3D Electron Microscopy: a collection (intro) of methods...

1. Little EM History
2. SEM vs. TEM...
3. LM vs EM / confocal?...
4. 3D EM methods for beam transparent (thin specimen) - volume and surfaces
5. 3D EM methods for thick specimen
   - small volume vs. large volume
   - destructive vs. preservative
6. Discussion

History:
Geschichte der Elektronenmikroskopie....
began in Berlin 1931...

Ernst Ruska
* 1906 in Heidelberg
† 1988 in Berlin
Nobel Price in Physics 1986
History Electron Microscopy....
1933...das “Übermikroskop”....

1936-40: $\mu m$.....$nm$!!!

Modern Electron Microscope....

Tools for Studying the Nano-Cosmos:
- Scanning-force & Scanning-tunneling Microscope (SPM)
- Field-Ion Microscope/ Atom Probe (1955 E.W. Müller - first image of an atom!)
- X-ray diffraction &-microscope
- Ion (He-) Microscope

Scanning Electron Microscopy: SEM

A virtual image built pixel by pixel is formed!
- Surface morphology (length, surface, width, depth, height)
- Element/Chemistry (X-ray, Auger, EBSD)

Transmission electron microscope (TEM)

A real image is formed by lenses....
- Internal morphology (length, surface, width, depth, relation)
- Element/Chemistry (X-ray, EELS, Auger)
Wavelengths of Electrons

<table>
<thead>
<tr>
<th>$V_{\text{acc}}$ / kV</th>
<th>Nonrelativistic wavelength [nm]</th>
<th>Relativistic wavelength [nm]</th>
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<tr>
<td>1</td>
<td>0.0388</td>
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<tr>
<td>40</td>
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<tr>
<td>1000</td>
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<td>0.00087</td>
</tr>
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</table>

(Atomic distances: ~ 0.1 nm (Å))

**Imaging Modes - LM vs. EM:** *(Light vs. Electron Optics)*

**Ernst Abbe: Resolution Power**

\[
d = \frac{\lambda}{2n \sin \alpha}
\]

Angular aperture of the lens - The aperture thus controls the ability of the lens to gather information about the object e.g. the eye at 25 cm corresponding to an angle of about 0.9° for a 4 mm exit pupil diameter of the eye lens; a typical LM with an oil immersion objective lens has 2α of ~175°). For EM typically 8-10mrad (0.5-0.9°)

![Image](https://www.microscopyu.com)

$\lambda_{\text{LM}}/\lambda_{\text{EM}} = 100'000x$  \(-\text{Resolution only 1000x better}\)

3D - Beam Transparent: Confocal Imaging ->
optical sectioning in Light Microscopy....for EM?

**EM:**

- you need a high convergent beam -> Cs Corr.
- a “beam transparent” specimen (<50-100nm)
- high contrast sample....

**z-slice imaging possible for solid state material**


=> for all other samples we need other approaches
EM in Life-Science: Cellular & Molecular....

3D Electron Microscopy

- "Thin" e-transparent => "Tomography" (various angle views..)
- "Thin" section
- tilt series
- virtual image stack

"Thin" section

- "Thick" not e-transparent => serial section
- real section or "en-bloc"
- section projections or bloc-face view
- Image Stack

3D - Beam Transparent EM

- **EM:**
  - macromolecular complexes (helices...)
  - 2D crystals (protein crystals)
  - symmetrical objects (icosahedral viral particle)
  - single particle (isolated > 100k Da)
  - tomographic reconstruction - tilt series

- **History of Electron Microscopy and 3D Reconstruction Methods**
  - 1950s: membrane topology of cellular structures, e.g. mitochondria
  - 1950s: (Crick, Klug et al) FT of helical structures, selection rules
  - 1964: (Parson and Martinus) high resolution electron diffraction on fibers
  - 1968: (DeRosier and Klug) first 3D structure determination of T4 Bacteriophage tail based on helical reconstruction
  - 1970: (Crowther et al) first icosahedral viruses
  - 1972 (Matricardi et al), 1974 (Taylor and Glaeser), 1975 (Unwin and Henderson): 2D crystals
  - 1983 (Kaisers et al): ribosome 3D reconstruction (asymmetric single particle)
  - 1990 (Henderson et al): atomic resolution of bacteriorhodopsin (2D crystal)

W. Wriggers....
3D - Beam Transparent EM

EM: macromolecular complexes (helices...)

