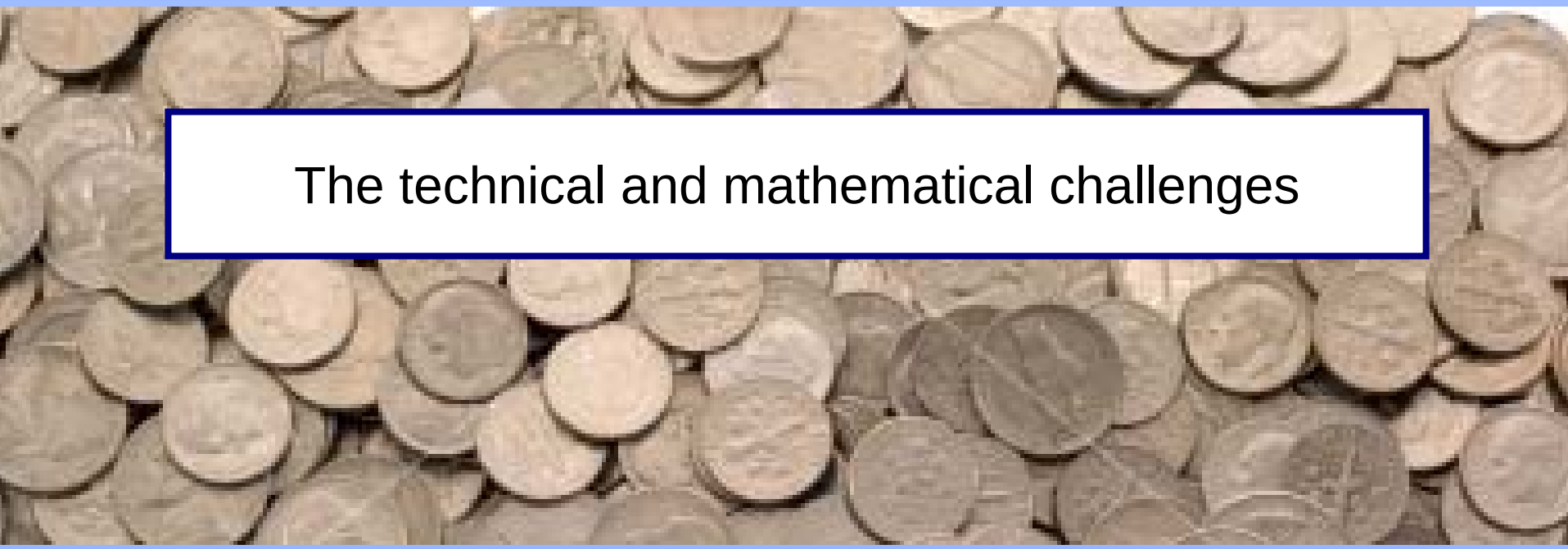

The technical and mathematical challenges

What are the goals that set the challenges?

We want maps that can yield an atomic model with a known accuracy, and we want to visualize conformational variability.

We want tomograms of cells in which we can identify, sort and average pieces of cell machinery at molecular resolution or better.

We want total automation so that anyone with a structural question can get answers without having to take a course like this one; the technology should be as easy to use as that of the light microscope.

Let’s begin with a list of things that need improvement:

Fusion proteins with concatenated MT are able to bind gold similar to previous studies. In this work, incubated MBP-MT samples acquired about 13 gold atoms while MBP-MT2 samples acquired about 38 gold atoms as determined at their mass spectrometry peak amplitudes. This is in agreement with past studies that showed a range of metal binding stoichiometries. This likely arises because MT tries to accommodate as many metal atoms as possible and thus it form a distribution metal binding stoichiometries. Similarly, in this work distributions associated with these peaks indicate a range of gold binding including from only a few atoms to values of It is difficult to resolve the difference in MT gold cluster appearance with TEM and STEM. Although both techniques show metal accumulation, clusters viewed by TEM appear more compact then their STEM counterparts. The more compact TEM appearance may result from the decreased signal to noise of TEM imaging as compared to STEM. Furthermore, resolution information from single TEM images is limited due to the small defocuses used to induce phase contrast. When viewing nanometer-sized complexes, these limitations may make small, close-proximity clusters appear as one, or, in the case of extended structures, may make small clusters undetectable against a carbon background. If the gold-induce MBP-MT2 structures are not extended in TEM, there are two likely reasons that may account for their altered appearance in STEM. First, samples for TEM were made soon after column elution, while STEM samples were shipped to Brookhaven national Laboratories. While samples appeared stable in the laboratory, it is unknown whether conditions shipping condition had an affect. Secondly, for both techniques protein samples are dried on the grid. TEM, samples were quickly absorbed and dried at room temperature on to EM grids. In contrast, STEM samples were blotted, cryo-plunged, and then freeze-dried on to grids. Perhaps quick drying allows for more compact structures, such as by rearrangements during dehydration. Alternatively, the quick freezing and slow dehydration process used for STEM may alter our gold-bound proteins. Even with these discrepancies in imaging, concatenated MT-containing complexes that can accumulate enough gold for direct visualization by each method. Visualization of concatenated MT-containing fusion proteins in biological complexes has proven difficult. Attempts to visualize MT-fusions to other proteins in two systems able to make filamentous structures have not yet been possible. It is unclear whether these issues result from the general difficulty of making protein fusions or is there a specific problem with MT fusions. Chromatography of MT-fused MBP protein (Figure 2A and 2C) did show oligomerization, which could easily explain problems with other systems. This oligomerization most likely implicates the large number of cysteines in MT. If this is the case, preparation of MT with strong binding, oxygen insensitive metals such as gold or cadmium, as well as more rigorous purification steps should provide more stable material. Without a complex more favourable for interpreting, in this work we have been able to create a simple complex with gold-bound MBP-MT2 and monoclonal MBP-antibody. The characteristic appearance and ease of formation of these complexes (Figure 4C and 4D) leads us to believe that the incubations used to fill gold binding sited in MT have not adversely affected this protein. At times, cryo-EM images hint at more strongly scattering densities associated with the extended arms of the antigen. These are consistent with the size and expected location of gold clusters in the complex. Unfortunately, the small size and flexibility of the complex make detection of identifiable views and image averaging near impossible. Even though more evidence is needed to make a definitive claim, these images of these antibody complexes show the potential of this method. Fusion proteins with concatenated MT are able to bind gold similar to previous studies. In this work, incubated MBP-MT samples acquired about 13 gold atoms while MBP-MT2 samples acquired about 38 gold atoms as determined at their mass spectrometry peak amplitudes. This is in agreement with past studies that showed a range of metal binding stoichiometries. This likely arises because MT tries to accommodate as many metal atoms as possible and thus it form a distribution metal binding stoichiometries. Similarly, in this work distributions associated with these peaks indicate a range of gold binding including from only a few atoms to values of It is difficult to resolve the difference in MT gold cluster appearance with TEM and STEM. 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Maybe it’s better to consider the things that do not need work:

Given that everything needs work, are there one or two real bottlenecks?

It doesn’t seem so. The answer is we have to

nickel and dime the methodology and

we need to bring lots of change!

