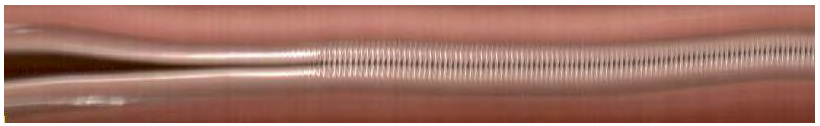
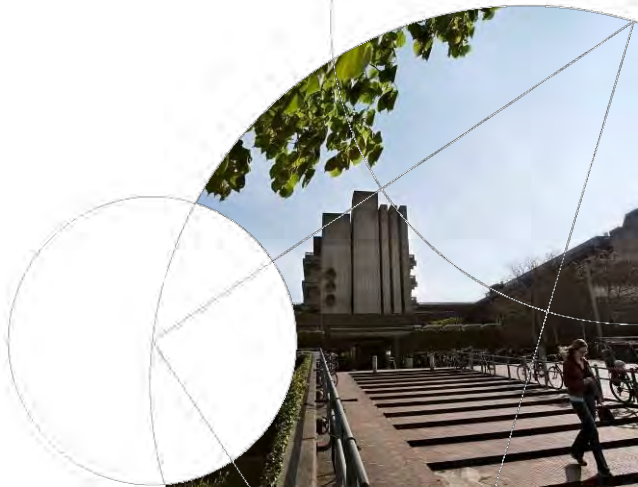
Ph.d. Morten Friis, Dr. Mette Pedersen and Professor Klaus Qvortrup

Faculty of Health Sciences

Advanced microscanning (in laryngology)

Future implication of new advanced microscopy in relation to high speed films





Advanced technology in head and neck surgery and otolaryngology Orlando 2012

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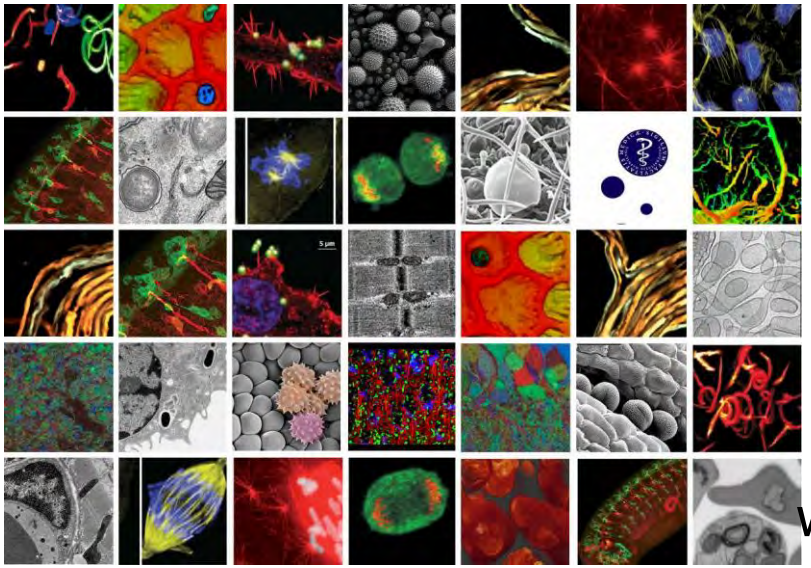
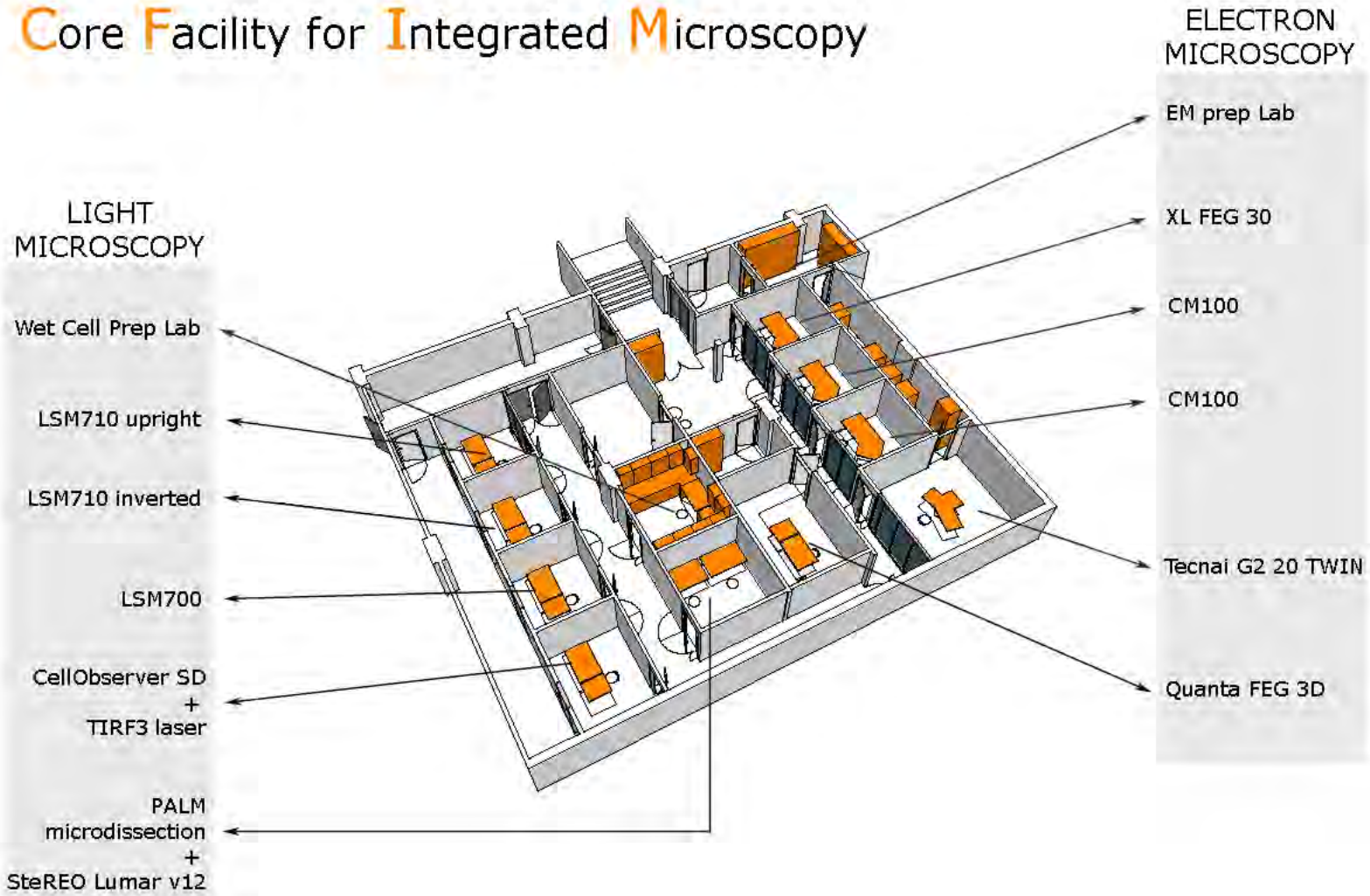
For the published paper: see www.mpedersen.org