- -> cylindrical coordinate (real and reciprocal space)
- -> different view angle immanent to helical structure arrangement (periodic in axial rise, pitch and repeat)
- -> selection of layer lines and use of Bessel function

Real space / Fourier space

References - Helical Reconstruction

- Cochrane, Crick, & Vand, 1962 (FT of helix)
- Klug, Crick, & Wyckoff, 1958 (selection rule, n-l plot)
- DeRosier & Klug, 1968 (first ever 3D reconstruction from EM)
- Stewart, 1988 (great review of helical reconstruction technique)
- Moody, 1990 (of course)
- 3D - Beam Transparent EM
- EM: - 2D crystals (protein crystals)
  - e-diffraction (amplitude) or FT of real images (amplitude & phase)...
  - periodic structure (real and reciprocal space)
  - collect different view angle - tilt series
  - add in fourier space the layers to a 3D frequence representation

Literature

- Henderson et al. (1984) Ultramicroscopy, 19, 147
Diffraction of a coherent parallel light beam by a regular object generates parallel beams emanating off-axis.

A filter, adjusted to the regular spots, separates their light from the noise. A second lens creates the filtered image.

Focusing of parallel waves in-phase, corresponding to regular structure on the picture, leads to small regularly arranged spots in the focus plane, while noise is focused elsewhere.

Diffraction of a coherent parallel light beam by a regular object generates parallel beams emanating off-axis.

A filter, adjusted to the regular spots, separates their light from the noise. A second lens creates the filtered image.

Focusing of parallel waves in phase, corresponding to regular structure on the picture, leads to small regularly arranged spots in the focus plane, while noise is focused elsewhere.

**Image enhancement:** signal, noise and averaging

improving the signal-to-noise ratio

**Real Space Filtering (in-silico)**

**Fourier Filtering in-silico**

- Analog

- "optical" Fourier Filtering...

- "in-silico" (Computer)

- contour map of structure

- Fourier Filtering in-silico

- Fourier coefficients [amplitudes and phases]

- Fourier transform

- inverse Fourier transform

- AMPLITUDES OF PHASES OF REFLECTIONS ABOVE BACKGROUND LEVEL

- AVERAGING OF REPETITIVE MOTIFS BY CRYSTALLOGRAPHIC METHODS

- e.g. TEM, Negative Film

- NEGATIVE FILM

- Optical diffraction

- Laser, FT

- well ordered areas

- optical pattern

- well ordered areas

- real space

- Laser, FT

- optical pattern
First 3D-reconstruction from a protein-complex (in fourier space)

Already 1975 Henderson et al used a combination of averaging technique by the use of 2D protein-crystals → to enhance the signal to noise ration to "see" one single unit in one projection view...

- By tilting such 2D protein-crystals they were able to collect over more than 7-15 years enough data with high resolution of the "identical" protein with good S/N ration from various tilt angles...

- By collecting different tilt angle -70° to +70° they were able to use tomographic reconstructions to reconstruct a 3D view...

Baker, Henderson (2001)

Tomography

The word tomography is composed of the greek words tomé (to section) and gráphein (to write, to draw) and means recording an image of a section through an object.

Tomography is a mathematical technique that reconstructs a certain property of the object from a series of integrals of this property. (e.g. Z-scattering or phase shift properties in transmission images of the object)

Schematic diagram to illustrate the principle of 3D-reconstruction (Fourier space)

- by TEM imaging...
- in-silico (PC)...

