

Lecture 9
Helen Saibil

Image acquisition, noise, particle selection, alignment

- Signal and noise
- Representation of digital images
- Detectors
- Particle selection
- Alignment



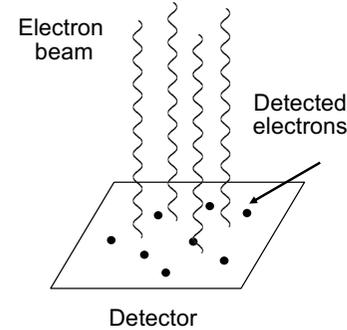
Image processing for cryo microscopy

2 - 12 September 2019

EMBO
Practical Course
Birkbeck College London

What is noise ?

In structural biology, radiation damage limits the useful electron dose from ice-embedded specimens to ~**10-30 electrons per Å²**. Stochastic or **counting noise** arises from variations in the number of electrons which arrive at a particular point, and can be described by a Poisson distribution. The signal to noise ratio improves by a factor of \sqrt{N} as the electron dose increases. However, the dose must be kept low to minimise **radiation damage** to the specimen. Electrons can transfer energy to the specimen, breaking bonds and causing mass loss in biological molecules. For analysis, we assume that the image is the **sum** of the structure information plus statistical noise.



electron dose = average (N) ± √N

N = 100 electrons per unit area

108	90	103
102	95	114
94	105	89

$\sqrt{N} / N = 10\%$

N = 1000 electrons per unit area

1025	1007	980
967	894	1016
1010	1046	964

$\sqrt{N} / N = 3.3\%$

Signal, noise and detection

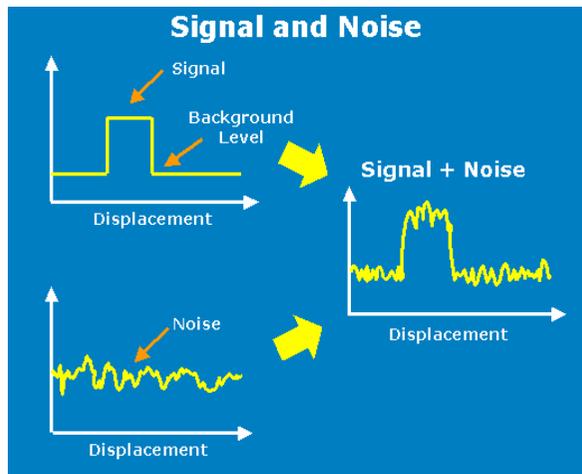
SNR: Signal-to-noise ratio

DQE: detective quantum efficiency

DQE gives a measure of how faithfully the signal is transmitted by the imaging system and is $= \frac{\text{SNR}_{\text{output}}^2}{\text{SNR}_{\text{input}}^2}$

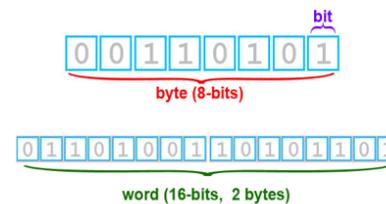
where output = digital image, input = electrons.

If DQE = 0.5, effectively half the electrons have been transmitted.



Digital images

Computer storage



Each picture element stored in the computer, with its own grey level, is called a **pixel**. A pixel can be 8 bits ($2^8 = 256$ grey levels), 10 bits, 12 bits or more, depending on detector and storage formats.

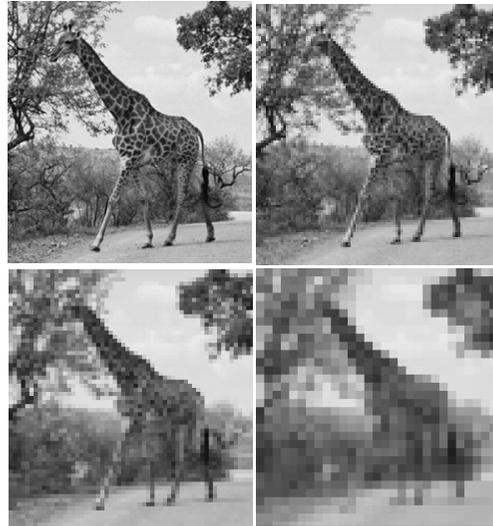
Pixel grey levels

Black to white is 0-255 for an 8-bit image.



Sampling

Pixel resolution is detector pixel size divided by magnification. e.g., for a detector pixel size of $6\ \mu\text{m}$ and 42kx magnification, pixel resolution in \AA is $60000/42000 = 1.43$.

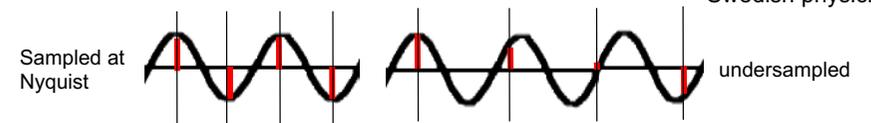


The Nyquist resolution limit in digital FTs

Images can be represented as the sum of a series of sinusoidal waves. The highest frequency wave term defines the resolution limit. In a digital image, the wave components are sampled at an interval defined by the pixel size. The highest frequency term that can be represented is **half** the sampling frequency. This is known as the **Nyquist frequency**: to be accurately represented, each sinusoidal repeat must be sampled at two points.

