Sample Preparation

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Motivation

PDB search results:
Entries with min 4 different chains (complexes) make up only ~1% of the PDB data base.

This is in contrast to the fact that:
Most proteins act in complexes with an average size of ~10 components (chains).

~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
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

GraFix test: Spliceosomal B Complex
Long adsorption times with GraFix

A

Spliceosomal B complex

B

U4/U6.U5 tri-snRNP

particles per area vs. time (min)

particles per area vs. time (min)
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
GraFix
A combined Gradient centrifugation and Fixation method

There is some concern that GraFix may create artefacts:

• 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. ✓

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, 2006
What actually happens - the simple case
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?

[Diagram showing a process with labels $K_{dis}$ and $K_{unf}$]

[Graphs showing temperature $T$ in K and distance $D$ in AU over time $t$]
Thermofluor Analyzer Interface - Graphs

- Textmasterformat bearbeiten
  - Zweite Ebene
  - Dritte Ebene
  - Vierte Ebene
  - Fünfte Ebene
Thermofluor Analyzer Interface - Input

Textmasterformat bearbeiten

Zweite Ebene

Dritte Ebene

Vierte Ebene

» Fünfte Ebene
Thermofluor Analyzer Interface - Results

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

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

Remember the starting material
Stabilization of BgHb (snail hemoglobin)

Standard purified Sample: Tris

Result buffer Imidazole pH 6

GraFix treated sample

Crystals
<table>
<thead>
<tr>
<th>Protein complex</th>
<th>ProteoPerfect result buffer</th>
<th>Crystallization buffer</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFG-Ribosome complex</td>
<td>MES, pH 6.5</td>
<td>MES, pH 6.5</td>
<td>Gao et al. Science 2009 326, no5953, 694-699</td>
</tr>
<tr>
<td>P97</td>
<td>HEPES, pH 6.8</td>
<td>HEPES, pH 7.0</td>
<td>Brünger et al JMB 2005 347,437-452</td>
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<tr>
<td>7S</td>
<td>Tris, pH 8</td>
<td>Tris, pH 7.6-8.2</td>
<td>Zhang et al. Cell 2011 146(3):384-95</td>
</tr>
<tr>
<td>6S/8S</td>
<td>Hepes, pH 6.8</td>
<td>HEPES, pH 7</td>
<td>Grimm et al. 2012 (Manuscript under revision)</td>
</tr>
<tr>
<td>Crm1</td>
<td>HEPES, pH 8</td>
<td>HEPES, pH 8</td>
<td>Monecke et al. PNAS (2012) under revision</td>
</tr>
<tr>
<td>80S Ribosome</td>
<td>Imidazole, pH 6.8</td>
<td>Imidazole, pH 6.8</td>
<td>Personal communication (Nenad Ban lab)</td>
</tr>
</tbody>
</table>
Possible Workflow for Sample Preparation

1. Complex Preparation
2. Buffer Optimization
3. Ligand Screen
4. GraFix

Complex with maximum stability

Cryo-EM

X-ray
What about Conformational Heterogeneity?

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

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

Can we crosslink at low temperature?
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