Better specimens – duh!

Biochemical control over conformational variations

Ribbons of undistorted, frozen-hydrated sections
from well-preserved, thick samples
screened and analyzed by cryo light microscopy.

Conducting embedding medium.

Better films for grids

Flat, stiff, sturdy, patterned holes, and non-stick

Conductive at very low temperatures (liquid helium).

Joe Wall proposed the use of poly-pyrrole films, which are strong and conduct well at LN_2 temperatures: Simon, M. N., Lin, B. Y., Lee, H. S., Skotheim, T. A., & Wall, J. S. (1990). Conducting polymer films as EM substrates. In G. W. Bailey (Ed.), In Proc. 12th International Congress for Electron Microscopy, (pp. 290-291). San Francisco Press.

Clonable heavy metal label EM analog of GFP

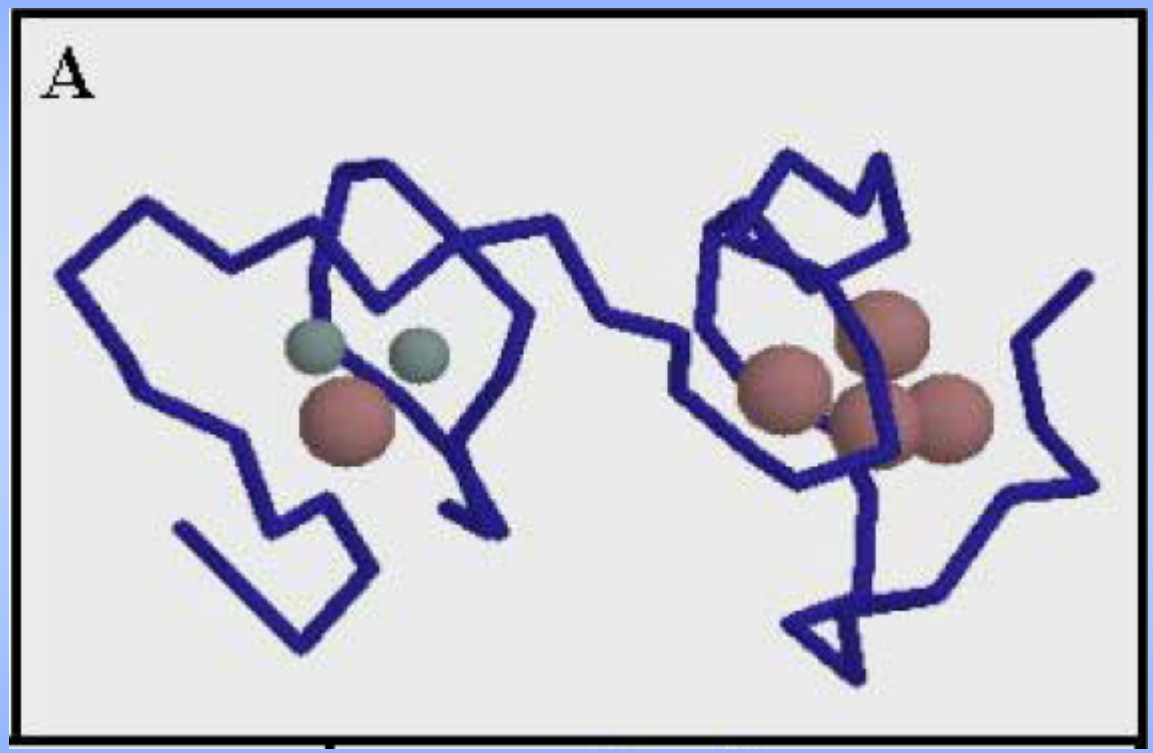
Protein tag upon which we can grow a gold cluster atom by atom inside an intact cell.

Gold label will be specific and will label every tag.

Metallothionein shows promise but needs work.

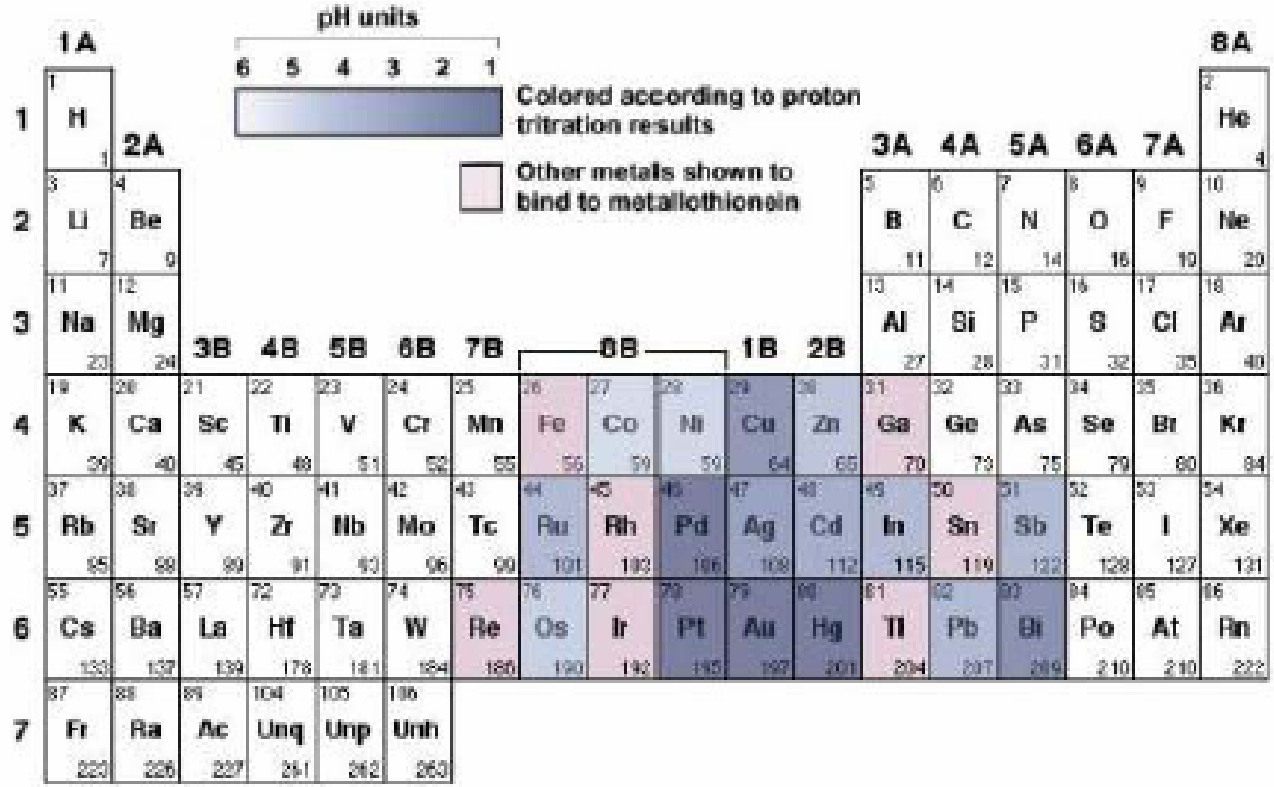
MT as clonable gold label

(Chris Mercogliano, JMB 355, 211, 2006; J. Struct. Biol. 160, 70, 2007)

