What can we apply MSA / classifications to?

- 2D classification (reference-free alignment: only centered data, not rotationally aligned)
- alignment by classification (alignment against class averages or a typical eigenimage)
- analysis of symmetry (through symmetry in the eigenimages)
- local MSA (focus on an area with high structural variability)
- re-classification of class averages belonging to an object view
- size-classification (e.g. White et al., *J. Mol. Biol.* 336 (2004) 453-460).
- 3D classification of structures (separation of mixed particle populations):

  particles:3D-SC, sub-tomograms
- classification of powerspectra (sorting of defocus classes)

Important to do before MSA:

- normalisation
- filtering
- centered data (aligned if for structure refinement)
- define MSA area: MSA mask

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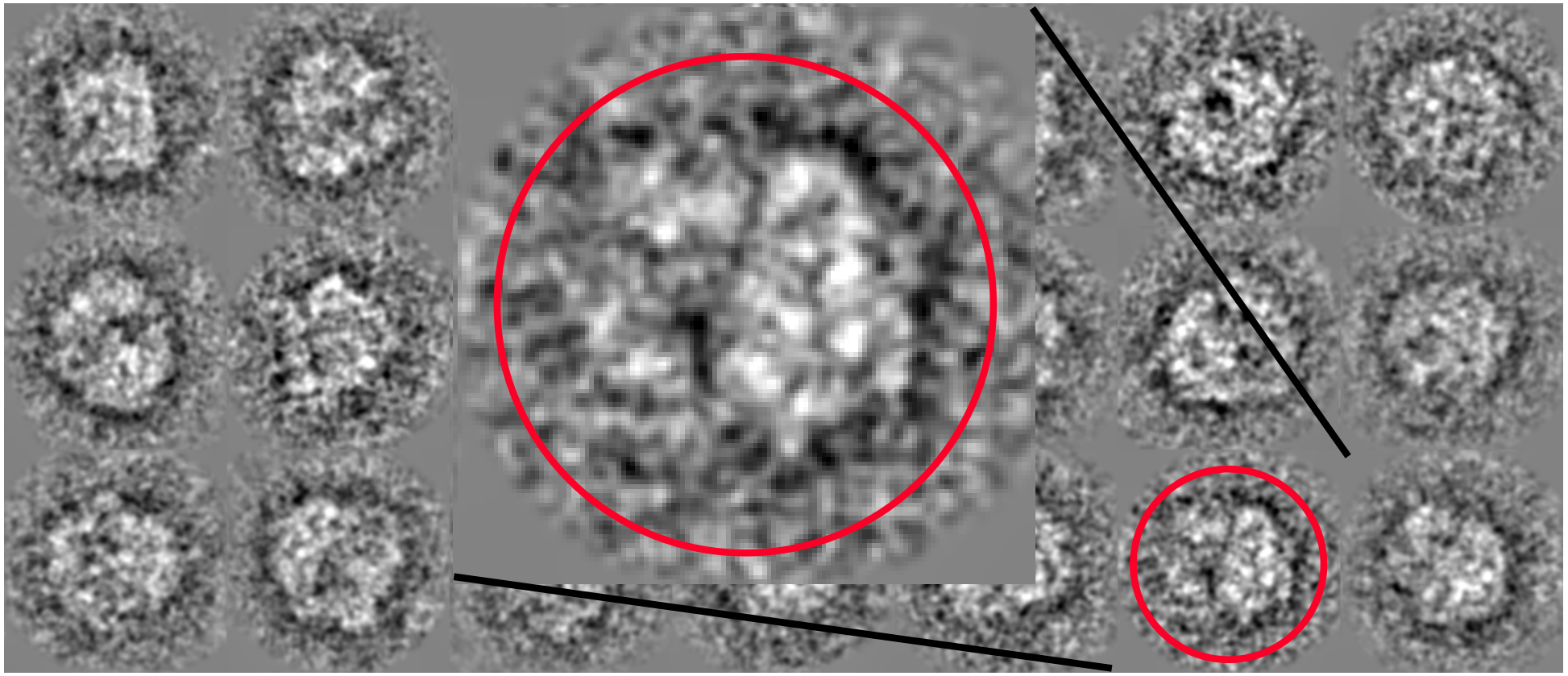
Example of initial class averages:



70S ribosome data set, 500 particles, 50 classes, ~10 members per class

band-pass filter: 300Å - 12Å; *Imagic*

Now find your Euler angles and start the structure…!

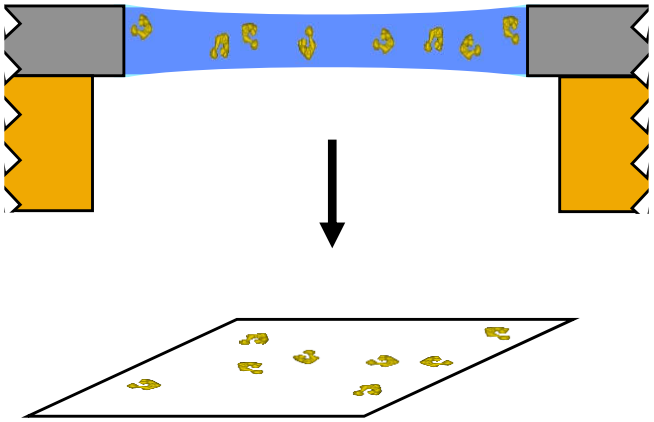
But will it work?

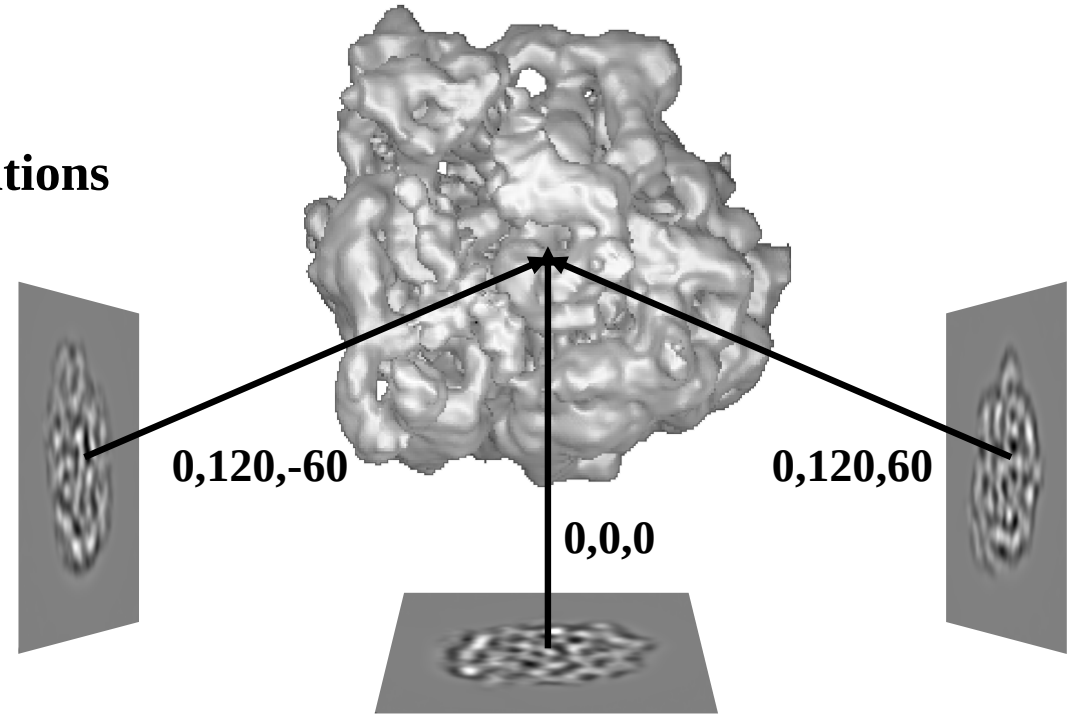
Structure determination by 3D reconstruction

Assumptions?

3D reconstruction of single particles:

unique particle type in random orientations





And what if we have different structures in the sample…?

Conformational changes of cats?

correlated movements?

Different conformations? → Cannot be averaged

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What means homogeneity?

- same composition

- same functional state

- same structural state, i.e. same conformational state

Determining structures of multiple conformational states in a single sample

Homogeneity of multi-component system / macromolecular complexes is tricky to get:

- multiple subunits / components (proteins, RNA, DNA,…)

- flexibility of the core structure

Additional, dynamic components:

- factors such as proteins, RNA, DNA binding transiently

- nucleotides (GTP +/- hydrolysable)

- ligands

Determining structures of multiple conformational states in a single sample

Sample heterogeneity:

- **typical problem of multi-component systems, composition & flexibility**
- **structure determination difficult / limits resolution**
- **detection of the problem: disordered 3D map; MSA**
- **advantage: analysis of dynamics**

Structure sorting has important implications for high-resolution structural studies and allows converting the problem of heterogeneity into an advantage by describing structure ensembles to provide insights into the dynamics of multi-component macromolecular assemblies.

see Klaholz, *Open J. Statistics*, 2015.

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Determining structures of multiple conformational states in a single sample

How to sort out heterogeneity?

→ particle sorting, advanced image processing

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

Determining structures of multiple conformational states in a single sample

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

"supervised classification":
uses projections calculated from a known 3D template (i.e. external reference)
for comparison with particle images,
assign group according to best cross-correlation coefficients, i.e. projection matching

if class membership and orientation parameters are estimated simultaneously:
***"unsupervised* classification" (K-means clustering, etc.)**

Practically:
use projections from several known 3D structures as references
(careful with the usage of crystal structures! use a strongly band-passed version),
run multi-reference alignment,
after a few cycles of refinement: add a reference (e.g. apo-form of a complex), iterate

e.g. Gao *et al.*, 2004; Connell *et al.*, 2008; etc.

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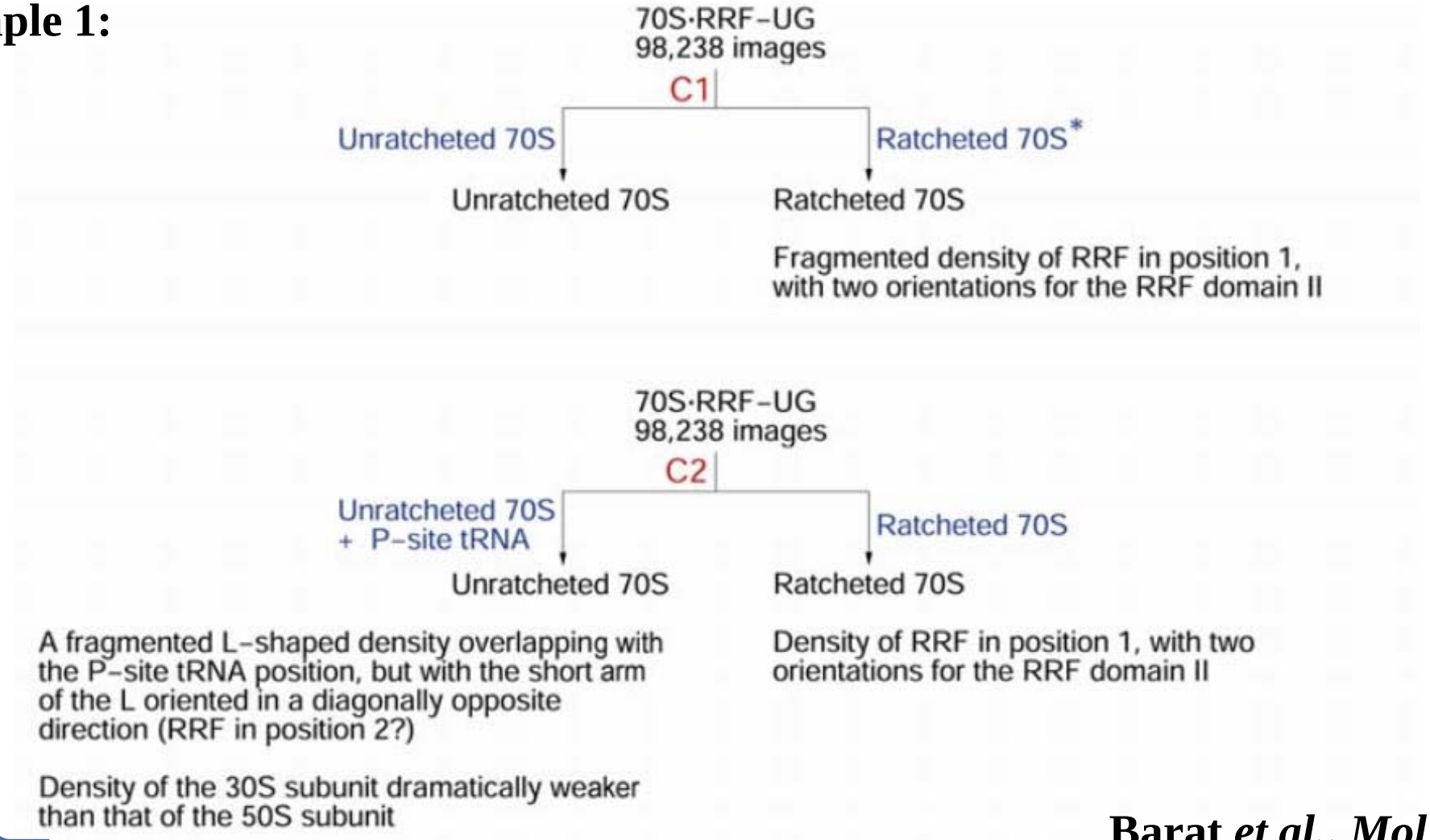
Determining structures of multiple conformational states in a single sample

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

example 1:



Barat *et al.*, *Mol. Cell* 2007.

