3D structure determination of dynamic macromolecular complexes

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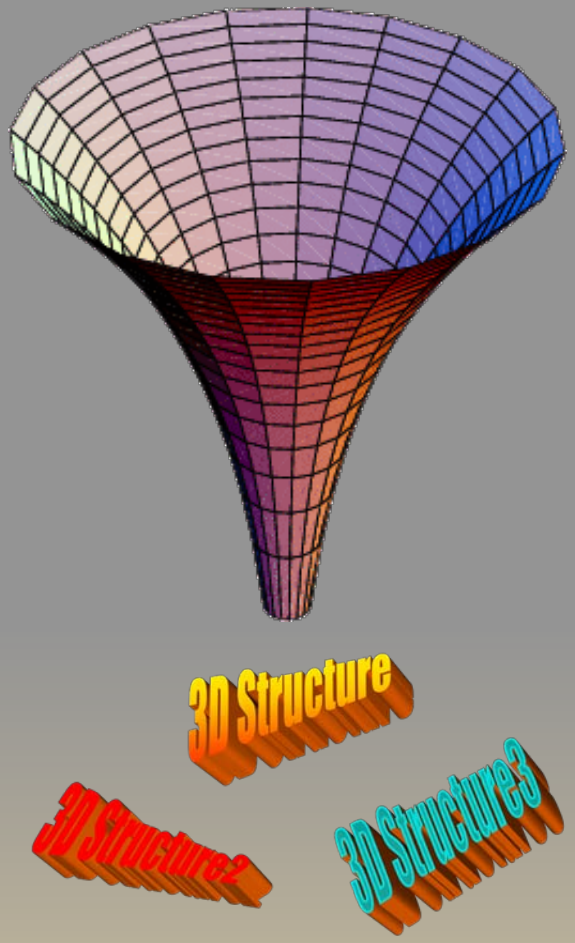
Single Particle Cryo-EM

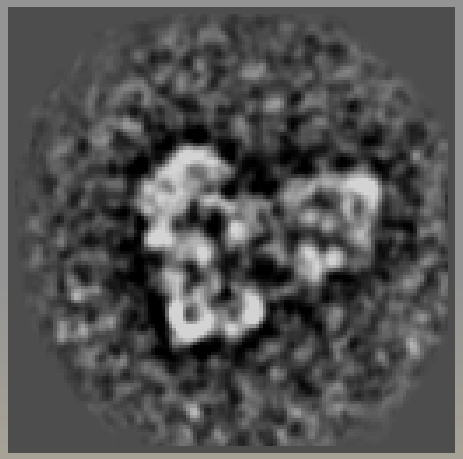
Millions ?

Hundred thousands ?

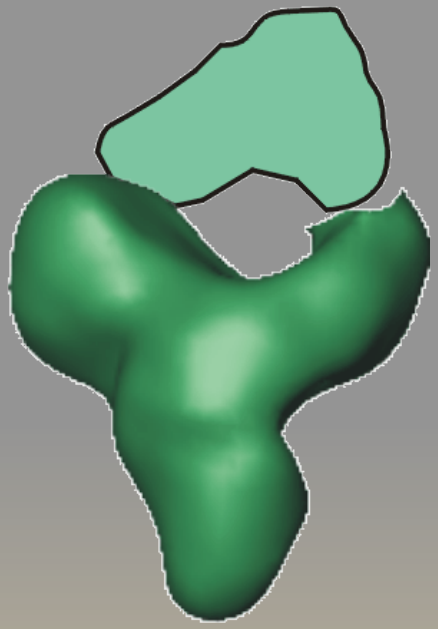


…works well for homogeneous complexes in defined structural/functional states…

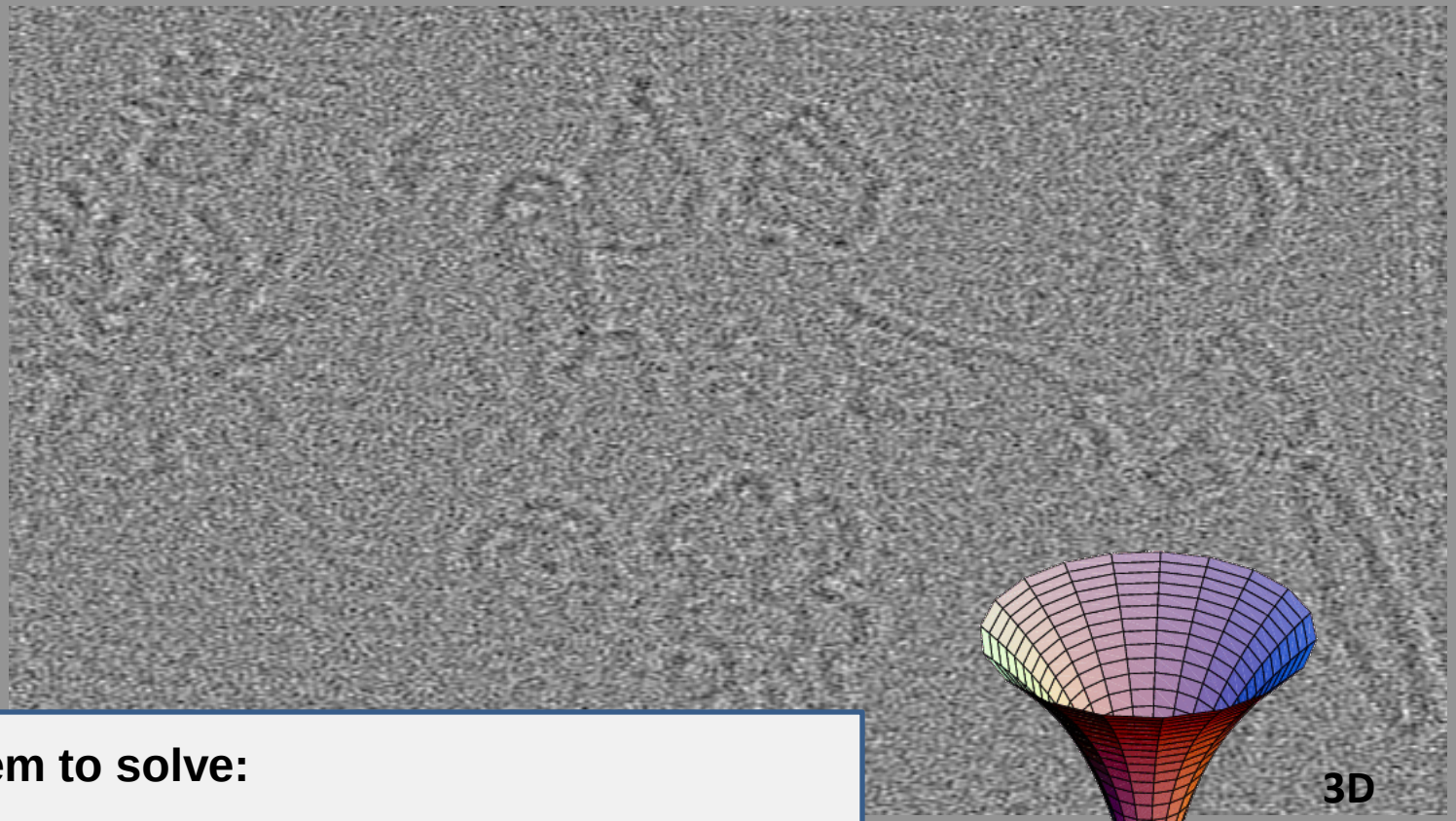
…but what about this…



Anaphase Promoting Complex



Spliceosome (complex B)



Problem to solve:

- 3 translational parameter
- 3 rotational parameter
- Unknown number of conformational parameter

....plus noise!

3D reconstruction

Sample Heterogeneity

Structural Heterogeneity

- Variations in P… …on
- Dam… …e to
  …eparation

GraFix – chemical stabilization
ECAD – analysis of stabilized complexes

Biochemistry and improved specimen preparation

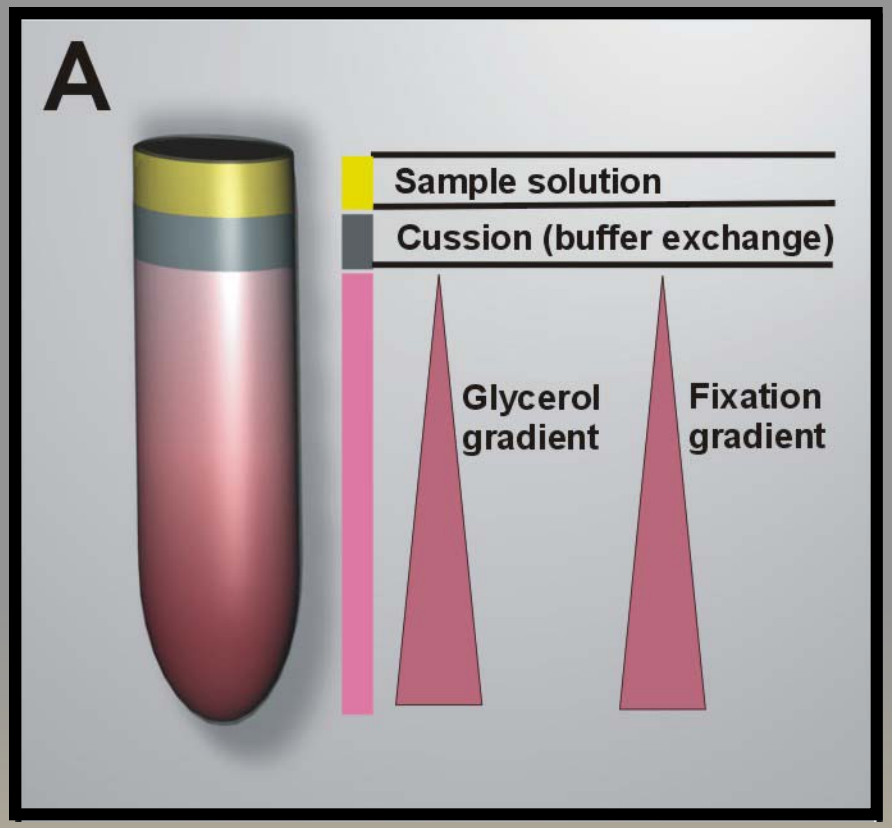
Conformational Heterogeneity

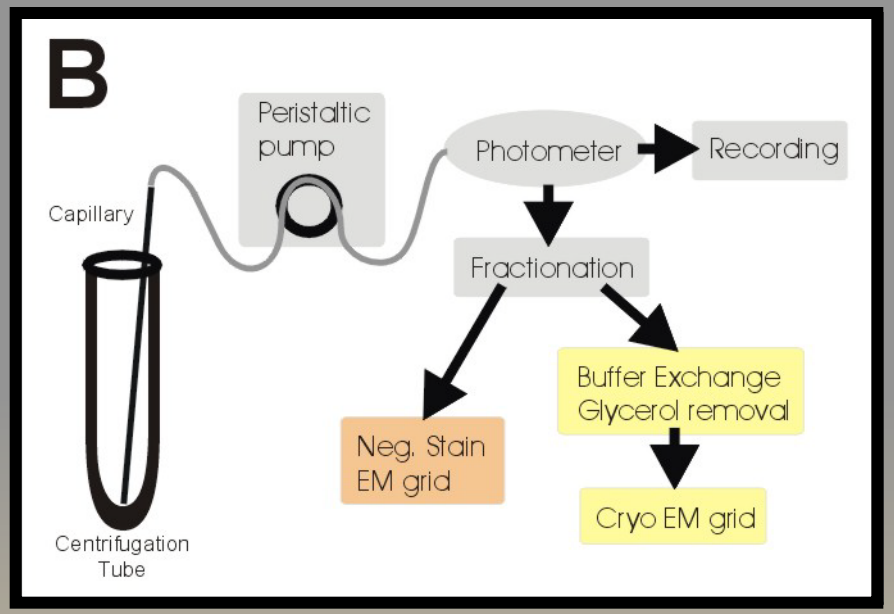
- Flexible Domains
- Mixture of different functional states

