CNRS
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1) Short summary of general concepts

2) Single particle image processing and 3D reconstruction

→ **getting prepared for the practicals**

Bruno Klaholz 2016, ReNaFoBis, Oléron School
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Electron microscopy: application examples - Summary

Negative staining	**2D observation** + 3D reconstruction
Spreading	**2D observation only**
Shadowing	**2D observation only**
Cryo-EM	(2D observation +) **3D reconstruction**
2D crystals	(2D observation +) **3D reconstruction**
Tomography of cellular structures	**(2D observation +) 3D reconstruction**
Freeze-fracture	**2D observation only**

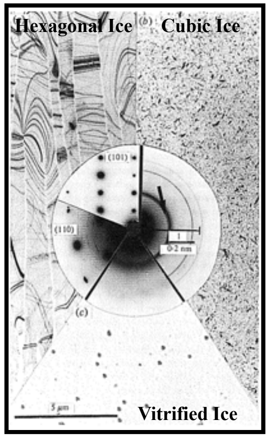
The importance of cryo-approaches

Vitreous ice:

forms by flash-cooling, is metastable and converts to crystalline ice modifications:

- cubic ice, forms when vitreous ice is warmed up above -135°C → keep samples below ~-135°C

- hexagonal ice, forms when water is (relatively slowly) cooled down at atmospheric pressure (is typical source of contamination in cryo-EM)

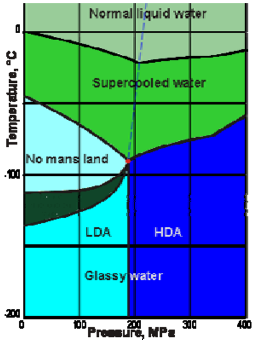


Dubochet *et al.*, 1988

cooling rate required to obtain vitreous ice: ~10^4 K / s

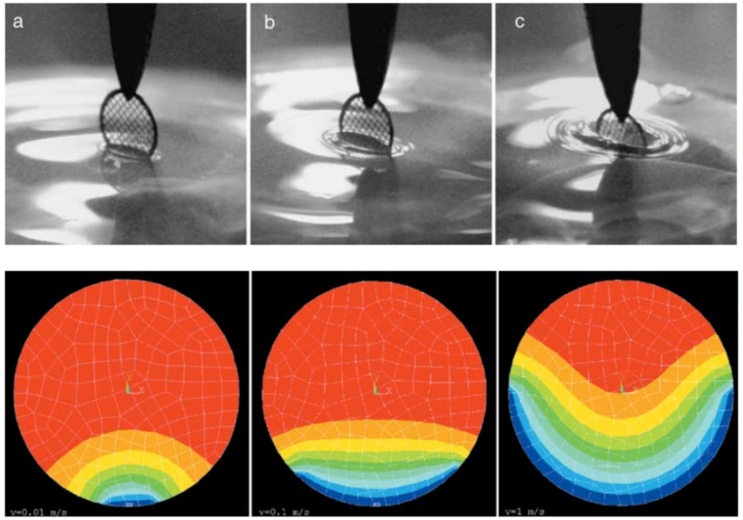
Boiling and melting points of liquid ethane: -88.7 °C / -183.3 °C, temperature of liquid nitrogen: -196 °C

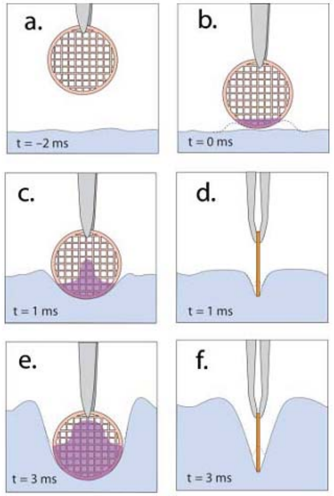
phase diagram of water



http://www1.lsbu.ac.uk/water/amorph.html

The importance of cryo-approaches in cryo-EM





Kasaset al. & Adrian, Journal of Microscopy, 2003

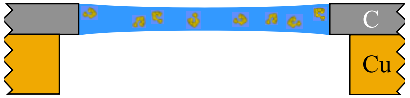
Benefits?

Flash-freezing:
→ vitrified water (amorphous ice)
→ specimen conservation (frozen-hydrated)
→ very weak ice sublimation in the vacuum of the microscope
→ fixation of particle orientations

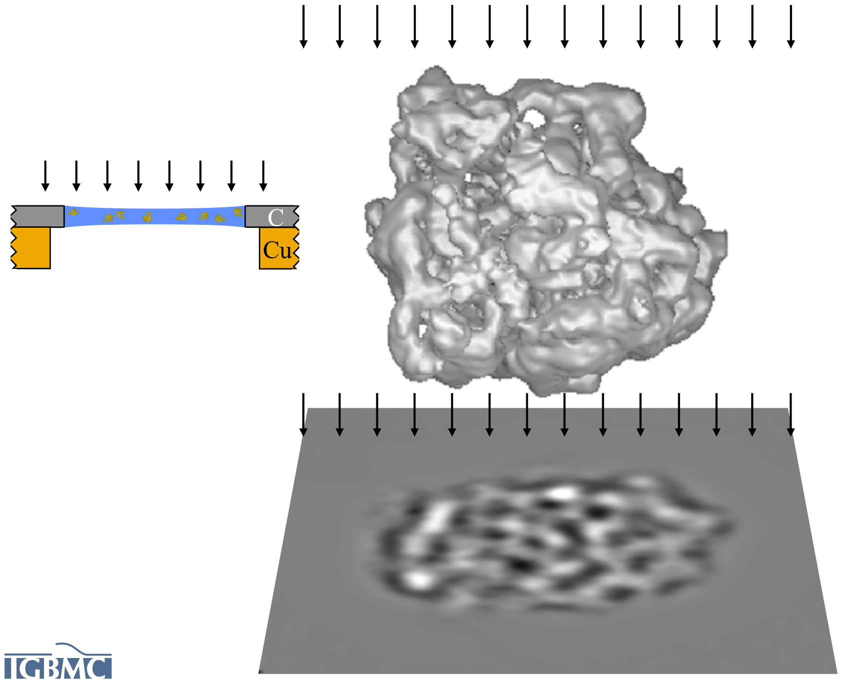
Sample preparation

A new research project – steps to cross for single particle cryo-EM:

- functional studies
- purification and biophysical characterization of complexes (very important)
- optimization of the sample for imaging:
    - fast freezing (vitreous ice)
    - buffer composition
    - support type (holey carbon vs continuous carbon film)
    - concentration (~0.5 mg/ml)
    - ice thickness
    - absence of contamination



Transmission electron microscopy



electrons

object (3D)

scattered electrons

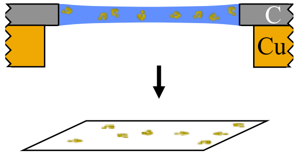
projection (2D)

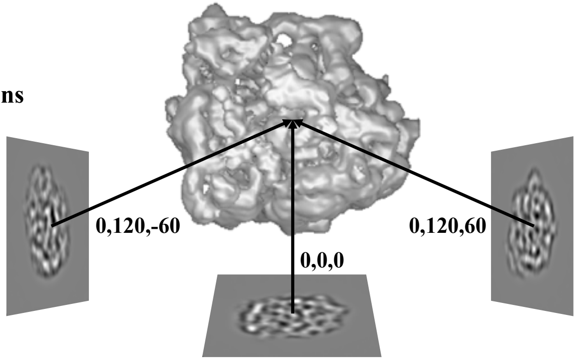
Structure determination by 3D reconstruction

Assumptions?

3D reconstruction of single particles:

unique particle type in random orientations





Reconstruction provides a 3D density map, i.e. a structure with all internal features

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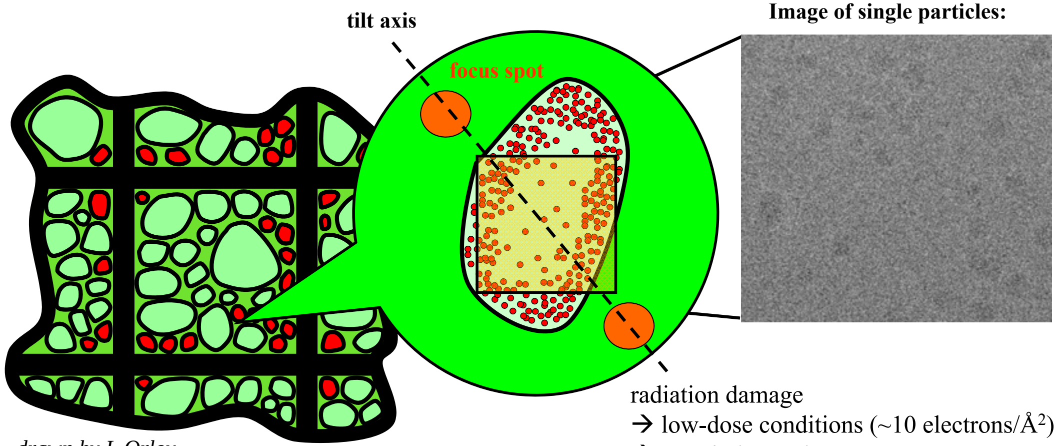
Sample irradiation

Concept of "low-dose": reduce sample irradiation



drawn by I. Orlov

radiation damage

→ low-dose conditions (~10 electrons/Å^2)

→**low I/σ in the images**

→**use averaging techniques to recover the signal**

Requirements for high-resolution data collection and structure determination:

- optimization of the sample for imaging (ice quality)
- optimal imaging conditions (beam alignment, parallel light)
- specimen stability (no drift)
- environment: low magnetic fields, weak vibrations, weak acustic noise, stable temperature
- image data collection & quality assessment
- pre-processing (particle selection, CTF-correction)
- large data sets
- high-resolution image processing procedures for structure determination / refinement
- strong & dedicated computing resources

Single particle image processing and 3D reconstruction.

Single particle image processing and 3D reconstruction.

I. Pre-processing

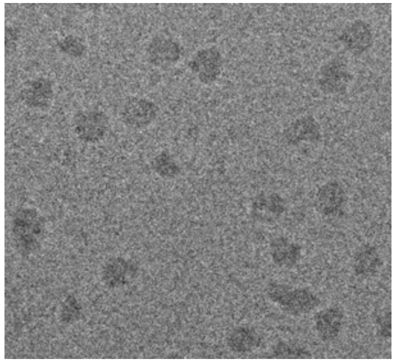
- Digitization of micrographs (negatives); not needed if CCD images
- particle selection, « boxing »
- correction of the contrast transfer function
- band-pass filtering and normalisation of particle images

II. Structure determination

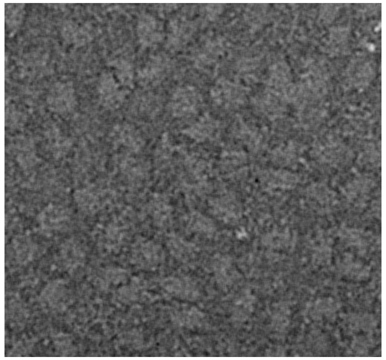
- particle centering / alignments
- MSA (multivariate statistical analysis) + classification
- angle assignment
    - angular reconstitution
    - projection matching
- 3D reconstruction (first map)
- structure refinement
- resolution assessment
- map interpretation; fitting of known structures, <u>atomic model</u> building…

I. Pre-processing

- Digitization of micrographs (negatives); not needed if CCD/CMOS images



CCD image (positive contrast)

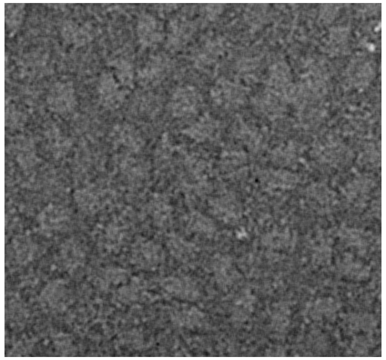


micrograph (negative contrast)

I. Pre-processing

- Digitization of micrographs (negatives)

high-resolution scanner (5000 dpi)



Sampling = Pixel size / Magnification

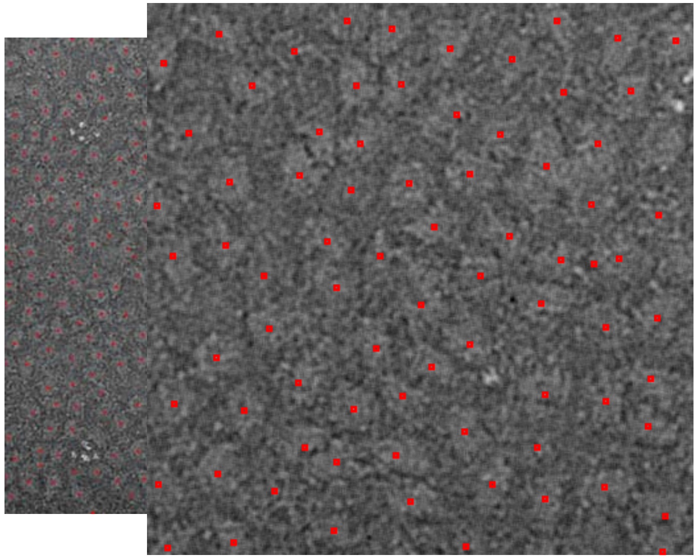
5 µm / 50 000 = 1 Å / pixel at specimen level

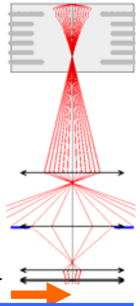
I. Pre-processing

- particle selection, « boxing »

What is important when selecting particles?

