Validation Methods

Simons Electron Microscopy Center
Winter EM Course 2016-17

The dark side of single-particle EM

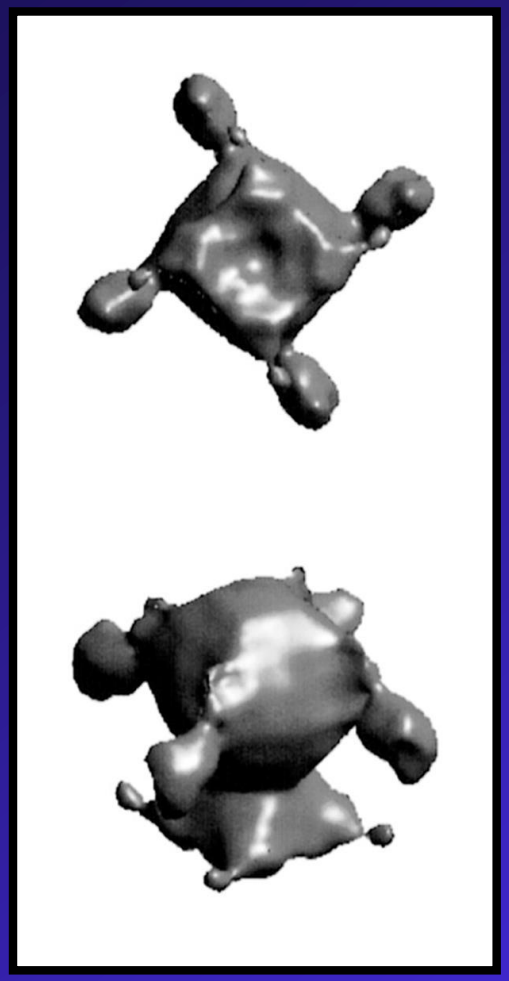
The great thing about single-particle EM:
Every data set and processing approach yields a 3D structure !

The bad thing about single-particle EM:
Every data set and processing approach yields a 3D structure !

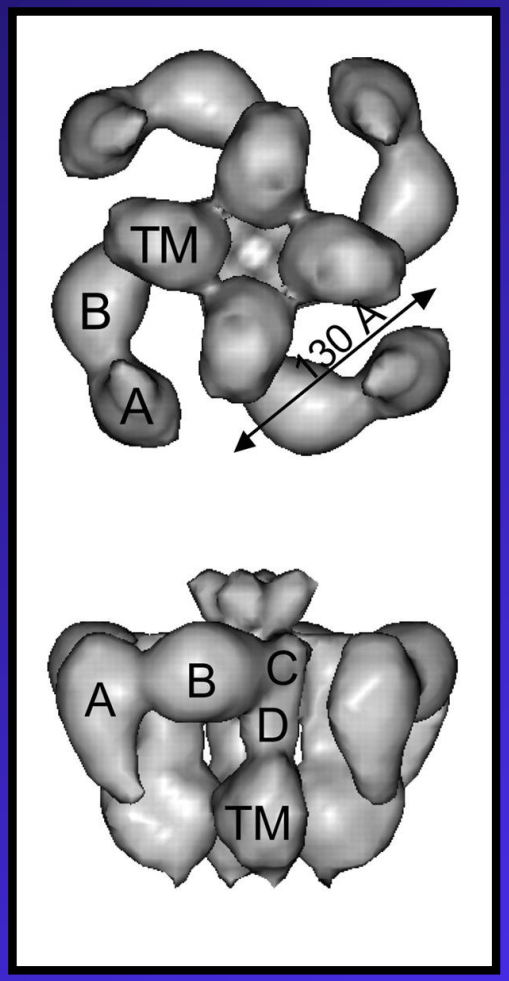
But is it correct ???

Particularly problematic
for low-resolution maps

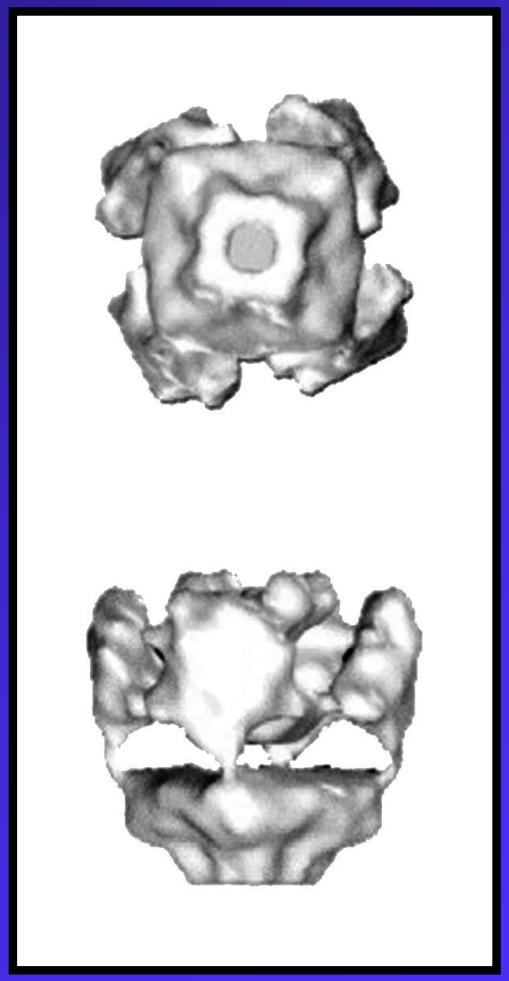
The issue: Structures of the IP3 receptor as determined by single-particle EM



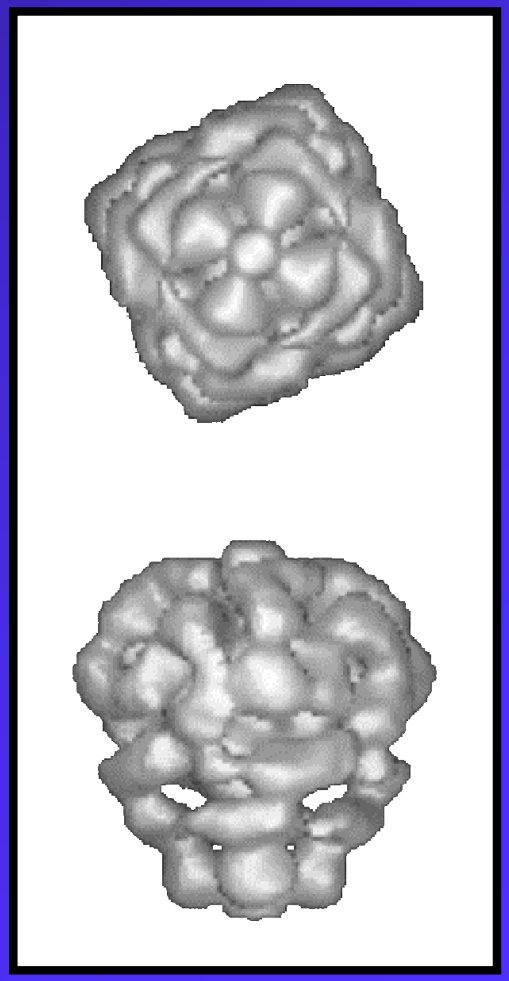
Jiang et al., 2002



Serysheva et al., 2003

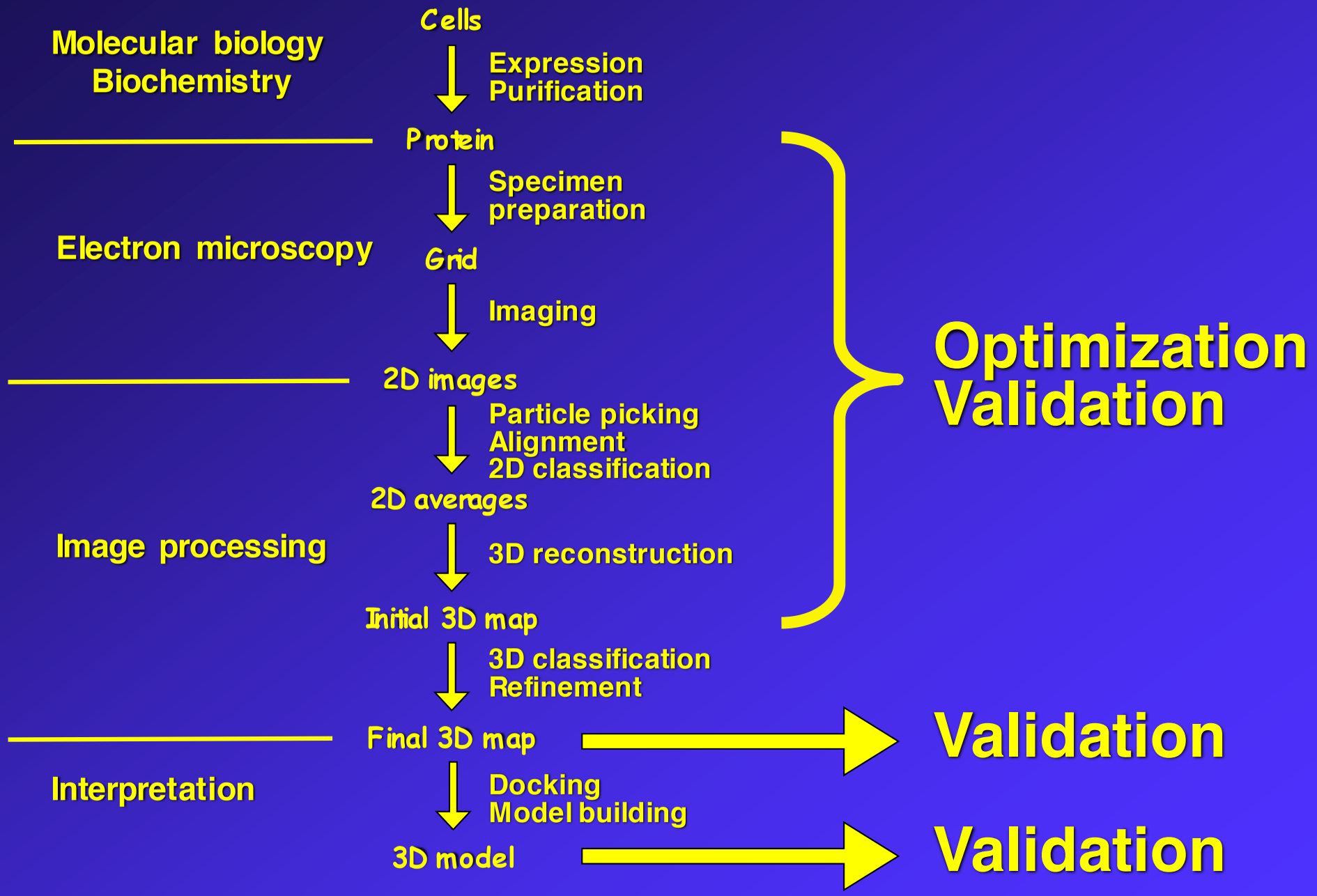


Jiang et al., 2003

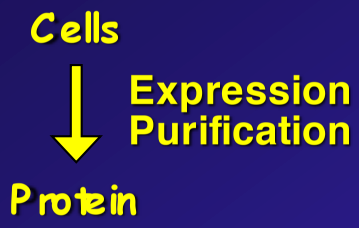


Sato et al., 2004

Structure determination by single-particle EM



Structure determination by single-particle EM



Potential issues:

Heterogeneity
- Compositional
- Conformational
  - Discrete states
  - Continuous movement

Effect of cross-linking

Structure determination by single-particle EM

Potential issues with samples

Before attempting structure determination –
Understand and optimize your sample !

Prepare negatively stained specimens:
Good contrast and preferred orientations
→ Easy to assess heterogeneity

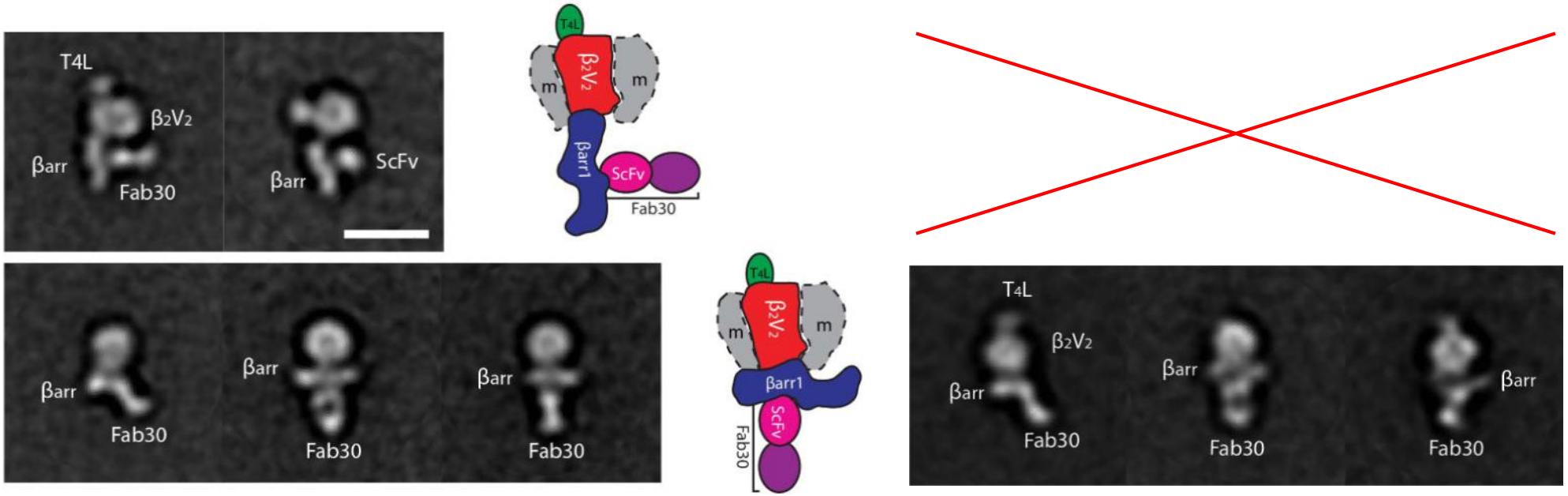
If particles look heterogeneous:
Calculate class averages
→ Assess type and degree of heterogeneity
→ Minimize heterogeneity by any means possible

If chemical fixation was used:
Look at unfixed sample to assess effect of cross-linking
→ Assess whether structure of cross-linked sample is meaningful

Effect of cross-linking: The β_2V_2R–βarrestin1–Fab30 complex

Native

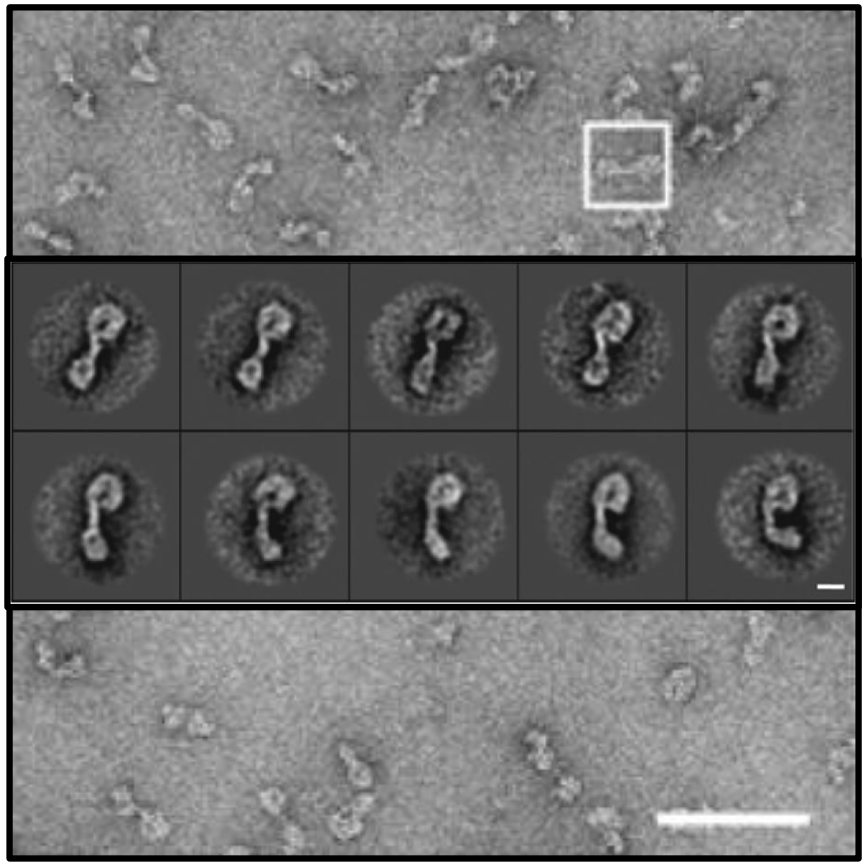
Cross-linked



Shukla et al. (2014) Nature 512: 218-222

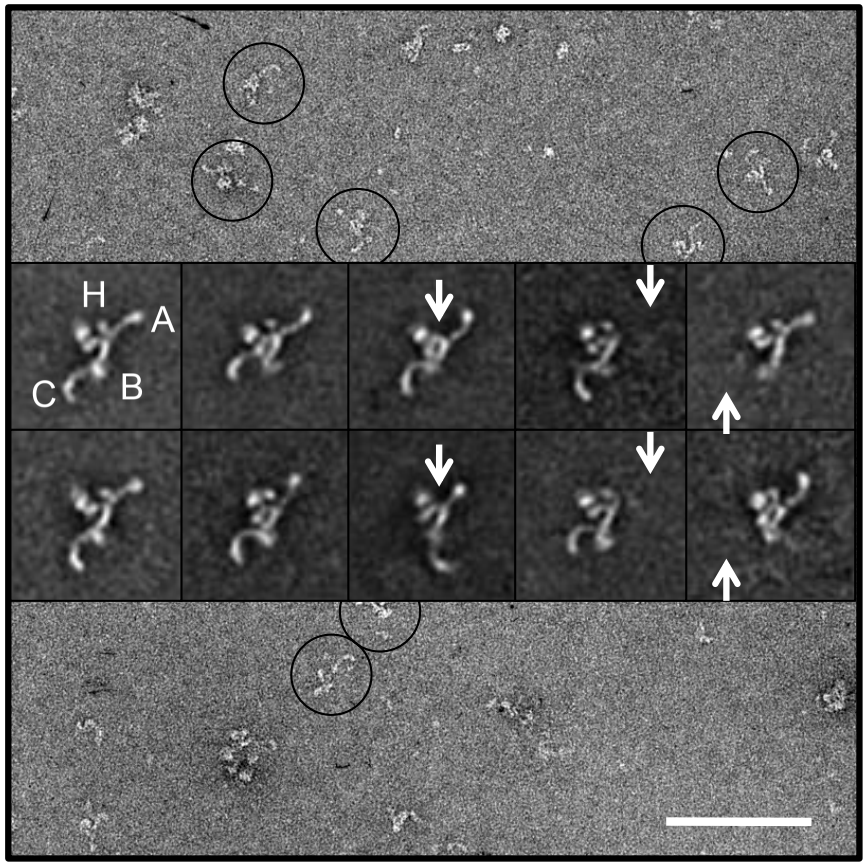
Effect of cross-linking: The HOPS tethering complex

Cross-linked



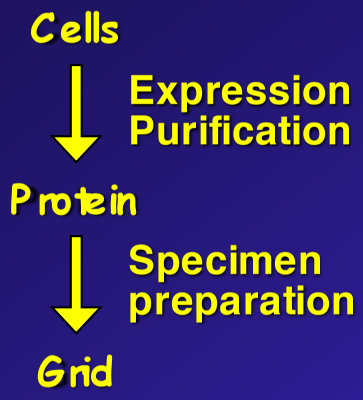
Bröcker et al. (2012)
PNAS 109:1991-1996

Native



Chou et al. (2016)
NSMB 23:761-763

Structure determination by single-particle EM



Potential issues:

- No particles
- Preferred orientations

Structure determination by single-particle EM

Potential issues with grids

No particles (particles bind to carbon and avoid holes)

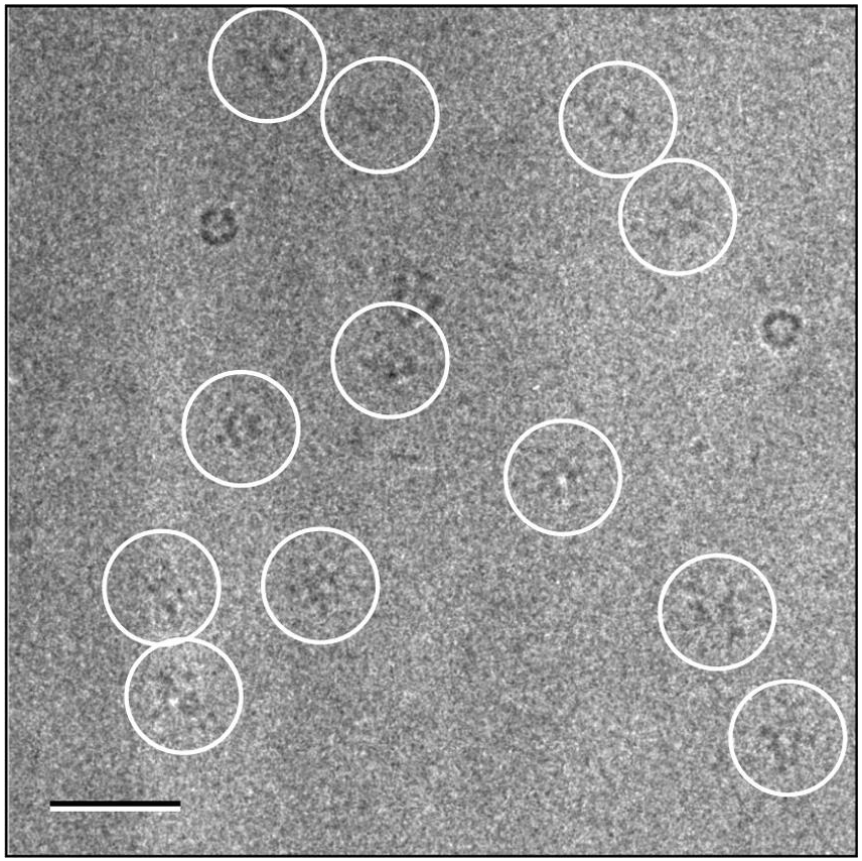
- Increase protein concentration
- Double blotting
- PEG treatment of grid
- Use thin carbon film

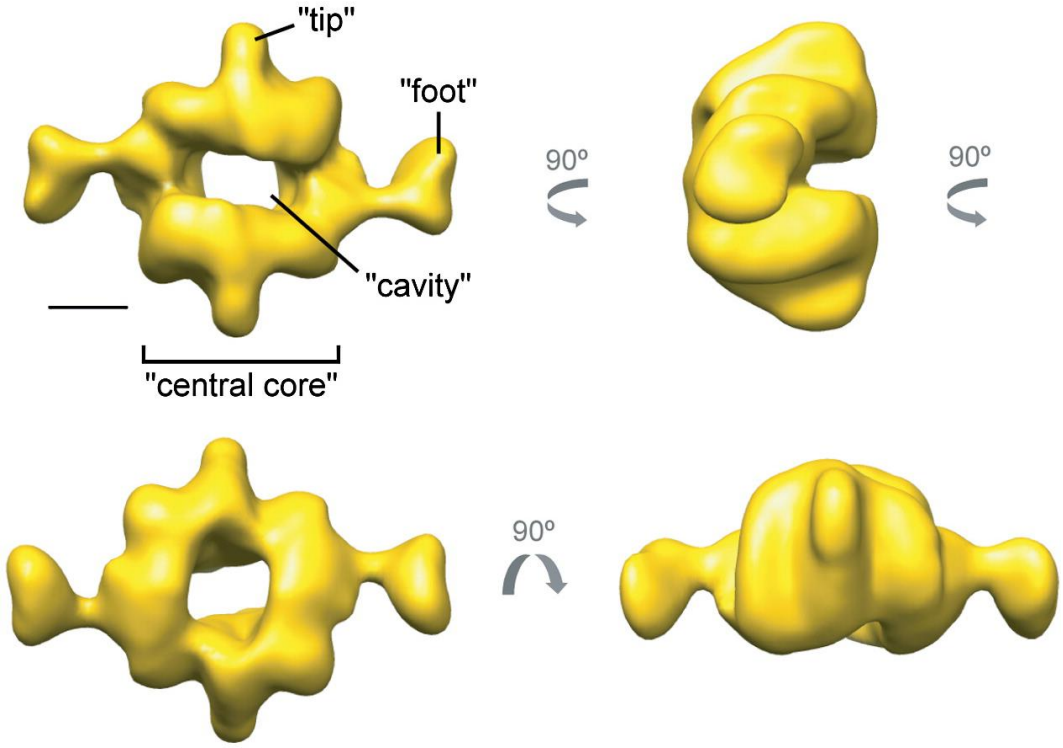
Preferred orientation (particles align at air/water interface)

Lack of views will result in:

- non-isotropic resolution of the density map

The mTOR1 complex





Yip et al. (2010) Mol. Cell 38:768-774

Structure determination by single-particle EM

Potential issues with grids

No particles (particles bind to carbon and avoid holes)

- Increase protein concentration
- Double blotting
- PEG treatment of grid
- Use thin carbon film

