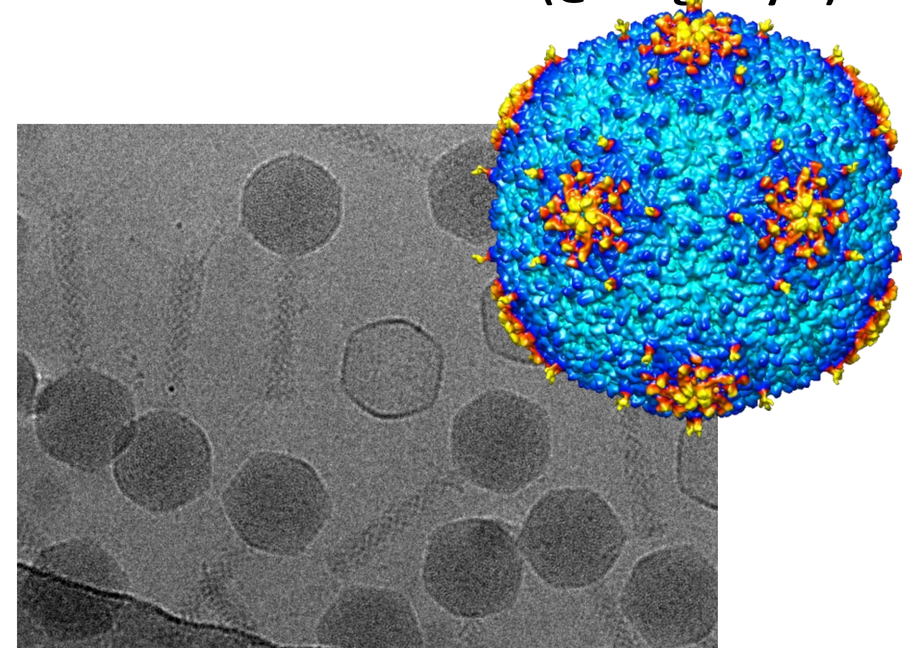
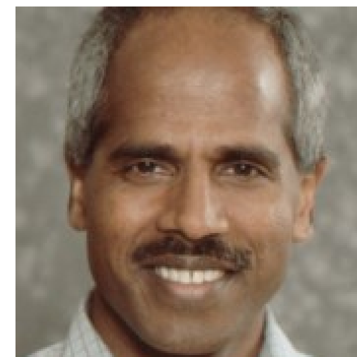
Those from whom I stole

Dr. Kristin Parent
("KP", course coordinator)

Dr. Sundharraman Subramanian
("Sundhar")

Research in the Parent lab (@Phage4Lyfe)





Dr. Kaillathe Padmanabhan
("Pappan")

Trivial, But Important: Size Matters

1 nm = 1×10^{-9} meter = one billionth of a meter

1 nm = 10 Å (Ångstrom)

1 µm = 1×10^{-6} meter

1 µm = 1000 nm

1 µm = 10,000Å

Trivial, But Important: Size Matters

.5 nm | 4 nm. | 20 nm. | 2000 nm | $7x10^6$nm | $8x10^7$ nm | $1.8x10^9$ nm

10^1 10^4 10^8 10^{12} 10^{16} 10^{20} 10^{24} 10^{28} Daltons

X-ray

NMR

Light

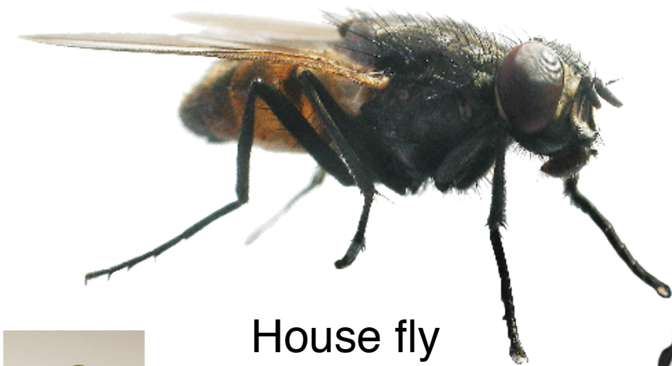
Electrons

Cryo-electron microscopy

From Subramaniam (2005) Fig. 1, p.2

How big/little specimens really are

Scale: 2^7
128X



House fly

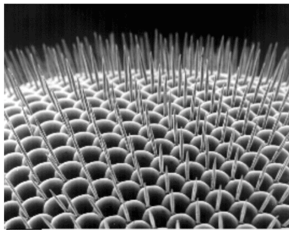
Needle and thread

5.0 mm

How big/little specimens really are

Scale: 2^{12}
4096X

Bed bug


Drosophila eye

Drosophila

200 μm

How big/little specimens really are

Scale: 2^{16}
65,536X

RBC

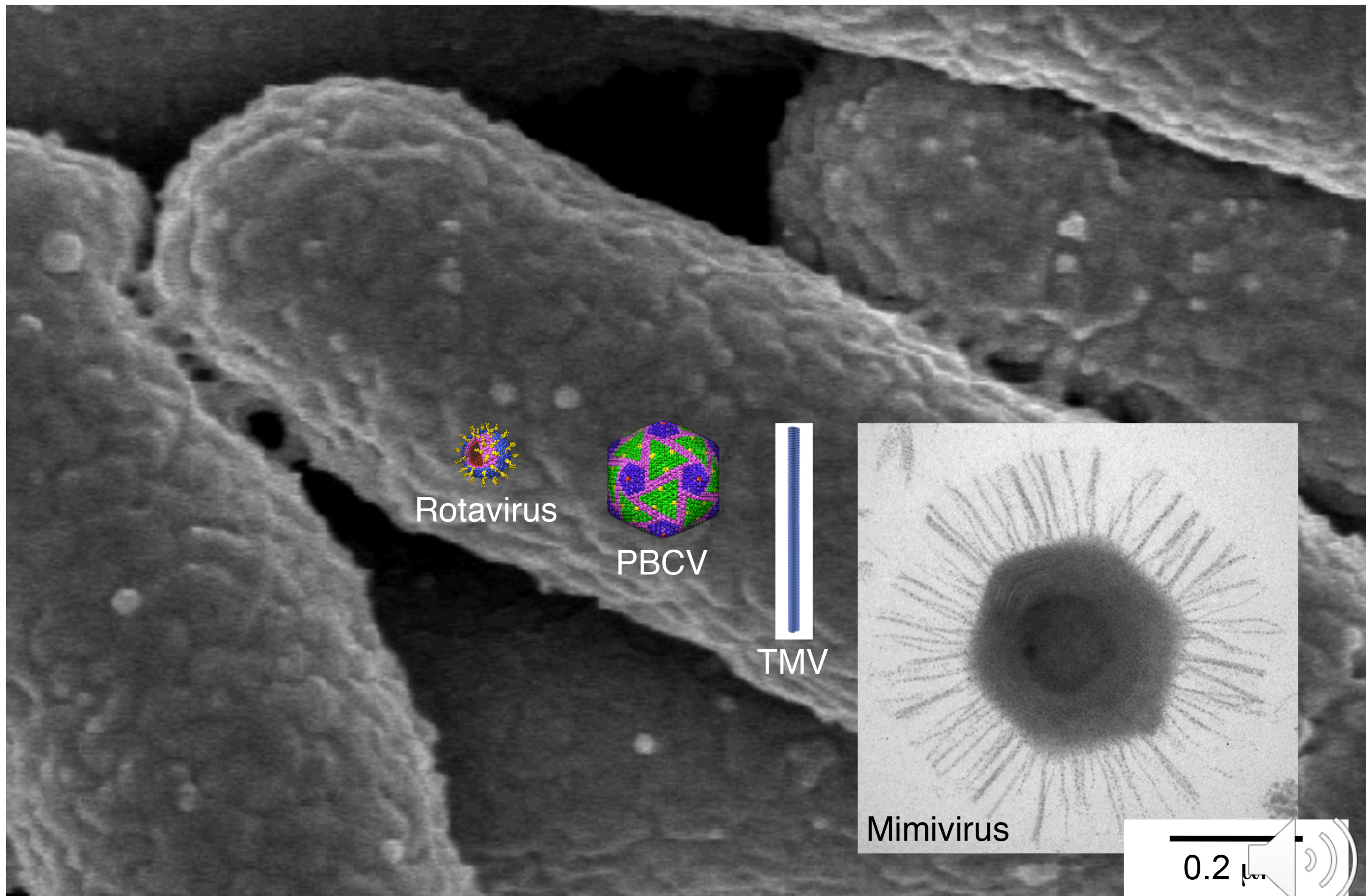
E. Coli

Drosophila eye

10 μm

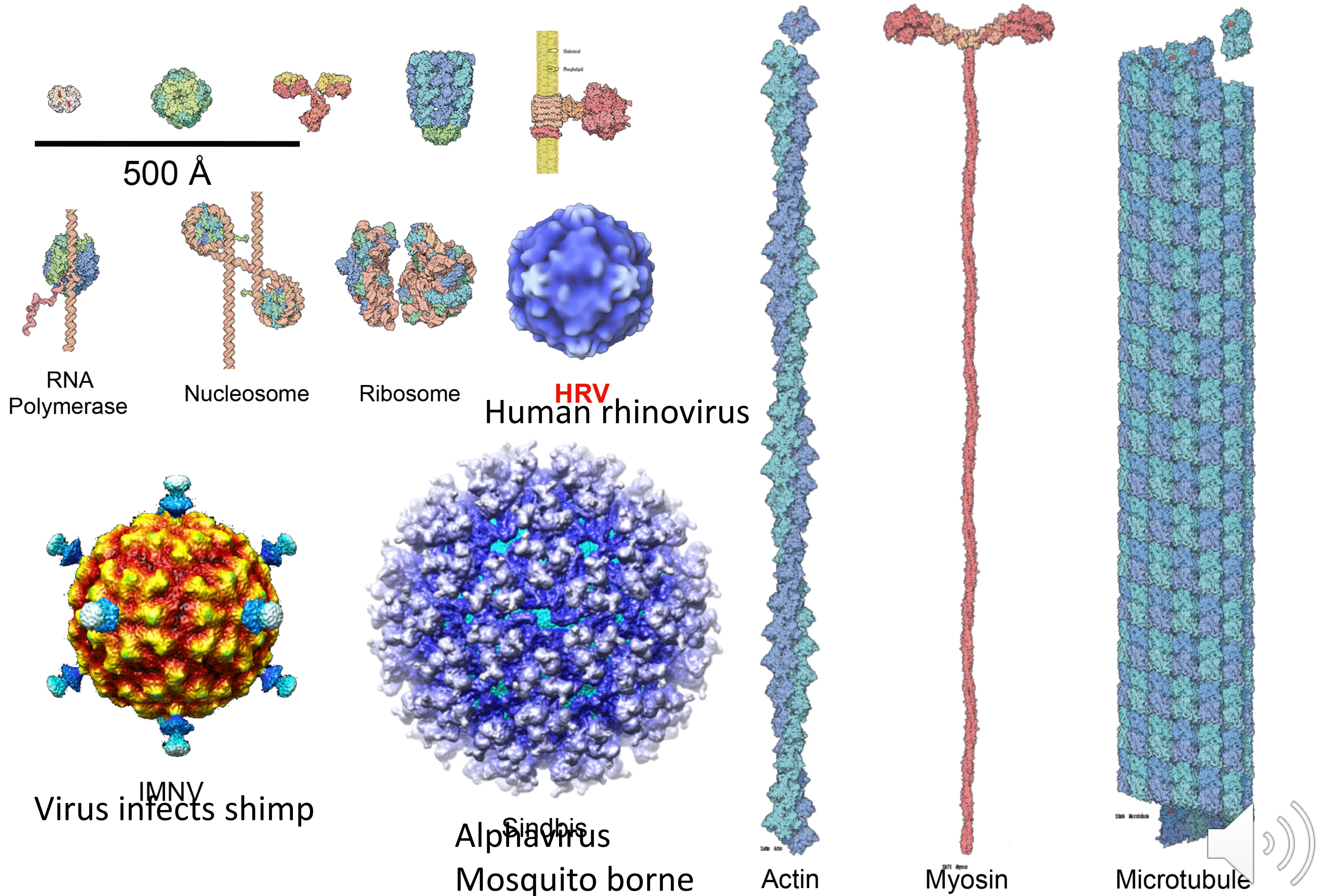
How big/little specimens really are

Scale: 2^{21}
2,097,152X



How big/little specimens really are

Scale: 2^{24}
16,777,216X



How can we visualize objects in that size range?

Principles: Comparison of Optical and Electron Microscopy

Key Concept:
Electrons and photons have common properties!
Both can be used to form images
Because both can be **Focused.**
To focus the beam must be ***Bent.***
X-rays cannot be focused!

Principles: Comparison of Optical and Electron Microscopy

Lamp

Source

Filament

ILLUMINATION SYSTEM

Condenser Lens(es)

SPECIMEN STAGE

Objective Lens

IMAGING SYSTEM

Projector Lens(es)

Viewing Screen

VIEWING AND IMAGE RECORDING SYSTEM

Light Microscope

Electron Microscope

Similarities between Optical and Electron Microscopes

Illumination system

Radiation source directed towards specimen

Condenser lenses to focus illumination beam

Specimen stage

Positions specimen between illumination and imaging system

Imaging system

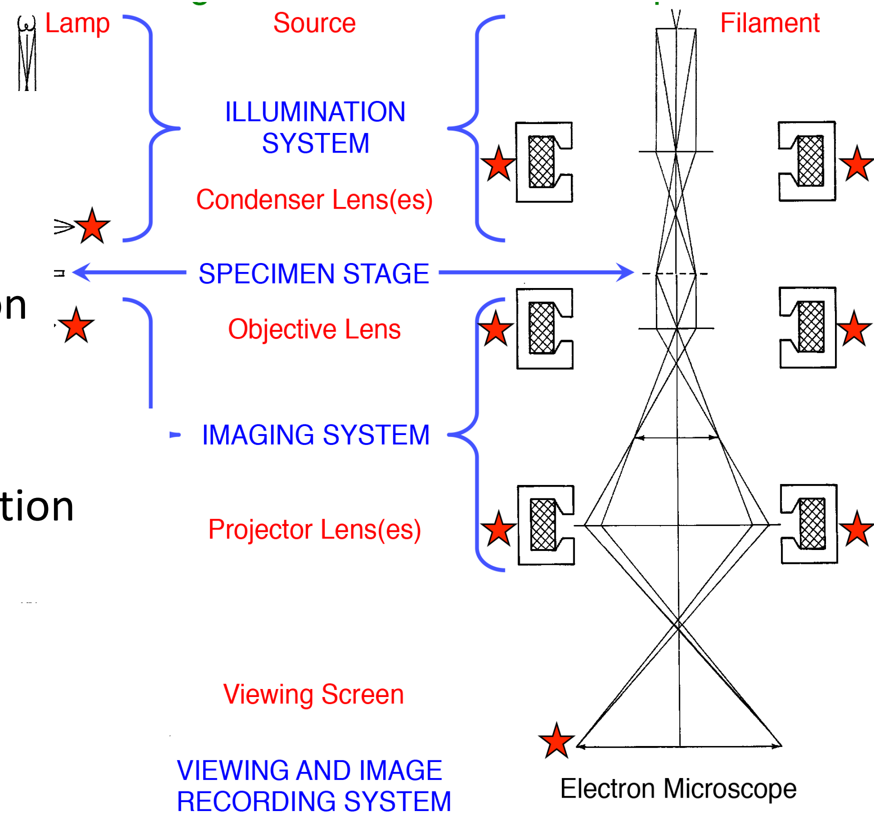
Lenses that produce the final image

Objective lens

Projector lenses

Converts radiation to permanent image

Some camera type to capture image



How is instrument resolution determined?