Baker, Henderson (2001)

3D-volume reconstruction: 2D-crystals

Purple membrane (biological solar cell)

Halo-bacterium halobium

15Å

1µ

3D-model of Bacteriorhodopsin
Tilt series reconstruction resolution: x,y: 3.5Å; z: 5Å
Unwin und Henderson 1975-1990
3D - Beam Transparent EM

EM: single particle (isolated > 100k Da) & tomographic reconstruction - “tilt series”
- collect as many images and projection of your sample (real)
- > 100'000 images of single particle (statistics)
- Multivariate statistics selects “classes” of different projection views
- > average n particles per class -> merge 2D transfers in 3D in Fourier space -> back-transformation (rFFT)

Electron Tomography - macromolecular complexes

2D-projections of single particles
- a random series of 2D-images aligned by man/computer selection
- selection of different projection classes of images
- Tomographic reconstruction
Electron Tomography - macromolecular complexes..

2D-projections of single particles
- a random series of 2D-images
  - aligned by man/computer selection
  - selection of different projection classes of images
  - Tomographic reconstruction

2D-projection tilt series
- by tilting the specimen stage...
  - TEM Tomography
  - selection of different projection classes of images
  - Tomographic reconstruction

"weighted back projection (real space)
- generate direct tilt series...(S/N!!)

2D-projections tilt series
- by tilting the specimen stage...
  - TEM Tomography
  - question of S/N...

3D-reconstruction
- to reconstruct the 3D-object all the backprojection bodies are summed
  - by Computer (in-silico)

W. Baumeister, MPI Martinsried -> more by Ohad Medalia

Crowther criterion
Elongation factor

d \_s = \alpha N \frac{D}{N}

\epsilon_\alpha = \sqrt{\frac{\alpha}{\alpha - \sin^2 \alpha}}

\eta = \frac{\alpha}{\sin^2 \alpha}

Density resolution
Elongation factor

d \_s = \eta \epsilon_\alpha

Missing wedge

Lit.: see also S.Nickel et al., Nature Reviews Molecular Cell Biology....
Classical Tomography:
only images along one rotation axis....

Single particle imaging Tomography:
images randomly over the entire spheres ......

3D - Beam Transparent EM
- macromolecular complexes (helices...)
- 2D crystals (protein crystals)
- symmetrical objects (icosahedral viral particle)
- single particle (isolated > 100k Da)
- tomographic reconstruction - tilt series

collect as many view angle as possible - use Fourier space maths or tomographic procedure to reconstruct 3D volume

Flow diagram 3D (cryo-) TEM
from sample preparation to 3D-map interpretation

Tomography (cellular TEM)
tilt series (same specimen area) averaging not possible resolution: 100-50Å

Single particles
MW >250kD tilting not necessary averaging (after classification) resolution: 20-10Å

1D-crystals (helices) tilting not necessary averaging resolution: 30-10Å

2D-crystals tilt series (different crystals) averaging resolution: 20-3Å

Alternative ways to extract 3D structure on macro-molecular complexes... ...
Surface relief reconstruction - TEM

T4-Polyhead: freeze-dried and unidirectionally shadowed with TaW (5 Å)

Surface relief reconstruction from SEM data

Pseudoreplica (freeze-dried coated or stained) of macromolecular complexes...SEM images

Basic’s to understand:
- principles of diffraction-based methods
- grounding by understanding only simple algebra
- visual representation & explanations
- cryo-EM & X-ray crystallography
- crystals (3D/2D), helices, viral geometry & single particle
The context matters...

“The Art lies in the context....”

Why imaging?
Nano-bio-morphology: Morphomics

Nano-bio-morphology: Morphomics

Why EM for Life Science..... but please not only Science Hollywood.....

EM in Life-Science: Cellular & Molecular....

