

Cryogenic Electron Microscopy (cryo-EM)

CS/CME/BioE/Biophys/BMI 279

Nov. 11, 16 and 18, 2021

Ron Dror



Nature,
Sept. 10, 2015

THE REVOLUTION WILL NOT BE CRYSTALLIZED

**MOVE OVER X-RAY CRYSTALLOGRAPHY.
CRYO-ELECTRON MICROSCOPY IS
KICKING UP A STORM IN STRUCTURAL
BIOLOGY BY REVEALING THE HIDDEN
MACHINERY OF THE CELL.**

BY EWEN CALLAWAY

In a basement room, deep in the bowels of a steel-clad building in Cambridge, a major insurgency is under way.

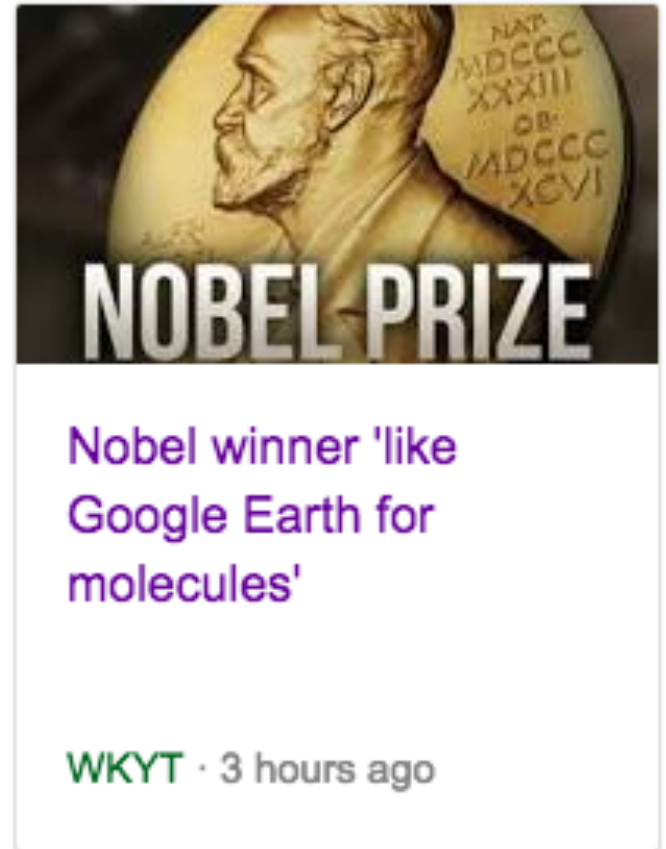
A hulking metal box, some three metres tall, is quietly beaming terabytes' worth of data through thick orange cables that disappear off through the ceiling. It is one of the world's most advanced cryo-electron microscopes: a device that uses electron beams to photograph frozen biological molecules and lay bare their molecular shapes. The microscope is so sensitive that a shout can ruin an experiment, says Sjors Scheres, a structural biologist at the UK Medical Research Council Laboratory of Molecular Biology (LMB), as he stands dwarfed beside the £5-million (US\$7.7-million) piece of equipment. "The UK needs many more of these, because there's going to be a boom," he predicts.

In labs around the world, cryo-electron microscopes such as this one are sending tremors through the field of structural biology. In the past three years, they have revealed exquisite details of protein-making ribosomes, quivering membrane proteins and other key cell molecules,

ILLUSTRATION BY MIKHAILOV

2017 Nobel Prize

Awarded to Jacques Dubochet, Joachim Frank and Richard Henderson and "For developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution"



Outline

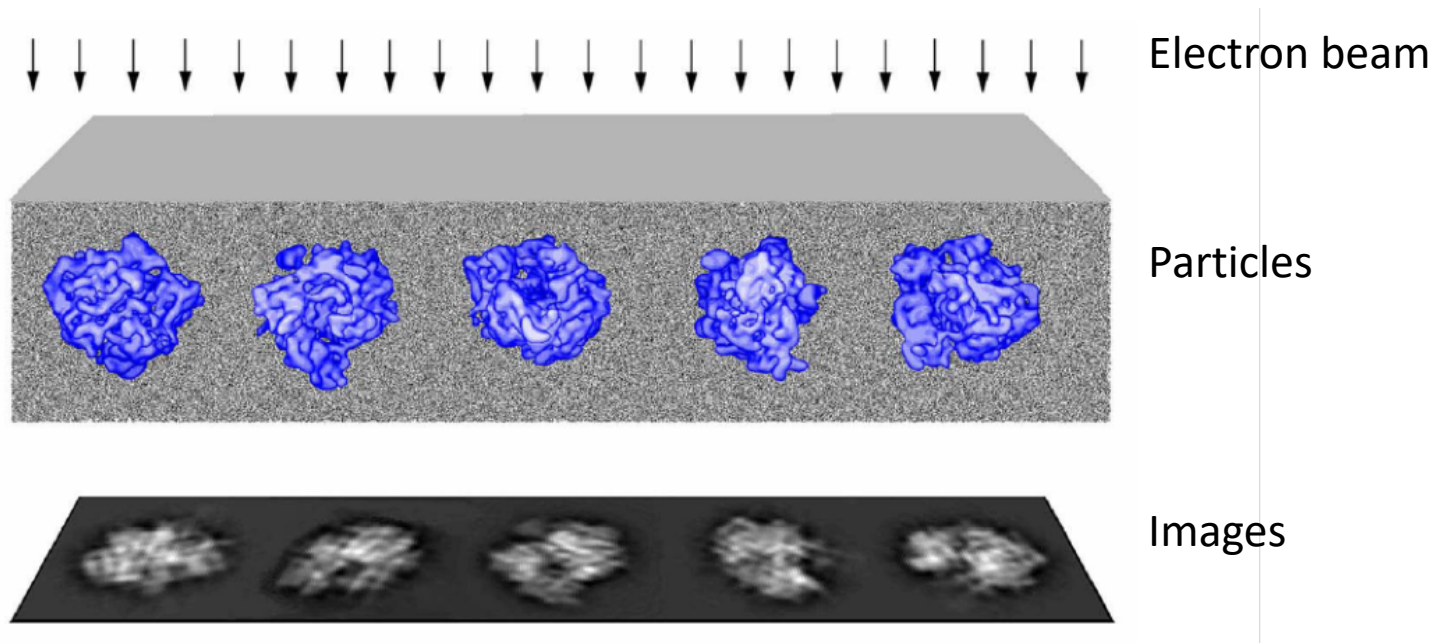
NOTE: CryoEM avoids the need to form crystals, which in many cases can be challenging for large molecules/ molecule complexes

- Overview of cryo-electron microscopy (cryo-EM)
- Cryo-EM images are *projections*
- Sample preparation
- Computational reconstruction methods
 - 2D image analysis
 - Image preprocessing
 - Particle picking
 - Image clustering and class averaging
 - 3D reconstruction
 - Reconstruction with known view angles
 - Structure refinement with unknown view angles
 - Calculating an initial structure
 - Fitting atomic-resolution models to lower-resolution EM structures
 - Capturing multiple conformations

Overview of cryo-electron microscopy (cryo-EM)

The basic idea

- We want the structure of a “particle”: a molecule (e.g., protein) or a well-defined complex composed of many molecules (e.g., a ribosome)
- We spread identical particles out on a film, and image them using an electron microscope
- The images are two-dimensional (2D), and each particle is positioned with a different, unknown orientation.
- Given enough 2D images of particles, we can computationally reconstruct the 3D shape of the particle





A high-end cryo-electron microscope

3 meters tall, costing millions of dollars!
Here's a pic of Ron for comparison.



Dramatic recent improvements

- Cryo-EM has been around for decades, but it has improved *dramatically* in the last several years due to:
 - Invention of better cameras
 - Until recently, electrons were detected either by photographic film, or by scintillator-based digital cameras which converted electrons to photons for detection
 - New “direct-electron detectors” can detect electrons directly, substantially improving image resolution and quality
 - Better computational reconstruction techniques
- Cryo-EM is thus coming into much wider use, and may challenge crystallography as the dominant experimental method for determining molecular structure.

Comparison to x-ray crystallography

- Cryo-EM's major advantage over crystallography is that it does not require formation of a crystal
 - Particularly advantageous for large complexes, which are usually difficult to crystallize
 - Also avoids structural artifacts due to packing in a crystal lattice. In EM, particles are in a more natural environment.
- On the other hand:
 - Cryo-EM's resolution is usually worse than that of crystallography
 - Reconstructing structures of small proteins from EM images is difficult, because images from different orientations look similar (i.e., “a blob”)
- Bottom line: Cryo-EM is particularly advantageous for larger complexes/molecules, because:
 - Large complexes tend to be harder to crystallize
 - The computational reconstruction problem in cryo-EM is usually easier to solve for large, asymmetric particles than for small ones

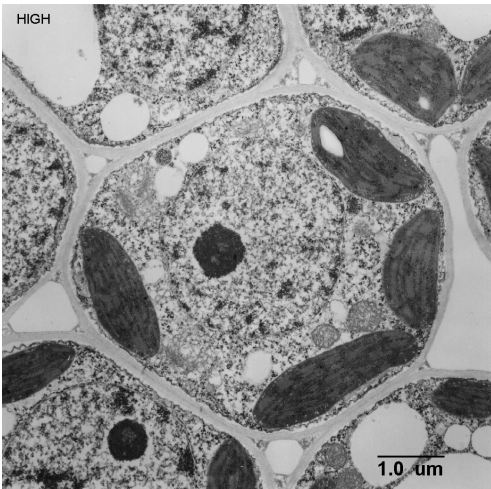
Cryo-EM images are *projections*

Cryo-EM uses *transmission* electron microscopy

In transmission EM, the source and of electrons and camera are on opposite sides

- In transmission electron microscopy, a beam of electrons pass through a thin sample before forming an image

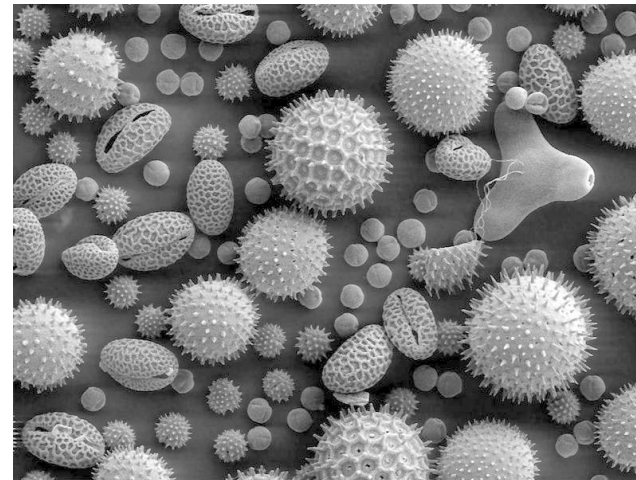
Transmission electron microscopy



http://www.cas.miamioh.edu/~meicenrd/ANATOMY/Ch2_Ultrastructure/Tempcell.htm

In scanning EM, you get particles that are reflected off surface of sample. So the source of electrons and camera are on same side.

