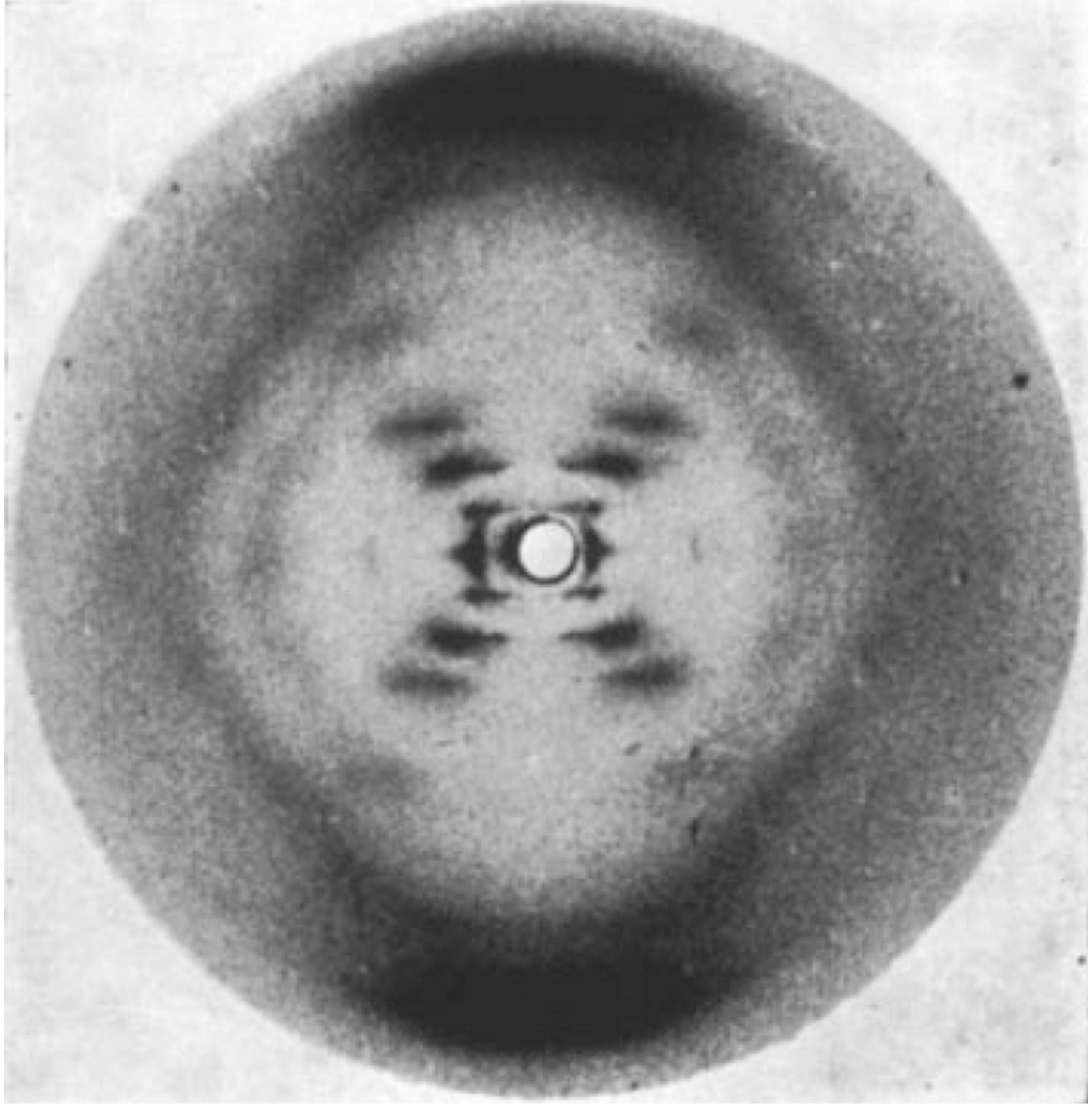
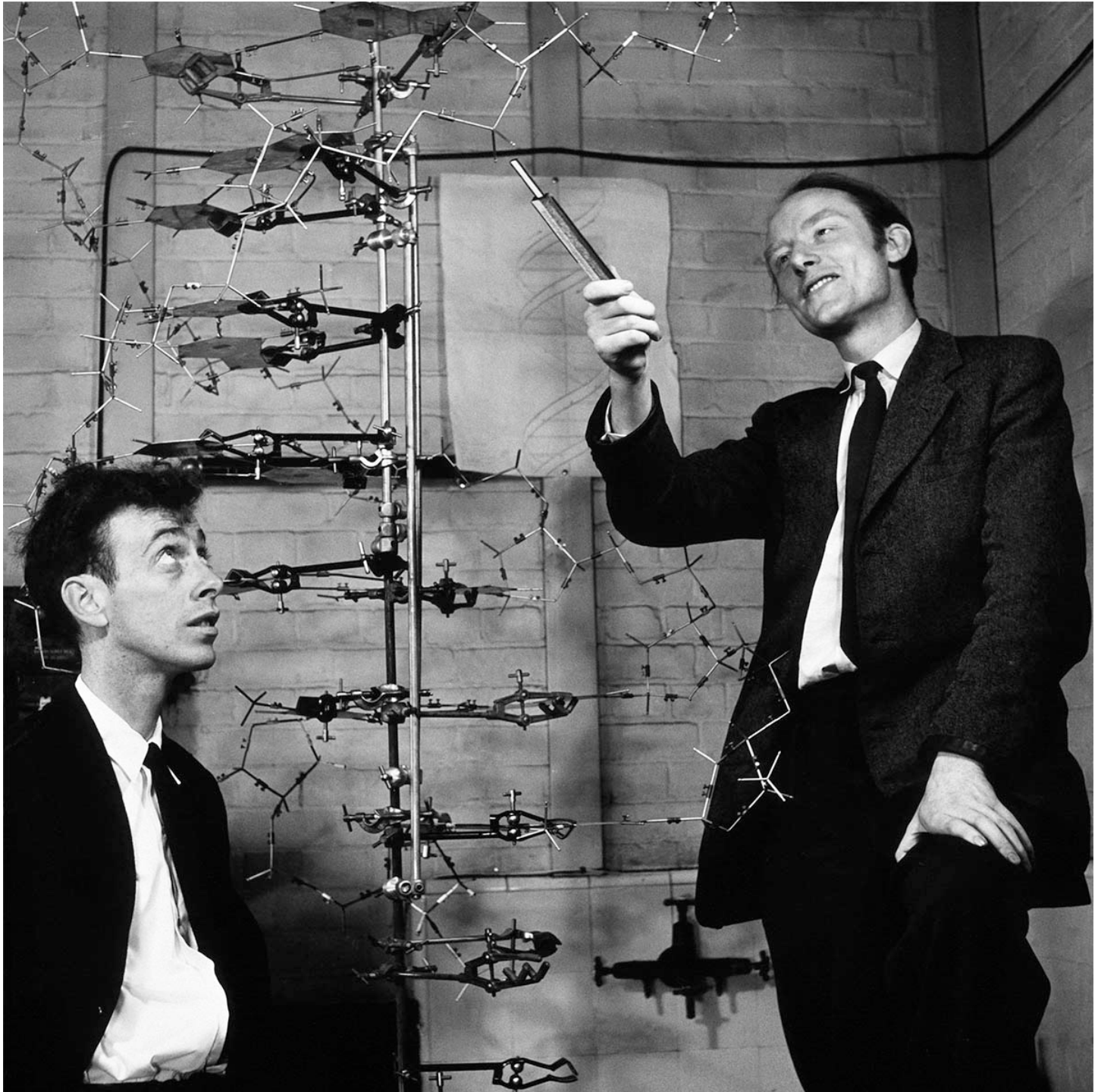
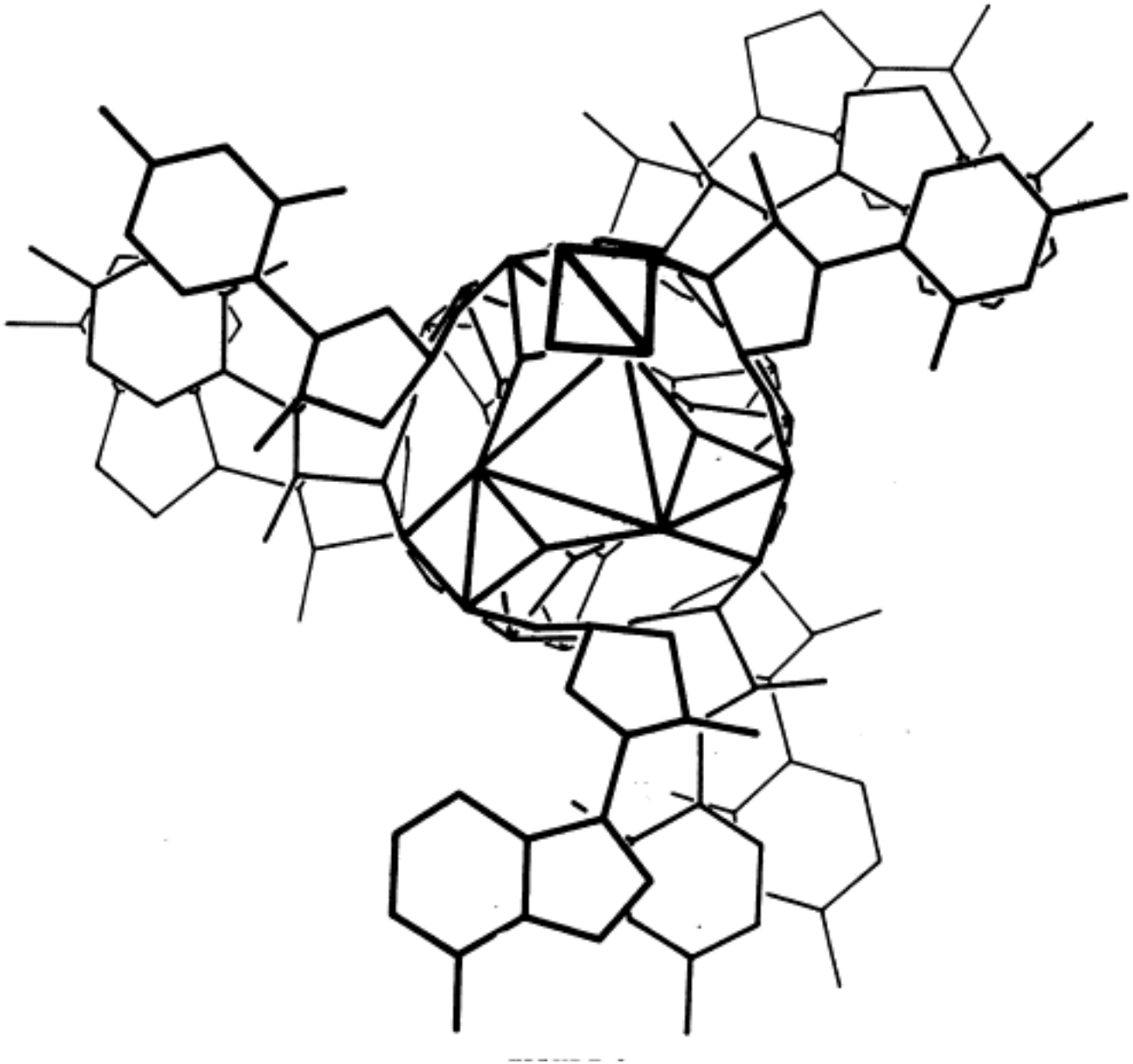


"Photo 51 x-ray diffraction image" by Raymond Gosling/King's College London





"A proposed structure for the nucleic acids." Linus Pauling 1953

**Validation methods have become much better established over the last couple of years.**

**What are the methods that are being used?**

**In what resolution realms are they useful?**

**Do we need more tools?**
**How do we avoid mistakes?**

**Is validation at very high resolution easier than at intermediate resolutions?**

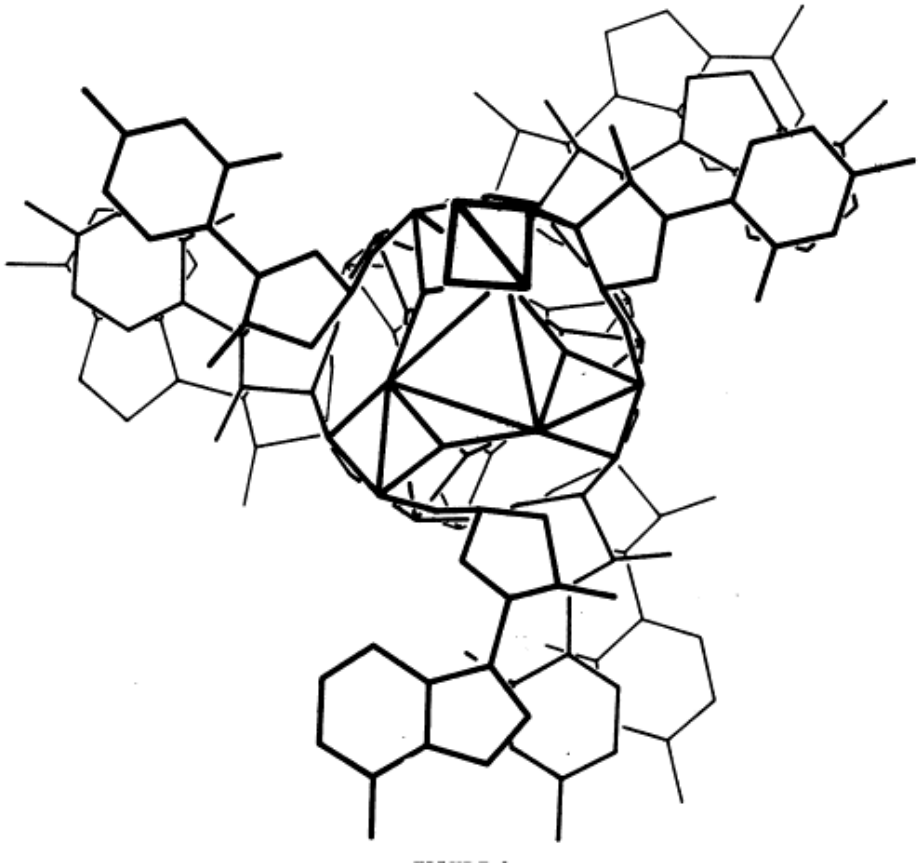
**What about highly heterogeneous  datasets?**

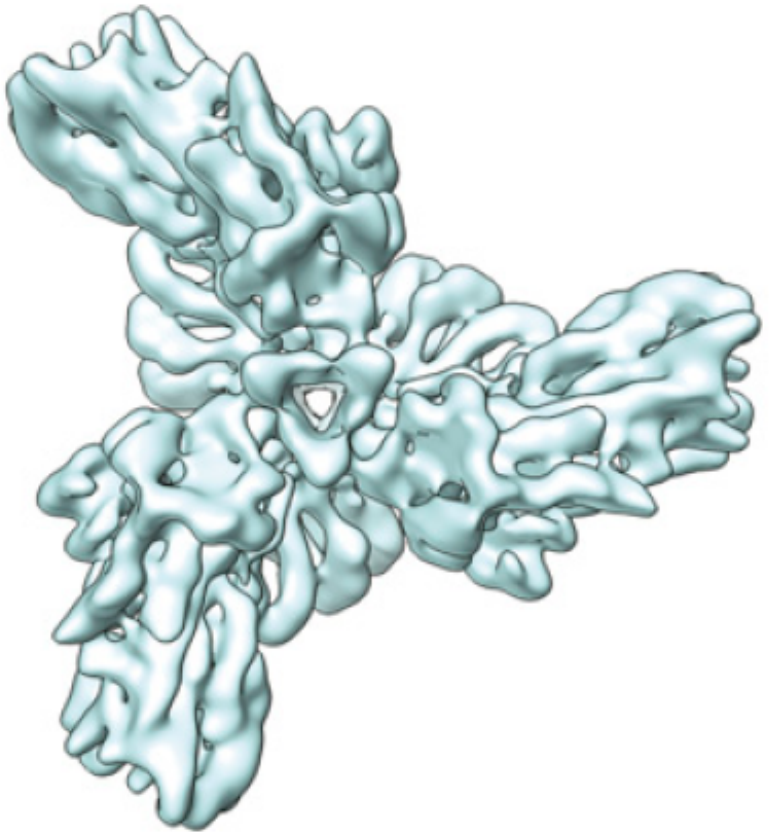
**Why is validation important?**

- Map validation
- Model validation
- Validation tests often reveal problems that can be resolved

**Why is data deposition and exchange important?**

- Allows others to check whether research claims are true
- Allows extraction of more information from same data
- Allows structure to be used as input to related projects



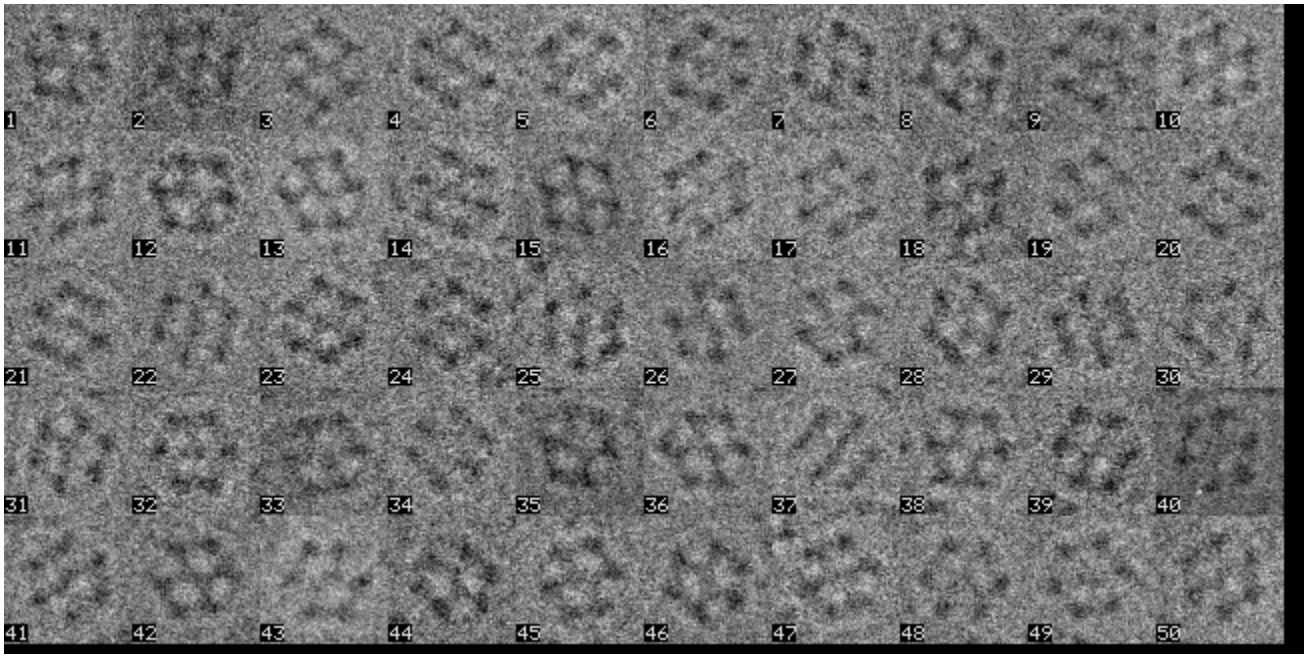


Mao et al PNAS
(2013) **110**, 12438

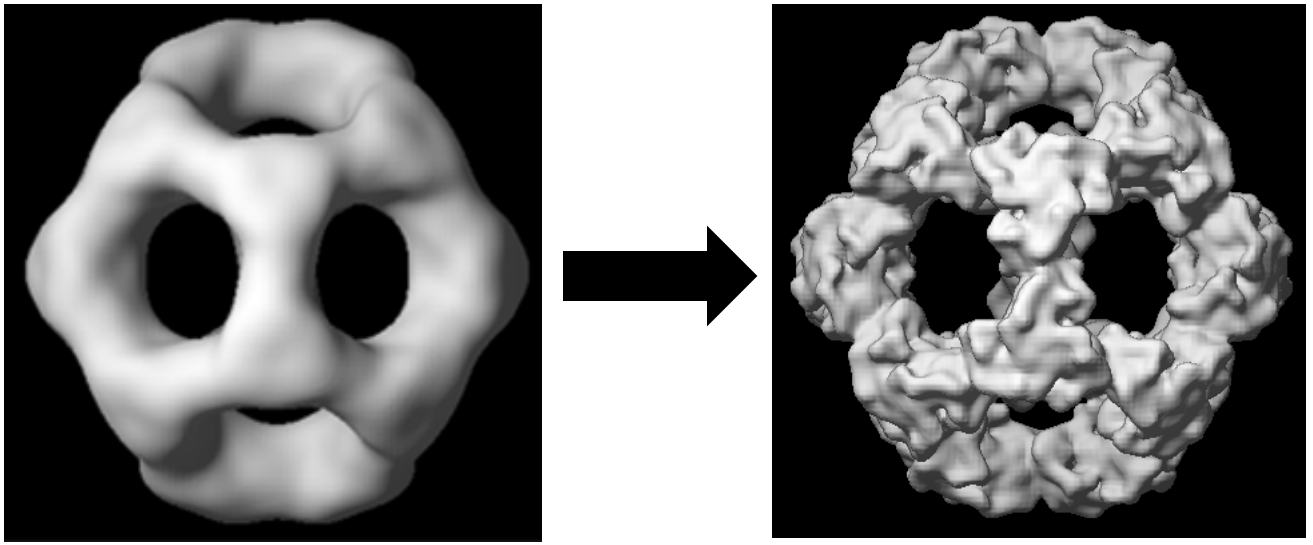
# Map Validation

- Is map correct (incorrect) at low resolution?
  (Tilt-pairs for optimization & validation)
- Is resolution assessment exaggerated?
- Simple Tests to Demonstrate Validity of Map
- User still decides how to process the data

PARTICLE IMAGES




STARTING MODEL



# *De novo* determination of "Starting Map"

Map projections agree with individual raw images and class averages (reference free)
Distribution of particle orientations

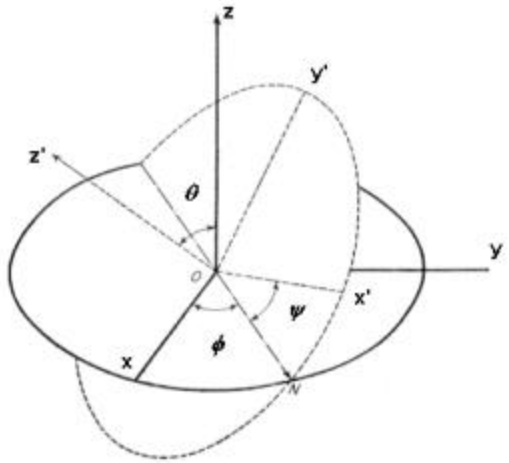
Absolute Hand (Experimental Determination-Tilting)

**Other sources for starting map:**

Derived from another highly similar structure?

