method of the Year 2015

The end of 'blob-ology': single-particle cryo-electron microscopy (cryo-EM) is now being used to solve macromolecular structures at high resolution.

# La révolution de la résolution en ME



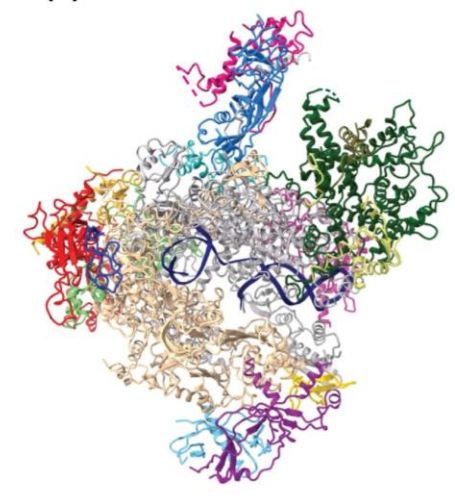
Apo Pol III  
17 Å resolution



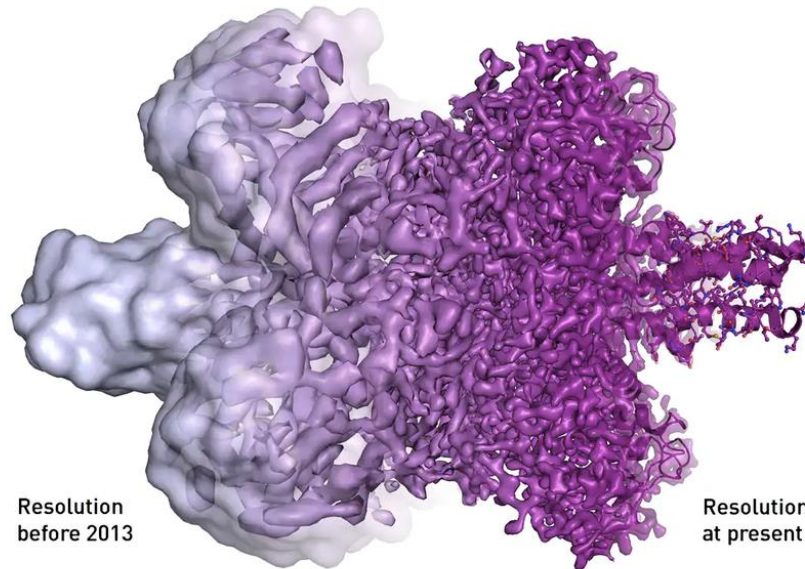
Apo Pol III  
10 Å resolution



Pol III elongation complex  
3.9 Å resolution



Current Opinion in Structural Biology

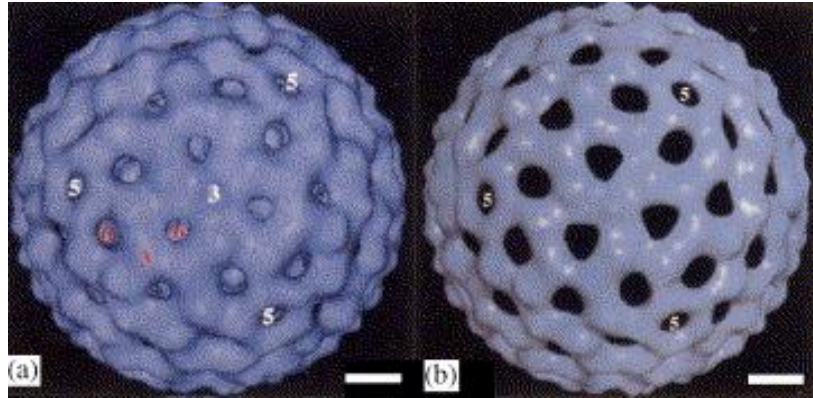


Resolution  
before 2013

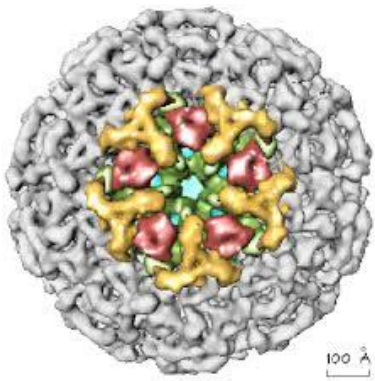
Resolution  
at present

# De la Bio-biologie à la biologie structurale

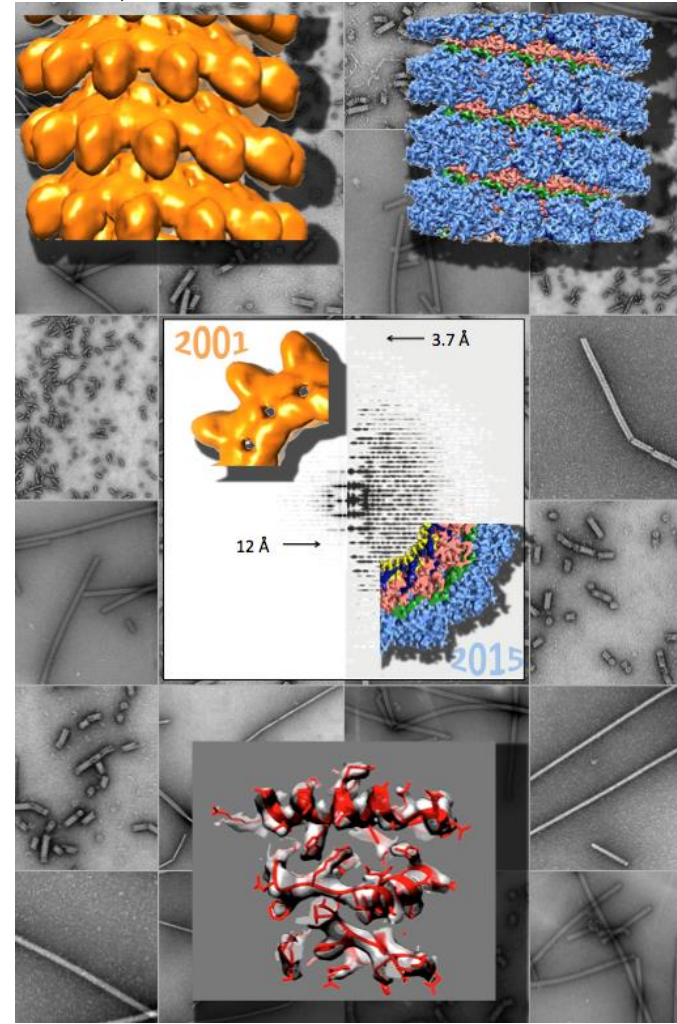
Postdoc à EMBL Grenoble



M2 : 1994 : 45 Å



Thesis : 1997 : 23 Å



# Les microscopes



# Les progrès techniques au niveau des instruments

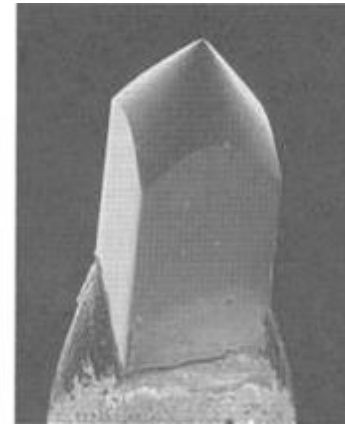
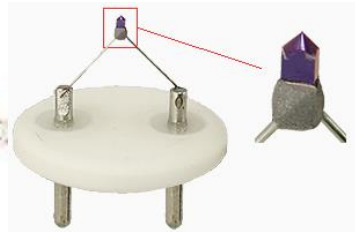
- Tension d'accélération(300 kV)
- Nature du canon à électrons
- Les lentilles
- Le vide
- Stabilité de l'étage froid
- Automatisation
- Les détecteurs d'électrons



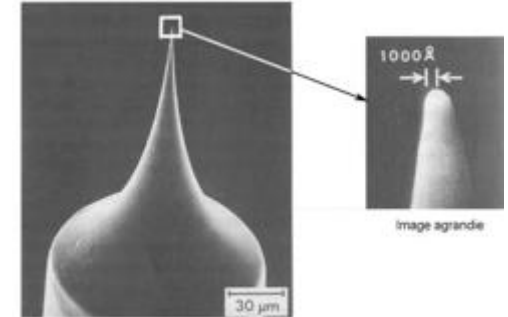
# Nature du canon à électrons



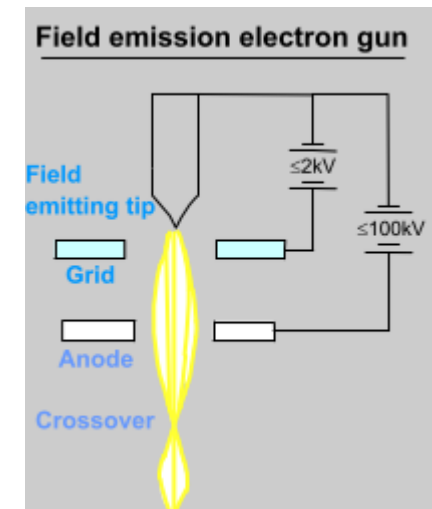
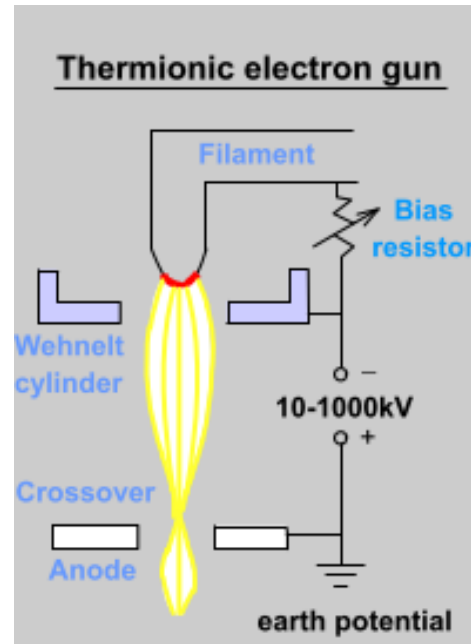
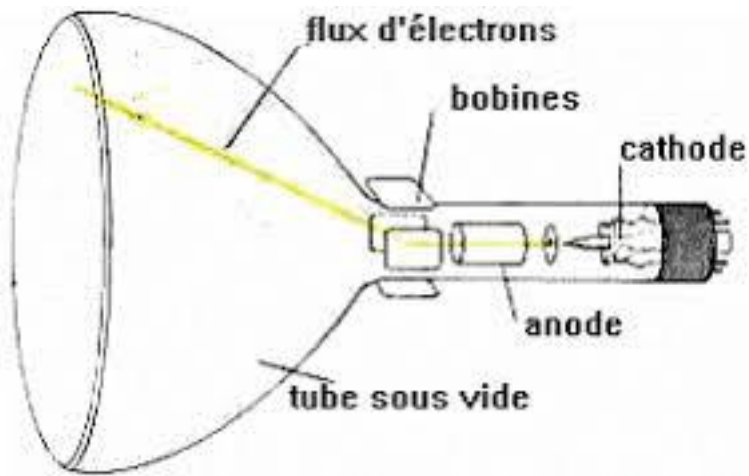
Filament de tungstène



Filament en  
hexaborure de lanthane

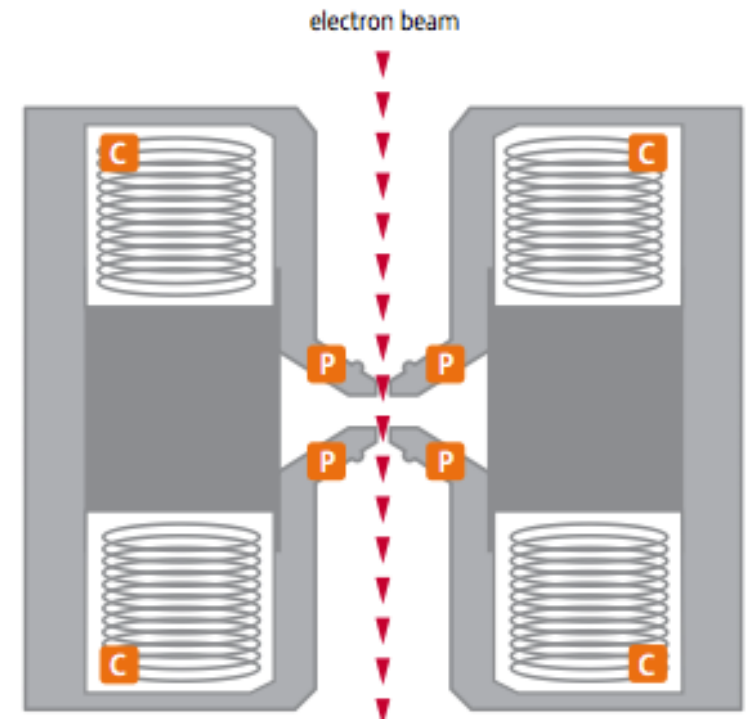


Canon à  
émission de champs



Très cohérent et très brillant

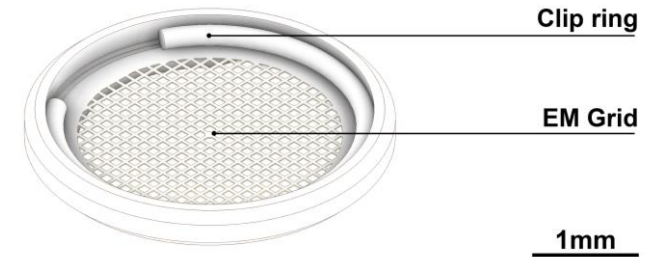
# Les lentilles électromagnétiques



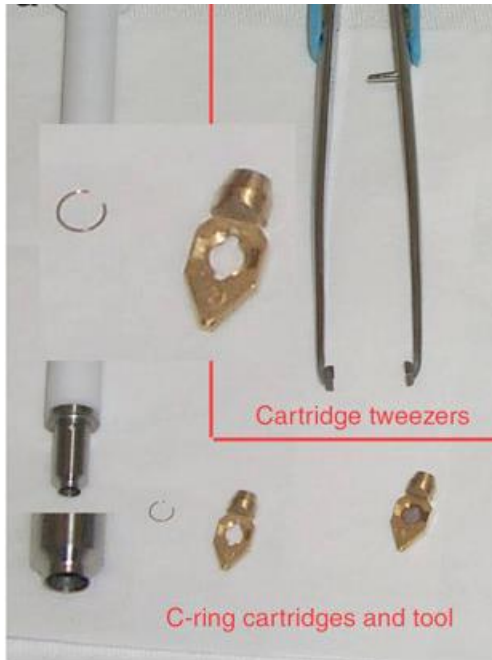
# L'étage froid



1ère génération



Dernière génération

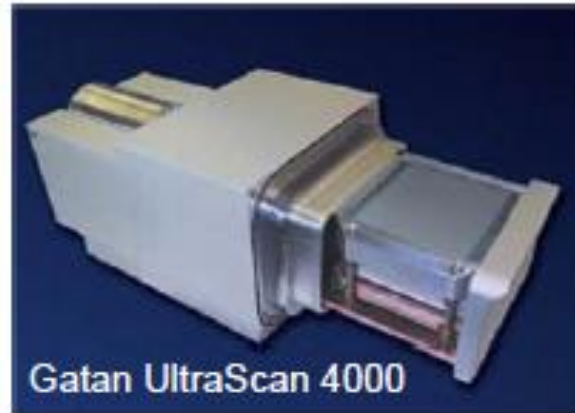


2ème génération

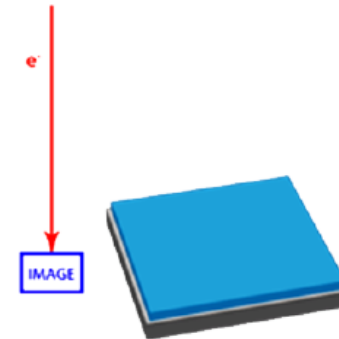


# Les détecteurs d'électrons

Film



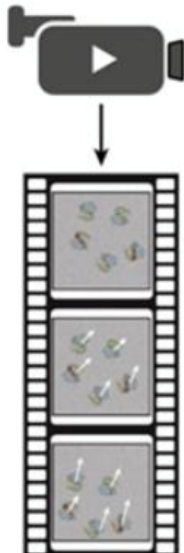
Direct e- detector



**Before**



**After**



Direct Electron Detection



Realignment of movies

Direct e- detector,  
2 main advantages

#1/ improved signal-to-noise

#2/ movie mode (fast read-out rate)  
-correct for motion  
-choose best frames

# Caractéristiques d'un microscope électronique

## Wavelengths of electrons ( $\lambda$ )

0.0349 Å at 120kV,

0.025 Å at 200 kV,

0.01969 Å at 300 kV

compare with wavelength of light: 2000 Å  
or X-rays:

1.5 Å for Cu K $\alpha$

1 Å synchrotron radiation

diffraction limit for  
resolution of  
any optical system:  
 $D = \lambda/2$

Quel est le problème avec les MET ?

Les lentilles ne sont pas parfaites : aberration (sphéricité; chromaticité).

Les échantillons biologiques sont sensibles aux électrons.

La stabilité mécanique n'est pas parfaite.

Le microscope n'est pas une loupe parfaite.

