Single-particle electron microscopy (cryo-electron microscopy)

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Ron Dror
Last month’s Nobel Prize in Chemistry

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"
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 bulging 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 Siros Scheres, a structural biologist at the UK Medical Research Council Laboratory of Molecular Biology (LMB), as he stands dwarfed beside the 45-million ($US87.7-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.

Outline

- Overview of single-particle electron microscopy (EM)
- Single-particle 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

The images are very noisy. First, clean up the 2D images. Then try to recreate the 3D structure whose projection created those 2D images.
Overview of single-particle electron microscopy (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
Stanford has recently purchased at least 3 of these machines.

A high-end cryo-electron microscope
Dramatic recent improvements

• Single-particle EM has been around for decades, but it has improved *dramatically* in the last five 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
• Single-particle EM is thus coming into much wider use, and may challenge crystallography as the dominant way to determine experimental structures
Comparison to x-ray crystallography

• Single-particle 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:
  – Single-particle EM’s resolution is (typically) lower 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: single-particle EM is particularly advantageous for large complexes, because:
  – Large complexes tend to be harder to crystallize
  – The computational reconstruction problem in single-particle EM is usually easier to solve for large particles than for small ones
Single-particle EM images are projections
Single-particle EM uses transmission electron microscopy

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

Transmission electron microscopy

Scanning electron microscopy detects surfaces, so it more closely mimics how we normally see.

Scanning electron microscopy

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

Single-particle 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
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), the 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
    - 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 cryo-electron microscopy (cryo-EM)
Usually you’ll perform negative staining to check your protein sample, make sure everything is okay. Then you’ll move to vitreous ice to take high resolution images.

Negative stain. Particles are easy to pick out.

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

Frank, 2006

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
Overview of computational methods

This is a good review, check it out!

Computational reconstruction methods

2D image analysis
Particles are very low contrast, meaning they don’t stand out very well from the background. Actually, this is an especially clean example!

The raw images don’t look so good

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

It wasn’t until recently that detectors were fast enough to record a movie.
Image preprocessing

• **Problem 2:** Overall brightness is often non-uniform (due to uneven illumination or sample thickness)

• **Solution:** high-pass filter the image

Because uneven illumination is a very low frequency effect.

There is blurring that happens when an image is recorded on the electron microscope, but we have a good model of how the blurring occurs, so we can partially correct for it.

- **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)

Frequencies corresponding to bright pixels here have strong signal under the microscope; frequencies corresponding to dark pixels are poorly detected under the microscope.

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

https://en.wikipedia.org/wiki/Contrast_transfer_function
Computational reconstruction methods

2D image analysis

Particle picking
Pick out the particles in the 2D images

It’s like you snap a bunch of blurry images of an anthill, and then you want to reconstruct the model of an ant.
Particle picking results

In reality, you’ll have snapshots of at least 10,000 different particles, sometimes 100s of thousands.

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

• Often this is still done manually, at least to seed automated methods with suitable templates

Doing a manual annotation helps you make sure your data look okay. This also provides templates that teaches the software what a particle should roughly look like.
Computational reconstruction methods

2D image analysis

Image clustering and class averaging
Averaging similar images reduces noise

• The images in each row above represent the same ideal image but with different corrupting noise

• If we average the images in each row (in the sense of averaging corresponding pixels), we end up with a less noisy image, because the noise in the different images tends to cancel out. Noise will average out because the noise between different images are random and not correlated with each other.

In practice, the particles under the microscope will be in different orientations, and these orientations aren’t told to you in advance, so you need a way to group particles with a similar orientation. (Imagine taking thousands of pictures of a water bottle floating in space — your pictures will capture all possible orientations of the water bottle.)

This is like grouping smiley faces with similar expressions as shown to the left.

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

*k* is decided in advance.

- 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

On the left is an example of *k*-means clustering iteration from [http://stanford.edu/~cpiech/cs221/handouts/kmeans.html](http://stanford.edu/~cpiech/cs221/handouts/kmeans.html), which also gives a good mathematical explanation.
Standard approach: $k$-means clustering

$k$ is decided in advance.

- 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
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.

This is essentially because the alignment step biases the random pixels towards positions that match the template image of the little girl, so when these images are averaged you reproduce the image of the little girl.
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.

This can be a big problem experimentally! In these examples, you can “reconstruct” an image that wasn’t there to begin with.
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

ISAC essentially computes a whole bunch of different k-means and combines the results to produce a more robust solution.

You’re not responsible for these methods
Class averaging results

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?

• 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.
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)

• The simplest approach would be \textit{back-projection}: reverse the projection process by “smearing” each projection back across the reconstructed image
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*.
Why does filtered back-projection work?  

You're not responsible for this

- 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.
Why does filtered back-projection work?

You’re not responsible for this

• 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.

Ideal filter shape grows linearly with frequency.

Filtered back-projection is a common technique, but there are several alternatives, including direct Fourier-domain reconstruction.
This carries over to the 3D case

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.

You’re not responsible for this
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
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.
The first iteration was based on a model so it had some detail, but the detail was slightly wrong, so iteration 2 looks more “blobby”
Final reconstruction

Protein: GroEL
6.5 Å resolution

Ignore the color coding
A high-resolution single-particle EM structure

You can see alpha helices! And you can see electron densities corresponding to each side-chain. The best EM structures are still not as high-resolution as the best crystal structures, but it’s remarkable that you can achieve atomic-resolution structures with this approach.

A 3.3 Å resolution EM structure

Li et al., Nature Methods 10:584 (2013)
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
    • Some use Bayesian approaches (e.g., RELION software)
    • Some of the most important recent algorithmic developments in single-particle EM are in this area.
    • You’re not responsible for these methods.
Computational reconstruction methods

3D reconstruction

Calculating an initial structure
How do we get an initial structural model?

*You’re not responsible for this*

- Multiple options:
  - We might have one from prior experimental work
  - Conduct specialized experiments, often at lower resolution
    - Example: *random canonical tilt* approach, which requires collecting each image twice, from different angles
  - Direct computational solution
    - Common lines method: relies on the fact that Fourier transforms of different 2D projections share a common line
    - Stochastic hill climbing: a robust projection matching (refinement) approach that often allows random initialization

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Frank, 2006
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.

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.

https://www.youtube.com/watch?v=6Knykqcxzfg