

Validation Methods

**Simons Electron Microscopy Center
Winter EM Course 2018-19**

The dark side of single-particle EM

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

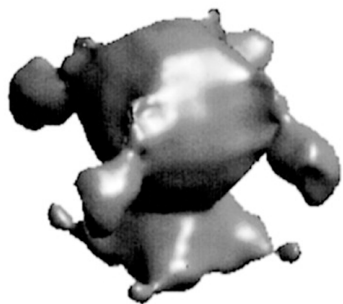
The bad thing about single-particle EM:
Every data set and processing approach yields a 3D map !

But is it correct ???

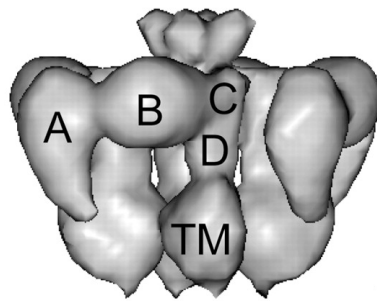
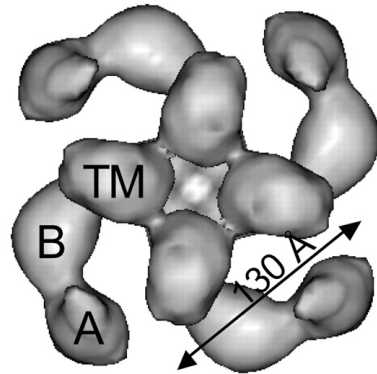


Particularly problematic
for low-resolution maps

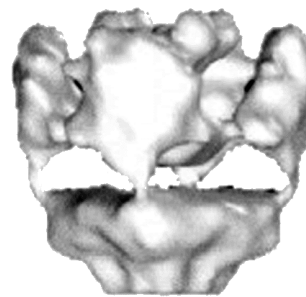
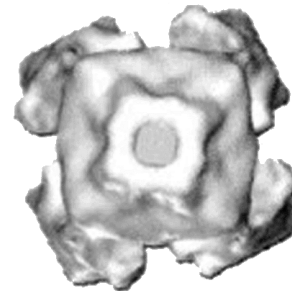
The issue: Structures of the IP3 receptor as determined by single-particle EM



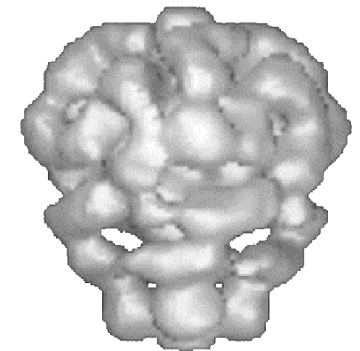
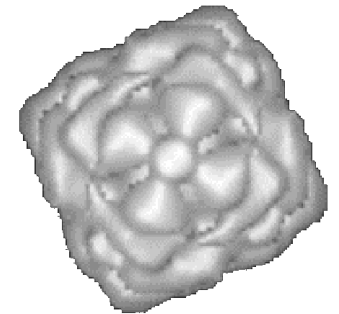
Jiang *et al.*,
2002



Serysheva *et al.*,
2003

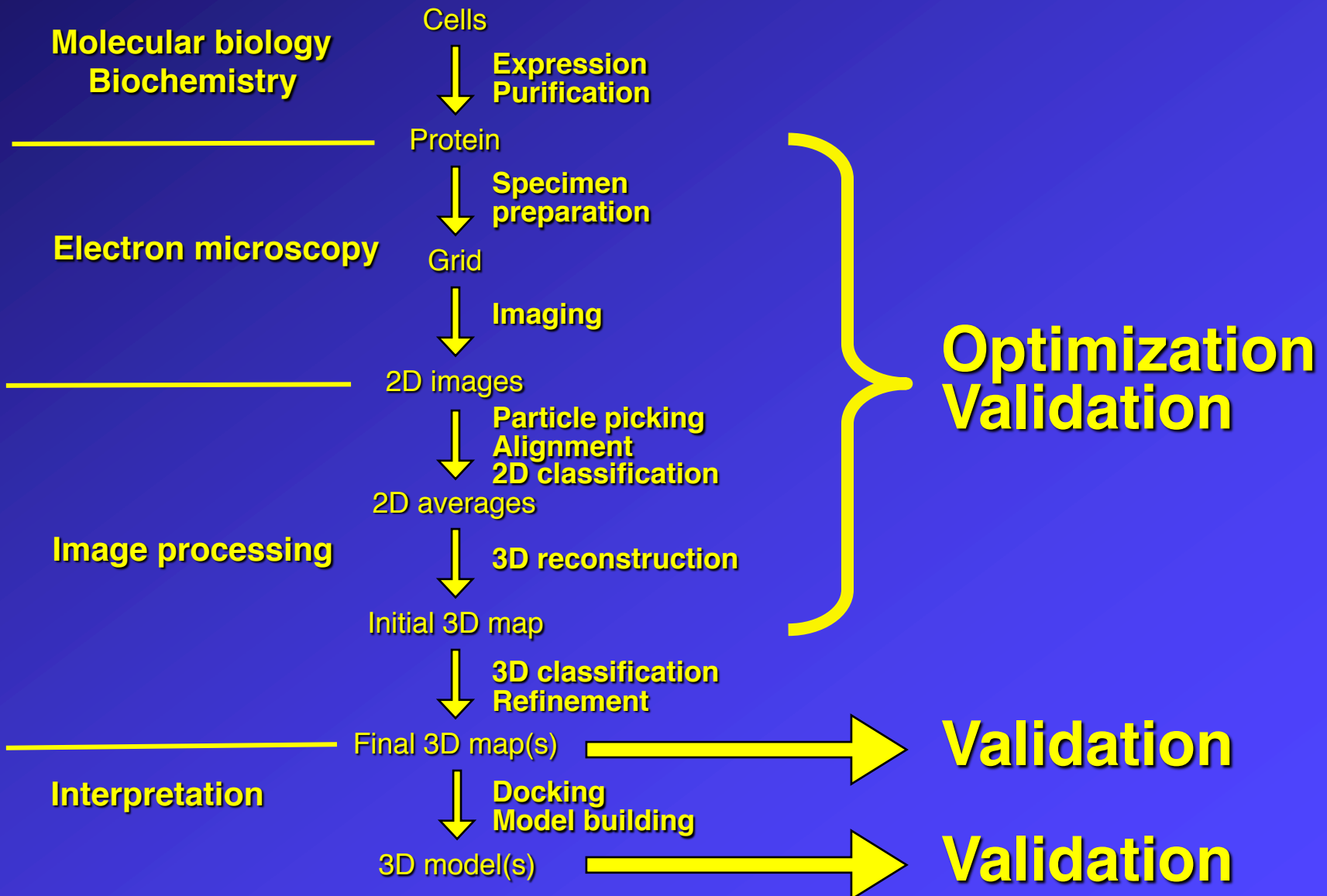


Jiang *et al.*,
2003



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 !

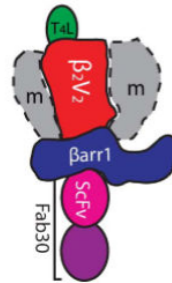
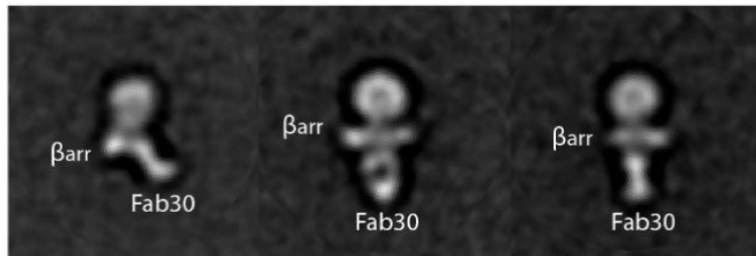
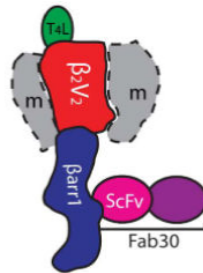
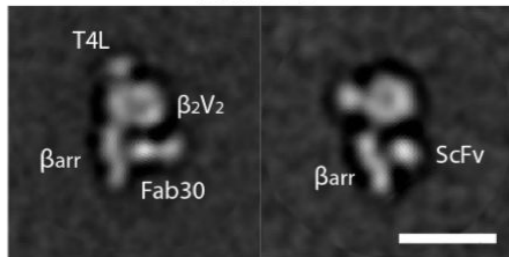
Prepare negatively stained specimens:
Good contrast and preferred orientations
→ Easy to assess heterogeneity

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

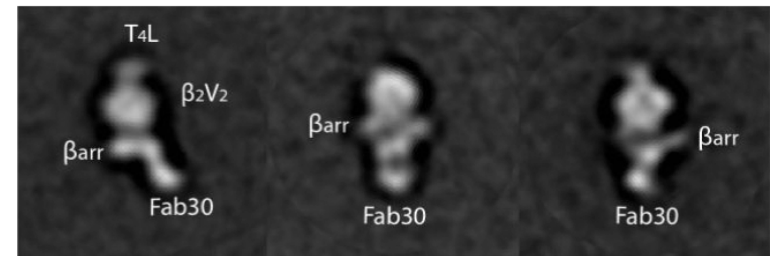
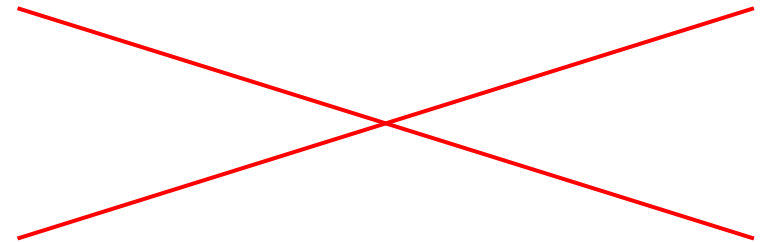
If chemical fixation was used:
Look at unfixed sample to assess effect of cross-linking
→ Assess whether structure of cross-linked sample is meaningful

Effect of cross-linking: The β_2V_2R - β arrestin1-Fab30 complex

Native



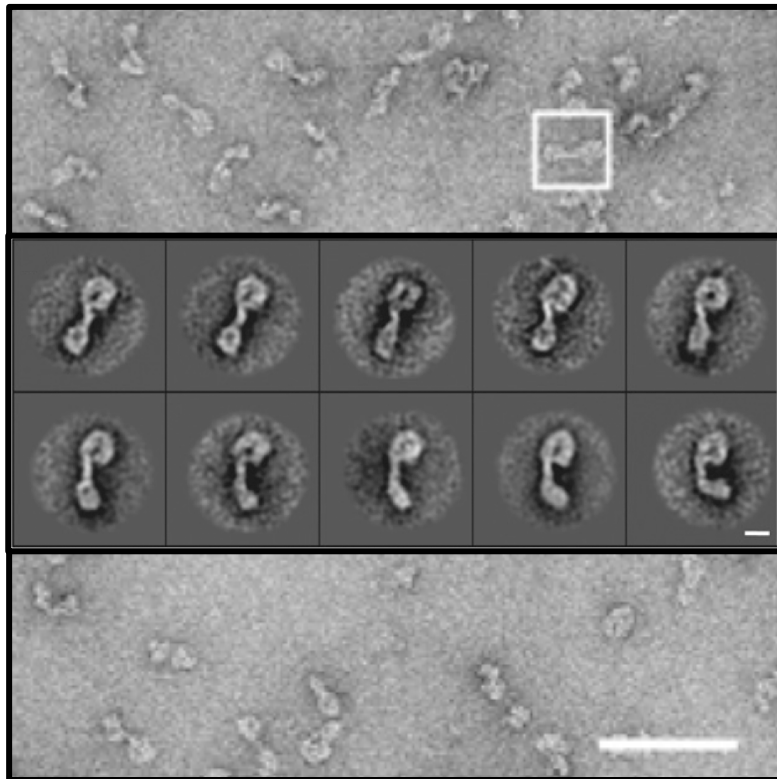
Cross-linked



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

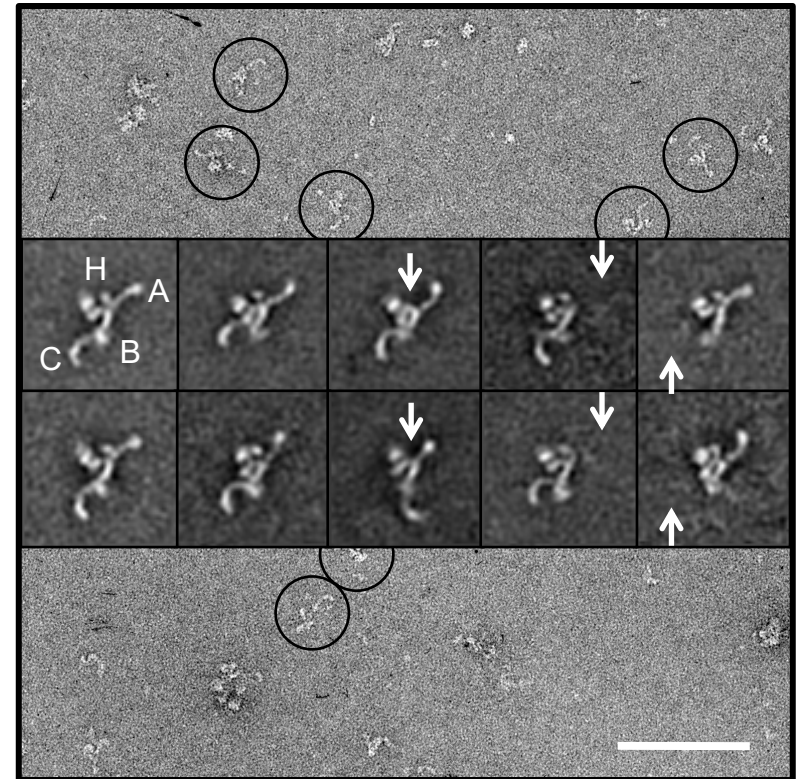
Effect of cross-linking: The HOPS tethering complex

Cross-linked



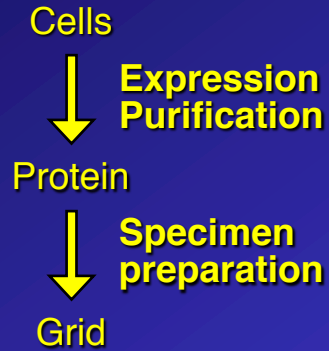
Bröcker *et al.* (2012)
PNAS 109: 1991-1996

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)

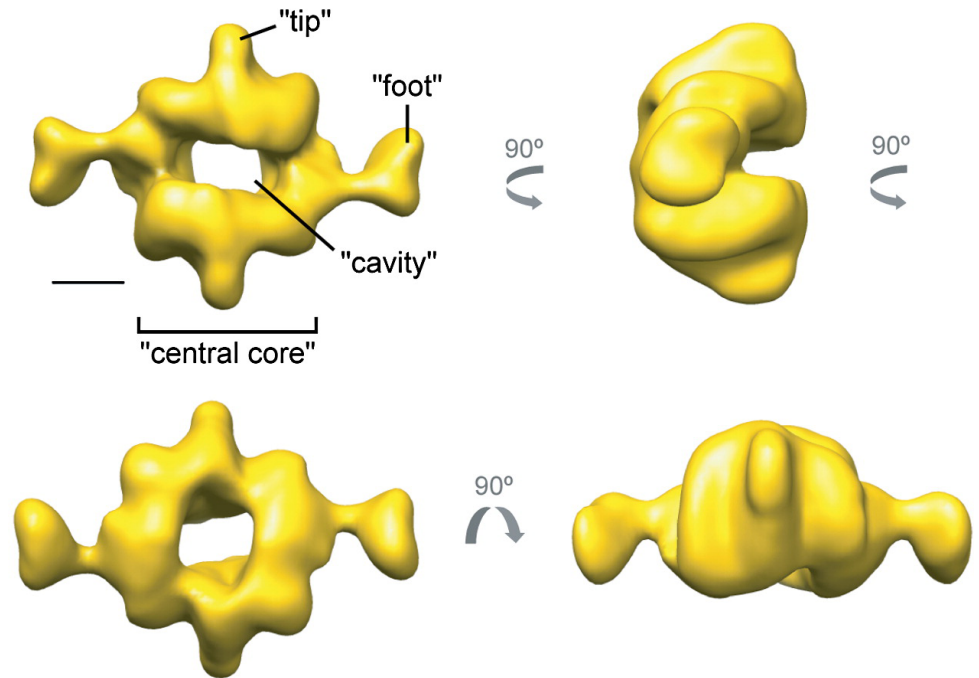
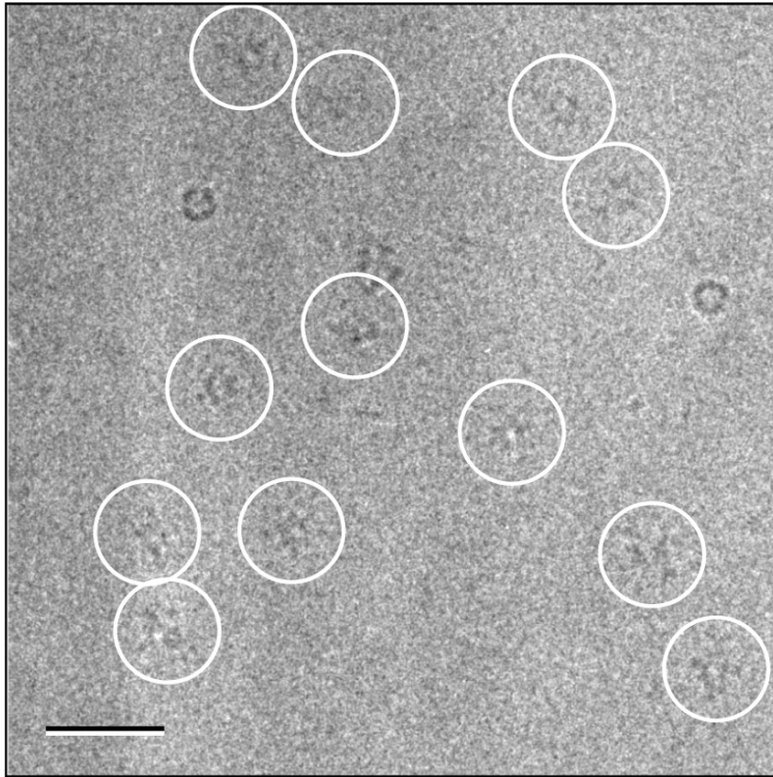
- Increase protein concentration
- Double blotting
- PEG treatment of grid
- Use different grids (gold, lacey carbon)
- Use thin carbon film

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)

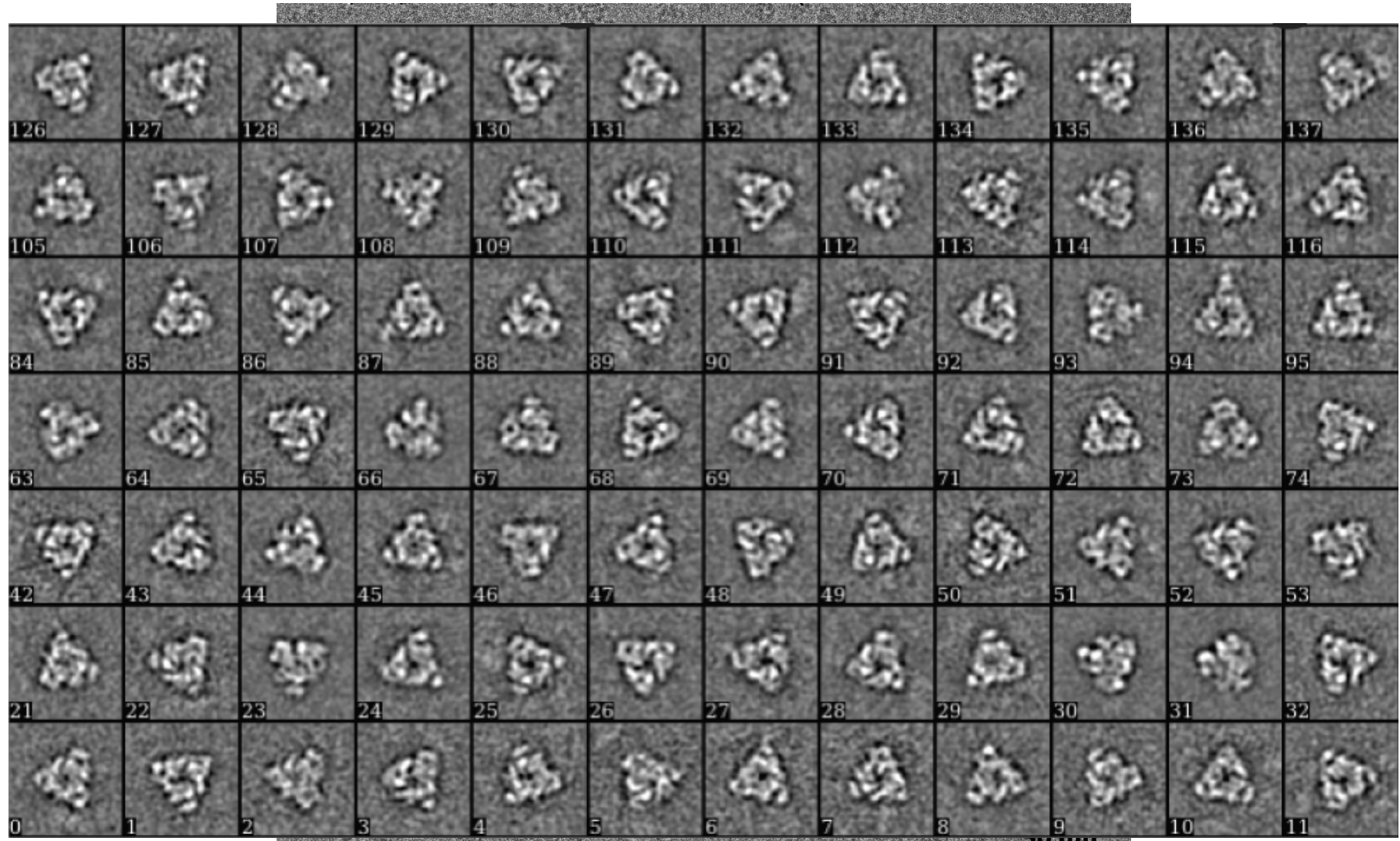
Lack of views will result in:

- 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)

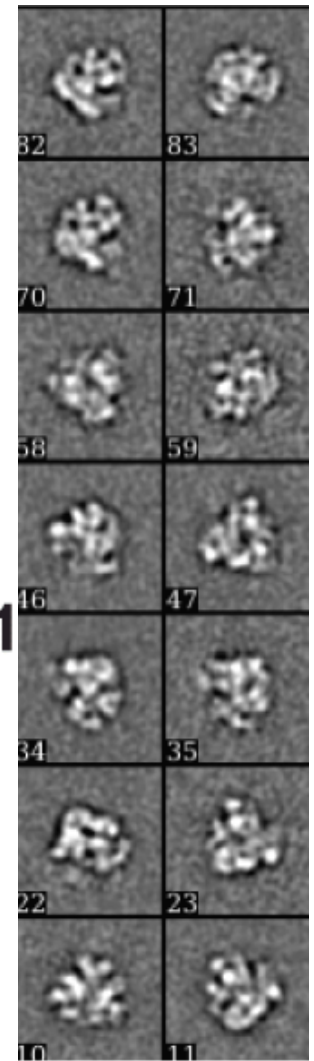
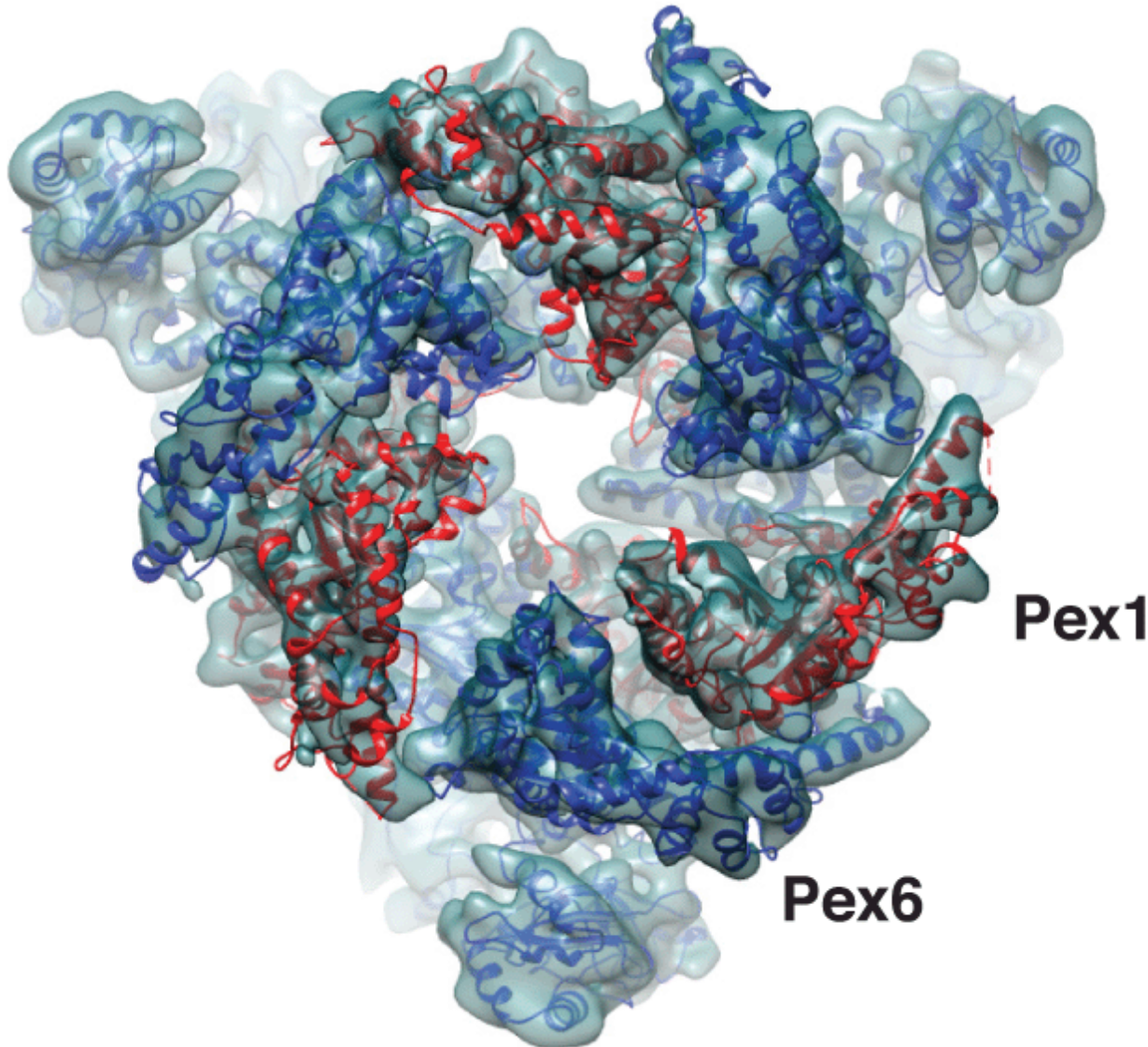
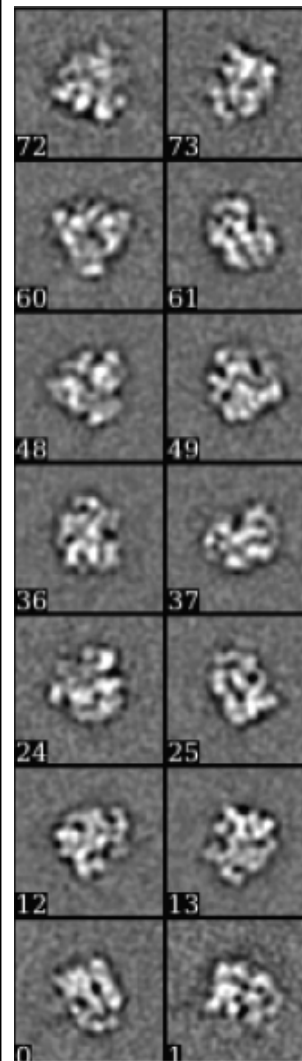
Spotiton

Preferred orientations: Pex1/6 complex

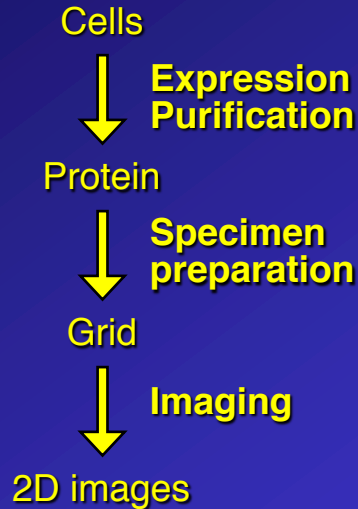
Without detergent



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



Beam sensitivity
→ low electron dose

→ Poor SNR can be fixed
by averaging

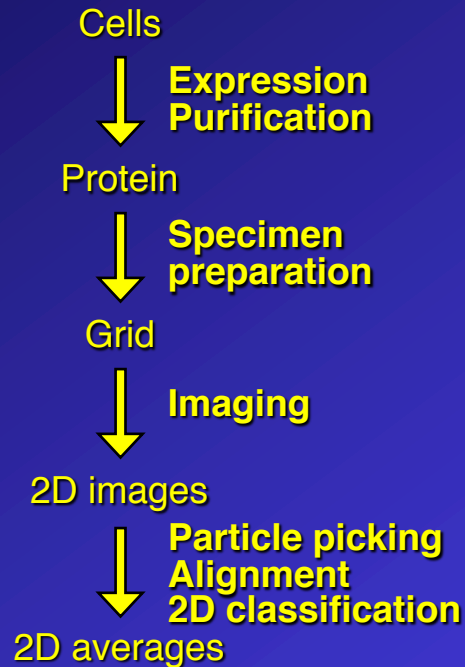
→ Loss of information
cannot be fixed

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

Problem fixed by DDD cameras

- Collect long movies
- Add frames with resolution filter
(removes damaged high-resolution information
retains low-resolution information for good SNR)

Structure determination by single-particle EM



Potential issues:

Particle picking:

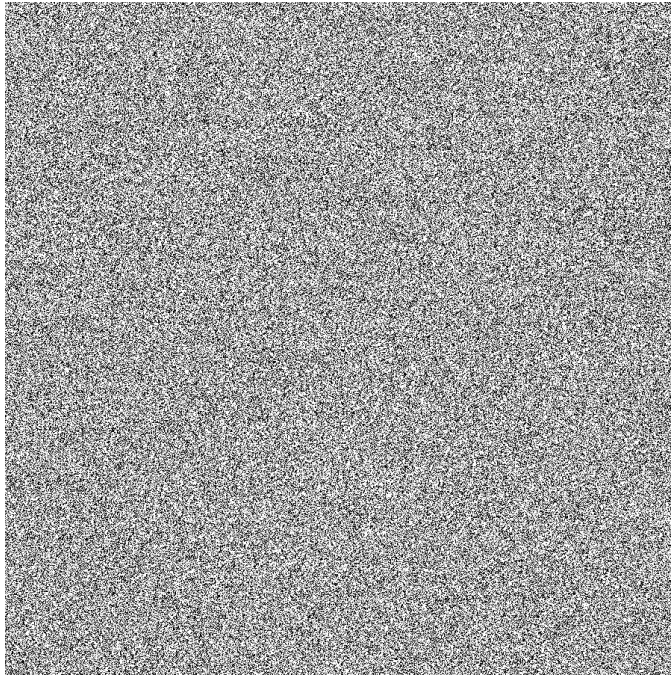
- Model/reference bias

2D classification:

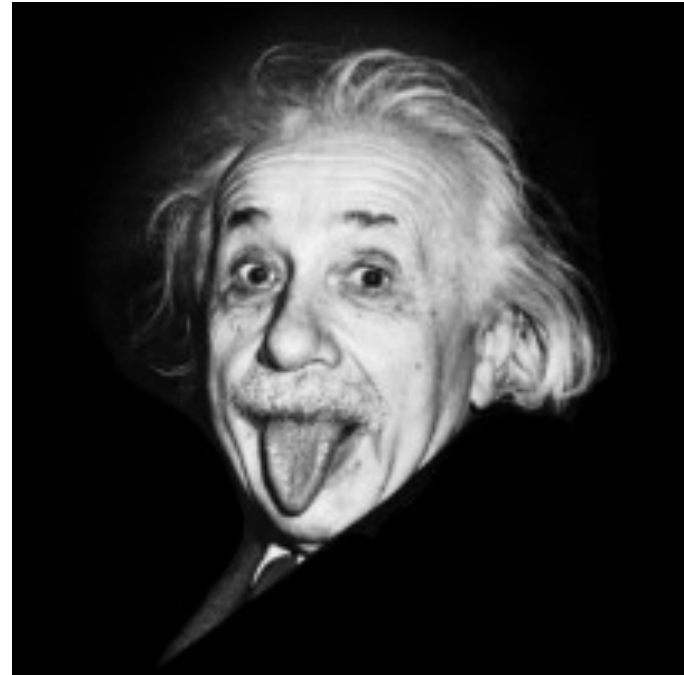
- Model/reference bias
- Number of classes
- Heterogeneous classes
- Disappearing classes

Structure determination by single-particle EM

Potential issues with particle picking



1,000 images containing
pure white noise



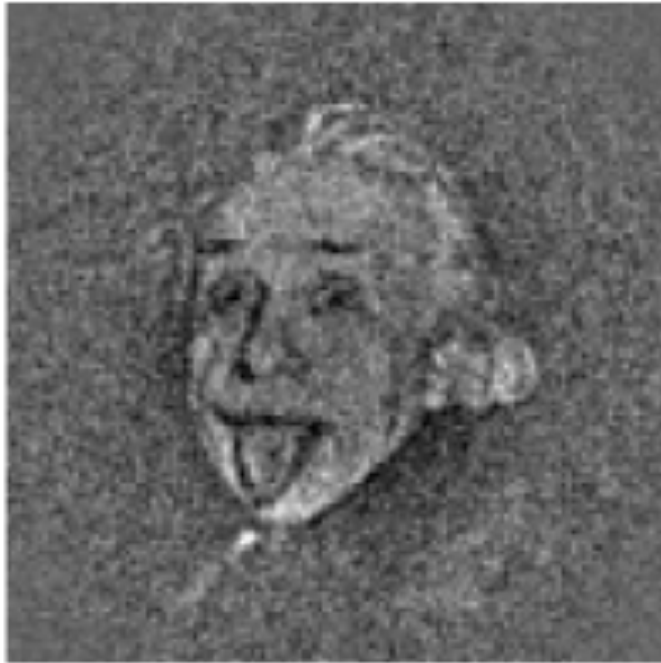
Reference:
Albert Einstein

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

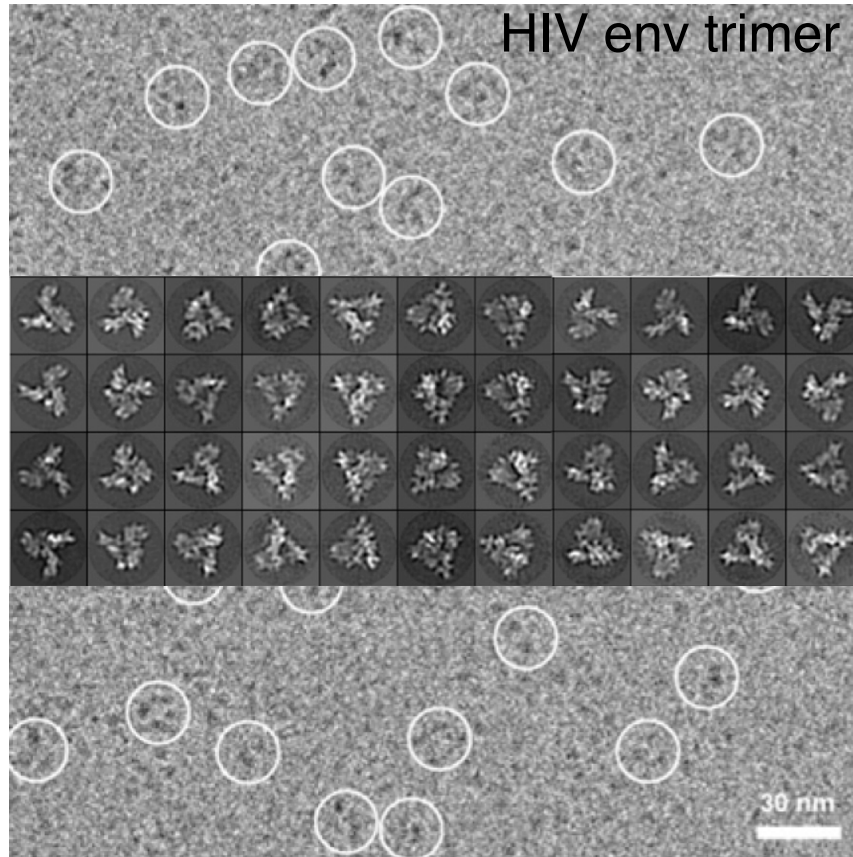
→ Einstein from noise

Shatsky *et al.* (2009) *J. Struct. Biol.* 166: 67-78

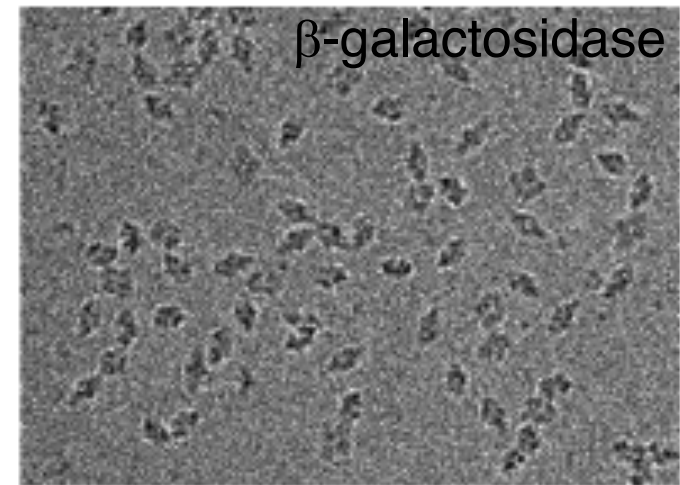
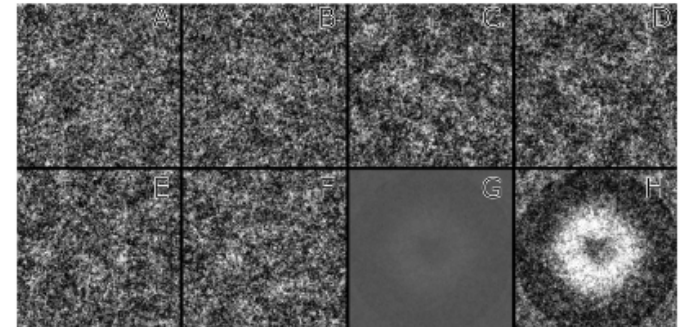
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Structure determination by single-particle EM

Potential issues with particle picking



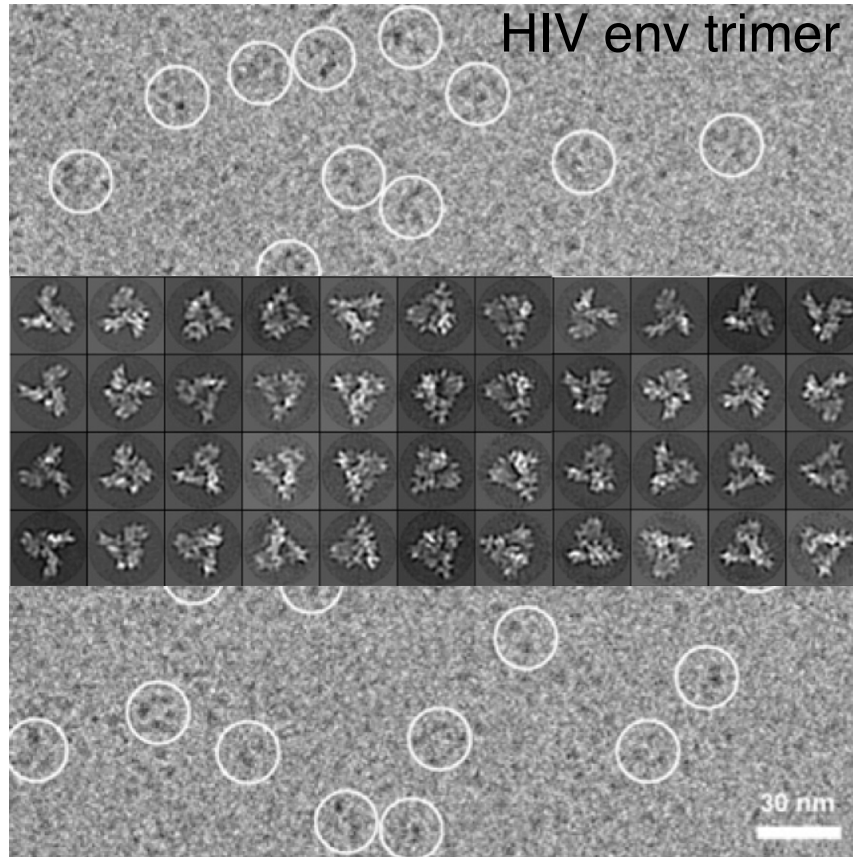
Mao *et al.* (2013)
PNAS 110: 12438-12443



Henderson (2013)
PNAS 110: 18037-18041

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

→ Better to first pick some images by hand and use resulting averages as templates for entire dataset

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

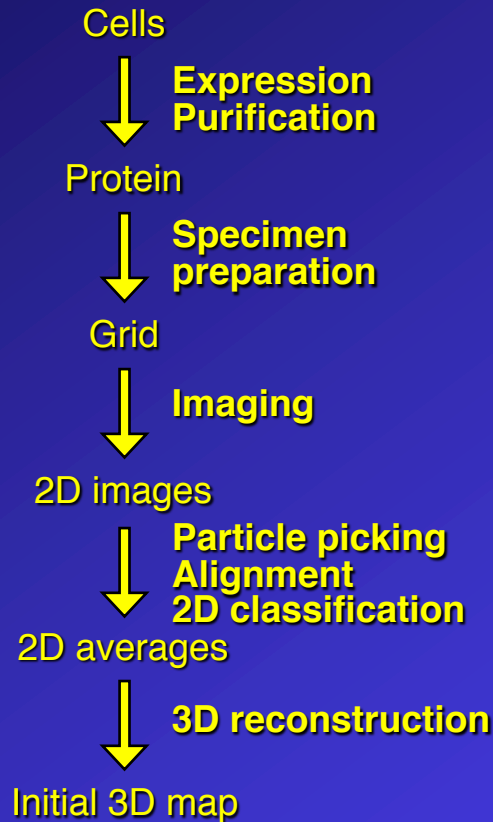
Potential issues with 2D classification (K-means)

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

The latest version of ISAC (as implemented in SPHIRE)
does not produce reliable classes anymore – use old SPARX version !

