Biochemistry 9001
“Protein structure determination by 3D Electron Microscopy”

Tommi A. White, Ph.D.
Associate Director, Electron Microscopy Core Facility
Assistant Research Professor, Biochemistry
Goals of this course

• Introduce and familiarize you to concepts related to 3D Electron Microscopy and protein structure determination

• Provide hands on training and techniques to assist you in protein structure determination using electron microscopy
Instructors

Tommi A. White, Ph.D.

- Ph.D. Biochemistry, University of Missouri 2002-2007
  - Jack Tanner – “Structural Studies of Bacterial Proline Catabolic Enzymes”
- Post-doctoral Fellowship, National Cancer Institute at NIH 2007-2012
  - Sriram Subramaniam – “Cryo-Electron Tomography of SIV Envelope Glycoproteins”
- Associate Director, Electron Microscopy Core 2012-present
- Assistant Research Professor in Biochemistry 2012-present

Narahari Akkaladevi, Ph.D.

- Ph.D. Biochemistry, Montana State
- Post-doctoral Fellowship, Kansas University Medical Center
  - Mark Fisher – “Structural Studies of Anthrax Toxin Pores in Nanodiscs”
- Post-doctoral Fellowship, University of Missouri
  - Gerald Hazelbauer – “Structural Studies of Chemoreceptors in Nanodiscs”
Course structure

• 5 lectures
• 5 labs & lab reports
• 2 weeks for individual projects
  • Project Abstract
  • Project Presentation
  • Project report
Lectures

• Mondays: 10:30 am – noon, LSC 121
• Theory, examples from literature and EMC
Labs

• 2 hours: in EMC, W125-W137 Veterinary Medicine
• One-on-one individual time (sign up!)
  • Monday: 1-3pm
  • Tuesday: 9-11, 11-1, 3-5
  • Wednesday: 11-1
• Hands on applications, demonstration and Labs

• Register in “Facility Online Manager”
  • http://emc.missouri.edu/policies-info/
  • http://emc.missouri.edu/reservations/
Early 90’s had electron microscopes in
- Geological Sciences
- CAFNR
- School of Medicine
- Veterinary medicine
1995 combined into Electron Microscopy Core
- Hosted by Veterinary Pathology
- Located in Veterinary Medicine Building

Electron Microscopy Core (EMC)

- Materials Prep
  - FEI Scios FIBSEM
  - Hitachi S-4700 SEM
- JEOL 1400 TEM
- Staff Office
- Staff Specimen Preparation

Cryo Prep/Mechanical
- FEI Tecnai F30 TEM
- Client Sample Preparation
- Microtomy
- FEI Quanta 600 ESEM

Stairs
Projects

• before July 8th – Project consultation
• July 11th – Project Abstract, 3 slides
• July 11-22 – Project Data Collection/Processing
• July 22nd – Project Presentation, 15 min
• July 29th – Project Report, 2 page Microscopy & Microanalysis Paper format
High Performance Computation

• Apply for lewis3 account
  • [https://doit.missouri.edu/wp-content/uploads/2015/09/UMBC_Account_Request_Form.doc](https://doit.missouri.edu/wp-content/uploads/2015/09/UMBC_Account_Request_Form.doc)
  • UMBC Account Request Form and send to me by 6/10/2015
    • Software = EMAN2.1, >4 GB RAM/core, 12 cores

• Purchase a thumb drive
  • USB3, 16 GB
  • Boot from drive (Window start up screen, ex. “F12” button)
  • Set up username
    • First initial, last name
  • Generate ssh keys
    • [http://docs.rnet.missouri.edu/HowTo/ssh](http://docs.rnet.missouri.edu/HowTo/ssh)
    • Share only your PUBLIC key, never share your private

• Tutorials – Wed at 11 am in 1 Stewart Hall
Why do we need 3D?
What is 3DEM?
What is 3DEM?

• 3-Dimensional Electron Microscopy
  • Using electron microscopy to determine a structure in 3-dimensions
  • Proteins are embedded to maintain their shape
    • Heavy metal stain
    • Sugar (trehalose)
    • Ice (non-crystalline)
  • Examples:
    • Single Particle Reconstruction (Single Particle Analysis, Chen Lab)
    • Random Conical Tilt (RCT, Radermacher Lab)
    • Electron Tomography (Subvolume Averaging, Subramaniam)
    • Electron Crystallography (MicroED, Gonen Lab)
    • Serial Block Face Imaging (FIBSEM Tomography, Denk Lab)
3DEM Examples: Single Particle Reconstruction

Ribosome structures to near-atomic (4.1 Å) resolution from thirty thousand cryo-EM particles (2013)
Xiao-chen Bai, Israel S Fernandez, Greg McMullan, Sjors HW Scheres
3DEM Examples: Cryo-electron Tomography
3DEM Examples: Serial Block Face imaging
2.2 Å resolution cryo-EM structure of β-galactosidase in complex with a cell-permeant inhibitor
Nature’s Method of the Year 2015: Cryo-TEM

Slides Compliments of Jeff Lengyel, FEI Co.
THE REVOLUTION WILL NOT BE CRYSTALLIZED

NATURE | VOL 525 | 10 SEPTEMBER 2015
Structural Biology in the Post Genomics Era

Table 2  Success rates for major steps in structure determination

<table>
<thead>
<tr>
<th>Status</th>
<th>Total number of targets</th>
<th>% Success (step)</th>
<th>% Success (overall)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloned</td>
<td>125,316</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Expressed</td>
<td>83,115</td>
<td>66.3</td>
<td>66.3</td>
</tr>
<tr>
<td>Purified</td>
<td>29,409</td>
<td>35.4</td>
<td>23.5</td>
</tr>
<tr>
<td>Diffraction-quality crystals or NMR spectra</td>
<td>8,690</td>
<td>29.5</td>
<td>6.9</td>
</tr>
<tr>
<td>In PDB</td>
<td>5,811</td>
<td>66.9</td>
<td>4.6</td>
</tr>
</tbody>
</table>
Proteins Act in Complexes

A Human Protein-Protein Interaction Network

- automated Yeast two-hybrid system
- 3186 mostly novel protein interactions among 1705 proteins
- large highly connected network
- resulted in 911 high-confidence interactions among 401 proteins

The vast majority of these interactions involve dynamic protein complexes with sizes between 50 - 500kD.
Added Value of Cryo-TEM

