3D-Repertoire - Quality Control

Why quality control by Electron Microscopy

Protein complexes can be purified by various strategies. The success is usually monitored by SDS-PAGE. This gives information on the subunit composition of the purified complex and major contaminations but not of the condition of the complex. In general, for structural work a complex is required, which is homogeneous in size and free of aggregates. Traditionally, such information is acquired by standard biophysical methods like analytical ultra-centrifugation, analytical gel-filtration or dynamical light scattering. All these techniques need substantial amounts of protein at relatively high concentration making these methods unsuitable for routine characterization of scarce protein complexes. Here electron microscopy provides an excellent alternative. As little as 2 µl of protein solution at a concentration of about 10 µg/ml can already be sufficient to give valuable information on homogeneity of the population of particles and the state of aggregation. Other than with conventional methods the condition of the protein can be directly assessed by visual inspection without further post-processing of the data (seeing is believing). At the same time information on size and shape of the complex are obtained.

What are the experiences with TAP-complexes so far?

In a pilot project in 2002/2003 together with Cellzome we looked at about 140 TAP-complexes after the fist purification step.

Almost all TAP-complexes showed substantial aggregation of protein and a heterogeneous particle population. Common contaminations such as viral capsids and fatty acid synthesases were frequently observed. Very rarely (ca. 5%) the majority (>80%) of particles represented a single homogeneous complex.

Some of the complexes were analyzed after the second step of TAP. Basically the same variation in shapes and sizes were observed but the sample was much more dilute.

These observations came as a surprise, because SDS-PAGE indicated that the complexes were reasonable pure.

Some selected TAP complexes were further optimized (Hurt-group, University Heidelberg). A SMART gel-filtration step usually removed all aggregates and generated complexes, which had a homogenous appearance. Astonishingly, the complex identified after the SMART step had not been recognized after TAP only, because it comprised a minor part of the population of particles.

What can we offer as quality control in 3D-Repertoire?

1. Standard Quality assessment

The main aim in quality control is to judge whether a purified complex is suitable for further structural investigations (homogenous particle population, no major aggregations). In addition, a systematic evaluation of complexes prepared by different methods will allow identifying the optimal strategy for complex purification for structural studies.

We will prepare 4 grids of a purified complex for electron microscopy using two different stains. One stain will be uranyl acetate, which in our hands gives best staining for most applications and has some fixative effect on the protein. However, it has a low pH (pH 4.5). The second stain will be sodium-phosphotungstate at pH 7.0, to avoid the pH problems.
The grids will be screened with the FEI Morgagni. CCD-Images (wide angle port) will be taken at three different magnifications. At low magnification a picture of a whole mesh (about 30 µm square) will show size and frequency of aggregations. At intermediate magnification, CCD-images will show the particle spread and the general homogeneity of the complexes. High magnification images will provide information on whether the particles represent a single complex (size and shape).
Because staining is variable across the grid, we will look at a minimum of three well-stained grid squares per grid. A minimum of three micrographs at medium and high magnification will be recorded. If the first grid is not satisfactory, the second grid will also be considered. CCD-Images will be recorded for both stains.
For a good assessment of the homogeneity of the particle population it is a prerequisite that the particles have optimal packing density. If particles are too densely packed, more grids will be prepared at different dilutions (PBS). If samples are too dilute, no further validation will be carried out.

The CCD-images will be evaluated and a short report together with the image-files will be sent back to the sender describing the main properties of the sample and annotating one or two of the micrographs to demonstrate the properties, if necessary.

For each stain and each magnification one typical micrograph will be selected and deposited in the central databank together with standardized statements on aggregation, homogeneity of particles and approximate size of particles. The homogeneity will be classified in groups: 1) more than 90% of particles represent one complex; 2) 70-90% represent a single complex 3) 50-70 % represent one complex; 4) less than 50% of the particles are likely to form the same complex.

