Cryogenic Electron Microscopy (cryo-EM)

CS/CME/BioE/Biophys/BMI 279

Nov. 30 and Dec. 5, 2023

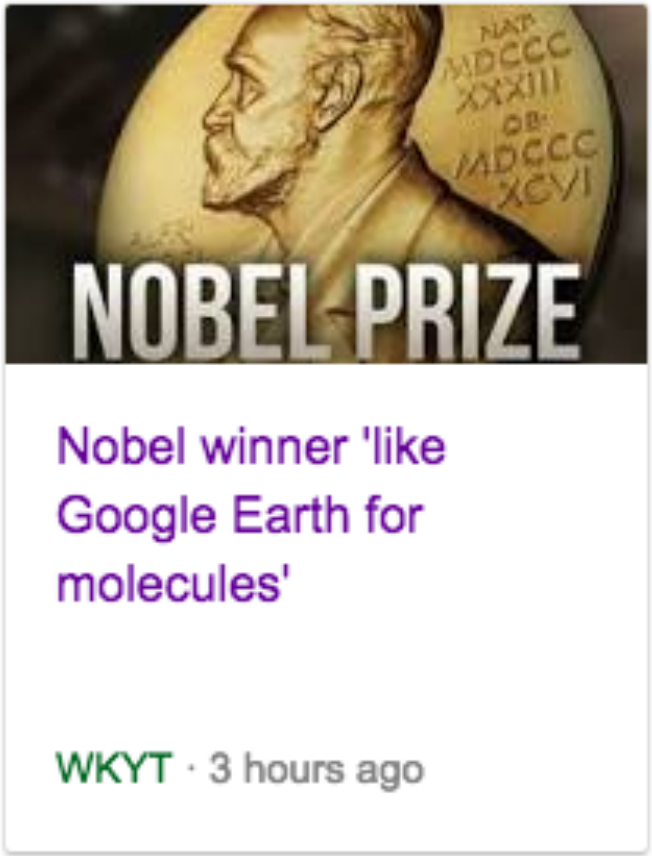
Ron Dror

Exam info

- December 14, 12:15-3:15 pm, Bishop Auditorium
  - 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) on course web site
  - Most questions will require a sentence or two to answer
  - You will not need to code during the exam
- Closed book, but you can bring one double-sided or two single-sided pages of notes

2017 Nobel Prize

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



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 VIKTOR KOEN

Nature, 2015

Outline

- Overview of cryo-electron microscopy (cryo-EM)
- Cryo-EM images are *projections*
- 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
    - 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

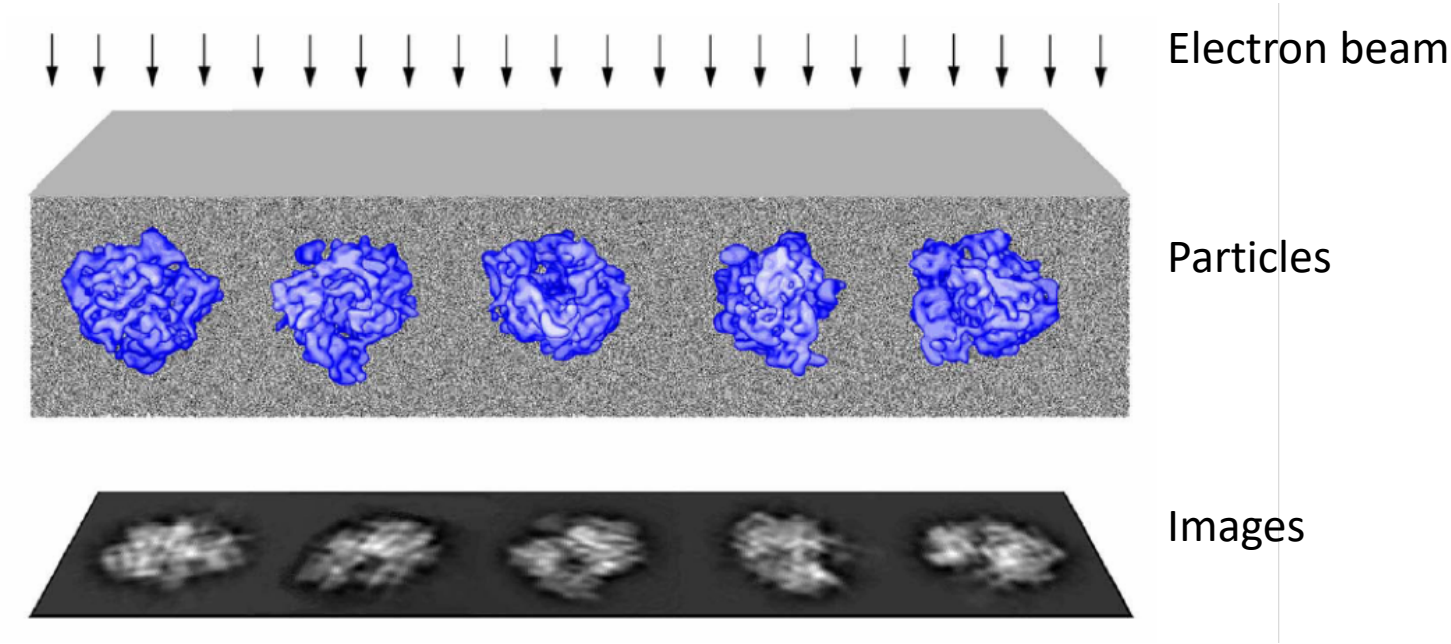


Image from Joachim Frank
http://biomachina.org/courses/structures/091.pdf

A high-end cryo-electron microscope

Dramatic recent improvements

- Cryo-EM has been around for decades, but it has improved *dramatically* in recent years due to:
  - Invention of better cameras
    - Until around 2008, electrons were detected either by photographic film, or by scintillator-based digital cameras that 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")

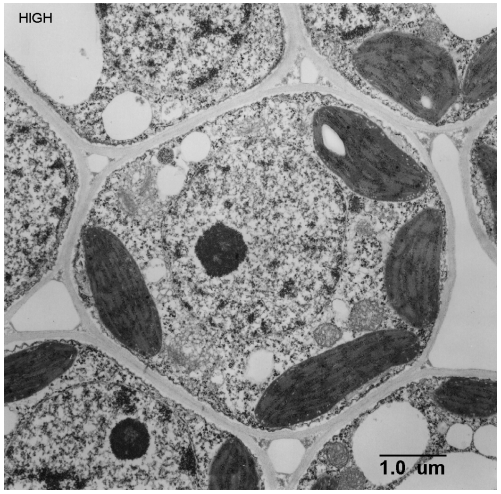
- Bottom line: Cryo-EM is particularly advantageous for larger complexes/molecules, because:
  - They tend to be harder to crystallize
  - The computational reconstruction problem in cryo-EM is usually easier to solve for large, asymmetric particles than for small ones

Cryo-EM images are *projections*

Cryo-EM uses *transmission* electron microscopy

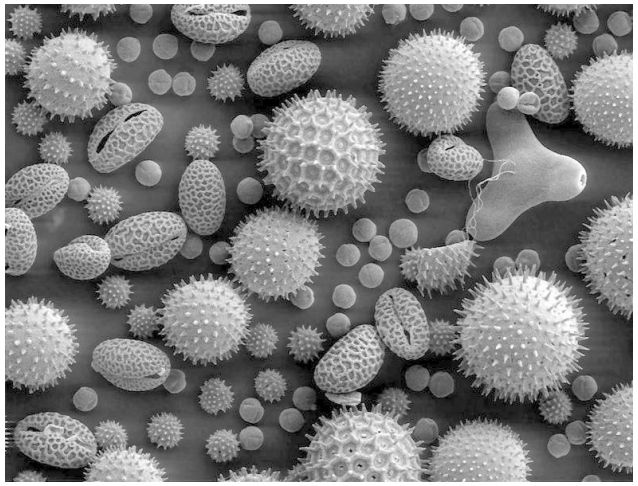
- In transmission electron microscopy, a beam of electrons passes through a thin sample before forming an image

