3D Electron Microscopy:

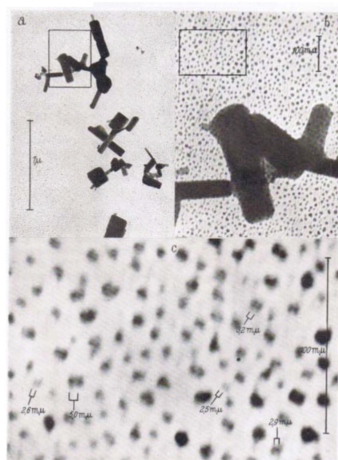
a collection of methods...

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

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

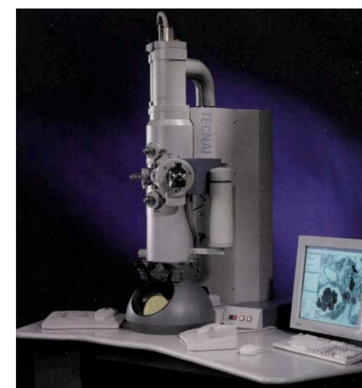
Ernst Ruska Max Knoll Bodo von Borries

History Electron Microscopy.... 1933...das "Übermikroskop"....



1936-40: mμ.....nm!!!

Modern Electron Microscope....



Tools for Studying the Nano-Cosmos:

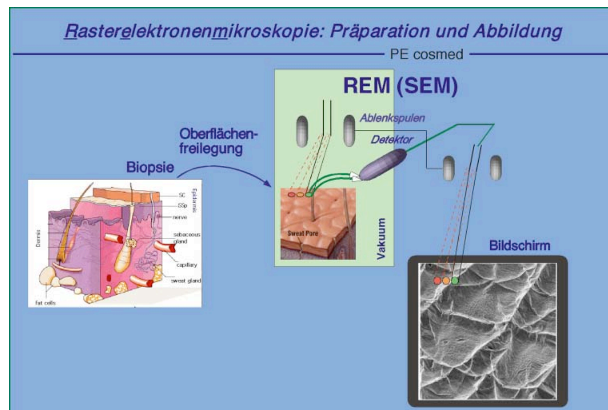
Scanning-force & Scanning-tunneling Microscope (SPM)

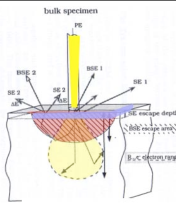
Field-Ion Microscope
(1955 E.W. Müller -
first image of an atom!)

X-ray diffraction &-microscope

Ion (He-) Microscope

Scanning Electron Microscopy: SEM

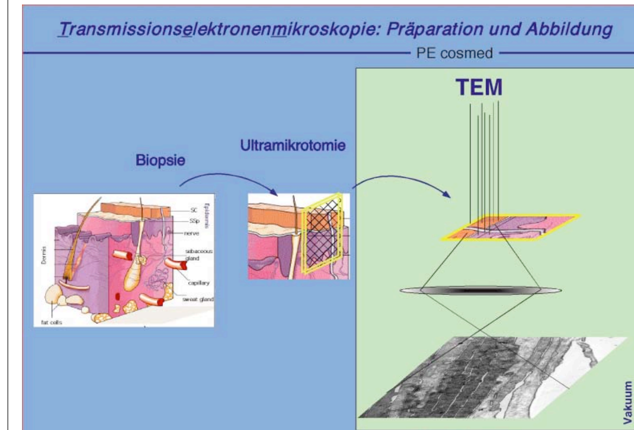


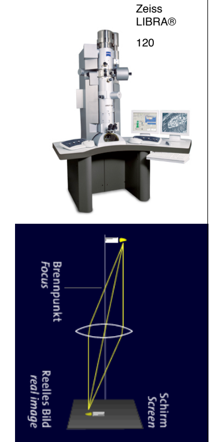


A virtual image built pixel by pixel is formed!

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

Transmission electron microscope (TEM)





A real image is formed by lenses....

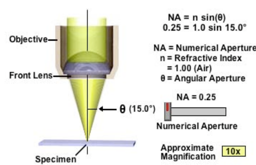
- Internal morphology (length, surface, width, depth, relation)
- Element/Chemistry (X-ray, EELS, Auger)

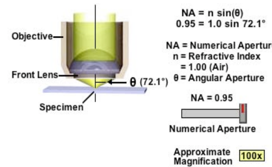
Imaging Modes - LM vs. EM: *(Light vs. Electron Optics)*

- **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°)





www.microscopyu.com

λ 400nm:

d_{eye} = 0,02-0.1mm

d_{LM} = λ/2 (200nm)

λ 0,004nm (100keV):

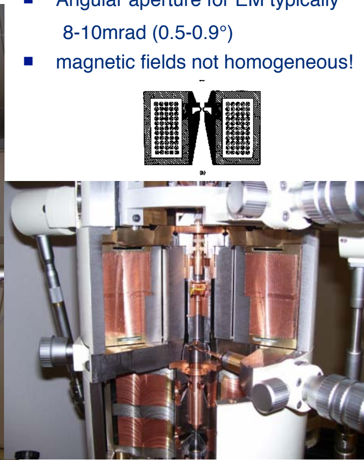
d EM = 0,2-0,1 nm!!!

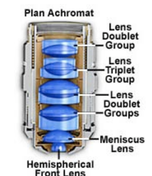
$\lambda_{LM}/\lambda_{EM}$ = 100'000x -> Resolution only 1000x better

Obtainable resolution: *(Electron vs. Light Optics)*

$$d = \frac{\lambda}{2n \sin \alpha}$$

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





LM perfect objective lens

λ 400nm:

d_{eye} = 0,02-0.1mm

d_{LM} = λ/2 (200nm)

λ 0,004nm (100keV):

d EM = 0,2nm!!!

Sub-Angstrom (corrected EM)

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

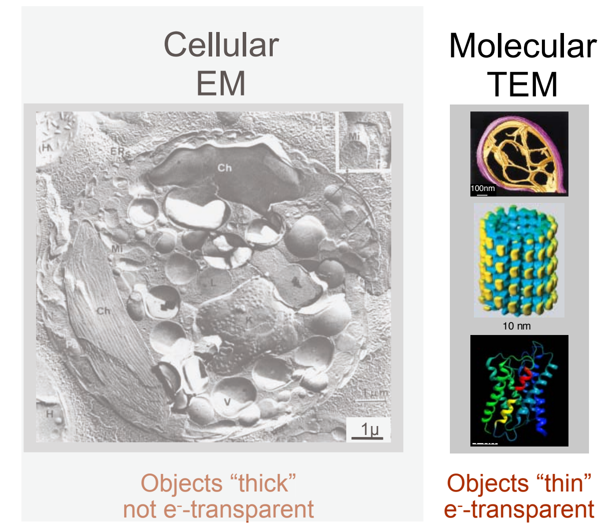
EMEZ Electron Microscopy ETH Zurich

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

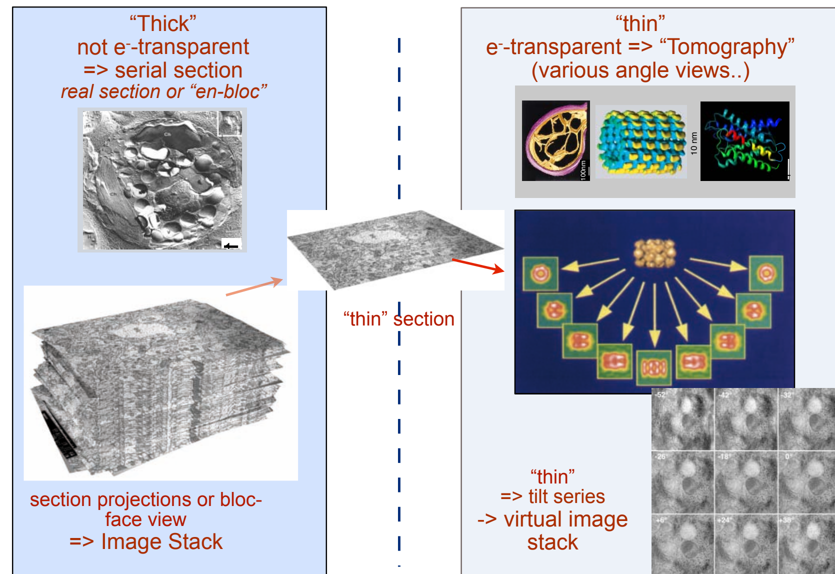
- EM:
  - you need a high convergent beam -> Cs Corr.
  - a "beam transparent" specimen (<50-100nm)
  - high contrast sample....
- z-slice imaging possible for solid state material

  Lit: J.J. Einspahr, P.M. Voyles Ultramicroscopy 106, 2006 "Prospects for 3D, nanometer-resolution imaging by confocal STEM" & M. Varela et al. Annual Review of Material Research 35, 2005 "Material characterization in the aberration-corrected STEM"
- => for all other samples we need other approaches