Structure determination by single-particle EM



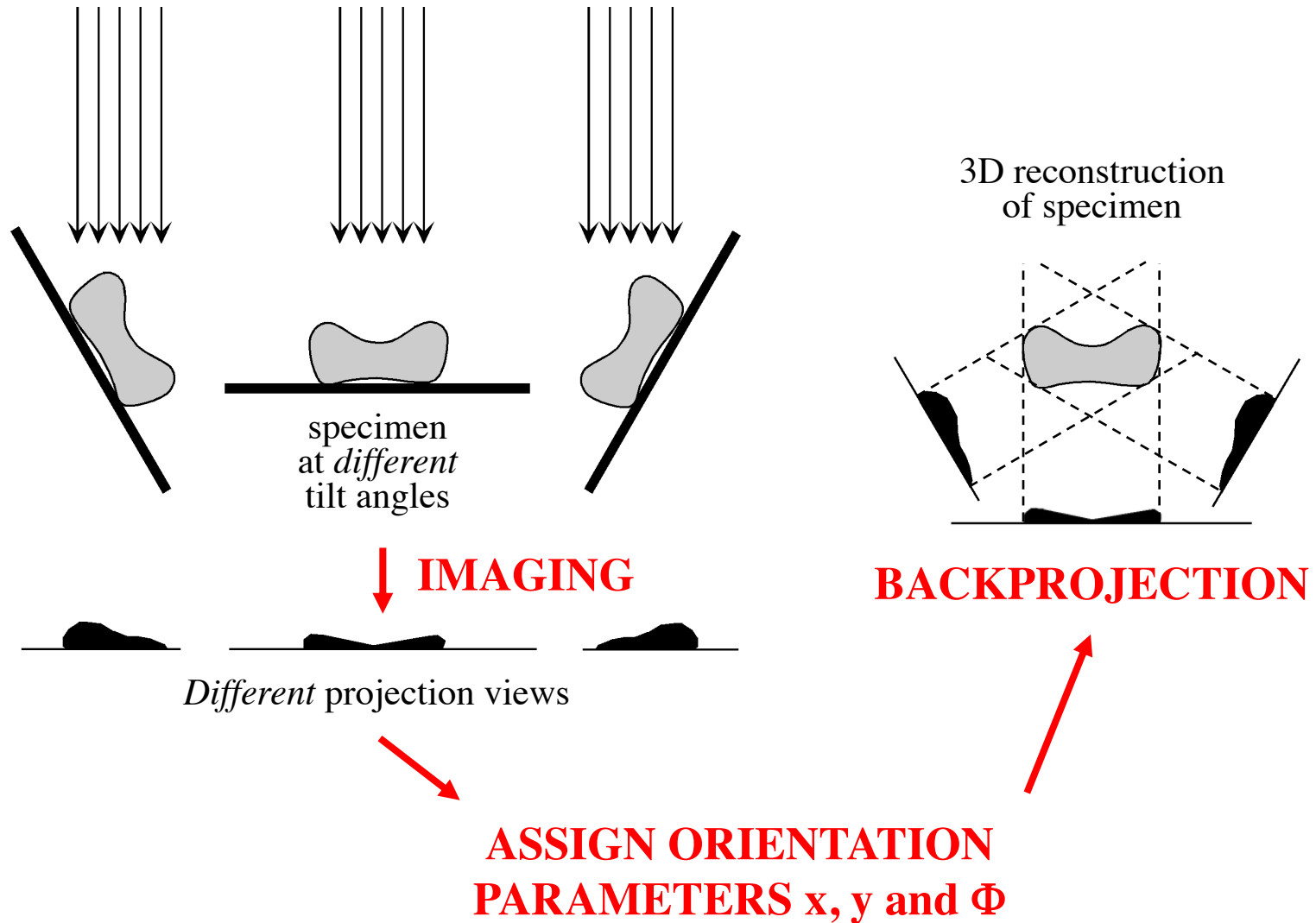
Potential issues:

Incorrect map

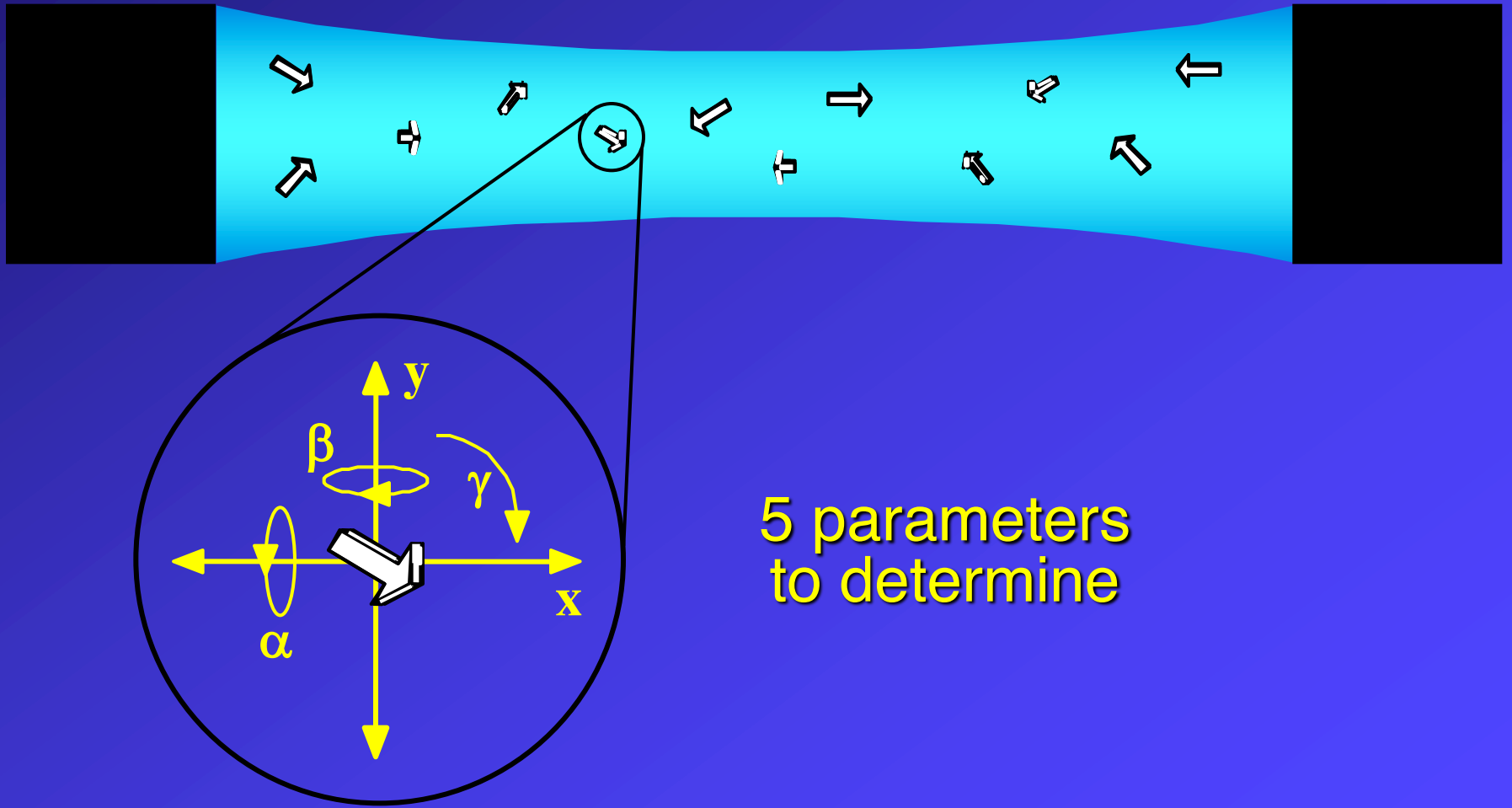
Because of:

- Heterogeneous sample
- Missing views
- Incorrect solution

Random conical tilt reconstruction



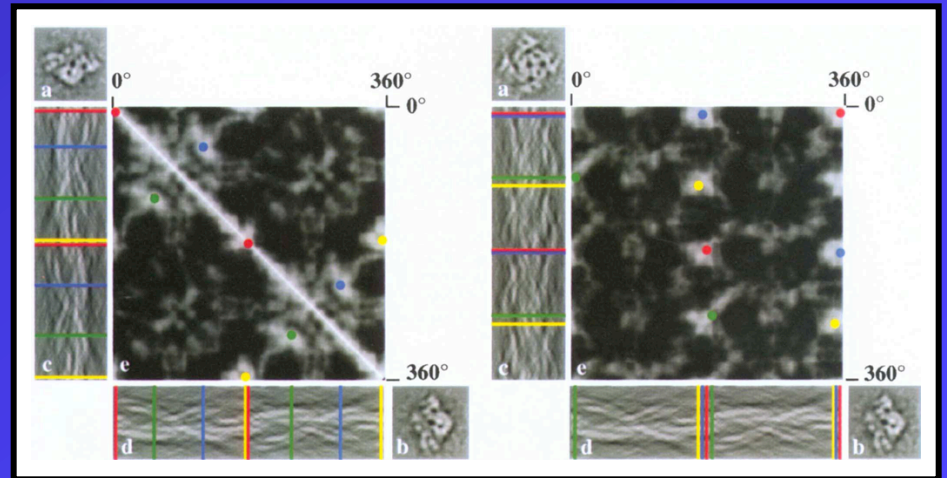
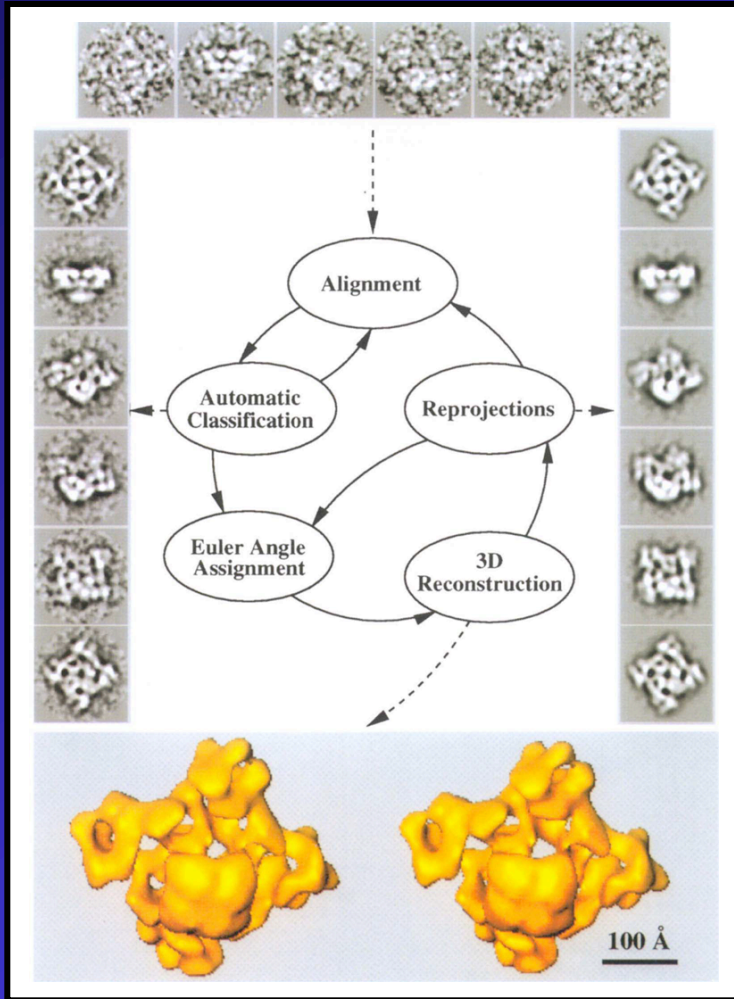
Single particles in ice



Angular reconstitution

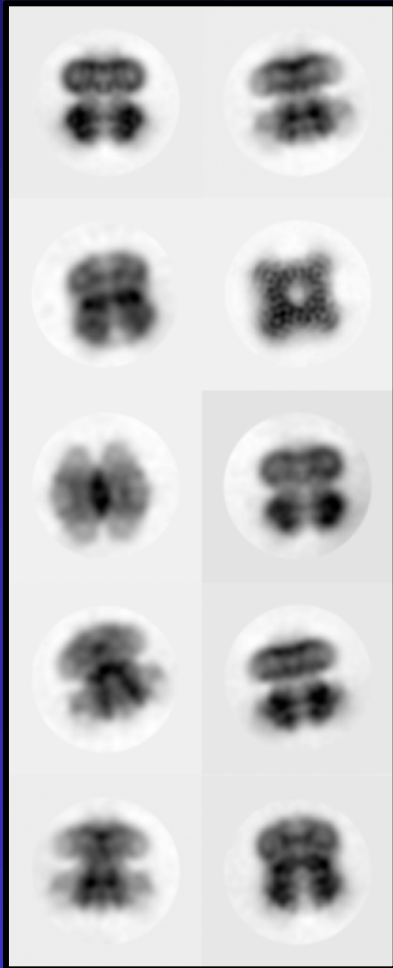
van Heel, 1987

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

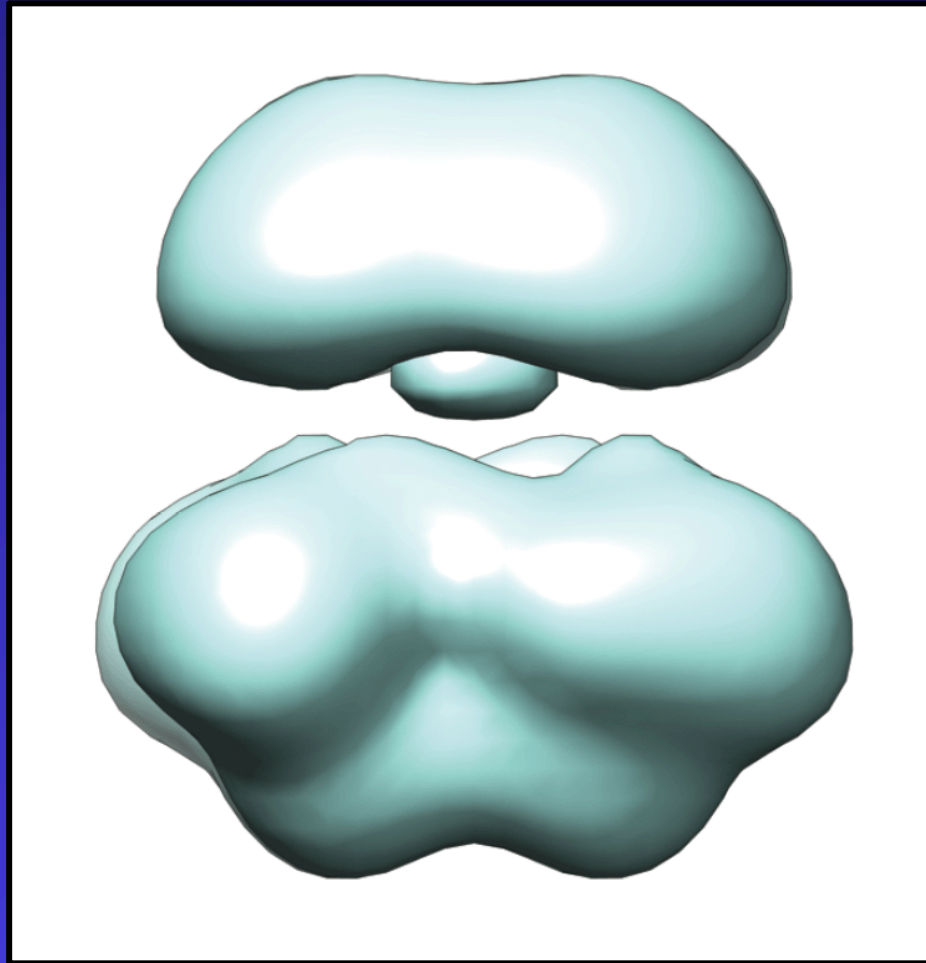


Serysheva *et al.*, 1995

Chicken Slo2.2 in the absence of Na^+



Class averages



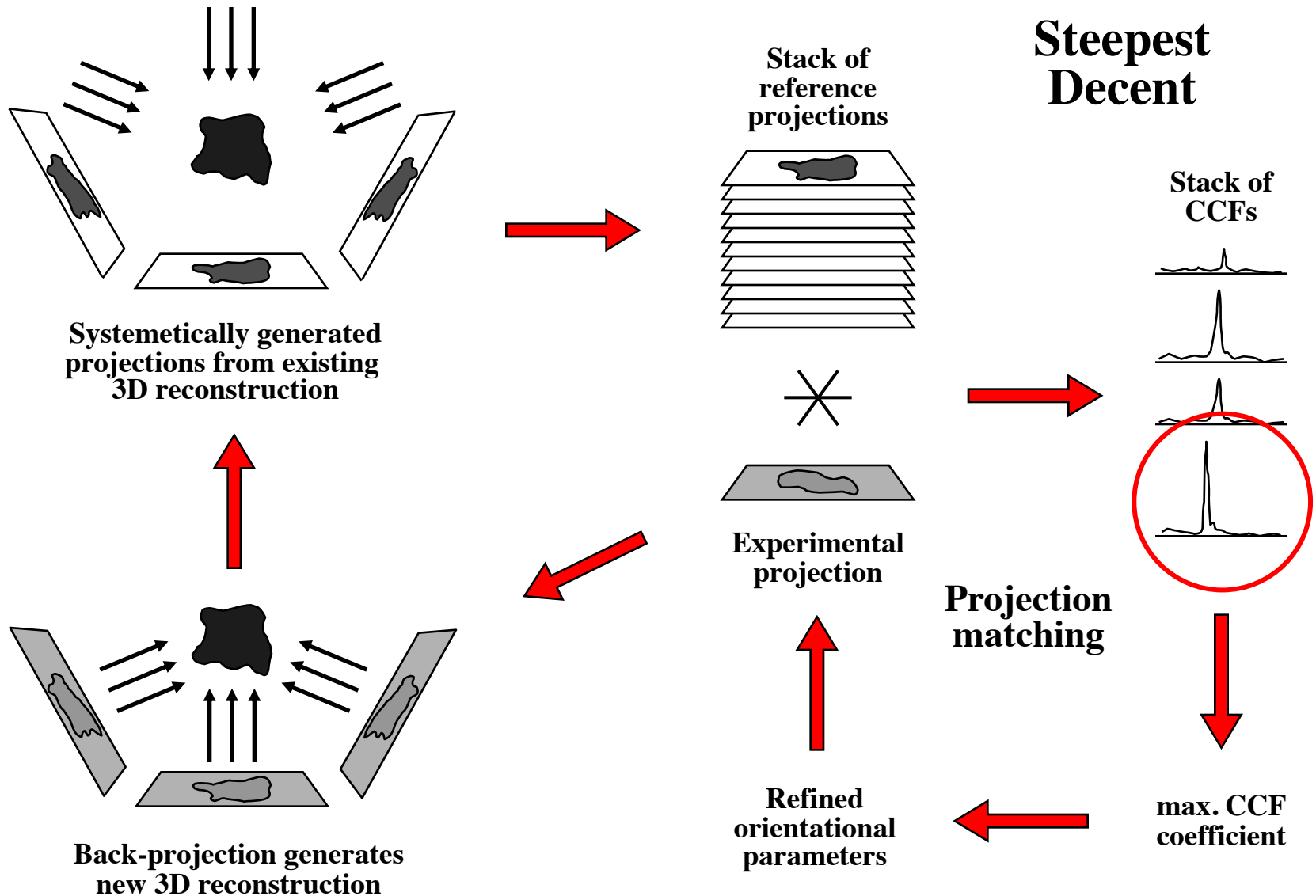
Initial model (obtained with VIPER)

VIPER

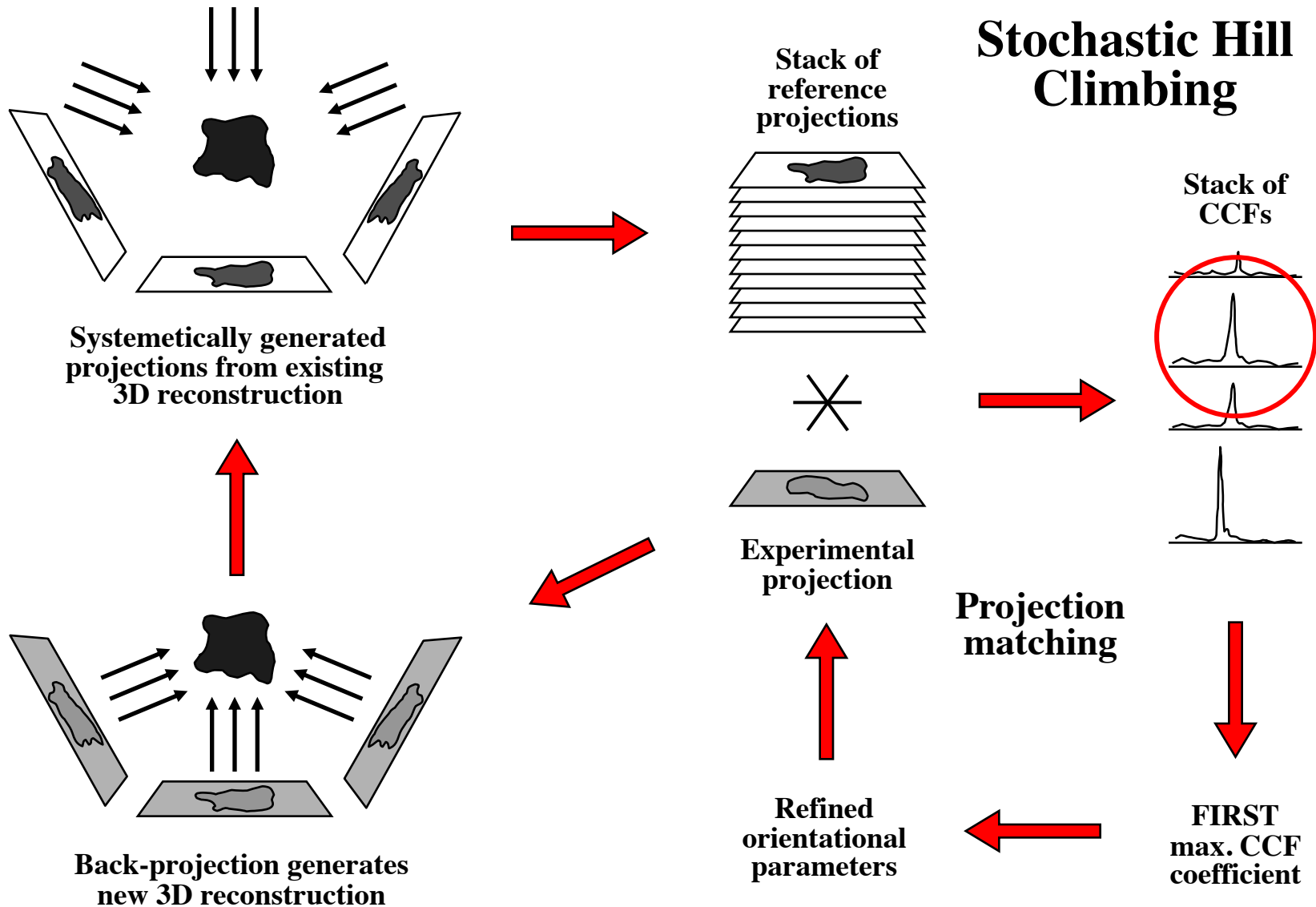
**Stochastic
Hill Climbing**

(initially introduced
in program SIMPLE)

