CNRS
INSERM
Université de Strasbourg

Parc d'Innovation - 1, rue Laurent Fries - ILLKIRCH - C.U. DE STRASBOURG - FRANCE

I. Resolution estimation

II. Atomic model building into cryo-EM maps

III. Common concepts & similarities between structural biology methods

IV. Role of cryo-EM in multi-scale multi-resolution integration

V. Considerations important to improve cryo-EM reconstructions

VI. Point group symmetries

VII. Pushing resolution by structure sorting, detectors & movie processing

Master M2S3 level, Brazil school & ISB workshop, ***Oléron school***

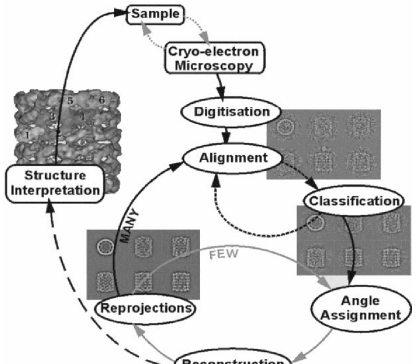
Bruno Klaholz,

Centre for Integrative Biology, IGBMC, Illkirch

2017

http://igbmc.fr/Klaholz

II. Structure determination
- overview



- improve quality of angle assignment

- improve quality of particle alignment

van Heel *et al.*, Quart. Rev. Biophys. 33, 4 (2000).

Some softwares:
(for single particle cryo-EM image processing)

- Imagic (van Heel lab) https://www.imagescience.de/index.html
- Spider (Frank lab) https://spider.wadsworth.org
- Frealign (Grigorieff lab) http://grigoriefflab.janelia.org/frealign
- EMAN (Ludtke lab) http://blake.bcm.tmc.edu/EMAN2
- XMIPP (Carazo lab) http://xmipp.cnb.csic.es
- Relion (Scheres lab) http://www2.mrc-lmb.cam.ac.uk/relion
- Scipion (Carazo lab) http://scipion.cnb.csic.es/m/home
- Sphire (Raunser lab) http://sphire.mpg.de/
- COW (Stark lab) www.cow-em.de
- cisTEM (Grigorieff lab, to be announced soon)
- …

Plus other specific software for helical reconstructions, viruses etc.
IHRSR (Egelman lab), SPRING (Sachse lab), Relion (Scheres lab)…
Baker lab, Zhou lab, etc.

I. Resolution estimation

II. Structure determination
- resolution assessment

Particle data set

split into 2 halves, refine independently

3D 3D

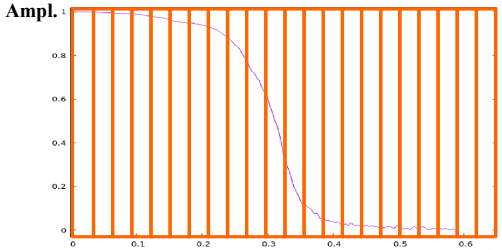
FT FT

calculate cross-correlation

by shells in Fourier space

Fourier Shell Correlation (FSC)

FSC curve



Cross correlation is calculated in Fourier space as a function of frequency (i.e. resolution shells)

Fourier Shell Correlation (FSC): van Heel *et al.*, 1982, Saxton & Baumeister, 1982.

II. Structure determination
- resolution assessment

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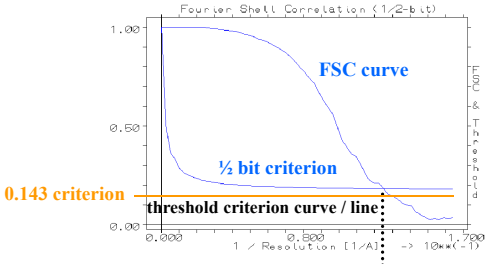
Fourier Shell Correlation (FSC)



1 / 1.25 x 1 / 10 Å = 8 Å

Rosenthal & Henderson, 2003 (0.143 criterion)
van Heel & Schatz, 2005 (1/2 bit criterion)

II. Structure determination
- resolution assessment

Particle data set

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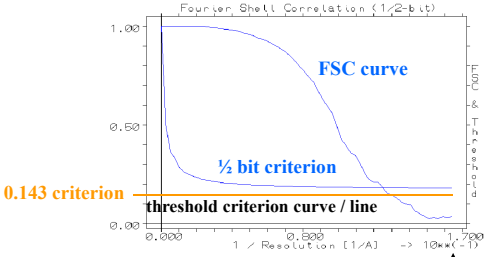
Fourier Shell Correlation (FSC)



Take care to
- do not cut off spectrum before Nyquist limit
- be generally careful with fixed threshold criteria
- masking can give artefacts at high frequencies
- max. resol. possible ~2/3 Nyquist (unless over-sampling is used)

II. Structure determination
- resolution assessment

Particle data set

split into 2 halves, refine independently

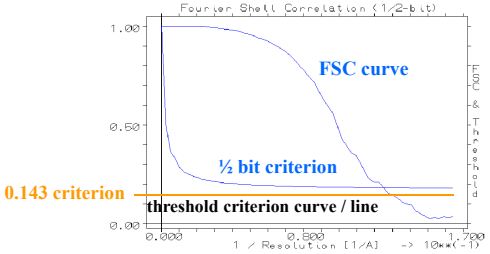
3D 3D

FT FT

calculate cross-correlation

by shells in Fourier space

Fourier Shell Correlation (FSC)



Keep in mind: resolution is what you can resolve in the 3D map!

Some basic concepts of cryo electron microscopy

Basic aspects:

- "resolution" corresponds to "spatial frequency" in image processing (1/ Å)
- Nyquist frequency is = 2 x pixel size, e.g. 1 Å / pixel → Nyquist = 2 Å
- interpolations during 2D image alignment and 3D reconstruction limit the possible resolution to about 2/3 of the Nyquist frequency, i.e. here ~ 3 Å *(exception: super-reso)*

pixels in 3D: "voxel"

Consider:

- any correlation calculation (e.g. alignment) is biased by the reference used
- resolution estimation, criteria used:
  - 0.5, arbitrary, historically from the virus field, tends to underestimate resolution
  - **0.143 (Henderson) and ½ bit (van Heel)**
  - 3 σ, not used anymore (over-estimation; useful for noise estimation)
  - **features in the map: can we see dsRNA helices (~10-12 Å resolution), α-helices (~8 Å), ß-sheets (~5 Å) or side chains (4-2.5 Å, depending on size)?**

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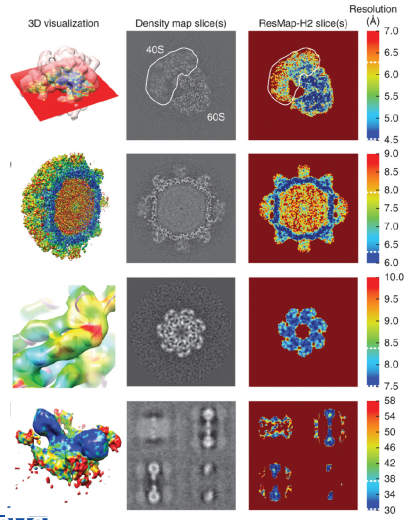
Local resolution analysis



Some softwares:

Bsoft (Heymann *et al.*, 2007)

ResMap (Kucukelbir *et al.*, 2014)

Local resolution can be different from average resolution (FSC) due to structural disorder

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II. Atomic model building into cryo-EM maps

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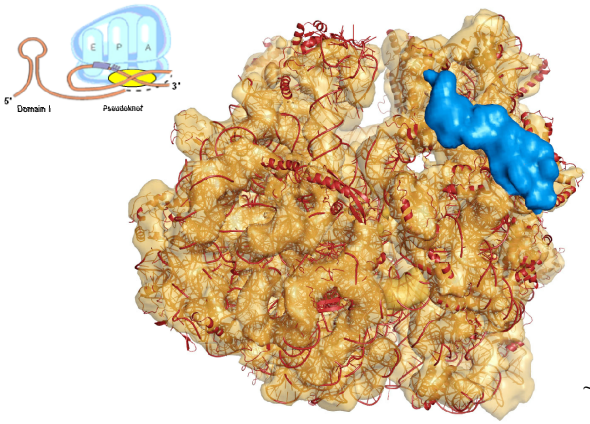
II. Atomic interpretation of cryo-EM maps



~12 Å resolution

Marzi *et al.*, *Cell* 2007.

