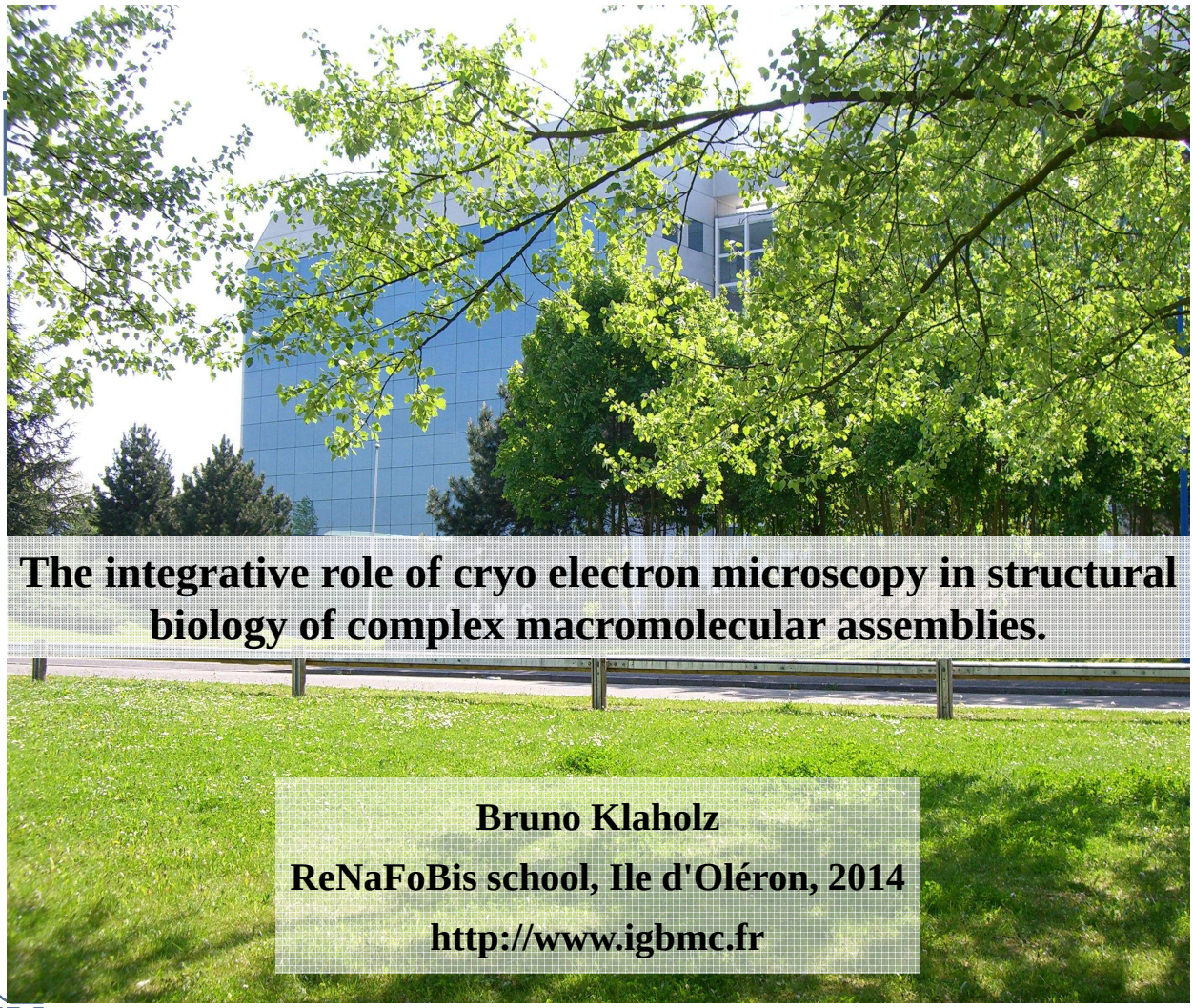

The integrative role of cryo electron microscopy in structural biology of complex macromolecular assemblies.

Bruno Klaholz

ReNaFoBis school, Ile d'Oléron, 2014

http://www.igbmc.fr

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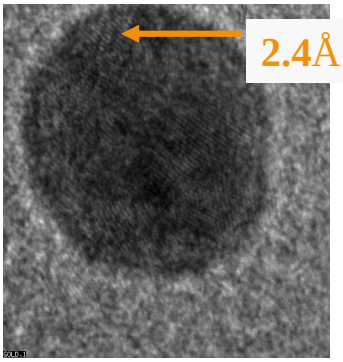
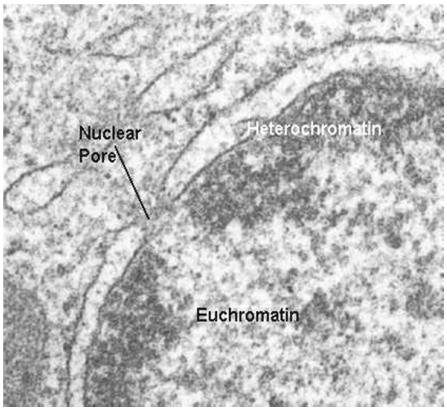
Electron Microscopy:

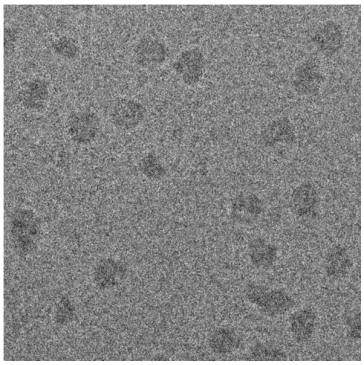
A) material sciences

B) cellular biology (cell sections)

C) molecular biology (extracted, purified single molecules)







Visual assignment of sample quality, visual annotation of cellular structure

Direct visualization, and more… **3D reconstruction!**
Involves a lot of image processing

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Plan:

I. Some basic concepts of cryo electron microscopy

II. Similarities between structural biology methods

III. Integrated structural biology examples using cryo-EM

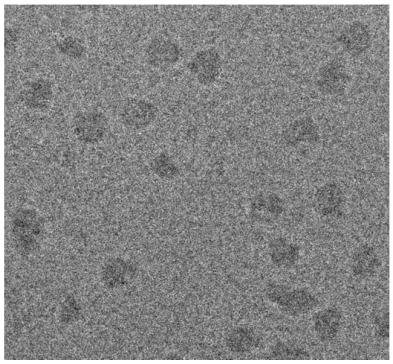
IV. Current & future challenges in cryo-EM

V. Instrumentation & technical highlights towards multi-scale integration

Some basic concepts of cryo electron microscopy

- visualize biological complexes in a hydrated, functional state
- images are 2D projections of a 3D object, i.e. they contain all internal features
- requirement: see the object under different angles to be able to reconstruct it

particles in ~ random orientations

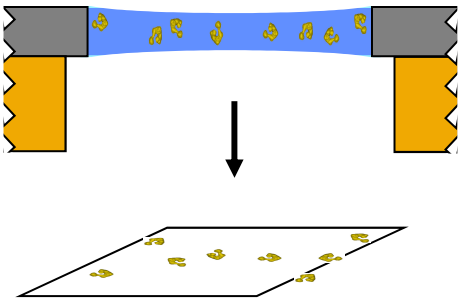


Some basic concepts of cryo electron microscopy

extracted, purified complexes,

preserved in hydrated state:

flash-frozen in the buffer



sample conc.: **~ 0.5 mg/ml**
[compare 3D crystallization: ~2-20mg/ml]

<u>Prioritize cryo-EM over negative staining EM:</u>

- avoids artifacts and limitation in resolution (~30Å) due to staining artifacts and flattening of the structures

- **cryo-EM provides best specimen preservation:**

no adsorption, no drying

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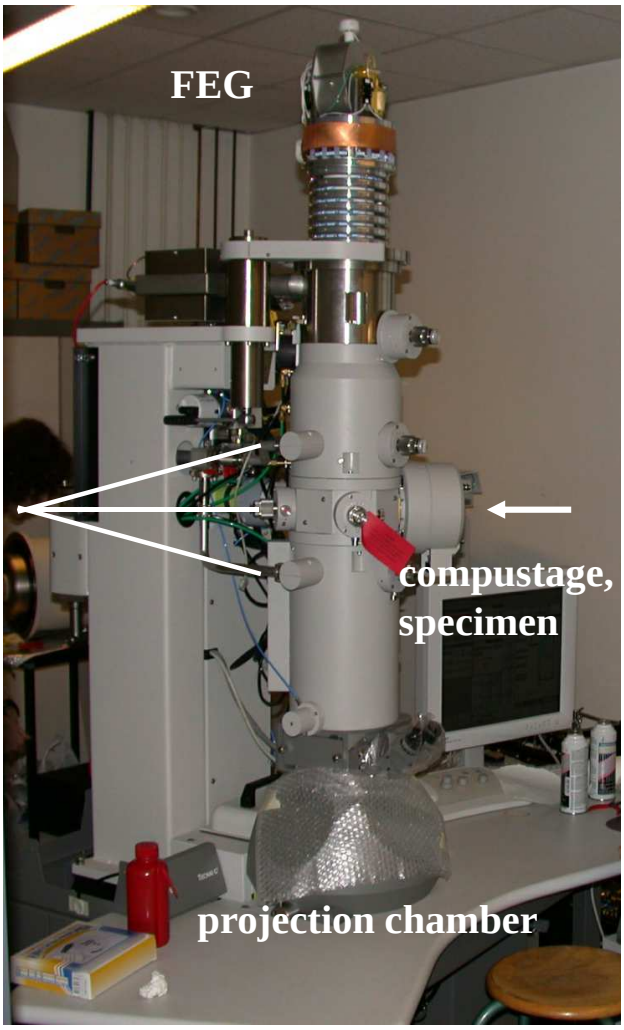
A transmission electron microscope (TEM)



source

sample

image acquisition

- **vacuum: ~10^{-6} Pa**
- **potentially high electron dose**
- **potentially high resolution**
  (λ≈ 0.025Å at 200kV)
  i.e. <u>not limited by the wavelength</u> or the optical system

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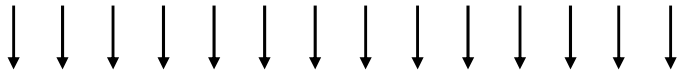
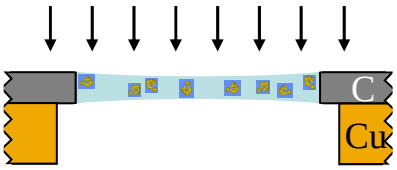
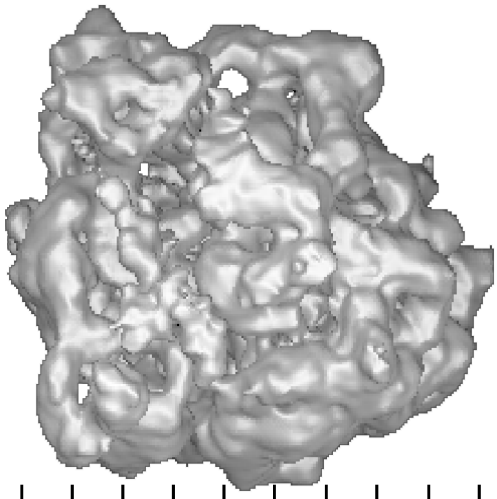
<u>Field emission gun (FEG) electron microscope (Tecnai20, IGBMC)</u>



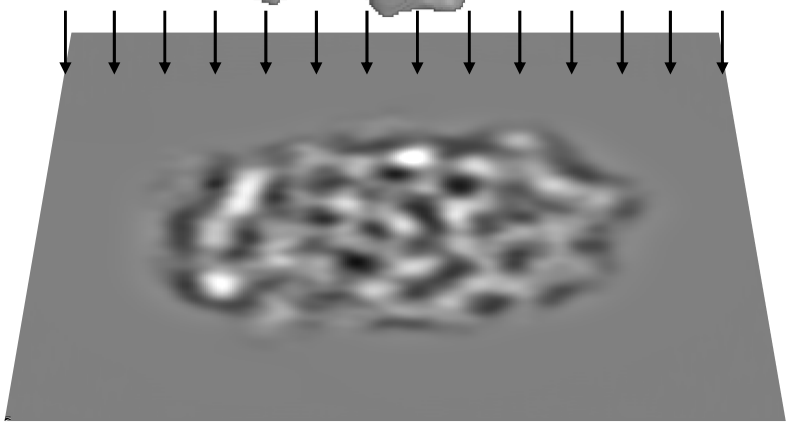
electrons



transmission
electron microscopy!



object (3D)



scattered electrons

projection (2D)

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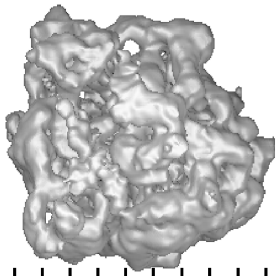
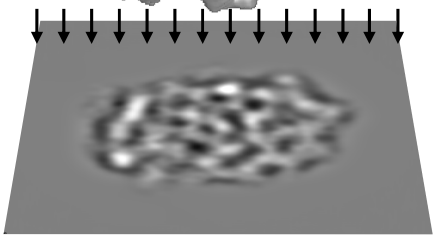
Transmission electron microscopy

electrons



object (3D)

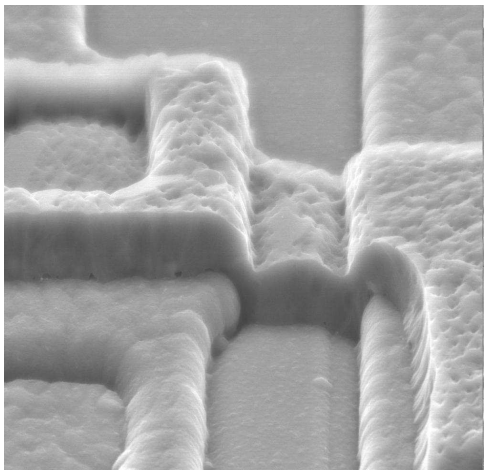


scattered electrons

projection (2D)

All *internal* features in a 2D view!