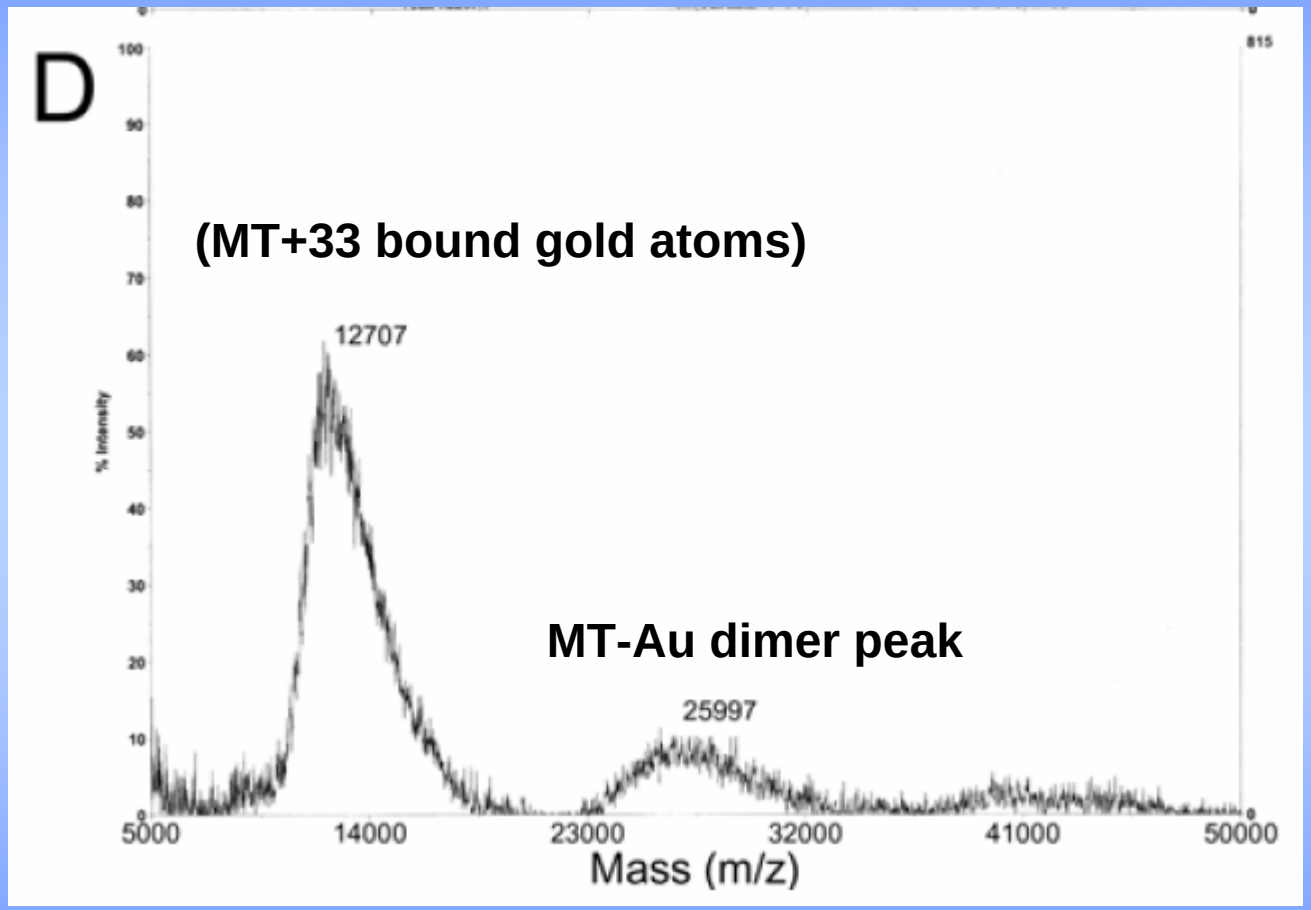


Metallothionein is a heavy-metal binding, ~60 aa protein having 20 cysteine residues

