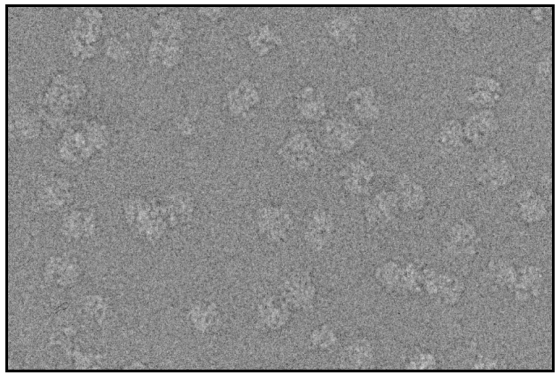
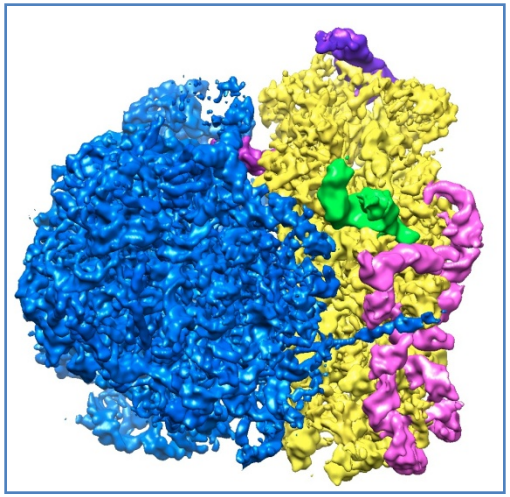
Single-Particle Reconstruction





How to get from here over here to there

Joachim Frank

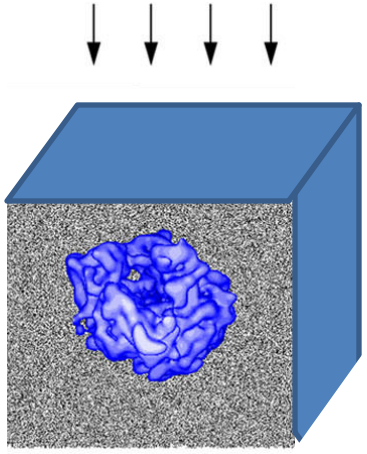
Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, and Department of Biological Sciences, Columbia University

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

Imaging in the Transmission Electron Microscope

Electrons

generated in cathode



3D object

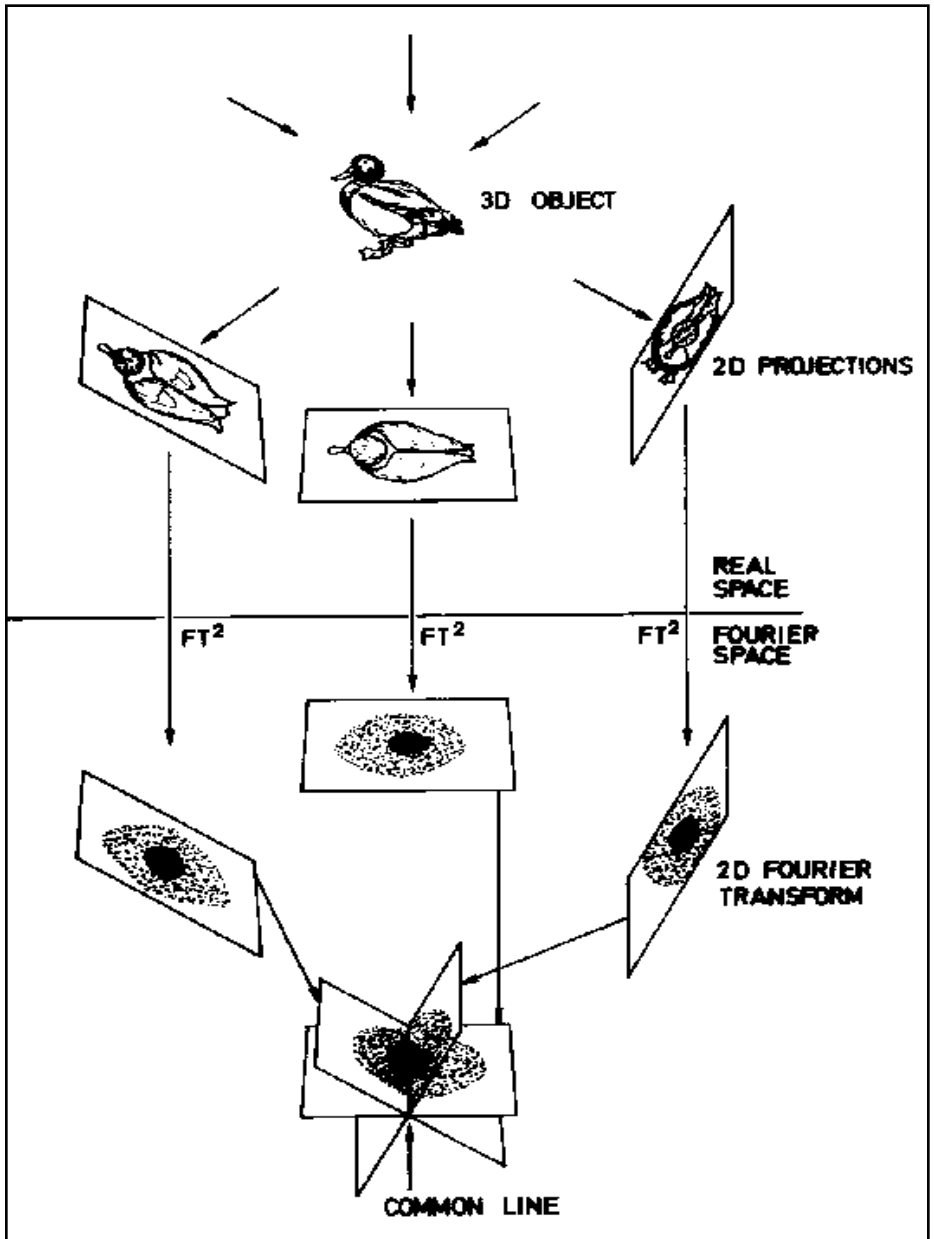
molecule embedded in vitreous ice

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.

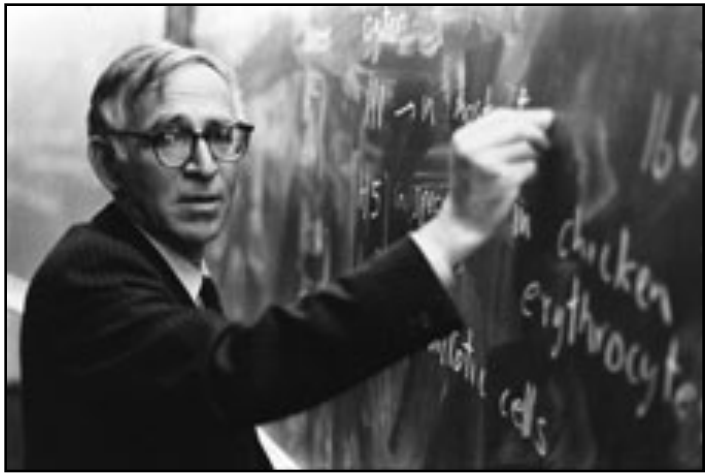
2) The Projection Theorem:

"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."

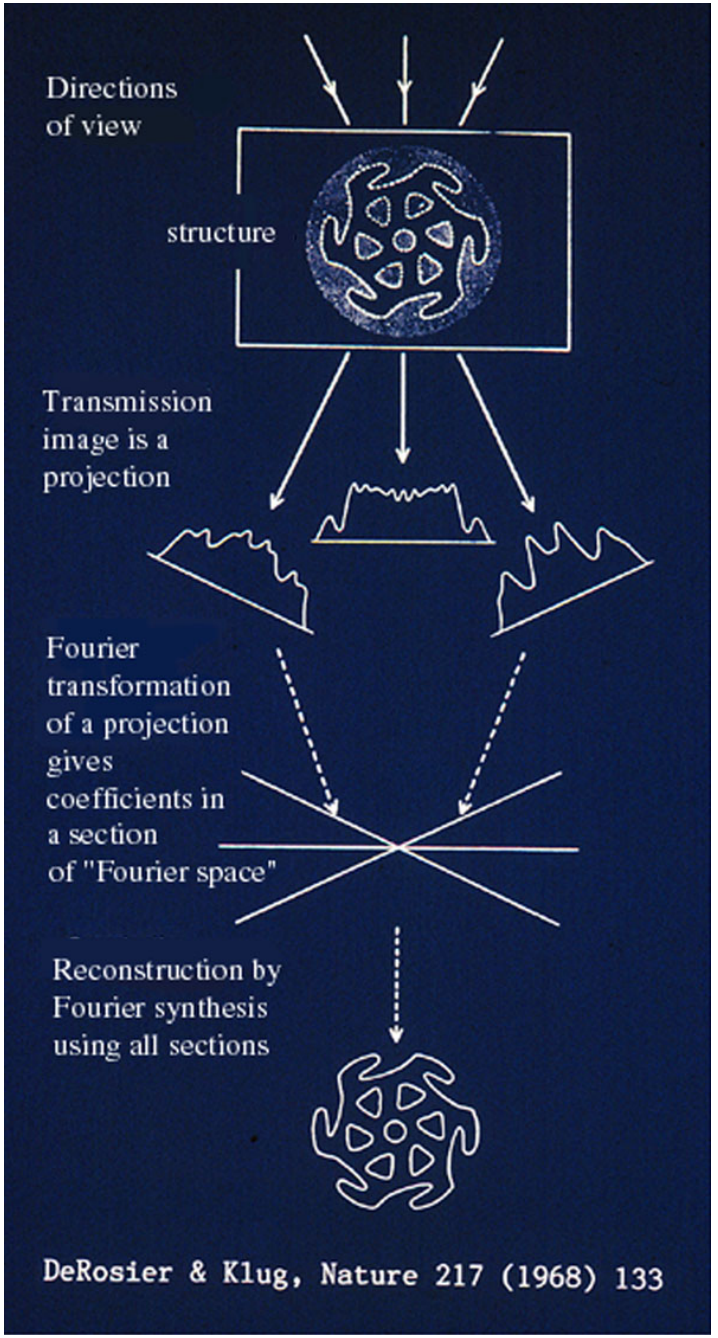
3) It is necessary to collect a sufficient number of projections over a large angular range. From these projections, the object's density distribution can be reconstructed.

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

1968

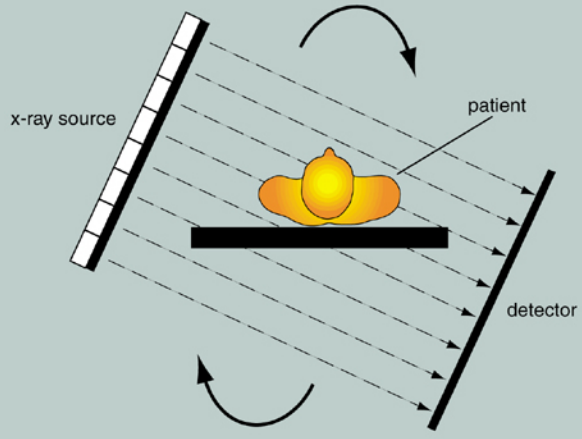


Aaron Klug and David DeRosier,
Laboratory for Molecular Biology, MRC,
Cambridge



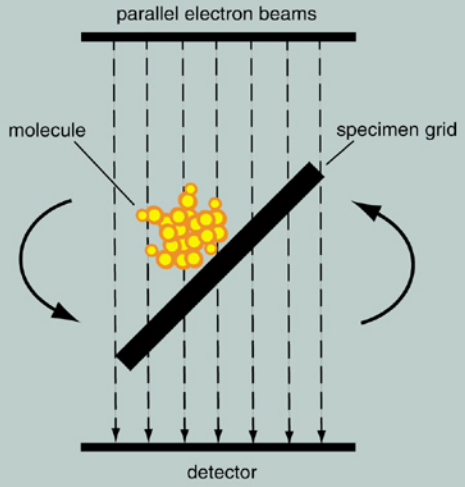
How . . . do we collect the projections?

Three data collection strategies for 3D reconstruction:



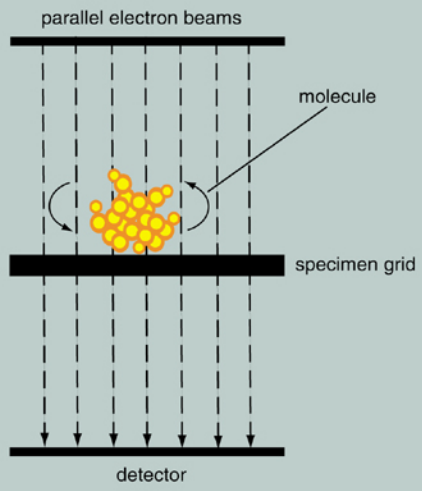
CAT - scan

- beam rotating
- patient stationary



Electron Tomography

- molecule rotating
- beam stationary

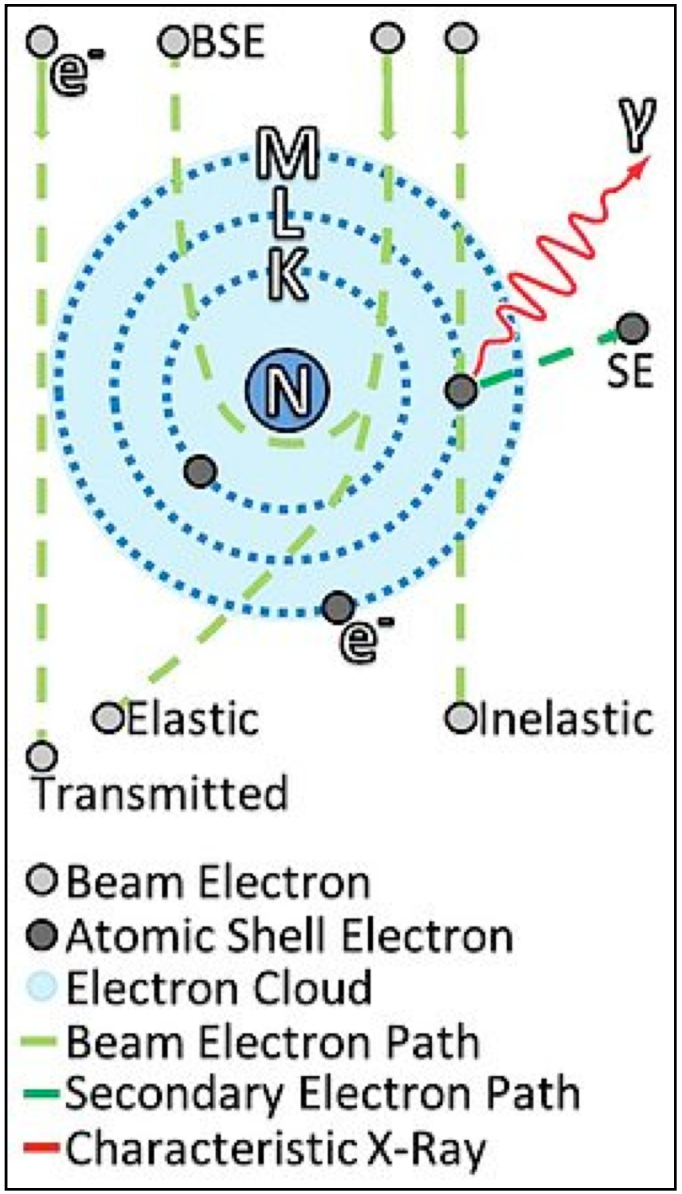


Single particle reconstruction

- molecule "rotating"
- beam stationary

Interactions of electrons with biological matter at 100 – 300 kV

- Elastic (high-res signal) vs. inelastic scattering (low-res, delocalized signal)
- Transmission electron microscopy: maximum thickness is ~0.25 μ = 2500 Å
- Larger thickness leads to multiple scattering and, eventually, total absorption
-



RADIATION DAMAGE

- Biological molecules are being destroyed by the electron beam. Electrons are an ionizing
radiation, splitting bonds, which results in the creation of free radicals.

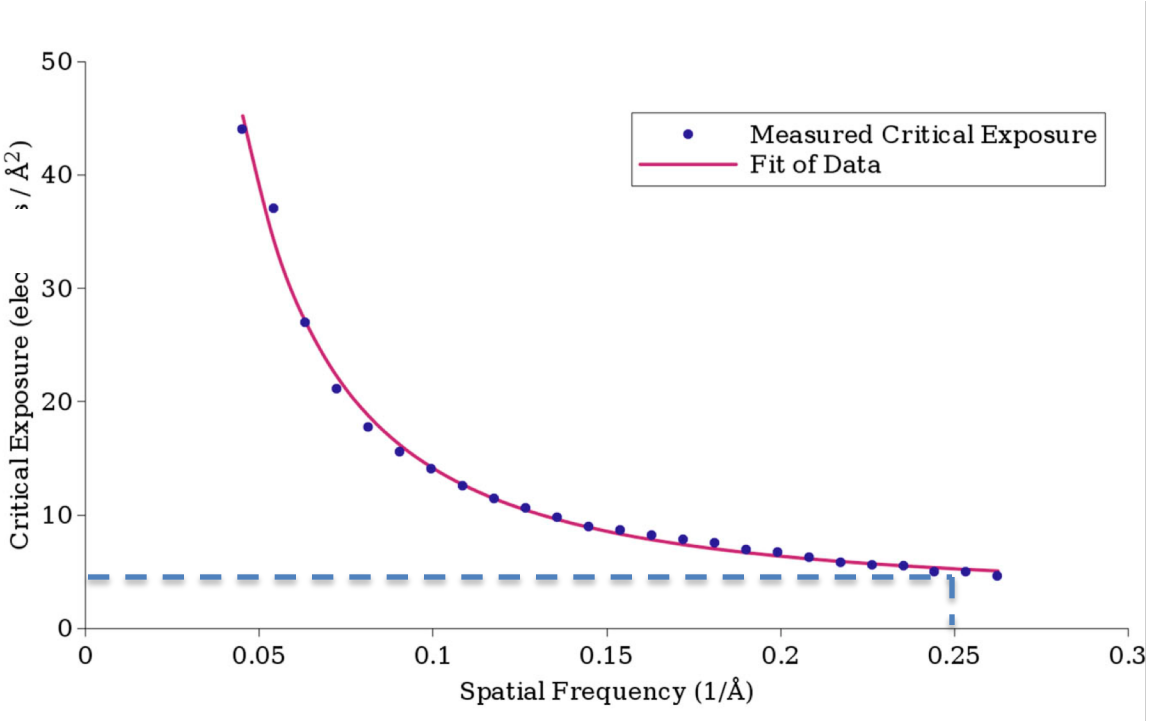
- These free radicals cause further damage as they migrate from the original site to other
sites of the molecule.

- Cooling to liquid nitrogen traps the free radicals, and thereby reduces radiation damage.

- Radiation damage affects small, high-resolution features more strongly than features at low resolution.

-

RADIATION DAMAGE



Critical exposure N_e as a function of spatial frequency
Grant & Grigorieff, eLife 2015

Example: to get 4Å resolution, the exposure should be below 5 electrons/Å²

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:

- no crystal needed
- 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):

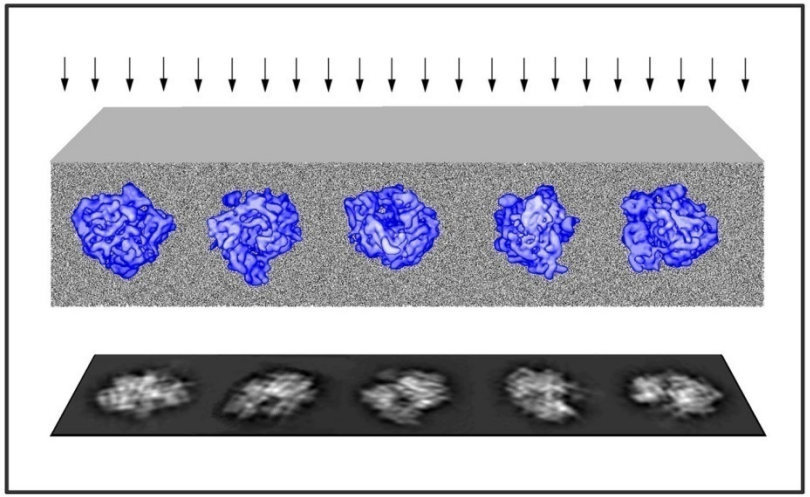
- large computational challenges
- atomic resolution difficult to achieve for particles lacking symmetries

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.



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 (~1000 Å) 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

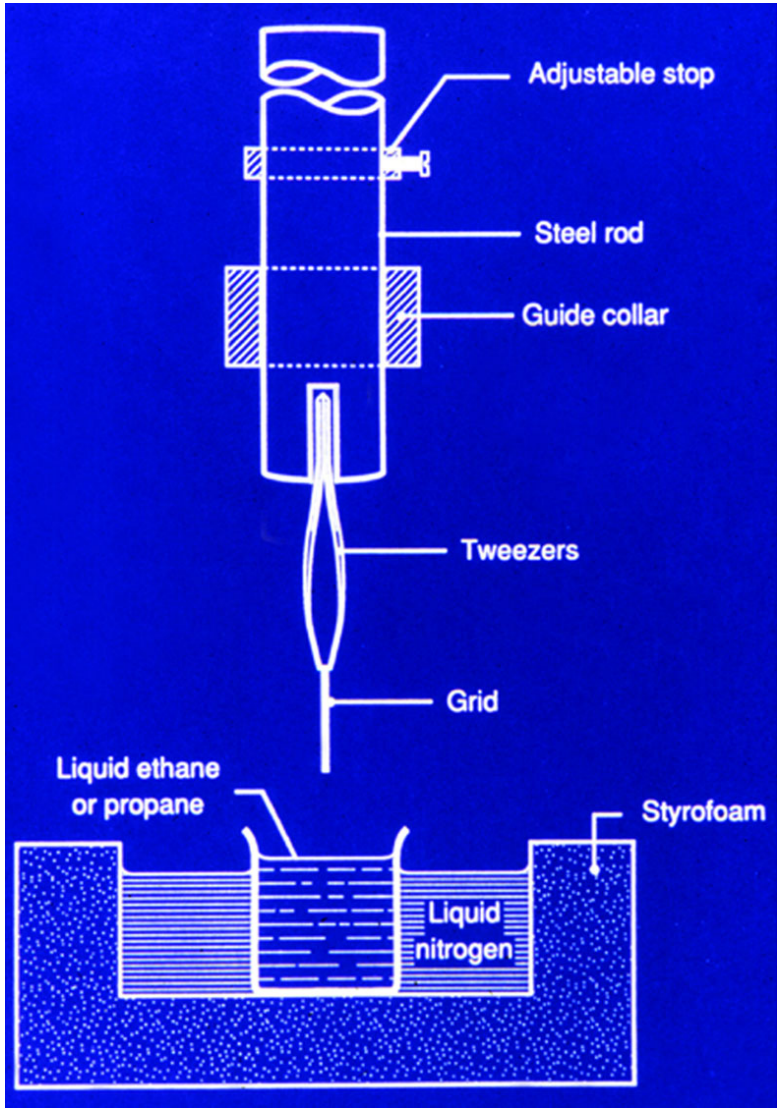
  (2) geometry and makeup of metal grid -- copper, molybdenum, gold

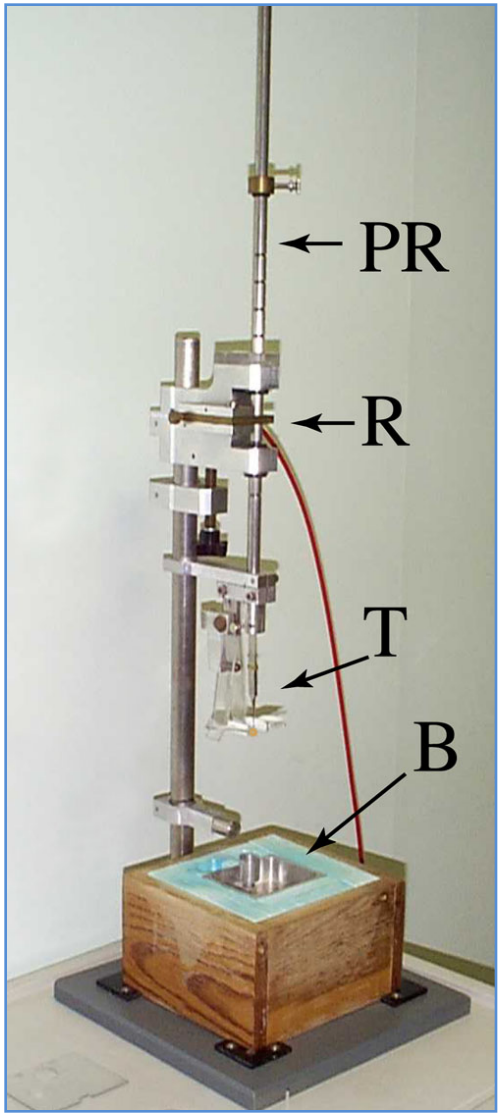
copper (traditional), **molybdenum** (match heat expansion of carbon), **gold** (avoid charge-induced vibrations)

Plunge-freezer to prepare samples for cryo-EM

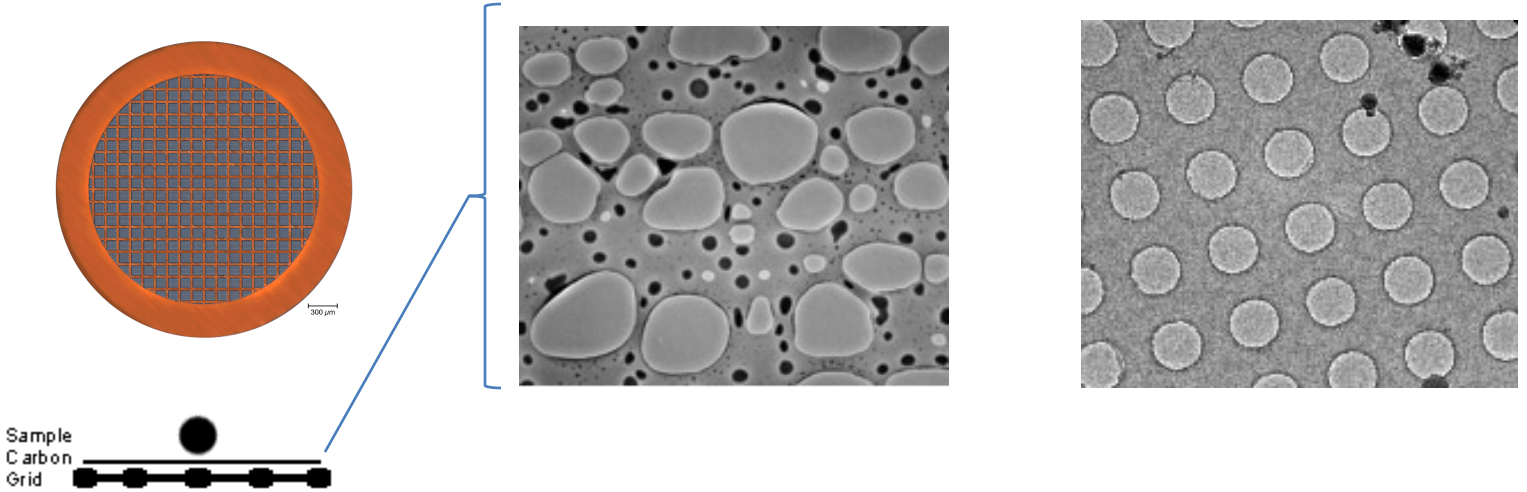
Manual

automated, climatized





Specimen support



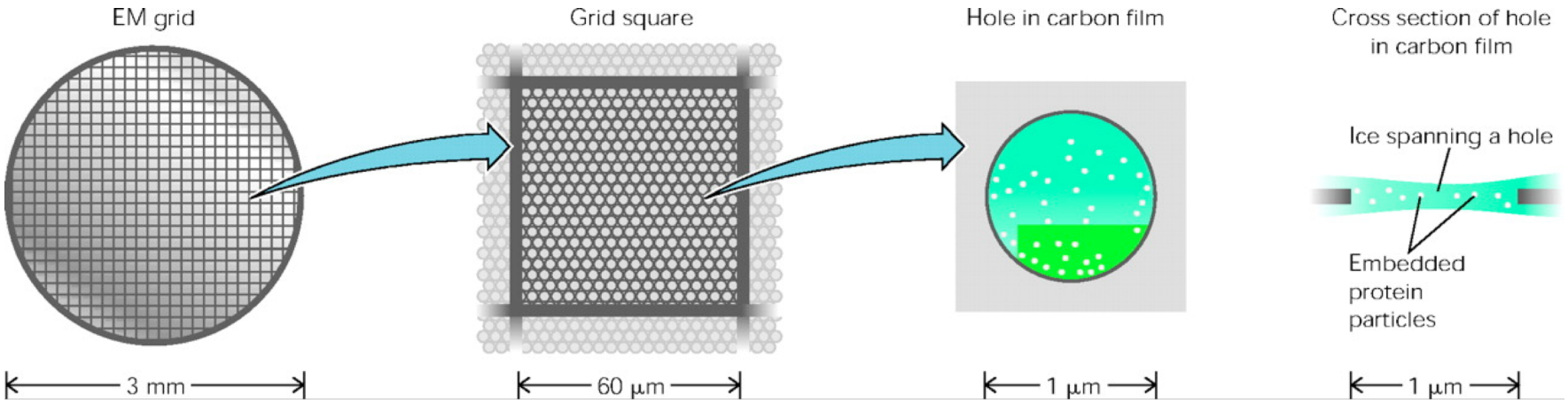
Home-made

Quantifoil or C-flat

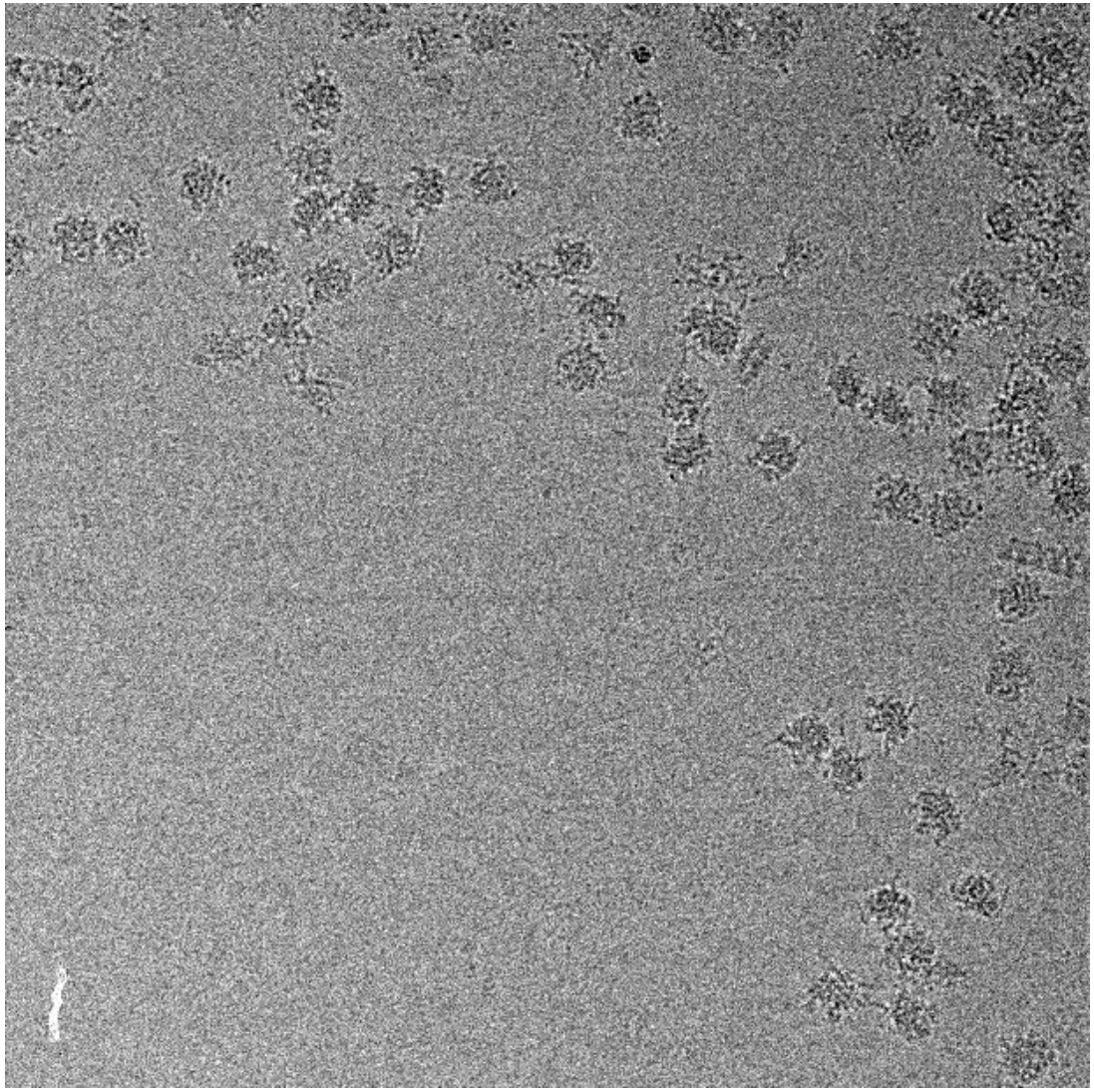
EM grid, Copper, 3 mm

Carbon layer on EM grid

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

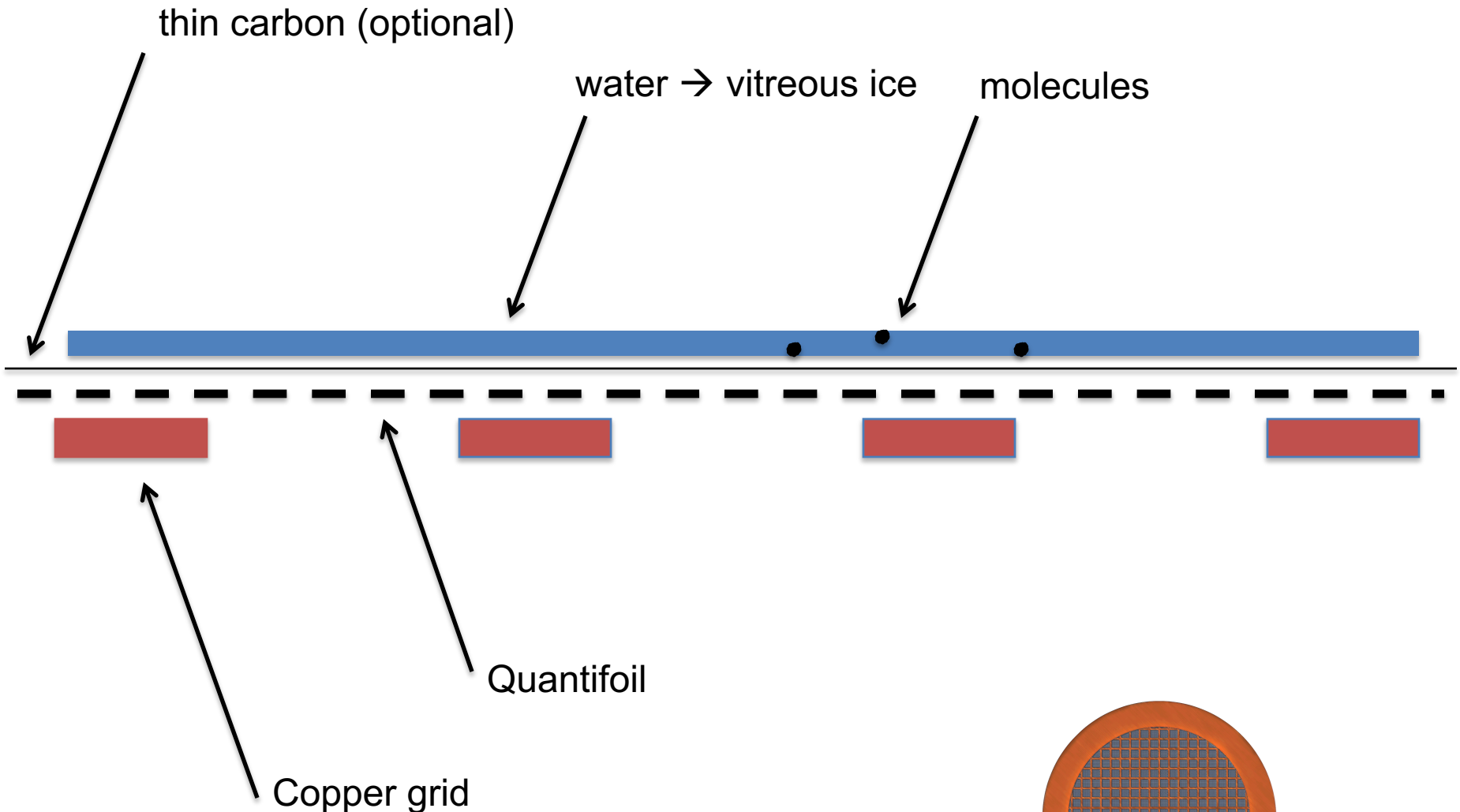

EM grid
Grid square
Hole in carbon film
Cross section of hole in carbon film
Ice spanning a hole
Embedded protein particles
3 mm
60 μm
1 μm
1 μm

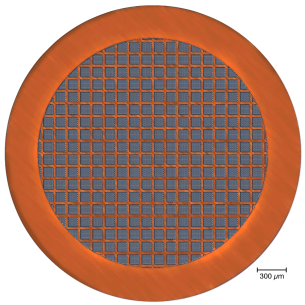
EXAMPLE OF MENISCUS EFFECT: MOLECULES ACCUMULATE NEAR EDGE OF HOLE



Thick ice

Thin ice

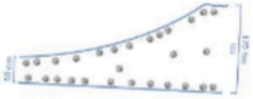
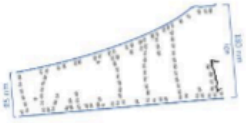
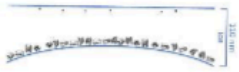




OPTIONAL: Thin carbon (~100 Å), produced by evaporation on mica, floated onto Quantifoil-coated grid.