<u>Proper centering!</u>





I. Pre-processing

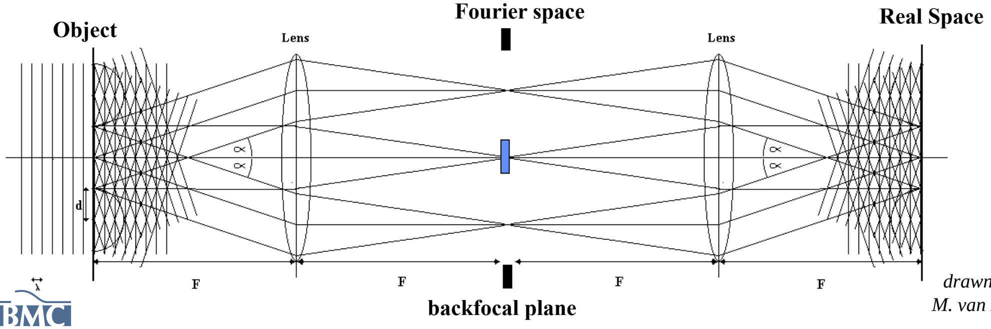
- correction of the contrast transfer function

Background: **Phase Contrast Microscope**

- Small phase difference difficult to observe
- Limited to study of thin specimens (<1000Å)
- **Phase contrast is converted to amplitude contrast by defocusing the specimen**
- Same technique used in light microscopy to study unstained specimens
- Staining provides strong amplitude contrast but may affect the macromolecular structure

Scherzer focus

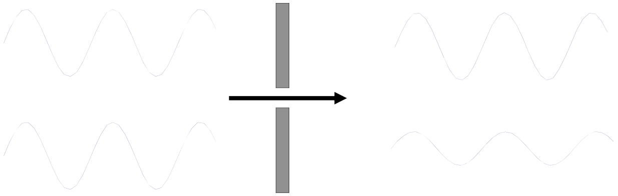
under-focus (e.g. -2 µm)



drawn by M. van Heel

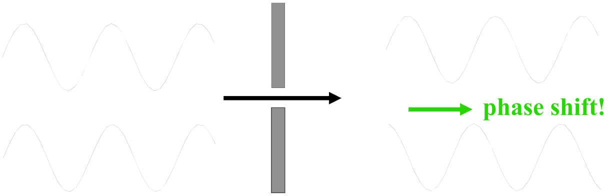
Specimen contrast

Amplitude contrast (inelastic scattering, absorption)



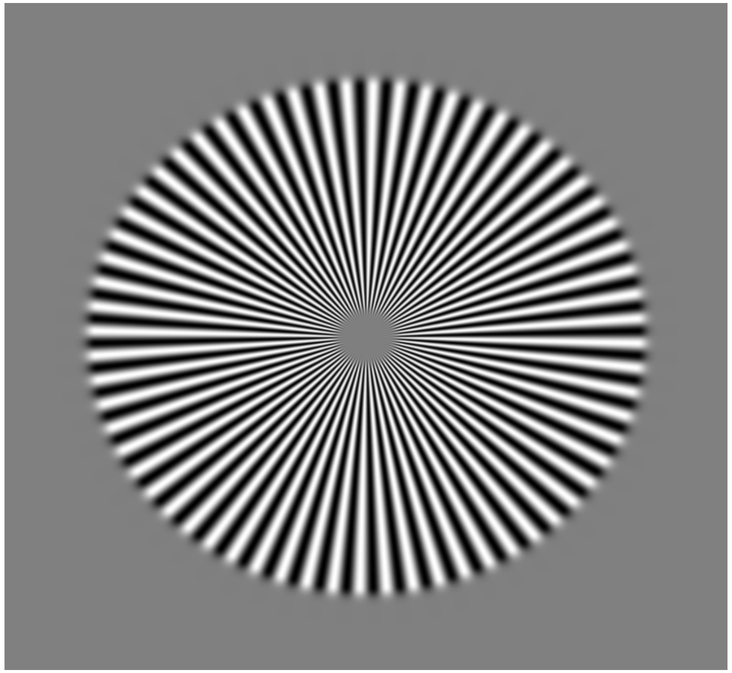
amplitude change! (absorption by sample)

Phase contrast (elastic scattering)



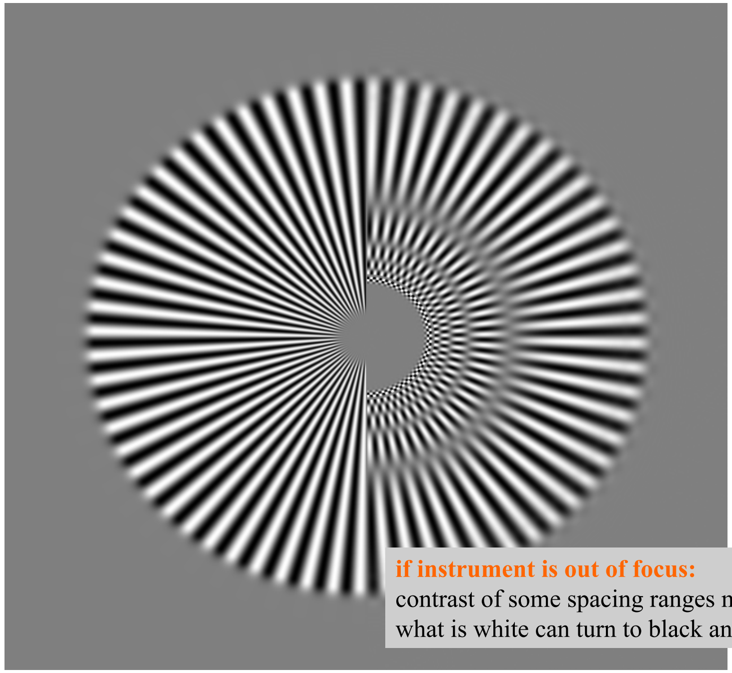
phase shift! (delay through sample)

Siemens stars: a whole range of spacings / frequencies / resolution in a single image



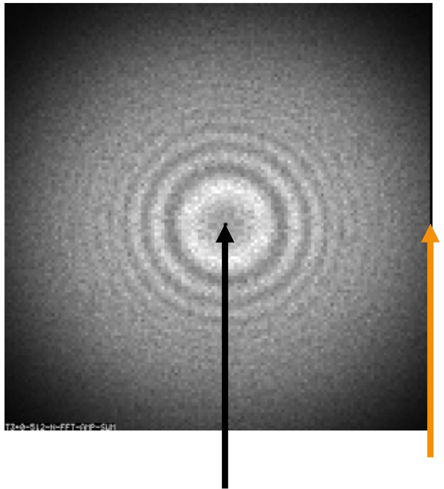
drawn by
M. van Heel

Calculated effect of an electron microscopical PhCTF on the image of a Siemens star



if instrument is out of focus:
contrast of some spacing ranges may be reversed, what is white can turn to black and vice versa.

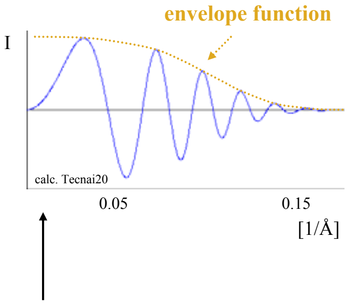
Powerspectrum = Fourier Transformation of the image



low resolution
low frequency

high resolution
high frequency

Profile of the intensity distribution



very low resolution difficult to measure

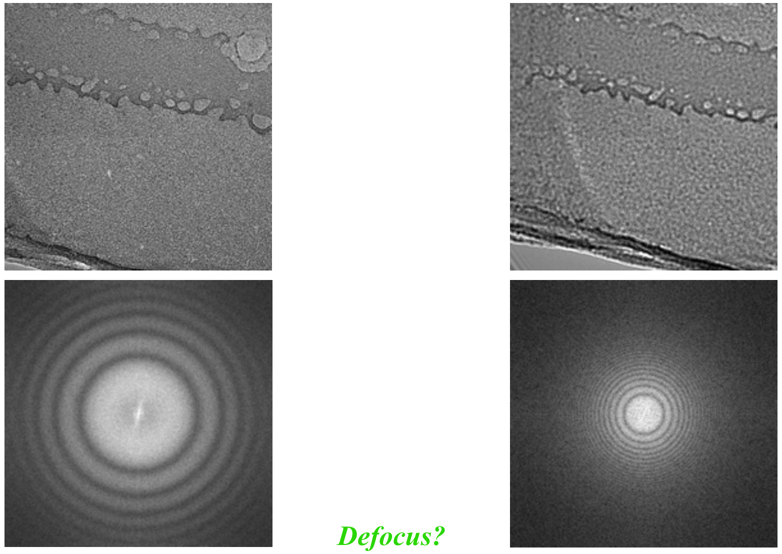
→ averaging techniques

Defocusing: effect on contrast, power-spectrum and max. resolution with FEG

defocus -1 µm **defocus -10 µm**

image

power-spectrum



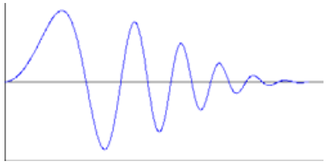
Defocus?

Focal point is <u>after</u> the sample (<u>under</u>-focus)

Images: continuous carbon area from holey carbon grid, cryo, Tecnai20, CCD 2Kx2K, 1024 pixel mode

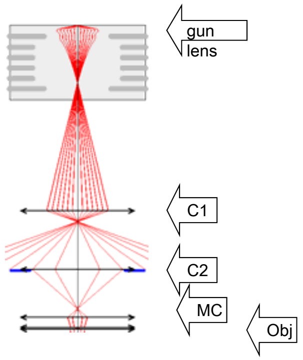
I. Pre-processing

- correction of the contrast transfer function



Weak-phase biological specimen → weak-contrast image

CTF function: $$T(k) = -\sin(\pi/2\, C_s \lambda^3 k^4 + \pi\, \Delta f\, \lambda\, k^2)$$



CTF depends on parameters of imaging system:

- ***λ***, wavelength defined by accelerating voltage (e.g. high tension 200 kV --> λ = 0.025 Å)
- ***Cs***, spherical aberration coefficient, determines the quality of objective lens
- other, constant parameters from electron microscope

constant

- ***Δf***, the **defocus value**, (offset from focus, "Scherzer" focus, underfocus: **focal point is below the sample**)

variable parameter

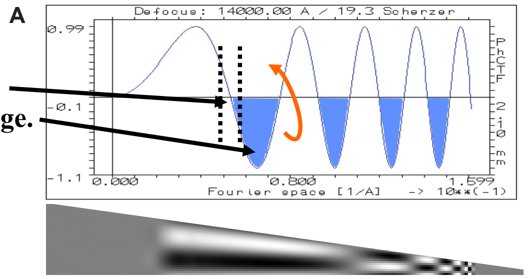
- ***k***, spatial frequency

I. Pre-processing

- correction of the **contrast transfer function (CTF)**

No information transfer at the zero crossing;
CTF leads to contrast inversion within the image.