Transmission electron microscopy



http://www.cas.miamioh.edu/~meicenrd/ANATOMY/Ch2 Ultrastructure/Tempcell.htm

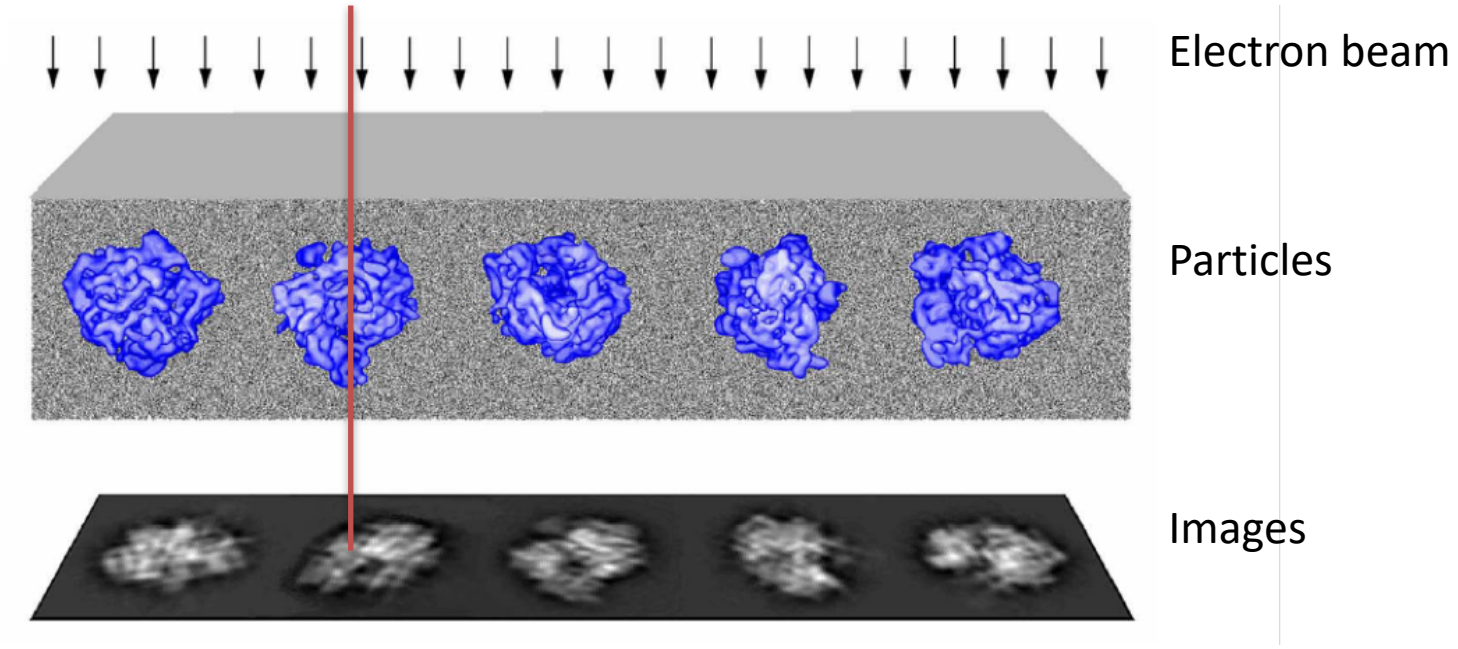
Scanning electron microsopy

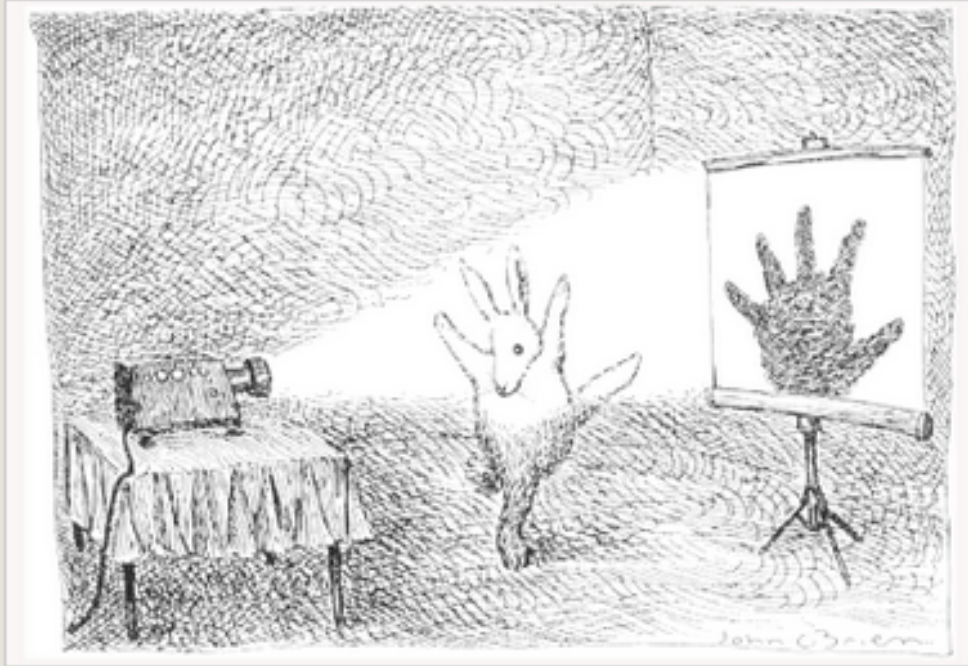


http://www.newscientist.com/data/images/ns/cms/dn14136/dn14136-1_788.jpg

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

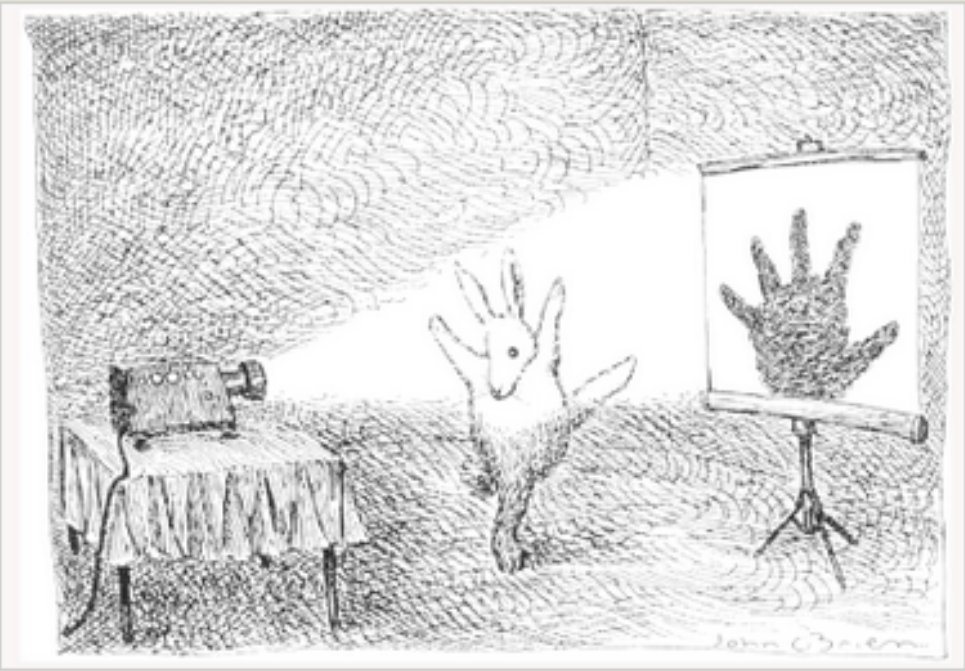


Figure 5.1 A single projection image is plainly insufficient to infer the structure of an object. (Note, though, that TEM projections do not merely give the outline, as in this drawing, but internal features, too—the bones and internal organs of the rabbit, which we would see if the projector were to 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

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

Vitrification

- To survive in the electron microscope (in a vacuum, under electron bombardment), particles are embedded in ice
- The sample is cooled extremely quickly (“flash frozen”), so the ice is “vitreous” (i.e., not crystalline)
- High-resolution single-particle EM relies on this “vitrification” process and is thus referred to as cryogenic electron microscopy (cryo-EM)