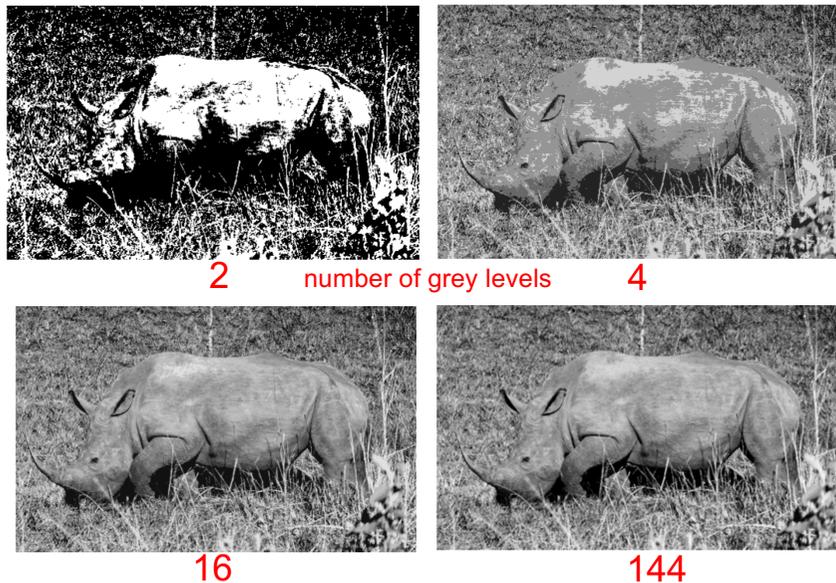


Harry Nyquist, Swedish physicist



For example, the reconstruction of a $3\ \text{\AA}$ map requires a sampling of $1.5\ \text{\AA}/\text{pixel}$ although in practice it is advisable to sample at $1/3$ the required resolution, $1\ \text{\AA}$, to avoid interpolation errors.

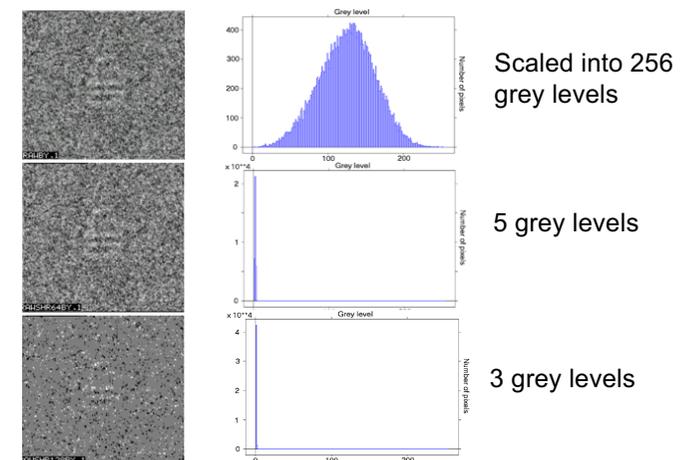
Grey scale resolution



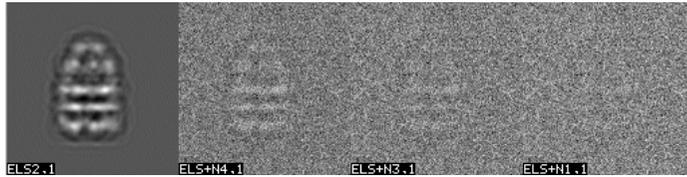
Grey scale resolution

Images are stored as 8 bits (256 grey levels) or more. But if data collection or conversion are done incorrectly this can produce a digital image represented by reduced number of grey levels, causing degradation of the image and loss of information.

Histogram of densities should be examined to ensure all the grey levels are represented.



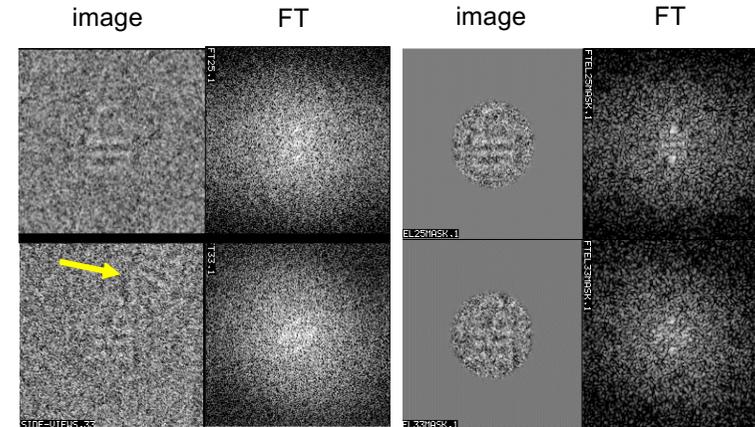
How much noise can we tolerate in single particle analysis?



Model image Signal:Noise Signal:Noise Signal:Noise
 2 μ m defocus 4:1 2:1 1:1

At SNR of 1:1, we can barely detect the particle. Alignment programs will not work well under such conditions as noise will correlate as well as signal.

Noise reduction by real space masking

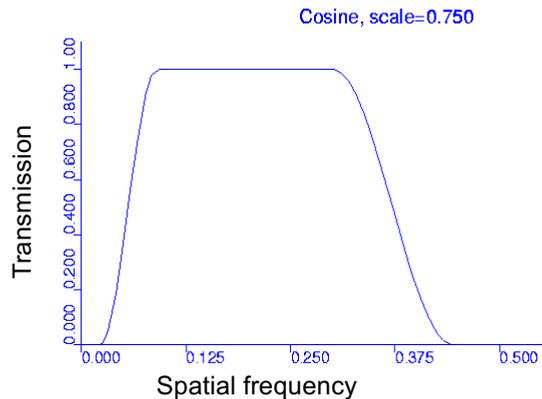


Information from neighbouring molecules contribute to the Fourier transform but can be removed by masking, leading to better alignment. The mask must not be too tight and its edges must be softened by a Gaussian or similar to prevent ripple effects which may introduce new alignment problems.

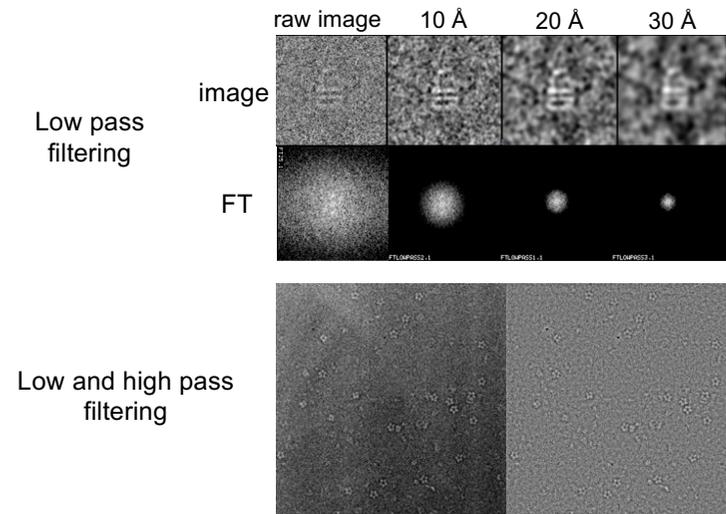
Noise reduction by Fourier bandpass filtration

This is used to remove low and high frequency noise such as uneven illumination (low) and detector noise (high). Selected frequencies of the Fourier transform of an image can be removed before back-transformation.