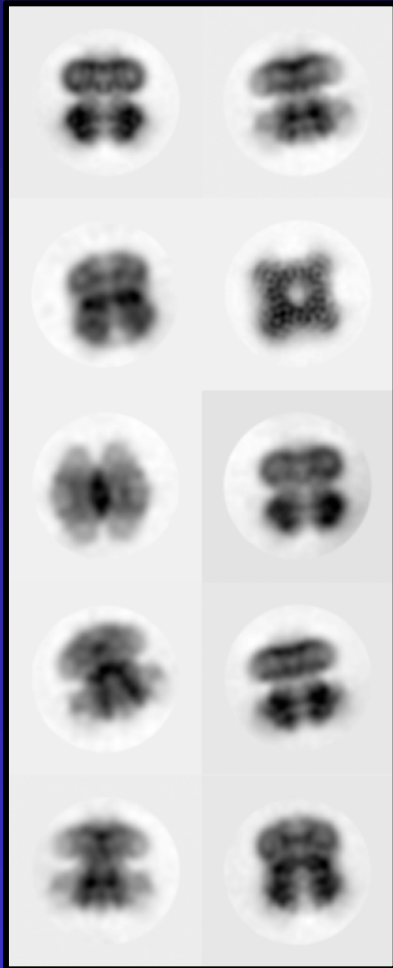
Angular refinement



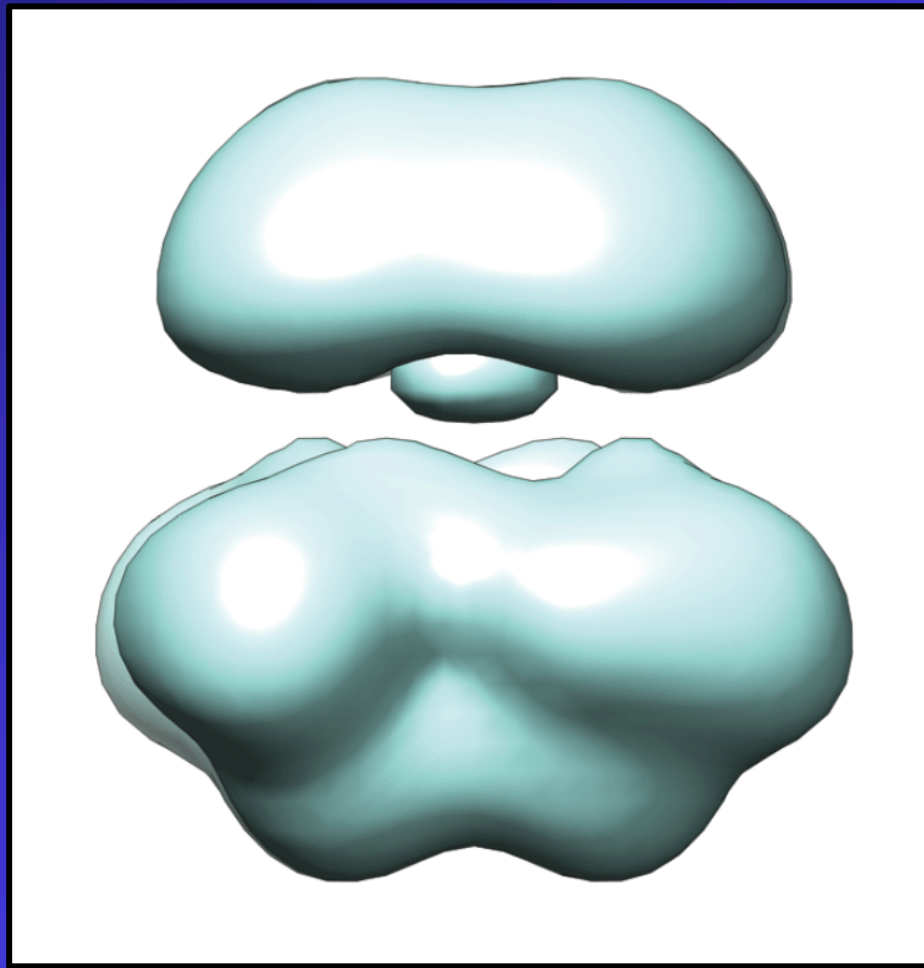
Angular refinement



Chicken Slo2.2 in the absence of Na⁺



Class averages



Initial model (obtained with VIPER)

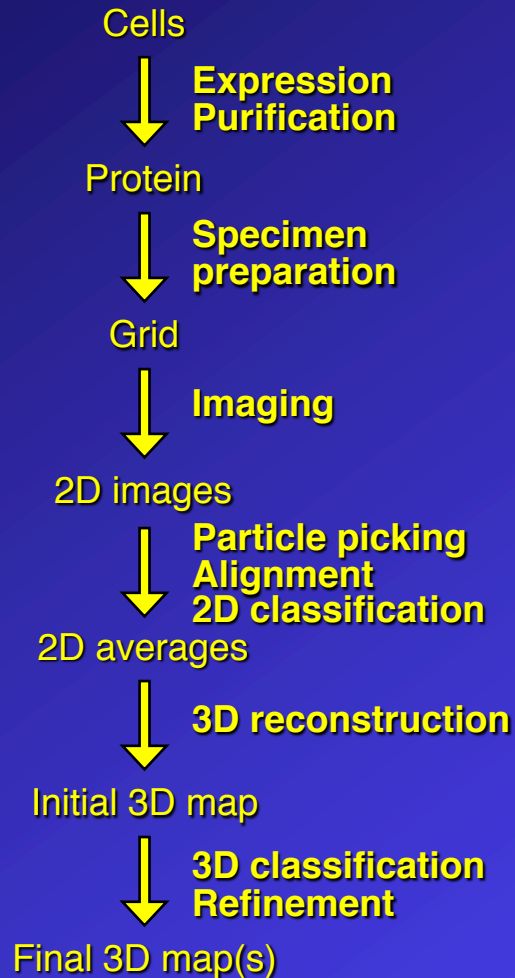
VIPER

**Stochastic
hill climbing**

Similar principles
as used in ISAC:
– stability and
reproducibility
assessments

cryoSPARC

Structure determination by single-particle EM



Potential issues:

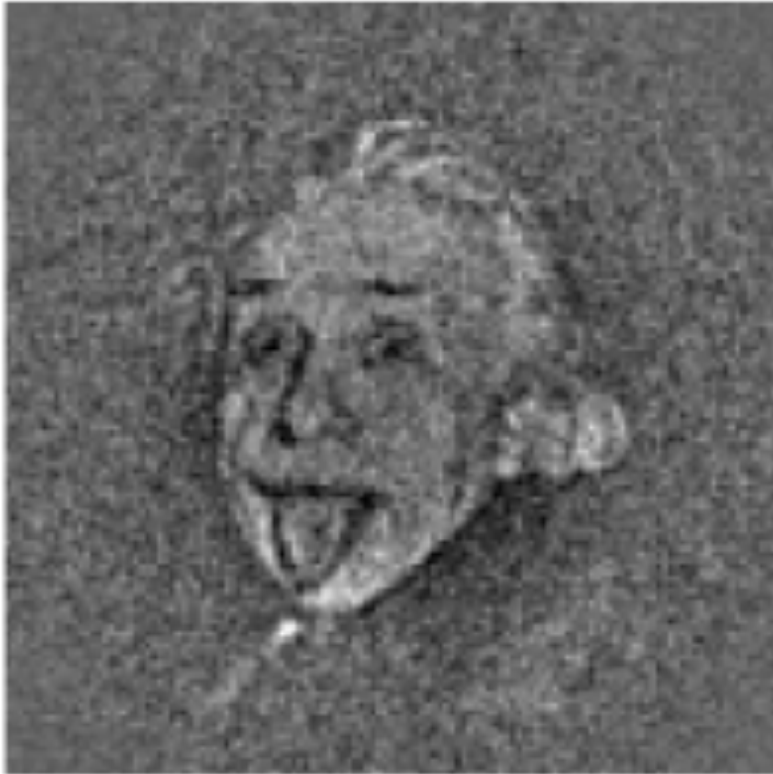
Reference bias

Overfitting

Resolution assessment

Structure determination by single-particle EM

Potential issues with density map



Model/reference bias

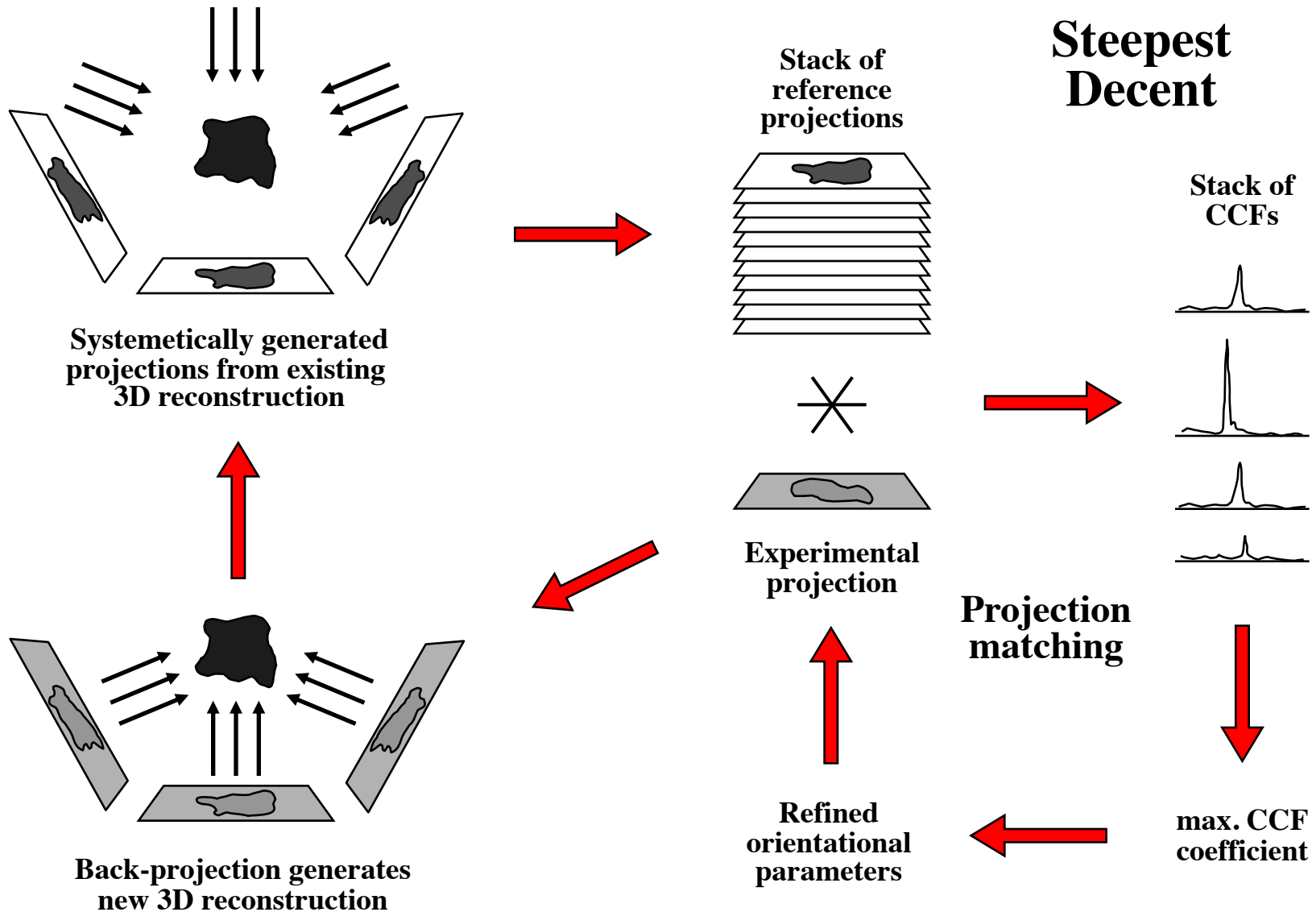
Average of 1,000 images containing pure white noise after alignment to an image of Albert Einstein

→ 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

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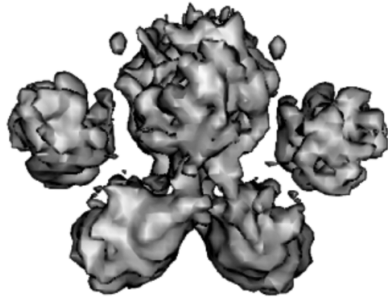
Over-fitting results in spurious high-resolution features due to alignment of noise

Shatsky *et al.* (2009) *J. Struct. Biol.* 166: 67-78

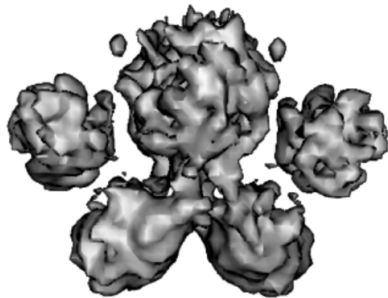
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Structure determination by single-particle EM

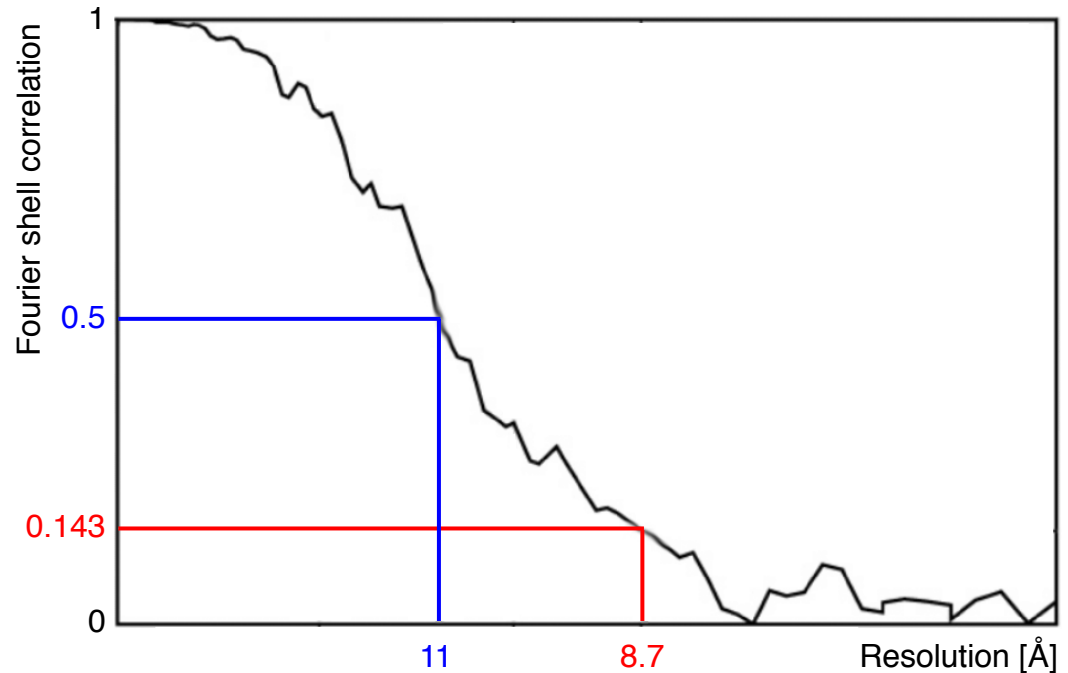
Resolution assessment



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



Maps have to be independent !



FSC = 0.5 Signal = Noise

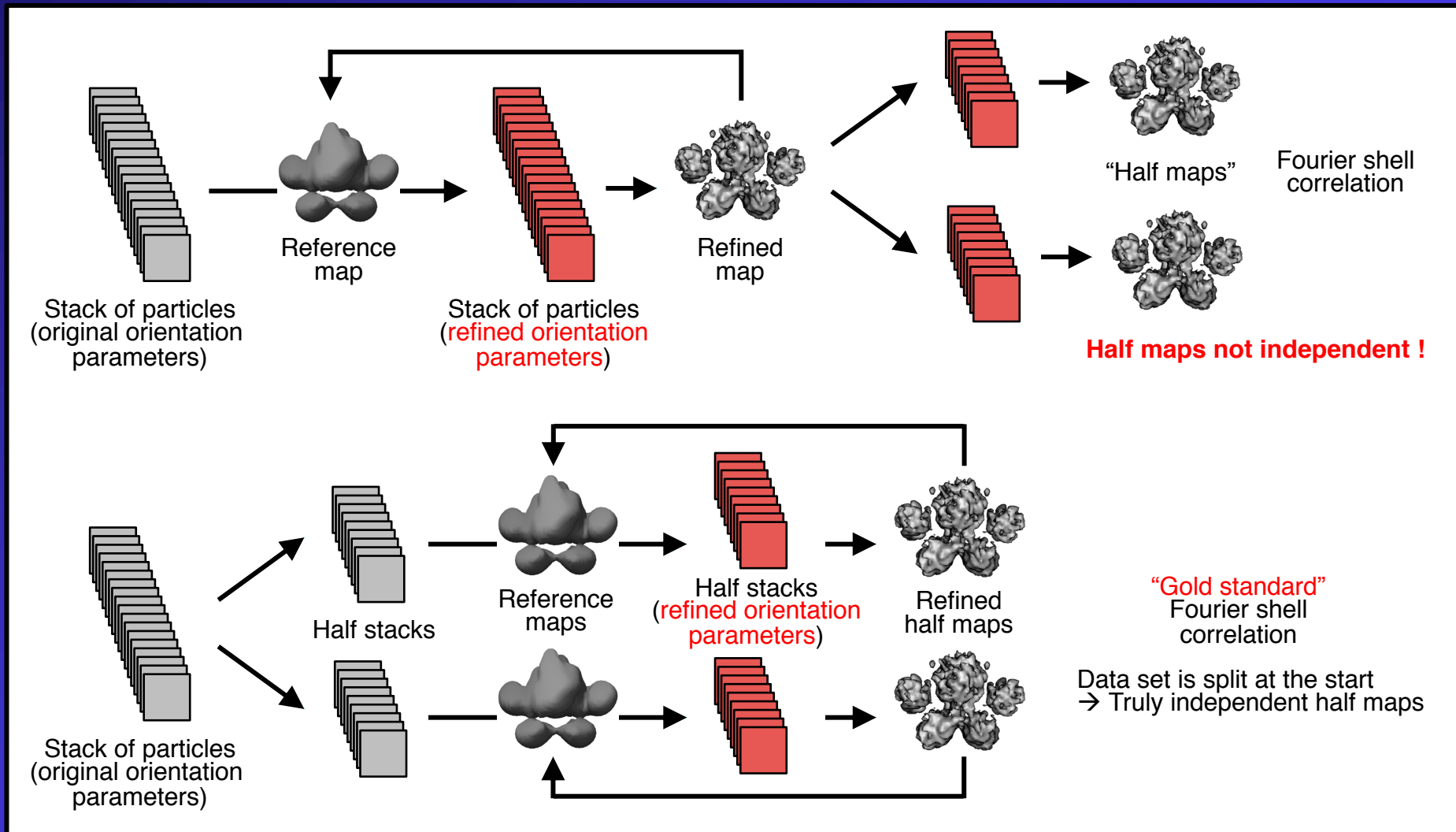
Böttcher *et al.* (1997) *Nature* 386: 88-91

FSC = 0.143 Phase error = 60°

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

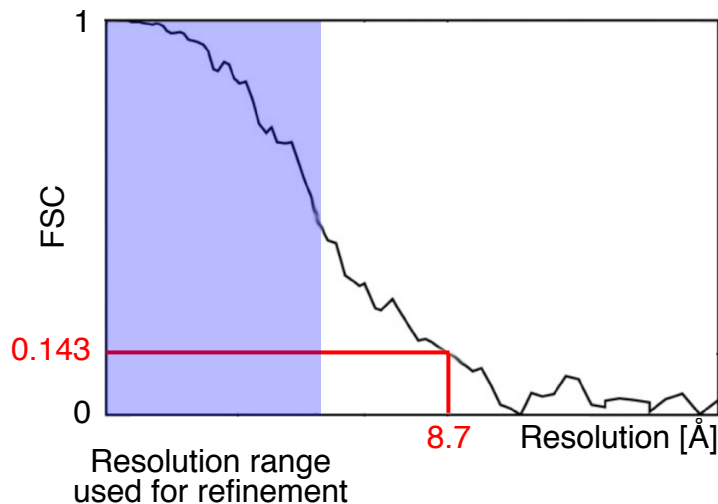
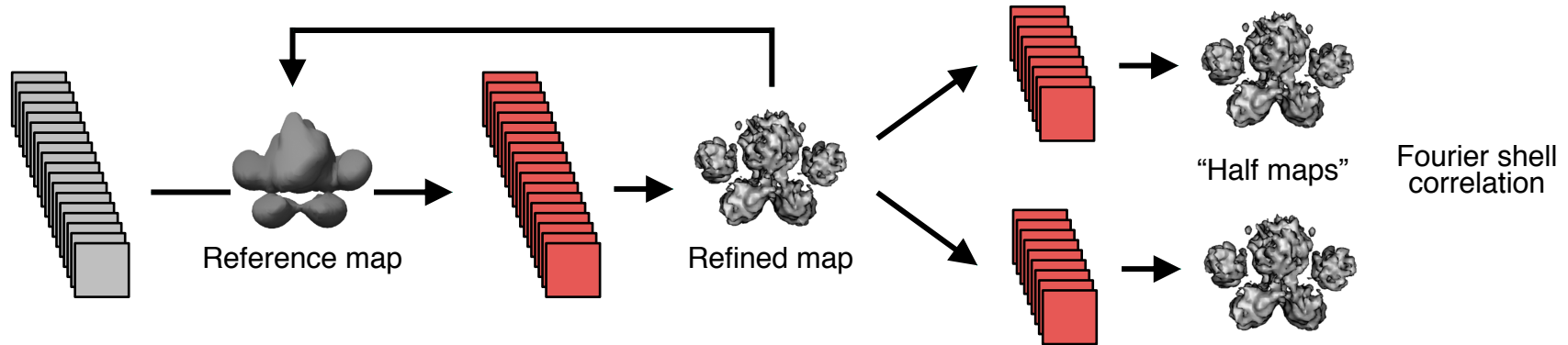
Structure determination by single-particle EM

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

What should be resolved ?