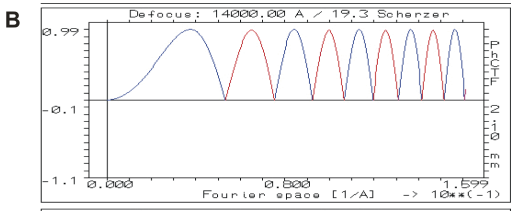
A



Radial segment of Siemens star

CTF-correction? By "phase flipping":

B



Therefore: data collection over range of defocus values, e.g. under-focus -1.0 – 3.0 µm

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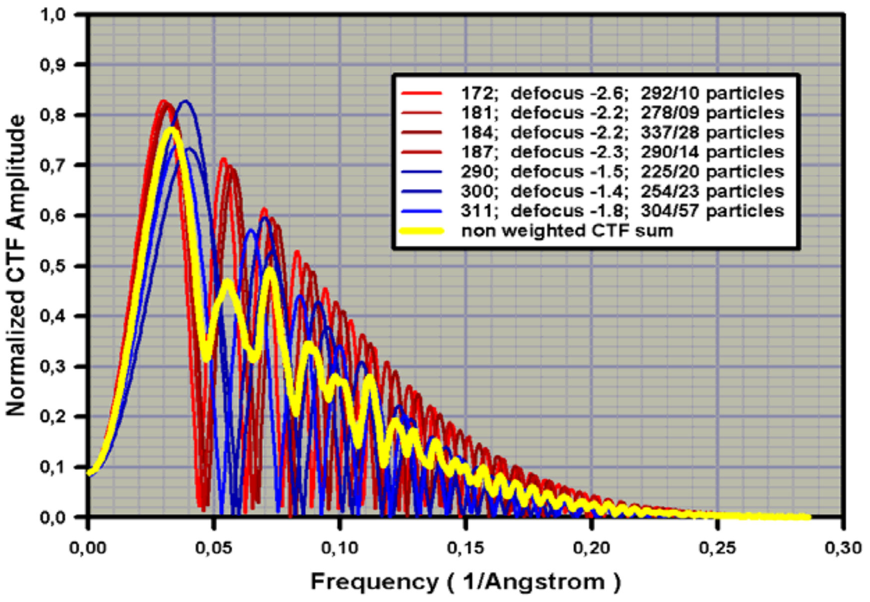
Combination of powerspectra from different defocus images

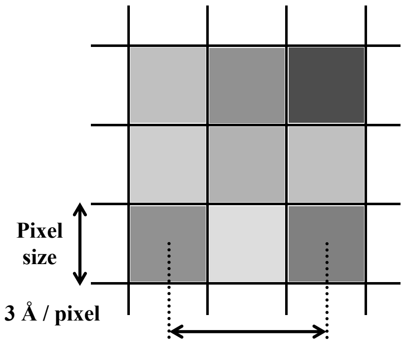


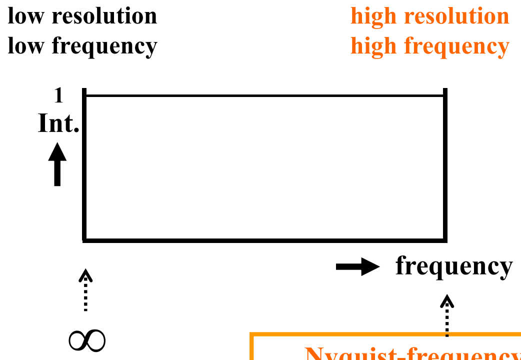
(spectra also take the envelope function into account)

I. Pre-processing

- band-pass filtering and normalisation of particle images

Grey values in pixels:



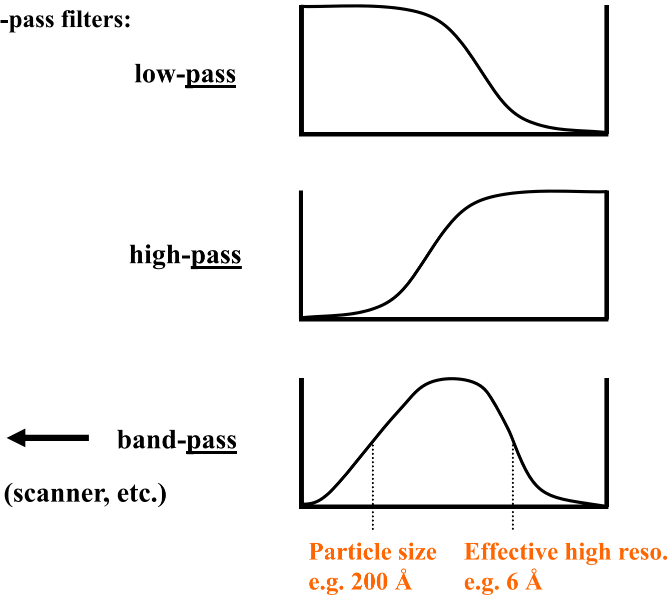


Maximum resolution = 2 x pixel size = Nyquist-frequency

Nyquist-frequency
= 2 x sampling
e.g. 2 x 3 Å / pixel = 6 Å

I. Pre-processing
- band-pass filtering and normalisation of particle images

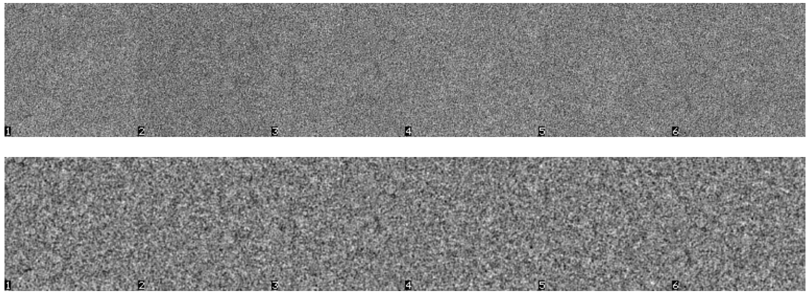
Combination of high-pass and low-pass filters:



Removes:
- low frequency contribution (scanner, etc.)
- high frequency noise

I. Pre-processing
- band-pass filtering and normalisation of particle images

Effect of bandpass filter:



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Single particle image processing and 3D reconstruction.

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II. Structure determination
- particle centering / alignments
- MSA (multivariate statistical analysis) + classification
- angle assignment
  - angular reconstitution
  - projection matching
- 3D reconstruction
- structure refinement
- resolution assessment
- map interpretation ; fitting of know structures, atomic model building…

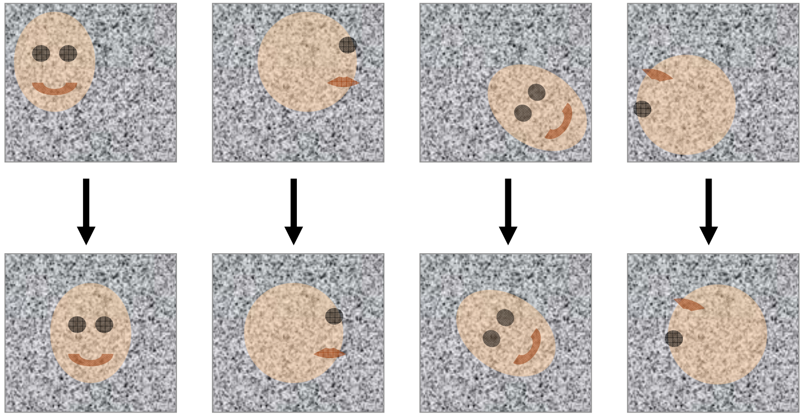
Overview of the concept:
align – classify – reconstruct in 3D

II. Structure determination
- particle centering / alignments

“reference-free” alignment (if structure unknown)
(or multiple reference alignment, if similar structure already known)

Translational Alignment

- Requires reference image(s) to align to



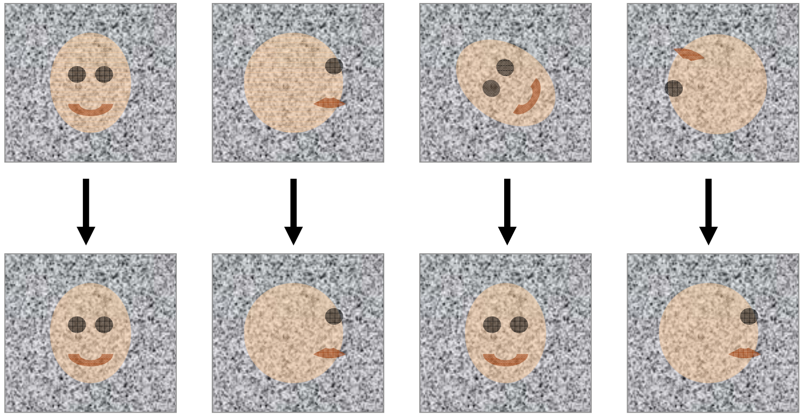
drawn by
A. Patwardhan

Overview of the concept:
align – classify – reconstruct in 3D

II. Structure determination
- particle centering / alignments

Rotational Alignment

- Requires reference image(s) to align to



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II. Structure determination
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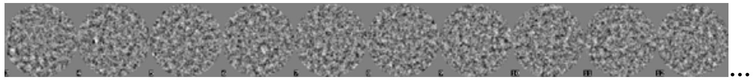
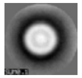
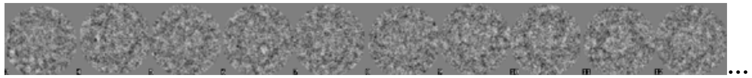


aligned to total sum of particles



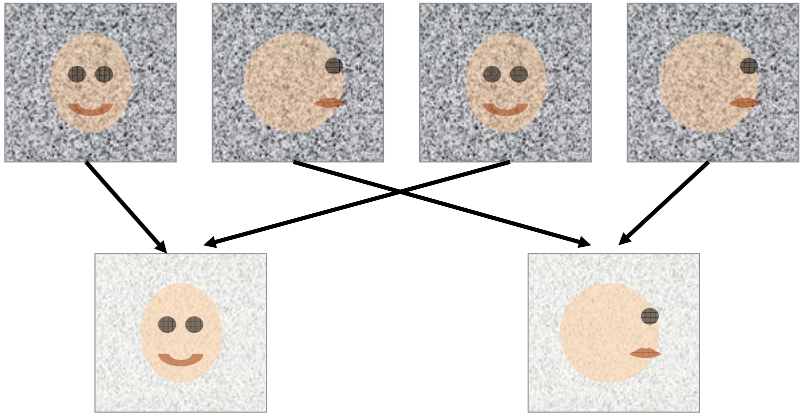
provides centered particle images:



Overview of the concept:
align – classify – reconstruct in 3D

II. Structure determination
- MSA (multivariate statistical analysis) + classification

- Combine like views to improve signal to noise: **how?** **Requires statistical analysis of the pixel intensities**



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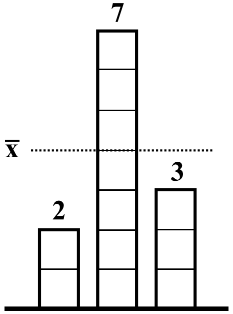
Some "basic statistics"

example for 3 values (measurements)



"how variable are the data"

$n = 3$

$\Sigma x_i = 2+7+3 = 12$

$\bar{x} = 1/n\ \Sigma x_i = 1/3\ (2+7+3) = 4$

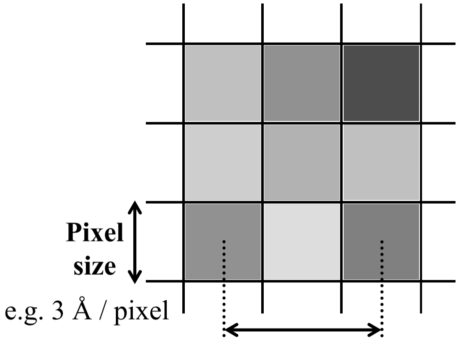
$\boxed{\sigma^2 = 1/n\ \Sigma (x_i - \bar{x})^2} = 1/3\ [(2-4)^2 + (7-4)^2 + (3-4)^2] = 14/3$ **variance**

$\sigma = \sqrt{1/n\ \Sigma (x_i - \bar{x})^2} = \sqrt{14/3} = 2.16$ **standard deviation**

"normalization"

Images are composed of pixels:

Grey values in the pixels, each is a measurement:



Maximum resolution = 2 x pixel size (**Nyquist frequency!**)

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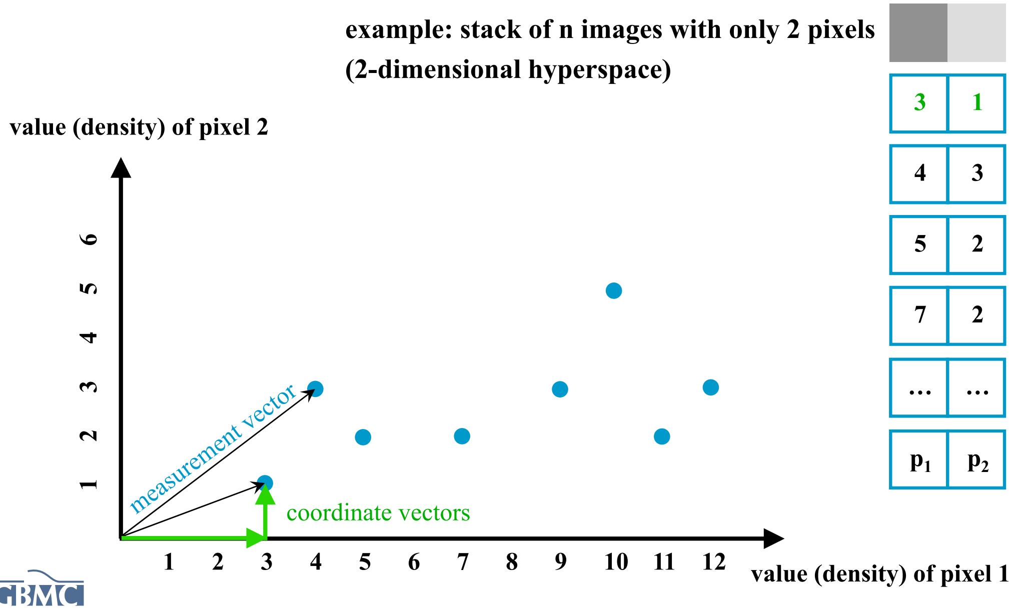
example: stack of n images with only 2 pixels (2-dimensional hyperspace)



