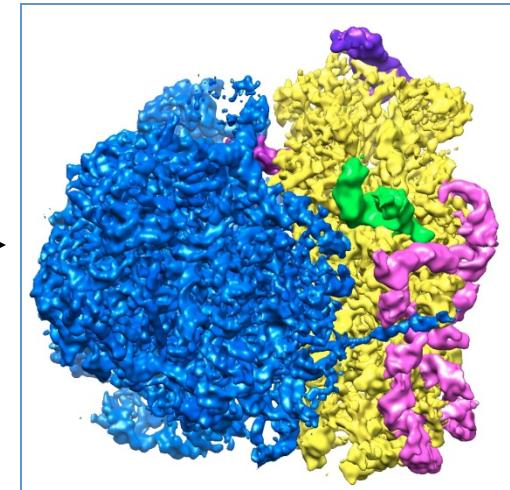
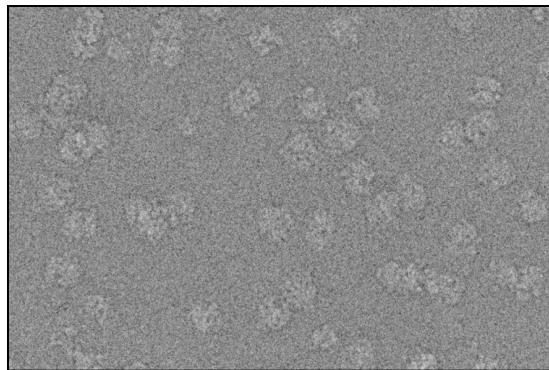


# Single-Particle Reconstruction



How to get from here

over here

to there

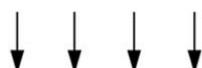
**Joachim Frank**

Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics,  
and Department of Biological Sciences, Columbia University

Supported by HHMI, NIH/NCRR, NIH R01 GM55440, and NIH R01 GM29169

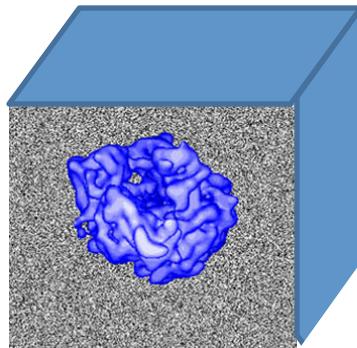
# Imaging in the Transmission Electron Microscope

Electrons



generated in cathode

3D object



molecule embedded  
in vitreous ice

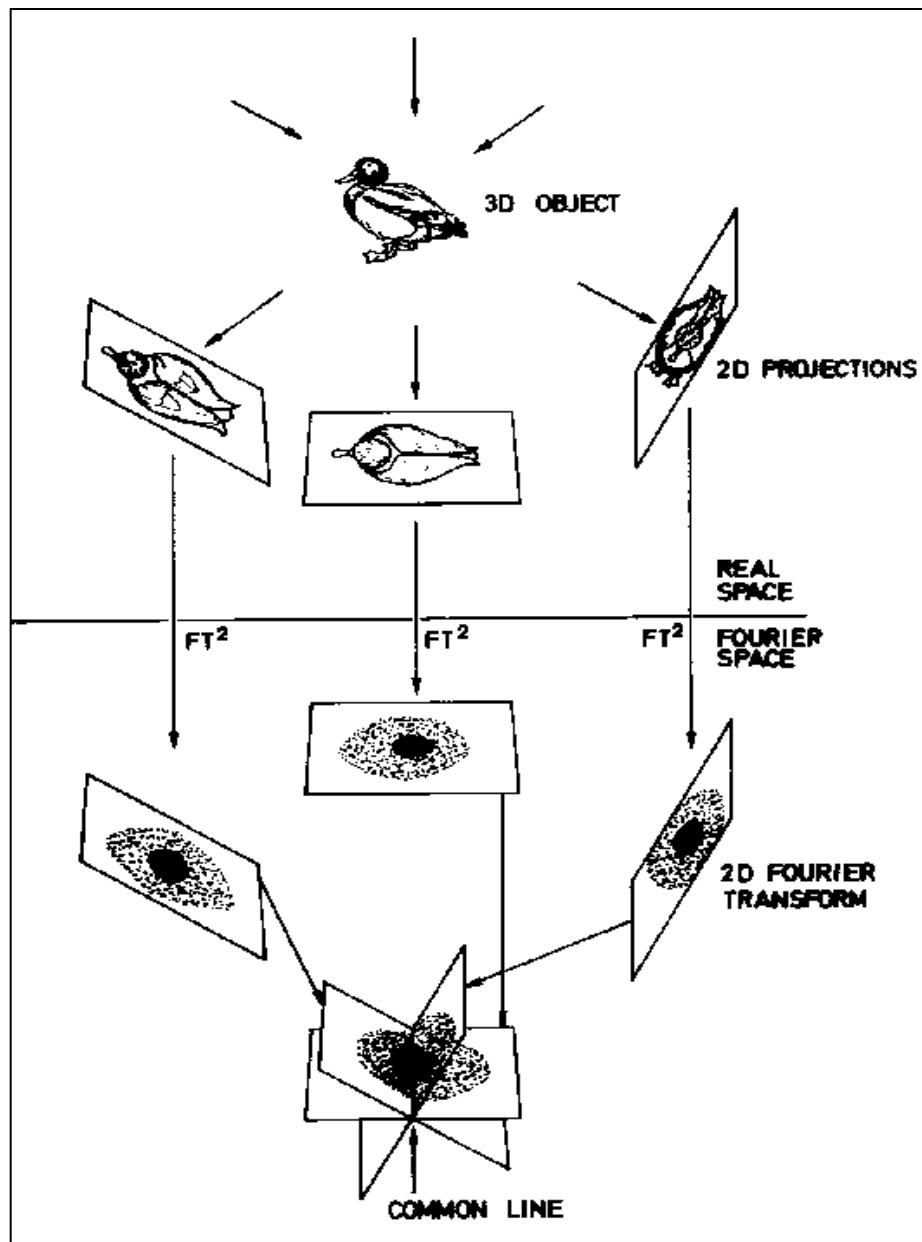
2D projection



recorded on film or digital  
camera

Transmission means that the signal is generated from electrons passing through the specimen. We see a 2D projection = line integral over the 3D density along the beam.

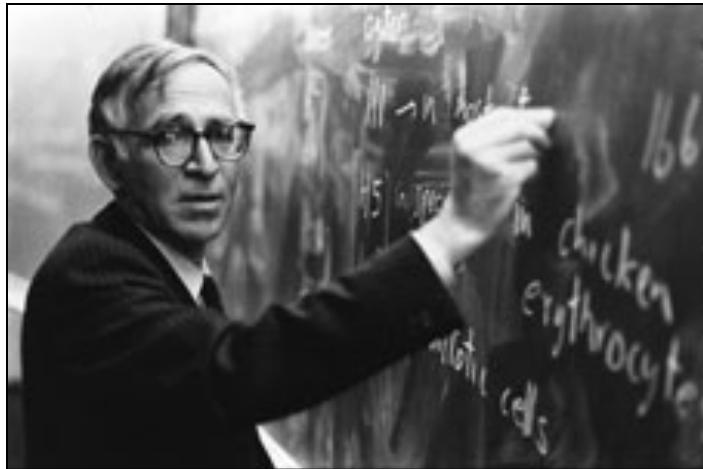
# How to get from 2D to 3D: The Projection Theorem



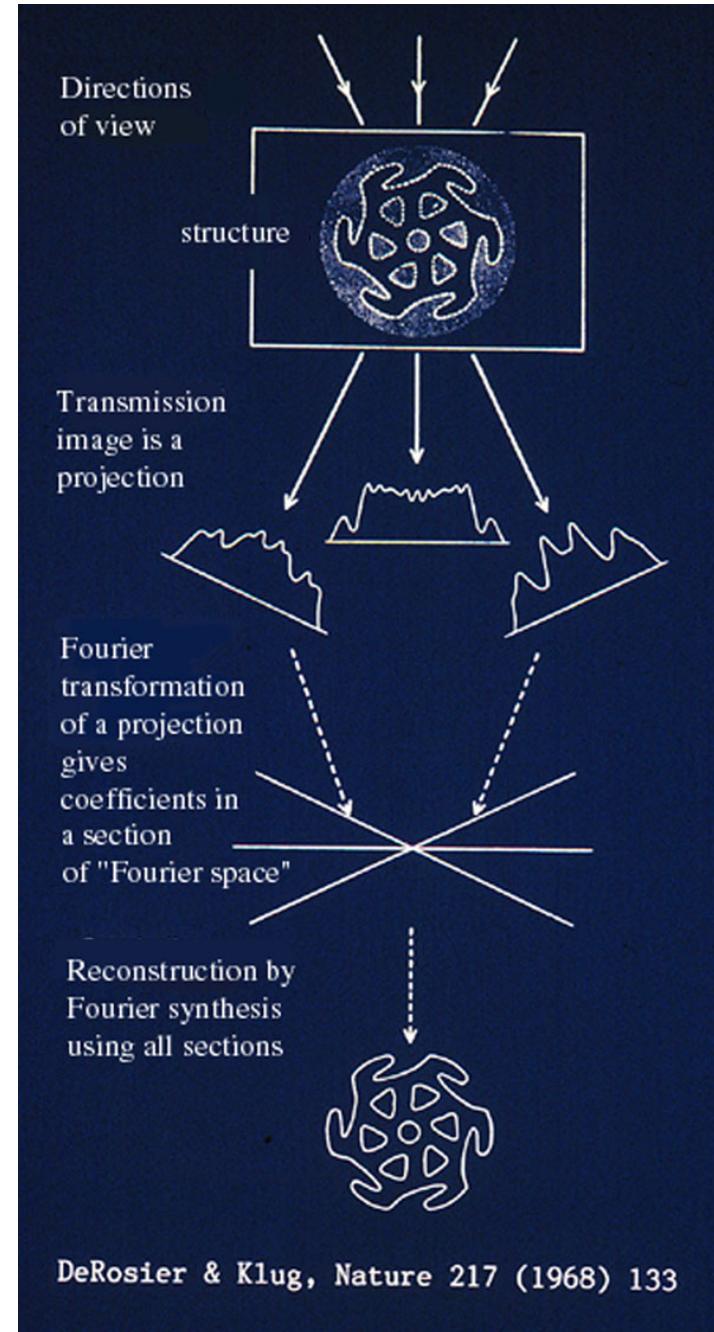
- 1) The transmission electron microscope forms projections of the 3D object.
- 2) The Projection Theorem:  
"The 2D Fourier transform of the projection of a 3D density is a *central section* of the 3D Fourier transform of the density, *perpendicular* to the direction of projection."
- 3) It is necessary to collect a sufficient number of projections over a large angular range. From these projections, the object's density distribution can be reconstructed.

# First 3D reconstruction from EM images: 3D reconstruction of bacteriophage Tail using the Fourier-Bessel approach

1968

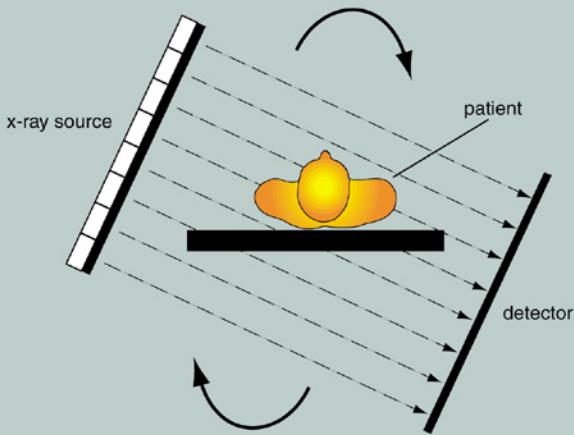


Aaron Klug and David DeRosier,  
Laboratory for Molecular Biology, MRC,  
Cambridge



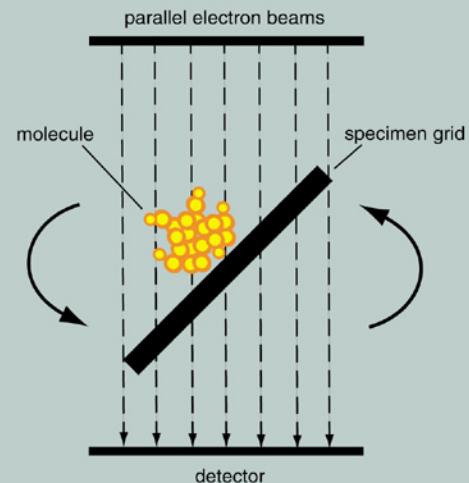
# How do we collect the projections?

## Three data collection strategies for 3D reconstruction:



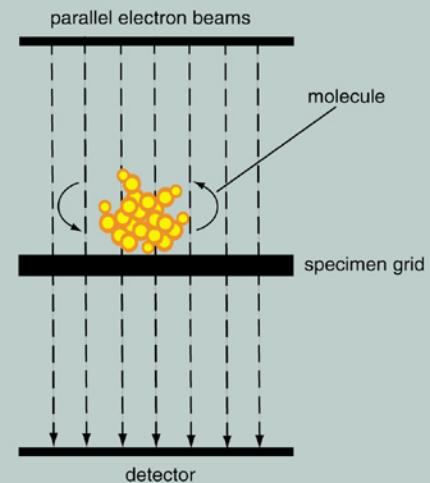
**CAT - scan**

- beam rotating
- patient stationary



**Electron Tomography**

- molecule rotating
- beam stationary



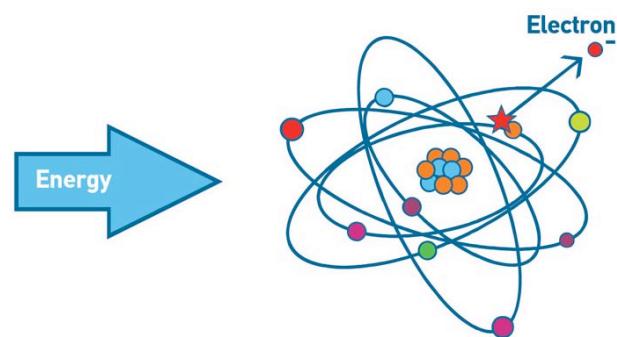
**Single particle reconstruction**

- molecule "rotating"
- beam stationary

Why single-particle data collection --  
Why not tomography?

### RADIATION DAMAGE

- Elastic scattering -- no energy transferred; no radiation damage; useful for image formation
- Inelastic scattering – leads to ionization, bond breaking, formation of radicals

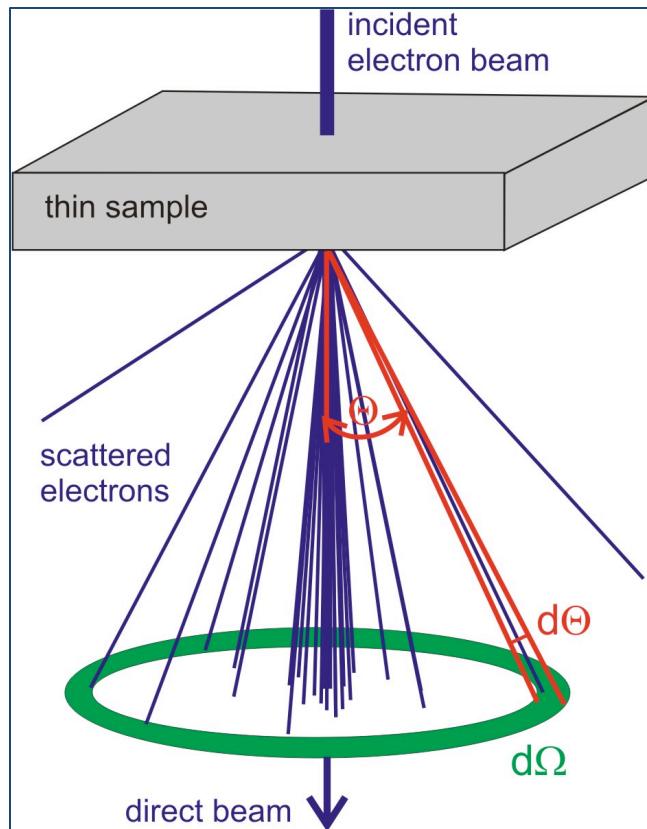


Why low temperature?

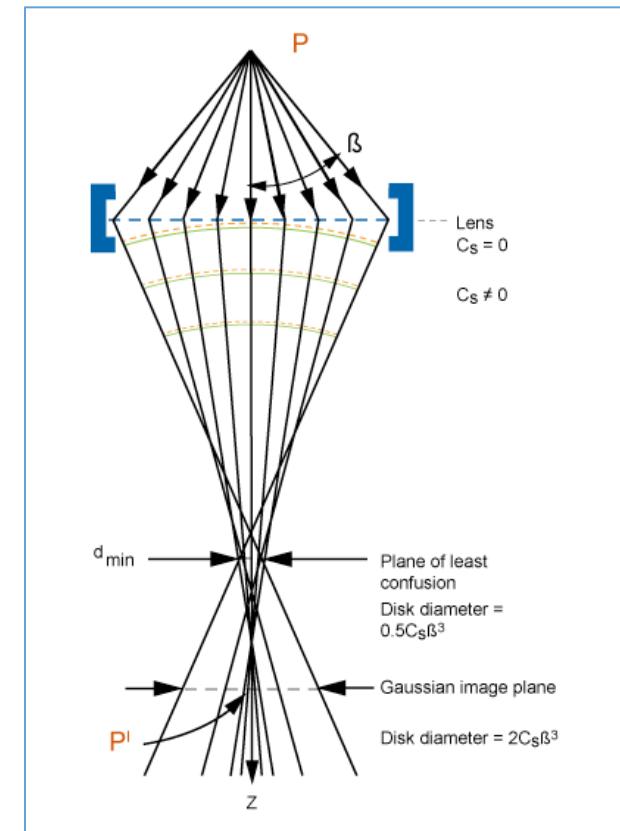
### DOUBLE PURPOSE:

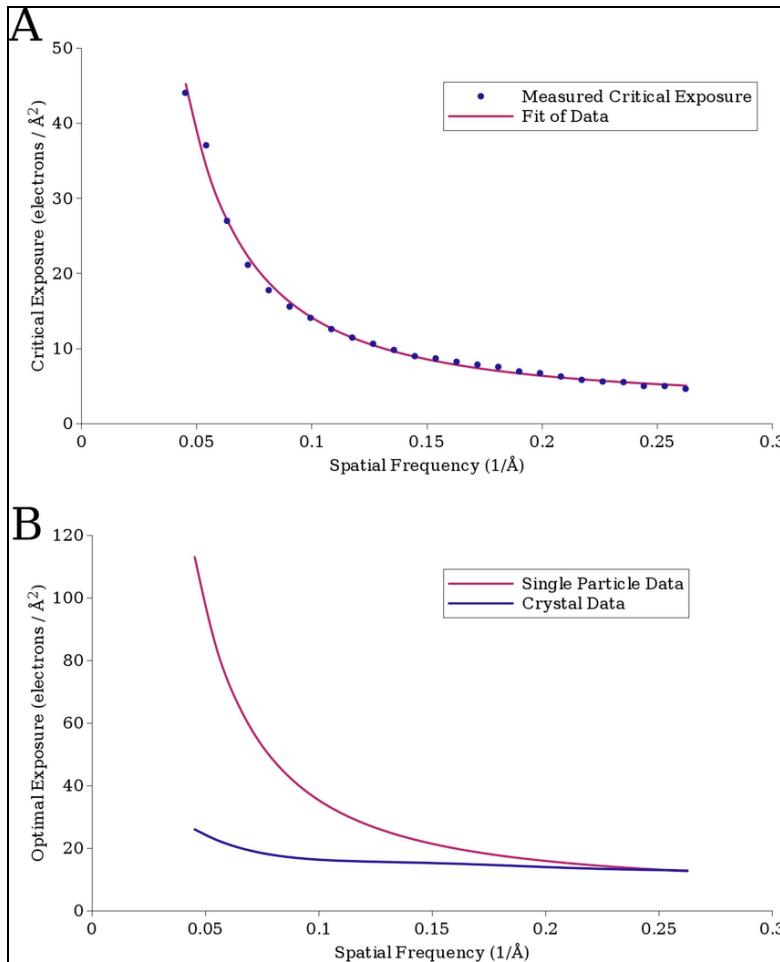
- (1) to present molecules in close-to-native environment in high vacuum to the beam
- (2) to trap damaged molecules and keep radicals from migrating, causing further damage

- The image is formed from elastically scattered electrons
- The lens gathers all rays scattered from a single point into an (imperfect) point in the image
- Large scattering angles – high resolution
- Small scattering angles – low resolution



low      high  
resolution →





Grant & Grigorieff, eLife 2015

Amplitude of crystal reflections affected by both damage to molecules and increasing disorder

**Damage affects small features first, then larger features**  
 Critical exposure  $N_e$  as a function of spatial frequency

Example: according to the curve, to get  $4\text{\AA}$  resolution, the exposure should be below  $5 \text{ electrons}/\text{\AA}^2$

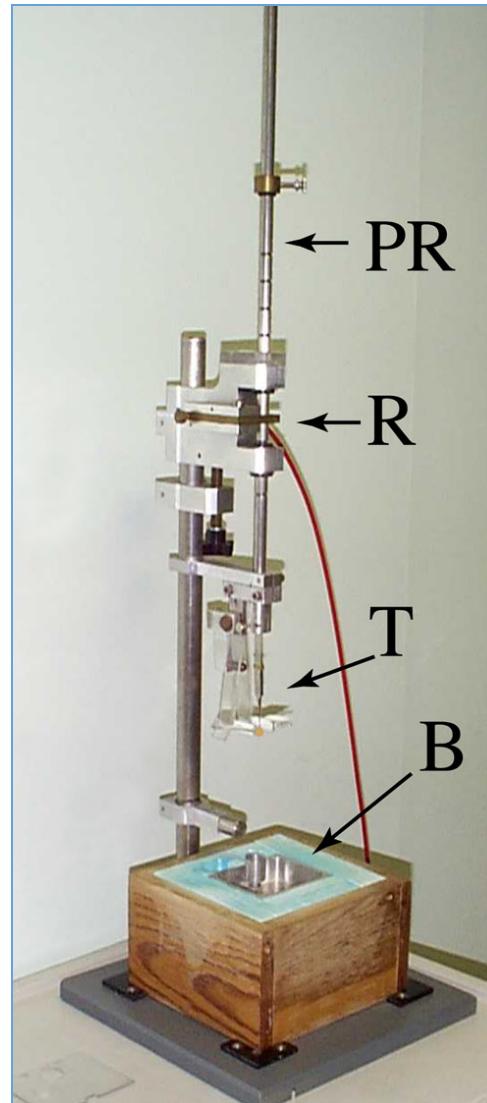
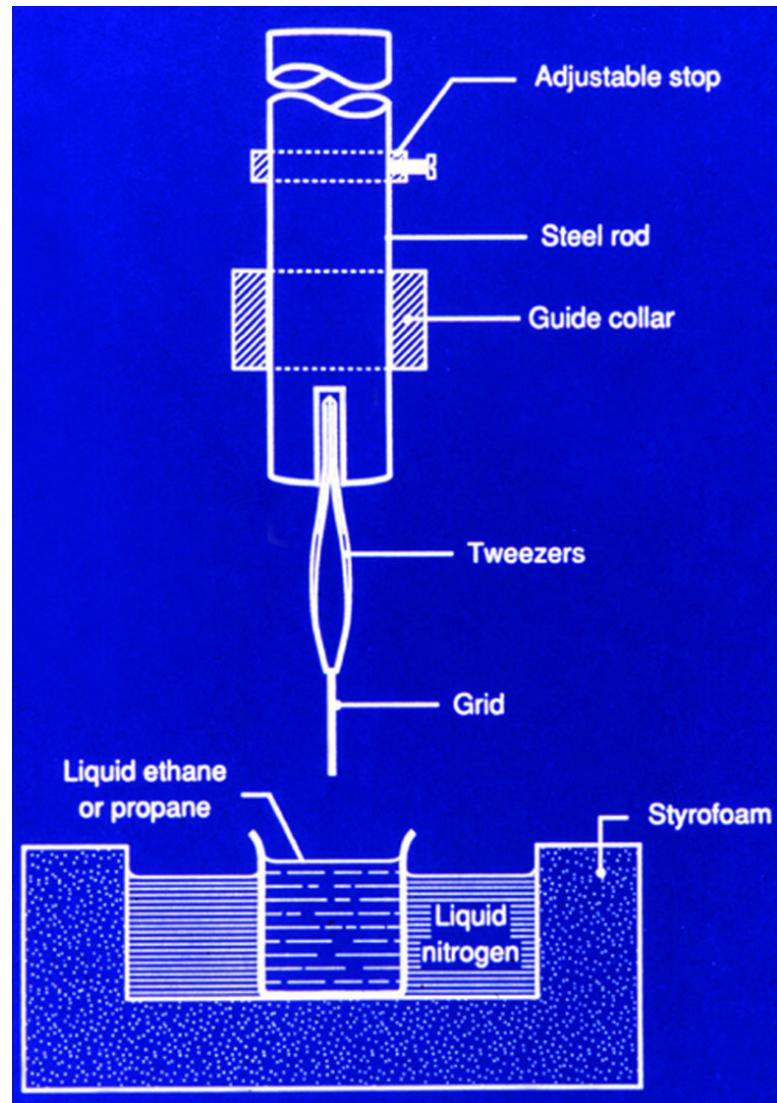
# Specimen preparation

- Purified sample – standards of purity have changed with the advent of classification (“computational purification”). In some experiments it is even desirable to admit molecules in different conformational and compositional states.
- Apply sample to EM grid as a thin film ( $\sim 1000 \text{ \AA}$ ) suspended over holes.
- Carefully controlled blotting is a critical step – control blotting force and blotting time
- Coverage with molecules is determined by:
  - (1) sample concentration
  - (2) geometry and makeup of metal grid -- copper, molybdenum, gold  
**copper** (traditional), **molybdenum** (match heat expansion of carbon), **gold** (avoid charge-induced vibrations)
  - (3) geometry of the overlaid carbon grid (Quantifoil) – size of holes, thickness of film
  - (4) (optional) overlaid thin carbon film

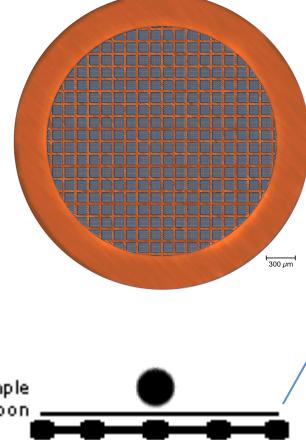
# Plunge-freezer to prepare samples for cryo-EM

Manual

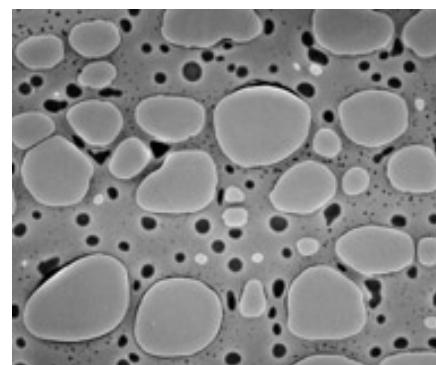
automated, climatized



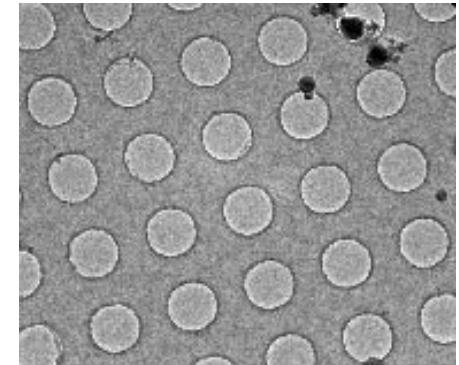
# Specimen support



EM grid, Copper, 3 mm



Home-made

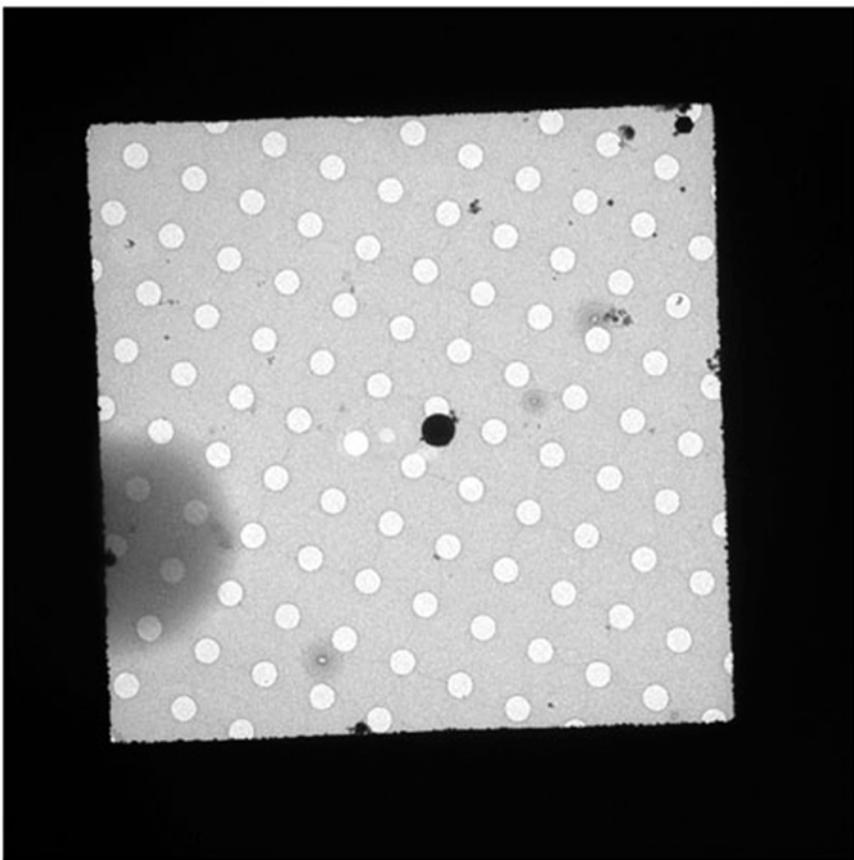


Quantifoil or C-flat

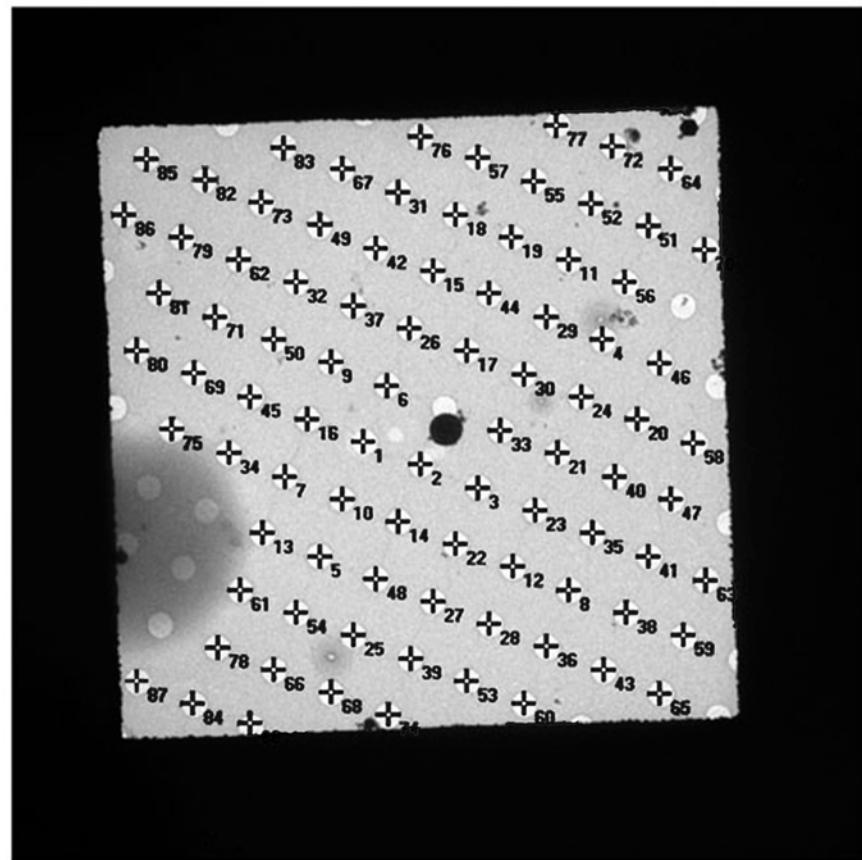
Carbon layer on EM grid

Quantifoil vs. C-flat grids: different edges, different thickness, different geometry for meniscus

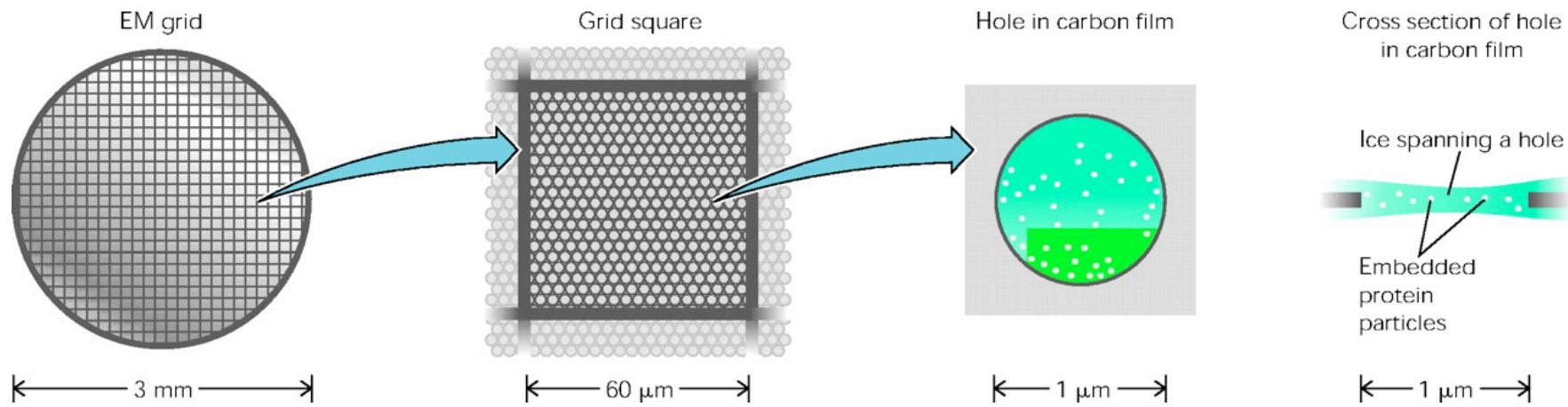
(a)



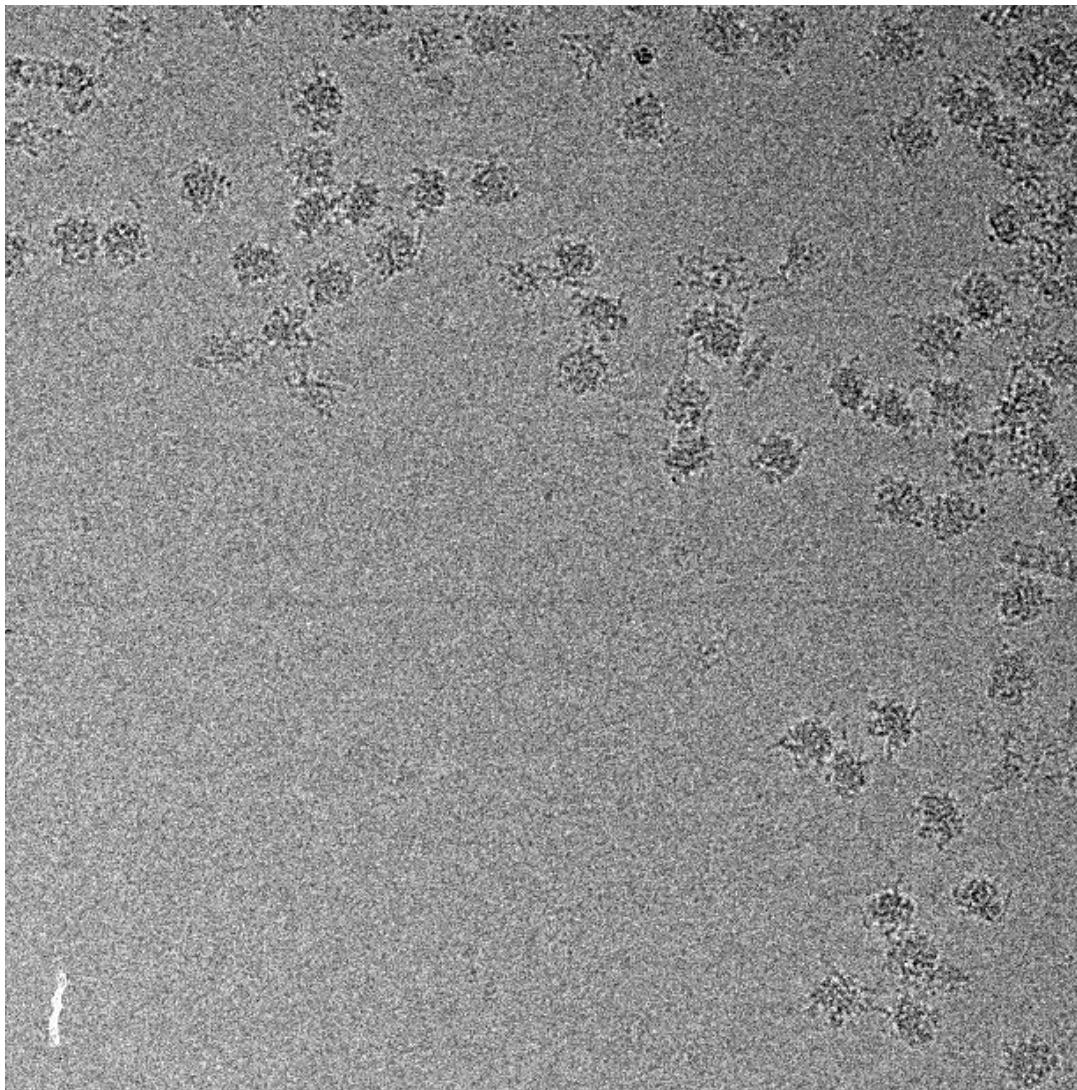
(b)



Holes regularly arranged on a Quantifoil grid enable fully automated data acquisition  
e.g. with LEGION



## EXAMPLE OF MENISCUS EFFECT: MOLECULES ACCUMULATE NEAR EDGE OF HOLE



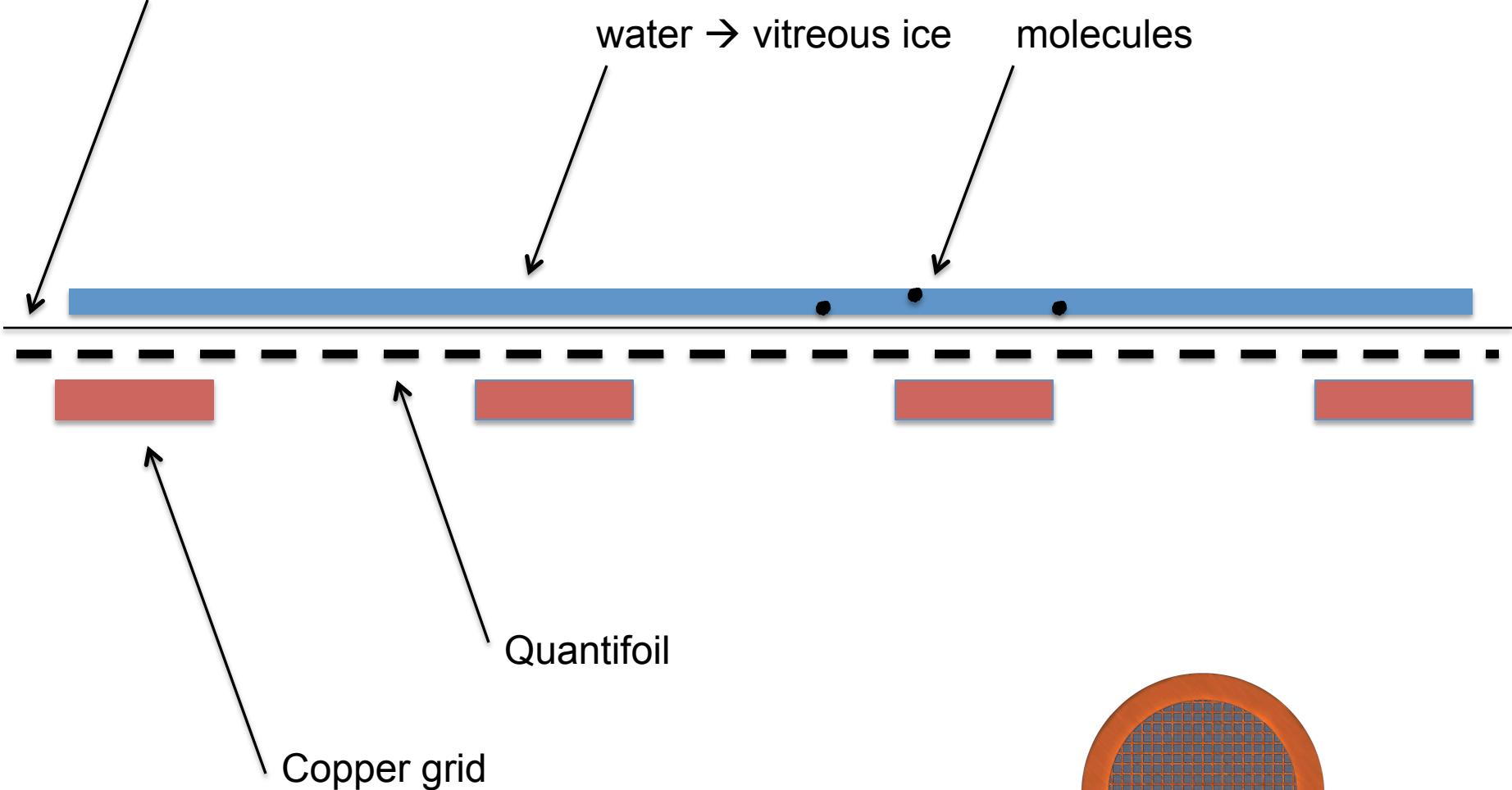
Thin ice

Thick ice

thin carbon (optional)

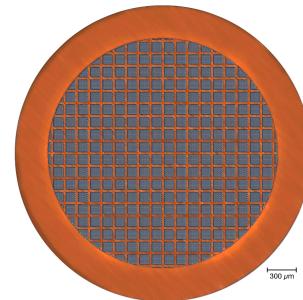
water → vitreous ice

molecules



OPTIONAL: Thin carbon (~100 Å), produced by evaporation on mica, floated onto Quantifoil-coated grid.

