Sample Preparation

Holger Stark
MPI for biophysical Chemistry
Göttingen
Germany

Motivation

PDB search results:

s with min 4 different chains (complexes) make up only ~1% of the PDB

s in contrast to the fact that:

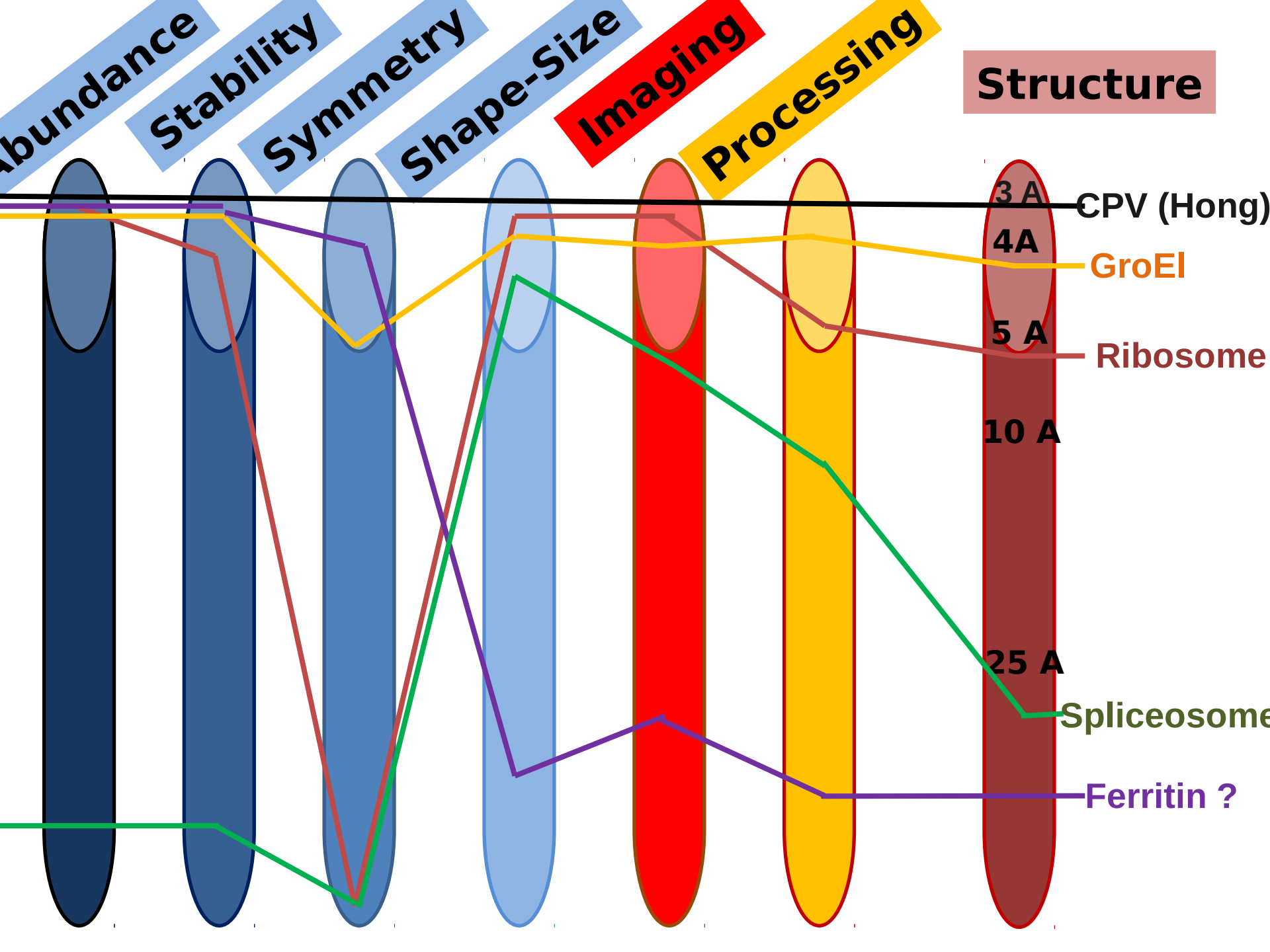
proteins act in complexes with an average size of ~10 components (cha

~1% of the PDB entries with >3 chains:
Most of these entries are well behaved and stable complexes such as ribosomes, GroEl, proteasome.

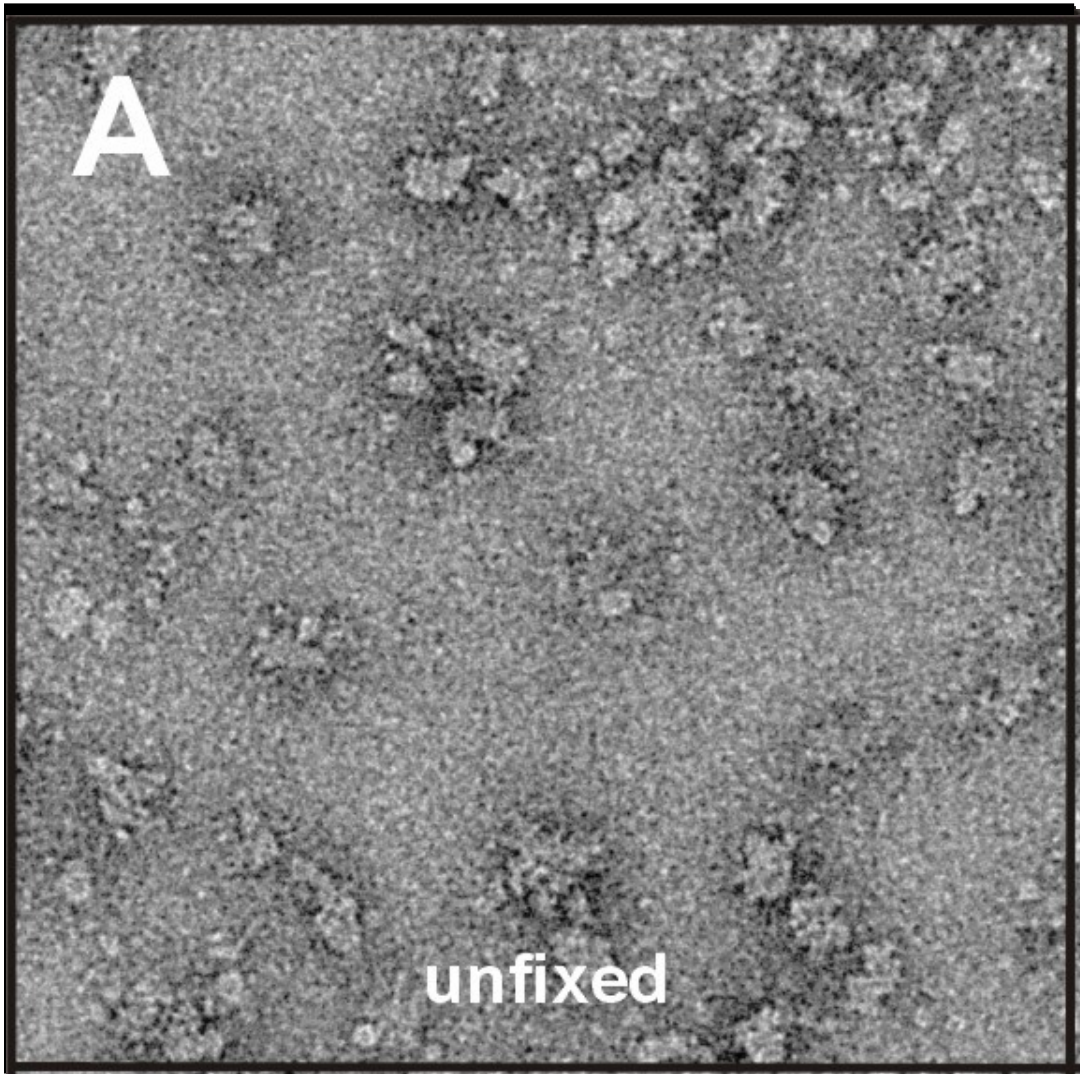
There are numerous of large (>150kDa) and asymmetric complexes in the cell and their structure is not known!

Challenging Complexes

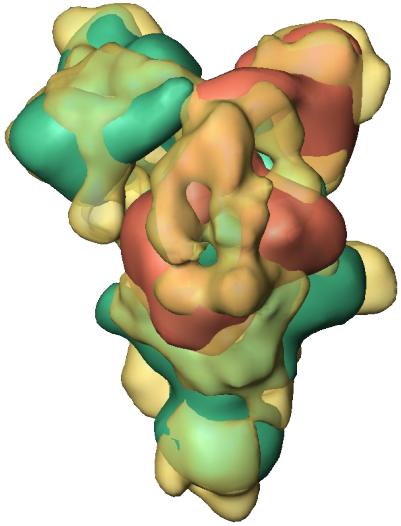
- Abundance – copy numbers
- Biochemical Purification difficult to optimize
- Stability
- Aggregation
- Structural Heterogeneity
- Conformational Heterogeneity


Abundance
Stability
Symmetry
Shape-Size
Imaging
Processing
Structure
3 A
CPV (Hong)
4A
GroEl
5 A
Ribosome
10 A
25 A
Spliceosome
Ferritin ?