# Quel est le problème avec le TEM: Ce n'est pas une loupe parfaite



Objet initial

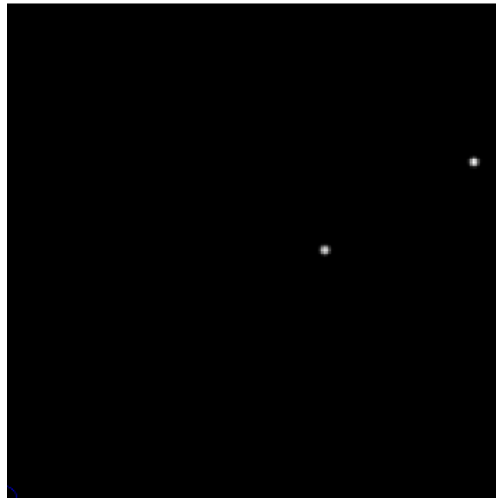


Microscope parfait



Image obtenue

# Quel est le problème avec le TEM: Ce n'est pas une loupe parfaite



Objet initial



Microscope électronique

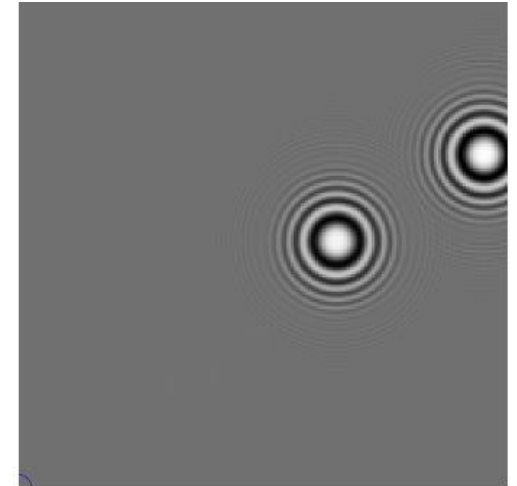
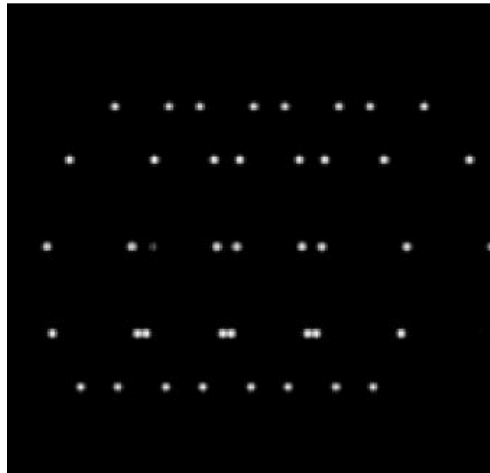


Image obtenue

# Quel est le problème avec le TEM: Ce n'est pas une loupe parfaite



Objet initial



Microscope électronique

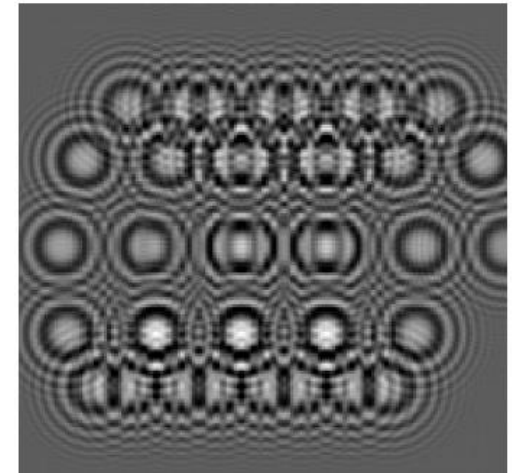
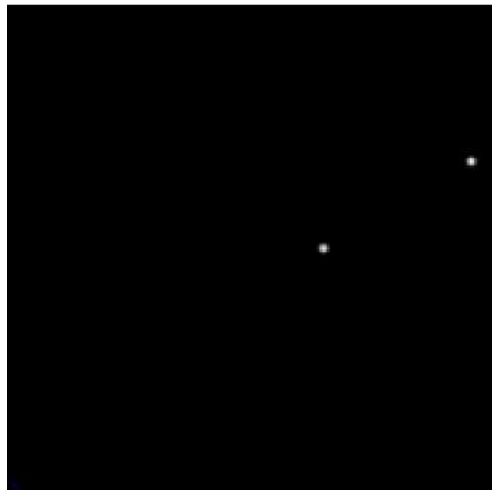
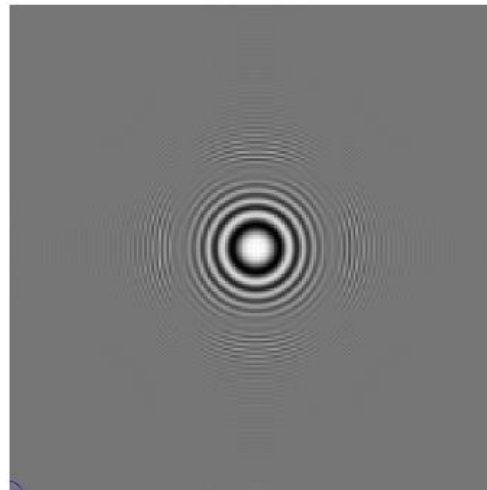


Image obtenue

# L'explication



Objet initial



Fonction propre du MET

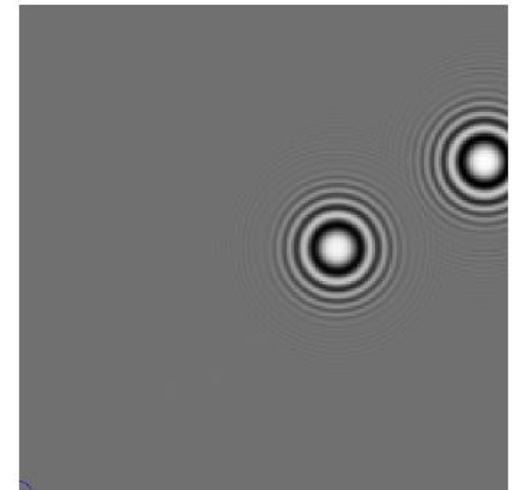


Image obtenue

C'est une convolution

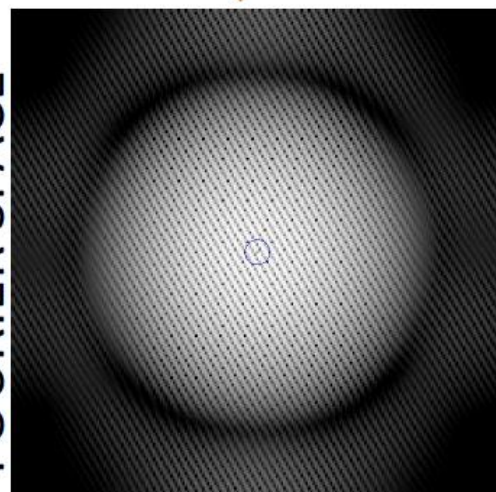
# Qu'est ce qu'une convolution ?



FOURIER SPACE



FT  
↓



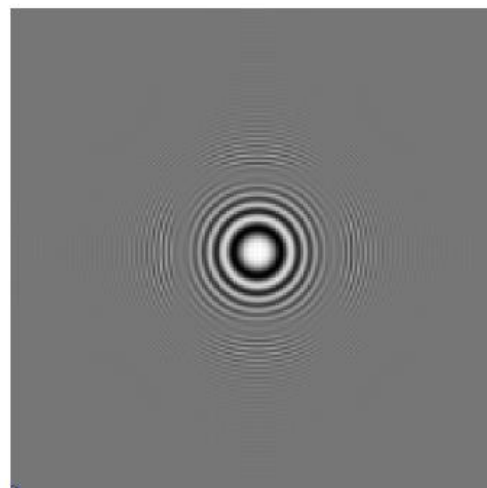
Transformée de Fourier  
de l'objet de départ

Adaptée d' A Desfosses

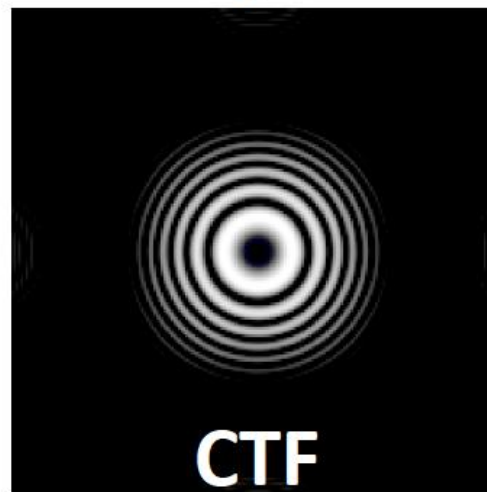


FT<sup>-1</sup>

X



FT  
↓



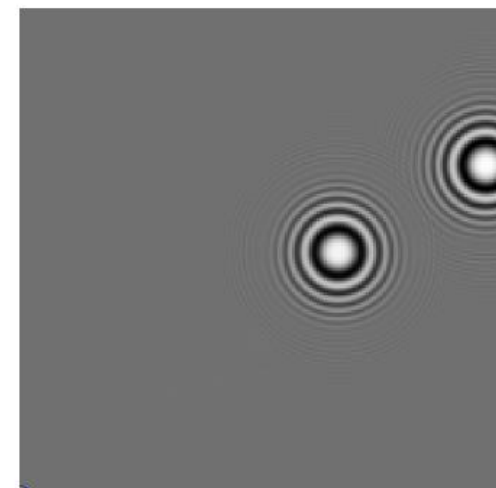
Fonction de transfert de  
contraste

=

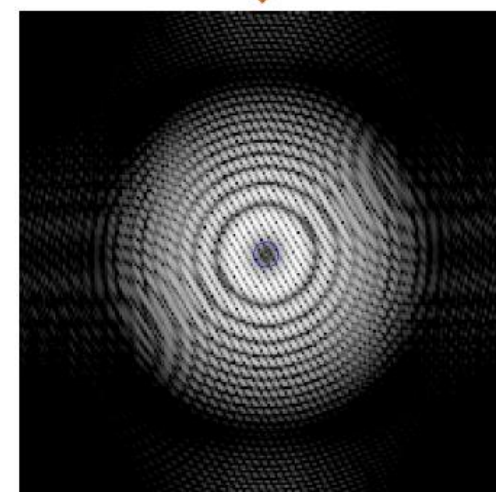


FT<sup>-1</sup>

=



FT  
↓

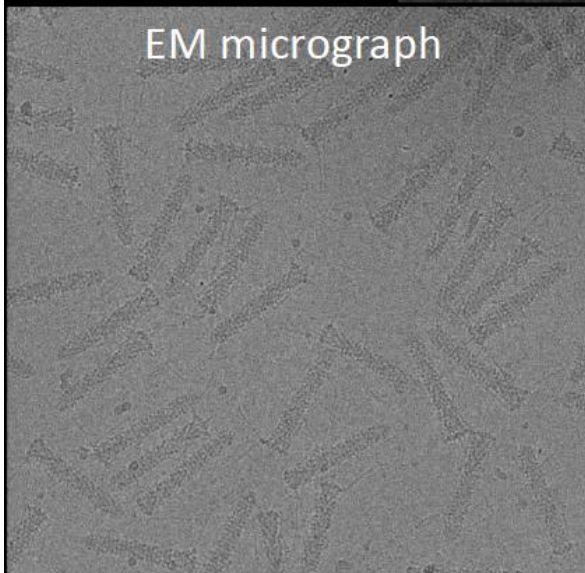


Transformée de Fourier  
de l'image obtenue

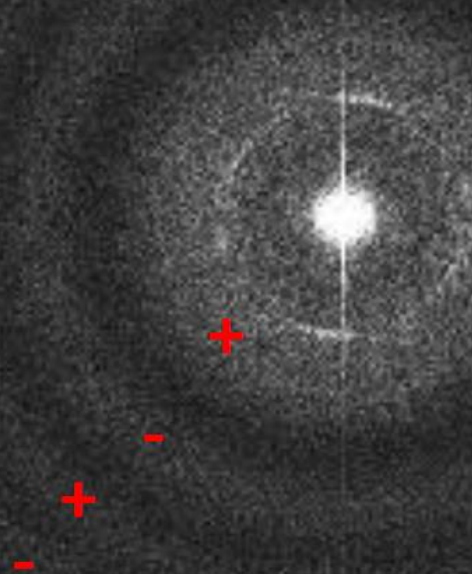
# CTF determination/correction

## A simple way : phase-flipping

Thon rings : zeros of the CTF



FT  
→

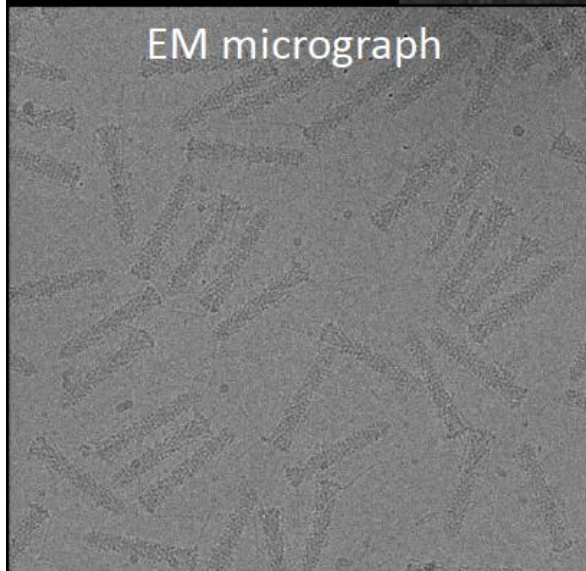


Fourier Transform of micrograph

# CTF determination/correction

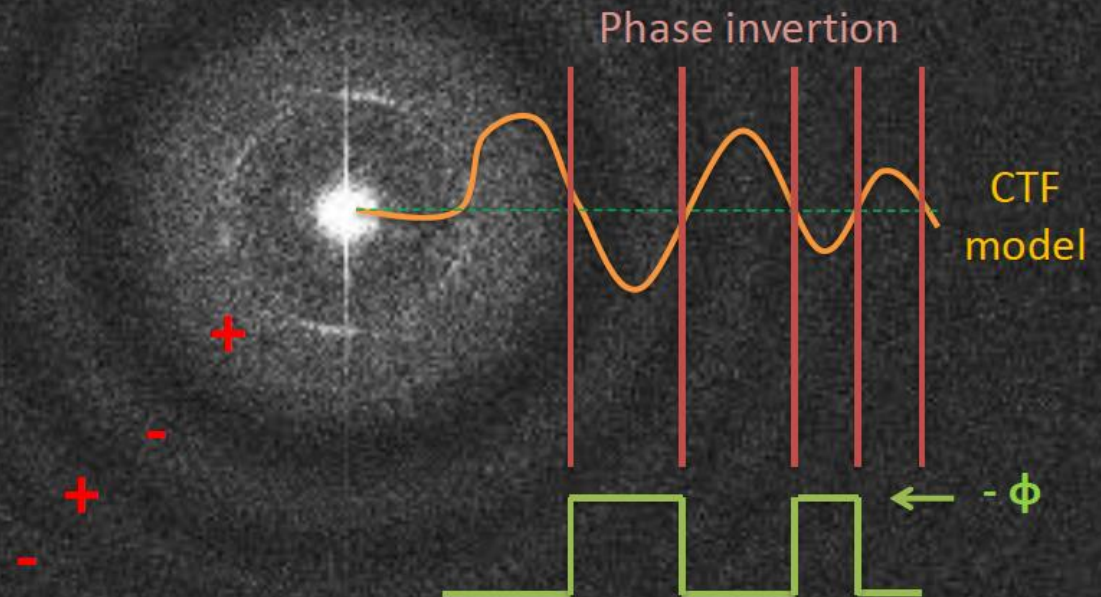
## A simple way : phase-flipping

Thon rings : zeros of the CTF



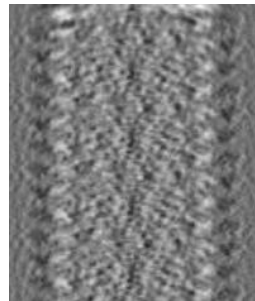
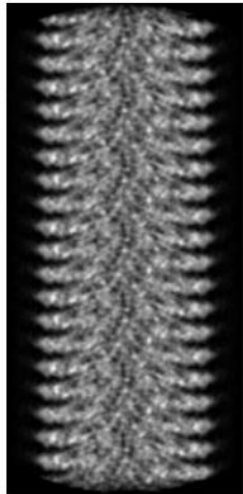
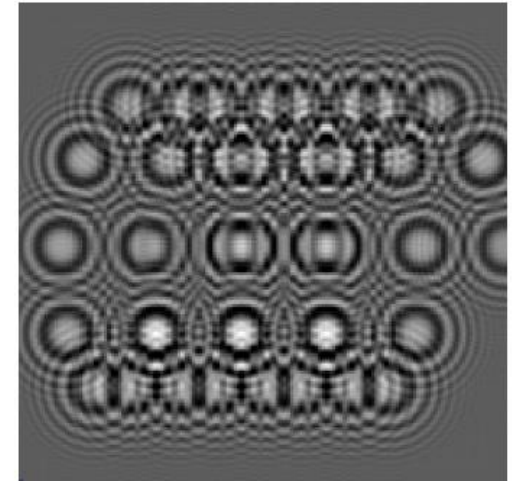
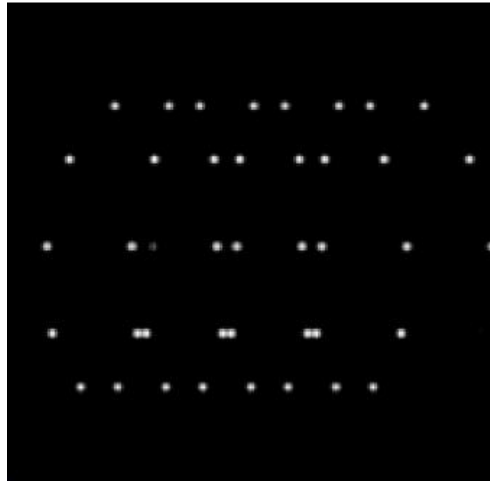
EM micrograph