Photons and electrons behave as **particles** and **waves**

Any moving particle has a wavelength associated with it

TEM: **electrons** travel very fast (near speed of light) and have **short wavelengths**

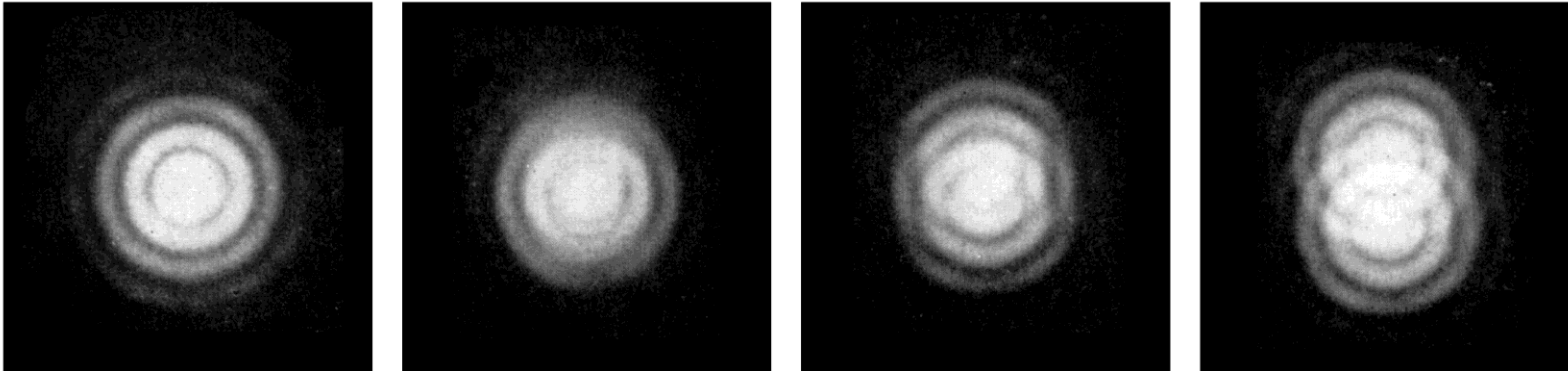
Resolution: ability to distinguish objects or object details

Instrument resolution: *limited by wavelength of radiation*

Practical limits of resolution

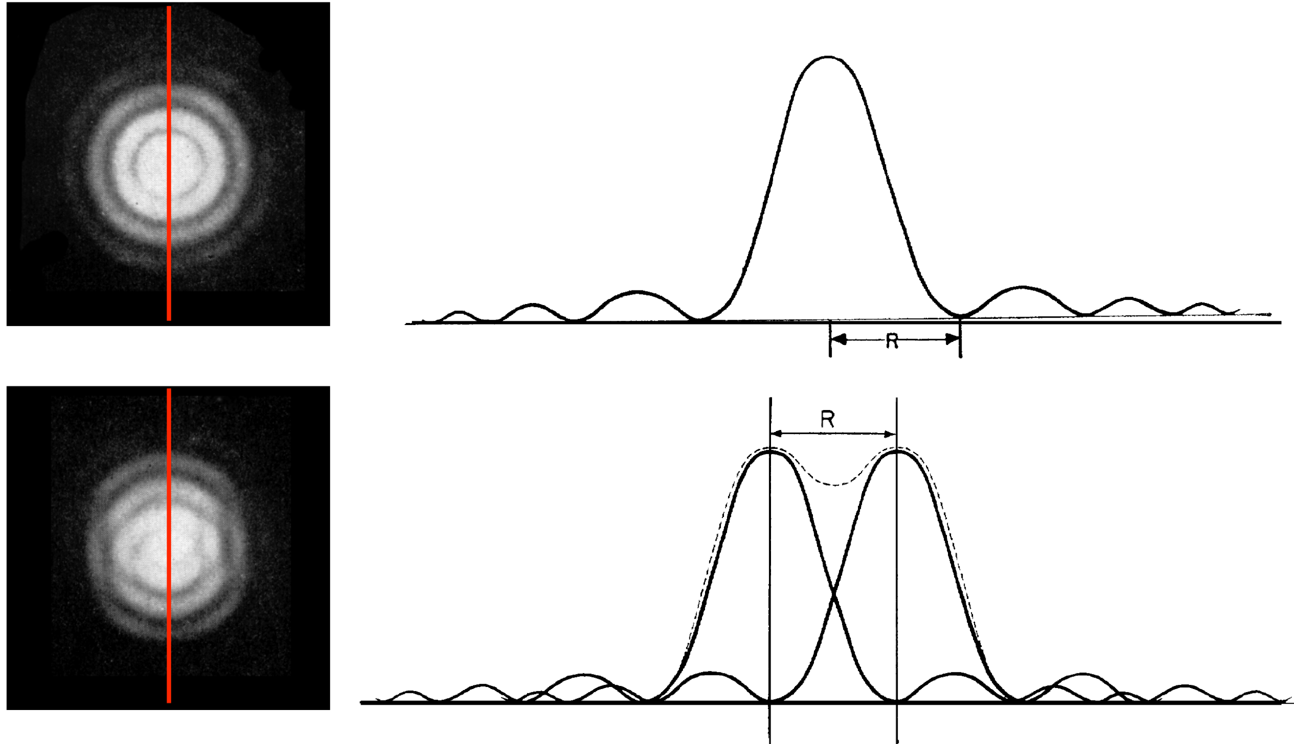
Ideal lens: each point in an object is a point

Real lens: each point in an object is spread out (Airy disk)



Airy disk: Caused by diffraction of the light wave.
See constructive and destructive interference pattern
Determined by the wavelength of the irradiation!

Practical limits of resolution



From Sjostrand, Fig. IV.18, p.115

The shortest distance between 2 Airy disks at which the two appear partially separated ~ ½ the width of the disks

Width of disk determined by wavelength of irradiation.

How do we get to high resolution?

The **shortest distance** between two Airy disks at which they appear partially separated corresponds to about 1/2 the width of the disks

The distance, *d*, in **object space** is given by the **Abbe Equation**:

$$d = \frac{0.612\lambda}{n \cdot \sin\alpha}$$

λ = wavelength of the radiation
n = refractive index of the media
α = lens semi-angular aperture

Note: $n \sin\alpha$ = lens numerical aperture (N.A.)

TEM outperforms light microscopes

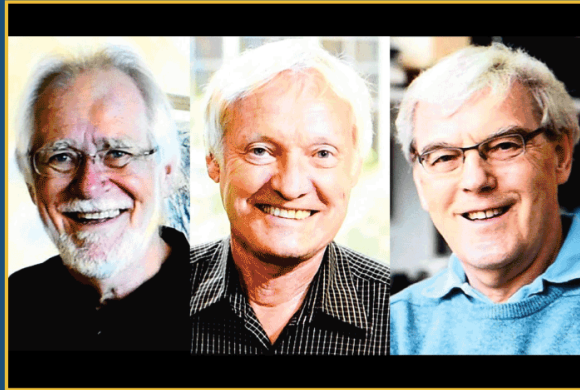
$$d = \frac{0.612\lambda}{n \cdot \sin\alpha}$$

To maximize resolving power (*i.e.* aim to get d as small as possible), **λ** must be decreased, *n* increased, or α increased

	n	*sin* α	λ*	*d*
LM	1.5	0.87	400 nm	~ 0.2 μm
TEM	1.0	0.01	0.0037 nm	0.23 nm

* λ = 400 nm for violet light
= 0.0037 nm for 100kV electrons

Nobel Prize in Chemistry



Scientists *Jacques Dubochet, Joachim Frank* and *Richard Henderson* were honoured for developing cryo-electron microscopy which simplifies and improves the imaging of biomolecules

1968 — First 3D-reconstruction DeRosier and Klug

1974 — Birth of cryo-EM Taylor and Glaeser

1982 — Vitrification technology Dubochet *et al.*

1987 — Single particle: random conical tilt Radermacher, Frank *et al.*

Single particle: angular reconstitution van Heel

1990 — Atomic structure of bacteriorhodopsin by electron crystallography Henderson *et al.*

1994 — Projection matching Penczek, Frank *et al.*

1997 — ≤10-Å virus structure Böttcher, Crowther *et al.*; Conway, McDowell *et al.*

1998 — 2D maximum-likelihood Sigworth

2008 — Virus *de novo* tracing Zhou *et al.*

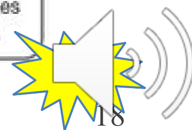
2012 — Direct electron-detection cameras used for motion correction Grigorieff *et al.*

2013 — Near-atomic maps of 30,000 ribosomes Scheres *et al.*

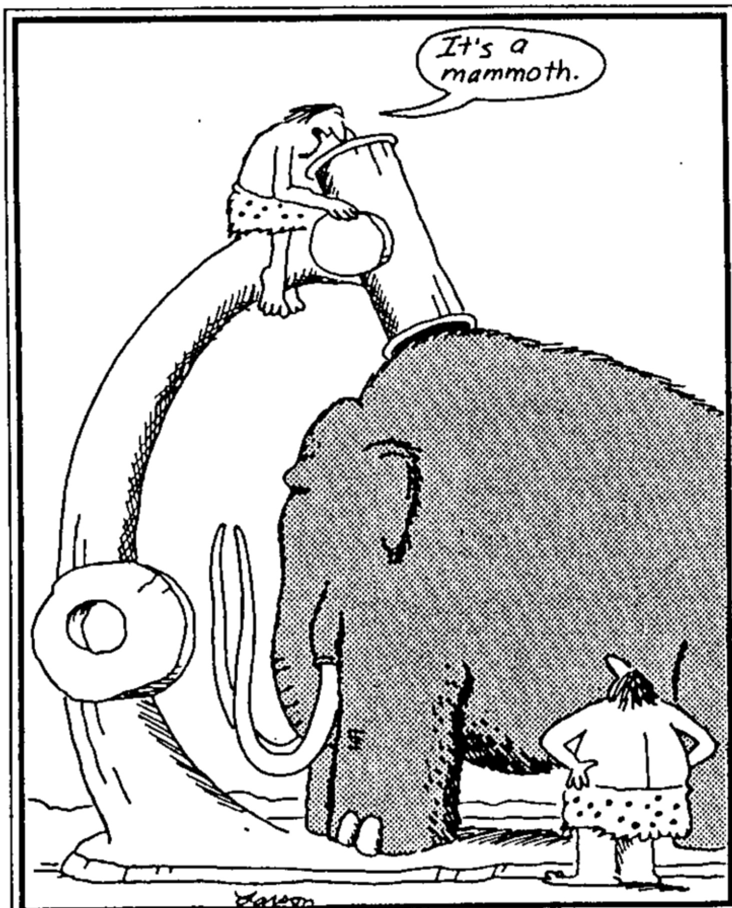
2015 — <3-Å structures Carragher, Potter *et al.*; Subramaniam and colleagues

De novo tracing <200 kDa Scheres, Shi *et al.*

gales, Nature Methods, 2015



The cryo-EM revolution is due to three main improvements



Early microscope

Magnified view of an object to visualize details using an **Optical Instrument**
****** hardware advancements with instrumentation***

Need to **Form** and **Record** an image
****** hardware advancements with cameras.***
Direct electron camera. The real gamechanger.

Ability to process **Big Data**
****** computation improvements- algorithms and data management Terabytes of image data.***

Differences between Optical and Electron Microscopes

Lenses

Optical (glass, FIXED focal length)

Electron (ferromagnetic, can adjust magnification with current)

Depth of field********

Optical: SMALL

Electron: BIG (whole specimen is in focus at once)

Specimen state

Optical: can be kept alive

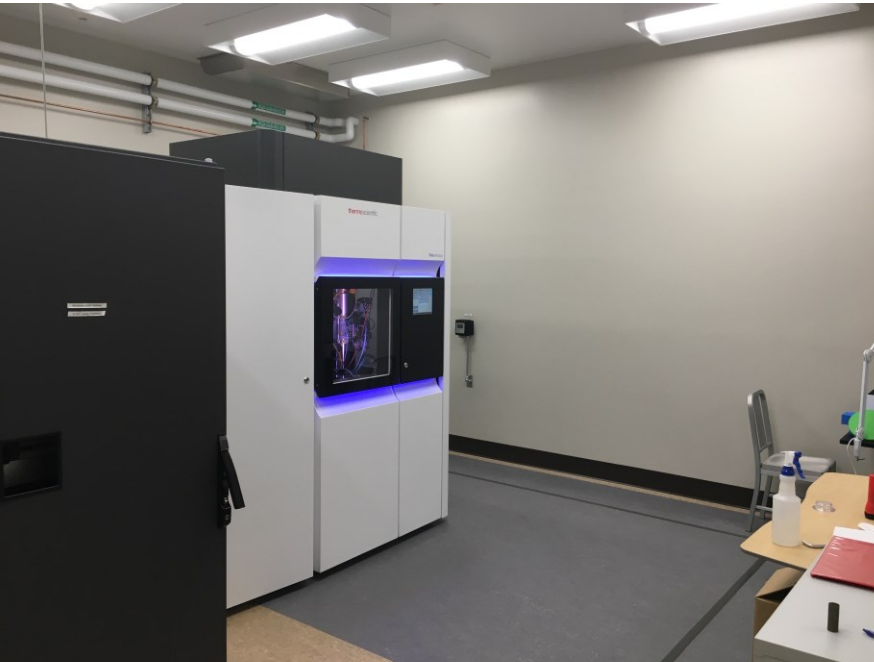
Electron: high vacuum, heavy metal stain, electron beam damage: DEAD ☹