Core Facility for Integrated Microscopy

- The facility of integrated microscopy offers a wide range of state-of-the-art light and electron microscopes for scientists and students
- Purpose built facility, ready for cell analysis
- User support in 3 levels: Minimal and Full support
- PhD courses in Light- and Electron Microscopy
- Booking of microscopes from www.cfim.ku.dk
- Support: Wet cell prep lab, sample prep lab and image post-processing workstations

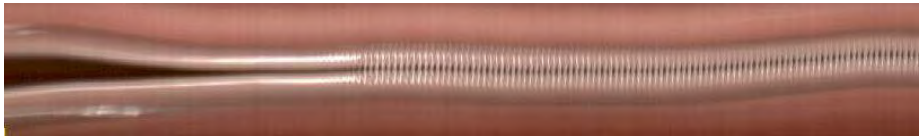


Core Facility for Integrated Microscopy

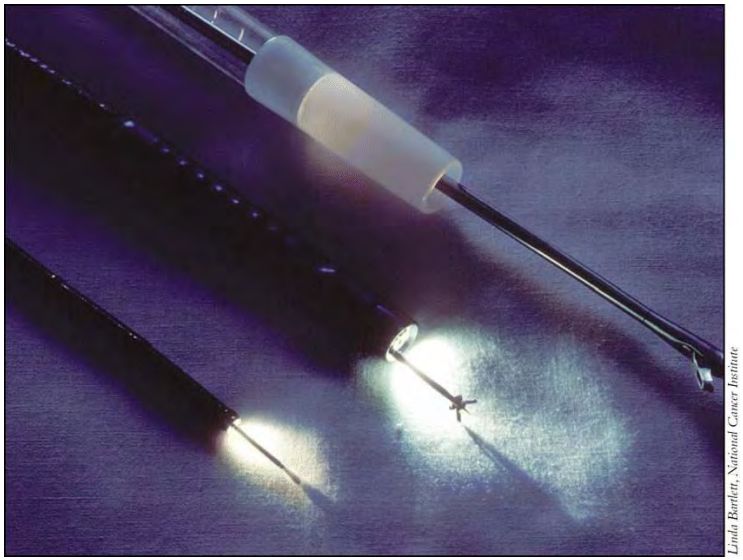


Endoscopic tools used by Laryngologists

Highspeed endocam



Kymography of vocal cords (in-tonation)



Light emitting diodes used together with ex. highspeed endocam.

So how can laryngology and findings in high speed films benefit from advanced microscopy?

- CFIM can investigate tissue by electron microscopy and light microscopy.The tissue analysis will be based on the high speed films and the pathology related phenomena, e.g. allergy, infection, vocal strain, swallowing problems, hormonel, age related, and neurological disorders.

Light Microscopy

The equipment in combination with electron microscopy can be used for wide field fluorescence microscopy:

- Confocal microscopy (z-sectioning of fixed or living specimens)
- Flourescence resonance energy transfer (FREIT)
- Flourescence recovery after photo bleching (FRAP)
- Flourescence loss in photobleaching (FLIP)
- Flourescence-lifetime imaging microscopy (FLIM) experiments
- Photoactivation and photoconversion experiments
- Spectral imaging
- Advanced unmixing of emission spectra
- Total internal reflection flourescence (TIRF) microscopy
- Integrated spectral flourescence correlation spectroscopy (FCS)
- Raster image correlation spectroscopy

Light microscopy

- The number of flourophores and flourescent proteins available is increasing every day
- Therefore, the factor that limits the number of proteins that can be labelled in samples with different flourophores has a capability to overcome the excitation and emission cross-talk between them
- Overlapping flourecent signals can be separated, like the ones originated by Green Flourescent protein (GFP) and yellow flourescent protein (YFP), or even separate flourescent labels from autoflourescence

Light microscopy

In order to perform emission fingerprinting, it is needed to:

- Acquire lambda stick of the sample of interest
- Load reference spectra for each of the flourescence emitting elements in the sample
- Ask the software to perform the unmixing of the lambda stick

Electron microscopy

The electron microscopes of the laboratories in combination with light microscopy:

- Transmission electron microscope (TEM)
- Scanning electron microscope (SEM imaging)
- 3D reconstruction of TEM images
- Unattended TEM sample preparation
- Cross-sectioning (SEM)
- Single particle analysis
- Tomography analysis

Electron Microscopy

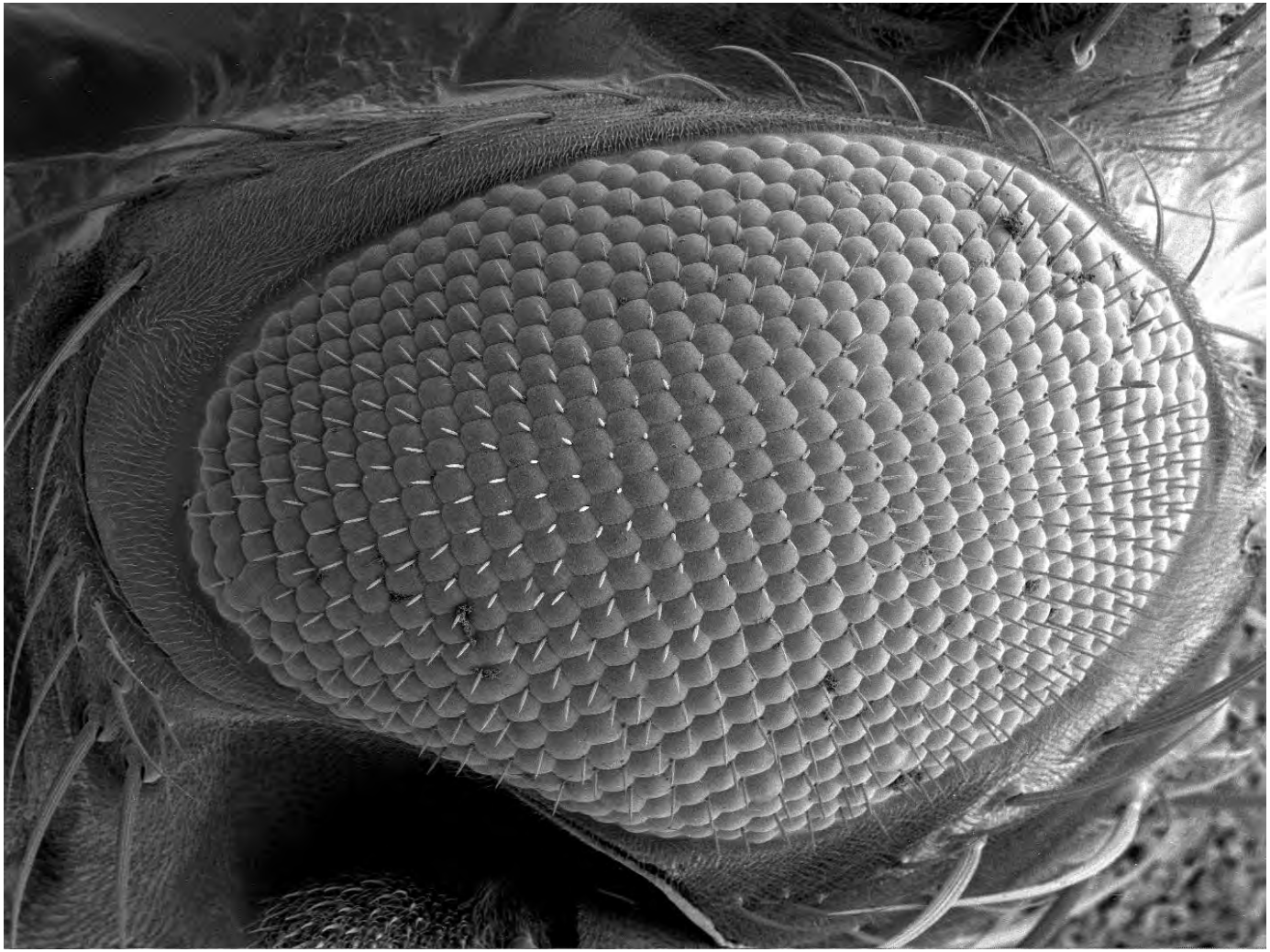
- TEM Tecnai G20 (cryo + tomography)
- TEM CM 100 (2)
- SEM Quanta 3D (cryo +dual beam)
- SEM FEG30