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

The raw images don’t look so good

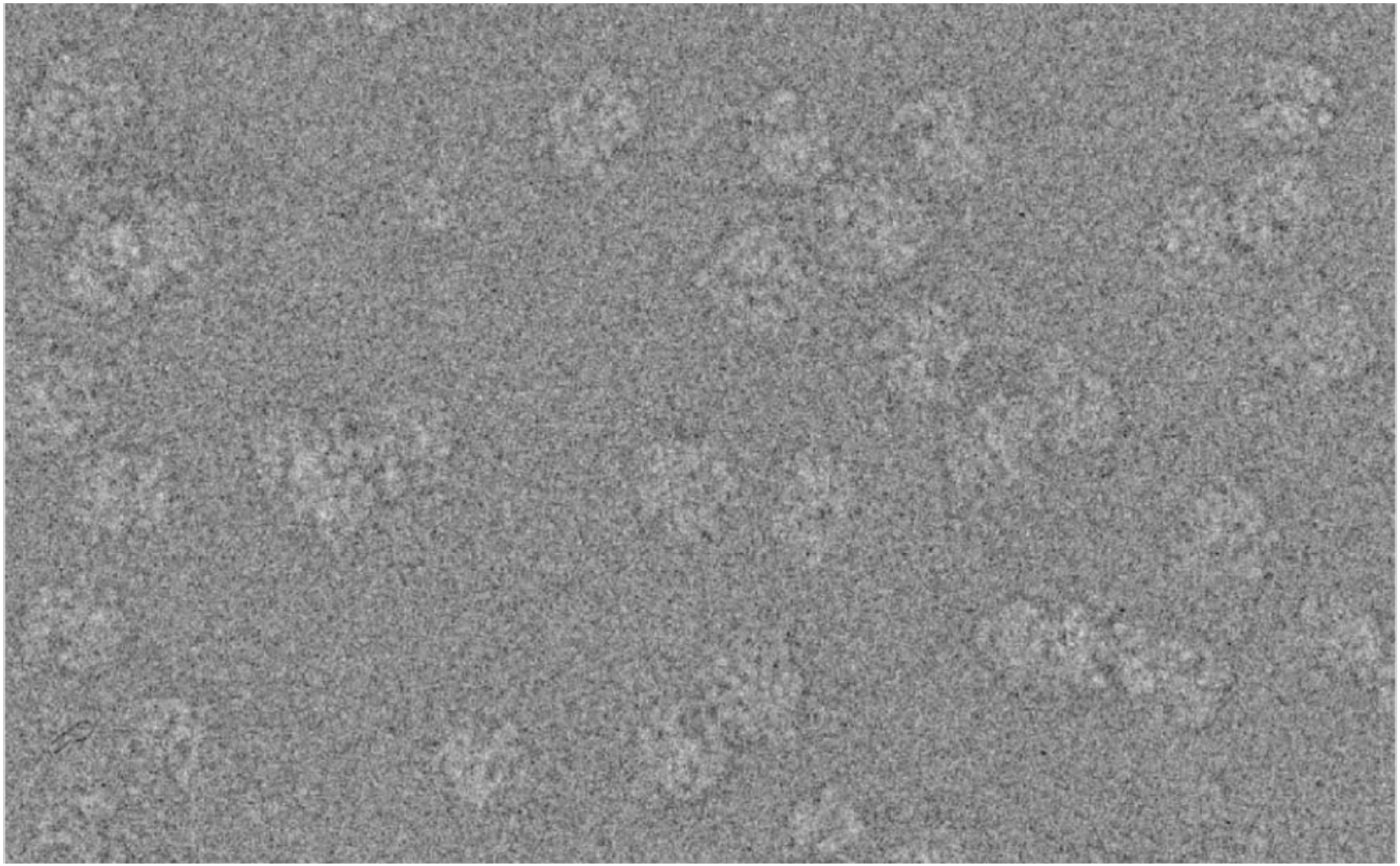


Image from Joachim Frank
http://biomachina.org/courses/structures/091.pdf

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

Computational reconstruction methods

2D image analysis

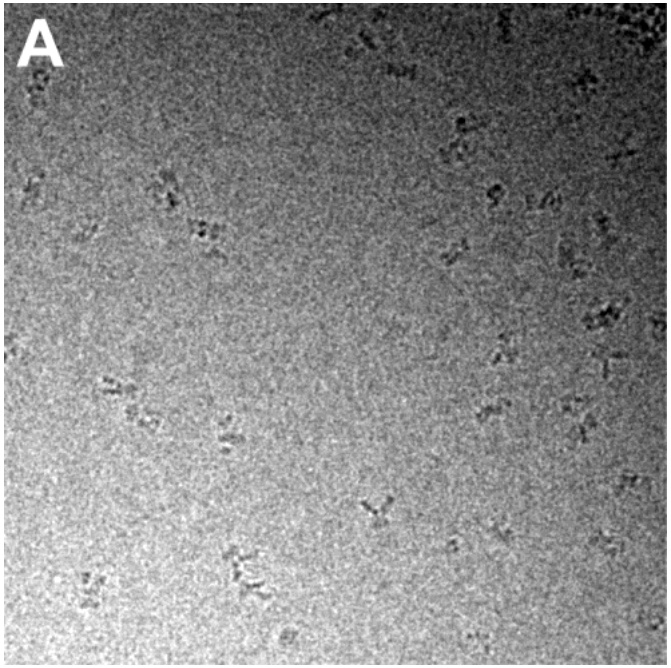
Image preprocessing

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

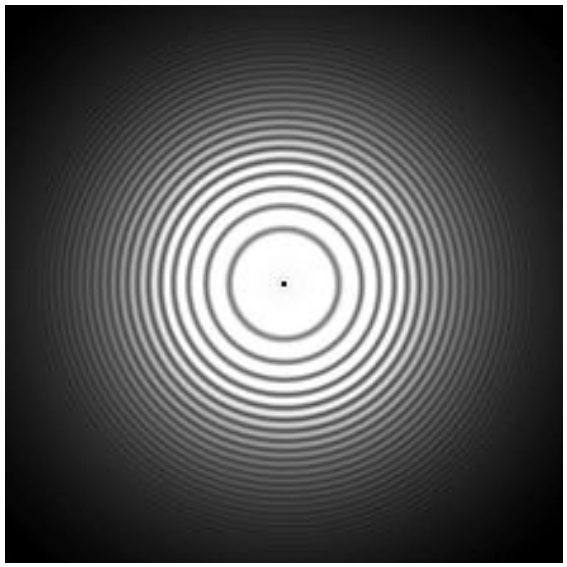
- **Problem 2**: Overall brightness is often non-uniform (due to uneven illumination or sample thickness)
- **Solution**: high-pass filter the image



Cheng et al., *Cell* 161:438 (2015)

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)

https://en.wikipedia.org/wiki/Contrast_transfer_function

Computational reconstruction methods

2D image analysis

Particle picking

Pick out the particles in the 2D images

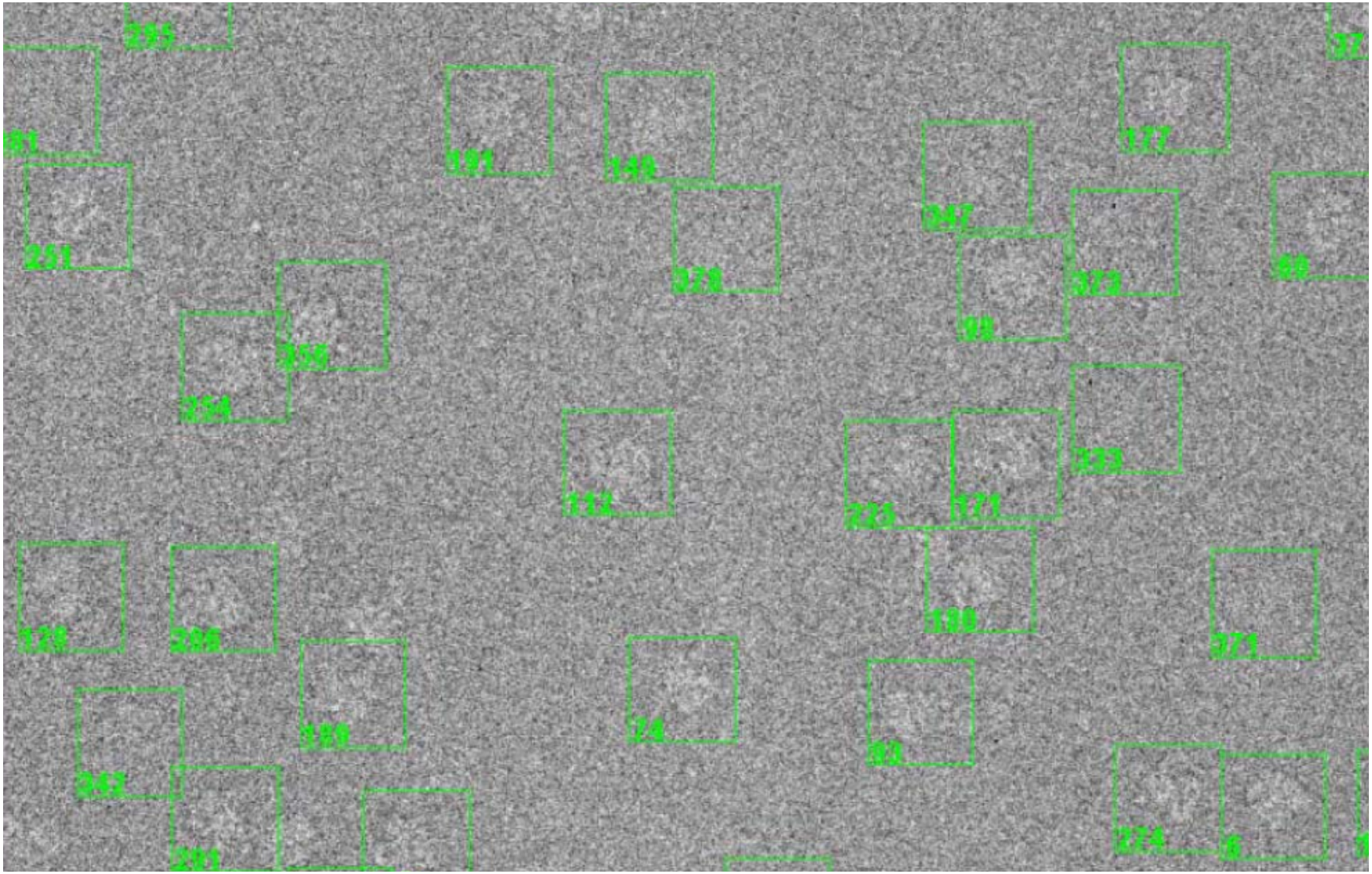


Image from Joachim Frank
http://biomachina.org/courses/structures/091.pdf

Particle picking results

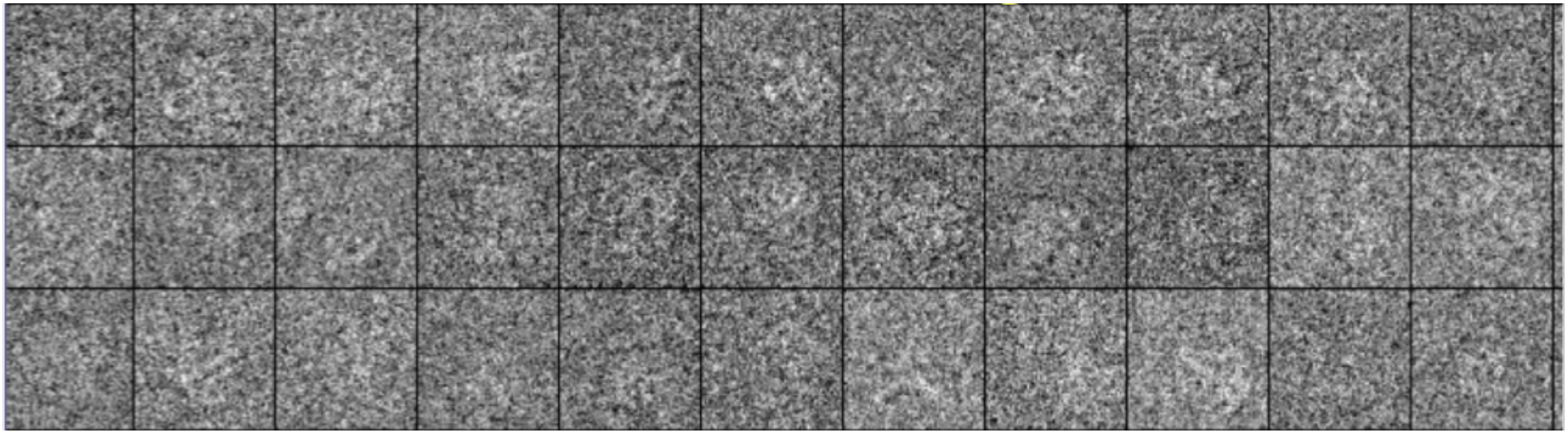
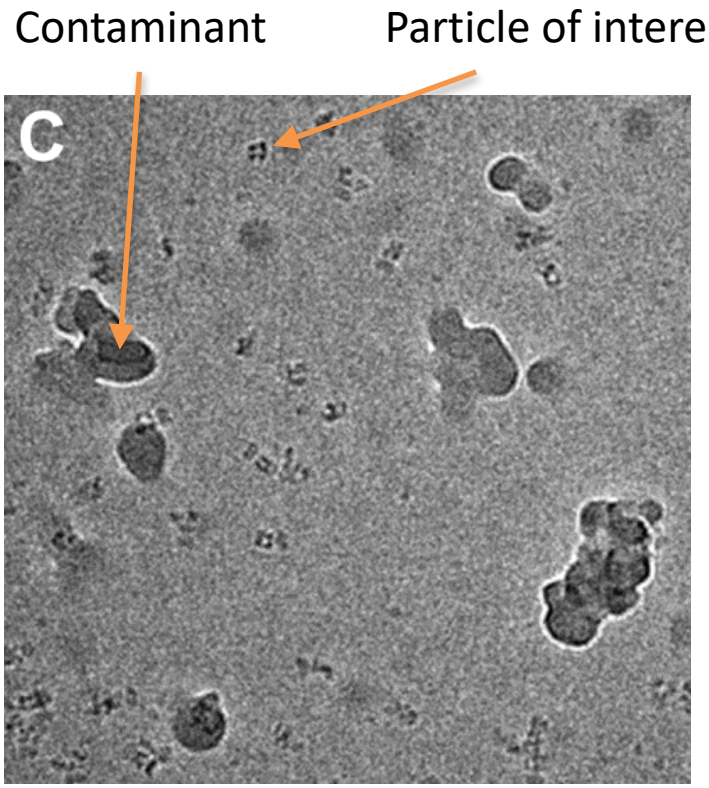


