SPH RE

SParx for HIgh Resolution Electron Microscopy

Reliability and Reproducibility

UTMB Workshop

May 8, 2019

SPHIRE Workflow

PROJECT
Project Settings

CTER
CTER CTF Estimation
CTF Assessment

WINDOW
crYOLO Particle Picking
Particle Extraction

ISAC
ISAC2 2D Clustering
Beautifier

VIPER
Initial 3D Modeling

MERIDIEN
MERIDIEN 3D Refinement
3D Refinement Assessment
PostRefiner

SORT3D
Variability Estimation
SORT3D 3D Clustering

LOCALRES
Local Resolution
3D Local Filter

MOVIE (optional)
Movie Alignment
Drift Assessment

Utilities
Utility commands

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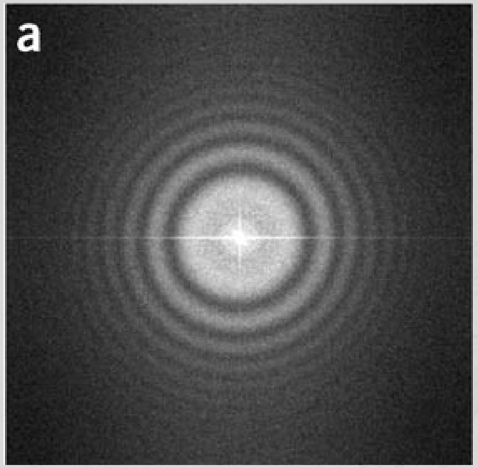
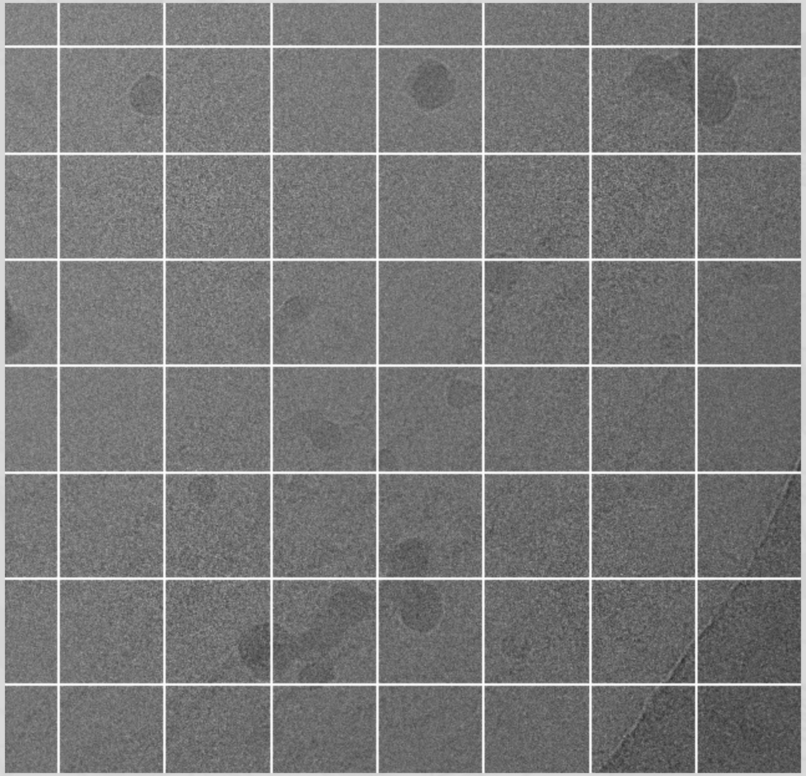
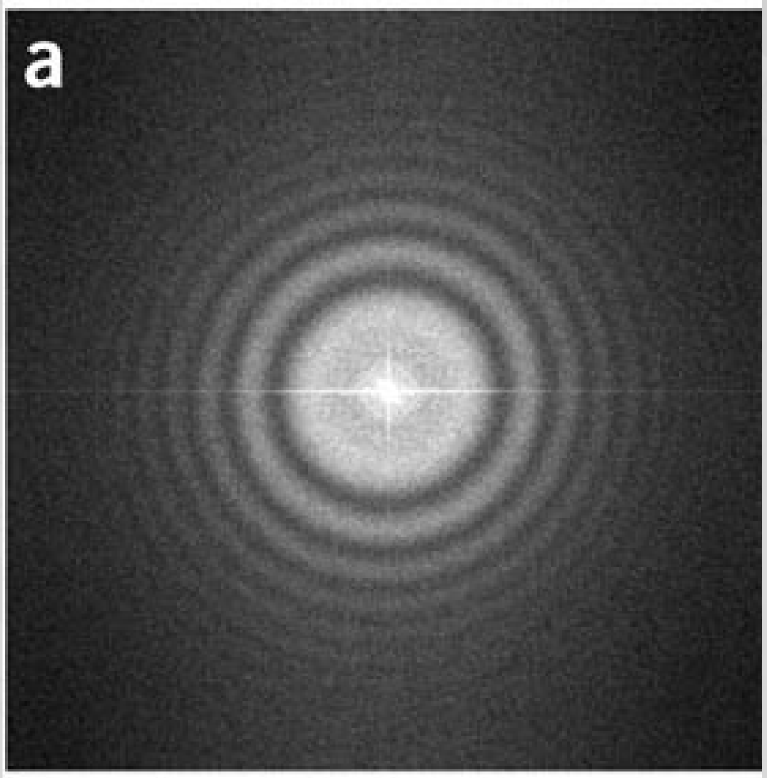


SPHIRE

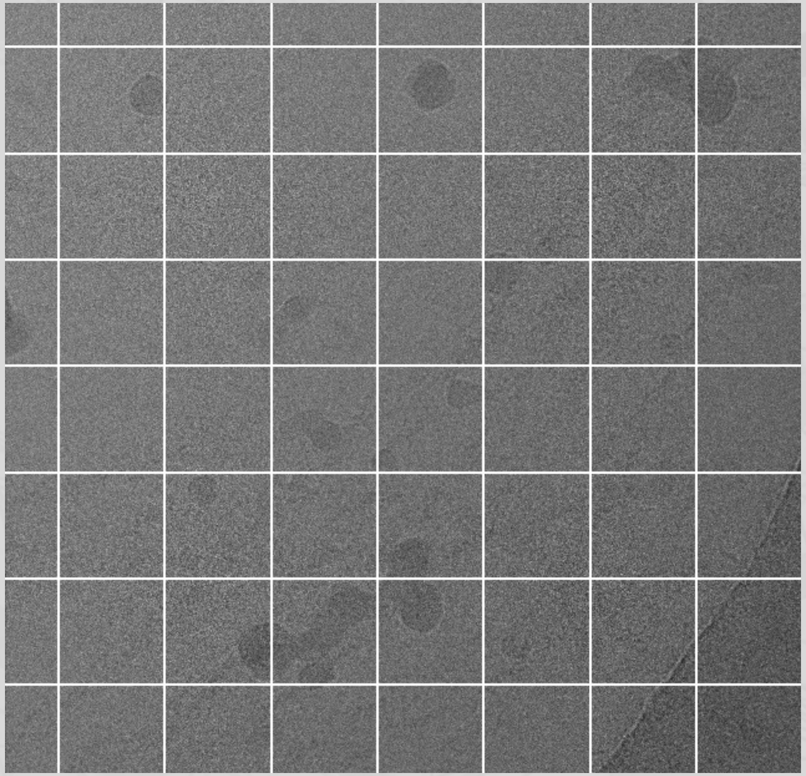
CTF Estimation

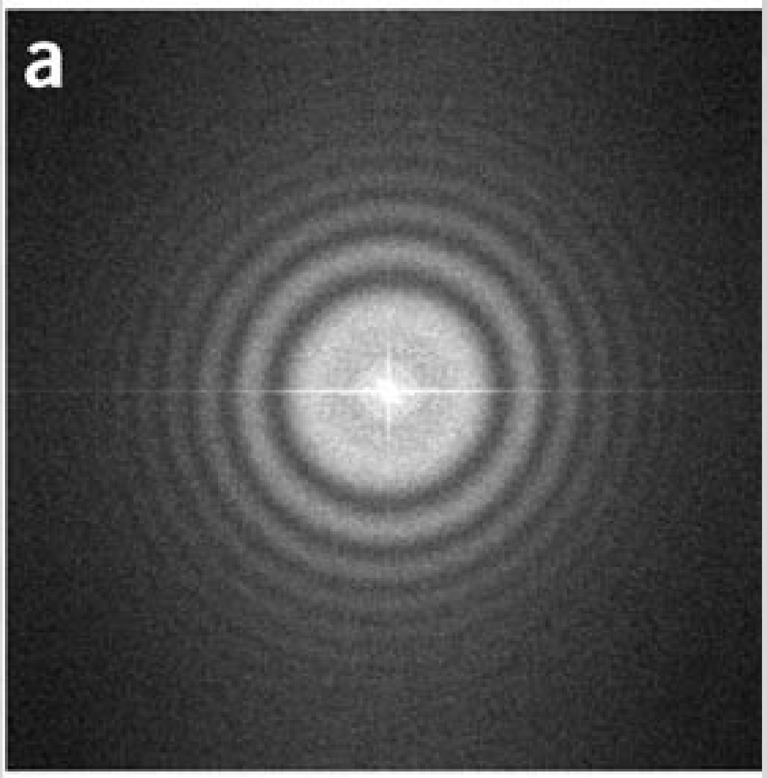




To estimate the error for the estimated parameters CTER does the estimation multiple times for random subsets of the tiles.

