

Validation Methods

A blast from the past ...

**NYSBC-NCCAT 2022
Single-particle cryo-EM Course**

The dark side of single-particle EM

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

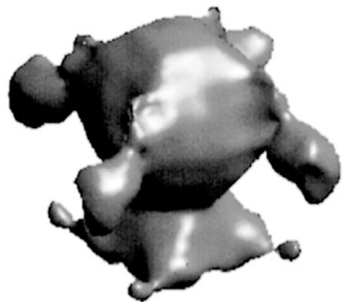
The bad thing about single-particle EM:
Every dataset and processing approach yields a 3D map !

But is it correct ???

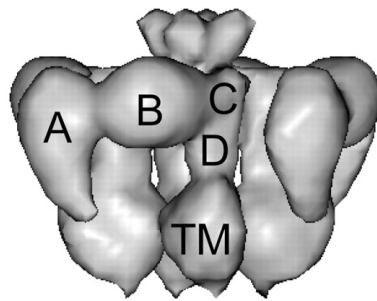
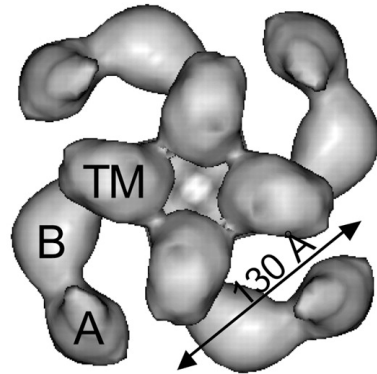


Particularly problematic
for low-resolution maps

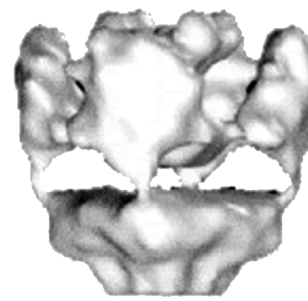
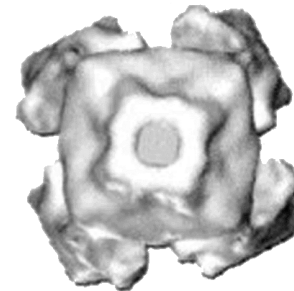
The issue: Structures of the IP3 receptor as determined by single-particle EM



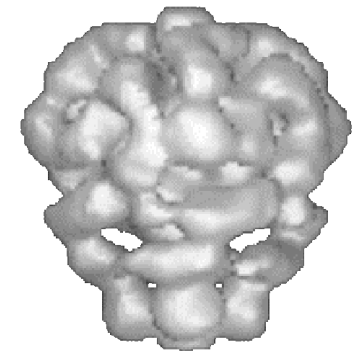
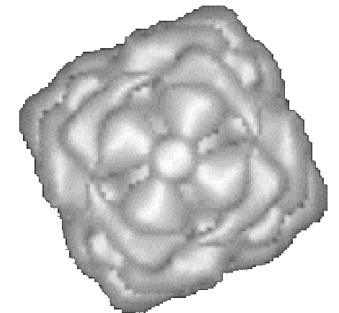
Jiang *et al.*,
2002



Serysheva *et al.*,
2003

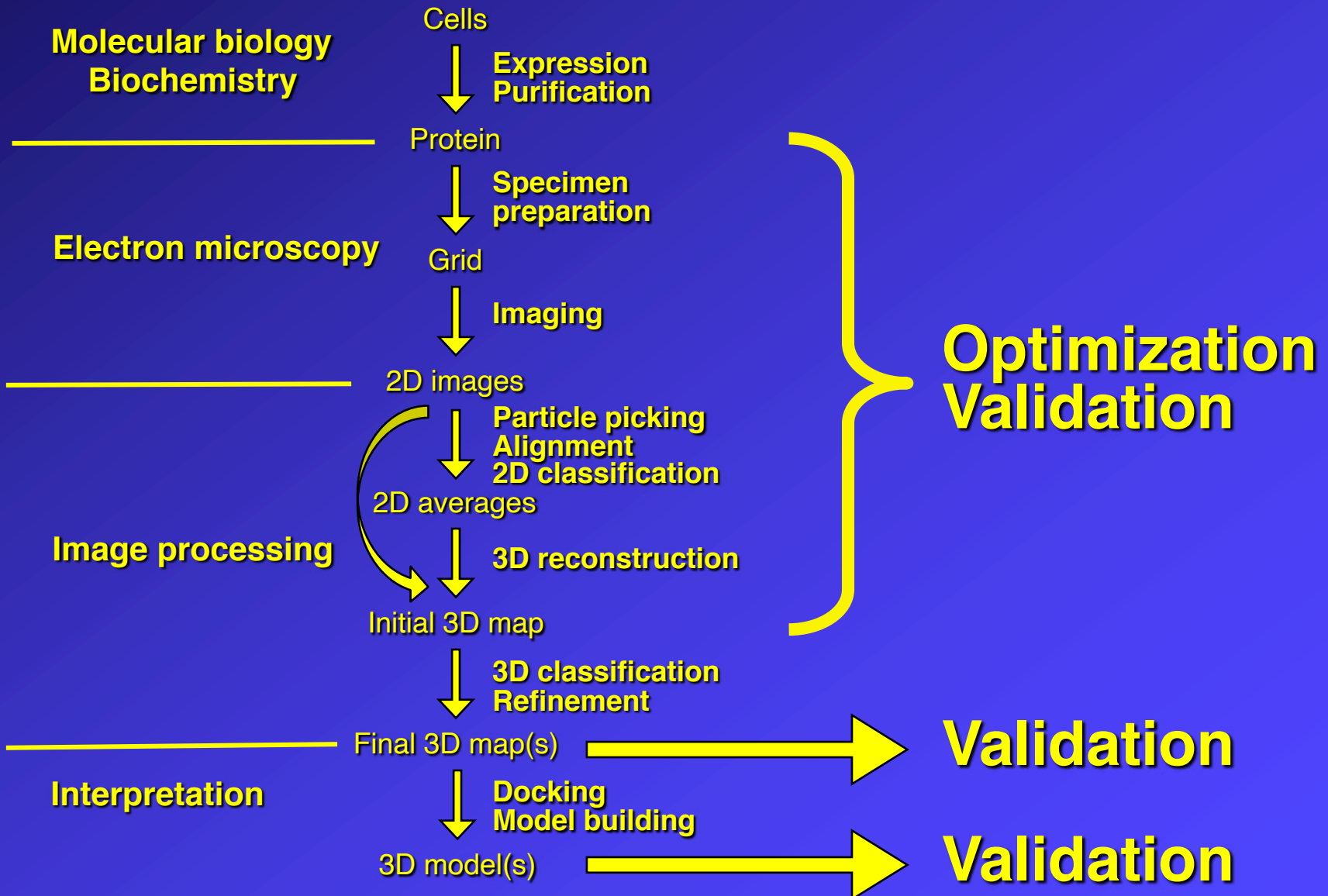


Jiang *et al.*,
2003



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 !

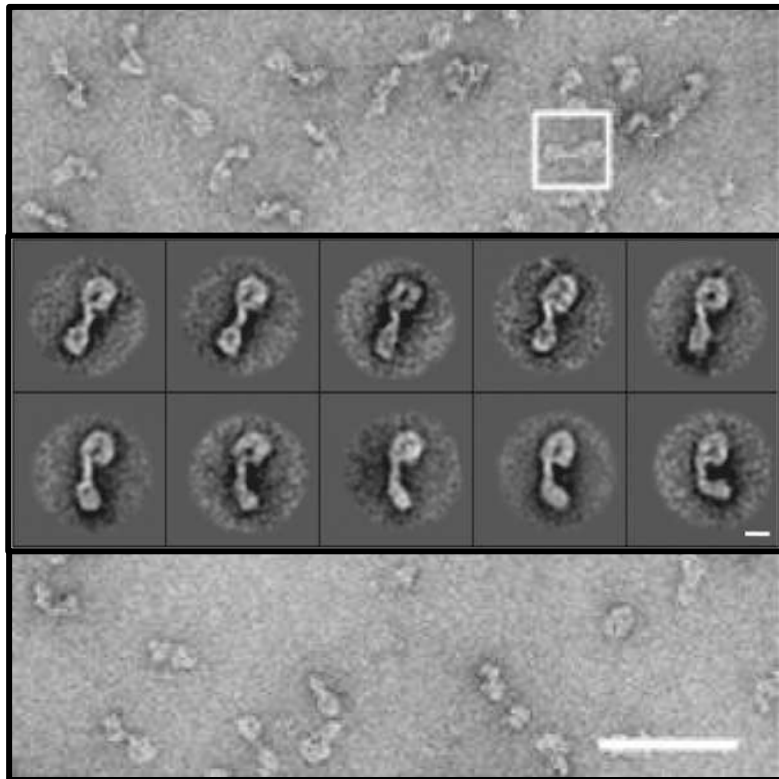
Prepare negatively stained specimens:
Good contrast and preferred orientations
→ Easy to assess heterogeneity

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

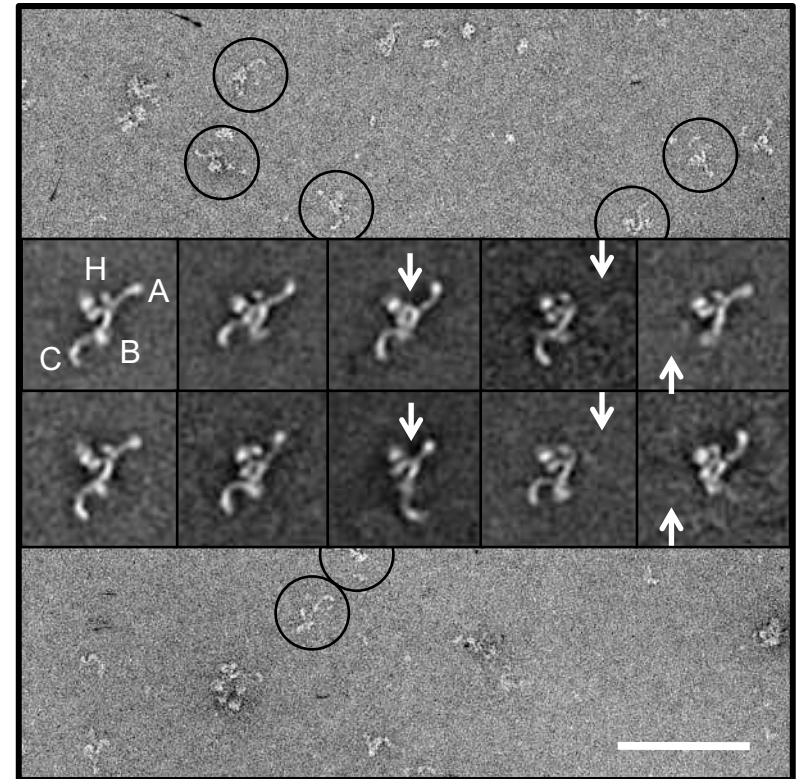
Effect of cross-linking: The HOPS tethering complex

Cross-linked



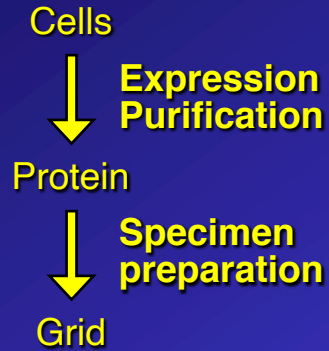
Bröcker *et al.* (2012)
PNAS 109: 1991-1996

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
- Use thin support film (carbon or graphene oxide)
- Use different grids, e.g., PEG-treated or gold grids

Preferred orientation (particles align at air/water interface)

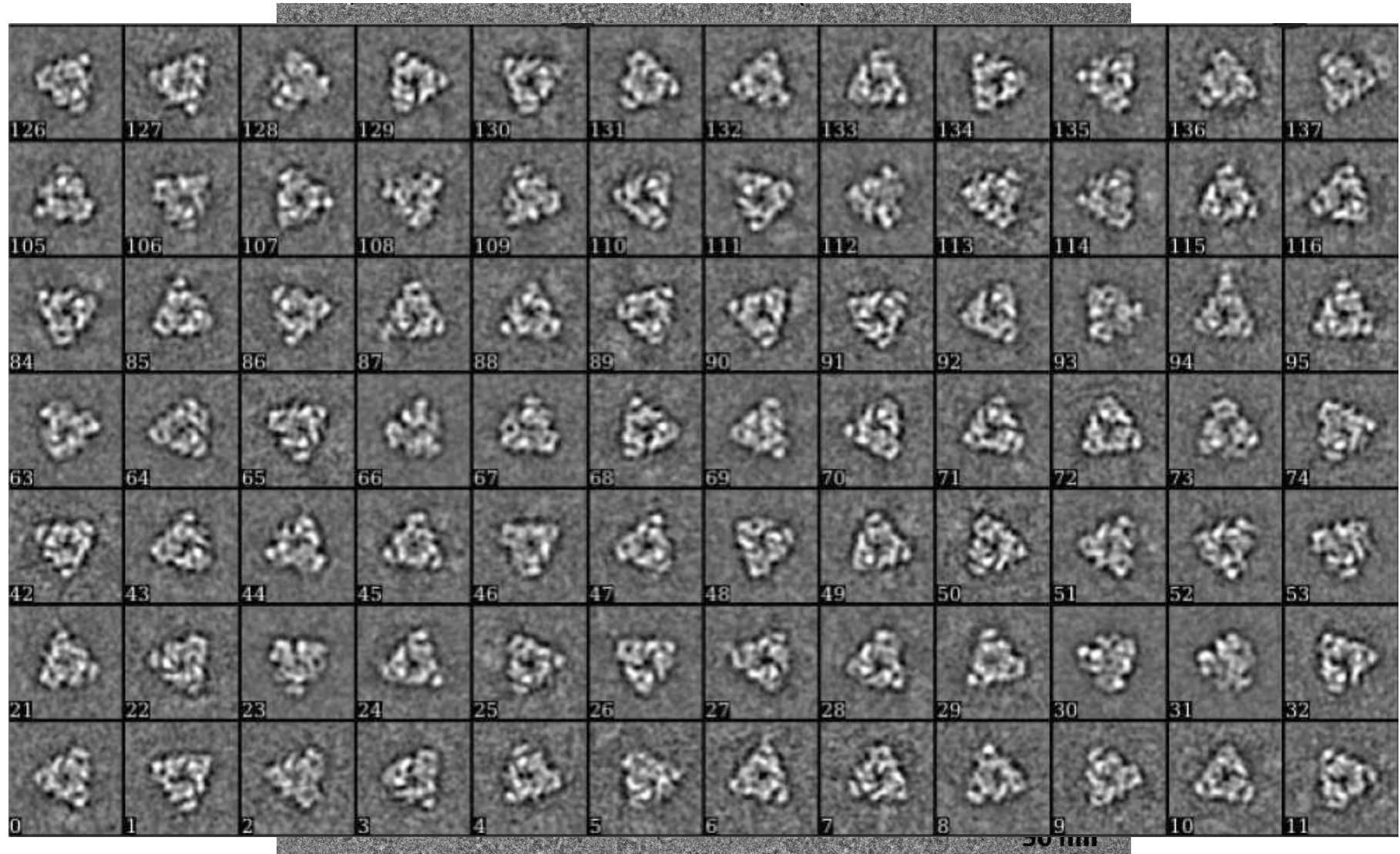
Lack of views will result in:

- non-isotropic resolution of the density map
- can potentially lead to an incorrect density map
- Use low concentration of detergent (changes surface tension)
- Use thin carbon film (commonly used for ribosome samples)
- Use gold grids

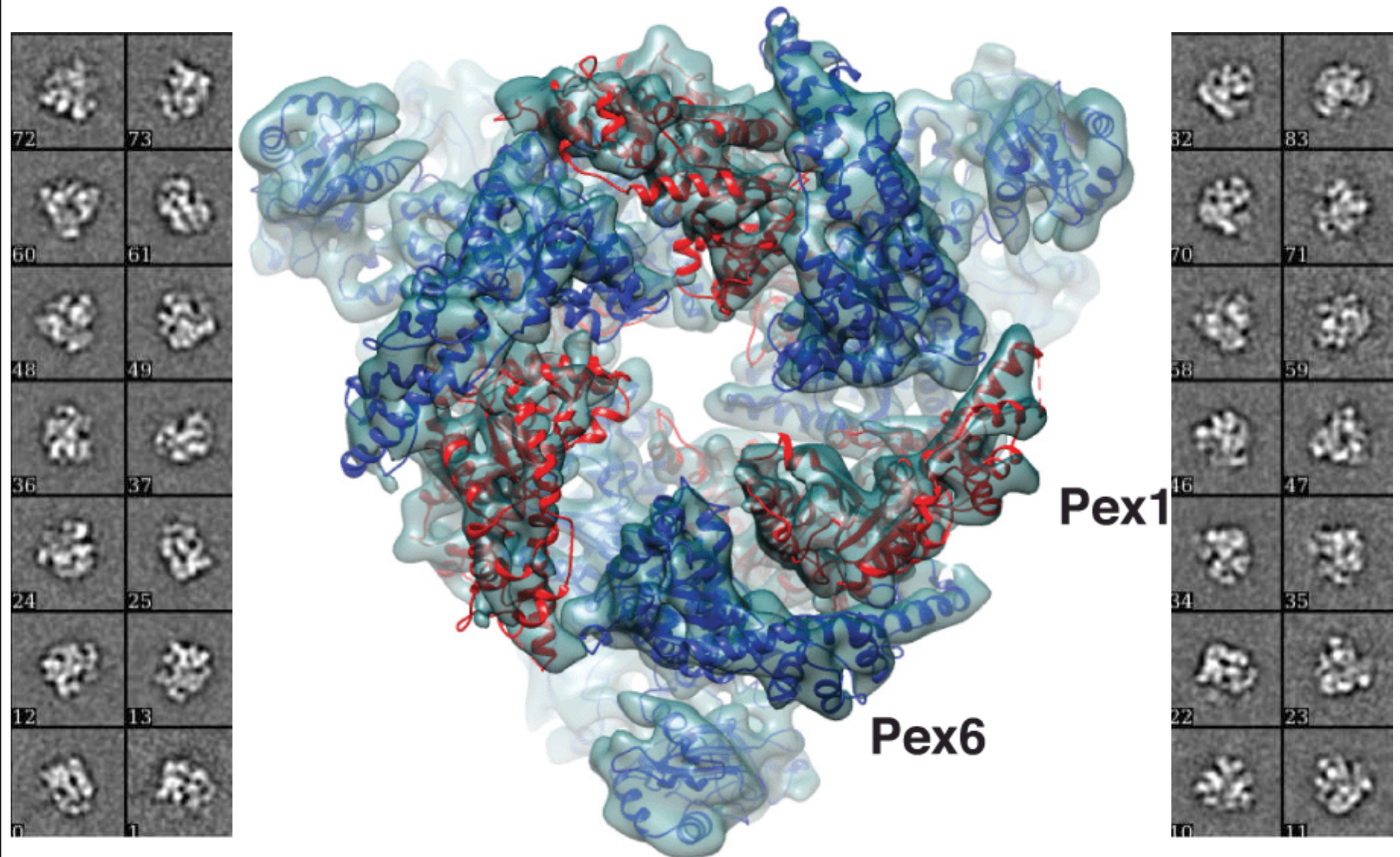
Different sample preparation approach (e.g., Spotiton)

Collect images from tilted specimens

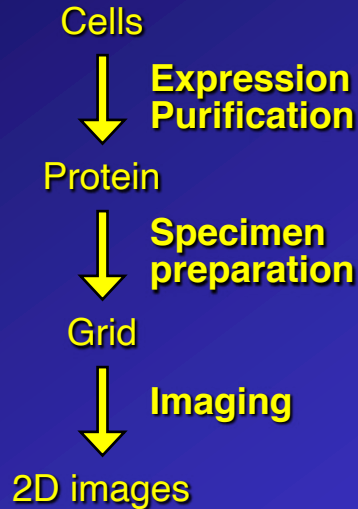
Preferred orientations: Pex1/6 complex Without detergent



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

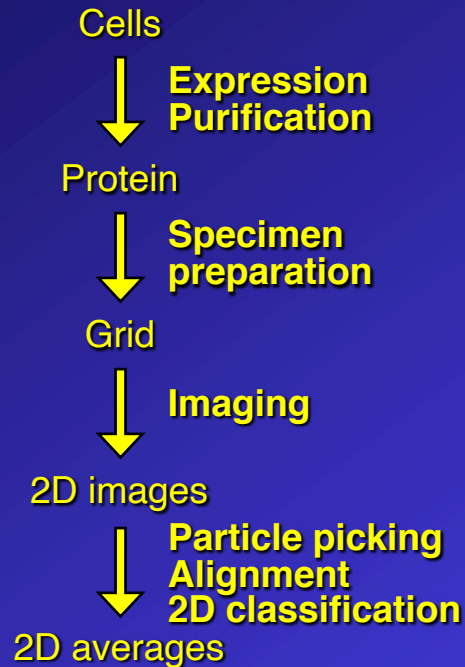
→ Loss of information
cannot be fixed

→ 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:

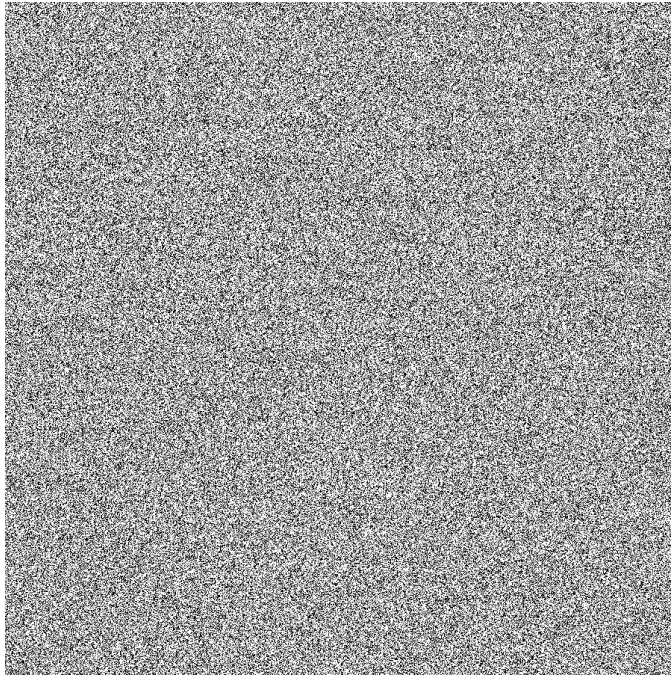
- Model/reference bias

2D classification:

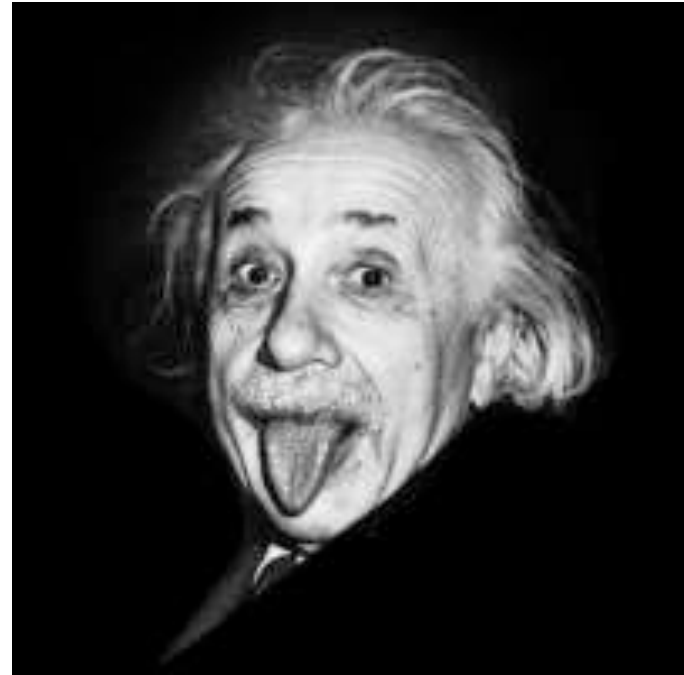
- Model/reference bias
- Number of classes
- Heterogeneous classes
- Disappearing classes

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

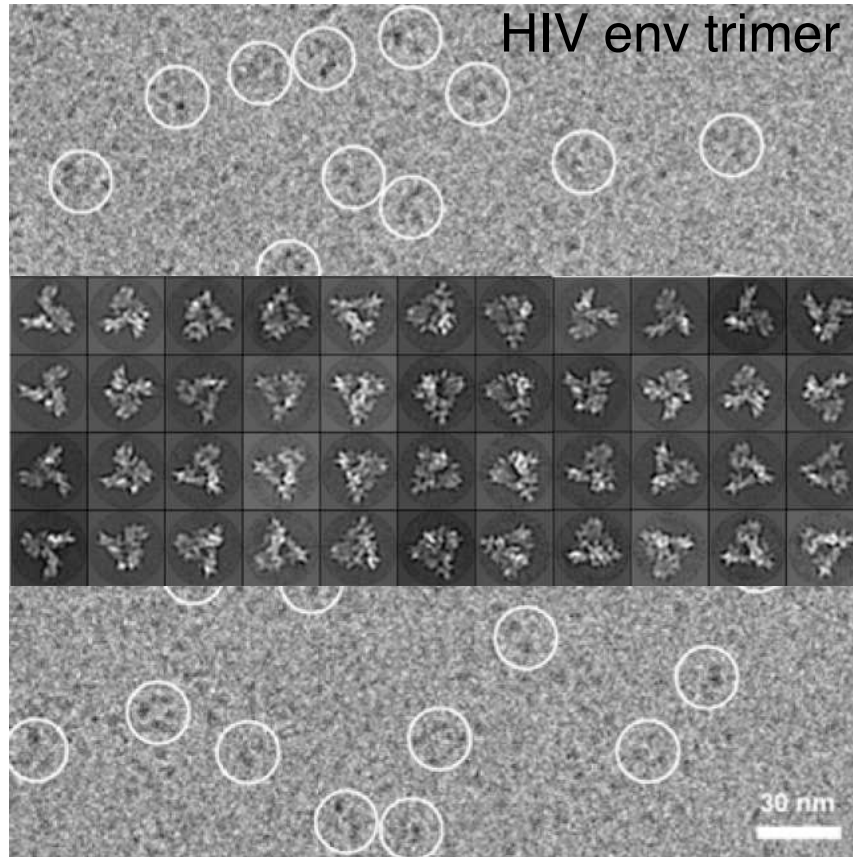
→ Einstein from noise

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

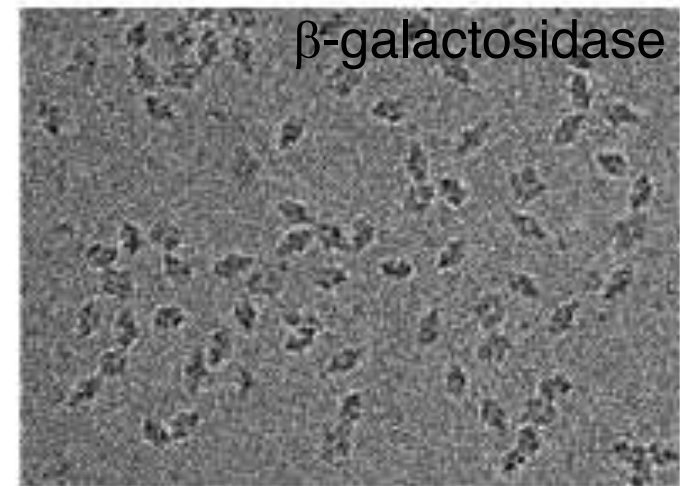
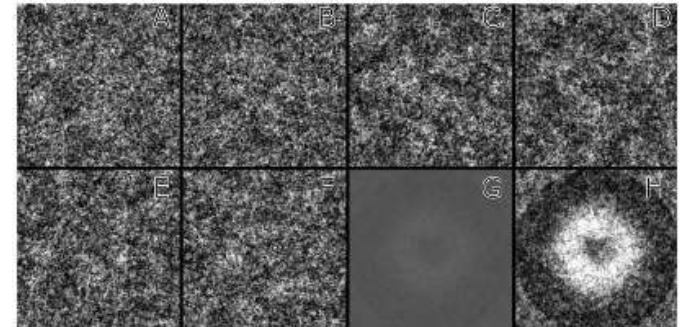
Henderson (2013) *Proc. Natl. Acad. Sci. USA* 110: 18037-18041

Structure determination by single-particle EM

Potential issues with particle picking



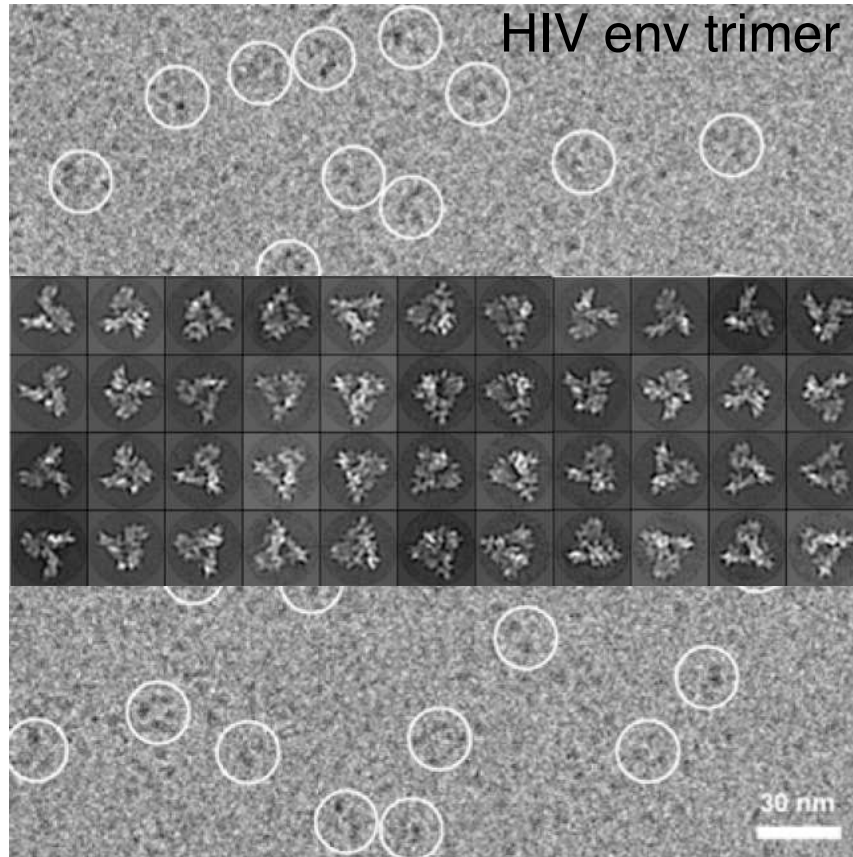
Mao *et al.* (2013)
PNAS 110: 12438-12443



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

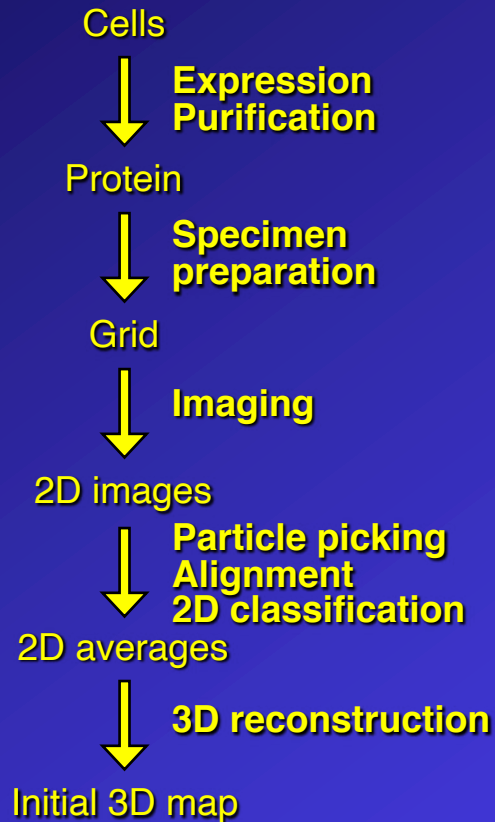
Using template matching to pick particles from very noisy images is dangerous

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

→ Better to first pick images without templates and use resulting averages as templates for re-picking

Henderson (2013)
PNAS 110: 18037-18041

Structure determination by single-particle EM



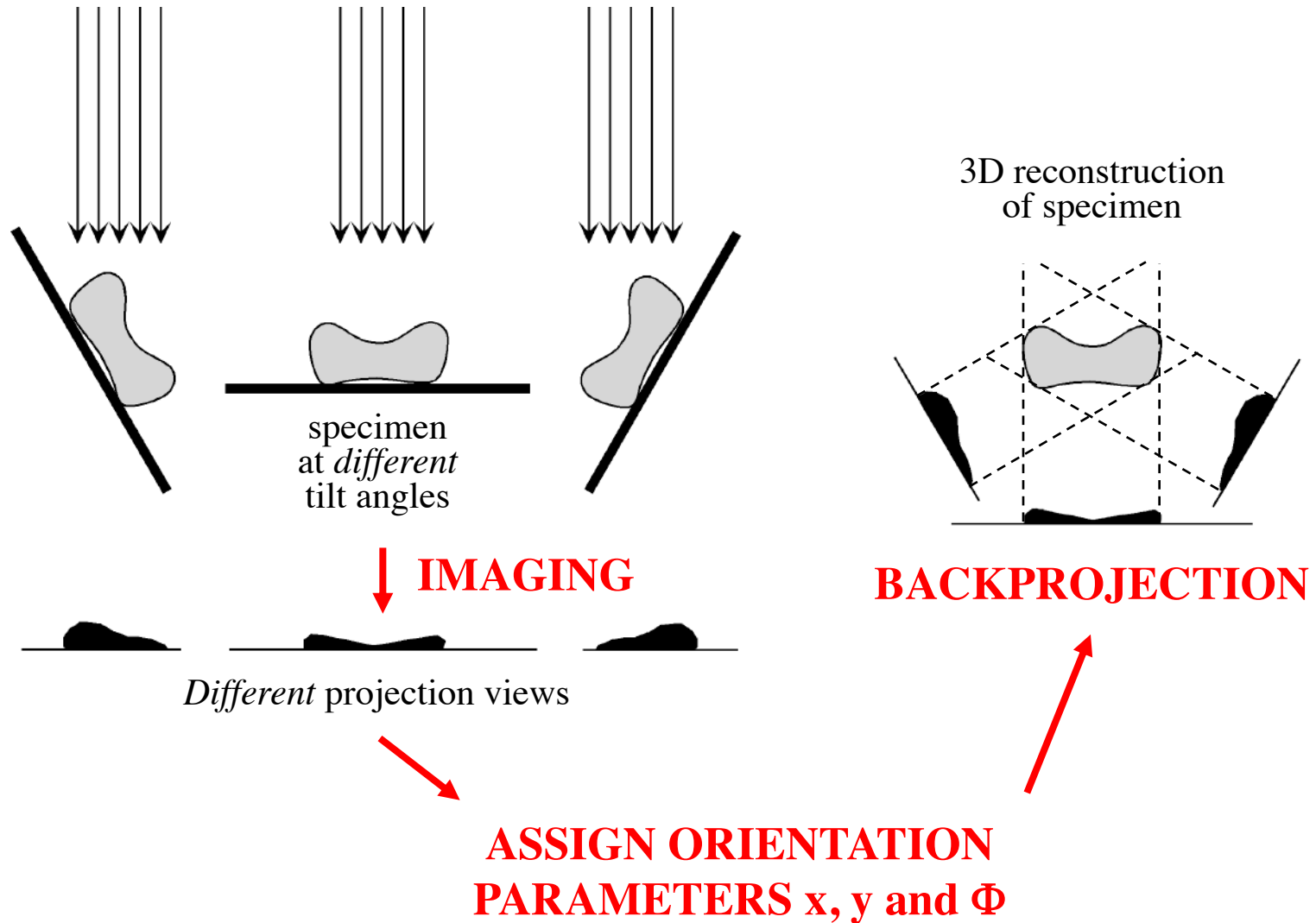
Potential issues:

Incorrect map

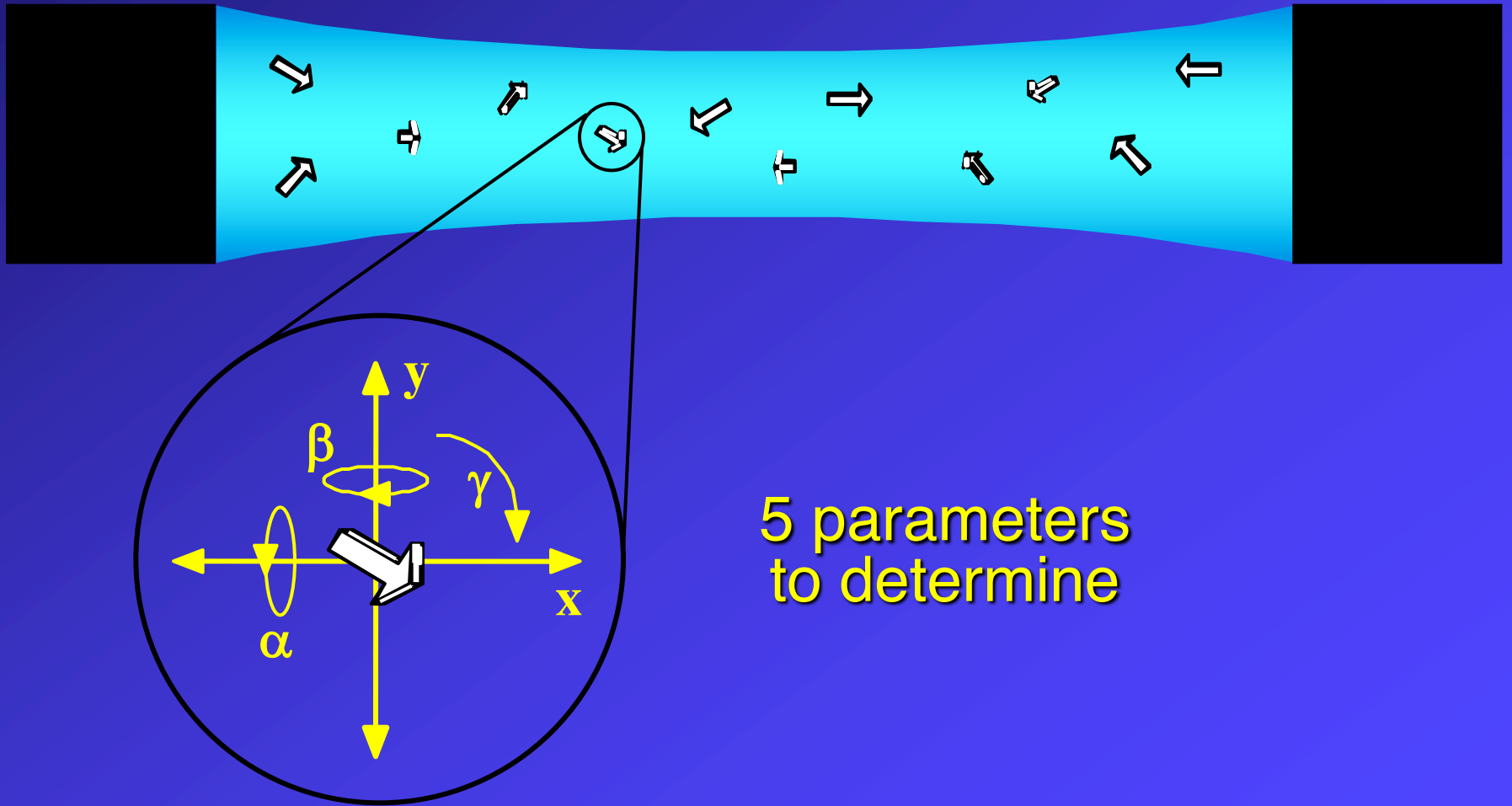
Because of:

- Heterogeneous sample
- Missing views
- Incorrect solution

Random conical tilt reconstruction



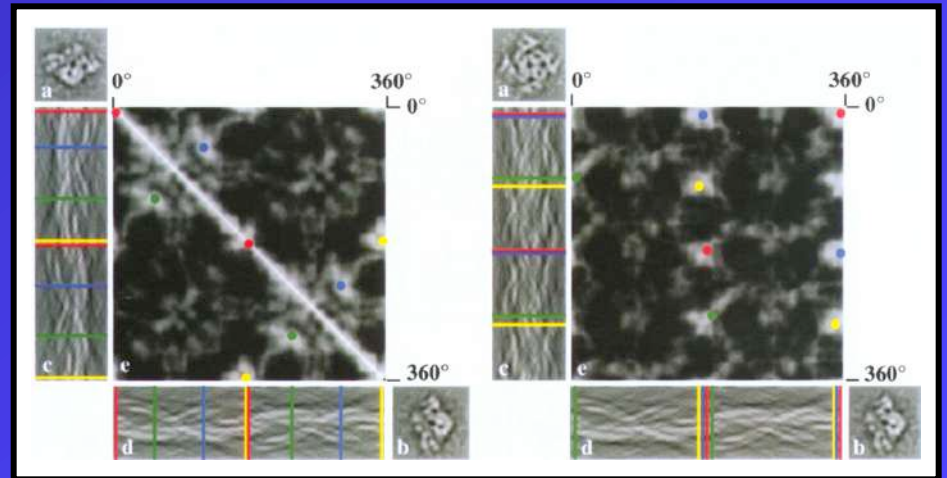
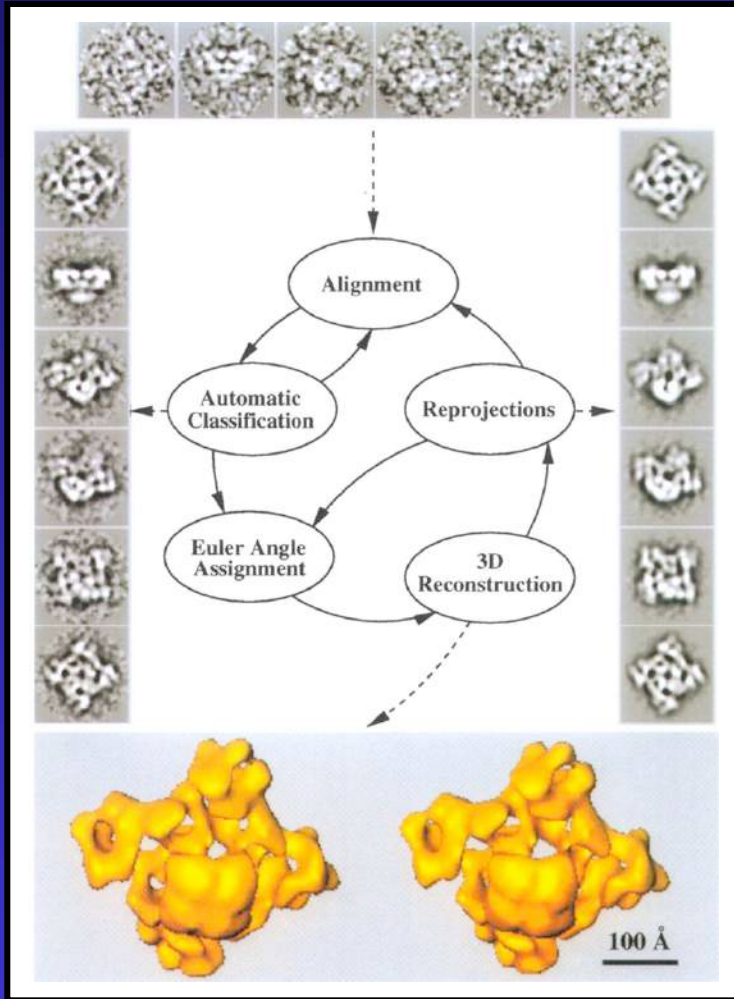
Single particles in ice



