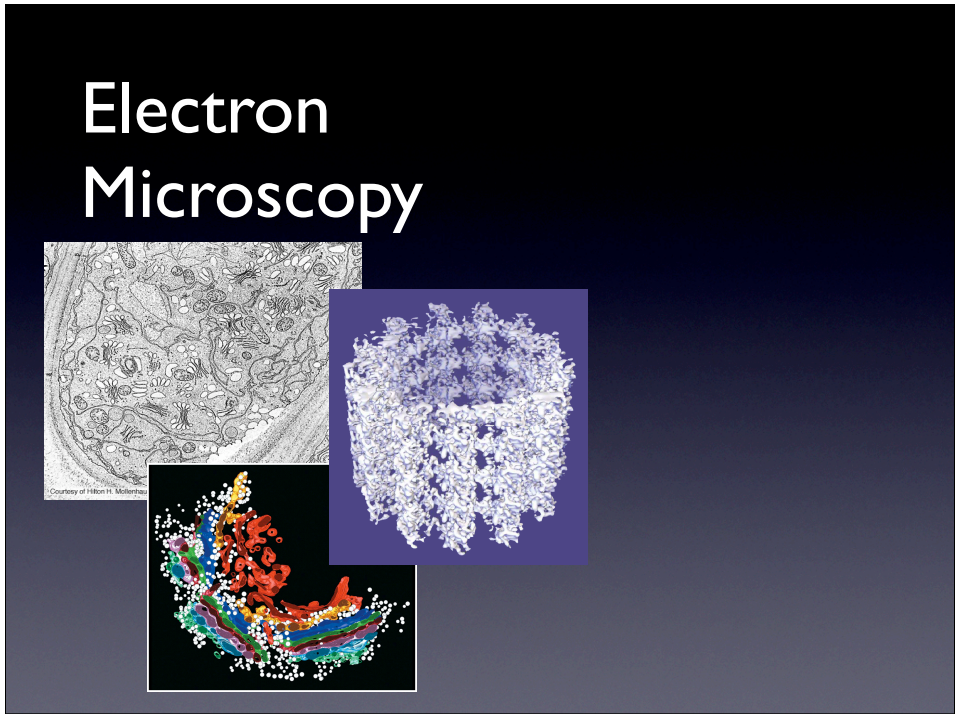
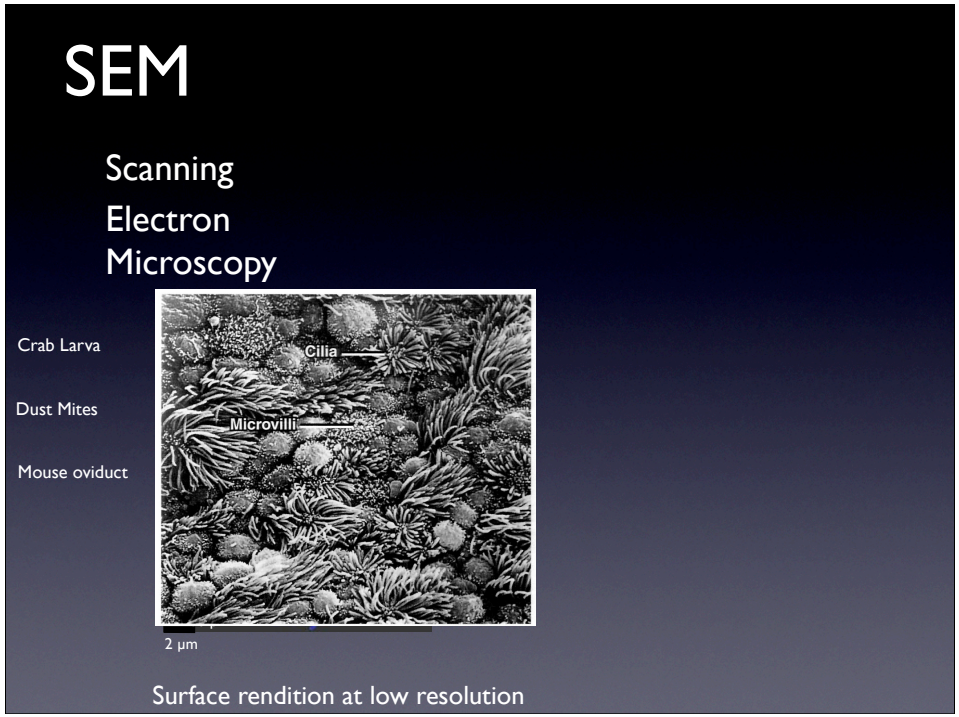
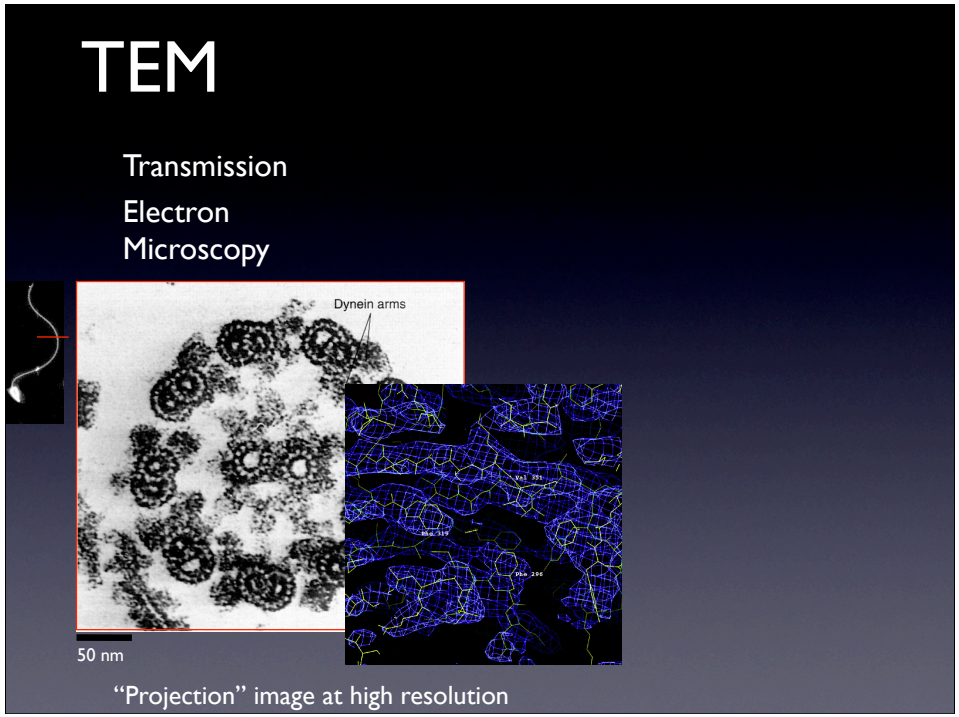


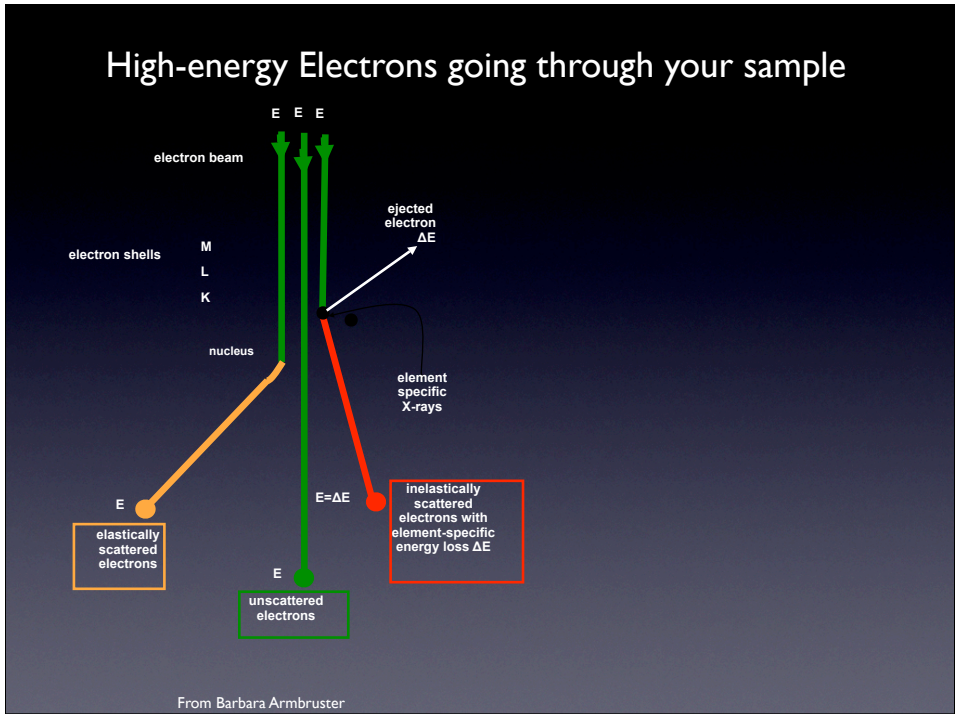
My name is Eva Nogales, and today I would like to give you a brief introduction to one of my favorite visualization techniques in molecular and cell biology: Electron Microscopy.