Structural Heterogeneity may be artificially created by standard EM Sample Preparation Procedures



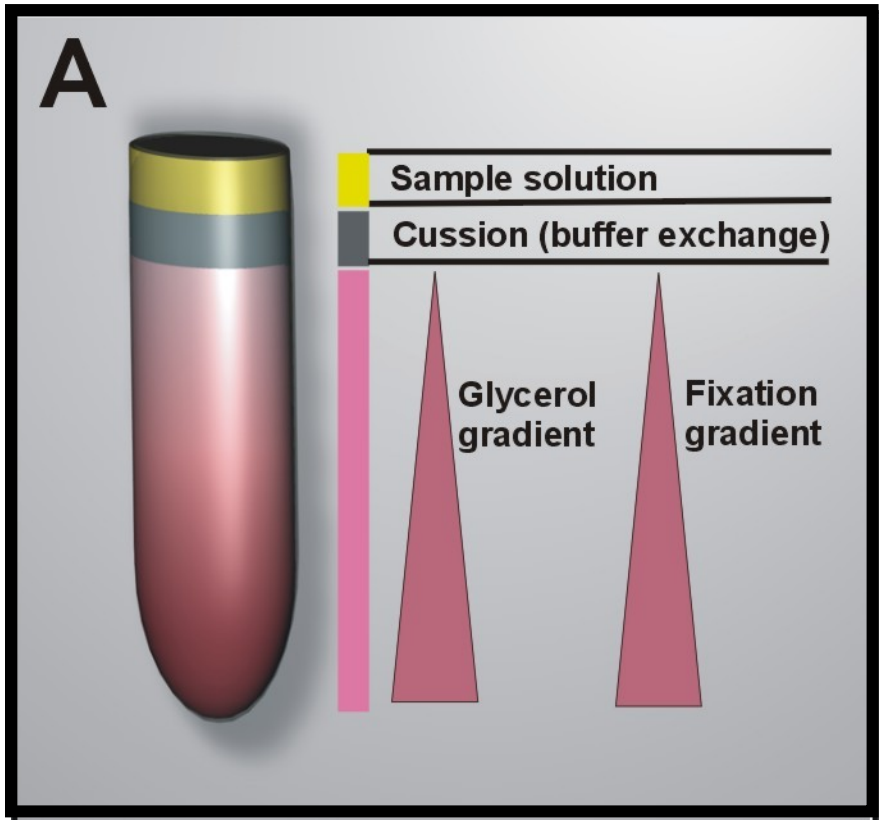
U4/U6.U5 tri-snRNP

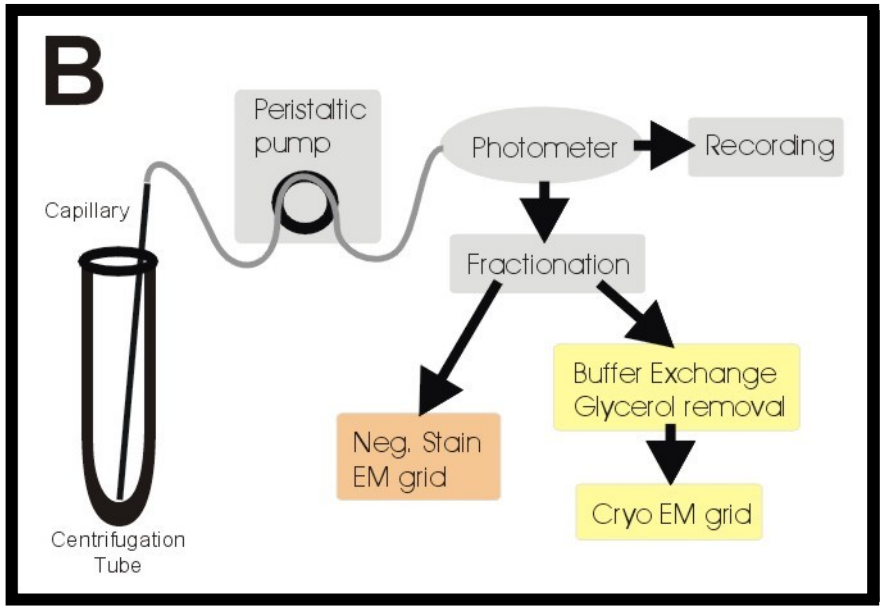


Sander et al., Mol Cell, 2006

GraFix

A combined Gradient centrifugation and Fixation method

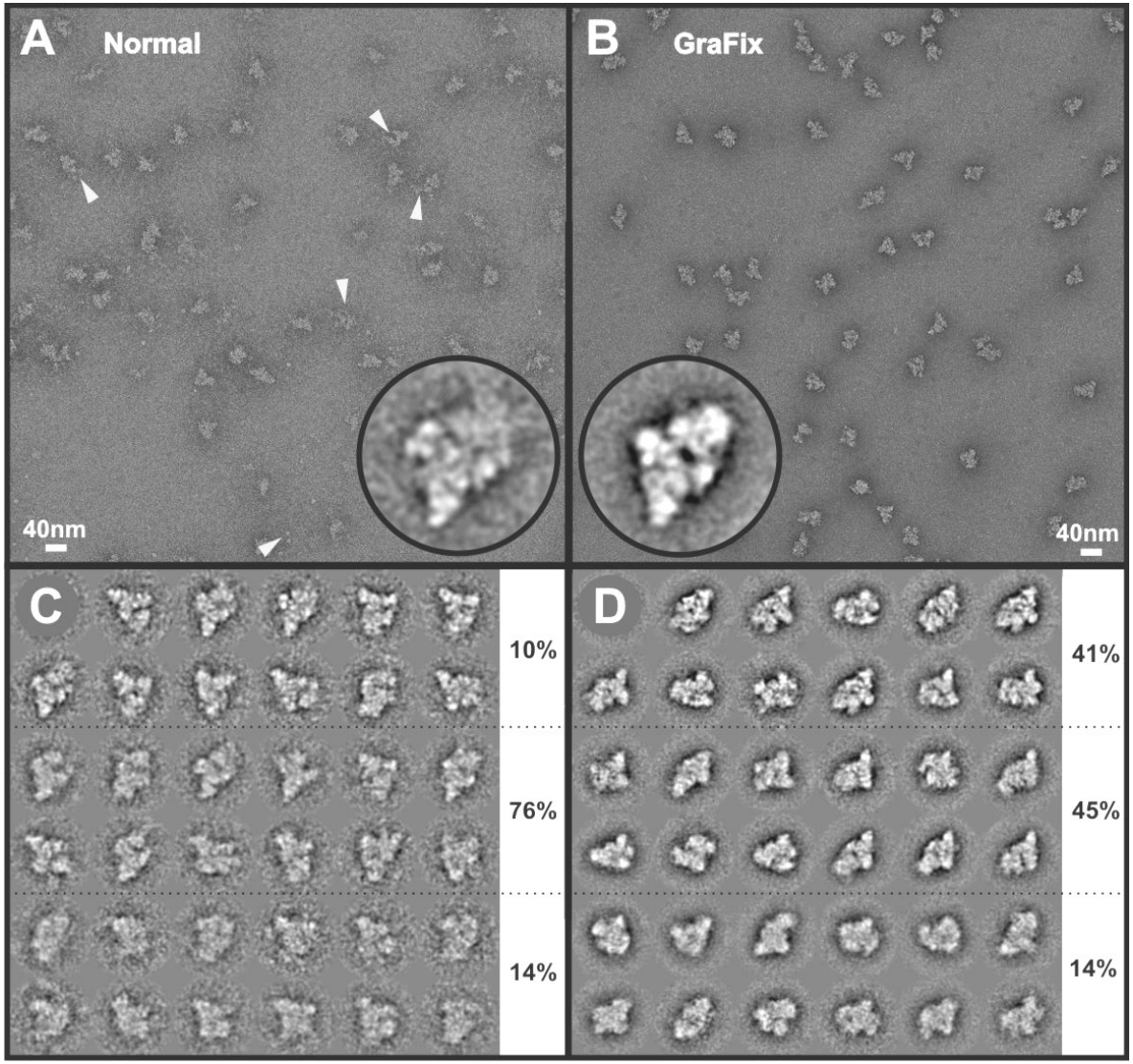


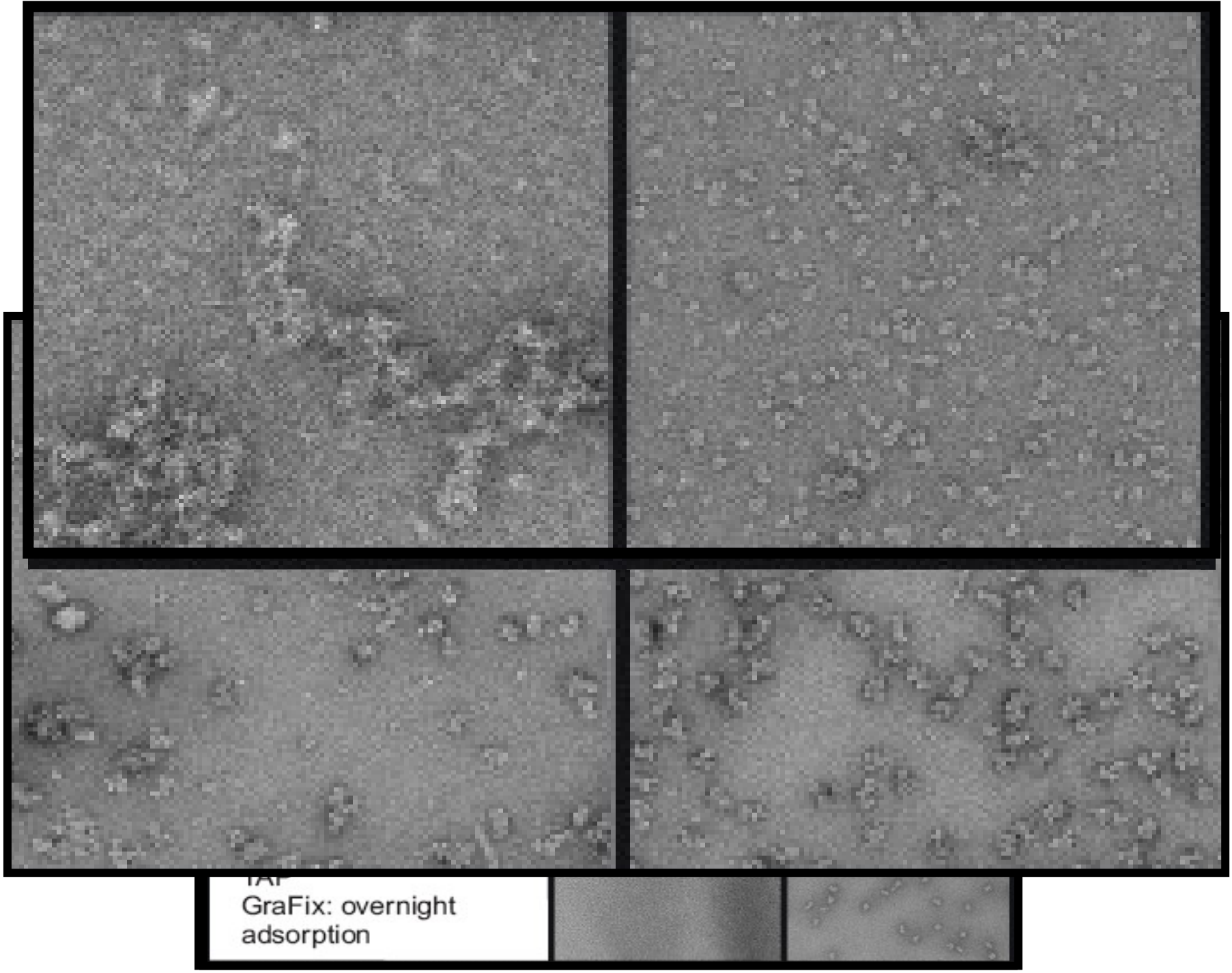


Typically
0 – 0.15% glutaraldehyde

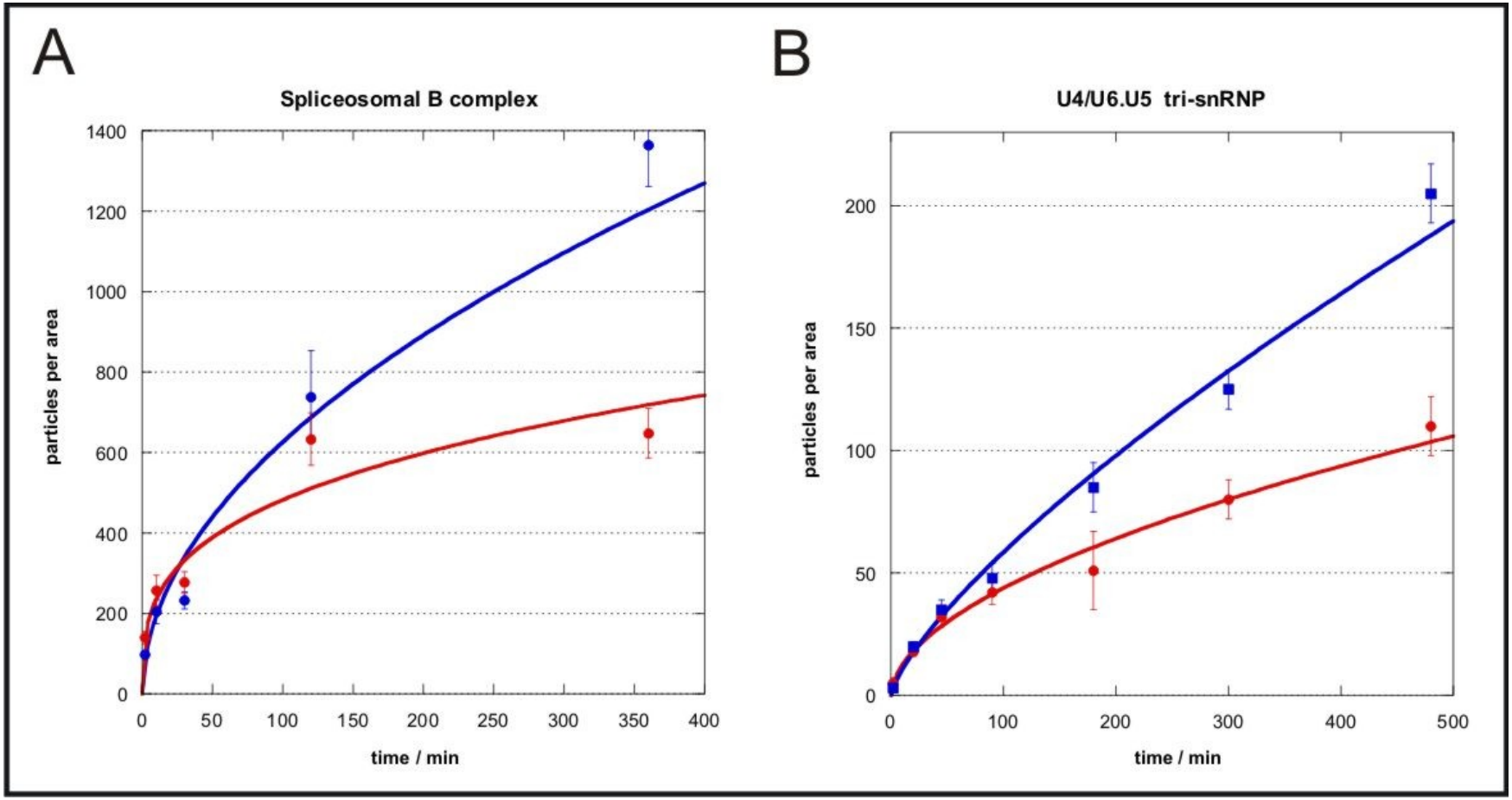
Kastner et al. Nature Methods, 2008

GraFix test: Spliceosomal B Complex




GraFix: overnight
adsorption