Günther Blobel: Electron microscopy is the molecular eye of science.
Excellent electron microscopists of the future should have the duty to produce authentic images of the molecular interaction network, in context and in detail.
These pictures should fill the textbooks of young molecular biologists and support their visual thinking.

http://multimedia.mcb.harvard.edu/media.html
Aim: The Cellular Nano-Morphology at the life-like State

Intact biological System
Preparation for EM
Electron Microscopy
Image Interpretation & Reconstruction
Structure Simulation

Sample extraction:
- Micro-Biopsy
- fast
- controlled
- physiological conditions

Freezing:
- freezing in ms
- life-like preservation
- vitrified water
- no ice crystals

Cryopreparation:
- selective dehybridation
- no shrinkage
- no loss & displacement
- antigen preservation

TEM (SEM):
- projection maps
- pattern recognition
- tomography
- low dose

Image processing:
- selection
- distortion
- calibration
- signal/distortion
- volume recovering

Computer model:
- selection
- reduction
- complexity
- rendering
- animation

Information Transfer System (Information Transfer Function...)

3D Electron Microscopy

"Thick" not e-transparent => serial section real section or "en-bloc"

"thin" section
- "replica" freeze fracture
- section projections or bloc-face view => Image Stack

"thin" section
- tilt series
- virtual image stack

Large Volume 3D EM methods...

- 3D - Data from sections....
  - classical serial sectioning...-> TEM
  - serial sectioning (arrays) for SEM
  - serial sectioning in the SEM...->

- serial sectioning in the FIB/SEM...->

- tomographic view of section volume...-> TEM Tomo

- serial section TEM-tomography...

ScopeM

K.L. Briggman & D.D. Bocks 2012;
Section Imaging  Block Face Imaging
Structure accessibility for SEM & TEM: serial sectioning...

50-100nm sections...

From serial sections to 3-D model:

Serial Sections 3D Reconstruction

Selected Area of Interest

High Pressure Frozen, Freeze-Substituted Paramecia, HM20 embedded, RT serial sections, TEM

Paramecia 3-D reconstruction:

Context embedded 3D models of entities of interest

■ 3D - Data from sections....

Serial section array
SEM imaging:
K. D. Micheva, S.J. Smith
Neuron 55, 2007
Can SEM replace classical TEM application for ultrastructure research?

Sample preparation

**In-vivo**

**Sample Preparation**

- CLSM
- FIB/SEM
  - (->TEM)

**LM/CLSM biopsy / extraction**

- labelling/staining

**BF/DF LM**

**CAT**

**FLM**

**FLM**

**BF/DF LM**

**serial section**

**block-face**

**determination of ROI** & serial sectioning

**CLSM**

**40x**

**Tissue**

- cell culture
- microorganisms
- tissue (biopsy)
- plants material
- ...

**C. elegans**

**Soy bean plant**

**Cell culture on sapphire disc**

+ UAc and/or OsO₄
+ Fluorescent dyes
e.g. DiIC₁₈

**Sample preparation**

**High-pressure Freezing**

**Freeze substitution & low temp. embedding**

**SEM tomography**

**Katja Kawaschinski; 2000; Biel et al., J Microsc 212 (2003)**

**Merchan_Perez, 2009**

**FIB/SEM**

**Beckmann Inst. TEM**

**TEM section on ITO glass slides - dwell time vs. S/N**

- 1.75nm/pixel
- 10kx; WD 2.3mm
- BSE (Esb Det.)
- SE (in-lens)
- => max speed
- SE: 50nsec/pixel
- BSE: 400nsec/pixel

**Ip=855pA**

- 45.8s/frame
- 1.6µs/pixel
- 1.75nm/pixel

**Ip=855pA**

- 23.1s/frame
- 0.817µs/pixel

**Ip=232pA**

- 1.5min/frame
- 3.2µs/pixel
Sample preparation

High-pressure Freezing → Freeze substitution & low temp. embedding → Determination of ROI & 3D imaging → 3D FIB-SEM tomography

e.g. cell culture + UAc and/or OsO₄ + Fluorescent dyes e.g. DiIC₁₈
microorganisms

Soy bean plant

Cell culture on sapphire disc + UAc and/or OsO₄ + Fluorescent dyes e.g. DiIC₁₈

C. elegans

3D - Data from sections....