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



3D Electron Microscopy



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- 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*
- *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: (Parson 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 (Matricardi *et al*), 1974 (Taylor and Glaeser), 1975 (Unwin and Henderson): 2D crystals
- 1983 (Knauer *et al*): ribosome 3D reconstruction (asymmetric single particle)
- 1990 (Henderson *et al*): atomic resolution of bacteriorhodopsin (2D crystal)

W. Wriggers....

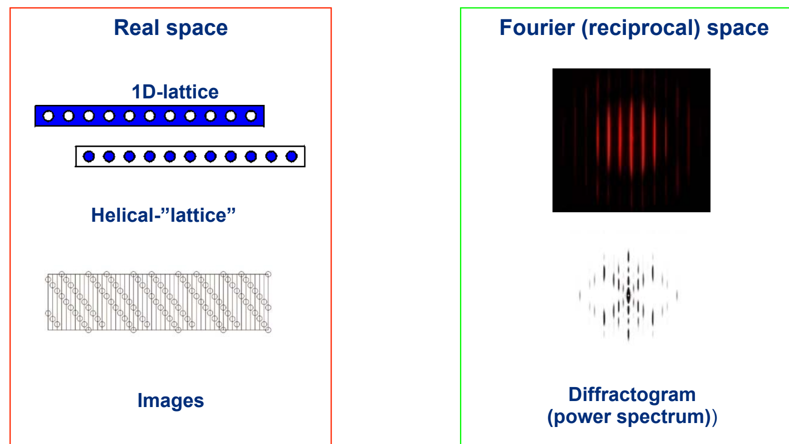
3D - Beam Transparent EM

- 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

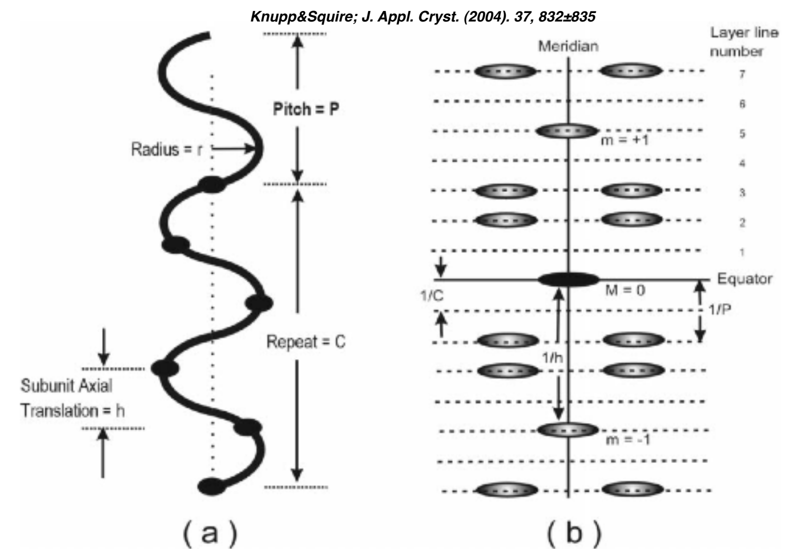
References-Helical Reconstruction

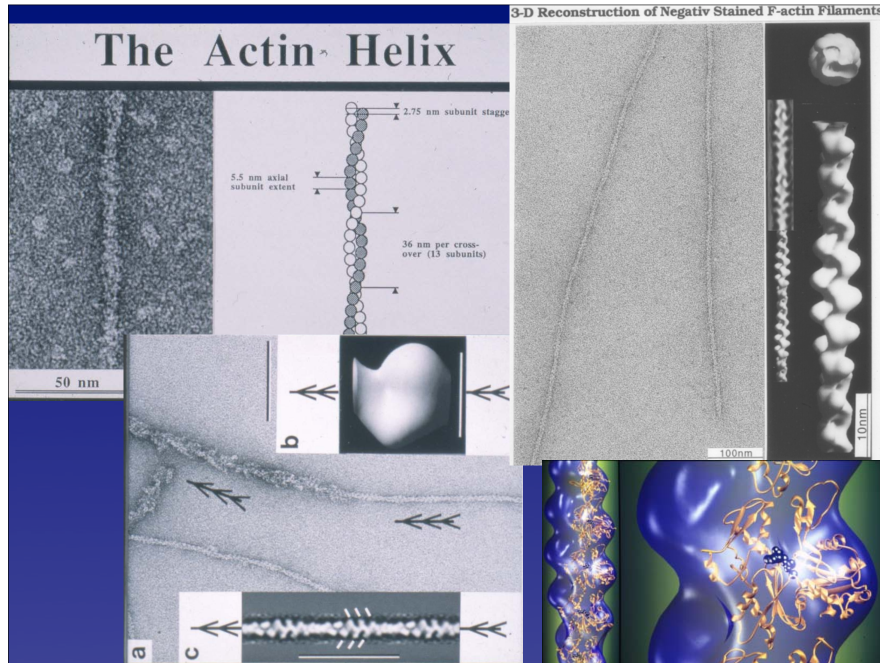
- Cochran, Crick, & Vand, 1952 (FT of helix)
- Klug, Crick, & Wyckoff, 1958 (selection rule, n-l plot)
- DeRosier & Klug, 1968 (first ever 3D reconstruction from EM)
- Stewart, 1988 (great review of helical reconstruction technique)
- Moody, 1990 (of course)