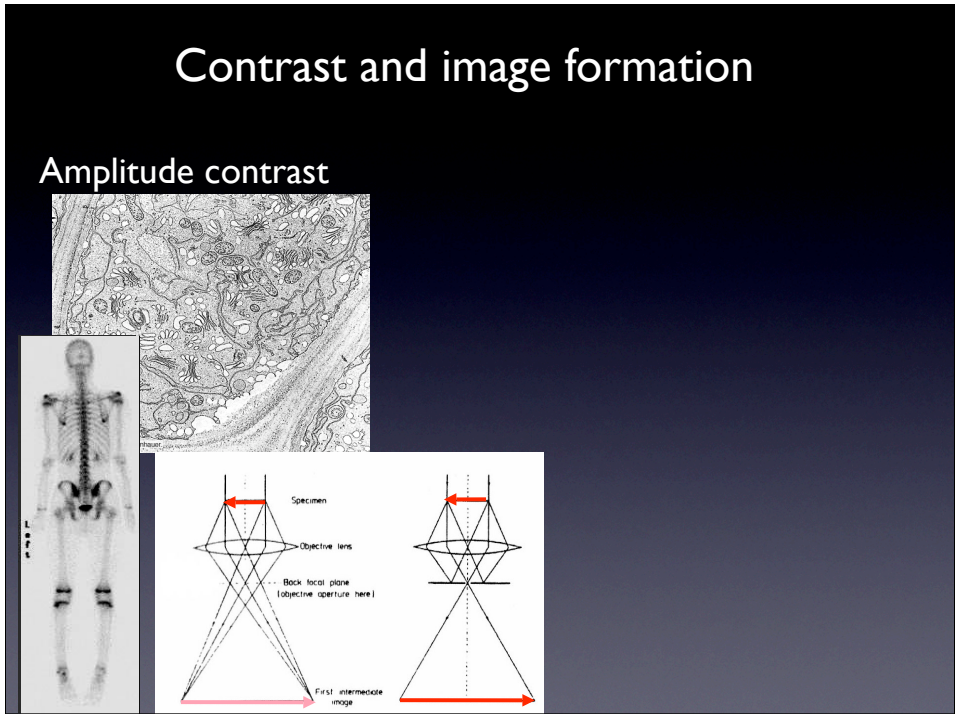
MIcroscopes and the images they produce come in two main flavors. SEM or Scanning Electron Microscopes, use focused beams of low-energy electrons to raster along a large object to give you a surface rendition at low resolution. This is an SEM image of a crab larva. What you see here is false color (there are no color in electron microscopy!). The real image looks like this. This is another from nasty dust mites that I am allergic too. Notice the scale of 200 ﾅms. The SEM can use higher magnification and look at individual cells. This is an image of a mouse oviduct where cells have cilia to generate currents that help the egg and sperm run to each other. Notice that the scale bar here is 2, rather than 200 ﾅm.



The method that I would like to concentrate on today is TEM, or Transmission Electron Microscopy. The physical principles for obtaining the image are very different here. Images corresponds to projections of a thin sample and can, in principle, give atomic resolution information of your sample.This is an example showing a thin slice cut out of the flagella of a sperm. What you see is a cross section view of microtubules forming a beautiful structure call the axoneme that run along the full length of the flagella. Notice the scale bar now is 50 nanometers! Microtubules are cytoskeletal elements present in all cells, and one of my favorite subjects of study. They are made by self-assembly of a protein called tubulin. This TEM image shows individual microtubules that have been formed in test tube with tubulin purified from cow brains. TEM has been actually used to visualized, at the atomic level the structure of tubulin. Every square in the blue mesh describing the density in the protein correspond to 1 Å. More on tubulin and microtubules later.

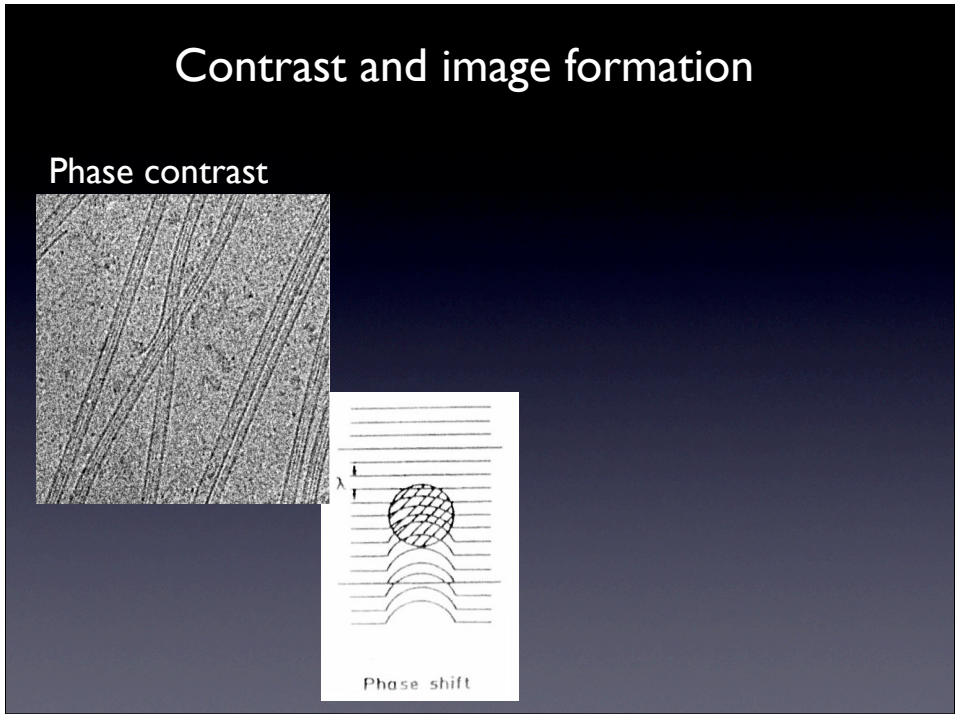


In TEM electrons have very high energies, hundreds of kilo electron volts, that make them travel down the microspore and right through your sample at close to the speed of light. In fact, most of them will not even realize they are going through matter and pass right through. The few that do interact will bounce of the nucleus of the atoms in the sample, very much like snooker balls bouncing off the edge of a snooker table, changing direction in a process called elastic scattering. We refer to this as “good” scattering events as they are going to be the ones important for the formation of the image. As they pass through the sample the electrons in the beam can also interact with electrons in the sample and lose energy in the process. This is known as inelastic scattering and is the equivalent of your snooker ball hitting another and slowing down while the other starts moving. We call this “bad” scattering both because it deteriorates the image and because it damages the sample.



The electron microscope can visualized your sample by generating contrast that reflects how much elastic scattering occurred at any given point. There are two major ways to do this. One is most typically used when looking at slices of cells, and is referred to as amplitude contrast. It works very much like an x-ray image that you get at the doctors. Your bones absorve more X-rays, so they appear darker in the image, as less X-rays get to hit the film emulsion. By the way, the image that the doctor shows you is the negative, which looks like this.

In the electron microscope electrons can either be absorbed by the sample or otherwise those that have scattered can be removed from the image by an aperture located after the sample that only lets through electron that have not scattered. Electrons that have gone through thinner regions of the object would scatter less and that region will appear brighter in the image. This contrast does work very well to generate images of organelles within cells, but falls short of generating high resolution information when looking at an individual macromolecule, like a protein.



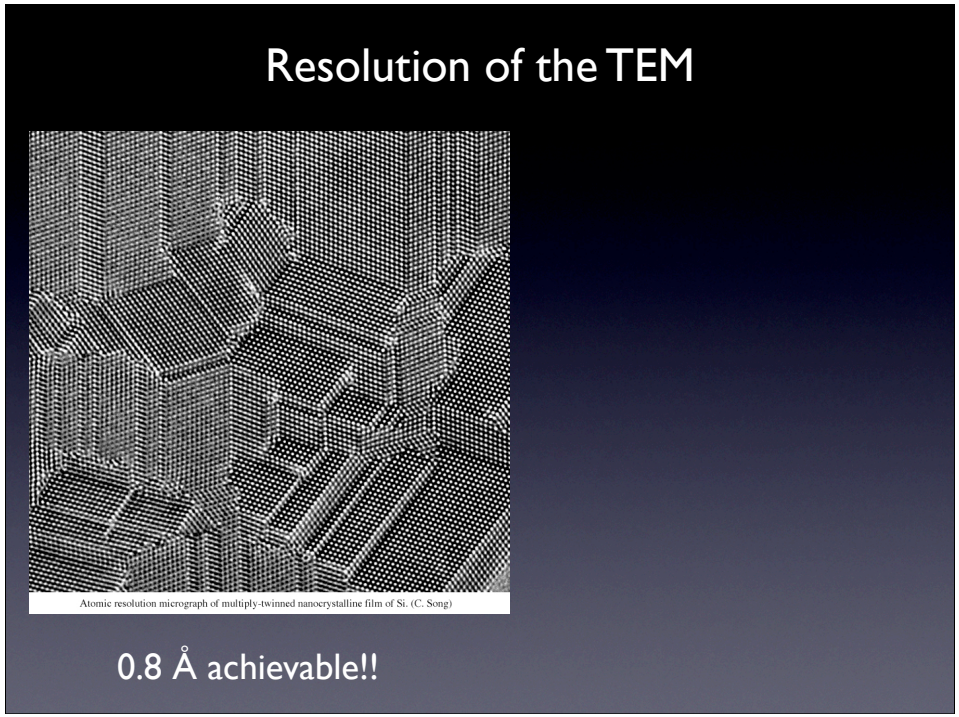
In that case the major contribution to the signal in the image is obtained using phase contrast. In this case the scattered electrons are let to go through all the way to form the image, and the objective lense is used to make them interfere with the unscattered electrons, a process that relies on the wave character of electrons moving at relativistic speed and the fact that their interaction with mater can be seen as a phase shift of the wave front.