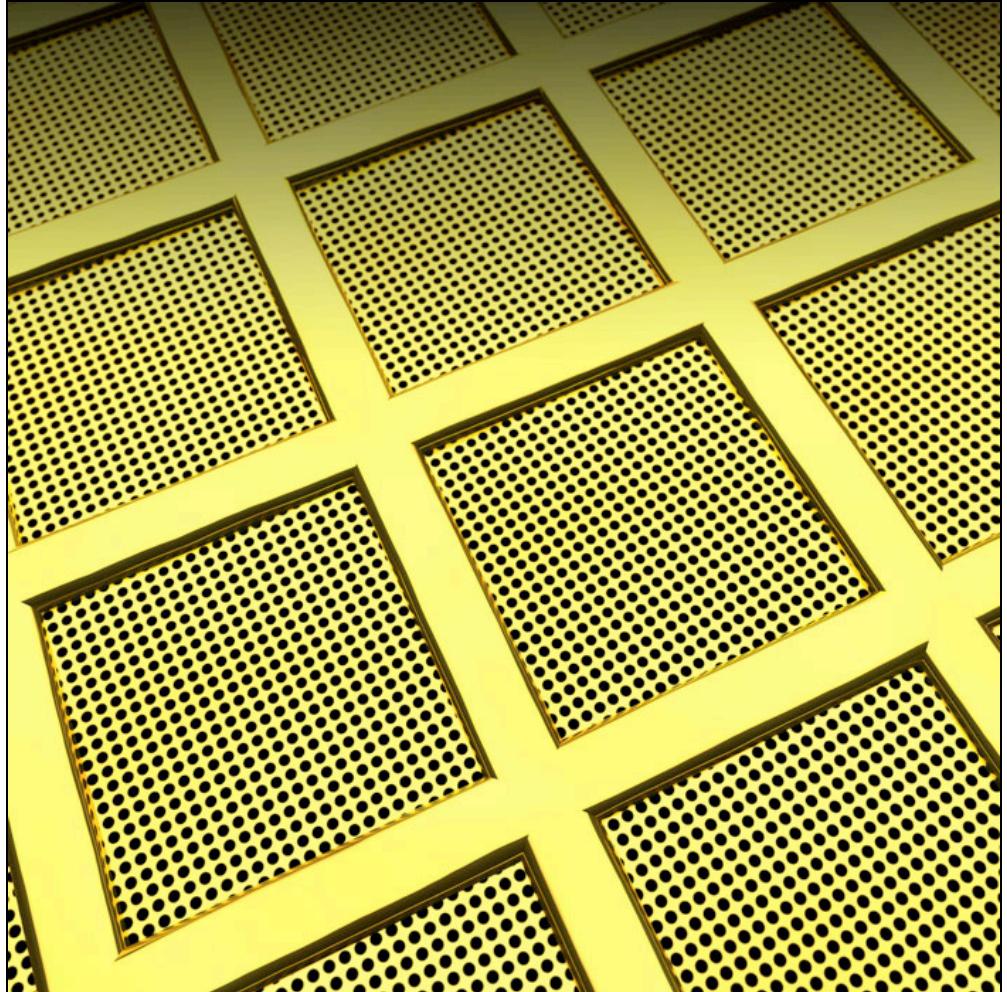
- (1) Enhance signal of power spectrum, for CTF determination
- (2) Induce more even coverage of orientations for some molecules (e.g., ribosomes)



## GOLD GRIDS

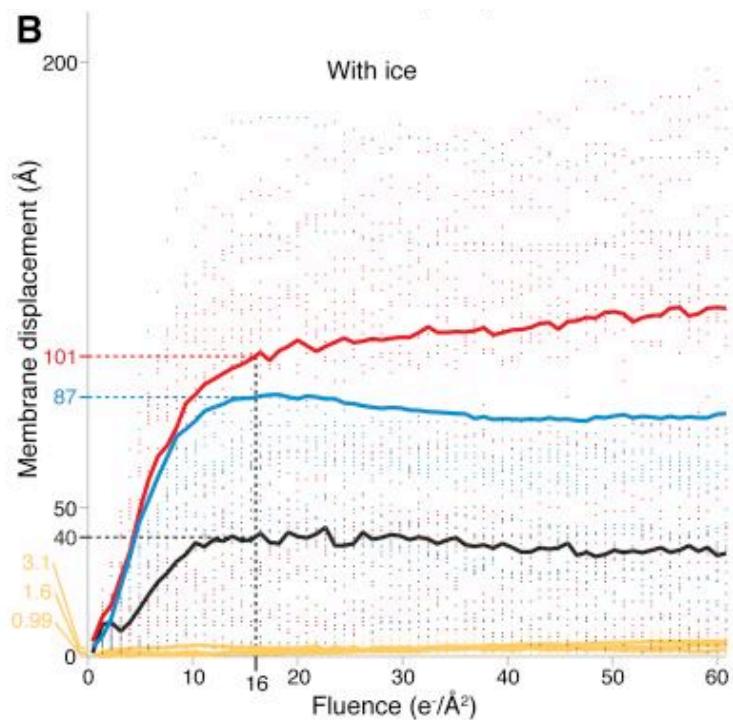
John Russo and Lori Passmore discovered that the carbon over the grid square oscillates like a drum, moving up and down. There is a sideways component, as well.

Gold grids reduce this effect 50-fold.



Russo and Passmore, Science 2014

## GOLD GRIDS VERSUS CARBON GRIDS: VIBRATION IN Z-DIRECTION



# The Single-Particle Approach to Averaging and Reconstruction in EM of Macromolecules

“Single” = unattached, free from contacts with other molecules.

This affects methodology of specimen preparation, electron microscopy, and image processing.

## *Why single particles?*

### **Advantages:**

- native conformation, unaffected by crystal packing
- functionally meaningful states can be visualized
- no part of the molecule needs to be chopped off for visualization
- multiple states visualized from the same sample
- ideal for looking at the dynamics of a molecular machine

### **Disadvantages (up to 2012):**

- large computational challenges
- atomic resolution difficult to achieve for particles lacking symmetries

# The Single-Particle Approach to Averaging and Reconstruction in EM of Macromolecules

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- no part of the molecule needs to be chopped off for visualization
- multiple states visualized from the same sample
- ideal for looking at the dynamics of a molecular machine

### **Disadvantages (2012 onwards):**

- large computational challenges
- ~~atomic resolution is as yet difficult to achieve without symmetries~~ (DED cameras)

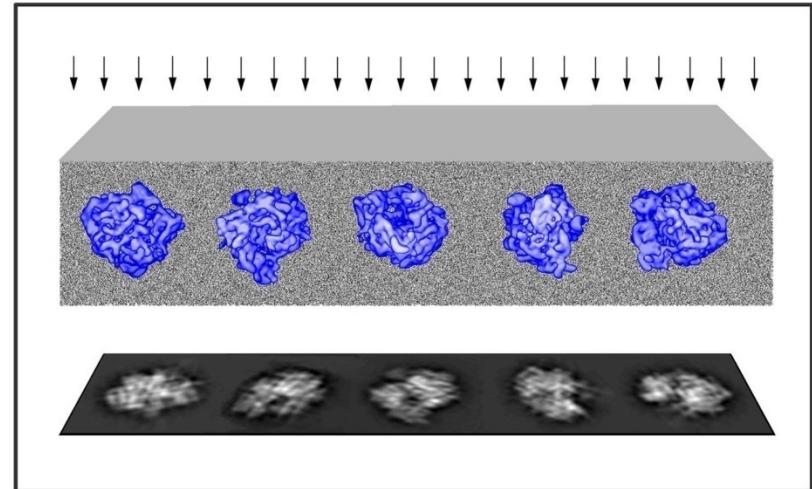
# Single-Particle Reconstruction

## Main initial assumptions in signal processing:

- 1) All particles in the specimen have (approx.) identical structure
- 2) All are linked by 3D rigid body transformations (rotations, translations)
- 3) Particle images are interpreted as a “signal” part (= the projection of the common structure) plus “noise”

*Important requirement:*

even angular coverage, without major gaps.

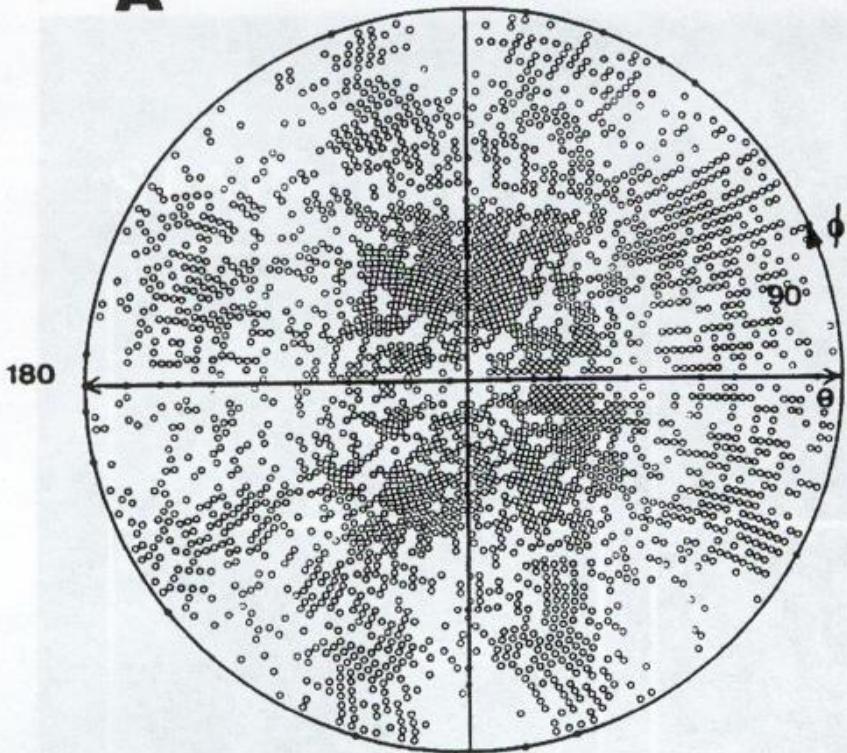


# 3D reconstruction requires even angular coverage

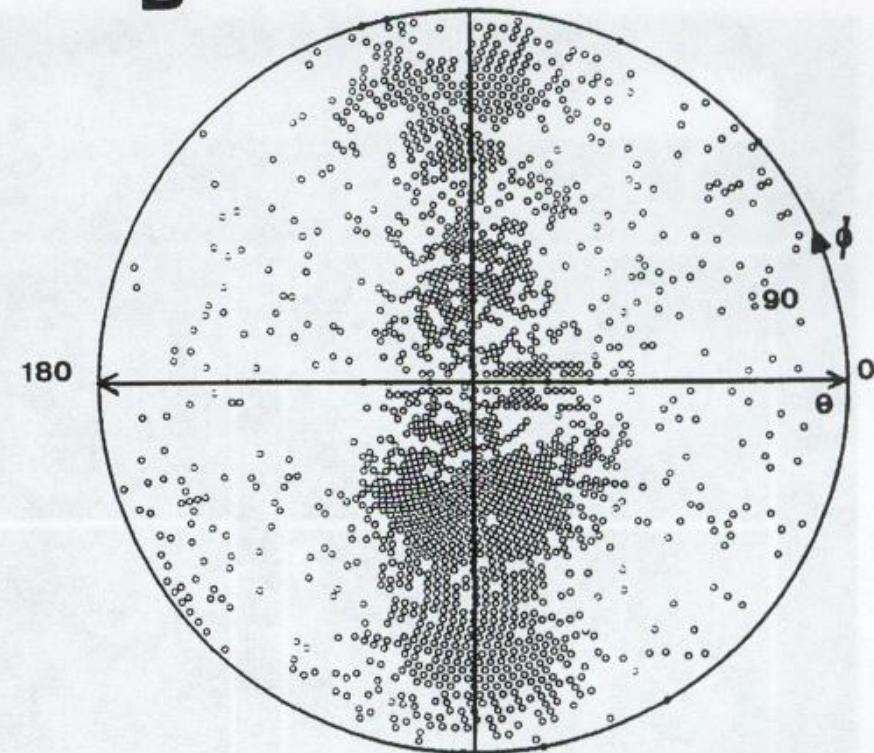
good

bad

**A**



**B**



“global coverage”

“single-axis-like coverage”

# Angular coverage

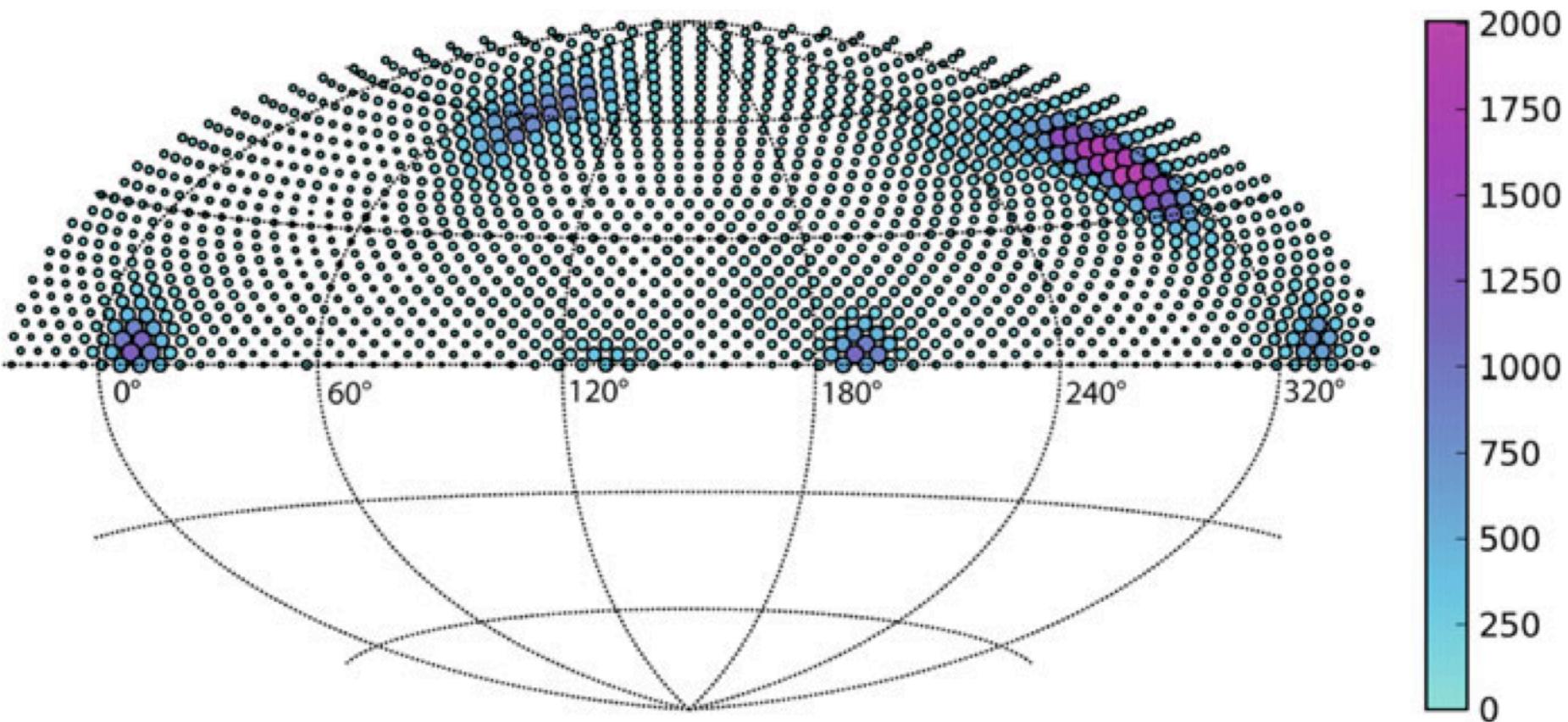
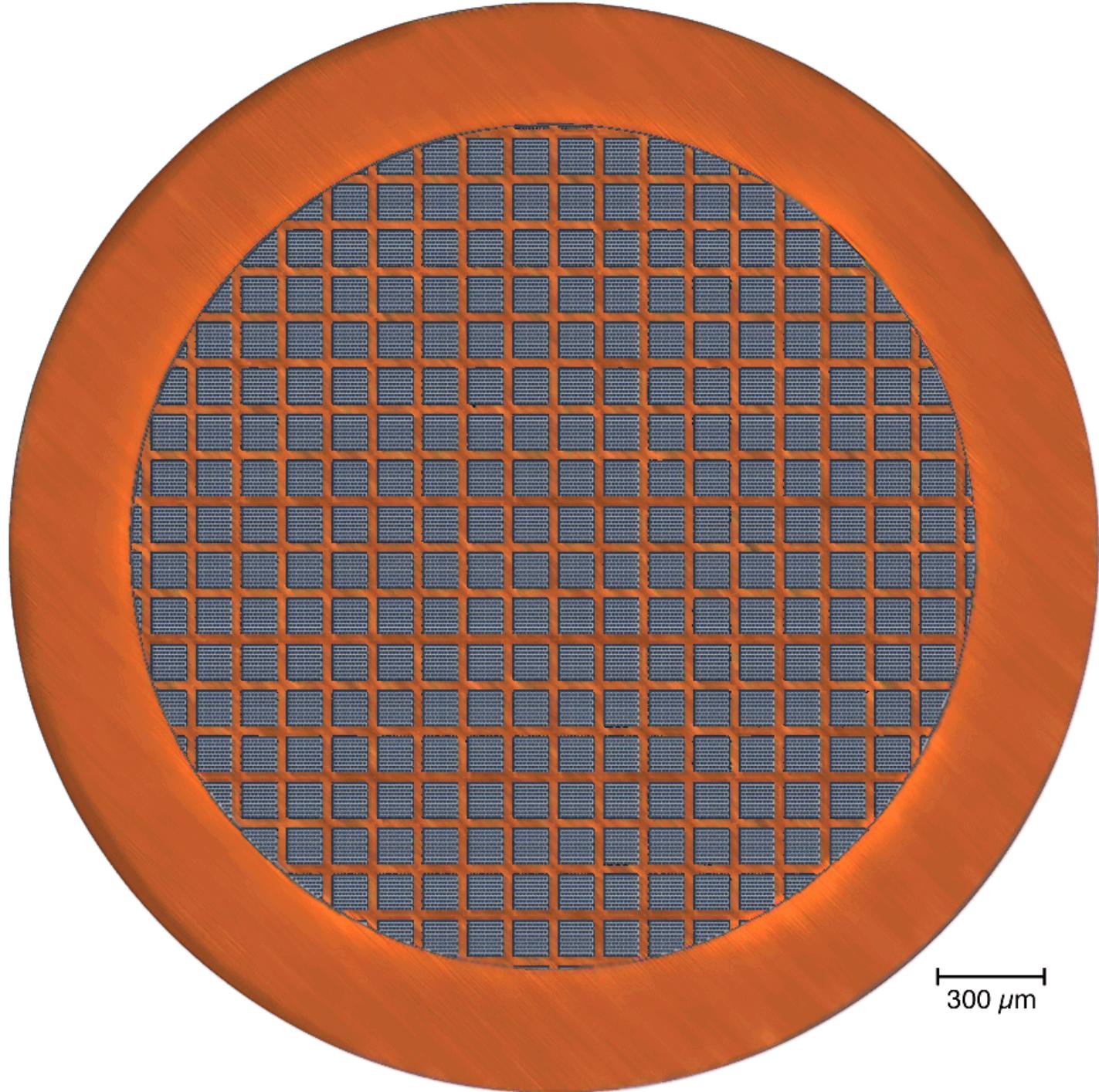
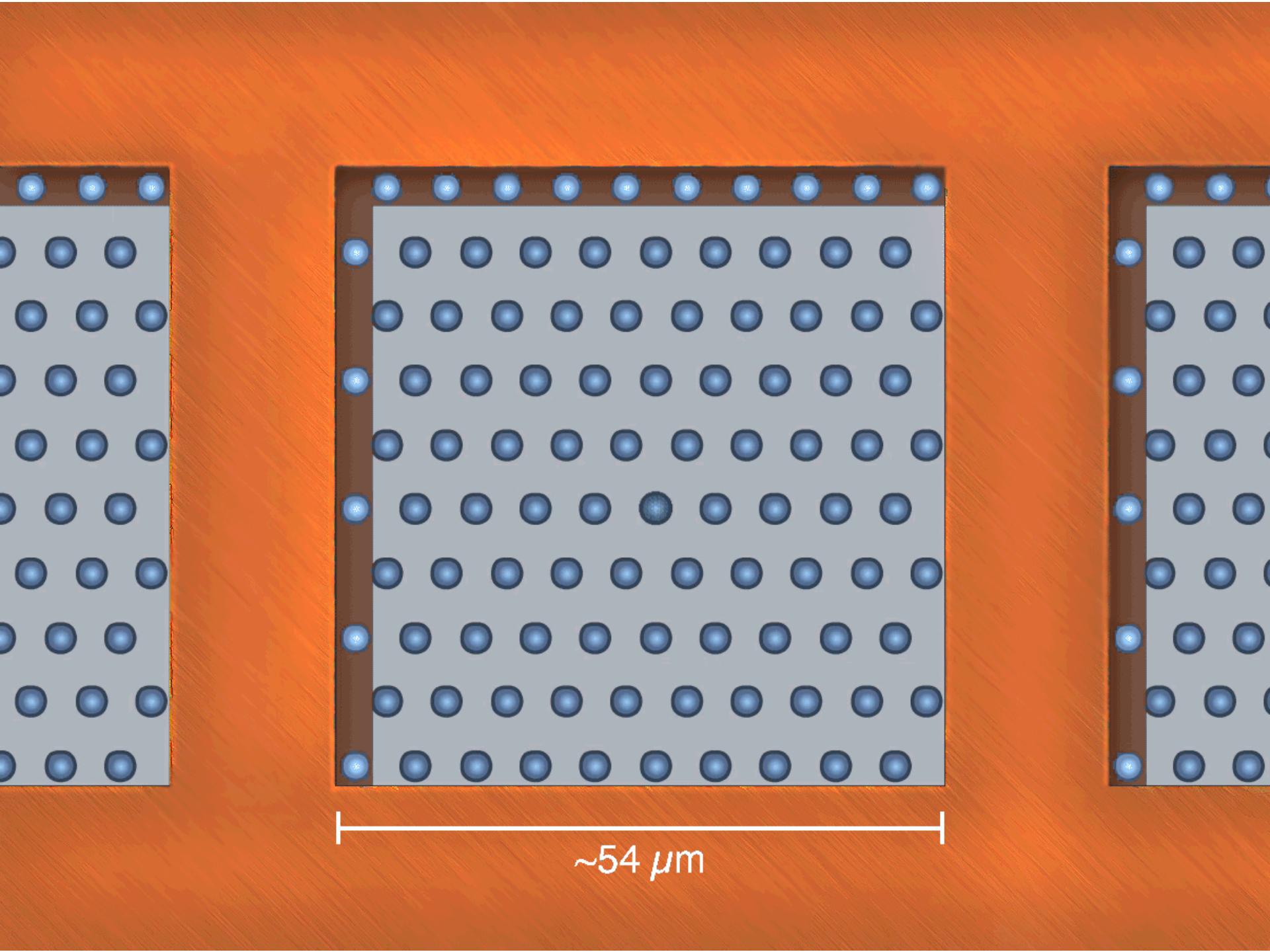


Illustration of sample on grid:

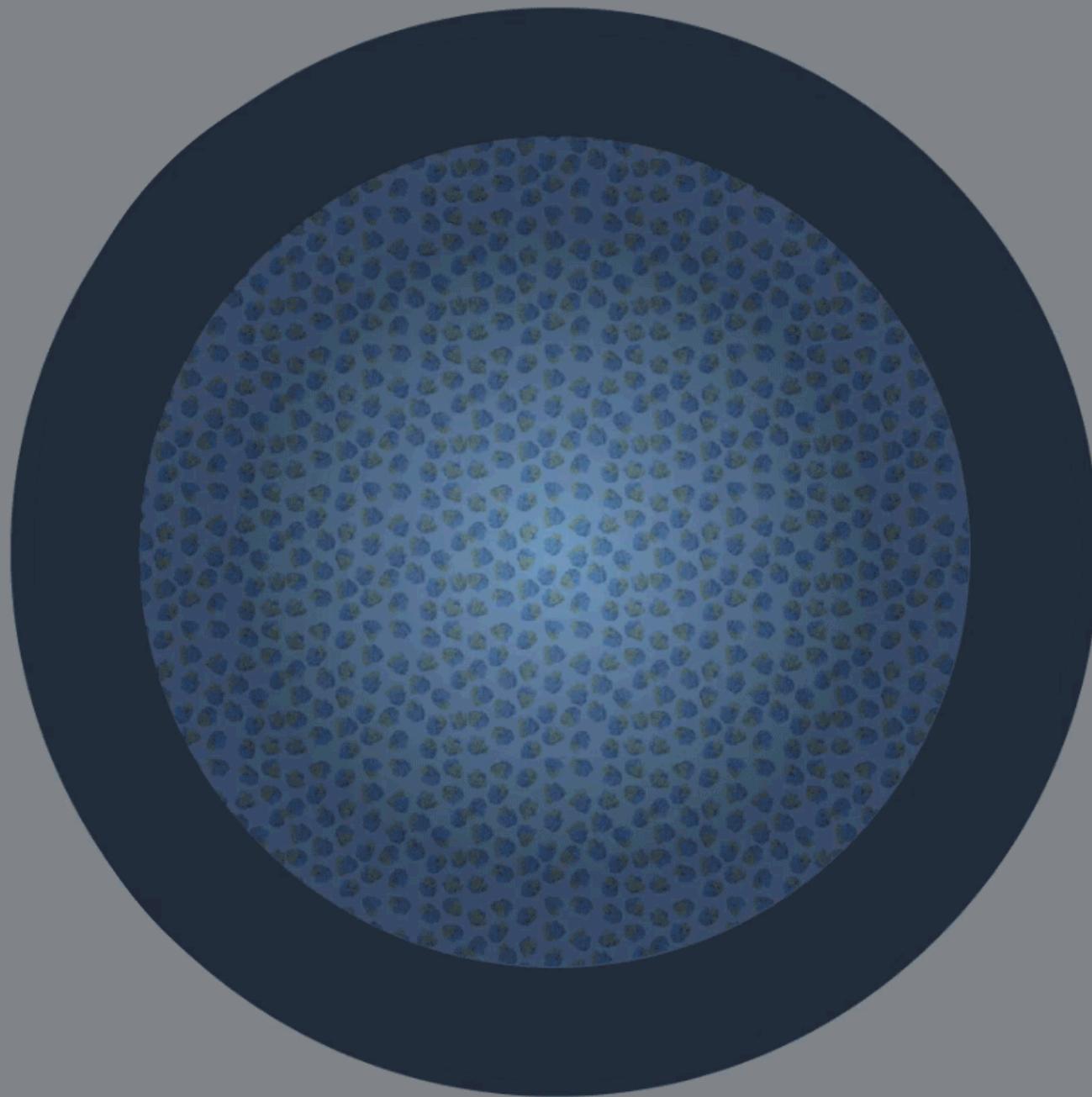
After blotting, the grid is covered with thin layer of liquid containing molecules