New Image Processing Software

GraFix

A combined Gradient Centrifugation and Fixation method

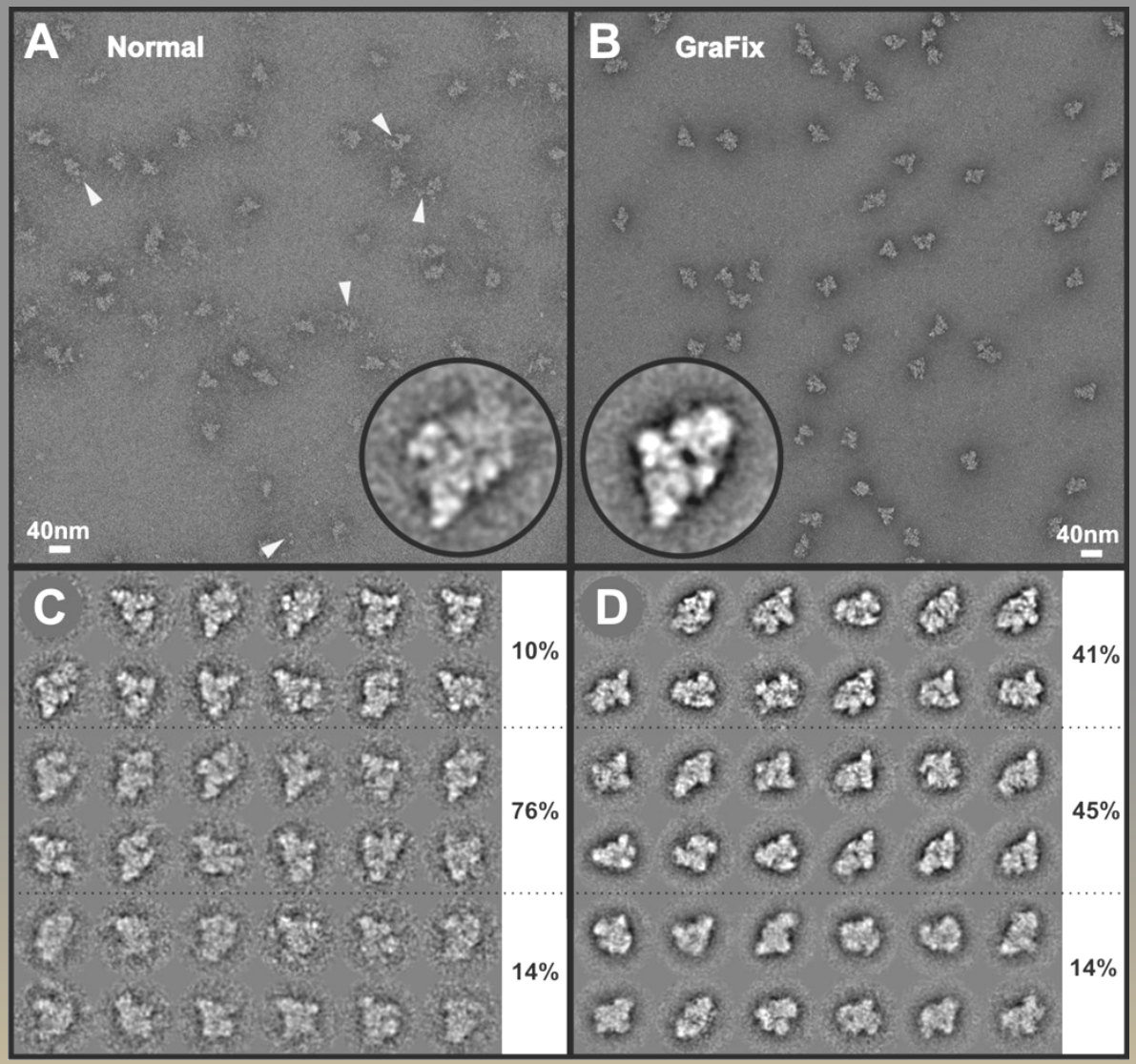




Typically
0 – 0.15% glutaraldehyde

Kastner et al, Nature Methods, 2008

GraFix test: Spliceosomal B Complex

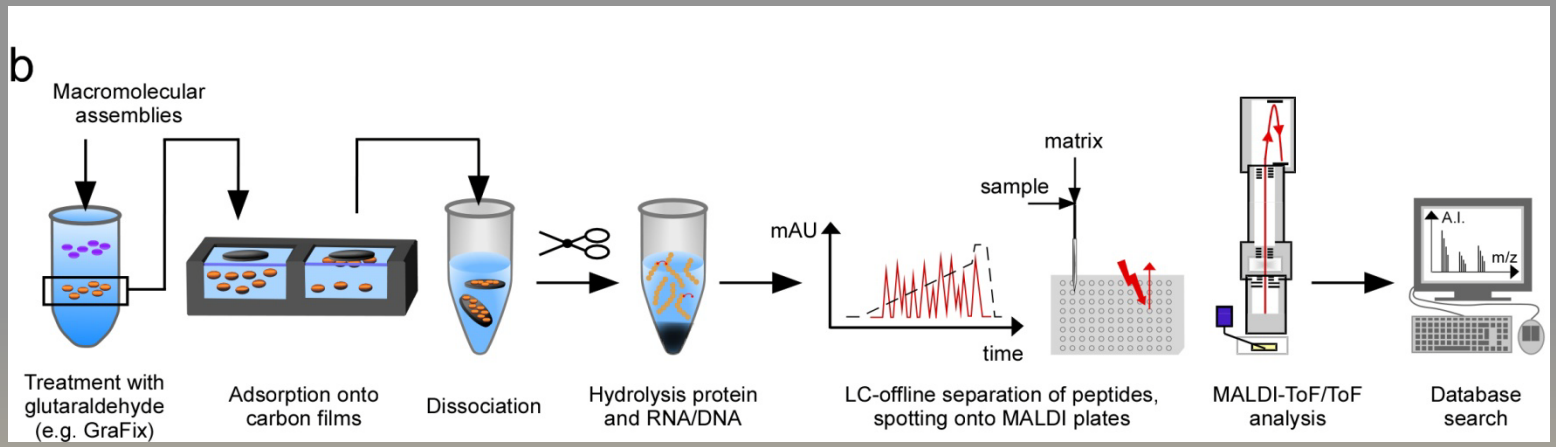


How to analyze chemically stabilized complexes?

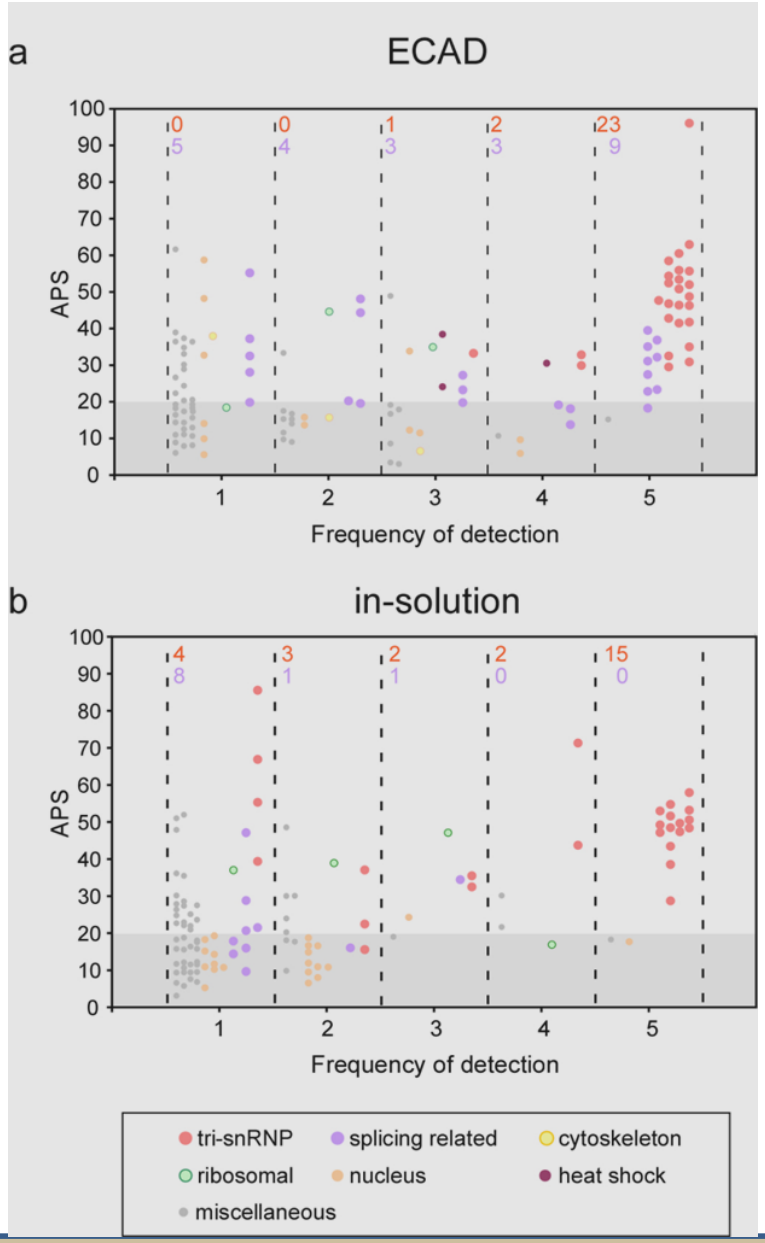
Problem :

Chemically stabilized macromolecules cannot be analyzed by SDS gel analysis

-> GraFix samples can be analyzed by Mass Spec
(ECAD, EM Carbon-film-Assisted endoproteinase Digestion)



ECAD



Higher sensitivity !

Preference to detect Peptides located at Interface regions

Reproducible detection of substoichiometric or transiently bound factors

Direct correlation of Mass Spec and Structure Determination

Collaboration with Florian Richter and Henning Urlaub , MPI Göttingen

How to determine reliable initial 3D model(s)

How to determine reliable initial 3D model(s)

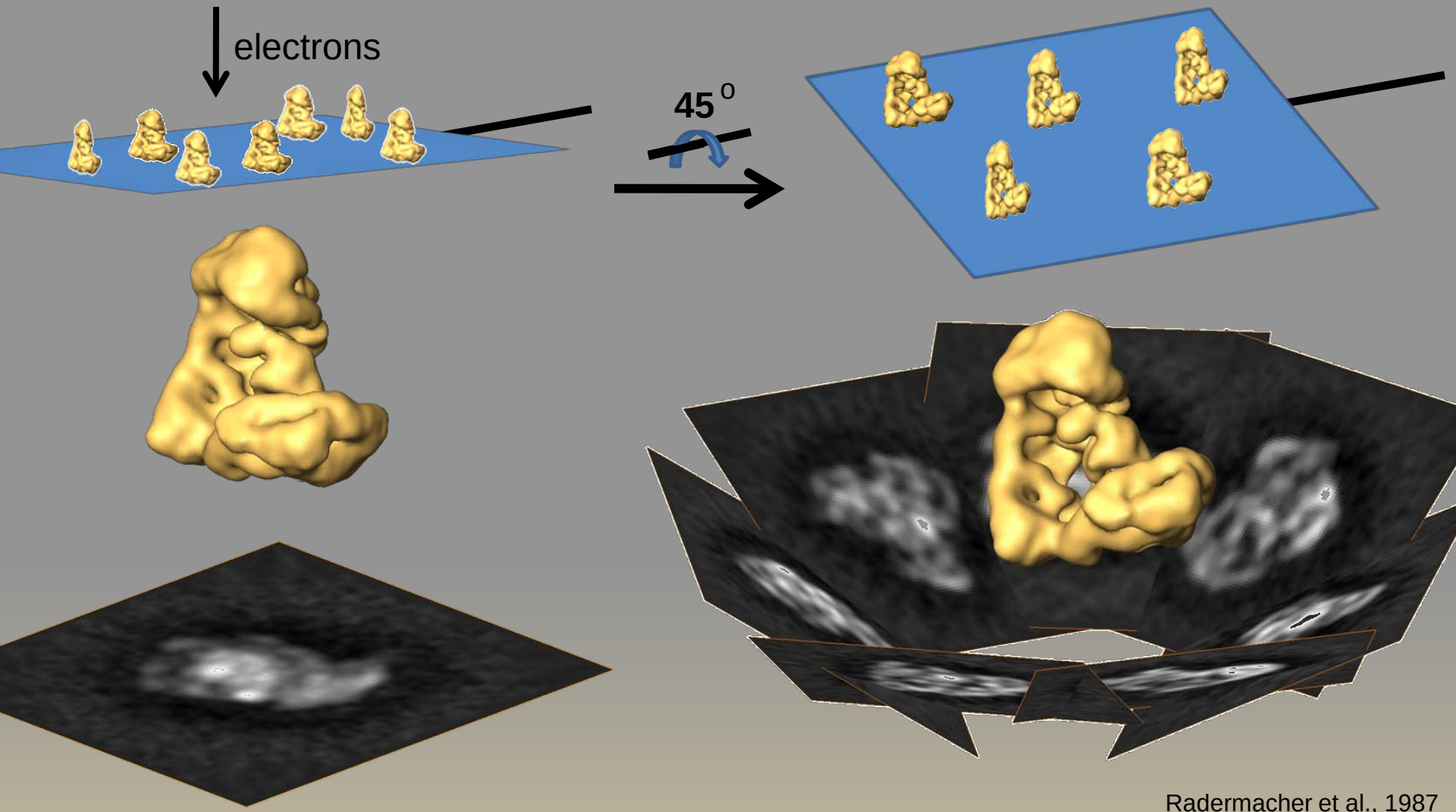
Problem to solve:

3 translational parameter
3 rotational parameter

Unknown number of conformational parameter
....plus noise!