Fourier bandpass filters should have smooth edges (e.g. Gaussian, Cosine bell, Cauchy) to avoid introducing ripple artifacts.



Effects of low and high pass Fourier bandpass filtration



Calculation of filter radii

Processing programs need **filter radii** in transform pixels or fractional units :

$$R = S \times N / F$$

where **S** is sampling in Å/pixel

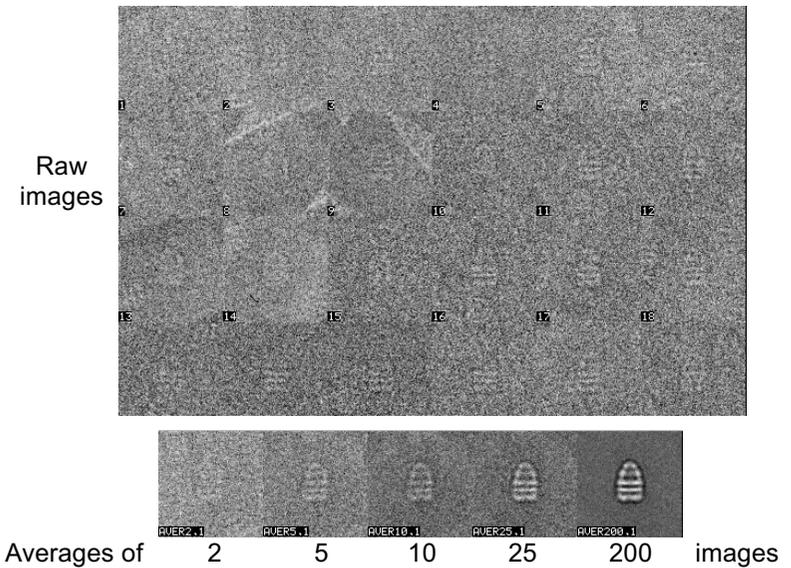
N is box size

F is required resolution in Å

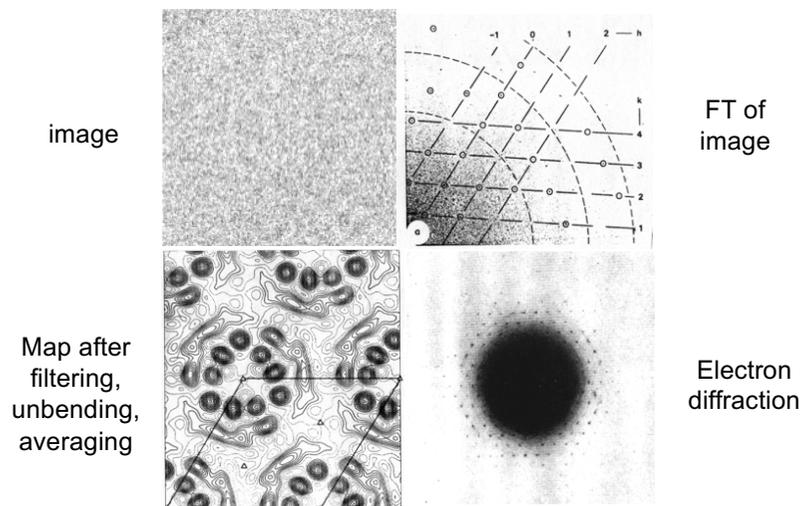
The maximum radius is half the box size, which corresponds to the Nyquist frequency, the highest spatial frequency that can be accurately represented by the digital transform. The fractional value for **R/N** or **S/F**, depending on program convention, uses the range 0 – 0.5 (e.g. in EMAN) or 0 – 1.

The **high pass filter** reduces background gradients: its radius must exceed the spacing corresponding to particle diameter, ie have a *smaller* value in *reciprocal* space than the reciprocal of particle diameter. The resolution of the analysis will be restricted by the **low pass filter**. For example, with a pixel size of 1 Å and maximum particle diameter 150 Å, you might choose a high pass filter radius corresponding to a spacing of 180 Å and low pass to 4 Å (fractional units $1/180 = .0056$ and $1/4.0 = 0.25$ respectively, using the convention that $r_{\max} = 1$).

Noise reduction by averaging

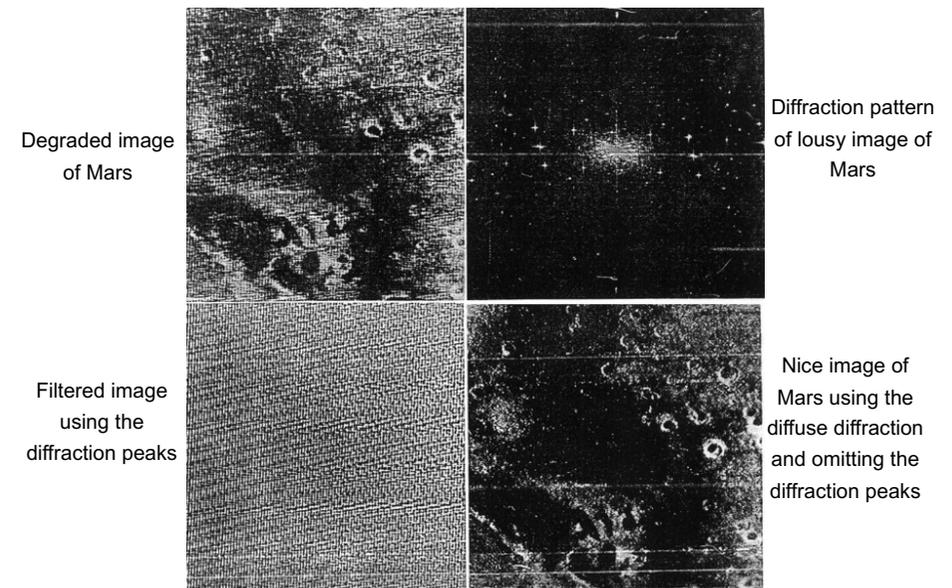


Noise reduction by filtering out everything except diffraction peaks - Bacteriorhodopsin

