Comment se lancer dans la microscopie électronique 3D avec des moyens simples

How to get started in 3D electron microscopy with simple means

François Orange

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Journées thématiques du GN-MEBA – 9-10 juin 2022 – Aix en Provence

CCMA : the Electron microscopy facility of Université Côte d'Azur

Centre Commun de Microscopie Appliquée

Common Facility for Applied Microscopy

Location :
Campus Valrose
(under the University Library)

The team :

- Director : Sandra Lacas-Gervais (MCF)
- François Orange (IR)
- Sophie Pagnotta (IE)
- Christelle Boscagli (Tech)

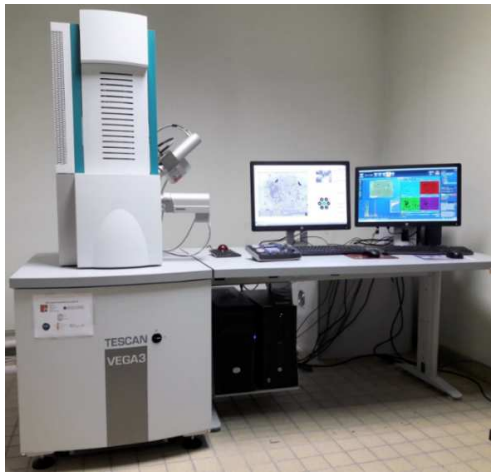
- 35 years of expertise in electron microscopy
- Open to all laboratories or private companies
- Multidisciplinary : mostly life sciences, but also provides local solutions for material sciences

CCMA : the Electron microscopy facility of Université Côte d'Azur

2 Scanning Electron Microscopes

- **JEOL JSM-6700F** (2004)
- Field emission gun
- Resolution 1 nm
- Cryo-SEM module

- **Tescan Vega 3 XMU** (2016)
- Tungsten filament
- Resolution 3 nm
- Large specimen chamber
- EDX detector for elemental analyses
- Low vacuum mode

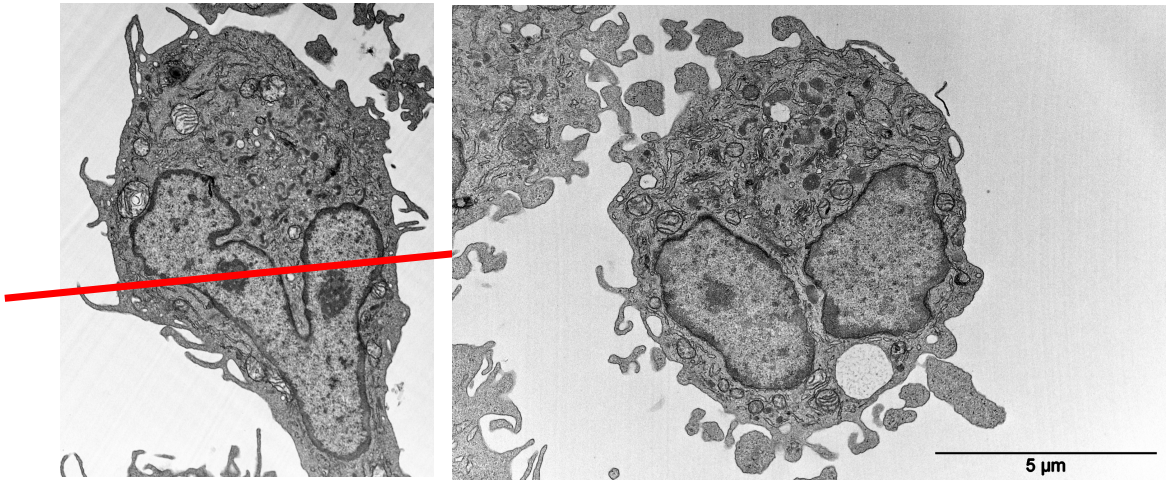


1 Transmission Electron Microscope

- **JEOL JEM-1400** (2009)
-120 kV
- Resolution 0.4 nm
- Optimized for biological samples (ultra-thin sections)

Limitations of two-dimensional observations

- Observation of thin sections in the TEM gives only a 2D visualization
- 2D can lead to limitation or difficulties for the understanding of the morphology of the observed structures

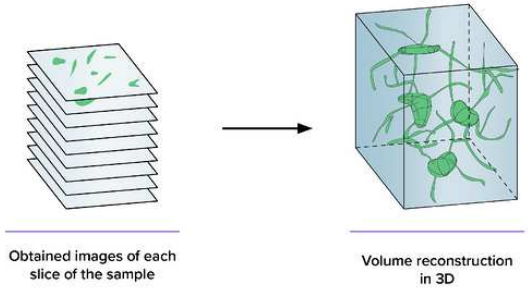


TEM images of monocytes

To gain access to the third dimension :

- Observation within a section
- Observation of serial sections (similar to a confocal microscope)

Recent years have seen major advances in 3D electron microscopy (EM) techniques, which allow three-dimensional visualization of biological ultrastructures at high resolution within large volumes.

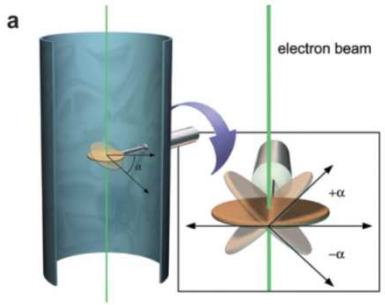


Techniques for 3D electron microscopy

Electron tomography

☑ Resolution

☒ Small structures only

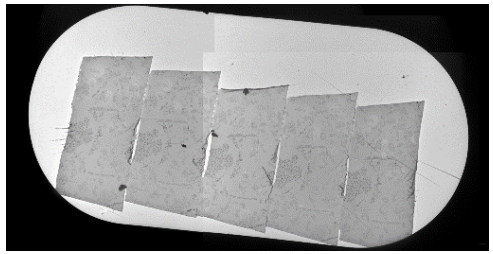


Subramaniam et al. (2003)

Serial section TEM

☑ Resolution, large X/Y area

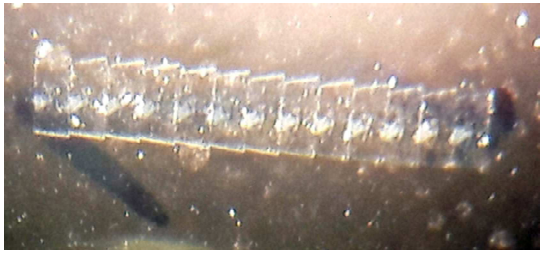
☒ Collection of sections, fragile



Array Tomography (serial section SEM)

☑ large X/Y area, conservation of sections

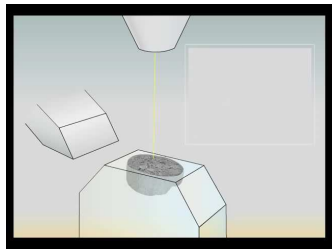
☒ Collection of sections



Serial Blockface SEM

☑ Large X/Y area, automation

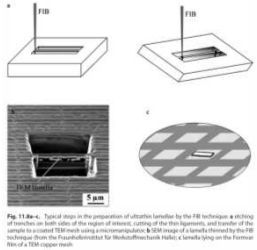
☒ Destructive, dedicated instrument, charging effects



FIB-SEM

☑ Z-resolution, automation

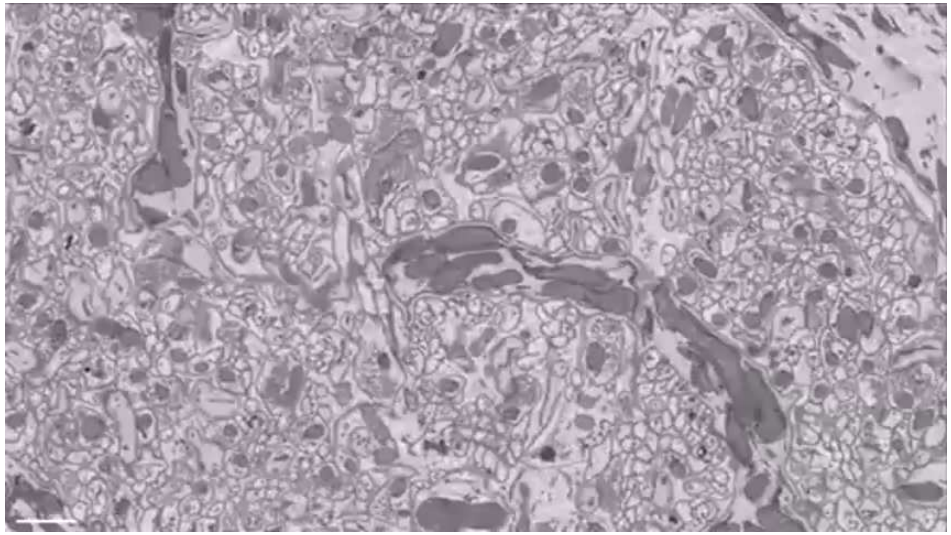
☒ Limited X/Y area, destructive, dedicated instrument



"There is no best volume EM method"

Briggman KL, Bock DD (2012) Volume electron microscopy for neuronal circuit reconstruction. Current Opinion in Neurobiology, 22,154–161

How to get started in volume electron microscopy ?