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

Cross-section of ice layer A. Nobel, NYSBC, biorxiv 2017

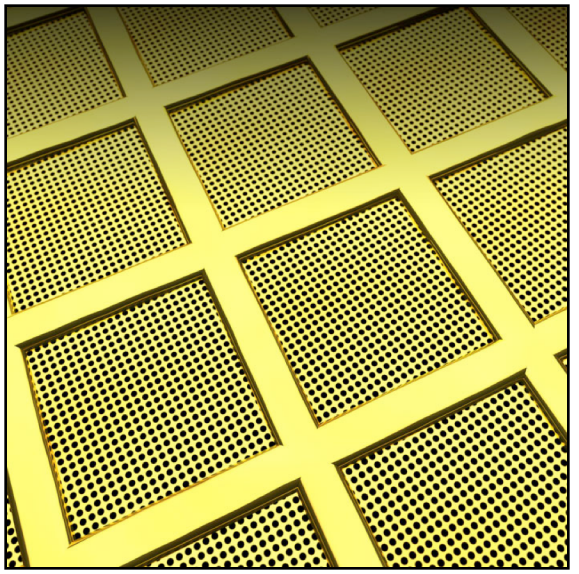
ectional gram	Sample # Name	Example cross-sectional schematic diagram	Sample # Name	Example cross-sectional schematic diagram	Sample # Name	Example cross-sectional schematic diagram
	14* Neural Receptor		**27*** IDE		**38*†** Apoferritin (0.5 mg/mL)	
	17* Protein with Bound Lipids (deglycosylated)		**30*†** GDH		**39*†** Apoferritin with 0.5 mM TCEP	
	18 Protein with Bound Lipids (glycosylated)		**31*†** GDH		**40** Protein with Carbon Over Holes	
	19* Lipo-protein		**32*†** GDH + 0.001% DDM (2.5 mg/mL)		**41** Protein and DNA Strands with Carbon Over Holes	
	20 GPCR		**33*†** DNAB Helicase-helicase Loader		**42*†** T20S Proteasome	
	21*† Rabbit Muscle Aldolase (1mg/mL)		**34*†** Apoferritin		**43*†** T20S Proteasome	
	22*† Rabbit Muscle Aldolase		**35*†** Apoferritin		**44*†** T20S Proteasome	

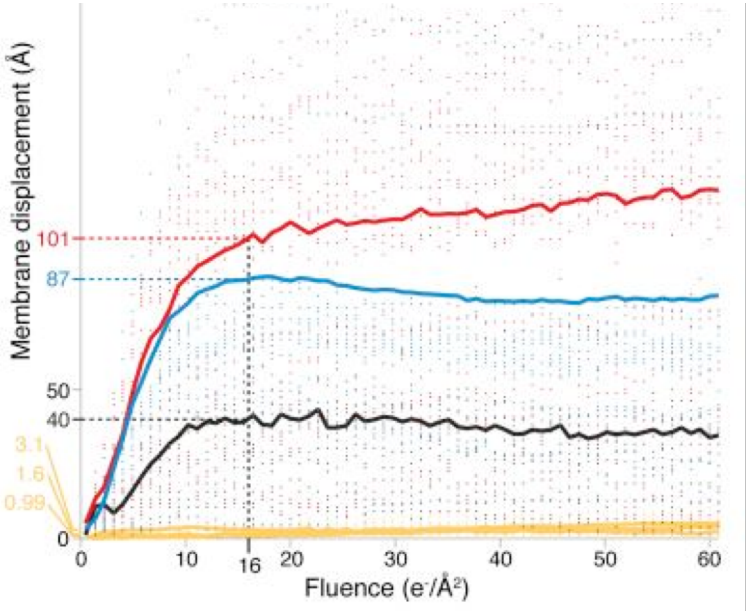
GOLD GRIDS

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.

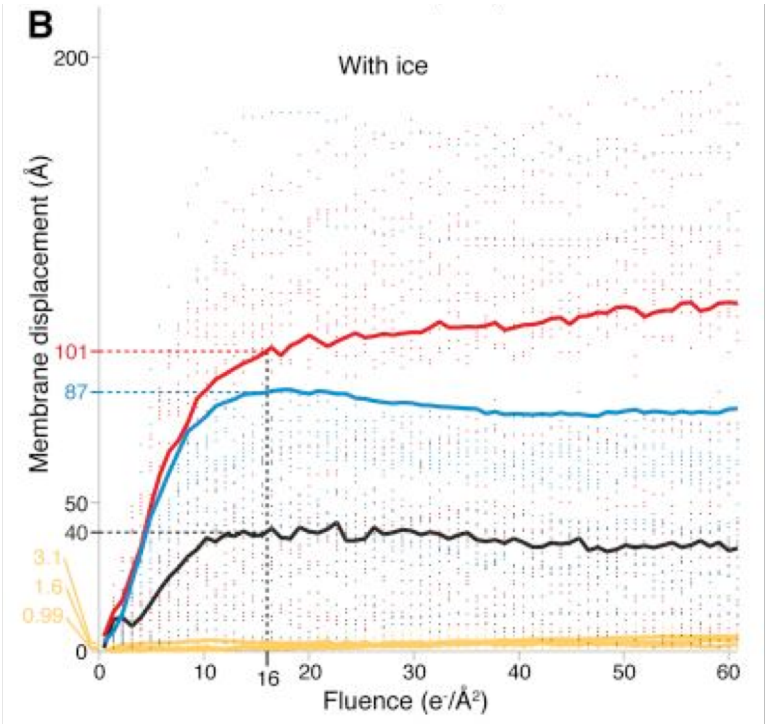
Gold grids reduce this effect 50-fold.





Russo and Passmore, Science 2014

GOLD GRIDS VERSUS CARBON GRIDS: VIBRATION IN Z-DIRECTION

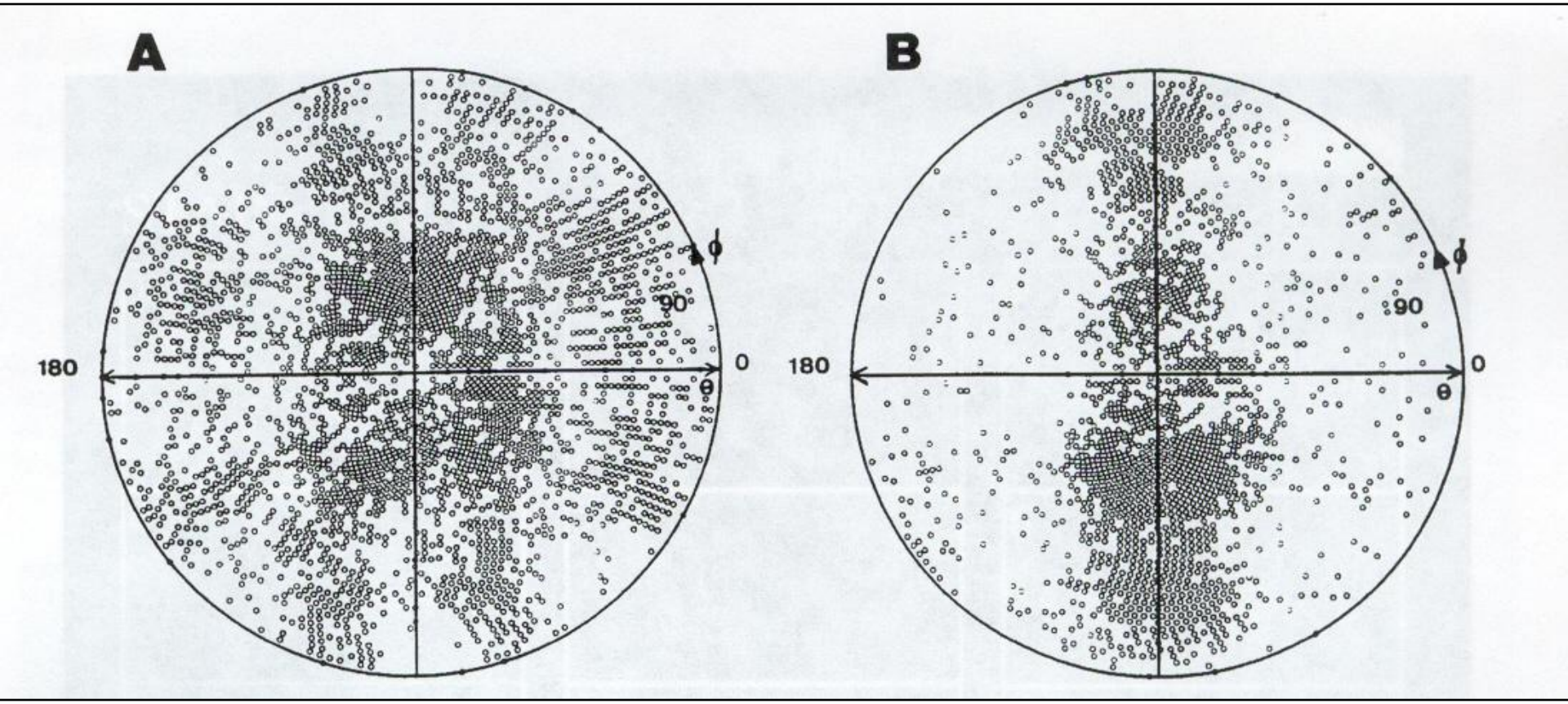


Russo and Passmore,
Science 2014

3D reconstruction requires even angular coverage

good

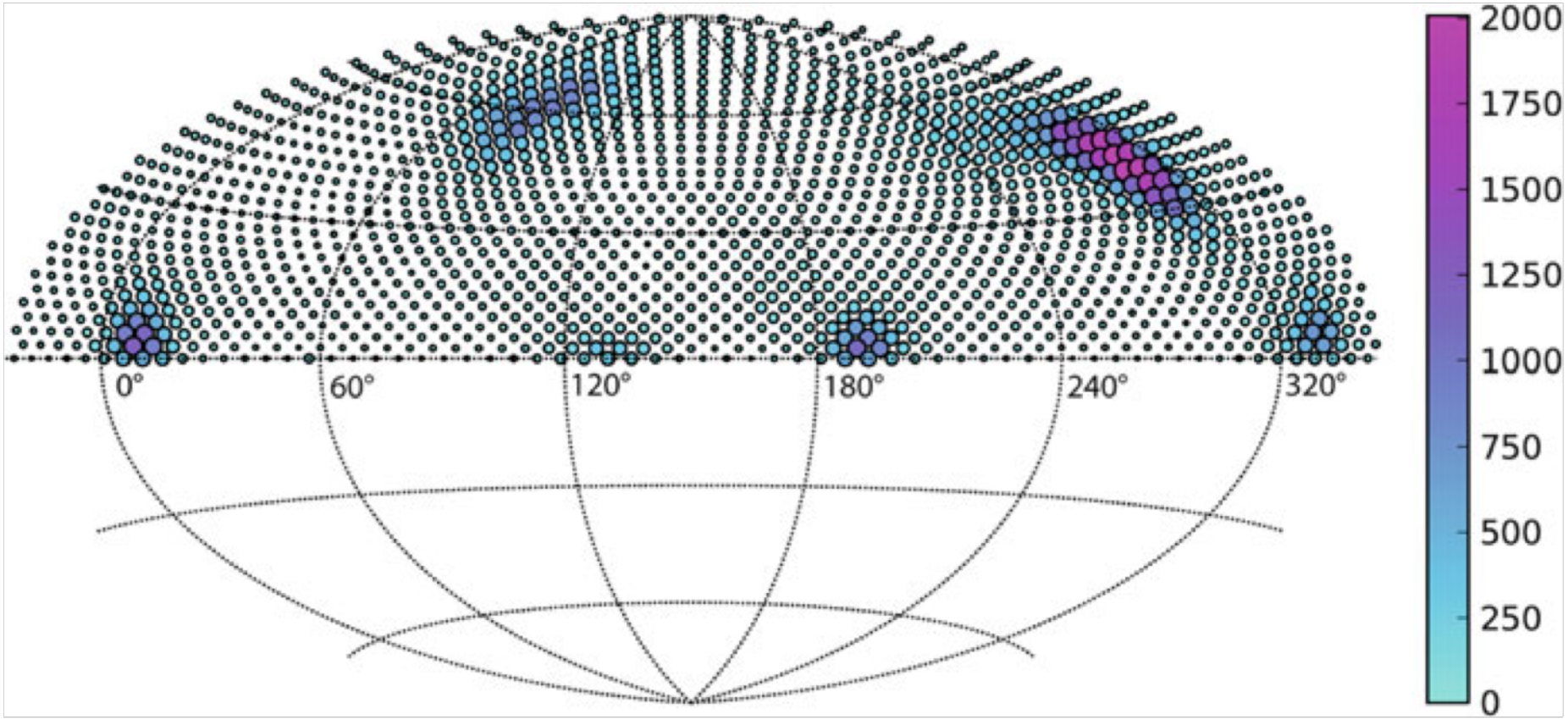
bad



“global coverage”

“single-axis-like coverage”

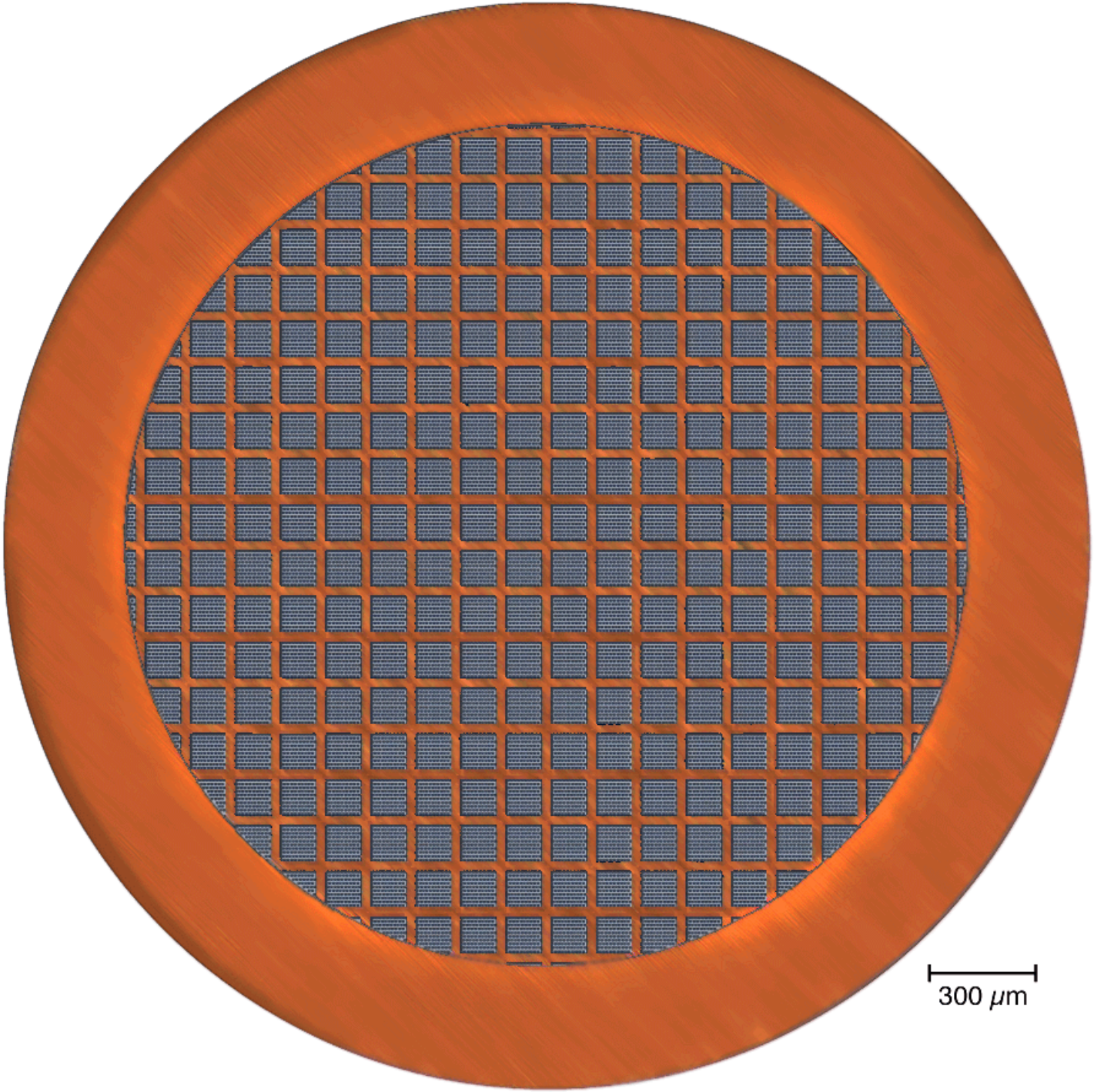
Angular coverage

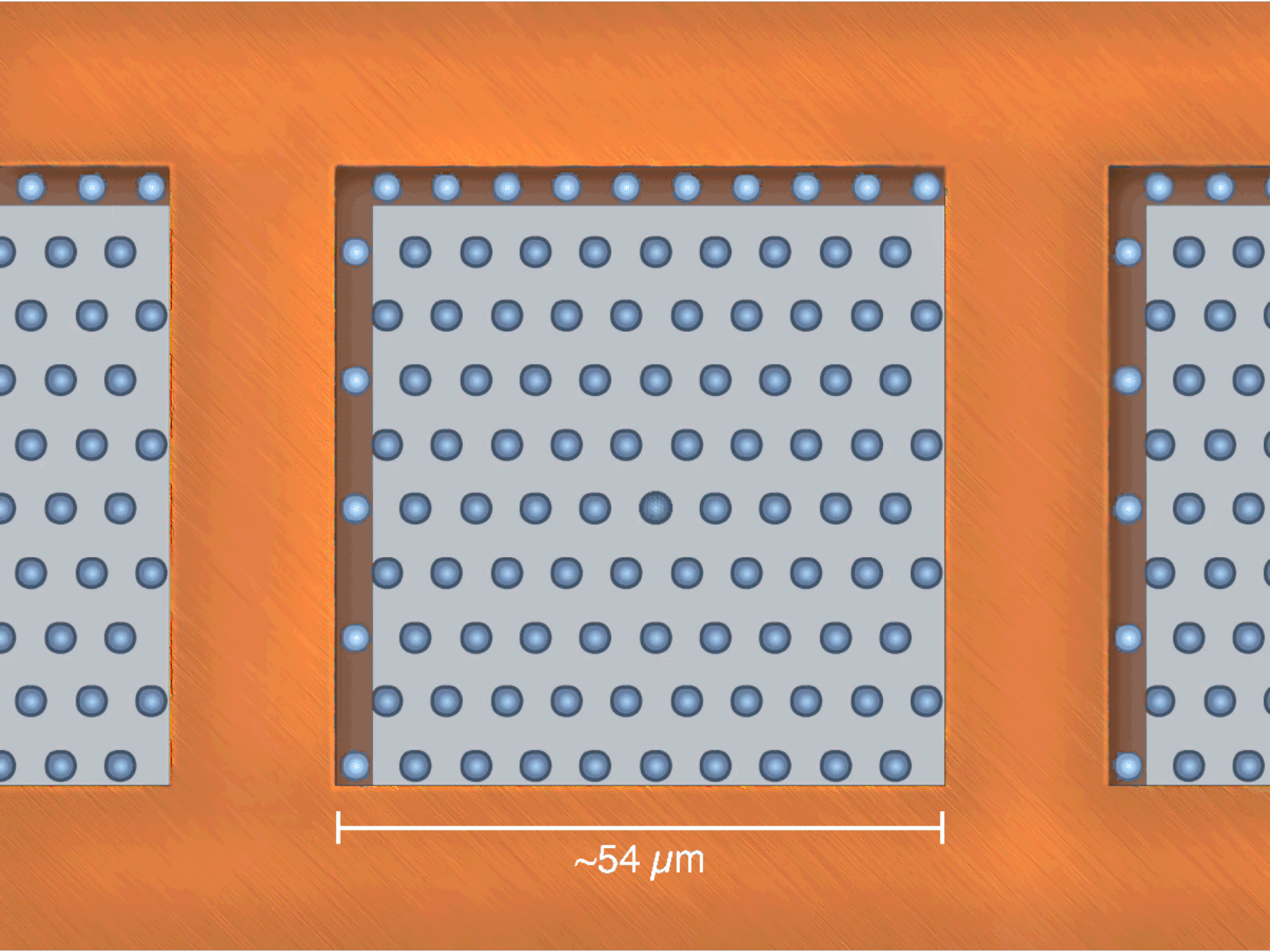


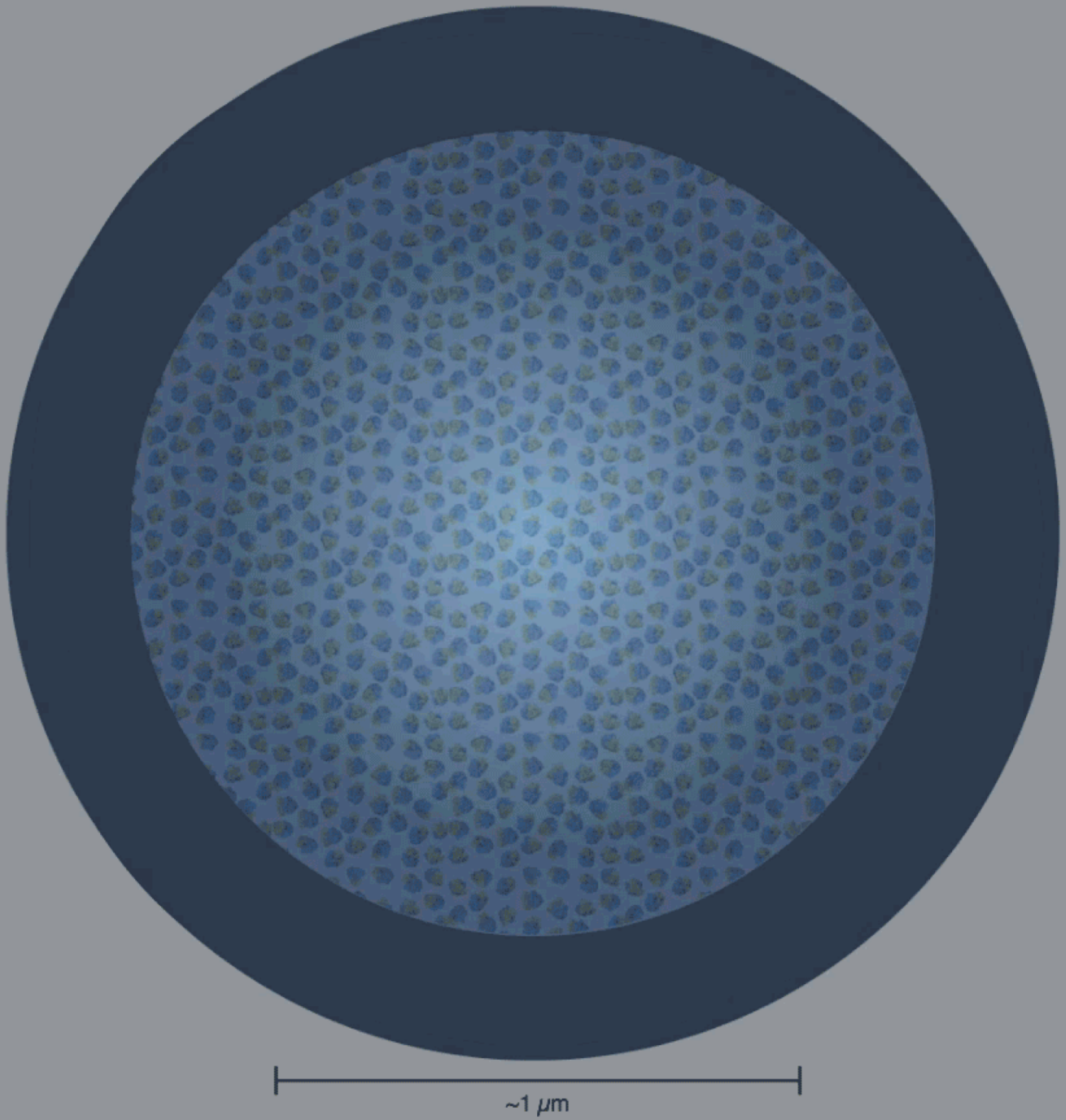
Des Georges et al., book chapter, 2013

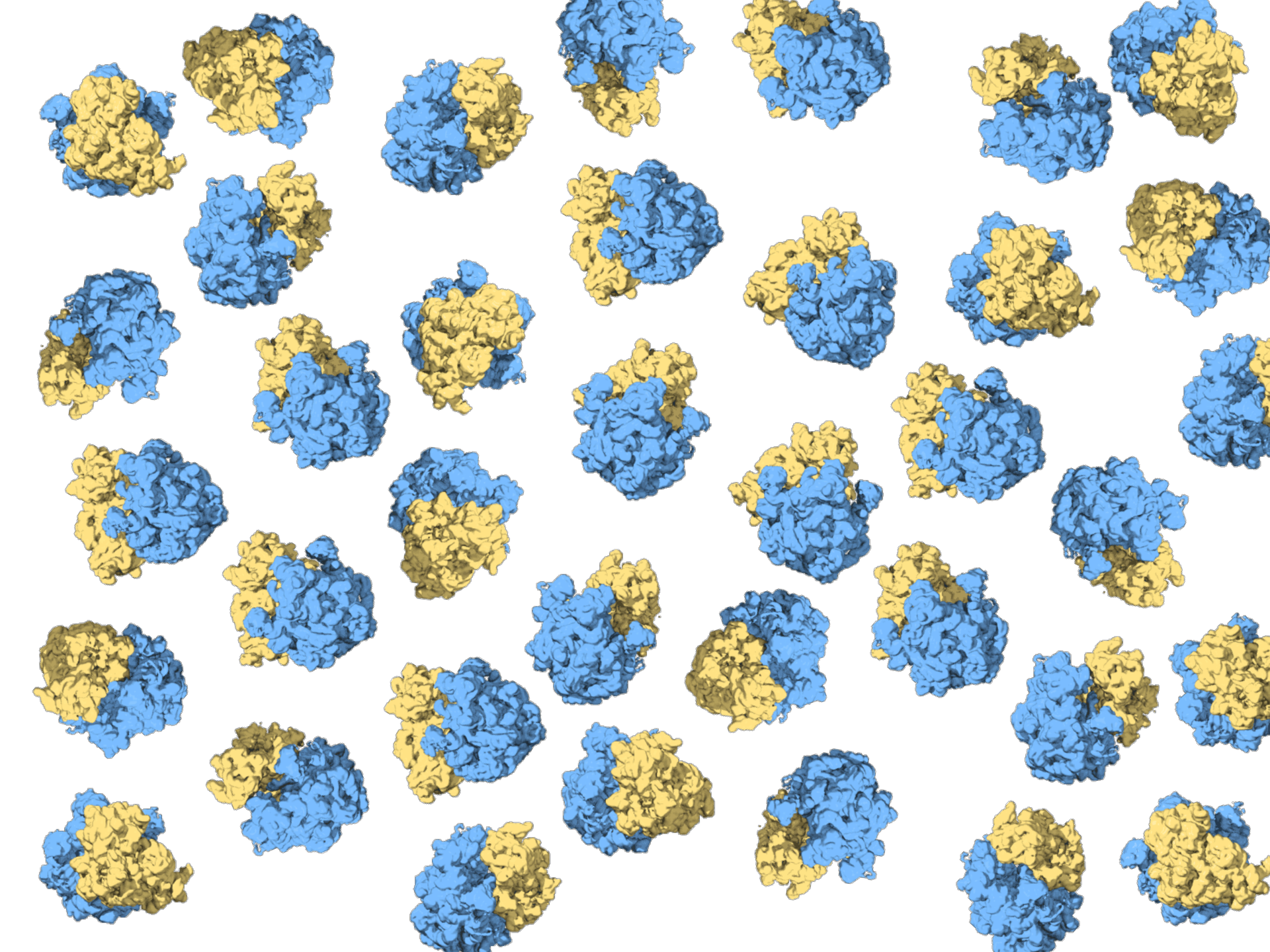
Illustration of sample on grid:

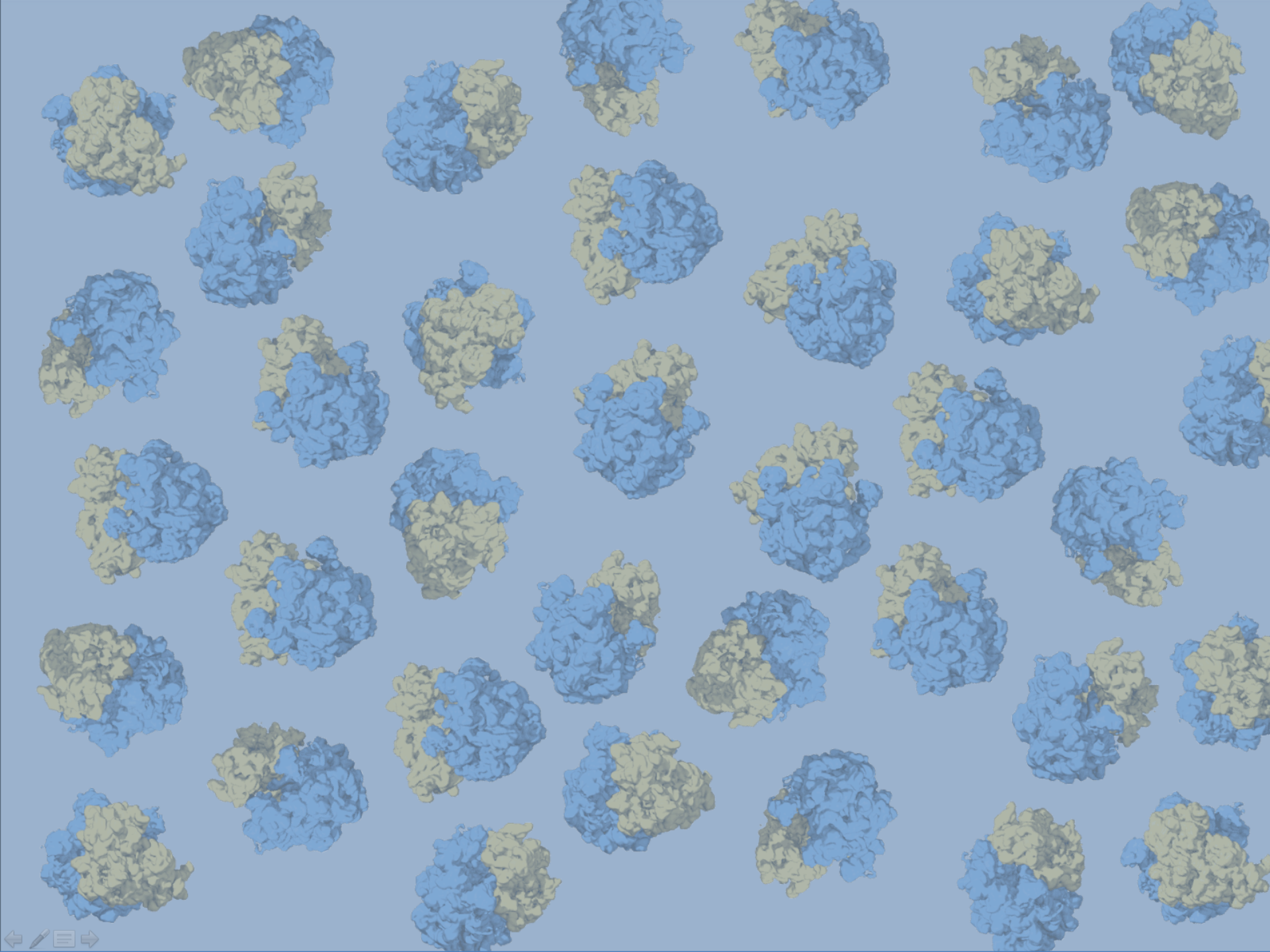
After blotting, the grid is covered with thin layer of liquid containing molecules

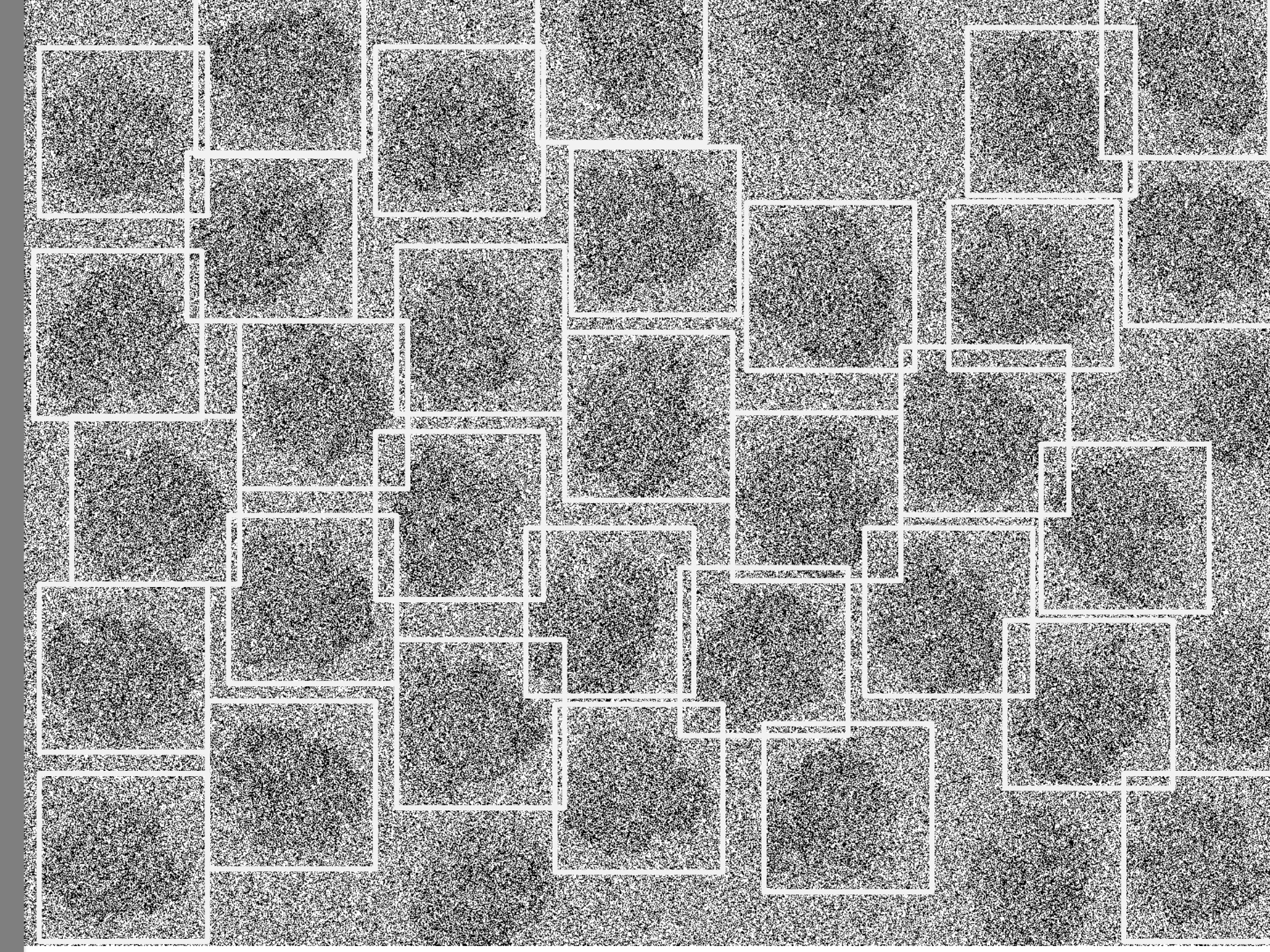

300 μm

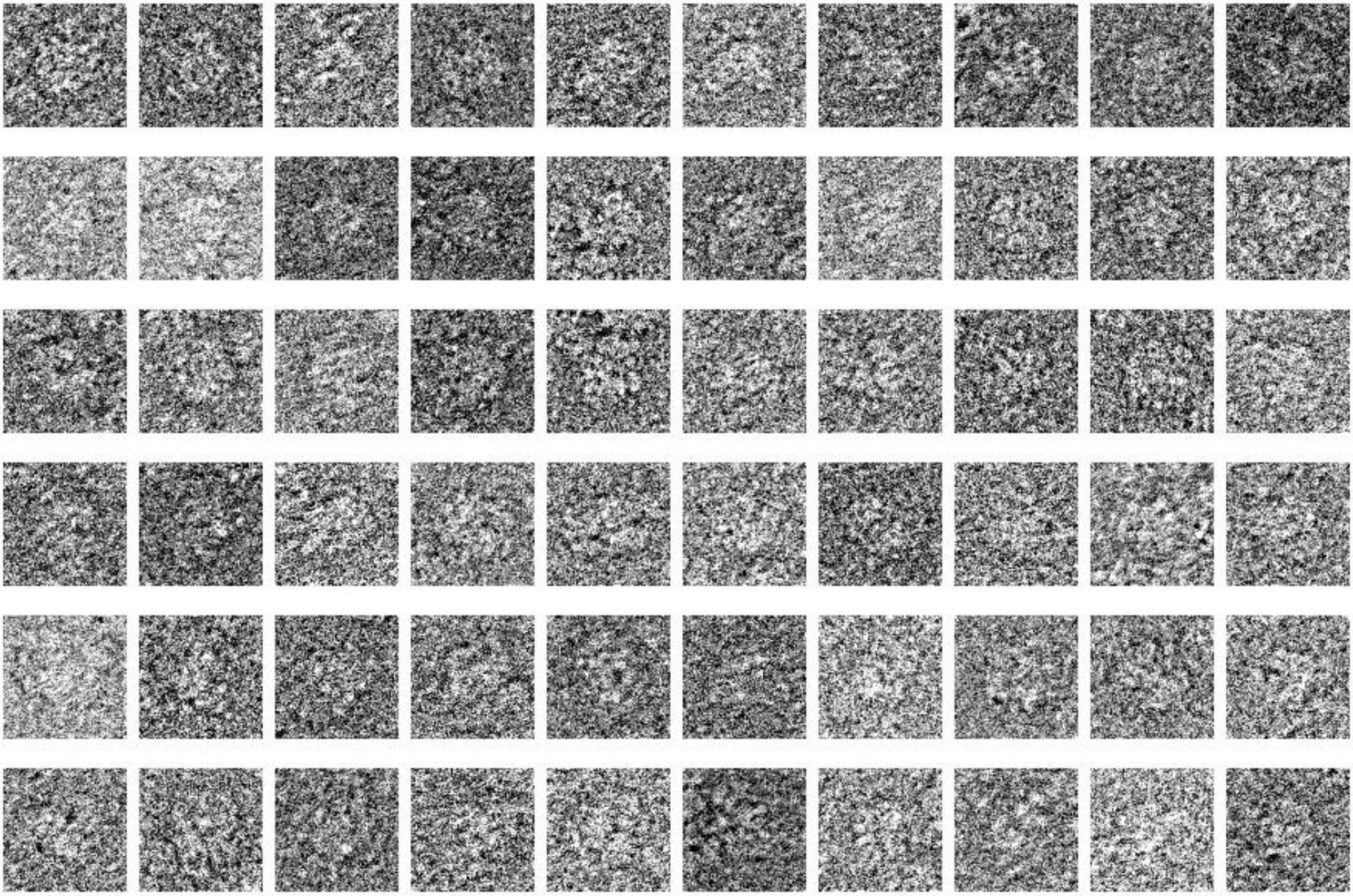

~54 μm

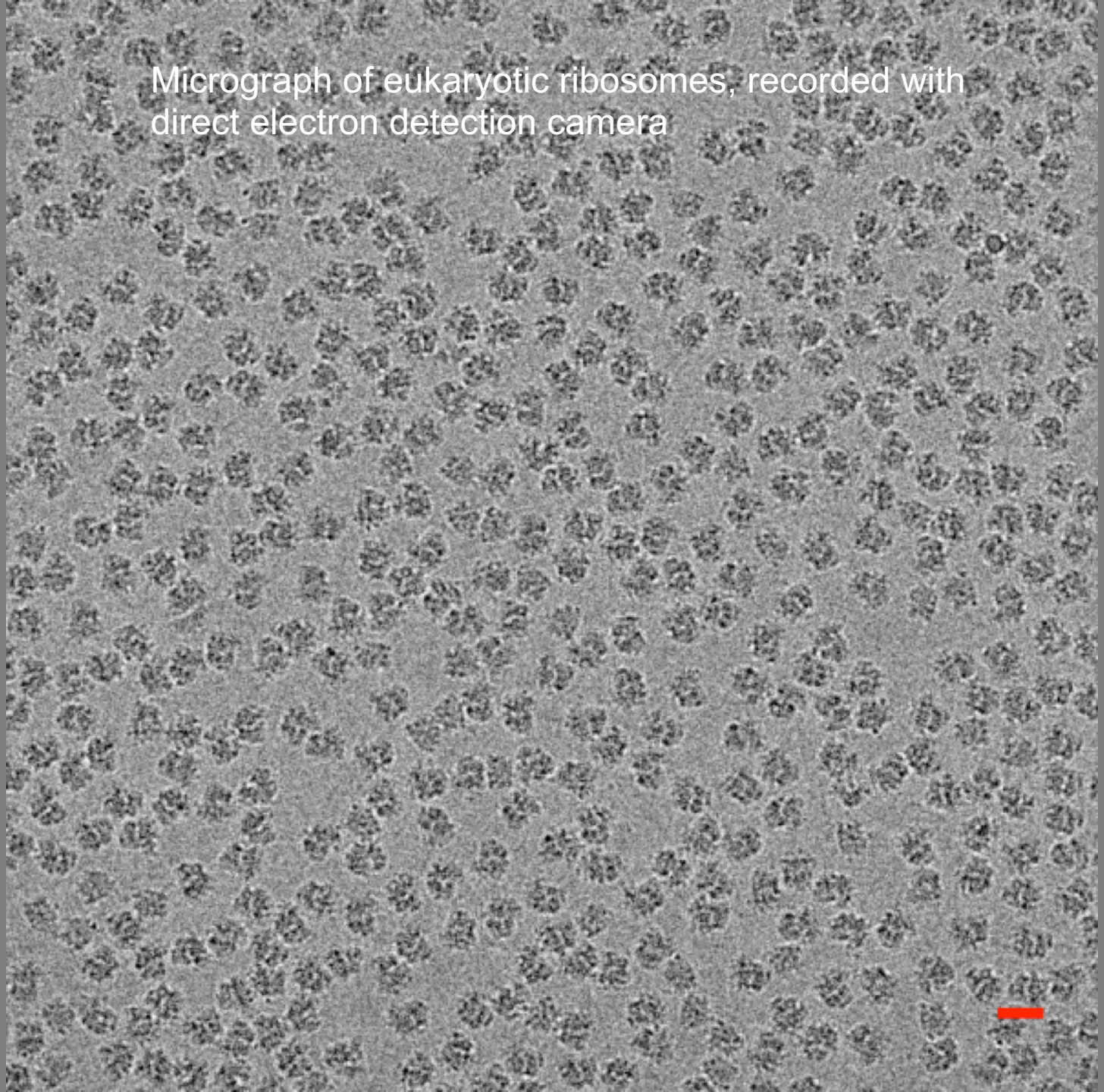

~1 μm










Micrograph of eukaryotic ribosomes, recorded with direct electron detection camera