Fitting of 70S crystal structure from *E. coli* (Schuwirth *et al.*, 2005; *Science*, 310, 827-834)

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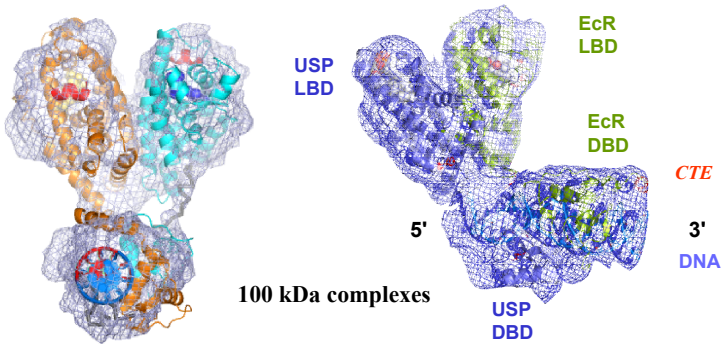
II. Structure determination
- map interpretation



100 kDa complexes

Orlov *et al.*, *EMBO J.* 2012. Maletta *et al.*, *Nature Communications, 2014.*

Fitting of crystal structures from the various protein domains

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Map interpretation, structure determination, atomic model building

Fitting procedures:
- manual fitting (e.g. O, Coot, Pymol, Chimera…)
- real space fitting
- reciprocal space fitting

1) global search
2) refinement

<u>At ~8-20 Å resolution:</u>

- fit complete structures, protein or RNA domains, factors; usually backbone is enough.

Rigid body or flexible fitting (e.g. Situs, MDFF, Flex-EM, iMODfit, …)

- use full maps or difference maps

<u>At ~3-5 Å resolution:</u>

- atomic model building: start with poly-Ala model, check register (position of Cα atom), check secondary structure elements (e.g. direction of α-helices), refine with crystallography programs (CNS, Buster, Phenix, CCP4,…), add side-chains if clearly visible,

use information from multi-sequence alignments; check geometry with Ramachandran plot

In general: be careful with local minima and over-fitting/over-interpretation!

modelling

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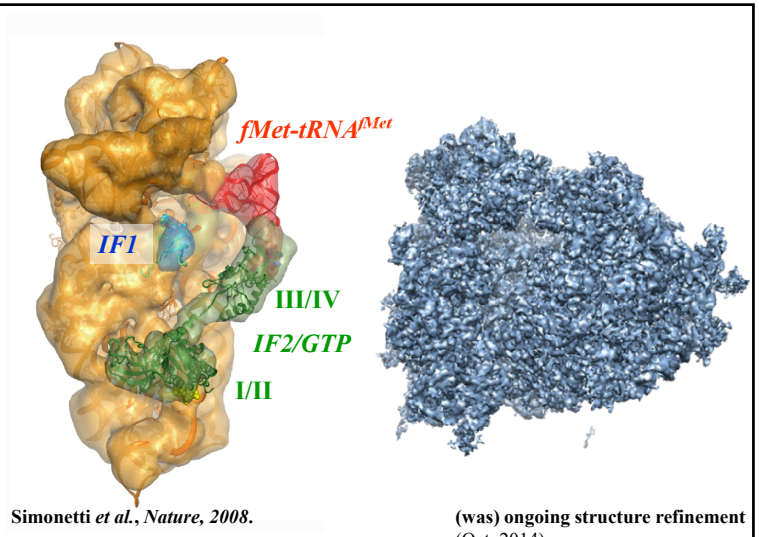

Simonetti *et al.*, *Nature, 2008.*

(was) ongoing structure refinement
(Oct. 2014)

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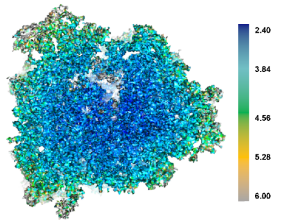
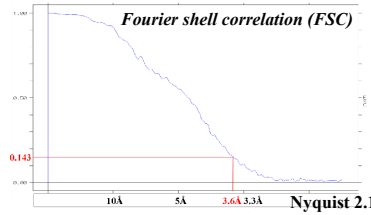
Structure determination at ~ 3 Å resolution by single particle cryo-EM

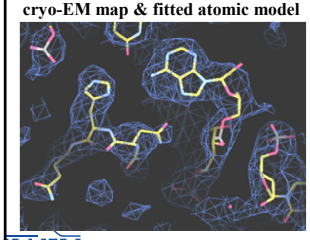


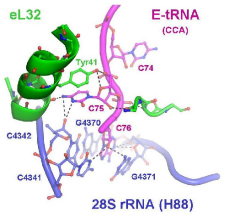


Nyquist 2.16Å

→ local resolution can be notably better than average resolution

cryo-EM map & fitted atomic model





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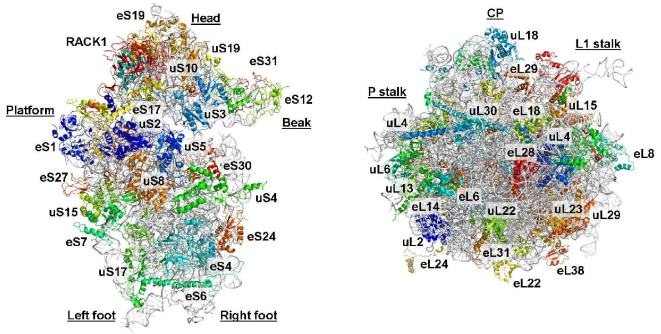
Khatter *et al.*, *Nature, 2015.*

High-resolution cryo-EM allows full atomic model building

Structure of the human ribosome

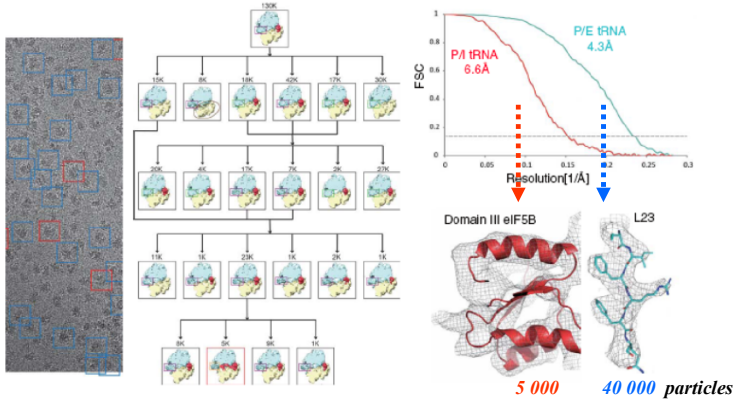
Overall structure: 80 proteins and 4 rRNA's (28S, 5S, 5.8S and 18S),

~220 000 atoms (5866 nucleotides, ~11590 amino acids)



Khatter *et al.*, *Nature* 2015.

II. Atomic interpretation of cryo-EM maps

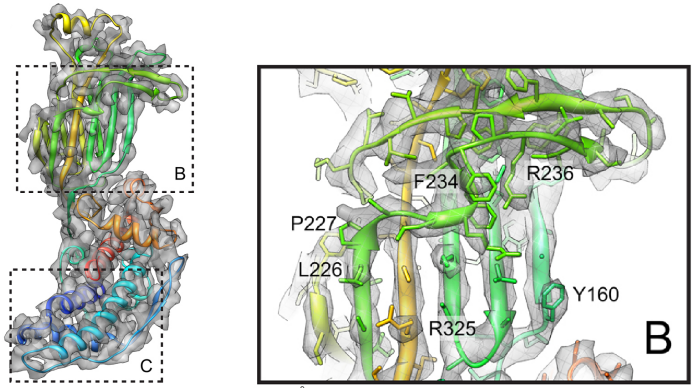


5 000 ***40 000*** ***particles***

Strong heterogeneity of a reconstituted eukaryotic translation initiation (eIF5B) complex: sorting → 5143 particles, representing 3% of the population in the sample, 6.6 Å reconstruction.