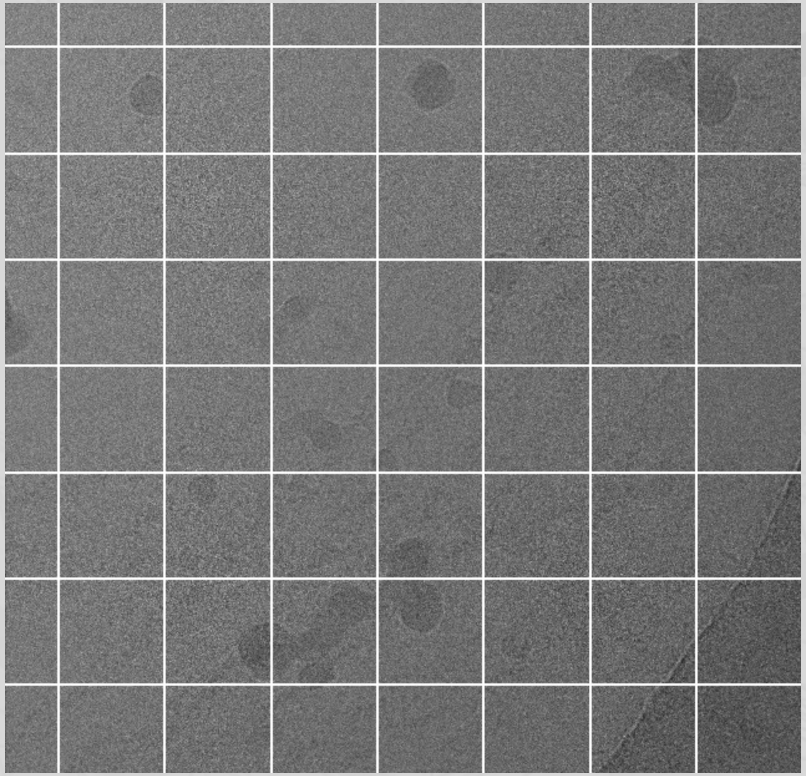
CTF Estimation

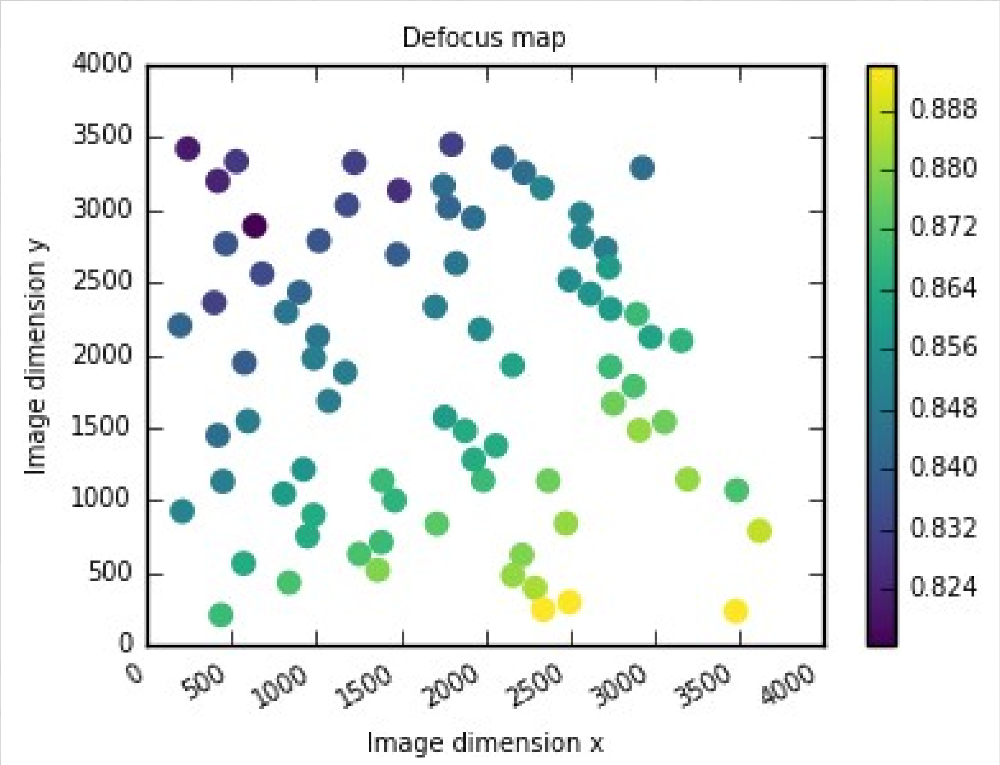




Assumptions:
1. The main contributors to scattering are your macromolecules of interest.
2. The grid is flat, in the *xy*-plane.

CTF Estimation

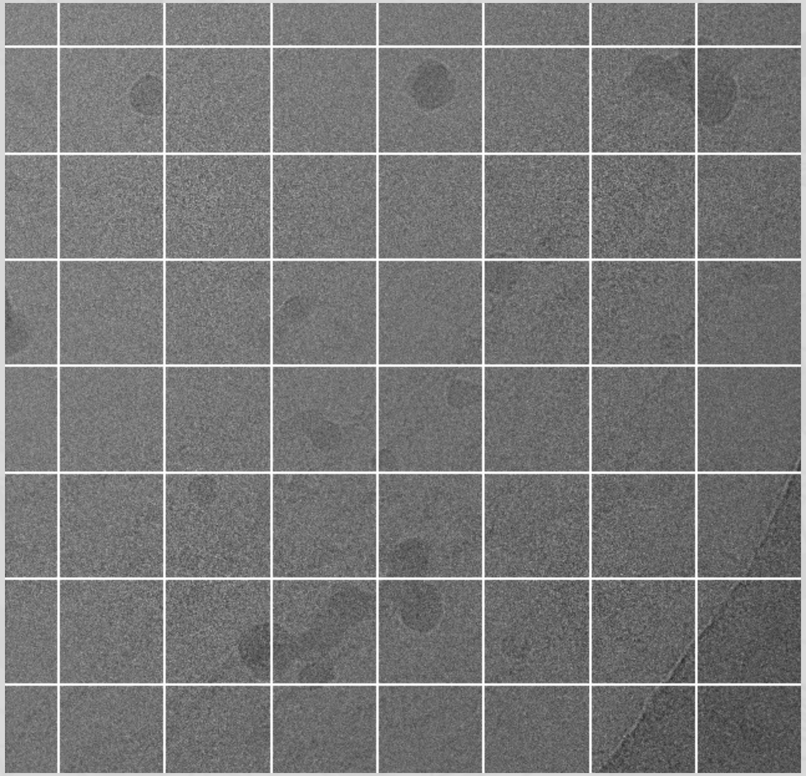


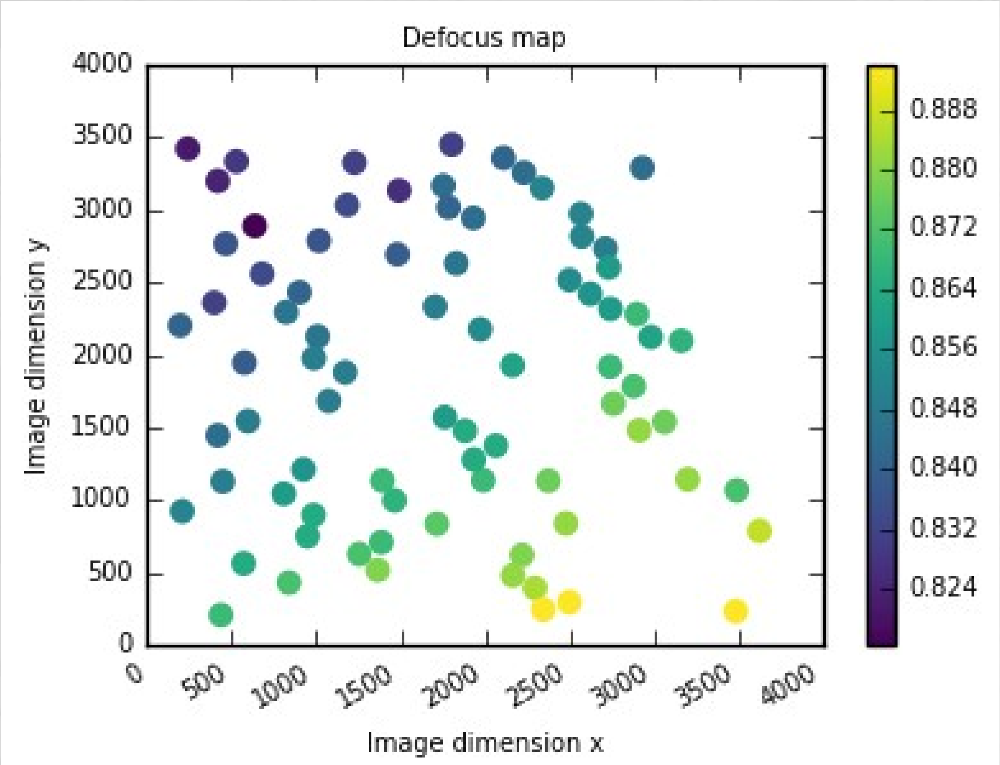


Reality:

1. Carbon, etc., will contribute to the power spectrum.
2. The grid is not flat.

CTF Estimation





Solution:

- CTF refinement
  - Probably won’t make a difference until beyond 4Å resolution

Should I worry?

- Accurate estimation of CTF parameters is very important.
  - More later, in the refinement/reference-based alignment section…
- Is CTF refinement helpful?
  - Yes, if you have tilted micrographs
  - For high-resolution (beyond 4Å), it may help.
- Is reproducibility a problem?

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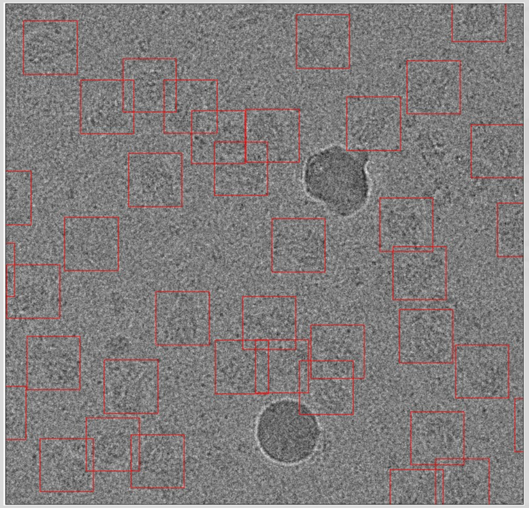
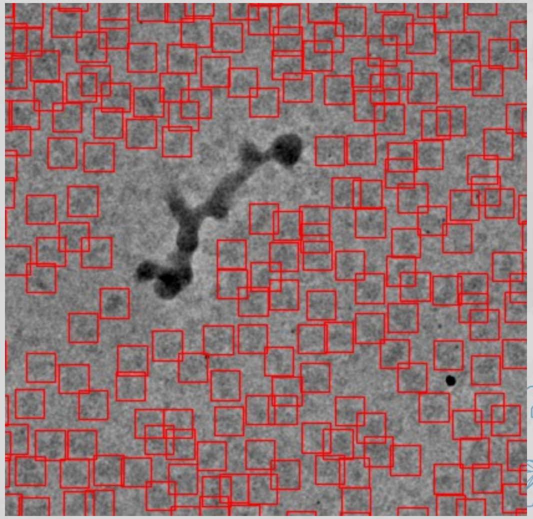