Long adsorption times with GraFix

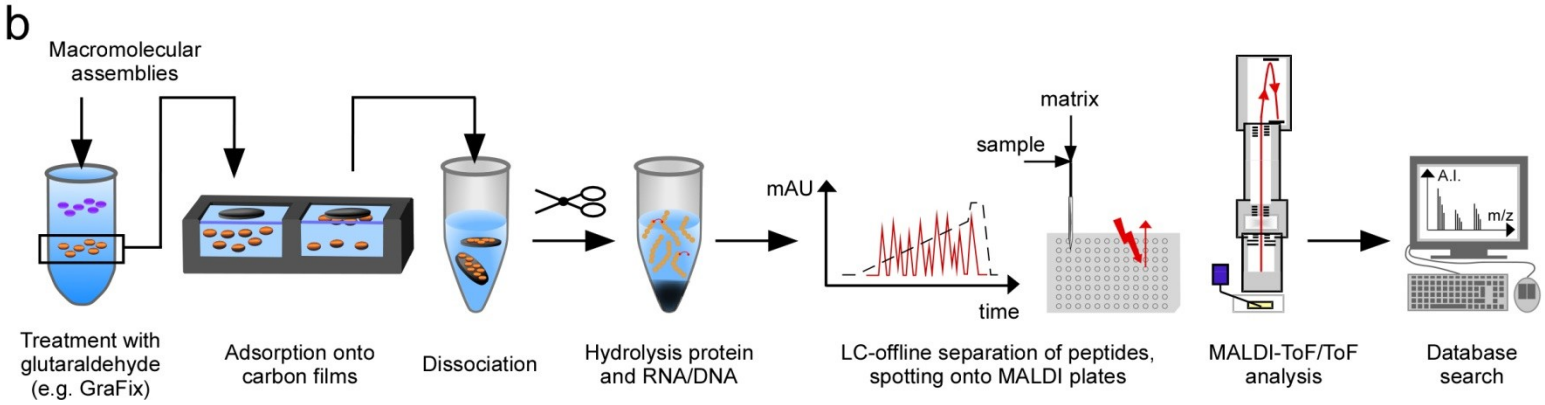


low to analyze chemically stabilized complexe

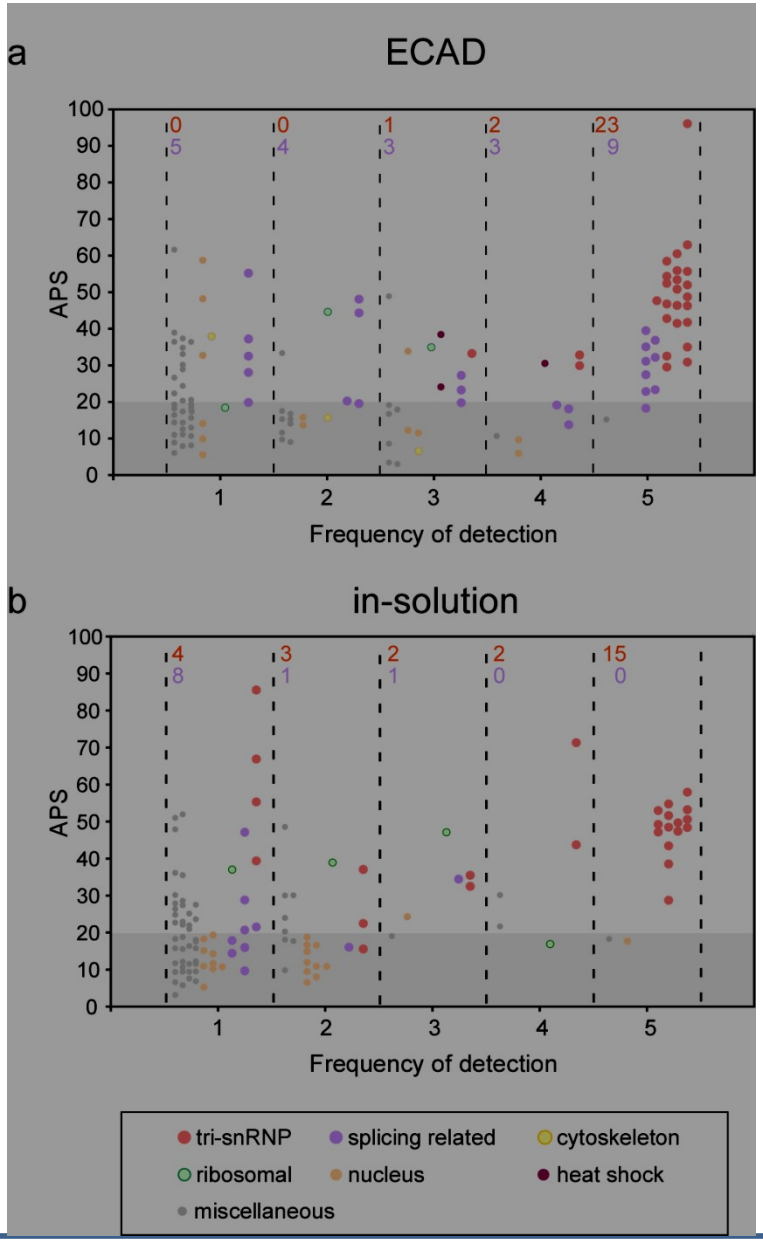
Problem :

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

GraFix

A combined Gradient centrifugation and Fixation method

---

here is some concern that GraFix may create artefact

- ribosomes and snRNPs did not reveal any difference in structure upon GraFix treatment.
- GraFix treated complexes can still be crystallized (Ferritin) !!
- The 3D structure of Ferritin and Ferritingrafix are identical !!!