Image from Joachim Frank
http://biomachina.org/courses/structures/091.pdf

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

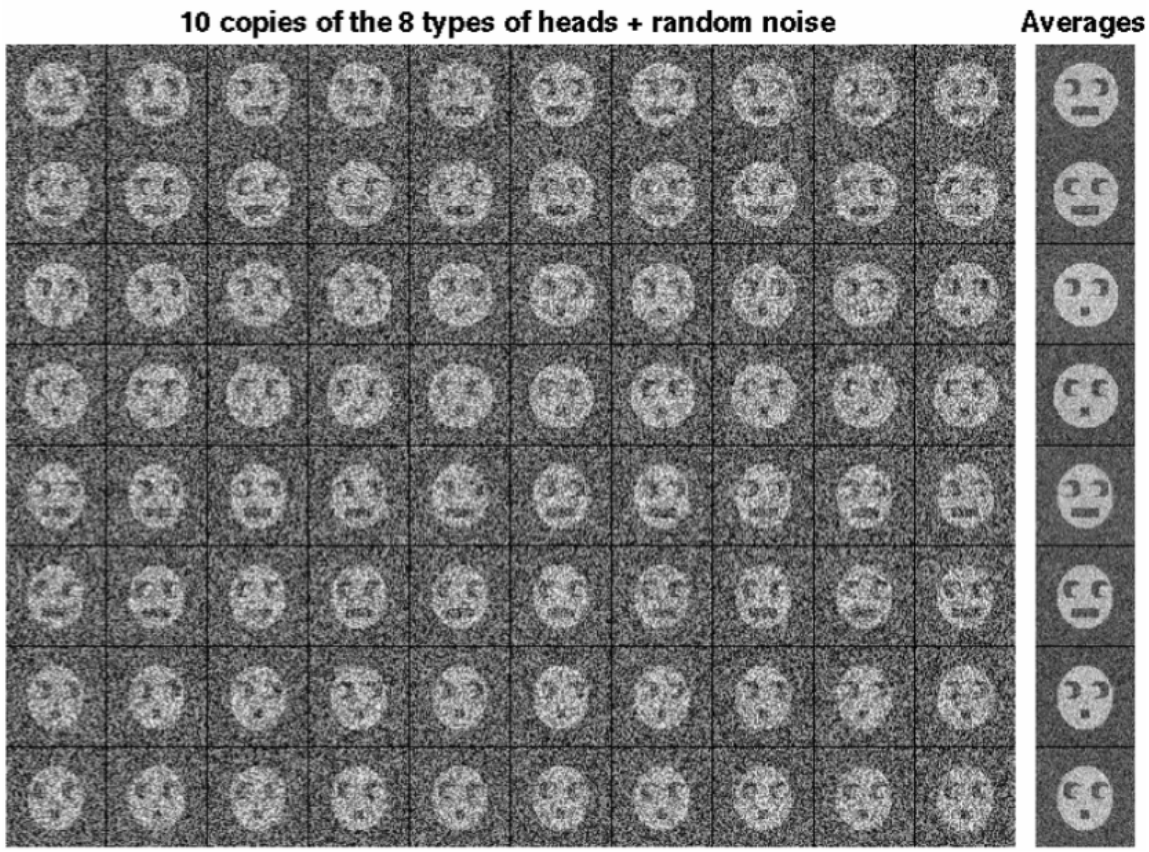


Image from Joachim Frank
http://biomachina.org/courses/structures/091.pdf

- 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”

Standard approach: *k*-means clustering

- 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

Caveat: Potential model bias in clustering/alignment

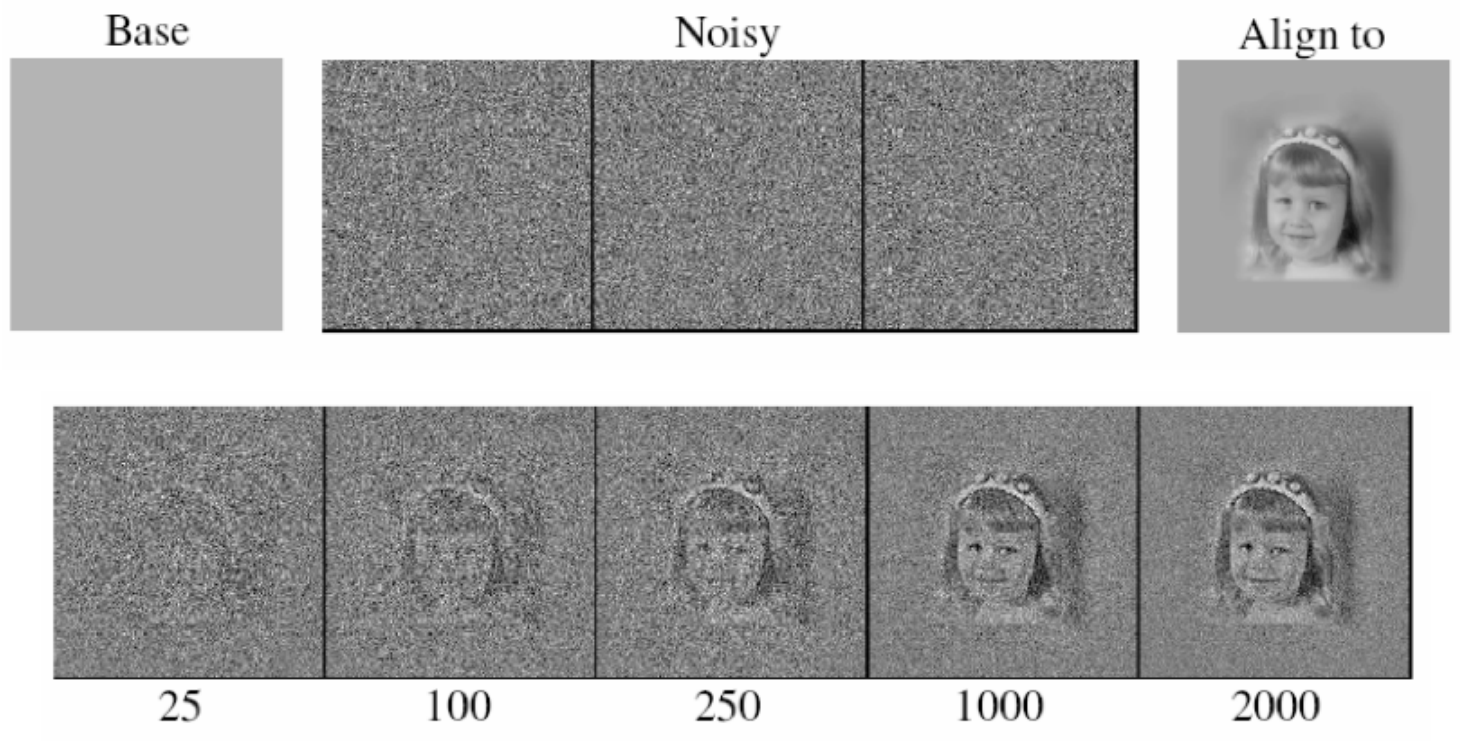


Image from Steve Ludtke
http://biomachina.org/courses/structures/091.pdf

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.

Avoiding these problems

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

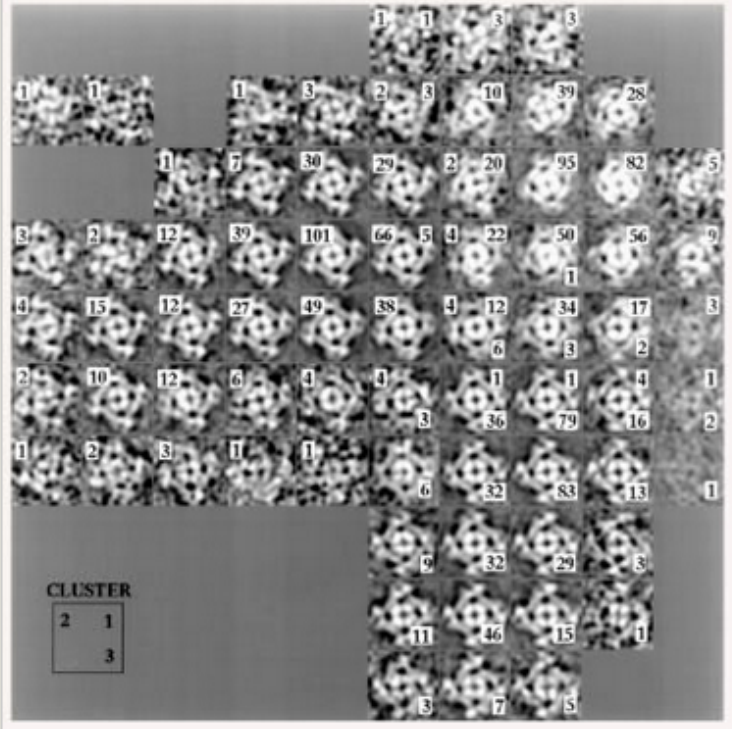
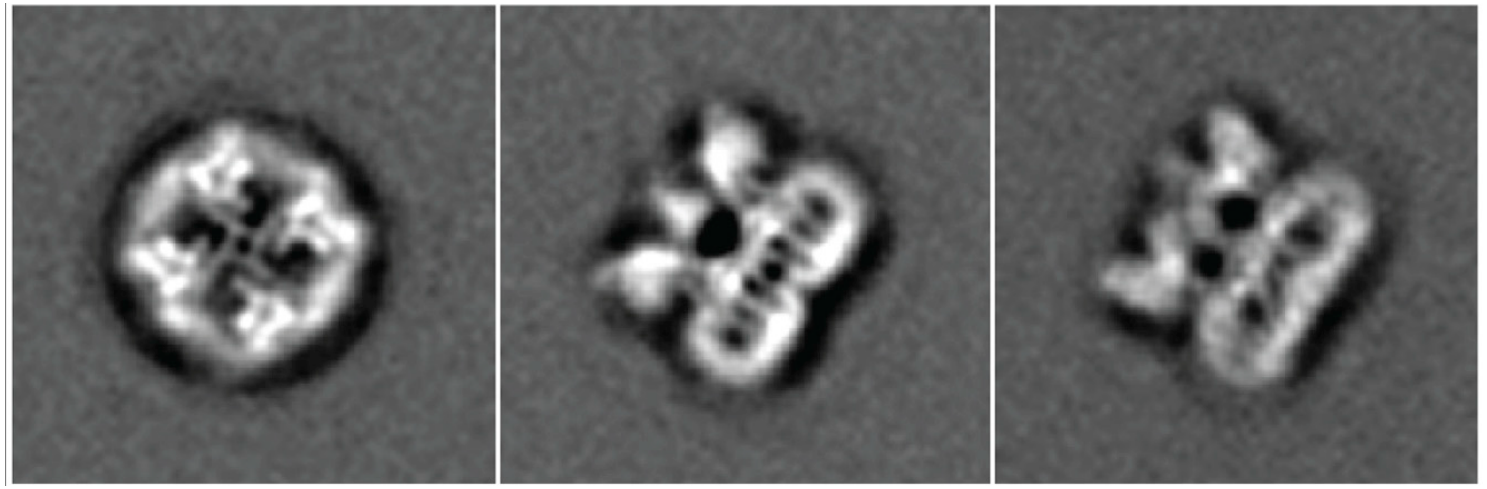


Figure 4.14 Checkerboard display of local averages (calcium release channel), each computed from images falling on a grid in factor space (factors 1 versus 2). The number on top of each average indicates the number of images falling into that grid space. Empty regions are bare of images. The distinction 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.

