Workshop on Advanced Topics in EM Structure Determination: Optimization and Validation

The Biological Challenges

The Scripps Research Institute
La Jolla, November 2012

Or to be more precise …

Sunday November 11 **Theme: Setting the Goals**

4:00 pm ***Registration***

4:45 pm **Welcome** **Bridget Carragher**

Session Chair: Clint Potter

5:00 pm **The Biological Challenges** **Tom Walz**

This introductory talk will describe the big picture: define what we mean by Optimization and Validation in the various subsections of our discipline: 2D crystallography, helical, icosahedral, single particle and tomography. What are the problems in each area; past, present and future of what might be possible. Perhaps compare and contrast with other methodologies (X-ray, NMR, EM, etc.), and why do we need to pay attention especially to validation in some areas. In particular why is single particle work so problematic? Perhaps illustrate these points with real successes and embarrassing failures (without offending people). Do the problems get bigger as we approach atomic resolution? What are the challenges in looking at intermediate states? How do we identify bad images? What is the current state of the art and the future prospects? What should we pay attention to and what should we NOT do? We expect the talk will focus on single particles which is where the likelihood of making mistakes is greatest and where there seems to be the most interest in the field right now.

In 60 minutes!

What I will actually try to cover …

ultra-brief history and future of different approaches as well as aspects of optimization and validation

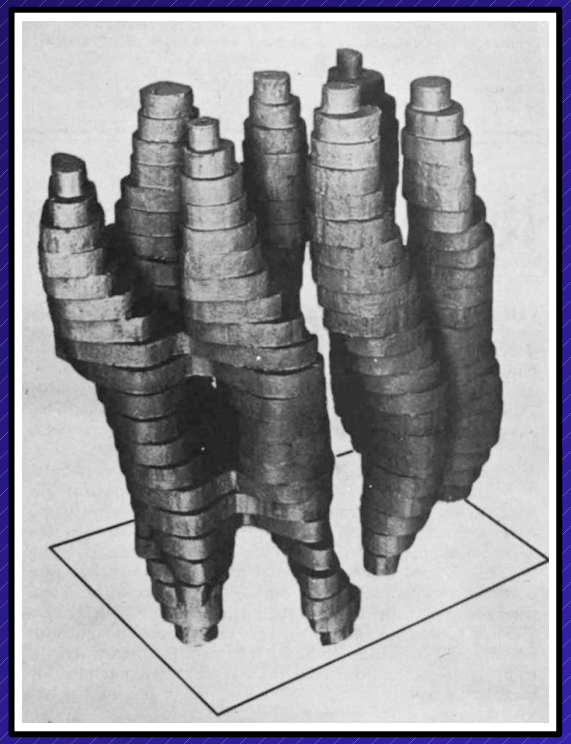
– electron crystallography
– helical reconstruction
– icosahedral symmetry
– electron tomography

– single-particle EM

– ultra-brief history
– some success stories
– future of single-particle EM (short, more from Niko)
– the dark side of single-particle EM (why so problematic?)
– sample heterogeneity
– DOs and DON'Ts in single-particle EM
– validation of EM maps

Electron crystallography – a bit of history

– pioneered by Richard Henderson and Nigel Unwin
using purple membrane (naturally occurring 2D array)



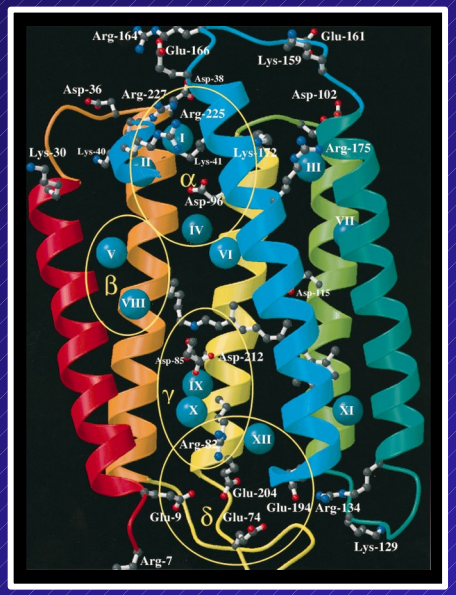
Henderson & Unwin (1975)
Nature 257: 28-32

density map at 7 Å resolution of bacteriorhodopsin
– first visualization of transmembrane α-helices

– sugar embedding for specimen preparation
– first software for 2D data processing (MRC)

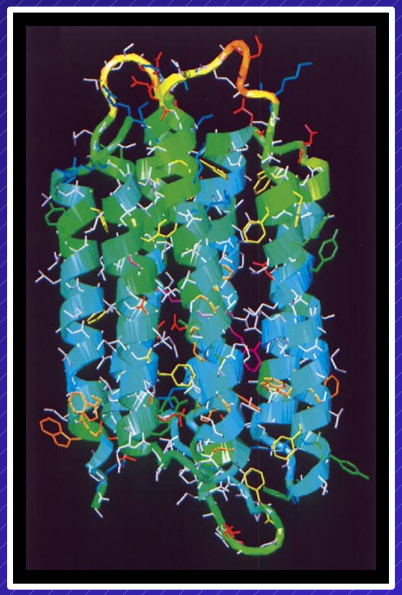
Electron crystallography – a bit of history

– further developed by Richard Henderson, Bob Glaeser and Yoshi Fujiyoshi (and contributions by many others)



Henderson *et al.* (1990)
JMB 213: 899-929

Grigorieff *et al.* (1996)
JMB 259: 393-421



Kimura *et al.* (1997)
Nature 389: 206-211

Mitsuoka *et al.* (1999)
JMB 286: 861-882

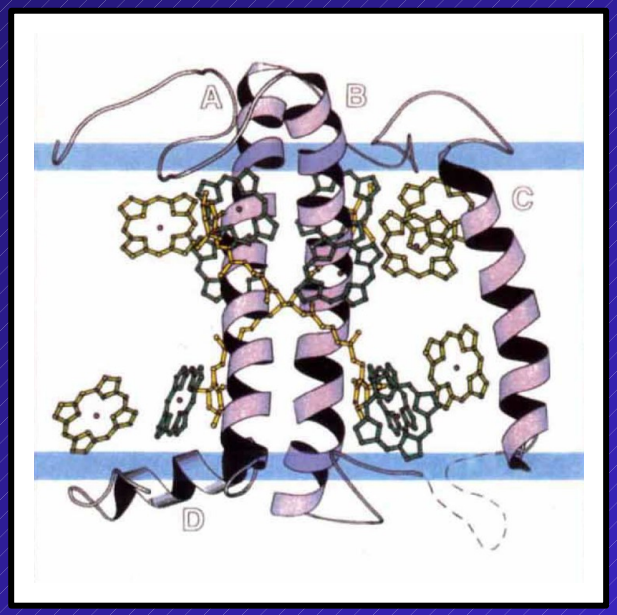
atomic model of bacteriorhodopsin
mechanistic insights in H+ transport

– low-dose imaging
– cryo-EM and specimen holder
– field emission electron source
– CCD camera
– intermediate voltage EM
– structure refinement
– top-entry specimen stage
– He cooling

Electron crystallography – a bit of history

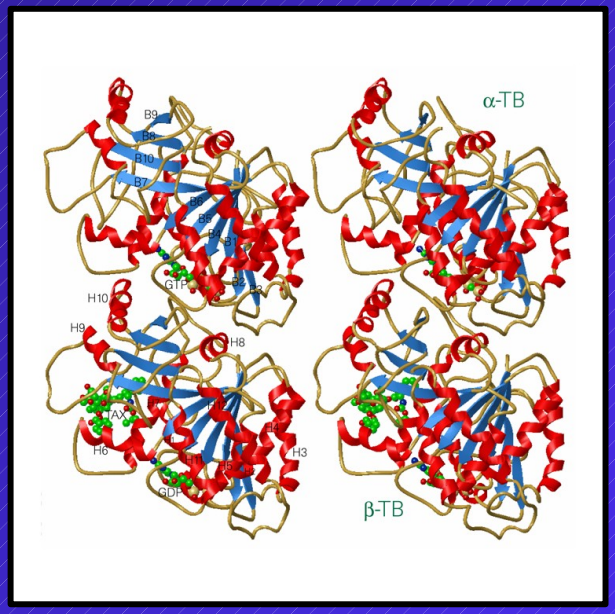
– first atomic models from artificially formed 2D crystals

light-harvesting complex 2
– membrane protein
(Werner Kühlbrandt)



Kühlbrandt *et al.* (1994)
Nature 367: 614-621

α/β tubulin dimer
– soluble protein
(Ken Downing)



Nogales *et al.* (1998)
Nature 391: 199-203

Electron crystallography – a bit of history

more atomic structures

– Aquaporins
  – AQP1 (Engel & Fujiyoshi)
    (Mitra)
  – AQP4 (Fujiyoshi)
  – AQP0 (Walz & Fujiyoshi)

– Several MAPEG proteins
  (Hans Hebert & Fujiyoshi/Mitsuoka)

many intermediate-resolution structures

reviewed in: Abeyrathne *et al.* (2012) *Comprehensive Biophysics* Volume 1

→ should be possible to extend to high resolution

Advances:

– new 2D crystallization methods
  – BioBeads (Rigaud)
  – dilution, chelation (Engel)
– new specimen preparation techniques
  – tannic acid (Downing, Kühlbrandt)
  – trehalose, carbon sandwich (Fujiyoshi)
– automated 2D crystallization screens (Engel, Stokes)
– automated specimen preparation and screening (Engel, Stokes)
– different phasing approaches
  – molecular replacement (Walz)
  – projective constraint optimization (Stahlberg)
  – fragment-based phase extension (Gonen)
– new software
  – 2dx (Stahlberg)
  – IPLT (Engel)

Electron crystallography – the future (if there is one)

Serious competition from X-ray crystallography

– needs less material (liquid handling robots)
– can handle smaller crystals (microdiffraction)
– membrane proteins can be stabilized through mutations (GPCRs)
– crystallization in lipidic cubic phase, lipidic bicelles, and presence of lipids can provide native environment
– very fast data collection and data processing
– highly automated

Some competition from NMR

– solid-state NMR is (slowly) advancing
– fragment searching method (UCP2 – Berardi *et al.* (2011) *Nature* 476: 109-113)

BUT:

– any kind of 2D crystal can provide structural information
– combination with single-particle techniques should be powerful

Electron crystallography – optimization & validation

Optimization

– optimize 2D crystallization → screening robots
– optimize specimen preparation (flatness, embedding)
– optimize data collection → movies (automation)
– optimize data processing → new algorithms
e.g., single-particle approach for poorly ordered crystals
profile fitting for diffraction patterns of vesicular crystals
real-space approach for stacked 2D crystals
→ make best use of every crystal

Validation

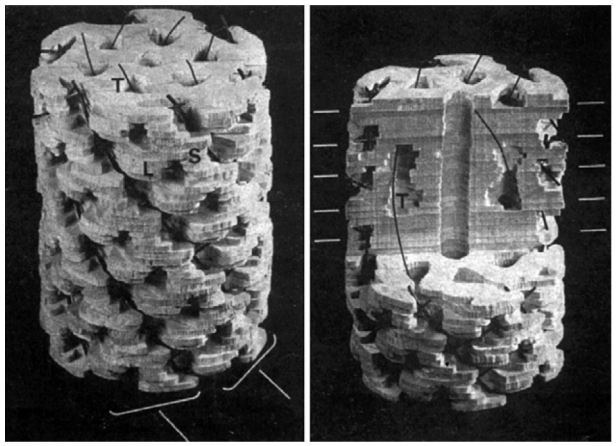
– not critical – crystallographic approach
– handedness can still be an issue at intermediate resolution

Helical reconstruction – a bit of history

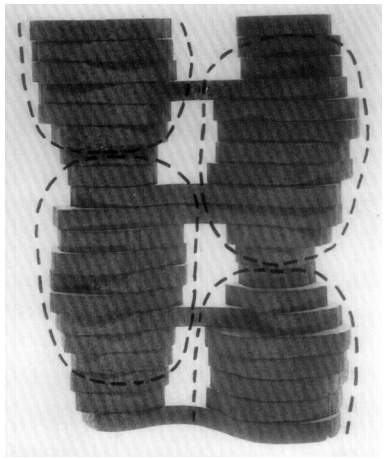
– pioneered by David DeRosier with Aaron Klug
using helical virus samples (TMV, T4 phage tail)

density maps of helical virus and actin structures

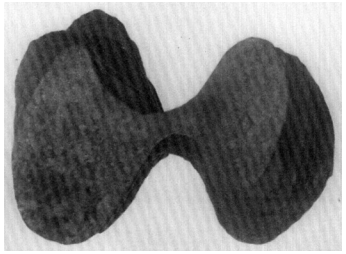
T4 phage tail



DeRosier & Klug (1968)
Nature 217: 130-134



actin



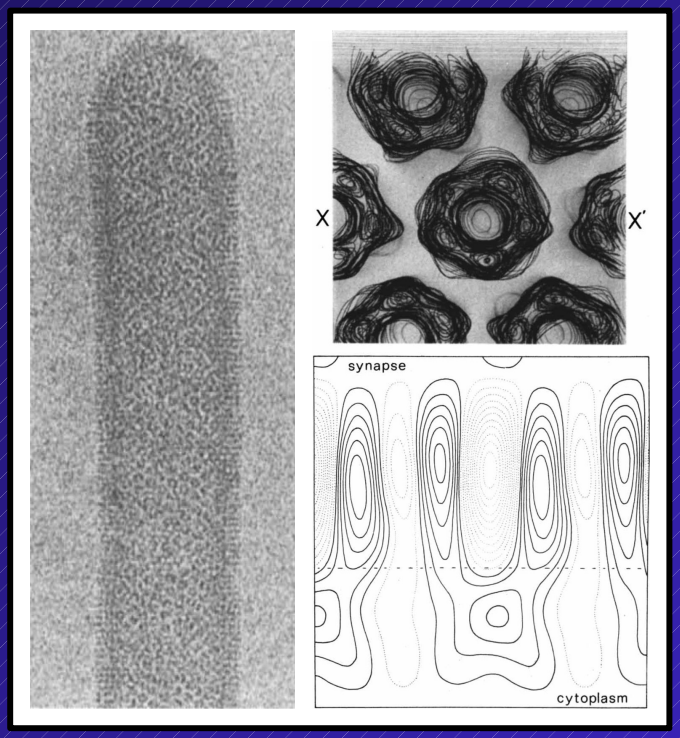
Moore *et al.* (1970)
JMB 50: 279-295

– first computational FFT
– first 3D reconstruction algorithm
(Klug & DeRosier (1966) *Nature* 212: 29-32)

Helical reconstruction – a bit of history

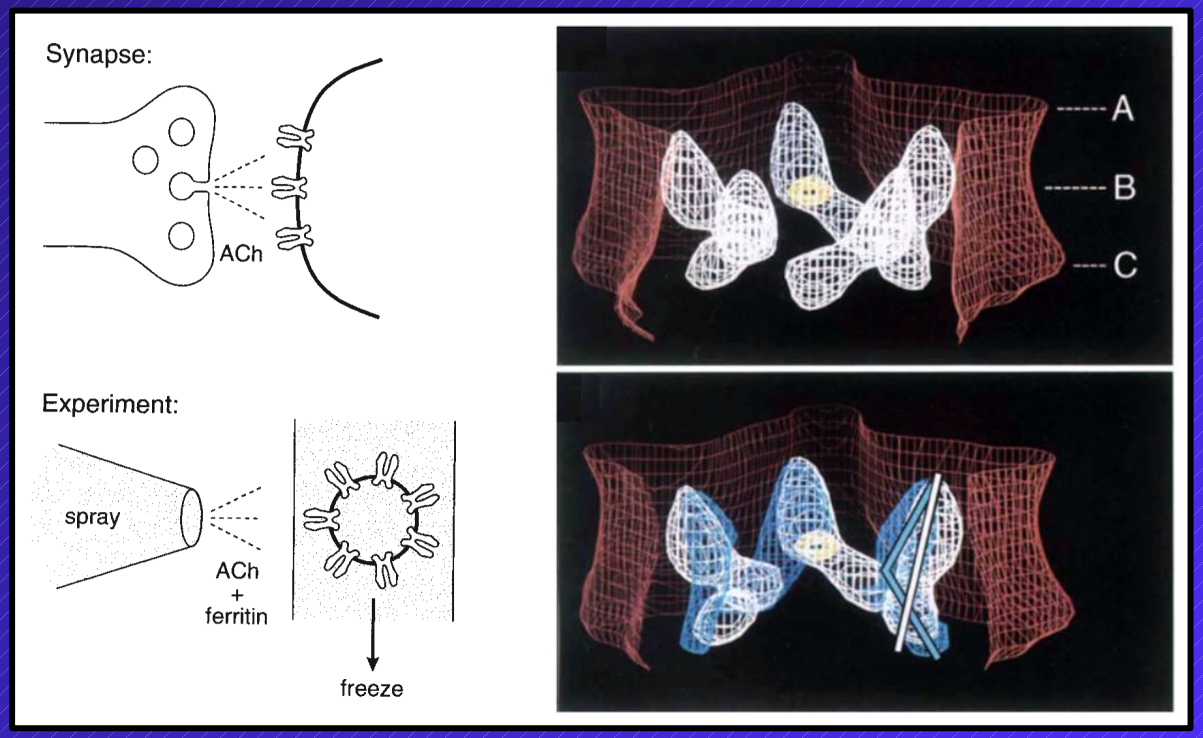
– applied to tubular crystals by Nigel Unwin
using nicotinic acetylcholine receptor

use of vitrified specimen



Brisson & Unwin (1985)
Nature 315: 474-477

time-resolved EM

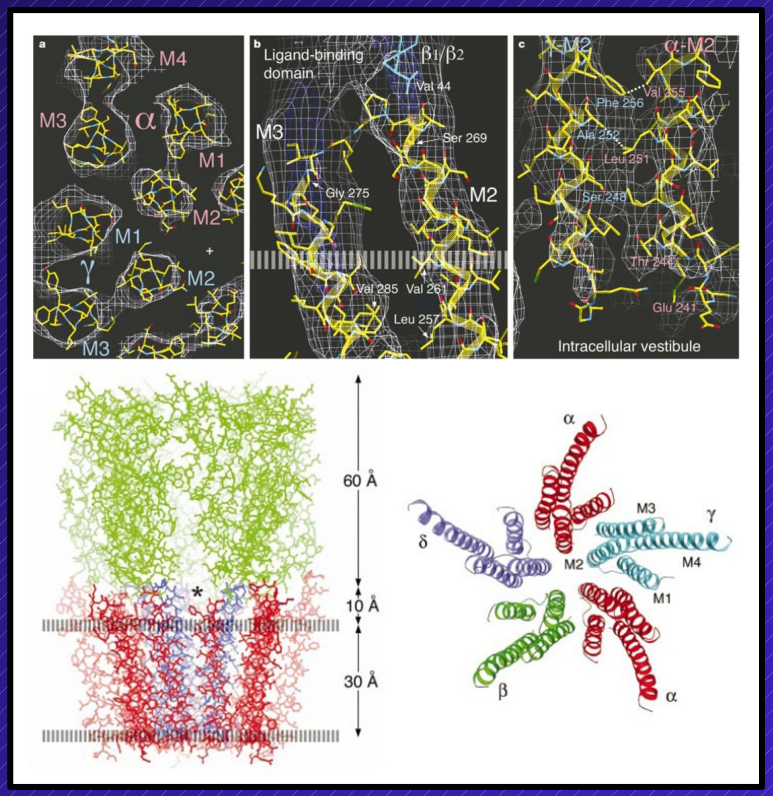


Unwin (1993)
Nature 50: 279-295

Helical reconstruction – a bit of history

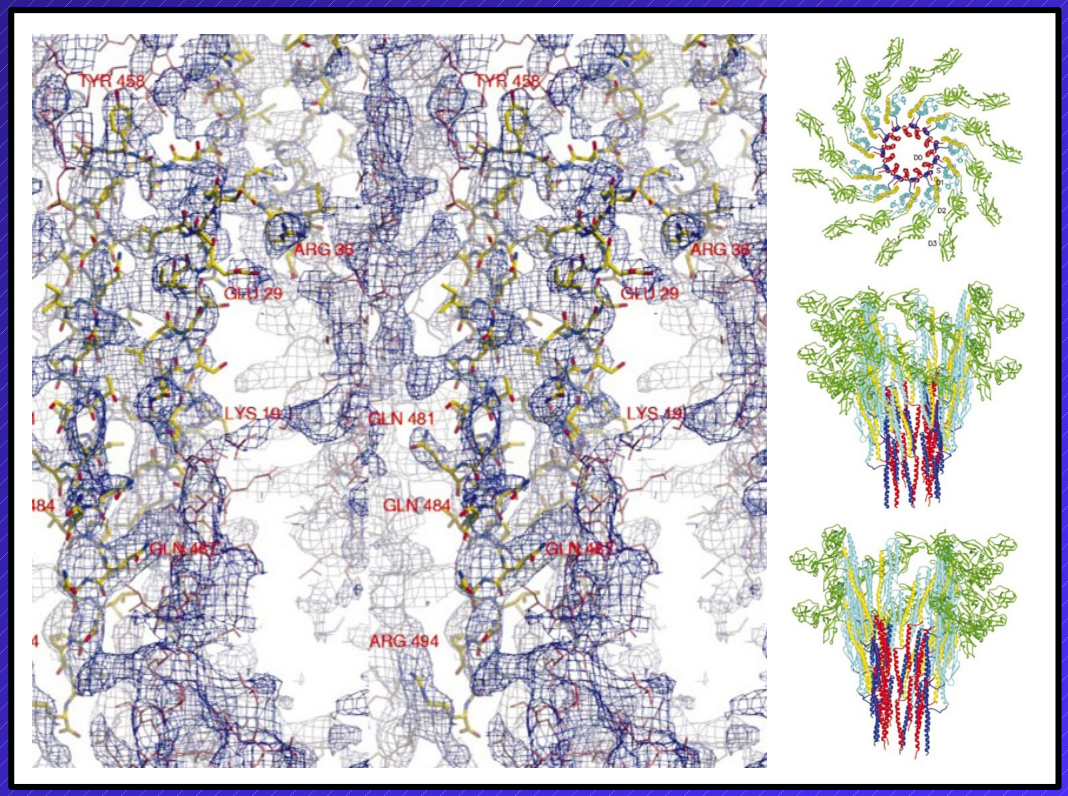
– first atomic models obtained with helical specimens

acetylcholine receptor at ~4 Å



Miyazawa *et al.* (2003)
Nature 423: 949-955

bacterial flagellar filament at ~4 Å



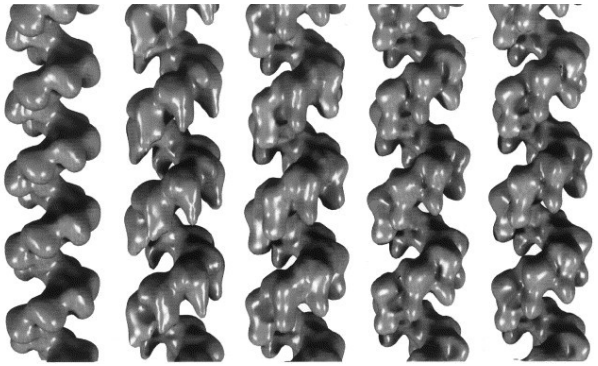
Yonekura *et al.* (2003)
Nature 424: 643-650

Helical reconstruction – a bit of history

– new approaches

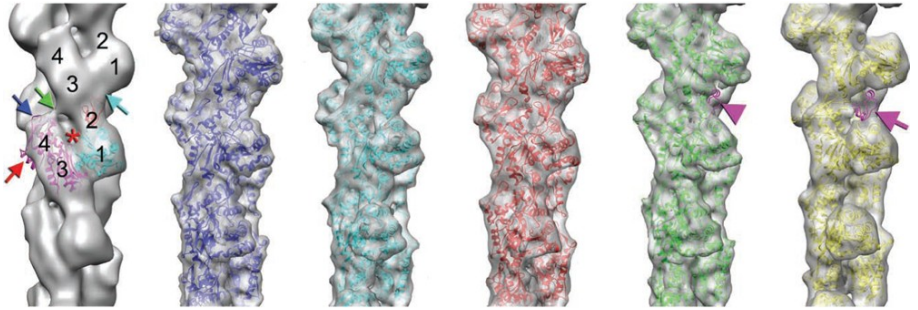
iterative helical real-space reconstruction

bacterial RecA filament



Egelman (2000) *Ultramicroscopy* 85: 225-234

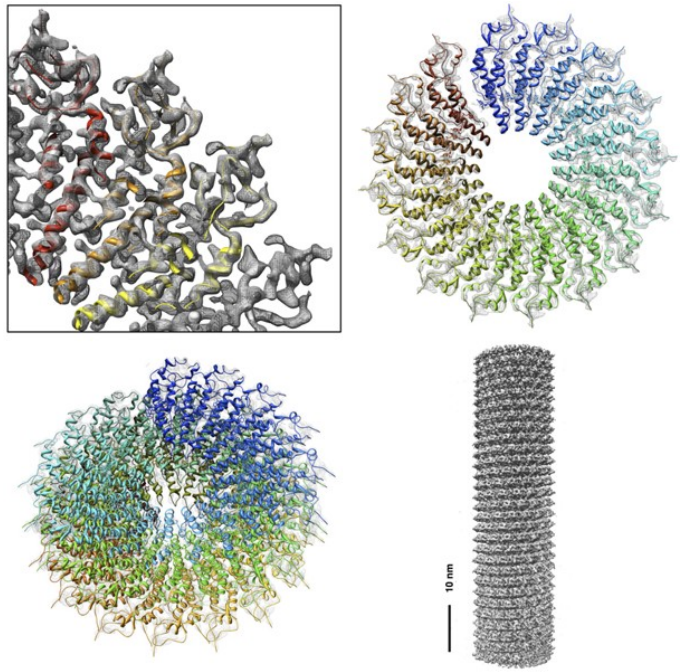
structural polymorphism in F-actin at ~10 Å



Galkin *et al.* (2010) *NSMB* 11: 1318-1324

real-space refinement (FREALIX)

TMV at ~4.5 Å



Sachse *et al.* (2007)
JMB 371: 812-835

Helical reconstruction – the future (looking pretty good)

No need to tilt & increasing number of samples with helical symmetry

– cytoskeletal proteins (in particular bacterial homologs) and associated proteins
– many DNA- and RNA-binding proteins
– amyloid-forming proteins
– tubular 2D crystals

What needs to get done:

– for tubular 2D crystals: same as for planar 2D crystals
– for any helical specimen:
  – optimize data collection by automation and recording movies
    → higher yield of useful data
  – robust method to create initial model (or define helical selection rule)
  – further improve software for alignment and classification

Helical reconstruction – optimization & validation

Optimization

– for tubular crystals: optimize 2D crystallization → screening robots
– specimen preparation – vitrification does not always work → ?
– optimize data collection → automation, movies
– optimize data processing → improve algorithms

Validation

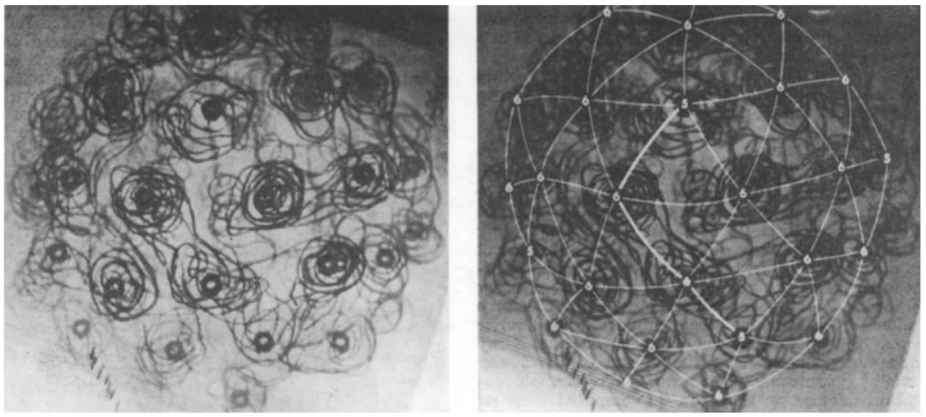
– important for low-resolution structures (due to difficulties in determining the correct helical selection rule from FFTs)
– convergence of structure (even in IHRSR) not sufficient !
– currently only possible if atomic structure is known – for protein (or at least part of it)

Icosahedral reconstruction – a bit of history

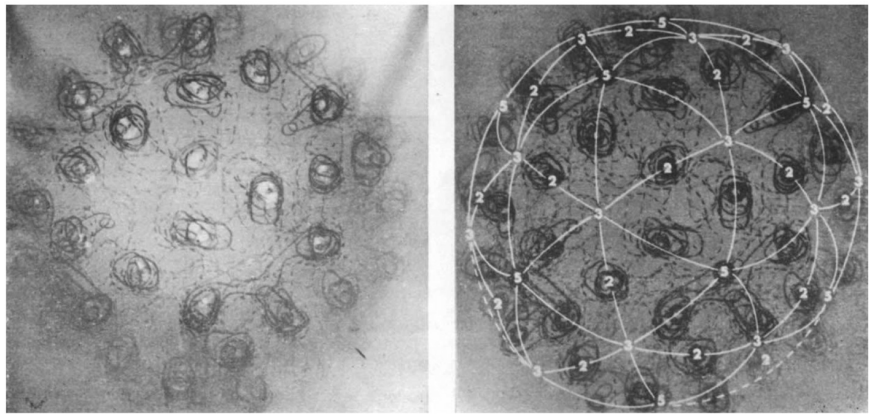
– pioneered by Tony Crowther (with David DeRosier and Aaron Klug) using human wart virus and tomato bushy stunt virus

density maps of icosahedral viruses

human wart virus



tomato bushy stunt virus

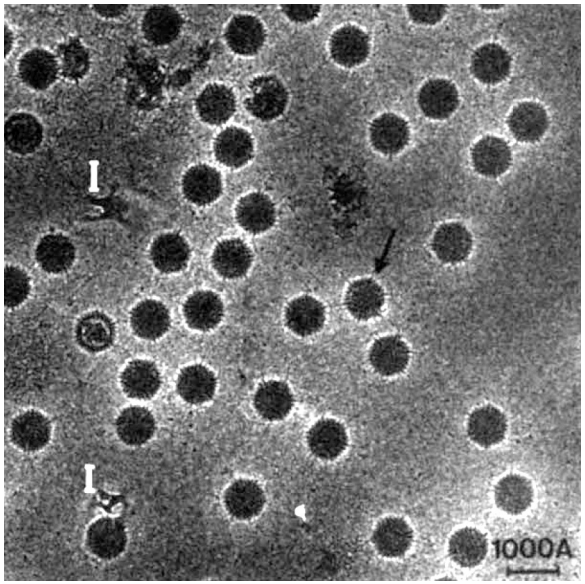


Crowther *et al.* (1970) *Nature* 226: 421-425

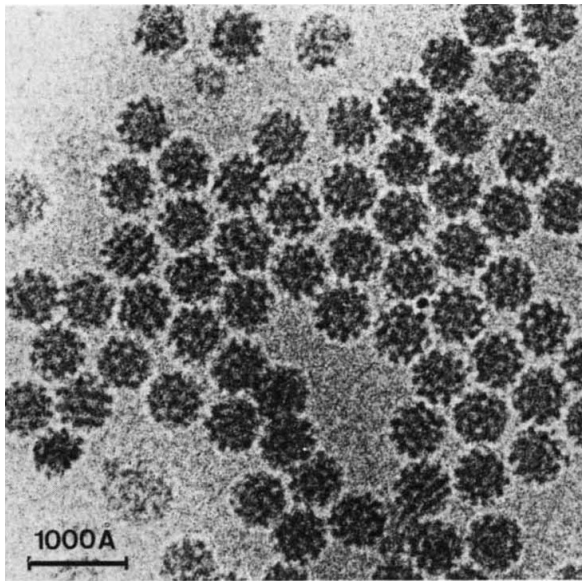
– first algorithms to reconstruct icosahedral specimens
(Crowther *et al.* (1970) *Proc. R. Soc. Lond.* 317: 319-340
(Crowther (1971) *Phil. Trans. R. Soc. Lond. B* 261: 221-230)