3	1
4	3
5	2
7	2
…	…
p_1	p_2

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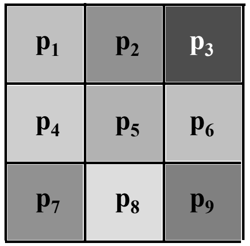
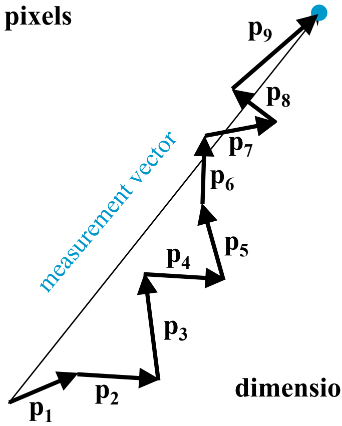
extension to more dimensions,
example: image with 3 x 3 pixels





dimensionality in image space: p^2

length of each coordinate vector $\hat{=}$ intensity of pixel p_i

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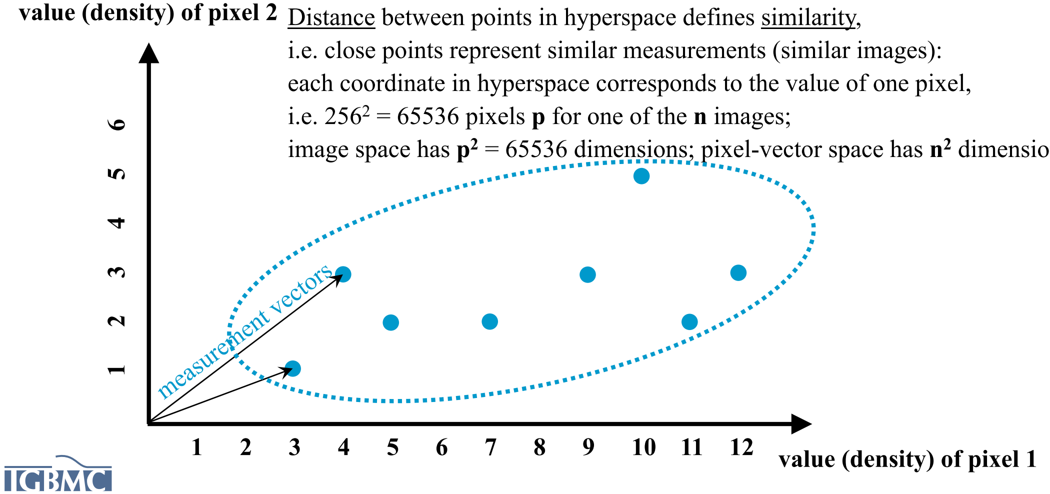
Large number of <u>comparable</u> measurements: stack of n images with the same size (e.g. 256x256)

Describe image pixels as a vector of numbers (individual pixel values / densities).

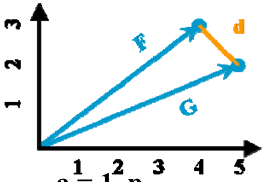
This vector is positioned in hyperspace:

→ each measurement corresponds to a point in hyperspace,
all measurements thus create a ***data cloud***.

<u>Distance</u> between points in hyperspace defines <u>similarity</u>,
i.e. close points represent similar measurements (similar images):
each coordinate in hyperspace corresponds to the value of one pixel,
i.e. $256^2 = 65536$ pixels **p** for one of the **n** images;
image space has $\mathbf{p}^2 = 65536$ dimensions; pixel-vector space has $\mathbf{n}^2$ dimensions.



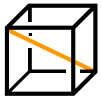
Similarity between two measurement vectors F and G:
inner product of the vectors (= correlation = covariance):



$C_{FG} = 1/p\ \Sigma\ F_a \cdot G_a$ **covariance** $a = 1, p$

$C_{FF} = 1/p\ \Sigma\ F_a \cdot F_a = 1/p\ \Sigma\ F_a^2$ **variance** $a = 1, p$

Euclidian square distance: *e.g. unit cube:*



$d = \sqrt{1^2+1^2+1^2} = \sqrt{3}$

$D^2_{FG} = \Sigma\ (F_a - G_a)^2$

$= \Sigma\ F_a^2 + \Sigma\ G_a^2 - 2\ \Sigma\ F_a \cdot G_a$ $a = 1, p$

(variances in F and G) minus (2 · correlation between F and G):

short distance means high correlation

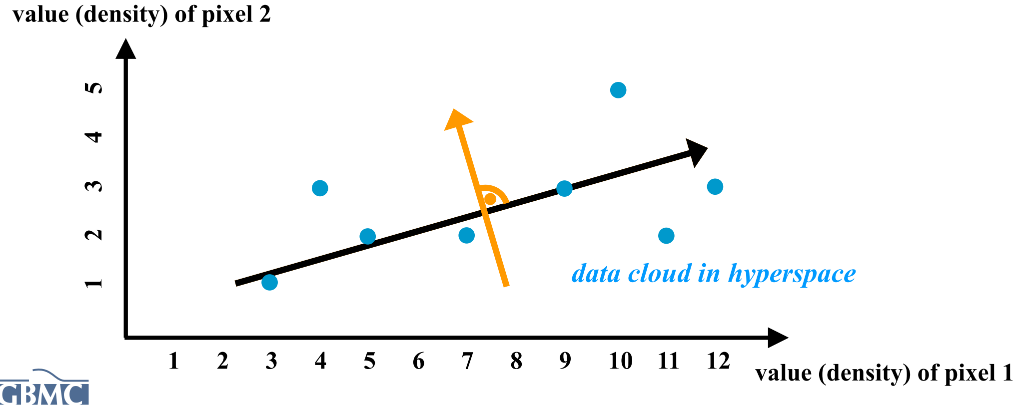
PCA metric (principal component analysis)

[for comparison: discrete correlation function: $C_{FG} = \Sigma\ F_a \cdot G_{(a-x)}$, **with shift x;**
cross-correlation coefficient CCC = $\Sigma\ F_a \cdot G_a / \mathrm{sqr}\ (\Sigma\ F_a^2 \cdot \Sigma G_a^2)$ **]**

Aim of MSA: adapt the coordinate system of the hyperspace to the shape of the data cloud.

First axis of the rotated coordinate system will correspond to the largest elongation of the data cloud, **i.e. highest variance**; the next (orthogonal) axis corresponds to the next-strongest variance.

Data reduction: use first few components that describe the strongest variations / main components;
reduction of dimensionality: e.g. 20 or 50 instead of p^2 (here 65536): gain factor **>1000**;
reduction of noise: by omitting higher components which describe only variance in noise;
main directions of variations: given by eigenvectors



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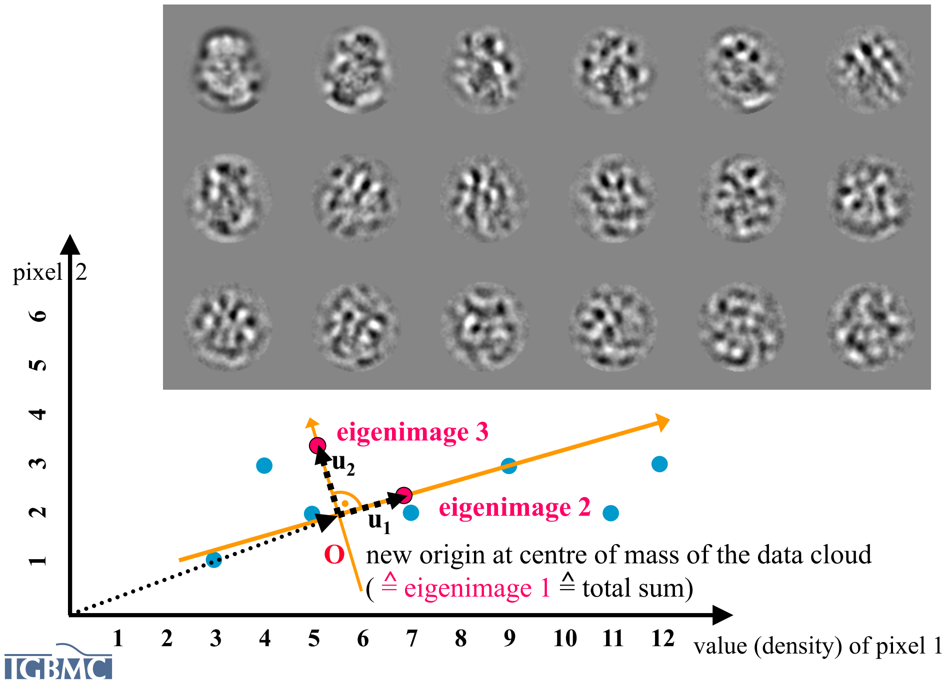
example: eigenimages



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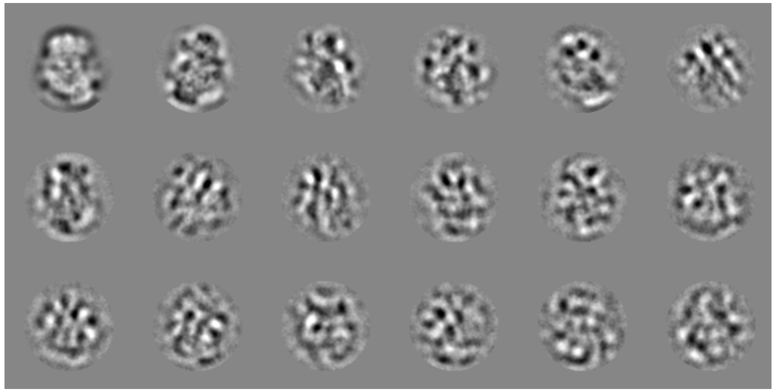
example: eigenimages



example: first 18 eigenimages of a data set

The entire data set can be reconstituted from a linear combination of the eigenimages, or simply approximated by a small subset of eigenimages (data reduction!)

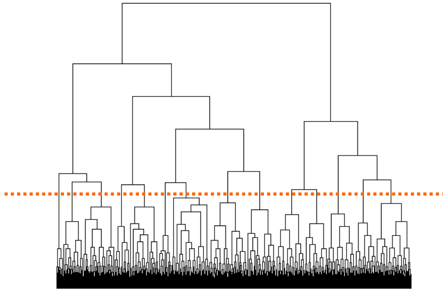
$$\text{"}a \cdot u_1 + b \cdot u_2 + \ldots\text{"}$$

II. Structure determination
- MSA (multivariate statistical analysis) + classification

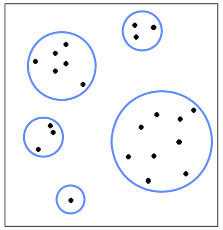
multivariate statistical analysis (MSA), related to principal component analysis: data set can be represented as a linear combination of images, each describing the highest differences within the data

→ data compression

→ images with statistically similar pixel intensity distribution can be grouped = classified into groups of images describing similar views of the 3D object

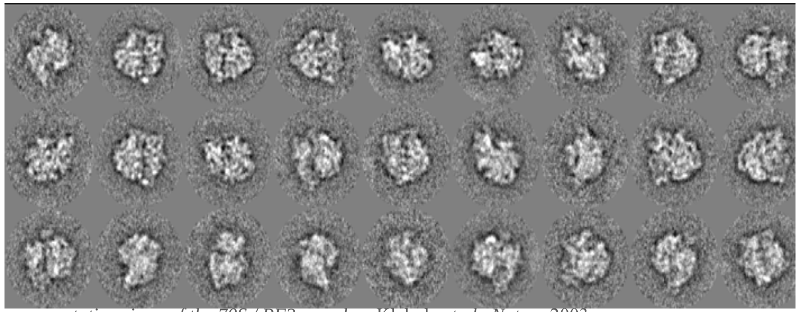


hierarchical ascendant classification



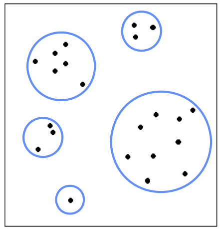
II. Structure determination
- MSA (multivariate statistical analysis) + classification

signal enhancement after
- classification by MSA
- hierarchical ascendant classification
- averaging of particles representing same views into class averages:



representative views of the 70S / RF2 complex; Klaholz et al., *Nature* 2003.

Typical class averages of ribosome particle images



Average the images of each class

Correct terms are important:

A classification is based on a statistical analysis:

- multivariate statistical analysis (MSA) provides information on variance (variability) which serves to merge similar images into class averages (classes); is *independent* of a reference

- classes *are NOT*: the sum of images that correlate best with a reference (through a multi-reference alignment)

Some references

- L. Borland & M. van Heel; Classification of image data in conjugate representation spaces, *J. Optic. Soc. Am.* A, 7 (1990) 601-610.
- M. van Heel *et al.;* Single-particle cryo electron microscopy: towards atomic resolution; Quart. Rev. Biophys. 33 (2000) 307-369.
- M. van Heel, Multivariate Statistical Classification of Noisy Images (Randomly Oriented Biological Macromolecules) *Ultramicroscopy* 13 (1984) 165-183.
- M. van Heel, Classification of very large electron microscopical image data sets, *Optik* 82 (1989) 114-126.
- E.R. Malinowski, *Factor Analysis in Chemistry*, 3rd ed. (2002)
- Benzécri J.-P. , L'Analyse des Données Vol 2, L'analyse des correspondances (1973-1980) Dunod Paris.
- Frank J: Three-Dimensional Electron Microscopy of Macromolecular Assemblies, Oxford University Press (2006).
- van Heel M, Frank J: Use of multivariate statistics in analyzing the images of biological macromolecules, *Ultramicroscopy* 6 (1981) 187-194.
- van Heel M: Multivariate Statistical Classification of Noisy Images (Randomly Oriented Biological Macromolecules), *Ultramicroscopy* 13 (1984a,)165-183.
- Ward JH: Hierarchical grouping to optimize an objective function. *J. Amer. Statist. Assoc*. 58 (1982) 236-244.