Determine orientations, 3D Reconstruction

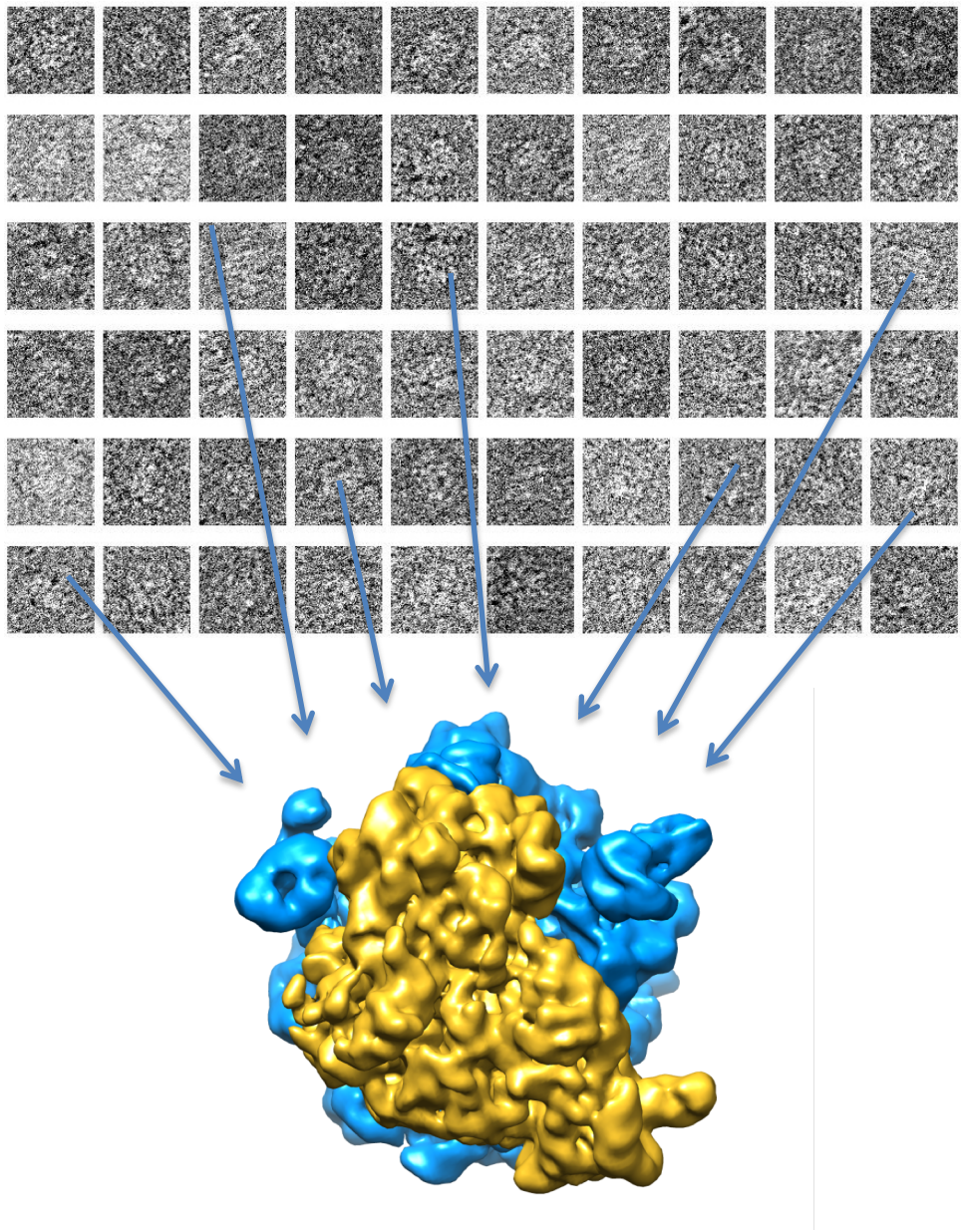
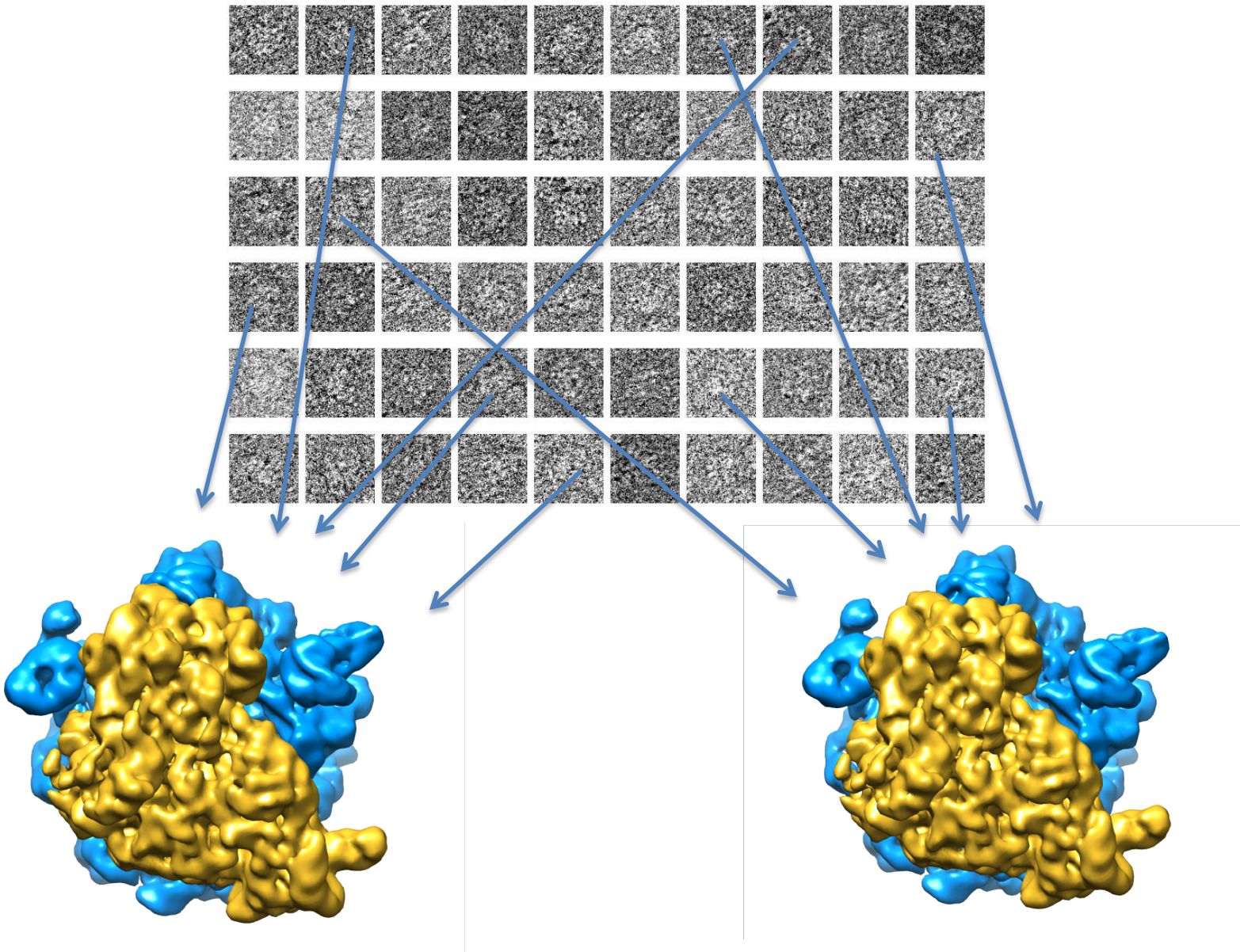


Image classification



Signal and noise -- definition of SNR

- Signal $s(\mathbf{r})$ (predictable, deterministic, originating from the object)

  versus

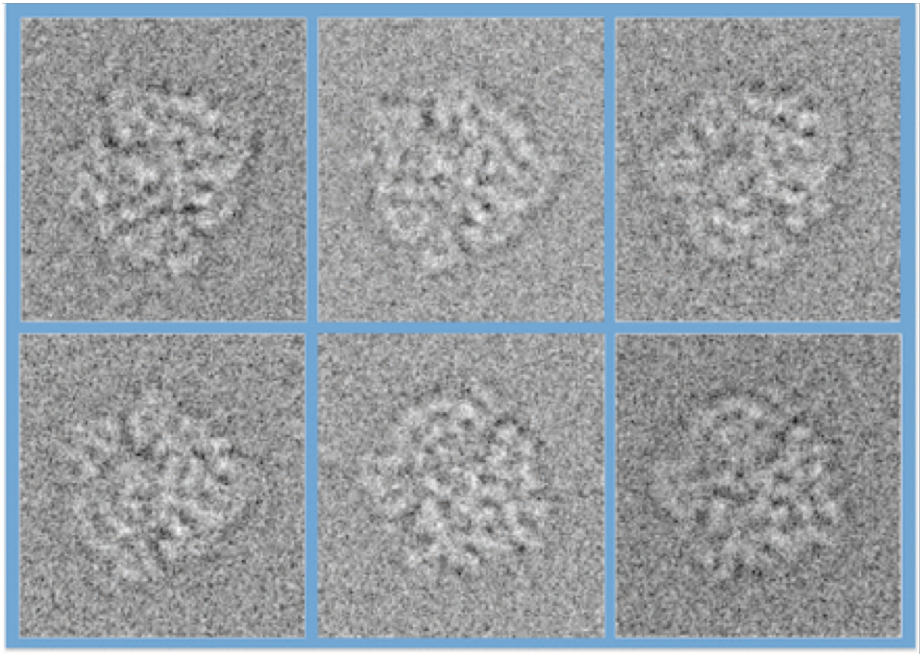
- Noise $n(\mathbf{r})$ (stochastic; unrelated to the signal; aperiodic [no two realisations are the same])
- Signal-to-noise ratio (SNR) = signal variance/noise variance

- 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 e/Å^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.

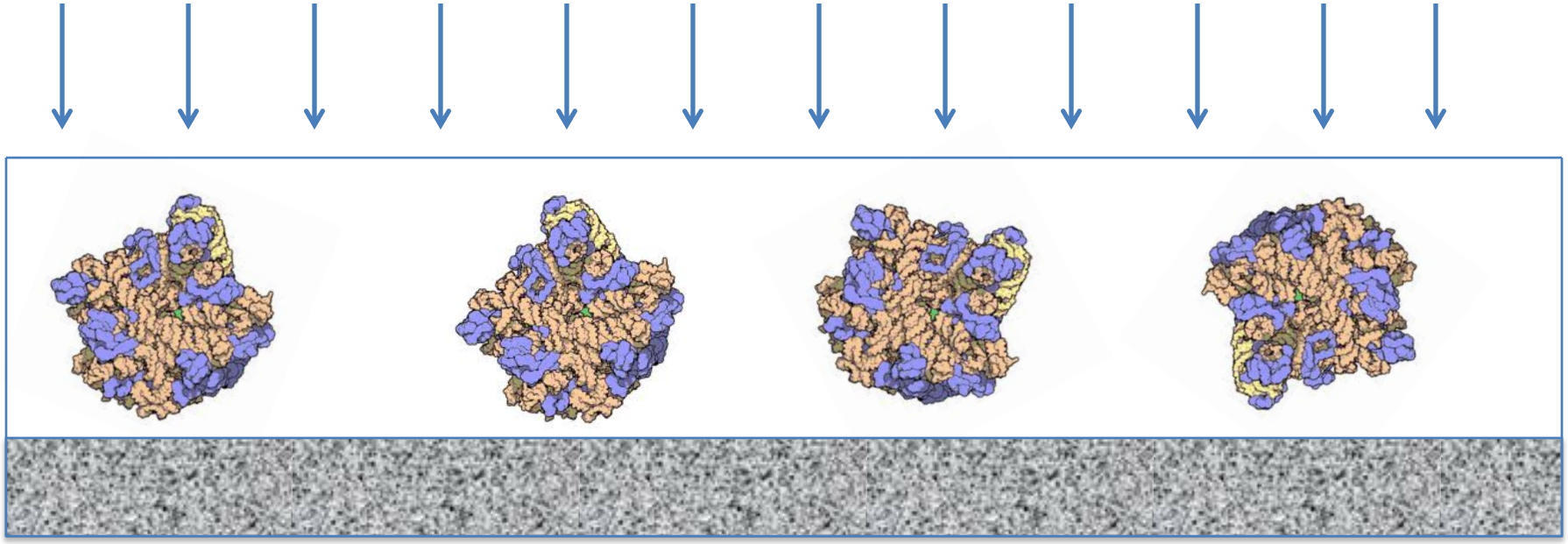
Only by averaging over a sufficient number of particle projections can the original signal be retrieved.



Simulated images of ribosome at SNR ~ 0.1

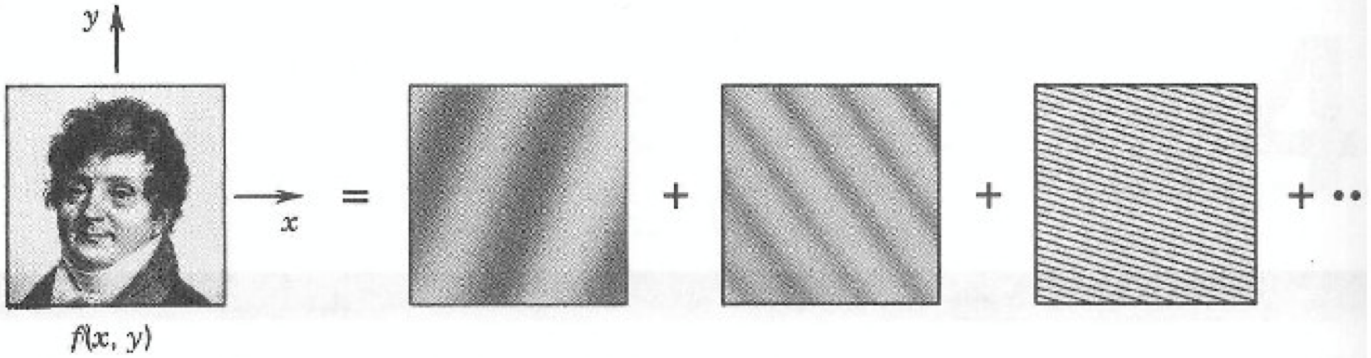
Schwander et al., Phil. Trans. Roy. Soc. 2014

“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..

2D Fourier transform



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

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:

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

$$u = 0,1,\ldots,M-1, \qquad v = 0,1,\ldots,N-1$$

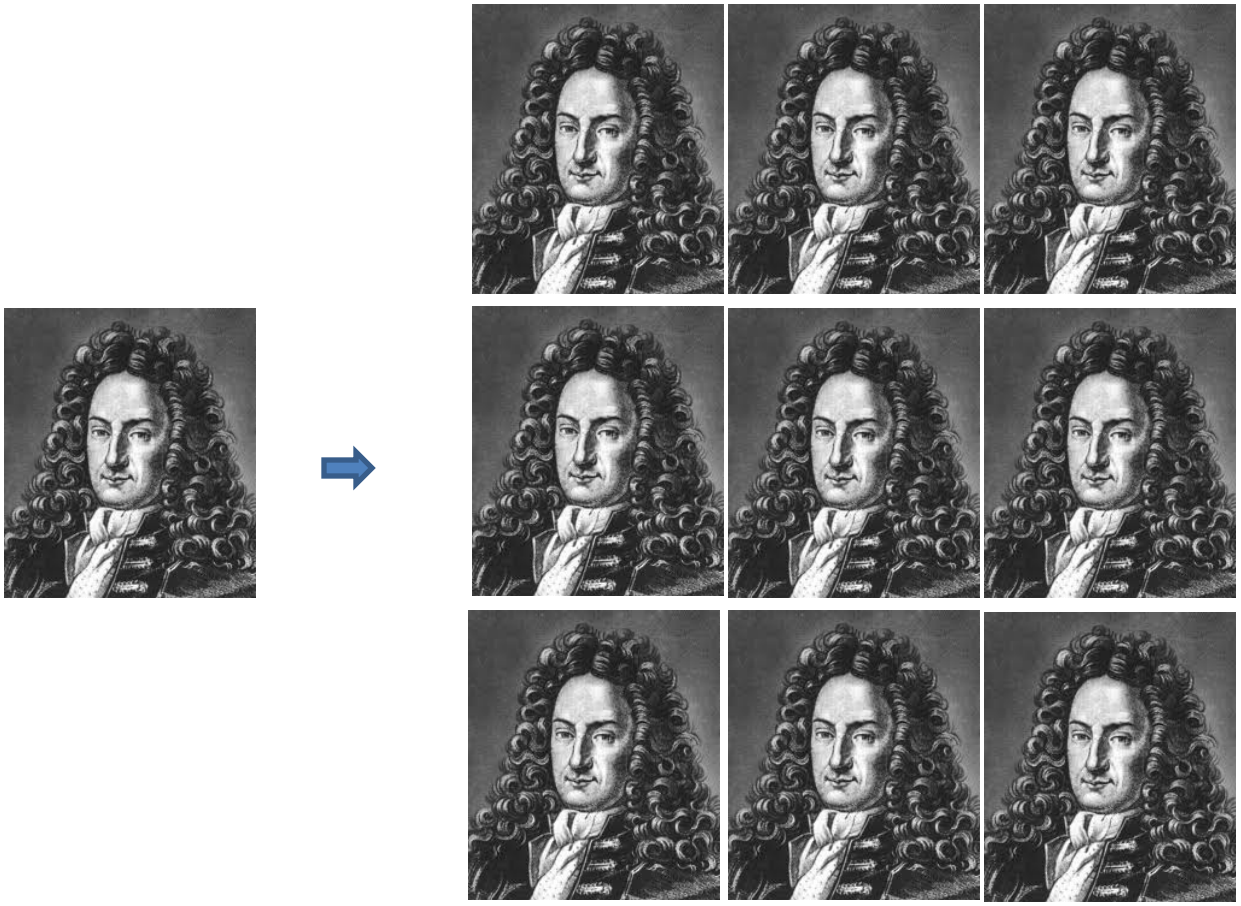
The inverse 2D DFT is given by:

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

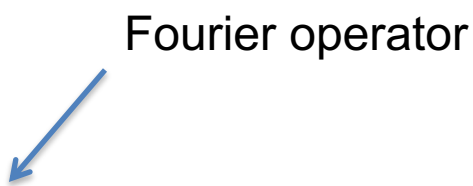
$$m = 0,1,\ldots,M-1, \qquad n = 0,1,\ldots,N-1$$

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

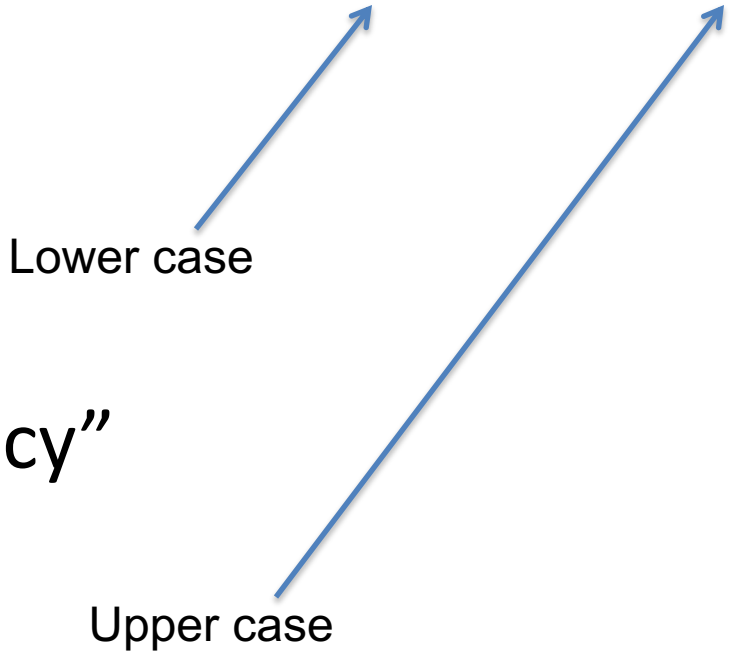


Notations:



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"

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

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

definition: $P(\mathbf{k}) = |F(\mathbf{k})|^2$ is the "Power spectrum"

Total power is the same in real and Fourier space:

$$\int_{\emptyset} |F(\mathbf{k})|^2 \, d\mathbf{k} = \int |i(\mathbf{r}) - avrg|^2 \, d\mathbf{r}$$

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

and subscript $_{\emptyset}$ indicates "exclude origin in the integration"

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

$$SNR = \int_{\emptyset} |S(\mathbf{k})|^2 \, d\mathbf{k} / \int_{\emptyset} |N(\mathbf{k})|^2 \, d\mathbf{k}$$

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

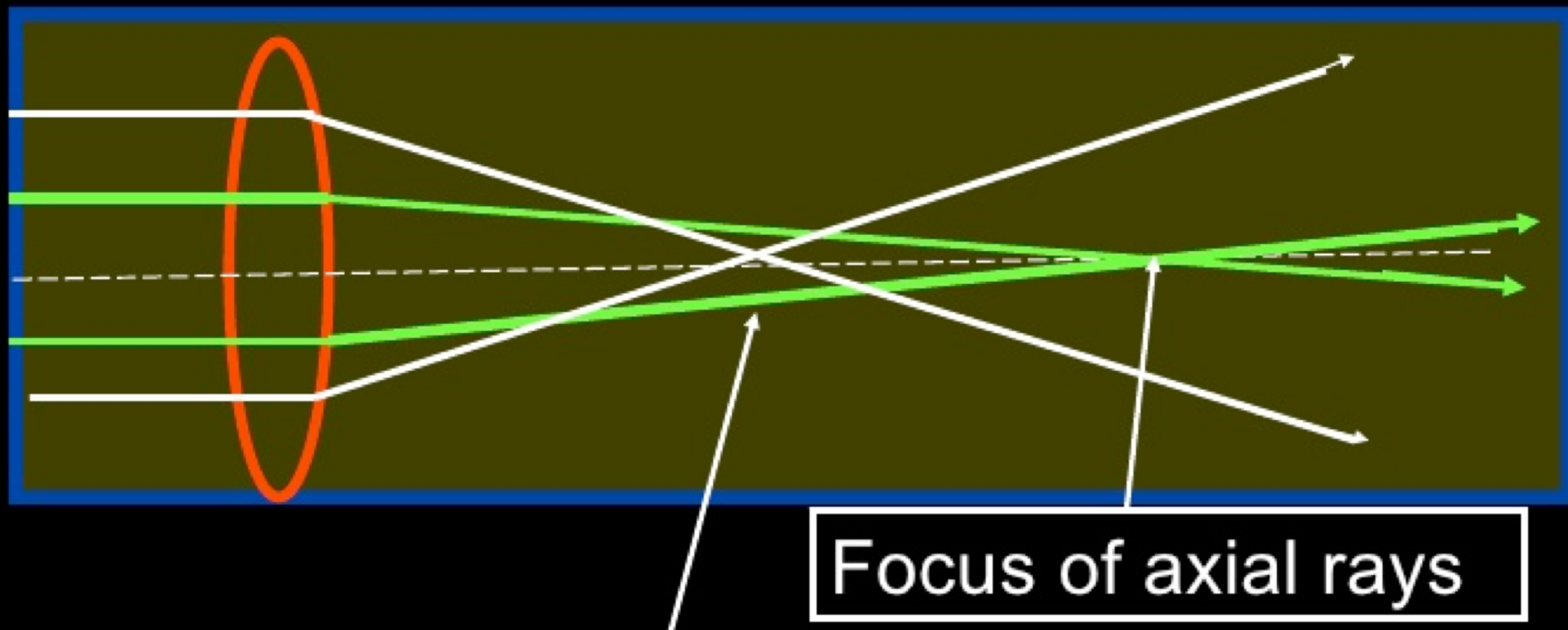
$$\gamma(\mathbf{k}) = -\pi\lambda\left[\Delta z + \frac{z_a}{2} sin2(\phi - \phi_0)\right] k^2 + \frac{1}{4}\lambda^3 C_s k^4$$

wave aberration function

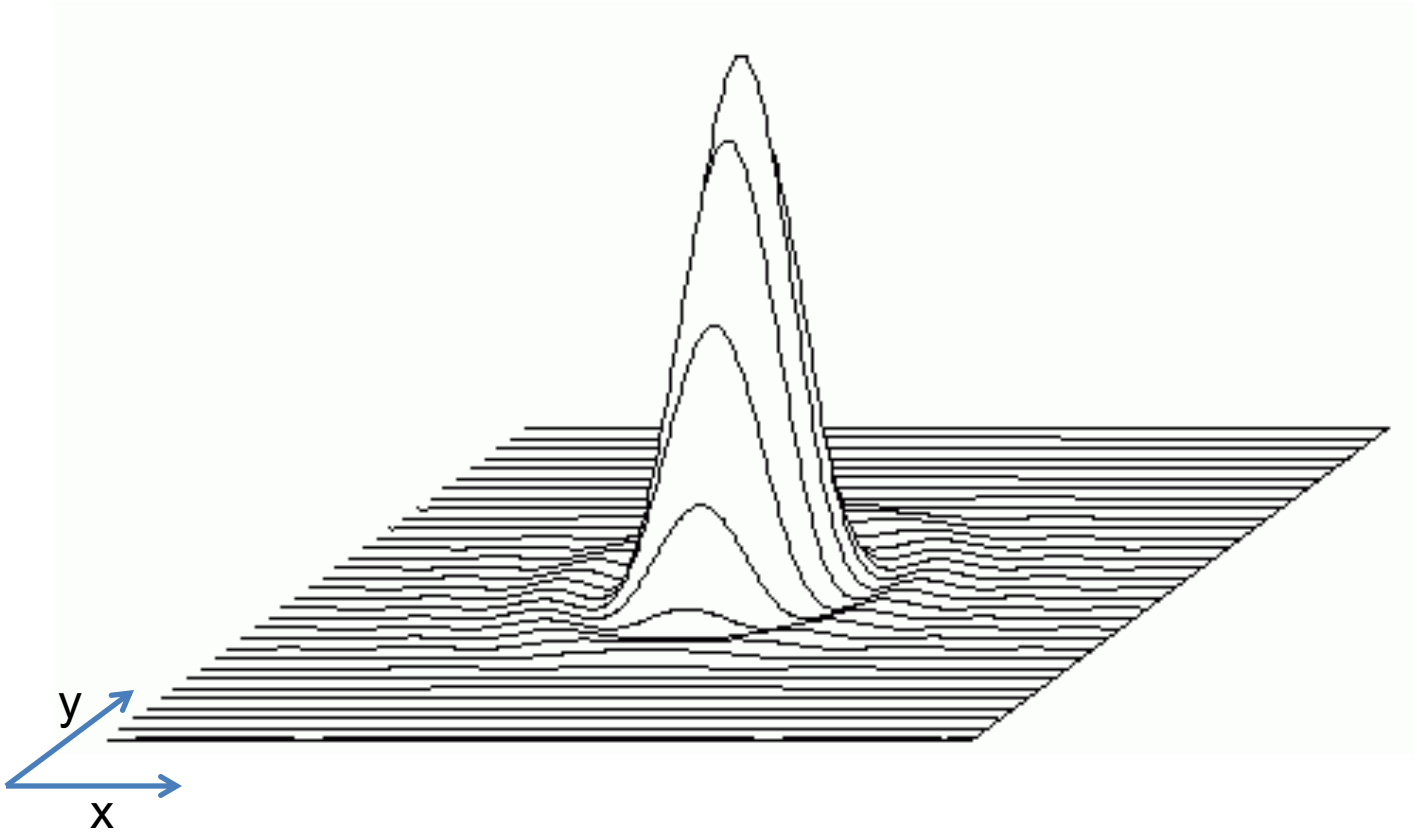
defocus ax. astigmatism spherical aberration

k = spatial frequency vector; *k* = length of this vector

- Spherical aberration



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

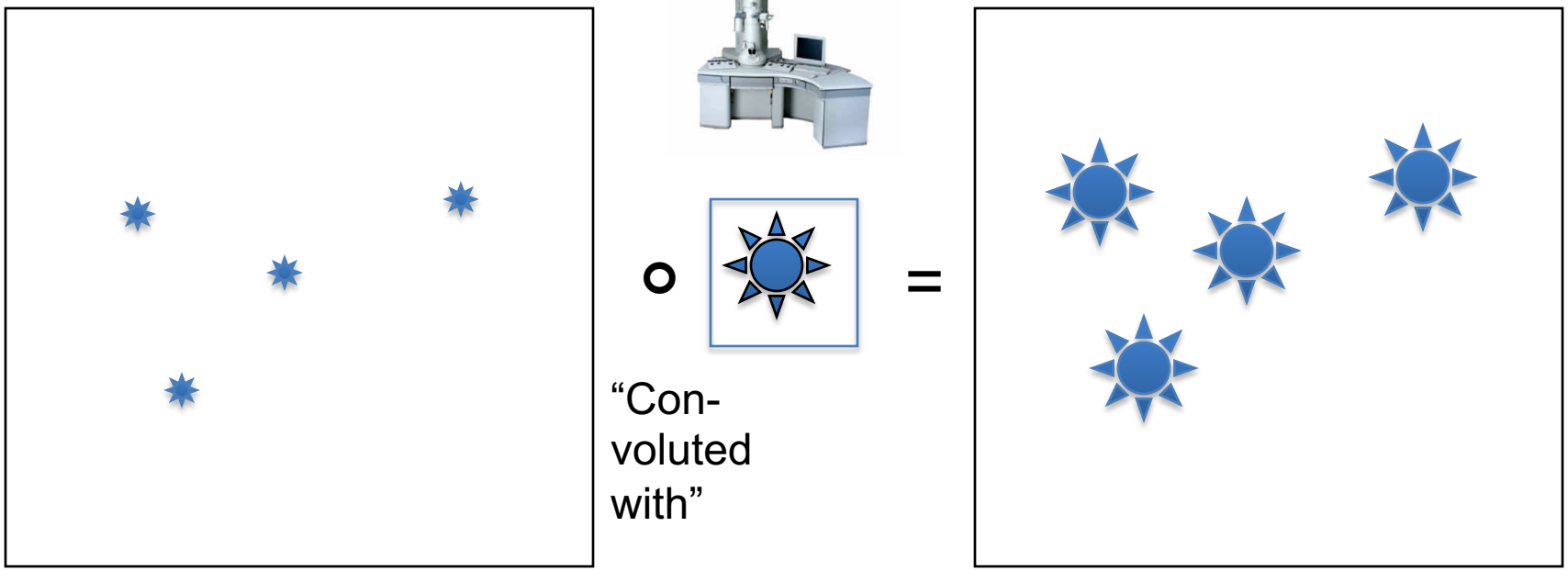


The point spread function has finite width, and is centered at the location that the point would have in the image formed by an ideal instrument.

