Challenging Macromolecules

(for single particle EM – the big picture)

Workshop on EM Structure Determination of Challenging Macromolecules

The Scripps Research Institute
La Jolla, November 2009

Part I – general introduction

What is single particle EM good for ?
What can NOT be done by the single particle approach ?
How does single particle EM intersect with other methods ?
Why is single particle EM difficult ?

Part II – a bit of history

What means "challenging" ? past
Where single particle EM was ~1995
What means "challenging" ? present
Success stories of single particle EM
Current developments and prospects for single particle EM

Part III – other considerations

What resolution is useful ?
What should be shown in a publication ?

Part I
general introduction

What is single particle EM good for ?

Single particle EM can provide structural information without the need to grow crystals !
(different from X-ray and electron crystallography)

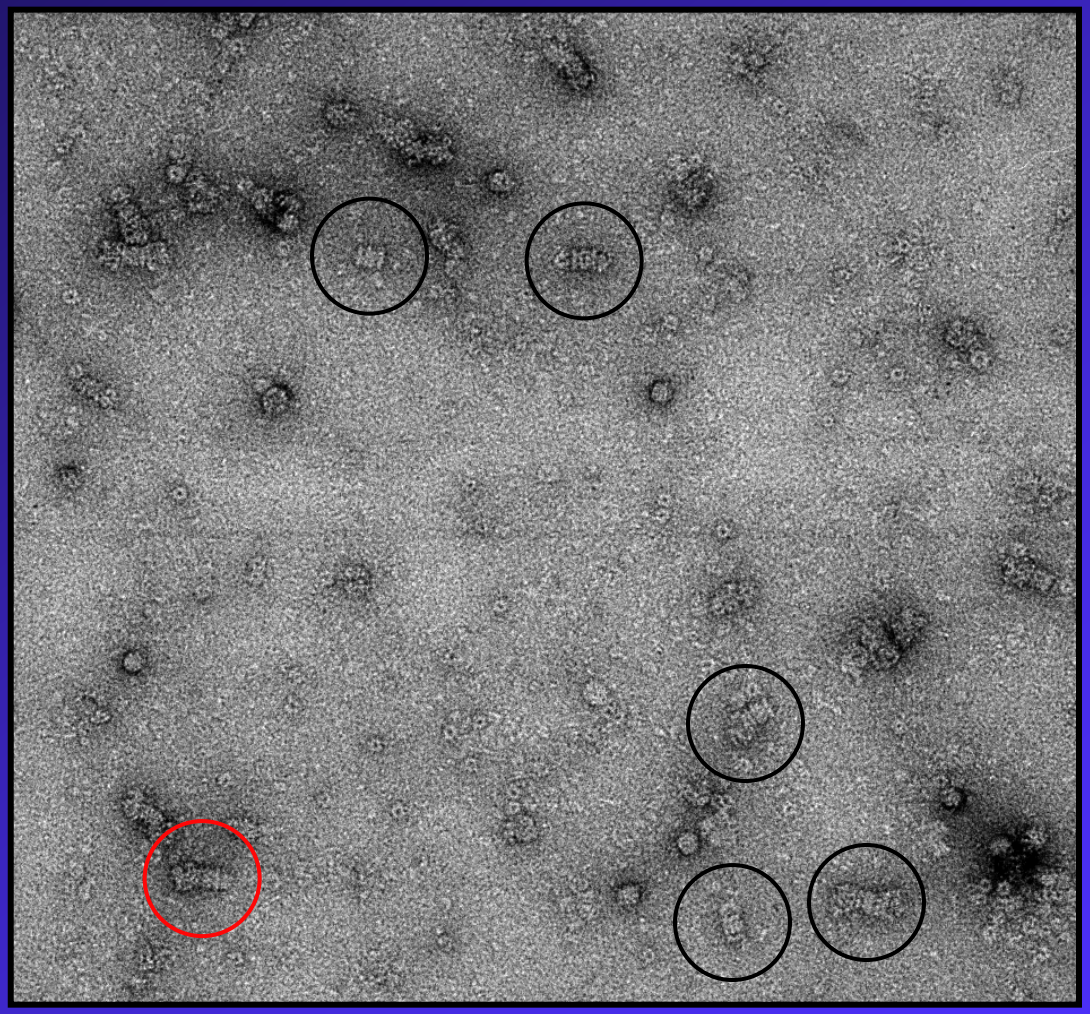
no crystallization issues

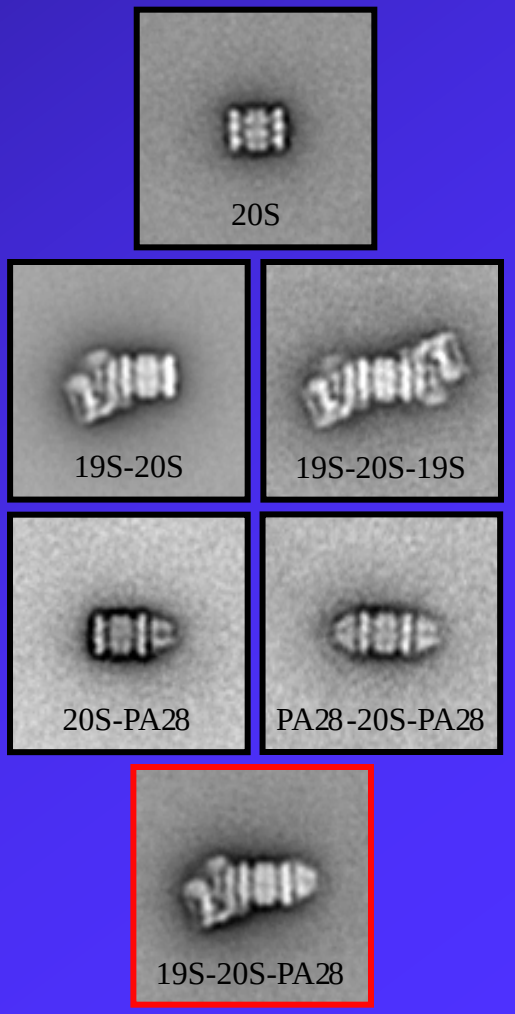
– overcomes the time-consuming task of trying to grow 2D or 3D crystals (but, if possible, crystals are "better")

– very useful to visualize molecules that do not crystallize (e.g., labile complexes, membrane proteins, small quantity, "difficult" samples)

What is single particle EM good for ?

Difficult samples: the PA28-26S complex (heterogeneous, minor component)

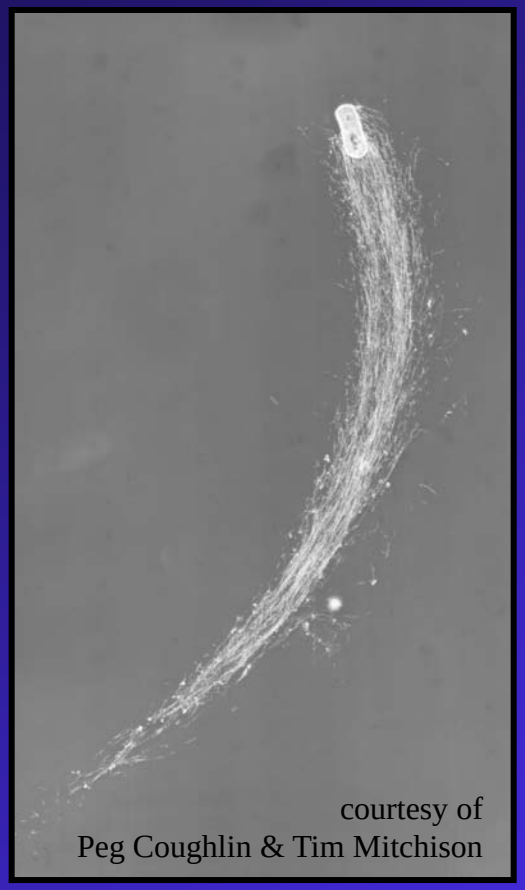




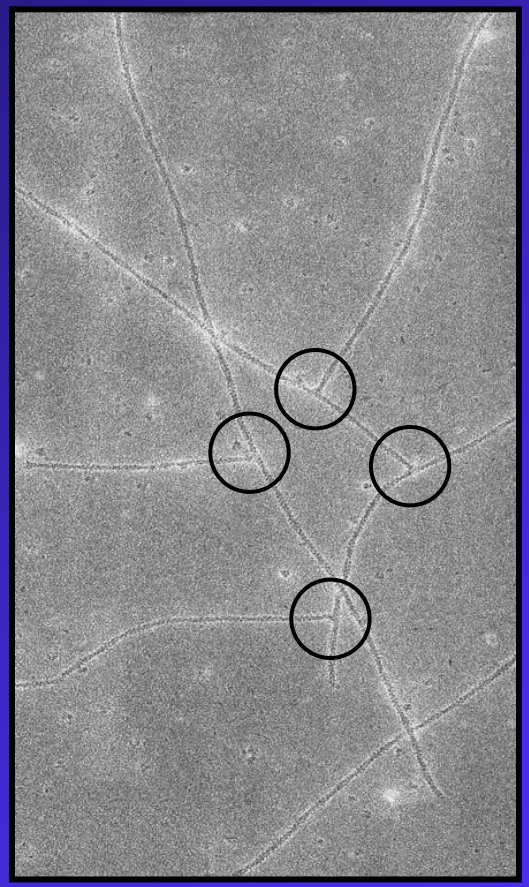
Cascio *et al.* (2002)
EMBO J. 21: 2636-2645

What is single particle EM good for ?

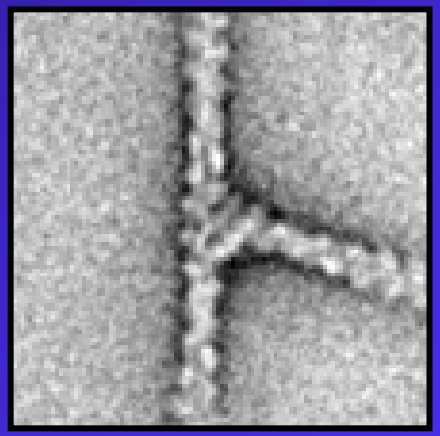
Difficult samples: branched actin filaments (Arp2/3 complex) (substructure)



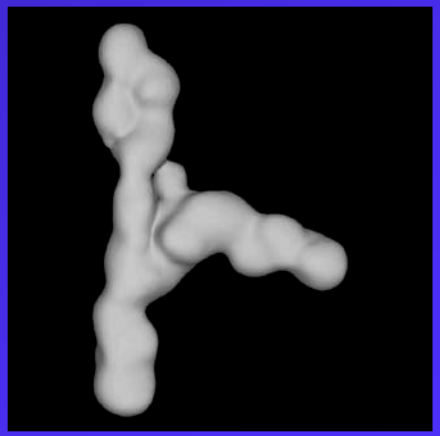
Listeria comet tail



in vitro reaction



projection average



3D map

What is single particle EM good for ?

Single particle EM can provide structural information without the need to grow crystals !
(different from X-ray and electron crystallography)

no crystallization issues

– overcomes the time-consuming task of trying to grow 2D or 3D crystals (but, if possible, crystals are "better")

– very useful to visualize molecules that do not crystallize (e.g., labile complexes, membrane proteins, small quantity, "difficult" samples)

no bias or artifacts due to crystallization

– shows the molecule "in solution"

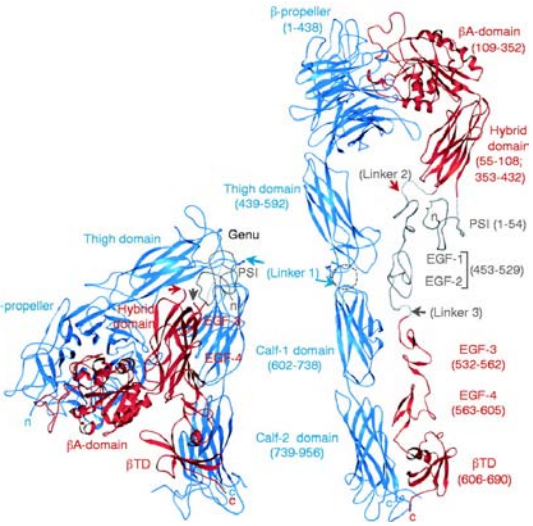
– no artifacts due to crystallization conditions or crystal packing

– reveals all conformations (crystals select just one)

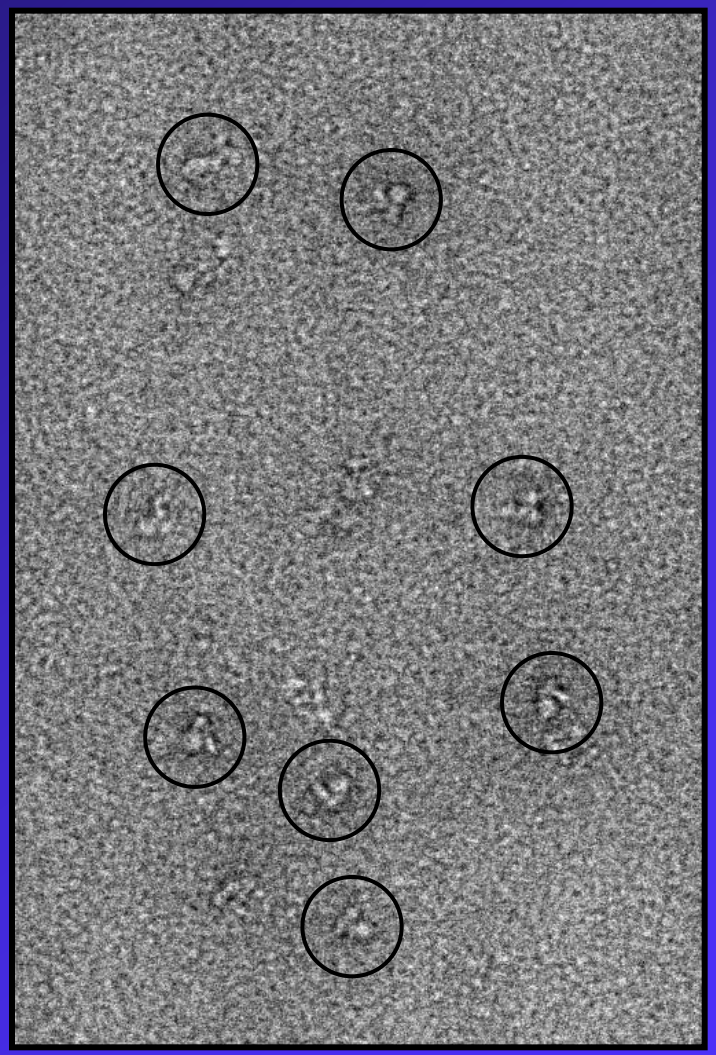
What is single particle EM good for ?

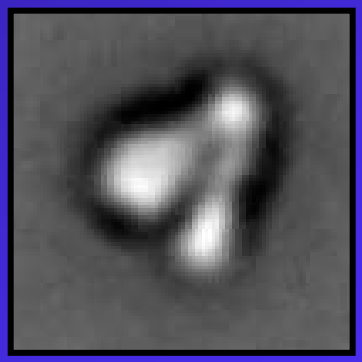
Molecules in solution: the bent structure of $\alpha_V\beta_3$

crystal structure of the extracellular segment of integrin $\alpha_V\beta_3$

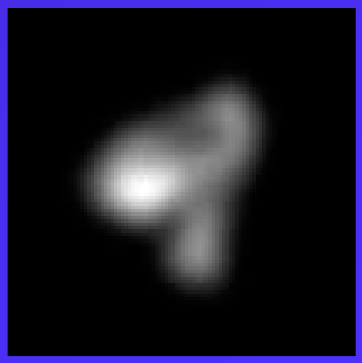


Xiong *et al.* (2001)
Science 294: 339-345





class average
determined by EM

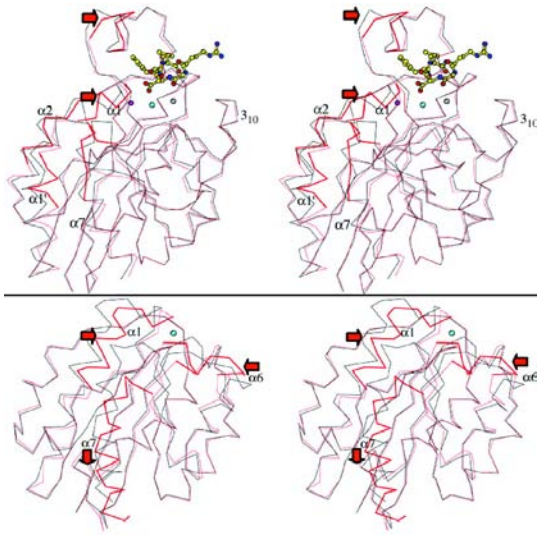


2D projection
from X-ray model

What is single particle EM good for ?

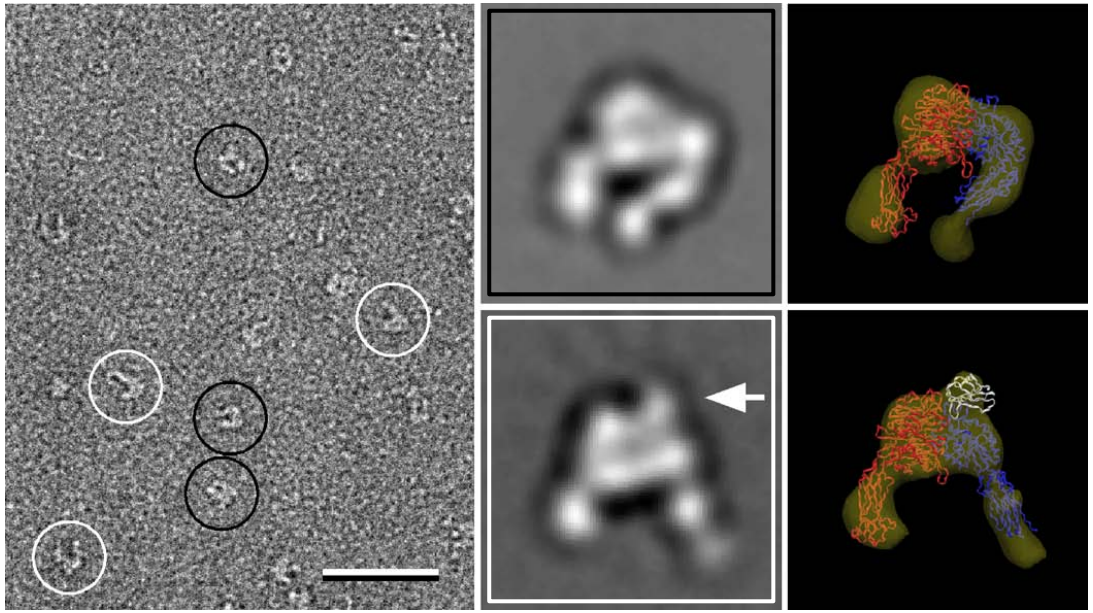
Crystal packing: activation of $\alpha_V\beta_3$

crystal structure of the extracellular segment of integrin $\alpha_V\beta_3$ in complex with an RGD ligand



Xiong *et al.* (2002)
Science 296: 151-155

single particle EM of the extracellular segment of integrin $\alpha_5\beta_1$ in complex with an Fn9-10 fragment



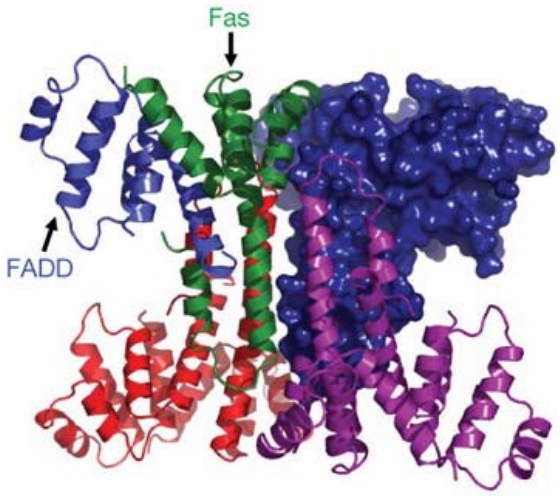
Takagi *et al.* (2003)
EMBO J. 22: 4607-4615

What is single particle EM good for ?

