Scanning Transmission Electron Microscopy (STEM)

S. A. Müller & A. Engel 2009

Dimensions in Life Sciences

[Diagram showing the scale from 1 cm to 1 Å, with categories for plant cells, animal cells, bacteria, viruses, ribosomes, small molecules, proteins, and atoms, indicating the use of light microscopes, electron microscopes, and scanning probe microscopes.]
Electrons require vacuum

Types of pump:
• Roughing pumps.
• High vacuum pumps that need backing by a roughing pump.
  e.g., diffusion pumps
• High vacuum pumps that do not need backing.
  e.g., ion pumps

also
• Cryogenic pumps; used at high vacuum.
  e.g., ‘cold finger’ in the objective pole piece; reduces sample contamination during imaging.

Electron Sources

Heated filament
Field emission gun
Pressure < 10\(^{-10}\) mBar
Field Emission Tip

Scanning electron microscope image of a field emission tip formed from a single tungsten crystal.

Electrons are focused by magnetic fields

From http://www.matter.org.uk/tem/lenses/electromagnetic_lenses.htm
Electromagnetic Electon Lens (cont)

STEM

TEM

field emission gun
condenser lens
scan coils
diffraction aperture
objective lens
specimen
elastic dark-field detector

objective aperture
descan coils

bright-field aperture

inelastic dark-field & bright-field detector

electron gun
wound coil filament
condenser 1
condenser 2
aperture C1
aperture C2

specimen
objective aperture
selected area aperture
diffraction

intermediate

projector

viewing screen

optics

camera

4
Hardware

TEM

The Hardware: VG HB-5 STEM
Specimen Stage

Pressure $\sim 10^{-8}$ mBar

Pressure $< 10^{-10}$ mBar
The STEM Electronics Console

Specimen Preparation

e⁻

Microscopy Grid

Carbon-Film
Elastic scattering

The Coulomb force $F$ deflects an electron of charge $-e$ passing close to a nucleus of charge $+Ze$, where $Z$ is the atomic number and $\varepsilon_0$ the permitivity constant:

$$F = -\frac{e^2 Z}{4 \pi \varepsilon_0 r^2}$$

(1)

Using Eq 1, the probability for deflection into an angular interval can be calculated. The coulomb field is screened by bound electrons, i.e., depends on the chemical nature of the scattering atom.

Electrons that pass through an area $d\sigma$ are scattered through an angle $\theta$ into a solid angle $d\Omega$. $d\sigma_{\text{elastic}} / d\Omega$ is the differential cross-section and is dependent on the angle $\theta$. 

Classical particle model of electron scattering at a nucleus.
Elastic scattering

The differential cross-section $\frac{d\sigma}{d\Omega}$ can also be explained by the wave model.

$$\frac{d\sigma_{\text{elastic}}}{d\Omega} = |f(\theta)|^2$$

where $f(\theta)$ is the amplitude of the scattered wave.

This equation allows the total elastic scattering cross section to be calculated.

The scattering probability $p_{\text{scat}}$ of an area $A$ that is irradiated by $N_0$ electrons can be calculated by,

$$p_{\text{scat}} = \frac{N_{\text{scat}}}{N_0} = \sigma \frac{N_{\text{atom}}}{A}$$

where $\sigma$ is the total elastic scattering cross section of the atoms in the area $A$.

Elastic scattering amplitudes

$$\frac{d\sigma_{\text{elastic}}}{d\Omega} = |f(\theta)|^2$$

$$\sigma_{\text{elastic}} = 1.5 \times 10^{-4} \frac{Z^{3/2}}{\beta^2}$$

$$\beta^2 = \left(\frac{v}{c}\right)^2 = 1 - \left[\frac{m_e c^2}{(m_e c^2 + E)}\right]^2$$
Most inelastically scattered electrons carry no high resolution information and create beam damage: we have to live with this!

\[ \frac{\sigma_{\text{elastic}}}{\sigma_{\text{inelastic}}} \sim Z \]

\[ \sigma_{\text{inelastic}} = 1.5 \times 10^{-4} \, Z^{1/2} \ln(2/\theta_{\text{E}})/\beta^2 \]

\[ \theta_{\text{E}} = \Delta E / (\beta^2 (m_0 c^2 + E)) \]

\[ \frac{d\sigma}{dE} \]

0 20 E (V)

The most probable energy loss is 20 eV (> ionisation energy); the corresponding scattering angle is only a few mrad i.e., mostly forward scattering.