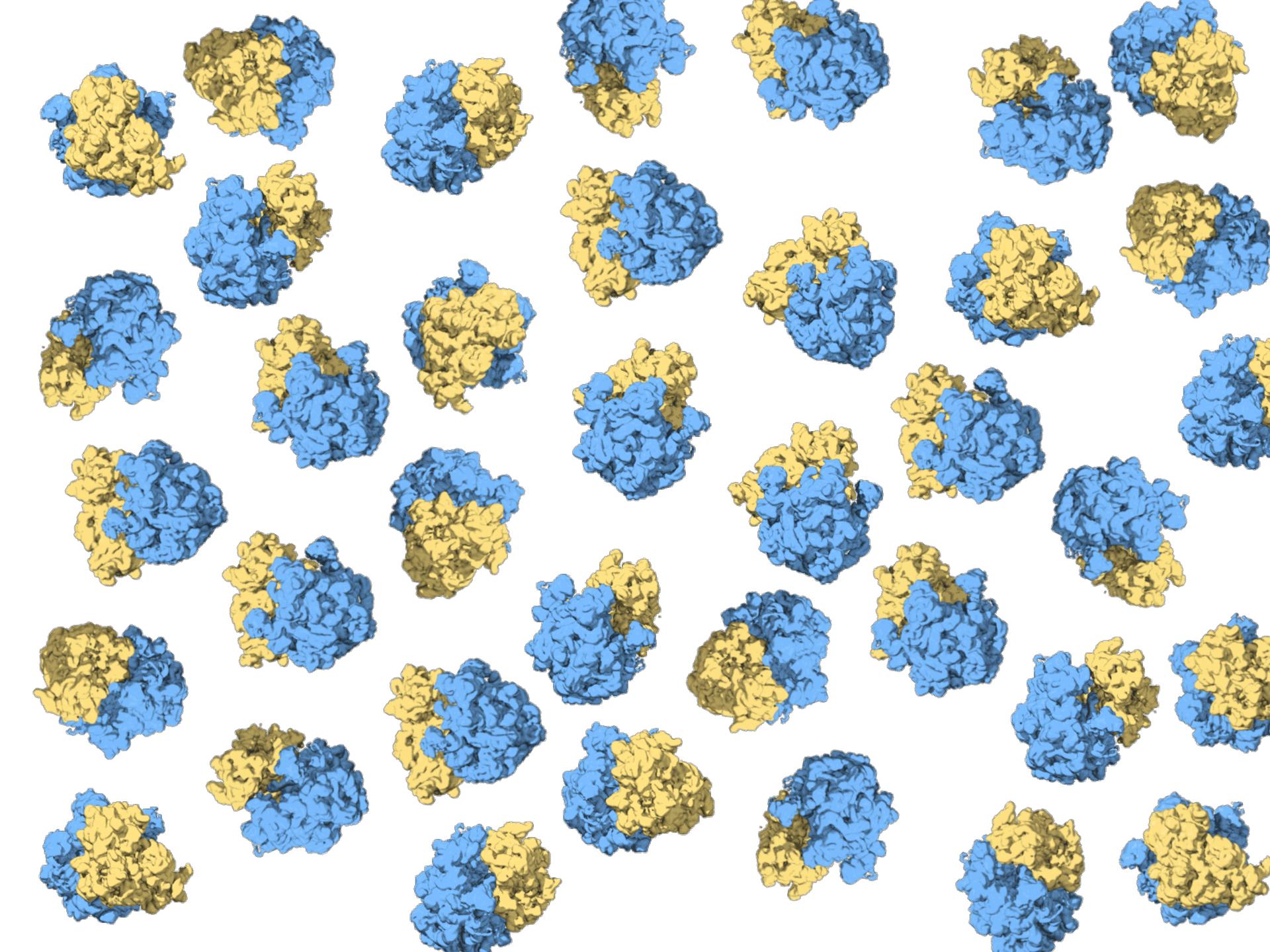
300  $\mu$ m

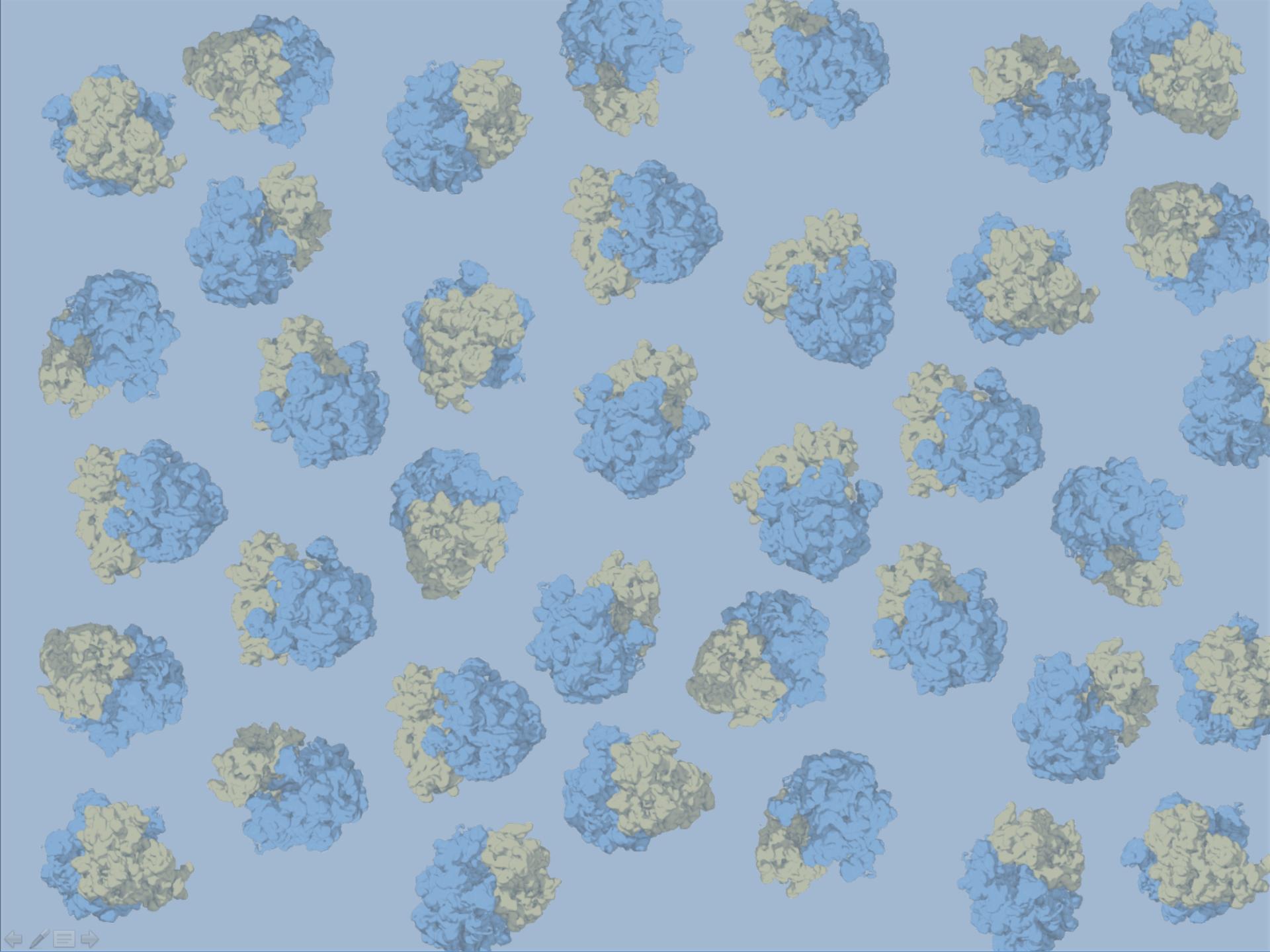


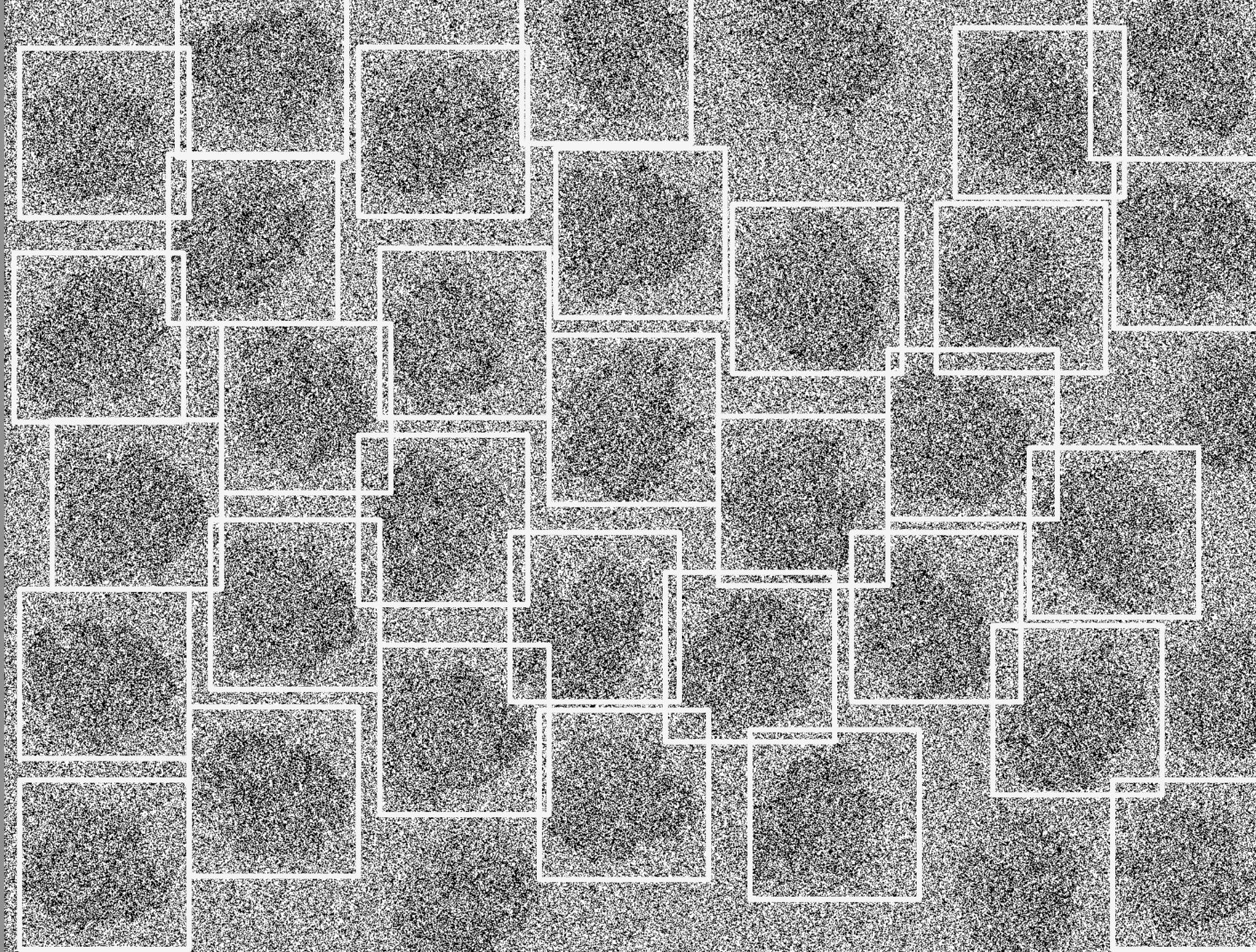
$\sim 54 \mu\text{m}$

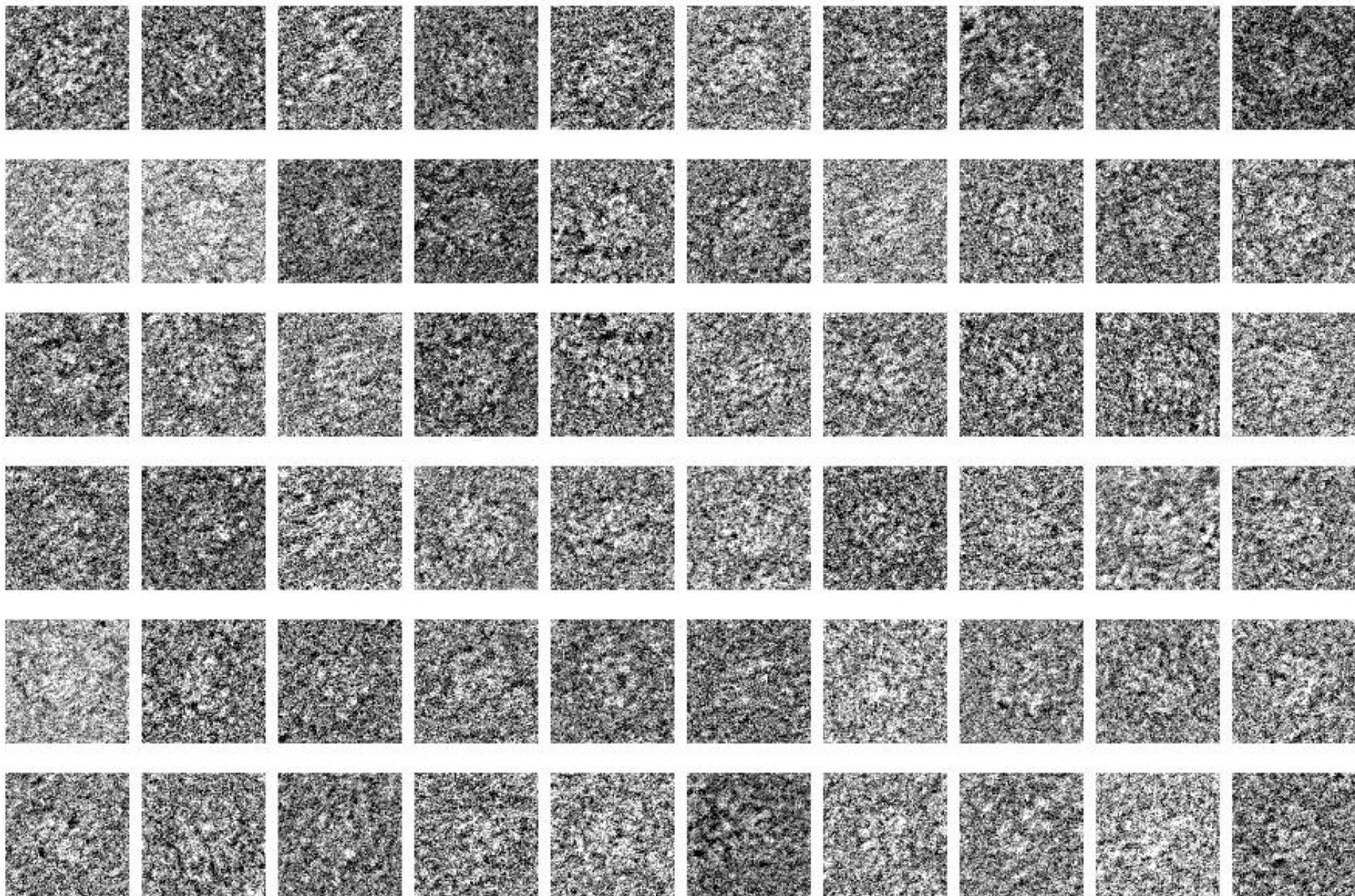


~1  $\mu\text{m}$

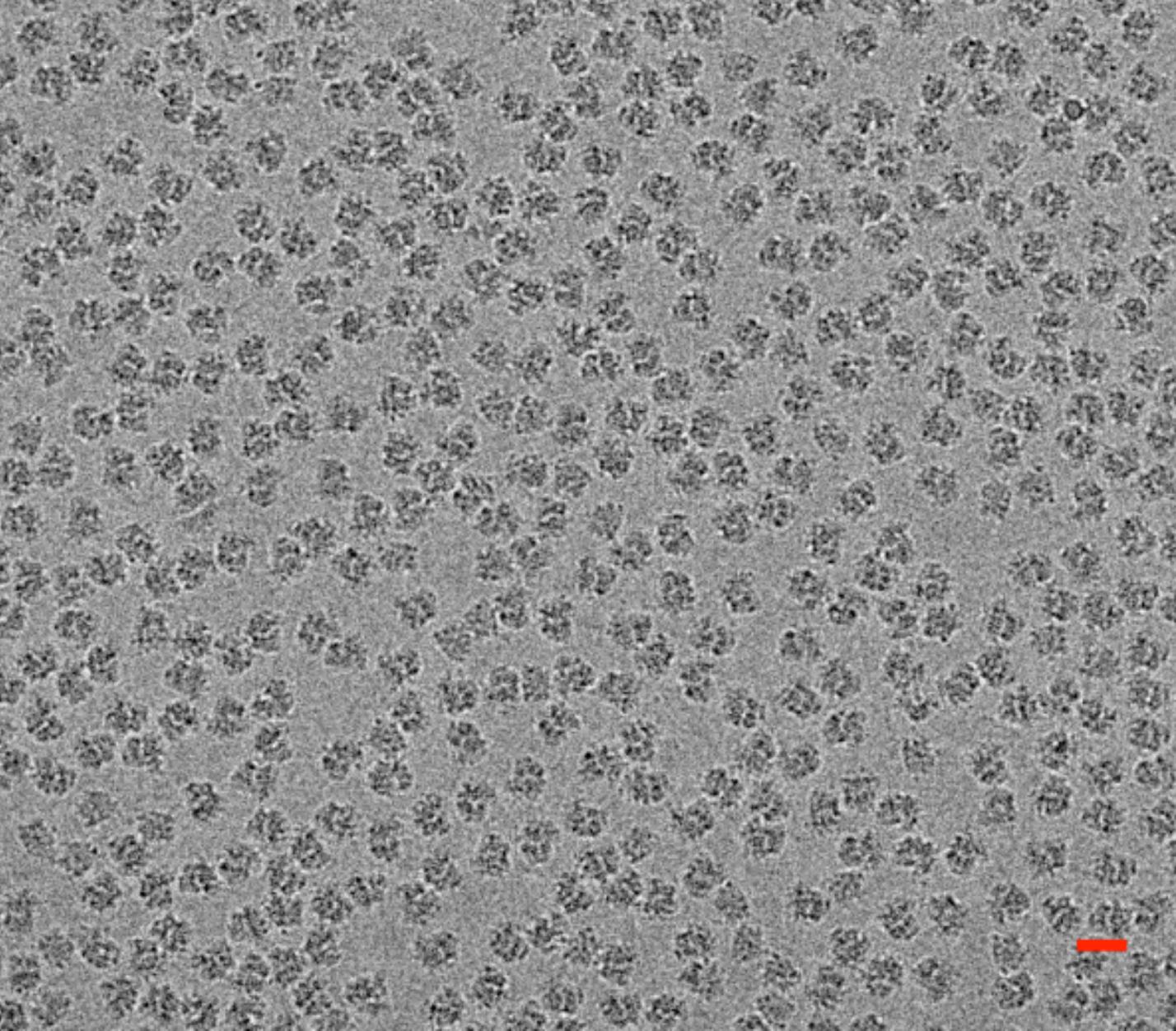




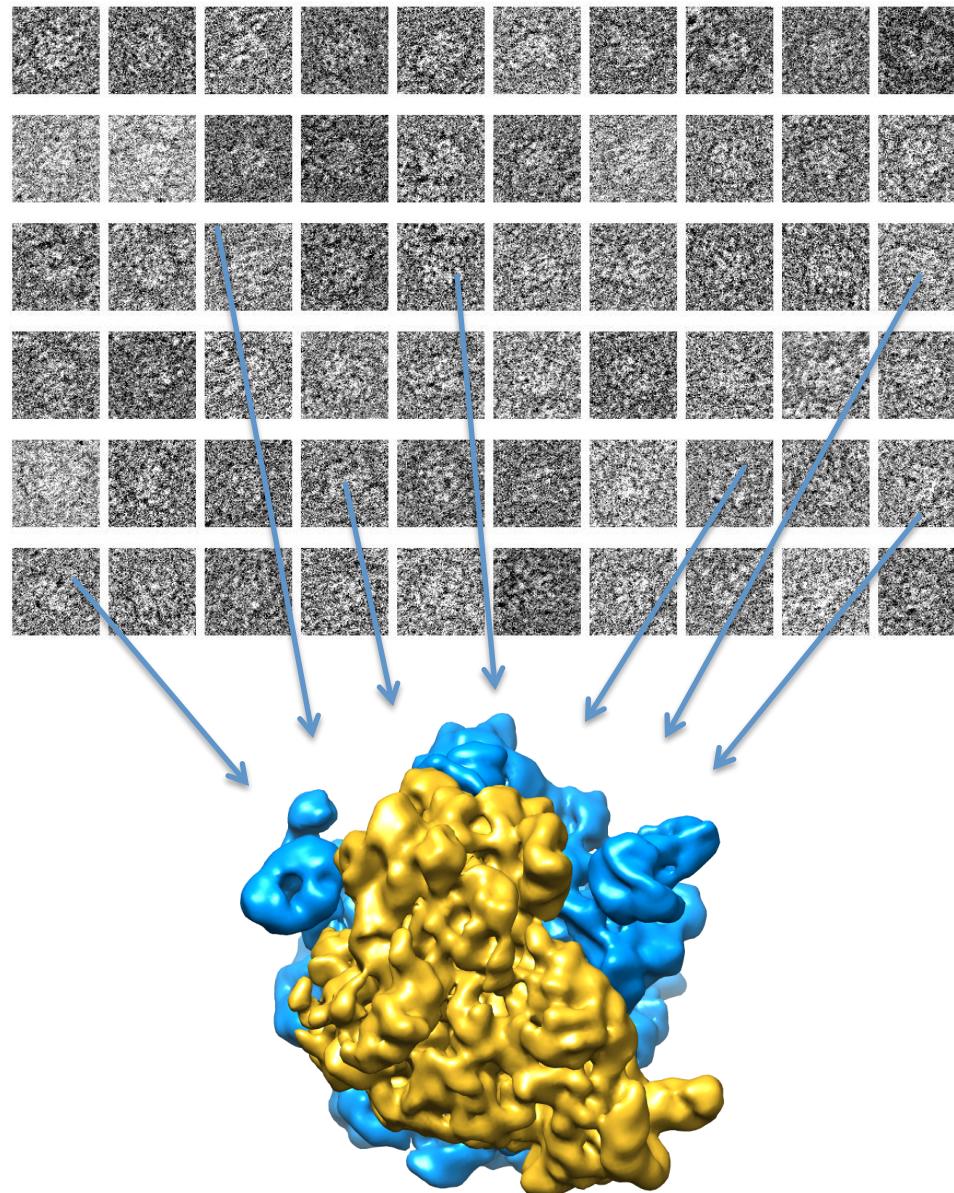




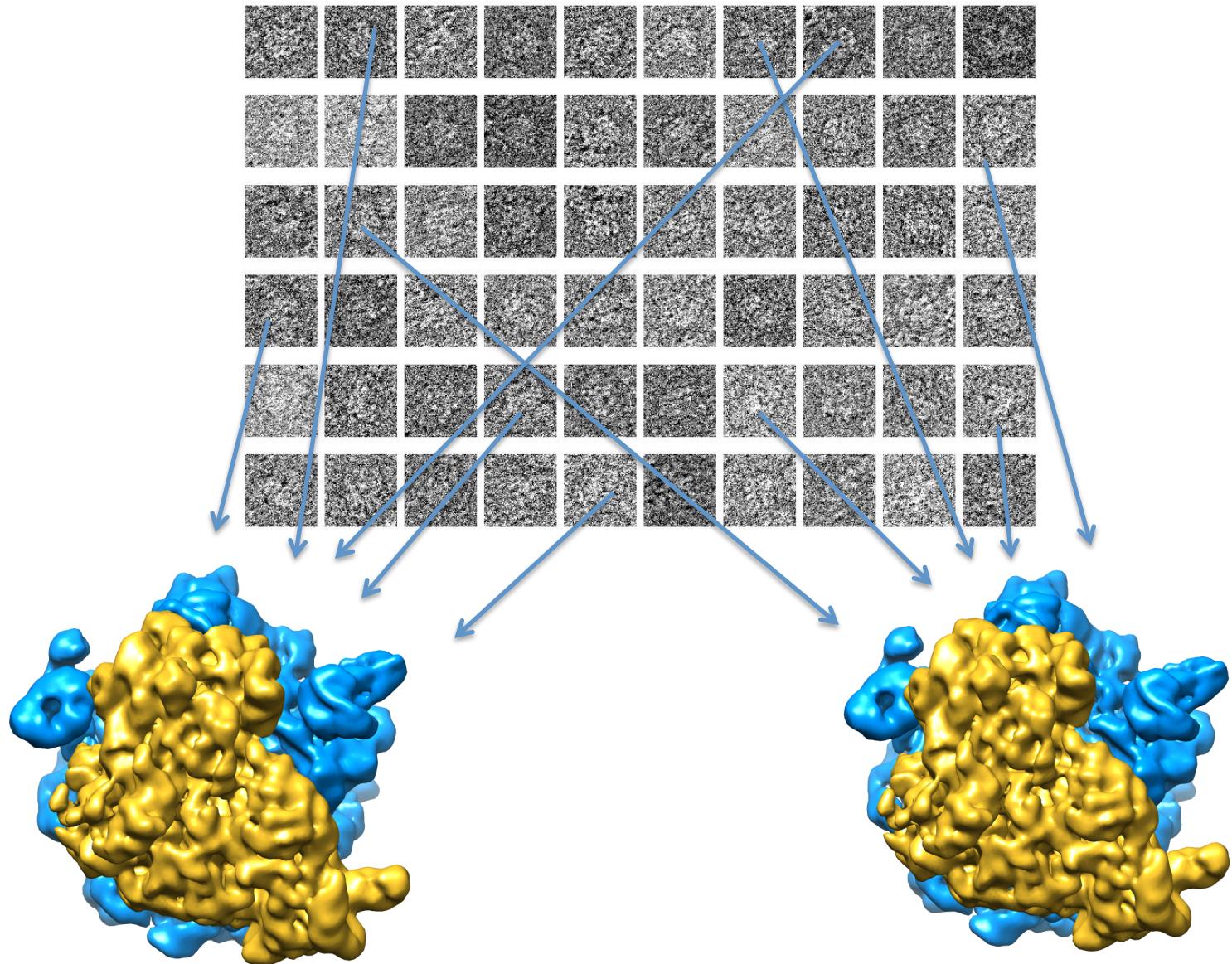
Micrograph of eukaryotic ribosomes, recorded with direct electron detection camera



# Determine orientations, 3D Reconstruction



# Image classification



# Single-Particle Methods are also used for ordered assemblies

Ordered assemblies never strictly follow the symmetries, so image processing relying entirely on the exact validity of the symmetries will fail to retrieve the high-resolution information. Applications of single particle methods:

- Helical order (e.g., acetylcholine receptor, actin-myosin fiber) – Ed Egelman's (and Pawel Penczek's) newer methods.
- 2D crystalline order (e.g., purple membrane protein) – “unbending” approach vs “patch averaging” method.
- Icosahedral order (e.g., adenovirus) – radical departure from initial MRC-based analytical methods.

*General principle: (1) use symmetry to roughly locate the repeats, then (2) refine position by using the cross-correlation function (CCF)*

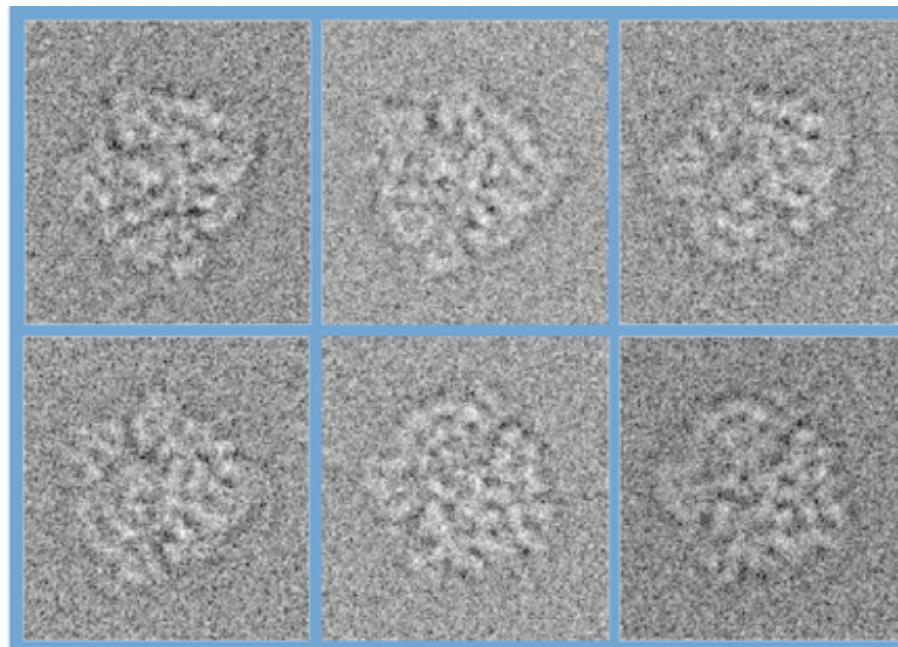
# Signal and noise -- definition of SNR

- Signal  $s(r)$  (predictable, deterministic, originating from the object)  
versus
- Noise  $n(r)$  (stochastic; unrelated to the signal; aperiodic [no two realisations are the same])
- Signal-to-noise ratio (SNR) = signal variance/noise variance
- Averaging over  $N$  noisy realizations of a signal increases the SNR by a factor of  $N$
- *Note that what is signal and what is noise in a given experiment depends on the way the experiment is designed.*

## “Shot Noise”

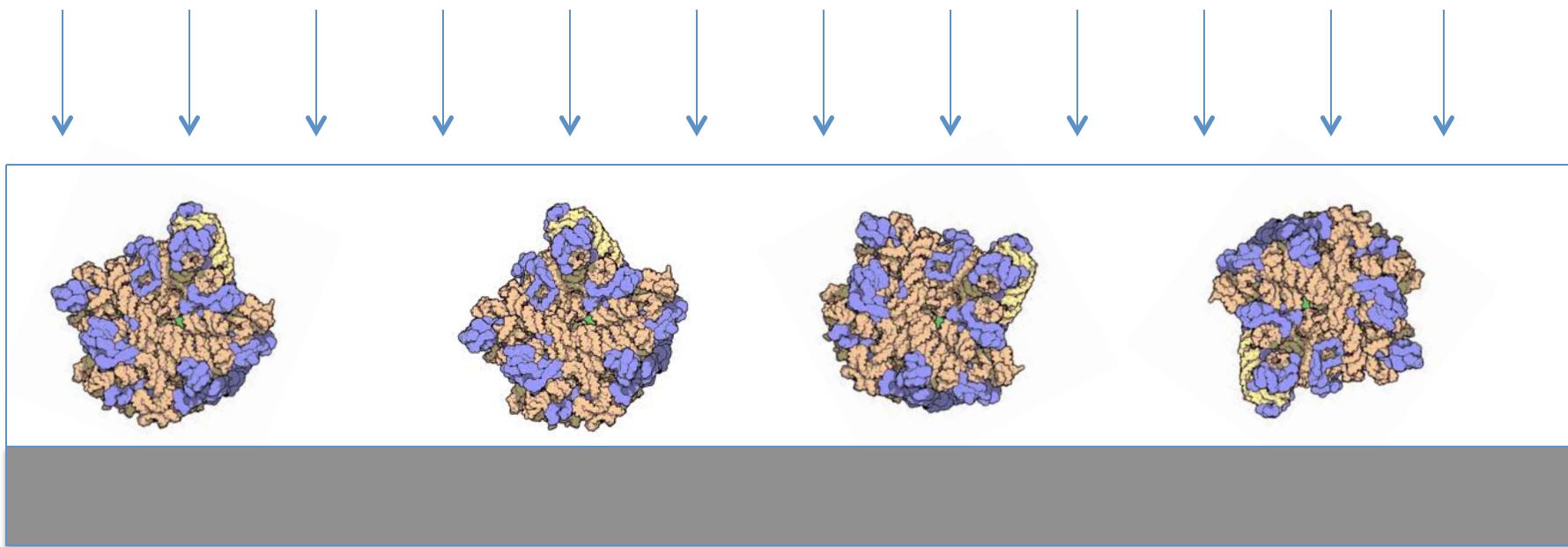
At the low exposure settings (e.g.,  $10 \text{ e}/\text{\AA}^2$ ), required to avoid radiation damage, the fluctuations of the electron distribution is a serious source of noise, called shot noise. Low-exposure images typically have an SNR of 0.1 (signal variance is only one tenth of noise variance).

Only by averaging over a sufficient number of particle projections can the original signal be retrieved.



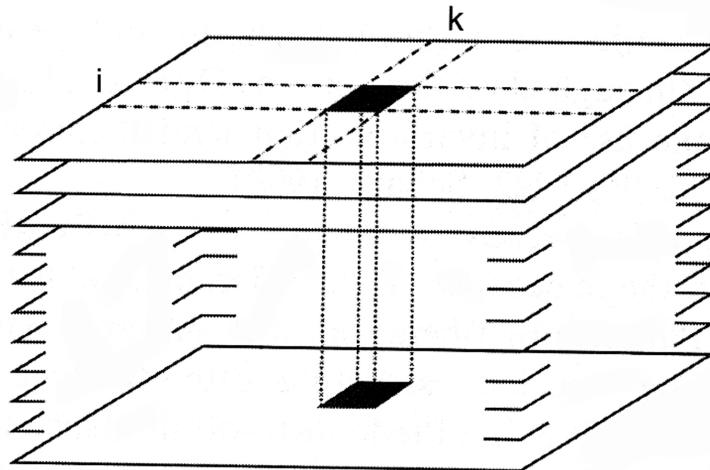
Simulated images of ribosome at SNR  $\sim 0.1$

## “Structural Noise”



The matrix of ice, and carbon deposit (if used) has a unique structure which is superimposed when a projection image is formed. When images of particles are averaged, the superimposed structure of the surrounding must be considered “noise” since it is not reproducible from one particle to the next..

## Two-dimensional processing: averaging of like images to eliminate noise



Averaging requires the images to be aligned: in the array, each image element, or pixel, must refer to the same point of the object.

Note: averaging in 2D only makes sense for molecules presenting the exact same view

# Averaging, to improve the SNR

Averaging can be done in one of two ways:

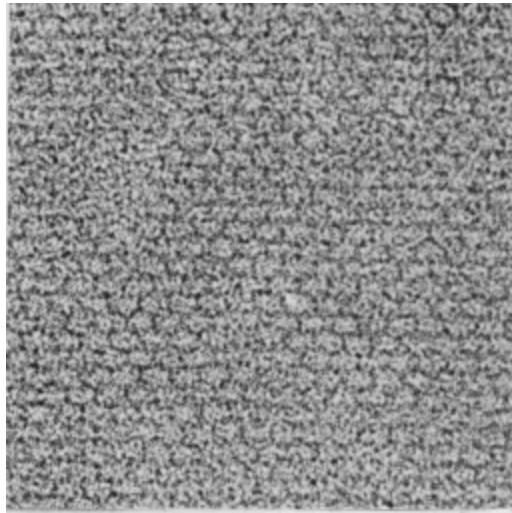
-- either --

make use of order or symmetries to locate repeats

– or –

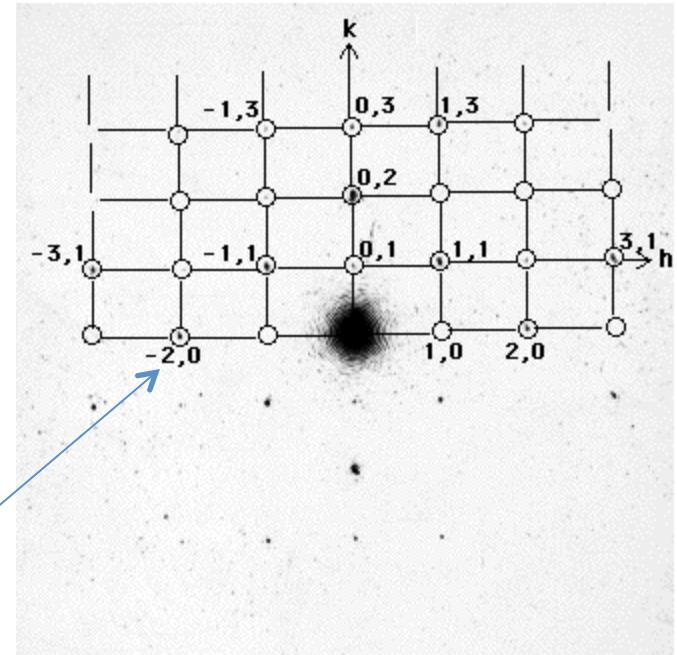
make use of cross-correlation search to locate repeats

In the first case, Fourier methods can be used instead of real-space averaging



Projection image of molecule  
ordered on a periodic lattice.  
**Noise is intermixed.**

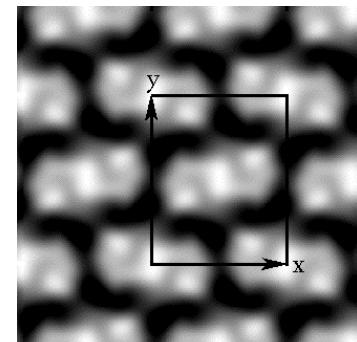
*2D Fourier transform*



Fourier transform is concentrated in spots  
on the reciprocal lattice. **Noise is separated.**

Indices define position on  
reciprocal grid  
Fourier transform is complex,  
each spot described by  
an **amplitude** and a **phase** of  
a component wave

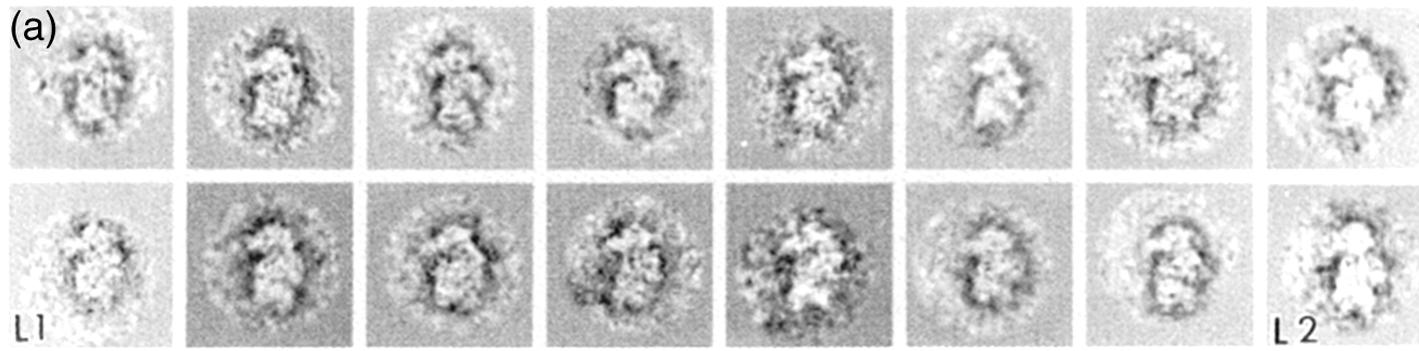
*Inverse Fourier  
transform*



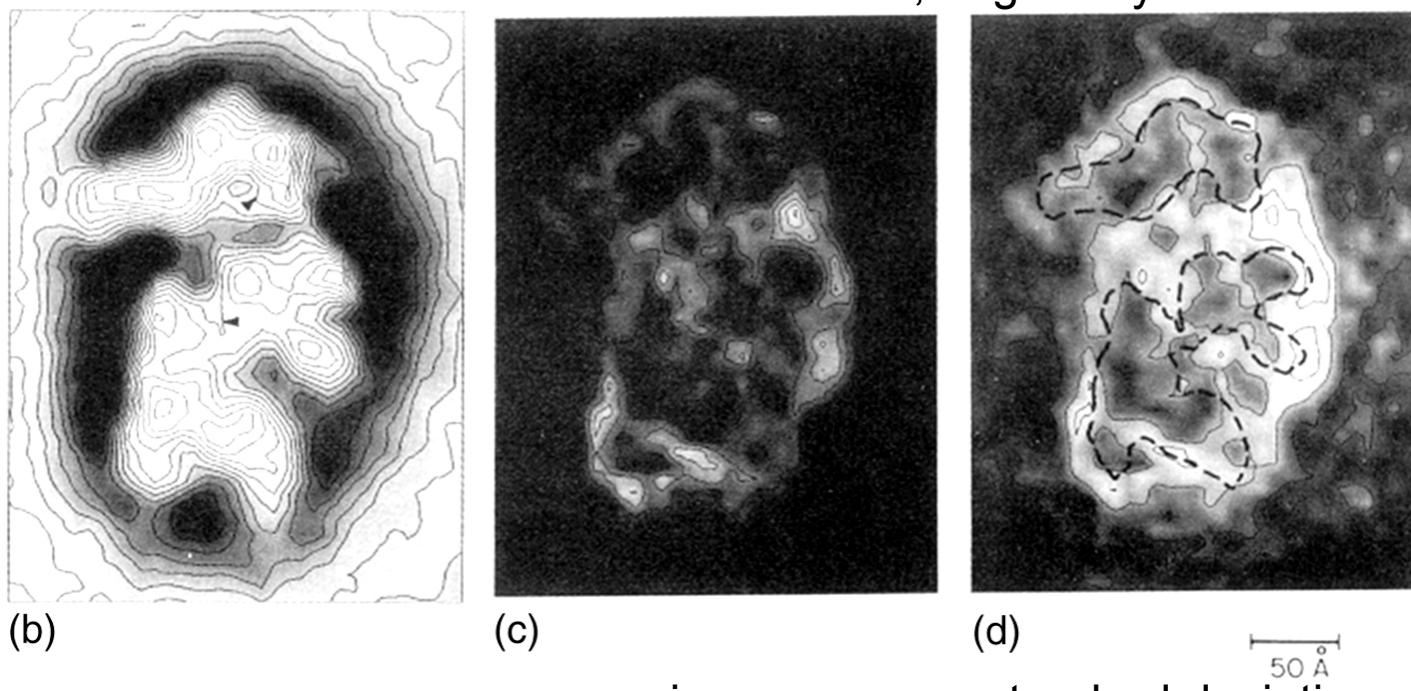
**1) make use of order or symmetries to  
locate repeats**

Noise-free projection image

2) make use of cross-correlation search  
to locate repeats.



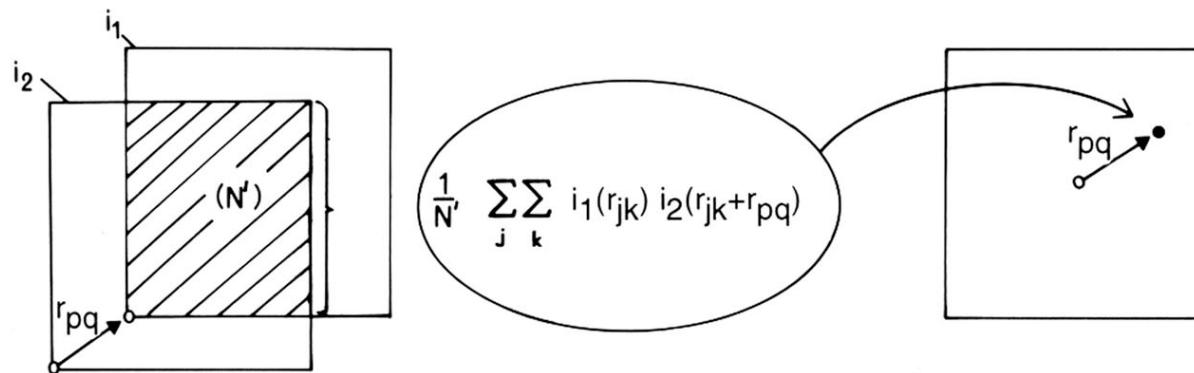
40S ribosomal subunits of HeLa cells, negatively stained



# Alignment of single-particle projections (“particles”) is achieved by cross-correlation

- Translational cross-correlation function (CCF)

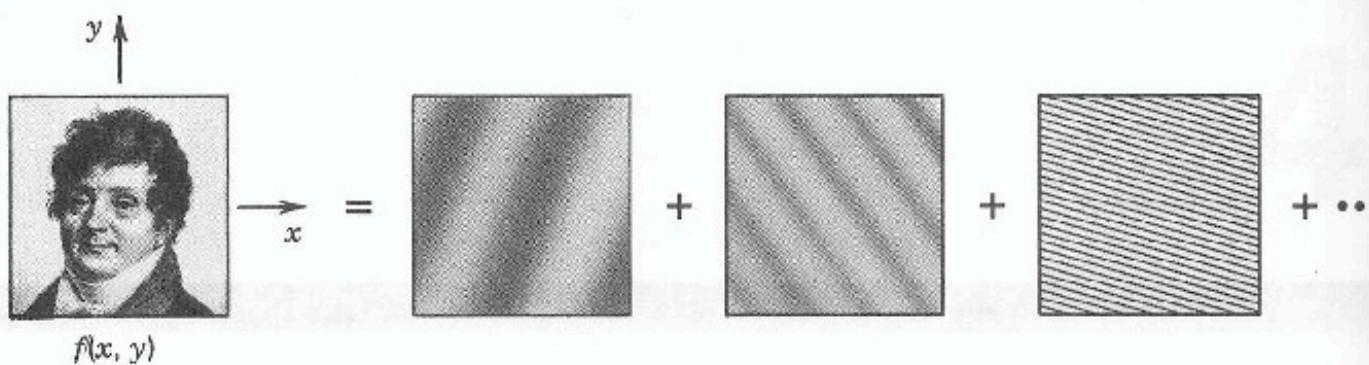
Discrete, unnormalized version:



**Fig. 3.8.** Definition of the cross-correlation function. Image 1 is shifted with respect to image 2 by vector  $r_{pq}$ . In this shifted position, the scalar product of the two images arrays is formed and put into the CCF matrix at position  $(p, q)$ . The vector  $r_{pq}$  is now allowed to assume all positions on the sampling grid. In the end, the CCF matrix has an entry in each position. From Frank (1980). Reproduced with permission of Springer-Verlag, New York.

- Rotational CCF – the same summation as above, but this time one image is rotated with respect to the other

## 2D Fourier transform



$$F(K_x, K_y) = \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} f(x, y) \exp(i(K_x x + K_y y)) dx dy$$

An image can be considered a superposition of sine waves of different spatial frequencies running in different directions. Each sine wave is characterized by an **amplitude** and a **phase**.

**Alternative representation** (as in this diagram) employ complex exponential functions with complex coefficients.

**The Discrete Fourier Transform (DFT) in 2D can be defined as:**

$$F(u, v) = \frac{1}{MN} \sum_{m=0}^{M-1} \sum_{n=0}^{N-1} f(m, n) \exp \left[ -2\pi i \left( \frac{mu}{M} + \frac{nv}{N} \right) \right]$$
$$u = 0, 1, \dots, M - 1, \quad v = 0, 1, \dots, N - 1$$

**The inverse 2D DFT is given by:**

$$f(m, n) = \sum_{u=0}^{M-1} \sum_{v=0}^{N-1} F(u, v) \exp \left[ 2\pi i \left( \frac{mu}{M} + \frac{nv}{N} \right) \right]$$
$$m = 0, 1, \dots, M - 1, \quad n = 0, 1, \dots, N - 1$$

An image  $f(m, n)$  is represented as a finite series of 2D exponentials with complex coefficients  $F(u, v)$ .

The discrete Fourier representation implies repetition of the image on an infinite lattice



## Notations:

Fourier transform:  $F(\mathbf{k}) = \mathcal{F} \{f(\mathbf{r})\}$

Inverse Fourier transform:  $f(\mathbf{r}) = \mathcal{F}^{-1} \{F(\mathbf{k})\}$

$\mathbf{r} = (x, y)$

$\mathbf{k} = (k_x, k_y)$

“spatial frequency”

Fourier operator

Lower case

Upper case

# Parseval's Theorem -- conservation of power, or conservation of information content

$$F(k) = \mathcal{F} \{i(r)\}$$

definition:  $P(k) = |F(k)|^2$  is the “Power spectrum”

*Total power is the same in real and Fourier space:*

$$\int_{\phi} |F(k)|^2 dk = \int |i(r) - \text{avrg}|^2 dr$$

where  $\text{avrg} = 1/\text{area} \times \int i(r) dr$

and subscript  $\phi$  indicates “exclude origin in the integration”

**Application: Signal-to-Noise ratio can be computed in Fourier space:**

$$\text{SNR} = \int_{\phi} |S(k)|^2 dk / \int_{\phi} |N(k)|^2 dk$$

# Point spread function and Contrast transfer function

In an optical instrument, the aperture limit, the aberrations of the lens and other imperfections have the effect that a single point in the object is imaged as an extended 2D function, the so-called Point Spread Function (PSF)

The Fourier transform of the PSF in EM is the **Contrast Transfer Function (CTF)**.

In the Transmission EM, the CTF is given by an analytical expression:

$$CTF = \sin(\gamma(\mathbf{k}))$$

where

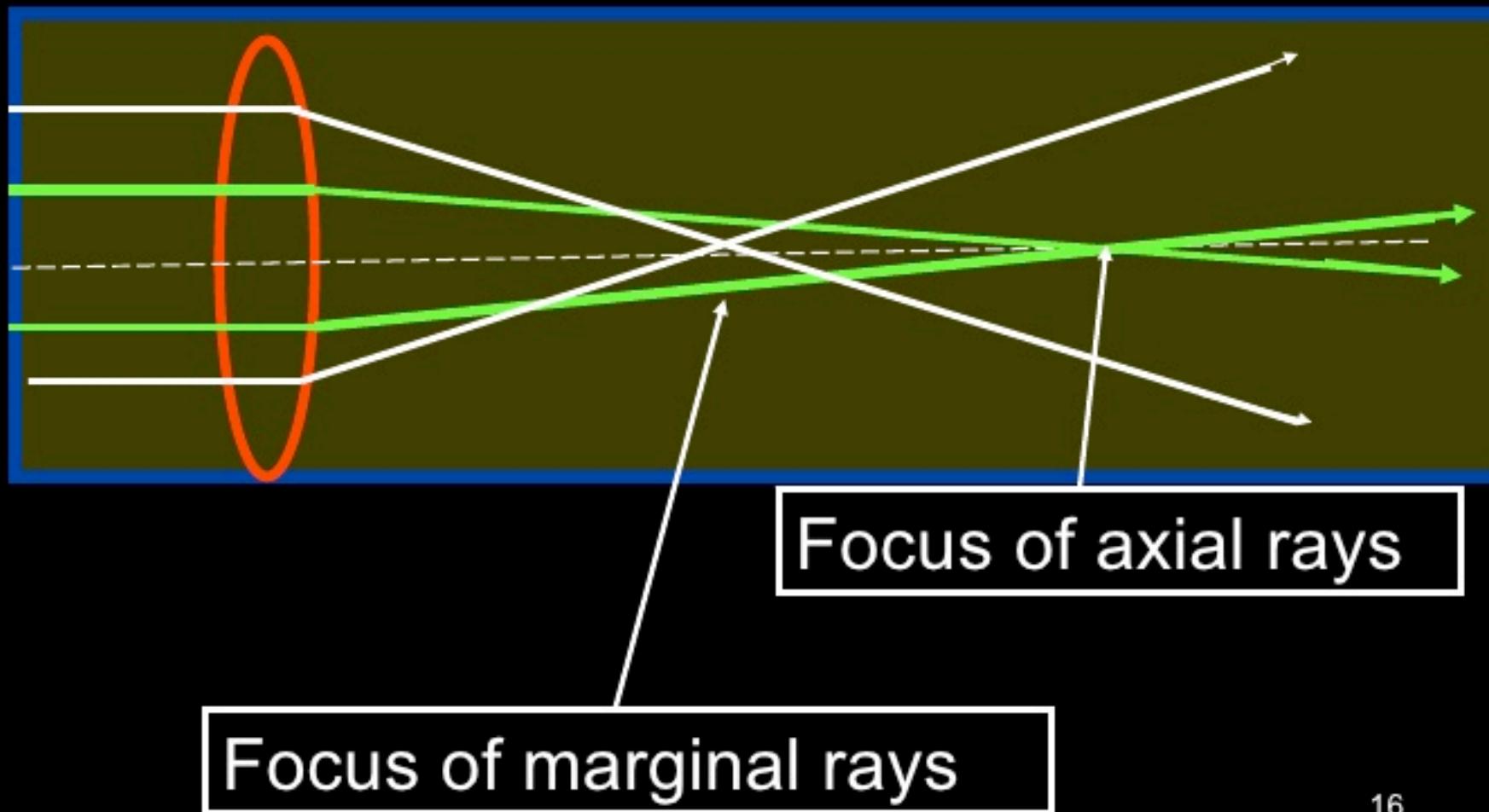
$$\gamma(\mathbf{k}) = -\pi\lambda \left[ \Delta z + \frac{z_a}{2} \sin 2(\phi - \phi_0) \right] k^2 + \frac{1}{4} \lambda^3 C_s k^4 \quad \text{wave aberration function}$$

defocus      ax. astigmatism      spherical aberration

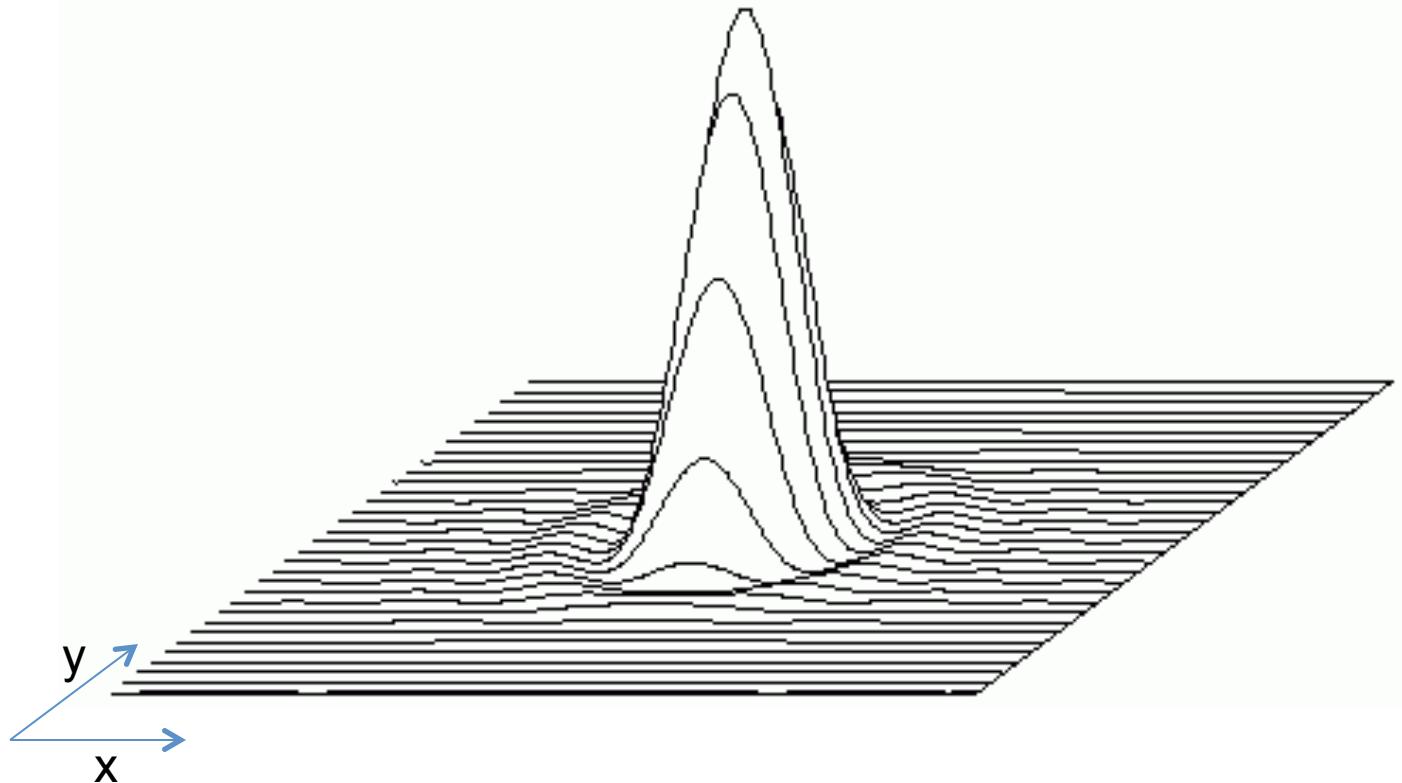


$\mathbf{k}$  = spatial frequency vector;  $k$  = length of this vector

- Spherical aberration

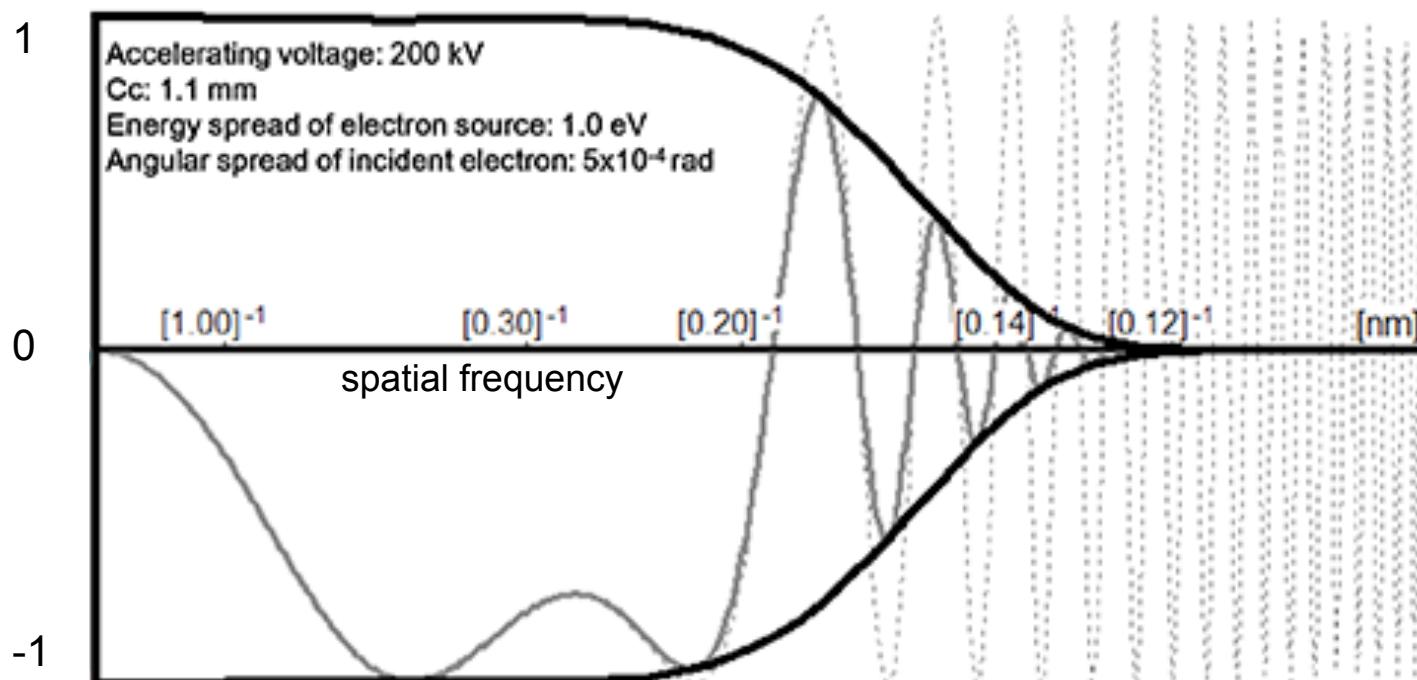


Point-Spread Function =  
Response of the optical instrument to a point object



The point spread function has finite width, and is centered at the location that the point would have in the image formed by an ideal instrument.