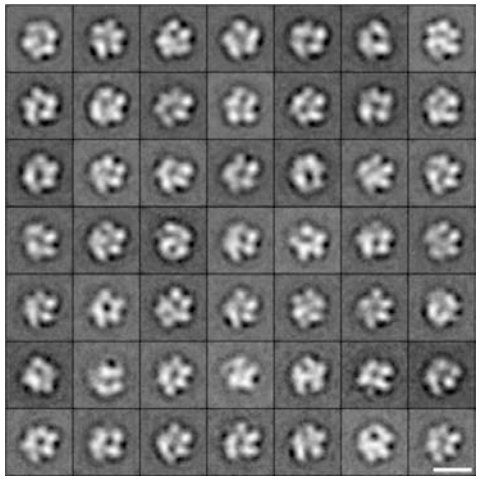
Crystallization conditions: Fas–FADD death domain complex

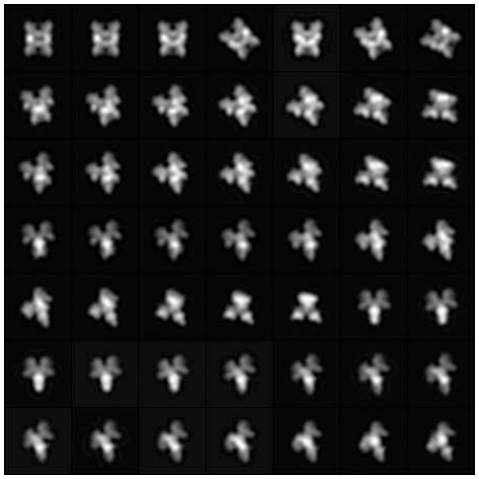
crystal structure of the Fas-FADD DD complex

Scott *et al.* (2009)
Nature 457: 1019-1022



4 Fas DD : 4 FADD DD
but:
crystallized at pH 4 !





7 Fas DD : 5 FADD DD
at 7 Å resolution when
crystallized at pH 8.5 !

~ 140 kDa

consistent with
known
mutations

similar to crystal structure
of
PIDD–RAID DD complex

What is single particle EM good for ?

Crystallization conditions: Fas–FADD death domain complex

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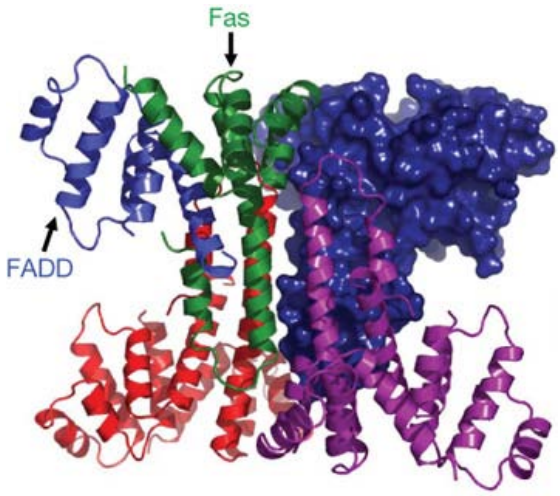

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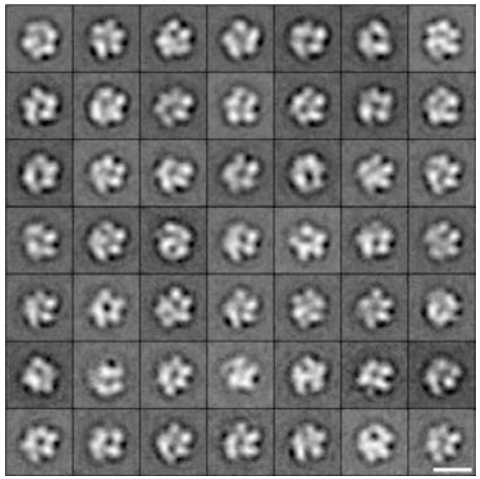
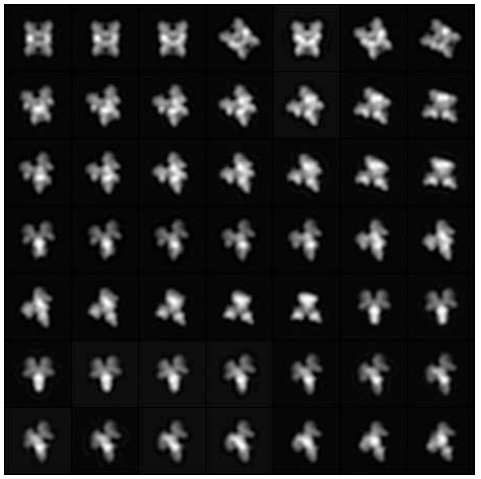
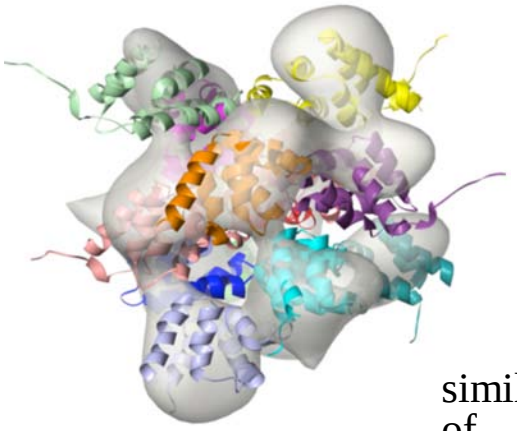






7 Fas DD : 5 FADD DD
at 7 Å resolution when
crystallized at pH 8.5 !

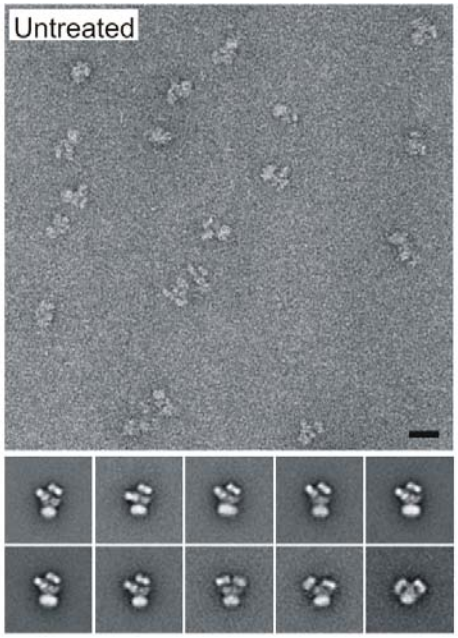
~ 140 kDa

consistent with known mutations

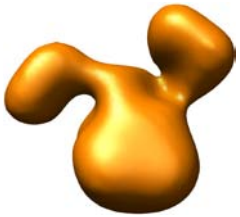
similar to crystal structure of
PIDD–RAID DD complex

What is single particle EM good for ?

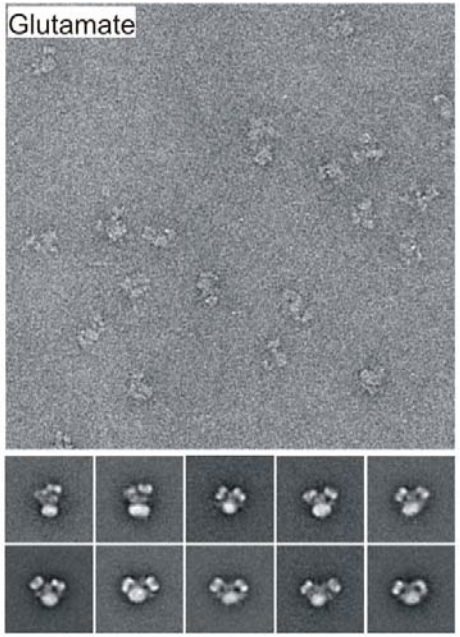
Reveals all conformations: desensitization of AMPA receptor



Type I particles



Type II particles



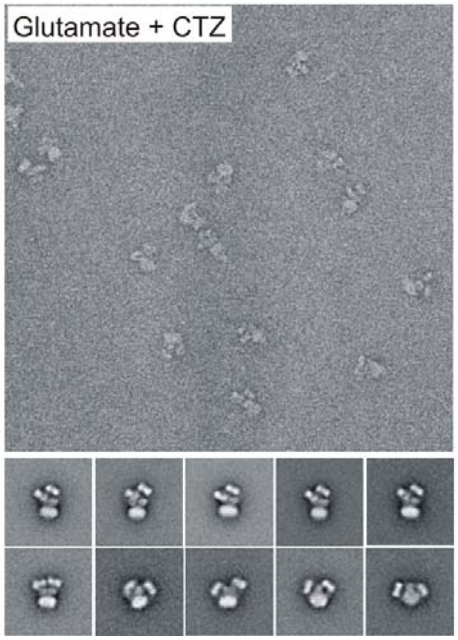


Table of classified particles

	Type I	Type II	Total particles
Untreated	60%	40%	10005
Glu	3%	97%	10053
Glu + CTZ	49%	51%	9104

Nakagawa *et al.* (2005)
Nature 433: 545-549

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no crystallization issues
– overcomes the time-consuming task of trying to grow 2D or 3D crystals (but, if possible, crystals are "better")
– very useful to visualize molecules that do not crystallize (e.g., labile complexes, membrane proteins, small quantity, "difficult" samples)

no bias or artifacts due to crystallization
– shows the molecule "in solution"
– no artifacts due to crystallization conditions or crystal packing
– reveals all conformations (crystals select just one)

Single particle EM can provide structural information for very large complexes !
(different from NMR spectroscopy)

What can NOT be done by the single particle approach ?

Single particle approach is very versatile and provides structural information from low to high resolution

Only two requirements:

1. molecule must be visible in the images

2. molecule must exist in many identical copies (each conformation in many copies)

any specimen that meets these two criteria can be studied by single particle EM

What can NOT be done by the single particle approach ?

1. **molecule must be visible in the images**
   (high contrast helps picking, alignment and classification)

   – ***mass and shape (mass/area) of the molecule is crucial***
   a globular domain is easier to see than
   a thin, extended domain of the same mass

   – ***specimen preparation***
   smaller molecules can be seen in negative stain
   than in vitrified ice

   – ***imaging conditions & instrumentation***
   high defocus, energy filter, phase plate
   all help to see small molecules

What can NOT be done by the single particle approach ?

2. **molecule must exist in many identical copies** (necessary for averaging)

   – ***must be chemically and structurally identical***
   subunits dissociating from complexes
   conformational equilibrium

   – ***the greater the heterogeneity, the greater the problem***
   limited heterogeneity: cryo-EM still possible
   significant heterogeneity: (cryo-)negative stain EM
   unique objects: electron tomography (if possible)

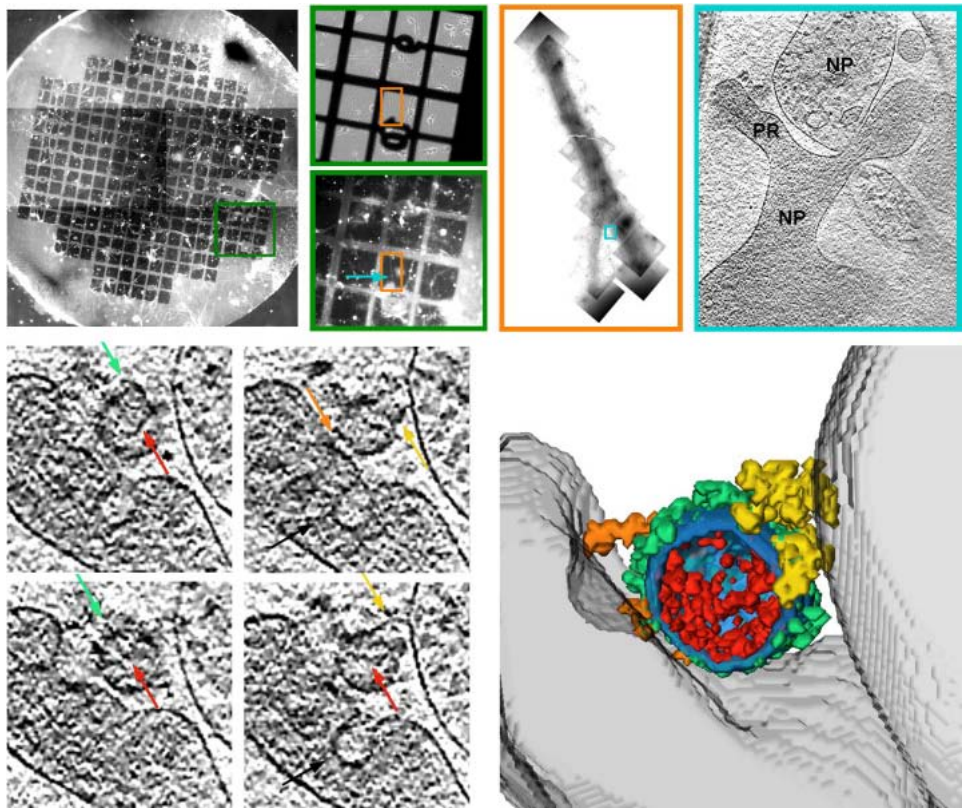
How does single particle EM intersect with other methods ?

light microscopy

no intersections

light microscopy –
thin section EM &
electron tomography

correlative microscopy



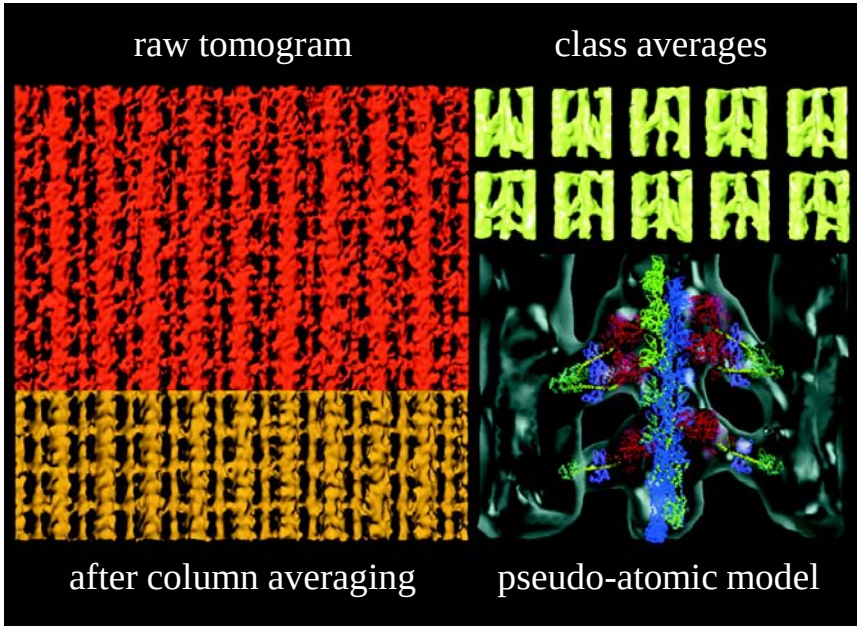
Lučić *et al.* (2007) *J. Struct. Biol.* 160: 146-156

How does single particle EM intersect with other methods ?

electron tomography

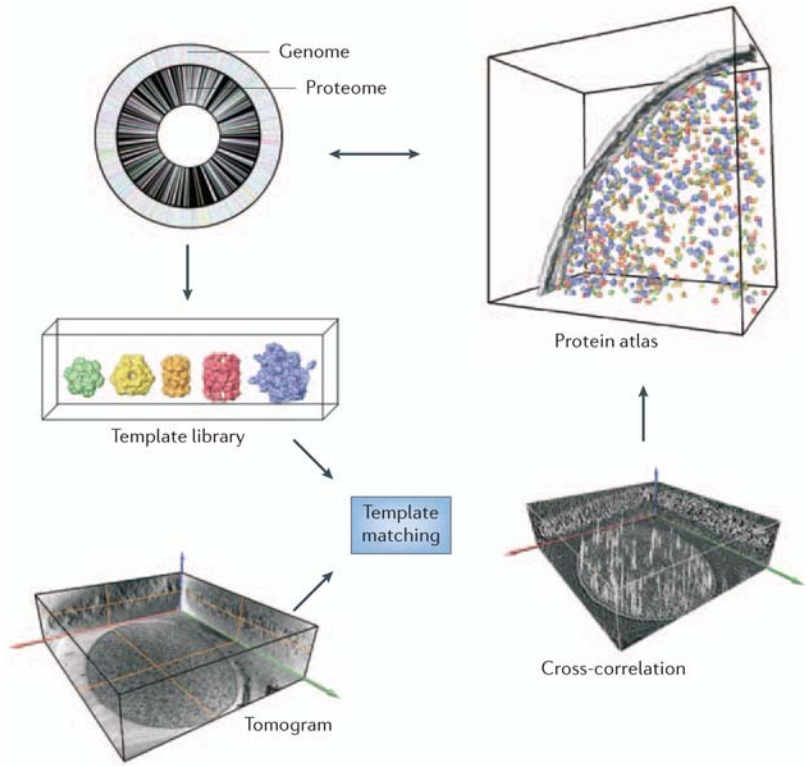
sub-tomogram averaging

insect flight muscle
Ken Taylor (FSU)



Baumeister (2002) *Curr. Opin. Struct. Biol.* 12: 679-684

structural proteomics



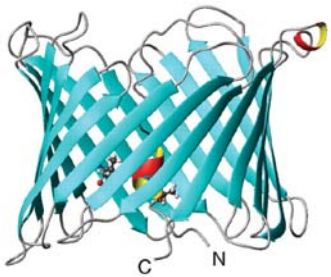
Nickell *et al.* (2006) *Nat. Rev. Mol. Cell Biol.* 7: 225-230

How does single particle EM intersect with other methods ?

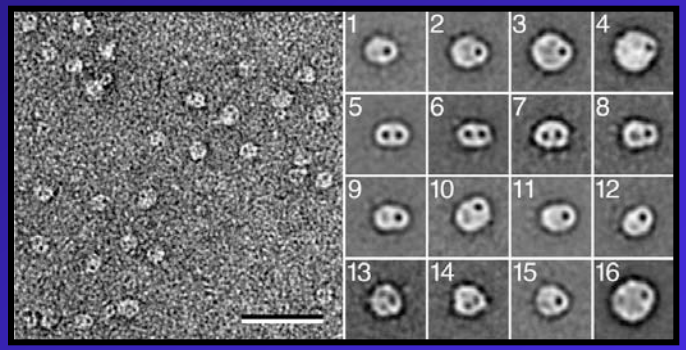
NMR spectroscopy

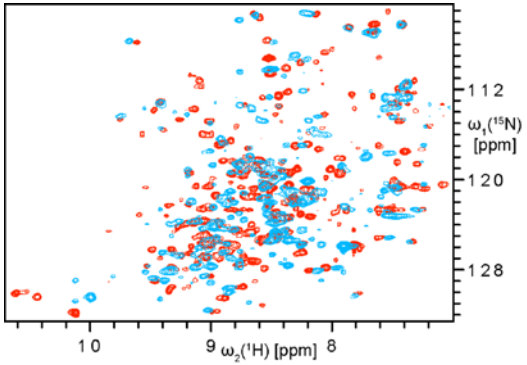
no intersections

NMR solution structure of human VDAC-1



Hiller *et al.* (2008)
Science 321: 1206-1210





2D [^{15}N,^{1}H]-TROSY spectra of [U-^{2}H,^{15}N]-VDAC-1

DMPC nanodiscs
LDAO micelles

Raschle *et al., submitted*

NMR spectroscopy – 2D crystals (electron crystallography) solid state NMR

Hiller *et al.* (2005)
Chembiochem. 6: 1679-1684
Solid-state magic-angle spinning NMR of outer-membrane protein G from *Escherichia coli*.

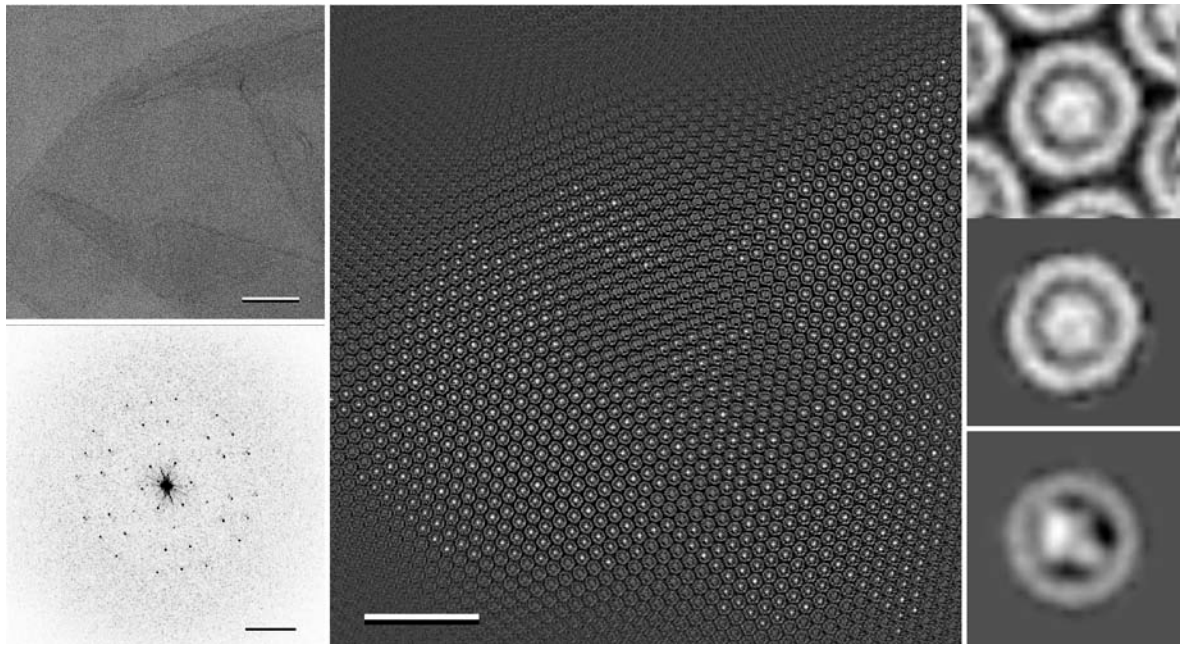
Hiller *et al.* (2008)
J. Am. Chem. Soc. 130: 408-409
[2,3-^{13}C]-labeling of aromatic residues – getting a head start in the magic-angle-spinning NMR assignment of membrane proteins.