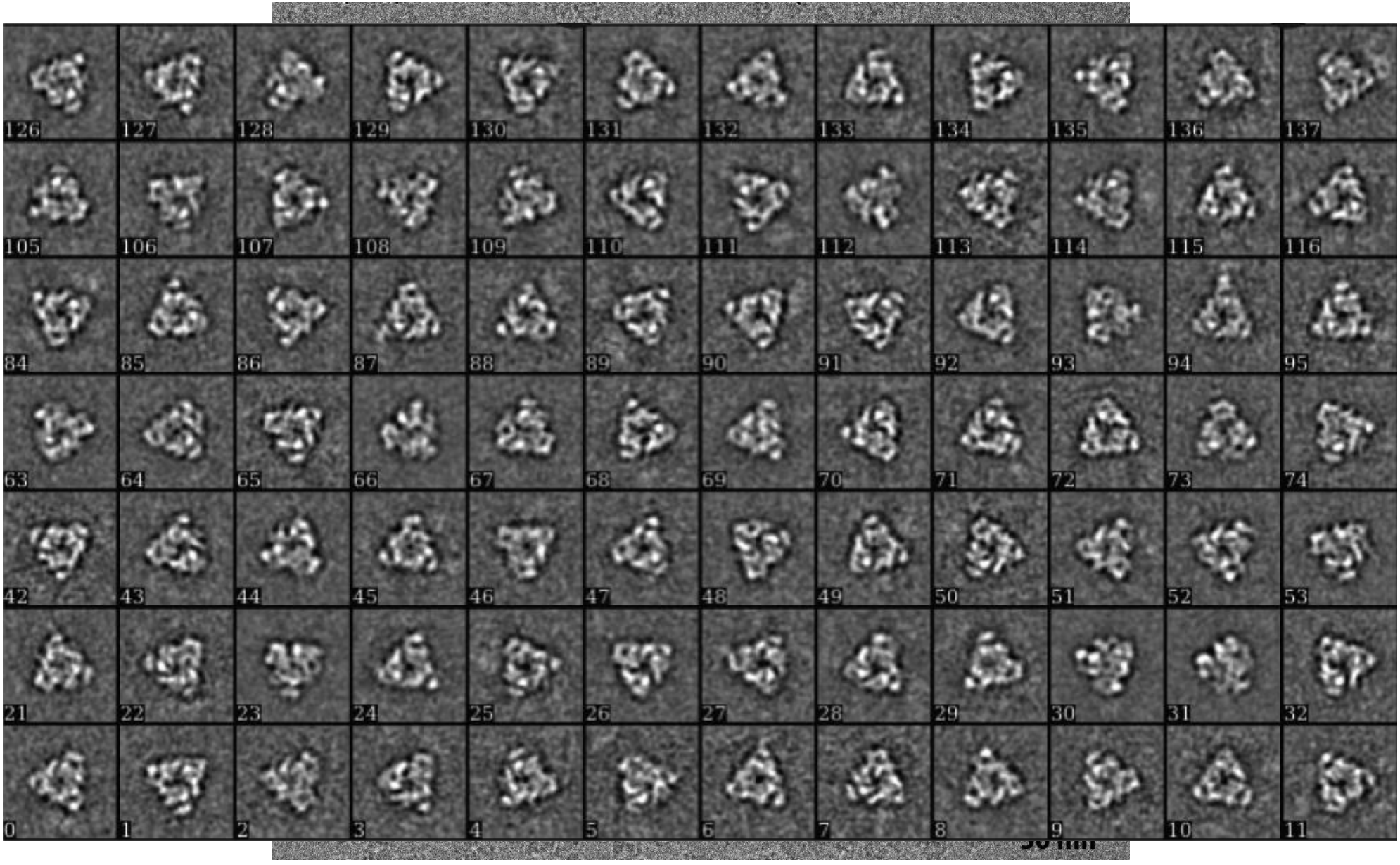
Preferred orientation (particles align at air/water interface)

Lack of views will result in:

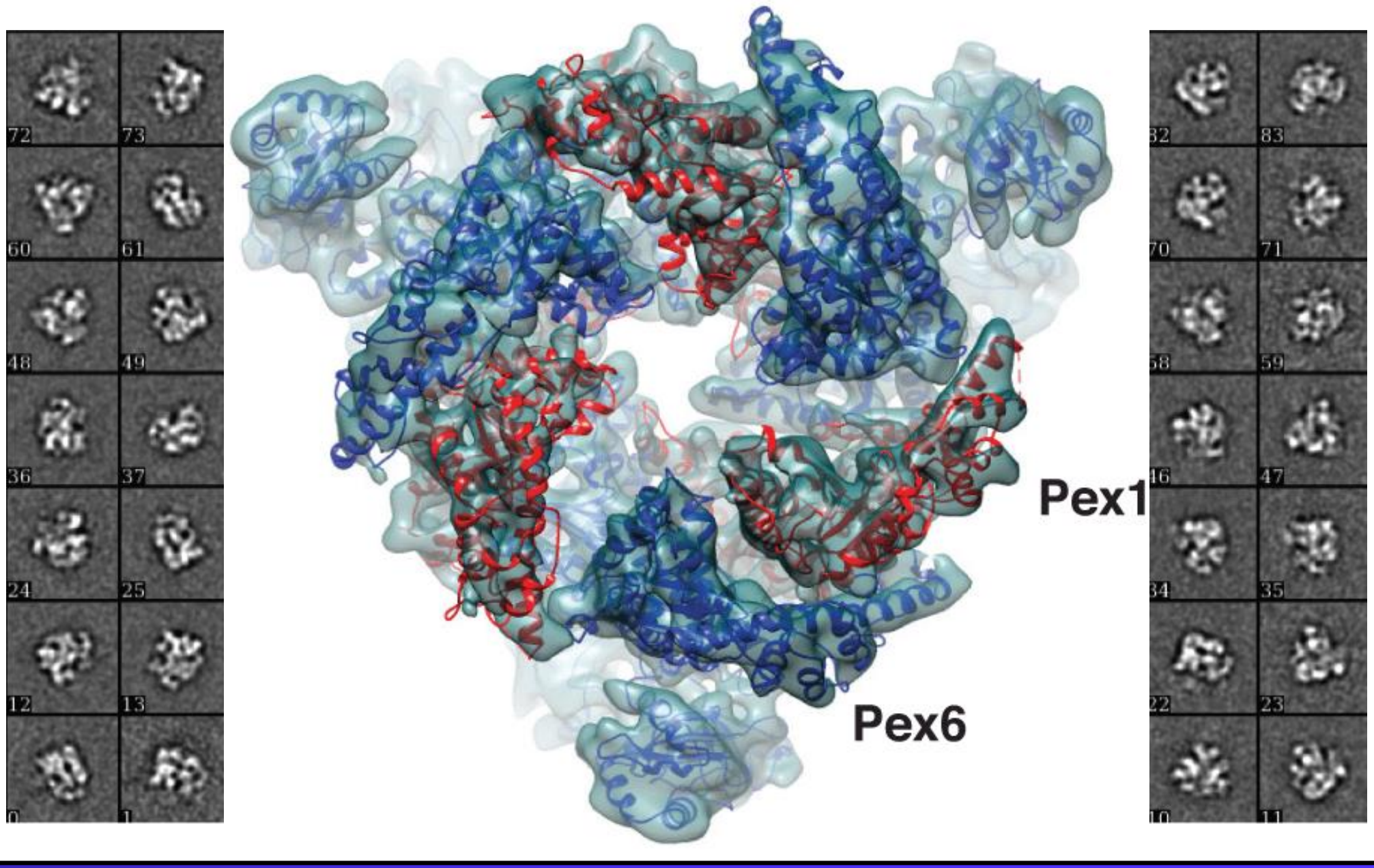
- non-isotropic resolution of the density map
- can potentially lead to an incorrect density map

- Use thicker (or thinner) ice
- Use low concentration of detergent (changes surface tension)
- Use thin carbon film (commonly used for ribosome samples)
- Use gold grids (Russo & Passmore (2014) Science 346: 1377-1380)

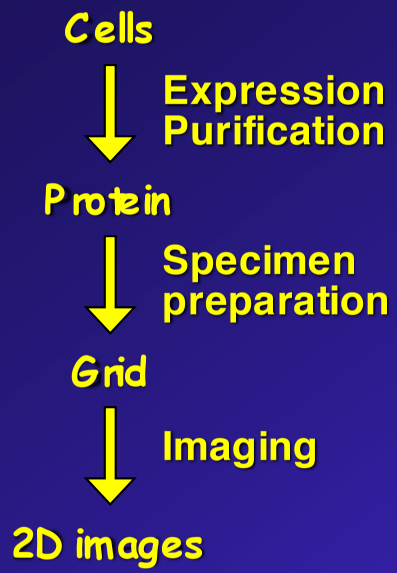
Preferred orientations: Pex1/6 complex Without detergent



Preferred orientations: Pex1/6 complex With detergent



Structure determination by single-particle EM



Potential issues:

- Low contrast
- Beam damage

Structure determination by single-particle EM

Potential issues with images

Poor electron scattering
→ high electron dose

→ Poor SNR can be fixed by averaging

Beam sensitivity
→ low electron dose

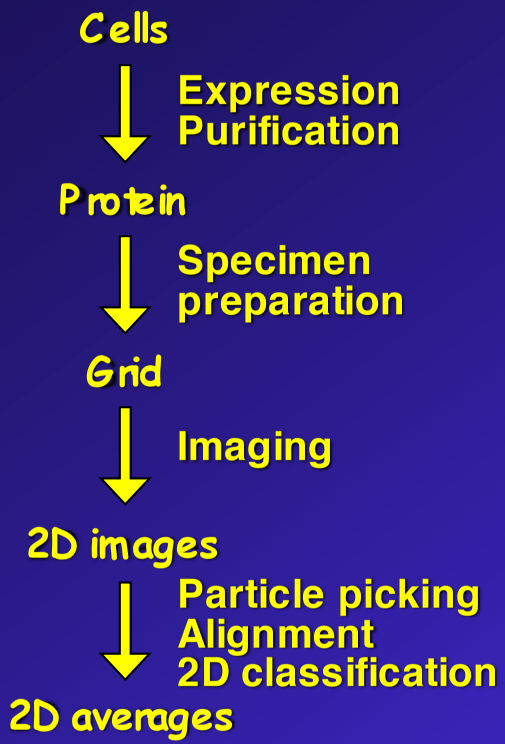
→ Loss of information cannot be fixed

→ Electron micrographs recorded with low electron doses
→ Particles hard too see, especially small ones

Problem fixed by DDD cameras

→ Collect long movies
→ Add frames with resolution filter

Structure determination by single-particle EM



Potential issues:

Particle picking:

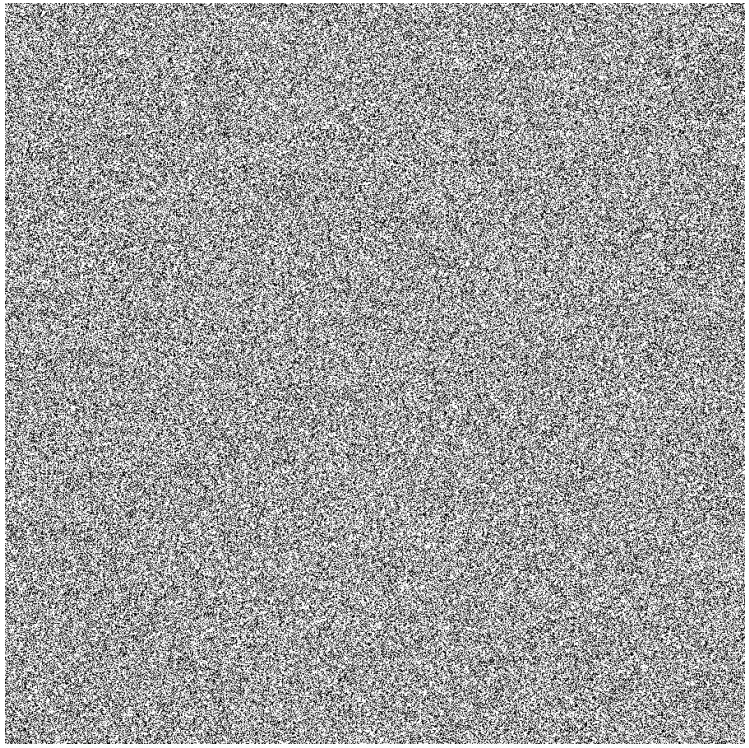
- Model/reference bias

2D classification:

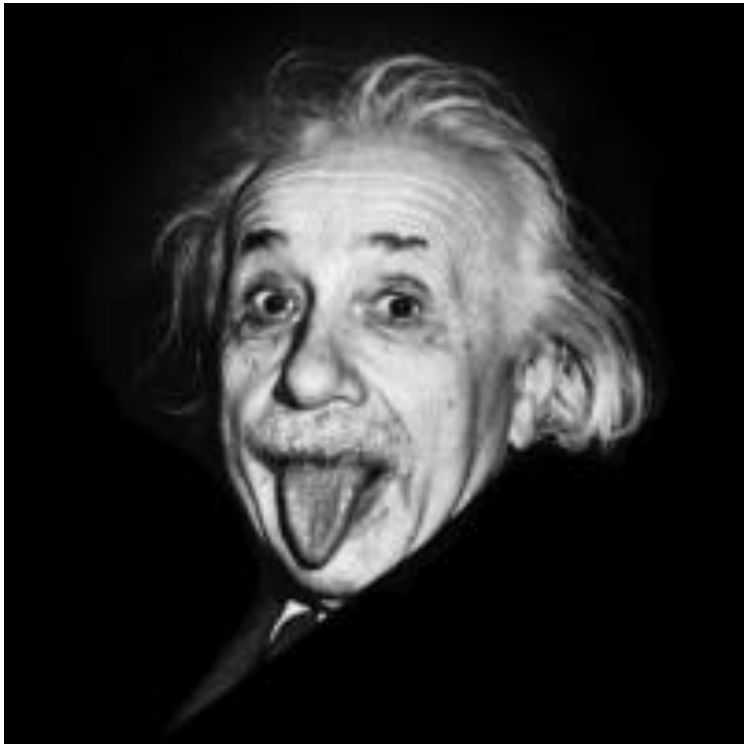
- Model/reference bias
- Number of classes
- Heterogeneous classes
- Disappearing classes

Structure determination by single-particle EM

Potential issues with particle picking



1,000 images containing
pure white noise



Reference:
Albert Einstein

Shatsky et al. (2009) J. Struct. Biol. 166:67-78
Henderson (2013) Proc. Natl. Acad. Sci. USA 110:18037-18041

Structure determination by single-particle EM

Potential issues with particle picking

Model/reference bias

Average of 1,000 images containing pure white noise after alignment to an image of Albert Einstein

→ Einstein from noise

Shatsky et al. (2009) J. Struct. Biol. 166: 67-78
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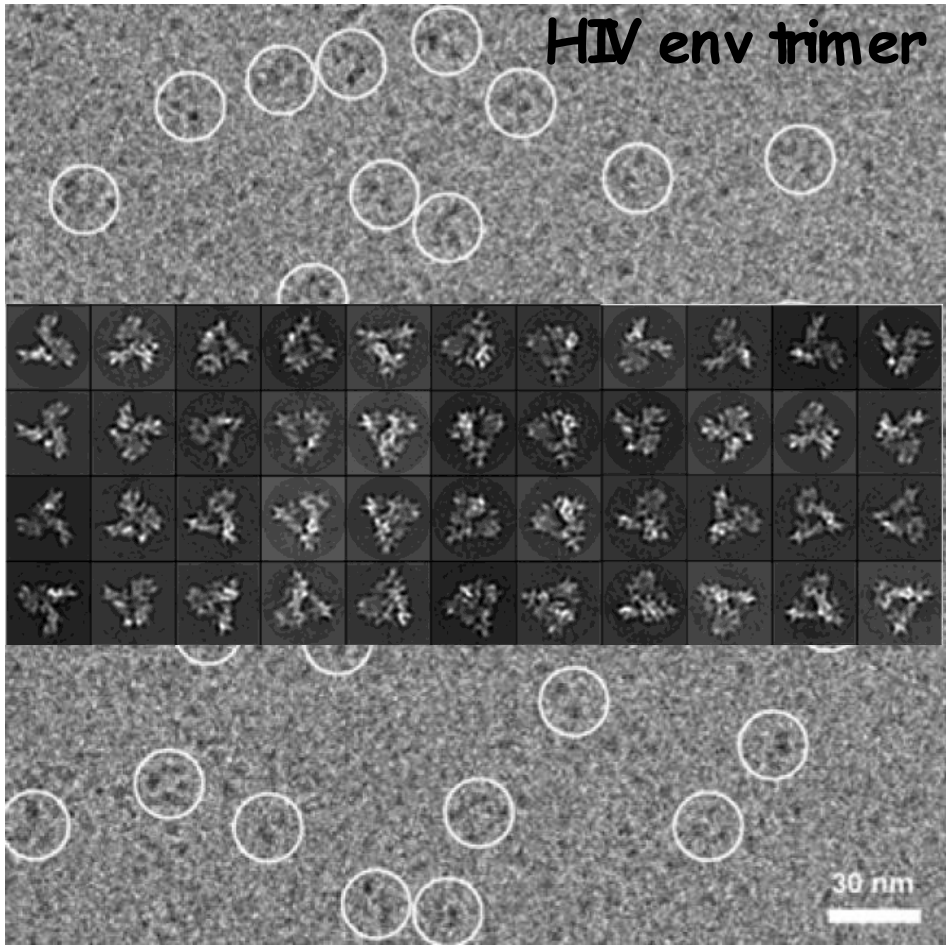
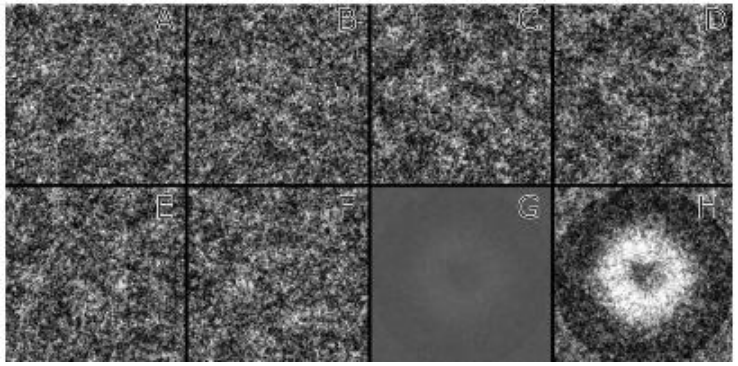
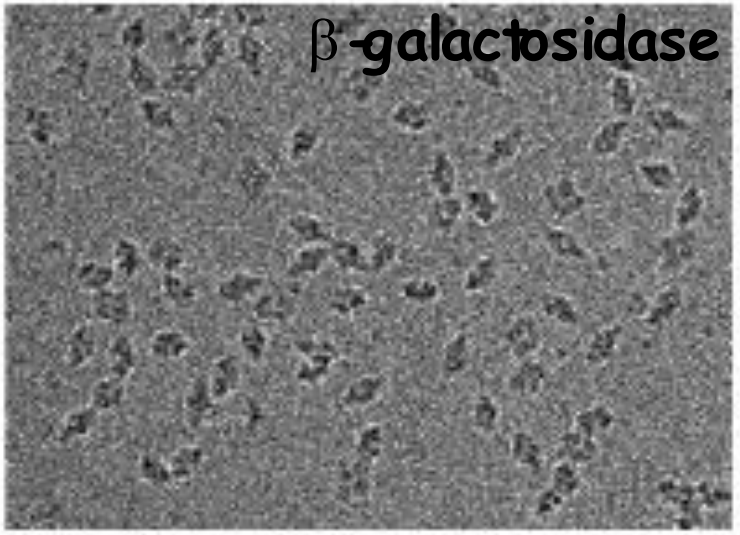
Structure determination by single-particle EM

Potential issues with particle picking



Mao et al. (2013)
PNAS 110:12438-12443





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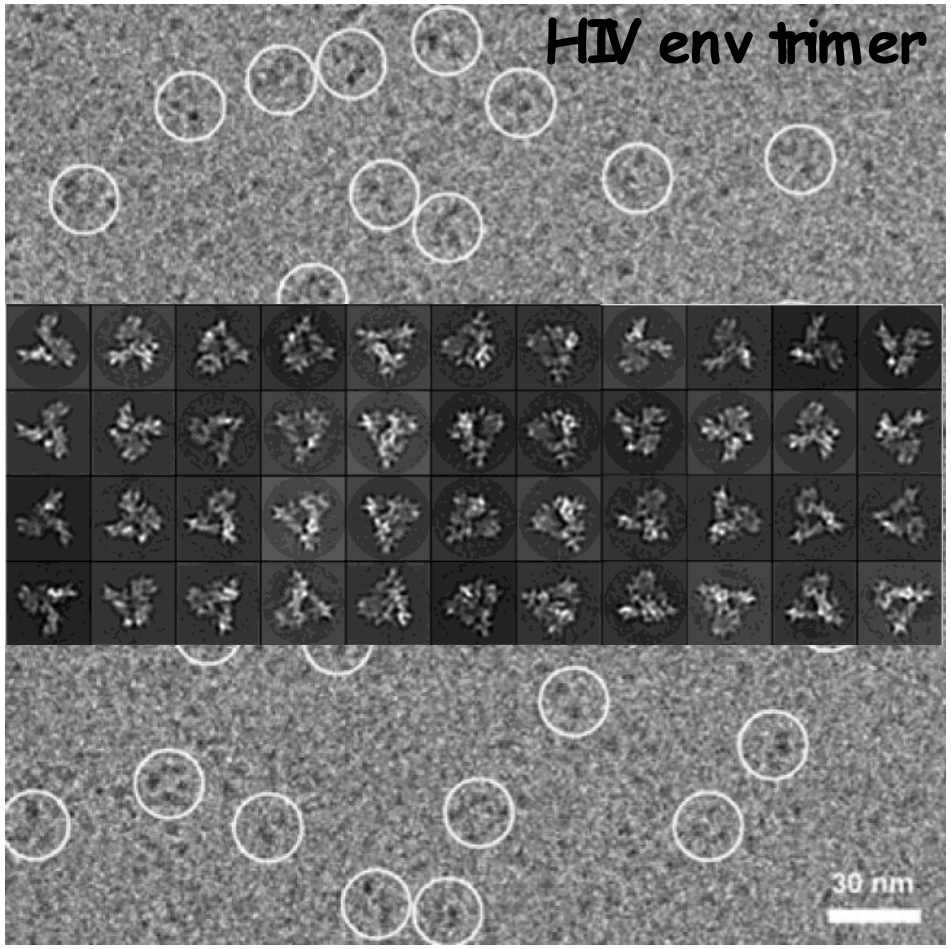
Structure determination by single-particle EM

Potential issues with particle picking



Mao et al. (2013)
PNAS 110: 12438-12443

Using template matching to pick particles from very noisy images is dangerous

→ Averages will end up looking like templates used for particle picking

Henderson (2013)
PNAS 110: 18037-18041

Structure determination by single-particle EM

Potential issues with 2D classification (K-means)

K-means classification needs to be initialized with
a number of classes K

- Deterministic initialization
  - K templates are provided
    (supervised classification, multi-reference classification)
    → reference bias → Einstein from noise

- Random initialization
  - K images are randomly chosen and used as references
  - data set is randomly split into K classes and class averages are used
    → results tend to be unstable (different results for different repeats)

Structure determination by single-particle EM

Potential issues with 2D classification (K-means)

Properties / issues of K-means classification

- the algorithm always converges, but not necessarily to the global optimum (the best possible solution)
- outliers (rare objects whose appearance is partially or entirely unrelated to that of the bulk of the data) have a very negative impact on the outcome
- problem of "group collapse", i.e., the possibility of a group losing its members to the point of vanishing
- if the number of groups is not guessed correctly and the groups are not well separable (always the case for very noisy data), the result depends dramatically on the initialization

Structure determination by single-particle EM

Potential issues with 2D classification (K-means)

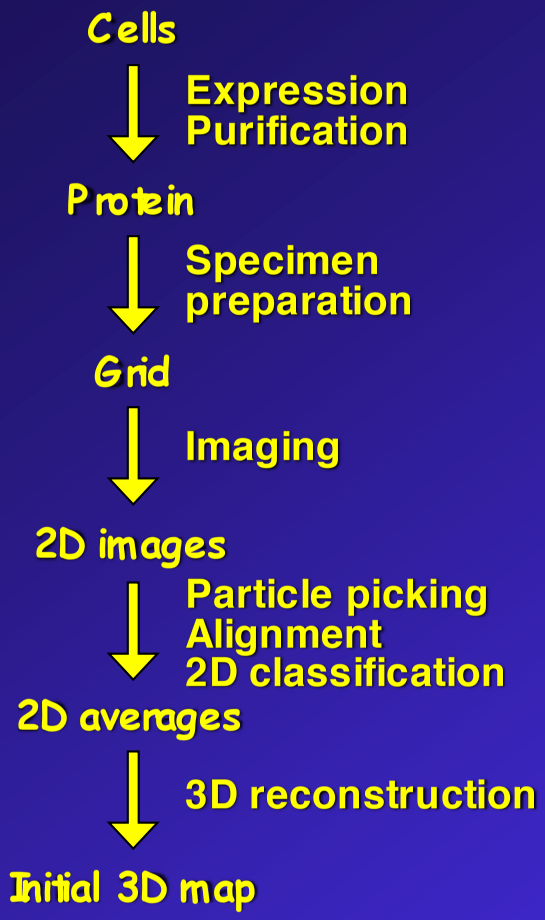
Iterative stable alignment and clustering (ISAC) procedure
Yang et al. (2012) Structure 20: 237-247

- Equal-size group K-means classification
  → prevents group collapse

- Assessment that alignment parameters for images in a cluster are stable (below a pixel error threshold) in repetitions
- Assessment that classes are reproducible in repetitions
  → classes are stable and reproducible
  → classes are homogeneous = good for 3D reconstruction

  - Only a fraction of the data set is assigned to classes
  - Computationally very expensive