What can we apply MSA to?

- **2D classification (reference-free alignment: only centered data, not rotationally aligned)**
- **alignment by classification (alignment against class averages or a typical eigenimage)**
- **analysis of symmetry (through symmetry in the eigenimages)**
- **local MSA (focus on an area with high structural variability)**
- **re-classification of class averages belonging to an object view**
- **size-classification (e.g.** White et al., *J. Mol. Biol.* **336** (2004) 453-460).
- **3D classification of structures (separation of mixed particle populations):**
  particles:3D-SC, sub-tomograms
- **classification of powerspectra (sorting of defocus classes)**

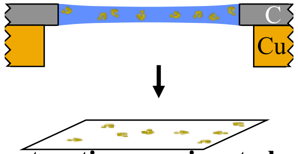
<u>Important to do before MSA:</u>
- **normalisation**
- **filtering**
- **centered data (aligned if for structure refinement)**
- **define MSA area: MSA mask**

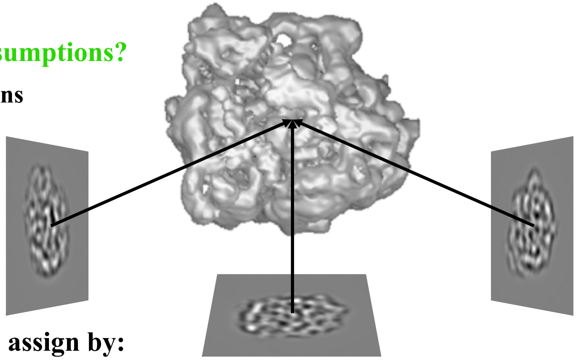
Determining structures of multiple conformational states in a single sample

II. Structure determination
- **angle assignment**
  - **angular reconstitution (in early stage of structure determination)**
  - **projection matching (if structure already well refined): find best correlation between input image and reference images from 3D re-projections)**

3D reconstruction of single particles: assumptions?

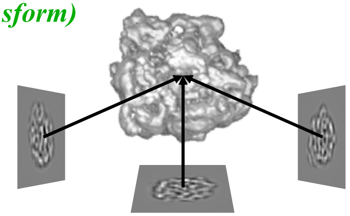
unique particle type in random orientations

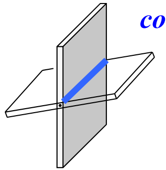




Reconstruction requires to have angles assign by:
- **<u>angular reconstitution</u> (in early stage of structure determination), or**
- **<u>projection matching</u> (if structure already refined; reference-dependent; bias), or**
- **<u>maximum likelihood</u> parameter assignment/refinement**

	Real space		*Fourier space*
map	**3d density distribution**	→ **3D FFT** ⇠	**3D Fourier transform**
	back-project ↑ ↓ **project**		↑ ↓ **extract central section** insert
class average	**2D projection**	→ **2D FFT** ⇠	**2D central section**
	back-project ↑ ↓ **project to line**		↑ ↓ **extract central line** insert
sinogram line *(Radon transform)*	**1D projection**	→ **1D FFT** ⇠	**1D central line**





<u>common line</u> projections theorem
Theorem of the central section.

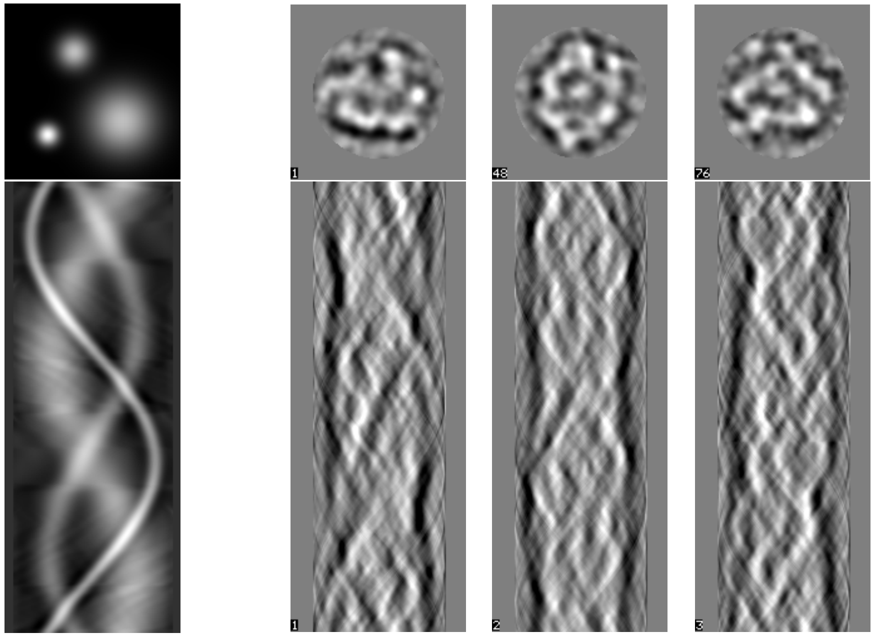
II. Structure determination

- angle assignment
  - angular reconstitution

amplitude-square-root filtered

sinogram = **line-projection** of the 2D image (also called Radon transform)

Select 3 clearly different views (here: class average numbers 1,48,76):



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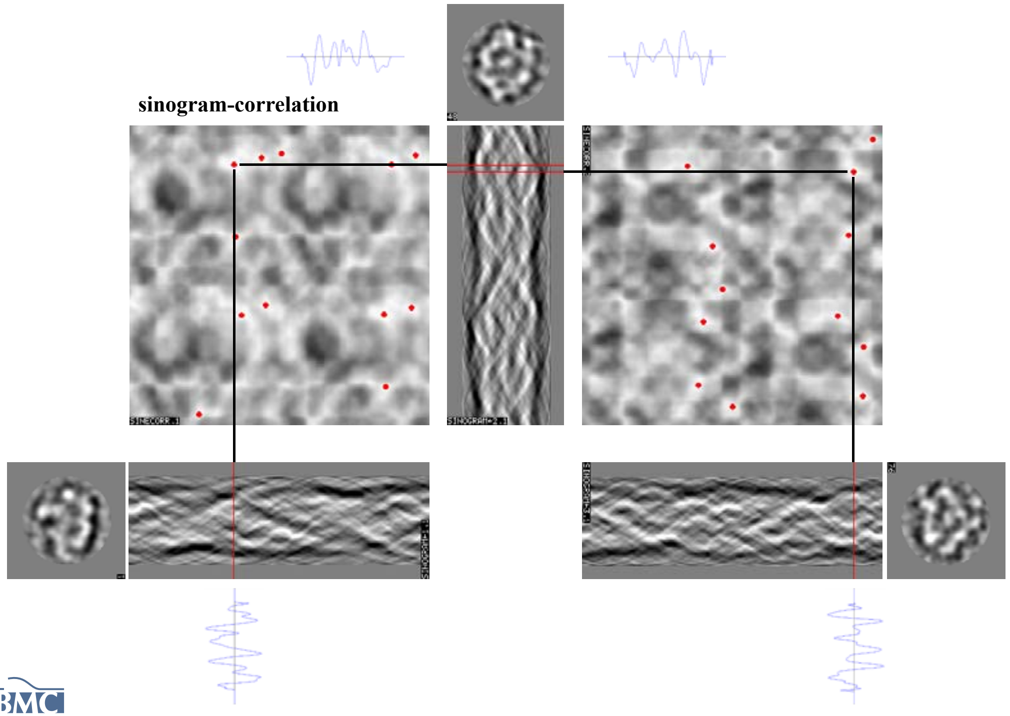
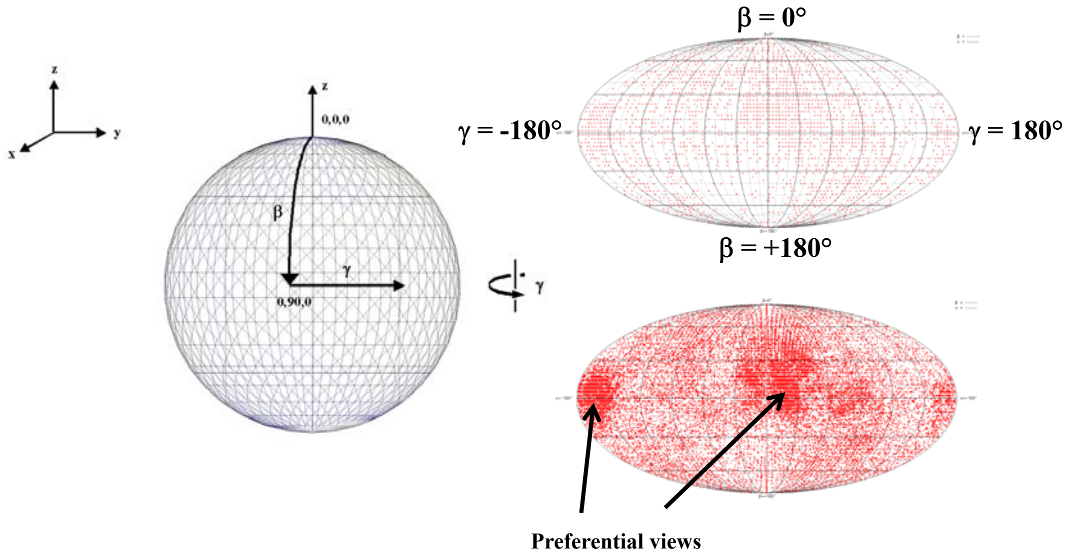
sinogram-correlation



II. Structure determination

- angle assignment
  - angular reconstitution

Particle angles plotted on sphere:



II. Structure determination

- angle assignment
    - angular reconstitution

In case of *ab initio* structure determination by

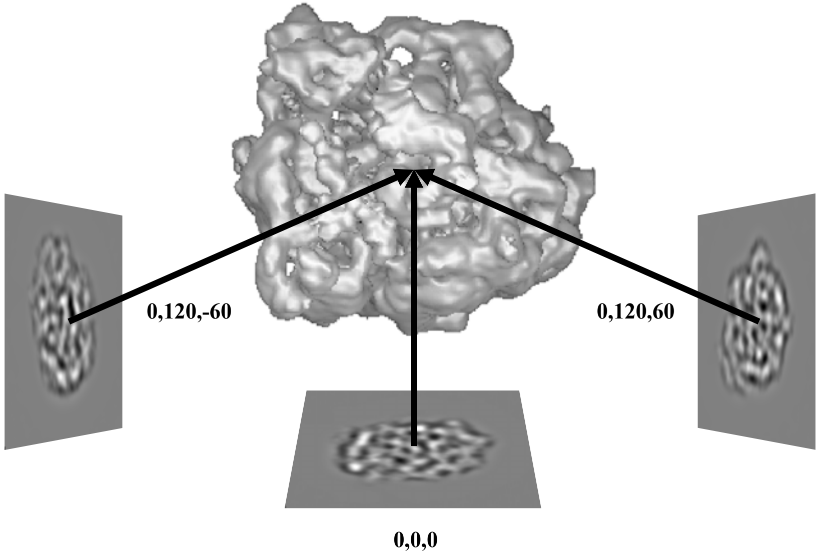
reference-free alignment and angular reconstitution:

Does not allow to determine handedness, requires either:

- random conical tilt (Radermacher *et al.*, J. Microsc. 1987)
- tomography
- phase residual error using a tilt pair (Rosenthal & Henderson, JMB 2003)
- fitting of crystal structures

II. Structure determination

- 3D reconstruction

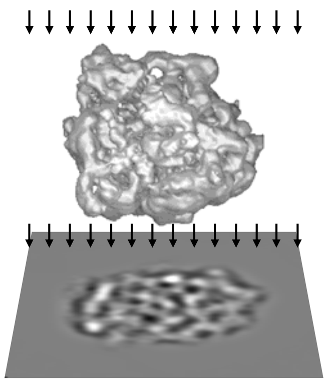


back-projection → 3D reconstruction

II. Structure determination

- 3D reconstruction

Transmission electron microscopy

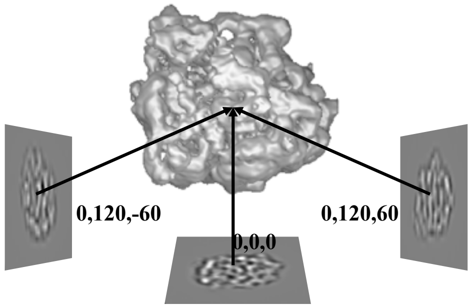


electrons

object (3D)

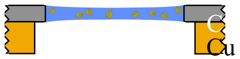
scattered electrons

projection (2D)



back-projection → 3D reconstruction

All *internal* features in a 2D view!



