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 three-dimensional image processing and 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 5, 2010)
§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 7, 2010)

Coherence: defines variance in $\lambda$ and phase of component waves
Resolution: ability to distinguish objects or object details
Instrument resolving power: limited by wavelength of radiation; at best $= 1/2 \lambda$
Image resolution: always $\leq$ resolving power of instrument
Maximum useful magnification of instrument is limited by $\lambda$
Electrons: can resolve finer object details than can 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

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
LECTURE 2 (Jan 7, 2010) Continued

THIN LENS EQUATION

\[ \frac{1}{f} = \left( \frac{1}{o} \right) + \left( \frac{1}{i} \right) \]

MAGNIFICATION

\[ M = \left| \frac{i}{o} \right| \]

LECTURE 3 (Jan 12, 2010)

The **lens aperture** determines the amount of radiation arriving from 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.

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.

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

**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.

LECTURE 4 (Jan 14, 2010)

**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).
Lecture 4 (Jan 14, 2010) Continued

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).

Depth of focus ($D_1$): distance along the optical axis where the IMAGE appears essentially the same.

Depth of field ($D_0$): 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.

Lecture 5 (Jan 19, 2010)

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.

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 $\times$ thickness. Scattering probability increases as mass thickness increases.
LECTURE 5 (Jan 19, 2010) Continued

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 6 (Jan 21, 2010)

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

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

Microscope CTF arises from the objective lens focal setting AND from the spherical aberration present in all electromagnetic lenses

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 underfocusing 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
LECTURE 6 (Jan 21, 2010) Continued

**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 TEM**

**Accelerating voltage:** usually best to increase (reduces specimen damage, improves depth of field and diffraction limited resolution, etc. etc.)

LECTURE 7 (Jan 26, 2010)

**Operation of 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

**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); goal is to obtain a complete, faithful, and permanent record of details contained in the electron image; photographic emulsions respond differently to electrons (single-hit) and photons (multiple-hit)

**Optical Density:** quantitative measure of blackening of the photographic emulsion

\[
OD = \log_{10} \frac{I_0}{I}
\]

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

**MIDTERM EXAM #1 (Jan 28, 2010)**
LECTURE 8 (Feb 2, 2010)

Operation of TEM (Cont'd)

Recording Images Photographically (on film):
Each e⁻ passes through several halide grains on its way through a typical, 20 µm 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.

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 are replacing film as the principle recording media in TEM (DDDs will eventually make CCDs seem archaic)

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.

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

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)
LECTURE 8 (Feb 2, 2010) Continued

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.

LECTURE 9 (Feb 4, 2010)

§II. THE SPECIMEN

Specimen Preparation

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)

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 (< 1mm³ best)

Dehydration/Embedding: Goal of dehydration is to remove H₂O 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)
LECTURE 9 (Feb 4, 2010) Continued

Specimen Preparation (Cont’d)

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 10 (Feb 9, 2010)

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 is 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⁻/Å² at RT.

Damage is proportional to TOTAL DOSE (= dose rate X exposure time), measured as electron flux and reported in units of in units of coulombs/cm² or e⁻/nm² or e⁻/Å² (1 e⁻ = 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⁻/Å².
Radiation Effects (Continued)

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/Å²) 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/Å² dose before fine features are lost in the images; At slightly higher doses (~30-40e/Å²) bubbles appear in regions where there is carbon support film, signifying that too many electrons have been used.

§III. THE STRUCTURE

Image analysis and processing: lots of steps involved; method employed generally depends on the type of specimen under investigation (asymmetric or symmetric single particle; helical (1D) filaments/particles; 2D crystals; thick (tomographic) specimens (cells, organelles, large macromolecular complexes)

Real vs. reciprocal (Fourier) space methods: real verses reciprocal dimensions; Fourier space methods are quantitative and quite powerful.

3D Reconstruction: 3D object (specimen) -> 2D projection images -> 3D structure (reconstructed density map).

Fourier Transforms: The Fourier transform provides another, characteristic means of representing an object.