Structure determination by single-particle EM



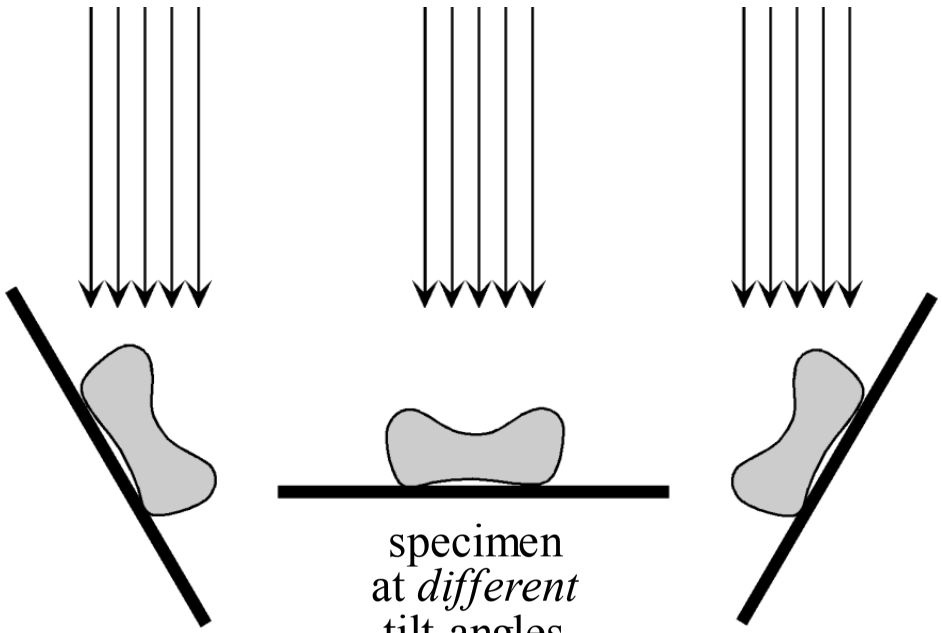
Potential issues:

Incorrect map

Because of:

- Heterogeneous sample
- Missing views
- Incorrect solution

Random conical tilt reconstruction

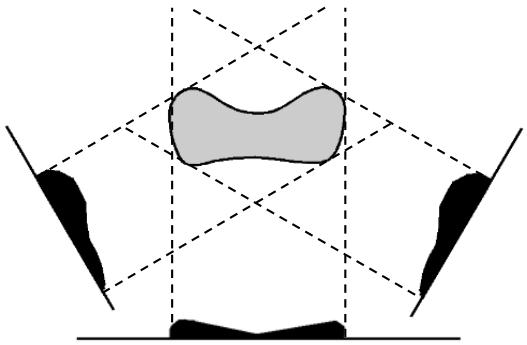


IMAGING

Different projection views

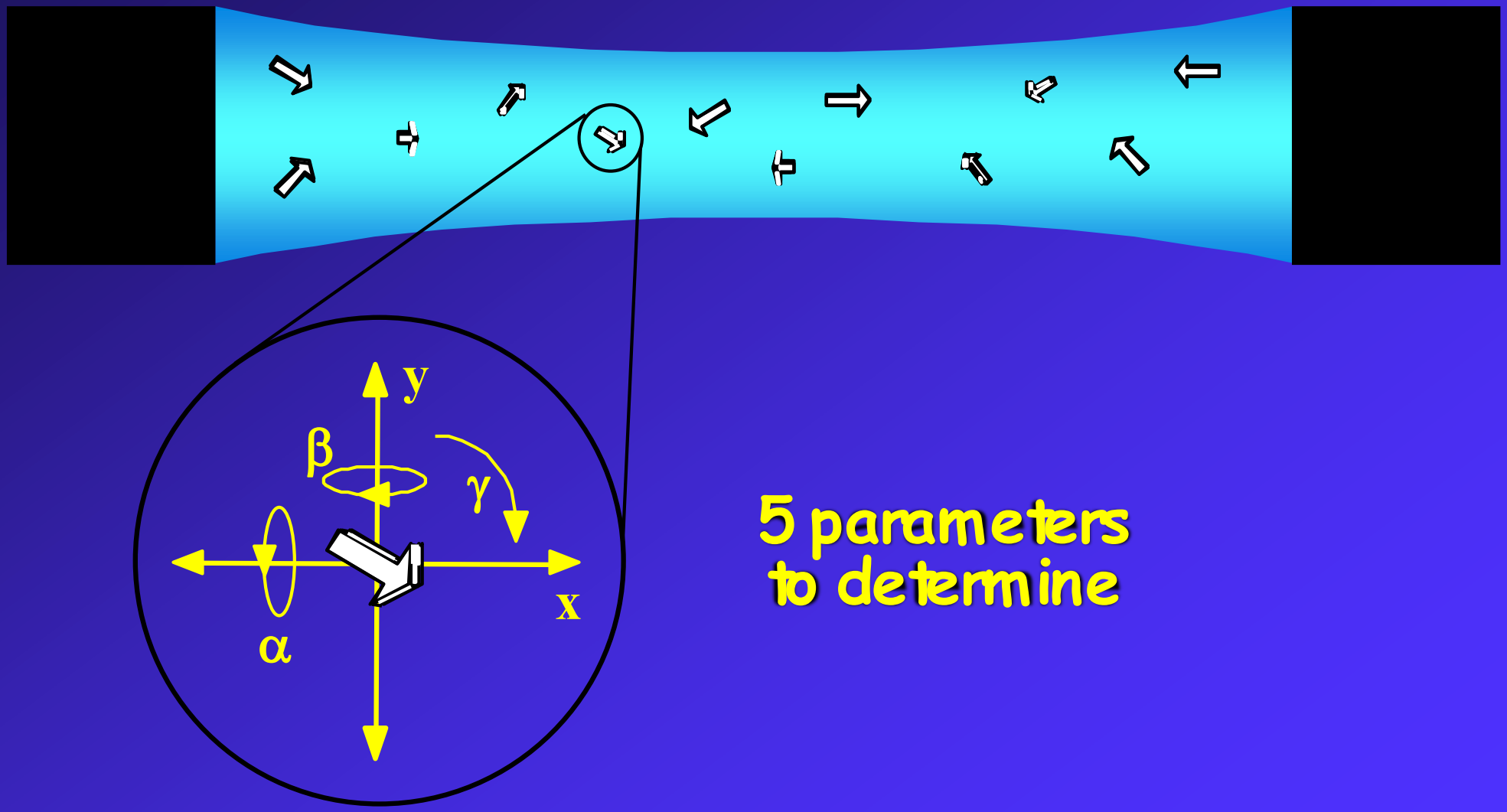
ASSIGN ORIENTATION
PARAMETERS x, y and Φ

3D reconstruction
of specimen



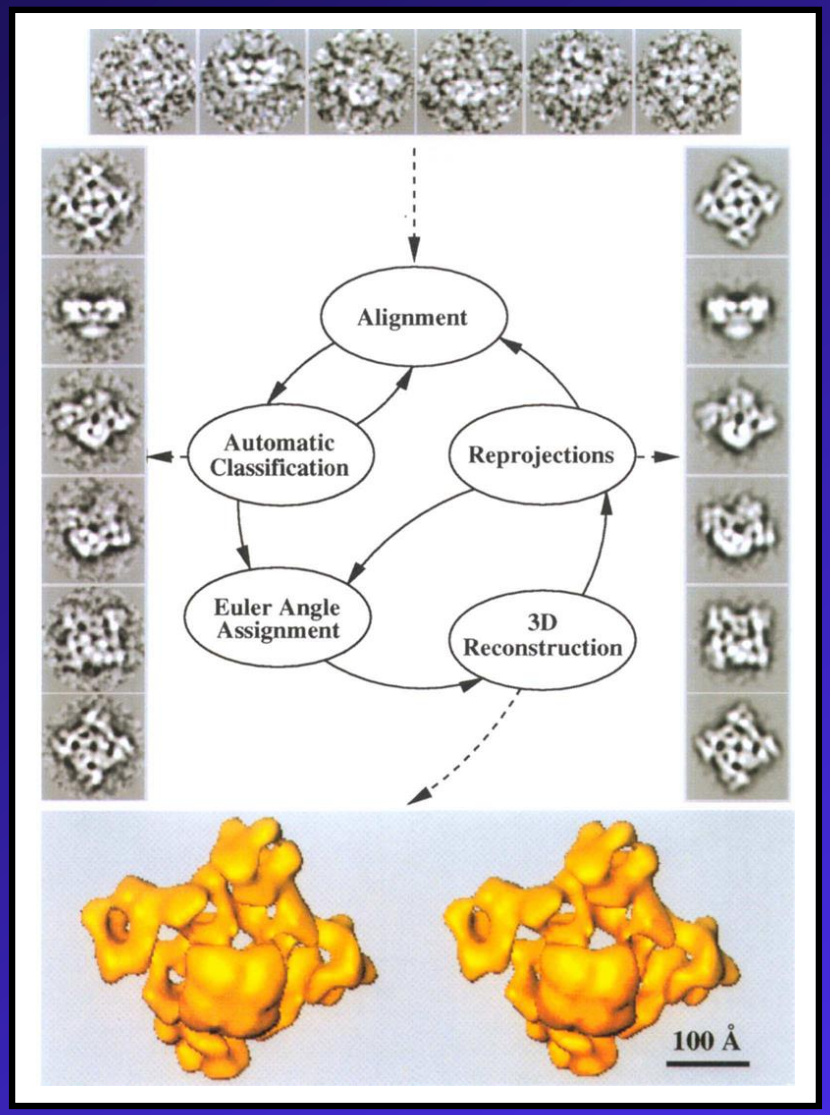
BACKPROJECTION

Single particles in ice



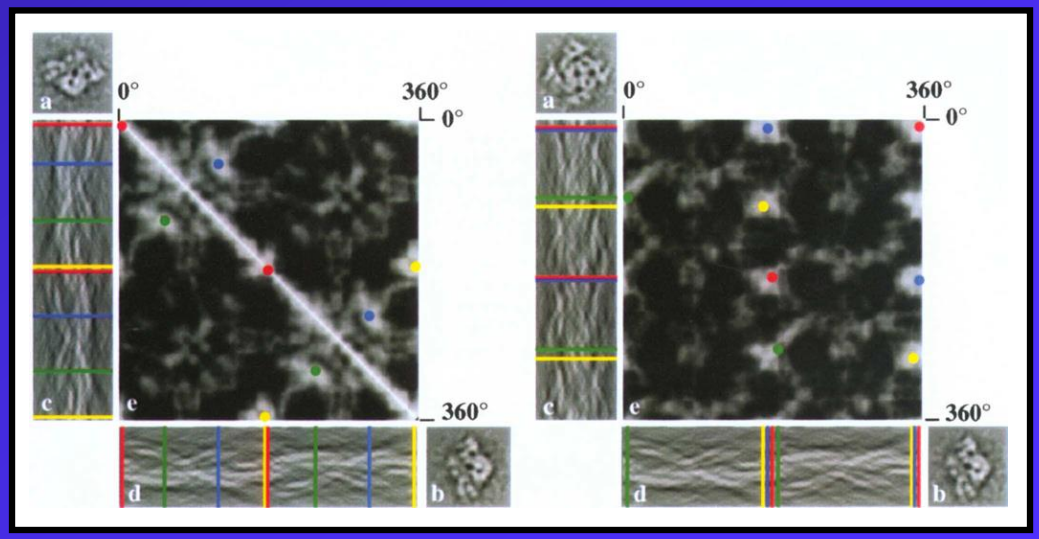
5 parameters
to determine

Angular reconstitution



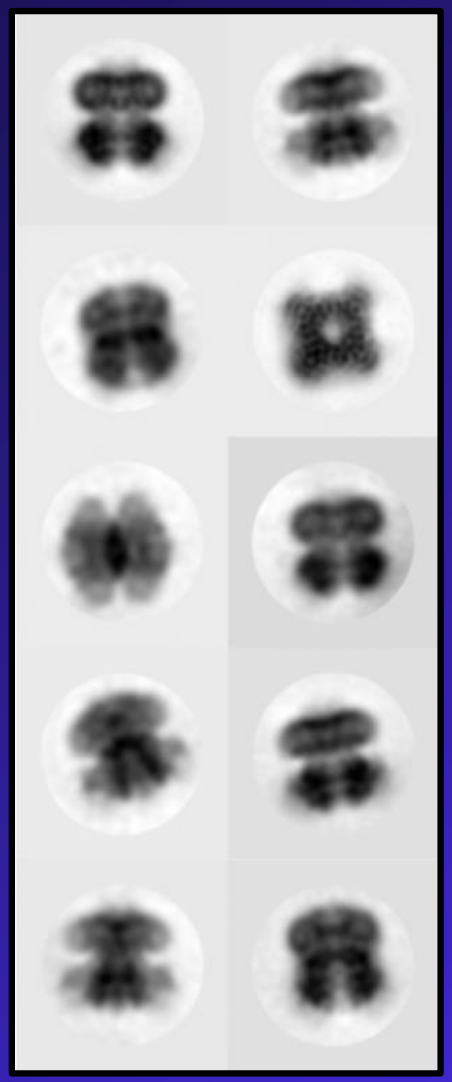
van Heel, 1987

1. choose 3 projection images that are perpendicular views of the particle (anchor set)
2. add in further projections and keep refining

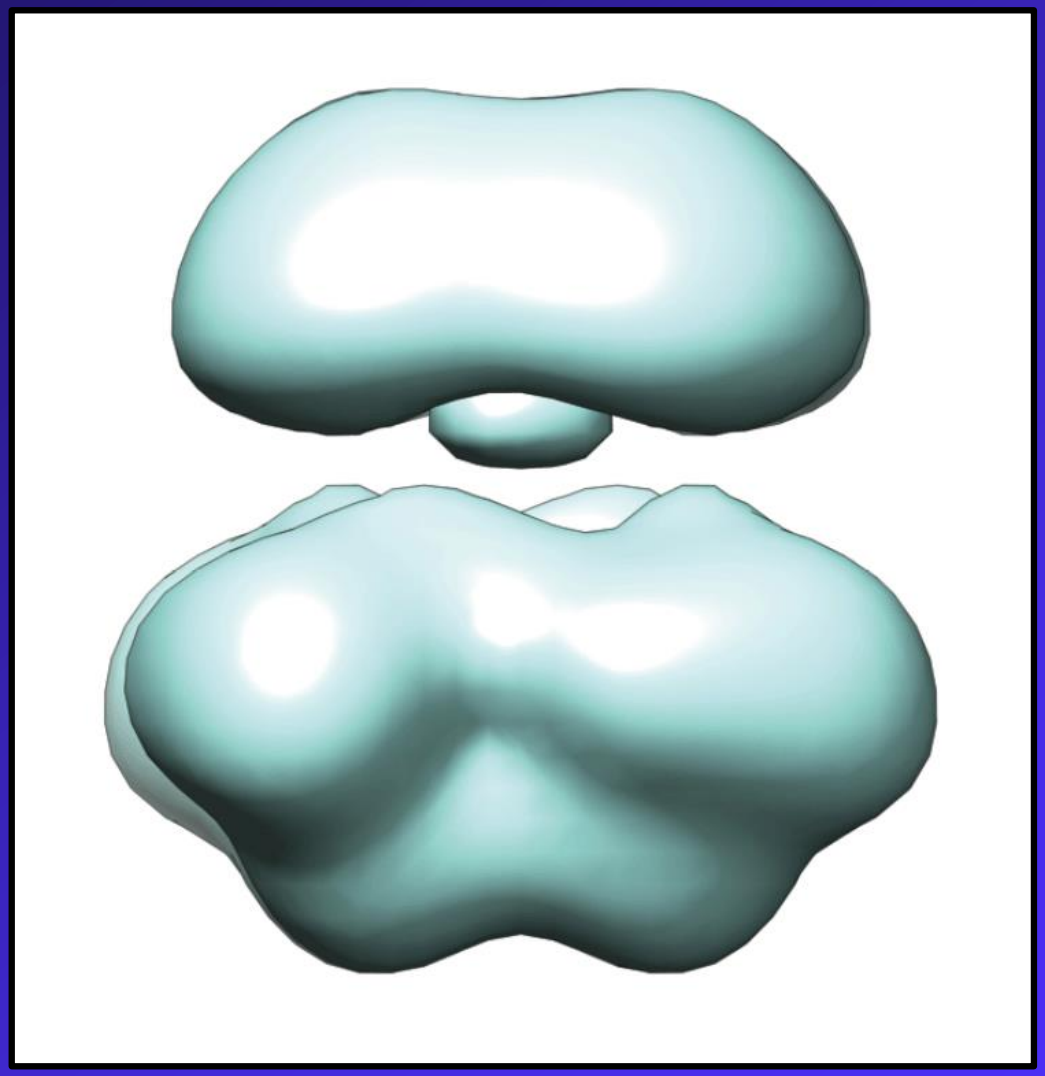


Serysheva et al., 1995

Chicken Slo2.2 in the absence of Na^+



Class averages



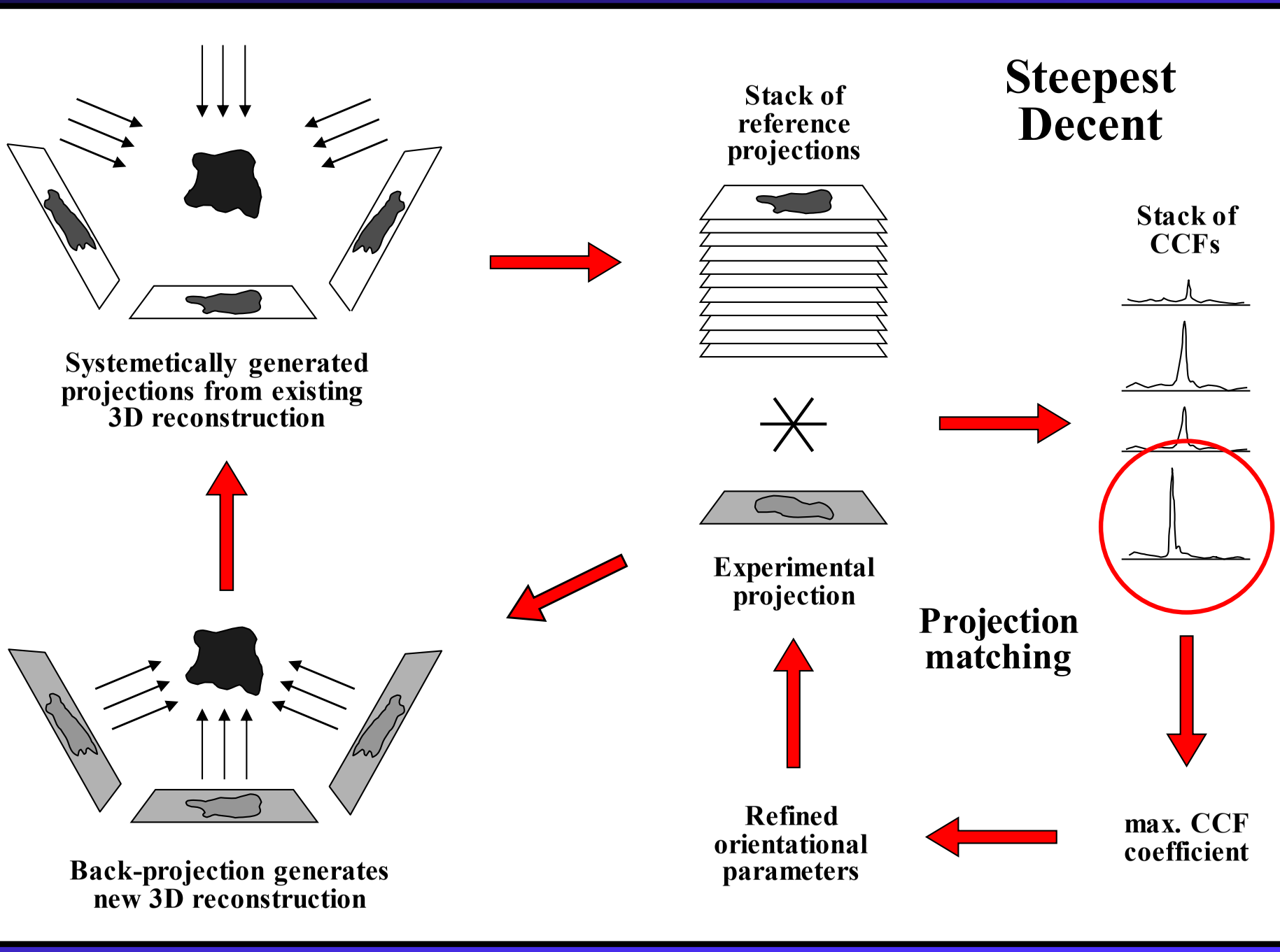
Initial model (obtained with VIPER)

VIPER

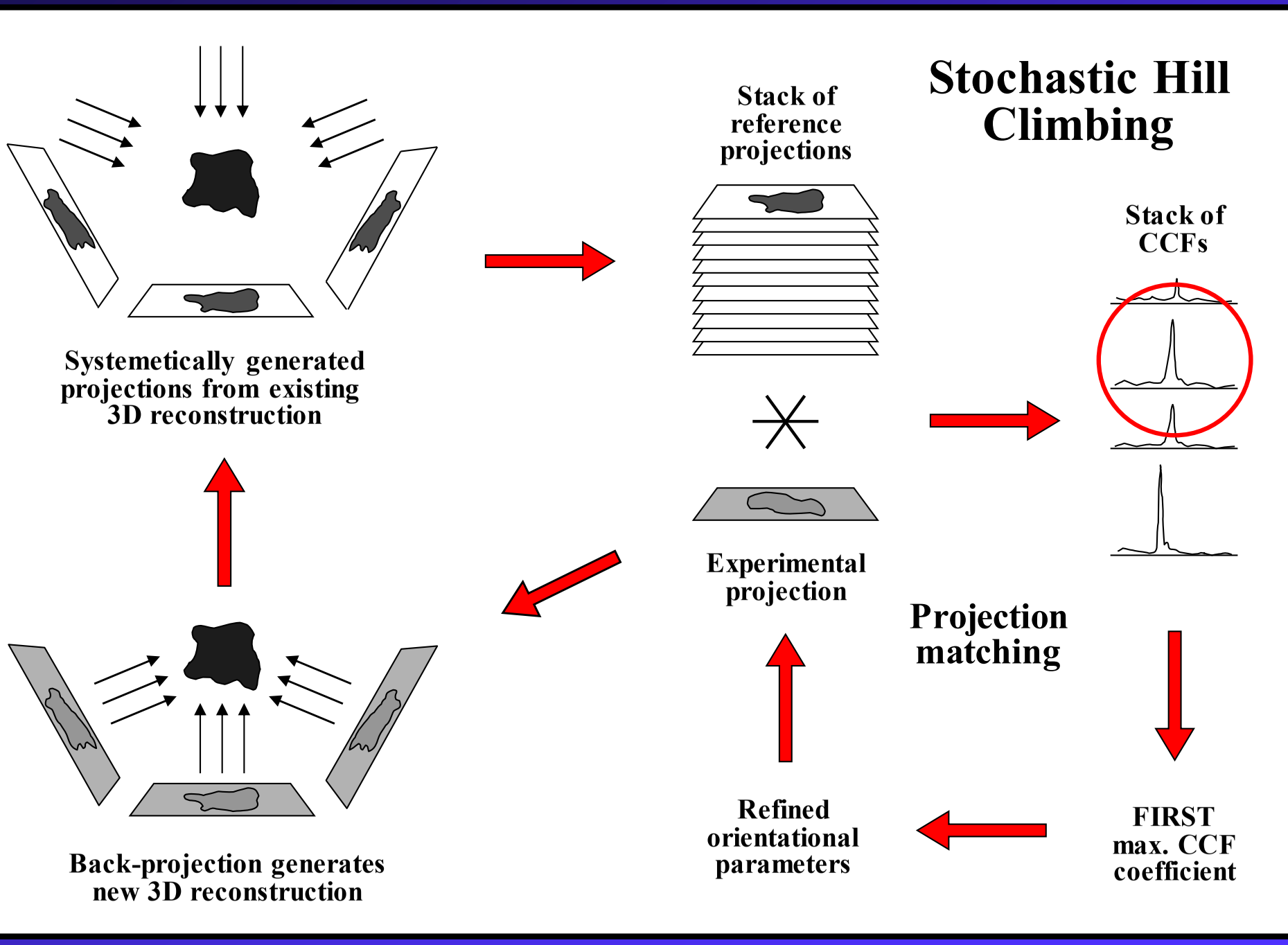
Stochastic Hill Climbing

(initially introduced in program SIMPLE)