Gatan : Serial images of mouse brain

My starting point : when I saw all these beautiful 3D modelisation, I wanted to do the same !!

But…

Problem : People might be reluctant to get involved in volume EM, since it is often considered as a highly complex and time consuming approach (acquisition, segmentation and reconstruction time), requiring expensive equipment.

- FIB-SEM and SBF provide hundreds to thousands of sections
- Time + cost : we cannot afford it at the CCMA

How to get started in volume electron microscopy ?

Array tomography = excellent entry point to volume EM

- Collection of a few dozens of ultrathin sections on a substrate : enough to obtain valuable data within a reasonable amount of time and at low cost

- Observed with a SEM using backscattered electrons (BSE)

- Only a few hours to produce, image and process

- Performed with instruments commonly found in EM facilities

<u>This presentation :</u>

- **Implementation of Array Tomography at the CCMA**
- **To show you that it is possible to get into 3D electron microscopy with equipment commonly found in EM facilities, and with limited investment (only a little bit of time and motivation)**

Array Tomography (or Serial Section SEM)

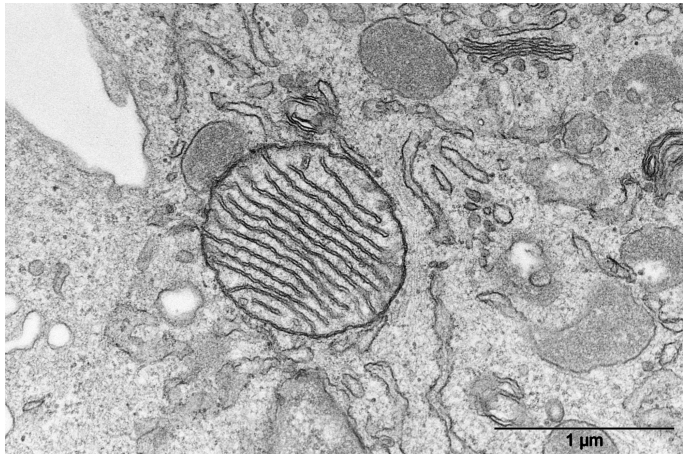
- Instead of using a TEM grid, **sections are collected on a flat surface** (e.g. silicon wafer, glass slide, tape) and **observed in the SEM**, using back-scattered electrons (BSE).

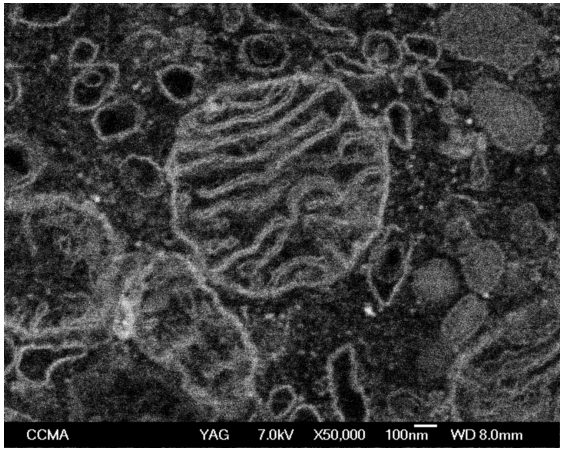
- How does it work :

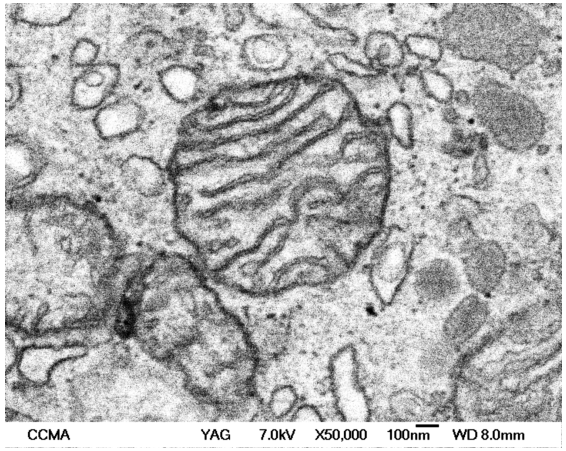
  - BSE : chemical contrast. **Brighter areas are the areas with heavier elements.**
  - Contrasting agent used for TEM use heavy metals : osmium, lead, uranium

➢ Ultrathin section can be observed in the SEM

TEM **SEM**







- Pros : possibility to collect a higher number of serial sections, less fragile than slot grids : long term conservation
- Cons : less resolution than TEM

Array Tomography (or Serial Section SEM)

- With the development of digital images and software : possibility to do segmentation and 3D reconstruction

- Some people have not waited for that !!

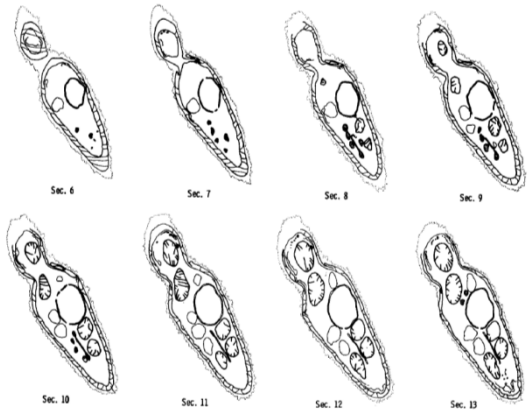
260 J. ULTRASTRUCTURE RESEARCH 29, 260–275 (1969)

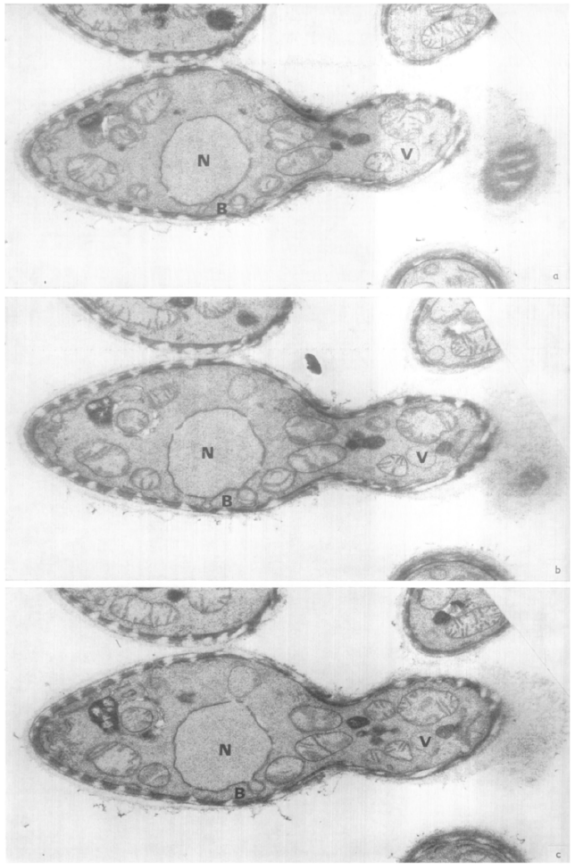
Three-Dimensional Reconstruction of *Pityrosporum* Yeast Cells Based on Serial Section Electron Microscopy[1]