How does single particle EM intersect with other methods ?

electron crystallography

single particle averaging of unit cells

RC-LHI photounit from *Rhodobacter sphaeroides*



Walz *et al.* (1998) *J. Mol. Biol.* 282: 833-845

method extended to maximum likelihood

Zheng *et al.* (2007)
J. Struct. Biol. 160: 362-374
A maximum likelihood approach to two-dimensional crystals.

3.5 Å resolution !

assess homogeneity of sample to be used for 2D crystallization

How does single particle EM intersect with other methods ?

X-ray crystallography

assess homogeneity of sample to be used for 3D crystallization	easy and fast – can speed up crystallization screens
use density map as phase start for phasing X-ray intensity data set	ribosome, viruses …
use single particle EM to assess accuracy of crystal structures	integrin conformation integrin activation Fas-FADD complex …

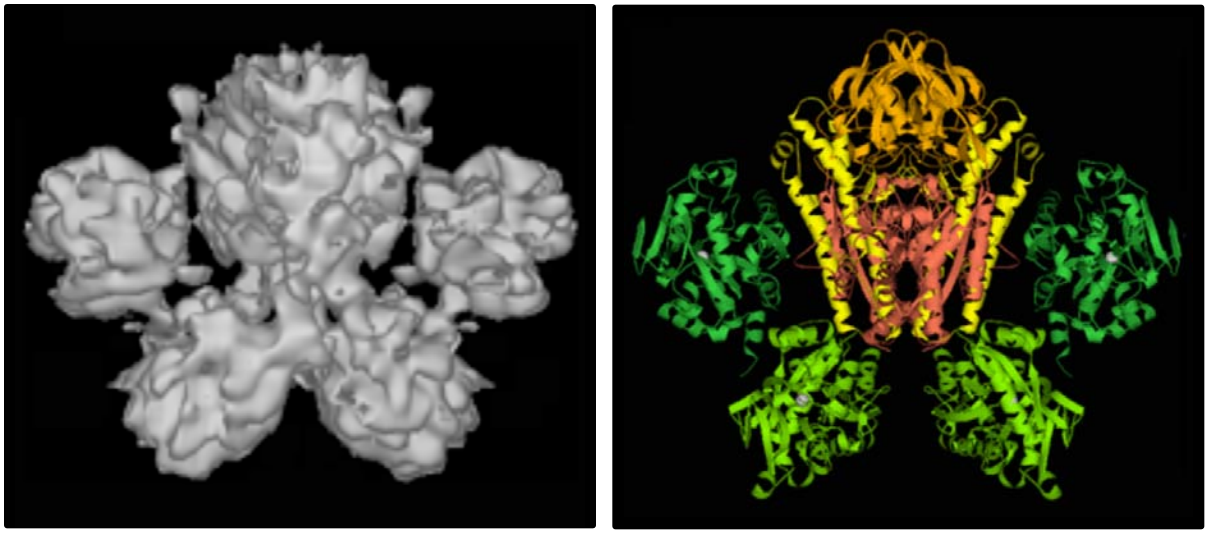
How does single particle EM intersect with other methods ?

any atomic structures

pseudo-atomic models !!!

(dock atomic models of subunits into EM map of complex)

Tf-TfR complex (290 kDa, ~8Å)



Cheng *et al.* (2004) *Cell.* 116: 565-576

Map of any resolution can be used, but:
the higher the resolution, the better the model !

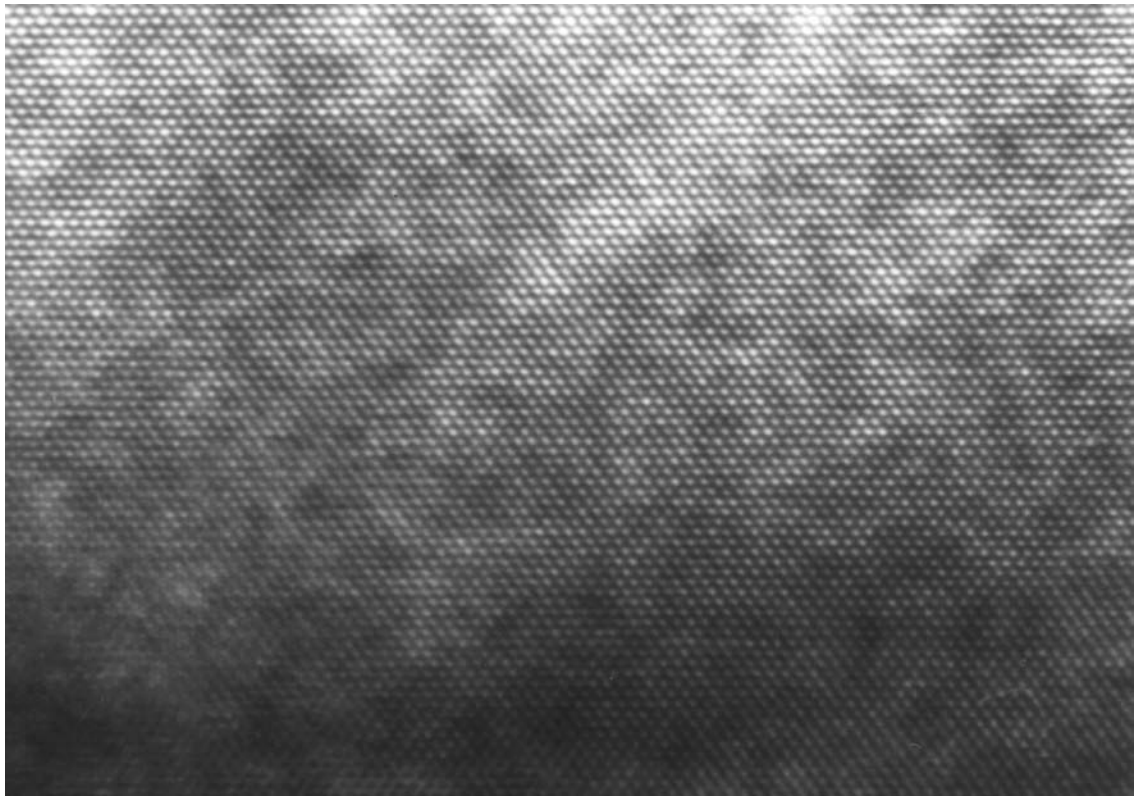
Pseudo-atomic models are still models, thus:
always good to verify !
(any additional info, mutagenesis, etc.)

Part II
a bit of history

Why is single particle EM difficult ?

EM can provide images at atomic resolution

$In_{0.53}Ga_{0.47}As$ crystal



Grigorieff *et al.* (1993) *Philos. Mag. A* 68:121-136

Why is single particle EM difficult ?

Problems specific to biological specimens

biological specimens consist of up to 80% of water

→ COLLAPSE OF STRUCTURE because of dehydration in vacuum of EM

requires specimen preparation (metal shadowing, negative staining, vitrification)

biological specimens consist of light atoms, such as C, N, O, H

→ LOW CONTRAST because electron scattering ~ atomic number Z

requires contrast enhancement (stain, defocus, energy filter, phase plate, **averaging**)

→ BEAM DAMAGE because $\sigma_{el} / \sigma_{in} = Z / 19$ (~2 inelastic per elastic scattering event)

requires protection (low electron dose, low temperature)

imaging is difficult (specimen movement deteriorates quality)
images have low signal-to-noise ratio (noisy images)

Why is single particle EM difficult ?

How to record good images ?

– top-entry specimen stages are more stable than side-entry stages (let stabilize)

– have the beam hit the edge of the carbon film (conductance)

– LINDA ? Other tricks ?

How to identify bad images ?

– check Fourier transform (Thon rings) – different ways to do it
– not easy because of low signal !

– during refinement (assess correlation of particle images with model)

the higher the resolution to be achieved,
the better the image quality has to be !

Why is single particle EM difficult ?

only noisy, distorted projection images

need to determine CTF parameters (defocus, astigmatism)

need to determine orientation parameters (x, y, Euler angles Φ, Ψ, Θ)

need to determine similarity (classification)

the higher the resolution to be achieved,
the more accurate the parameters have to be determined !

no criterion to assess correctness of a 3D reconstruction

image processing always produces a density map (no matter how inappropriate)

even resolution determination is ambiguous

difficult to verify the accuracy of a 3D reconstruction,
especially for low-resolution density maps

What means "challenging" ?

past: before subnanometer resolution (~1995)

Every molecule was challenging …
methodology had to be developed from scratch

Main problems:

beam damage – low-dose techniques
– specimen preparation / data collection

low signal-to-noise images – averaging
– alignment / classification

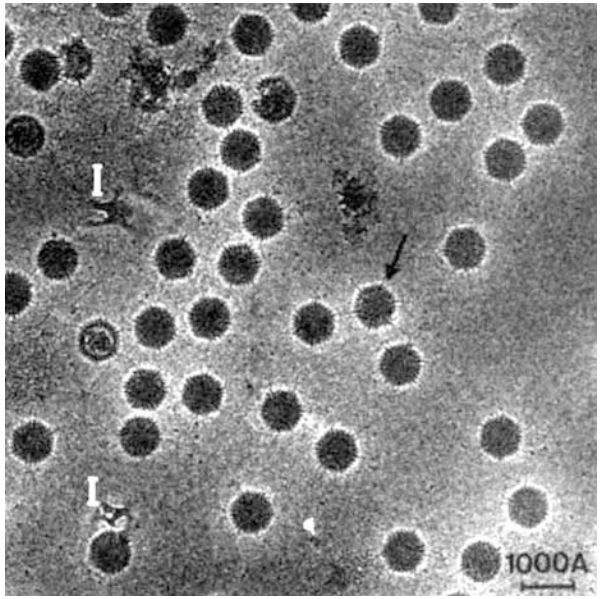
only projection information (tilting)
– orientation determination
– 3D reconstruction
– Refinement

What means "challenging" ? past

Specimen preparation

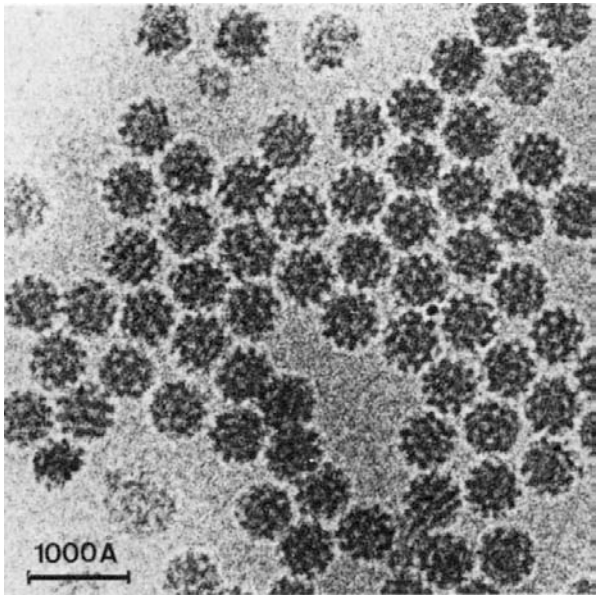
Negative staining *good contrast, but*
– resolution limited to ~20 Å
– artifacts (flattening, deformation, incomplete embedding)

Vitrification *native preservation, but*
– poor contrast (size limitation)
– "random orientations" (heterogeneity)



adenovirus type 2

Semliki Forest virus



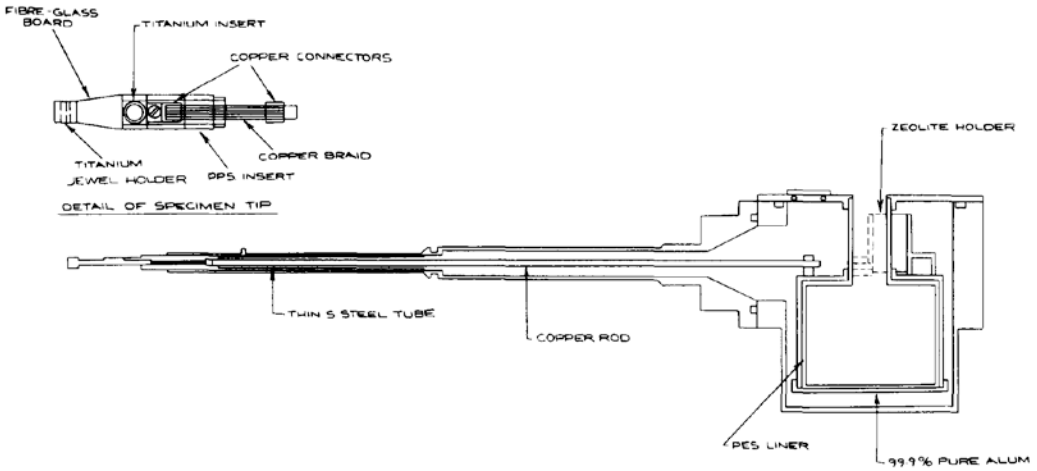
Adrian *et al.* (1984)
Nature 308: 32-36

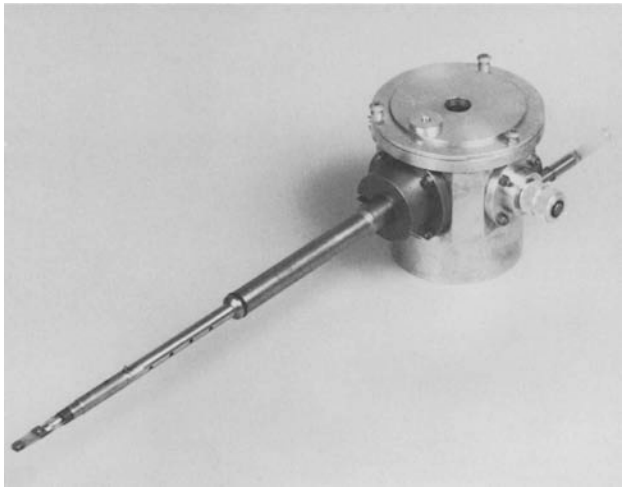
What means "challenging" ? past

Data collection

Low-dose procedures

Specimen holders – *stable goniometers*
– *cryo-transfer holders*





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

What means "challenging" ? past

Alignment / classification

Multivariate statistical analysis
(Frank, Ludtke, Penzcek, Radermacher, van Heel, …)

Orientation determination / 3D reconstruction

Single-axis tilt series (Hoppe)
Common lines (Crowther)
Random conical tilt (Radermacher & Frank)
Angular reconstitution (van Heel)

Refinement

Projection matching

What means "challenging" ? past

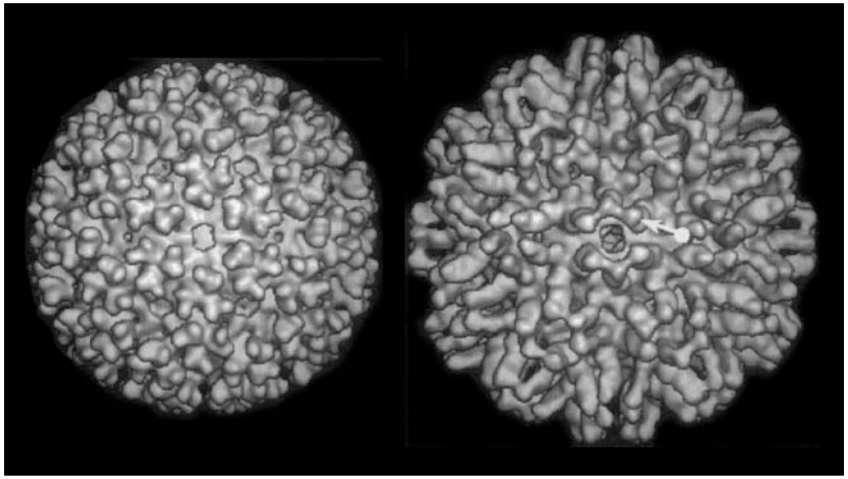
What was possible ?

3D reconstructions at molecular (~25 Å) resolution of large, "homogeneous" complexes, preferably with symmetry
(ribosome, ryanodine receptor, GroEL, clathrin coat, viruses)

Where single particle EM was ~1995

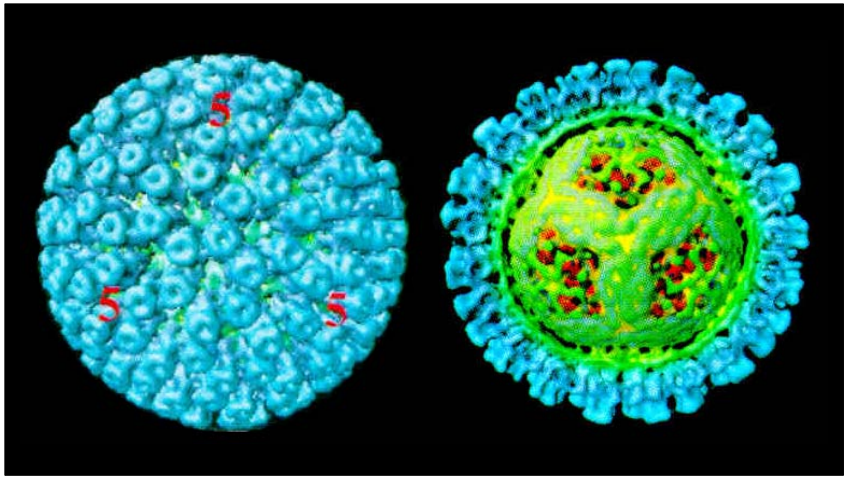
Viruses at ~25 Å

Ross River virus – Fab binding



Smith *et al.* (1995)
Proc. Natl. Acad. Sci. USA 92: 10648-10652

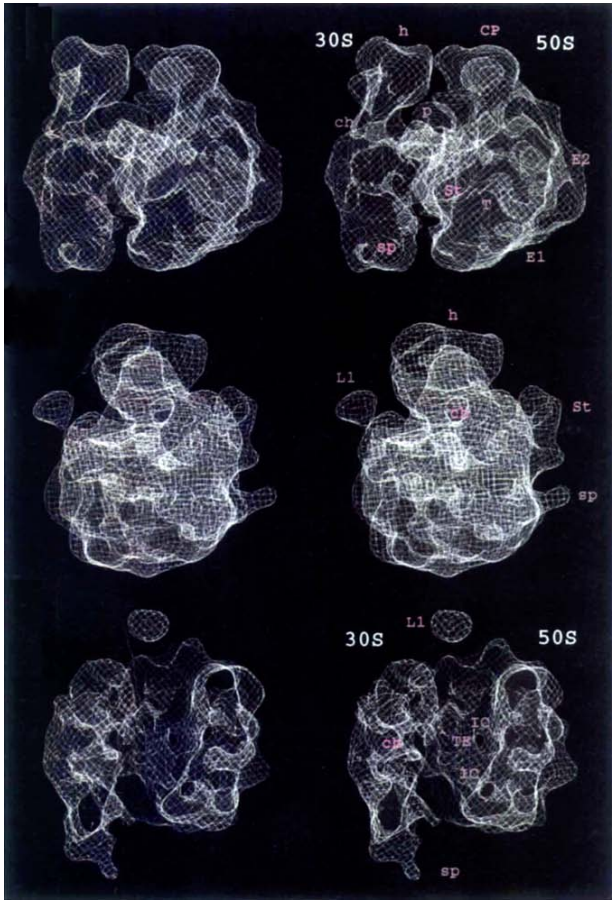
Rotavirus DLP – RNA organization

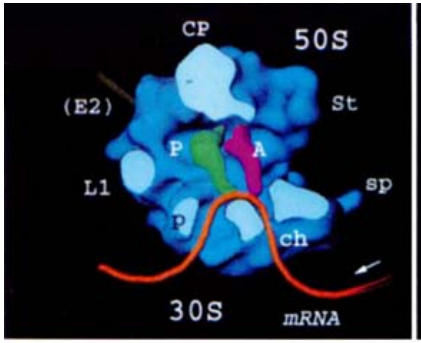


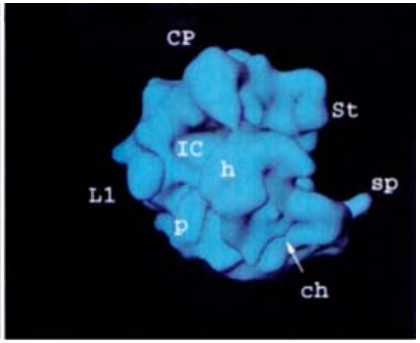
Prasad *et al.* (1996)
Nature 382: 471-473