Zero Tilt Imaging

Random Conical Tilt Imaging



Radermacher et al., 1987

DETERMINATION OF DE NOVO 3D MODELS

- **Random conical tilt reconstructions**

  5000-40000 tilted image pairs, CCD camera, neg stain and cryo
  10-40 images/3D structure

Few hundred noisy RCT 3D volumes

- **Alignment of RCT 3D reconstructions by rotational**

  3D „Maximum Likelihood“-like alignment

  reference free 3D alignment

  (according to Sigworth, JSB, 1998)

Alignment of all Volumes in 3D

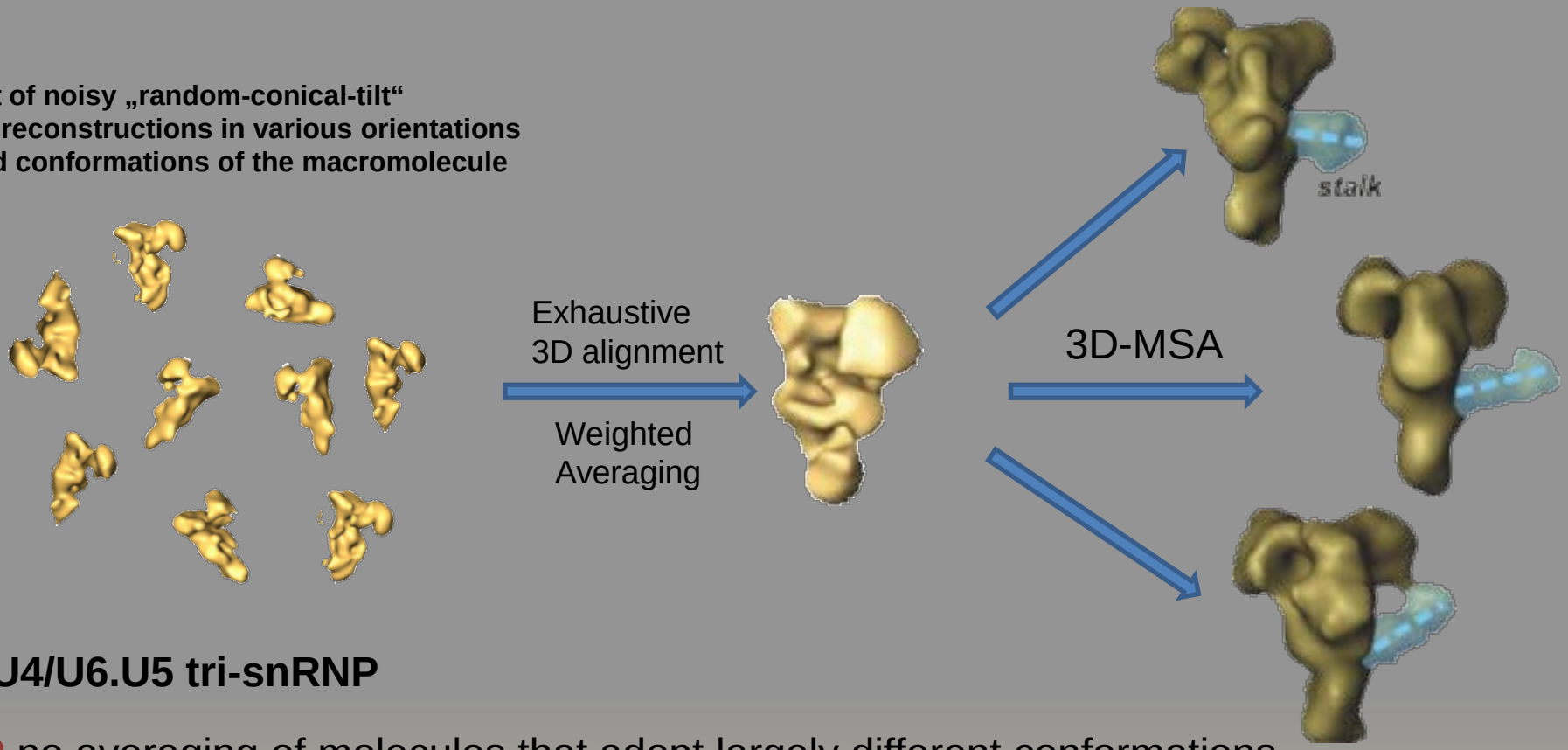
- **3D MSA and classification**

  new MSA implementation – faster and more reliable at low SNR

Find Similar 3D volumes

3D Structure Determination of „DYNAMIC" Macromolecules

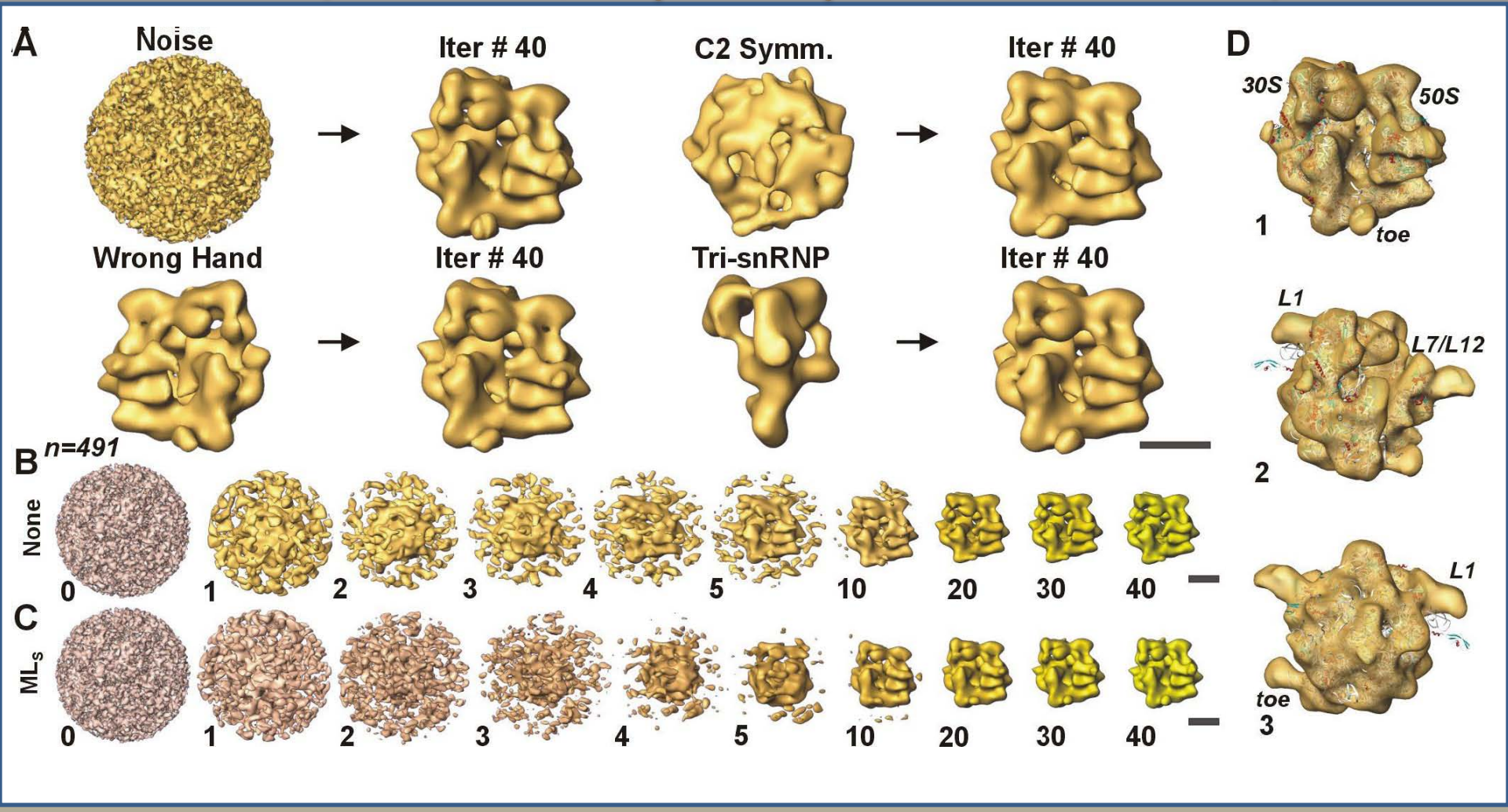
Set of noisy „random-conical-tilt"
3D reconstructions in various orientations
and conformations of the macromolecule