Optional material

Class averaging results



Cheng, *Cell* 161:450 (2015)

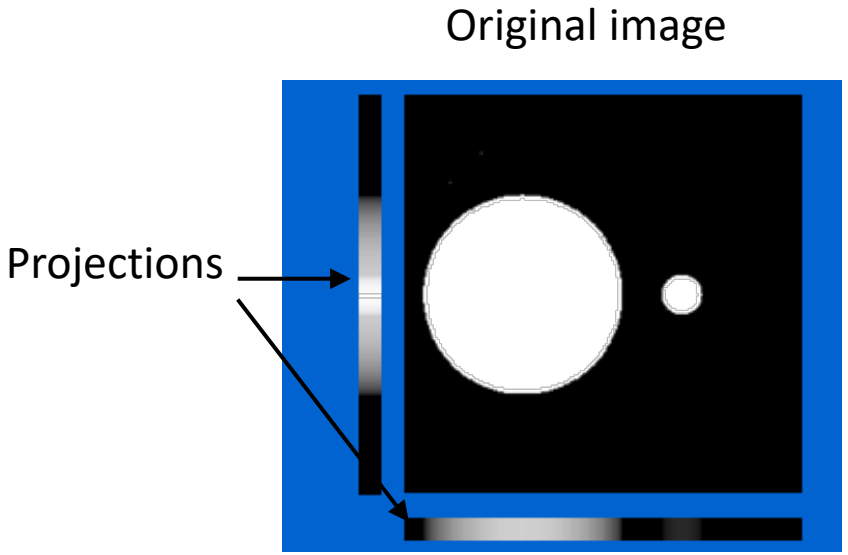
These are considered good class averages
(from a high-resolution single-particle EM study)

Computational reconstruction methods

3D reconstruction

Problem

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



- Discuss:
  - How could you do this if you know the view angle for each projection?
  - How could you do this if you don't know the view angles?

Computational reconstruction methods

3D reconstruction

Background: Reconstruction with known view angles

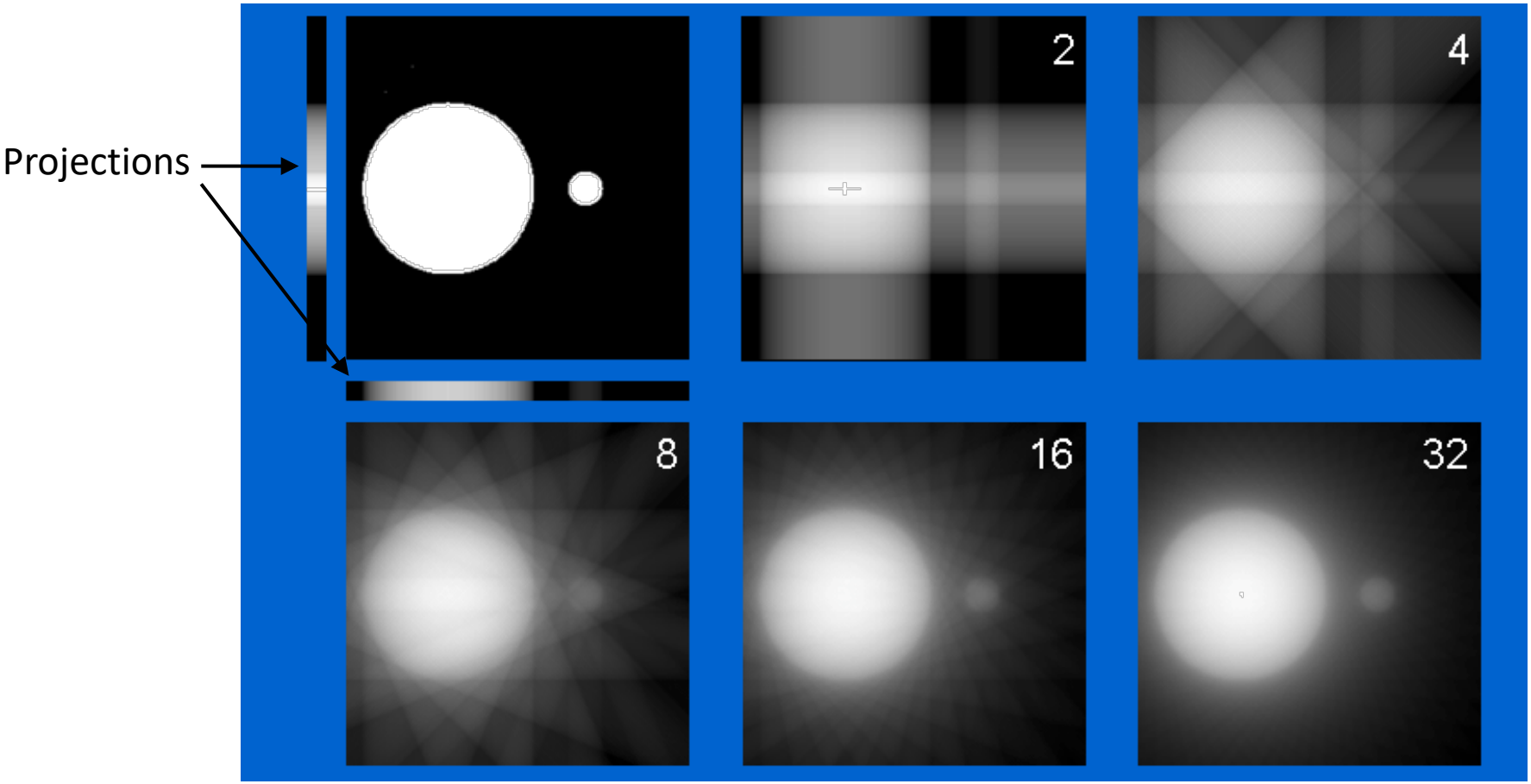
Suppose you knew the view angle for each particle image

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

Back-projection

Original image

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

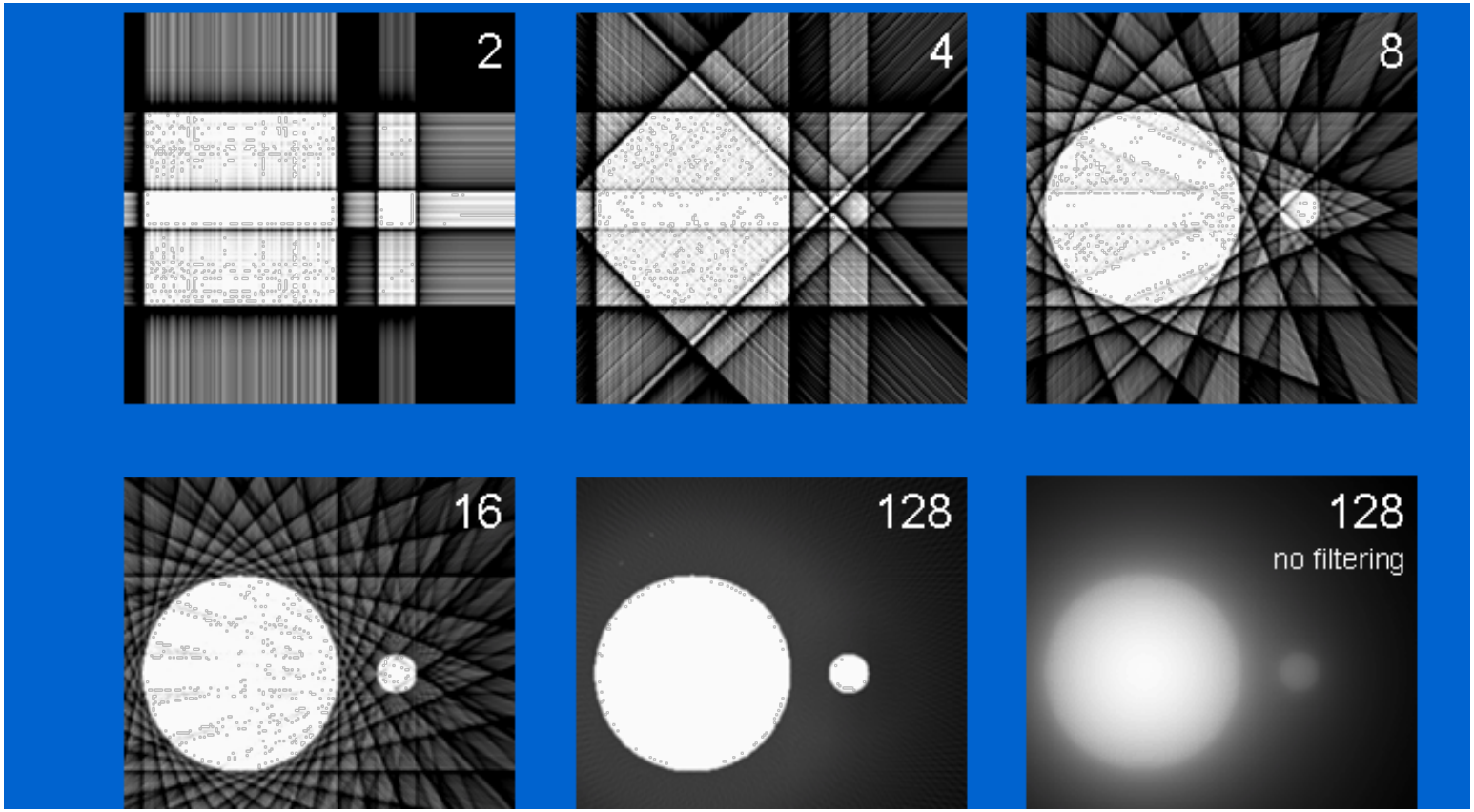


http://www.impactscan.org/slides/impactcourse/basic_principles_of_ct/img12.html

The result of back-projection is a *blurred* version of the original image.
How can we fix this?