3ex

<u>Support</u>

- Optical bench, diffraction
- Sputter coaters, carbon evaporators etc.
- Cryo-lab

Image of Drosophila eye produced by scanning electron microscopy



Electron microscopy

- Electron and ion microscopes use a beam of particles instead of light, and use *electromagnetic or electrostatic lenses* focus the particles
- They can see features as small as *one tenth of a nanometer*, including individual atoms
- Scanning probe microscopes allow researcers to image, characterize and even manipulate material structures at exceedingly small scales including features of atomic proportions
- Scanning probe microscopes use no lenses, but a very sharp probe that interacts with the sample surface. It maps various forces and interactions that occurs between the probe and the sample to create an image. Scanning electron microscopy is a high-resolution tool perfect for rendering the surface of a specimen

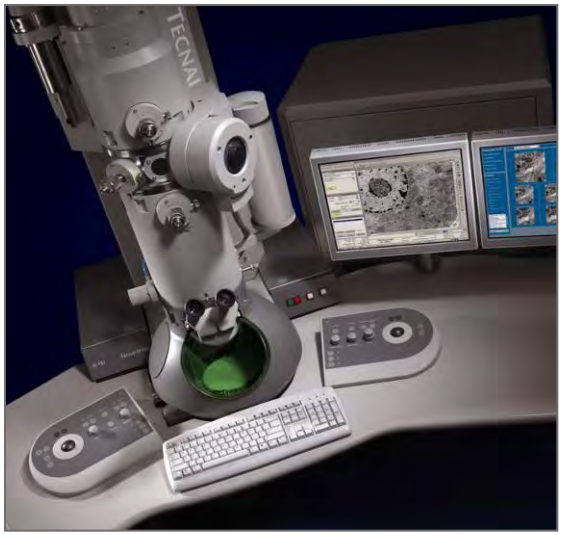
Electron microscopy

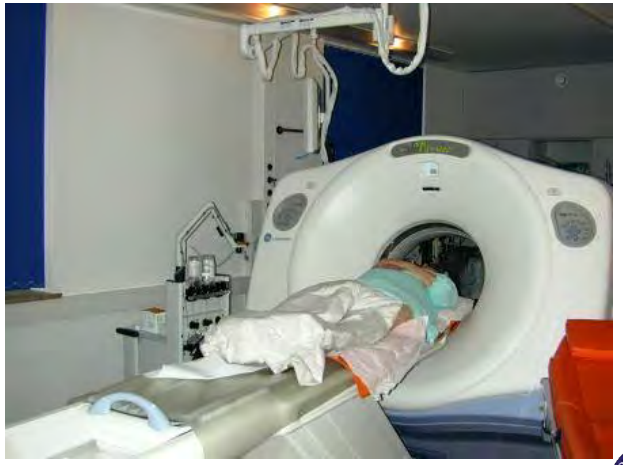
- Electron tomography (ET) is a tomograpy technique for obtaining detailed 3D structures of subcellular macromolecular objects
- Electron tomography is an extension of traditional transmission electron microscopy and uses a transmission electron microscope to collect the data. In the process, a beam of electrons is passed through the sample at incremental degrees of rotation around the centre of the target sample
- The information is collected and used to assemble a three dimensional image of the target
- Tomography is found in many disciplines and it is useful in exploring *intra cordal structures of the vocal chords* or laryngesl tissue-blocks e.g. of the arytenoid region
- Current resolutions of ET systems are in the 5-20 nm range, siutable for examining supra-molecular multi-protein structures

Tomography, what's in a word?

Definition Tomography….
Tomos = section, slice, cutting

<u>Different types of tomography</u>:
- Electron Tomography
- Computer Tomography
- X-ray Tomography
- *Poly*tomography