Success rate

99% of all PDB XRC structures

New biological Insights

Statistical analysis of PDB entries by end 2012

New Era for Structural Biology

from protein monomer to complexes

99% of all PDB XRC structures

New biological Insights

Statistical analysis of PDB entries by end 2012

New Era for Structural Biology

from protein monomer to complexes
Complementarity of XRC, NMR and Cryo-TEM

Towards an integrative structural biology approach!!
Cryo-TEM is Unique

- protein complexes and molecular machines/assemblies
- molecular motions and dynamic processes
- heterogeneous samples
- no crystallization needed
Dramatic growth in cryo-TEM structures solved

Graph from the Protein Databank (PDB)
Ion Channel TRPV1 at 3.4Å Resolution

Maofu Liao, Erhu Cao, David Julius & Yifan Cheng
Nature 2013 504:: 107-112
Ribosome (2014):

Wong W, et al. eLife (2014) 3
Fernandez IS, Cell (2014) 157 pp. 823-831

And at least one more coming: 12 high impact publications: 1 /month only on ribosomes!
Membrane proteins:

TRPV1 (300 kD)

ABC-transporter (135 kD)

γ-secretase (170 kD)

Also membrane proteins are HOT!

Filaments:

Egelman group: actin, *Structure Cell* (accepted)

(M)any high resolution structure(s) seems to be a ticket for a high impact publication!
In-silico purification allows for high resolution structure determination of very low population ribosome translation initiation complex

6.6Å Eukaryotic Translational Initiation Complex. Low population of complex prevents (<3%) crystallization

Data collected on an FEI Tecnai Polara G2 with a Falcon II direct electron detector
Why?...

- Autoloading of samples
- Brighter electron sources
- Superior optics
- Superior vacuum
- Contrast enhancement
- Breakthrough cameras
Technology Breakthrough Meets Market Demands

Direct Electron Detection

Added Value of Cryo-TEM

The Resolution Revolution

Werner Kühlbrandt

SCIENCE VOL 343 28 MARCH 2014
Why electrons?
Serial Block Face Imaging
Tomography
Single Particle Analysis

Subramaniam 2005 *Curr Opin Microbiol.*, modified TAW
Electrons

- Involved in electricity, magnetism, thermal conductivity
- A moving electron creates a magnetic field
- Wave-particle duality
- Small particle
  - $9 \times 10^{-28}$ grams
- Negatively charged
  - $1.6 \times 10^{-19}$ Coulomb
- Higher wavelength
  - ~2 picometers @ 200 keV
- 90% the speed of light
  - 270,000 km/s
- Easily accelerated
  - resting energy 0.511 MeV
Single Particle Reconstruction

• “quantitative way of determining the structure of macromolecules from micrographs, showing them as a collection of isolated, unattached particles”

• Uses not one, but thousands of macromolecules

• Multiple orientations or “Views” in 2D projections

• Group same views together, align and average

• Reconstruct 3D from multiple 2D views

Frank, 2009, QrevBiophys
Comparison to X-ray Crystallography

• To determine the structure factors, need both
  • amplitudes
  • phase

• “Phase problem”
  • In diffraction patterns, you can only measure the intensity/amplitudes of the planes

• “Phase measuring diffractometer” - W. Hoppe, 1983
  • With TEM images, we measure both amplitude and phase
Transmission Electron Microscopy (TEM)
Transmission Electron Microscopy (TEM)

Diagram showing the components of TEM, including:
- Illumination Source
- Condenser Lens
- Specimen
- Objective Lens
- Projection Lens(es)
- Image Plane
- Fluorescent Screen
- LM

Legend:
- LM
- TEM
Filament (LaB6, FEG)

Condenser Lenses (C1 – beam size, C2 – intensity)

Condenser Aperture (reduces scattering)

Objective Lens (upper/lower pole piece)

Objective Aperture (contrast)

Projection Lens (3)

Fluorescent Screen

Digital Camera (CCD)
Vacuum

- Electron microscopes have high vacuums...why?
  - Electrons are very easily scattered
  - Want to control their trajectories
- Challenge for biological specimens
  - Most are hydrated (esp. proteins)
  - How to work around?
## Vacuum: Pumps

<table>
<thead>
<tr>
<th>Type</th>
<th>Range (mbar)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical</td>
<td>atm - $10^{-2}$</td>
<td>roughing, backing</td>
</tr>
<tr>
<td>Oil Diffusion</td>
<td>$10^{-1} - 10^{-9}$</td>
<td>main, column</td>
</tr>
<tr>
<td>Turbomolecular</td>
<td>$10^{-1} - 10^{-9}$</td>
<td>main, column</td>
</tr>
<tr>
<td>Ion</td>
<td>$10^{-5} - 10^{-12}$</td>
<td>column, gun</td>
</tr>
</tbody>
</table>
Filaments

Lanthanum Hexaboride

Schottky Field Emitter
Field Emission Gun: Types

**LaB6**
- JEOL JEM 1400
- Lanthanum Hexaboride
- Vacuum \(10^{-4}\)
- Crossover = 10 micron
- $1K

**Schottky FEG**
- FEI Tecnai F30 Twin
- Zirconium Oxide
- High Vacuum \(<10^{-6}\)
- 0.015 micron
- $12K
Spatial Coherence

- Perfect spatial coherence would be all electrons emit from the same point
- Smaller tips
  - more coherent
  - Better phase contrast
  - Better resolution
- Which tip has better spatial coherence?
Field Emission Gun (FEG)

- Source of electrons = the “gun”
  - Provides large stable current in a small electron beam
  - Source determines resolution
  - Tungsten (W) or ZrO$_2$

W&C p. 80
Field Emission Gun: How does it work?

- FEG = cathode (-300 kV)
- Anode 1 = “extraction voltage”
  - Positive by a few kV
  - Intense electric field
  - Electrons tunnel out
- Anode 2 = accelerates electrons
  - TEM = 50-300 kV

Produces electrons upon application of a large electric potential

Produces a “refined” electrostatic lens for crossover

W&C p.80
Lenses: purpose

- Magnify/Demagnify
- Focus
- Illumination
Lenses: what are they?

- Electromagnets
  - Vary...
    - current using a coil around a soft iron core
    - Affects resultant magnetic field

W&C p.97
Lenses

LENSES ARE FINITE
All lenses are imperfect insofar as they cannot gather all the radiation emitted by an object and so can never create a perfect image.