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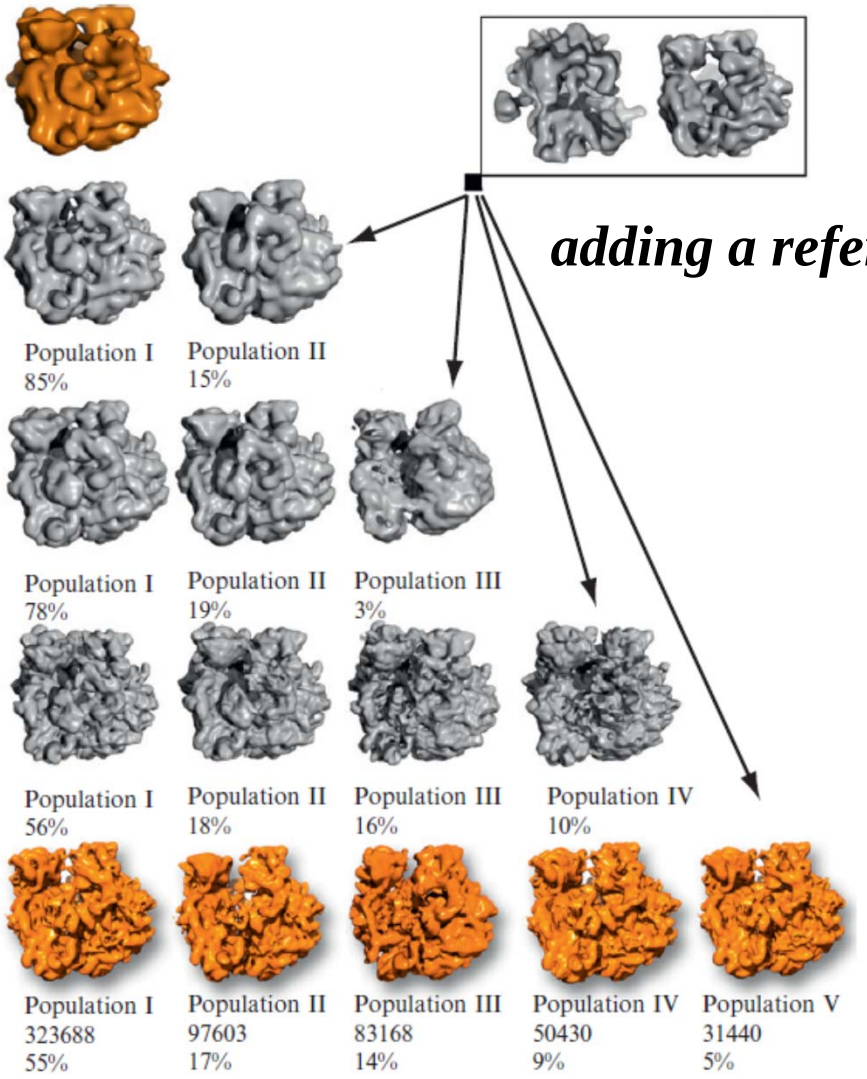
Determining structures of multiple conformational states in a single sample

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

example 2



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

Determining structures of multiple conformational states in a single sample

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

distinguish: <u>orientational</u> classification and <u>conformational</u> classification

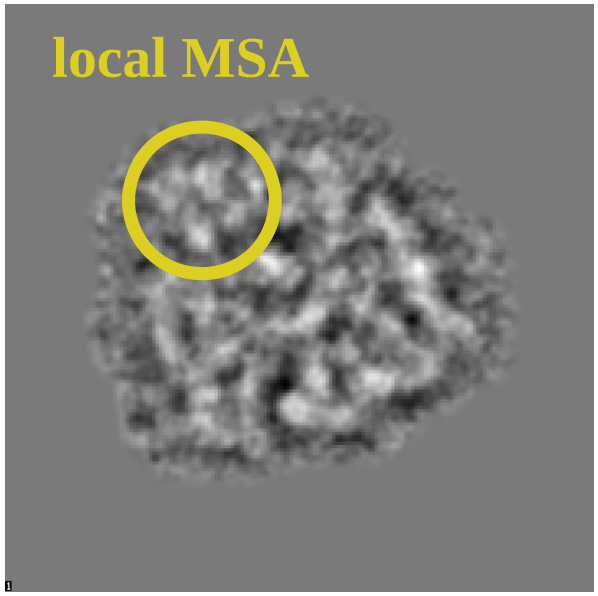
Determining structures of multiple conformational states in a single sample

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

distinguish: <u>orientational</u> classification and <u>conformational</u> classification

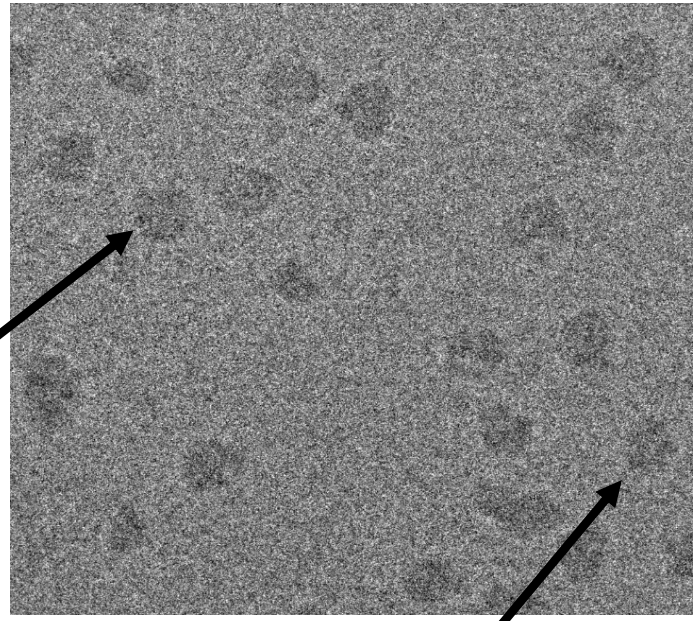
Determining structures of multiple conformational states in a single sample

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.

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

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Determining structures of multiple conformational states in a single sample

Local / focused classification

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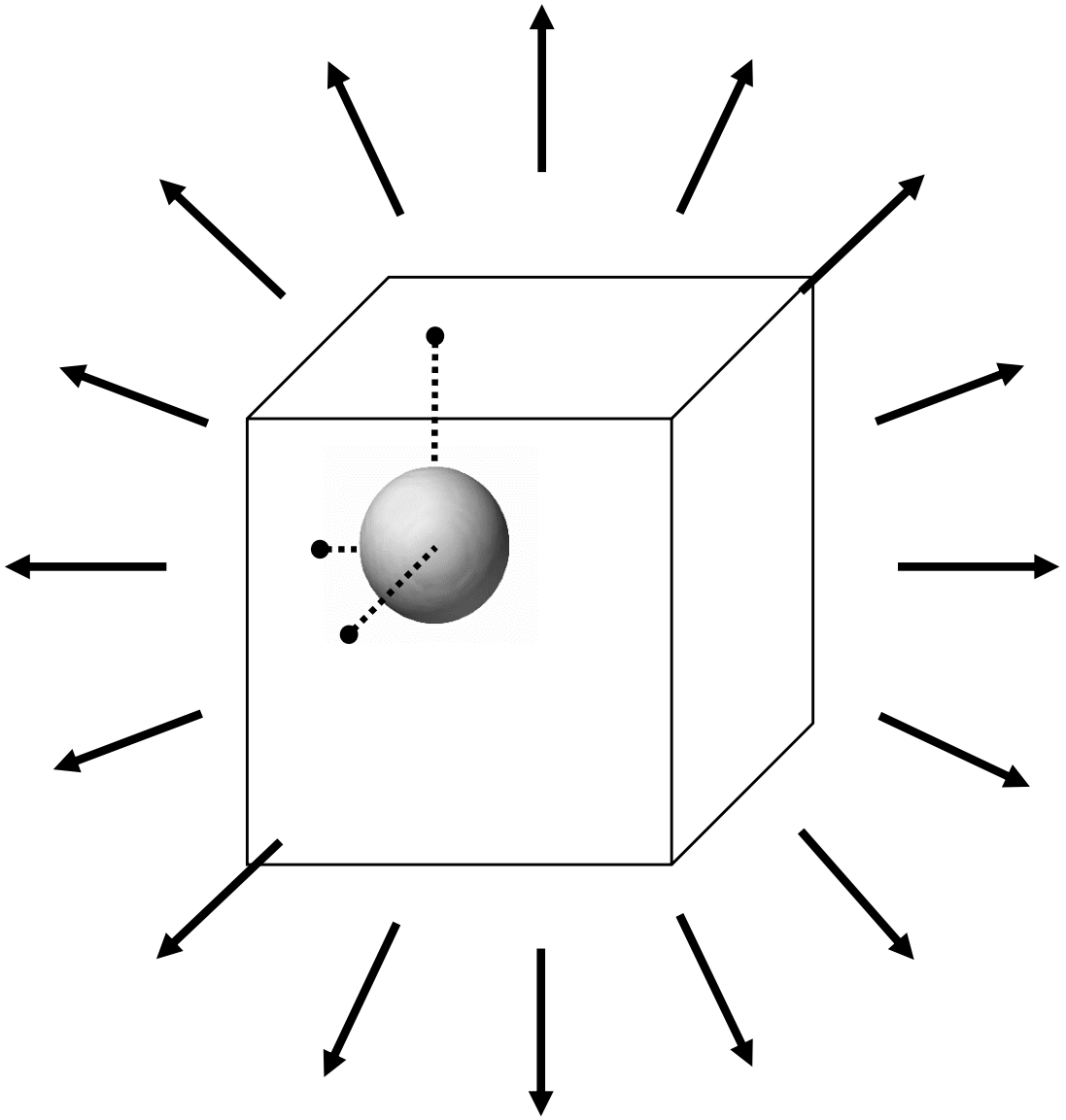
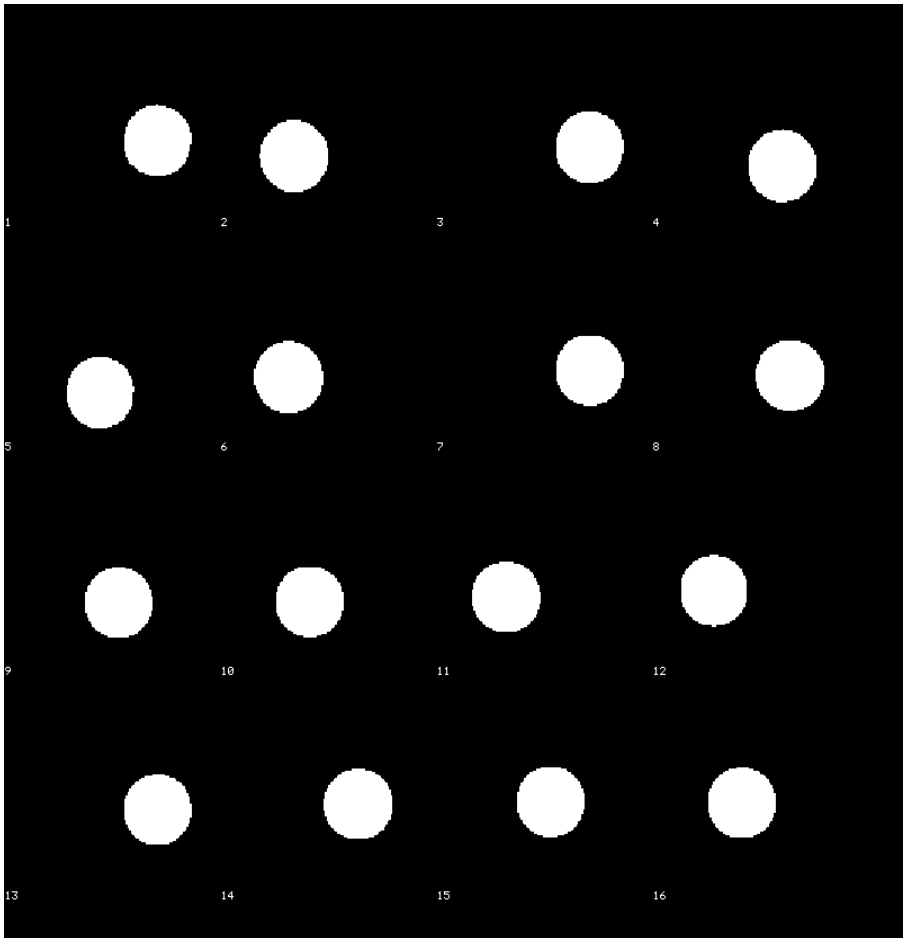
Determining structures of multiple conformational states in a single sample





Klaholz, *Open J. Stat.* (2015).

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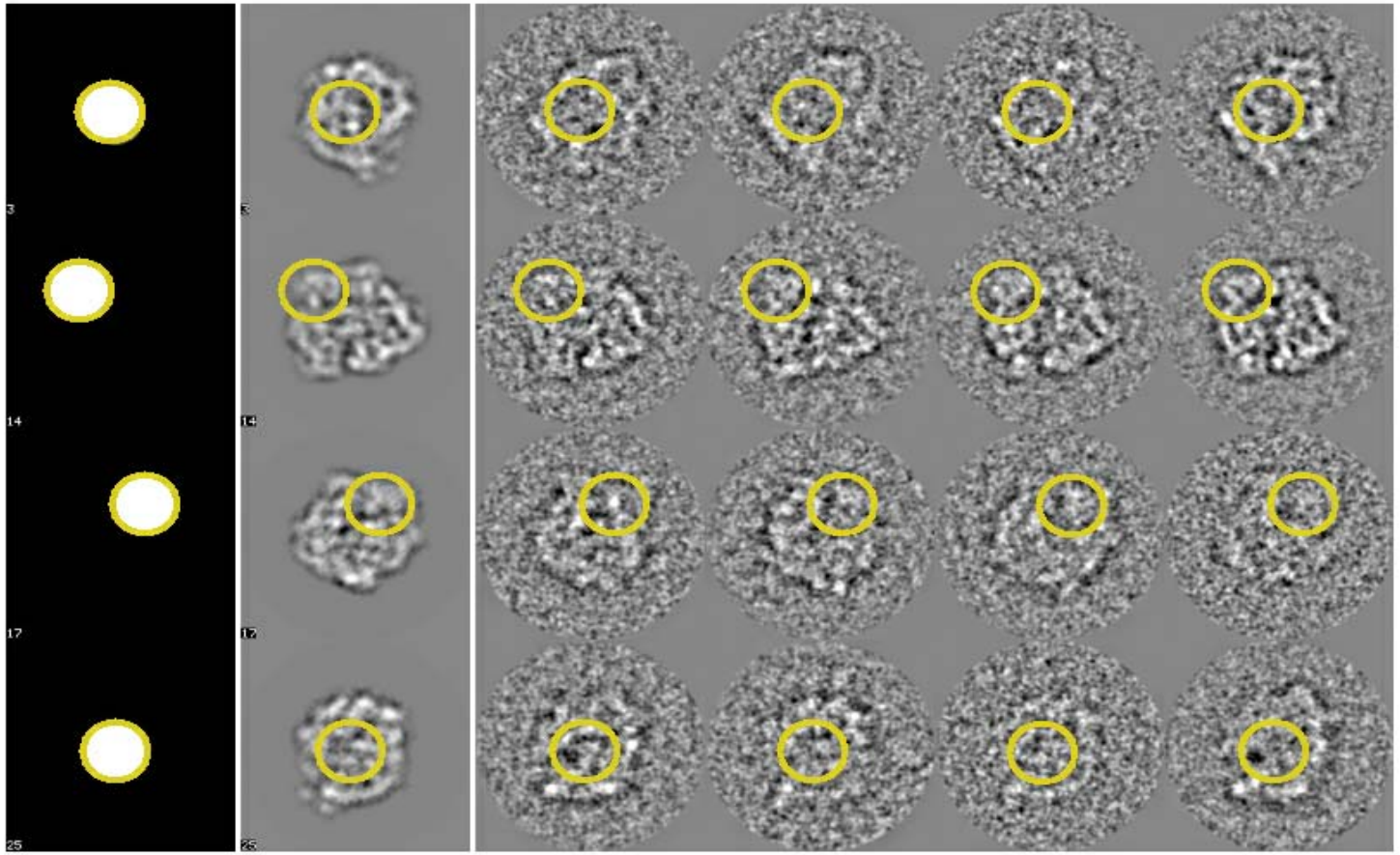
Determining structures of multiple conformational states in a single sample

local 2D MSA

series of MSA's on particle views (class averages or extracted particles):