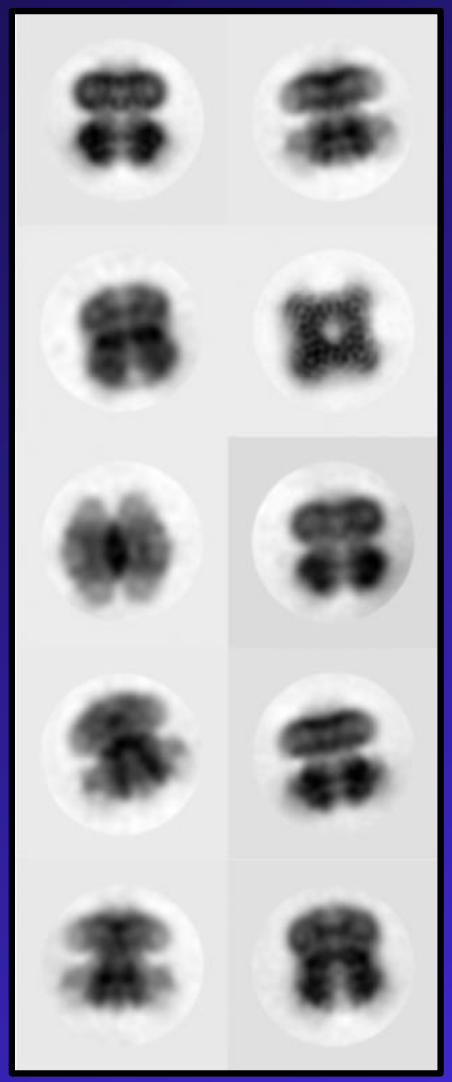
Angular refinement



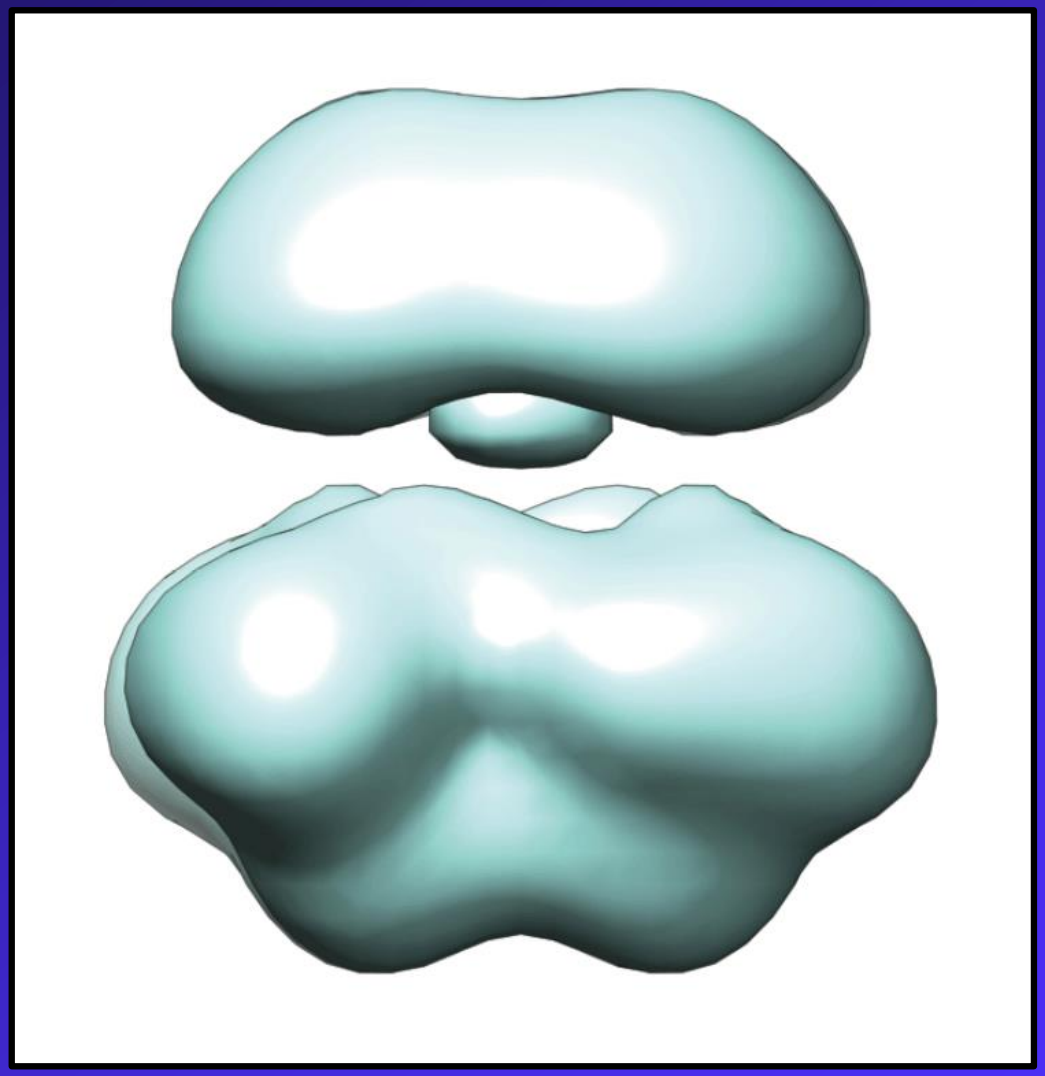
Angular refinement



Chicken Slo2.2 in the absence of Na^+



Class averages



Initial model (obtained with VIPER)

VIPER

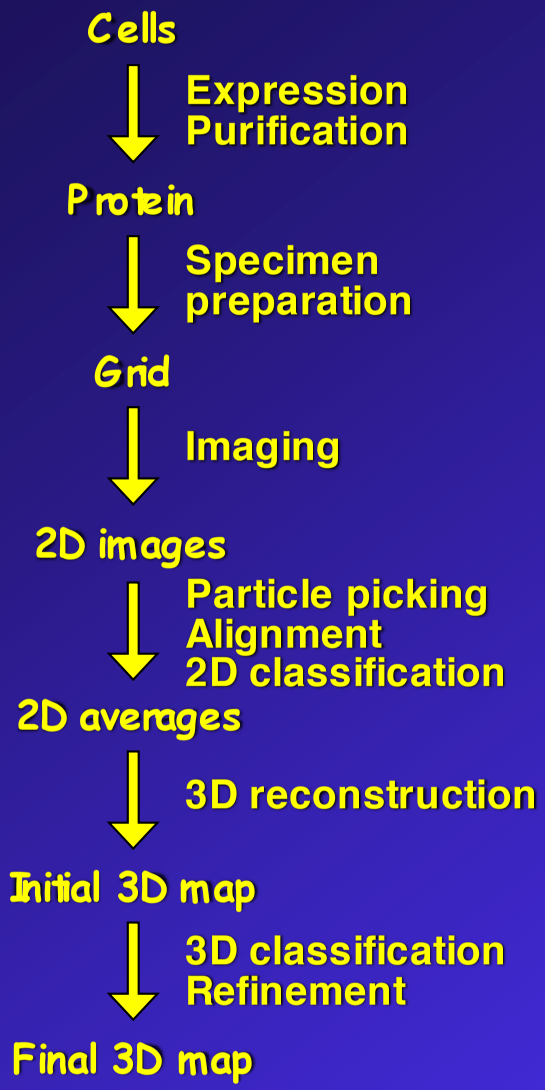
Stochastic hill climbing

Similar principles as used in ISAC:

– stability and reproducibility assessments

cryoSPARC

Structure determination by single-particle EM



Potential issues:

Reference bias

Overfitting

Resolution assessment

Structure determination by single-particle EM

Potential issues with density map

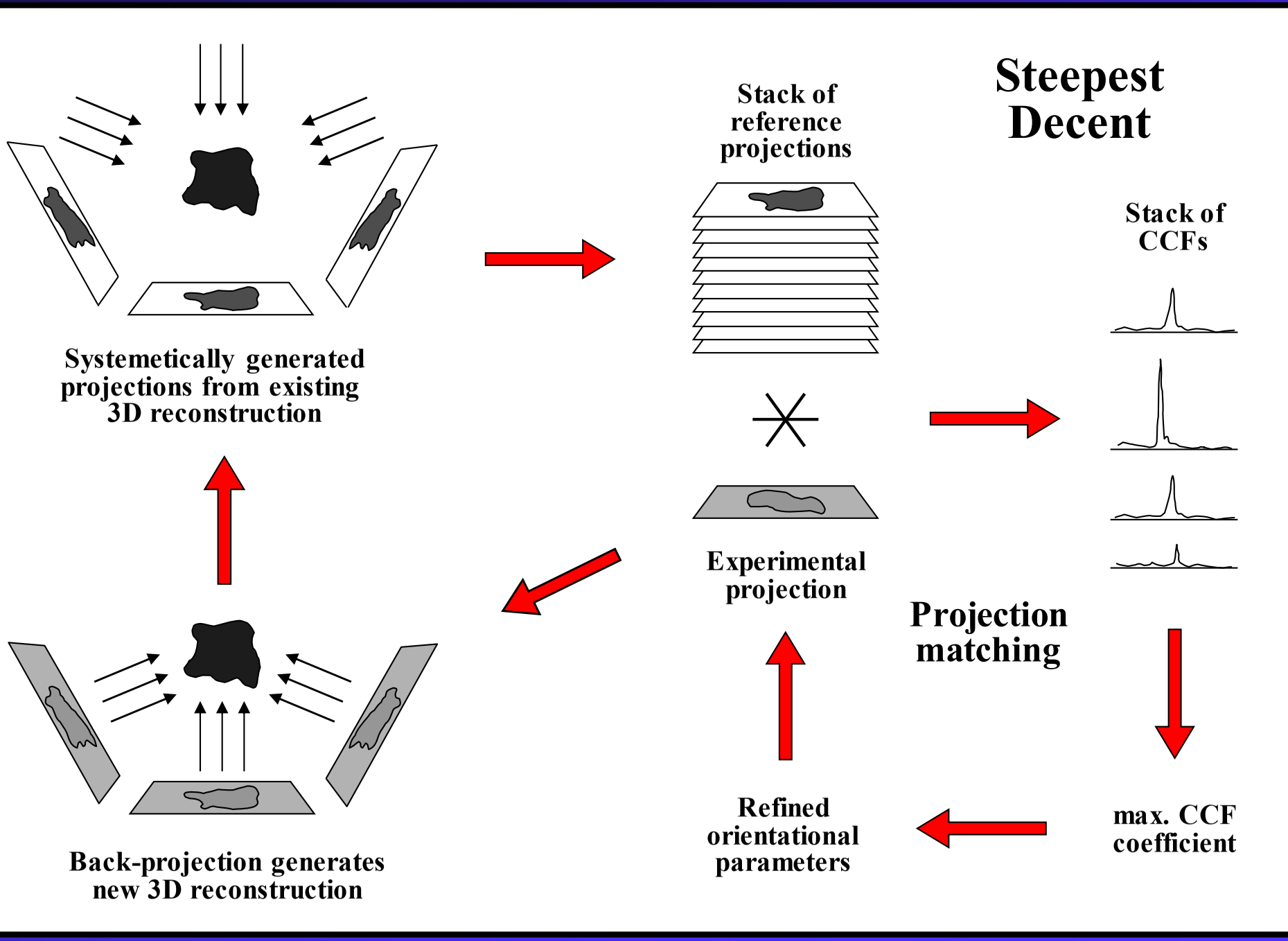
Model/reference bias

Average of 1,000 images containing pure white noise after alignment to an image of Albert Einstein

→ Einstein from noise

Shatsky et al. (2009) J. Struct. Biol. 166: 67-78
Henderson (2013) Proc. Natl. Acad. Sci. USA 110: 18037-18041

Angular refinement



Structure determination by single-particle EM

Potential issues with density map

Model/reference bias

Average of 1,000 images containing pure white noise after alignment to an image of Albert Einstein

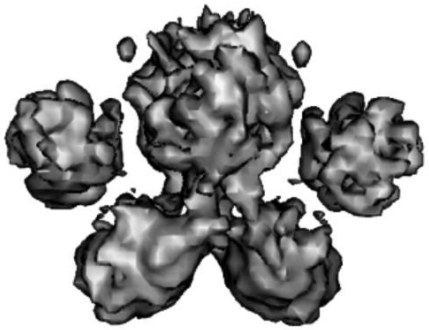
→ Einstein from noise

Over-fitting results in spurious high-resolution features due to alignment of noise

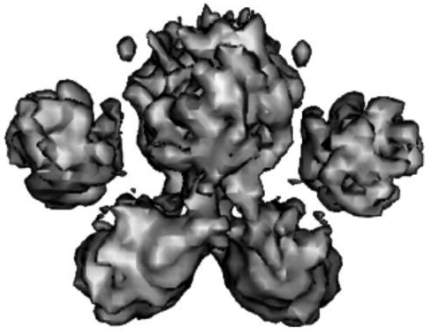
Shatsky et al. (2009) J. Struct. Biol. 166:67-78
Henderson (2013) Proc. Natl. Acad. Sci. USA 110:18037-18041

Structure determination by single-particle EM

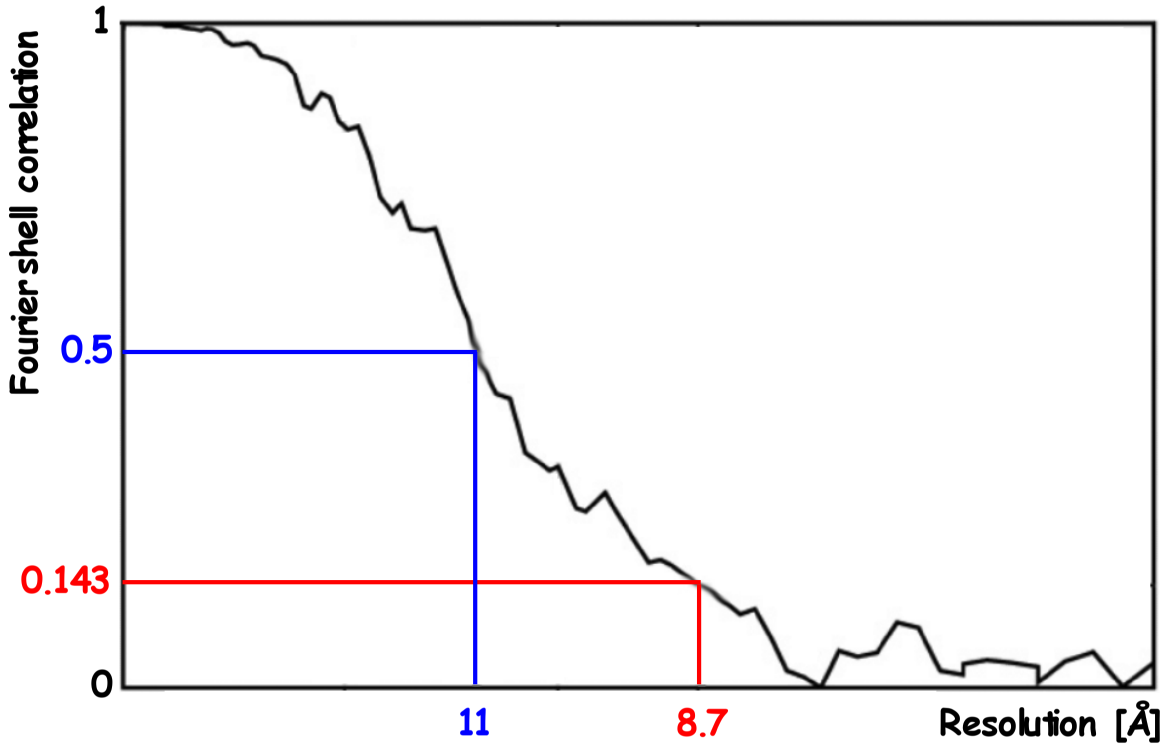
Resolution assessment



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



Maps have to be independent !



FSC = 0.5 Signal = Noise

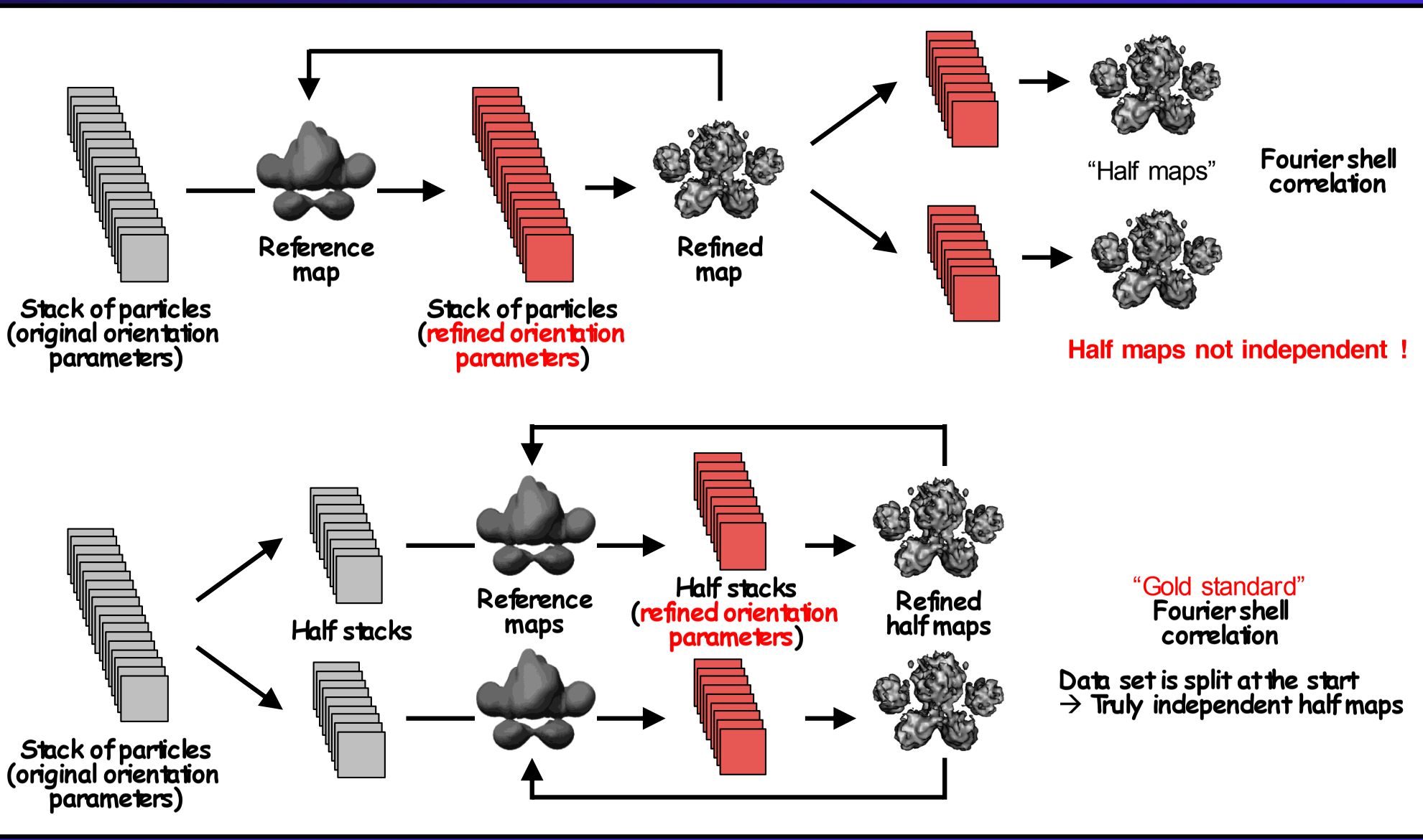
Böttcher et al. (1997) Nature 386: 88-91

FSC = 0.143 Phase error = 60°

Rosenthal & Henderson (2003) J. Mol. Biol. 333: 721-745

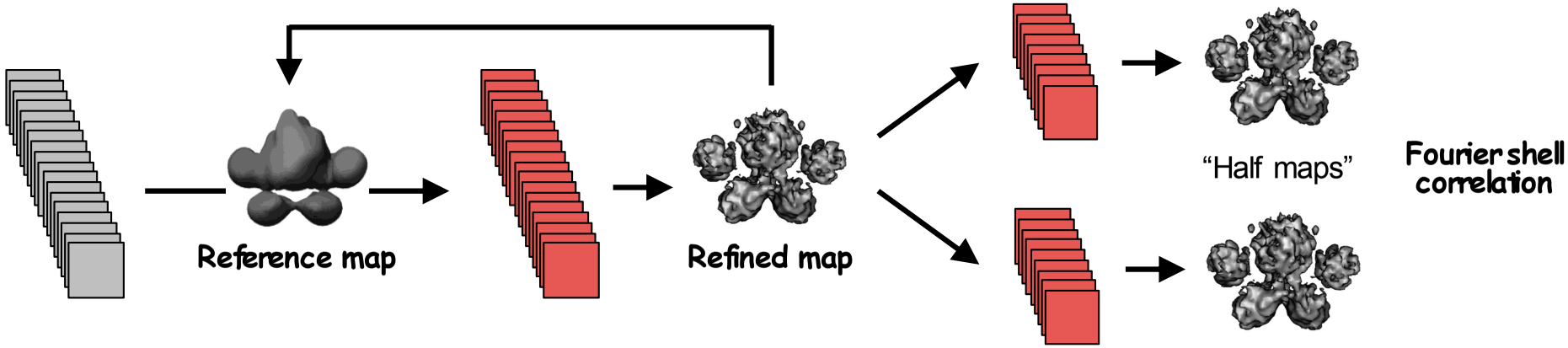
Structure determination by single-particle EM

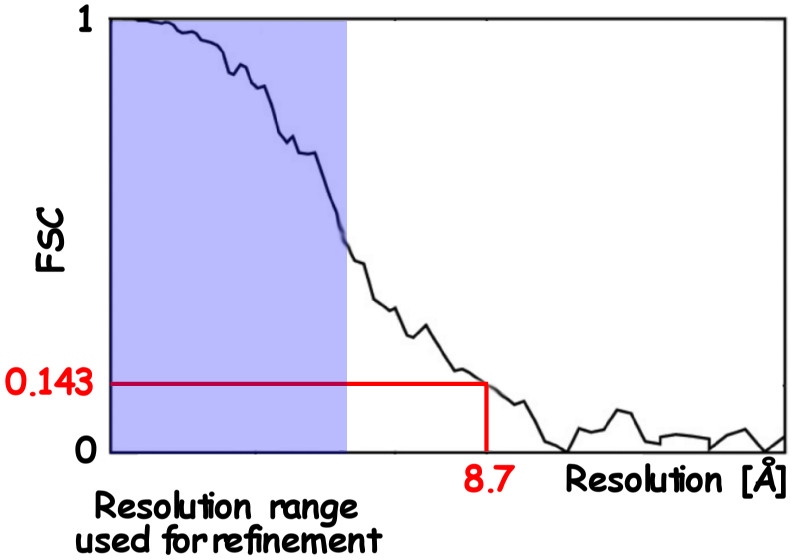
Resolution assessment



Structure determination by single-particle EM

Resolution assessment





"Gold standard" FSC is not the **only valid resolution assessment**

Even "gold standard" FSC can **give overestimated resolution**

Resolution is just a number

Local resolution

Structure determination by single-particle EM

Resolution assessment

Whatshould be resolved ?