Inelastic scattering

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**Image comparison**

Image of gold islands on a carbon layer

Adapted from http://www.matter.org.uk/tem/stem_images.htm

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**STEM**

**optics:**

**incoherent imaging using a coherent point source**

Phase contrast coherent

Scattering contrast incoherent
Specimen preparation

Adsorb from solution to the carbon film of an EM grid.

Use a negative stain.

Use a positive stain
i.e., stain and then wash; chemically bound stain remains.
Application: E.g., visualization of DNA by dark-field TEM or STEM.

Leave unstained. Application: Mass measurement by STEM.

Cryo-TEM methods not considered.
STEM Dark-field images

Unstained

Negatively stained

Positively stained

50 nm

Carbon support & protein

Carbon, protein surrounded by negative stain (uranyl acetate)

Carbon, protein with bound stain (uranyl acetate)

Quantitative Biological Scanning Transmission Electron Microscopy

*from Protein...*  
*to picture...*  
*to Mass*
Mass measurements can only be made on unstained proteins free from buffer salts, and images are recorded with a low electron dose.

**Special requirements**

- **Grids that are inert to buffer salts** to be sure that the specimen is salt free i.e., gold coated copper or titanium grids.

- **Clean, thin, non-contaminating carbon support films** to obtain maximum contrast on the images i.e., thin carbon films prepared in an oil-free vacuum.

**Note that:**

- Negative or positive stains **cannot** be used.

- All buffer salts must be removed by **washing the grid after protein adsorption either with pure water (quartz double-distilled) or with a volatile buffer e.g., ammonium acetate or ammonium bicarbonate.**

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**Specimen Preparation**

Fenestrated carbon foil
Thin (3nm thick) carbon film

Sample adsorbed from solution, grid washed, ± negative stain
**Freeze-dried if unstained**
Specimen prep. : Freeze-drying

Cool the cartridges
Blot and plunge-freeze the sample grid
Replace the cartridge cap
Transfer the cartridges
Cooled block with cartridges in position

TEM & STEM: Calibrate the magnification

i.e., determine the size of the image & in particular for STEM, the size of the irradiated picture elements (pixels) of the scan.
Quantitative STEM 1

Beam current

Single crystal tungsten
Extraction voltage: 0.5 to 4.5 kV
Accelerating voltage: up to 100 kV

Quantitative STEM 2

Collection Efficiency

Aluminum-coated, plastic scintillator
(Nuclear Enterprises, NE 160)
Collection angle 13 - 100 mrad
Aluminum-coated glass tube
→ photocathode
How to relate counted electrons to sample mass

The mass is calculated from the average scattering cross section $<\sigma_e>$, average atomic weight $<m>$, collection efficiency $\varepsilon$, counted electrons $N_e$ and dose $D$.

$$N_e = N_0 \cdot n \cdot \varepsilon \cdot \sigma_e / A$$  \hspace{1cm} (1)

or

$$N_e = n \cdot \varepsilon \cdot \sigma_e \cdot D$$  \hspace{1cm} (2)

where $N_0$ is the number of incident electrons and $D$ is the incident electron dose, $N_0/A$.

From (2) we calculate the number $n$ of atoms within $A$. Multiplying $n$ by the average atomic mass $<m>$ yields

$$M = N_e \cdot <m> / (\varepsilon \cdot \sigma_e \cdot D)$$  \hspace{1cm} (3)
Efficiency of the dark-field detector ‘on the day’

Counting efficiency

Beam current $< 2 \times 10^{-12}$ A
Etching and Contamination in STEM

Central region multiply scanned at high magnification, image recorded at lower magnification.

