THE BOTTOM LINE

This document is designed to help students focus their attention on basic concepts that are important for understanding the fundamental principles of transmission electron microscopy, biological specimen preparation, and image processing and three-dimensional reconstruction. Once these concepts are understood, it will be much easier to gain a deeper and lasting comprehension of relevant and related topics.

LECTURE 1 (Jan 8, 2013)

§I. THE MICROSCOPE

Arrangement and function of components in LMs and TEMs are similar
Photons and electrons behave as particles and waves
According to de Broglie: a wavelength is associated with any moving particle
In TEM, electrons travel *very* fast and have *very* short wavelengths
Diffraction refers to the bending of the path of radiation caused by 'obstacles'
Diffraction of light and electrons illustrates their wave nature
Interference occurs when diffracted and undiffracted waves combine
Ideal lens: images each object point as a point in the image plane
Real lens: images each object point as an Airy disk in the image plane
Size of the Airy disk is inversely proportional to the lens aperture (opening)

LECTURE 2 (Jan 10, 2013)

Coherence: defines variance in λ and phase of component waves
Resolution: ability to distinguish objects or object details
Resolution limit: Smallest separation of points that can be recognized as distinct
Instrument resolving power: Resolution achieved by a particular instrument under optimum viewing conditions; limited by wavelength of radiation; at best = 1/2 λ according to Abbe's simple criterion; more realistic estimate given by Rayleigh Criterion (using Abbe Equation), which takes into account the lens numerical aperture in addition to the wavelength of the radiation used to form images
Image resolution: always $\leq$ resolving power of instrument
Maximum useful magnification of instrument is limited by λ of radiation used to form the images
Electrons: can resolve finer object details than photons
Classical (Photon) verses Electron Optics:
Refractive index changes abruptly at glass surfaces (classical optics)
Refractive index changes gradually in electric or magnetic fields (electron optics)
Photons travel in broken straight lines; electrons follow curved trajectories
Basic Laws of Classical Geometrical Optics:
1: Rectilinear propagation of light
2: Law of reflection
3. Law of refraction (Snell's Law)
4. Independence of rays
All of these hold for electrons with exception of #4 (but see p.17 of lecture notes)

LECTURE 2 (Jan 10, 2013) Continued

Geometrical (Ideal) verses Physical (Real) Optics:

Geometrical: Specifies **ray paths** through lenses and apertures

Physical: Accounts for diffraction and interference effects

Construction of ray diagrams:

1: All rays entering a converging lens **parallel to the lens axis** are brought to a common point on the axis, the **focal point**

2: All rays passing through the geometrical center of a lens pass straight on

3: Principle of reversibility: if the direction of a ray is reversed in any lens system, the ray exactly retraces its path through the system

Real and Virtual Images

In a real image, rays physically reunite at the image plane where a photographic plate can be exposed

In a virtual image, rays diverge and are not physically reunited at the image plane so a photographic plate cannot be exposed

THIN LENS EQUATION

$$1/f = (1/o) + (1/i)$$

f = focal length of lens
o = distance of object in front of lens
i = distance of object behind lens

MAGNIFICATION

$$M = \left| i/o \right|$$

The **lens aperture** determines the amount of radiation arriving from the object that can be focused to form an image; the larger it can be made, the more information the lens can gather and transmit into the image

High magnification imaging generally requires three or more lenses; the total magnification is the product of all separate magnifications; image formed by each lens becomes the object for the subsequent lens, whether or not a real, intermediate image is formed

Thermionic emission creates a source of electrons

LECTURE 3 (Jan 15, 2013)

A charged object produces an electric field

The path of an electron passing through an electric or magnetic field is bent or refracted in a series of gradual steps at the equipotential surfaces. The net result is fundamentally the same as given by Snell's Law of refraction (light optics). Curved equipotential surfaces exhibit the properties of a lens.

Focal length of an electromagnetic lens is determined by field strength and electron speed.

$$f = \frac{KV_r}{(N \cdot I)^2}$$

LECTURE 3 (Jan 15, 2013) Continued

Focusing an image in the TEM is achieved by varying current by small amounts in the **OBJECTIVE** lens

Wehnelt shield (gun cap) controls beam shape and emission in the electron gun.