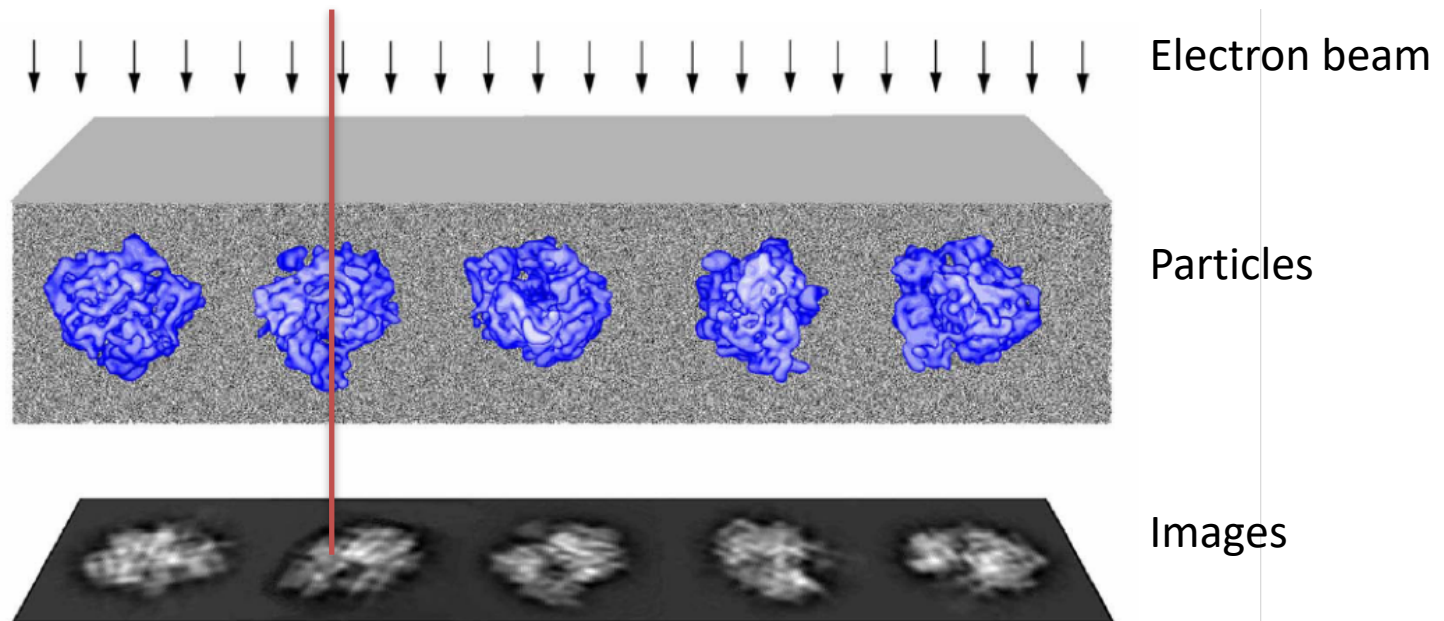
Scanning electron microscopy

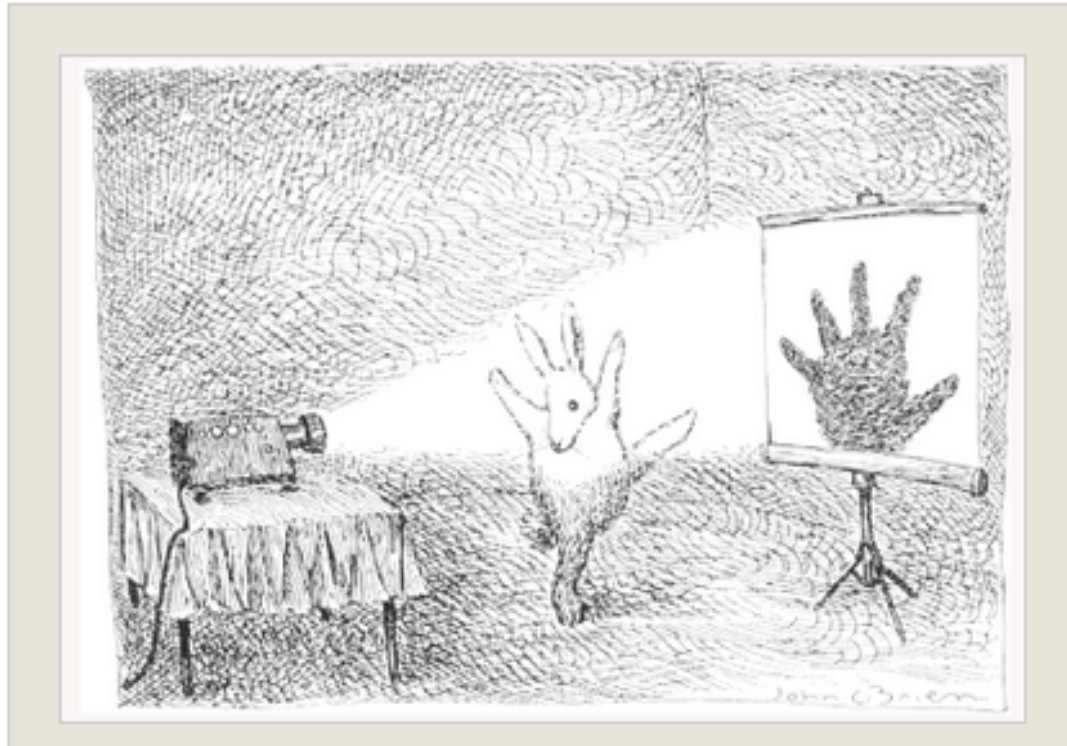


http://www.newscientist.com/data/images/ns/cms/dn14136/dn14136-1_788.jpg

Cryo-EM images are projections

- Each recorded 2D image is thus a projection of the 3D shape (density) we want to reconstruct
 - That is, we can think of each pixel value in the 2D image as a sum of the values along a line through the 3D sample (in the direction of the electron beam)





From Joachim Frank, *Three-dimensional electron microscopy of macromolecular assemblies: Visualization of biological molecules in their native state*, 2006

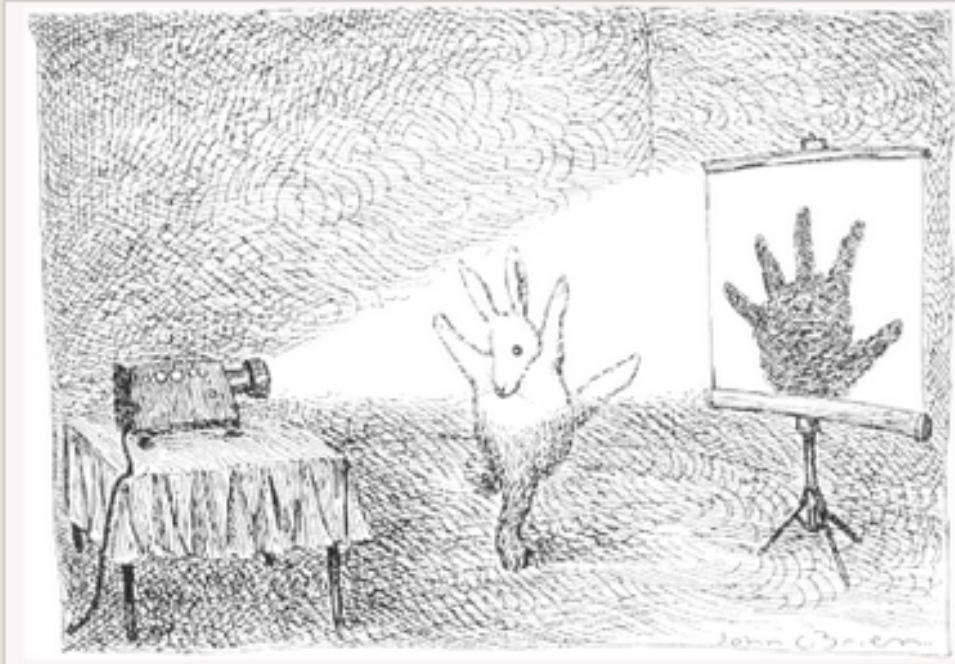


Figure 5.1 A single projection image is plainly insufficient to infer the structure of an object. (Note, though, that TEM projections do not merely give the outline, as in this drawing, but internal features, too—the bones and internal organs of the rabbit, which we would see if the projector were to emit X-rays.) (Drawing by John O'Brien; © 1991 *The New Yorker*.)

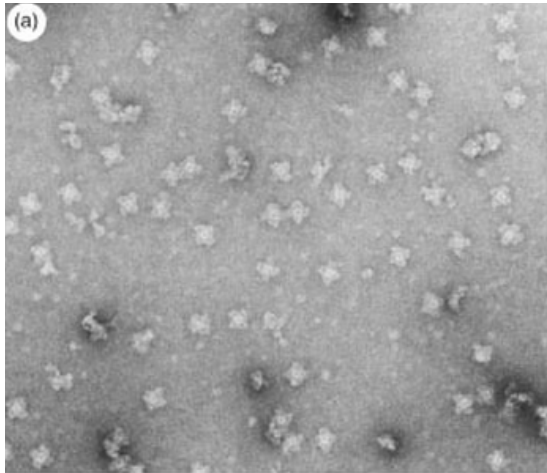
In transmission EM, the image would look more like an “x-ray” of the bunny than a shadow of the bunny

Sample preparation

Sample preparation

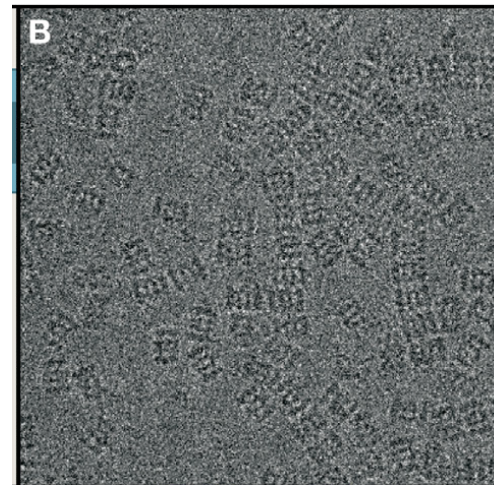
- To survive in the electron microscope (in a vacuum, under electron bombardment), particles are usually prepared in one of two ways:
 - Negative staining
 - Coat particles with heavy metal salt crystals
 - This increases contrast (particles are easy to pick out from background)
 - It limits resolution to ~ 20 Å and can introduce artifacts
 - Vitrification
 - Flash frozen means very quickly frozen. The purpose is to preserve the conformation
 - Particles are embedded in ice (vitreous ice: flash frozen, not crystalline)
 - This gives less contrast, but enables much higher resolution (below 4 Å)
 - High-resolution single-particle EM relies on vitrification and is thus referred to as cryogenic electron microscopy (cryo-EM)

Here we see images are quite noisy with small contrast between particles and background



Negative stain.
Particles are easy to pick out.

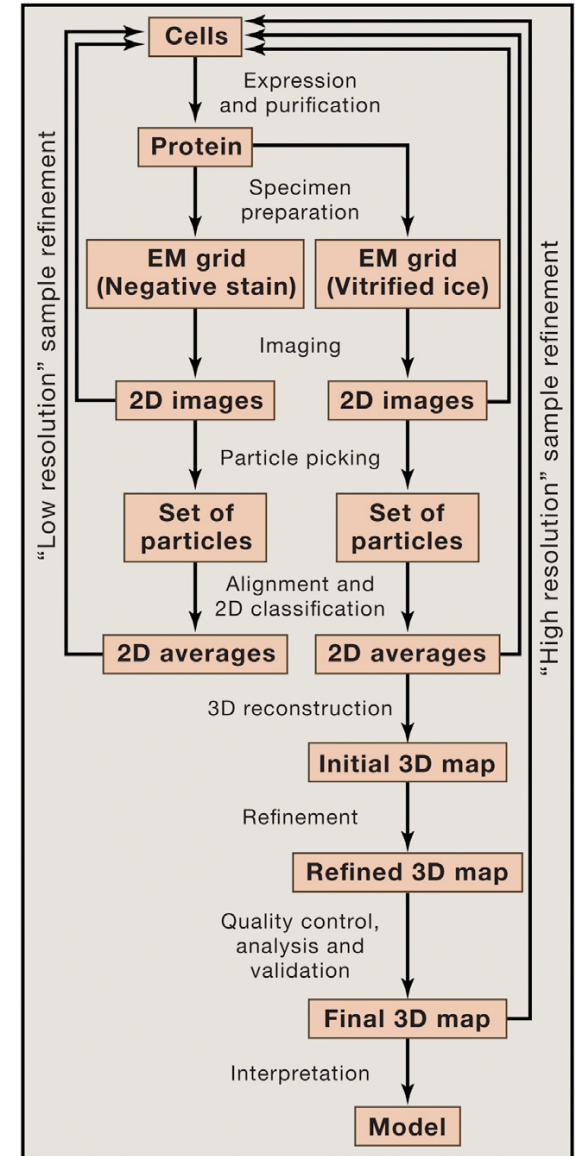
Frank, 2006



Vitreous ice.
Particles are harder to pick out,
even though this is a very good
("easy") case.