- classical serial sectioning...→ TEM
- serial sectioning (arrays) for SEM
- serial sectioning in the SEM...→ W. Denk


- serial sectioning in the FIB/SEM...→ a new way to section embedded sample (resin and cryo...)

FIB: Focussed Ion Beam - the “ion knife”

'-DualBeam’: Sample illumination by electron and/or ion beams

in situ sample preparation...

SEM mode FIB mode

“CrossBeam”/ “Dual Beam”

 Acquisition of 3D image stacks with FIB-SEM

1. Deposition of protecting C-layer
2. Milling of a trench, milling current 6.5 - 13nA
3. Polish the cross section, milling current 1.5nA
4. Imaging with SEM (ESB)
5. Cut again a slice away with ion beam
6. Repeat 4.-5. for acquisition of a 3D serial section stack (fully automatized)
Acquisition of 3D stack by FIB / SEM

FIB-SEM: ROI extraction of a lamella for HR-TEM Tomography...

FIB/SEM vs section: limited volume by FIB/SEM....
Can we look at larger sample area’s by SEM?

Two alternatives:
- in-situ microtome (3View or Denkatome - computer controlled)
- array tomography (CAT - correlative/computer controlled)

Sample preparation

High-pressure Freezing → Freeze substitution & low temp. embedding → Determination of ROI & serial sectioning → SEM tomography

- e.g. cell culture
- microorganisms
- tissue (biopsy)
- plant material...

+ UAc and/ or OsO₄
+ fluorescent dyes
  e.g. DiIC18

Determination of ROI & serial sectioning

CLSM (40x)

SEM

3D data with an in situ ultramicrotome.....

(Denkatome...Denk&Horstmann 2004 PLOS Vol 2)

May be combined with CLSM of pre-embedding antibody labeling, or diffusible dyes.....
- fast for large volumes
- destructive
- large amount of heavy metal needed
- e-beam induced hardening during operation

Gatan 3View....
3D data with an in situ ultramicrotome.....
(Denkatome....Denk&Horstmann 2004 PLOS Vol 2)

May be combined with CLSM of pre-embedding antibody labeling, or diffusible dyes....
- fast for large volumes
- destructive
- large amount of heavy metal needed
- e-beam induced hardening during operation

Ultra thin section imaging by SEM

- array tomography (CAT - correlative/computer controlled)

SEM section imaging for ultrastructure (TEM quality....)

- highly flexible for LM modes
- not limited in size and orientation
- no Cu-bar covering
  “the most interesting region”
- immunolabeling possible...
Large number ultra-thin section imaging easier in SEM....

Wilke et al, 2008

TEM (Grid...)

SEM conquers TEM ultrastructure domain (Life-Science)

3D LM/SEM - Data from sections...Array Tomography

Serial section array
SEM imaging:
K. D. Micheva, S.J. Smith
Neuron 55, 2007

1. Chemical Fixation
2. Dehydration
3. Embedding
4. Serial sections
5. Post-embedding labeling
6. Multilabeling...
7. Histo or SEM
-> Au-BSE localisation???

Correlative Microscopy 2 - Array Tomography:
- 60 Sections (80nm)
- 310μm x 230μm; 5nm pixel size; 4μsec dwell time...
- Matrix of 4 x 3 images each 16384 x 16384 pixels

=> 3’221’225’472 pixel per ROI ! => Acquisition time is per ROI 4h 9min!