Don’t worry, no wave theory or relativity today. Let me instead show you how an electron microscope looks like..

Movie at the Tecnai 12

This is what I would called a medium of the range microscope (I'll show you an state-of-the art one in a minute). Up there is the source that emits electrons, it is called an electron gun. Some electron guns work very much like a bulb, where a filament, typically made of tungsten, is heated up and emits thermionic electrons that are just spit out by the filament as it gets hot. An improved version is a field emission gun, where the quantum effect of tunneling is used to generate electrons with very well defined energy coming from a very sharp point in the gun. This really improves the quality of the images! The electrons travel down the column, which is maintained at ultrahigh vacuum by a complex system of pumps, to make sure the electrons do not interact with air. Two electro-magnetic lenses that can change the direction of the electrons as they travel down, are used to control the illumination on your sample, say how bright the electron beam is, and how large an area you illuminate at a time. Here is where the sample is located, typically in the middle of the most important lens in the scope, the objective lense that recombines the scattered and unscattered electrons to give rise to an image of the object. That image can then be magnified by a system of intermediate lenses that allow you to choose your magnification, from 50 times to 400,000 times!! Images can them be obtained, by means of a fast TV camera to survey the sample, or recorded on either photographic film or on a CCD camera for further analysis.

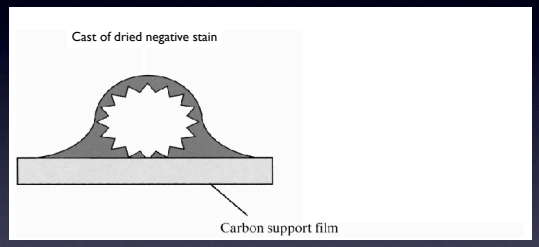
You need very little sample to do electron microscopy. A single drop of a very diluted protein sample is placed on a metal EM grid covered with a thin layer of carbon. This grid will have millions of copies of the protein you are interested in, enough to keep you taking pictures for a while!



I often get asked what kind of resolution can TEM reach. Microsopes like the one I just showed you are capable of producing images where individual atoms can be seen. However that happens only when the sample you are looking at does not get damage by the electrons. In that case you can pass many electrons through it and generate enough contrast for atom by atom visualization. Unfortunately organic matter is very sensitive to high energy electrons. In addition to the nice elastic scattering that I told you about and that is used to generate contrast in the image, a nasty process also occurs. The electrons in the beam can interact with other electrons in the sample. In this case the first pass energy to your sample and are able to kick electrons out of the sample and ionize it. Ionized molecules then move around breaking bonds in the sample and basically destroying it. In fact not many years ago people thought that TEM would never be able to produce high resolution structures of proteins because they would be totally destroyed by the time the electron went thought them, so that only ashes would be left to see. Well, if that had been true I would not have a job today. Fortunately there are two ways in which one can look at biological macromolecules and get representative images of the healthy sample. As always, each one comes at a price.

The Problem with Biological Matter

- Biological samples hate vacuum
- They have low intrinsic contrast
- They are very Radiation Sensitive



Solution:

- Negative Staining
  - + High contrast
  - + Fast and easy
  - - Artifacts
  - - Limited resolution

A routine and relatively simple way to look at biological material in the electron microscope is by embedding it in a cast that scatters electrons very strongly. This is known as negative staining. This method deals with the three main issues concerning biological samples: preserving them in the vacuum of the scope, generating contrast in the image, and making sure that what you image is not a pile of ashes. As long as the cast (or stain) reproduces the shape of the protein you are in business. Your protein may have vaporized, but the cast would be a good representation. The high contrast comes from the use of very heavy atoms in a solution that covers the samples and dries out before you inserted into the microscope column. Typically Uranyl salts are used. The large atomic number of the uranium warranties good scattering of your electron by the large nucleus. By this method you generate images with very good contrast. However the resolution is limited, both by the preservation of the shape by the cast and ultimately, by the grain size of the dried stain solution. It is call 渡egative because you protein will appear brighter, as it is the part that excludes the stain.

The Problem with Biological Matter

Biological samples hate vacuum

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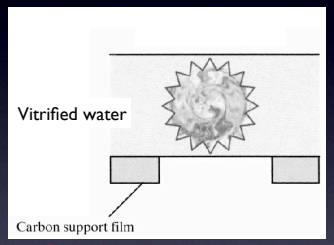


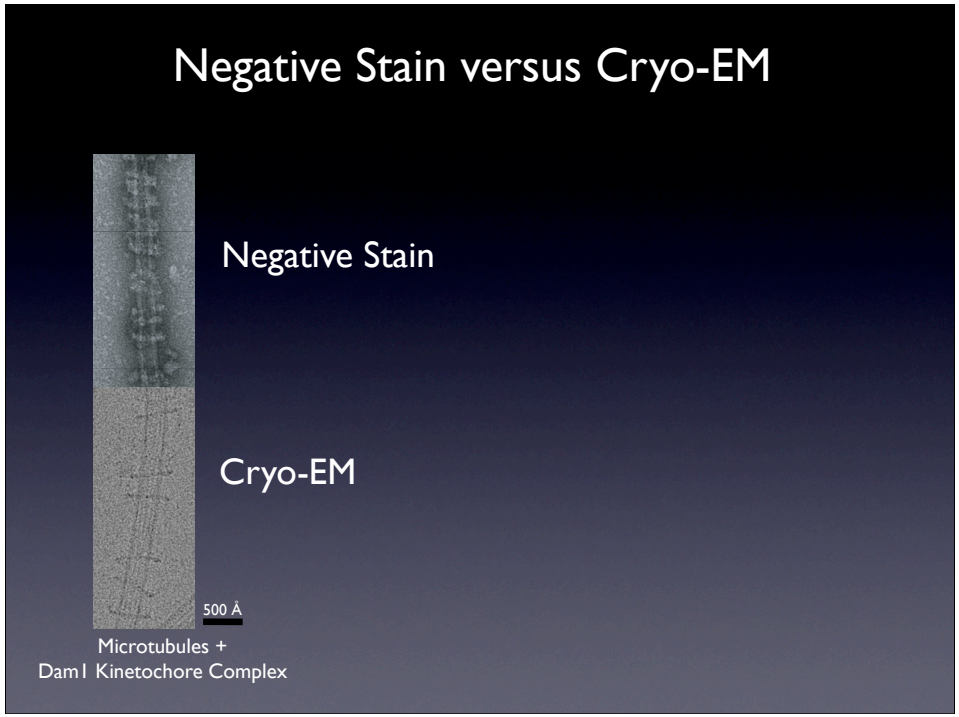
Solution:

- Frozen-hydrated Samples (CRYO-EM)

+ Good Preservation
+ High resolution
+ Time resolution

- Technically demanding
-/+ Low contrast
-/+ Minimization of Radiation Damage.
Still, Low-dose (10 $e^-/Å^2$)

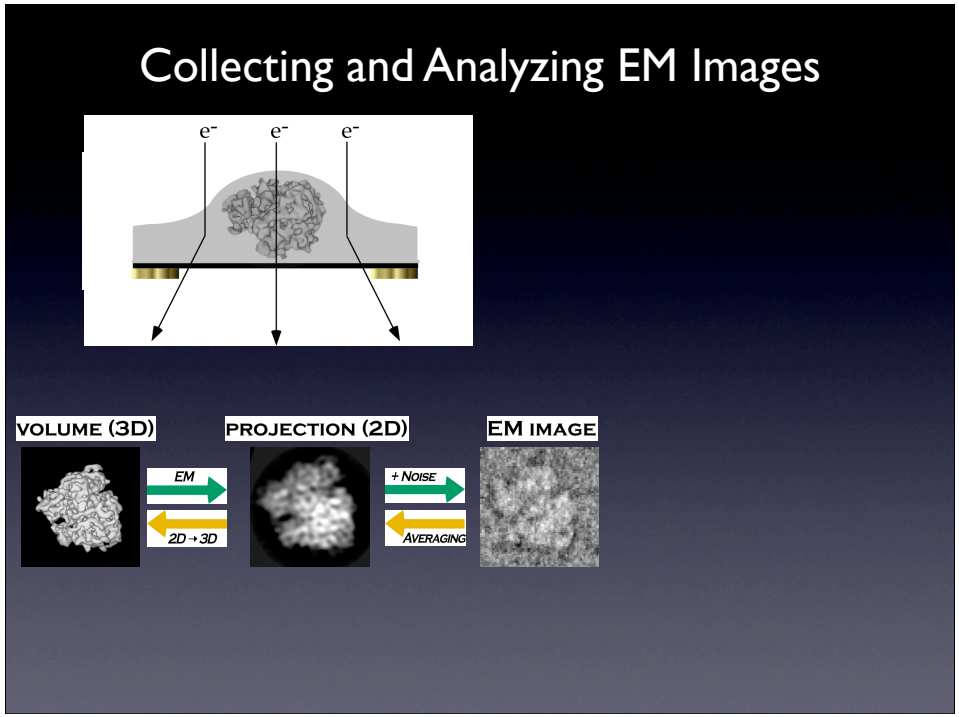
An alternative that improves preservation and potential resolution is cryo-electron microscopy. A thin aqueous layer of tyour sample is very quickly frozen and then inserted into the scope for examination. If the sample is kept under liquid nitrogen temperature (that is, about -170 C), it remains a solid in the vacuum of the scope. In this case the sample is not stained in any way, so contrast has to be enhanced by the optics of the microscope, and it is always much less than that obtained with stains. However, because you are imaging the object directly, you are sure that artifacts related to poor coverage of the sample by the stain, or the collapse of the cast during the drying process do not occur. Preserving the sample requires that the freezing process is done very fast, at about one million degrees per second. Most importantly, maintaining the sample at very low temperatures minimizes radiation damage in the sample, as the low thermal energy of the molecules in the frozen solution does not allowed harmful radicals to move very far. Still, very low doses need to be use before the sample burns up in the electron beam. The combination of low contrast of the sample and low illumination result in really noisy images. Computers and image analysis will come to the rescue, but before I get into that, let me show you a really mighty scope.