Cheng, *Cell* 161:450 (2015)

Negative stain is often used as an initial step in the experimental work, but we'll focus on the cryo-EM data (i.e., images from vitrification) —specifically, on using that data to calculate the 3D structure



Computational reconstruction methods

Overview of computational methods

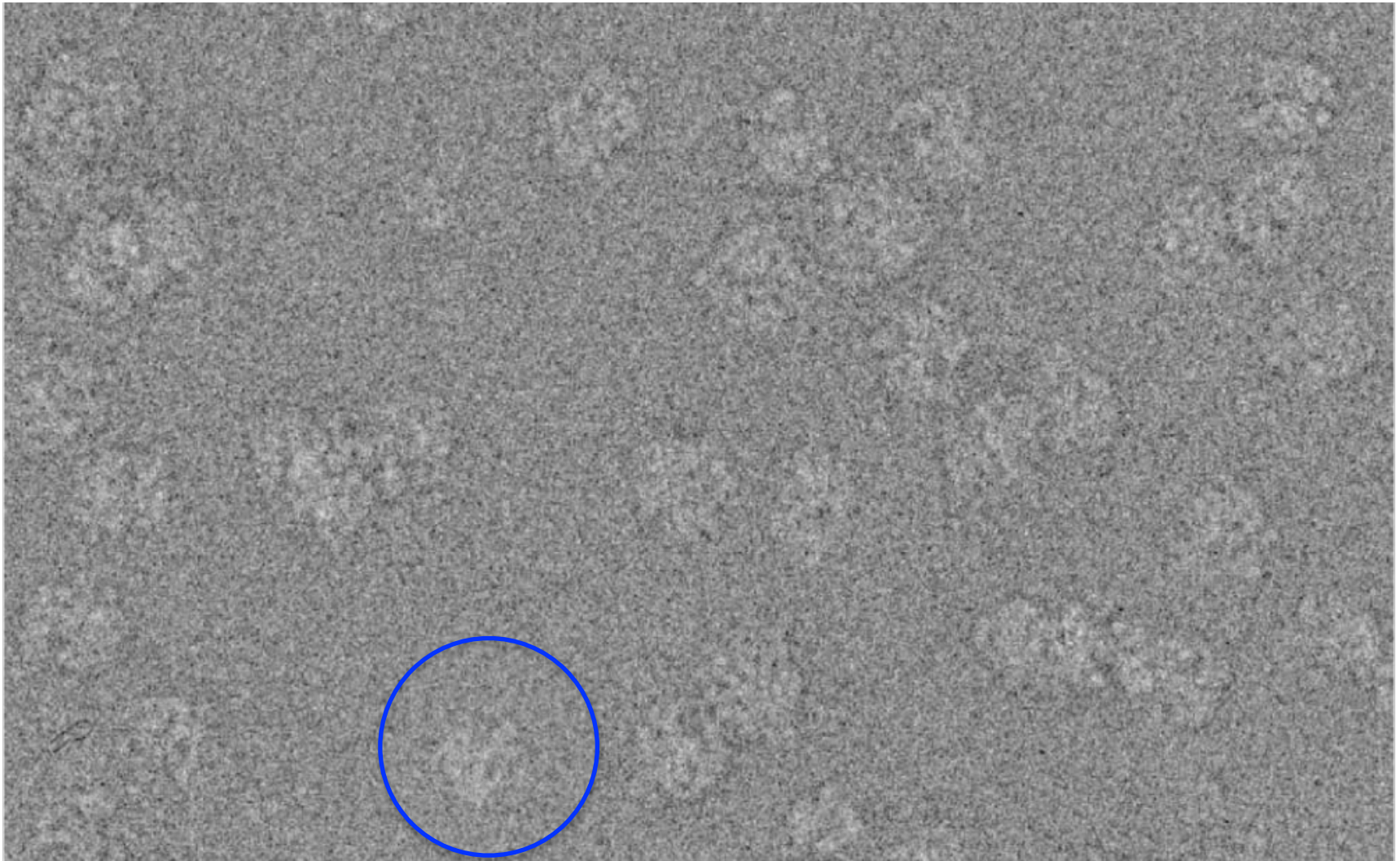
- **2D image analysis:** First, go from raw image data to higher-resolution 2D projections
 - Image preprocessing
 - Particle picking
 - Image clustering and class averaging
- **3D reconstruction:** Then use these higher-resolution projections to build a 3D model
 - Background: Reconstruction with known view angles
 - Structure refinement with unknown view angles
 - Calculating an initial structure
 - Fitting atomic-resolution models to lower-resolution EM structures
 - Capturing multiple conformations

Computational reconstruction methods

2D image analysis

Very noisy image.

The raw images don't look so good



In the circle is a particle

Image from Joachim Frank

<http://biomachina.org/courses/structures/091.pdf> 22

Before attempting any 3D reconstruction, we do several types of processing on the images

Computational reconstruction methods

2D image analysis

Image preprocessing

Image preprocessing

- **Problem 1:** The sample tends to move slightly during imaging, blurring the image
- **Solution**
 - Direct electron detectors are fast enough to record a movie instead of a single image
 - Align the movie frames computationally, then average them together

Image preprocessing

- **Problem 2:** Overall brightness is often non-uniform (due to uneven illumination or sample thickness)
- **Solution:** high-pass filter the image

The same high pass filter we saw in previous lectures on image analysis

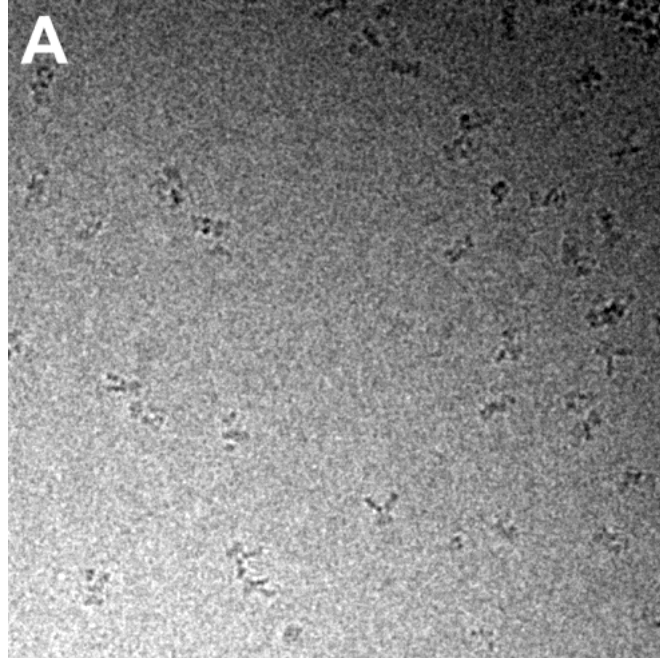
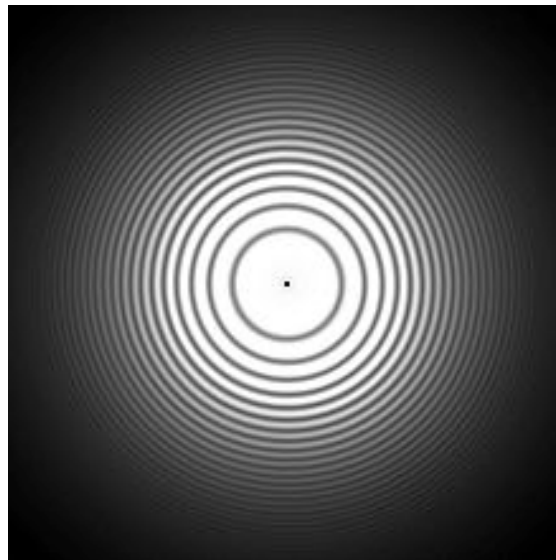


Image preprocessing

- **Problem 3:** The optics cause the recorded image to be a blurred version of the ideal image
 - This blurring is a convolution, and can thus be expressed as a multiplication in the frequency domain, where the ideal image is multiplied by the “contrast transfer function”
- **Solution:** Estimate parameters of the contrast transfer function, then correct for it
 - Some of the parameters are known (from the optics), while others are estimated from the images
 - Correction is generally done in the frequency domain



A typical contrast transfer function, in the frequency domain (zero frequency at the center)

The contrast transfer function is not perfect, so some information does get lost; however, it does an overall good job at unblurring

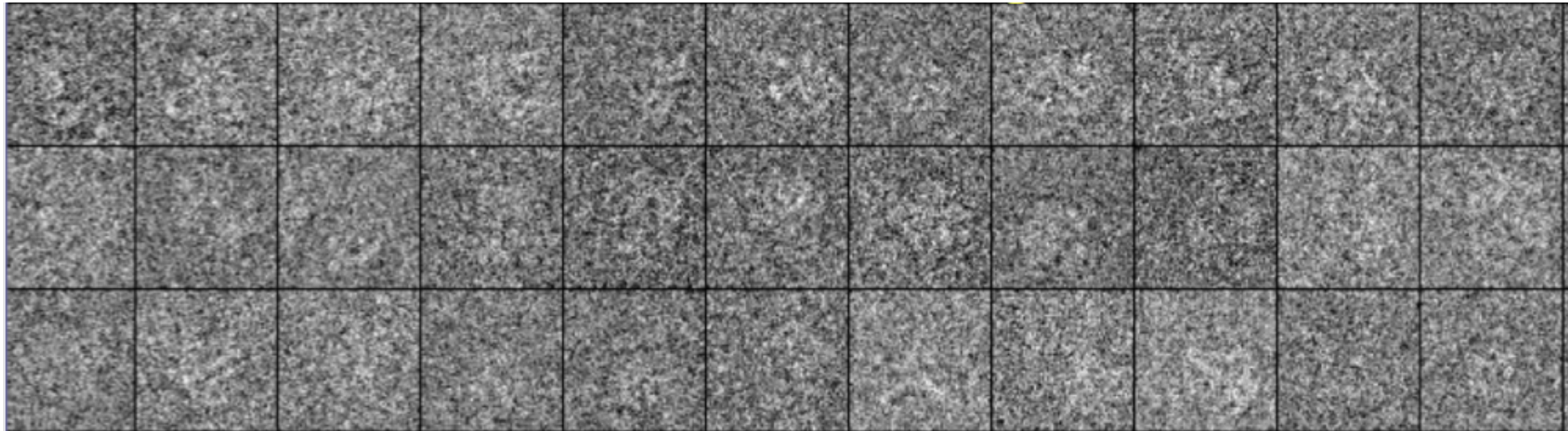
You're not responsible for the particular form of the contrast transfer function

Computational reconstruction methods

2D image analysis

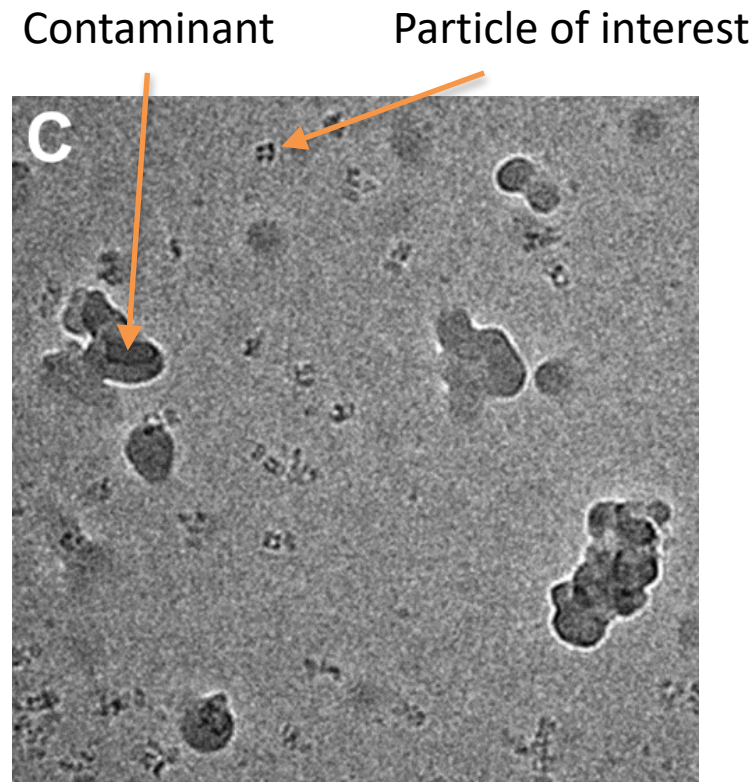
Particle picking

Particle picking results



Particle picking methods

- Particle picking can be difficult, because the images are low-contrast and noisy
 - Images may also have contaminants that should be ignored
- A variety of automated and semi-automated methods have been developed
 - For example, matching to templates, or picking out high-contrast regions
 - Some particles are often still packed manually to seed automated methods with suitable templates