Scanning electron microscopy



Only surface!

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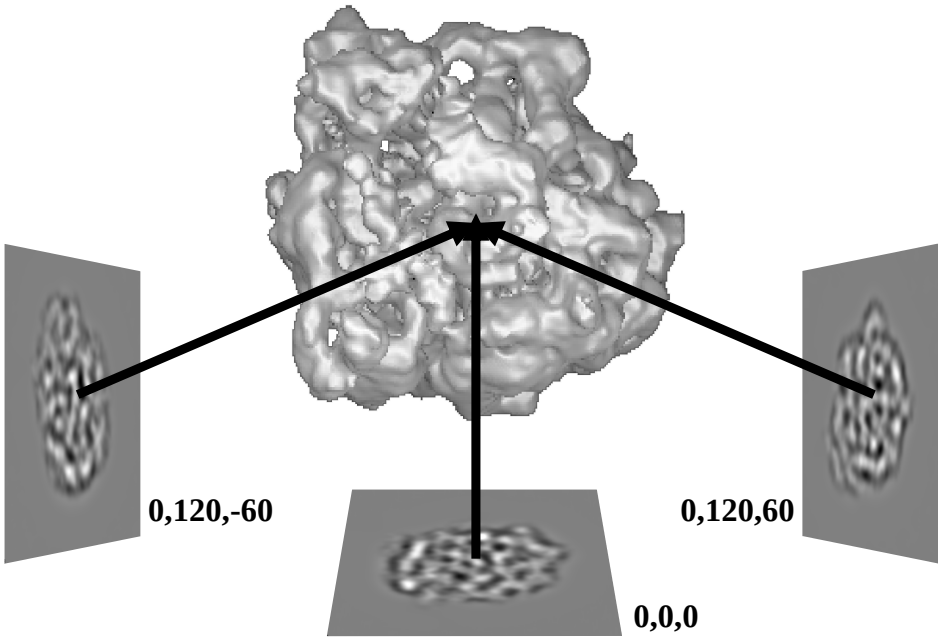
Concept of 3D reconstruction: back-projection (franc.: rétro-projection)



Reconstruction provides a 3D density map, i.e. a **structure** with all internal features

Requires to have **angles** assign by:

- **angular reconstitution (in early stage of structure determination), or**
- **projection matching (if structure already refined; reference-dependent; bias), or**
- **maximum likelihood**

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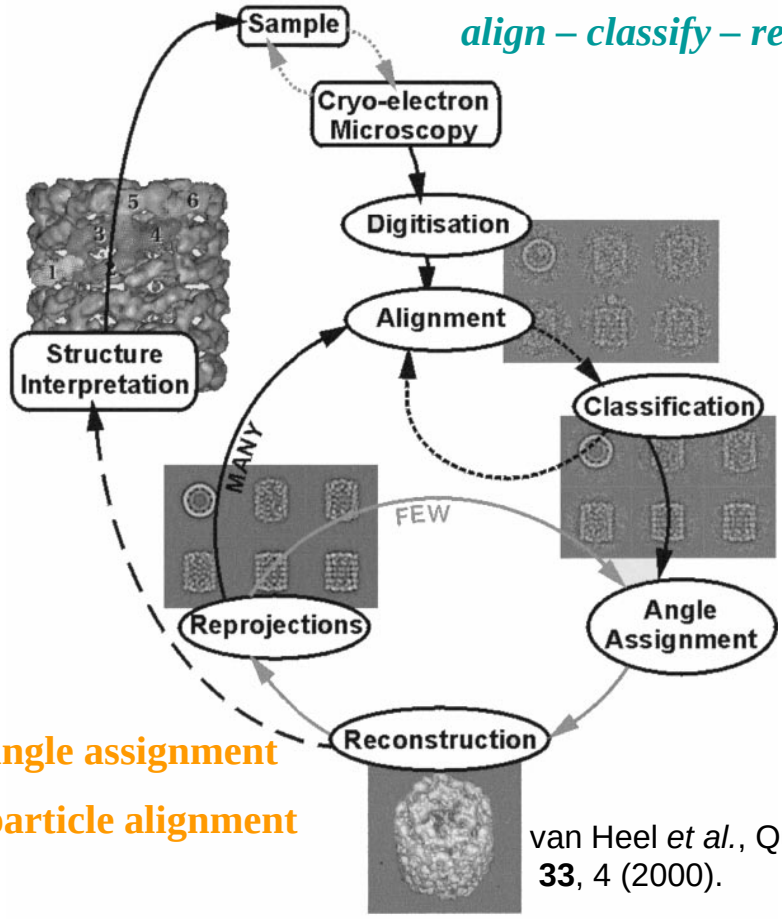
Structure determination and refinement in cryo-EM



align – classify – reconstruct in 3D

- **improve quality of angle assignment**
- **improve quality of particle alignment**

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

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Some basic concepts of cryo electron microscopy

Correct terms are important (be precise and rigorous in science):

By cryo-EM, we obtain:

- a "3D reconstruction" (initial or refined)
- a "cryo-EM map" or "density"
- a "structure"

technically:

- back-projection
- angular reconstitution
- random conical tilt
- tilt series / tomogram

NOT:

- an "envelope" (would be SAXS or neg. stain. EM)
- a "volume", units would be Å^3 (e.g. volume of a pocket, volume x density = mol. mass)
- a "surface", units would be Å^2 (e.g. interaction surface between 2 proteins)
- a "model", would be a molecular model *fitted to* the map (crystallography/cryo-EM)
  or a model *compatible with* SAXS data or NMR restraints;
  other models: "homology model", "hypothetical model", "working model"

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Some basic concepts of cryo electron microscopy

Correct terms are important:

A classification is based on a statistical analysis:

- multivariate statistical analysis (MSA) provides information on variance (variability) which serves to merge similar images into class averages (classes); is *independent* of a reference
- classes *are NOT*: the sum of images that correlate best with a reference (through a multi-reference alignment)

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Some basic concepts of cryo electron microscopy

Basic aspects:

- "resolution" is called "frequency" in image processing
- 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

Consider: *pixels in 3D: "voxel"*

- 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 resolutio
  - 0.143 (Henderson) and ½ bit (van Heel)
  - 3 σ, not used anymore (over-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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Single particle cryo-EM image processing and 3D reconstruction

I. Pre-processing

- Digitization of micrographs (negatives); not needed if CCD images
- particle selection, « boxing »
- correction of the contrast transfer function
- band-pass filtering and normalisation of particle images

II. Structure determination

- particle centering / alignments
- MSA (multivariate statistical analysis) + classification
- angle assignment
  - angular reconstitution
  - projection matching
- 3D reconstruction
- structure refinement
- resolution assessment: criteria + what you can resolve in the 3D map!
- map interpretation ; fitting of crystal or NMR structures, …

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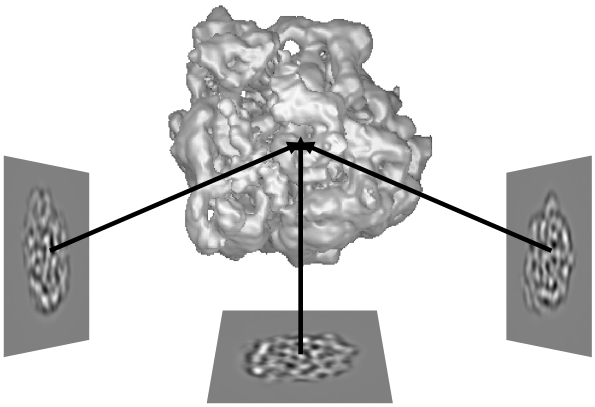
II. Similarities between structural biology methods

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

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The importance of cryo-approaches

For both crystallography and cryo-EM:

- preservation of the hydrated, functional state

- reduction of irradiation damage

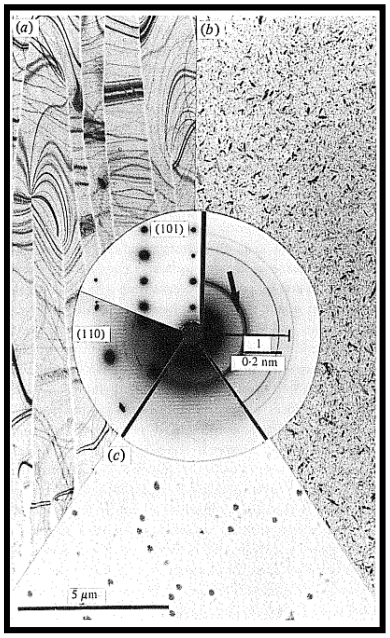
- mechanical stabilization of the sample

cryo-EM:

flash-freezing,

low salt, no cryo-protectants

(would reduce image contrast)



Dubochet *et al.*, 1988

crystallography:

cryo-protectants

glycerol, PEG, high salt, oil, etc.

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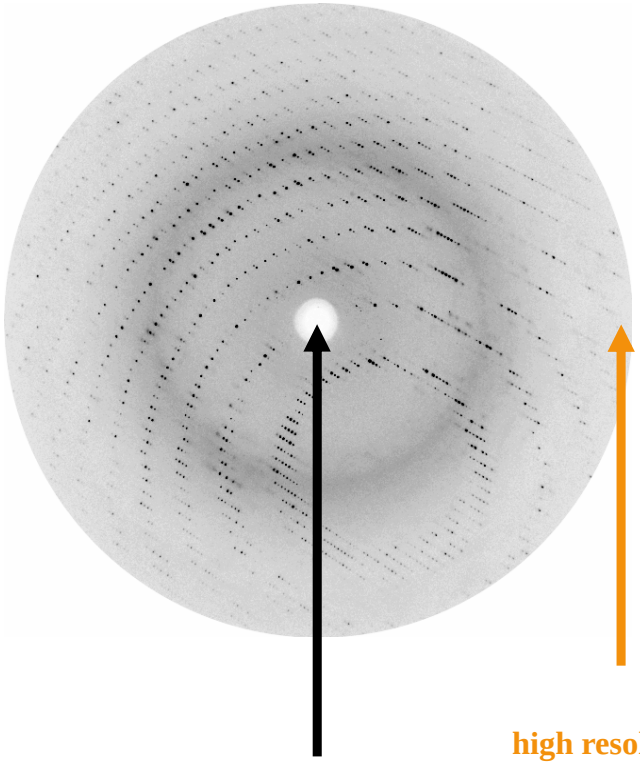
Complementarity of structural approaches: similarities between methods