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

→ allows re-classification after orientational classification

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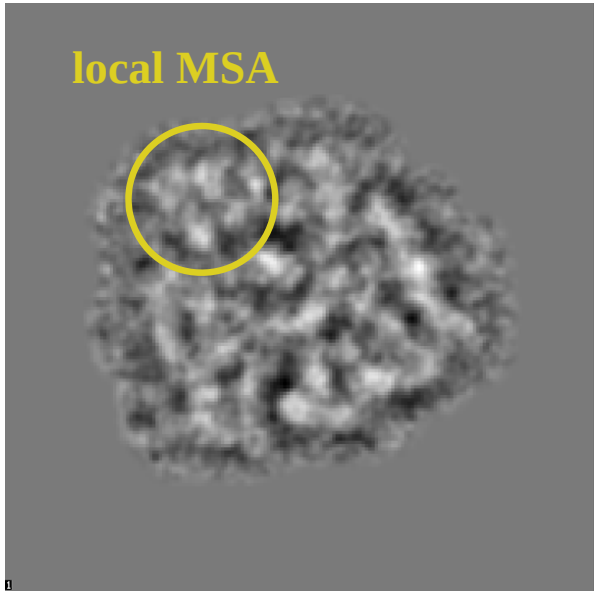
Determining structures of multiple conformational states in a single sample

local 2D MSA



30S 50S

"seeding" with different structures, from the sample (no external refs)

Image processing procedure

initial (merged) structure:
localise 3D area of disorder

↓

msa of classums-particles on restricted area

↙ ↘ cross-validation of group assignment

3D reconstruction 3D reconstruction

↘ ↙

reproject and append reprojections (reference 1 + 2)

↓ MRA

sort particles according to reference number

↙ ↘

MSA, AR, 3D MSA, AR, 3D

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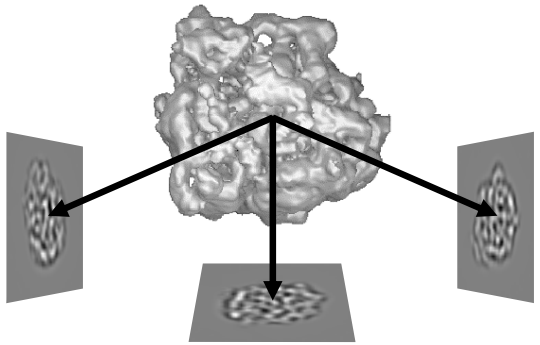
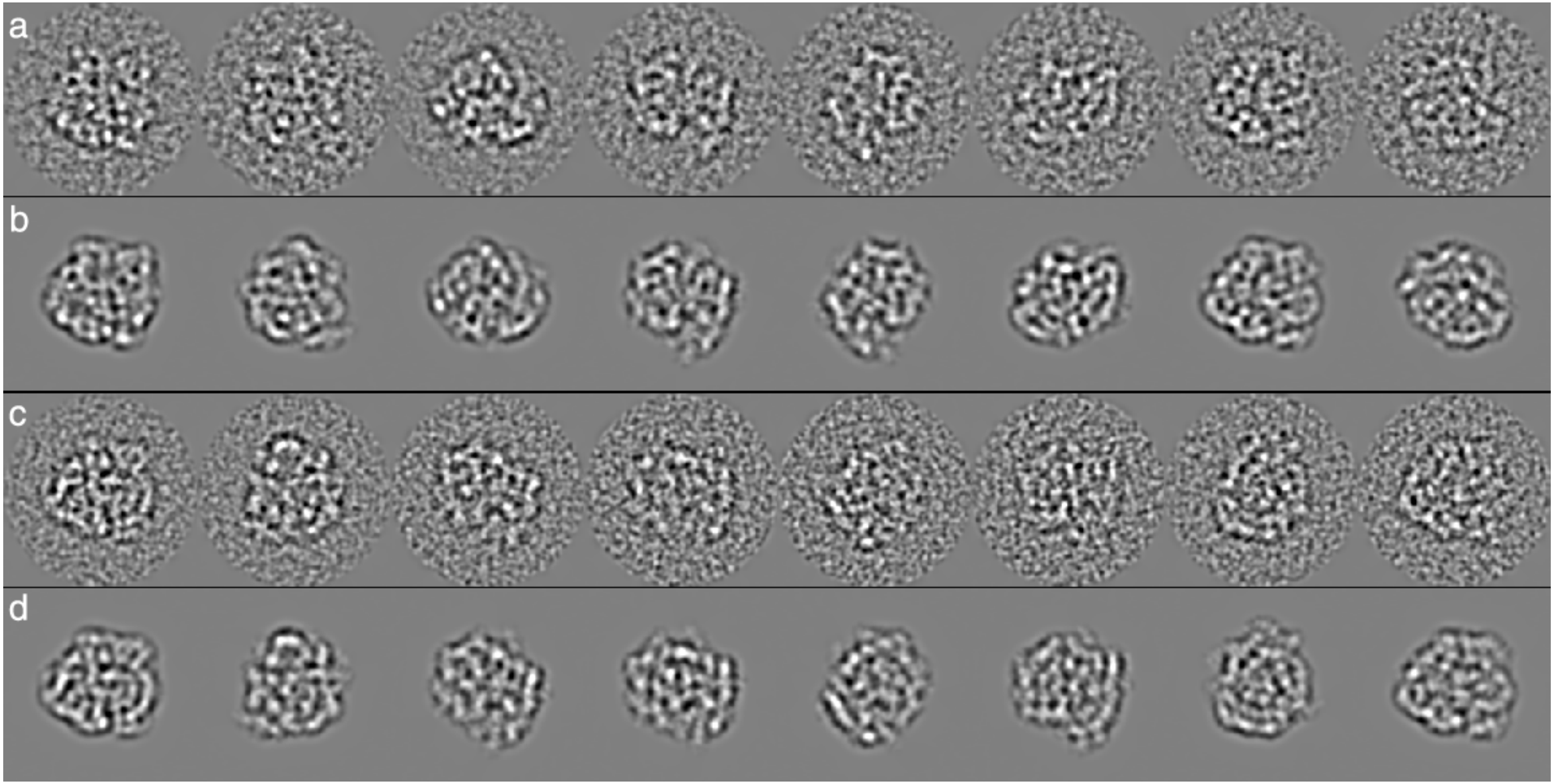
group assignment by iterative cross-validation with corresponding re-projections





set 1

set 2

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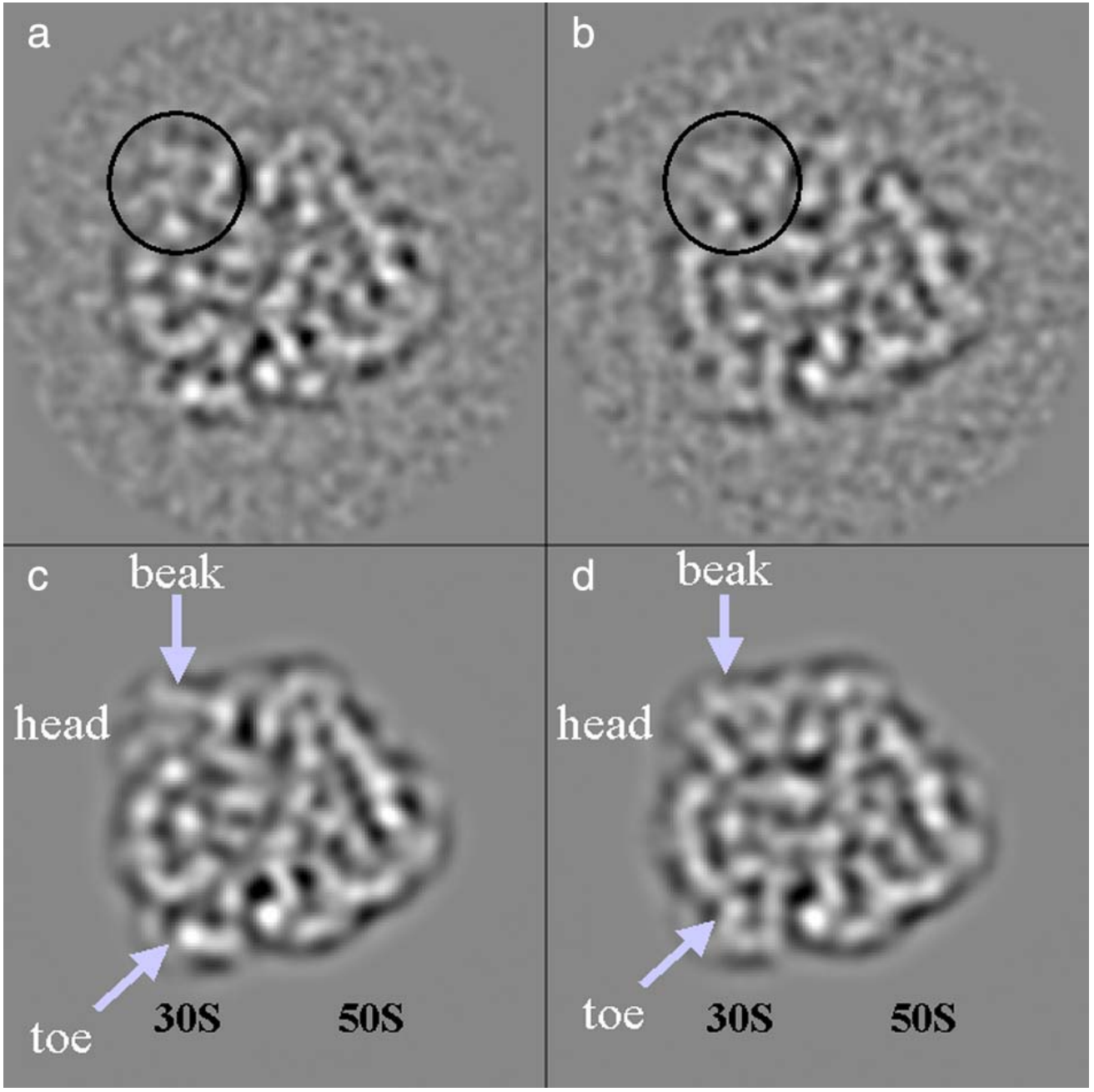
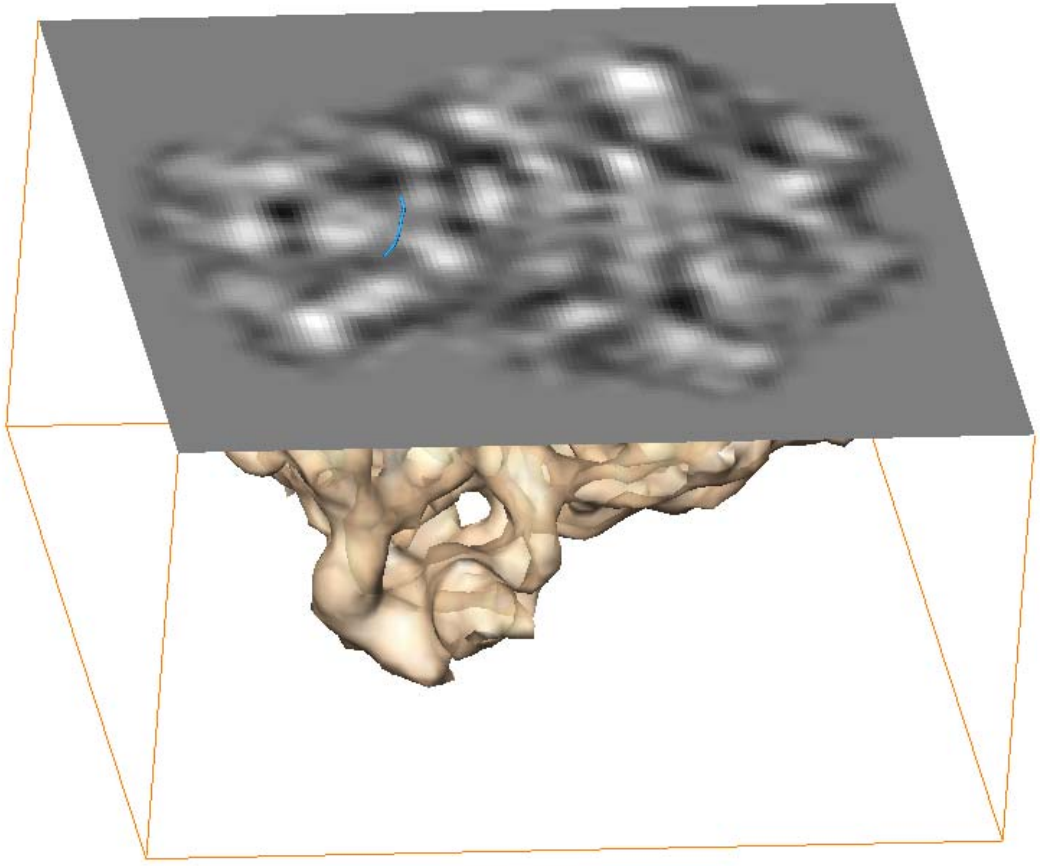
Procedure applied to distinct areas of the ribosome: conformational changes are correlated:

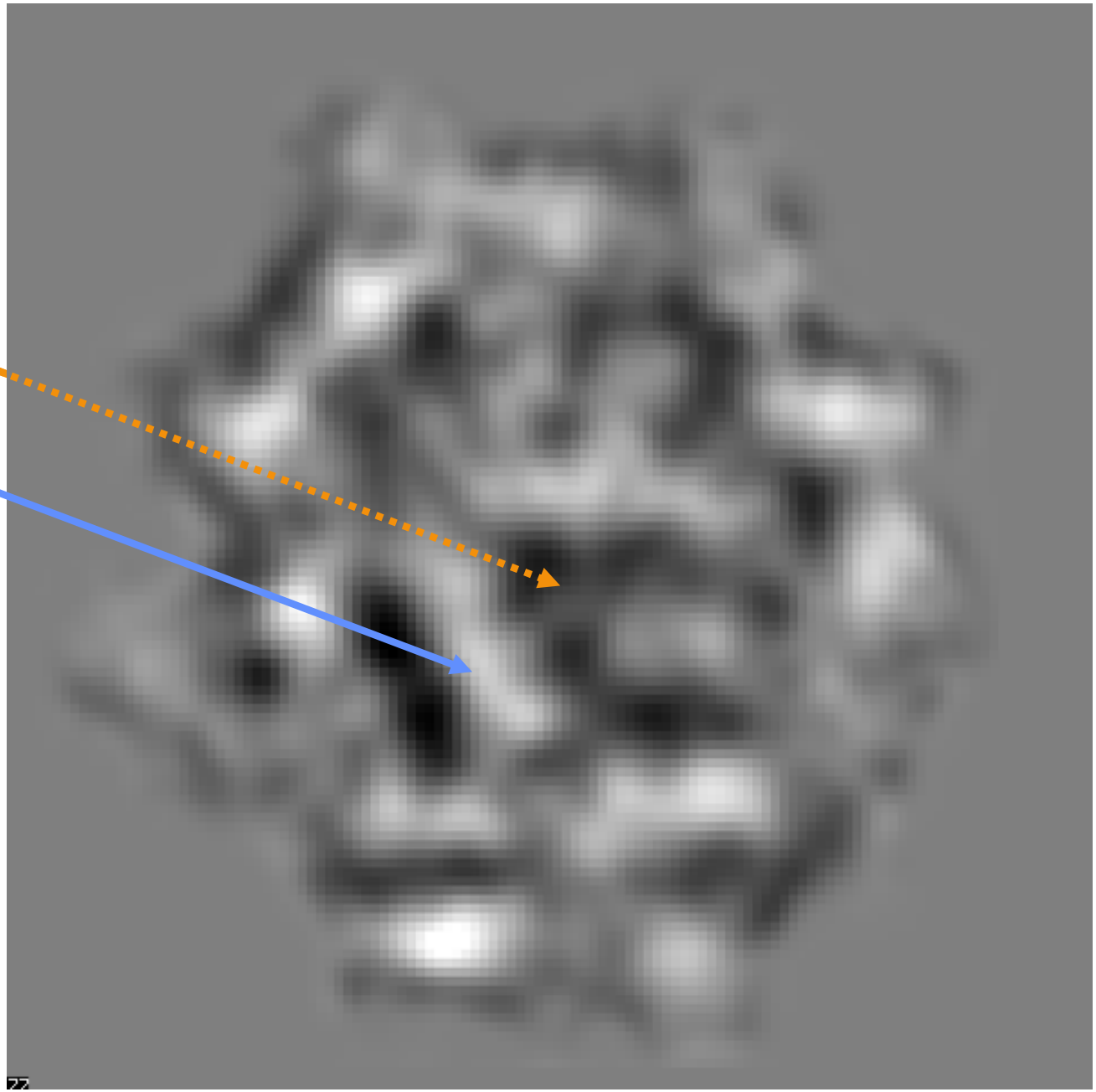


Sections through the 3D map:



P-site tRNA

E-site tRNA



Concept of structure sorting is true at 10-20 Å resolution and needs to be validated at that resolution,

but
also at 2 Å resolution
(e.g. multiple side-chain conformations seen in crystal structures)

tRNA translocation

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Intrinsic limitations of 2D-based particle sorting:

(i) usually requires user-knowledge of the structure because some typical molecular views are needed to visually detect structural heterogeneity; solution: use variance map

(ii) it harbours the problem of assigning a particle image to a precise group (i.e. one structural state or another) across different viewing angles (addressed in part by automatic iteration of the cross-validation with re-projections);

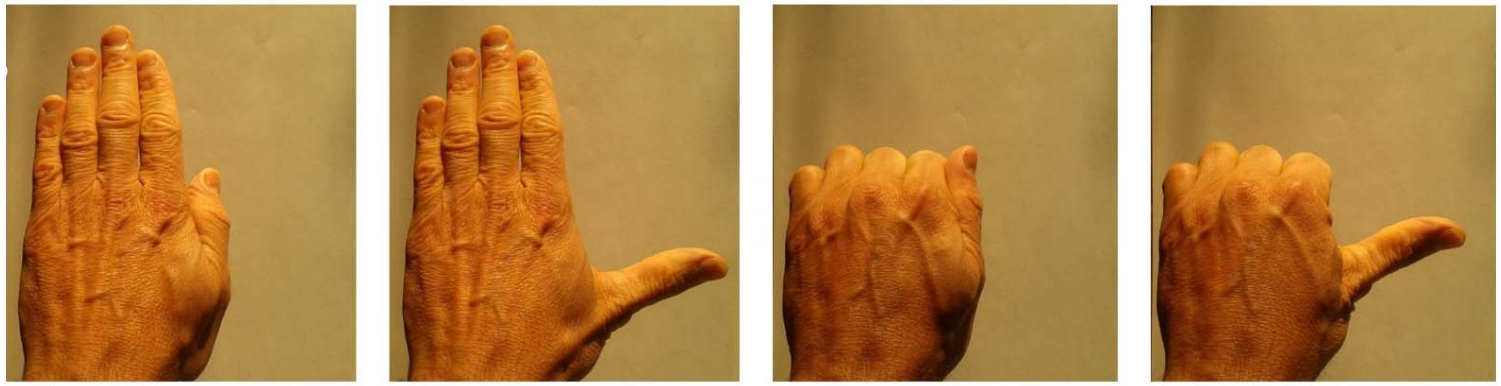
(iii) the procedure is difficult to extend to more than two different states.

MSA-based 3D classification

Determining structures of multiple conformational states in a single sample

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

different conformations of the 3D objects



(here: conformational variability within a given orientation)

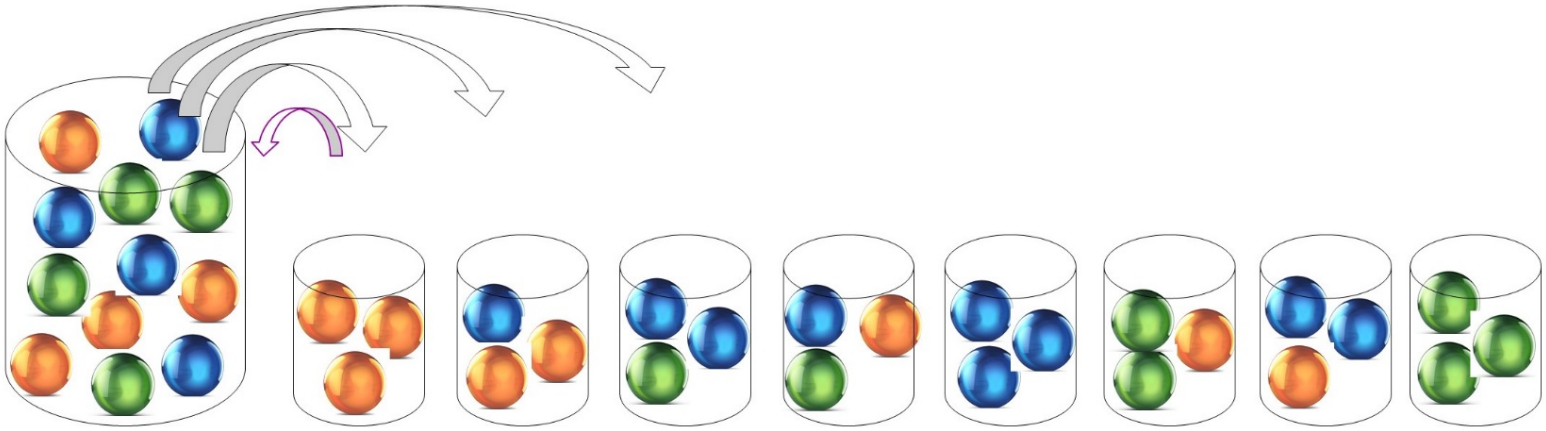
Klaholz, *Open J. Statistics*, 2015.

Determining structures of multiple conformational states in a single sample

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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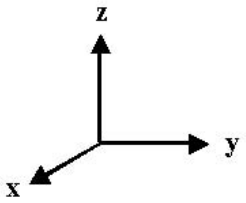
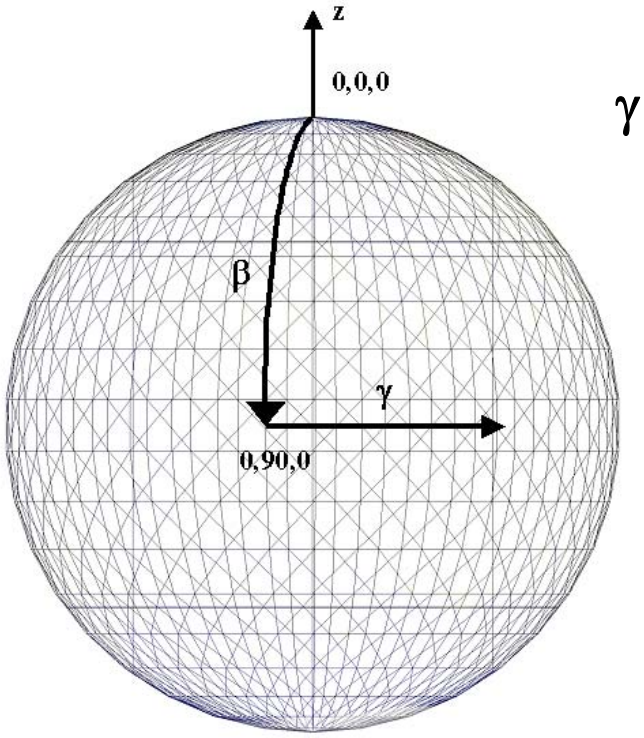
Determining structures of multiple conformational states in a single sample

3D MSA

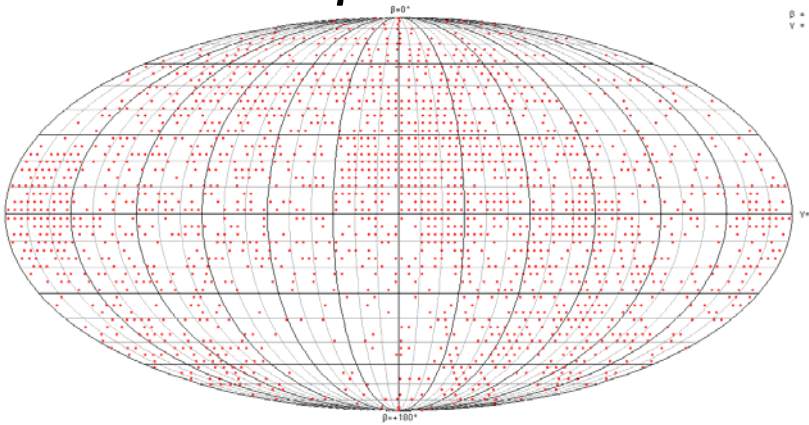




Particle angles plotted on sphere:

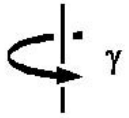
β = 0°

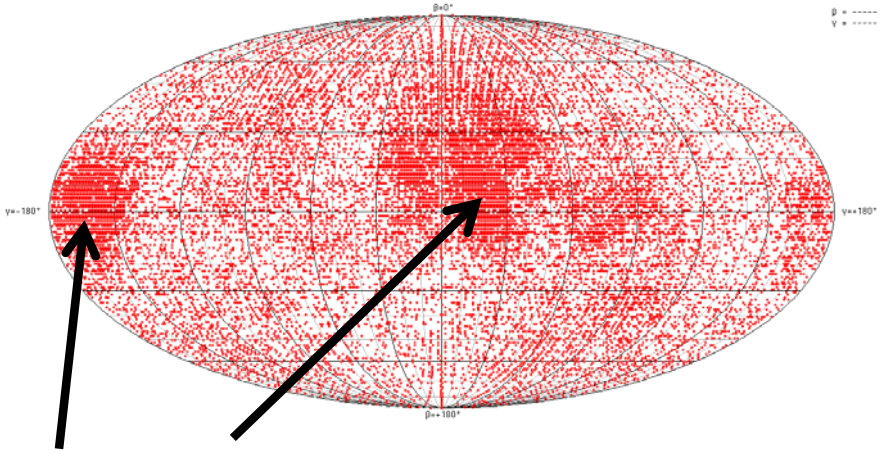
γ = -180°



γ = 180°

β = +180°





(preferential views)