Gun-crossover considered the actual **source of e^-** for the TEM

Condenser lens system **focuses / concentrates** the electron beam onto the specimen to give optimal **illumination** for viewing and recording the image

Double condenser lens system has several advantages, including: more flexible control of illumination, wider range of intensities, reduces area of object irradiated and specimen contamination, improves image contrast, and increases filament life.

Double condenser lens system uses two apertures: C1A – large, fixed; C2A – small, adjustable.

Electromagnetic lenses are 'crummy': they suffer from spherical and chromatic aberration and lens asymmetry along with many other problems.

Spherical aberration is the principal factor that limits TEM resolving power; does so more than diffraction effects or chromatic aberration.

Chromatic aberration occurs because lens focal length varies with electron/phonton wavelength; images that include CA are the combination (superposition) of a series of images (with different focal planes, rotations, and magnifications).

Lens asymmetry occurs for many reasons, but primarily because no real lens can be manufactured that is perfectly axially symmetric. Result: focal length varies with direction. Condenser and objective lens astigmatism are controlled (i.e minimized) by the microscopist.

Objective lens is the most critical lens in the TEM. It performs the first stage of imaging and determines instrument resolving power and contrast. Specimen, aperture, anticontaminator, and stigmators lie inside or close to the lens field.

Objective aperture sits at the back focal plane of the objective lens and intercepts electrons scattered by the specimen through large angles (giving rise to scattering contrast).

LECTURE 4 (Jan 17, 2013)

Depth of focus (D_i): distance along the optical axis where the **IMAGE** appears essentially the same.

Depth of field (D_o): distance along the optical axis over which the **OBJECT** could be moved and still give essentially the same image at a fixed plane.

Consequence of large relative depth of field: TEM images are (to a first approximation) **PROJECTIONS** of the entire contents of a specimen.

Projection images are ***NOT*** shadow-graphs (no transmission through the object) but are similar to X-ray photos.

Electron image typically recorded on a CCD camera or a photographic emulsion

Photographic recording must be done at a magnification sufficient to capture resolution in the **electron** image

Resolution of CCD or photographic emulsion is superior to the fluorescent screen

Microscope vacuum: needed primarily (1) so electron beam can travel through microscope without interacting with anything but the actual specimen and (2) so specimen contamination does not become a problem.

LECTURE 4 (Jan 17, 2013) Continued

Anticontaminator: a cooled surface that, when placed close to the specimen, traps residual gases in the column and prevents them from interacting unfavorably with the specimen. Most anticontaminators are cooled with **liquid N_2**.

Electrical System: Current supplies for each lens must be very stable, otherwise the recorded image will be blurred. The same is true for the accelerating voltage.

Specimens (made of atoms) are mostly empty space. The imaging electron beam in a TEM interacts with (*i.e.* scatters from) specimen atoms.

Image contrast in TEM arises from both **electron scattering** (particle nature) **AND interference** (wave nature) and depends on both the specimen (**inherent contrast**) and the microscope (**instrumental contrast**).

Resolution in TEM images of biological specimens is normally **limited by contrast NOT by lack of resolving power** (Recall: resolving power is limited by the optics).

Contrast: relative difference in intensity between an image point and its surroundings

Amount of electron scattering from a finite region of a specimen depends on specimen density AND overall thickness in the direction of the beam.

Mass thickness = mass X thickness. Scattering probability increases as mass thickness increases.

Biological specimens have **low inherent contrast** (mainly composed of light atoms: C, N, O, H), which is why weak contrast is a limiting problem in imaging biological specimens (or any *very* thin specimen). To increase inherent specimen contrast, materials of **high atomic number** are added (forms the basis of many biological specimen preparation procedures).

Paths of electrons in beam are affected primarily by electrostatic interactions with specimen atoms (the atomic nuclei and the atomic electron "cloud")

Primary types of electron/specimen scatter: elastic (no energy loss), inelastic (some energy loss, which causes specimen damage), and none (no energy loss).

