3D Electron Microscopy: 
a collection of methods...

History Electron Microscopy....
1933...das “Übermikroskop”....

Modern Electron Microscope....

Tools for Studying the Nano-Cosmos:

- Scanning-force & Scanning-tunneling Microscope (SPM)
- Field-Ion Microscope (1955 E.W. Müller - first image of an atom!)
- X-ray diffraction &-microscope
- Ion (He-) Microscope

Electron Microscopy ETH Zurich (EMEZ), Roger.Wepf@emez.ethz.ch
Scanning Electron Microscopy: SEM

- Surface morphology (length, surface, width, depth, height)
- Element/Chemistry (X-ray, Auger, EBSD)

Transmission electron microscope (TEM)

- Internal morphology (length, surface, width, depth, relation)
- Element/Chemistry (X-ray, EELS, Auger)

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 2\(\alpha\) of ~175°). For EM typically 8-10mrad (0.5-0.9°)

Obtainable resolution: (Electron vs. Light Optics)

Angular aperture for EM typically 8-10mrad (0.5-0.9°)
- Magnetic fields not homogeneous!

\[ \lambda \text{400nm: } d_{\text{eye}} = 0.02-0.1\,\text{nm} \]
\[ d_{\text{LM}} = \frac{\lambda}{2} (200\,\text{nm}) \]
\[ \lambda \text{0.004nm (100keV): } d_{\text{EM}} = 0.2-0.1\,\text{nm} !!! \]

\[ \lambda \text{400nm: } d_{\text{EM}} = 0.2,2-0.1\,\text{nm}!!! \]

\[ \lambda \text{0.004nm (100keV): } d_{\text{EM}} = 0.2,2-0.1\,\text{nm}!!! \]

\[ \frac{\lambda_{\text{LM}}}{\lambda_{\text{EM}}} = 100'000x \quad \rightarrow \text{Resolution only 1000x better} \]

\[ \lambda_{\text{LM}/\lambda_{\text{EM}}} = 100'000x \quad \rightarrow \text{Resolution only 1000x better} \]
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....

- **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
3D - Beam Transparent EM

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: (Person and Martius) 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 (Matsushita et al), 1974 (Taylor and Glaser), 1975 (Unwin and Henderson): 2D crystals
- 1983 (Kruis et al): ribosome 3D reconstruction (asymmetric single particle)
- 1990 (Henderson et al): atomic resolution of bacteriorhodopsin (2D crystal)

References-Helical Reconstruction

- Cochran, Crick, & Vand, 1952 (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)

Real space / Fourier space

Real space

Fourier (reciprocal) space

Diffractogram (power spectrum)
- 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 frequency representation

**Literature**
- Electron tomography processing
  Baldwin & Henderson, (1994) Ultramicroscopy, 14, 319
- Image processing
  Henderson et al. (1985) Ultramicroscopy, 19, 147
- Processing of fibres images and data merging
  Henderson et al. (1990) J. Mol. Biol. 213, 929
- Refinement
  Gregoret et al. (1990) J. Mol. Biol. 233, 421
Schematic diagram to illustrate the principle of 3D-reconstruction (Fourier space)

Baker, Henderson (2001)

3D-volume reconstruction: 2D-crystals
Purple membrane (biological solar cell)

Unwin und Henderson 1975-1990

Contour map of the projected structure

Electron Tomography - macromolecular complexes...

Orlova, Wah Chiu (1996)

3D-volume reconstruction: single particles
Ca-release channel (2'400 kD)

preparation: "frozen-hydrated" TEM: -180°C, high defocus

2D-projection tilt series
by using inherent tilted specimen entities (helical structures) or by collecting tilt series (2D crystals).

-> TEM "Tomography"
**3D-volume reconstruction: single particles**

Overview of the various iterative refinements

- 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)

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**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

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**EM Tomo: resolution and weighting - limited tilting!**

Crowther criterion

\[ d_r = \pi \frac{D}{N} \]

Elongation factor

\[ e_r = \sqrt{\frac{\alpha + \text{steric incidence}}{\alpha - \text{steric incidence}}} \]

\[ d_r = d_x \cdot e_r \]

- Fourier Space

- Missing wedge

\[ d = \text{lateral resolution} \]
\[ D = \text{thickness of sample} \]
\[ N = \text{number of projections} \]
\[ \alpha = \text{missing wedge angle} \]

Lit.: see also S.Nickel et al., Nature Reviews Molecular Cell Biology...
**Back Projection**

**Classical Tomography:**
...only images along one rotation axis....

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

Hoppe & Tiptoe; 1979 in: Advances in Structure Research by Diffraction Methods...