Density from X-ray model? Low-pass filtered

A spherical or cylindrical blob? Icosahedral or helical symmetry

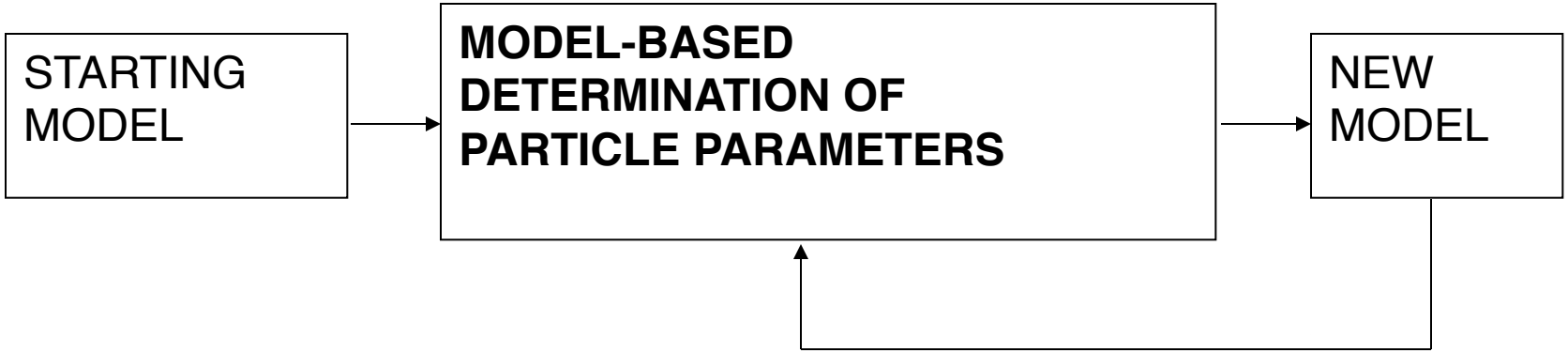



PARTICLE ORIENTATION

ψ,θ,φ,x,y

MICROSCOPE PARAMETERS

Defocus(3), magnification

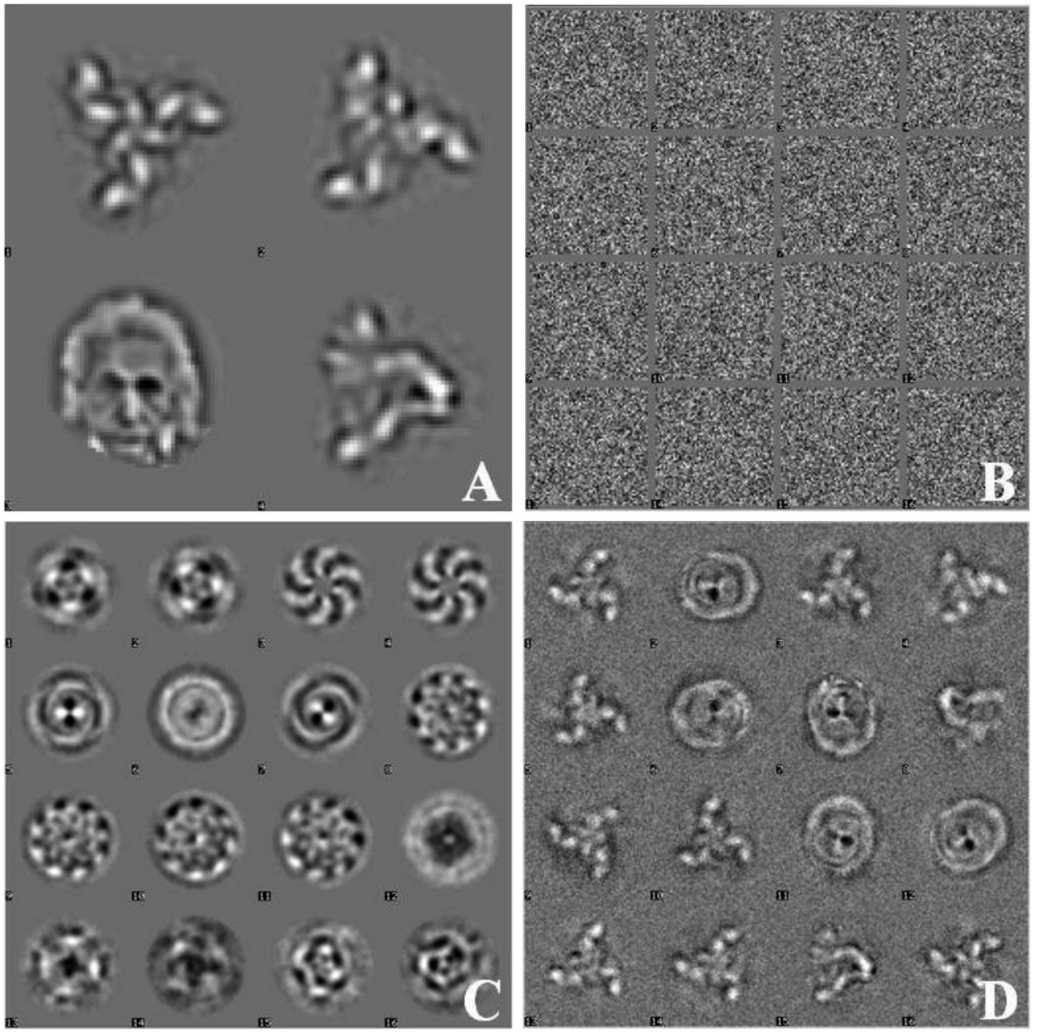



MEASURE OF AGREEMENT BETWEEN CALCULATED MODEL PROJECTION AND IMAGE

"MINIMIZE PHASE RESIDUAL"

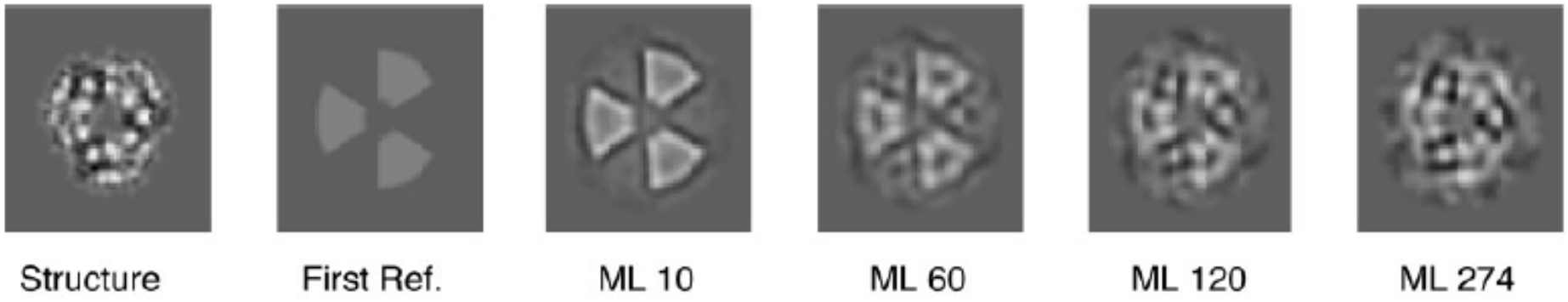
$$PR = \frac{\sum_i |\Delta\phi_i F_i|}{\sum_i |F_i|}$$

**(A) Four reference images (each 64 × 64 pixels) used for picking from 1,024 random noise images (of 1,024 × 1,024 pixels).**



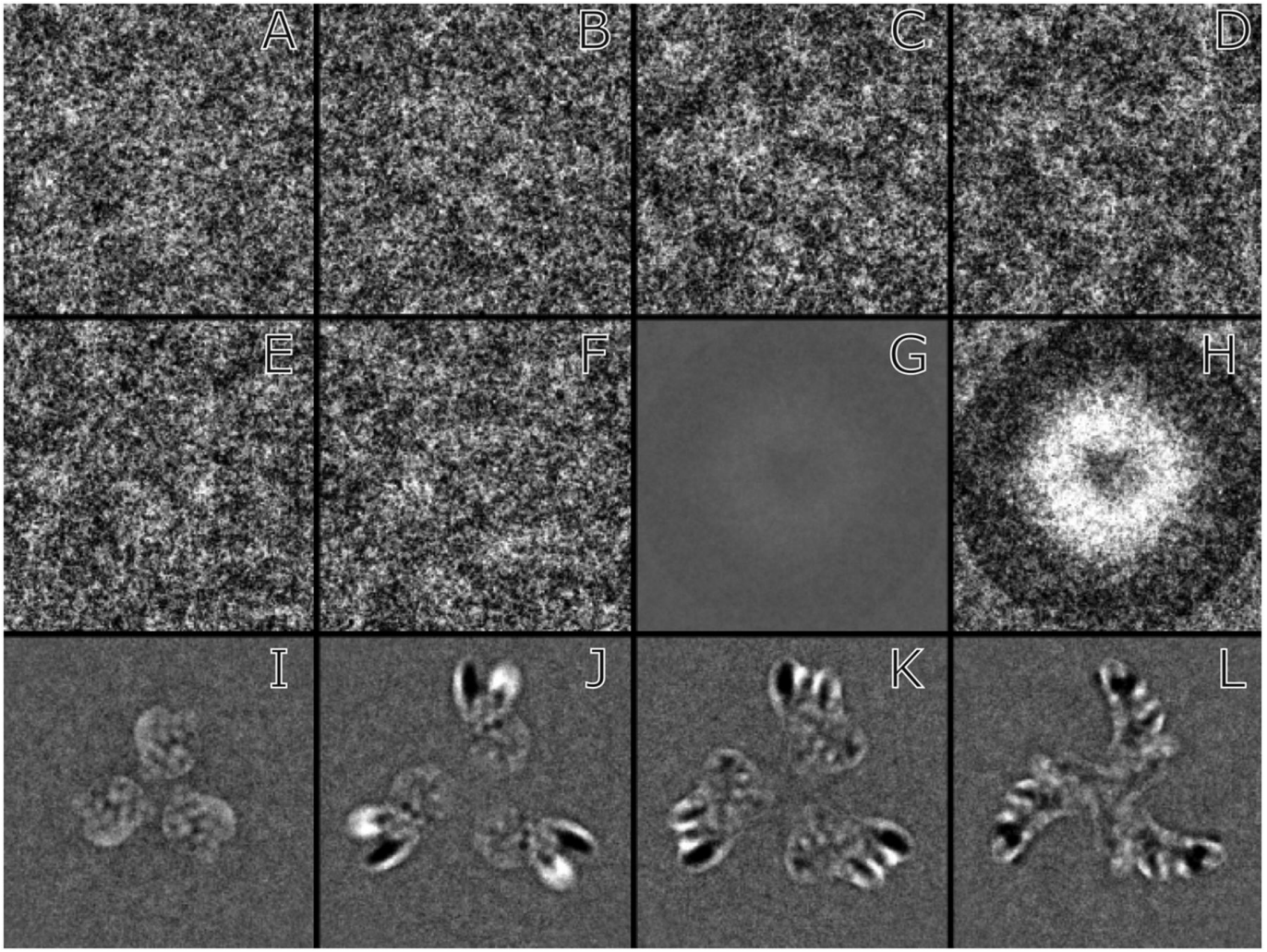

van Heel M PNAS 2013;110:E4175-E4177

# Maximum Likelihood (ML) vs. CC (Align)

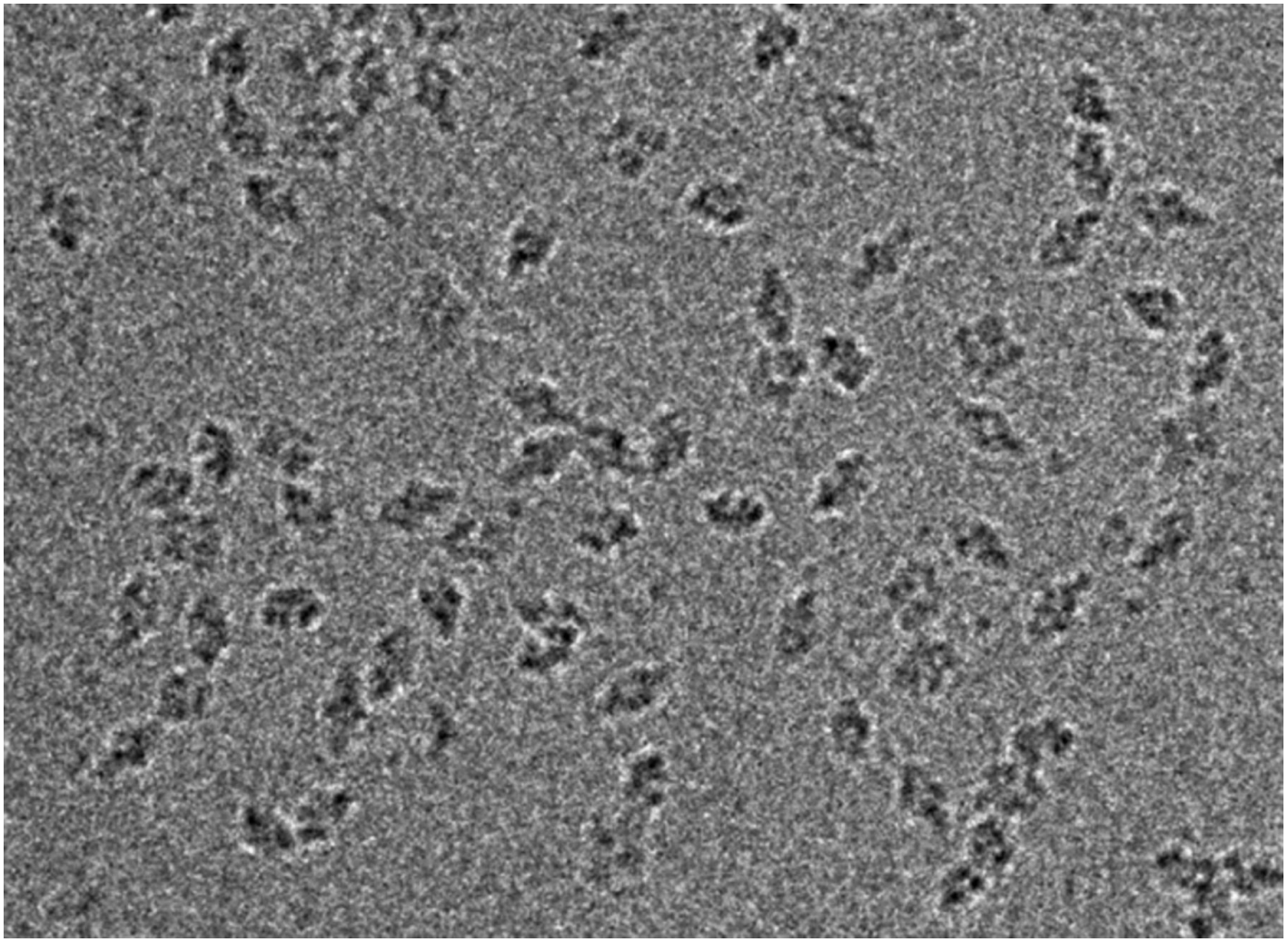

F.J. Sigworth (1998) JSB, 328-339

**(A–F) Six individual windowed images from the stack of 423 that was supplied by the authors (21).**

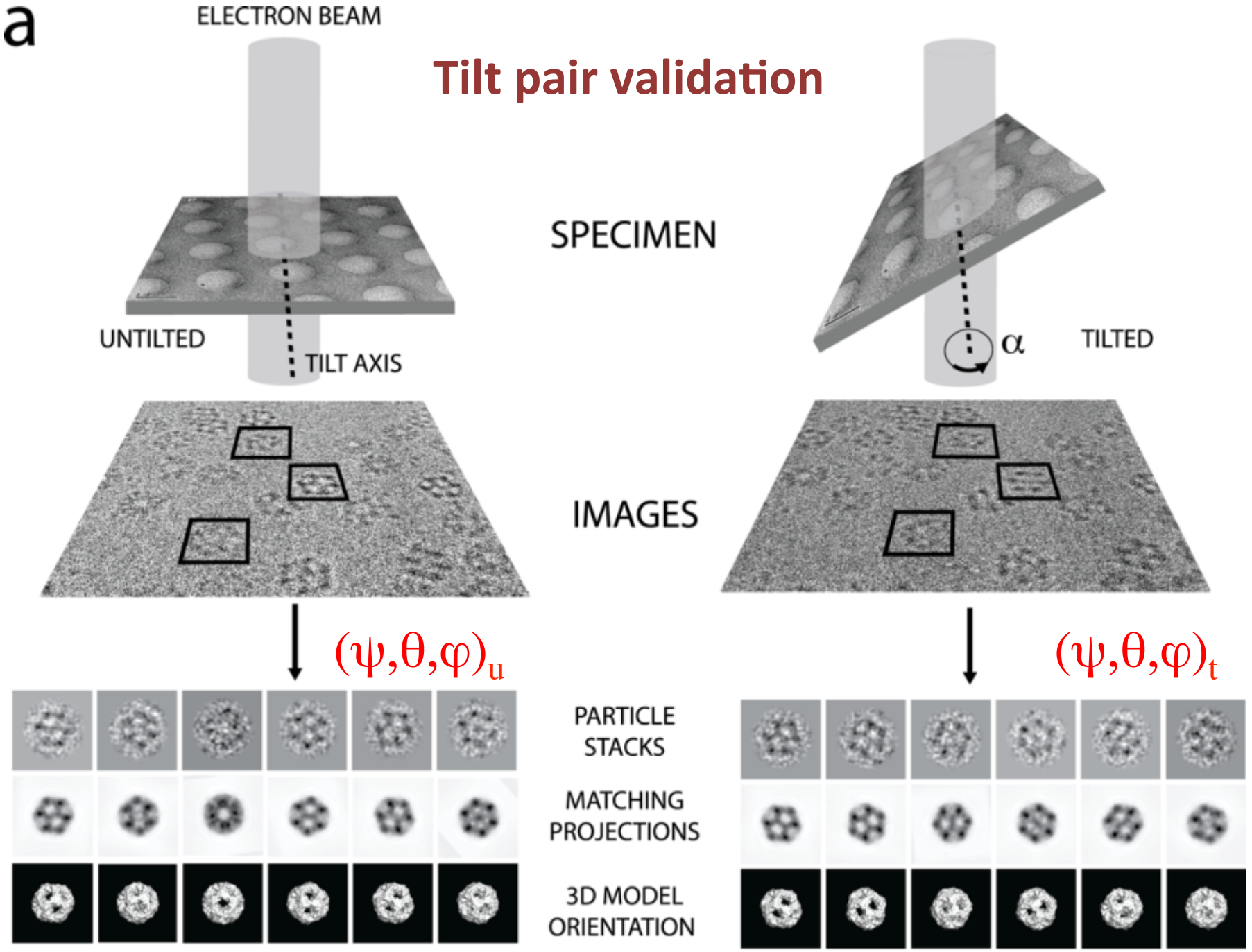



**Henderson R PNAS 2013;110:18037-18041**

**Cryo-EM image of a field of view of β-galactosidase single particles (molecular weight, 450 kDa).**



**Henderson R PNAS 2013;110:18037-18041**

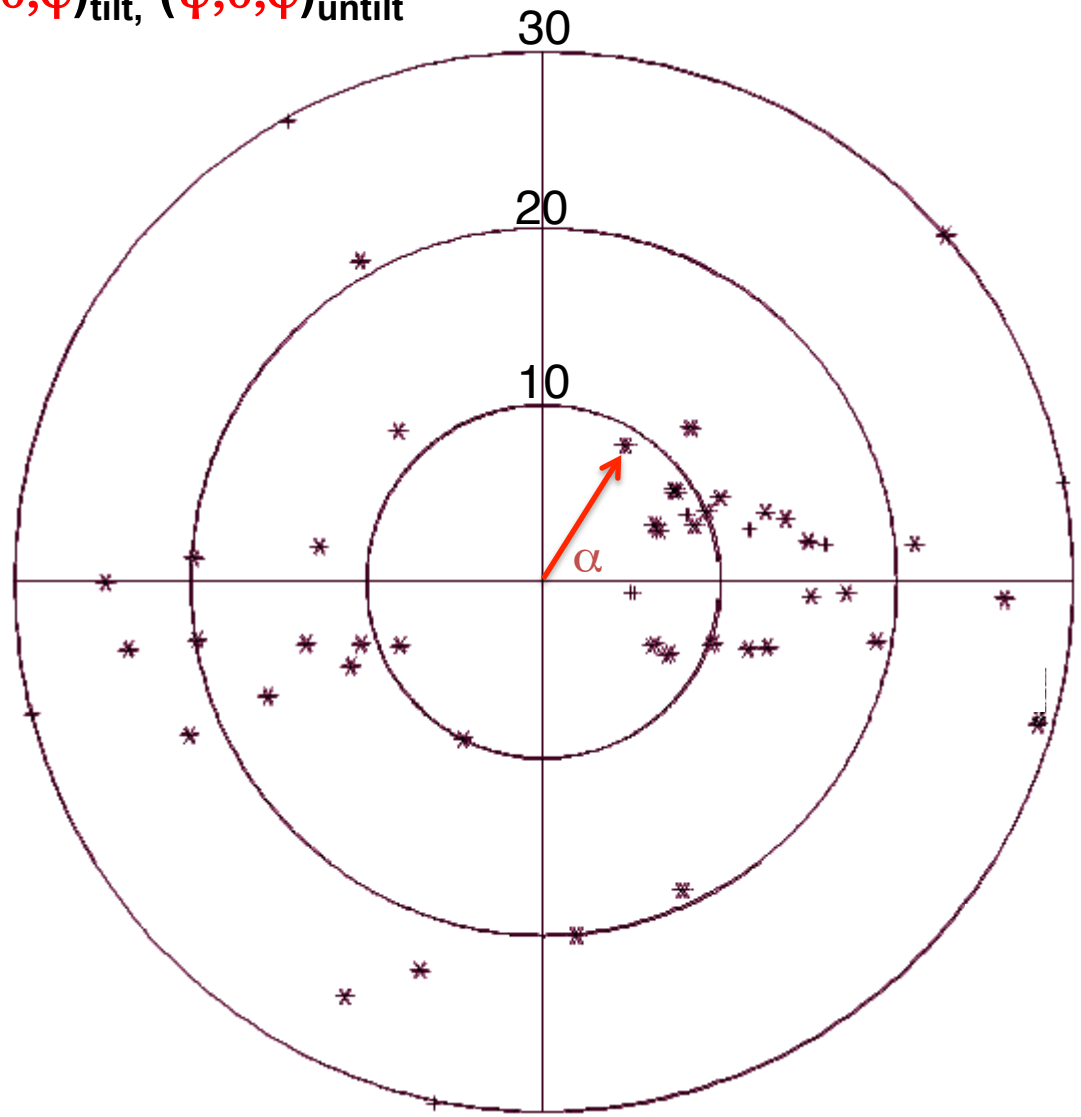

a
ELECTRON BEAM
Tilt pair validation
SPECIMEN
UNTILTED
TILT AXIS
α
TILTED
IMAGES
$(\psi,\theta,\varphi)_u$
$(\psi,\theta,\varphi)_t$
PARTICLE STACKS
MATCHING PROJECTIONS
3D MODEL ORIENTATION

# TILT AXIS FOR EACH PARTICLE PAIR

**CALCULATED FROM $(\psi,\theta,\varphi)_{tilt}$, $(\psi,\theta,\varphi)_{untilt}$**

**RADIUS IS TILT ANGLE**

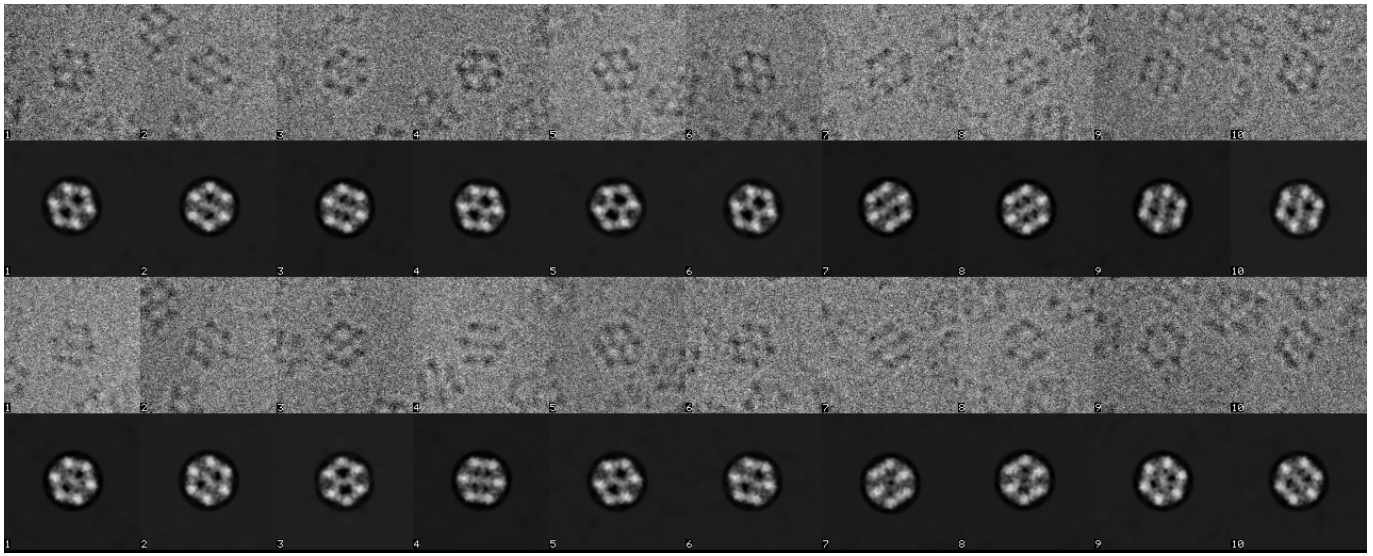
**TILT AXIS DIRECTION $\alpha$**




**FOR PERFECT DATA**
**ALL POINTS COINCIDE**

# PHASE RESIDUAL SCORE FOR ALL POSSIBLE TILT AXES

$(\psi,\theta,\varphi)_u$



UNTILTED
$(\psi,\theta,\varphi)_u$

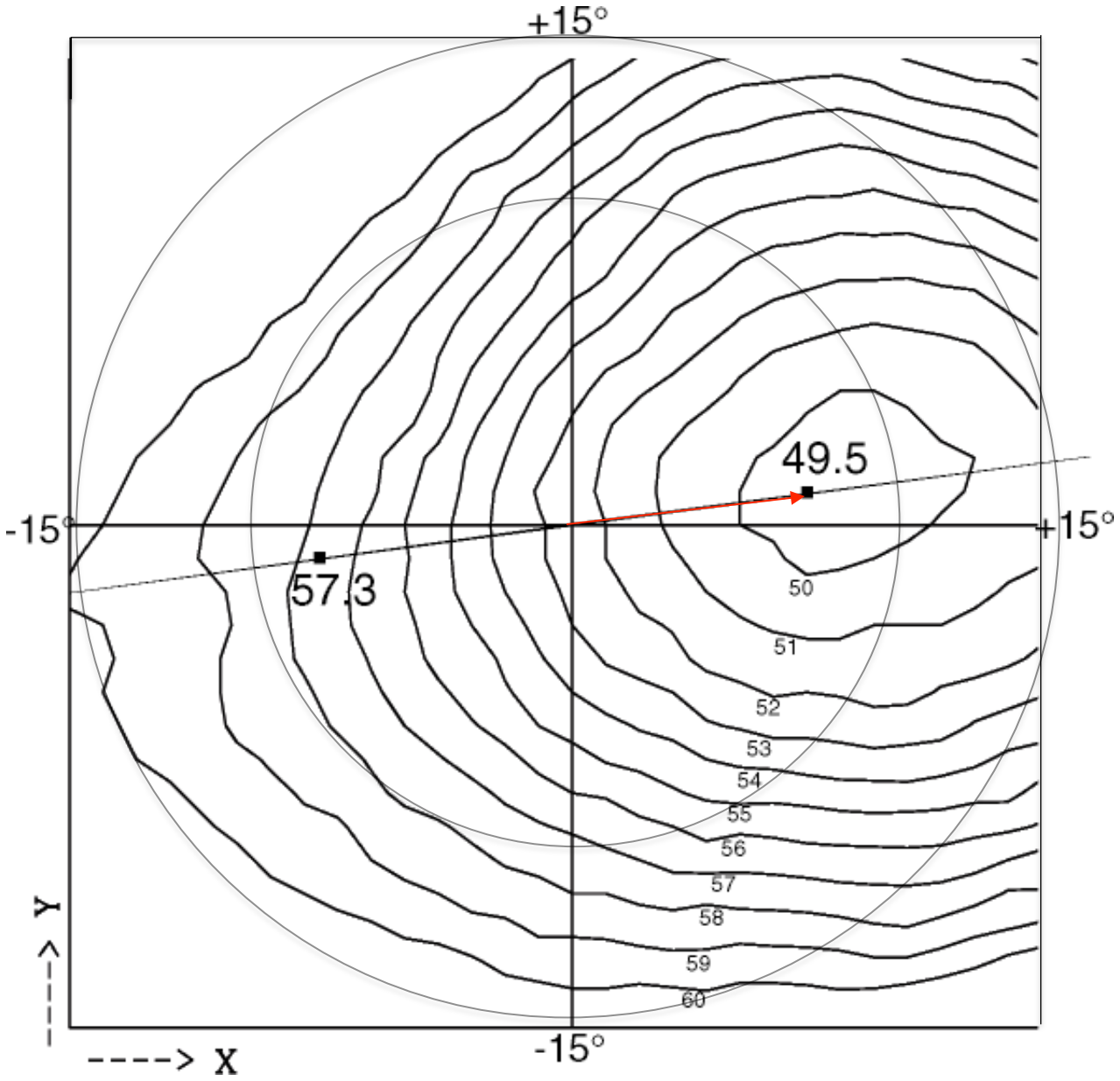
APPLY TILT

$(\psi,\theta,\varphi)_t$

SCORE AGREEMENT WITH TILTED IMAGE "PHASE RESIDUAL"