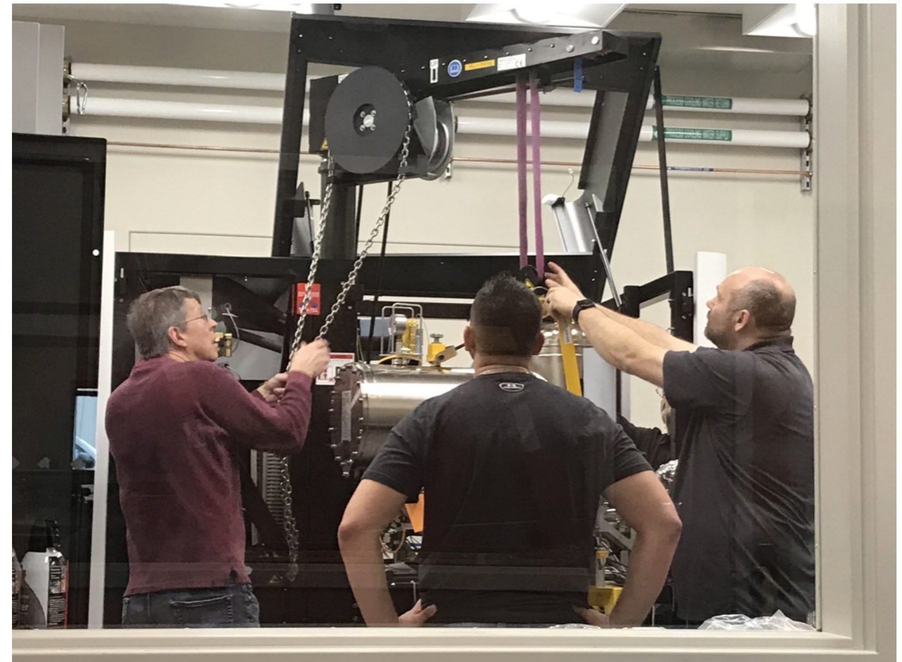
Price tag

Optical- thousand to tens of thousands

Electron- ***millions (our facility is worth about over $14 M)***

Cryo-Electron Microscope at MSU





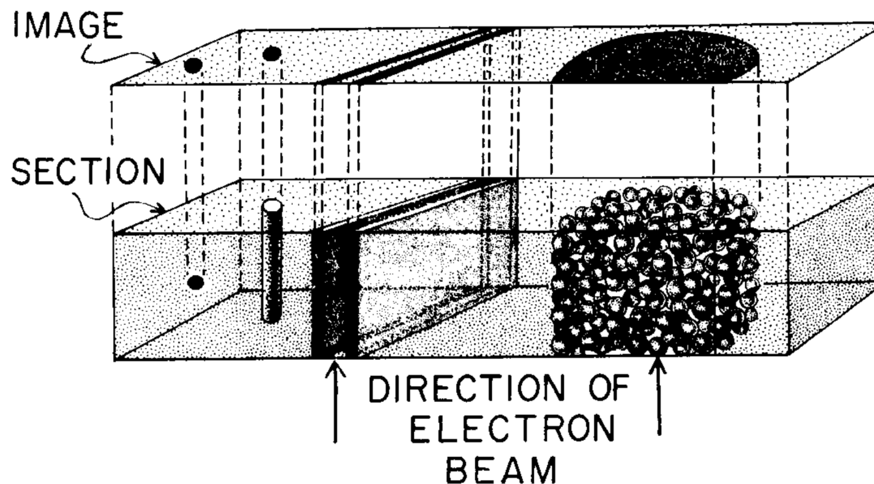
https://cryo-em.natsci.msu.edu/

Talos Arctica (200 keV)
Falcon 3 DDD
Autoloader

So… what makes these so awesome?

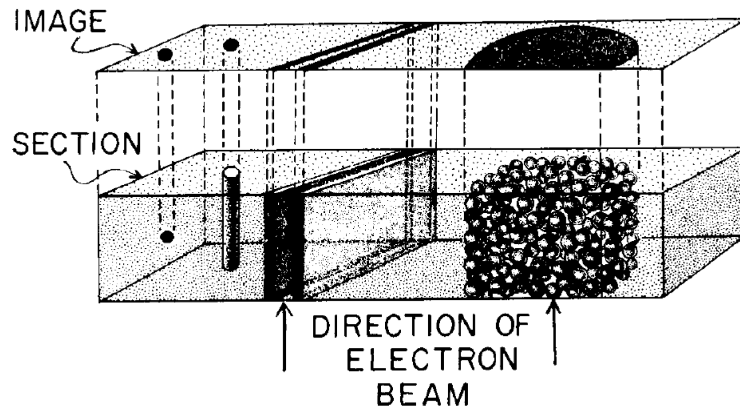
Depth of Field is BIG in TEM

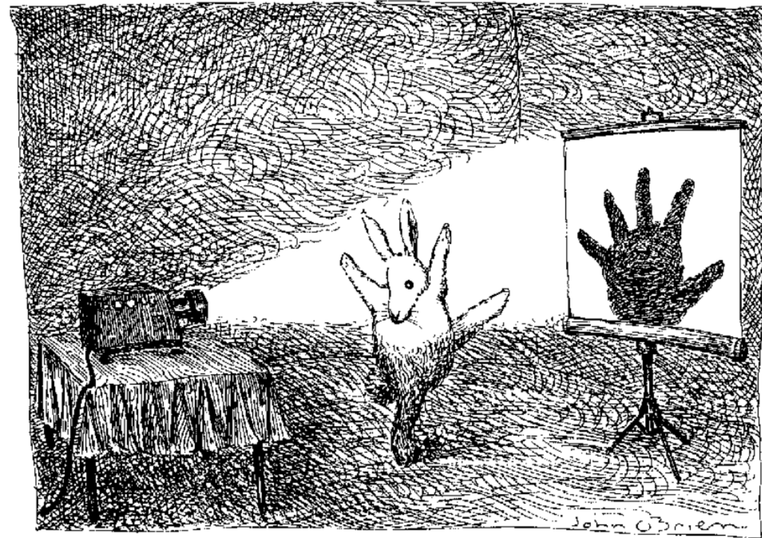
Images are **projections** of the **entire contents** of a specimen



Each part of the **2D** image represents **projected** contributions from a **3D** object in the direction of the electron beam

More about Depth of Field

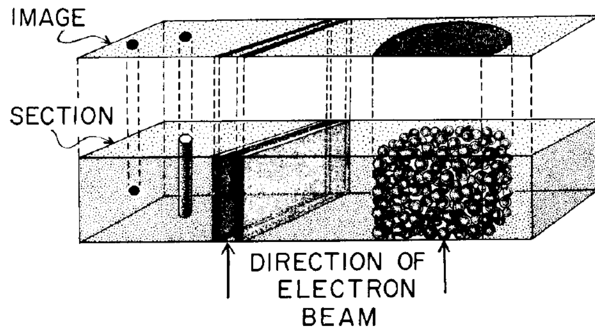




Projection images are **NOT** “shadow-graphs”

Radiation **IS** transmitted through a TEM image

TEM images are **NOT** shadow-graphs, but more like X-ray images



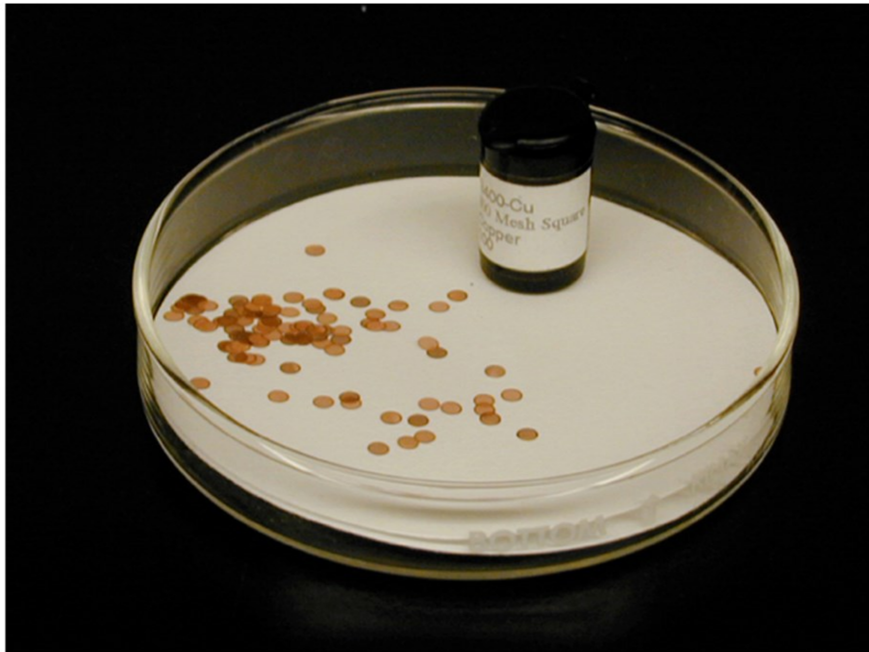
The type of information we obtain depends heavily on **Specimen Preparation**

There are a variety of TEM imaging types

Each has pros and cons

- Thin section TEM
- Negative staining
- Metal shadowing
- **Cryo-EM**

Specimen Support Material



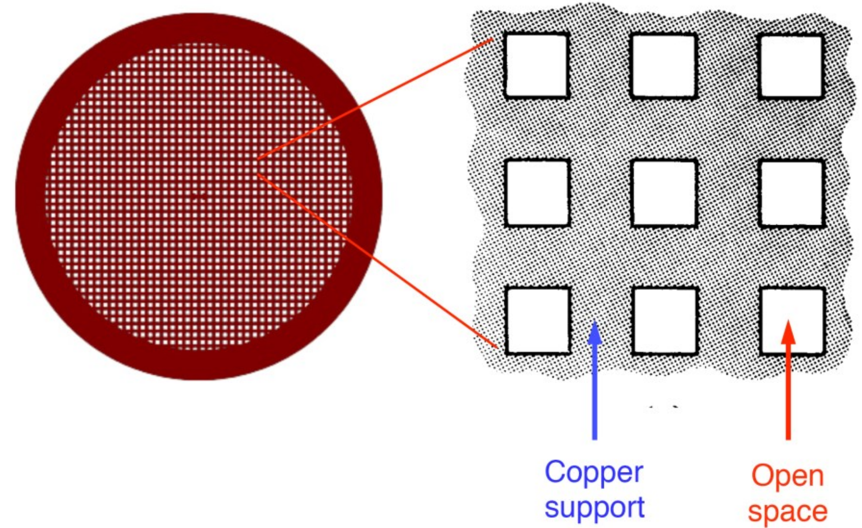
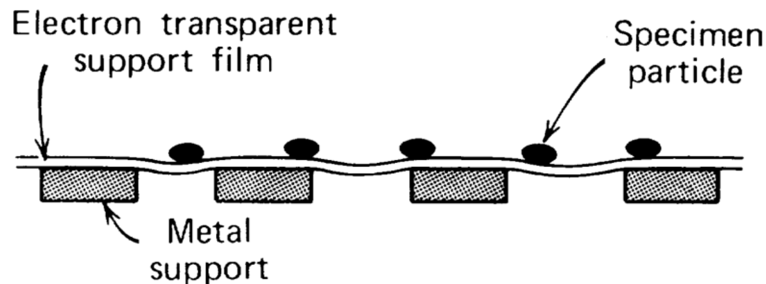


Image courtesy of P. Chipman (2004)



Surface to deposit sample

Adds **physical strength** to grid

Heat dissipation in e^- beam

Carbon or formvar (**amorphous**)

Thin section examples:

Retroviruses budding from a leukemia cell

From Bozzolla and Russell, Fig. 19-129, p.48

Thin section examples:

Ultra thin section of gram positive bacteria

Streptococcus mutans
(causes tooth decay)

0.25 μm

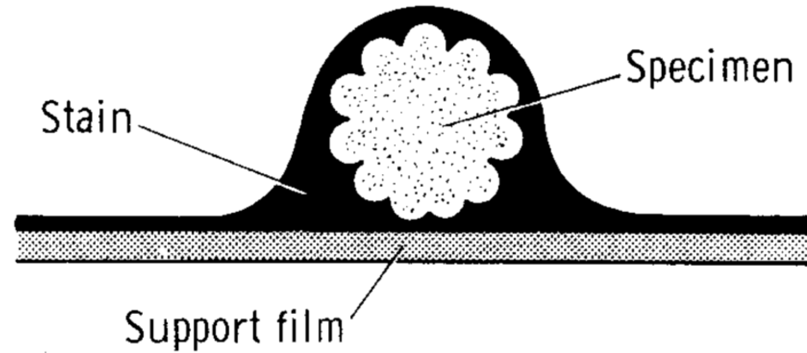
From Bozzolla and Russell, Fig. 19-119, p.482

Negative staining

- Pros
  - Stains provide **high contrast**
  - Can assess concentration and homogeneity easily
  - Great for small particulate specimens (proteins, macromolecular complexes, 20 kDa-100 MDa)
  - Information comes from a **single particle**
  - **Rapid!** (~15 min prep)
- Cons
  - **Dehydrates** the specimens (terrible for membranes!)
  - **Lower resolution** information obtained (12-40Å)
  - Not everything stains the same

* Fairly common technique

Negative staining (mostly particulate samples)



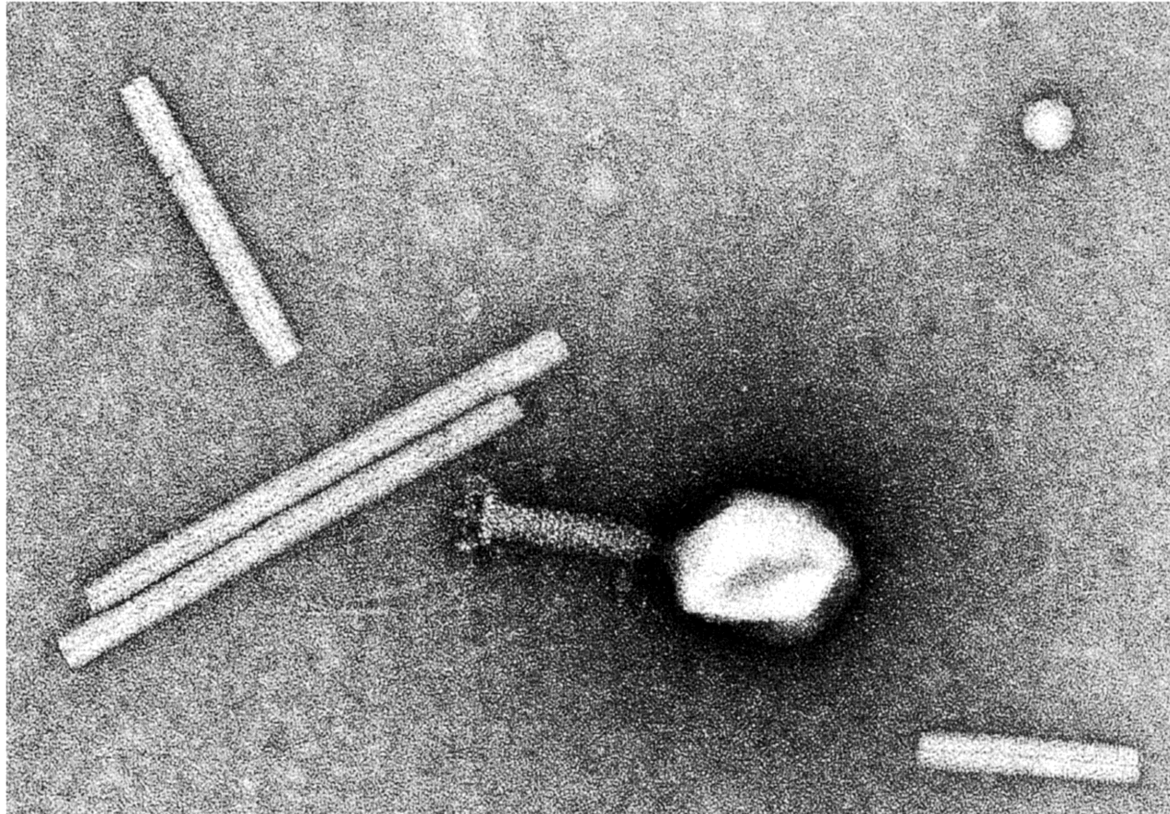
Sample is embedded in **heavy metal salt** (fast and easy!)