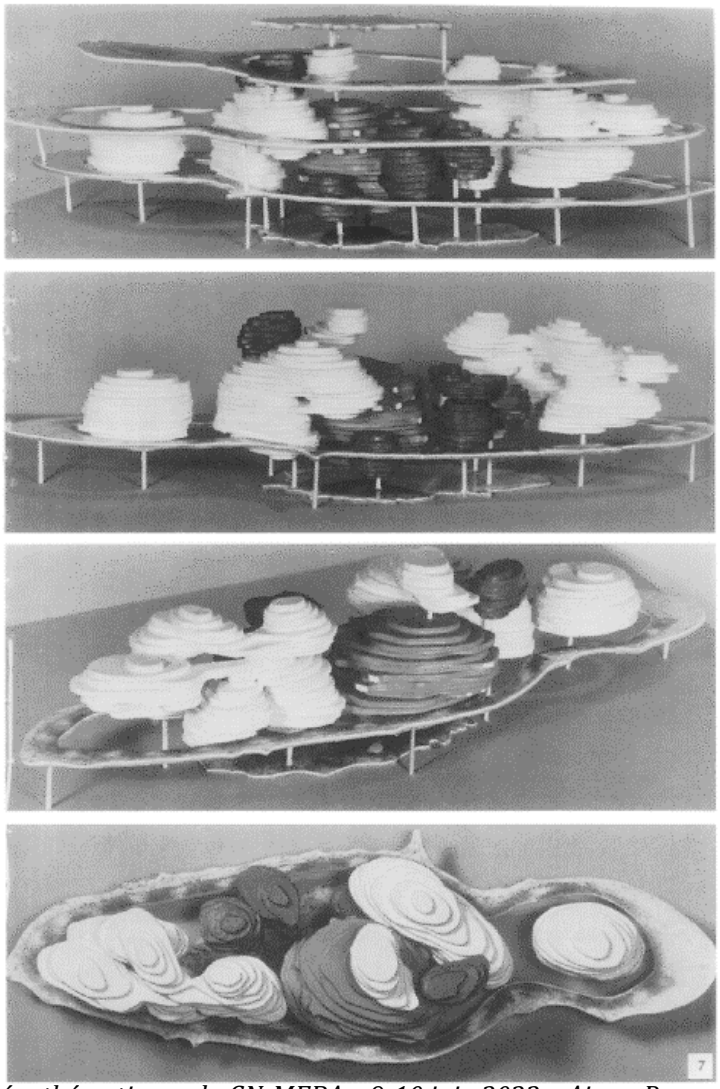
FRANCES M. KEDDIE and LUCIANO BARAJAS

Department of Medicine, Division of Dermatology, Center for the Health Sciences and Department of Zoology, University of California Los Angeles, California 90024

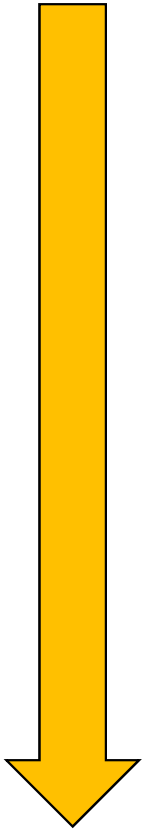
Received April 2, 1969







Workflow



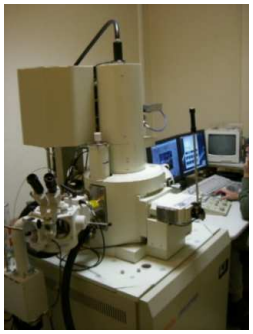
- **Sample preparation**
- **Section collection**
- **SEM observations**
- **Images alignment**
- **Segmentation**

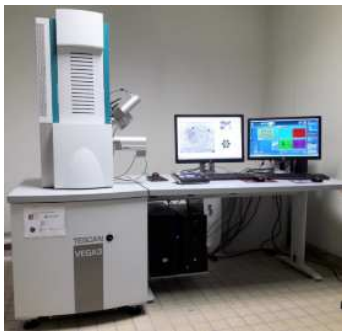
Workflow : Instruments used

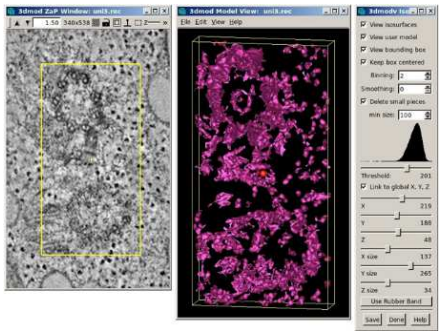
- **FEG-SEM** (Jeol JSM-6700F, 2004) with YAG BSE detector
- **W-SEM** (Tescan Vega 3, 2016) with YAG BSE detector
    - These are not the most efficient detectors for ultrathin section imaging, but it works

- **Ultramicrotome** (Reichert Ultracut S) equipped with a common diamond knife (3mm, 45°)

- **Carbon coater** (Edwards Auto 306)





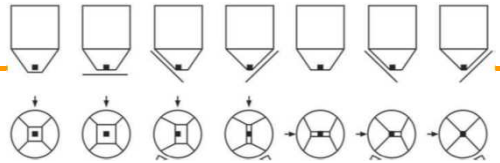


- **IMOD software** (open-source) on a laptop (Windows 7, Intel i5 processor)

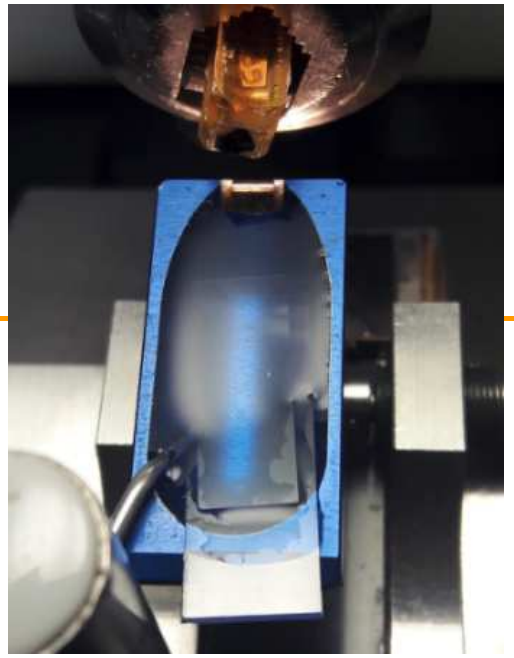
Kremer JR, D.N. Mastronarde DN, McIntosh JR (1996)

➢ **State-of-the-art equipment is not required !**

Workflow : Sample preparation



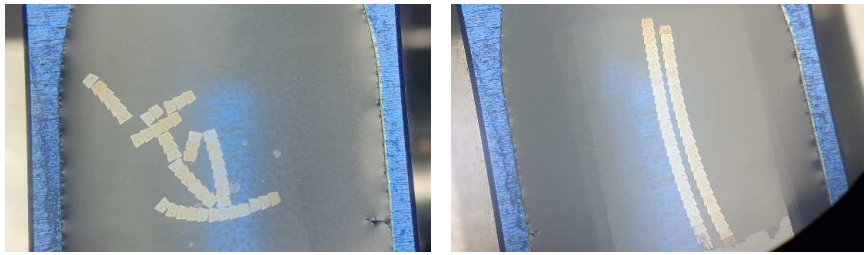
- **Standard resin blocks used for ultrastuctural studies**
- **We have not used a specific protocol**
- Routine procedure: GA fixation, OsO4 post-fixation, Epon resin



Workflow : Section collection

The aim is to obtain a nice ribbon of section on the ultramicrotome

- Requires a well trimmed block face
- Tip : put glue to the resin block (neoprene glue + xylene)
- In the diamond knife boat : partially immersed glass slide, made hydrophilic (glow discharge)
- A ~20-30 ultrathin sections (50 - 150 nm) ribbon is obtained, then moved towards the glass slide and the first section attached to the water / glass slide limit
- The water level is very slowly decreased using the peristaltic pump
- The coverslip is removed and let to dry



Without and with glue on the resin block

François Orange

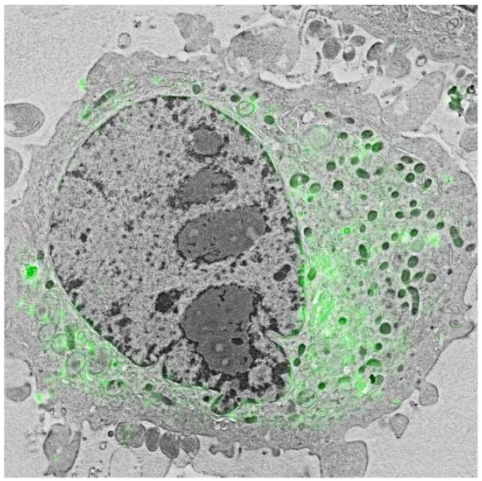
Workflow : Section collection

Why collect the sections on a glass slide ?