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.*

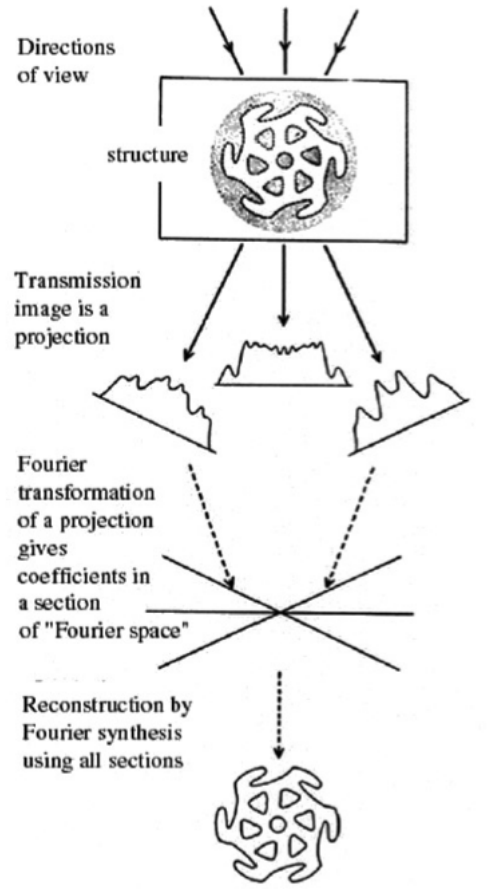


http://www.impactscan.org/slides/impactcourse/basic_principles_of_ct/img15.gif

Why does filtered back-projection work?

(Optional Material)

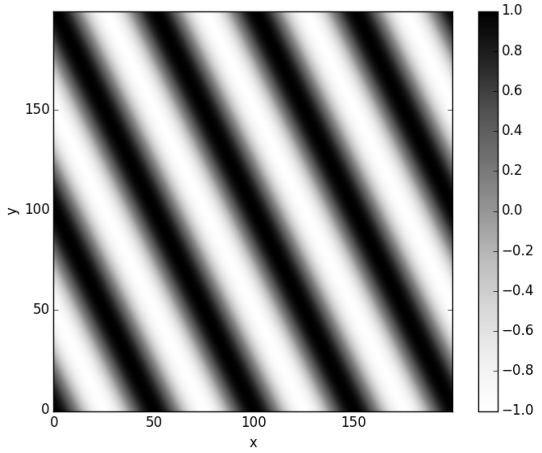
- To answer this, use the *projection slice theorem*



DeRosier & Klug, Nature 217 (1968) 133

Projection slice theorem (2D version):
The 1D Fourier transform of any 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



Why does filtered back-projection work?

(Optional Material)

- 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.

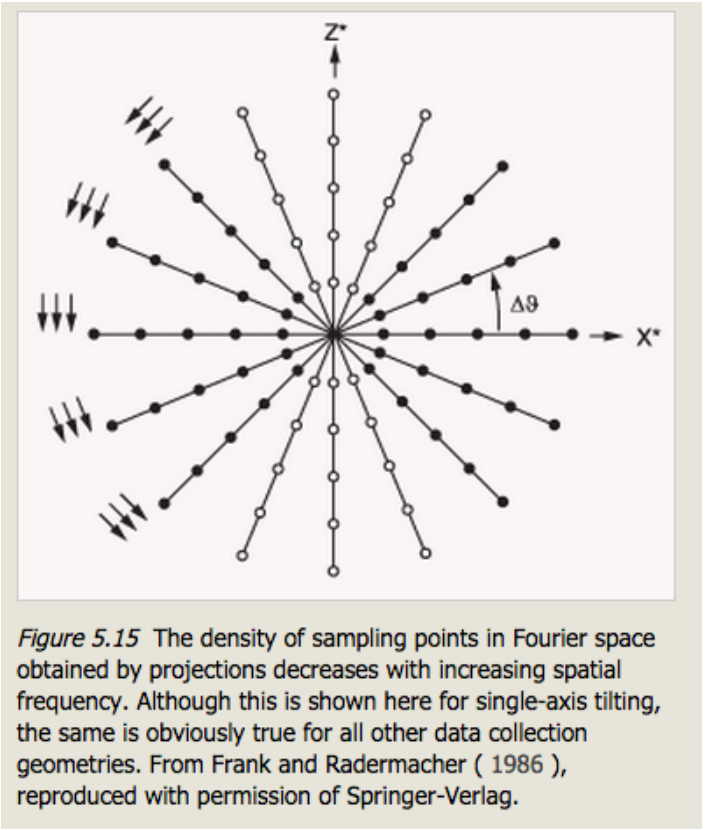
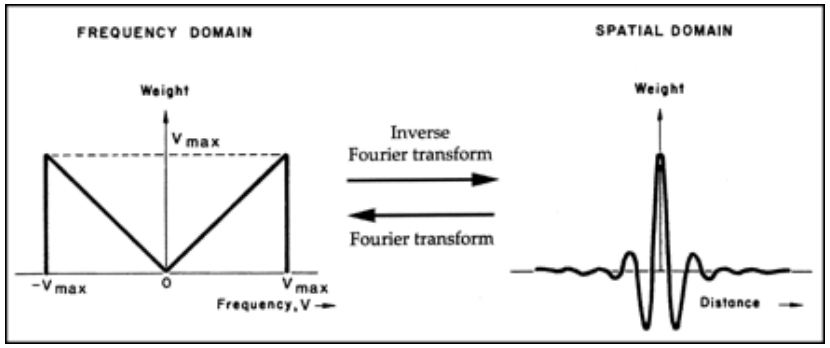


Figure 5.15 The density of sampling points in Fourier space obtained by projections decreases with increasing spatial frequency. Although this is shown here for single-axis tilting, the same is obviously true for all other data collection geometries. From Frank and Radermacher (1986), reproduced with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

Frank, 2006

Ideal filter shape grows linearly with frequency.



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

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

This carries over to the 3D case

(Optional Material)

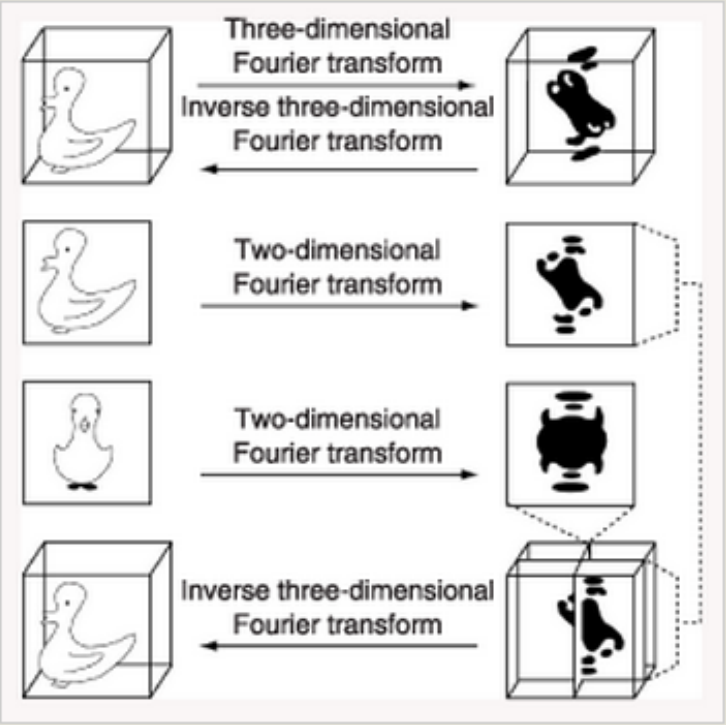


Figure 5.2 Illustration of the projection theorem and its use in 3D reconstruction. From Lake (1971), reproduced with permission of Academic Press Ltd.

Frank, 2006

Projection slice theorem (3D version): The 2D Fourier transform of any 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*

An example

Class averages (starting point for reconstruction)