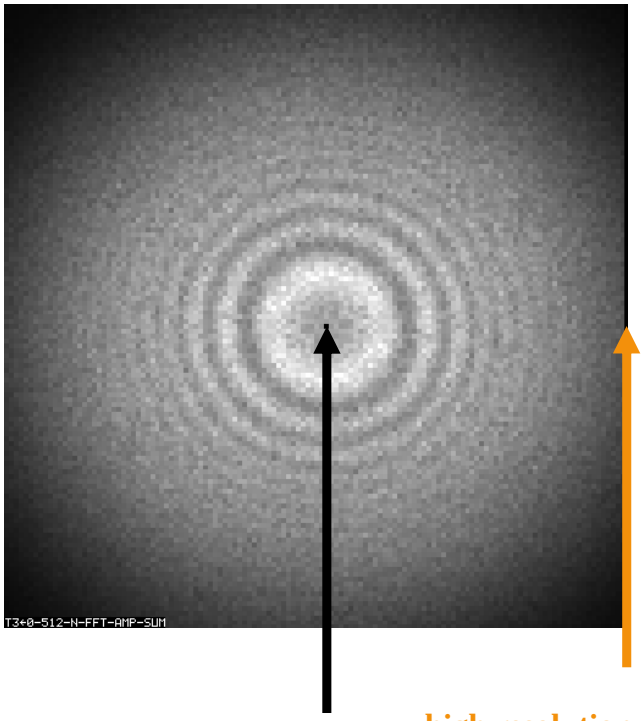
crystallography



low resolution

high resolution

cryo-EM



low resolution
low frequency

high resolution
high frequency

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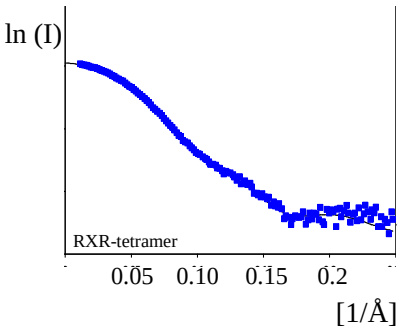
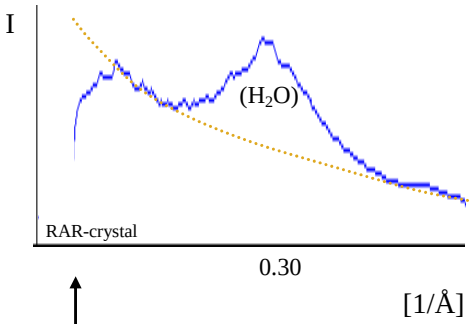
Complementarity of structural approaches: similarities between methods

Profile of the intensity distribution

SAXS

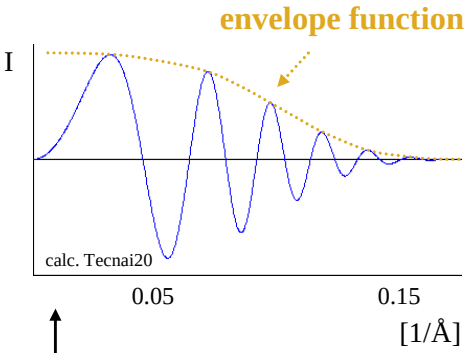


crystal diffraction



beamstop

cryo-EM



very low resolution difficult to measure

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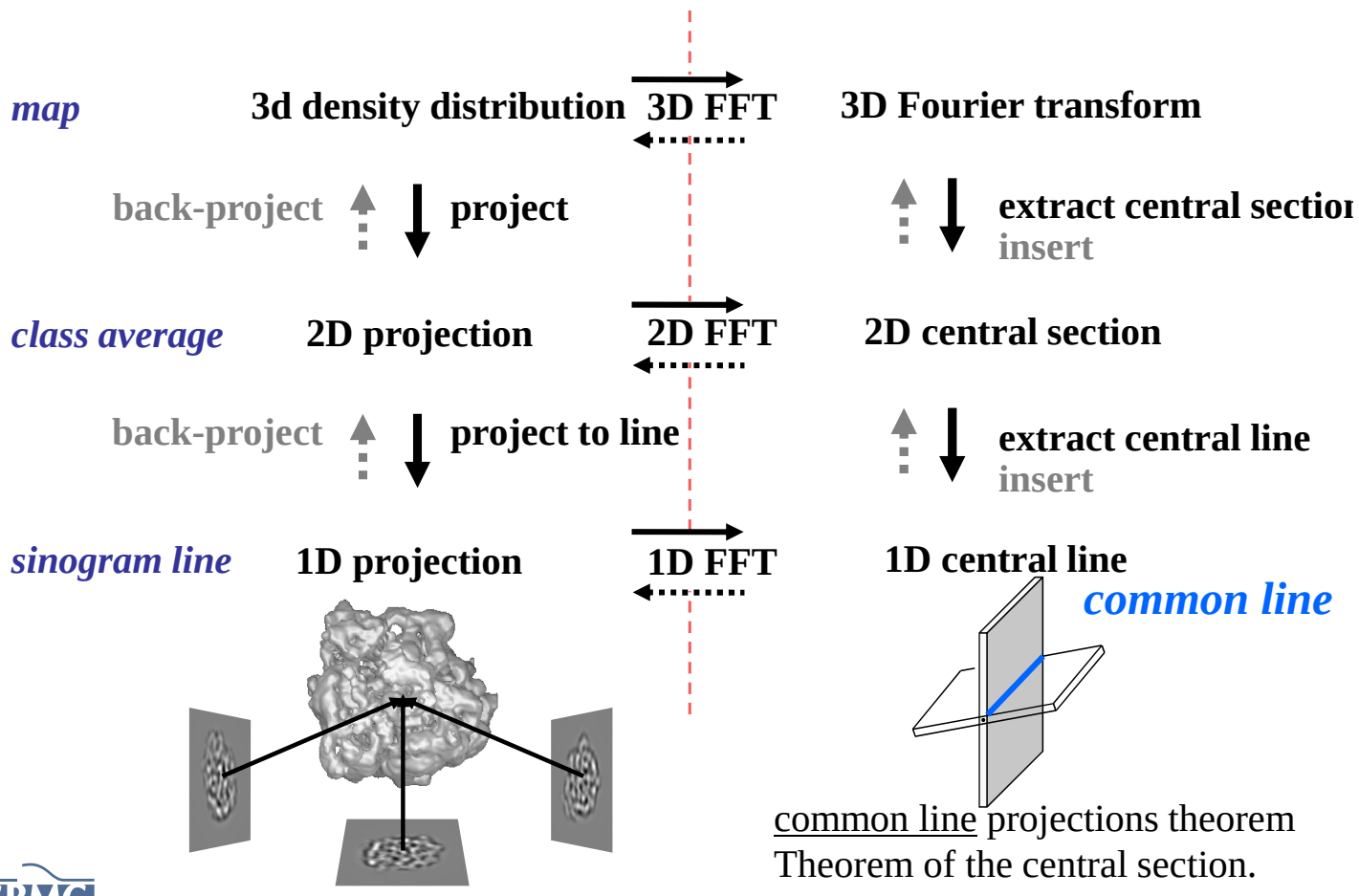

common line projections theorem
Theorem of the central section.

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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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III. Integrated structural biology examples using cryo-EM

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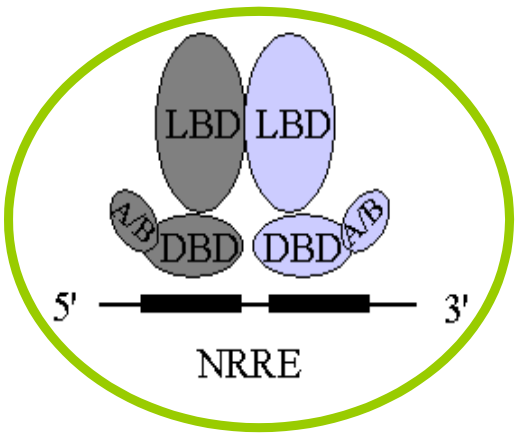
Structure and function of full nuclear receptors

- architecture of full-length DNA-bound NR complexes
- topology of full-length NR's bound to different response elements
- mechanism of ligand- and DNA-dependent activation and co-regulator recruitment
- important targets for biomedical research (steroids, vitamin D etc.)



"textbook drawing"

Possible structural approaches:

- NMR
- crystallography
- SAXS
- cryo-EM

molecular weight: ~100-150kDa

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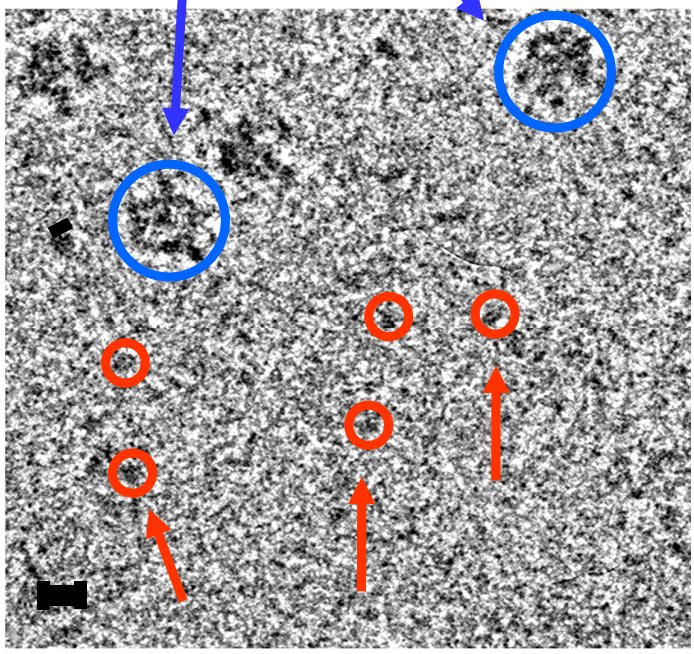
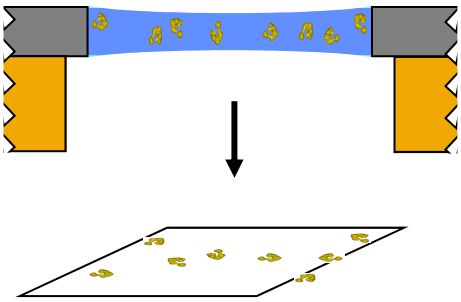
Architecture of the RXR/VDR DR3 DNA complex

50S ribosomal subunit

~1.3MDa



flash-frozen in the buffer

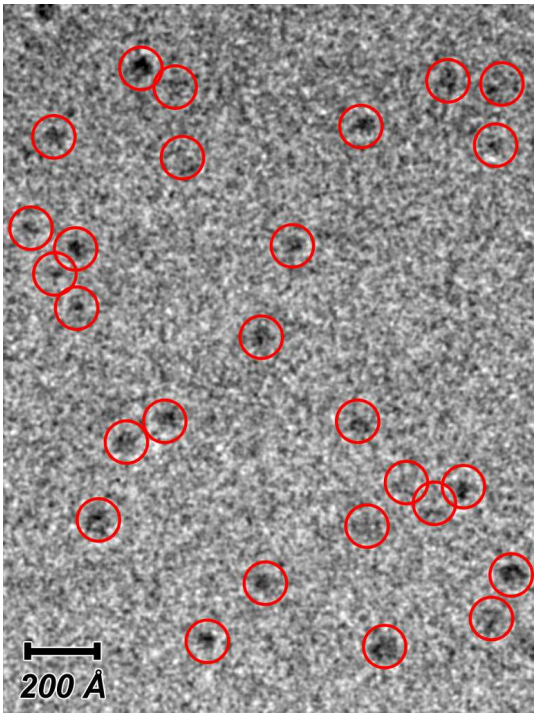


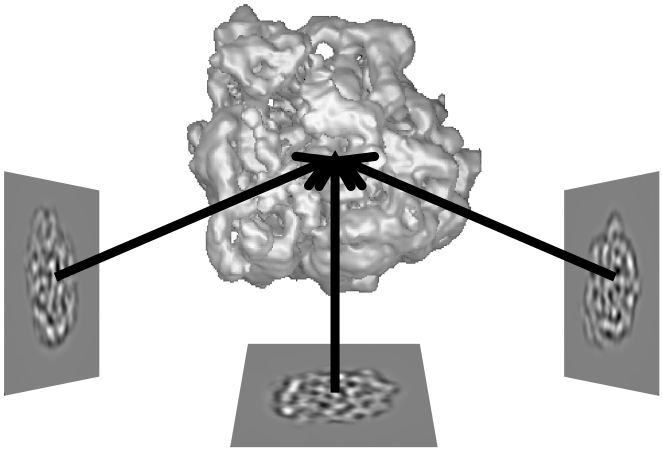
100 Å **NR complex ~100 kDa** ***200kV data***

Avoid a priories…:

was usually considered too small for cryo-EM… (limit >250kDa)