W&C p.93
Lens Abberations

Spherical

Chromatic

\[ C_s = 0 \]

\[ C_s \neq 0 \]

\[ d_{\text{min}} \]

\[ \beta \]

\[ \text{Disk diameter} = 0.5C_s\beta^3 \]

\[ \text{Disk diameter} = 2C_s\beta^3 \]

\[ \text{Plane of least confusion} \]

\[ \text{Gaussian image plane} \]

\[ \text{Energy-loss electrons} \]

\[ \text{No-loss electrons} \]

\[ \text{Plane of least confusion} \]

\[ \text{Gaussian image plane} \]
Lens Abberations: Astigmatism

Circular Cross Section Of The Electron Beam

Elliptical, Astigmatic Cross Section Of The Electron Beam

Weak Magnetic Field

Top View Of Electromagnetic Lens

Strong Magnetic Field

Astigmatic Electron Beam

Images a, b, c, d showing differences in electron beam cross-sections under varying magnetic fields.
Lens aberrations: Astigmatism

Astigmatism

Corrected Astigmatism
Apertures

• Strip of metal
• 4 Holes drilled with decreasing size
• Limits undesirable scattering, leading to noise in images.
Digital Camera

[Diagram of a digital camera with labels for various components such as Focal Point, Fluorescent screen and projection, Focusing screen, CCD camera, and Processor.]
Detector Quantum Efficiency

\[ DQE = \left( \frac{S_{\text{out}}}{N_{\text{out}}} \right)^2 \left( \frac{S_{\text{in}}}{N_{\text{in}}} \right)^2 \]

- Metric to compare efficiency of detection
- Where...
  - \( S = \) signal (electrons)
  - \( N = \) Noise (background)
- A perfect detector would have a DQE of 1
- Detectors have <1
Charge Coupled Device (CCD)

- Metal-insulator-silicon devices that store charge
- Each pixel in an array
  - is an individual capacitor
  - Isolated from each other
  - Collect charge in the well
  - Proportional to incident radiation intensity
- Our CCDs
  - JEOL = 2K x 2K
  - F30 = 4K x 4K
Renaissance in imaging...

- Direct electron Detection
- Revolutionizing cryo-EM
- Take movies and compensate for drift over course of acquisition
Dose

• TEM is Electro-magnetic Radiation

• Damage to sample
  • “knock-on” – incoming electron interacts with atomic nucleus
  • Mass loss (decrease in sample thickness)
  • Bubbling (hydrogen bonds broken)

• Limit doses given to sample
  • “Low Dose”
  • 20-80 e-/Å²
TEM Contrast mechanisms

Thicknes

Mass

Diffraction
Beam-specimen interactions

Elastic Scattering
• No Energy loss
• High angle (>5°)
• Interactions with nucleus or inner valence electrons

Inelastic Scattering
• Energy Loss
• Low angle <5°
• Interactions with outer valence electrons
• Lost energy as
  • X-rays
  • Plasmons
  • Beam damage
  • Heat/bubbling
Biochemistry 9001 – Lecture 2

“Protein structure determination by 3D Electron Microscopy”

Tommi A. White, Ph.D.
Associate Director, Electron Microscopy Core Facility
Assistant Research Professor, Biochemistry
Overview of Lecture 2

• Electron Optics
• Signal and Image formation
• Resolution
• Negative Staining
Electron Optics
How does a Lens work?

**MAGNIFY**

- Arrow object
- Parallel rays brought to a focus
- Arrow image

**FOCUS**

- Optic axis
- Point object
- Point image
- $\alpha$, $\beta$
Magnification

- Object plane
- Focal Plane
- Image Plane

- Magnification = \( \frac{d_i}{d_o} \)

- Thin Lens Criterion
  \[ \frac{1}{f} = \frac{1}{d_i} + \frac{1}{d_o} \]
Focusing – ray diagram

(A) Overfocus

(B) focus

(C) Underfocus

Object

Lens

Image plane

$\alpha_1$

$\alpha_2$
Signals and Image Formation
Focusing – Real Space (Images)

Overfocus  focus  Underfocus

Fresnel fringe = phase contrast mechanism
Focusing – Reciprocal Space (FFT)

- Overfocus
- Focus
- Underfocus

Thon Rings
Real vs. Reciprocal Space

- Reciprocal imaging projects the “focal plane”
- Real space imaging projects the “image plane”
Real vs Reciprocal Space

Real Space (nm)

Reciprocal (1/nm)

Measurements are in “nanometers”

Measurements are in “inverse nanometers”
Real vs. Reciprocal – Crystalline Specimens
Fast Fourier Transform

- Signals as sine waves
- Define a function describe a wave containing:
  - Amplitude
  - Phase
  - Frequency/Wavelength
  - Direction

**FOURIER TRANSFORM**
The Fourier transform of a function is an expression of that function as a ‘sum’ of frequencies; it is the frequency-domain representation of the function.
What does FFT show you?

- 2D complex sine wave that has been decomposed
- Frequency domain
  - High spatial frequency = small distances
Music and Hearing

- Each note = sine wave
- Chord = complex additive sine wave
- Ear receives chord
  - Decomposes each sine wave into a note
  - Ear performs a “FFT” on chord
Defocus

• Increasing defocus
• Increases contrast, low resolution terms

LaB₆

-30 nm

-66 nm

-190 nm

FEG
Next lecture...

- More information about FFT & CTF
- Also see Grant Jensen’s “Intro to Cryo-EM” Part 2 on YouTube

https://www.youtube.com/playlist?list=PL8_xPU5epJdctoHdQjpfHmd_z9WvGxK8-
Resolution
Resolution = Detector

- Pixels = “picture element”
- each pixel is “physically” 14 micron$^2$ on the CCD
  - Makes a 29 mm$^2$ array
- Depending on magnification, now each pixel correlates with a new unit of measure based on magnification calibrations.