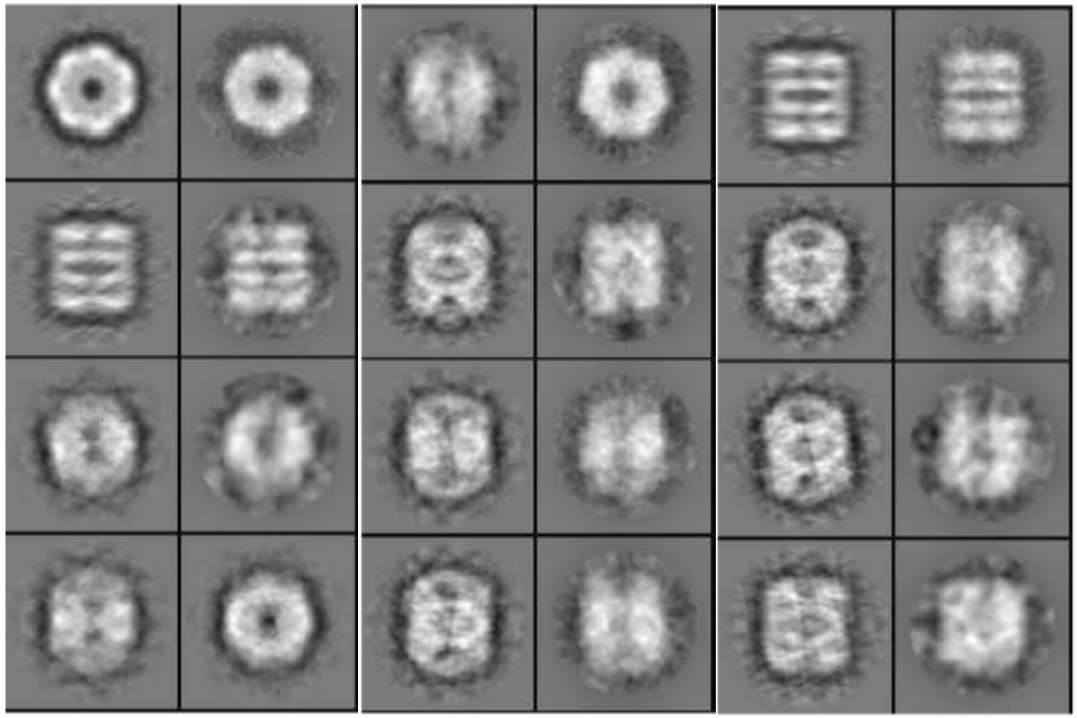
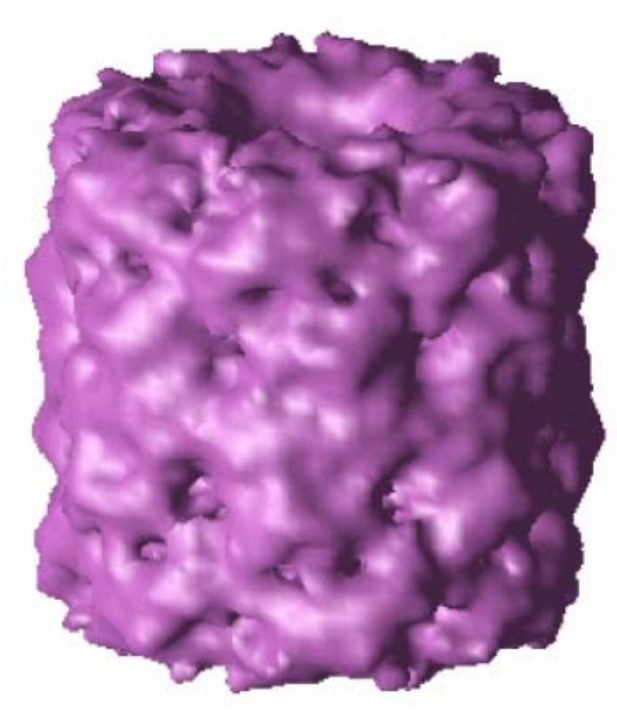


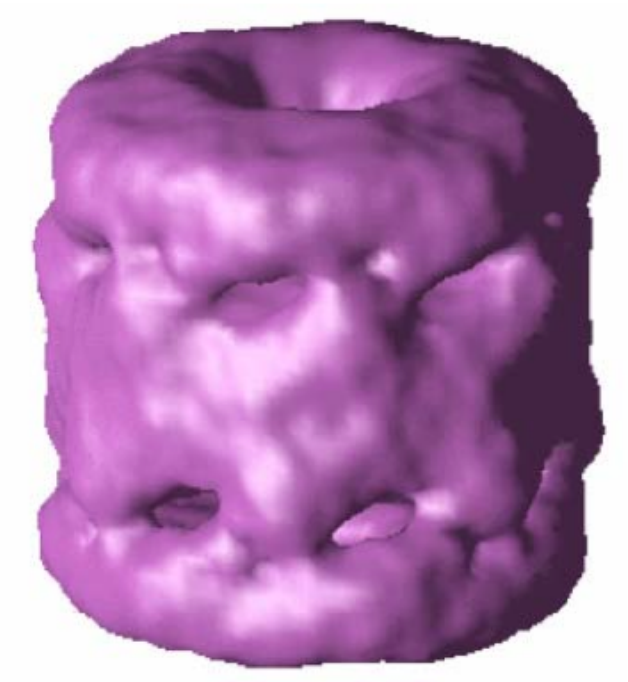
Image from Steve Ludtke
http://biomachina.org/courses/structures/091.pdf

Iteration 1

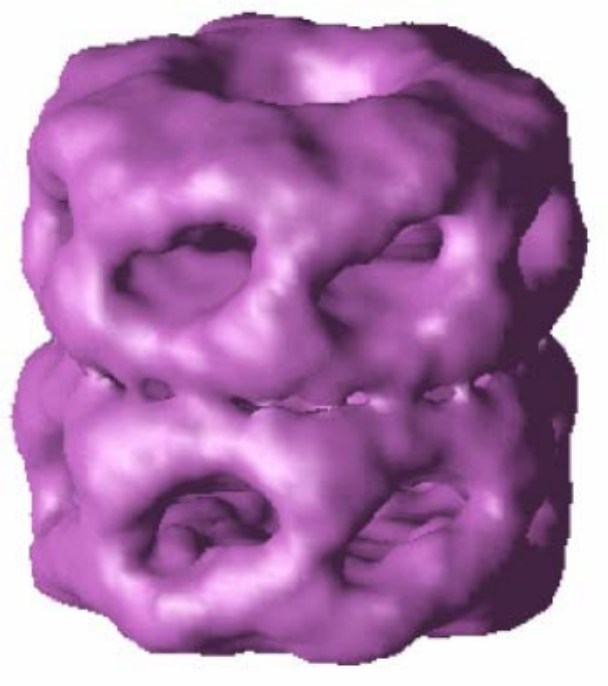


This surface is a contour map. Estimated density is greater than a threshold value inside the surface and less than that value outside it. “Density” here corresponds roughly (not precisely) to electron density.