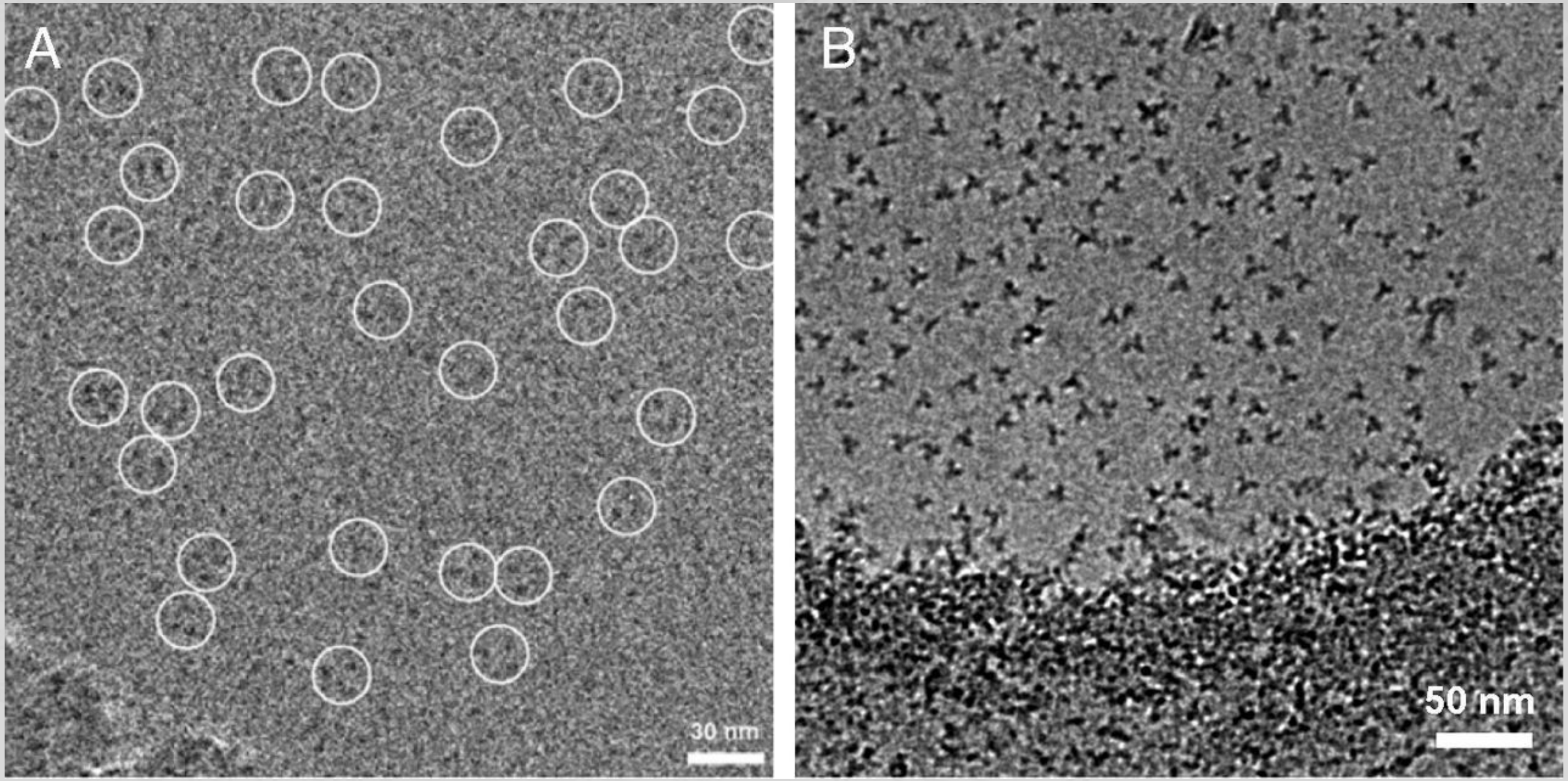


What might go wrong during picking

- Can you actually see the particles?
  - Don’t blindly trust automatic pickers.

Case of HIV-1 envelope trimer

From Mao et al. (2013) From Harris et al. (2011)



Letter by Sriram Subramaniam (2013) PNAS

What might go wrong during picking

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  - Do the templates reflect all views of your particle?

What might go wrong during picking

- Can you actually see the particles?
  - Don't blindly trust automatic pickers.
- If using template-based picking:
  - Do the templates reflect all views of your particle?
- If picking manually:
  - Are you subconsciously biasing your picks to recognizable views?
  - One solution: Pick generously, and hope 2D classification picks out unexpected views.

Specific for neural-network pickers

During training, does the network simply become good at matching the training picks?

- In which case, the network would fare poorly on any unseen data.

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Solutions:

- Augmentation – The training data are duplicated, with some modifications.
  - Rotation, e.g., by multiples of 90 degrees
  - Adding noise
  - Random contrast changes
  - Multiplication/addition/subtraction of pixel values
  - Dropout – set random number of particles to the mean value

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  - Adding noise
  - Random contrast changes
  - Multiplication/addition/subtraction of pixel values
  - Dropout – set random number of particles to the mean value
- Validation
  - Some fraction of the training data (e.g., 20%) are set aside.
  - Training is performed on the other 80%.
  - The network is tested on the 20% validation set.

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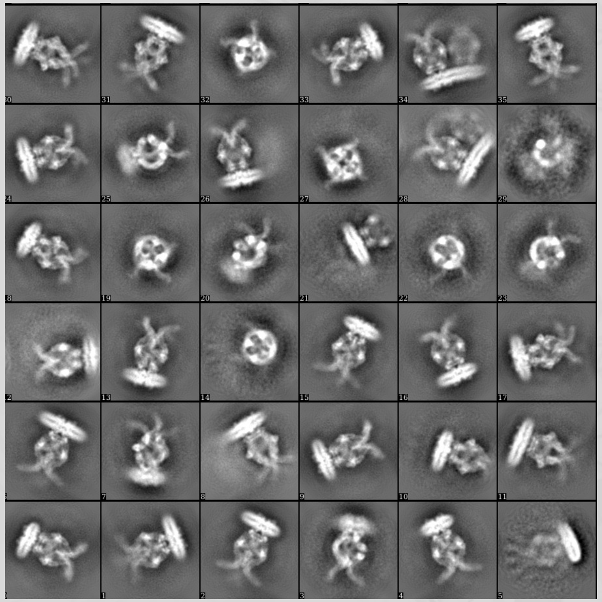
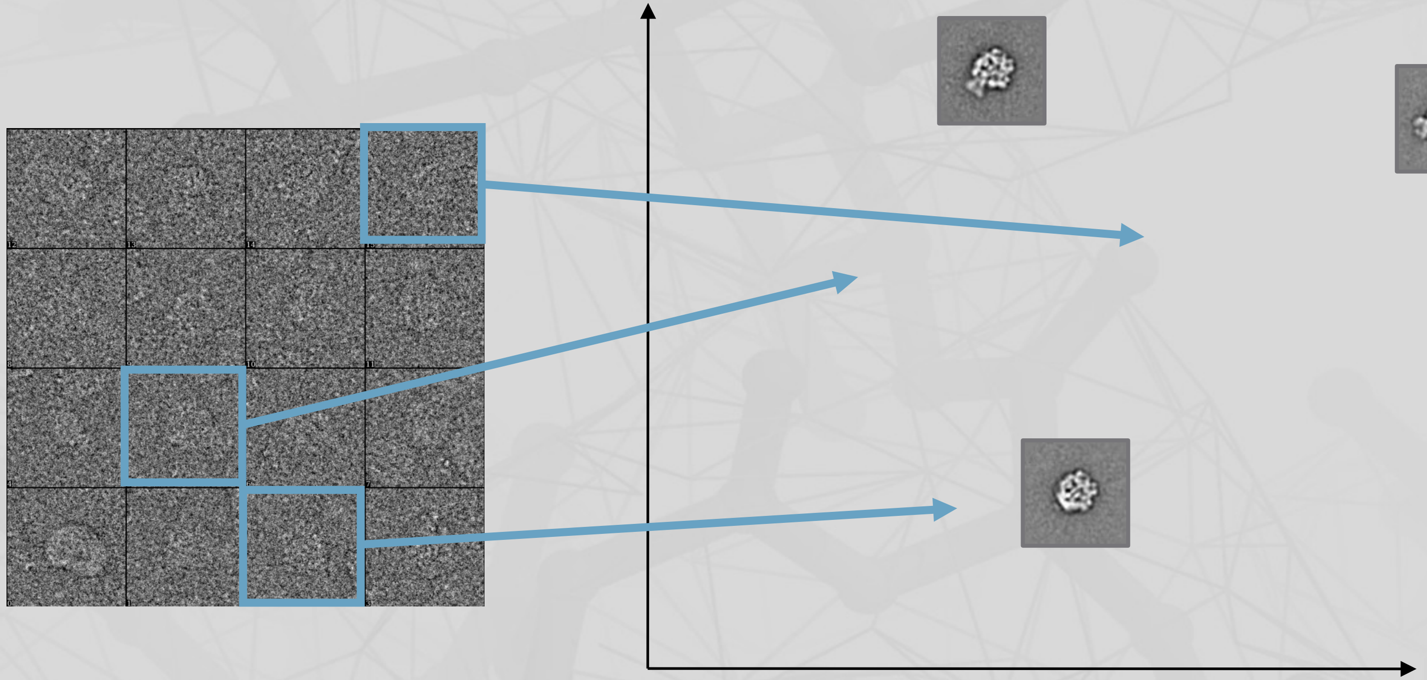


2D classification: When is it used?

- Initial-model generation
- “Cleaning“ of data sets
  - Removing non-particles
- First look at your macromolecule of interest

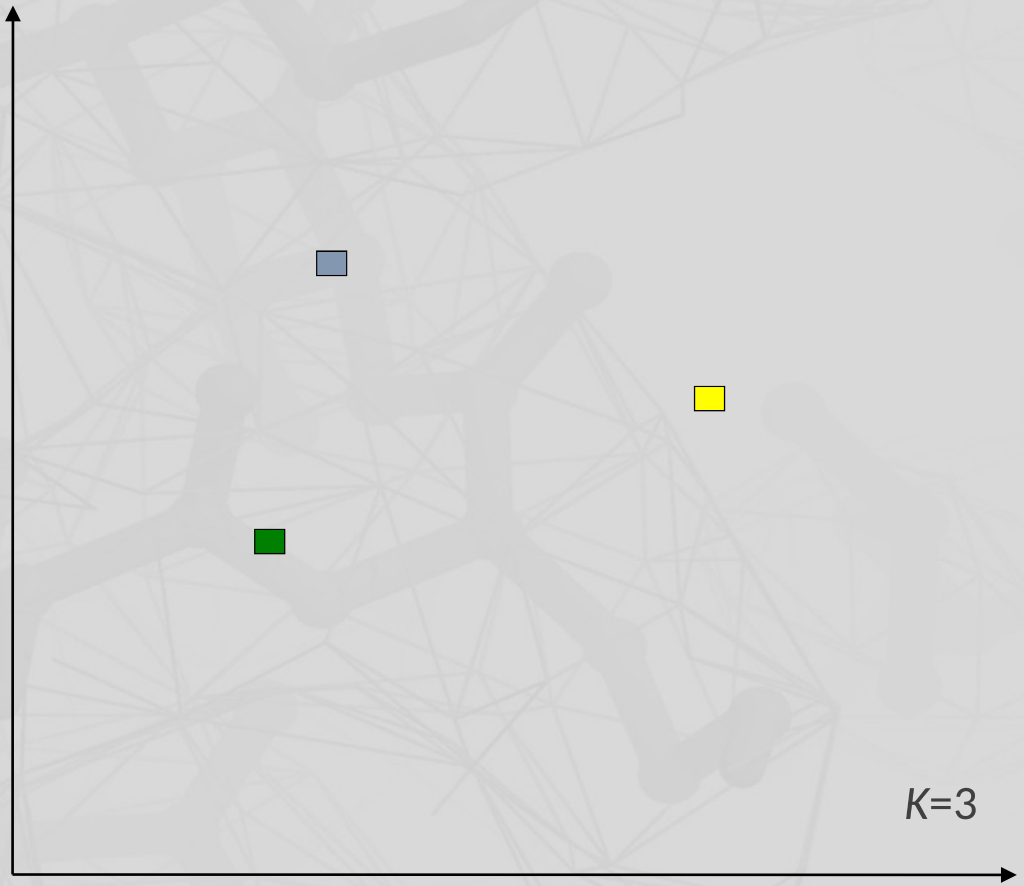
K-means clustering



Every image with N pixels
can be considered a point in a
N-dimensional coordinate system

The higher the similarity of
a pair of images, the closer
the representing points are.