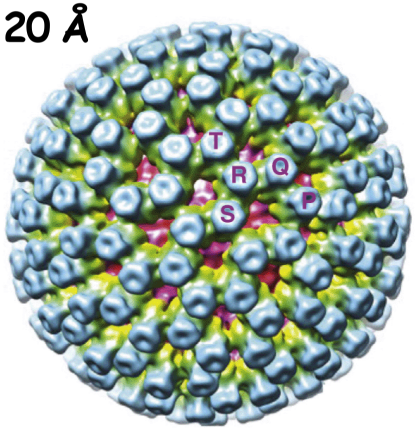
> 20 Å
protein envelope

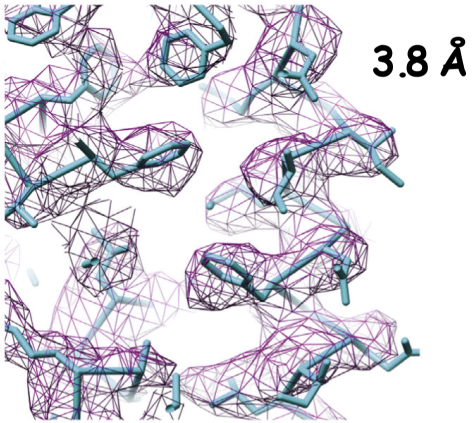
~ 9-10 Å
α-helices

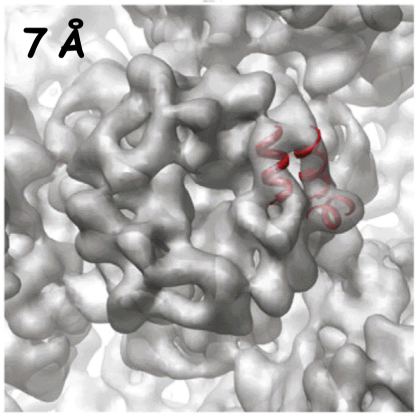
< 4.8 Å
β-sheets

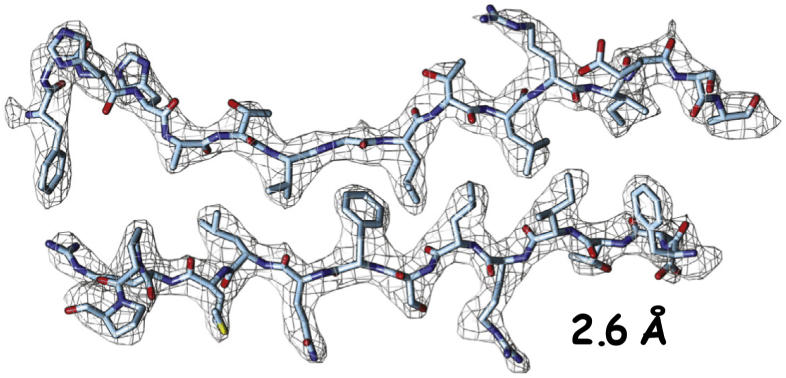
~ 4 Å
bulky side chains

Rotavirus double-layered particle



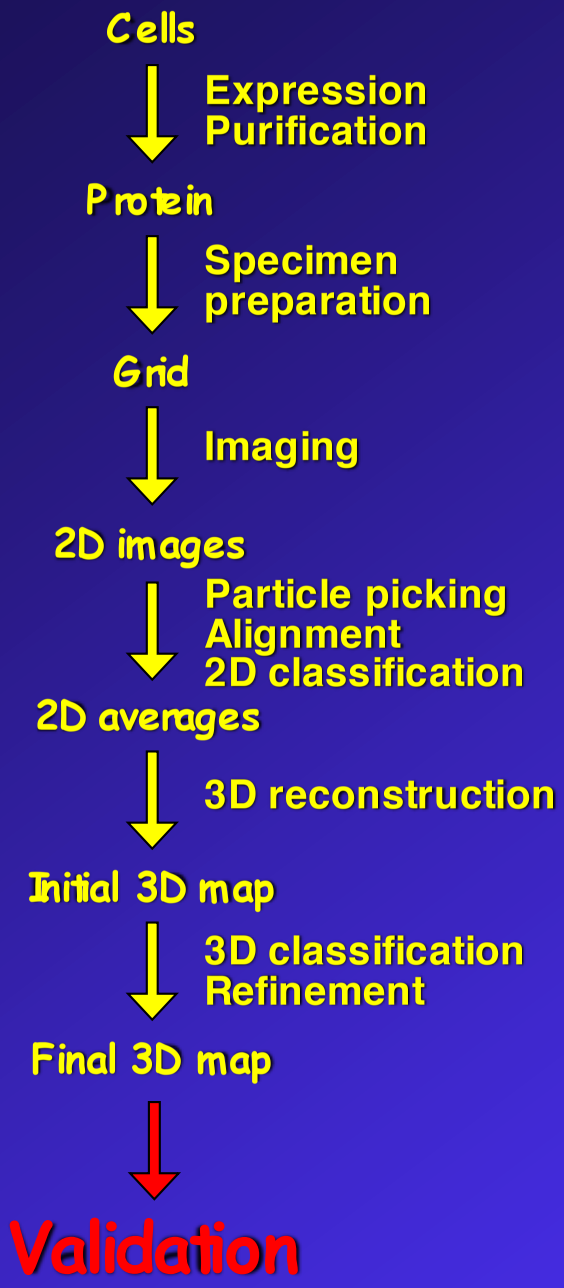




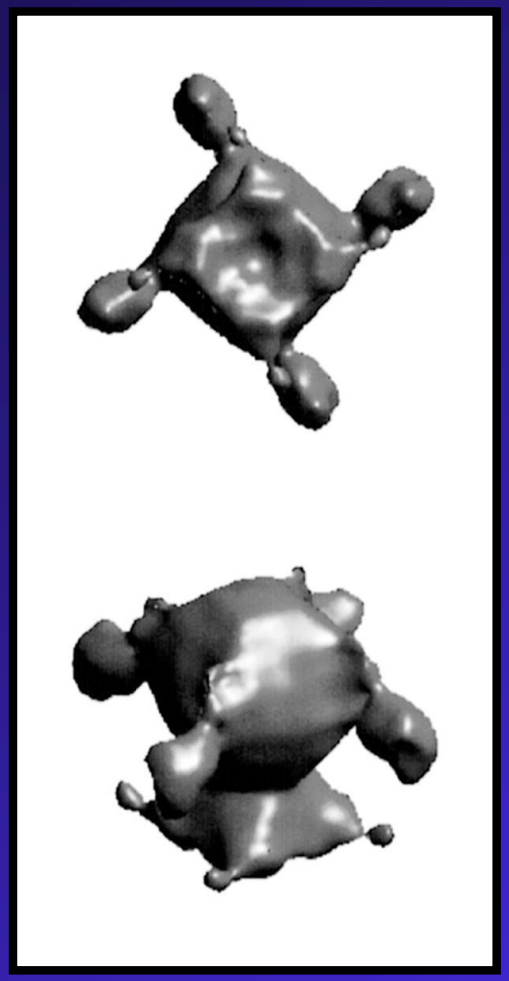


Rosenthal & Rubinstein (2015) Curr. Opin. Struct. Biol. 34: 135-144

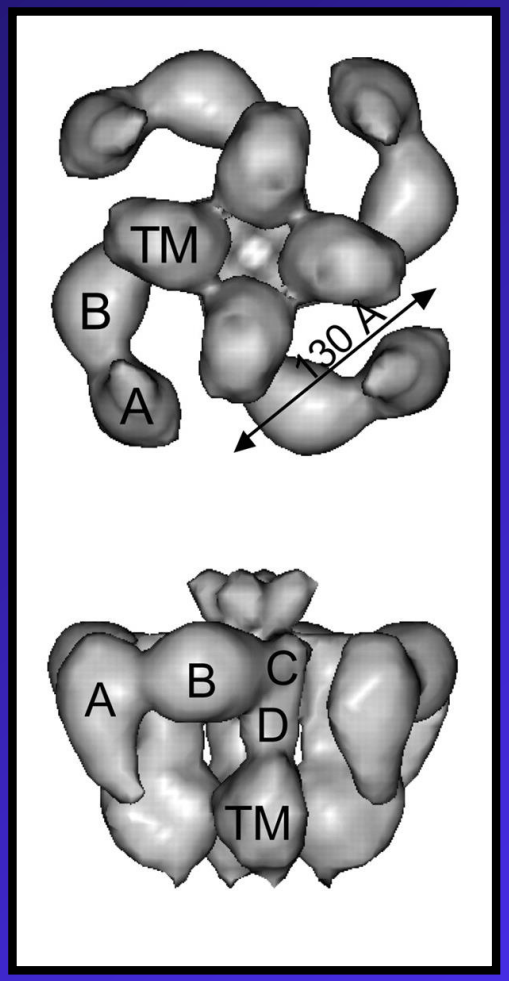
Structure determination by single-particle EM



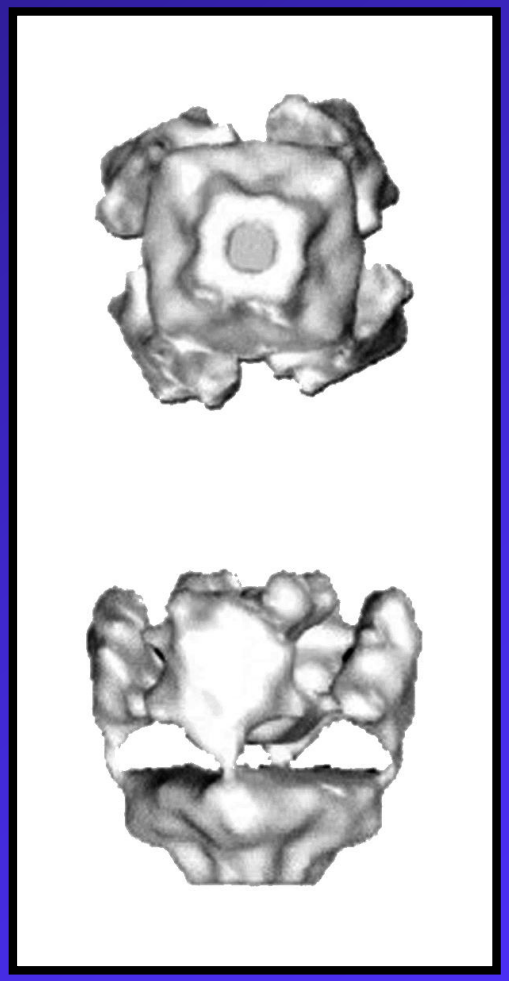
The issue: Structures of the IP3 receptor as determined by single-particle EM



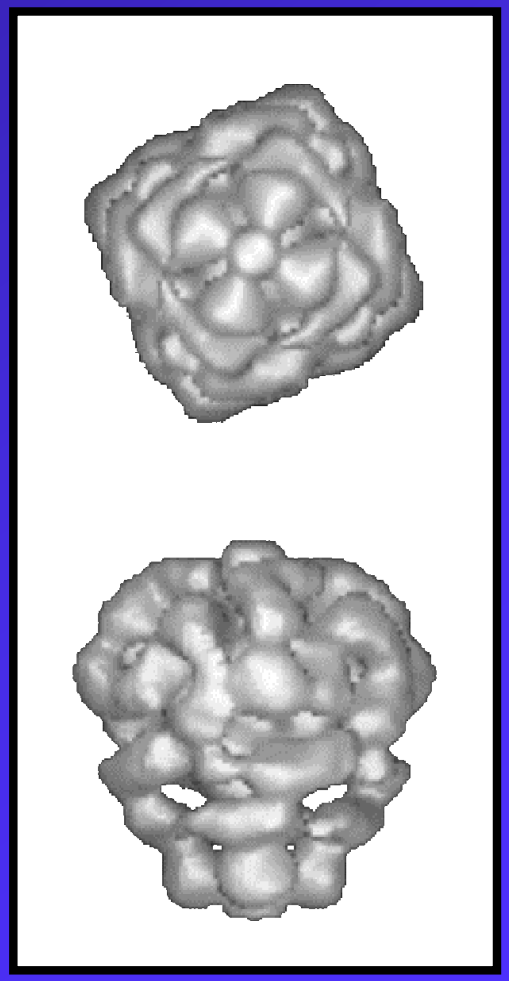
Jiang et al., 2002



Serysheva et al., 2003



Jiang et al., 2003



Sato et al., 2004

Map validation

Meeting of experts in 2010 to come up with standards for map validation

Outcome summarized in 2012:

Structure
Meeting Review

Outcome of the First Electron Microscopy Validation Task Force Meeting

Richard Henderson,[1] Andrej Sali,[2] Matthew L. Baker,[3] Bridget Carragher,[4] Batsal Devkota,[5] Kenneth H. Downing,[6] Edward H. Egelman,[7] Zukang Feng,[5] Joachim Frank,[8,9] Nikolaus Grigorieff,[10] Wen Jiang,[11] Steven J. Ludtke,[3] Ohad Medalia,[12,21] Pawel A. Penczek,[13] Peter B. Rosenthal,[14] Michael G. Rossmann,[15] Michael F. Schmid,[3] Gunnar F. Schröder,[16] Alasdair C. Steven,[17] David L. Stokes,[18] John D. Westbrook,[5] Willy Wriggers,[19] Huanwang Yang,[5] Jasmine Young,[5] Helen M. Berman,[5] Wah Chiu,[3] Gerard J. Kleywegt,[20] and Catherine L. Lawson[5,*]

Henderson et al. (2012) Structure 20: 205-214

Map validation

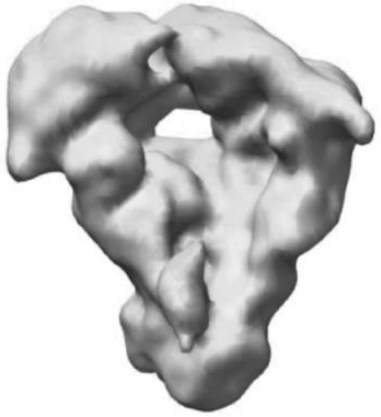
– Compare reference-free averages with projections

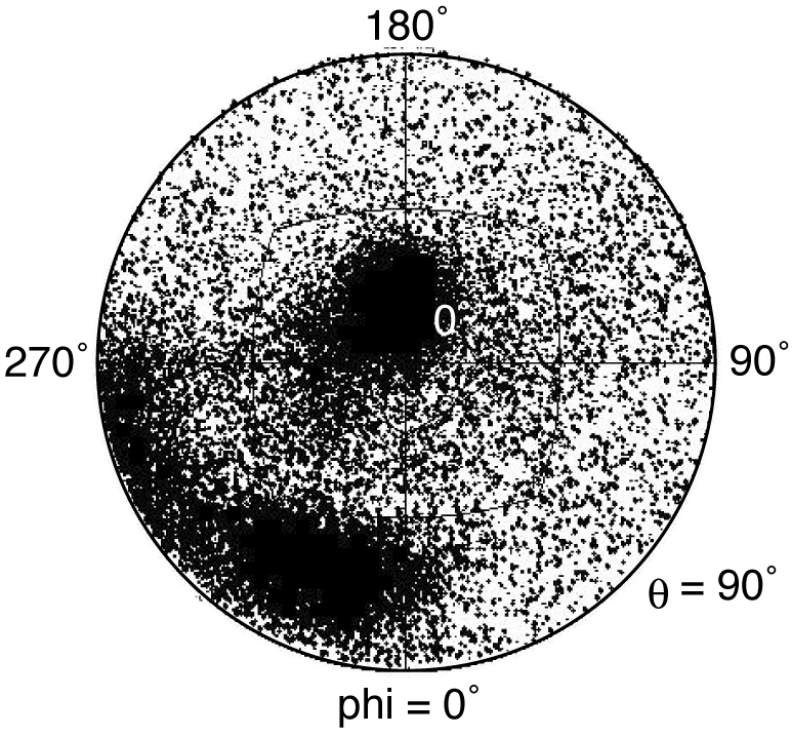
Henderson et al. (2012) Structure 20: 205-214

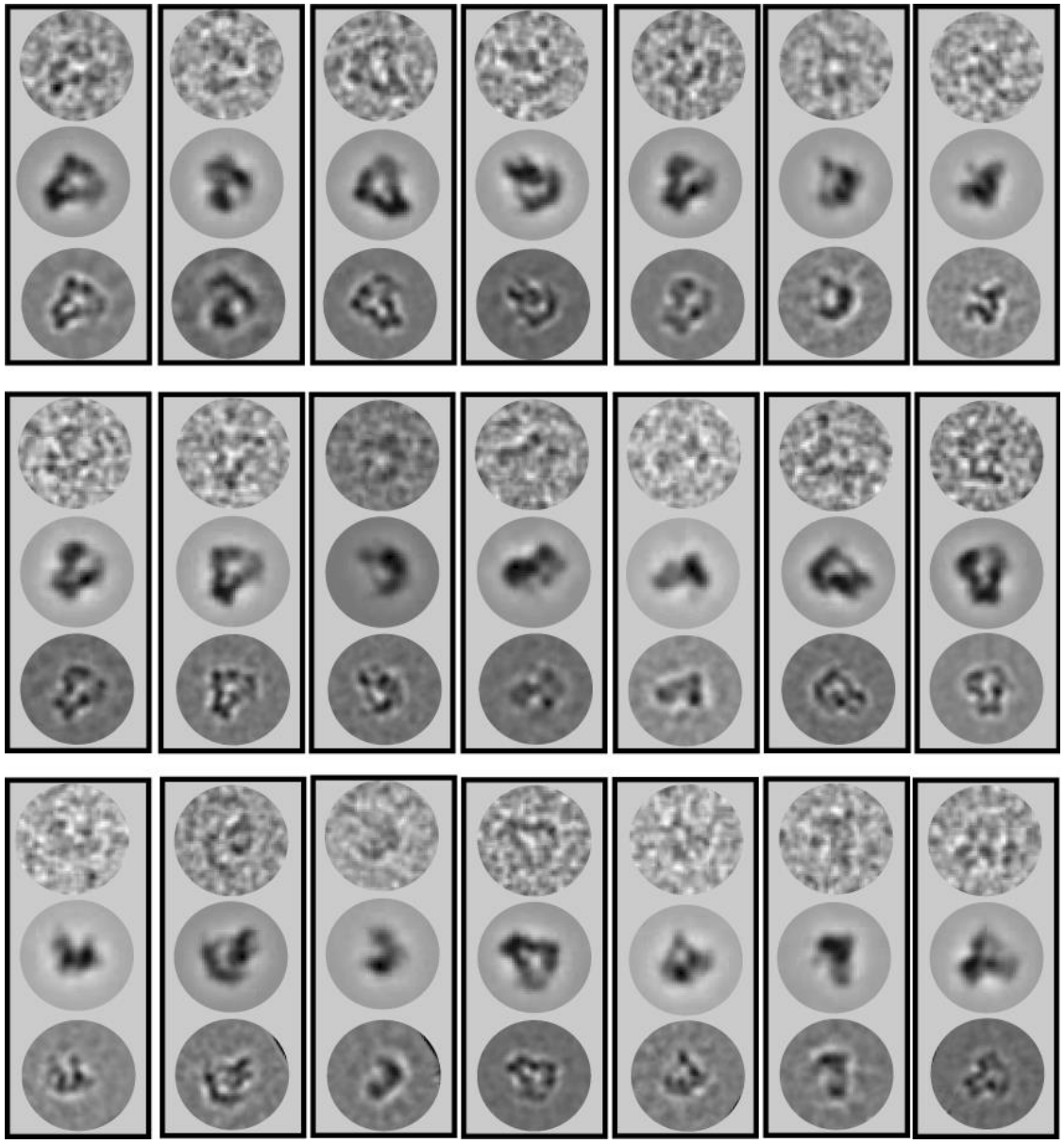
Map validation

Re-projections and angular distribution

Anaphase promoting complex







Map validation

- Compare reference-free averages with projections
  - only checks consistency of 3D map with 2D data
  - also check angle distribution
- Tilt-pair analysis

Henderson et al. (2012) Structure 20: 205-214

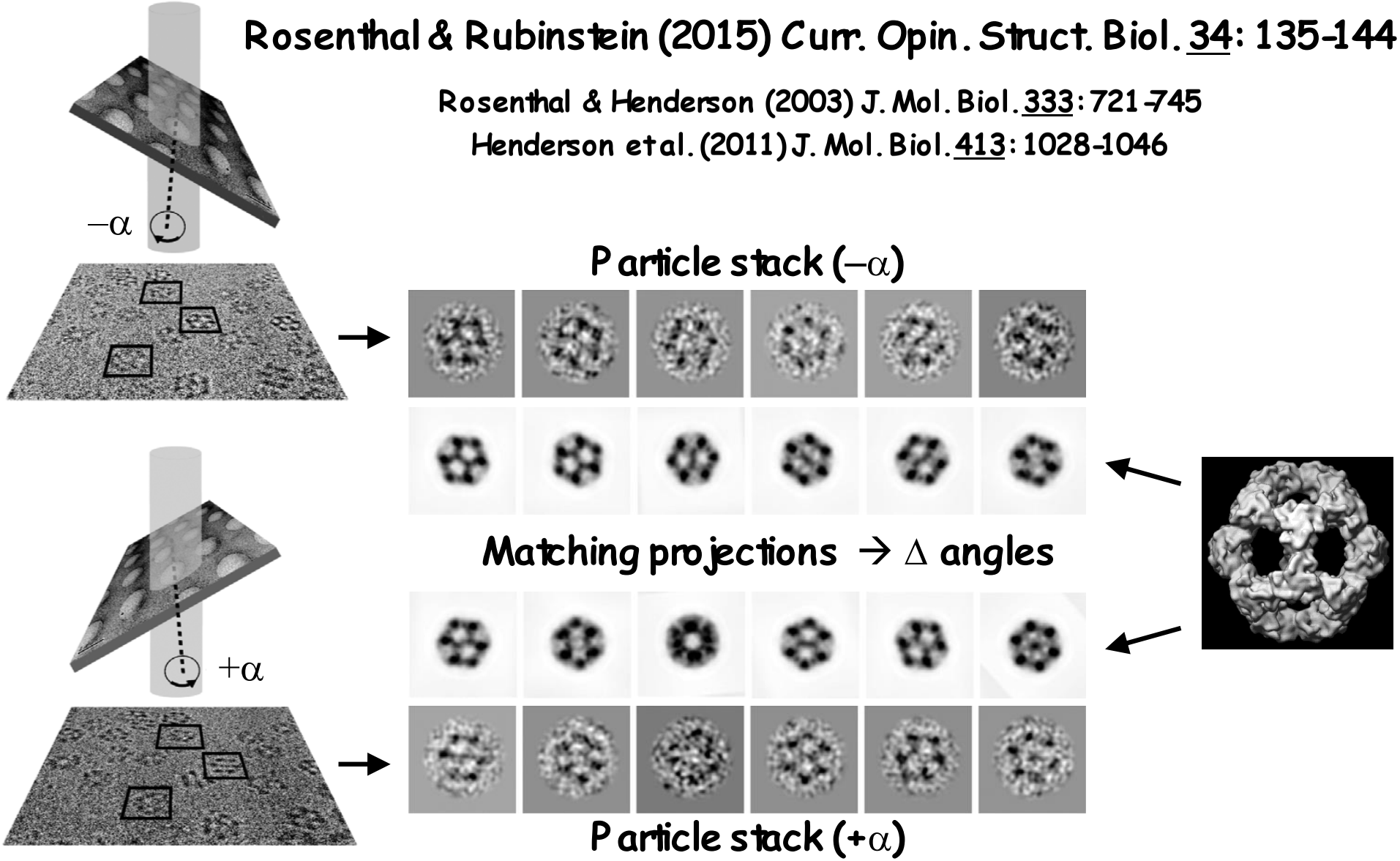
Map validation

Tilt-pair analysis

Rosenthal & Rubinstein (2015) Curr. Opin. Struct. Biol. 34: 135-144

Rosenthal & Henderson (2003) J. Mol. Biol. 333: 721-745

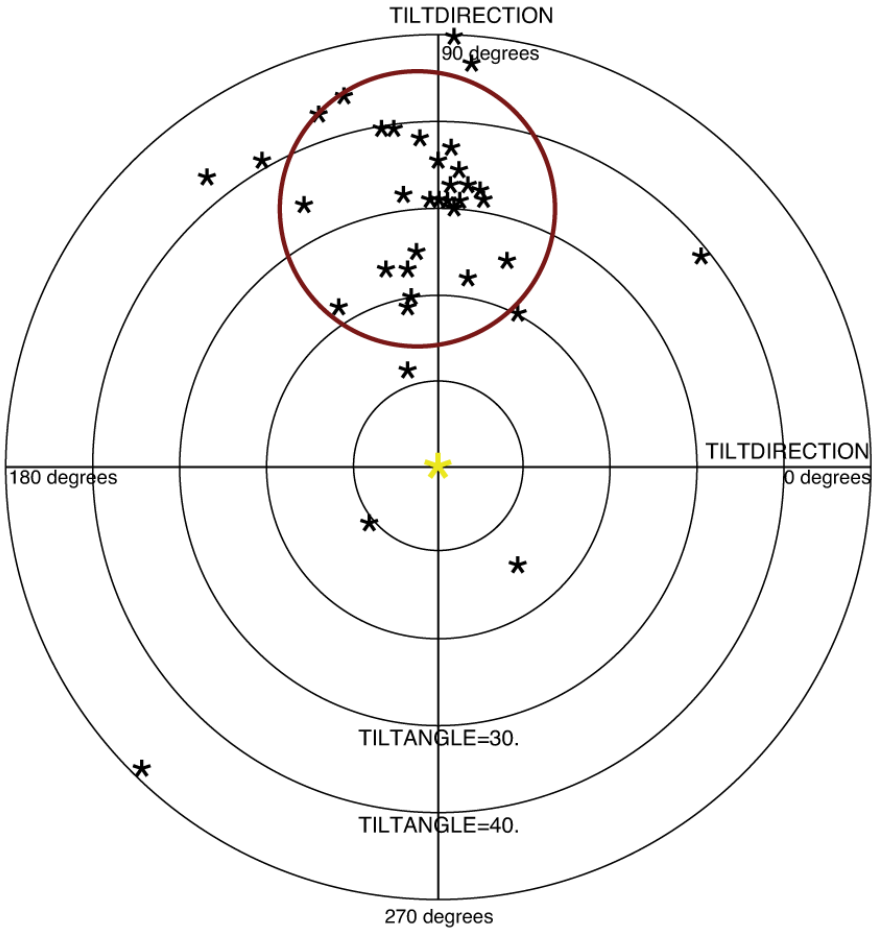
Henderson et al. (2011) J. Mol. Biol. 413: 1028-1046



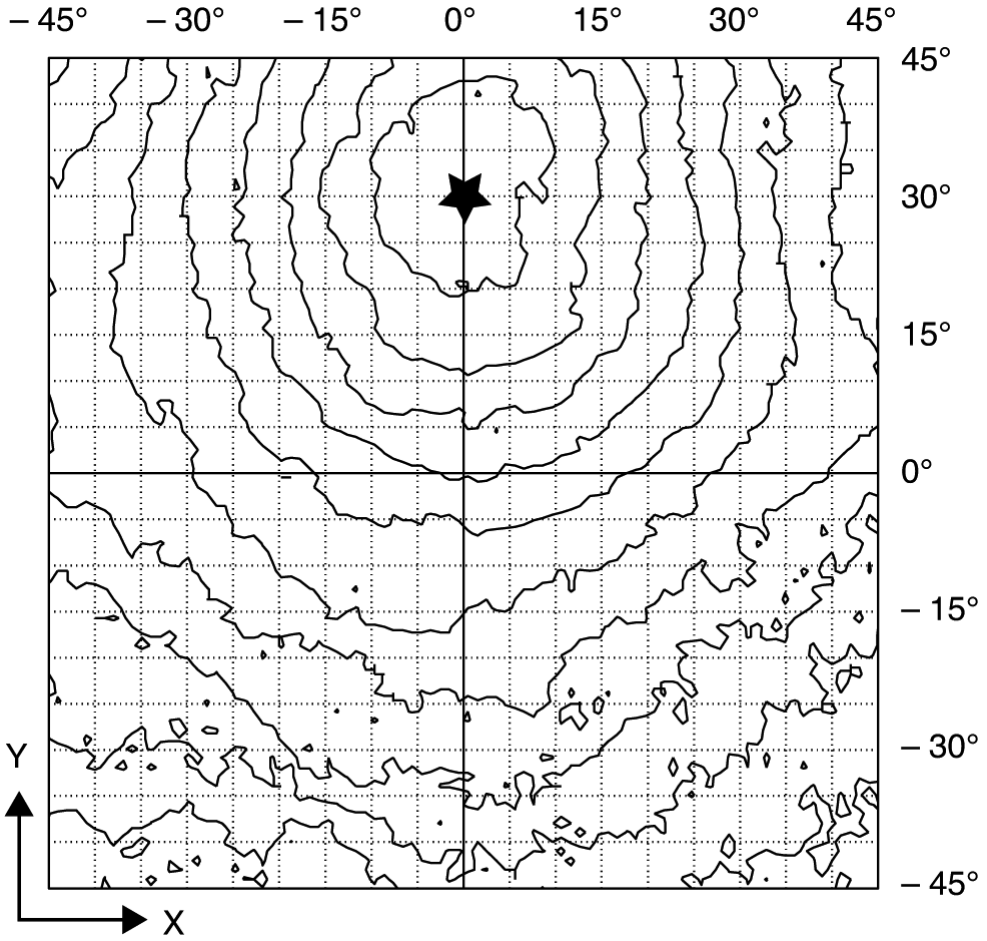
Map validation

Tilt-pair analysis

Tilt-pair parameter plot



Tilt-pair phase residual plot



Rosenthal & Rubinstein (2015) Curr. Opin. Struct. Biol. 34: 135-144

Map validation

Tilt-pair analysis

Henderson et al. (2011) J. Mol. Biol. 413: 1028-1046

Table 1. Overview of tilt-pair statistics

Specimen	Symmetry	Particle size (Å)	Molecular mass (MDa)	Number of tilt pairs	Number of particles	Successful alignment (%)	Angular error (°)	
							Mean	Maximum
Rotavirus DLP	*I*2	700	50	10	95	100/100	0.25	1.0
CAV	*I*2	255	2.7	1	45	62/82	2.5	3.5
70S ribosomes	*C*1	270×260	2.6	12	220	45/75	4.0	5.0
FAS	*D*3	260×220	2.6	2	44	59/95	4.0	6.0
PDH-E2CD	*I*1	280	1.6	1	50	62/94	3.0	4.0
Thermus V-ATPase	*C*1	250×140	0.6	1	50	54/80	10.0	16.0
Bovine F-ATPase	*C*1	250×140	0.6	1	29	52/79	20.0	25.0
DNA-PKcs	*C*1	150×120	0.47	14	108	44/81	15.0	17.0
β-Galactosidase	*D*2	180×130×95	0.45	2	119	74/91	10.0	14.0