GraFix does not „repair“ previously damaged complexes, therefore buffer optimization is also required.

Classical optimization

1) Consumes substantial amounts of sample (≈ 10 mg).

2) Is tedious owing to purification capacity and readout.

3) For practical reasons limited to a small set of conditions.

The ideal optimization setup

1) Consumes little amounts of sample.

2) Should be highly sensitive.

3) Has high-throughput and samples a comprehensive set of conditions.

4) Utilizes simple instrumentation/ chemistry.

The Thermofluor™ method

1) Consumes little amounts of sample (≈1000 pmol/ 96 conditions). ✔

2) Should be highly sensitive. ✔

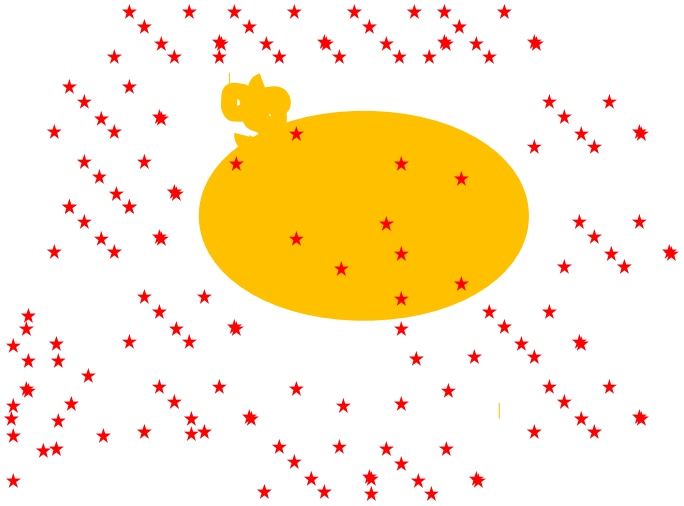
Er

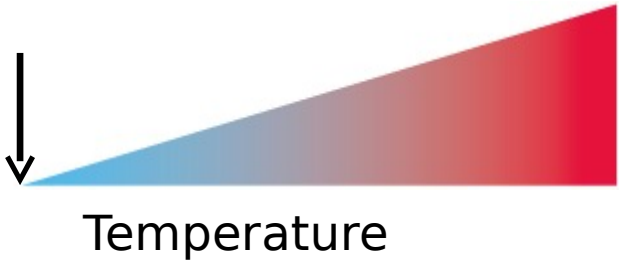
3) Has high-throughput and samples a comprehensive set of conditions (90 conditions in one screen). ✔

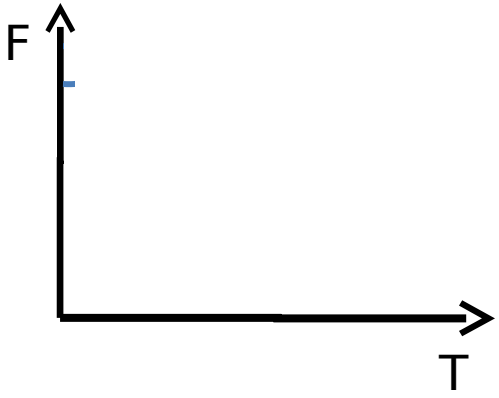
4) Utilizes simple instrumentation/ chemistry. ✔

Ericsson et al, Analytical Biochemistry, 20

What actually happens - the simple case







Journal of Biomolecular Screening
http://jbx.sagepub.com/

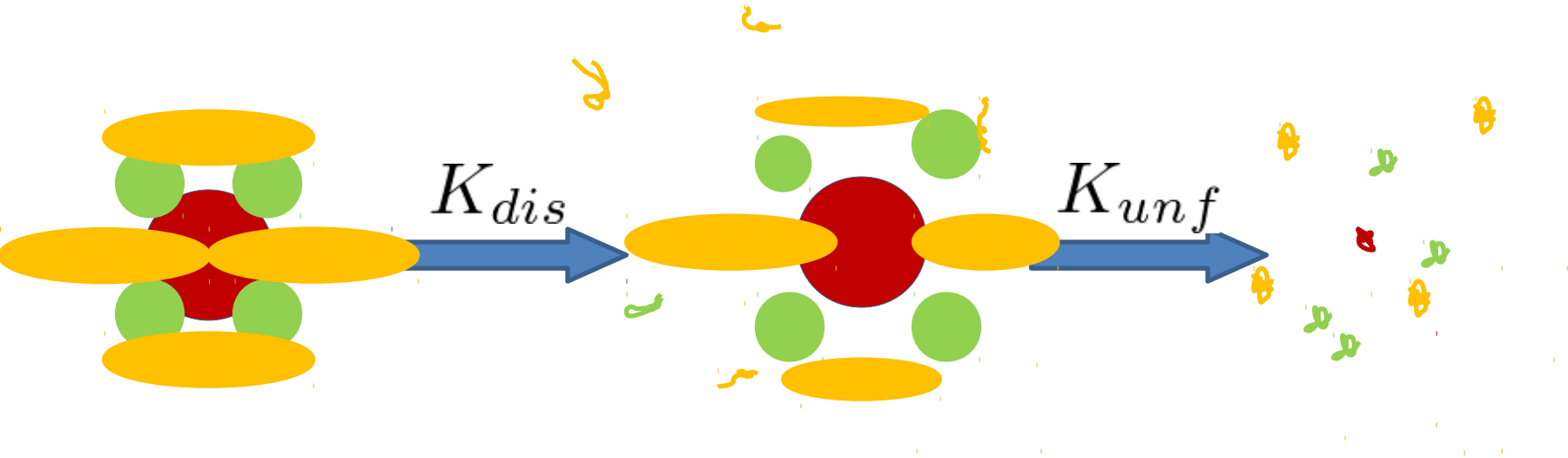
Universal Screening Methods and Applications of ThermoFluor®

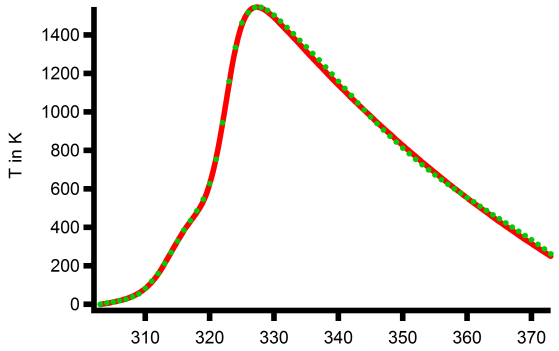
Maxwell D. Cummings, Michael A. Farnum and Marina I. Nelen

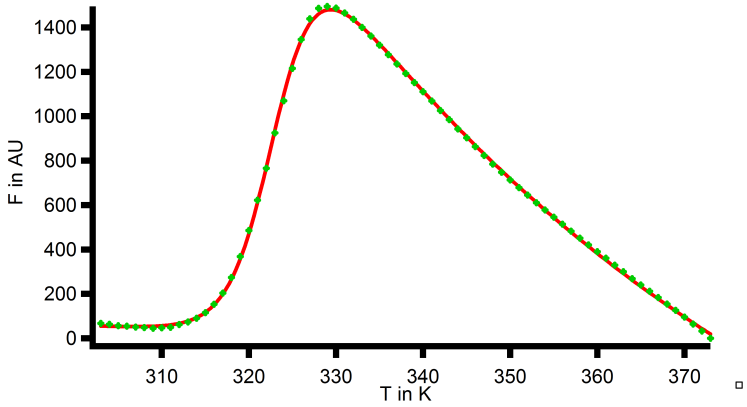
J Biomol Screen 2006 11: 854
DOI: 10.1177/1087057106292746

The relatively complex melting curves characteristic of multidomain proteins (or systems) currently preclude application of ThermoFluor® to screening of these systems. A group at Roche has also recently reported the discovery of reasonably

How to interpret data?