14 micron

Pixel

14 micron

500 nm

Pixel @ 5000x

500 nm

50 nm

Pixel @ 50,000x

50 nm

5 nm

Pixel @ 500,000x

5 nm
Resolution = Focusing

- Closer to focus = higher spatial frequency, higher resolution
Resolution = Microscope

• Alignments (should be realigned at least daily)
• Apertures (smaller, decreases scattering)
• Abberations (Cs, Cc, Astigmatism, Distortions)
Resolution = Specimen

- Media
  - Negative staining; Stains have limited resolution
  - Vitreous ice; increase scattering with increased thickness

- Dose
  - Beam damage to specimen with increasing dose
Negative Staining
Examples of Negative Stain

70S Ribosome, 15 nM, 2% UA

KLH, 10 µg/mL 2% UA
Theory of Negative staining

T. Ruiz, Methods Mol Bio. 2006
Theory of Negative Staining

T. Ruiz, Methods Mol Bio. 2006
Negative stain

• Heavy metal stains
  • Uranyl Acetate
  • Uranyl Formate
  • Methyl Amine Tungstate
  • Vanadium
  • Phosphotungstic Acid
  • Ammonium Molybdate
Types of Negative Stains

**Specimen preparation**

- **Negative Staining**
  - Deep staining
  - Stains:
    - **Uranyl Acetate (Ted Pella, Powder)**
      - Concentration: 0.5-4%
      - Shelf life: ~ 6 months
      - Commonly used pH: ~ 4
      - Grids Life: ~ 1 year
    - **Uranyl Formate (Ted Pella, Powder)**
      - Concentration: 0.5-1%
      - Shelf life: make fresh
      - Commonly used pH: ~ 4-5,
      - Grids Life: N.A.
    - **Phosphotungstic Acid (Ted Pella, Powder)**
      - Concentration: 2%
      - Shelf life: ~ 3 weeks
      - Commonly used pH: ~ 7 (adjust pH NaOH or KOH)
      - Grids Life: ~ 4 weeks
    - **Ammonium Molybdate (Ted Pella, Powder)**
      - Concentration: 2%
      - Shelf life: ~ 2 weeks
      - Commonly used pH: ~ 7 (adjust with NH₄OH)
      - Grids Life: ~ 3 weeks
    - **Nanovan (Nanoprobes, Ready to use)**
      - Concentration: 2%
      - Shelf life: 1 year
      - Commonly used pH: ~ 7.5-8
      - E vanadate = 23; can substitute for GTP or ATP
    - **NanoW (Nanoprobes, Ready to use)**
      - Concentration: 2%
      - Shelf life: 1 year
      - Commonly used pH: ~ 7.5-8
      - Grids Life: ~ at least 1 year

T. Ruiz, Methods Mol Bio. 2006
Steps of Negative Staining

- Classical staining
  - Support: continuous carbon or continuous plastic coated with a carbon layer

- Apply a surface charge to the carbon film
  - Glow discharge
    - Plasma treatment
  - Negative (air)
  - Positive (methyl amine)
Steps of Negative Staining

1. Sample
2. Blot for 30 s - 1 min
3. Stain with 1%-2% Uranyl Acetate
4. Do not let dry
5. Blot
6. Dry fast
7. Rinse with buffer (w/ salt or stain)
Exploring Staining

Example: Phosphofructokinase

Methyl Amine Tungstate  Uranyl Acetate  Ammonium Molybdate
Difficult Specimens

- Difficult samples: Tricks
  - Rinse with stain droplets (5-10 droplets)
Difficult Specimens

- Wash over stain drops (3-5 drops)

50mM phosphate, see crystals falling

Good for Phosphate and Detergent containing buffers

If see change in droplet surface tension, add an extra drop of stain to wash

©Teresa Ruiz, Microscopy and Microanalysis Meeting 2013; Page 17
Negative Staining Advice

- Try more than one stain
  - Stains have charges and they can interact with your sample

- Simplify your buffer maintaining the sample integrity
  - Small impurities can have deleterious effects

- A good starting concentration
  - 10-20ug/ml for ~600 kDa, 40ug/ml for 1 MDa

- Magnification calibration
  - Add to your sample TMV (15-30ug/ml)

- How to recognize good areas in deep stain
  - Similar to ice

T. Ruiz, Methods Mol Bio. 2006
Lab 2: Negative Staining - KLH

• Explore Protein Concentration
• Staining methods
• Stain types

• Many variables, some you control others you don’t.
CTF Continued

- **CTF Explorer**
Biochemistry 9001 – Lecture 3

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Narahari Akkaladevi, Ph.D.
Postdoctoral fellow, Biochemistry

&

Tommi A. White, Ph.D.
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Assistant Research Professor, Biochemistry
## Overview from last week

- Image formation
- FFT of image
- Negative staining and data collection

## Overview of Lecture-3

- Particle picking & CTF correction
- 2D classification
- 3D reconstruction
Real vs Reciprocal Space

Real Space (nm)

Measurements are in “nanometers”

Reciprocal (1/nm)

Measurements are in “inverse nanometers”
Contrast Transfer Function (CTF)

- CTF is the function which modulates the amplitudes and phases of the electron diffraction pattern formed in the back focal plane of the objective lens.

Image formation

CTF function

Spatial frequency

Thon rings

FT

F{O} • CTF

FT^{-1}

FT

Object

lens

bfp

Image
Contrast Transfer Function

\[ CTF = T(k) = -\sin \left[ \frac{\pi}{2} C_s \lambda^3 k^4 + \pi \Delta f \lambda k^2 \right] \]

It is complicated curve which will depend on:
- \( C_s \) (the quality of objective lens defined by spherical aberration coefficient)
- \( \lambda \) (wave-length defined by accelerating voltage)
- \( \Delta f \) (the defocus value)
- \( k \) (spatial frequency)

Contrast Transfer Function-correction

\[ F\{I\} = F\{O\} \cdot CTF \]
\[ F\{O\} = F\{I\}/CTF \]

Take inverse F (\( F^{-1} \))

\[ O = F^{-1}[F\{I\}/CTF] \] → **CTF correction**
Image formation and CTF correction

Object (O) \[ \xrightarrow{\text{FT}} \] \[ \text{FT}(O) \cdot \text{CTF} \] \[ \xrightarrow{\text{FT}^{-1}} \] \[ \text{FT}^{-1}[\text{FT}(O) \cdot \text{CTF}] \]

Image formation

Image formation and CTF correction

\[ O = \text{FT}^{-1}[\text{FT}(I)/\text{CTF}] \]
(a) CTF curves, for a single defocus, (b) overlaid for two different defocus values. (c) shows multiple defocus values. (d) The sum of amplitude absolute values of all curves in (c), showing the overall transfer of spatial frequency components in a data set with the defocus distribution shown.