Elastic scattering: beam electron changes its trajectory when passing close to a specimen atom **nucleus**

Inelastic scattering: beam electron changes its trajectory when passing close to a specimen atom **'stationary' electron**

Multiple scattering occurs in thick specimens and can be a serious source of chromatic aberration and loss of resolution in TEM images

Scattering (amplitude / aperture) contrast verses interference (phase) contrast:

Aperture contrast: arises from loss of electrons from imaging beam (particle nature) when highly scattered electrons fall outside the objective lens or are stopped by the objective aperture. Primarily affected by mass thickness of specimen (can be controlled to some extent by preparation procedures). Dominant source of contrast for thick or stained specimens.

Interference contrast: images with Fresnel fringes occur when diffracted electron waves constructively and destructively combine at the image plane. Affected by **objective lens defocus** (under operator control) and **spherical aberration** (not controllable by operator). Dominant source of contrast for thin or unstained specimens. Essential for **high resolution** work.

LECTURE 5 (Jan 22, 2013)

Contrast transfer function (CTF): describes relationship between image and specimen

CTF is characteristic of or influenced by (1) particular microscope used, (2) conditions of imaging, and (3) type of specimen.

Microscope CTF arises from the objective lens focal setting AND from the spherical aberration present in all electromagnetic lenses. It affects the way information about the specimen is stored in the electron image. Mostly controlled by the microscopist's choice of defocus and voltage (electron wavelength); also influenced by the spherical aberration of the objective lens, but this is a constant for a given microscope, and hence is fixed. The series of images of Mickey Mouse, simulated as if they were formed in a TEM, illustrate how details in the specimen get distorted by the imaging system of the instrument. The simulation still falls short of depicting a 'real' situation with a more typical TEM specimen given that "Mickey Mouse" is a simple, binary object, consisting of only two density levels (pure white or pure black) and one that is noise free (we will define and describe "noise" later).

TEM alignment: affects resolving power and convenience of operation

Goal of alignment: make optical elements of TEM coaxial

Principle for aligning any electromagnetic lens based on image rotations caused by fluctuating the current (or voltage) in lenses

Small changes in objective lens current used to focus electron image

True / near / exact / dead focus: where phase contrast in the electron mage is minimal. Microscopist needs to learn how to identify this focus setting. Nonetheless, slight **under**focusing gives optimum results with most biological specimens (enhances interference contrast without seriously affecting image resolution)

Disturbances to Microscope Performance

Contamination: causes astigmatism, drift, and decreased contrast

Image drift and mechanical instabilities: caused by instabilities in specimen holder, stage assembly, and specimen

Electrical and magnetic instabilities: TEM needs shielding

Image astigmatism: caused by asymmetrical field in objective lens; use stigmators to minimize.

Focal drift: caused by micro-discharges in gun

Operation of the TEM

Accelerating voltage: usually best to increase (reduces specimen damage, improves depth of field and diffraction limited resolution, etc. etc.) up to a certain point, because higher voltage leads to lower amplitude/aperture contrast

LECTURE 6 (Jan 24, 2013)

Operation of the TEM (Cont'd)

Apertures: Smaller is better……up to a point

Condenser Aperture: Small apertures are best for high resolution imaging but, as aperture size is reduced, fewer electrons are available to illuminate the specimen

Objective Aperture: Small apertures improve **scattering** contrast and reduce spherical and chromatic aberrations, but, as size is reduced, diffraction limited resolution becomes poorer and apertures are harder to align, keep aligned, and more sensitive to effects of contamination

LECTURE 6 (Jan 24, 2013) Continued

Operation of the TEM (Cont'd)

Magnification: depends on the nature of the specimen and experiment; trade offs are field of view, potential resolution, and radiation damage; need to consider the resolution of the recording medium

Focusing: set by making **small** changes in **objective** lens current; slight **underfocusing** gives optimum results; use wobbler to focus at low mags and minimum contrast method (minimize support film phase contrast) at high mags

Magnification Calibration: **nominal** magnification settings in TEM can't be trusted; record images of **calibration standards**

Resolution Tests: Record micrographs of suitable test specimens to check microscope performance; measure actual resolution achieved in the **recorded** image