Image processing: particle selection, classification, structure determination





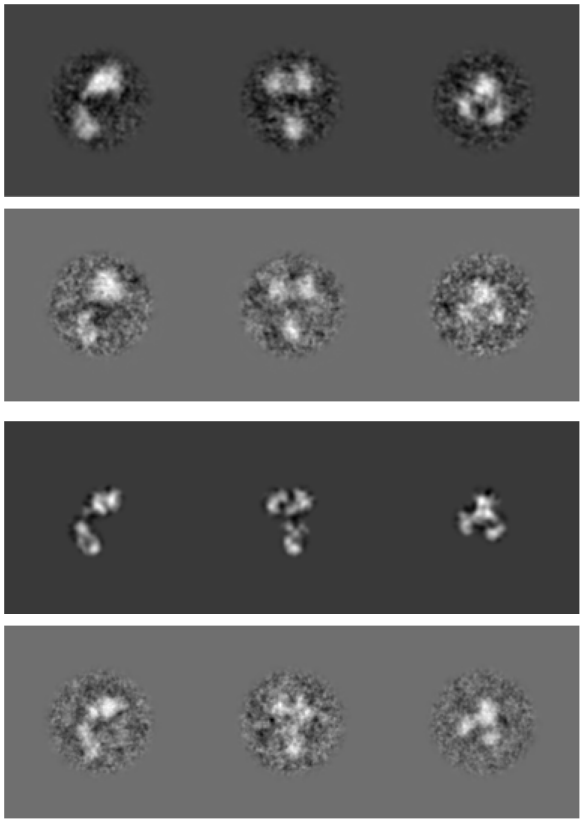
angle determination and 3D reconstruction

20 000 particles selected, defocus used: -2 to -4 µm

MSA, classification, common-line angle assignment and refinement (Imagic)

(no projection matching)

Two independent *ab initio* structures:



100kV data
Re-projections

4k CCD, Polara

100kV data
Class averages

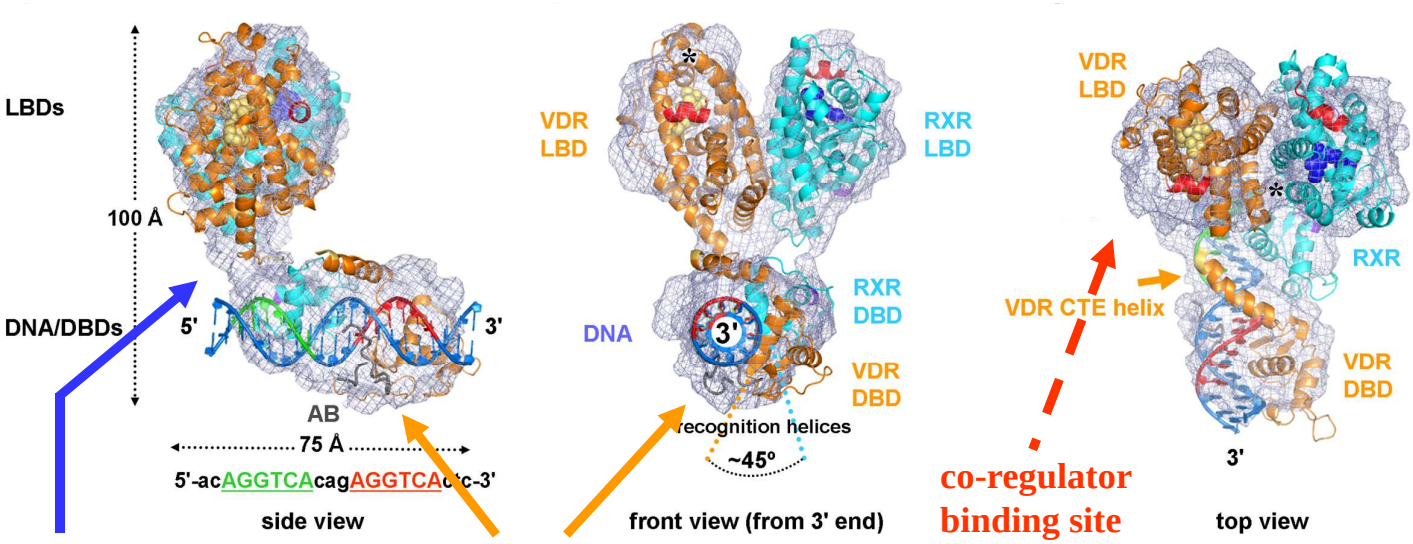
200kV data
Re-projections

film, Tecnai20

200kV data
Class averages

→ 3D correlation ~90%!

Architecture of the RXR/VDR DR3 DNA complex



linker region (hinges) visible

A/B domain - DNA contact

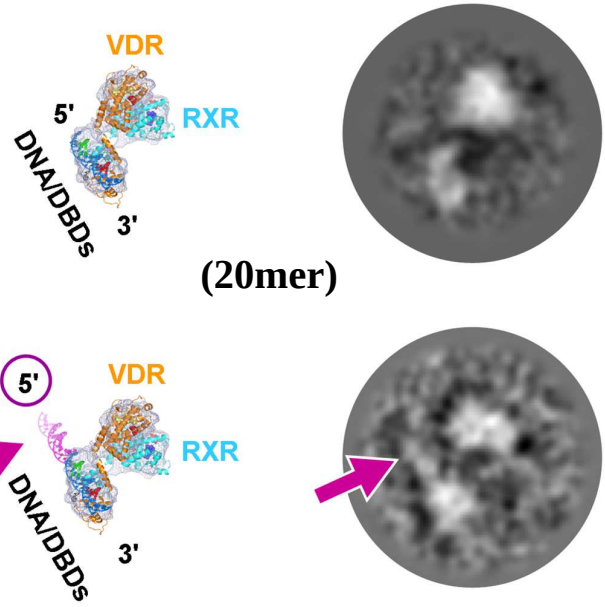
co-regulator binding site

Orlov *et al.*, *EMBO J.* 2012.

key concepts: - asymmetric topology
- open conformation

Architecture of the RXR/VDR DR3 DNA complex

Assignment of DNA polarity:

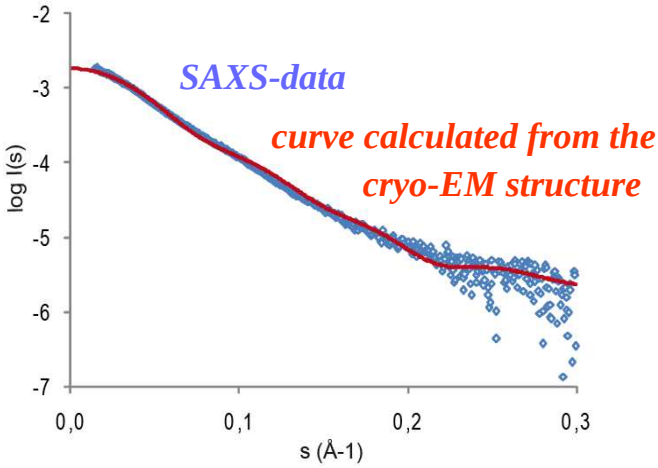


5'-extended DNA

(35mer)

→ *independent structure validation*

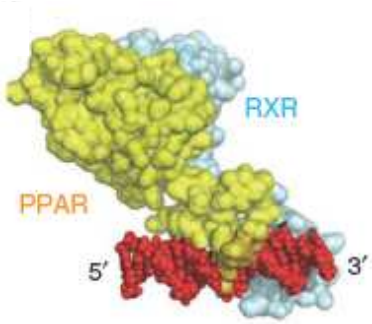
Consistency of cryo-EM and SAXS data:



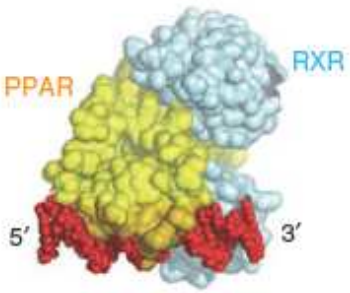
Orlov *et al.*, *EMBO J.* 2012.

Architecture of nuclear receptors on DR1 response elements

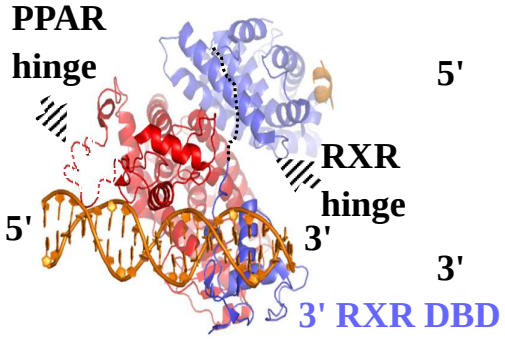
PPAR/RXR DR1



solution



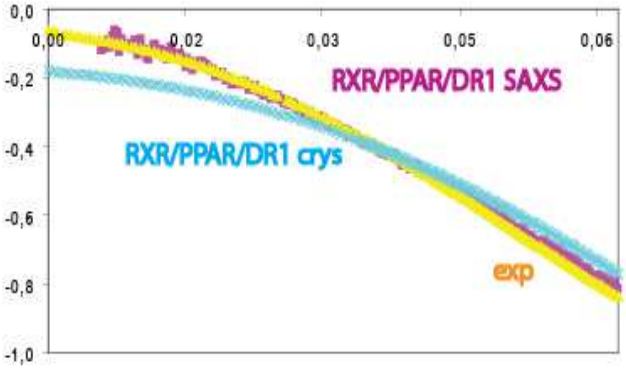
crystal



PPAR/RXR DR1 crystal structure

(Chandra *et al.*, *Nature* 2008)

SAXS-derived PPAR/RXR model



different protein conformation in the crystal and in solution

Rochel et al., Nat. Struct. Mol. Biol., 2011.

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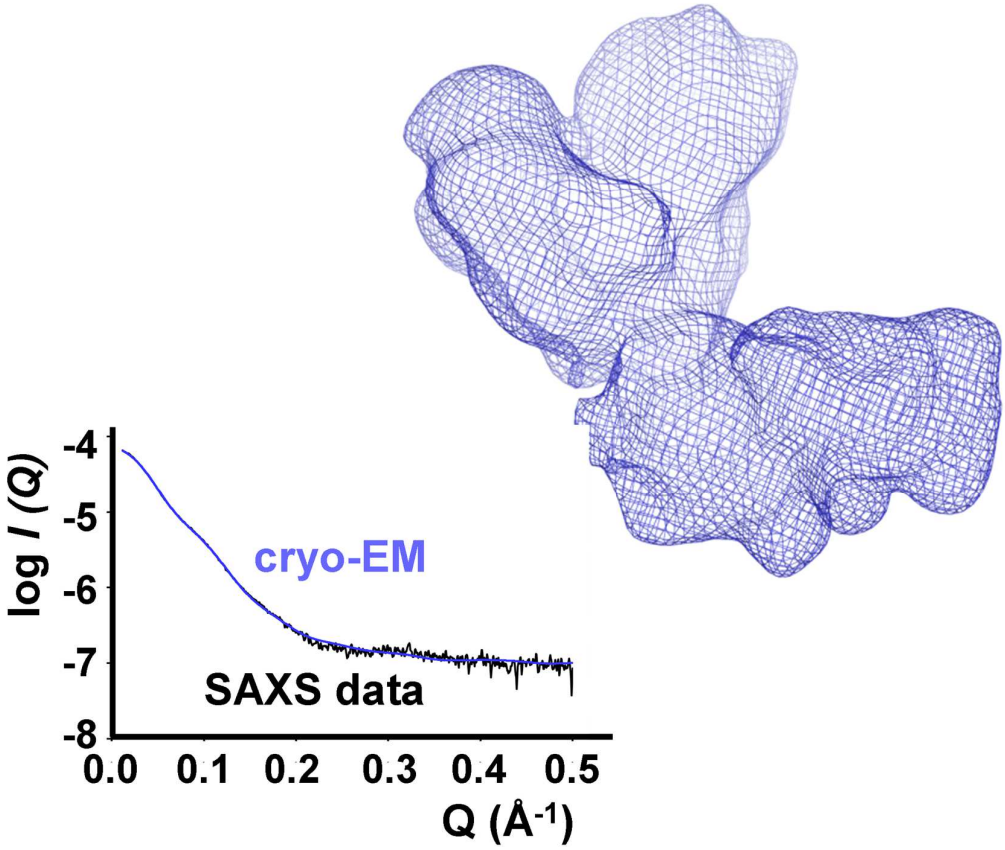
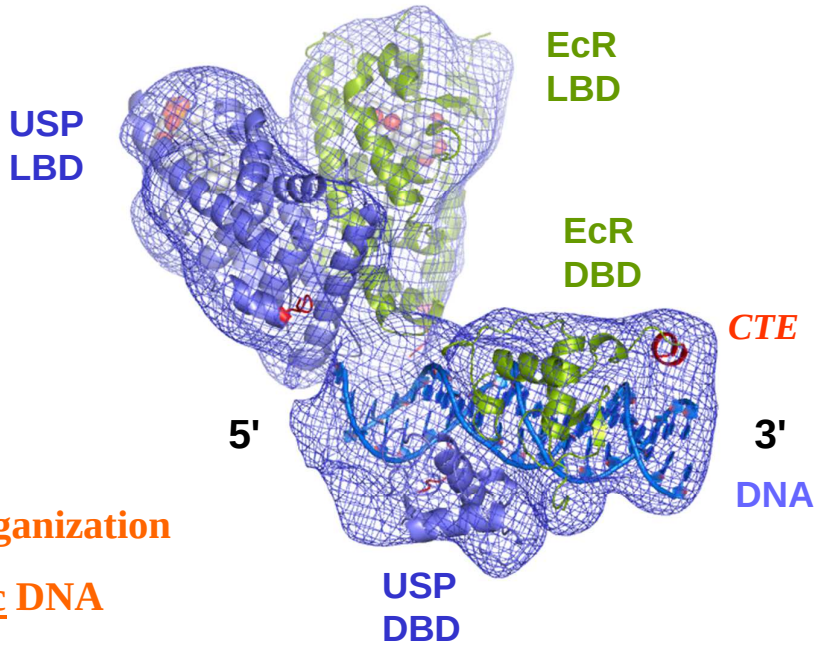
Structure of the USP/EcR complex on a natural DNA inverted repeat (IR1)