So, data collection need to be done at few different defocus values

Orlova et al. 2011
Particle picking

(1) Manual picking or (2) Automated picking using reference particle.

Boxing particles
Ideal box size is 2x particle size

Stack of particles

CTF Correction
Classification and 2D averaging

- Classification strategies are divided into "supervised" and "unsupervised"

- **Supervised** (Model-based classification): divide according to similarity with "reference"

- **Unsupervised** (Reference-free classification): divide according to intrinsic properties.
Reference free 2D class averages of GroEL using EMAN2
3D Reconstruction—Projection theorem

FT(projection of an object) = a central section of the 3D FT(object)
Orientation of section changes in the same way as that of the projection

FT (duck) = 3D FT in reciprocal space

FT (2D EM image of duck)
= 2D FT
= one central section of 3D FT

FT (90° projection of duck)
= central section of 3D FT 90° from the first 2D FT

Putting the 2D FT’s together in the correct angular orientation gives partial reconstruction of 3D FT

Enough different central sections
=> good approximation of 3D FT
=> 3D reconstruction by FT$^{-1}$
Three known methods

(i) Multiple common lines

(ii) Random-conical reconstruction

(iii) Projection matching.

First two approaches are described here to obtain an *ab initio* structure: random-conical tilt (RCT) and common lines.

Third one is when an initial 3D model exists, reference-based alignment can be used.
Surface rendered views, projections, and transform sections of a structure, with a common line intersection illustrated in reciprocal space. The structure has rotational symmetry, and there are several symmetry-related common lines. From the angles between common line projections of different views, the relative Euler-angle orientations of a set of projections can be determined.

Orlova et al. 2011
Common lines

**Advantage:** More useful in high-symmetry specimens, like icosahedral viruses

**Problem:** Very difficult in noisy images, especially in particles with low symmetry
Random Conical Tilt

(a) The original object, with arrows indicating the angular directions of the data projections. (b) Representation of the sections in Fourier space corresponding to data projections collected within this cone of angles. (c) A central cross section of the planes in Fourier space is shown in. (d) A surface view of the resulting conical tilt reconstruction, surrounded by the eight projections corresponding to the original view directions.

Limitations

- Technically difficult to get good quality images at high tilt, because of specimen thickness and microscope stage stability, especially for cryo-EM.
- The tilted images will have a gradient of defocus.
**Projection matching procedure.** A set of images is compared to a set of references from an initial model (low resolution). Once the best matching found between the image and one of the references (reprojections), based on the height of the correlation peak, the shift relative to the matching reference and angles of that reference are assigned to the image. Images 1 and 6 have the best correlation with model projection a (red arrows), while Images 2 and 5 match image e (blue arrows).

*Orlova et al. 2011*
Projection Matching

Advantages:

- Only one set of images from each field needed
- Can use all projections represented in the image: no initial sorting of one type only
- If an adequately wide range of viewing directions is present, no missing cone
- Procedure yields a method for refinement of the reconstruction (same one used for all other methods of making a reconstruction)

Disadvantages:

- Need to use some information or assumption to model: potential for bias toward initial model
- If only a limited number of different projections are present in the micrograph, cannot get the complete angular range required
The Steps Involved in Structure Determination by Single-Particle Cryo-EM

A single-particle project should start with a characterization of the specimen in negative stain (left arm of the workflow).

Only once the EM images, or potentially 2D class averages, are satisfactory, i.e., the particles are mono-disperse and show little aggregation and a manageable degree of heterogeneity (low-resolution sample refinement), is the sample ready for analysis by cryo-EM (right arm of the workflow).

The images, 2D class averages and 3D maps obtained with vitrified specimens may indicate that the sample requires further improvement to reach near-atomic resolution (high-resolution sample refinement).

Cheng et al. 2015
Fourier Shell (Ring) Correlation

Fourier Ring/Shell Correlation, FRC (2D)/FSC (3D)

Split of data into two halves, average or reconstruction calculated, but measure is correlation of F’s (amp & phase) with each other, averaged over the ring or shell

$$FSC = \frac{\sum(F_1 \cdot F_2)}{\big(\sum F_1^2 \cdot \sum F_2^2\big)^{1/2}}$$

Correlation ~1 at low resolution, drops with resolution, ideally sharply

Calculation: FT(avg or recon) divided radially into increments of resolution
- calculate a measure of similarity between corresponding pixels or voxels of the two halves

Criteria for resolution cut-off:
FSC = 0.5 : arbitrary but “sensible” threshold
Biochemistry 9001 – Lecture 4

“Protein structure determination by 3D Electron Microscopy”

Tommi A. White, Ph.D.
Associate Director, Electron Microscopy Core Facility
Assistant Research Professor, Biochemistry
Overview of Lecture 4

• Review of Contrast Transfer Function
• Cryo-EM
• Vitrification
• Beam Damage & Low Dose
Review of CTF
What does CTF describe?

• Electron beam interacts with specimen
• Exits specimen
  • some electrons have shifted phase
• Passes through lenses
• Forms image
If everything was perfect...

- Specimen = 3D arrangement of atoms = ρ(x,y,z) = ρ(r)
- Scattering from specimen = FT[ρ(r)] = F(S)
- Scattered and unscattered electrons captured by lens, which performs an inverse FT on them = image formation

Focused unscattered electrons => bright background
Focused scattered electrons => FT⁻¹[F(S)] = r(x,y) = 2D image (intensity variations from background) = projection of densities in r(x,y,z)
Note: F(S) contains both scattering amplitude and phase information, unlike I(S):
Both AMPLITUDE & PHASE are needed for (and contained in) the image r(x,y)
Real-world image formation

- Electron optical defects (primarily $C_s$, beam incoherence) prevent perfect operation of lens
- Imperfections grouped into a “contrast transfer function” (CTF) that is inextricably linked to the objective lens
- $F(S)$ is multiplied by the CTF before the action of the lens ($FT^{-1}$)

![Diagram showing electron beam, scattering, focusing, and resulting image with noise]

- Effect of CTF is seen in image as changes from correct features of image
  - get $\rho'(x,y)$ instead of $\rho(x,y)$
- Also have noise in image due to sub-optimal dose: less than ideal S/N statistics
CTF “terms”

Contrast Transfer Function

\[ \text{CTF} = T(k) = - \sin \left( \frac{\pi}{2} C_s \lambda^3 k^4 + \pi \Delta f \lambda k^2 \right) \]

It is complicated curve which will depend on:

- \( C_s \) (the quality of objective lens defined by spherical aberration coefficient)
- \( \lambda \) (wavelength defined by accelerating voltage)
- \( \Delta f \) (the defocus value)
- \( k \) (spatial frequency)
CTF Continued

- CTF Explorer
- http://www.maxsiderov.com/ctfexplorer/
Cryo-EM
Why Cryo-EM?