Array Tomography & Immunolocalisation (lipids):
- primary antibody against Glucosyl-Ceramid 3; secondary antibody Cy3

Cloud Microscopy: www.cubicice.de

SEM: 197 GB

LM Stack: 440 MB

SEM Stack: 197 GB

Cloud Microscopy: www.cubicice.de

Gemini 1530

310μm x 230μm x 4.8μm

Cloud Microscopy: www.cubicice.de
Correlative Microscopy 2 - Array Tomography:

- 60 Sections (80nm)
- 310 µm x 230 µm; 5nm pixelsize; 4 µsec dwell time...
- Matrix of 4 x 3 images each 16384 x 16384 pixels

=> 3'221'225'472 pixel per ROI ! => Acquisition time is per ROI 4h 9min!

3D - Data from sections in TEM....

tomographic view of section volume...->TEM Tomo

serial section TEM-tomography...

TEM Tomography:
multilamellar bodies in the Stratum Granulosum..

80nm HM20 section from a sample freeze-substituted 1999

Resin embedded samples are a "Storage" device for "morphomic data"

-> "Data block" & "Data slices"

Reinvestigated 2002 by TEM Tomography...
-> membrane visibility!

Membrane Visibility in TEM sections - basic morphology

80nm HM20 section from a sample freeze-substituted 1999

-> "Data block" & "Data slices"

Tilt: -60deg to +60deg
3-D reconstruction of Golgi-TGN from TEM Tomography:

http://bio3d.colorado.edu/pubs/Golgi/GolgiAnalysis.html

3-D reconstruction of whole cells (B.Marsh):
from 46 and 27 sections - each reconstructed from a TEM Tomogram... -> 3D statistical data

Expedited approaches to whole cell electron tomography and organelle mark-up in situ in high-pressure frozen pancreatic islets
Andrew B. Noske a,b, Adam J. Costin a,1, Garry P. Morgan a,1, Brad J. Marsh a,b,c,* JSB 2007

Summary: 3D tissue imaging....

<table>
<thead>
<tr>
<th>Method</th>
<th>x-y Resolution</th>
<th>z Resolution</th>
<th>Volume Limit (µm³)</th>
<th>Contrast</th>
<th>Automated Acquisition + Alignment</th>
<th>Immunocytochemistry is possible</th>
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<td>EM/TEM</td>
<td>&gt;2 nm</td>
<td>40 - 90 nm</td>
<td>&gt; 20 × 20 × 20</td>
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<td>difficult</td>
<td>pre and post embedding</td>
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<td>HRF/SEM</td>
<td>&gt;5 nm</td>
<td>&gt;10 mm</td>
<td>&gt;20 × 20 × 20</td>
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<td>yes</td>
<td>pre-embedding</td>
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<tr>
<td>EM/SEM</td>
<td>&gt;55 nm</td>
<td>30 nm</td>
<td>&gt; 300 × 300 × 300</td>
<td>++</td>
<td>yes</td>
<td>pre-embedding</td>
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<tr>
<td>Serial Serial section LM/SEM</td>
<td>&gt;20nm</td>
<td>30-50µm</td>
<td>&gt; 600 × 500 × 500</td>
<td>(+)++</td>
<td>yes</td>
<td>pre- &amp; post embedding</td>
</tr>
</tbody>
</table>

EM-Holography? ... mainly for solid state materials not yet successful in LS

3D Electron Microscopy

The LM/SEM (FIB) world

“Thick” not e-transparent => serial section real section or “en-bloc”

Cryo-SEM

The LM/TEM world

“thin” e-transparent => “Tomography” (various angle views...)

serial section LM/SEM

section projections or bloc-face view

=> Image Stack

“thin” section

=> TEM “Tomo”

“thin” => tilt series

-> virtual image stack
How to Read 3D EM data...

- see. Lit: Saibil, HR (2007) How to read papers on three-dimensional structure determination by electron microscopy. in Evaluating techniques in biomedical research, Cell Press

Some further reading on 3D EM data...

**Reviews**


**Cellular tomography**