Metals Able to Bind to Metallothionein

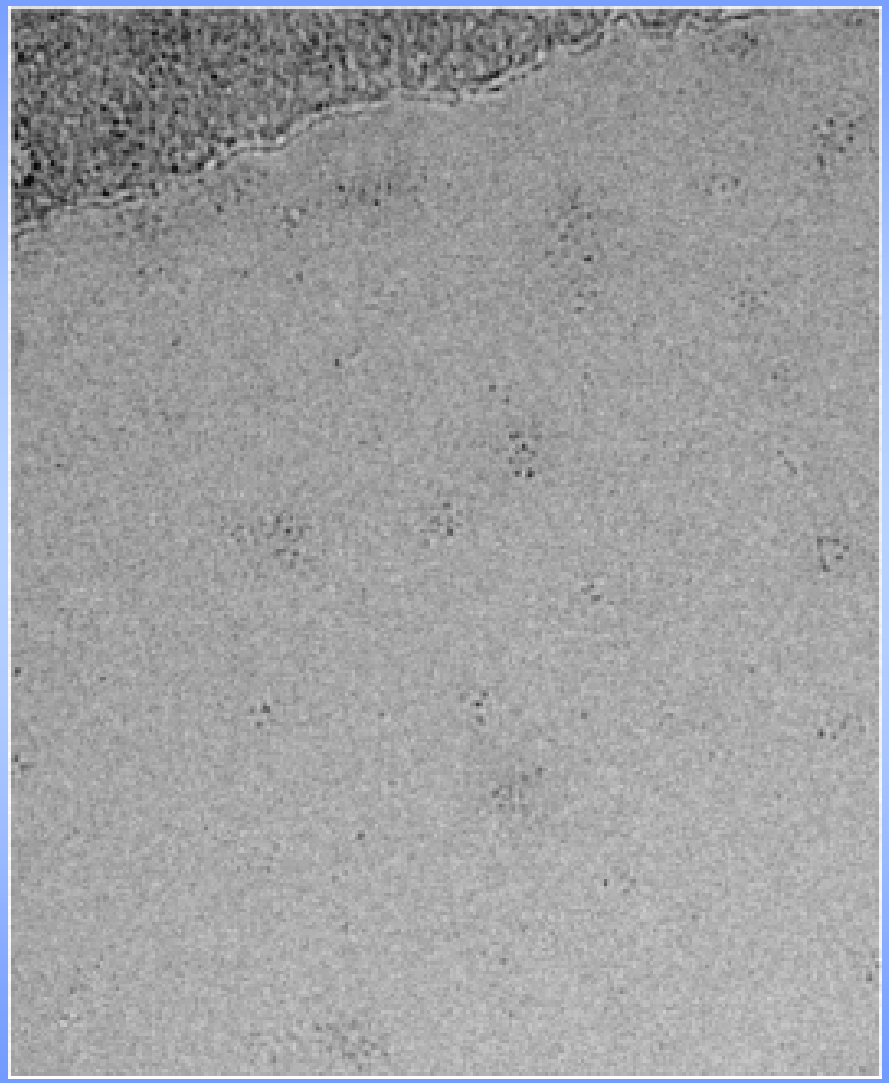


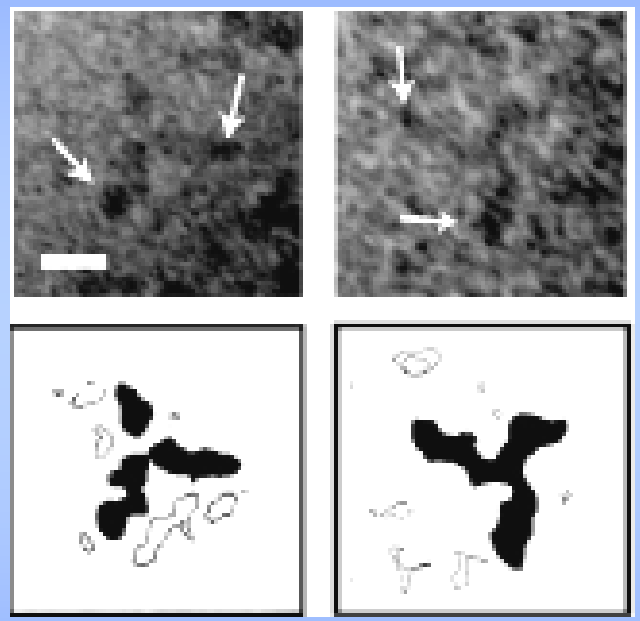
MT can bind up to 40 Au but more typically ~18-20 per copy.



Maldi mass spectrum

Frozen hydrated image of chimera MBP (maltose binding protein)–MT_2 reacted with antibody against MBP





Improvements to equipment

Phase plate + no spherical aberration

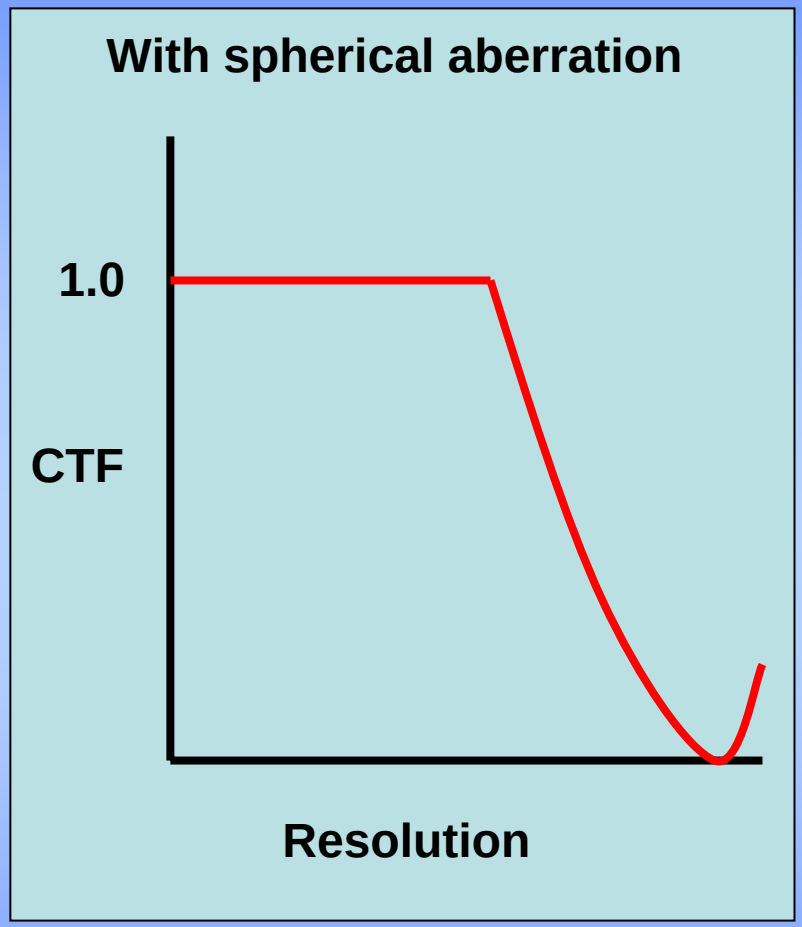
DQE=1, MTF=1 digital imaging system

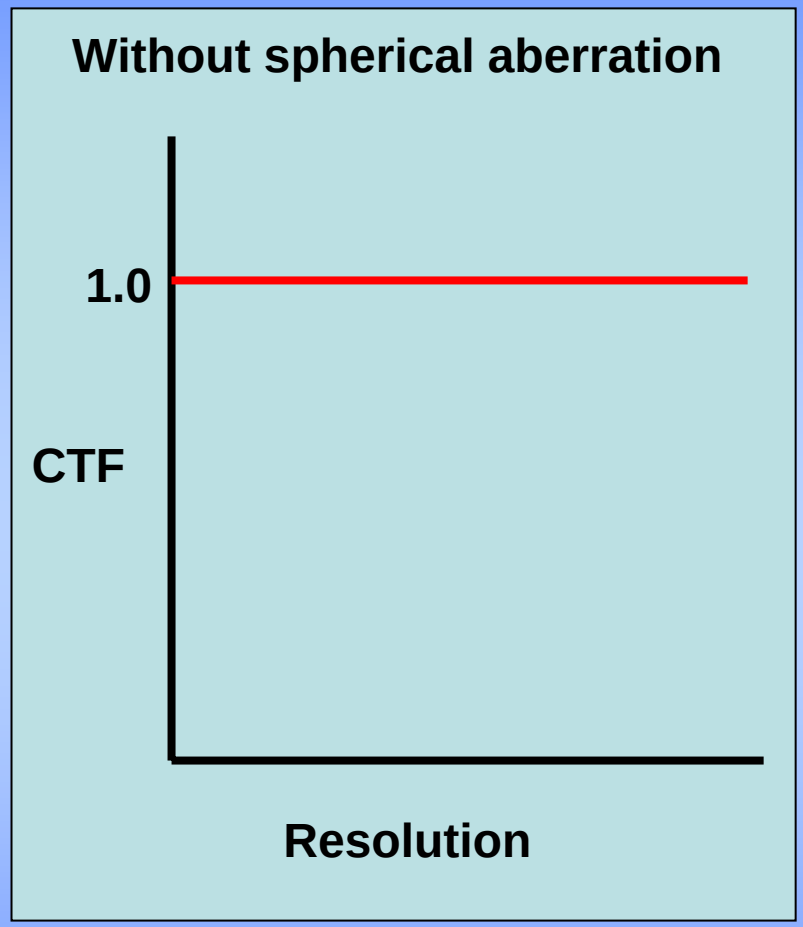
Energy filter

Drift & vibration free, double tilt cryo holder

360^{o} cylindrical cryo holder

Phase plate + no spherical aberration





The amplitude variations leaving the specimen faithfully reflect the details in the structure at every level of detail.

DQE=1 & MTF=1 digital imaging system

The electrons arriving at the image plane contain the information leaving the specimen.
The recording system generally degrades that information.

DQE (detector quantum efficiency)=SNR_{in} /SNR_{out} =1 means that in the recorded image every electron is detected without introducing additional noise.
SNR=signal to noise ratio.

