S²C² Workshop – Cryo-EM Imaging Process workshop
In 1968, Klug and I realized that EM images could be treated as projections of the 3D structure. By collecting a set of views, we were able to make the first 3D reconstruction of a structure from electron micrographs.

**IF ONLY ... the images were perfect projections of the desired 3D structure, a lot of what follows in my talk would not be necessary.** Here are the problems:

Electrons damage the specimen.

Underfocus affects amplitudes and phases.

Digitization and boxing of the image affects and limits amplitudes and phases.

Coherence of the electron beam limits resolution.

Beam induced motion limits resolution.

Image distortion by the lenses affects phases.

Interpolation reduces high resolution amplitudes.

Beam tilt alters phases.

Lack of plane parallel illumination alters phases.

Insufficient depth of field alters amplitudes and phases.

Multiple scattering alters amplitudes and phases.

Not all scattered electrons are imaged.
The Fourier transform (FT) is used to characterize images.

The FT of a sample describes the outcome of all diffraction experiments* at any wavelength and in any direction of the incident radiation.

The Ewald sphere describes the portion of the FT seen in any one experiment:

* in the linear approximation
To examine what amplitude and phase (if recorded) are measured by a diffracted ray, draw an arrow from the center of the sphere in the direction of the diffracted ray. The point in the FT where the arrow intersects the Ewald sphere is the element of the FT sampled.

By sweeping the direction of illuminating radiation through all angles, one can measure the FT out to a distance of $2/\lambda$. Hence the highest possible Fourier (not Rayleigh) resolution is $\lambda/2$.

Draw a sphere that passes through the origin as follows:
- Radius of the sphere = $1/\lambda$
- Arrow from circle center to the origin $X,Y,Z=0$ points in the direction of the illuminating radiation.
If $\Delta x$ is 0.75 Å and $N$ is 128, then $\Delta X = 1/(128 \times 0.75) = 0.0104 \text{ Å}^{-1}$.

The radius of the ring ($=1/2\Delta x$) defines the Nyquist limit, which gives the highest feasible resolution of $(1/(2 \times 0.75) = 1/1.5 \text{ Å}^{-1}$ or 1.5 Å resolution).
The FT is a mathematical operation, which I will not describe.

To get a FT, you feed your digitized image into a FT program.

The question I address is what you do with a FT.

It has information about your sample and about the imaging conditions.
Optical FT

“short” exposure

“longer” exposure

Thon rings
<table>
<thead>
<tr>
<th>What you see.</th>
<th>What you get</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spots</td>
<td>You know the specimen is crystalline</td>
</tr>
<tr>
<td>Spot positions</td>
<td>Unit cell size and shape</td>
</tr>
<tr>
<td>Spot size</td>
<td>Size of coherent domains</td>
</tr>
<tr>
<td>Intensity relative to background</td>
<td>Signal/noise ratio</td>
</tr>
<tr>
<td>Distance to farthest spot</td>
<td>Resolution</td>
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<td>Amplitude and phases of spots</td>
<td>Structure of molecules</td>
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**Positions of Thon rings**  
**Amount of defocus**
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<tr>
<td><strong>Ellipticity of Thon rings</strong></td>
<td><strong>Amount &amp; direction of astigmatism</strong></td>
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Stronger here

Weaker here

darker

lighter

darker

lighter
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<td><strong>Asymmetric intensity of Thon rings</strong></td>
<td><strong>Amount &amp; direction of instability</strong></td>
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Figure 1.
An example of Fourier transforms of 300 kV images from an exposure series of a catalase crystal at 100 K. Only a quarter-plane of the Fourier-space is shown, with the origin of each Fourier transform in the bottom-right of each frame. Each image was collected with 10 e⁻/Å².
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<tr>
<td>Fall off of spot intensity with dose</td>
<td>Maximum electron dose</td>
</tr>
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</table>
Resolution and radiation damage

Resolution is limited by the wavelength, $\lambda$, of the radiation. At best, resolution is $\sim \lambda/2$.