U4/U6.U5 tri-snRNP

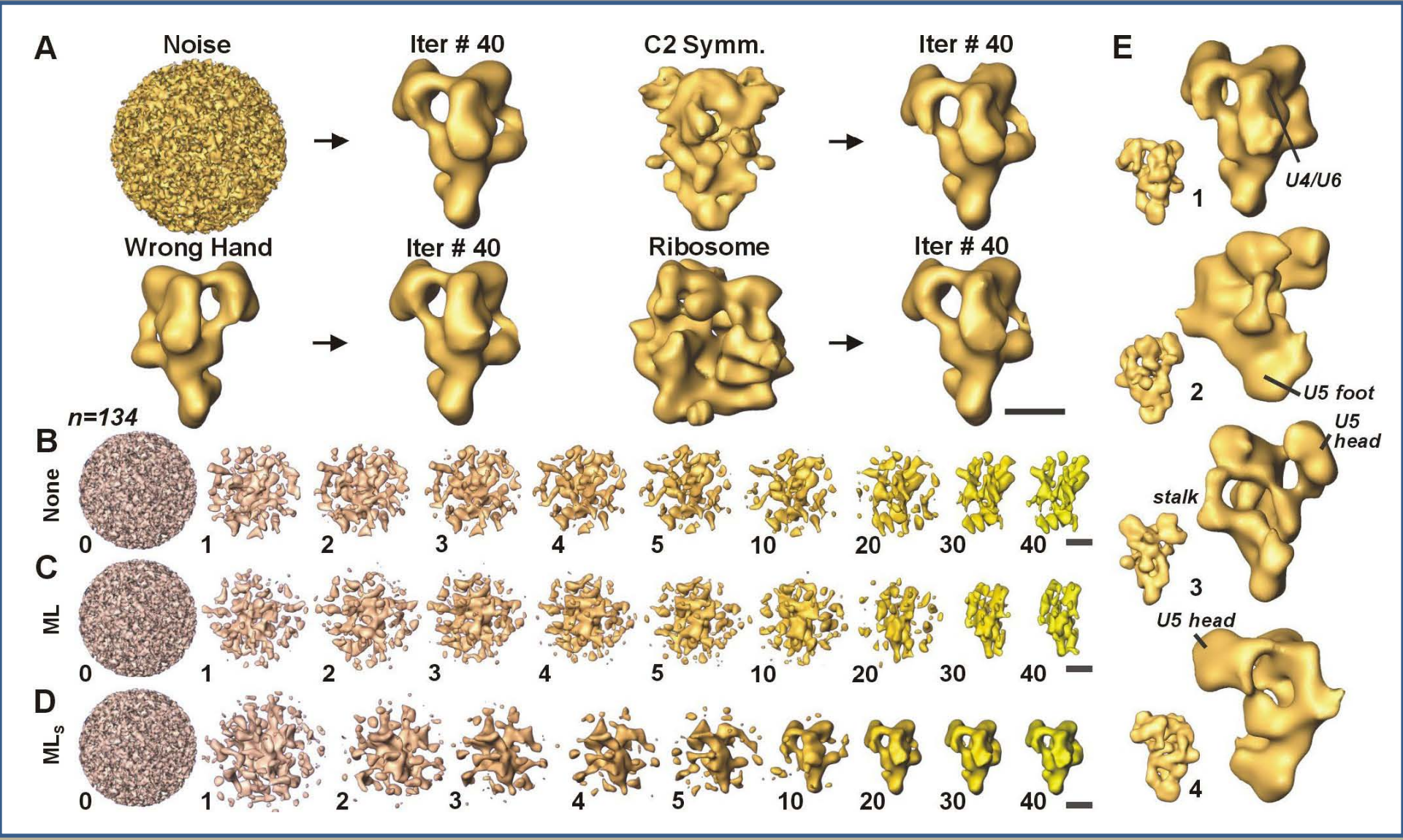
- no averaging of molecules that adopt largely different conformations
- no model bias!
- user independant, automated
- computationally not too demanding!!

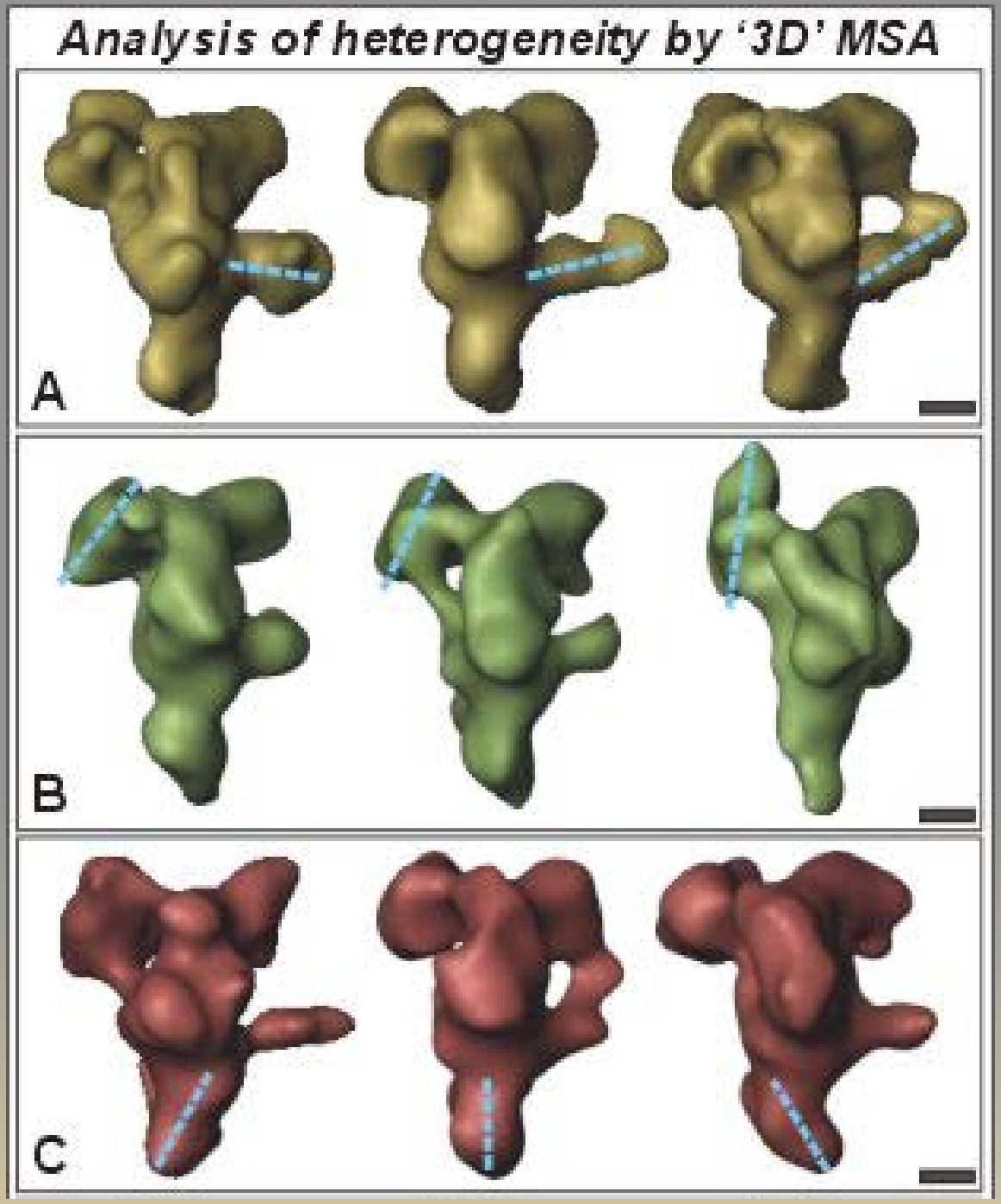
70S Ribosome Data
(real data, cryo tilt pairs, CM200 FEG)



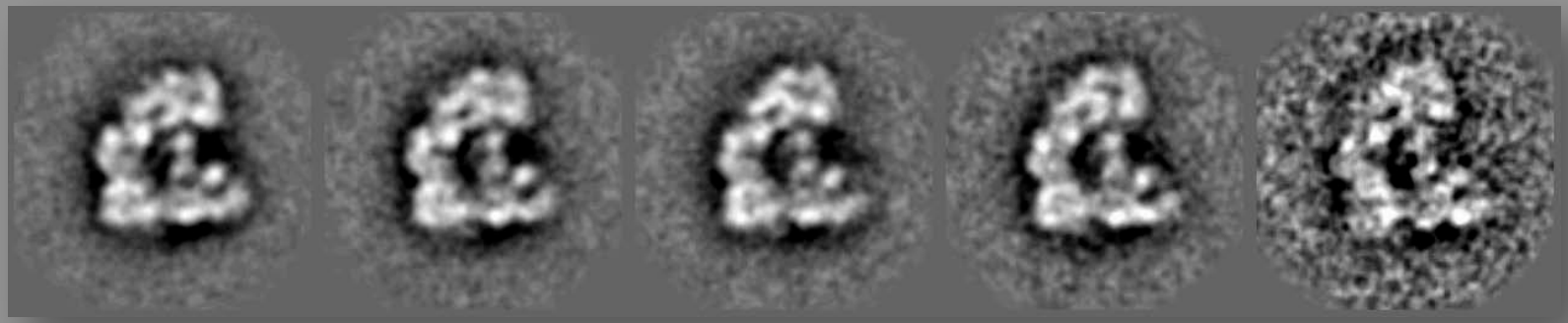
~26Å resolution, no user interaction!

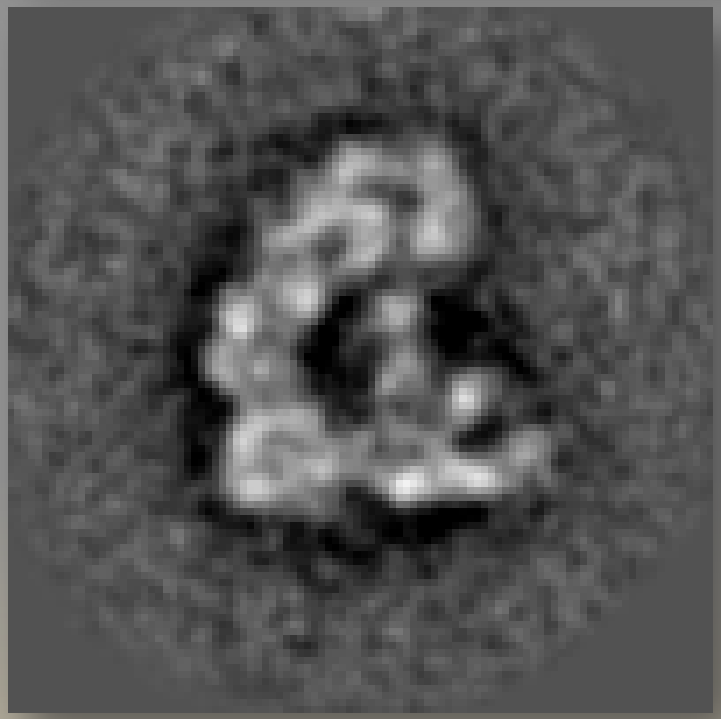
SPLICEOSOMAL U4/U6.U5 TRI-SNRNP



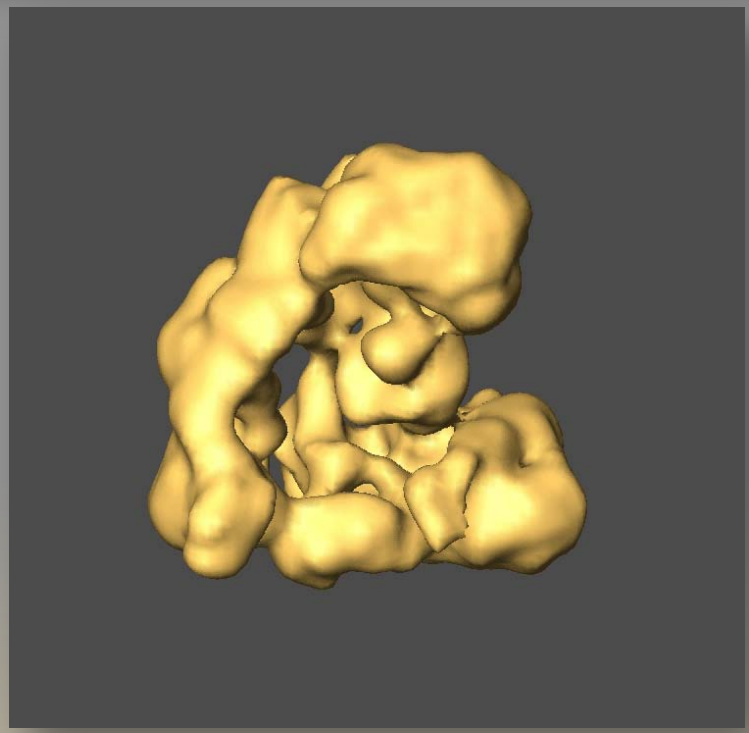

Analysis of heterogeneity by '3D' MSA
A
B
C

Anaphase Promoting Complex





Different orientations or
Different conformations?



Herzog et al., Science 2009

Size related problems…

• Random conical tilt data collection in cryo is technically challenging, especially for MW of <1 MDa

• Our technique also works in stain but tilt images in negative stain are prone to image artefacts due to flattening and inhomogeneous staining. The smallest macromolecule we did so far is ~400 kDa.