FT  
→

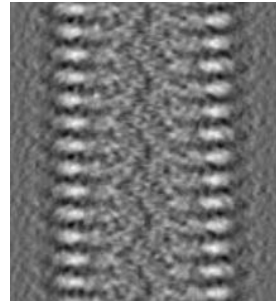


Invert the phase behind  
every second thon ring  
and back-FT

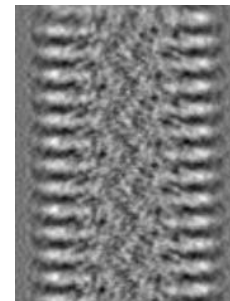
Fourier Transform of micrograph



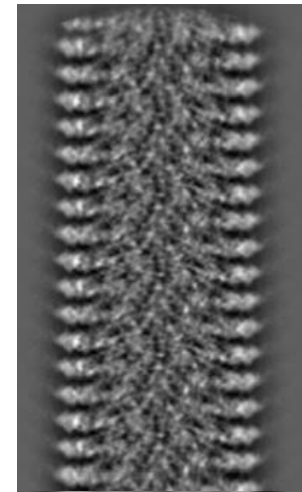
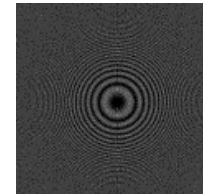
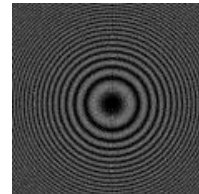
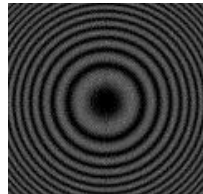
-0.6  $\mu\text{m}$



-1.2  $\mu\text{m}$

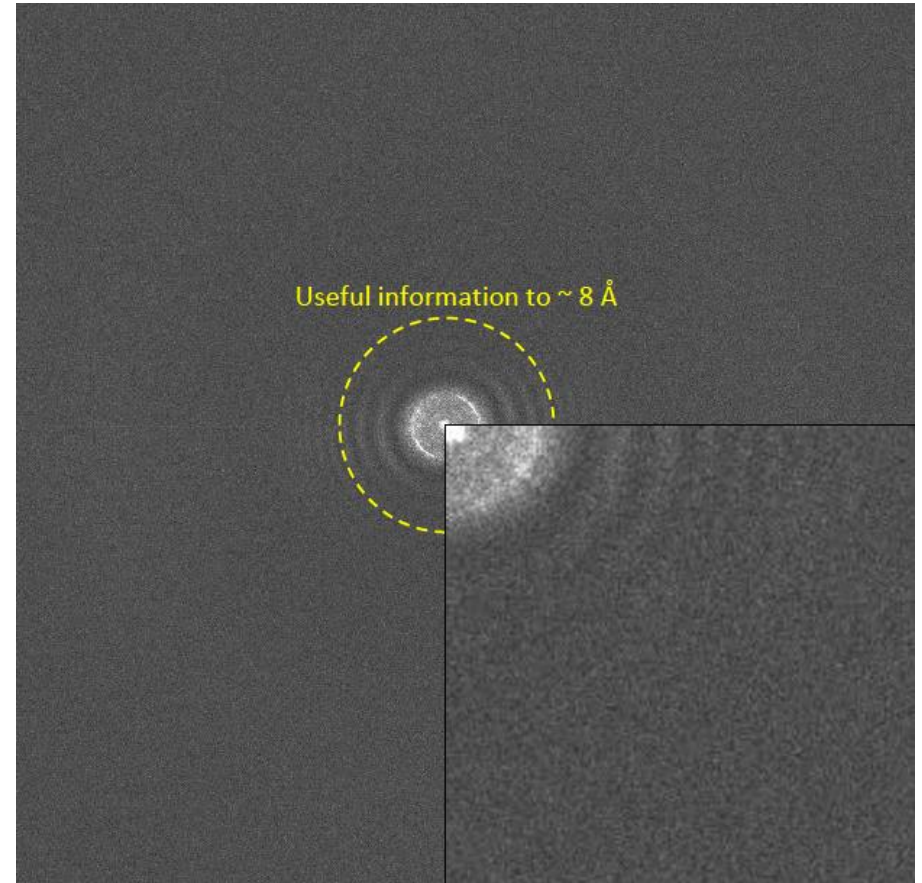


-2.15  $\mu\text{m}$

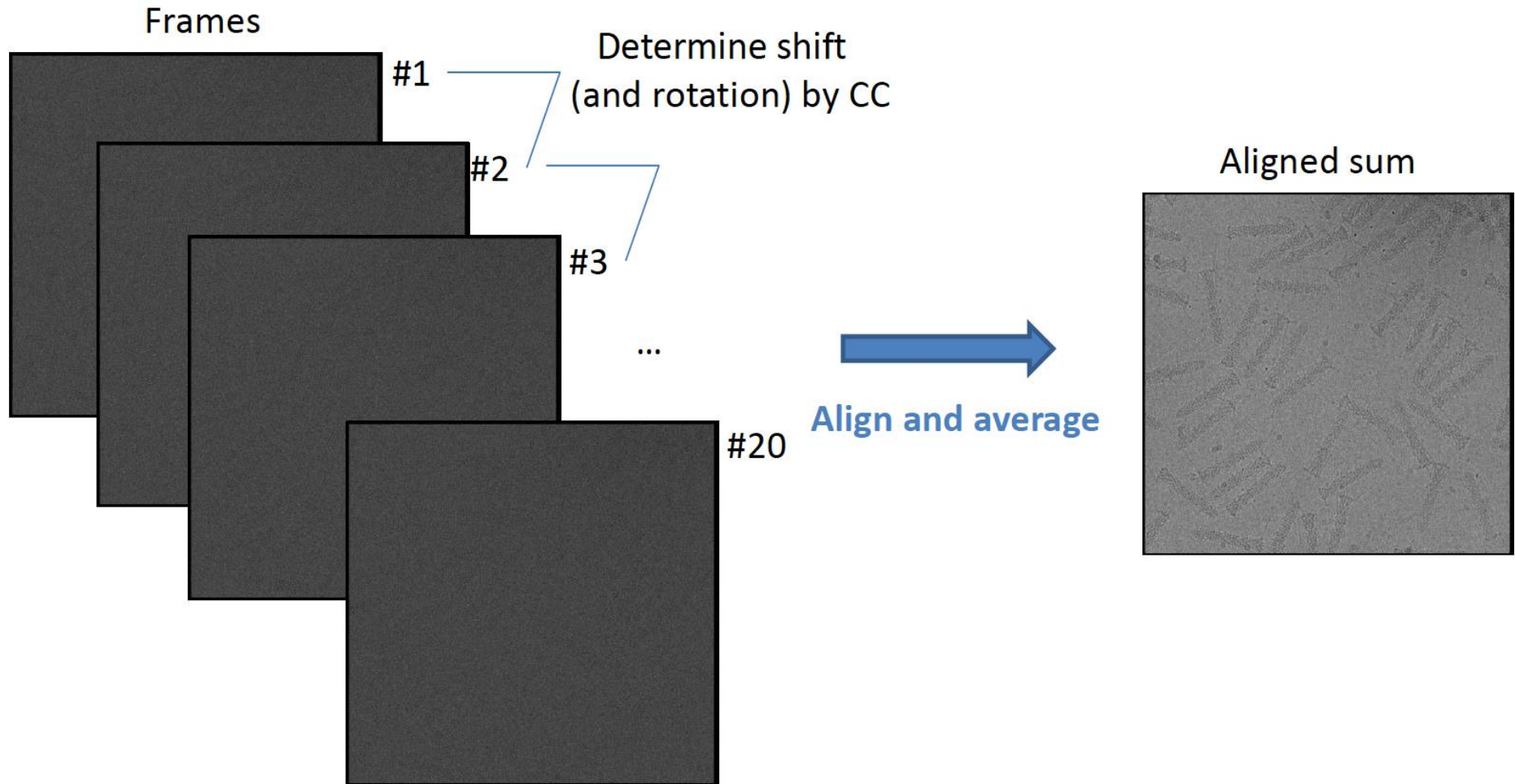


Adapted from A Desfosses

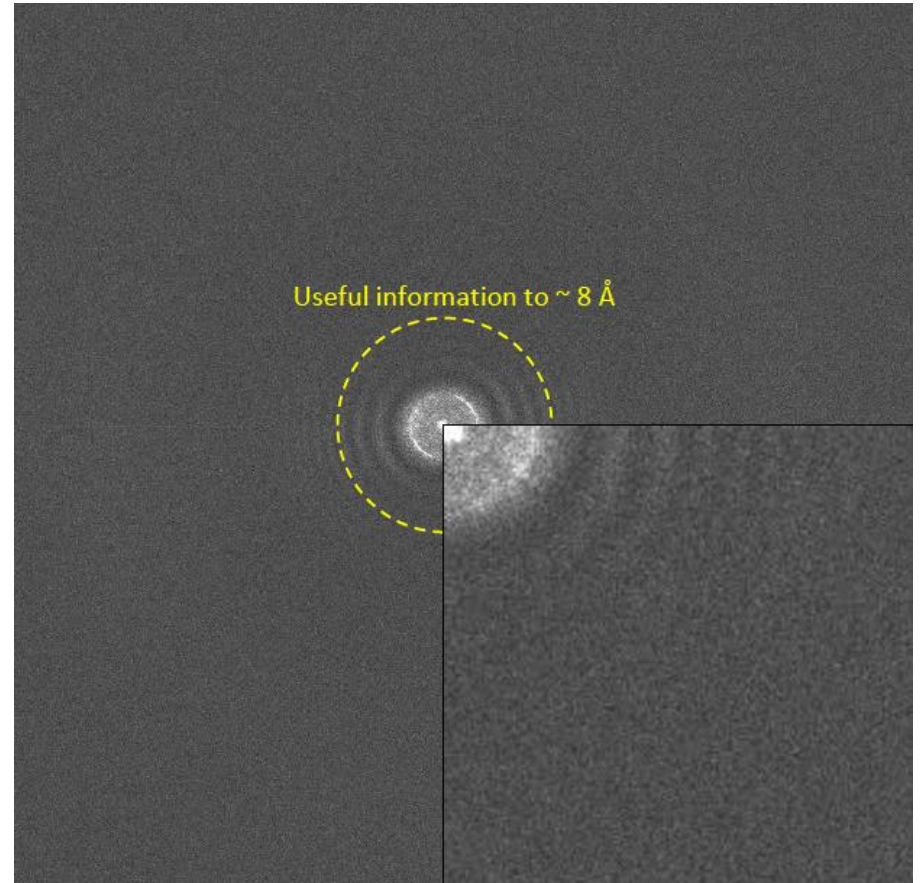
# L'échantillon/microscope ne sont pas parfaitement stables