K-means clustering

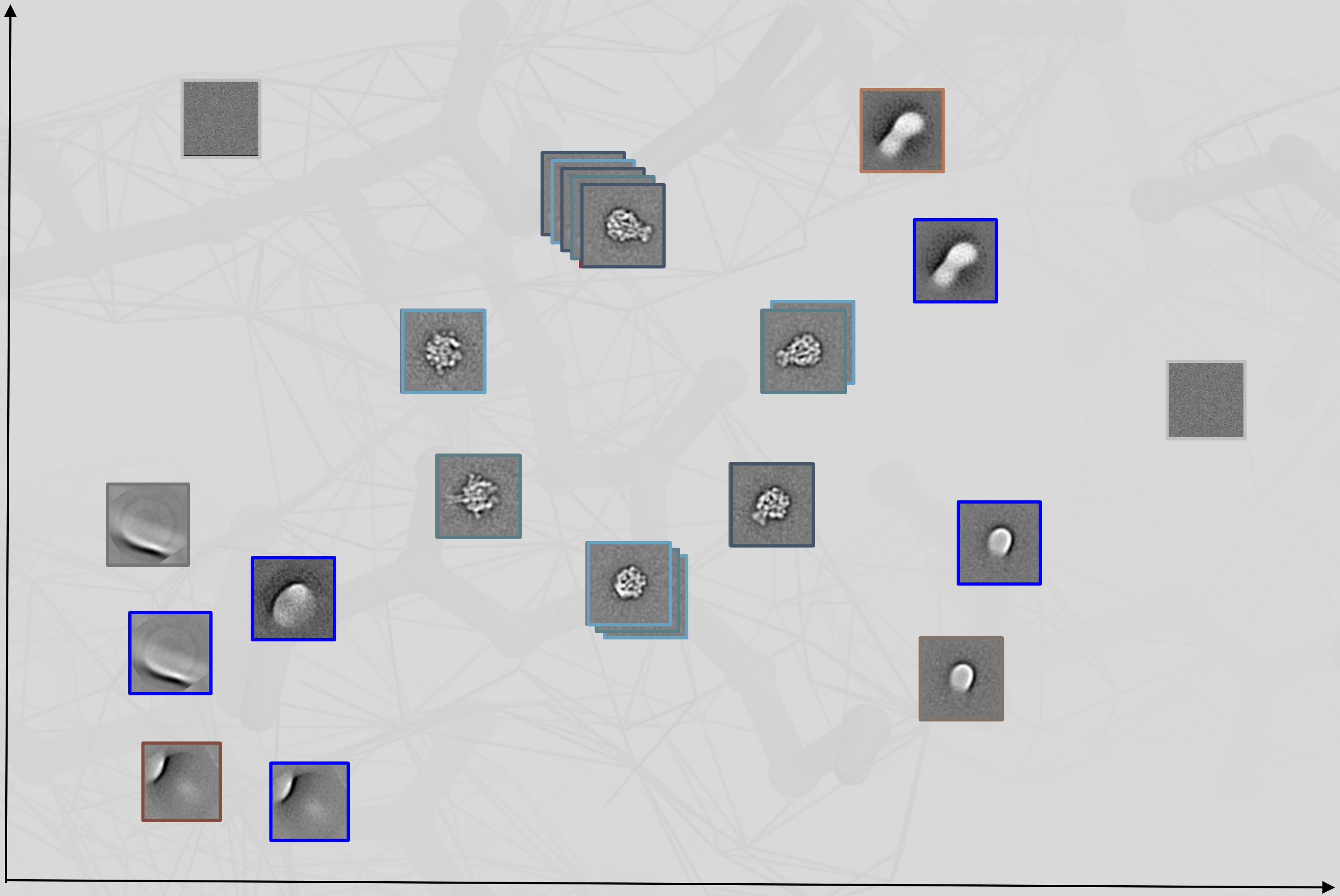


The real cryo-EM world is far too noisy for K-means

100 particles
img_per_grp=10,
minimum_grp_size=3

Expected K=10

Returned K=17



ISAC can handle the real cryo-EM world!

Weaknesses of K-means

K-Means clustering is a good algorithm because it is simple and fast.
However, it is not perfect…

Need to guess the number of clusters K	The number of clusters is a critical parameter and can affect results considerably
Sensitive to initial condition	Results dramatically depend on the initialization. The algorithm may be trapped in the local optimum ➜ Model bias problem
Not robust to outliers	Data points far from the centroid may pull the centroid away from the center - Weakness of arithmetic mean ➜ Especially problematic for **preferred orientations**
Limited to circular clusters of similar size	K-means can hardly handle clusters of variable size/density

ISAC: Iterative Stable Alignment and Clustering

What can ISAC do better to overcome problems of K-means?

Need to guess the number of clusters K	Ask for **number of images per group** instead ➜ Equal-Size K-means
Sensitive to initial condition	**Run 2D clustering multiple times** starting from different initial conditions ➜ Keep reproducible classes only
Not robust to outliers	**Multiple 2D alignments within each cluster** to identify heterogeneous clusters and outliers, which have high variation in alignment results ➜ Keep stable classes only
Limited to circular clusters of similar size	**Reject too small clusters** typical for outliers and **limit maximum size**

2D classification: Take home messages

- 2D classification is useful.
- If it doesn‘t perform well, don’t be satisfied with it.

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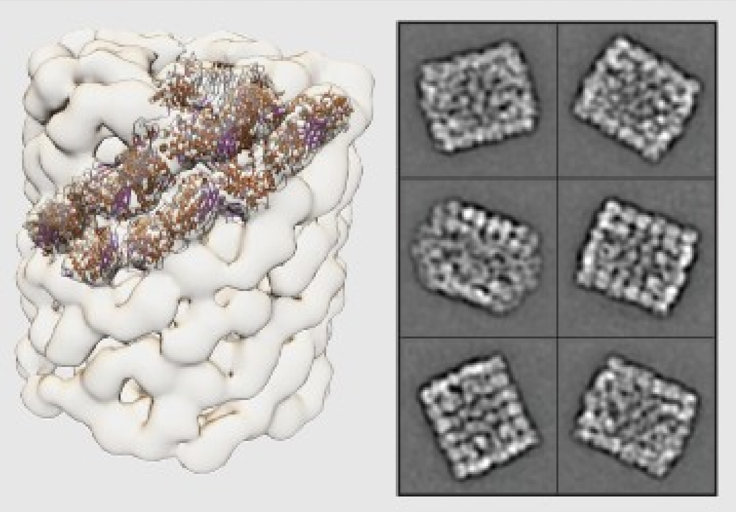
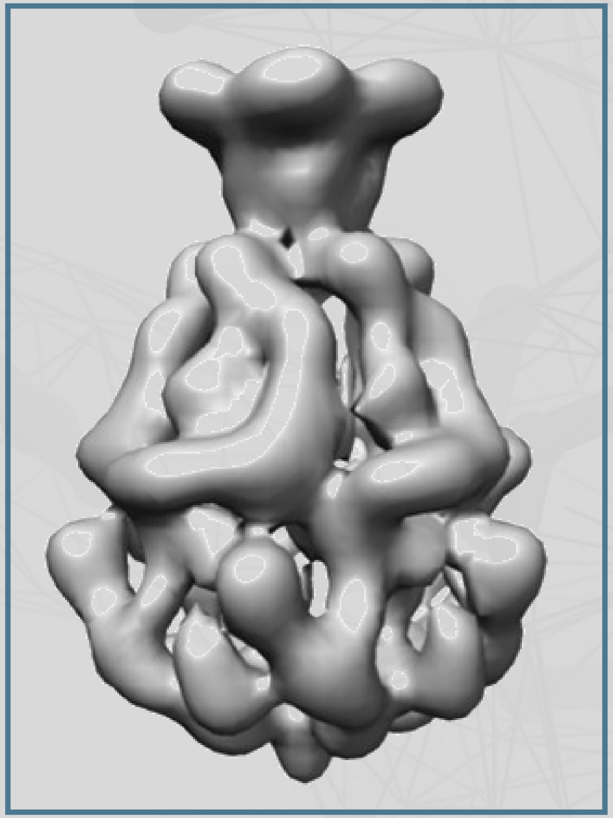
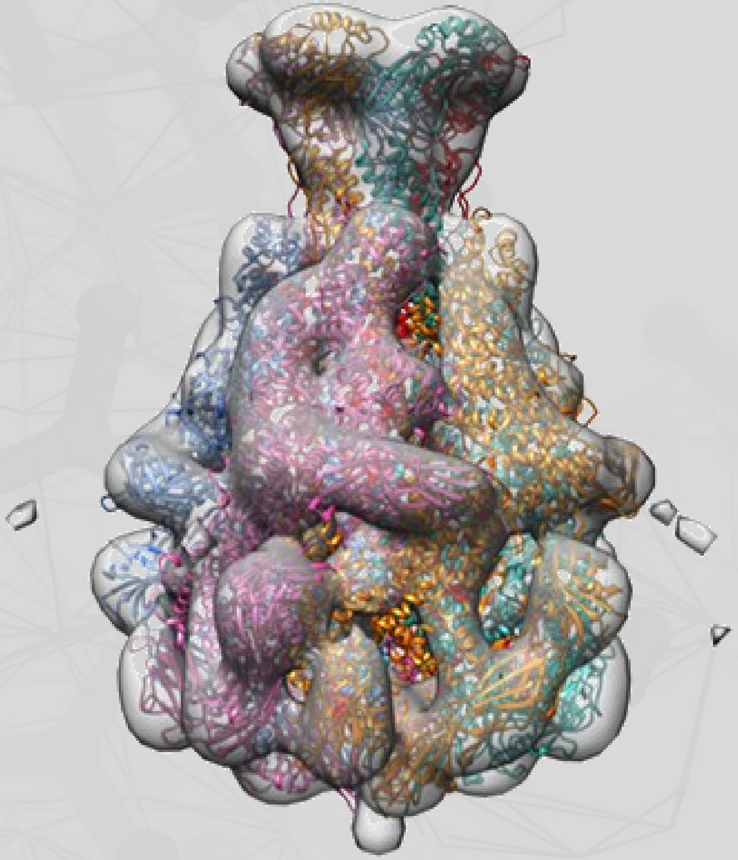


What can go wrong? Handedness

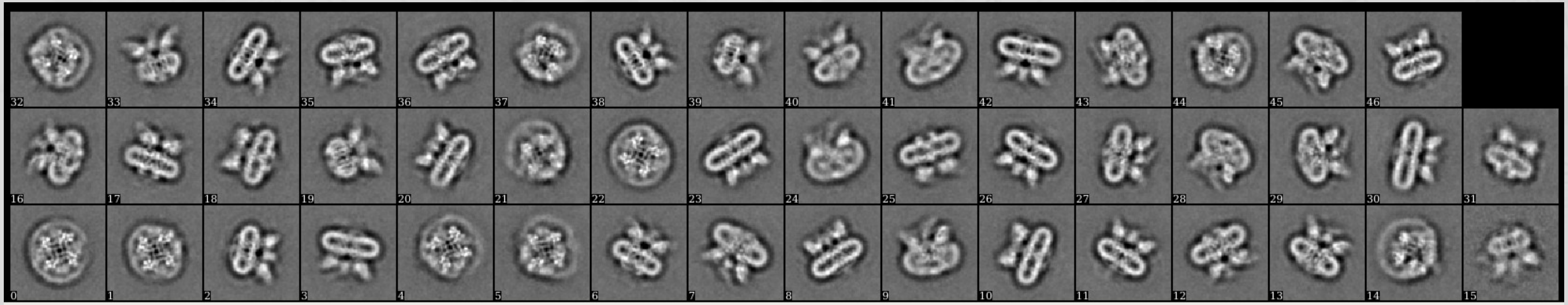
Handedness: You have a 50% chance of getting the right handedness from a VIPER run. It should not matter for further image processing.