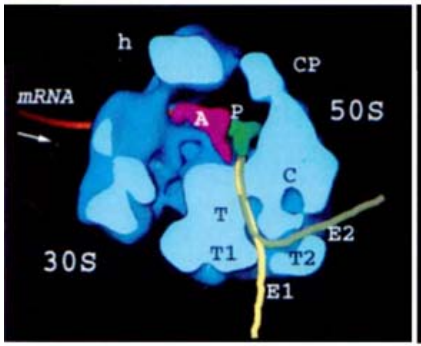
Where single particle EM was ~1995

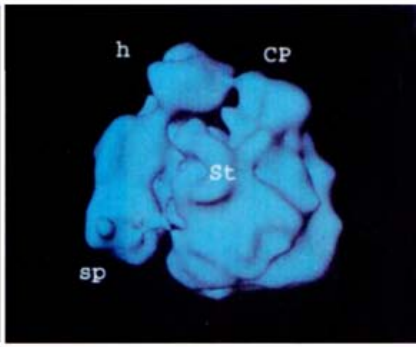
E. coli ribosome at 25 Å

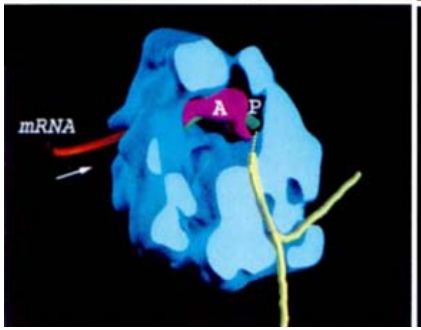


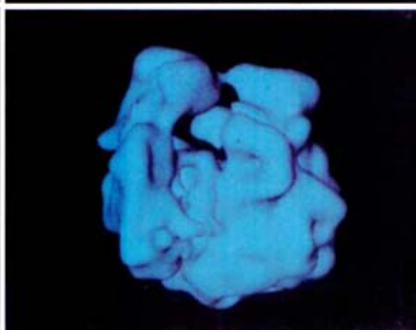








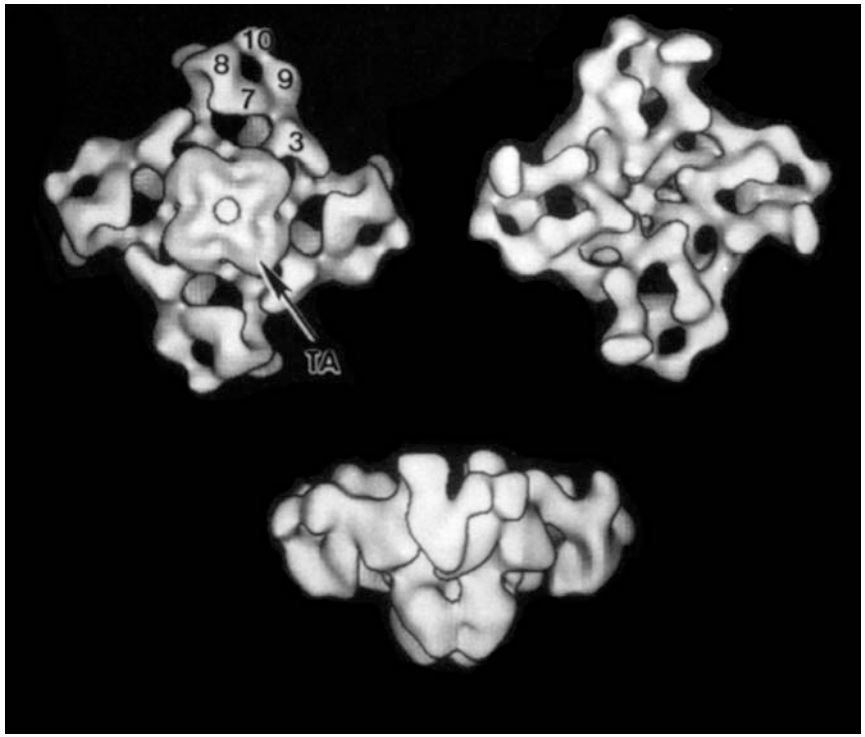




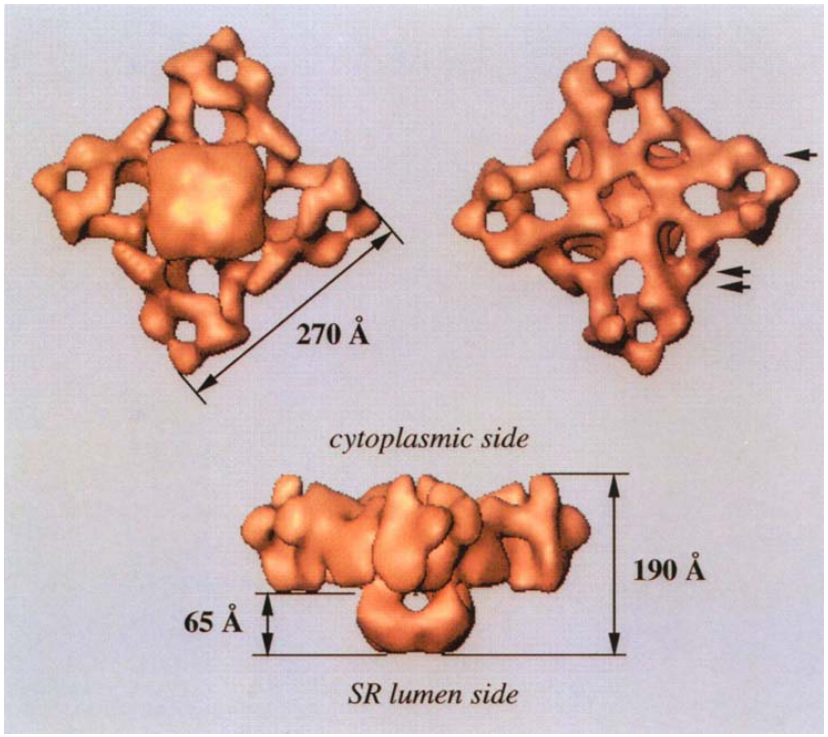
Frank *et al.* (1995) *Nature* 376: 441-444

Where single particle EM was ~1995

Ryanodine receptor at 30 Å



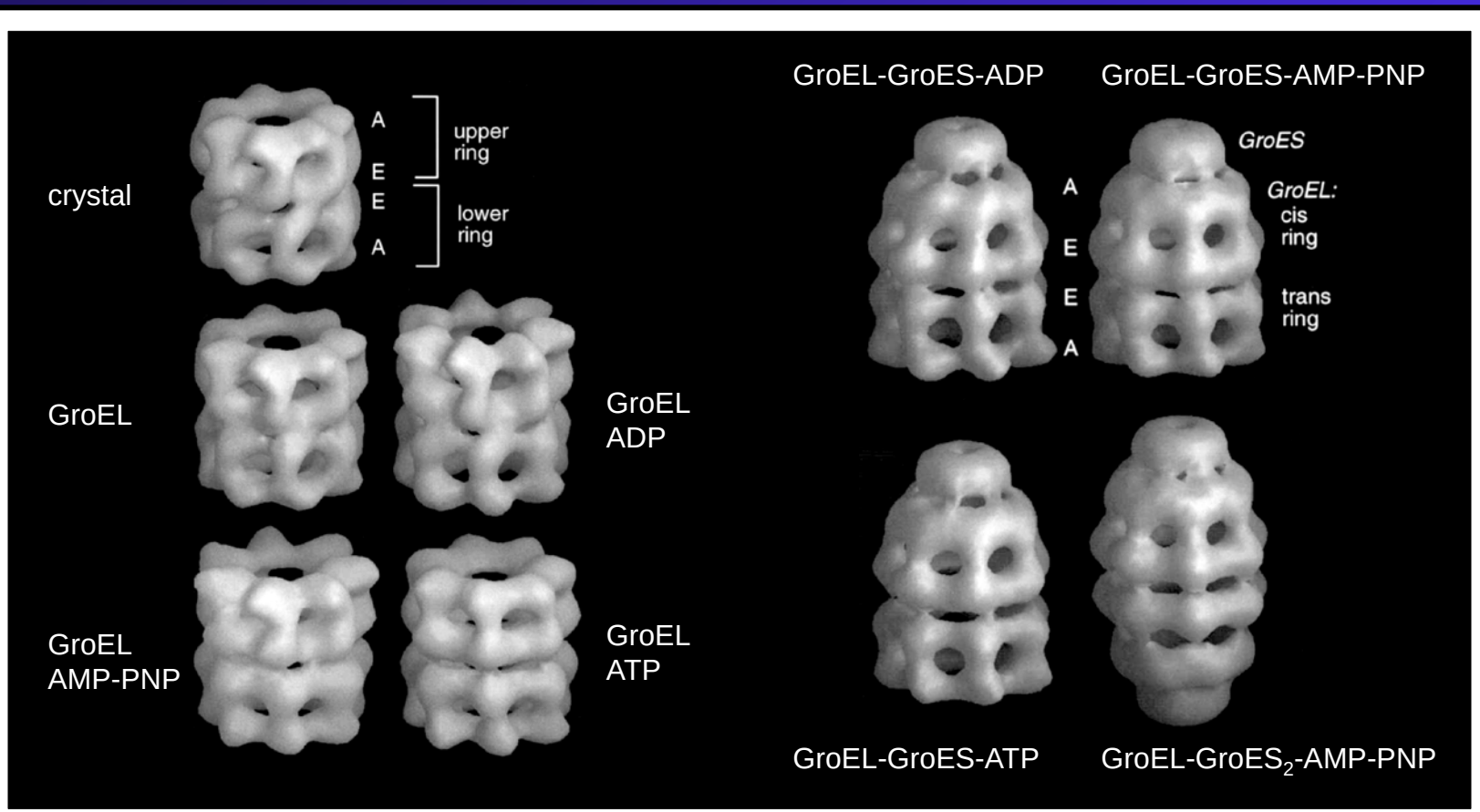
Radermacher *et al.* (1994)
J. Cell Biol. 127: 411-423



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

Where single particle EM was ~1995

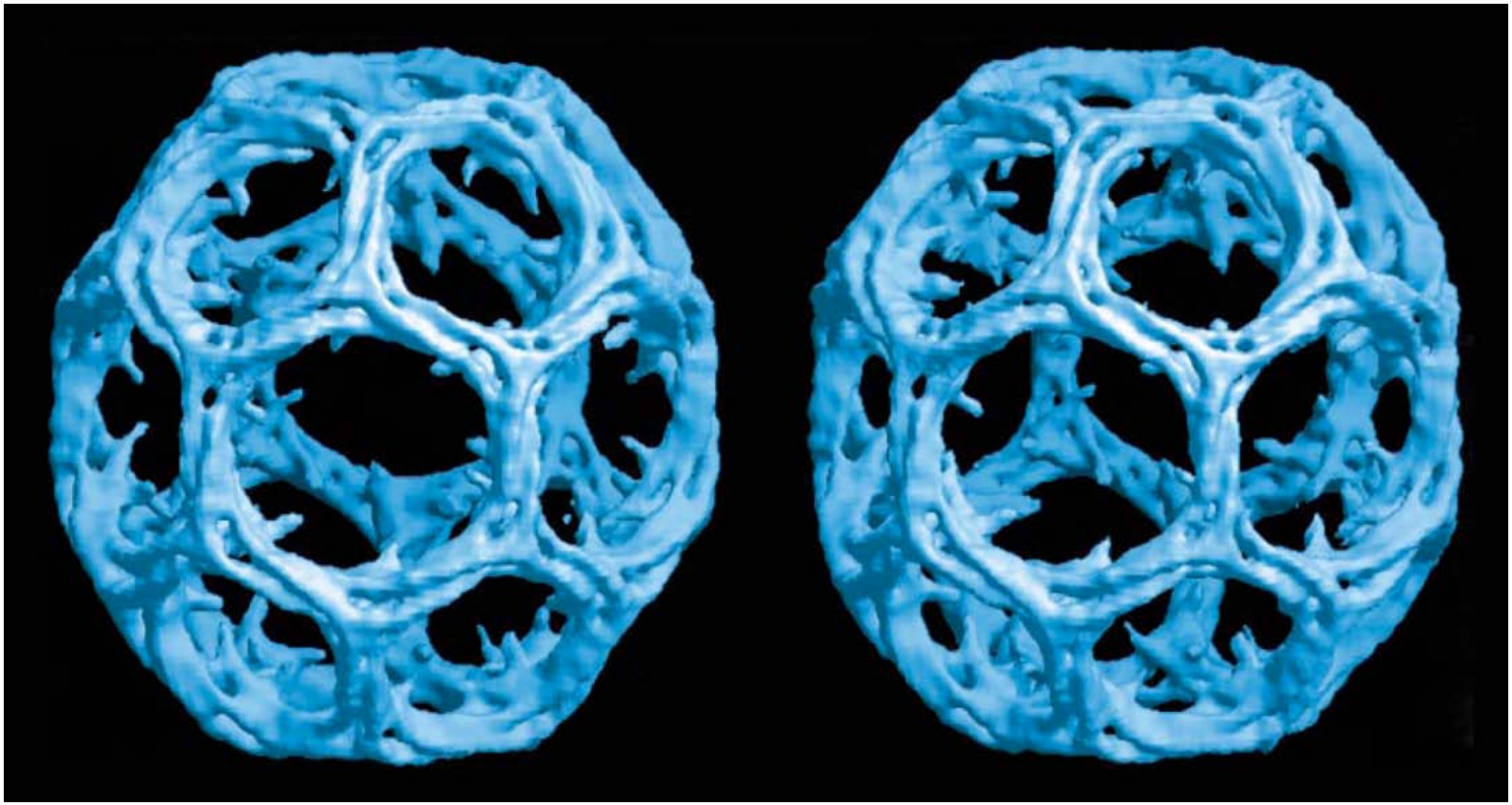
GroEL-GroES complexes at 30 Å



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

Where single particle EM was ~1995

Clathrin coat at 21 Å



Smith *et al.* (1998) *EMBO J.* 17: 4943-4953

What means "challenging" ? past

What was possible ?

3D reconstructions at molecular (~25 Å) resolution of large, "homogeneous" complexes, preferably with symmetry
(ribosome, ryanodine receptor, GroEL, clathrin coat, viruses)

What was challenging ?

– *better than molecular resolution for "ideal" (large, stable, homogeneous) complexes*

– *any 3D reconstruction of small, asymmetric complexes and complexes with structural heterogeneity*

Computing power was a major limitation !

Richard's prophecy in 1995

Quarterly Reviews of Biophysics **28**, 2 (1995), pp. 171–193
Printed in Great Britain

The potential and limitations of neutrons, electrons and X-rays for atomic resolution microscopy of unstained biological molecules

RICHARD HENDERSON
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

10. CONCLUSION

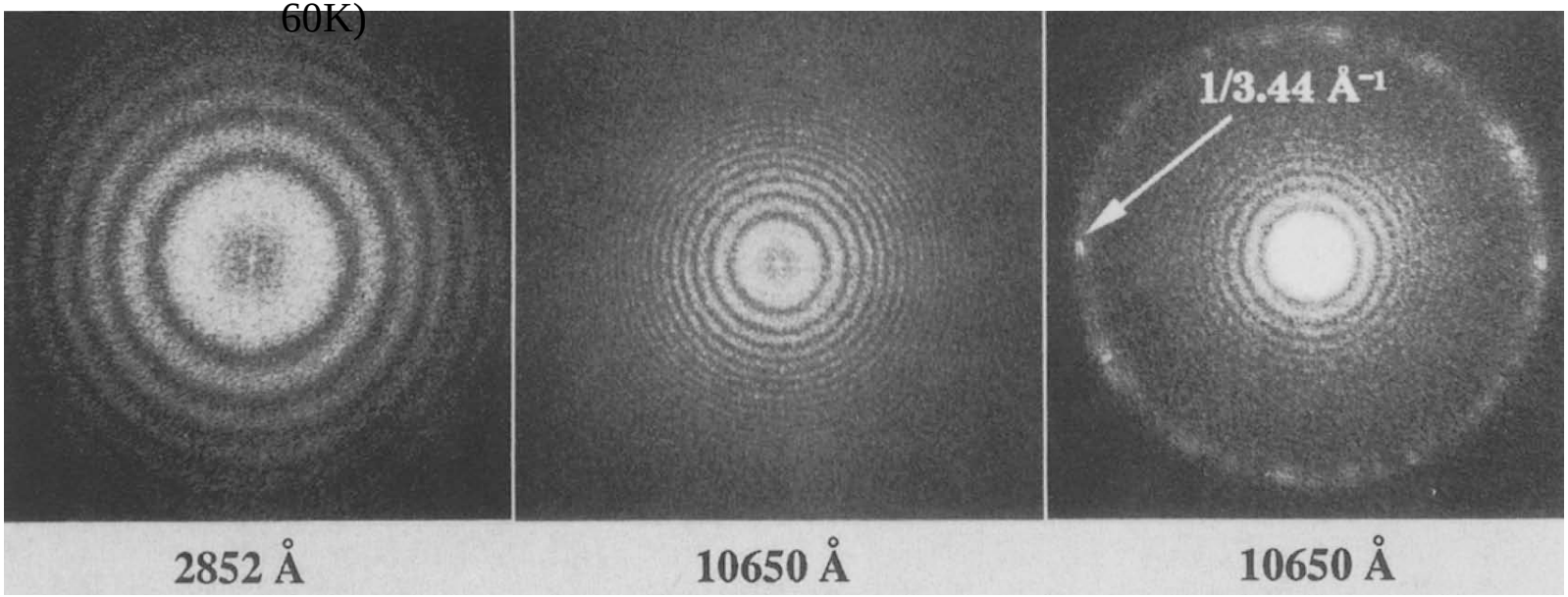
Electron microscopy offers great scope for immediate improvements in capability by addressing the practical problems of specimen movement and charging of specimens embedded in amorphous ice (Henderson, 1992). Images of molecules or molecular assemblies of molecular weights of $\sim 10^5$ and above should contain enough information to determine the orientation and alignment of the particle being observed, so that subsequent image averaging methods may be used to determine the atomic structure. The number of particle images which must be averaged is at least 10000 (Table 2), making a large but manageable computing task. Surprisingly, the number of independent images required is independent of the particle size, being greater than 10000 in all cases. This drops to about 4000 at 10 Å resolution, and about 2000 at 20 Å resolution (see Appendix). Of course, in practice, the quality of the data is likely to be less than perfect, so it will be necessary to average considerably more than this to produce satisfactory maps of the structures. In the three-dimensional density map of bacteriorhodopsin (Henderson *et al.* 1990), the images of approximately 5 million molecules were averaged to produce recognizable amino acid side chains. In a similar analysis of

What means "challenging" ?

transition: first subnanometer resolution maps

FEG: *better coherence / envelope function*
higher resolution, CTF correction

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

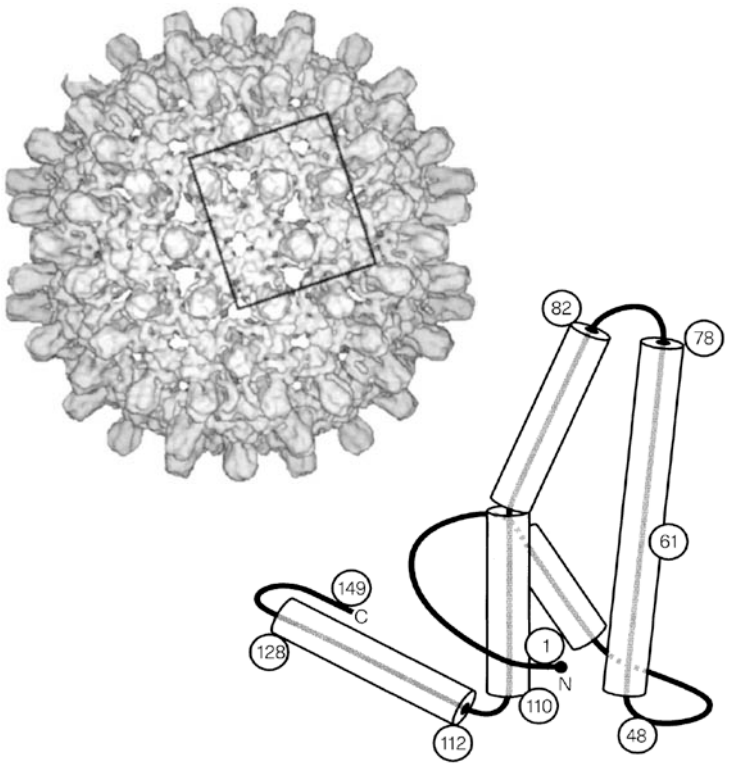


Zhou & Chiu (1993) Prospects for using an IVEM with a FEG for imaging macromolecules towards atomic resolution. *Ultramicroscopy* 49: 407-416

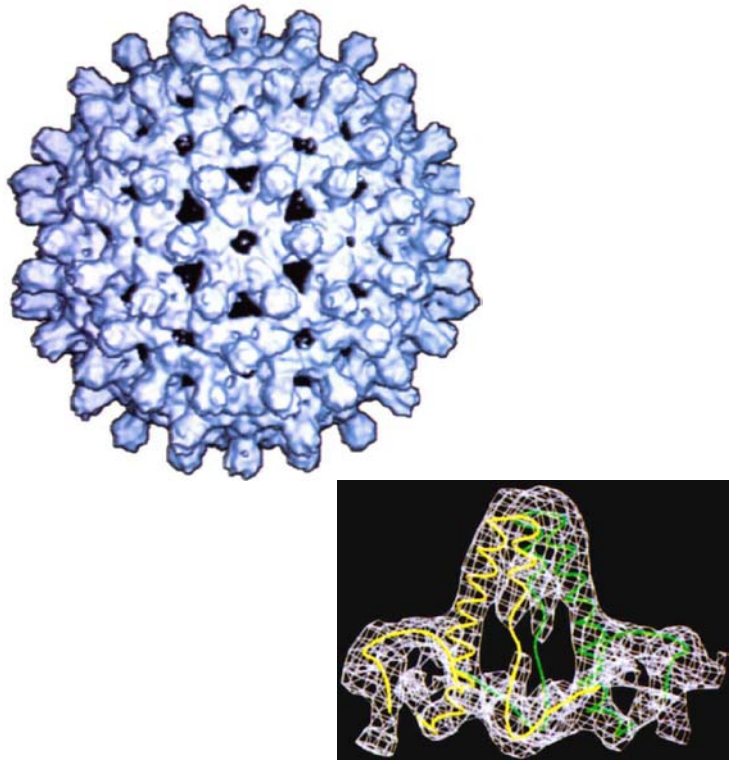
What means "challenging" ?

transition: first subnanometer resolution maps

Hepatitis B virus capsid (1997)



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



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

What means "challenging" ?

present: after first subnanometer resolution maps (1997)
"new" developments

Main problems: *beam damage*
– specimen preparation / data collection

low signal-to-noise images *– averaging*
– alignment / classification

only projection information *– tilting*
– orientation determination / 3D reconstruction

What means "challenging" ? present

Specimen preparation

Cryo-negative staining (Dubochet, Stark)

– high contrast of stain but few artifacts due to freezing
– resolution still limited
– very tedious and difficult !