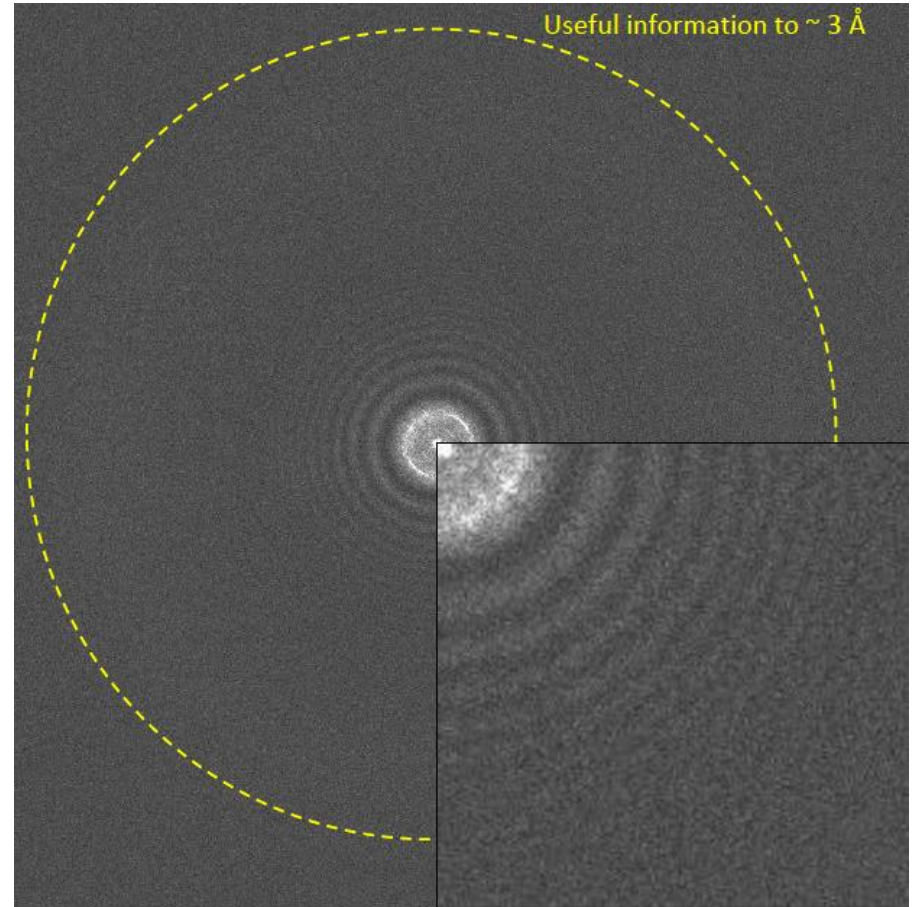
# Correction des images



# Avant correction



# Après correction

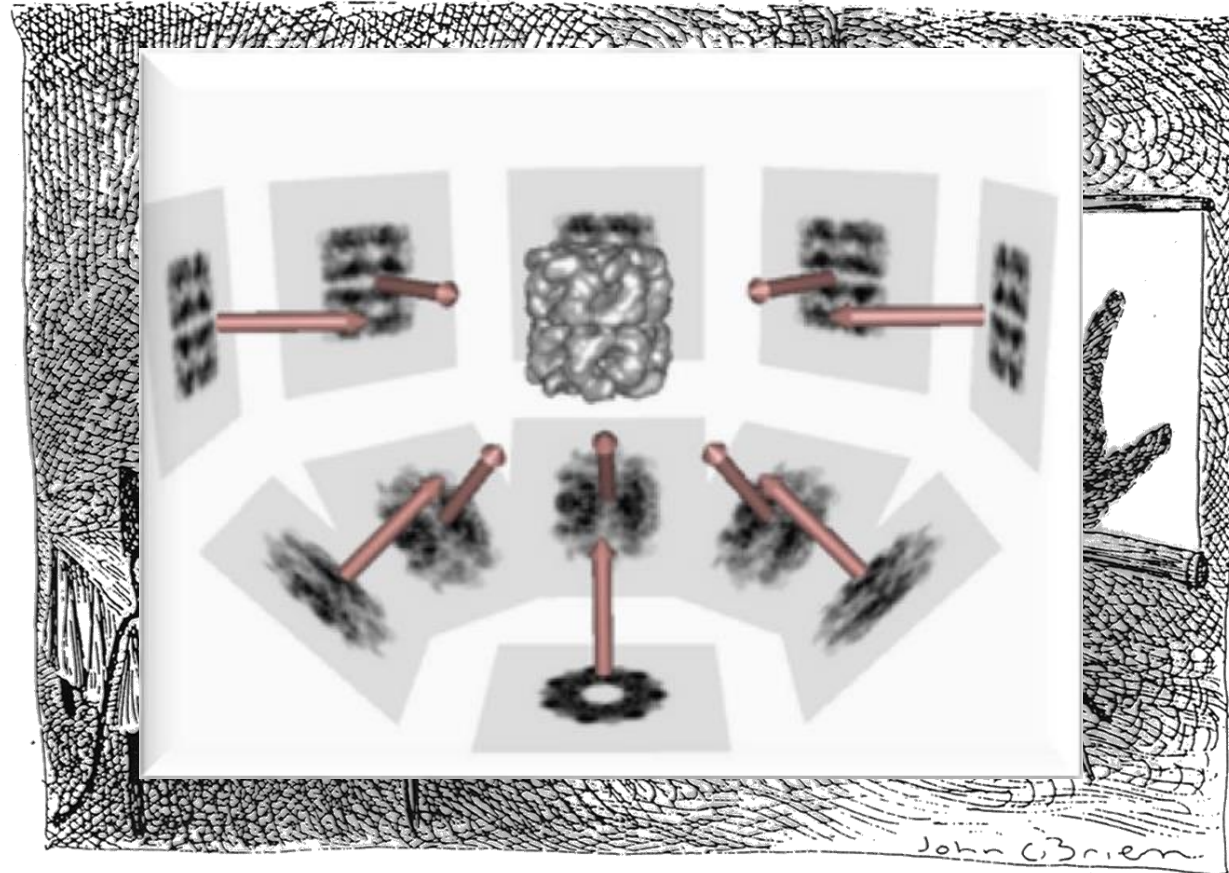


# Production de données

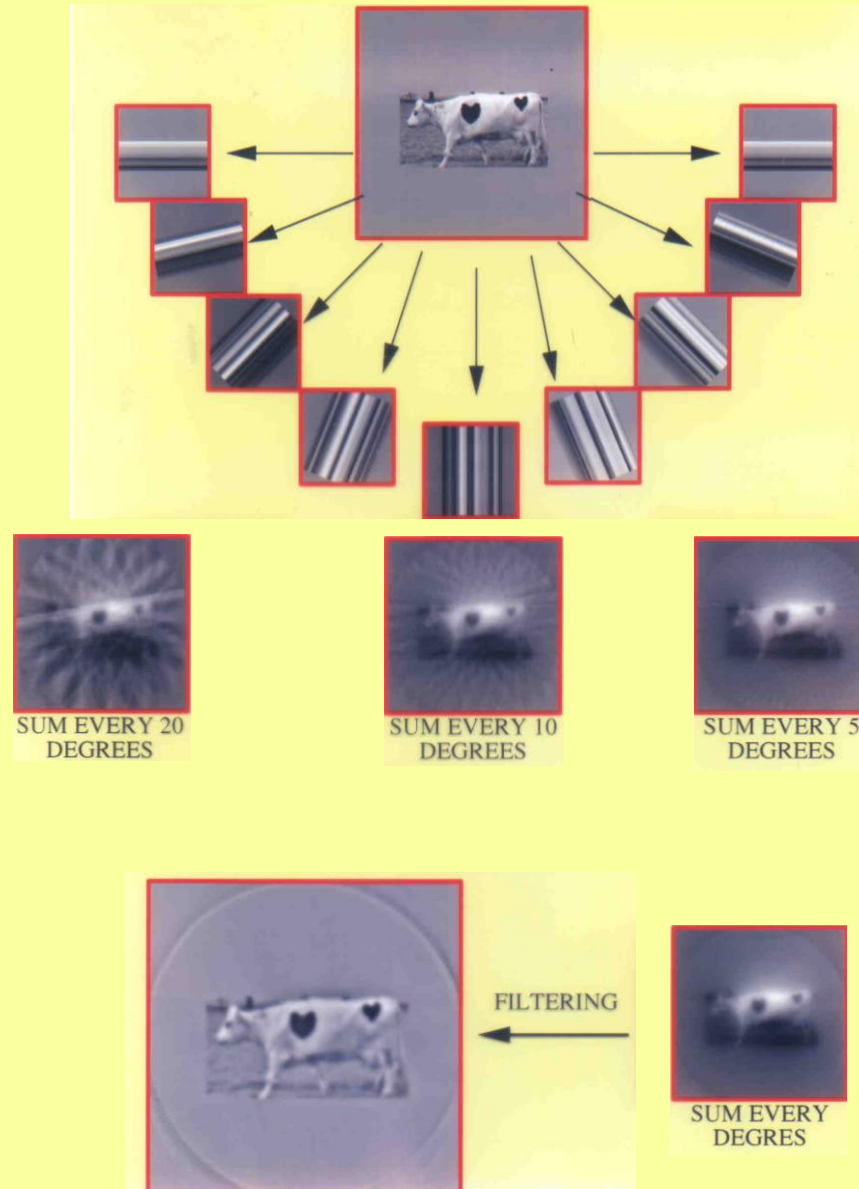


Jusqu'à 9 Gb / image (films) et 800 films par jour

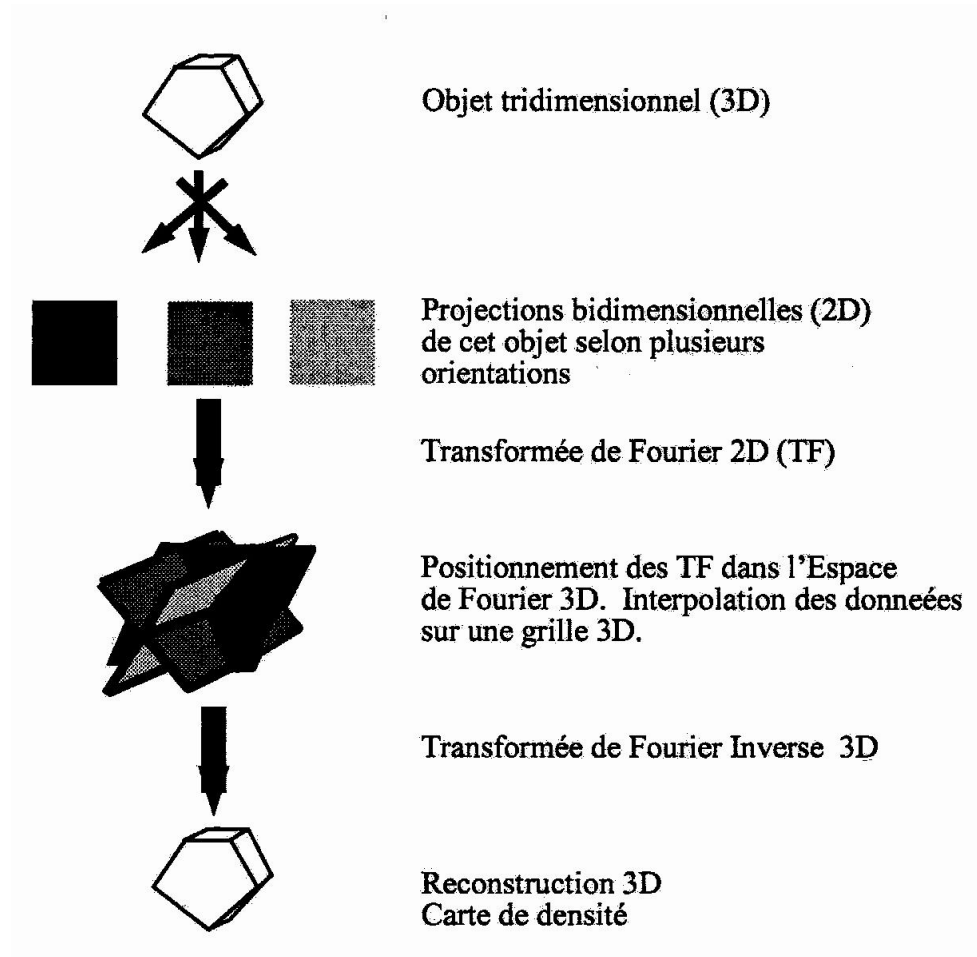
# Analyse d'images



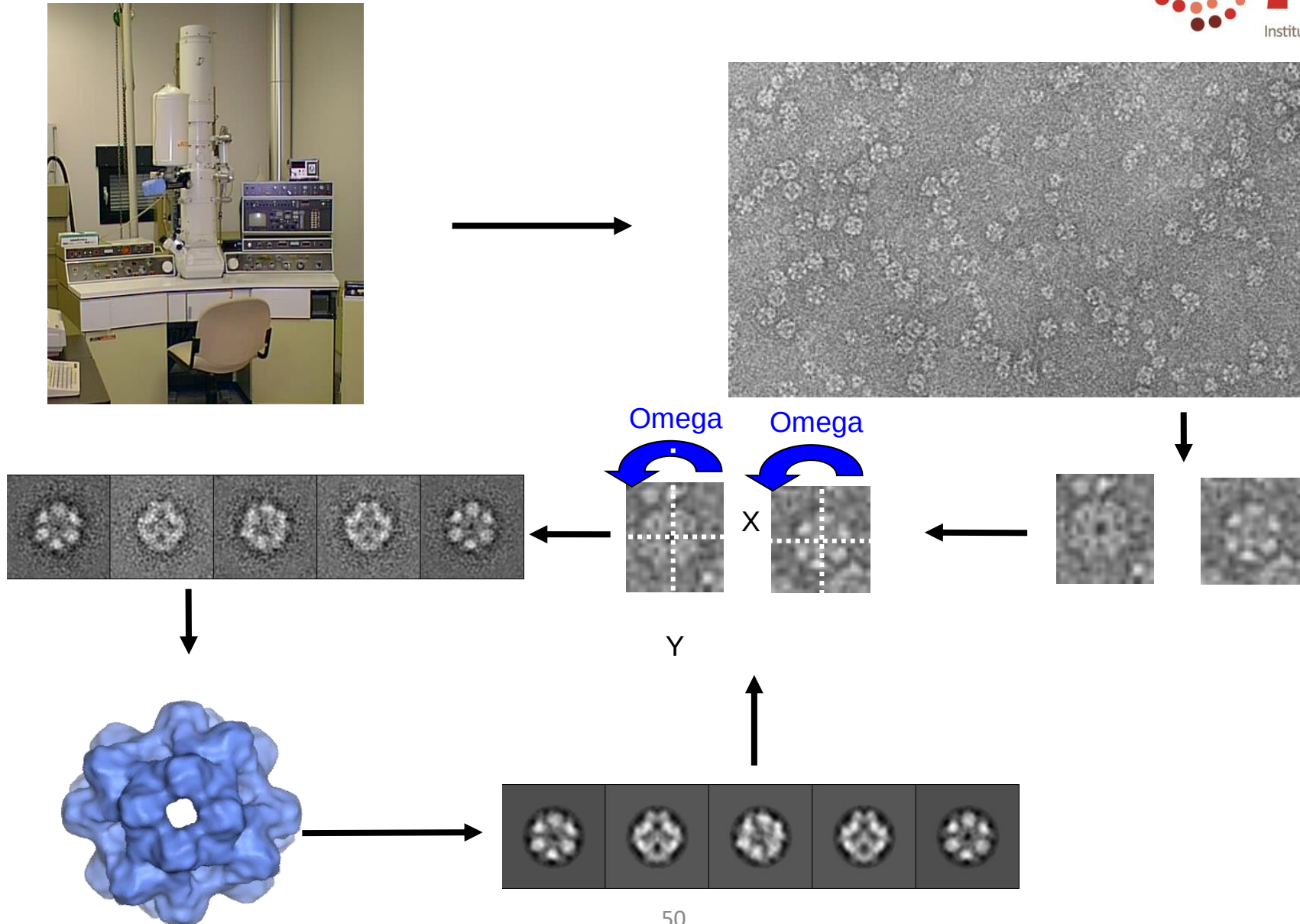
# Reconstruction tridimensionnelle



# Reconstruction tridimensionnelle



# Reconstruction tridimensionnelle



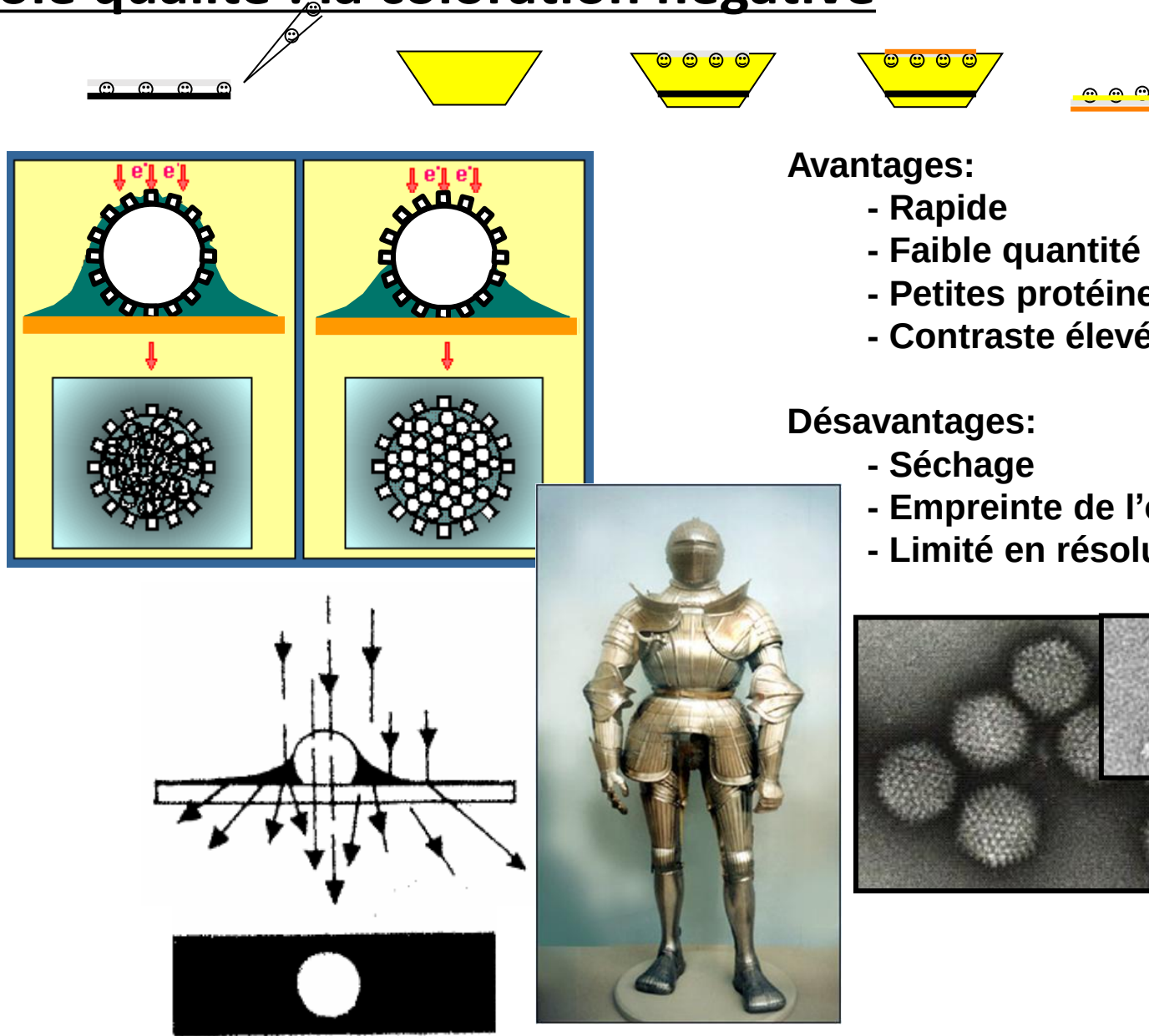
# Préparation des échantillons biologiques pour la microscopie électronique

Les échantillons doivent :

- résister au vide et aux électrons
- garder leur forme native



# Contrôle qualité : la coloration négative



## Avantages:

- Rapide
- Faible quantité d'échantillon
- Petites protéines
- Contraste élevé

## Désavantages:

- Séchage
- Empreinte de l'échantillon
- Limité en résolution (15 Å)

## Cryo-electron microscopy of viruses

**Marc Adrian, Jacques Dubochet, Jean Lepault & Alasdair W. McDowell**

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG

*Thin vitrified layers of unfixed, unstained and unsupported virus suspensions can be prepared for observation by cryo-electron microscopy in easily controlled conditions. The viral particles appear free from the kind of damage caused by dehydration, freezing or adsorption to a support that is encountered in preparing biological samples for conventional electron microscopy. Cryo-electron microscopy of vitrified specimens offers possibilities for high resolution observations that compare favourably with any other electron microscopical method.*

*Quarterly Review of Biophysics* **21**, 2 (1988), pp. 129-228

*Printed in Great Britain*

129

## Cryo-electron microscopy of vitrified specimens

JACQUES DUBOCHET<sup>1</sup>, MARC ADRIAN<sup>2</sup>, JIIN-JU CHANG<sup>3</sup>,  
JEAN-CLAUDE HOMO<sup>4</sup>, JEAN LEPAULT<sup>5</sup>,  
ALASDAIR W. MCDOWALL<sup>6</sup> AND PATRICK SCHULTZ<sup>4</sup>

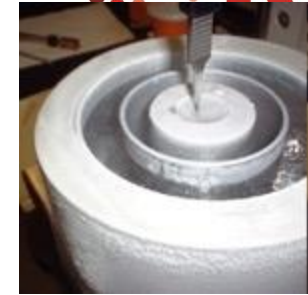
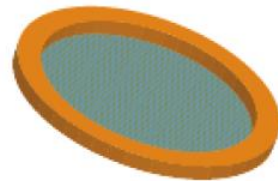
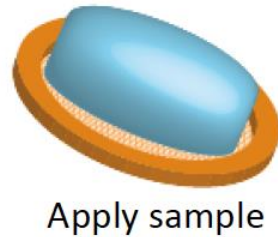
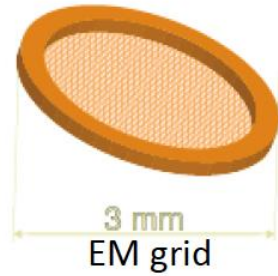
*European Molecular Biology Laboratory (EMBL), Postfach 10. 2209, D-6900 Heidelberg, FRG*



Jacques Dubochet



# Cryo-microscopie électronique

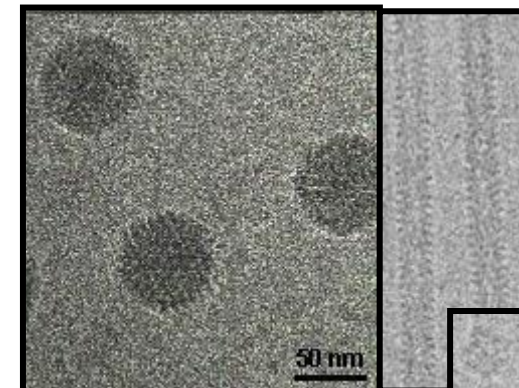
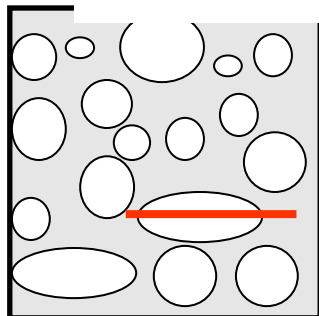
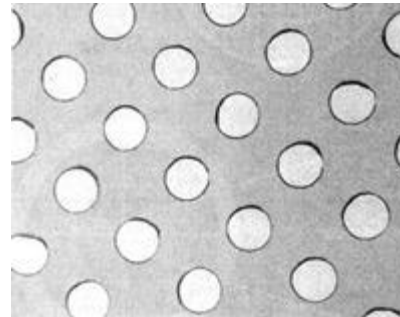


## Avantages:

- Etat natif de l'échantillon

## Désavantages:

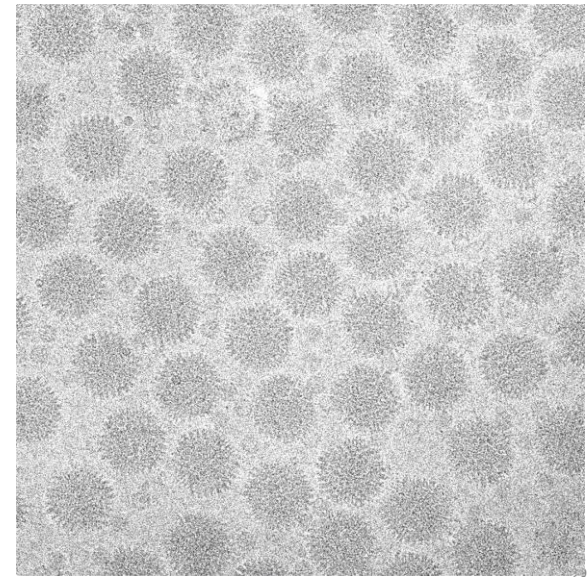
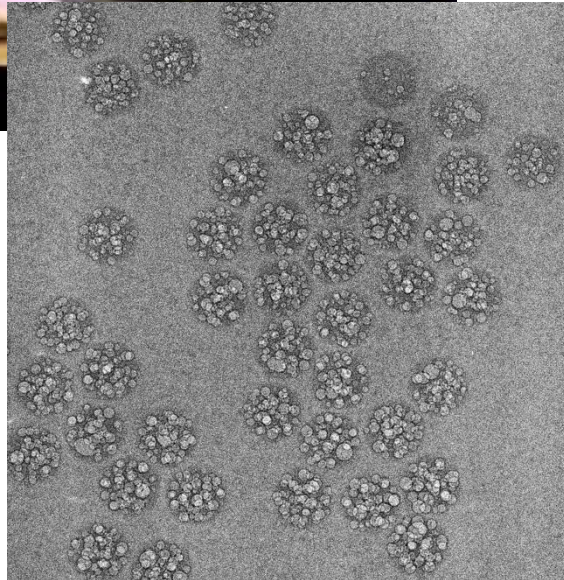
- Faible contraste
- Sensibilité aux électrons
- Taille limitée



# Sensibilité vis-à-vis des électrons



3s d'exposition au faisceau d'électrons correspond à la dose présente au centre d'une explosion atomique.