IGBMC

Structure of the USP/EcR complex on a natural DNA inverted repeat (IR1)



key concept:

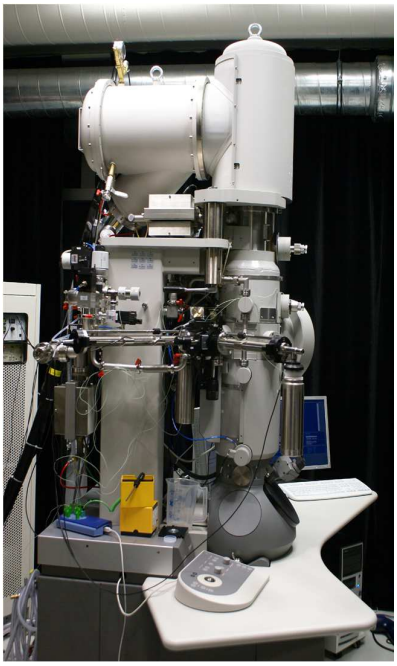
asymmetric organization

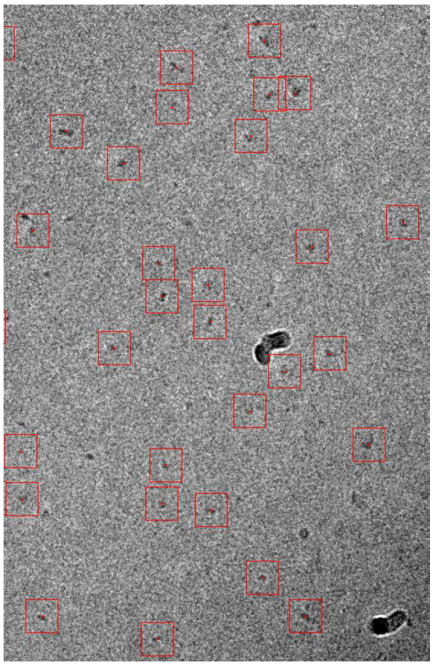
on a symmetric DNA

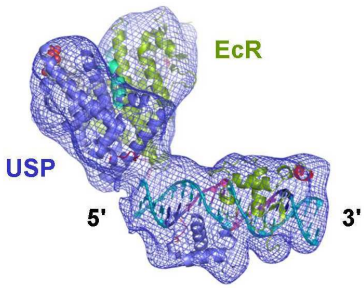
IR1:
hsp27

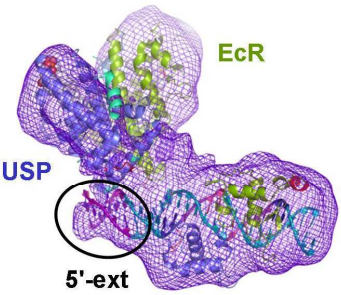
CAAGGGTTCAATGCACTTGT
GTTCCCAAGTTACGTGAACA

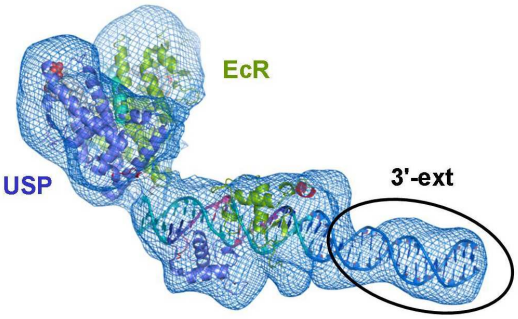
3 cryo-EM structures:









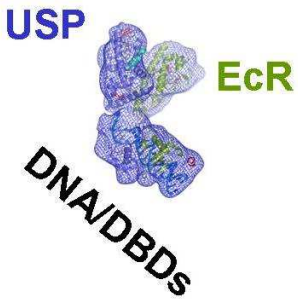


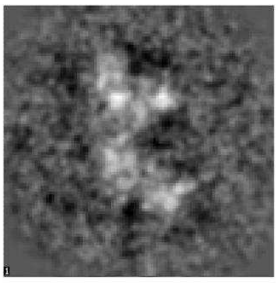
Polara electron microscope data, CCD, 100kV, 59k, 50 000 particles

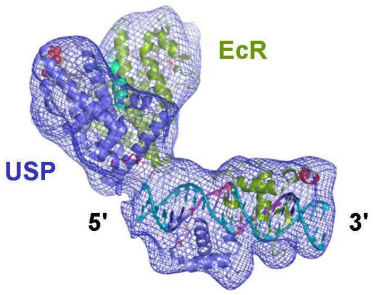
→ ***independent structure validation***

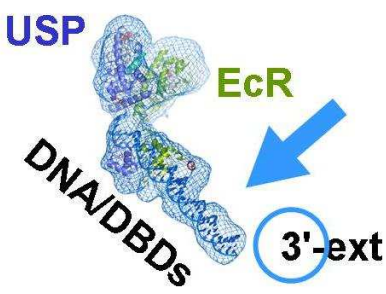
Maletta *et al.*, *Nature Communications, 2014, in press.*

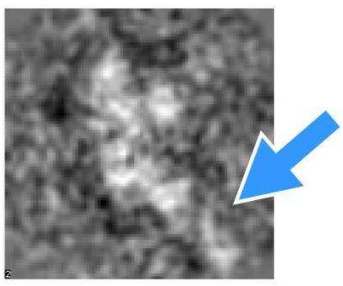
Assignment of the polarity on the DNA:

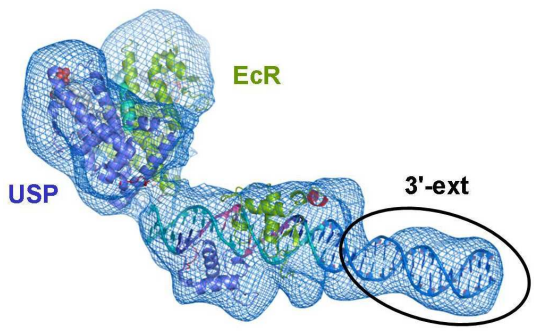








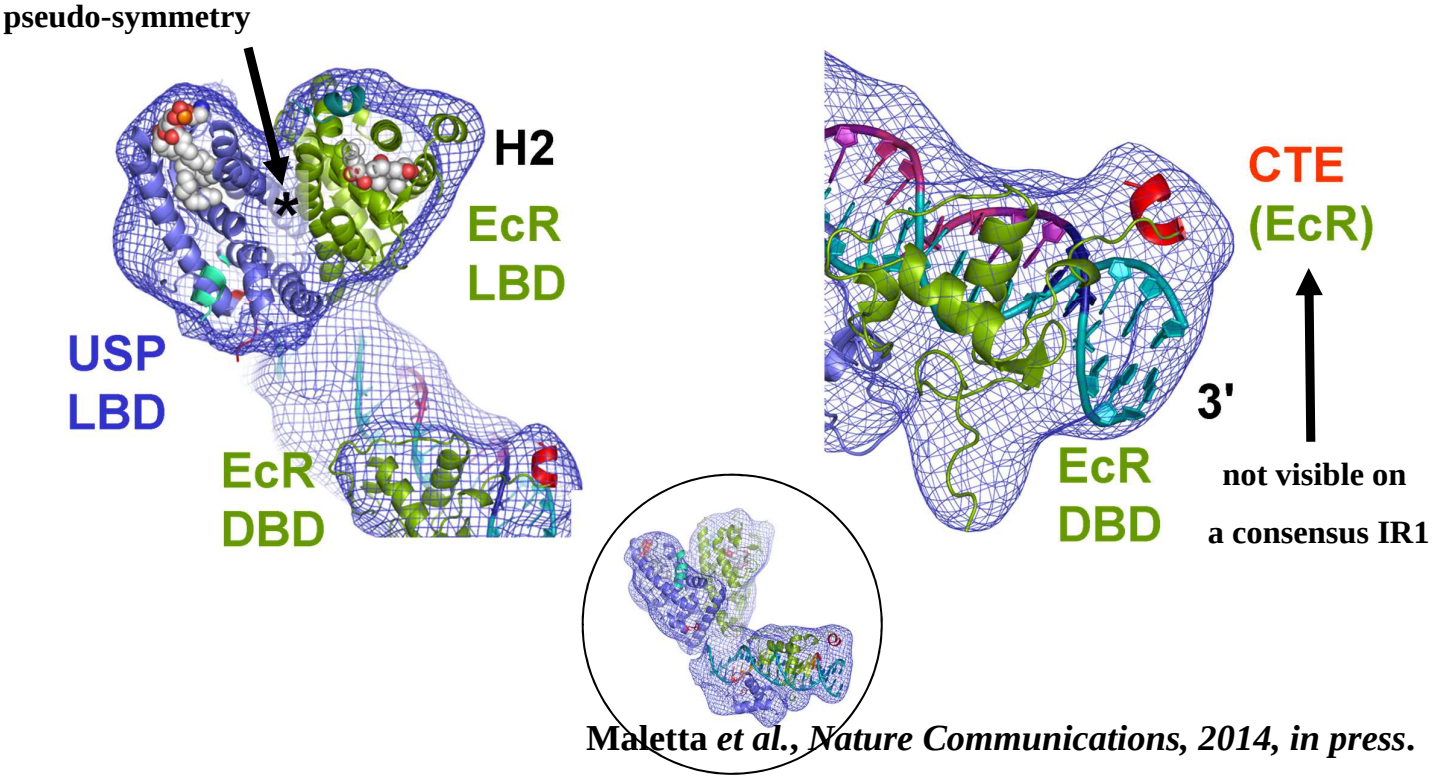




3'-DNA extended complex (35mer, +18mer)

Maletta *et al.*, *Nature Communications, 2014, in press.*

Identification of key structural features in the USP/EcR complex



Maletta *et al., Nature Communications, 2014, in press.*

IV. Current & future challenges in cryo-EM:

- how to push resolution to the atomic level?

- how to analyze flexible complexes?

- how to integrate towards the cellular level?

→ instrumentation

→ software developments for image processing

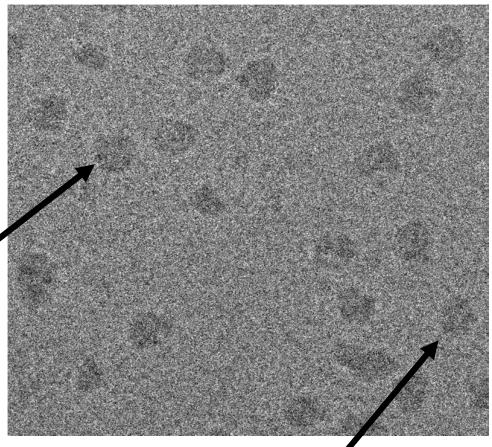
Conformational changes of cats?

Determining structures of multiple conformational states in a single sample

local 2D MSA (multi-variate statistical analysis)

30S **50S**

70S particle without factors?

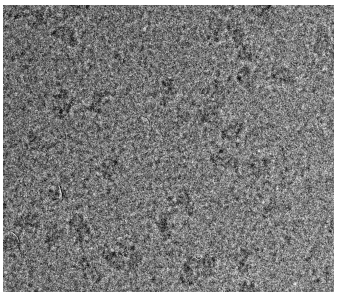


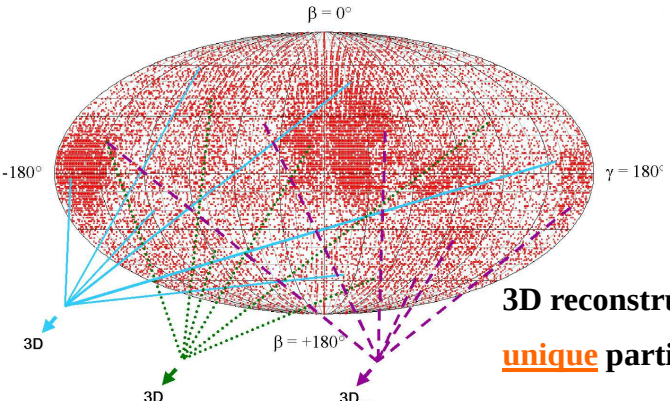
50S view of the 70S **or** **50S particle?**

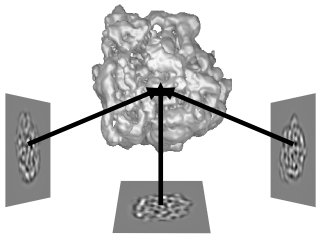
Klaholz et al., *Nature* 2004; see Suppl. Mat.