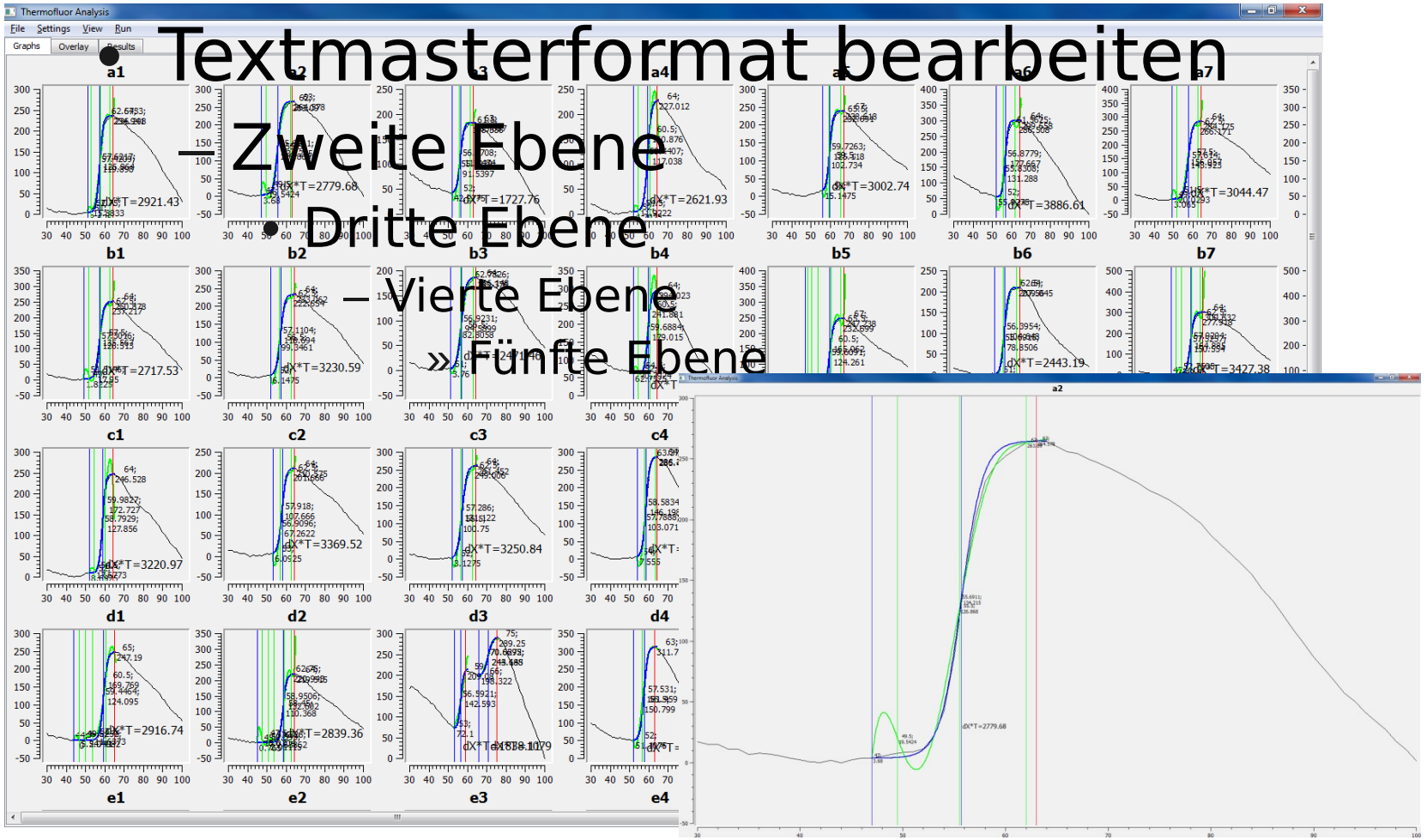






Thermofluor Analyzer Interface - Graphs

- Textmasterformat bearbeiten
  - Zweite Ebene
    - Dritte Ebene
      - Vierte Ebene
        - Fünfte Ebene



Thermofluor Analyzer Interface - Input

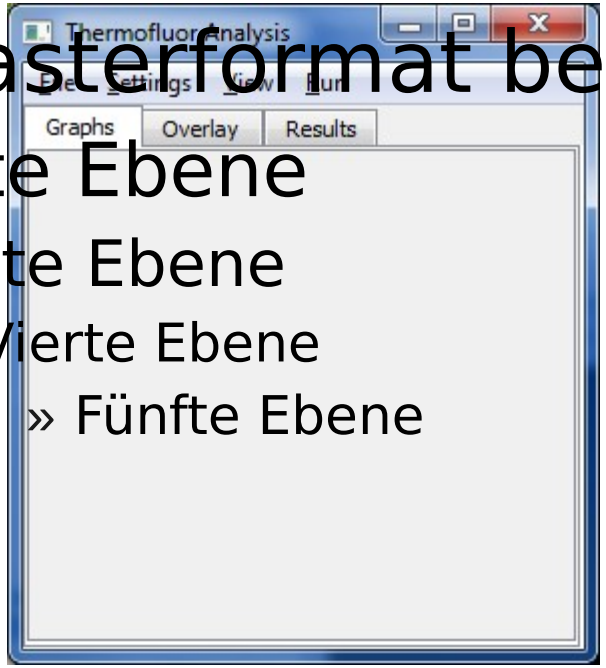
tmasterformat bearbeiten

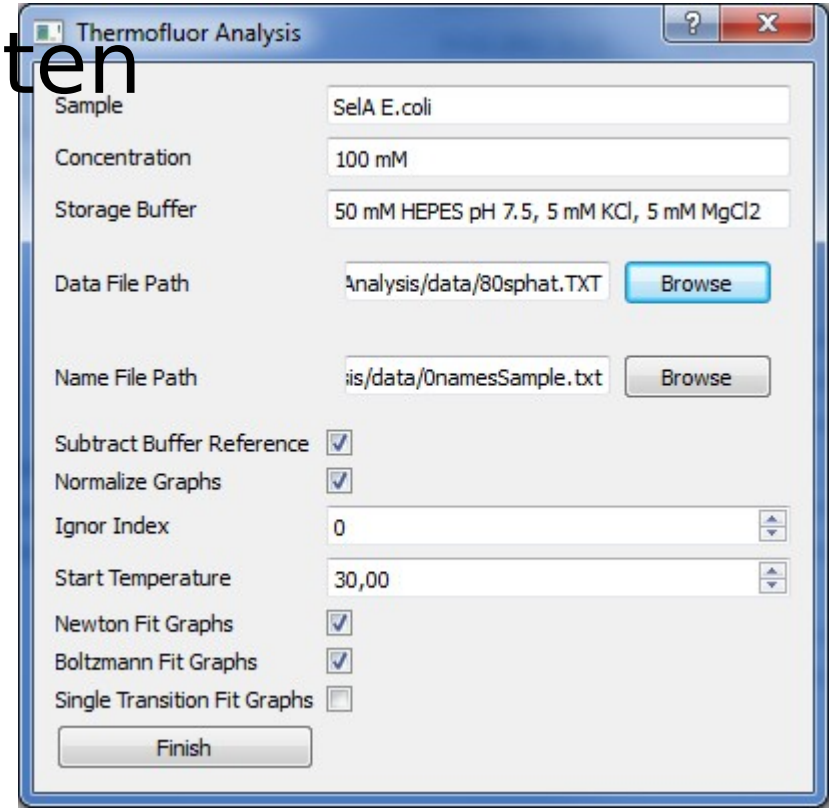
weite Ebene

Dritte Ebene

– Vierte Ebene

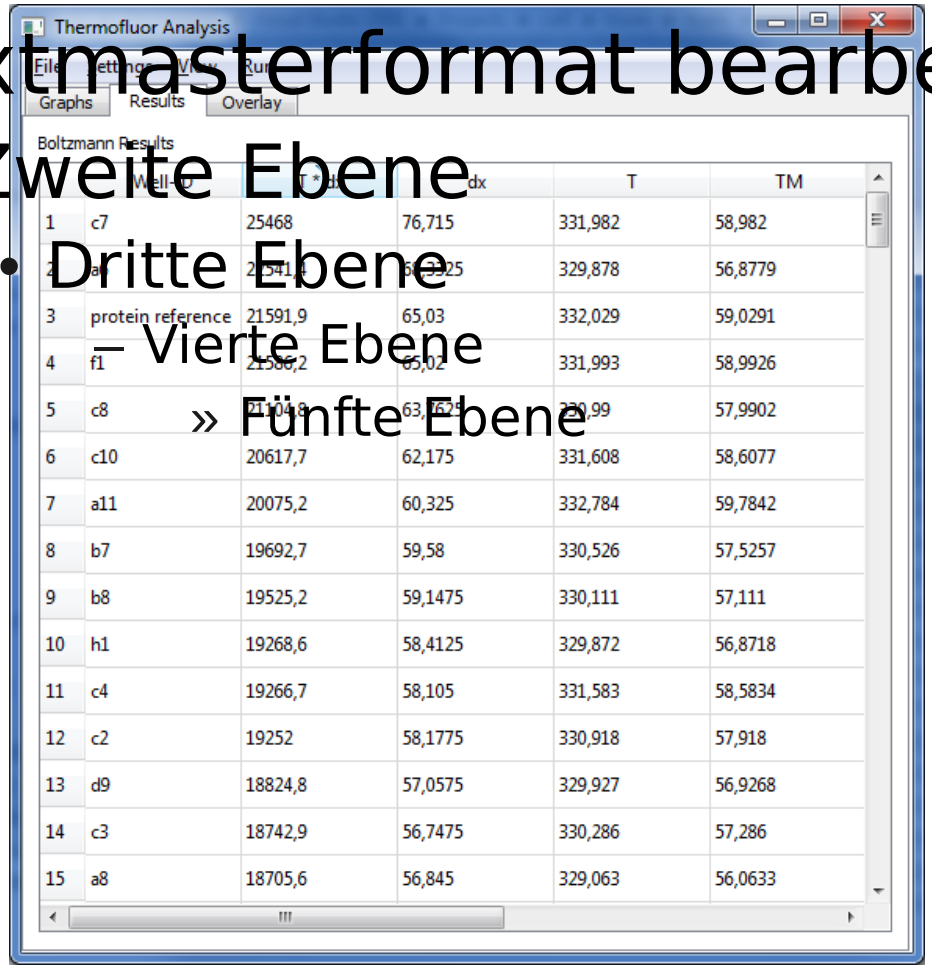
» Fünfte Ebene





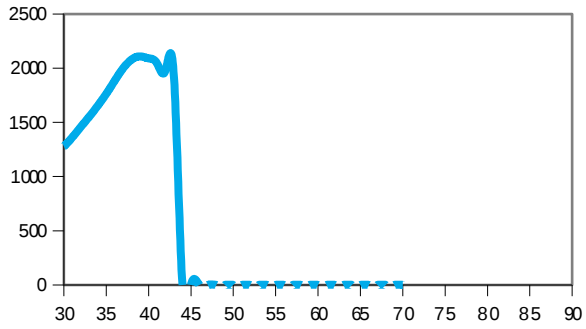
Thermofluor Analyzer Interface - Results

- Textmasterformat bearbeiten
  - Zweite Ebene
    - Dritte Ebene
      - Vierte Ebene
        - » Fünfte Ebene