Species from the vacuum and the grid, are drawn into the beam, break down under it, deposit on the irradiated area and cross-link to form polymers.

http://em-outreach.ucsd.edu/web-course/Sec-II.B.1-B.9/Sec-II.B.1-B.9.html#Effects1

Mass loss

Tobacco mosaic virus sample
What makes STEM mass analysis special?

The image

STEM is not a bulk technique. Measurements possible:

- **Total mass of protein complexes**
  The individual complexes are measured
  Analysis of heterogeneous samples possible
  Huge mass range: 100 kDa to > 100 MDa

- **Mass-per-length of individual filaments**

- **Mass-per-area of sheets**

- **Mass maps**
  Mass can be linked to shape and appearance
The importance of an image

Example: Supraspliceosome complexes

Questions:
What is the total mass of the complexes?
Is this mass compatible with the presence of 4 spliceosomes?

Heterogeneous sample.
The brighter the particle the heavier it is.


Spliceosome Complexes: Mass analysis

The mass histograms can only be interpreted by inspecting the particles.
Supraspliceosome complexes

Negative stain

Positive stain

Contrast reversed to mimic a TEM bright-field image


Aquaporin 1

Aquaporin 1 tetramers

Walz et al. (1994) EMBO J. 13, 2985-2993

2D crystals of aquaporin 1

From the mass per-area:
- Primarily single layers.
- Two aquaporin 1 tetramers per unit cell.

Walz et al. (1994) J. Biol. Chem. 269, 1583-1586
Actin filaments and myosin thick filaments

From Molecular Biology of the Cell

Actin

Au$_{11}$ cluster 0.8 nm in diameter
3D reconstructions from STEM dark-field images

Supports the Holmes-Lorenze atomic model of the actin filament.

Mass maps 1: Myosin thick filaments

Scale bar:
400nm on images
200nm on profiles

a) Intact TF
b) TF core remaining after treatment with 0.4M NaCl
c) Partially dissociated untreated TF

Mass variation along myosin TFs and TF cores

Conclusion: Cores are formed from 7 sub-filaments each containing 4 strands of paramyosin rather than the 2 originally proposed.


The nuclear pore complex

Mass Maps 2: The nuclear pore complex

Intact pores embedded in the nuclear envelope

NPC has a mass of 120 MDa

Heavy ring (cytoplasmic)

Light ring (nucleoplasmic)

After detergent treatment

Dark-field STEM: Negatively stained samples

Image contrast inverted to show protein in bright shades

Philippe Ringler
Molecular transfer across membranes

Shigella

Yersinia spp.

Three translocators: YopB, YopD, LcrV

Host cell

LcrV?

Link between Needle and Pore?

YopB/YopD

YopB/YopD

Host cell

Link between Needle and Pore?

OM

IM

YscF

Yersinia

Cordes et al. 2003

Blocker et al. 2001

Guy Cornelis

Anti-LcrV antibodies specifically label the tip complex

Mueller et al. Science 2005
What you should know

- Elastic electrons: high-resolution information
- Inelastic electrons: lower resolution, beam damage
- STEM Dark Field: no phase, only scattering contrast: ideal for imaging single molecules & for mass determination
- By measuring probe current, magnification & scattered electrons, mass mapping of biological samples is feasible
- How the mass of a complex is calculated
- Why each electron is counted by the dark field detector
- The sensitivity is such that any dirt (detergent, small solutes, protein fragments) must be eliminated

Mass maps 3: The surface layer protein tetrabrachion

The bacterial toxin ClyA

Complexes formed in the presence of detergent. **Stoichiometry?**

- ClyA monomer: 34.45 kDa
- Single complex: 475 ± 25 kDa
- Double complex: 947± 49 kDa

Indicates 13 monomers and a small amount of detergent.

N.Eifler et al. (2006) EMBO J.