Fernández *et al.*, Science 2013; V. Ramakrishnan & S. Scheres.

Atomic model building examples in cryo-EM



Rotavirus VP6 cryo-EM structure; 3.8 Å resolution; α-helices, β-sheets, bulky side-chains;

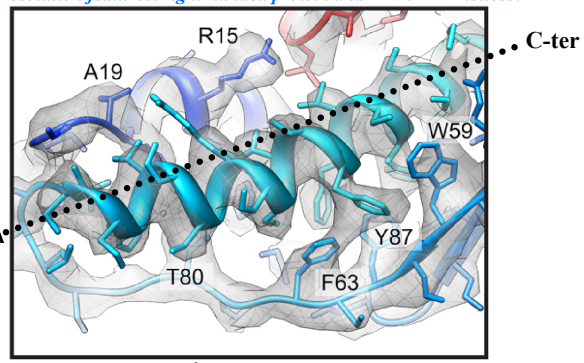
Individual stands in the β-sheet region are separated, loops connecting the strands are defined.

Near-atomic-resolution cryo-EM for molecular virology.

Hryc CF, Chen DH, Chiu W. Curr Opin Virol. 2011.

Atomic model building examples in cryo-EM

high-resolution features: right-handed protein and DNA/ARN helices!



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Near-atomic-resolution cryo-EM for molecular virology. Hryc, Chen, Chiu W., Curr Opin Virol. 2011.

See also tools from Wah Chiu lab on atomic model building: Curr. Op. Struct. Biol. 2015

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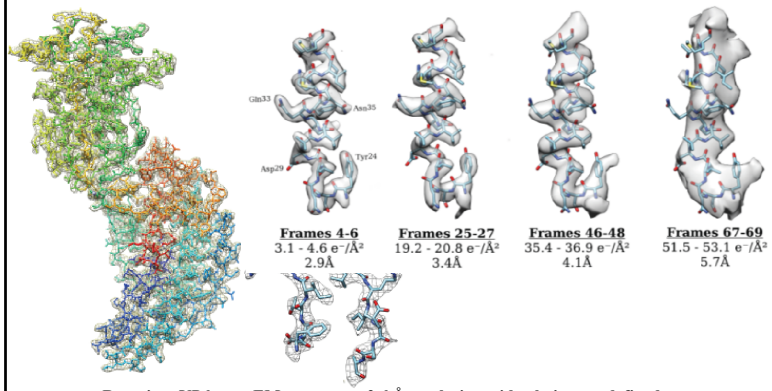


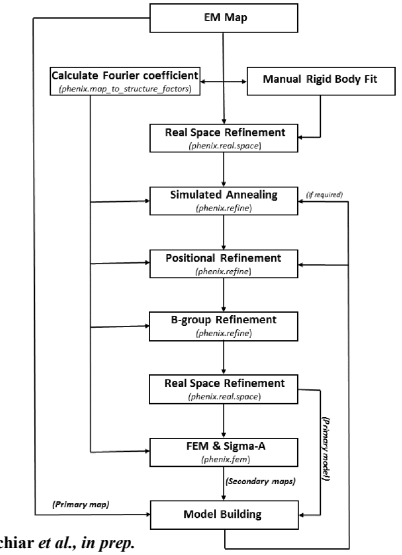
Rotavirus VP6 cryo-EM structure; 2.6 Å resolution; side-chains are defined.

optimize exposure dose to select movie frames

Grant, T., Grigorieff, N., *eLife*, 2015.

Atomic model building:

combining cryo-EM and X-ray crystallography refinement procedures



Natchiar *et al., in prep.*

III. Common concepts & similarities between structural biology methods

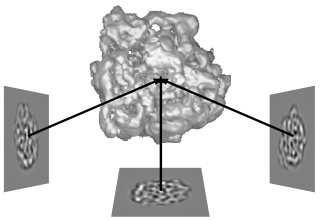
III. Similarities between structural biology methods

Is the purified sample homogeneous?

What means homogeneity?
- same composition
- same functional state
- same structural state, i.e. same conformational state

Why do we need homogeneity?

--> **most structural biology approaches are <u>averaging techniques</u>**:
- crystallography
- SAXS
- NMR
- EM and 3D reconstruction

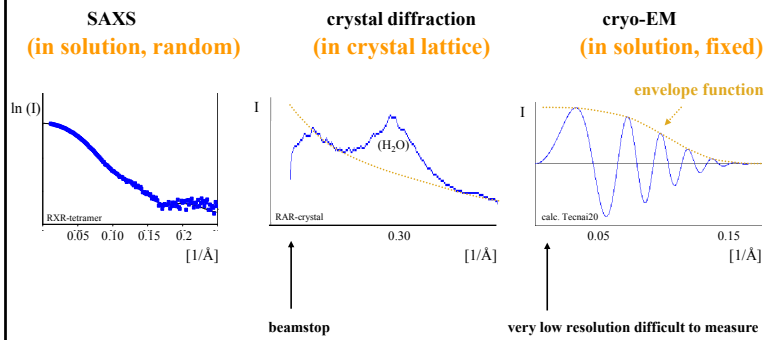


- mass spectrometry (MALDI-TOF etc.)
- dynamic light scattering
- protein / RNA gel electrophoresis
- kinetic studies

exceptions:
- electron tomography
- other single molecule experiments

Complementarity of structural approaches: similarities between methods

Profile of the intensity distribution



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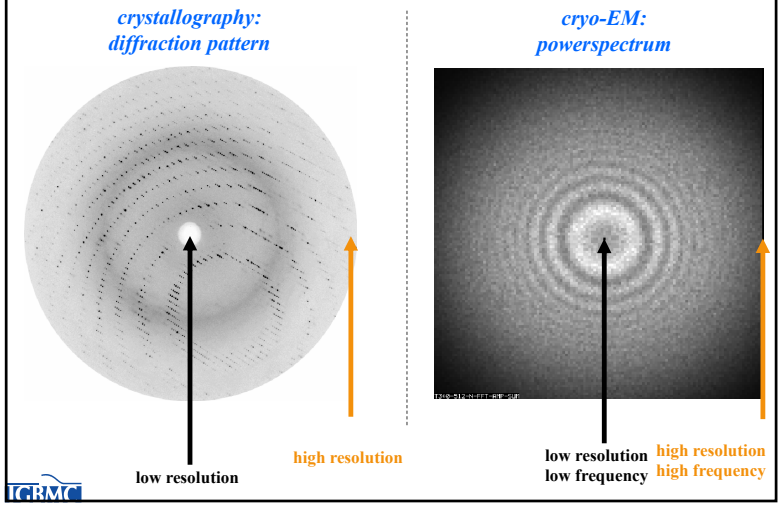
Complementarity of structural approaches: similarities between methods



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Related concepts in crystallography / cryo-EM

- molecular replacement (MR) using a known crystal structure and importance of low resolution data collection // importance of low frequencies in cryo-EM to *see* the object (high DQE of detector), i.e. align particles and get initial 3D reconstruction
- phase extension in crystallography // gradual increase of high-frequency components during structure refinement in cryo-EM (increasing precision of particle alignment during image processing, refine with restrained alignment)
- solvent flattening in crystallography // masking of entire structure, focused refinement
- wavelength λ = ~1-2 Å, diffraction-limited // λ = ~0.022 Å, no limitation; Ewald sphere flat
- high B factors in crystallography // less resolved regions in the cryo-EM map
- resolution estimation: cc1/2 (~0.1-0.2) // FSC in cryo-EM (0.143;1/2 bit); half data sets
- maximum likelihood parameter refinement of atomic models in crystallography // ML-based parameter refinement in cryo-EM (transl./rot., defocus etc.)
- Monte Carlo approaches / molecular dynamics simulations / simulated annealing // resampling-based structure sorting in cryo-EM
- group / sub-group relationships; space group symmetries (lattice symmetry) // point group symmetries (internal particle symmetry)

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Common problem: determination of the handedness

- crystallography: depends on correct processing of phased reflections
- SAXS: ambiguity cannot be resolved easily (unless clear fitting of crystal structure etc.)
- NMR: ambiguity solved by using chirality constraints
- single (cryo-)EM images are projections, i.e. mirrors are indistinguishable

Determination of handedness in EM:

- random conical tilt (Radermacher *et al.*, J. Microsc. 1987)
- tomography (technically tricky on single particles)
- phase residual error using a tilt pair (Rosenthal & Henderson, JMB 2003)
- fitting of crystal structures (requires reasonable resolution)
- high-resolution features: right-handed protein and DNA/ARN helices!