GraFix (Stark)

– stabilizes complexes
– not good for every specimen

Monolayer purification Affinity Grid (Walz)

– fast and easy
– protection from air/water interface
– not good for every specimen

What means "challenging" ? present

Data collection

CCD camera

Energy filter

Top-entry stage & helium cooling
(Fujiyoshi)

LINDA (dose-rate effect, long exposures, somewhat controversial)
(Grigorieff)

Automation (Leginon)
(Carragher, Potter)

What means "challenging" ? present

Image processing

Heterogeneity (Frank, Penczek, Ludtke, Stark, …)

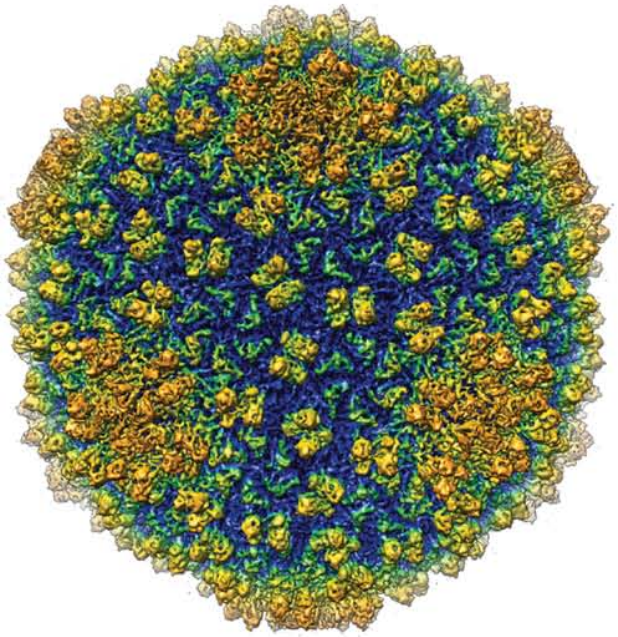
Maximum likelihood (Sigworth, Carazo, …)

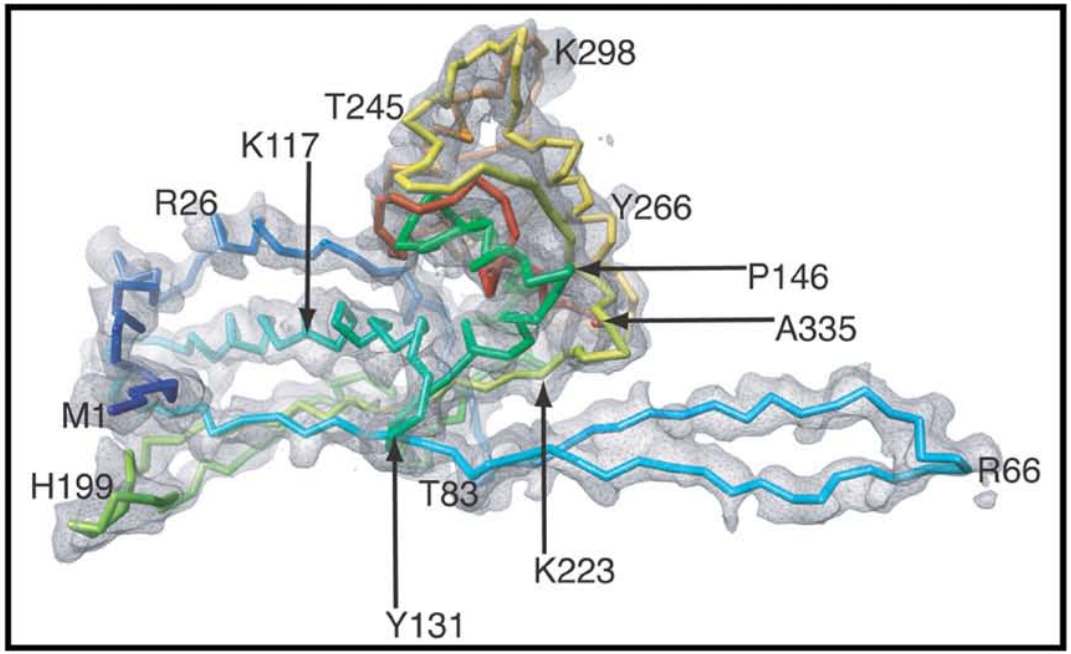
GPU processing (Stark, Frank, Cheng, Stahlberg, …)

Automation (Carragher, Potter, …)

Success stories of single particle EM

Viruses – ε15 virus capsid at 4.5 Å

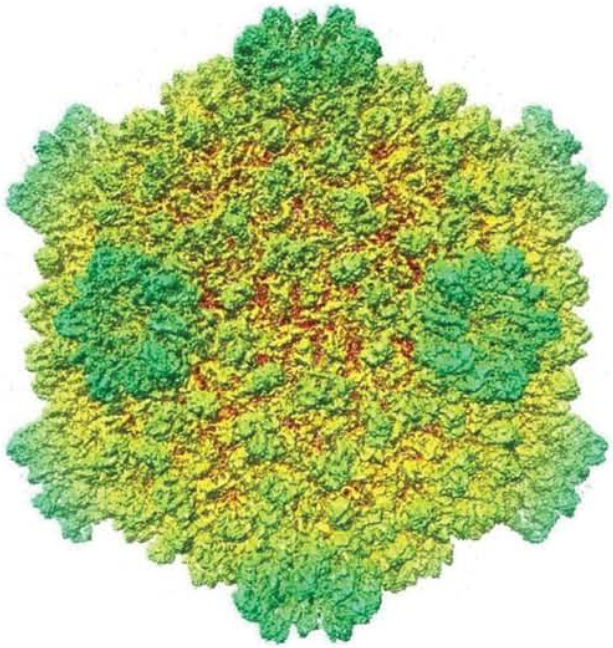


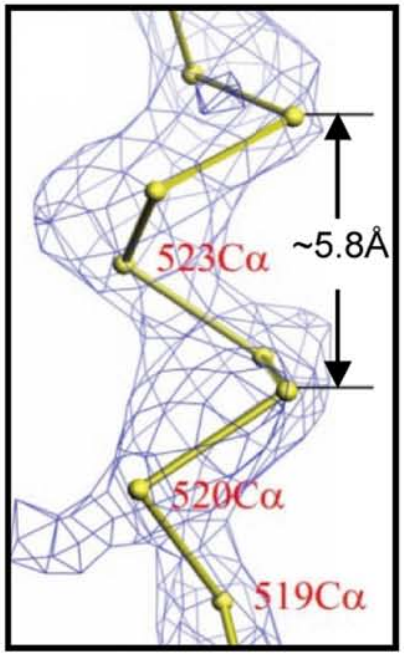


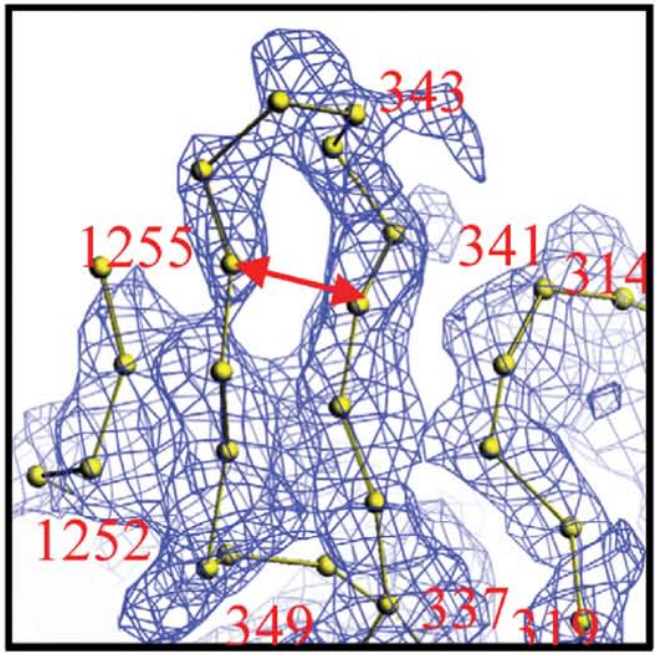
Jiang *et al.* (2008) *Nature* 451: 1130-1134

Success stories of single particle EM

Viruses – cytoplasmic polyhedrosis virus at 3.88 Å



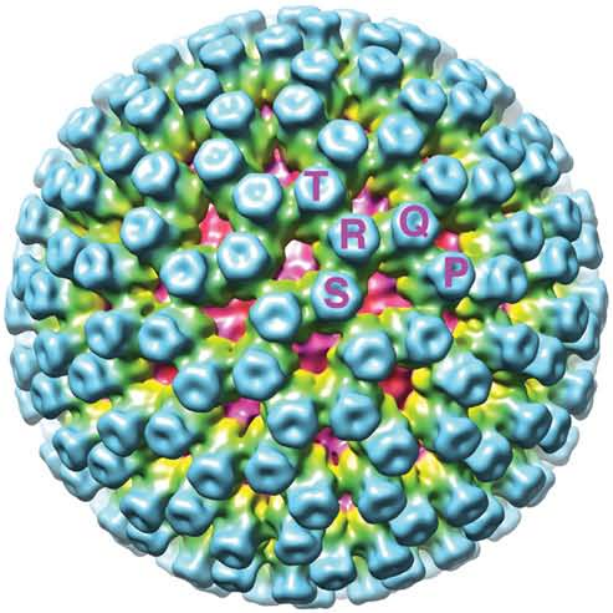


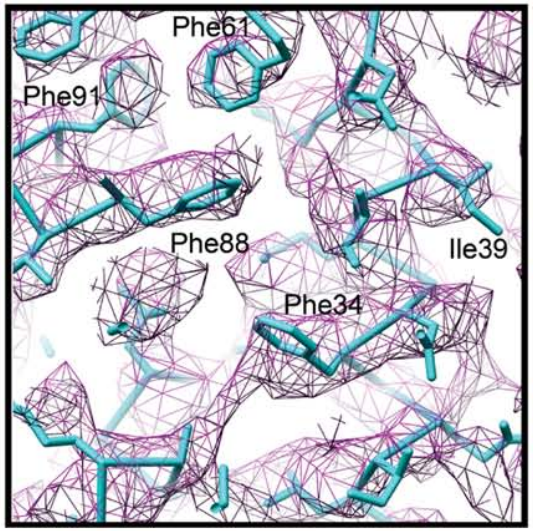


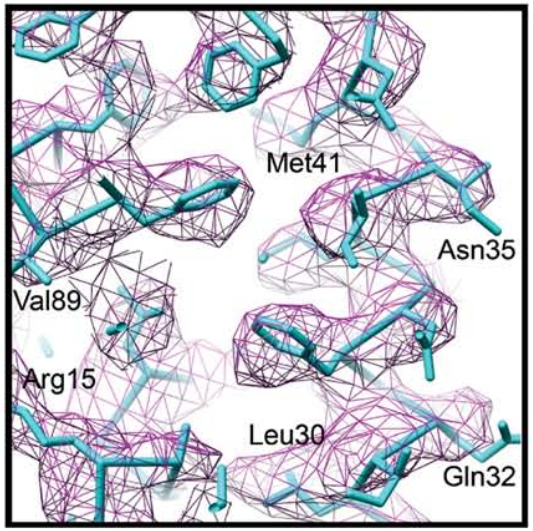
Yue *et al.* (2008) *Nature* 453: 415-419

Success stories of single particle EM

Viruses – rotavirus DLP at 3.8 Å







Zhang *et al.* (2008) *Proc. Natl. Acad. Sci. USA* 105: 1867-1872

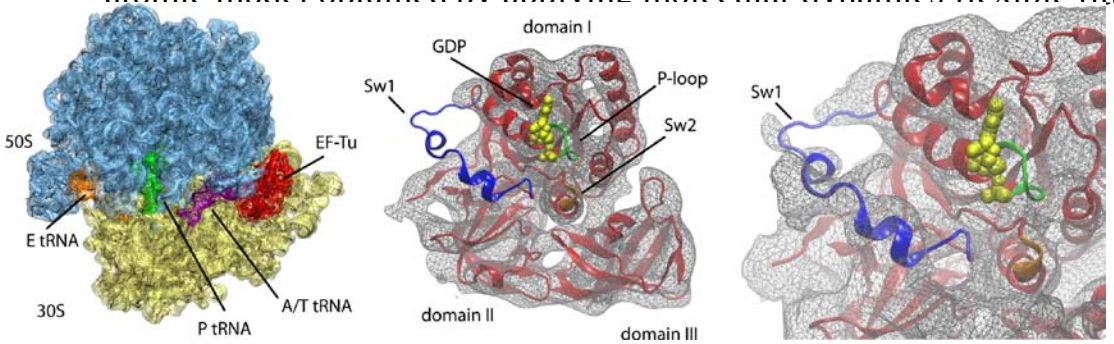
Success stories of single particle EM

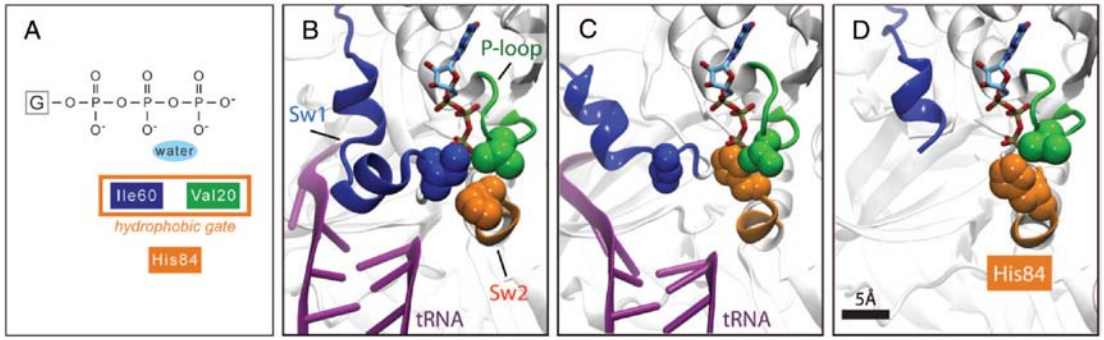
Ribosome at 6.7 Å (molecular dynamic flexible fitting)

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

70S ribosome with Phe-tRNA^{Phe}□EF-Tu□GDP ternary complex stalled by kirromycin at 6.7 Å resolution

atomic model obtained by applying molecular dynamics flexible fitting (MDFF)



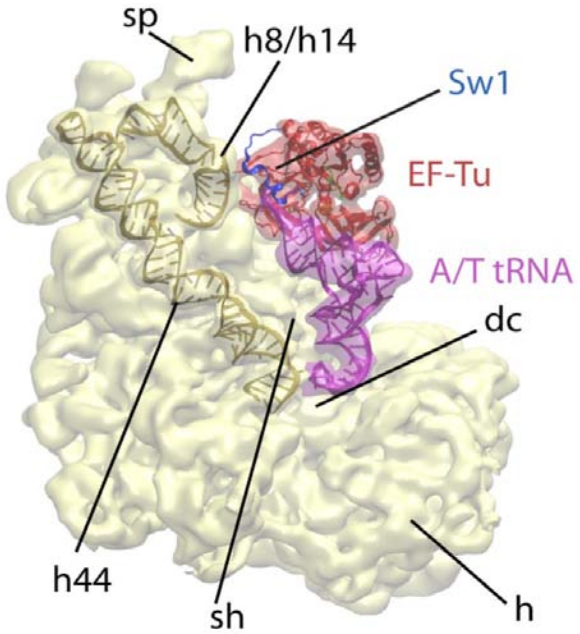


hydrophobic gate

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

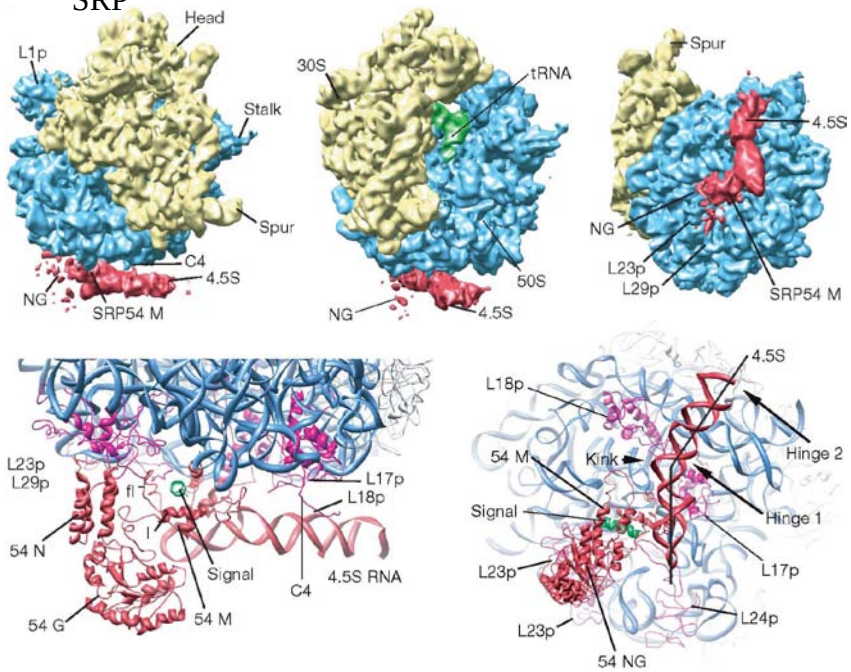
Success stories of single particle EM

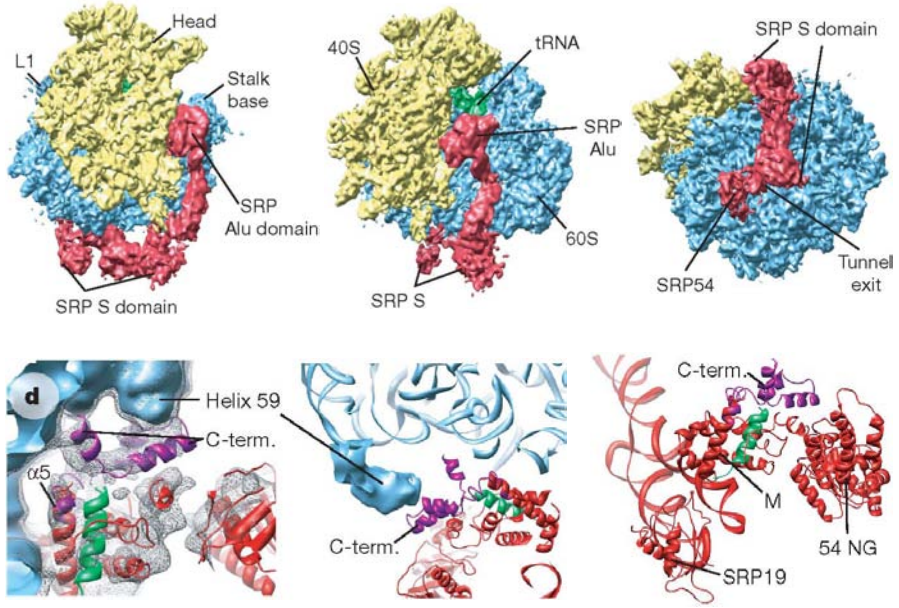
Ribosome at ~9 Å (signal recognition particle)