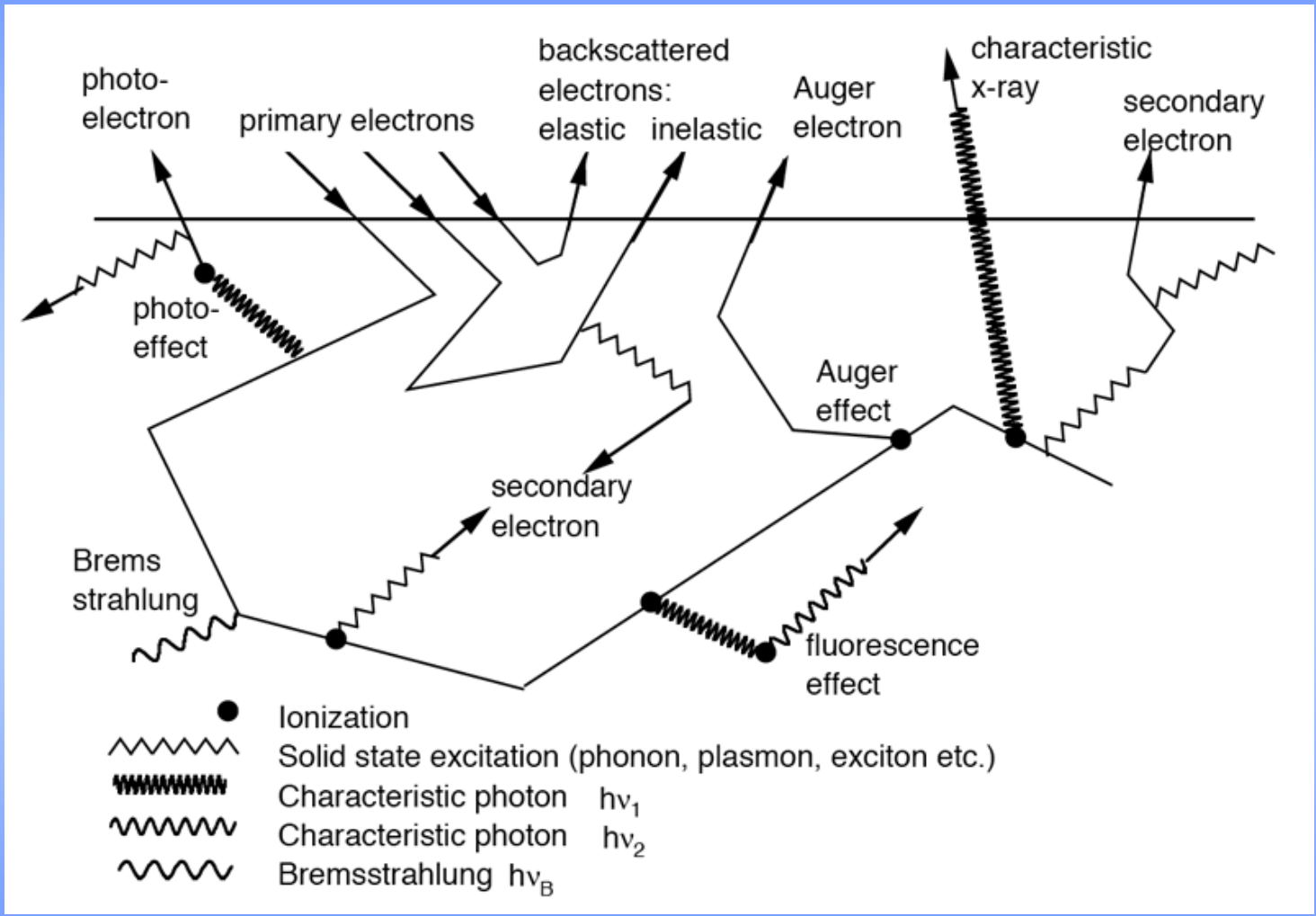
IF MTF(R)=modulation transfer function=1 for all spatial frequencies, then the recorded image will faithfully capture all the detail arriving at the image plane.

Why aren’t images so faithful?

Electrons interact with matter
much as a superball bounces around a room.

At every collision, it can generate
secondary electrons, Auger electrons, photons etc.

What happens as the electron interacts with matter:

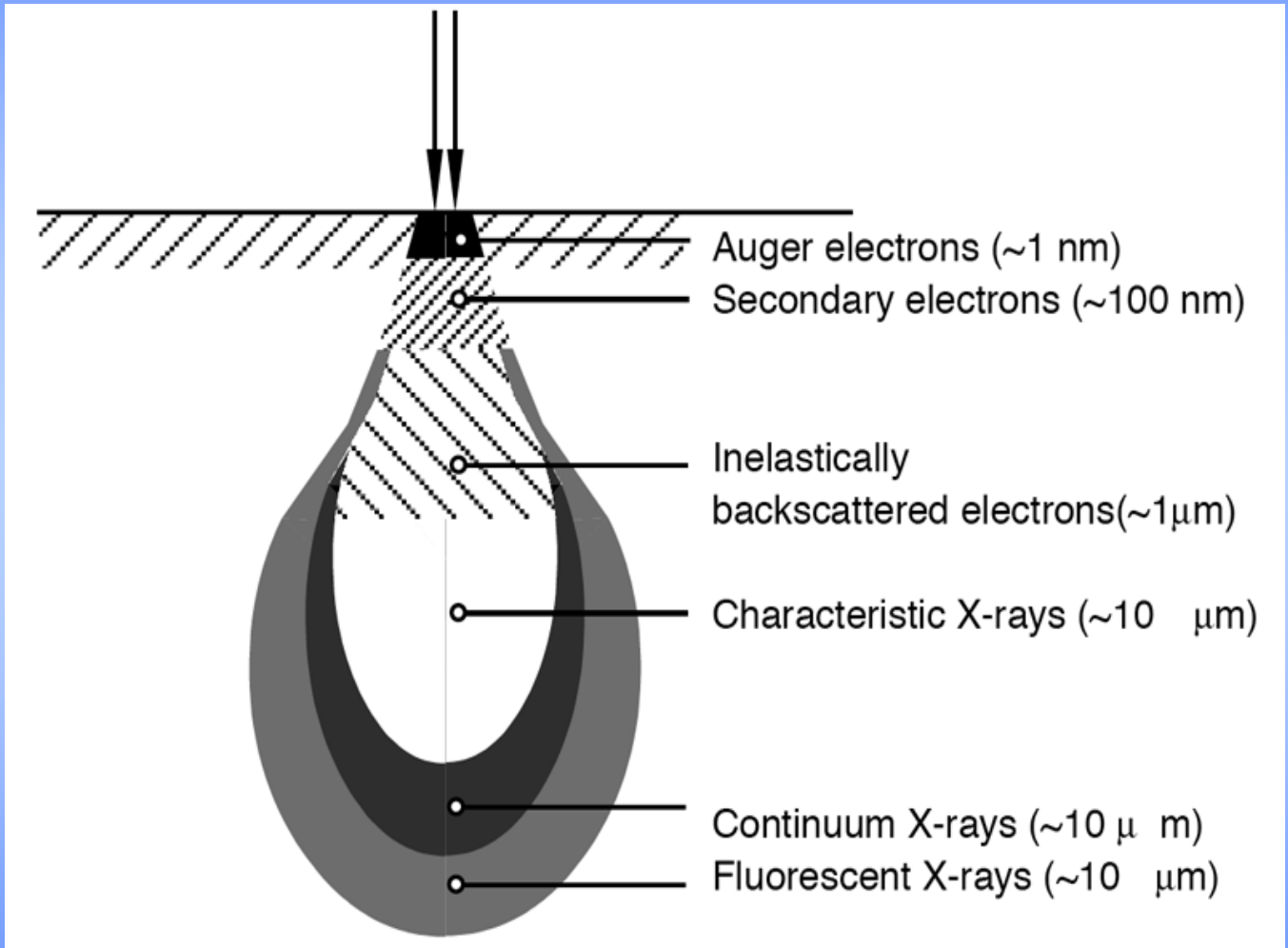


"courtesy of Wolfgang Werner
http://www.iap.tuwien.ac.at/~werner/qes_tut_interact.html "

The electron loses most of its energy at the end of its run.

The volume the electron explores is a kind of hanging drop with little energy lost at the region where it enters the detecting layer.

This shows the interaction volume of a beam of electrons with matter; the electron wanders as it penetrates matter.



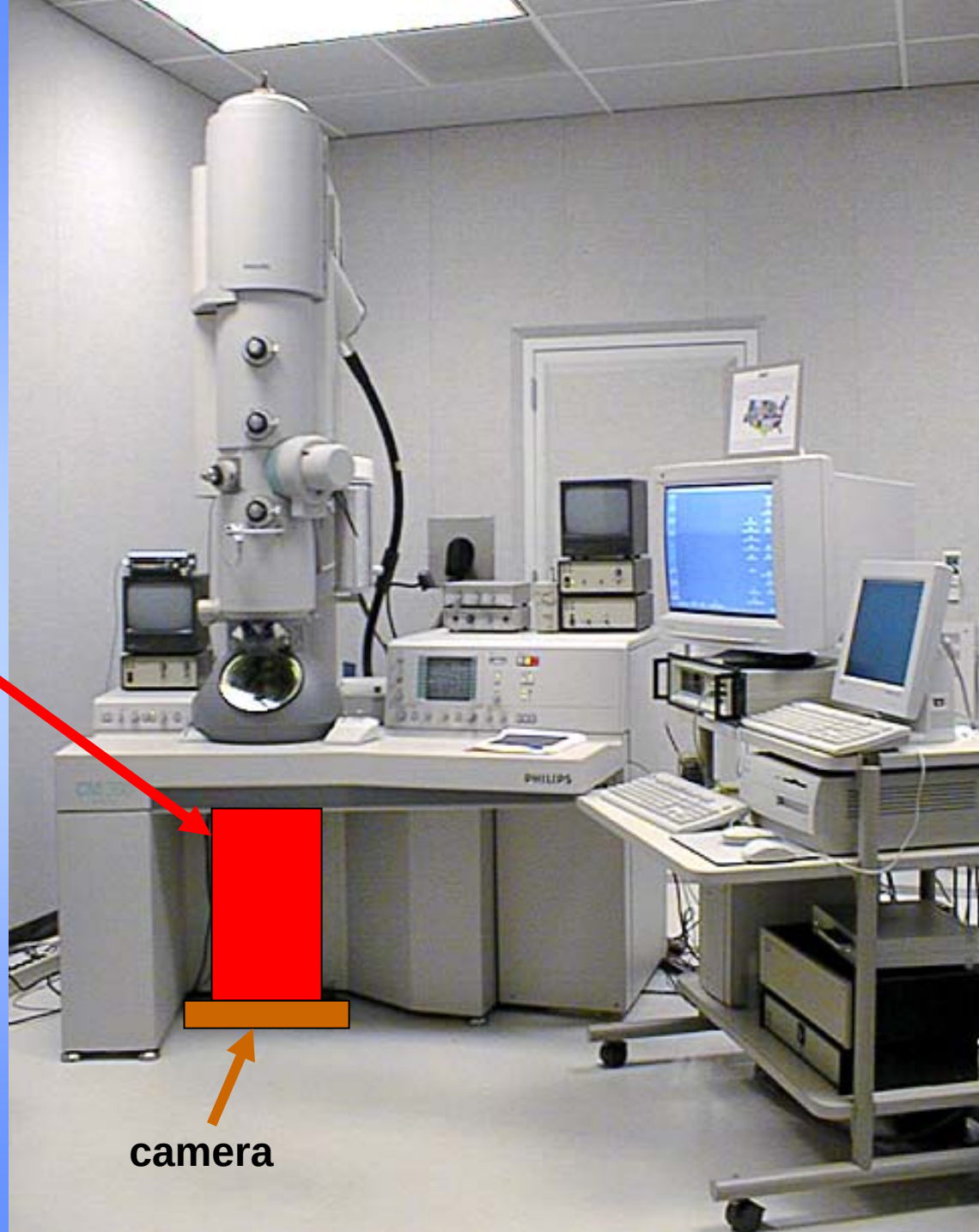
"courtesy of Wolfgang Werner
http://www.iap.tuwien.ac.at/~werner/qes_tut_interact.html "

Thus if one uses a thin detector,
the potential spread of the electron beam
in the detector material (e.g., fluorescent screen)
is minimized (MTF~1),
but because the electrons lose little of their energy
at the beginning of their track,
some pass through without being detected (DQE<1).

If one uses a thick detector,
the potential spread of the electron beam
in the detector material (e.g., fluorescent screen)
is maximal (MTF<1),
but because the electrons lose most of their energy
In the thicker material,
They are essentially all detected (DQE~1).

The problem is worse the higher the energy of the electrons.

Ken Downing has been constructing an electron decelerator.
The idea is to slow down the electrons, which will increase
both the DQE and MTF.



Energy filter

For biological macromolecules, the ratio of inelastic to elastic scattering is about 3.
That mean for every elastically scattered electron, which are the ones carrying the information, there are 3 inelastically scattered electrons generating a noisy background and damaging the specimen.

The inelastically scattered electron have lost energy and can removed by an energy filter.