This is an example of the same biological sample, microtubules decorated with a protein ring that function to attach microtubules to chromosomes. In the negative stain sample the contrast is so high that we can even see the individual pieces that assemble into the ring in the background. Those are hardly visible in the cryo-em image, but here the cylindrical shape of the microtubule and the circular shape of the ring are perfectly mantained.

Movie at the JEOL 3100

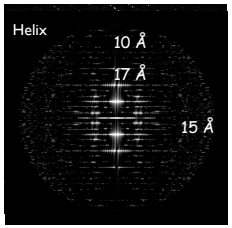
This beast uses 300 keV electrons generated with a field emission gun. The column is much wider because you need more powerful electromagnetic lenses to deflect higher energy electrons. Most importantly, it has a stage for the mounting of the sample that is kept at very close to absolute zero temperature (that is about -270 C) by liquid helium. This temperature reduces the damage in the sample, and the mechanical design assures that the sample moves very little while being imaged (you know what happens when you take an picture of someone moving!). The reason why this column is not only much wider, but also much taller, is that it has an extra part right here. This is an in-coulnm energy filter. It acts very much like a prism for electron, spraiding them in a rainbow depending on their energies. Remember what I told you about inelastic scattering, the process that damages the sample. Well, it also damages the image. When the electrons going through the sample loose energy, they are affected by the electro-magnetic lenses different from those that did not giving rise to a defect in the image called chromatin aberration, that generally degrades it and adds noise. The energy filter is used to remove those electrons so that they do not contribute to the image. This is particularly important when you are looking a thick sections of frozen cells, where the probability of inelastic scattering is really high.



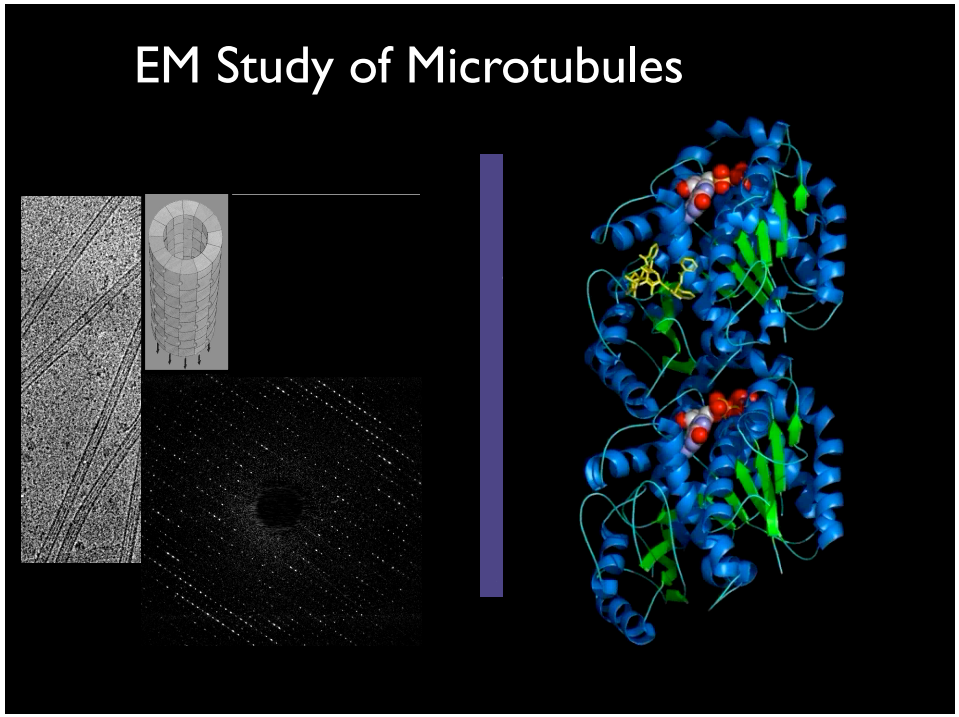
Let's go back to basic principles now and review a few concepts. The sample, typically a purified macromolecular complex in the size of a few 100 kDa to a few Mega Daltons, is placed on a substrate, typically a thin layer of carbon, and is either embedded in a thin layer of negative stain or a thin layer of vitrified water. Electrons are passed through it and some are scattered by the sample. It is very important to remember that what we obtained from our 3-Dimensionla object using TEM is a 2D projection, containing information of the whole object, but condensed into a single layer. When looking at biological materials, because they have poor contrast and are illuminated for very short times with limited electron dose, the image is actually very noisy. So, how do we recover our 3D object from noise 2D images? This is done computationally and it involves two steps: one in which images of the same view of the object are identified and averaged together to increase contrast, and one in which different averaged views are combined together in the right way to recover the 3D information. This is what we call 迭econstruction. How these two steps are carried out and how experimentally or computationally intense the process is depends on the type of sample you are looking at.

Different Methods for Different Types of Sample

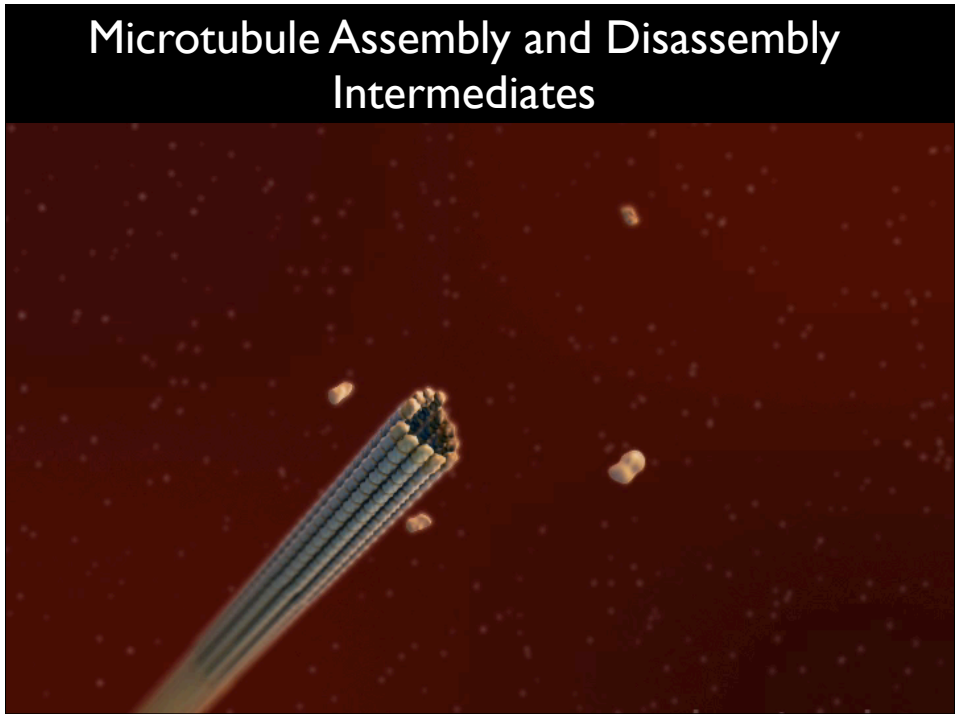
2D Crystals



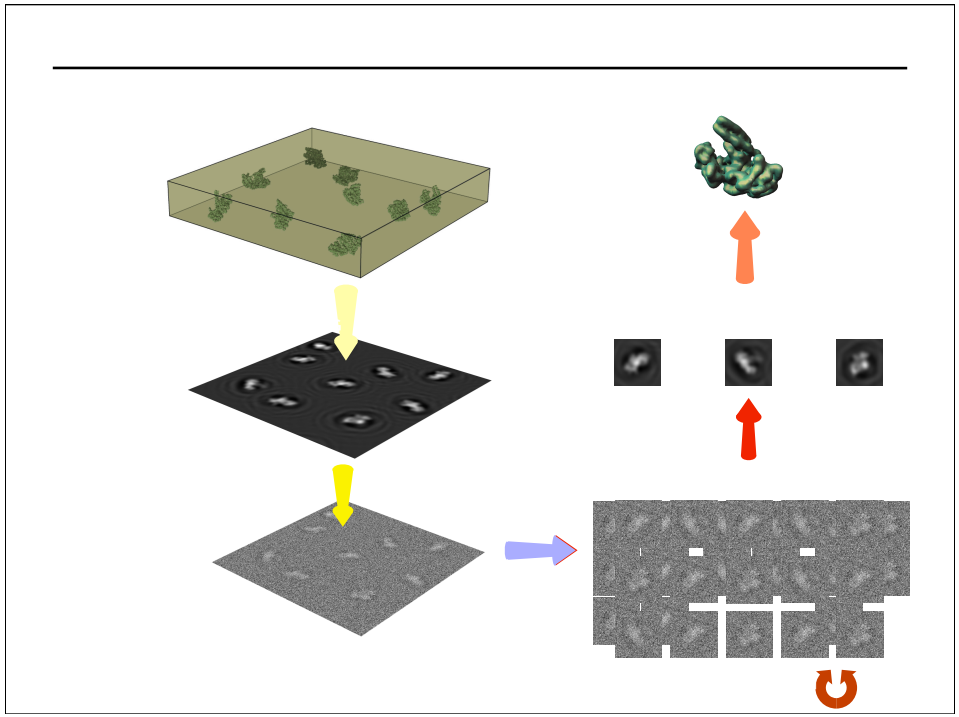
An ideal but rare type of sample for TEM is a 2D crystal of a protein. The crystal is always on the same orientation on the EM carbon support, so tilting of the specimen, where the stage where the sample is mounted is rotated with respect to the electron beam, is absolutely required. This is experimentally complex but computationally the data is very easy to analyze due to the intrinsic order of the sample. Medium (10 A) to high (3 A) resolution is typically obtained from this type of sample. Another excellent type of specimen is a naturally existing or artificially formed helix, where a single image contains the repeating unit in many orientations, making it possible to obtain a full 3D reconstruction without the need to tilt. Low to high resolution structures are obtained for this type of sample. More generally the macromolecular of interest is not in a helical arrangement, neither can be crystallized in 2 dimensions. These specimens are studied using what are called single particle methods, which are the most computationally involved. The resolution reach varied from low, especially in the case of particles without internal symmetry and with flexible regions,. To high for particles with high symmetry, like viruses. Let me show you a few examples.