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



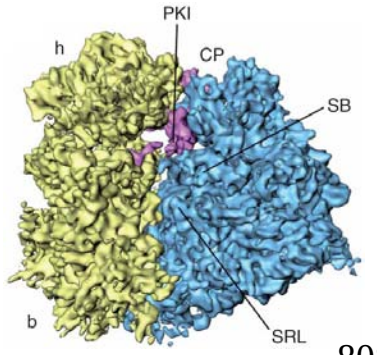


Halic *et al.* (2006) *Nature* 444: 507-511

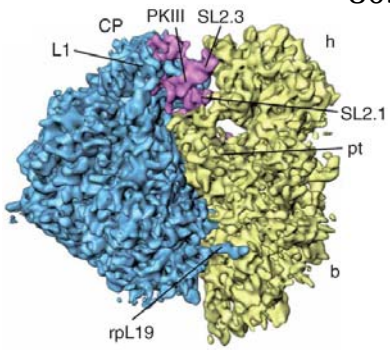
Success stories of single particle EM

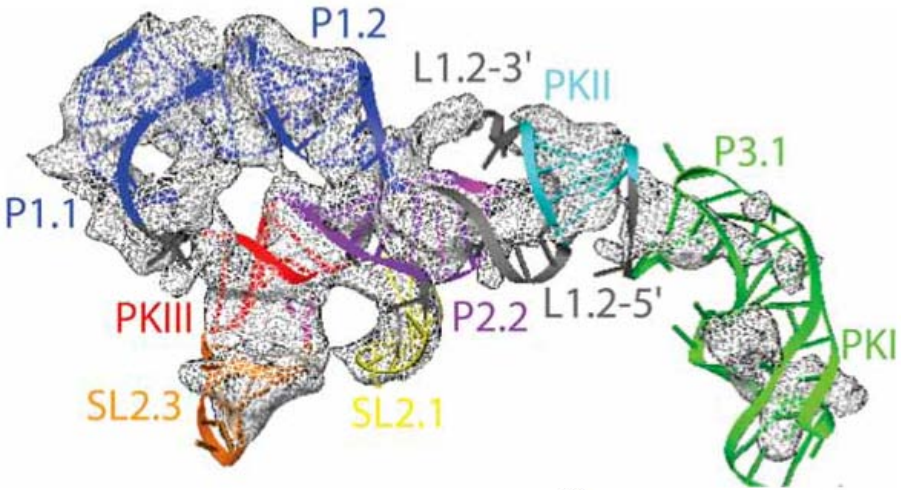
Ribosome at 7.3 Å (IRES RNA fold)

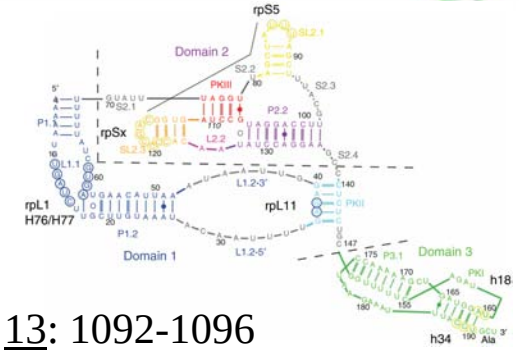
Structure of the ribosome-bound cricket paralysis virus IRES RNA



80S



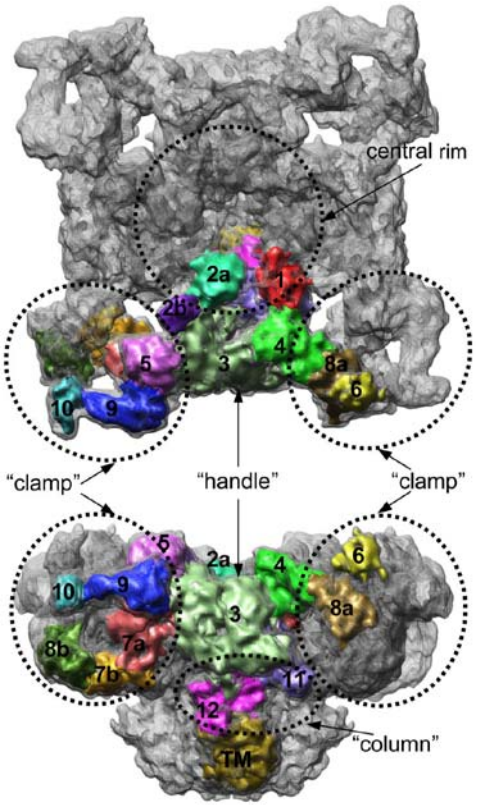


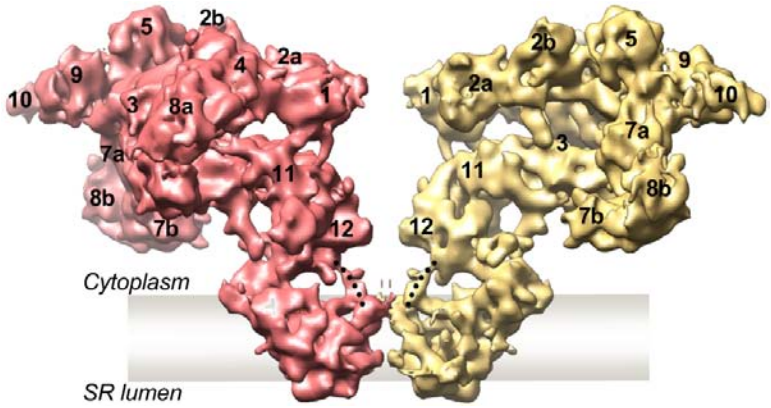


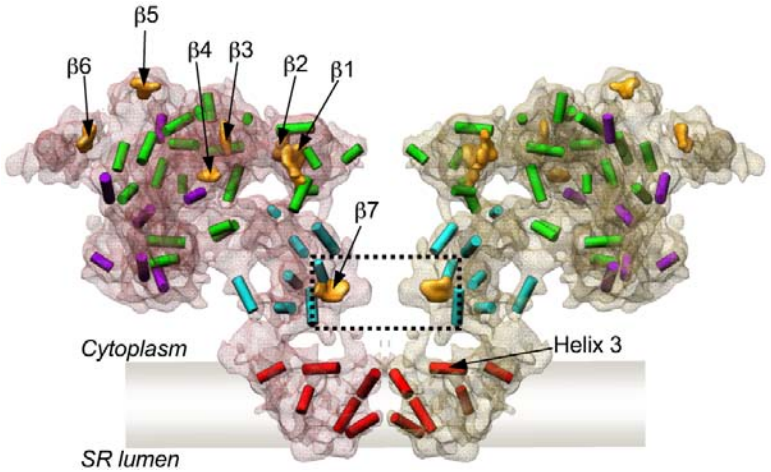
Schüler *et al.* (2006) *Nat. Struct. Mol. Biol.* 13: 1092-1096

Success stories of single particle EM

Ryanodine receptor at 9.6 Å (secondary structure)



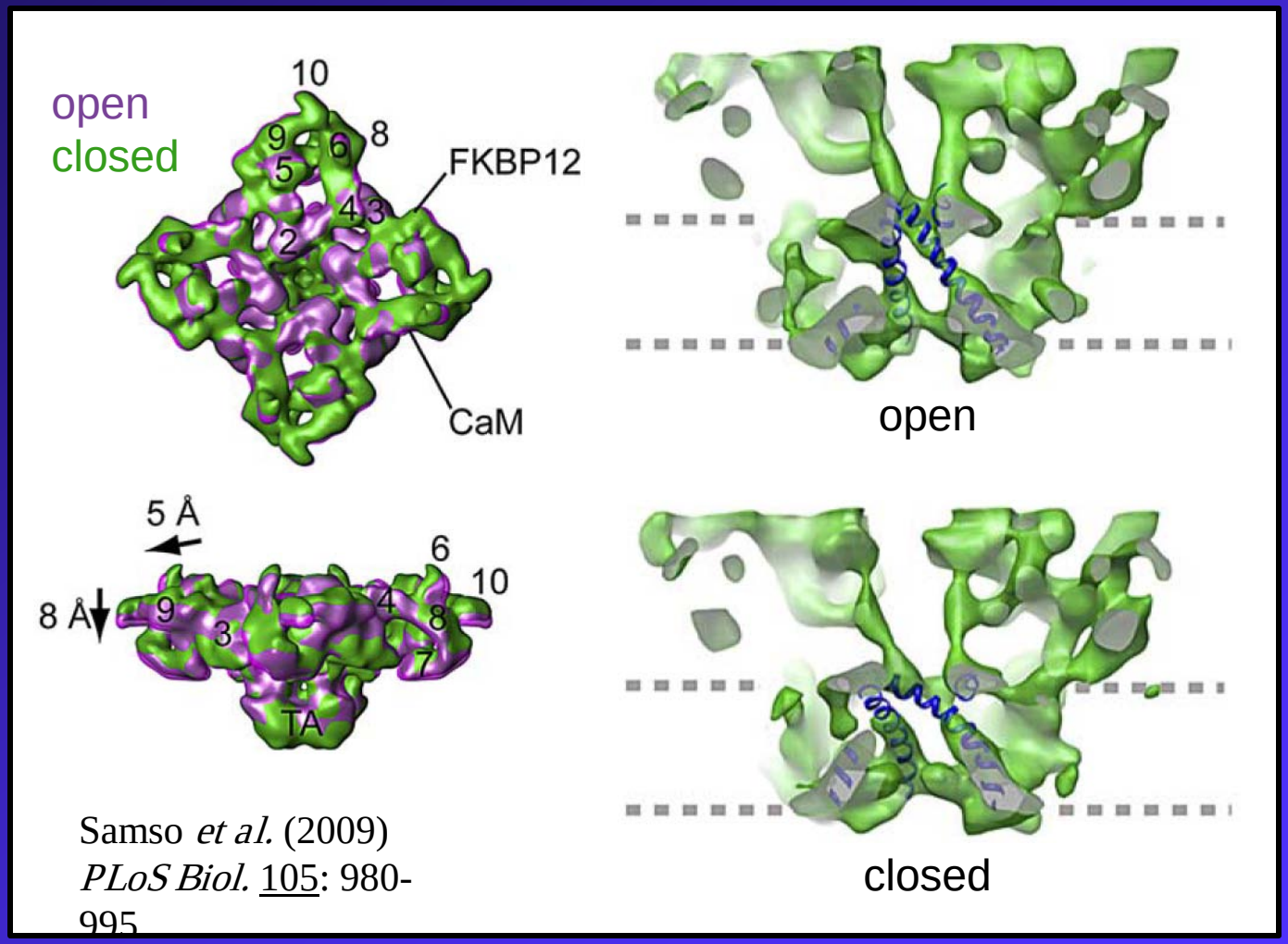




Serysheva *et al.*
(2008)
PNAS 105: 9610-9615

Success stories of single particle EM

Ryanodine receptor at 10.2 Å (channel gating)

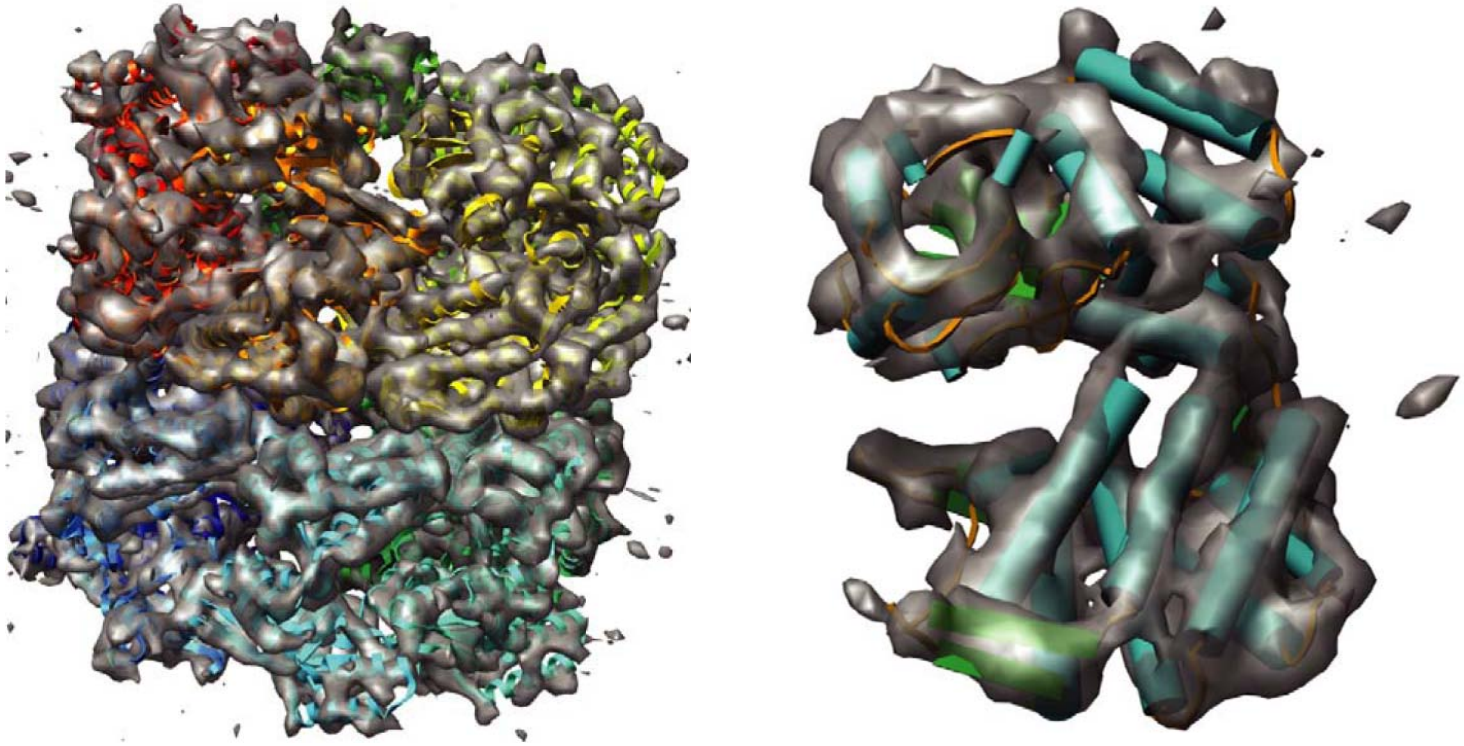


Samso *et al.* (2009) *PLoS Biol.* 105: 980-995

Success stories of single particle EM

GroEL at 7.8 Å (automation)

Automated cryoEM data acquisition and analysis of 284742 particles of GroEL

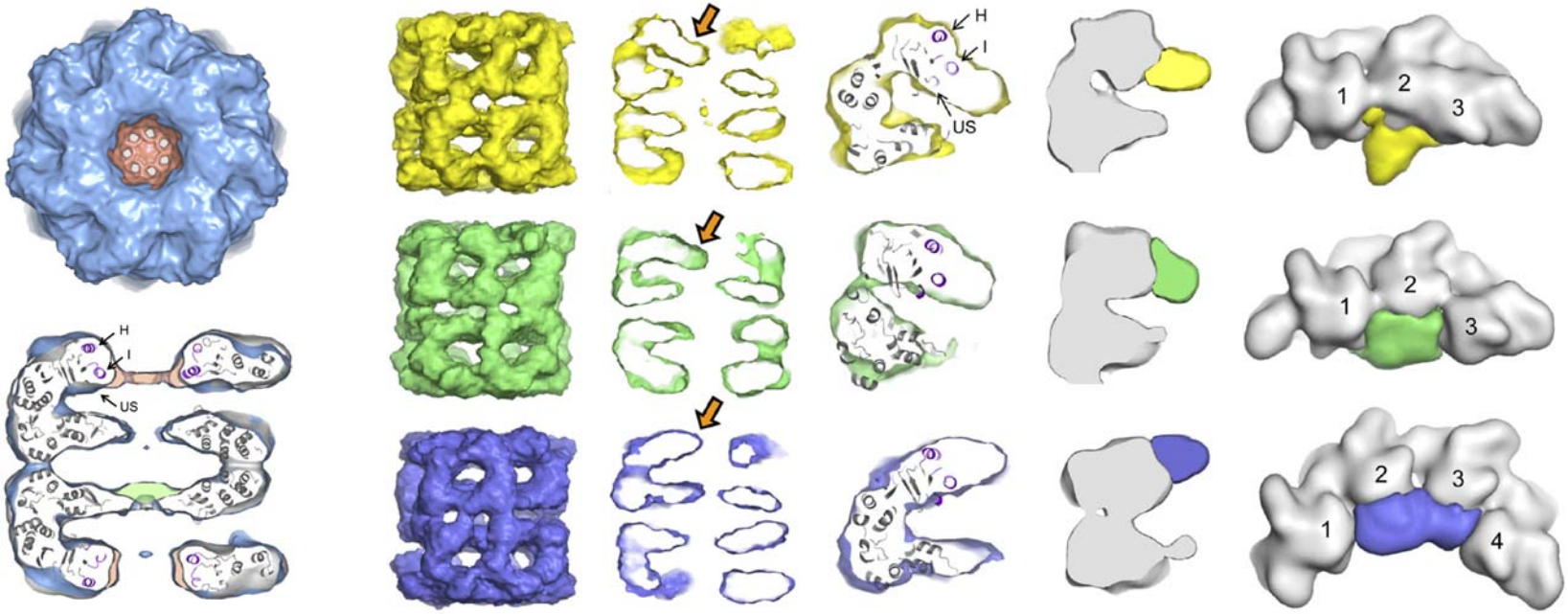


Stagg *et al.* (2006) *J. Struct. Biol.* 155: 470-481

Success stories of single particle EM

GroEL at ~10 Å (substrate binding)

Topologies of a substrate protein bound to the chaperonin GroEL



7-fold symmetrized

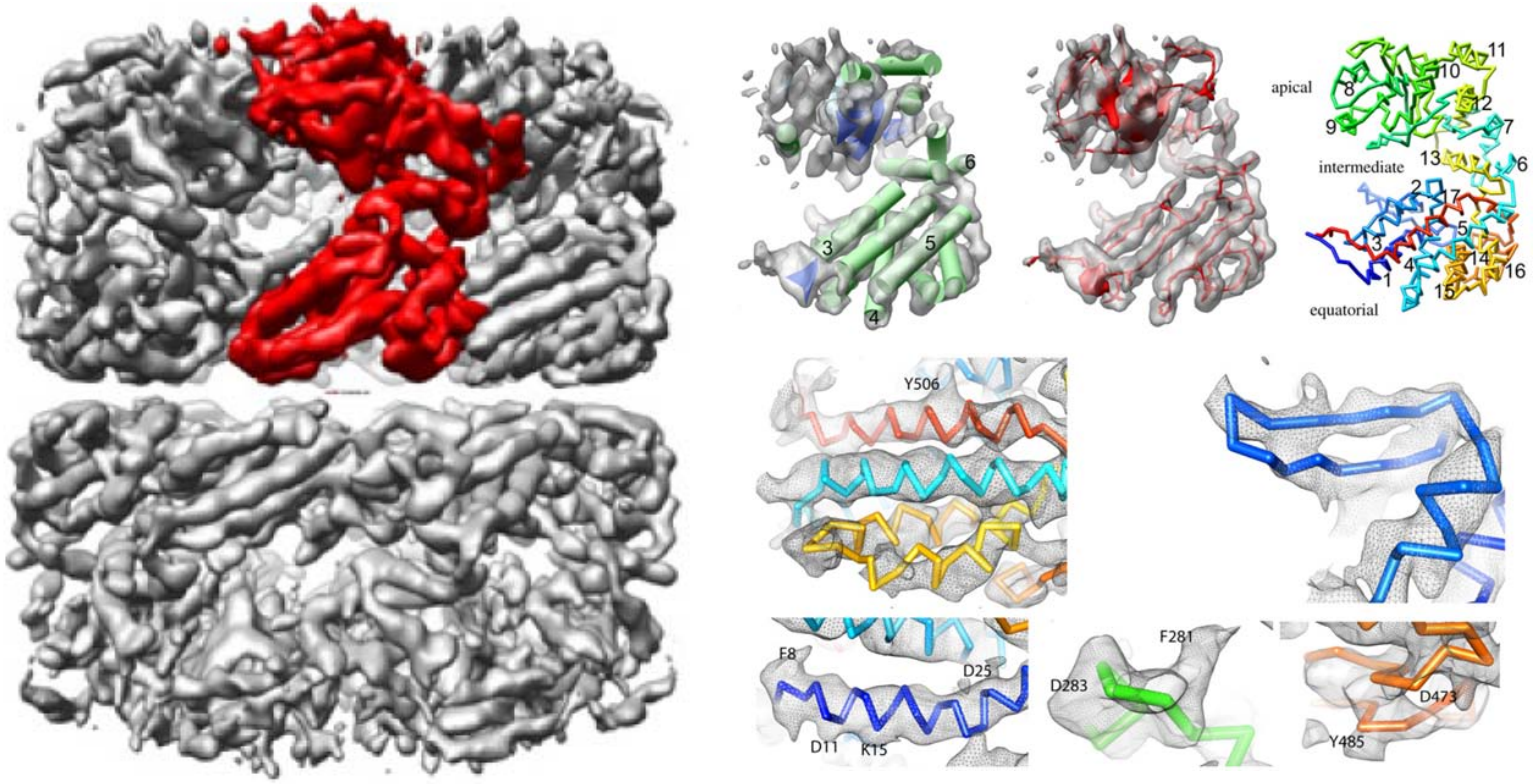
reconstructions without symmetry enforced