Icosahedral reconstruction – a bit of history

– vitrification pioneered by Jacques Dubochet (with Marc Adrian)
  - specimen preservation in near-native environment



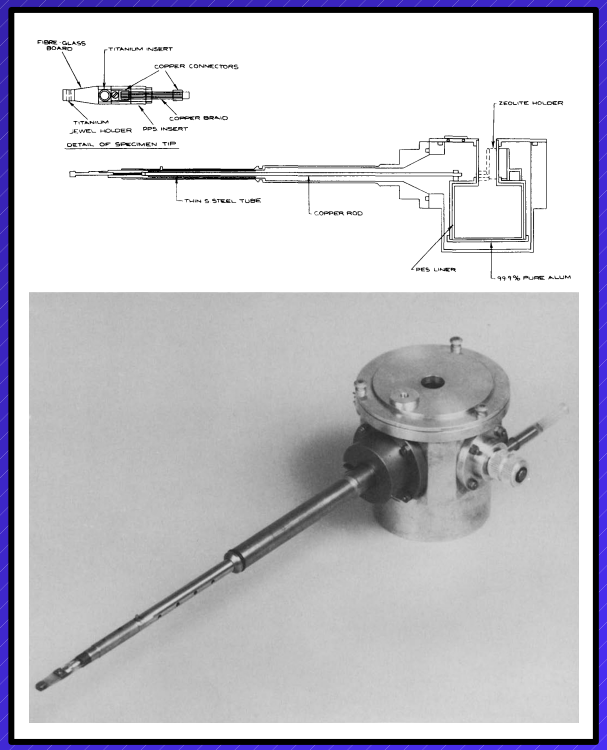
adenovirus type 2



Semliki Forest virus

Adrian *et al.* (1984) *Nature* 308: 32-36

cryo-specimen holder

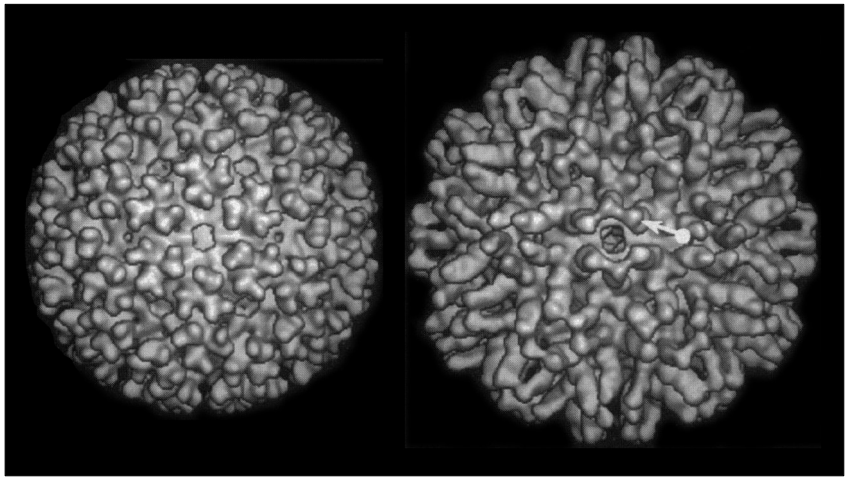


Henderson *et al.* (1991) *Ultramicroscopy* 35: 45-53

Icosahedral reconstruction – a bit of history

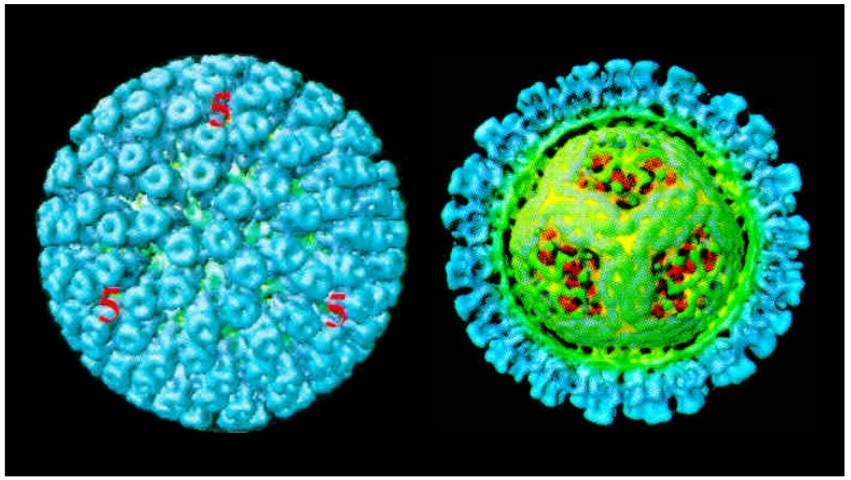
– many virus structures at ~25 Å
– subunit organization
– receptor and antibody binding
– genome organization

Ross River virus – Fab binding



Smith *et al.* (1995)
PNAS 92: 10648-10652

Rotavirus DLP – RNA organization

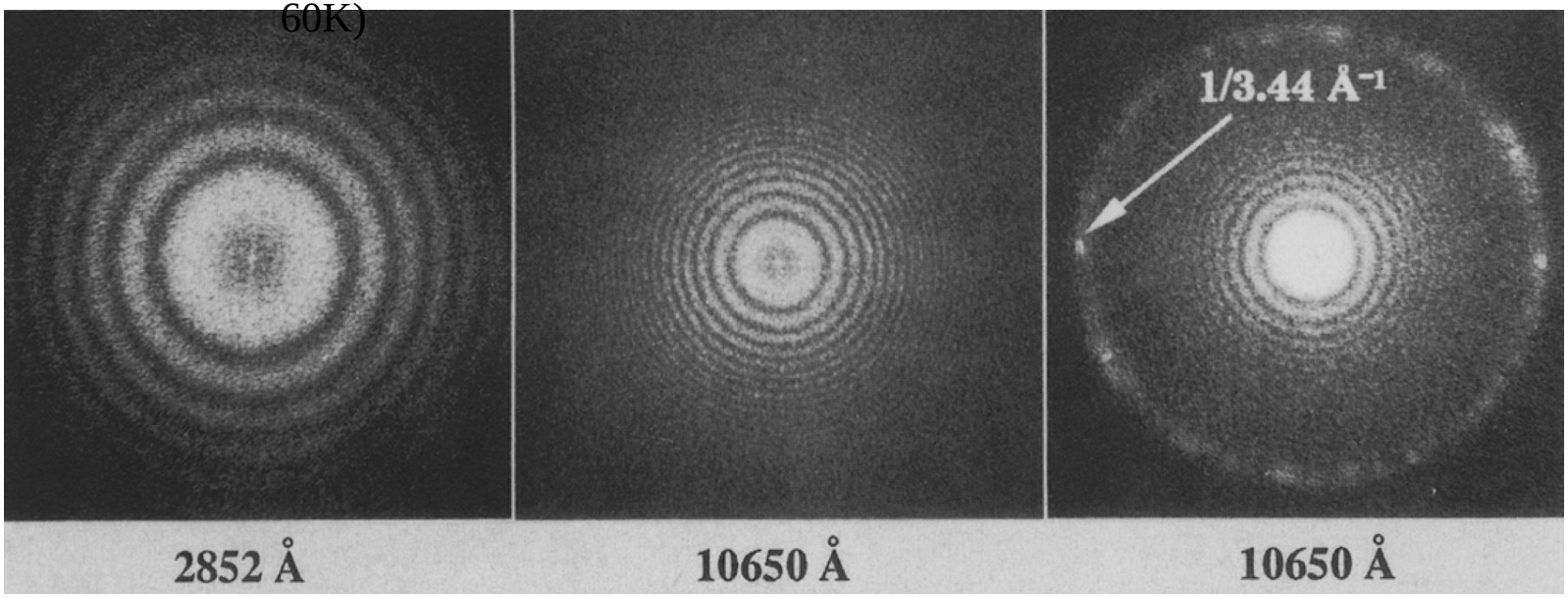


Prasad *et al.* (1996)
Nature 382: 471-473

Icosahedral reconstruction – a bit of history

– introduction of FEG instruments – better coherence / envelope function
- higher resolution, CTF correction

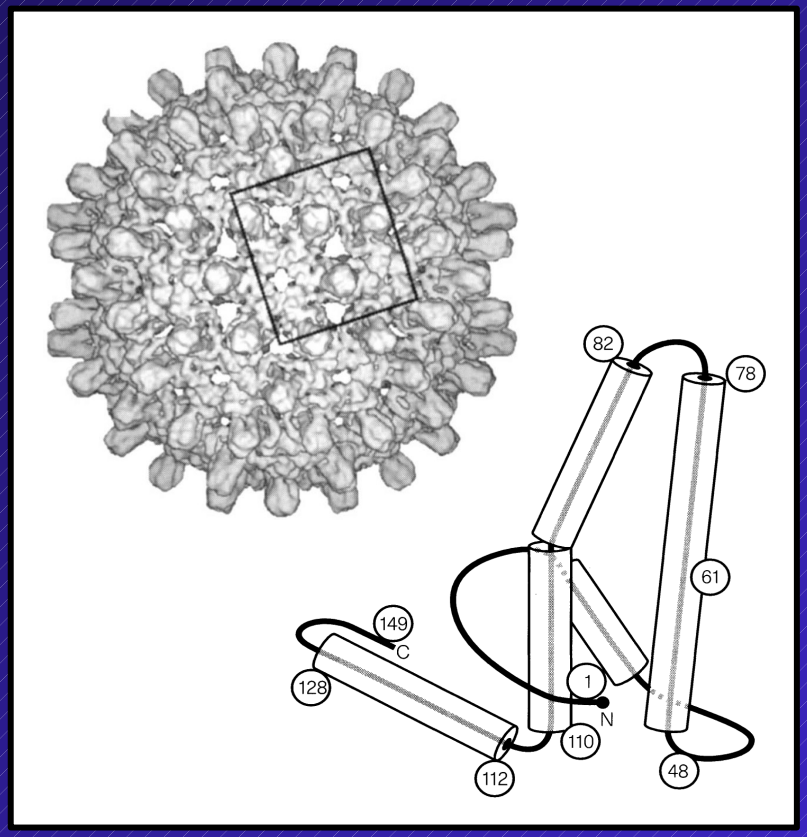
Hitachi HF2000 with cold field emission gun (200 kV, 60K)



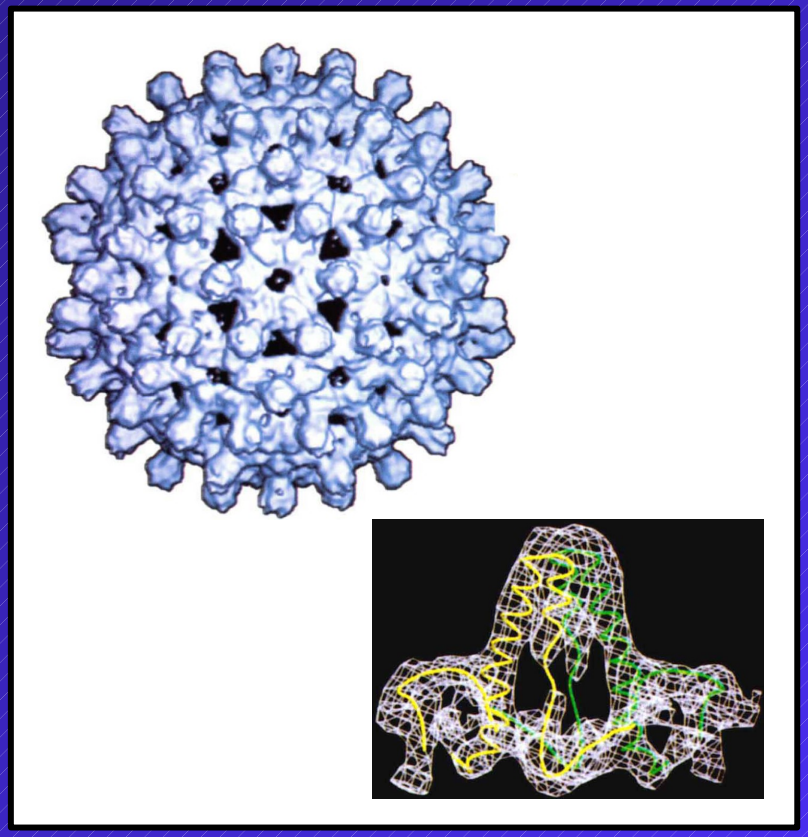
Zhou & Chiu (1993)
Ultramicroscopy 49: 407-416

Icosahedral reconstruction – a bit of history

– 1997: first sub-nanometer resolution structures: Hepatitis B virus capsid



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



Conway *et al.* (1997)
Nature 386: 91-94

Icosahedral reconstruction – a bit of history

– 2008: first atomic models obtained with icosahedral specimens

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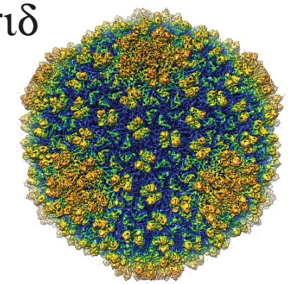
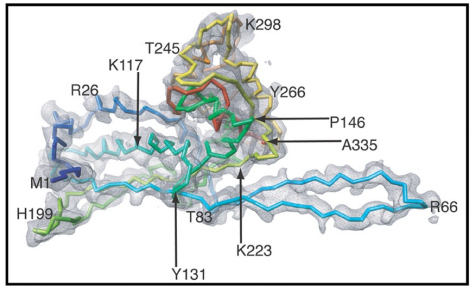
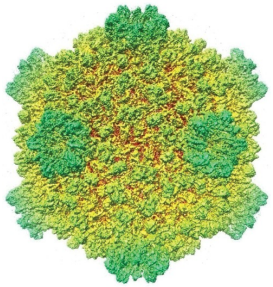
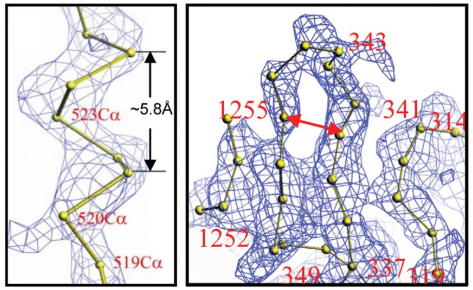




Jiang *et al.* (2008)
Nature 451: 1130-1134

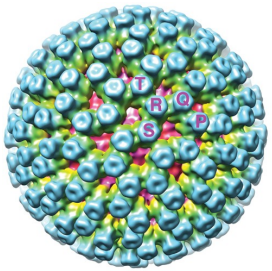
cytoplasmic polyhedrosis virus at 3.88 Å

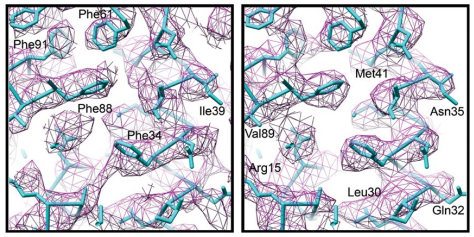




Yue *et al.* (2008)
Nature 453: 415-419

rotavirus DLP at 3.8 Å

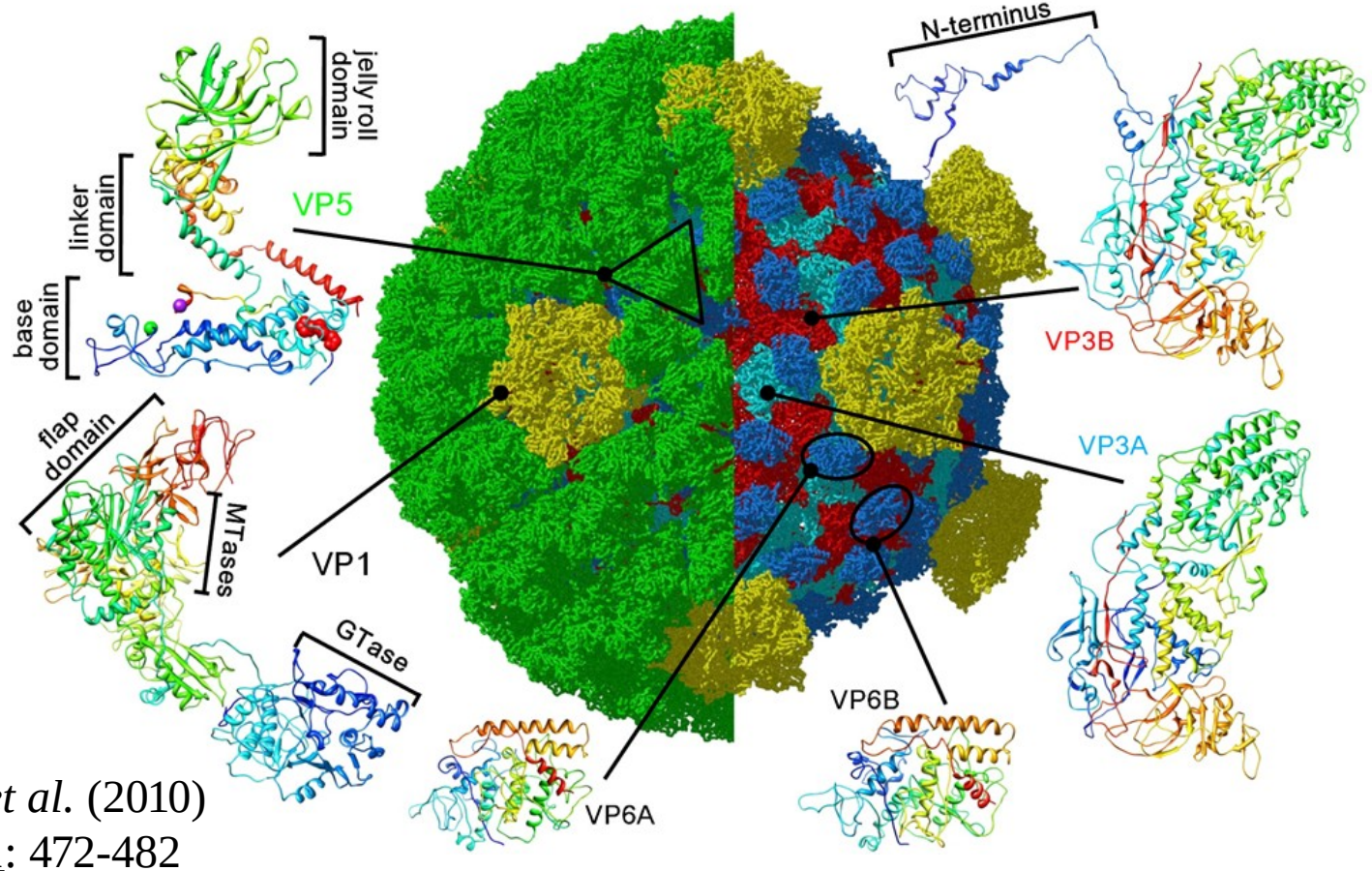




Zhang *et al.* (2008)
PNAS 105: 1867-1872

Icosahedral reconstruction – a bit of history

Primed infectious subvirion particle of aquareovirus at 3.3 Å resolution



Zhang *et al.* (2010)
Cell 141: 472-482

Icosahedral reconstruction – the future (already here)

No need to tilt & many samples with icosahedral symmetry

– viruses
– virus-like particles
– complexes with receptors, co-receptors and antibodies

What needs to get done:

– optimize data collection by automation and recording movies
→ higher yield of useful data
ALREADY HAPPENING !

– optimize data processing by automation
ALREADY HAPPENING !

Icosahedral reconstruction – optimization & validation

Optimization

– not much left to optimize ! (except image quality → Niko)
– optimize data processing → improve algorithms

Validation

– not critical – icosahedral symmetry
– handedness can still be an issue at intermediate resolution

Electron tomography – a bit of history

– pioneered by Walter Hoppe, Wolfgang Baumeister and David Agard

– automation of data collection
(Koster *et al.* (1992) *Ultramicroscopy* 46: 207-227

– electron tomography of vitrified specimen
(Dierksen *et al.* (1995) *Biophys. J.* 68: 1416-1422)

– use of energy filter
(Grimm *et al.* (1997) *Biophys. J.* 72: 482-489)

– electron tomography of eukaryotic cells
(Medalia *et al.* (2002) *Science* 298: 1209-1213)

– double-tilt data collection
– sub-tomogram averaging
– correlative microscopy

Electron tomography – the future (looking bright)

Unlimited number of specimens

What needs to get done:

– simplify cryo-sectioning

– develop *in situ* / clonable label

– improve correlative microscopy for area selection

– maybe optimize data collection by recording movies
→ higher yield of useful data

– improve phase plates

– Cc corrector (?)

– improve software (alignment, segmentation, subtomogram averaging etc.)

Electron tomography – optimization & validation

Optimization

– specimen preparation (cryo-sectioning)
– software (segmentation, subtomogram averaging etc.)

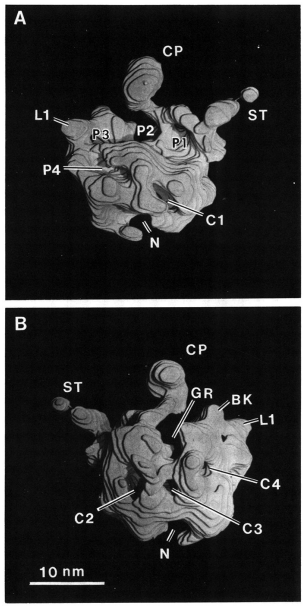
Validation

– difficult
  – resolution of a tomogram unclear
  – accuracy of segmentation unclear (poor SNR, missing wedge/pyramid)

Single-particle EM – a bit of history

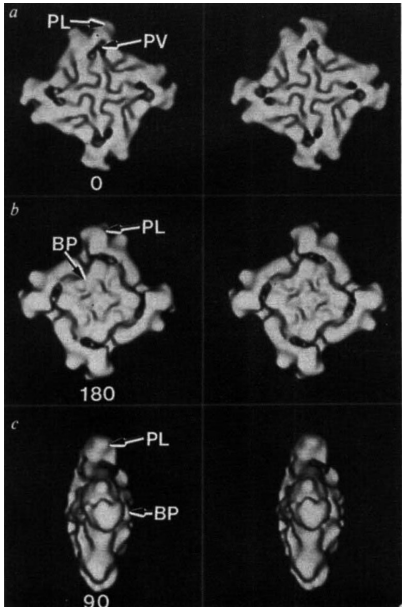
– pioneered by Joachim Frank (SPIDER) and Marin van Heel (IMAGIC), Steve Ludtke/Wah Chiu (EMAN) and Jose-Maria Carazo (XMIPP)

E. coli 50S ribosomal subunit
negative stain – ~20 Å resolution



Radermacher *et al.* (1987)
EMBO J. 6: 1107-1114

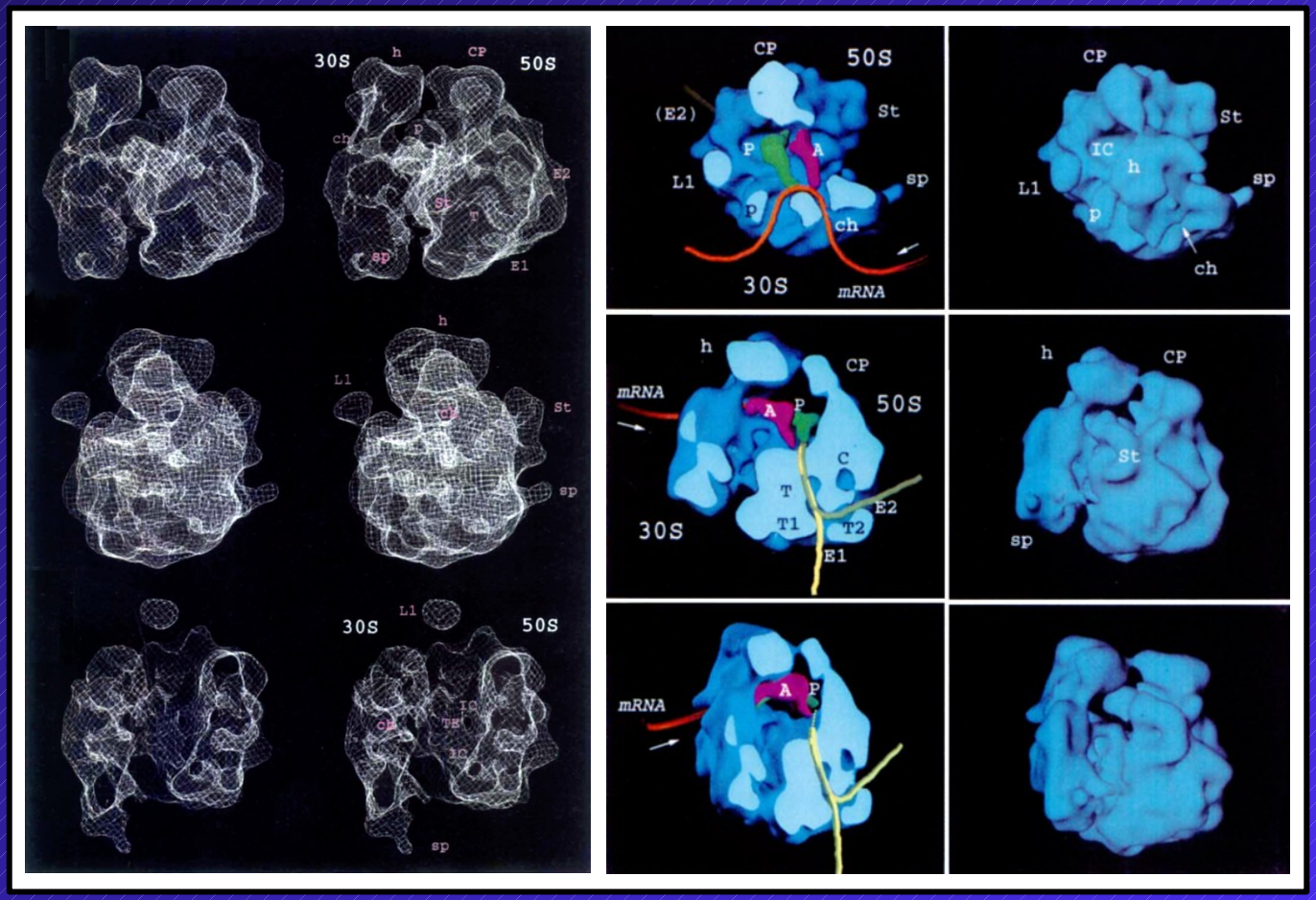
Ryanodine receptor
negative stain – 38 Å resolution



Wagenknecht *et al.* (1989)
Nature 388: 167-170

Single-particle EM – a bit of history

Cryo-EM of *E. coli* ribosome – 25 Å resolution

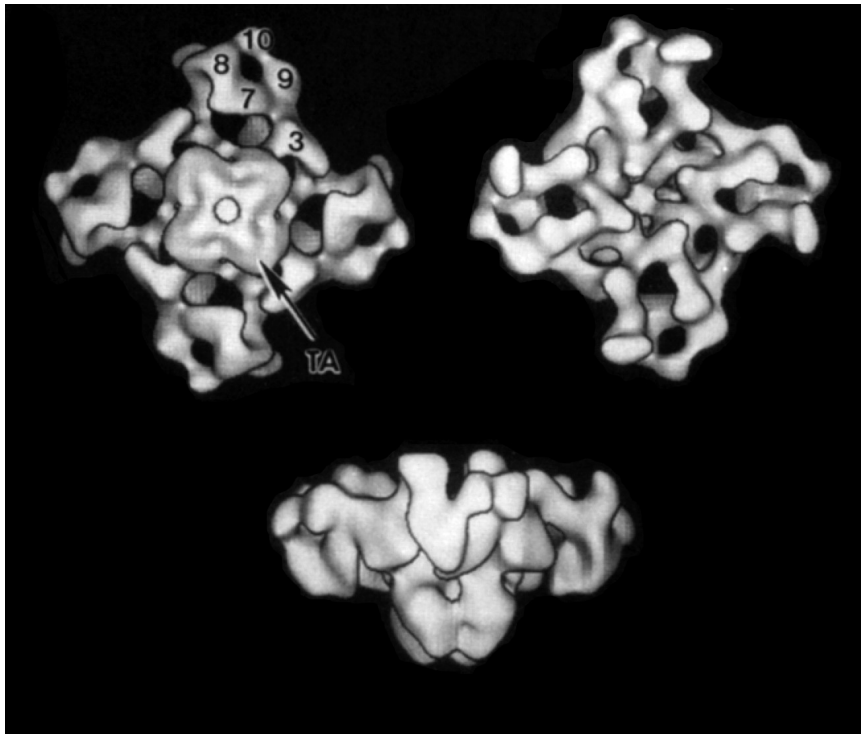


Frank *et al.* (1995) *Nature* 376: 441-444

Single-particle EM – a bit of history

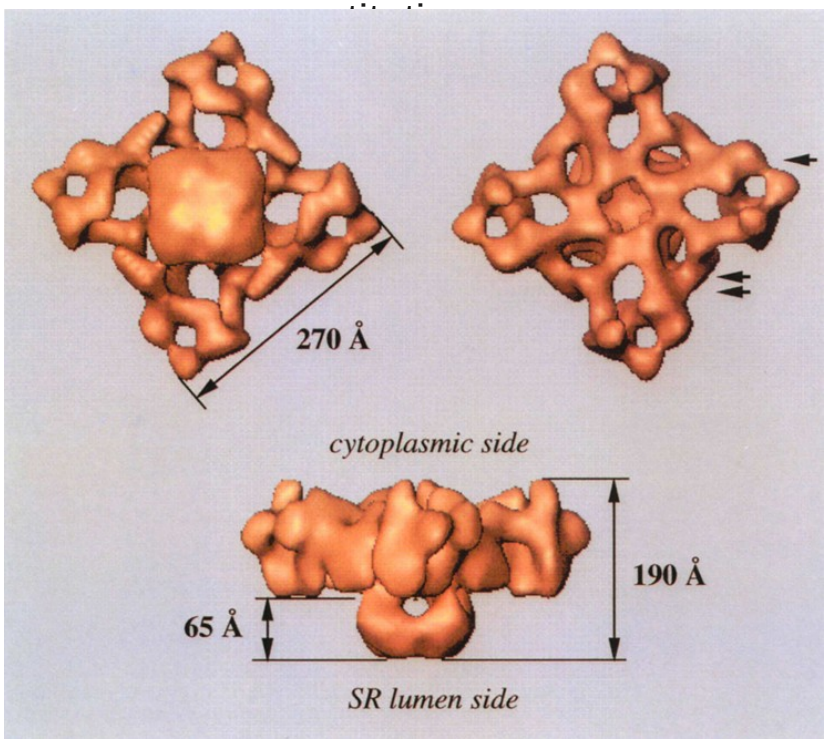
Cryo-EM of ryanodine receptor – ~30 Å resolution

Random conical tilt



Radermacher *et al.* (1994)
J. Cell Biol. 127: 411-423

Angular



Serysheva *et al.* (1995)
Nat. Struct. Biol.. 2: 18-24

Single-particle EM – a bit of history

many advances !!!