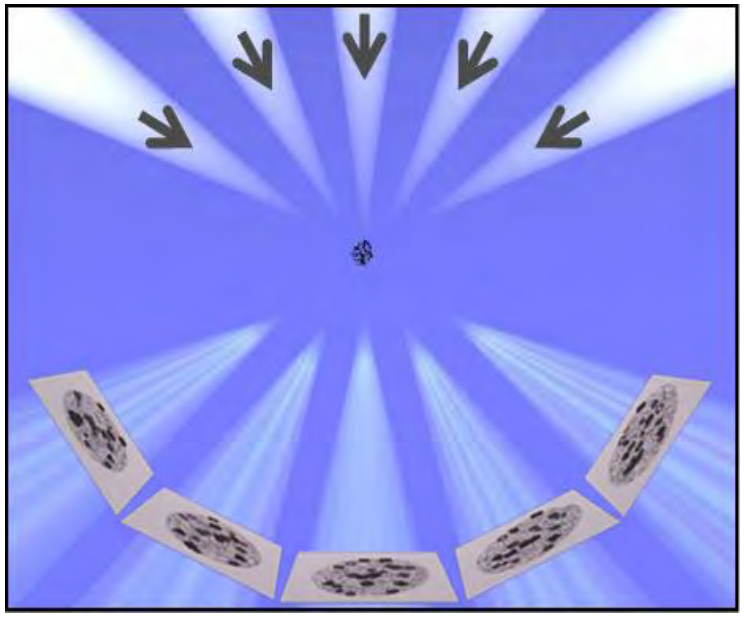




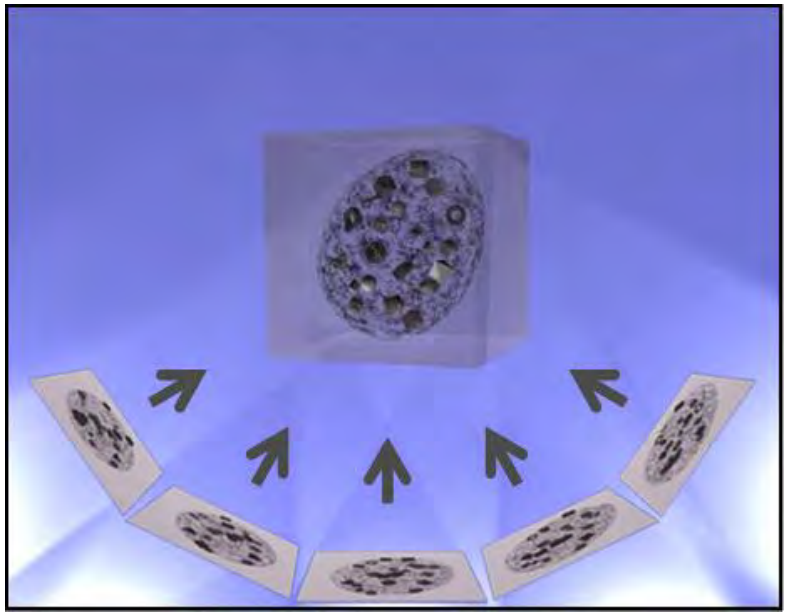
Electron Tomography

Principle

3D-object => set of 2D-projections

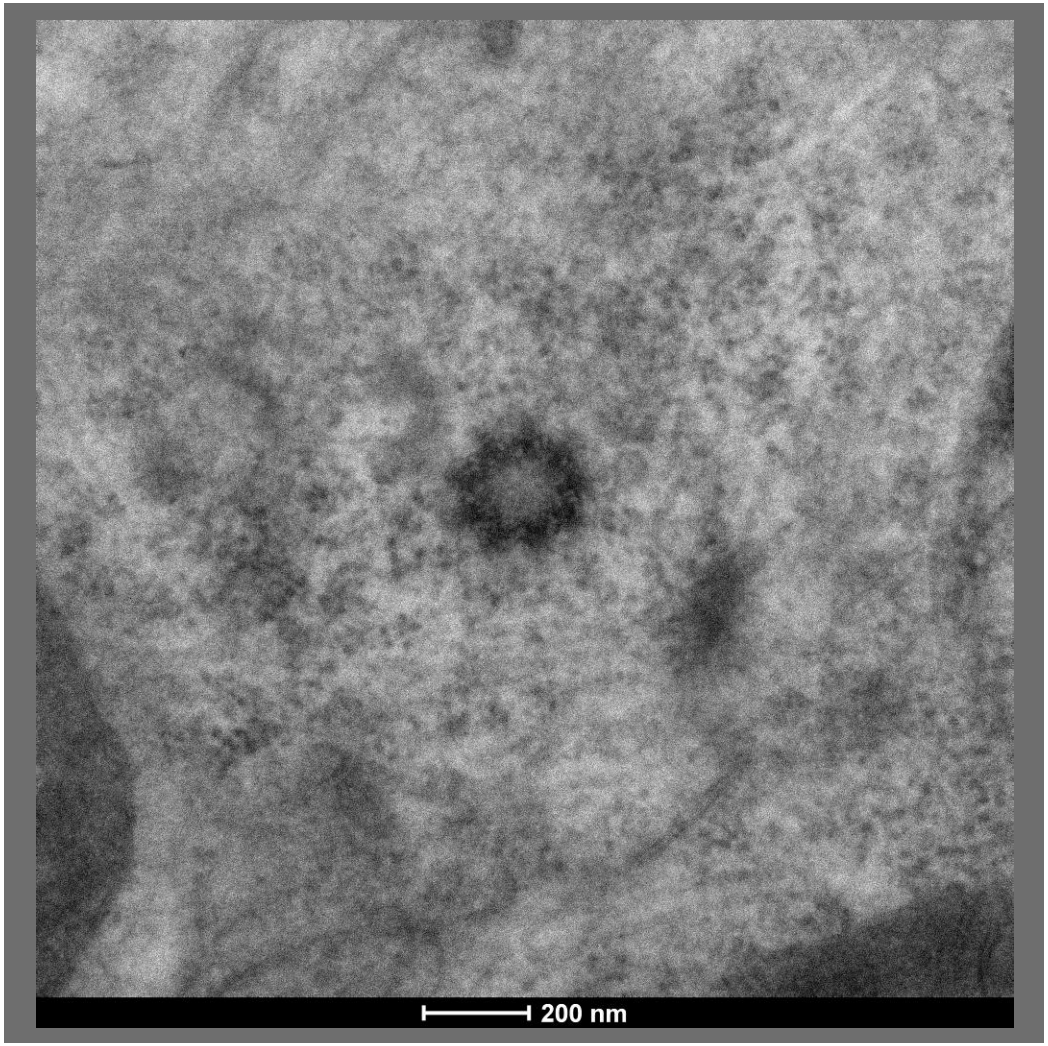


2D-projections => 3D-reconstruction

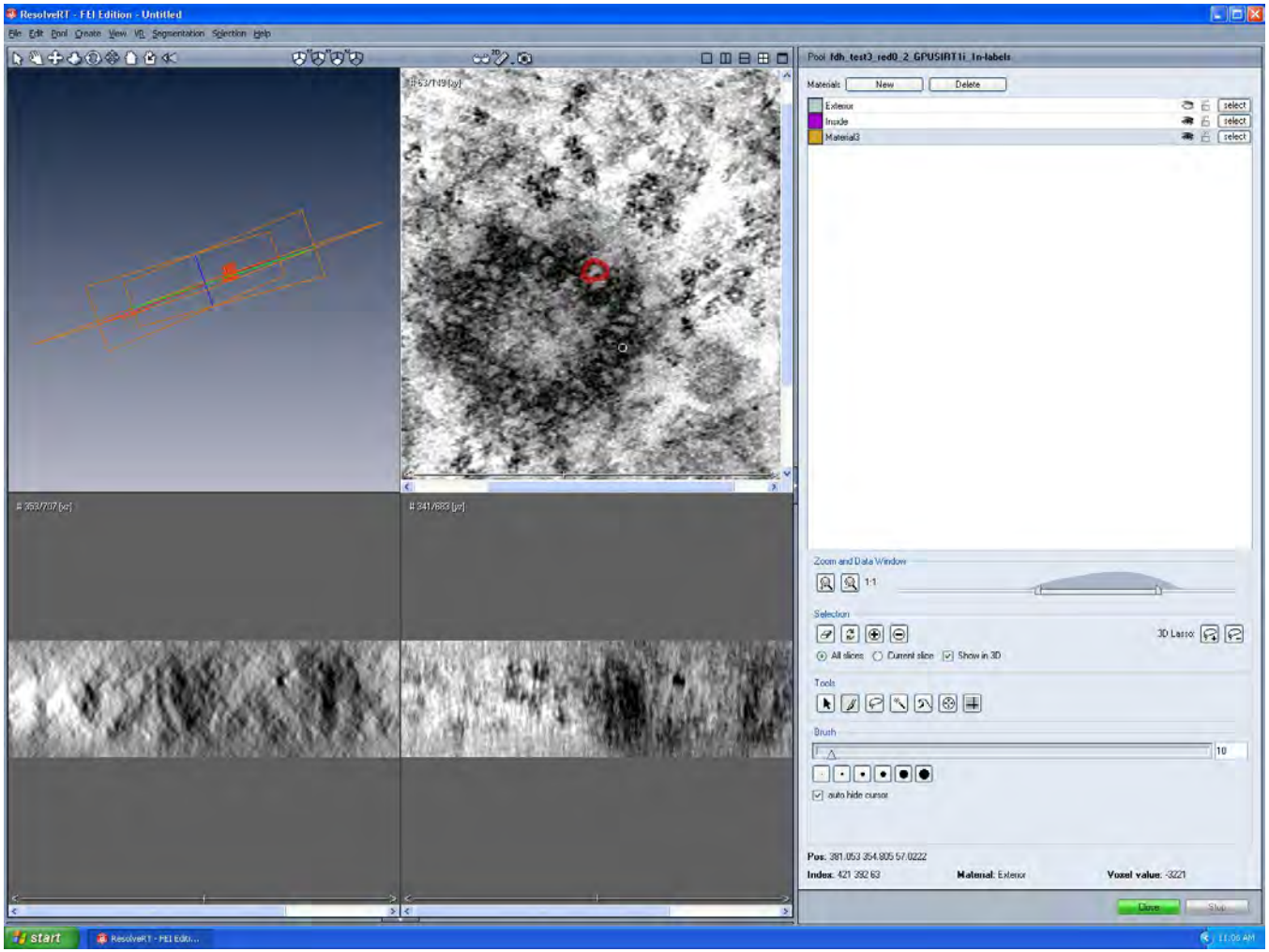