Elad *et al.* (2007) *Mol. Cell* 26: 415-426

Success stories of single particle EM

GroEL at ~4 Å (backbone trace)

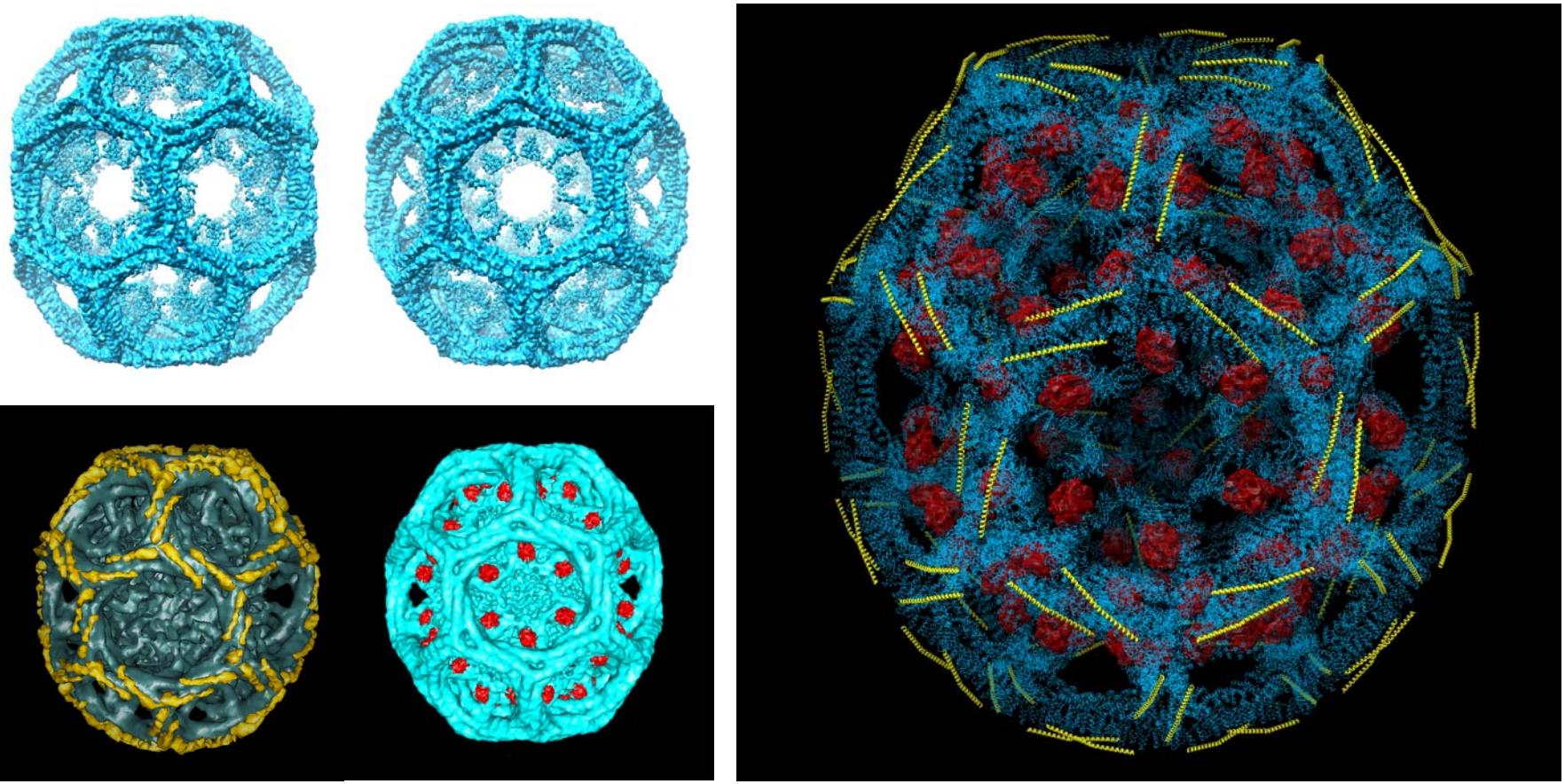
De novo backbone trace of GroEL from single particle electron cryomicroscopy



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

Success stories of single particle EM

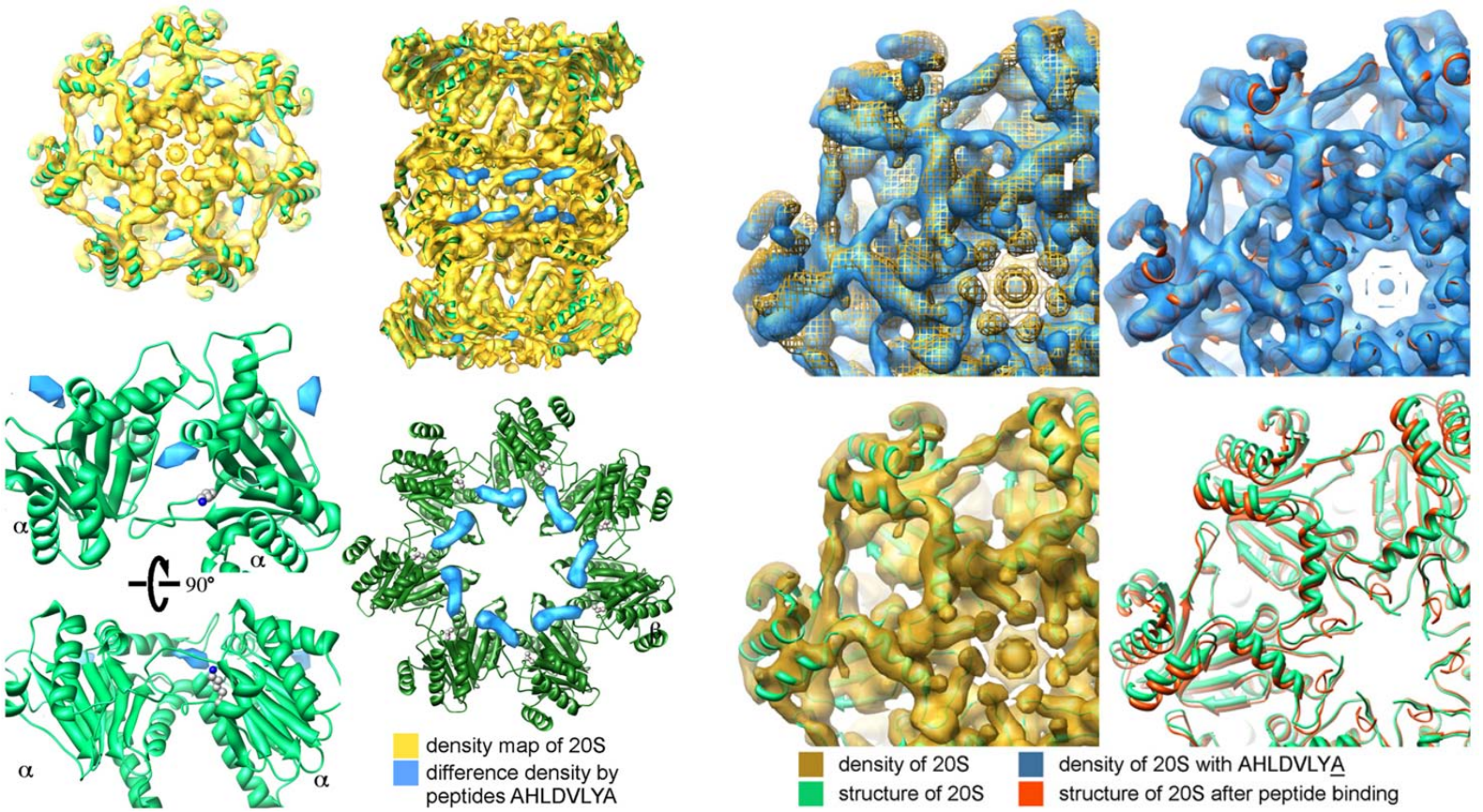
Clathrin cage at 7.9 Å



Fotin *et al.* (2004) *Nature* 432: 573-579; Fotin *et al.* (2004) *Nature* 432: 649-653

Success stories of single particle EM

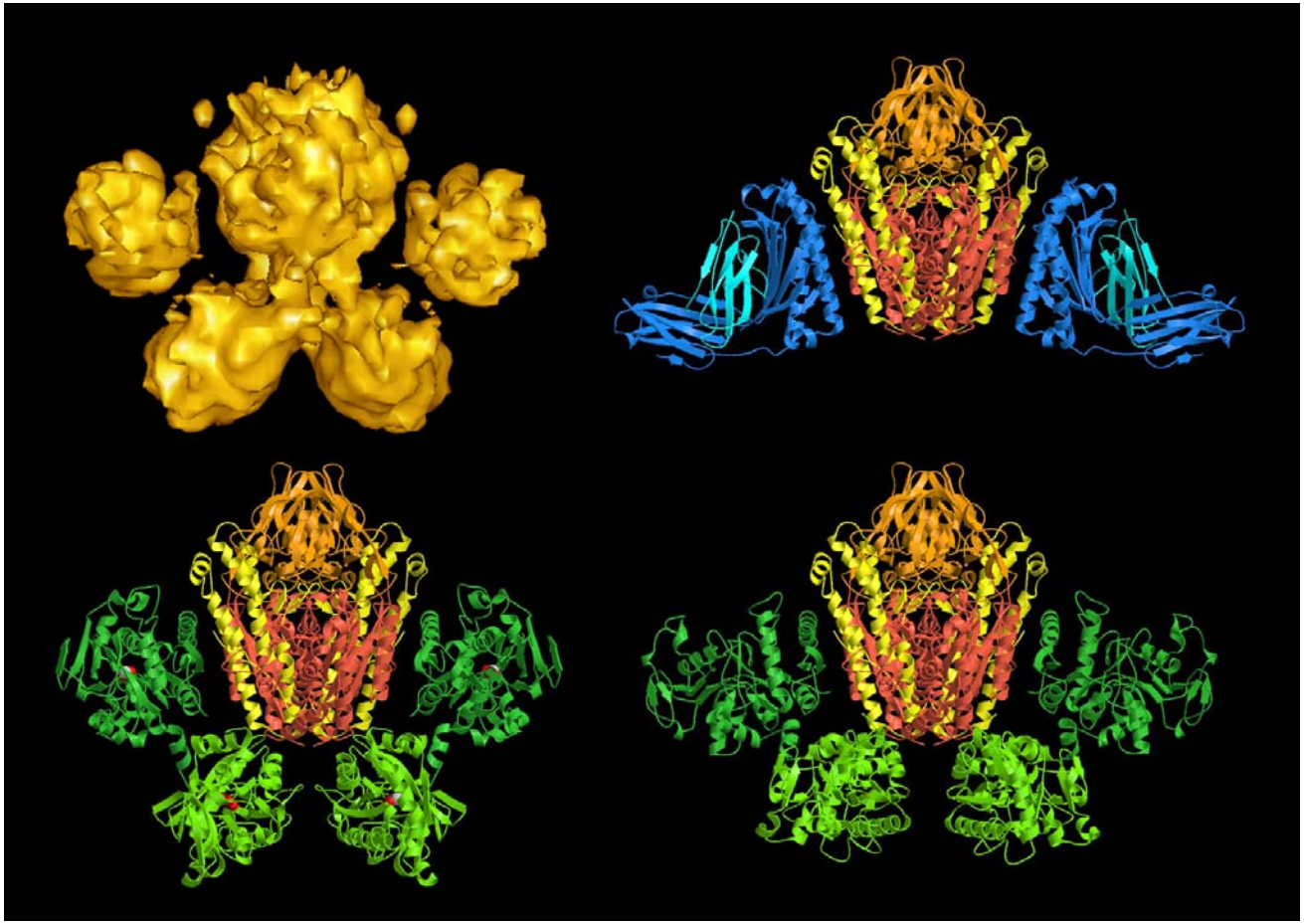
20S proteasome at ~6 Å (8 aa peptide)



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

Success stories of single particle EM

Tf-TfR complex at 7.5 Å (~280 kDa)



Cheng *et al.* (2004) *Cell* 116: 565-576

What means "challenging" ? present

What is possible now (state-of-the-art) ?

– 3D reconstructions at near-atomic resolution of viruses

– 3D reconstructions at subnanometer resolution of "homogeneous" complexes, preferably with symmetry

– 3D reconstructions at molecular resolution (or better) of "heterogeneous" complexes

What remains challenging ?

– 3D reconstructions at near-atomic resolution of complexes that do not have high symmetry

– 3D reconstructions at subnanometer resolution of "heterogeneous" (especially conformational continuum) and/or small complexes

What are current developments ?

new detectors – better signal-to-noise images

phase plate – better signal-to-noise images

dynamic TEM – less beam damage

humongous data sets – deal with heterogeneous samples

new image processing algorithms
– every step from particle picking to map verification

What we desperately need:
– reliable resolution criterion (maybe Rmeasure)
– objective accuracy criterion (like Rfree)

Prospects of single particle EM

ask Richard !

Viruses are leading the way
molecular → subnanometer → near-atomic resolution

Other macromolecules will follow
– large molecules with high symmetry (e.g. GroEL)
– large molecules with low symmetry (e.g. ribosome)
– smaller molecules
– very heterogeneous molecules (further in the future)

Maybe still the major role of single particle EM in the future:
envelopes of complexes at molecular or subnanometer resolution
– to produce pseudo-atomic models by docking X-ray structures
– to characterize conformational changes for functional insights

Part III
other considerations

What resolution is useful ?
What INFORMATION is useful ?

Resolution steps:	*> 15 Å*	*molecular envelopes*
	~ 10 Å	*α-helices*
	~ 4.5 Å	*β-sheets*
	~ 3.5 Å	*near-atomic resolution*
	~ 1.5 Å	*atomic resolution*

2D averages or 3D density maps

Specimen preparation:	*negative staining*
	vitrification
	cryo-negative staining
	chemical fixation

What resolution is useful ?
What INFORMATION is useful ?

3D density maps at near-atomic resolution of native specimen in vitrified ice

BUT: – not always (actually rarely) possible
– not always necessary

Useful information depends exclusively on the biological question to be answered

some times, projection averages of negatively stained samples is all you can do or need to do to answer a biological question *

* Disclaimer: This is a personal opinion – not shared by most referees that will review your papers

What should be shown in a publication ?

Results

– **Raw data** (vitrified and negatively stained specimens)
– **Class averages** (all of them in Supplementary Material)
– **3D reconstructions** (potentially initial models, e.g. RCT)

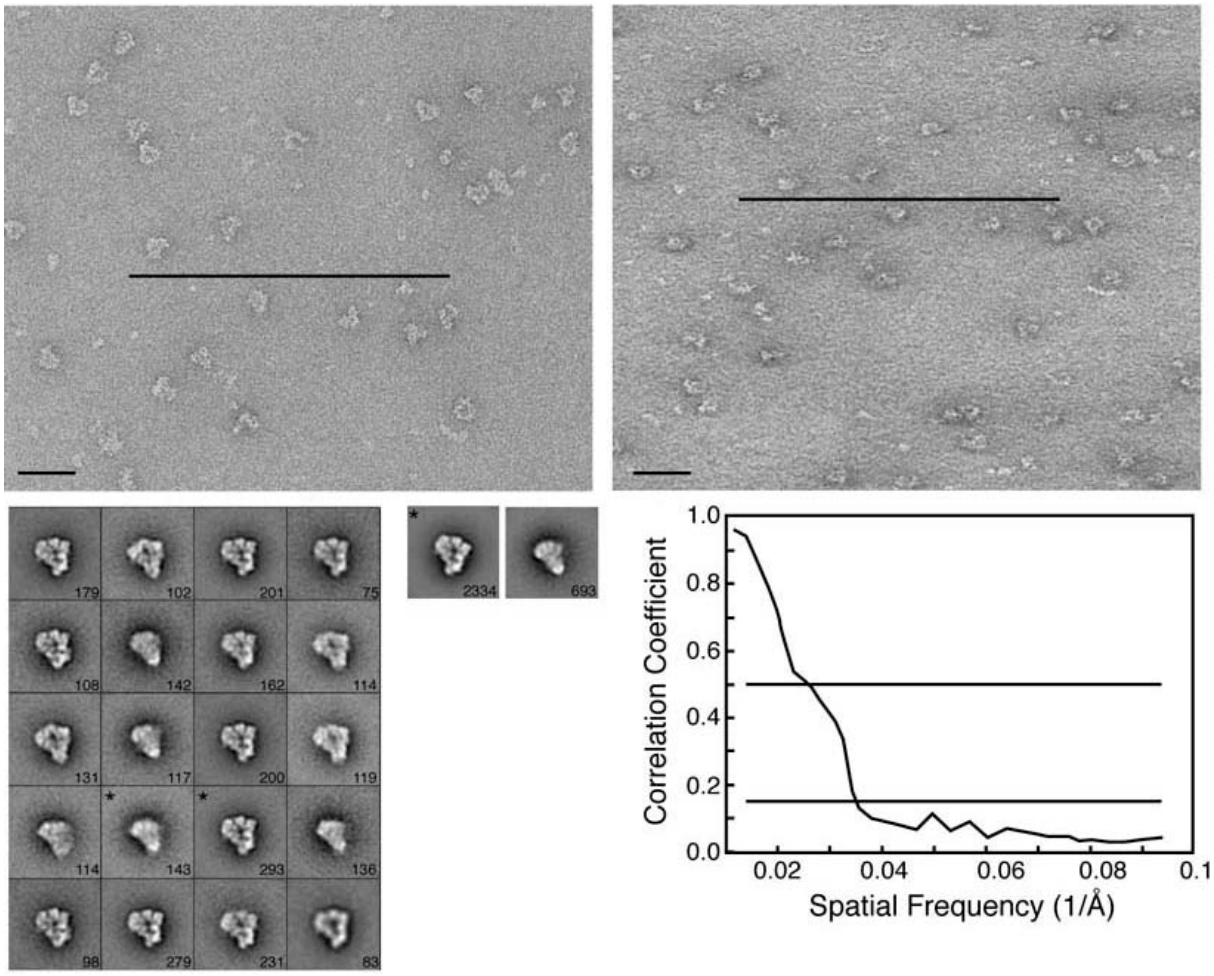
Quality control

– **FSC curve / Rmeasure**
– **Angular distribution**
– **Comparisons** (raw images, class averages, reprojections)

Interpretations

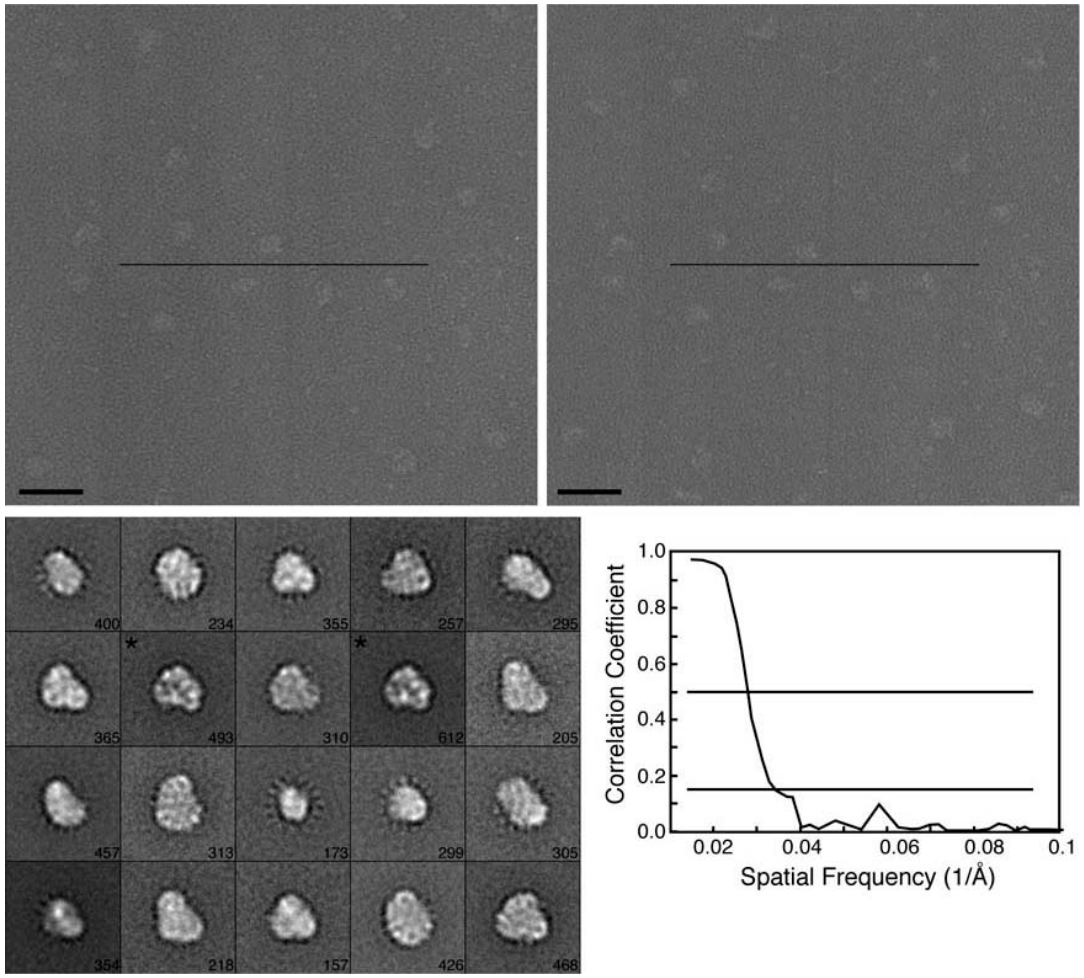
– **docking**
– **labeling**
– **variance maps, etc.**

What should be shown in a publication ?