Instrumentation:
– field emission electron source
– CCD camera
– energy filter
– top-entry specimen stages
etc.

Software:
– multivariate statistical analysis
– maximum likelihood
– refinement strategies
– flexible fitting
– secondary structure identification
etc.

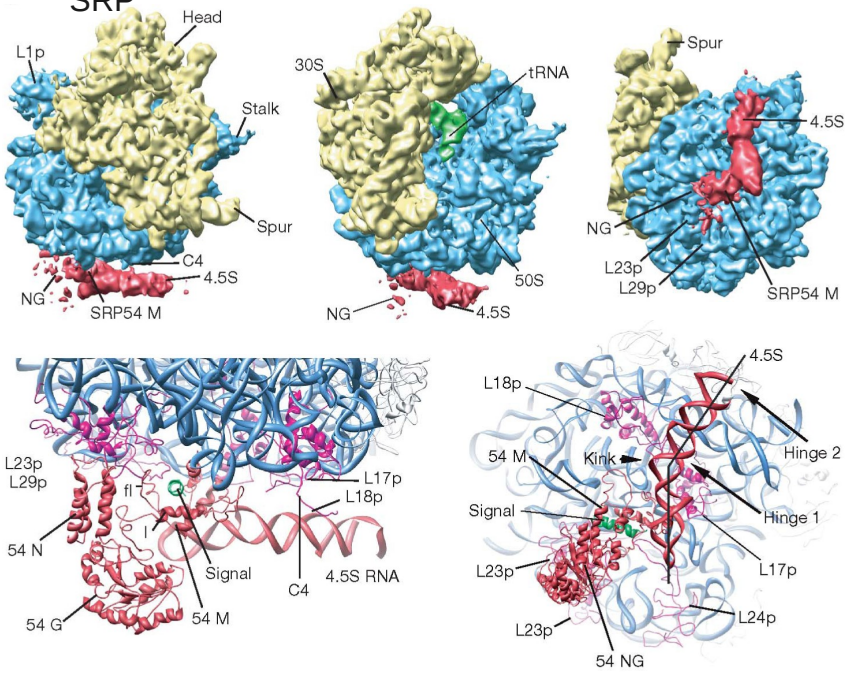
Automation of data collection and image processing

Success stories of single-particle EM

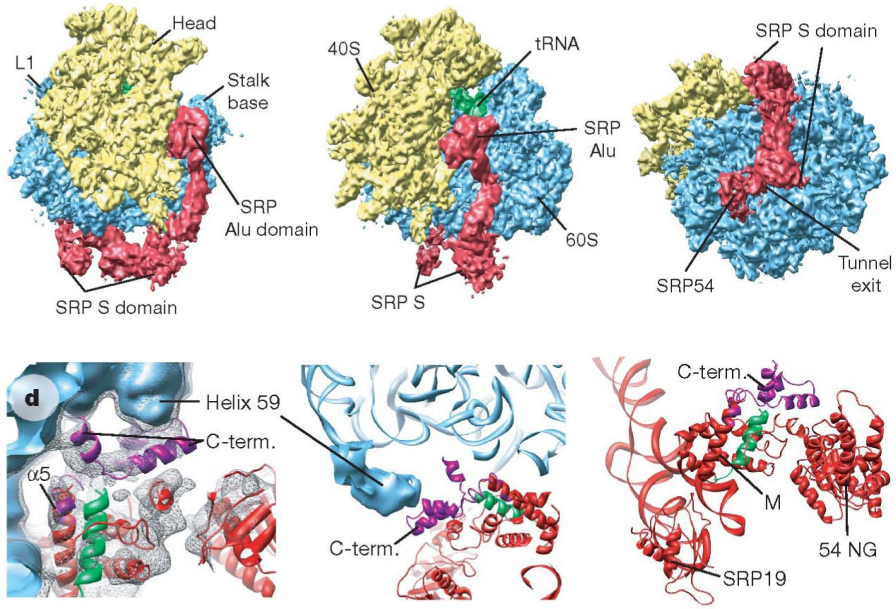
Ribosome at ~9 Å (signal recognition particle)

Following the signal sequence from ribosomal tunnel exit to signal recognition particle

70S ribosome–nascent-chain complex with *E. coli* SRP



80S ribosome–nascent-chain complex with mammalian SRP

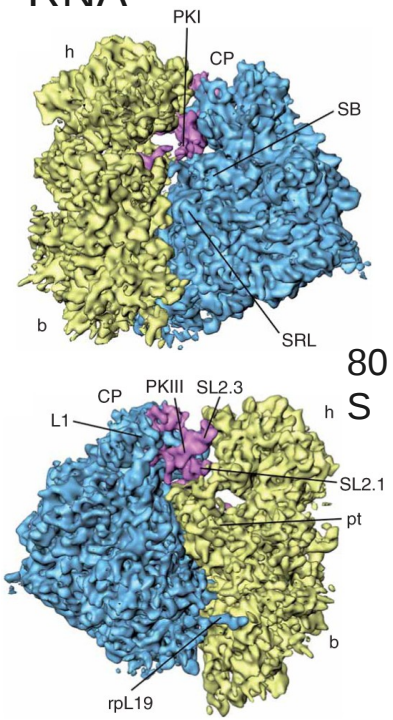


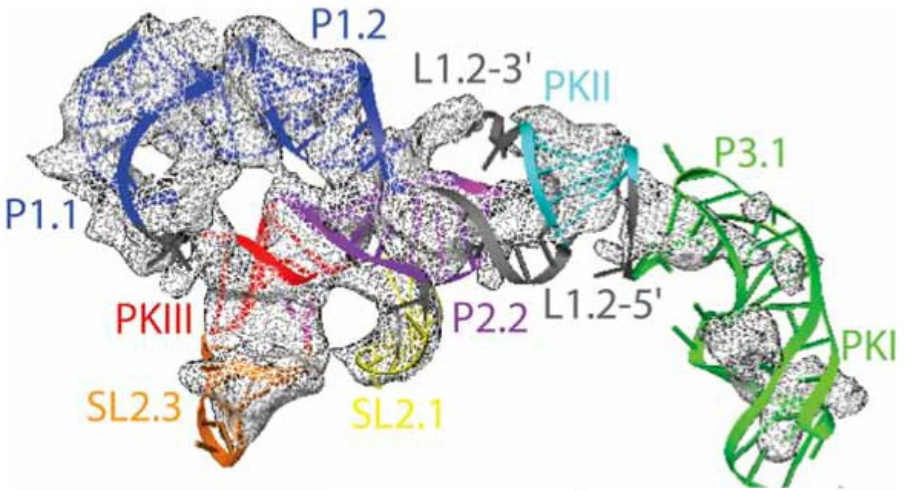
Halic *et al.* (2006) *Nature* 444: 507-511

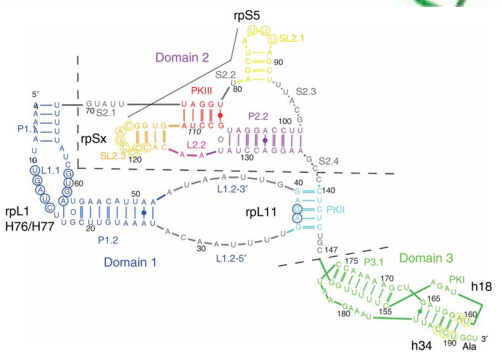
Success stories of single-particle EM

Ribosome at 7.3 Å (IRES RNA fold)

Structure of the ribosome-bound cricket paralysis virus IRES RNA







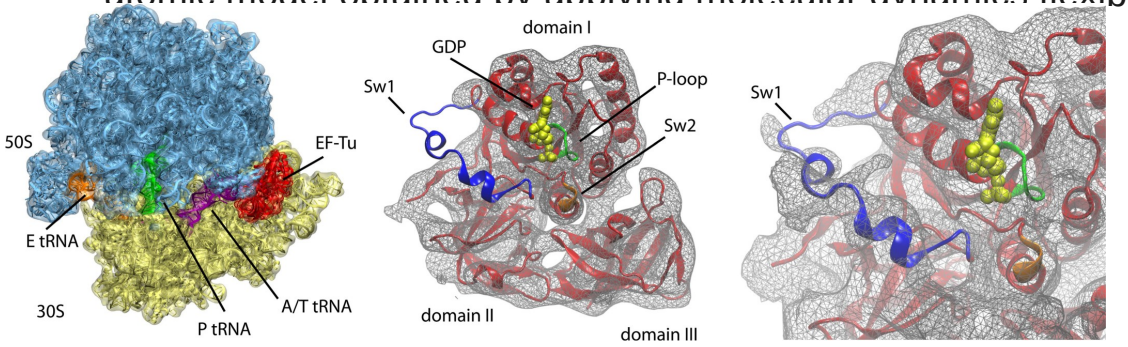
Schüler *et al.* (2006) *NSMB* 13: 1092-1096

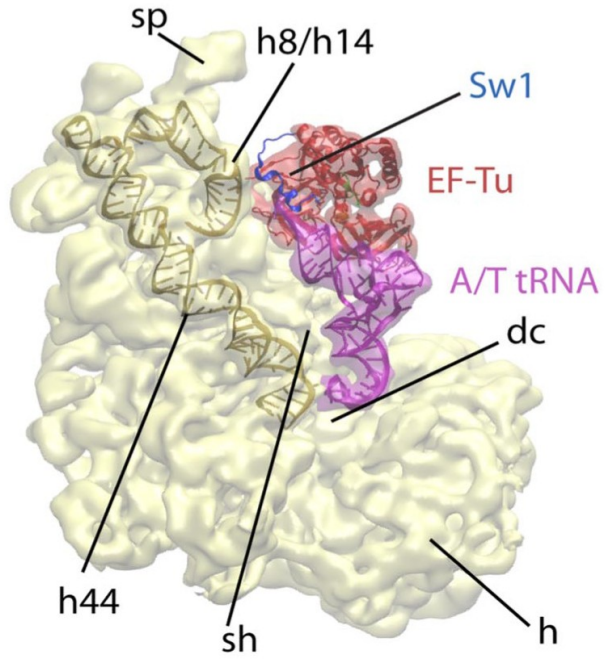
Success stories of single-particle EM

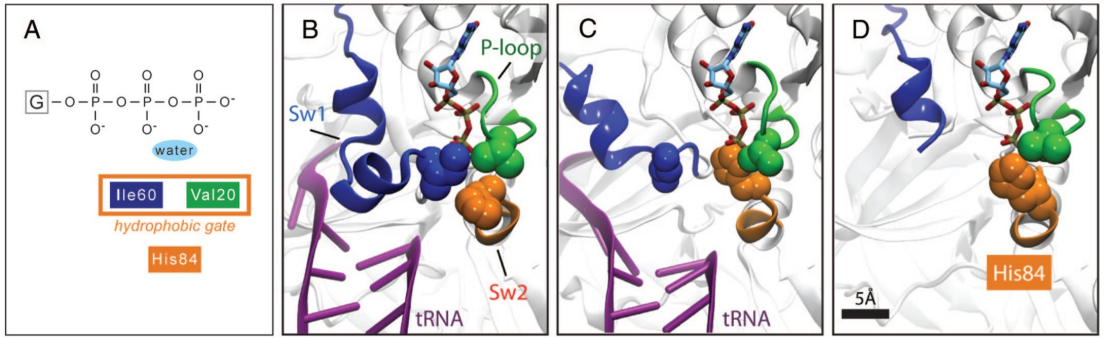
Ribosome at 6.7 Å (molecular dynamics flexible fitting)

Ribosome-induced changes in elongation factor Tu conformation control GTP hydrolysis

- 70S ribosome with Phe-tRNAPhe·· EF-Tu·· GDP ternary complex stalled by kirromycin at 6.7 Å resolution
- atomic model obtained by applying molecular dynamics flexible fitting (MDFF)







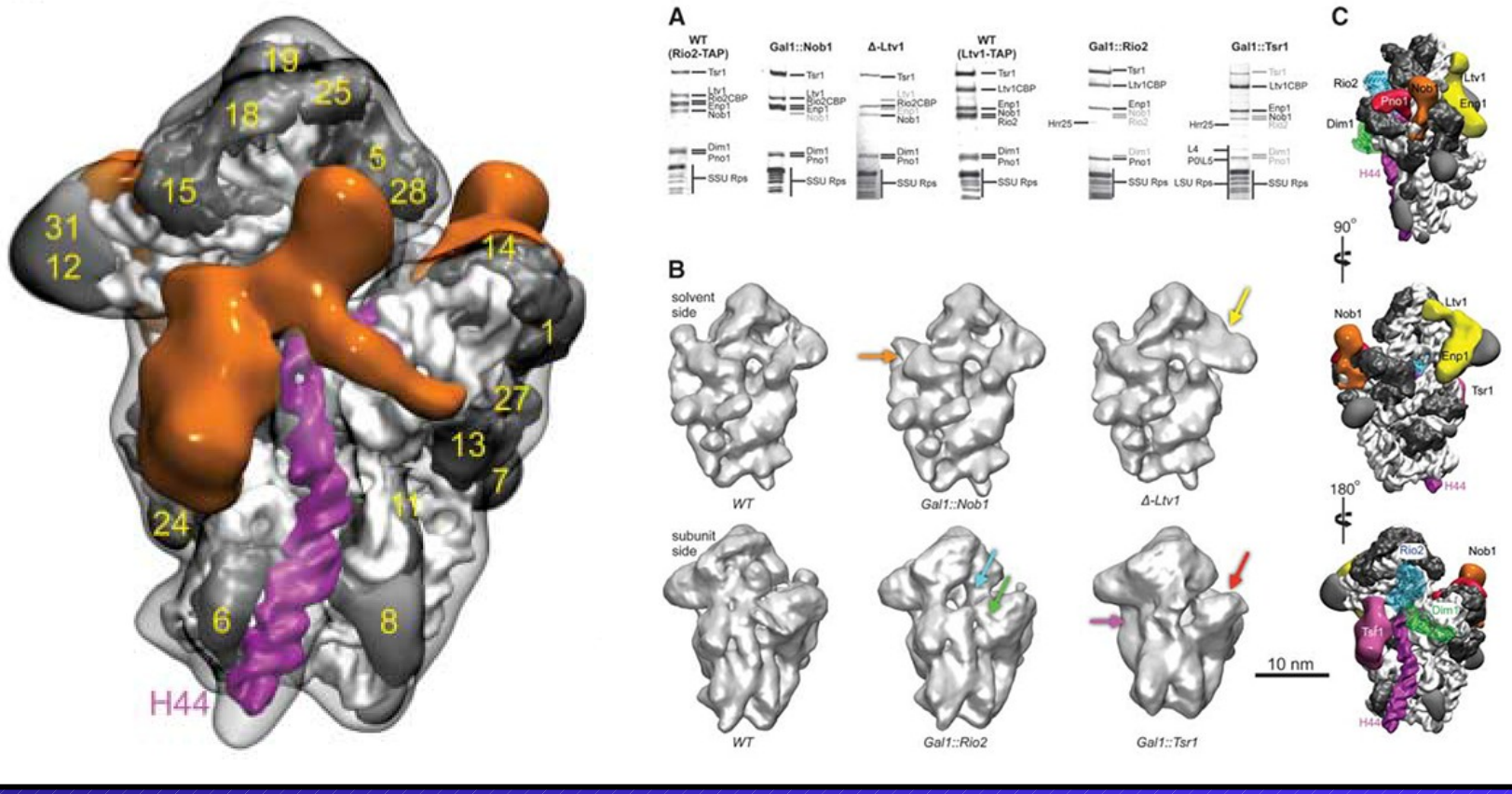
hydrophobic gate | crystal structure with kirromycin | EM map & MDFF with kirromycin | crystal structure of aurodox-bound EF-Tu (open gate)

Villa *et al.* (2009) *PNAS* 106: 1063-1068

Success stories of single-particle EM

Late pre-40S ribosome assembly intermediate (molecular dynamics flexible fitting)

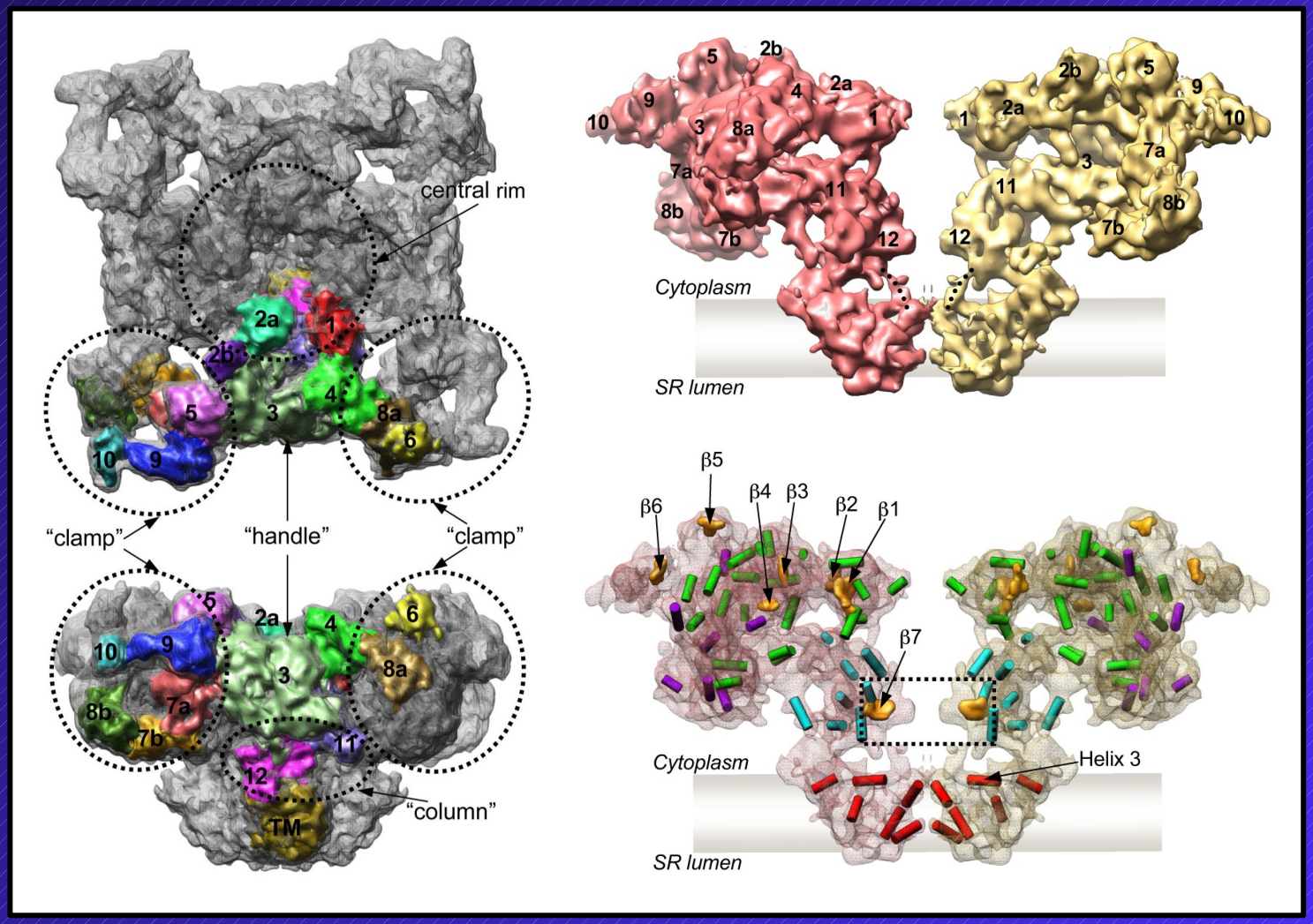
Ribosome assembly factors prevent premature translation initiation by 40S assembly intermediates



Strunk *et al.* (2011) *Science* 333: 1449-1453

Success stories of single-particle EM

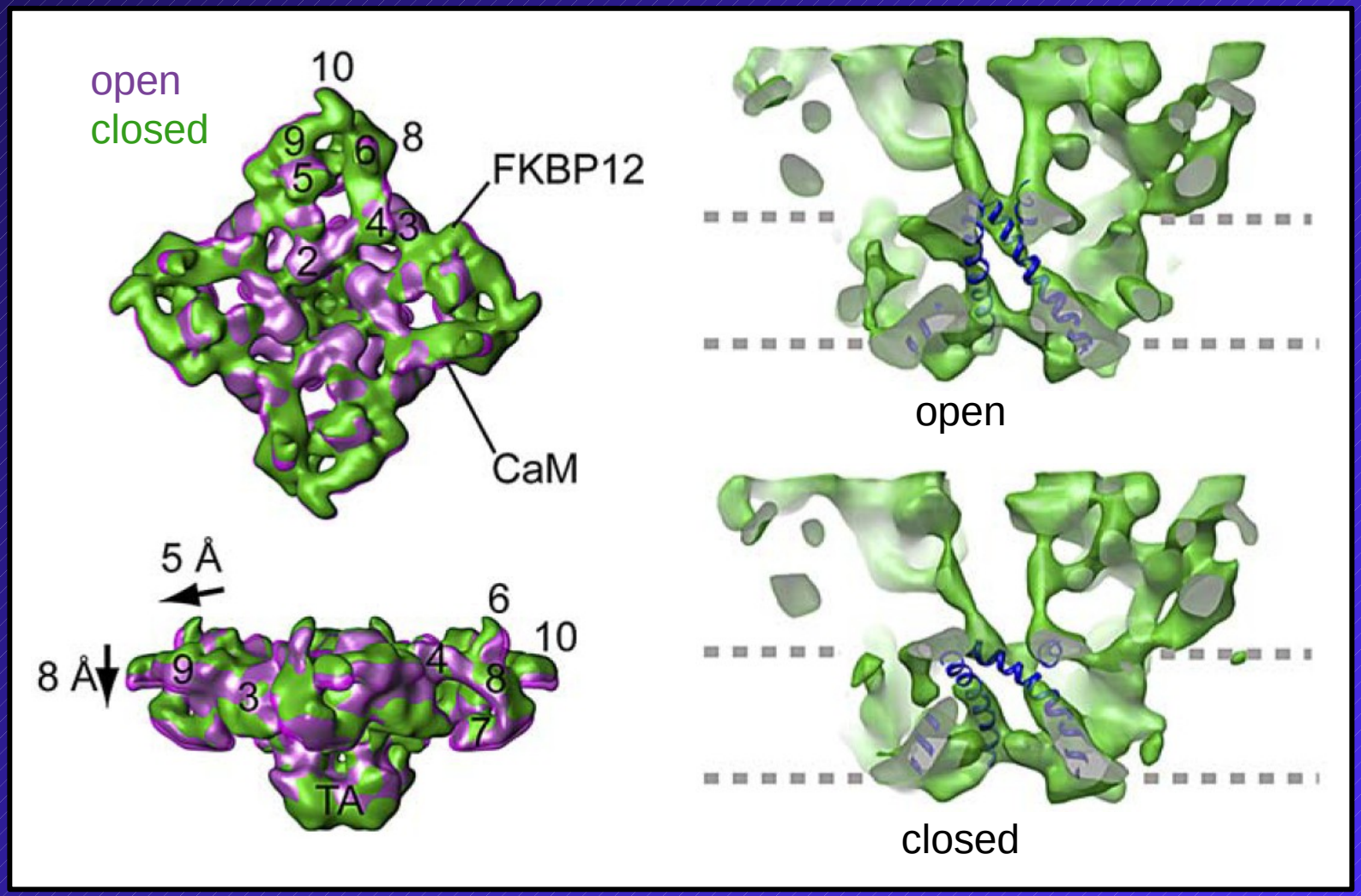
Ryanodine receptor at 9.6 Å (secondary structure assignment)



Serysheva *et al.* (2008) *PNAS* 105: 9610-9615

Success stories of single-particle EM

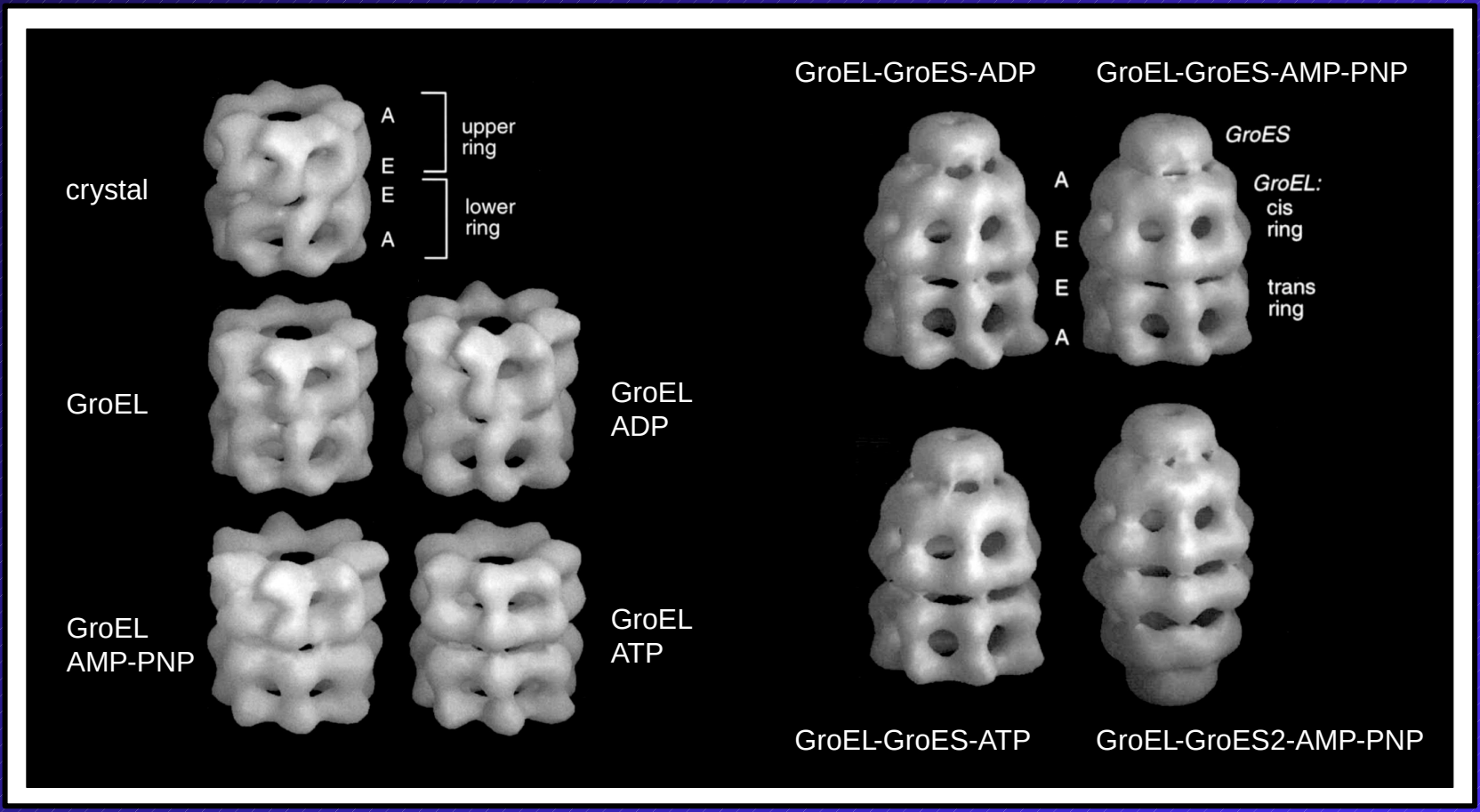
Ryanodine receptor at 10.2 Å (channel gating)