S. Nickell, C. Kofler, A. Leis, W. Baumeister: Nature Reviews Molecular Cell Biology

Aligned tomogram image taken during the demonstration of Rat brain plastic embedded tissue sections



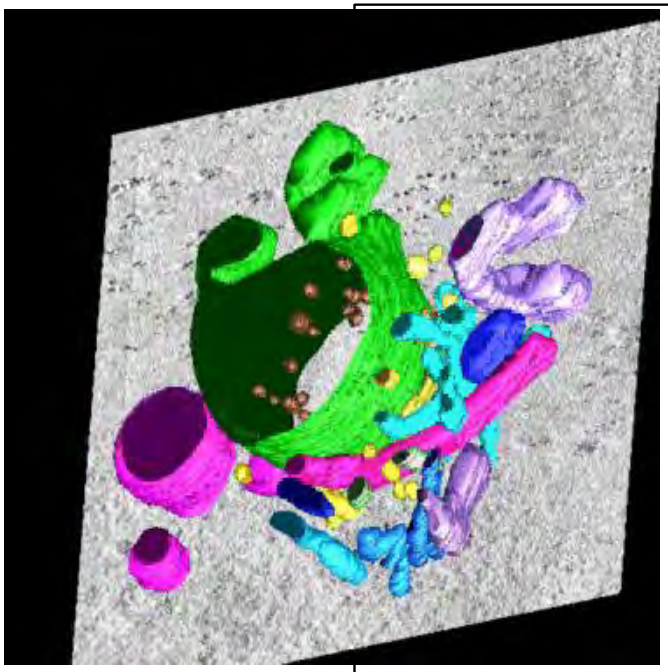
Visualized 3D reconstruction in ResolveRT using Label fielding allows the user to accentuate particular structures