- determines whether overall 3D map is correct at 15-20 Å resolution (but not high-resolution features)
- allows determination of handedness
- can be used to refine parameters used for orientation determination → can thus be used to improve the map
- validates orientation parameters (but not microscope parameters, i.e., defocus, magnification)

"If less than 60% of particles show a single cluster, the basis for poor orientation parameters should be investigated"

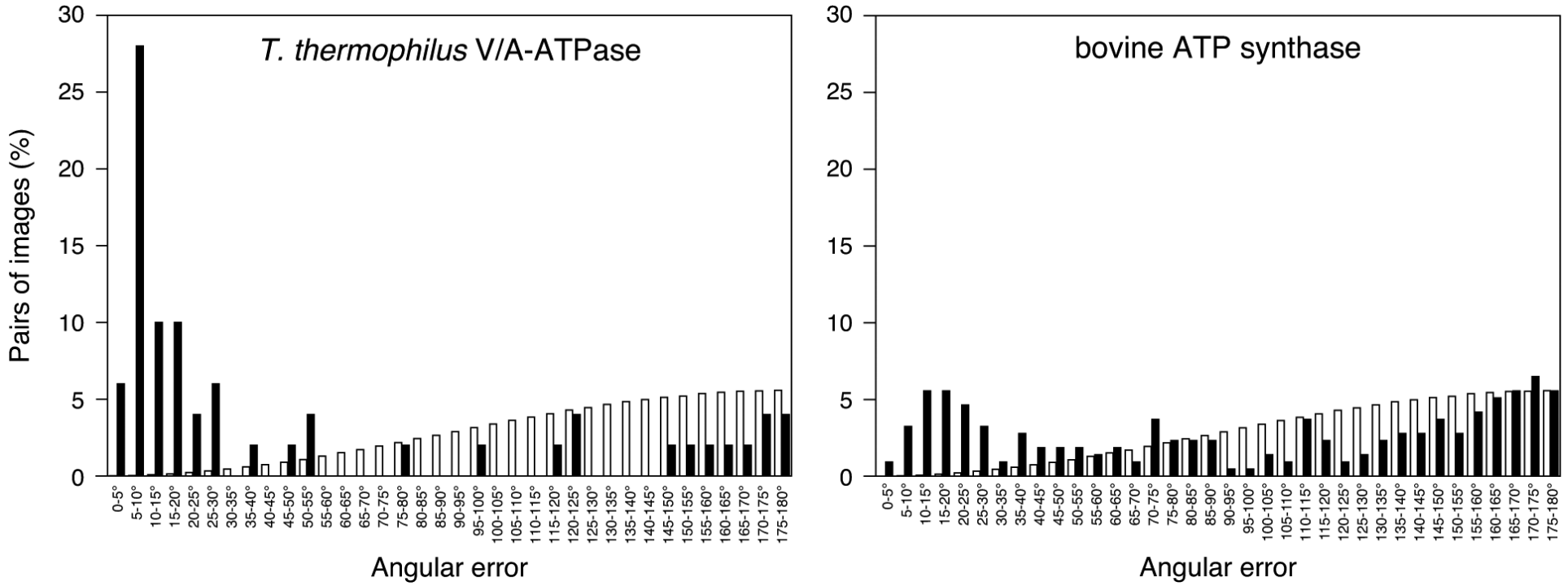
Map validation

Tilt-pair analysis

Tilt-pair alignment test

■ angular errors for determination of the tilt transformation of each particle pair

□ expected for random orientations



Rosenthal & Rubinstein (2015) Curr. Opin. Struct. Biol. 34: 135-144

Baker et al. (2012) Proc. Natl Acad. Sci. USA 109: 11675-11680

Russo & Passmore (2014) J. Struct. Biol. 187: 112-118

Map validation

Tilt-pair web server

Input

Load previous settings

Micrograph Parameters

Magnification: 4.98 A/px	Defocus 1: 58626.0 A	Tilt search range: 20.0 degrees
Voltage: 300.0 kV	Defocus 2: 59084.0 A	Min resolution: 100.0 A
Particle size (radius): 30.0 pixels	Angle astigmatism: 55.7 degrees	Max resolution: 30.0 A

Disable CTF correction

Note: Positive defocus value equals underfocus.

Input data

3D model: Browse.. e2map.mrc

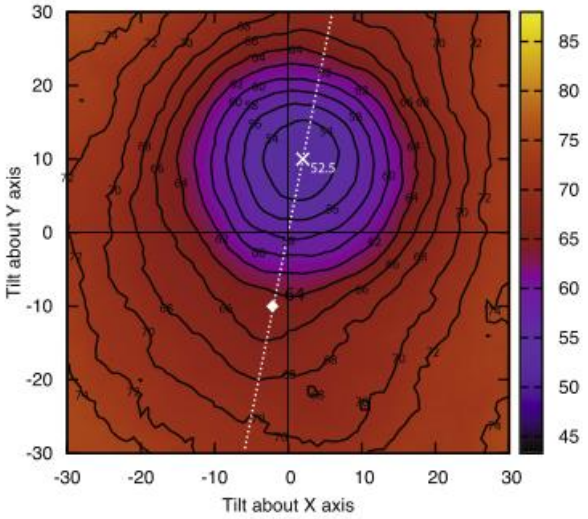
Stack 1 (max 300 particles): Browse.. stack1.mrc

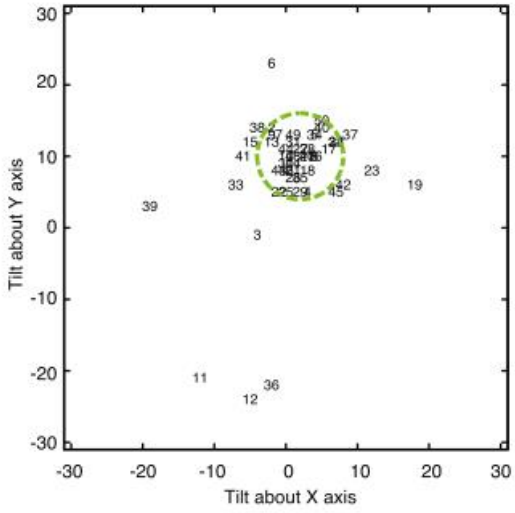
Stack 2 (max 300 particles): Browse.. stack2.mrc

Parameters for the Stack 1: Browse.. stack1.par

Parameters format: Frealign

Output





Parameters:

Magnification	4.98 (effective: 9.96) A/px
Defocus	58626 ; 59084
Astigmatism	55.7
Voltage	300 kV
Resolution Range	100.0 - 30.0 A
Tilt Range	30
Particle radius	20 (effective: 10) px
Optimized box size (after binning)	46
Effective binning:	2

Summary of the results for all submitted particles:

Minimal Phase Residual: **52.53°**
Minimum at the position: **2.0°, 10.0°**
Tilt axis (angle with respect to the X axis): **78.7°**
Tilt angle: **10.2°**
Hand Phase Difference: **12.48°**
Average distance to the global minimum: **5.24°**

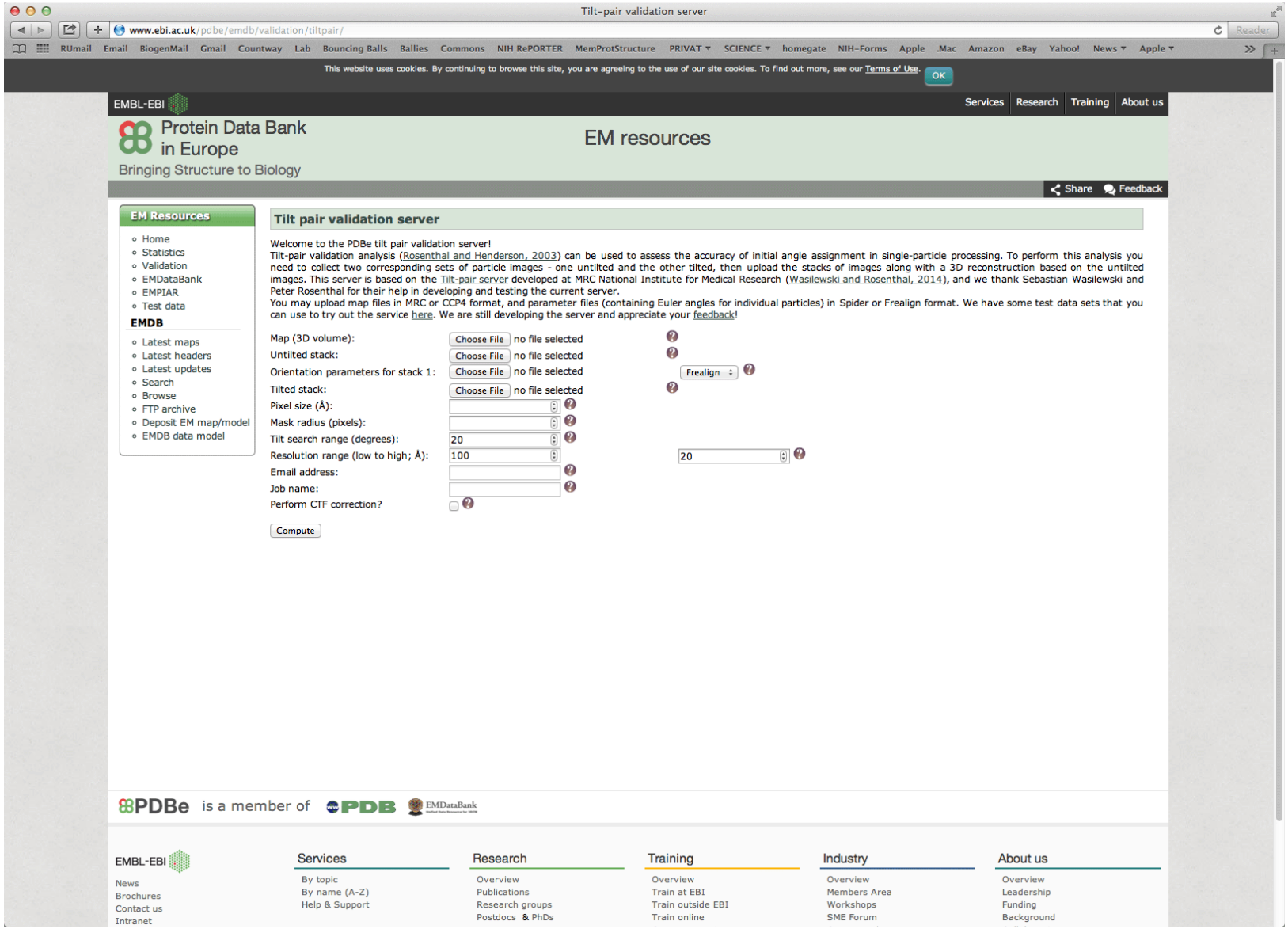
Particles in the cluster (0.5σ - **6.13°**) near the minimum average phase residual:
1 2 4 5 7 8 9 10 13 14 16 17 18 20 21 22 25 26 27 28 29 30 31 34 35 43 44 46 47 48 49

Particles outside the cluster:
3 6 11 12 15 19 23 24 32 33 36 37 38 39 40 41 42 45 50

Wasilewski & Rosenthal (2014) J. Struct. Biol. 186: 122-131

Map validation

http://www.ebi.ac.uk/pdbe/emdb/validation/tiltpair/



Map validation

- Compare reference-free averages with projections
  - only checks consistency of 3D map with 2D data
  - also check angle distribution
- Tilt-pair analysis
  - excellent, also establishes handedness
- "Gold standard" FSC
  - not necessarily needed (but certainly not bad)
- Randomize phases

Henderson et al. (2012) Structure 20: 205-214

Map validation

Randomize phases

Rosenthal & Rubinstein (2015) Curr. Opin. Struct. Biol. 34: 135-144

Chen et al. (2013) Ultramicroscopy 135: 24-35

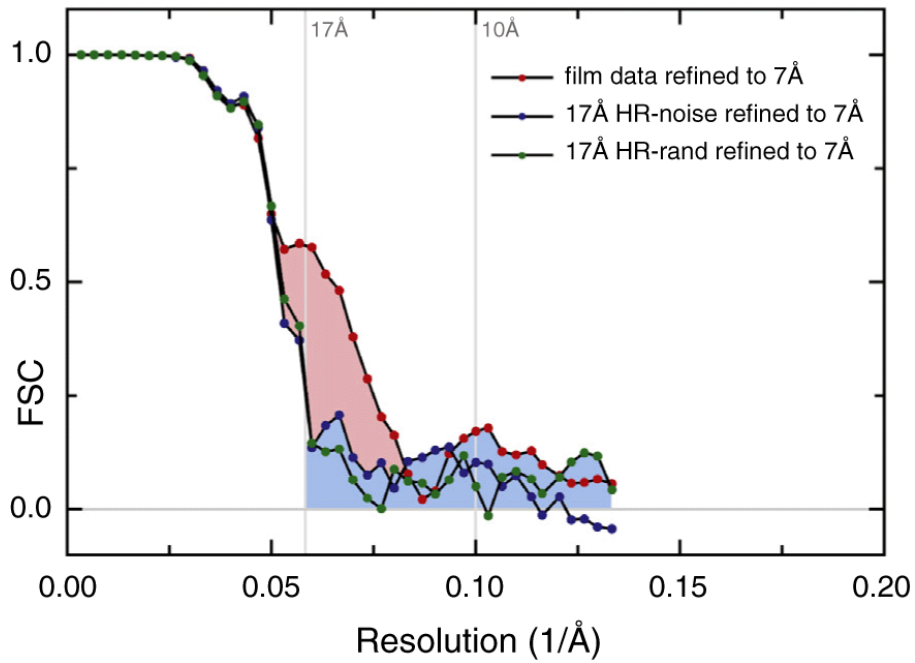
- Do single-particle reconstruction / refinement
- Determine resolution (FSC)
- Take raw data, randomize phases beyond which FSC_T falls below a threshold (75 or 80%)
- Redo the same analysis and recalculate FSC curve
- Any signal in region of randomized phases indicates issues with noise alignment in that region
- Can be implemented in any package

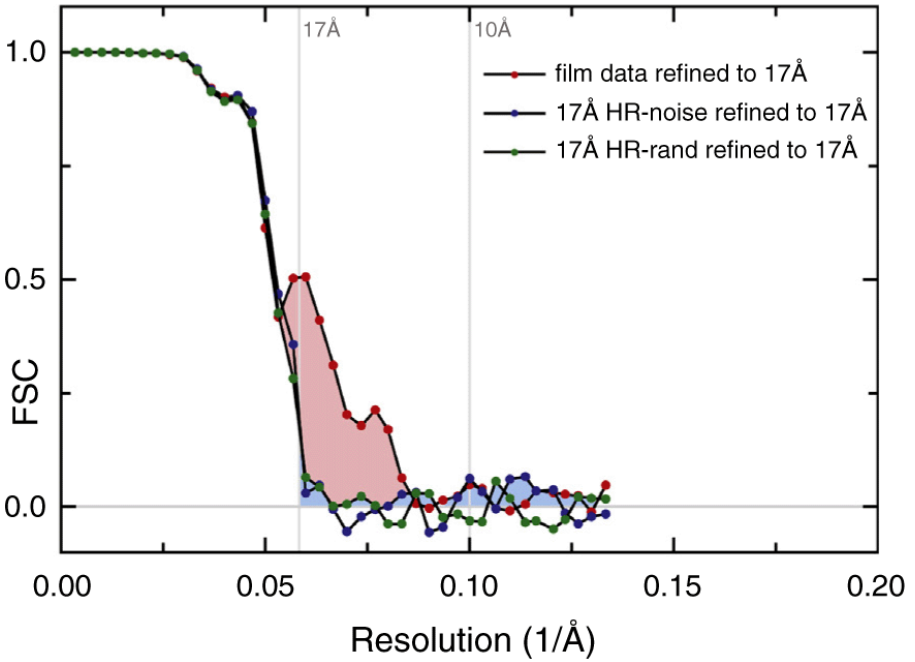
Map validation

Randomize phases

Rosenthal & Rubinstein (2015) Curr. Opin. Struct. Biol. 34: 135-144

Chen et al. (2013) Ultramicroscopy 135: 24-35





FSC signal due to over-fitting (noise)

FSC signal due to true structural information

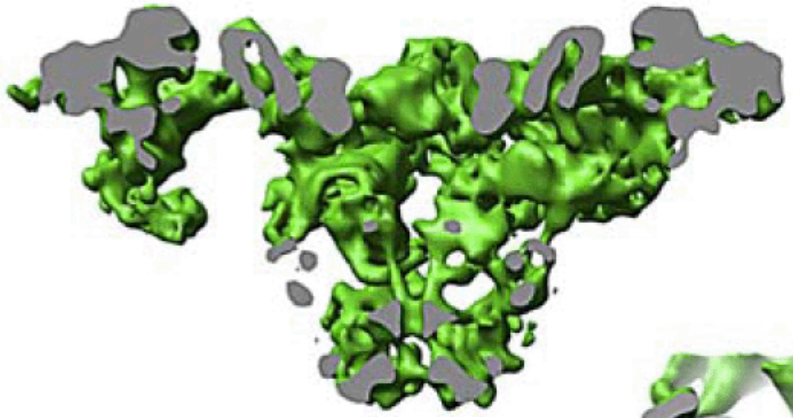
Map validation

- Compare reference-free averages with projections
  - only checks consistency of 3D map with 2D data
  - also check angle distribution
- Tilt-pair analysis
  - excellent, also establishes handedness
- "Gold standard" FSC
  - not necessarily (but certainly not bad)
- Randomize phases
  - excellent, but not commonly used
- Appearance of expected secondary structure elements

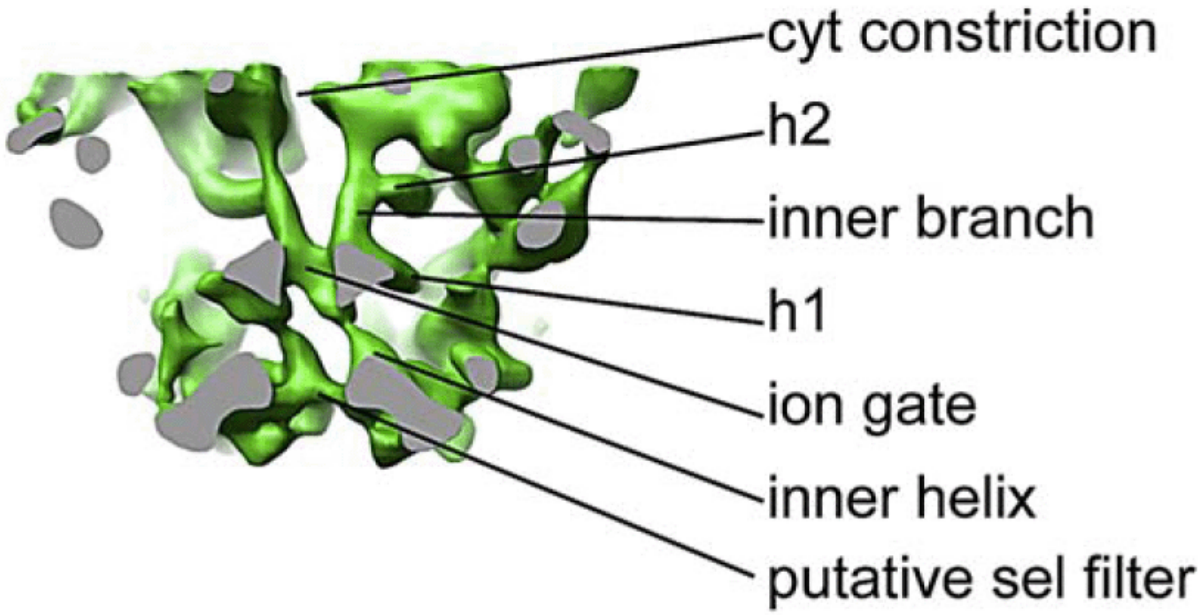
Henderson et al. (2012) Structure 20: 205-214

Map validation

Expected secondary structure



Ryanodine receptor 1
at 10.2 Å resolution



Samso et al. (2009) PLoS Biol. 7: e1000085

Map validation

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  - also check angle distribution
- Tilt-pair analysis
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- "Gold standard" FSC
  - not necessarily (but certainly not bad)
- Randomize phases
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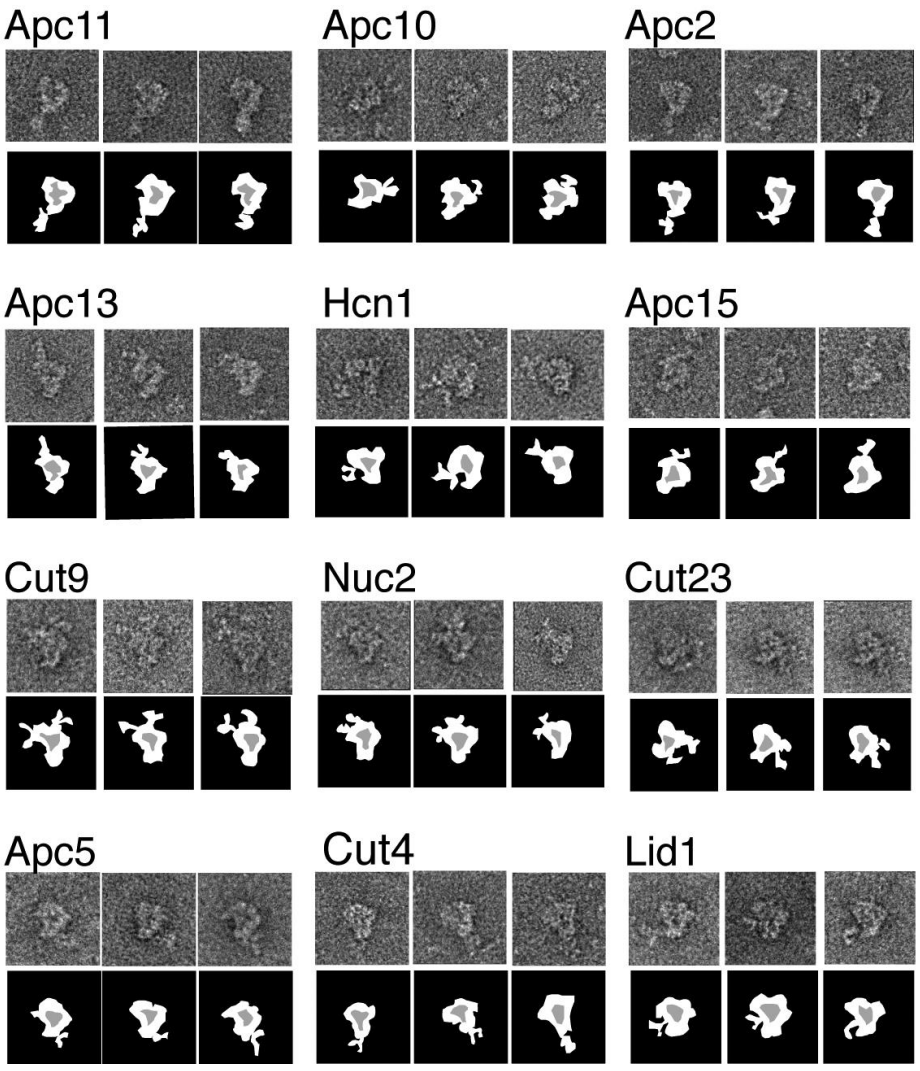
- Appearance of expected secondary structure elements
- Evaluate with published information

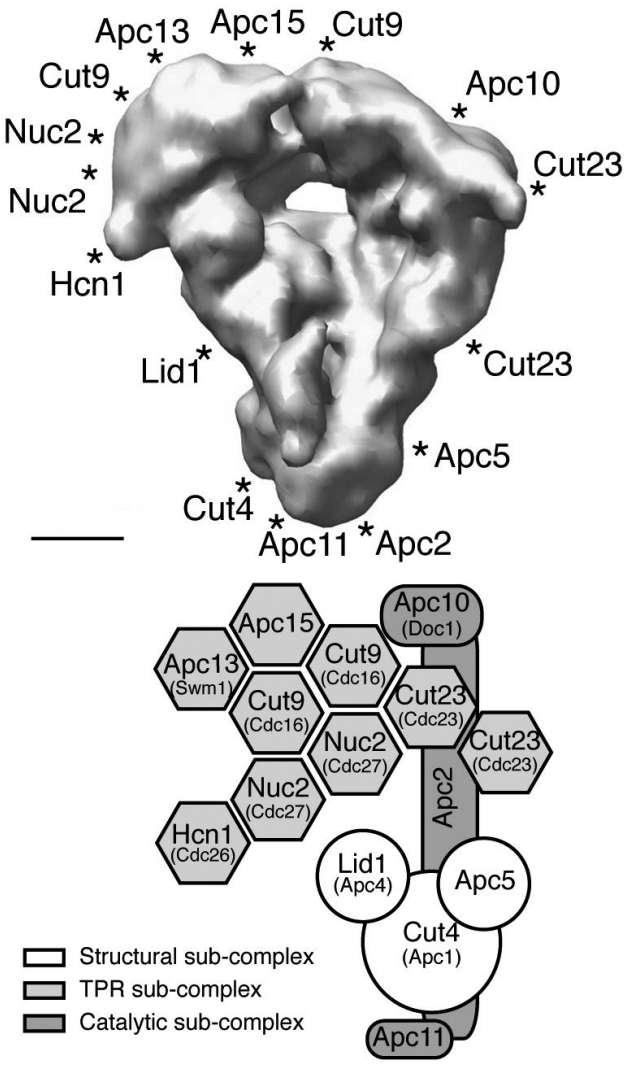
Henderson et al. (2012) Structure 20: 205-214