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IV. Role of cryo-EM in multi-scale multi-resolution integration

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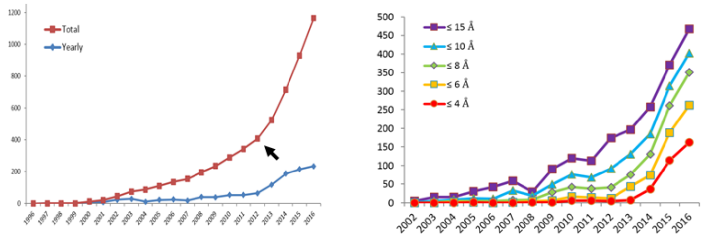
Contribution of cryo-EM to structural biology databases

- largely thanks to high-sensitivity cameras: direct electron detectors
- advanced image processing including movie processing and 2D & 3D classifications
- is part of a "revolution" in cryo-EM and structural biology



Orlov *et al.*, *Biology of the Cell*, 2016.

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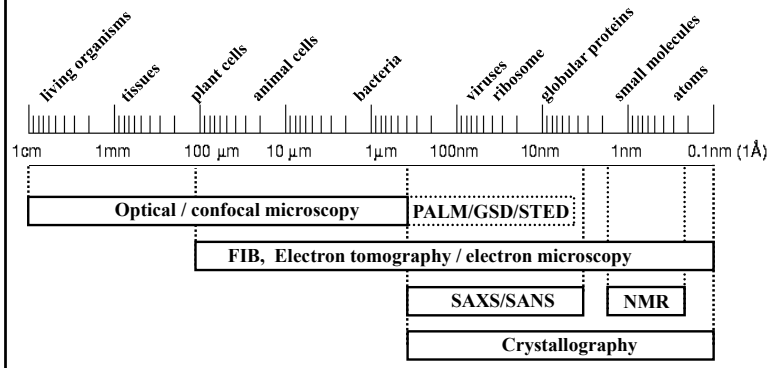

→ *challenging objects require multi-scale multi-resolution integration and R & D*

Ménétret *et al.*, RNA structure and folding (book), de Gruyter, 2013.

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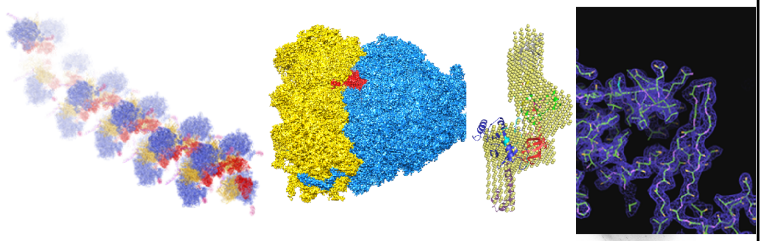
Multi-resolution integration of ribosome complexes



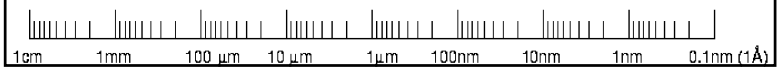
Molecular assemblies ← Single particles → Crystal structure

Myasnikov *et al.*, *Ultramicroscopy* 2013
Myasnikov *et al.*, *Nat. Comm. 2014.*

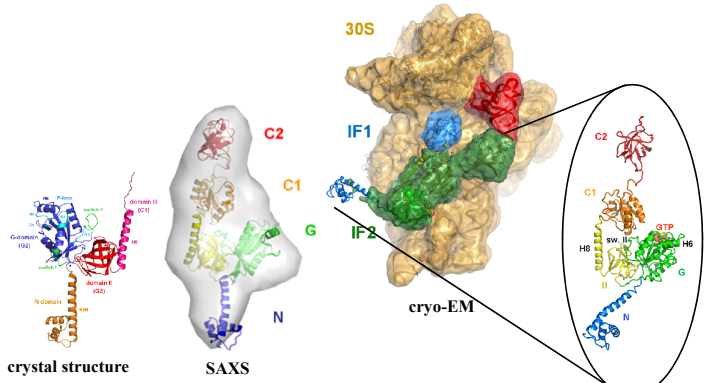
Khatter *et al.*, *Nature* 2015.
Myasnikov *et al.*, *Nat. Comm.* 2016

Simonetti *et al.*, *Acta Cryst. 2013*
Simonetti *et al.*, *PNAS 2013.*

fluorescence microscopy | *cryo electron tomography* | *cryo-EM* | *SAXS* | *crystallography*



Involvement of IF2 N domain in ribosomal subunit joining revealed from architecture and function of the full-length initiation factor



Simonetti *et al.*, *Acta Cryst D, 2013.*

Simonetti *et al.*, *PNAS, 2013.*
Eiler *et al.*, *PNAS, 2013.*

crystallography, SAXS, cryo-EM, kinetics and single molecule fluorescence

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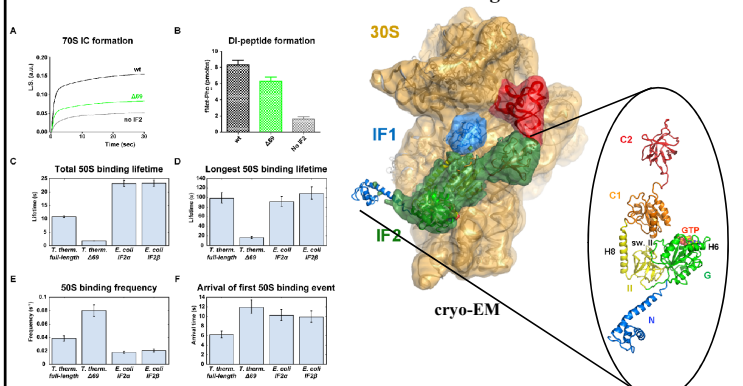

Simonetti *et al.*, *Acta Cryst D, 2013.*

Simonetti *et al.*, *PNAS, 2013.*
Eiler *et al.*, *PNAS, 2013.*

crystallography, SAXS, cryo-EM, kinetics and single molecule fluorescence

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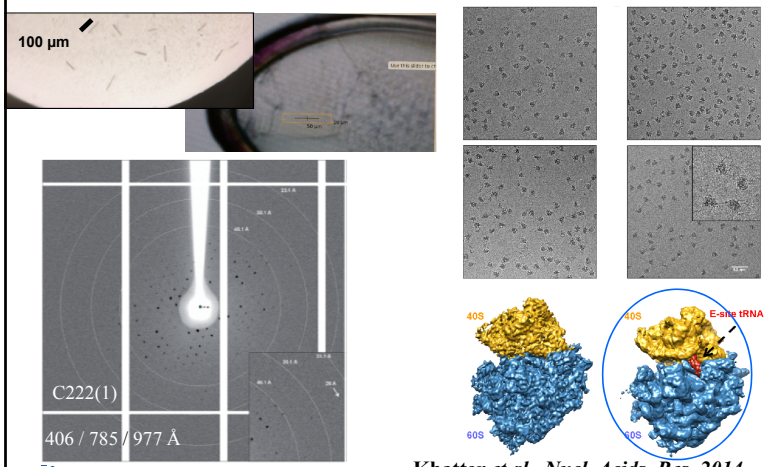
Crystallography of (large) macromolecular complexes:

use cryo-EM for sample optimization: first human 80S ribosome crystals



SLS

Khatter *et al.*, *Nucl. Acids. Res. 2014.*

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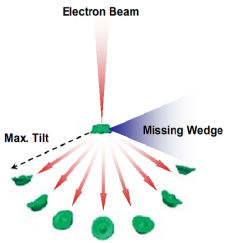
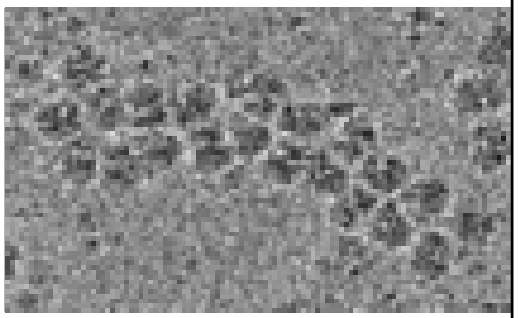
Towards higher complexity: molecular assemblies