Real space / Fourier space

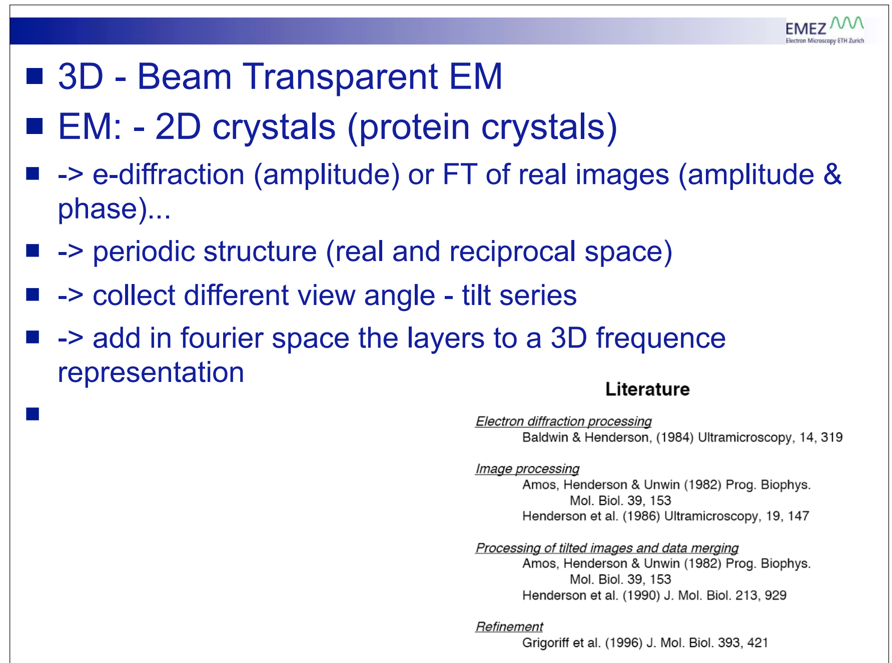


Real space / Fourier space

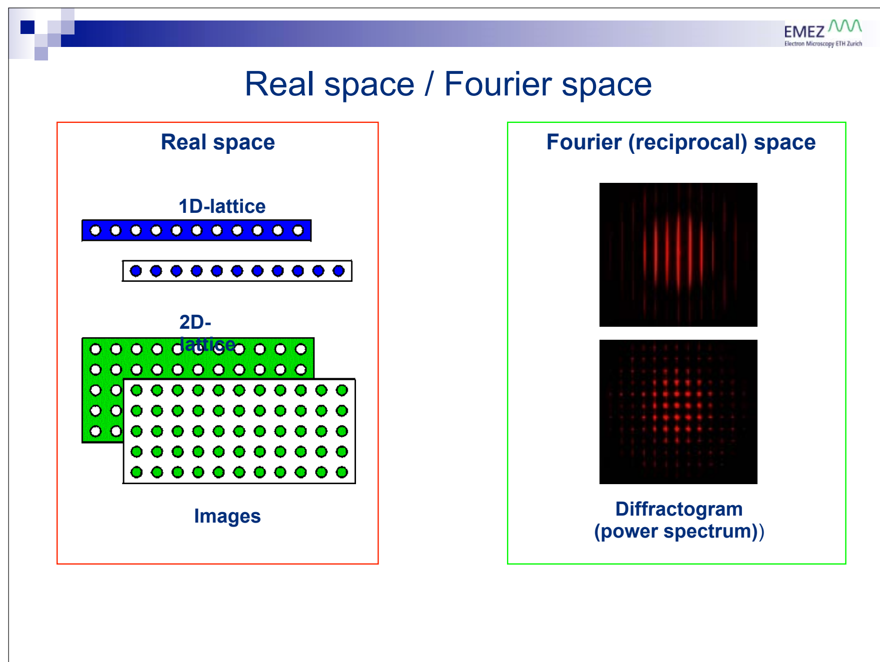




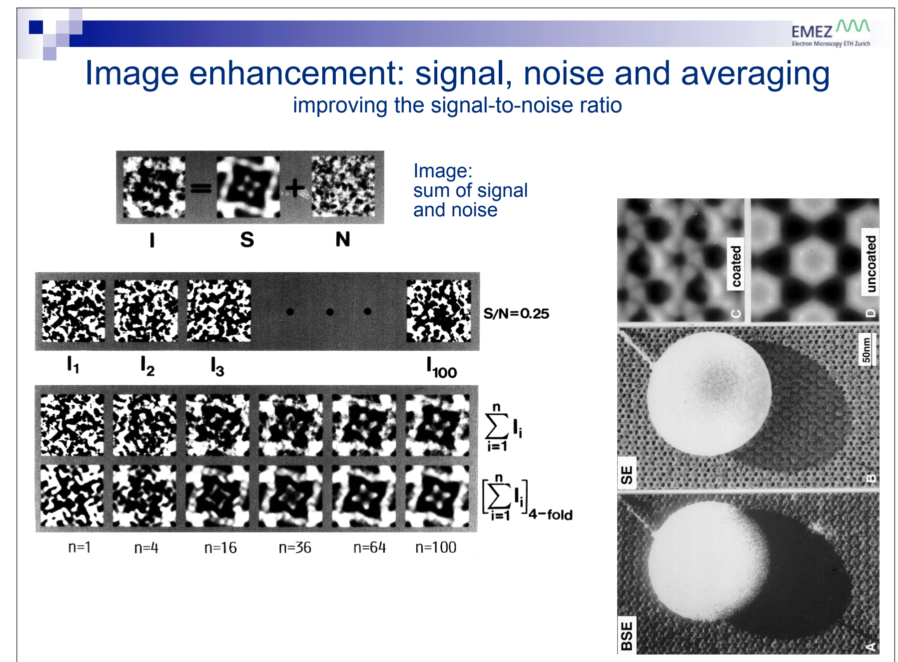
The Actin Helix
3-D Reconstruction of Negativ Stained F-actin Filaments
2.75 nm subunit stagger
5.5 nm axial subunit extent
36 nm per cross-over (13 subunits)
50 nm
100nm
10nm



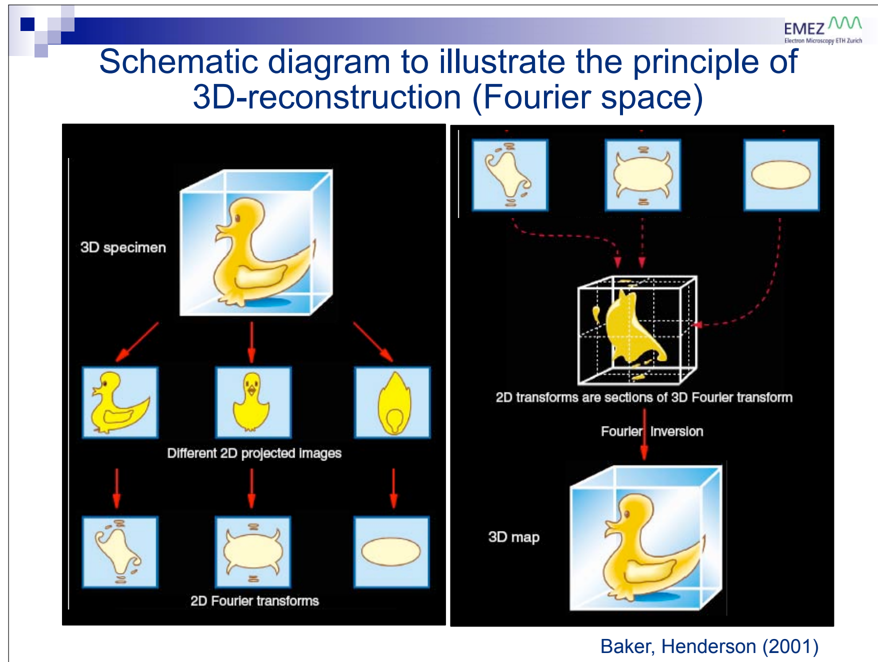
EMEZ
3D - Beam Transparent EM
EM: - 2D crystals (protein crystals)
-> e-diffraction (amplitude) or FT of real images (amplitude & phase)...
-> periodic structure (real and reciprocal space)
-> collect different view angle - tilt series
-> add in fourier space the layers to a 3D frequence representation
Literature
Electron diffraction processing
Baldwin & Henderson, (1984) Ultramicroscopy, 14, 319
Image processing
Amos, Henderson & Unwin (1982) Prog. Biophys. Mol. Biol. 39, 153
Henderson et al. (1986) Ultramicroscopy, 19, 147
Processing of tilted images and data merging
Amos, Henderson & Unwin (1982) Prog. Biophys. Mol. Biol. 39, 153
Henderson et al. (1990) J. Mol. Biol. 213, 929
Refinement
Grigorieff et al. (1996) J. Mol. Biol. 393, 421

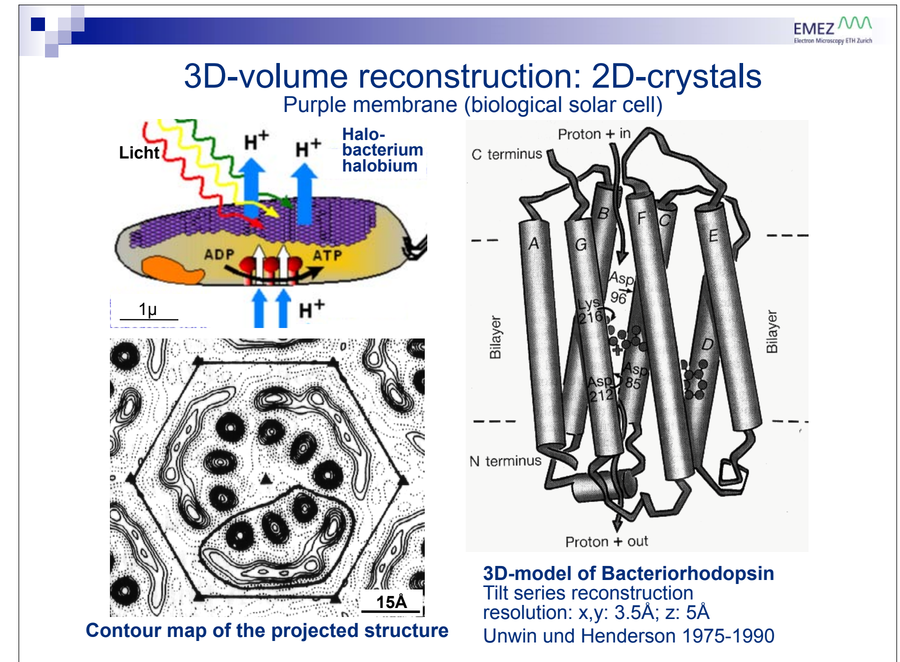


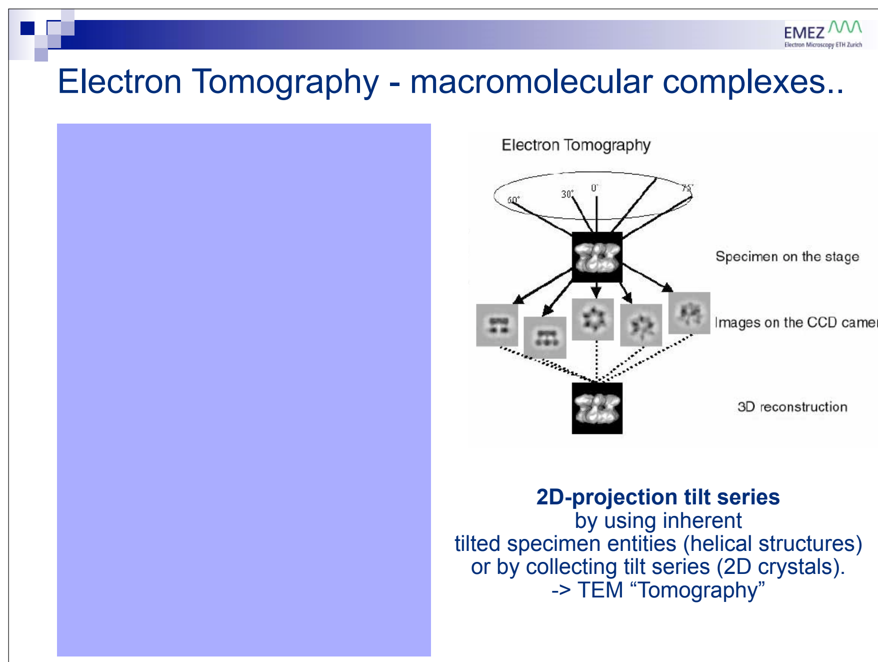
EMEZ
Real space / Fourier space
Real space
1D-lattice
2D-lattice
Images
Fourier (reciprocal) space
Diffractogram
(power spectrum))