Angular reconstitution

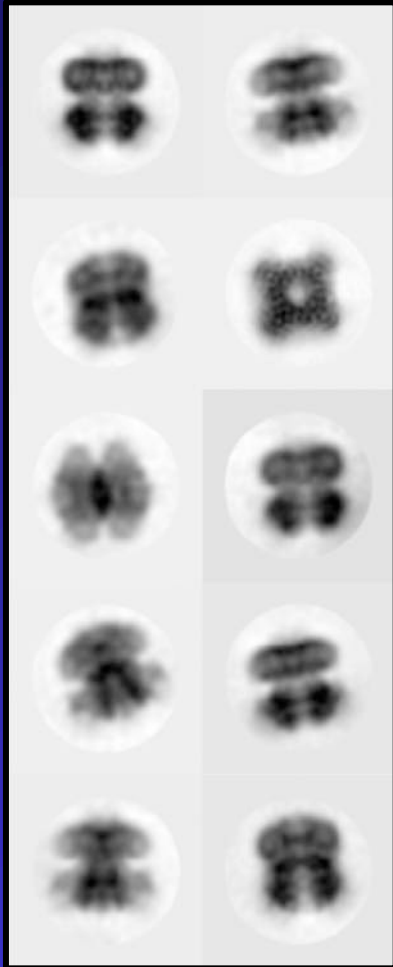
van Heel, 1987

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

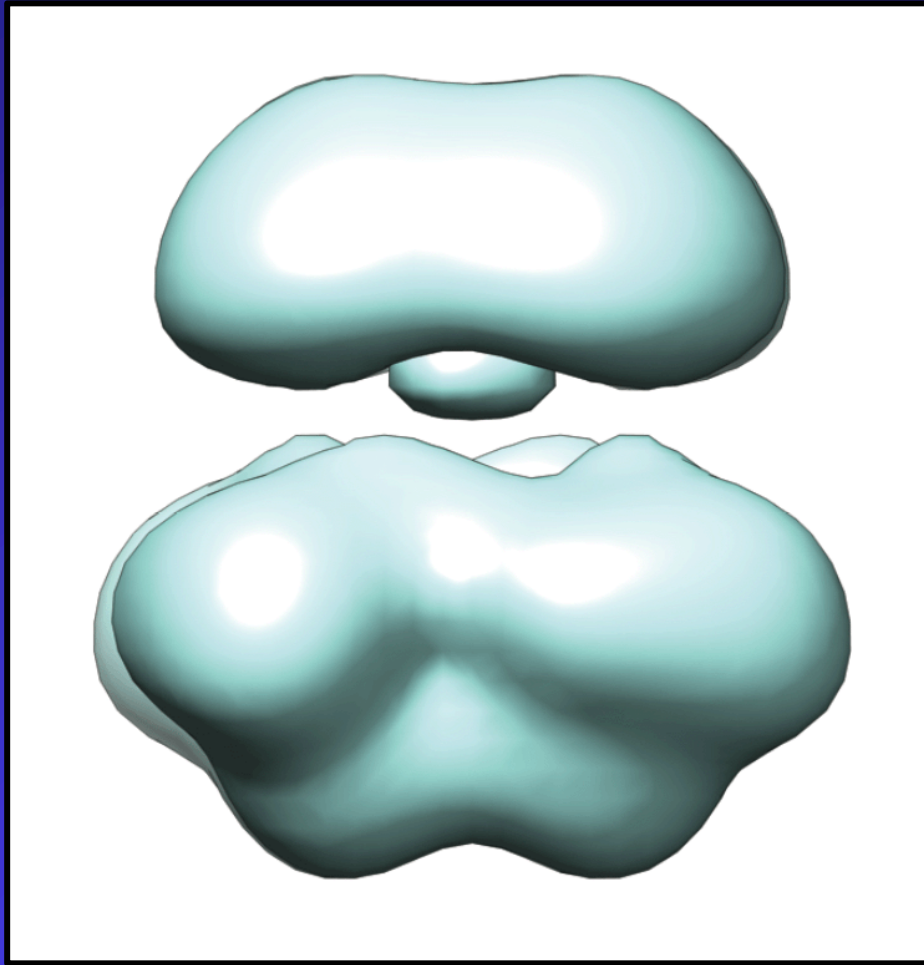


Serysheva *et al.*, 1995

Chicken Slo2.2 in the absence of Na^+



Class averages



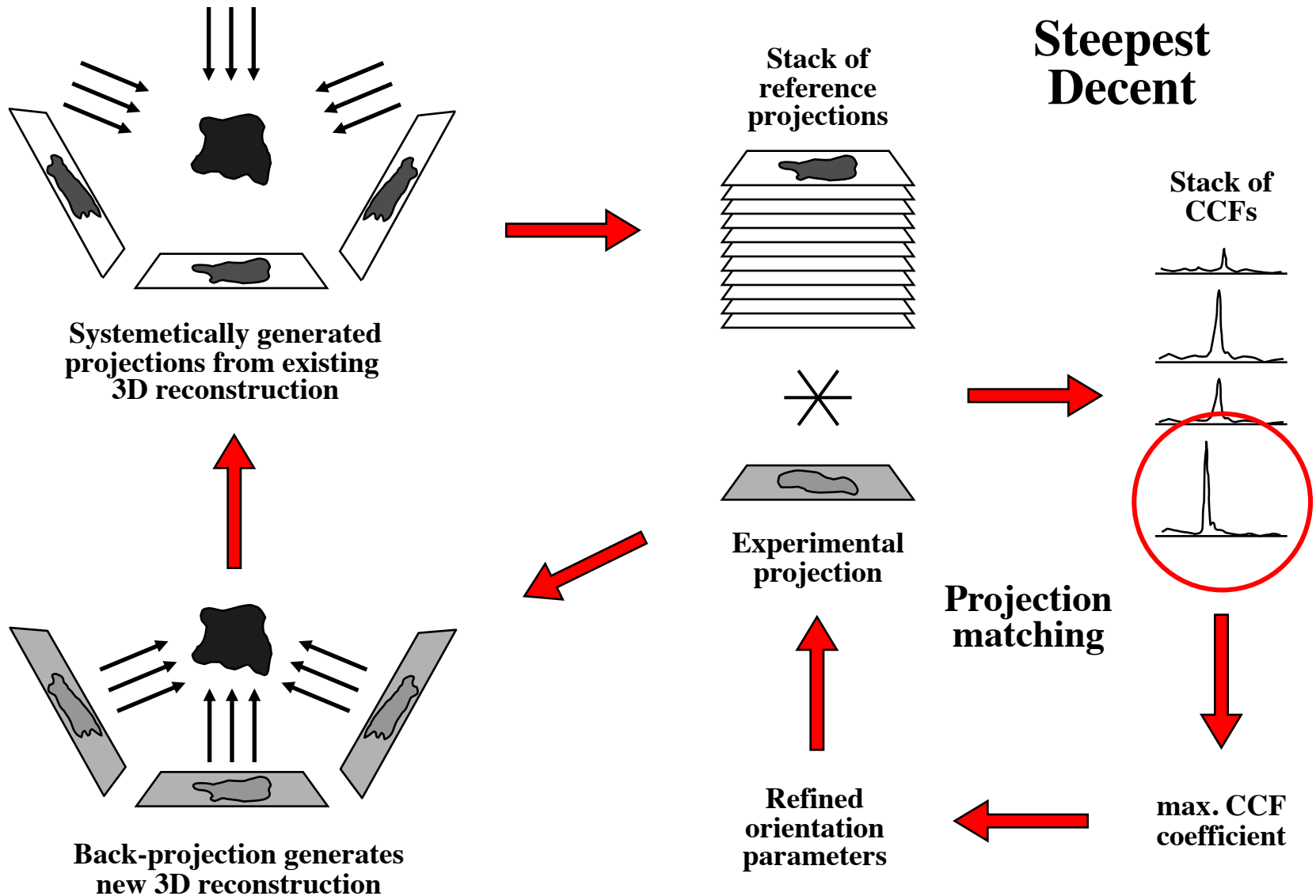
Initial model (obtained with VIPER)

VIPER

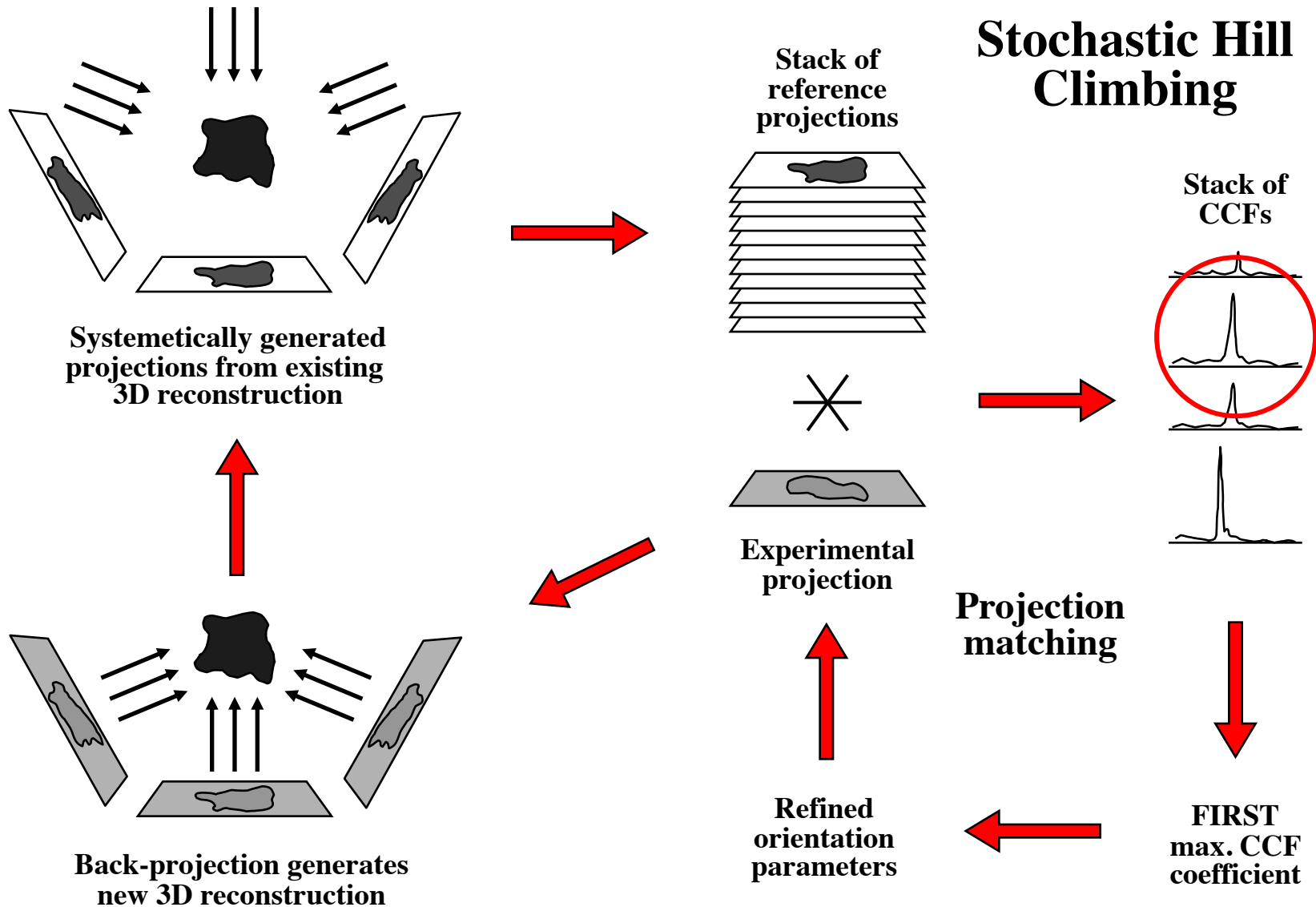
**Stochastic
Hill Climbing**

(initially introduced
in program SIMPLE)

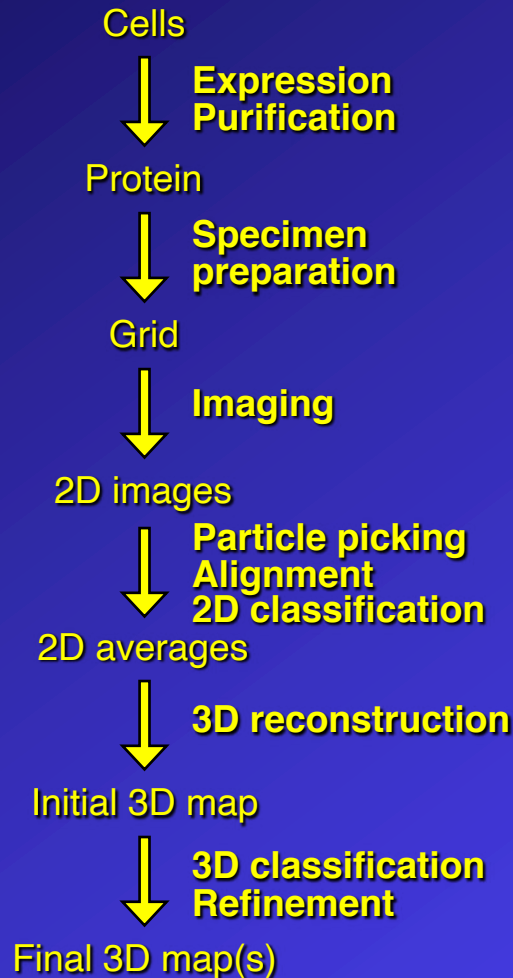
Angular refinement



Angular refinement



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

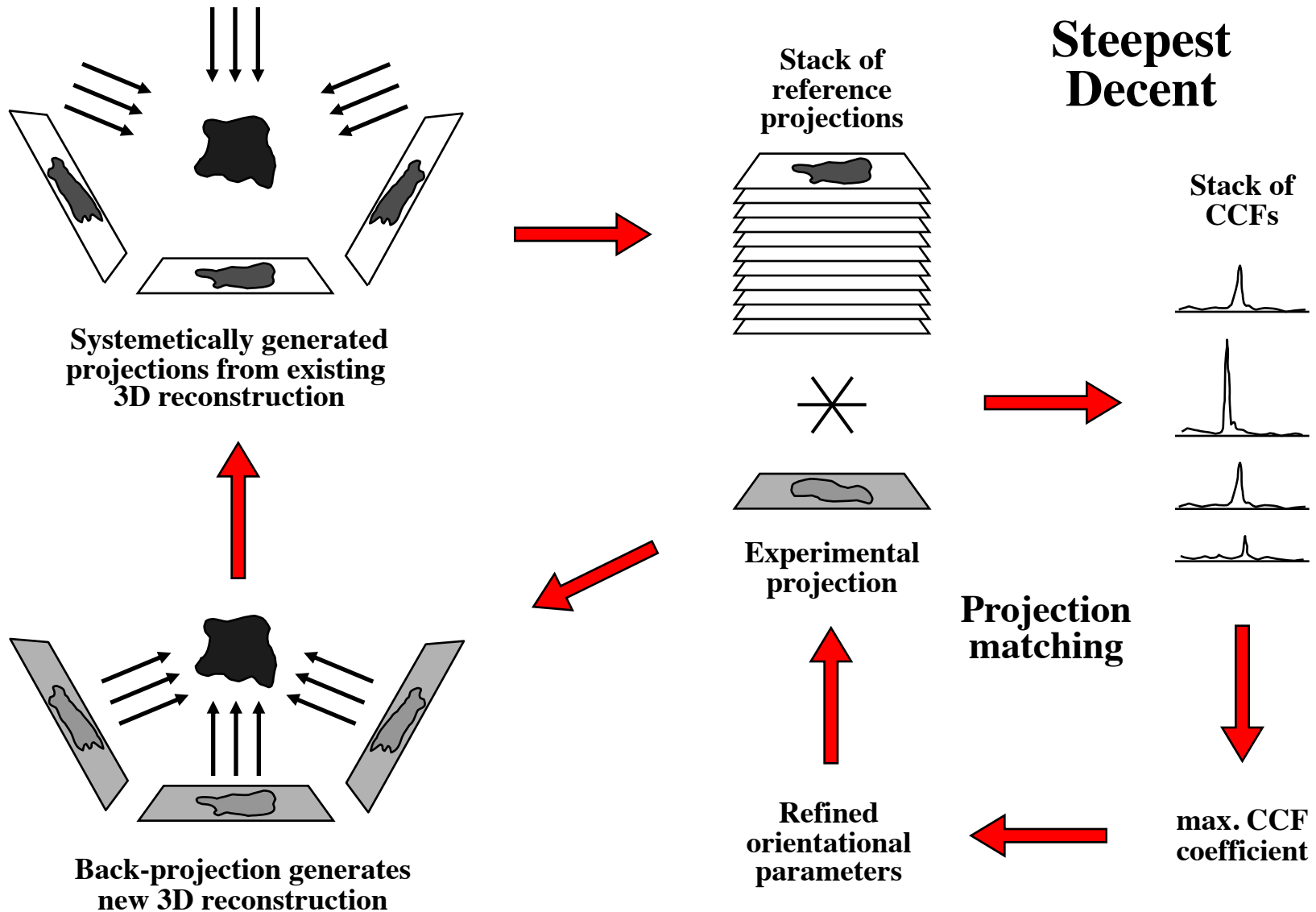
Average of 1,000 images containing pure white noise after alignment to an image of Albert Einstein

→ Einstein from noise

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

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Angular refinement



Structure determination by single-particle EM

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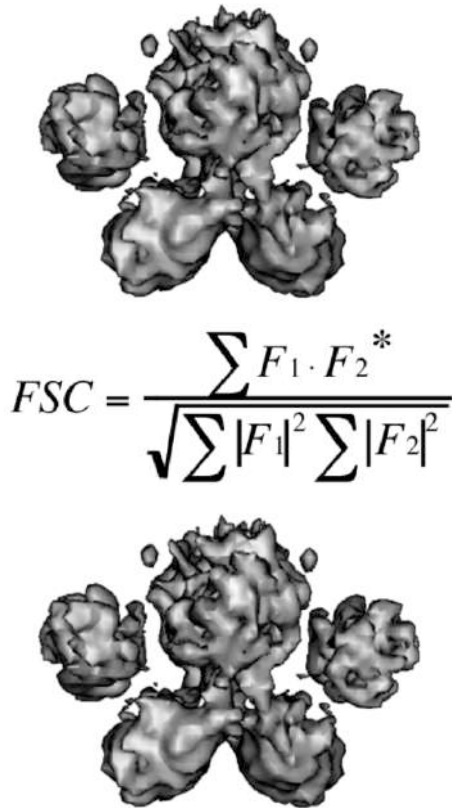
Over-fitting results in spurious high-resolution features due to alignment of noise

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

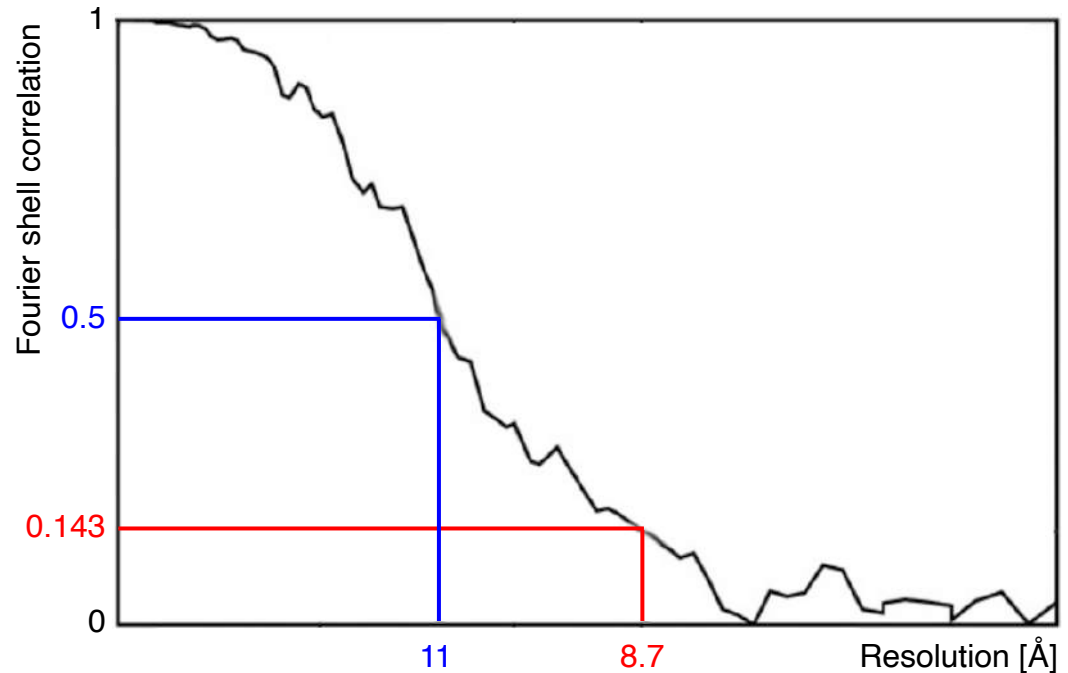
Henderson (2013) *Proc. Natl. Acad. Sci. USA* 110: 18037-18041

Structure determination by single-particle EM

Resolution assessment



Maps have to be independent !