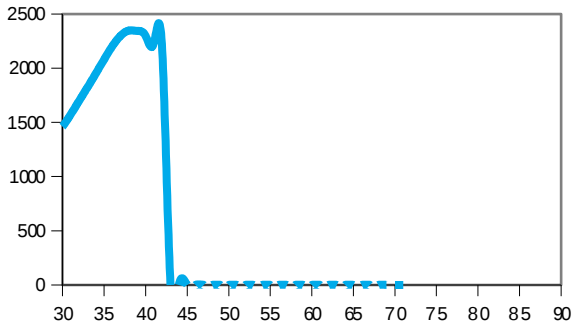


	Well ID	T*	dx	T	TM
1	c7	25468	76,715	331,982	58,982
2	a7	25411,9	65,625	329,878	56,8779
3	protein reference	21591,9	65,03	332,029	59,0291
4	f1	21586,2	65,02	331,993	58,9926
5	c8	21104,8	63,7625	330,99	57,9902
6	c10	20617,7	62,175	331,608	58,6077
7	a11	20075,2	60,325	332,784	59,7842
8	b7	19692,7	59,58	330,526	57,5257
9	b8	19525,2	59,1475	330,111	57,111
10	h1	19268,6	58,4125	329,872	56,8718
11	c4	19266,7	58,105	331,583	58,5834
12	c2	19252	58,1775	330,918	57,918
13	d9	18824,8	57,0575	329,927	56,9268
14	c3	18742,9	56,7475	330,286	57,286
15	a8	18705,6	56,845	329,063	56,0633

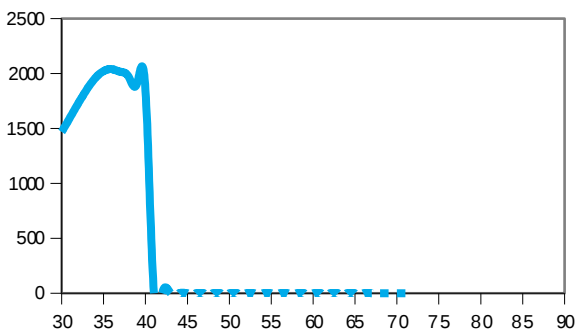
Proof of principle



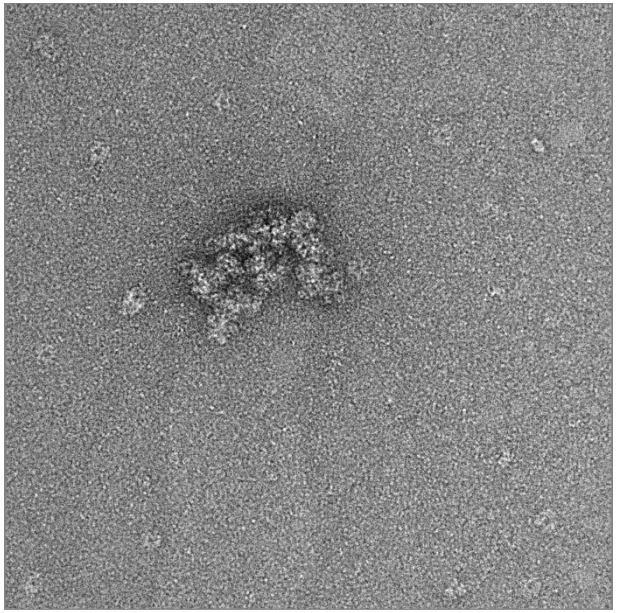
Imidazole pH 5.4

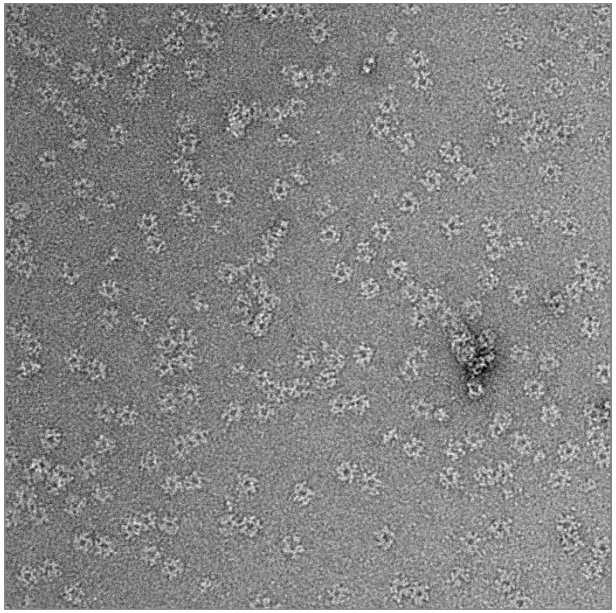


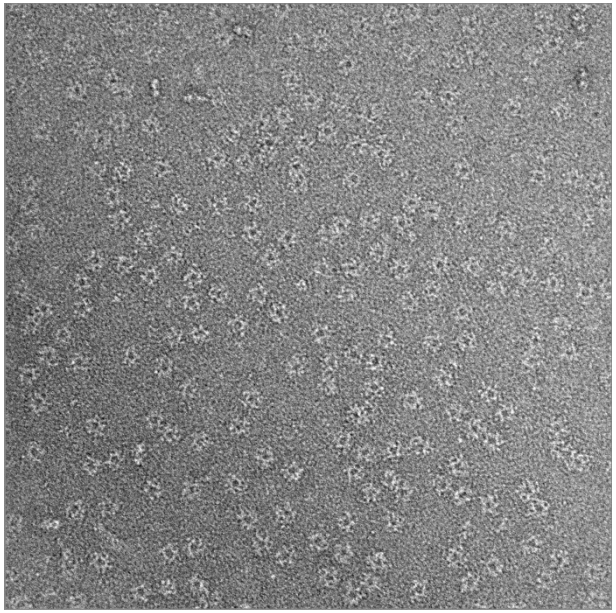
Imidazole pH 6.6



Imidazole pH 8.0

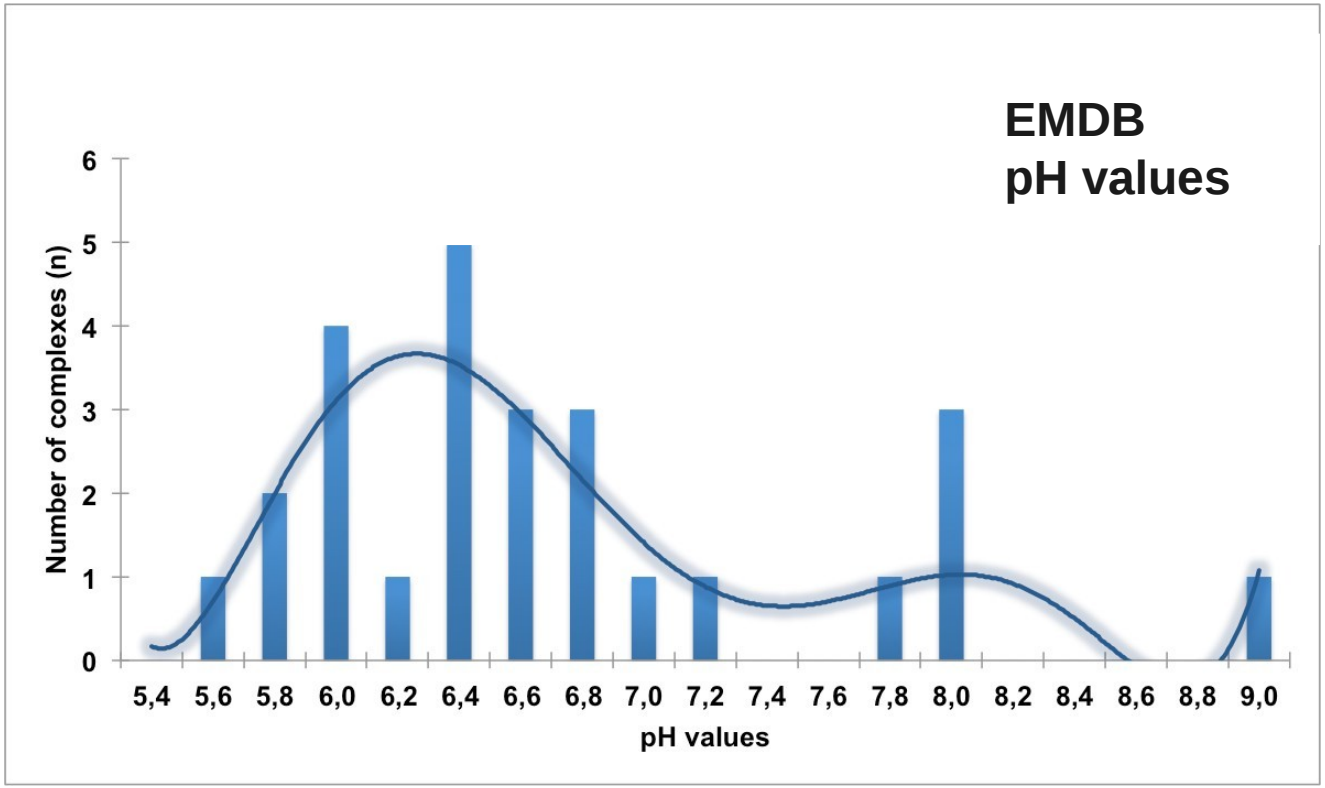






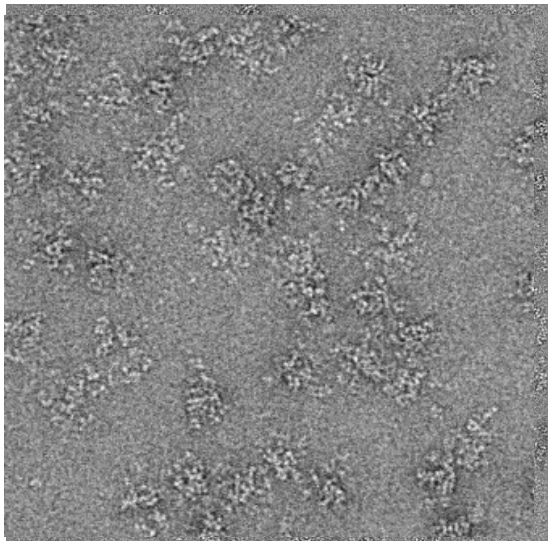
Dramatic pH Effect on Stabilization

pH dependent stabilization profile of macromolecular complexes (n=30 complexes)

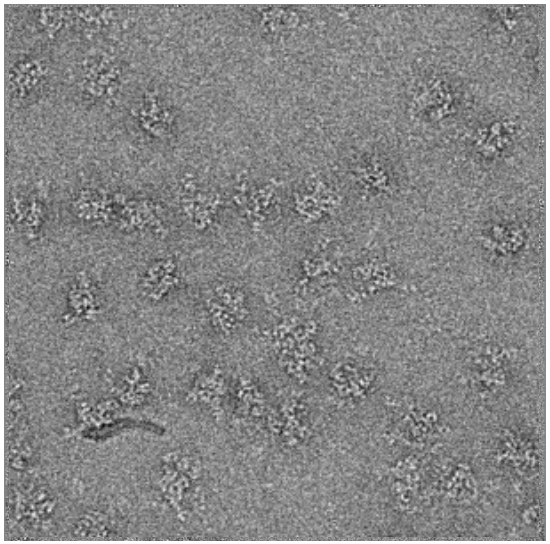


Most complexes studied so far were probably not treated under the best conditions. Lots of room for improvement !!!