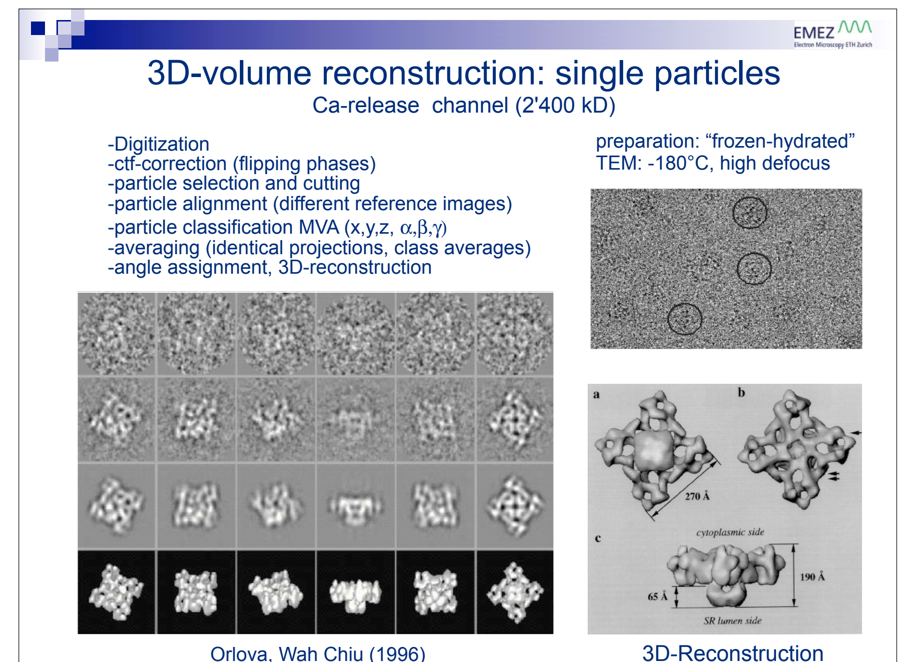


EMEZ
Image enhancement: signal, noise and averaging
improving the signal-to-noise ratio
Image:
sum of signal
and noise
I
S
N
S/N=0.25
I1
I2
I3
I100
n=1
n=4
n=16
n=36
n=64
n=100
4-fold
coated
uncoated
SE
BSE
50nm


EMEZ
Schematic diagram to illustrate the principle of 3D-reconstruction (Fourier space)
3D specimen
Different 2D projected images
2D Fourier transforms
2D transforms are sections of 3D Fourier transform
Fourier Inversion
3D map
Baker, Henderson (2001)

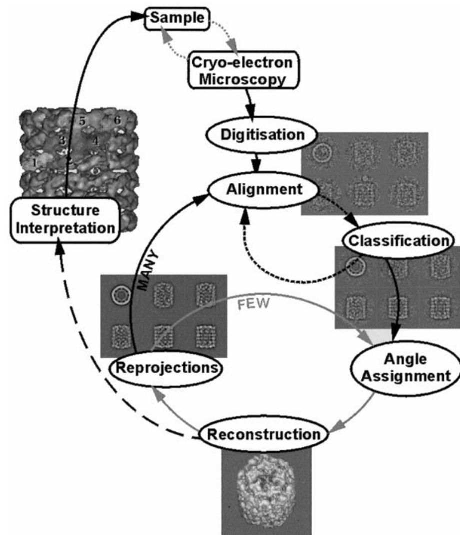

EMEZ
3D-volume reconstruction: 2D-crystals
Purple membrane (biological solar cell)
Licht
Halo-
bacterium
halobium
ADP
ATP
1μ
15Å
Contour map of the projected structure
Proton + in
C terminus
N terminus
Bilayer
Proton + out
3D-model of Bacteriorhodopsin
Tilt series reconstruction
resolution: x,y: 3.5Å; z: 5Å
Unwin und Henderson 1975-1990


EMEZ
Electron Tomography - macromolecular complexes..
Electron Tomography
Specimen on the stage
Images on the CCD camera
3D reconstruction
2D-projection tilt series
by using inherent
tilted specimen entities (helical structures)
or by collecting tilt series (2D crystals).
-> TEM "Tomography"


EMEZ
3D-volume reconstruction: single particles
Ca-release channel (2'400 kD)
-Digitization
-ctf-correction (flipping phases)
-particle selection and cutting
-particle alignment (different reference images)
-particle classification MVA (x,y,z, α,β,γ)
-averaging (identical projections, class averages)
-angle assignment, 3D-reconstruction
preparation: "frozen-hydrated"
TEM: -180°C, high defocus
270 Å
cytoplasmic side
190 Å
65 Å
SR lumen side
Orlova, Wah Chiu (1996)
3D-Reconstruction

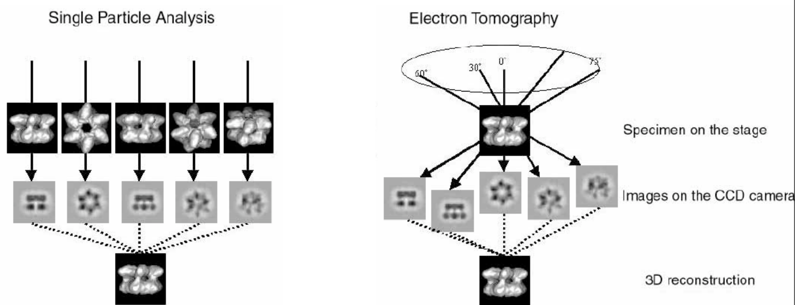
3D-volume reconstruction: single particles

overview of the various iterative refinements



- 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 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

EM Tomo: resolution and weighting - limited tilting!

Crowther criterion

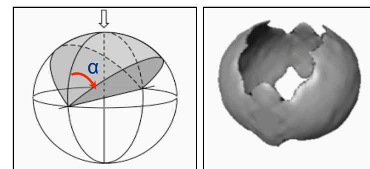
$$d_y = \pi \times \frac{D}{N}.$$

Elongation factor

$$e_{yz} = \sqrt{\frac{\alpha + \sin\alpha \times \cos\alpha}{\alpha - \sin\alpha \times \cos\alpha}}.$$

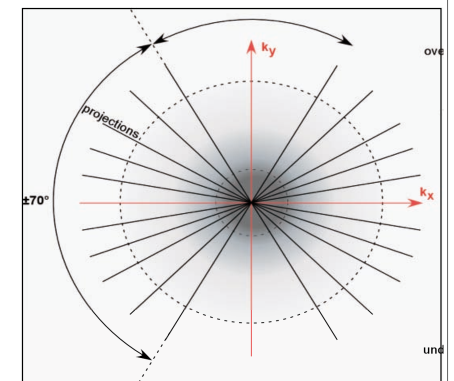
$$d_z = d_y \times e_{yz}.$$

Missing wedge

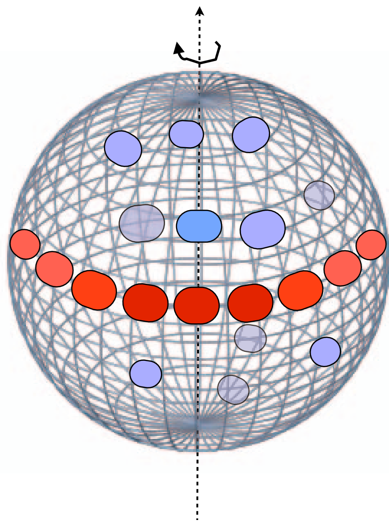


d = lateral resolution
D = thickness of sample
N = number of projections
α = missing wedge angle

Fourier Space



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



Back Projection.....

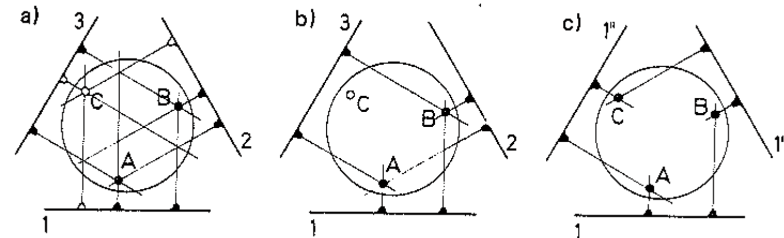
Classical Tomography:

...only images along one rotation axis....