AVERAGE 50 PARTICLES

# Average Phase Residual (PR) 50 Particles




TILT ANGLE
7.5 °
ΔPR=7.8 °

# REFINE PARAMETERS

## USED TO DETERMINE ORIENTATION

- SEARCH PROCEDURE
- MODEL QUALITY
- RESOLUTION RANGE
- RESOLUTION WEIGHTS (TEMPERATURE FACTOR)

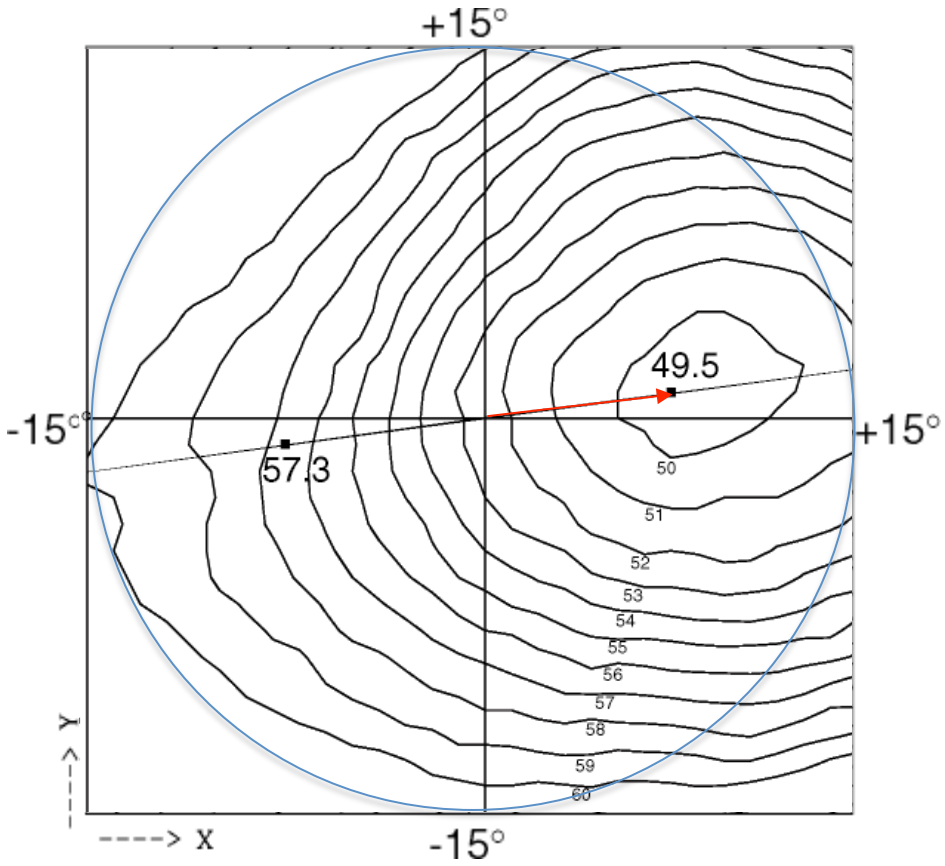
- DEFOCUS VALUES
- RECONSTRUCTION RADIUS
- ETC.

## RESOLUTION RANGE

| | PR+ | PR- | ANGLE |
|---|---|---|---|
| 100-35 Å | 48.3 | 53.5 | 6.0deg |
| 80-25 Å | 43.4 | 60.2 | 11.5deg |

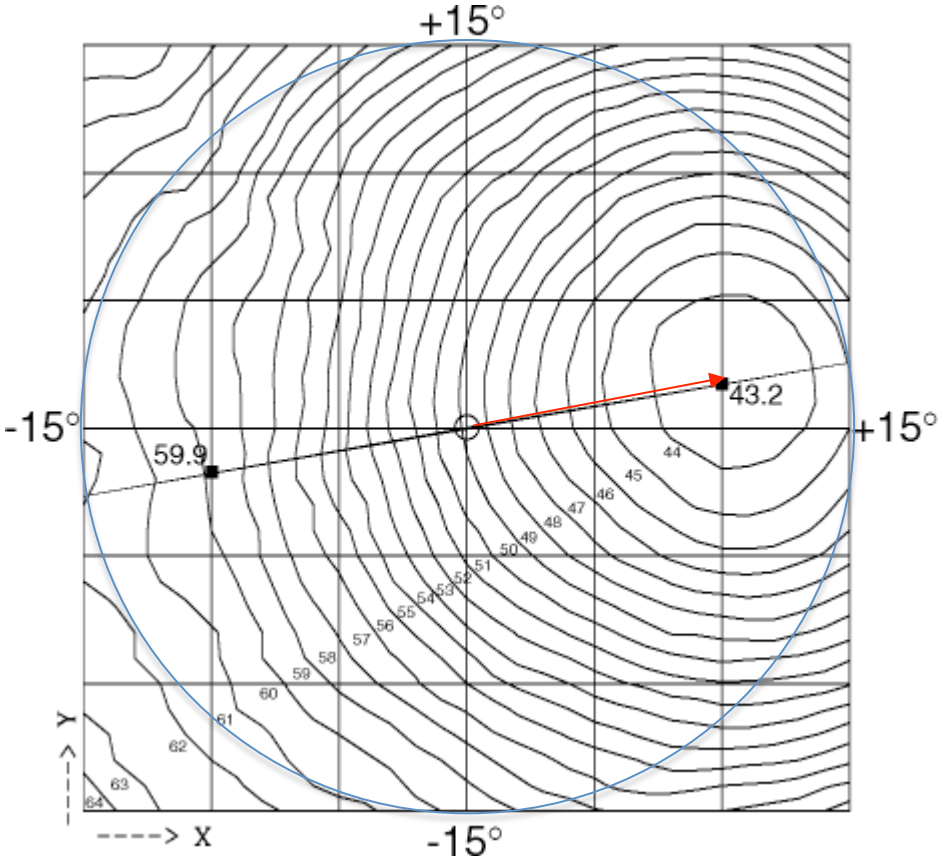
# Average Phase Residual 50 Particles

BEFORE




TILT ANGLE 7.5 º
ΔPR=7.8 º

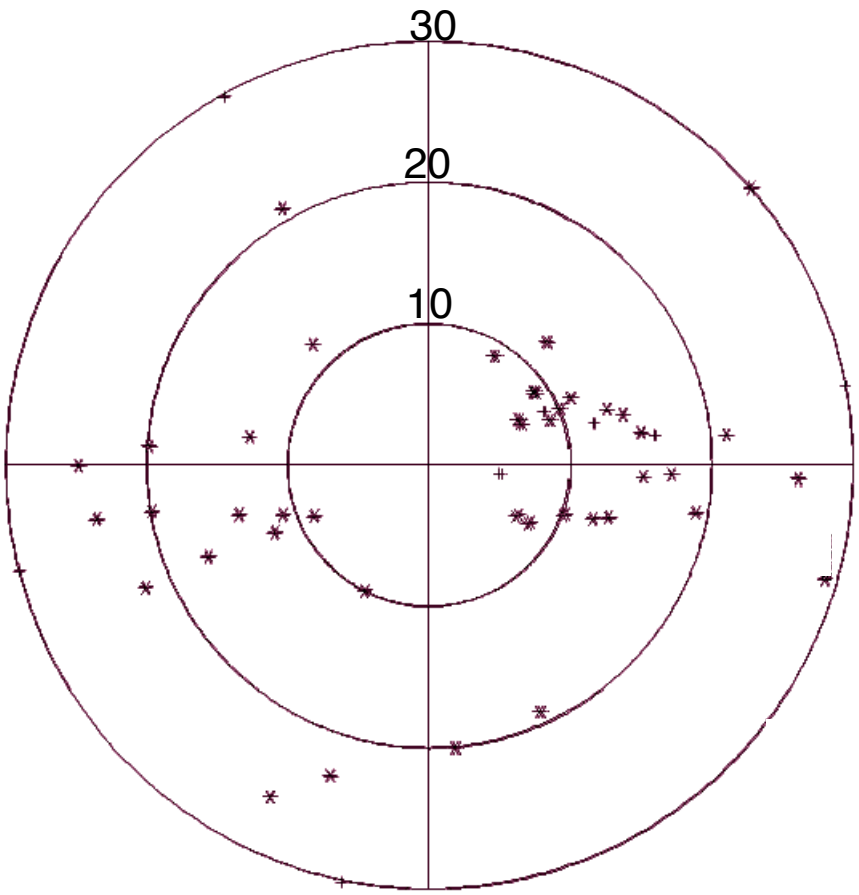
AFTER



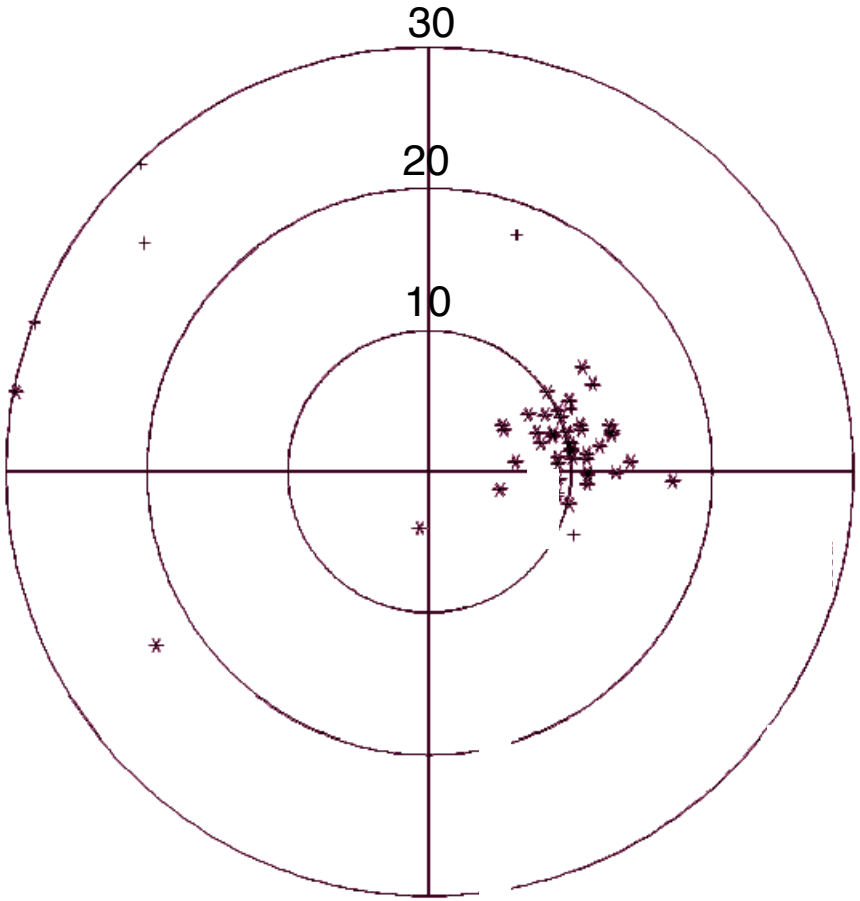

TILT ANGLE 10 º
ΔPR=16.7 º

# TILT AXIS FOR EACH PARTICLE PAIR

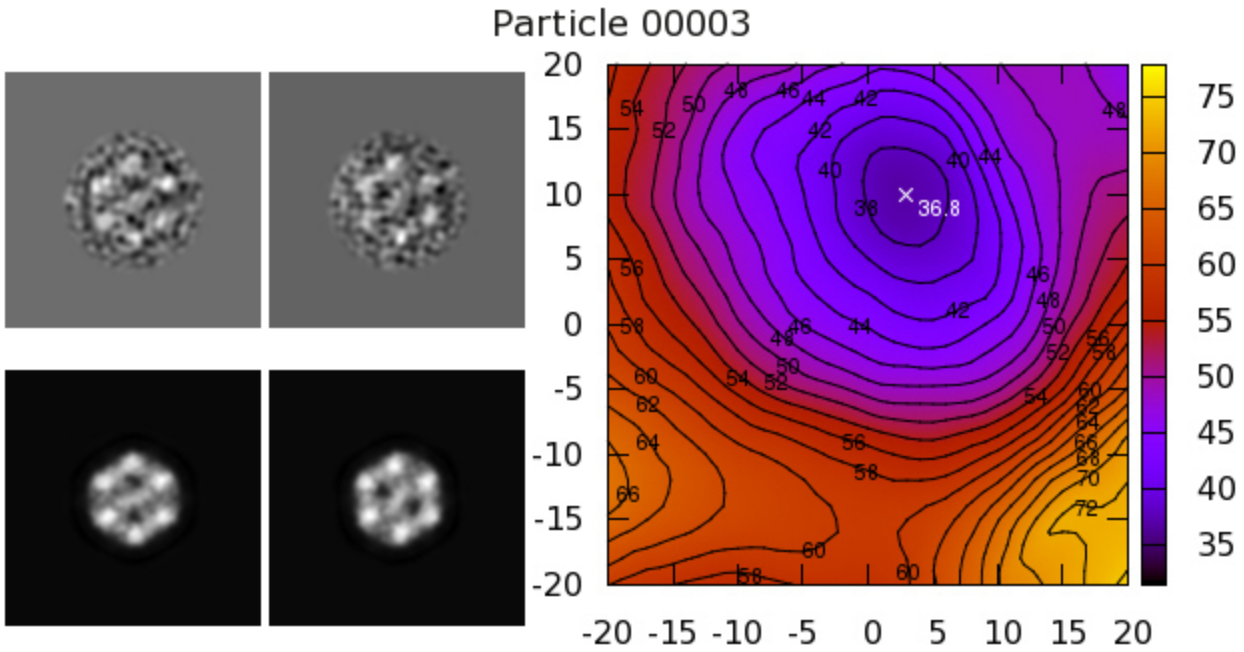
BEFORE OPTIMIZATION




AFTER OPTIMIZATION



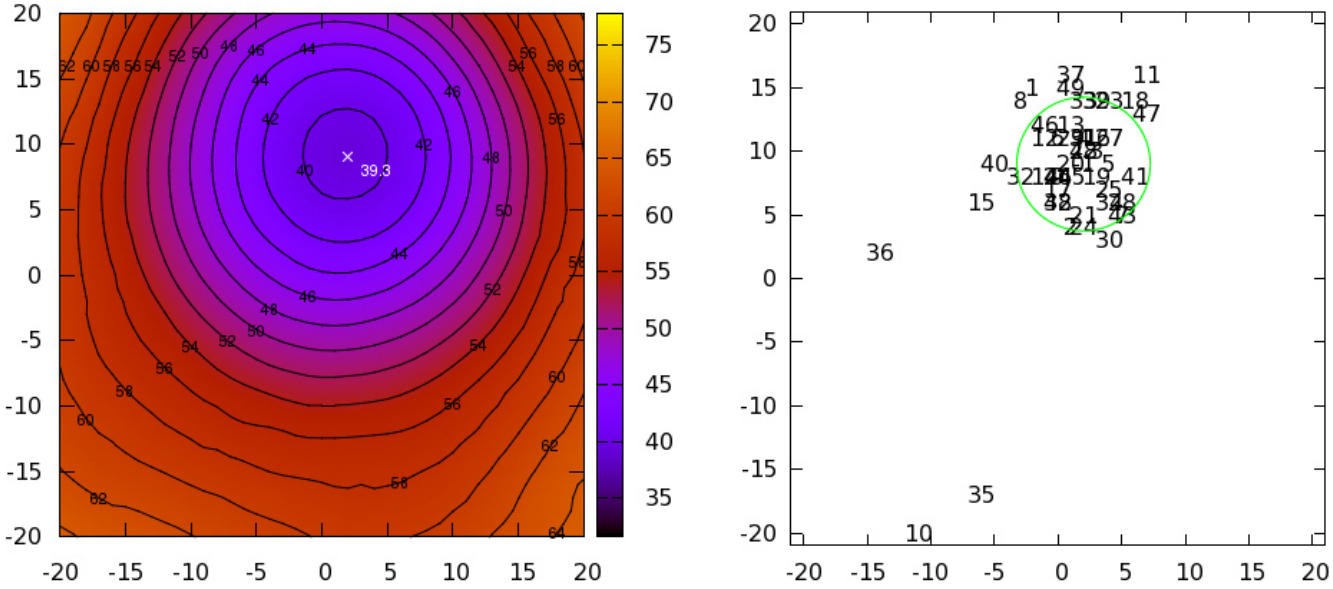
# Report for Each Particle




Minimal Phase Residue: **36.77 °**
Minimum at position: **3.0°, 10.0°**
Hand Phase Difference: **21.03 °**

Save as archive

| | |
|---|---|
| 3D model: | /home/swasile/Hand/combine_22av_halfp.map2k.mrc |
| Untilted stack: | /home/swasile/Hand/e2f301982.partpadred.mrc |
| Tilted stack: | /home/swasile/Hand/e2f301983.partpadred.mrc |
| Parameters file: | /home/swasile/Hand/e2_1982u_96.par |
| Experiment identifier: | Sample demo job |




| | |
|---|---|
| Magnification | 4.98 A/px |
| Defocus | 58626 ; 59084 |
| Astigmatism | 55.7 |
| Voltage | 300 kV |
| Resolution Interval | 100.0 - 30.0 A |
| Tilt Interval | 20 |
| Particle radius | 20 px |
| Optimized box size : | 128 |
| Effective binning: | 1 |

Average for all particles submited:

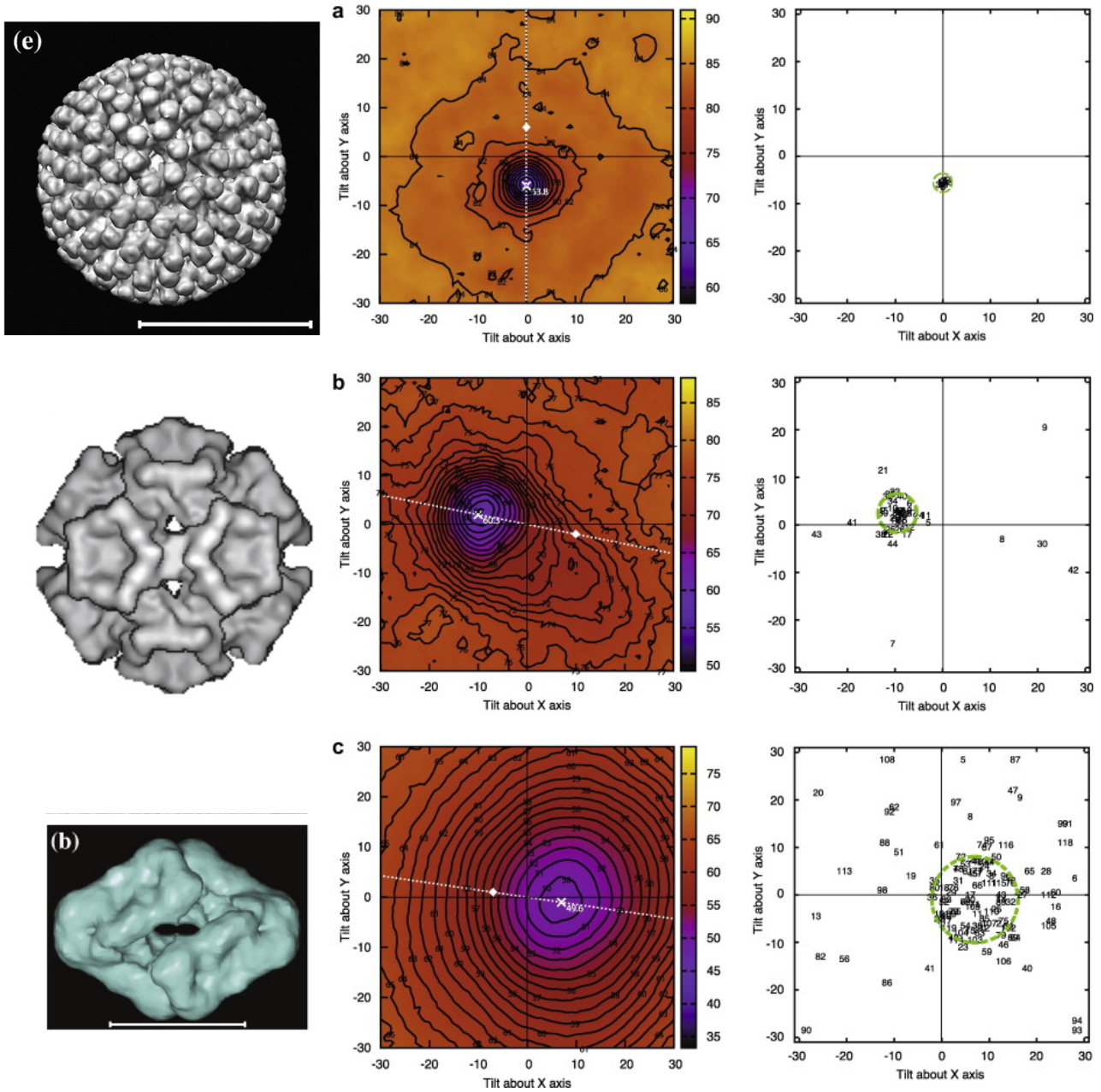
Minimal Phase Residue: **39.26 °**
Minimum at position: **2.0°, 9.0°**
Hand Phase Difference: **14.13 °**
Average distance from the mean minima: **5.25 °**

Particles with the hand difference below the average:
2 7 9 11 12 14 15 17 19 20 21 24 26 30 32 35 36 38 41 45

Particles with minima distant from the determined tilt transfromation:
1 8 10 11 15 18 23 30 35 36 37 40 47 49

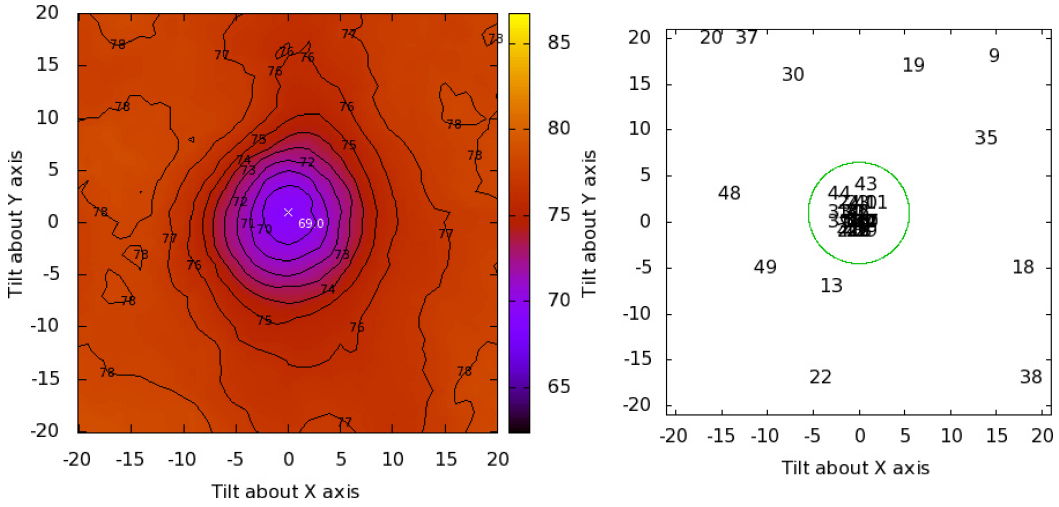
Particles contributing to the determined minimum:
0 3 4 5 6 13 16 22 25 27 28 29 31 33 34 39 42 43 44 46 48

VIEW DETAILED REPORT

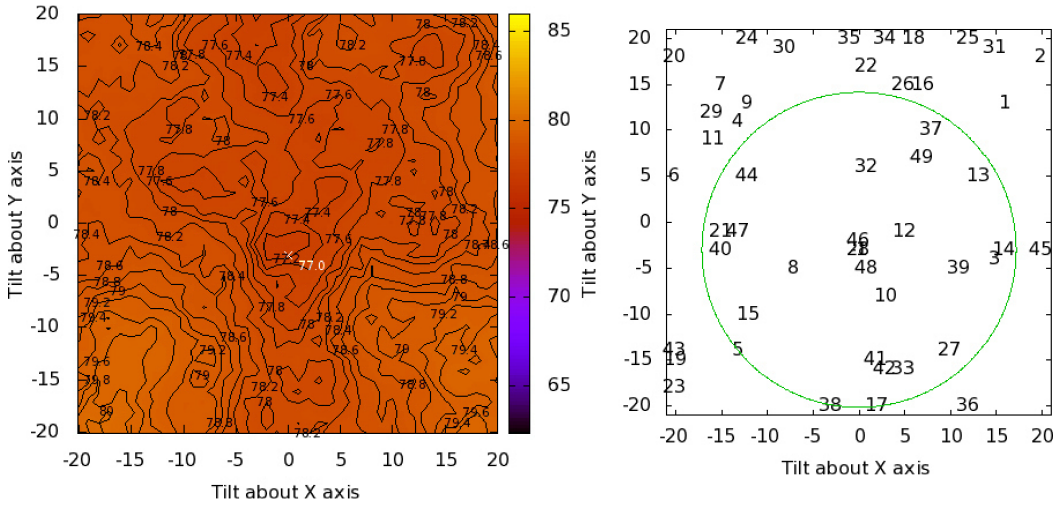

(e)
(b)
a
b
c
Tilt about X axis
Tilt about Y axis

# An incorrect model

"Self"
Untilted orientations
vs. untilted images



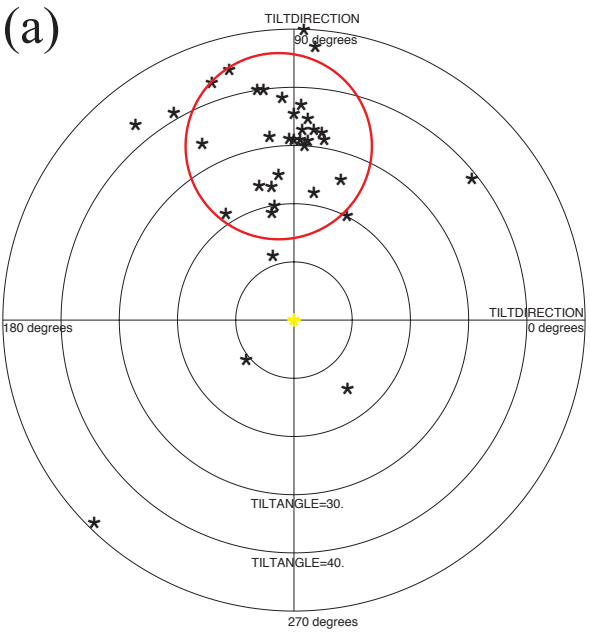

"Tilt"