➢ Correlative Light Electron Microscopy (CLEM) in mind

➢ To have the possibility in a near future to observe the sections on both fluorescence and electron microscope

- The best results among other surfaces tested (carbon tape, plastic tape, mica sheet…)
- Other prefered solution : silicon wafers



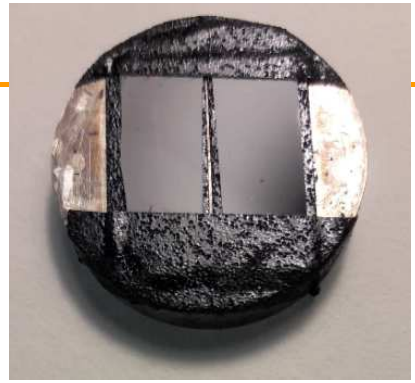
Delmic, C.J. Peddie

Workflow : Post-treatment (staining, mounting, carbon coating)

Quite simple steps

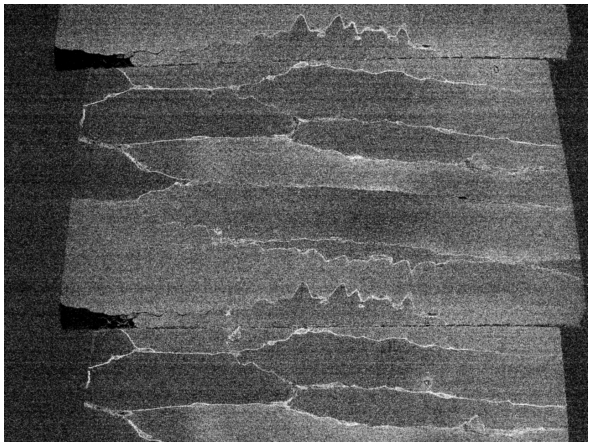
- Sections are stained with uranyle acetate and lead citrate (same procedure as for TEM grids)

- Glass slides are mounted on a SEM stub and carbon coated

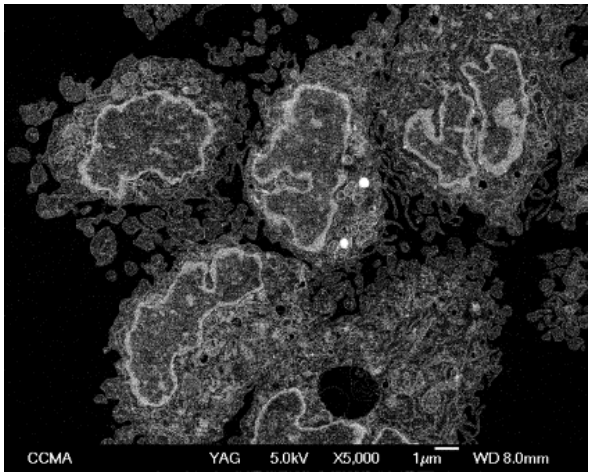


Workflow : SEM observations

- Observations in BSE mode (remember = negative of a TEM image !)
- Region of Interest (ROI) is found manually in each sections



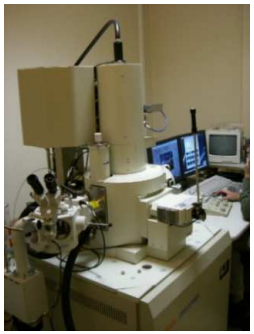
- Imaging at high resolution : ~2-3 hours for 30 sections
- **Non destructive technique** : Once all the images are acquired, the SEM stub with the glass slide on it can be kept for future observations
- Possibilty to make a survey at low magnification to better detect the ROI



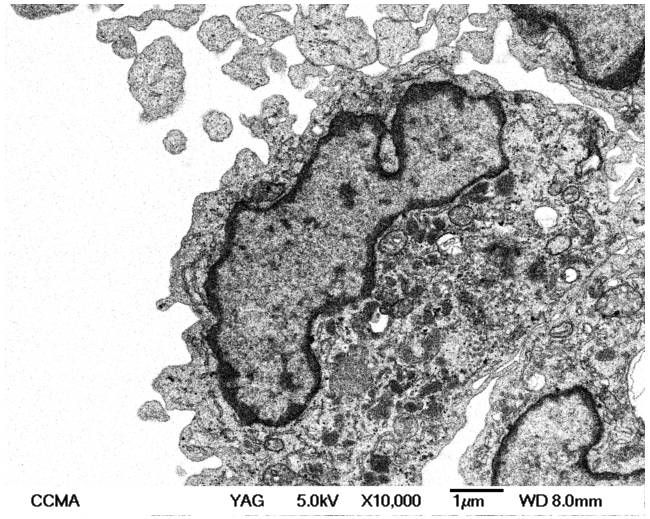
- As for TEM, all the common sectioning artifacts are there : staining precipitates, folds, distorsion, missing sections…

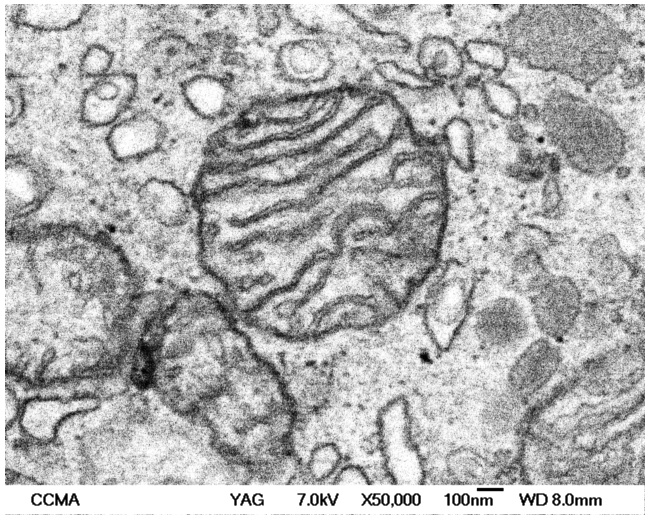
- **Flexible technique** : Depending on the scientific objectives and the type of samples, Array Tomography procedure can be adapted at every steps (e.g. sections thickness, number of sections, staining, SEM magnification…)

Workflow : SEM observations

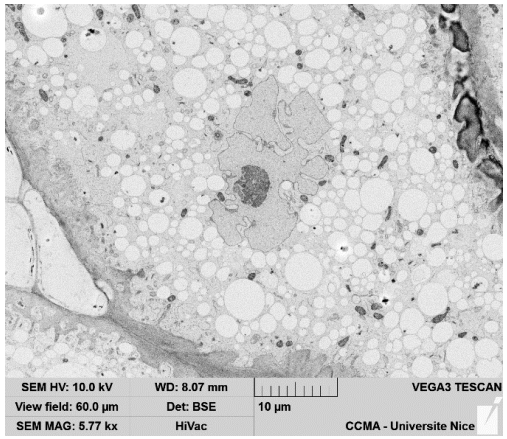


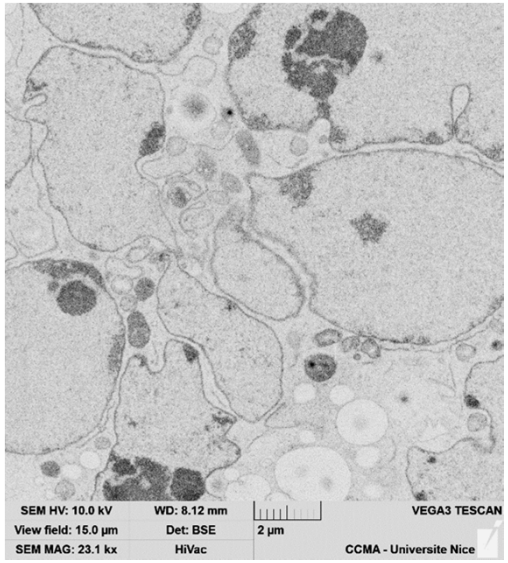
FEG-SEM

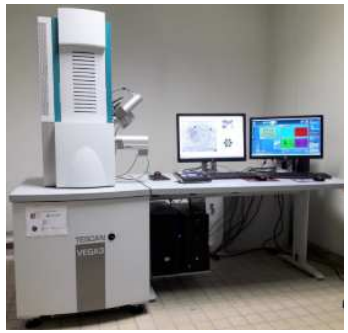




W-SEM







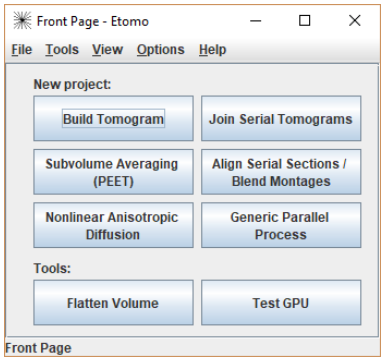
- Our BSE detectors (in chamber, YAG) are not the most efficient detectors
- Less signal than In Lens detectors
- Need to adapt : an image will take several minutes instead of several seconds
- **Observations possible with a tungsten SEM**