• A form of electron microscopy
• Specimen is studied at cryogenic temperatures (-170 C)
• Rapid freezing (“Cryo-fixation”)
• Native “frozen, hydrated” state
Cryo-EM

• Biological specimens are hydrated
• Electron microscopes have high vacuums

• Water ≠ Vacuums

• What to do? Freeze them...
Cryo-EM

- Requires “ice” formation
- But not just any kind of ice...
Water Phase diagram

Robards & Sleytr, 1985
Vitrification

• Rapid Freezing...

Freezing rate $= \frac{1}{\text{specimen size}}$
(Specific) Heat Capacity of Water

Specific Heat Capacity ($c_p$) = amount of heat needed to raise the temperature of a unit of mass by 1 degree

<table>
<thead>
<tr>
<th>State</th>
<th>$c_p$ (J/mol/K)</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid H2O</td>
<td>75</td>
<td>-35</td>
</tr>
<tr>
<td>Crystal H2O</td>
<td>33</td>
<td>-35</td>
</tr>
<tr>
<td>Supercooled H2O</td>
<td>105</td>
<td>-35</td>
</tr>
</tbody>
</table>

Robards & Sleytr, 1985
Vitrification

• Rapid Freezing...

\[
\text{Freezing rate} = \frac{1}{\text{specimen size}}
\]

• Prevent assembly of water molecules into a crystalline lattice
Crystalline forms of ice

- Tetragonal
- Hexagonal

J. Dubochet, 2009
Robards & Sleytr, 1985
Crystalline forms of ice

Three forms of ice as imaged in TEM at -170 C (outer). Inner shows X-ray diffraction of those ice forms.

J. Dubochet, 1988
How to achieve vitrification?

• Plunge freezing
• High Pressure Freezing
• Adding cryo-protectants
Plunge freezing

• Mayer & Bruggeller 1980 produced vitrified water
• Jacques Dubochet & McDowall 1981 produced a thin layer of vitrified liquid water

How????
Need something to vitrify water

• High boiling point
• High heat capacity
  • Prevent a layer of vapor forming between the sample and cryogen (Bellare, 1999)
• High thermal conductivity
  • A rate of $10^5$ K/s (Dubochet & McDowall, 1981)
  • Pull heat away from specimen
• A freezing point below specimen vitrification temp

Dobro, et al. 2010 Meth Enzymol
Ethane or Propane

• High boiling point
  • -89 C

• High heat capacity
  • 65 J/mol/K at -179 C

• High thermal conductivity
  • A rate of 13,000 K/s (Dubochet & McDowall, 1981)

• A freezing point below specimen vitrification temp
  • -179 C
Plunge Freezing
Side view

Incoming electrons

carbon

ice

carbon

30 nm
Another way to vitrify: High pressure freezing (HPF) drastically reduces nucleation and thus water crystal growth rate

- Lowering the freezing point of water to $<-90 \, ^\circ C$
- Rapid freezing of samples under high pressure ($>2000 \, \text{bar}$)
  - Cooling time from room temperature to $-100 \, ^\circ C < 15 \, \text{ms}$

Studer et al. 2008
The problem of cryo-fixation: Ice crystal formation

- Fast freezing at the surface
- Increasing size of ice crystals due to slower freezing rates in the depth of the sample
Low Dose & Radiation Damage
Biological Specimens are sensitive

• Radiation damage
  • Hydrolysis
  • Protein denaturation

• Increased by decreasing voltage
  • Electrons have more time to interact with specimen
  • Larger contrast with more interaction

• Apply controlled dose
  • SPR = 20-30 electrons/Angstrom$^2$
  • Tomography = 50-80 electrons/Angstrom$^2$
How to apply controlled dose

• **Search** at low magnification with
  • beam a low currents (increase “spot size”)

• **Focus** area
  • Perform all alignments, corrections, and defocusing
  • As close to specimen as possible, but beam not overlap
  • Same settings as “Exposure” only offset a certain amount

• **Exposure** area
  • One shot
  • After determining all settings and performing all alignments, corrections and defocusing
  • Take timed exposure with known dose
FEI LowDose panel

Low dose

Status: LD on, Focus state 2

Search

Low Dose  Blank  Peek

Focus

TEM Mi  3960x  Spot 5  Int 62.26  x 0.000 um  y 0.000 um

TEM SA  97200x  Spot 5  Int 66.46  4.31 um  156.8°

Exposure

43200x  Spot 2  Int 66.46  1.0 s

Exposure time (sec)  1.0

Use spotscan

Dim Screen

Wait (sec) after plate in  0

Pre-expose (sec)  0.0

Wait after pre-exposure  0.0

Series

Start

Start
FEI LowDose panel: Search Mode

Status: LD on, Focus state 2

TEM Mi
4200x
Spot 6
Int 62.26
x 0.000 um
y 0.000 um

TEM SA
31000x
Spot 4
Int 66.46
4.31 um
156.8°
1.0 s

Exposure time (sec) 1.0
Wait (sec) after plate in 0
Pre-expose (sec) 0.0
Wait after pre-exposure 0.0
FEI LowDose panel: Exposure Mode
3.2.2 Focus panel

Focus distance (um)

Focus angle (degrees)
Effect of objective aperture: enhances contrast!
Grids

- Quantifoil
  - 100 nm carbon
- Protochips Cflat
  - 50 nm carbon
Quantifoil MultiA
3.2.2 Focus panel

Focus distance (um)

Focus angle (degrees)
Dose Calculations

• In vacuum (hole)
• See the total counts (10,000)
• Convert “counts to e” (26 counts/electron 200kV)
• Divide by pixels (4096 x 4096, no binning)
• Divide by length of exposure (1 sec)