# Contrast transfer function



Envelope function due to  
energy spread  
and angular spread  
“partial coherence”

# Effects of energy spread and angular spread

- Energy spread: voltage changes → wavelength changes
- Defocus spread: defocus changes have approx. same effect as voltage changes

Envelope function due to energy spread/defocus spread is independent of defocus

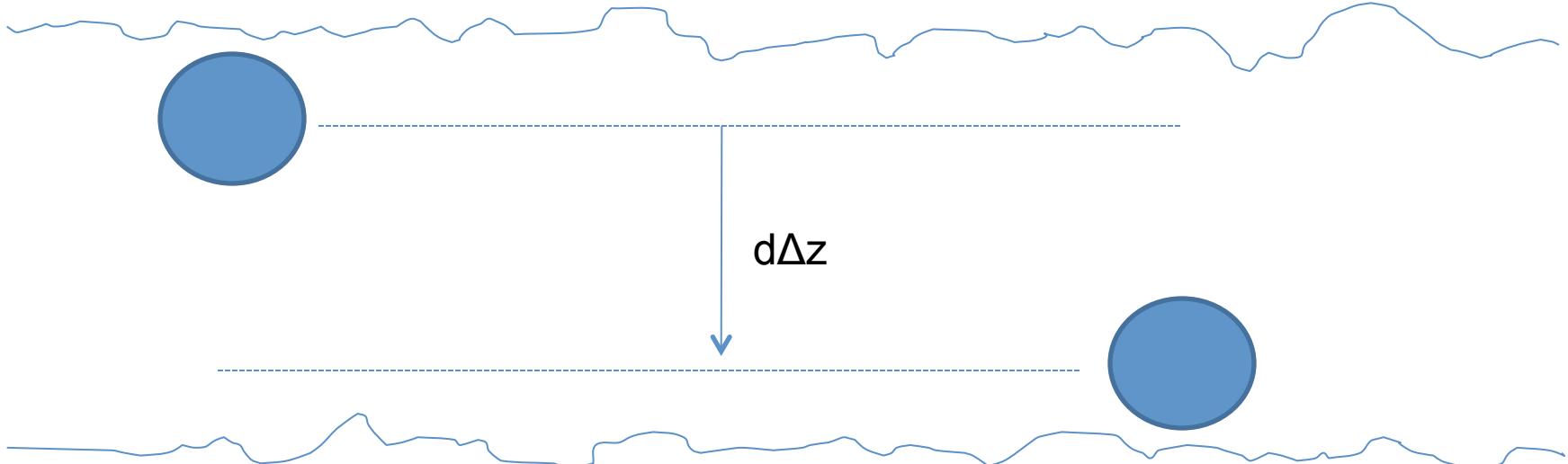
- Angular spread: point source replaced by extended source – convergent (non-parallel) illumination

Envelope function is defocus-dependent

$$\text{CTF}(k) = \text{CTF}_{\text{ideal}}(k) \times E_{\text{energy spread}}(k) \times E_{\text{angular spread}}(k)$$

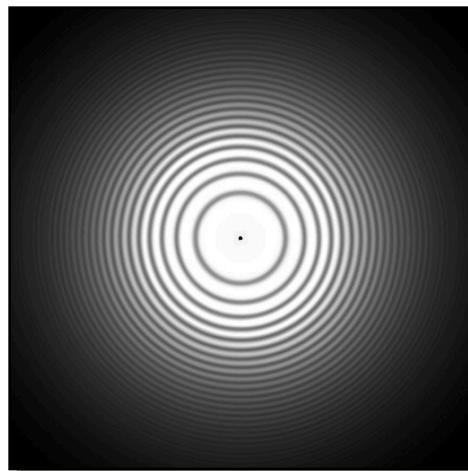
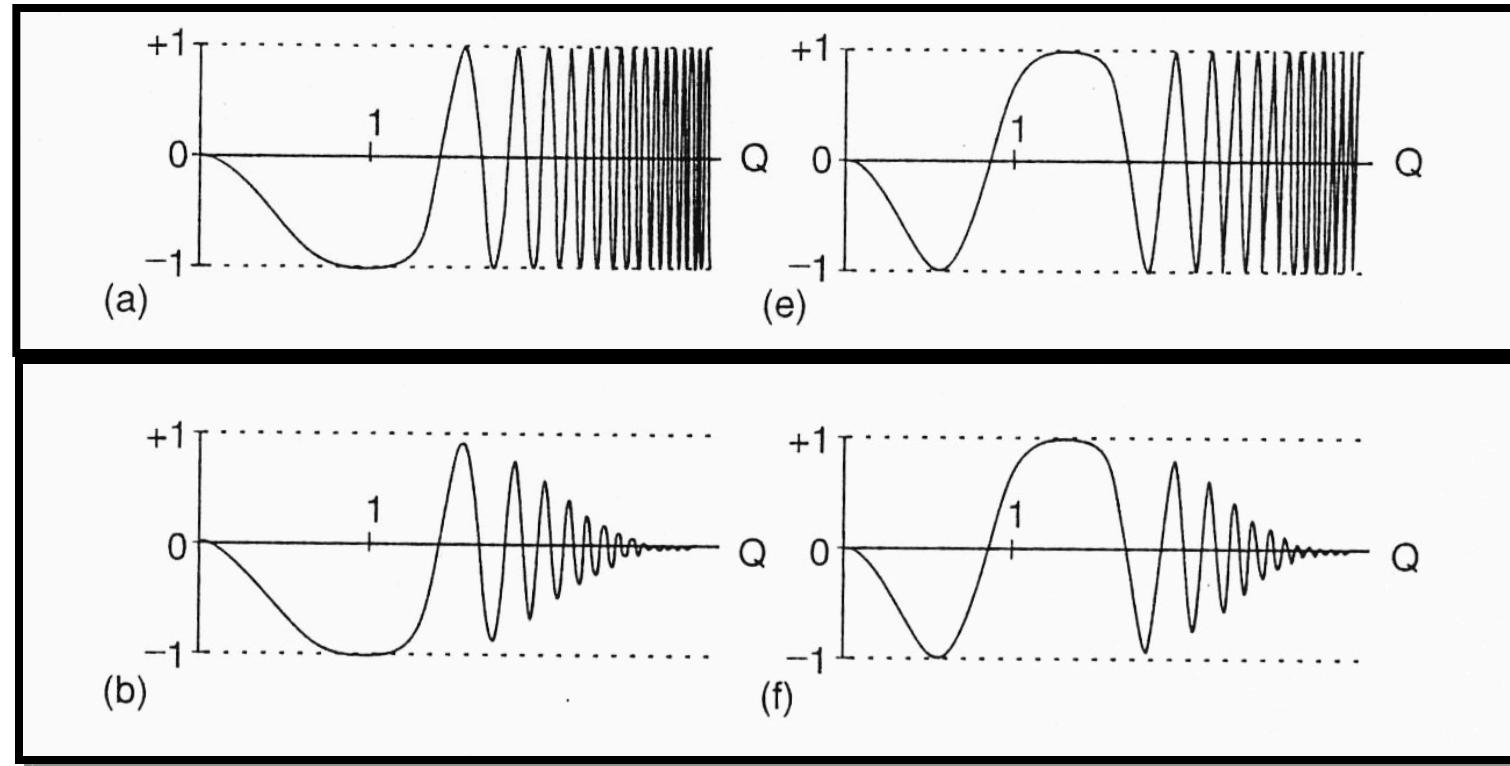
Defocus (and hence the CTF) is affected by the particle's z-position within the ice layer.

Ideally, defocus should be measured for each particle separately, but the signal is often not strong enough.



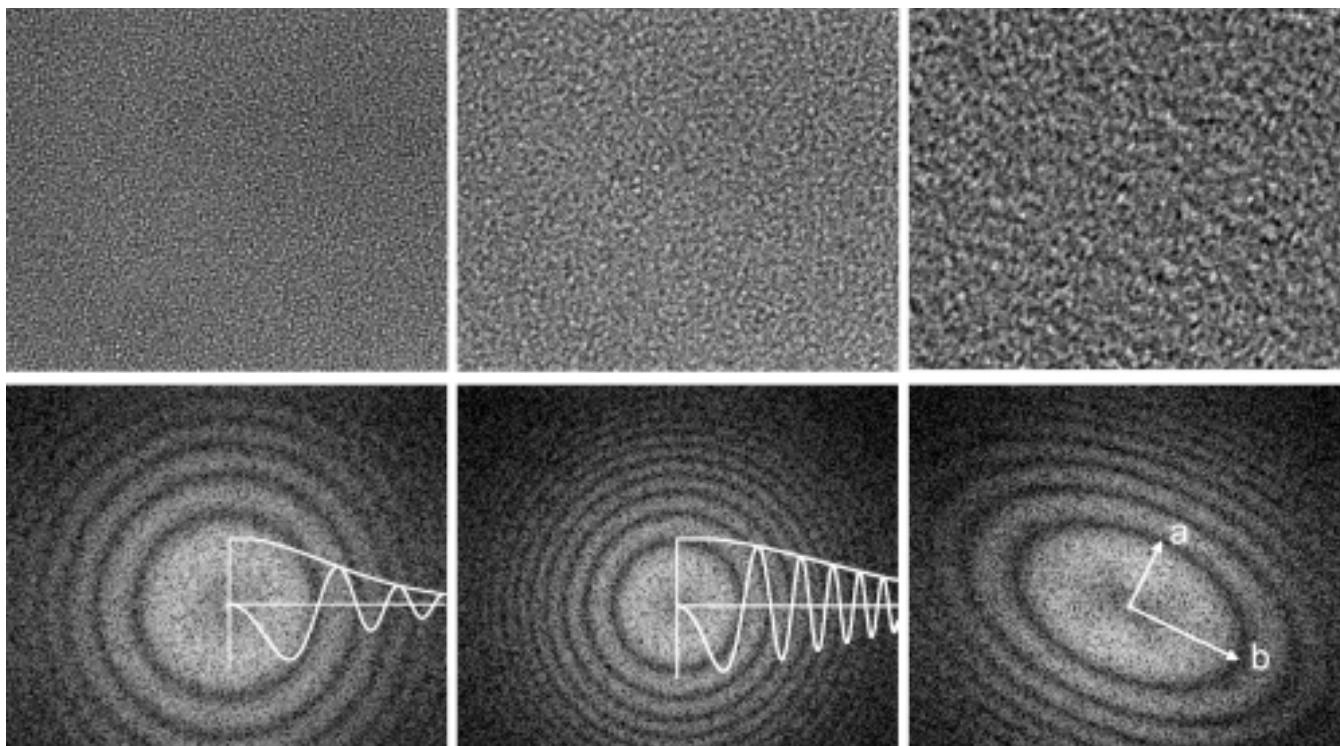
Ice layer large compared with particle diameter

# Contrast transfer function



Power spectrum  
“Thon rings”

after Fritz Thon, a pioneer  
in optical diffraction analysis



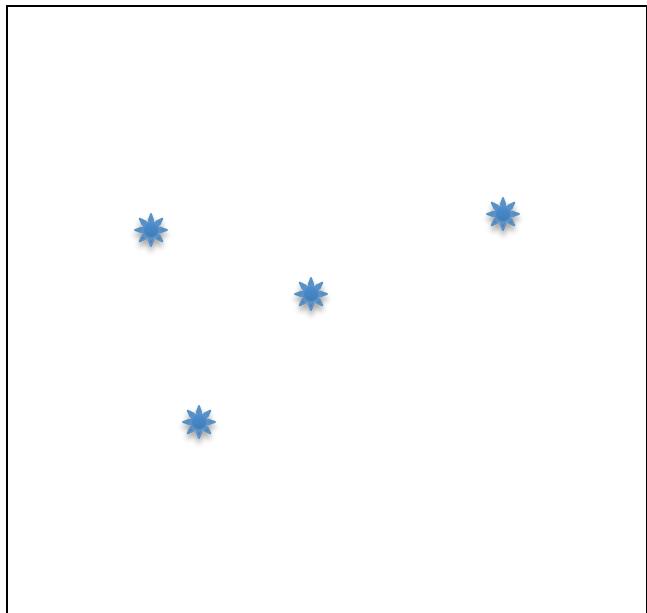
### What Thon rings show:

- 1) how far the information transmitted ranges in Fourier space
- 2) whether the lens is astigmatic (CTF depends on angle in the plane)

Why do we see rings? Because for an amorphous object, such as carbon, the amplitudes of Fourier components are roughly the same throughout Fourier space. Without CTF, we would see a uniform (white) disk up to the radius that corresponds to the resolution limit.

Instead we see concentric white rings separated by black lines (zero transitions).

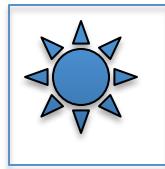
OBJECT



PSF

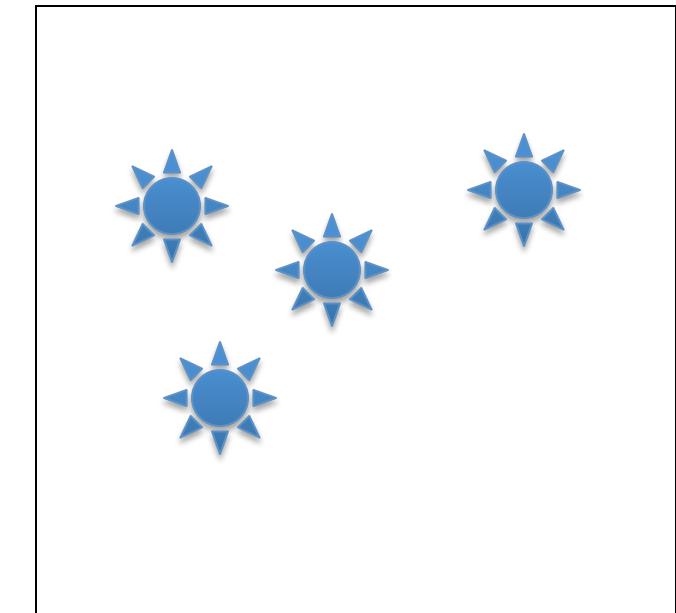


o



“Con-  
voluted  
with”

IMAGE = CONVOLUTION  
PRODUCT



An object consisting of points, convoluted with the point spread function of the optical instrument, results in an image in which each point is replaced by the PSF..

# Convolution theorem

# Convolution Theorem

$o(\mathbf{r})$  = “2D object” or “2D projection of a 3D object”

$s(\mathbf{r})$  = “signal resulting from EM imaging”

$h(\mathbf{r})$  = “point-spread function”

$s(\mathbf{r}) = o(\mathbf{r}) \circ h(\mathbf{r})$  = “convolution product of  $o(\mathbf{r})$  with  $h(\mathbf{r})$ ”

stands for

$$s(x) = \iint o(x', y') h(x - x', y - y') dx' dy'$$

or

$$s(\mathbf{r}) = \int o(\mathbf{r}') h(\mathbf{r} - \mathbf{r}') d\mathbf{r}'$$

- Convolution
- ⊗ Correlation
- × Scalar product

Special case:  $o(\mathbf{r}) \circ \delta(\mathbf{r}) = s(\mathbf{r})$     convolution with a delta function is an identity operation

Convolution Theorem says:  $S(\mathbf{k}) = \mathcal{F}\{s(\mathbf{r})\} = O(\mathbf{k})H(\mathbf{k})$ , where  $S(\mathbf{k}) = \mathcal{F}\{o(\mathbf{r})\}$ ,  $O(\mathbf{k}) = \mathcal{F}\{o(\mathbf{r})\}$ ,  
and  $H(\mathbf{k}) = \mathcal{F}\{h(\mathbf{r})\}$

# Correlation Theorem

$s(\mathbf{r})$  = “image signal” – two noisy versions:  $s_1(\mathbf{r}) = s(\mathbf{r}) + n_1(\mathbf{r})$  and  $s_2(\mathbf{r}) = s(\mathbf{r}) + n_2(\mathbf{r})$

$CCF(\mathbf{r}) = s_1(\mathbf{r}) \circledast s_2(\mathbf{r})$  = “correlation function of  $s_1(\mathbf{r})$  with  $s_2(\mathbf{r})$ ”

stands for

$$CCF(x, y) = \iint s_1(x', y') s_2(x + x', y + y') dx' dy'$$

or

$$CCF(\mathbf{r}) = \int s(\mathbf{r}') s(\mathbf{r} + \mathbf{r}') d\mathbf{r}' + noise\_term$$

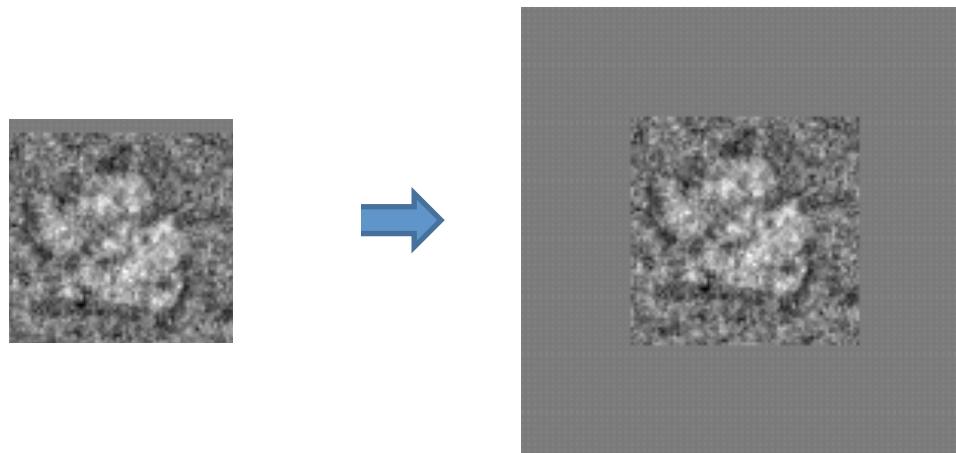
- Convolution
- ⊗ Correlation
- × Scalar product

Special case:  $s(\mathbf{r}) \circledast s(\mathbf{r})$  autocorrelation function

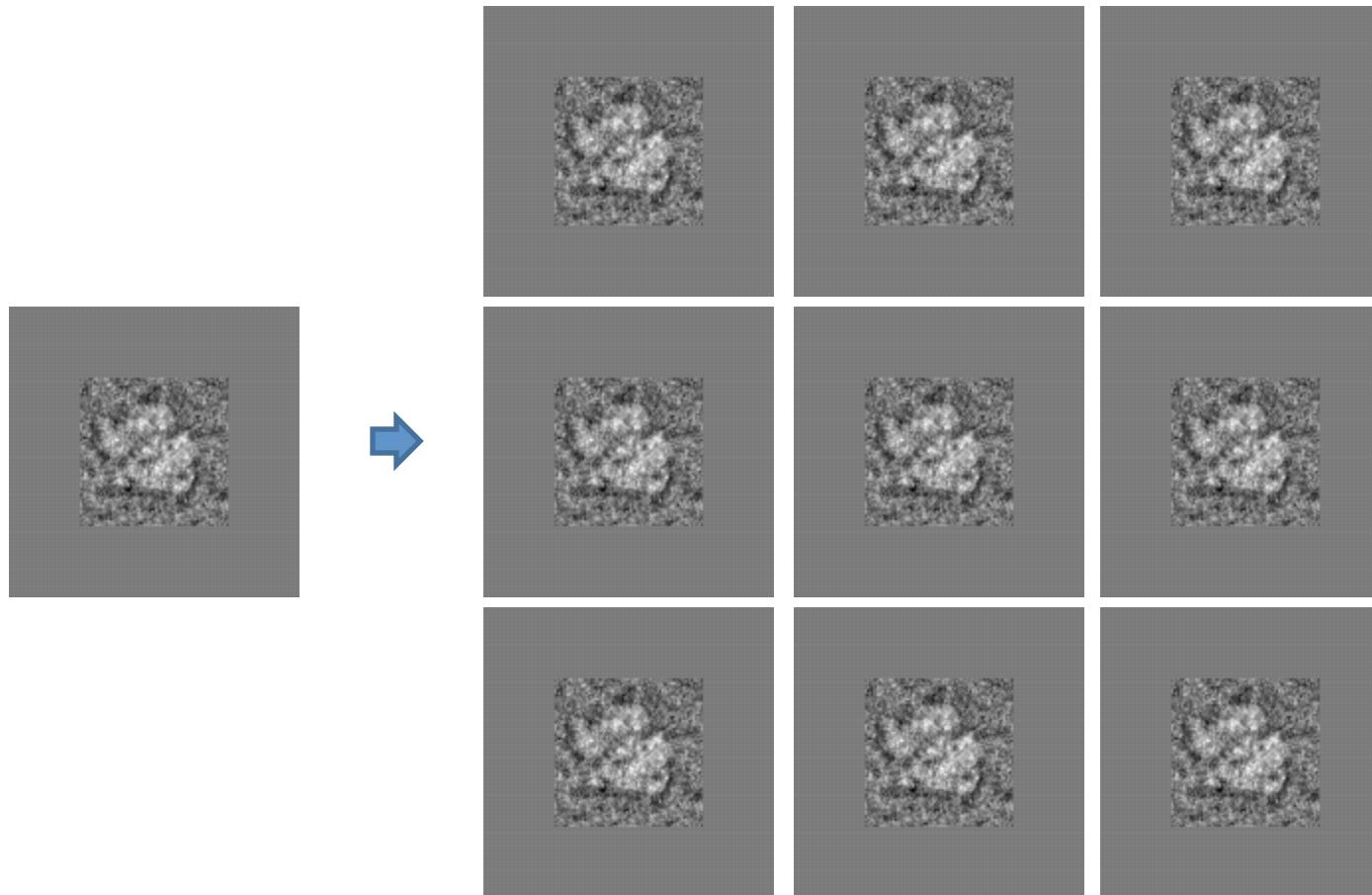
Correlation Theorem says:  $\Phi(\mathbf{k}) = \mathcal{F}\{CCF(\mathbf{r})\} = S_1(\mathbf{k}) S_2^*(\mathbf{k})$ ,

where  $S_1(\mathbf{k}) = \mathcal{F}\{s_1(\mathbf{r})\}$ ,  $S_2(\mathbf{k}) = \mathcal{F}\{s_2(\mathbf{r})\}$

Padding is needed when CCF is computed via Fourier methods:



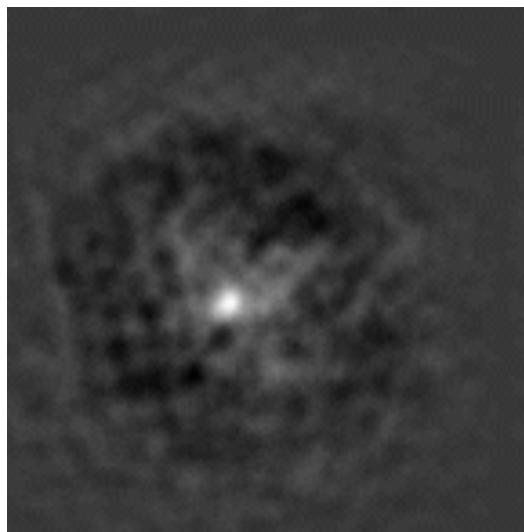
Need for padding follows from  
the discrete Fourier representation:



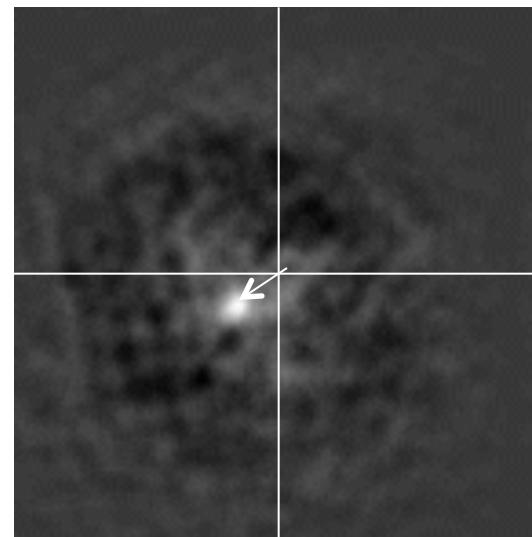
Nonpadded image would get superimposed on a copy of itself

# Translational alignment using the CCF – a practical example

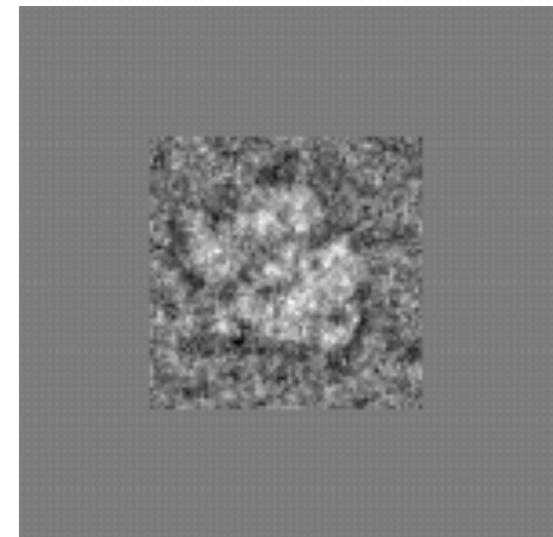
The peak indicates position of perfect alignment of two images of the same molecule



CCF



shift of peak from origin  
indicates relative shift of images



one of the images,  
padded

Example for solving a problem using both convolution and correlation Fourier theorems:

## CCF of two EM images of an object with different CTFs:

CCF of two images  $s_1(\mathbf{r})$ ,  $s_2(\mathbf{r})$  of the same object signal  $o(\mathbf{r})$  with different CTFs,  $H_1(\mathbf{k})$  and  $H_2(\mathbf{k})$

(using *correlation* and *convolution theorems*).

$$s_1(\mathbf{r}) = o(\mathbf{r}) \circ h_1(\mathbf{r}); \quad s_2 = o(\mathbf{r} + \Delta\mathbf{r}) \circ h_2(\mathbf{r})$$

$$\text{CCF}(\mathbf{r}) = s_1(\mathbf{r}) \circledast s_2(\mathbf{r})$$

$$\begin{aligned} S_1(\mathbf{k}) \times S_2^*(\mathbf{k}) &= O(\mathbf{k}) \times H_1(\mathbf{k}) \times \{O(\mathbf{k}) \times \exp[2\pi i \mathbf{k} \Delta \mathbf{r}] \times H_2(\mathbf{k})\}^* \\ &= O(\mathbf{k}) \times O^*(\mathbf{k}) \times \exp[-2\pi i \mathbf{k} \Delta \mathbf{r}] \times H_1(\mathbf{k}) \times H_2^*(\mathbf{k}) \end{aligned}$$

- Convolution
- ⊗ Correlation
- × Scalar product

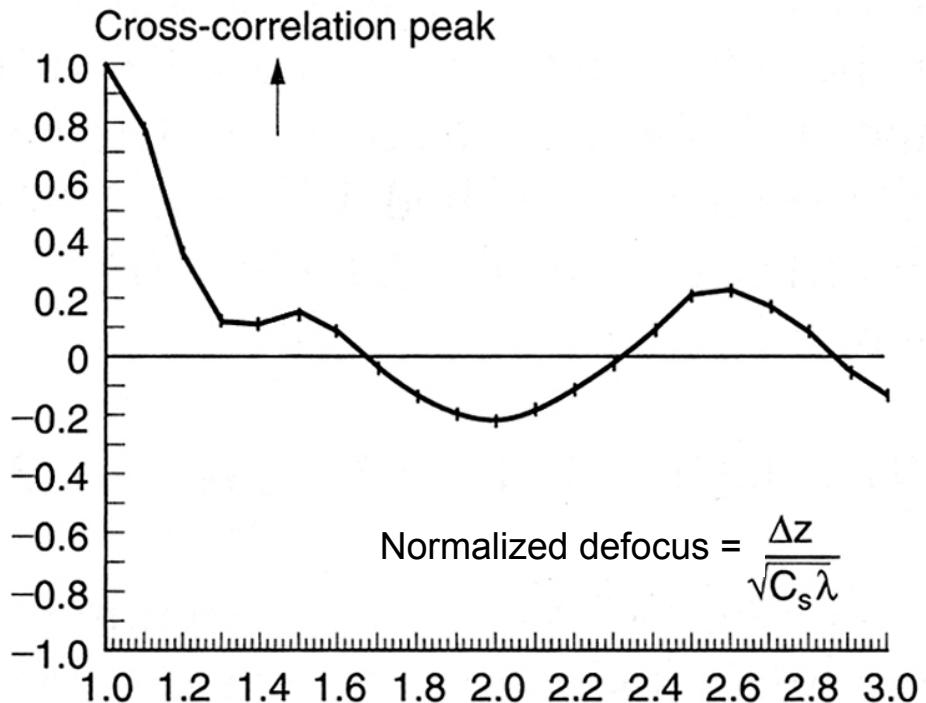
Now back in real space:

$$\text{CCF}(\mathbf{r}) = [o(\mathbf{r}) \circledast o(\mathbf{r})] \quad \circ \quad [h_1(\mathbf{r}) \circledast h_2(\mathbf{r})] \quad \circ \quad \delta(\mathbf{r} - \Delta\mathbf{r})$$

auto-correlation function of signal    CCF of point spread functions    shifted by  $\Delta\mathbf{r}$

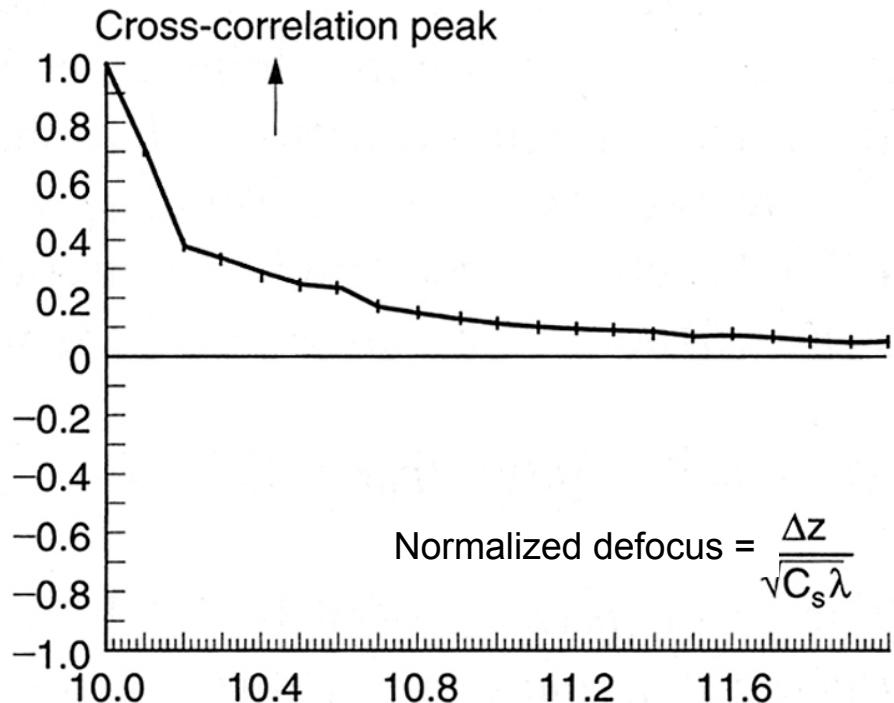
(Scherzer) normalized defocus = 1

(a)



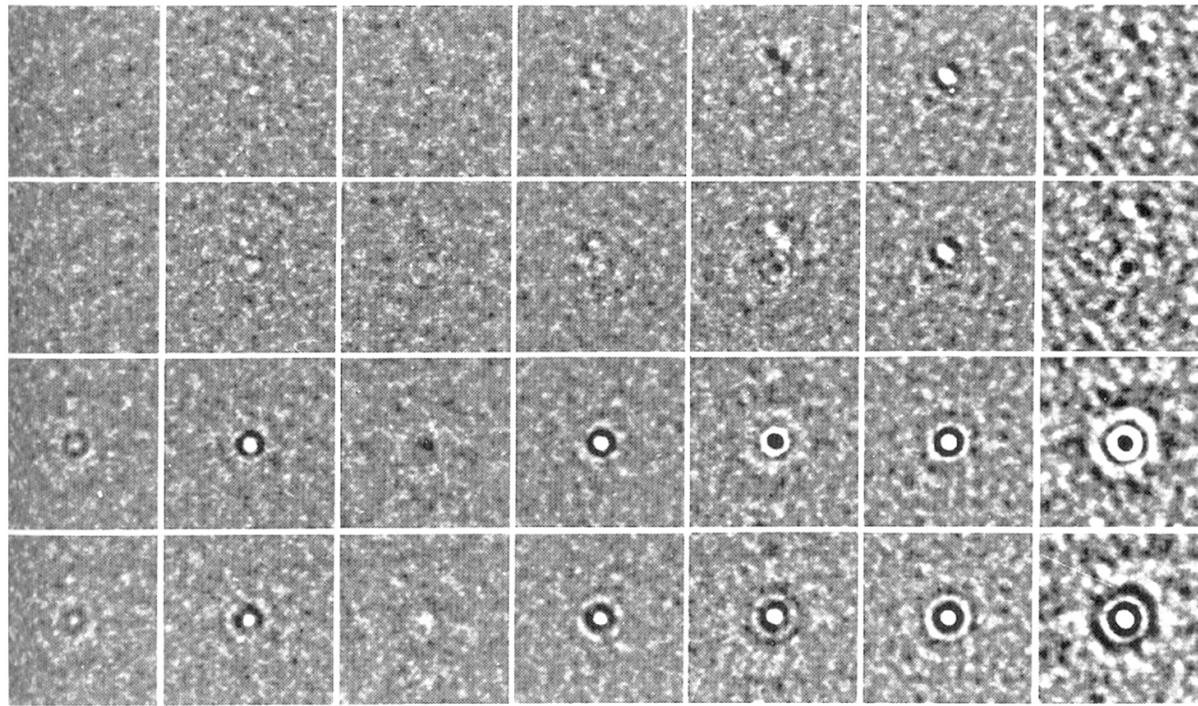
normalized defocus = 10

(b)



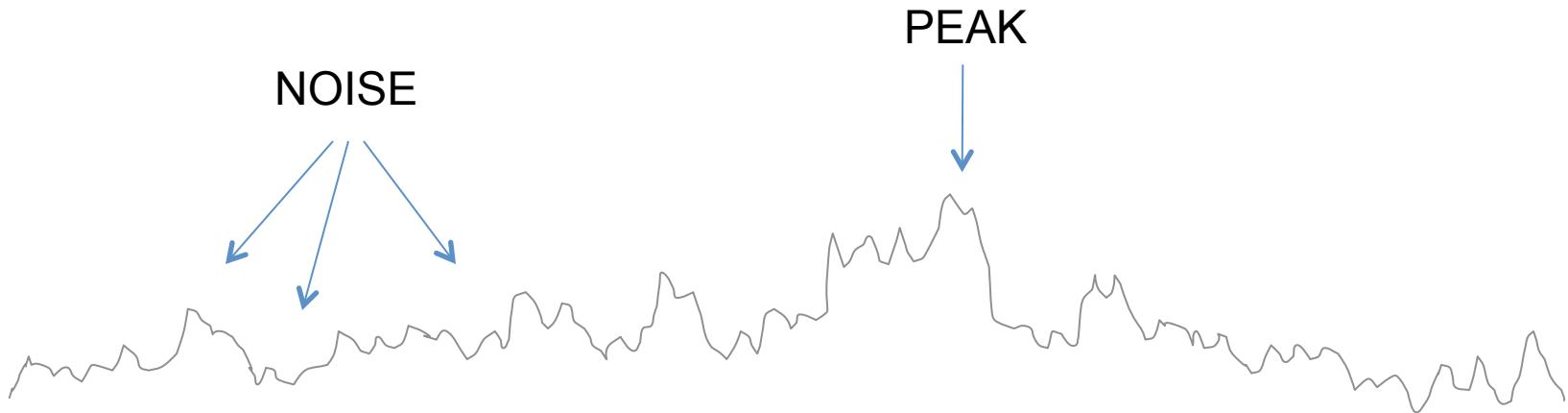
Value of normalized CCF peak as a function  
of the difference in focus,  $\delta\Delta z$

*Tools:* CCFs of micrographs of the same specimen, as a function of  $\delta\Delta z$



To get sharpest CCF peak (yielding highest alignment accuracy), both images to be aligned should have the same defocus

The cross-correlation function has a peak standing out from a noisy background



The SNR in the CCF determines whether we will be successful in finding the correct peak and its x,y coordinates. What factors affect the size of the SNR?

# Criterion for detection of CCF peak: feasibility of alignment

Because of the low signal-to-noise ratio in the images, there exists a critical threshold for the feasibility of alignment of two raw images of a molecule.

The critical parameters are:

$p_{crit}$  -- maximum exposure [electrons/unit area] the molecule can tolerate

$D$  -- particle size

$c$  – contrast

$d$  – resolution (in real space)

Particle size D should satisfy

$$D \geq \frac{3}{c^2 d p_{crit}}$$

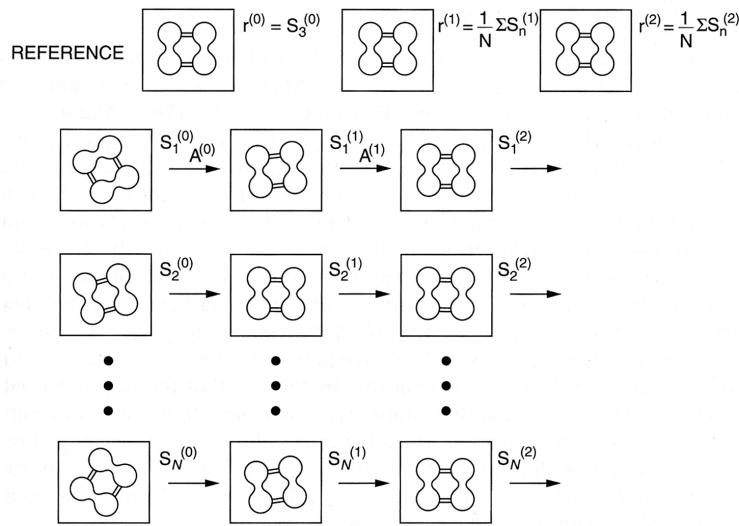
Saxton and Frank (Ultramicroscopy 1976)

also see Henderson (Quart. Rev. Biophys. 1995): number of molecules of a given size required to reach 3A resolution, based on scattering data for electrons, X-rays, and neutrons.

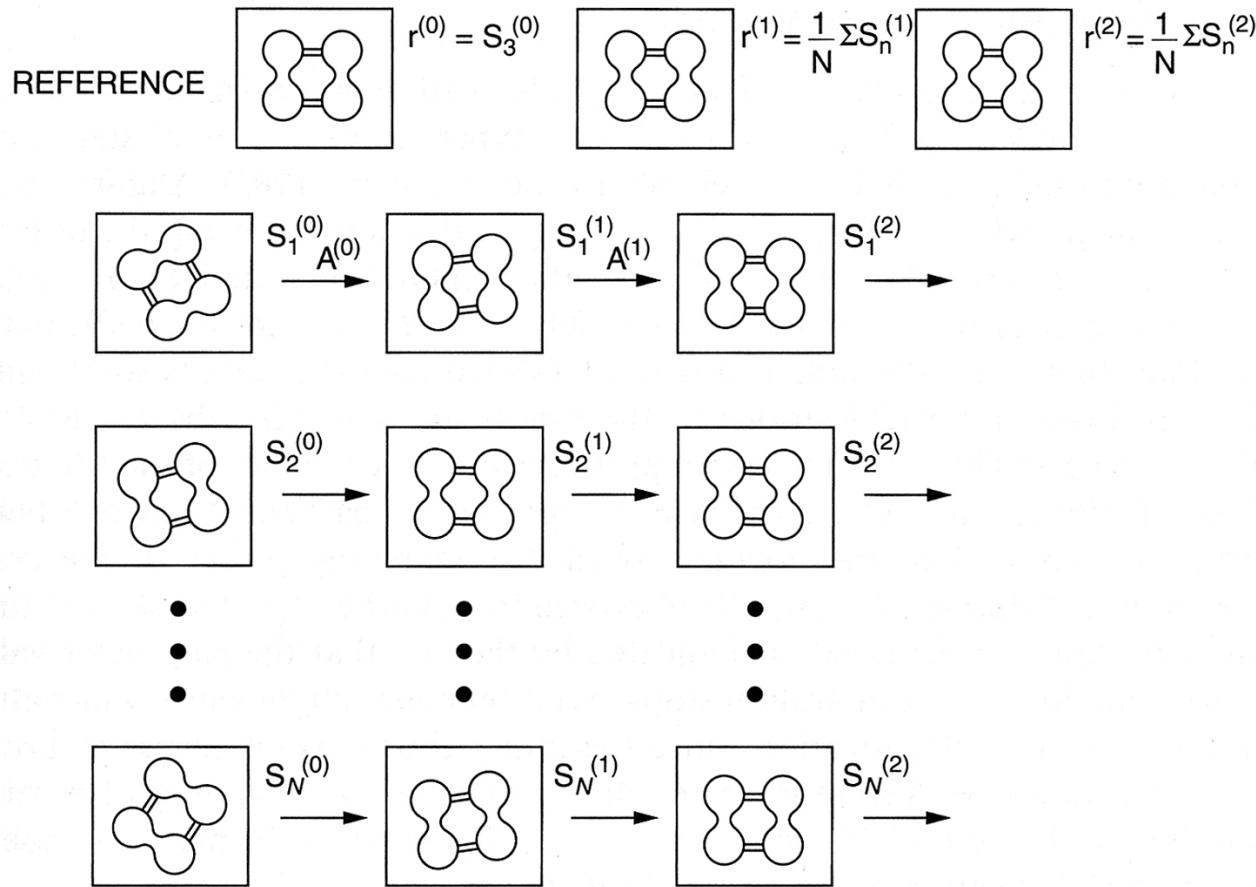
# 2D alignment strategies:

reference-based  
versus reference-free

A variant of the reference-based method updates the reference as it goes along, and becomes thus less dependent on the initial choice of reference.