Samso *et al.* (2009) *PLoS Biol.* 7: e85

Success stories of single-particle EM

GroEL-GroES complex at 30 Å

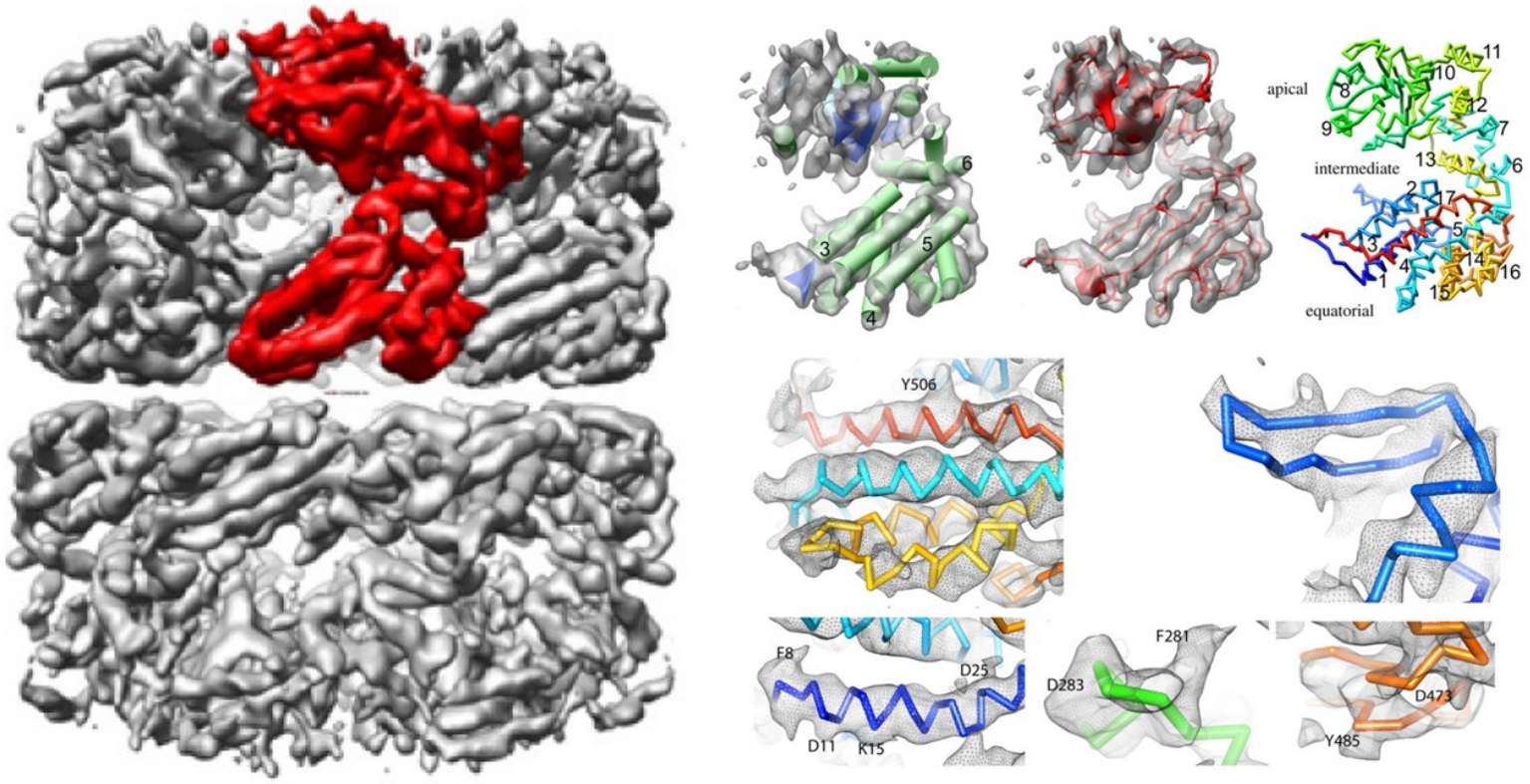


Roseman *et al.* (1996) *Cell* 87: 241-251

Success stories of single-particle EM

GroEL at ~4 Å (backbone trace)

De novo backbone trace of GroEL from single particle electron cryomicroscopy

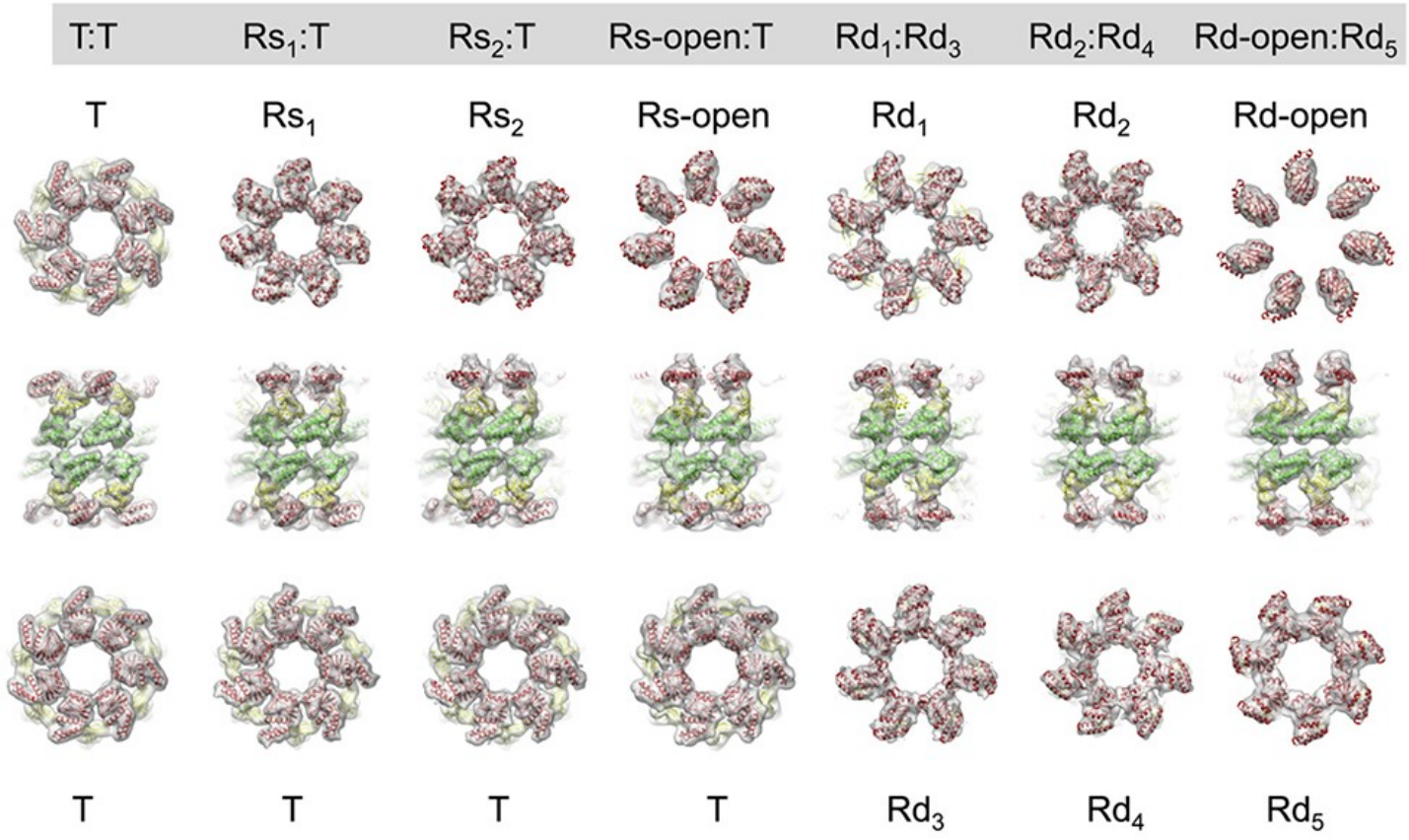


Ludtke *et al.* (2008) *Structure* 16: 441-448

Success stories of single-particle EM

GroEL at ~8 Å (conformational states)

ATP-triggered conformational changes delineate substrate-binding and –folding mechanics of the GroEL chaperonin

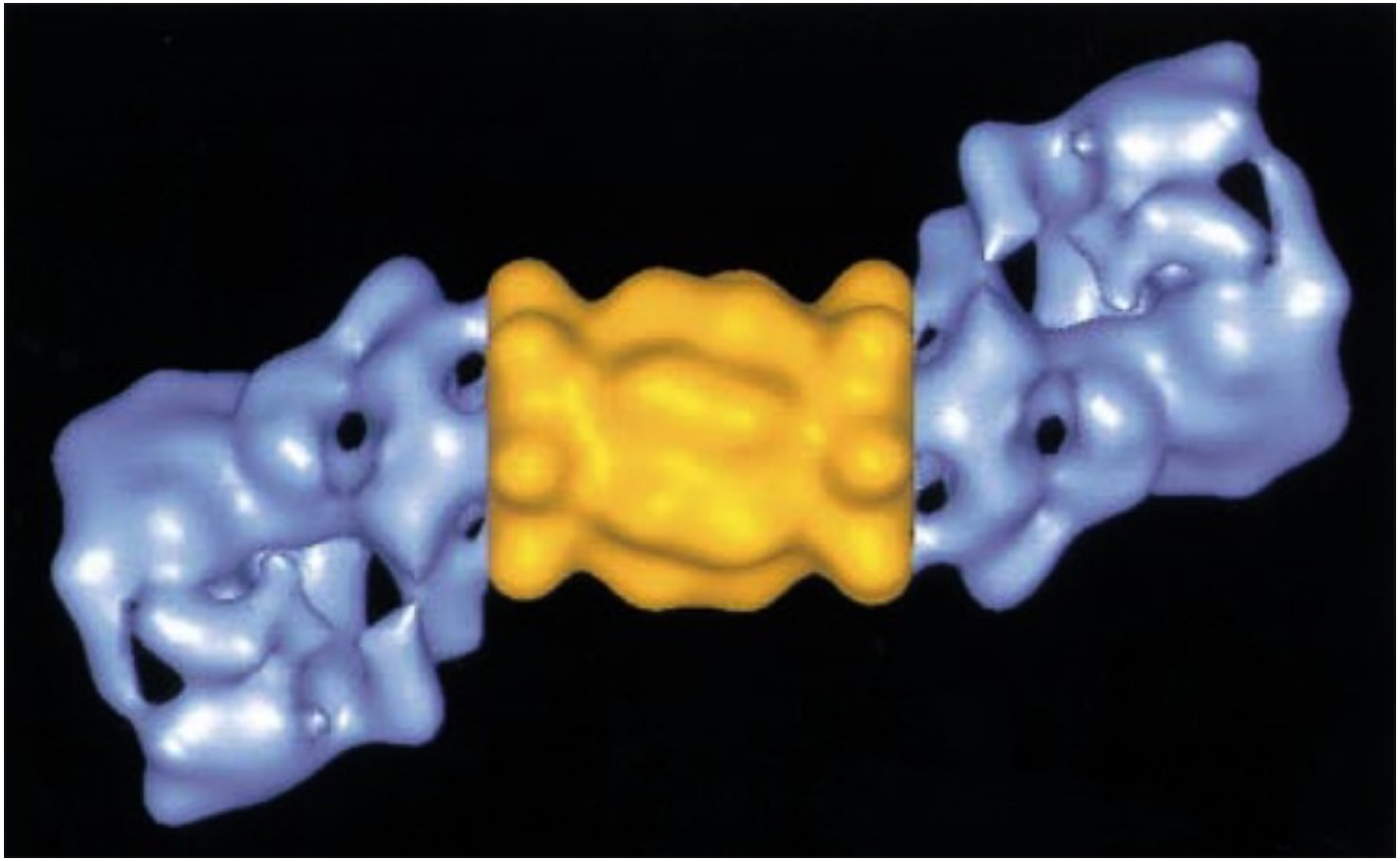


Clare *et al.* (2012) *Cell* 149: 113-123

Success stories of single-particle EM

26S proteasome at 28 Å

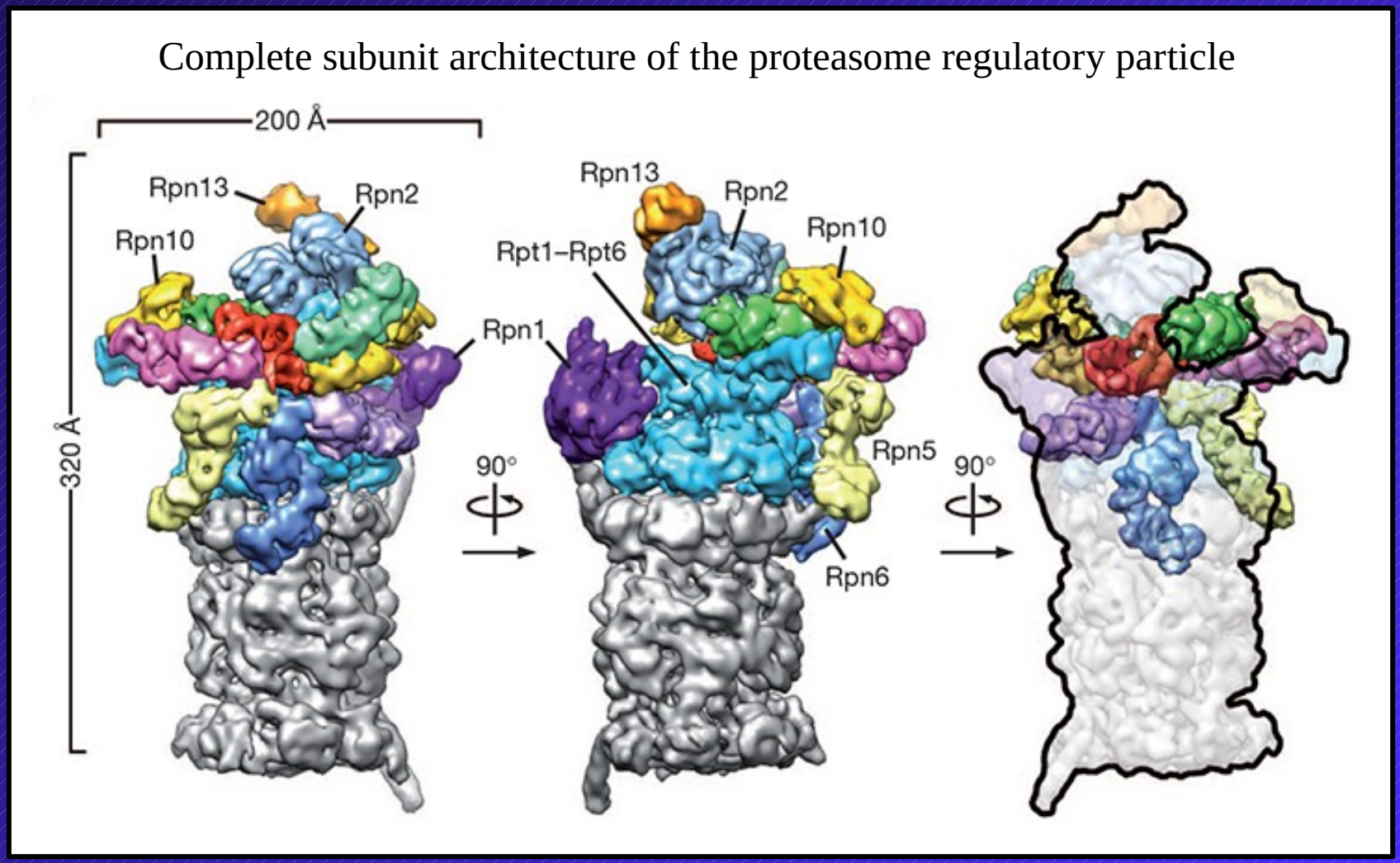
26S proteasome structure revealed by three-dimensional electron microscopy



Walz *et al.* (1998) *JSB* 121: 19-29

Success stories of single-particle EM

26S proteasome at 9 Å (subunit organization)

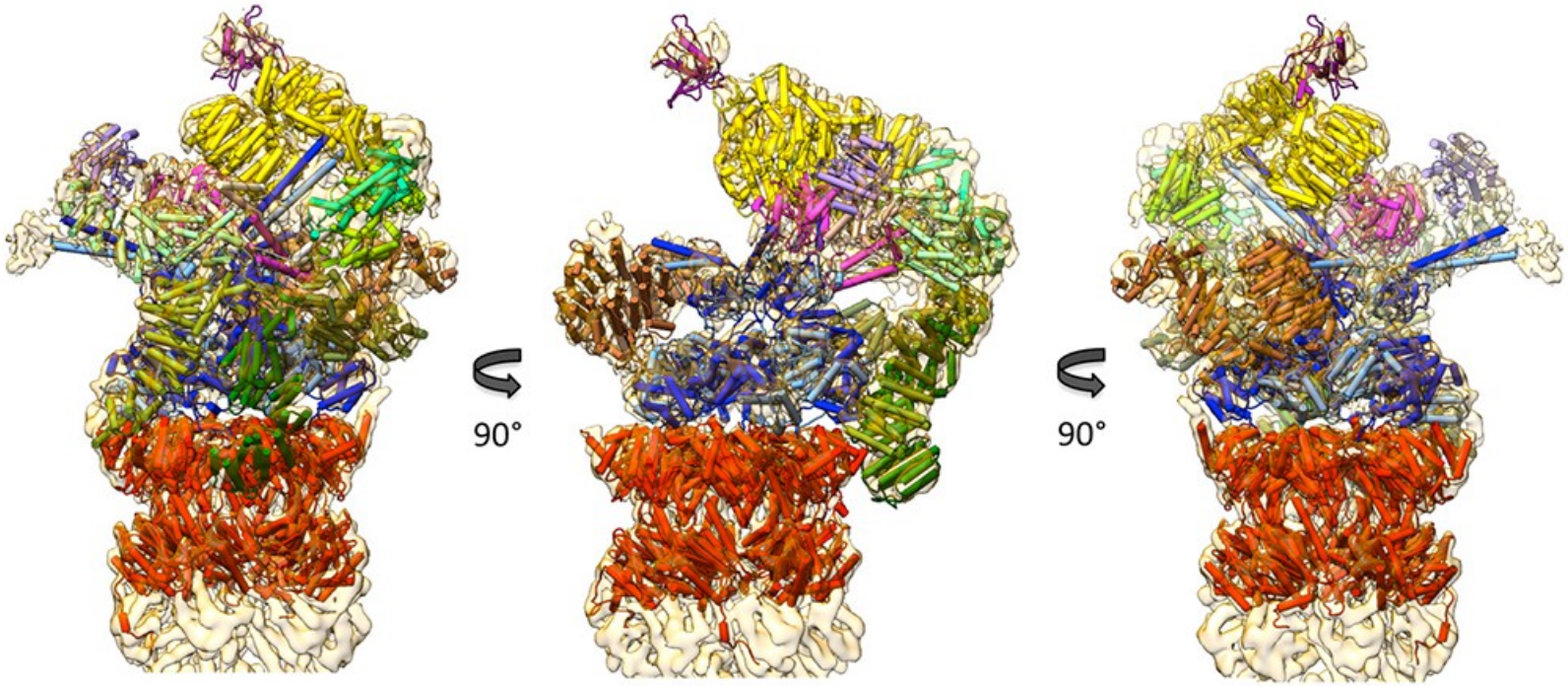


Lander *et al.* (2012) *Nature* 482: 186-191

Success stories of single-particle EM

26S proteasome at ~7 Å (pseudo-atomic model)

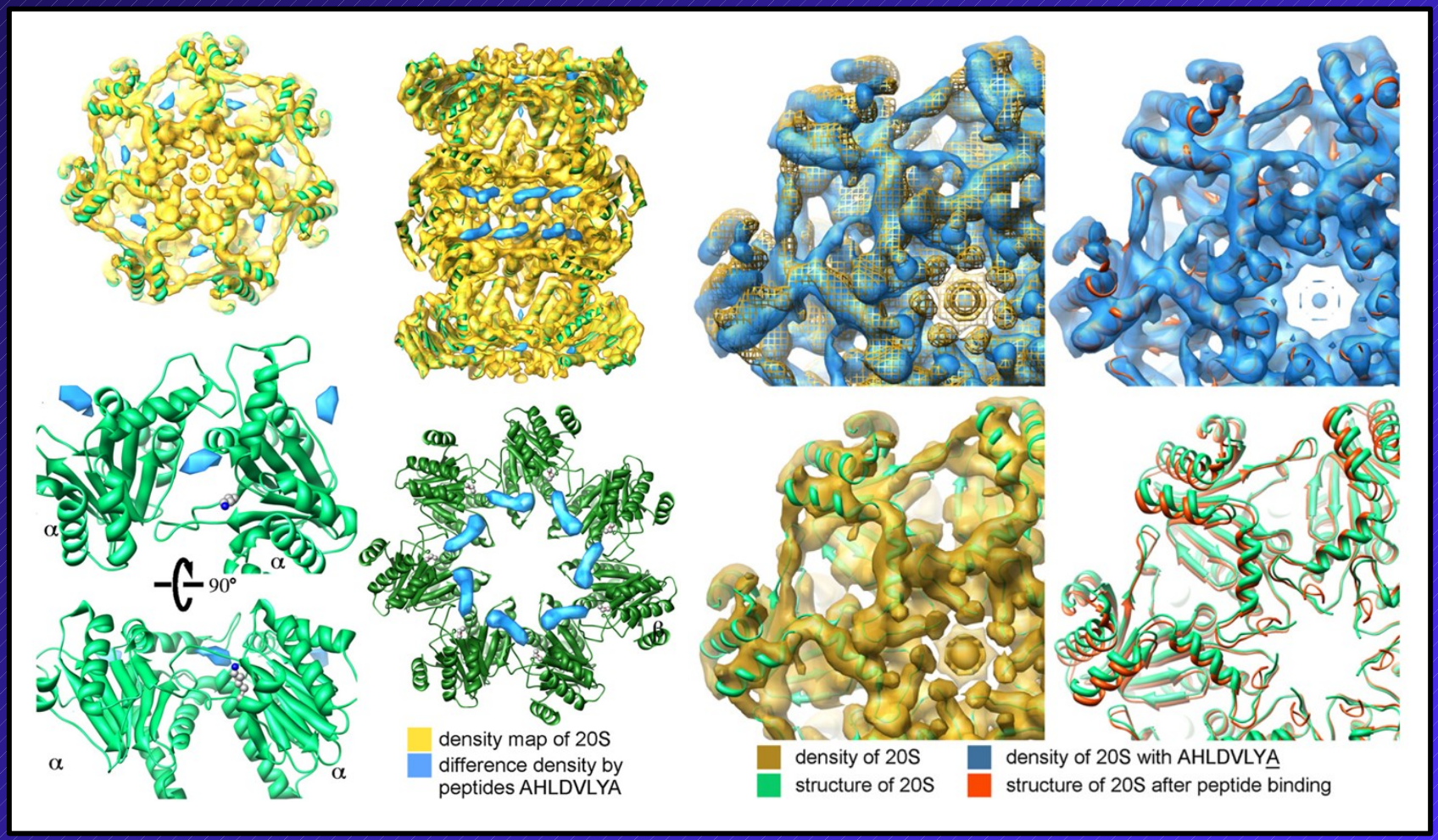
Near-atomic resolution structural model of the yeast 26S proteasome



Beck *et al.* (2012) *PNAS* 109: 14870-14875

Success stories of single-particle EM

20S proteasome at ~6 Å (8 aa peptide)



Rabl *et al.* (2004) *Mol. Cell* 30: 360-368

The “future” of single-particle EM

Automation

– pioneered by Bridget Carragher and Clint Potter
  → Leginon – data collection
  → Appion – data processing

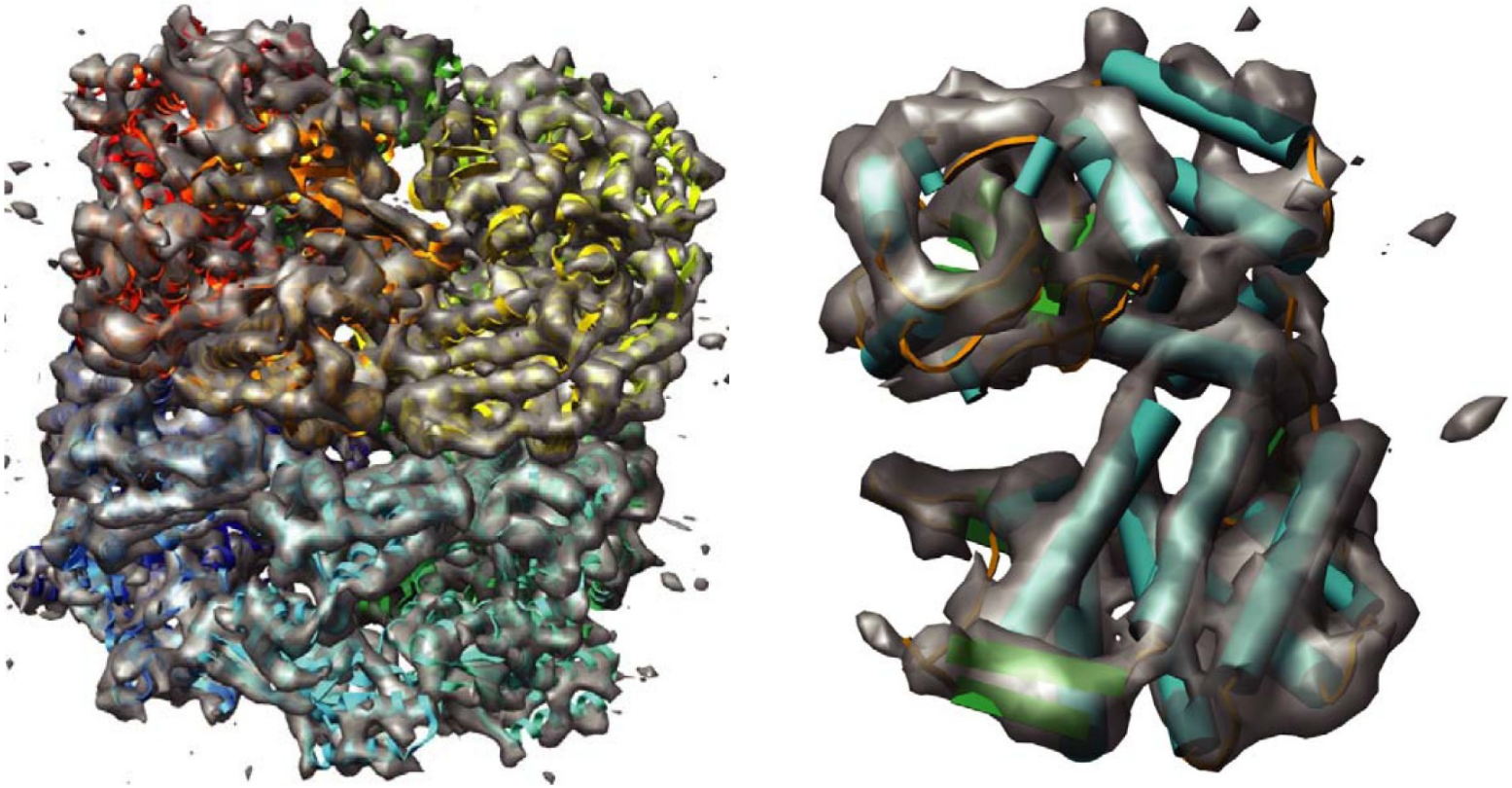
– also semi-automation – David Mastronarde
– David Agard and Yifan Cheng

– allows collection of humongous data sets without or little user input
  → speeds up structure determination
  (especially if image processing also automated)
  → allows more stringent selection of “good” particles
  → opens up new possibilities to study heterogeneous particle populations
  – conformational heterogeneity
  – time-dependent processes

The “future” of single-particle EM

First integrated automation – GroEL at 7.8 Å

Automated cryoEM data acquisition and analysis of 284742 particles of GroEL

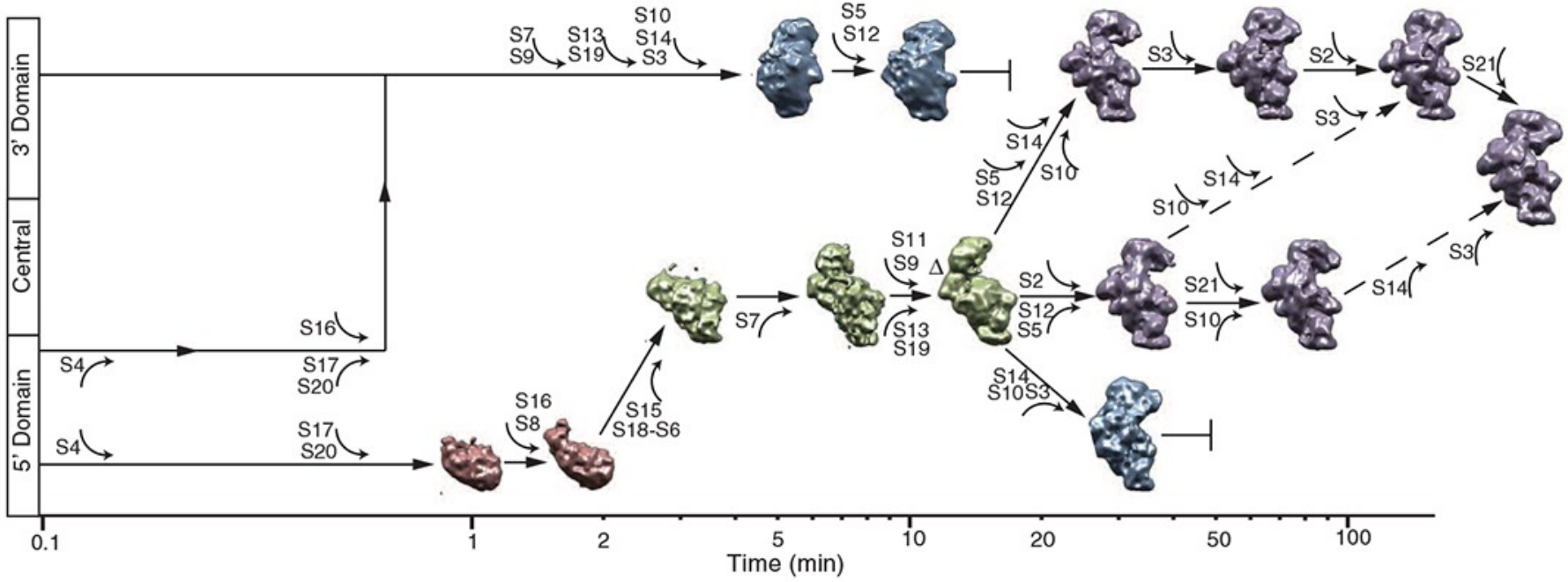


Stagg *et al.* (2006) JSB 155: 470-481

The “future” of single-particle EM

Automation – Ribosome biogenesis (time-resolved, >1,000,000 particles classified)