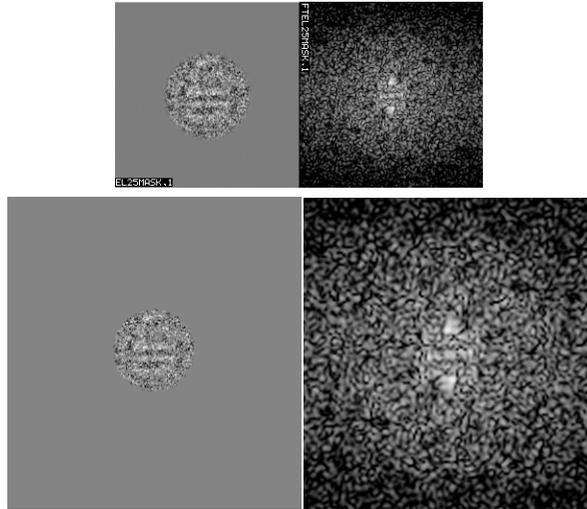


Henderson & Unwin, 1974

One person's signal is another person's noise



Padding images increases the sampling of the transform



Adding a frame of background around the image samples the transform more finely and reduces rounding errors during interpolation. This is important for cross correlation calculations used in alignment.

What is normalization and why is it important?

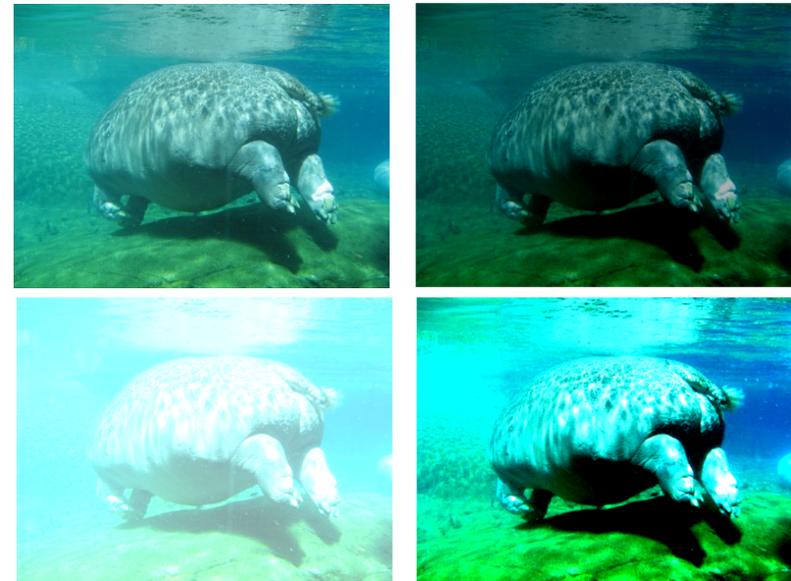
For an individual image, the range of pixel values may not matter because display programs scale the image values to the screen, but what if you are doing a series of cross correlations to align images to a set of references? If some references have very big numerical values, they will give higher cross correlation peaks and will falsely appear to provide the best match.

Moreover, the value of the FT at the origin is equal to the mean value of the image, so a high mean value will give a huge central peak on the FT which is unrelated to the structural information.

You **MUST normalize** all images and references used for cross-correlation.

This means rescaling their pixel values to a **mean value of zero** and to the same **standard deviation**.

What is normalization and why is it important?

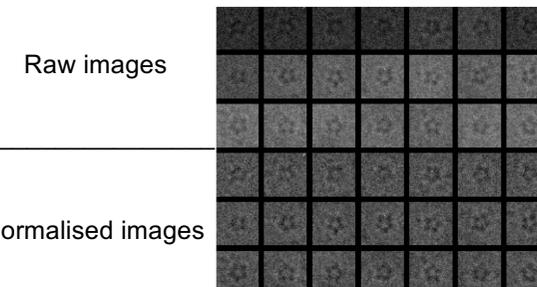


Normalisation

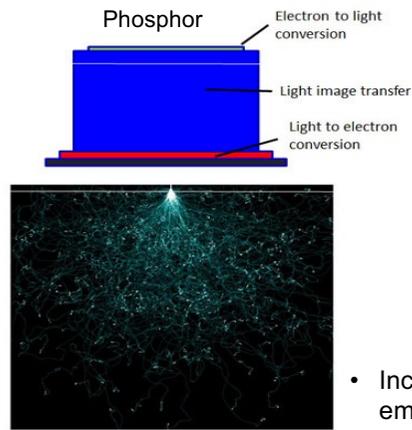
$$X_{\text{norm}} = (X - m)/s$$

X, pixel density; m, mean; s, standard deviation

Normalisation scales the data so the variation in each particle is equivalent.

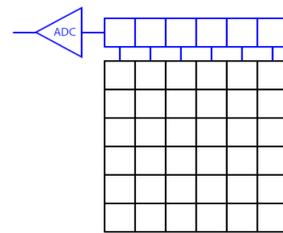


Revolution in electron detectors: the old



Sensor pixel size 15-25 μm

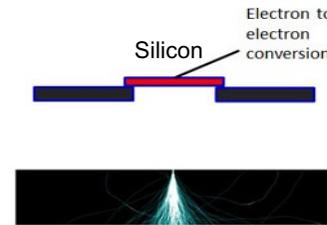
DE website



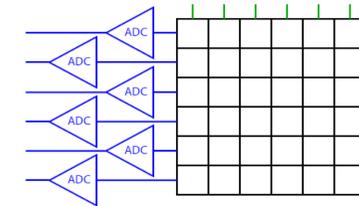
CCD/CMOS

- Incident electrons excite phosphor screen to emit photons, which are converted to charge by a semiconductor device
- Charge readout faster with CMOS (complementary metal-oxide semiconductor) than CCD (charge-coupled device)

