CRYOEM 001:
COLLECTING DATA AND OTHER MATTERS

February 8, 2023
WHAT BROUGHT ABOUT THE RESOLUTION REVOLUTION (~2012-2014)

- **Hardware**
  - Microscopes
  - Direct Detectors
  - Computers

- 2012->2017
  - Cost reduced by 100x
Example TEM schematic
One of many types of TEMs

- Filament
- Wehnelt Anode
  - Gun deflectors
  - Condensor lens 1
  - Condensor stigmator
  - Condensor lens 2
  - Beam deflectors
  - Objective stigmator
  - Objective lens upper
  - Objective lens lower
  - Image deflectors
- Intermediate lens
  - Diffraction stigmator
  - Projector lens 1
  - Projector lens 2
- Beam axis
- Viewing screen

- CCD camera
- Sample holder
- Objective aperture
- Selected area aperture
  (or immediate aperture)
- Fixed aperture
- “Gun”
- Condensor aperture 1
  (may be adjustable by user depending on TEM type)
THREE-DIMENSIONAL RECONSTRUCTION:  
STRUCTURES WITH HELICAL SYMMETRY. 1968  
(sample prep: negative staining)  
Pioneering work: 3D reconstruction of a  
bacteriophage tail using the Fourier-Bessel  
approach, 1968  
Application of the Projection-Slice Theorem

Aaron Klug and David DeRosier, LMB/MRC Cambridge
DIRECTIONAL INFORMATION LOSS

\[ \Delta \theta = 2^\circ \quad \theta_{\text{max}} = 90^\circ \]
\[ \Delta \theta = 5^\circ \quad \theta_{\text{max}} = 60^\circ \]

Baumeister et al. (1999) Trends Cell Biol. 9:81
Iterative angular refinement

J. Frank, in *Molecular Machines in Biology* 2011
NEW ERA (SINCE 2012): DIRECT ELECTRON DETECTING CAMERAS
DETECTORS

Digital Cameras for TEM

Photon converted

Direct sensing

- CCD: Charge Coupled Device
- CMOS: Complementary Metal Oxide Semiconductor

phosphor fiber optic plate

high dose rate

Direct Detectors
DETECTORS

Detector Performance Characterization

MTF (Modulation Transfer Transform) contribute to signal envelope

DQE (Detector Quantum Efficiency) S/N over spatial frequency range

Counting
DETECTORS

Detector Performance Characterization

dectris.com

Ruskin, et al JSB
# K3 SPECS

<table>
<thead>
<tr>
<th>Specifications</th>
<th>K3</th>
<th>K3 Base</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM operating voltage (kV)</td>
<td>200 / 300</td>
<td></td>
</tr>
<tr>
<td>Sensor size (pixels)</td>
<td>5,760 x 4,096</td>
<td>3,456 x 4,096</td>
</tr>
<tr>
<td>Readout modes</td>
<td>Counting Super-resolution</td>
<td>Counting</td>
</tr>
<tr>
<td>Max. image size (pixels)</td>
<td>11,520 x 8,184</td>
<td>3,456 x 4,096</td>
</tr>
<tr>
<td>Performance relative to physical Nyquist (DQE)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 0.5</td>
<td>&gt;0.87 / &gt;0.83</td>
<td>&gt;0.8</td>
</tr>
<tr>
<td></td>
<td>&gt;0.53 / &gt;0.53</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Sensor read-out (full fps)</td>
<td>&gt;1500</td>
<td></td>
</tr>
<tr>
<td>Transfer speed to computer (full fps)</td>
<td>&gt;75</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Motion correction</td>
<td>Inline</td>
<td></td>
</tr>
<tr>
<td>Gatan Microscopy Suite® software</td>
<td>Included</td>
<td></td>
</tr>
<tr>
<td>Automation support</td>
<td>Latitude and other third-party software</td>
<td></td>
</tr>
</tbody>
</table>

Specifications are subject to change without notice.
COUNTING MODE

Electron enters detector.

Electron signal is scattered.

Charge collects in each pixel.

Events reduced to highest charge pixels.

K3 lowers Read Noise with Correlated Double Sampling (CDS)

Standard mode

- Reset
- Read$_1$

Pixel charge

Net readout = Read$_1$

Time

CDS mode

- Reset
- Read$_0$

Pixel charge

Net readout = Read$_1$ - Read$_0$

Time $\times$ 2

https://www.gatan.com/
CTF: WHY IS MONITORING THE CTF IMPORTANT IN OUR DATA COLLECTION?

a  
b  
c  
Astigmatism  
Drift
The contrast transfer function (CTF) mathematically describes how aberrations in a transmission electron microscope (TEM) modify the image of a sample.