Map validation

Evaluation with published information

Anaphase promoting complex





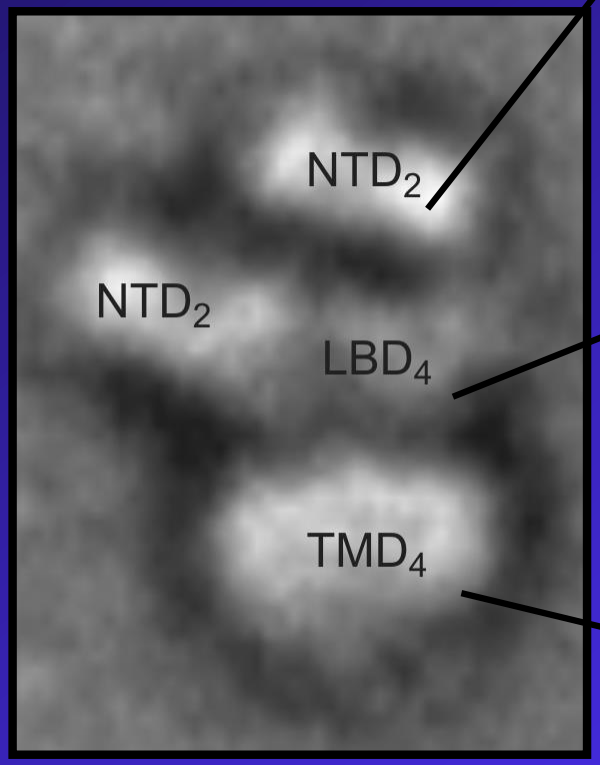
Map validation

- Compare reference-free averages with projections
  - only checks consistency of 3D map with 2D data
  - also check angle distribution
- Tilt-pair analysis
  - excellent, also establishes handedness
- "Gold standard" FSC
  - not necessarily (but certainly not bad)
- Randomize phases
  - excellent, but not commonly used
- Appearance of expected secondary structure elements
- Evaluate with published information
  - yeast two-hybrid analysis
  - pull-down experiments
  - cross-link mass spectrometry
- Dock known atomic structures into map

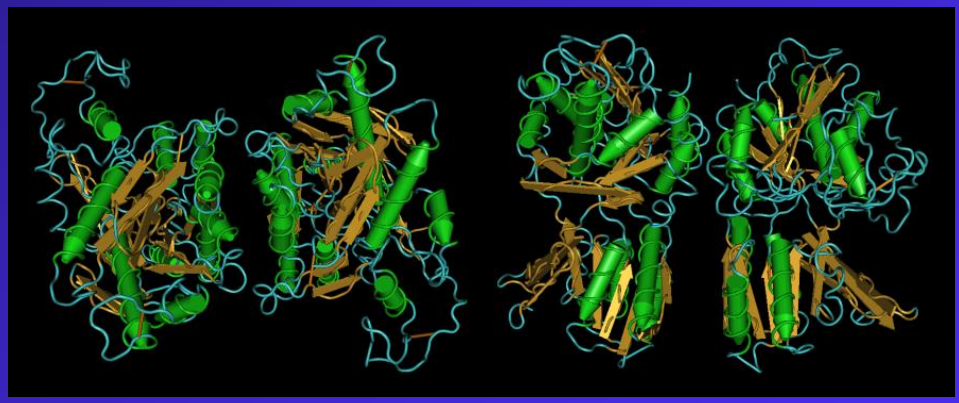
Henderson et al. (2012) Structure 20: 205-214

Map validation

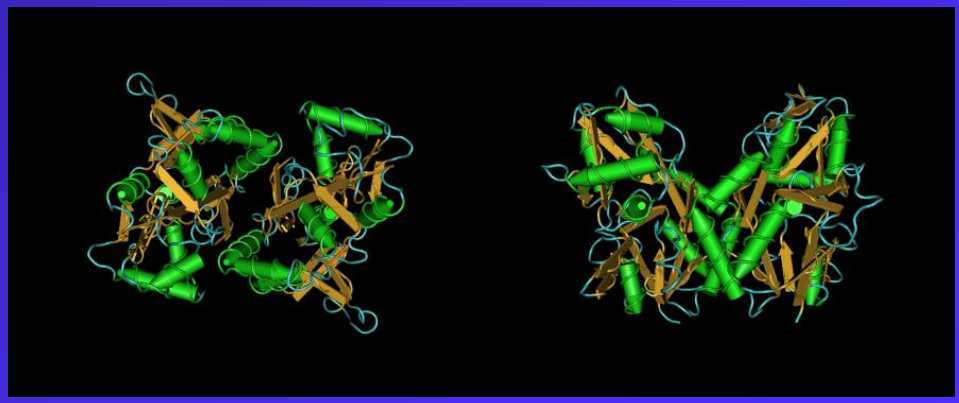
Docking of atomic models



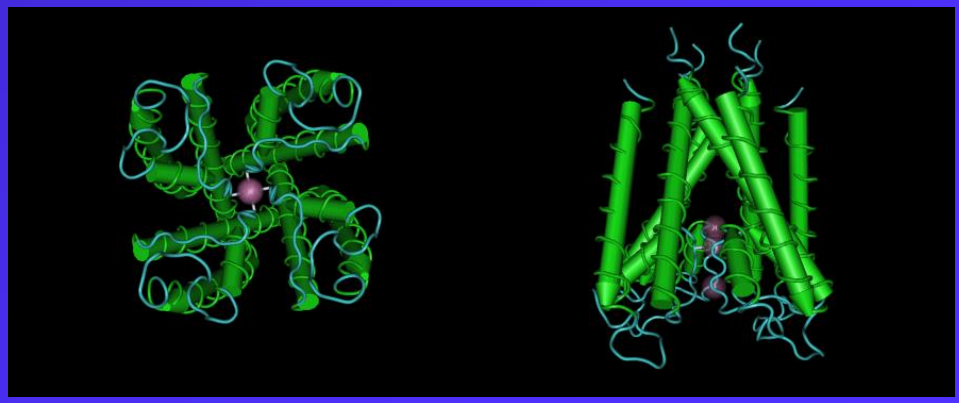
mGluR1
Kunishima et al. 2000
(K. Morikawa)



GluR2
Armstrong et al. 2000
(E. Gouaux)

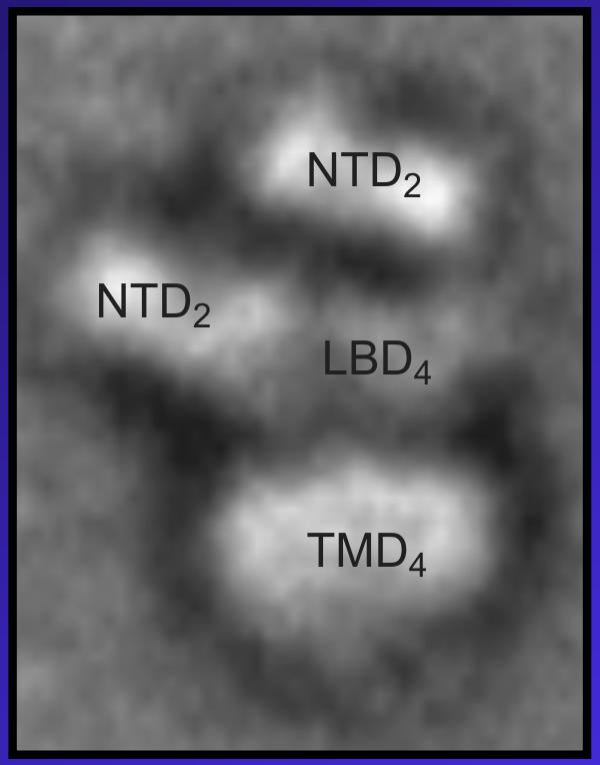


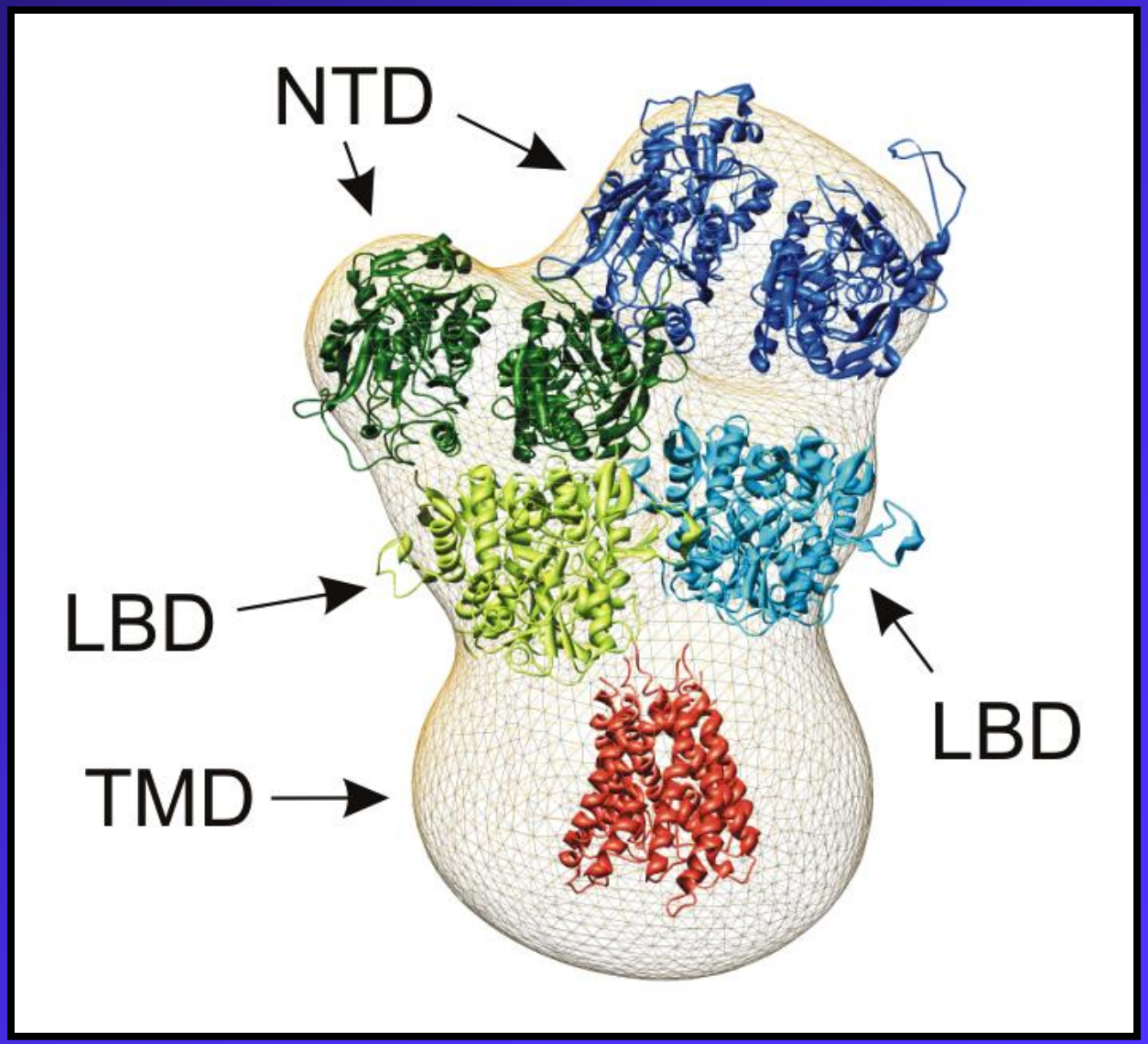
KcsA
Doyle et al. 1998
(R. MacKinnon)



Map validation

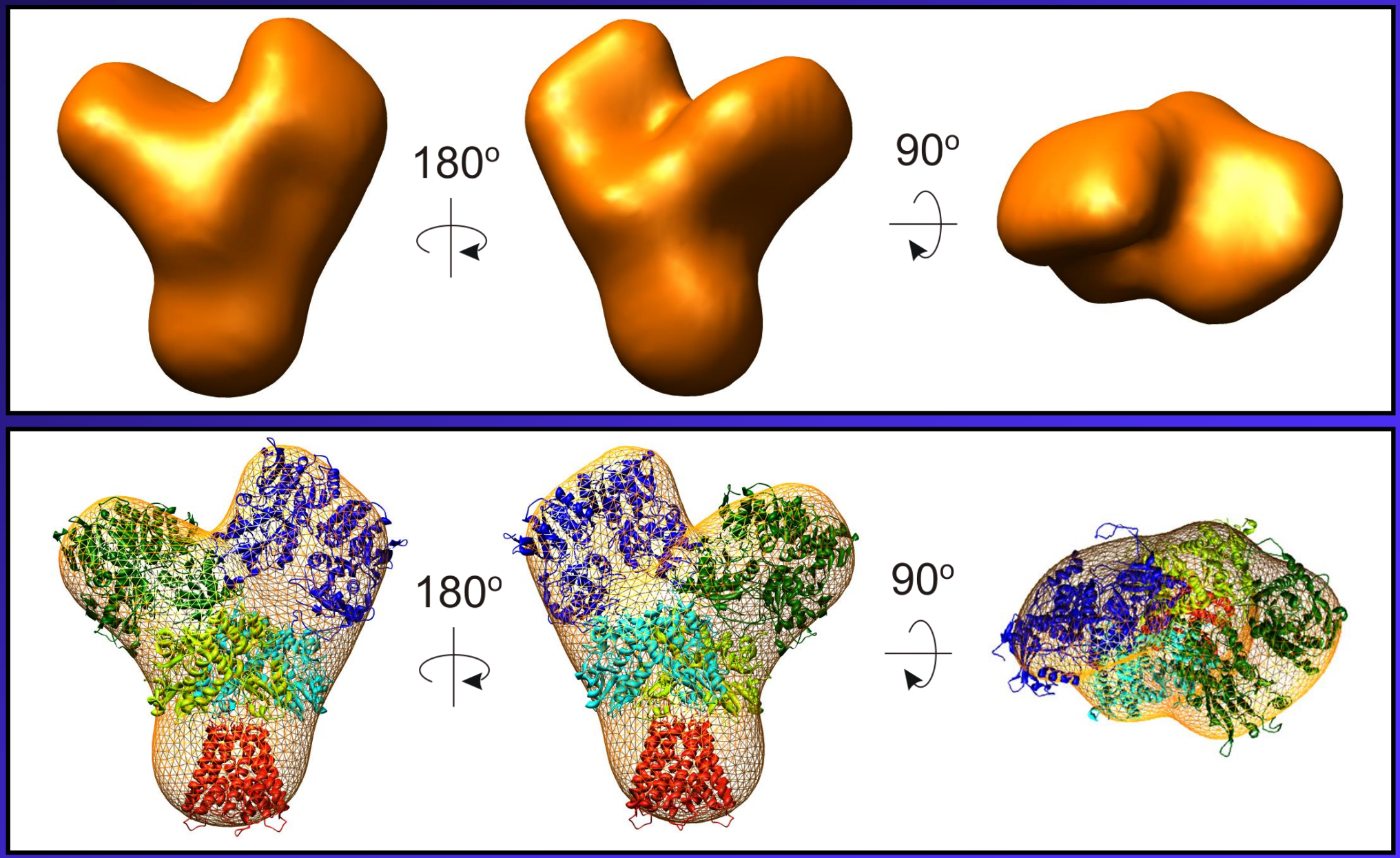
Docking of atomic models





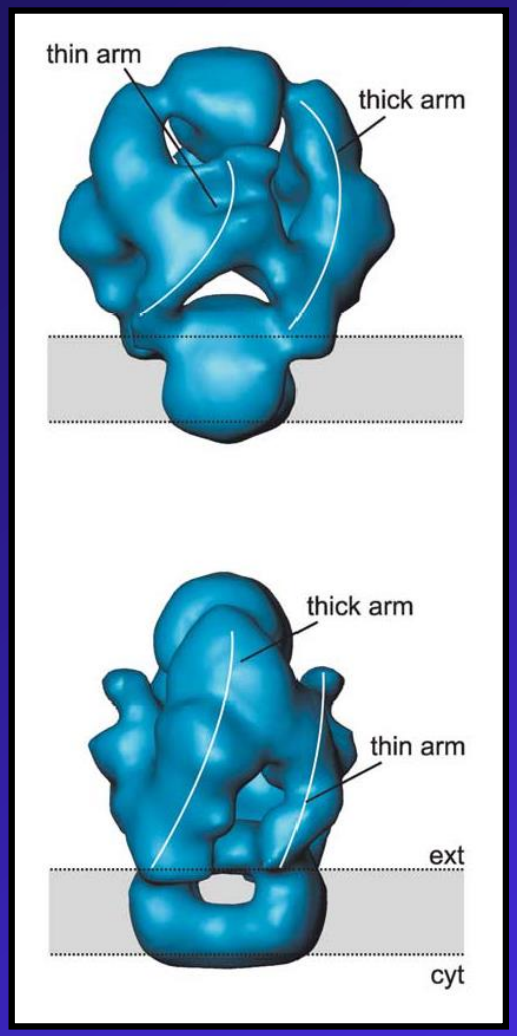
Map validation

Docking of atomic models



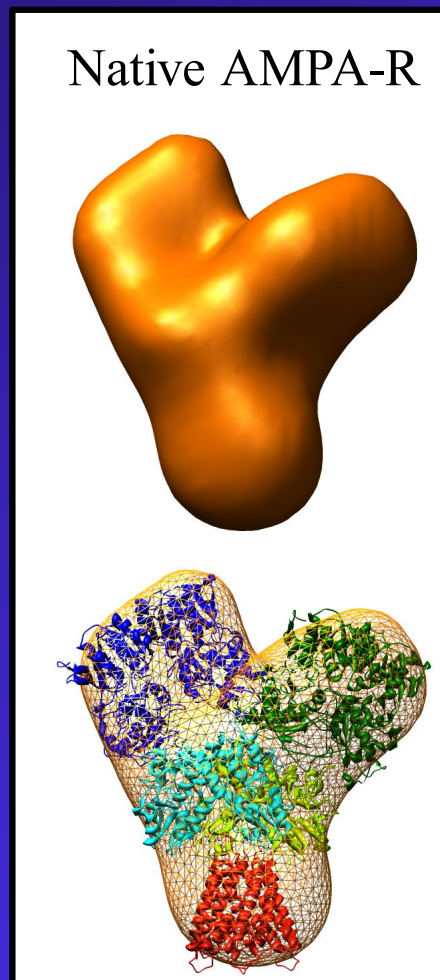
Map validation

Docking of atomic models



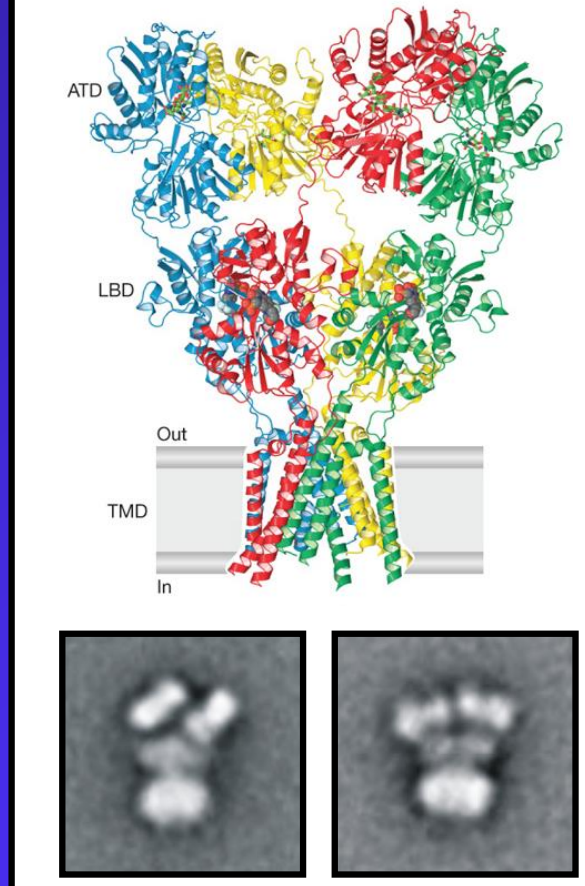
Tichelaar *et al.* (2004)
JMB 344: 435-442

Native AMPA-R



Nakagawa *et al.* (2006)
Biol. Chem. 387: 179-187

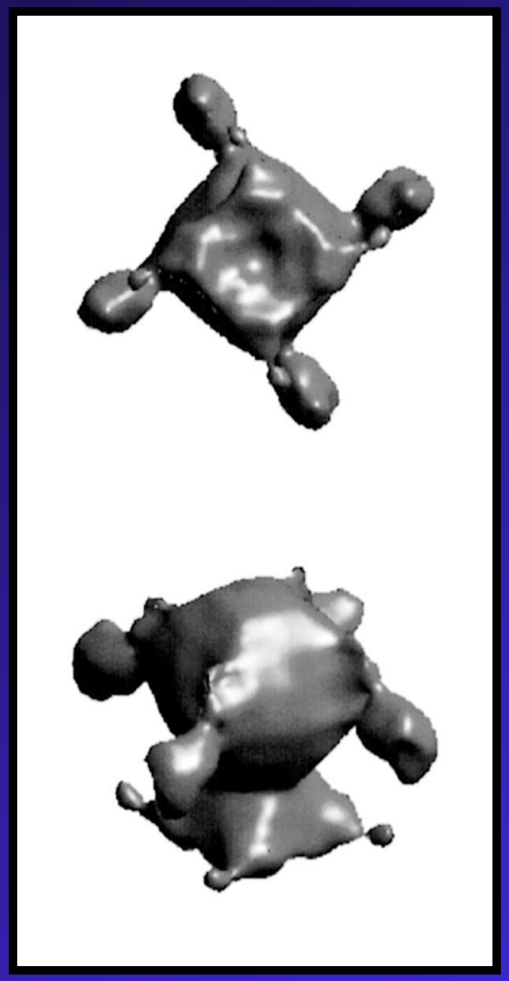
Engineered GluA2



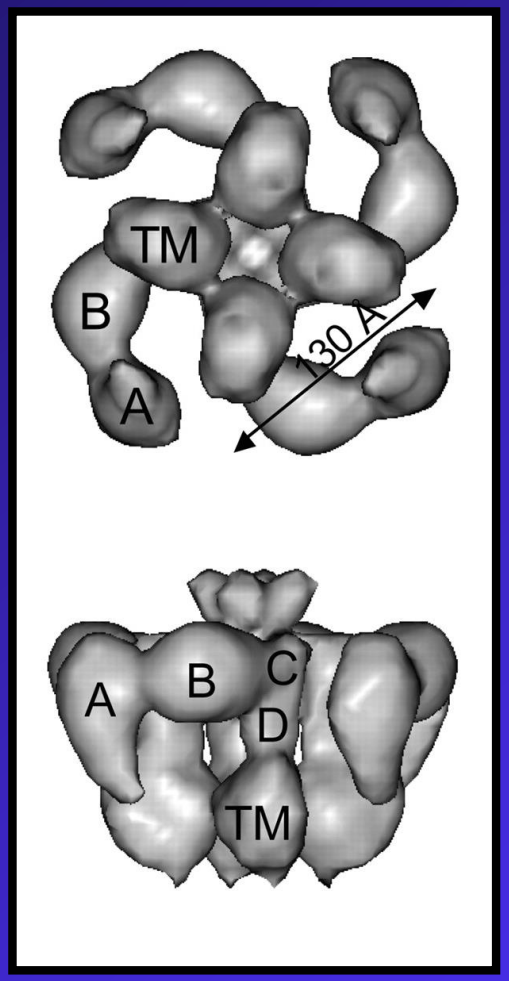
Sobolevsky *et al.* (2009)
Nature 462: 745-758