→ tomogram
(i.e. a 3D reconstruction from tilt series)
see pioneering work by W. Baumeister

Cryo electron tomography (cryo-ET) of

- purified complexes
- cell sections

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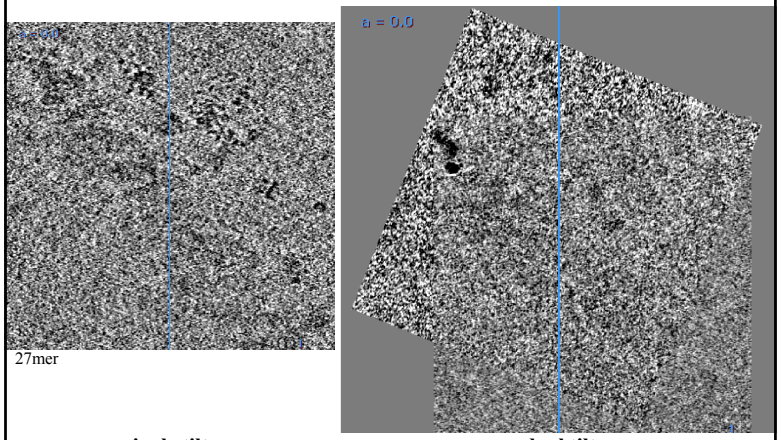
3D polysome reconstruction from single- / dual-tilt cryo electron tomography

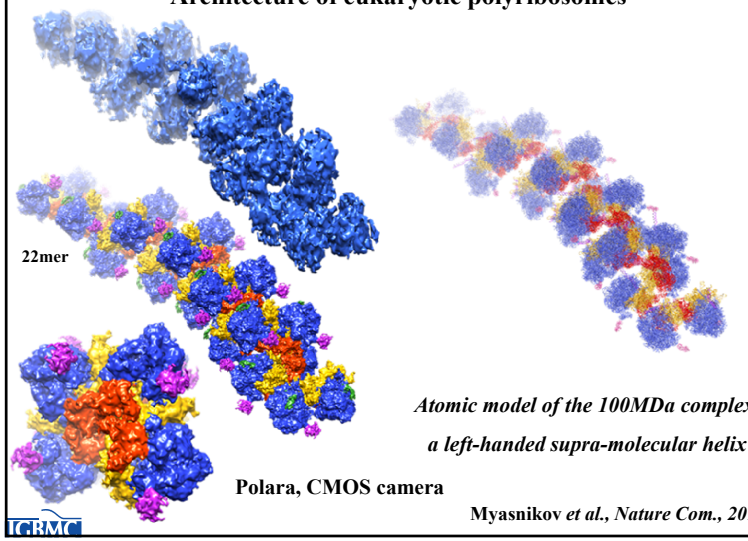


27mer

single tilt *Polara, 4k CCD, 150kV* dual tilt

[like a κ-goniometer setup] *inspect3D, IMOD* Myasnikov *et al.*, *Ultramicroscopy* 2013.

Architecture of eukaryotic polyribosomes



22mer

Atomic model of the 100MDa complex,

a left-handed supra-molecular helix

Polara, CMOS camera

Myasnikov *et al.*, *Nature Com.*, *2014.*

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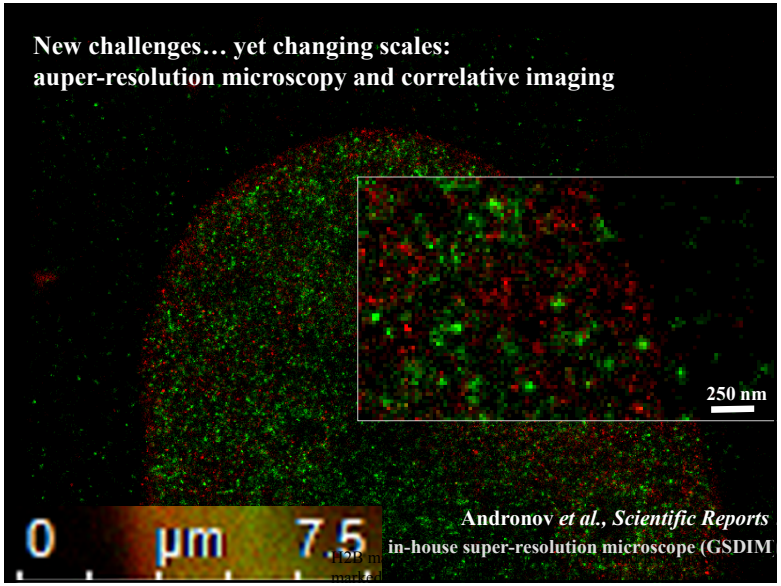
New challenges… yet changing scales: auper-resolution microscopy and correlative imaging



Andronov *et al.*, *Scientific Reports*

in-house super-resolution microscope (GSDIM

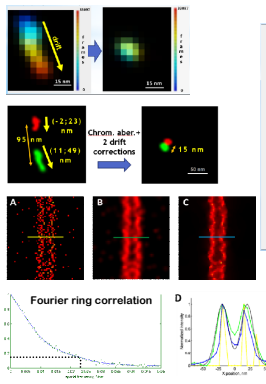
Super-resolution imaging of histones within chromatin complexes:

SharpViSu, a pipeline for processing of super-resolution data

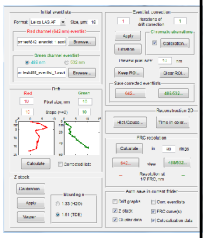
SharpViSu:

A pipeline for:

- Drift correction
- Chromatic aberration correction
- Voronoi-weighted image representation
- Resolution estimation (FRC)



User interface:



Andronov *et al.*, *Bioinformatics, 2016.*

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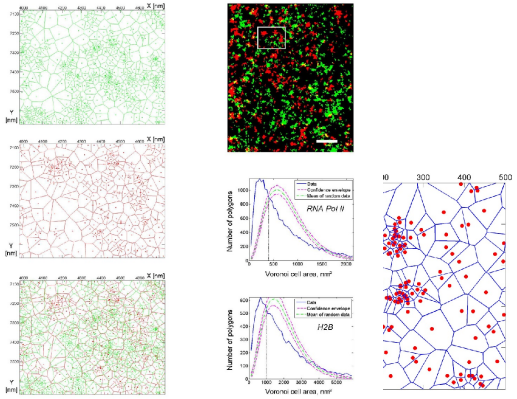
Super-resolution imaging of histones within chromatin complexes:

SharpViSu, a pipeline for processing of super-resolution data

ClusterViSu:

clustering analysis

- **Voronoi-diagram based**
- **clustering analysis**
- **statistical quantification**