FSC = 0.5 Signal = Noise

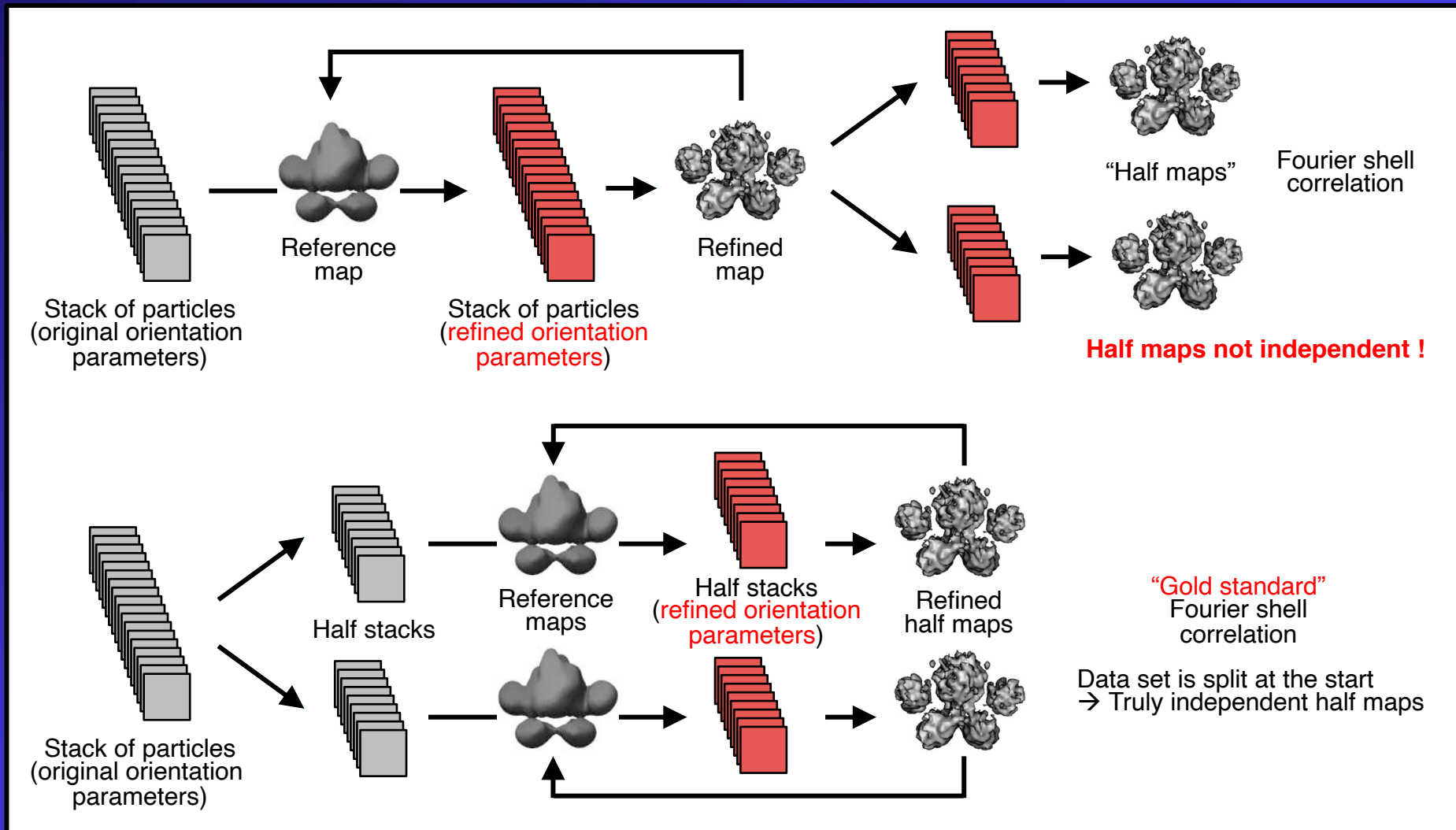
Böttcher *et al.* (1997) *Nature* 386: 88-91

FSC = 0.143 Phase error = 60°

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

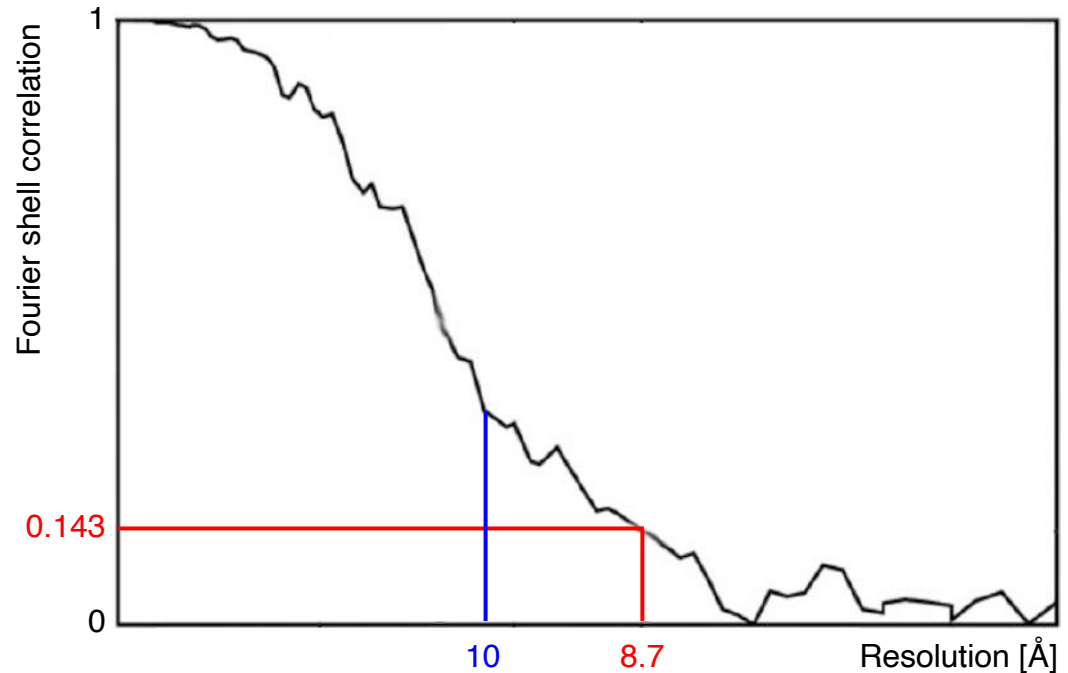
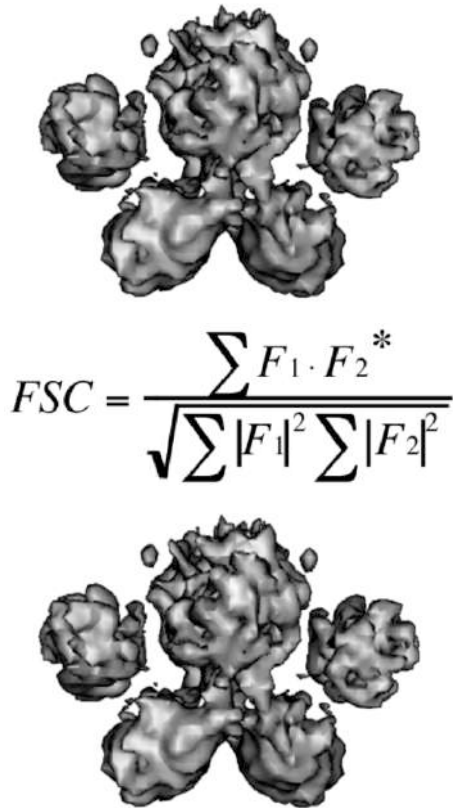
Structure determination by single-particle EM

Resolution assessment



Structure determination by single-particle EM

Resolution assessment



If the entire dataset is refined together against a reference resolution-filtered to 10 Å

FSC = 0.143 criterion still meaningful as long as FSC shows correlation beyond resolution of reference (10 Å)

Structure determination by single-particle EM

Resolution assessment

The 2016 map challenge

December 2018 Special Issue of *J. Struct. Biol.* with contributions regarding the map and model challenges (Lawson & Chiu, Heymann *et al.*)

Current procedure to estimate resolution by FSC is not sufficiently standardized

Several variables (e.g., map box size, voxel size, filtering and masking practice and threshold value for interpretation) can substantially impact the determined resolution

Archives could independently estimate the resolution of maps by FSC from deposited unmasked, minimally filtered half-maps

Still does not take into account local resolution differences !

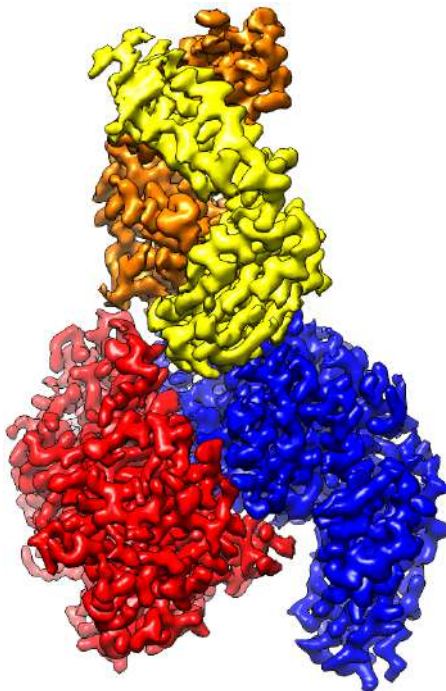
Structure determination by single-particle EM

Local resolution

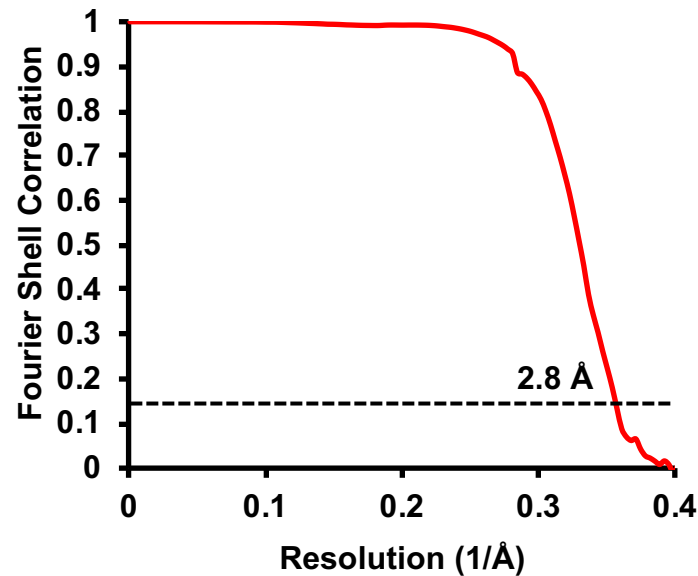
Abciximab

heavy chain

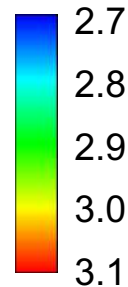
light chain



$\alpha\text{IIb}\beta 3$

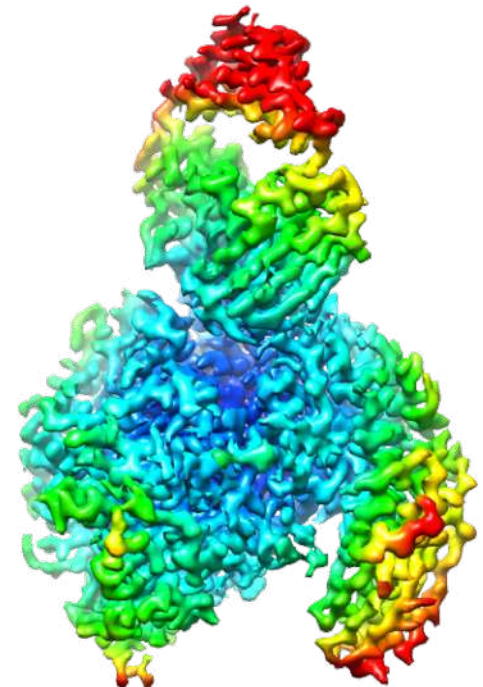


Reliable model can
be built *de novo* for
the entire complex



ResMap

Kucukelbir *et al.* (2014)
Nat. Methods 11: 63-65



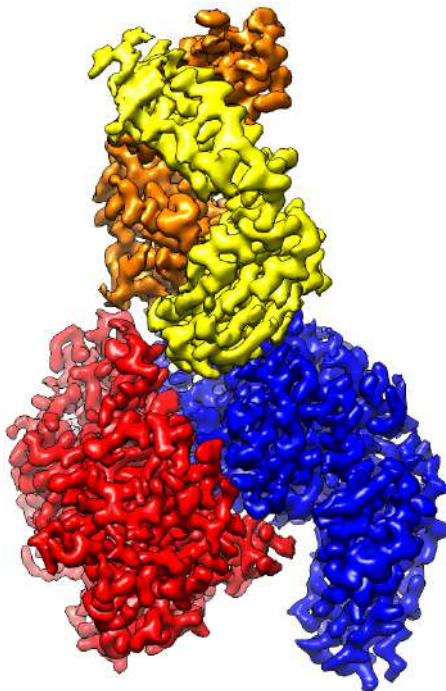
Structure determination by single-particle EM

Local resolution

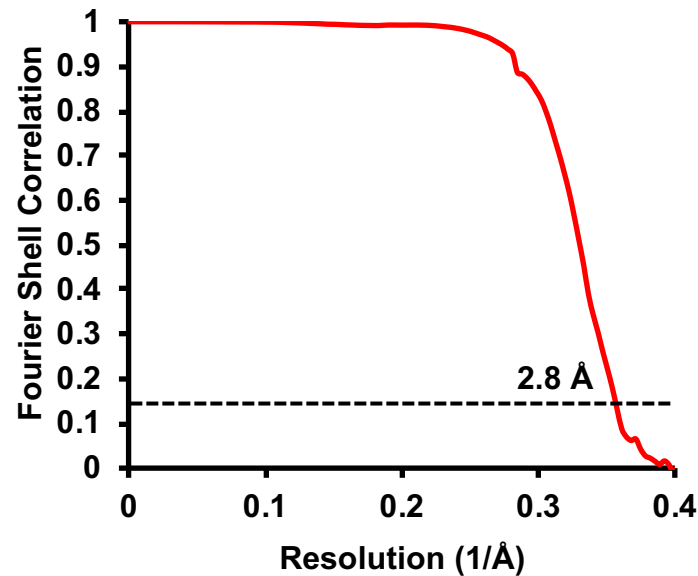
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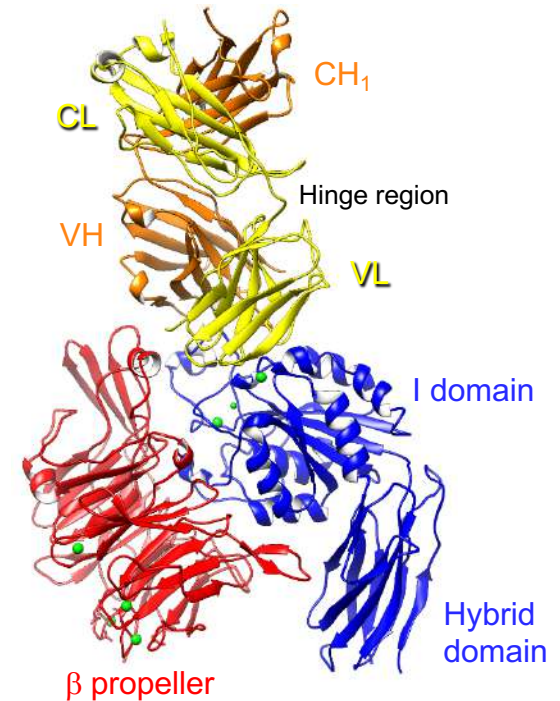
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$\alpha IIb\beta 3$



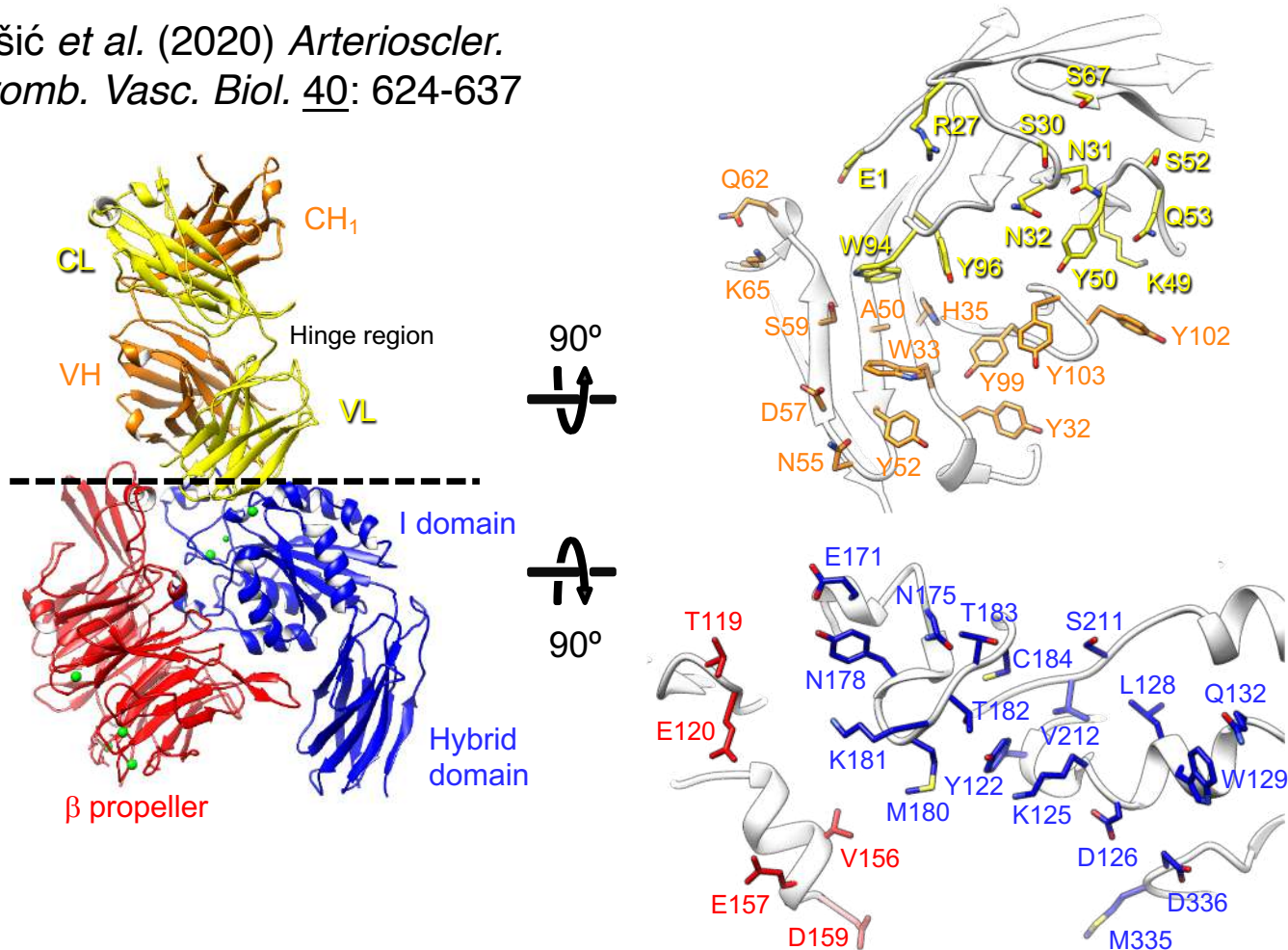
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Structure determination by single-particle EM