Single particle imaging Tomography:

...randomly images over the entire spheres .....

Backprojection principle...& use of symmetry information to improve final dataset....



Projection & Backprojection...

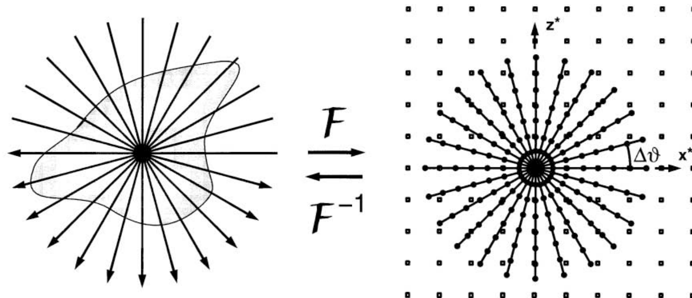
Backprojection only of stained particles...(A, B)

Symmetry used to "Reconstruct" missing "Density" ->Backprojection of all particles... (A, B & C)

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

Basic of 3D-Reconstruction of beam transparent specimen.....

accumulation of information from different views



The "projection theorem"

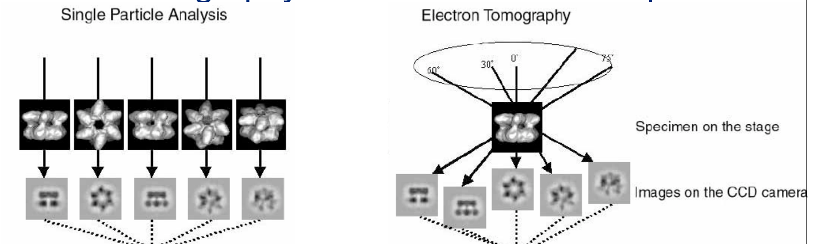
A 2D projection of a 3D object corresponds to a central section of the 3D Fourier transform of the object.

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..

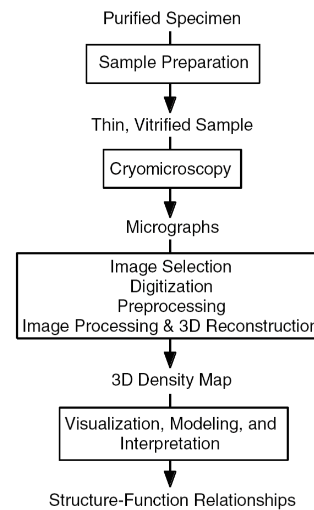


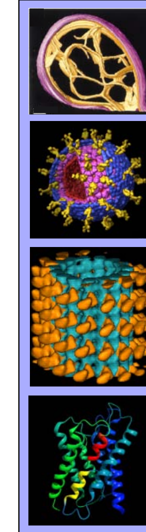
	Single particle analysis	Electron tomography
Principle to obtain multiple views	Merged many particles with various views in solution	Take micrographs of one particle tilted at various angles in the microscope
Crystallization	Not needed	Not needed
Structural heterogeneity	Averaged out	Visualized individually
Current resolution	High (up to 8 Å)	Low (30 Å)
Missing information	None / Missing cone	Missing wedge/pyramid

- 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 **grápheín (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: 30-10Å

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

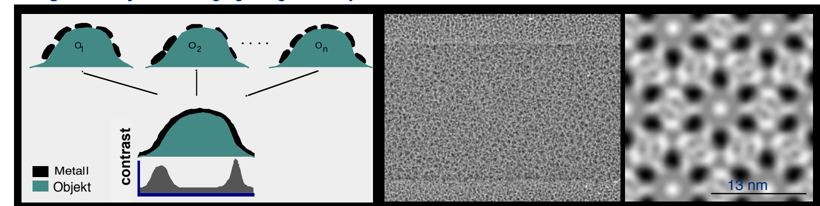
H. Gross...

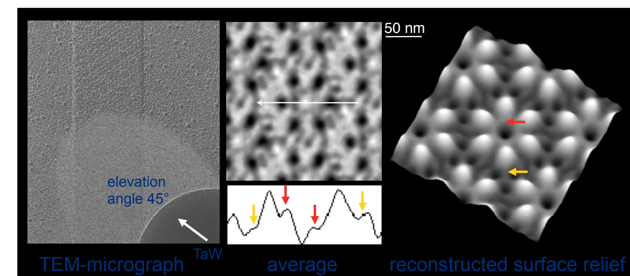
Alternative ways to extract 3D structure on macromolecluar complexes... ...

Surface relief reconstruction - TEM

"optimal granularity" -statistical nucleation -averaging out granularity

Polyhead freeze-dried and rotary shadowed (30°)

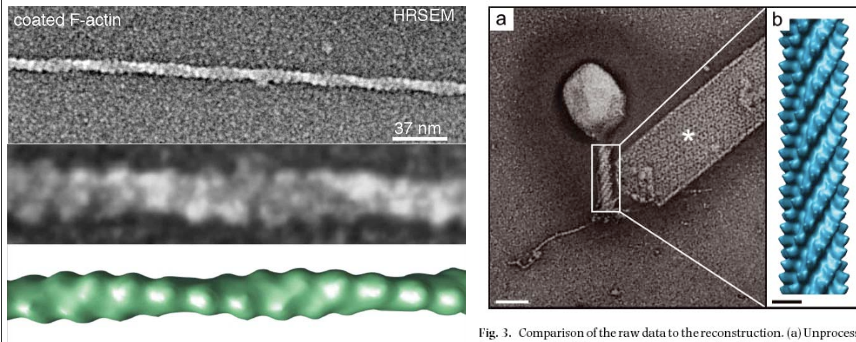


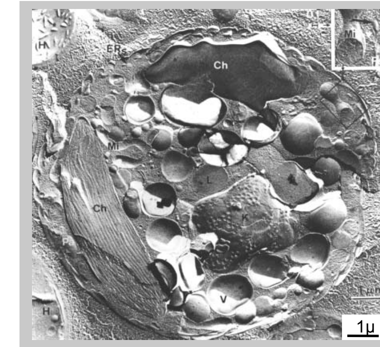


T4-Polyhead: freeze-dried and unidirectionally shadowed with TaW (5Å)

H. Gross...

Surface relief reconstruction from SEM data....





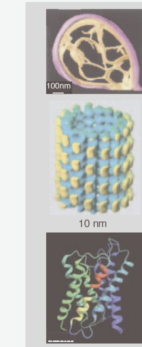


Fig. 3. Comparison of the raw data to the reconstruction. (a) Unprocessed in-lens cryo-FESEM micrograph of negatively stained bacteriophage T4: a polyhead structure can be seen on the right (*). A darker colour in

freeze-dried and shadowed with 1nm W or

from negative staining...

J.D. Woodward...2009

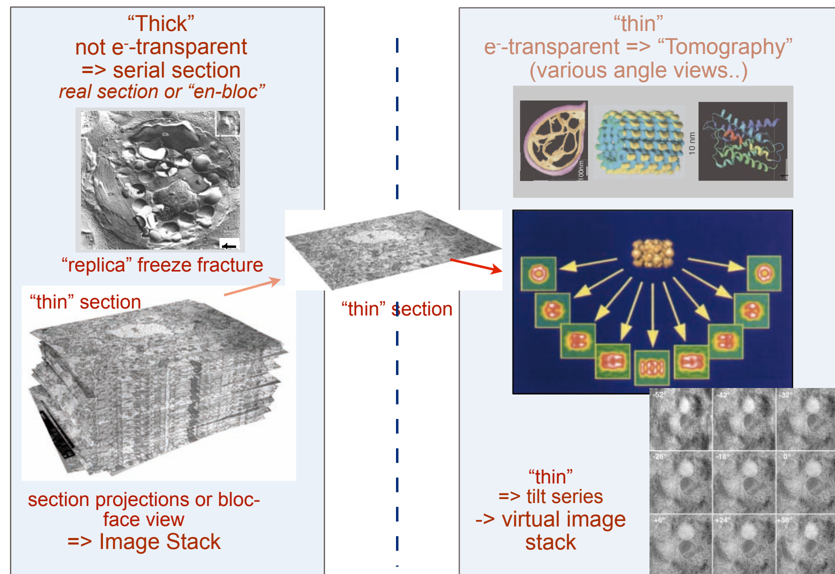
EM in Life-Science: Cellular & Molecular....