Sorting out heterogeneity of the complexes:

3D statistical analysis and 3D classification:
3D sampling and classification (3D-SC)







3D reconstruction of single particles:

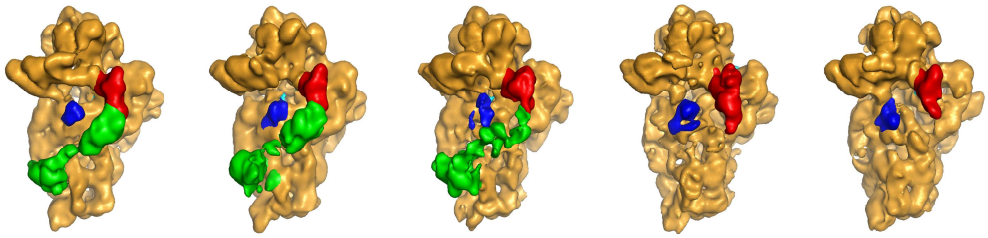
unique particle type in different orientations

2D → 3D → 4D

200kV FEG data;

total 80 000 particles

resolution of 3D's: 9Å

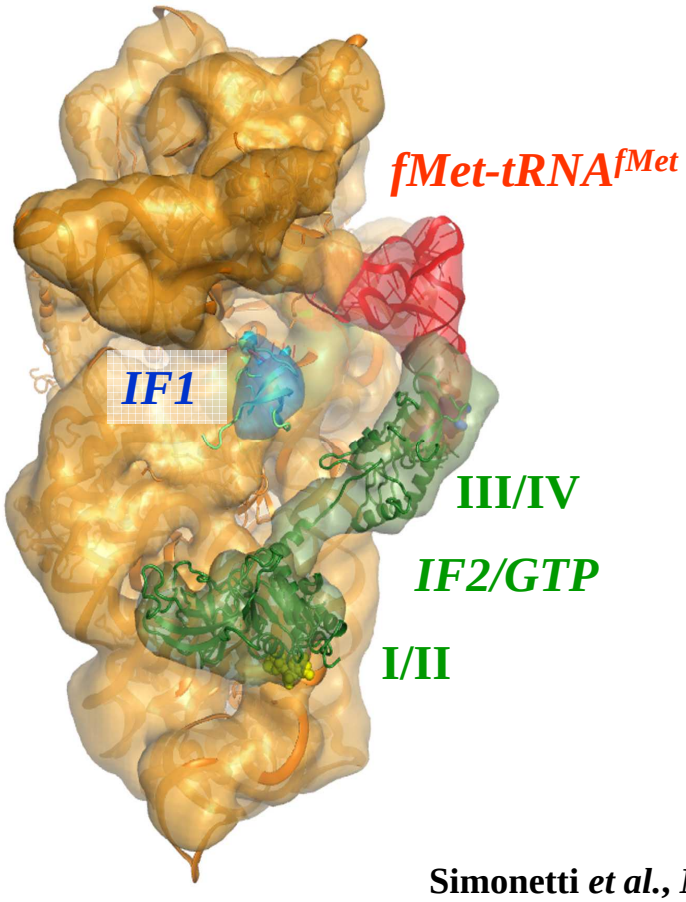


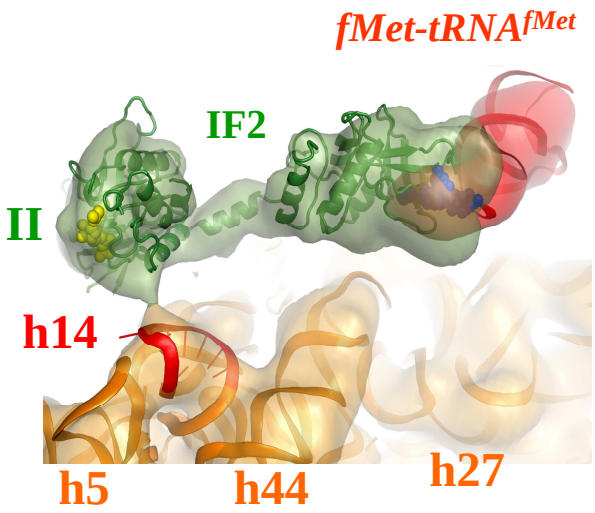
40 % **28 %** **10 %** **8 %** **14 %**

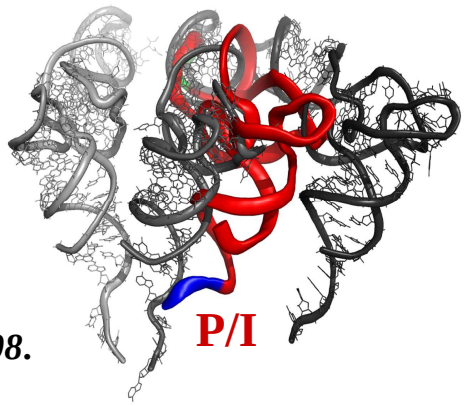
Simonetti *et al.*, *Nature*, 2008.

used by Fischer et al., *Nature*, 2010; Papai et al., *Nature* 2010.

Cooperative binding of the initiator tRNA and IF2 in the 30S initiation complex

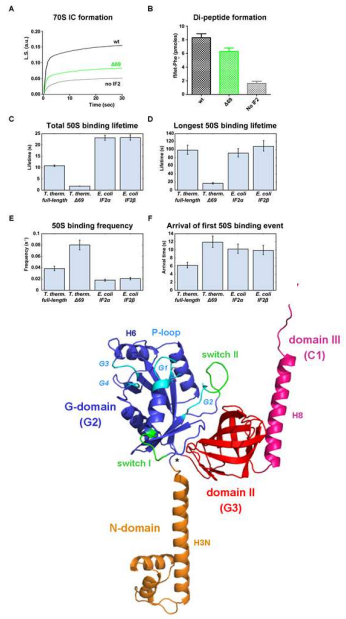




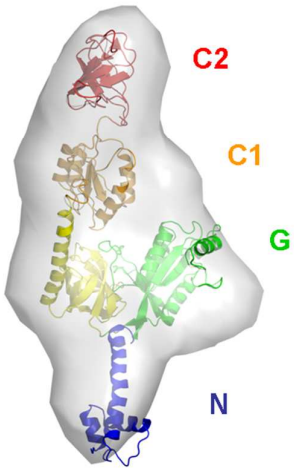


Simonetti *et al.*, *Nature*, *2008*.

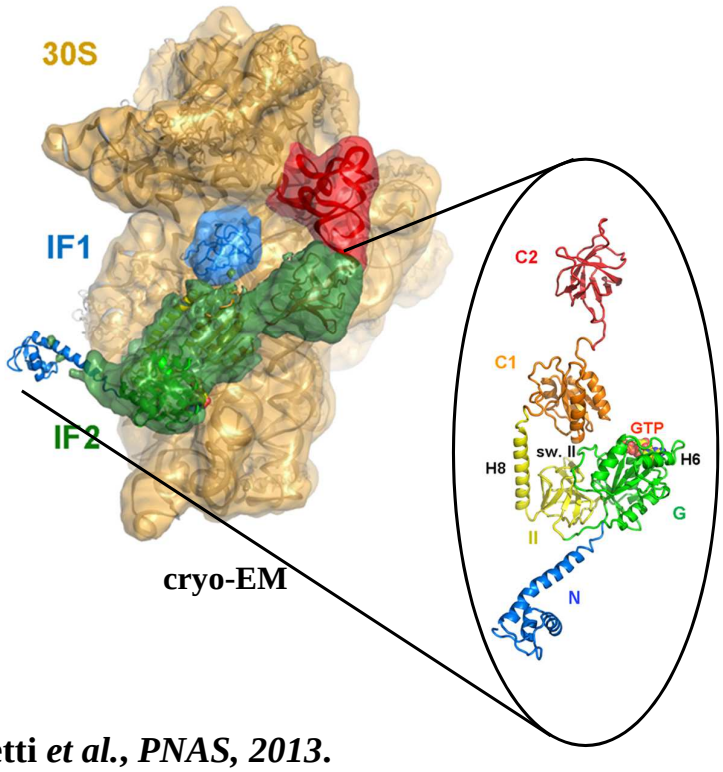
Involvement of IF2 N domain in ribosomal subunit joining revealed from architecture and function of the full-length initiation factor



crystal structure



SAXS



cryo-EM

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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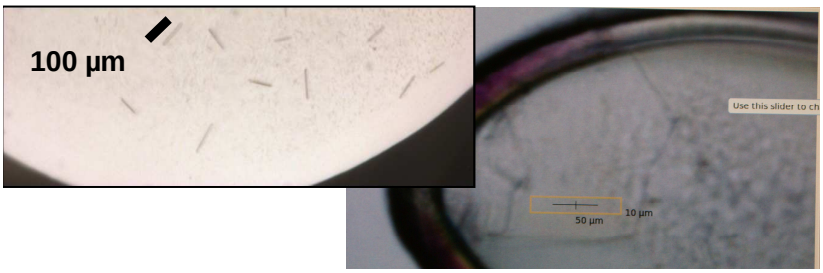
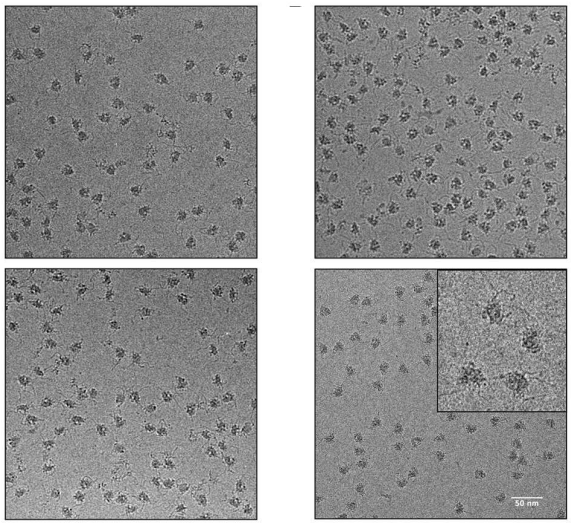
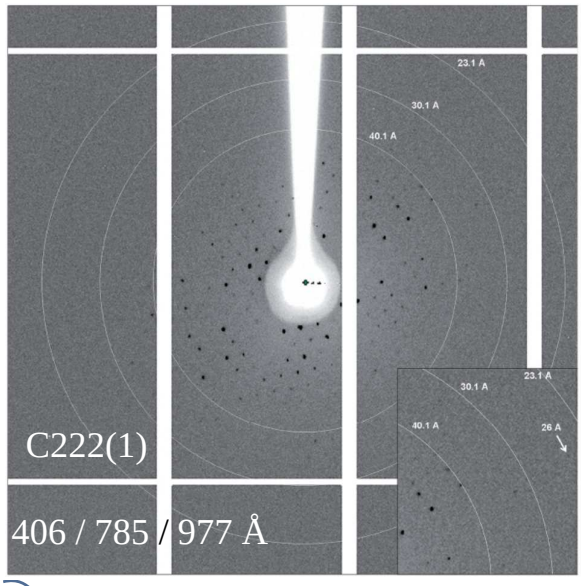
Crystallography of (large) macromolecular complexes:

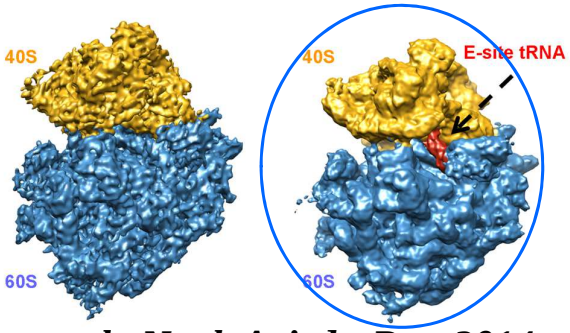
use cryo-EM for sample optimization: first human 80S ribosome crystals