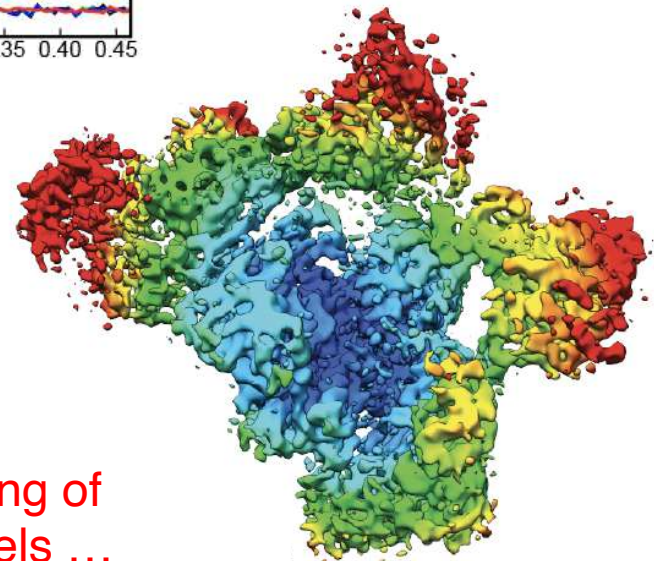
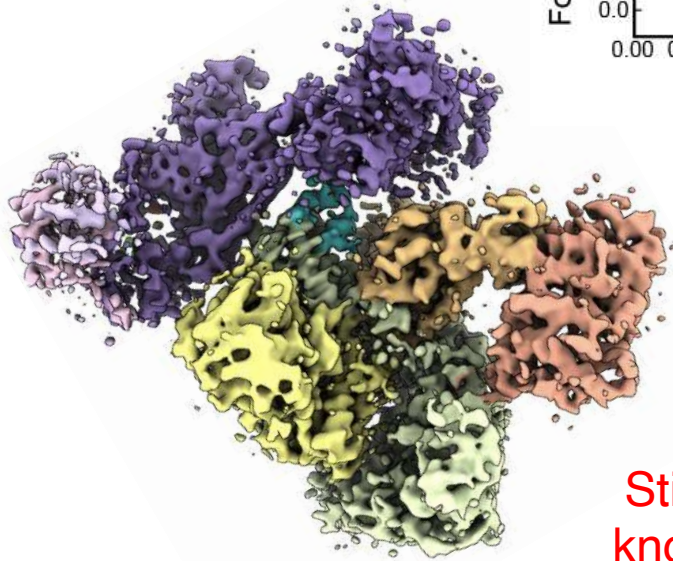
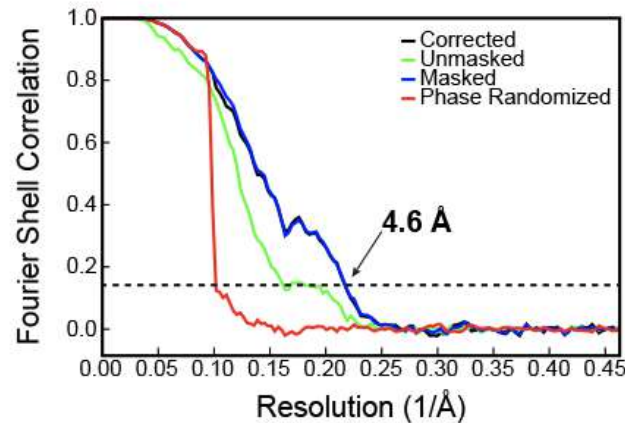
Local resolution

Nešić *et al.* (2020) *Arterioscler. Thromb. Vasc. Biol.* 40: 624-637



Structure determination by single-particle EM

Local resolution



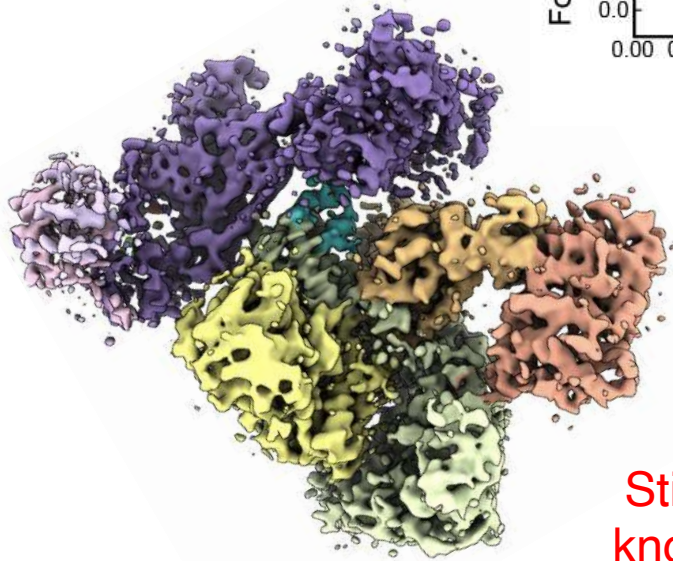
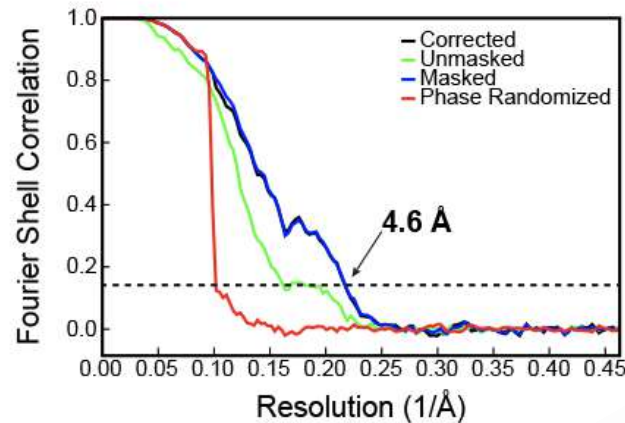
Still good for docking of
known atomic models ...

... and there is now AlphaFold2 !



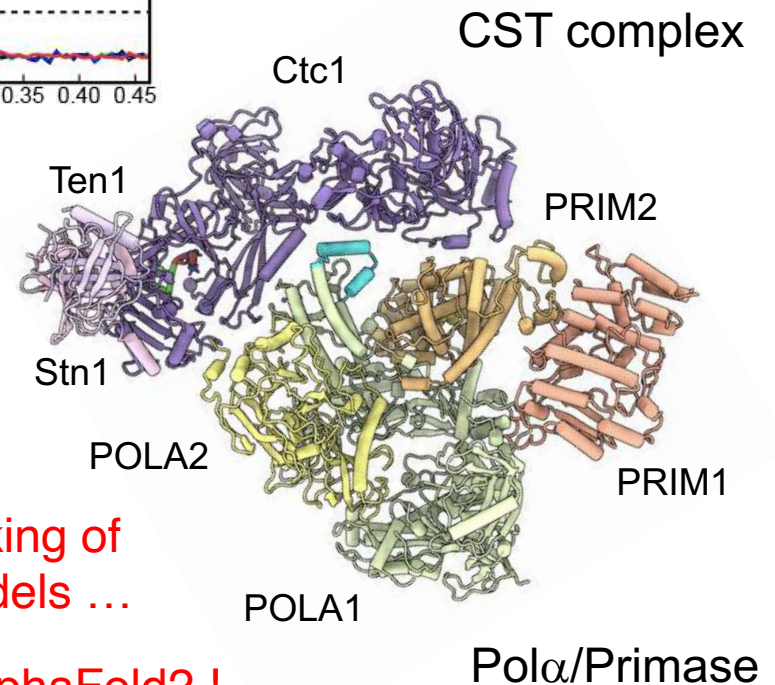
Structure determination by single-particle EM

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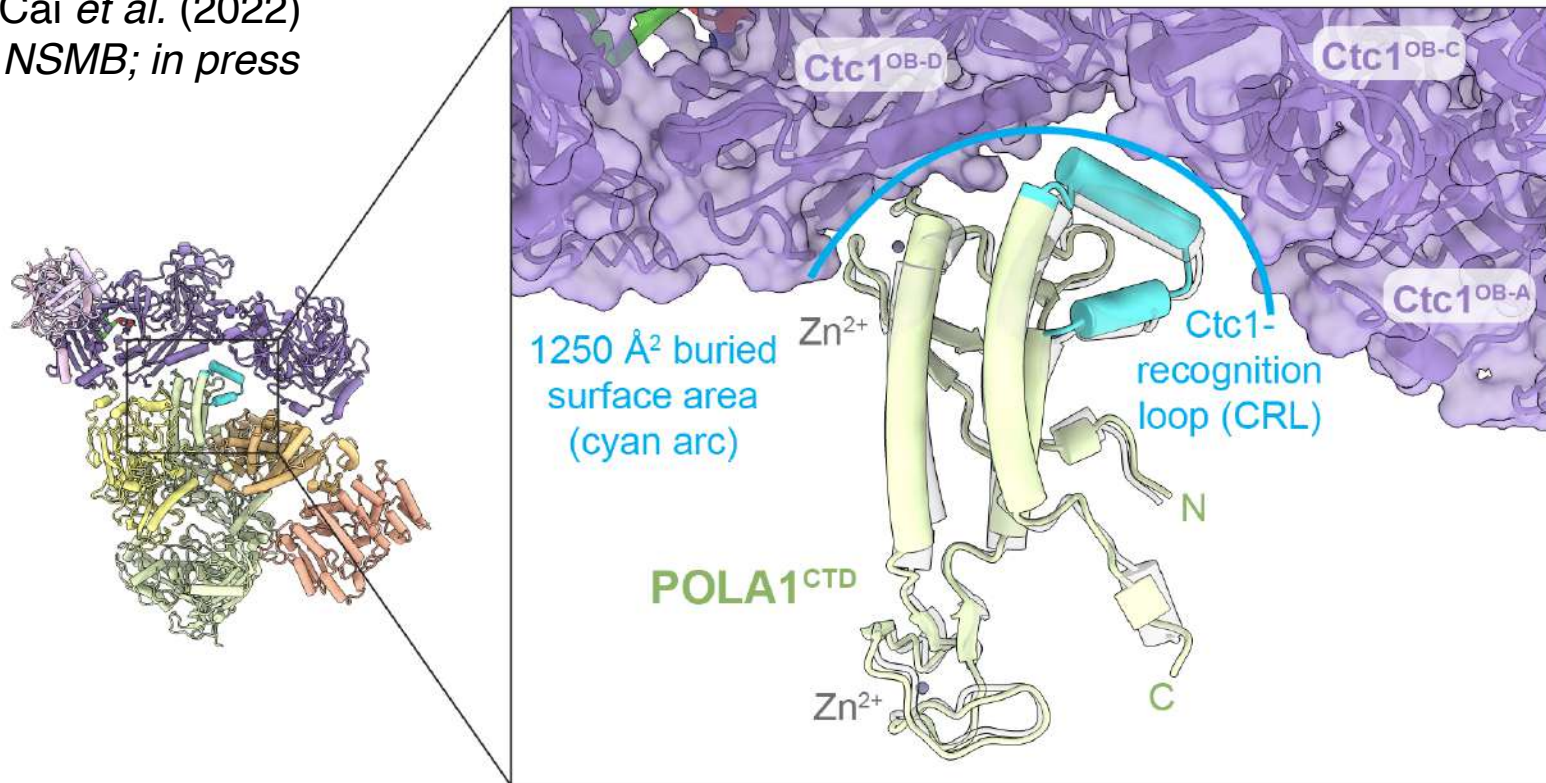
... and there is now AlphaFold2 !



Structure determination by single-particle EM

Local resolution

Cai *et al.* (2022)
NSMB; in press



Structure determination by single-particle EM

Resolution assessment

What should be resolved ?

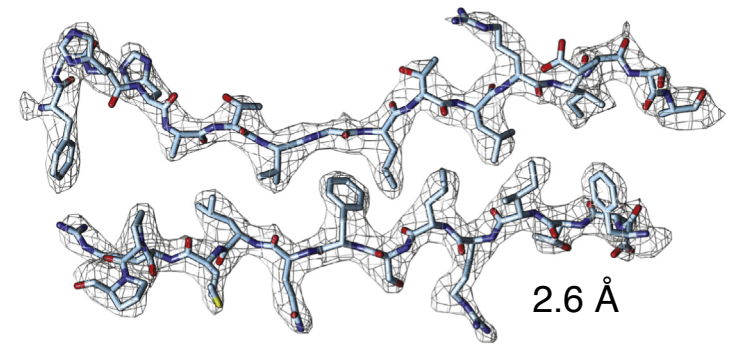
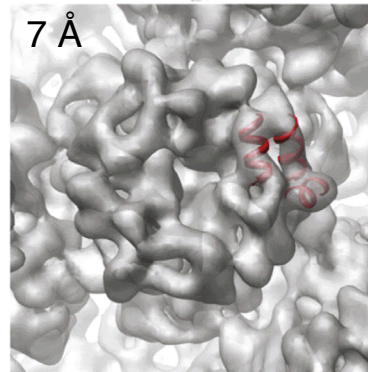
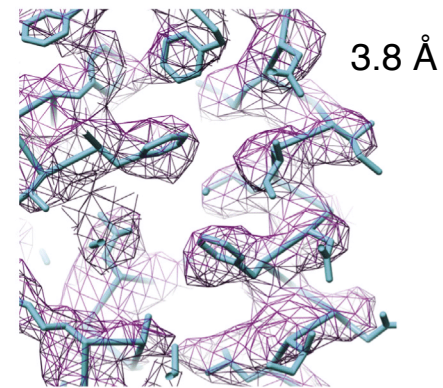
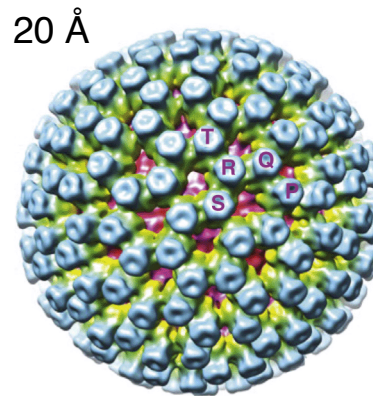
> 20 Å
protein envelope

~ 9-10 Å
 α -helices

< 4.8 Å
 β -sheets

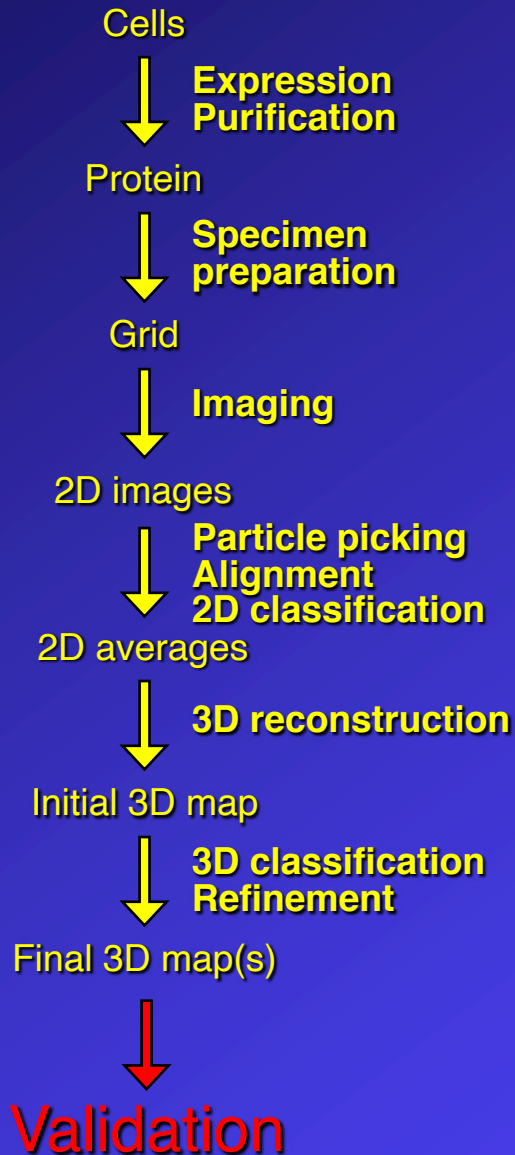
~ 4 Å
bulky side chains

Rotavirus double-layered particle

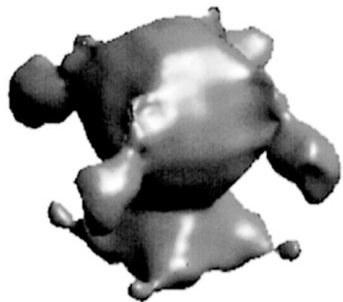


Rosenthal & Rubinstein (2015) *Curr. Opin. Struct. Biol.* 34: 135-144

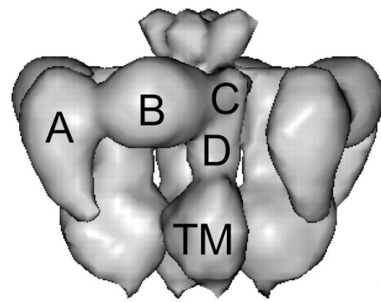
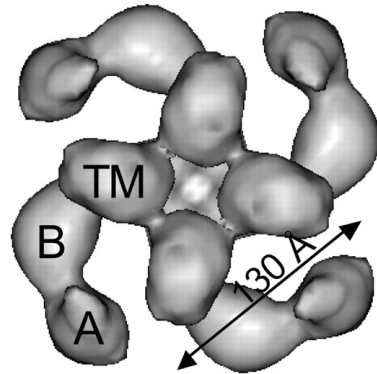
Structure determination by single-particle EM



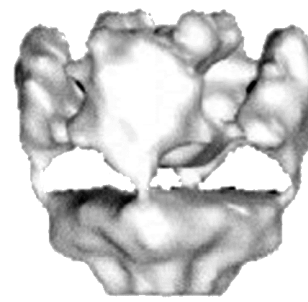
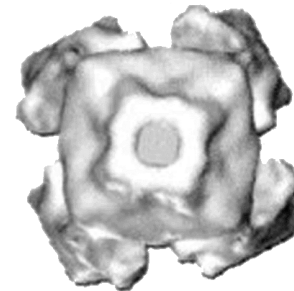
The issue: Structures of the IP3 receptor as determined by single-particle EM



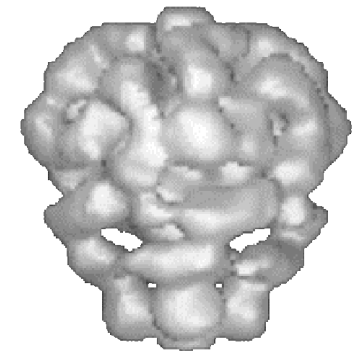
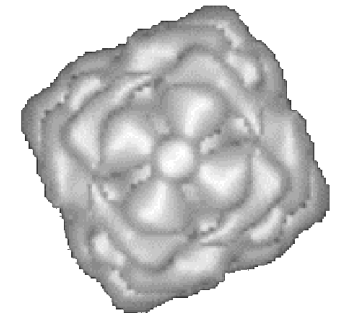
Jiang *et al.*,
2002