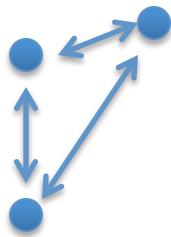
# Multi-Step Reference-Based Alignment



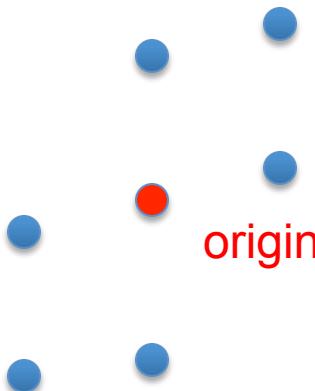
How to combine translational and rotational alignment: use of invariants, such as the autocorrelation function

# Properties of the Autocorrelation Function

- The autocorrelation function of an image preserves directional features of the image
- For instance, correlations, within the same image, between distinct maxima separated by a vector
- The ACF is centro-symmetric
- Example: an image consisting of three dots:

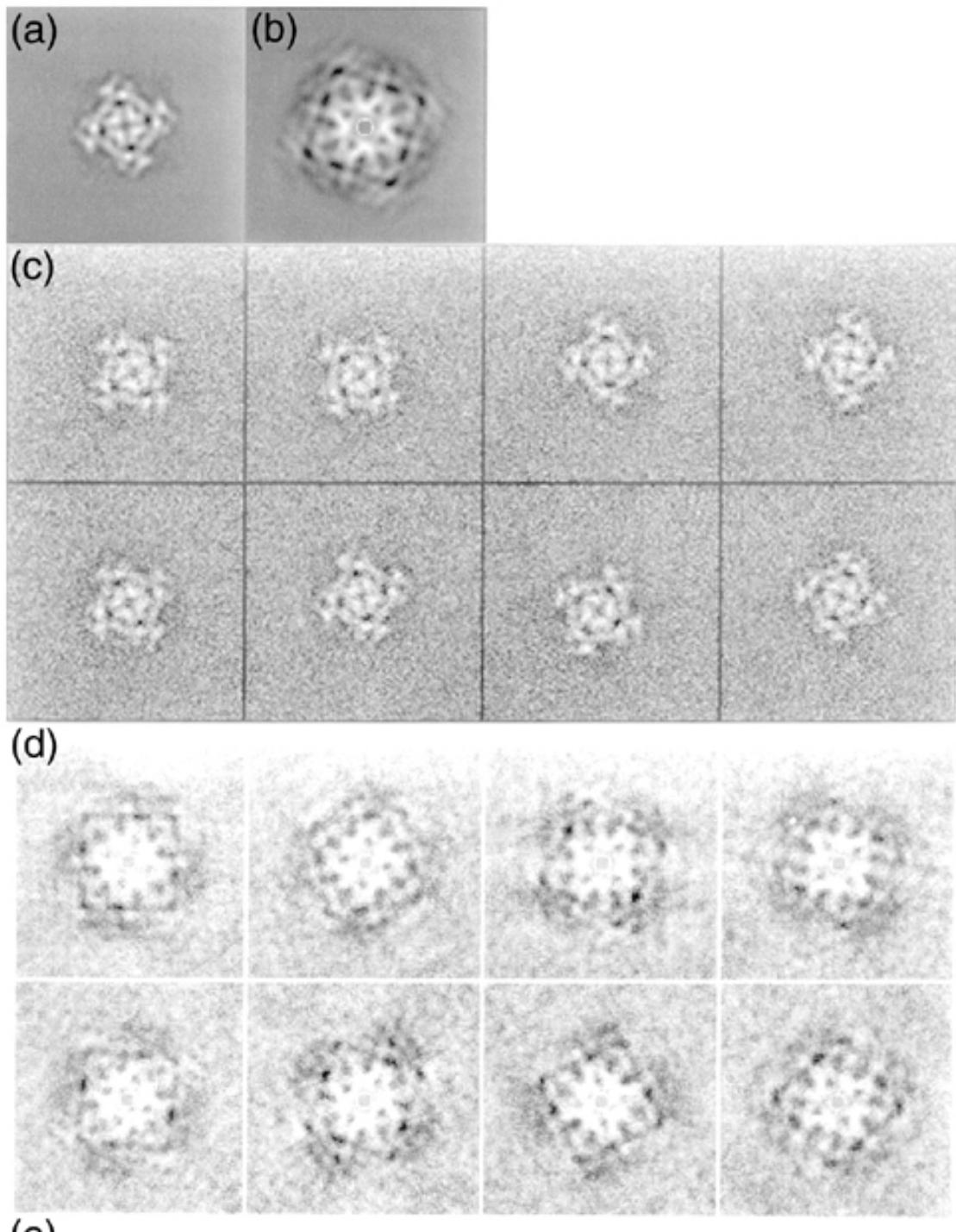


IMAGE



AUTOCORRELATION FUNCTION

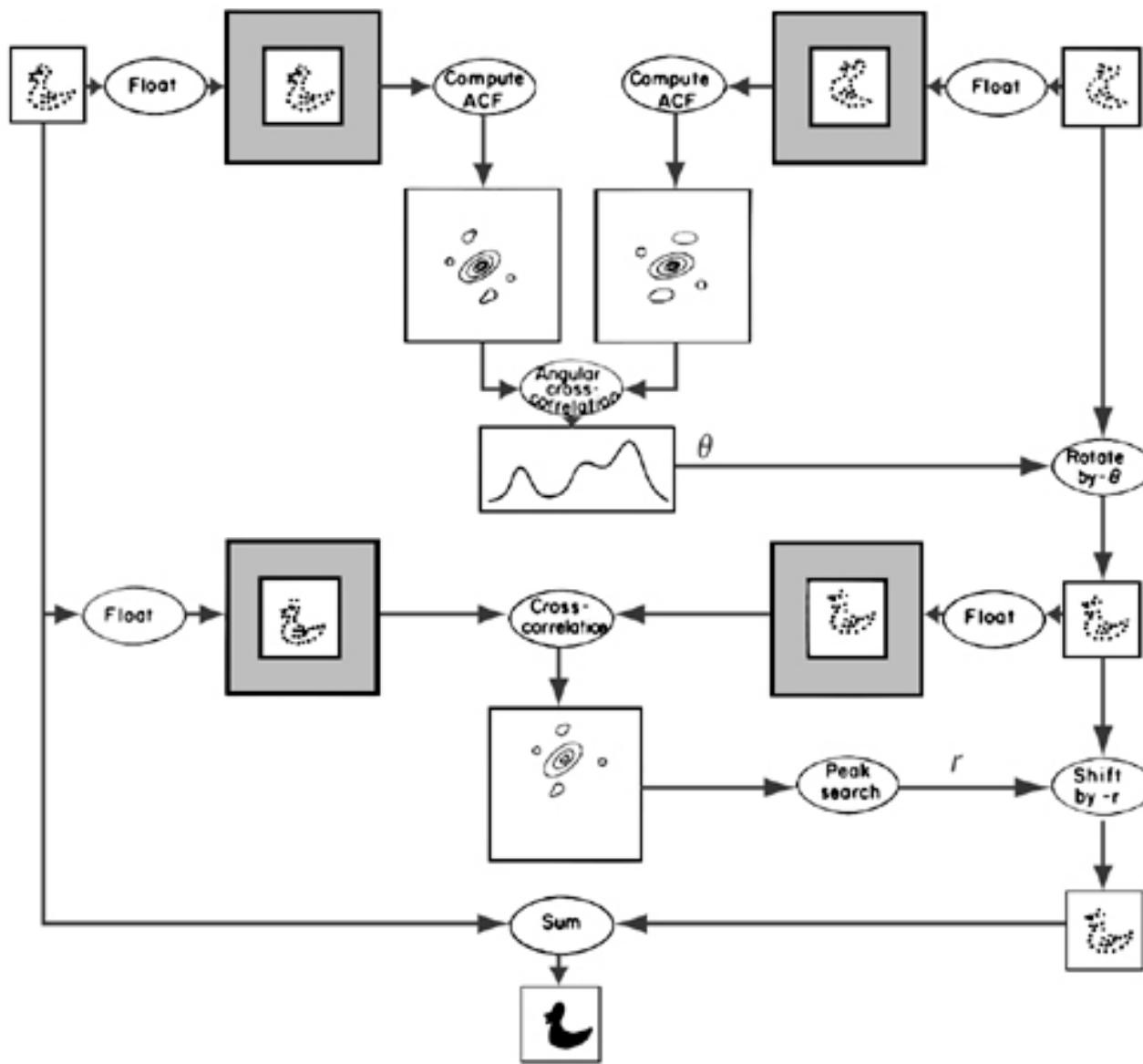
Noise-free molecule and  
its ACF



The molecule in arbitrary  
positions

ACFs of molecules above

# ACF – based alignment method



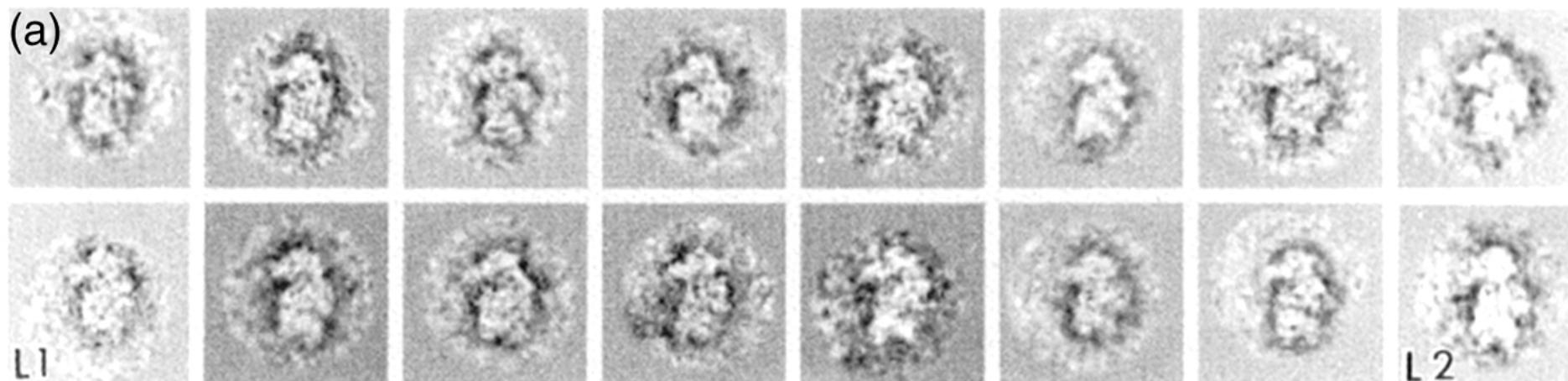
# Variance map

- The variance map is a “byproduct” of the averaging. It can be used to find the regions where the images, on average, differ maximally.
- It is also the yardstick that helps determine whether or not a density in a difference map is significant.

$$v_{(N)}(\mathbf{r}_j) = \frac{1}{(N-1)} \sum_{i=1}^N [p_i(\mathbf{r}_j) - \bar{p}_{(N)}(\mathbf{r}_j)]^2$$

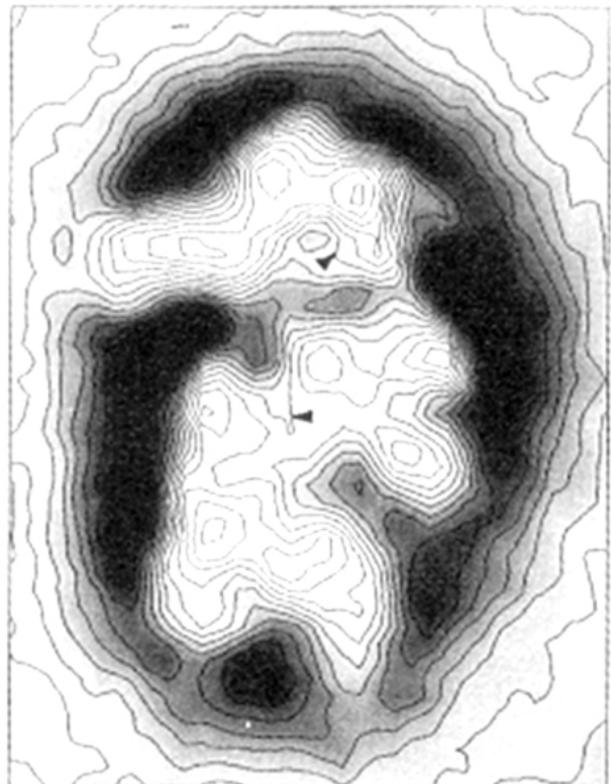
particle image      average image

(a)



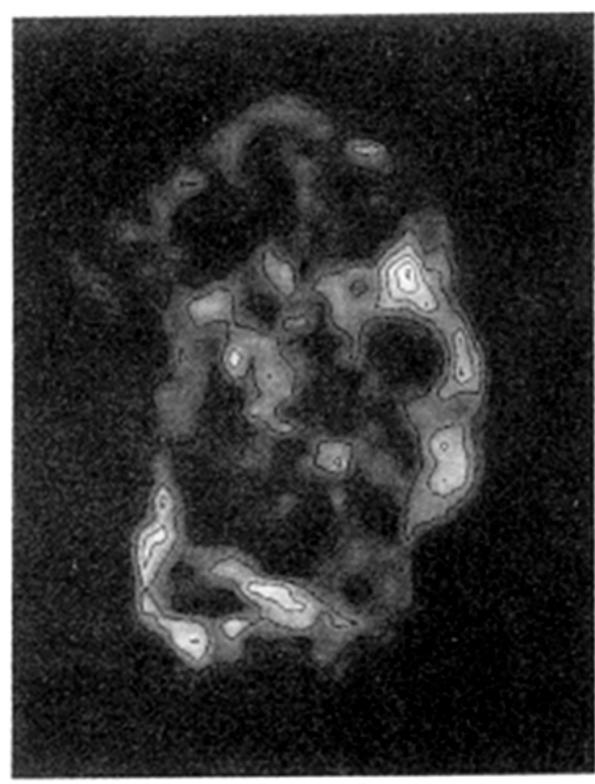
L1

L2



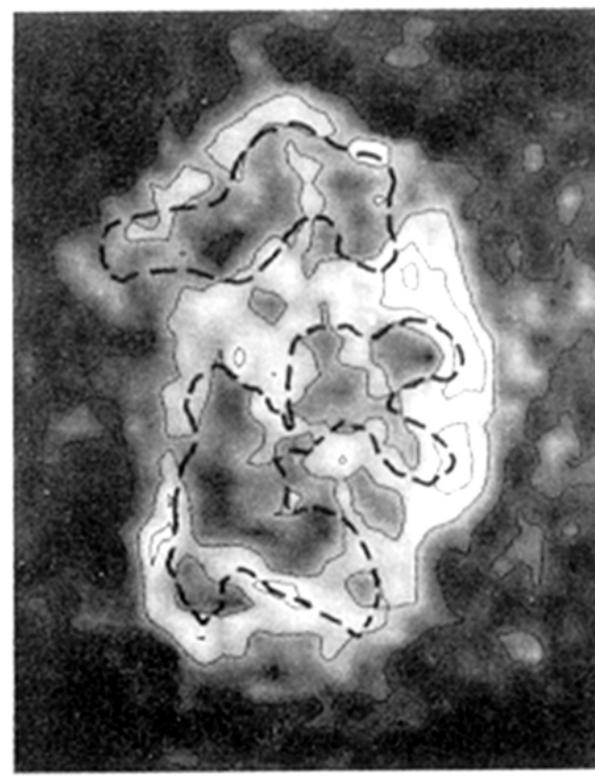
(b)

average



(c)

variance map



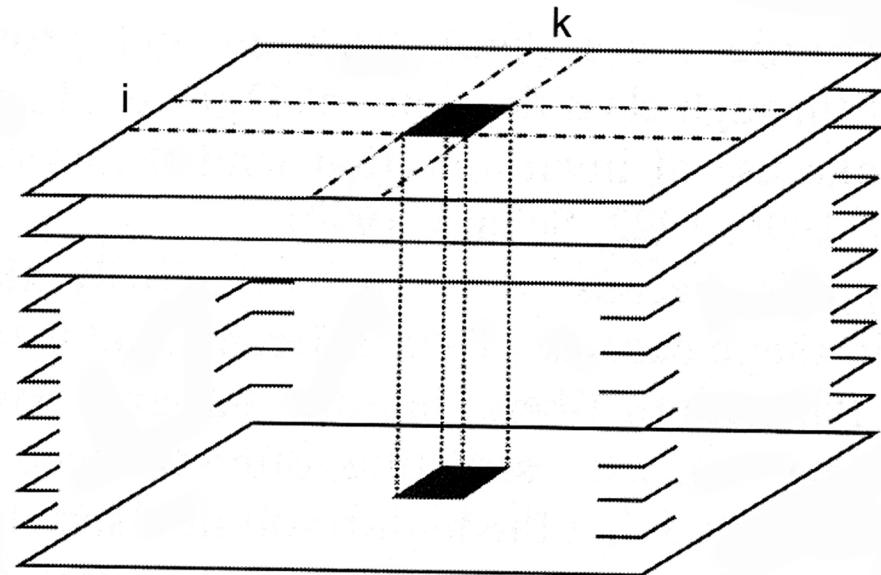
(d)

s.d. map

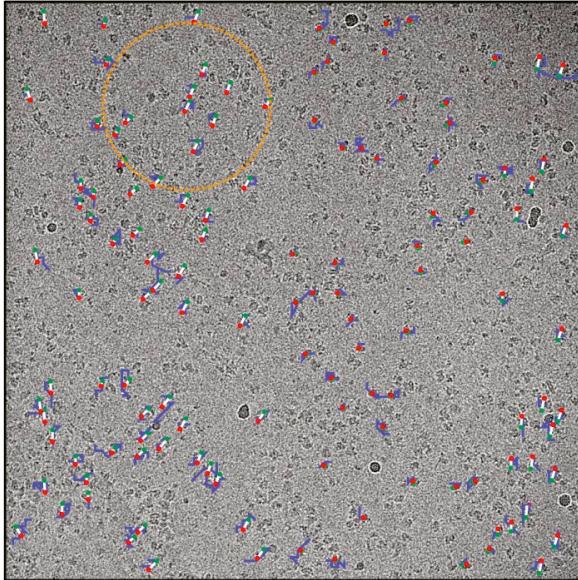
50 Å

# Alignment of frames in movie mode of Direct Electron Detection cameras

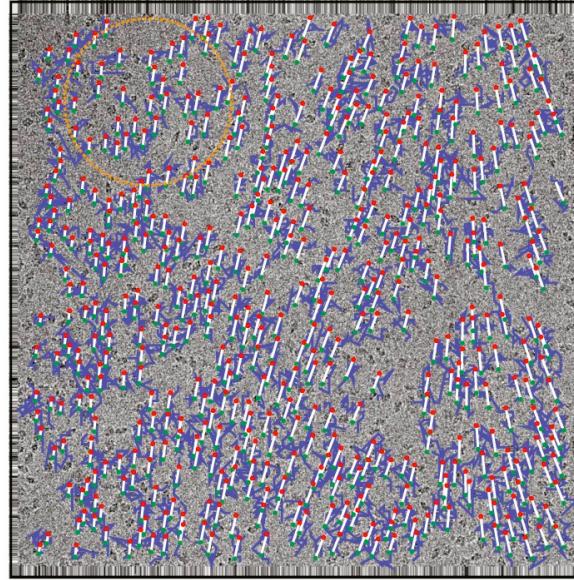
Most DED cameras allow data to be collected in multiple frames. This makes it possible to correct for drift, even on the level of single particles.



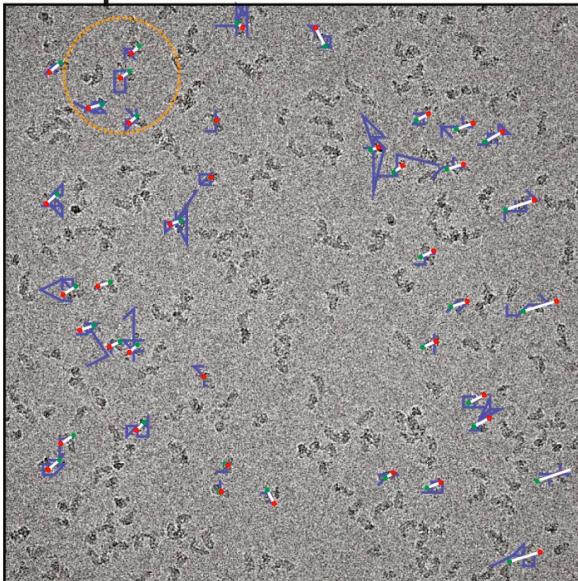
$\gamma$ -secretase



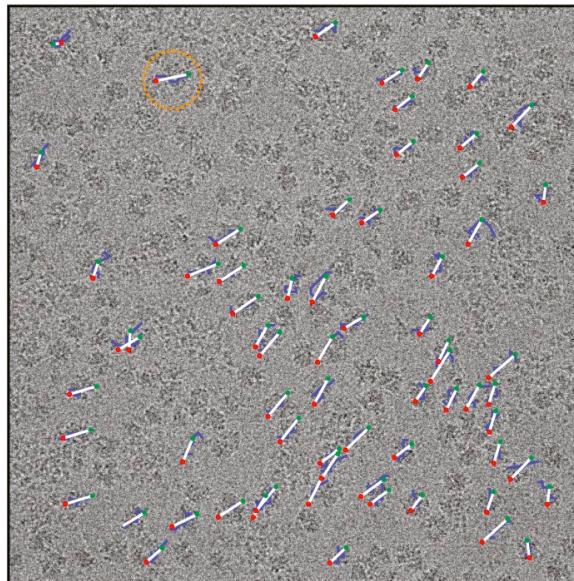
$\beta$ -galactosidase



complex-I



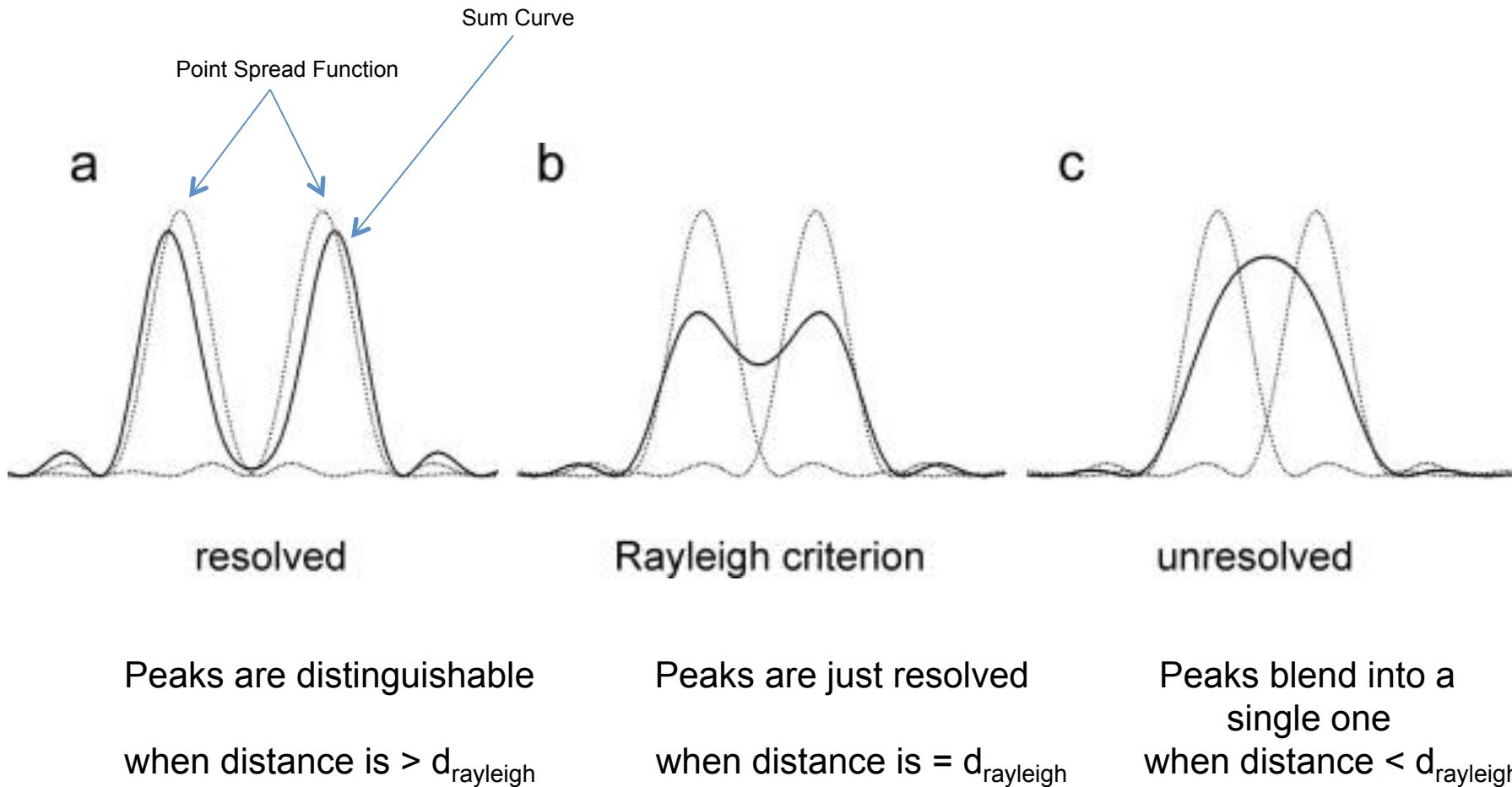
mitoribosome



(vectors of motion are exaggerated)

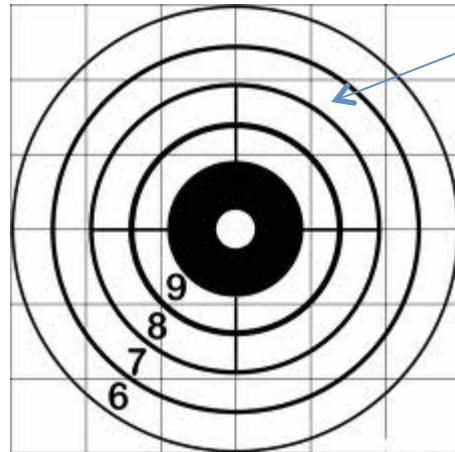
S. Scheres, eLife 2014

## Resolution criterion: Images of two points, as function of their separation



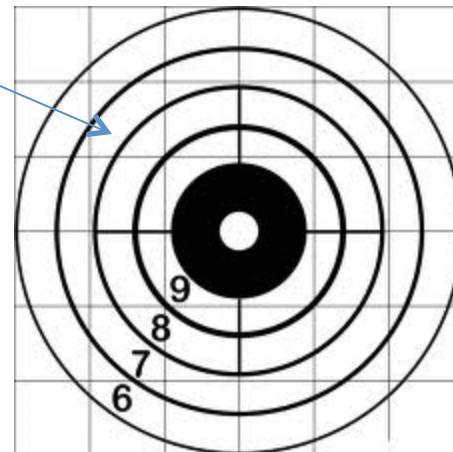
# Resolution definition, determination in Fourier space

- Resolution is a reciprocal quantity, measured in Fourier space
- Defined as the spatial frequency [1/Å] up to which information is reproducible, by some measure of reproducibility
- Decomposition of information, by Fourier rings
- Randomly picked halfsets (e.g., odd- vs. even-numbered images)
- Compare averages [reconstructions] from halfsets over rings (shells) in Fourier space



$F_1(\mathbf{k})$

$k, \Delta k$   
 $k$  ring radius  
 $\Delta k$  ring width



$F_2(\mathbf{k})$

# Resolution measures & criteria: Fourier ring/shell correlation

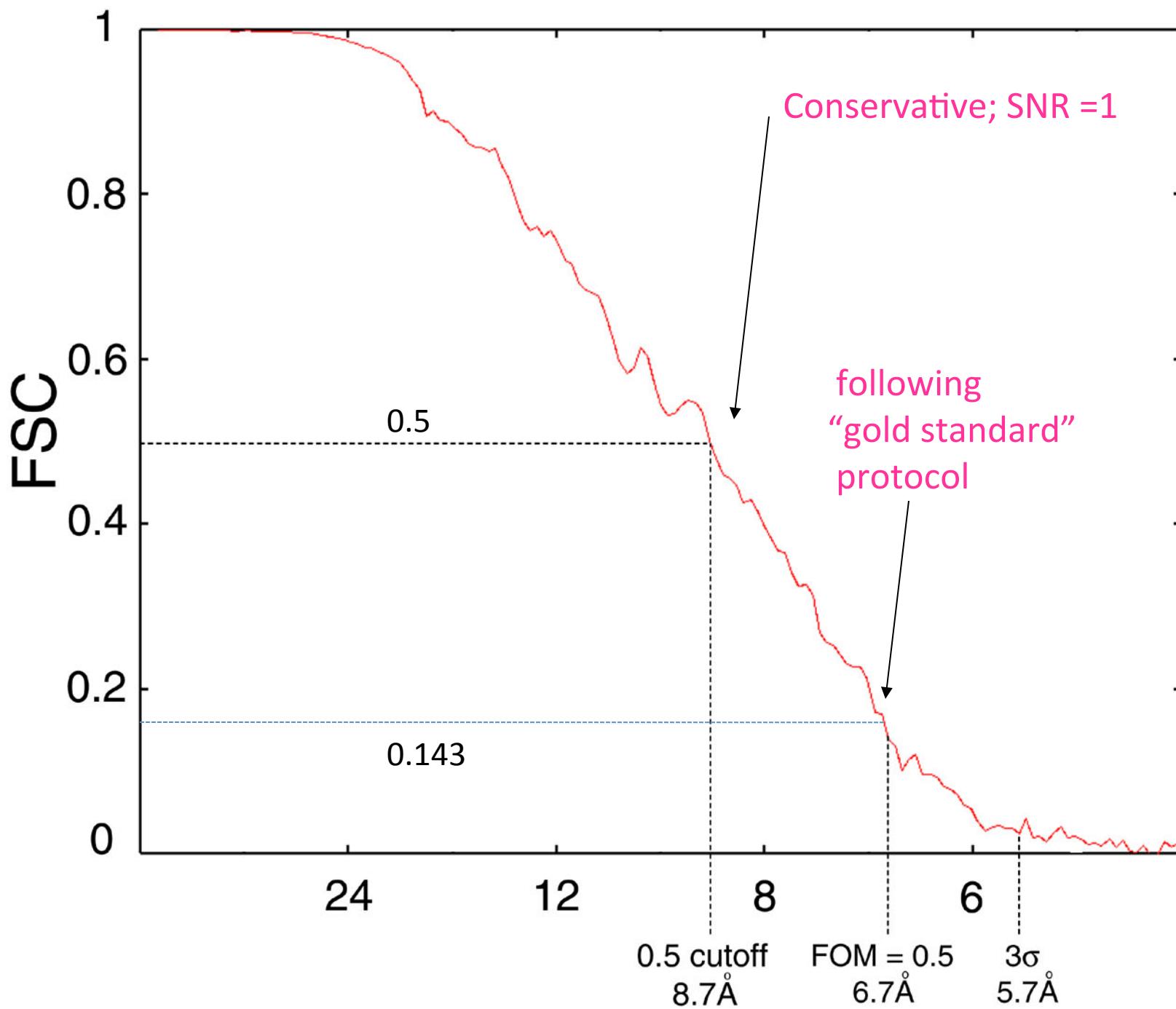
$F_1(\mathbf{k}), F_2(\mathbf{k})$  Fourier transforms of halfset averages  
(or halfset reconstructions)

$$FSC(k, \Delta k) = \frac{\text{Re} \left\{ \sum_{(k, \Delta k)} F_1(\mathbf{k}) F_2(\mathbf{k}) \right\}}{\left\{ \sum_{(k, \Delta k)} |F_1(\mathbf{k})|^2 |F_2(\mathbf{k})|^2 \right\}^{1/2}}$$

$\mathbf{k}$  = *spatial frequency vector*

$k$  =  $|\mathbf{k}|$  *abs. size of spatial frequency*

$\Delta k$  = *ring width* or (in 3D) *shell thickness*



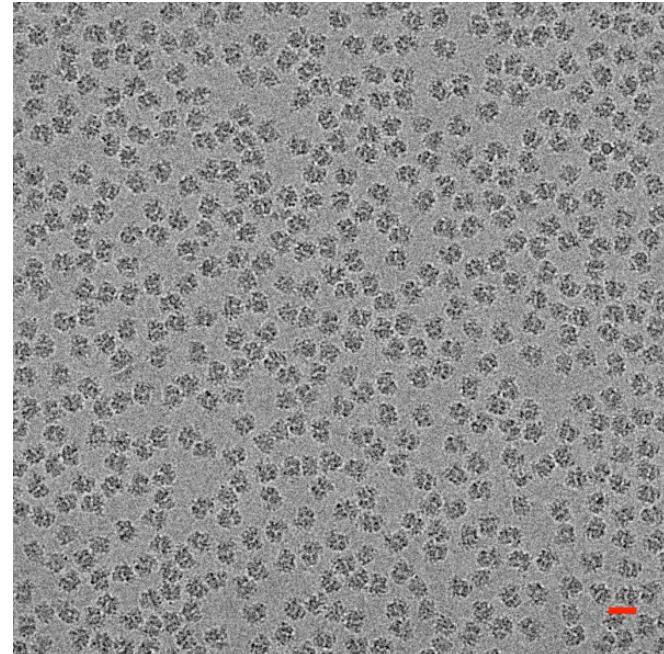
# Multivariate Data Analysis and Classification

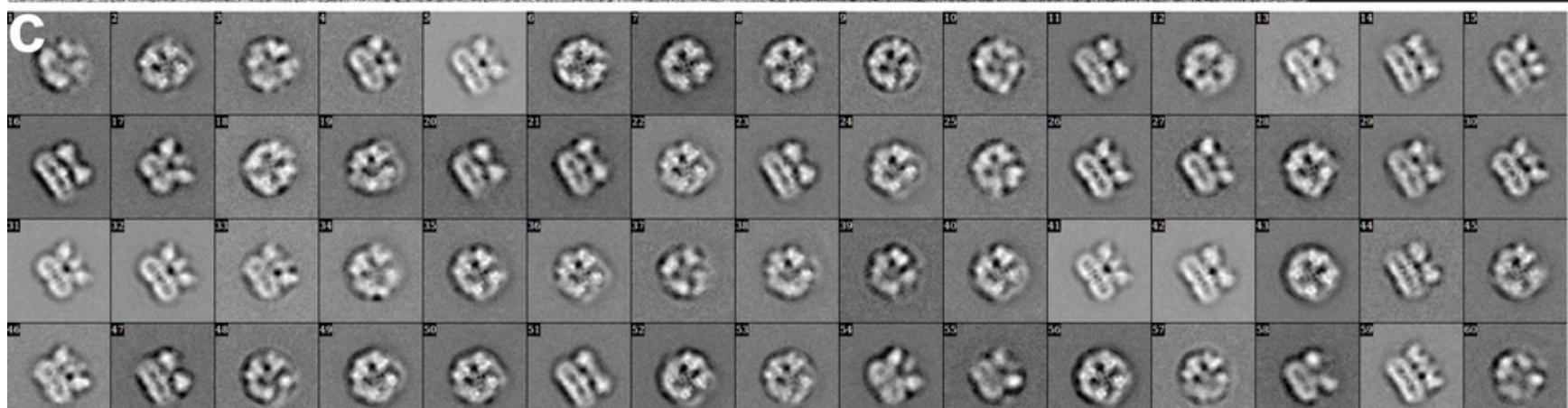
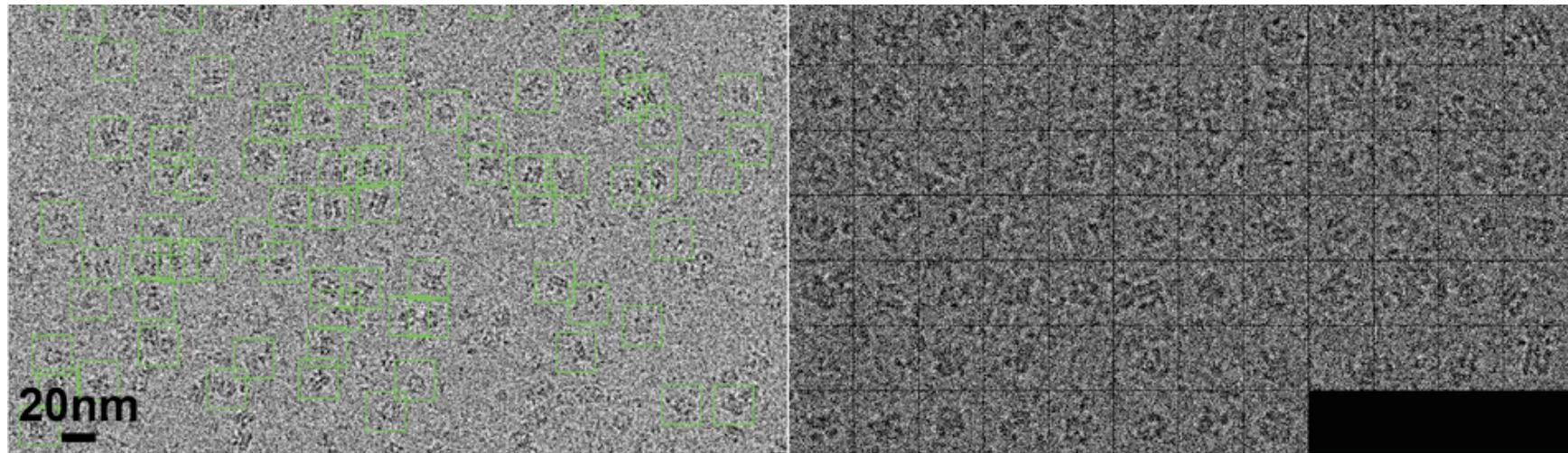
- Images often need to be sorted into classes
- Heterogeneity is due to (1) different viewing angle and (2) different conformations of the molecules
- Sorting them visually only works in the simplest cases
- Multivariate analysis reduces the dimensionality of the classification problem

## Classification in 2D

RATIONALE:

Inventory of existing views

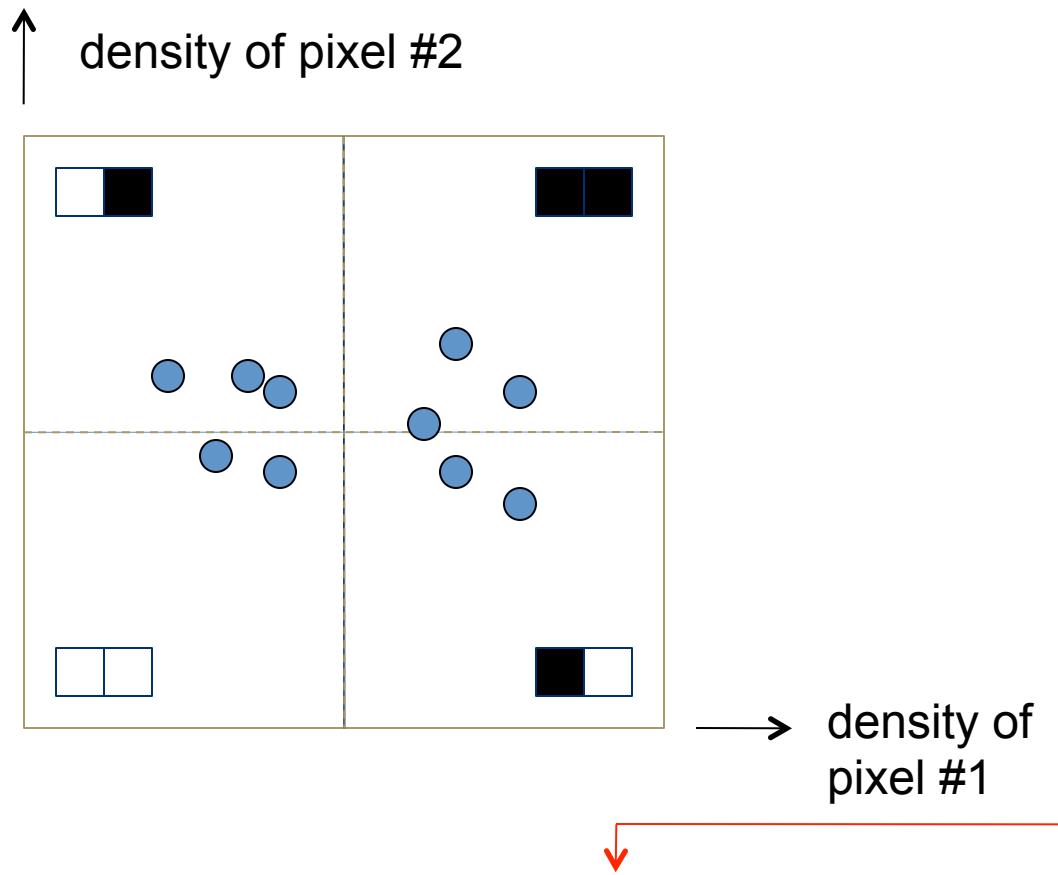




# An image represented in a high-dimensional Euclidean space.

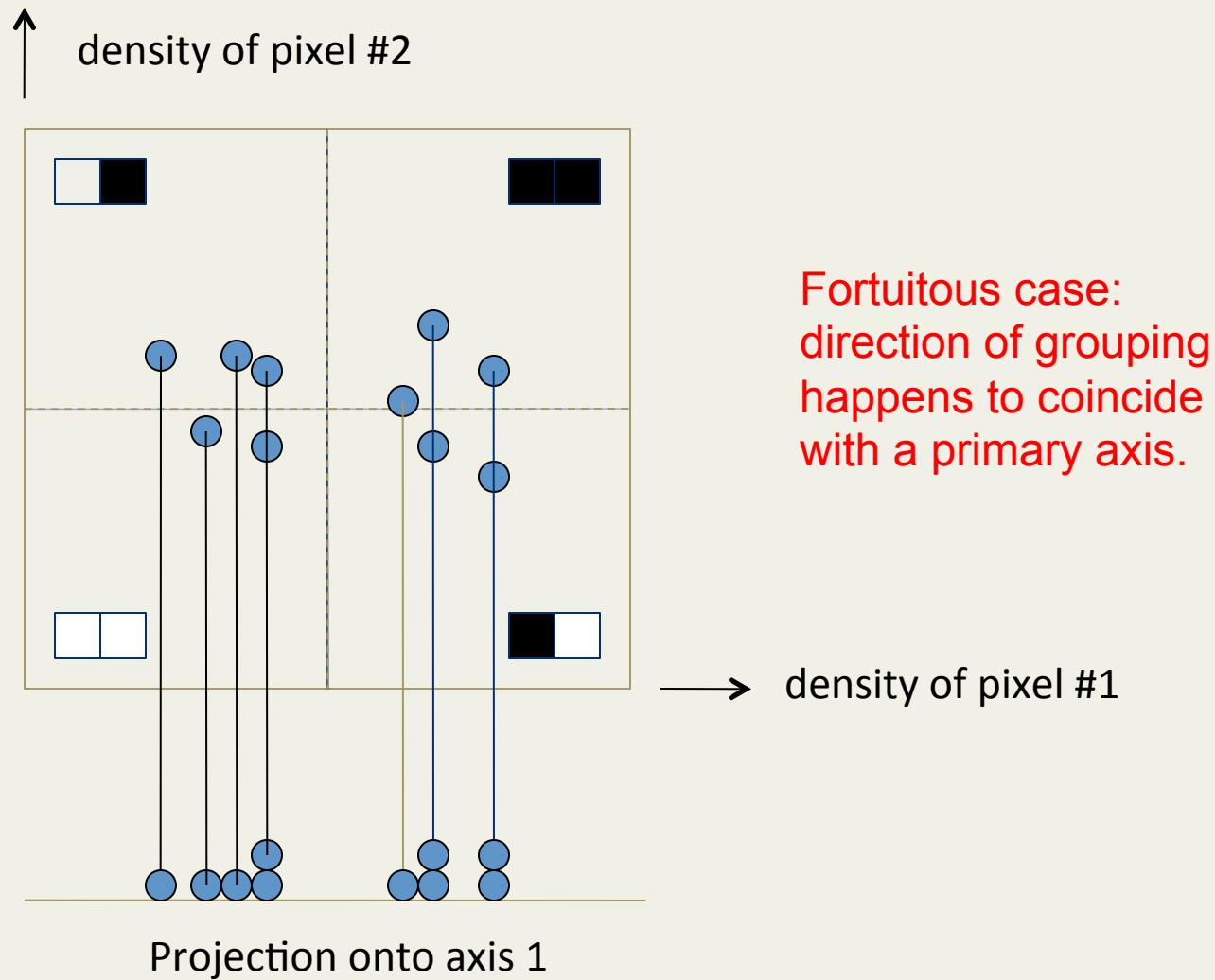
- An image represented by an array of  $N \times M$  pixels can be thought as a vector in a (generalized) Euclidean space with  $N \times M$  dimensions
- For example, an image of  $64 \times 64$  pixels is a vector in a 4096-dimensional space
- If two images are “similar” it means the distance between the vectors representing them is small. That is, the vector end points lie close together
- Groups of similar images form clusters in the generalized Euclidean space
- To show the concept, and introduce an important tool for classification, I will use a simplistic image containing only two pixels

Introducing: a set of images, each consisting of **2** pixels

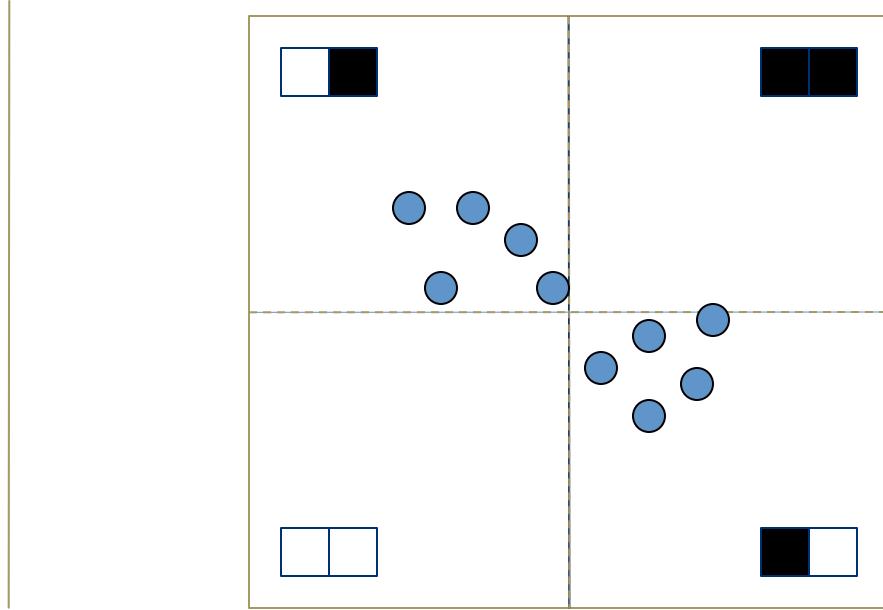


*Similarity = closeness in 2-D Euclidean space*  
*Two images are similar if their (generalized) Euclidean distance is small*

## A set of images consisting of two pixels: Intro into classification



density of pixel #2



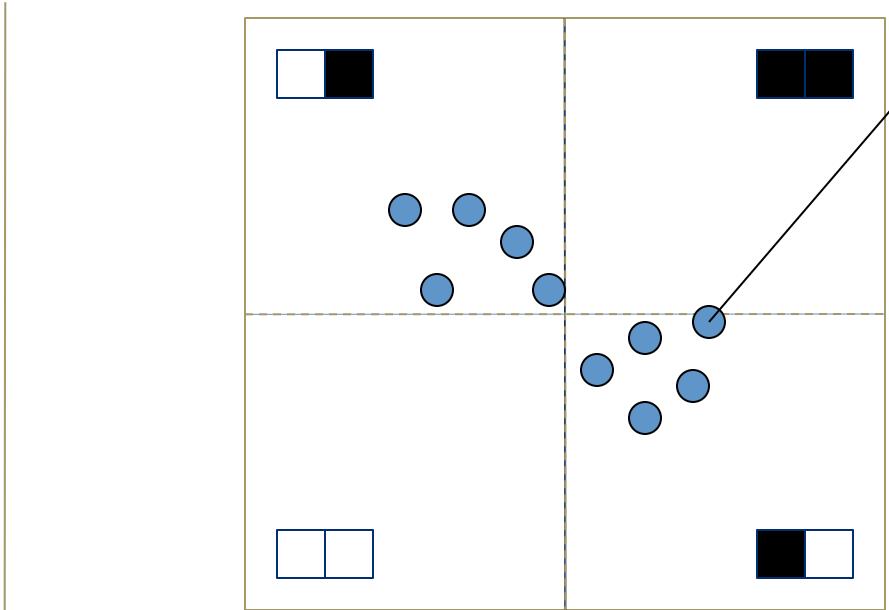
General case:  
grouping is in a direction  
that does not coincide  
with a primary axis.