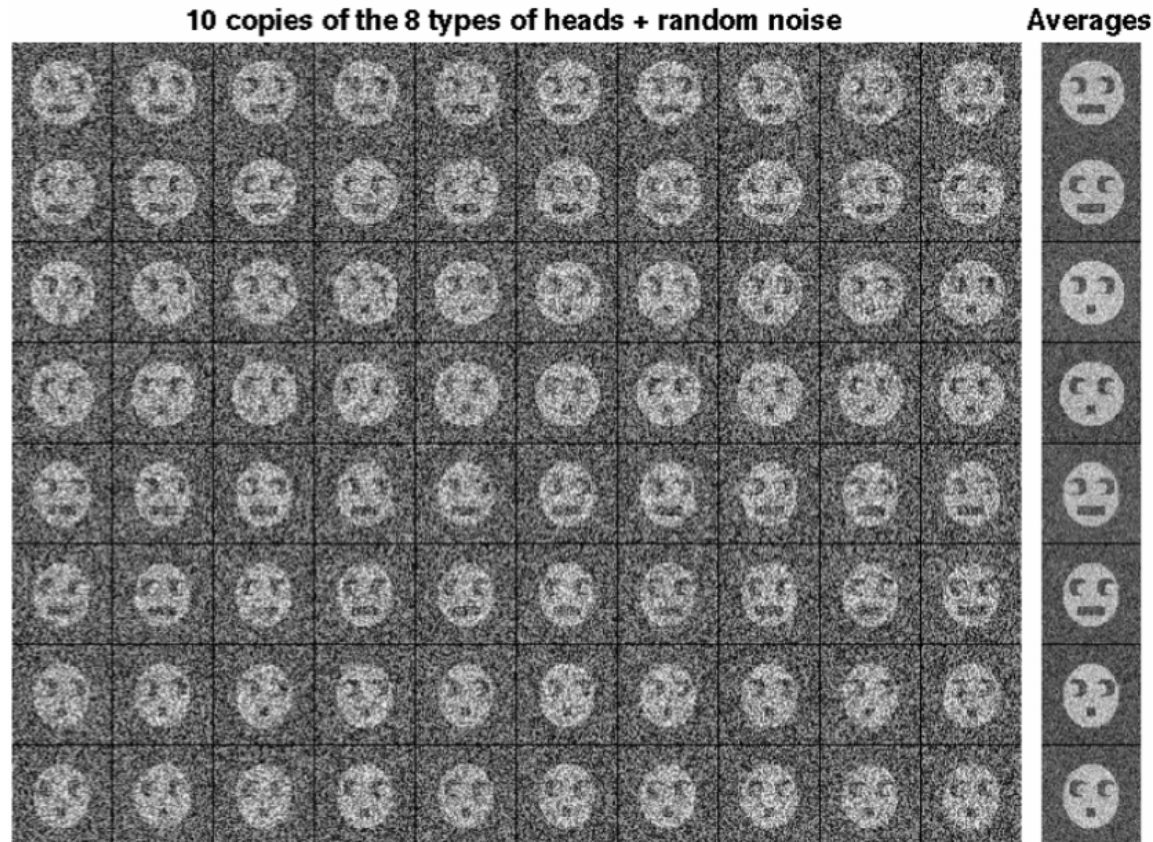
Cheng et al., *Cell* 2015

Computational reconstruction methods

2D image analysis

Image clustering and class averaging

Averaging similar images reduces noise



The challenge here is finding particles with the same orientation, meaning they are taken from the same angle.

Image from Joachim Frank
<http://biomachina.org/courses/structures/091.pdf>

- The images in each row above represent the same ideal image but with different corrupting noise
- If we average the images in each row (that is, average corresponding pixels), we end up with a less noisy image, because the noise in the different images tends to cancel out

Goal: cluster the particle images into classes of similar images

- Group together images with similar view angles
 - Then align them to one another and average them together to reduce noise
- To do this, divide images into several classes (with each class representing a set of similar view angles)
- We need to determine both what the classes are and which images should be assigned to each class
- This is a *clustering* problem
 - Group images such that the images within a group are similar, but images in different groups are different
 - In machine learning terminology, this is “unsupervised learning” We don’t know what the classes/labels are, hence “unsupervised”

Standard approach: k -means clustering

In the context of image analysis, the number k refers to how many angles our image is taken from

- Pick k random images as class exemplars
- Then iterate the following:
 - Assign each image to the closest exemplar
 - Average all the images in each class to determine a new class exemplar
- Notes:
 - In the assignment step, we need to align each particle image against the exemplar images
 - We need to specify the number of classes (k) in advance, or experiment with different values of k
 - k -means clustering is guaranteed to converge, but not guaranteed to find a globally optimal solution
 - Indeed, the solution may depend heavily on the initialization conditions, and may be heavily suboptimal

We repeat this process multiple times to get a reliable solution

Caveat: Potential model bias in clustering/alignment

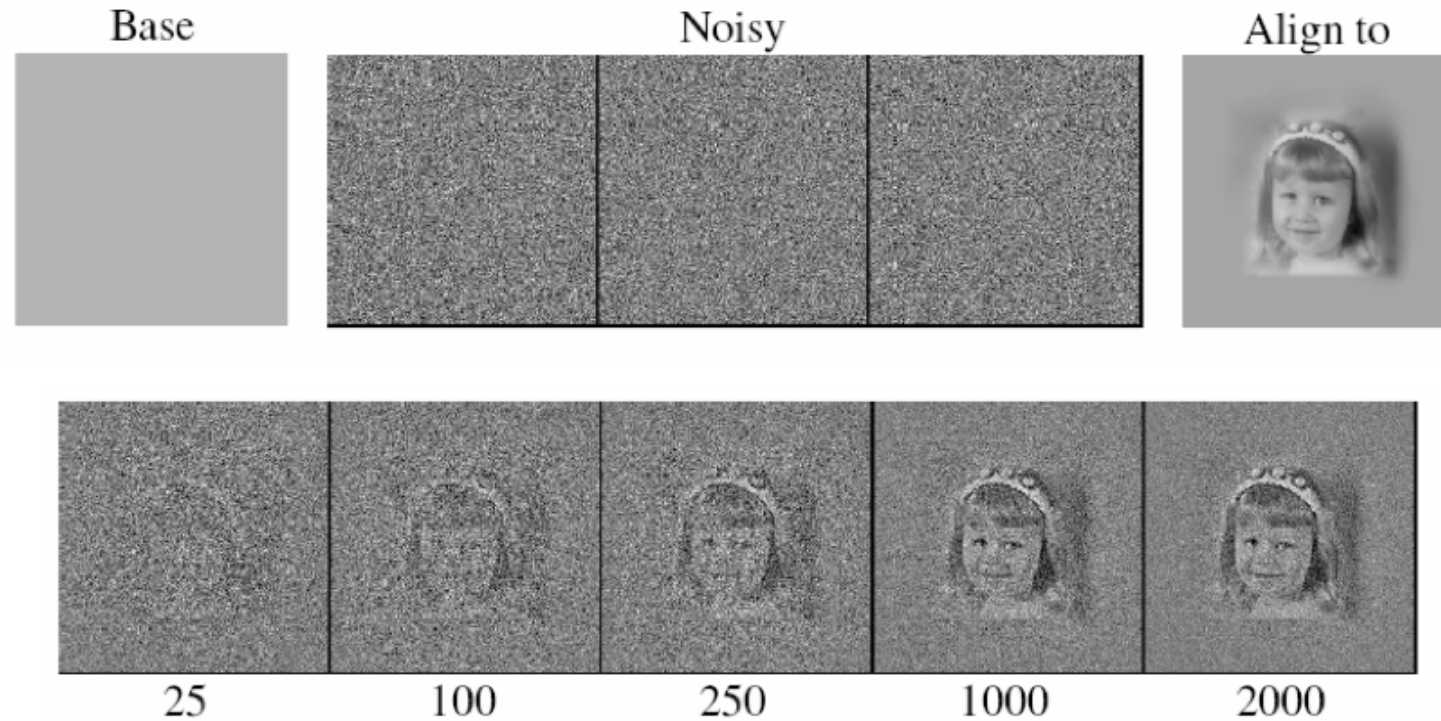


Image from Steve Ludtke

<http://biomachina.org/courses/structures/091.pdf>

In this case, the images are just noise, but by selecting images and alignments that best match a given template, we get a class average that looks like the template.

Caveat: Potential model bias in clustering/alignment

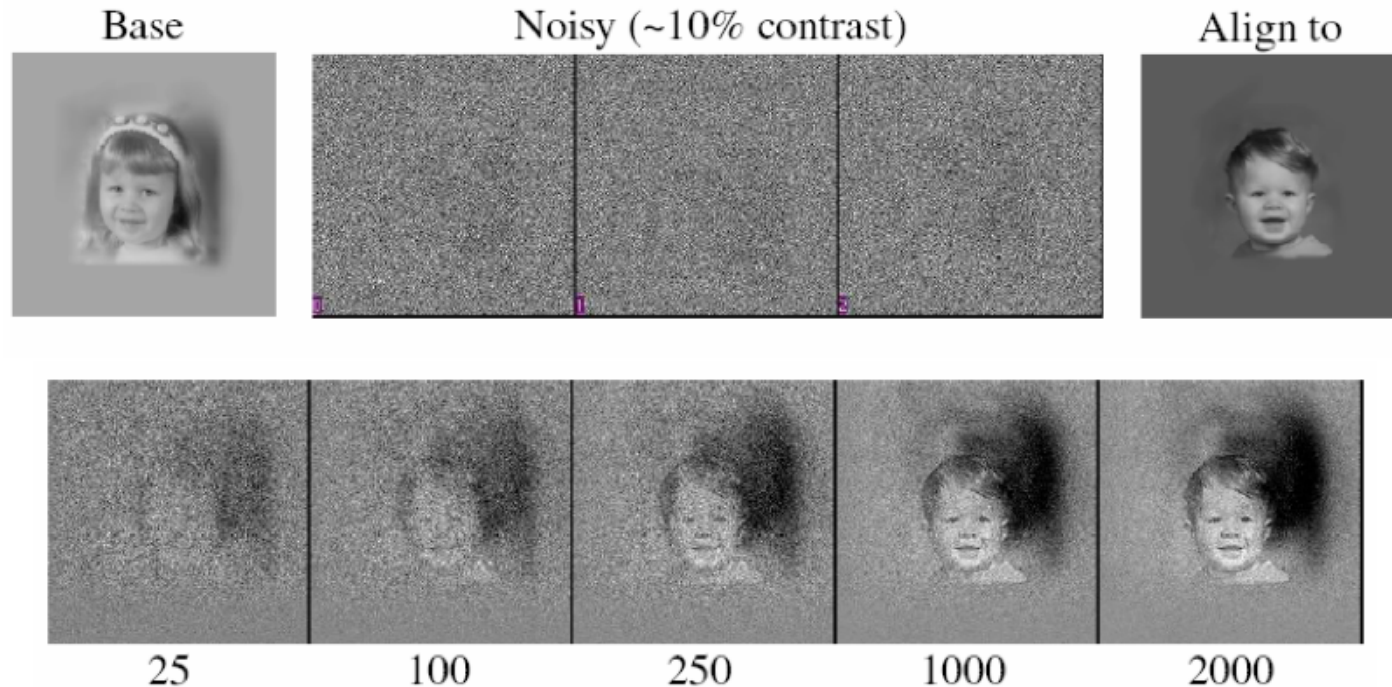


Image from Steve Ludtke

<http://biomachina.org/courses/structures/091.pdf>

In this case, the images are noisy versions of one face, but by selecting images and alignments that best match a second face, we get a class average that looks like the second face.