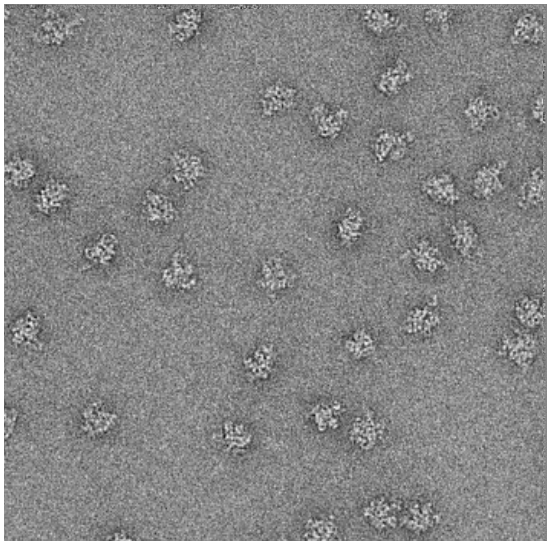
Stabilization of BgHb (snail hemoglobin)



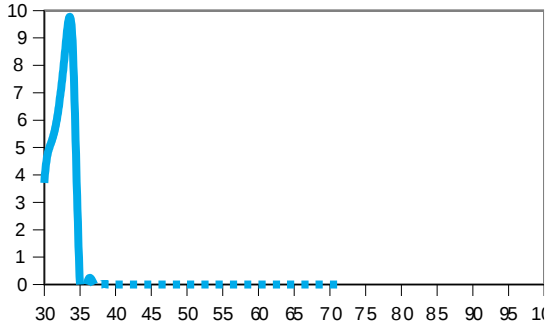
Standard purified sample

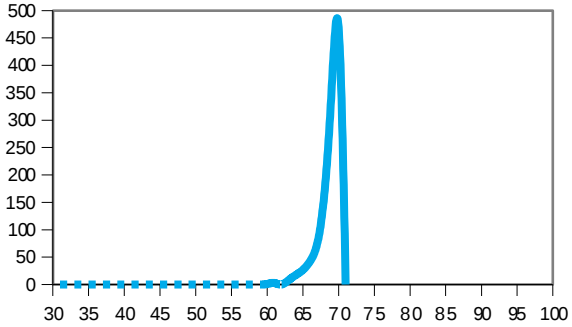


Sample rebuffered into Imidazole pH 6



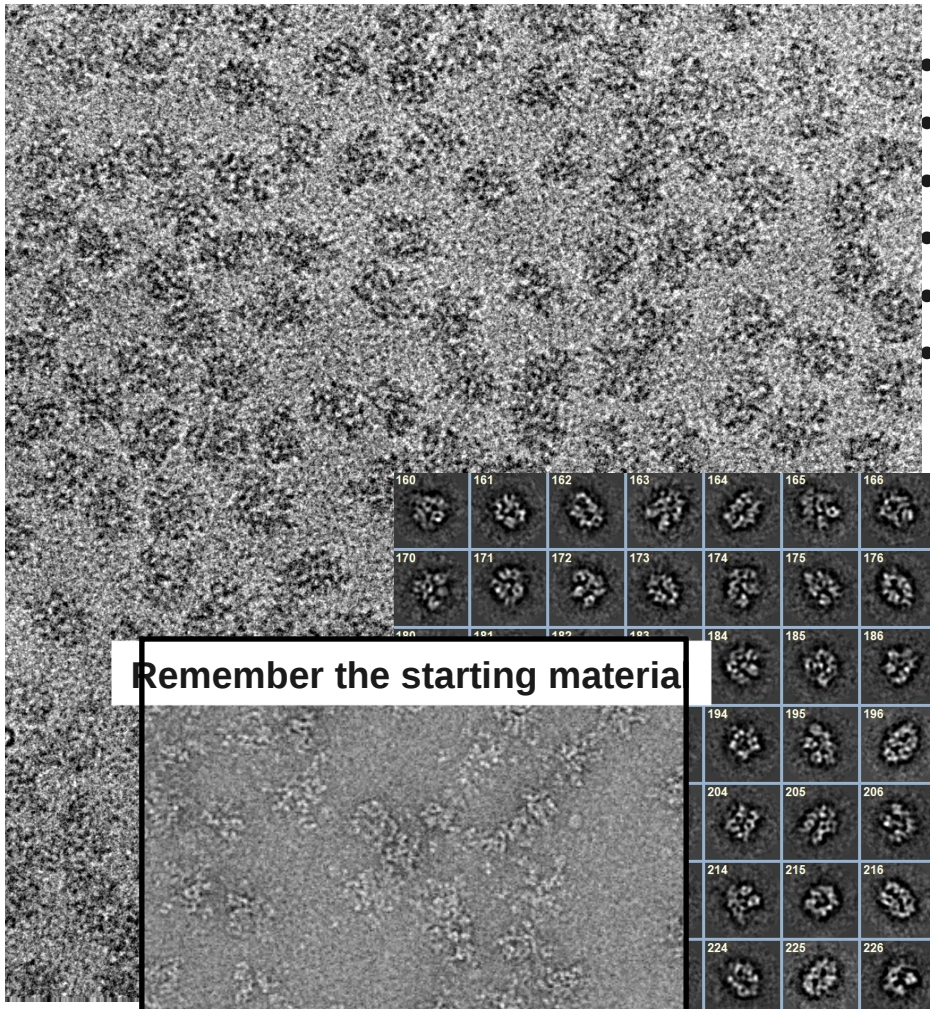
GraFix treated sample



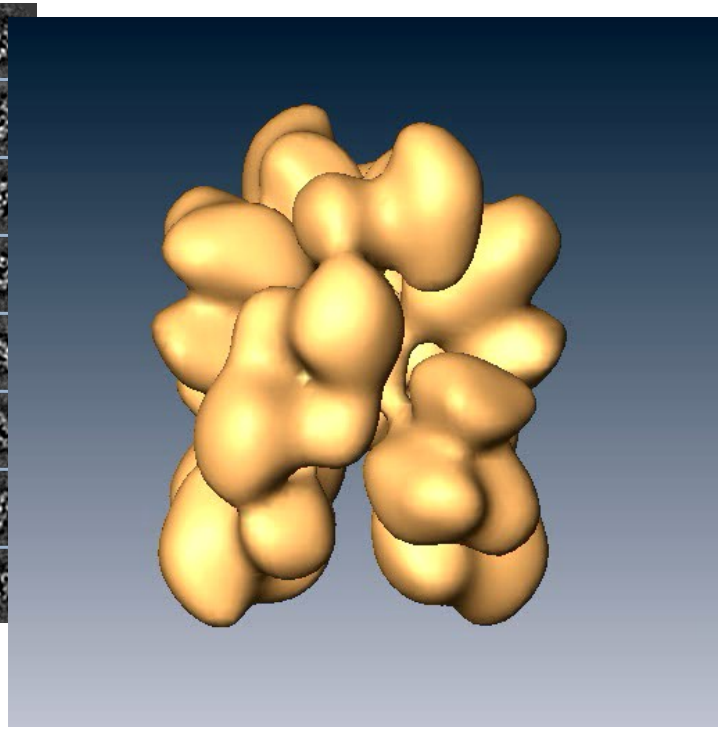


Vanessa Möller, Jürgen Markl

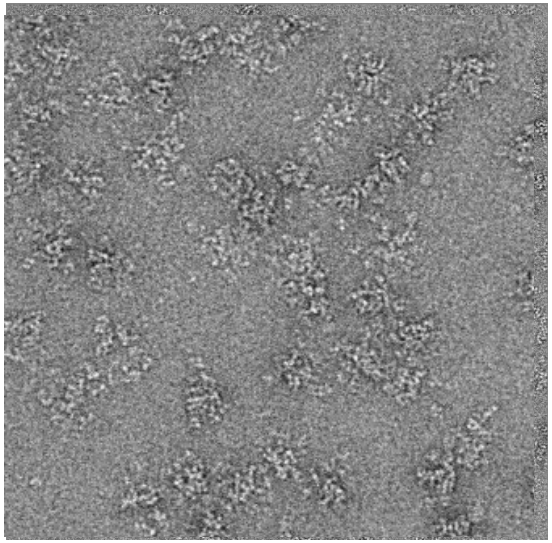
BgHb optimzation!