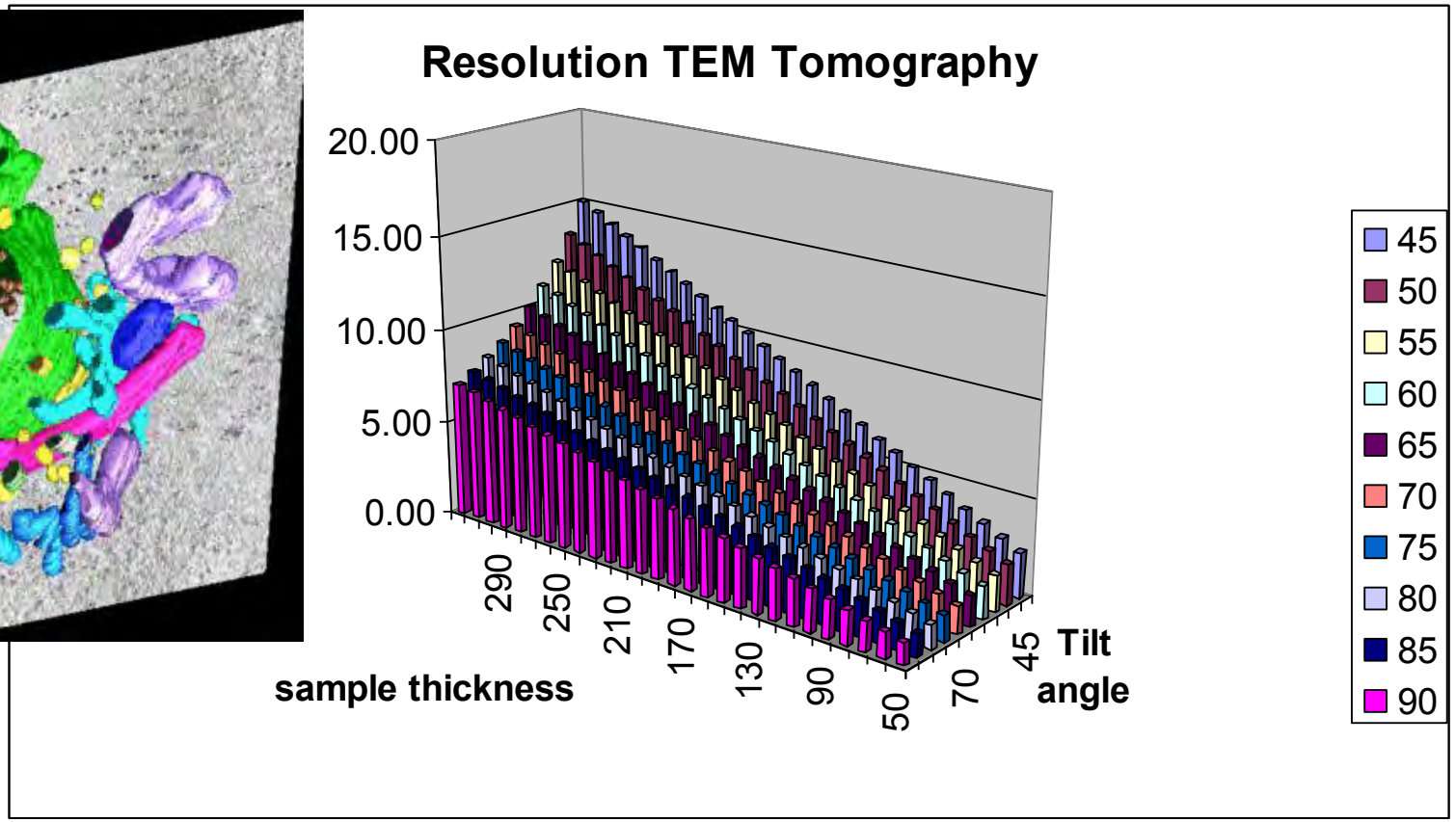


Three-dimensional imaging

- Three-dimensional reconstrution enables visualization of the size, shape and spatial relationship between anatomical structures
- Three-dimensional imaging can be computer generated or directly visualized by stereomicroscopy
- The tissue needs to be optimally preserved by perfusion fixation, to describe surfaces and intracellular features accurately (Glutaraldehyde solution)
- The stereo lumar v12 can be used for any of the diverse applications in conventional stereomicroscopy. It also can acquire highresolution three-dimensional images in the largest specimen field in its class, and be used for flourescence imaging of light microscopy

Resolution Transmission electron Microscope Tomography





$$d = \frac{\pi . D}{N} \sqrt{\frac{(\alpha + \sin\alpha\cos\alpha)}{(\alpha - \sin\alpha\cos\alpha)}}$$