Direct Electron Detectors



Sensor pixel size 5-15 μm



CMOS detectors

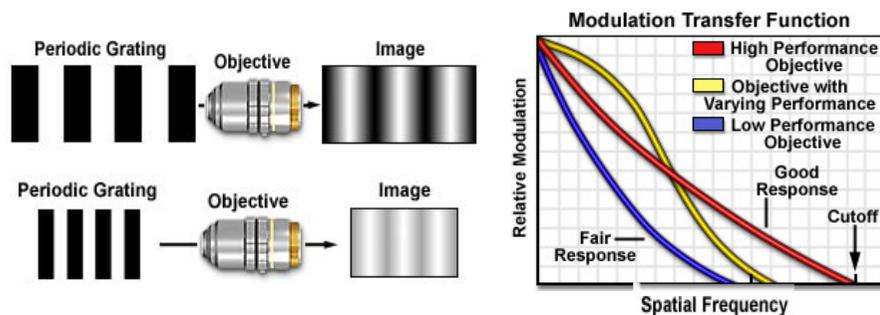
- Incident electrons excite semiconductor electrons, generating a charge
- Charge read and converted to voltage
- Technical issues solved: radiation hardness, fast readout, back thinning

Optical performance as a function of resolution

MTF: modulation transfer function gauges resolution and performance. It gives a measure of the contrast transmitted from the object to the image at a particular resolution.

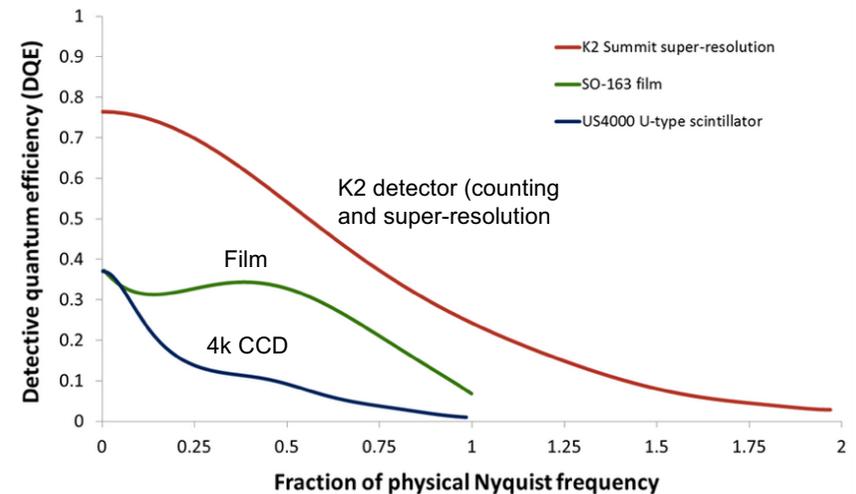
MTF = Image modulation/Object modulation

The equivalent in cryo EM is the **Contrast Transfer Function**



The objective modifies the image by a **point spread function**

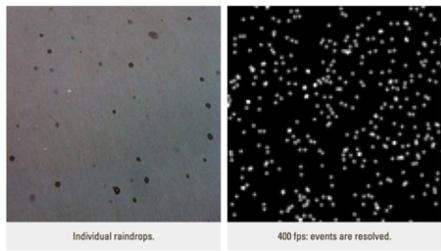
$$DQE = SNR_{\text{output}}^2 / SNR_{\text{input}}^2$$



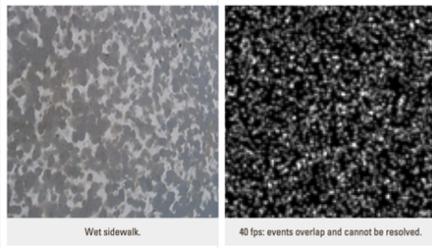
Gatan web site

Electron Counting

Sparse arrivals



Individual counts not resolved at high dose rate



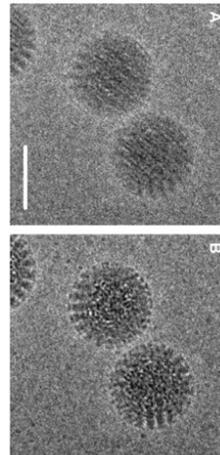
Super resolution



Gatan web site

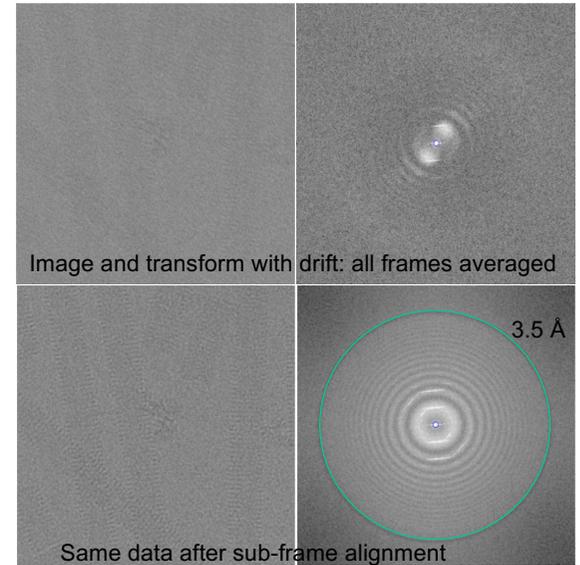
Fast frame rate of direct detectors enables motion correction