**Projection & Backprojection**

- Backprojection only of stained particles (A, B)
- Symmetry used to “Reconstruct” missing “Density” -> Backprojection of all particles (A, B & C)

**Basic of 3D-Reconstruction of beam transparent specimen**..... accumulation of information from different views

The “projection theorem”
A 2D projection of a 3D object corresponds to a central section of the 3D Fourier transform of the object.
For a 3D-reconstruction as many as possible different projections are needed (fill the 3D Fourier space)
For example: electron tomography (cells, tissues)
- tilt-range +/- 60-70°, tilt-increments 2-5°

**Electron Tomography - macromolecular complexes..**

<table>
<thead>
<tr>
<th>Principle to obtain multiple views</th>
<th>Electron tomography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merged many particles with various views in solution</td>
<td>Take micrographs of one particle tilted at various angles in the microscope</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Crystallization</th>
<th>Not needed</th>
<th>Not needed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural heterogeneity</td>
<td>Averaged out</td>
<td>Visualized individually</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Current resolution</th>
<th>Electron tomography</th>
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<tbody>
<tr>
<td>High (up to 8 Å)</td>
<td>Low (30 Å)</td>
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</table>

<table>
<thead>
<tr>
<th>Missing information</th>
<th>Electron tomography</th>
</tr>
</thead>
<tbody>
<tr>
<td>None / Missing cone</td>
<td>Missing wedge/pyramid</td>
</tr>
</tbody>
</table>
### 3D - Beam Transparent EM

- **TEM:**
  - 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**

- 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)

### 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: 20-3Å

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

### Surface relief reconstruction - TEM

- "optimal granularity" - statistical nucleation
- averaging out granularity
- Polyhead freeze-dried and rotary shadowed (30°)

### Alternative ways to extract 3D structure on macromolecular complexes... ...
Surface relief reconstruction from SEM data....

freeze-dried and shadowed with 1nm W or from negative staining...

J.D. Woodward...2009

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

Cellular EM

Objects "thick" not e-transparent

Molecular TEM

Objects "thin" e-transparent

3D Electron Microscopy

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

"replica" freeze fracture

"thin" section

section projections or bloc-face view => Image Stack

"thin" e-transparent => "Tomography"
(Various angle views..)

"thin" section => tilt series -> virtual image stack

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...
Structure accessibility for SEM & TEM: serial sectioning...

From serial sections to 3-D model:

Serial Sections

3D Reconstruction

Selected Area of Interest

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
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...)

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)

3D - Data from sections....

- tomographic view of section volume...-> TEM Tomo
- serial section TEM-tomography...
**Electron tomography**

*weighted back projection (real space)*

-> generate direct tilt series...(S/N!!!)

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**2D-projections**

a 3D-object is projected at various tilt angles into a series of 2D-images

-> by TEM Imaging!

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**3D-reconstruction**

to reconstruct the 3D-object all the backprojection bodies are summed

-> by Computer (in-silico)

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W. Baumeister, MPI Martinsried

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**TEM Tomography:**

*multilamellar bodies in the Stratum Granulosum*..

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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!

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• Stratum Corneum lipids are synthesised in the TGN (GluCer) and exported in Multivesicular lamellar bodies into the intercellular space (Cer)...

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3-D reconstruction of Golgi-TGN from TEM Tomography:

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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, Adam J. Costin a, Garry P. Morgan a, Brad J. Marsh a,b,c,* JSB 2007

Correlating FM and cryo-ET: Full Correlation Cycle

LM

3D Electron Microscopy

The LM/TEM world

"Thin" + not e-transparent => "Tomography" (various angle views...)

"Thin" section

=> TEM "Tomo"

serial section LM/SEM

The LM/SEM (FIB) world

"Thick" section projections or bloc-face view

=> Image Stack

serial section LM/SEM

3D - Data from sections....

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

A good biological EM Lab needs:
- a Fluorescence LM
- a (Cryo)-HR-SEM
- a Ultramicrotome

3D - Data from sections....

NG108 neuroblastoma/glioma hybrid cell line

Some further reading on 3D EM data...

**Single particles**

**Helical reconstruction**

**Electron crystallography**


**Some further reading on 3D EM data...**

**A.** Surface views

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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</thead>
<tbody>
<tr>
<td>Surface views</td>
<td>Section</td>
<td></td>
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<tr>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
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<tbody>
<tr>
<td>Projections</td>
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</table>

Some further reading on 3D EM data...

**Reviews**

**Cylindrical tomography**


http://multimedia.mcb.harvard.edu/media.html
comprehensible
“Nano-cosmos”
in its natural context
Thanks for your attention ...