2) Stability tests for samples in category 1 and 2

For structural studies, the sample does not only need to be homogeneous but also stable and concentrated. Concentrating a dilute sample can be cumbersome and often ends with aggregation and loss of most of the protein. Furthermore, complexes are often only stable for a short period of time and do not withstand freezing without additives.

We offer to follow stability over prolonged storage at 4°C. A grid will be prepared and evaluated immediately, after 1 day, 3 days, 7 days, 14 days, and 30 days. Here we will use the optimal conditions determined in the initial quality control.

To test the stability for freezing, the sample will be snap-frozen (liquid nitrogen) and thawed 10 times. Samples will be prepared after freezing cycle 1, 3 and 10. If sensitivity against freezing is detected, we will test 10% and 30% sucrose as additives for cryo-protection.

Concentrating very dilute samples is often tricky. Severe aggregation and loss of most of the sample is a common problem. We offer concentrating a dilute sample in a microcon (cutoff 100 kDa or 30 kDa according to size of the complex). Grids will be

prepared before concentrating, at 5-fold concentration and at maximal concentration (20-fold). The concentration factor will be estimated by the reduction in volume. Grids of the concentrated sample will be prepared after dilution for compensating the calculated concentration effect. This will give a rough estimate on how much protein is lost during concentration, whether aggregation occurs and if the complex keeps its integrity. This information will be deposited in the central databank.

3) Determination of characteristic views for samples of category 1 and 2

Homogeneous samples with sufficient particle density (min 100 particles per µm²) will be further analyzed by image processing. Small data sets of 1000 to 2000 particle images will be selected from 10-20 CCD-images (2Kx2K CCD camera, 5.2 Å/Pixel). Characteristic views will be calculated by standard image processing methods (IMAGIC) using the alignment by classification strategy followed by multiple rounds of alignment and classification (up to 6 rounds).
Analysis of the eigenimages in the classification of the none-aligned data will also allow determination of symmetries. Characteristic well-defined views and information on obvious symmetries will be deposited in the central databank.

4) Electron Cryo-microscopy of complexes of category 1 and 2

Selected large complexes (>= 400 kDa) of category 1 and 2, which have passed the concentration stability-test, will be further investigated by electron cryo-microscopy of vitrified samples. This will establish, whether particles spread nicely over holes or have a preference to attach to the carbon support film, which will influence the way how to freeze grids best. If particle spread and particle density is good, further standard image processing will be carried out, determining characteristic views, for comparison to the stained views and eventually a three-dimensional map, using the three-dimensional map calculated from the negatively stained data (see below, things to develop) as first reference in reference based refinement.

What are the requirements for EM-screening

- The complex should be not smaller than 150 kDa for quality control. More reliable results are expected for larger complexes (ideally more than 500 kDa). Electron cryo-microscopy of vitrified samples (4.) will be carried out for complexes larger than 500 kDa.
- The Complex should be at least 80% pure according to SDS (send scanned gel)
- The protein concentration should be 10 µg/ml or above (protein concentration should be stated). For smaller protein concentrations success is less likely (below 1 µg/ml statistics will be insufficient for accessing quality). For electron cryo-microscopy of vitrified samples (4.) protein concentrations need to be between 2 and 5 mg/ml depending on the size of the complex (in exceptional cases, when strong interaction with surfaces occurs, smaller amounts can be tolerable).
- We require a minimum of 16 µl of protein sample for screen 1, 3 and 4 provided in two aliquots. This is sufficient for preparing 8 Grids. For screen 2, ca. 1 ml is required.
- The sample should be free of detergents. In the presence of detergents the preparation of grids for electron microscopy becomes less reliable (provide more volume, or higher concentrations). Problems in the concentrating screen

will arise from increasing detergent concentrations. Electron cryo-microscopy of detergent containing samples is not possible in the high-throughput set up.

