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Contents

List of Figures ........................................ viii
List of Tables ......................................... ix
List of Abbreviations ................................. x

Summary ................................................... xii

1 Introduction .......................................... 1
  1.1 Pre-mRNA splicing .................................. 1
    1.1.1 The splicing reaction .......................... 1
    1.1.2 Structure of introns ............................ 2
    1.1.3 The splicing cycle .............................. 3
    1.1.4 Architecture and function of the [U4/U6.U5] tri-snRNP and
        its subunits [U4/U6] and U5 ..................... 5
  1.2 Electron Microscopy and Image Processing .......... 12
    1.2.1 Image formation in the transmission electron microscope (TEM) 12
    1.2.2 Amplitude contrast and phase contrast .......... 12
    1.2.3 Phase contrast transfer function (PhCTF) .......... 13
    1.2.4 Digital image processing for structure determination ...... 16
  1.3 Aim of the work .................................. 29

2 Materials and Methods .............................. 30
  2.1 Materials .......................................... 30
    2.1.1 Software ....................................... 30
    2.1.2 Chemicals .................................... 30
    2.1.3 Laboratory materials .......................... 31
    2.1.4 Special Equipments ............................ 31
    2.1.5 Samples for methodological tests ............... 32
  2.2 Electron microscopy .............................. 32
    2.2.1 Preparation of holey carbon copper grids ......... 32
3 Results

3.1 Developments for improvement of reliability of de novo 3D structures 40
3.1.1 Advantages of a CCD detector for 3D de novo structure determination of single particles 40
3.1.2 Development and characterization of a 3D maximum-likelihood based weighted averaging algorithm for 3D startup model computation 59

3.2 Developments for improvement of resolution and speed 73
3.2.1 Development and characterization of an automatic procedure for CTF correction 74
3.2.2 Development of corrilm-based exhaustive multi-reference alignment via polar coordinates and comparison to direct alignment 91

3.3 Structural analysis of the tri-snRNP and its subunits 108
3.3.1 2D electron-microscopic analysis of the snRNPs [U4, U6, U5], [U4, U6], and U5 108
3.3.2 Computation of initial 3D models via the RCT ML method 110
3.3.3 3D structural analysis of human native [U4, U6, U5] tri-snRNPs 111
3.3.4 3D structural analysis of human native U5 snRNPs 114
3.3.5 3D structural analysis of human native [U4, U6] di-snRNPs 117
3.3.6 Rigid body fitting of U5 snRNP into [U4, U6, U5] tri-snRNP 118
3.3.7 Rigid body fitting of U4, U6 di-snRNP into [U4, U6, U5] triple snRNP 121
3.3.8 Euler angle distribution of projection views 124

4 Discussion
4.1 Importance of proper recording conditions in *de novo* single-particle structure determination ........................................... 128

4.2 Advantages of ML-based 3D weighted averaging for computation of initial 3D models and analysis of structural heterogeneity .................. 131

4.3 Influence of CTF evaluation and correction and on the quality of 3D reconstructions ................................................................. 132

4.4 Influence of the type of alignment algorithm on the quality of 3D reconstructions ................................................................. 133


4.6 Outlook ................................................................................. 138

References ............................................................................. 140

Danksagung ........................................................................... 153

Erklärung ................................................................................. 155

Lebenslauf .............................................................................. 157
List of Figures

1.1 The splicing reaction ........................................... 2
1.2 Intronic consensus sequence .................................. 3
1.3 Pre-mRNA splicing cycle ..................................... 4
1.4 Structure of tri-snRNP snRNAs ............................... 6
1.5 Comparison of molecular weights ............................ 11
1.6 Electron microscopic phase contrast ........................ 14
1.7 Phase contrast transmission function ........................ 15
1.8 Euler angles .................................................. 25
1.9 3D reconstruction ............................................ 28