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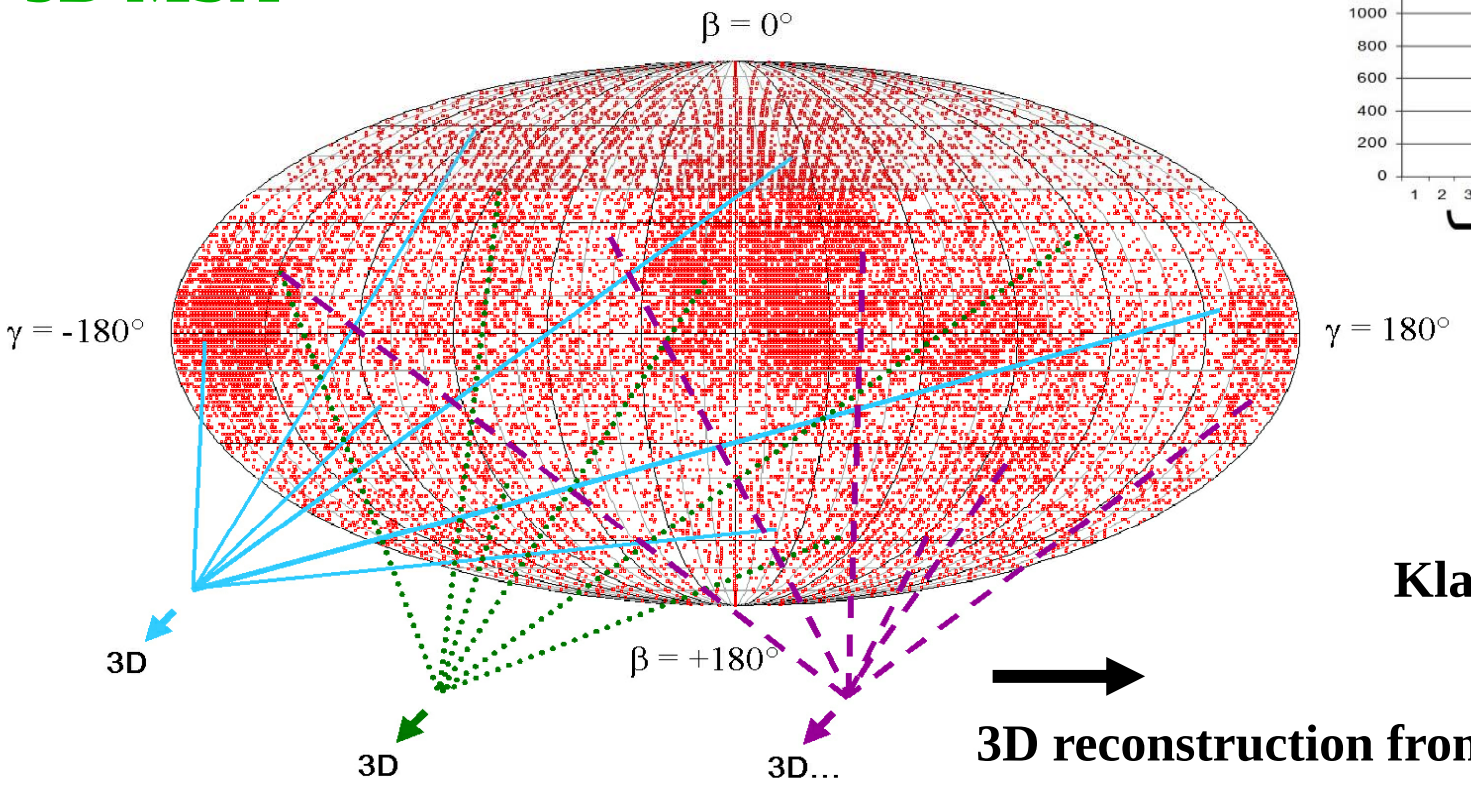
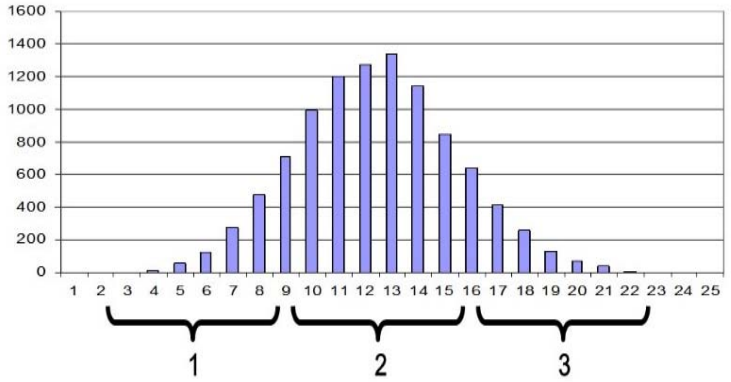
Determining structures of multiple conformational states in a single sample

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, 3D variance map;
see also work by P. Penczek (bootstrapping (re-sampling), used primarily to find region of variance, i.e. estimation of 3D variance)

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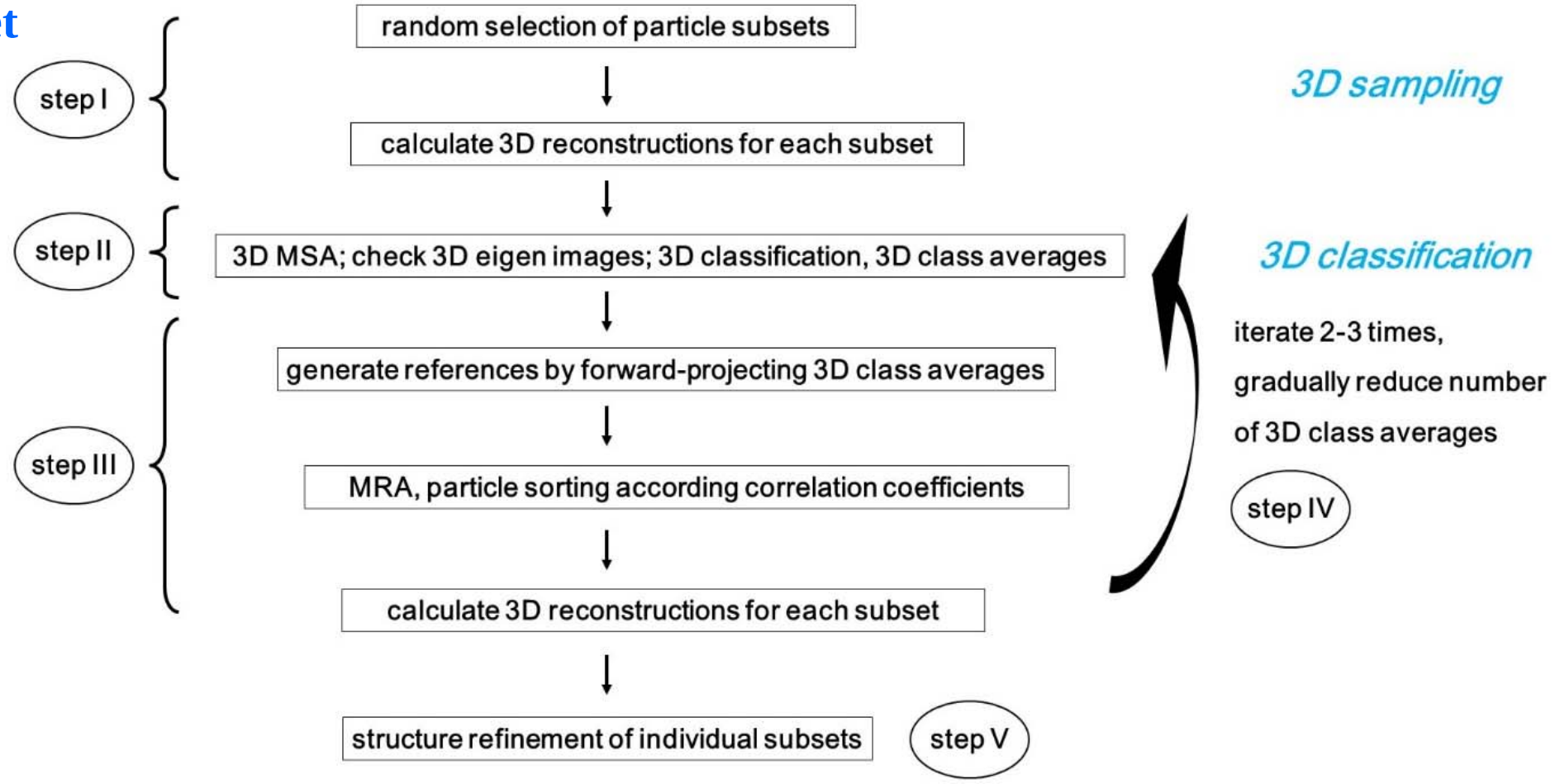
Determining structures of multiple conformational states in a single sample

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

10-50 particles per set

e.g. 10 000 structures



initially use coarsened (binned) data to speed up the process

Klaholz, *Open J. Statistics*, 2015.

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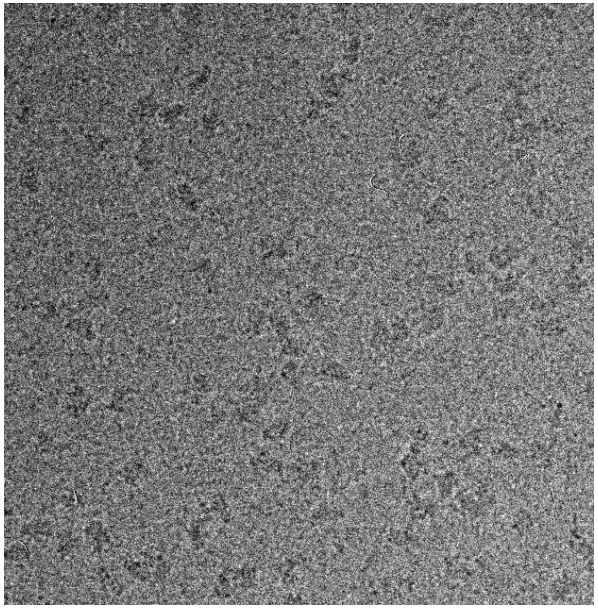
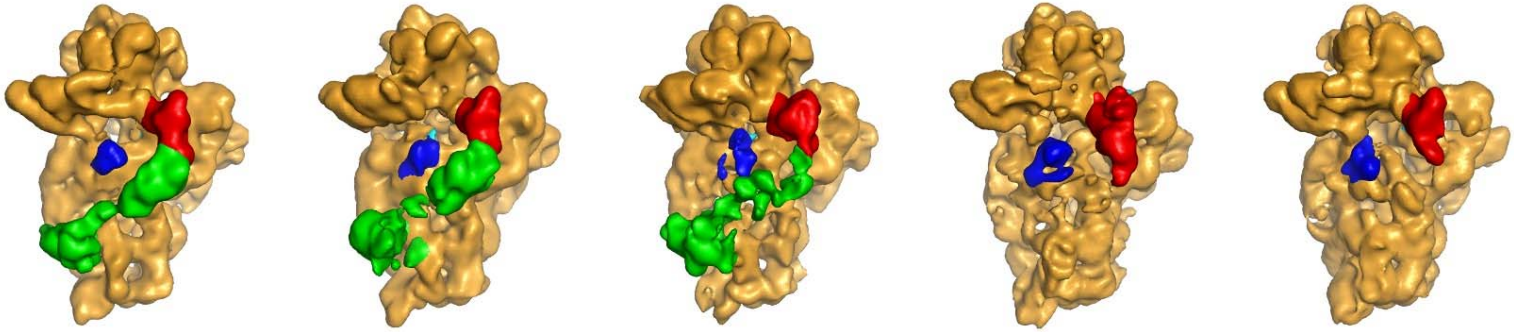
Determining structures of multiple conformational states in a single sample

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

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200kV FEG data;

total 80 000 particles

resolution of 3D's: 9Å

2D → 3D → 4D

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

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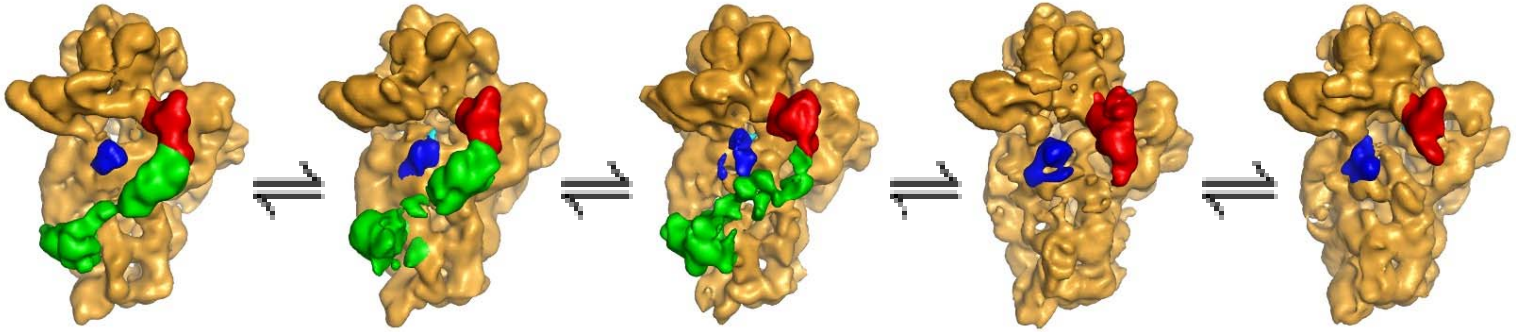
Determining structures of multiple conformational states in a single sample

3D MSA

Addressing the structural state of reaction intermediates that are in equilibrium with each other!

particle populations



$\alpha A + \beta B \rightleftharpoons \sigma S + \tau T$ **40 %** **28 %** **10 %** **8 %** **14 %**

forward reaction rate = $k_+ A^\alpha B^\beta$

backward reaction rate = $k_- S^\omega T^\iota$

→ equilibrium constant: $K = \frac{k_+}{k_-} = \frac{\{S\}^\sigma \{T\}^\tau}{\{A\}^\alpha \{B\}^\beta}$

The 3D classification procedure 3D-SC has been used since by other groups also:
(Papai *et al.*, *Nature* 2010; Fischer *et al.*, *Nature* 2010). **Simonetti *et al.*, *Nature*, 2008.**