Visualizing ribosome biogenesis: parallel assembly pathways for the 30S subunit

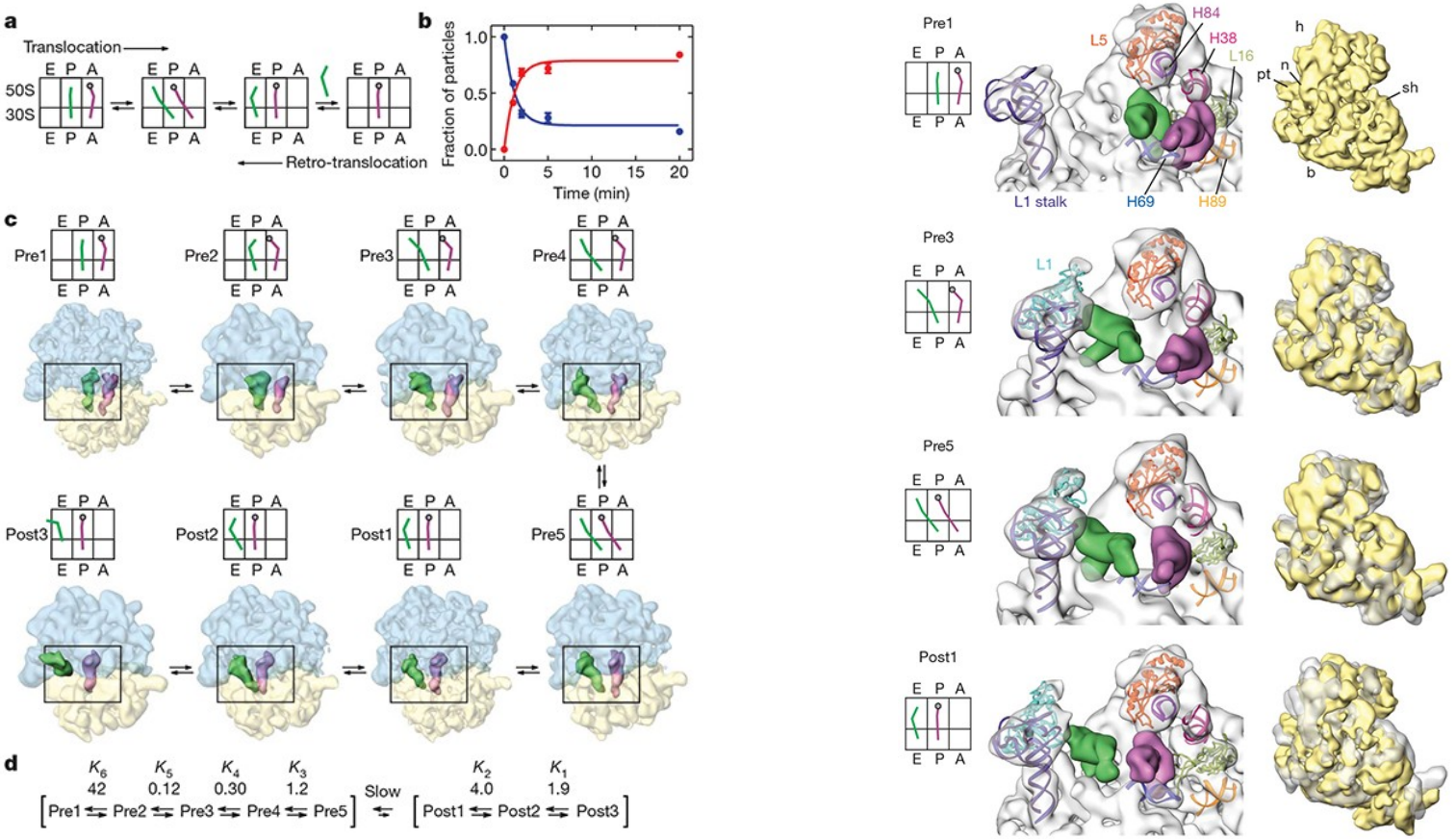


Mulder *et al.* (2010) *Science* 330: 673-677

The “future” of single-particle EM

Automation – Ribosome translation (2,004,547 particles classified)

Ribosome dynamics and tRNA movement by time-resolved electron cryomicroscopy



Fischer *et al.* (2010) *Nature* 466: 329-333

The “future” of single-particle EM

Phase plates

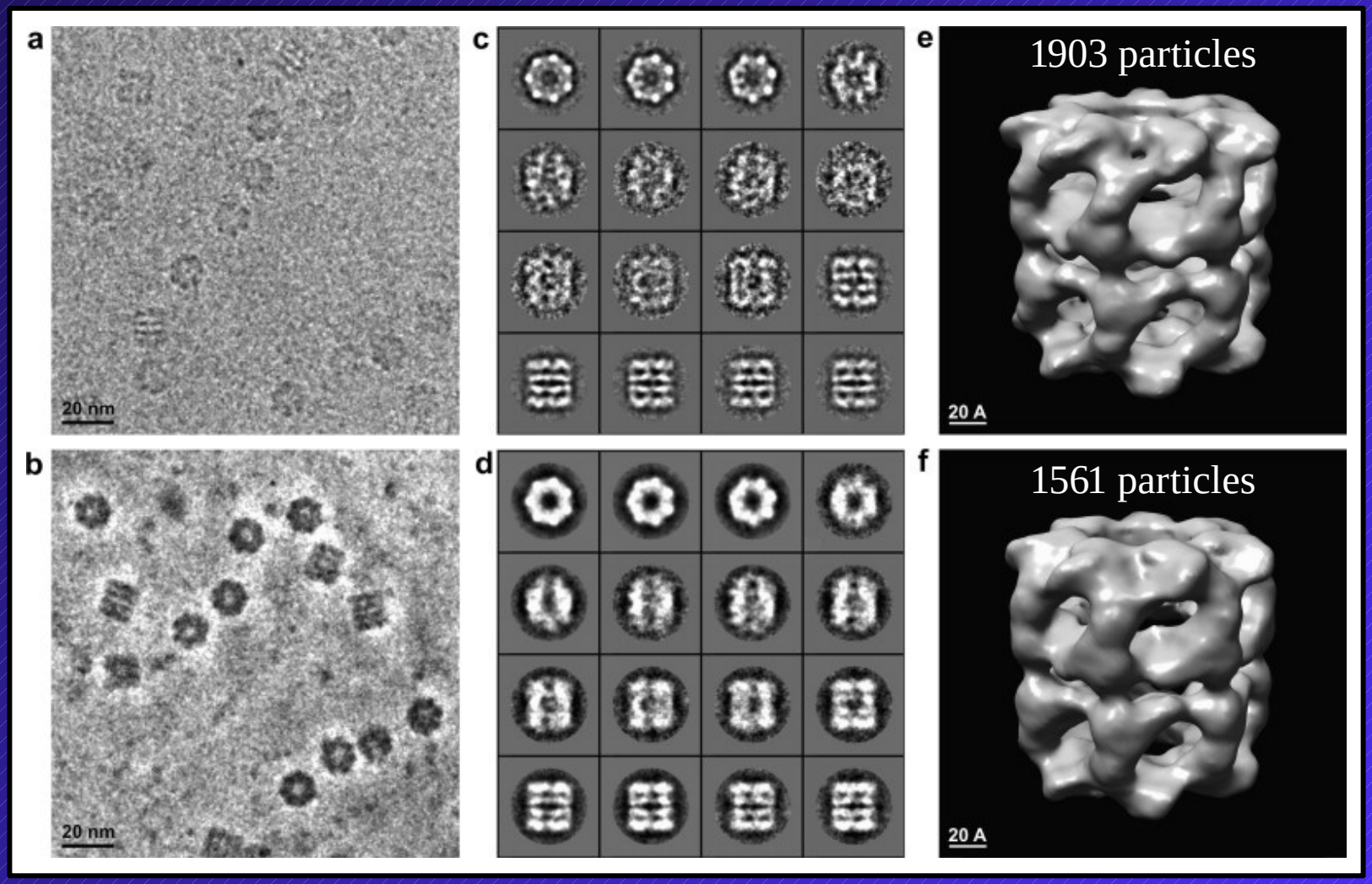
– several attempts from 1960-1980 to develop phase plates for EM, but failed due to practical issues, such as manufacturing and charging of phase plates

– phase plates revived by Kuniaki Nagayama
Nagayama (2005) *Adv. Imaging Electr. Phys* 138: 69-146

– technical issues remain – development/testing continues in several groups:
Kuniaki Nagayama
Bob Glaeser
Wah Chiu
Rasmus Schröder
Werner Kühlbrandt

The “future” of single-particle EM

First 3D reconstructions with phase plate data – GroEL at 12 Å



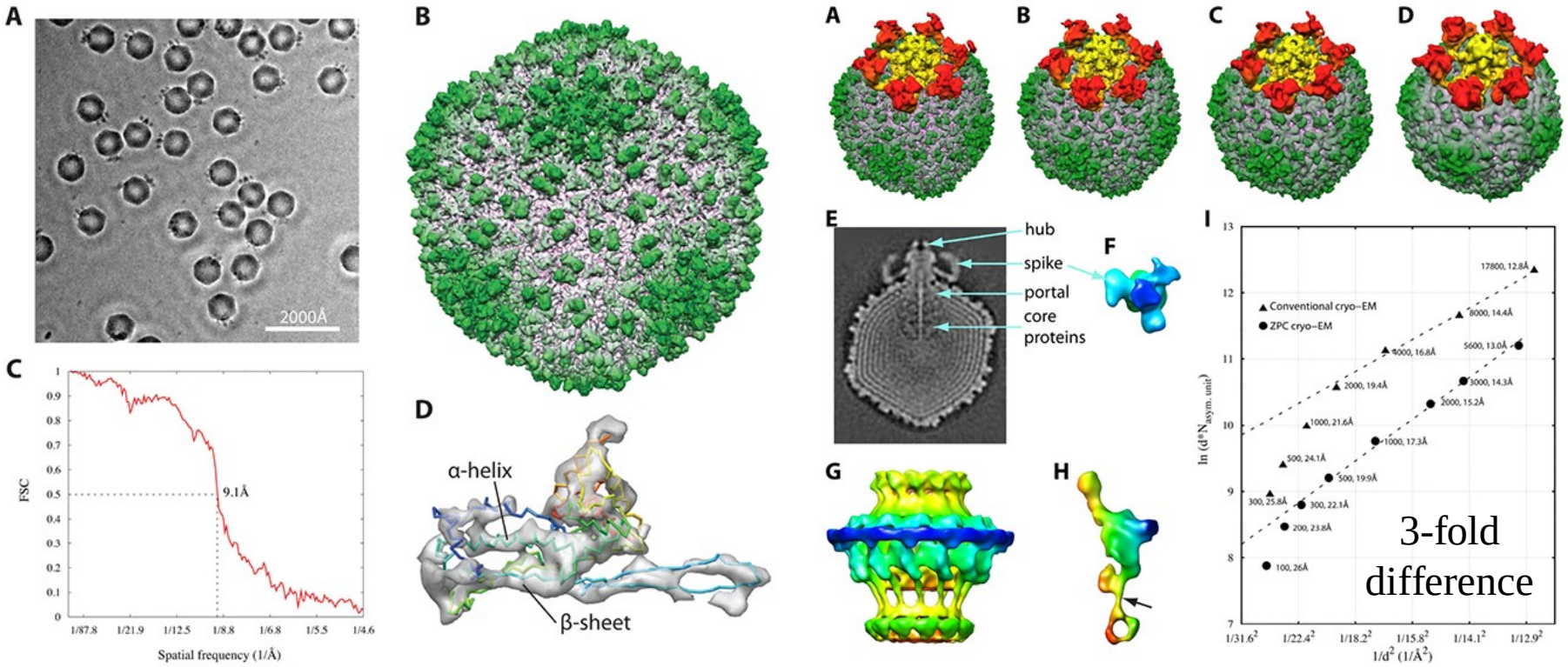
Danev & Nagayama (2008) *JSB* 161: 211-218

The “future” of single-particle EM

First 3D reconstructions with phase plate data – ε15 bacteriophage

Symmetry imposed
2,900 particles, ~9 Å resolution

No symmetry imposed
5,600 particles, ~13 Å resolution



Murata *et al.* (2010) *Structure* 18: 903-912

The “future” of single-particle EM

Direct detector device (DDD) cameras

– eliminate scintillators with fiber optics or lenses
  → eliminates image artifacts (distortion, fixed patterns, gain variations)
– direct detection sensors have small point spread function
  → allows for small pixels possible (more pixels/area & large detectors)
– significantly enhanced detection efficiency of incoming electrons
  → each primary electron creates large signal
– fast read-out time

→ great DQE (better than film!)
→ can record “movies”

Mark Ellisman
Clint Potter
Bridget Carragher

Richard Henderson
Wasi Faruqi

David Agard
Peter Denes

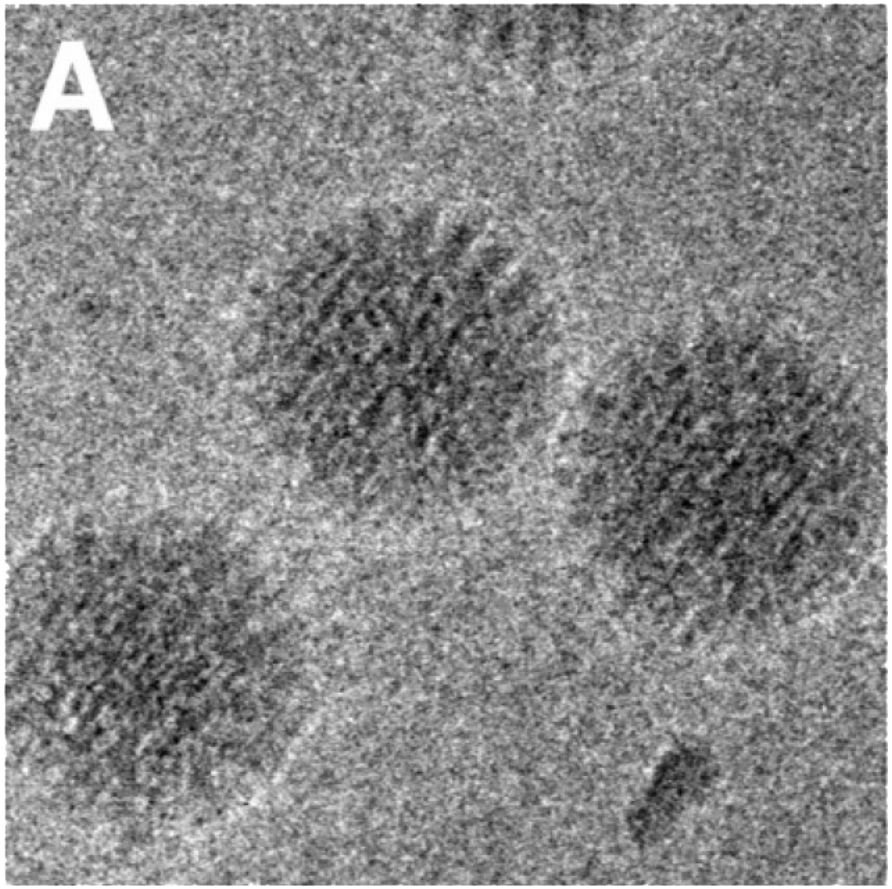
Direct Electron
DE-12 & DE-20

FEI
Falcon

Gatan
K2 (Base & Summit)

The “future” of single-particle EM

DDD camera – recording movies



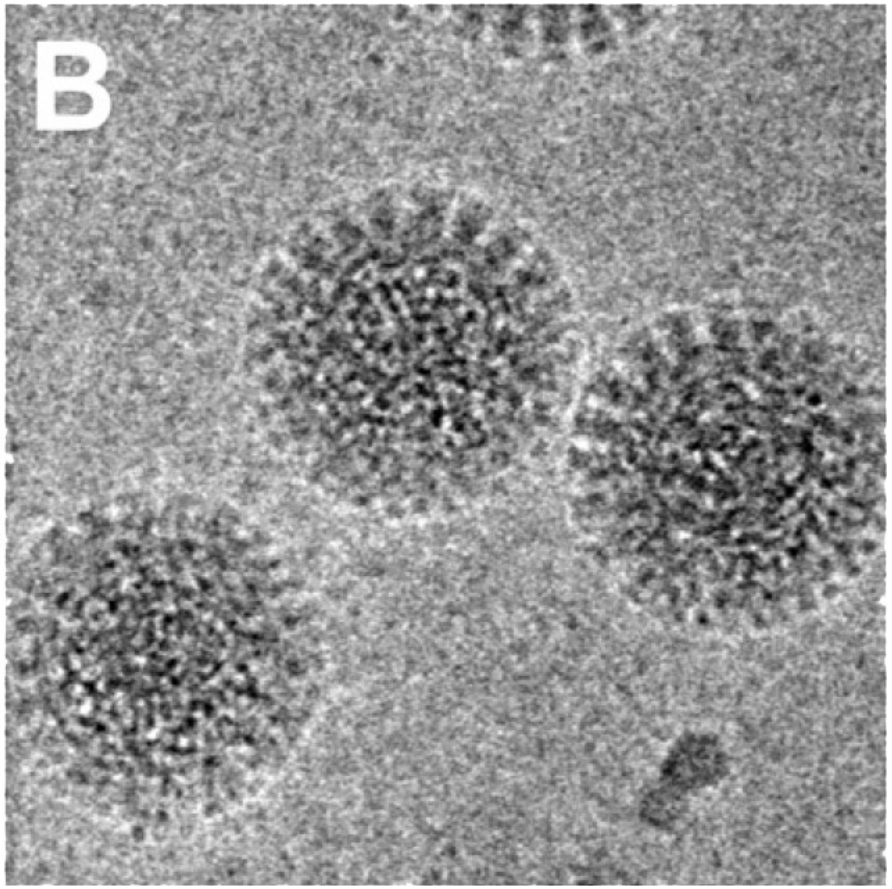


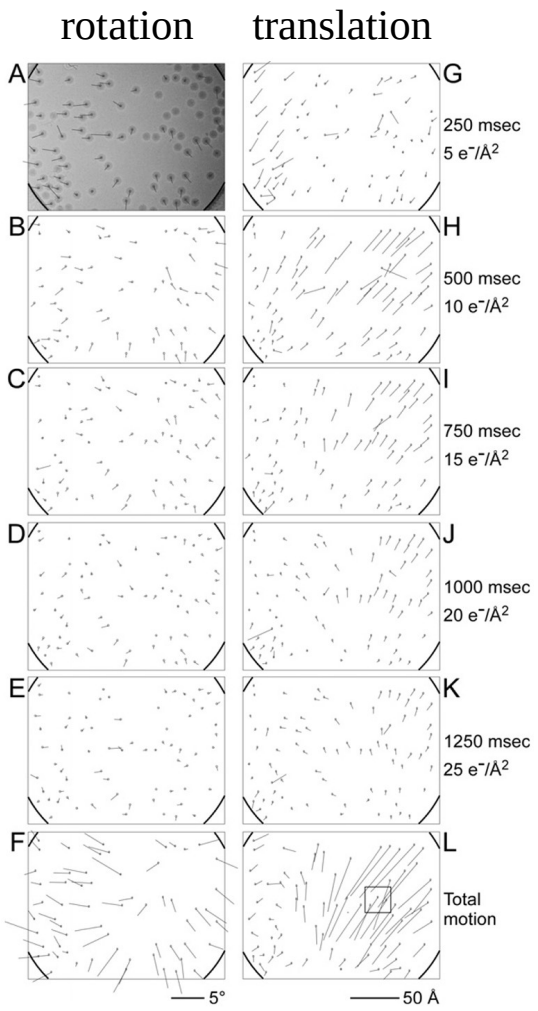
Image quality close to perfect !

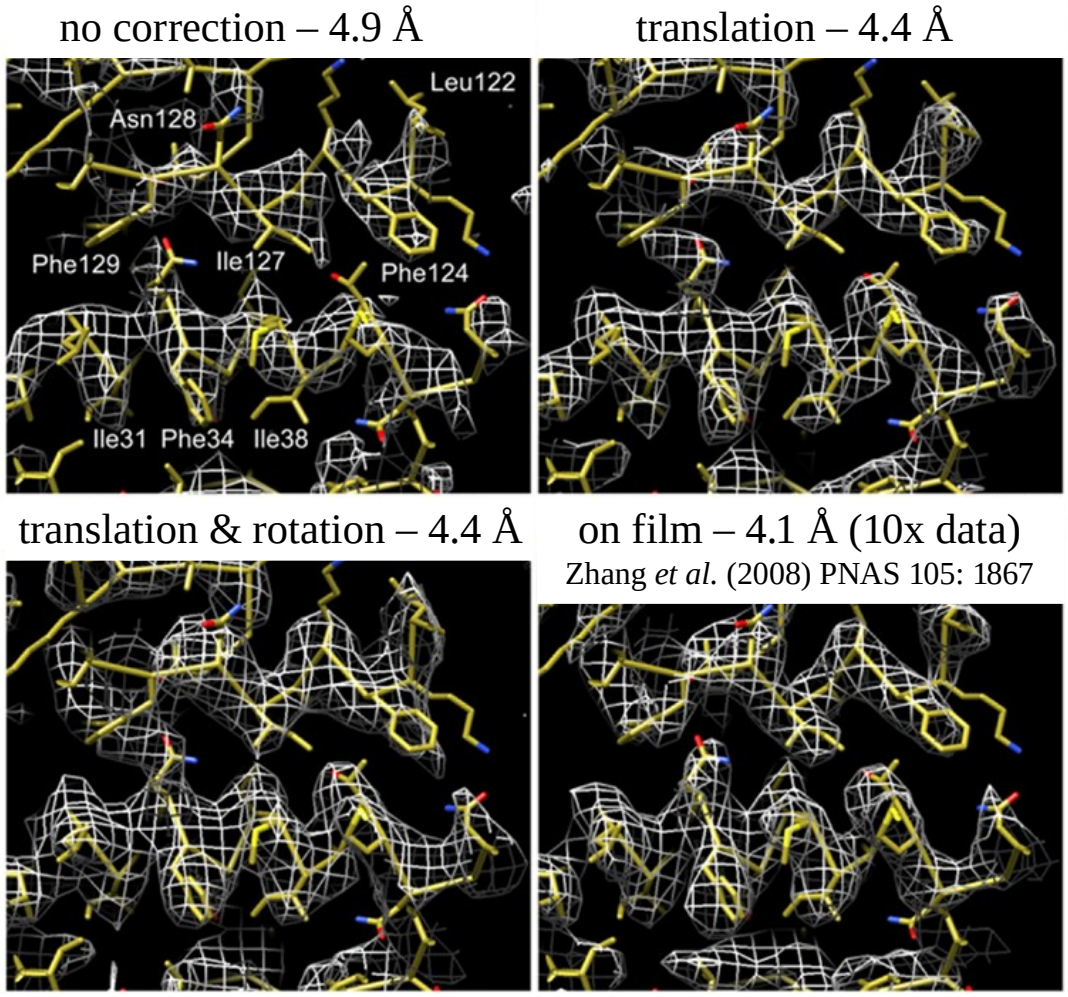
changes the way we assess and ensure image quality

Brilot *et al.* (2012) *JSB* 177: 630-637 and Campbell *et al.* (2012) *Structure* 20: 1-6

The “future” of single-particle EM

DDD camera – recording movies





Brilot *et al.* (2012) *JSB* 177: 630-637 and Campbell *et al.* (2012) *Structure* 20: 1-6

The “future” of single-particle EM

DDD camera combined with automation

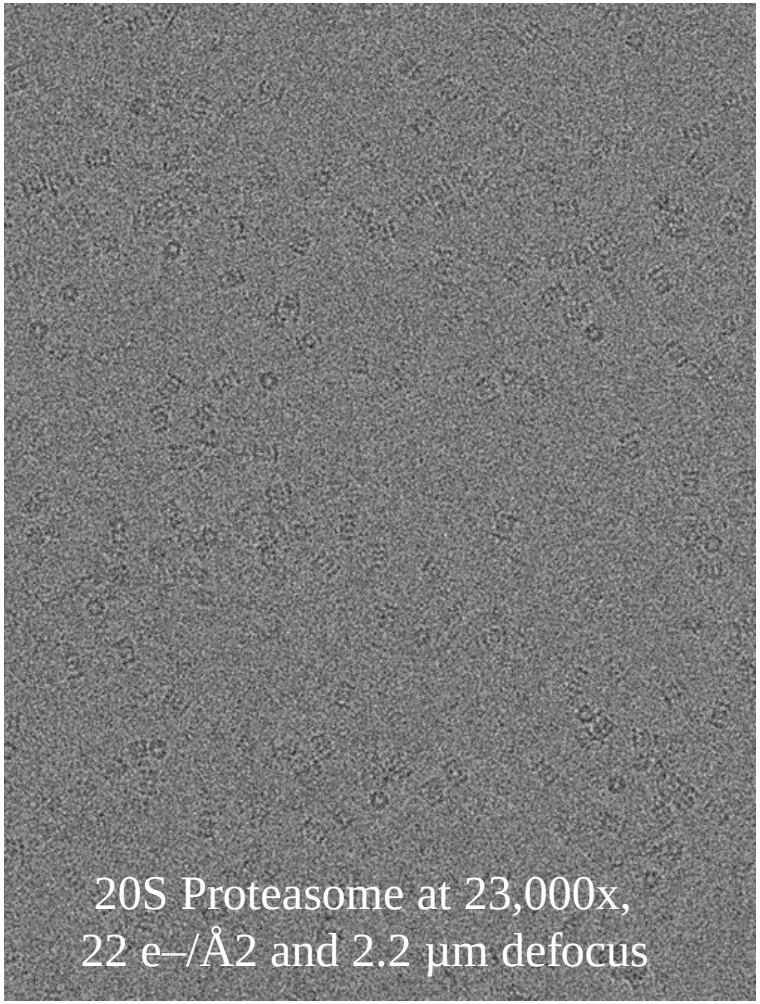
2012 GRC on Three-dimensional Electron Microscopy

Poster Session A (Mon/Tue)

Name	Affiliation	Poster Title
BUTCHER, SARAH	UNIVERSITY OF HELSINKI	TBD
CARAZO GARCIA, JOSE MARIA	NATIONAL CENTER FOR BIOTECHNOLOGY	Xmipp 3.0: Advances in image processing for 3D Electron Microscopy of Single Particles.
CHEN, JAMES	MIT	A Direct Classification Method for Single-Particle Electron Microscopy
CHENG, YIFAN	UNIVERSITY OF CALIFORNIA SAN FRANCI	A Near atomic resolution 3D reconstruction in 5 days made possible by the first K2 camera
COMOLLI, LUIS	LAWRENCE BERKELEY NATIONAL LABORATO	Cryo-ET and Cryo-EM image analysis of a surface layer protein self-assembly in 2D lattices and higher order 3D structures in solution
DE MARCO, ALEX	EUROPEAN MOLECULAR BIOLOGY LABORATO	Insights in subtomogram averaging: validations, and transversal measurements
EFREMOV, ROUSLAN	MAX-PLANCK-INSTITUTE FOR MOLECULAR	Potential of lipid nanodiscs for single particle cryo EM of membrane protein complexes

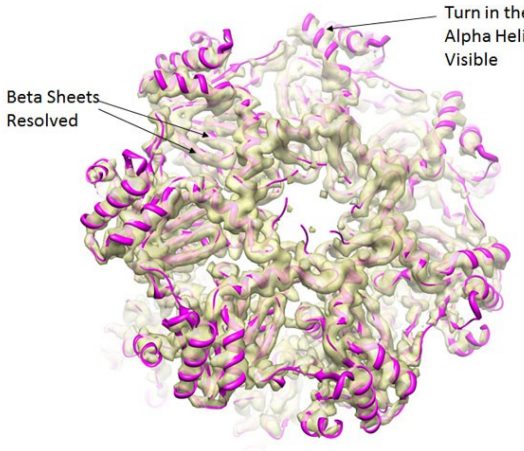
The “future” of single-particle EM

DDD camera combined with automation

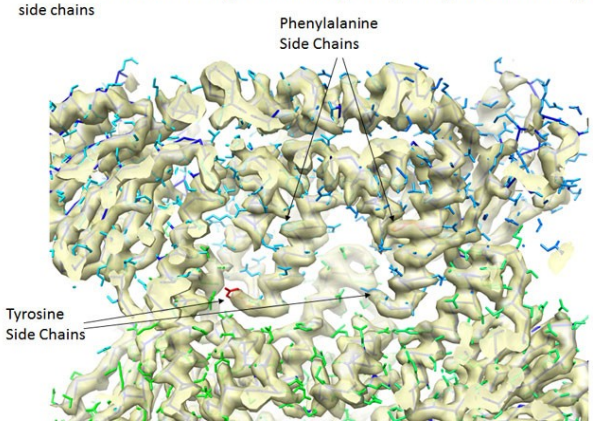


20S Proteasome at 23,000x,
22 e–/Å2 and 2.2 µm defocus

A recent reconstruction of the 20S Proteasome from K2 Summit™ Counting data shows estimated to be at 4.4 Å resolution (0.5 FSC). Å resolution shows both beta sheet and alpha helices.