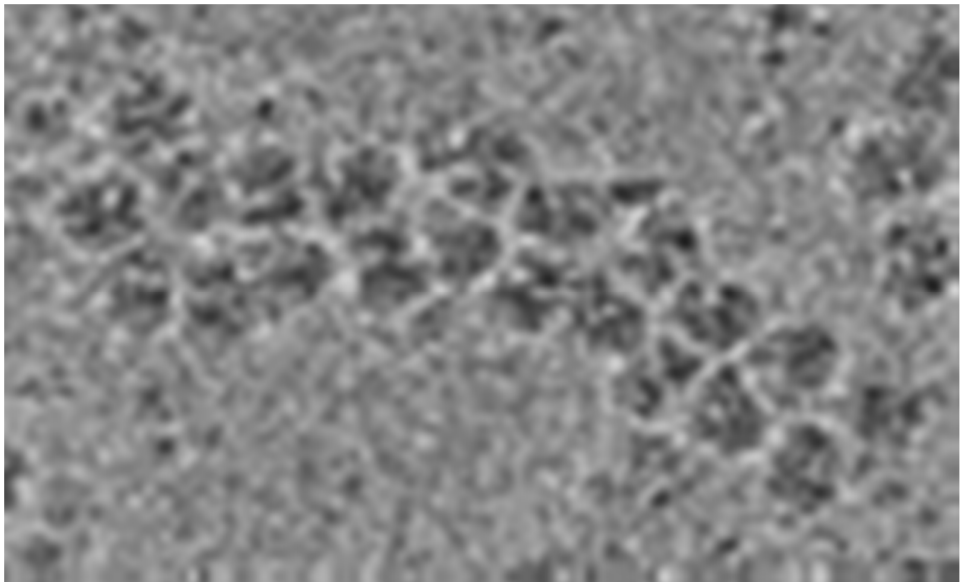
SLS



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

IGBMC

Towards higher complexity: molecular assemblies

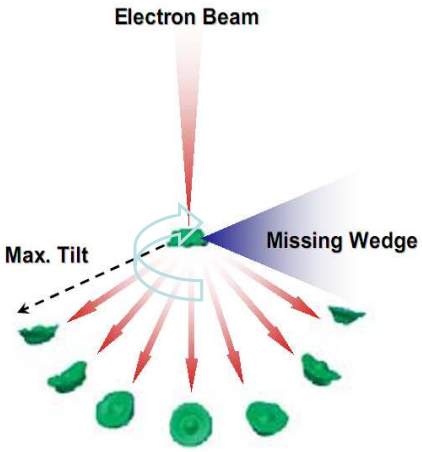


Cryo electron tomography (cryo-ET) of

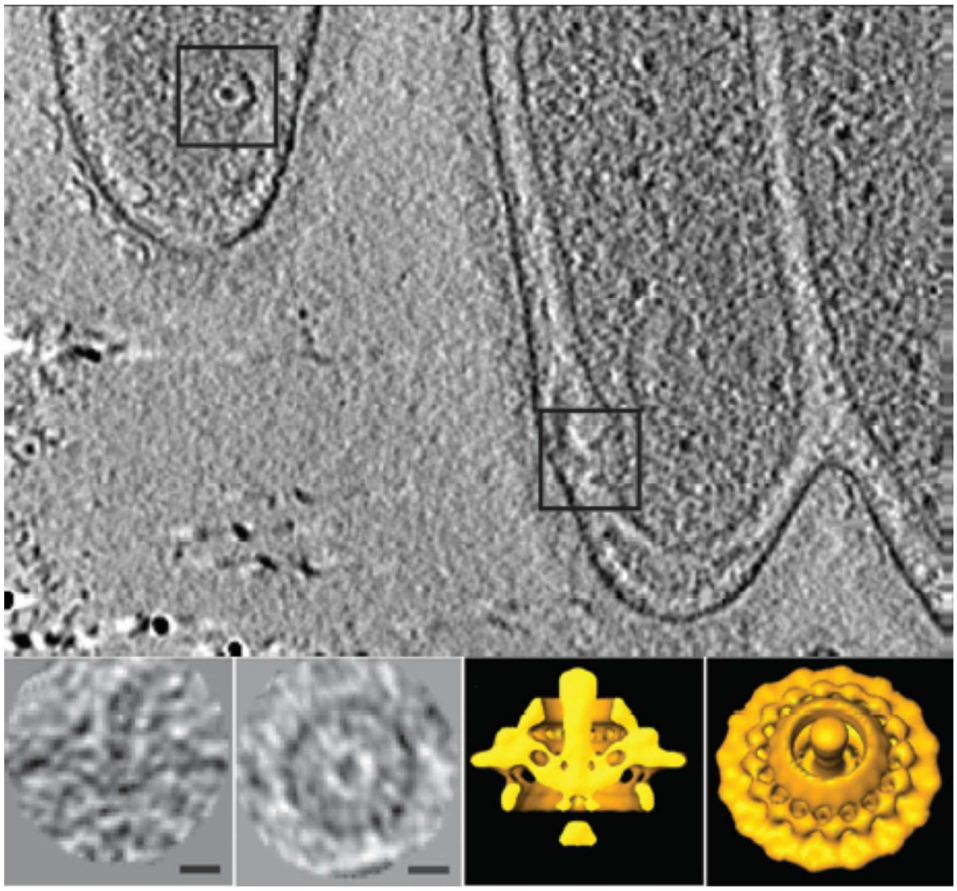
- purified complexes

- cell sections

Cryo electron tomography



→ tomogram
(i.e. a 3D reconstruction from tilt images)

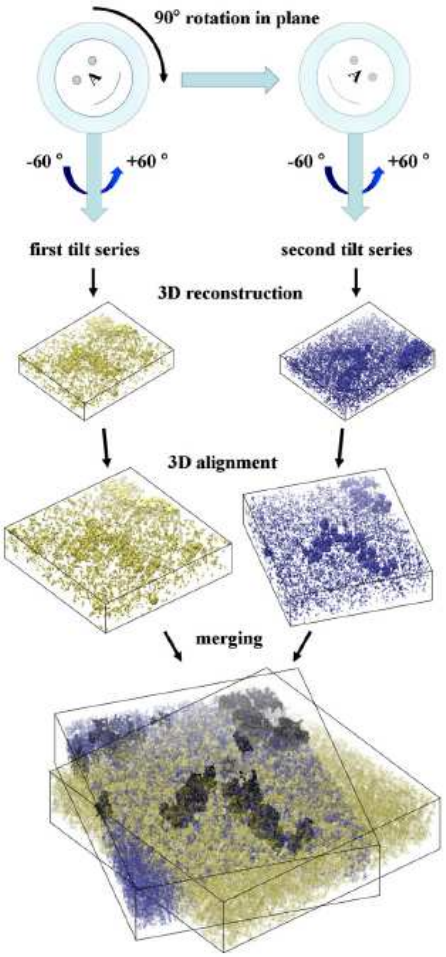


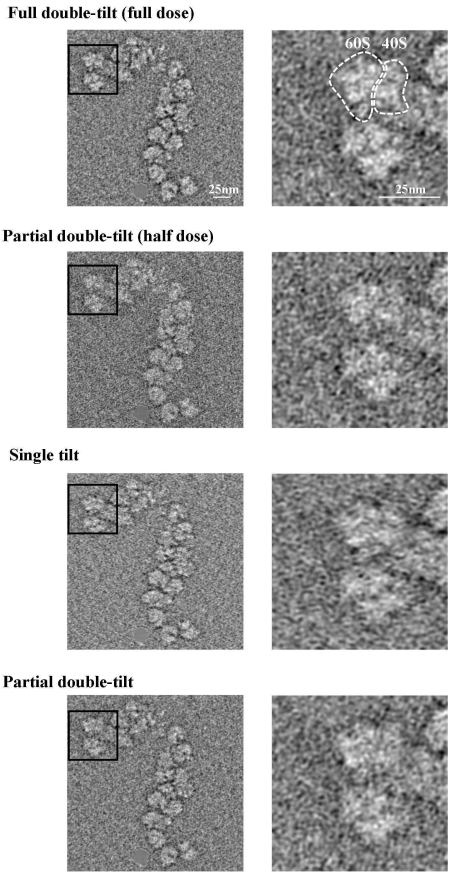
flagellar motor

Murphy et al,. Nature 2006, 442, 1062–1064.

Tomography of cellular structures

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





full dual-tilt

partial dual-tilt (half-dose)

single-tilt

partial dual-tilt (3° versus 12°)

Myasnikov *et al.*, *Ultramicroscopy* 2013.

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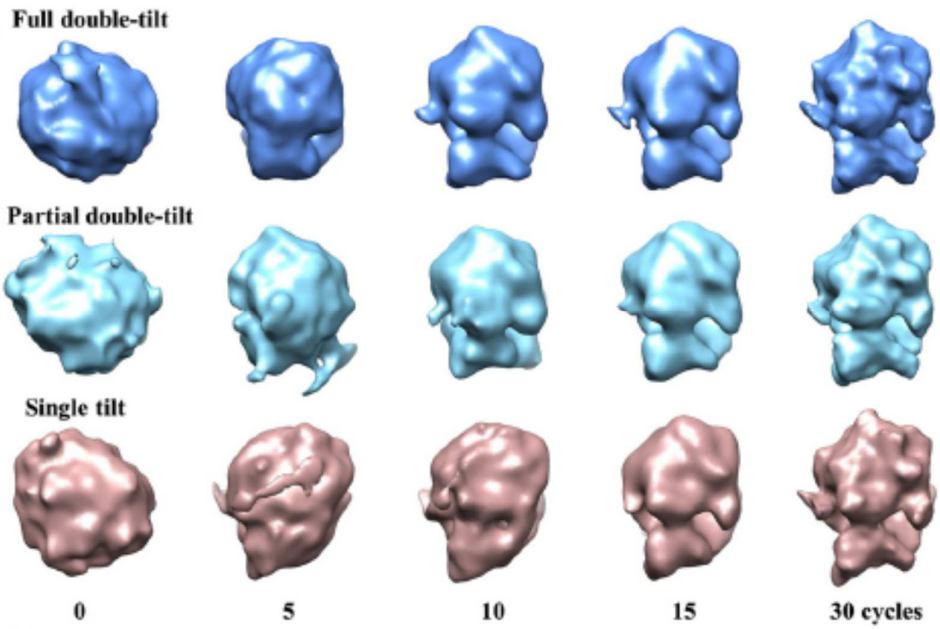
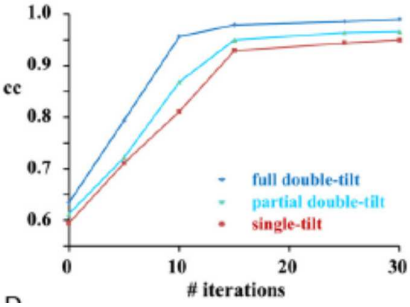
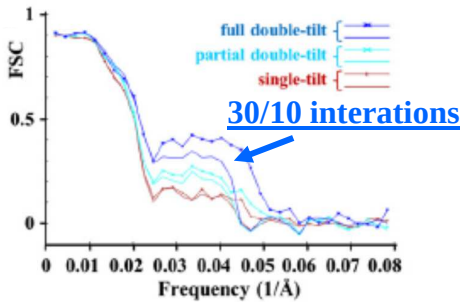
3D polysome reconstruction from single- / dual-tilt cryo electron tomography







→ faster convergence of sub-tomogram averaging when using dual-axis data (even partial)

Myasnikov *et al.*, *Ultramicroscopy* 2013.