Avoiding these problems

- A variety of more sophisticated clustering methods ameliorate these problems
 - Some involve modifications to k-means (including the recently developed Iterative Stable Alignment and Clustering method)
 - Some involve principal components analysis or other dimensionality reduction techniques
 - Some recent methods eliminate this averaging step

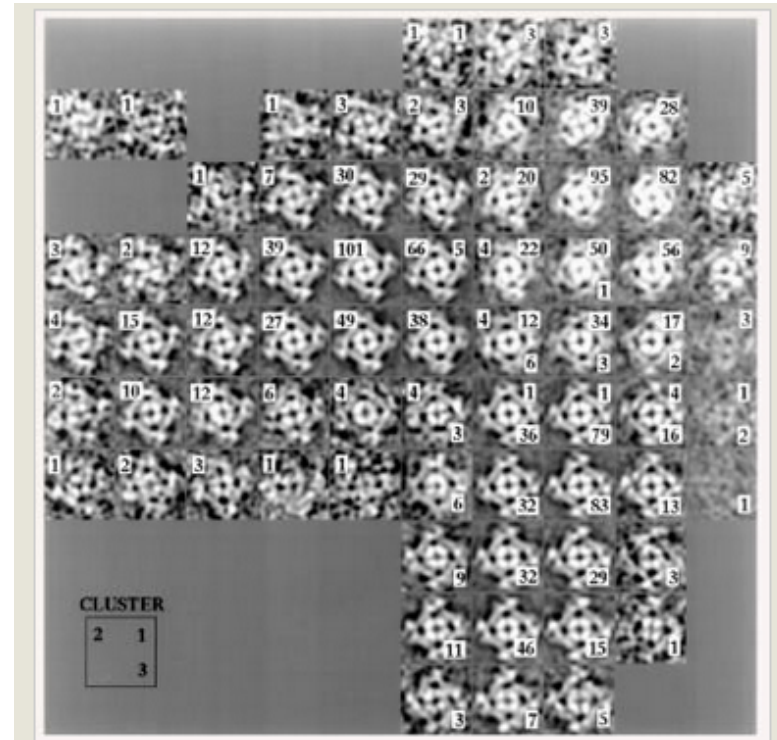
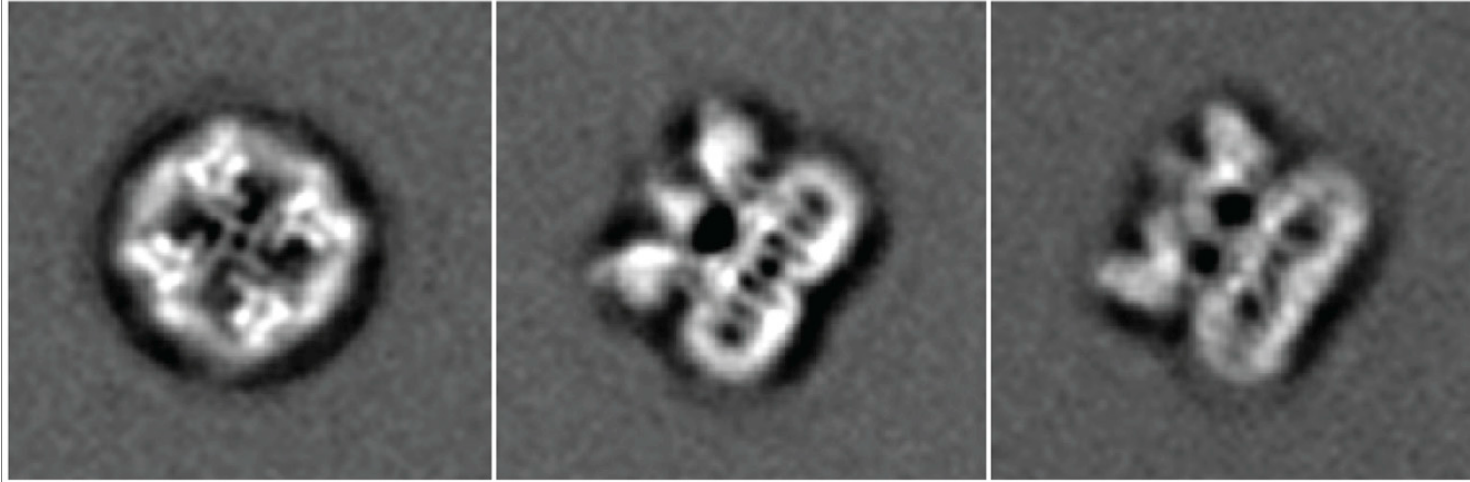


Figure 4.14 Checkerboard display of local averages (calcium release channel), each computed from images falling on a grid in factor space (factors 1 versus 2). The number on top of each average indicates the number of images falling into that grid space. Empty regions are bare of images. The distinction of main interest is between molecules lying in different orientations, related by flipping. It is seen from the peripheral pinwheel features pointing either clockwise (on the left) or counterclockwise (on the right). From Frank et al. (1996), reproduced with permission of Elsevier.

You're not responsible for these methods

Class averaging results



Cheng, *Cell* 161:450 (2015)

These are considered good class averages
(from a high-resolution single-particle EM study)

Different angles of the same particle

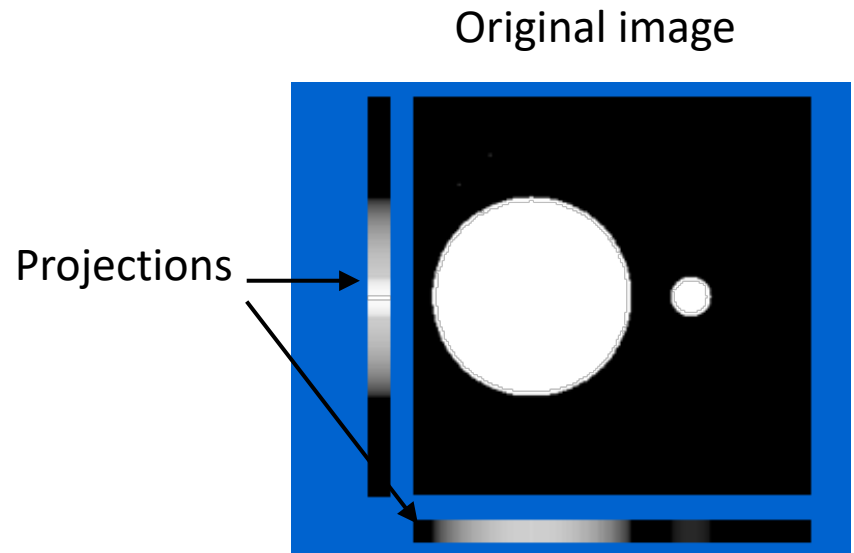
Computational reconstruction methods

3D reconstruction

How to go from 2D images with unknown viewing angles to a 3D shape

Problem

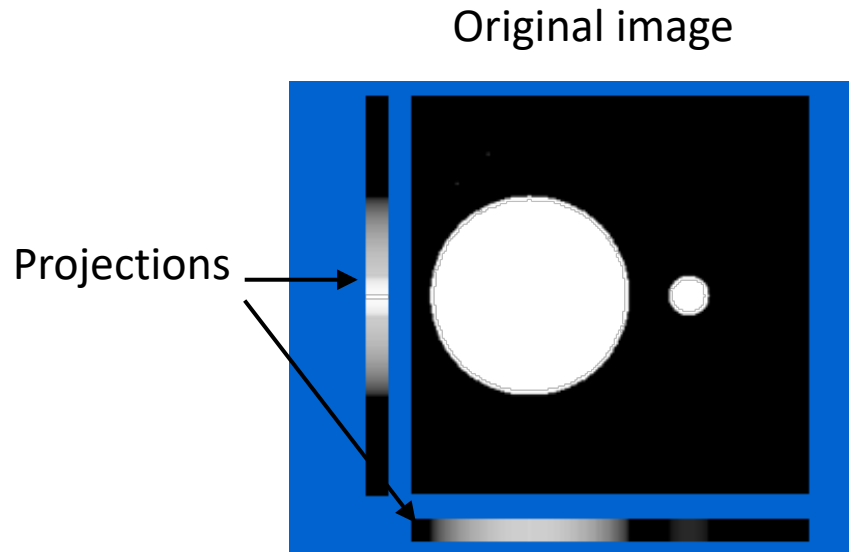
- Suppose you're given many projections of a 2D image, and you want to reconstruct the original image. How would you do it?



- Working with your neighbor, try to come up with one way to do it if you know the view angle for each projection, and another if you don't
 - Don't look at the next few slides. If you already have, try to come up with approaches that are not on those slides.

Problem

- Suppose you're given many projections of a 2D image, and you want to reconstruct the original image. How would you do it?



- How would you do it if you know the view angle for each projection?
- How would you do it if you *don't* know the view angle for each projection (as is the case for cryo-EM)?

Computational reconstruction methods

3D reconstruction

**Background: Reconstruction with known
view angles**

Suppose you knew the view angle for each particle image

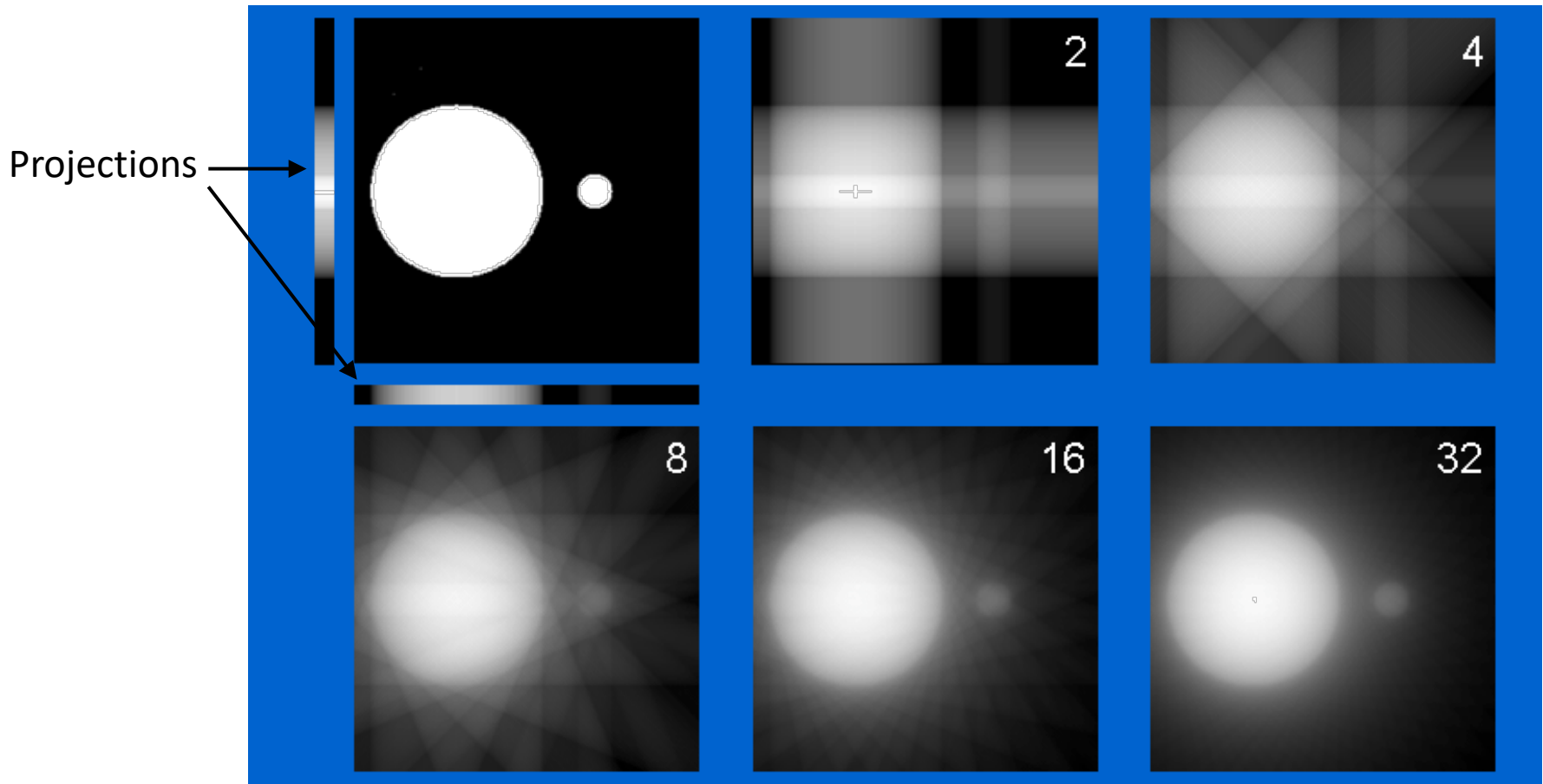
- How would you reconstruct the 3D density map from 2D projections? CT = computed tomography
 - Same problem is encountered in medical imaging (e.g. in CT scans, which are basically 3D x-rays)
- The simplest approach would be *back-projection*: reverse the projection process by “smearing” each projection back across the reconstructed image

Back-projection

Reconstructed image appears to improve as you increase the number of projects.