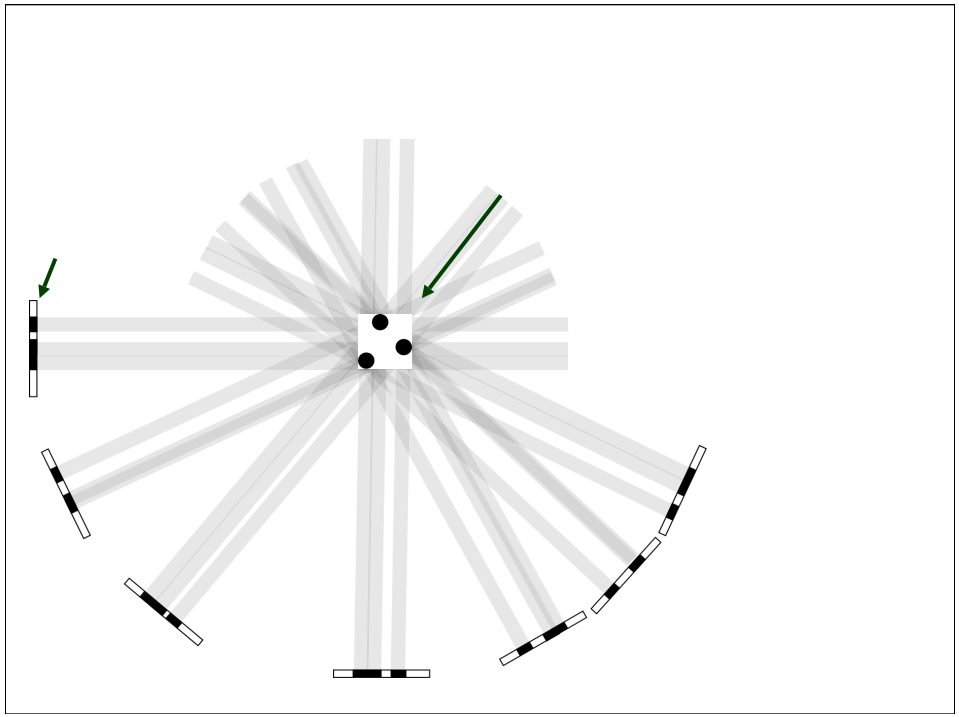
Cytosketal self-assembly systems, like microtubules, are examples of naturally occurring helices. In microtubules a and b tubulin subunits interact head to tail making protofilaments that then associate laterally making a tube. Because one protofilament is slightly staggered with respect to the next, the tubulin subunits make a helical pattern on the wall of the microtubule. From images like this of frozen-hydrated samples one can reconstruct the whole microtubule with a resolution of about 8 A, which allows for the visualization of alpha helical elements in the protein. Tubulin can be tricked to assemble in a different way, in which the protofilaments interact in an antiparallel way and do not close into tubes but form large 2D sheets, ideal for electron crystallographic studies, where the resolution obtain is better than 4 A, allowing the traced of the polypeptide chain and thus the atomic description of the protein. This is the structure of tubulin obtained by this method, displayed using a ribbon diagram that describes the path of the polypeptide chain.



Interestingly microtubules are very dynamic in the cell and grow and shrink in a complex matter that it is possible to study by electron microscopy this is an animation that describe the process.

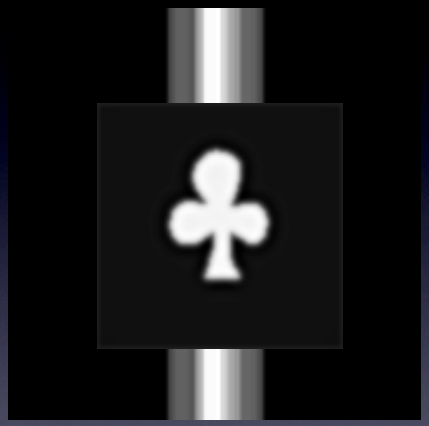


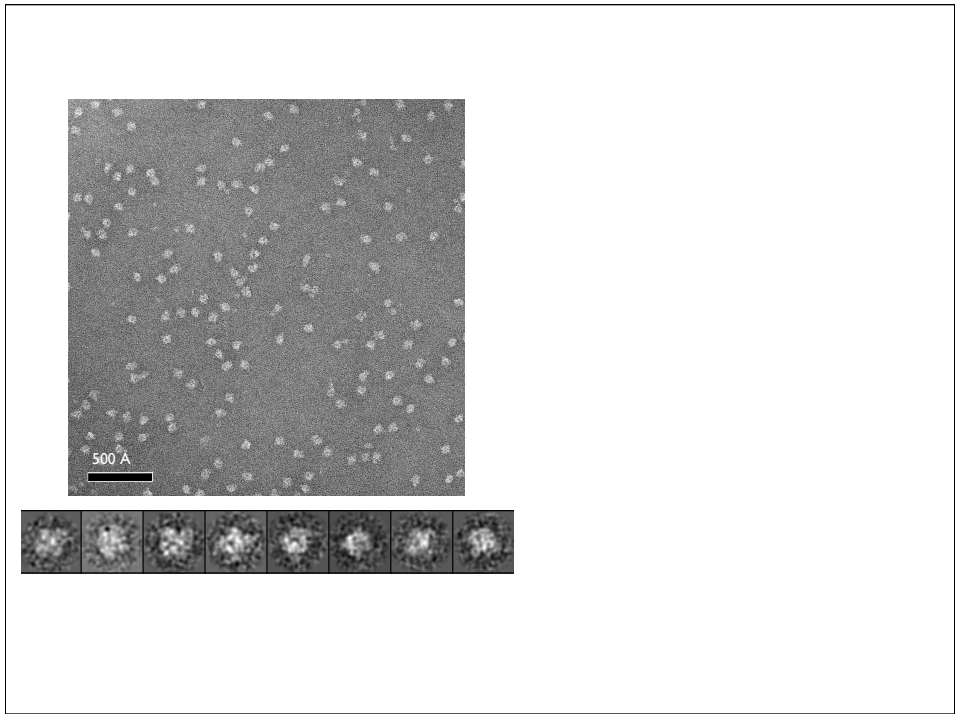
Let me summarize what the analysis of Single Particle Reconstruction involves.



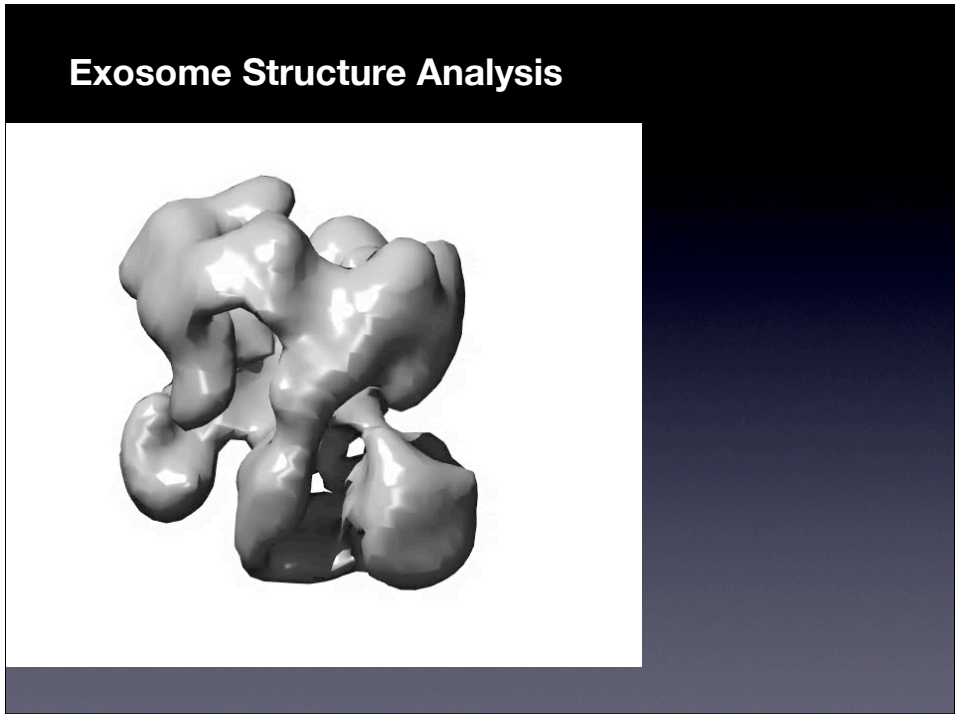
Once relative angles between the different views of the sample have been obtained, a three #d reconstruction of the object is calculated by a computational process called back projection, where the projections are made to interfere constructively defining the object. The following movie shows this in a little more animated way and shows you how the reconstructed object becomes more and more clear as more views are included in the backprojection process.