Heavy metal salt adds considerable **contrast**

Artifacts?

Most are low-med pH (3-7) and high [salt] (~20% final)
Dehydration/flattening
Sample may be "positively stained"

Examples of negatively stained images



TMV and bacteriophages T4 and ϕX174

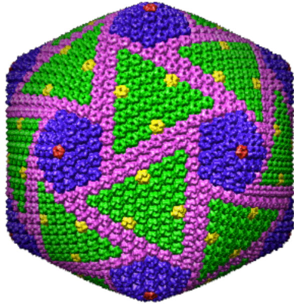
Image taken by F. Eiserling

Note contrast is reversed from thin sections! **(NEGATIVELY stained)**

Good way to count phage/virus isolated from various environments

How big/little specimens really are

Scale: 2^{22}
4,194,304X



PBCV-1

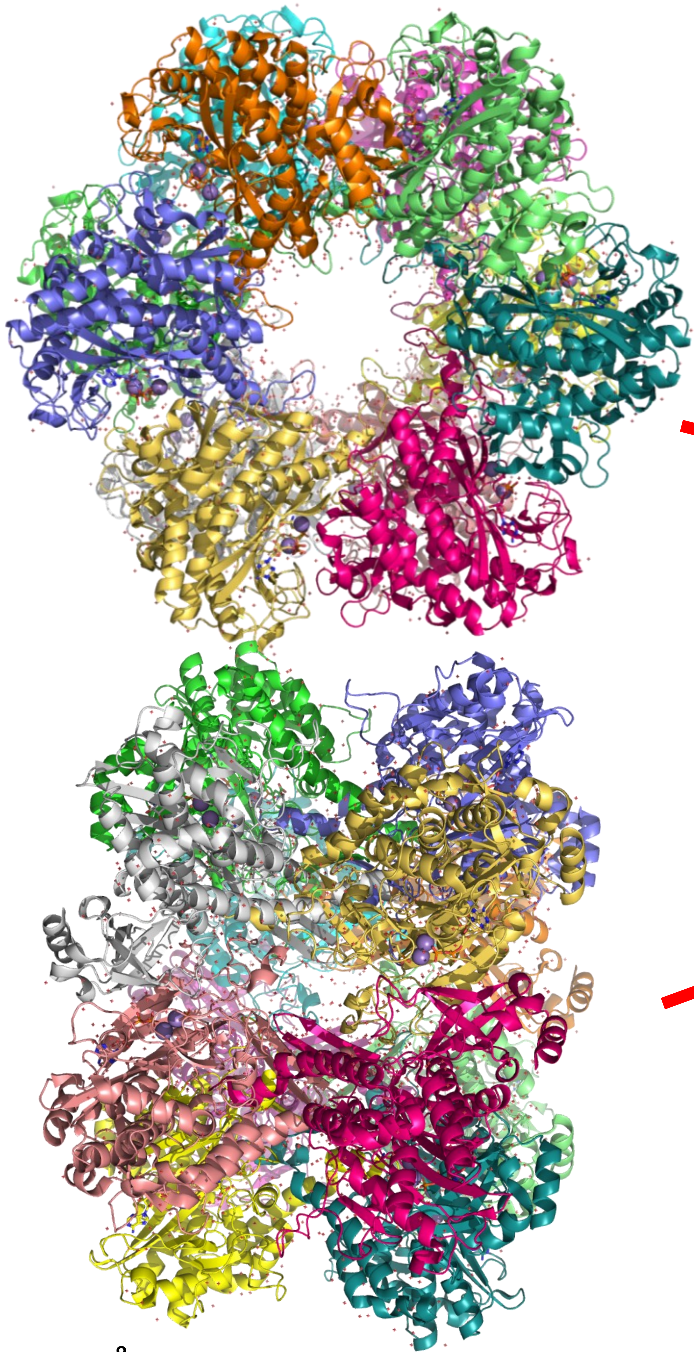
TMV

Mimivirus

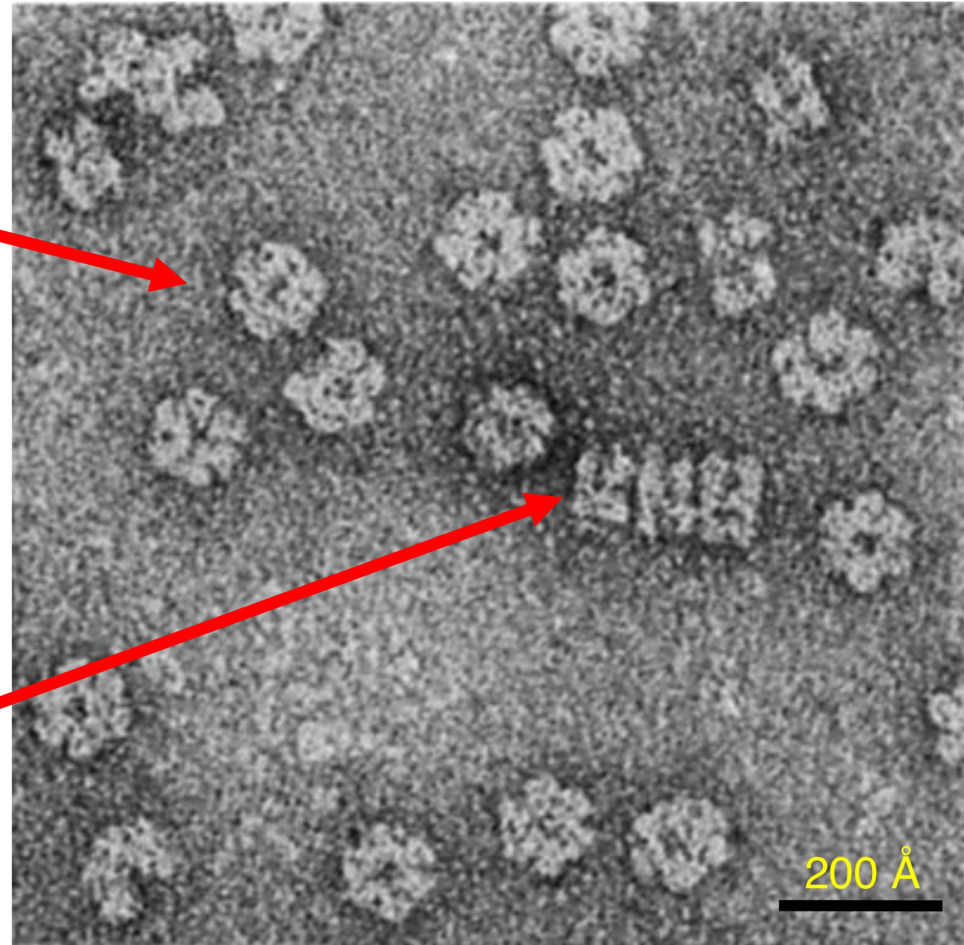
2000 Å

www.stanford.edu/group/virus/mimi/2005/index.htm

Examples of negatively stained images



1FPY 2.9 Å X ray

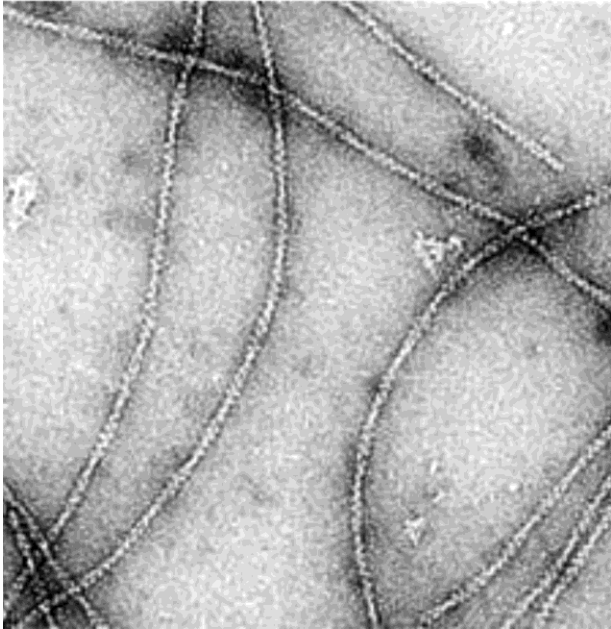


Glutamine synthetase

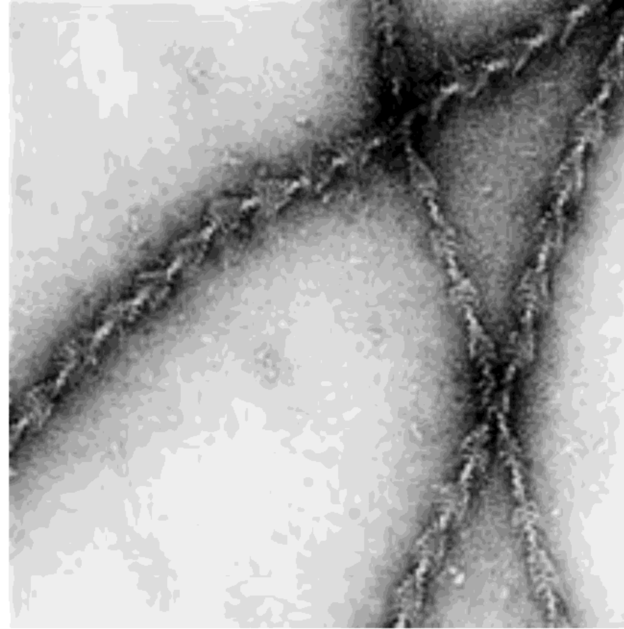
(2001) Biochemistry **40**: 1903-1912

Examples of negatively stained images

Actin

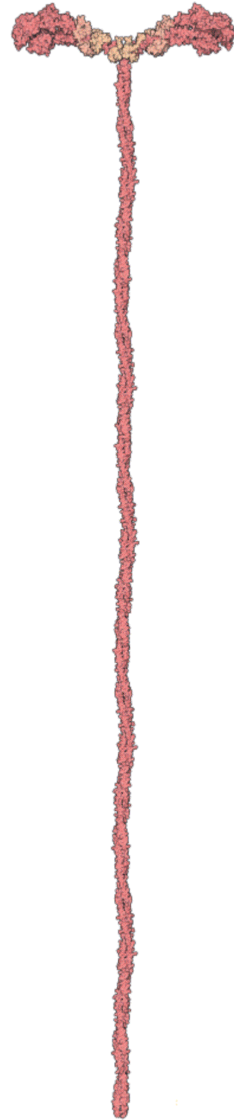


Actin filament



Actomyosin filament

Actin



Myosin

Images taken by R. Graig (see www.umassmed.edu/cemf/negstain.aspx)

Cryo-EM

- Pros
  - High resolution (can obtain 1.5 Å in rare cases)
  - Native like state
- Cons
  - Very low contrast
  - Computationally intensive
  - Expensive

* Much more common technique recently

Vitrification: blotting and plunge freezing
Just like crystal freezing. Avoid ice formation

Filter paper
EM grid
Sample
Filter paper
Ethane slush

More recently, robotic versions available

Pros:

- Reliable
- Consistency
    - Timing
    - Temperature
    - Humidity
    - Blot Force

Cons:
- Big price tag ($80k-0.6M)

How to find and image a specimen?

Too thick

Dry - no ice

Just right

Radiation damages the specimen (one shot only to image)

Low dose microscopy

…rch" at low mag, with …exposure (<1 $e^-/Å^2$)

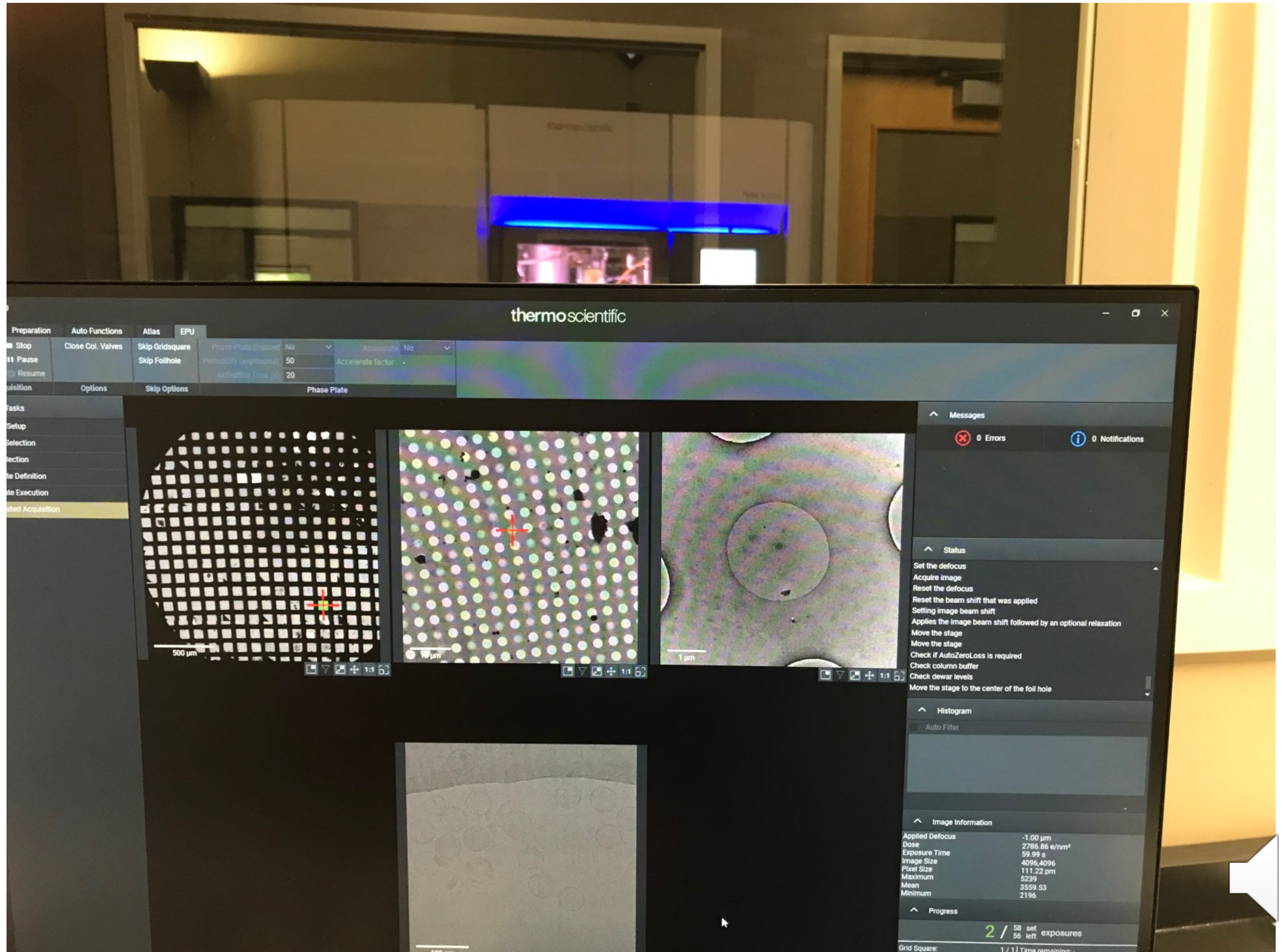
…us" off target

…ge" at high mag and …that does not destroy …ble (20-24 $e^-/Å^2$)

A **2-sec** exposure delivers energy at t… …equivalent to the energy that we would experience if a 10-megaton hydrogen bomb detonated **30 meters outside this room!!**

**** Only possible since relatively recent advances in computation!

Modern day support films cryo-EM: Quantifoil and automated software



Final image has low contrast but high resolution info!