The phase shift (phase distortion function) due to the objective lens can be combined into a single phase factor $\chi$, given by,

$$
\chi (|g|) = \left( \frac{1}{2} \pi C_s \lambda^3 |g|^4 - \pi \Delta f \alpha^2 |g|^2 \right) \quad [4236a.a]
$$

$$
\chi = \frac{2\pi}{\lambda} \left( \frac{1}{4} C_s \alpha^4 - \frac{1}{2} \Delta f \alpha^2 \right) \quad [4236a.b]
$$

where,
- $C_s$ -- The spherical aberration coefficient, defining the quality of objective lens,
- $\lambda$ -- The wave-length,
- $\Delta f$ -- The defocus value,
- $|g|$ -- The spatial frequency,
- $\alpha$ -- The convergence semi-angle.

https://www.globalsino.com/EM/page4236.html
Most cryo-EM data are acquired using defocus contrast

- At high defocus, high-resolution information in the image is strongly delocalized.
- Image processing can re-localize the signals, but at most only about half of the theoretical contrast is preserved by defocusing.
- “Underfocus” means decreasing the strength of the objective lens, effectively focusing above the specimen.
Modeling an image

\[ X = CA + N \]

Deconvolution:
\[ \tilde{A} = X/C \]
How to undo the CTF effects?

1. Phase flipping

\[ \tilde{A} = \text{sgn}(C)X \]
How to undo the CTF effects?

1. Phase flipping

\[ \tilde{A} = \text{sgn}(C)X \]

2. Wiener filter

\[ \tilde{A} = \frac{CX}{C^2 + k} \]
3. Wiener from multiple images

\[
\tilde{A} = \frac{\sum_i^N C_i X_i}{k + \sum_i^N C_i^2}
\]

\[k = \frac{1}{\text{SNR}}\]

\[= \frac{|N|^2}{|A|^2}\]
Join a discussion on sub-2.5 Å cryo-EM structure determination of GPCRs for drug design
Wed, Sep 9, 2020, 8 p.m. EDT | 5 p.m. PDT | 10 a.m. AEST | 9 a.m. JST

Attend our upcoming Ask the Experts Q&A session on routine sub-2.5 Å cryo-EM structure determination of GPCRs for drug design. This rapidly developing field is constantly producing new and exciting biological and pharmacological discoveries. Ask questions and get answers from leading academic investigators in the field.

You’ll learn about:
- GPCR biochemistry and purification for cryo-EM
- GPCR sample preparation for cryo-EM
- High-resolution single-particle cryo-EM imaging and 3D reconstruction of GPCRs
TIPS AND TRICKS FOR

Cryo-EM sample preparation

- The quality of the cryo-EM sample governs the outcome of the experiment!
- We optimized the plunging parameters for ice thickness consistency and grid coverage
- Our blot time is relatively long: 10 s
  - For every new sample, depending on the initial concentration, we prepare 2-3 grids
  - with 2x dilution in-between
  - GPCR sample concentrations in the range 3 – 7 mg/ml work best.
- Avoid as much as possible lower concentrations!
- Gold foil grids (UltrAuFoil) improved the consistency of getting uniformly thin ice and reduce beam-induced motion.
DATA COLLECTION

Cryo-EM data acquisition strategy

- Collect on the thinnest possible ice that still has good particle coverage!
- We used $3 \times 3 = 9$-hole beam-image shift data acquisition scheme, 1 image/hole, realized with home-made scripts in SerialEM
  - Defocus range: $0.5 - 1.5 \ \mu m$. Start at the high end on the first hole and reduce the defocus step-wise for each hole in the pattern, e.g. $1.4 \rightarrow 1.3 \rightarrow 1.2 \rightarrow \ldots \rightarrow 0.6$
  - Use an energy filter with $<15 \ \text{eV}$ slit
- Do not use super-res (K3), select pixel size $\approx 1/3$ the resolution you are hoping to get. Use EER with Falcon 4.
- Throughput: 1 sample/day $\approx$ 1 structure/day; $\sim$5,500 movies
- Collect non-gain-normalized compressed TIFFs/EER. Prepare your own gain reference with Relion.
WHAT FACTORS MATTER

Performance factors

- Do not use VPP; use zero-loss filtering; defocus <1.5 μm; total exposure ≥60 e/Å²

![Graph showing performance factors](image.png)
IMPACT OF GRIDS

Benefits of Au foil grids

- More consistent grid quality – many squares with uniformly thin ice; support does not break