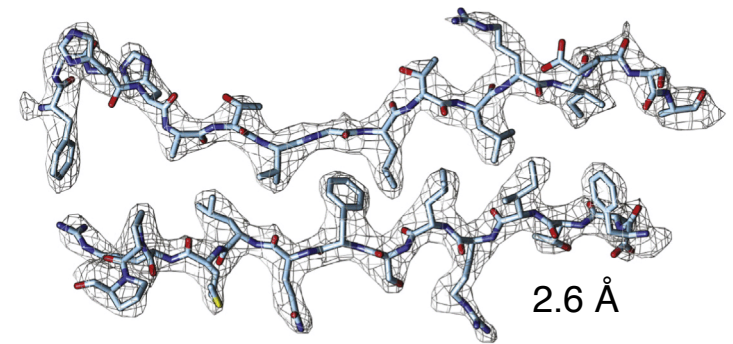
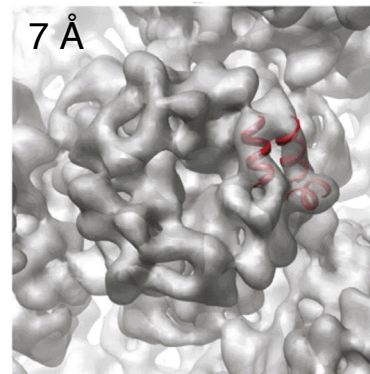
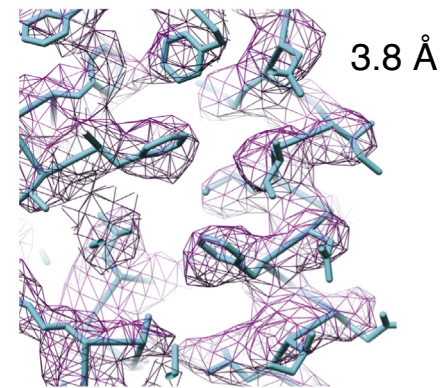
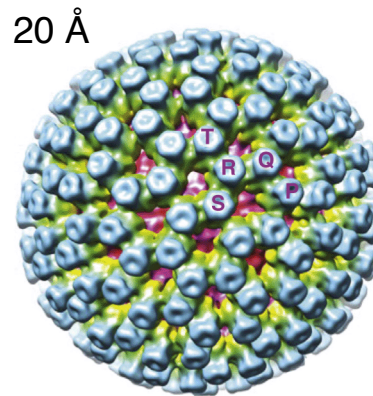
> 20 Å
protein envelope

~ 9-10 Å
 α -helices

< 4.8 Å
 β -sheets

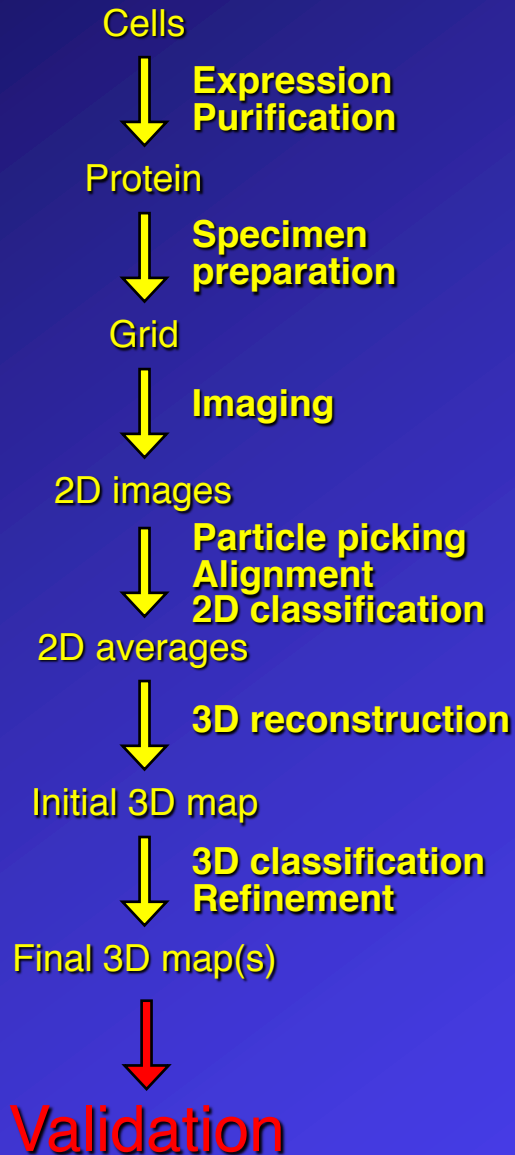
~ 4 Å
bulky side chains

Rotavirus double-layered particle

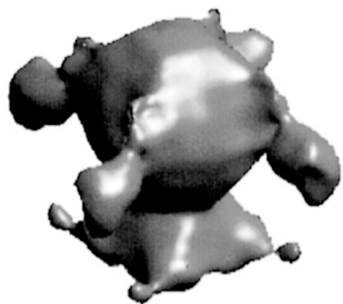
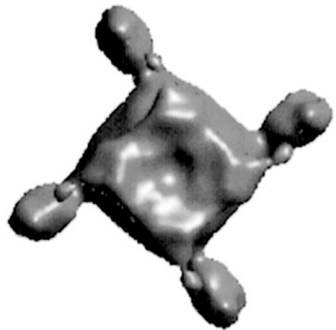


Rosenthal & Rubinstein (2015) *Curr. Opin. Struct. Biol.* 34: 135-144

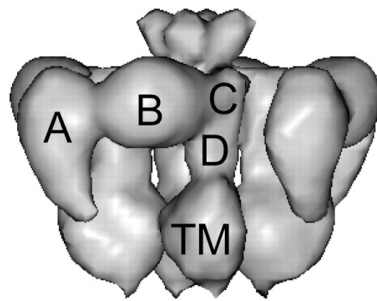
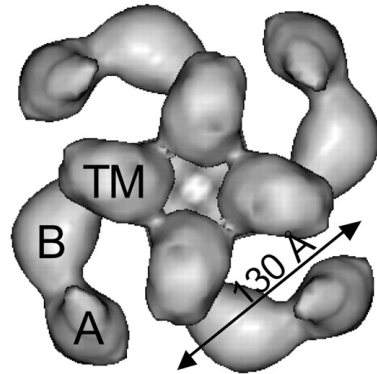
Structure determination by single-particle EM



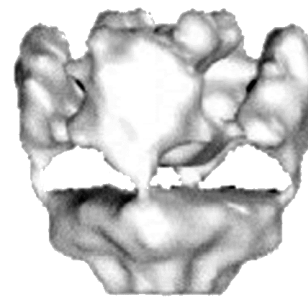
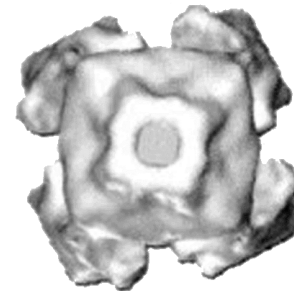
The issue: Structures of the IP3 receptor as determined by single-particle EM



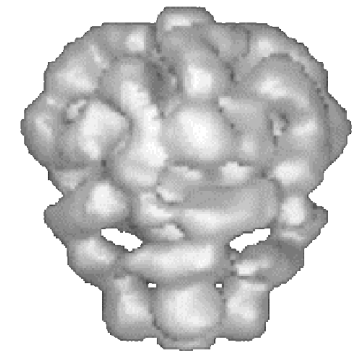
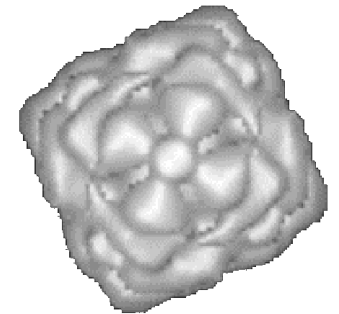
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2004

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

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

Map validation

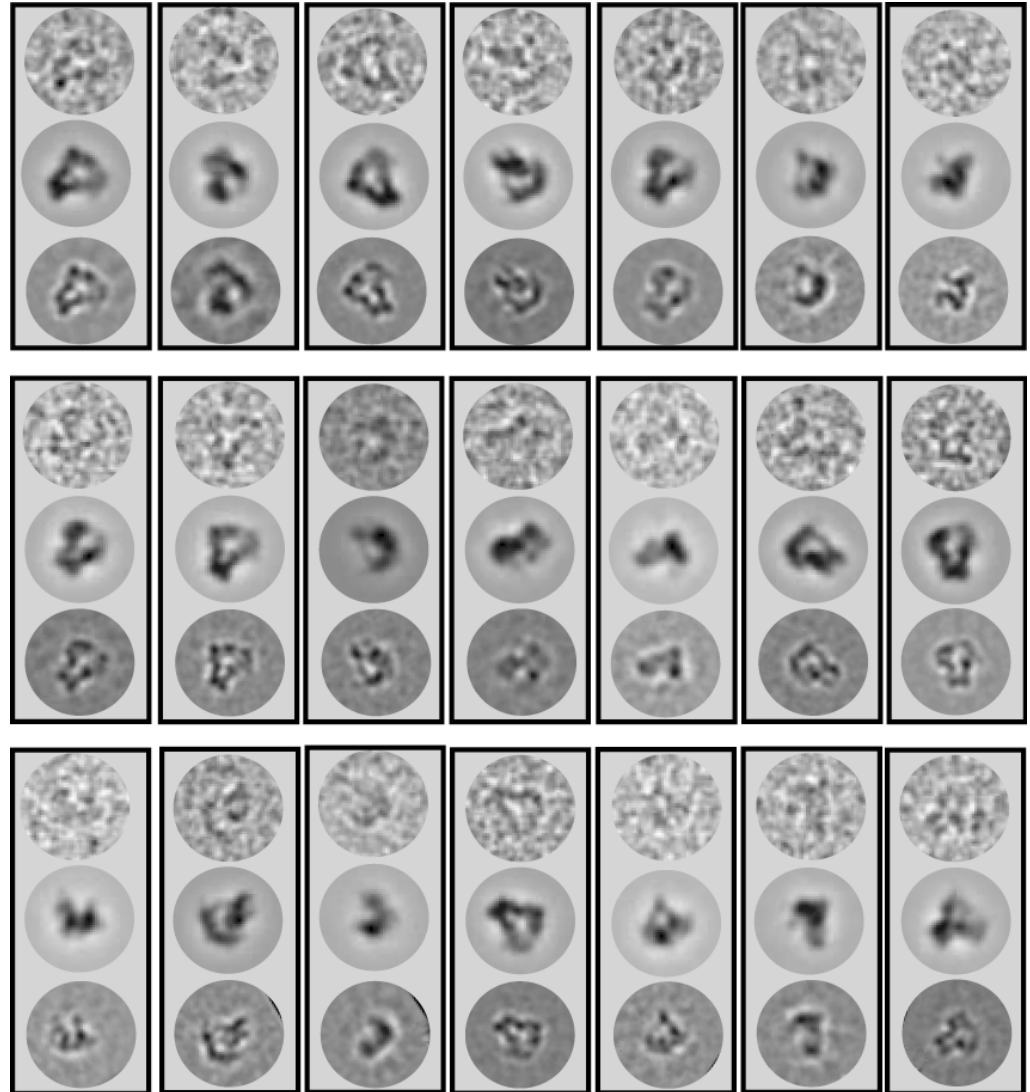
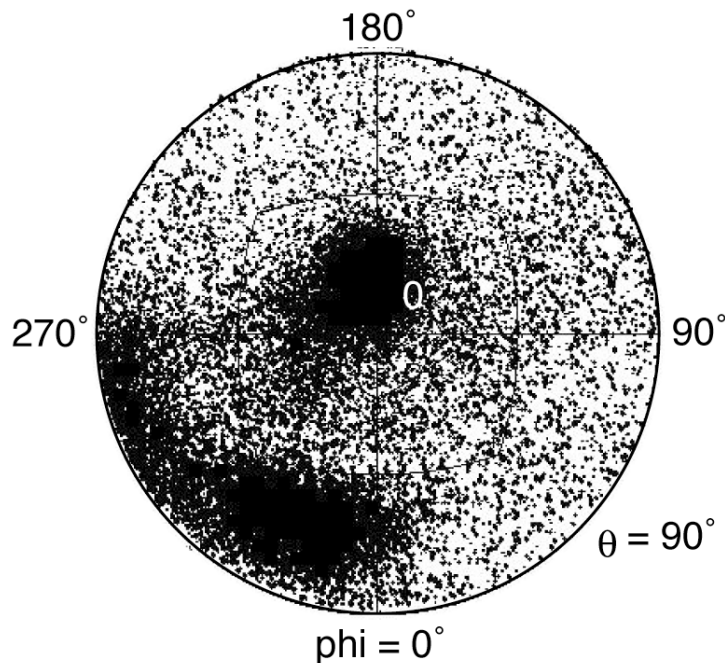
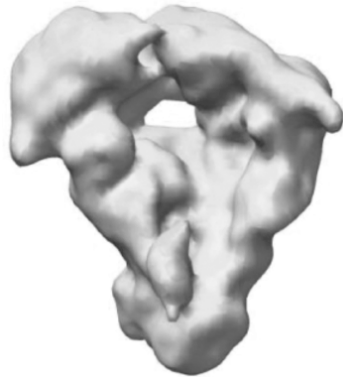
- Compare reference-free averages with projections

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

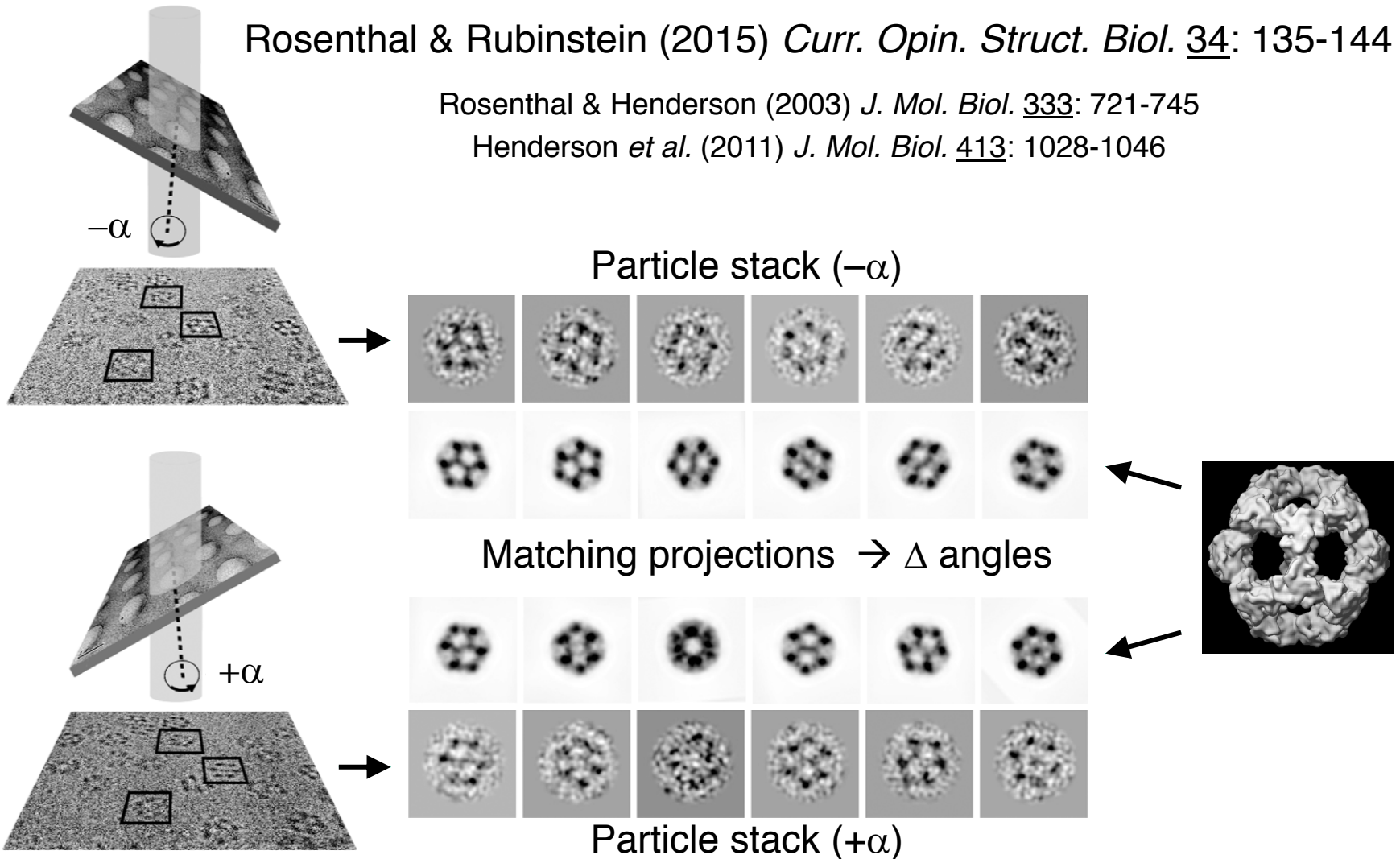
Map validation

Tilt-pair analysis

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

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

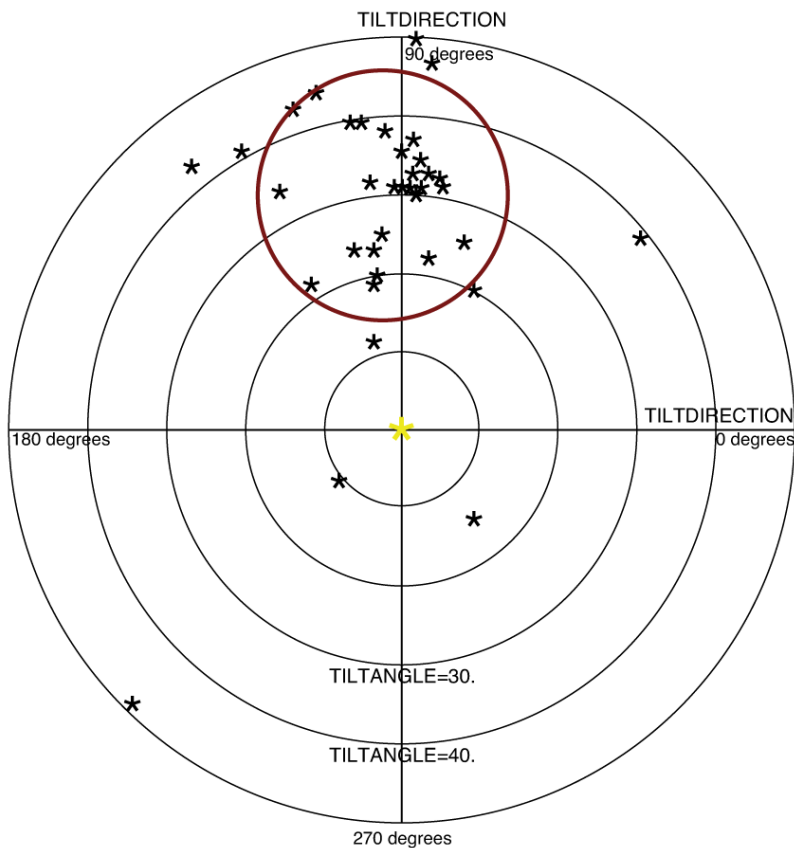
Henderson *et al.* (2011) *J. Mol. Biol.* 413: 1028-1046