Low dose image of Simian Virus 40 (Baker lab, UCSD)

What features are evident at various resolutions in cryo-EM?

Sample Type	Method	Resolution Range	Features you can see
Larger, heterogenous	Tomography	~20 - 40 Å	Ultra structure mainly
Smaller, homogenous	Single particle analysis	~1.5 - 60 Å	Depends* • > 10 Å; overall envelope • 6-10 Å, alpha helices • 4-5 Å, beta sheet strands • <3.5 Å, amino acid side chains • <2.0 Å water and metal atoms

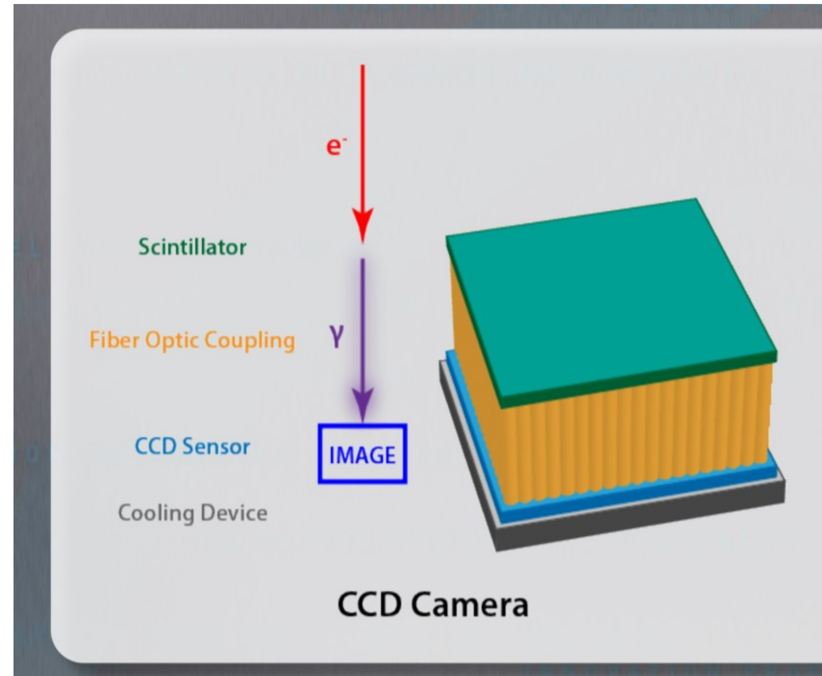
About 900 Cryo-EM structures under 2.5 Å in the PDB.

Okay, we have a microscope and know how to take pictures, but how do we store the information?

Three choices:

1. Film
2. CCD camera (Charge Coupled Device)
3. DDD (Direct Detection Device)

CCD Cameras (Charged coupled device)



Advantages:

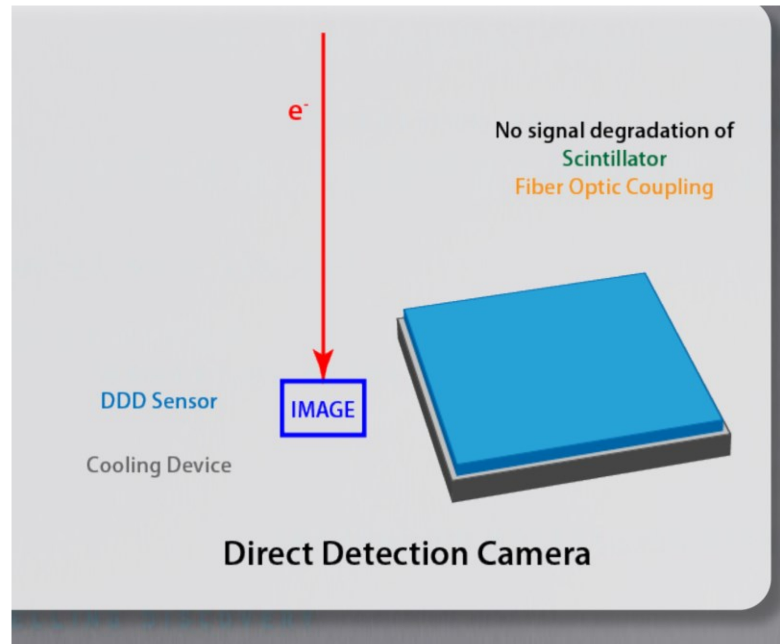
1. Immediate image access
2. Good for automated data collection
3. Good for cryo-tomography

Disadvantages:

1. Poorer resolution and less real estate ($4k^2$ pixels, ~15um)
2. $200k typical cost for $4k^2$

*mostly used for screening these days

DDD (Direct Detection Device)



Advantages:

1. Immediate image access
2. Good for automated data collection
3. Good for cryo-tomography
4. MOVIE mode
5. pixel size and detector sizes almost as good as film
6. better sensitivity per pixel

Disadvantages:

1. HUGE amounts of data (each image = 1GB)
2. High cost (~$1M)

*best for high resolution imaging

How do we get a 3D structure?

Main choices:

1. Single Particle Analysis (SPA)*
2. Tomography*
3. Micro-ED (electron diffraction)

*We will explore 1 & 2 analysis techniques in this course

Single Particle Cryo-EM (3D reconstructions from averaging many particles together)

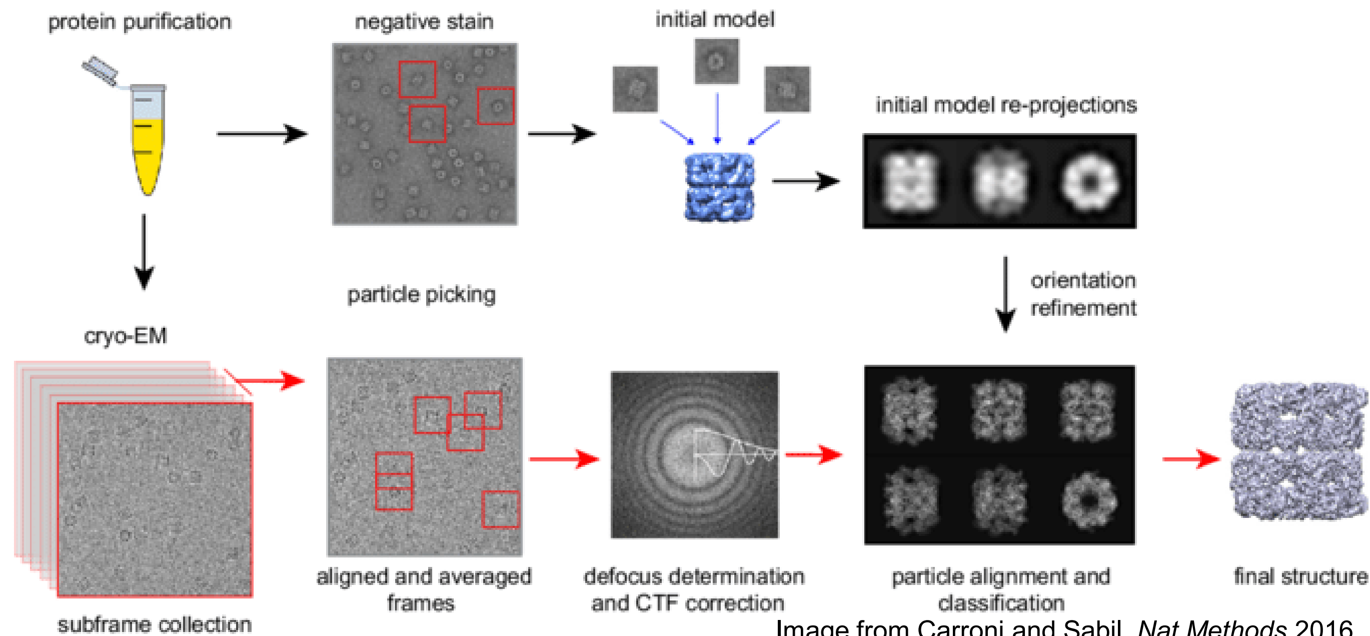


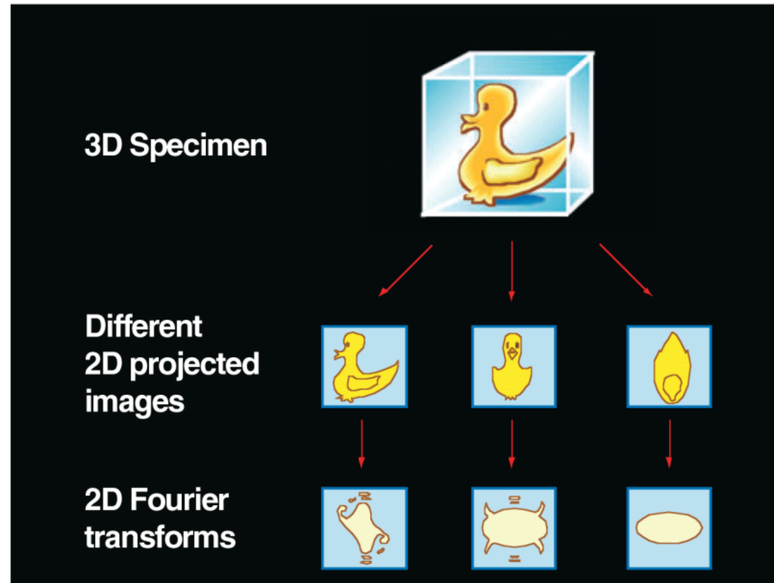
Image from Carroni and Sabil, *Nat Methods* 2016

Good for **homogeneous** samples

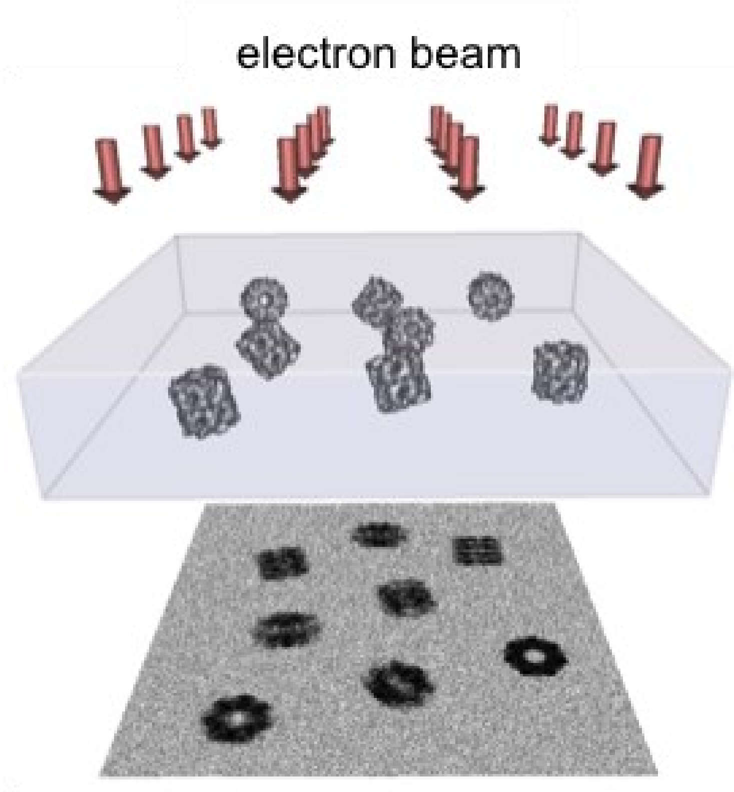
Currently routinely achieving atomic level resolution (3 Å)

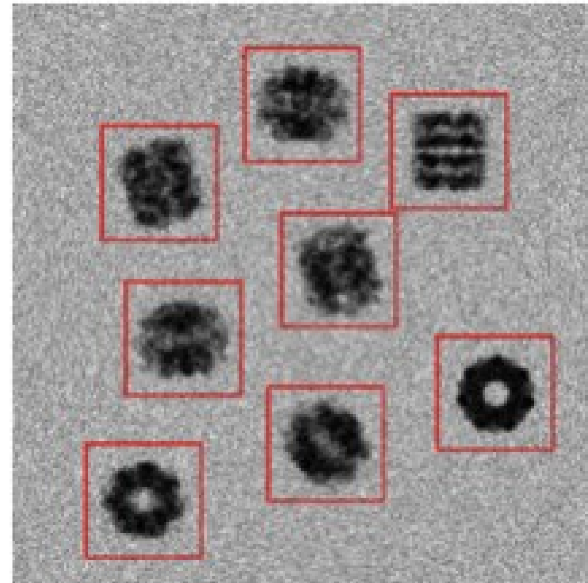
Total dose is low (<24 $e^-/Å^2$)

How do you go from 2D projections to 3D reconstructions?