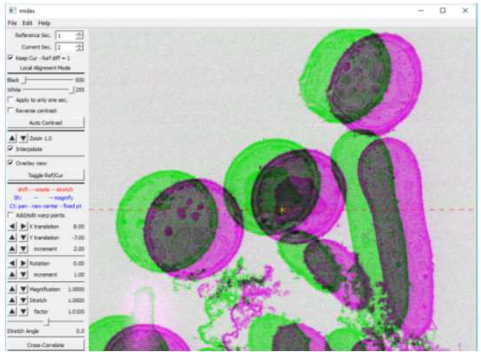
Workflow : Alignment

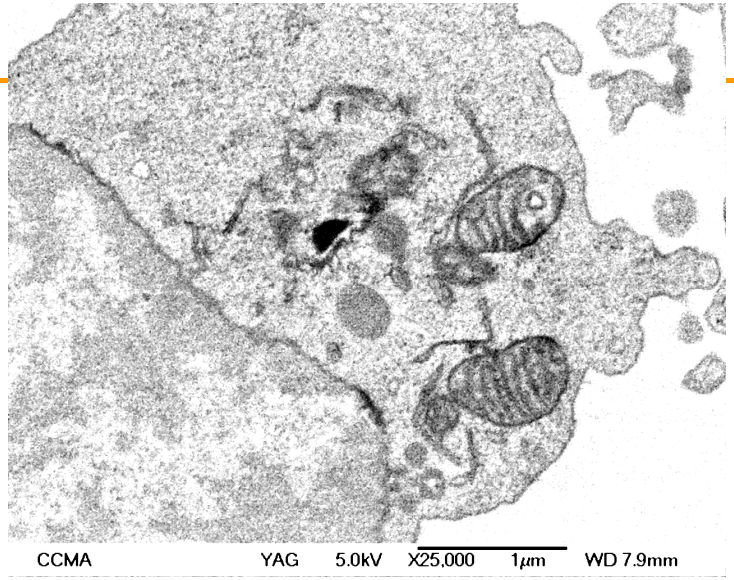
- Images processing : all the steps can be done with **IMOD**
    - Open-source software
    - Work smoothly on a laptop

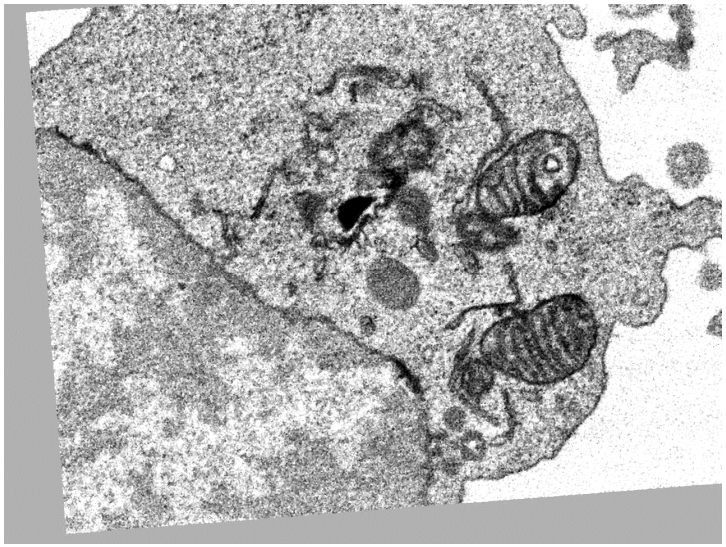
- All the images acquired need to be aligned
    - Important step in Array Tomography : need to move between each section, distorsions, folds, sectioning artifacts…

- MIDAS module : semi-automatic method
    - Efficient for alignment of serial section



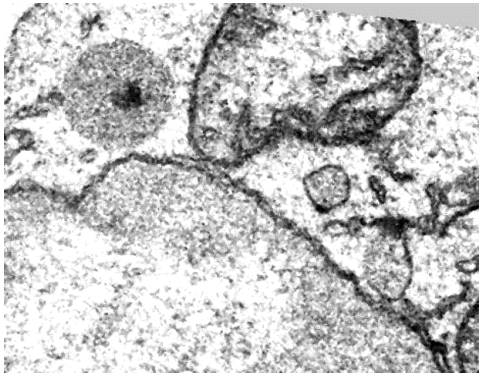


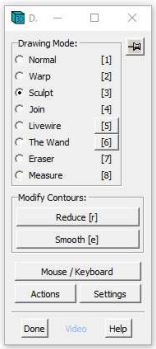


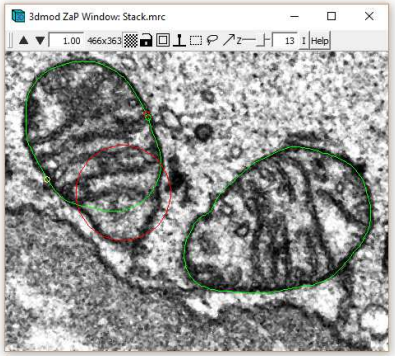


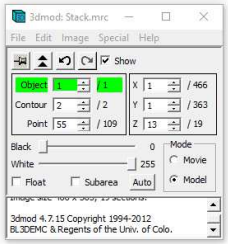
Workflow : Segmentation

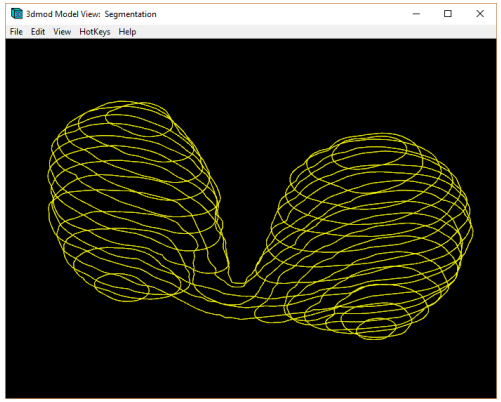
- Segmentation : often rate limiting step of the entire process
- The time needed depends of what needs to be modelized
- IMOD software (3dmod module) : many tools to help during segmentation !