The current structure is starting to show density corresponding to some of the larger side chains

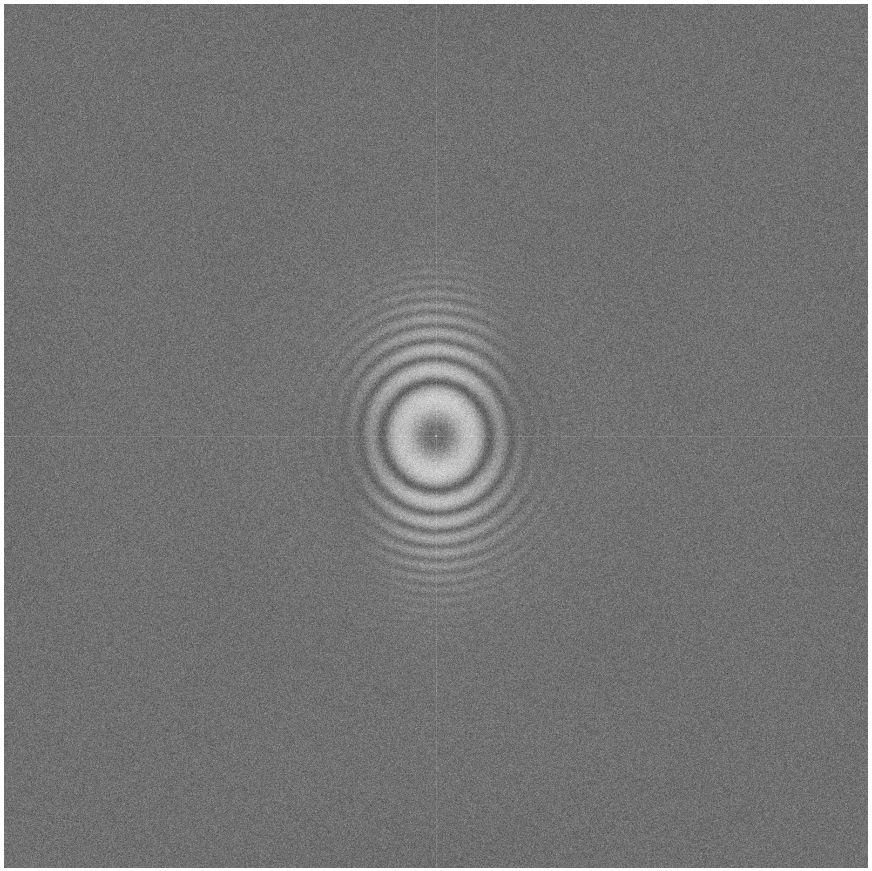


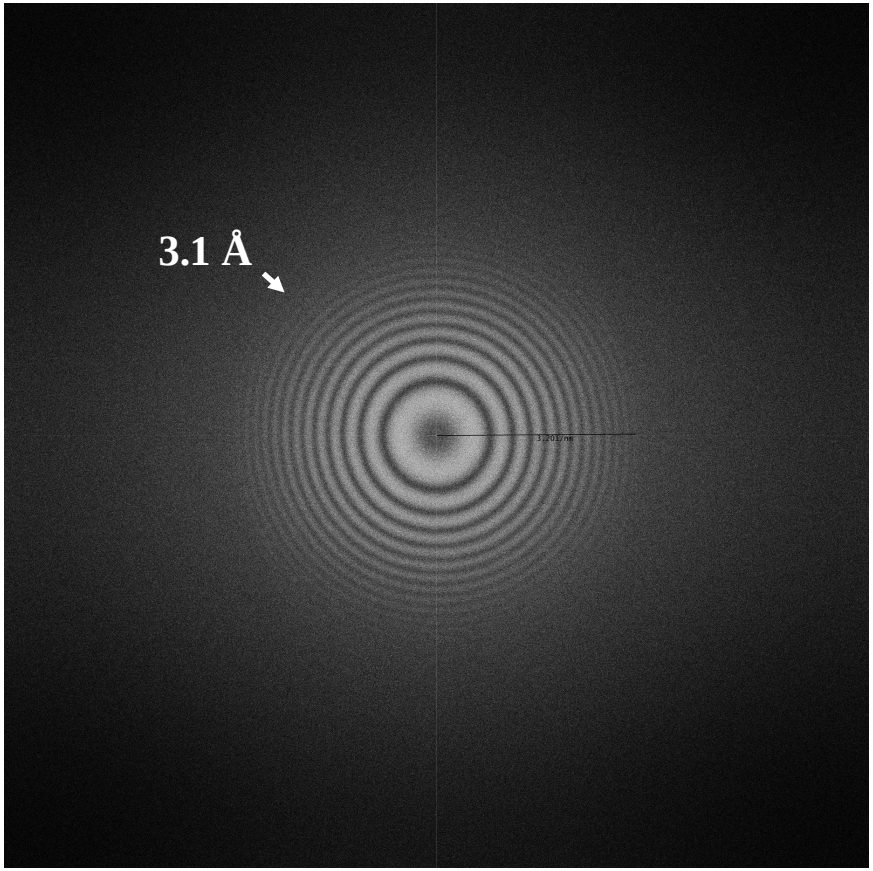
Gatan web page – Data from Xueming Li & Yifan Cheng

The “future” of single-particle EM

DDD camera on a Tecnai F20 (side-entry holder)

Data recorded on Tecnai F20 @ 200 kV, 25,000x in super resolution mode
Exposure time: 10x 0.5s (total time: 5s) / Dose rate: 2.433 e–/pixel/s (total dose: ~36 e–/Å2)

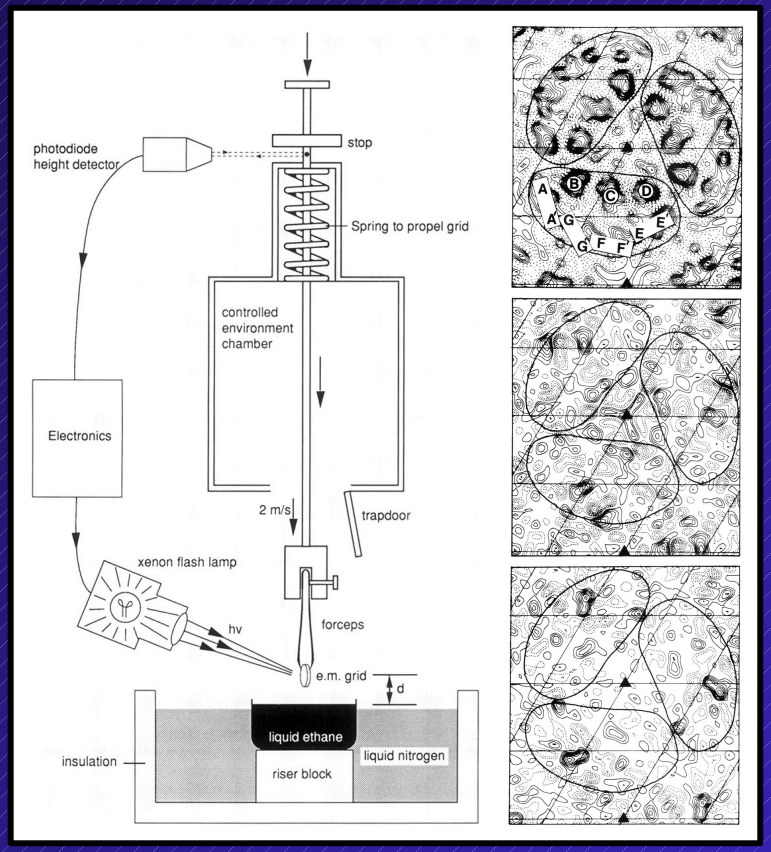




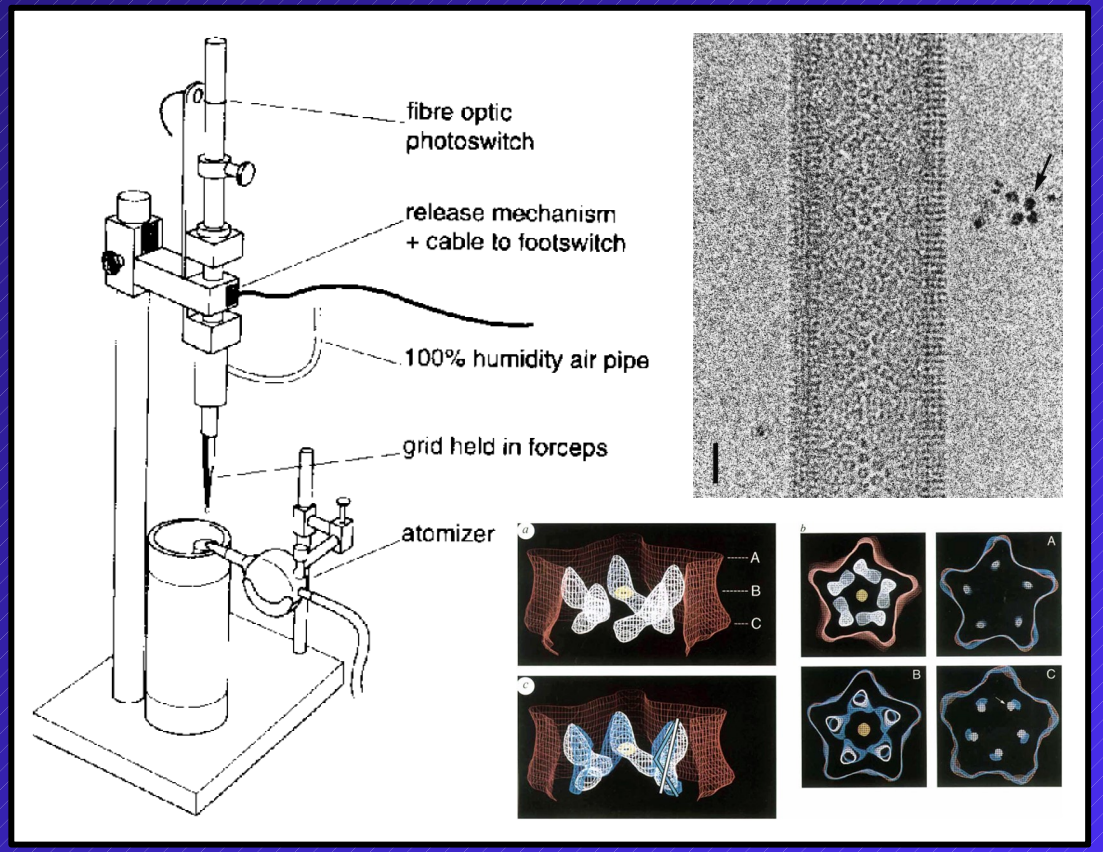
Can compensate for drift as well as beam-induced specimen movement !

The “future” of single-particle EM

Time-resolved EM



Subramaniam *et al.* (1993)
EMBO J. 12: 1-8



Berriman & Unwin (1994) *Ultramicroscopy* 56: 241-252
Unwin (1995) *Nature* 373: 37-43

Joachim Frank: Rapid mixing apparatus

The dark side of single-particle EM

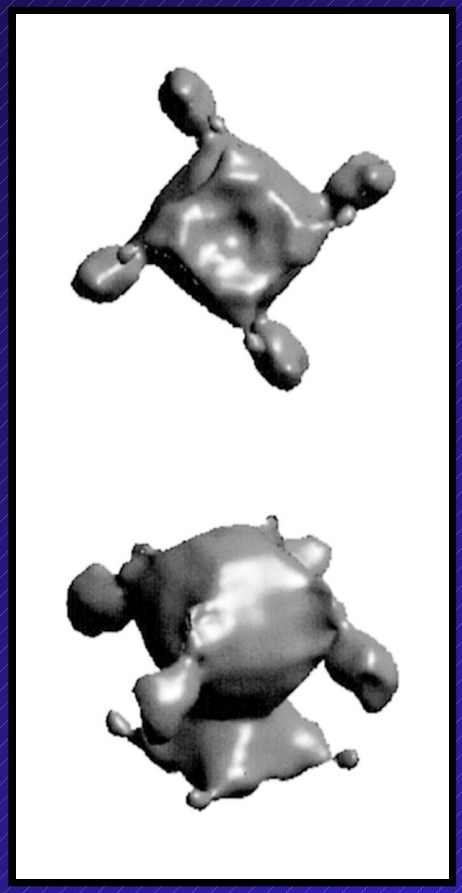
The great thing about single-particle EM:
Every data set and processing approach yields a 3D structure !

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

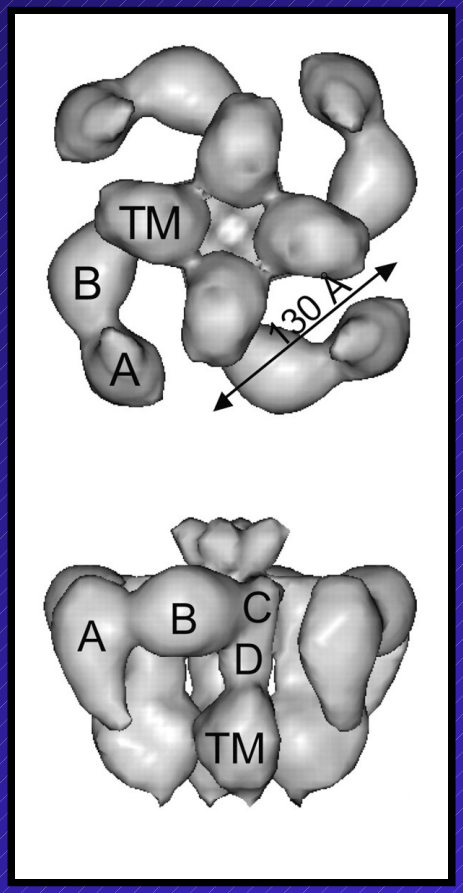
But is it correct ???

The dark side of single-particle EM

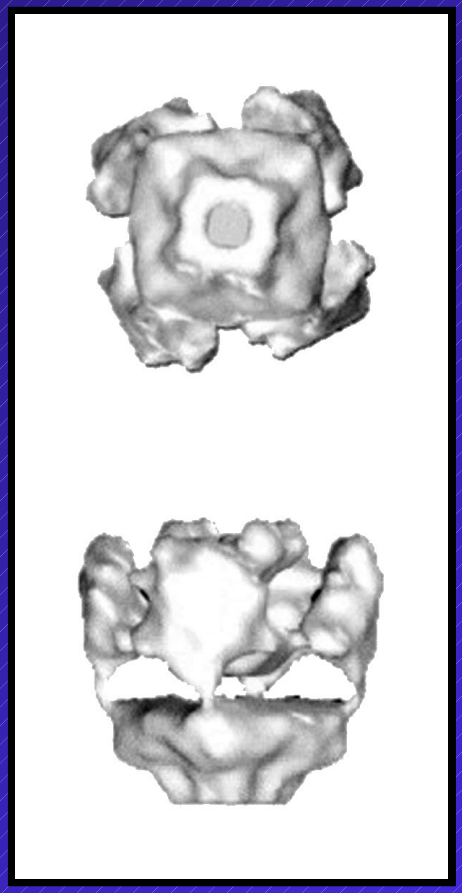
3D maps of the IP3 receptor



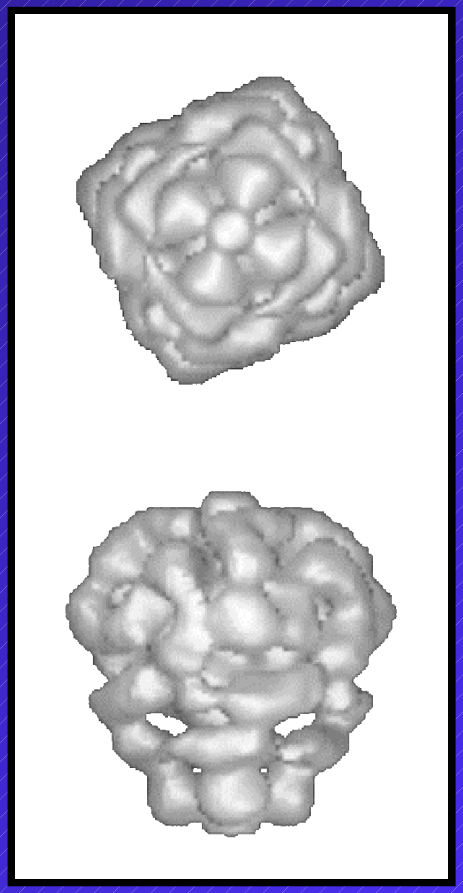
Jiang *et al.* (2002)
EMBO J. 21: 3575-3581



Serysheva *et al.* (2003)
JBC 278: 21319-21322



Da Fonseca *et al.* (2003)
PNAS 100: 3936-3941



Sato *et al.* (2004)
JMB 336: 155-164

What can go wrong in single-particle EM

Every single step !

Sample: can be heterogeneous
→ If not taken into account, 3D map will be a mixture of different structures

Sample preparation: negative staining can introduce artifacts
→ 3D map will be flattened and/or distorted

Data collection: non-randomly distributed orientations
→ 3D map will be less defined (lack resolution) in certain directions

Initial model generation: may not reflect actual structure
→ 3D map may end up having spurious features or be completely incorrect

Map refinement: risk of over-refinement (alignment of noise)
→ 3D map may end up having spurious features (and artificially high resolution)

Resolution measurement: problems of over-refinement and cut-off
→ resolution may not be appropriate to follow improvement of 3D map

Map interpretation: map may not have sufficient or may have incorrect details
→ incorrect pseudo-atomic model / risk of flexible fitting

→ Push button software carries risks !
→ Importance of structure validation !

Sample heterogeneity

To obtain a meaningful average/3D map,
the imaged particles have to be structurally identical

Heterogeneity is a problem, in particular because it is difficult for particles with randomly distributed orientations to distinguish between projection images of two particles in different orientations and two particles with different structures

Many ways a sample can be heterogeneous

Discrete heterogeneity

– dirty protein preparations:
  – contaminants, degradation products
  – mixtures (e.g., different oligomeric states)

– unstable complexes:
  – complexes with/without subunits
  – proteins/complexes with/without binding partners (substrates, activators, Fabs etc.)

– proteins/complexes with different but well-defined conformational states

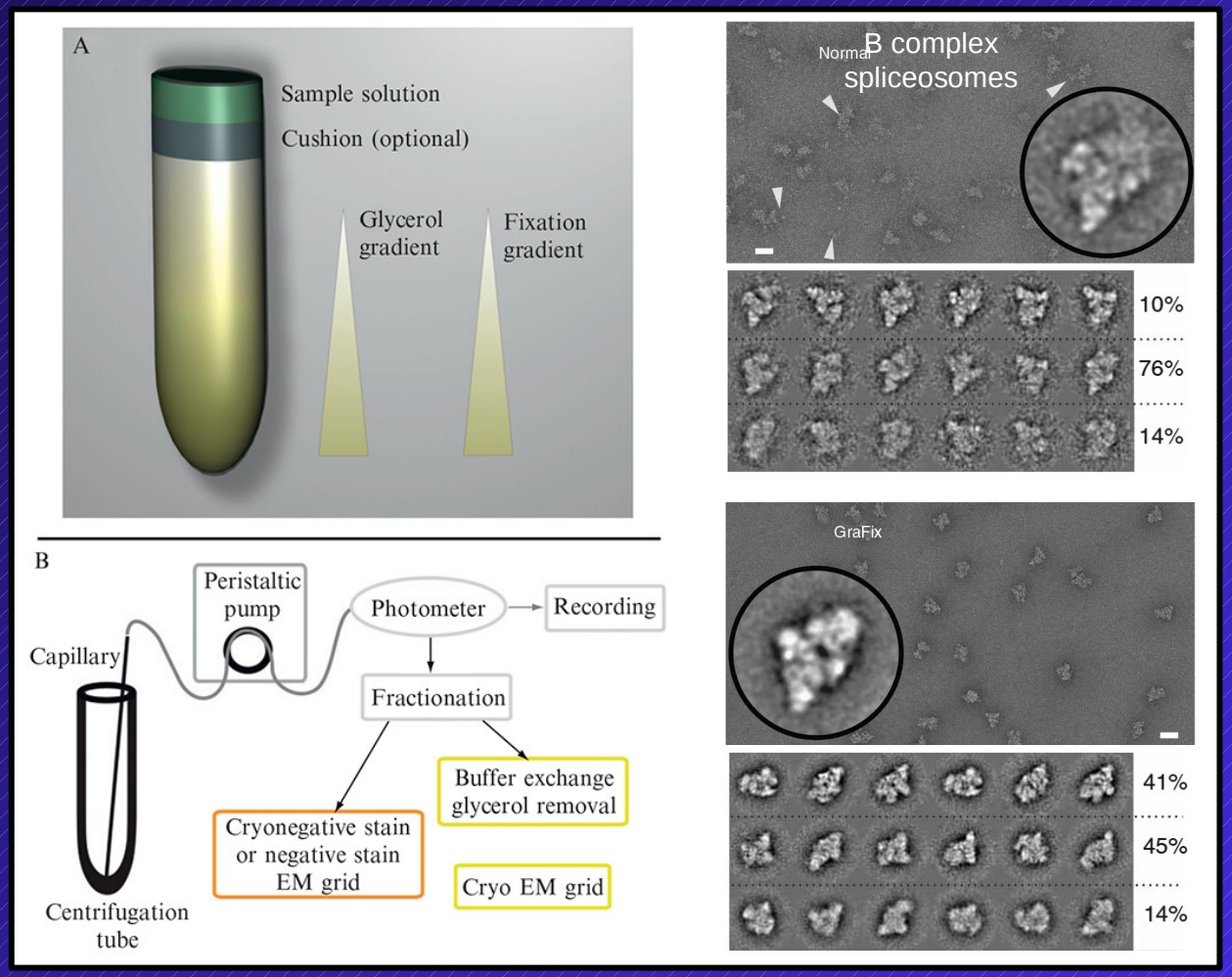
Continuous heterogeneity

– flexible overall structure
– flexibly tethered domains

Different types of heterogeneity require different approaches

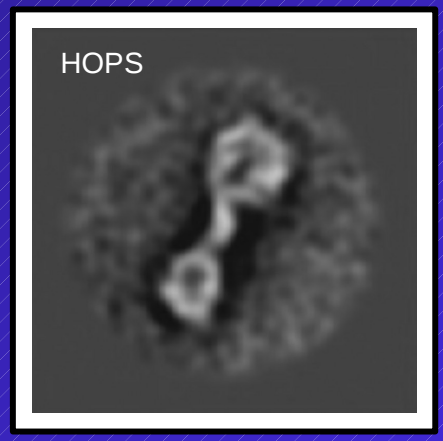
Discrete heterogeneity

Labile complexes: chemical fixation



Stark (2010)
Methods Enzymol. 481: 109-126

Uchtenhagen *et al.* (2008)
Nat. Methods 5: 53-55

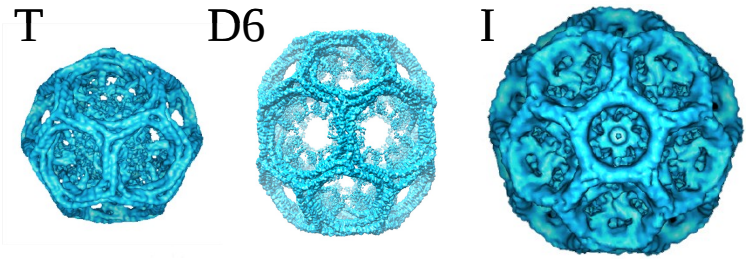


Bröcker *et al.* (2012)
PNAS 109: 1991-1996

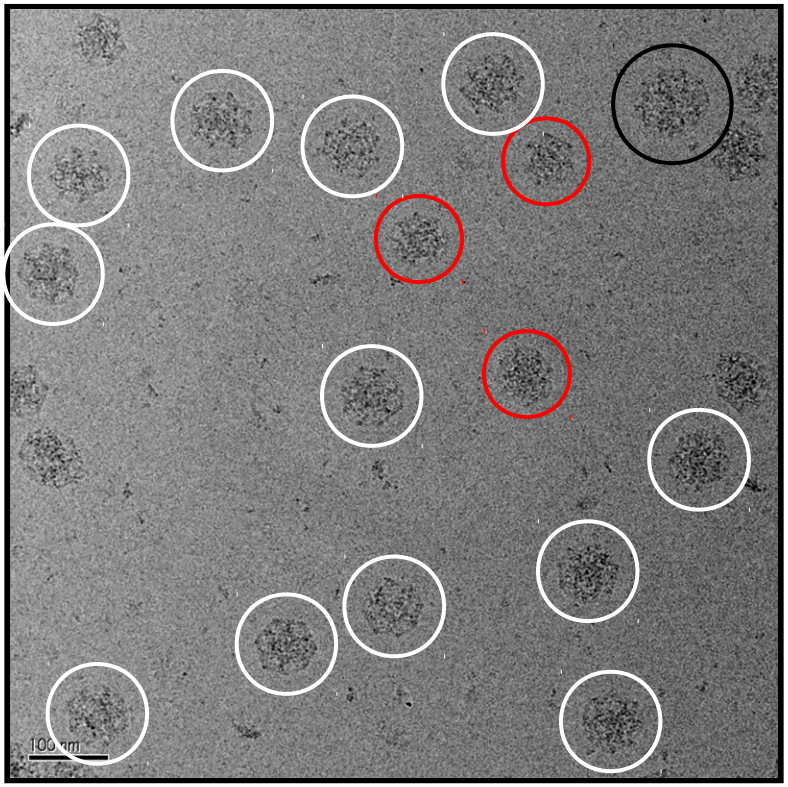
Discrete heterogeneity

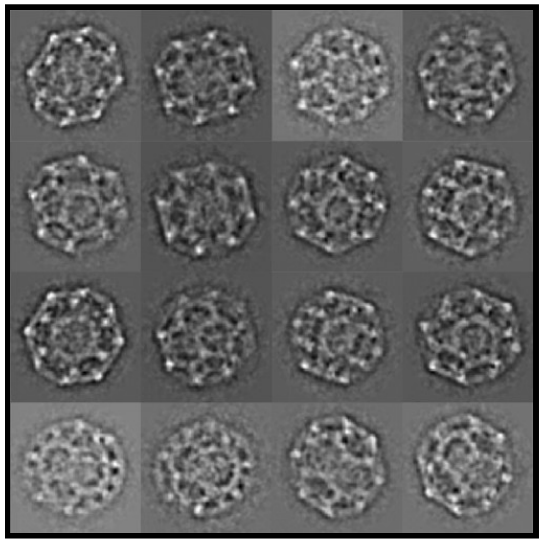
Classification of 2D projections followed by 3D reconstruction

clathrin coat types

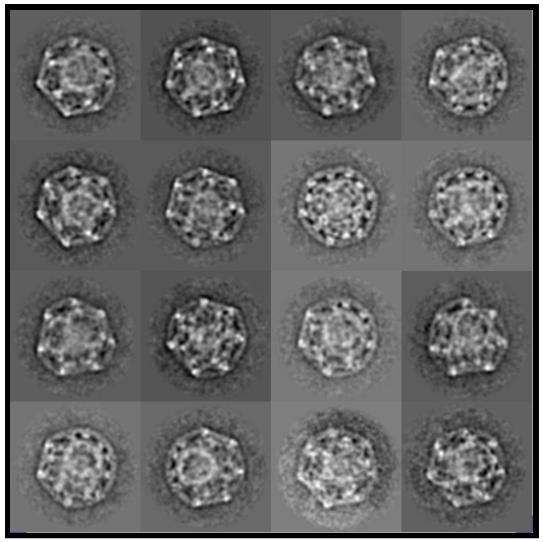


raw image (ice)





averages of D6 coats

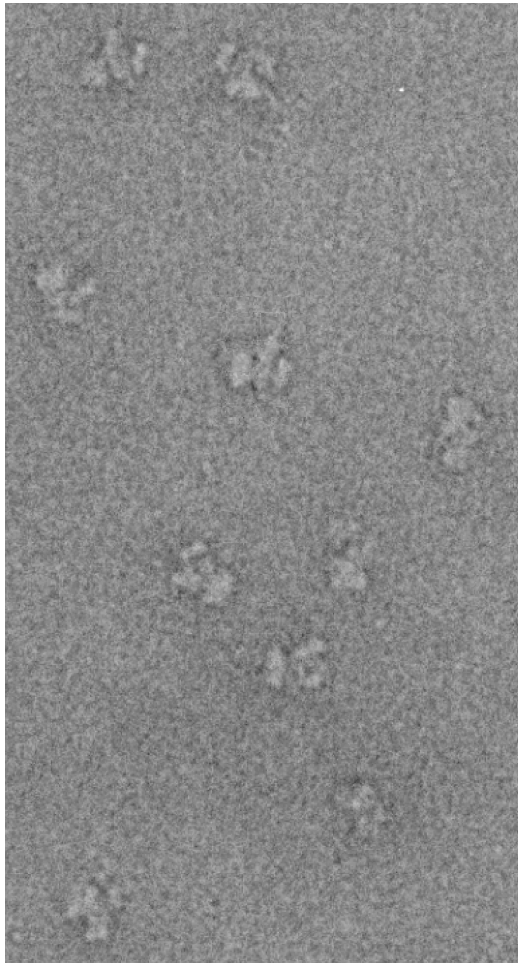


averages of T coats

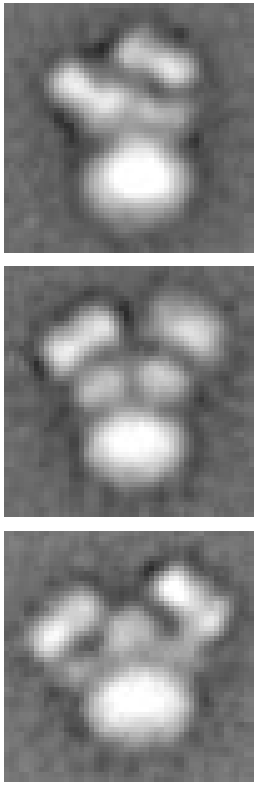
Discrete heterogeneity

Classification of 2D projections followed by 3D reconstruction

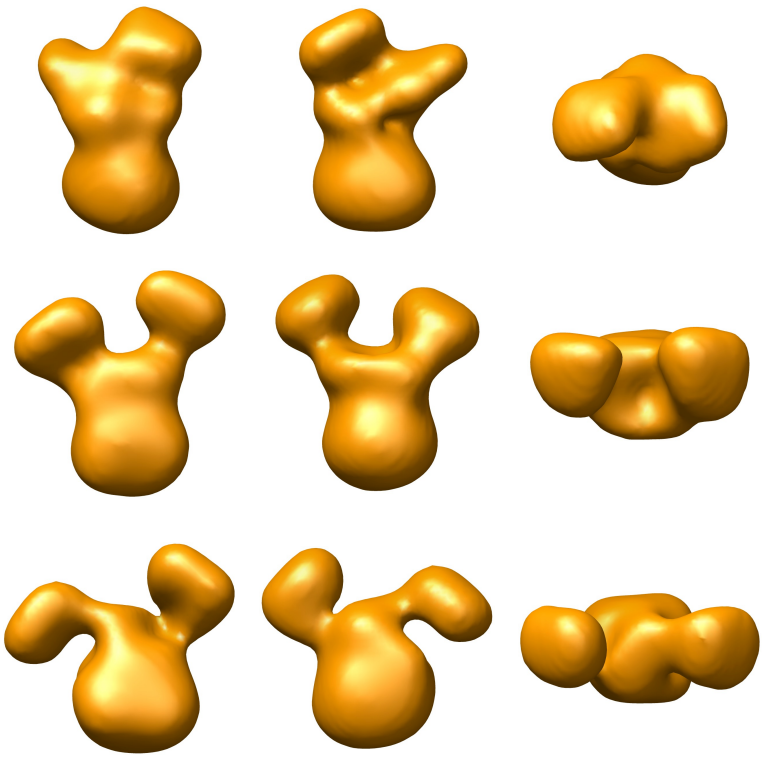
Collect 0º/60º tilt pairs (negative stain)