The wavelength of a moving particle, like an electron, is given by $\lambda = h/p$ (h is Planck’s constant and p is the relativistic momentum). For a 300 keV electron, the wavelength is $\sim 0.02$ Å.

If this green ball (28 gm) is dropped from the height of one meter, its wavelength is $\sim 5 \times 10^{-23}$ Å. With a green ball microscope, we should see the quarks ($10^{-11}$ Å ?) in a proton ($10^{-5}$ Å).

In grad school, I asked my professor: Why can’t we make a microscope using, for example, green balls?

My professor said my question was frivolous and dilatory, but was it?
What is wrong with $\lambda = h/p$?

Nothing, but wavelength is not the only consideration.

The interaction of a particle with a sample involves two cross-sections: the elastic and the inelastic cross-sections or probabilities.

In an elastic interaction, the electron is scattered but does not lose energy. These electrons remain coherent and contribute to the image of one’s structure.

In an inelastic collision, the electron transfers energy to the structure and loses its coherence. These electrons damage the specimen and contribute to the background/noise.

For every elastically scattered electron, which leaves the sample unchanged, there are ~5 inelastically-scattered electrons that transfer energy to the sample and damage it. Controlling damage is essential to high resolution cryo-microscopy.

The answer is that the elastic to inelastic scattering ratio for green balls landing on an EM grid is ~0.
The probability that an electron is scattered is a function of **sample thickness**. Thinner is better.

Mean free path is the distance in which fraction of unscattered electrons is $\approx e^{-1} = 0.37$

**Electron Crystallography of Biological Molecules**, Glaeser, Downing DeRosier, Chiu, Frank
Questions or comments about Fourier Transforms of images and their use?
Scattering contrast is a form of amplitude contrast

Heavy metals are strong scatterers of electrons and produce contrast by scattering electrons outside the imaging system.

The thicker the heavy metal layer the fewer the number of electrons at that position in the image. Shadowing with platinum, osmium staining of sections, and negative staining with uranyl salts are ways of using heavy metals to produce contrast.

In negative stain, the structure is seen as a hole in the layer of stain. (NOTE: The contrast is opposite to that in frozen hydrated images.)
Negative stain is particulate, which prevents detail finer than ~15 Å from being seen.

The beautiful and important internal features are not seen.
Negative stain

**Pro**
- Fast
- Easy
- Acts as a fixative
- Generates high contrast
- Any TEM will do
- Generally gives faithful info on molecular detail (>15Å)
- Is radiation resistant

**Con**
- Cannot see particle itself
- Cannot see secondary structure
- Distorts/shrinks/flattens structure
- Has opposite contrast to ice images
- Some reviewers wrongly do not accept negative stain results
Questions or comments about negative stain?
Cryo-samples are treated as transparent objects.

A plane parallel electron wavefront approaches transparent particles embedded in a transparent medium.

The electron wavefront is distorted by the sample. The parts passing through the particles are advanced.

These ‘bumps’ in the wavefront give rise to the scattered and unscattered beams that are brought into focus by a lens.

Image formation of particles in ice.
Image formation by a perfect lens

The transparent object scatters or diffracts pairs of beams at symmetric angles.

These beams are redirected by the lens to recombine with the unscattered beam at the image plane.

The optical path lengths for the scattered and unscattered beams are the same so that the original wave front is regenerated.
Thus, the imaged wavefront has the same bumps that arose when the plane wavefront passed through the sample. They are bumps in wavefront shape and not in wavefront amplitude. Thus an in-focus image has no contrast.

In the light microscope, we use a phase contrast objective to generate contrast in a transparent specimen.

An ordinary objective gives some contrast because the lens is not a perfect lens.

A phase contrast objective turns differences in phase into differences in amplitude.

How can phase contrast be generated in the electron microscope?
How to think about phase contrast.

A wave passing through a particle is advanced compared to the unscattered wave.

The deformed or advanced part of the wavefront can be described by \( \cos(wt - d) \) compared to \( \cos(wt) \) for the undeformed or rest of the wavefront. (\( w \) is frequency, \( t \) is time and \( d \) is the advance)

The deformed part is the sum of the unscattered wave + the scattered wave:
\[
\cos(wt-d) \approx \cos(wt) + d \sin(wt) \text{ when } d \text{ is small}
\]

\( \cos(wt) \) is the unscattered beam, and \( d \sin(wt) \) is the scattered beam.