Electrons
Angstrom^2 * second
Work from my mentor: Dan Shi

• Effects of various parameters on vitreous ice
Optimize data quality for low contrast particles

Contrast vs. ice thickness

$\alpha$-$2\epsilon$-2 subunit of ACDS

Ice thickness 30 nm – 40 nm

Ice thickness thicker than 70 nm

Defocus value -2.63 $\mu$m
Contrast vs. Dosage

Nucleosome complex (NCP)

First NCP images of 15 e/Å² and Second image of ~45 e/Å² with -2.63 μm defocus
Contrast vs. High tension
HIV gp140 and gp140+fabs

- Gp140+fabs, Polara FEG 200 kv
- gp140+fabs, Krios FEG 80 kv
Particle contrast varied in different salt concentration buffers

HM image of frozen-hydrated sample in high salt concentration (>200 mM)

HM image from frozen-hydrated sample in low salt concentration (<30 mM)
Electron sources with different coherences

E2CD core

- FEG filament, 120 kV, x59k
- LaB$_6$ filament, 120 kV, x52k
Good References

- Low Temperature Methods in Biological Electron Microscopy
  - A. W. Robards and U. B. Sleytr

- Handbook of Cryo-Preparation Methods for Electron Microscopy
  - Edited by Annie Cavalier, Danièle Spehner, Bruno M. Humbel
Biochemistry 9001 – Lecture 5

“Protein structure determination by 3D Electron Microscopy”

Tommi A. White, Ph.D.
Associate Director, Electron Microscopy Core Facility
Assistant Research Professor, Biochemistry
Overview of Lecture 5: Advanced Topics

- Hybrid Methods
- Direct Detectors
- Phase plates
- Review of literature
Hybrid Methods
What are hybrid methods?

- Random Conical Tilt
- Cryo-electron Tomography
- Single Particle Tomography
- Random Conical Tilt
- 2D Crystallography
- Micro-electron diffraction (Electron Crystallography Tomography)
Data Collection

Single Particle Reconstruction
- One image per particle
- \( \sim 20-50 \text{ e/Å}^2 \)
- Need many particles \((10^5)\)

Electron beam

Sample

Detector

Electron Tomography
- Multiple images per particle
- \( \sim 1-2 \text{ e/Å}^2 \)
- Need fewer particles \((100-1000)\)

Janic, et al. 2008 J Microscopy
Central Slice Theorem

The section/projection theorem:

$$\text{FT}[ \int f(x,y,z) dx ] = F(0,Y,Z)$$

USE: The Fourier transform of a projection of a 3D object is equal to a central section of the 3D Fourier transform of the object.

An electron micrograph is a projection of a 3D object.

Its transform provides one slice of the 3D transform of the 3D object.

By combining the transforms of different views, one builds up the 3D transform section by section.

One then uses the IFT to convert the 3D transform into a 3D image.
Cryo-electron Tomography

(a) Virus
(b) Pipette
(c) Forceps with EM grid loaded
   Vitrification apparatus
   Liquid ethane bath
(d) Cartridges loaded with EM grids under liquid nitrogen
(e) Microscope column
   Tilt images captured
(f) Tomogram
Unaligned raw tilt series
Aligned raw tilt series
Tomogram Reconstruction

- automated fiducial based tilt series alignment (Amat, et al. 2010)
- weighted backprojection (Kremer, et al. 1996)
Cryo-ET Subvolume averaging (Single Particle Tomography)
Subvolumes

White, et al. 2010 *PLoS Pathogens*
Iterative alignment, classification and averaging

• automatically select spikes (template-matching)
• Starting model
  • $360^\circ$ symmetrized reference composed of all spikes
  • Minimizes model bias
• Use to center and align individual spikes
Why subvolume averaging in Cryo-ET?

1. Boost SNR
   – Biological material sensitive to radiation
   – Individual volumes not enough SNR for direct structural interpretation

2. Get rid of occlusions (missing wedge)
   – Obtain complete representation (without gaps) by stitching pieces together
Missing Wedge Effects on real space reconstruction

Subramaniam, et al. 2007 Curr Opin Struct Biol
Alignment through classification strategy
Accounting for missing wedge:
alignment and classification

1\textsuperscript{st} Volume 1D Signal (Fourier space)

2\textsuperscript{nd} Volume 1D Signal (Fourier space)

Signal overlap

Iterative alignment, classification and averaging

Iteration 1 (2,3)

- Classification (use PCA to divide into 10 classes)
  - select most trimeric class in gp120
- Refine alignments
- 50-60% most trimeric classes used as reference for next iteration
Iterative alignment, classification and averaging

Iteration 4

- Impose 3-fold symmetry
- Classification (10 classes)
- Refine alignments
- 50-60% most trimeric classes as reference for next iteration
sCD4-bound SIV Env

unliganded

SIV\textsubscript{mne} E11s
SIV\textsubscript{mac} 239
SIV CP-MAC

White, et al. 2010 JVI
Resolution $\sim 21 \, \text{Å}$
7D3-bound CP-MAC

White, et al. 2010 PLoS Pathogens
White, et al. 2011 JVI
Tilt pairs (Random Conical Tilt)

• Required to validate structure
  • “Tilt Pair Validation”

• Tilting to known angle, imaging, then assigning angles

• See Radermacher, Meth Mol Bio v. 319 for detailed protocol
2D Electron Crystallography

• Membrane proteins
• Assemble into 2D lattice

H. Stahlberg, cyclic nucleotide gated potassium channel MloK1
Electron crystallography

Crystalline vs. non-ordered specimens:

1. all information in **diffraction pattern** or **FT(image)** is concentrated in discrete points => high S/N

2. Ease of averaging: precise spatial relationship of molecules with respect to one another => no alignment problem

**Optical (or pseudo-optical) Fourier filtration:** Spatial separation of signal from (most) of noise in FT allows filtration to greatly reduce noise

---

**Lattice of ducks**

Add noise (lots of it)

Calculate FT & mask to keep only spots

Calculate FT⁻¹ of masked FT

Most noise gone, Some features lost
FT of a 2D crystal

FT of a 2D lattice is a **2D lattice of rods**:
- **crystalline sampling** in the plane of the lattice
- **continuous FT normal** to the plane

Diffraction pattern of a 2D crystal recorded by a beam normal to the crystal plate is a 2D lattice of spots, on the reciprocal lattice of the crystal lattice

This 2D lattice is the intersection of the 3D FT of the crystal with the Ewald sphere
  (for a very short wavelength of radiation, like 100KV electrons, the Ewald sphere is approximated by a plane, except at very high resolution)

These same spots are obtained either by:
  1. Collecting a diffraction pattern, or
  2. Calculating the FT of an image
Collecting 3D information

3D data is collected by **tilting the crystal** (like XRD), and collecting the diffraction intensity at the intersection of each reciprocal space rod with the Ewald plane.