Roles of image analysis / processing: Assess and adjust conditions of microscopy and measure image resolution; Perform image enhancement and restoration procedures; Examine, assess, and enhance specimen features

Noise: Goal of IP is to reduce noise as much as possible and provide a clear view of specimen structure; Caused by: the specimen, the support film, the microscope, and the recording device

Biomacromolecules are often symmetrical or periodic and hence readily studied by diffraction (i.e. Fourier-based) methods; Diffraction theory is at the heart of understanding the “how and why” of image processing

Crystal: Regular arrangement of atoms, ions, or molecules; Continuing translational repetition of some structural pattern (unit cell)
Lecture 1

2D unit cell: defined by two edge lengths \((a,b)\) and one interaxial angle \((\gamma)\)

3D unit cell: defined by three edge lengths \((a,b,c)\) and three interaxial angles \((\alpha,\beta,\gamma)\)

Lattice: a rule for translation (defines an infinite array of imaginary points); In a lattice each point is identical to every other point; 5 2D lattices and 14 3D lattices

Crystal structure: array of objects placed at lattice points; built by placing a motif at every lattice point

Crystal lattice: array of imaginary, infinitely small points; Five 2D lattices and fourteen 3D (Bravais) lattices

Motif: object that is translated (may be symmetric or asymmetric)

Crystal structure, crystal lattice, and motif: all restricted in the symmetries they can display; biomacromolecular assemblies themselves are NOT restricted

Asymmetric Unit (ASU): part of the symmetric object from which the whole is built up by repeats

Symmetry: An object is symmetrical if it is indistinguishable from its initial appearance when spatially manipulated; Biological objects may display symmetry about a point or along a line; Symmetry of any object is described by some combination of symmetry operations

Symmetry Operations (lead to superimposition of an object on itself): Rotation, translation, reflection, inversion; biological aggregates or crystals only described by rotation or translation operations (or both)

Symmetry Element: Geometrical entity such as a point, line, or plane about which a symmetry operation is performed

Point Group: Collection of symmetry operations that define the symmetry about a point; types of operations include: Rotational \((n)\), Mirror or Reflection \((m)\), Inversion \((i)\), and Improper Rotations.

Point Group Types: Cyclic \((Cn\ or \ n)\), dihedral \((Dn\ or \ n2\ if \ n\ odd\ or \ n22\ if \ n\ even)\), cubic \((T\ or\ 23; \ O\ or\ 432; \ I\ or\ 532)\).

Notation Systems: S (Schoenflies) and H-M (Hermann-Mauguin)

Translational Symmetry: Symmetry operation of shifting object a given distance in a given direction

Lecture 2

Screw Axis Symmetry: combines translation and rotation operations to produce a structure with helical symmetry; Screw axes found in crystals only include: \(2_1, 3_1, 3_2, 4_1, 4_2, 4_3, 6_1, 6_2, 6_3, 6_4,\) and \(6_5\)

Periodic Structure: Conceptually, built up in two steps. 1) A motif is generated from the ASU by the symmetry operations of the point group, and 2) The structure is generated from the motif by the translational symmetry operations of the lattice

Glide Plane Symmetry: Translation followed by a mirror operation (or vice versa)

Biological molecules display point group, line group (screw axis), plane group, and space group symmetries.

Diffraction methods: powerful means to determine molecular structure; Characteristic of diffraction: Each point in a diffraction pattern arises from interference of waves that have scattered from all irradiated portions of the object
Structure determination by diffraction methods: Involves measuring or calculating structure factors ($F_S$) at discrete points in the diffraction pattern; each $F$ is described by two quantities, an amplitude (strength of interference at a particular point) and a phase (relative time of arrival of scattered wave at a particular point)

Fourier transform: just a different way to represent an object; in mathematical terms, it describes the distribution of amplitude and phase in different directions, for all possible directions of radiation scattered by an object

Inversion theorem: the Fourier transform of the Fourier transform of an object is simply the original object!!! Analogous to Abbe's treatment of image formation (see below).

Fourier synthesis: Any periodic object can be represented mathematically as a summation of sinusoidal waves

Fourier analysis: Any periodic object can be decomposed mathematically into a series of sinusoidal waves

Image formation is considered a double diffraction process: An image is the diffraction pattern of the diffraction pattern of an object.

First Stage: parallel beam of rays incident on an object is scattered and the Fraunhofer diffraction pattern appears in focus at the back focal plane of the lens; called “forward Fourier transformation” stage, which is analogous to Fourier analysis

Second Stage: Scattered radiation passes beyond the back focal plane of lens and interferes (recombines) to form an image; Called “back” or “inverse Fourier transformation” stage, which is analogous to Fourier synthesis

Inverse relationship: Object <---- Transform

Bragg’s Law: visualizes diffraction as arising from reflection of radiation from 2D planes in 3D crystals, or 1D lines in 2D crystals; provides a simple conceptual basis for describing diffraction from crystals: $n\lambda = 2d_{hkl}\sin\theta_{hkl}$

LECTURE 13 (Feb 18, 2010)

Structure factor ($F$): is a complex number, described by an amplitude and a phase or by an A-part and a B-part; each $F$ contains contributions from all of the unit cell contents; each $F$ represents a sinusoidal wave in real space that can be summed with other $F$s in a Fourier synthesis to mathematically represent a periodic structure

Argand diagram: graphical representation of $F$

Convolution theorem: Provides a precise way to describe the relationship between objects (real space) and transforms (reciprocal space).