# Comparaison coloration négative – cryo-microscopie électronique



# Les échantillons



Rubbish in

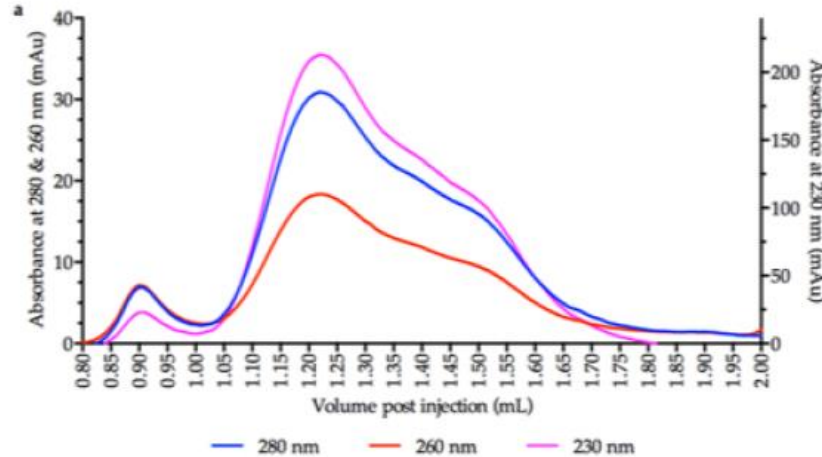


Meilleur microscope  
au monde

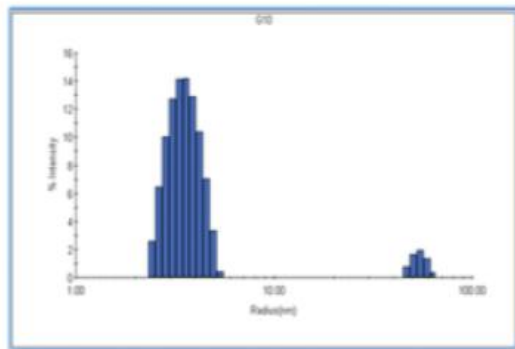


Rubbish out

# Optimisation des échantillons

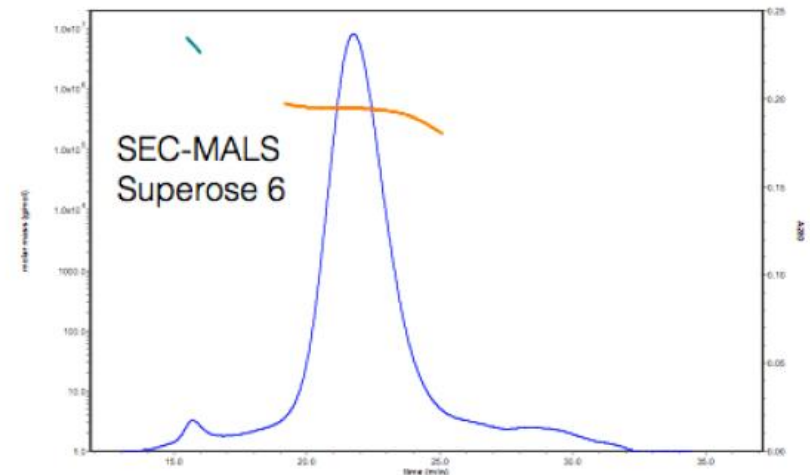


Size exclusion chromatography



DLS  
(Dynamic light scattering)

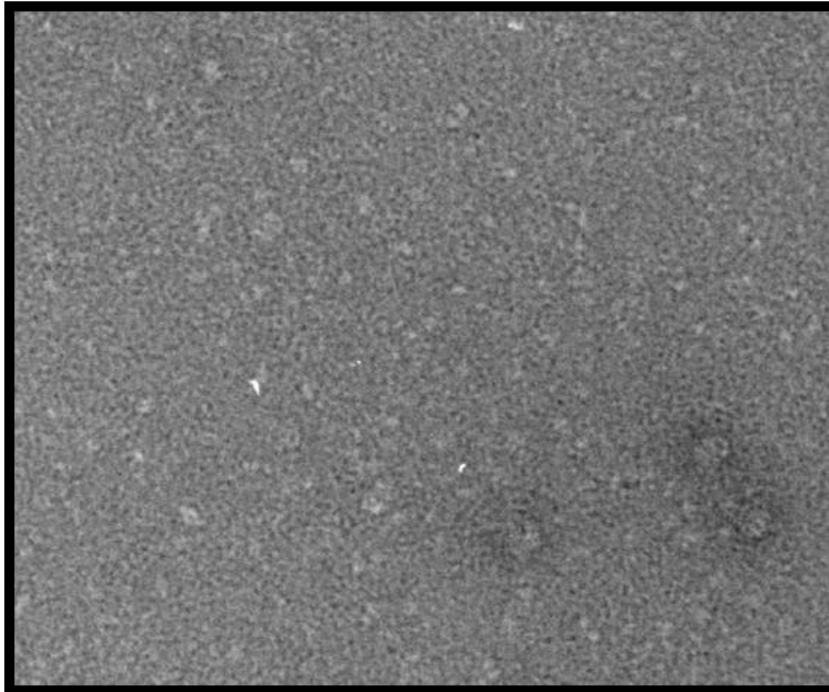
**\*Optimise buffer conditions  
(salt, pH, detergent, etc)**



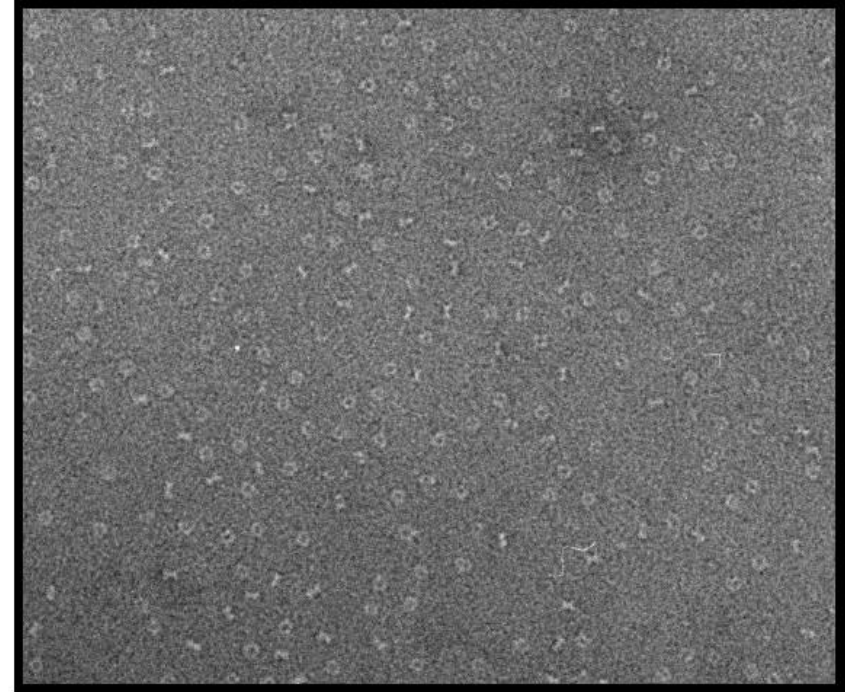
SEC MALS  
(Size exclusion chromatography  
coupled to multi-angle light  
scattering)

# Optimisation des échantillons

Clonage, expression, purification jusqu'à l'homogénéité



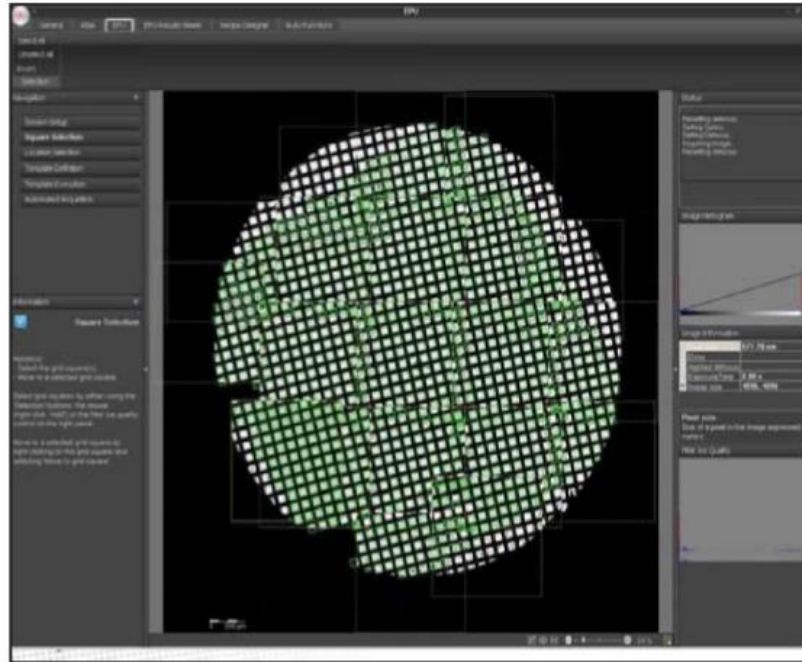
Hétérogène



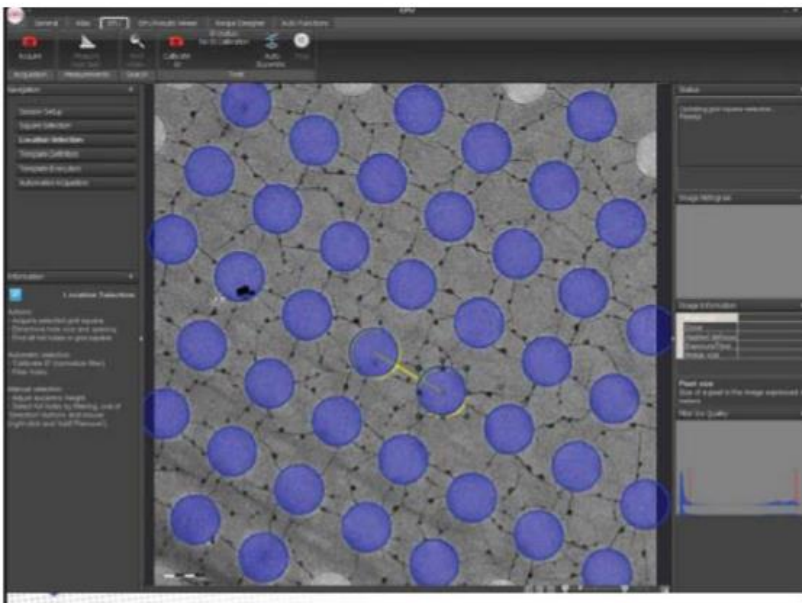
Homogène

150 kDa

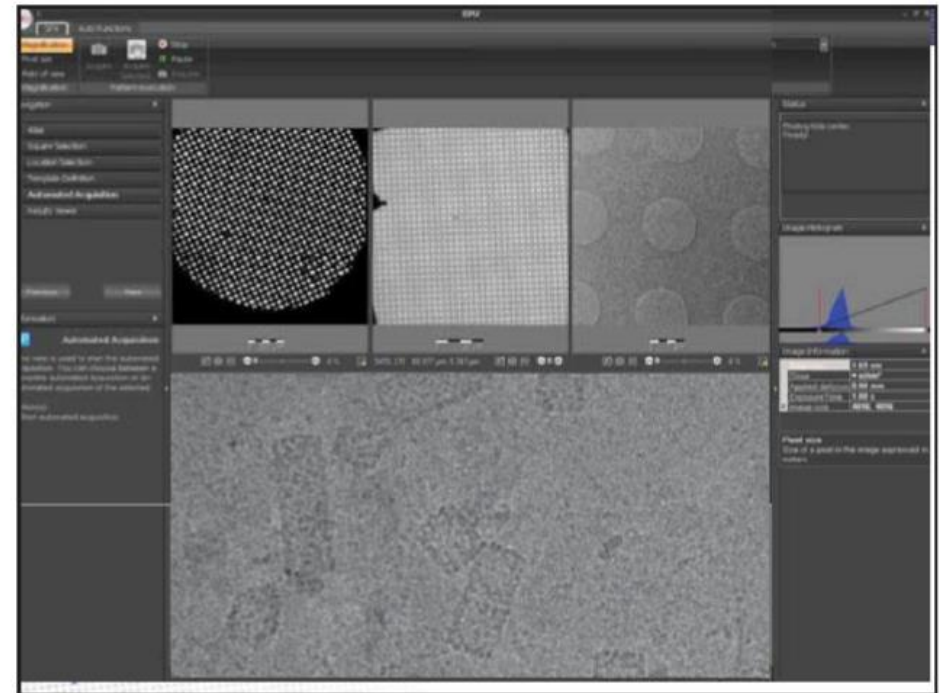
# Collecte des données



1/ Grid square selection



2/ Assisted hole selection



3/ Automated acquisition  
(autofocus, drift correction, ...)

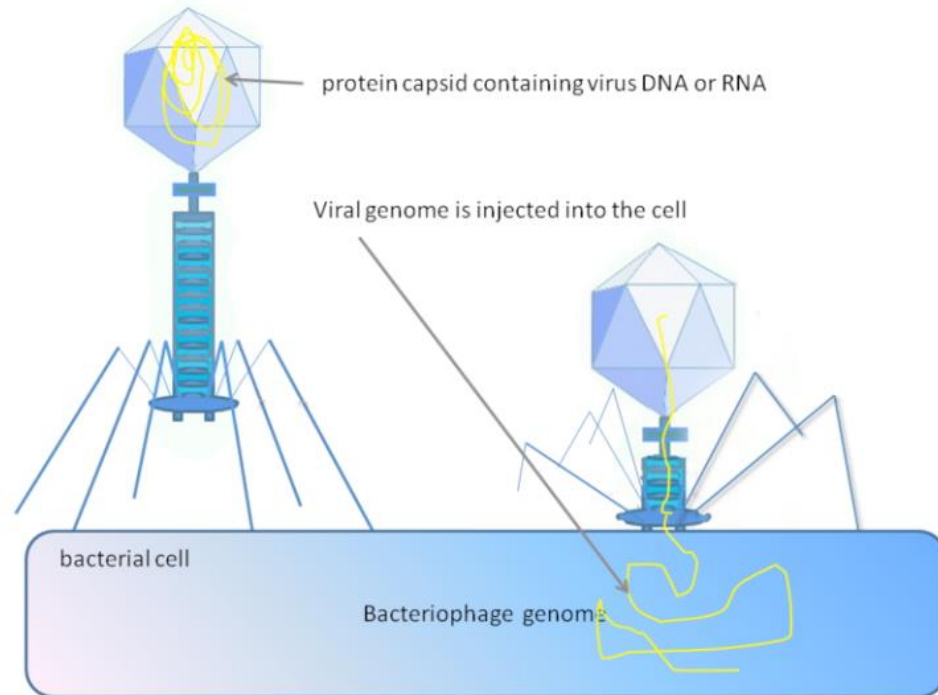
# Bactériophages

Charles Arnaud, Romain Linares, Grégory Effantin, Takashi Yamada, cécile Breyton



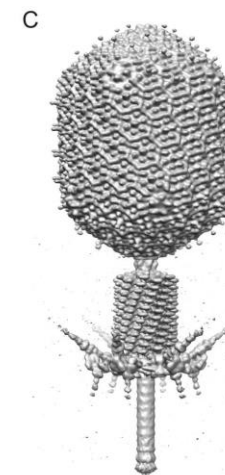
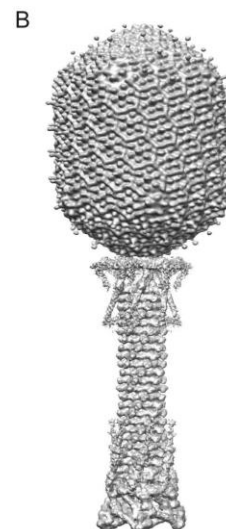
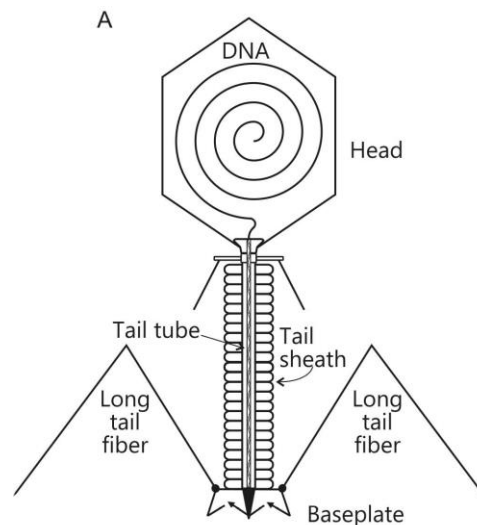
- Virus qui infectent les procaryotes (bactéries)
- Micro-organismes les plus abondants, diversifié sur Terre:  $10^{31}$  particules. Présent dans tous les biotopes
- Rôle dans la régulation, diversité et l'évolution des populations procaryotes (incluant ceux de notre microbiome)
- Rôle dans l'acquisition de certains traits de virulence pour les bactéries
- Alternative aux antibiotiques (phagothérapie)
- ~80% des gènes de bactériophage n'ont pas d'homologues protéiques actuellement connus
- Taille élevée