Image Intensifier / TV Displays: convenient way to view, focus, and stigmate images at very low electron fluxes

Microscope maintenance: pay the big bucks for a service contract

Photography: analog (film) vs. digital (CCD or DDD); goal is to obtain a complete, faithful, and permanent record of details contained in the electron image

Recording Images Photographically (on film):

Photographic emulsions respond differently to electrons (**single-hit**) and photons (**multiple-hit**)

Optical Density: quantitative measure of blackening of the photographic emulsion

$$OD = \log_{10} \frac{I_0}{T}$$

Exposure of a photographic emulsion to electrons is a **single-hit** process; virtually **every** halide crystal hit by an e^- is rendered developable; subsequent hits of same crystal by other electrons **irrelevant**

Each e^- passes through **several** halide grains on its way through a typical, 20μm thick photographic emulsion, losing some energy in each

Photographic graininess: a statistical phenomenon caused by **"electron noise"; NOT** a defect in the emulsion

Graininess is caused by 1) the random arrival of e^- quanta at the recording device and 2) granularity in the emulsion (distribution of silver halide crystals)

Electron noise: distribution of e^- "particles" in the beam at any moment is random

Reducing graininess (increasing S/N) requires increased exposure. This can be accomplished by using: 1) **more electrons**, 2) **image processing** to average many images together, or 3) chemical **development strategies**. First is OK for most routine TEM but not for radiation sensitive samples. Second is very powerful way to increase S/N in images. Third is more a darkroom 'trick' and not typically used.

LECTURE 6 (Jan 24, 2013) Continued

Operation of the TEM (Cont'd)

Photography: analog (film) vs. digital (CCD or DDD) **(Cont'd)**

Recording Images Photographically (on film): (Cont'd)

Resolution of emulsion is limited: electron track through emulsion includes sideways scatter (electron diffusion), which blurs image details and reduces contrast; **Resolution** in the FINAL (*i.e.* recorded) image is always POORER THAN the resolution achieved in the electron image; use magnification high enough to make sure details in the electron image are captured by the emulsion, but not too high or excessive radiation damage can occur and field of view will be restricted.

Recording Images Digitally (on CCD):

CCD cameras have replaced film as the principle recording media in most TEMs

DDDs will eventually replace film for high resolution work and may even replace CCDs at some point

Advantages of CCD: Immediate image access; large dynamic range; strict linear response with electron dose; amenable to numerous automated microscopy tasks; pixel binning operations are straightforward and quite useful for specimens searches, focusing, astigmatism corrections, etc.; can easily and rapidly manipulate the contrast range of a digital image

Disadvantages of CCD: Poorer pixel resolution than film (15 μm vs. ~ 5-10 μm for film); Limited number of pixels (e.g. 4k by 4k vs. ~16k by 20k for film), hence **small field of view**; high upfront cost

Basic CCD designs: Lens-coupled and fiber-optic coupled. In the first, the CCD array sits outside the microscope vacuum; in the second, it sits inside the vacuum.

LECTURE 7 (Jan 29, 2013)

Recording Images Digitally (on CCD) (Cont'd):

Nyquist Criterion: finest detail (highest spatial frequency) we can capture in a digital image is TWICE the size of one pixel; necessary to sample (digitize) the image at a step size AT LEAST two times finer than the desired or expected resolution

Recording Images Digitally (on DDD):

Based on CMOS technology (**C**omplementary **M**etal **O**xide **S**emi-conductor), which has widespread use in numerous imaging applications.

Advantages of DDD: Detects electrons directly as opposed to indirectly in a CCD; **i**mmediate image access; large dynamic range; strict linear response with electron dose; amenable to numerous automated microscopy tasks; pixel resolution comparable to film (5-6 μm vs. ~ 5-10 μm for film); much faster readout than CCDs (40 frames per second in some commercially available DDDs versus 1-4 sec for available CCDs).