The Fourier transform of the convolution of two functions is the product of their Fourier transforms: $T(f \cdot g) = F \cdot G$

The Fourier transform of the product of two functions is equal to the convolution of the Fourier transforms of the individual functions: $T(f \times g) = F \cdot G$

Transforms are like fingerprints: Simple, symmetric structures ⇒ simple, symmetric transforms; Asymmetric structures ⇒ complex transforms; simple inspection of most transforms does NOT lead directly to a unique determination of structure

Crystal structure: Equivalent to the convolution of the contents of the unit cell ($f_1$) with a finite, real space lattice ($f_2$); $f_3 = f_1 \cdot f_2$

Transform of crystal structure: Equivalent to the FT of the unit cell contents ($T(f_1) = F_1$) sampled by (i.e. multiplied by) the FT of the crystal lattice ($T(f_2) = F_2$); $F_3 = F_1 \cdot F_2$. 
Transforms are like fingerprints: Simple, symmetric structures ⇒ simple, symmetric transforms; Asymmetric structures ⇒ complex transforms; simple inspection of most transforms does NOT lead directly to a unique determination of structure.

Common transforms: transform of a line is another line at right angles to the first one (the object); transform of a circle is an Airy disk; transform of a rectangle is “sin(x)/x” type function (a “perpendicular cross”).

**Reciprocity:** Dimensions in object (real space) are inversely related to dimensions in the transform (reciprocal space).

**Resolution:** Outer regions in a FT arise from fine (high resolution) details in the object; coarse (low resolution) object features contribute near the central region of the FT.

**Low-pass filter:** low-resolution Fourier components are allowed to “pass” through filter and form an image while high resolution features are removed.

**High-pass filter:** high resolution Fourier components are allowed to “pass” through filter and form an image while low resolution components are removed.

Geometry and spacings of the crystal and reciprocal lattices obey a reciprocal relationship.

The **intensity distribution** in a transform is determined by the **motif structure**, NOT by the spacings or geometry of a crystal lattice; spacings and geometry of crystal lattice only determine where the motif transform is sampled.

**Projection Theorem:** FT of the projected structure of a 3D object is equivalent to a 2D central section of the 3D FT of the object; each central section intersects the origin (i.e., center) of the 3D transform and is perpendicular to the direction of projection.

**Friedel’s Law:** Diffraction pattern from the projected structure of a real object has an inversion center in the intensity distribution; amplitudes are the same: \(|F_{hkl}| = |F_{-h,-k,-l}|\); phases are opposite: \(\alpha_{hkl} = -\alpha_{-h,-k,-l}\).

**Optical Diffraction:** Objective way to assess and reveal periodic structural information; Lots of other uses (e.g., to determine micrograph defocus, astigmatism, specimen drift, etc.)

**Optical Filtering:** Means to remove non-periodic noise contributions from micrographs to give clearer image of specimen structure.

**Indexing:** *most important* step of any filtering experiment; determine reciprocal lattice and thereby distinguish signal and noise components in specimen image.

**Indexing:** for most well-ordered, crystalline specimens, the OD pattern is a lattice of strong spots (Bragg reflections) against a weaker background of noise; noise generally appears everywhere in the OD pattern.

**Filtration:** Place filter mask (opaque material with holes positioned to allow unobstructed passage of diffraction spots at lattice points) in back focal plane of optical diffractometer, and position translationally and rotationally to allow unobstructed passage of all spots at the lattice points; filtering reduces image noise by averaging unit cell images in a periodic array; size of holes in filter mask determine how much noise is removed and the **extent of local averaging** (as hole size decreases, averaging increases and vice versa).
LECTURE 14 (Feb 23, 2010) (Continued)

Computer Image Analysis: many advantages to digital processing, including ability to manipulate and assess data quantitatively (e.g. remove or suppress image aberrations, specimen distortions, average separate 2D or 3D reconstructions). Most importantly, 3D reconstruction is possible.