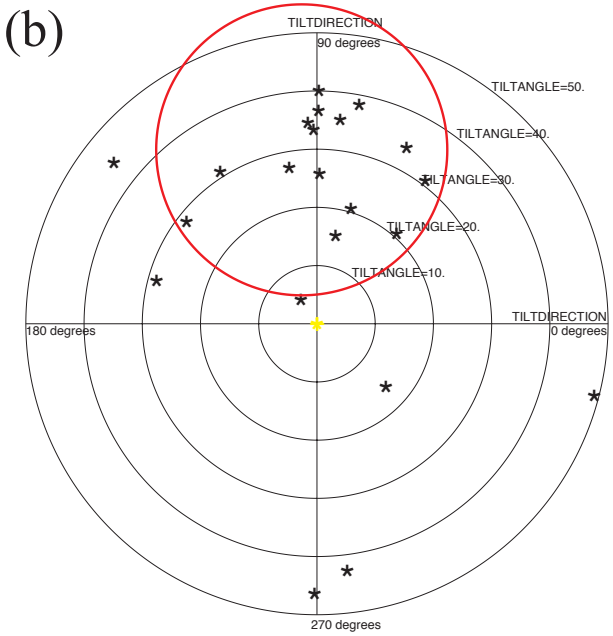


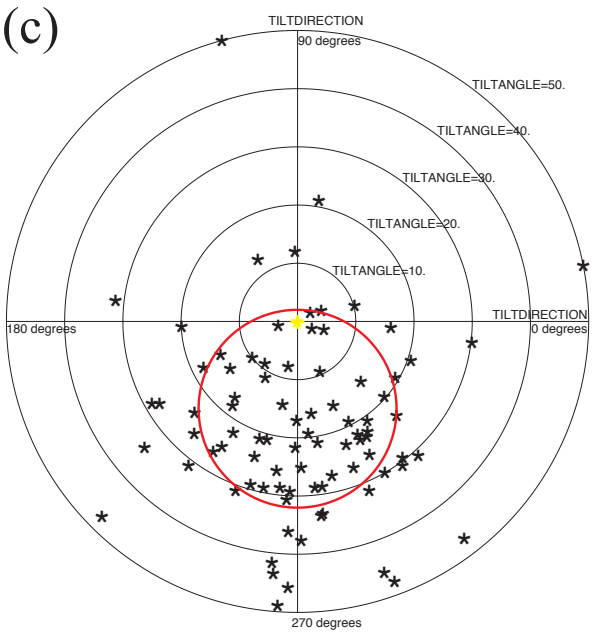
*T.thermophilus* V-type ATPase

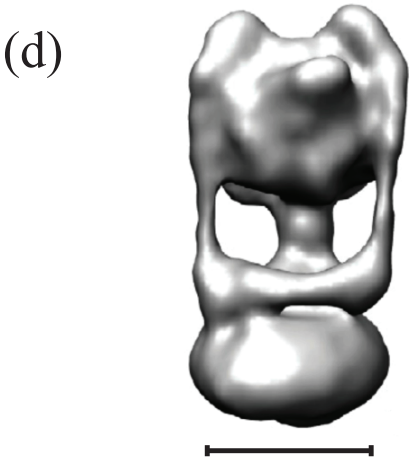
bovine mitochondrial F-type ATPase

DNA-PKcs

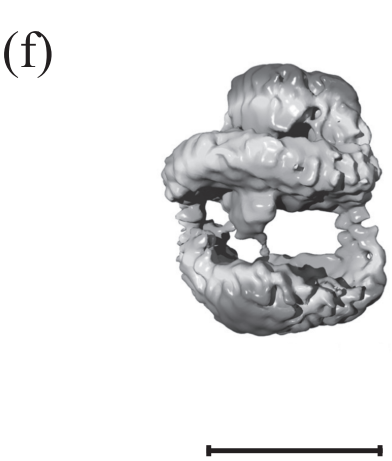


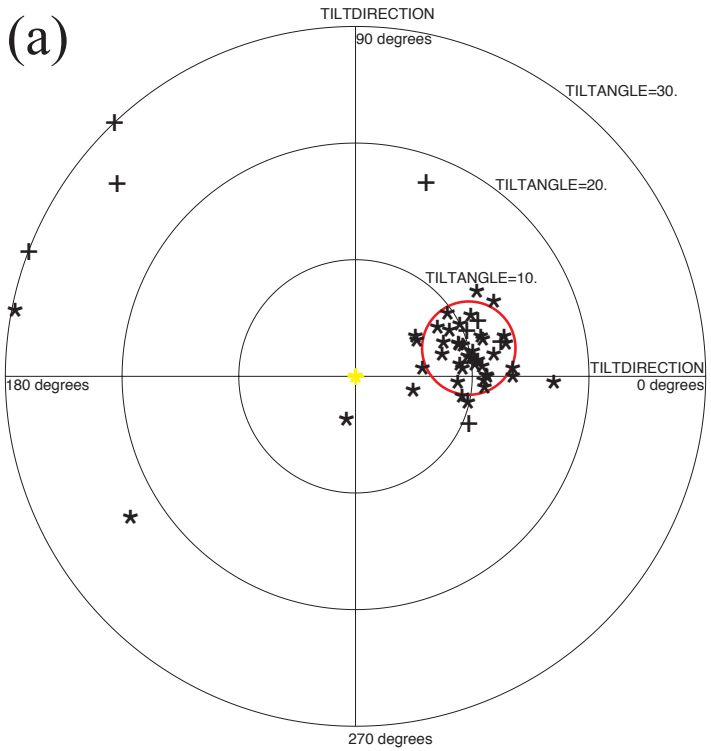


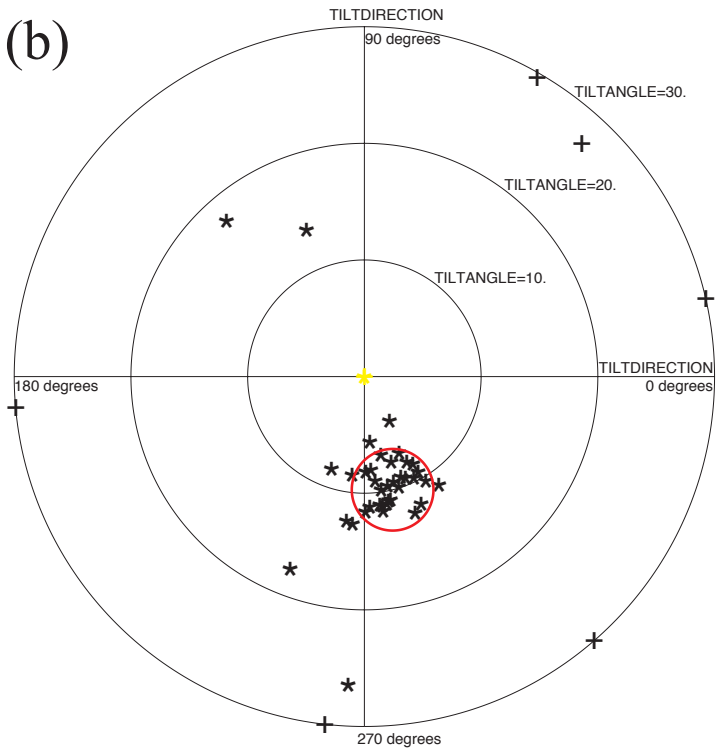


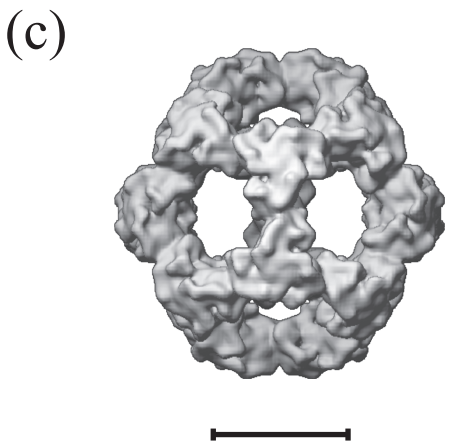

Data from Lau & Rubinstein (2010); Rubinstein et al (2003); Williams et al (2007)

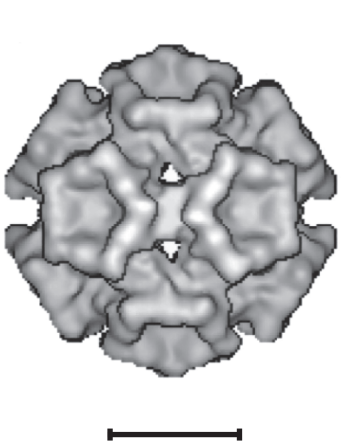
Pyruvate dehydrogenase, E2CD

Chicken anemia virus, CAV



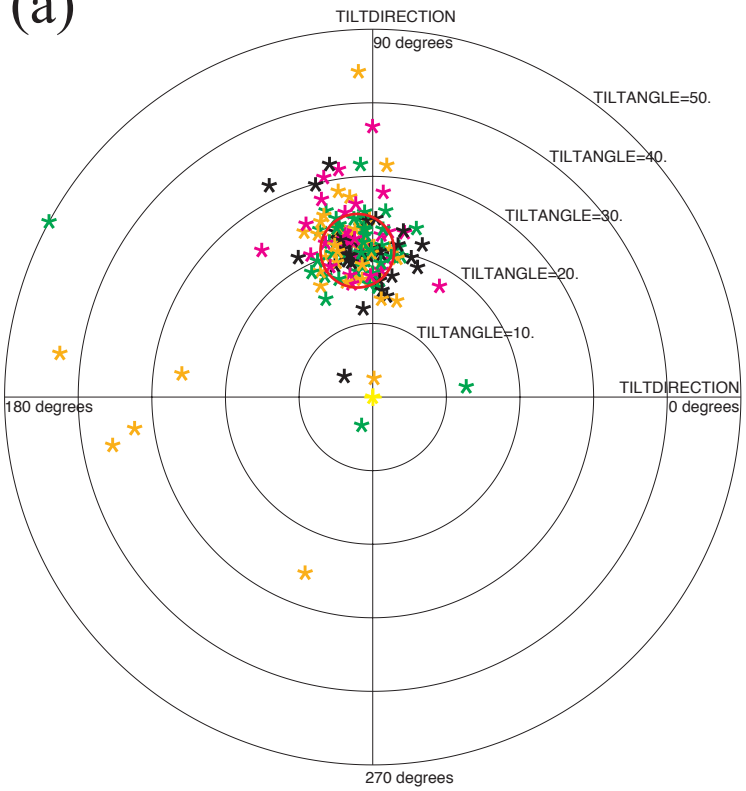




Data from Rosenthal & Henderson (2003);

Crowther et al (2003)

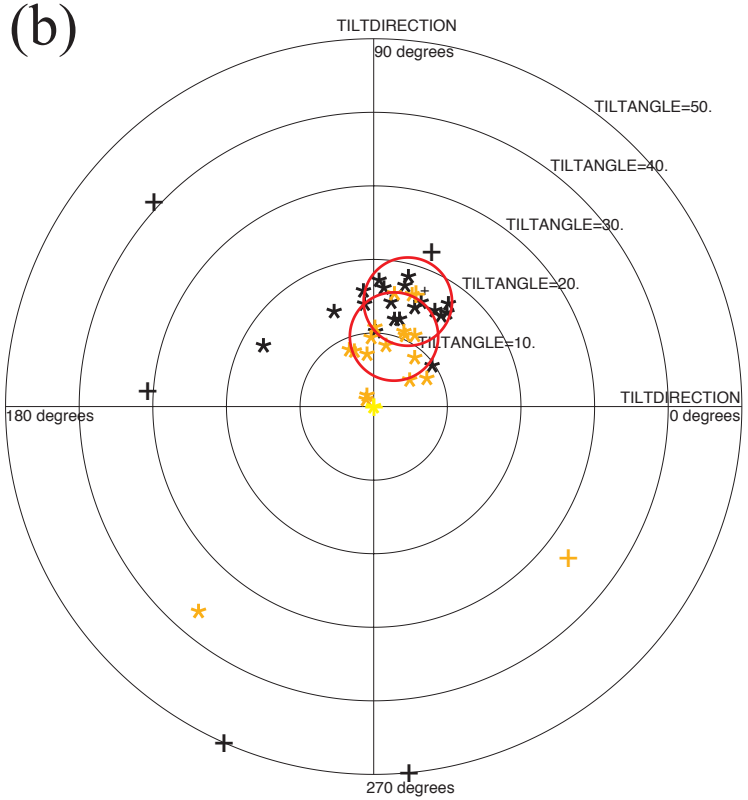
*E.coli* 70S ribosome

(a)

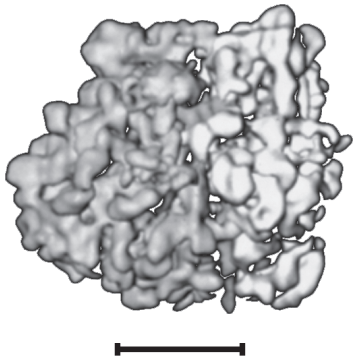



Yeast fatty acid synthetase (FAS)

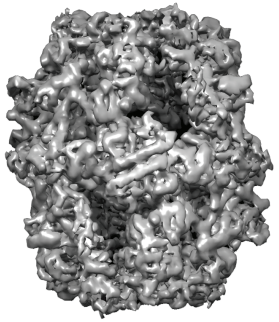
(b)




(c)

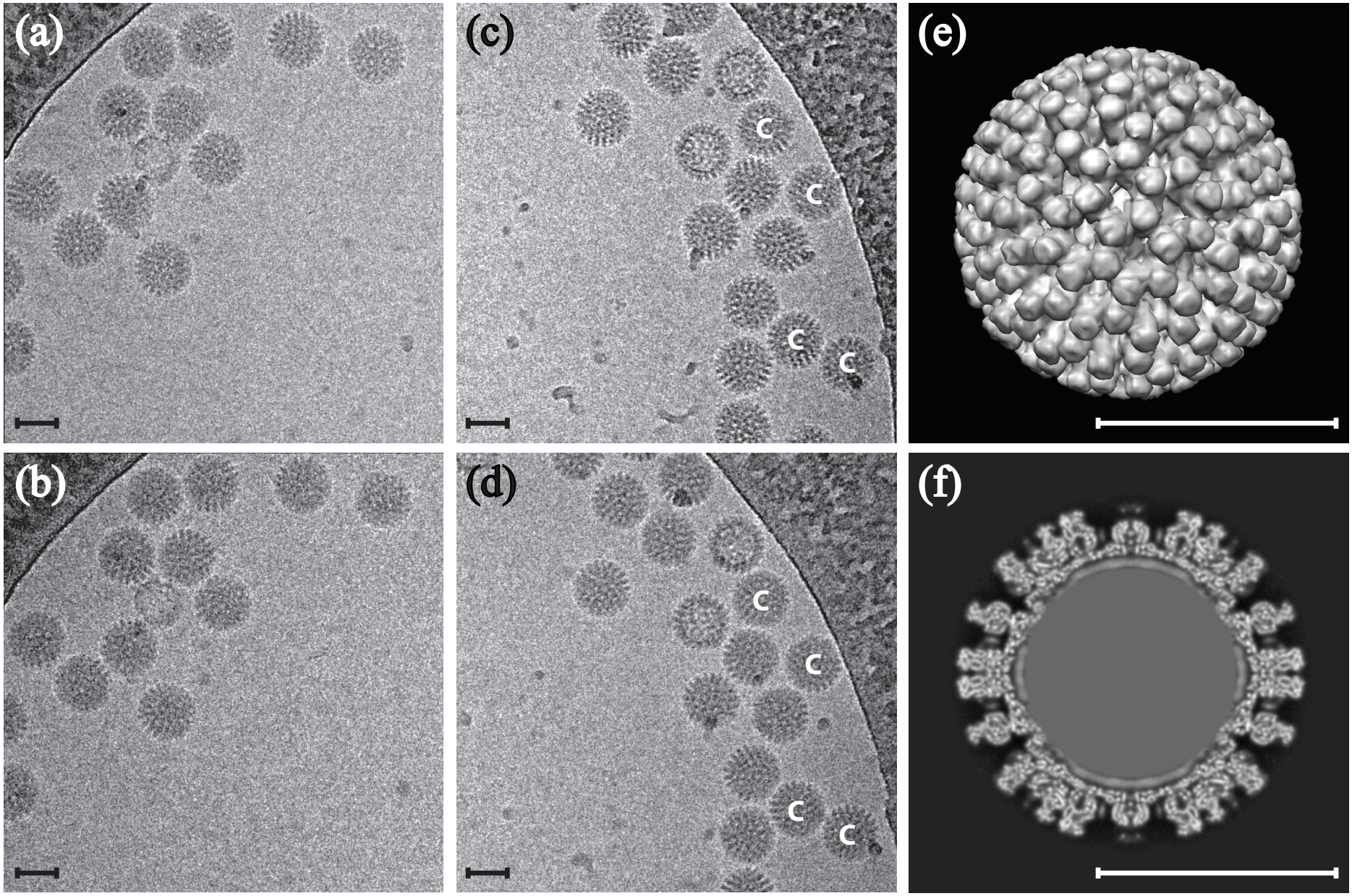


(d)

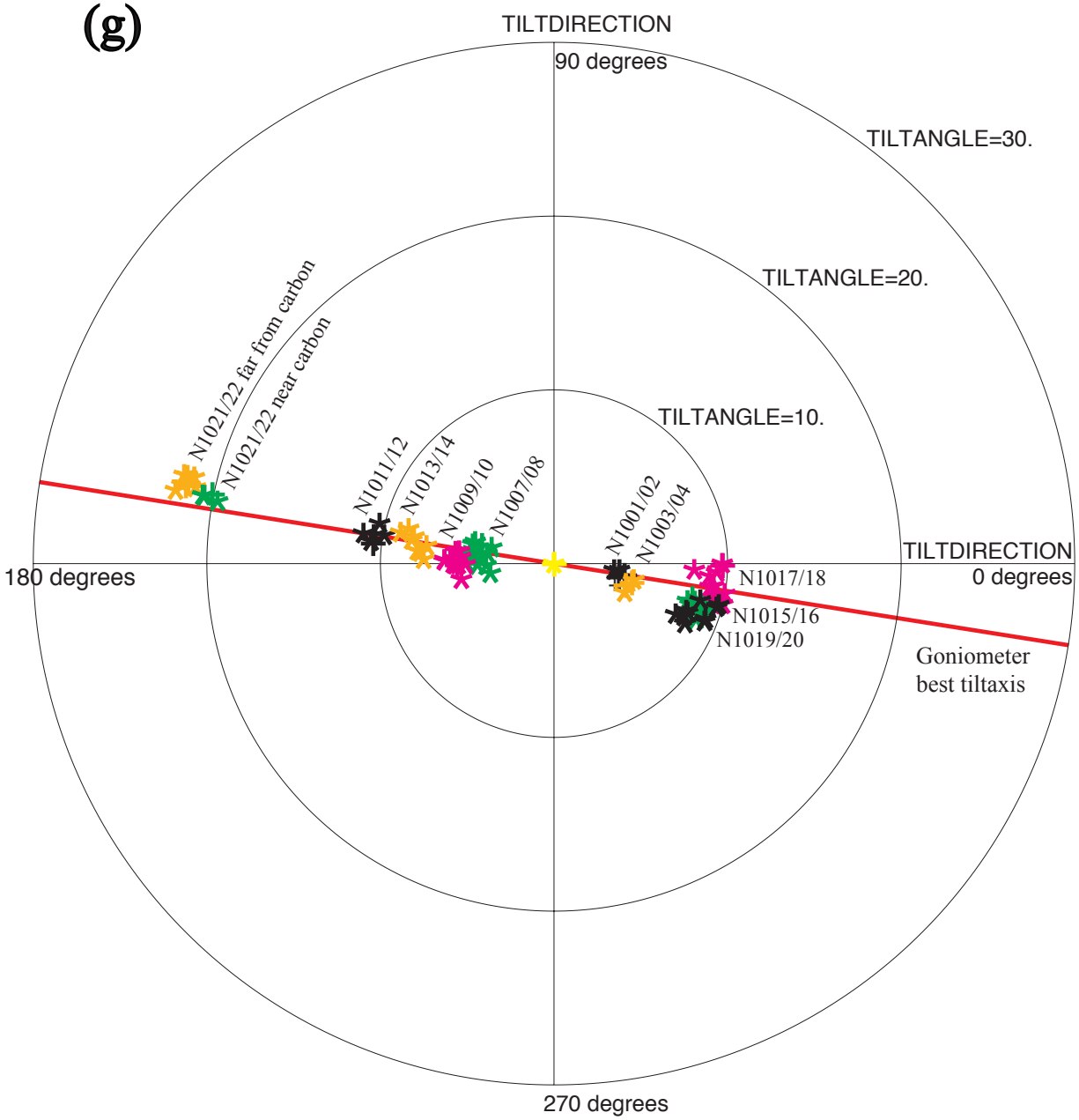


Specimens and tilt pairs by Lori Passmore (empty 70S) and Luciano Ciccarelli (FAS)

Rotavirus (T=13, MW 50MDa) tilt pair images: James Chen & Niko Grigorieff, Brandeis



# Rotavirus 50MDa : 10 tilt pairs, Chen & Grigorieff

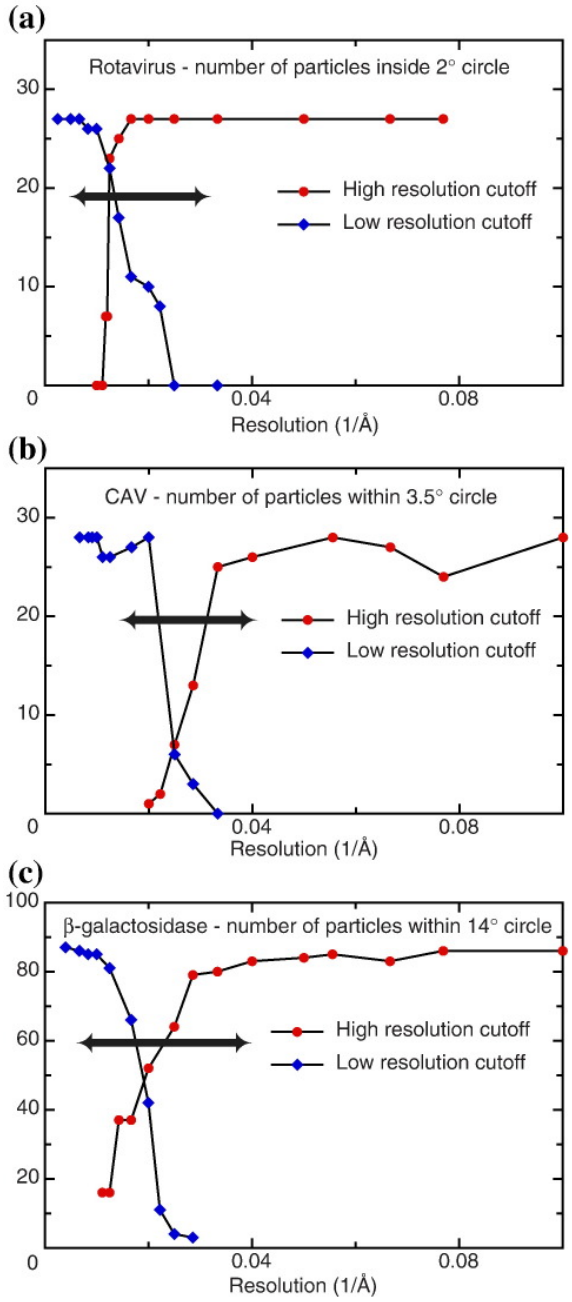



| Film pair | <TANG> (sd) | Nom. TANG |
|---|---|---|
| N1001/2 | +3.83 (±0.20) | +5.0 |
| N1003/4 | +4.50 (±0.21) | +5.0 |
| N1007/8 | -4.24 (±0.39) | -5.0 |
| N1009/10 | -5.67 (±0.33) | -5.0 |
| N1011/12 | -10.4 (±0.44) | -10.0 |
| N1013/14 | -8.07 (±0.63) | -10.0 |
| N1015/16 | +8.67 (±0.45) | +10.0 |
| N1017/18 | +9.34 (±0.53) | +10.0 |
| N1019/20 | +8.83 (±0.81) | +10.0 |
| N1021/22 | -21.14 (±0.95) | -20.0 |