Cellular EM

Molecular TEM

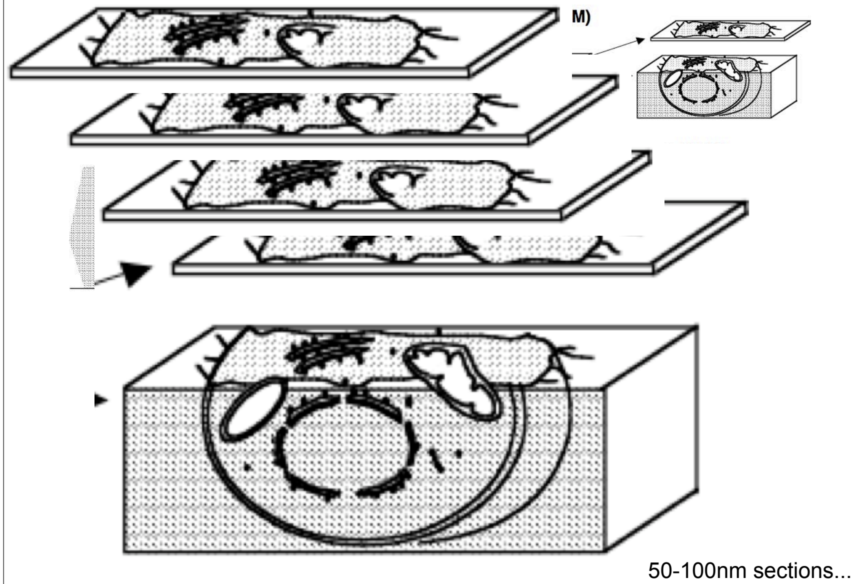
Objects "thick" not e⁻-transparent

Objects "thin" e⁻-transparent



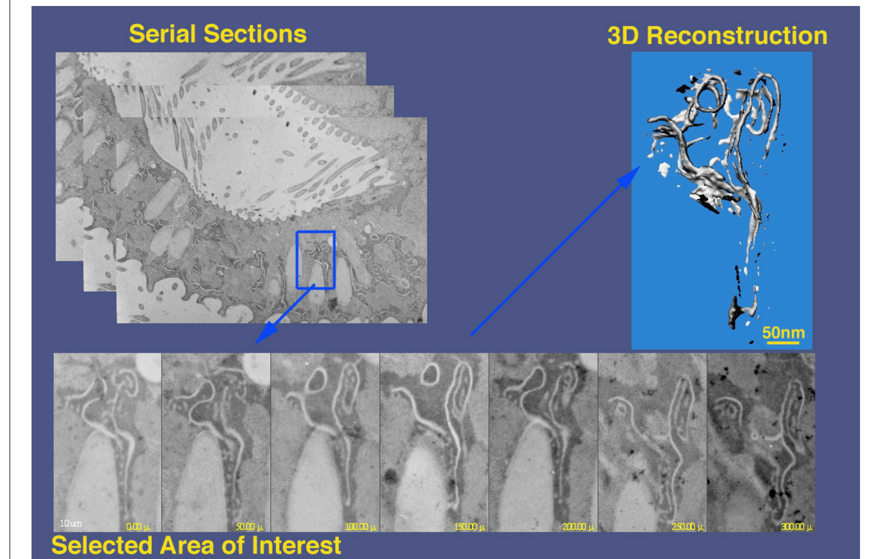
- 3D - Data from sections....
- *classical serial sectioning...-> TEM*
- *serial sectioning (arrays) for SEM*
- *serial sectioning in the SEM...->*
- *serial sectioning in the FIB/SEM...->*
- *tomographic view of section volume...->TEM Tomo*
- *serial section TEM-tomography...*

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

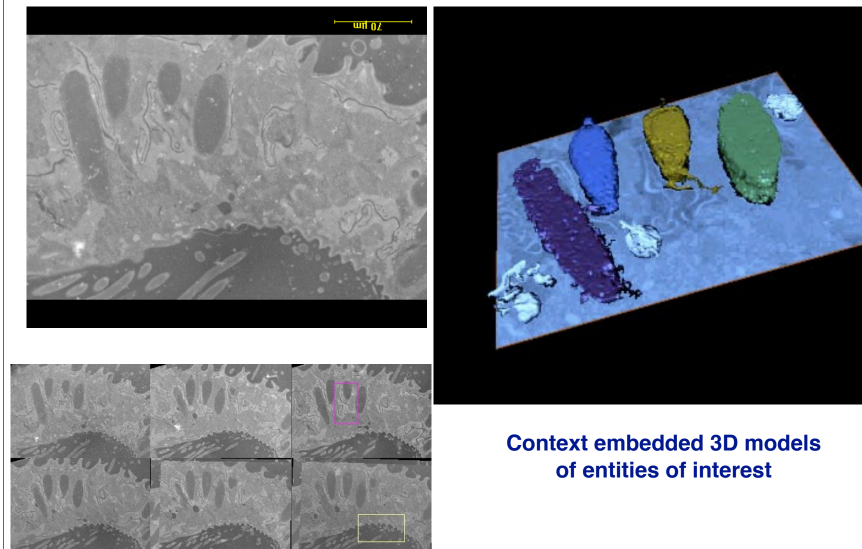


50-100nm sections...

From serial sections to 3-D model:



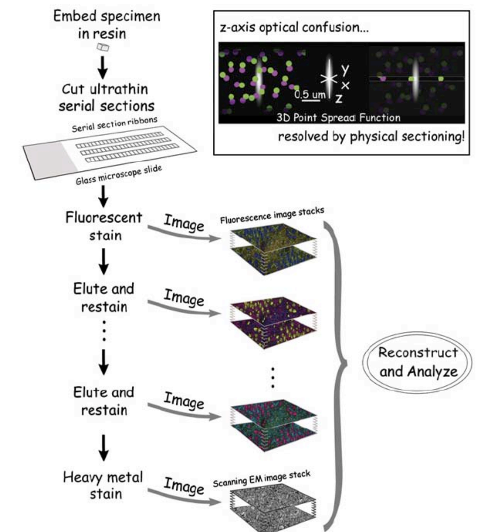
Paramecia 3-D reconstruction:



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



EMEZ

3D - Data from sections....

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

Lit: W. Denk & H. Horstmann, 2004, PLos Biol.2, "Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure"

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

ETH Eidgenössische Technische Hochschule Zürich Swiss Federal Institute of Technology Zurich — EMEZ Electron Microscopy ETH Zurich

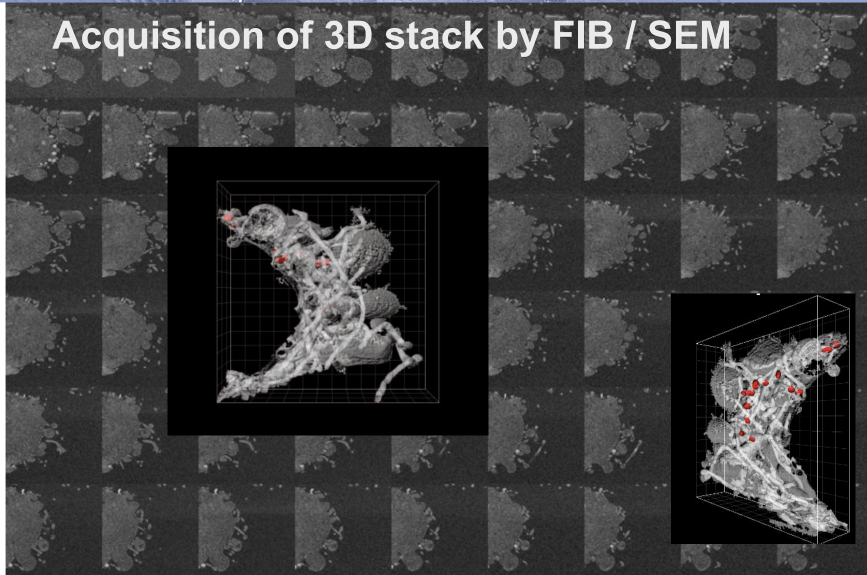
Acquisition of 3D image stacks with FIB-SEM

Ion-beam

e-beam

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)

miriam.lucas@emez.ethz.ch

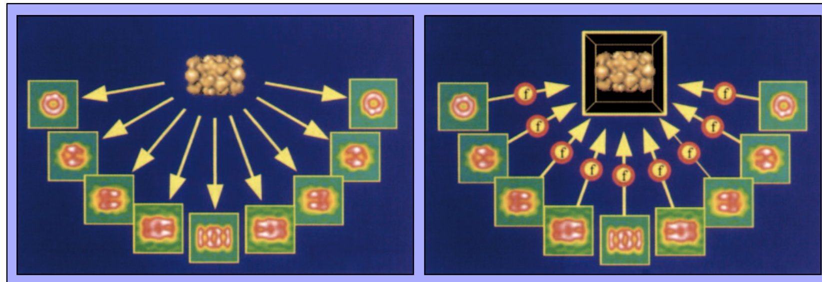


EMEZ

3D - Data from sections....