Motion correction on rotavirus



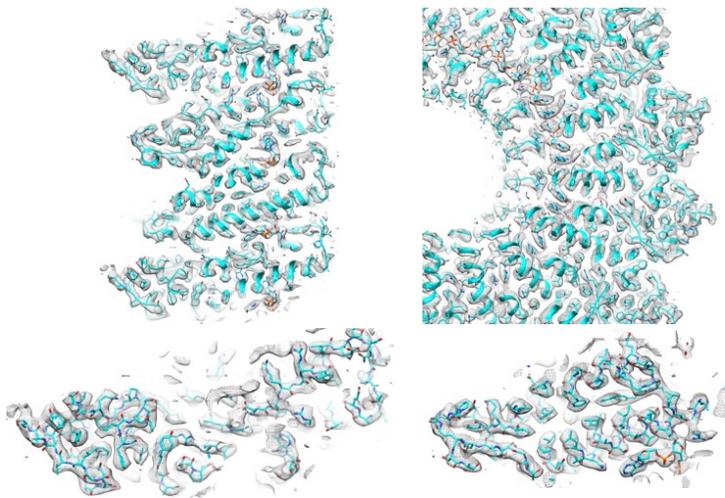
Brilot et al JSB (2012)

Direct electron detector: TMV on carbon film



Dan Clare

TMV reconstruction with fitted coordinates

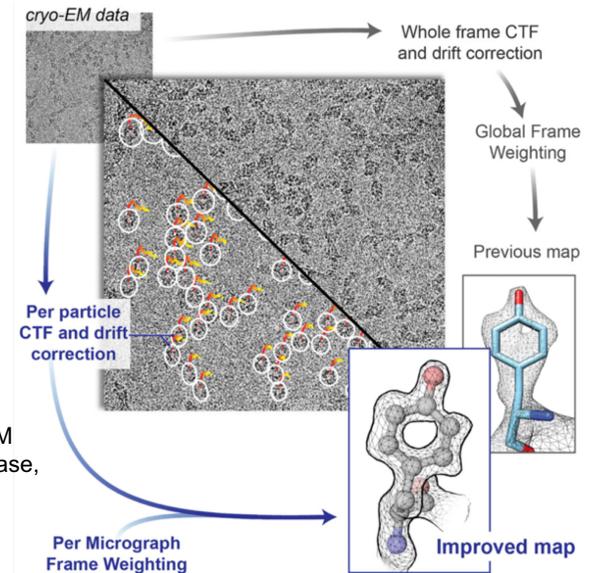


90% of TMV side chains defined; 3.3 Å resolution

Gatan K2

Dan Clare

The resolution revolution in cryo EM

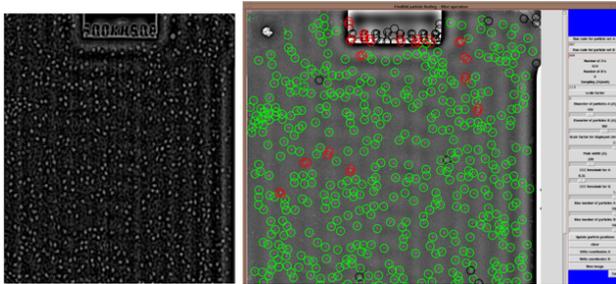


Atomic resolution cryo-EM structure of β -Galactosidase, Bartesaghi et al (2018) Structure 26, 848-856.

Finding the particles: Correlation based template matching

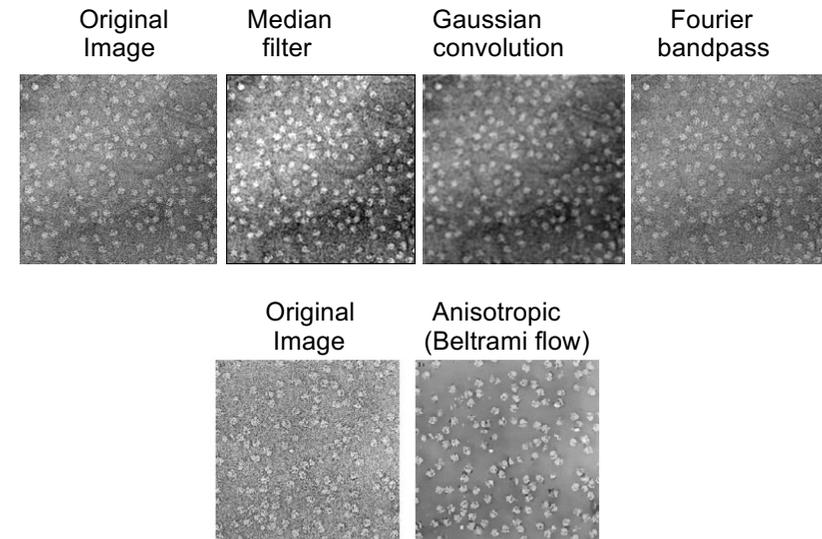
Reduce noise by Gaussian convolution, anisotropic diffusion or Fourier filtering. Calculate correlation map between micrograph image and templates in various orientations. Find peaks, select particles from the unfiltered micrograph according to adjustable thresholds.

- Depends on quality of template(s)
- Sensitive to noise



Huang & Penczek(2004), Rath & Frank(2004), Roseman(2003), Sigworth(2004), Wong et al., Ludtke(1999)

Noise reduction techniques for particle picking

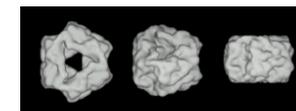


Software packages for automatic particle detection

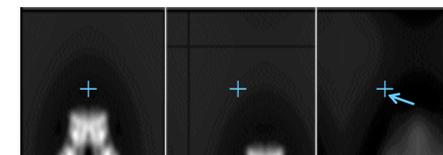
- Signature (Chen) Correlation/template matching
- EMAN boxer (Ludtke) Correlation/template matching
- FindEM (Roseman) Correlation based, template matching
- DoG picker/Tiltpicker (Carragher) Difference of Gaussians/thresholding
- Relion (Scheres) Correlation/template matching
- Gautomatch (Zhang) Correlation/template matching
- CrYOLO (Sphire)
- Topaz (Noble)
- Particle picking in Warp (Tegunov, Cramer)