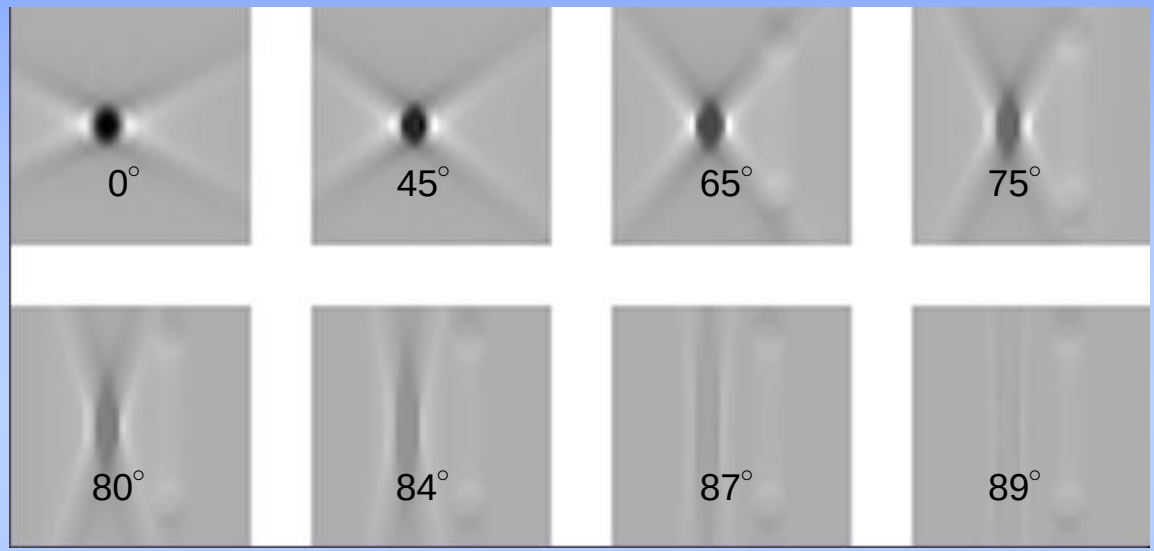
Such filters exist but we are not routinely used because we lack confidence that their use improves the SNR. More research needed.

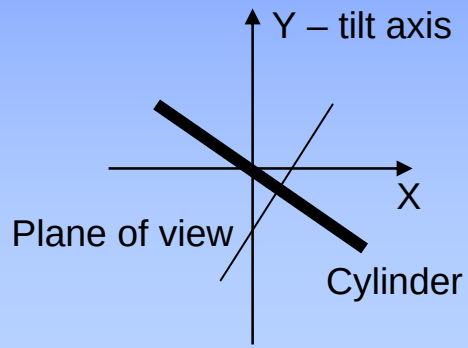
Drift & vibration free, double tilt cryo holder

In tomography, we get 3D data by tilting the specimen.
Because we cannot tilt extended specimens by +/- 90^{o}, the missing data causes a loss of resolution along the (axial) direction of the electron beam.

If we collect data by tilting first around the x-axis and then around the y-axis, the resolution is significantly improved in the axial direction.

Line Spread Functions for Single Axis Tomography

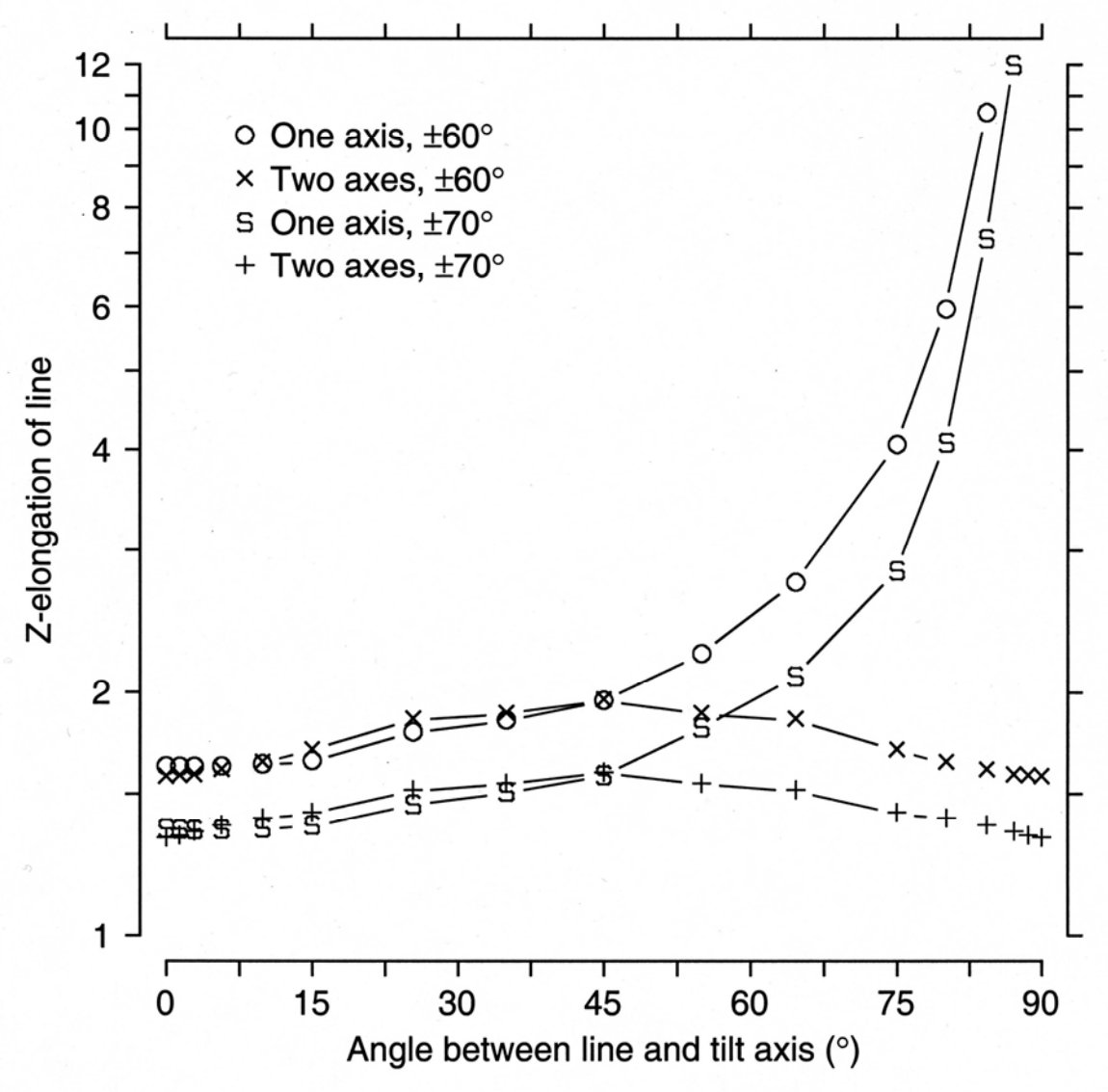




A model cylinder was projected and reconstructed by single axis tomography. These are cross-sectional views of the cylinder, for various angles between the cylinder and the tilt axis.