![Diagram showing wave deformation and phase contrast](image)
How underfocusing generates phase contrast

We need only look at one of the pair of scattered images.

For simplicity, we will look at the central ray.

In underfocus, the image plane lies before the in-focus plane.

At the image plane, the scattered and unscattered beams arrive at the same time as expected.
At the underfocus plane, however, the scattered beam arrives ahead of the unscattered beam. (It has to arrive earlier because it has farther to go to get to the focal plane on time, that is, in phase.)
Because the scattered wave (red) arrives ahead of the unscattered wave (blue), they generate destructive interference. The particle appears dark. Here is why:

The sum of the red and blue waves gives a change in phase.

These are the two waves as they exit the sample plane or arrive at the in-focus plane.

These are those two waves as they arrive at the under-focus plane.

The sum of the red and blue waves now gives a change in amplitude.
The larger the scattering angle, the larger the path difference of the scattered wave relative to the unscattered wave. As a result the contrast varies with scattering angle.
How to interpret the Thon rings

Thon rings appear in the diffraction pattern of the image.

Increasing scattering angle:
- Destructive interference details are dark
- No contrast: no data
- Constructive interference details are light
Scattering contrast + a little underfocus phase contrast

negative stain

underfocus phase contrast

ice

JT Finch, J Gen Virol. 24, 359, 1974


By comparing the 1D curve of the CTF with the observed Thon rings, we can determine the amount of defocus and any astigmatism.

We may also have to determine the spherical aberration coefficient, Cs.
Questions or comments about defocus phase contrast?
Phase plate contrast

The unscattered beam passes through a hole while the scattered beam passes through a carbon film, which advances it relative to the unscattered beam.

The Volt Phase Plate (VPP) has a continuous layer of carbon. A negative voltage is generated at the center and delays the unscattered beam, which has the same effect as advancing the scattered beam in the Zernike phase plate.

![Images of lacey carbon film acquired with (A) a ZPP and (C) a VPP. The image in B is a fringe reduction software-filtered version of the ZPP image in A. (Scale bar: 20 nm.)](Fig. 5)

Even with the phase plate, there is defocus or spherical aberration, which must be corrected.
How defocus affects the Thon rings in the FT

The form of the Thon rings is given by the contrast transfer function (CTF):

For a phase object using defocus contrast:

$$ctf(X) = -A(X)\sin(\pi \lambda X^2 \Delta z - 0.5\pi \lambda^2 X^4 c_s)$$

Where $X$ is the reciprocal distance, $A$ is the envelope function, $\lambda$ is the wavelength, $\Delta z$ is the defocus, and $C_s$ is the spherical aberration coefficient.

For phase object using a perfect 90° phase plate:

$$ctf(X) = B(X)\cos(\pi \lambda X^2 \Delta z - 0.5\pi \lambda^2 X^4 c_s)$$

Questions or comments about phase plates?
Image defects
Corrections for defocus

Images are okay out to the first zero in the CTF.

But after the first zero, the features in the next zone add with the wrong contrast (the wrong sign). Such features will correlate negatively with the true structure. The change in contrast must be fixed!

The simplest fix is to change the sign of the phases in the zones of reversed contrast, which is called phase flipping.

The more complicated fix is to use a Weiner filter, which also tries to fix amplitudes:

$$F_{\text{corrected}} = F_{\text{observed}} \times \frac{\text{CTF}}{\text{CTF}^2 + \text{N/S}}$$

Where F is the observed complex Fourier amplitude and phase and N/S is the noise to signal ratio. The N/S term prevents the zeros of the CTF from amplifying noise.
How accurate does the determination of defocus need to be?

A typical defocus might be about 1 micron (= 10,000 Å). We need to refine that measure to about +/- 100 Å or 1%.