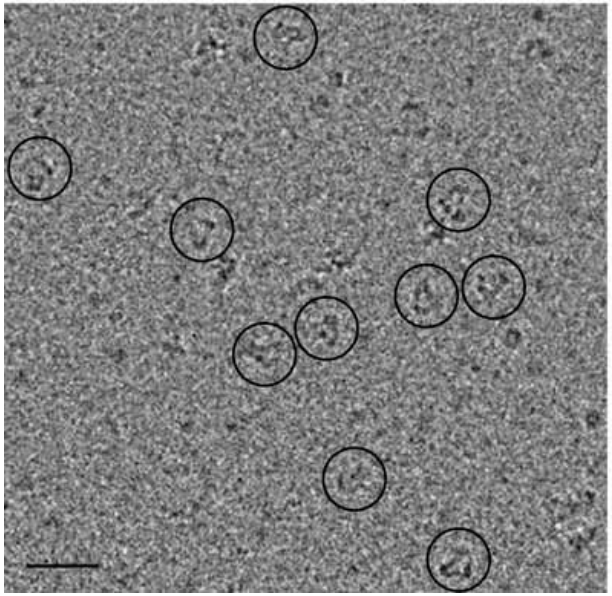
APC – negative staining

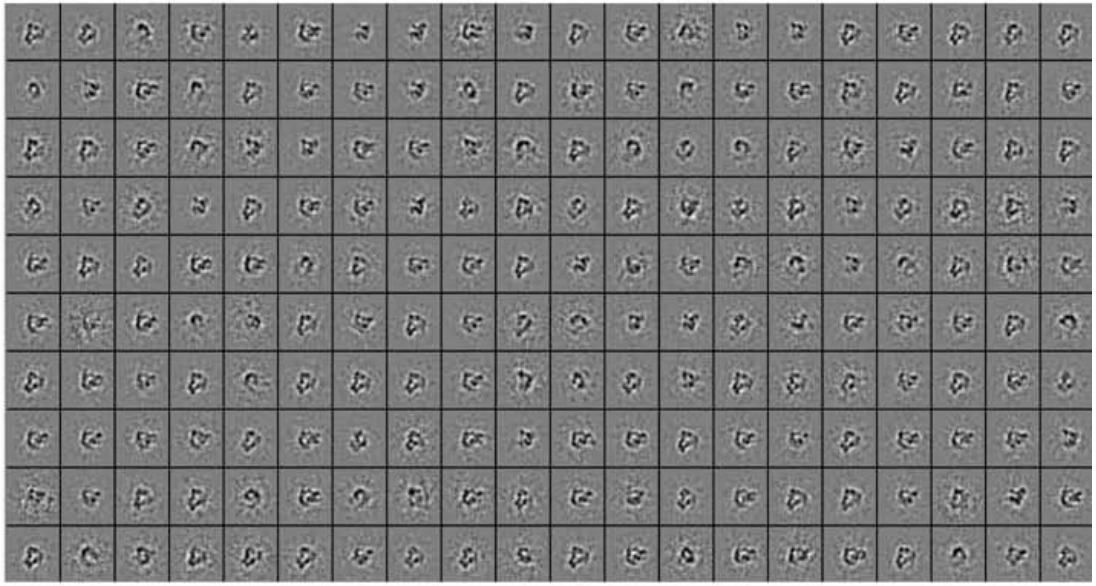
What should be shown in a publication ?



APC – cryo-negative staining

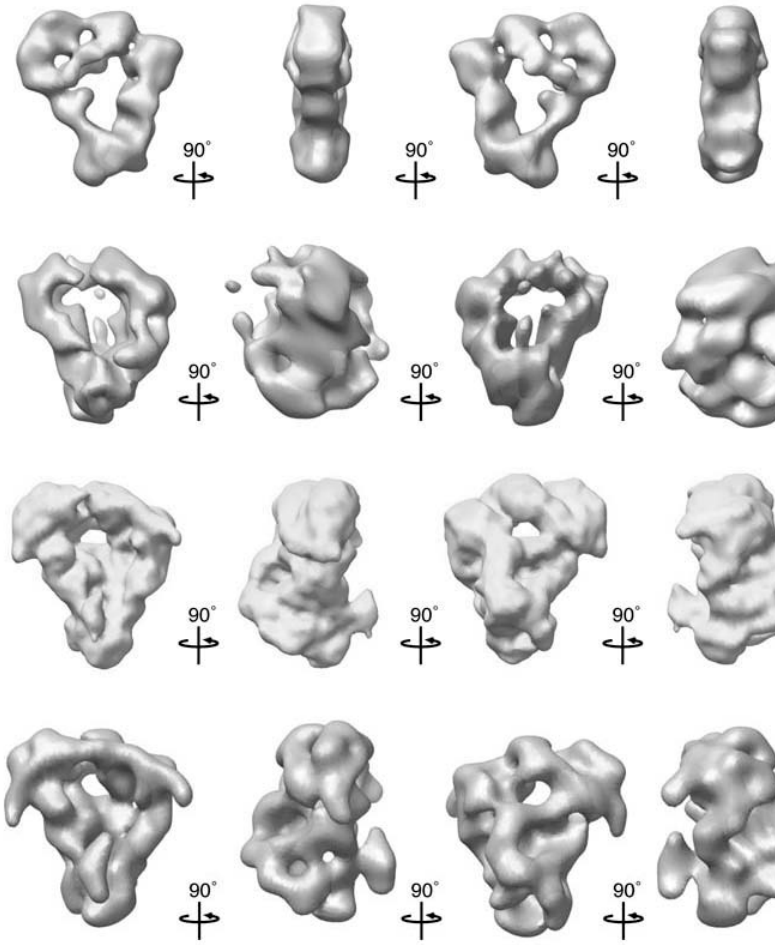
What should be shown in a publication ?





APC – vitrified ice

What should be shown in a publication ?



negative staining
RCT

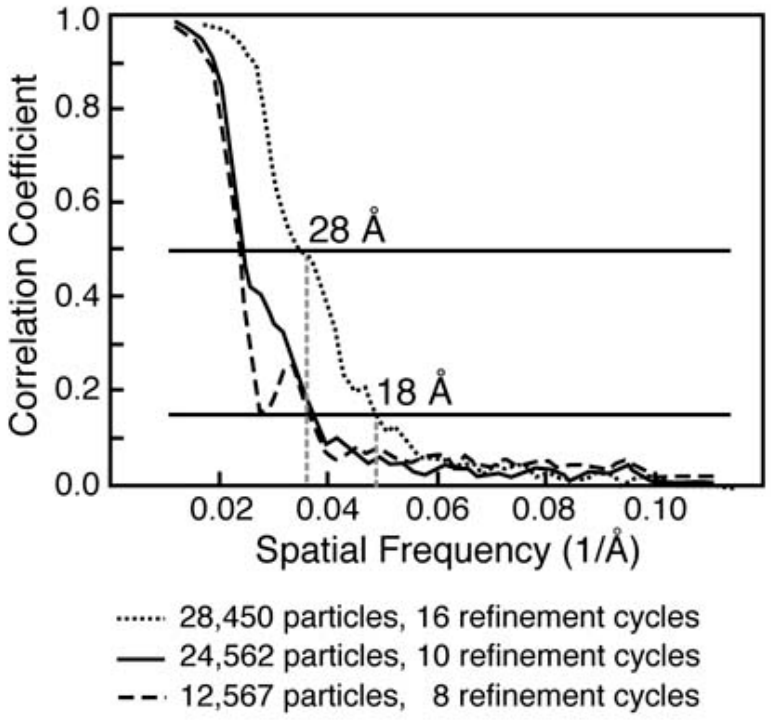
cryo-negative staining
RCT

vitrified ice
FREALIGN using
RCT reference

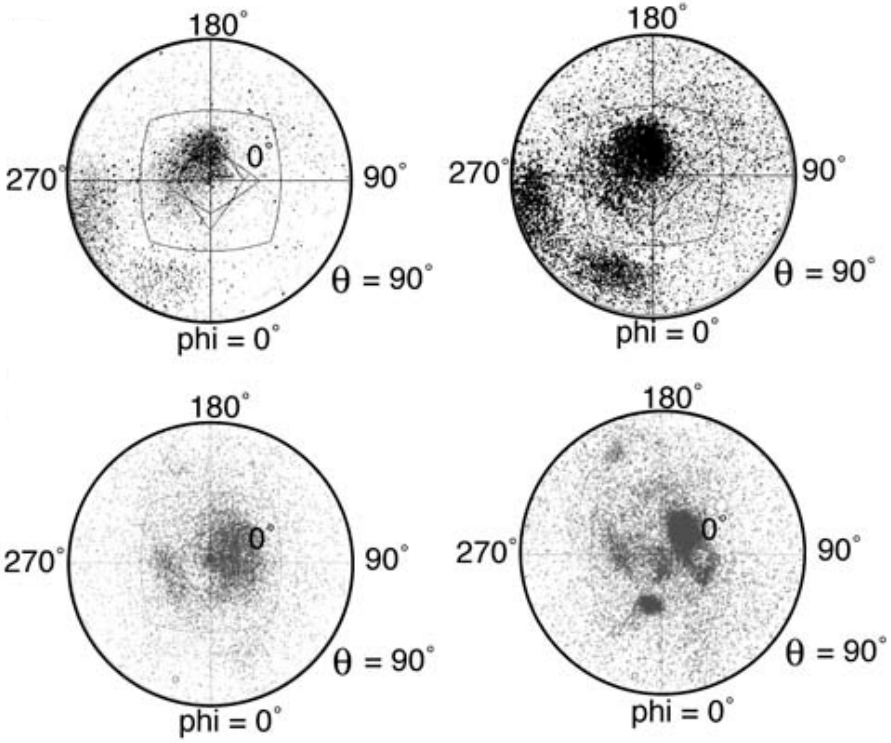
vitrified ice
de novo reconstruction
using OP command

APC – 3D
reconstructions

What should be shown in a publication ?

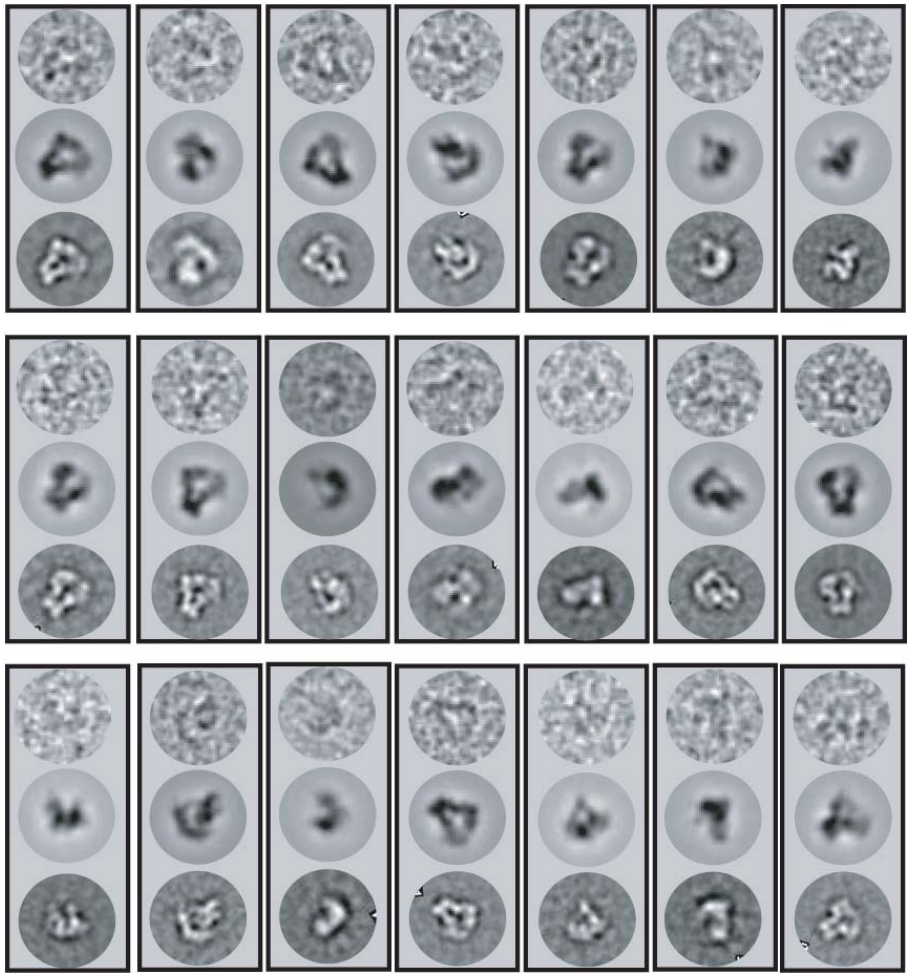


APC – FSC curves



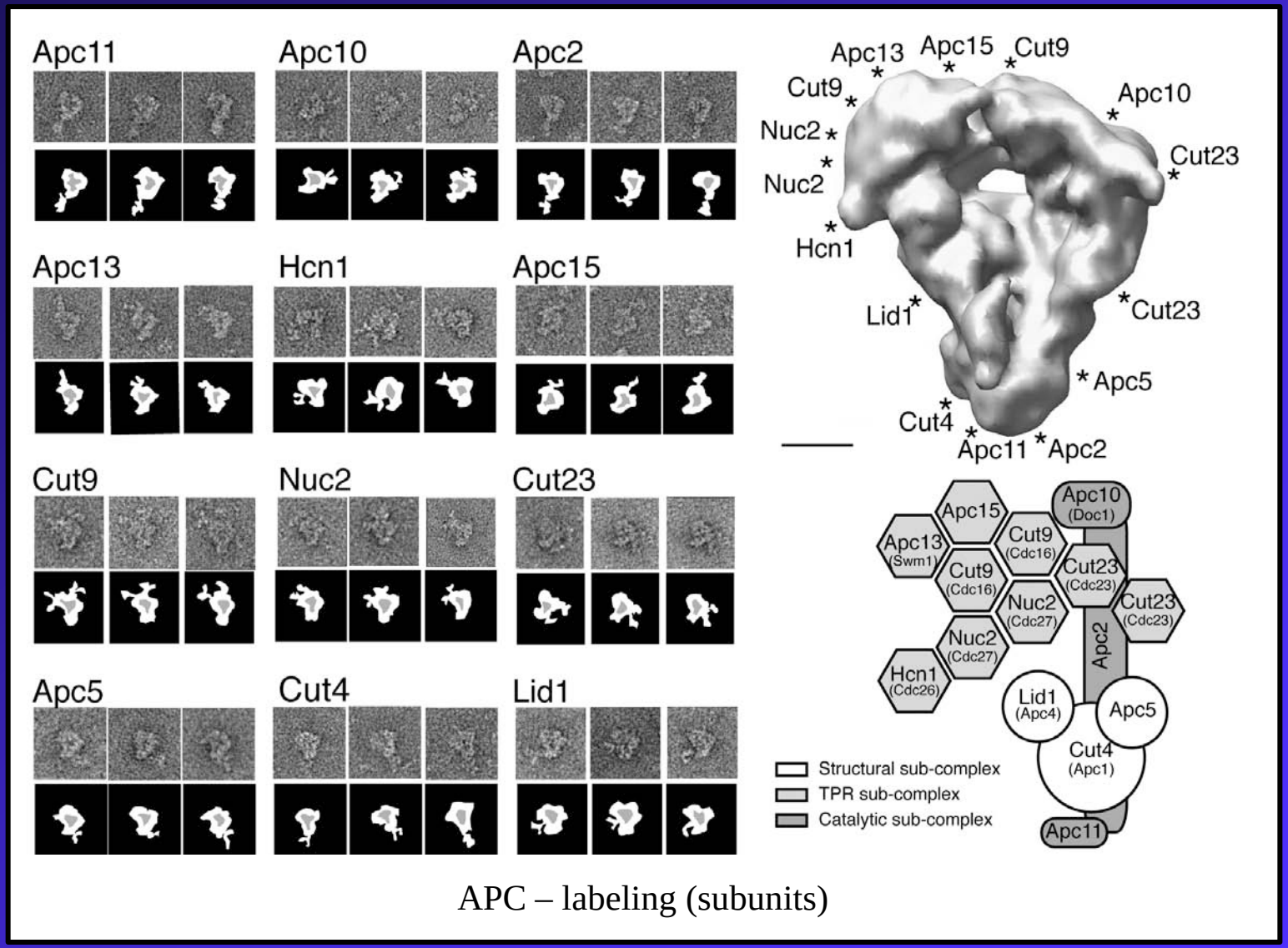
APC – angular distributions

What should be shown in a publication ?



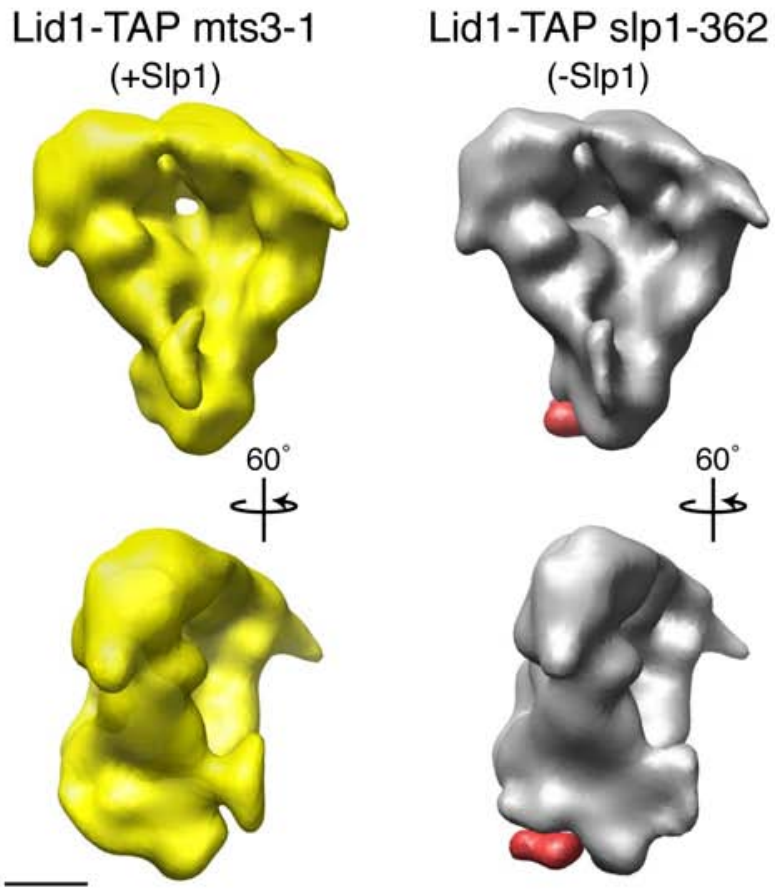
APC – comparisons

What should be shown in a publication ?

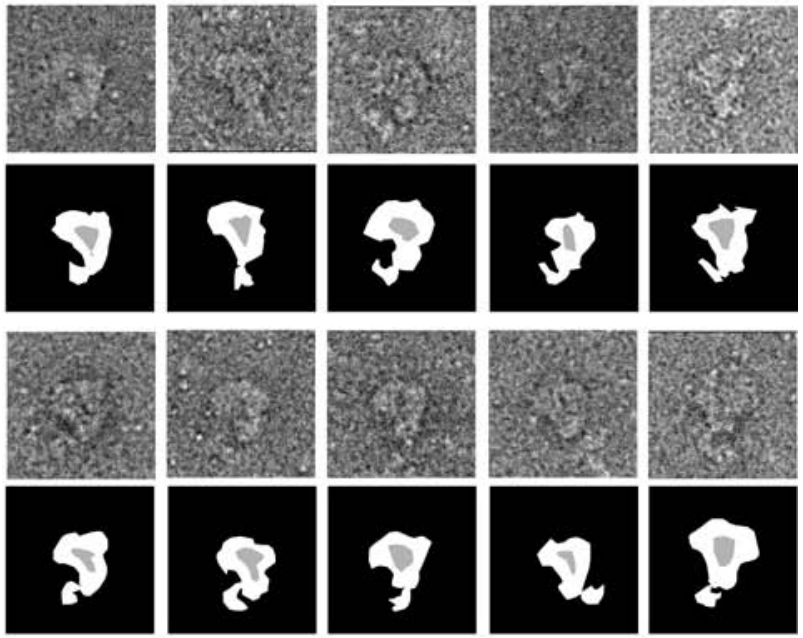


APC – labeling (subunits)

What should be shown in a publication ?



APC – 3D variance (activator)



APC – labeling (activator)