Alignment and cross-correlation

1. Translational alignment to a reference



Small hsp
with 32
symmetry



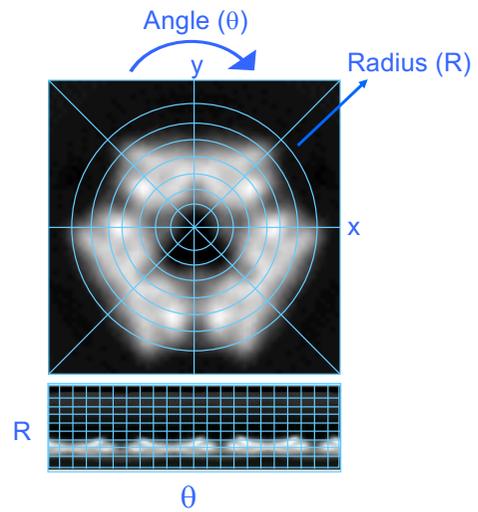
Shift of image 2
to match image 1

Image 1

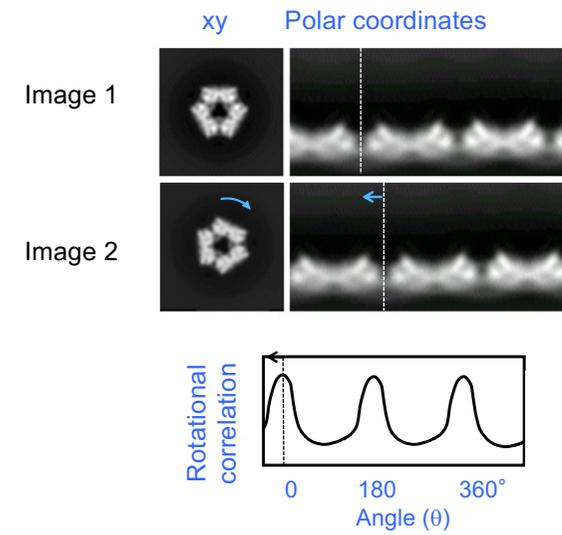
Image 2

Cross-
correlation

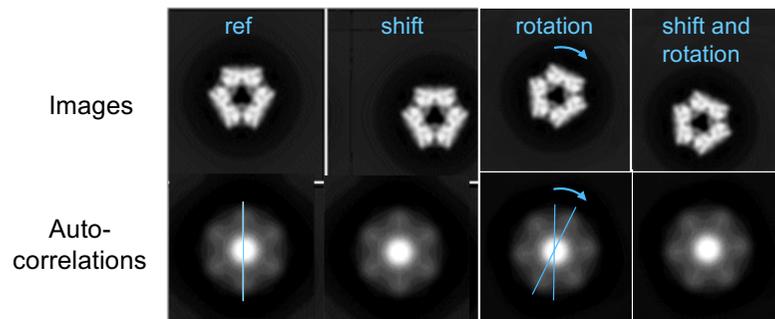
Polar coordinates



Rotational alignment to a reference

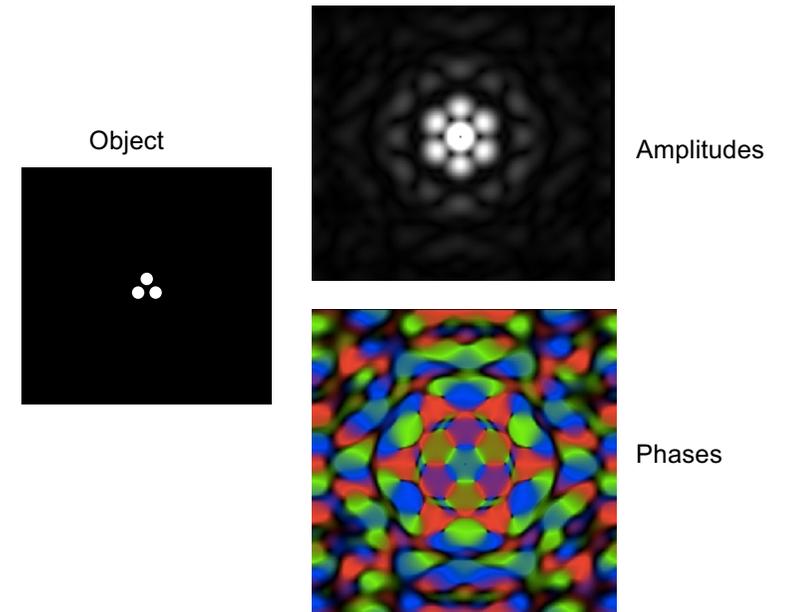


Autocorrelation



The autocorrelation function is the correlation of an image with itself. Since the 2 images being correlated are at the same position, the central peak is always at the origin and the autocorrelation does not vary when the object is shifted. Other features of the autocorrelation reflect features in the structure and rotate with the image. Note the additional 2-fold symmetry in the autocorrelation.

Phases reveal the true symmetry



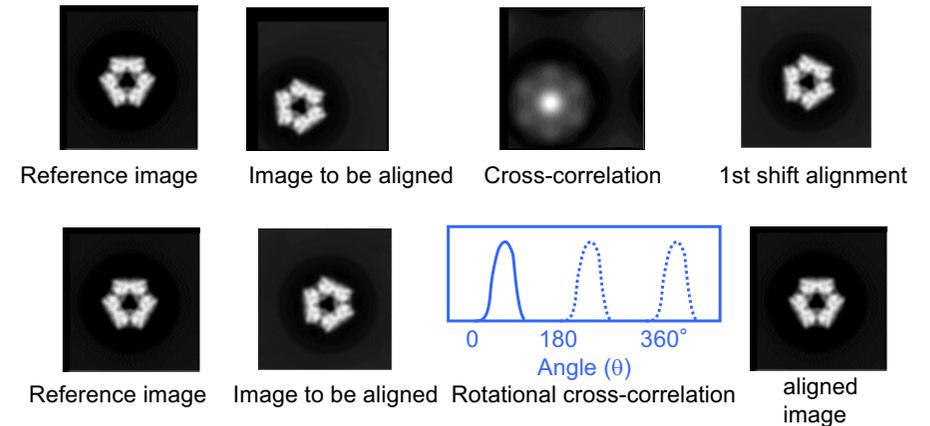
Autocorrelation of atoms

The **Patterson function** in crystallography is the autocorrelation function of an atomic structure. Each pair of atoms (similar density features) gives rise to a peak in the Patterson function, which is a collection of inter-atomic vectors. It is obtained by calculating the FT of the diffraction pattern. It does not contain any phase information, which is another way of saying that it does not change when the object is shifted.