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<th>200 kV</th>
<th>300 kV</th>
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<tr>
<td>2.0</td>
<td>54 Å</td>
<td>80 Å</td>
<td>102 Å</td>
<td>122 Å</td>
</tr>
<tr>
<td>3.0</td>
<td>122 Å</td>
<td>179 Å</td>
<td>228 Å</td>
<td>274 Å</td>
</tr>
<tr>
<td>4.0</td>
<td>216 Å</td>
<td>319 Å</td>
<td>406 Å</td>
<td>488 Å</td>
</tr>
<tr>
<td>7.0</td>
<td>662 Å</td>
<td>976 Å</td>
<td>1244 Å</td>
<td>1494 Å</td>
</tr>
</tbody>
</table>

Limiting factors in atomic resolution cryo electron microscopy: No simple tricks

Xing Zhang and Z. Hong Zhou

PMID: 21627992
Published online 2011 May 24. doi: 10.1016/j.jsb.2011.05.004
Coherence of the electron gun limits resolution

With a LaB$_6$ gun at 100 keV, it was hard to get below 10 Å resolution.

With the thermally-assisted field emission gun (FEG) at 300 keV, the spatial and temporal coherence are much improved over a LaB$_6$ gun at 100 keV.

With the availability of the FEG, resolutions dropped to 4 - 7Å, but this limitation was not due to the FEG but to beam induced motion.

The cold FEG on the JEOL microscope is even better apparently.
Beam induced motion and/or drift cause loss of resolution preventing resolutions below 4Å.

The direct electron detectors made it possible to break a single exposure into a movie of many frames and correct for the motion.

Movie

Recorded with direct electron detector DE-12 (Direct Electron)

Frame rate = 40 fps
Dose/frame = 0.5 e⁻/Å²
Duration = 1.5 s
No. of frames = 60
Total dose = 30 e⁻/Å²
Accumulating Average

Average shows emerging detail as noise decreases.

Particles are blurred later in the movie due to beam-induced movement.
Each averaged frame corresponds to 0.25 s.

Dose/frame = 5 e⁻/Å²
Beam induced motion causes blurring

Image corrected for beam induced motion

With these new cameras, resolution dropped from 4A to better than 2 A in some cases.
Distortions can affect phases

For a 150 Å particle being solved to 1.5 Å, variations in the magnification must be corrected to within ~ 0.25%

Capitani et al. Ultramicroscopy 106, 66, 2006
Digitizing the image

Digitizing an image limits the resolution of the data.

Digitizing the image can also cause aliasing in which high resolution Fourier terms are added into low resolution terms.
FT of the sampled image is a ‘crystal’ of FTs. The digital FT calculates one unit cell of the infinite FT.
Fixing the dimensions of the FT

There is a slight problem with looking at the FT of a non-square image. While the x,y spacings in the image are the same (0.75 A), the X,Y spacings in the transform are not the same size.

Suppose you have a digitized image that is sampled every 0.75 A and that is 128 by 256 pixels.

The FT is also 128 by 256 reciprocal pixels. The reciprocal pixel size in X is $1/(128*0.75) = 0.0104 \text{ A}^{-1}$. The pixel size in Y is $1/(256*0.75) = 0.0052$. Note: the reciprocal pixel sizes are different if the X and Y directions!
To make the reciprocal pixels have the same size, we can embed the image into a square array:

![Image diagram]

Note the big jumps in density at the edges.

The process is called padding, but there is a problem.
To get the input you want, you float the image by subtracting average density at the box’s edges.

This is what you want to input into the FT program.

But you also get the FT of this.

The particle density in the array seen in 1D.
This is a 1D version.

Floating may include removing a gradient of density.

This is what you want to input into the FT program.
Apodizing the image edges may also be necessary.

Padded image

Gradient-removed, floated, apodized, padded image
The effect of interpolation

To turn the image upright, we could interpolate the densities into an upright box.

But if we have a pixel size of 1 A, and we use bilinear interpolation, the amplitudes at a resolution of 3A will be reduced by a factor of 2 and the 2 A amplitudes are reduced to 0.

There are better ways to interpolate data, but I leave that to others to discuss.
The effect of incident beam tilt on diffracted beams.