# Bactériophages

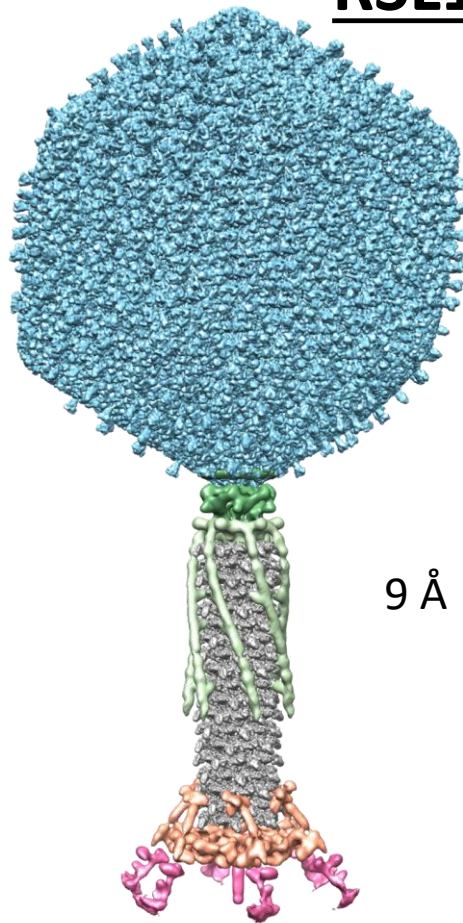
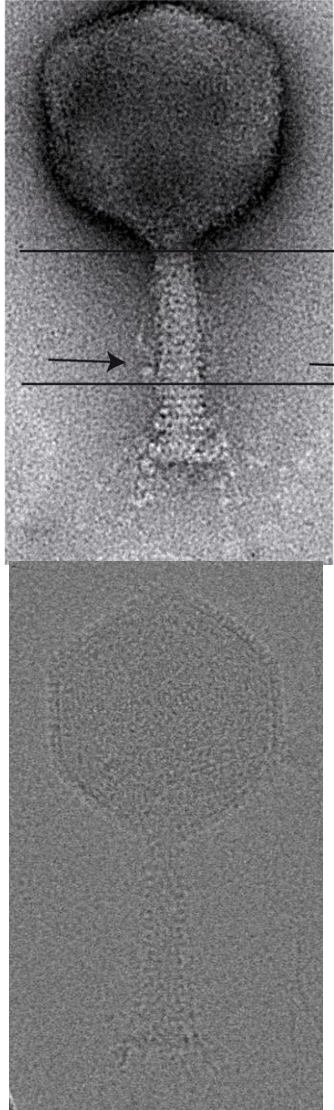


# Classification des bactériophages

| Order               | Family                  | Morphology                               | Nucleic acid    | Examples        |
|---------------------|-------------------------|------------------------------------------|-----------------|-----------------|
|                     | <i>Myoviridae</i>       | Non-enveloped, contractile tail          | Linear dsDNA    |                 |
| <i>Caudovirales</i> | <i>Siphoviridae</i>     | Non-enveloped, long non-contractile tail | Linear dsDNA    | $\lambda$ phage |
|                     | <i>Podoviridae</i>      | Non-enveloped, short noncontractile tail | Linear dsDNA    | T7 phage        |
|                     | <i>Tectiviridae</i>     | Non-enveloped, isometric                 | Linear dsDNA    |                 |
| Unassigned          | <i>Corticoviridae</i>   | Non-enveloped, isometric                 | Circular dsDNA  |                 |
|                     | <i>Lipothruxviridae</i> | Enveloped, rod-shaped                    | Linear dsDNA    |                 |
|                     | <i>Plasmaviridae</i>    | Enveloped, pleomorphic                   | Circular dsDNA  |                 |
|                     | <i>Rudiviridae</i>      | Non-enveloped, rod-shaped                | Linear dsDNA    |                 |
|                     | <i>Fuselloviridae</i>   | Non-enveloped, lemon-shaped              | Circular dsDNA  |                 |
|                     | <i>Inoviridae</i>       | Non-enveloped, filamentous               | Circular ssDNA  |                 |
|                     | <i>Microviridae</i>     | Non-enveloped, isometric                 | Circular ssDNA  |                 |
|                     | <i>Leviviridae</i>      | Non-enveloped, isometric                 | Linear ssRNA    |                 |
|                     | <i>Cystoviridae</i>     | Enveloped, spherical                     | Segmented dsRNA |                 |

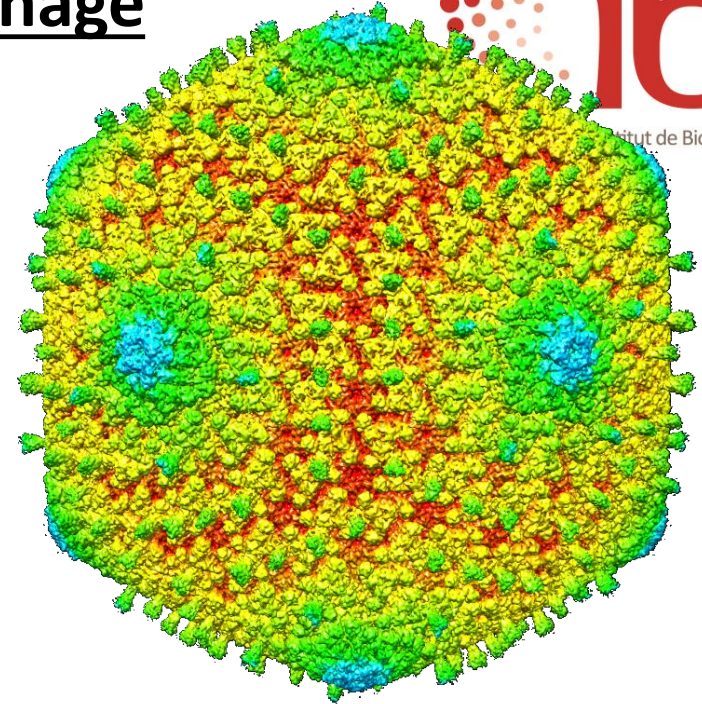


# RSL1 jumbo phage

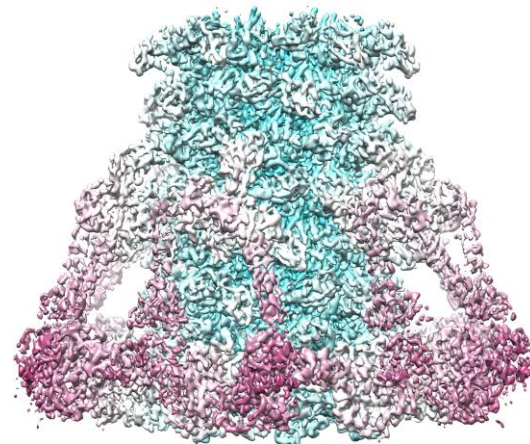


9 Å

2013



5.2 Å

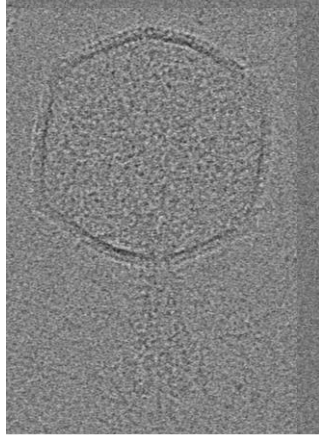


5.9 Å

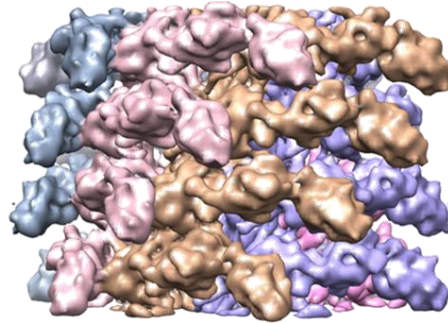
100 Å

En collaboration avec Hiroshima University

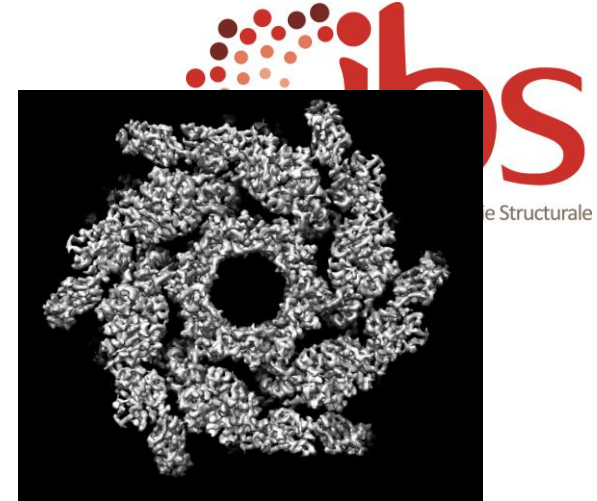
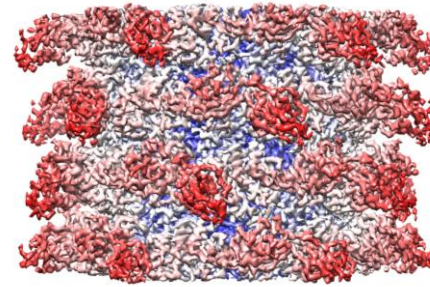
G Effantin



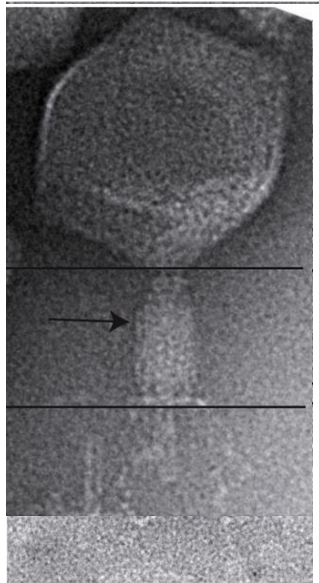
9 Å



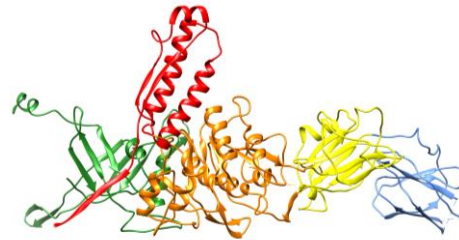
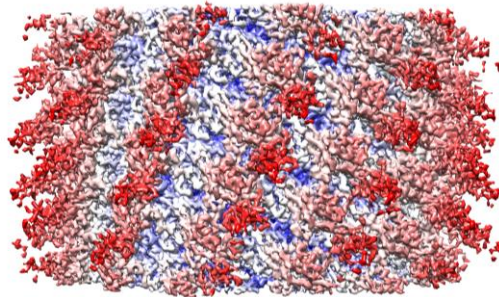
3.7 Å

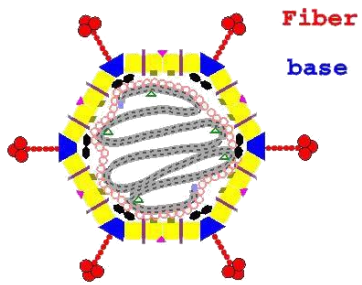


2013



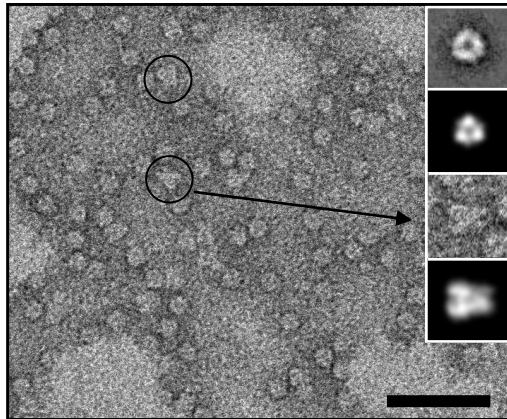
4.2 Å





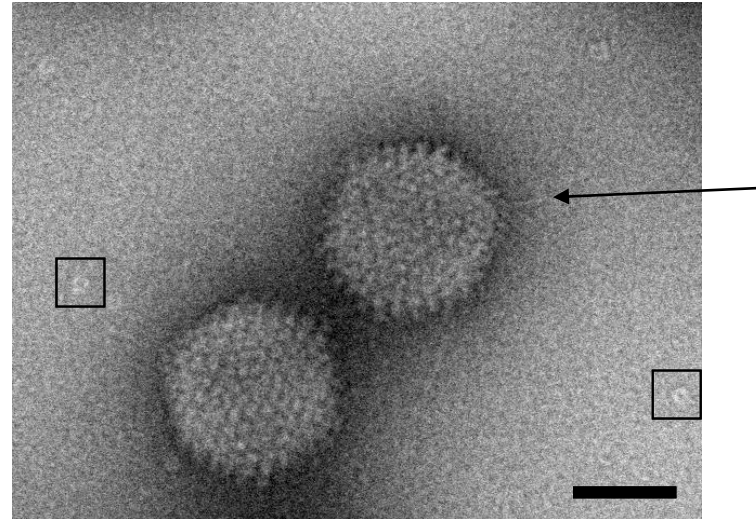
# Adénovirus

Emilie Stermann, Grégory Effantin, André Lieber, Pascal Fender

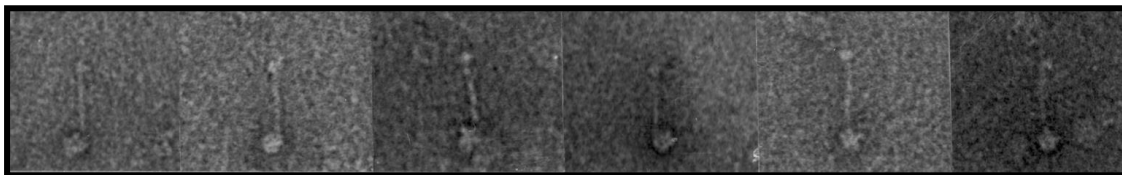


**Hexon**

**SST**

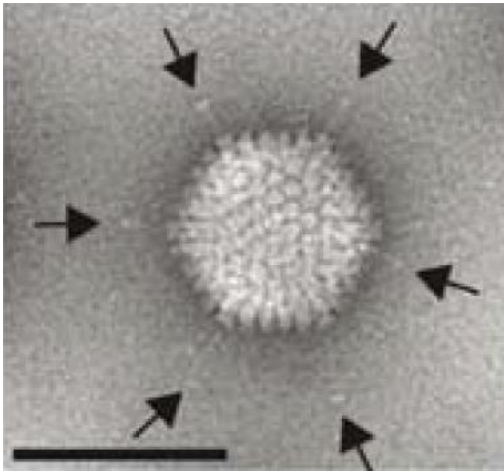


**Molybdate d'Ammonium**

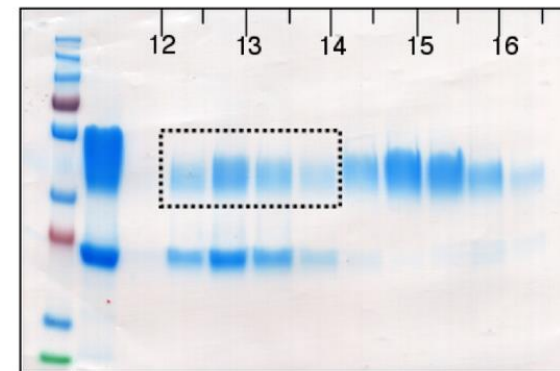
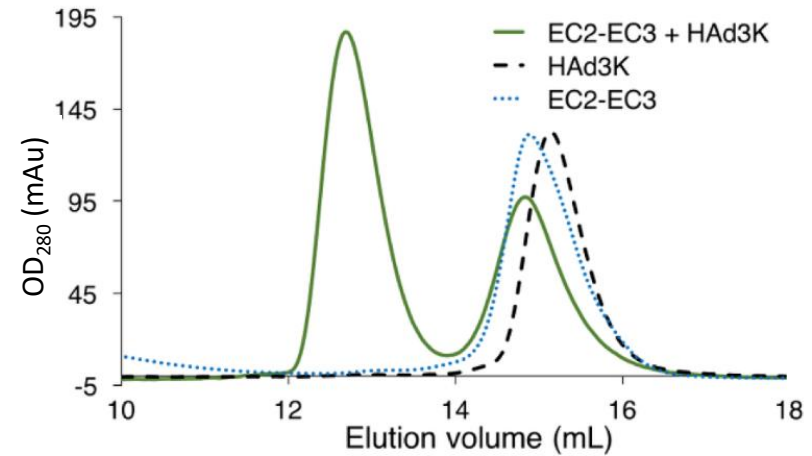