OBJECT

PSF

IMAGE = CONVOLUTION PRODUCT

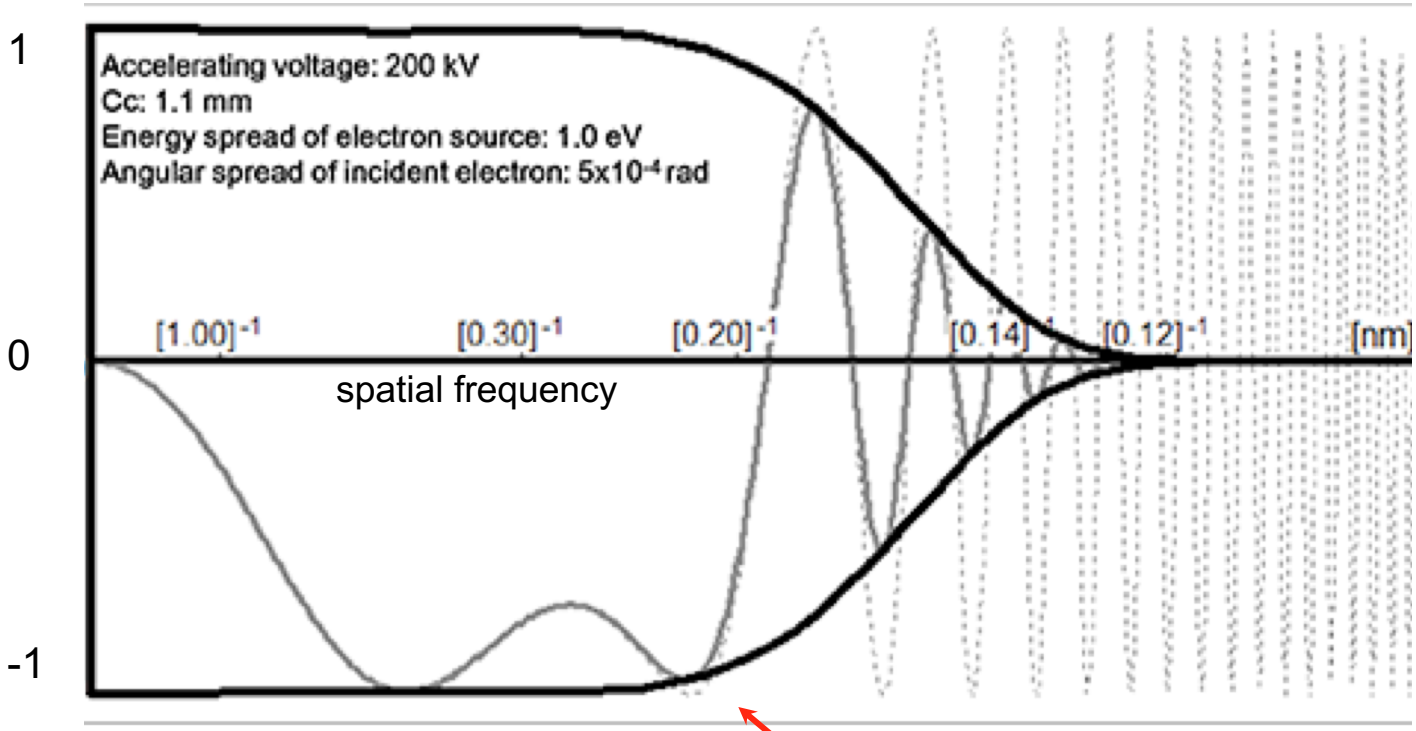


“Con-voluted with”

An object consisting of on arrangement 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

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

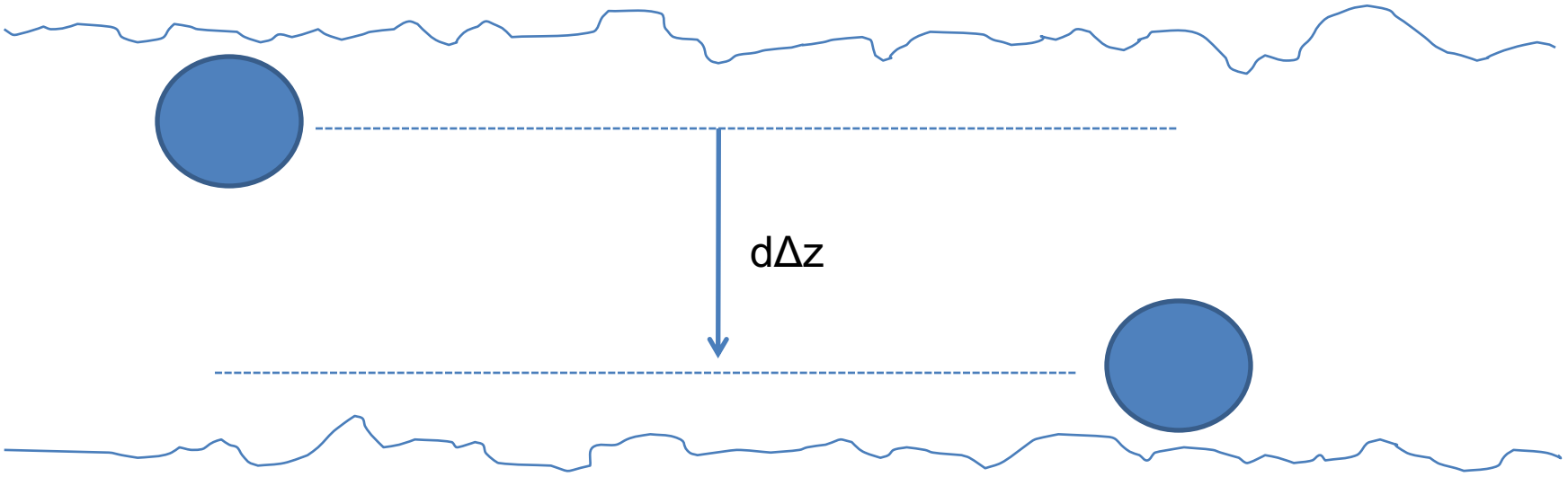
- Angular spread: point source replaced by extended source – convergent (non-parallel) illumination

  Envelope function is defocus-dependent

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

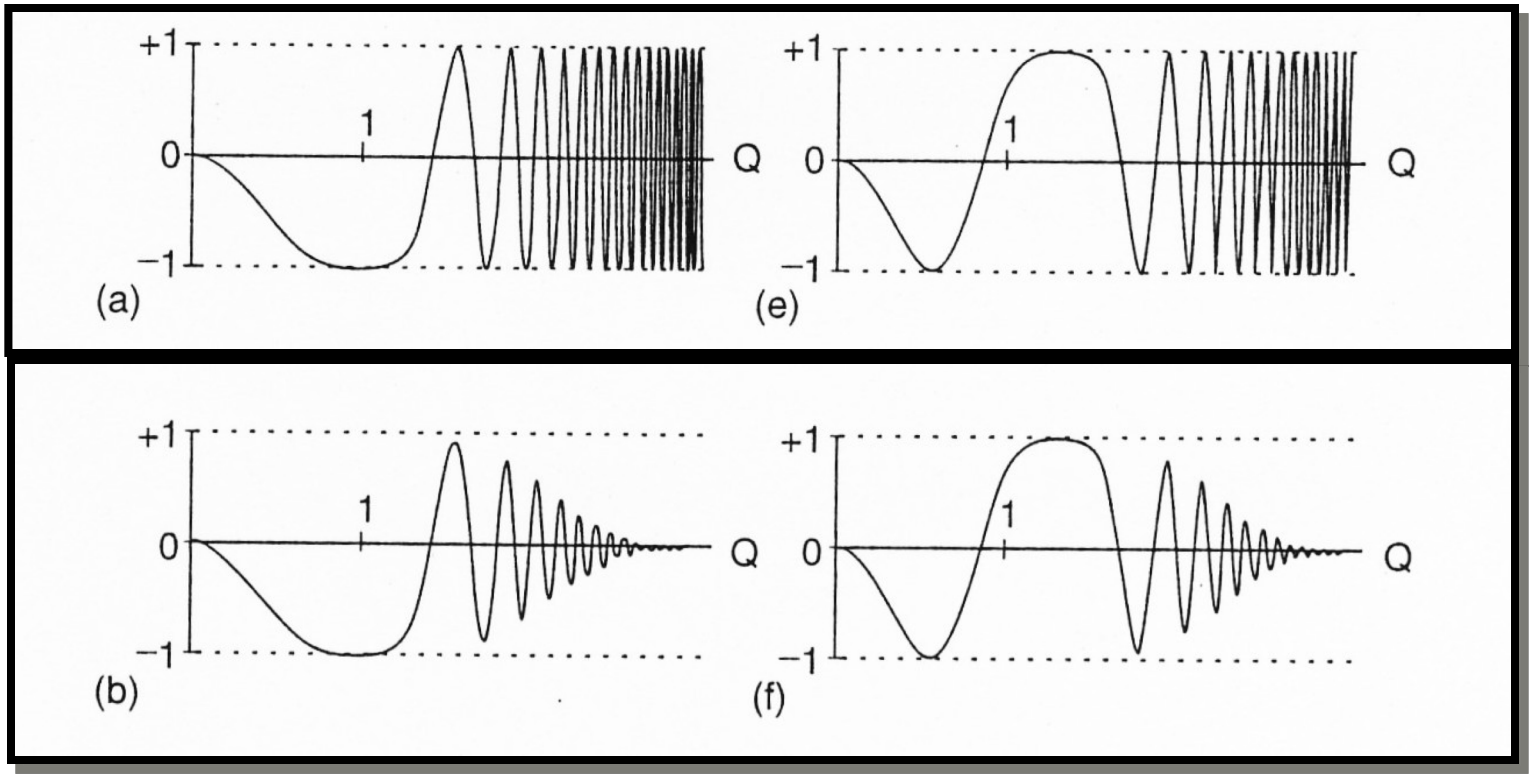
Defocus (and hence the CTF) is affected by the particle's z-position within the ice layer.

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



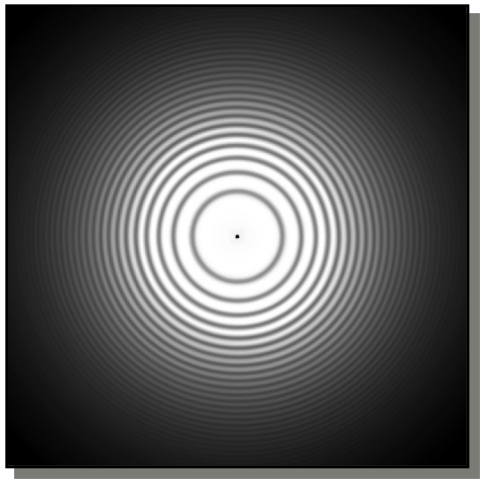
Ice layer large compared with particle diameter

Contrast transfer function



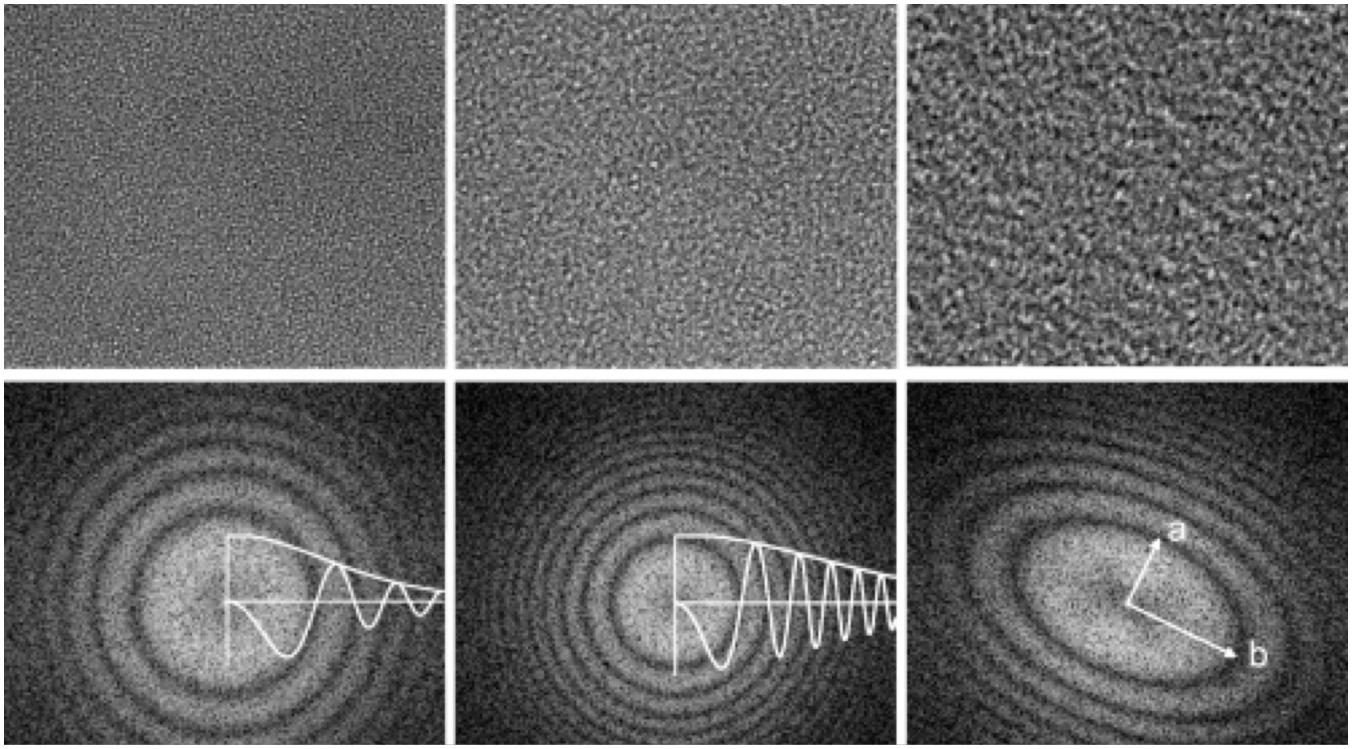
Coherent

Partially coherent



Power spectrum
“Thon rings”

after Fritz Thon, a pioneer in optical diffraction analysis

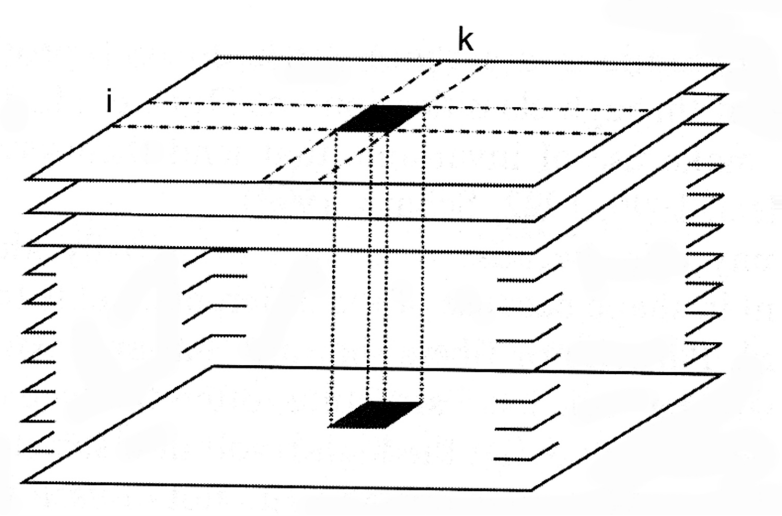


What Thon rings show:

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

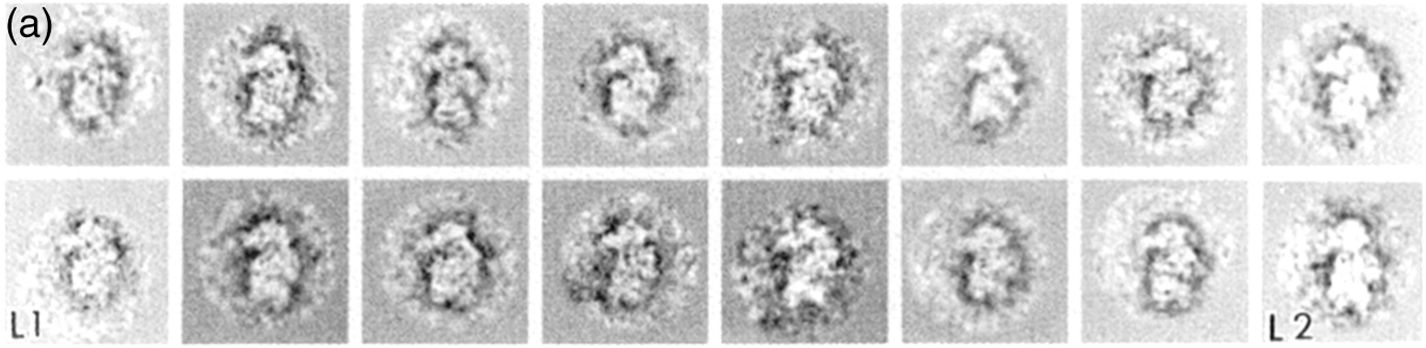
Why do we see rings? Because for an amorphous object, such as carbon, the amplitudes of Fourier components are roughly the same throughout Fourier space. Without CTF, we would see a uniform (white) disk up to the radius that corresponds to the resolution limit.
Instead we see concentric white rings separated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

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

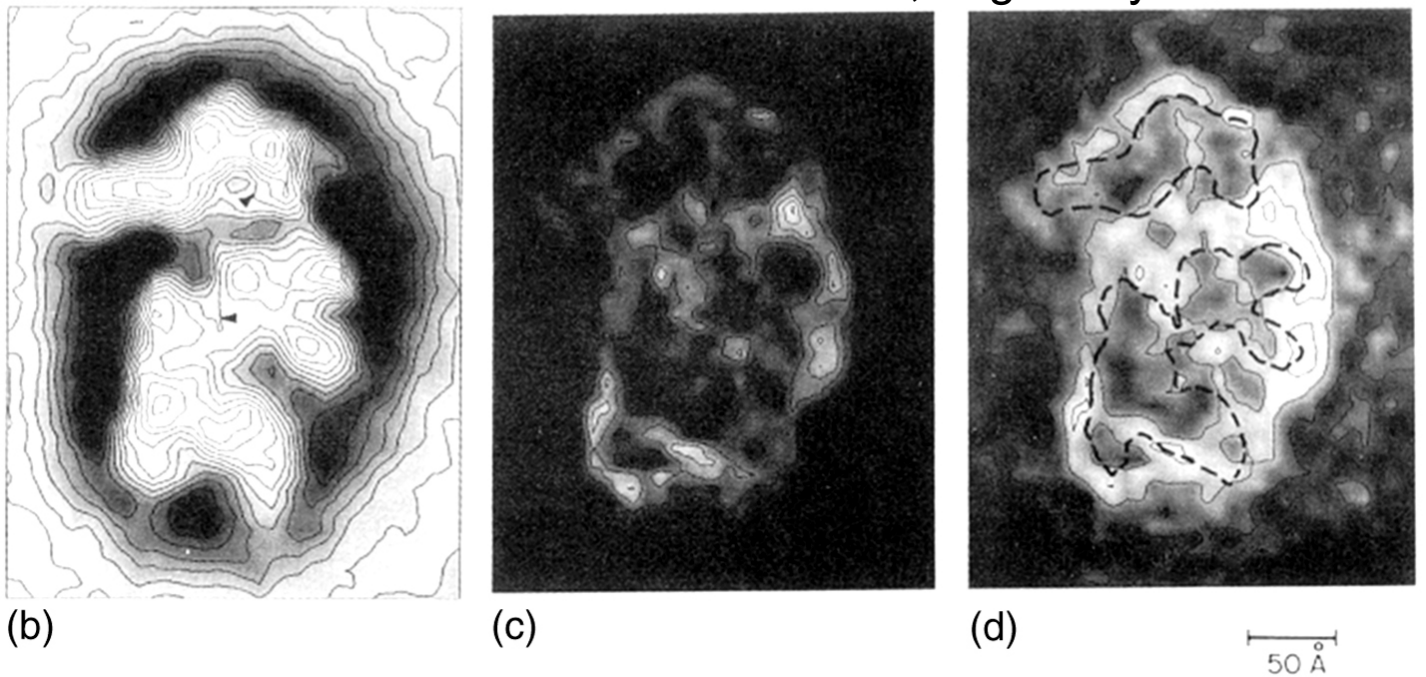


Averaging requires the images to be aligned: in the array, each image element, or pixel, must refer to the same point of the object.

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



40S ribosomal subunits of HeLa cells, negatively stained



average variance map standard deviation map

Frank et al., Science 1981

Alignment of single-particle projections ("particles") is achieved by cross-correlation

- Translational cross-correlation function (CCF)

  Discrete, unnormalized version:

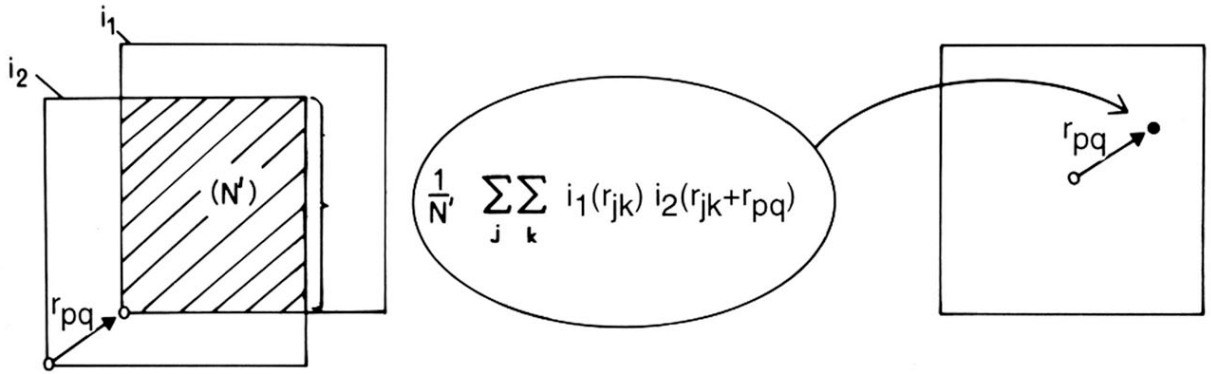


Fig. 3.8. Definition of the cross-correlation function. Image 1 is shifted with respect to image 2 by vector $\mathbf{r}_{pq}$. In this shifted position, the scalar product of the two images arrays is formed and put into the CCF matrix at position (p, q). The vector $\mathbf{r}_{pq}$ is now allowed to 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 CONVOLUTION

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

or

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

Short notation: s(r) = o(r) o h(r)

Fourier space: S(k) = O(k) H(k)

2D CROSS-CORRELATION

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

or

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

CCF(r) = $s_1(r)$ ★ $s_2(r)$

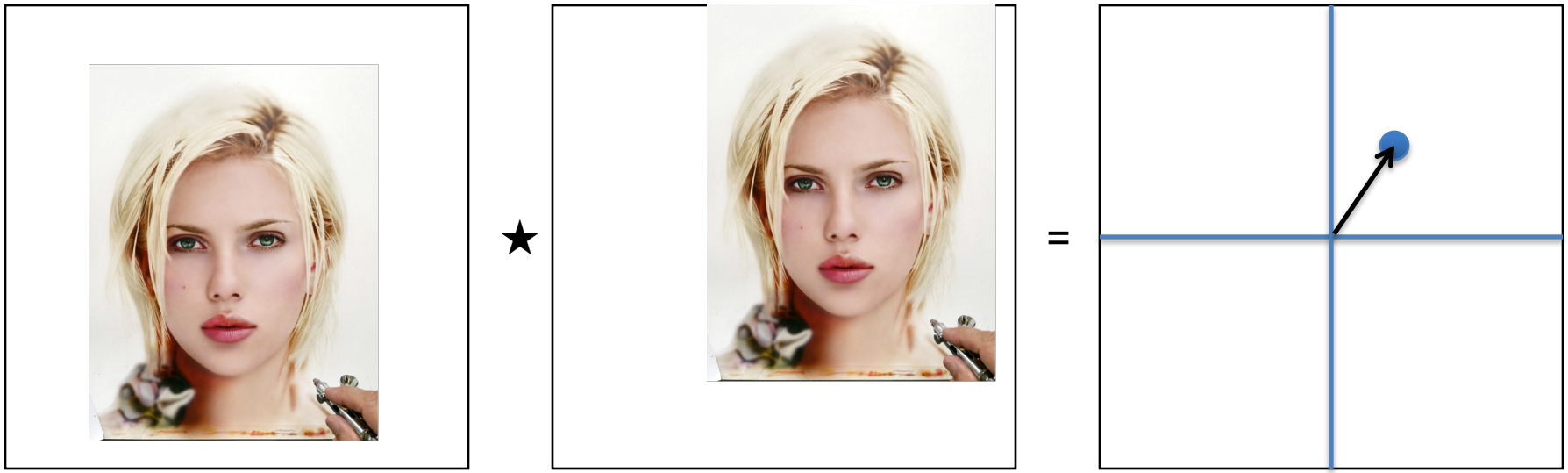
Φ(k) = $S_1(k)$ $S_2^*(k)$

In Fourier space, a real-space convolution becomes a scalar product,
a cross-correlation integral becomes a conjugate product

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

or

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



Images identical
CCF peak is sharp, well defined

Images dissimilar, but face is in the same place
CCF peak is unsharp, not well defined

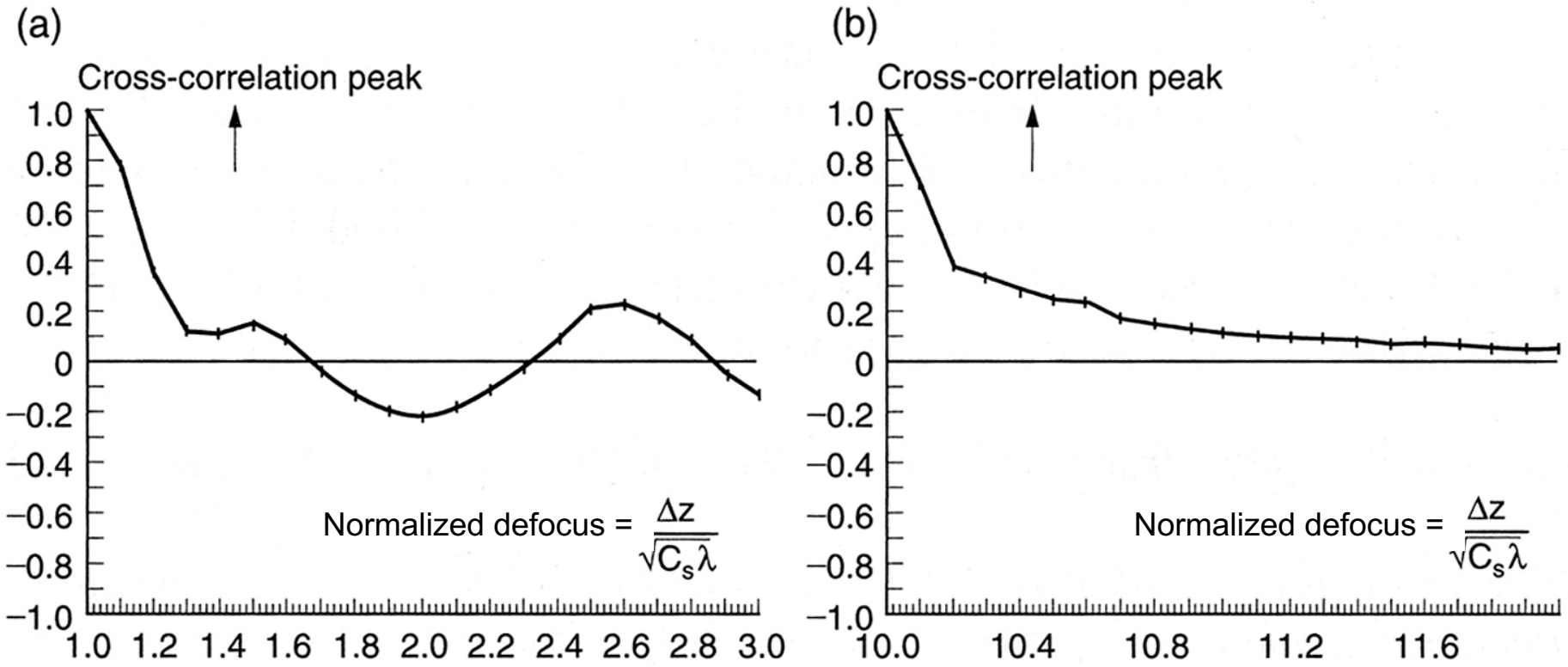
ACF of image: $(S\,H)\,(S\,H)^* = (S\,S^*)\,(H\,H^*)$ ACF of S convoluted with ACF of PSF

CCF of 2 images of same signal with different CTFs:

$(S_1\,H_1)\,(S_1\,H_2)^* = (S_1 S_1^*)\,(H_1\,H_2^*)$ ACF of S convoluted with CCF of PSFs

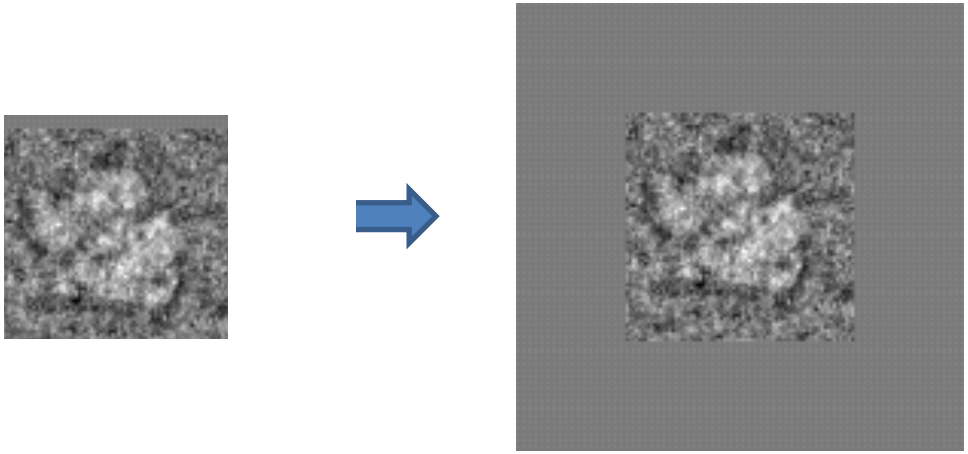
(Scherzer) normalized defocus =1

normalized defocus = 10

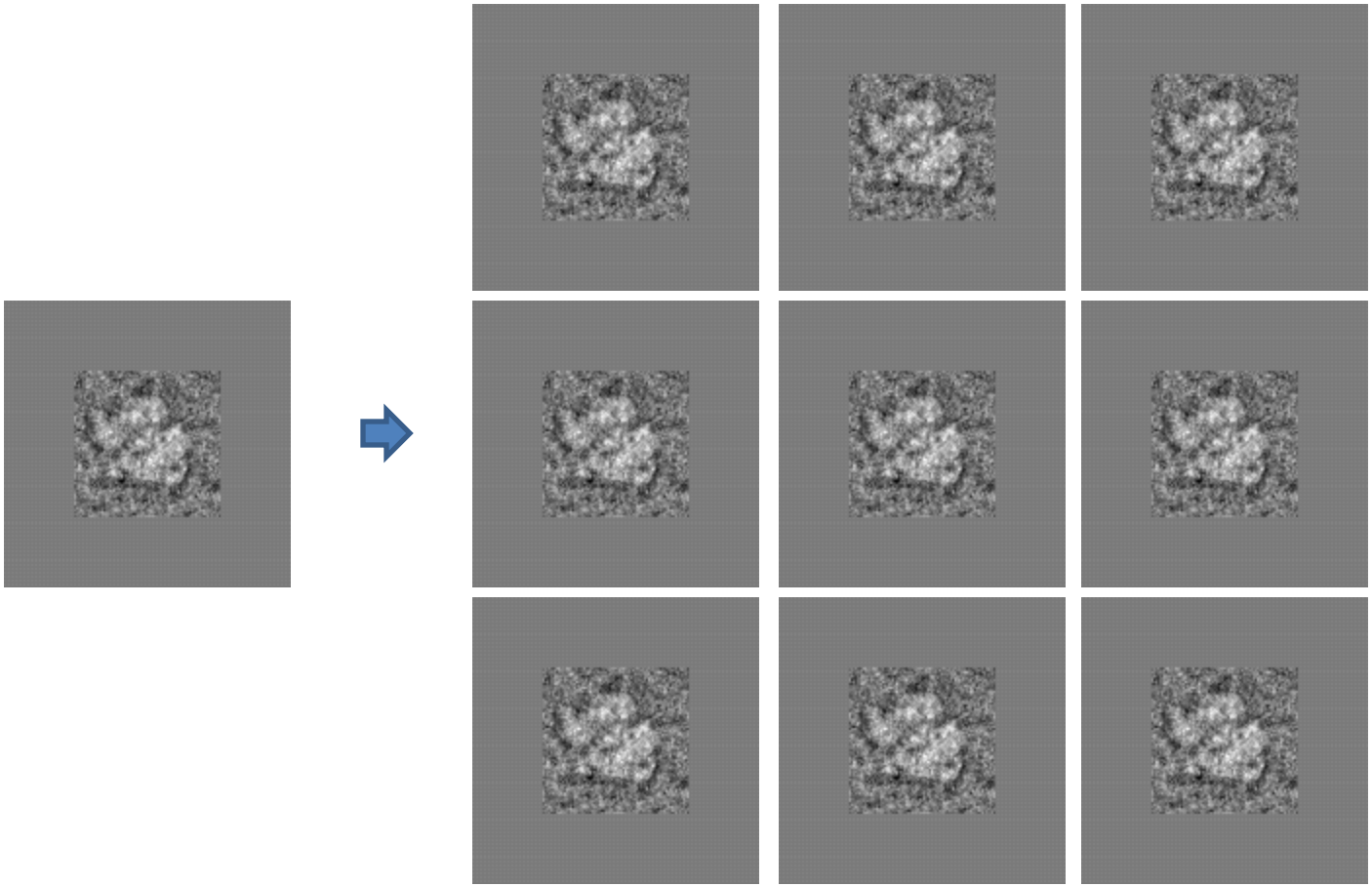


Value of normalized CCF peak as a function of the difference in focus, $\delta\Delta z$

Padding is needed when CCF is computed via Fourier methods:



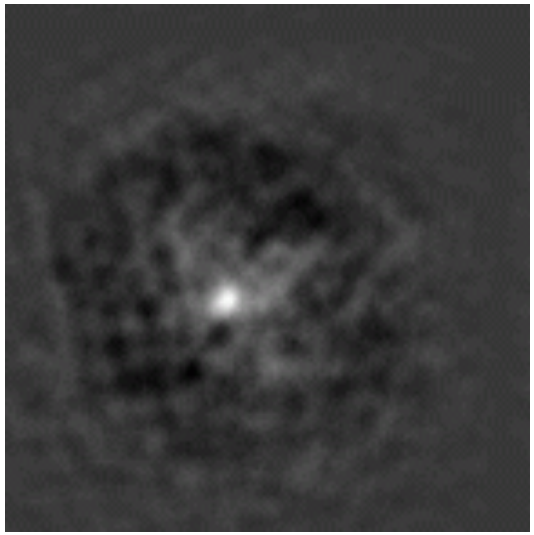
Need for padding follows from the discrete Fourier representation:



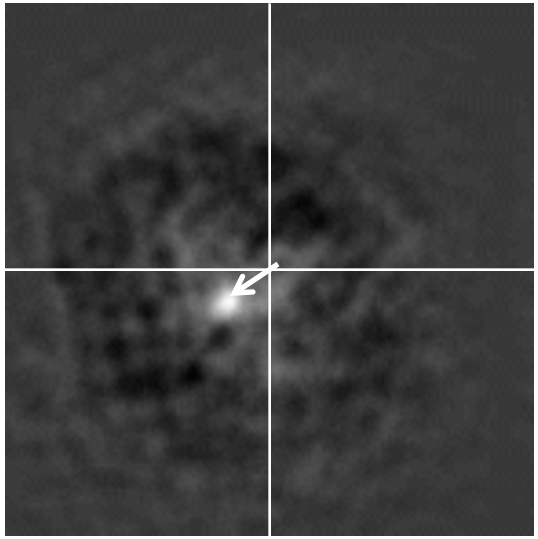
Nonpadded image would get superimposed on a copy of itself

Translational alignment using the CCF – a practical example

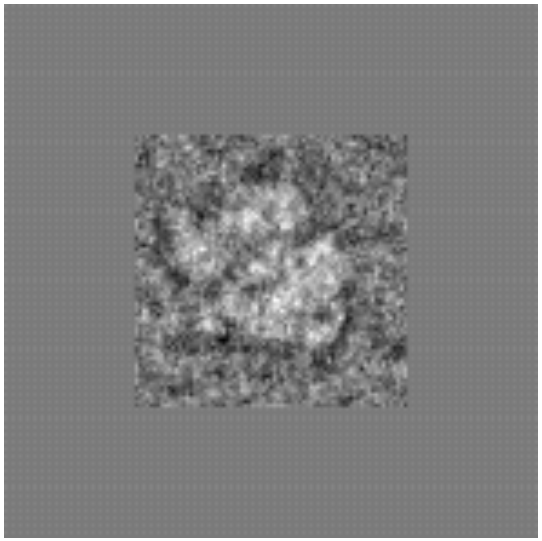
The peak indicates postion of perfect alignment of two images of the same molecule



CCF

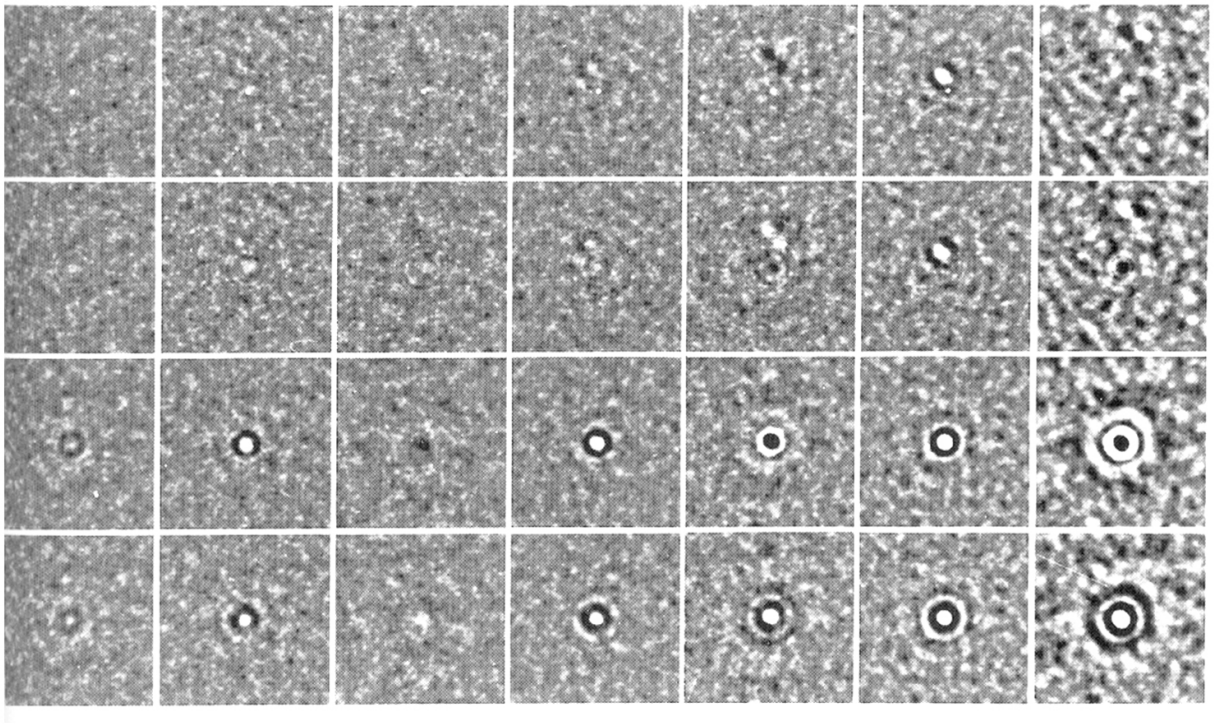


shift of peak from origin indicates relative shift of images



one of the images, padded

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

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 1977)

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.