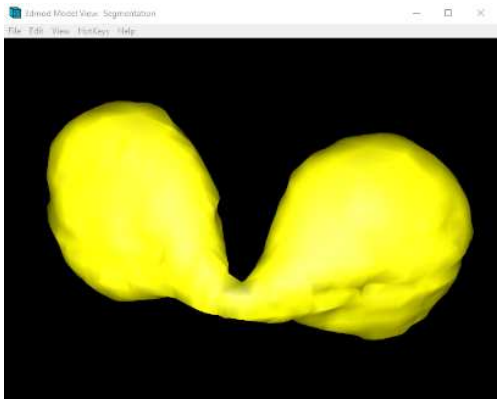


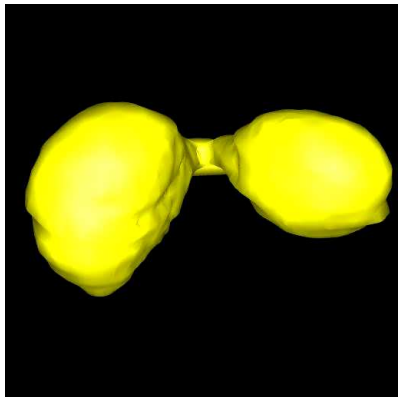










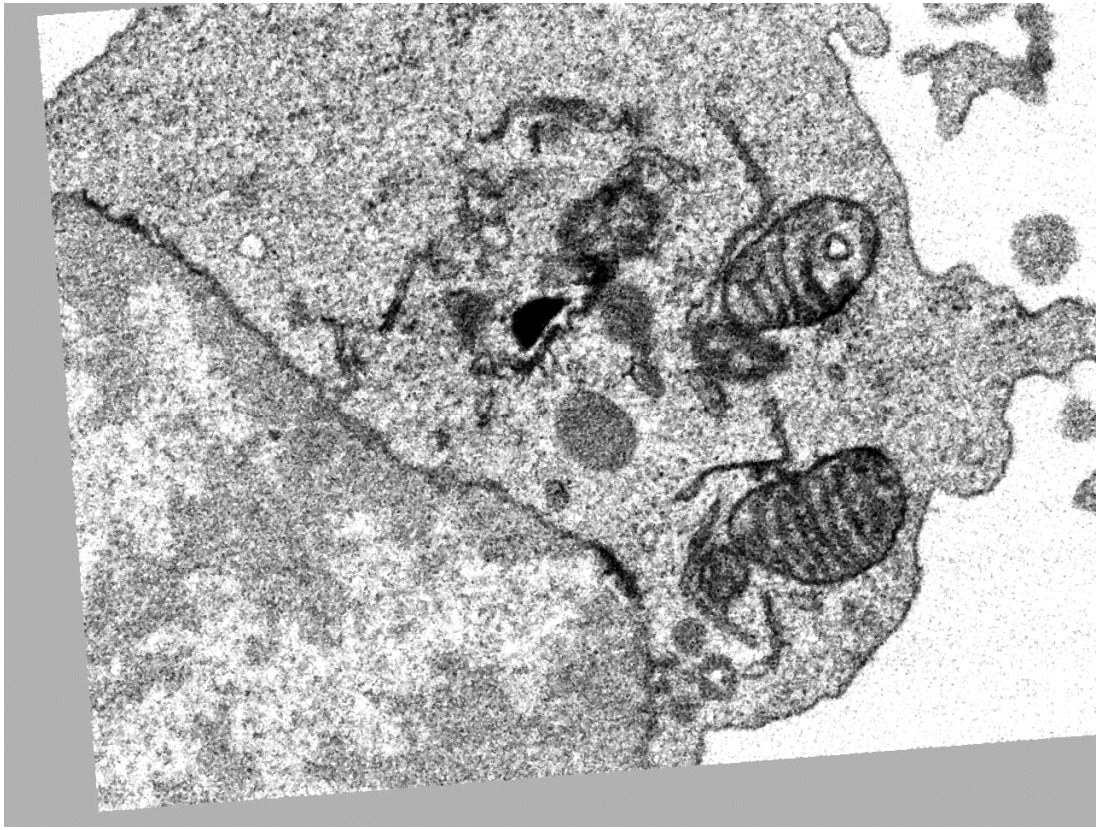


- IMOD : Tutorial available !

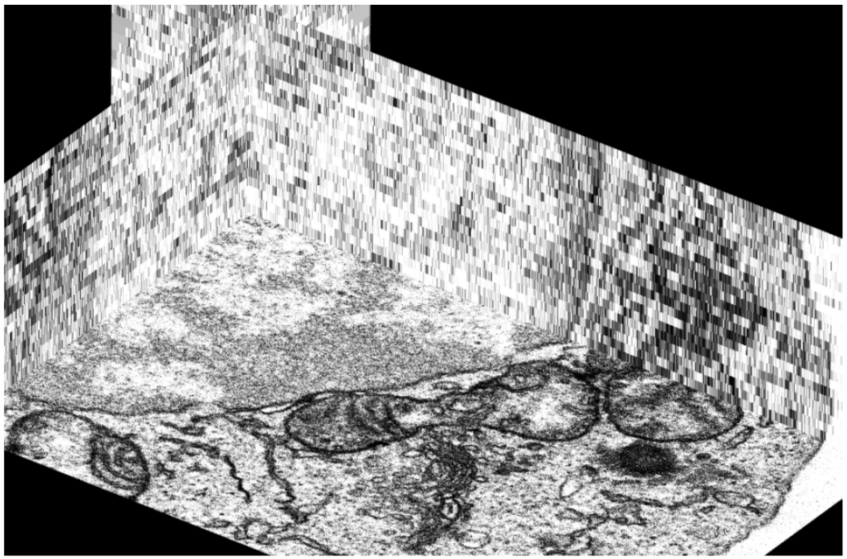
Example : Ultrastructure of macrophages

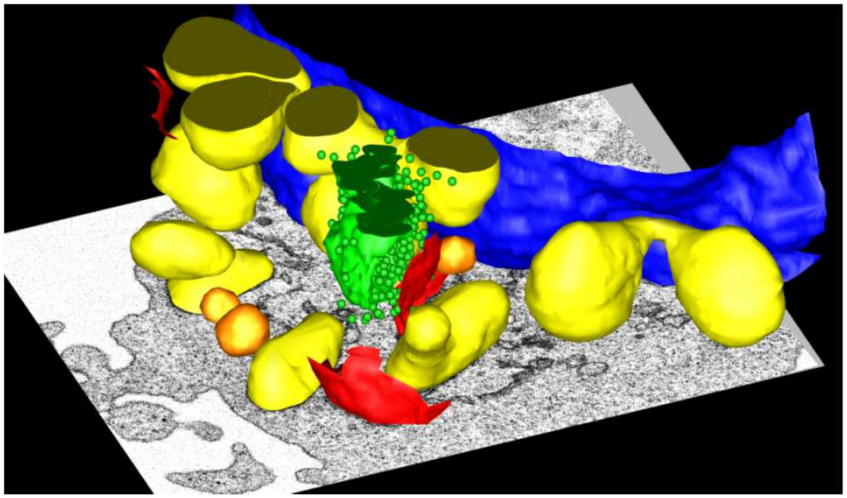
Collaboration with **Arnaud Jacquel, Sonia Boulakirba (Auberger Team, C3M, Nice)**

- Sections thickness : 50 nm
- Number of sections : 30
- Total thickness : 1.5 µm