Classify particles from 0º images



Calculate 3D reconstructions using the RCT approach



Use 3D maps to align and classify particles from cryo-EM images

Discrete heterogeneity

Classification and 3D reconstruction combined

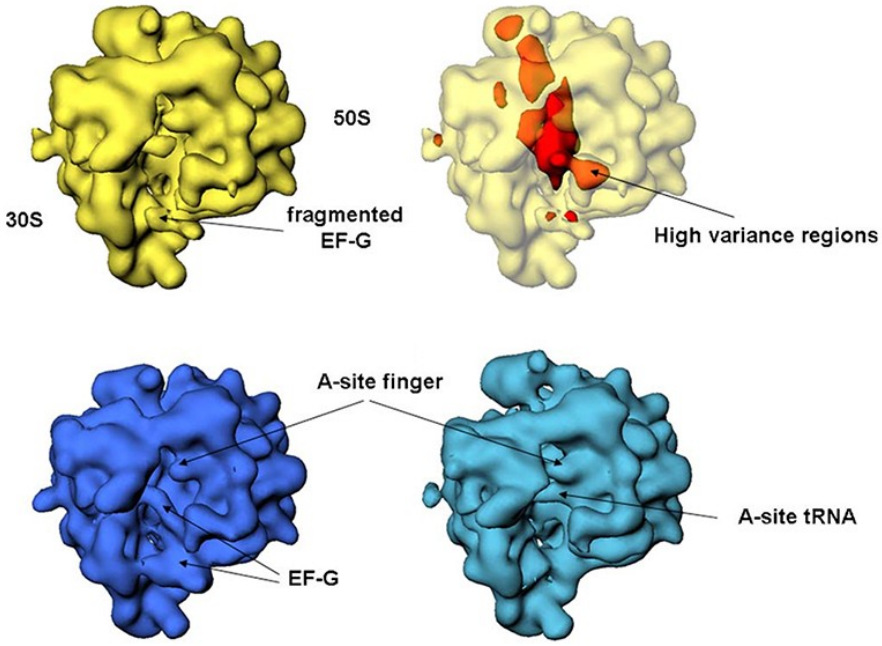
3D variance map followed by focused classification

Calculation of:
structure of the complex using the 3-D projection alignment technique
3-D variance and covariance maps using the bootstrap technique

Determination of *K* initial templates:
3-D spherical mask is placed in the location corresponding to the region of high variance and
projected into *I* quasi-evenly distributed angular directions yielding a set of 2-D binary mask
2-D masks are used to classify the 2-D projection data according to the average pixel density within regions outlined by respective masks

Refinement:
of *K* initial templates using the multireference 3-D projection alignment

3D reconstruction
using entire data set



high EF-G
occupancy

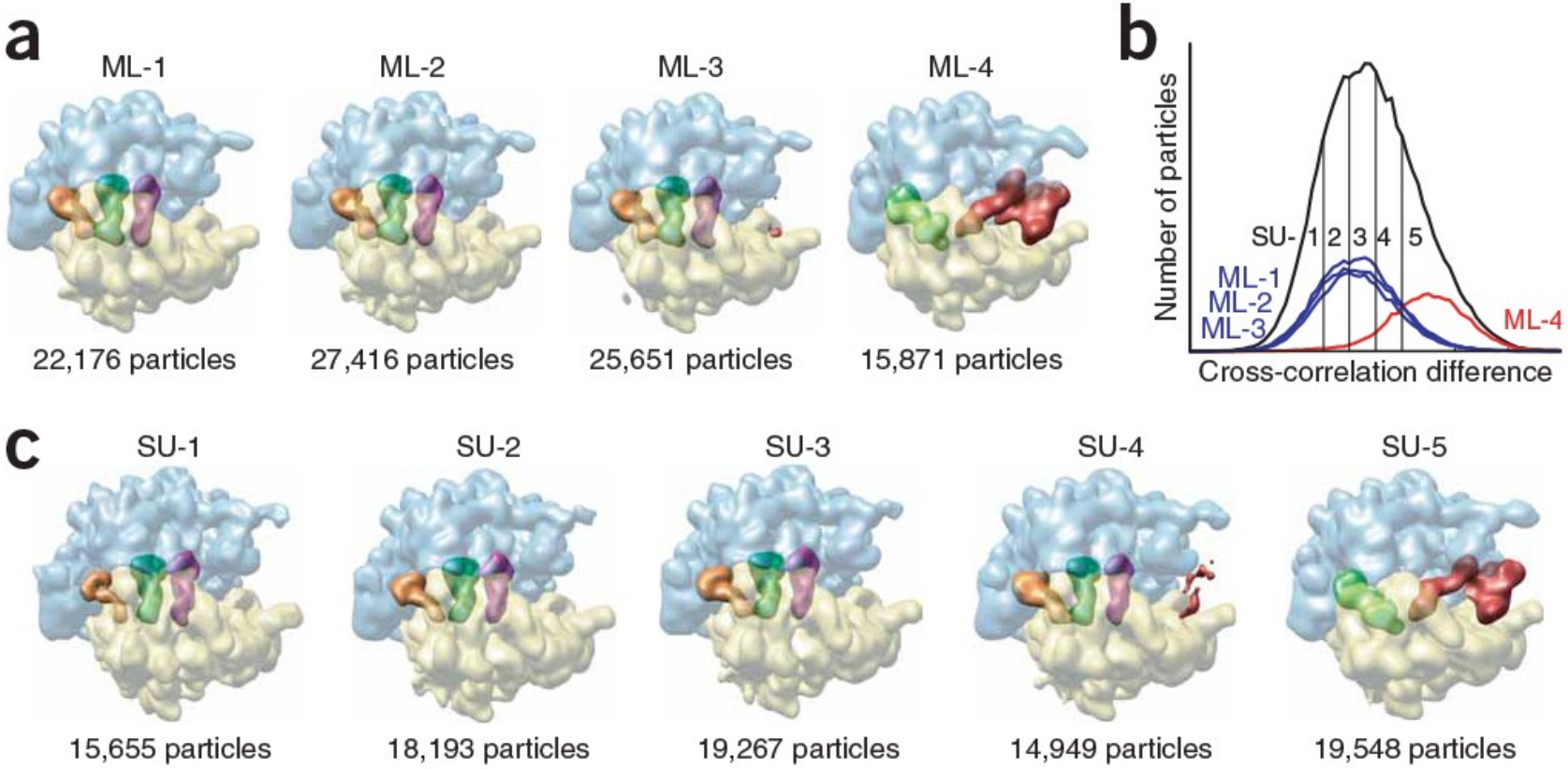
low EF-G
occupancy

Penczek *et al.* (2007) *JSB* 154: 184-194

Discrete heterogeneity

Classification and 3D reconstruction combined

Simultaneous refinement of multiple structures (using maximum likelihood)



Scheres *et al.* (2007) *Nat. Methods* 4: 27-29

Discrete heterogeneity

Classification and 3D reconstruction combined

2012 GRC on Three-dimensional Electron Microscopy

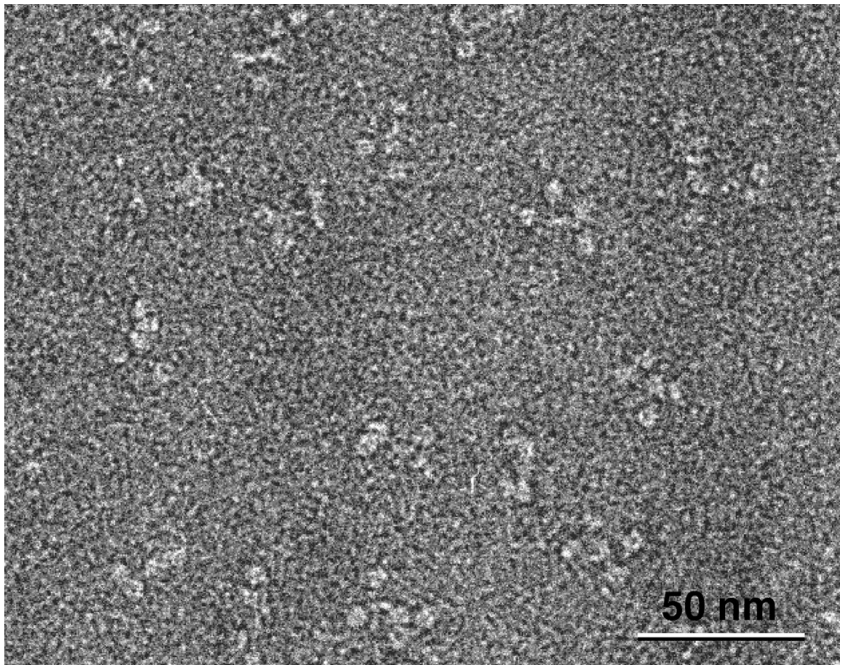
Poster Session A (Mon/Tue)

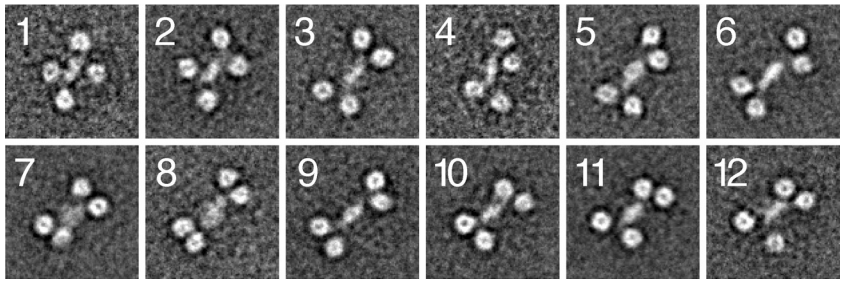
Name	Affiliation	Poster Title
ELMLUND, DOMINIKA	STANFORD UNIVERSITY	SIMPLE: software for ab initio reconstruction of flexible single-particles
ELMLUND, HANS	STANFORD UNIVERSITY	Single-particle ab initio reconstruction and heterogeneity analysis via bijective orientation search

Continuous heterogeneity

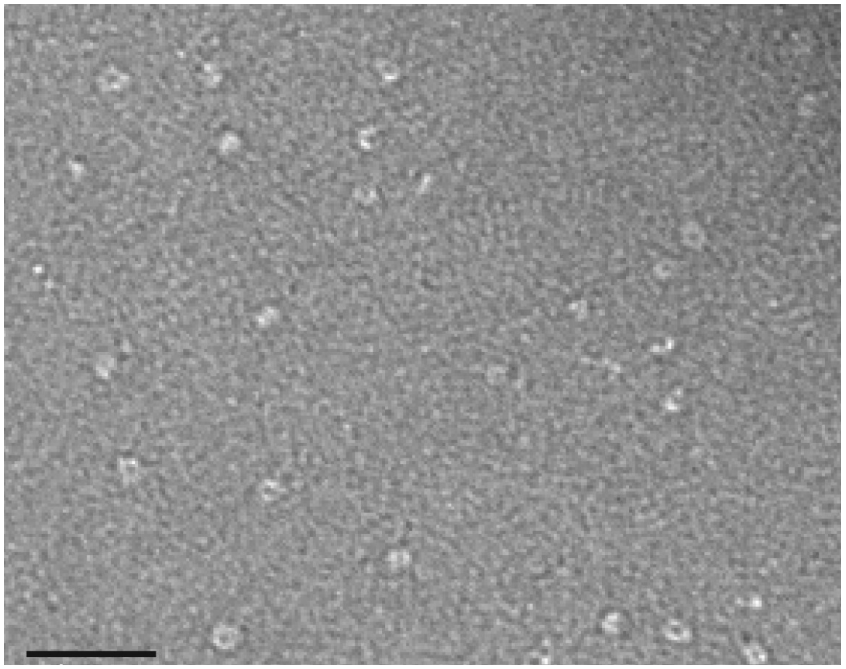
Flexibly tethered domains

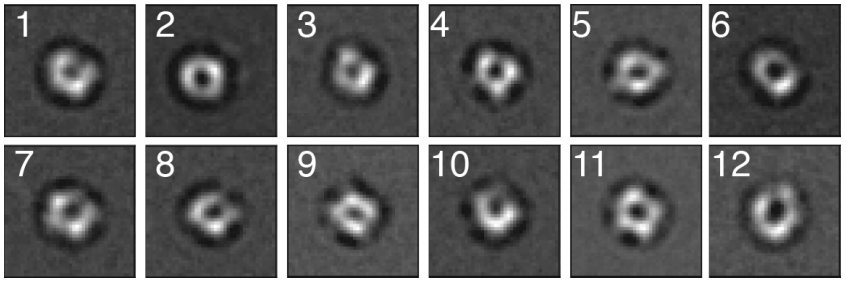
PRP19 (tetramer)





PRP19 WD40 domain

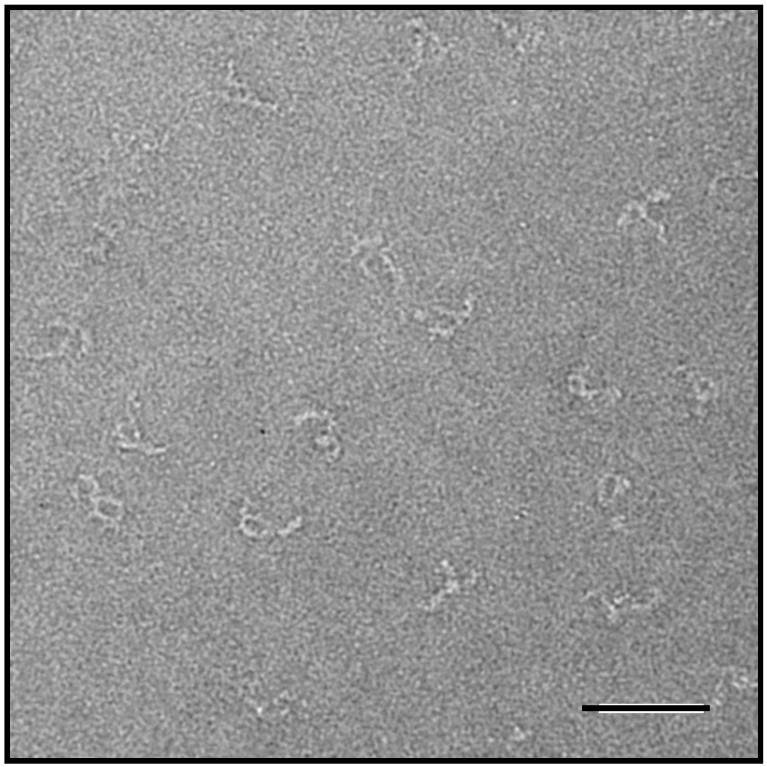




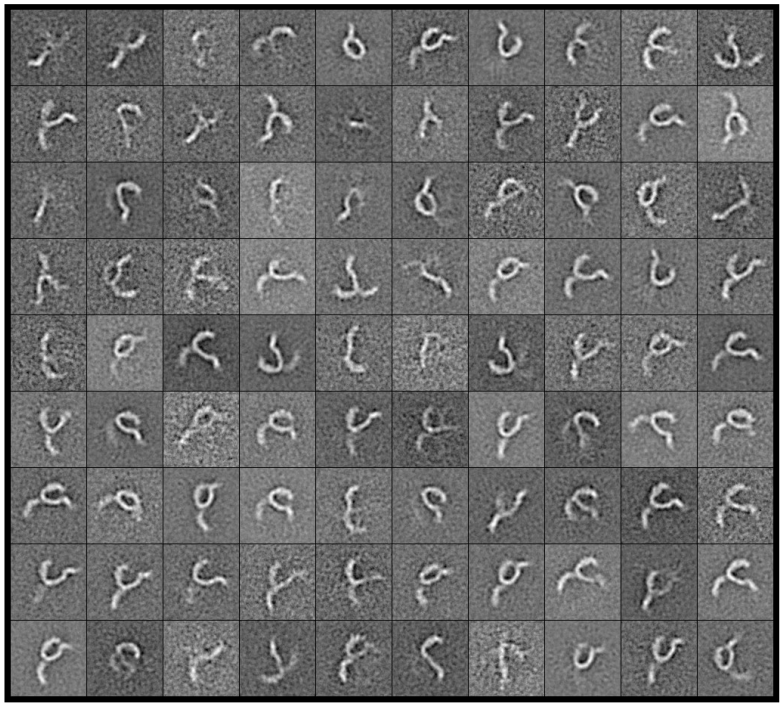
Continuous heterogeneity

Flexible overall structure (doable)

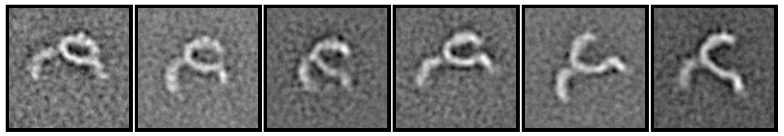
Cog1-4 sub-complex of COG



raw image (negative stain)



class averages

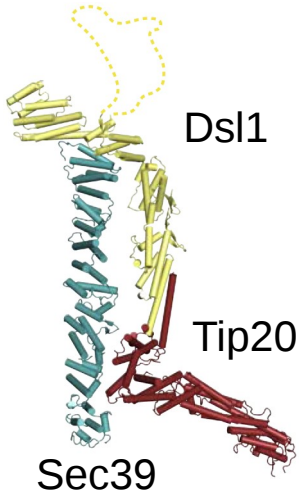


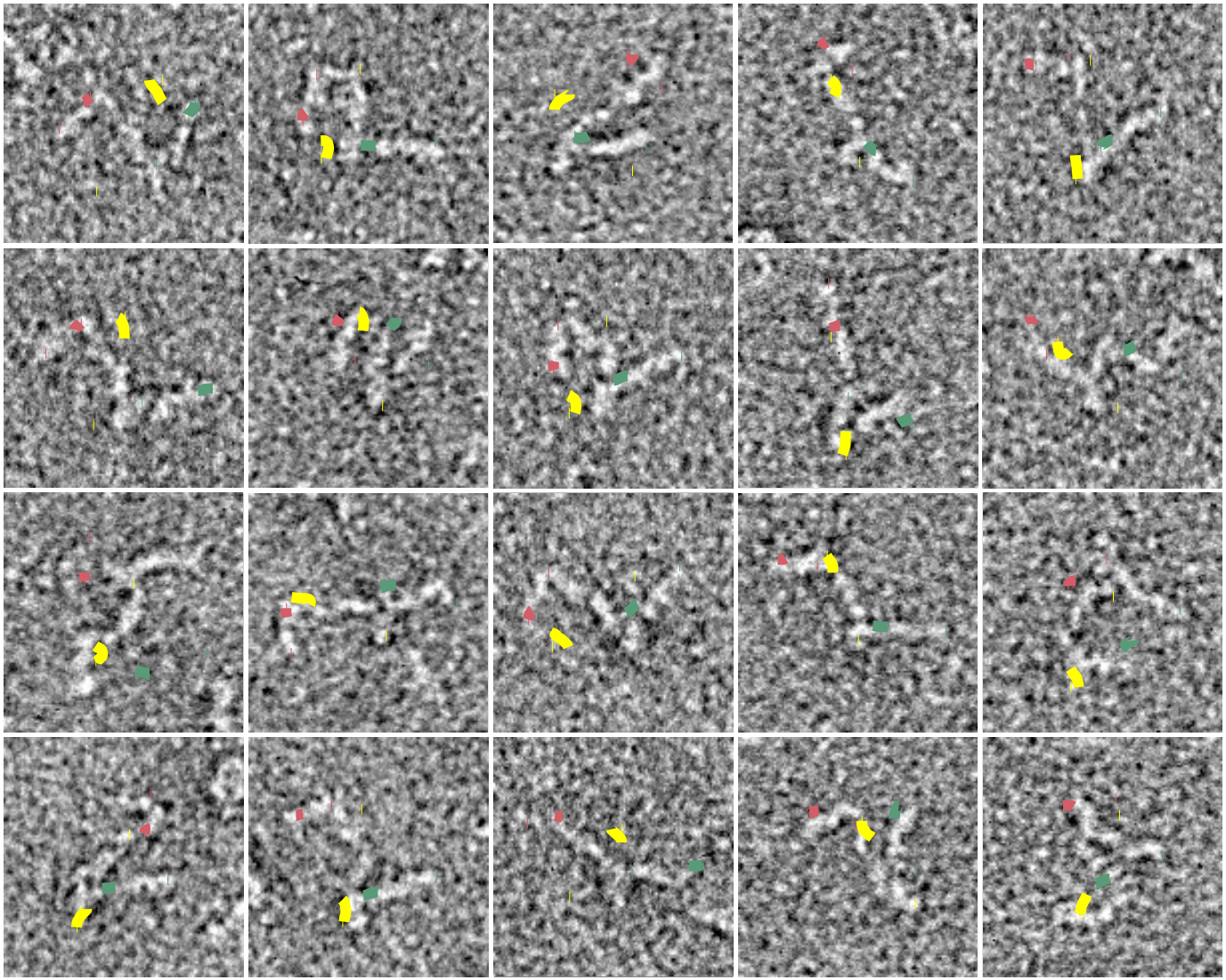
The finer the sampling of the conformational space, the more features can potentially be resolved, but the less contrast enhancement is achieved.

Continuous heterogeneity

Flexible overall structure (impossible)

Dsl1 complex



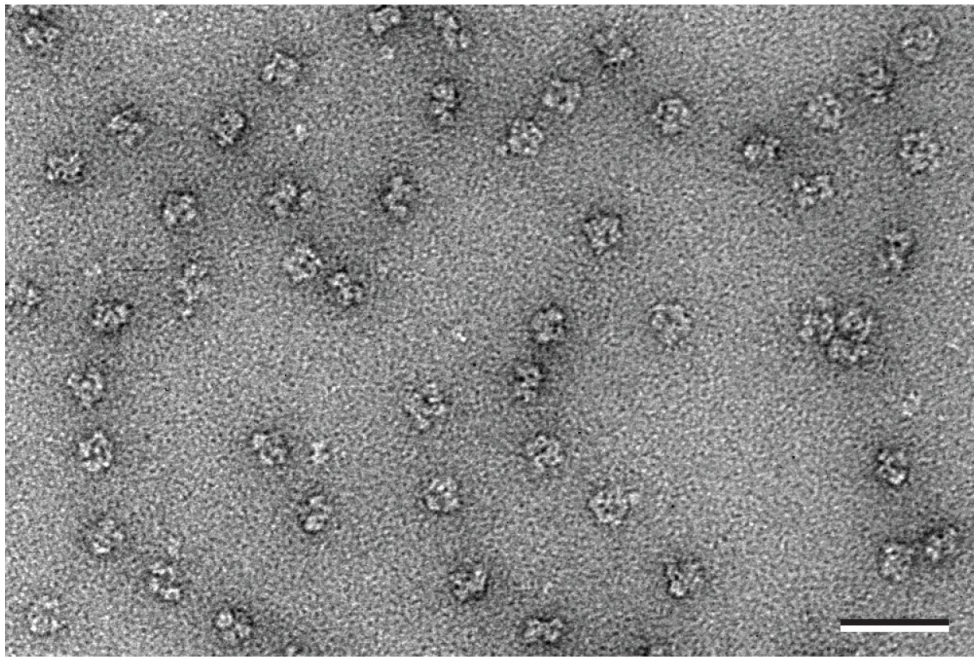


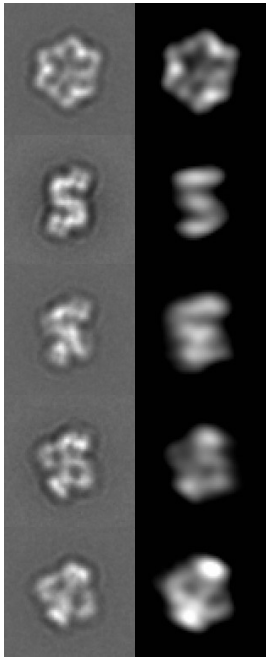
DOs and DON'Ts in single-particle EM ?

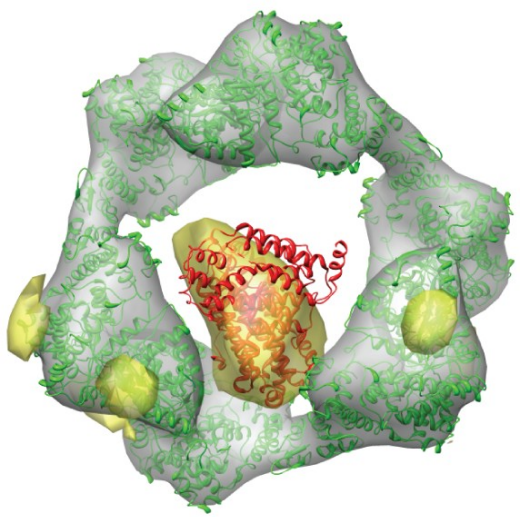
Sample

DO NOT assume that a sample is homogeneous
– even if it looks great biochemically (activity, gels etc.)
– virtually every sample has some degree/some kind of heterogeneity

DO check samples first by negative stain EM
– good contrast & usually preferred orientations
→ easier to assess quality and homogeneity of particles







DOs and DON'Ts in single-particle EM ?

Sample preparation

Negative staining
usually preferred orientations
→ tilting required
→ random conical tilt 3D reconstruction

Pros:
– good contrast, suitable for small molecules
– 3D reconstruction algorithm is reliable
– suitable for heterogeneous samples

Cons:
– limits achievable resolution to ~20 Å
– suffers from preparation artifacts (flattening, deformations, etc.)

Cryo-negative staining
(Holger Stark method)
same as negative staining, but minimizes preparation artifacts

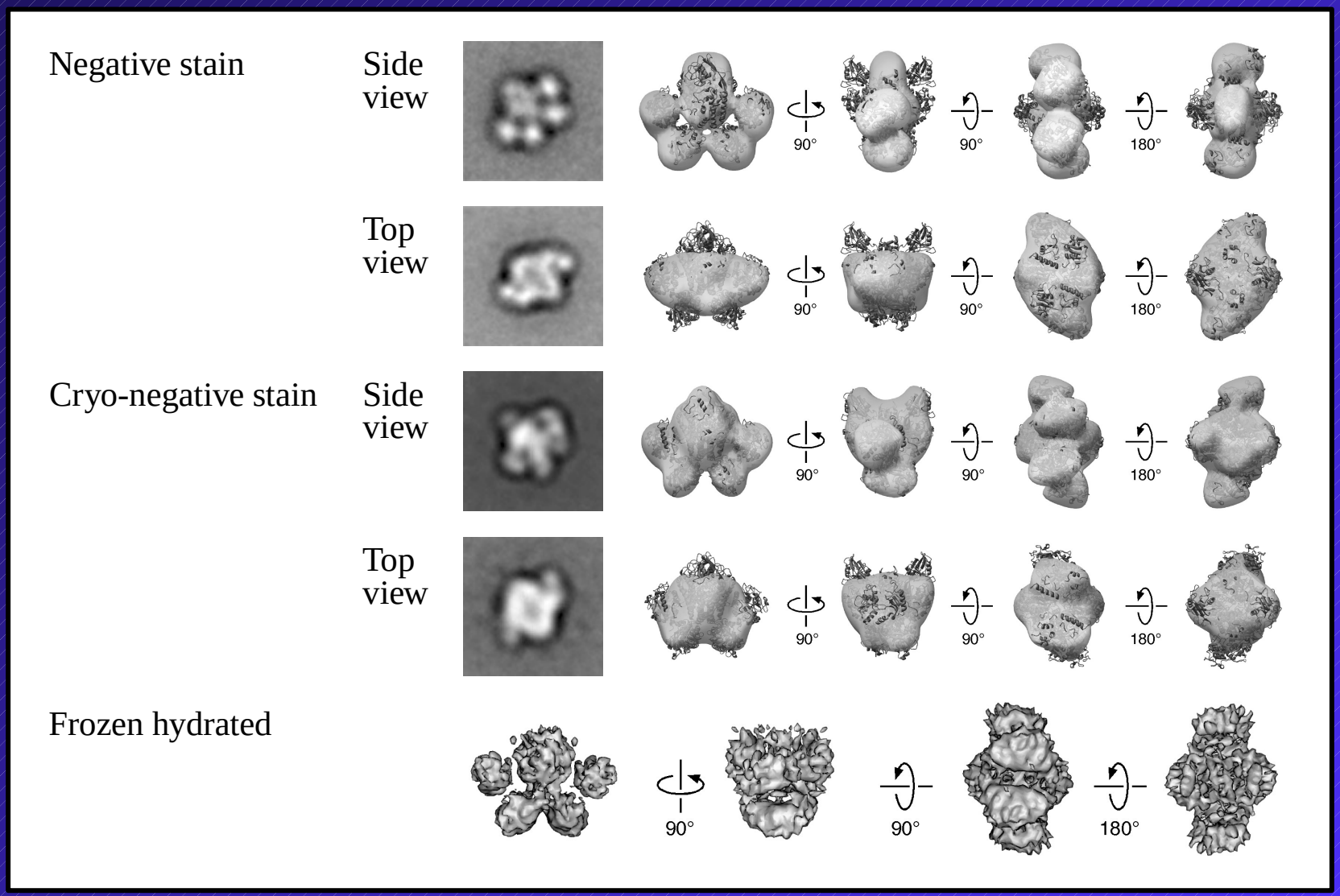
Vitrification
usually randomly distributed orientations
→ no tilting required
→ common line-based 3D reconstructions

Pros:
– no limitation of resolution
– best specimen preservation

Cons:
– poor contrast, unsuitable for small molecules (unless phase plate works)
– difficult for heterogeneous samples (not possible to distinguish between different view and different structure)
– common line-based 3D reconstructions not always reliable

DOs and DON'Ts in single-particle EM ?