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:
-

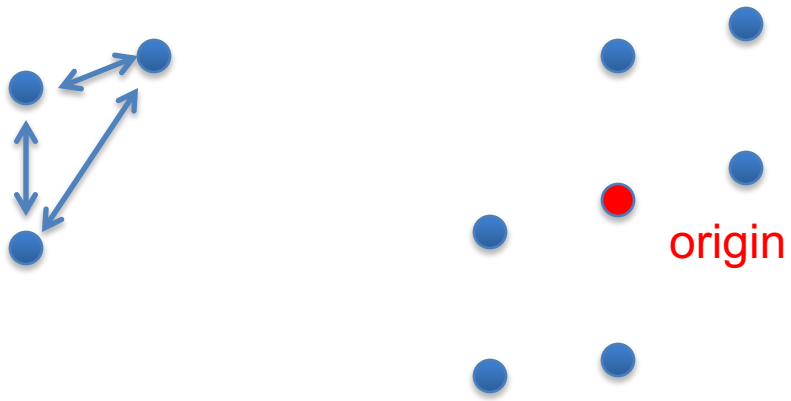
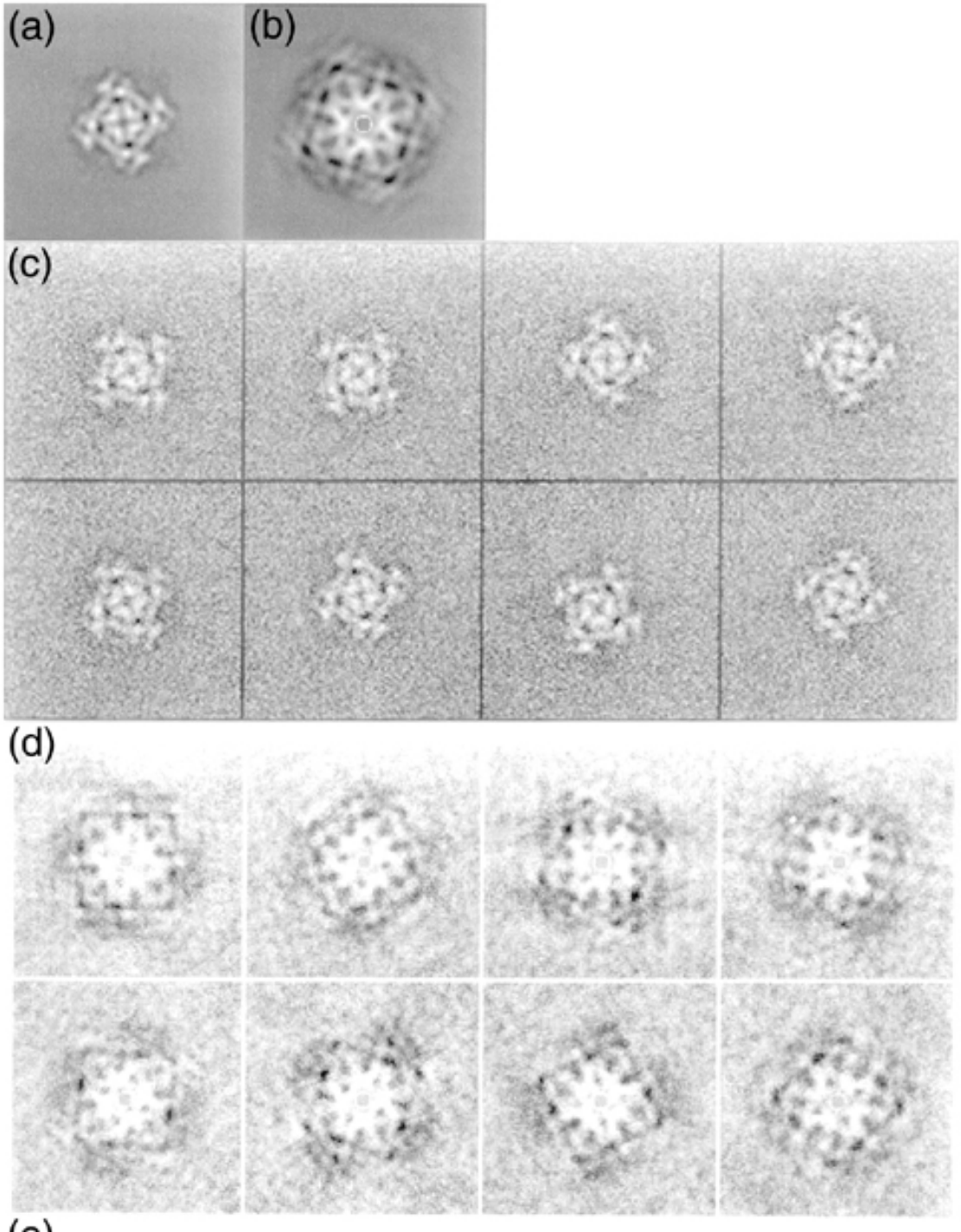


IMAGE AUTOCORRELATION FUNCTION

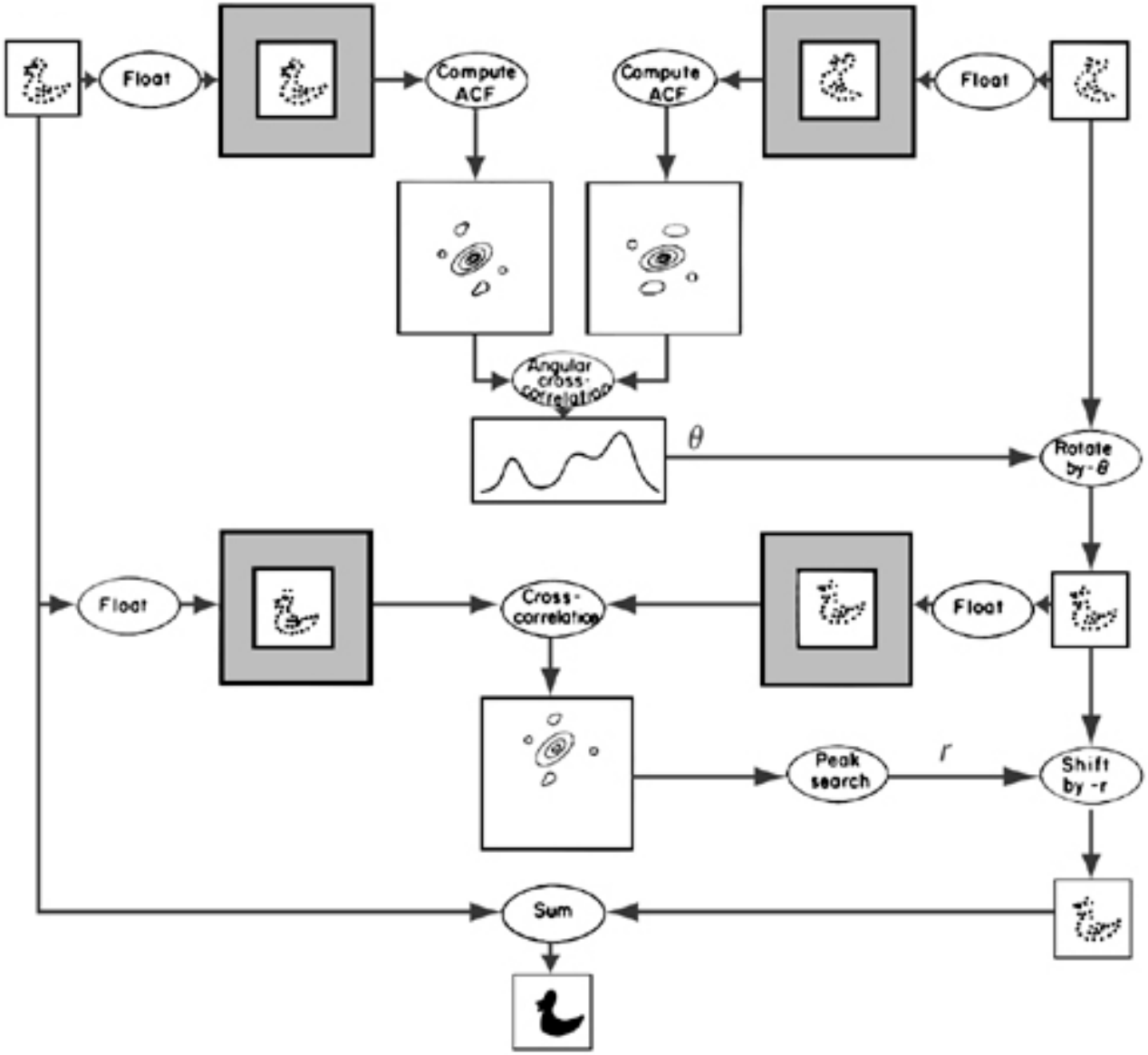
Noise-free molecule and its ACF

The molecule in arbitrary positions

ACFs of molecules above



ACF – based alignment method



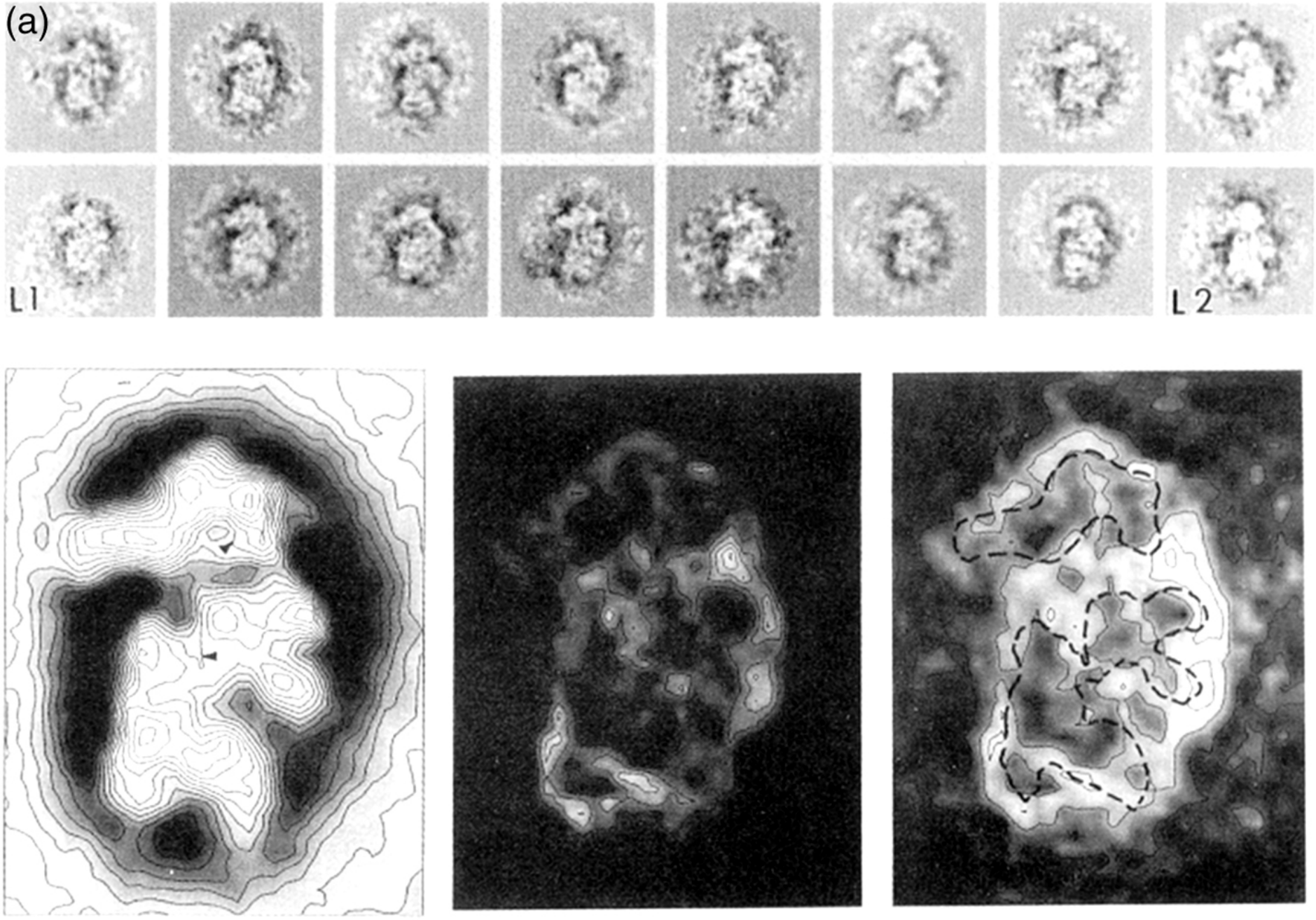
Variance map

- 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.

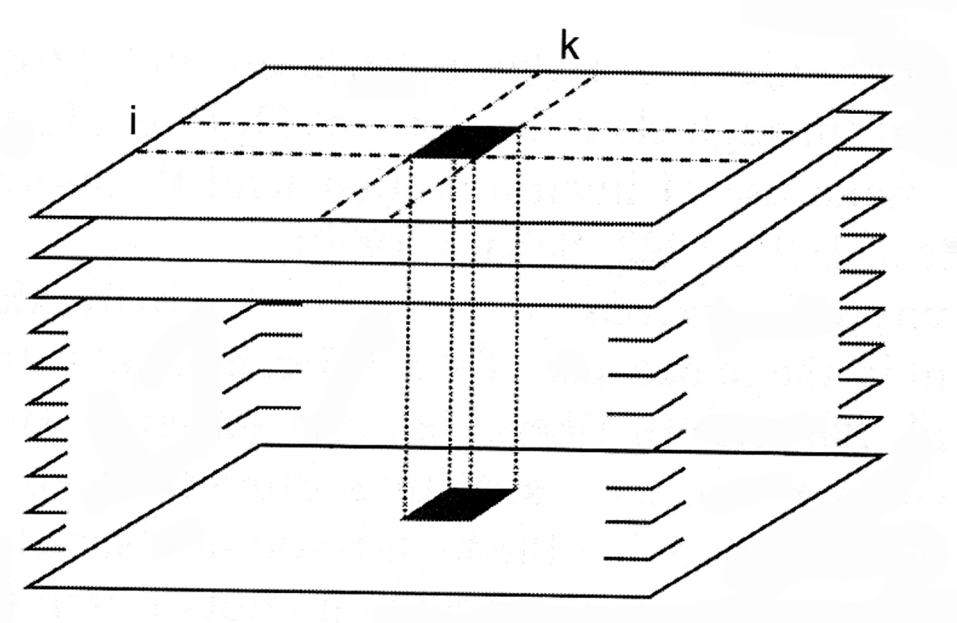
$$v_{(N)}(\mathbf{r}_j) = \frac{1}{(N-1)} \sum_{i=1}^{N} [p_i(\mathbf{r}_j) - \bar{p}_{(N)}(\mathbf{r}_j)]^2$$

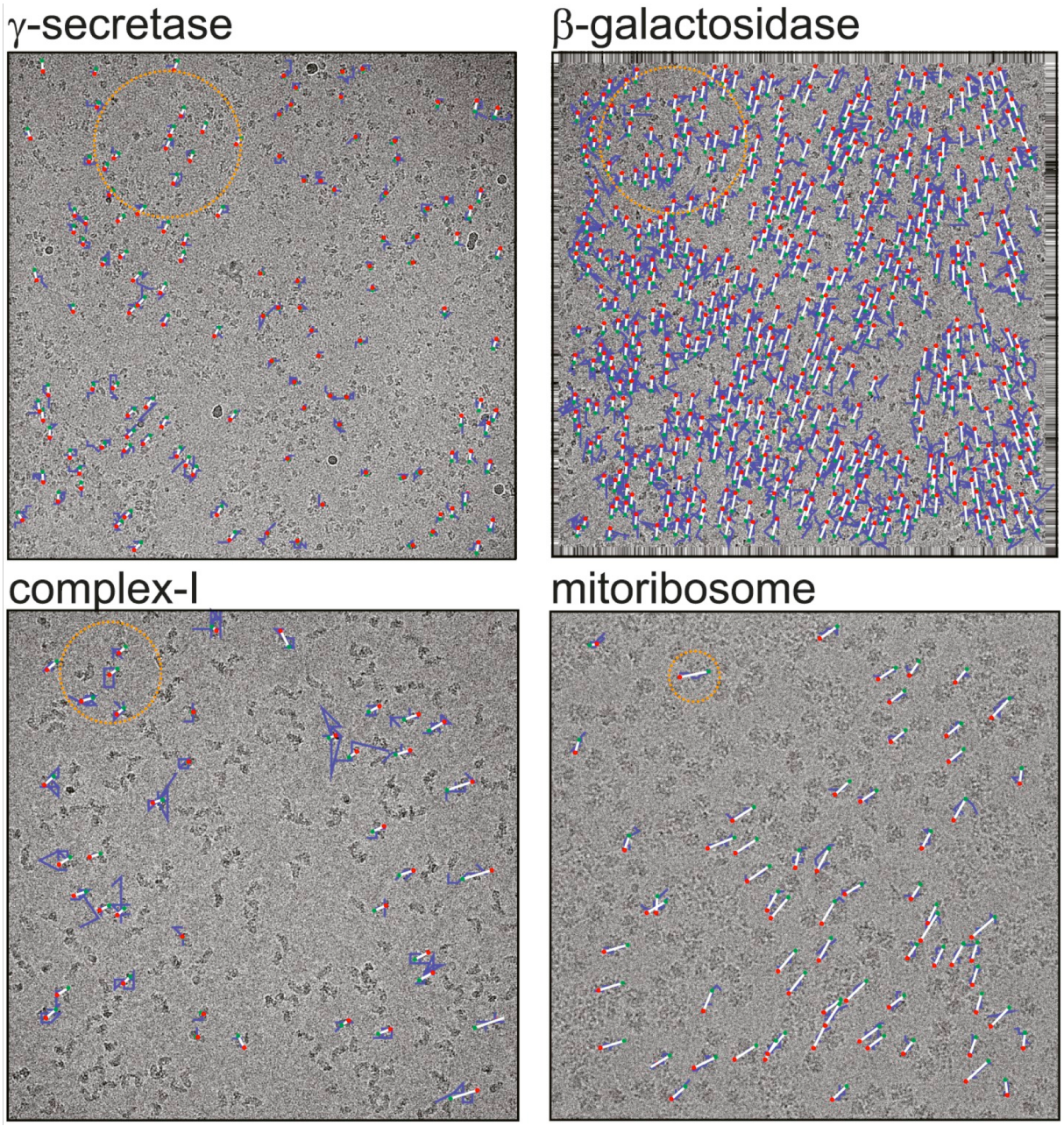
particle image average image


(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.

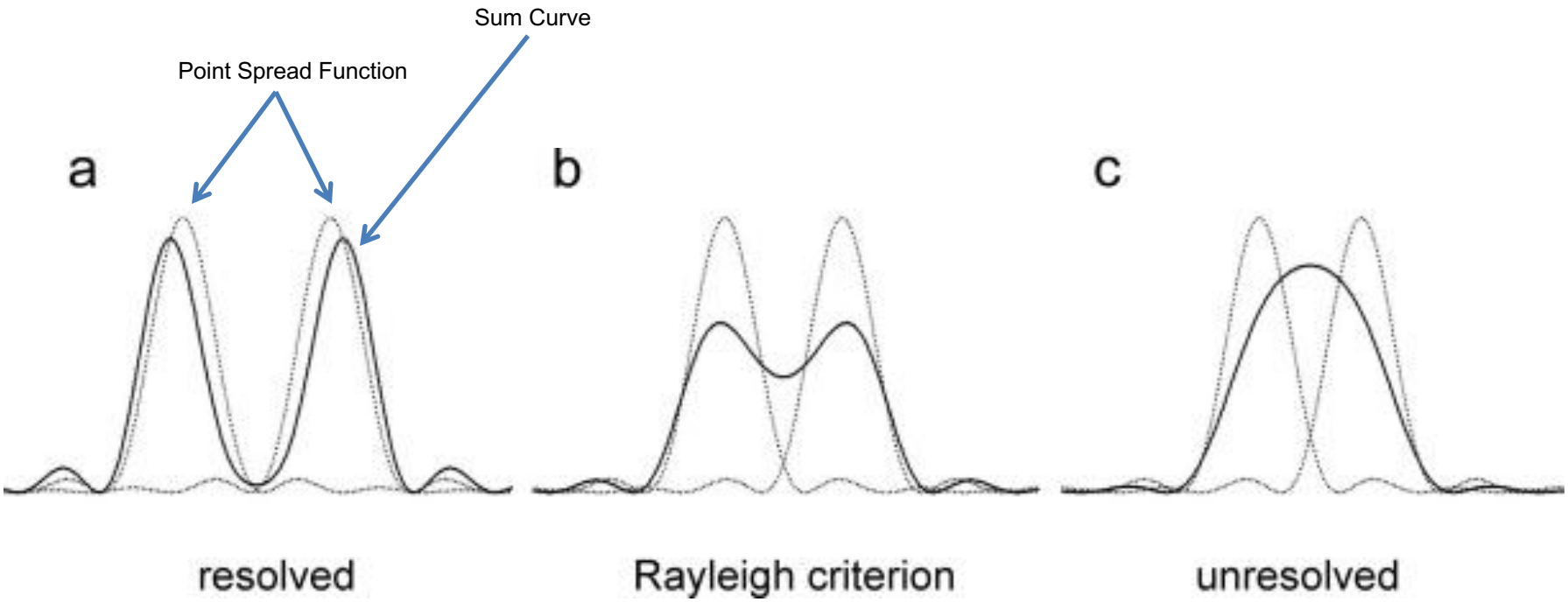




(vectors of motion are exaggerated)

S. Scheres, eLife 2014

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



Peaks are distinguishable

when distance is > $d_{rayleigh}$

Peaks are just resolved

when distance is = $d_{rayleigh}$

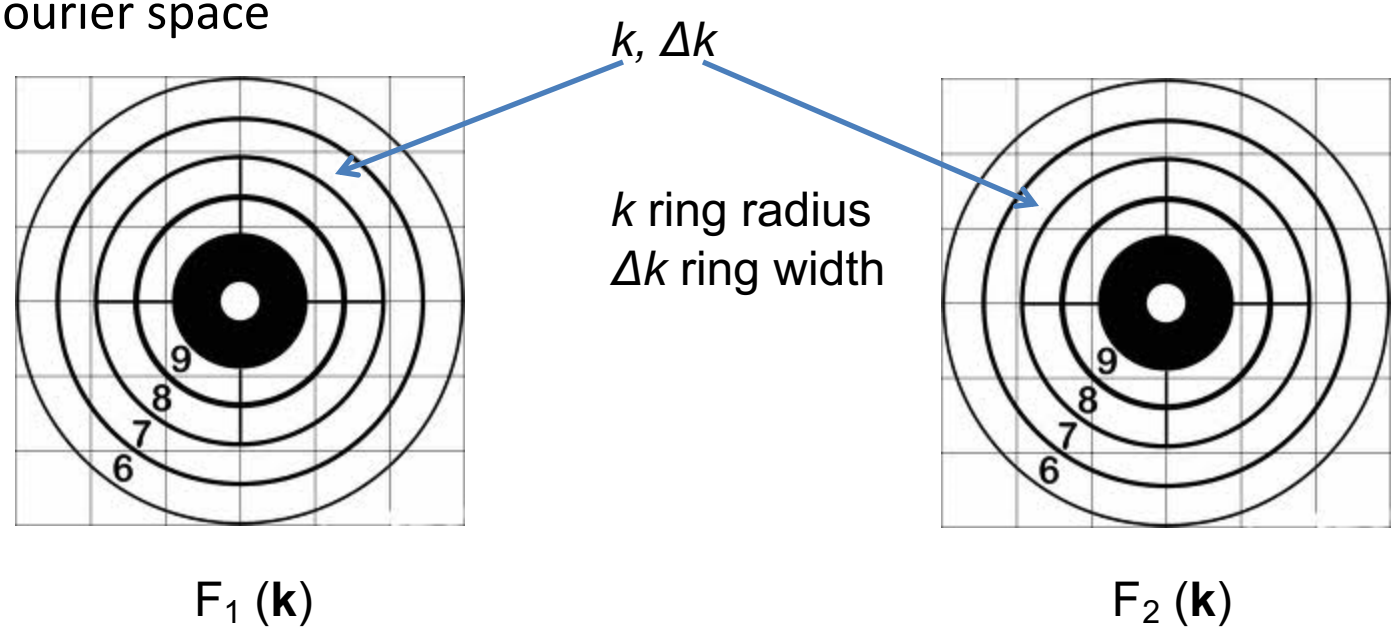
Peaks blend into a single one

when distance < $d_{rayleigh}$

E. V. Orlova and H. R. Saibil, in *Chemical Reviews* 2011

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



Resolution measures & criteria: Fourier ring/shell correlation

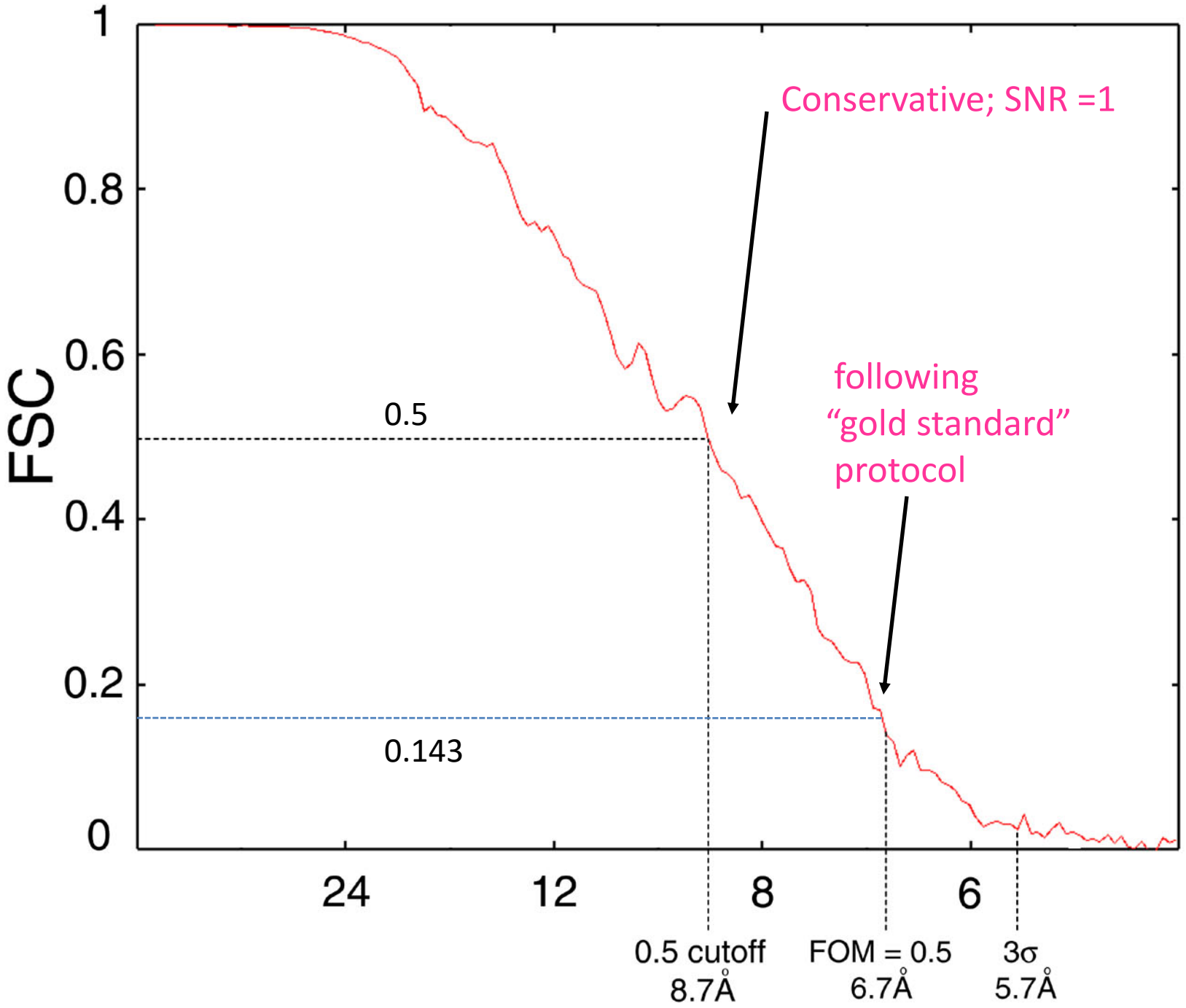
$F_1(\mathbf{k}), F_2(\mathbf{k})$ Fourier transforms of halfset averages (or halfset reconstructions)

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

k = *spatial frequency vector*

k = |**k**| *abs.* size of *spatial frequency*

Δ*k* = *ring width* or (in 3D) *shell thickness*


FSC
1
0.8
0.6
0.4
0.2
0
0.5
0.143
24
12
8
6
Conservative; SNR =1
following
“gold standard”
protocol
0.5 cutoff
8.7Å
FOM = 0.5
6.7Å
3σ
5.7Å

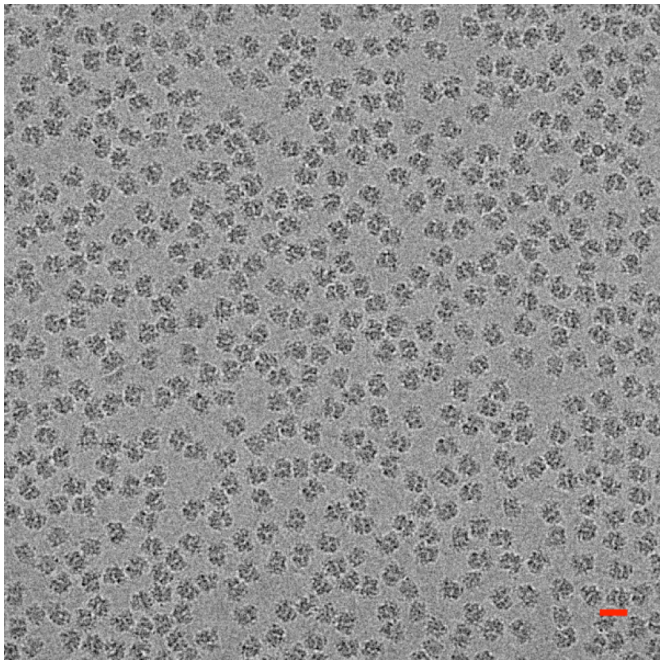
Multivariate Data Analysis and Classification

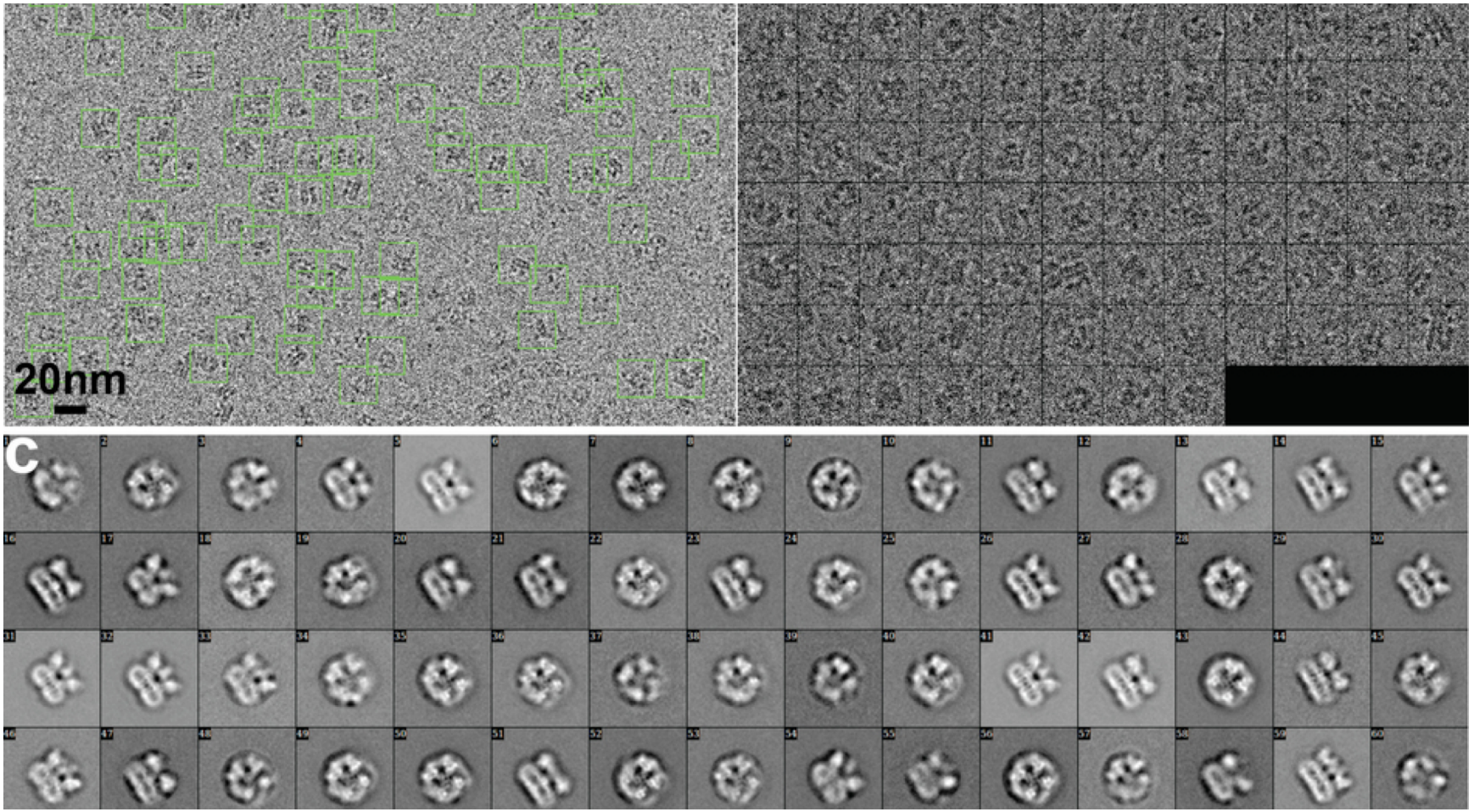
- Images often need to be sorted into classes
- Heterogeneity is due to (1) different viewing angle and (2) different conformations of the molecules
- Sorting them visually only works in the simplest cases
- Multivariate analysis reduces the dimensionality of the classification problem

Classification in 2D

RATIONALE:

Inventory of existing views



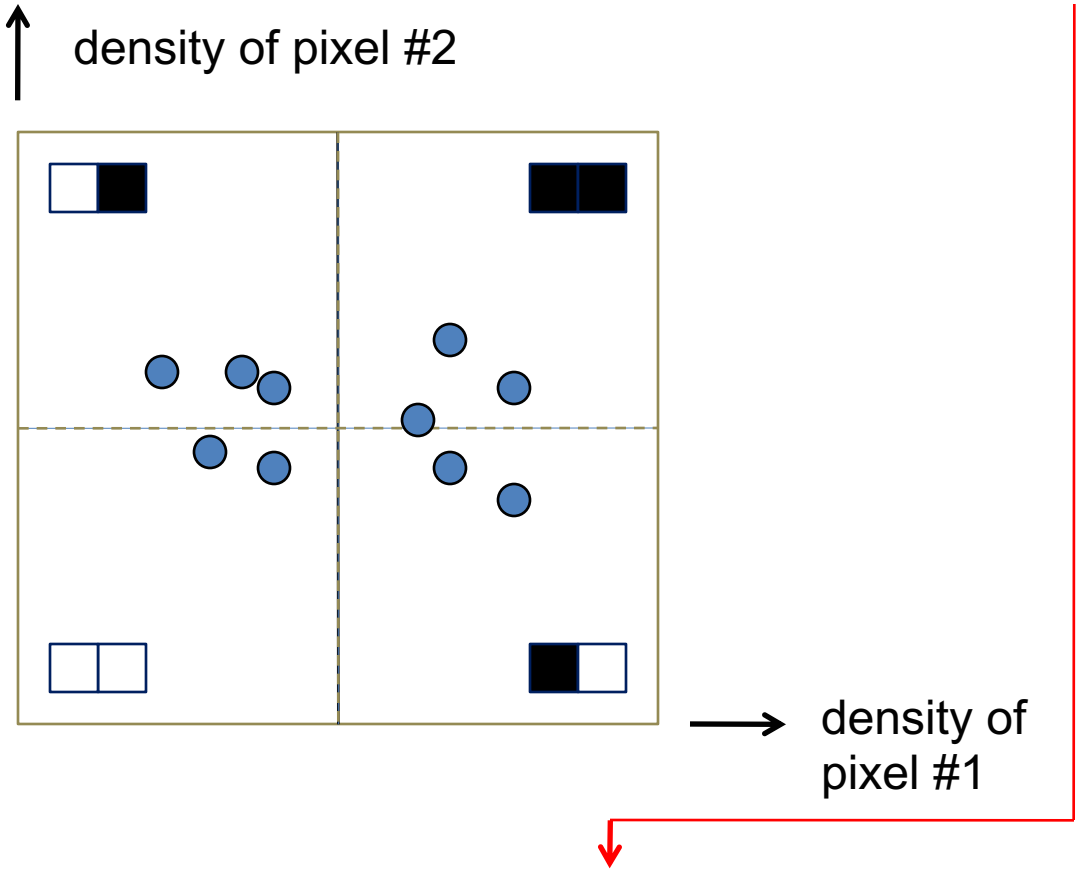


Liao et al., Nature 2013

An image represented in a high-dimensional Euclidean space.