Disadvantages of DDD: Limited number of pixels (e.g. 4k by 4k vs. ~16k by 20k for film), hence **small field of view**; high upfront cost

LECTURE 7 (Jan 29, 2013) Continued

Recording Images Digitally (on DDD): (Cont'd)

Basic DDD designs: Two basic designs: HPD (**H**ybrid **P**ixel **D**etector) and MAPS (**M**onolithic **A**ctive **P**ixel **S**ensor). HPDs are better suited for detecting X-rays and MAPS for detecting electrons. Both produce large signals and are capable of detecting single electron events. MAPS, with smaller pixel sizes (range from 5 to 14 μm in current commercial detectors compared to 55 μm for one HPD) and larger size arrays (~4k by 4k verses 256 by 256 for the HPD) are better suited for TEM imaging experiments compared to HPDs. HPDs employ two separate layers of silicon in each pixel to detect and readout the electron events whereas each pixel in a MAPS detector has the detector and readout electronics built into one layer.

Other Modes of TEM Operation

Electron Diffraction: study crystalline specimens (especially metals); patterns consist of series of rings (random oriented samples) or discrete lattice of sharp spots (single crystals)

Dark Field EM: images formed only from scattered electrons; much higher contrast than bright field images; intensity very low (longer exposure time / more radiation damage); difficult to focus and correct for astigmatism (no interference contrast)

High Resolution, High Voltage Microscopy TEM: Highest voltage microscopes designed to deliver highest resolution (short wavelength electron beam) and to study thick specimens (some biological sections as thick as a few microns).

Tilt and Stereo TEM: Tilt needed to see 'hidden' aspects of specimen (e.g. bilayer membranes); stereo mimics our human binocular vision and gives perception of depth but doesn't reveal full 3D structure of specimen; stereo glasses or ability to diverge or cross one's eyes needed to see a stereo pair correctly.

Low Temperature TEM: Described in detail in §II.A.

Electron Energy Loss Spectroscopy (EELS): Separates out different wavelength (i.e. different energy) inelastically scattered electrons to form a spectrum and images showing presence and location of specific kinds of atoms in the specimen.

X-ray Microanalysis: Scans very small electron probe back and forth across the specimen and measures the wavelengths of the emitted X-rays from the specimen as a function of location; Energy transmitted to specimen atoms by inelastic scattering events cause some specimen electrons to jump to higher energy orbitals, and, when they decay back to their ground state, they emit some of the lost energy in the form of X-rays whose wavelengths are specific to the transition.

§II. THE SPECIMEN

Biological Specimen Preparation Techniques

Goal: obtain TEM images that faithfully represent the specimen in its native state

Obstacles for any prep method: contrast, thickness, dehydration, radiation damage

Grids/Support Films: 3mm copper grids; need surface on which to deposit samples; most common support films are carbon and carbon-stabilized plastic; ideal qualities include: good conductor; adequate physical strength to withstand handling and vacuum conditions; low electron scattering so as not to reduce specimen contrast; be amorphous (structureless)

LECTURE 7 (Jan 29, 2013) Continued

Biological Specimen Preparation Techniques (Cont'd)

Thin-Section TEM: Mostly used with tissue samples; sectioning needed to get specimen thin enough for TEM. Procedure involves four major steps: fixation, dehydration and embedding, sectioning, and staining.

Fixation: Goal is to stabilize "normal" ultrastructure of specimen via chemical or physical preservation; glutaraldehyde is the primary fixative; osmium tetroxide is also often used. Fixation affected by: pH, buffer type, osmolarity, fixative concentration, temperature and time of fixation, specimen size (< $1mm^3$ best)

Dehdration/Embedding: Goal of dehydration is to remove H_2O to allow non-water-soluble embedding medium to infiltrate specimen. Goal of embedding is to infiltrate the tissue with a liquid polymer (e.g. epoxy resin) that is hardened after infiltration is complete. Once polymer is hardened, the specimen can be cut into thin sections

Microtomy: Goal is to cut sections that are *generally* 50 to 70 nm thick.