Limitations in quality control by EM

Samples are prepared by embedding the protein in a matrix of heavy metal salts and subsequent complete drying. These conditions are far from native and the treatment with heavy metal salts as well as the low pH of some of the common stains can introduce artifacts. Complexes, which are very sensitive to ionic strength might be destroyed by the staining. The same is true for sensitivity to low pH. However, according to our experience preservation is usually good enough to recognize a "good" sample and will probably result in less than 10% "false-negatives".
There is significant variability between different grids prepared for electron microscopy. This will be compensated by preparing at least 2 grids for each condition.

Open questions

Complexes are often very sensitive and cannot be frozen without severe damage to the sample. Furthermore, often they cannot be stored for prolonged times (more than 12 hours) at 4°C without degradation. This poses the question of how to ship the sample. One possibility might be to prepare grids, where the sample is purified and to ship the grids (see below).

Data from the evaluation should be archived centrally and be made accessible to all participants. This requires standardized image formats and standardized information. Which information should be given here and which naming conventions are useful? Ideally, this needs to be identified before data collection starts.

Future Perspectives

1) Making EM-grids hydrophilic without glow discharge

Complexes are often too labile for surviving freezing or prolonged storage at 4°C. Therefore, it would be advantageous to prepare the grids where the protein is purified. Within the network this would pose the problem that grids have to be prepared where no EM-facility exists. We can supply the different groups with carbon-coated grids. However, these grids age over time and become hydrophobic, which makes staining and adherence of the protein to the grids difficult and irreproducible. This problem is usually solved by glow discharging the grids immediately before use. Unfortunately, the necessary equipment is not common in wet-labs. We will test other ways such as washing with organic solvents and storage under UV light to find a way, to render grids hydrophilic with equipment available in a typical wet-lab. A 'kit' for preparing grids will then be developed and provided to interested groups.
In case of interest, a training course (1-2 days) for preparing grids and some theoretical background on electron microscopy and quality control can be organized by us at EMBL Heidelberg.

2) Localization of subunits in characteristic views

For a number of tags used for purification, commercial monoclonal antibodies are available. For complexes of category 1 and 2 these antibodies can be used to localize

the tags and therefore the respective subunits in the characteristic views. This will allow testing the proximity between subunits and validate the theoretical predictions.
For labeling we require more experience which protocols work best. The standard protocols for post-labeling on the grid and blocking sticky areas with BSA are not suitable for subsequent image processing and the determination of characteristic views. We will test and optimize different protocols using the V-ATPase from yeast, where we have already different tags attached to several subunits.
When we have established a reliable protocol, the localization of subunits in the characteristic views will be provided as "quality control" of complexes of category 1 and 2. Information on the mapping will be deposited in the central databank.

3) "High Throughput" 3D-Image Reconstruction

3D-image reconstruction is not always straight forward and often error prone. We will explore possibilities for generating more reliable 3D-starting models using tomography. One possibility we will investigate is the determination of initial models by doing a single tilt-series of a field of particles and calculating tomograms of 10-20 individual particles in different orientations. These 10-20 tomograms will be combined to an initial three-dimensional model without a missing wedge/cone. This initial 3D-model will serve as first reference in a model based refinement of the raw-data previously acquired for the determination of the characteristic views. If we reach good success rates for determining a three-dimensional map in a reasonable short time (less than one week for a refined image reconstruction) we will add 3D-image reconstruction as a general option for the quality assessment of complexes in category 1 and 2.
The resulting low resolution 3D-models will help testing the theoretical models (Rob Russell) of the complexes. Furthermore, the tomograms and the refined 3D-model will add detailed information on the homogeneity of the complexes and allowing an accurate prediction of whether structural work can be pursued to high resolution. Furthermore, these three-dimensional models can serve as starting models for analysis of vitrified samples.