The left, L, and right, R, diffracted waves travel different path lengths from each other and from the unscattered wave when the beam is tilted.

As a result, the these waves do not interfere as they should, and the measured phase is incorrect.

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<th>400 kV</th>
</tr>
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<tbody>
<tr>
<td>2.0</td>
<td>0.002°</td>
<td>0.005°</td>
<td>0.007°</td>
<td>0.011°</td>
</tr>
<tr>
<td>3.0</td>
<td>0.007°</td>
<td>0.016°</td>
<td>0.025°</td>
<td>0.036°</td>
</tr>
<tr>
<td>4.0</td>
<td>0.016°</td>
<td>0.036°</td>
<td>0.060°</td>
<td>0.085°</td>
</tr>
<tr>
<td>7.0</td>
<td>0.090°</td>
<td>0.195°</td>
<td>0.316°</td>
<td>0.457°</td>
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* Magnitudes of beam tilt resulting in a phase error of 45°.
If the incident beam is convergent or divergent, objects away from the center of the field suffer from beam tilt.

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<td>1075°</td>
<td>494°</td>
<td>305°</td>
<td>211°</td>
</tr>
<tr>
<td>3.0</td>
<td>319°</td>
<td>146°</td>
<td>90.4°</td>
<td>62.5°</td>
</tr>
<tr>
<td>4.0</td>
<td>134°</td>
<td>61.8°</td>
<td>38.1°</td>
<td>26.4°</td>
</tr>
<tr>
<td>7.0</td>
<td>25.1°</td>
<td>11.5°</td>
<td>7.1°</td>
<td>4.9°</td>
</tr>
</tbody>
</table>

Phase error is another way of thinking about the effect of beam tilt.
Lack of depth of field due to curvature of the Ewald sphere causes amplitude and phase errors.

This plane through the origin \(X,Y,Z=0\) corresponds to the FT of a projection.

Resolution Limit imposed by focus gradient across specimen depth.

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<tr>
<td>300</td>
<td>2.82 Å</td>
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<td>1.87 Å</td>
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<tr>
<td>600</td>
<td>3.98 Å</td>
<td>3.28 Å</td>
<td>2.91 Å</td>
<td>2.65 Å</td>
</tr>
<tr>
<td>900</td>
<td>4.88 Å</td>
<td>4.02 Å</td>
<td>3.56 Å</td>
<td>3.25 Å</td>
</tr>
<tr>
<td>1300</td>
<td>5.86 Å</td>
<td>4.83 Å</td>
<td>4.28 Å</td>
<td>3.90 Å</td>
</tr>
</tbody>
</table>

*Limitation based on formula by DeRosier (2000).*
Waves that are diffracted or scattered more than once have inaccurate amplitudes and phases.

If the probability of a scattering event is $p$, the probability of the beam being scattered twice is $\sim p^2$.

The amplitude and phase of the resulting beam is a mixture of two Fourier terms.
Questions?

My power point and a treatise I wrote on the FT are available.
The amplitude of the plane wave just before it hits the specimen can be described as

\[ A(-\delta,y) = 1 \]

After passing through a transparent specimen, the amplitude of the plane wave becomes

\[ A(0,y) = e^{i\rho(y)} \text{ where } \rho(y) \text{ describes the variation in path length along } y \]

If \( \rho \) is small (the thin phase object approximation), we can write:

\[ A(0,y) \approx 1 + i\rho(y) \]

Let us assume for simplicity that \( \rho(y) = a\sin(y) \), where \( a \ll 1 \) (i.e., \( \rho \) is small).

\[ A(0,y) \approx 1 + i a\sin(y) \]

At the perfect defocus plane for this periodicity, we would find destructive interference:

\[ A(\text{defocus},y) \approx 1 - a\sin(y). \text{ At } y = 0, \text{ we would see the maximum contrast.} \]

Suppose instead that \( \rho(y) = a\sin(y-c) \), then we would find

\[ A(\text{defocus},y) \approx 1 - a\sin(y-c). \text{ At } y = c, \text{ we would see the maximum contrast.} \]

The sine wave is shifted by \( c \)!