by David Mastronarde, Boulder Lab for 3D EM of Cells, CO

Elongation in Z of a Reconstructed Cylinder



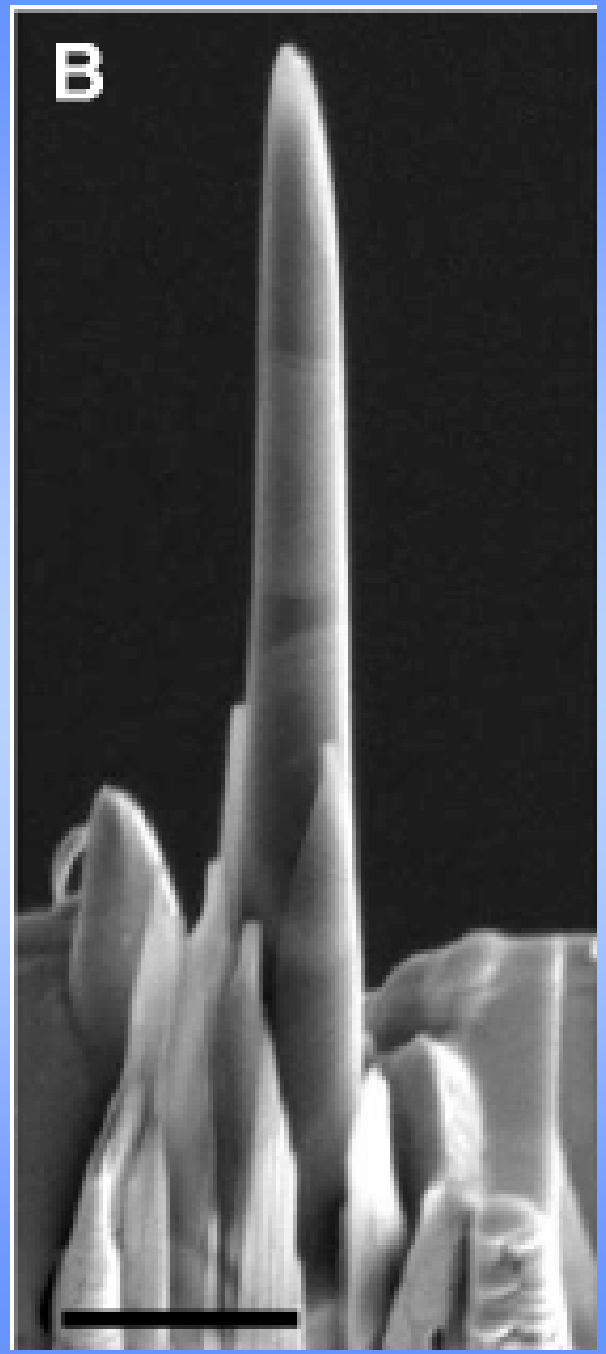
by David Mastronarde, Boulder Lab for 3D EM of Cells, CO

360^{o} cylindrical cryo holder

If we had a cylindrical specimen in a cylindrical cold stage, we could obtain tilts of +/- 90^{o} .
There would be no loss of resolution in the axial direction due to inability to collect a full 3-D data set.

Using an ion beam milling device, it is possible to generate a cylindrical specimen having a radial dimension of ~100 nm.

Is there someway to make and keep a frozen cylinder in a cryoEM?



Cylinder milled out a plastic embedded cells.
Heymann JA, Hayes M, Gestmann I, Giannuzzi LA, Lich B, & Subramaniam S.
J Struct Biol. 2006 Jul;155(1):63-73.
Bar = 2000 nm.

What we need to learn:

At what electron dose and dose rate do we get the best SNR?
(Chen and Grigorieff "slow dose results)

What is the best cryotemperature to use?

What image do we expect from a specimen when we include elastically, quasi-elastically, and inelastically scattered electrons over a fixed solid angle?

We want to know this because if we are trying to determine a model/map from a set of images, we should fit the model/map to the recorded, original images not to the images after background subtraction, filtering, etc.

Image analysis

Resolution seems to be improving with time.
In Grigorieff's group, it is in part a consequence of better image analysis: alignment, CTF determination and correction, and reconstruction

Determination of alignments parameters in the face of potential variations in particle conformation

Misalignment and averaging over conformational variations turn signal into ‘noise’.

What to do about variations in particle conformation?

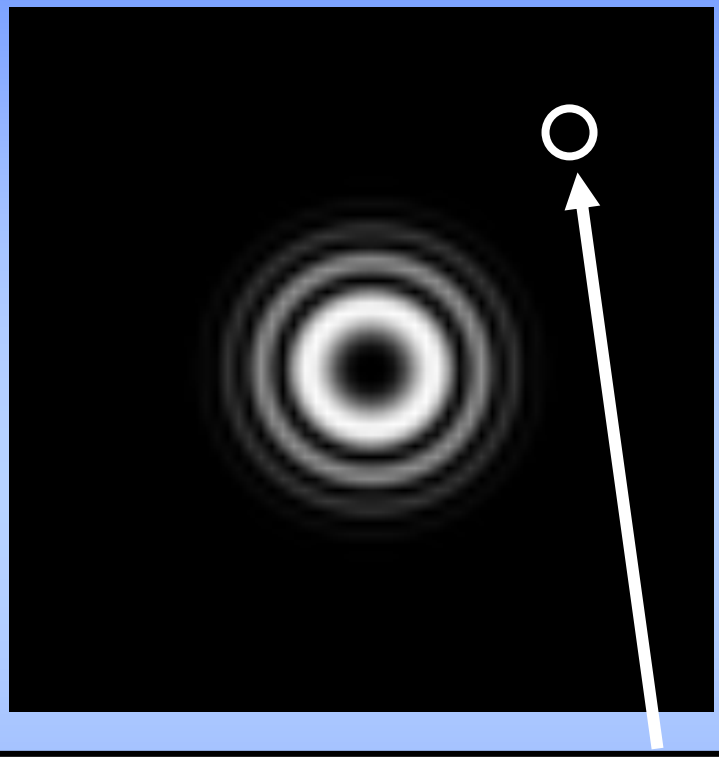
1. Classify images and sort looking for conformational variations

Are there bad images or only variations in particle conformation or both?

2. Predict conformations based on a preliminary model (e.g., normal mode analysis) and sort using them as reference images?

How small are the varying details we can detect given the inherent SNR?

Better CTF determination and correction if necessary
For depth of field (Ewald sphere correction).



If you do not get the positions of the higher resolution zeros correctly, you put in data with the wrong phase.

If you do not correct for the depth of field,
you turn signal into noise
(add in wrong amplitude and worse wrong phase).

Better reconstruction algorithms

Is maximum likelihood (Sigworth) really the best and is it practical?

In this method, all images are added in all orientations into the reconstruction but theyare weighted according to the probability that the image and the alignment are correct.

What should one do when the probability shows no peak for higher resolution data?

What algorithm should one use to generate the map?

There is evidence that careful selection of particles leads to higher resolution reconstructions but is this optimal?

What should one do: weight particles according to variance or throw out bad particles?

Best method for generating a map:
Back projection
SIRT
FREALIGN
Wavelets
Matrix inverse

Ideally you want to make a reconstruction that satisfies the images including inelastic, quasieleastic, and elastic scattering with account taken of the embedding matrix, but is it worth it?

You want to understand the noise so that you can optimize the map as rigorously as possible.

For tomograms, you want optimal strategies for sorting, classifying, aligning and averaging repeating substructures

By so doing you can increase the resolution of the substructure.

By so doing you can find changes in structure as Nicastro did for dyneins in different parts of the flagellum.

Once you have the best map possible you want to build an atomic structure if the resolution warrants it.

Is it possible to recognize a fold from a 3D map and use threading to fit the known sequence into the map?

With conformational adjustments to existing atomic models of the component parts, get an accurate model for a complex? How does resolution affect the accuracy?

We need to be able to build reliable interdomain interfaces.

We need experiments to test our models. Solid state NMR?

We need to automate everything.

END