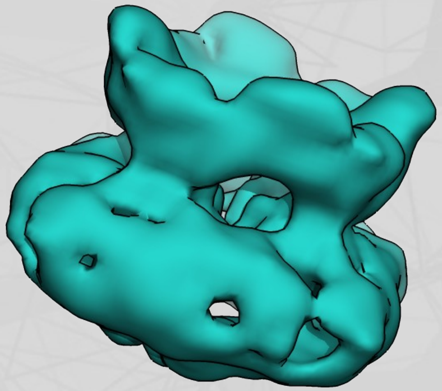


From pre-calculated results

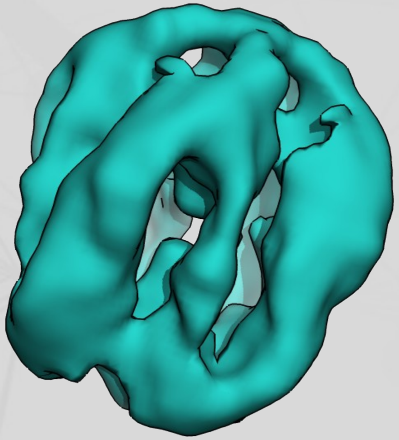


What can go wrong? Everything





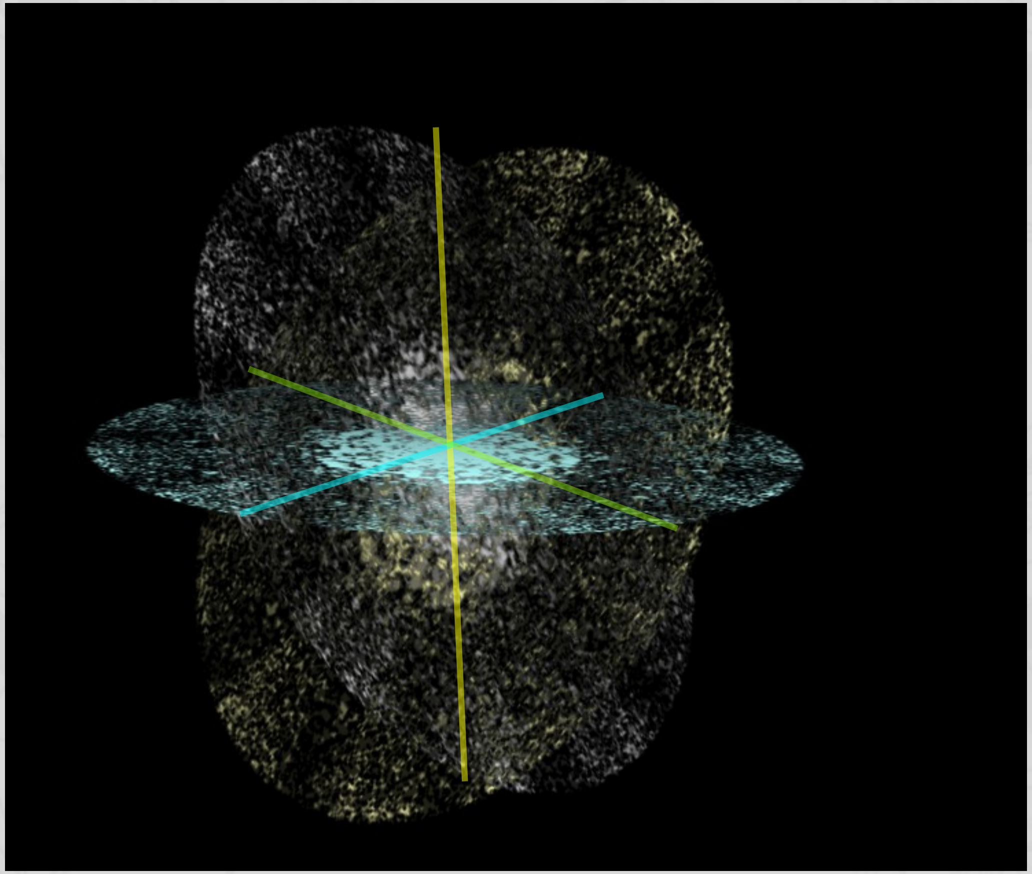
In some nice cases



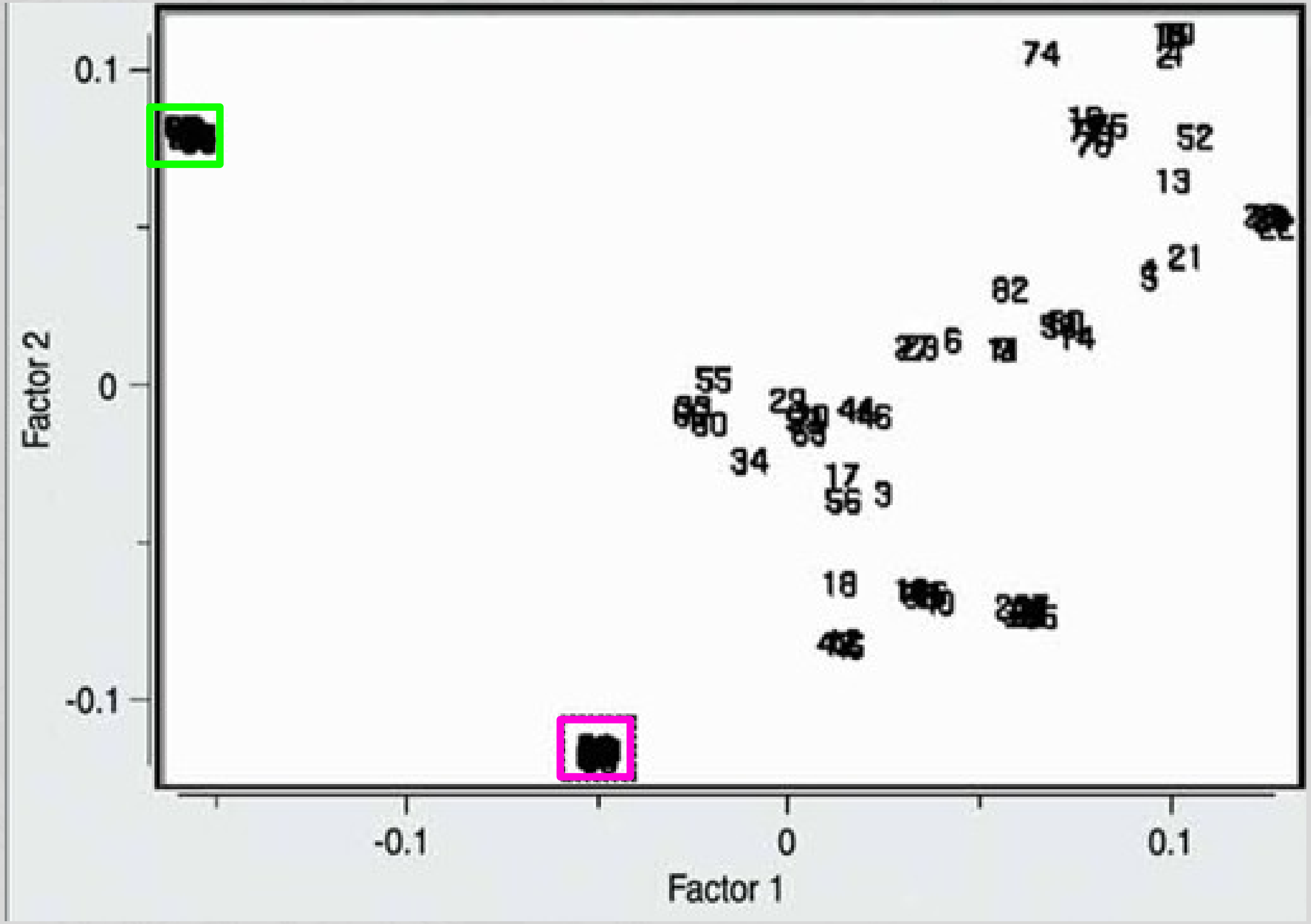
But sometimes...

Why so bad?

- Images are noisy.
  - With noise-free data, initial-model algorithms work great.
- Common lines are composed of a few hundred Fourier coefficients (~Fourier pixels).
  - of which, maybe a few dozen have a good SNR



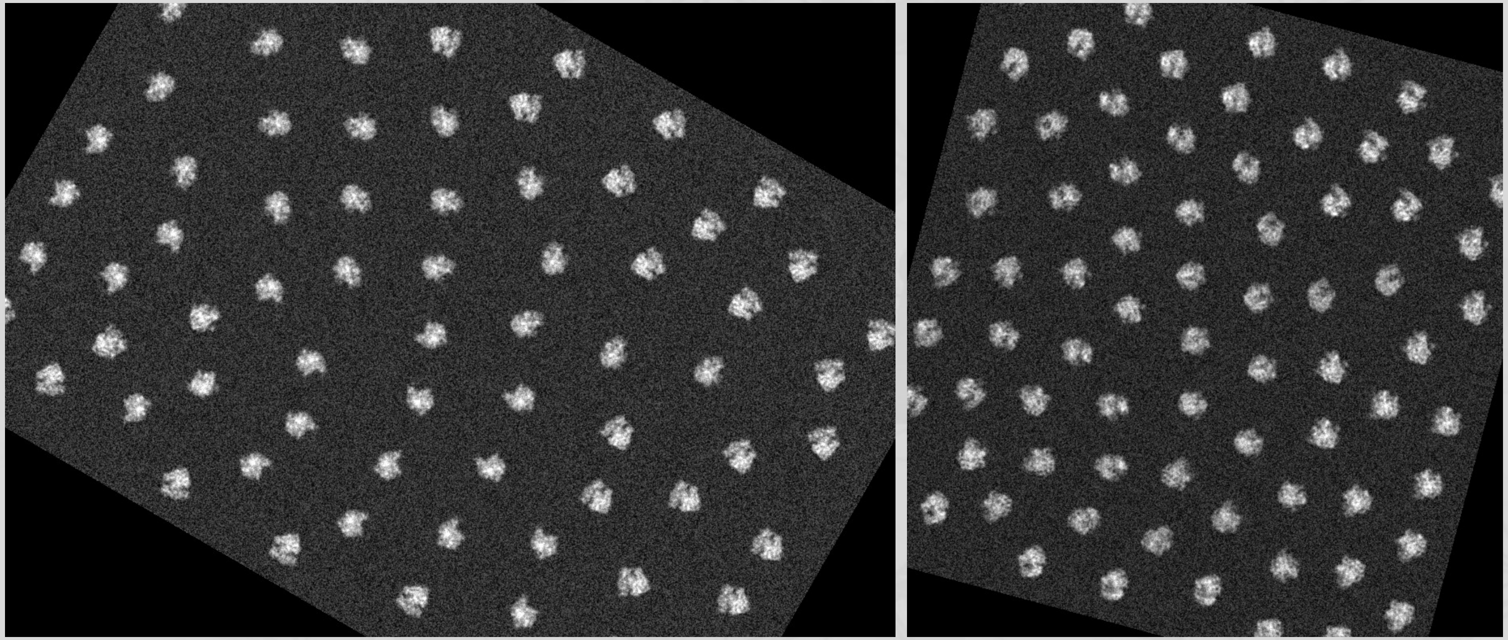
Reproducibility? Multivariate Statistical Analysis



Shaikh et al. (2008) Nature Protocols.