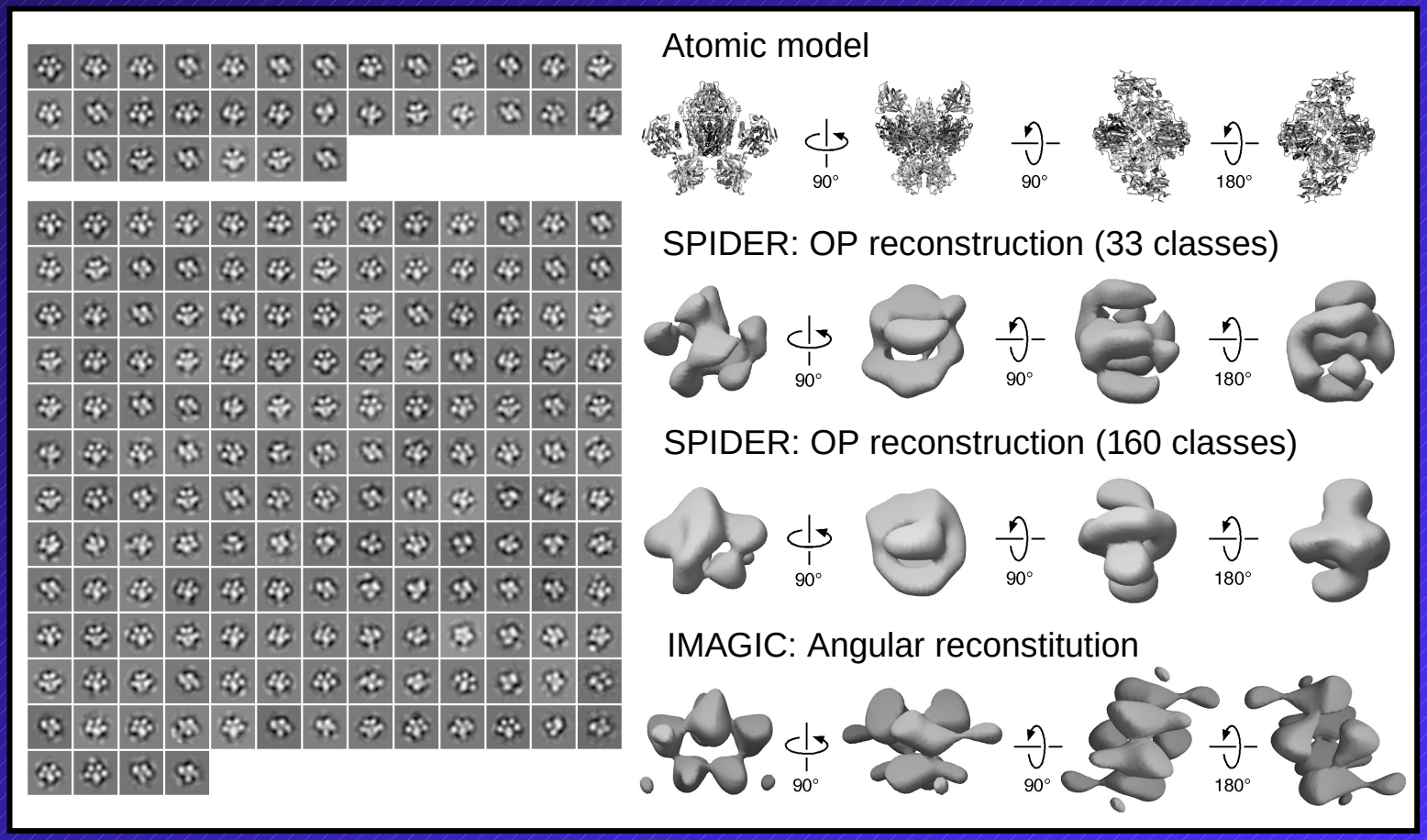
Sample preparation (Tf-TfR complex)



DOs and DON'Ts in single-particle EM ?

Initial model generation

DO NOT simply believe whatever the program generates
– random conical tilt reconstructions of negatively stained samples can suffer from distortions
– common line-based methods of vitrified samples may generate an inaccurate model



DOs and DON'Ts in single-particle EM ?

Initial model generation

DO NOT simply believe whatever the program generates
– random conical tilt reconstructions of negatively stained samples can suffer from distortions
– common line-based methods of vitrified samples may generate an inaccurate model

common line-based methods are continually being improved
and are becoming more reliable (if data set is homogeneous)

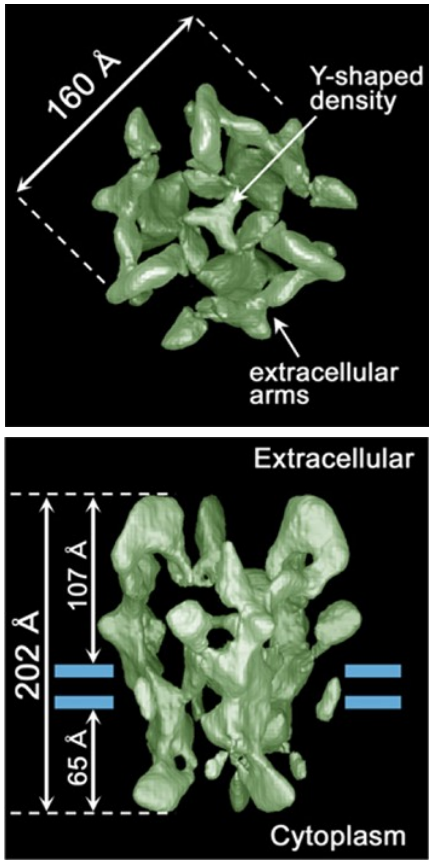
DOs and DON'Ts in single-particle EM ?

Software development

DO NOT develop or test new approaches or software tools
with uncharacterized test specimens

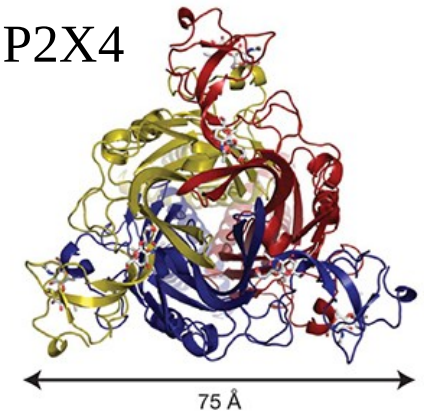
P2X2

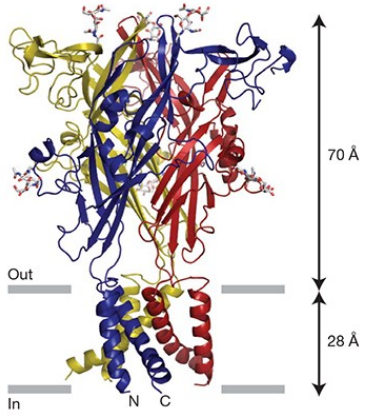
The P2X2 particles were picked up by a combination of two automatic programs: the autoaccumulation method using SA (Ogura and Sato, 2004a) and the three-layered neural network method (Ogura and Sato, 2001, 2004b), and the 3D structure was reconstructed with echo-correlated reconstruction methods using SA assuming C3 symmetry in our **s**ingle-**p**article **i**mage analysis method using **n**eural **n**etwork and **s**imulated annealing (SPINNS) (Yazawa et al., 2007) and other algorithms in the IMAGIC V software (van Heel et al., 1996)



Mio *et al.* (2009) *Structure* 17: 266-275

P2X4





Kawate *et al.* (2009) *Nature* 460: 592-8

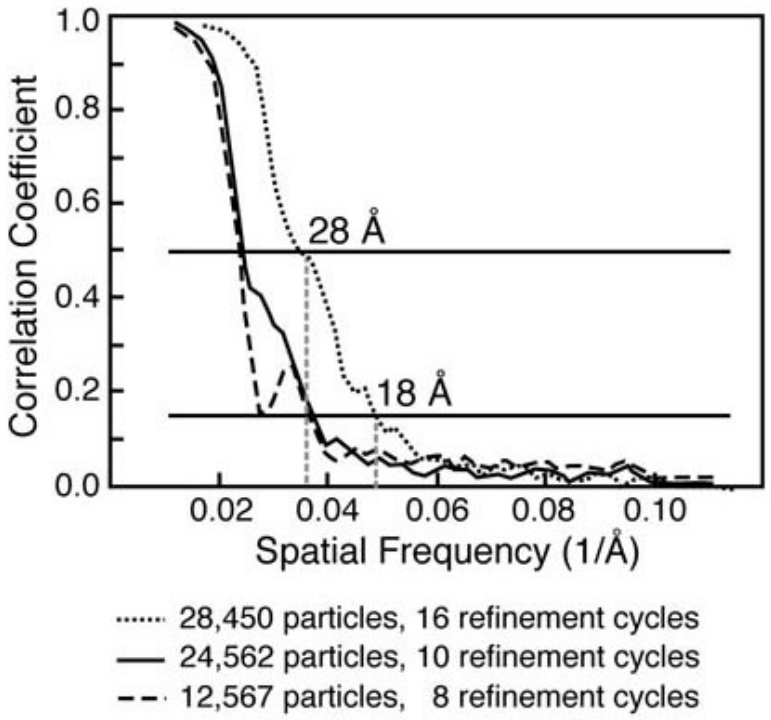
Validation of EM maps

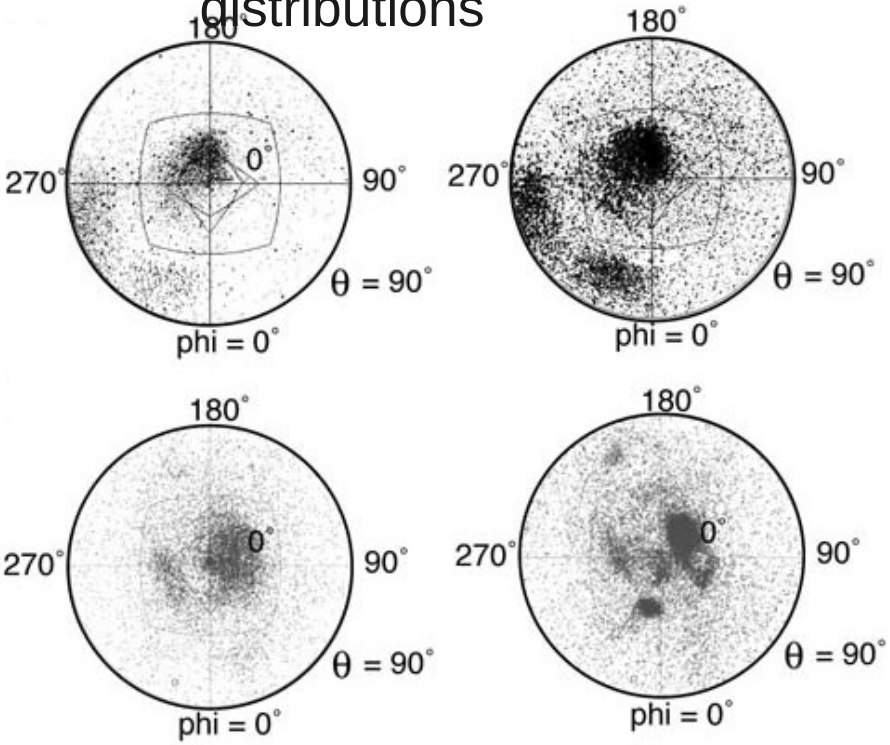
Quality control

Anaphase-promoting complex (APC)

FSC curves

Angular distributions

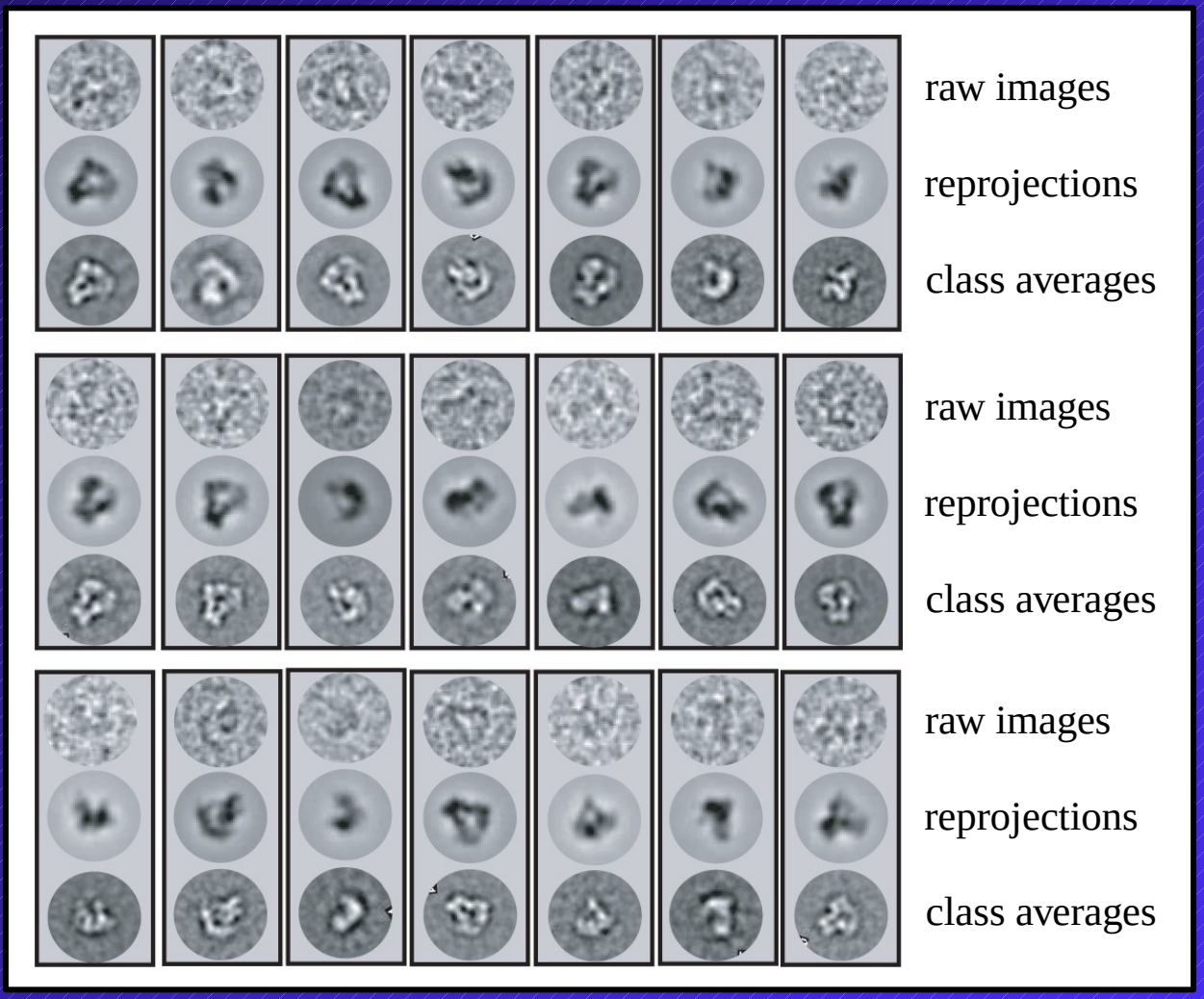




Validation of EM maps

Quality control

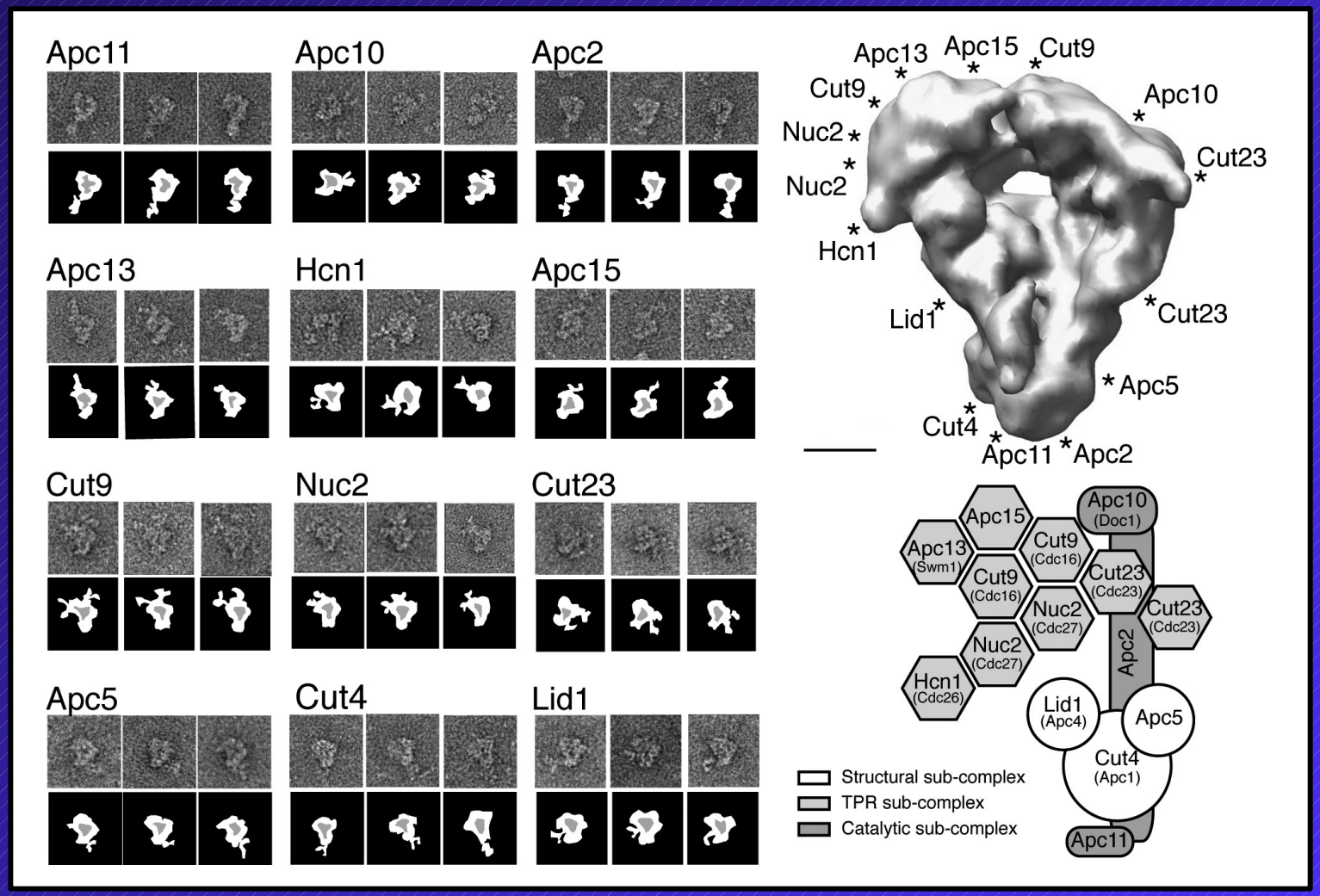
Anaphase-promoting complex (APC)



Validation of EM maps

Comparison with published information

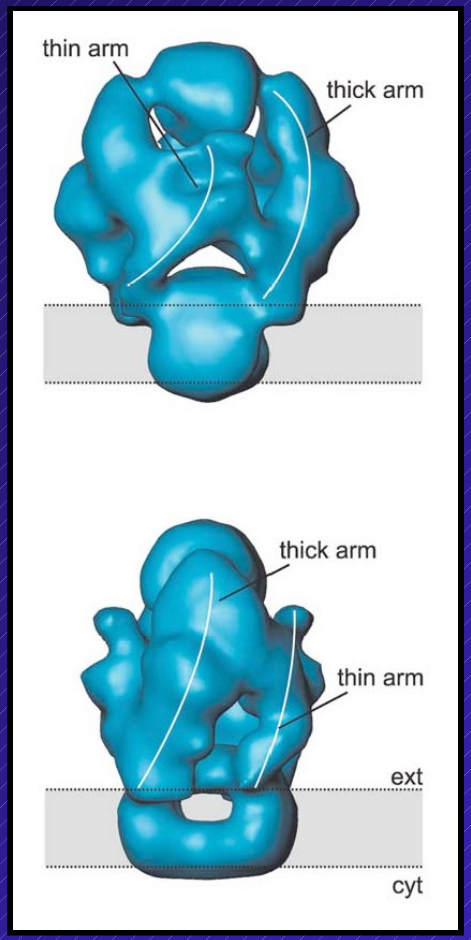
Anaphase-promoting complex (APC)



Validation of EM maps

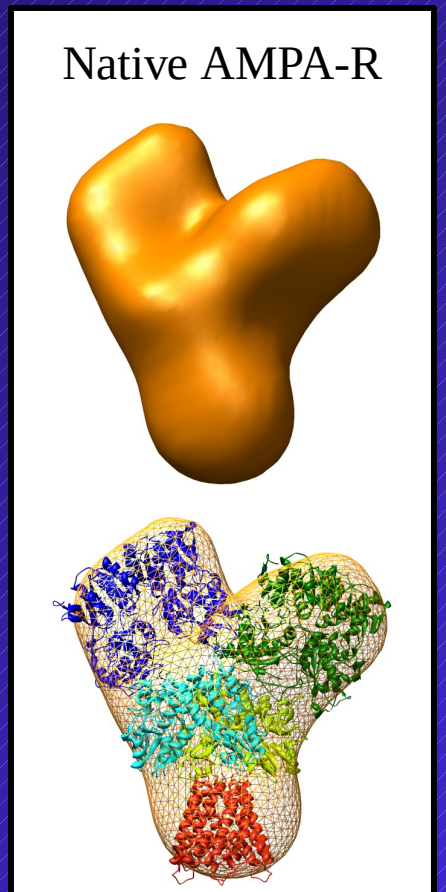
Docking of atomic models

AMPA receptor



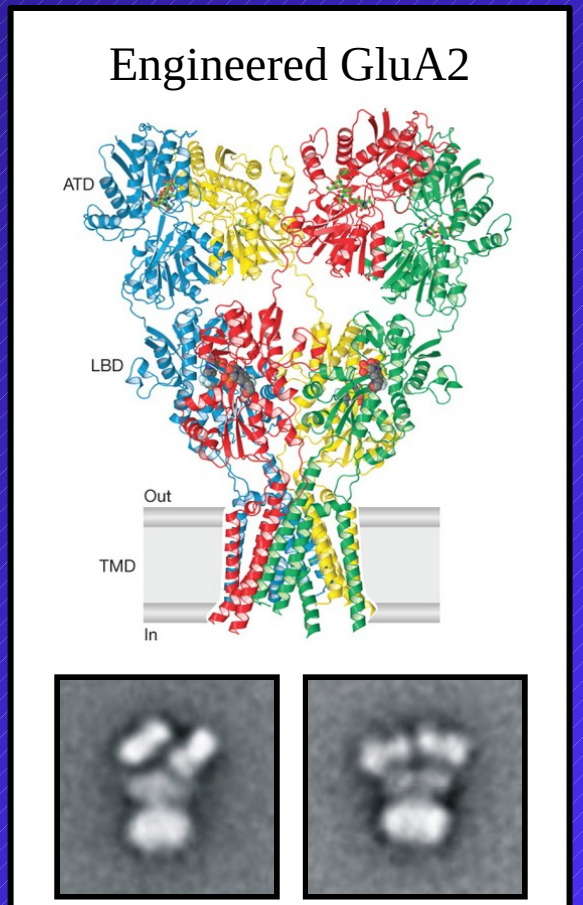
Tichelaar *et al.* (2004)
JMB 344: 435-442

Native AMPA-R



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

Engineered GluA2

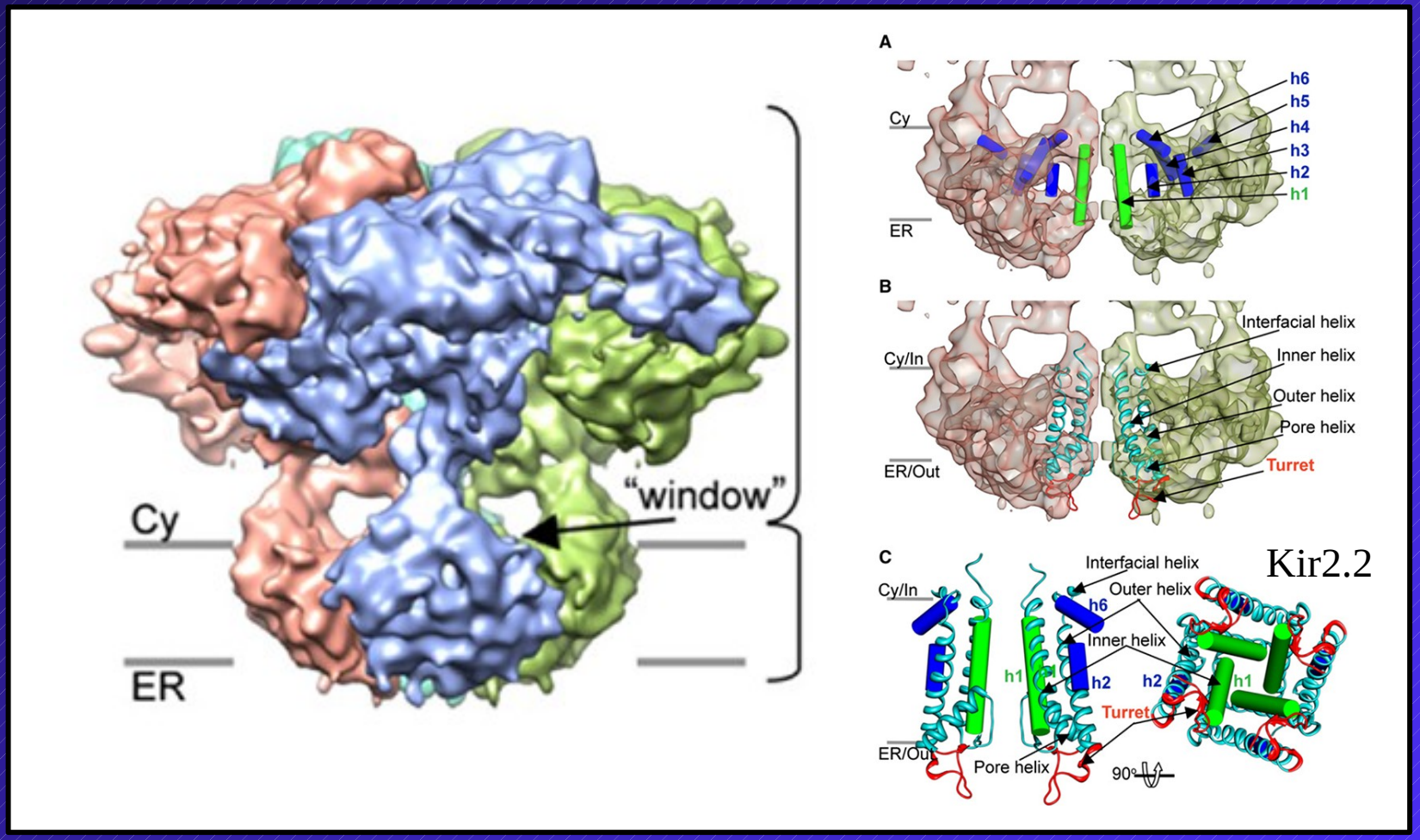


Sobolevsky *et al.* (2009)
Nature 462: 745-758

Validation of EM maps

Do features correspond to resolution and expectations ?

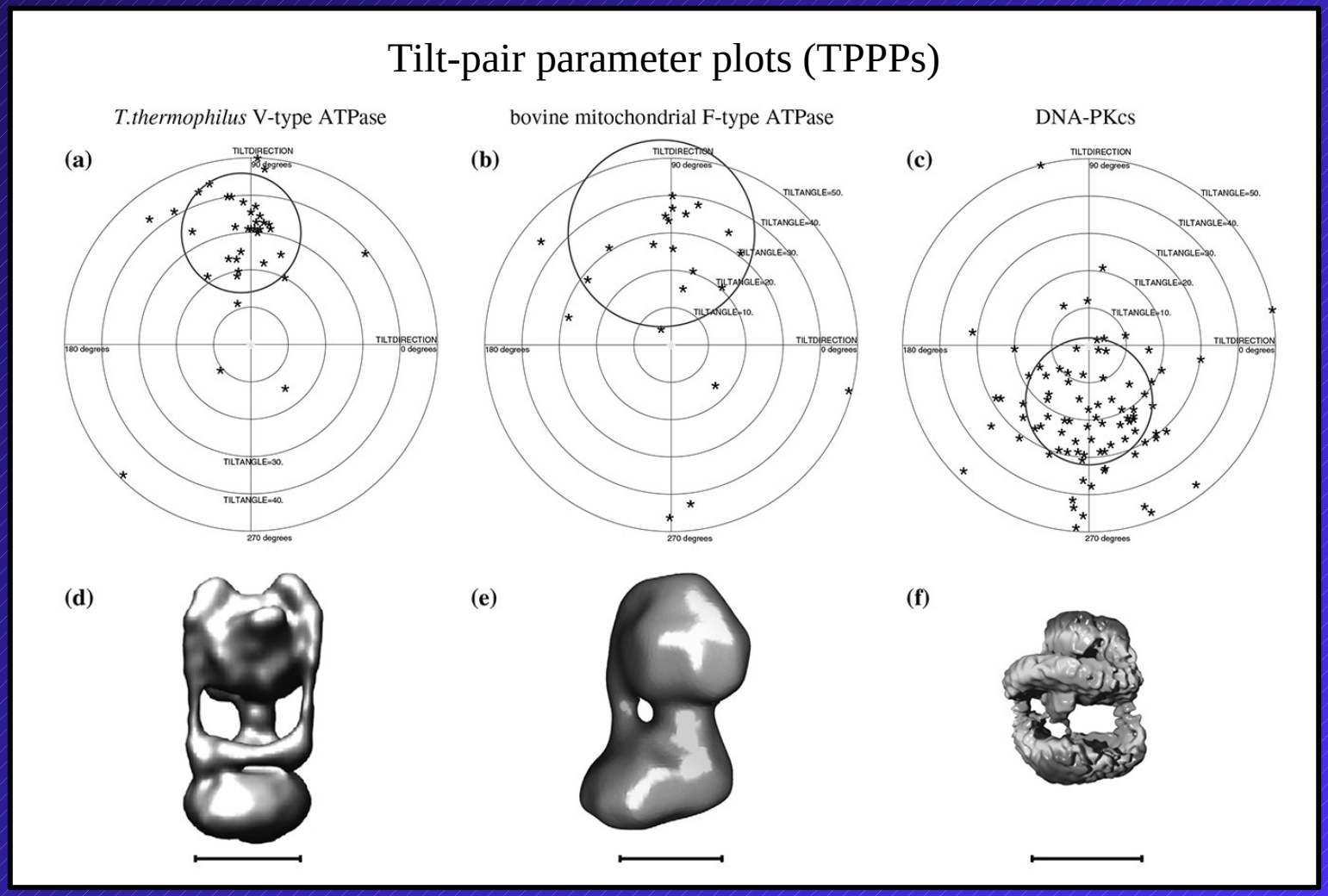
IP3 receptor (~1 nm resolution)



Ludtke *et al.* (2012) *Structure* 19: 1192-1199

Validation of EM maps

Gold standard: Tilt-pair analysis



Henderson *et al.* (2011) *JMB* 413: 1028-1046

Proteins are evil !

Optimization is helpful
Validation is essential

Remain vigilant and always validate your maps as best you can