Some basic concepts of cryo-EM & 3D reconstruction

Correct terms are important (be precise and rigorous in science :-)

By cryo-EM, we obtain:

- a "3D reconstruction" (initial or refined)
- a "cryo-EM map" or "density map"
- a "structure"

technically:

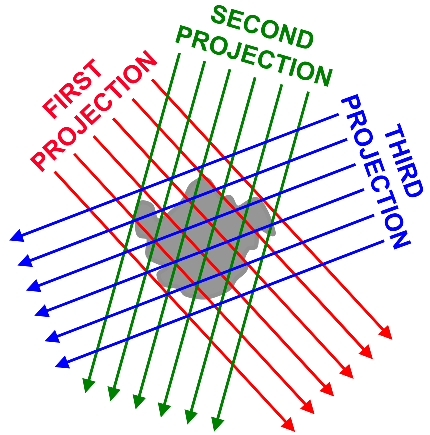
- back-projection
- angular reconstitution
- random conical tilt
- tilt series / tomogram

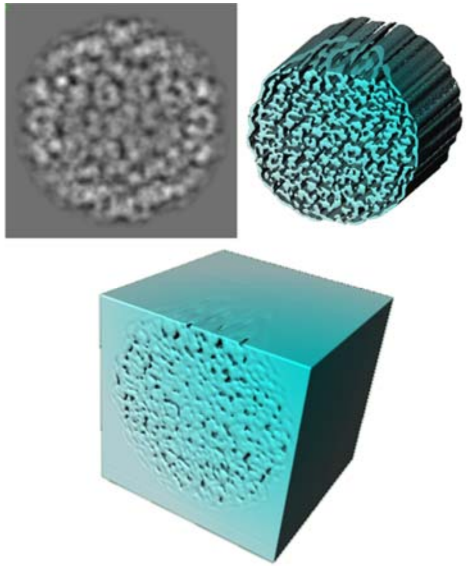
NOT:

- an "envelope" (would be SAXS or neg. stain. EM)
- a "volume", units would be Å^3 (e.g. volume of a pocket, volume x density = mol. mass)
- a "surface", units would be Å^2 (e.g. interaction surface between 2 proteins)
- a "model", would be a molecular model *fitted to* the map (crystallography/cryo-EM)
    or a model *compatible with* SAXS data or NMR restraints;
    other "models": "homology model", "hypothetical model", "working model"

II. Structure determination

- 3D reconstruction





drawn by I. Orlov

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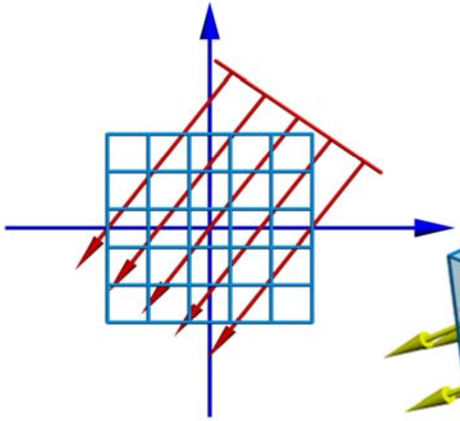
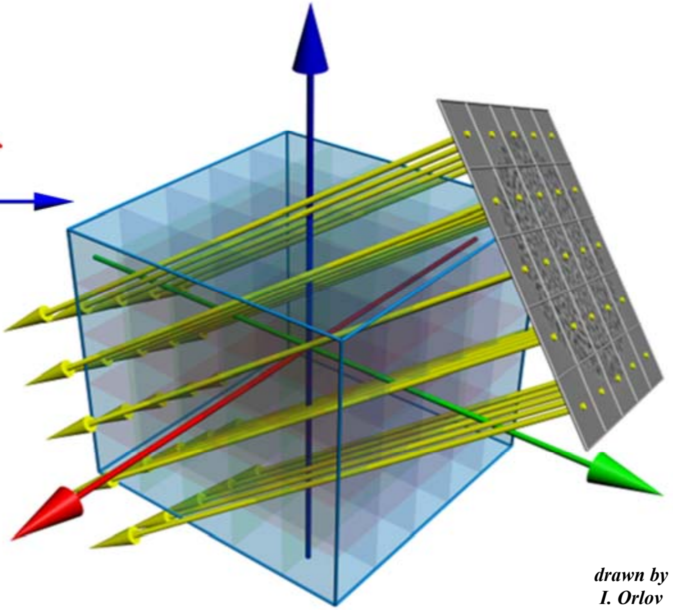
sun rays: projections

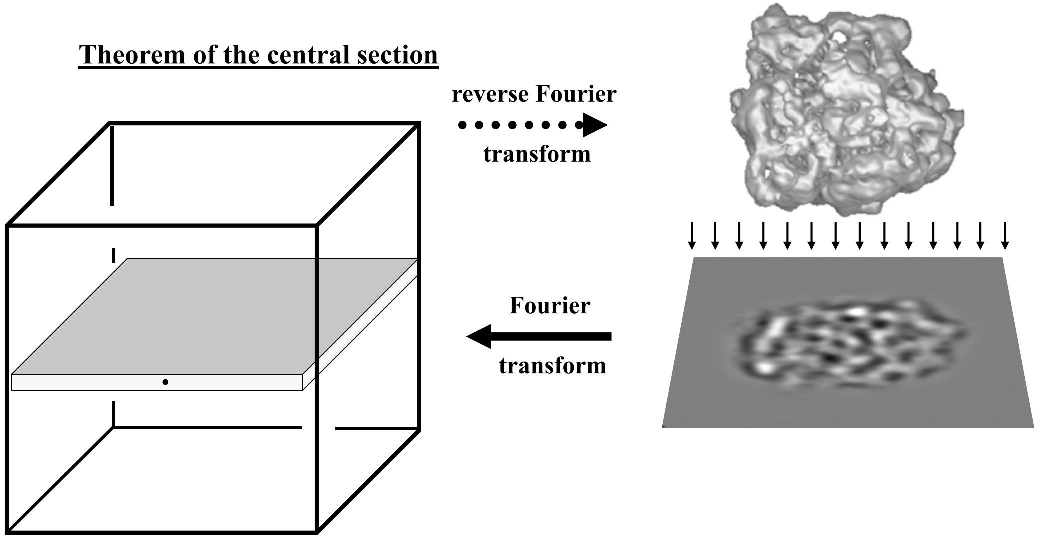
Back-Projections in 3D





drawn by I. Orlov

Theorem of the central section



reverse Fourier transform

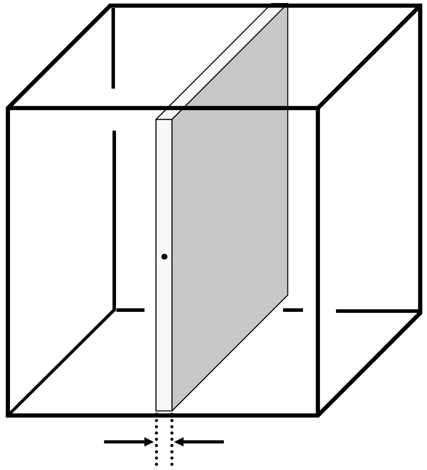
Fourier transform

central section in Fourier space

Theorem of the Central Section:
The FT of a 2D projection corresponds to the central section of the 3D Fourier transform of the density (i.e. plane perpendicular to the direction of the projection).

D. DeRosier & A. Klug, *Nature* 1968; R. G. Hart, *Science* 1968.

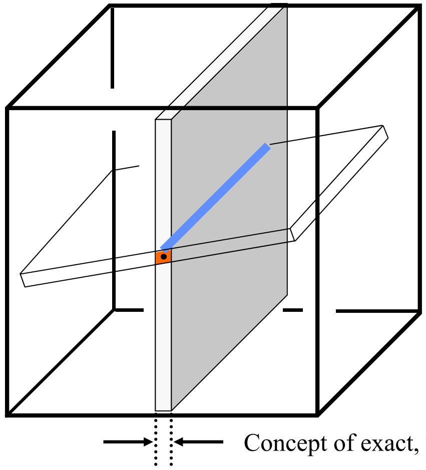
3D reconstruction by feeding in central sections in Fourier space



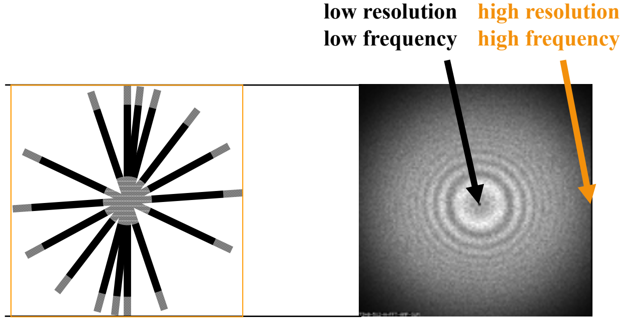
1/D, D particle size

(all representations in Fourier space)

3D reconstruction by feeding in central sections in Fourier space



Reminder: power-spectrum:

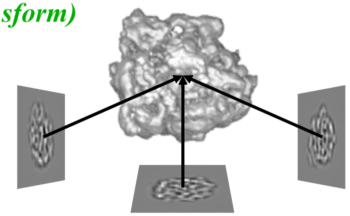


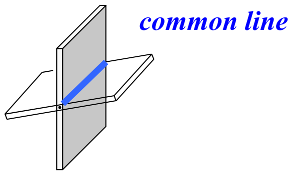
(representation in 2D for simplification)

Concept of exact, weighted filtered back-projection (WBP) reconstruction:
- back-projection
- filtered (partial removal of low frequencies to reduce overlap of central sections)
- weighting (down-weighting preferential orientations)

(Harauz & van Heel, 1986; Radermacher *et al.*, 1986)

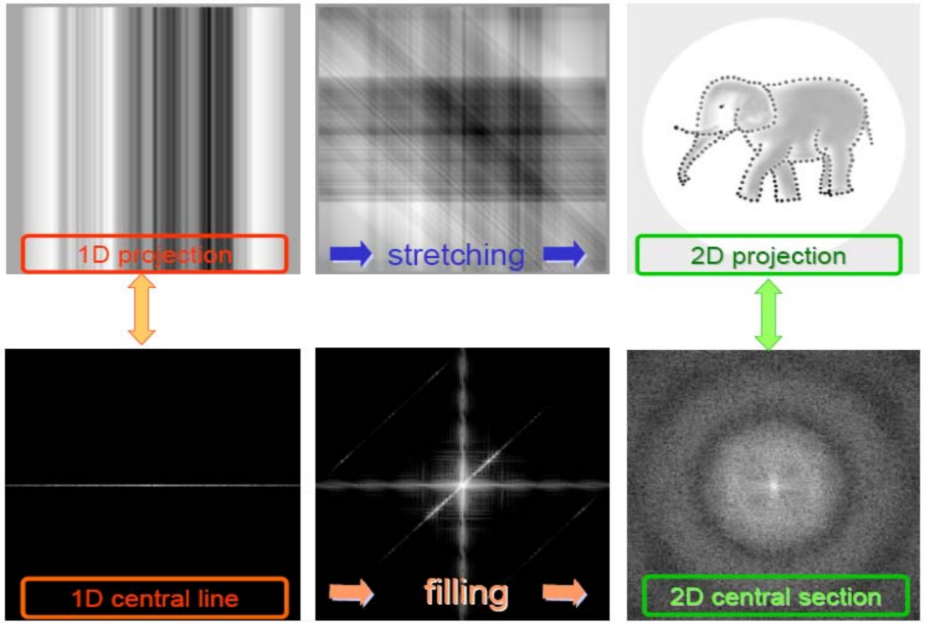
	Real space		*Fourier space*
map	**3d density distribution**	**3D FFT**	**3D Fourier transform**
	back-project ↑ ↓ **project**		↑ ↓ **extract central section** insert
class average	**2D projection**	**2D FFT**	**2D central section**
	back-project ↑ ↓ **project to line**		↑ ↓ **extract central line** insert
sinogram line *(Radon transform)*	**1D projection**	**1D FFT**	**1D central line**





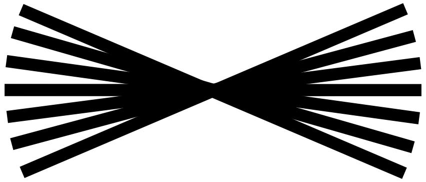
common line projections theorem
Theorem of the central section.