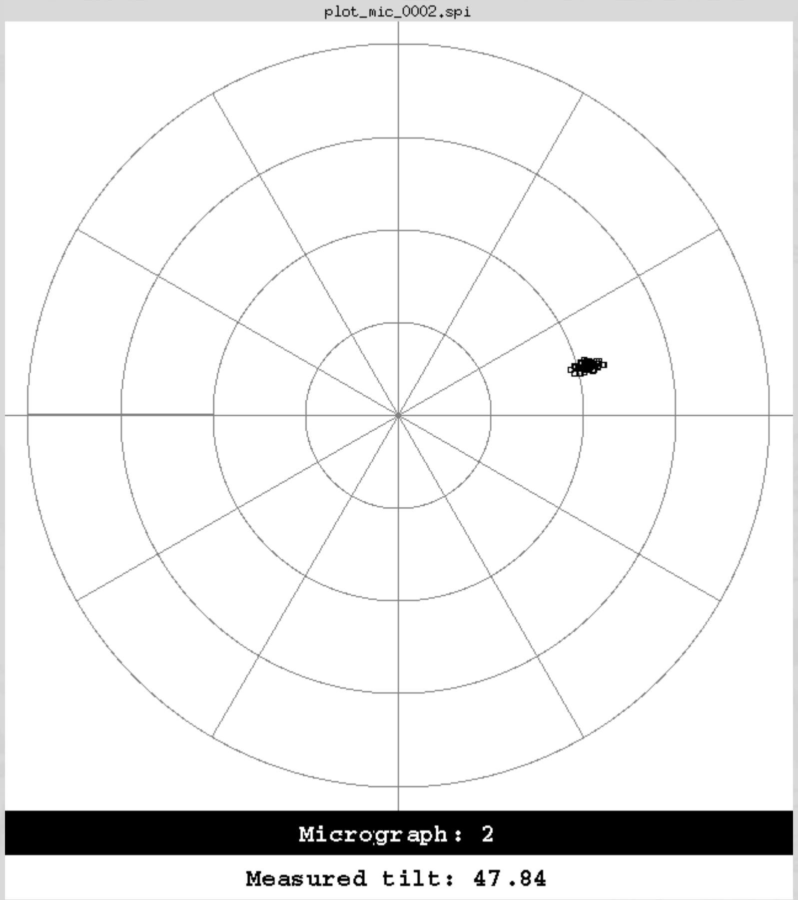
Reproducibility? Tilt validation

described in Rosenthal & Henderson (2003) JMB

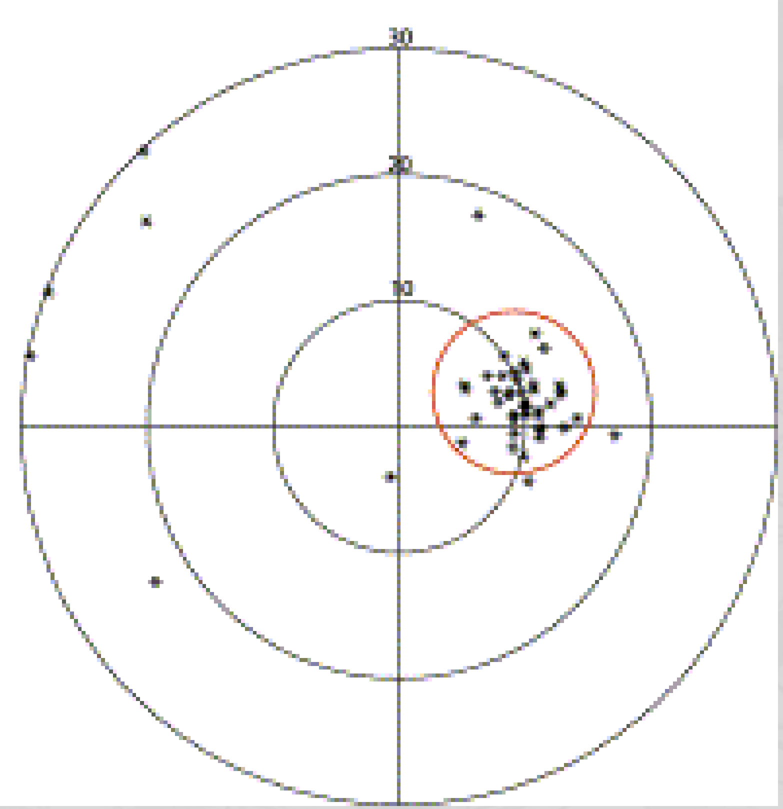


0° 48°

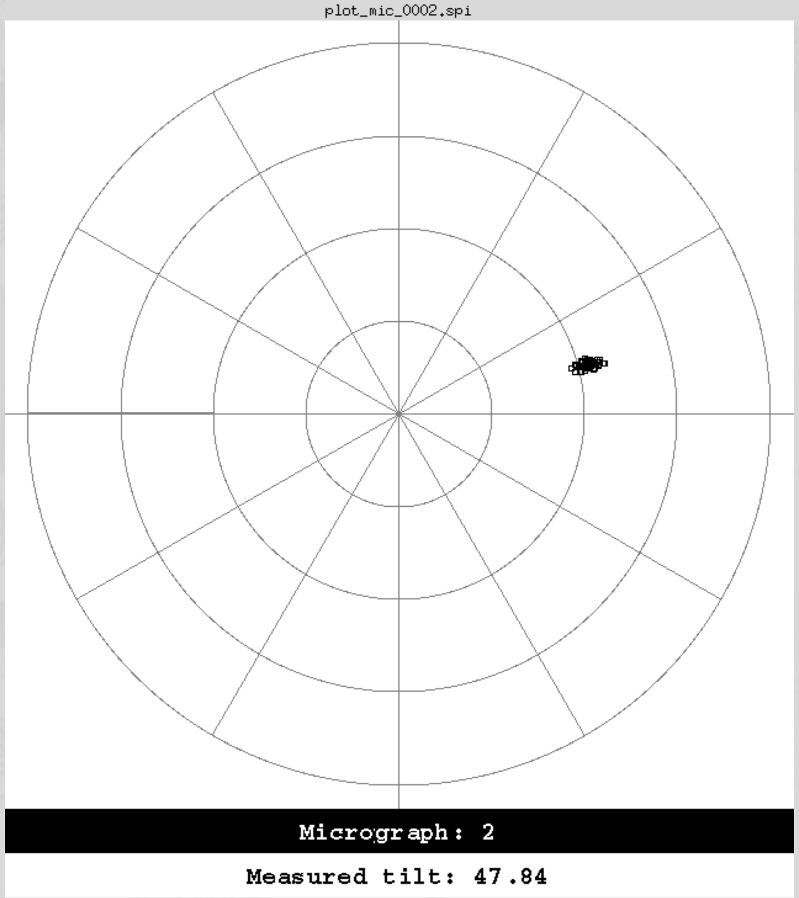
Artificial data



Reproducibility? Tilt validation



Real data
Rosenthal & Henderson (2003) JMB



SPHERE

Initial models: Take home messages

- Run initial-model generation many times, or use a program that generates many models.
- Validate!
- Random conical?

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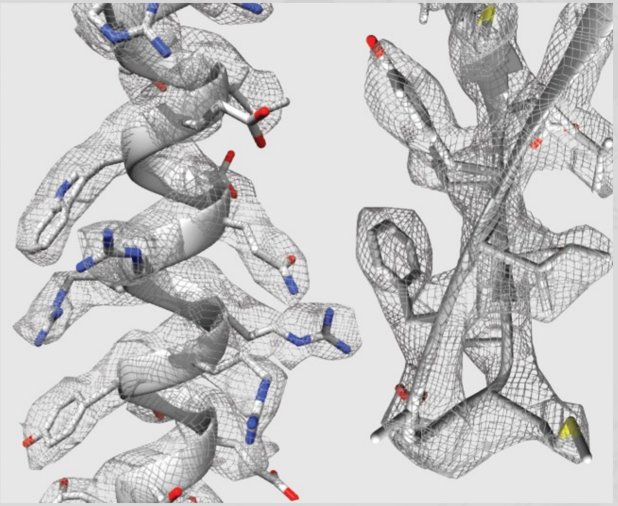
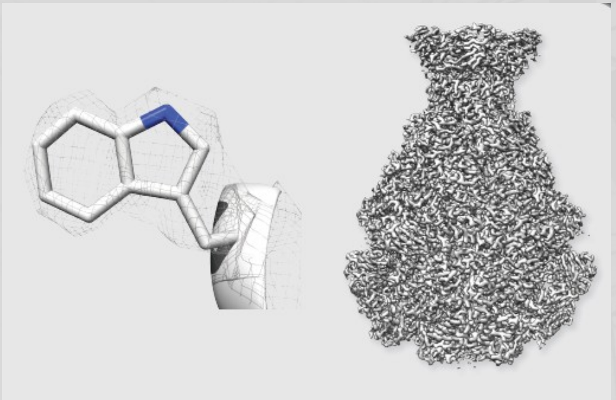
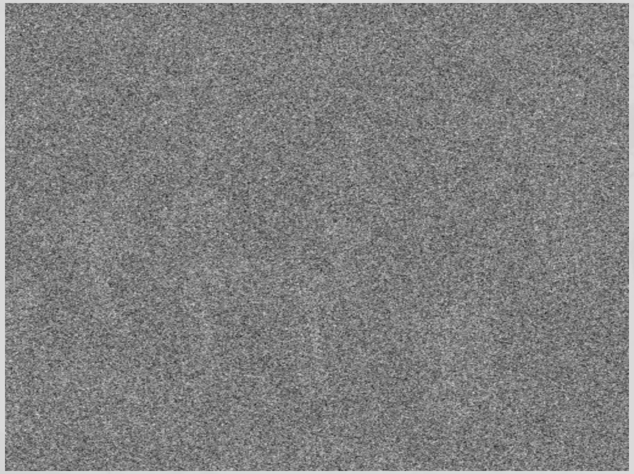