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

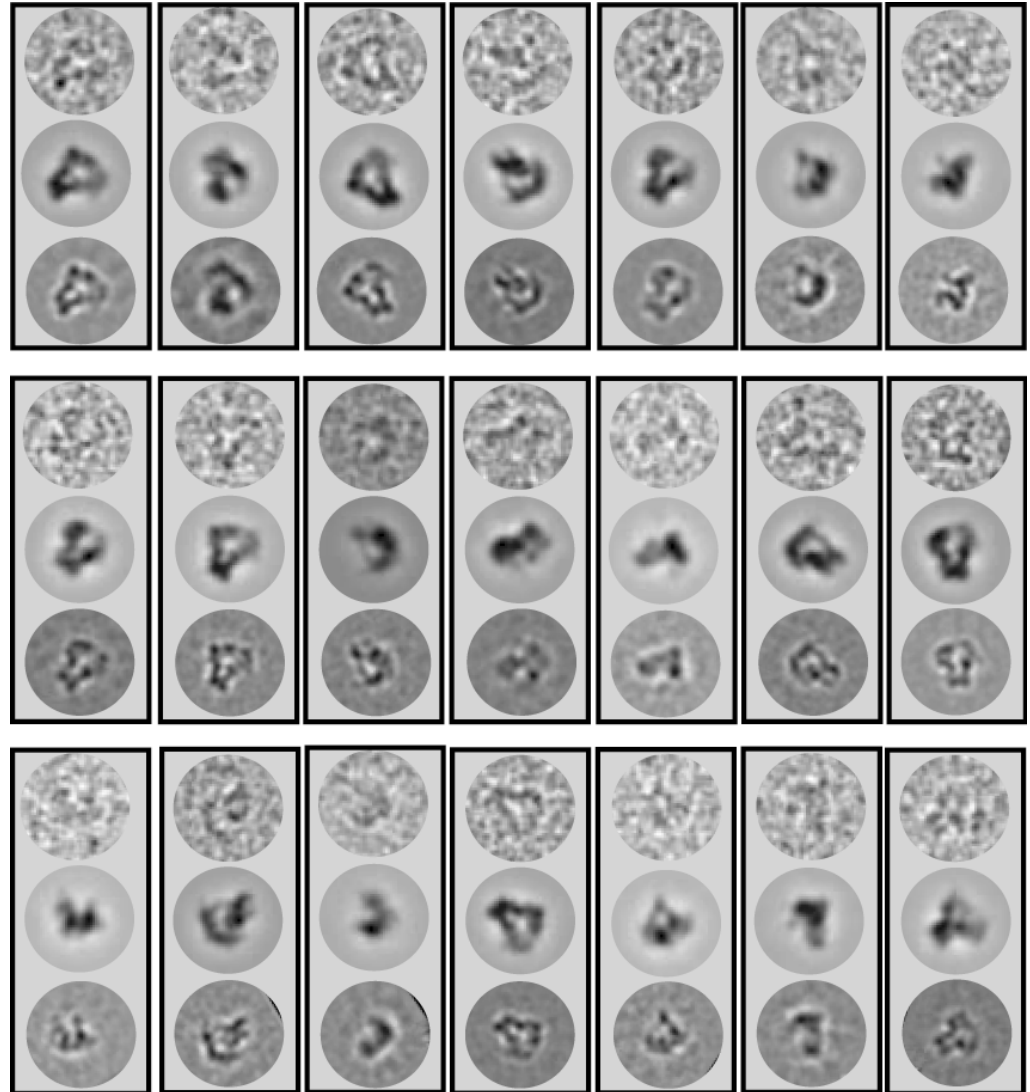
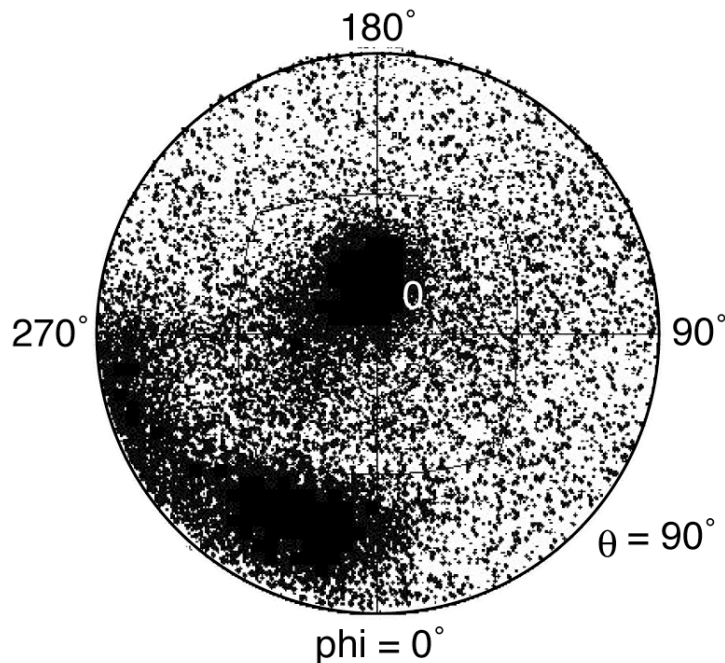
- Compare reference-free averages with projections

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

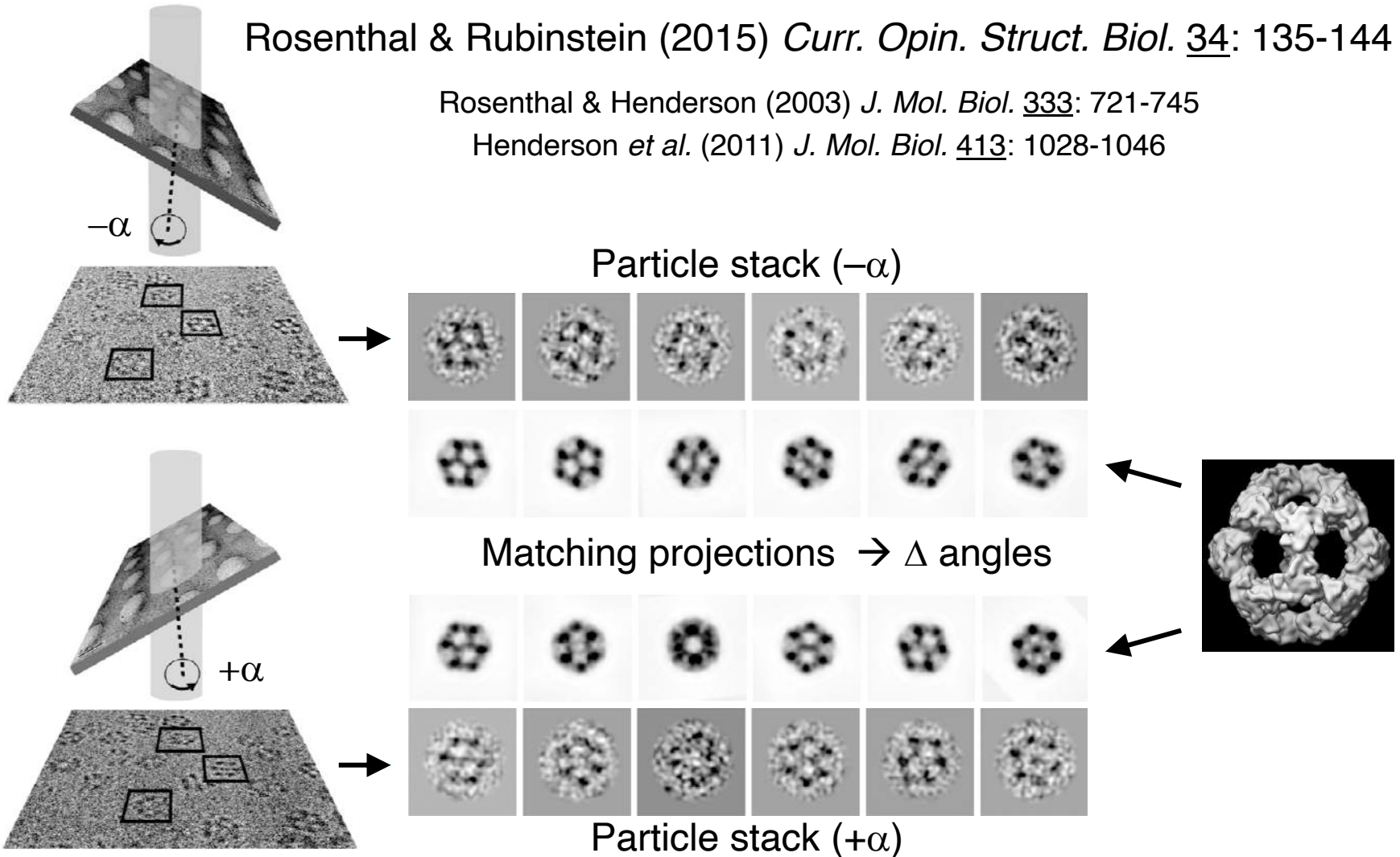
Map validation

Tilt-pair analysis

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

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

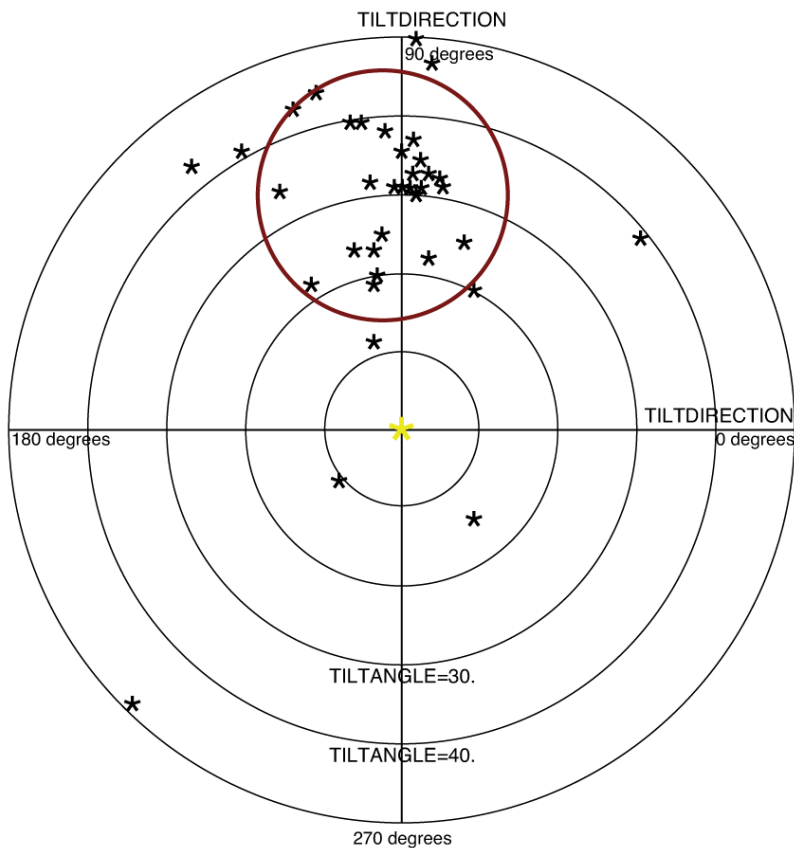
Henderson *et al.* (2011) *J. Mol. Biol.* 413: 1028-1046



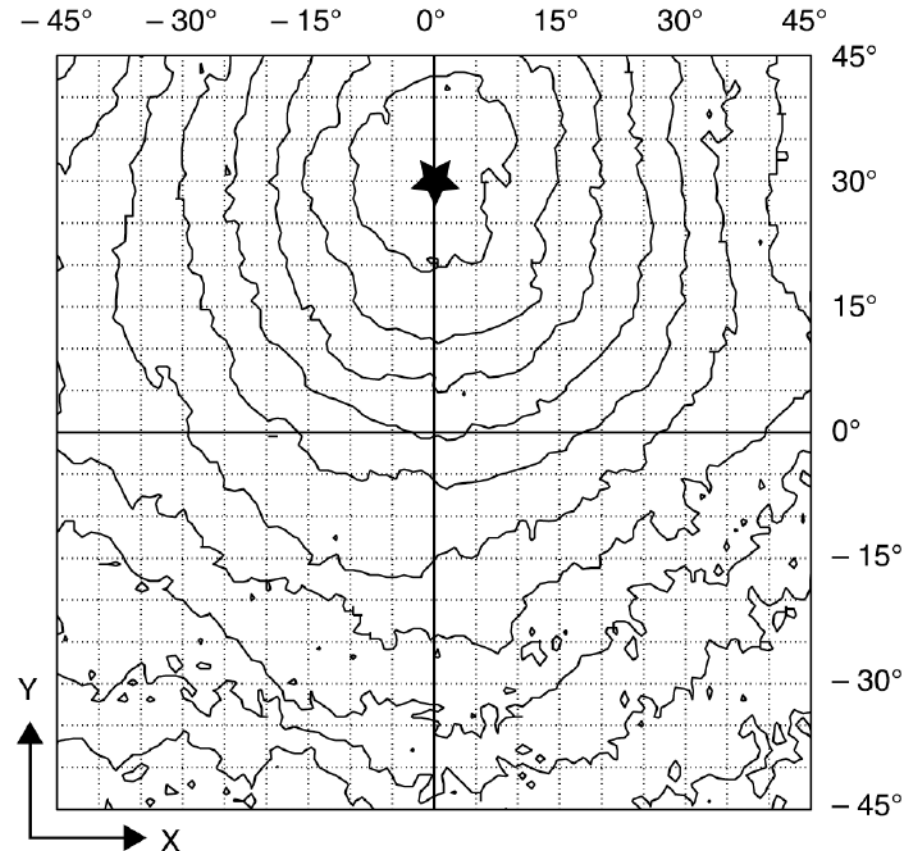
Map validation

Tilt-pair analysis

Tilt-pair parameter plot



Tilt-pair phase residual plot



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 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

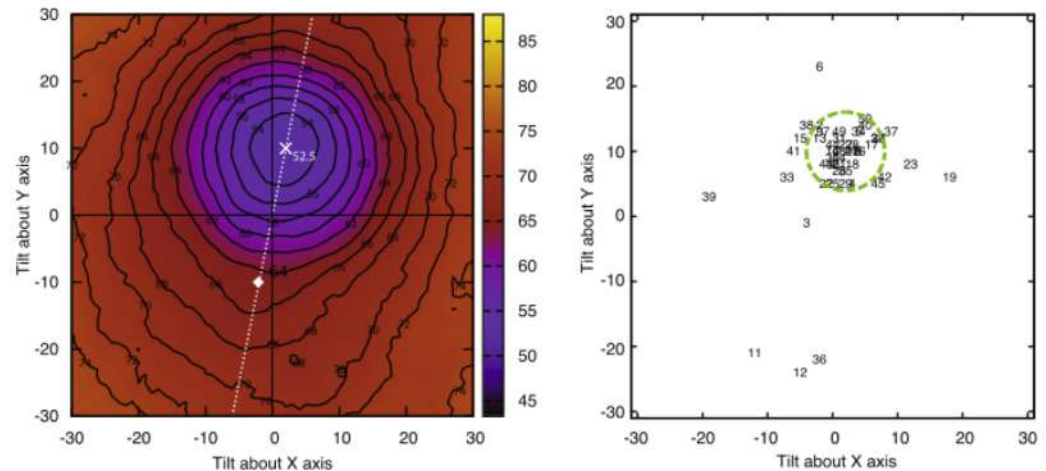
Stack 1 (max 300 particles)
Browse... stack1.mrc

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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EMDB

- Latest maps
- Latest headers
- Latest updates
- Search
- Browse
- 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? ☐

Frealign:

PDBe is a member of PDB EMDataBank

EMBL-EBI

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By name (A-Z)
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Postdocs & PhDs

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Train online

Industry

Overview
Members Area
Workshops
SME Forum

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Overview
Leadership
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 now pretty much default)
- 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

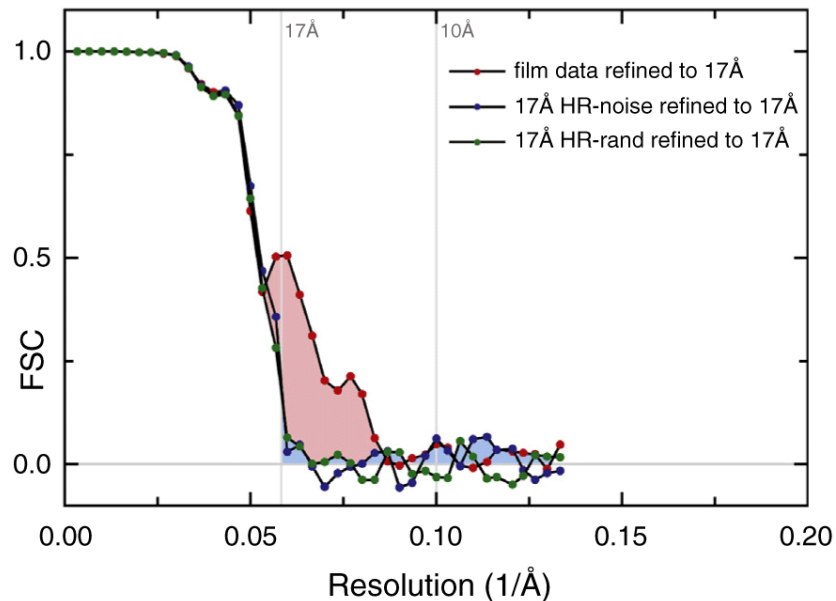
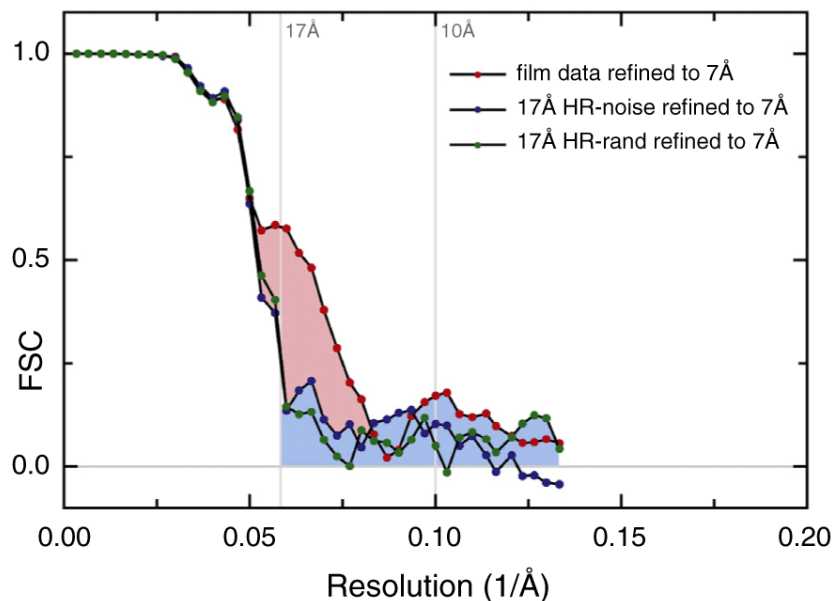
- Do single-particle reconstruction / refinement
- Determine resolution (FSC)
- Take raw data, randomize phases beyond which FSC_T falls below a threshold (75 or 80%)
- Redo the same analysis and recalculate FSC curve
- Any signal in region of randomized phases indicates issues with noise alignment in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

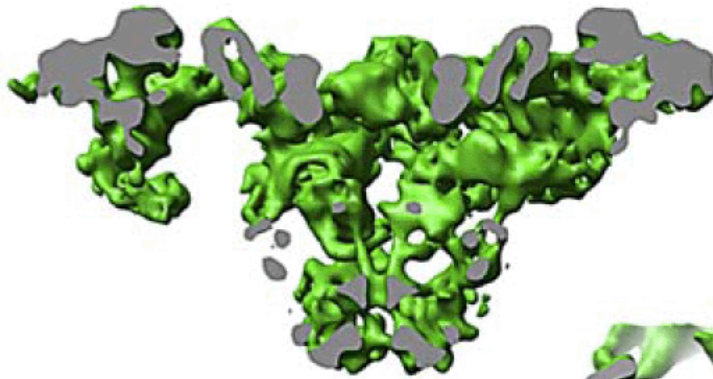
Map validation

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 - excellent (implemented in software packages)
- Appearance of expected secondary structure elements

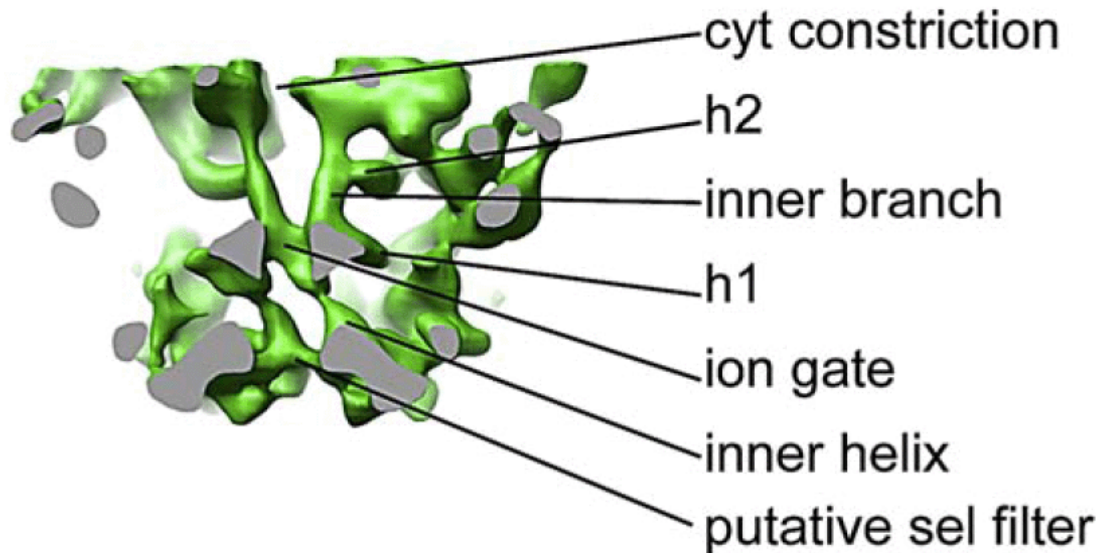
Henderson *et al.* (2012) *Structure* 20: 205-214