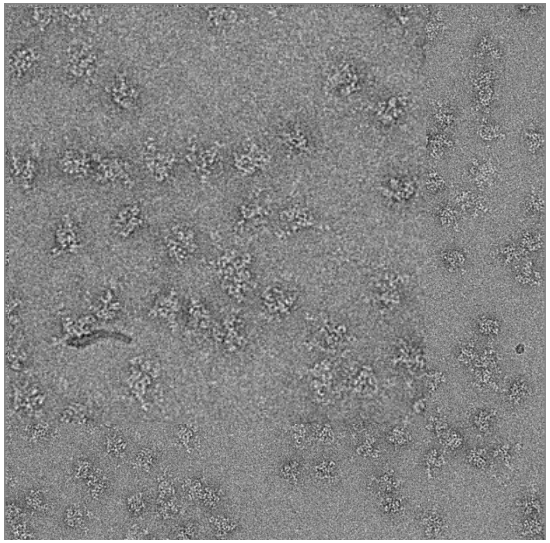


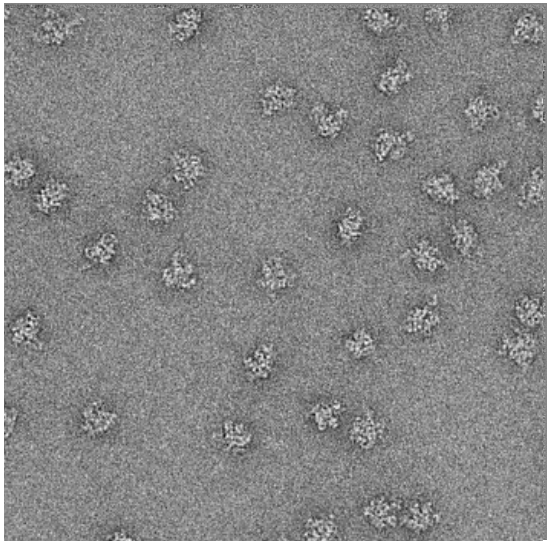
- Day1: buffer optimization
- Day2: GraFix run
- Day3: Preparation of cryo grids
- Day3/4: Imaging over night!
- Day5: Particle picking and 2D analy
- 3D after few weeks



Stabilization of BgHb (snail hemoglobin)



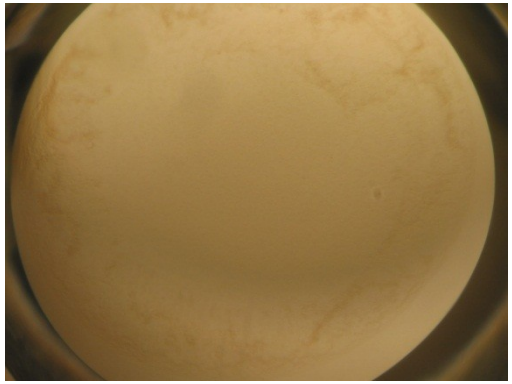


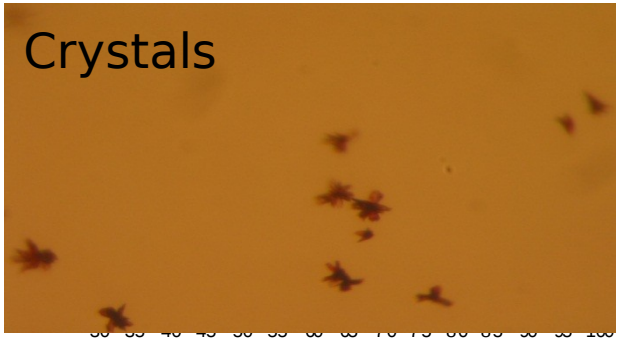


Standard purified Sample: Tris

Result buffer Imidazole pH 6

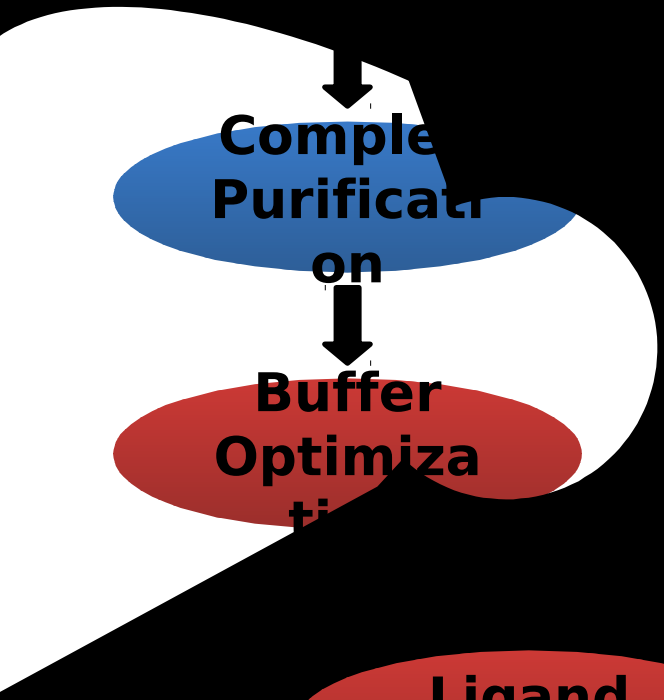
GraFix treaed sample





Perfect Correlation with Crystallization conditions

Protein complex	ProteoPerfect result buffer	Crystallization buffer	Citation
EFG-Ribosome complex	MES, pH 6.5	MES, pH 6.5	Gao et al. Science 2009 326, no5953, 694-699
P97	HEPES, pH 6.8	HEPES, pH 7.0	Brünger et al JMB 2005 347,437-452
7S	Tris, pH 8	Tris, pH 7.6-8.2	Zhang et al. Cell 2011 146(3):384-95
6S/8S	Hepes, pH 6.8	HEPES, pH 7	Grimm et al. 2012 (Manuscript under revision)
	HEPES, pH 8	HEPES, pH 8	
Crm1	HEPES, pH 7.5	HEPES, pH 7.5	Monecke et al. PNAS (2012) under revision
80S Ribosome	Imidazole, pH 6.8	Imidazole, pH 6.8	Personal communication



Cry
o-
EM

X-
ray

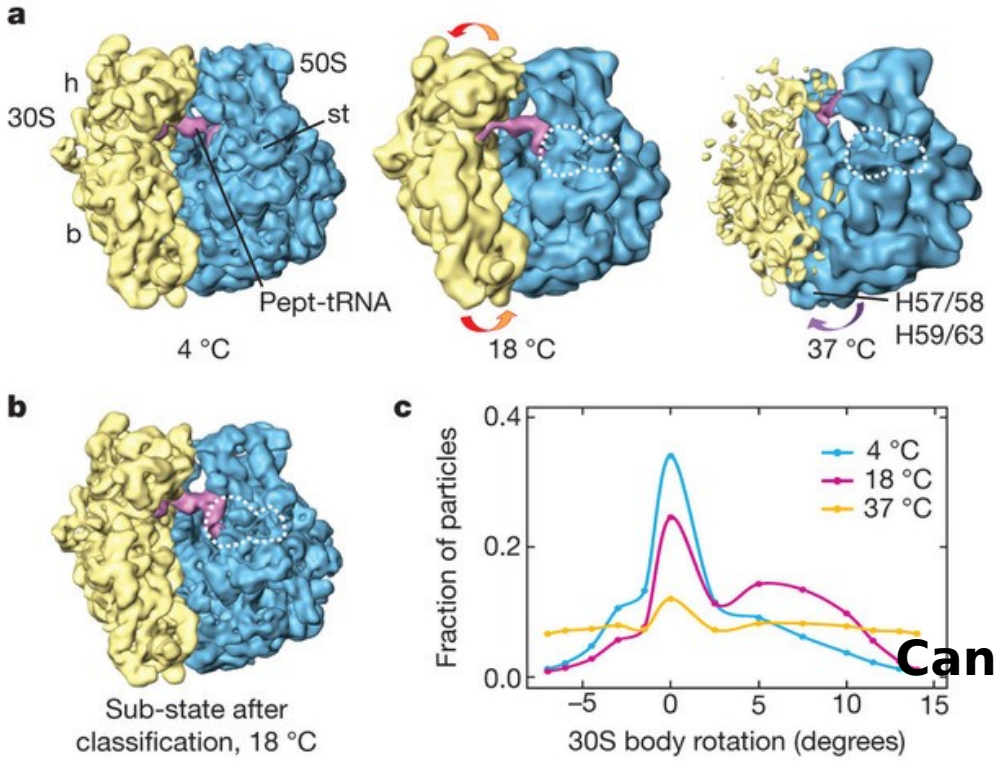
Compl
ex
With
maximum
stability

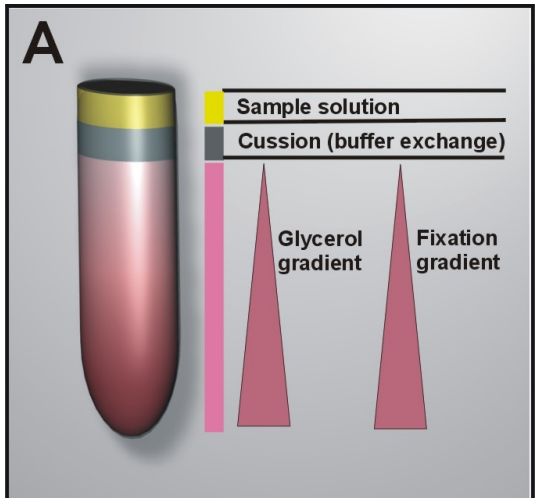
Ligand
Screen

GraFix

What about Conformational Heterogeneit

Cannot be avoided by biochemical stabilization.
Thermal energy is sufficient to generate conformational heterogeneity !





Can we crosslink at low temperatu

- **functional stabilization (not easy, not always possible)**
- **work with Thermophiles ?**
- **Cryo Fixation (Cool-GraFix)**

Acknowledgements

Ashwin Chari
David Haselbach
Jan-Martin Kirve
Niels Fischer

Wen-Ti Liu
Jil Schrader
Michael Hohns
Andrius Kraskauskas
Florian Hauer
Prakash Dube
Florian Platzmar
Boris Busche
Mario Lüttich
Tobias Koske
Frank Würriehausen