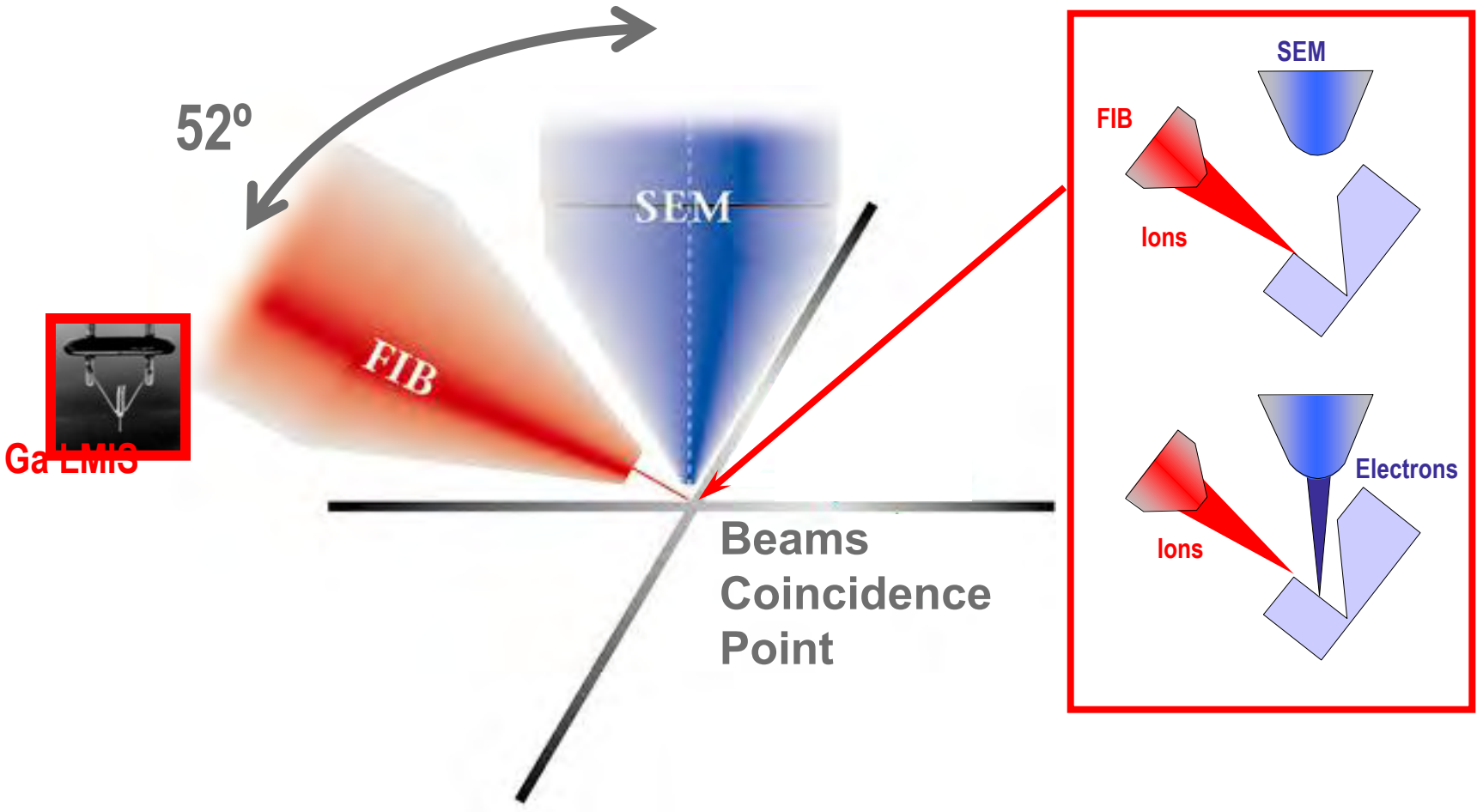
D = sample thickness

α = Max Tilt angle

N = 140 images

d = resolution

Principle of a DualBeam



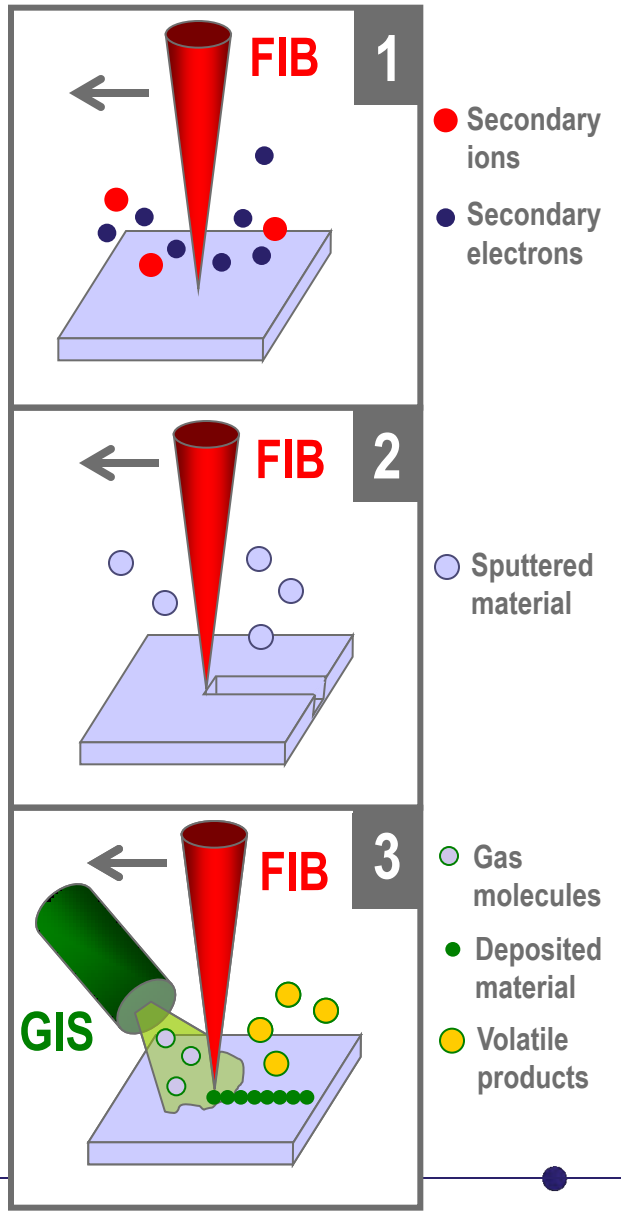
FIB : Three Basic Operating Modes

1. Emission of secondary electrons and ions
   - **FIB Imaging**
2. Sputtering of substrate atoms
   - **FIB milling**
3. Chemical interactions
   - **FIB deposition / enhanced etch**

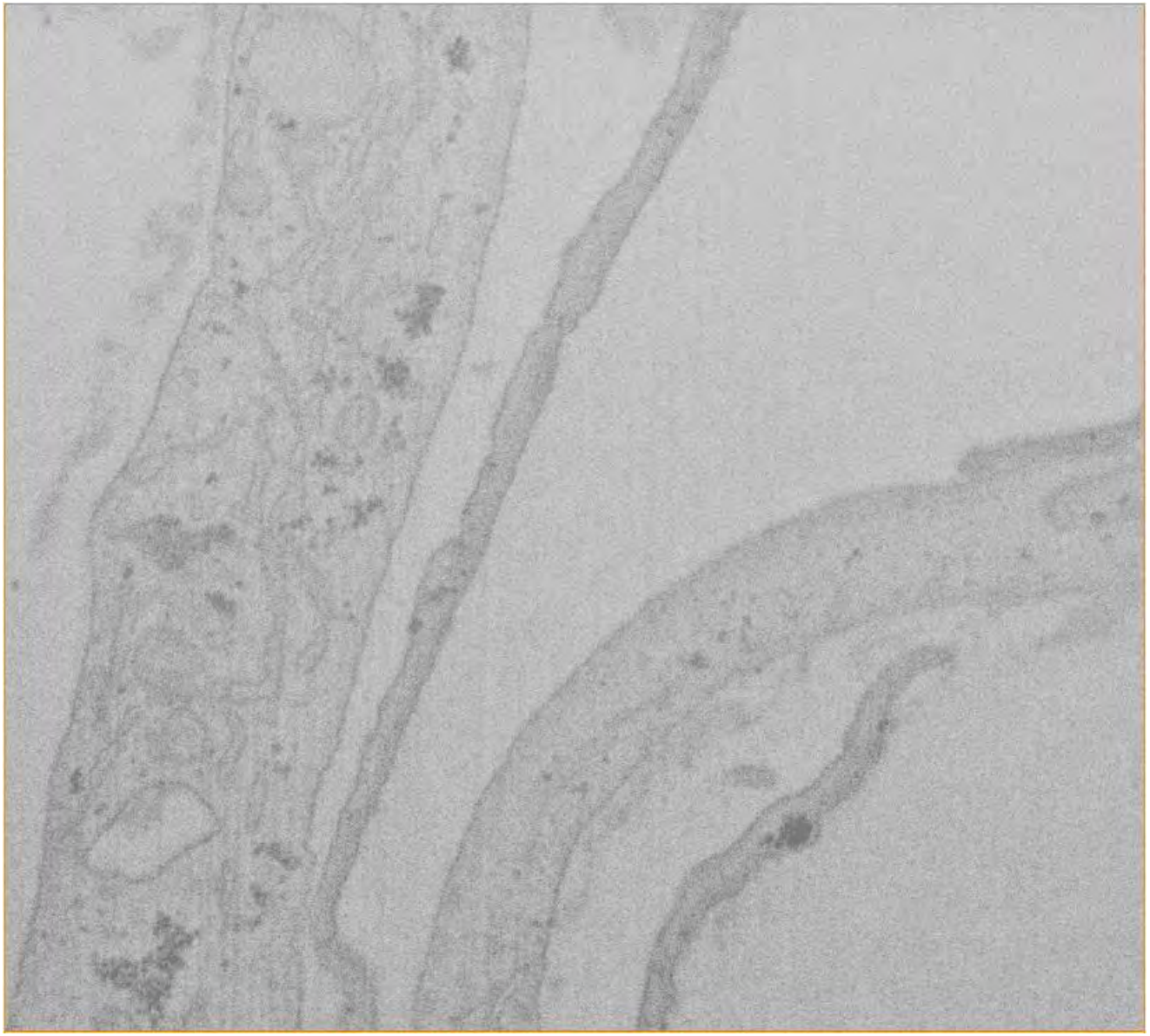
Other effects :