Fig.1

# Table 2 – overview of TPPP (tilt pair parameter plot) statistics

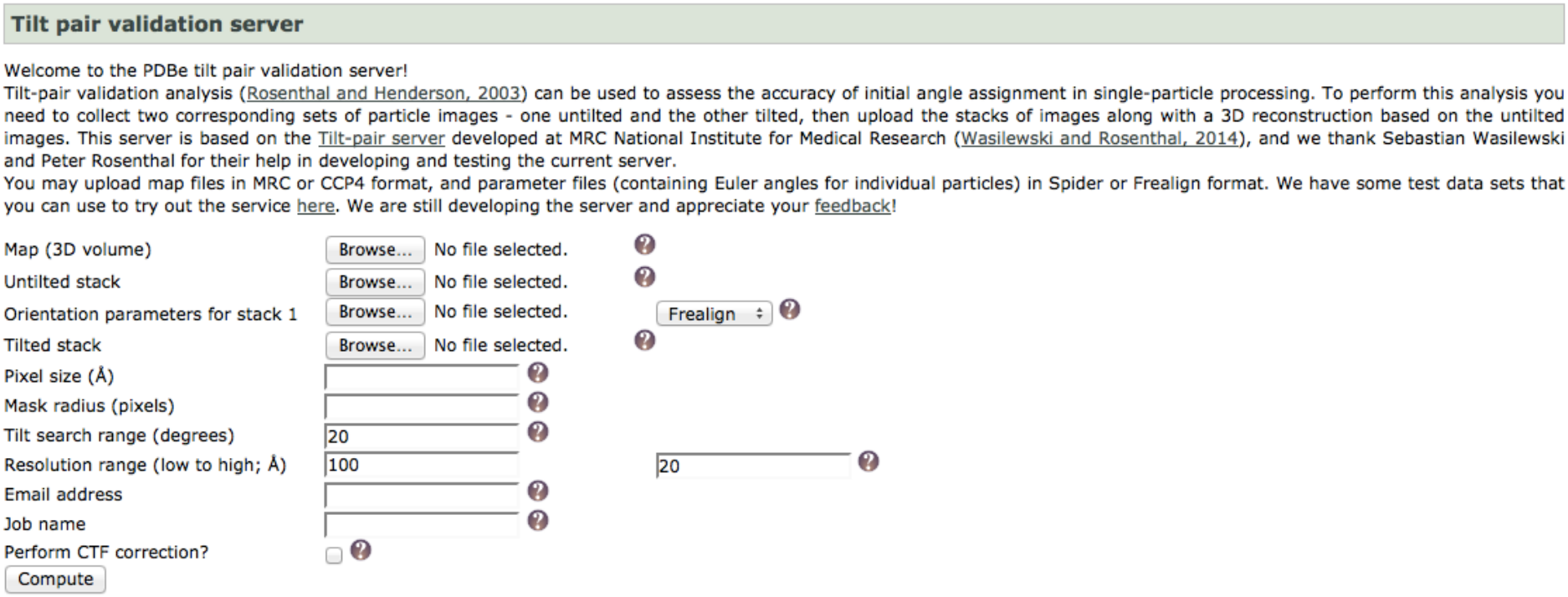
| Specimen | Symmetry | Particle size | Molecular Weight | Number of tilt pairs | Number of particles | Successful alignment (%) | Mean/maximum angular error (degs) | |
|---|---|---|---|---|---|---|---|---|
| Rotavirus DLP | I2 | 700 Å | 50 MDa | 10 | 95 | 100/100 | 0.25 | 1.0 |
| Norwalk virus | I1 | 420 Å | 10 MDa | 1 | 51 | 98 | 1.5 | 2.5 |
| HdH | D5 | 550 Å | 8 MDa | 3 | 45 | 78 | 1.5 | 3.0 |
| CAV | I2 | 255 Å | 2.7 MDa | 1 | 45 | 62/82 | 2.5 | 3.5 |
| FAS | D3 | 260x220 Å | 2.6 MDa | 2 | 44 | 59/95 | 4.0 | 6.0 |
| 70S ribosomes | C1 | 270x260 Å | 2.6 MDa | 12 | 220 | 45/75 | 4.0 | 5.0 |
| PDH-E2CD | I1 | 280 Å | 1.6 MDa | 1 | 50 | 62/94 | 3.0 | 4.0 |
| Thermus V-ATPase | C1 | 250x140 Å | 0.6 MDa | 1 | 50 | 54/80 | 10.0 | 16.0 |
| Bovine F-ATPase | C1 | 250x140 Å | 0.6 MDa | 1 | 29 | 52/79 | 20.0 | 25.0 |
| DNA-PKcs | C1 | 150x120 Å | 0.47 MDa | 14 | 108 | 44/81 | 15.0 | 17.0 |
| β-galactosidase | D2 | 180x130x95 Å | 0.45 MDa | 2 | 119 | 74/91 | 10.0 | 14.0 |



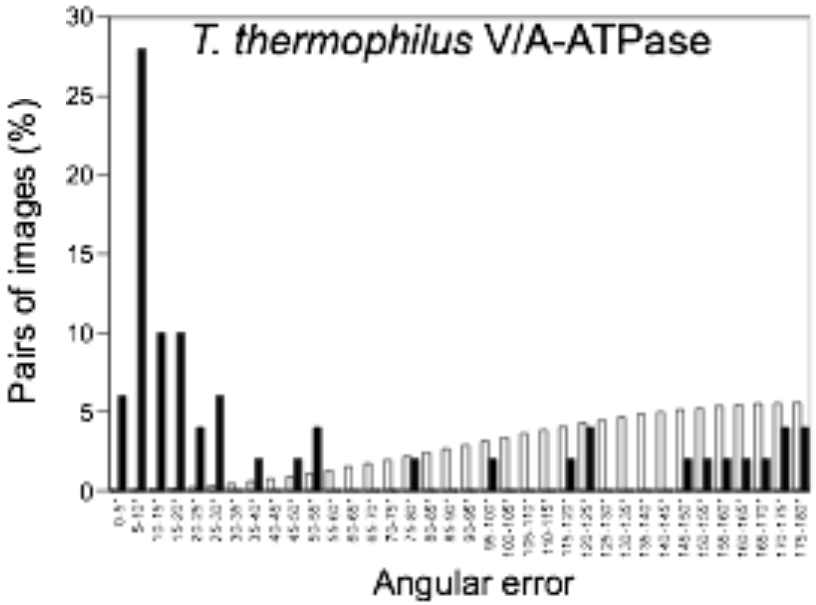

What resolution range should I use to reifne orientations?

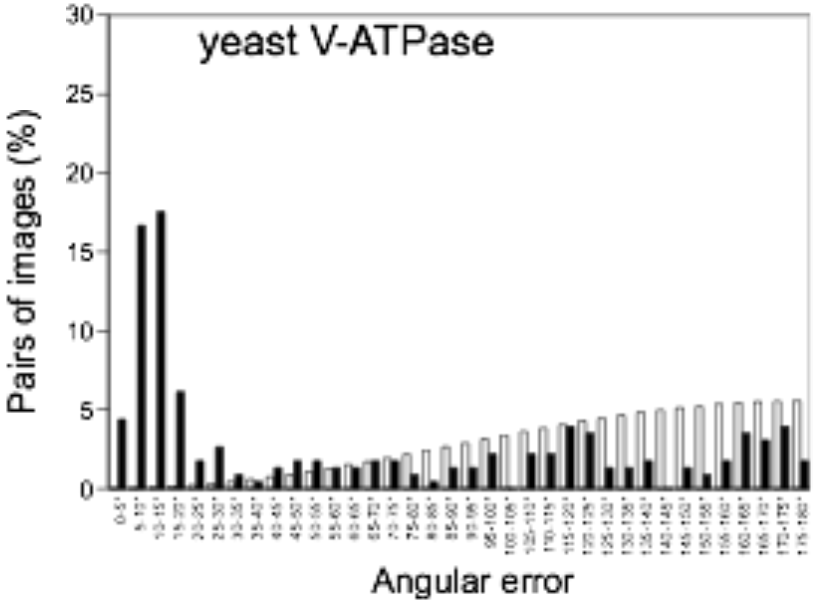
Frequency-limited refinement does not lead to worse orientations.

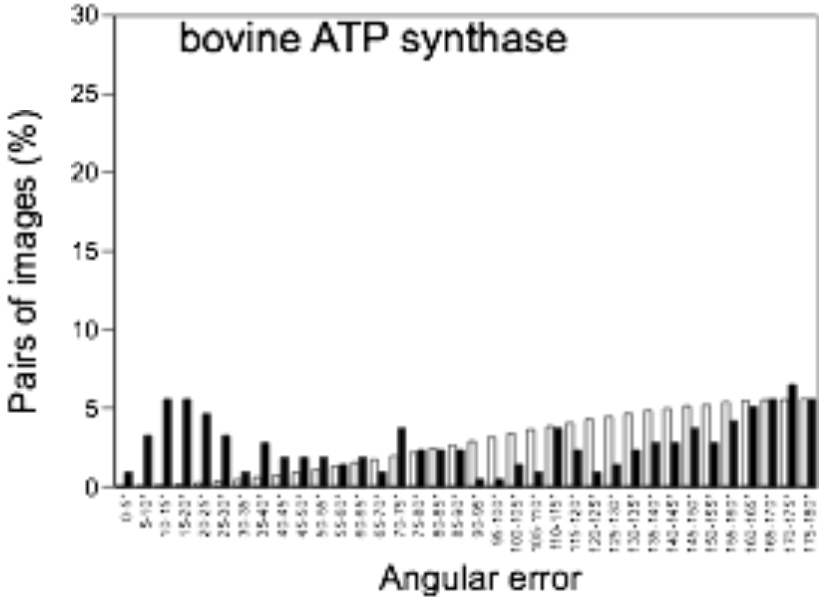
# PDBe Tilt-Server



# Accuracy of particle alignment (Baker & Rubinstein, PNAS 2012)



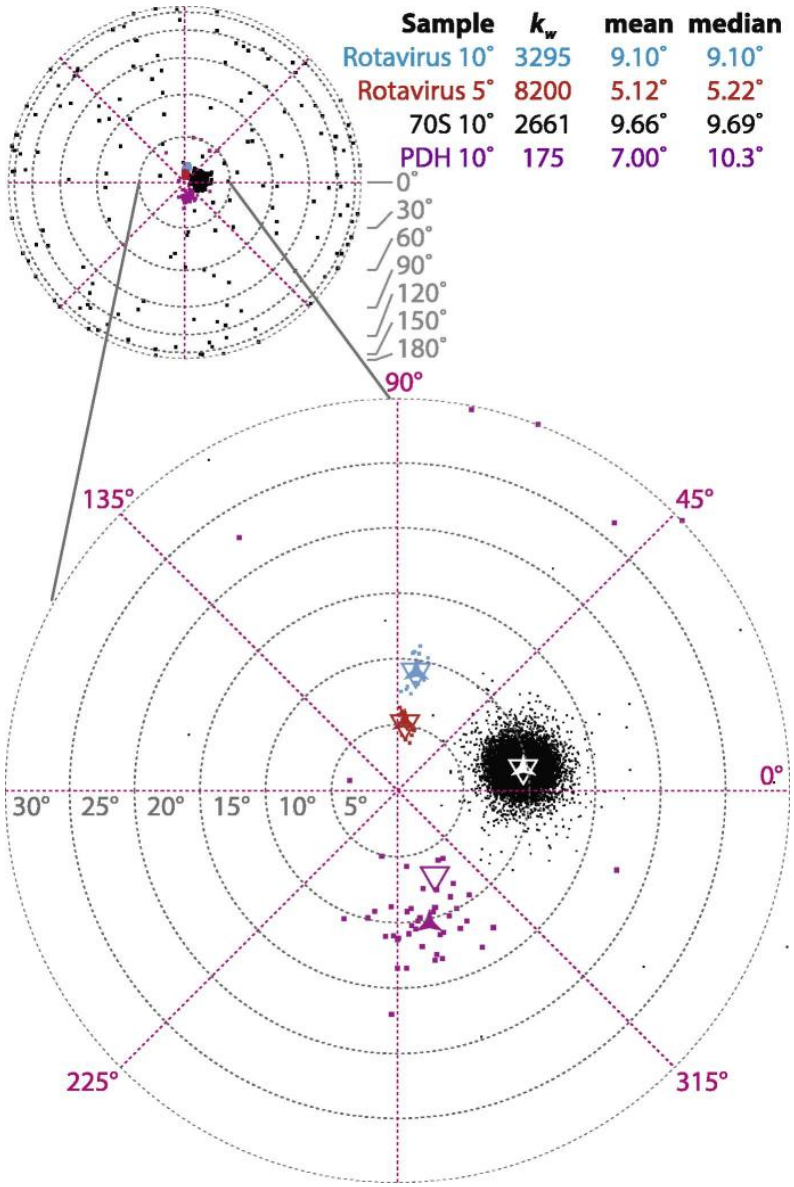



# TILTSTATS (Russo and Passmore, JSB 2014)

$$f(\omega) = e^{\kappa \cos \omega}$$

$K > 10$

| | |
|---|---|
| Rotavirus | 8200 |
| 70S | 2661 |
| PDH | 175 |



# Application of Tilt-Pairs

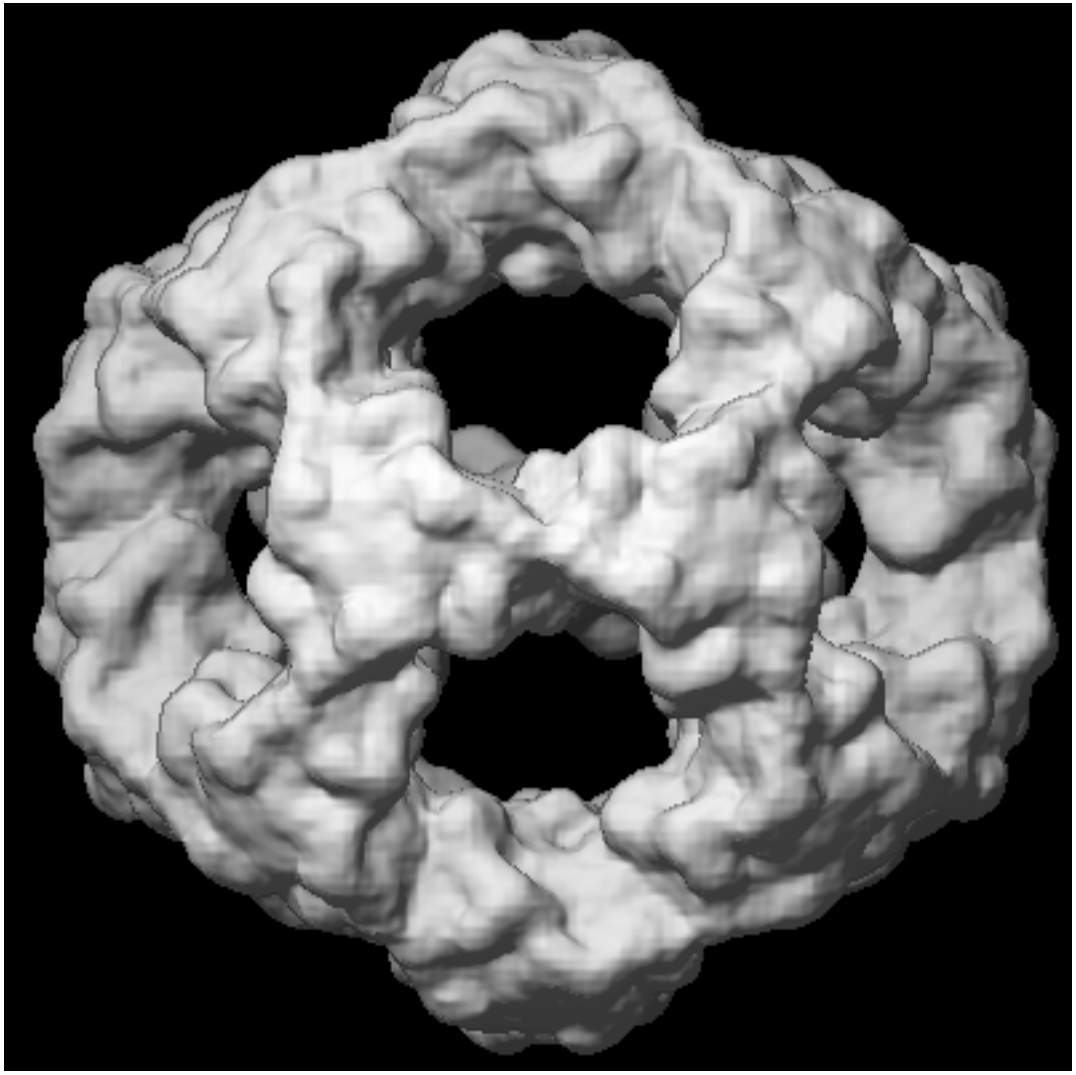
- Learn how to optimize orientation determination for your molecule, map, and images
- Does tilt-pair parameter plot match map resolution?
- Validate a map
- Tilt-pairs for whole dataset, e.g. heterogeneity
- Negative-stain problems

Do we need to do tilt-pairs at high resolution?

# MAP RESOLUTION

# Resolution Measures

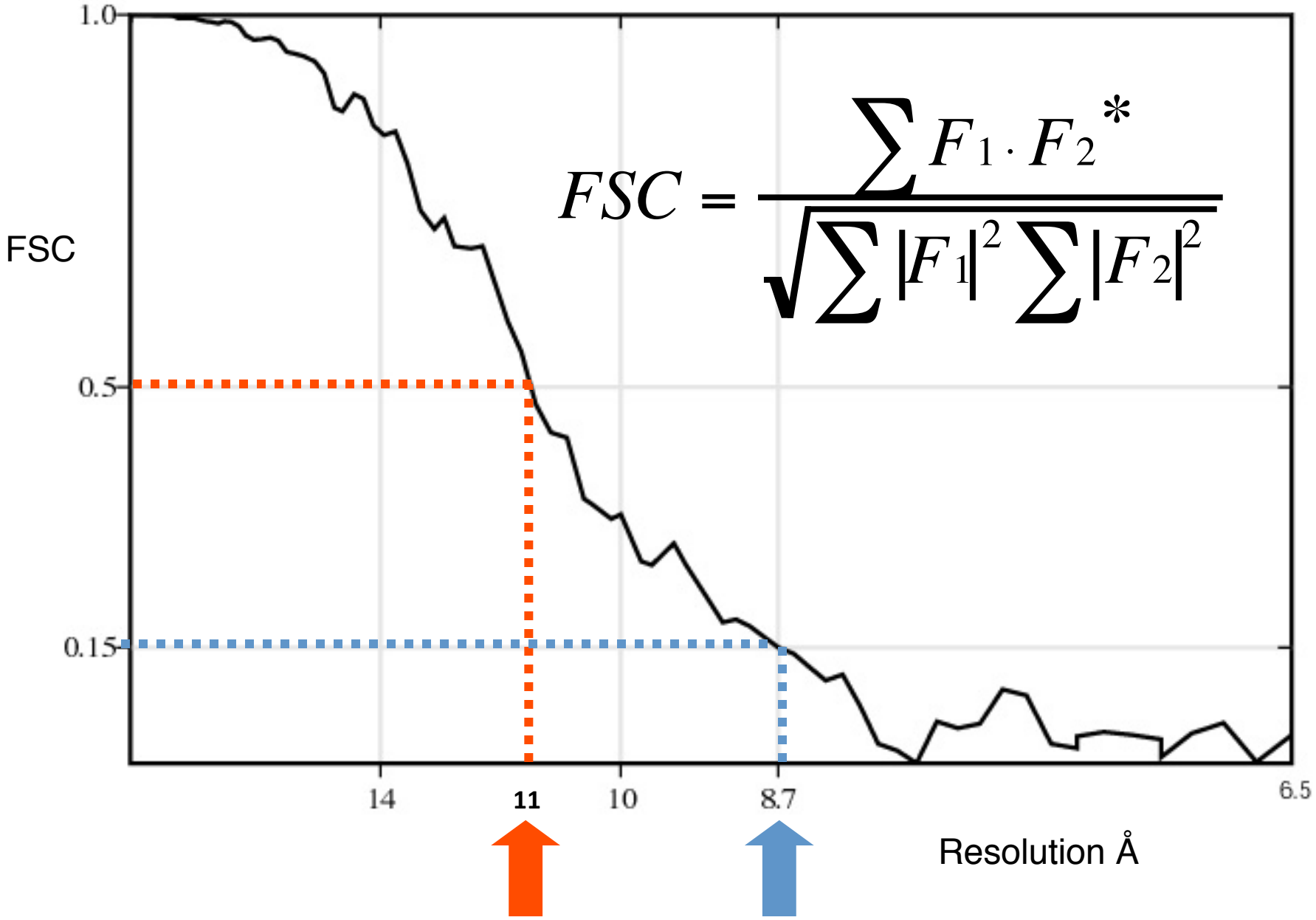
- FSC
- Rmeasure
- Resmap
- Bootstrap
- 3D variance estimates



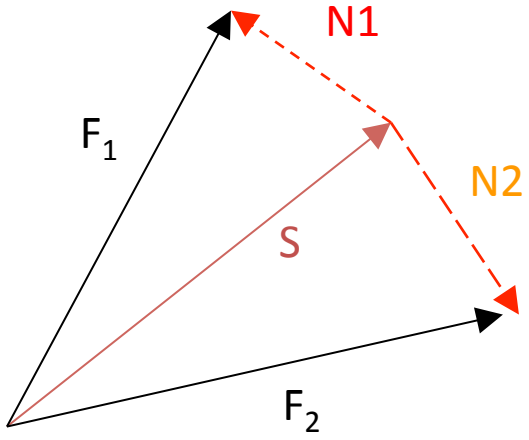
Map Resolution Should Be Reported, and Visible Structural Features Should Be in Accordance with the Claimed Resolution

Fourier Shell Correlation: Show the whole curve.

Visibility of expected features – α-helices visible at 9 Å resolution? β-strands at 4.8 Å resolution? Side-chains beyond 4 Å?


1.0
0.5
0.15
FSC
$FSC = \frac{\sum F_1 \cdot F_2^*}{\sqrt{\sum |F_1|^2 \sum |F_2|^2}}$
14
11
10
8.7
6.5
Resolution Å

$$FSC = \frac{\sum F_1 \cdot F_2^*}{\sqrt{\sum |F_1|^2 \sum |F_2|^2}}$$




Signal~Noise FSC=0.5

$$FSC_{full} = \frac{2FSC}{1+FSC}$$

# Correlation with a perfect reference

$$C_{ref} = \frac{\sum F_1 \cdot F_{ref}}{\sqrt{\sum |F_1|^2 \sum |F_{ref}|^2}} = \frac{\sum |F_1||F_{ref}|\cos(\Delta\varphi)}{\sqrt{\sum |F_1|^2 \sum |F_{ref}|^2}}$$

Looks like figure-of-merit (Blow and Crick,1959)