- An image represented by an array of N x M pixels can be thought as a vector in a (generalized) Euclidean space with N x M dimensions
- For example, an image of 64 x 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

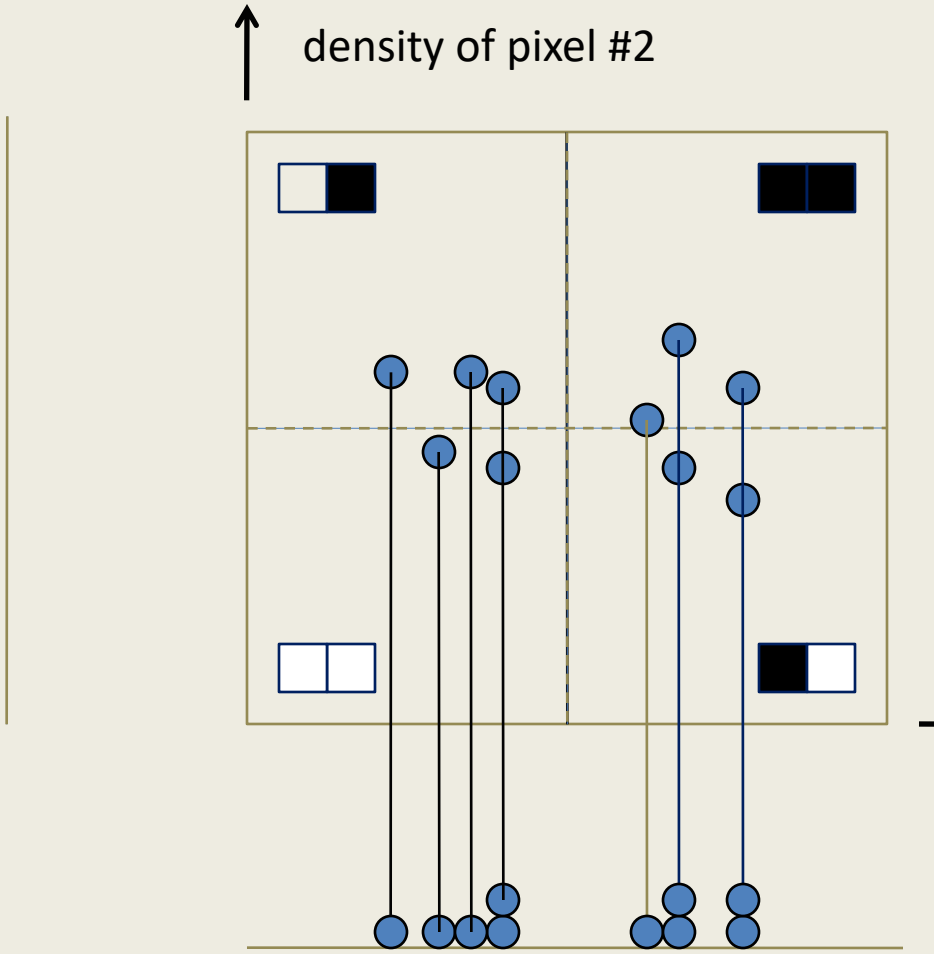
Introducing: a set of images, each consisting of 2 pixels



Similarity = closeness in 2-D Euclidean space

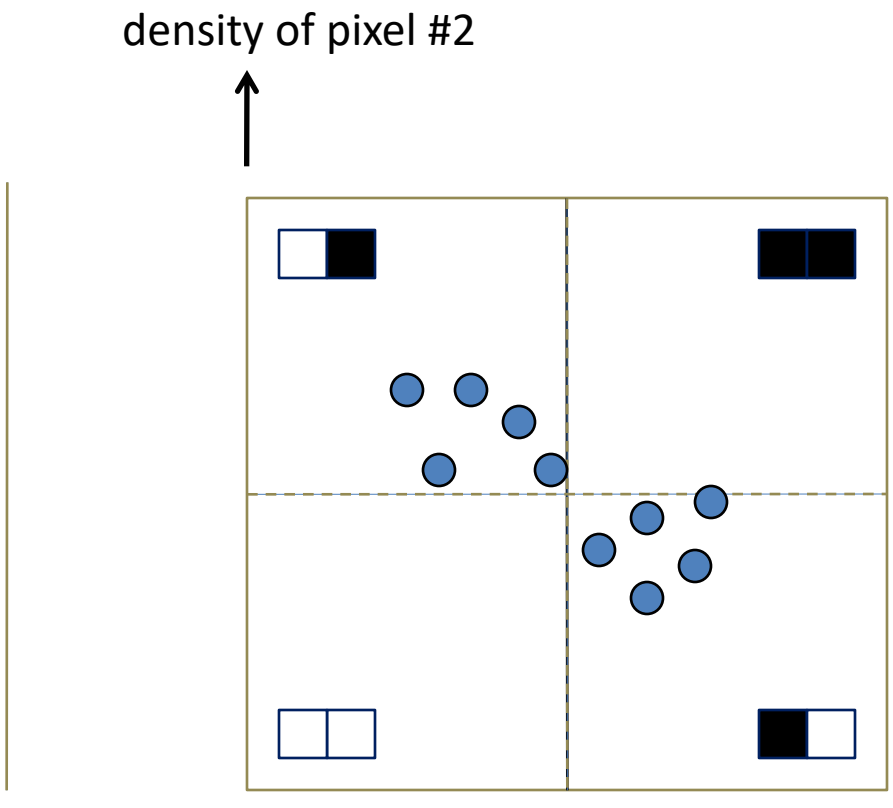
Two images are similar if their (generalized) Euclidean distance is small

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

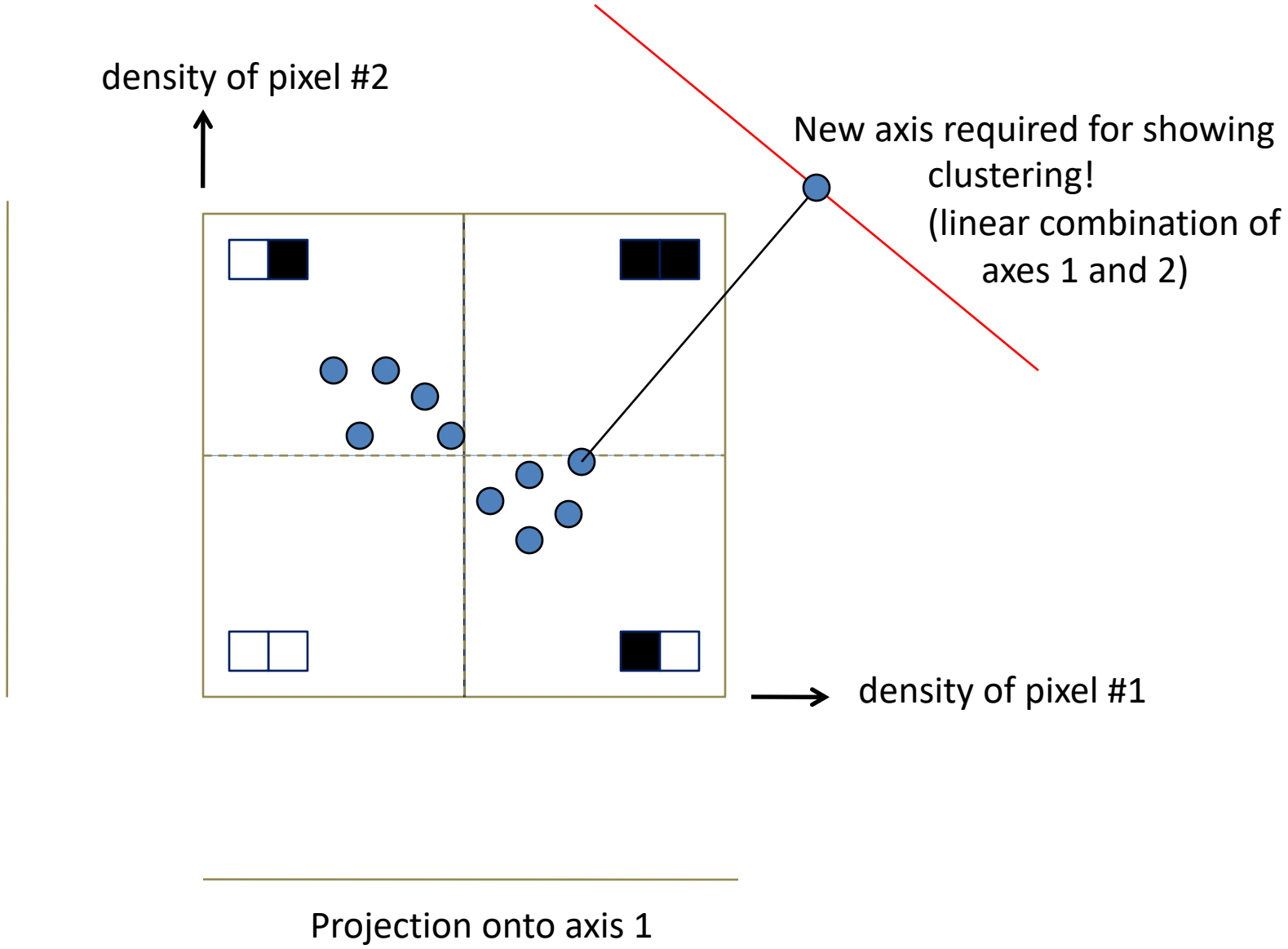


Fortuitous case: direction of grouping happens to coincide with a primary axis.

Projection onto axis 1



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


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

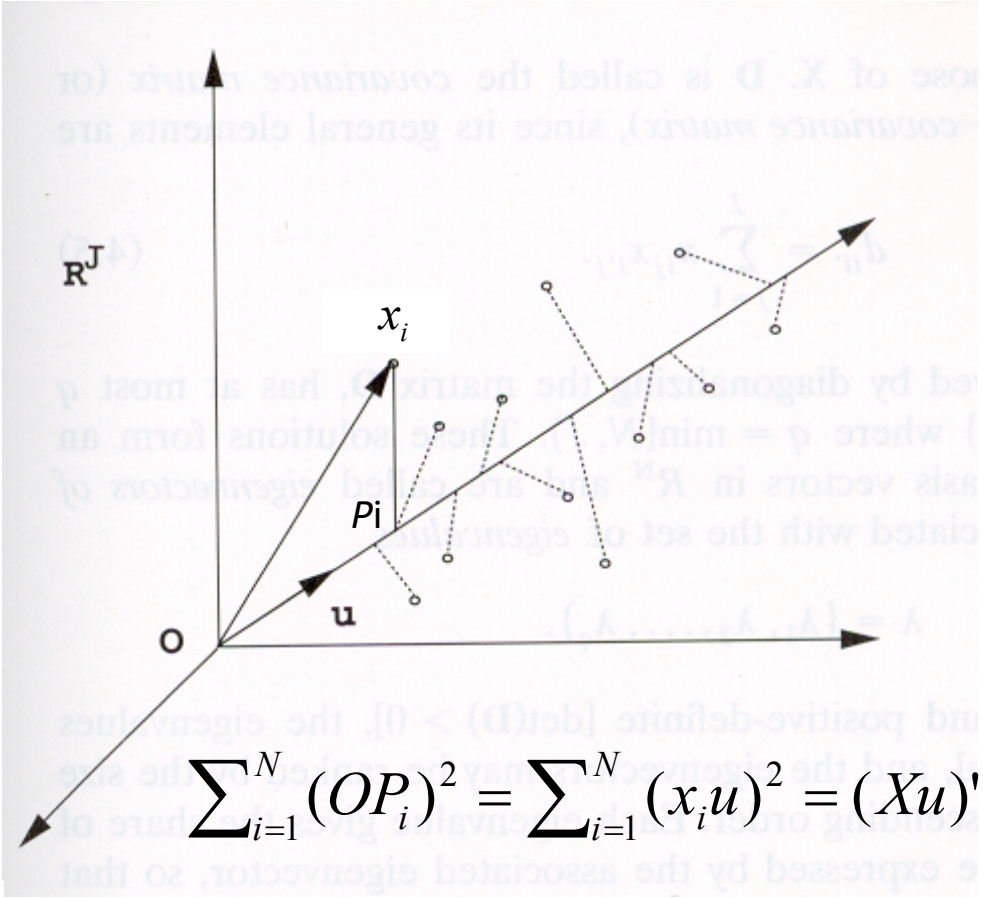
Tools: Classification, and the Role of MDA

- 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

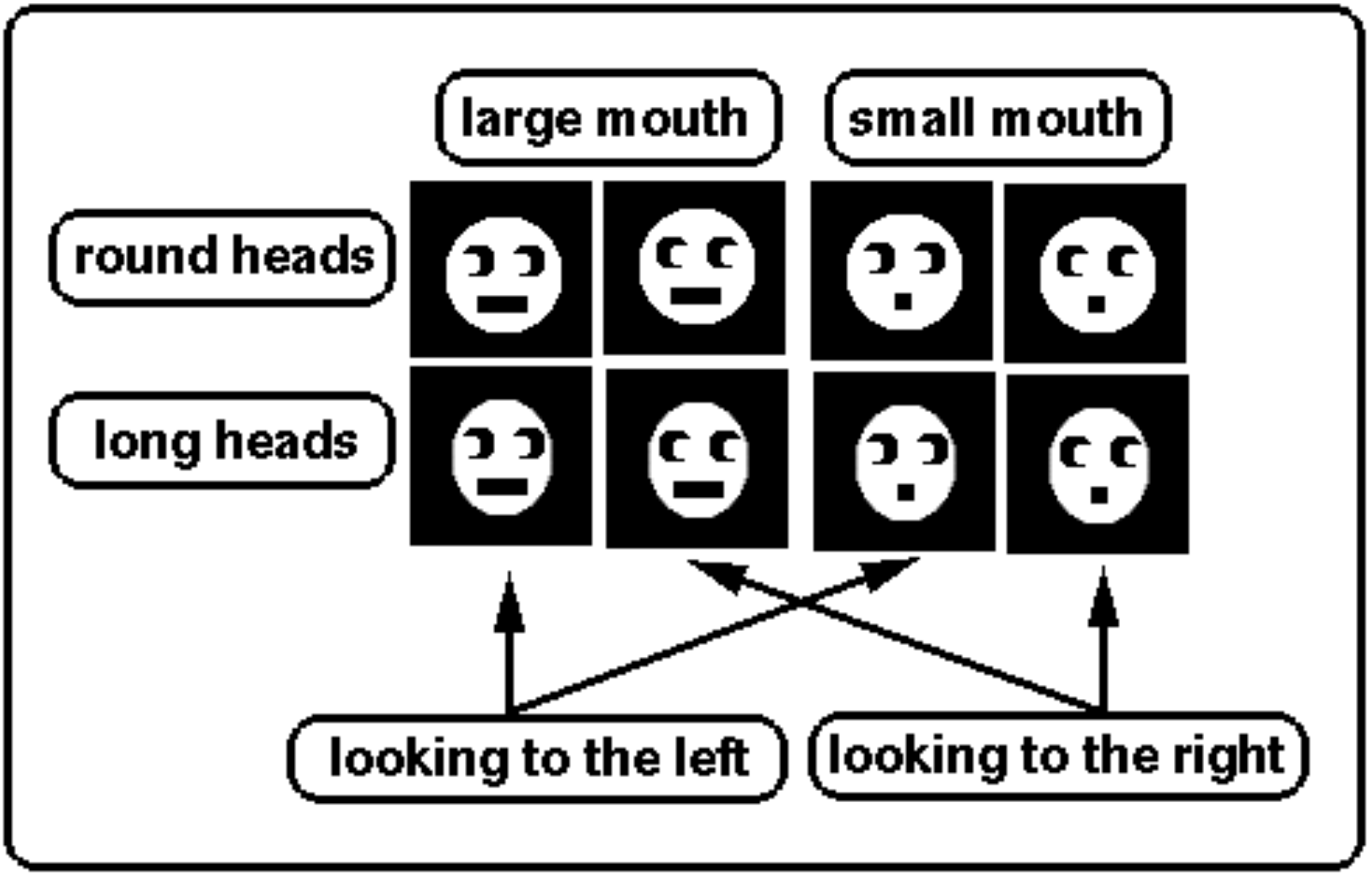


X= matrix containing N image vectors (each with J elements) as rows

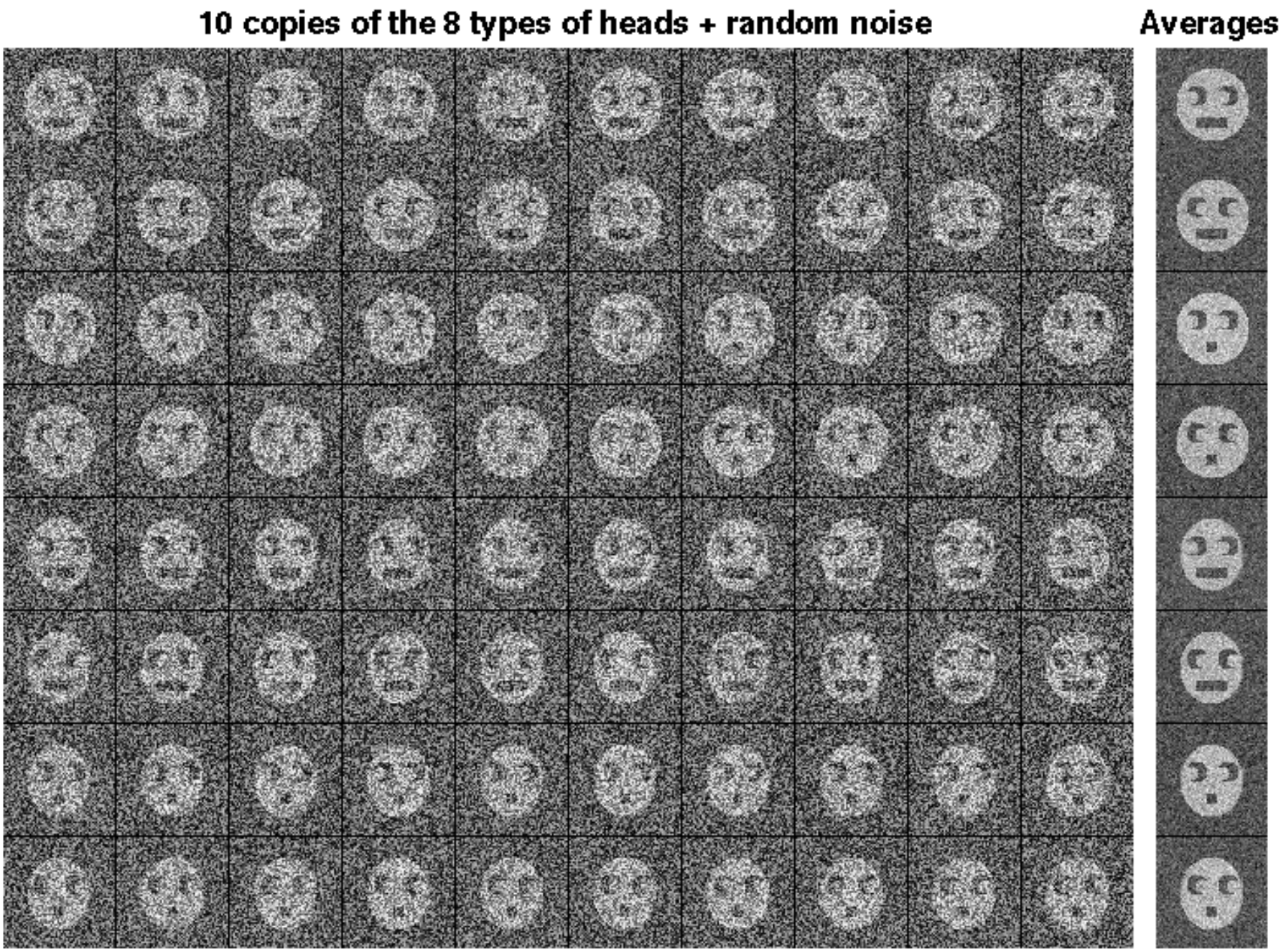
$$\sum_{i=1}^{N}(OP_i)^2 = \sum_{i=1}^{N}(x_i u)^2 = (Xu)'X'u = u'X'Xu \xrightarrow{!} \max$$

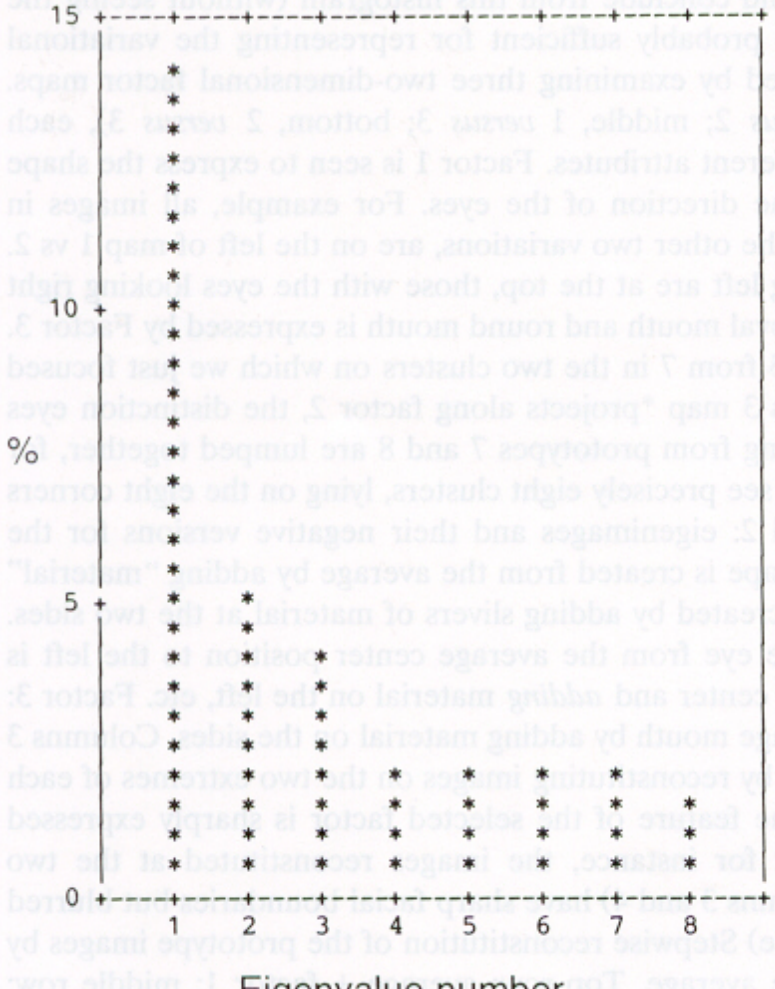
p. 151 [note error in book figure!]

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



Brétaudière JP and Frank J (1986) *J. Microsc.* **144**, 1-14


10 copies of the 8 types of heads + random noise
Averages



eigenvalue histogram

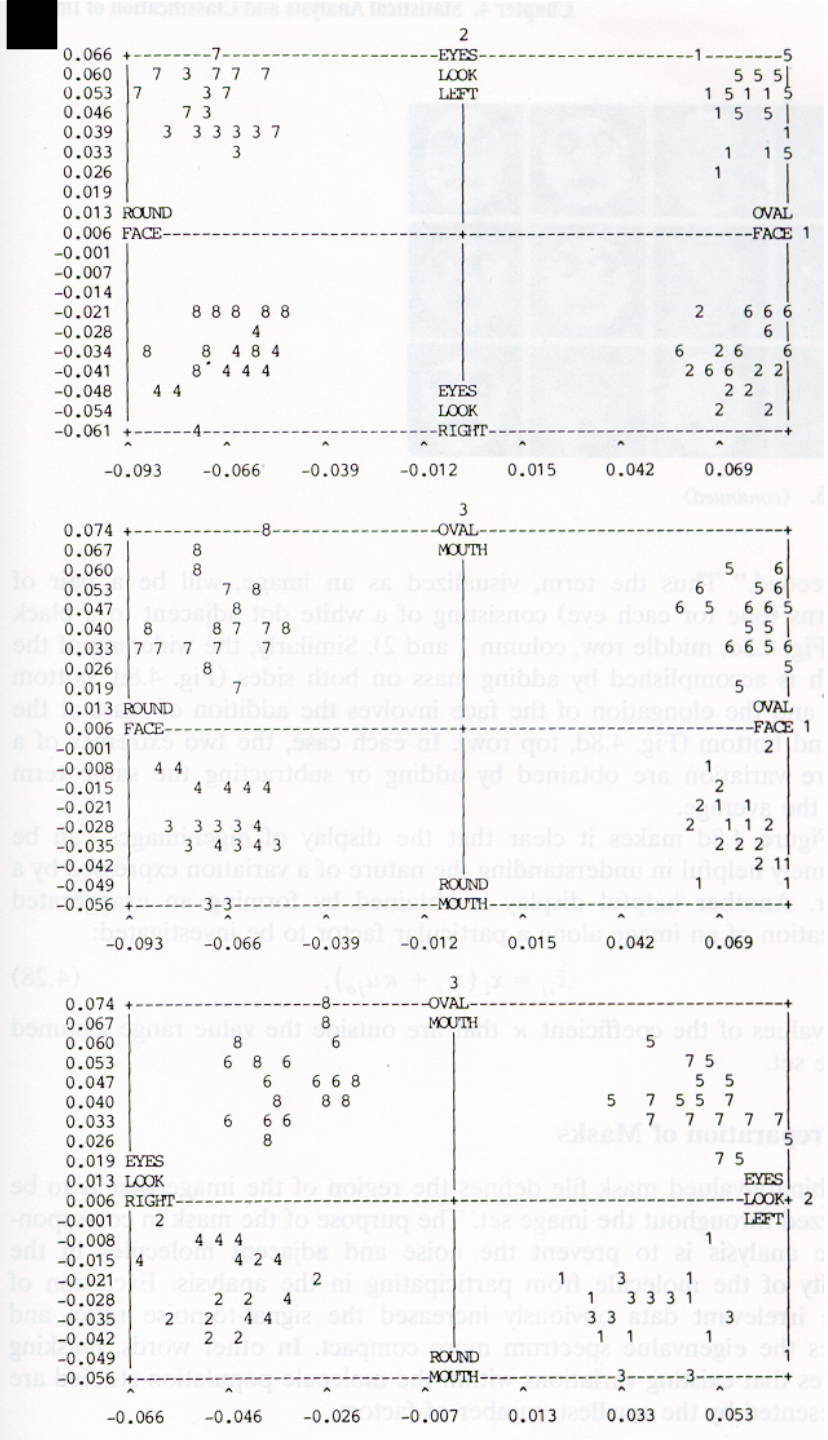
3 stand out,
i.e., 3 factors are sufficient.!

1 vs 2

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

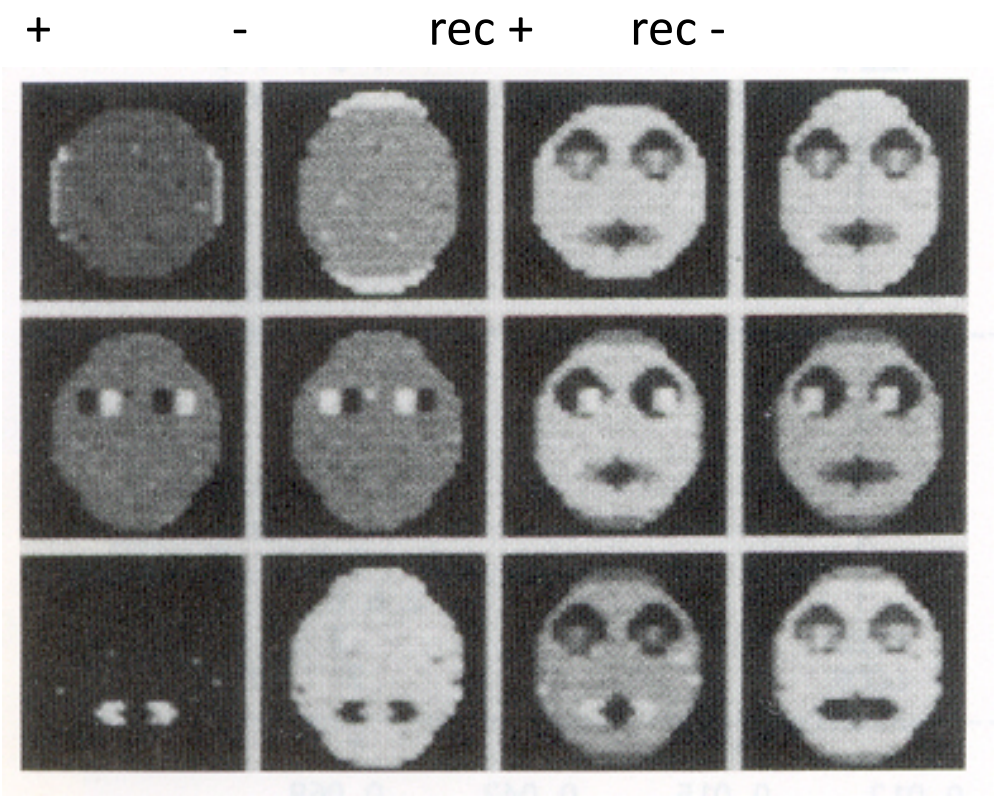
1 vs 3

2 vs 3



MDA: eigenimages

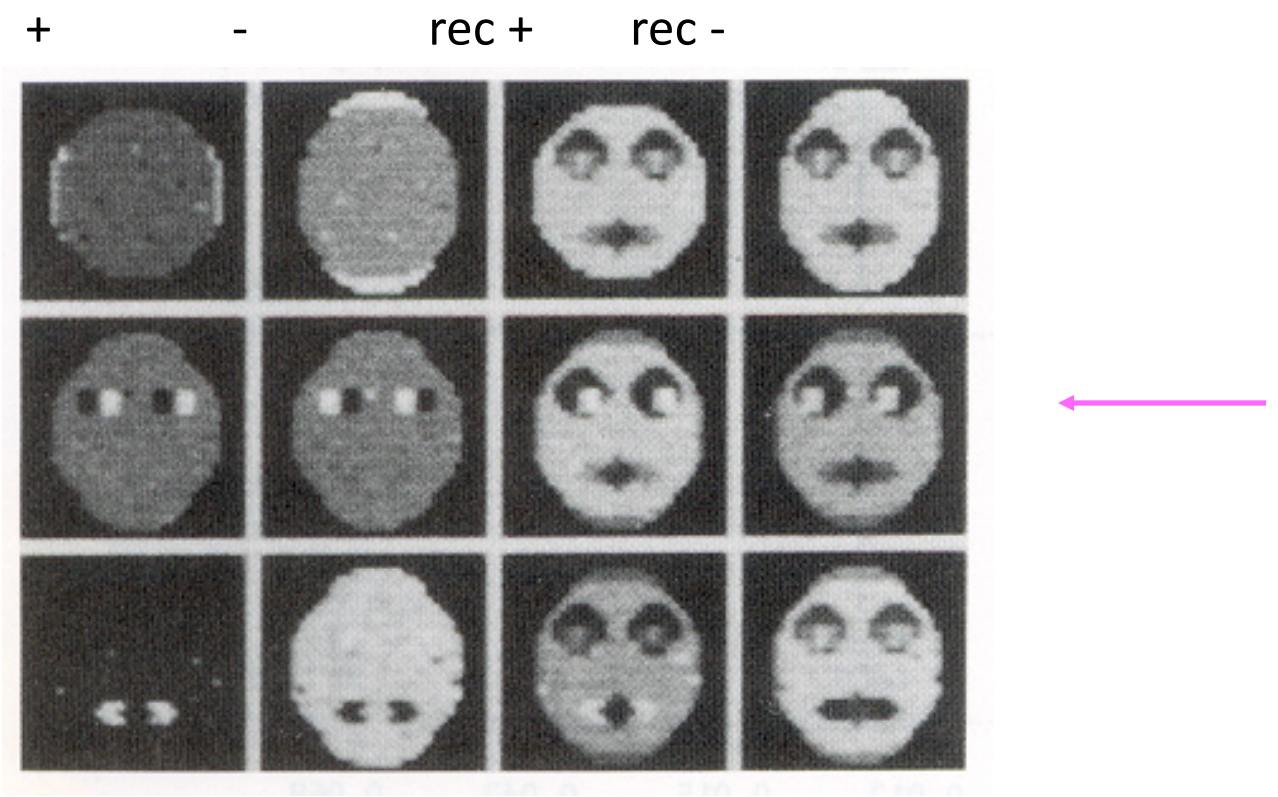
- Factor 1
- Factor 2
- Factor 3



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.

- Factor 1
- Factor 2
- Factor 3



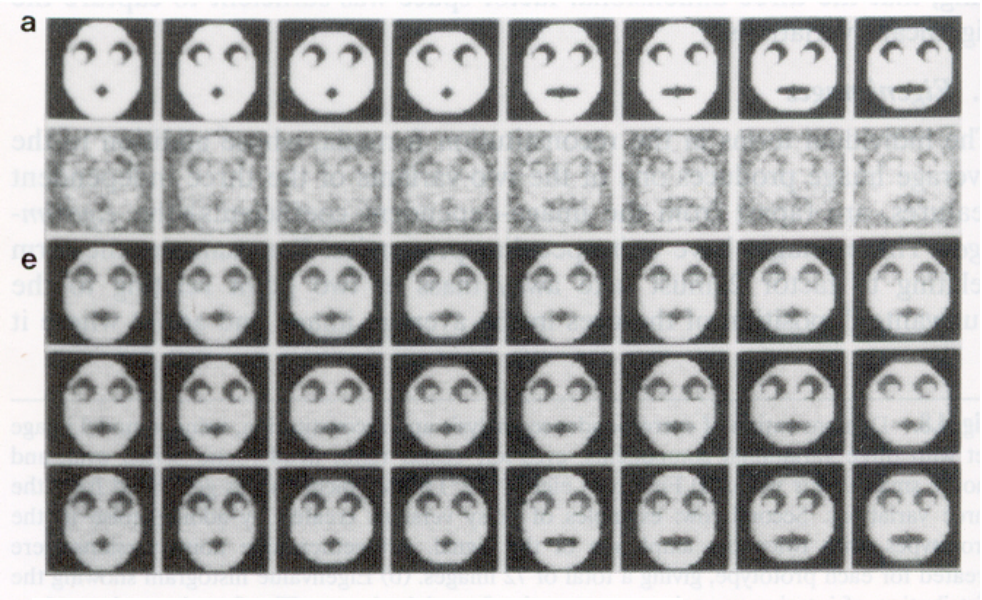
Class Averages

Instances

Total Avrg + F1

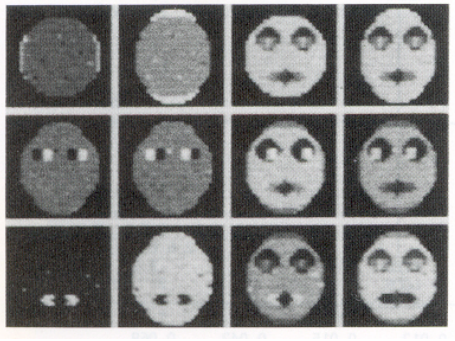
Total Avrg + F1+F2

Total Avrg + F1+F2+F3

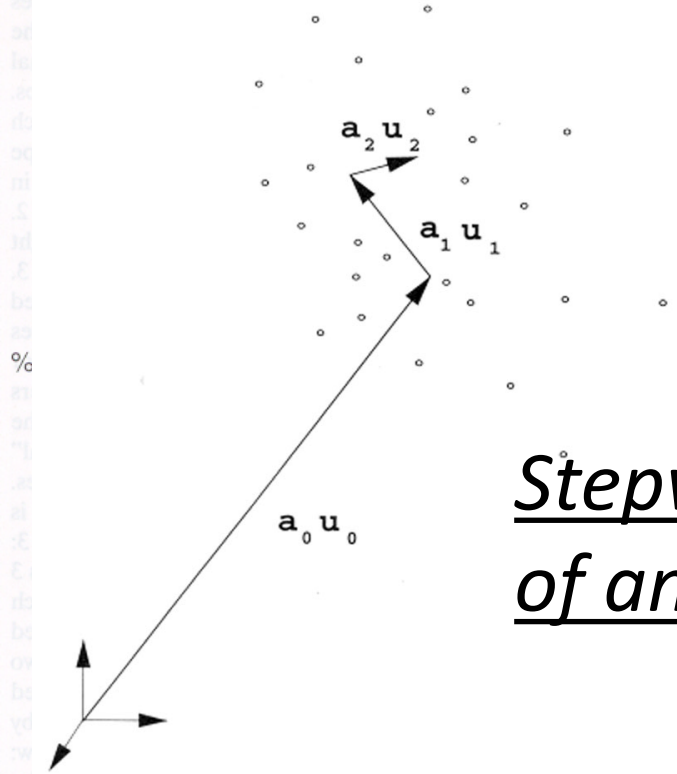


p. 176

+ -



p. 175



Stepwise reconstitution of an image

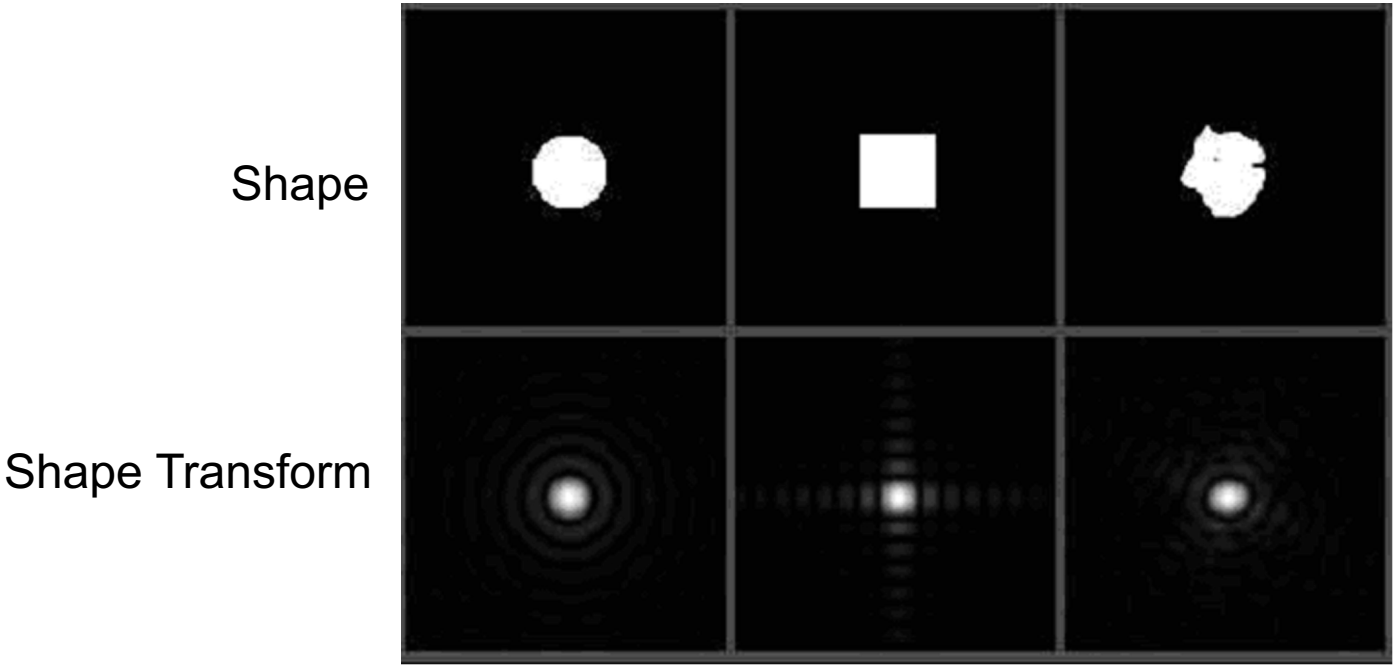
p. 159

3D reconstruction -- preliminaries

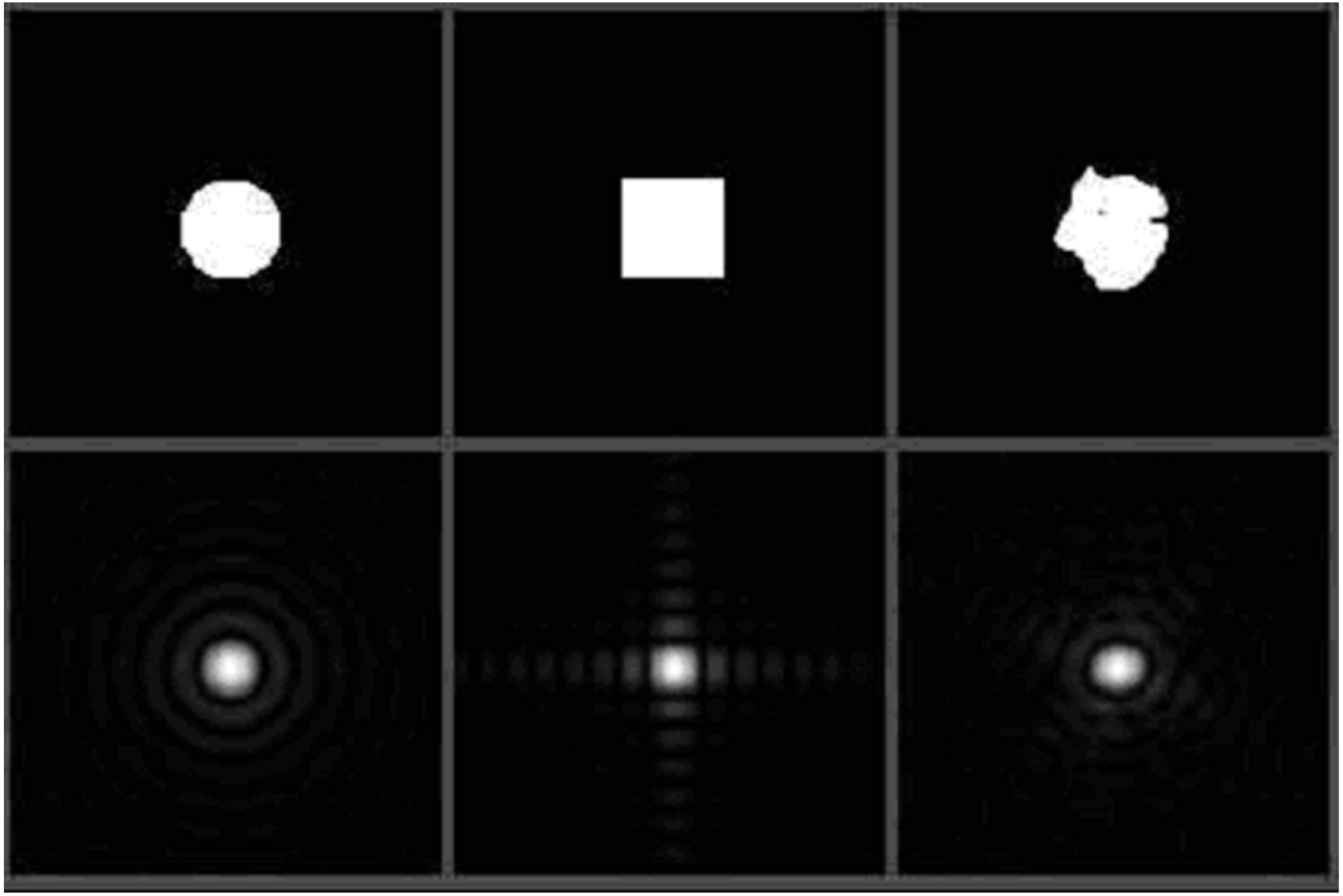
- Under what conditions are projections of an object similar to one another?
- Similarity ←→ closeness in high-dim E-space
  ←→ belonging to the same cluster
  ←→ high correlation

Shape Transform

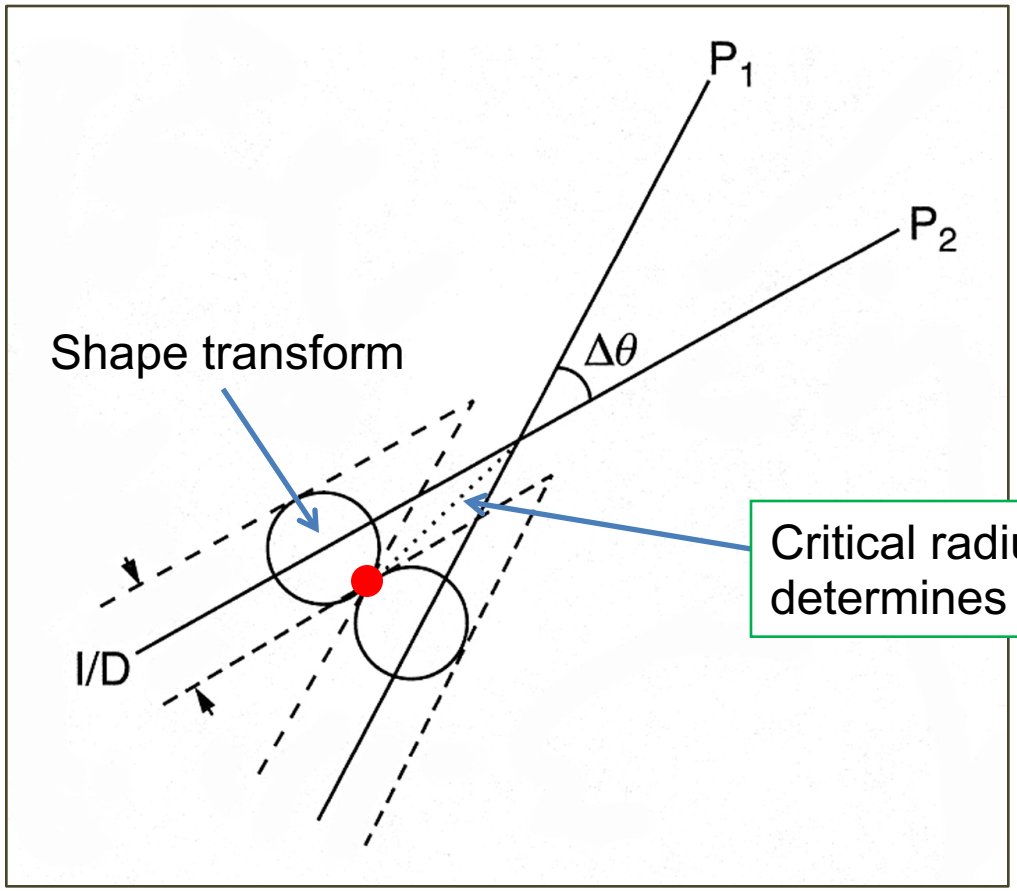
- The Shape Transform is the Fourier transform of a binary mask function (1 inside, 0 outside) whose shape is the shape of an object in 2D or 3D
- It indicates the size and shape of the local region in Fourier space within which Fourier coefficients are correlated/dependent.