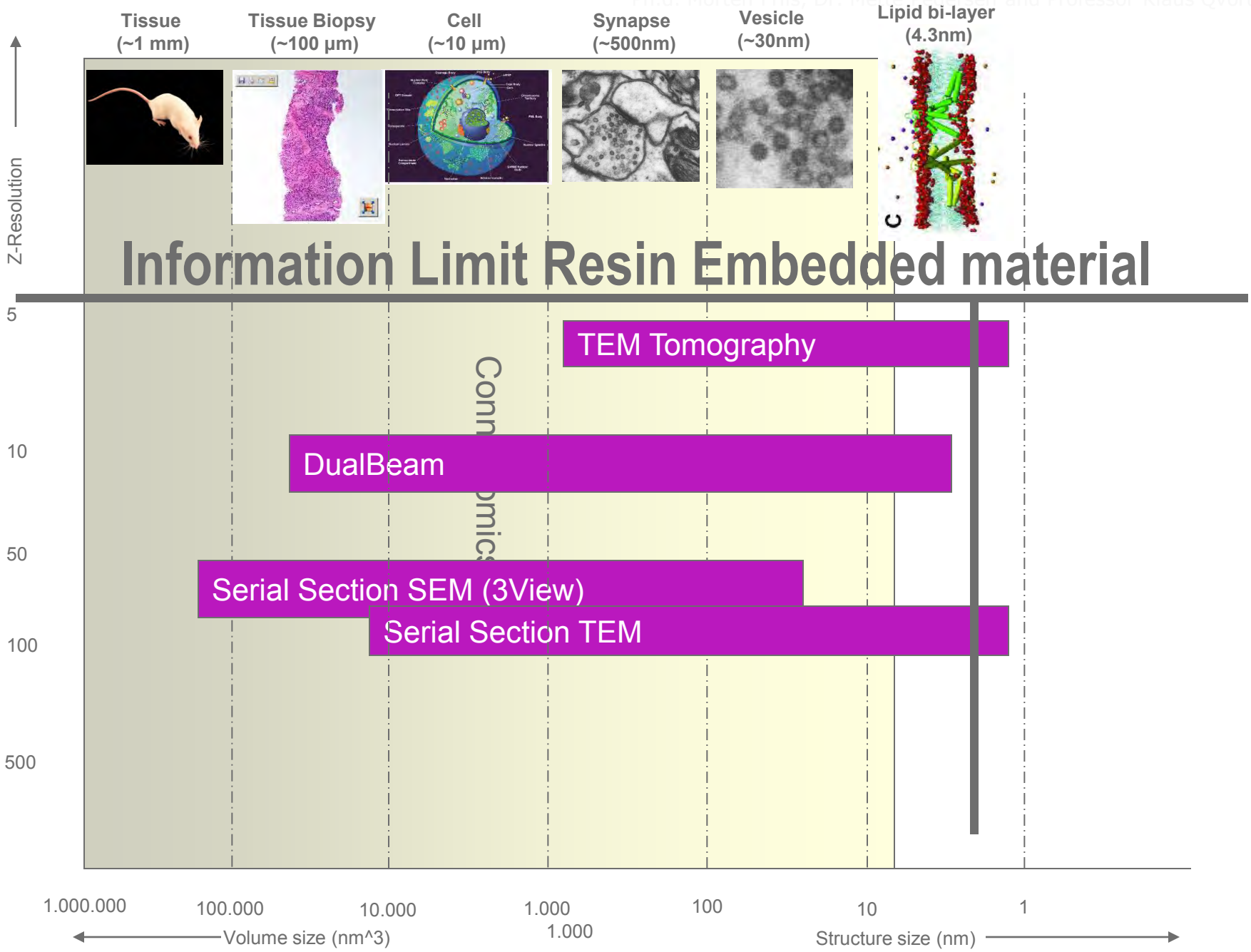
- Ion implantation
- Displacements of atoms in the solid (induced damages)
- Heating

Gas injection system, secondary electrons interaction with the gas



Slice'n'view



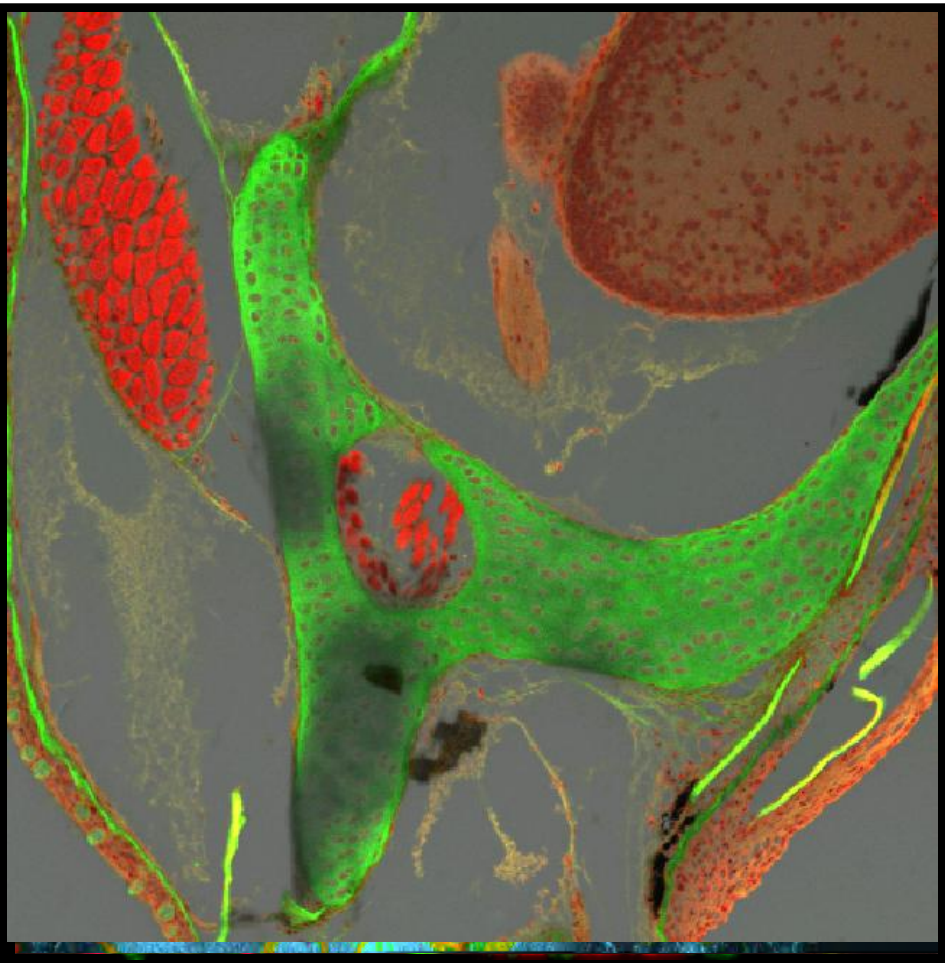

Tissue
(~1 mm)
Tissue Biopsy
(~100 µm)
Cell
(~10 µm)
Synapse
(~500nm)
Vesicle
(~30nm)
Lipid bi-layer
(4.3nm)
Z-Resolution
Information Limit Resin Embedded material
5
10
50
100
500
TEM Tomography
DualBeam
Serial Section SEM (3View)
Serial Section TEM
1.000.000
100.000
10.000
1.000
100
10
1
1.000
Volume size (nm^3)
Structure size (nm)

Applications in more than a wide field

Nearly all fields of Science...

Agricultural Research, Alzheimer, Cancer, Cell Science, Biochemistry, Botany, Immunology, Developmental Biology, Ecology, Epidemiological Diseases, Evolutionary Biology, Food design, Genetics, HIV, Material Quality Control, Material Sciences, Medicine, Membrane Research, Neurobiology , Parasitology, Pharmacology, Physics, Plant Biology, Proteomics, Signal Transduction, Virology...

benefit from Light Microscopy



.....including laryngology

Conclusion

- The coordinated light and eletron microscopy approach offers a wide range of state-of the art light and electron microscopes for scientists
- The number of flourophores and flouscent proteins available is increasing every day. Therefore, the factor that limits the number of proteins that can be labelled in samples with different flourophores has a capability to overcome the excitation and emission cross-talk between them. Overlapping flourescent signals can be seperated, like the ones originated by Green Flourescent Protein (GFP) and Yellow Flourescent Protein (YFP), or even separate flourescent labels from autoflourescenes.
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