→ density of pixel #1

---

Projection onto axis 1

density of pixel #2



New axis required for showing  
clustering!  
(linear combination of  
axes 1 and 2)

→ density of pixel #1

---

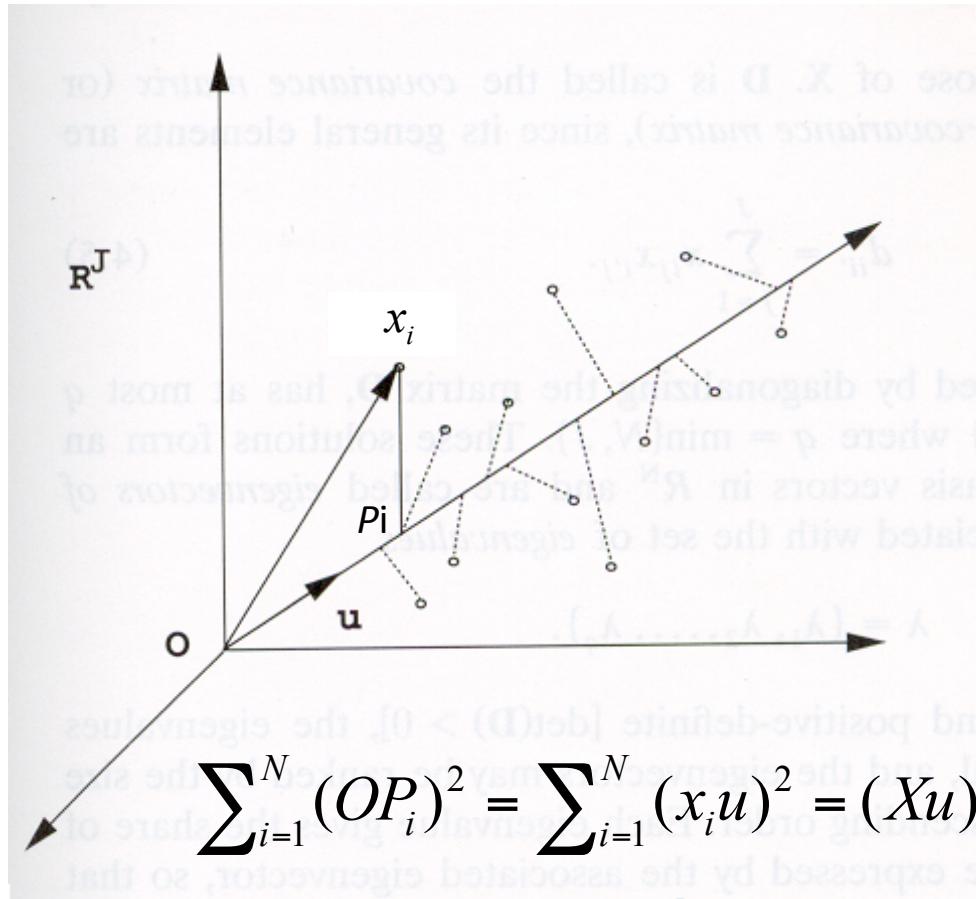
Projection onto axis 1

# *Tools:* Classification, and the Role of MDA

- Classification deals with “objects” in the space in which they are represented.
- For instance, a 64x64 image is an “object” in a 4096-dimensional space since, in principle, each of its pixels can vary independently.  
Let’s say we have 8000 such images. They would form a cloud with 8000 points in this space. This is an unwieldy problem.
- Unsupervised classification is a method that is designed to find clusters (regions of cohesiveness) in such a point cloud.
- Role of **Multivariate Data Analysis** (MDA): find a space (“factor space”) with ***reduced*** dimensionality for the representation of the “objects”. This greatly simplifies classification.
- Reasons for the fact that the space of representation can be ***much smaller*** than the original space: ***resolution limitation*** (neighborhoods behave the same), and ***lateral correlations*** due to the physical origin of the variations (e.g., movement of a structural component is represented by correlated additions and subtractions at the leading and trailing boundaries of the component).
-

# Principle of MDA:

Find new coordinate system, tailored to the data

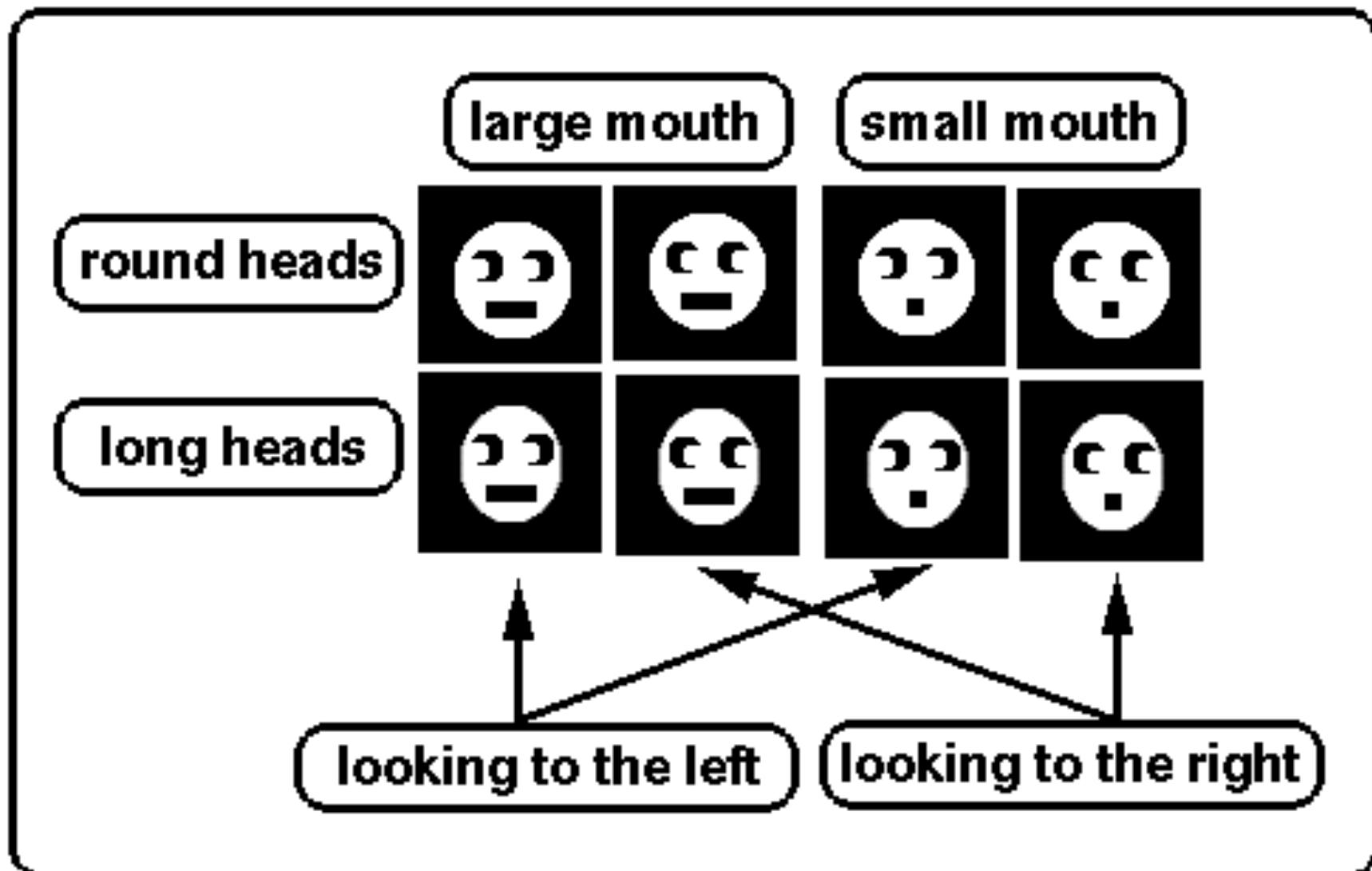


$X = \text{matrix containing } N \text{ image vectors (each with } J \text{ elements) as rows}$

$$\sum_{i=1}^N (OP_i)^2 = \sum_{i=1}^N (x_i u)^2 = (Xu)' X' u = u' X' X u \xrightarrow{!} \max$$

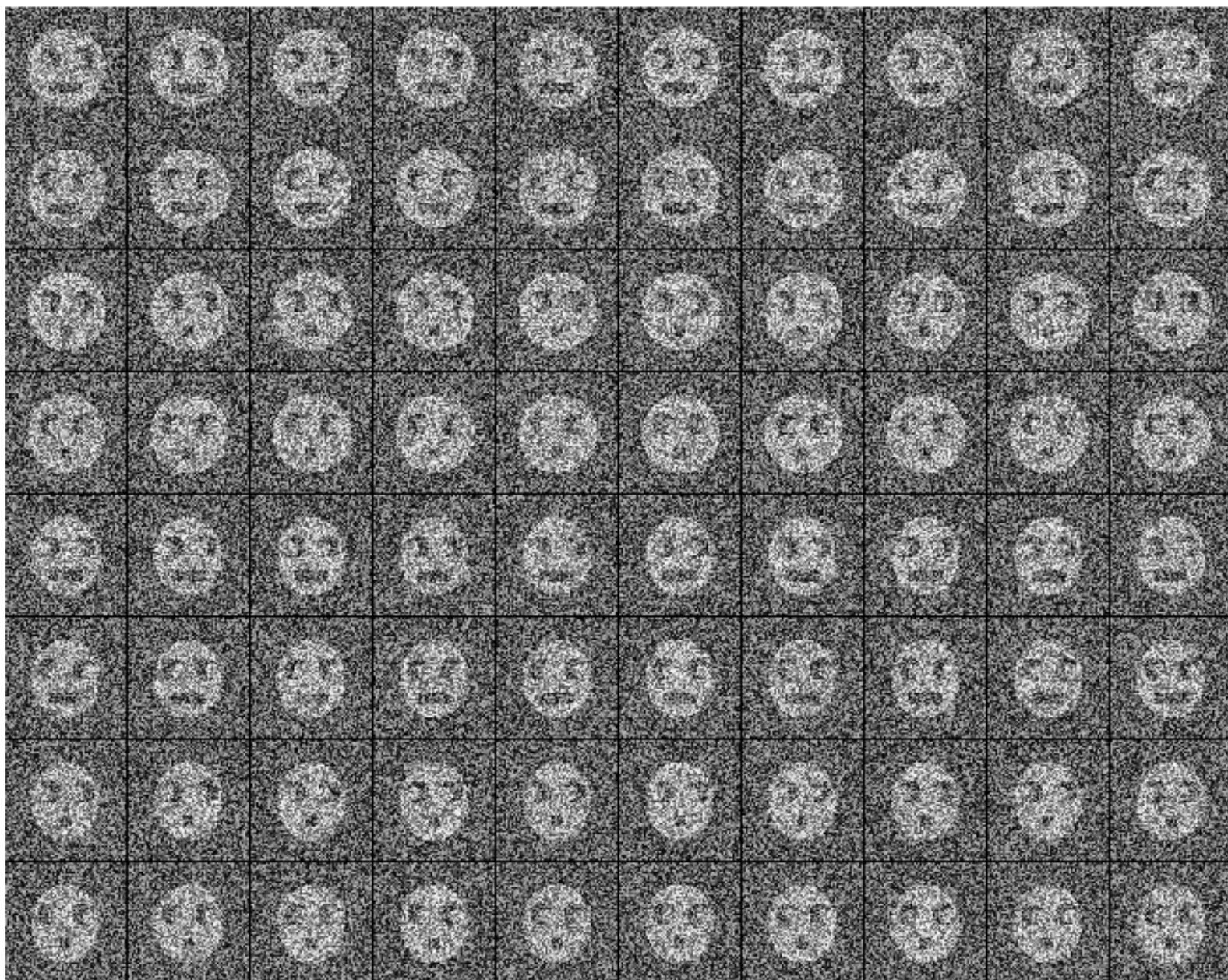
p. 151 [note error in book figure!]

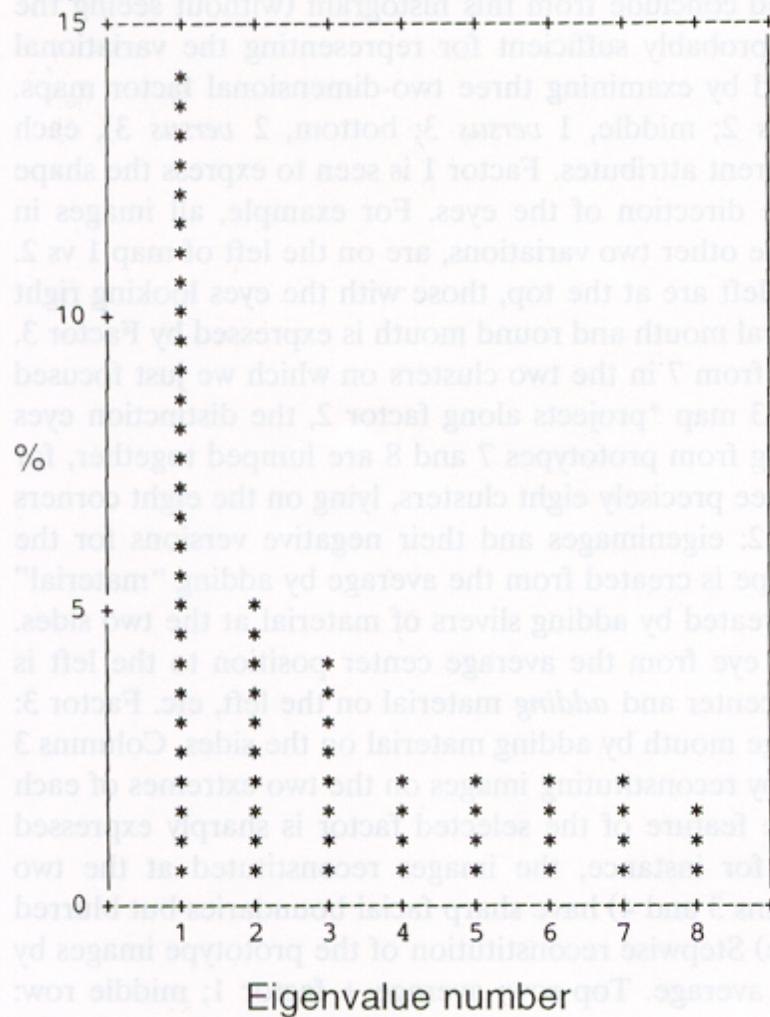
32 x 32 phantom images in 8 (=  $2^3$ ) varieties



10 copies of the 8 types of heads + random noise

Averages





## eigenvalue histogram

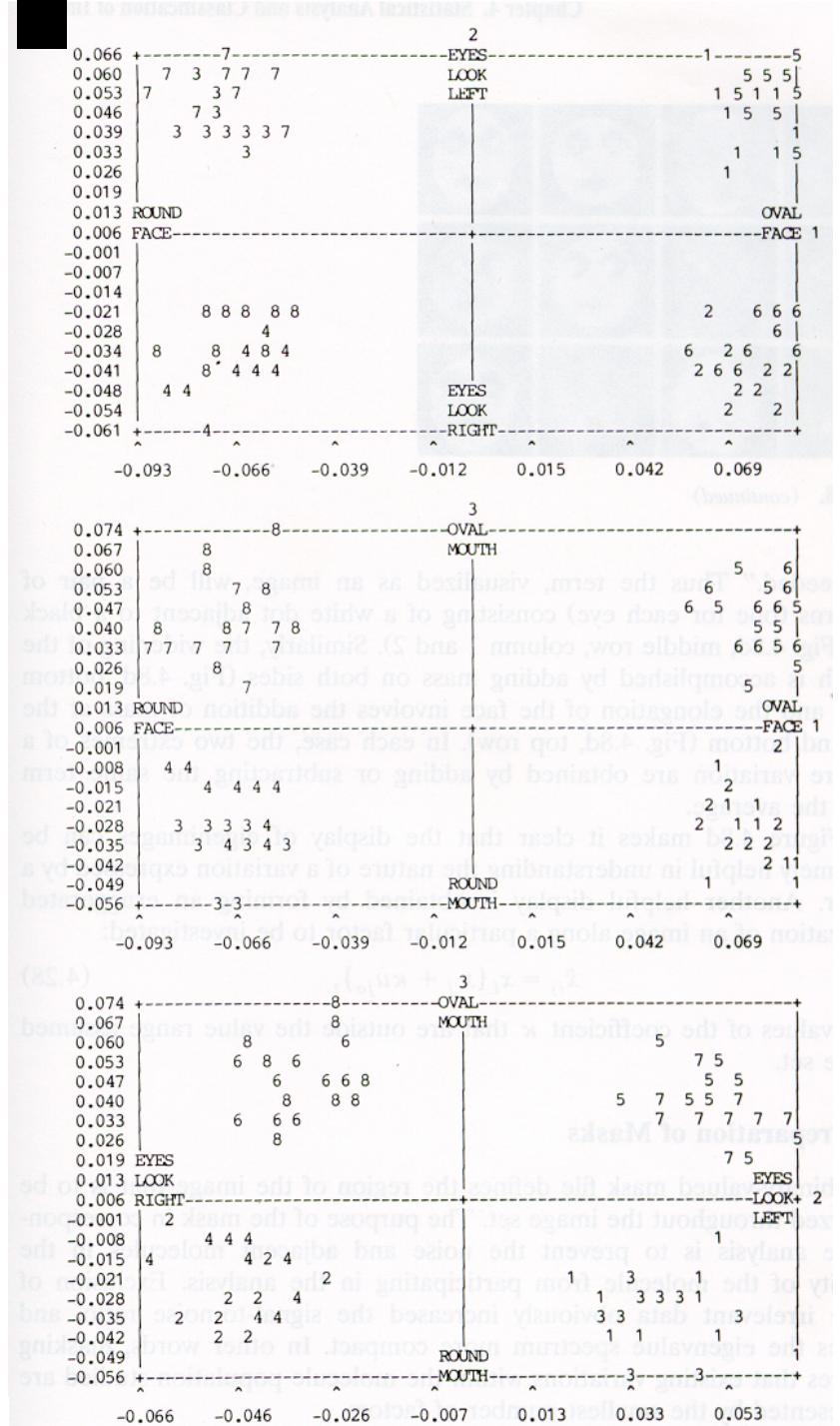
3 stand out,  
i.e., 3 factors are  
sufficient!

1 vs 2

Data are clustered in the eight corners of a 3D factor space formed by the first three factors.

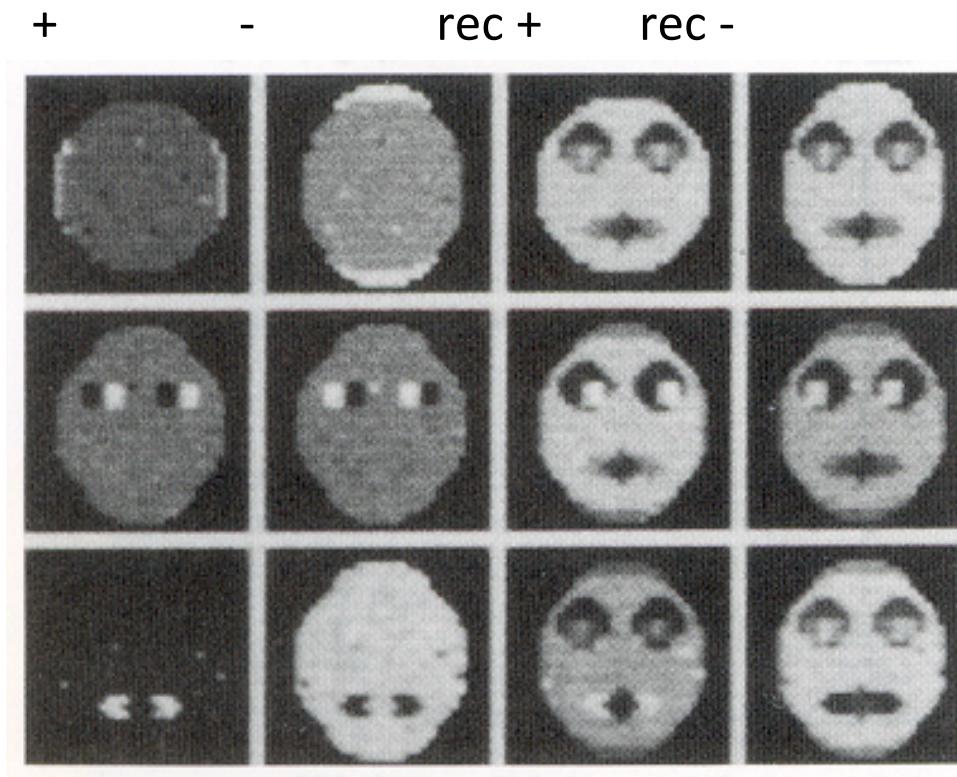
1 vs 3

2 vs 3



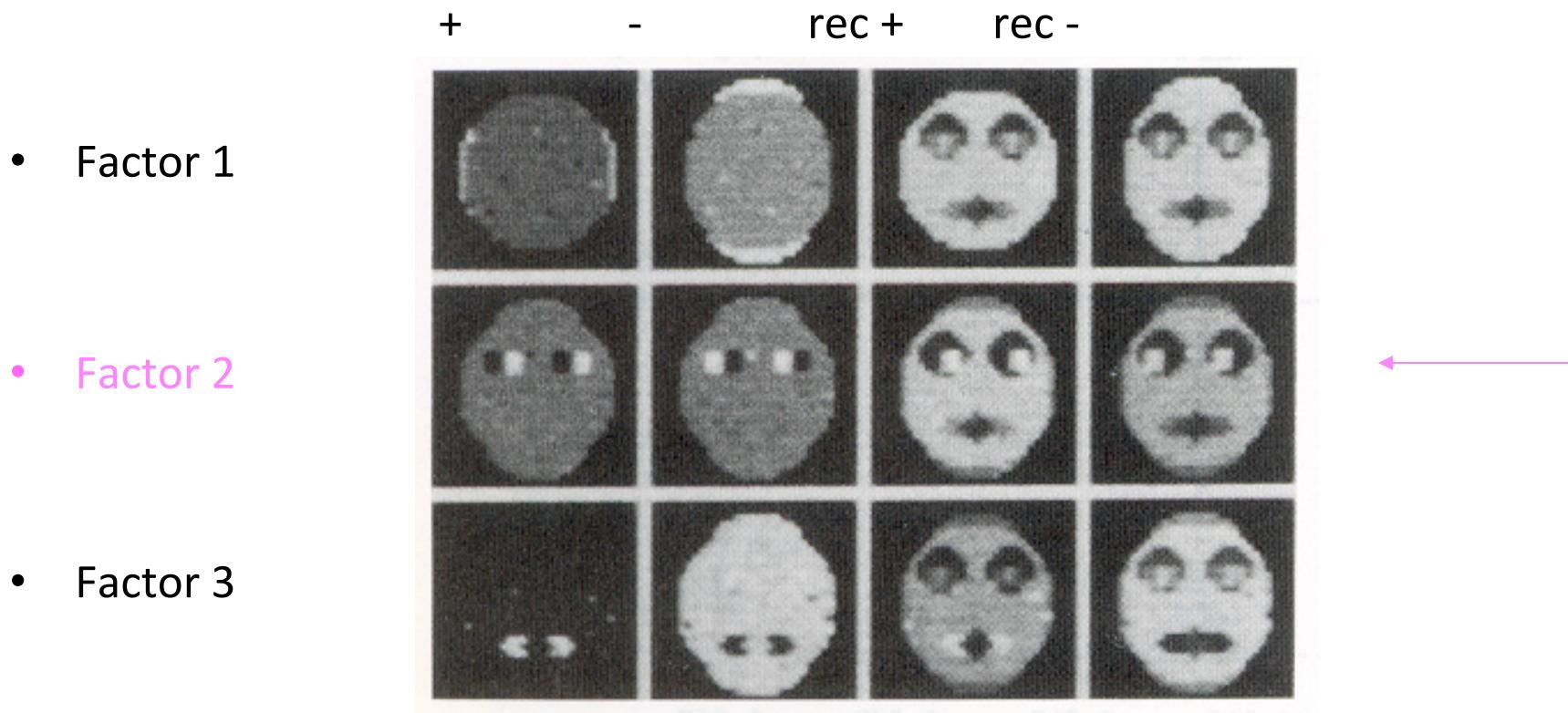
# MDA: eigenimages

- Factor 1
- Factor 2
- Factor 3



Example Factor 2: position of eyes. Average contains centered oval (from superposition of all images containing eyes shifted to right with those having eyes shifted to the left).

To express the eye-related features of the images, the eigenimage must either subtract density on the left and add it on the right, or vice-versa.



Class Averages

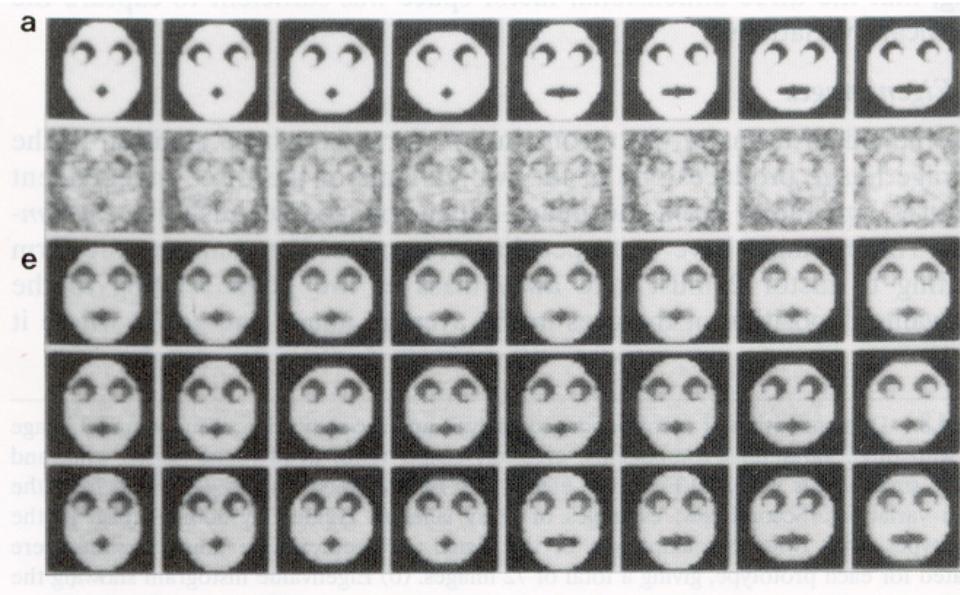
p. 176

Instances

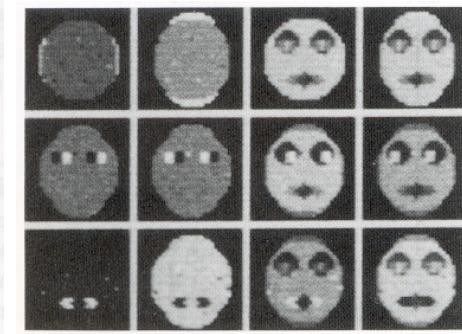
+

-

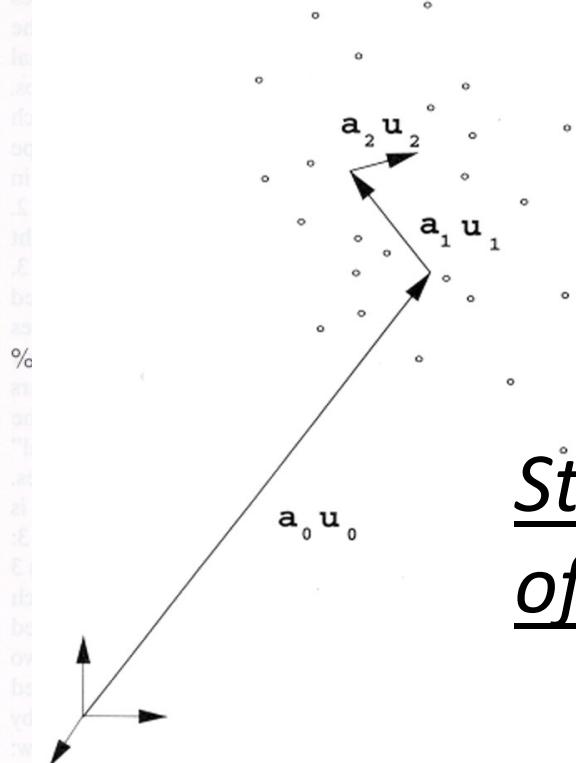
Total Avrg + F1



Total Avrg + F1+F2



Total Avrg + F1+F2+F3



Stepwise reconstitution  
of an image

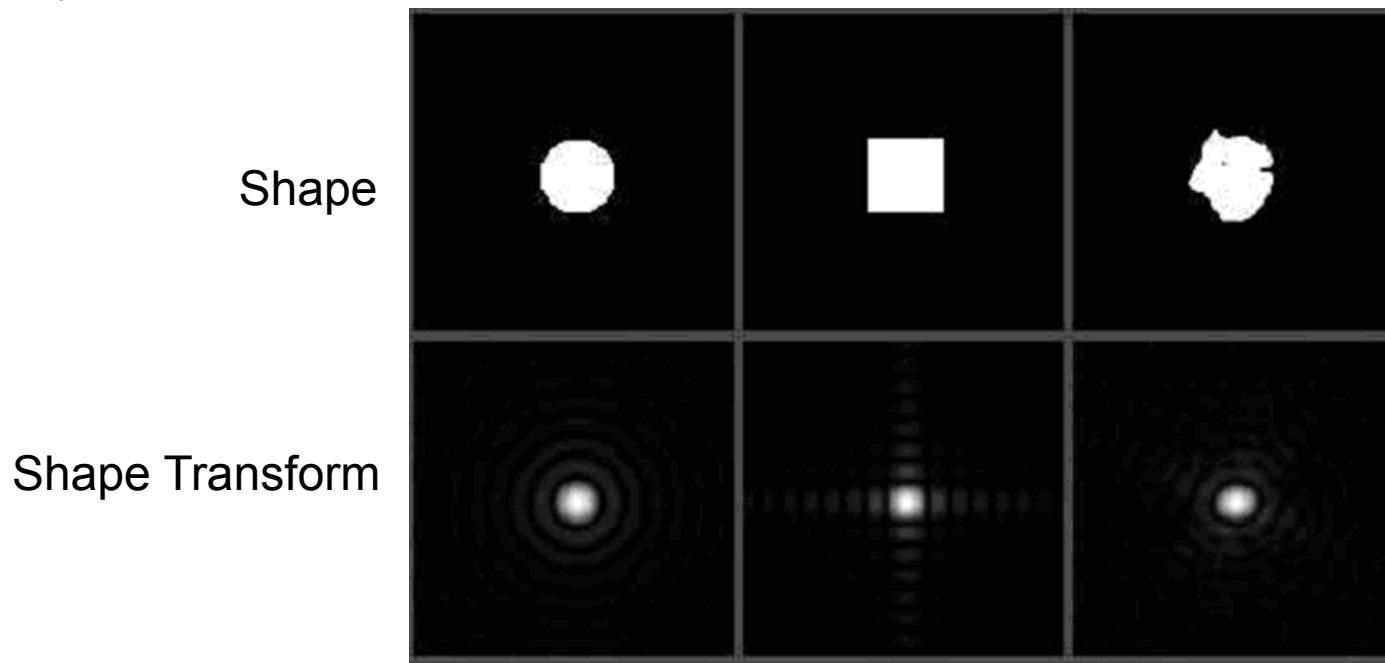
p. 159

# 3D reconstruction -- preliminaries

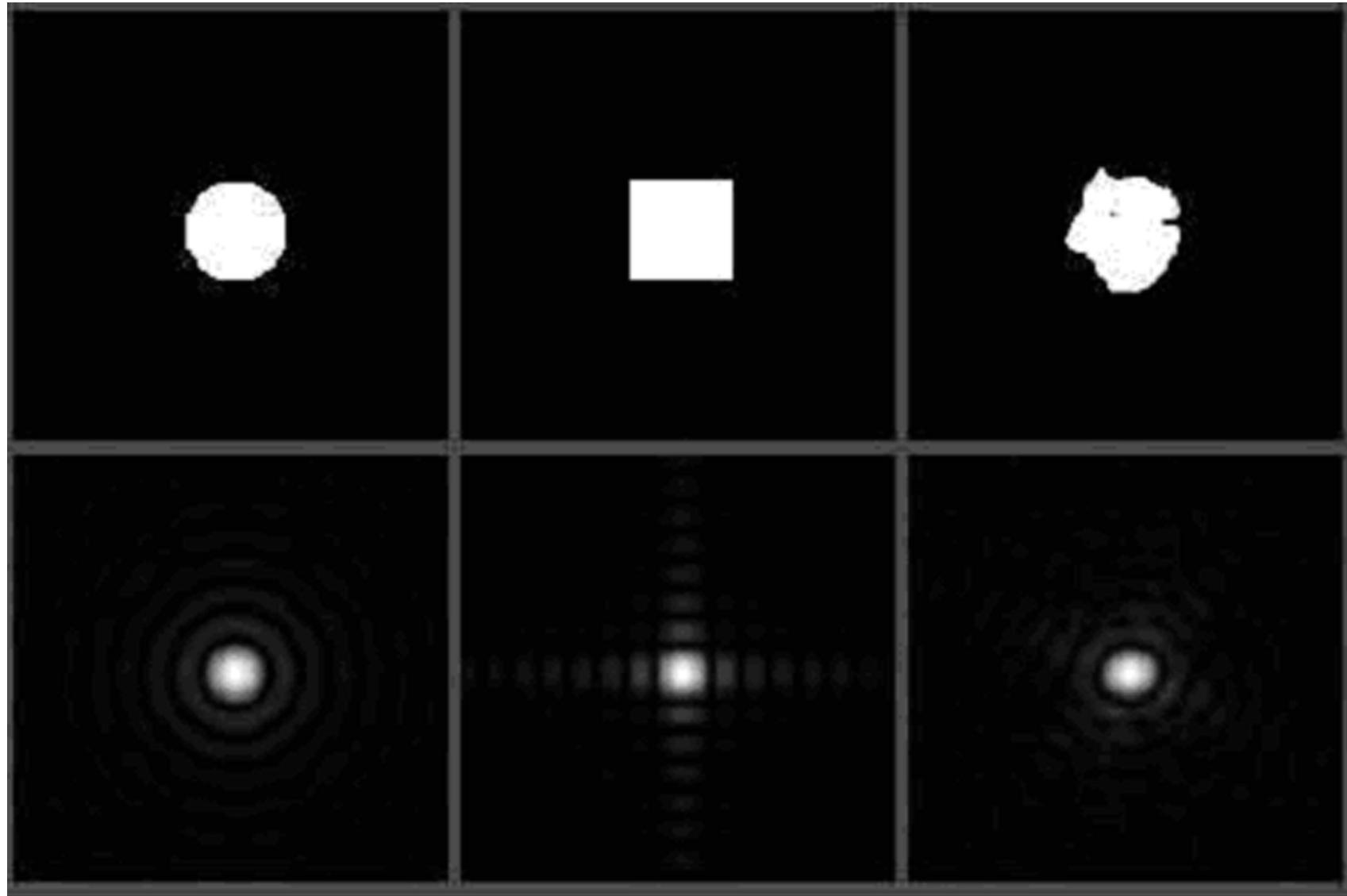
- Under what conditions are projections of an object similar to one another?
- Similarity  $\leftrightarrow$  closeness in high-dim E-space
  - $\leftrightarrow$  belonging to the same cluster
  - $\leftrightarrow$  high correlation

# Shape Transform

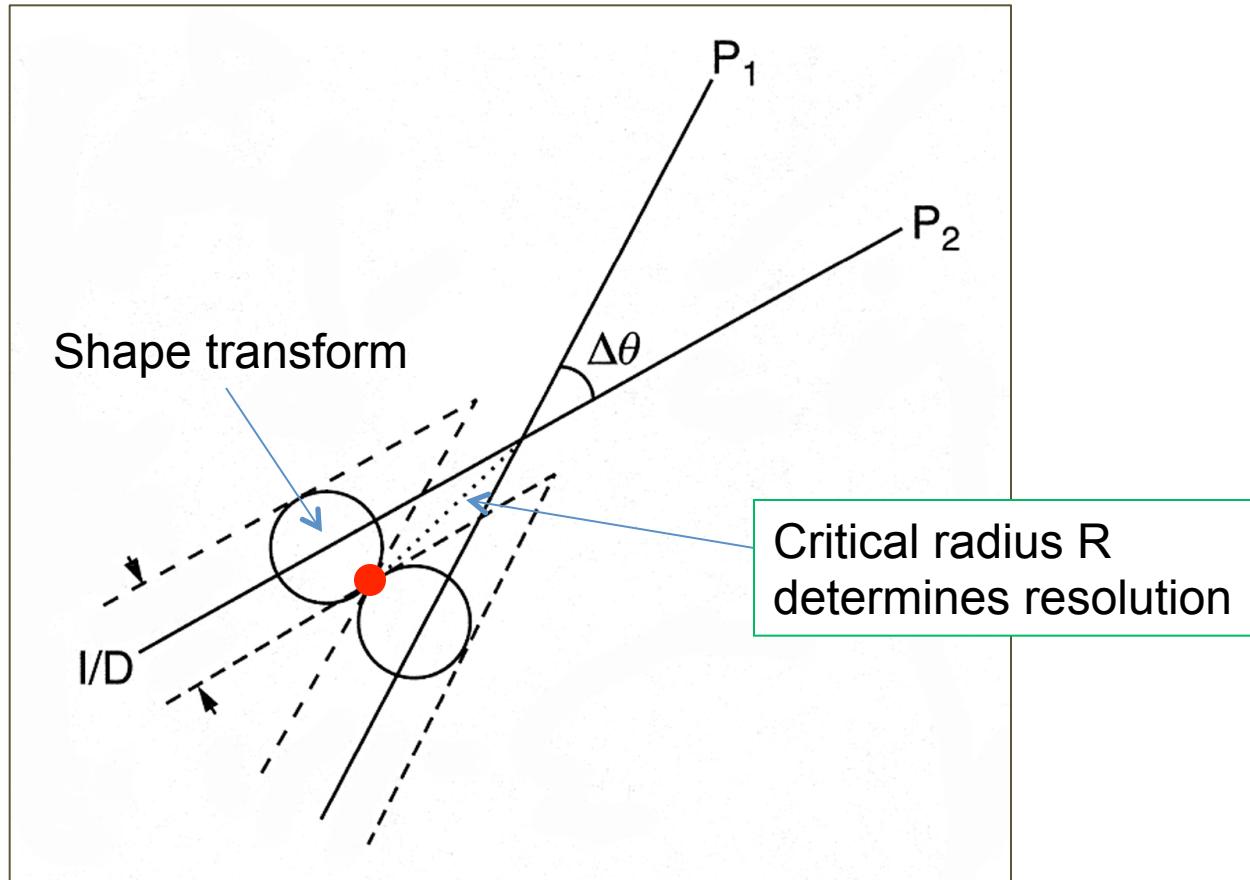
- The Shape Transform is the Fourier transform of a binary mask function (1 inside, 0 outside) whose shape is the shape of an object in 2D or 3D
- It indicates the size and shape of the local region in Fourier space within which Fourier coefficients are correlated/dependent.