**Penton**

# Interaction fibre de l'Adénovirus - Desmoglérine 2 (DSG2)



Human adenovirus



EC2-EC3 + HAd3K

Stermann et al., Sci Rep 2018

# Interaction fibre de l'Adénovirus - Desmogléine 2 (DSG2)

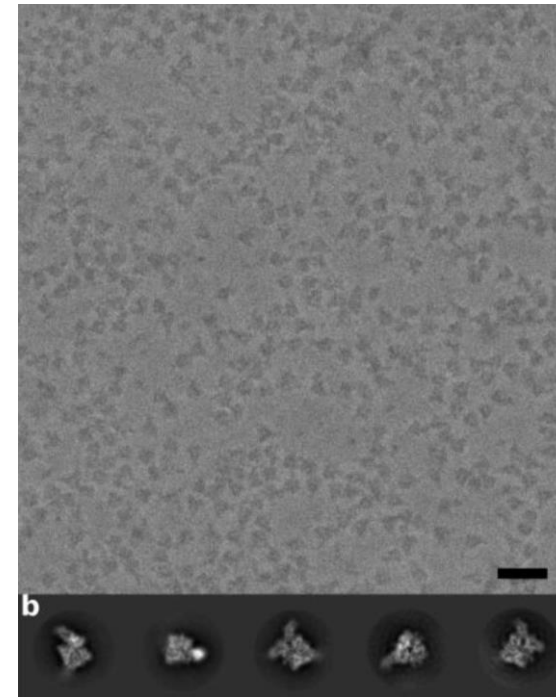
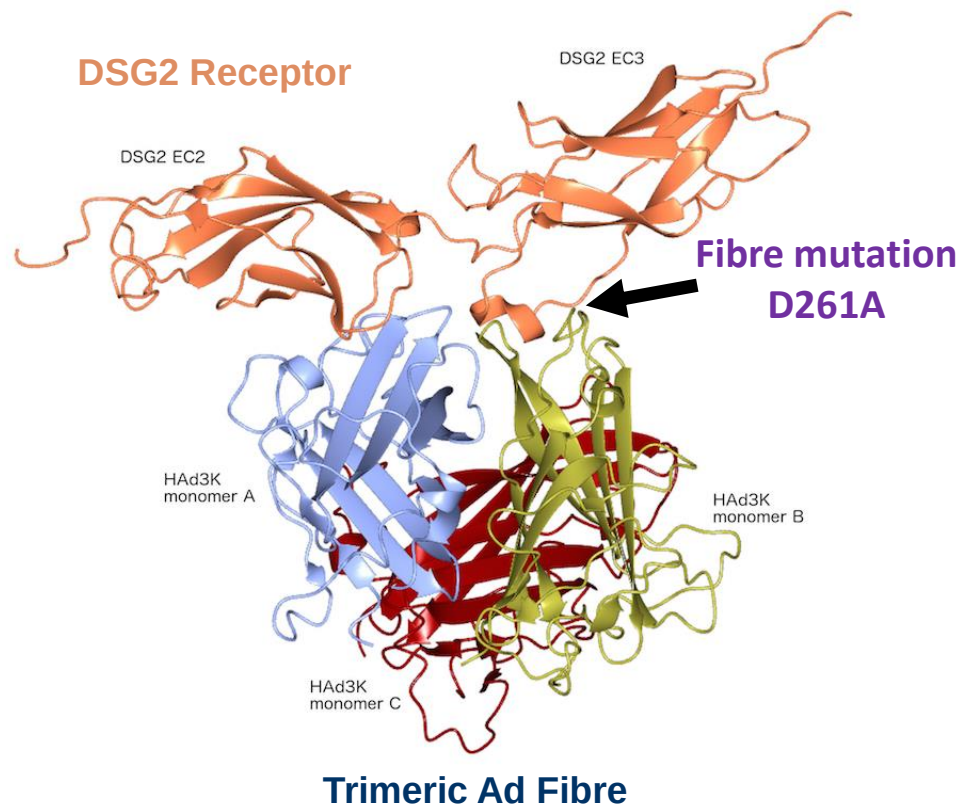


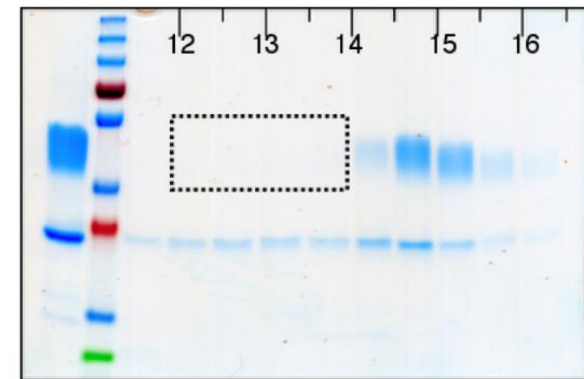
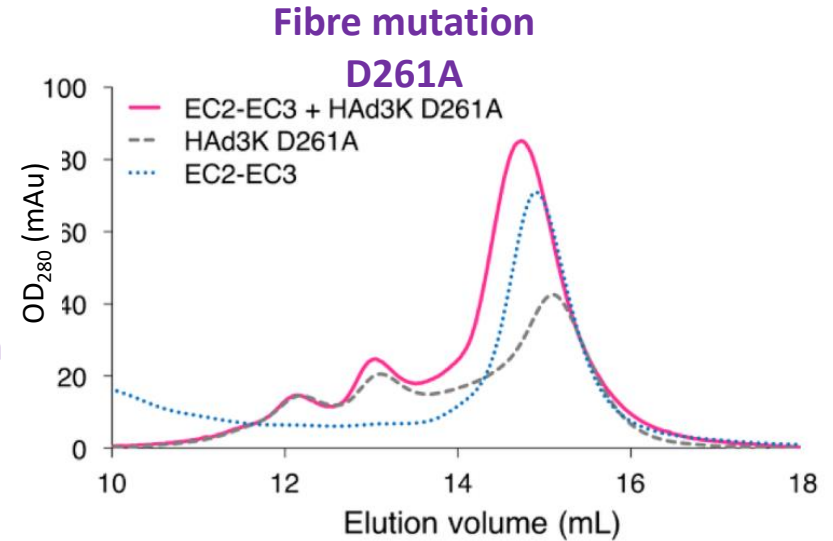
Image du complexe Ad+DSG2 (96kDa)  
obtenue à l'aide du microscope Krios (ESRF)

# Interaction fibre de l'Adénovirus - Desmogléine 2 (DSG2)



Complex (96kDa) determined @3.5Å

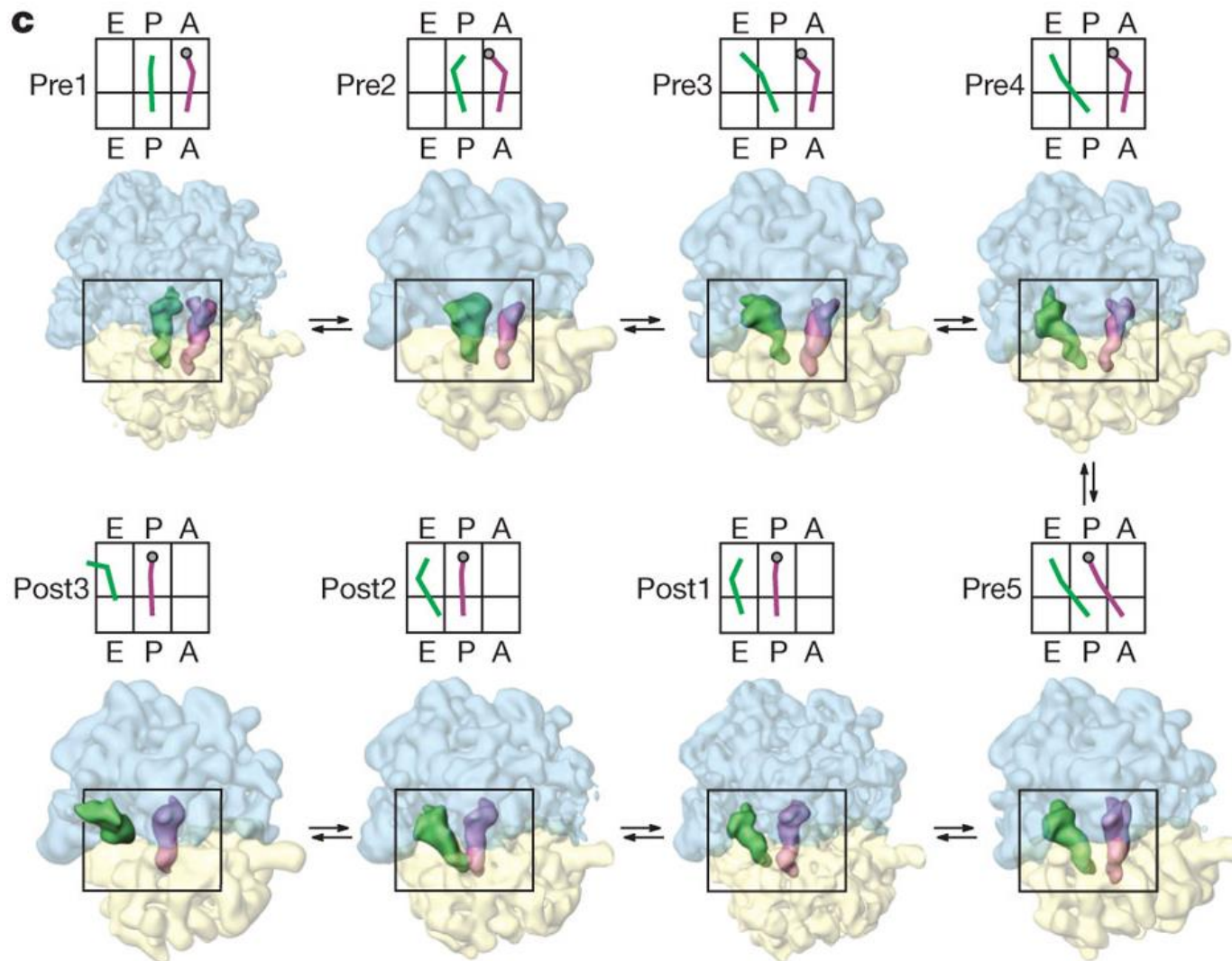
*G. Effantin*



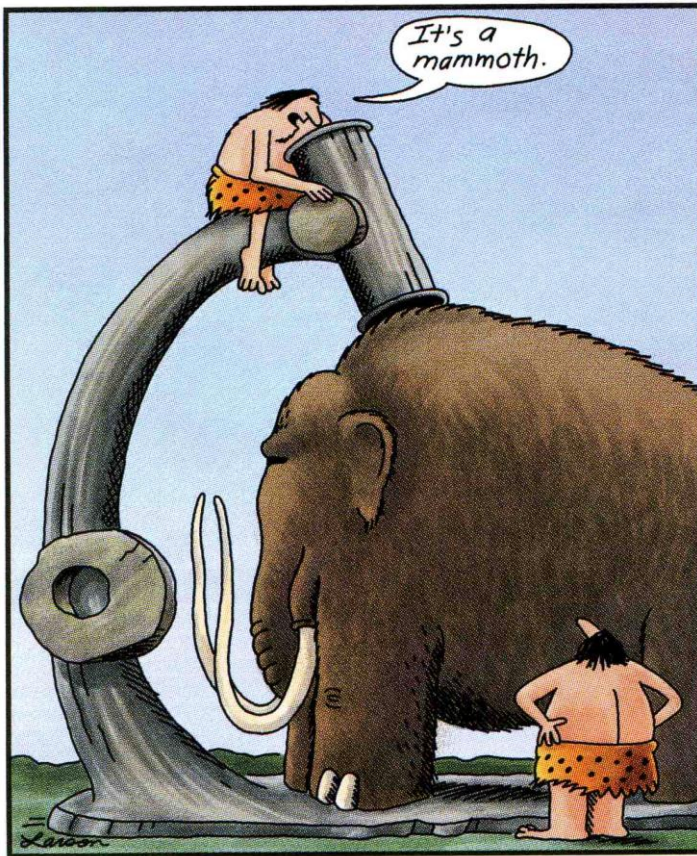
EC2-EC3 + HA3K D261A

Stermann et al., Nat Comm 2019

# Autre exemple

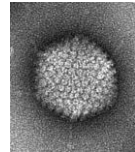
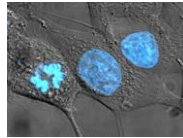


# Échantillons biologiques épais

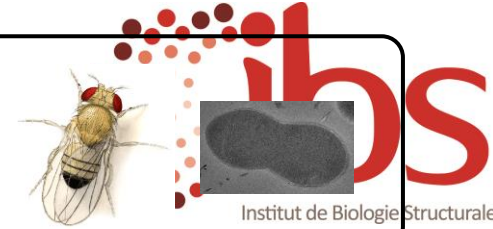


Early microscope





# Cellule



- Fixation  
(pour arrêter  
les processus cellulaires)

## Cryo fixation

*HPM100*



## Chemical fixation



- « Rigidification »

**CEMOVIS**

-FC7



**Freeze  
substitution**



→ Tokuyasu  
**RT gelatine  
embedding**

**RT resin  
embedding**

**PLT**



- Microtomie

**RT  
sectionning**



**Cryosections  
Immunogold**  
UC7-FC7



**RT  
sectionning**



**RT  
sectionning**



- Visualisation

**Cryo-TEM**

**TEM**

**TEM**

**TEM**

**TEM**

|                 |       |     |        |       |    |
|-----------------|-------|-----|--------|-------|----|
| Ultrastructure  | +++++ | +++ | + / ++ | ++    | ++ |
| Immunolabelling | -     | ++  | +++    | + / - | ++ |
| Difficulty      | +++   | ++  | ++(+)  | +     | +  |

## Protocole

- **Fixation chimique (ou par le froid)** : stabilise les échantillons grâce à des **aldéhydes** (formaldéhyde and glutaraldéhyde) ou au tétraoxyde d'osmium (lipides)
- **Déshydratation** ou remplacement de l'eau par des solvants organiques
- **Enrobage** à l'aide d'une résine époxy, par exemple Epon.
- **Ultramicrotomie** : coupes de 60–90 nm d'épaisseur à l'aide d'un couteau diamant.
- **Coloration (atomes lourds)**
- **Observations**

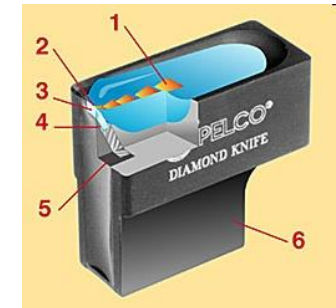
# Échantillons biologiques épais

Substitution de l'eau par du  
plastique (AFS)

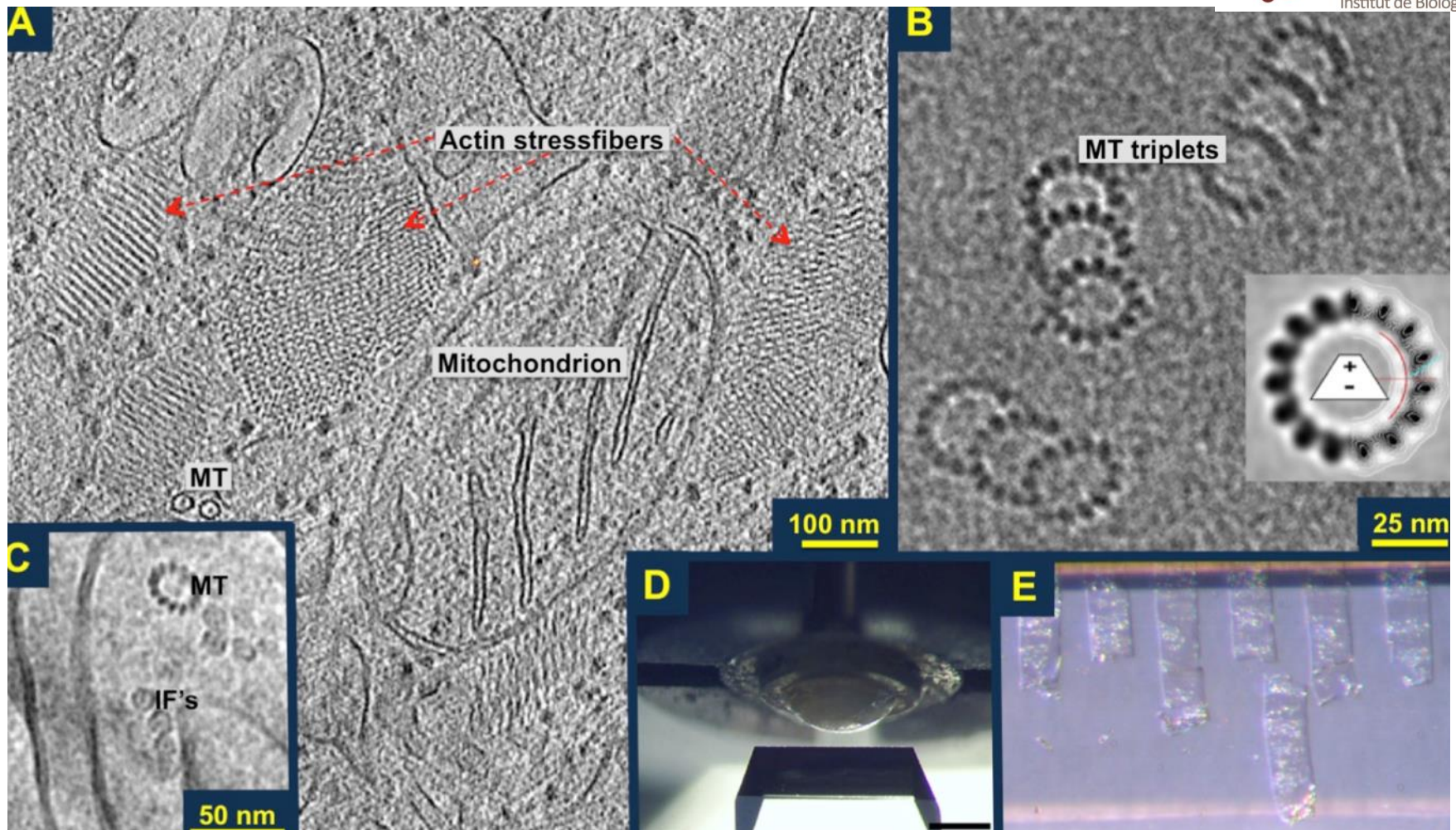
- Congélation haute pression pour empêcher la formation de glace dans les échantillons épais (HPM 100)