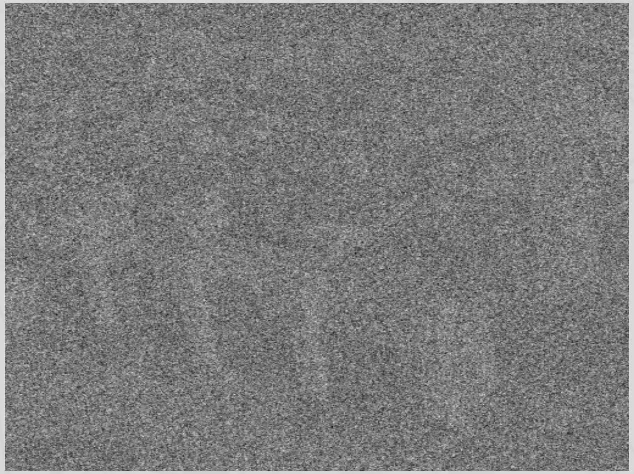
Reference-based alignment: Problems

- Reference bias

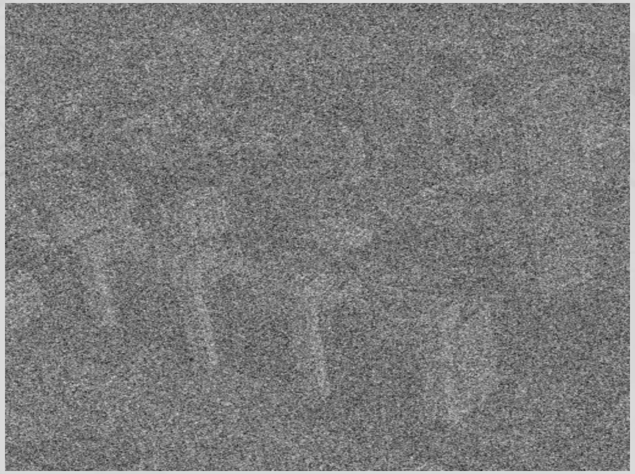
Reference-based alignment: Bias



N = 128



N = 256



N = 512

N = 1024

N = 2048

original

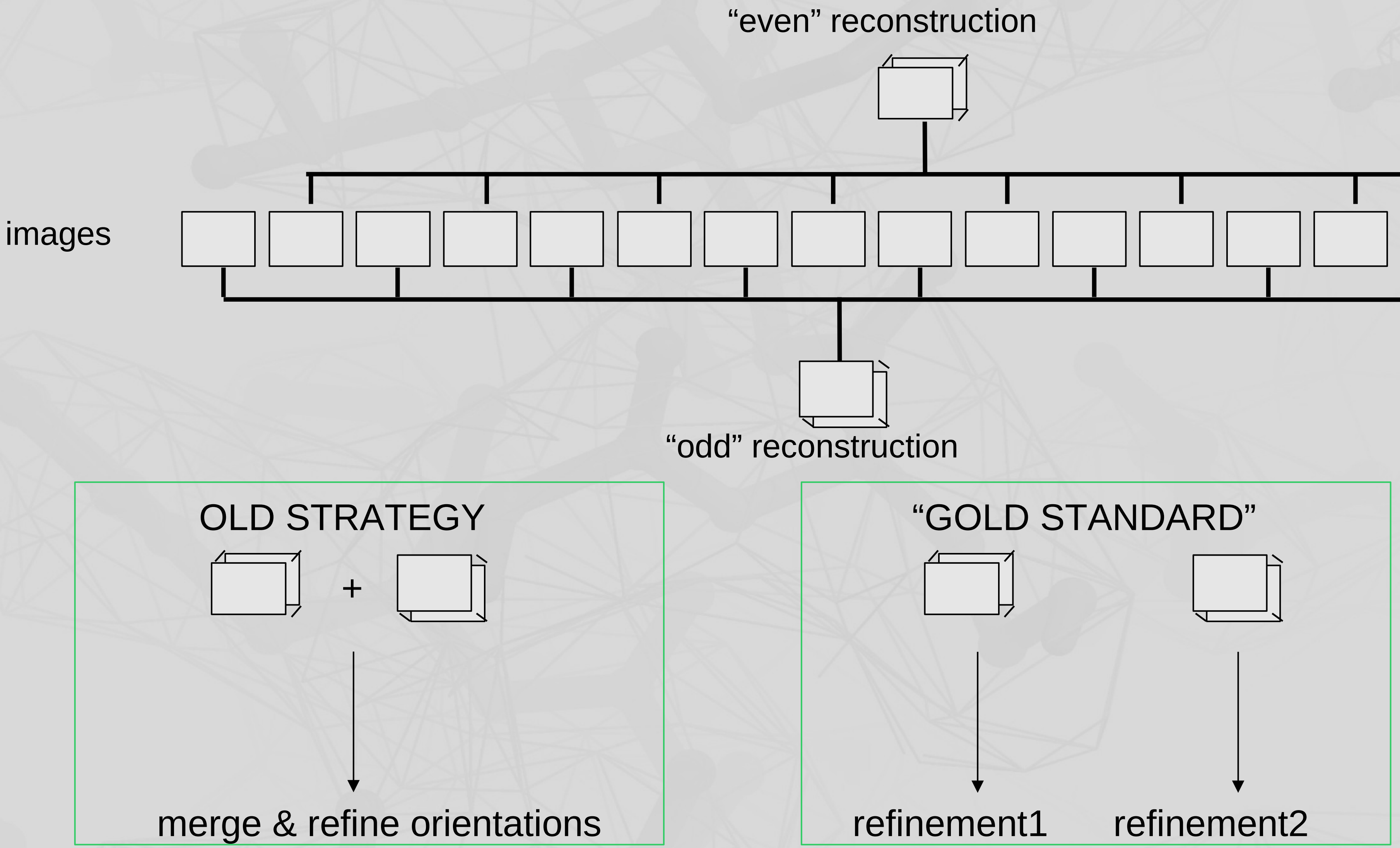
Reference bias: Solutions/defenses

- Internal reference-free alignment
  - Like in IMAGIC (?), EMAN2

Reference bias: Solutions/defenses

- Internal reference-free alignment
- “Gold” standard
  - Henderson et al. (2012) “Outcome of the First Electron Microscopy Validation Task Force Meeting.” Structure
  - Grigorieff (2000) “Resolution measurement in structures derived from single particles.“ Acta Cryst D

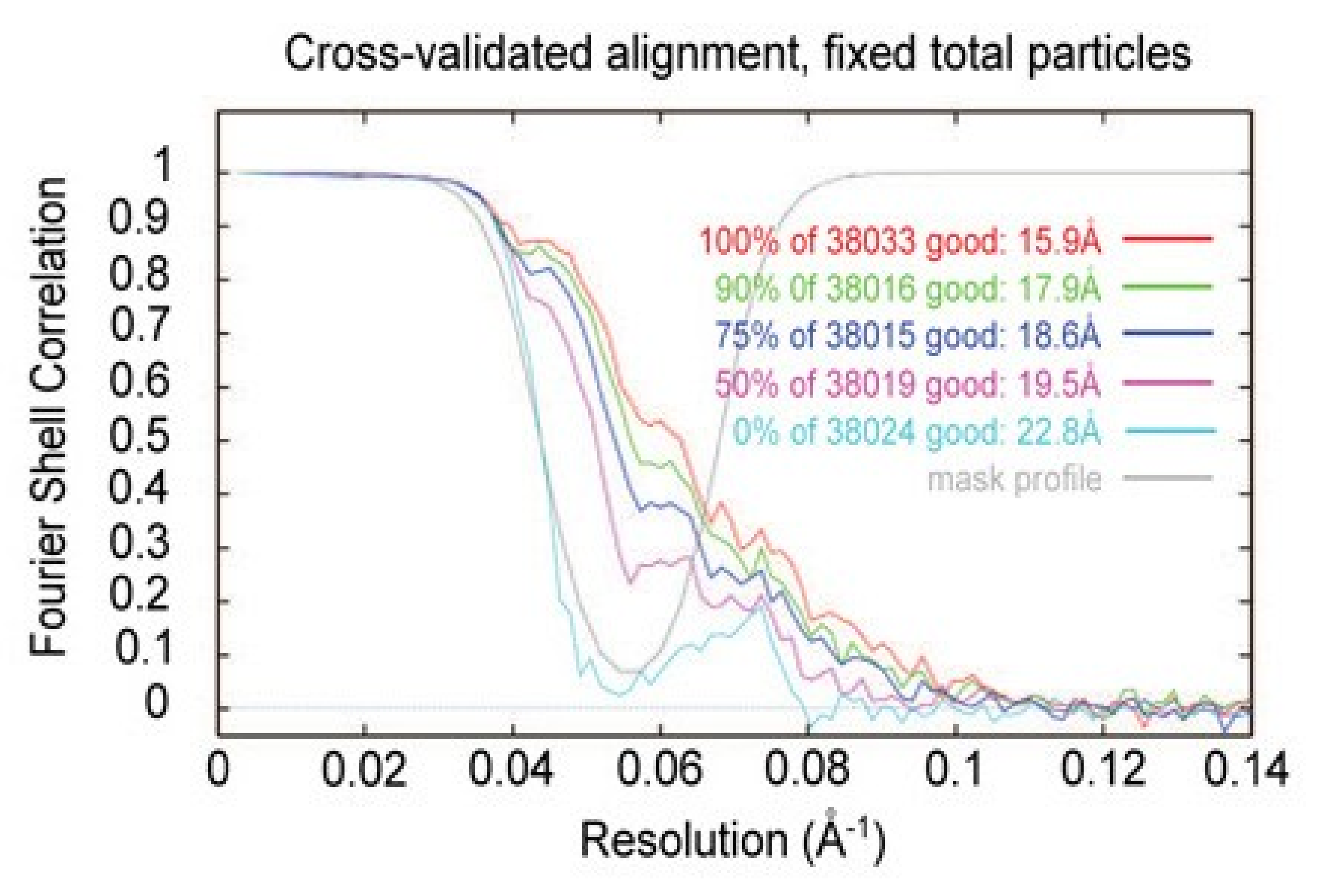
Reference-based alignment: “Gold” standard