Map validation

Expected secondary structure



Ryanodine receptor 1
at 10.2 Å resolution



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

Map validation

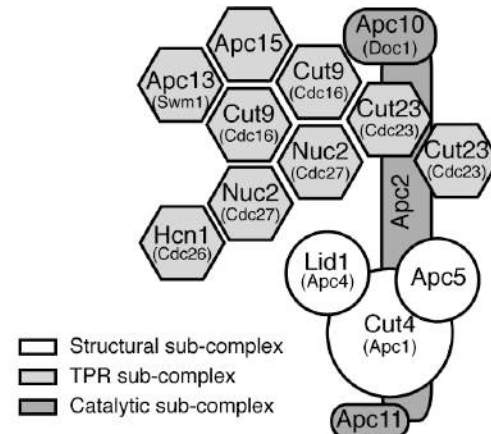
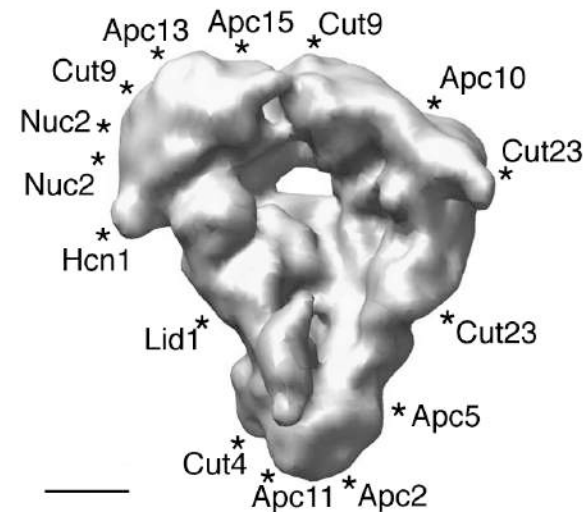
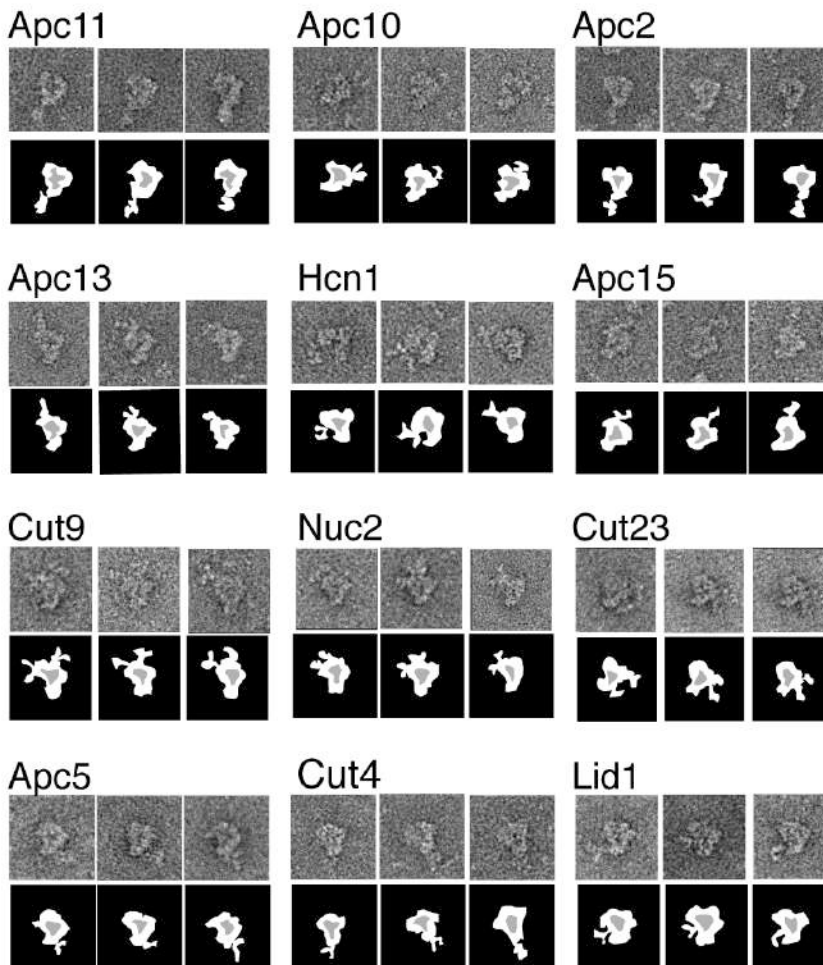
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 - excellent (implemented in software packages)
- Appearance of expected secondary structure elements
- Evaluate with published information

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 needed (but now pretty much default)
- Randomize phases
 - excellent (implemented in software packages)
- 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

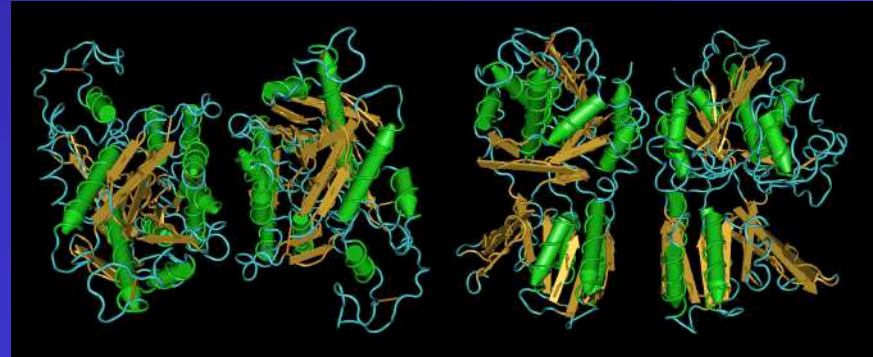
Henderson *et al.* (2012) *Structure* 20: 205-214

Map validation

Docking of atomic models

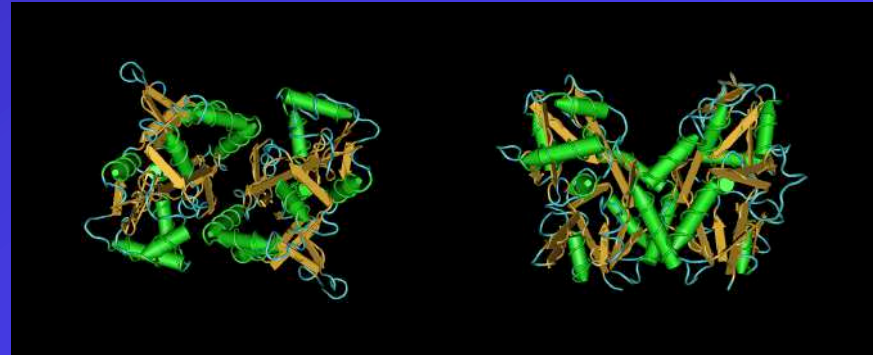
mGluR1

Kunishima et al. 2000
(K. Morikawa)



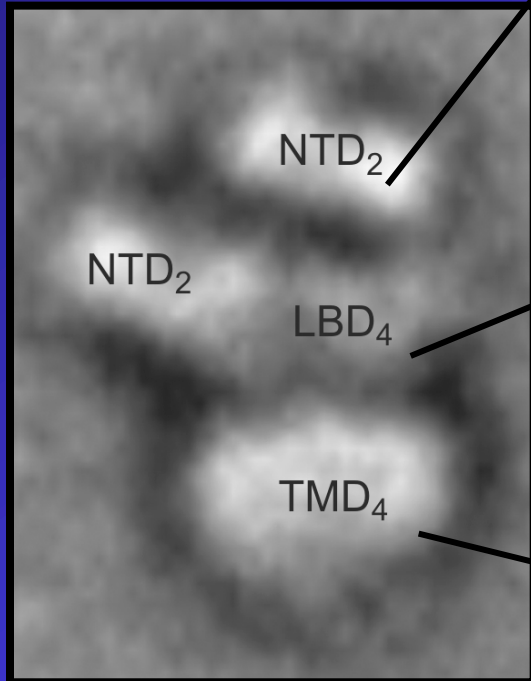
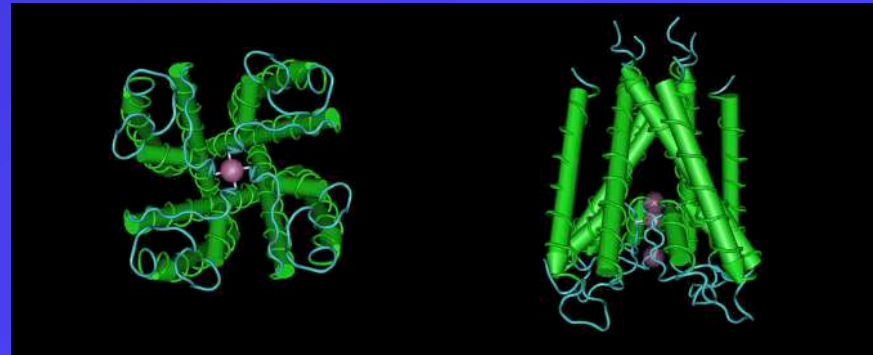
GluR2

Armstrong et al. 2000
(E. Gouaux)



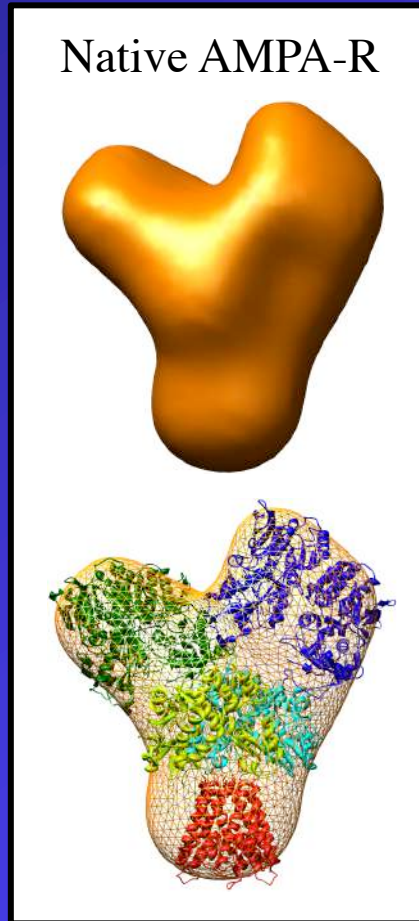
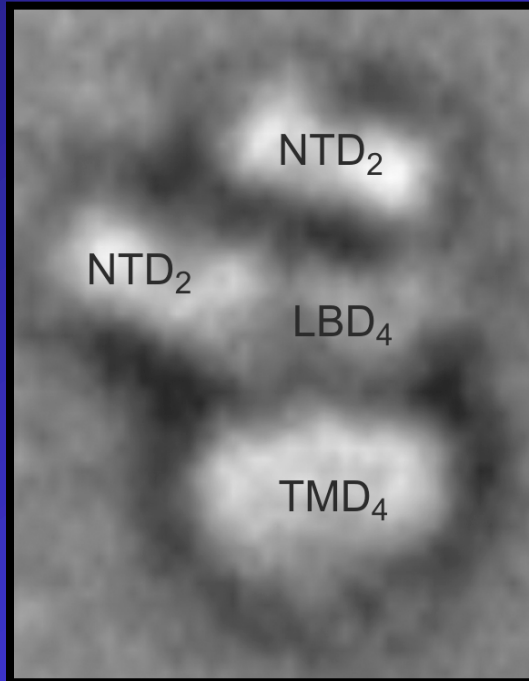
KcsA

Doyle et al. 1998
(R. MacKinnon)

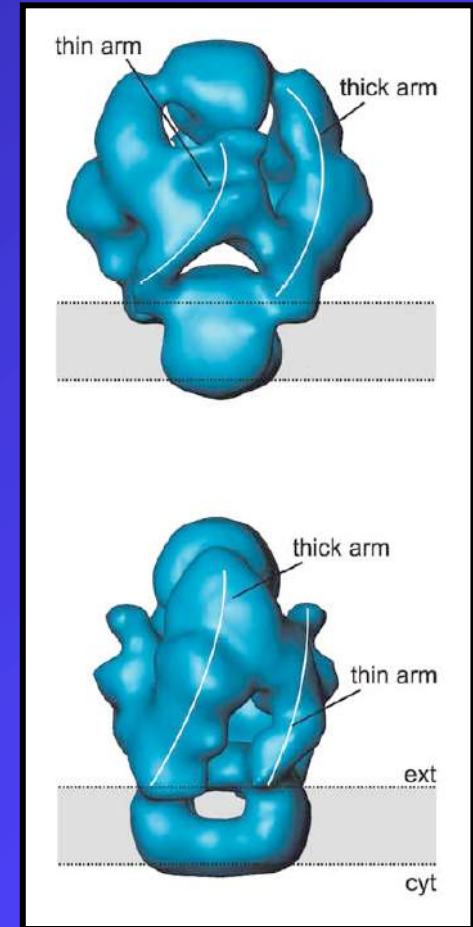


Map validation

Docking of atomic models



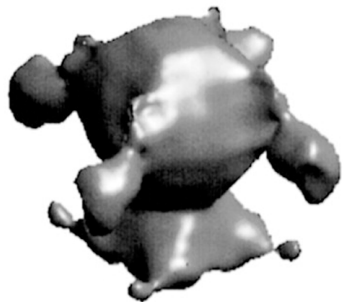
Nakagawa *et al.* (2006)
Biol. Chem. 387: 179-187



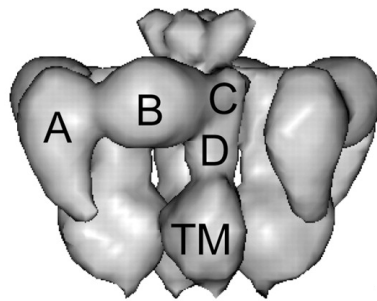
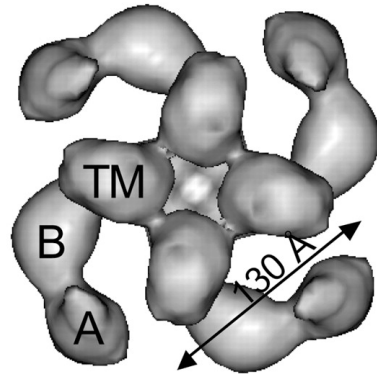
Tichelaar *et al.* (2004)
JMB 344: 435-442

Map validation - IP3 receptor

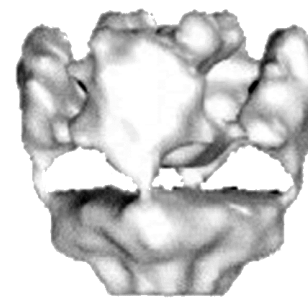
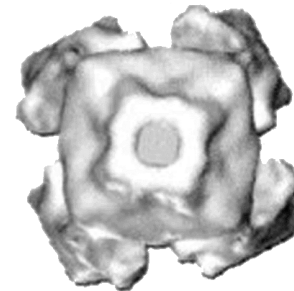
Different maps of the IP3 receptor



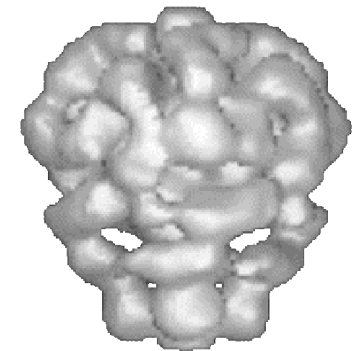
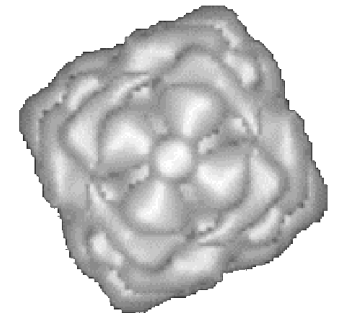
Jiang *et al.*,
2002



Serysheva *et al.*,
2003



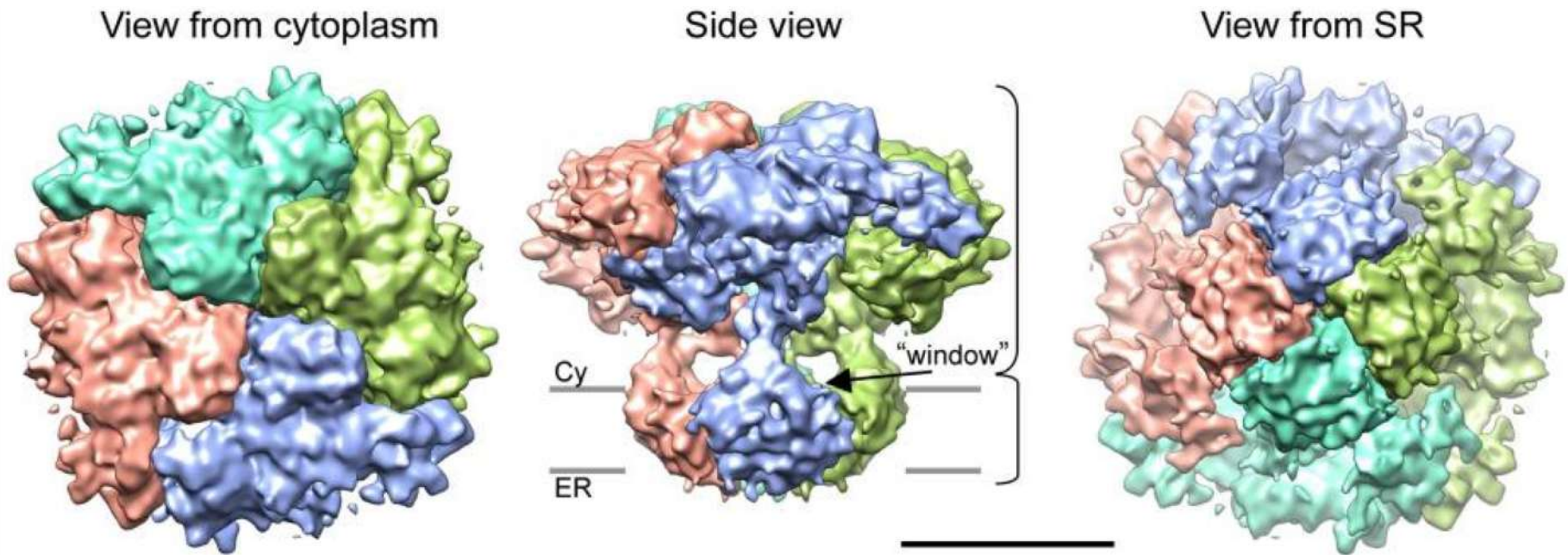
Jiang *et al.*,
2003



Sato *et al.*,
2004

Map validation - IP3 receptor

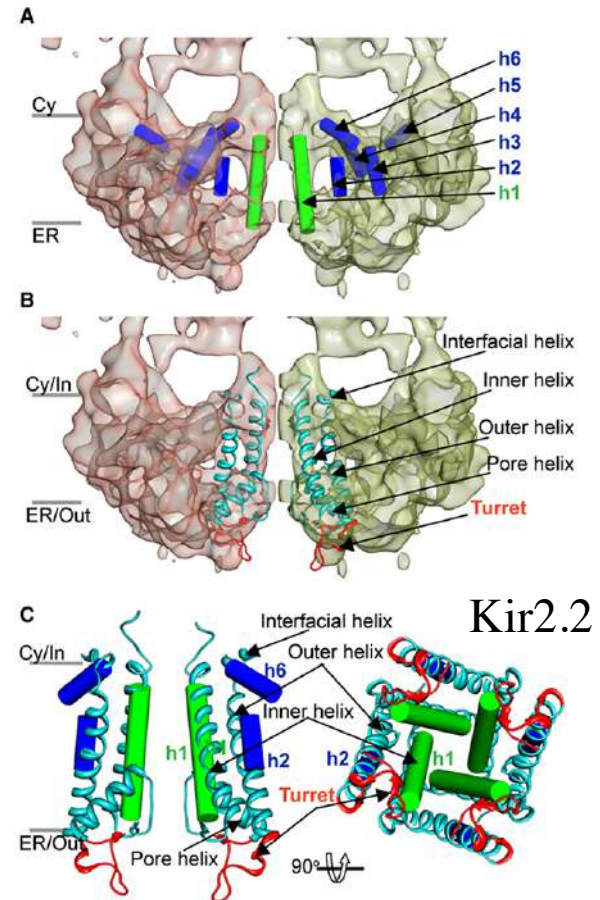
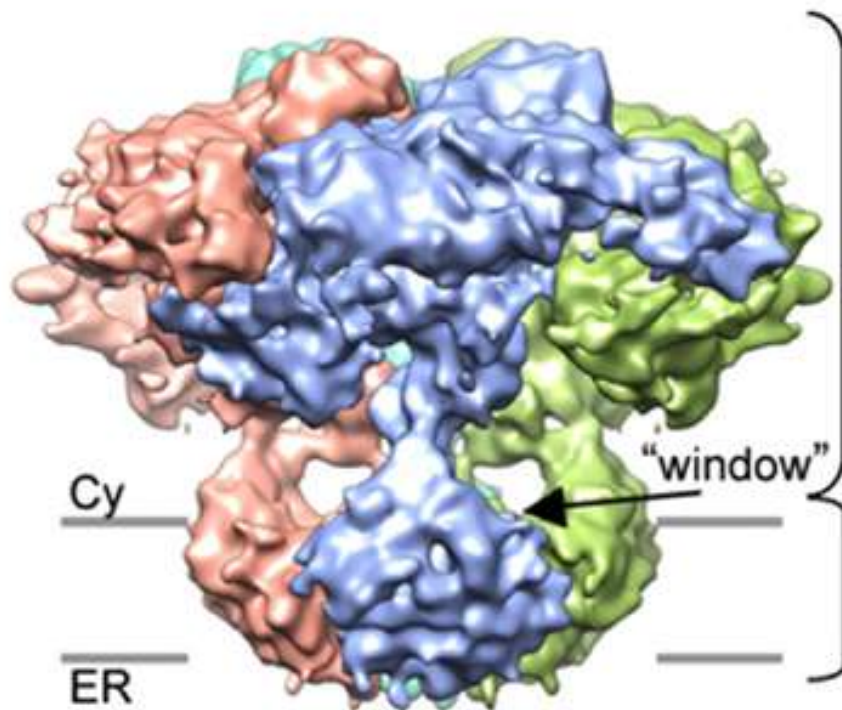
New density map in 2011 at 11 Å resolution