# Shape Transforms



# Similarity of projections, condition for 3D reconstruction, and “kissing” shape transforms

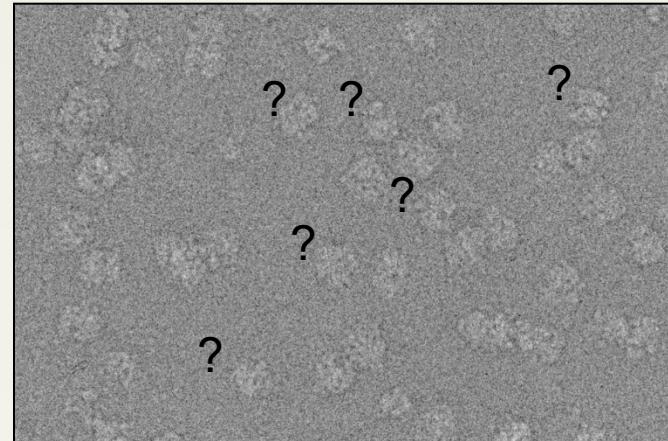


$P_1, P_2$  central sections in Fourier space.  
 $\Delta\theta$  angle subtended by  $P_1, P_2$   
 $D$  particle diameter

# Determination of Particle Orientations

(A) unknown structure -- bootstrap

- (1) Random-conical (uses unsupervised classification)
- (2) Common lines/ angular reconstitution  
(uses unsupervised classification)



(B) known structure – low-res map available

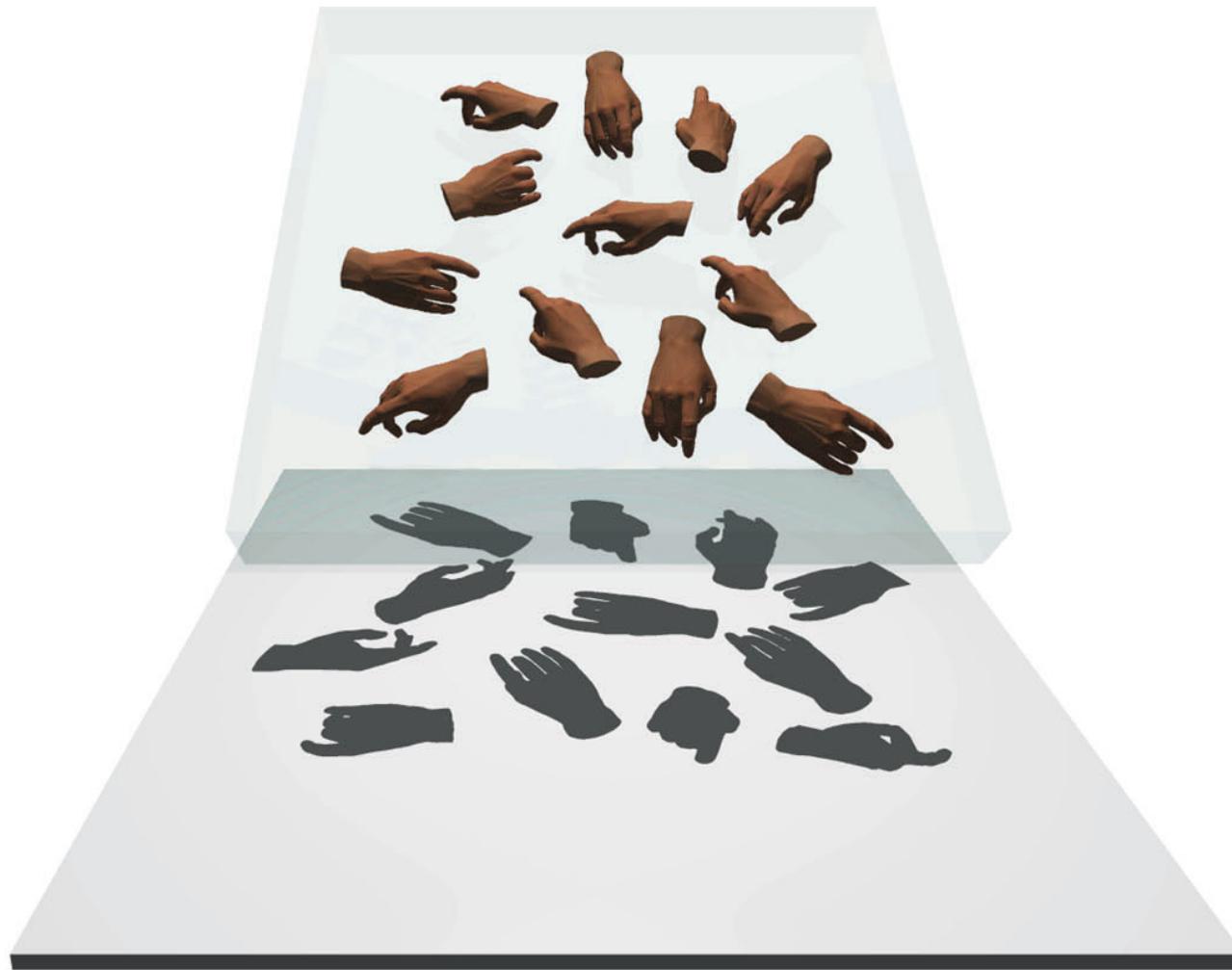
- (1) reference-based (3D projection matching  
= a form of supervised classification)
- (2) common lines/ angular reconstitution

# RANDOM CONICAL RECONSTRUCTION

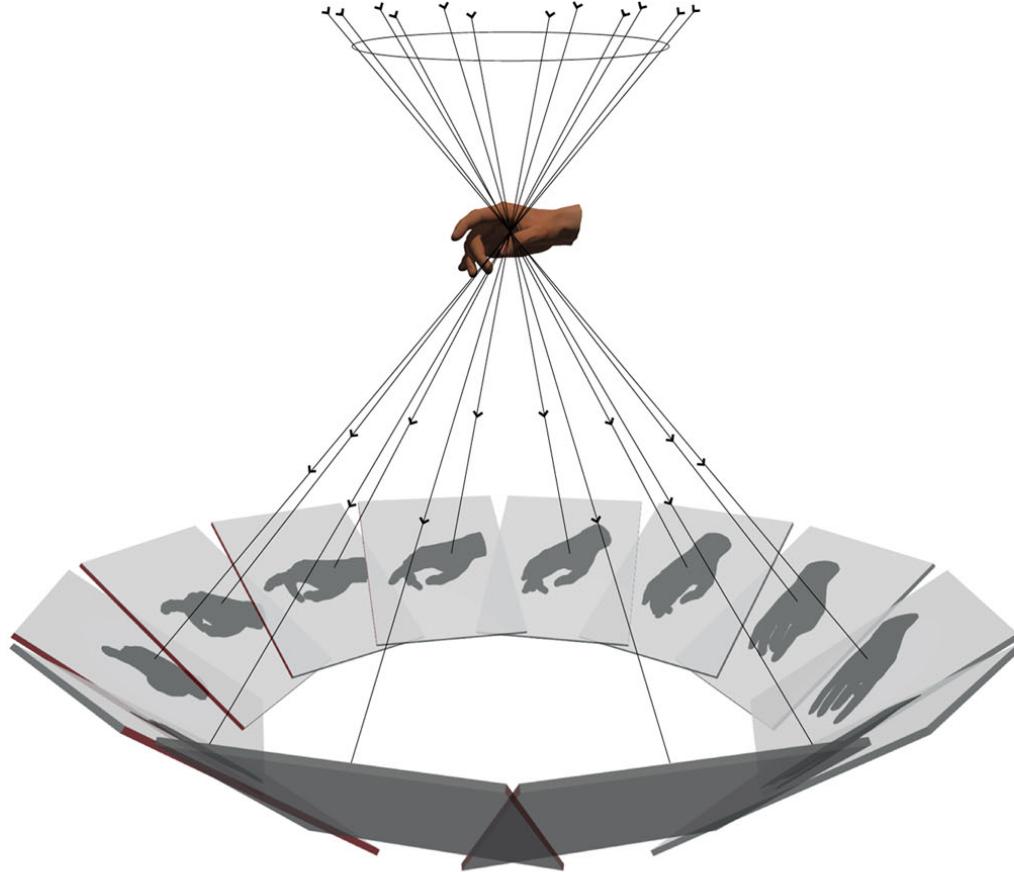
0-degree view



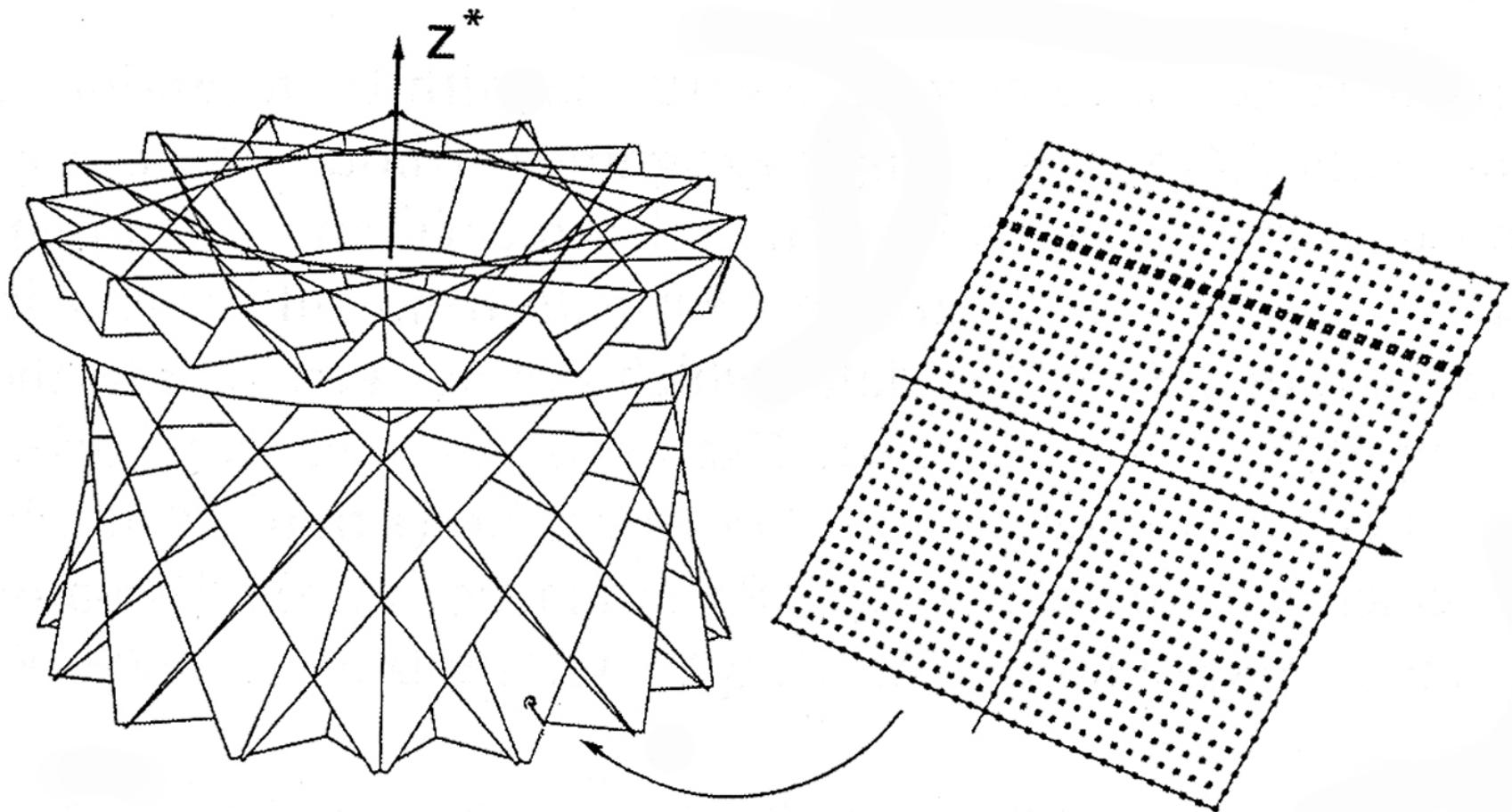
## 50-degree view



## Equivalent geometry in the coo system of the particle

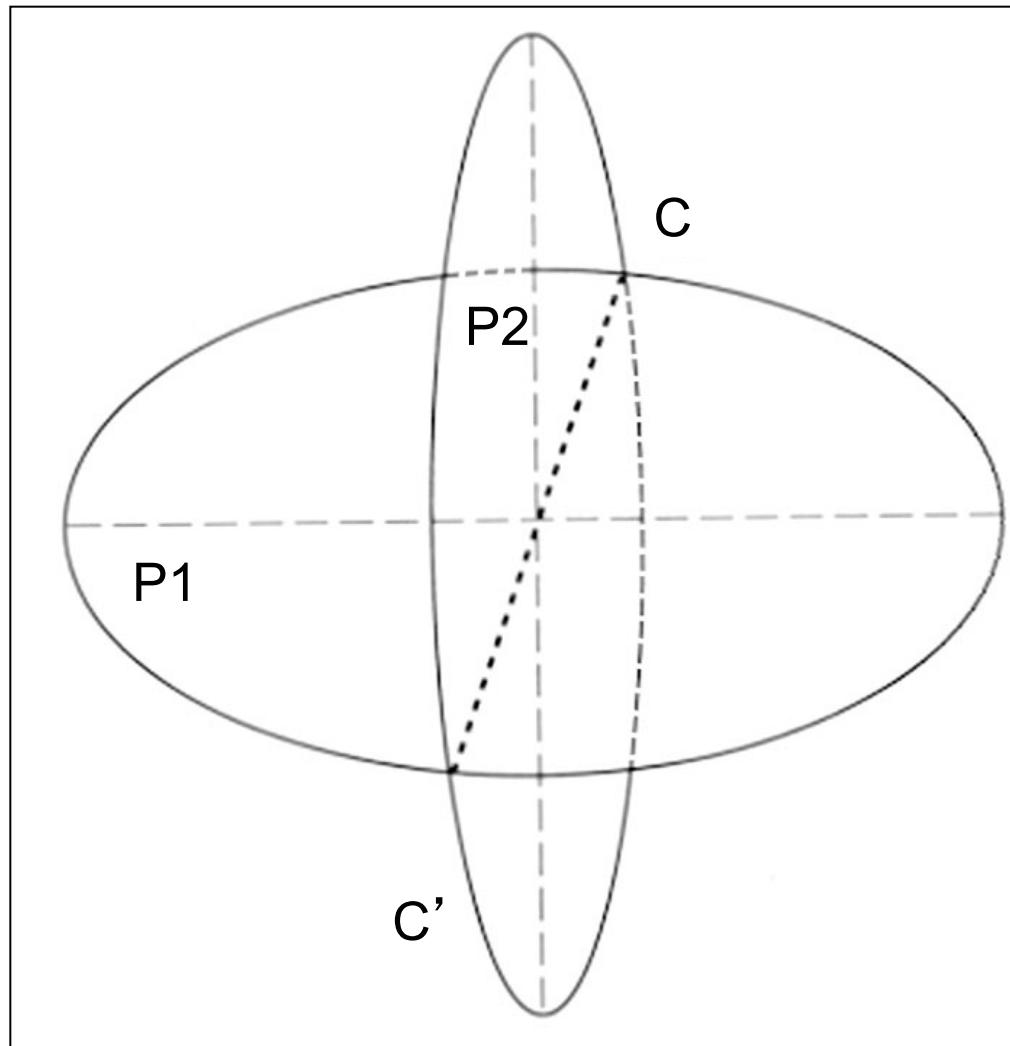


# Conical Data Collection Geometry in Fourier Space



Lanzavecchia et al.

# COMMON LINES APPROACH TO DETERMINING ORIENTATION



Common line C-C' of two projections represented by central sections P1 and P2

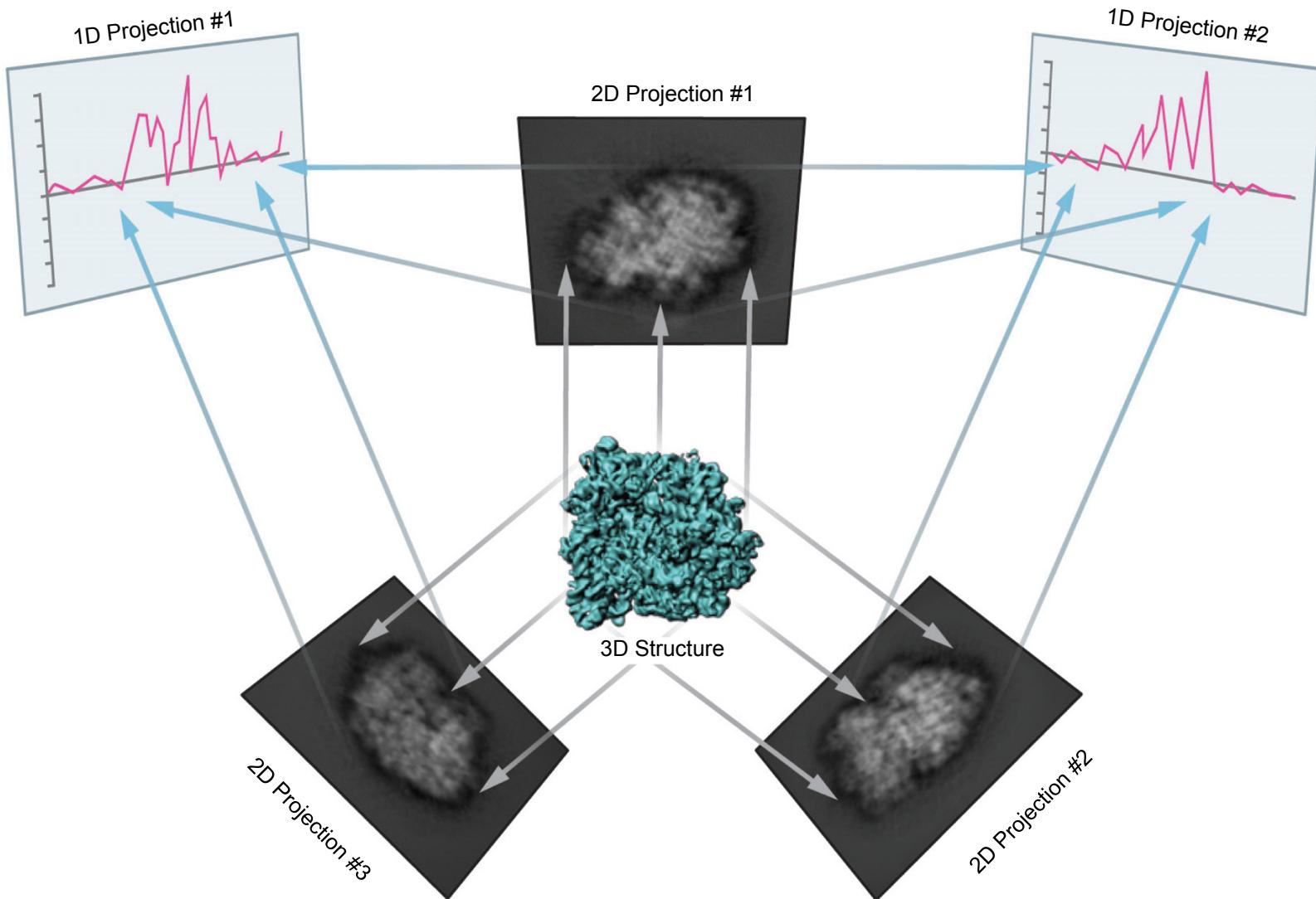
Two 2D projections of the same 3D object have in common:

in Fourier space: Fourier coefficients along the  
line of intersection: “common line”

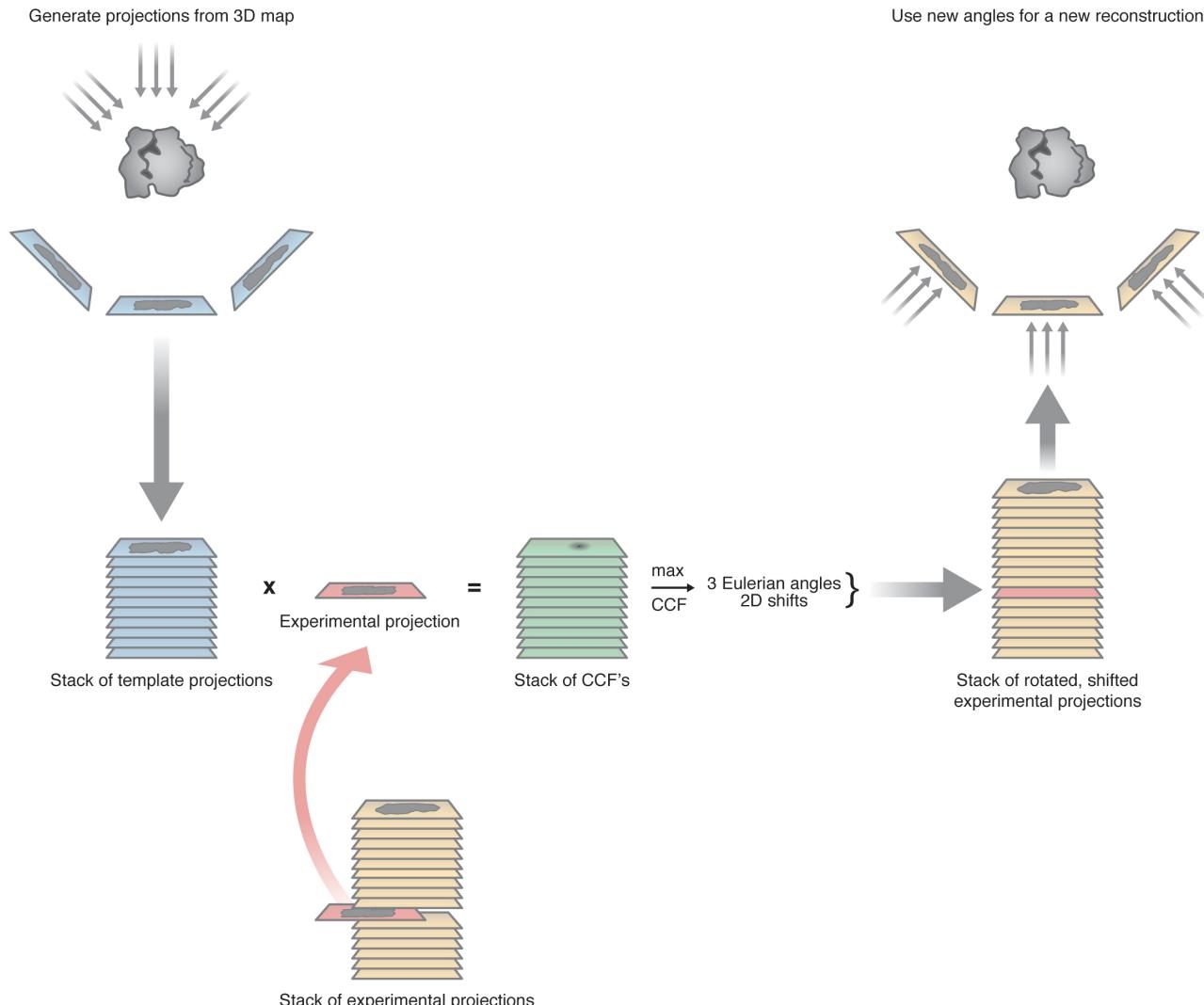
in real space: 1D projections in particular directions

The Sinogram (or Radon transform) of an image is an exhaustive ordered display of all of its 1-D projections

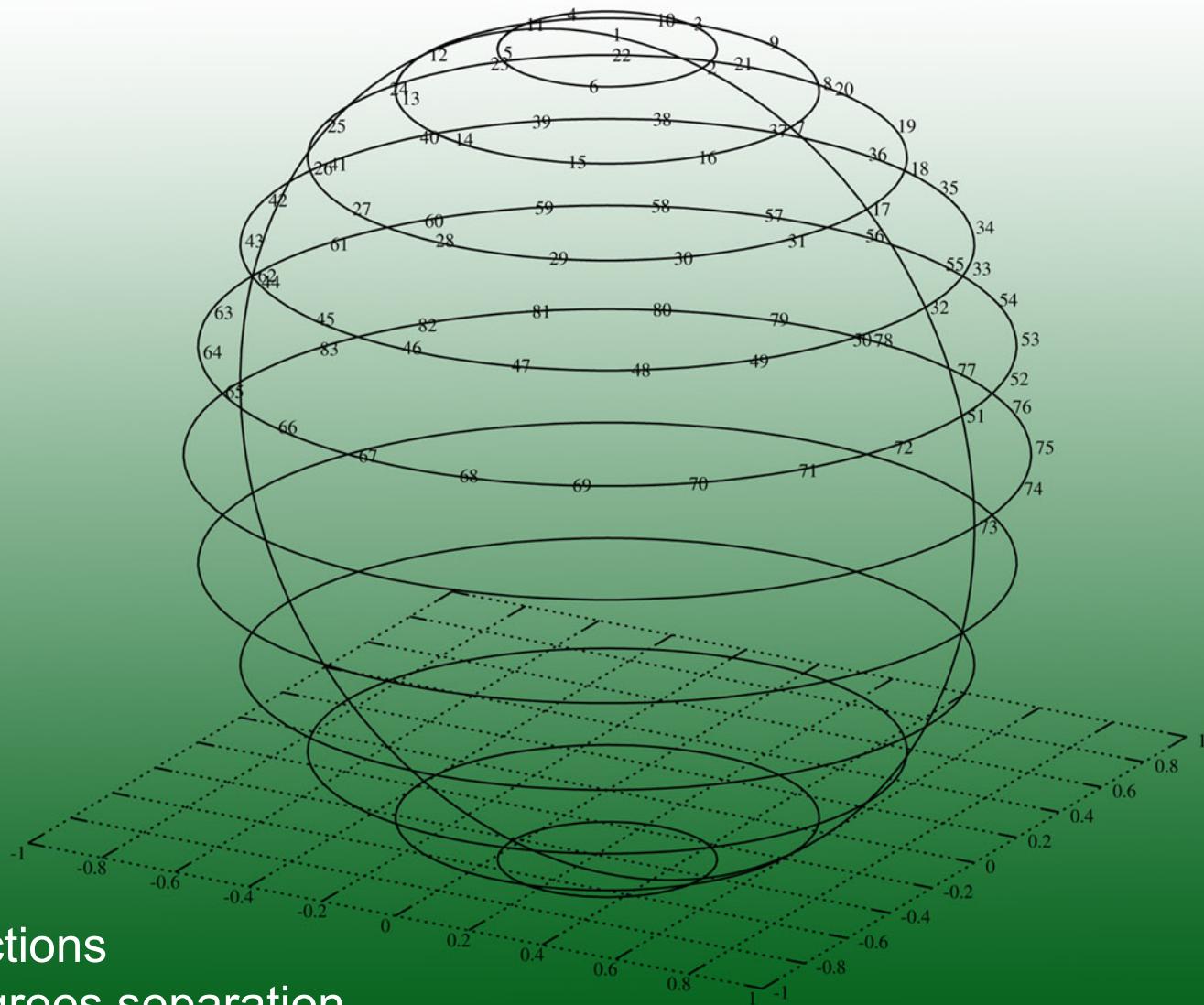
Comparing two sinograms, one can find the angles for which maximum agreement is reached

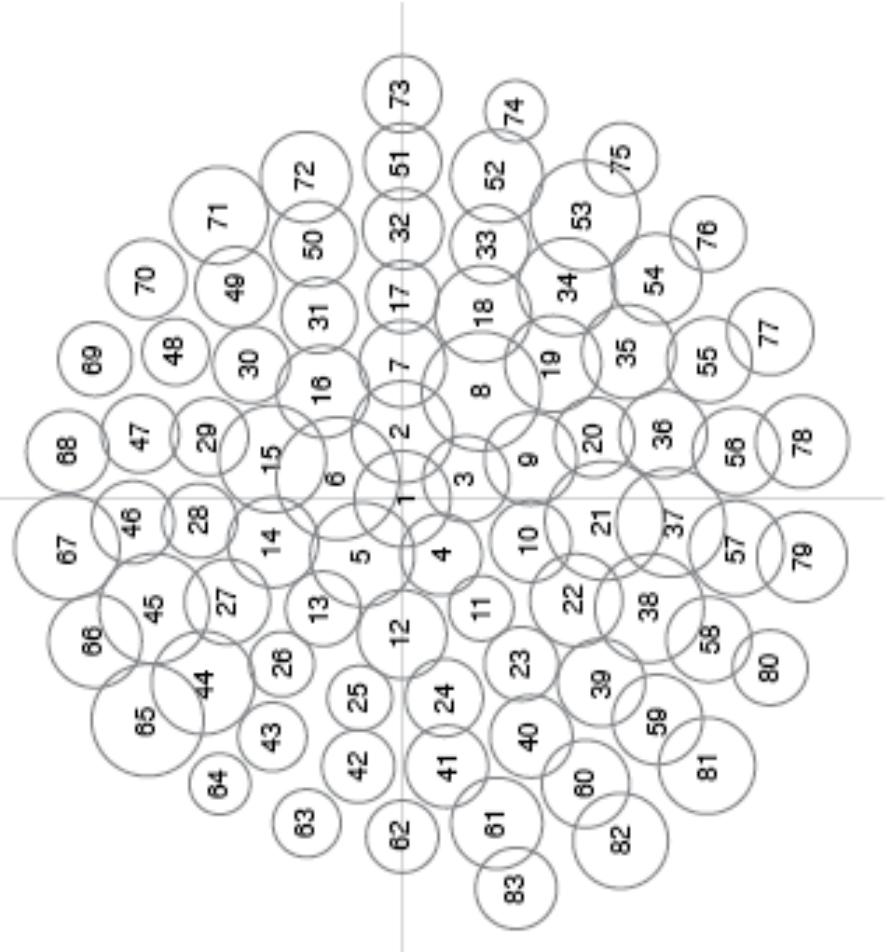


# Determination of orientation by projection matching

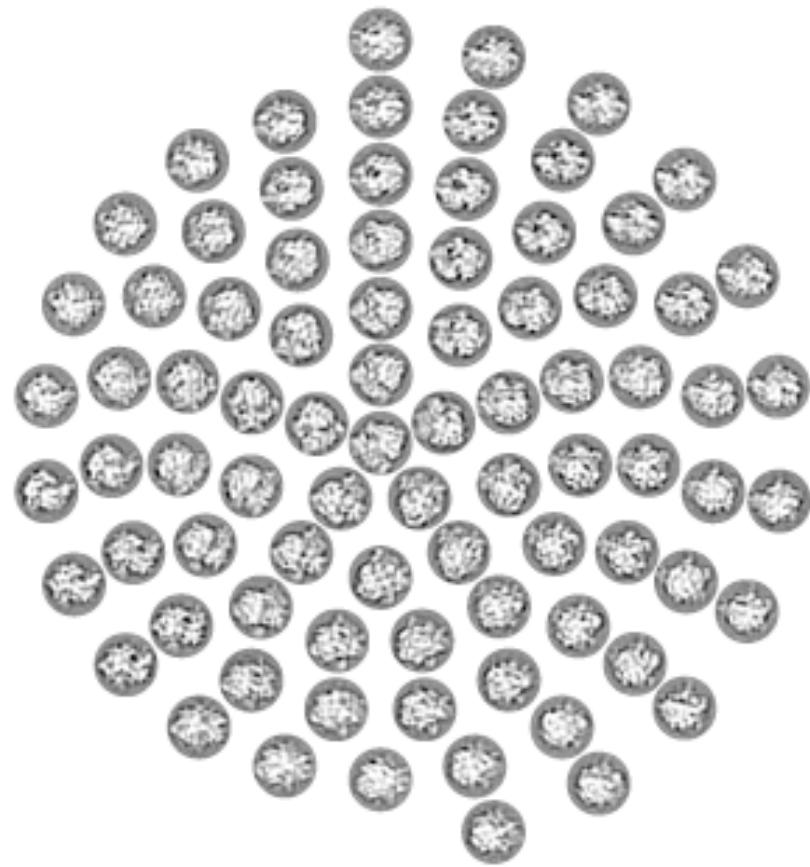


# Initial Angular Grid



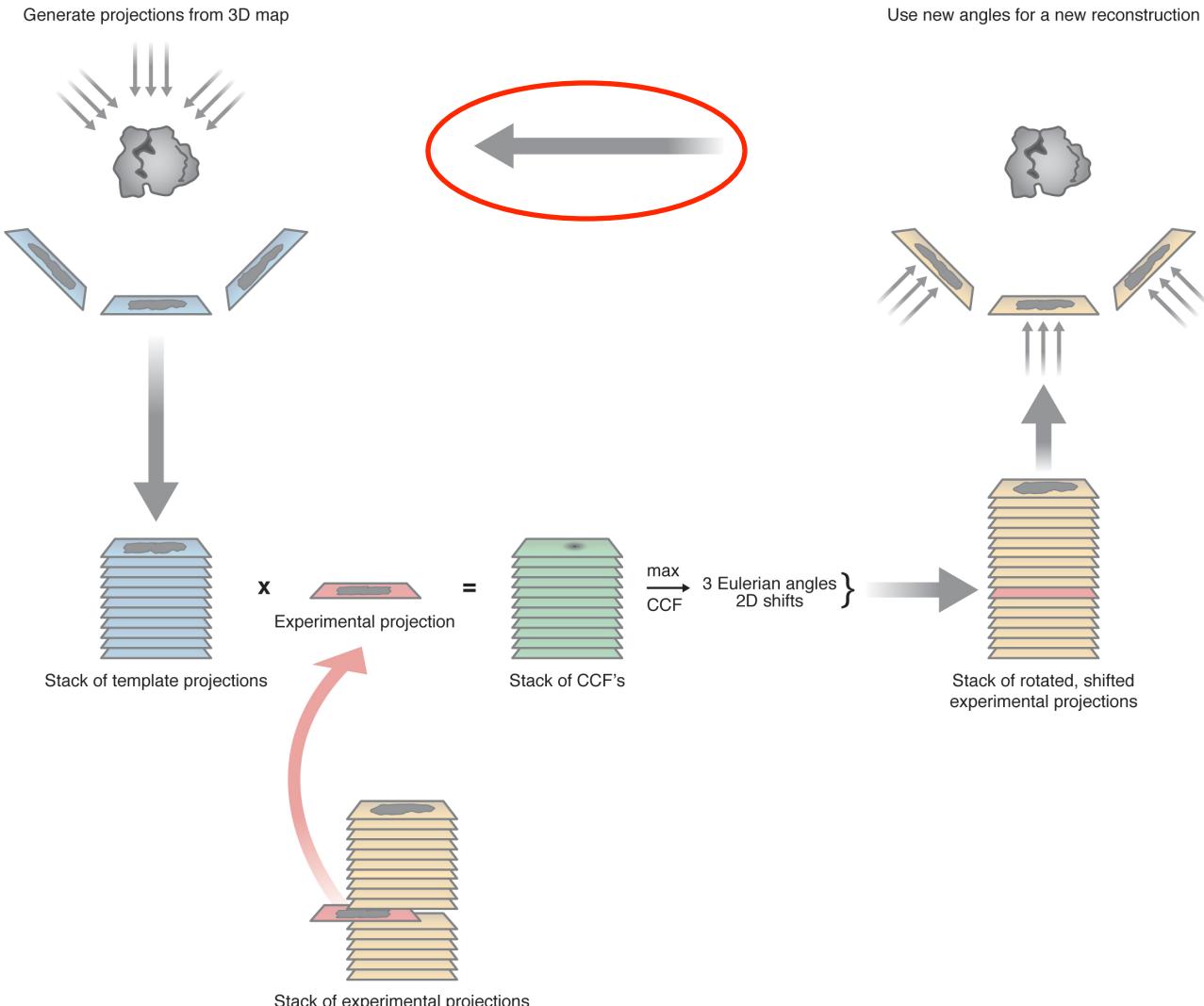


83-projection grid



averages of particles classified

# Iterative Angular Refinement



# Angular Refinement

Given an initial 3D reference,

Iterate the steps {3D projection matching + reconstruction}

Decrease angular grid size as you go on (range:  $15^\circ \rightarrow 0.5^\circ$ )

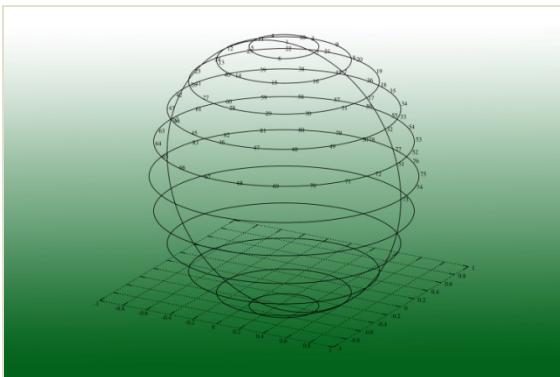
Convergence criteria:

- (1) convergence of particle angles
- (2) convergence of resolution (monitor progress with FSC)

“Rule of neighborhood” saves computing time

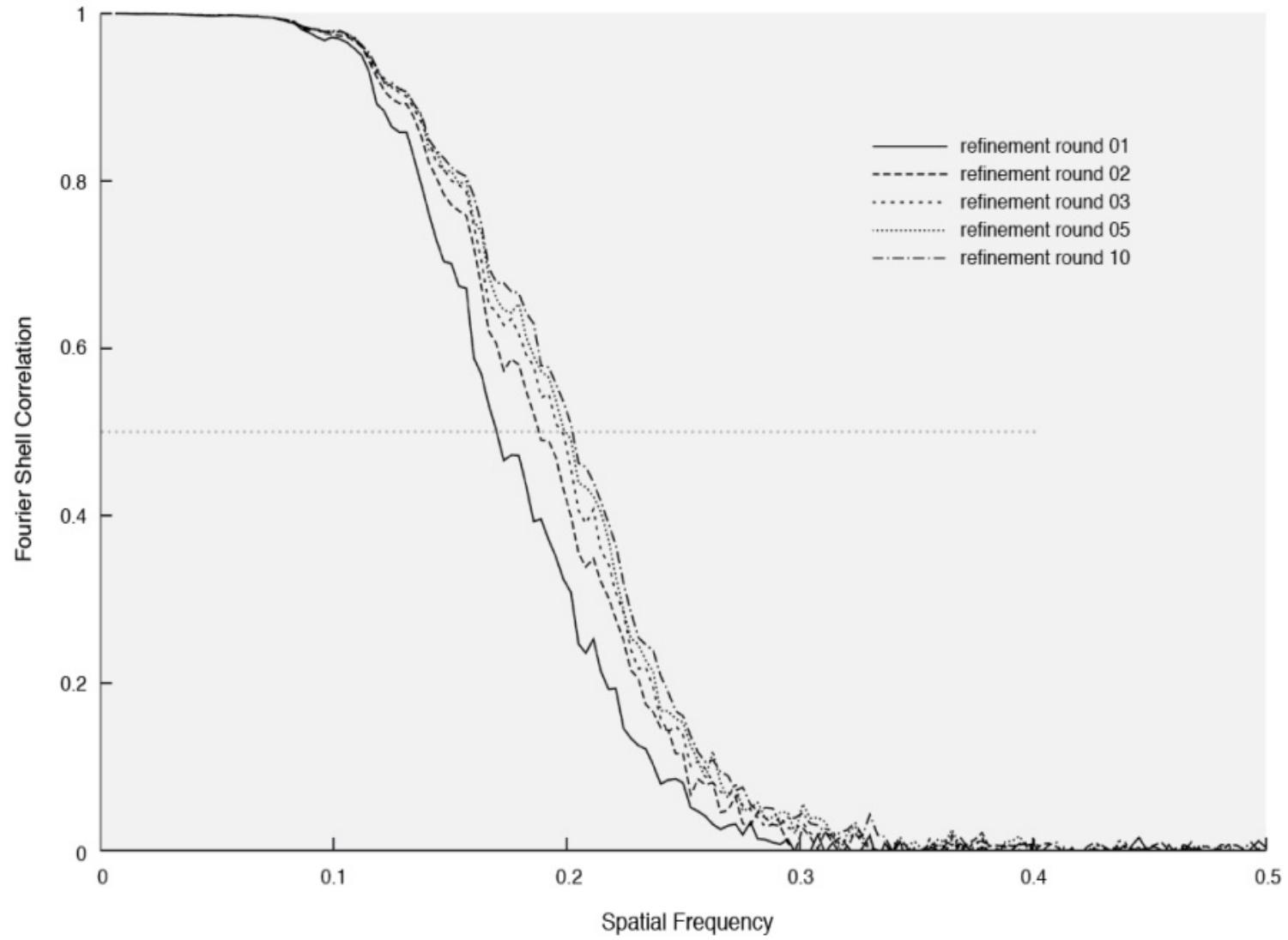
- questions

- Start with coarse grid (15 degrees)
- Decrease angular separation, down to 0.5 degrees
- At some point, switch from global coverage to local coverage of previously determined angles



Increasingly finer angular increments

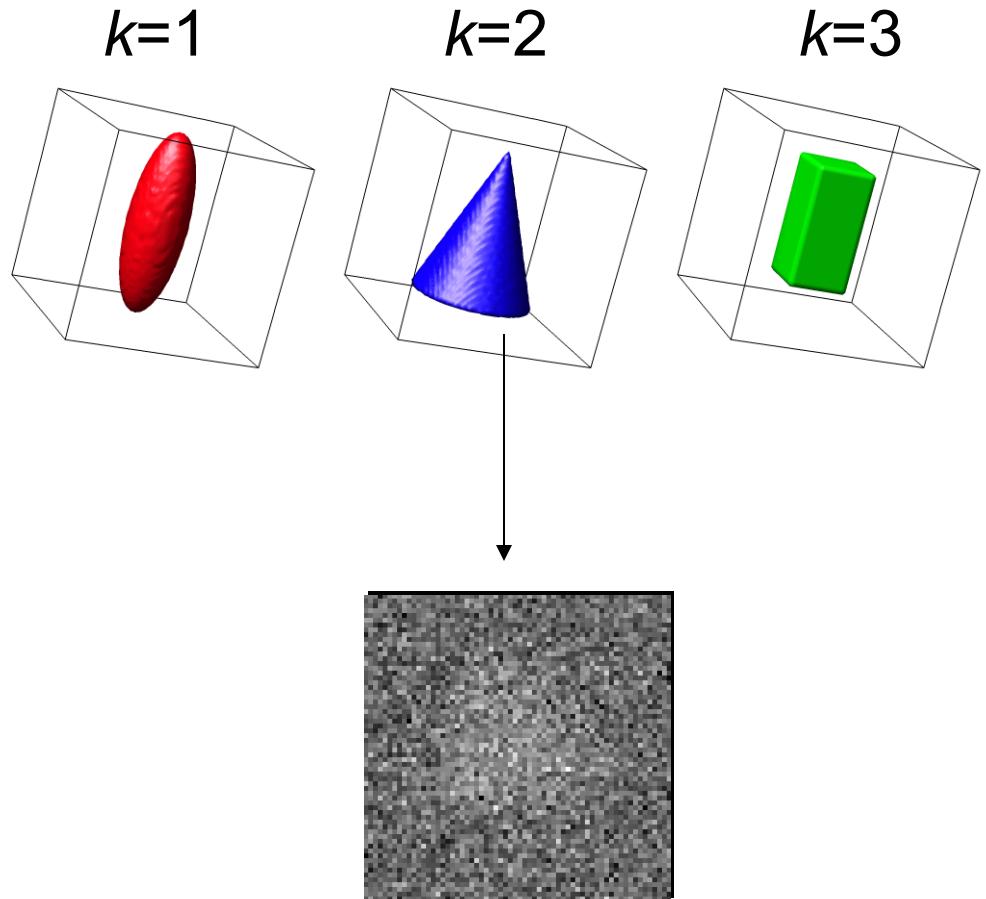




FSC following progress of refinement

# 3D Unsupervised Classification

Statistical model:  
each image is a projection of one  
of  $K$  underlying 3D objects,  $k$ .