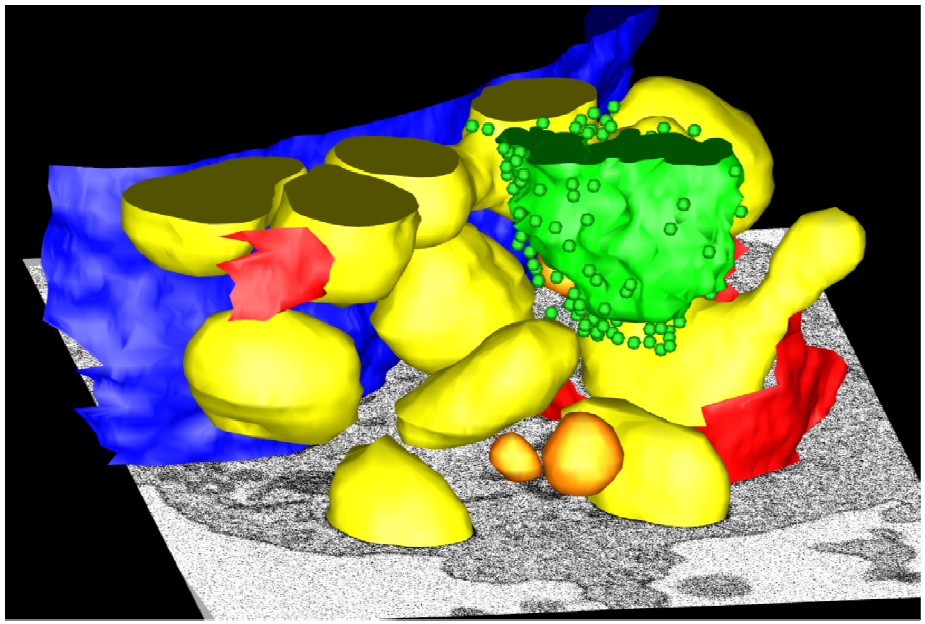


Example : Ultrastructure of macrophages

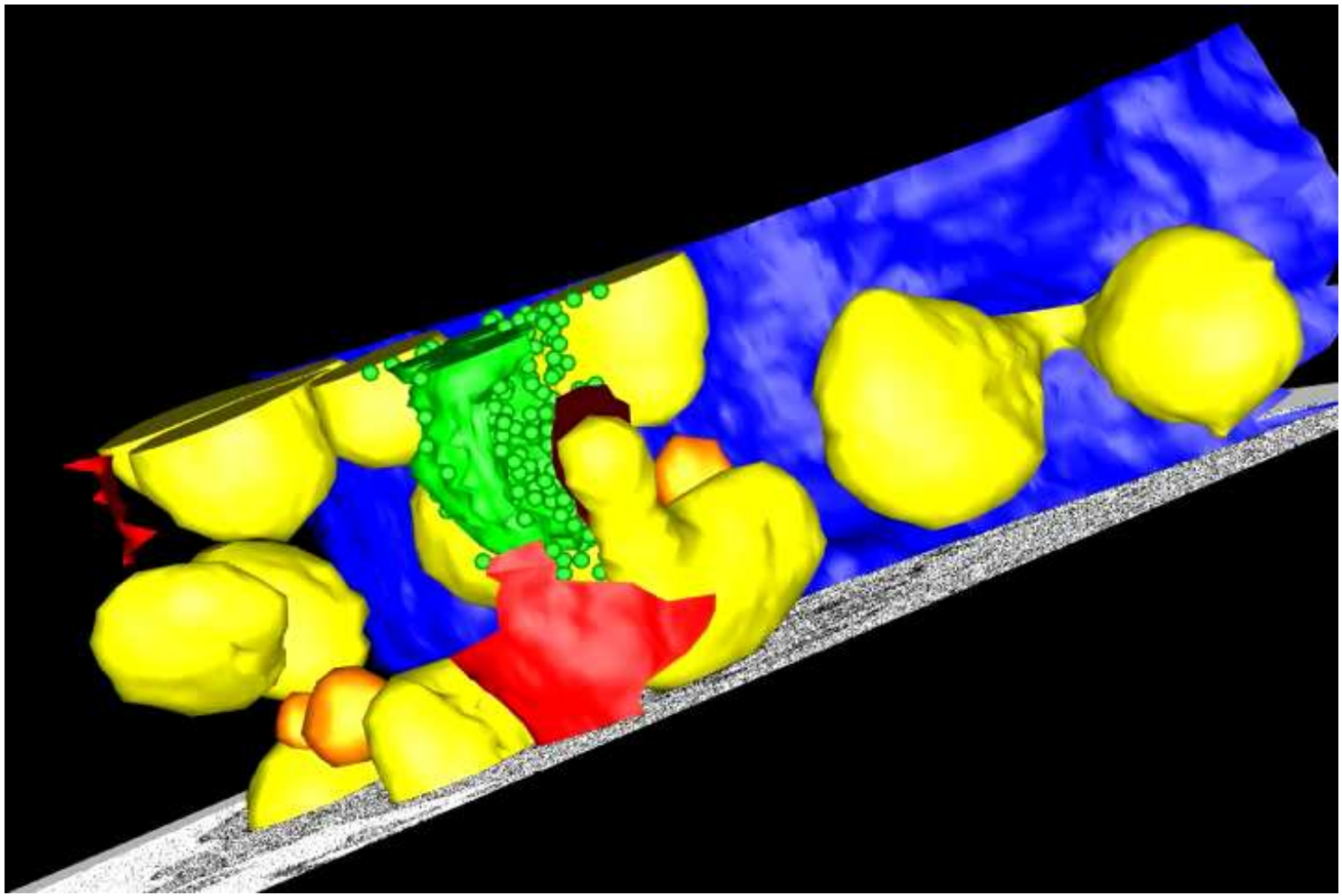




- Only a couple of hours needed at each step



Example : Ultrastructure of macrophages



Example : Ultrastructure of *Candida albicans*

Collaboration with **Allon Weiner (Arkowitz Team, IBV, Nice)**

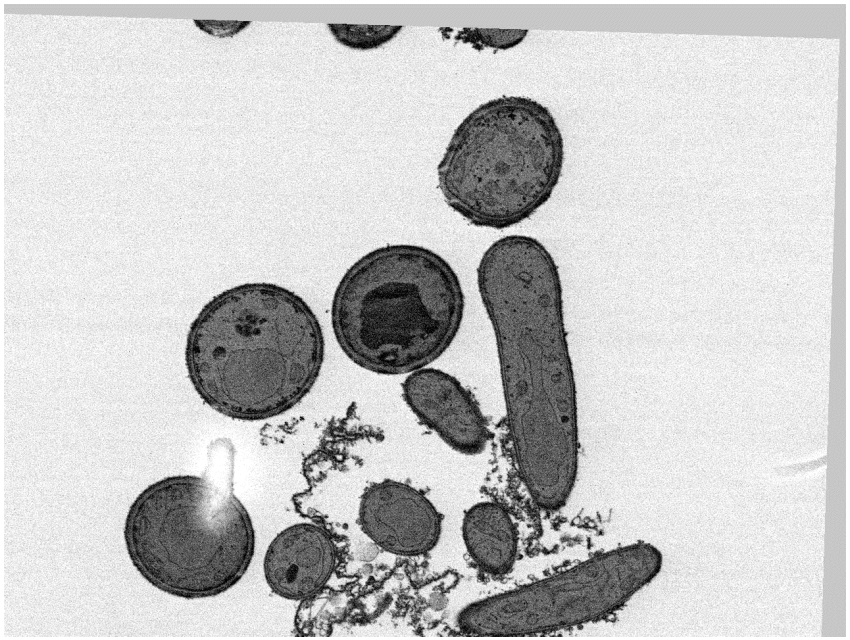
institut Valrose Biologie

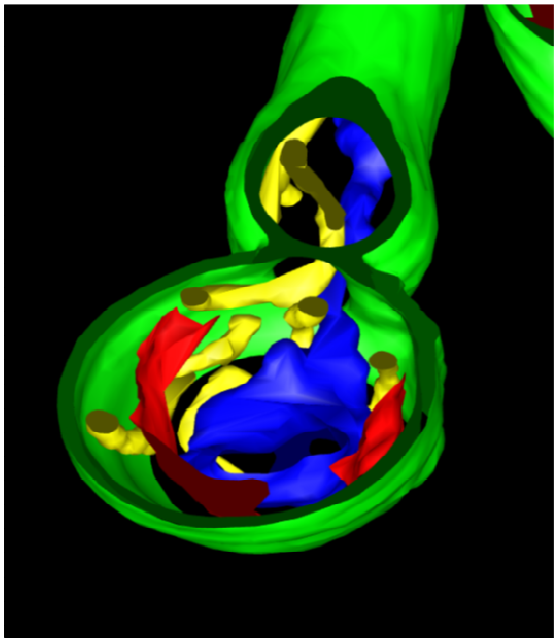
Serial sections imaging by both TEM and SEM

- **Post-fixation : OsO4 replaced by potassium permanganate**

- Section thickness : 100 nm

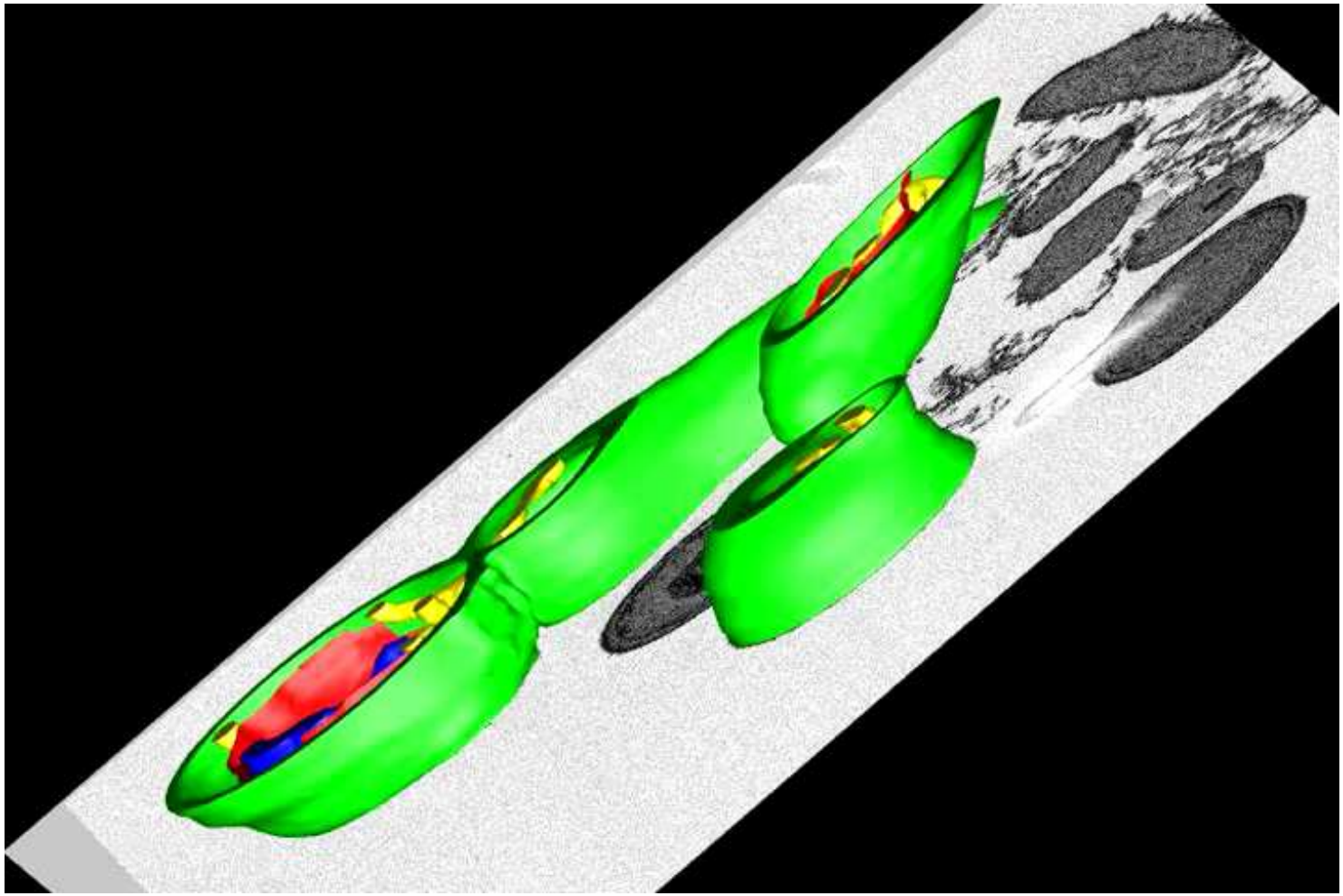
- Number of sections : ~15 (SEM and TEM)





Weiner, A, Orange, F, Lacas-Gervais, S, et al. On-site secretory vesicle delivery drives filamentous growth in the fungal pathogen Candida albicans. Cellular Microbiology. 2019; 21:e12963.