Original image

Reconstructions based on different numbers of projections (2, 4, 8, 16, 32)



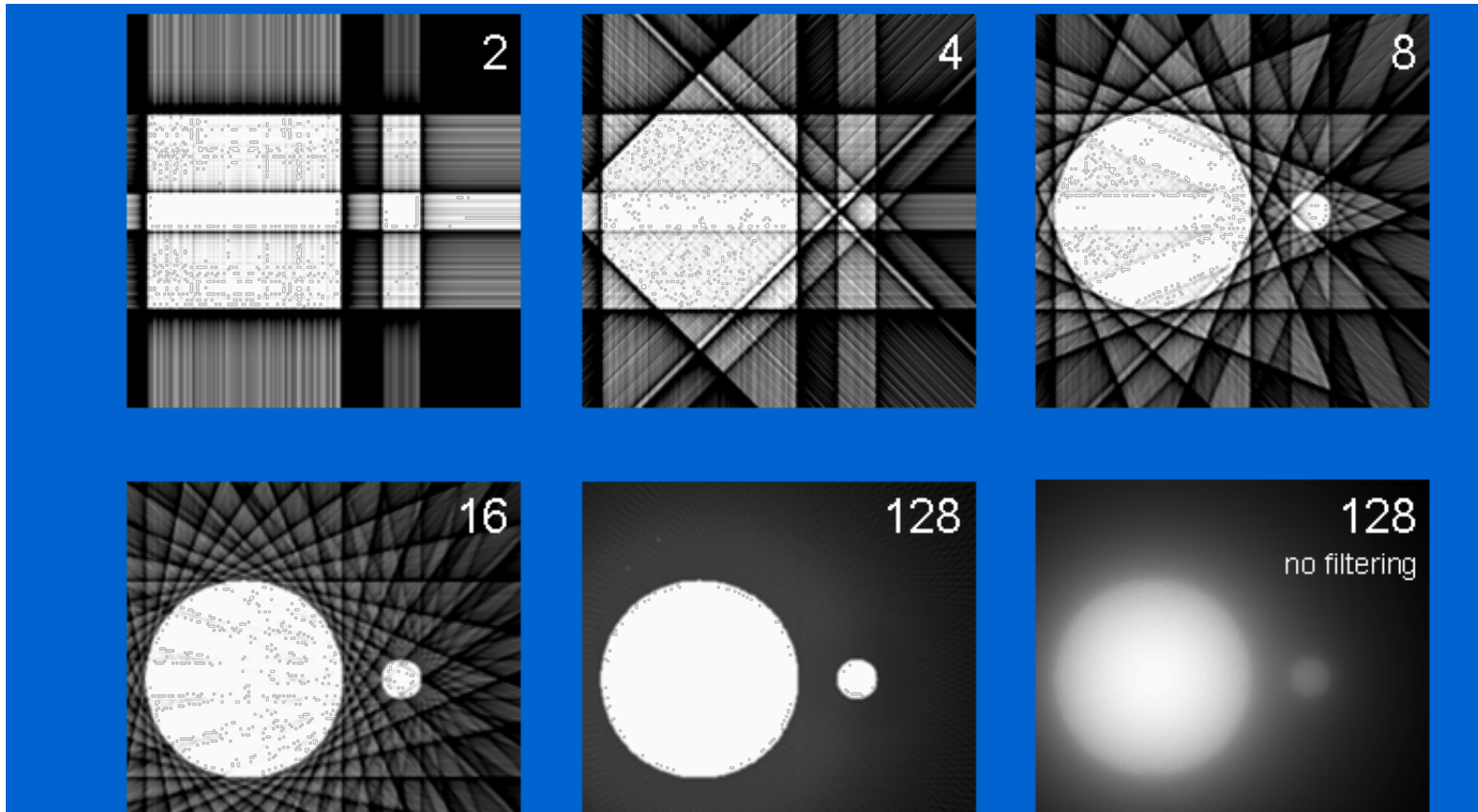
http://www.impactscan.org/slides/impactcourse/basic_principles_of_ct/img12.html

The result of back-projection is a *blurred* version of the original image. 44

How can we fix this? If you use a million projections, your image would improve but would still have blur

Filtered-back projection

- It turns out we can fix this problem by applying a specific high-pass filter to each image before back-projection. This is *filtered back-projection*.



Why does filtered back-projection work?

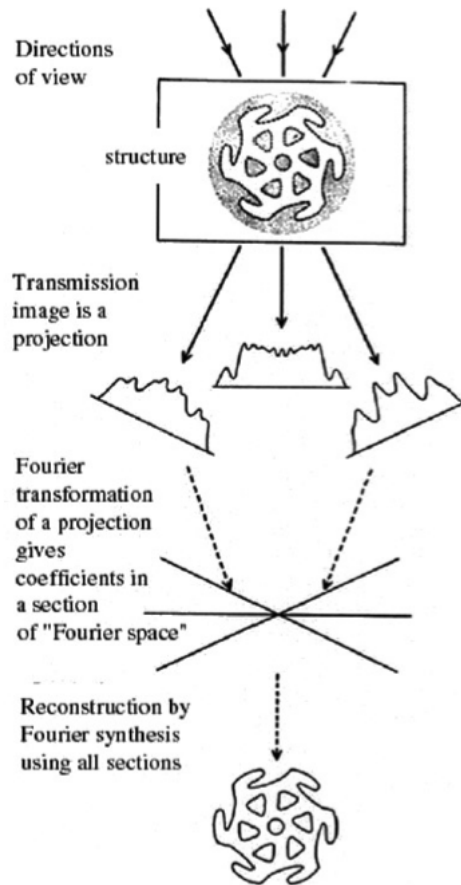
(Optional Material)

- To answer this, use the *projection slice theorem*

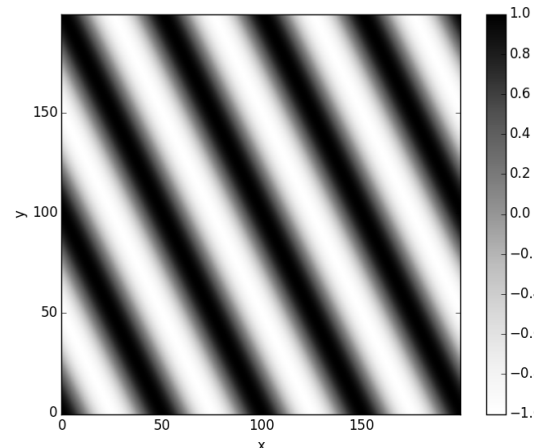
Projection slice theorem (2D version):

The 1D Fourier transform of the 1D projection of a 2D density is equal to the central section—perpendicular to the direction of projection—of the 2D Fourier transform of the density

This theorem holds because each of the 2D sinusoids used in the 2D Fourier transform is constant in one direction



DeRosier & Klug, Nature 217 (1968) 133



Why does filtered back-projection work?

- Back-projection is equivalent to filling in central sections in the Fourier domain
- The problem is that when reconstructing by back-projection, we overweight the low-frequency values (in the figure, the density of dots is greatest near the center)
- To fix this, reduce the weights on low-frequency components.

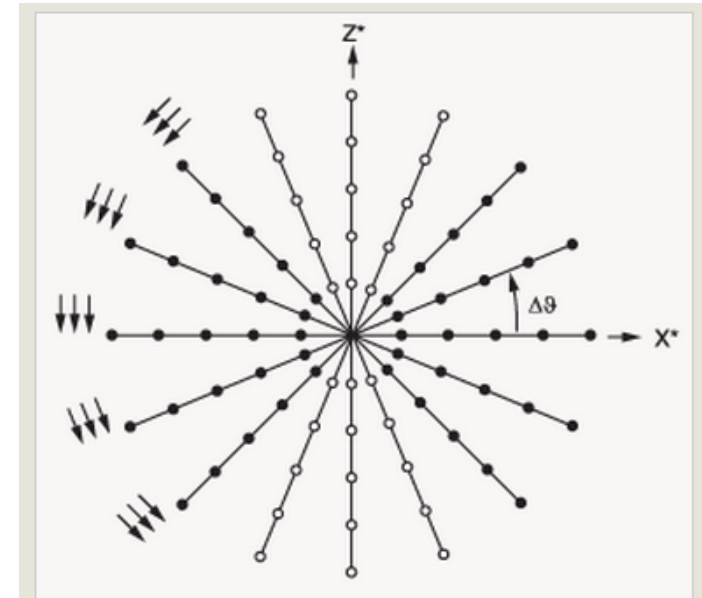
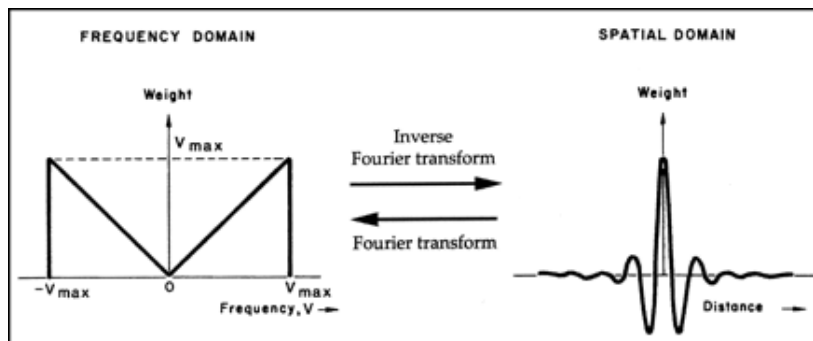


Figure 5.15 The density of sampling points in Fourier space obtained by projections decreases with increasing spatial frequency. Although this is shown here for single-axis tilting, the same is obviously true for all other data collection geometries. From Frank and Radermacher (1986), reproduced with permission of Springer-Verlag.

Frank, 2006

Filtered back-projection is a common technique, but there are several alternatives, including direct Fourier-domain reconstruction

Ideal filter shape grows linearly with frequency.



<http://jnm.snmjournals.org/cgi/content-nw/full/42/10/1499/F2>

This carries over to the 3D case

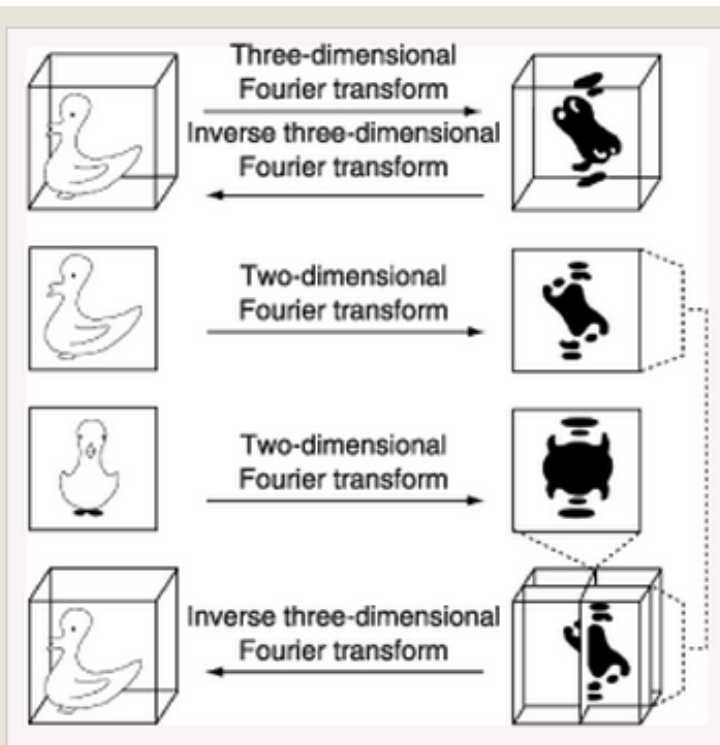


Figure 5.2 Illustration of the projection theorem and its use in 3D reconstruction. From Lake (1971), reproduced with permission of Academic Press Ltd.

Projection slice theorem (3D version):
The 2D Fourier transform of the 2D projection of a 3D density is equal to the central section—perpendicular to the direction of projection—of the 3D Fourier transform of the density

Computational reconstruction methods

3D reconstruction

**Structure refinement with unknown view
angles**

Refining a structure

- If we're not given the view angles for each particle, but we have a decent initial 3D model, then iterate the following steps to improve the model:
 - For each projection (i.e., each class average), find the view angle that best matches the 3D model
 - Given the newly estimated view angles, reconstruct a better 3D model (e.g., using filtered back-projection)
- This is called *3D projection matching*

The viewing angle estimates aren't perfect, but they allow you to improve the 3D model. You would then iterate this process until you get a good 3D model.