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

Map validation - IP3 receptor

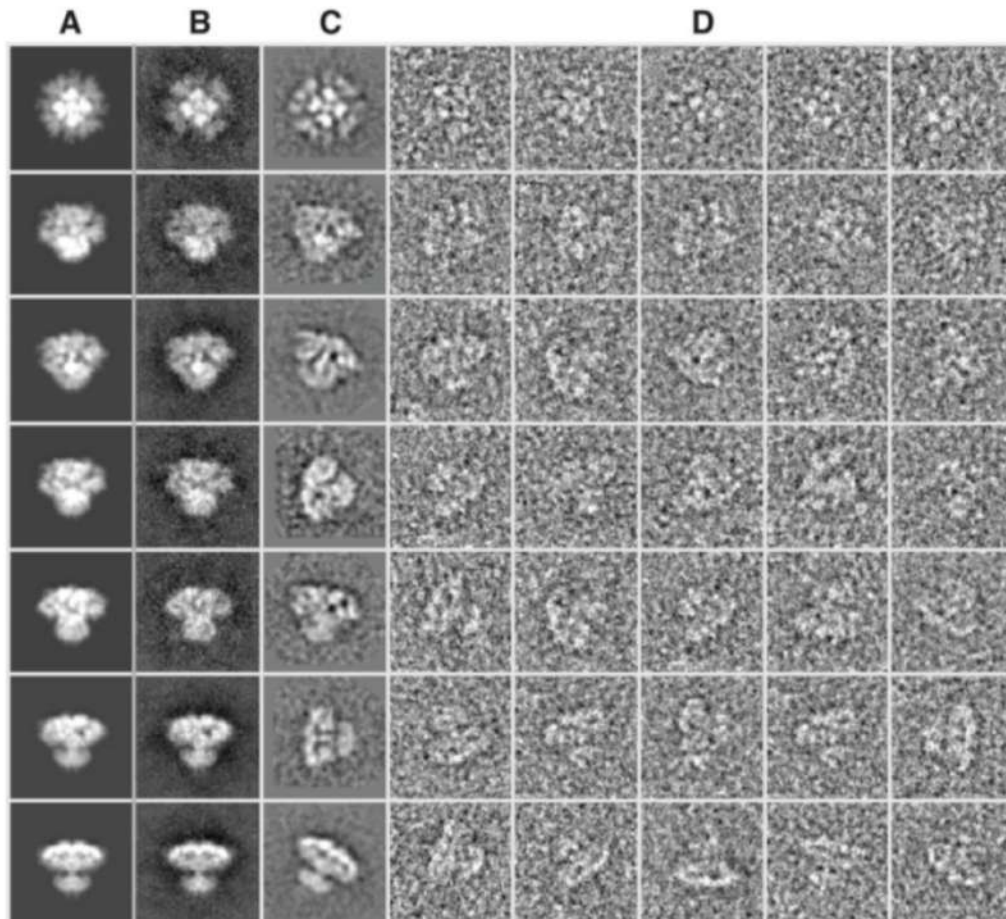
Expected secondary structure elements



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

Map validation - IP3 receptor

Comparison of reference-free averages with projections



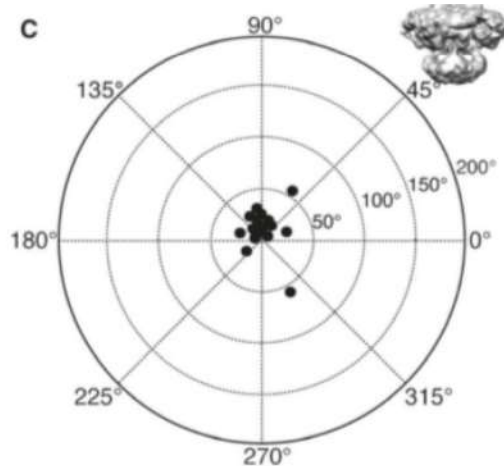
- A: Map projection
- B: Reference-based class average
- C: Reference-free class average
- D: Selected particles

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

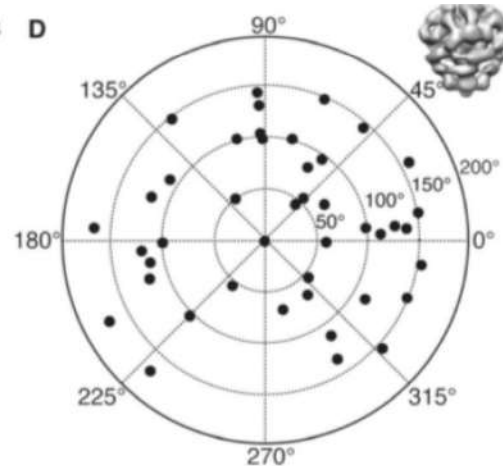
Map validation - IP3 receptor

Tilt pair test

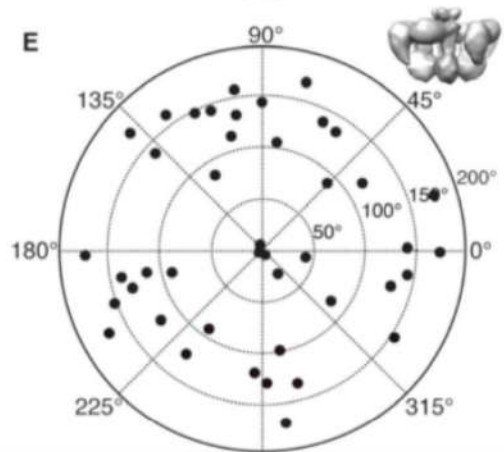
Ludtke *et al.*
2011



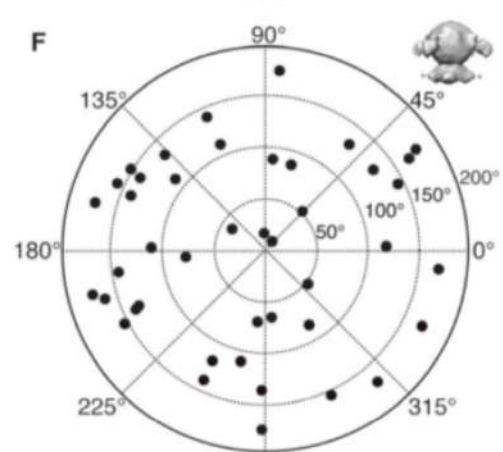
Sato *et al.*
2004



Serysheva *et al.*
2003



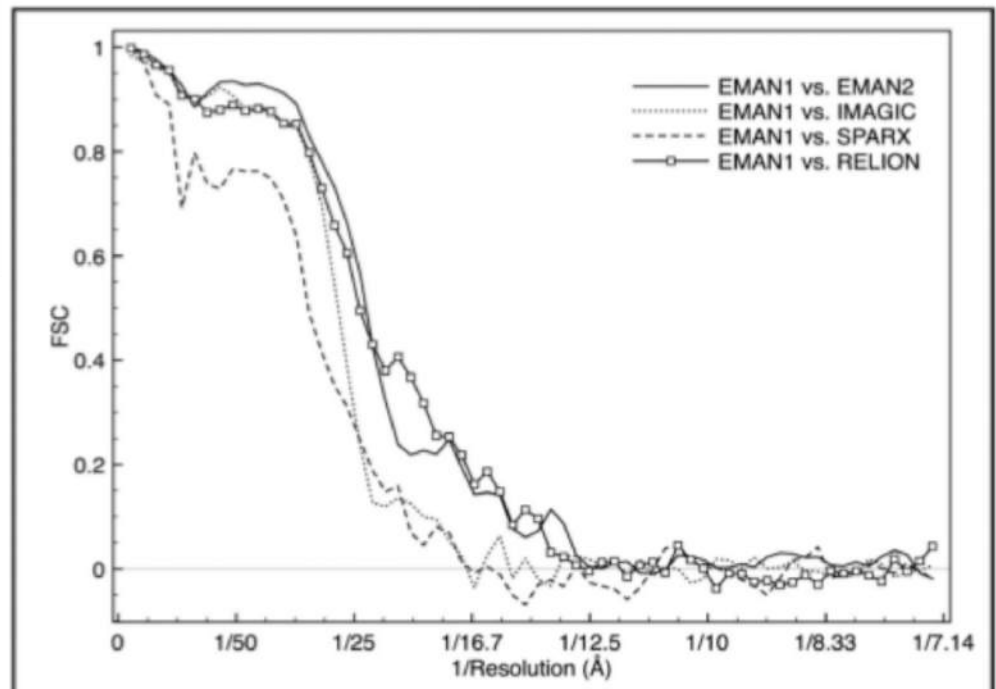
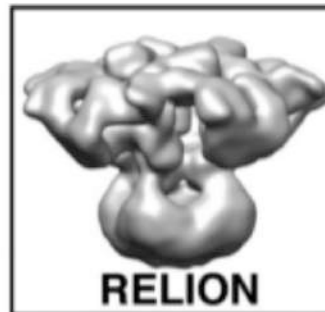
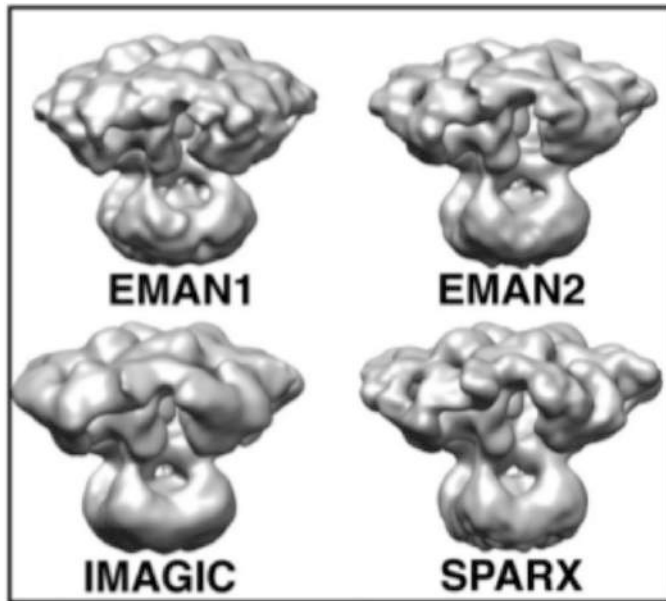
Jiang *et al.*
2002



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

Map validation - IP3 receptor

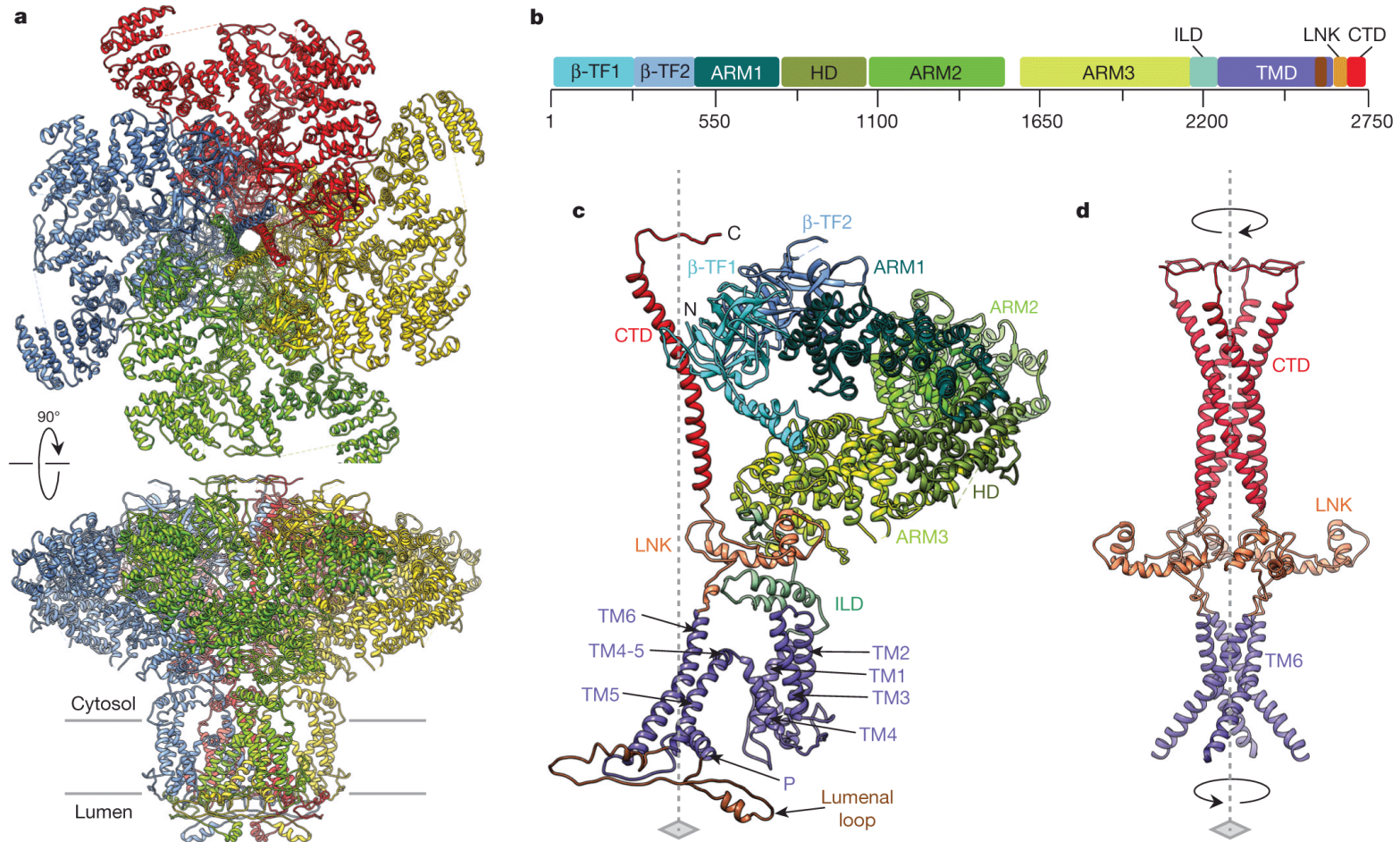
Comparison of maps from different programs



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

Map validation - IP3 receptor

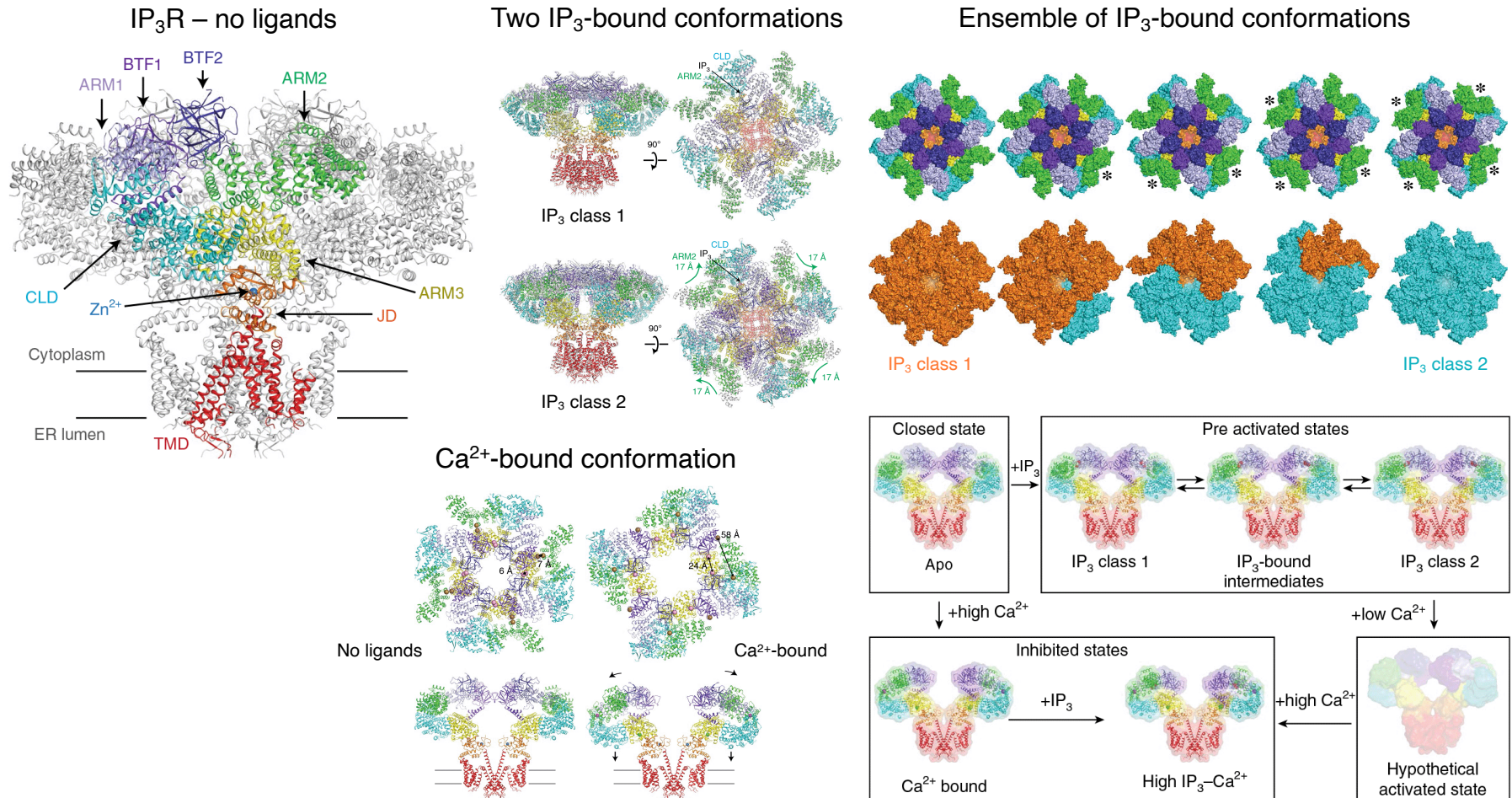
4.7 Å resolution structure (2015)



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

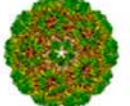
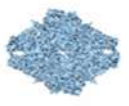
Map validation - IP₃ receptor

3.5 Å resolution structure (2018)



Map validation - the 2016 map challenge

Develop benchmark datasets, encourage development of best practices, evolve criteria for evaluation and validation, compare and contrast different approaches

target	1. GroEL <i>in silico</i>	2. T20S Proteasome	3. Apo-Ferritin	4. TRPV1 Channel	5. 80S Ribosome	6. Brome Mosaic Virus	7. β -Galactosidase
Reference EMD map entry	 --	 EMD-6287	 EMD-2788	 EMD-5778	 EMD-2660	 EMD-6000	 EMD-5995
Primary Citation	Vulovic et al	Campbell et al	Russo & Passmore	Liao et al	Wong et al	Wang et al	Bartesaghi et al
Reported Resolution ($\text{\AA}$)	~ 3	2.8	4.7	3.3	3.2	3.8	3.2

7 datasets: rigid particles that should be easy to reconstruct

Input are raw cryo-EM data (from EMPIAR)

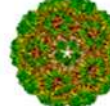
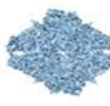
→ 27 members of the community submitted 66 maps

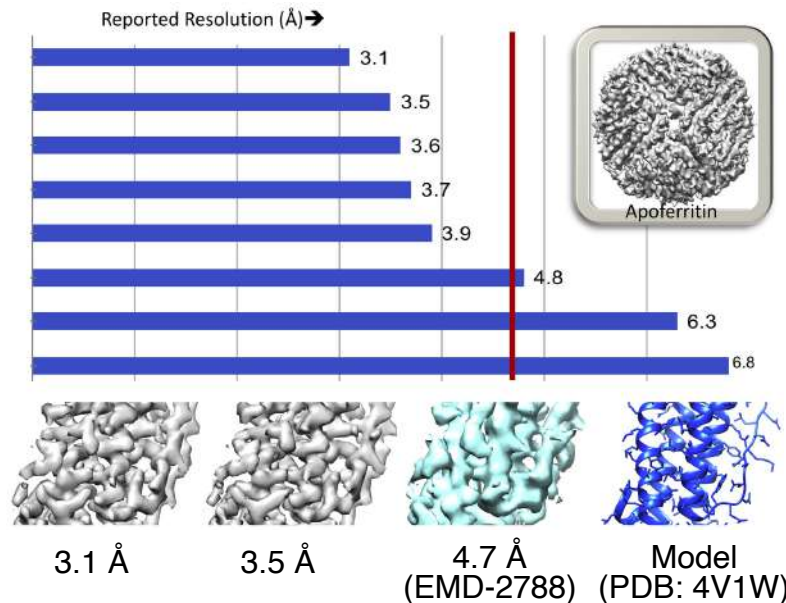
Assessors devised a range of analyses to evaluate the submitted maps, including visual impressions, Fourier shell correlation, pairwise similarity, and interpretation through modeling

December 2018 Special Issue of *J. Struct. Biol.* with contributions regarding the map and model challenges (Lawson & Chiu, Heymann *et al.*)

Map validation - the 2016 map challenge

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Current procedure to estimate resolution by FSC is not sufficiently standardized

Affected by factors such as map box size, voxel size, filtering and masking practice, and threshold value for interpretation

Map validation - the 2016 map challenge

Develop benchmark datasets, encourage development of best practices, evolve criteria for evaluation and validation, compare and contrast different approaches

Assessors found no strong trends.

No strong relationship between map quality and used software package or workflow.

The user's choices determine the map quality.

Future focus should be on promulgating best practices

processing of independent sets
proper resolution-limited alignment,
appropriate masking and map sharpening

and encapsulating these in the software.

Note that the maps had different qualities/resolutions,
BUT NONE WAS COMPLETELY WRONG !