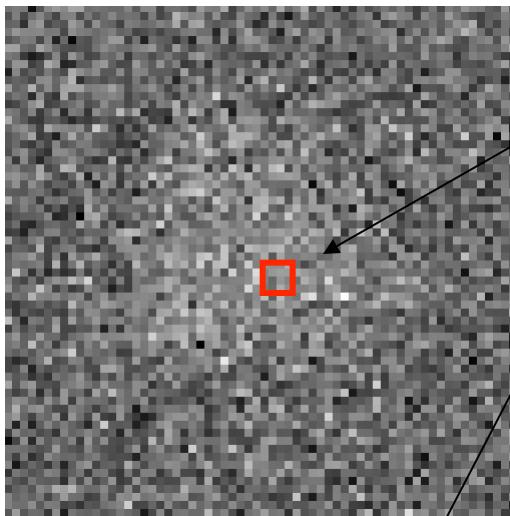


with addition of  
white Gaussian noise

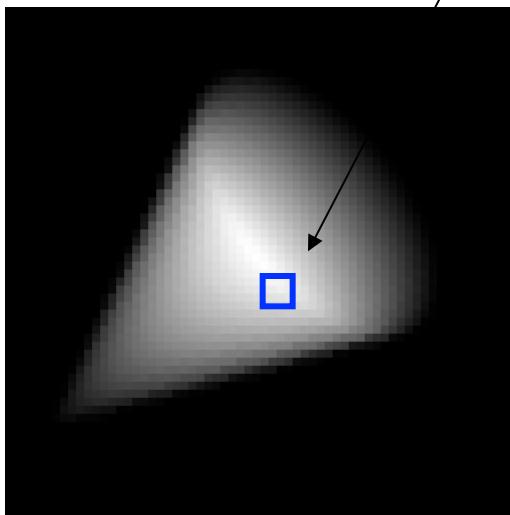
Unknowns: class numbers  $k$ , rotations, translations

*Statistical model: the probability that  $X_j$  is observed at pixel  $j$ , given the data model  $A_j$ , has Gaussian distribution centered on  $A_j$ , with halfwidth  $\sigma$*

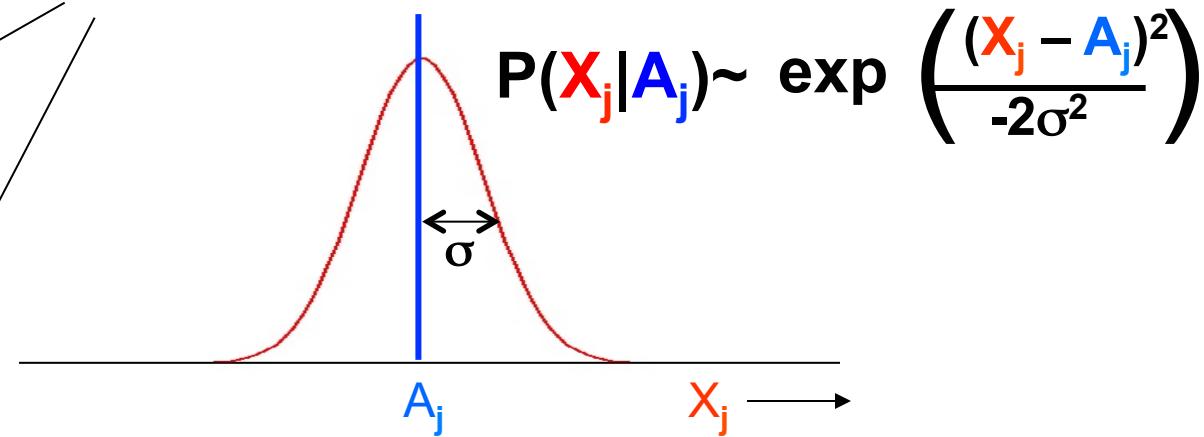
data



model



For each pixel  $j$ :



white noise =  
independence between pixels!

$$P(\text{data image}|\text{model image}) \sim$$

$$\prod_j P(X_j|A_j)$$

# Likelihood

- Find a model  $\Theta$  that optimizes the log-likelihood of observing the entire dataset:

$$L(\Theta) = \sum_{i=1}^N \ln \sum_{k=1}^K \sum_{rot} \sum_{trans} P(image_i | k, rot, trans, \Theta) P(k, rot, trans | \Theta)$$

class

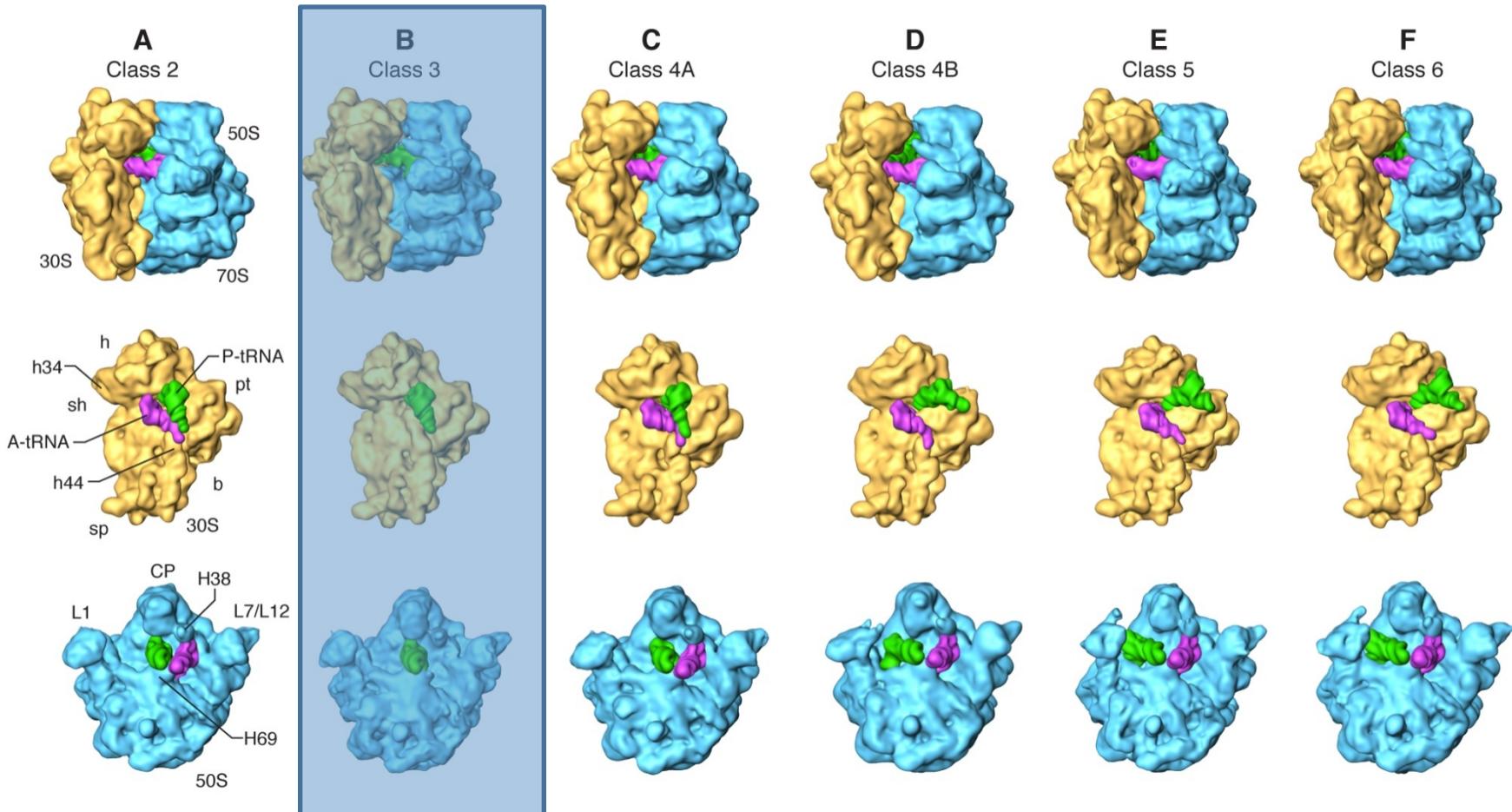
integrate over all **unknowns!**

The model  $\Theta$  comprises: estimates for 3D objects,  $\sigma$ , ...

Optimization algorithm: **Expectation Maximization**

# ML3D

no A-site tRNA



Pre-translocational states of wt 70S *E. coli* ribosome

QUESTIONS?

# Generalized Euclidean distance

Euclidean distance between two images  $f_1$  and  $f_2$ :

$$E^2_{12}(\alpha, r') = \sum_{j=1}^J |f_1(r_j) - f_2(R_\alpha r_j + r')|^2$$

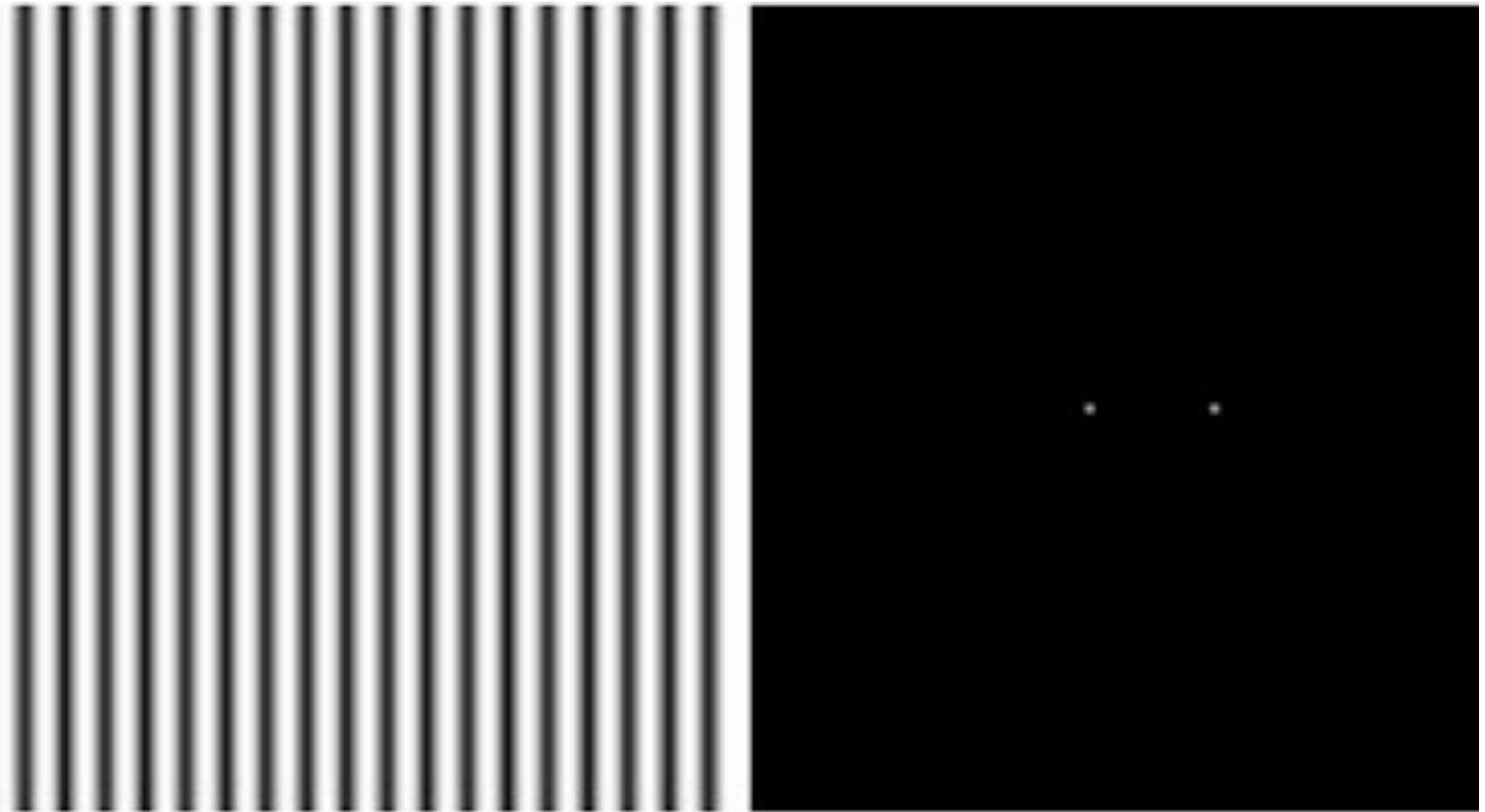
$$\begin{aligned} &= \sum_{j=1}^J |f_1(r_j)|^2 + \sum_{j=1}^J |f_2(R_\alpha r_j + r')|^2 - \sum_{j=1}^J |f_1(r_j)f_2(R_\alpha r_j + r')| \\ &\quad \text{const.} \qquad \qquad \text{const.} \qquad \qquad \text{cross-correlation} \end{aligned}$$

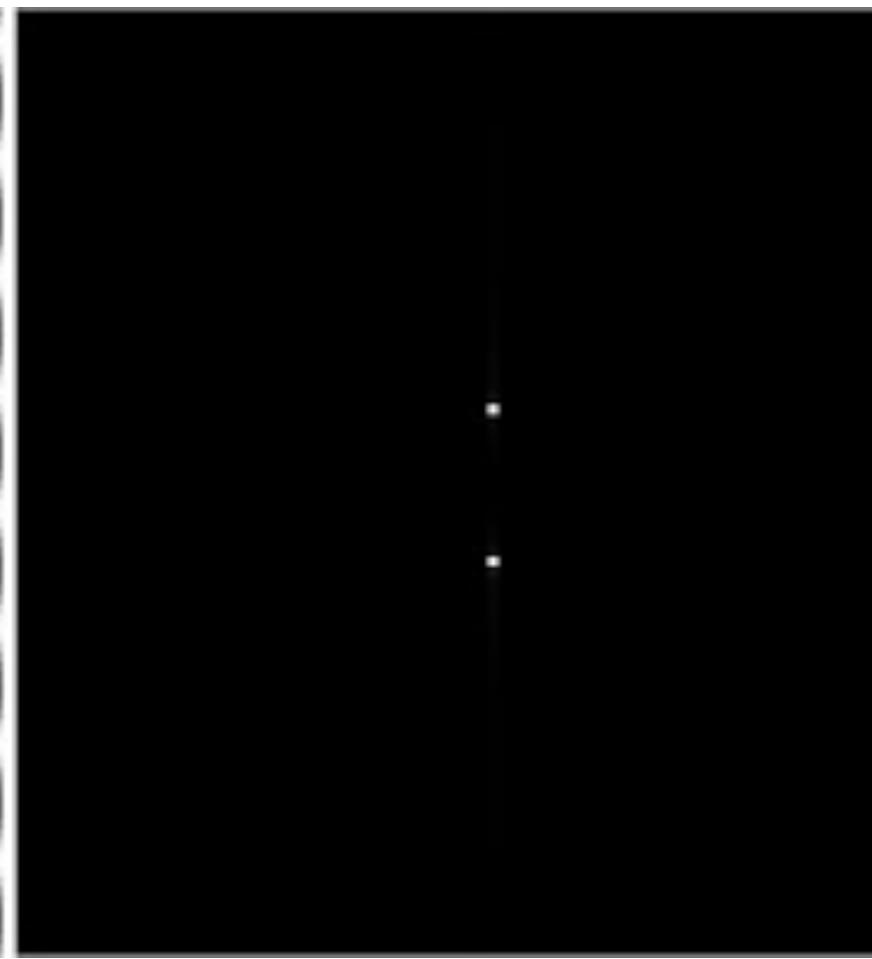
Similarity = closeness in high-dimensional Euclidean space = small E-distance  
→ large value of CCF peak at matching position

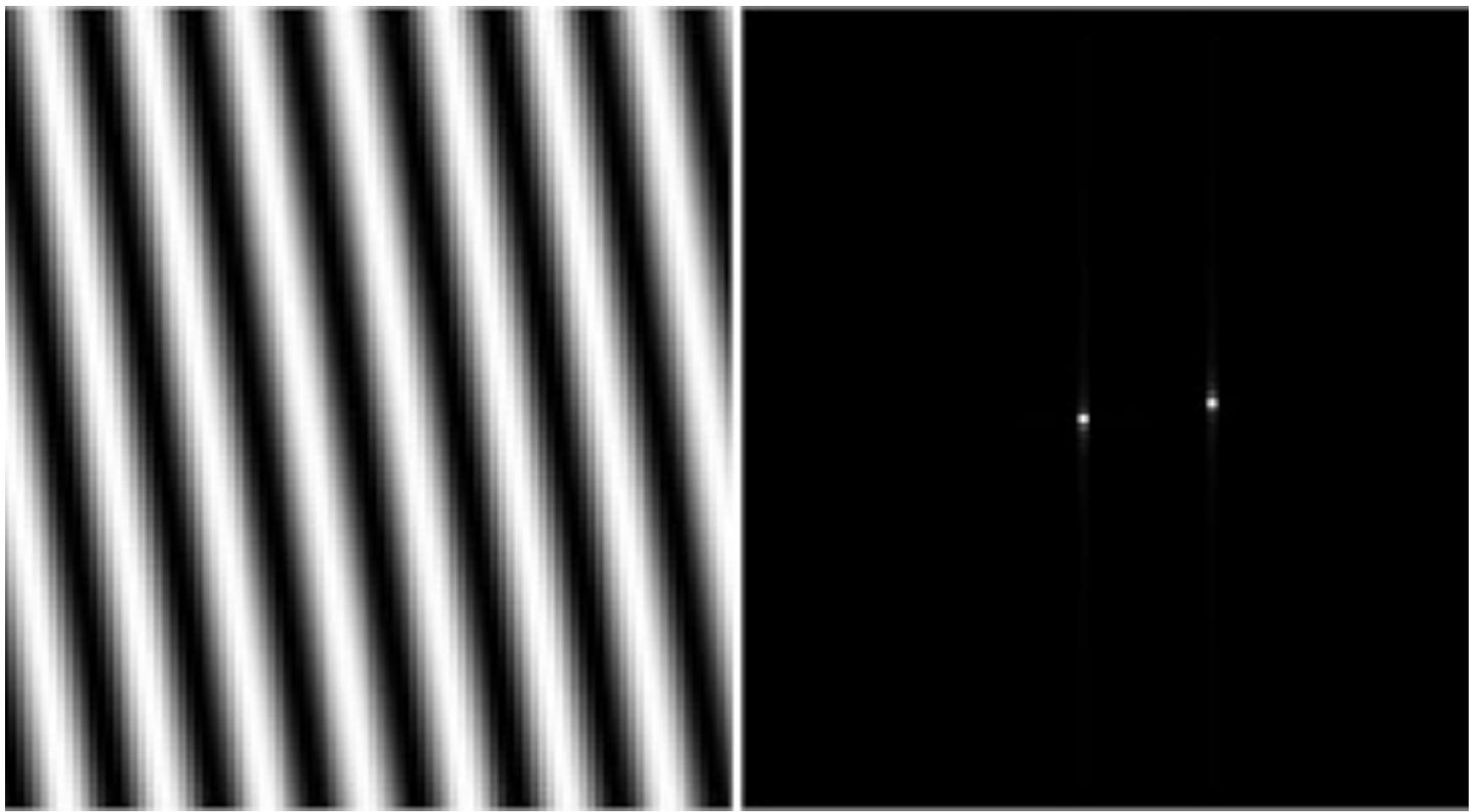
It's easily verified that the Fourier transform of any real-valued image has the following property:

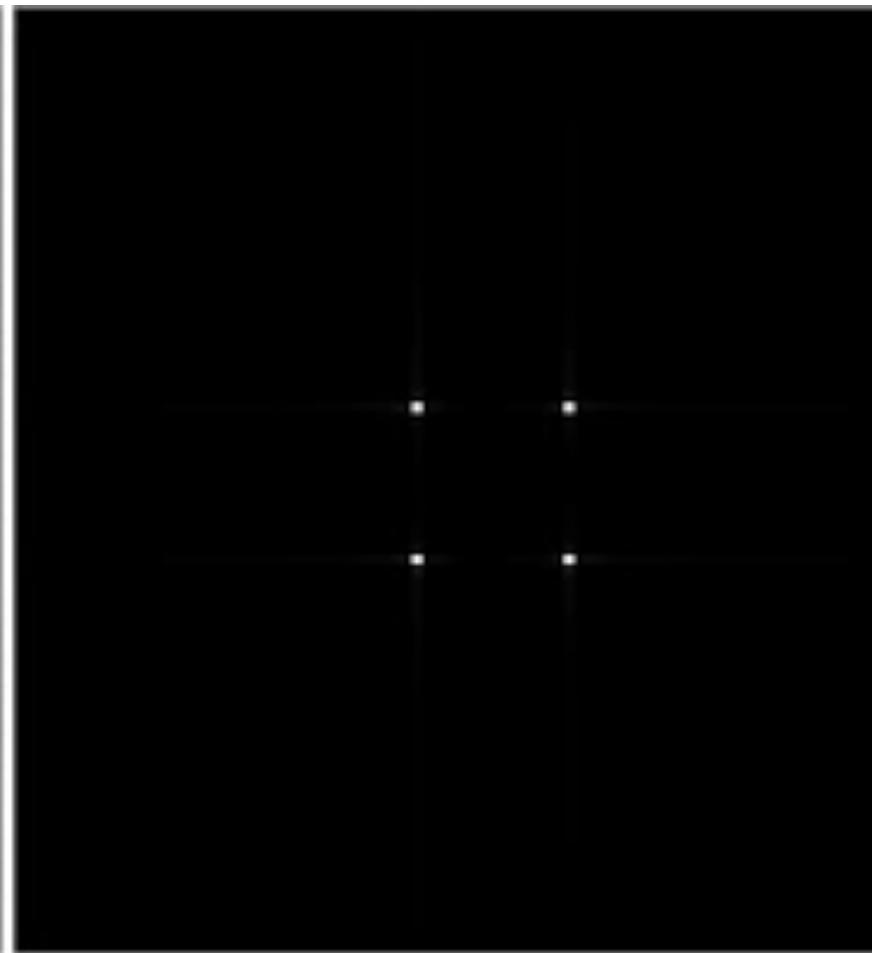
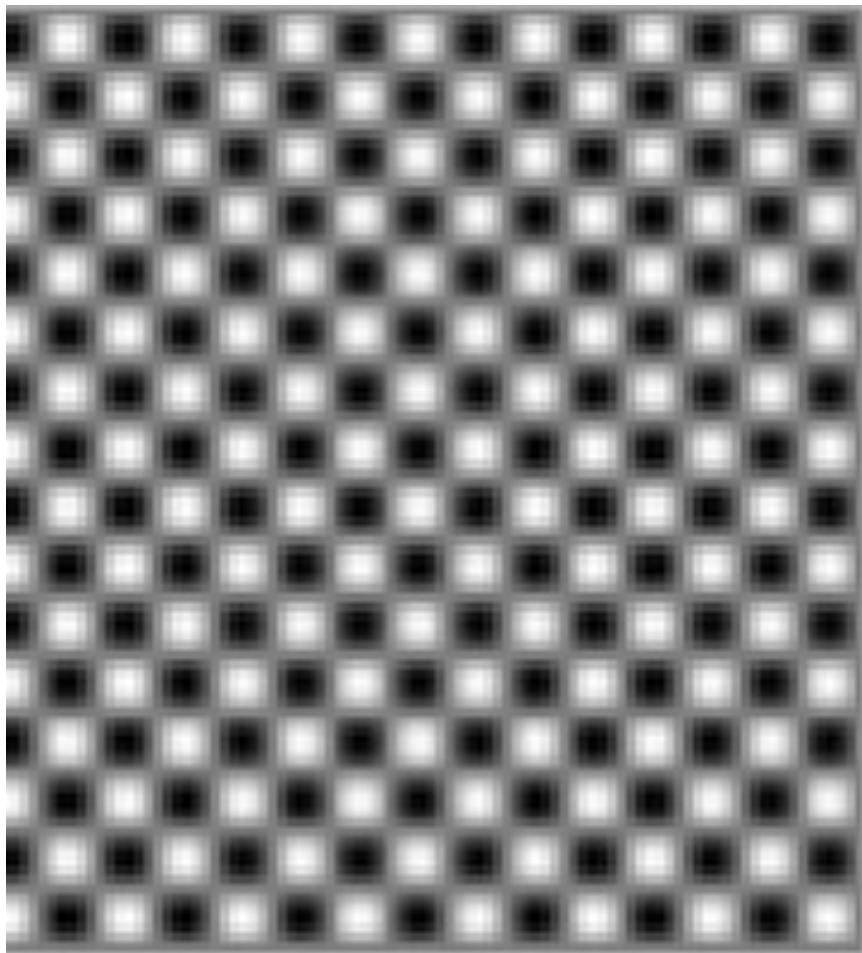
$$F(k_x, k_y) = F^*(-k_x, -k_y) \quad (\text{Friedel's Law})$$

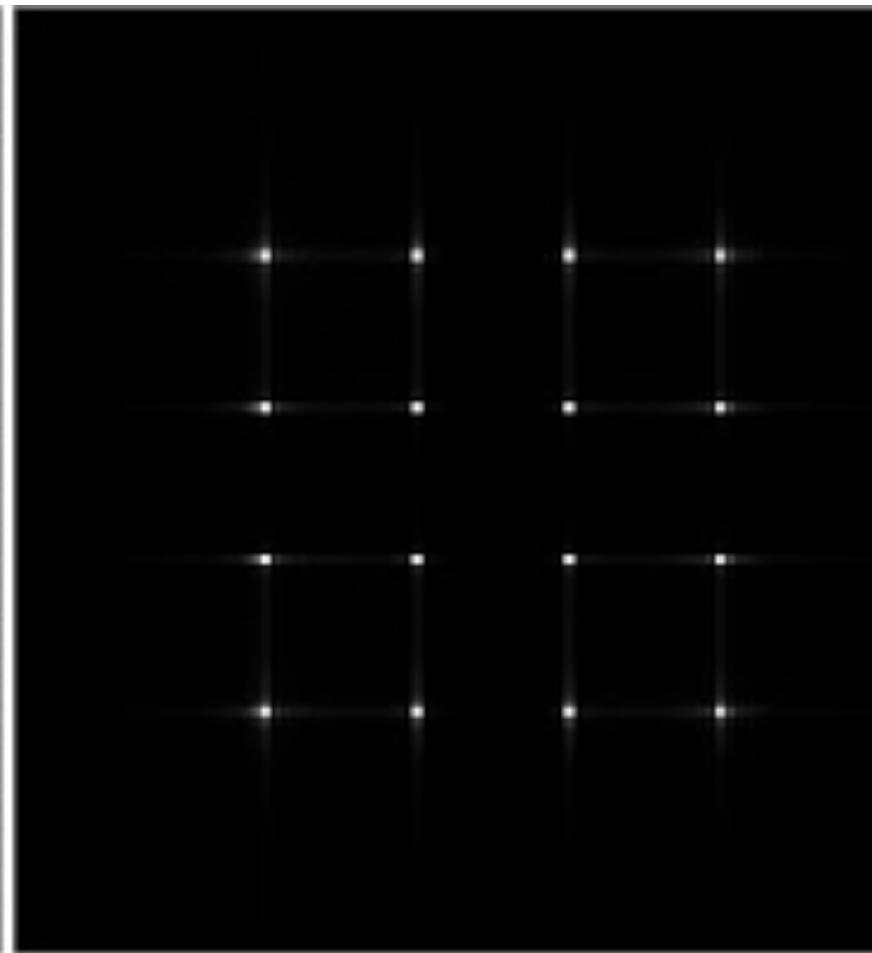
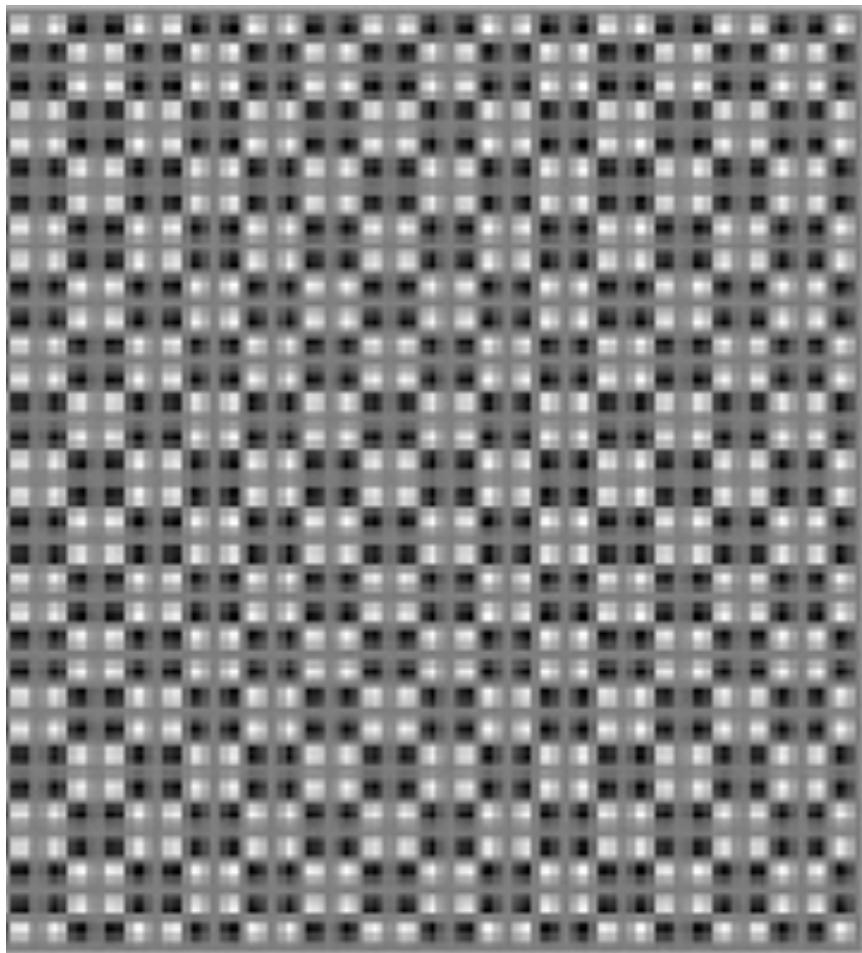
# Examples for Fourier transforms of simple functions:



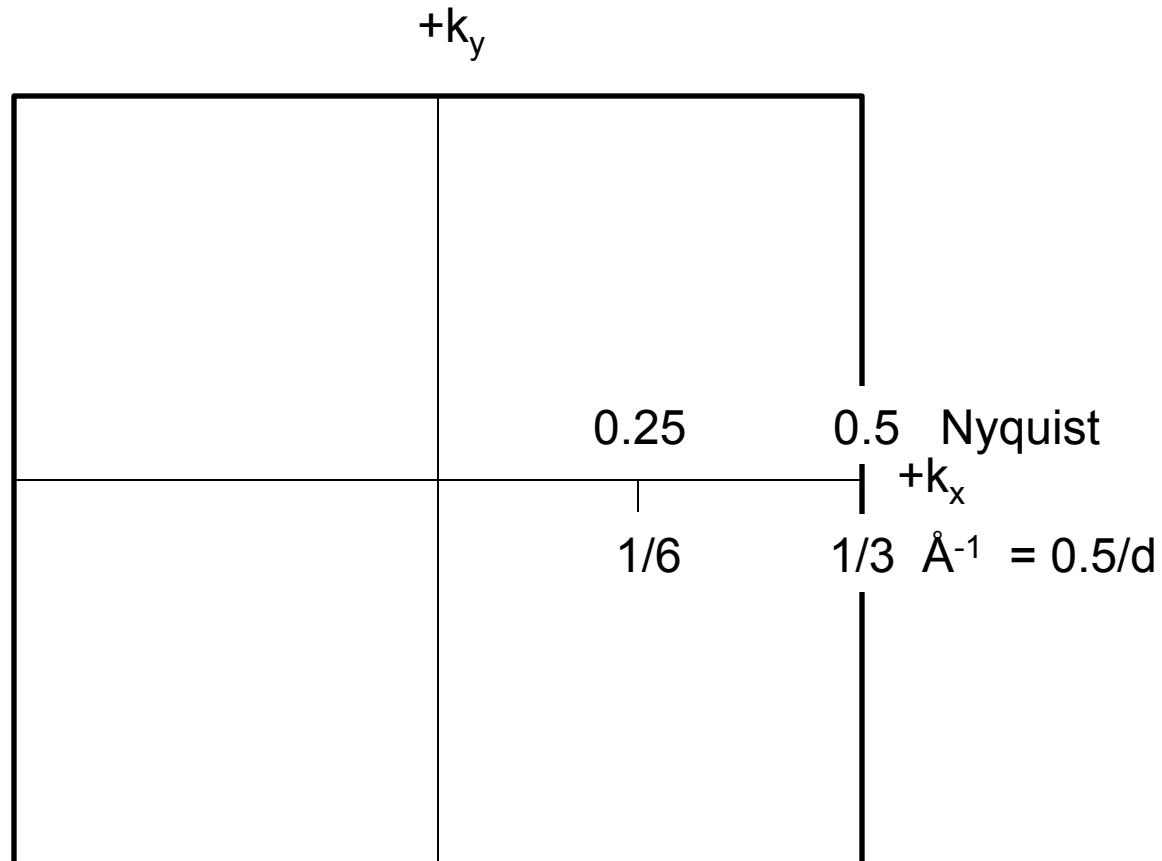




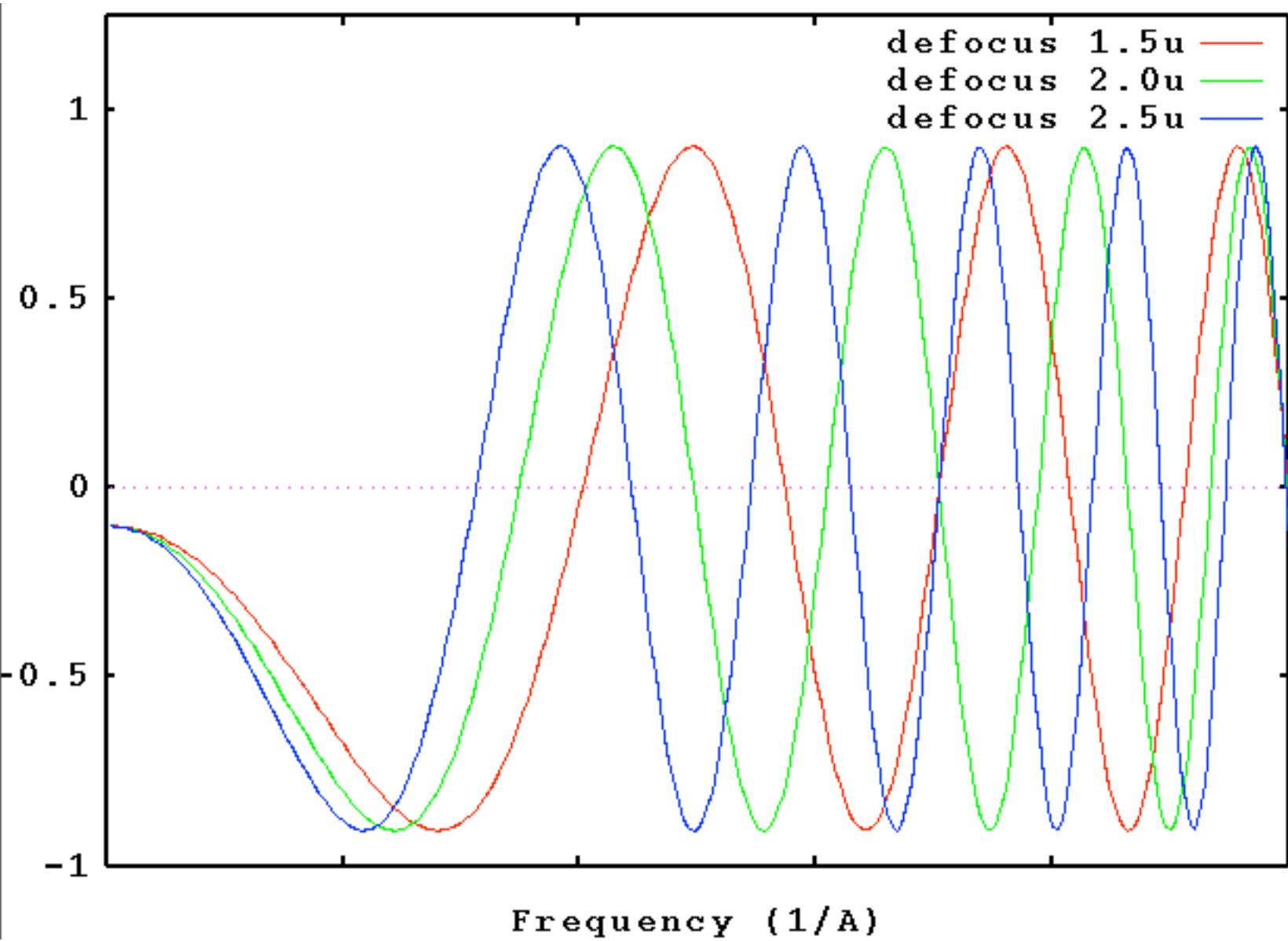


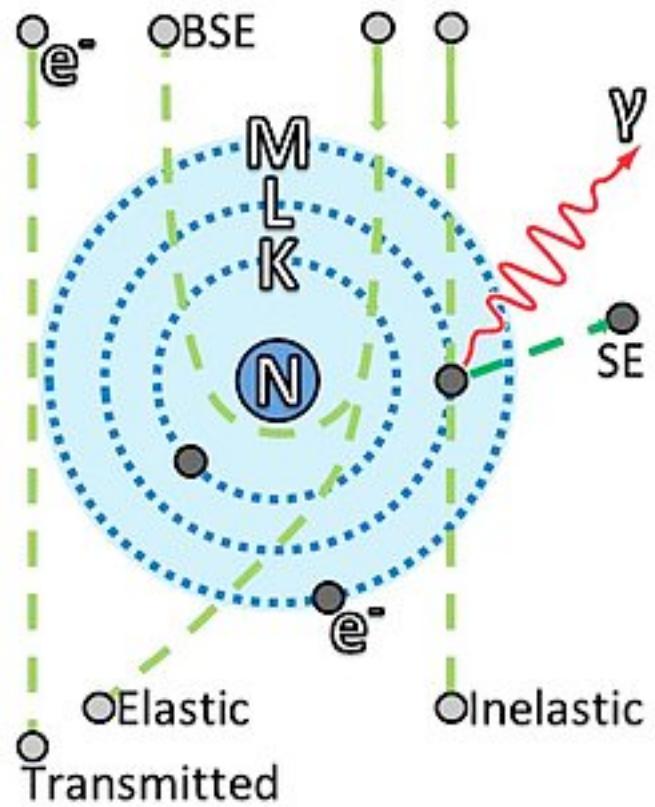


# Units of spatial frequency in 2D Fourier space



Spatial frequency is either in Nyquist units (0... 0.5) or in physical units  $0.5/d$  relating to the sampling step  $d$ . In above example, sampling step is  $d = 1.5 \text{ \AA}$





- Beam Electron
- Atomic Shell Electron
- Electron Cloud
- Beam Electron Path
- Secondary Electron Path
- Characteristic X-Ray

# Generalized Euclidean distance

Euclidean distance between two images  $f_1$  and  $f_2$ :

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$$\begin{aligned} &= \sum_{j=1}^J |f_1(r_j)|^2 + \sum_{j=1}^J |f_2(R_\alpha r_j + r')|^2 - \sum_{j=1}^J |f_1(r_j)f_2(R_\alpha r_j + r')| \\ &\quad \text{const.} \qquad \qquad \text{const.} \qquad \qquad \text{cross-correlation} \end{aligned}$$

Similarity = closeness in high-dimensional Euclidean space = small E-distance  
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