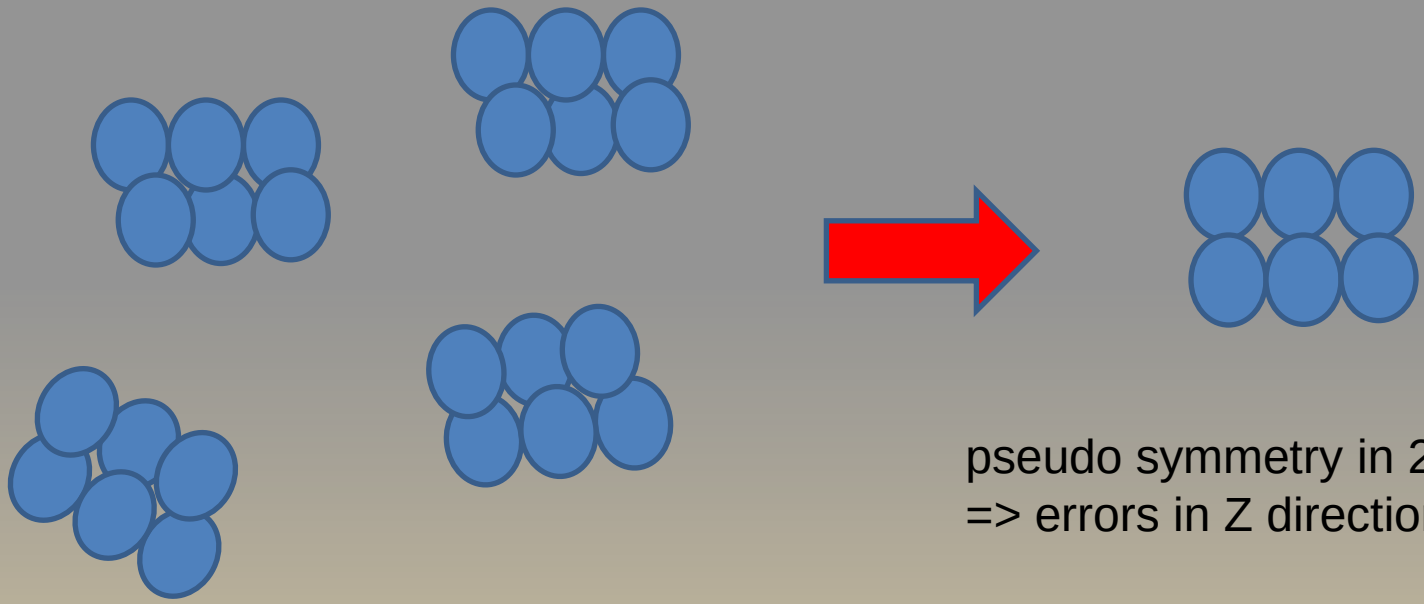
• low SNR – higher alignment errors - classification errors - wrong 3D models

• Solutions: high quality cryo images with excellent contrast (phase plates, better detectors, lower accelaration voltage)

We never do really well as long as we cannot determine the structural and conformational variability of the specimen in the initial structure determination phase!!!

In contrast to normal RCT...

- ...we use significant lower number of particle images per 3D structure. There is no reliable classification of images into classes comprising several hundred raw images!!!
- individual RCT 3D structures do suffer from the missing cone problem
- wrong 2D classification leads to pseudo symmetry
- wrong 2D classification leads to unreliable 3D models



pseudo symmetry in 2D plus flattening
=> errors in Z direction!!!

"High-resolution" refinement of wrong 3D models

• high-resolution refinement is usually done by projection matching!

• sometimes „wrong 3D models“ can easily be „refined“ to „high resolution“. Whenever there is little overlap in structural information of the raw data and the model, the noise in the raw images can be even more effectively aligned.
->overfitting of noisy data!!!

• wrong 3D startup models can easily be „refined“ to „high-resolution“ as judged by FSC curves

• ....this kind of „resolution“ depends mostly on image statistics, image filtering and available computer power...

• Example: we had a wrong exosome 3D model and „refined“ it to better than 5 Angstrom resolution by projection matching using ~250.000 raw images and a fine angular sampling of reference images.

We can now …

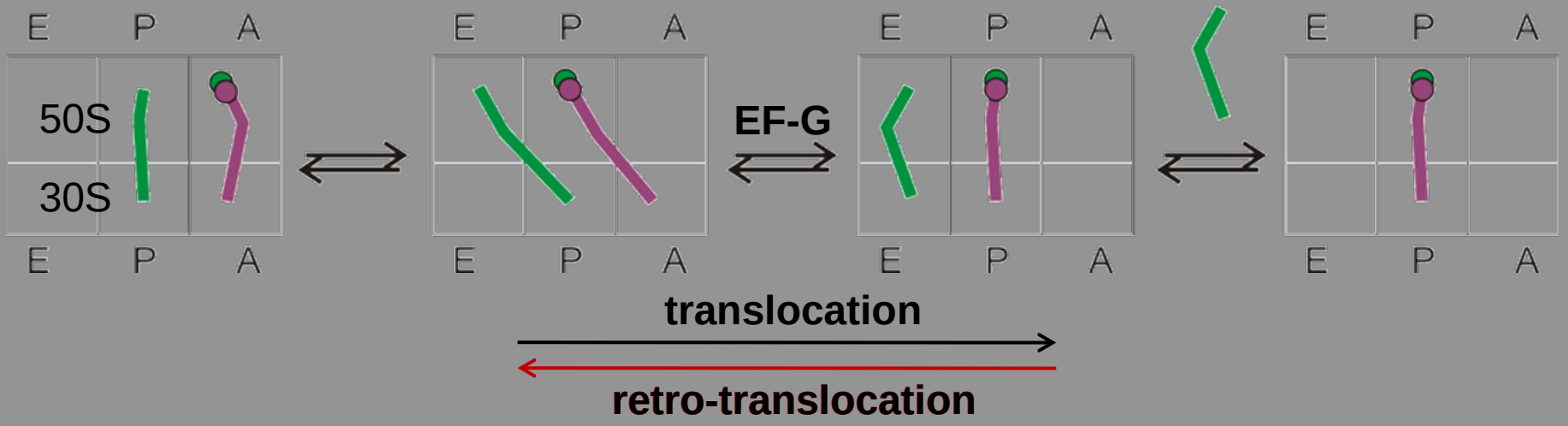
- ... determine bias free 3D structures of dynamic macromolecules at low resolution

- ... study the overall conformational space of macromolecules at low resolution

… can we do that also at higher resolution… ?

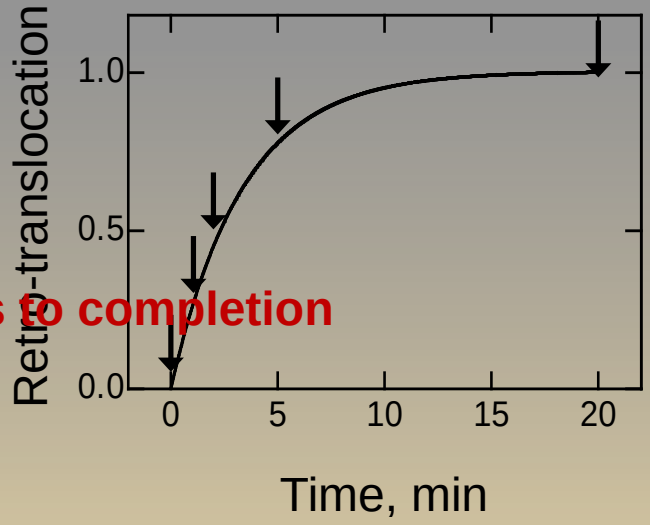
… and maybe even time-resolved?