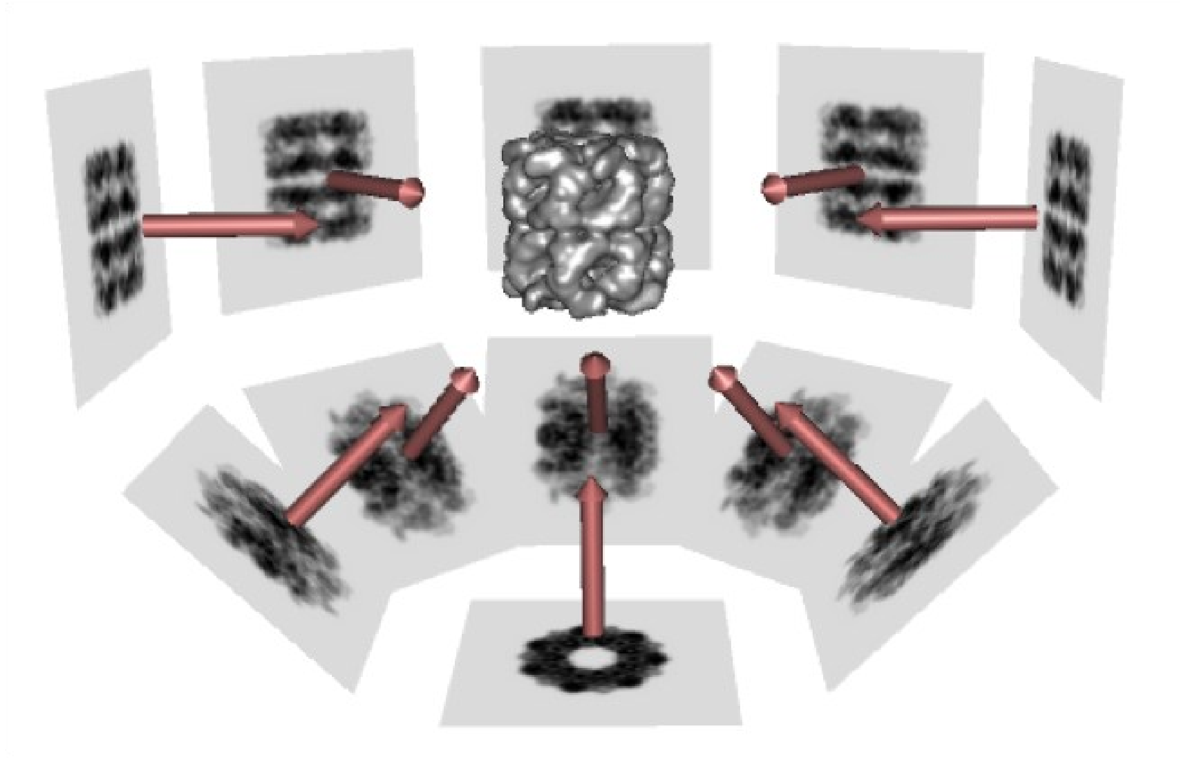
Single particle analysis





http://people.csail.mit.edu/gdp/cryoem.html

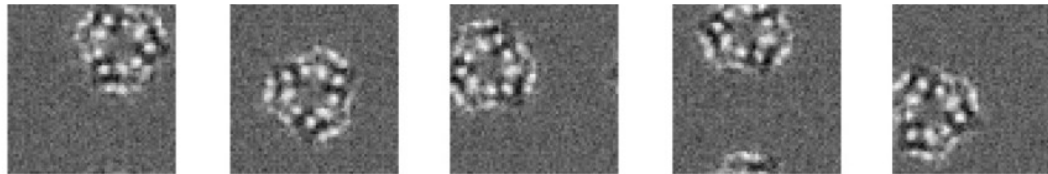
Single particle analysis



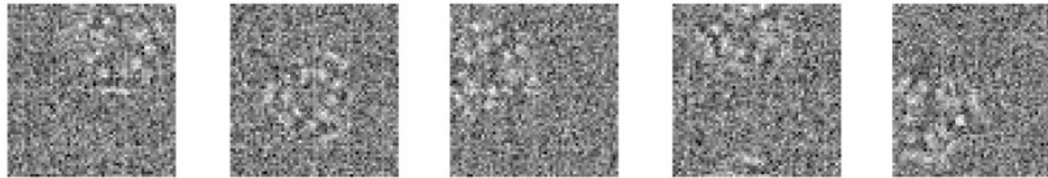
http://people.csail.mit.edu/gdp/cryoem.html

2D Classification

Goal is to group similar projections together and as a digital particle purification process



Features clearly visible



Features barely visible

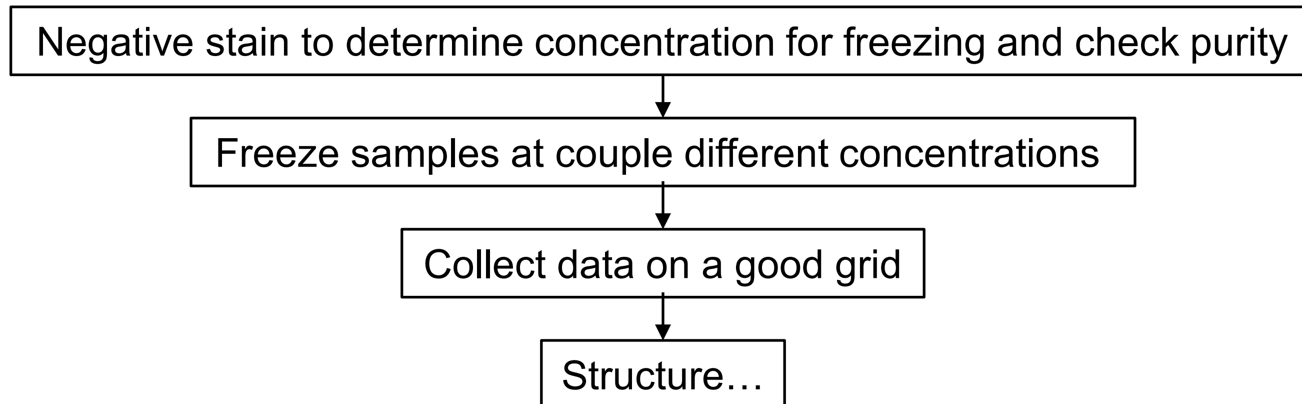
Due to very low signal to noise ratio, grouping projections based on features is difficult

Sigworth, Journal of Structural Biology 122, 328–339 (1998)

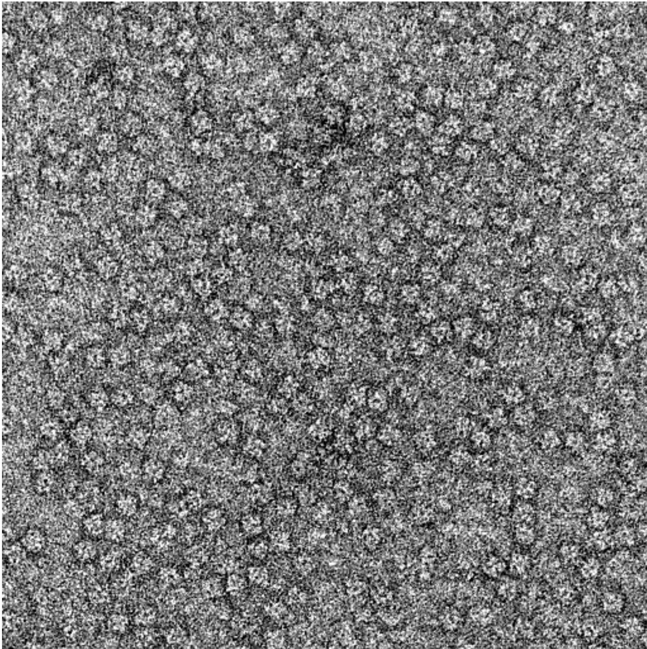
Example - LarA – Jian Hu Lab

Sample type: soluble protein
Number of amino acids: 495
Molecular weight: 56207.08 Da
Oligomeric State: May be a Hexamer or an Octamer
Amount of Sample required: ~5 µL per grid