→ RNA polymerase and H2B are independenly localized

Andronov *et al.*, *Sci. Rep.*, *2016.*

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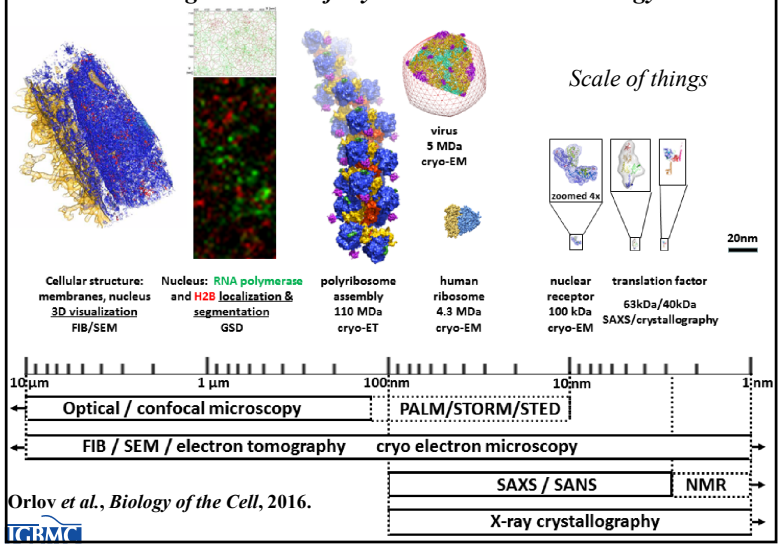
Integrative role of cryo-EM in structural biology



Orlov *et al.*, *Biology of the Cell*, 2016.

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V. Considerations important to improve cryo-EM reconstructions

1. **Address heterogeneity**
2. **Perform focused refinement**
3. **Take into account preferential views**
4. **Make sure symmetry is correctly determined**

Some tricks to improve the cryo-EM reconstruction and facilitate map interpretation:

1. Address heterogeneity:

1) reference-based, i.e. cross correlation with forward-projections of known structures

2) multivariate statistical analysis (MSA): 3D classification / bootstrapping, 3D resampling

3) maximum likelihood based class assignment

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Determining structures of multiple conformational states in a single sample

distinguish: orientational classification and conformational classification

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Determining structures of multiple conformational states in a single sample

distinguish: orientational classification and conformational classification

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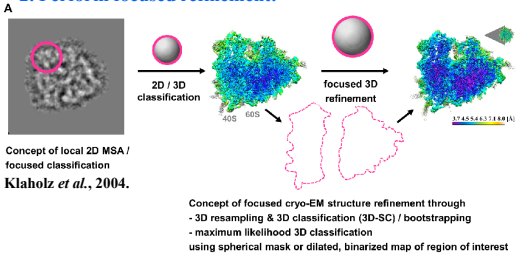
Some tricks to improve the cryo-EM reconstruction and facilitate map interpretation:

2. Perform focused refinement:



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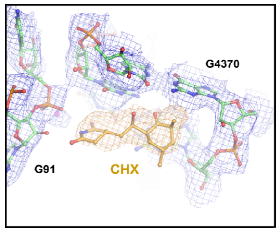
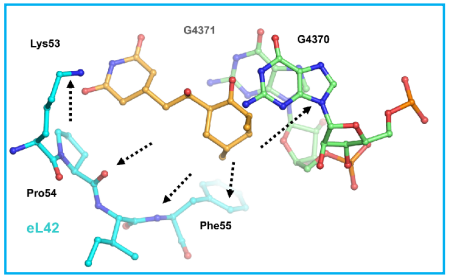
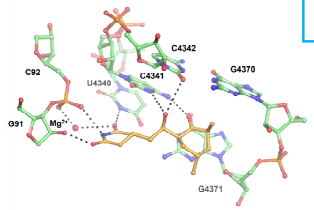
von Loeffelholz *et al.*, *Curr. Opin. Struct. Biol. 2017, under revision.*

Cryo-EM: a promising tool for structure-based drug design







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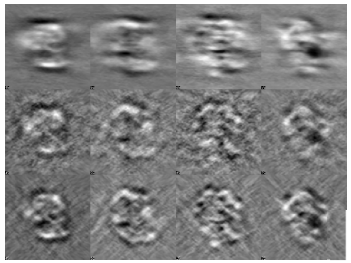
Myasnikov *et al.*, *Nat. Comm.*, 2016.

Some tricks to improve the cryo-EM reconstruction and facilitate map interpretation:

3. Take into account preferential views:

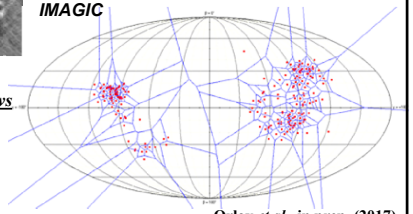


BKPR non-weighted

strong distortions/artifacts in 3D reconstruction

BKPR weighted

IMAGIC



sections of 3D's calculated from 250 class averages with strong preferential views

Orlov *et al.*, in prep. (2017).

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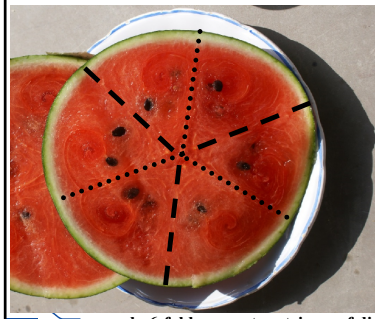
4. Make sure symmetry is correctly determined

VI. Point group symmetries
(internal symmetry of isolated objects)

Some tricks to improve the cryo-EM reconstruction and facilitate map interpretation:

symmetries in nature….:



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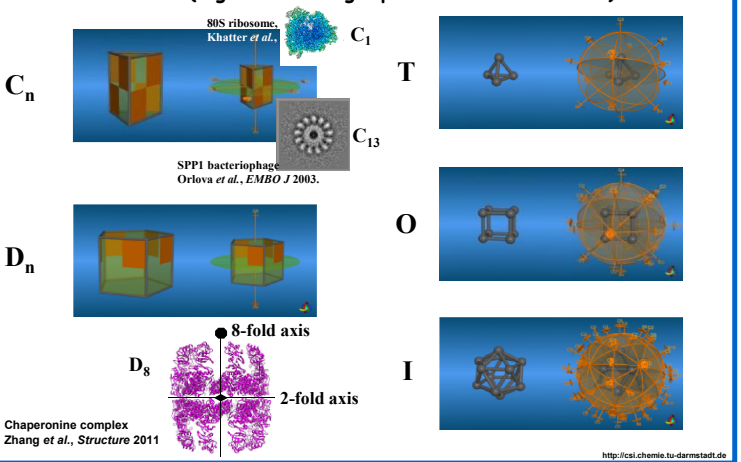
pseudo 6-fold symmetry: trimer of dimers!

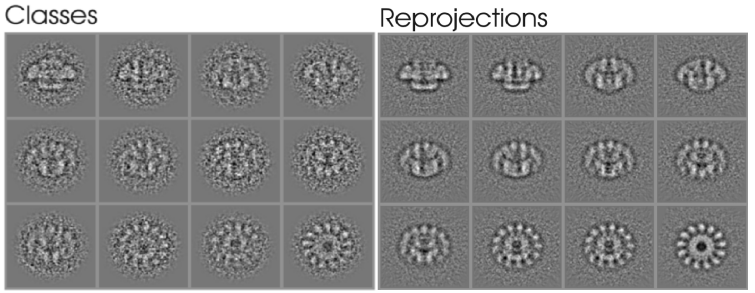
watermelon/flower photos: B. Klaholz, 2011/2017

Point group symmetries of biological objects (e.g. used in single particle reconstructions)



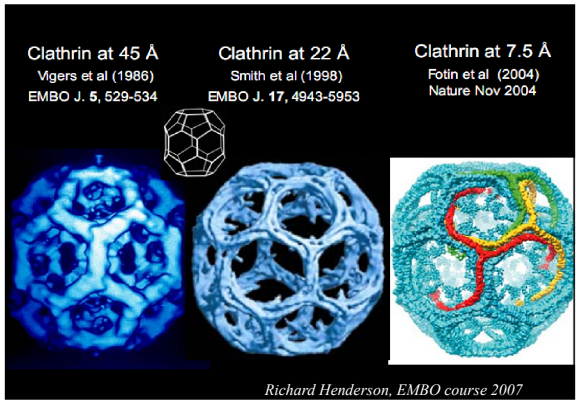
Practical Example – SPP1 Bacteriophage Portal Protein C_{13} point group symmetry

reverse of back-projection:
forward-projection along defined directions (angles)