Translocation:
tRNA movement through the ribosome

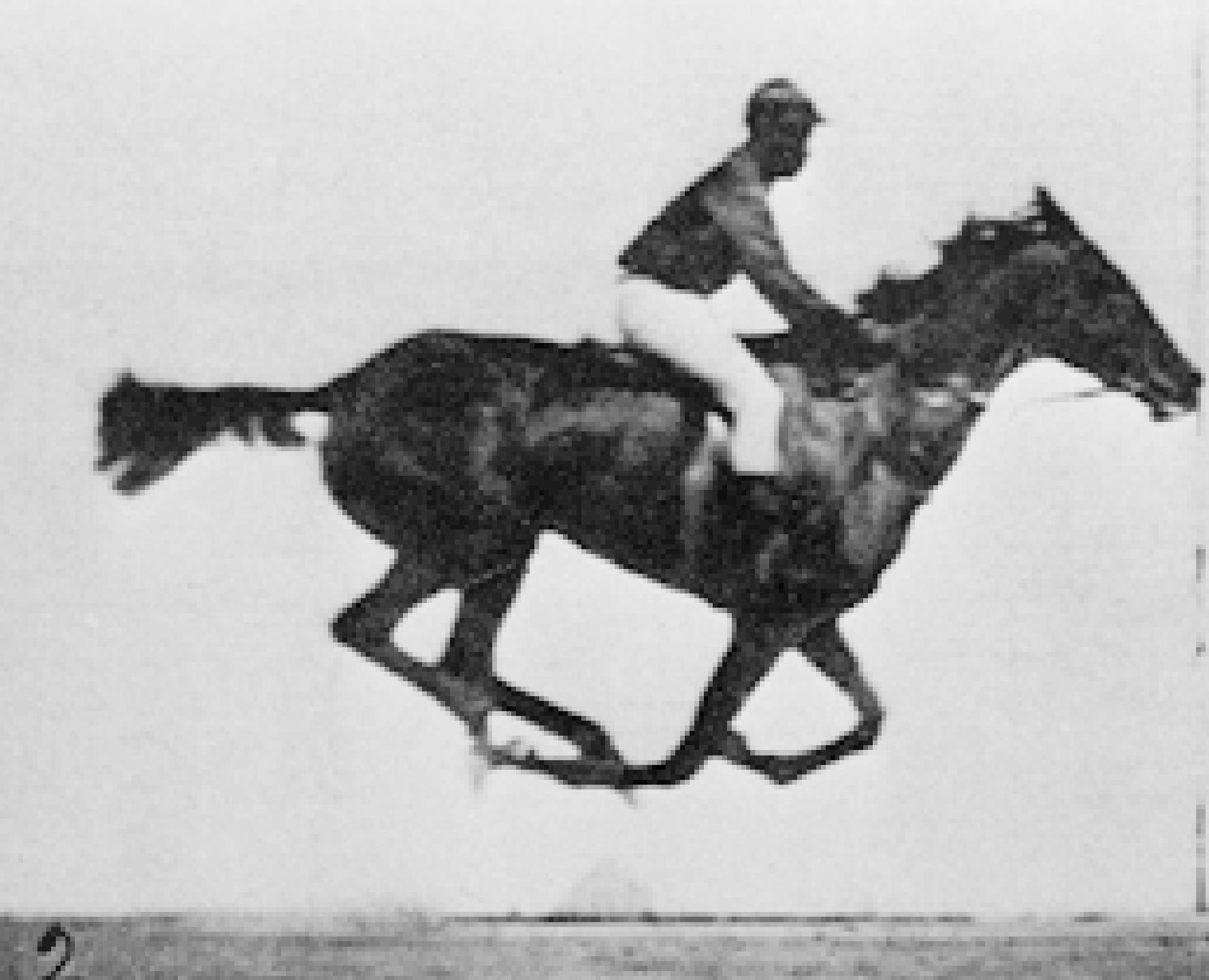


Time-resolved cryo-EM

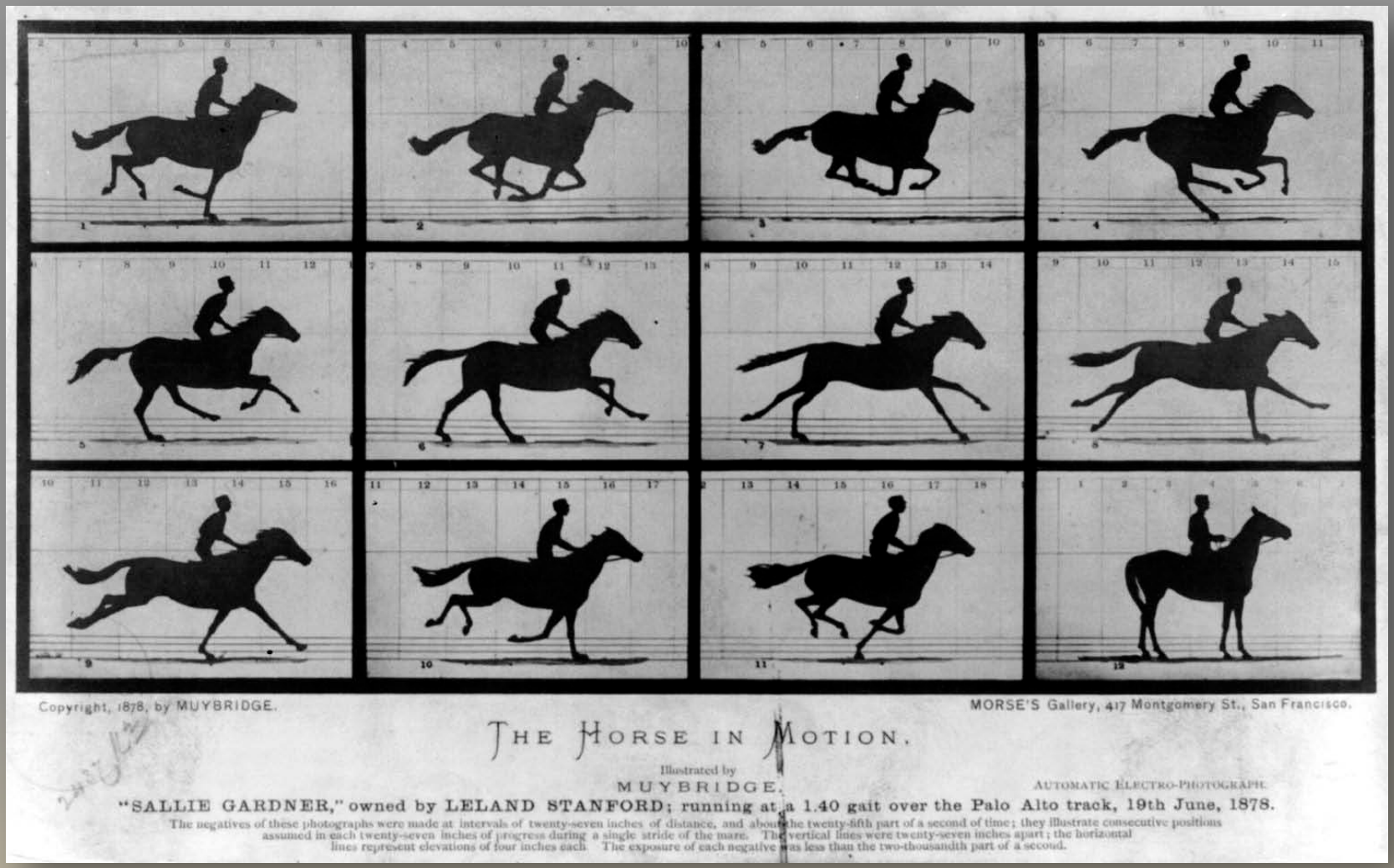
- EF-G catalysed translocation: ms time range
- Spontaneous forward translocation: inefficient
- **Retro-translocation: proceeds in 20 minutes to completion**
  - Data were collected at different time points (0, 1, 2, 5 and 20 minutes) at 18°C



Do horses "fly" while galloping?



They do "fly" !!!



Copyright, 1878, by MUYBRIDGE.

MORSE'S Gallery, 417 Montgomery St., San Francisco.

THE HORSE IN MOTION.

Illustrated by

MUYBRIDGE.

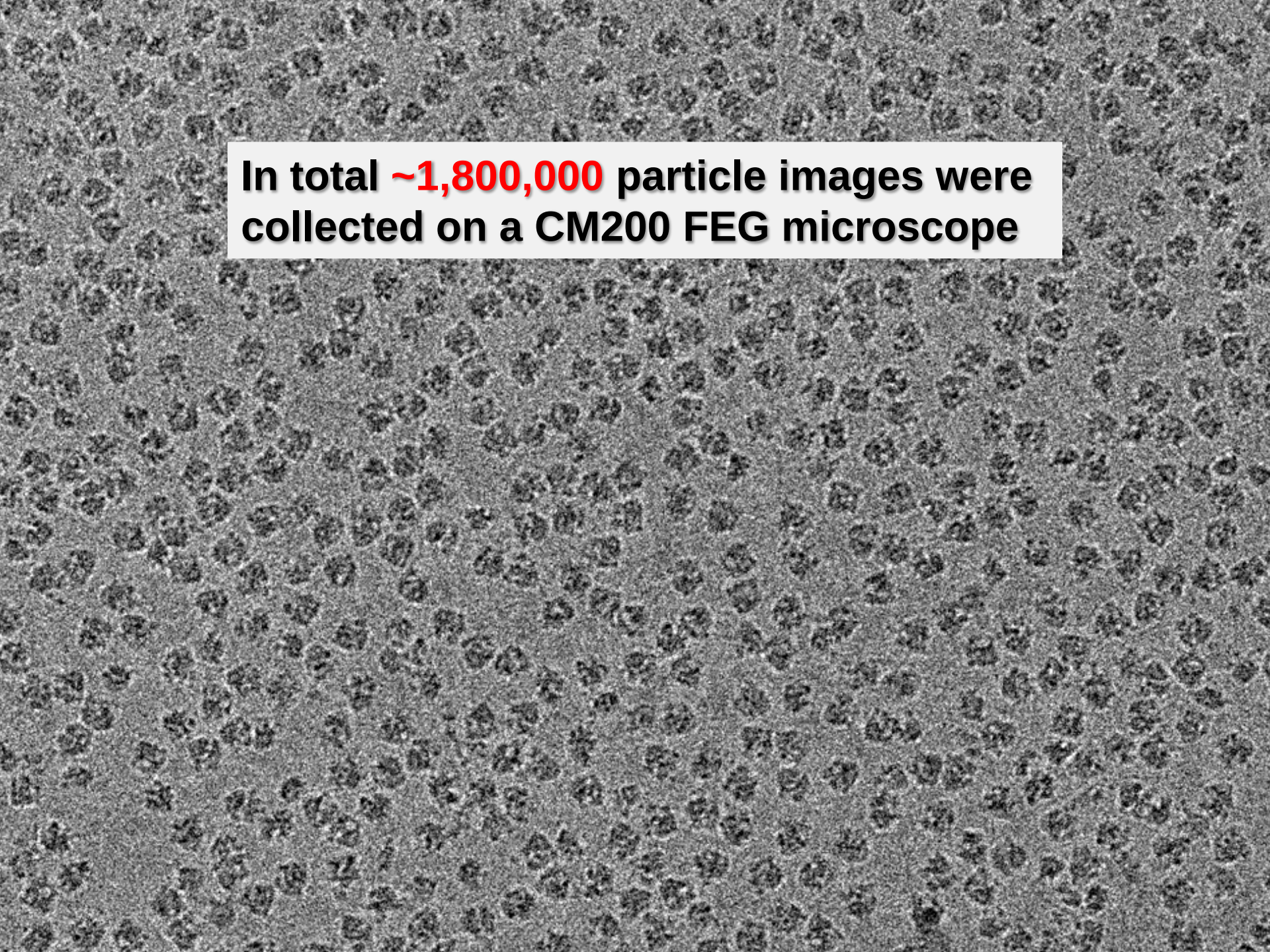
AUTOMATIC ELECTRO-PHOTOGRAPH.

"SALLIE GARDNER," owned by LELAND STANFORD; running at a 1.40 gait over the Palo Alto track, 19th June, 1878.

The negatives of these photographs were made at intervals of twenty-seven inches of distance, and about the twenty-fifth part of a second of time; they illustrate consecutive positions assumed in each twenty-seven inches of progress during a single stride of the mare. The vertical lines were twenty-seven inches apart; the horizontal lines represent elevations of four inches each. The exposure of each negative was less than the two-thousandth part of a second.

Horse problem: solved by a „single molecule technique“
far away from the thermodynamically favoured state