# Estimating $C_{ref}$

$$C_{ref} = \sqrt{\frac{2FSC}{1+FSC}}$$

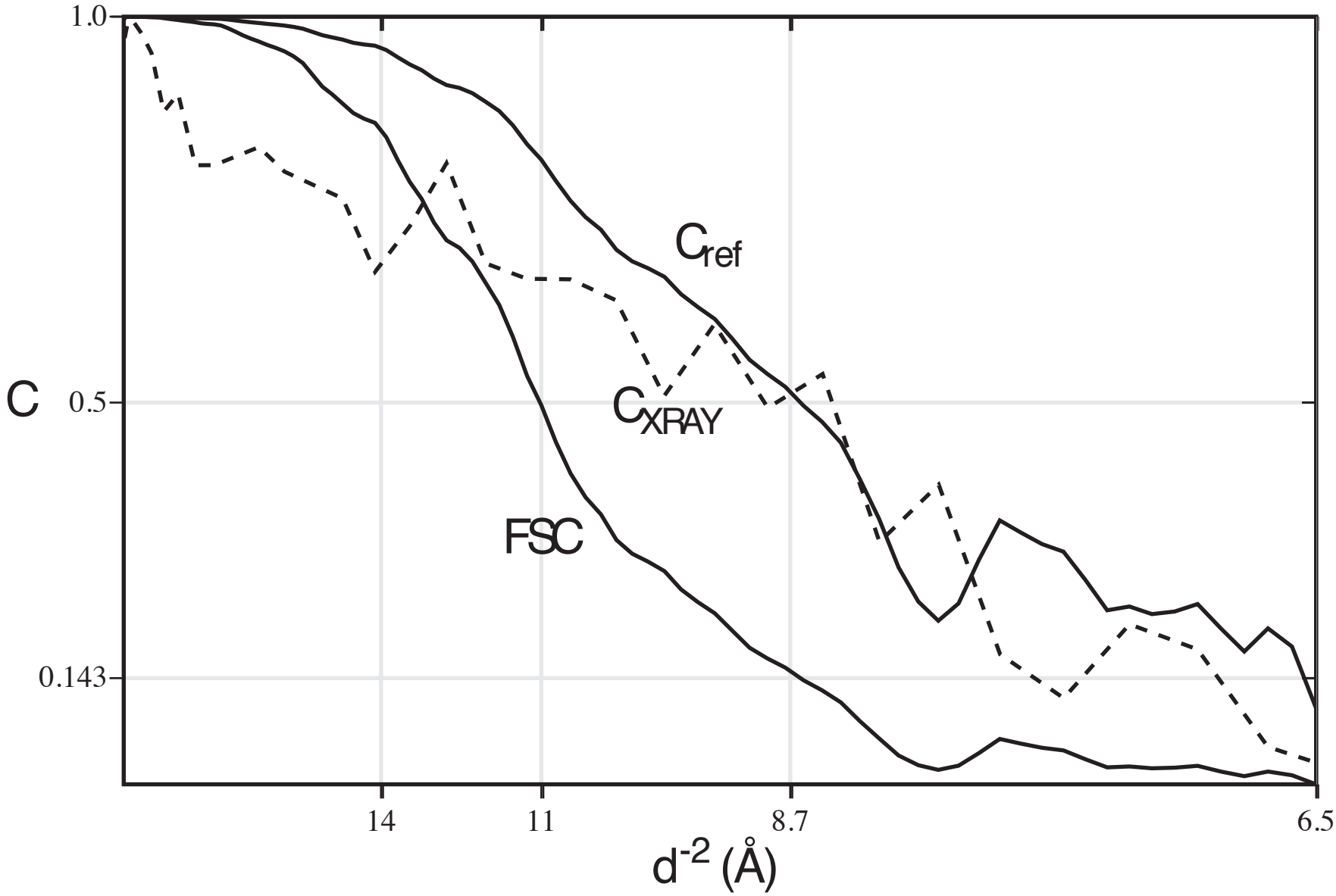
$C_{ref}$=0.5 FSC=0.14

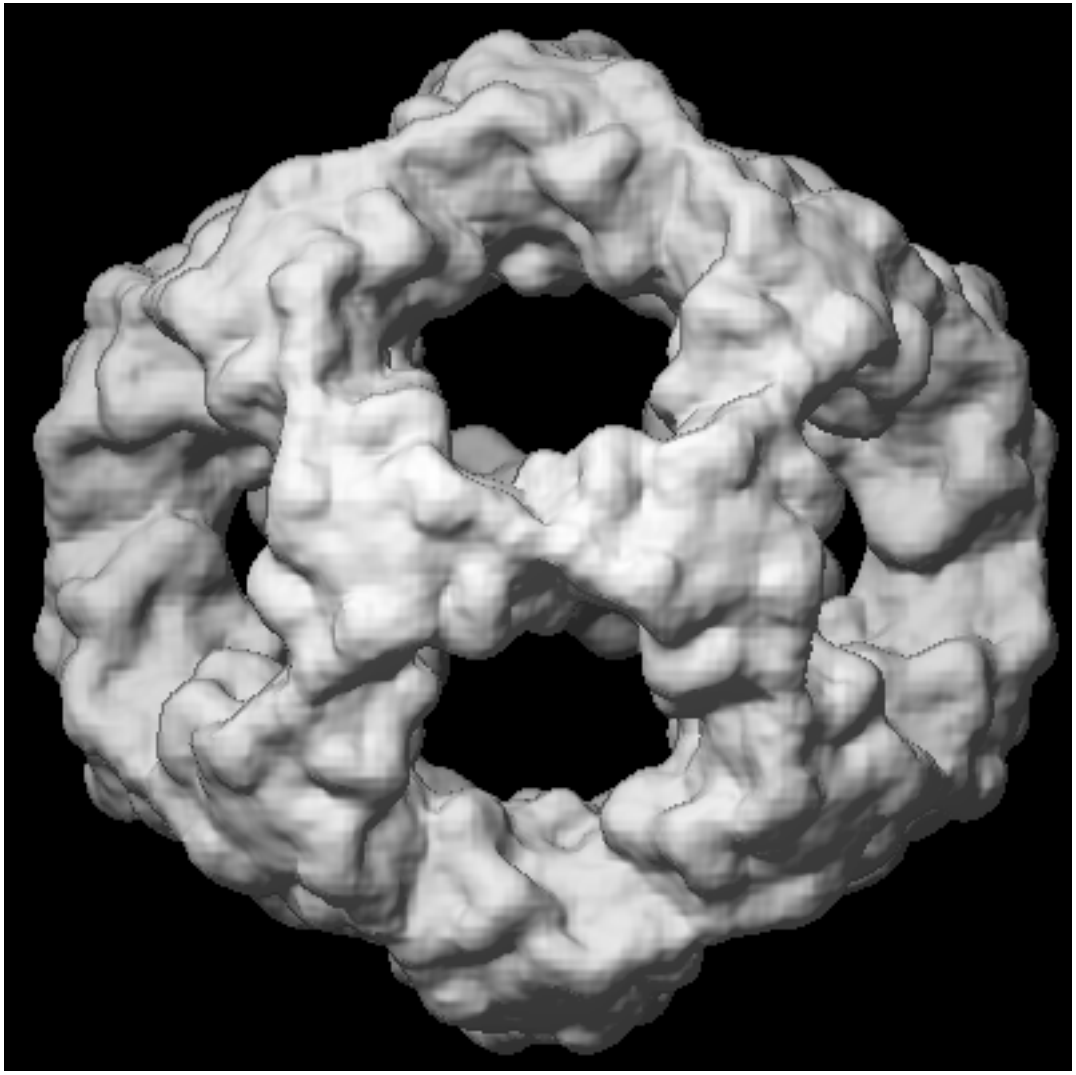
## $C_{ref}$ corresponds to crystallographic FOM "Figure of Merit"

$C_{ref}$=0.5 mean phase error 60 ° (last shell) interpretable by an atomic model

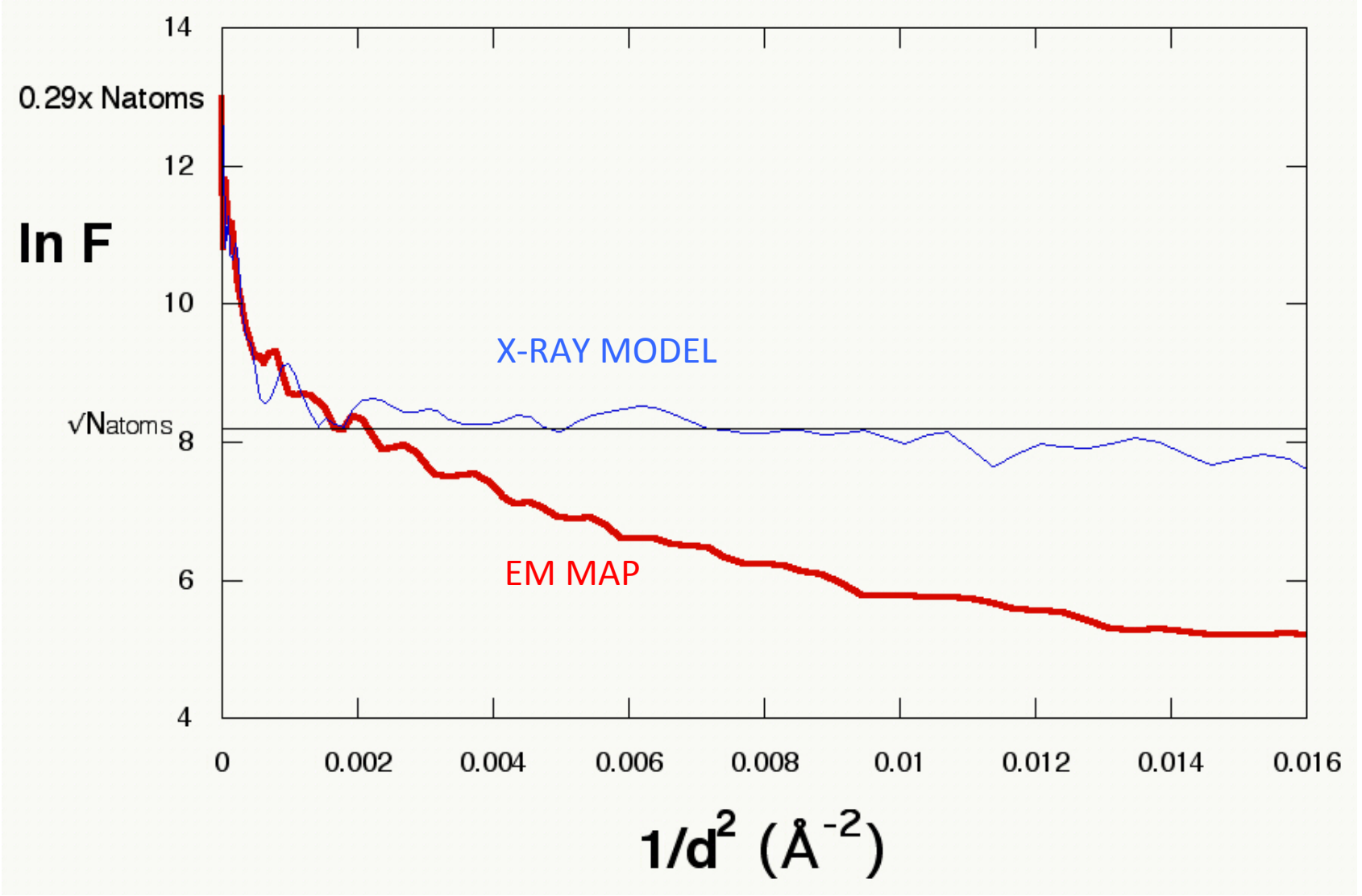
| FSC | $FSC_{FULL}$ | $C_{REF}$ | PHASE ERROR | $S/N_{1/2}$ |
|---|---|---|---|---|
| 0.50 | 0.67 | 0.82 | 35° | 1.00 |
| 0.33 | 0.50 | 0.71 | 45° | 0.71 |
| 0.14 | 0.25 | 0.50 | 60° | 0.41 |

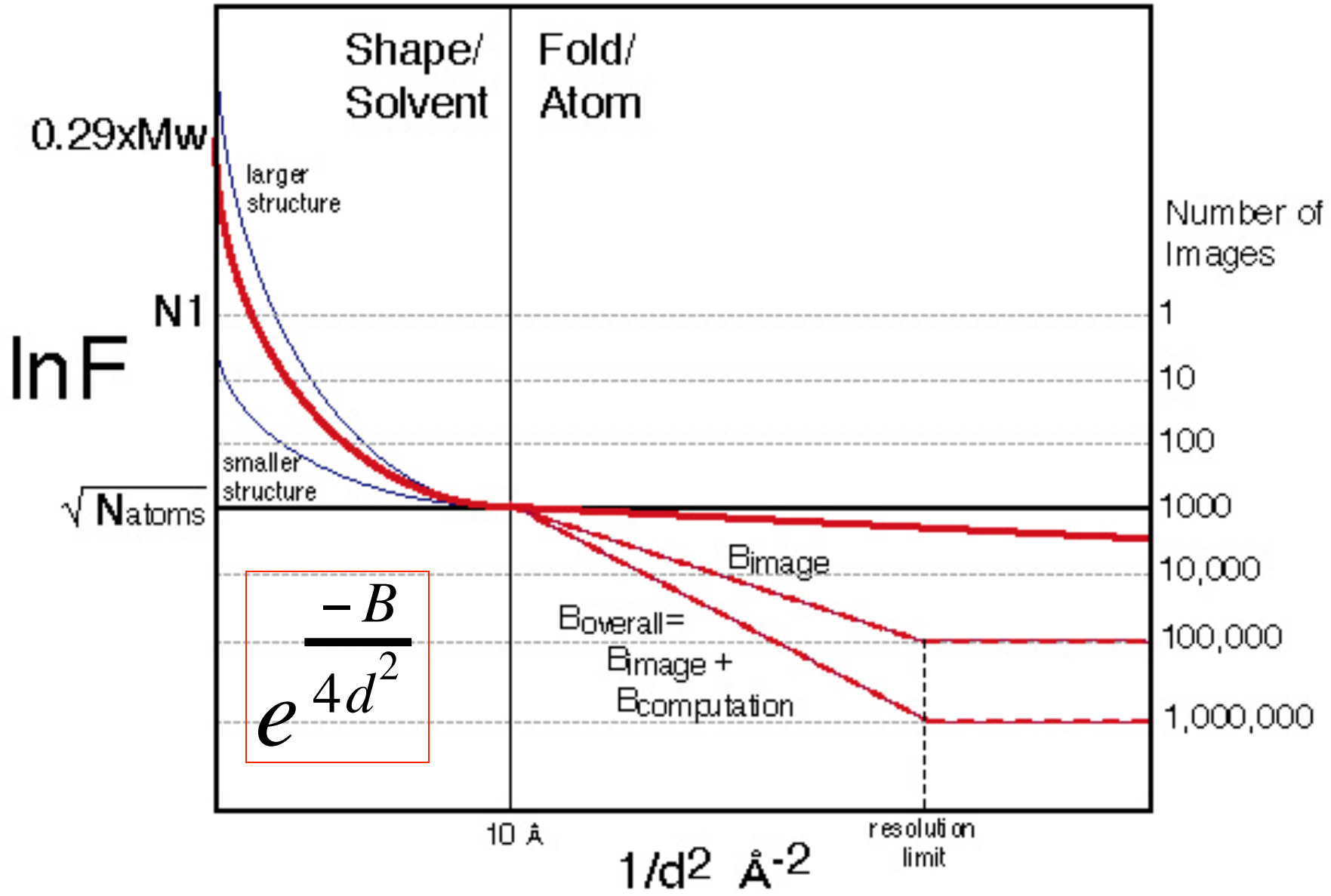
# FSC between Map and Model ($C_{ref}$)





# RADIALLY-AVERAGED EM MAP AMPLITUDES COMPARED TO X-RAY




Shape/
Solvent
Fold/
Atom
0.29xMw
larger
structure
N1
lnF
smaller
structure
√ Natoms
Number of
Images
1
10
100
1000
10,000
100,000
1,000,000
Bimage
Boverall=
Bimage +
Bcomputation
$e^{\frac{-B}{4d^2}}$
10 Å
resolution
limit
1/d2 Å-2

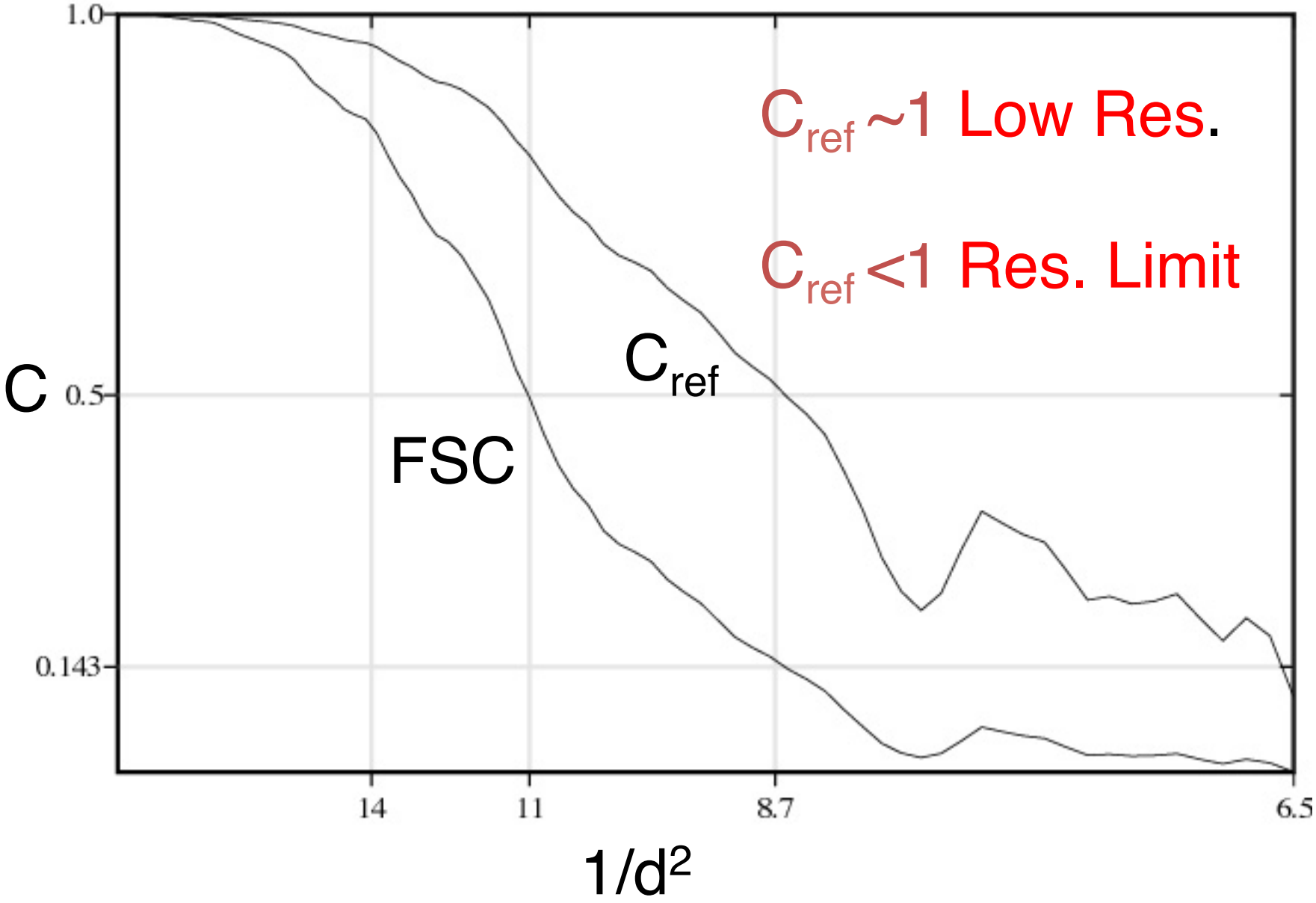
# Contrast Restoration

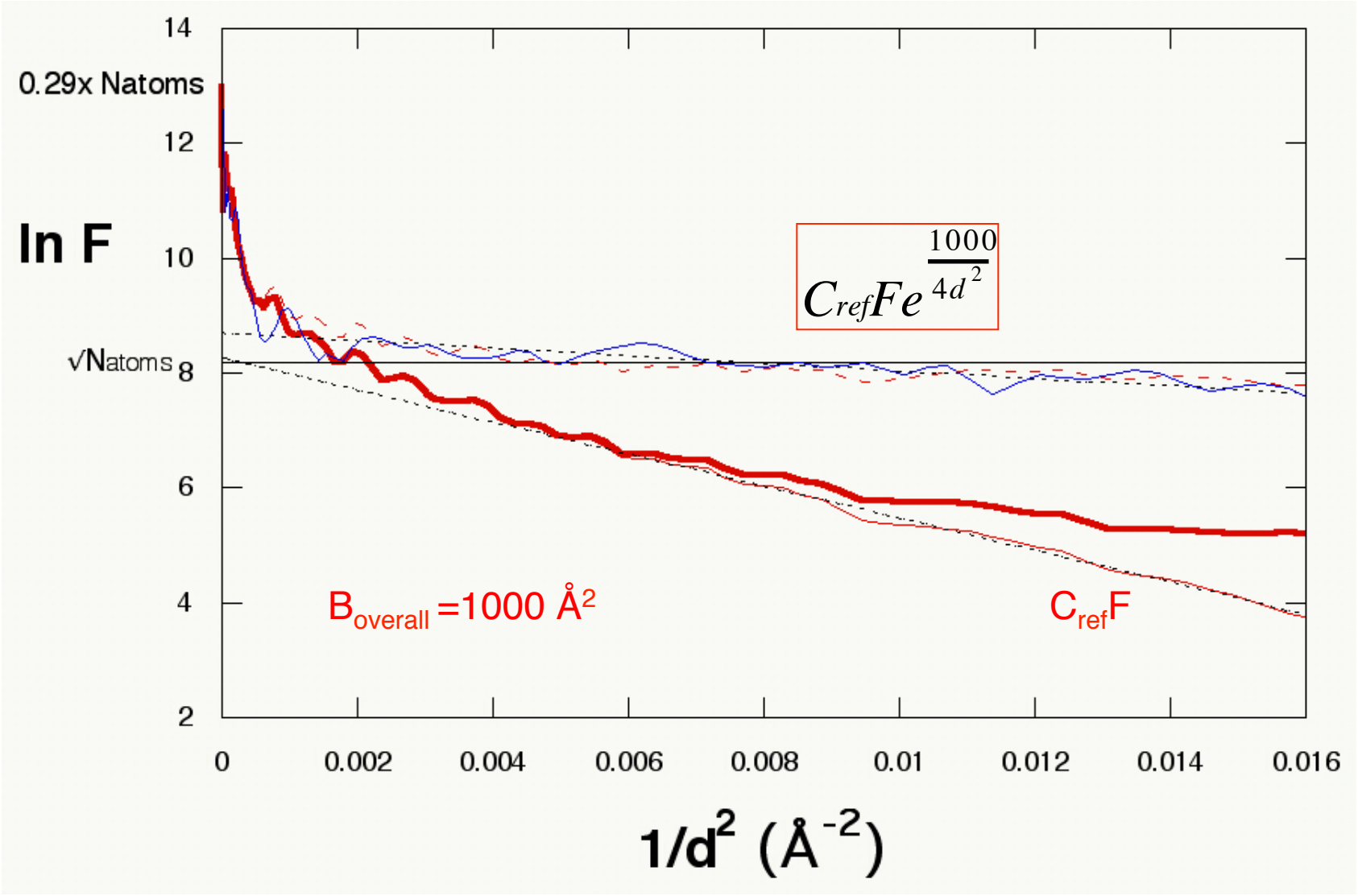
Incorrect Scaling of High and Low Resolution Amplitudes makes map looks featureless

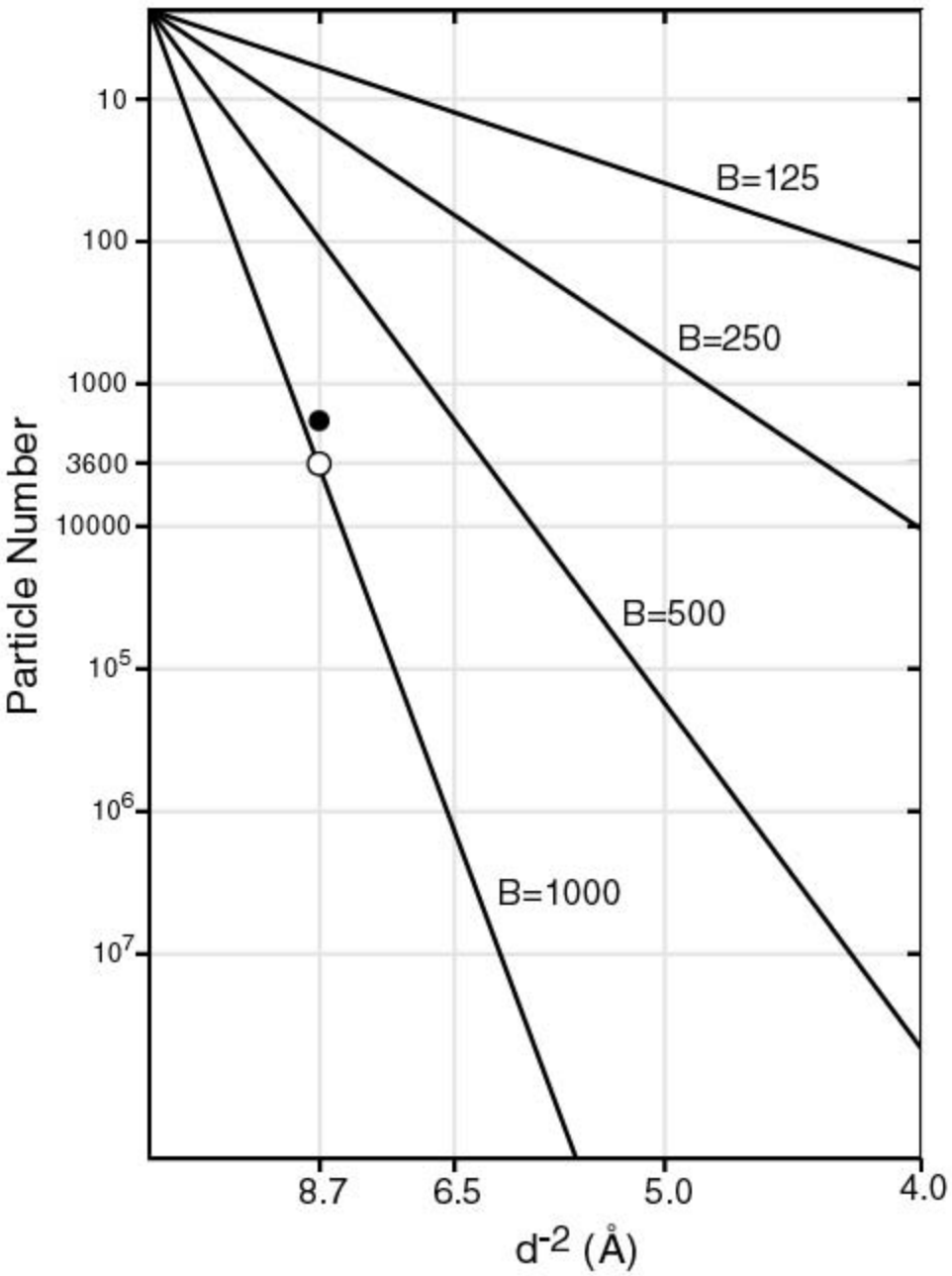
Problem: Application of a negative temperature factor amplifies both signal and noise

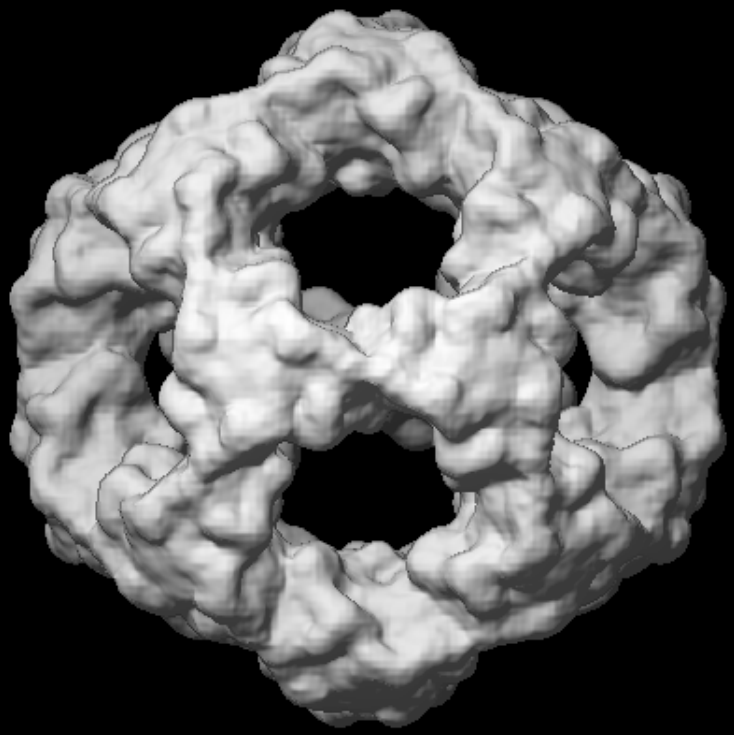
Suppress Noise by using
Noise-Weighted Structure Factors $C_{ref}F$
Similar to Figure-of-Merit weighting