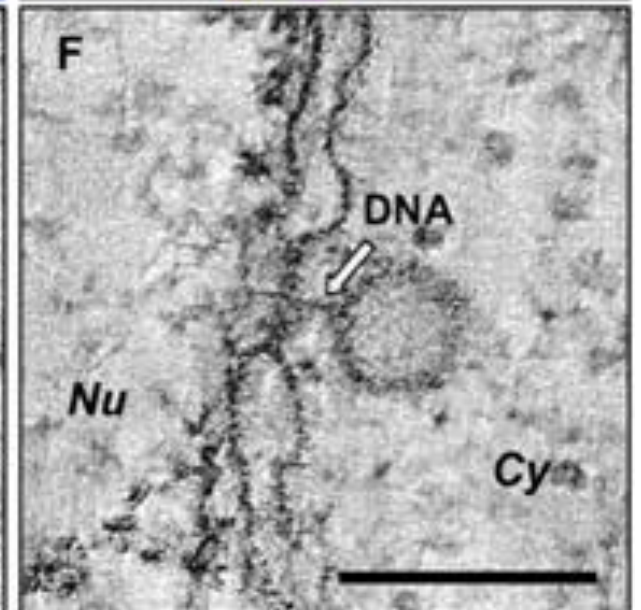
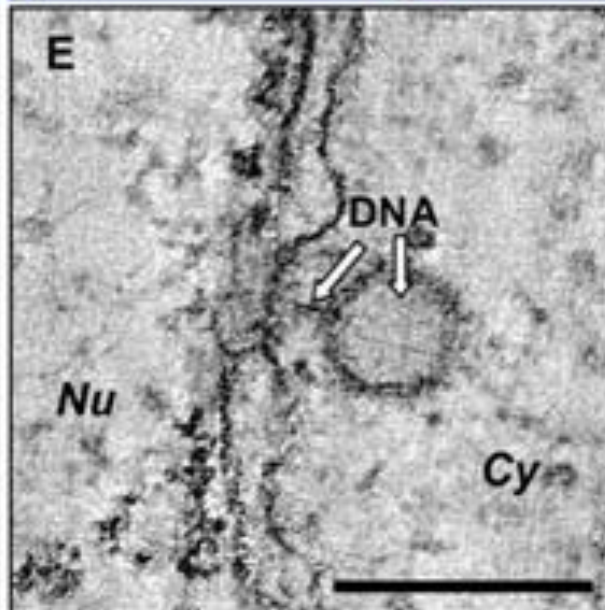
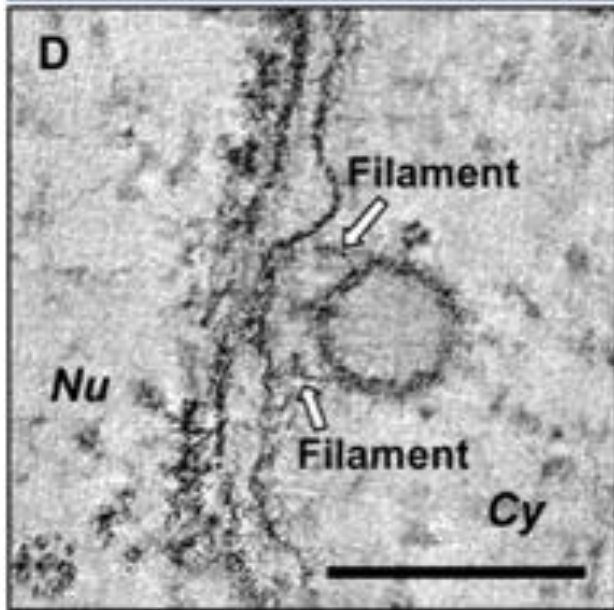
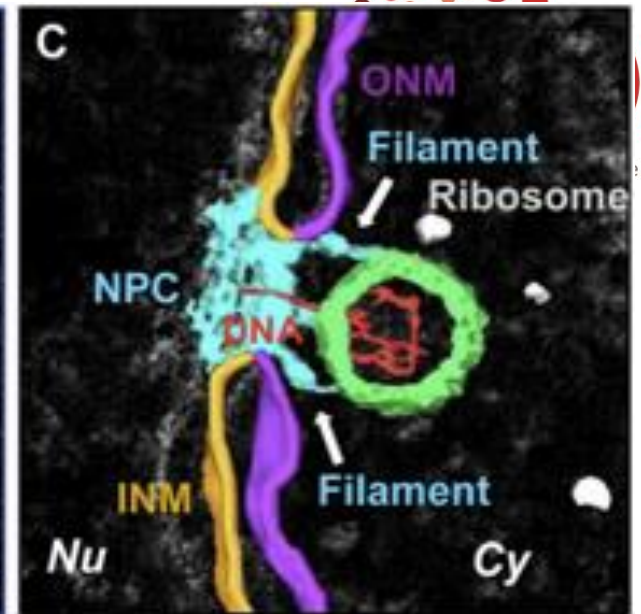
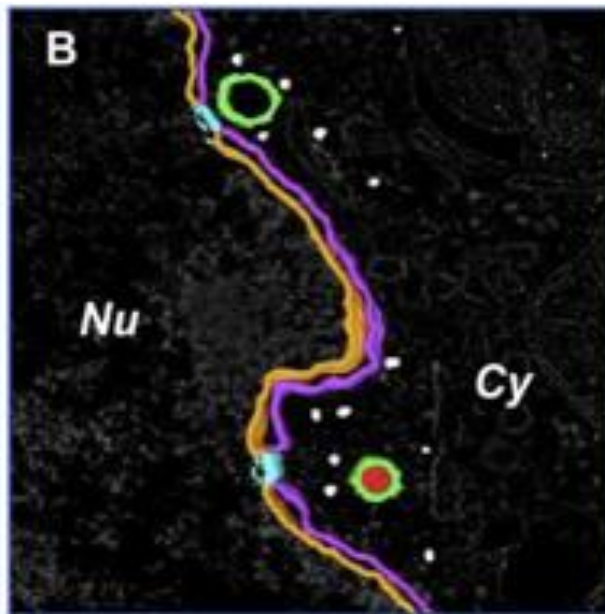
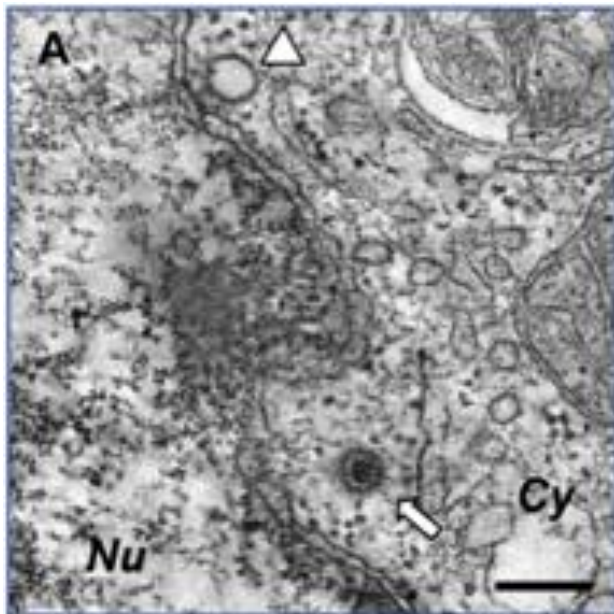


Coupe des cellules avec un  
couteau diamant à l'aide d'un  
ultramicrotome



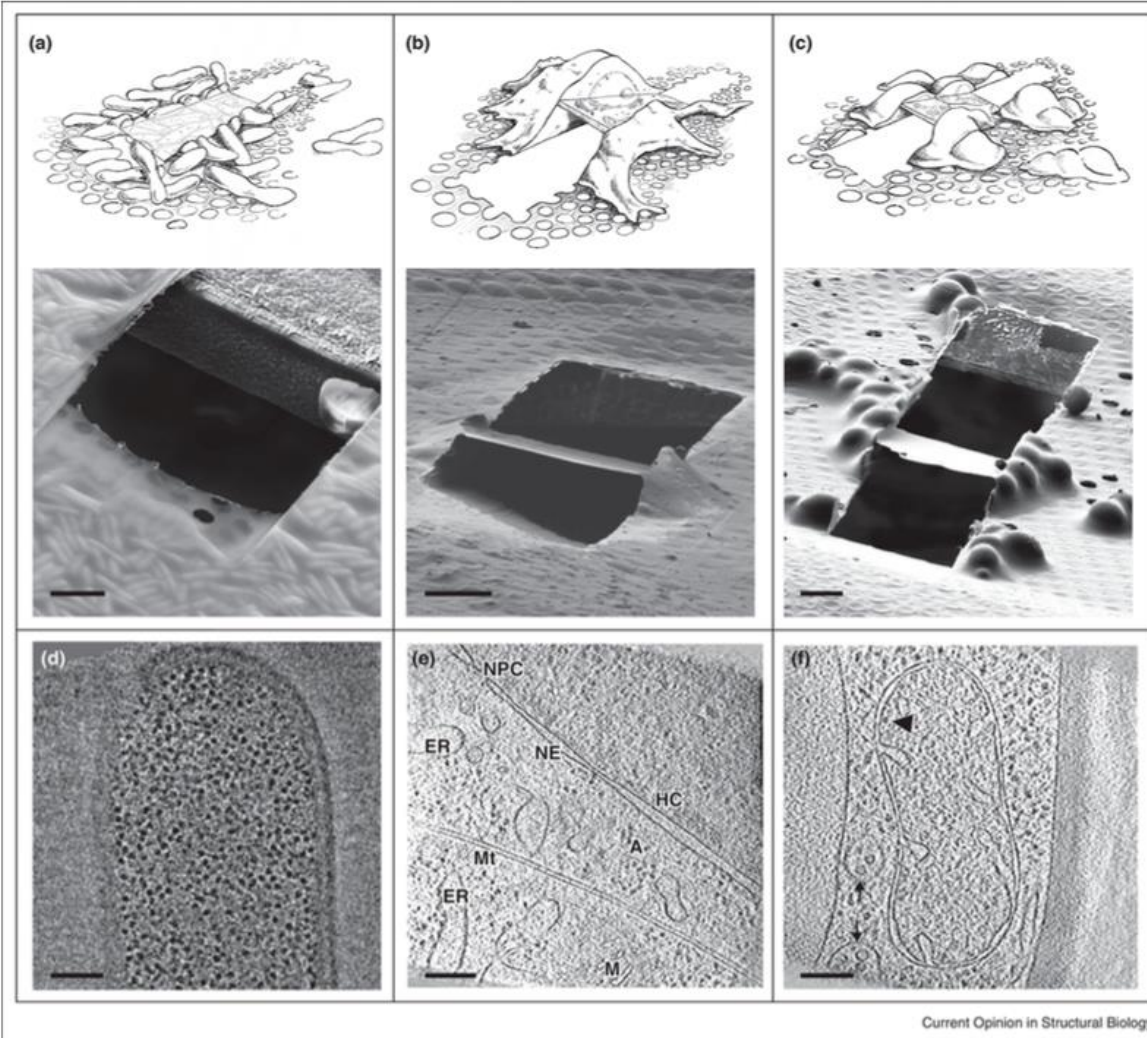
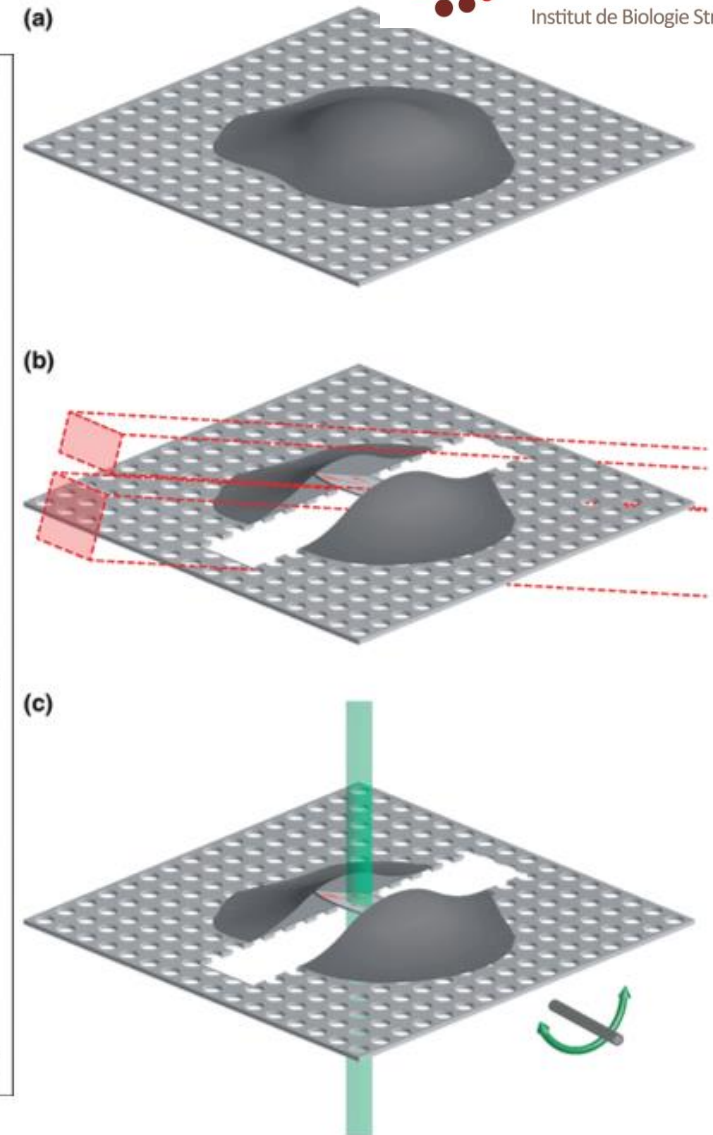
# Exemple de coupes cryo



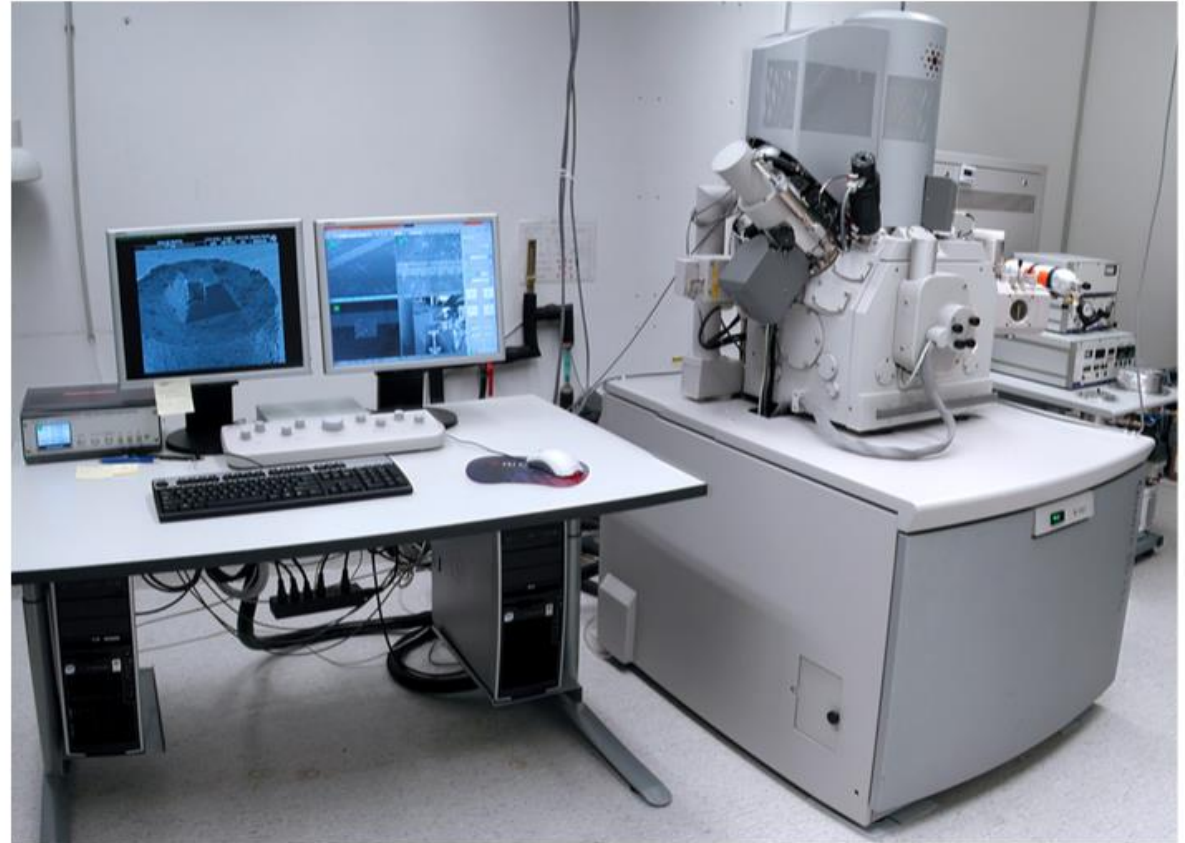
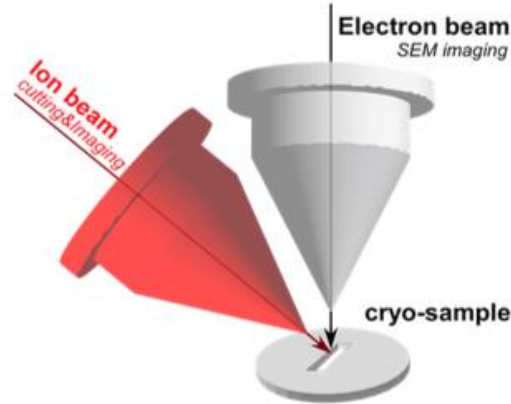


Virus at the nuclear pore; injection of the DNA  
Bars: 200 nm.

# Focused ion beam milling



# Focused Ion Beam – FIB



# Instruments



2009, 2018

T12



2013

F20



2013

Glacios



2019

K2 direct electron detector



**ibs**  
Institut de Biologie Structurale



Storage system  
150Tb  
Working space  
150 Tb

Cluster  
660 cores  
24 GPU to come

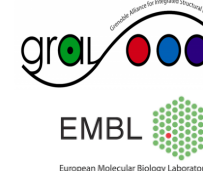


**MERCI !** **DES QUESTIONS ?**

## Les financeurs

## Les personnes impliquées

|            |            |
|------------|------------|
| B Arragain | R Linares  |
| G Effantin | WL Ling    |
| L Estrozi  | H Malet    |
| D Fenel    | C Moriscot |
| B Gallet   | E Neumann  |
| D Housset  |            |



Rhône-Alpes