Cryo-EM: statistical method, not an ensemble method
elevated temperature

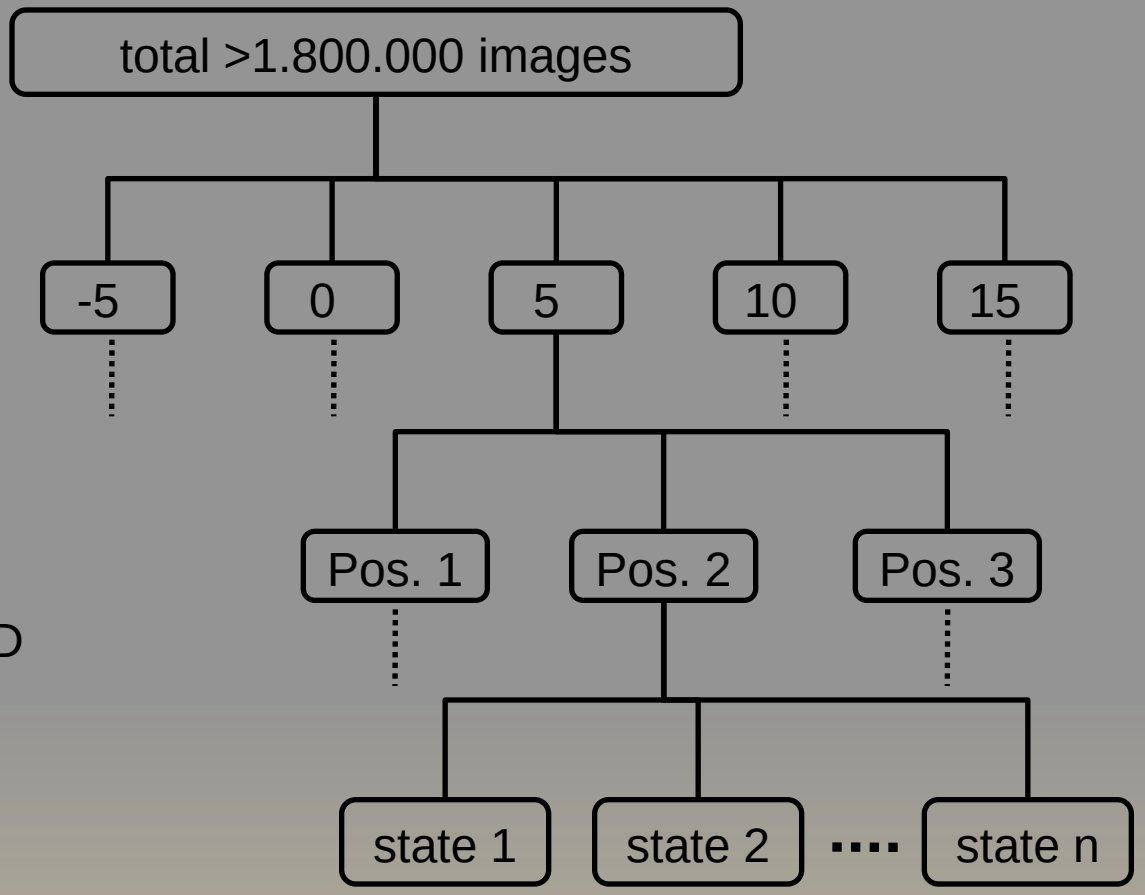

In total ~1,800,000 particle images were collected on a CM200 FEG microscope

Multi-step hierarchical classification

1. 30S body rotation
modeling by „relaxation“

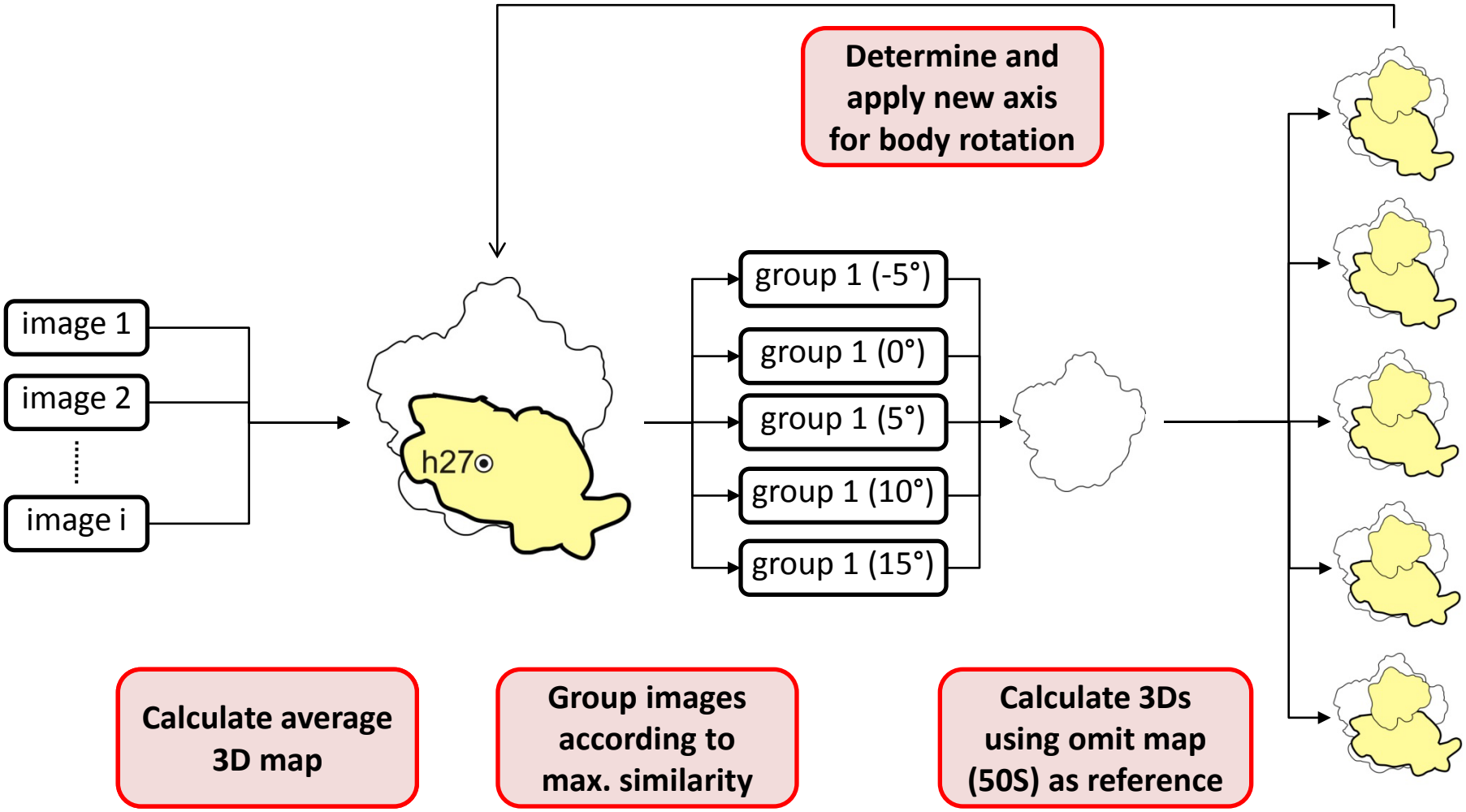
2. 30S head position
focused 3D MSA of bootstrap 3D volumes (Klaholz/ Penzcek)

3. tRNA densities
focused 3D MSA of bootstrap 3D volumes



⇒ 50 states/structures in total

Classification by 30S body rotation: Modeling by “relaxation”



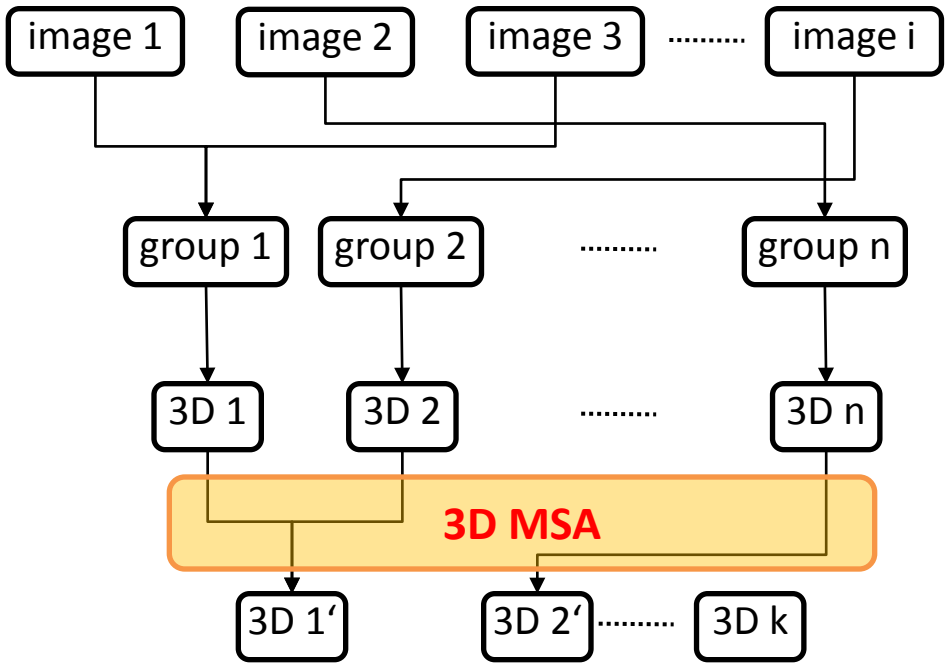
Classification by 30S head position and tRNA state: Focused 3D MSA (Klaholz/Penczek)

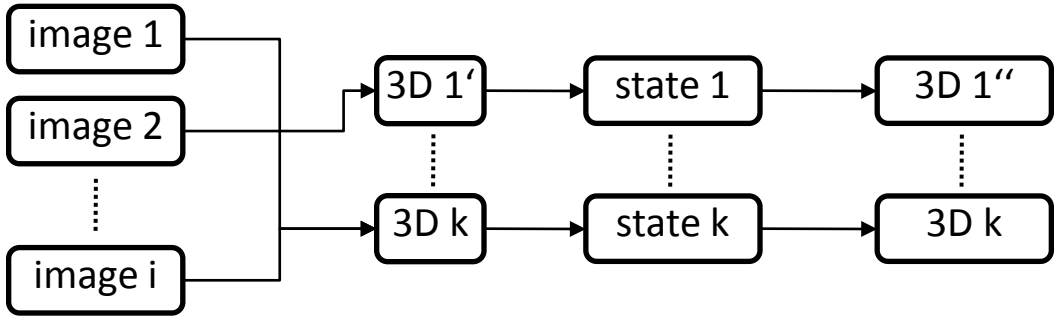
Bootstrapping

Group & Average 3Ds by similarity in confined area

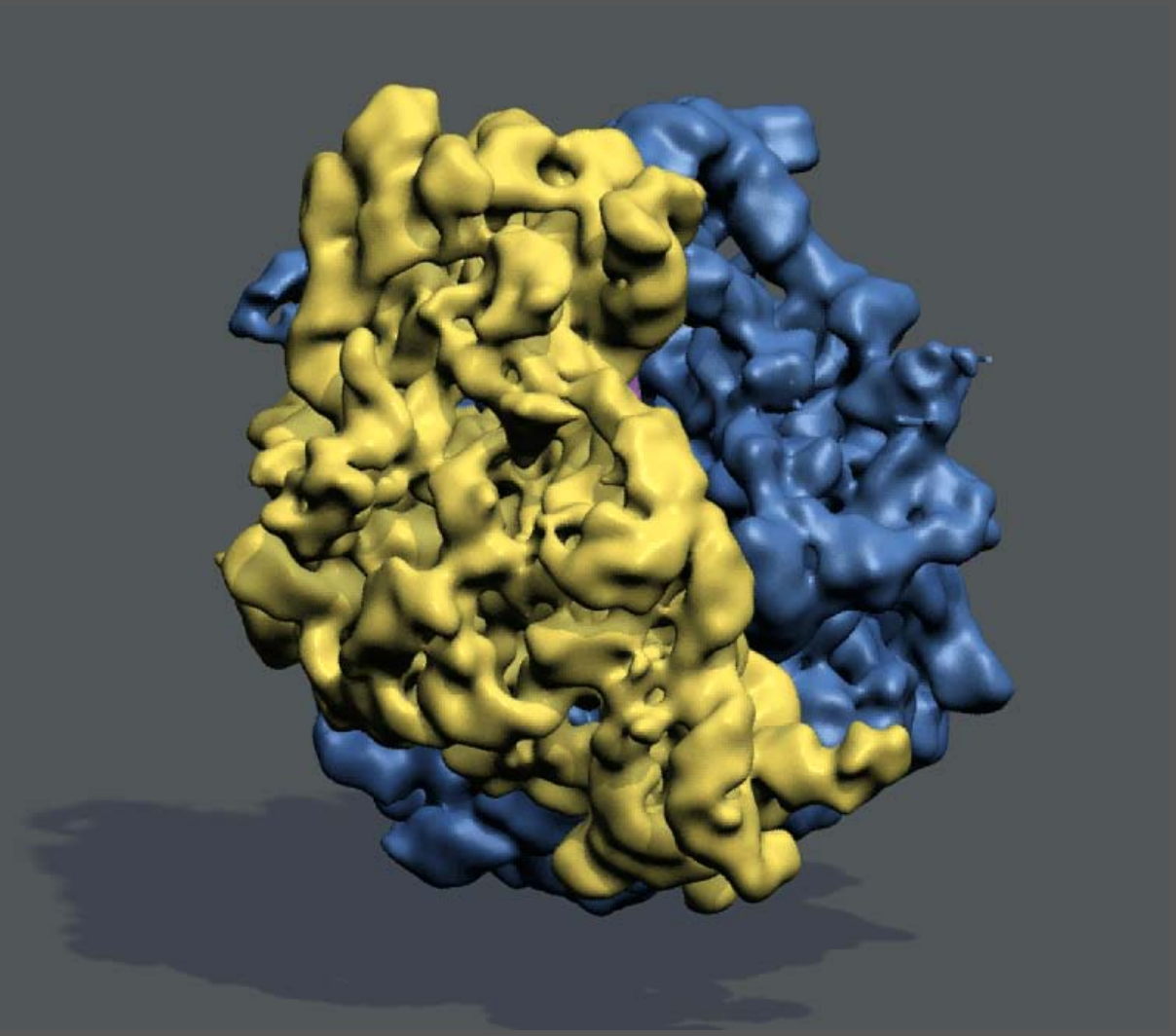
Sort images by similarity with class average 3Ds