Map validation - IP3 receptor

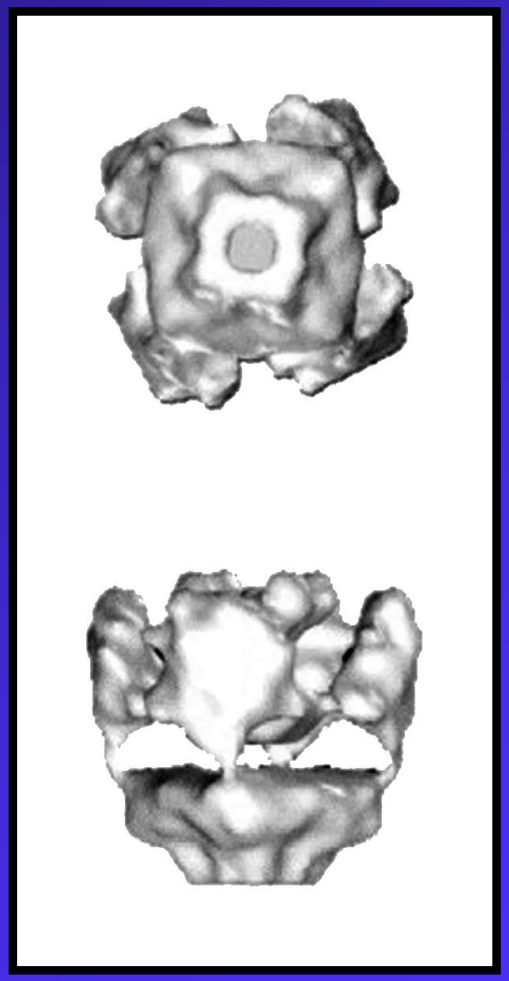
Different maps of the IP3 receptor



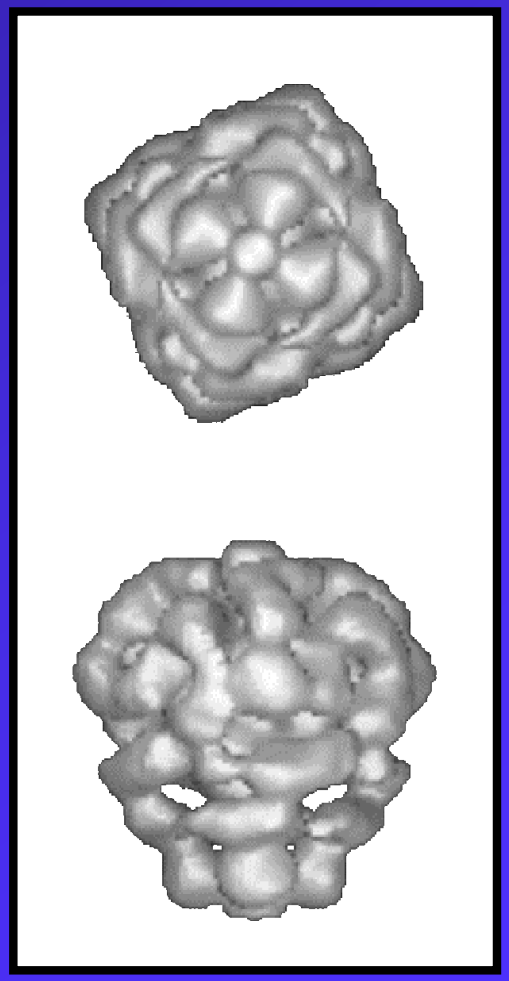
Jiang et al., 2002



Serysheva et al., 2003



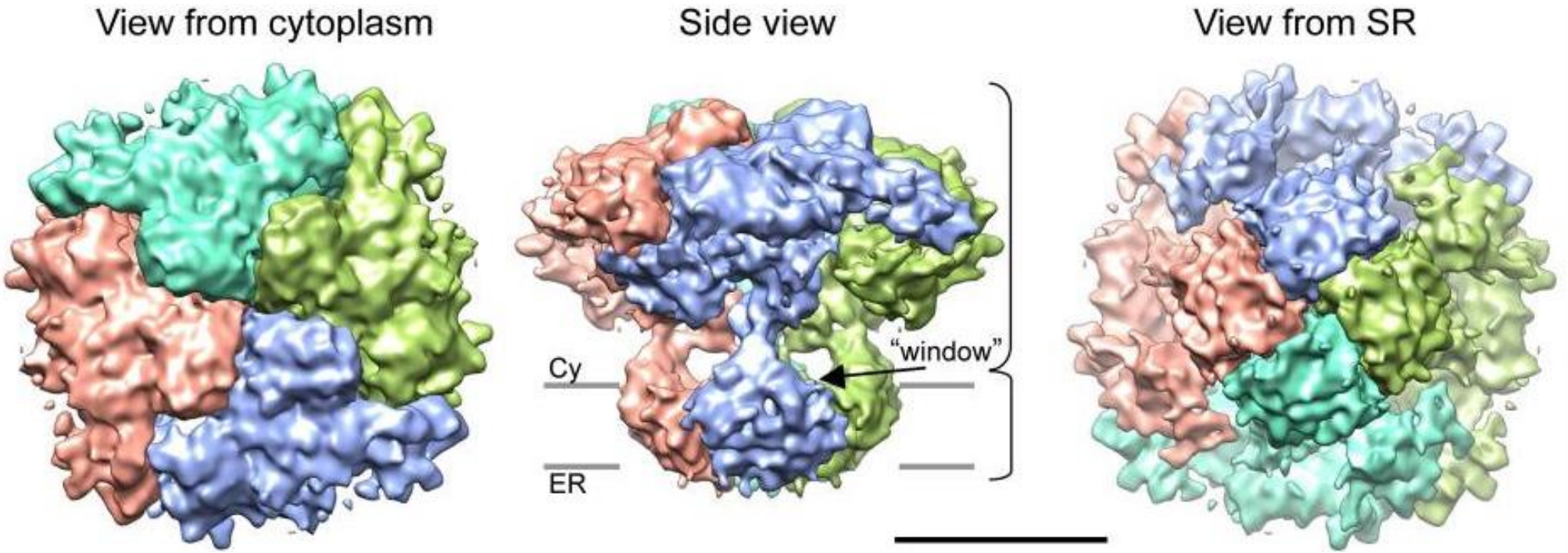
Jiang et al., 2003



Sato et al., 2004

Map validation – IP3 receptor

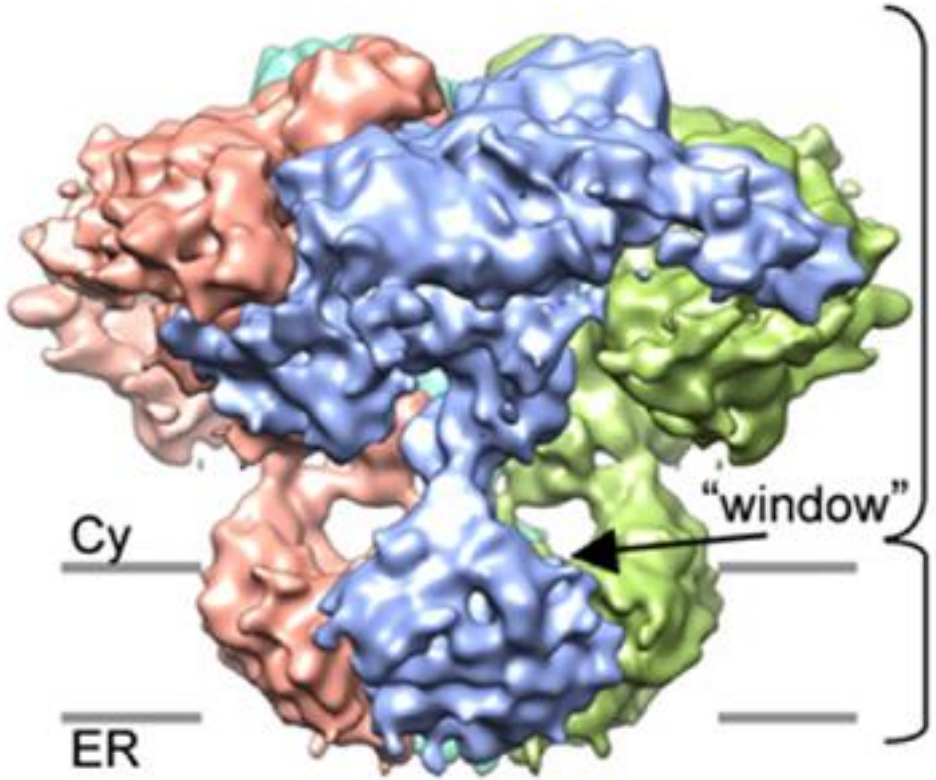
New density map in 2011 at 11 Å resolution

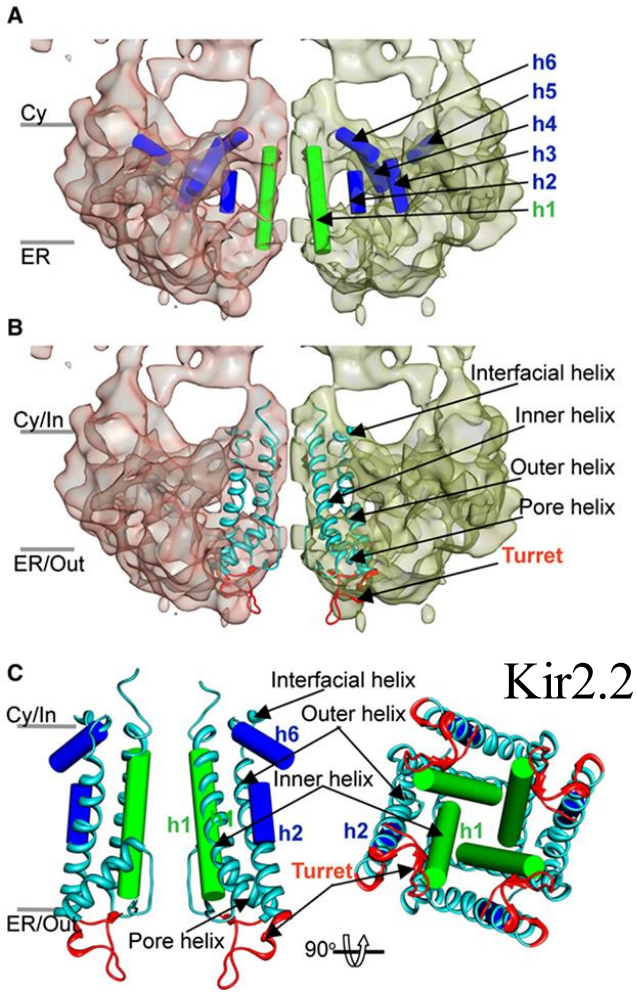


Ludtke et al. (2011) Structure 19: 1192-1199

Map validation - IP3 receptor

Expected secondary structure elements

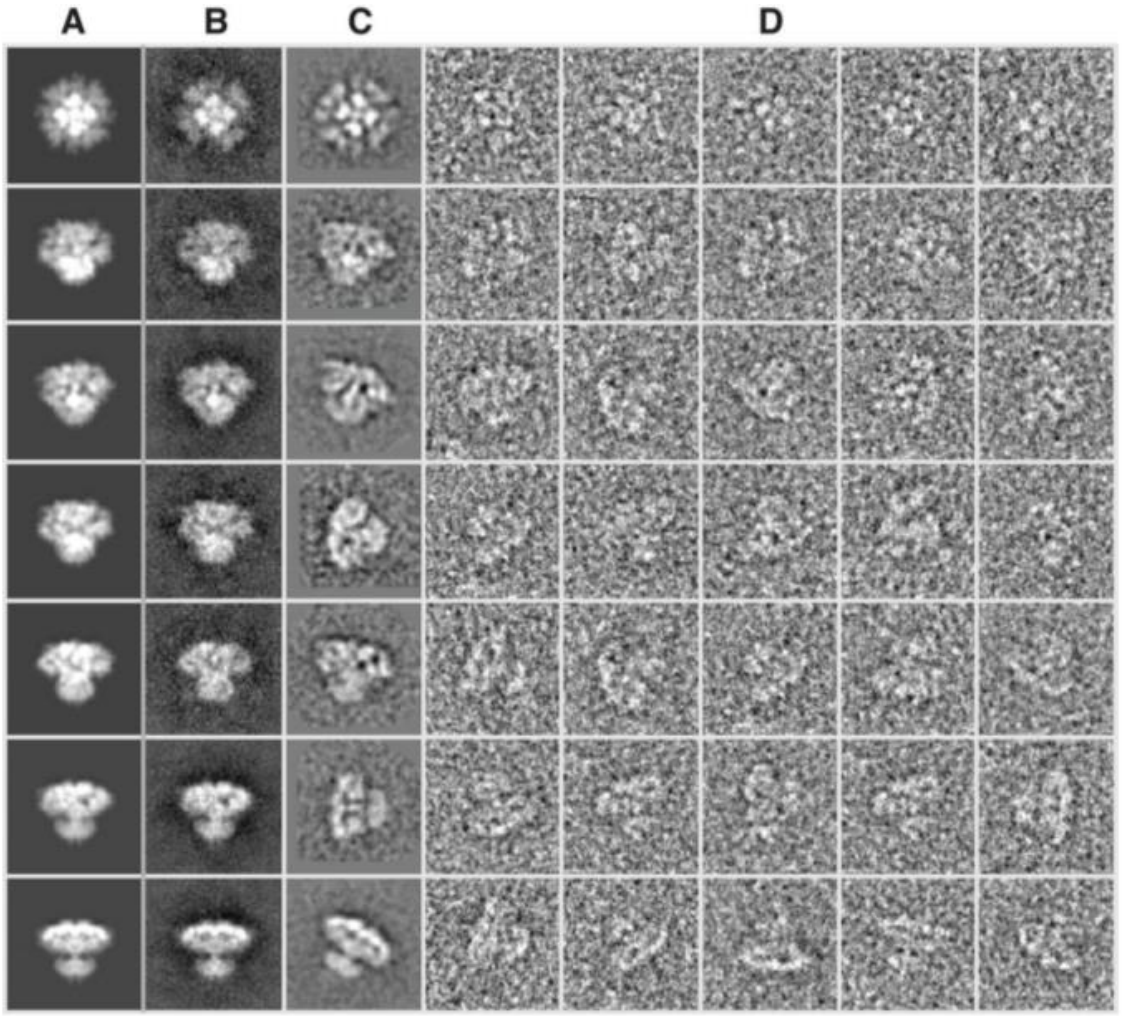




Ludtke et al. (2011) Structure 19: 1192-1199

Map validation - IP3 receptor

Comparison of reference-free averages with projections



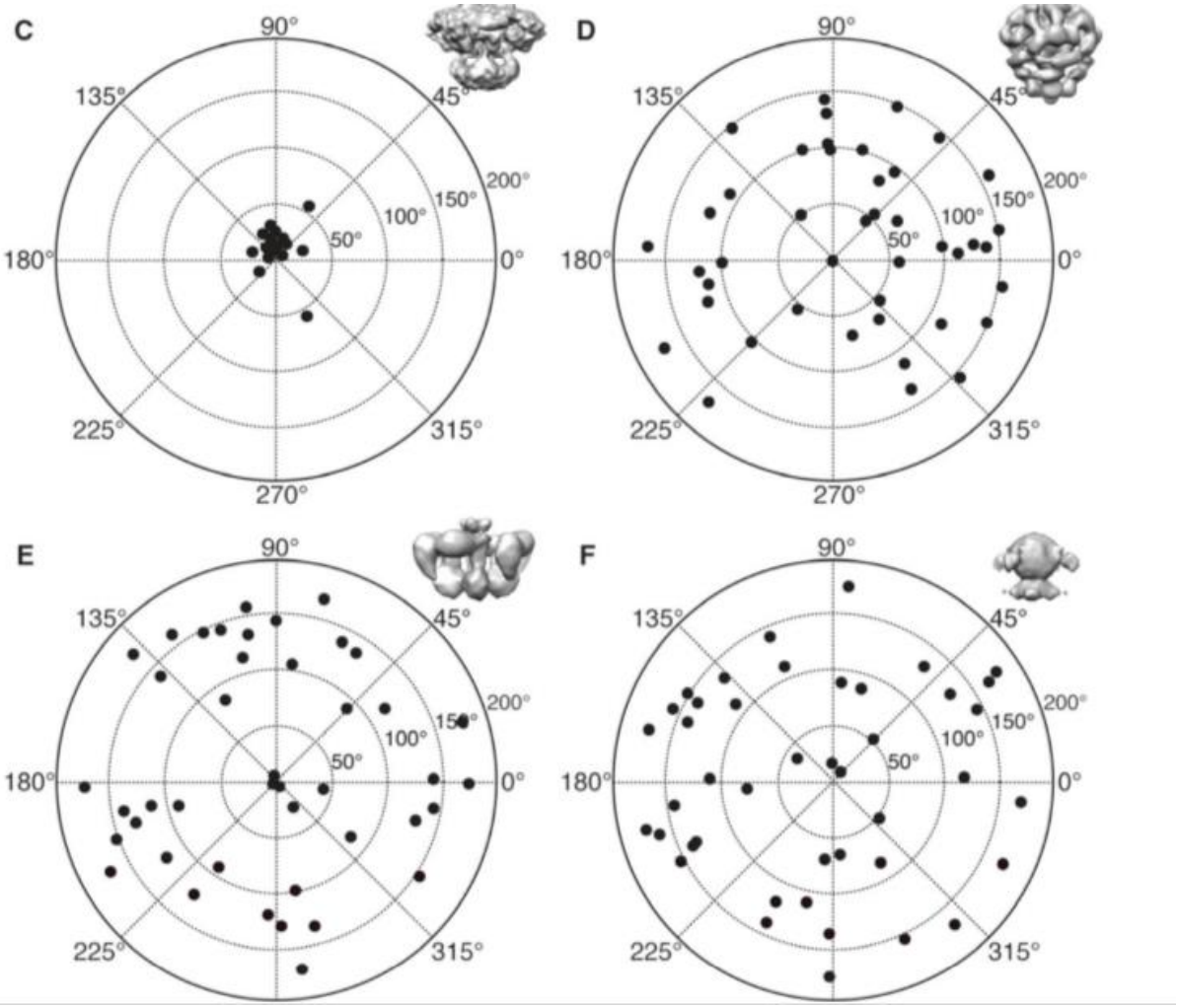
A: Map projection
B: Reference-based class average
C: Reference-free class average
D: Selected particles

Murray et al. (2013) Structure 21: 900-909

Map validation – IP3 receptor

Tilt pair test

Ludtke et al. 2011



Sato et al. 2004

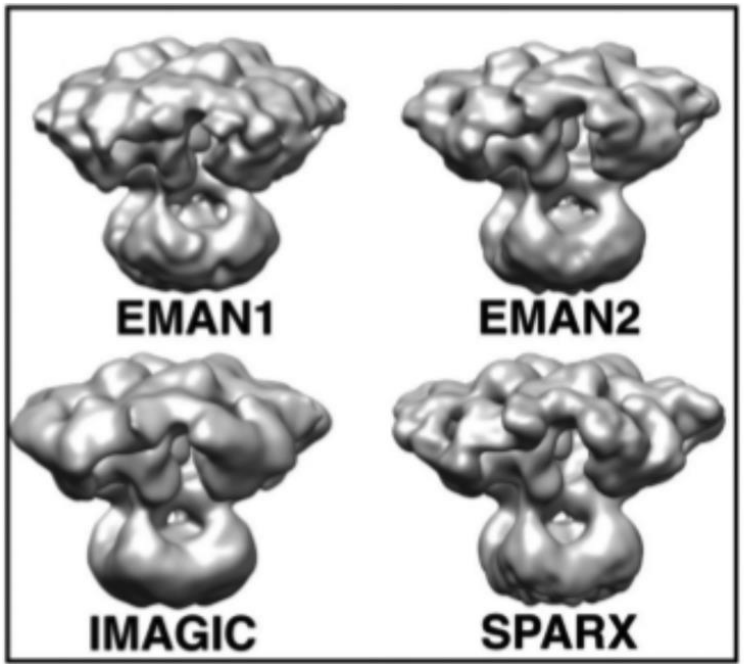
Serysheva et al. 2003

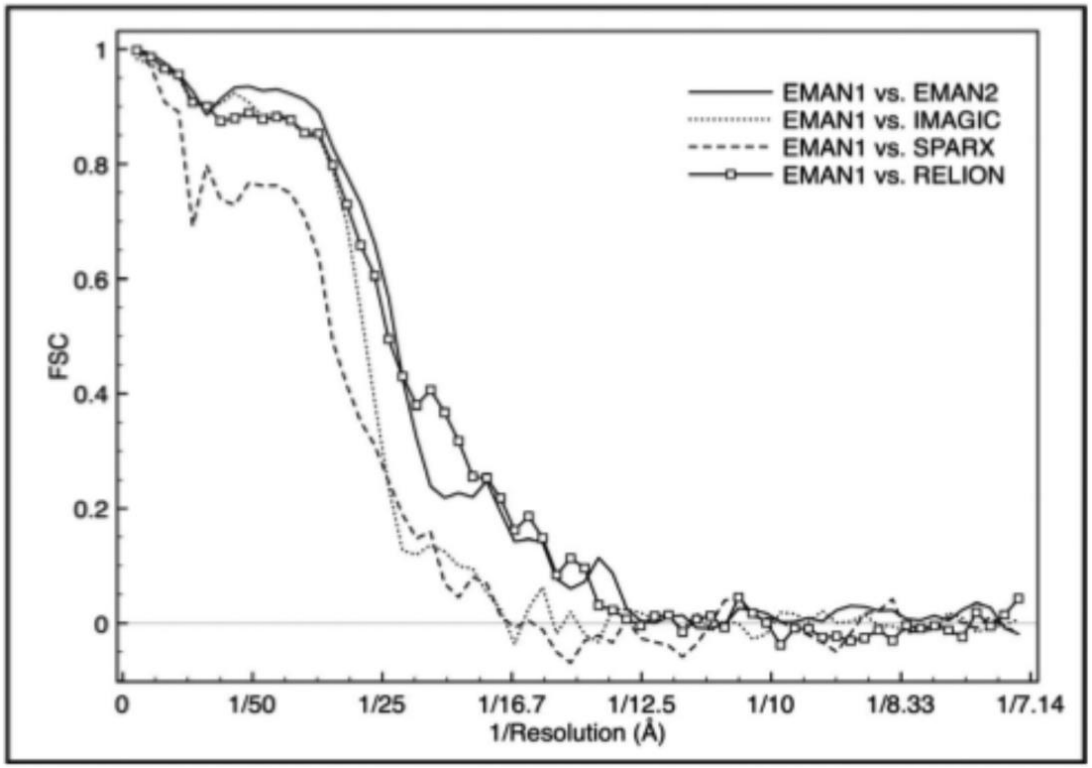
Jiang et al. 2002

Murray et al. (2013) Structure 21: 900-909

Map validation - IP3 receptor

Comparison of maps from different programs

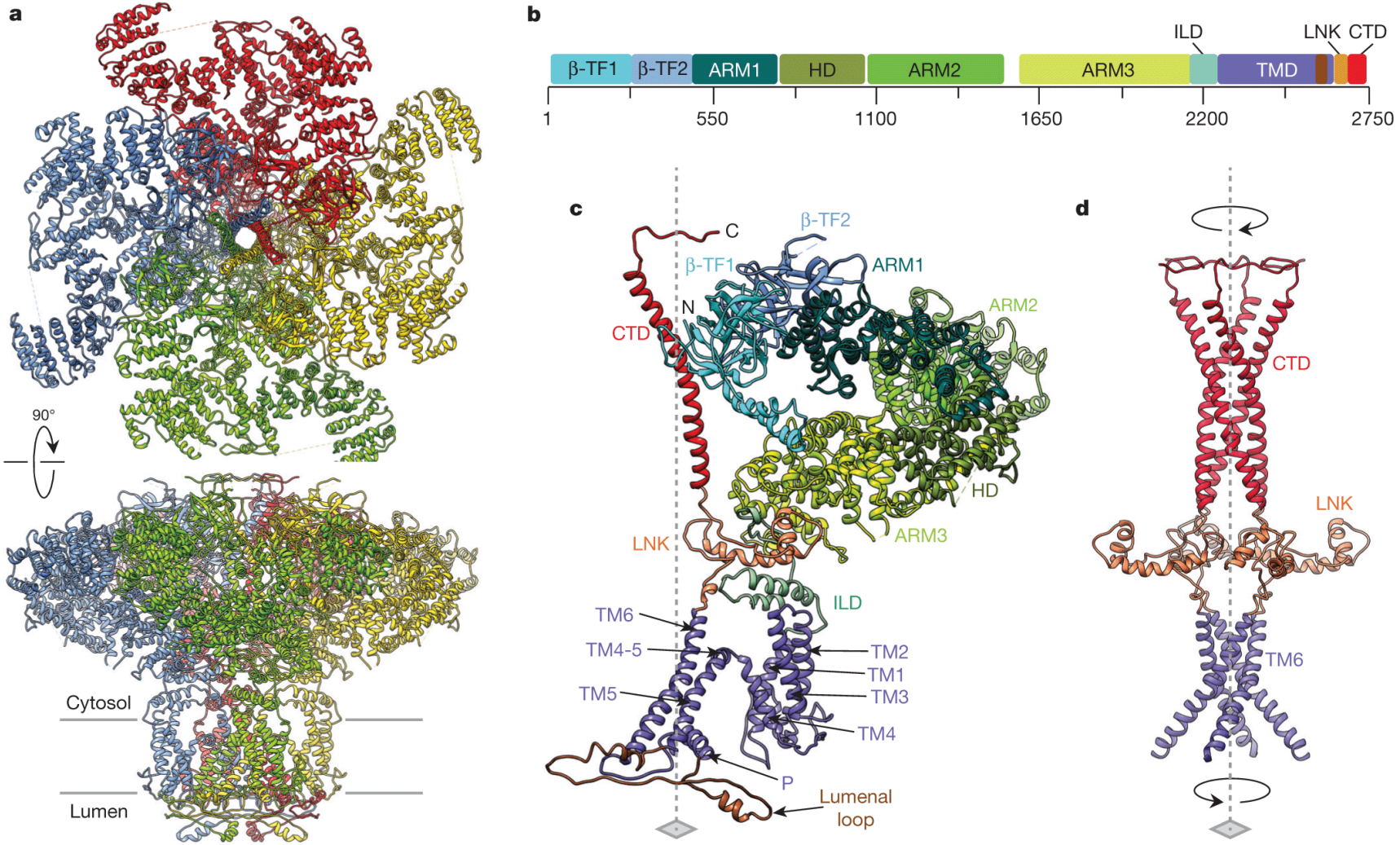




Murray et al. (2013)
Structure 21: 900-909

Map validation - IP3 receptor

4.7 Å resolution structure



Fan et al. (2015) Nature 527: 336-341