Reconstruction by Backprojection

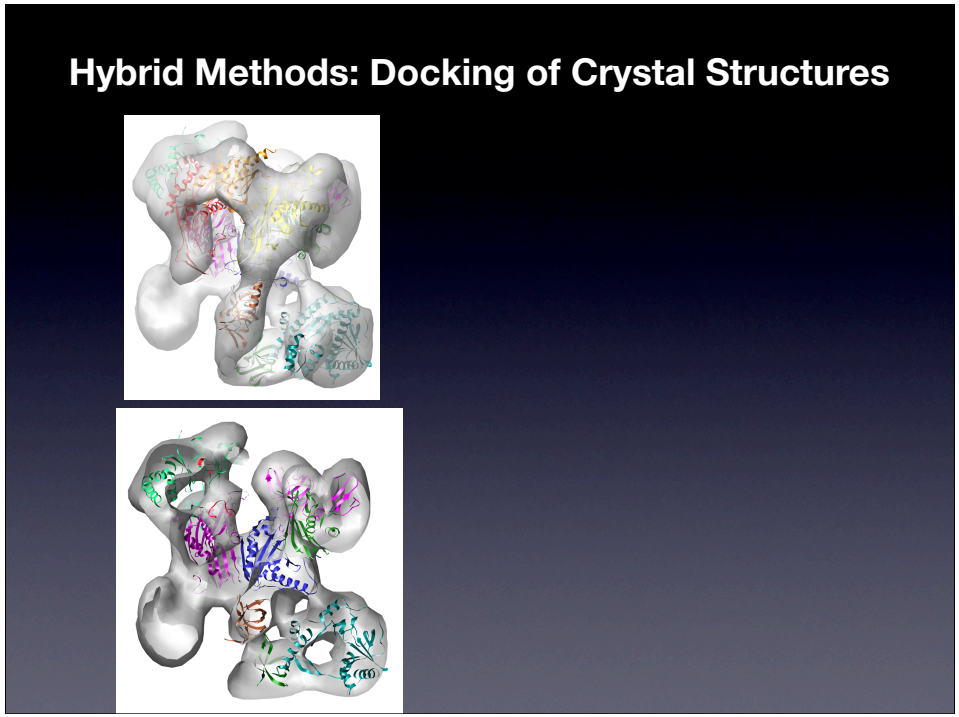




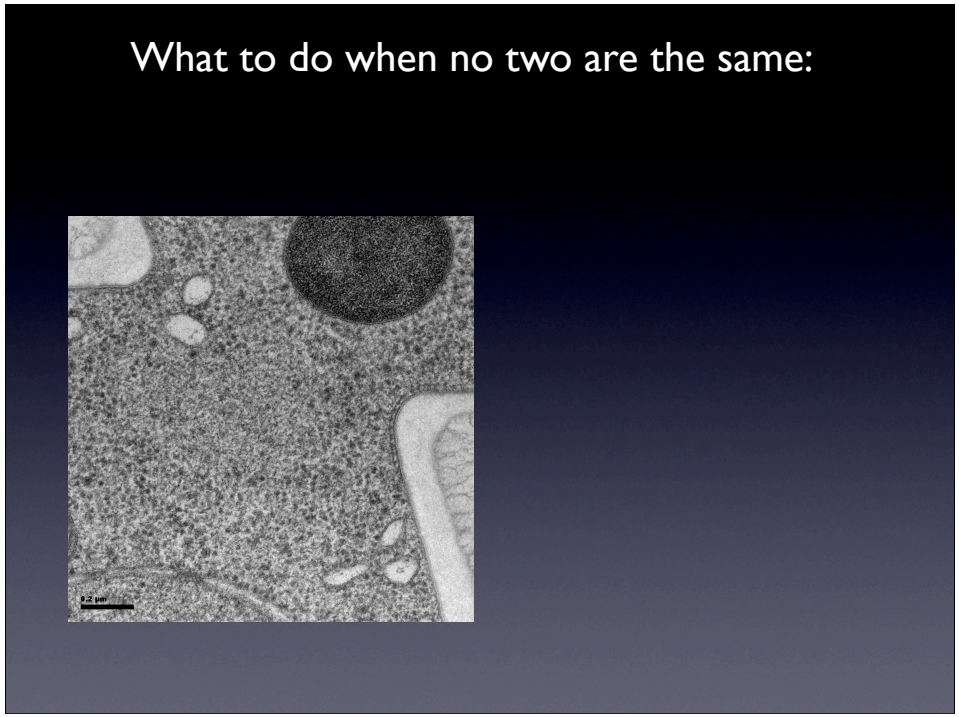
This is an image of a negatively stain sample of the yeast exosome, a molecular machine that breaks down RNA in the cell. The complex has a molecular mass of about 400 kDa (CHECK) and dimensions of about 150 A. This is how individual particles of the complex look like. This is how class averages, obtained from many copies of the same object look like, with marked improvement in the signal. Finding the right relative orientation of these class averages allow us to reconstruct the complex using back projection.



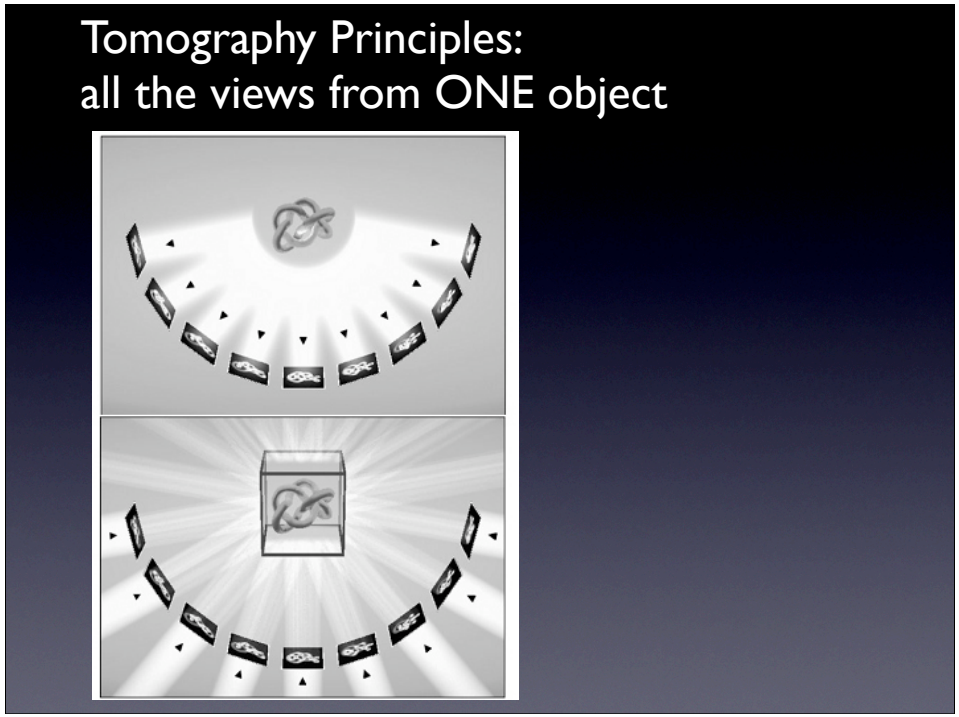
This is how the yeast exosome looks like at about 18 A resolution. We obtained two different reconstructions, one of the whole complex, shown in gray, the other of the core components, which actually lack the RNAse activity, shown in blue. When we subtract one from the other we see where the additional components are. Now, we were fortunate that the structure of the core section had been obtained at atomic resolution using X-ray crystallography for the human homolog. So, we were able to dock in that structure in our reconstruction. For the rest we used bioinformatics to identify structural homologs in the protein data bank and came out with most of the remaining components.



This hybrid methodology, which combines the atomic structure of components, with the lower resolution structure of full complexes, is very powerful and shows the beautiful complementarity of electron microscopy and X-ray crystallography. The interaction of components can now be described with pseudo atomic precision.