Calculate 3D for each state using omit reference map



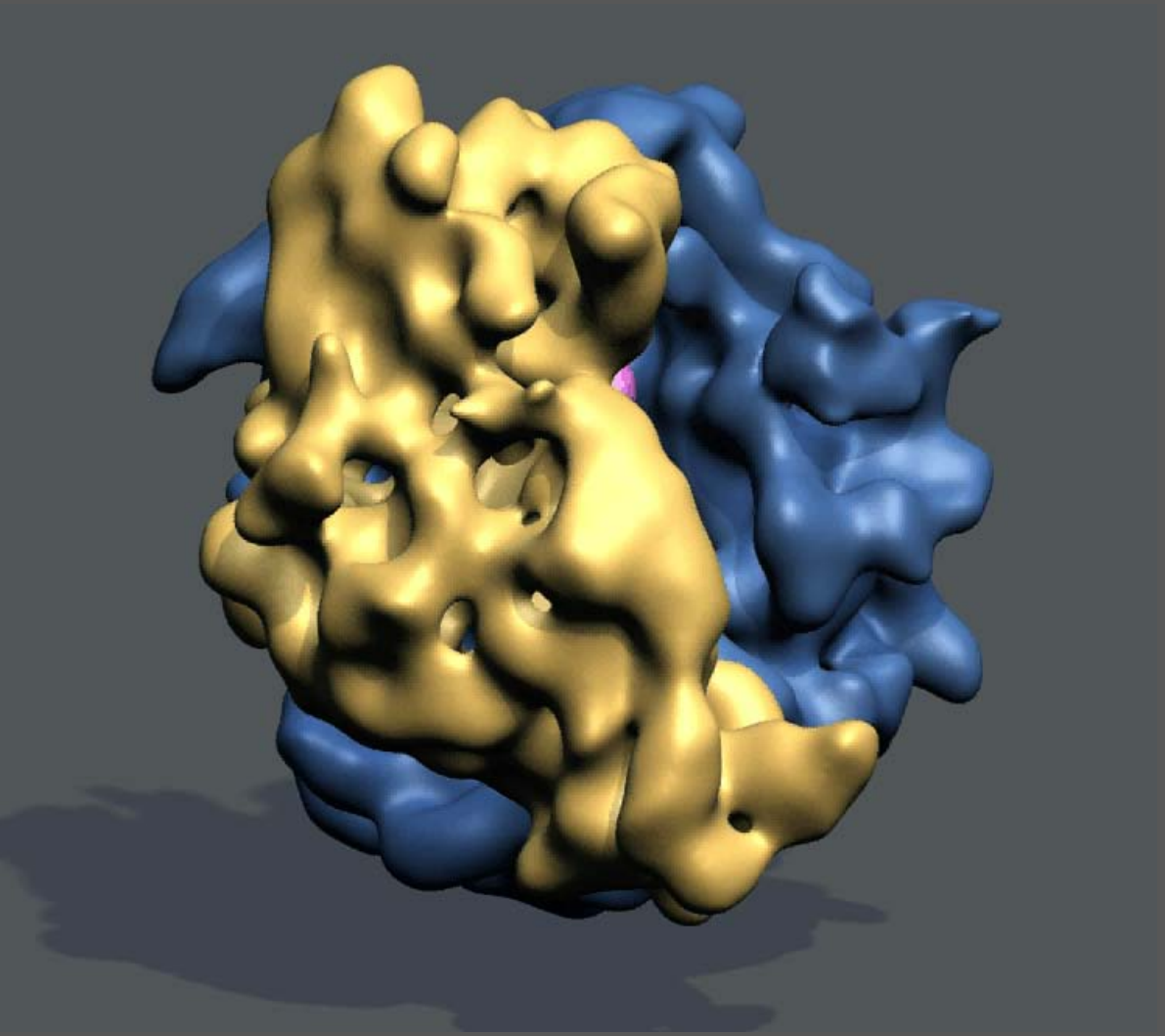


70S ribosome at 8-9 Å resolution



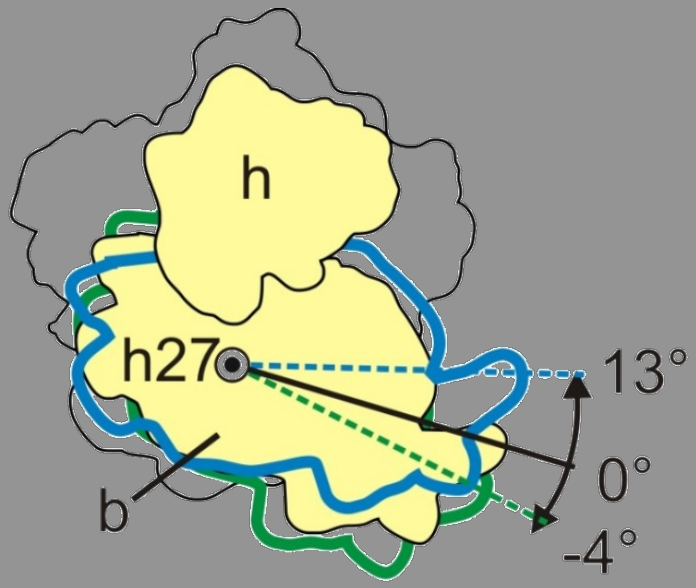
- only ~25.000 particle images
- isotropic resolution
- only limited by statistics...

New E-site tRNA position bound to the L1 stalk

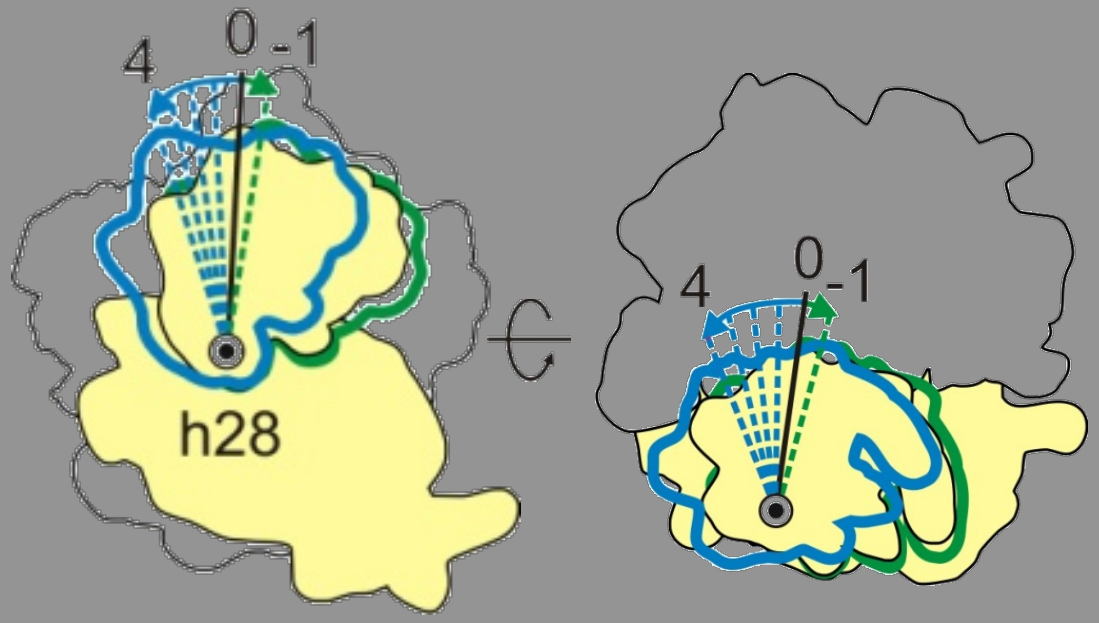


- only ~2.500 particle images
- less than 0.2 % of all images
- fully defined P-site tRNA

Quantifying 30S dynamics



30S body rotation



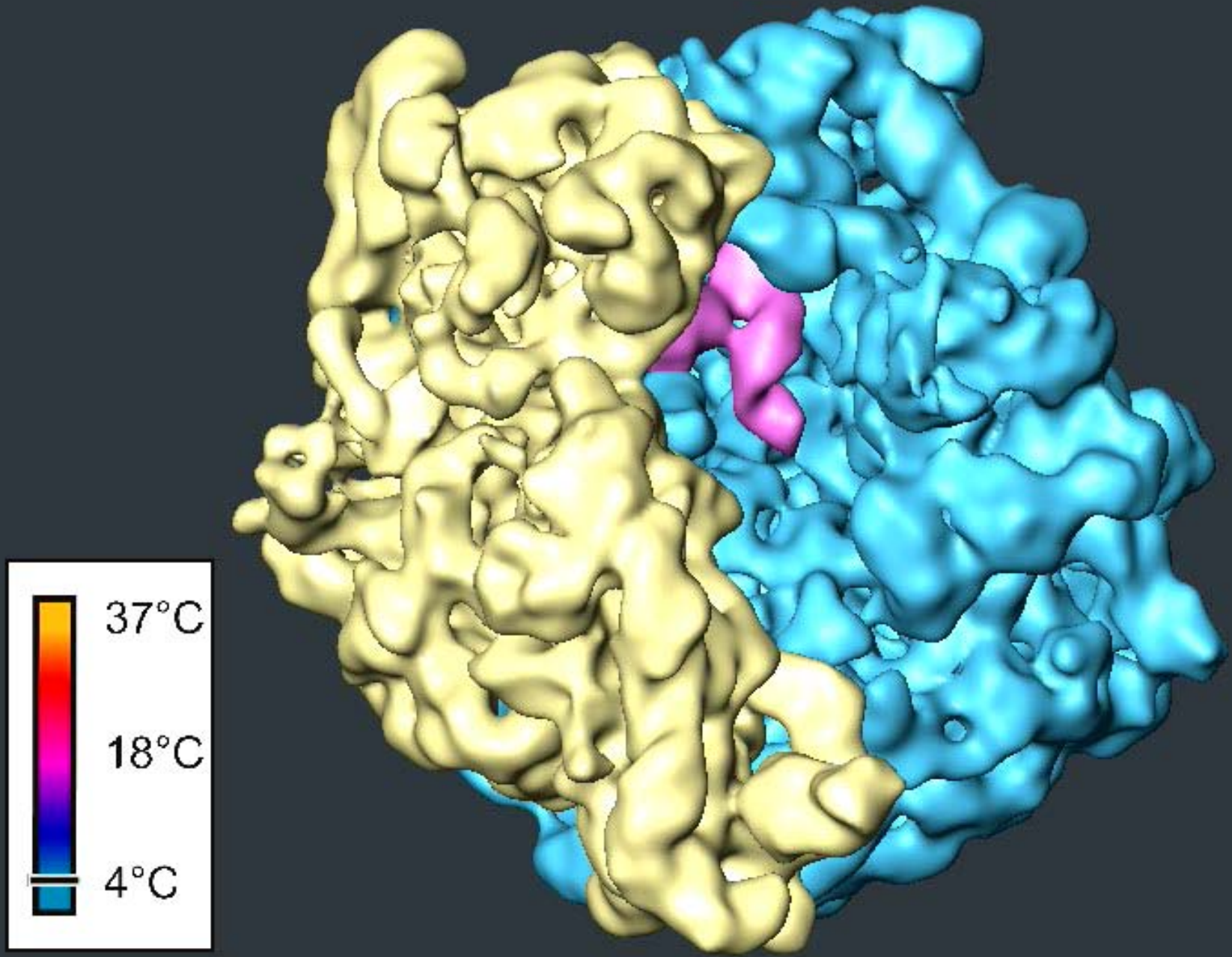
30S head movements

Temperature Dependence of Ribosome Dynamics

Various sample temperatures prior to vitrification :

4 °C, 18 °C, 37 °C

At time point zero (just one tRNA)
~25.000 images
Without computational sorting!


37°C
18°C
4°C

Chemical versus Thermal Energy

- A molecular motor that consumes 100-1000 ATPs per second has a chemical power of **10^{-16}** to **10^{-17}** W.

- The same motor moving through water is exposed to a thermal noise power of **10^{-8} W** (thermal energy kT at RT of 4x 10^{-21} J with a thermal relaxation time of ~10^{-13} s)

- 8-9 orders of magnitude higher noise power than power to drive directed motion.

- A Brownian motor can benefit from the thermal noise and convert it into directed motion by a mechanism for overcoming energy barriers.

Astumian & Hänggi, Physics Today, 2002

… are all "macromolecular machines" Brownian motors ?

- Chemical energy is negligible compared to thermal energy !
- „Macromolecular machines“ are in fact „thermal machines“
- Conformational transitions represent „micro ratchets“. The varying energy potential can be used to make the machines work following the principle of a Brownian motor.
- we can understand the true machine function of macromolecular complexes only by studying their dynamics at physiological temperature.

Summary

• Reliable 3D structure determination of dynamic macromolecules requires the **simultaneous** analysis of the structural variability.

• Time-resolved single particle cryo-EM can be done; applicable to other macromolecules.

• Computational sorting of images possible up to currently <1nm resolution for structural differences of 1%.

• Coupling of motion in macromolecules provides functionally important informtion.

• Kinetic rate constant and equlibrium constants from time-resolved cryo-EM data

• To study temperature dependent dynamics of macromolecular complexes is most probably important to fully understand the function of macromolecules.

Summary 2 – small complexes

No strict size limit!

Reliable structure determination is dependent on:

- size
- symmetry
- shape
- sample quality
- conformational homogeneity
- negative stain or cryo
- Image quality

Future improvements can be expected by:

- new detectors
- image phase plates
- improved computational tools
- maybe aberration correctors

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MPG

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