Time requirements

1. Standard Screening

Preparing grids of 1 sample (4 grids, 2 stains) takes approximately 30 min. If more samples can be done in parallel up to 4 complexes can be prepared per hour (16 grids in total).
Screening per grid (3 grid squares, 7 micrographs each (1 low, 3 intermediate, 3 large magnification) -> 21 CCD images) takes about 20 min. For screening all 4 grides of a sample (2 stains, 2 grids each) ca. 80 min are required.
Sorting, evaluating, annotating images and extracting information for databank and reporting back takes ca. 90-120 min/sample

160-230 min/sample

Shorter evaluation times are possible for very good samples or by compromising on the time spent for exploring samples and the number of images taken, as well as on the details in the evaluation report. This might be necessary in peak times when many samples come in or for very specific questions such as "Is my sample aggregated?" or "Did it improve compared to last time?".

abs. min 120 min/sample

Turnover/week 15-20 samples/ technician (also agrees with our experience with the Cellzome screen 20 samples/ week, only one stain, only one grid, 3 persons working part-time)

2. Stability tests

For the stability tests, the best conditions (stain and dilution) will be known from the tests before. For all tests (freezing, storage, concentrating) we will require one working day, scattered over 30 days. Samples can be done in parallel with a speed-up of max 10%. The time consuming steps are the evaluation and the microscopy

1 day/sample

3. Characteristic Views

Data-collection for the characteristic views is probably 2 hours/sample on CCD at the CM200-FEG. Selecting 1000-2000 particles interactively and doing standard processing takes ca. 1.5 working days.

2 days/sample

4. Mapping of one subunit in a characteristic view

Preparing grids at different ratios of antibody/protein and screening for the best conditions ca. 0.5 days. Collecting data and determination of characteristic views 2 days (see above)

2.5 days/sample

5. "High Throughput" Image Reconstruction

Collecting 1-2 tilt-series ca. 0.5 days. Calculating tomograms of 10-20 selected particles and merging volumes ca. 1 day. Using these volumes for model-based iterative projection matching using raw-data from the characteristic views (see above 3.) ca. 1 day.

2.5 days/sample

6. Electron Cryo-microscopy

Freezing and electron microscopy for evaluating particle spread and density will require 1-2 days. For further data-collection (10000 particles) and detailed image reconstruction 3-6 months will be required.

a) initial feasibility test 1-2 days
b) in depth analysis ca. 4 months/sample

Max. Turnover of complexes/week for a certain task

Task	**complexes/week**
1.Quality control	20
2.Stability-tests	5
3.Characteristic Views	2.5
4.Mapping of subunits	2
5.3D-Image reconstruction stained samples	2
6a.Electron Cryo-Microscopy feasibility study	5
6b.In depth image-analysis of cryo-data	0.06

Total time requirement for certain type of analysis

Analysis	**Analysis to be completed before**	**accumulated Time for Analysis of one complex /1day**
1.Quality control		0.5
2.Stability-tests	1.	1.5
3.Characteristic Views	1.	2.5
4. Mapping of one subunit	1,3	5.
5.3D-Image reconstruction stained samples	1.,3.	5.
6a.Electron Cryo-Microscopy feasibility study	1.,2.,3.,5.	6
6b.In depth image-analysis of cryo-data	1.,2.,3.,5.,6a	100-120

People involved:

3D-Repertoire/Quality Control:
1 Technician (Simone Prinz) Data collection, preparing of samples, basic image processing, standard assessment of quality (possible starting date 15.April 2005)

Further support by Böttcher-group:
Assessment of standard screens, selection of data for databank, establishing general criteria: Bettina Böttcher, later with clearer criteria and more experience handing over to Simone Prinz. In peak times the whole group will help with the evaluation.

"Development" of "high-Throughput" 3D-Image reconstruction: Bettina Böttcher and Meikel Diepholz (side project for Meikel Diepholz)

Support of image processing in quality control: Bettina Böttcher, Meikel Diepholz

Electron cryo-microscopy feasibility tests (Stephanie Kronenberg (3D-Repertoire, purification and structure determination of RNase P/MRP, starting August), Bettina Böttcher, Meikel Diepholz, Britta Gerlach)

Further image processing and analysis of vitrified samples: Bettina Böttcher, Meikel Diepholz (1 complex max), Britta Gerlach, (1 complex max)