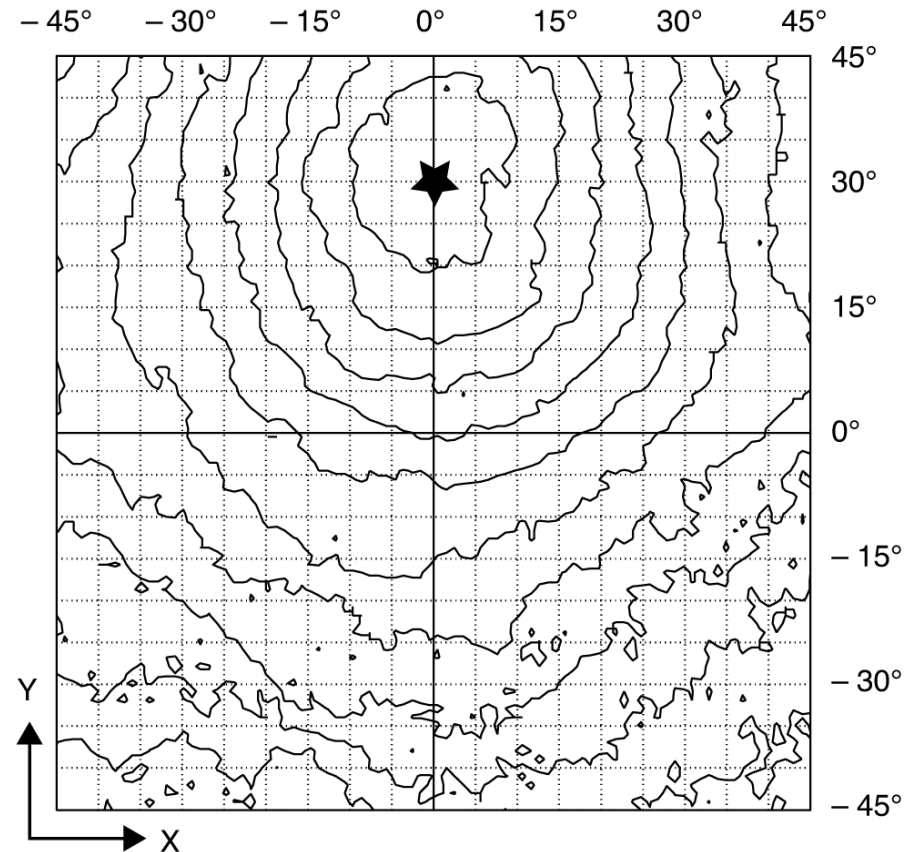
Map validation

Tilt-pair analysis

Tilt-pair parameter plot



Tilt-pair phase residual plot



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	I2	700	50	10	95	100/100	0.25	1.0
CAV	I2	255	2.7	1	45	62/82	2.5	3.5
70S ribosomes	C1	270×260	2.6	12	220	45/75	4.0	5.0
FAS	D3	260×220	2.6	2	44	59/95	4.0	6.0
PDH-E2CD	I1	280	1.6	1	50	62/94	3.0	4.0
<i>Thermus</i> V-ATPase	C1	250×140	0.6	1	50	54/80	10.0	16.0
Bovine F-ATPase	C1	250×140	0.6	1	29	52/79	20.0	25.0
DNA-PKcs	C1	150×120	0.47	14	108	44/81	15.0	17.0
β-Galactosidase	D2	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)

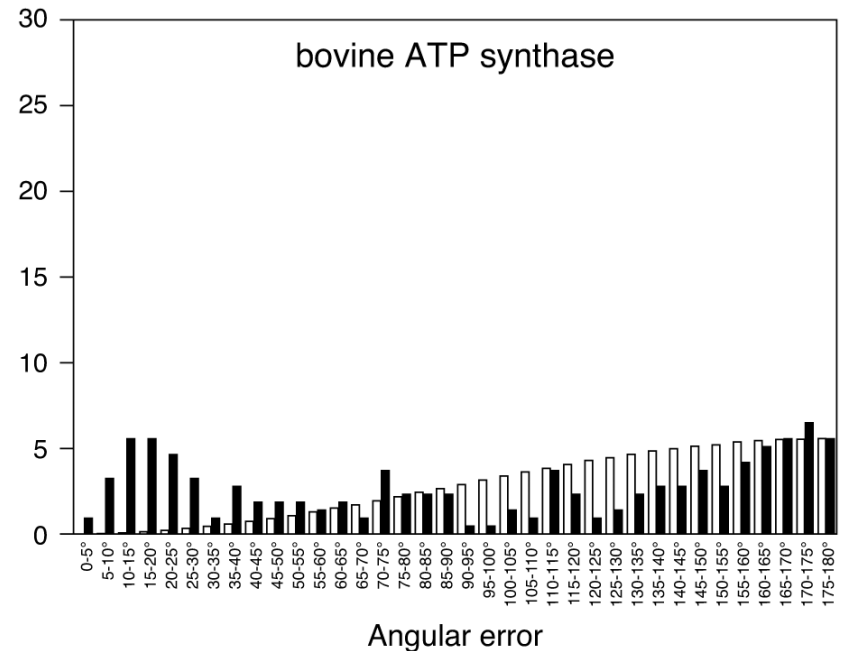
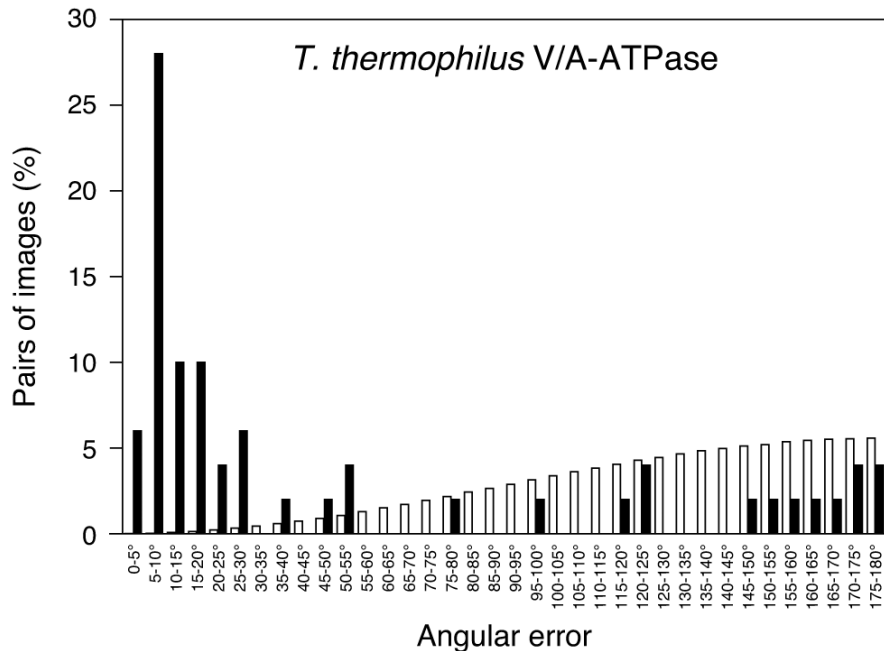
“If less than 60% of particles show a single cluster, the basis for poor orientation parameters should be investigated”

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

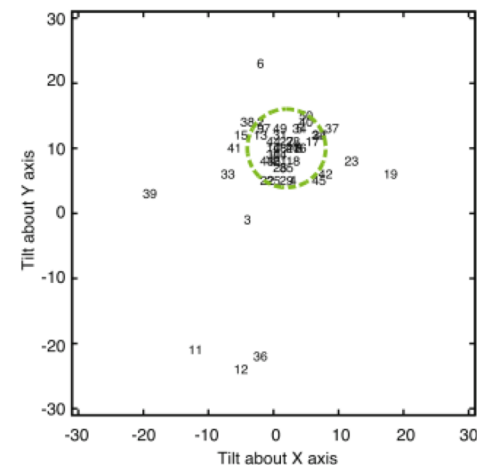
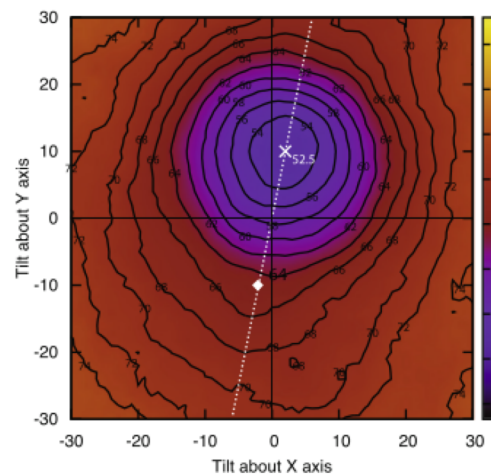
Stack 1 (max 300 particles)
[Browse...](#) stack1.mrc

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](#)

Map validation

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

Tilt-pair validation server

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

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- FTP archive
- Deposit EM map/model
- EMDB data model

Tilt pair validation server

Welcome to the PDBe tilt pair validation server!

Tilt-pair validation analysis (Rosenthal and Henderson, 2003) can be used to assess the accuracy of initial angle assignment in single-particle processing. To perform this analysis you need to collect two corresponding sets of particle images - one untitled and the other tilted, then upload the stacks of images along with a 3D reconstruction based on the untitled images. This server is based on the [Tilt-pair server](#) developed at MRC National Institute for Medical Research (Wasilewski and Rosenthal, 2014), and we thank Sebastian Wasilewski and Peter Rosenthal for their help in developing and testing the current server.

You may upload map files in MRC or CCP4 format, and parameter files (containing Euler angles for individual particles) in Spider or Frealign format. We have some test data sets that you can use to try out the service [here](#). We are still developing the server and appreciate your [feedback](#)!

Map (3D volume): no file selected

Untitled stack: no file selected

Orientation parameters for stack 1: no file selected

Tilted stack: no file selected

Pixel size (Å):

Mask radius (pixels):

Tilt search range (degrees):

Resolution range (low to high; Å):

Email address:

Job name:

Perform CTF correction? ☐

PDBe is a member of EMBL-EBI, PDB, and EMDataBank

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Overview
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Funding
Background

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

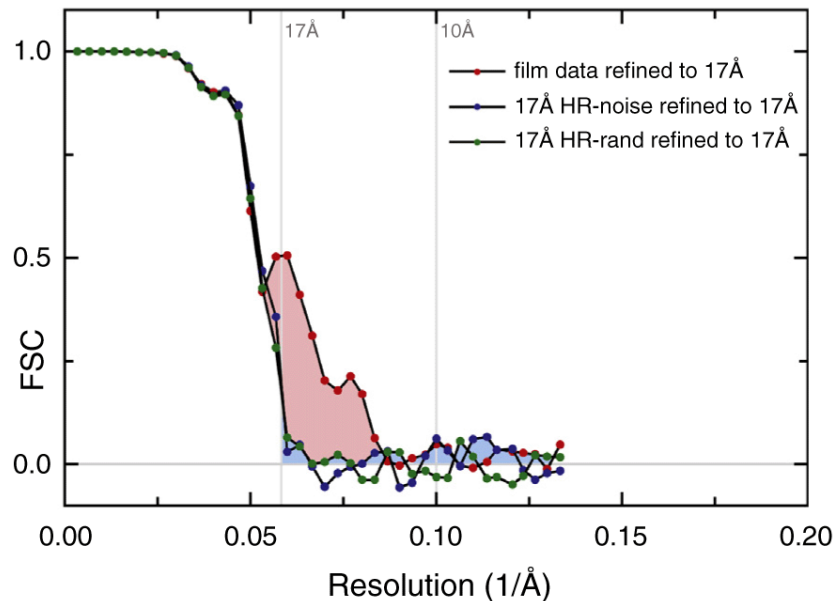
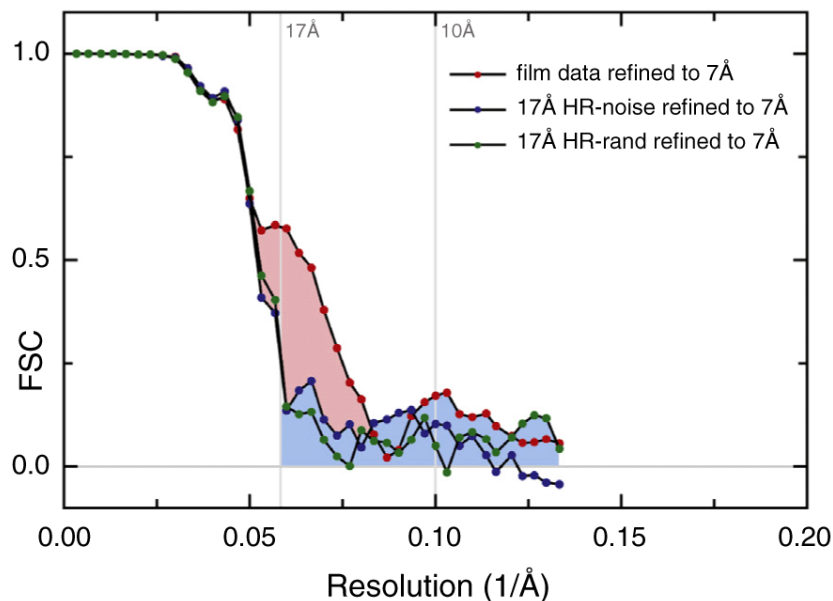
- Do single-particle reconstruction / refinement
- Determine resolution (FSC)
- Take raw data, randomize phases beyond which FSC_T falls below a threshold (75 or 80%)
- Redo the same analysis and recalculate FSC curve
- Any signal in region of randomized phases indicates issues with noise alignment in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

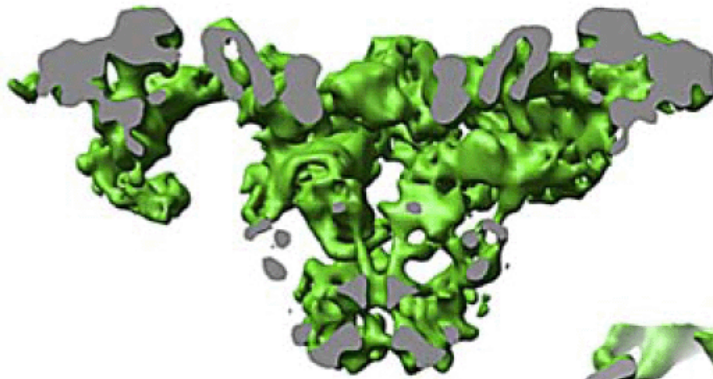
Map validation

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- Randomize phases
 - excellent, but not commonly used
- Appearance of expected secondary structure elements

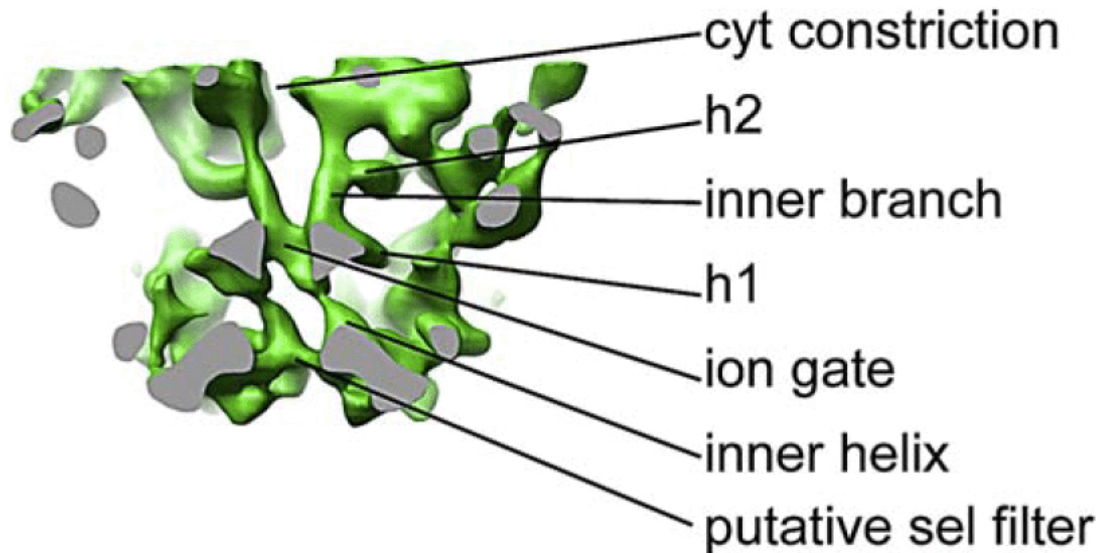
Henderson *et al.* (2012) *Structure* 20: 205-214

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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- Evaluate with published information

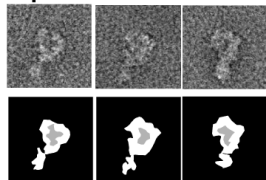
Henderson *et al.* (2012) *Structure* 20: 205-214

Map validation

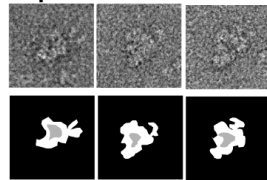
Evaluation with published information

Anaphase promoting complex

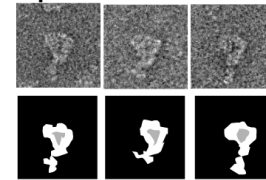
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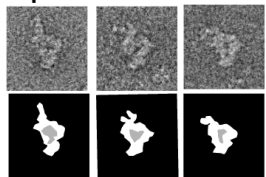
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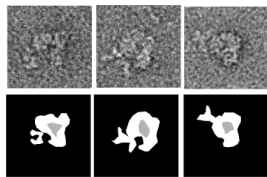
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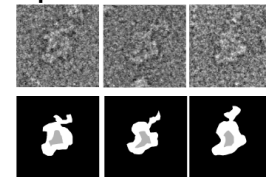
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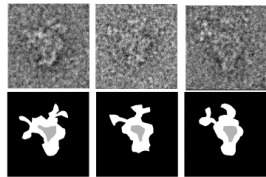
Hcn1



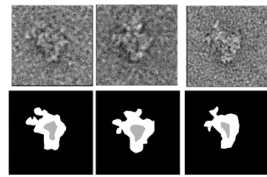
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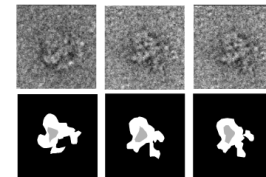
Cut9



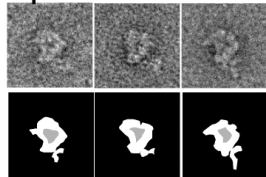
Nuc2



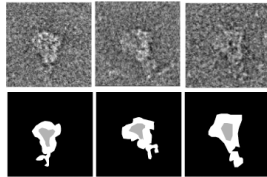
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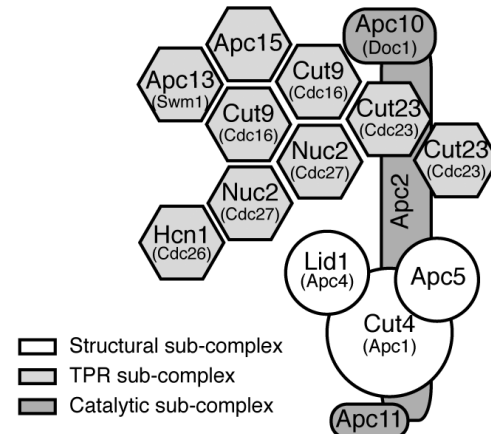
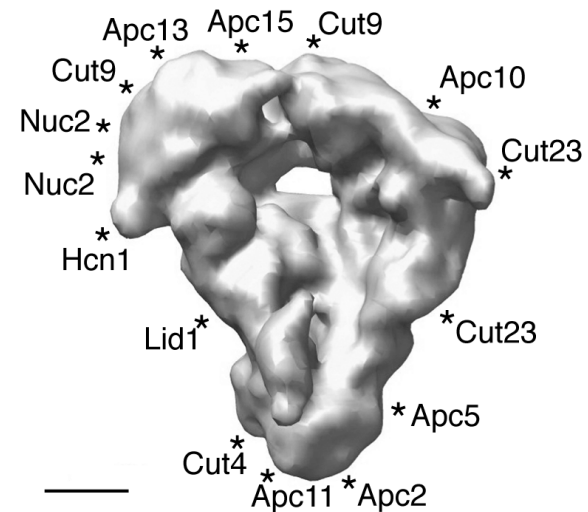
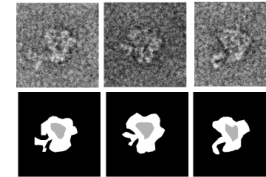
Apc5



Cut4



Lid1



- Structural sub-complex
- TPR sub-complex
- Catalytic sub-complex

Map validation

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 - 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

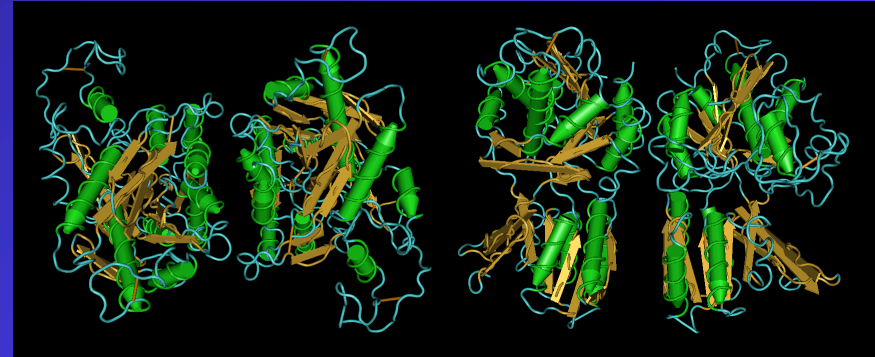
Henderson *et al.* (2012) *Structure* 20: 205-214

Map validation

Docking of atomic models

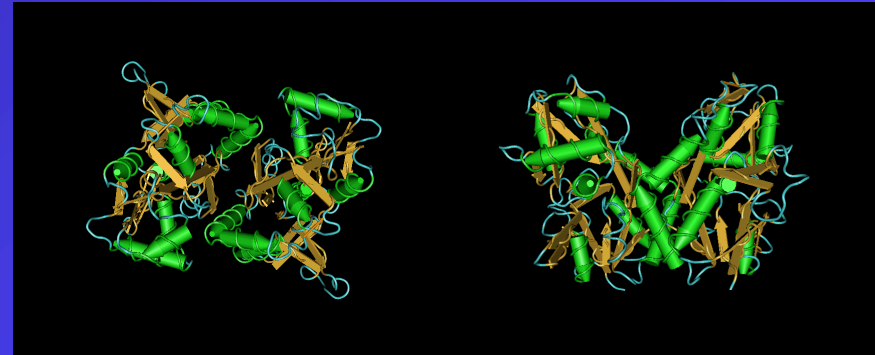
mGluR1

Kunishima et al. 2000
(K. Morikawa)