Ideal World Scenario



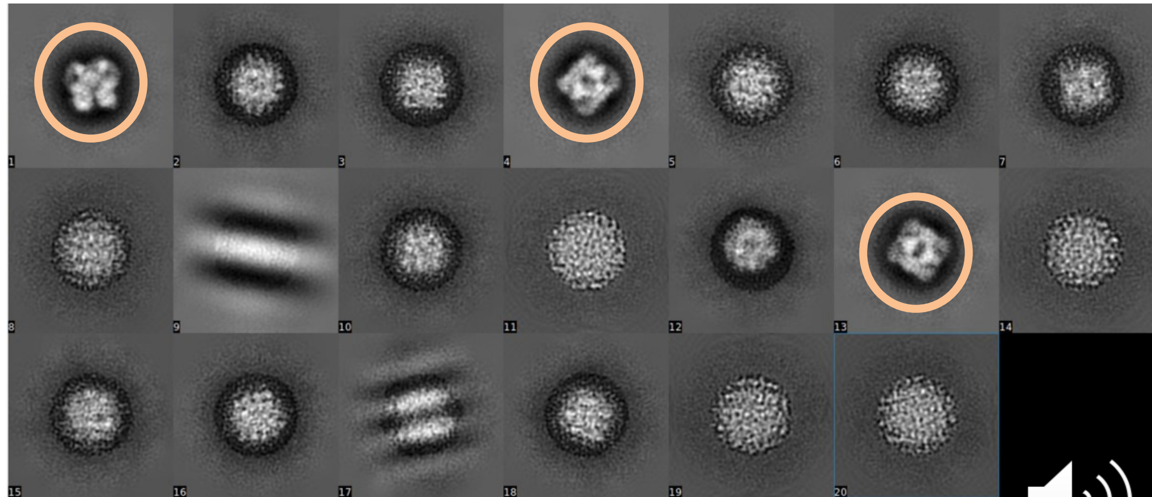
LarA – Negative Stain



Collected <1day of data for some initial 2D class averages

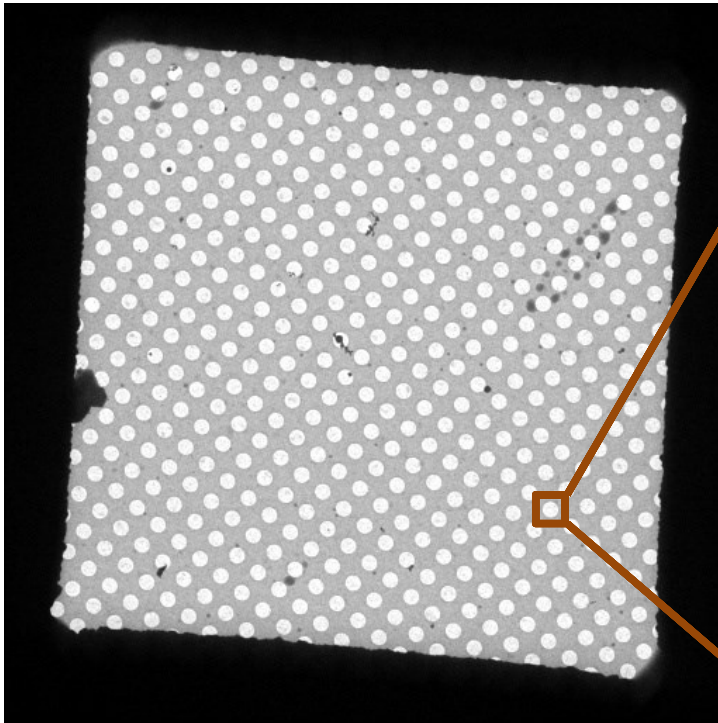
Particle picking

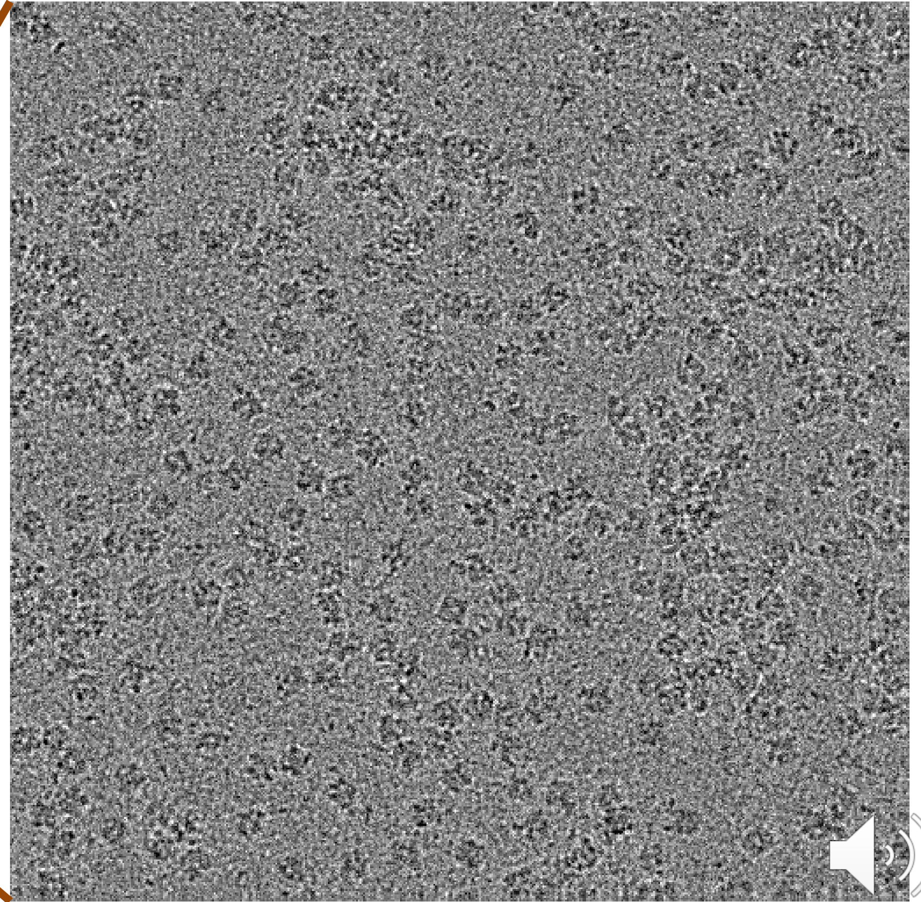
2D classification

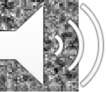


LarA – Ultrathin carbon coated Quantifoil grids

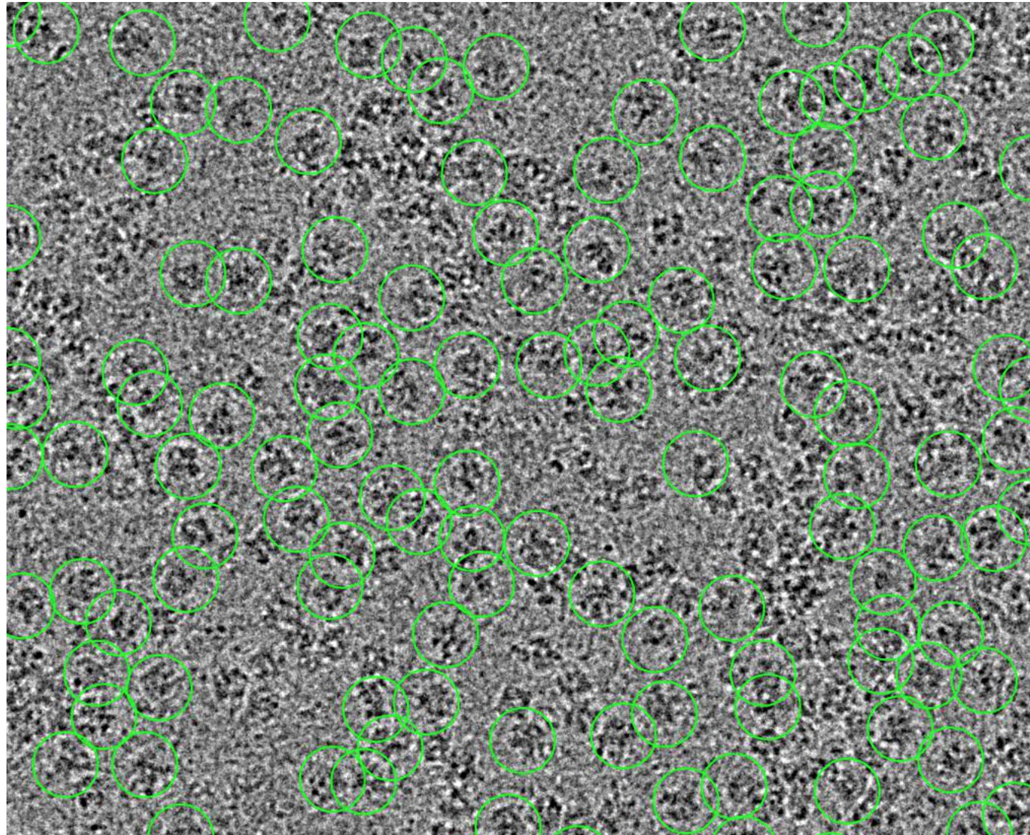
Gridsquare view







LarA – Particle picking and 2D classification

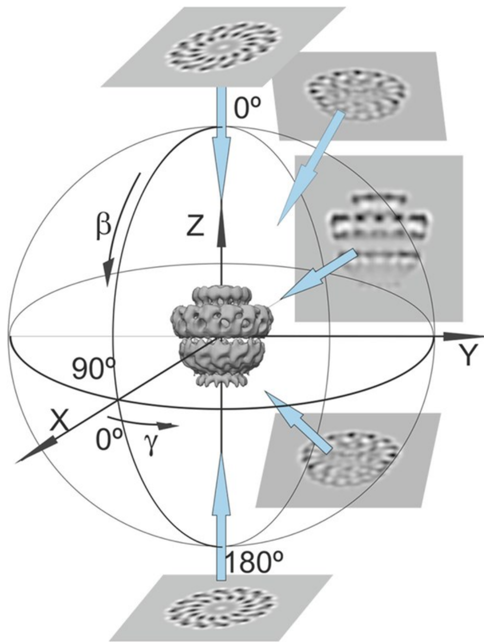


LarA – Particle picking and 2D classification

LarA – Good 2D classes

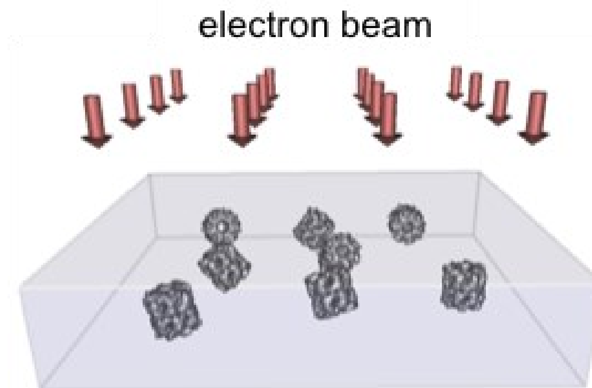
- Make sure the class averages are centered and have structural features
- Avoid classes with blurred averages

3D reconstruction



Total of 6 degrees of freedom

- Each projection can be defined by a set of Euler angles and the shift in X, Y in reference to a 3D structure
- Why not Z ?

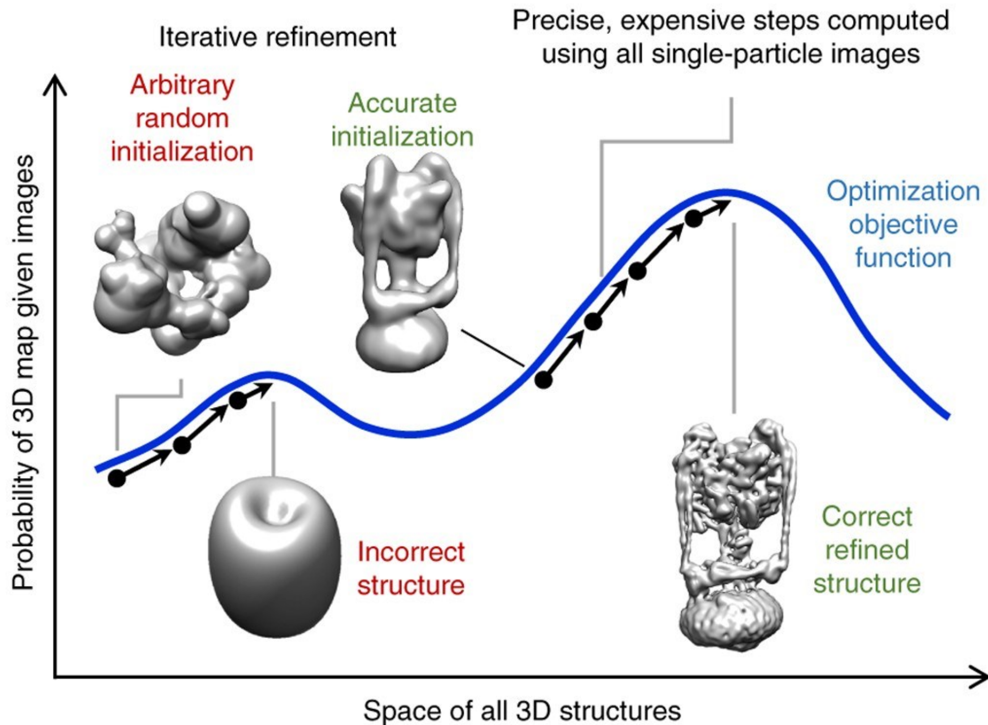


The shift in Z is negligible if one assumes that the electron beam is along Z

Costa et al, *Bacterial Protein Secretion Systems: Methods and Protocols*, Methods in Molecular Biology, vol. 1615, DOI 10.1007/978-1-4939-7033-9_28

http://people.csail.mit.edu/gdp/cryoem.html

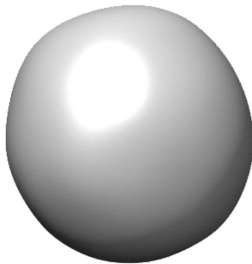
3D ab-initio

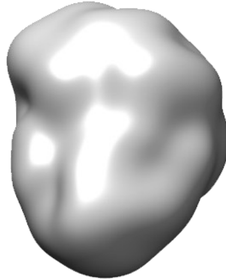


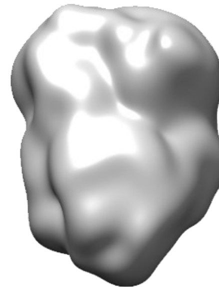
- Goal is to estimate an initial 3D model of your samples based on a given set of projections
- Can lead to incorrect initial model
- Computationally intensive and challenging
- Various algorithms have been developed to address this problem
- Common theme is start with random initial parameters and then iteratively refine them

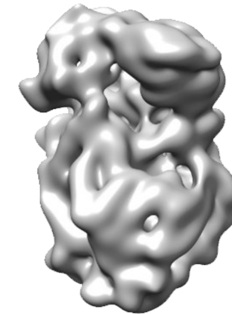
Punjani et al, Nat Methods, 2017; 14, 290–296

3D ab-initio – cisTEM


Start


Cycle 9

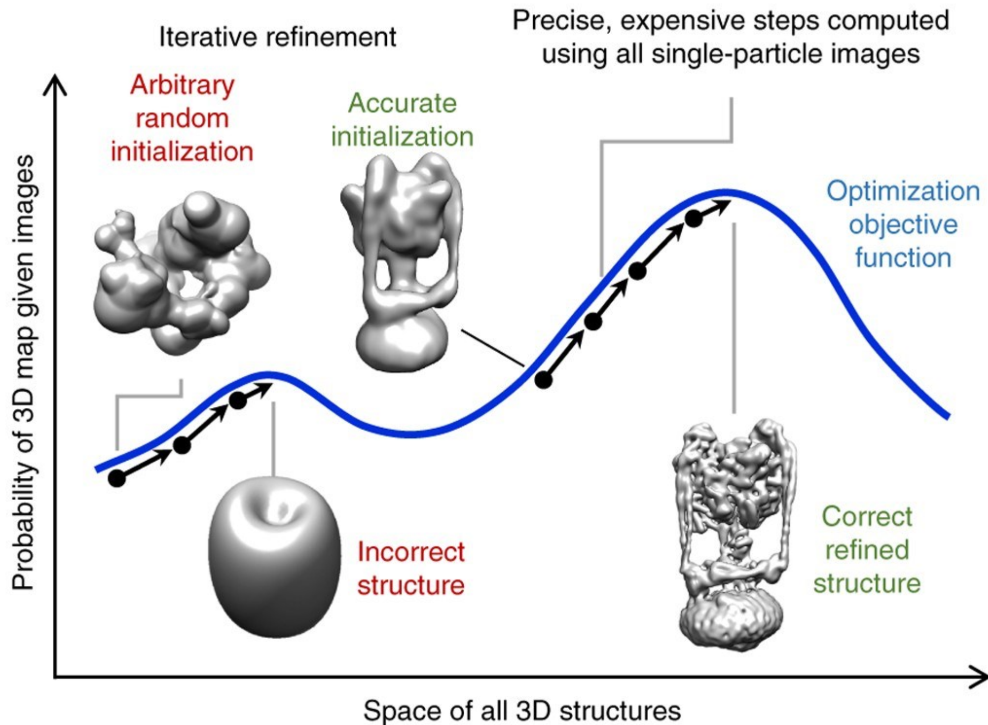

Cycle 27


Cycle 40

- Assign random Euler angles for each projection image
- Initial model will basically be a sphere with no features
- Iterative rounds of projection matching with Global searches (sampling the whole range of Euler angles)
- Utilize a subset of the particles and continually increase the number of particles used to generate the initial model

Grigorieff, Methods in Enzymology, Volume 579, 2016;

3D ab-initio



- Usually initial alignment is carried out at a lower resolution and then increased if improvement is achieved.
- If no features show up during the initial model and the resolution does not improve, then the approach needs to be optimized
- Spherical molecules like viruses and apoferritin for example need to be aligned at a higher resolution initially and more particles are utilized.

Punjani et al, Nat Methods, 2017; 14, 290–296

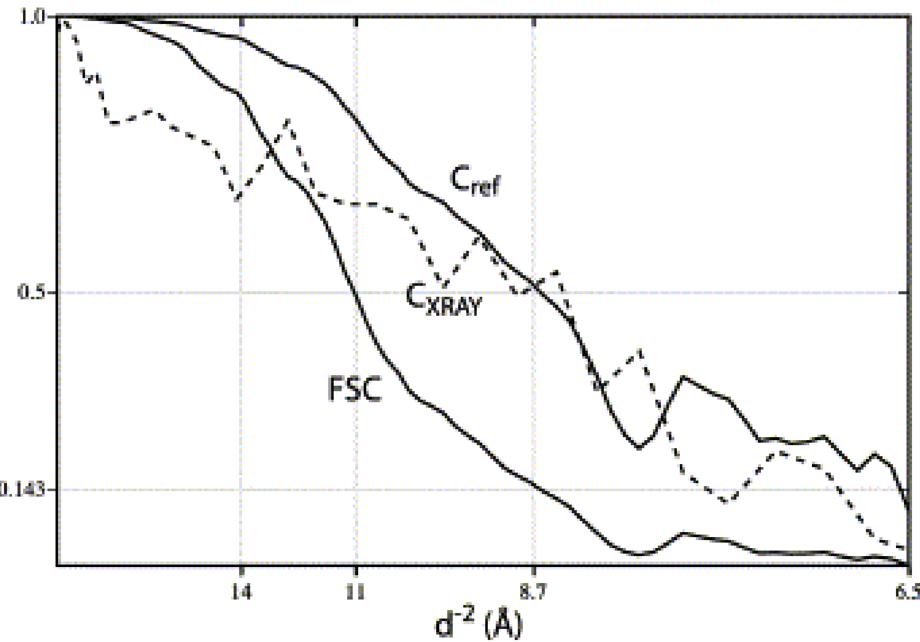
But how do you define the *resolution*?
The data is collected to the same resolution always
What determines the resolution?
The quality of the 3D reconstruction.
How do you evaluate that?
FSC curve

$$\mathrm{FSC} = \frac{\sum F_1 F_2^*}{\sqrt{\sum |F_1|^2 \sum |F_2|^2}}$$

- Take the images, split them randomly into two halves.
- Calculate an electron density map from each of the halves
- Apply the structure factor equation (the same one as crystallography)
- The FSC is the correlation between two independent maps, where each map is calculated from half the images. F1 and F2 are the structure factors of the two maps.