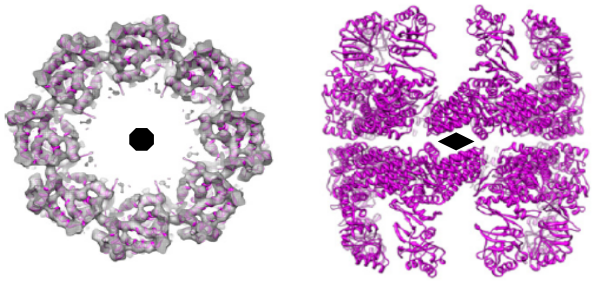


Orlova et al., *EMBO J.*, 6, 1255-62, 2003

Practical Example – Clathrin complex D_6 point group symmetry



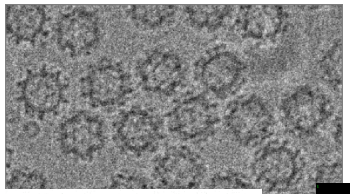
Practical Example – Chaperonin complex D_8 point group symmetry

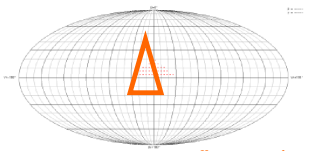


2-fold symmetry axis orthogonal to the main symmetry axis: dihedral point group symmetry

Zhang et al., *Structure*, 19, 633–639, 2011

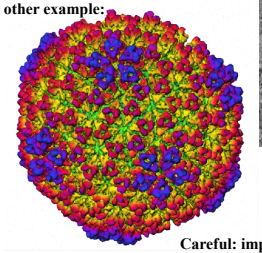
Practical Example – Icosahedral Reconstruction

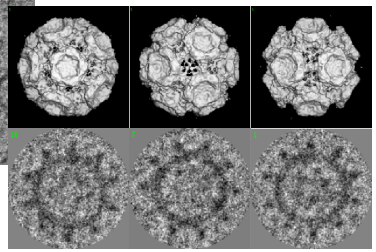




small asymmetric unit

other example:





Careful: imposition of inexplicit symmetry can happen…

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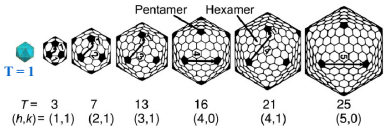
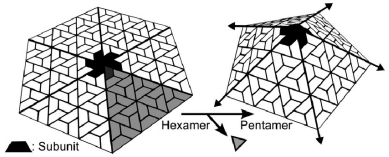
Icosahedral Triangulation Number

The T-number is calculated by
(1) applying a grid to the surface of the virus with coordinates *h* and *k*,
(2) counting the number of steps between successive pentagons on the virus surface,
(3) applying the formula:

$$T = h^2 + h \cdot k + k^2$$

gives the number of structural units per face,
= number of subunits in the asymmetric unit

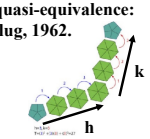




always 12 pentamers,
but variable hexamer insertion

Multi-symmetric polyhedra:
Goldberg, Tôhoku Math., 1937.

Concept of quasi-equivalence:
Caspar & Klug, 1962.



http://viralzone.expasy.org/all_by_protein/1057.htm

The two integers h and k describe the number of hexamers (h+k-1)
one has to "walk over" to get from one pentamer to an adjacent pentamer.

see also Johnson & Speir, *JMB* 1997. Mannige & Brooks, PLoS One, 2010

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VII. Pushing resolution by structure sorting, detectors & movie processing

→ *particle sorting, advanced image processing*

How to sort out heterogeneity?

3 different approaches:

1) reference-based, i.e. cross correlation with forward-projections of known structures

2) multivariate statistical analysis (MSA): 2D classification or 3D classification

3) maximum likelihood based class assignment

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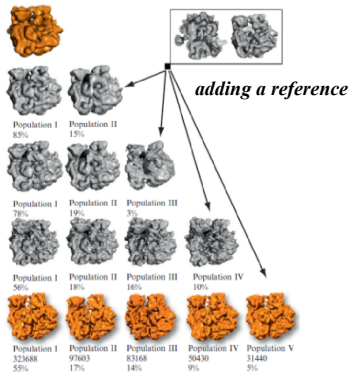
Determining structures of multiple conformational states in a single sample

1) reference-based, i.e. cross correlation with forward-projections of known structures

example:



Loerke *et al.*, Meth. Enzymol. 2010

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Determining structures of multiple conformational states in a single sample

2) multivariate statistical analysis (MSA): 2D classification, 3D classification

distinguish: orientational classification and conformational classification

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Determining structures of multiple conformational states in a single sample

2) multivariate statistical analysis (MSA): 2D classification, 3D classification

distinguish: orientational classification and conformational classification

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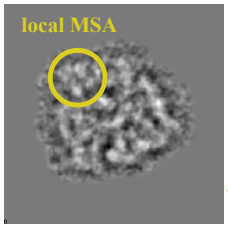
Determining structures of multiple conformational states in a single sample

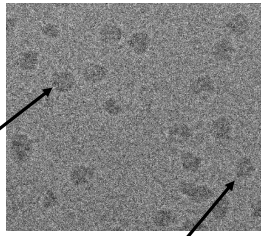
2) multivariate statistical analysis (MSA): 2D classification, 3D classification

local 2D MSA (focused classification)



30S 50S

70S particle without factors?



50S view of the 70S or 50S particle?

Perform 2 classifications:
(i) global MSA for classification according to particle orientations (i.e. classical class averages)
(ii) local MSA with a smaller mask for classification according to particle variability.

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Klaholz et al., *Nature* 2004; see Suppl. Mat.

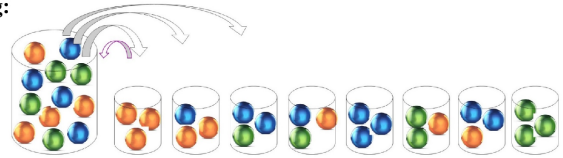
Determining structures of multiple conformational states in a single sample

2) multivariate statistical analysis (MSA): 2D classification, 3D classification

Concept of 3D re-sampling and classification (3D-SC)

statistical resampling:

jack-knifing / bootstrapping ("resampling")



- jack-knifing: selection of small subsets
- bootstrapping: random selection of small subsets, part of which can be re-selected (resampling with replacement;
repeated random resampling is a Monte Carlo approach)

see: Quenouille, 1949; Efron, 1979; Simon, 1969 / 1997; Good, 2005.

Klaholz, *Open J. Statistics,* 2015.

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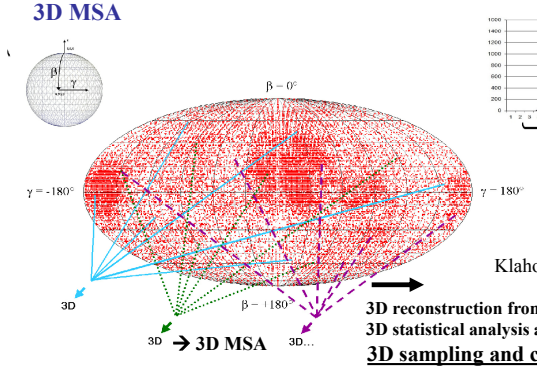
Determining structures of multiple conformational states in a single sample

2) multivariate statistical analysis (MSA): 2D classification, 3D classification

3D MSA



Klaholz, *Open J. Statistics,* 2015.

3D reconstruction from many subsets (resampling)
3D statistical analysis and 3D classification:
3D sampling and classification (3D-SC)

→ does both re-sampling and 3D classification;
see also work by P. Penczek (bootstrapping (re-sampling), used primarily to find region of variance)

Simonetti *et al.*, *Nature,* 2008.