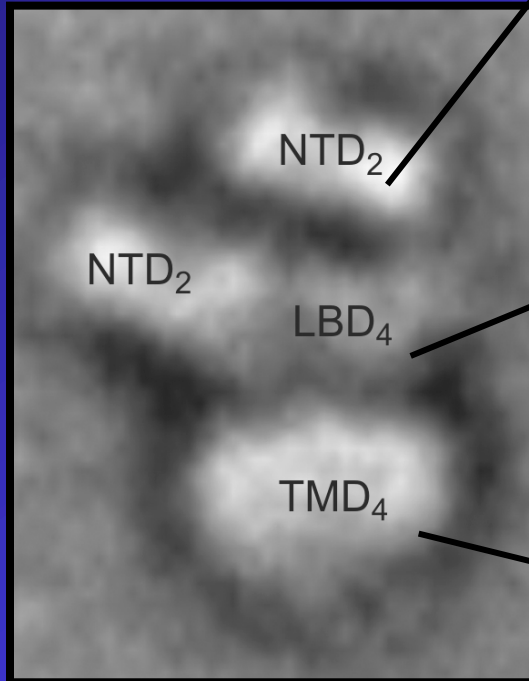
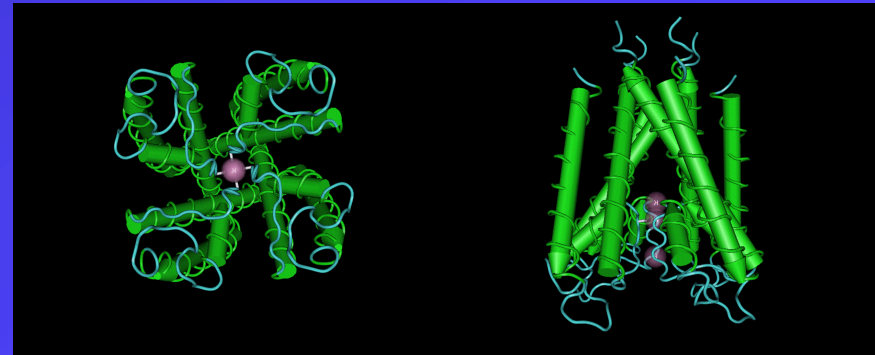
GluR2

Armstrong et al. 2000
(E. Gouaux)



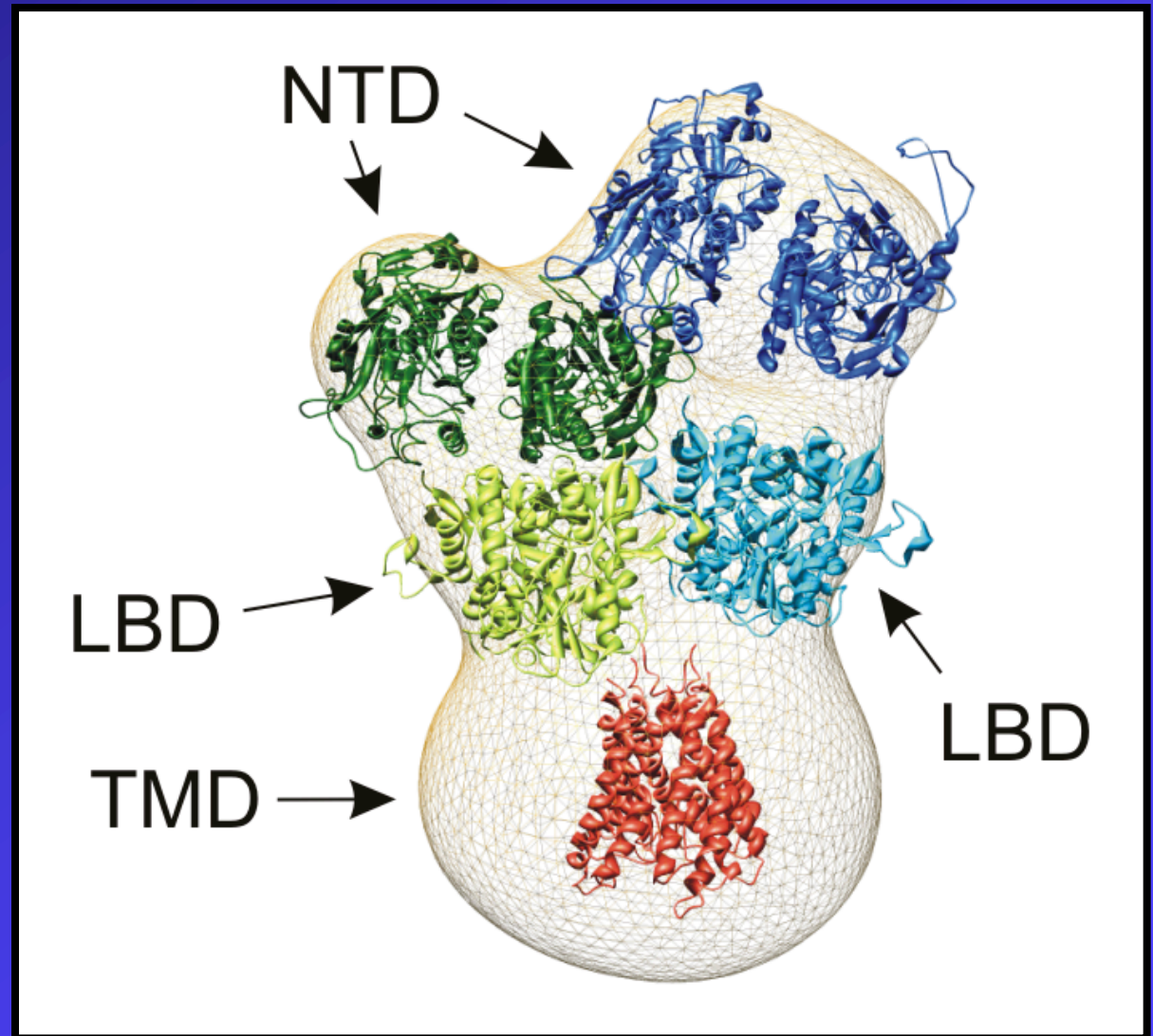
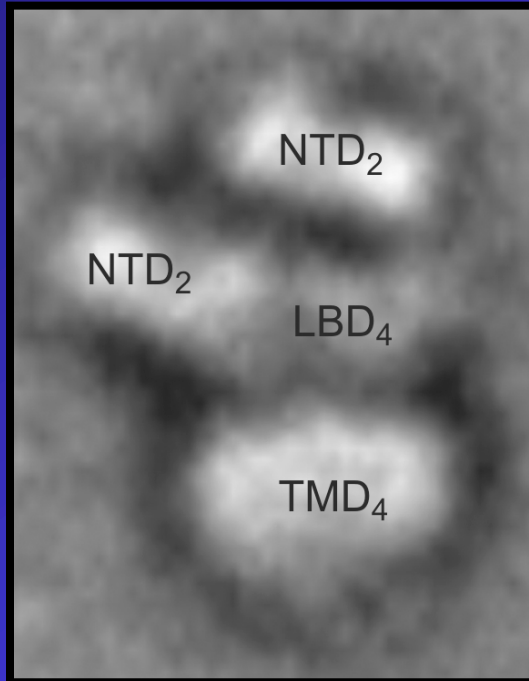
KcsA

Doyle et al. 1998
(R. MacKinnon)



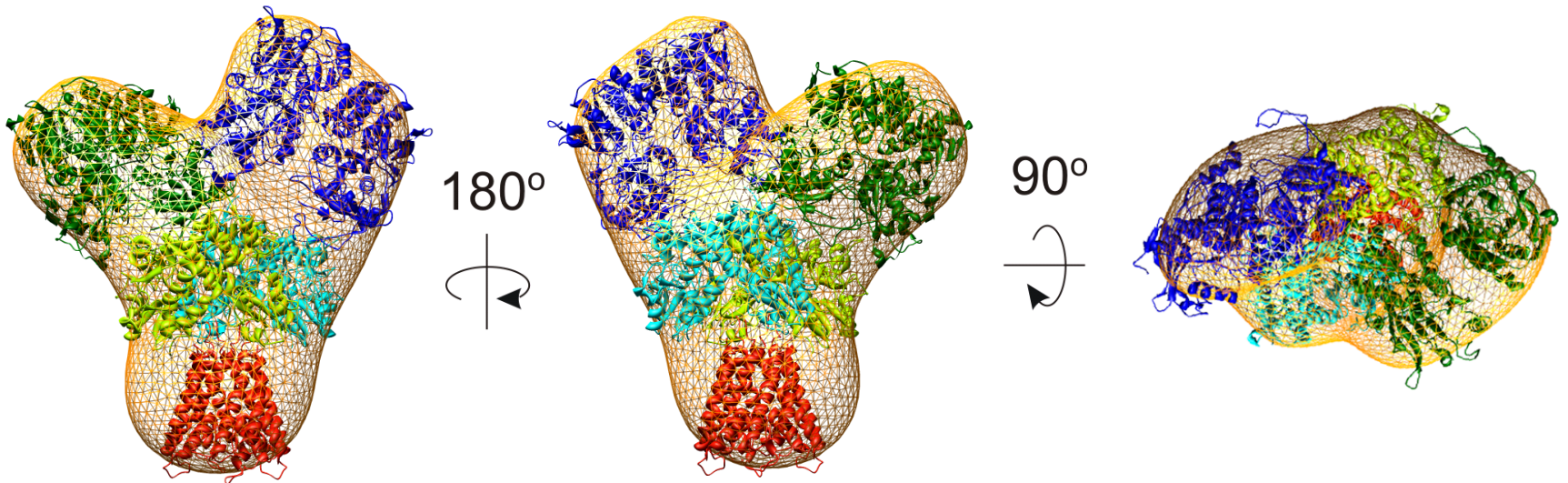
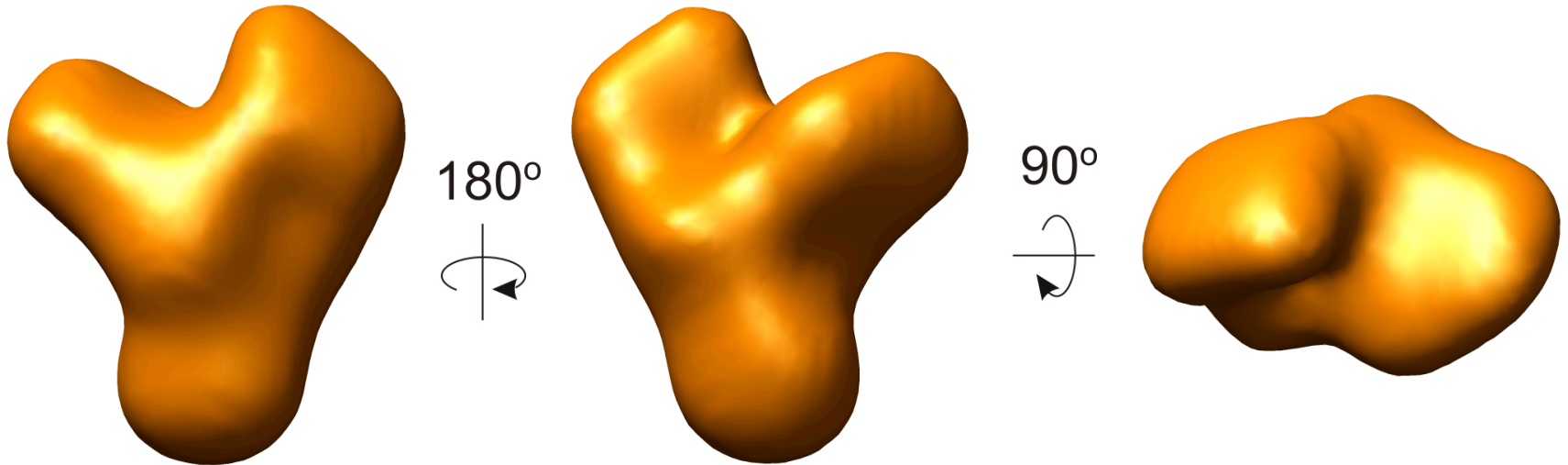
Map validation

Docking of atomic models



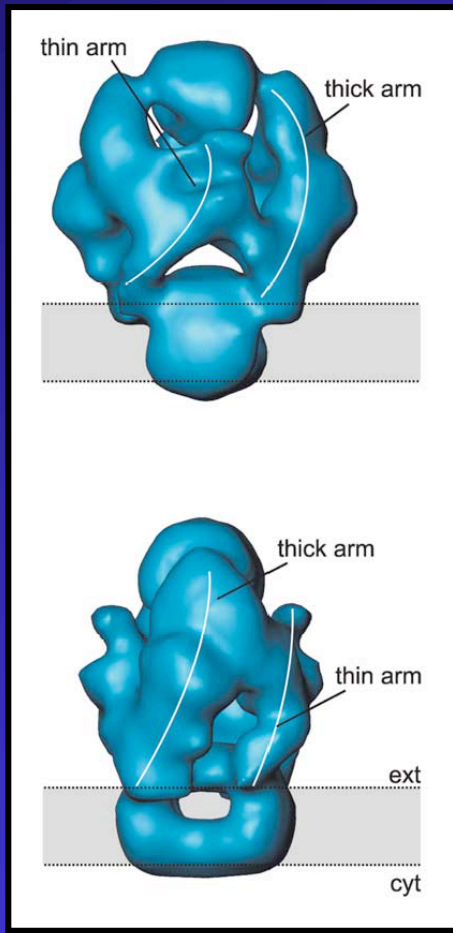
Map validation

Docking of atomic models

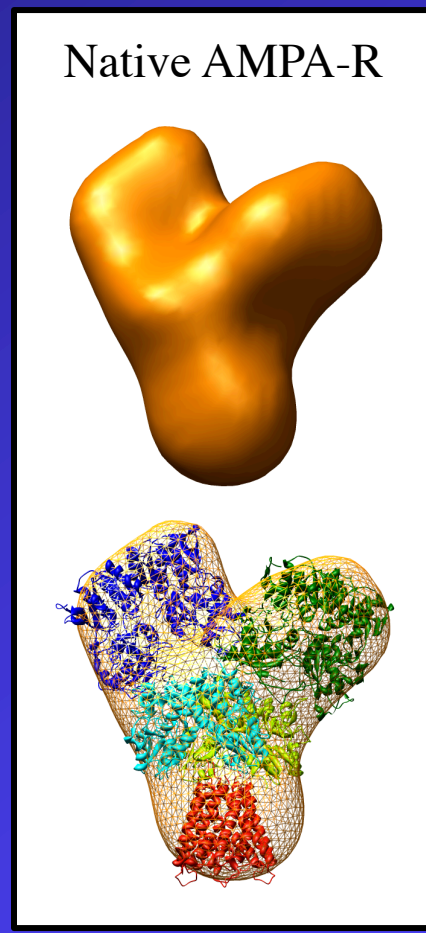


Map validation

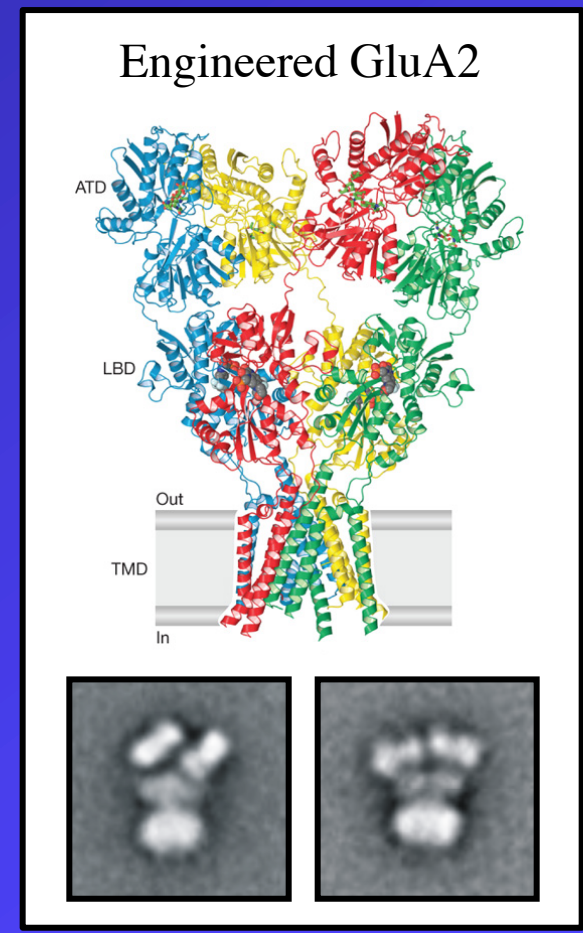
Docking of atomic models



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



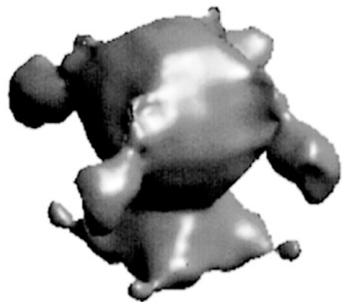
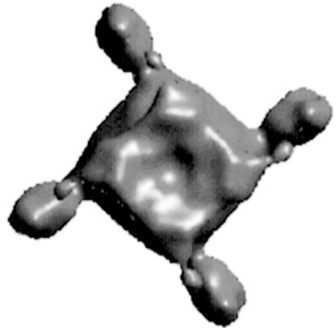
Nakagawa *et al.* (2006)
Biol. Chem. 387: 179-187



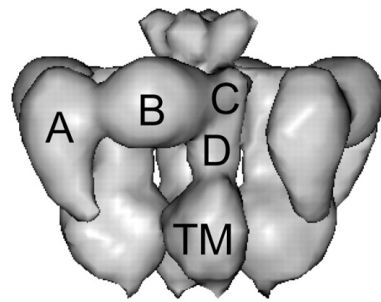
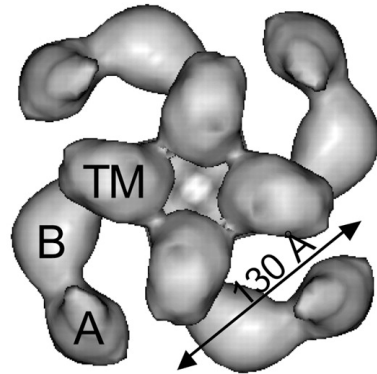
Sobolevsky *et al.* (2009)
Nature 462: 745-758

Map validation - IP3 receptor

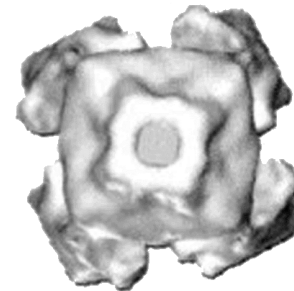
Different maps of the IP3 receptor



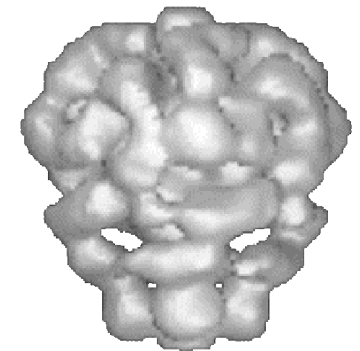
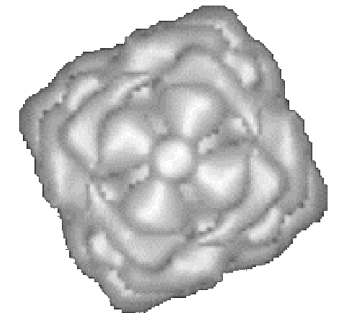
Jiang *et al.*,
2002