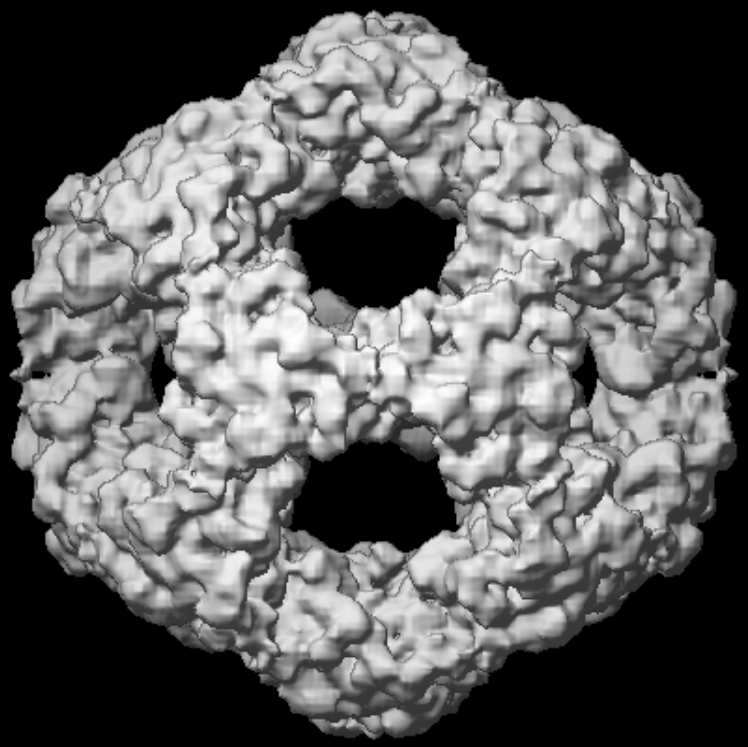
$$C_{ref} = \sqrt{\frac{2FSC}{1+FSC}}$$


1.0
0.5
0.143
C
14
11
8.7
6.5
1/d²
FSC
C_ref
C_ref ~1 Low Res.
C_ref <1 Res. Limit


14
0.29x Natoms
12
ln F
10
√Natoms
8
6
4
2
0
0.002
0.004
0.006
0.008
0.01
0.012
0.014
0.016
1/d² (Å⁻²)
$C_{ref}Fe^{\frac{1000}{4d^2}}$
B_overall =1000 Å²
C_ref F

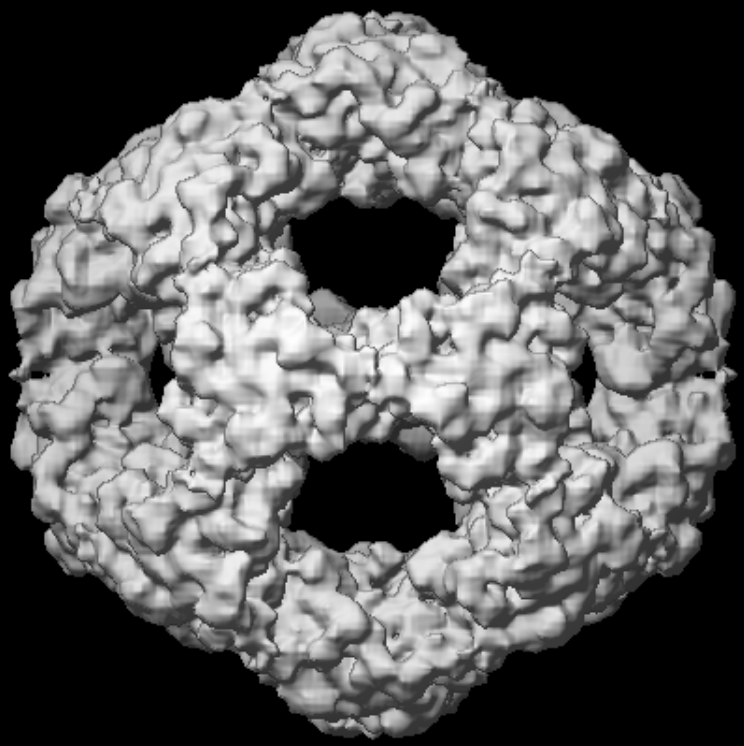

Particle Number
10
100
1000
3600
10000
$10^5$
$10^6$
$10^7$
B=125
B=250
B=500
B=1000
8.7
6.5
5.0
4.0
$d^{-2}$ (Å)

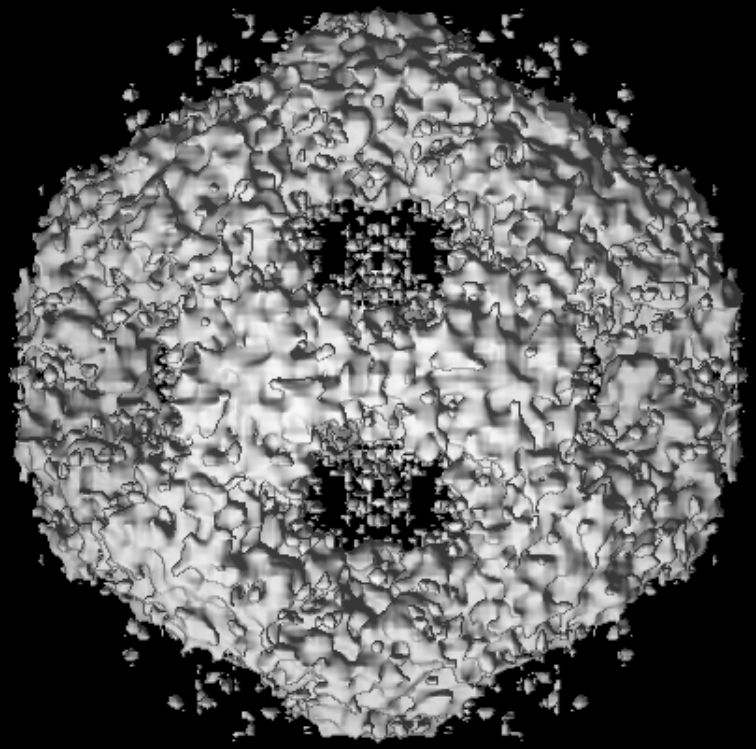




F

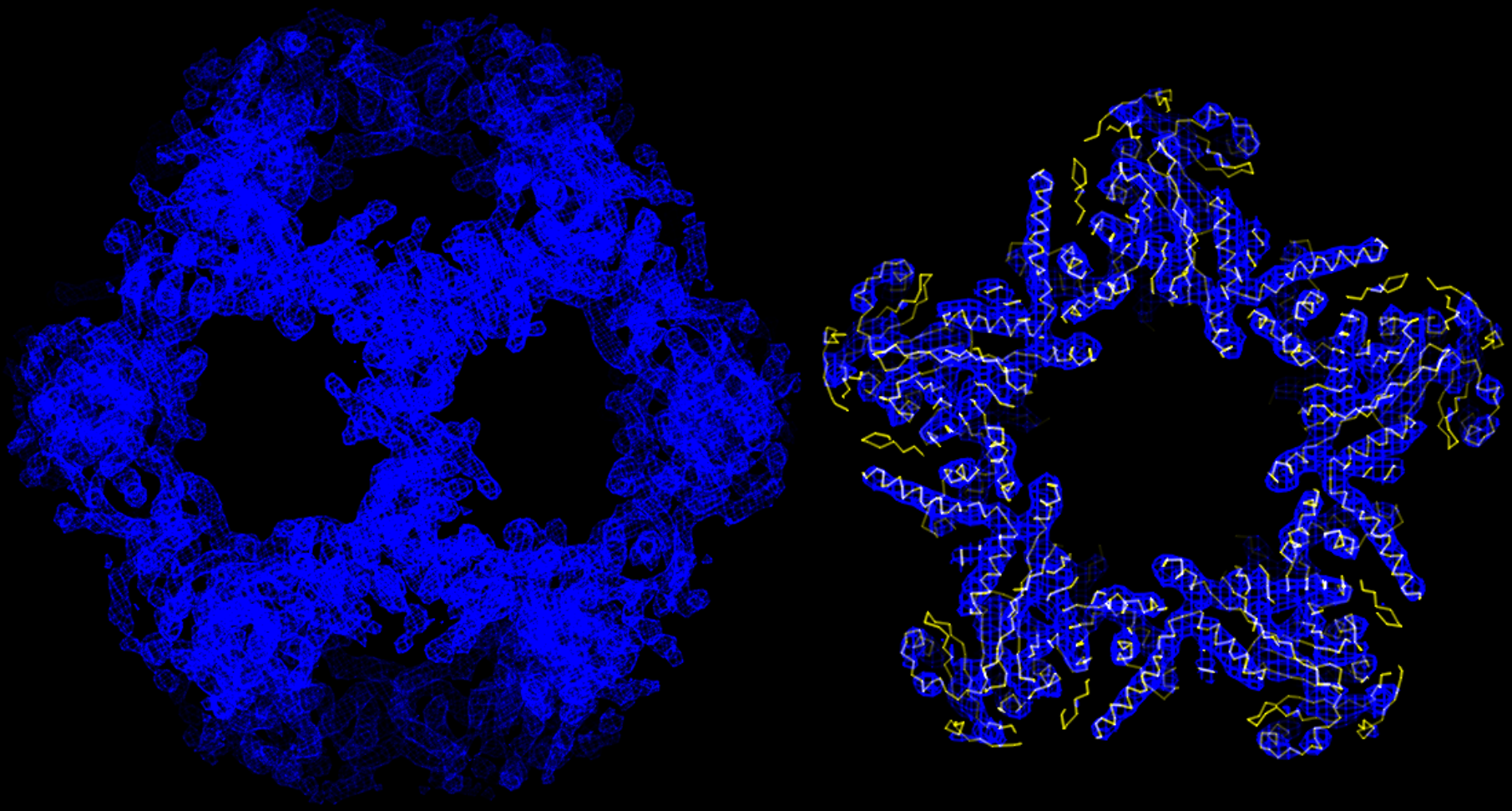
$C_{ref}Fe^{(1000/4d2)}$





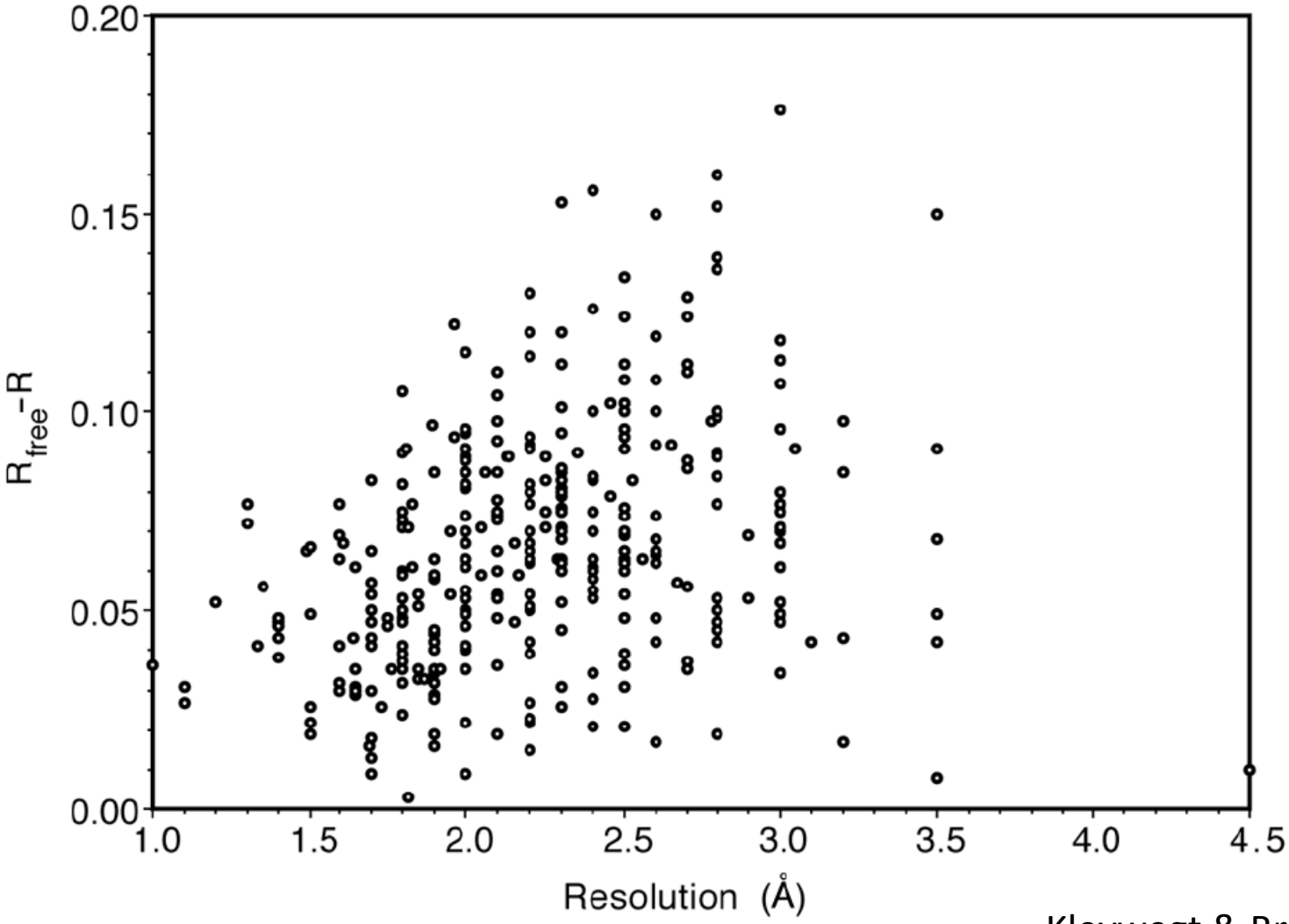
$C_{ref}Fe^{(1000/4d2)}$

$Fe^{(1000/4d2)}$



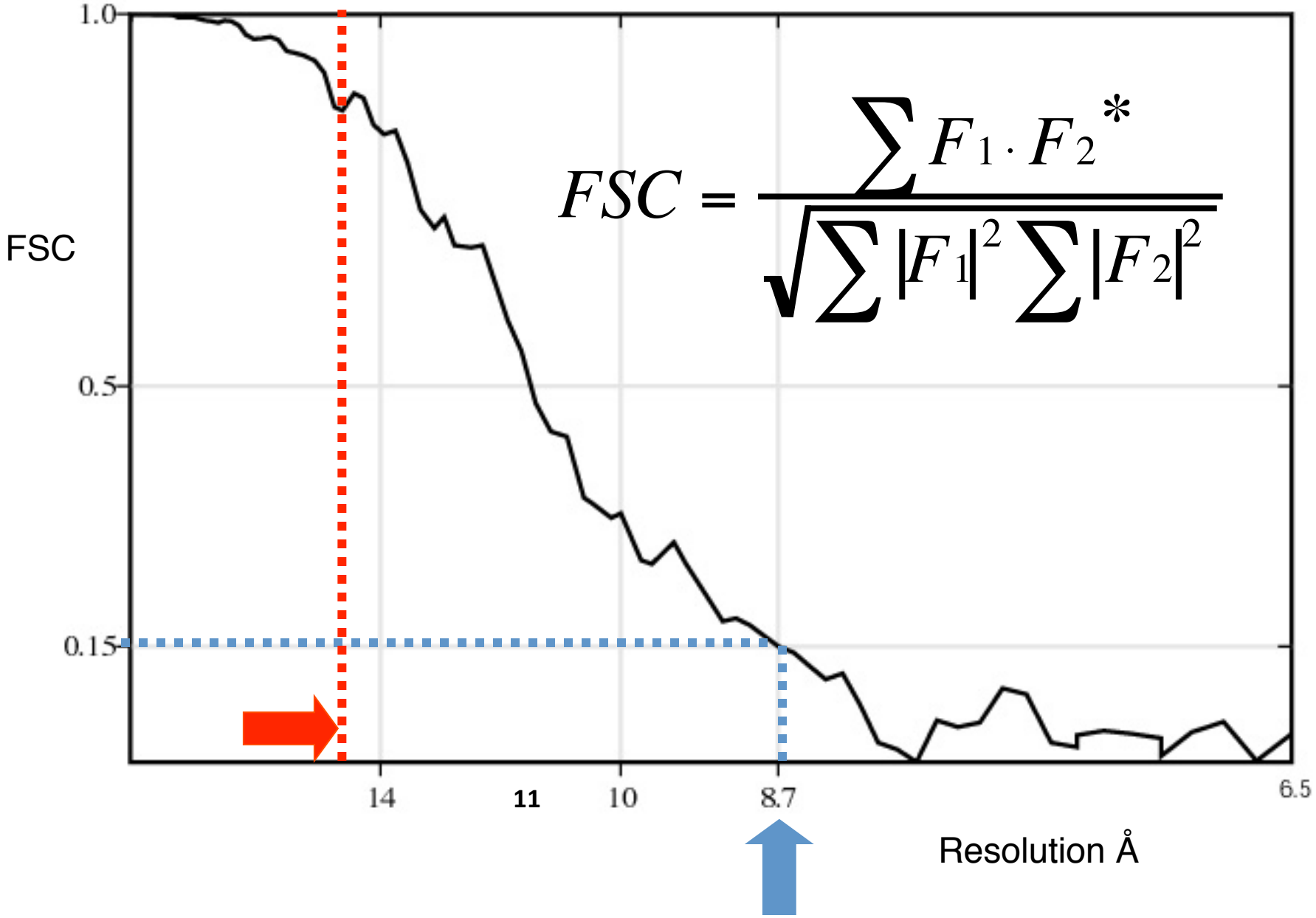
# PREVENTING AND DETECTING OVER-FITTING
# (Validating Resolution)

# $R_{free}$ in X-ray validation




Kleywegt & Brunger 1996

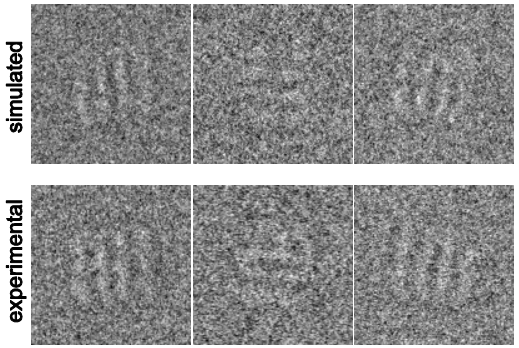
# Free Shells

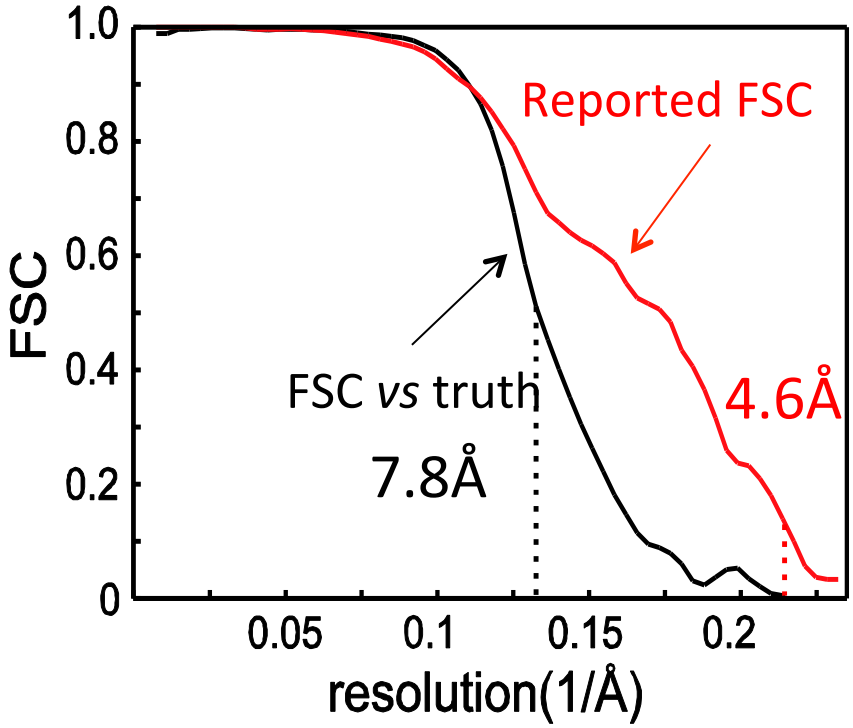

1.0
0.5
0.15
FSC
$FSC = \frac{\sum F_1 \cdot F_2^*}{\sqrt{\sum |F_1|^2 \sum |F_2|^2}}$
14
11
10
8.7
6.5
Resolution Å

# The pitfalls of undetected overfitting

(typical practice until ~2011)

- 20,000 simulated GroEL particles
- Conventional projection matching




FSC between map and (perfect) model at FSC = 0.5

FSC between two independent half data sets at FSC = 0.143

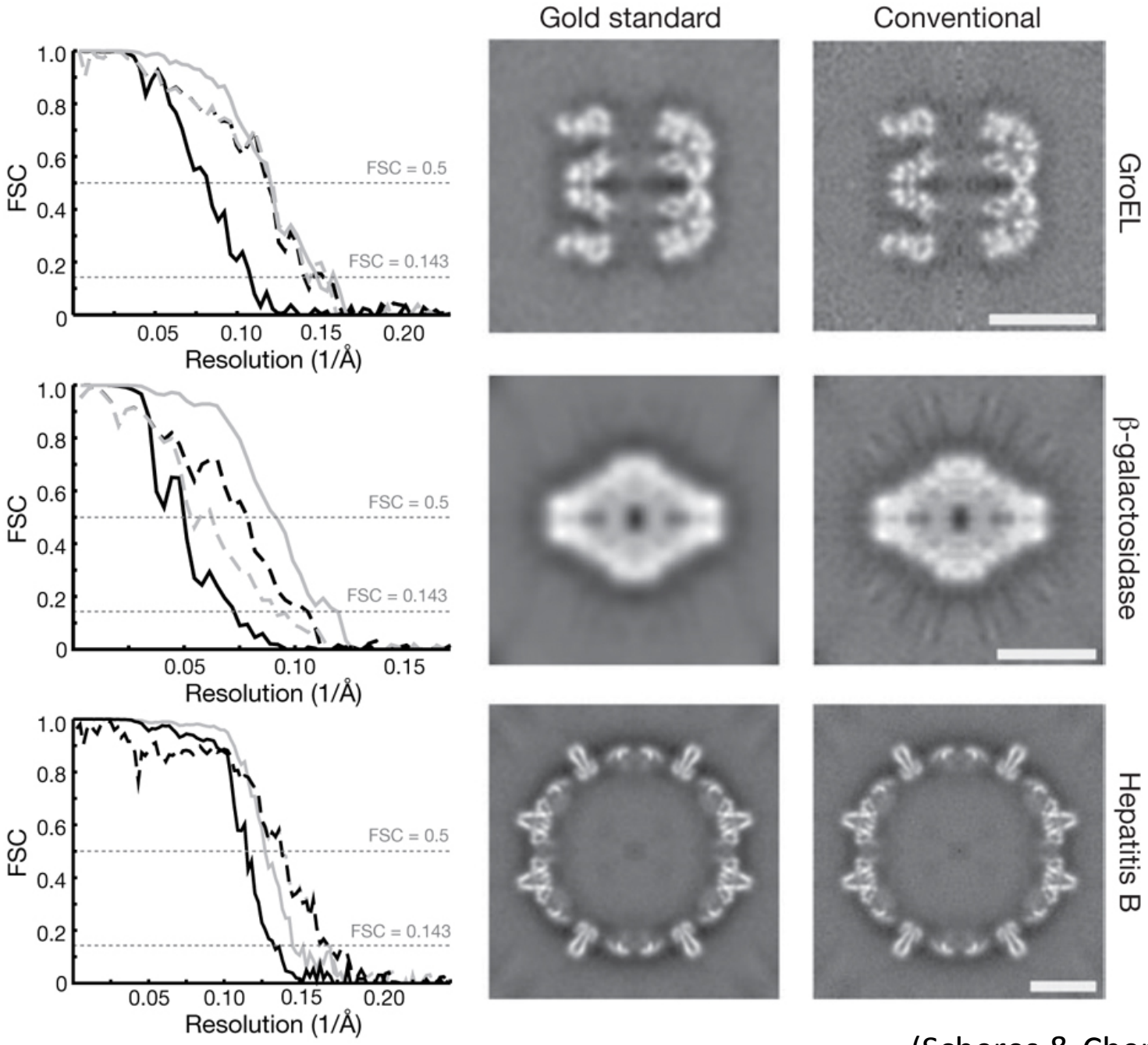
The New York Times | http://nyti.ms/1olthKI

U.S. | NYT NOW

# Facing Challenge to Execution, Texas Calls Its Process the Gold Standard

By MANNY FERNANDEZ and JOHN SCHWARTZ MAY 12, 2014

Use gold-standard FSC calculation (i.e. process data as two completely independent halves - this means independent starting models, refinement and masking)




(Scheres & Chen, 2012)

# Refinement of two independent models or frequency-limited refinement do not lead to worse orientations or resolutions.

Refinement of two independent models
or frequency-limited refinement do not lead
to worse orientations or resolutions.