MIDTERM EXAM #2 (Feb 25, 2010)

LECTURE 15 (Mar 2, 2010)

Computer Image Analysis (Cont’d):

'Typical' digital processing procedure: 1. Select a 'best' set of micrographs. 2. Digitize your best micrographs. 3. Box and float the digital images. 4. Fast Fourier transform the images. 5. Index the 2D lattice (for crystals). 6. Perform 2D filtering and/or 3D reconstruction

Densitometry (of film): goal is to convert optical densities (grey levels) in the photographic emulsion into digital form (a numerical array corresponding to the relative optical densities at all locations in the image); each density value in the digitzed image is represented as a pixel; Rule of thumb is to scan images at a step size at least twice as fine (2X = Nyquist limit) or finer (usually 3-4X) than the expected resolution in the image

Boxing: Zero everything outside the area of interest (equivalent to "masking" procedure in OD and OF experiments)

Floating: Determine mean intensity of pixels at box perimeter and subtract this value from ALL image intensities inside the masked area

"Pseudo-Optical" Filtering: Filter mask is generated in the computer with "holes" centered at the locations of an ideal reciprocal lattice

Fourier Averaging: all unit cells are averaged (i.e. not just a local average as one gets with pseudo optical filtering, which employs finite size, filter mask holes); specimen 2D crystal transform is idealized; a single structure factor is used to represent the data at each point of a perfect, reciprocal lattice; Fourier synthesis of this reduced set of structure factors gives the reconstructed structure of ONE unit cell (process is formally equivalent to performing filtering with infinitely small mask holes)

Assess and apply additional symmetry (if warranted): impose crystal and motif symmetries by averaging symmetry related reflections and enforcing phases to obey 'perfect' relationships

3D Reconstruction (Fourier Methods): Structure factor amplitudes and phases (Fs) determined at all points of a 3D transform by combining Fs from 2D transforms computed from many, unique views of the specimen

3D Reconstruction (Protocol): Specific rationale chosen for collecting and combining information from different views mostly depends on the type of specimen studied (symmetry & size); types include 2D crystals, 1D filaments or helical assemblies, singles particles (symmetric and asymmetric), cells and organelles and other large complexes that have unique structures (no two 'identical'); overall, there are many many 'issues' to consider when deciding exactly what to do to achieve success with a project (from specimen preparation and microscopy, to final analysis of a 3D reconstruction).
3D Reconstruction (Cont’d):

Planar (“2D”) Specimens: 2D crystal transform is a lattice of lines. Images of specimens tilted in the microscope are needed to generate 2D image transforms, from which SF data are obtained along each of the layer lines. Because one is only able to tilt specimens up to ±70° in most modern TEMs, this results in a ‘missing wedge’ or ‘missing pyramid’ in the 3D Fourier transform of the 2D crystal. Missing data from the 3D transform typically generates non-isotropic resolution in the reconstructed 3D structure (features become stretched or smeared in the Z-direction - i.e. perpendicular to the plane of the crystal).

Helical (“1D”) Specimens: are similar in some ways to 2D crystals, but are generally more difficult to analyze; a big advantage is that it is often possible to get a decent, low resolution 3D reconstruction from a SINGLE image of a helical particle since an image of one particle can contain many views of the asymmetric unit (#ASUs depends on the helical symmetry); 3D reconstructions of helical specimens follows one of two protocols (Fourier-Bessel or iterative real-space); helical structures can be mathematically represented by a series of helical (or cylindrical) waves.

Single Particles with Icosahedral Symmetry: 100s and often 1000s of particle images of unstained, vitrified icosahedral viruses are needed to obtain 3D reconstructions at sub-nanometer resolution; each image of a particle with 532 symmetry contains 60 unique views of its asymmetric unit; Basic assumptions: particles have stable, ‘identical’ structures and obey 532 symmetry. Processing scheme involves many steps and programs (several of which have been automated) that must be performed in a defined sequence. Most important is to define accurately the view orientation (three angles) and phase origin (point of reference given by x,y coordinates) for each imaged particle.

Single Particles with Icosahedral Symmetry: (Continued)

The cross-correlation (CC) of two functions is similar (but not identical) to the convolution of two functions. The CC gives a measure of the similarity of two functions. The highest value of CC (= 1.0) means the two are identical. A CC = 0.0 means that the two have nothing in common and a CC = -1.0 means the two are completely opposite (i.e. anti-correlated) and, for example, have completely opposite contrast.