Serysheva *et al.*,
2003



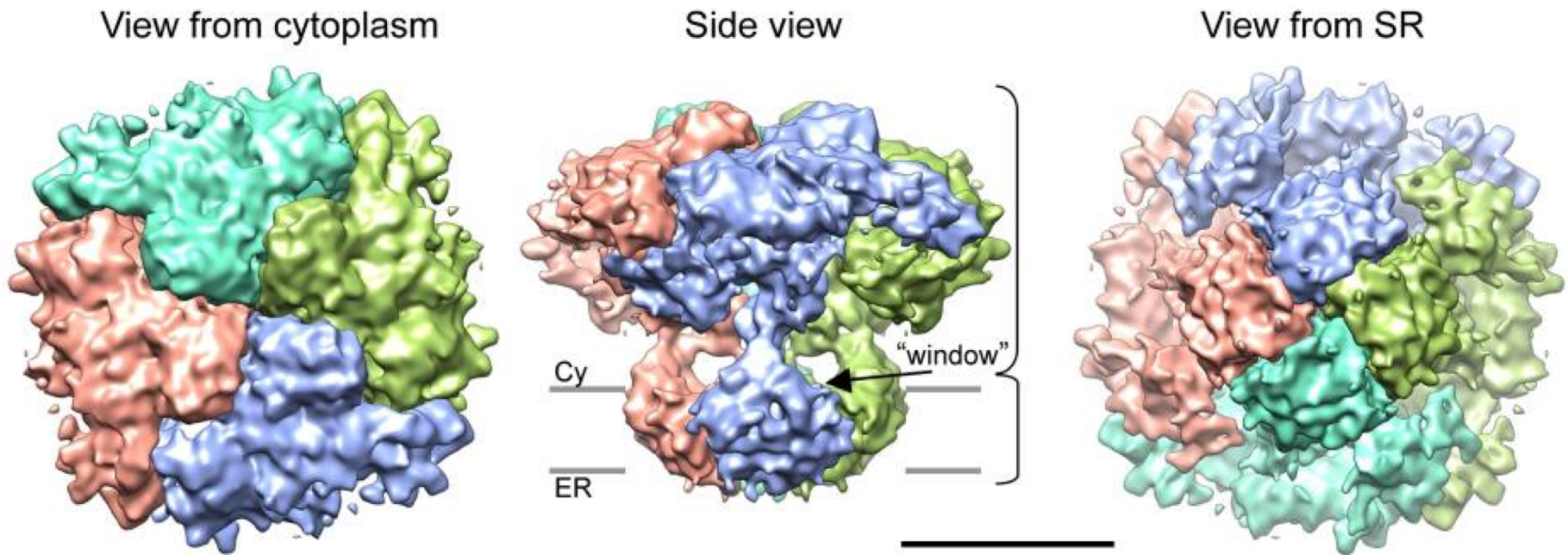
Jiang *et al.*,
2003



Sato *et al.*,
2004

Map validation - IP3 receptor

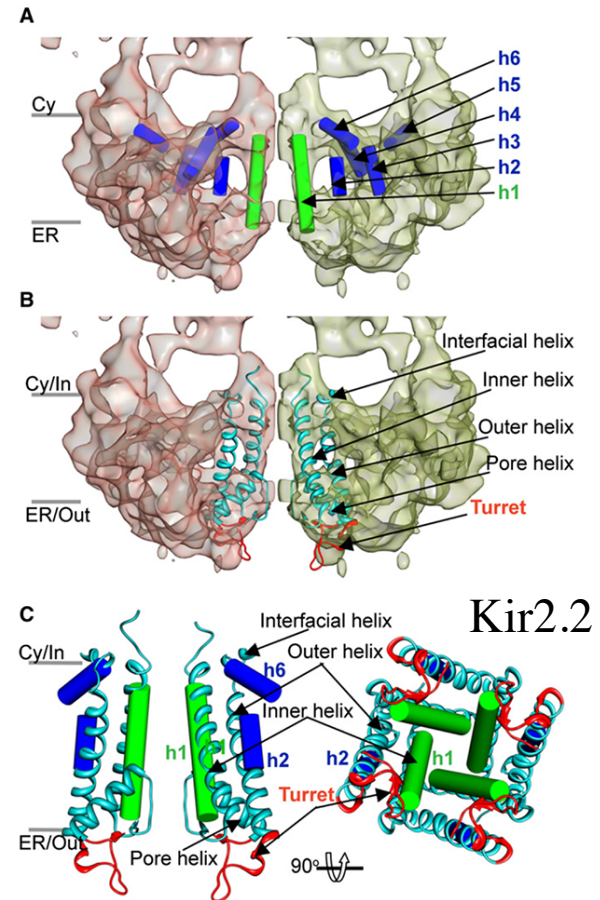
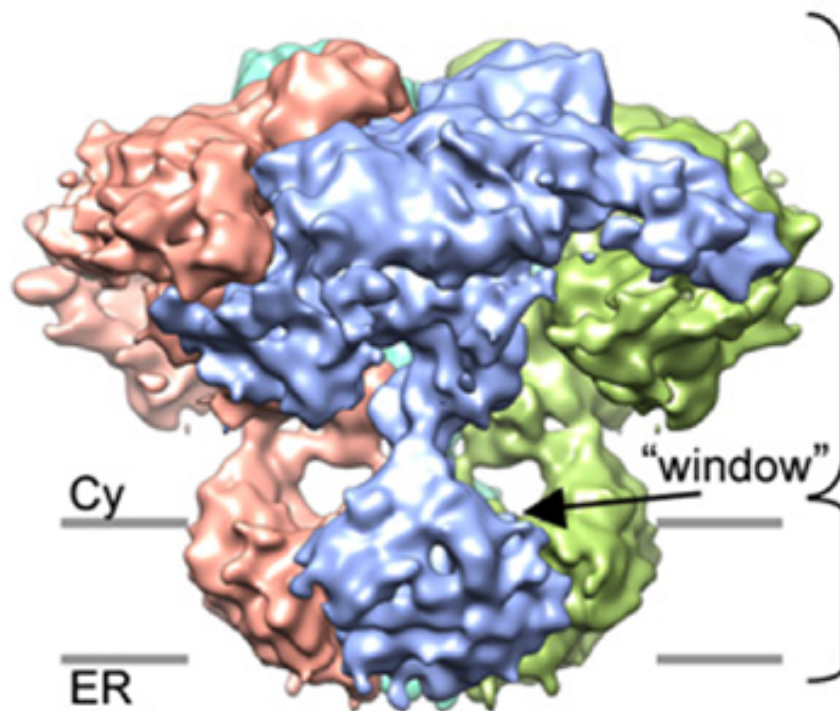
New density map in 2011 at 11 Å resolution



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

Map validation - IP3 receptor

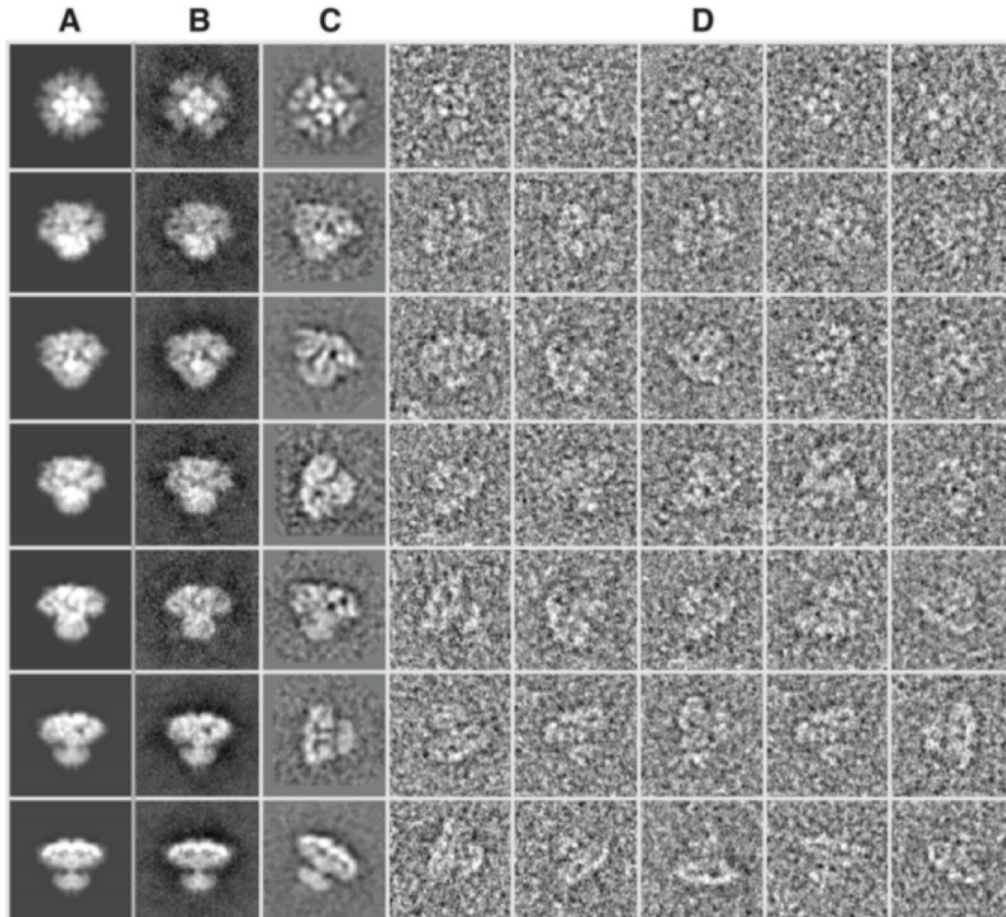
Expected secondary structure elements



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

Map validation - IP3 receptor

Comparison of reference-free averages with projections

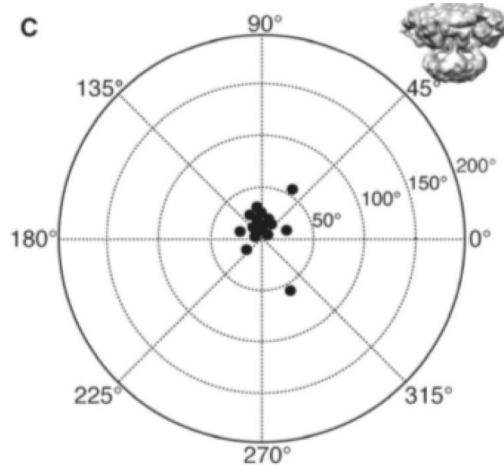


- A: Map projection
- B: Reference-based class average
- C: Reference-free class average
- D: Selected particles

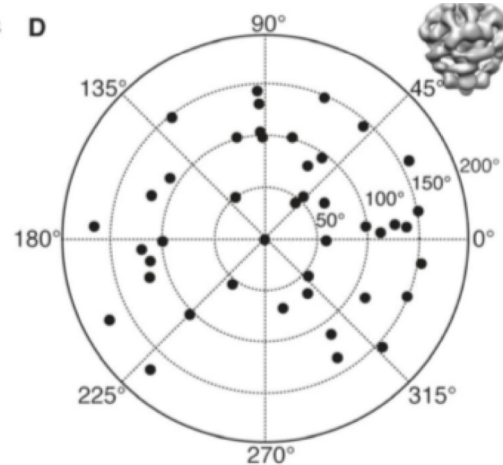
Map validation - IP3 receptor

Tilt pair test

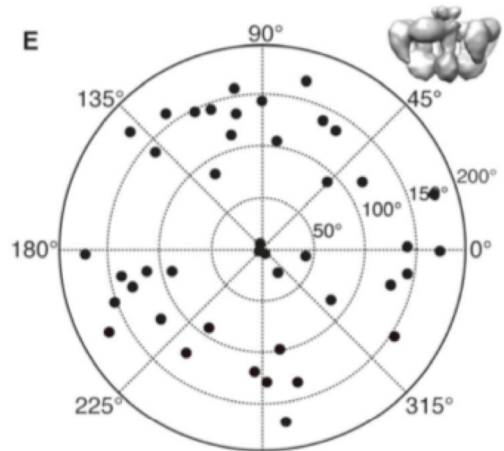
Ludtke *et al.*
2011



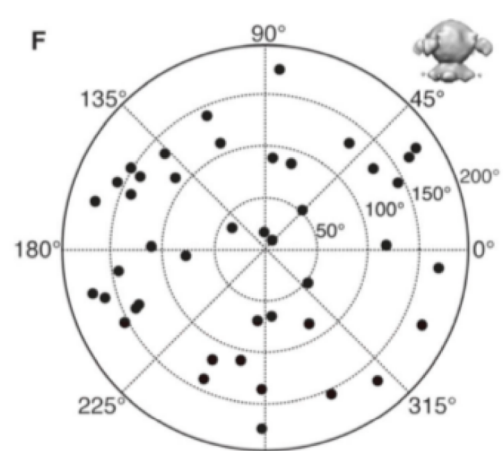
Sato *et al.*
2004



Serysheva *et al.*
2003



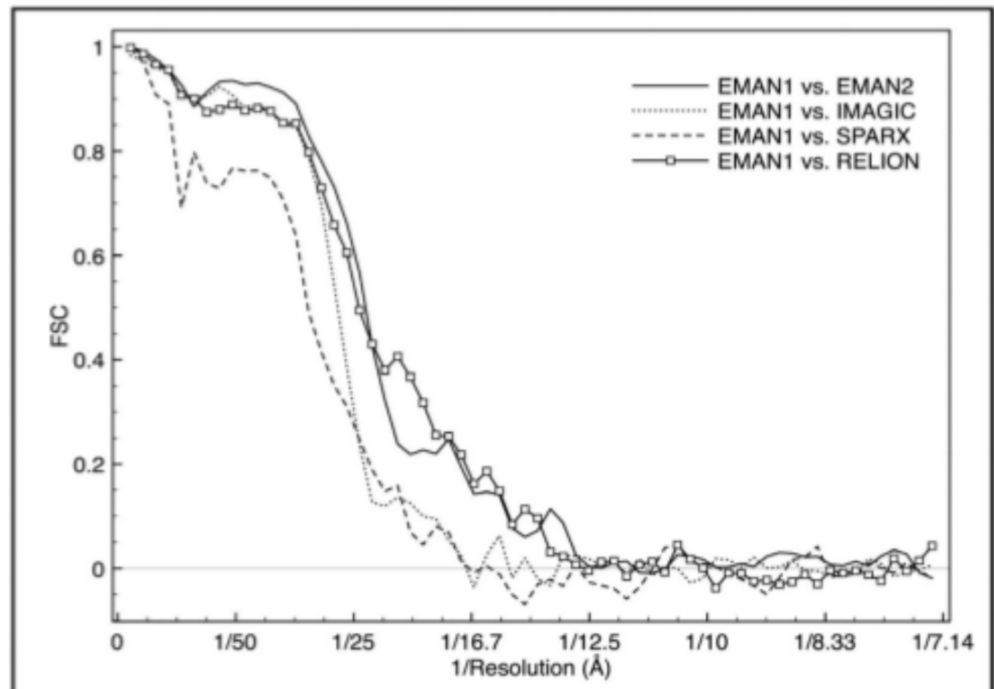
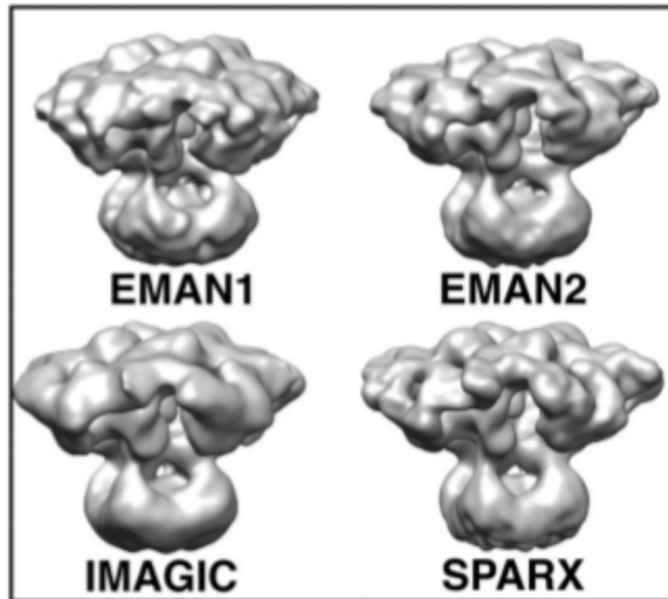
Jiang *et al.*
2002



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

Map validation - IP3 receptor

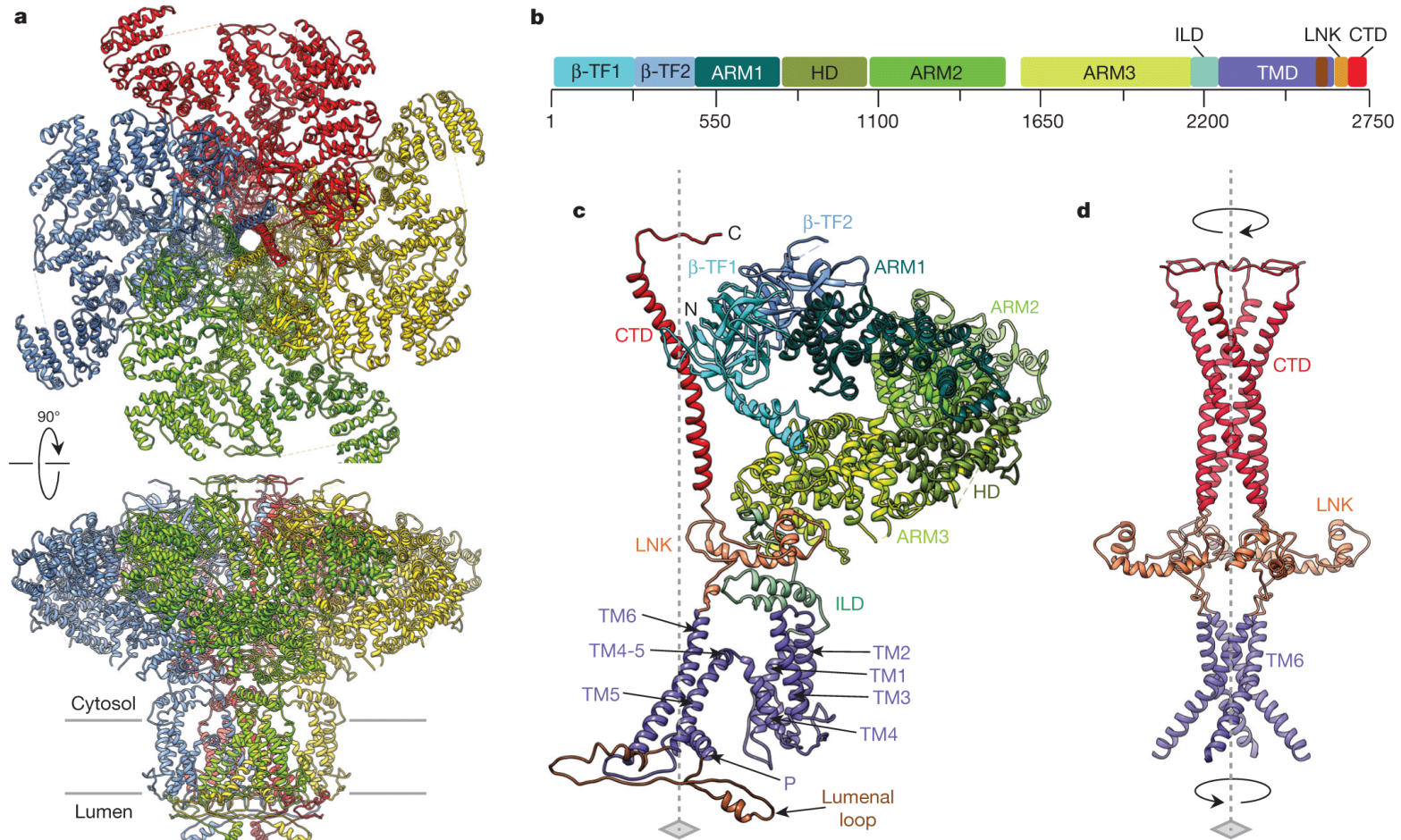
Comparison of maps from different programs



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

Map validation - IP3 receptor

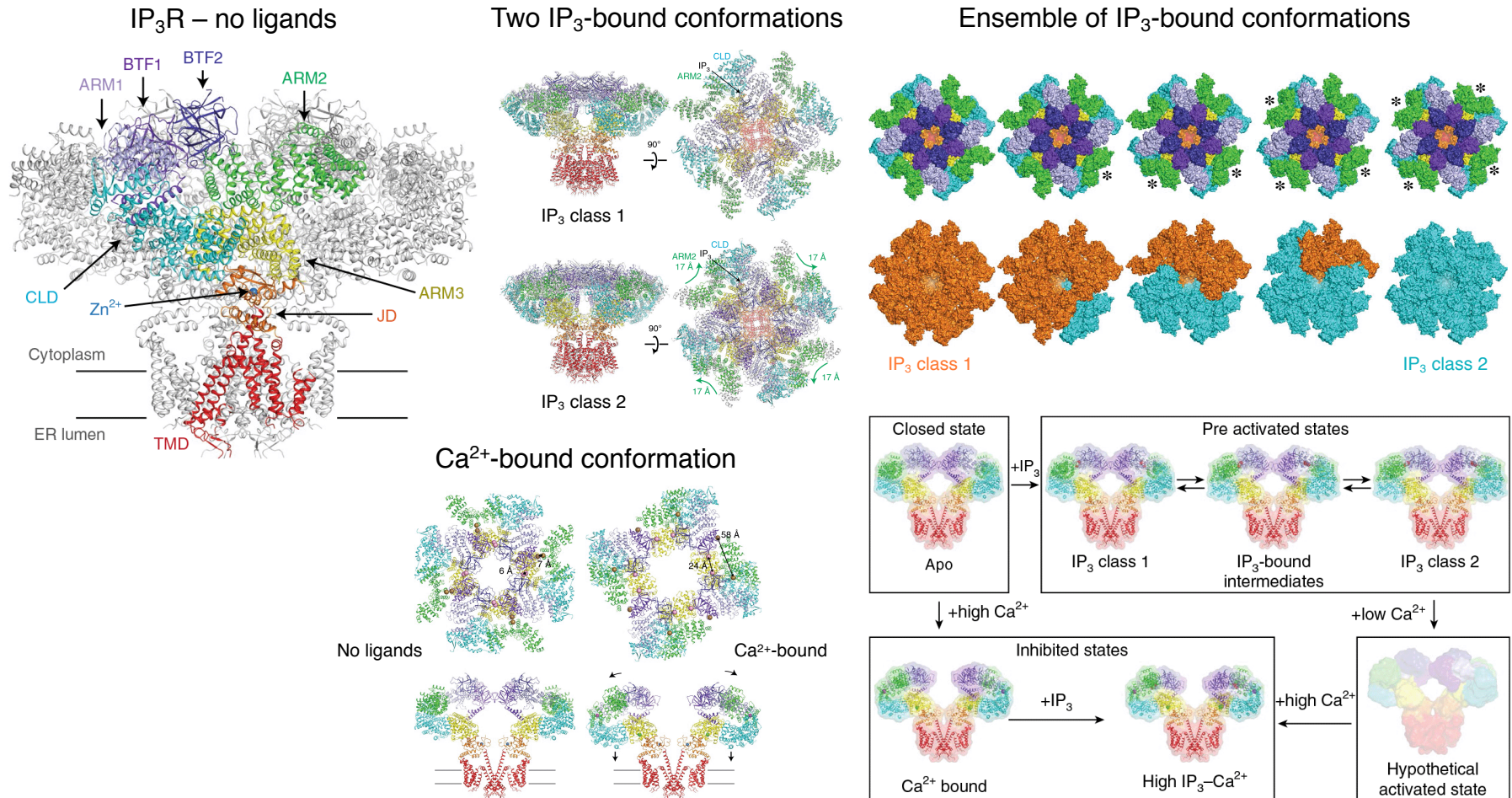
4.7 Å resolution structure (2015)



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

Map validation - IP₃ receptor

3.5 Å resolution structure (2018)



Paknejad & Hite (2018) *Nat. Struct. Mol. Biol.* 25: 660-668