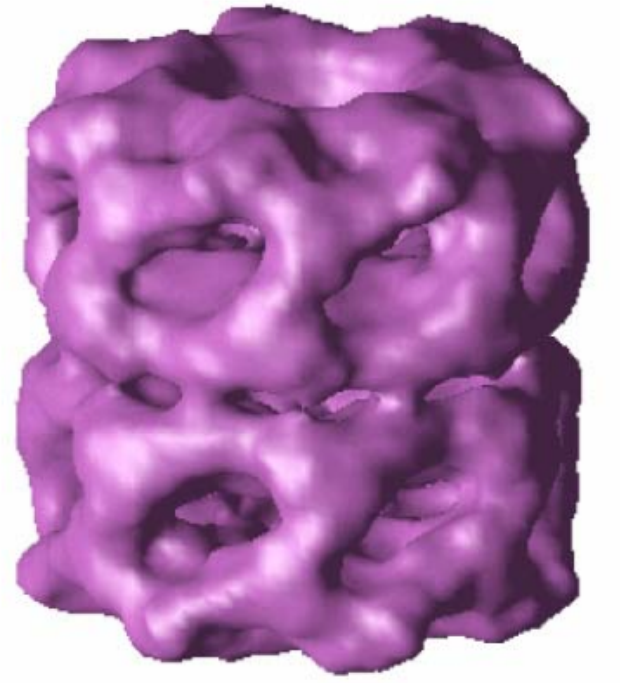
Iteration 2



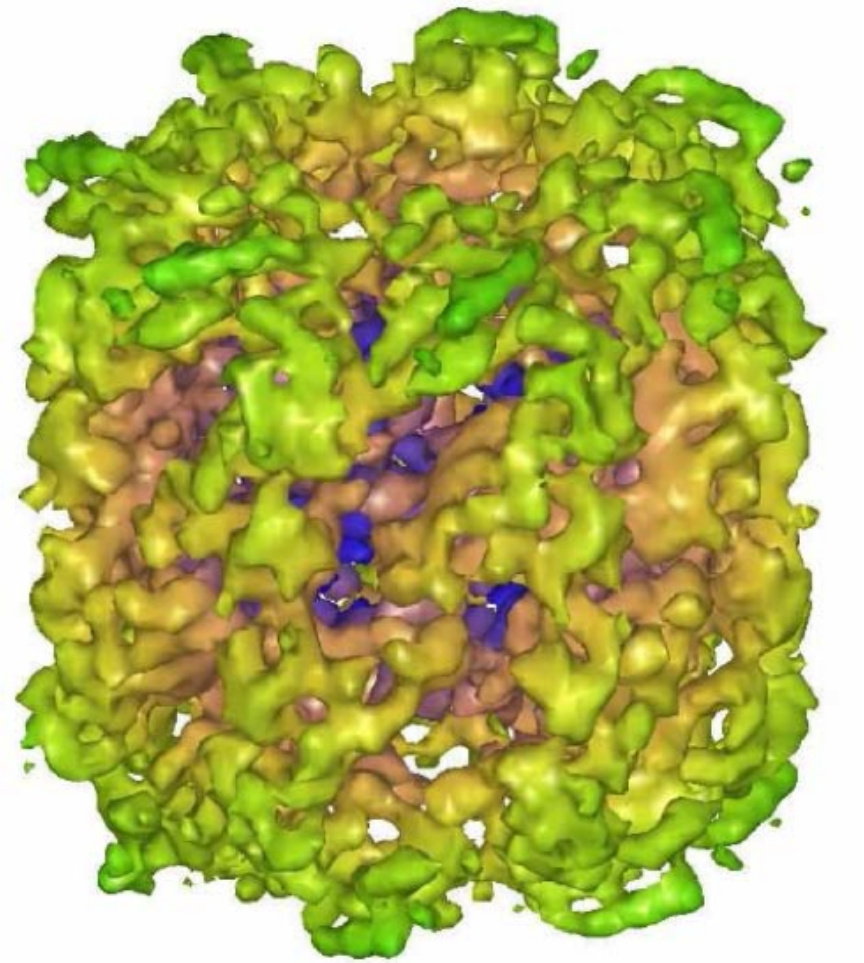
Iteration 3



Iteration 4



Final reconstruction



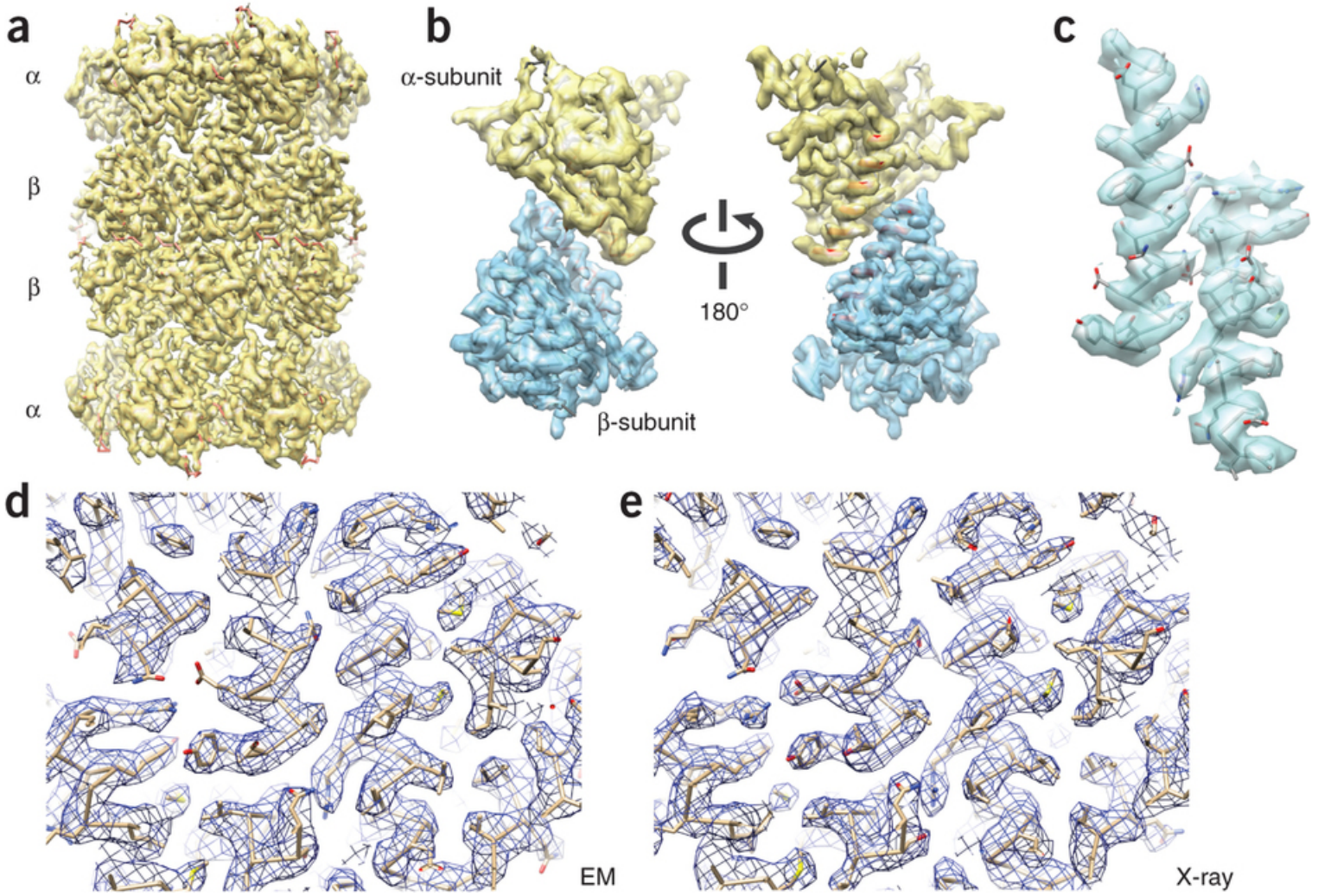
Protein: GroEL
6.5 Å resolution

Ignore the color coding

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
    - Many use Bayesian statistical approaches (e.g., RELION software)

A high-resolution cryo-EM structure



A 3.3 Å resolution EM structure

Li et al., Nature Methods 10:584 (2013)

A recent development: Atomic-resolution Cryo-EM

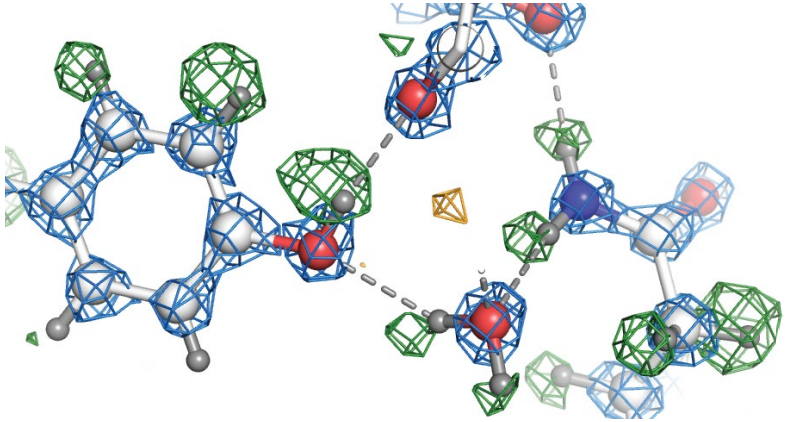
Nakane … Scheres, *Nature*, **Nov. 5, 2020**

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 in certain cases
  - This resolution allows one to see density for individual atoms, even hydrogen!



Nakane et al, *Nature*, 2020

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 camera angles

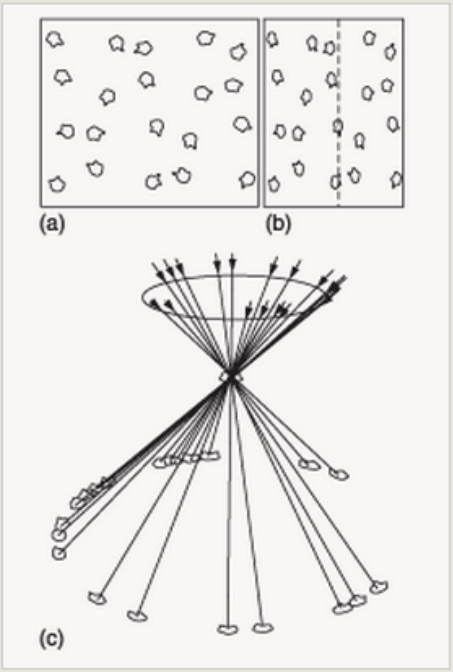
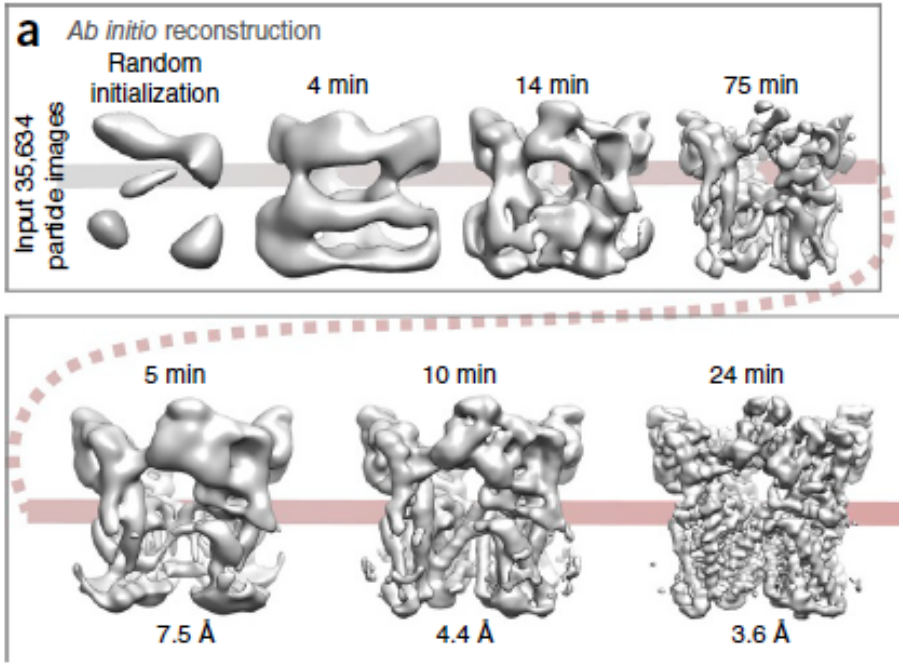


Figure 5.12 Principle of the random-conical data collection: (a) untilted; (b) tilted field with molecule attached to the support in a preferred orientation; (c) equivalent projection geometry. From Radermacher et al. (1987 b), reproduced with permission of Blackwell Science Ltd.

Frank, 2006

How do we get an initial structural model?

- Direct computational solutions are becoming practical!
- Example: stochastic gradient descent method
  - 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

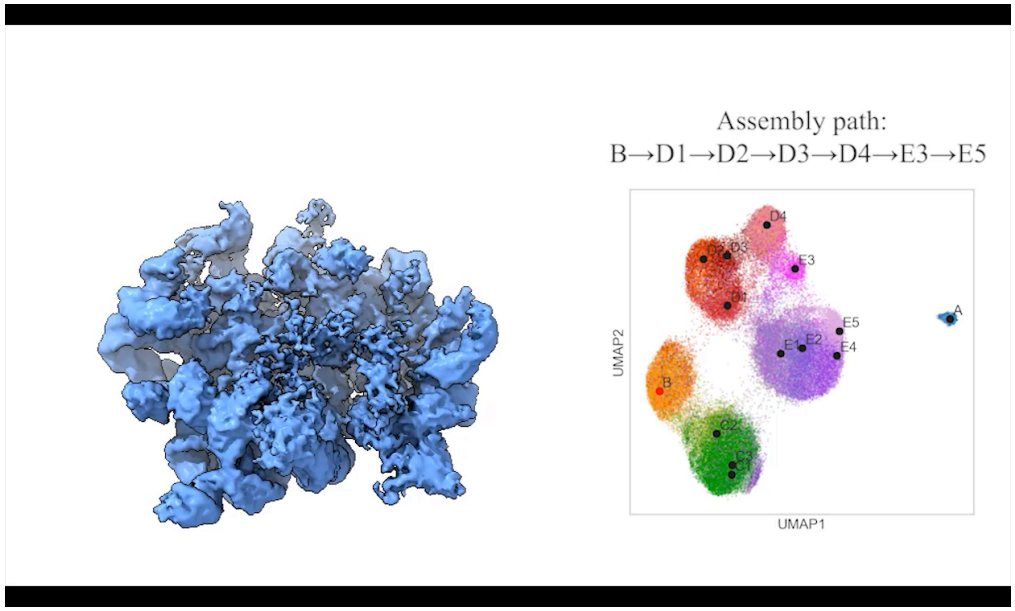
Capturing multiple conformations

Capturing multiple conformations from a single cryo-EM dataset

- Each particle is potentially flash-frozen in a different conformation—so in principle, one could reconstruct multiple conformations from a cryo-EM image dataset
- Challenge: We only have one image (projection) of each particle, and we don't know in advance which conformation that particle was in
- Traditional solution: separate particle images into multiple classes that appear to correspond to different conformations, 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* (2021)