Example : Ultrastructure of *Candida albicans*



Example : Mitochondria in muscle tissue

Collaboration with **Emmanuelle Genin, Baptiste Ropert (Paquis Team, IRCAN, Nice)**

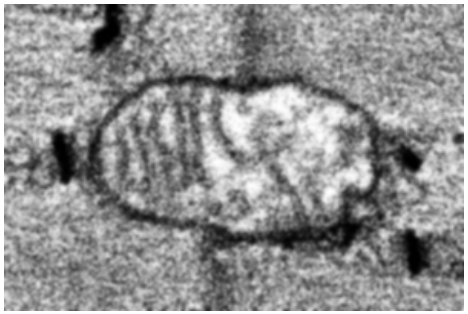
Difficulty to assess mitochondria condition from a single thin section

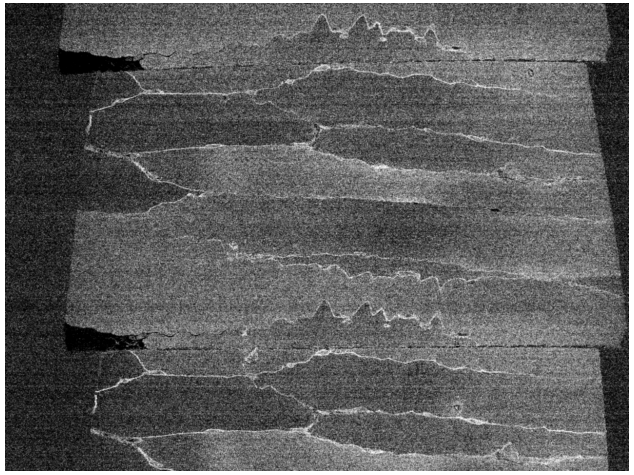
- Observation of serial sections for confirmation

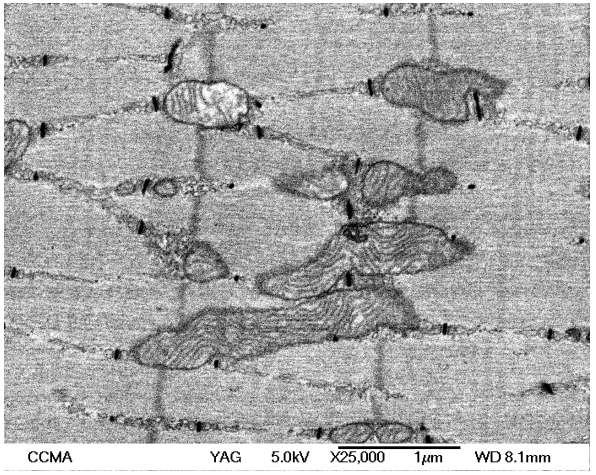
- In addition : test for resolution of BSE-SEM

- Section thickness : 50 nm

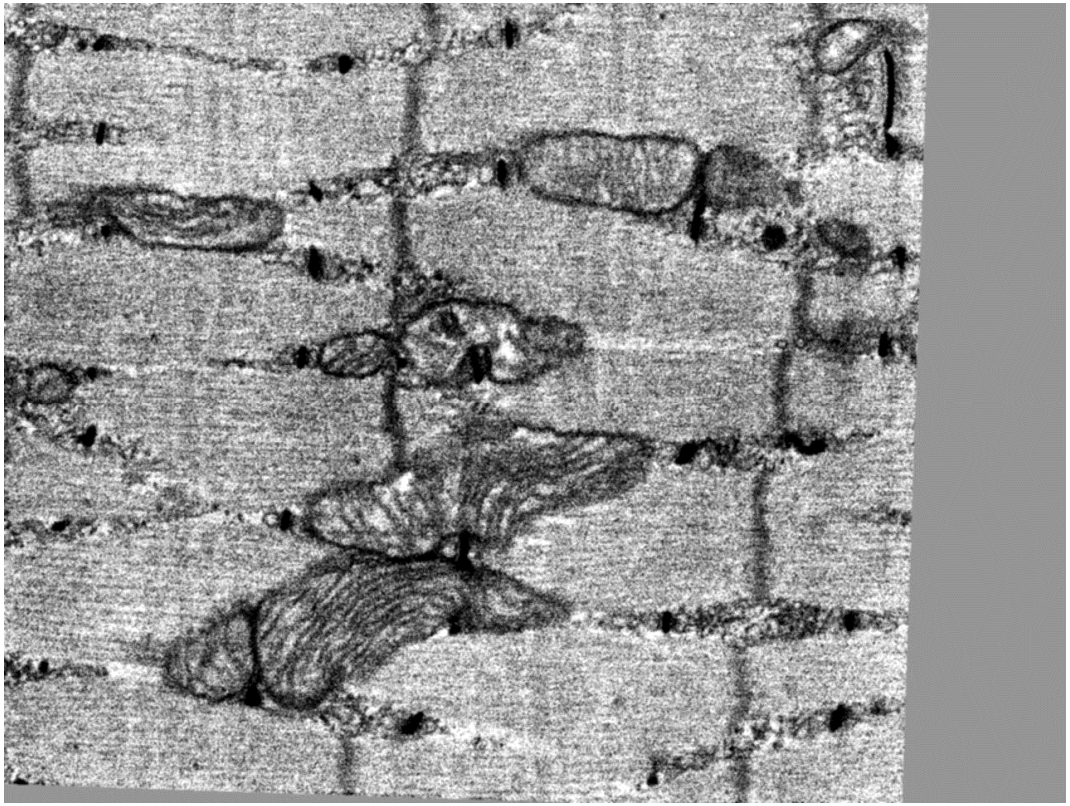
- Number of sections : 10

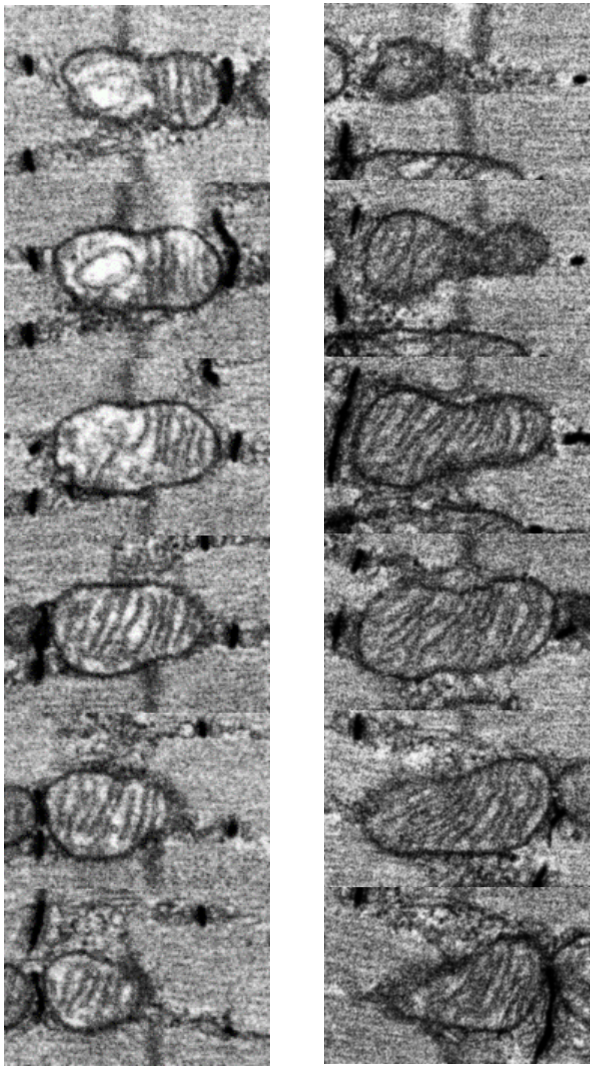






Example : Mitochondria in muscle tissue





No segmentation required

Conclusions

- The results shown are examples of what can be done

- **By focusing on a few dozens of sections, each step required only a few hours of work** (sectioning / images acquisition / alignement and segmentation)

- Depending on the scientific objectives and the type of samples, **Array Tomography procedure can be adapted at every steps** (e.g. sections thickness, number of sections, staining, SEM magnification…)

- A way to :
    - Get into 3D electron microscopy
    - Get confortable with the methodology and the different tools
    - Develop interest in these techniques

  - Before shifting to dedicating instrumentation and improved workflows