Shape Transforms

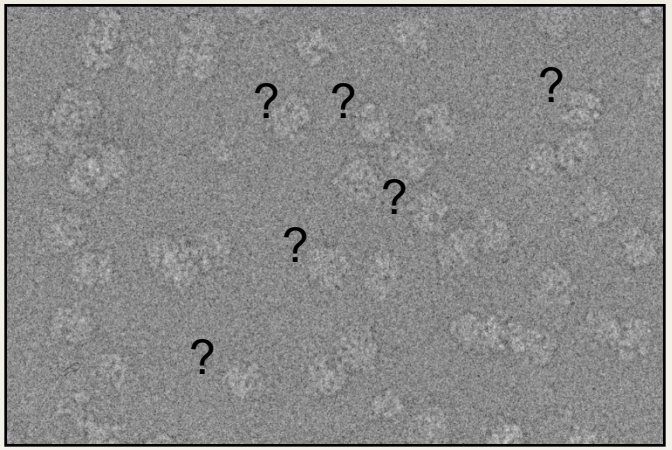


Similarity of projections, condition for 3D reconstruction, and "kissing" shape transforms



P1, P2 central sections in Fourier space.
Δθ angle subtended by P1, P2
D particle diameter

Determination of Particle Orientations

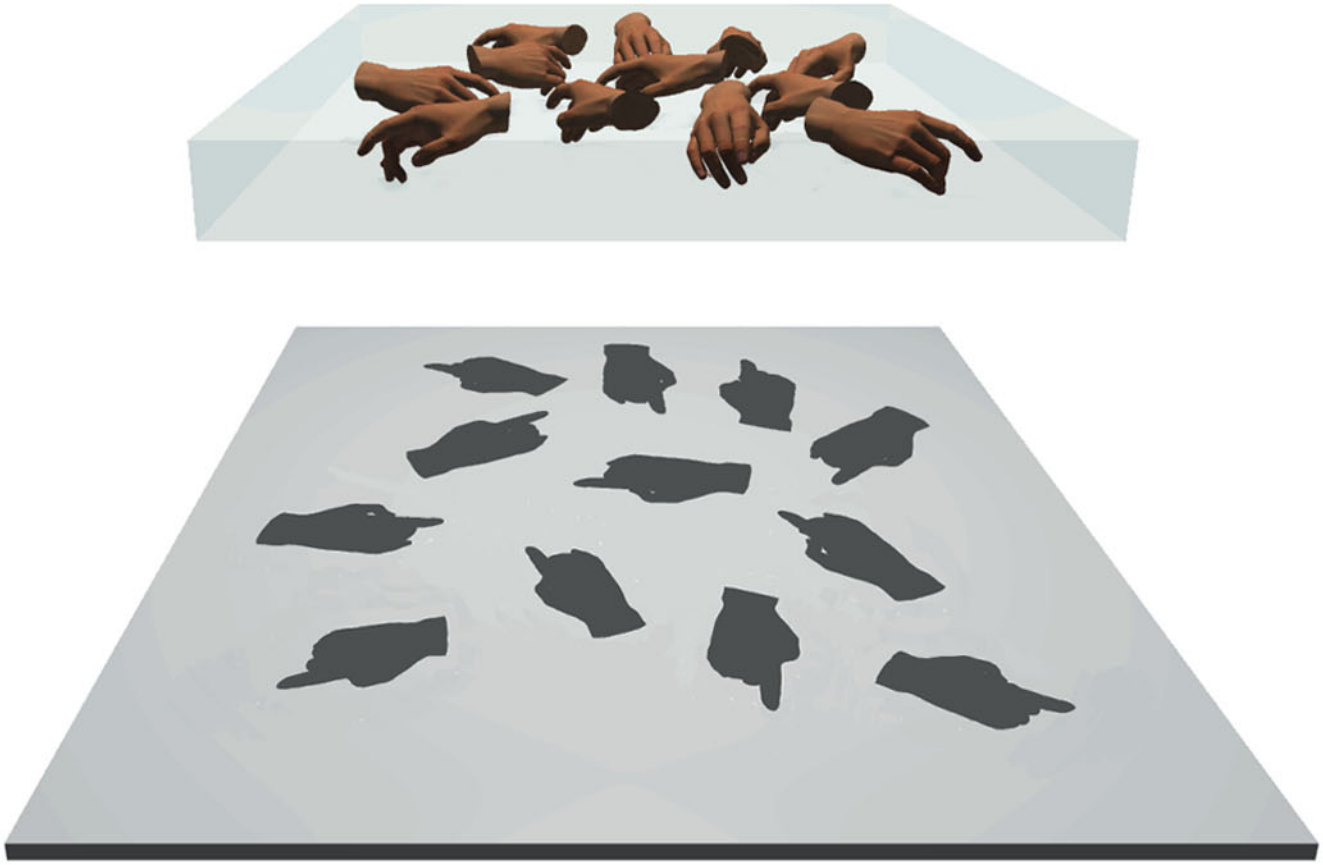


(A) unknown structure -- bootstrap
- (1) Random-conical (uses unsupervised classification)
- (2) Common lines/ angular reconstitution (uses unsupervised classification)

(B) known structure – low-res map available
- (1) reference-based (3D projection matching = a form of supervised classification)
- (2) common lines/ angular reconstitution

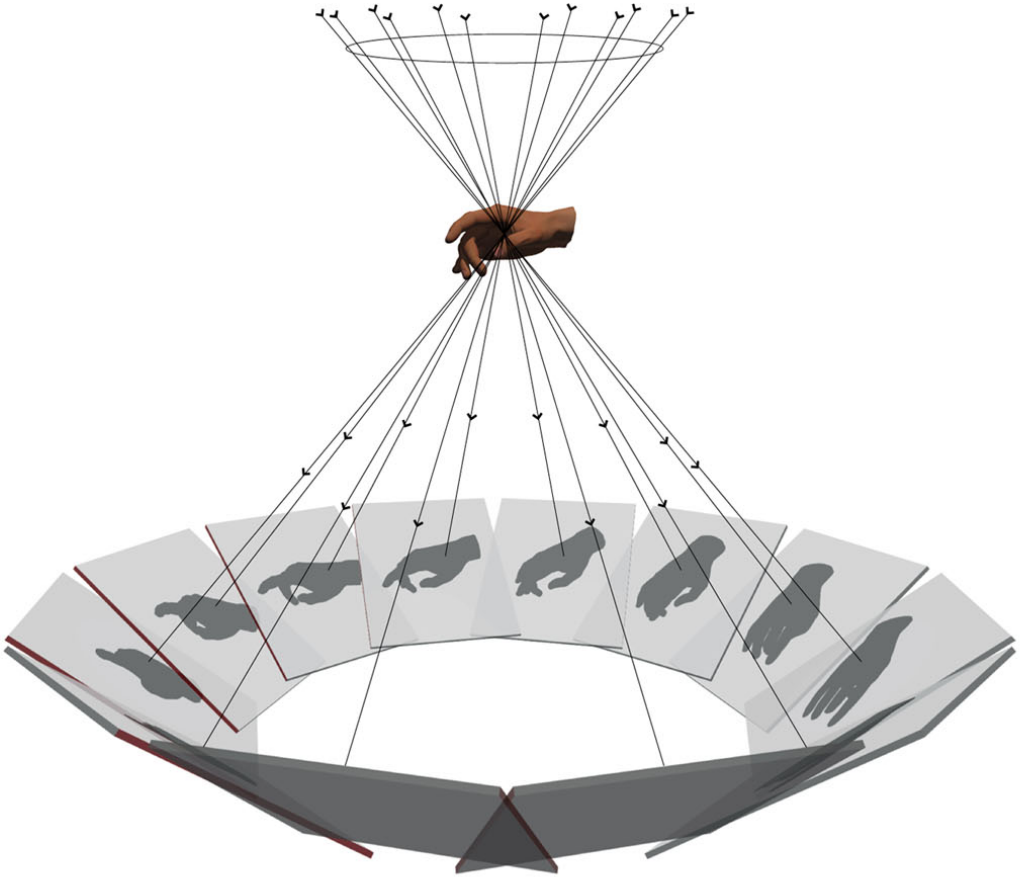
RANDOM CONICAL RECONSTRUCTION

0-degree view

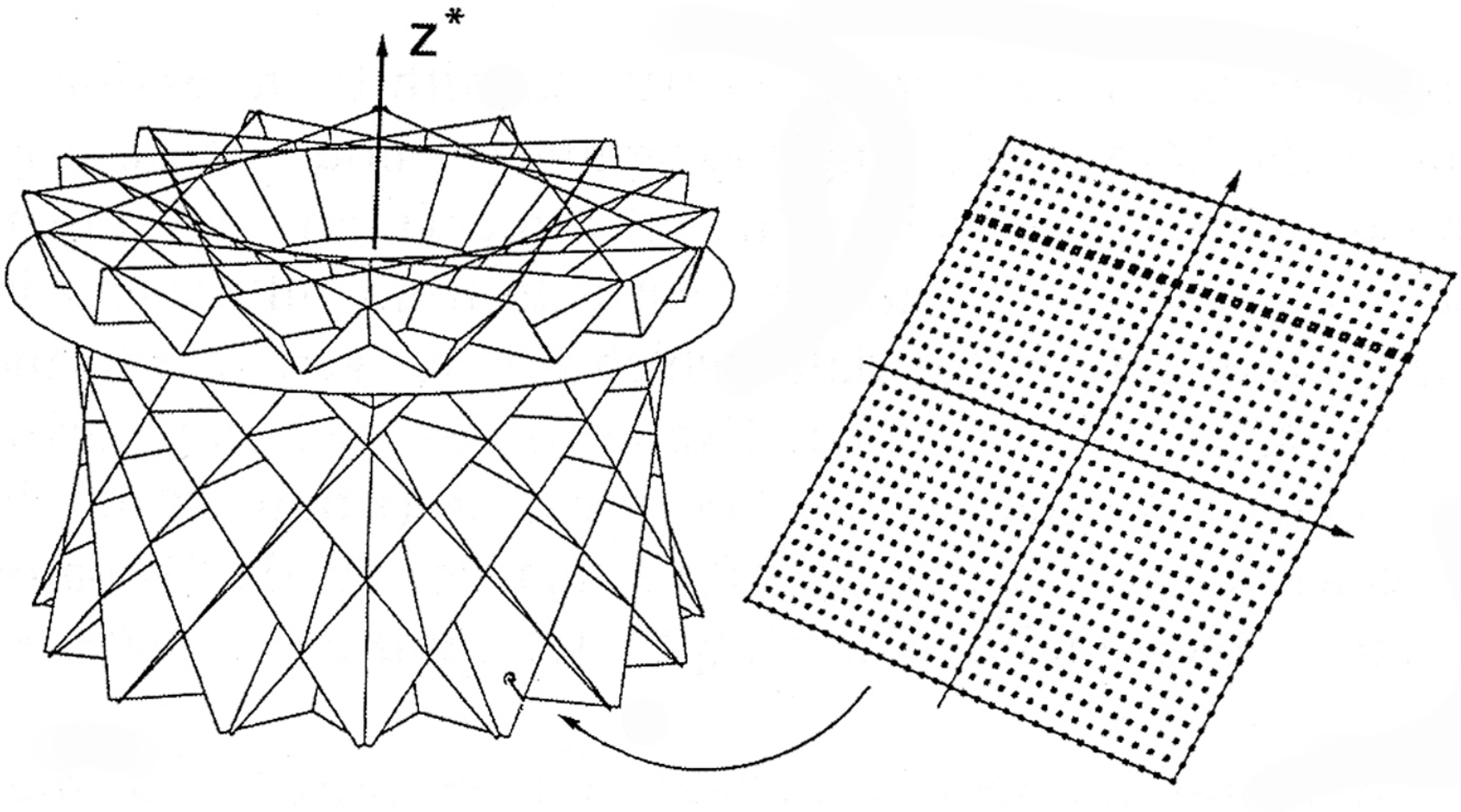


50-degree view

Equivalent geometry
in the coo system of the particle

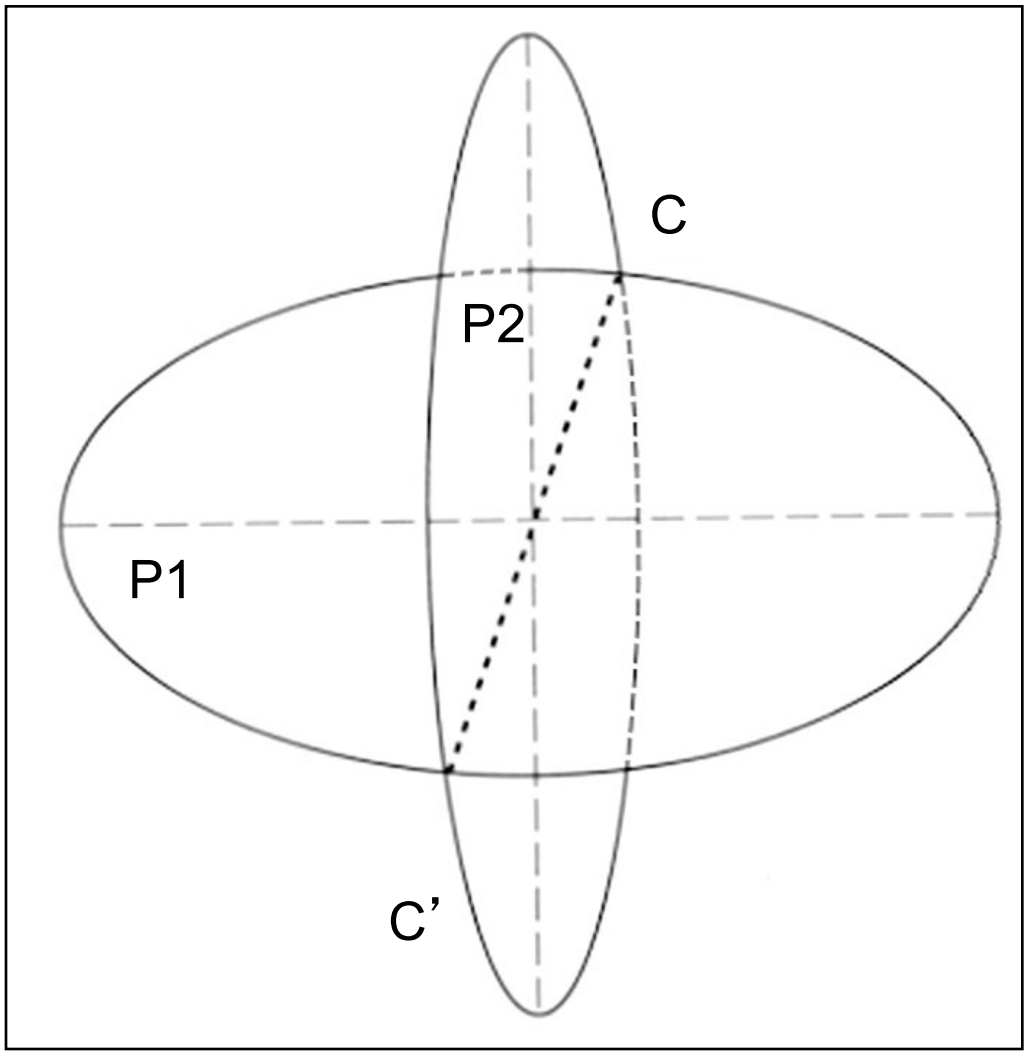


Conical Data Collection Geometry in Fourier Space



Lanzavecchia et al.

COMMON LINES APPROACH TO DETERMINING ORIENTA



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

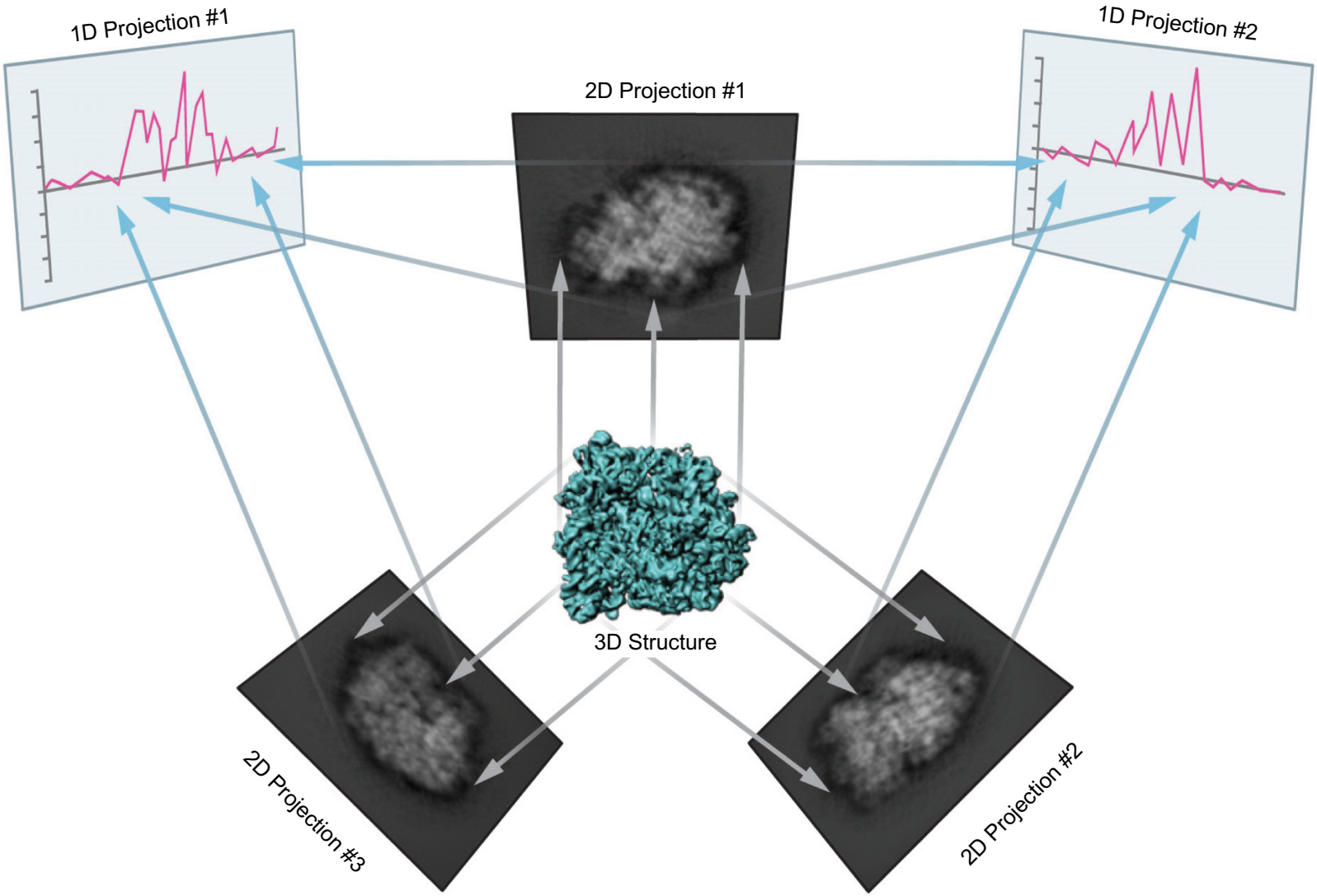
Two 2D projections of the same 3D object have in common:

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

in real space: 1D projections in particular directions

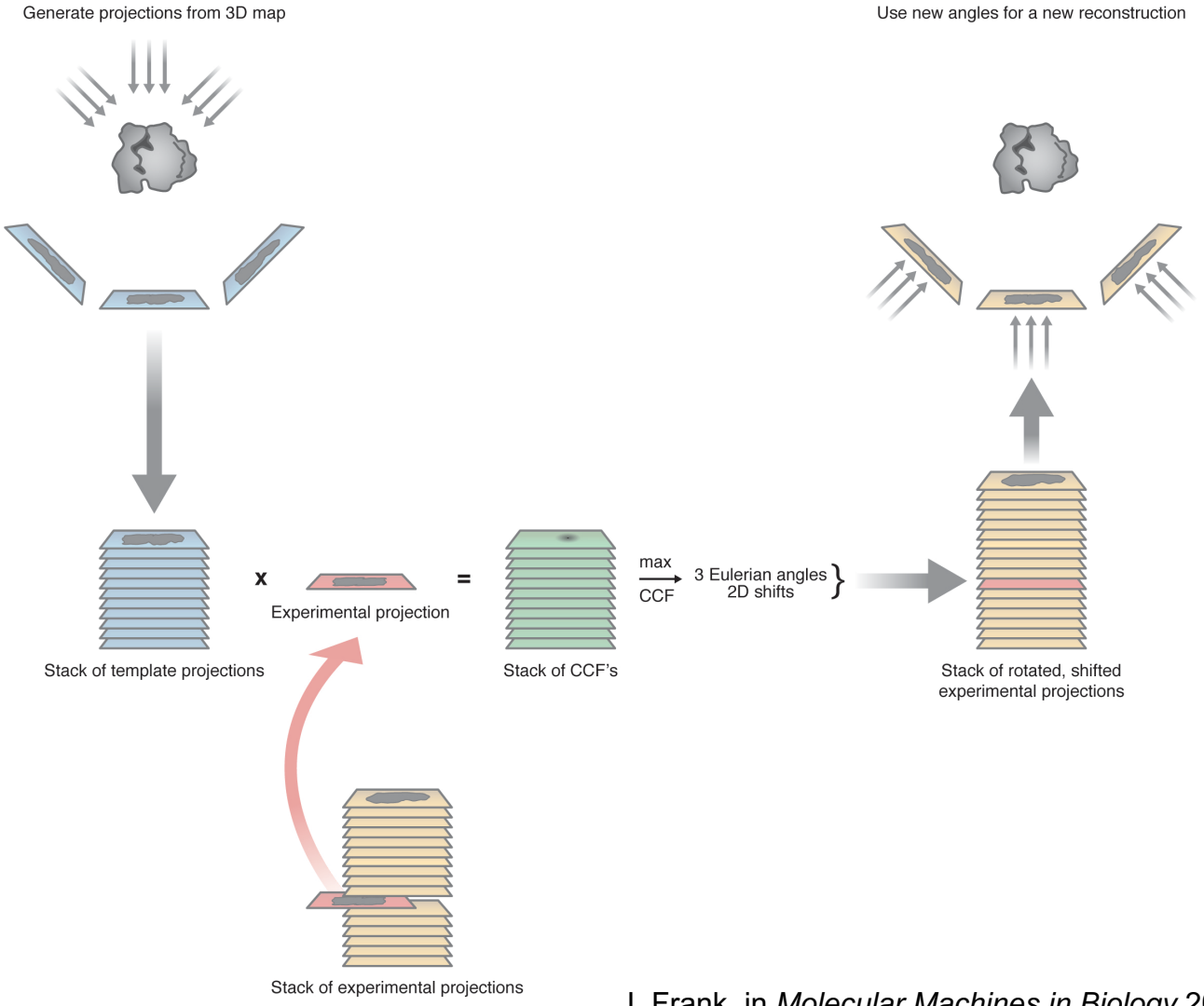
The Sinogram (or Radon transform) of an image is an exhaustive ordered display of all of its1-D projections

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



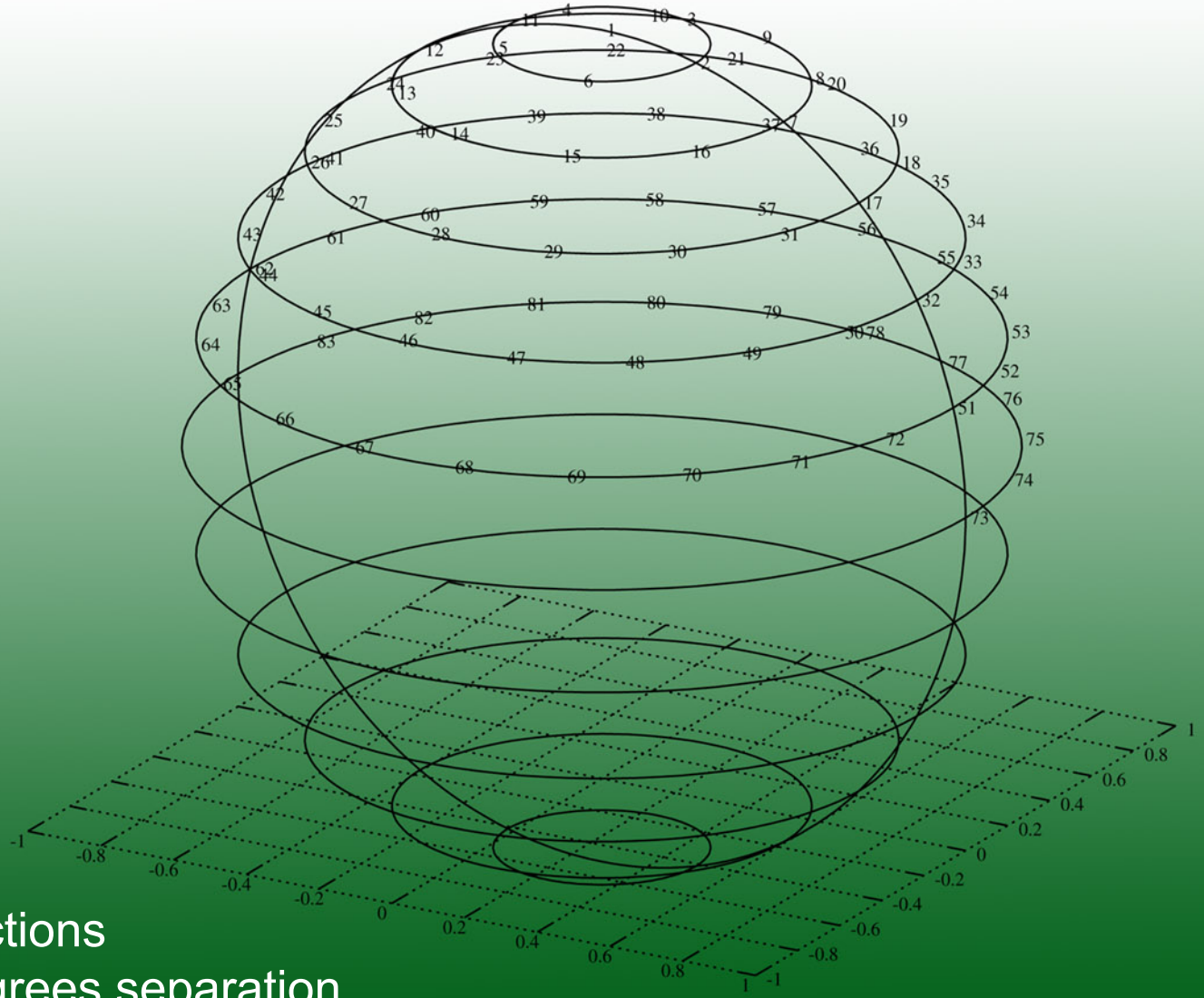
J. Frank, in *Molecular Machines in Biology* 2011

Determination of orientation by projection matching



J. Frank, in *Molecular Machines in Biology* 2011

Initial Angular Grid

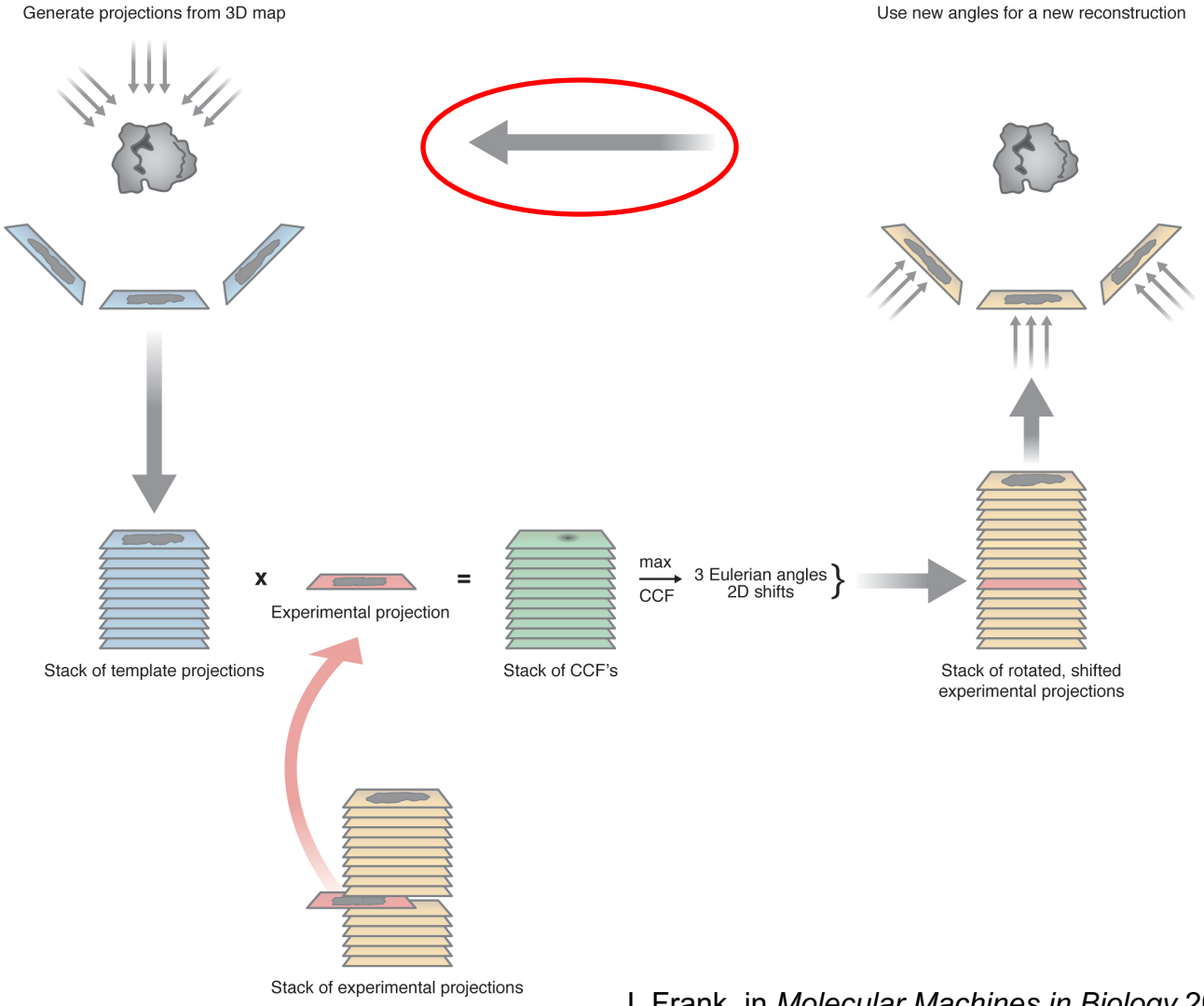


83 directions
~15 degrees separation

83-projection grid

averages of particles classified

Iterative Angular Refinement



J. Frank, in *Molecular Machines in Biology* 2011

Angular Refinement

Given an initial 3D reference,

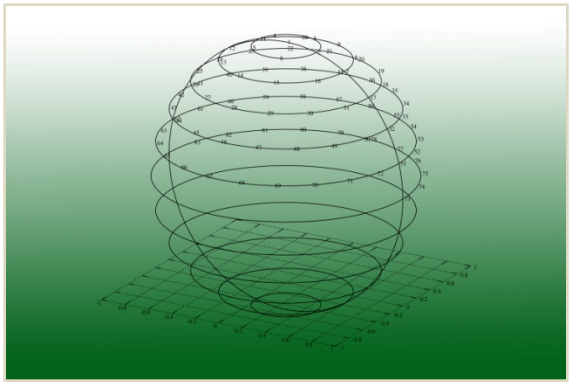
Iterate the steps {3D projection matching + reconstruction}
Decrease angular grid size as you go on (range: 15° ➔ 0.5°)

Convergence criteria:
(1) convergence of particle angles
(2) convergence of resolution (monitor progress with FSC)