Model-based (template matching) procedures are used to solve the general problem of determining the origin and view orientation of each particle image. ‘Bad’ images are identified by means of various correlation coefficients and only the ‘good’ ones are used to compute a 3D reconstruction. Reconstruction quality is assessed by means of a FSC (Fourier-Shell Correlation) plot. The refinement process is continued until no more progress can be made, assessed by the FSC curve and limited by the available image data.
Single Particles with Icosahedral Symmetry: (Continued)

Visualization and interpretation: The goal is to inspect and analyze the 3D density map in a variety of ways to better understand how virus structure dictates functional properties. Similar tools are used to examine all type of macromolecular specimens. Methods include shaded-surface representations (in color or black and white) to portray the density map as a solid surface at some chosen density threshold; planar density sections (e.g. right through the center of the virus showing internal as well as external features); spherical sections to highlight densities at a particular radius; and animations to illustrate the relative positions of subunit components, or to illustrate the docking and fitting of atomic models into portions of the density map, or to help guide an audience to a particular region of interest in the map, or to illustrate how certain features in a map can be modeled (e.g. tubes of density fit with cylinders to represent the path of an \(\alpha\)-helix in a protein chain).

Single Particles with Low or No Symmetry: Classic example is the ribosome. Many of the same principles used in icosahedral virus image processing are used to process images of macromolecules with lower or no symmetry. One expects to have a sample with particles randomly orientated in the microscope (so as not to require tilting); template matching is used to help determine and refine particle origin and orientation information; 1000s to 100s of 1000s of particle images may be needed to reach sub-nanometer resolutions; 3D density map data are visualized and analyzed with software similar to or identical to that used to study virus structures.

The general processing scheme includes particle boxing, particle image alignment and classification and formation of class averages, particle origin and orientation determination, 3D reconstruction, production of a set of projections images from the current 3D reconstruction to be used as templates for refining particle origins and orientations, and final display and interpretation of the 3D density map.

Alignment of images from particles that are similarly oriented (i.e. with two of three angles that define view the same) but whose origins (identical points of reference) are not in the same position relative to the box center and whose rotation around the view direction is not the same is based on the use of the autocorrelation function (ACF) of each image. ACFs are translation invariant (peak always occurs at center of pattern), hence the rotational cross-correlation of two image ACFs gives the absolute rotation angle between the two images. If one image is rotated to remove this difference, then the two images will only differ in translation, which can be identified by cross correlating one image with the rotation-adjusted second image. The peak in this CCF pattern provides the absolute difference in (x,y) position between the two images and is used to translate the second to conform with the first, in which case the two can now be summed to form an average. The process is repeated for all other such images that have been classified as belonging to one class (particles viewed in the same direction, but randomly oriented with respect to that direction).
Single Particles with Low or No Symmetry (Continued):

**MSA (Multivariate Statistical Analysis):** very powerful mathematical tool that can detect and rank order variations in data (e.g. digital images of presumed similar particles); It is multidimensional with the number of dimensions equal to the number of pixels in one image (i.e. would be 10,000 for a 100² image); Histogram plot of eigenvalues spectrum provides clues about data variability (Factor 1, Factor2, etc.); 2D plots of data ‘clouds’ (e.g. Factor 1 verses factor 2, or 2 vs. 3, or 1 vs. 3, etc) provide a quick assessment of clustering of image data set; HAC (Hierarchical Ascendant Classification) categorizes clusters of images that differ and can be separately averaged to form more and more sub-averages; These sub-averages can be used to determine relative orientations among the separate class averages, which is then used to produce a low resolution 3D reconstruction, which, in turn, can be used to begin an iterative, template-based, cross-correlation type refinement of particle origin and orientation parameters.

**Electron Tomography:** Particularly useful for examining large, individual macromolecular complexes, and small organelles and whole cells (usually limited to 2 µm or thinner)

Data collection involves recording several images of the same field of view with the specimen stage rotated about a tilt axis through ±60-70°. Main problem with this scheme is the “missing wedge” or “missing pyramid” (if a double tilt series is used) in Fourier space that leads to loss of resolution in the resulting 3D tomogram in the direction parallel to the optic axis of the microscope.

Tomographic data collection is performed in an automated fashion, using the microprocessor of the TEM to perform 4 main functions: search, track, focus, expose. Gold markers are often used as fiducials to help in the process of aligning the tomography set of images.

Segmentation is the process by which specific features (e.g. membranes) are identified in successive tomographic sections.

Cryo-tomography performed on particles or cells suspended and frozen in vitreous buffer, provide detailed structural information about individual particles (e.g. viruses like HIV or vaccinia in the process of uncoating inside an infected cell) or the organization of organelles and macromolecules within cells (e.g. distribution of actin filaments in the advancing filopodia of migrating cells).