Staining: Goal is to add mass thickness to otherwise 'invisible' sectioned material. Classic protocol uses uranyl acetate followed by lead citrate

Sectioning artifacts: Main ones include chatter, knife marks, and section compression, all of which are relatively distinct (but require different approaches to eliminate)

Negative Stain TEM: Mostly used with particulate samples (macromolecules and macromolecular complexes); quick and easy; increases mass thickness and gives excellent aperture contrast; yields good resolution (15-25 Å); specimen preservation is OK (better than sectioning but worse than cryoEM).

Metal Shadow TEM: Used with particulate samples, replicas, and freeze-fractured/etched cells to view surface features.

Freeze Drying/Etching/Fracture TEM: Mostly used with cells to view membranes and particle distributions in membranes. Preserves specimen much better than air-drying.

LECTURE 8 (Jan 31, 2013)

Biological Specimen Preparation Techniques (Cont'd)

Unstained and Frozen-Hydrated: Used to examine many kinds of macromolecules and macromolecular complexes, including asymmetric objects (e.g. ribosome), objects with rotational (e.g. icosahedral viruses) or helical symmetry (e.g. acto-myosin filaments), and 2D crystals (e.g. purple membrane); Vitrification provides excellent preservation of native specimen structure (eliminates need for chemical fixation and staining); Preparing samples manually or by robot is much more involved than negative staining; must use low dose ('shoot in the dark') methods to record images while minimizing beam damage to specimen; Predominant source of image contrast arises from defocusing the objective lens (very little aperture/scattering contrast since there is no heavy metal stain and changes in mass thickness vary only slightly throughout the specimen)

Radiation Effects

Radiation damage limits usefulness of TEM data regardless of initial quality of specimen

It rather than instrumental resolving power is MAIN limiting factor in obtaining high resolution images of biological molecules.

Most biological specimens tolerate an exposure of no more than ~1 $e^-/Å^2$ at RT.

LECTURE 8 (Jan 31, 2013) Continued

Damage is proportional to TOTAL DOSE (= dose rate X exposure time), measured as electron flux and reported in units of in units of coulombs/cm^2 or e^-/nm^2 or $e^-/Å^2$ ($1e^-$ = 1.6 x 10^{-19} coulomb)

Primary interactions between the electron beam and the specimen are excitation (raising of electron to higher energy orbital), ionization (formation of ions or radicals from loss of electrons), and displacement (knock-off of atoms); All are temperature independent and occur on the order of ~ 10^{-14} sec (= 10 femtoseconds)

Electron irradiation results in several secondary effects: Chemical and physical changes, mass loss and cross-linking, production of heat, charge effects, contamination and etching, and crystal structure damage.

Criteria used to measure radiation damage include total mass loss, loss of specific elements, loss of crystalline structure, and changes in the infrared, visible, or ultraviolet spectra.

Critical Dose: Dose at which the intensity of a given peak in an electron diffraction pattern falls to 1/exp (*i.e.* 37%) of its original value at zero dose; Critical dose for unstained protein crystals at room T irradiated with 100 KeV electrons is ~1 $e^-/Å^2$.

Procedures to reduce radiation damage: Most important include (1) reducing the number of electrons ("Low Dose") used to form images, (2) reducing specimen temperature, and (3) increasing the accelerating voltage of the imaging beam; Other methods include reducing contamination and etching, stabilizing specimens and support films with a thin layer of evaporated carbon, and, in the case of thin-sections, pre-irradiating the sections.

Relation between Contrast, Resolution and Radiation Damage: As exposure is reduced, statistical fluctuations from one picture element to another can be much greater than the inherent change in density in neighboring portions of the object; Low dose images exhibit a ***REALLY*** poor Signal-to-Noise (S/N) ratio; **Image averaging** needed to achieve high resolution.

Radiation Effects in Negatively-Stained Specimens: Stain condenses and crystallizes under influence of beam and may not contrast the specimen features as well as before irradiation starts; Minimal does procedures (<$20e^-/Å^2$) are used to preserve more detail in stained specimens (like grooves on outside of TMV).

Radiation Effects in Frozen-Hydrated Specimens: Vitrified specimens tolerate only 10-$20e^-/Å^2$ dose before fine features are lost in the images; At slightly higher doses (~30-$40e^-/Å^2$) bubbles appear in regions where there is carbon support film, signifying that too many electrons have been used.