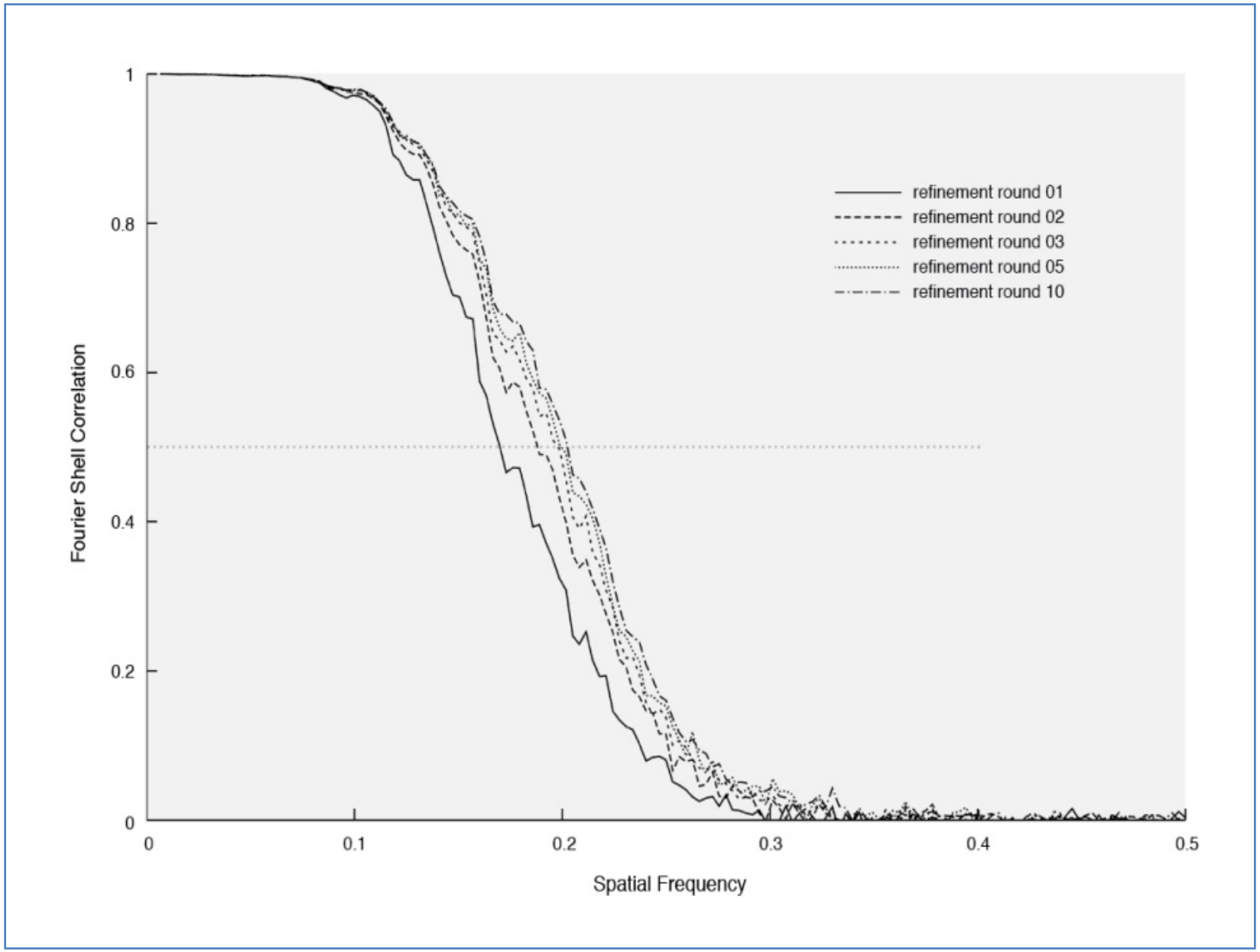
“Rule of neighborhood” saves computing time

- 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



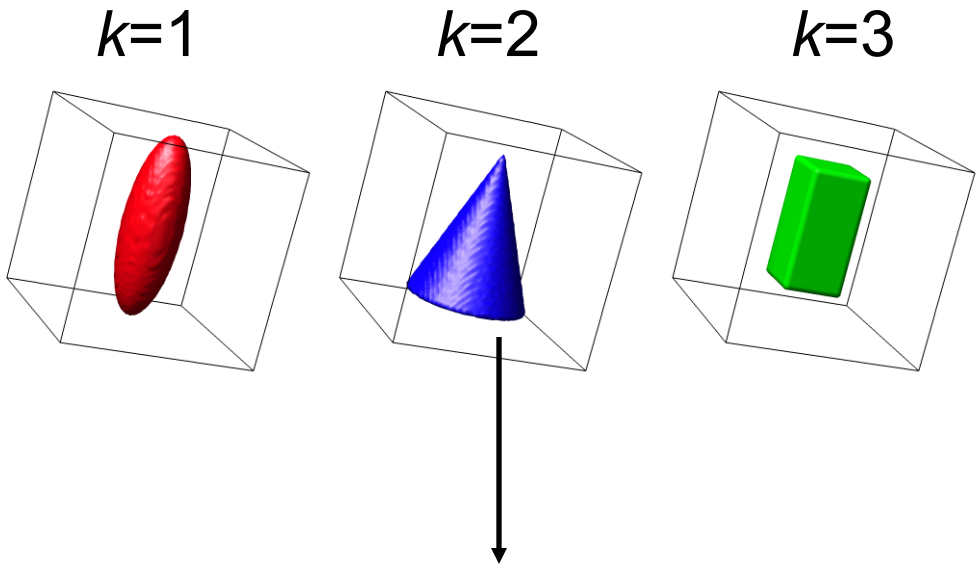
Increasingly finer angular increments



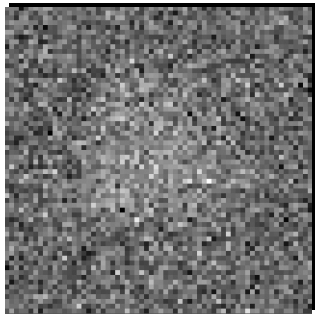
FSC following progress of refinement

3D Unsupervised Classification

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

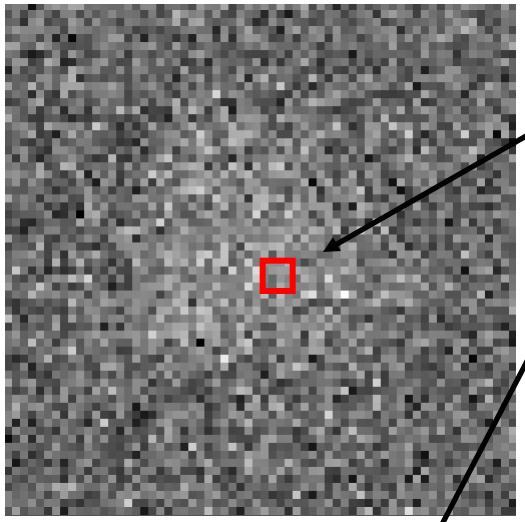


with addition of
white Gaussian noise

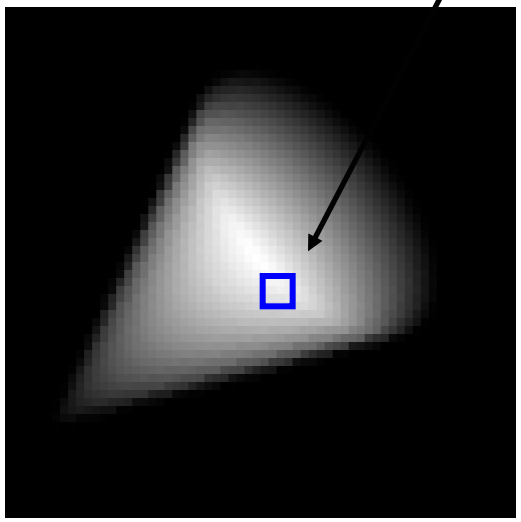


Unknowns: class numbers *k*, rotations, translations

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 σ

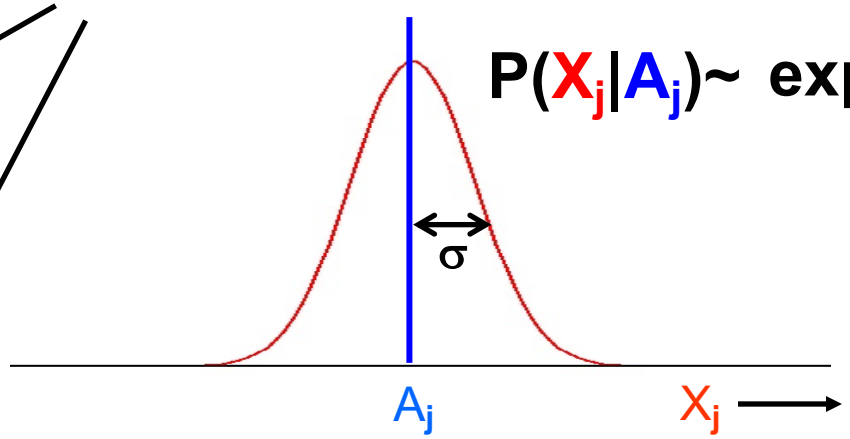


data



model

For each pixel j:



$$P(X_j|A_j) \sim \exp\left(\frac{(X_j - A_j)^2}{-2\sigma^2}\right)$$

white noise =
independence between pixels!

P(data image|model image) ~

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

Likelihood

- Find a model Θ that optimizes the log-likelihood of observing the entire dataset:

class

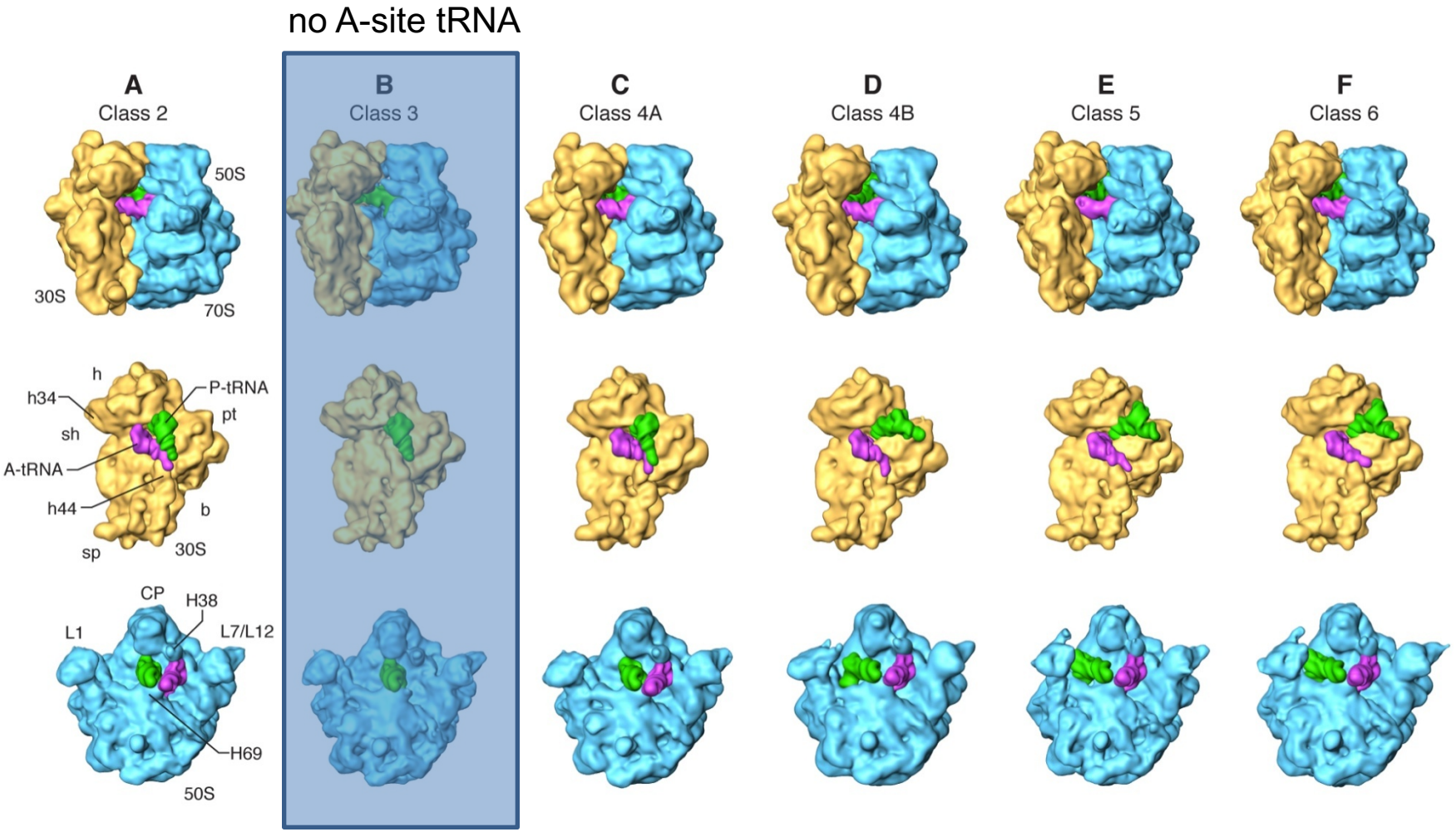
$$L(\Theta) = \sum_{i=1}^{N} \ln \sum_{k=1}^{K} \sum_{rot} \sum_{trans} P(image_i \mid k, rot, trans, \Theta) P(k, rot, trans \mid \Theta)$$

integrate over all unknowns!

The model Θ comprises: estimates for 3D objects, σ, …

Optimization algorithm: Expectation Maximization

ML3D



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

Agirrezabala et al., PNAS 2012

QUESTIONS?

Generalized Euclidean distance

Euclidean distance between two images f_1 and f_2:

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

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

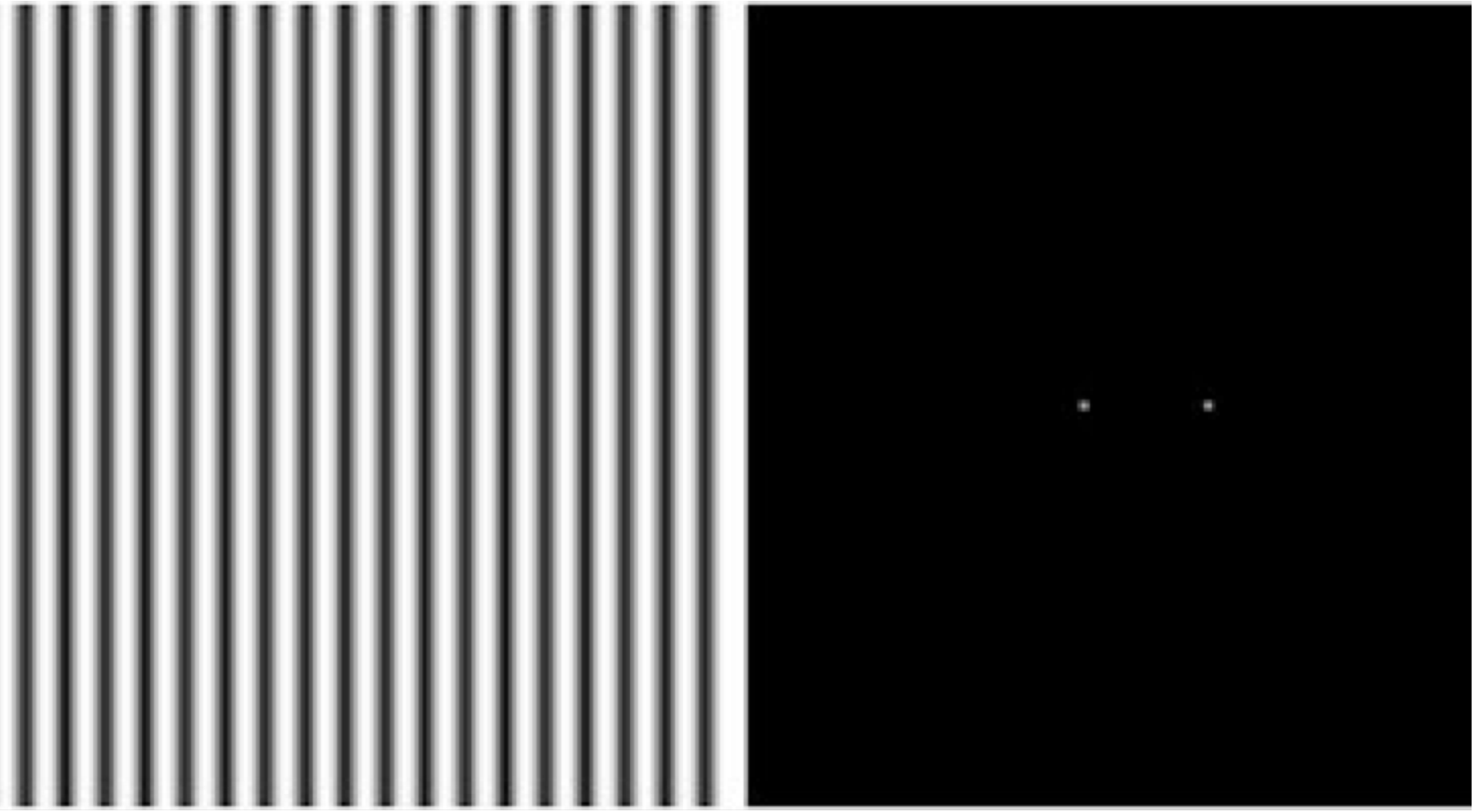
const. const. cross-correlation

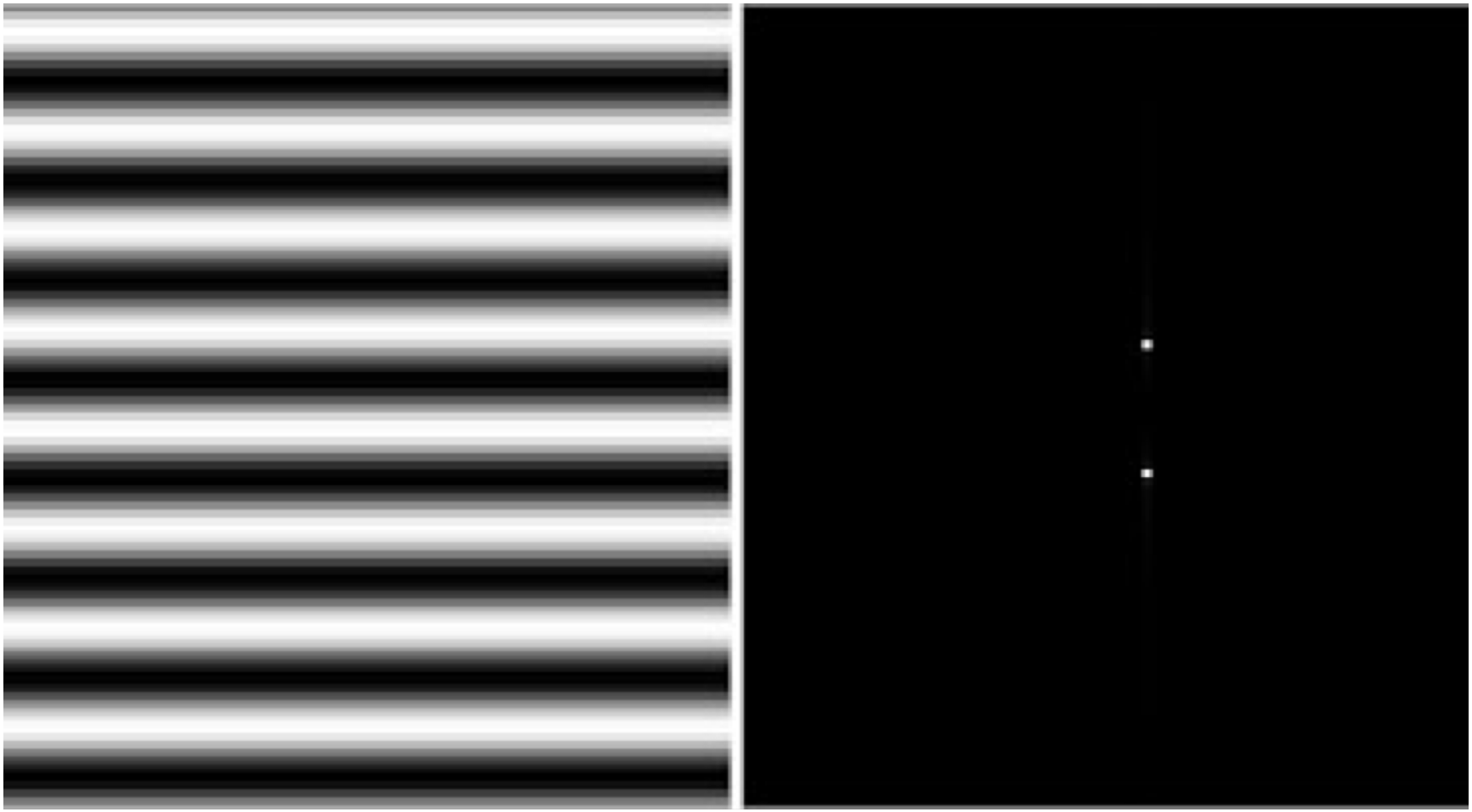
Similarity = closeness in high-dimensional Euclidean space = small E-distance
→ large value of CCF peak at matching position

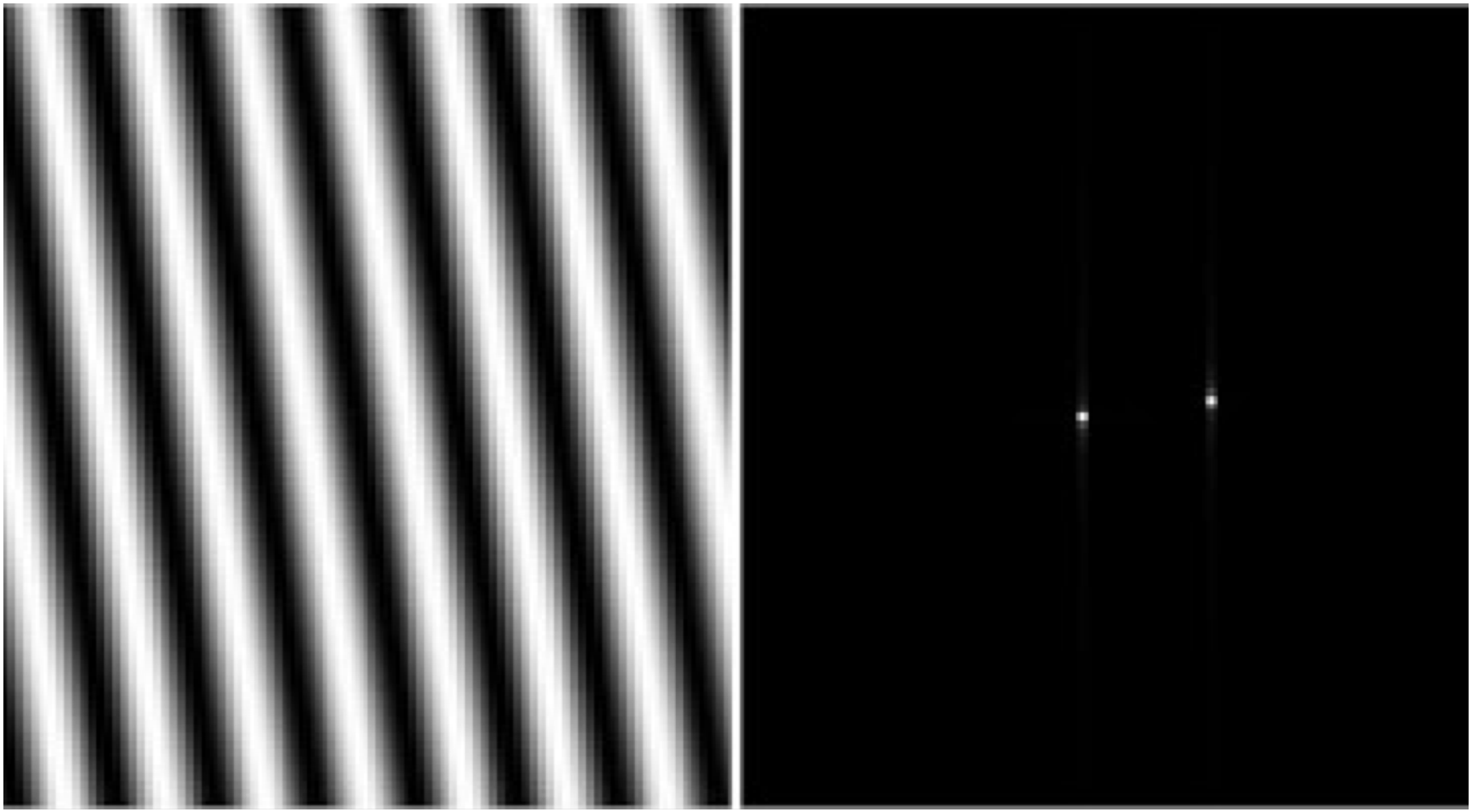
It's easily verified that the Fourier transform of any real-valued image has the following property:

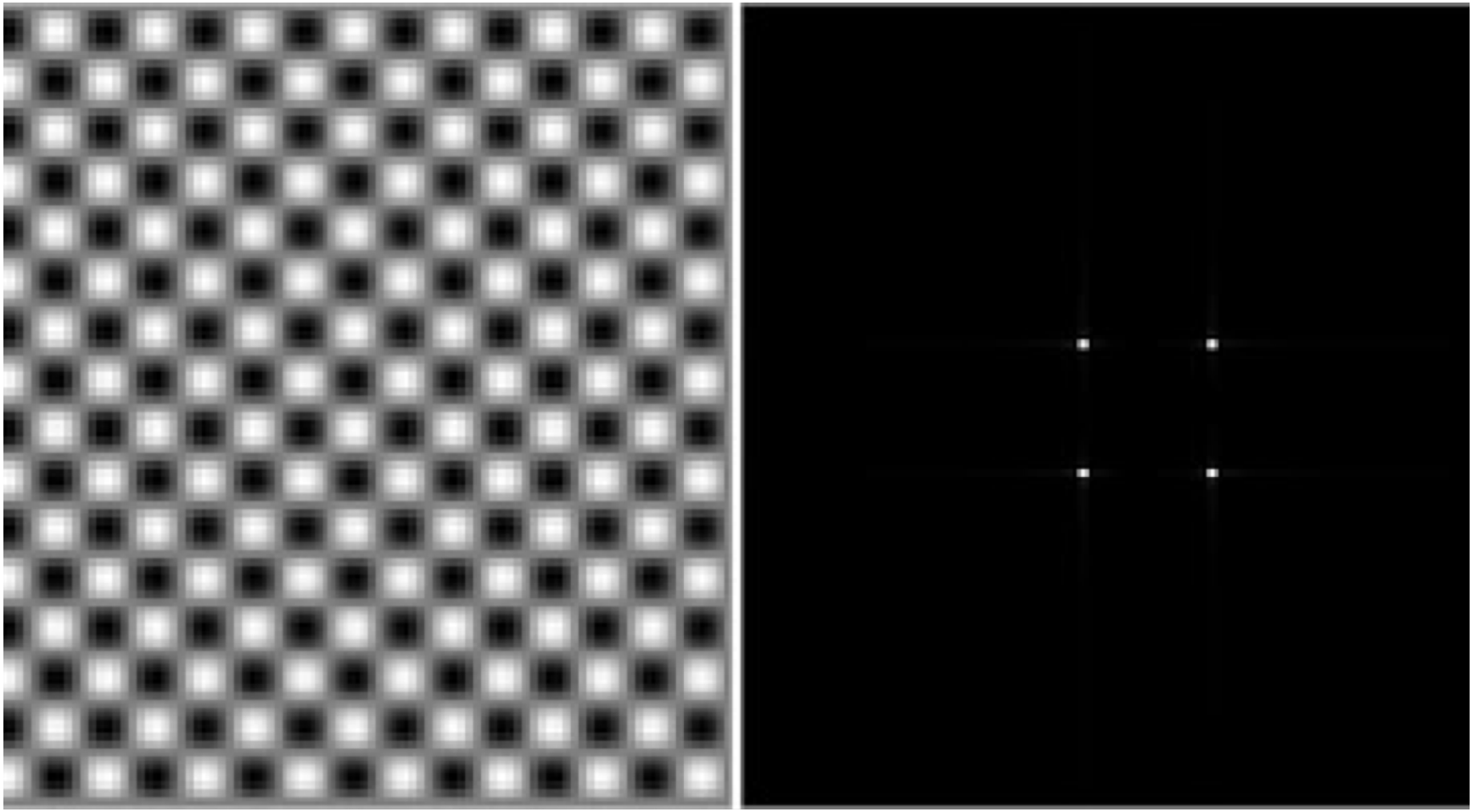
$$F(k_x,k_y) = F^*(-k_x,-k_y) \qquad \text{(Friedel's Law)}$$

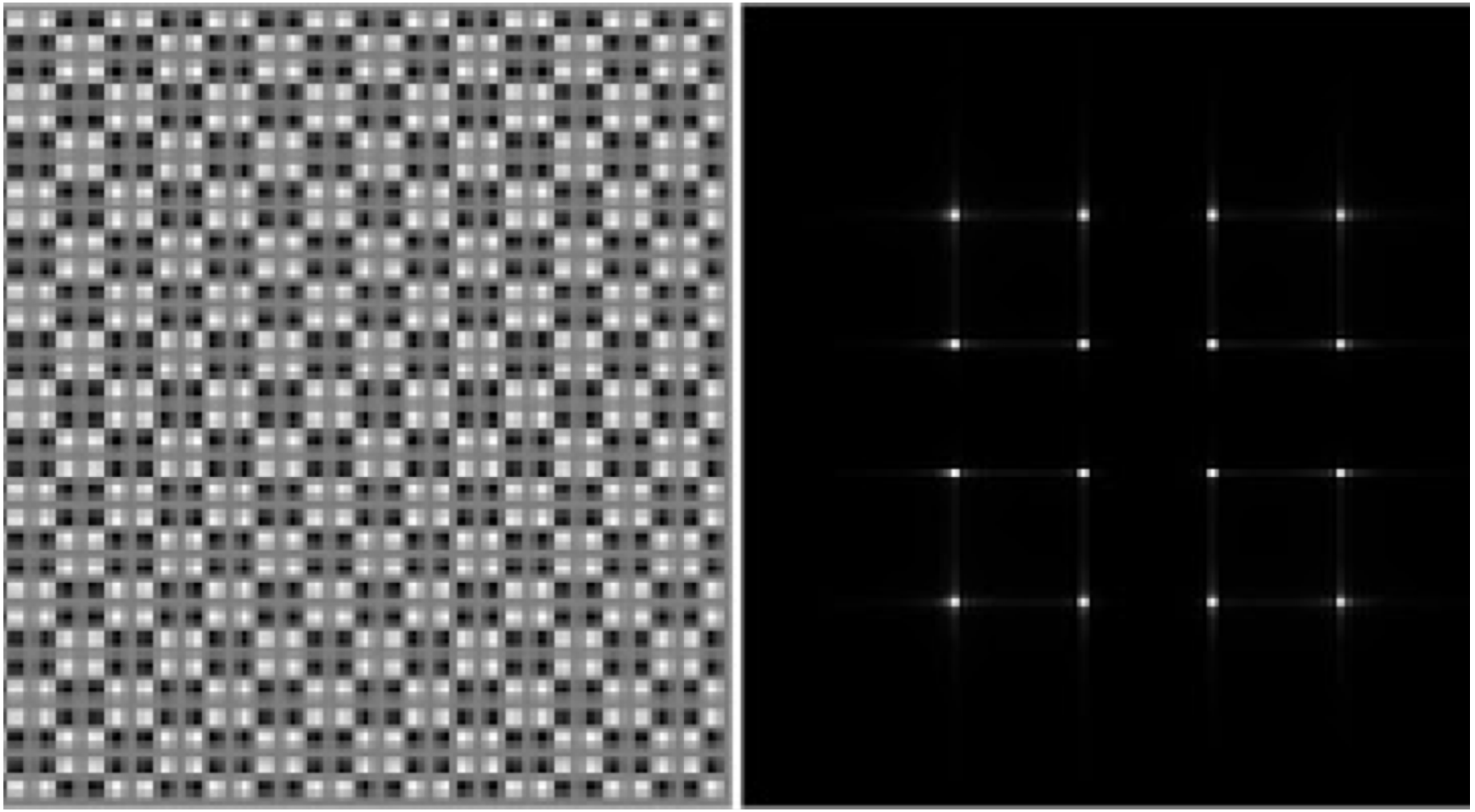
Examples for Fourier transforms of simple functions:



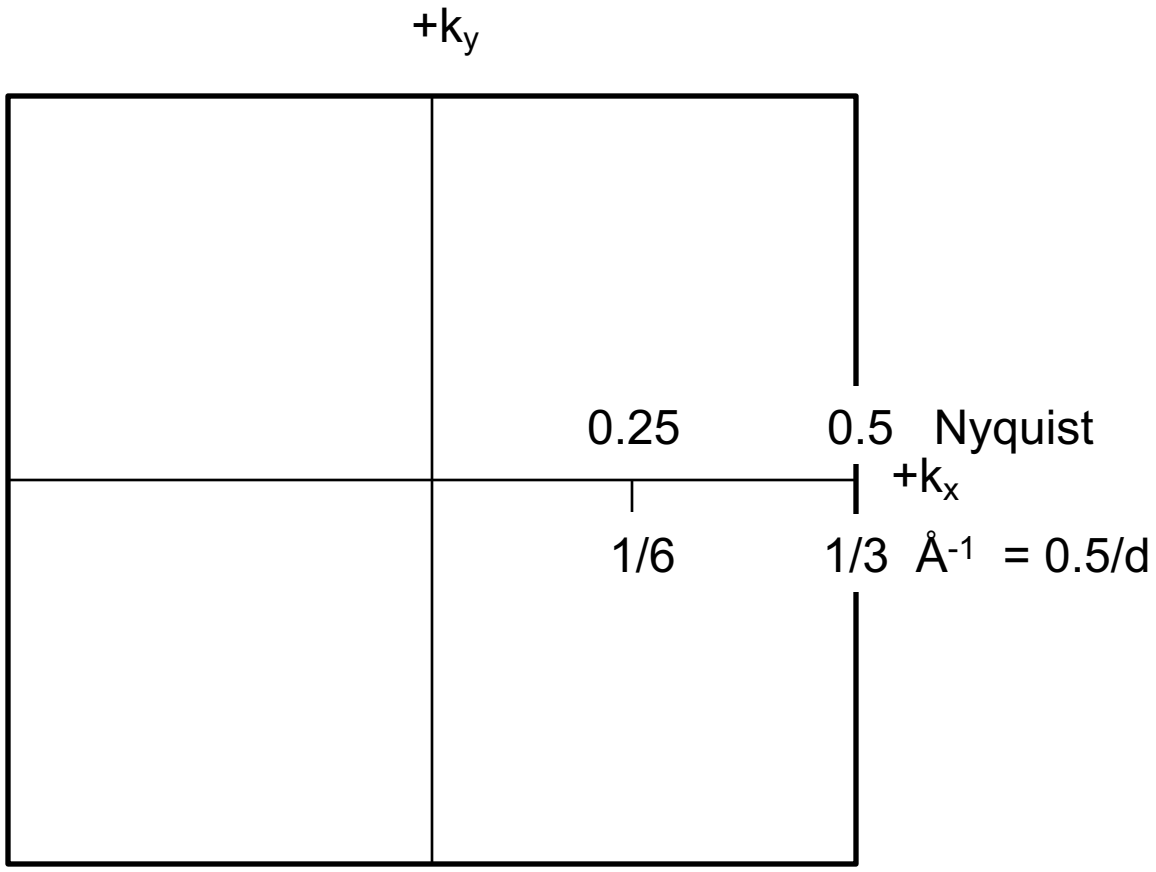




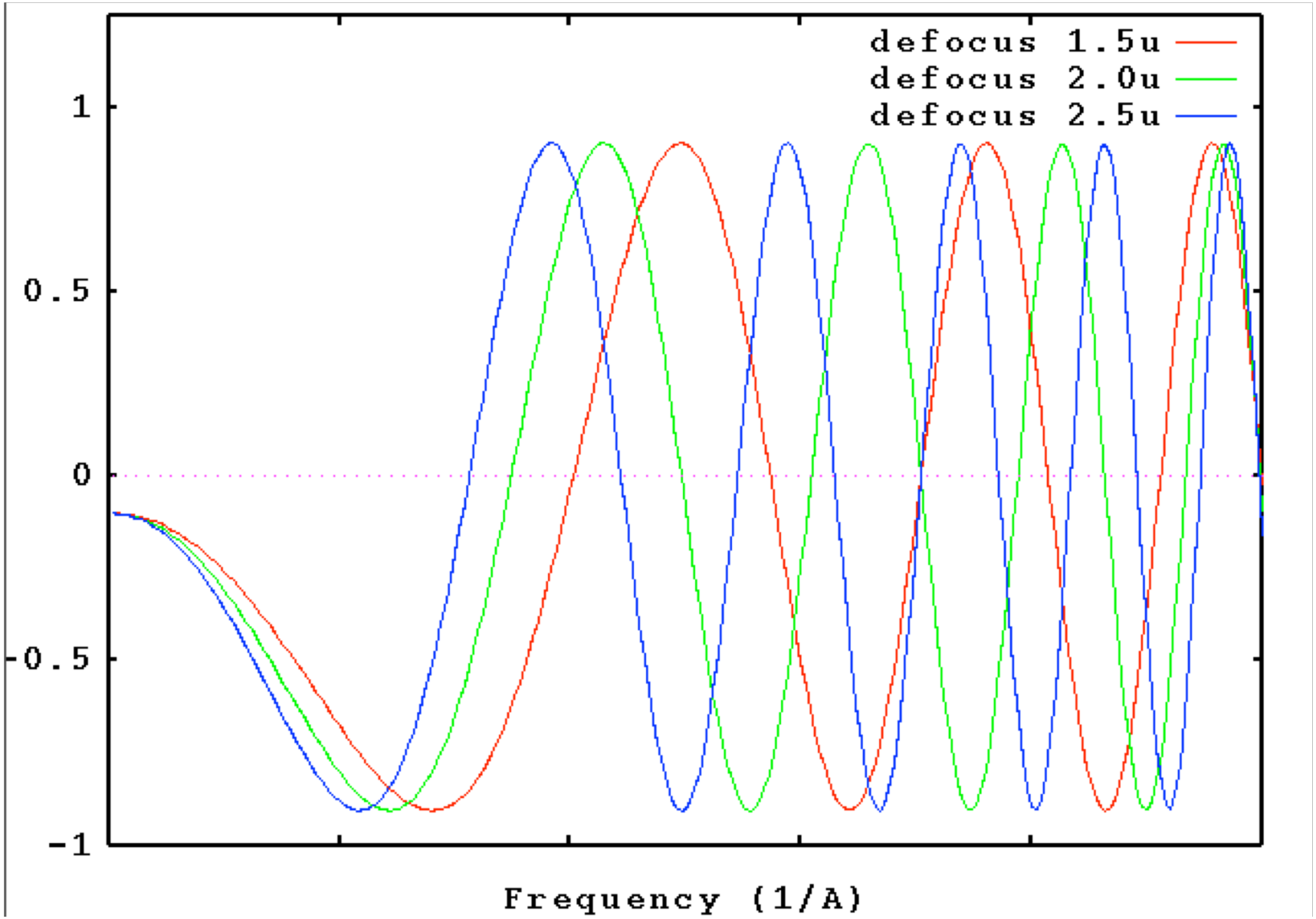




Units of spatial frequency in 2D Fourier space



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 Å


defocus 1.5u
defocus 2.0u
defocus 2.5u
1
0.5
0
-0.5
-1
Frequency (1/A)