An example

Class averages (starting point for reconstruction)

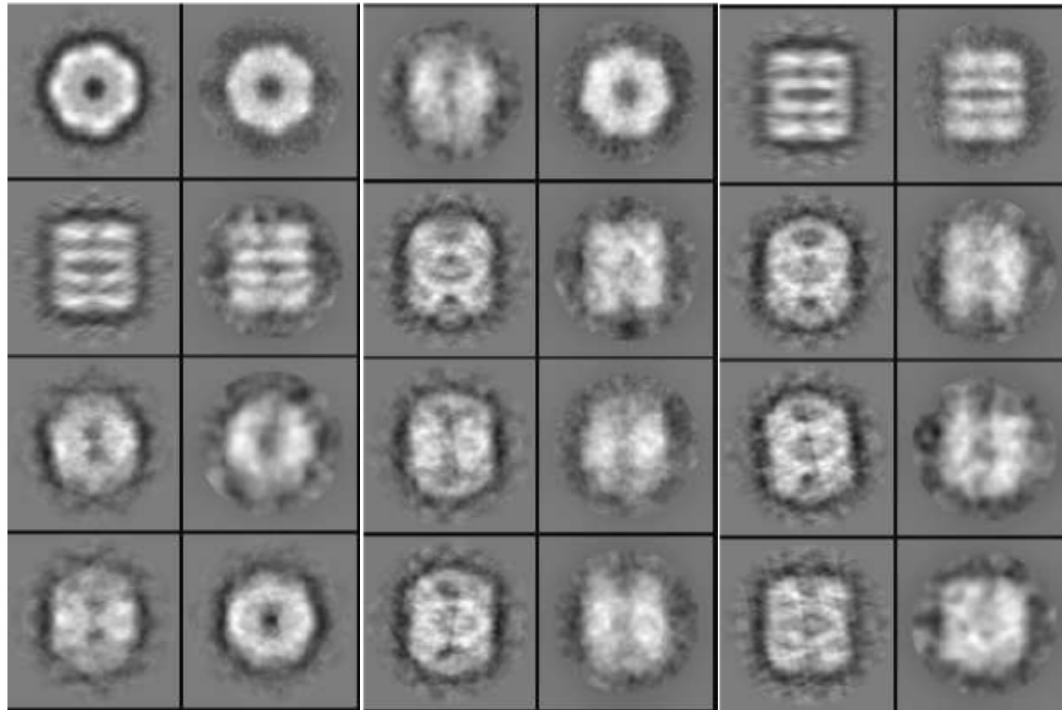
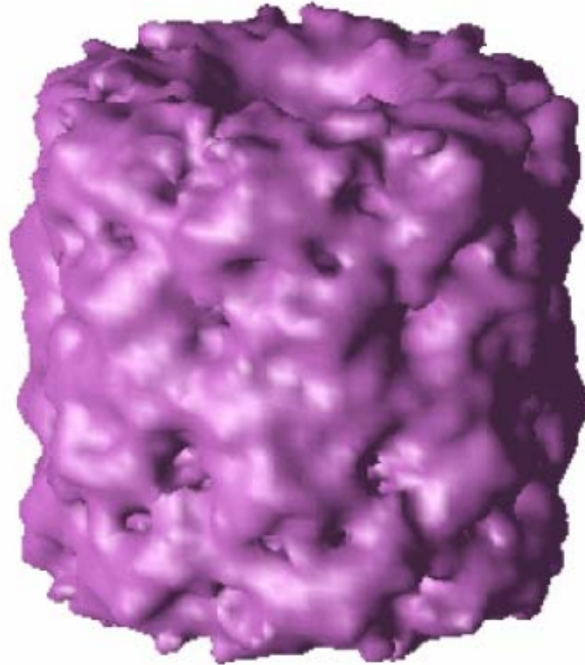


Image from Steve Ludtke

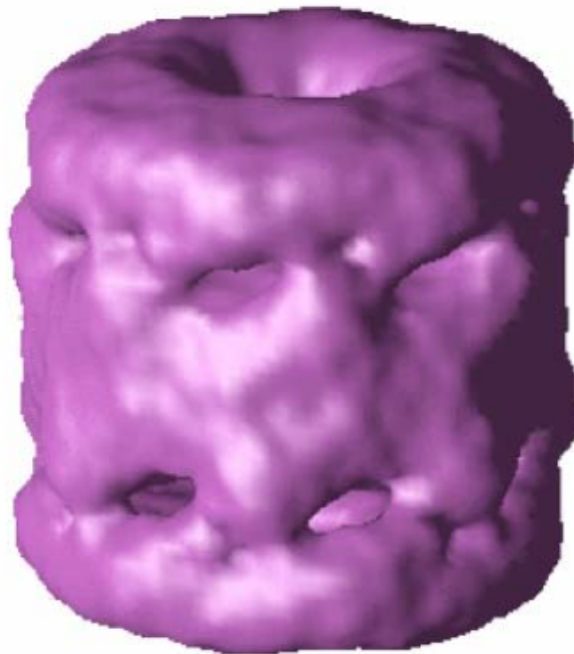
<http://biomachina.org/courses/structures/091.pdf>

Iteration 1

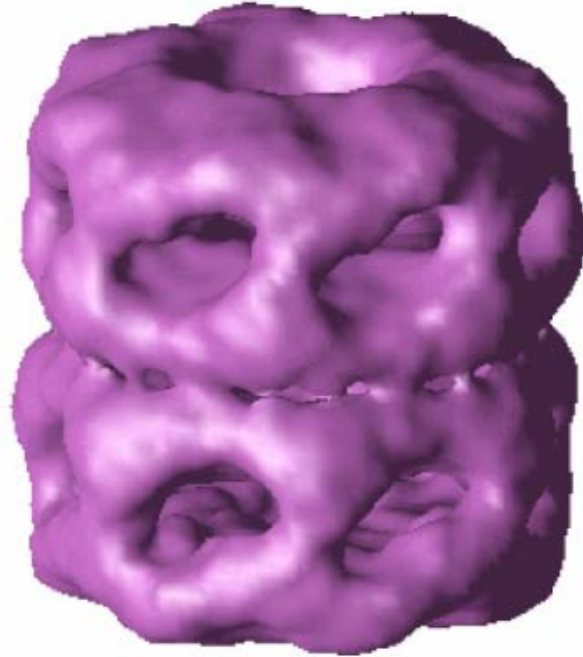


This surface is a contour map. Estimated density is greater than a threshold inside the surface and less than the threshold outside it. “Density” here corresponds roughly (not precisely) to electron density.

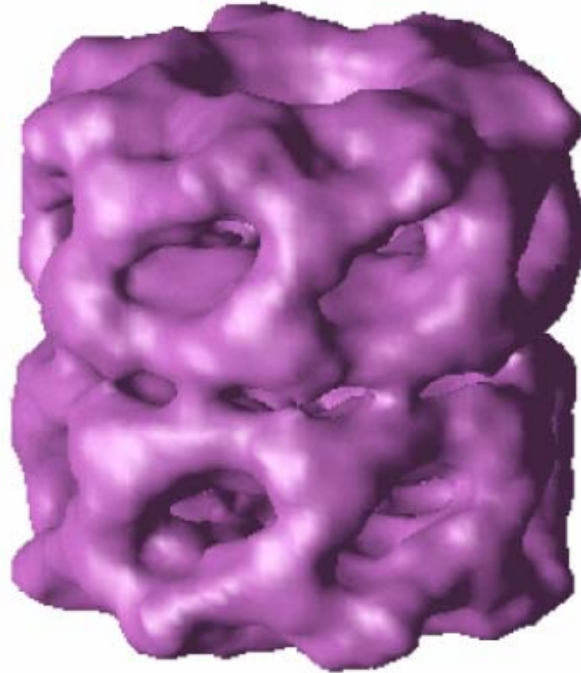
Iteration 2



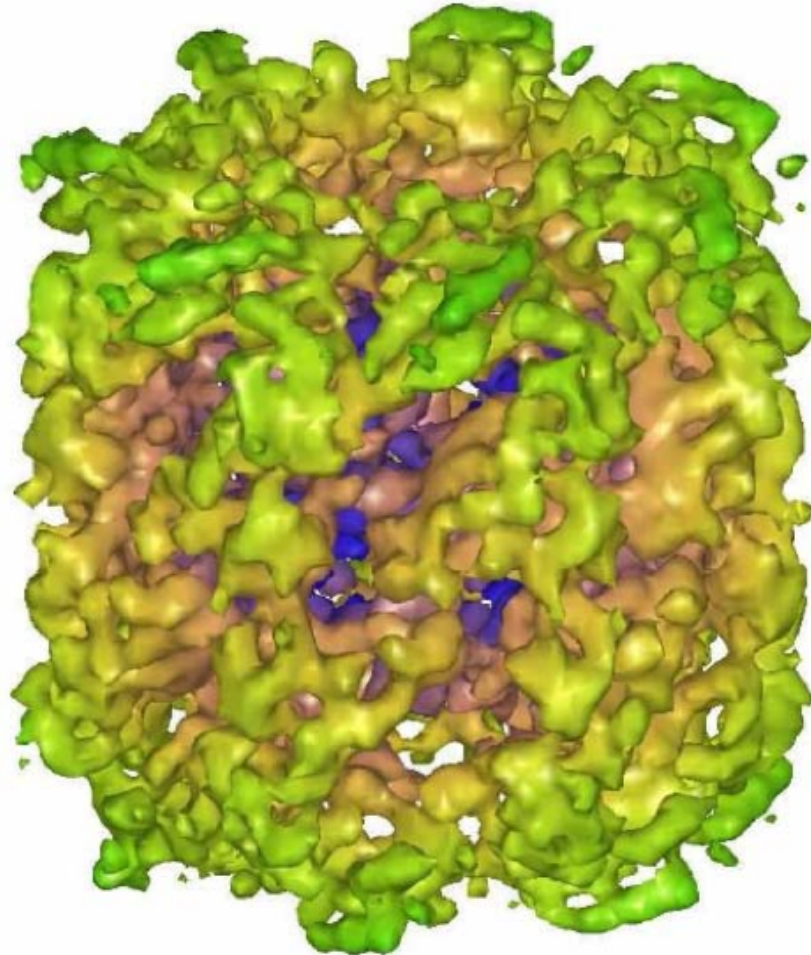
Iteration 3



Iteration 4



Final reconstruction

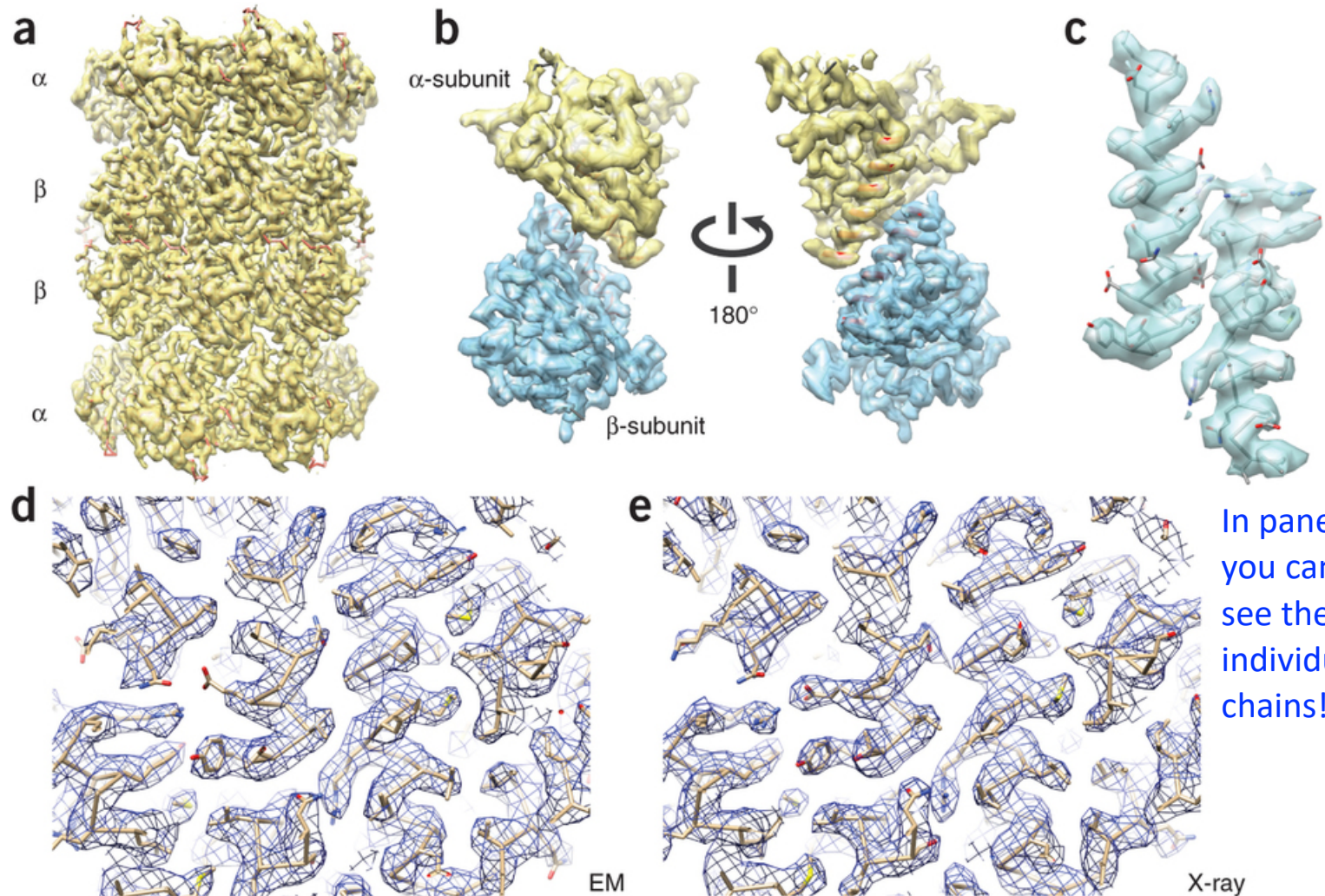


Protein: GroEL
6.5 Å resolution

Ignore the color
coding

When this was published in 2013, 6.5 angstroms was great resolution, but now in 2021 resolution has become much better.