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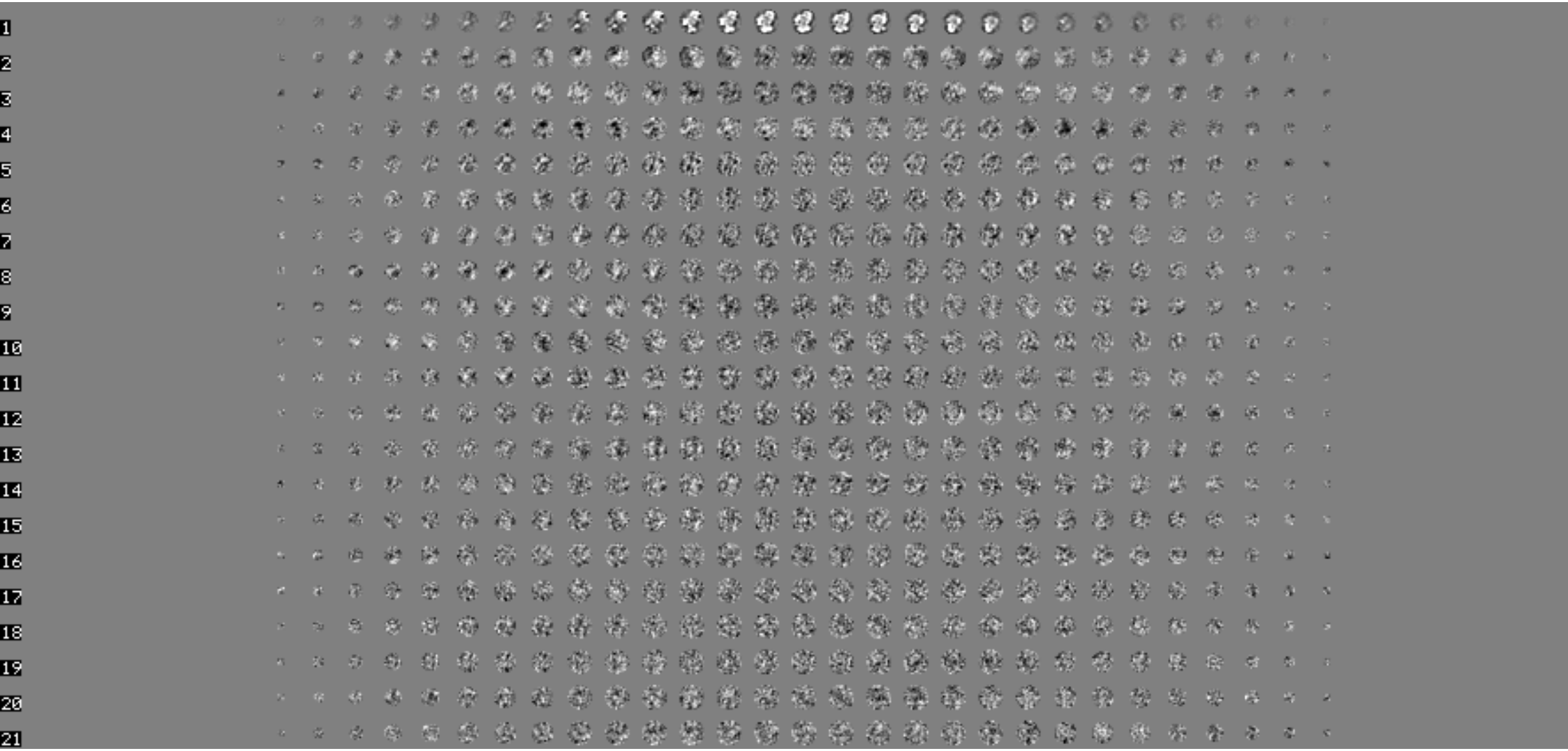
3D MSA

3D eigenimages: (30S)



Klaholz, *Open J. Statistics*, 2015.

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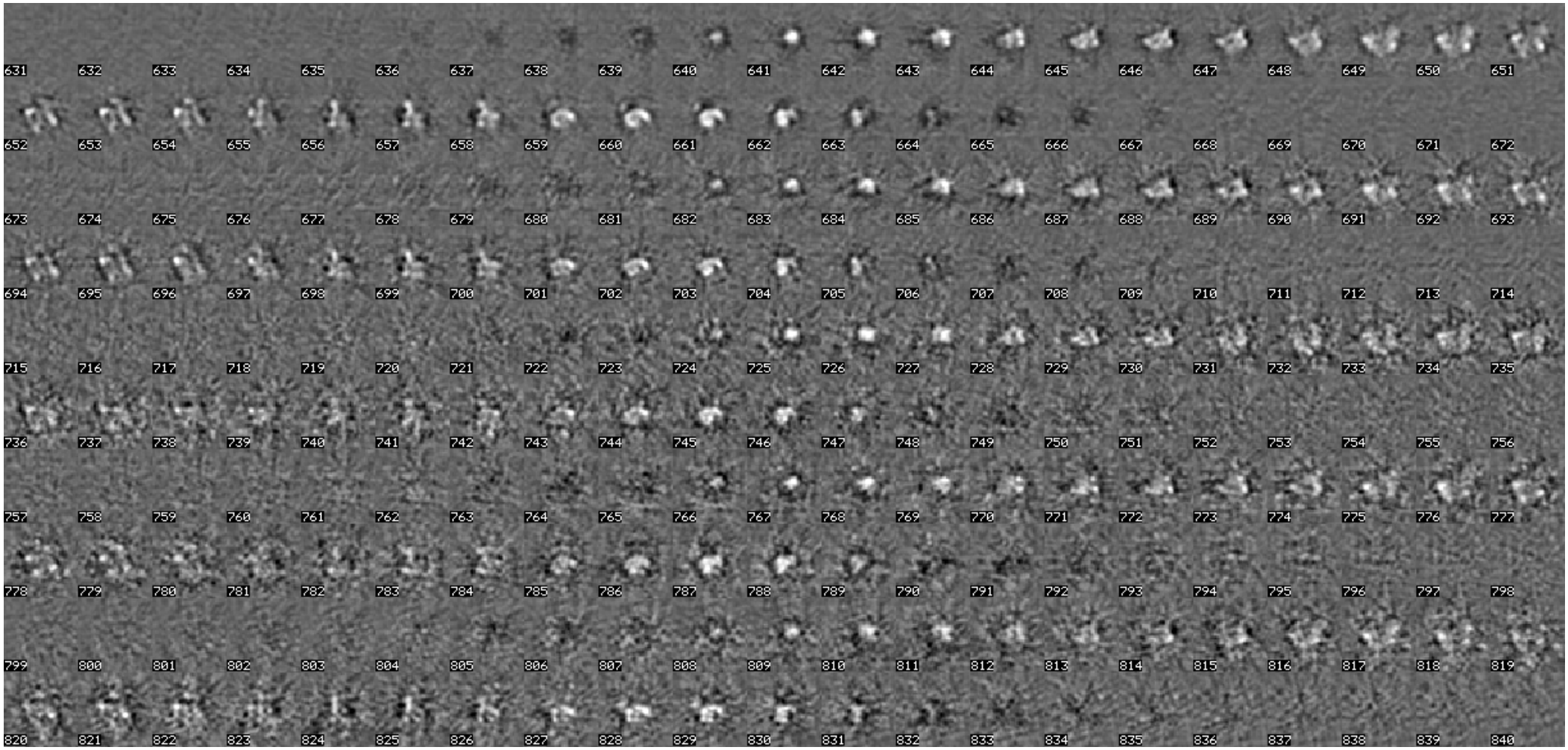
3D MSA

3D class averages: (30S)



3D #

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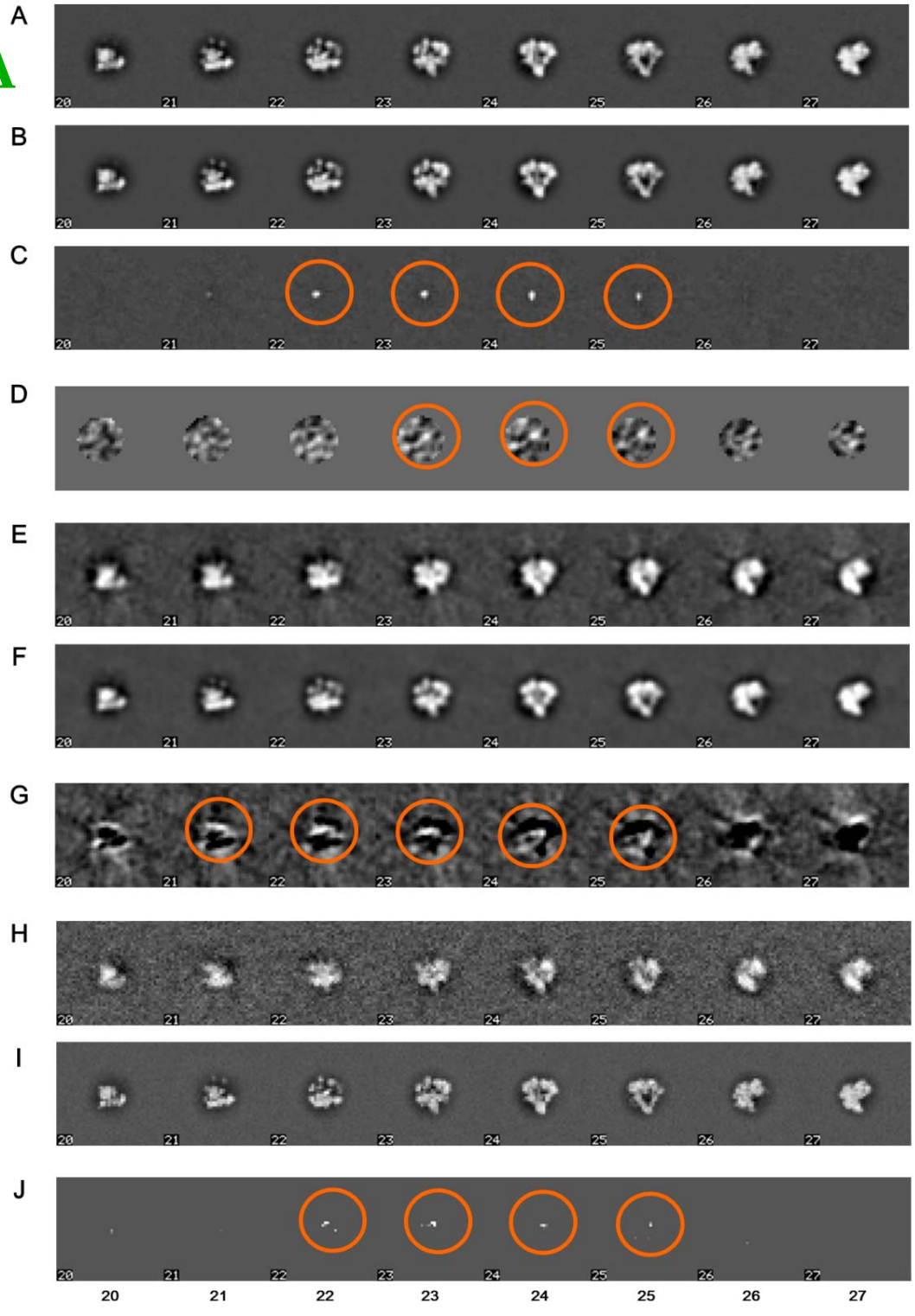
Klaholz, *Open J. Statistics*, 2015.

3D MSA



small difference found by 3D MSA (<1% of the total mass of the complex; modeled RNA Pol data)

Imagic

Klaholz, *Open J. Statistics*, 2015.

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Some examples based on 2D classification or on 3D classification (3D-SC / resampling / bootstrapping):

Klaholz *et al.*, 2004; White *et al.*, 2004 (size variation);

Penczek *et al.*, 2006; Cheng *et al.*, 2007;

Elad *et al.*, 2008; Simonetti *et al.*, 2008;

Wang et al., 2013;

Liao *et al.*, 2015.

focused classification / focused refinement:

concept implemented later into Relion, Frealign etc.

→ analyse regions, subunit structural variations

→ not limited by the number of different structural states in the sample

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 (max. expectation)

(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

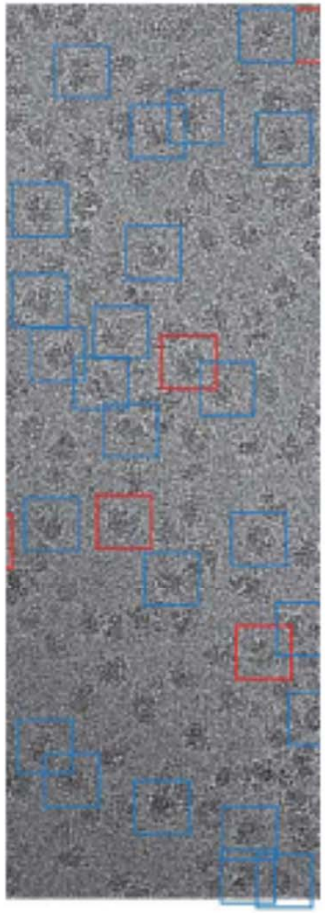
e.g. Scheres *et al.*, *JMB* 2005; *Meth. Enzymol.* 2010;

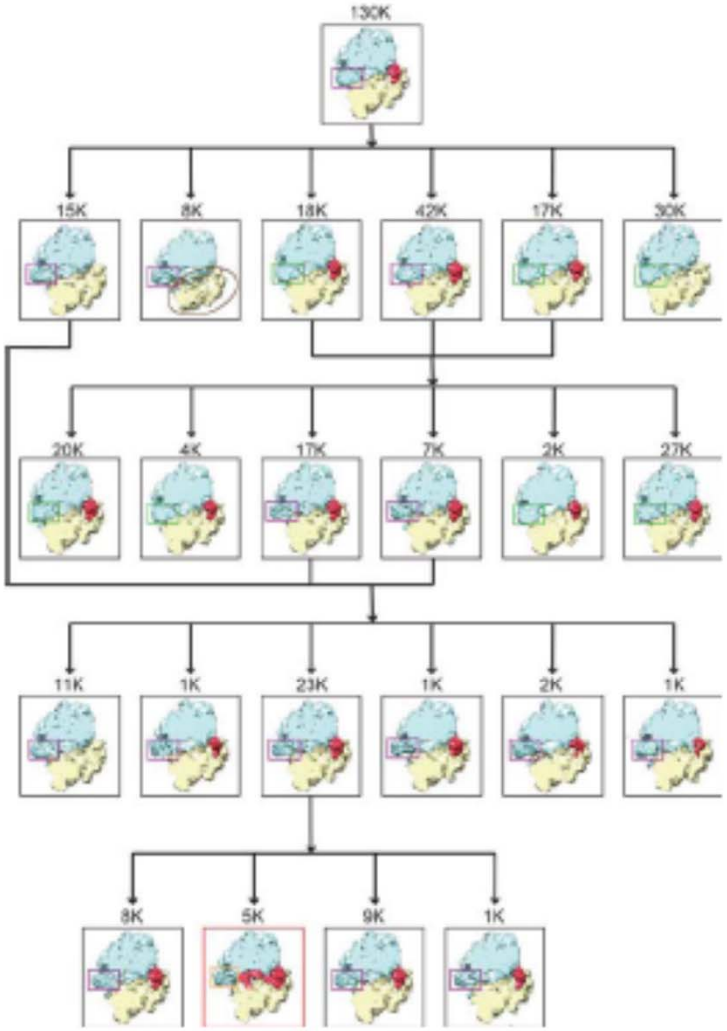
Lyumkis *et al.*, *JSB* 2013

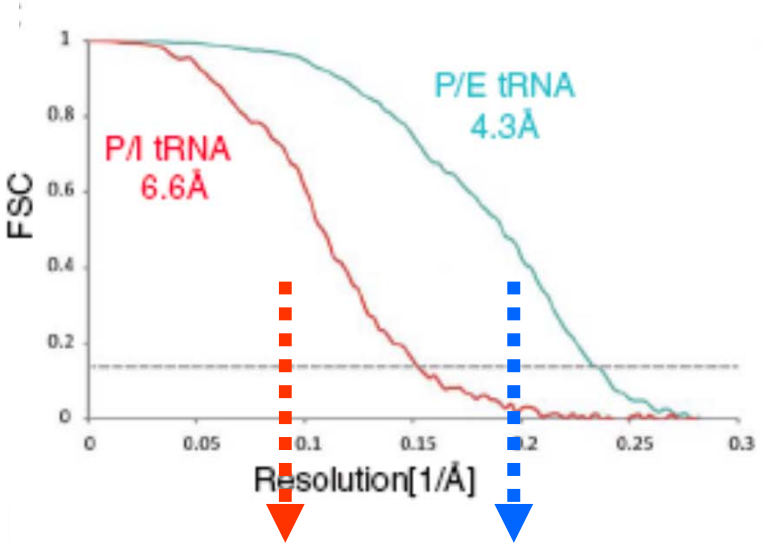
Introduction of the ML concept in cryo-EM: Sigworth, *JSB* 1998;