3D Reconstruction in REAL and FOURIER Spaces



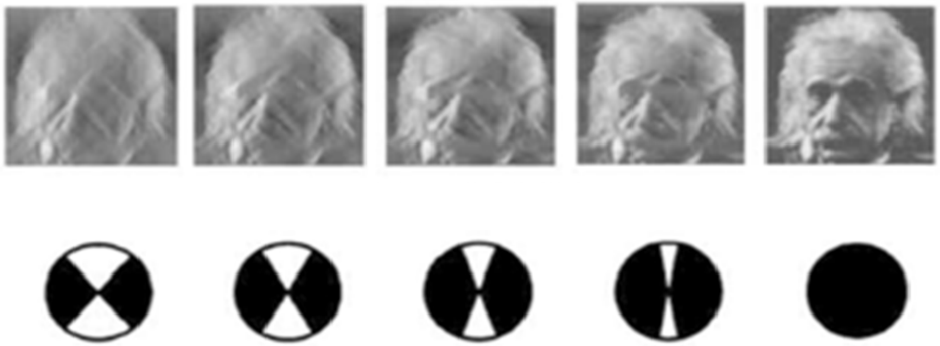
From E. Orlova EMBO course for Image Processing for Cryo Electron Microscopy, 2013

Back projection of individual images fills the reciprocal space by adding central sections:



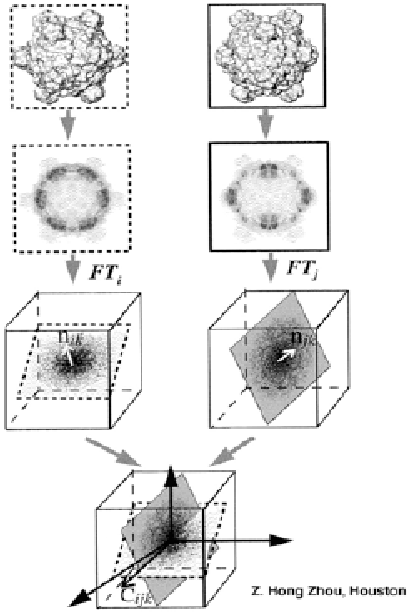
central sections in Fourier space

Filling Fourier space



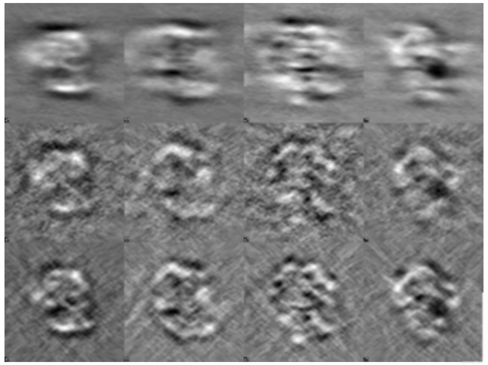
(example from tomography)

3D Reconstruction in REAL and FOURIER Spaces



Z. Hong Zhou, Houston

Importance of proper weighting in the case of preferential views



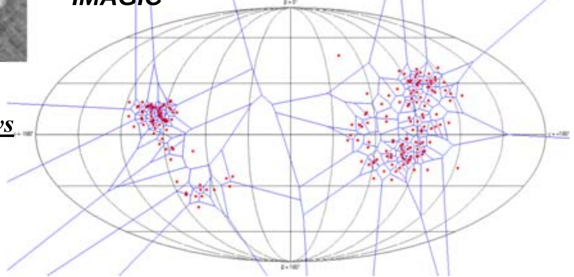
BKPR non-weighted

BKPR weighted

IMAGIC

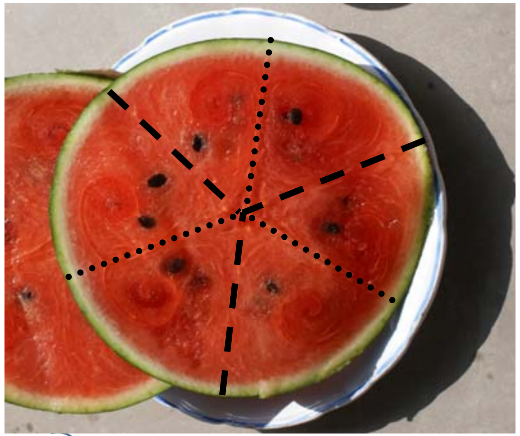
strong distortions/artifacts in 3D reconstruction

sections of 3D's calculated from 250 class averages with strong preferential views

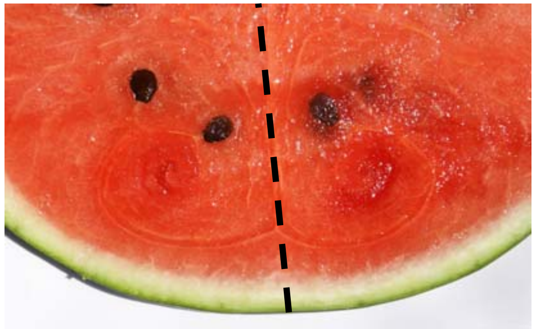


Orlov *et al.*, in prep. (2016).

symmetries in nature…



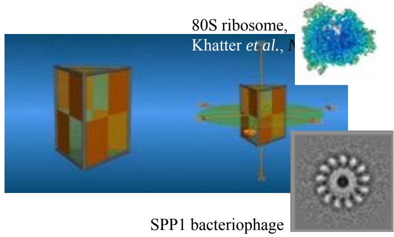
pseudo-symmetry: trimer of dimers!



watermelon photos: B. Klaholz, 2011

Point group symmetries of biological objects (e.g. used in single particle reconstructions)

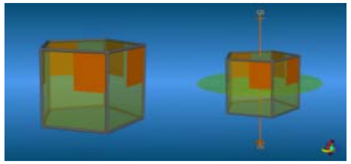
C$_n$



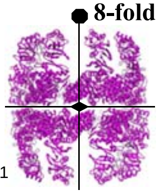
80S ribosome,
Khatter *et al.*, N

SPP1 bacteriophage
Orlova *et al.*, *EMBO J* 2003.

D$_n$

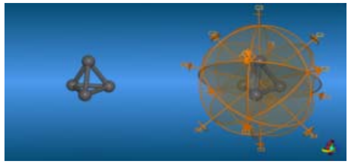


D$_8$



Chaperonine complex
Zhang *et al.*, *Structure* 2011

T



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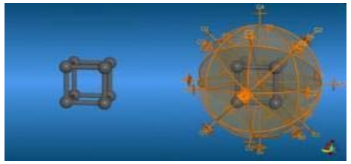
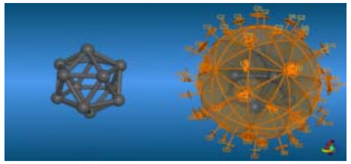
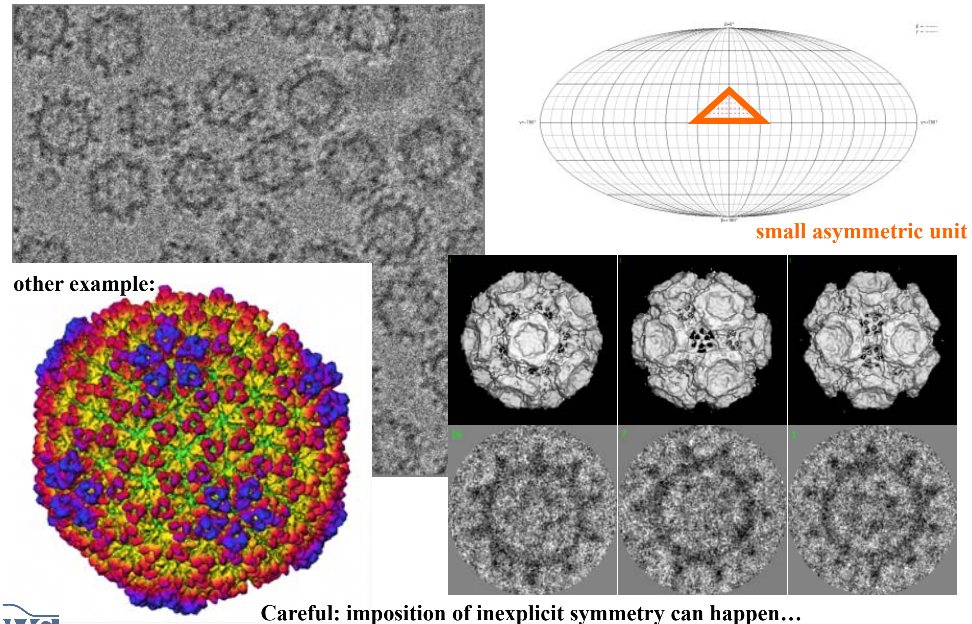


I



http://csi.chemie.tu-darmstadt.de

Practical Example – Icosahedral Reconstruction



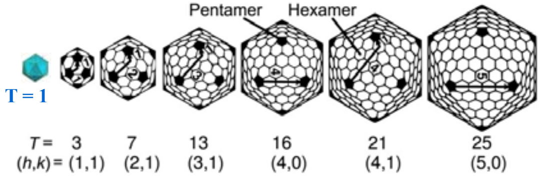
small asymmetric unit

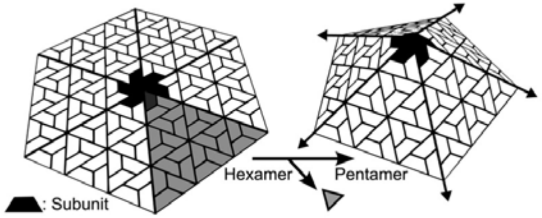
other example:

Careful: imposition of inexplicit symmetry can happen…

Icosahedral Triangulation Number

The T-number is calculated by
(1) applying a grid to the surface of the virus with coordinates *h* and *k*,
(2) counting the number of steps between successive pentagons on the virus surface,
(3) applying the formula: $T = h^2 + h \cdot k + k^2$ gives the number of structural units per face, = number of subunits in the asymmetric unit

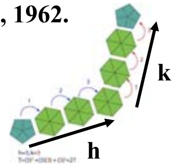




always 12 pentamers,
but variable hexamer insertion

Multi-symmetric polyhedra:
Goldberg, Tôhoku Math., 1937.

Concept of quasi-equivalence:
Caspar & Klug, 1962.



http://viralzone.expasy.org/all_by_protein/1057.html

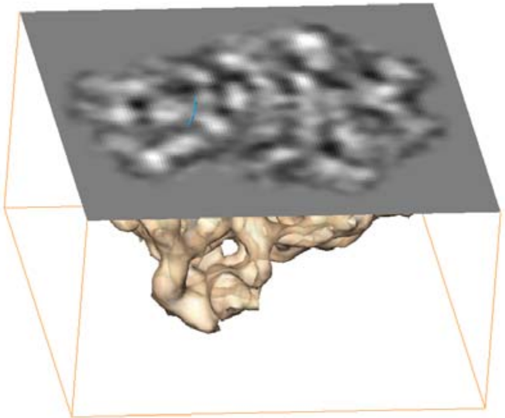
The two integers h and k describe the number of hexamers (h+k-1) one has to "walk over" to get from one pentamer to an adjacent pentamer.

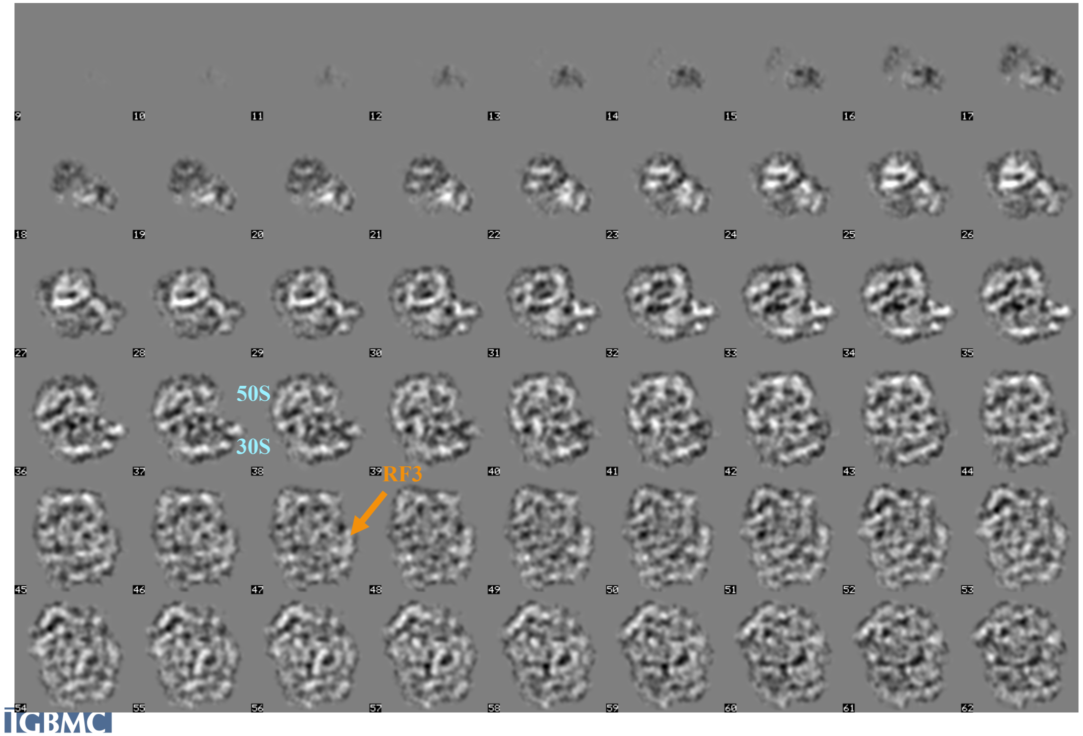
see also Johnson & Speir, *JMB* 1997.