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V. Instrumentation & technical highlights towards multi-scale integration

<u>High-resolution electron microscopes:</u>

- ultra-stable specimen holders,

- high-resolution optics,

- parallel electron beam,

- aberration correction,

- use lower voltage for better contrast of small complexes,

- automatic data collection for single particle cryo-EM and cryo elctron tomography (cryo-ET),

- standardize sample preparation (cryo-EM freezing, high-pressure freezing and ultra-microtomy for cell section)

<u>High-sensitivity cameras:</u>

direct electron detectors, CMOS camera, counting events;

is part of a "revolution" in cryo-EM and structural biology, like for Pilatus/Eiger detectors in X-ray crystallography

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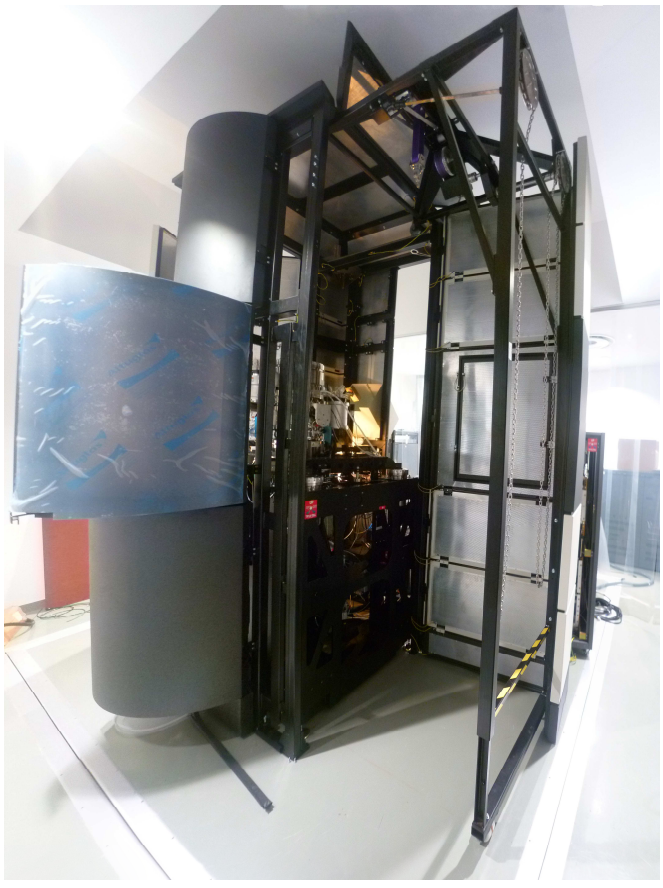
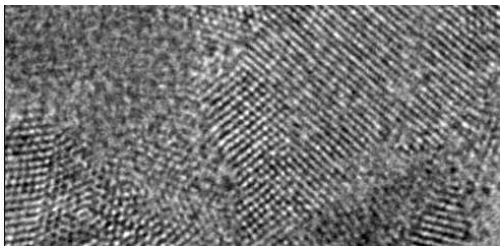
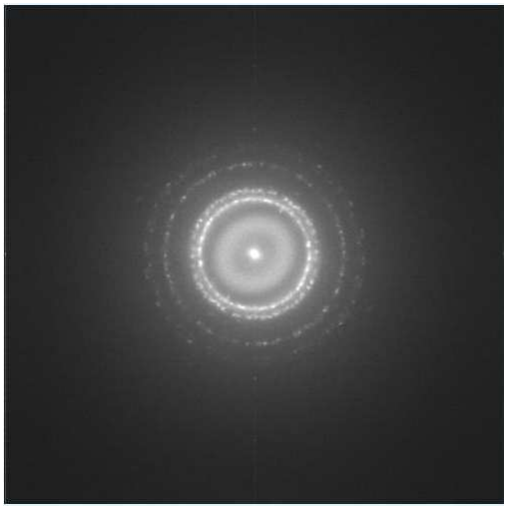


Titan Krios installation 10-12.2013, CBI





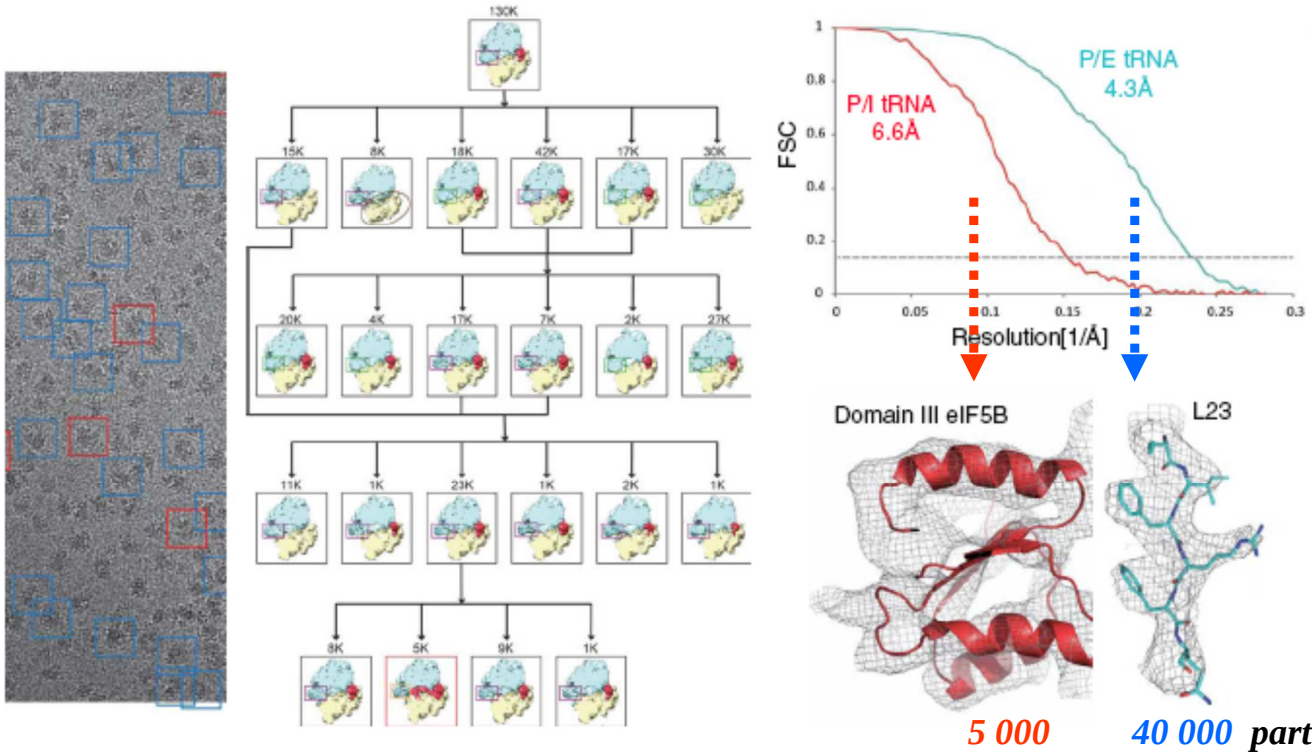
Reaching the atomic level…?

- large data sets,

- image processing to high-resolution

→ see data set for the cryo-EM practicals

V. Instrumentation & technical highlights towards multi-scale integration



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. Ramakrishan & S. Scheres.

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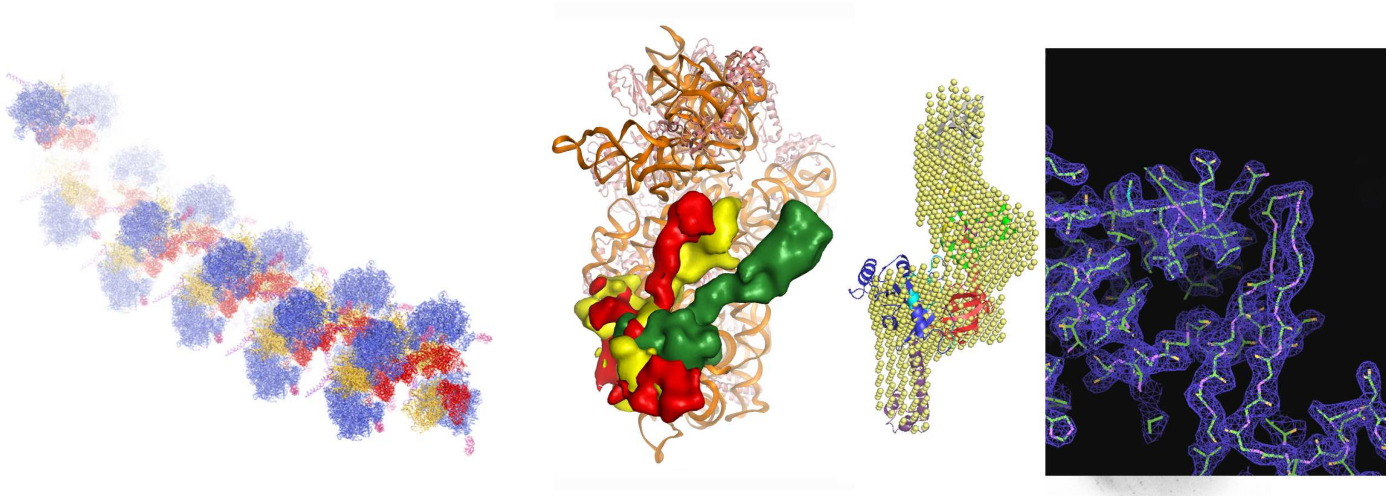
Multi-resolution integration of gene expression regulation



Molecular assemblies ← Single particles → Crystal structure

Myasnikov *et al.*, *Ultramicroscopy* 2013
Myasnikov *et al.*, *submitted*.

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

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

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

Integrative structural biology of (large) macromolecular complexes

synergy-core for data integration:

- **electron microscopy**
- **crystallography**
- **NMR**
- **SAXS**
- **bio-informatics**
- **biochemistry (purification, functional tests)**

structure ⇄ **function**

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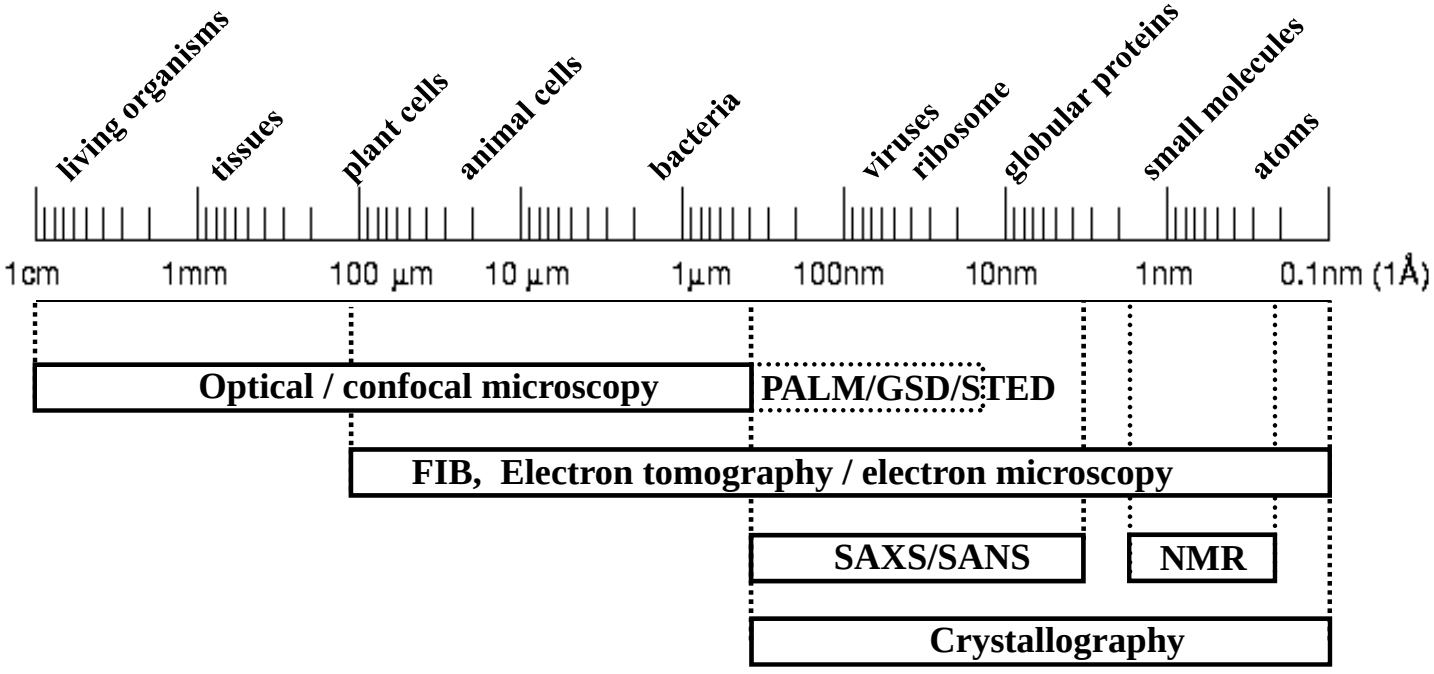
Gene expression regulation through an integrated structural biology approach



Challenging objects require multi-scale multi-resolution integration

Ménétret *et al.*, Springer, 2013.

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Leonid Andronov
Brice Beinsteiner
Isabelle Billas
Isabelle Hazemann
Heena Khatter
Jean-François Ménétret
Kareem Mohideen
Alexander Myasnikov
Kundhavai Natchiar
Igor Orlov
Karima Tazibt
Morgan Torchy
Alexander Urzhumtsev
Massimiliano Maletta
Stefano Stella
Angelita Simonetti
Srividhya KV
Sankar Manicka
Anne-Sophie Humm

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Dino Moras
Natacha Rochel, Judit, Maria, Anna, Yannick B.
Ali Hamiche
Jean Cavarelli
Daniele Spehner

IBMC
Pascale Romby
Stefano Marzi
Gilbert Eriani
Philippe Dumas

Camerino University
Claudio Gualerzi
Attilio Fabbretti

Pushchino
Alexander Spirin
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Zhanna Afonina

Pierre R., SOLEIL
Florian D. & Meitian W., SLS

Christian Cambillau, Marseille
Christophe Ritzenthaler, IBMP

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