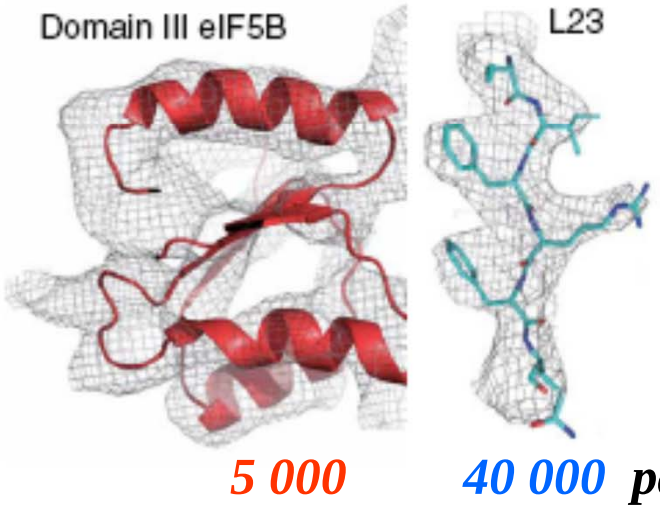
in X-ray crystallography: G. Bricogne, *Acta Cryst A*, 1991.

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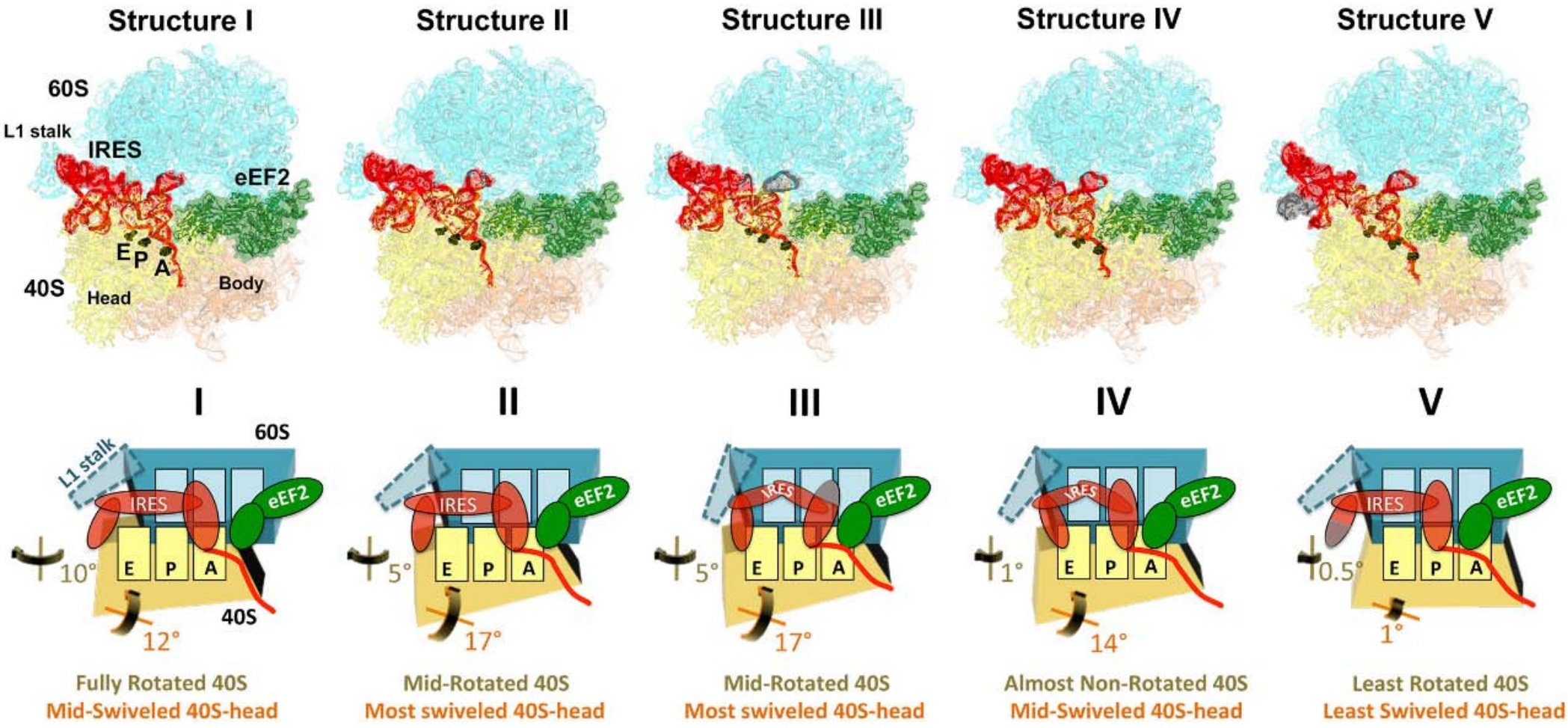
e.g. ML-based focused classification of 80S / TSV IRES complex with eEF2/GDP/sordarin



Abeyrathne *et al.*, *eLife* 2016

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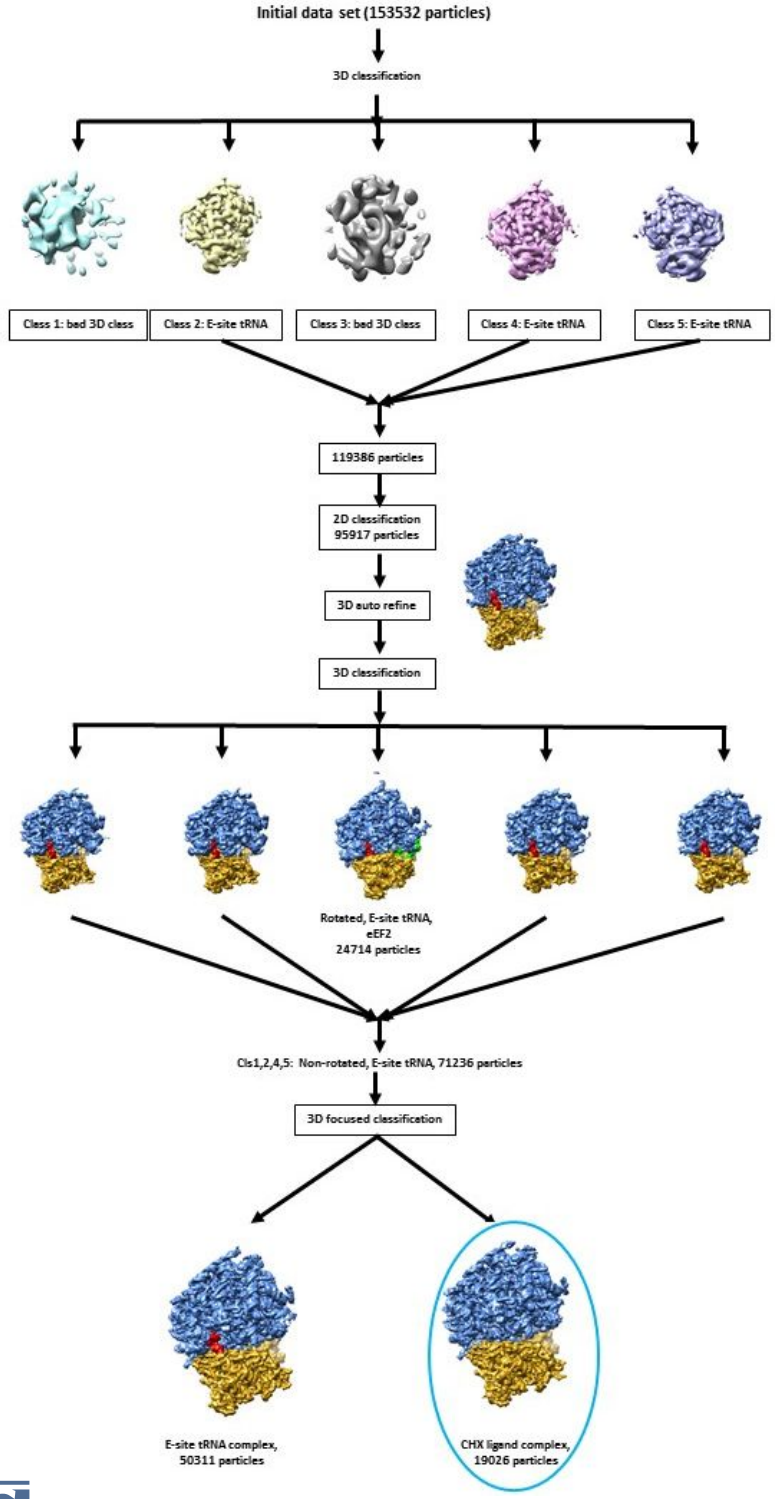
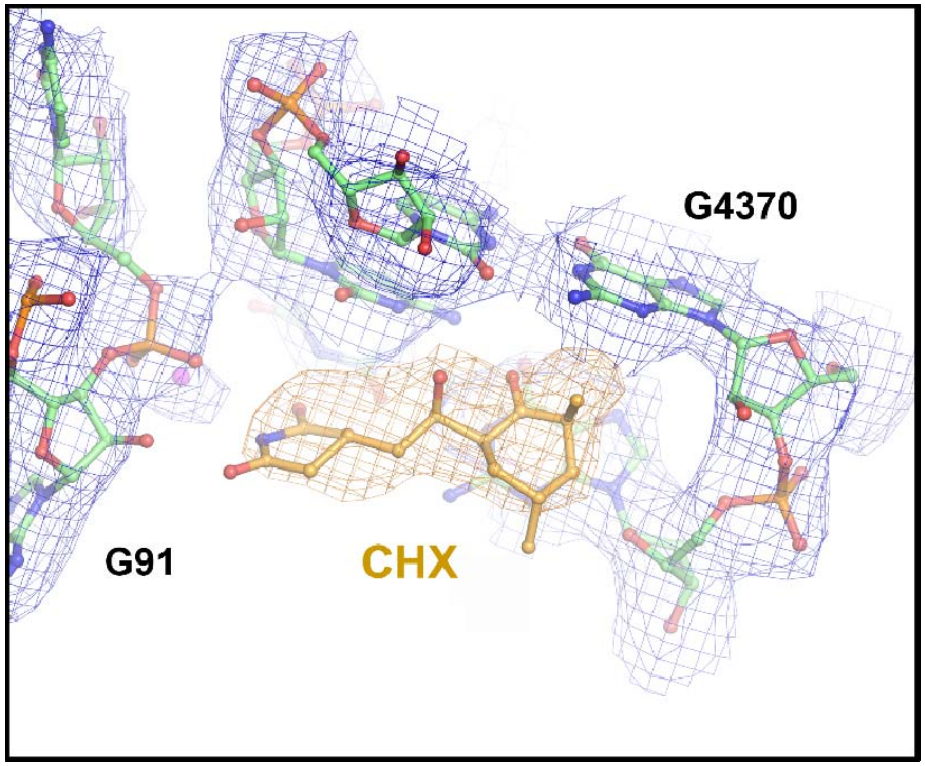


e.g. ML-based focused classification

sorting scheme for human 80S/antibiotic complex



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

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Summary:

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
variance analysis + resampling, bootstrapping, 3D resampling

3) maximum likelihood based class assignment

4) deep learning methods (coming)

see also review by White *et al.*, *Biomed. Res. Int.* 2017, 1032432.