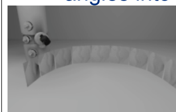
- *tomographic view of section volume...->TEM Tomo*
- *serial section TEM-tomography...*

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



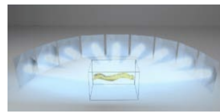
2D-projections
a 3D-object is projected at various tilt angles into a series of of 2D-images

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



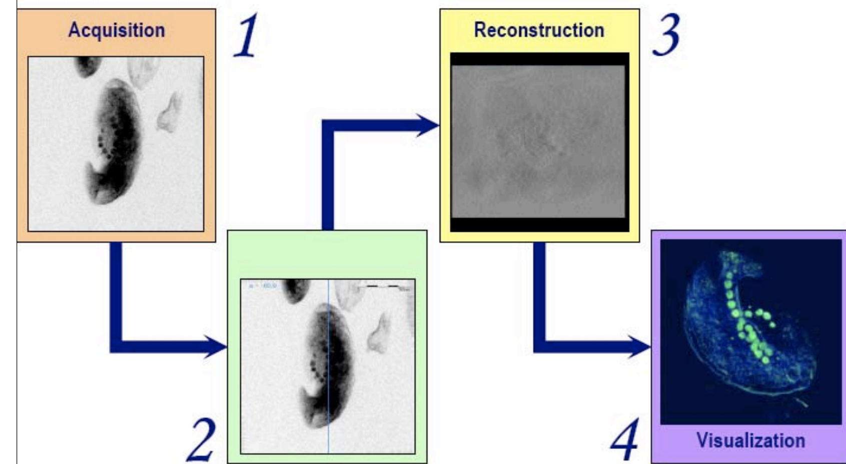
-> by TEM Imaging!

-> by Computer (in-silico)



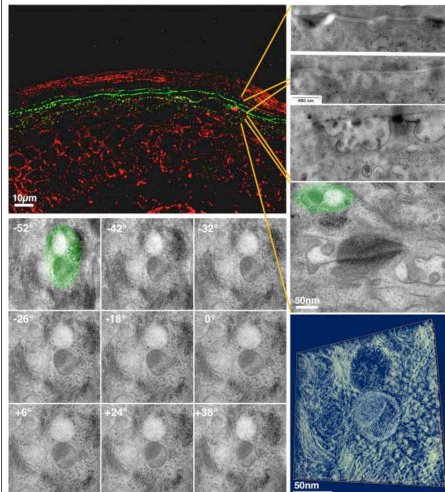
W. Baumeister, MPI Martinsried

Electron Tomography - Four Steps



Courtesy: Dr. Kobayashi, National Institute of Advanced Industrial Science and Technology, Osaka, Japan

TEM Tomography:
multilamellar bodies in the Stratum Granulosum..



80nm HM20 section from a sample freeze-substituted 1999

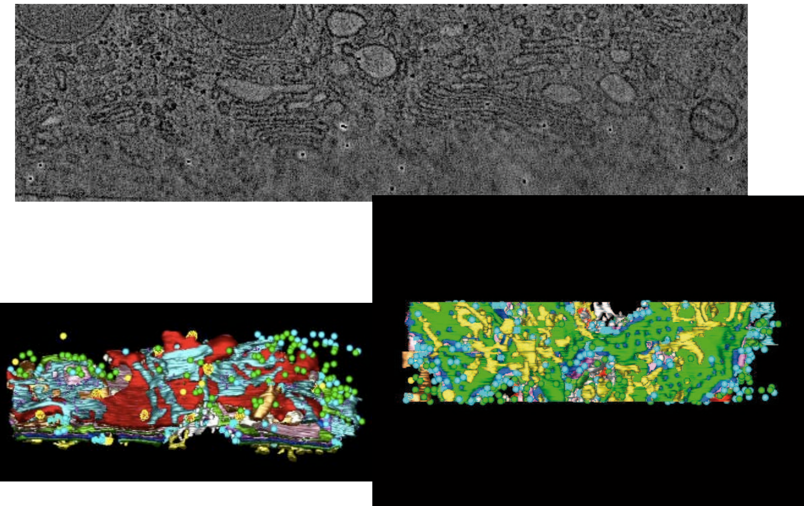
Resin embedded samples are a "Storage" device for "morphomic data"

-> "Data block" &
"Data slices"

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

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

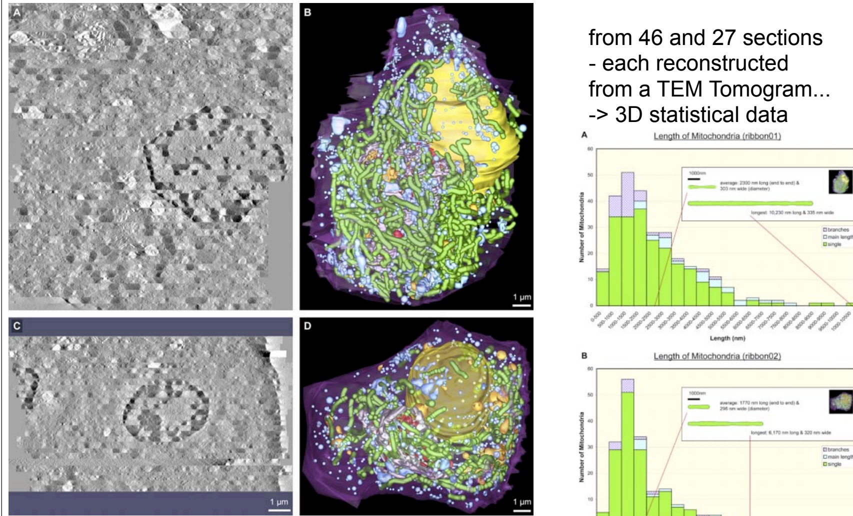
3-D reconstruction of Golgi-TGN from TEM Tomography:



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

Ladinsky et. al. (1999). Golgi structure in three dimensions: Functional insights from the NRK cell. *J. Cell Biol.*, **144**: 1135-1149.

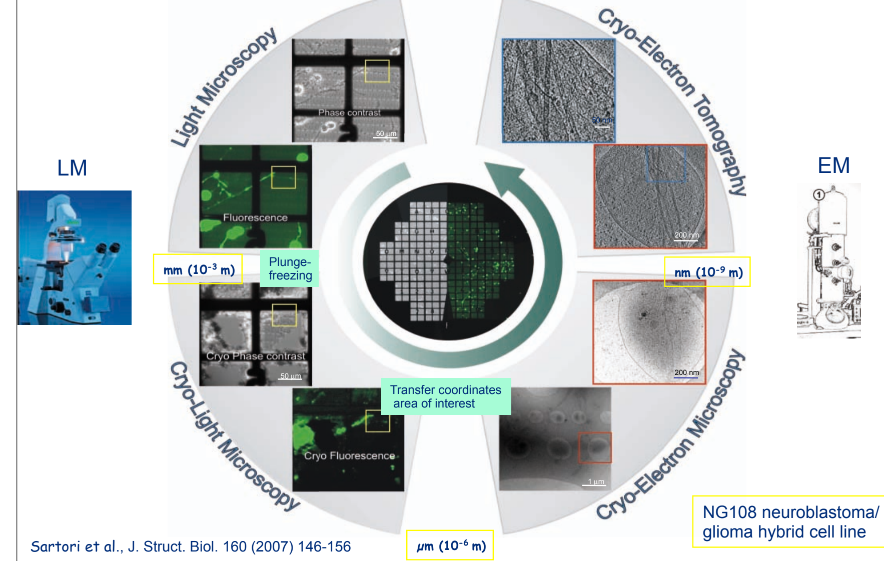
3-D reconstruction of whole cells (B.Marsh):



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

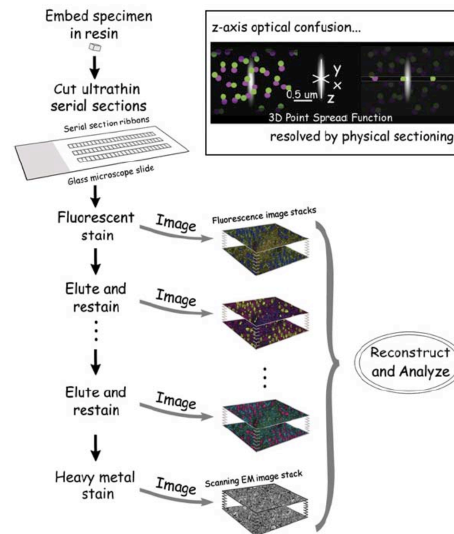
Correlating FM and cryo-ET: Full Correlation Cycle



Sartori et al., J. Struct. Biol. 160 (2007) 146-156

3D - Data from sections....