Rosenthal et al, J. Mol. Biol. (2003) 333, 721–745

FSC curve

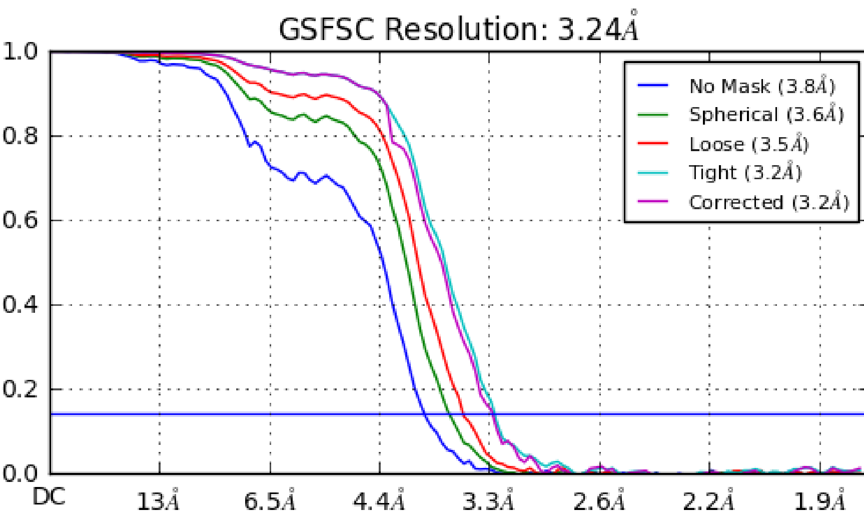


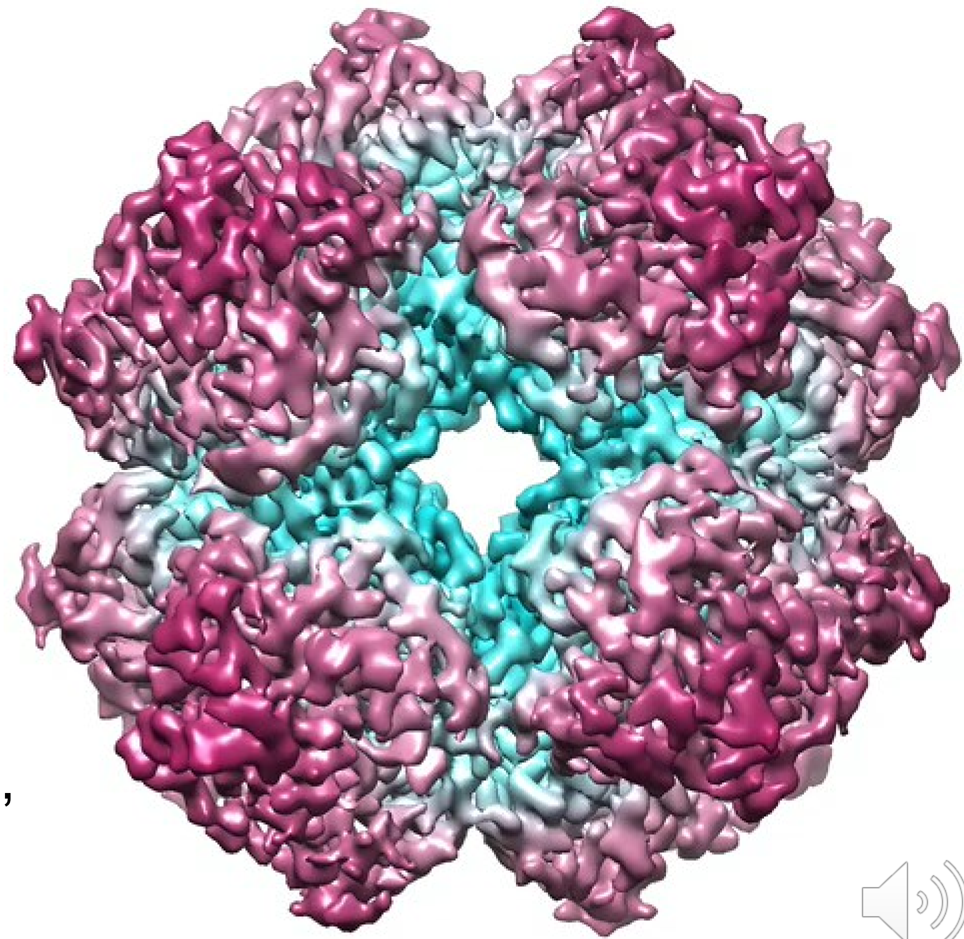
C_{REF} the estimated correlation between a density map calculated from all the data and a perfect reference map.
C_{XRAY} – Crystallographic Figure of Merit

Rosenthal et al, J. Mol. Biol. (2003) 333, 721–745

- **The resolution of the map is assigned at the point where the FSC crosses a threshold of 0.143.**
- Earlier 0.5 was used as the cutoff but its an underestimate because your final reconstruction contains both the halves of data.
- But there is so much debate still going on whether it should be 0.5 or 0.143.

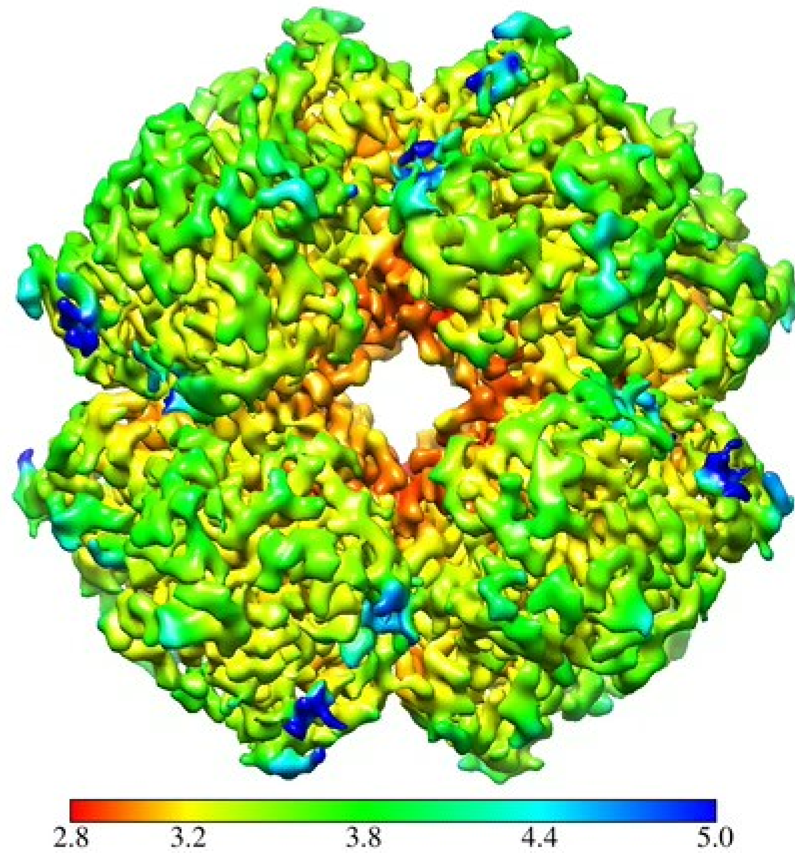
LarA – C1 symmetry



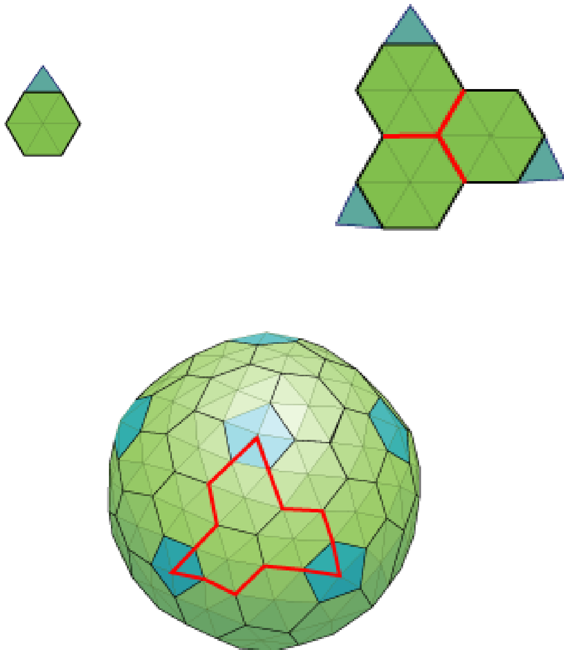


A total of **eight LarA monomers**, so higher symmetry possible.
C4 ? D2 ?

LarA – C1 Local Resolution



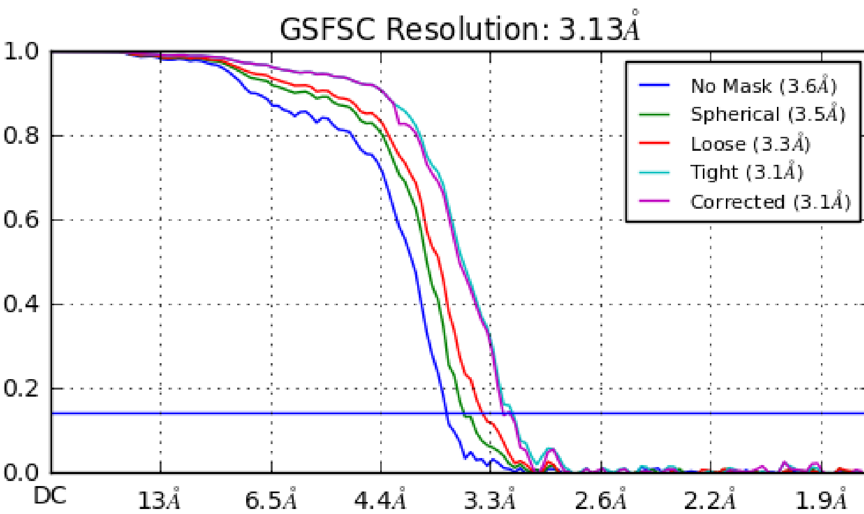
Symmetry averaging

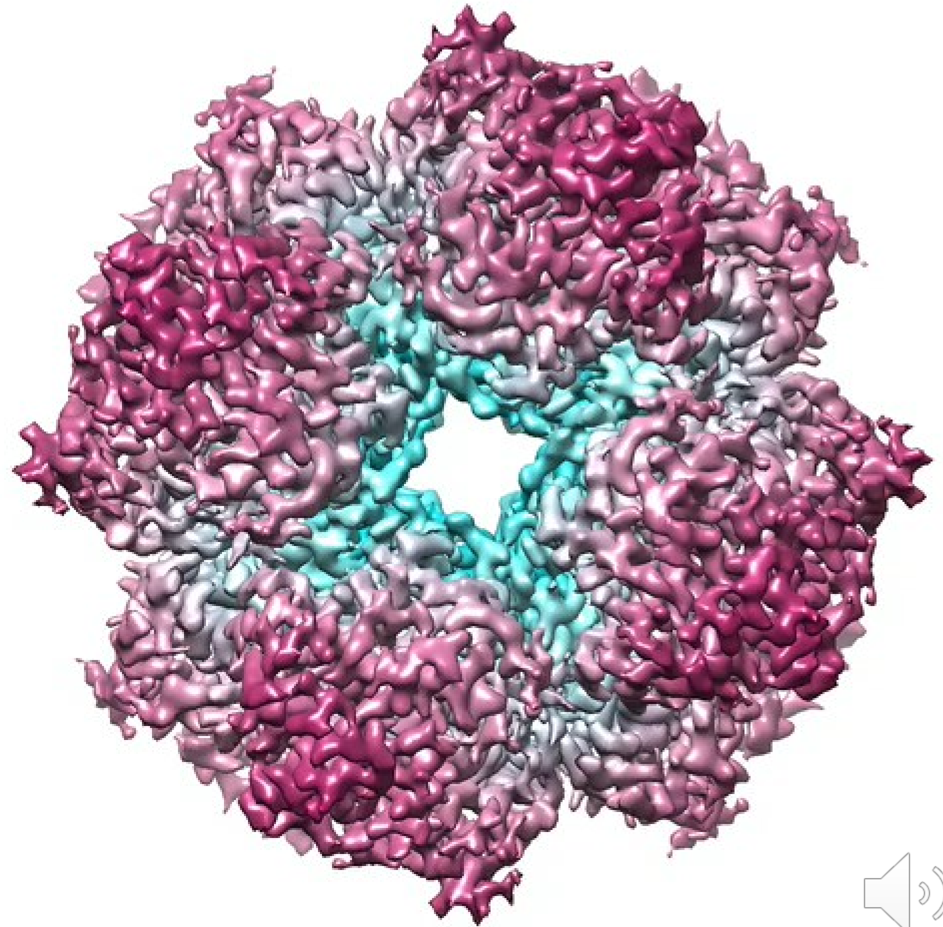


Mostly beneficial but….

- Certain biological molecules are oligomers that contain repeating units of a single monomer
- The monomers follow a certain pattern when they come together to form the oligomer
- Simple oligomeric assemblies include dimer, trimer and tetramers
- Certain viruses can form icosahedral structures that contain 60 sub-units
- **Symmetry helps with data processing as there is now extra parameters that can relate projections.**
- **The number of projections required to obtain a reconstruction is lowered when symmetry operators are involved.**

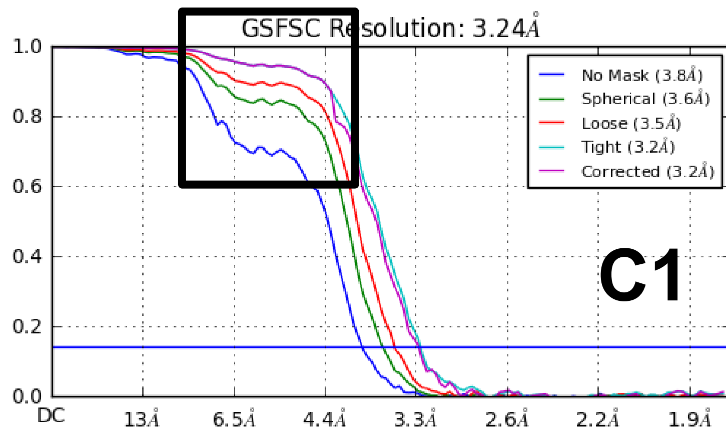
LarA – C4 symmetry

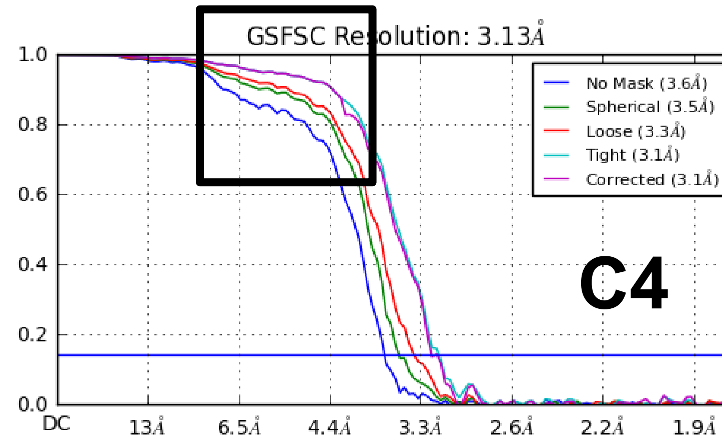


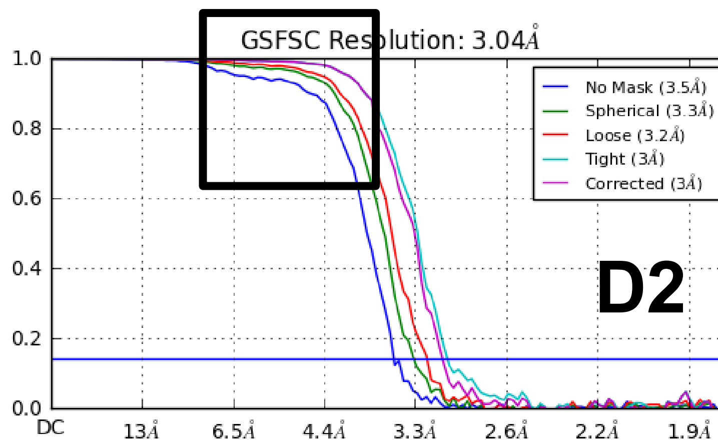


A total of eight LarA monomers, so higher symmetry possible. C4

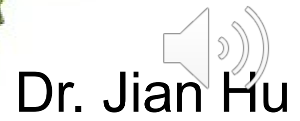
LarA – C1 (vs) C4 (vs) D2 symmetry







LarA – Model



Dr. Jian Hu

LarA – Closeup in coot

Dr. Jian Hu

Can reconstruct a lot of different specimen types with SPA

1000Å

100Å

a — 70S *E. coli* ribosome

b — Hepatitis B virus core

c — Actin-myosin filament

d — Light-harvesting 2D crystal

Current advances reach 1.5 Å resolution with this method!
Artifacts? Averaging losing signal from structure that is not homogenous

Take home messages

TEM is awesome and really powerful

More than one way to look at a specimen

Need to be aware of the artifacts/limitations

Microscopy is approaching crystallography to achieve near-atomic resolutions of biological structures