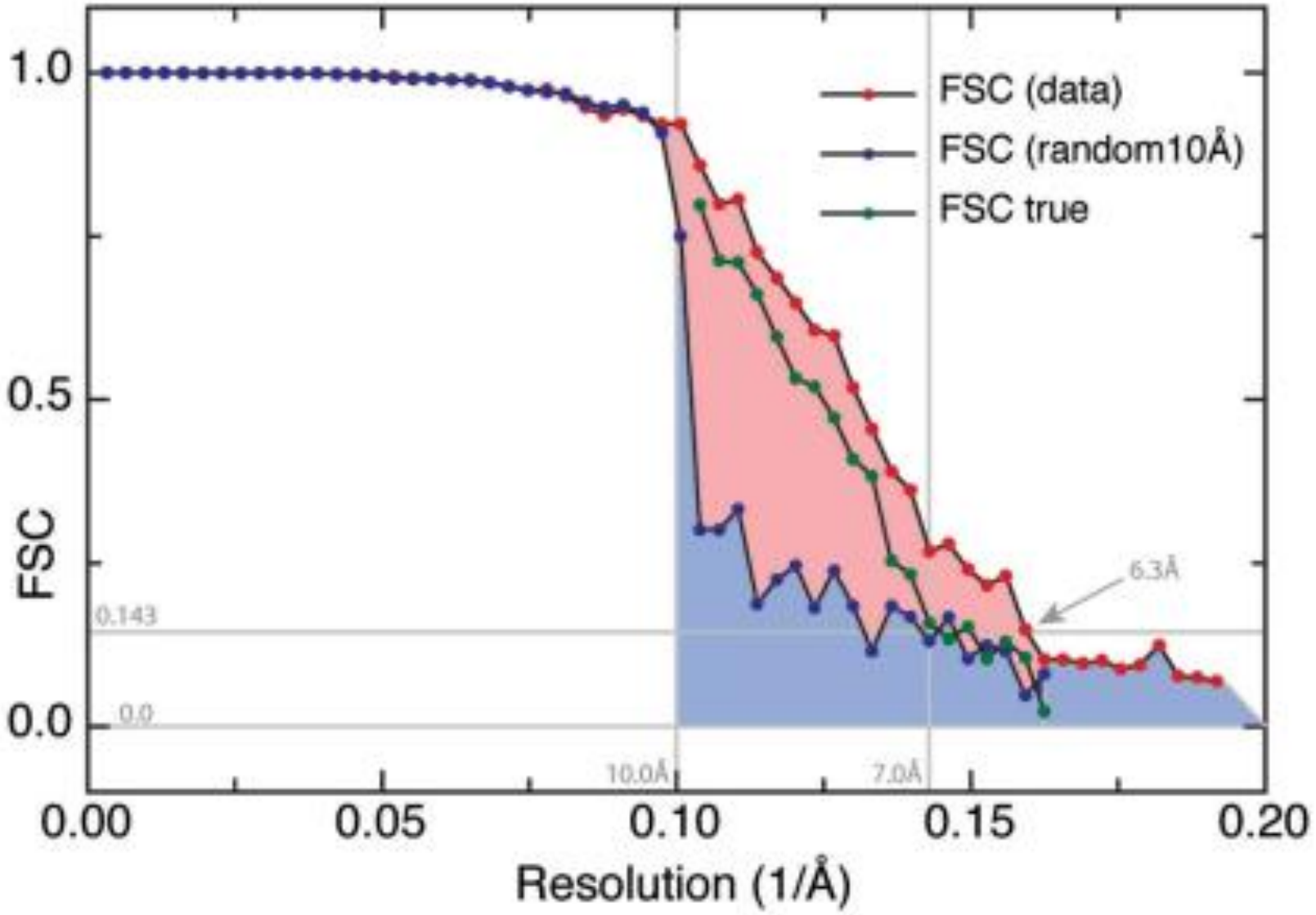
# HIGH RESOLUTION NOISE SUBSTITUTION

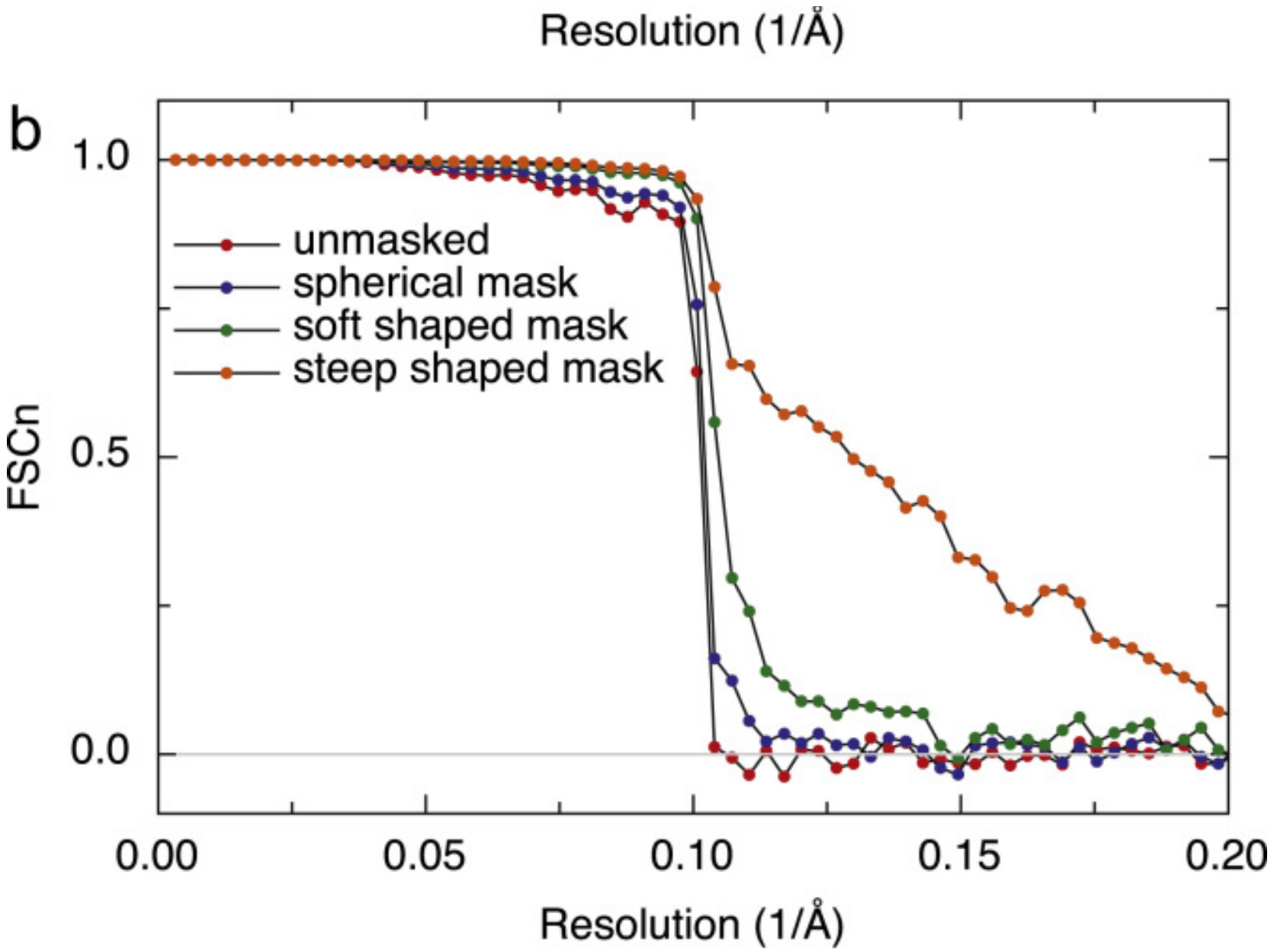
Perform Single Particle EM analysis.

Repeat but substitute random phases beyond a selected resolution (HR-noise)

Any overfitted noise will show up as non-zero FSC.

Genuine information will show up as the area between the two curves


1.0
0.5
0.0
FSC
FSC (data)
FSC (random10Å)
FSC true
6.3Å
0.143
0.0
10.0Å
7.0Å
0.00
0.05
0.10
0.15
0.20
Resolution (1/Å)


Resolution (1/Å)
b
1.0
0.5
0.0
FSCn
unmasked
spherical mask
soft shaped mask
steep shaped mask
0.00
0.05
0.10
0.15
0.20
Resolution (1/Å)

# MODELS

# Assessing Single Particle Model

- High Resolution Features Look Like Proteins
- Lower Resolution Agreement with an X-ray Structure
- Explanation of Experimental Data
- Biological Prediction
- Unbiased Procedures
- Cross Validation
- Communicating Results

Fitting one component X-ray model into EM map may define absolute hand and also validate the map calculation.
Fold/shape at lower resolution.

Fitting may be assisted by experimental absolute hand determination (include another specimen of known hand)

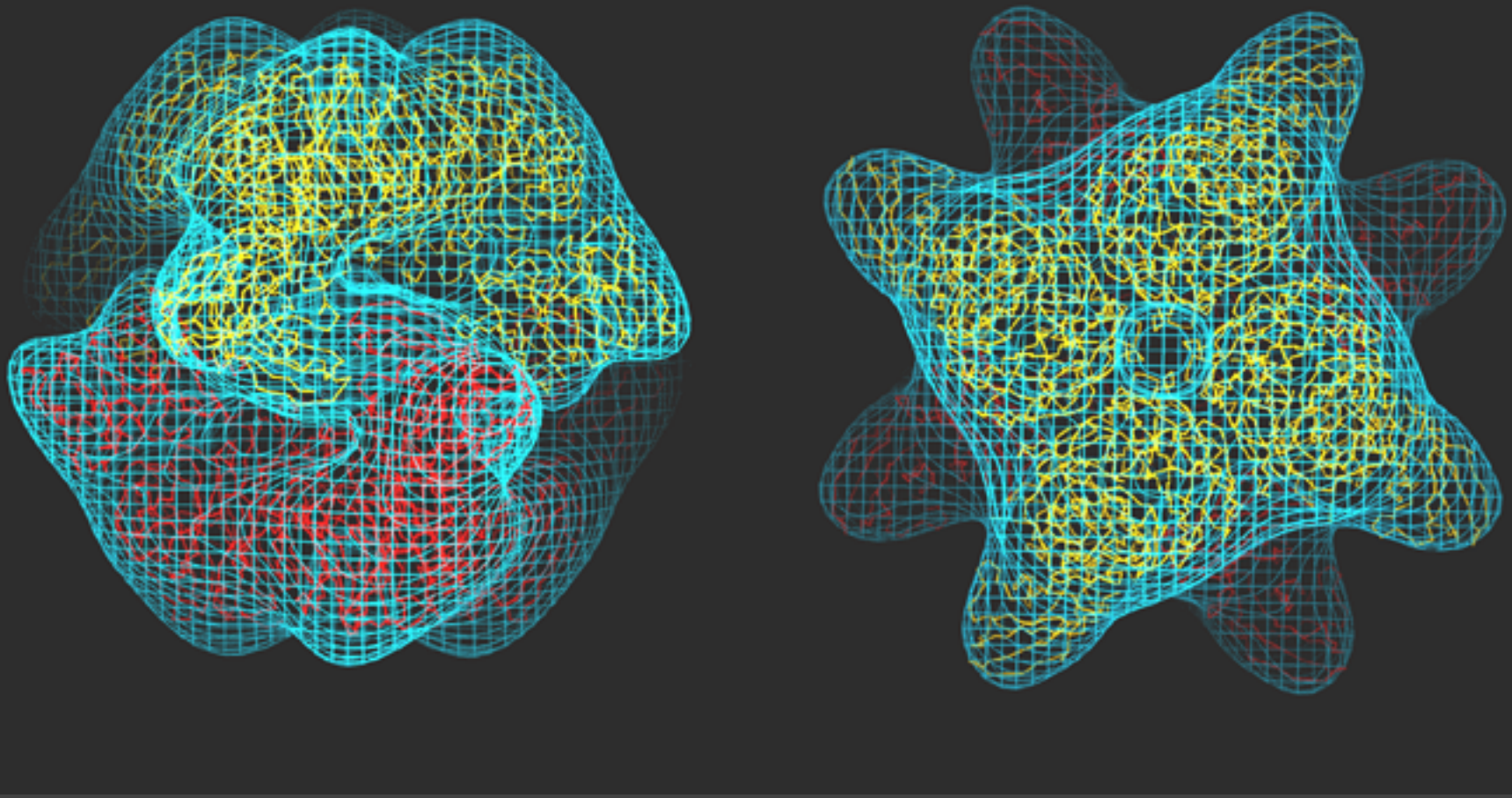
Backbone/Side chains

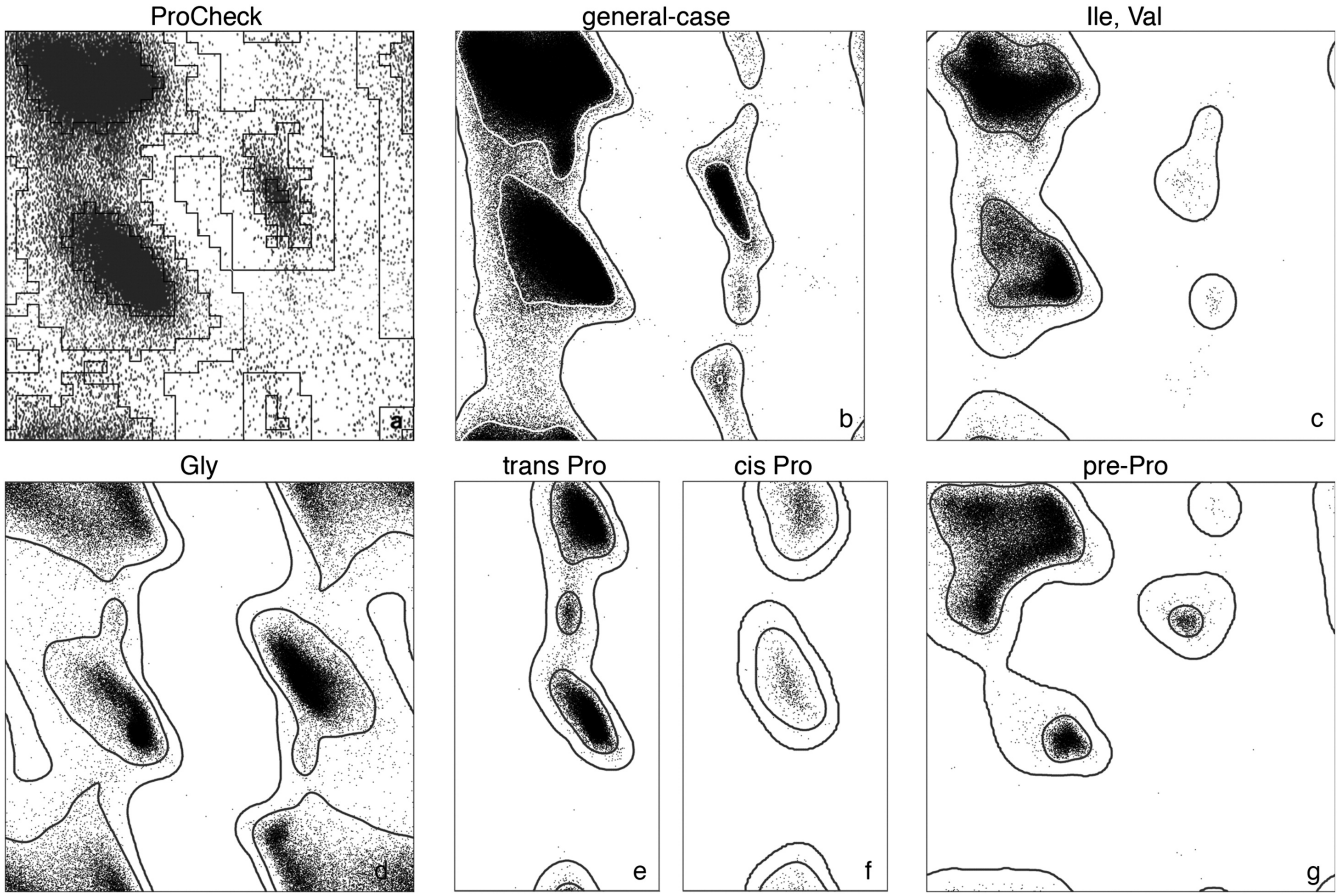
Refinement of protein model against map

- agreement with map
- stereochemistry of model
- several observation to refinable parameters

Connection to X-ray validation

# Fitting low resolution map with X-ray model





# CHALLENGES

# Heterogeneity

Good?

Do tomography on everything!

Is it OK to present a model that represents a small percentage of the particles?

What is going on with the rest of the dataset?

Tilt-pairs

# Tomograms

- Resolution Estimate
- Missing-wedge
- Sub-tomogram averaging

# Helices

- Power spectrum of sum of filaments

# Map validation

Visibility of expected features – α-helices visible at 9 Å resolution? β-strands at 4.8 Å resolution? Side-chains beyond 4 Å?

Tilt pair validation to show orientations are correct – check that orientation determination is working by collecting one or two tilt pairs and calculating a tilt pair parameter plot (TPPP) to show clear clustering of most particles around the tilt angle and axis used.

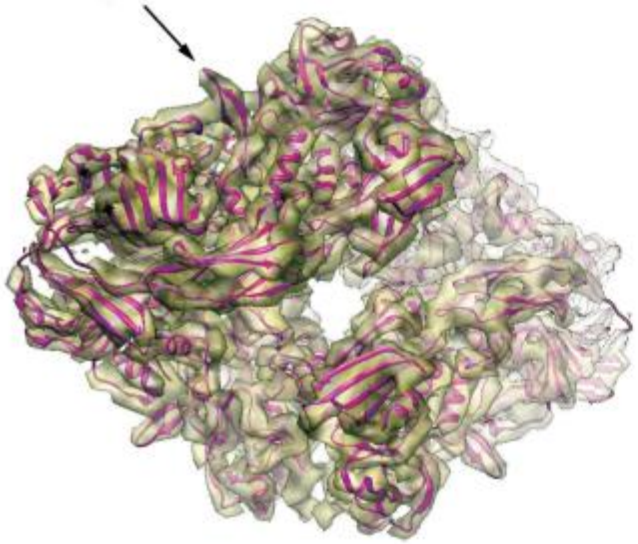
Resolution validation – (a) use gold-standard FSC calculation (i.e. process data as two completely independent halves - this means independent starting models, refinement and masking); (b) perform high resolution noise substitution and compare FSC curves of raw data with HR-noise substituted data.

Ensure you can "see" your particles – for small or very small particles, record initial images at high (5 µm) defocus and high (60-120 el/Å$^2$) dose. Avoid the "Einstein from noise" pitfall by initial manual or local-variance-based (i.e. not CCF) particle picking.

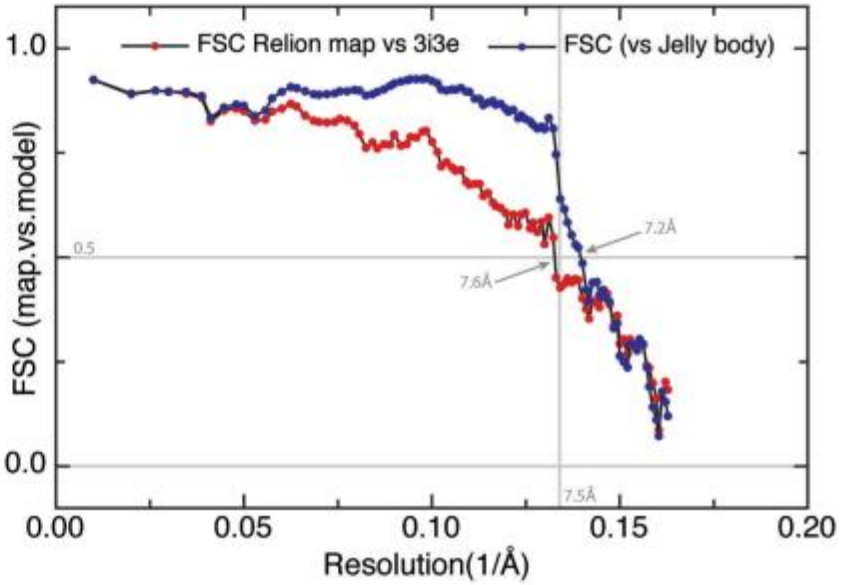
**DO DETECTORS CHANGE THINGS?**

# Four informative validation tests (from Chen et al, 2013)

(a) Map/model superposition

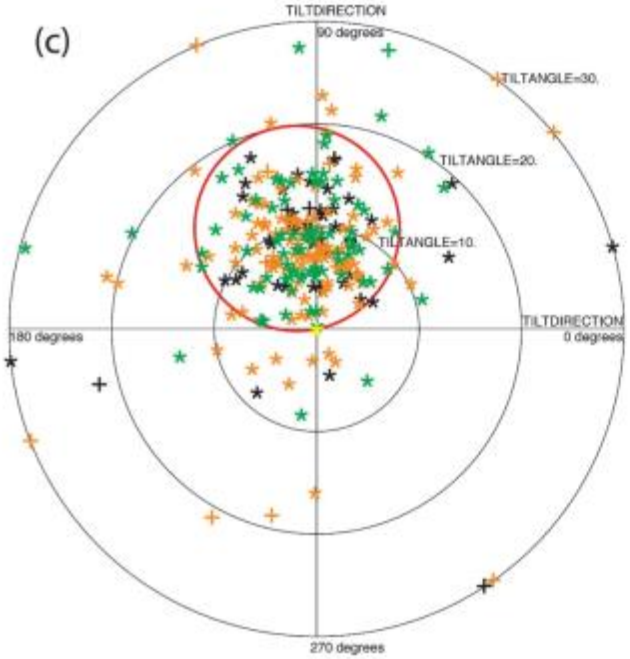


(b) Map/model FSC

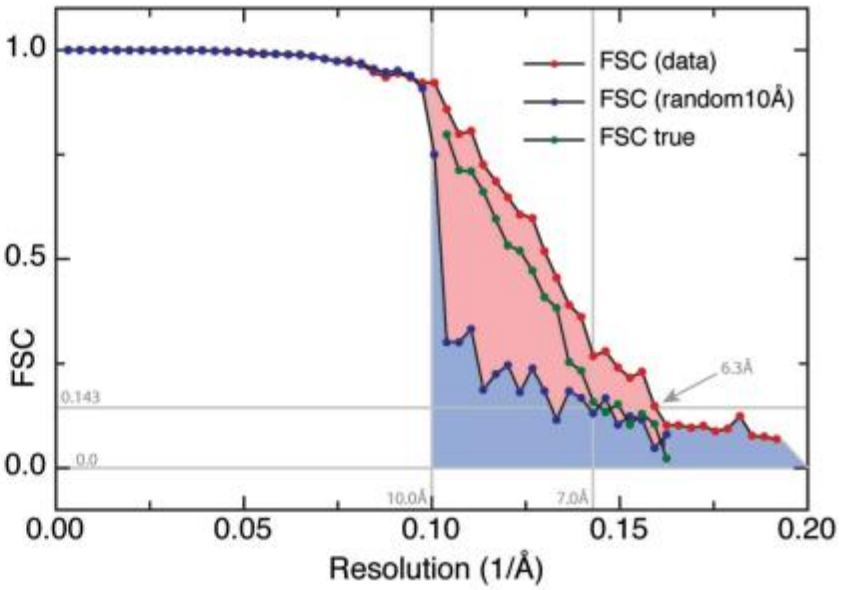



Tilt Pair Parameter Plot

(c)



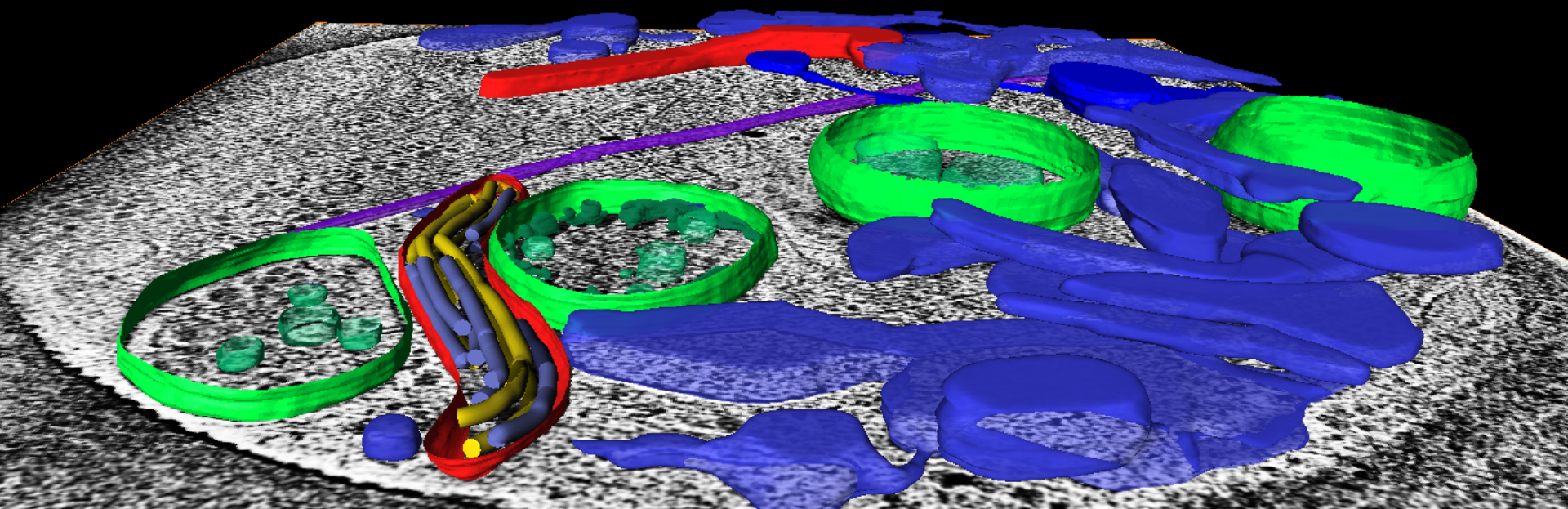

(d) High-resolution noise substitution



MRC National Institute for Medical Research
Mill Hill, London

MRC Laboratory Molecular Biology, Cambridge
Richard Henderson

PDBe, European Bioinformatics Institute
Ardan Patwardhan



Structure
Meeting Review

# Outcome of the First Electron Microscopy Validation Task Force Meeting

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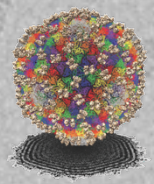
# 2015 Map and Model Challenges



EMDataBank
Unified Data Resource for 3DEM

- Goal: develop community-accepted map and model validation criteria
- Two working groups composed of 3DEM community members will define test cases and format of each challenge
  - Bridget Carragher: map validation chair
  - Paul Adams: model validation chair
- Test cases from deposited/publicly available data
- Formulation and announcement of the challenges by early 2015

# Four different procedures give four different amounts of "overfitting" and resolution overestimation