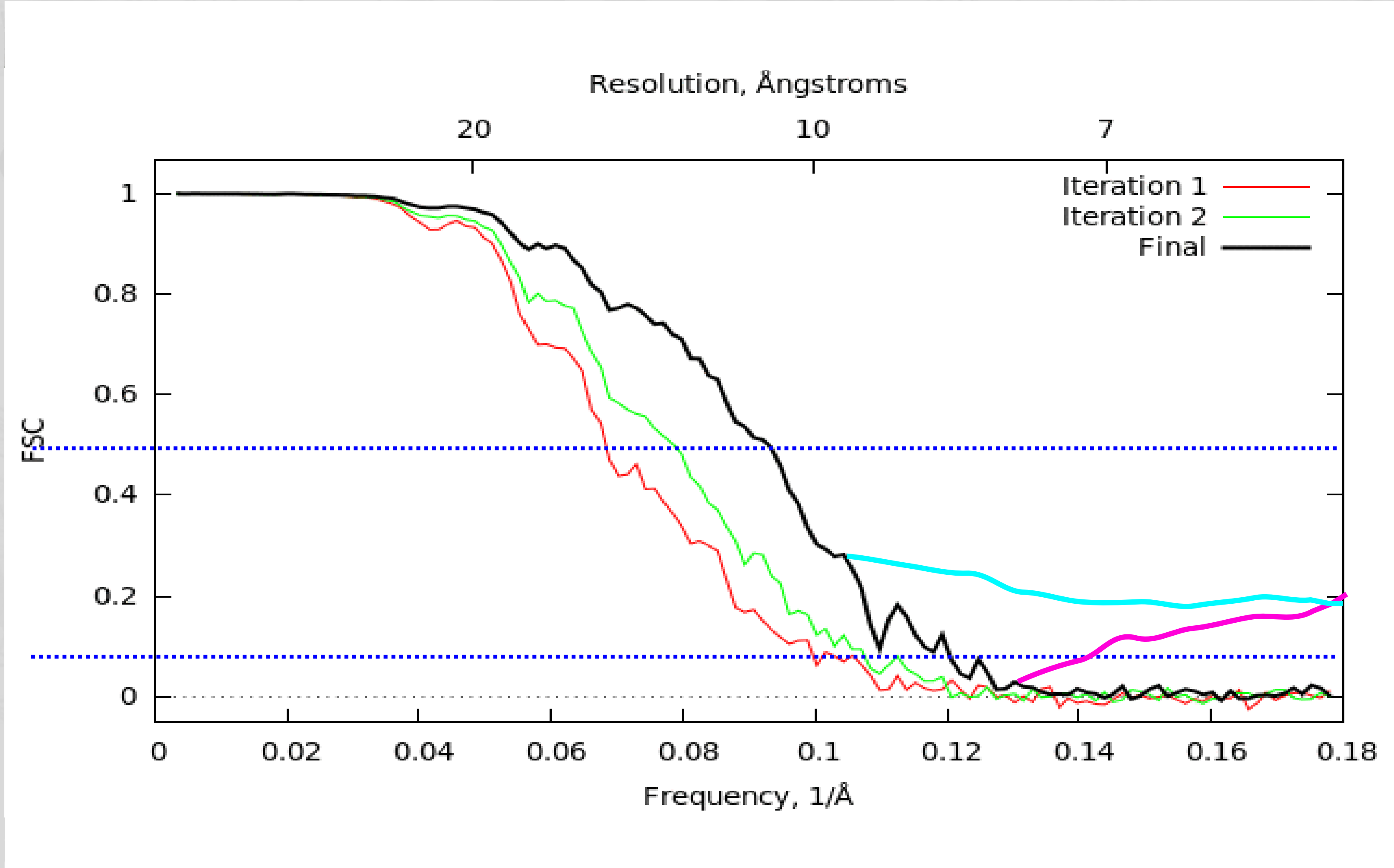
Reference bias: Solutions/defenses

- Internal reference-free alignment
- Gold standard
- Omit/alter data in reference
  - Shaikh et al (2003) "An approach to examining model dependence in EM reconstructions using cross-validation" JSB
  - Chen… Henderson (2013) "High-resolution noise substitution to measure overfitting and validate resolution in 3D structure determination by single particle electron cryomicroscopy." Ultramicroscopy

Reference-based alignment: Free FSC



Reference-based alignment: Weird FSC curves



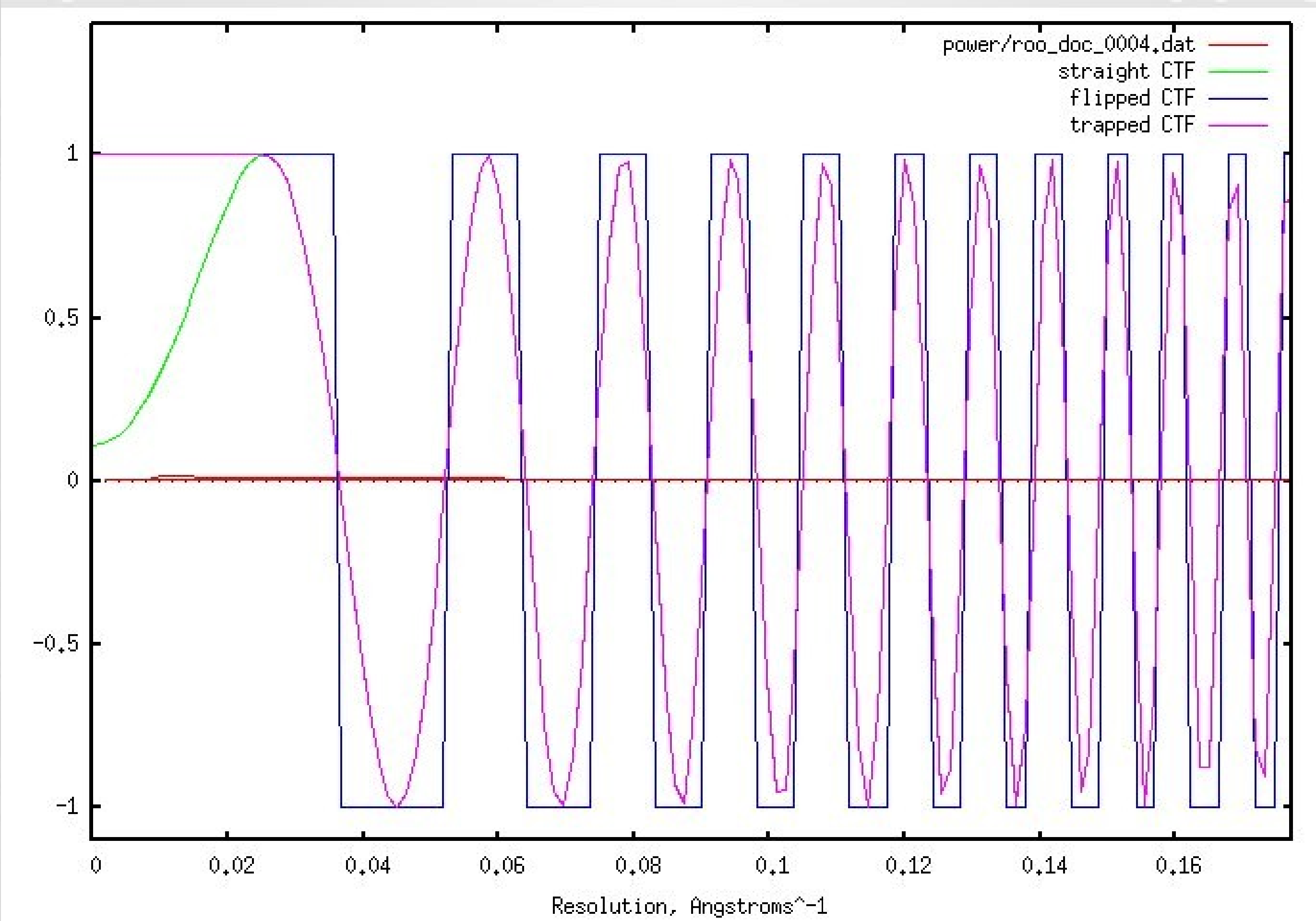
FSC=0.5

FSC=0.143

Possible causes

- Artifacts in reconstruction algorithm
- Particles accidentally duplicated in two half-sets
- Incorrect CTF estimation

CTF-correction: Phase-flipping

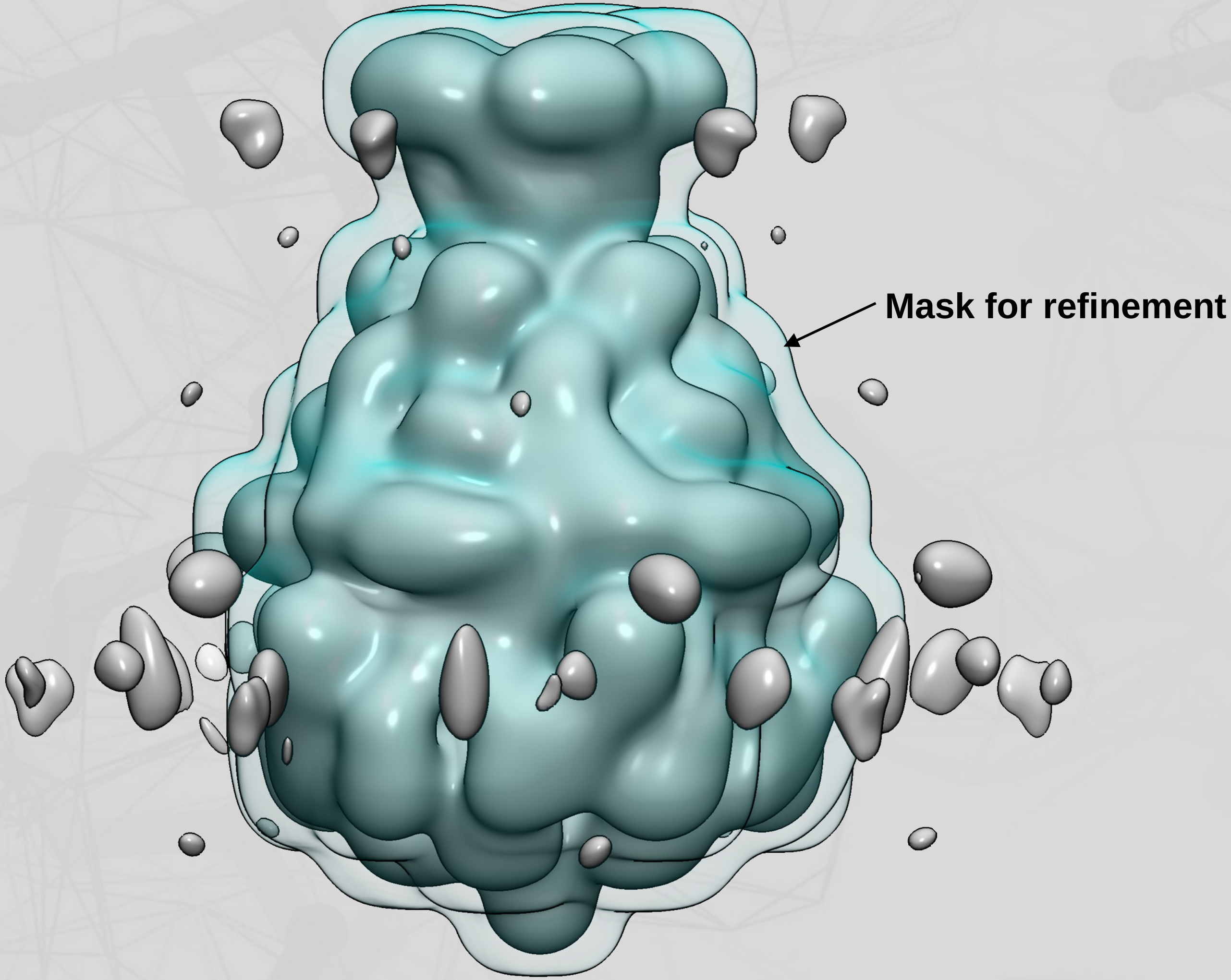


http://spider.wadsworth.org

Possible causes

- Artifacts in reconstruction algorithm
- Particles accidentally duplicated in two half-sets
- Incorrect CTF estimation
- Too tight masking
  - Or other edges or sharp features in map

Reference-based alignment: Masking



At the microscope

- Are the microscope settings really what you think they are (e.g., magnification)?
  - Calibration
  - Internal calibration standards, e.g., TMV

Before the microscope

- Is the sample really what you think it is?
  - Run a gel!
- Are the conditions really “native-like”?
  - Buffer conditions probably not physiological
  - Concentration probably higher than in vivo
  - Quaternary interactions: oligomerization state, binding partners
  - Interactions with grid
    - Air-water interface
    - Carbon, gold, graphene

Thank you for your attention