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



A good biological EM Lab needs:

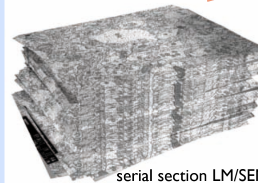
- a Fluorescence LM
- a (Cryo)-HR-SEM
- a Ultramicrotome

3D Electron Microscopy

The LM/SEM (FIB) world

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

Cryo-SEM



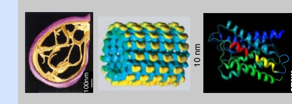
serial section LM/SEM

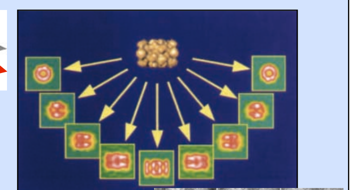
section projections or bloc-face view
=> Image Stack

"thin" section

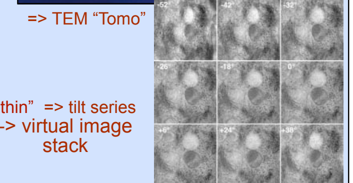
The LM/TEM world

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





=> 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

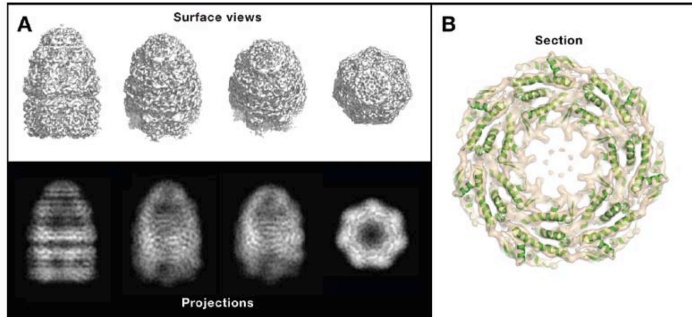


Figure 2. Surface Views, Density Projections, and Section of a 3D Cryo-EM Map of GroEL-GroES
(A) 3D surfaces and corresponding projections of the map density of the GroEL-GroES-ATP complex (Ranson et al., 2006).
(B) Section through the map showing the fit of α helices from the atomic structure of the equatorial domains. Figure reproduced from Ranson et al. (2006).

Some further reading on 3D EM data...

Reviews
Hawkes, P. & Valdre, U. (1990) Biophysical Electron Microscopy, Academic Press
Dubochet, J., Adrian, M., Chang, J.J., Homo, J.C., Lepault, J., McDowell, A.W. & Schultz, P. (1988). Quart. Rev. Biophys. 21, 129-228.
Henderson, R. (1995). The potential and limitations of neutrons, electrons and X-rays for atomic resolution microscopy of unstained biological molecules. Quart. Rev. Biophys. 28, 171-193.
Saibil, HR (2000) Macromolecular structure determination by cryo-electron microscopy. Acta Cryst. D 56, 1215-1222.
Chui, W, Baker, M, Almo, S (2006) Structural biology of cellular machines. Trends in Cell Biol 16, 144-150.
Frank, J (2006) Three dimensional electron microscopy of macromolecules. Oxford University Press.

Cellular tomography
McIntosh, R, Nicastro, D, Mastronarde, D (2005) New views of cells in 3D: an introduction to electron tomography. Trends in Cell BIol 215, 43-51.
Lucic, Forster & Baumeister (2005) Structural studies by electron tomography: from cells to molecules. Ann Rev Biochem 74, 833-865.

Hoffpauir, Pope and Spirou (2007); "Serial sectioning and electron microscopy of large tissue volumes for 3D analysis and reconstruction: a case study of the caly of Held, Nature Protocols; Vol.2 No.1

Some further reading on 3D EM data...

2

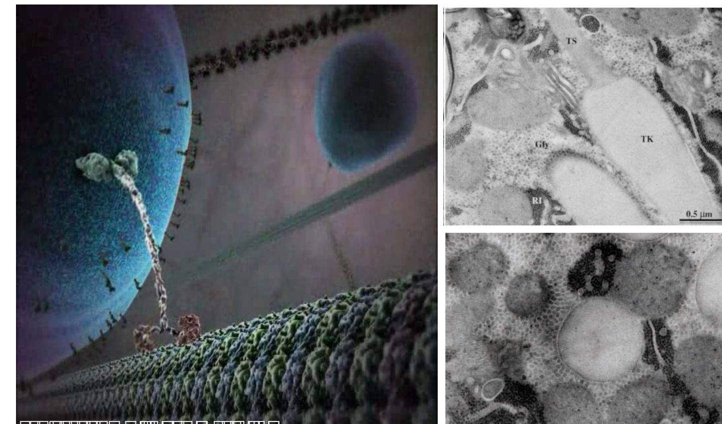
Single particles
Frank, J. (2002) single-particle imaging of macromolecules by cryo-electron microscopy. Annu. Rev. Biophys. Biomol. Struct. 31, 303–319.
van Heel, M., *et al* (2000) Single-particle electron cryo-microscopy: towards atomic resolution. Quart. Rev. Biophys. 33, 307–369.
Boettcher, B., Wynne, S. A., Crowther, R. A. (1997). Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. Nature 386, 88-91.
Lander et al (2006) The structure of an infectious P22 virion shows the signal for headful DNA packaging. Science 312, 1791-1795.

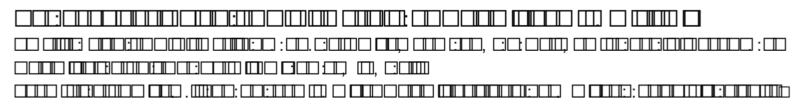
Helical reconstruction
DeRosier, D.J. and Klug, A. (1968) Reconstruction of 3-dimensional structures from electron micrographs. Nature 217, 130-134.
Yonekura, K Maki-Yonekura, S & Namba, K (2003) Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. Nature 424, 643-650.
Miyazawa, A, Fujiyoshi, Y & Unwin, N (2003) Structure and gating mechanism of acetylcholine receptor pore. Nature 423, 949-955.

Electron crystallography
Amos, L.A., Henderson, R., Unwin, P. N. T. (1982). Three-dimensional structure determination by electron microscopy of two-dimensional crystals. Progr. Biophys. Mol. Biol. 39, 183-231.
Henderson, R., *et al* (1990). Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. J. Mol. Biol. 213, 899-929.
Nogales, E, Wolf, SG. & Downing, KH (1998) Structure of the αβ tubulin dimer by electron crystallography. Nature 391, 199–203.

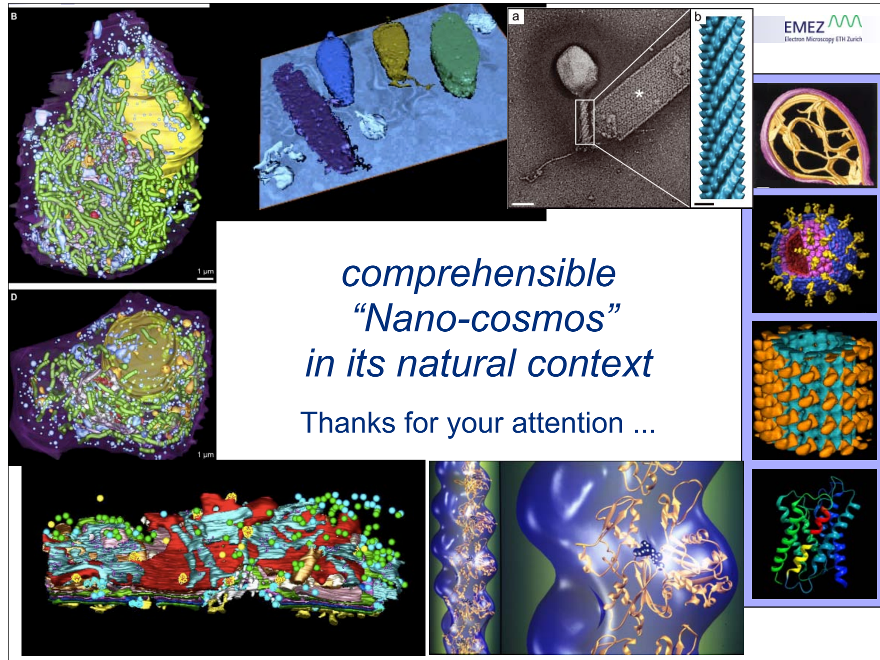
Golas, M. M., C. Boehm, B. Sander, K. Effenberger, M. Brecht, H. Stark and H. U. Goeringer: „Snapshots of the RNA editing machine in trypanosomes captured at different assembly stages in vivo. EMBO Journal 28, 766-778 (2009)

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





http://multimedia.mcb.harvard.edu/media.html


EMEZ
Electron Microscopy ETH Zurich
B
D
a
b
1 µm
1 µm
comprehensible
“Nano-cosmos”
in its natural context
Thanks for your attention ...