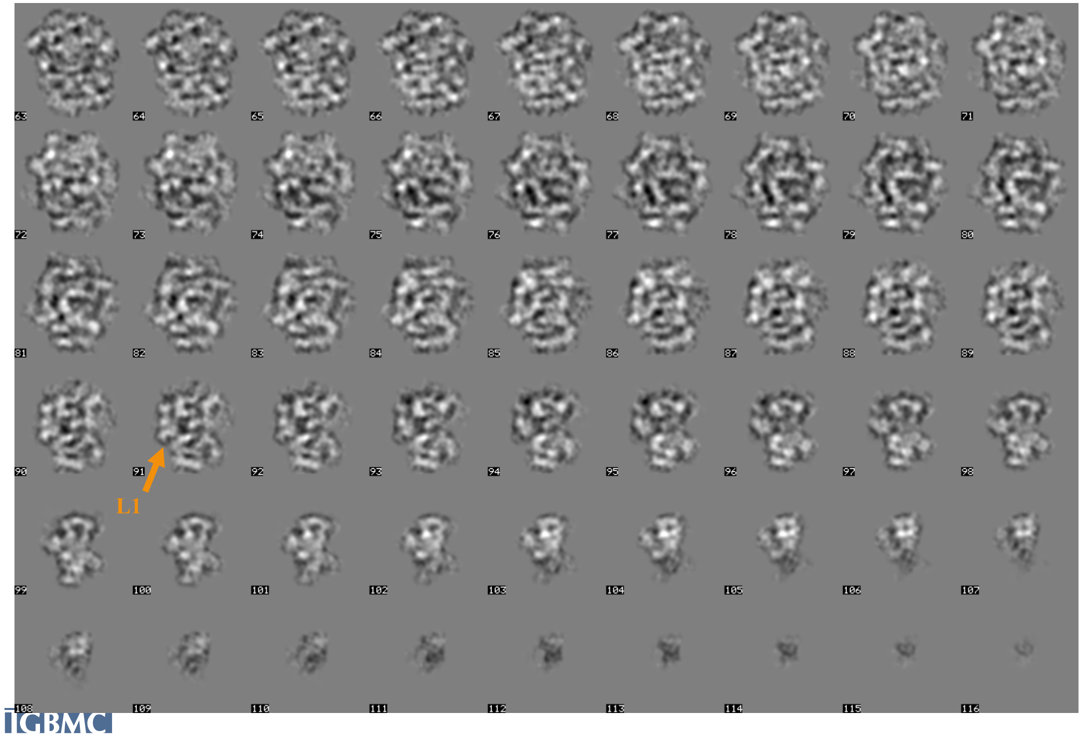
Mannige & Brooks, PLoS One, 2010

II. Structure determination
- 3D reconstruction

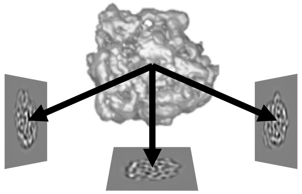
<u>Representing 3D structures</u> as consecutive sections through the 3D structure:



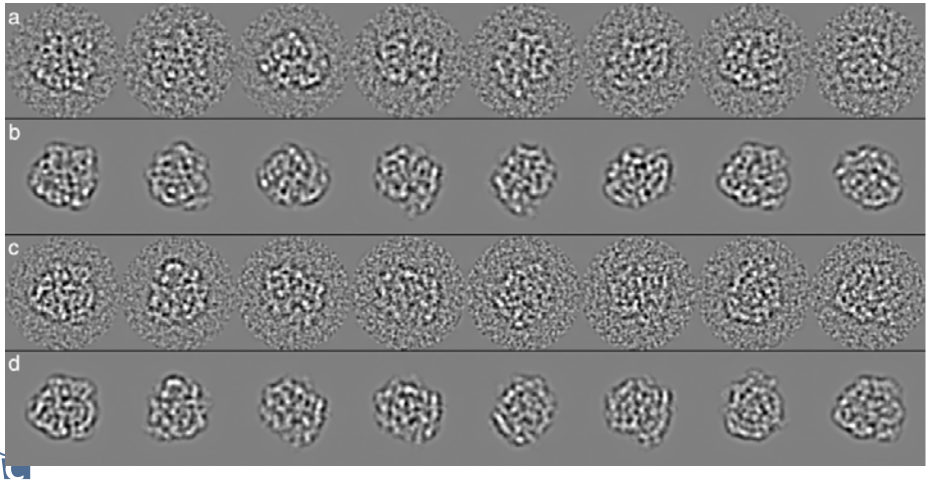




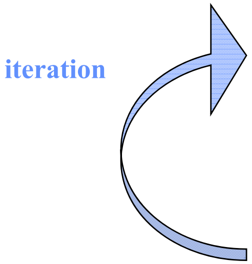
II. Structure determination
- 3D reconstruction



<u>cross-validation</u> of angle assignment and image quality by comparison with re-projections according to the same angles



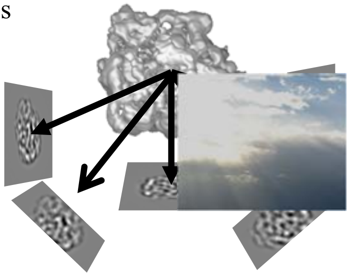
II. Structure determination
- structure refinement



- centering/alignment
- variance analysis + classification
- angle assignment
- angular reconstitution → 3d-reconstruction
- reprojections = new references

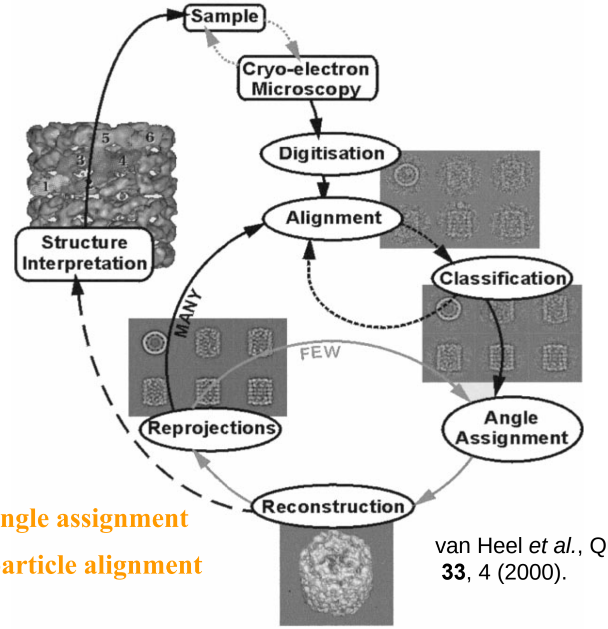
"phase" the particles by aligning/classifying them

- improve quality of angle assignment
- improve quality of particle alignment



equally distributed forward-projections
(re-projections)

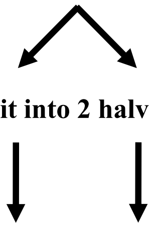
II. Structure determination
- overview



- improve quality of angle assignment
- improve quality of particle alignment

van Heel *et al.*, Quart. Rev. Biophys. **33**, 4 (2000).

II. Structure determination
- resolution assessment



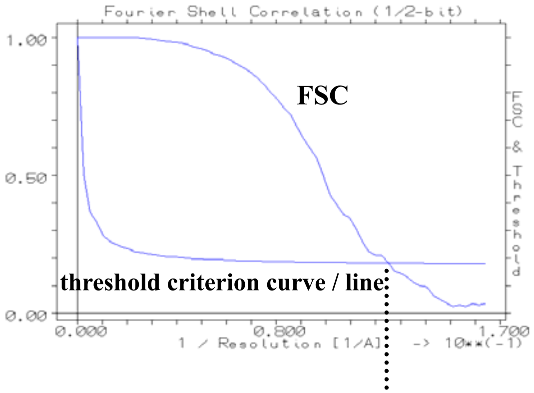
Particle data set

split into 2 halves

3D **3D**

calculate cross-correlation by shells in Fourier space

Fourier Shell Correlation (FSC)



1 / 1.25 x 1 / 10 Å = 8 Å

Keep in mind: resolution is what you can resolve in the 3D map!

Some basic concepts of cryo electron microscopy

Basic aspects:

- "resolution" corresponds to "spatial frequency" in image processing (1/ Å)

- Nyquist frequency is = 2 x pixel size, e.g. 1 Å / pixel → Nyquist = 2 Å

- interpolations during 2D image alignment and 3D reconstruction limit the possible resolution to about 2/3 of the Nyquist frequency, i.e. here ~ 3 Å *(exception: super-reso)*

pixels in 3D: "voxel"

Consider:

- any correlation calculation (e.g. alignment) is biased by the reference used

- resolution estimation, criteria used:

  - 0.5, arbitrary, historically from the virus field, tends to underestimate resolution

  - 0.143 (Henderson) and ½ bit (van Heel)

  - 3 σ, not used anymore (over-estimation)

  - features in the map: can we see dsRNA helices (~10-12 Å resolution), α-helices (~8 Å), ß-sheets (~5 Å) or side chains (4-2.5 Å depending on size)?

B. Klaholz, 2016

II. Structure determination
- map interpretation

II. Structure determination
- map interpretation ; fitting of crystal or NMR structures

Fitting procedures:

- **manual fitting (e.g. O, A. Jones, Acta Cryst. (1991))**
- **real space fitting**
- **reciprocal space fitting**

1) global search

2) refinement

e.g. torsion-angle molecular dynamics

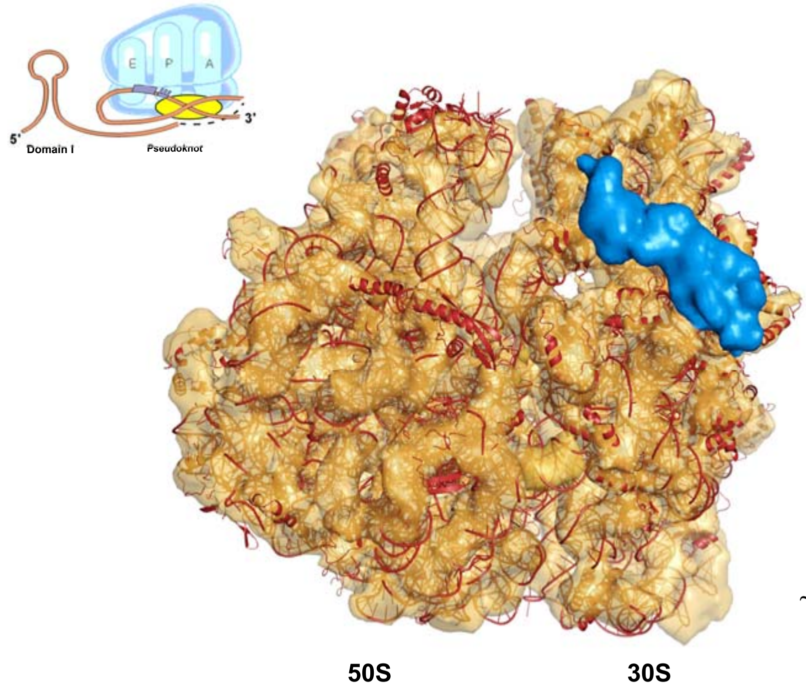
- fit complete structures, domains, factors;

Usually backbone is enough.

rigid body or flexible fitting

- use full maps or difference maps

Be careful with local minima and over-fitting!



~12 Å resolution

Marzi *et al.*, *Cell* 2007.

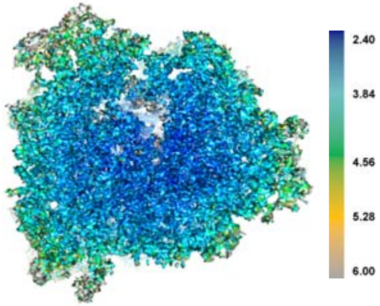
Fitting of 70S crystal structure from ***E. coli*** (Schuwirth *et al.*, 2005; *Science*, **310**, 827-834)

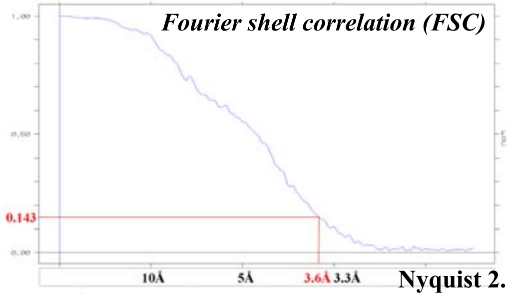
II. Structure determination
- map interpretation

Orlov *et al.*, *EMBO J.* 2012.

Atomic structure of the human ribosome

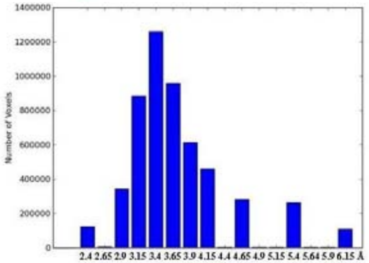
Structure determination at < 3 Å resolution by single particle cryo-EM

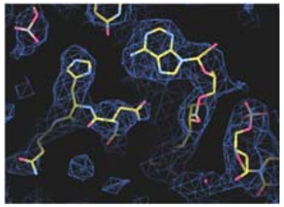




Nyquist 2.16Å

→ local resolution can be notably better than average resolution





cryo-EM map

Khatter *et al.*, *Nature, 2015.*

Some softwares:
(for single particle cryo-EM image processing)

- **Imagic**
- **Spider**
- **Frealign**
- **EMAN**
- **XMIPP**
- **Relion**
- **Scipion**
- **…**

Plus other specific software for helical reconstructions, viruses etc.