Summary:

Determining structures of multiple conformational states in a single sample

Conclusions & tips:

- 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)

- after 3D classification: go for focused refinement →

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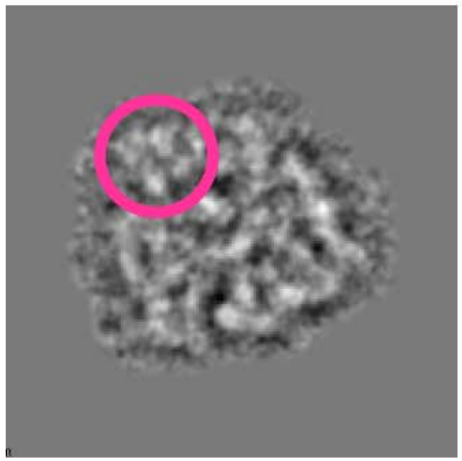
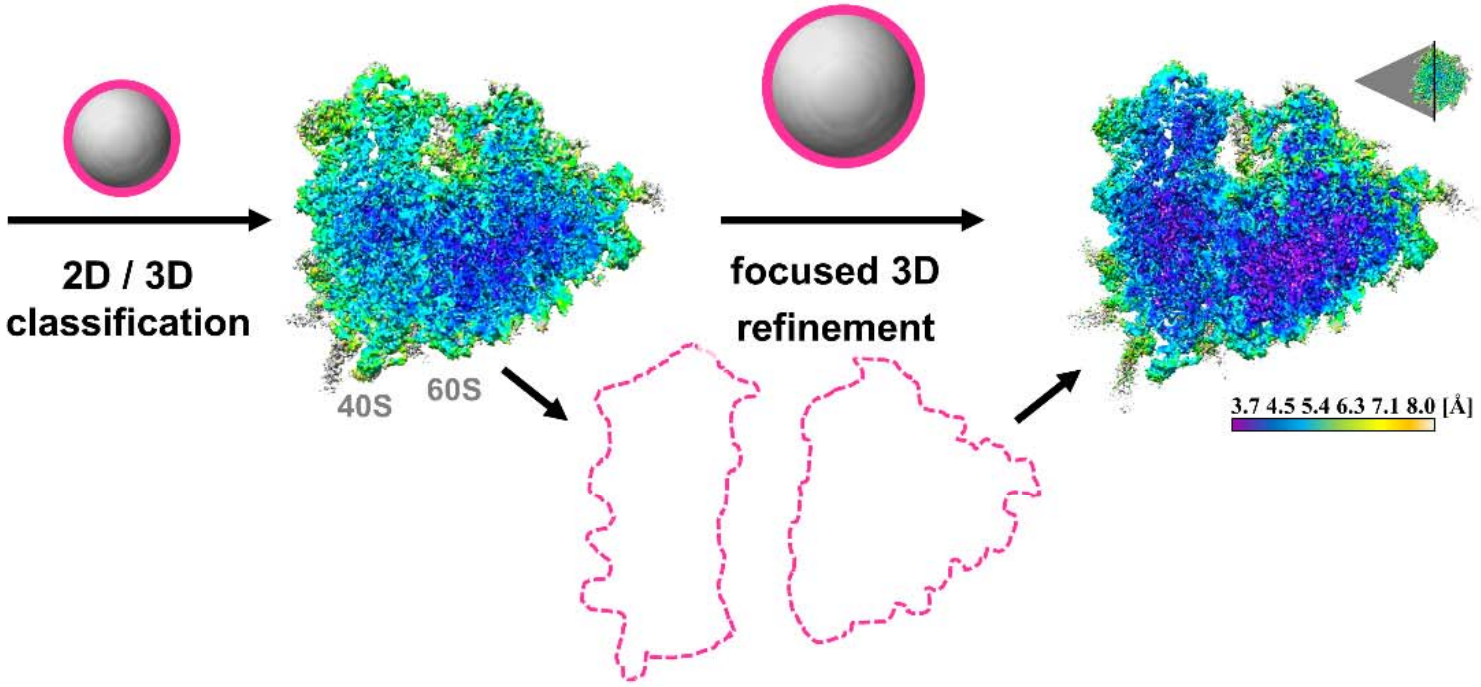
Local MSA / focused 2D/3D classification & focused refinement:



Concept of local 2D MSA / focused classification



Concept of focused cryo-EM structure refinement through
- 3D resampling & 3D classification (3D-SC) / bootstrapping
- maximum likelihood 3D classification
using spherical mask or dilated, binarized map of region of interest

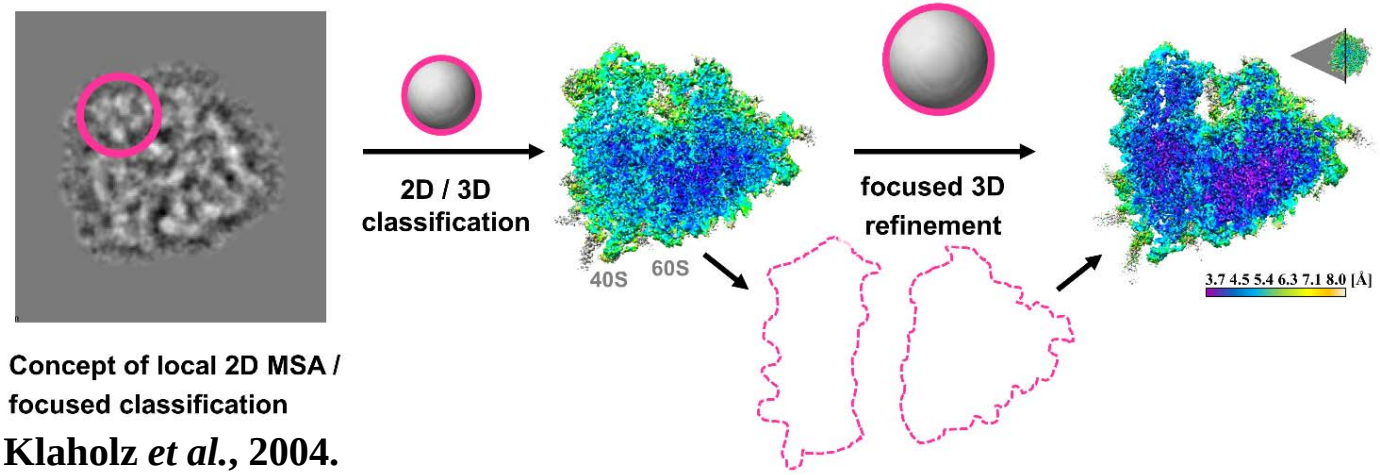
see also:
Klaholz *et al.*, *Nature* 2004;
White *et al.*, *JSB* 2004;
Penczek *et al.*, *JSB* 2006;
Wong *et al.*, *Elife* 2014;
…

Helps: use a slightly larger region than the region of interest, e.g. 30-50 Å in diameter

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

Advanced image processing to improve cryo-EM reconstructions and map interpretation

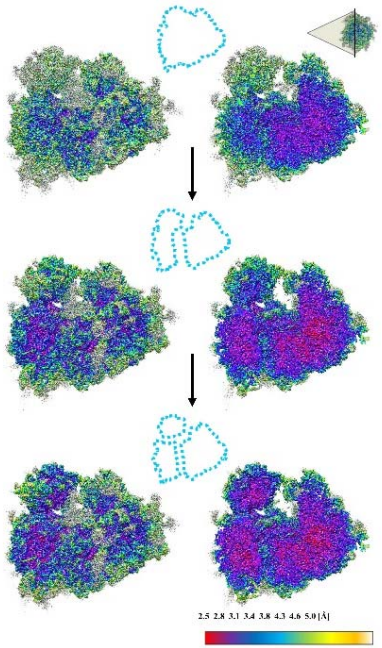
Local MSA / focused 2D/3D classification & focused refinement:



Concept of local 2D MSA / focused classification

Klaholz *et al.*, 2004.

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

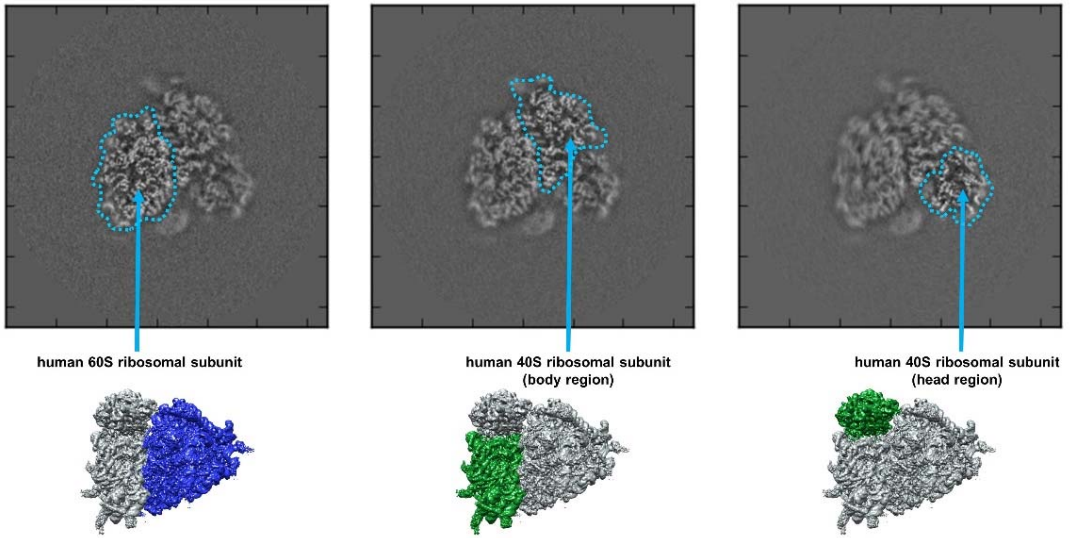


Focused refinement

global 80S refinement

refinement of the individual
60S and 40S ribosomal subunits

refinement of the individual
60S subunit, 40S body and 40S head regions



Natchiar *et al.*, *Nature* 2017.

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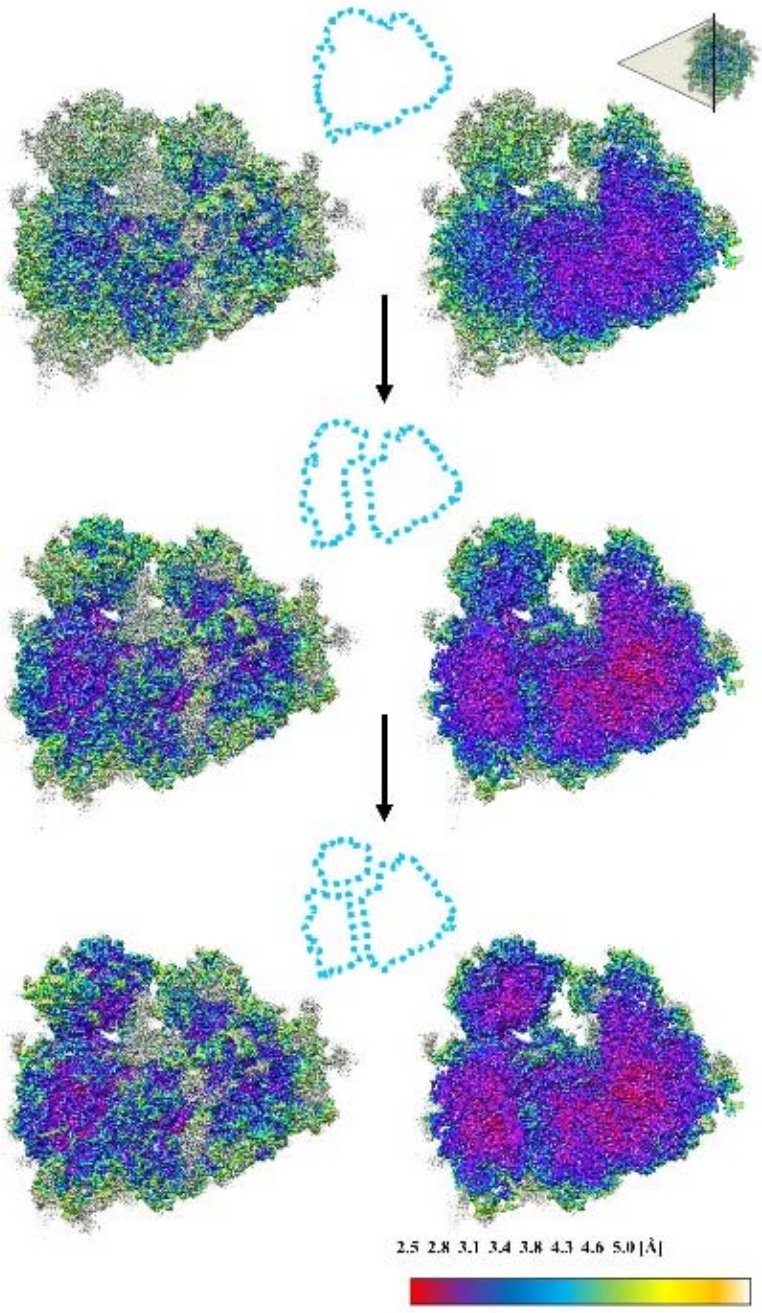
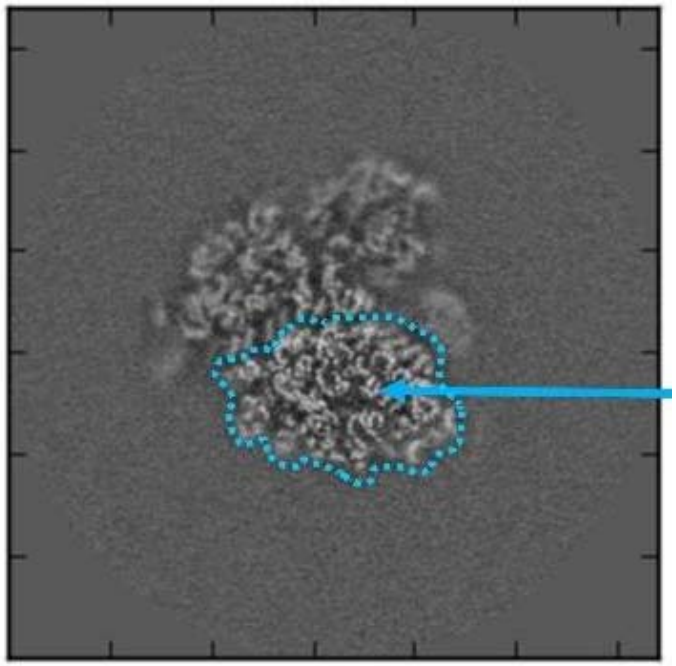
Focused refinement

(with subtraction, Relion)



global 80S refinement



refinement of the individual
60S and 40S ribosomal subunits

refinement of the individual
60S subunit, 40S body and 40S head regions

Natchiar *et al.*, *Nature* 2017.

See also: von Loeffelholz *et al.*, *Curr. Opin. Struct. Biol.* 2017.

Specific tips on focused refinement:

- works best after 3D classification / sorting

→ makes sure that it corresponds to a conformational / functional state, therefore the PDB requires to deposit the low-resolution map before focused refinement

→ provides a composite map

- further improves with partial signal subtraction (Bai *et al.*, 2015)
- localized reconstruction of subunits on viruses (Ilca *et al.*, 2015)
- subunit subtraction and focused refinement on GroEL (Roh *et al.*, 2017)
- re-centering focused region helps (alignment quality); e.g. Blees *et al.*, 2017
- dynamic signal subtraction (Schoebel *et al.*, 2017)
- multi-body refinement (Nakane *et al.*, 2018)

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