Shown below: a tilt of the specimen by an angle $\psi$ tilts the reciprocal space rods by $\psi$, yielding a non-zero value of $z^*$ along each rod.

- $z^*$ for any tilt **varies with the resolution** of the rod ($h$ or $k = 1,2,3\ldots$)

Note: $z^*$ is more limited for low-resolution lattice rods than for high-resolution rods.

Open circles show the sampling of the lattice rods at zero tilt angle ($\psi$ in the figure).
Micro-Electron Diffraction
MicroElectron Diffraction
Direct Electron Detectors
Charge Coupled Device (CCD)
Charge Coupled Device (CCD)

- Metal-insulator-silicon devices that store charge
- Each pixel in an array
  - is an individual capacitor
  - Isolated from each other
  - Collect charge in the well
  - Proportional to incident radiation intensity
- Our CCDs
  - JEOL = 2K x 2K
  - F30 = 4K x 4K
CCD - Indirect Electron detector

• Uses Phosphor to convert Electron energy to light

• “Counts per Electron”
  • Example: Ultrascan 4000 = 26 counts per electron at 200 kV

Mullan, et al. 2009 Ultramicroscopy
Detector Quantum Efficiency

- Metric to compare efficiency of detection
- Where...
  - $S = \text{signal (electrons)}$
  - $N = \text{Noise (background)}$
- A perfect detector would have a DQE of 1
- Detectors have $<1$

$$DQE = \left( \frac{S_{\text{out}}}{N_{\text{out}}} \right)^2 \left( \frac{S_{\text{in}}}{N_{\text{in}}} \right)^2$$
Graph showing the comparison of Detector quantum efficiency (DQE) between US4000 U-type scintillator and SO-163 film. The x-axis represents the fraction of physical Nyquist frequency, while the y-axis shows the DQE. The curve for US4000 U-type scintillator is purple, and the curve for SO-163 film is red.
Loss of high resolution information

• Great SNR at Low resolution
• Poor Spatial Resolution
  • At High Resolution (Or high spatial Frequency), there is decreased intensity variations (or decreased Modulation transfer function)
• Okay for Radiation insensitive specimens
  • Materials science
  • Increase Exposure time to gather more signal
• Bad for Radiation sensitive specimens
  • Biological Specimens
  • Increase In Exposure time causes damage to specimen
Modulation Transfer Function

\[
MTF = \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}} + I_{\text{min}}}
\]
What is direct electron detection?

• Complimentary Metal Oxide Semiconductor
  • Circuits
    • Use Metal-Oxide-SemiConductor Field Effect Transistors
    • Dissipate Less Power
    • Dominate current integrated circuit manufacturing

McMullan, et al. 2009 Ultramicroscopy
CCD Camera

- Scintillator
- Fiber Optic Coupling
- CCD Sensor
- Cooling Device

Direct Detection Camera

- DDD Sensor
- Cooling Device

No signal degradation of Scintillator Fiber Optic Coupling
Is it really just the CMOS?
Noisy images

• Low signal to noise

• Due to
  • Movement of specimen upon exposure (blurring)
  • Poor performance of cameras at low doses (too much = bubbling/burning)
Blur

Griegorieff, 2013, eLife
Not all the same trajectory
Movie Mode

• New Direct Electron Detectors acquire Movies
• 40-60 frames a second
• “Cherry-Pick” which frames to use
• Then align and averaging those Frames
Excellent sensitivity and SNR so that each raw frame contains usable information.

Direct detection delivers high resolution.

High frame rate with no dead time between frames.
Blurred vs. Aligned/Averaged
Manufacturers

• Gatan “K2”
• FEI “Falcon”
• Direct Electron
Phase Plates

X-ray Crystallography

Cryo-EM
Phase Plate – What is it?

- Low resolution contrast can be increased by applying high defocus (typically 1 micron), but as a consequence, there is contrast loss at high resolution (reduction of contrast envelope, many contrast inversions)
FEI Volta Phase Plate

Principle: Central beam of the diffraction plane sets up a “Volta Potential”

This potential shifts the phase of the unscattered electrons by roughly 90°. Due to this phase shift, the contrast in the image is improved significantly!

After a certain amount of images, the effect decreases, and a new area on the Phase Plate can be used (to be automated by the FEI Application Software).

Phase shift is “tunable” depending on the delivered electron dose.
Cryo-tomography - Synaptosomes

CTEM def. 5 um

VPP-TEM def. 1 um

Sample & data collection by Shoh
Cryo-tomography - Primary neuron culture

- Dose: ~ 100 e/A².
Sub-tomogram averaging of 26S Proteosomes

Cryo-tomography - Worm sperm

Sample & data collection by M. Khoshouei
SPA with VPP: 20S Proteosome

VPP
$\Delta Z = 0 \mu m$

Conventional TEM
$\Delta Z = -1.6 \mu m$

Scale = 50 nm; Danev & Baumeister, 2016
VPP Data collection

• Focus in “F”
  • Perform linear interpolation to determine defocus

• Collect in “D”
  • 4 images with direct detector

Danev & Baumeister, 2016
Principle: Central beam of the diffraction plane sets up a “Volta Potential”

This potential shifts the phase of the unscattered electrons by roughly 90°. Due to this phase shift, the contrast in the image is improved significantly!

After a certain amount of images, the effect decreases, and a new area on the Phase Plate can be used (to be automated by the FEI Application Software)

• The “Volta Potential” is continuously changing over use
• Thus the VPP is a “consumable”

Phase shift is “tunable” depending on the delivered electron dose.
• Need very careful focusing to collect “in focus” data
Abstract Presentations

• 5 – 10 minutes
• 3 slides
  • Slide 1 – Introduction
  • Slide 2 – Materials and Methods
  • Slide 3 – Anticipated Results, Caveats/Pitfalls
Gabriella Kiss, FEI Co.