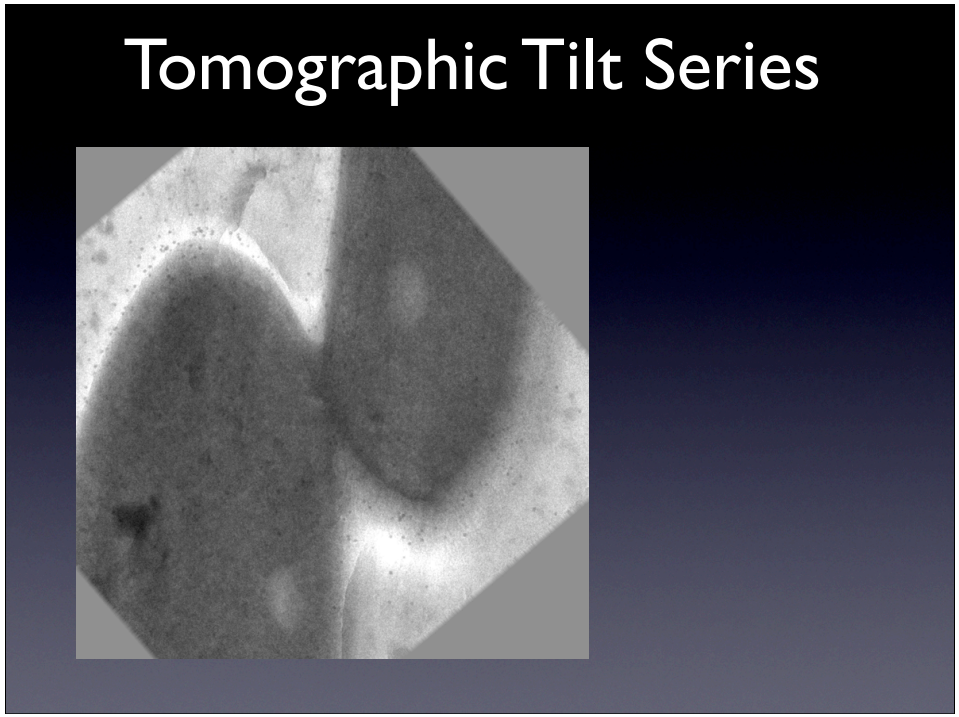


For all the previous examples shown, many identical copies of the specimen of interest are available, each is exposed to the electron beam only once, to minimize radiation damage, and then images are combine to gain signal and three dimensional information. But what about cases where not two identical object exist, like when we are studying cells or cellular components. In this case averaging is not possible. The methodology to use is called electron tomography.

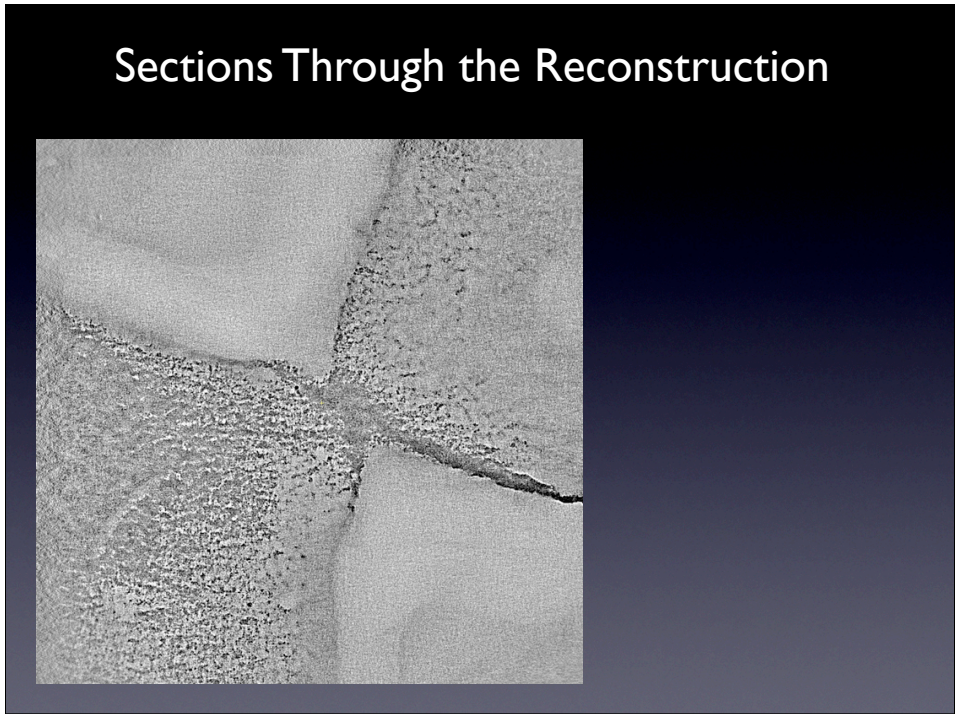


The principal idea behind tomography is that all the views that are needed to reconstruct your structure of interest have to come from the same object. Because the problem with radiation damage still holds, the total dose has to be fractionated to take the full set of images from a single object and thus the reconstruction suffers from very high noise. To compound thing even more, the interior of cells is very dense. So, while the data processing is the simplest of all the cases I've shown you, the interpretation of the data is the most complex, and scientists spent more than 90 % of their time trying to interpret these reconstructed tomographic volumes.

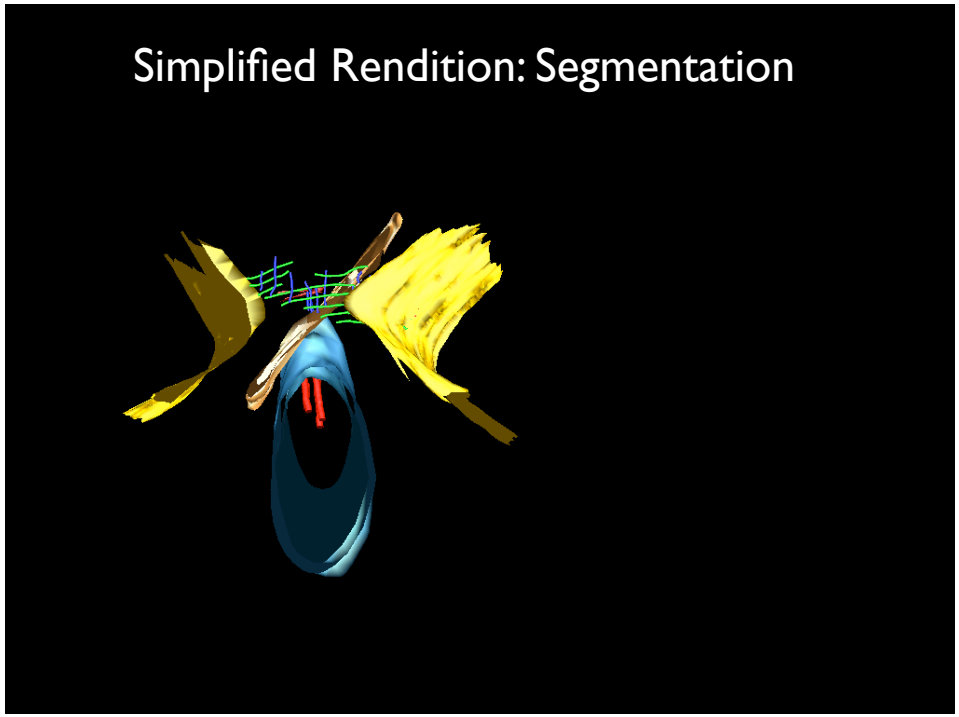
Let me give you an example from our own recent research. We are interested in septins, a cytoskeletal component that is essential in cytokinesys, the splitting of cells after segregation of chromosomes has taken place. We study these in yeast cells, where these proteins were first discovered. The septin filaments localize at the bud neck, which is the site of septation in budding yeast. This is an image of a section through a yeast, and this is a magnified area of the type of structures, close to the membrane, that we are looking for.



Once a good cells has been identified, images of the area are obtained at many different tilts of the object with respect to the electron beam. This is called a tomographic series and looks like this when they are shown one right after the other. This data is then used to obtain a 3D reconstruction by back projection.

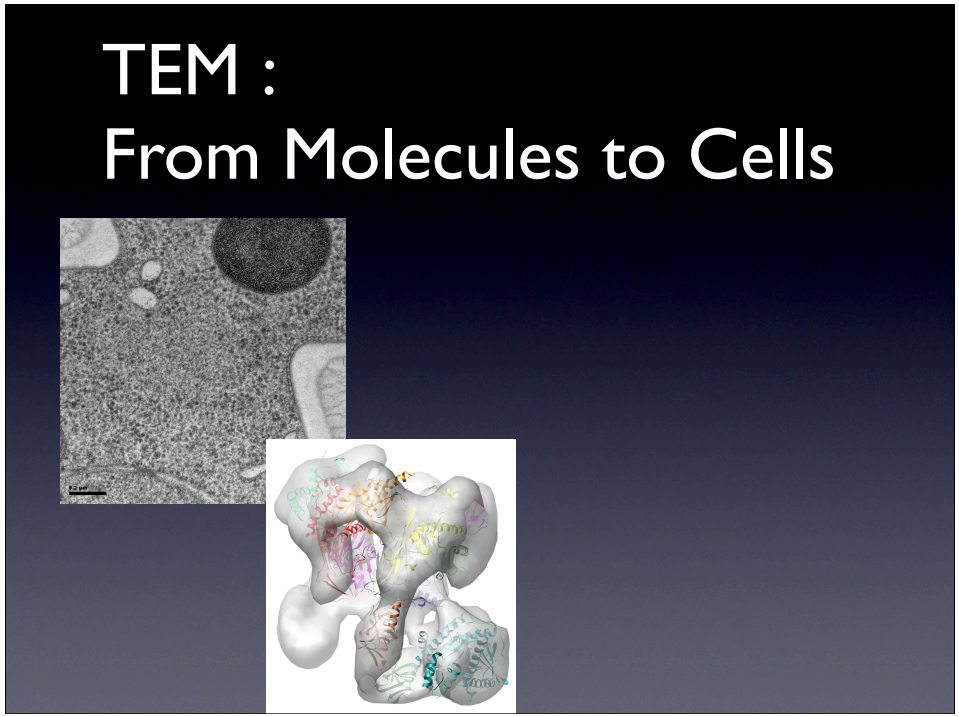


The complex tomogram (that is how the reconstruction is referred to) is typically shown as a number of sections through the specimen, just is if you were traveling through the sample. This still can be very confusing, so scientists use segmentation techniques to extract the most important features in the image and then represent them as simple surfaces.