For small molecules, the atomic positions can be deduced from the peaks in the Patterson function.

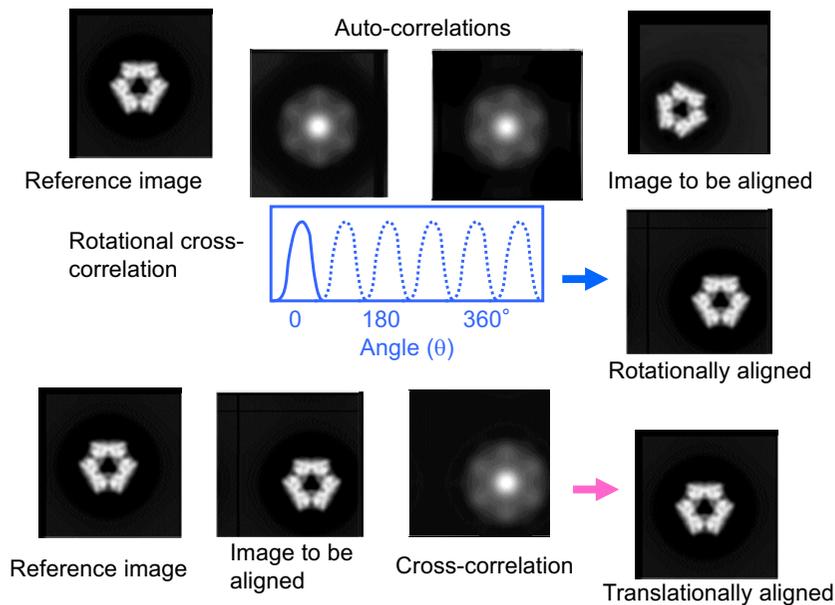
Alignment

Algorithm for alignment of individual images with respect to one reference image by an iterative sequence of translational and rotational alignments using cross-correlation functions. 3-5 iterations are sufficient to achieve good alignment.

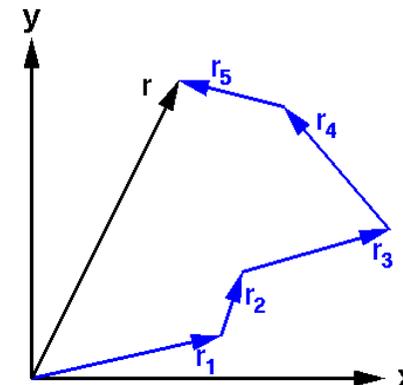


Steinkilberg, M. & Schramm, H.J. (1980) Eine verbesserte Drehkorrelationsmethode für die Strukturbestimmung biologischer Macromoleküle durch Mittelung elektronenmikroskopischer Bilder. Hopper-Seylers. Z. Physiol.Chem. 361, 1363-1369

2-step alignment using autocorrelations



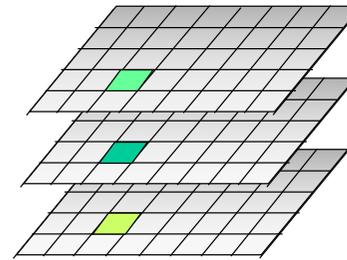
Vector addition of successive alignment parameters to avoid degradation by repeated interpolation of images



Alignment: How to assess it

- Clarity of averaged image
- Resemblance to raw data
- Improvement of correlation coefficients with successive iterations
- Low **variance** inside molecule projection
- Similar features after classification

Variance



For a stack of aligned images, the variance can be calculated for each pixel, to give a map of variations between the images in the data set. This can help to assess the reliability of features seen on the average image, and can reveal if images of different structures are mixed up in the same data set.

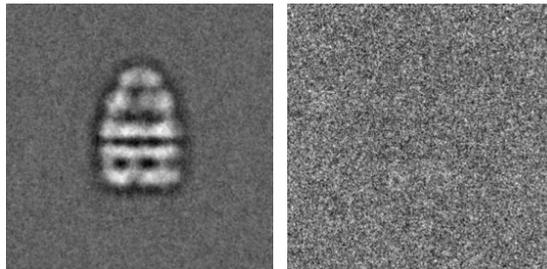
The variance is determined for each pixel as the difference between the pixel value in a given image and the average value of that pixel in all the images. This difference is squared and the sum of these squares is calculated for all the images in the stack.

$$\text{Variance} = [1/(N-1)] \sum_{i,j} [P_i(r_j) - P_{av}(r_j)]^2$$

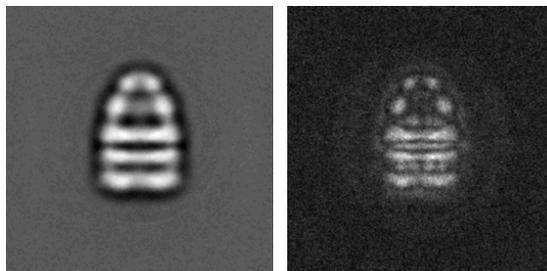
where $P_i(r_j)$ is the value of pixel j in image i and $P_{av}(r_j)$ is the average value of pixel j in all the images, for a set of N images.

Average and variance of homogeneous and mixed data sets

Set of views with same orientation



Mixture of side views - different orientations around 7-fold (vertical) axis



average

variance

Approaches in alignment and classification

- **Projection matching by cross correlation:** the conventional method is to simply assign the match according to the highest score, which is often not correct, due to noise or model limitations. **Maximum likelihood/Bayesian** methods, as in Relion and Frealign, include noise models and assign a distribution of matches with different weights.
- Projection matching can be done with class averages or single images, in real or Fourier space
- **Classification** to sort out heterogeneity: e.g. supervised classification (such as ribosome \pm factors), separation by multivariate statistical analysis in 2D or 3D, 3D variance calculation by bootstrapping, multiparticle refinement in 3D
- **Focused alignment and classification** on variable subregions

Model bias – the starting model must be appropriately filtered to remove features that are not known to be represented in the data:

Einstein from noise – alignment of random noise to an image



References

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