A high-resolution single-particle EM structure



A 3.3 Å resolution EM structure

Caveat

- Structure refinement methods are prone to *overfitting*
 - Converged model can show features that don't really exist and just reflect noise in the images (analogous to the issue with image clustering)
 - A variety of methods have been developed recently to deal with this issue [Overfitting is an issue that often comes up in machine learning](#)
 - Some use Bayesian approaches (e.g., RELION software)
 - Some of the most important recent algorithmic developments in cryo-EM are in this area.

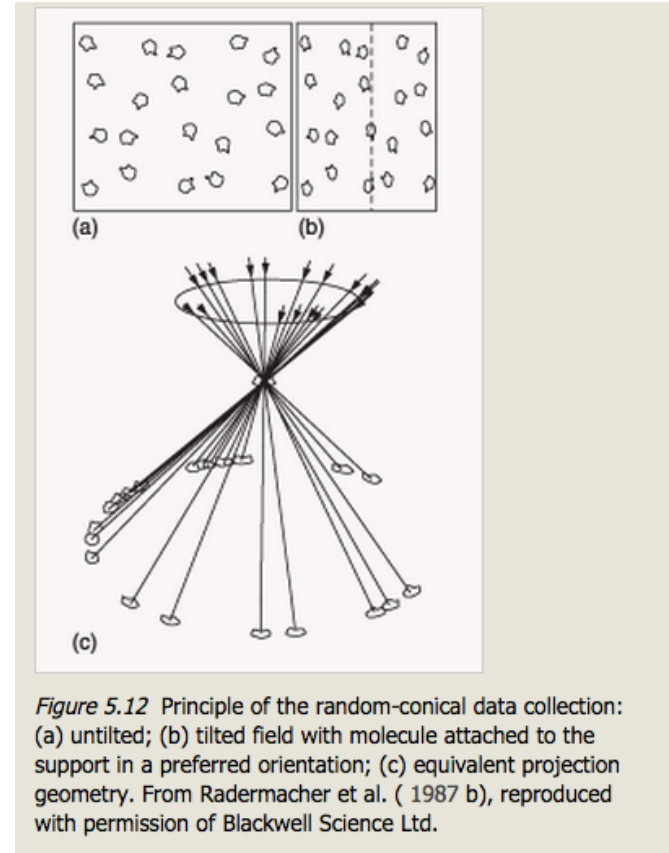
Computational reconstruction methods

3D reconstruction

Calculating an initial structure

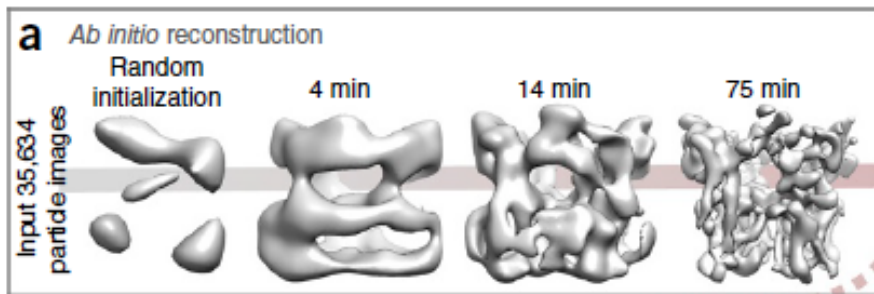
How do we get an initial structural model?

- Traditional options:
 - Might have an initial model from prior experimental work (e.g., a homologous protein)
 - Conduct specialized experiments, often at lower resolution
 - Example: *random canonical tilt* approach, which requires collecting each image twice, from different angles

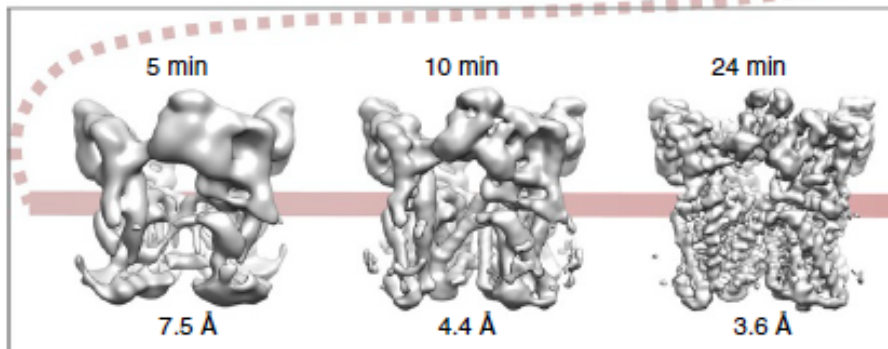


How do we get an initial structural model?

- Direct computational solutions are becoming practical!
- Example: stochastic gradient descent method: Today, stochastic gradient descent is the method to solve optimization problems in machine learning.
 - Choose a 3D model randomly
 - Repeat the following two steps:
 - Select a random subset of the images
 - Adjust the 3D model to maximize probability of observing the selected images



Stochastic
gradient
descent



Refinement

Punjani et al., cryoSPARC:
algorithms for rapid
unsupervised cryo-EM
structure determination,
Nature Methods (2017)

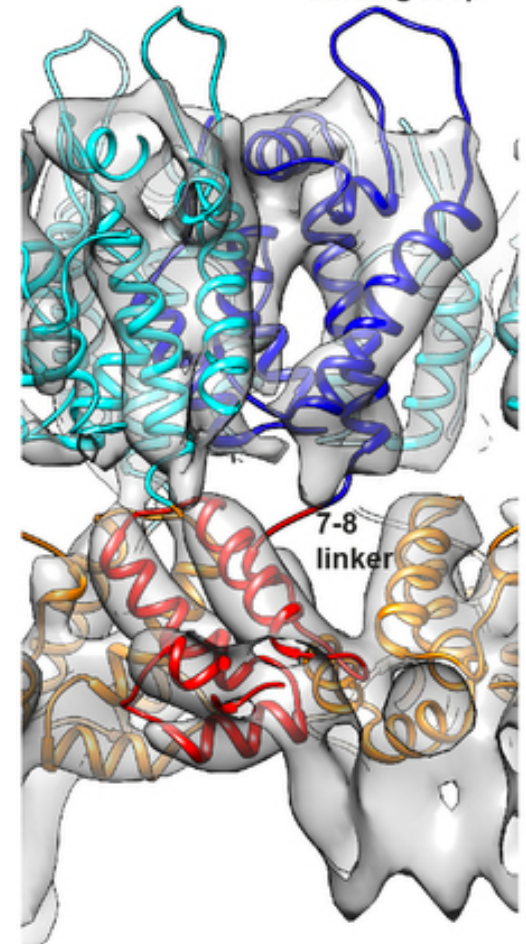
Computational reconstruction methods

3D reconstruction

**Fitting atomic-resolution models to
lower-resolution EM structure**

Obtaining atomic-resolution models from lower-resolution EM

- Often we have high-resolution x-ray crystallography structures of each individual protein in a complex whose low-resolution structure was determined by single-particle EM.
- We can fit the high-resolution structures into the EM density computationally.



Using only CryEM, we don't know how subunits bind together, and how the subunits adjust their conformations to fit together.

Obtaining atomic-resolution models from lower-resolution EM

- Approaches based on molecular dynamics simulations can be used to allow the proteins to relax away from their crystallographic structures to better fit the EM density.

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presents

Desktop MDFF

A recent development: Atomic-resolution Cryo-EM

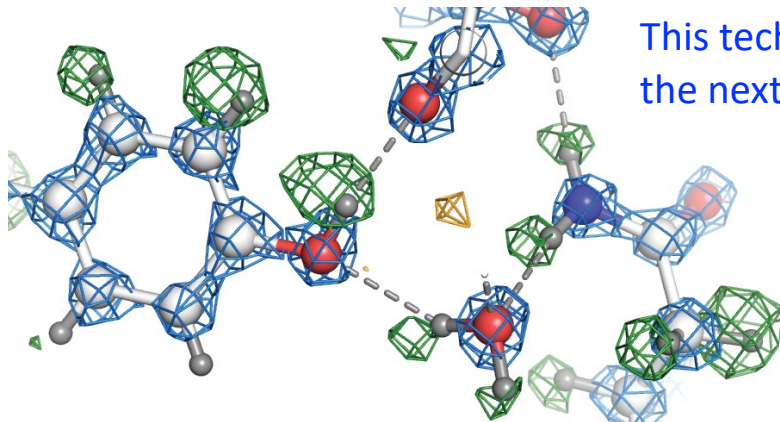
Nakane ... Scheres, *Nature*, Nov. 5, 2020

Single-particle cryo-EM at atomic resolution

Yip ... Stark, *Nature*, Nov. 5, 2020

Atomic-resolution protein structure determination by cryo-EM

- New technology (energy filter and new electron source, camera, software) allows resolution of 1.2 Å, at least for one protein
 - This resolution allows one to see density for individual atoms, even hydrogen!



This technology is still difficult to access; hopefully in the next several years, it will be more common

Nakane et al,
Nature, 2020

Computational reconstruction methods

3D reconstruction

Capturing multiple conformations

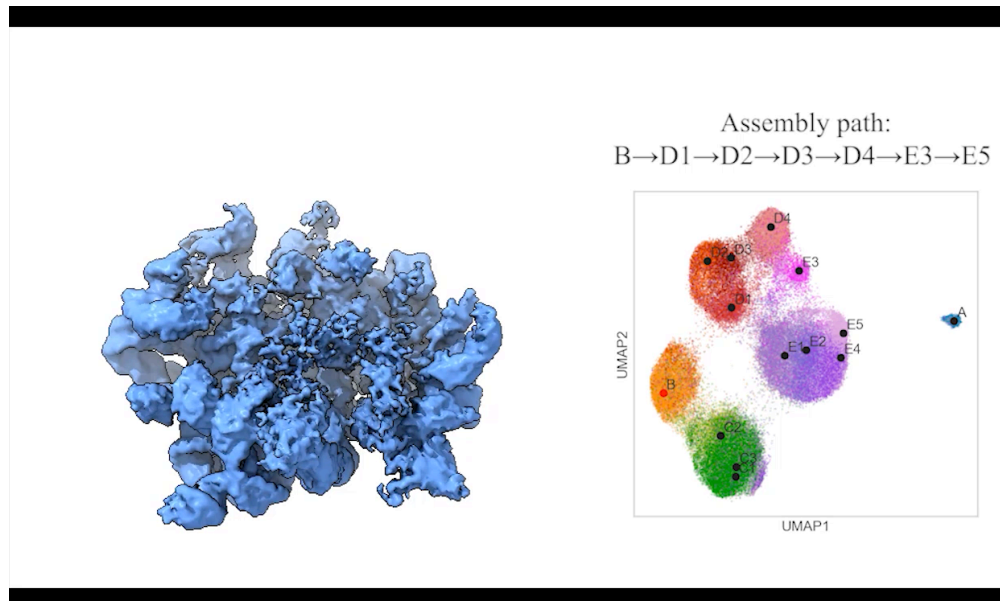
Capturing multiple conformations from a single cryo-EM dataset

- Each particle is potentially flash-frozen in a different conformation
- We only have one image (projection) of each particle
- Traditional solution: separate particle images into multiple classes that appear to correspond to different, then use images in each class to reconstruct a 3D model

Recent development: methods that reconstruct a continuous space of conformations

- Example: CryoDRGN

- Constructs a generative neural network in which several latent variables determine the 3D structure
 - Network parameters are optimized to maximize the likelihood of the observed images (calculated in Fourier space, taking advantage of the projection slice theorem)
- By varying the latent parameters, one can move through the predicted conformational space



Zhong et al.,
CryoDRGN:
reconstruction of
heterogeneous cryo-
EM structures using
neural networks,
Nature Methods 68
(2021)

Exam info

- Dec. 10 at 3:30 pm in 320-105
 - If you can't make this time, please let CAs know immediately to arrange an earlier time slot
 - You'll have three hours, but based on past experience, most students won't use that much time
- Practice exam questions (and solutions) posted on course web site
 - Most questions will require a sentence or two to answer
 - You will not need to code during the exam