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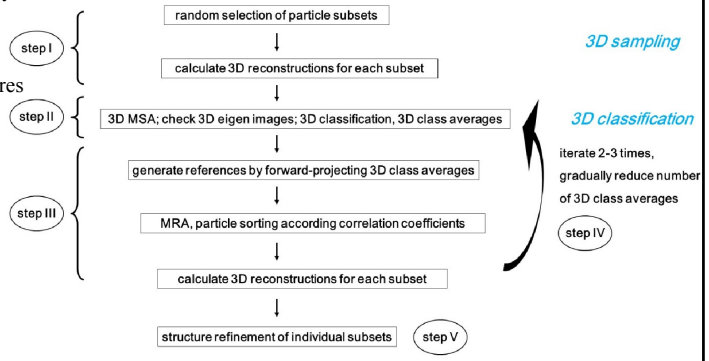
Determining structures of multiple conformational states in a single sample

2) multivariate statistical analysis (MSA): 2D classification, 3D classification

10-50 particles per set

Concept of 3D sampling and classification (3D-SC)

e.g. 10 000 structures



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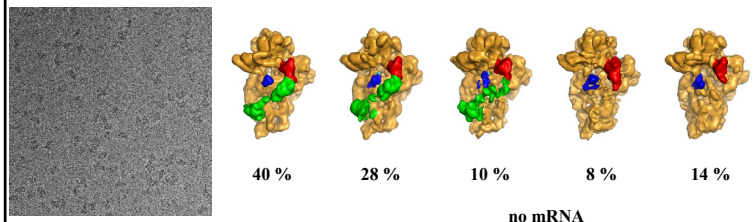
Determining structures of multiple conformational states in a single sample

2) multivariate statistical analysis (MSA): 2D classification, 3D classification

3D MSA

3D reconstruction from many subsets (resampling),
3D statistical analysis and 3D classification:
3D sampling and classification (3D-SC)

Multiple states in the 30S initiation complex



40 % **28 %** **10 %** **8 %** **14 %**

no mRNA

no IF2

200kV FEG data;
total 80 000 particles
resolution of 3D's: 9Å

2D → 3D → 4D

Simonetti *et al.*, *Nature,* 2008.

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Determining structures of multiple conformational states in a single sample

3) maximum likelihood based class assignment

→ assign particles to different 3D classes based on maximum likelihood

(probability distribution; uses randomly selected references + ML-weighting)

Practically:

random subsets are optimized and a low-resolution average structure is used as reference,

i.e. resampling is used in combination with likelihood optimization

e.g. Scheres *et al.*, *JMB* 2005; *Meth. Enzymol.* 2010;

Lyumkis *et al.*, *JSB* 2013

(ML concept in cryo-EM: Sigworth, *JSB* 1998;

in X-ray crystallography: G. Bricogne, *Acta Cryst A*, 1991**)**

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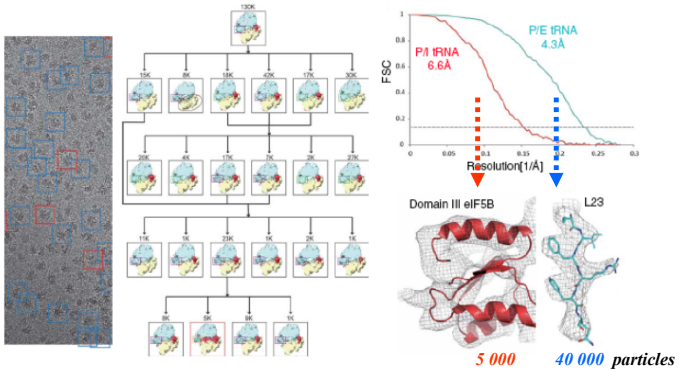
Examples of ML-based 3D classification



5 000 *40 000* ***particles***

Strong heterogeneity of a reconstituted eukaryotic translation initiation (eIF5B) complex: sorting → 5143 particles, representing 3% of the population in the sample, 6.6 Å reconstruction. Fernández *et al.*, *Science* 2013; V. Ramakrishnan & S. Scheres.

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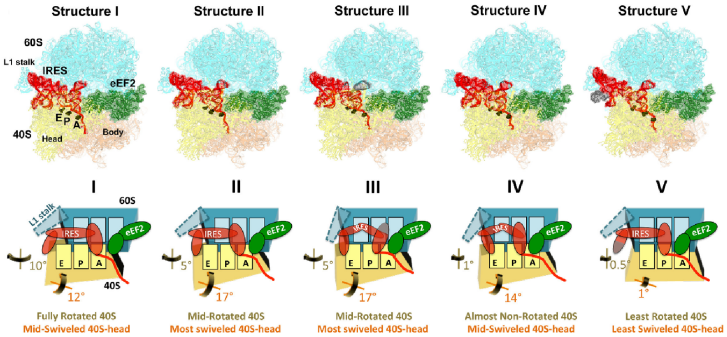
e.g. ML-based focused classification of 80S / TSV IRES complex with eEF2/GDP/sordarin



Abeyrathne *et al.*, *eLife* 2016

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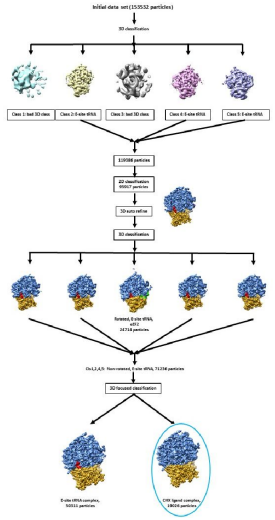
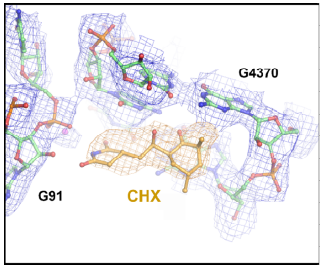


e.g. ML-based focused classification sorting scheme for human 80S/antibiotic complex



Myasnikov *et al.*, *Nat. Comm. 2016.*

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Conformational changes of cats?

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Determining structures of multiple conformational states in a single sample

Possibilities to address heterogeneity:

1) reference-based, i.e. cross correlation with forward-projections of known structures

2) multivariate statistical analysis (MSA): 2D classification or 3D classification

3) maximum likelihood based class assignment

Conclusions:

- **do not assume a single state in your sample / multi-subunit complex**
- **consider lower symmetry (viruses etc.) to see differences between subunits**
- **if to use local / focused classification/refinement: use slightly larger region**
- **also useful in sub-tomogram averaging and 3D classifications**
- **consider: any sub-ensembles will not be entirely homogeneous due to the statistical nature of the procedures (resampling and/or ML)**

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Pushing resolution in cryo-EM:

High-resolution electron microscopes, direct electron detectors & movie processing

- **back-thinning of Si-layer**
- **counting mode**
- **super-resolution mode**
- **dose fractionation**
- **motion correction (movie processing):**
  - **MotionCorr** } *pairwise*
  - **Unblur** } *to the average* } *whole frame*
  - **Optical Flow** } *to the average* } *whole frame*
  - **particle polishing**
  - **MotionCorr2**
- **beam-induced specimen drift**
- **exposure filtering (dose optimization / frame selection)**

Brilot *et al.*, *JSB*, 2012.
Campbell *et al.*, *Structure*, 2012.
Li *et al.*, *Nat Methods*, 2013.
Ruskin *et al.*, *JSB*, 2013.
Liao *et al.*, *Nature*, 2013.
Fernández *et al.*, Science 2013.
McMullan *et al.*, *Ultramicr.*, 2014.
Allegretti *et al.*, *eLife*, 2014.
Wong *et al.*, *eLife*, 2014.
Bartesaghi *et al.*, *PNAS*, 2015.
Scheres, *eLife*, 2014.
Fischer *et al.*, *Nature*, 2015.
Khatter *et al.*, *Nature*, 2015.
Greber *et al.*, Science, 2015.
Bartesaghi *et al.*, *Science*, 2015.
Grant *et al.*, *eLife*, 2015.
…

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see also publication series Methods in Enzymology, 2016