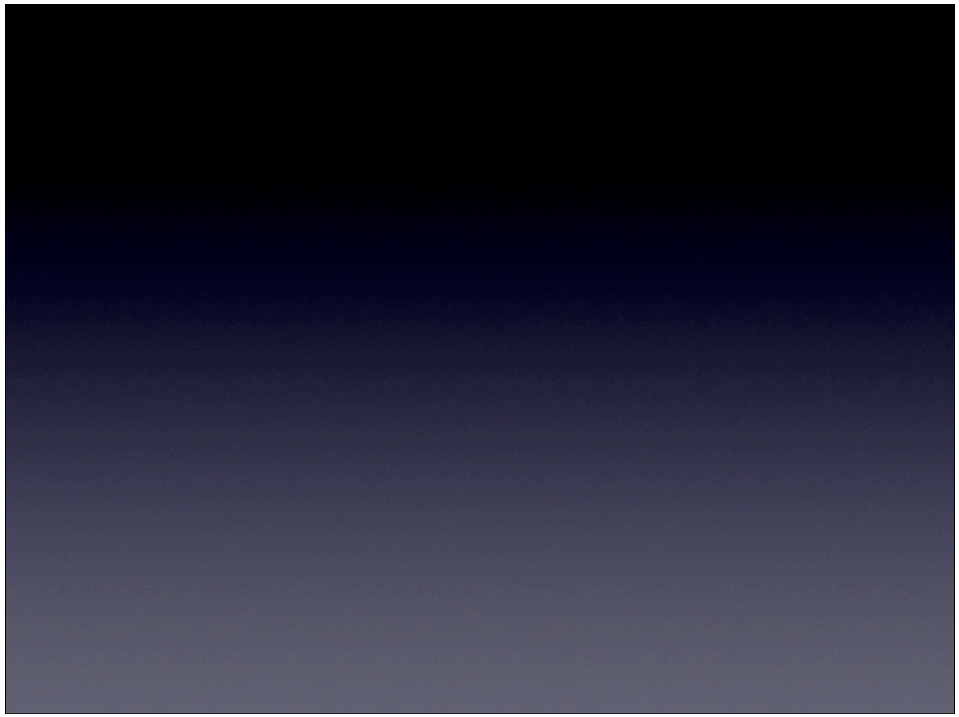


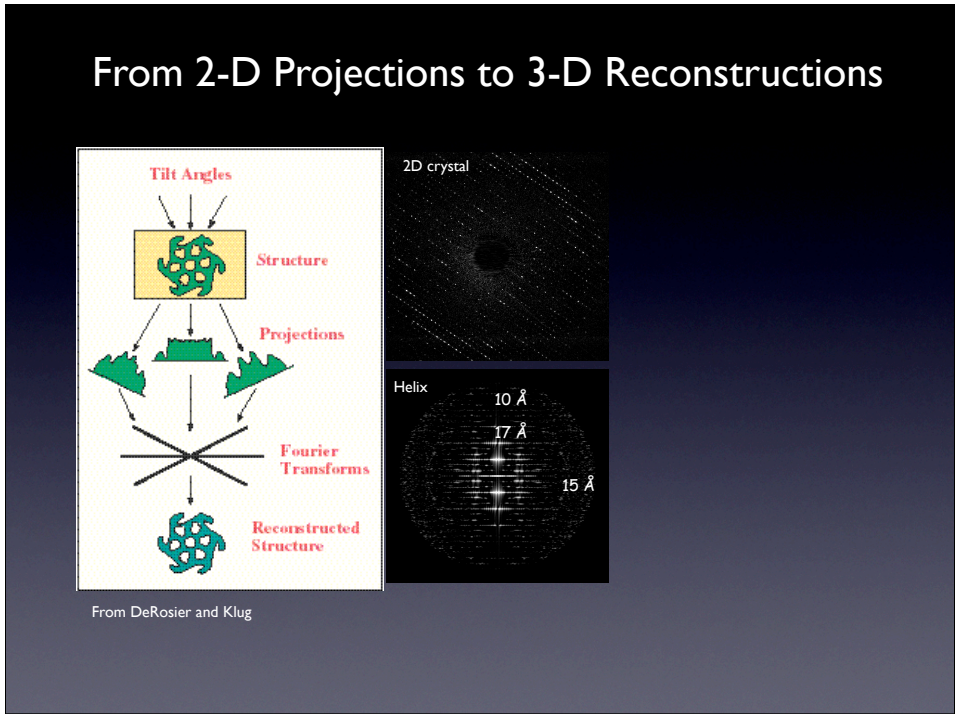
This is the segmented rendering of that tomogram. It shows the plasma membrane, the nucleus, endomembranes, microtubules inside the yeast nucleus that are part of the mitotic spindle separating the chromosomes, and the filaments adjacent to the membrane that we are interested in. Notice that we have colored them (again, artificial color!) differently depending on their direction with respect to the plane of the membrane.

Tomography offers the possibility of merging cellular and molecular studies, in a very unique fashion. Because the elements studies are not 都elected as they are in vitro structural studies where one protein complex is purified before visualization, or in fluorescent microscopy of the cell, where only one or a few components are visualized by placing flourophores, the richness of information is enormous, and the challenge is really in mining the information out of each tomogram. We are interested in the septins, but our tomogram could be used by people interested in nuclear envelop, endomembranes, mitotic microtubules, ribosomes, etc, etc. Hopefully tomography maps are made available publicly, just like X0-ray crystal structures or EM maps of purified components, so that the whole scientific community can benefit from these complex and fascinating 3D maps of the interior of cells.



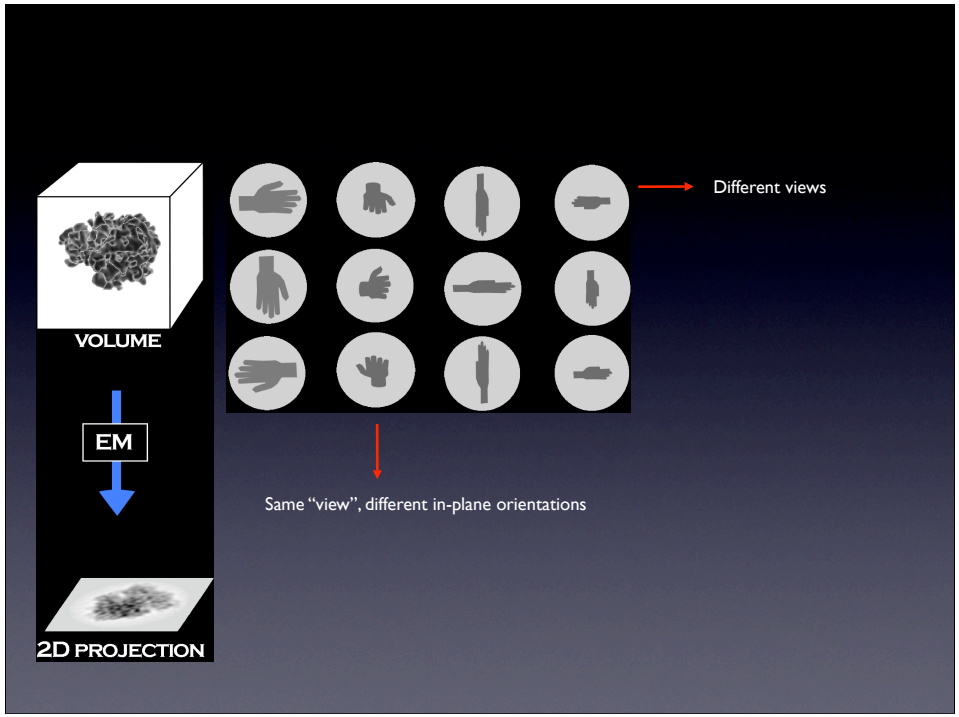
I hope in this brief introduction to the use of electron microscopy in biology I have been able to give you a taste of the possibilities and the challenges of the technique. Hopefully I have shown you the incredible breath of applications, from ultrastructure of whole cells, to high resolution chain tracing of individual proteins. There are very few things that this technique cannot give you information on. And although I do not have time to tell you, I just want to mention that a lot of new technique development is going on in the field and that both the generality of its applicability, and the resolution and reliability of the 3D reconstruction EM can produce are going to improve dramatically within the next few years, when you may be considering this the technique of choice for your own studies. I can't wait myself!





As already mentioned, the large depth of focus in the TEM means that every image is a projection of the 3-D density in the direction of the electron beam.
To recover 3-dimensional information, projections from different angles have to be collected.
This is done differently depending on the type of sample under study.

How are different projections related to one another?
The central theory states that the Fourier transform of a projection corresponds to a plane in the 3-D Fourier transform of the 3-D density, that includes the origin.



When studying single particles, which are not organized in higher order structures the reconstruction process is very involved. Images of the sample give you many occurrences of the molecule of interest in different in plane orientations (same view, but rotated, as well as different views of the object. So different computational classification methods, couple to alignment procedures are used to separate particles into those belonging to the sample view to generate class averages with good signal to noise ratio, then these views are combined in the right way, using either more geometrical or more analytical methods, that are beyond the scope of this presentation, to give the 3D structure of the object.