2.1 Illustration of the “sandwich” preparation technique ........ 34

3.1 Analysis of DPR, SSNR and signal decay of film compared to CCD 45
3.2 Dependence of predicted minimum DPR and maximum SSNR on the defocus ................................................. 46
3.3 Summary of DPR and SSNR for all CCD detector settings .... 48
3.4 Spatial frequency dependent signal decay for all CCD detector settings 49
3.5 Analysis of TMV images recorded on Kodak SO-163 film ........ 50
3.6 Analysis of TMV images recorded on 1x binned CCD detector .... 51
3.7 Analysis of TMV images recorded on 2x binned CCD detector .... 52
3.8 Analysis of TMV images recorded on 4x binned CCD detector .... 53
3.9 Influence of the sampling interval on the performance of alignment and classification .............................................. 54
3.10 Accuracy of the measurements of DPR, SSNR and B-Factor ........ 56
3.11 High-resolution capabilities of film compared to CCD ........... 57
3.12 Cross-correlation based stitching of overlapping CCD images .... 58
3.13 Flow chart of the ML-based 3D weighted averaging procedure ... 63
3.14 Characterization of ML-based weighted averaging for 2D images .. 66
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.15</td>
<td>Application of the 3D ML algorithm to a set of 133 70S ribosomal</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>RCT 3D densities</td>
<td></td>
</tr>
<tr>
<td>3.16</td>
<td>Application of the algorithm to the SF3b data set containing 62 3D</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>densities</td>
<td></td>
</tr>
<tr>
<td>3.17</td>
<td>Application of the RCT/ML algorithm followed by 3D MSA</td>
<td>72</td>
</tr>
<tr>
<td>3.18</td>
<td>Flow-chart for defocus correction</td>
<td>81</td>
</tr>
<tr>
<td>3.19</td>
<td>Multivariate statistical analysis of power spectra</td>
<td>82</td>
</tr>
<tr>
<td>3.20</td>
<td>Assessment of accuracy of defocus and astigmatism</td>
<td>83</td>
</tr>
<tr>
<td>3.21</td>
<td>Detection of defocus via correlation measurements between predicted</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>and measured power spectra</td>
<td></td>
</tr>
<tr>
<td>3.22</td>
<td>Quality of a theoretical CTF including B-Factor and unconvoluted</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>noise</td>
<td></td>
</tr>
<tr>
<td>3.23</td>
<td>Microscope-dependent signal decay</td>
<td>87</td>
</tr>
<tr>
<td>3.24</td>
<td>Measurement of accuracy of the defocus correction method</td>
<td>88</td>
</tr>
<tr>
<td>3.25</td>
<td>Variations in defocus and B factor occurring in micrographs</td>
<td>89</td>
</tr>
<tr>
<td>3.26</td>
<td>Correlation images (&quot;corrims&quot;)</td>
<td>97</td>
</tr>
<tr>
<td>3.27</td>
<td>Computed test images for characterization of alignment algorithms</td>
<td>98</td>
</tr>
<tr>
<td>3.28</td>
<td>Evaluation of corrim-based MRA on a computer cluster with 68 nodes</td>
<td>101</td>
</tr>
<tr>
<td>3.29</td>
<td>Appearance of corrims as a function of SNR</td>
<td>102</td>
</tr>
<tr>
<td>3.30</td>
<td>Behavior of MRA algorithms with respect to SNR and angular distance</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>between reference images</td>
<td></td>
</tr>
<tr>
<td>3.31</td>
<td>Accuracy of fast corrim-based MRA</td>
<td>105</td>
</tr>
<tr>
<td>3.32</td>
<td>Corrims for assessment of image quality</td>
<td>106</td>
</tr>
<tr>
<td>3.33</td>
<td>Consequences of errors contained in reference images</td>
<td>107</td>
</tr>
<tr>
<td>3.34</td>
<td>Electron-microscopic visualization of snRNPs</td>
<td>108</td>
</tr>
<tr>
<td>3.35</td>
<td>3D appearance of snRNPs based upon RCT/ML</td>
<td>110</td>
</tr>
<tr>
<td>3.36</td>
<td>tri-snRNP visualized under native cryo conditions</td>
<td>112</td>
</tr>
<tr>
<td>3.37</td>
<td>Quality of tri-snRNP 3D reconstruction</td>
<td>112</td>
</tr>
<tr>
<td>3.38</td>
<td>3D structure of tri-snRNP</td>
<td>114</td>
</tr>
<tr>
<td>3.39</td>
<td>U5 snRNP 3D reconstruction</td>
<td>115</td>
</tr>
<tr>
<td>3.40</td>
<td>3D structure of U5 snRNP</td>
<td>116</td>
</tr>
<tr>
<td>3.41</td>
<td>3D MSA analysis of high-salt resistant U5 snRNP</td>
<td>117</td>
</tr>
<tr>
<td>3.42</td>
<td>3D MSA analysis of U4 U6 di-snRNP</td>
<td>118</td>
</tr>
<tr>
<td>3.43</td>
<td>Main features of U5 snRNP and tri-snRNP</td>
<td>119</td>
</tr>
<tr>
<td>3.44</td>
<td>Rigid body fitting of U5 snRNP into tri-snRNP</td>
<td>120</td>
</tr>
<tr>
<td>3.45</td>
<td>Conformational flexibility of U5 snRNP head domain</td>
<td>122</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.46</td>
<td>Conformational flexibility of U5 snRNP head domain within the tri-snRNP</td>
<td>123</td>
</tr>
<tr>
<td>3.47</td>
<td>Rigid body fitting of U4/U6 di-snRNP into tri-snRNP</td>
<td>124</td>
</tr>
<tr>
<td>3.48</td>
<td>Contacts between Non-U5 snRNP proportion and U5 snRNP proportion</td>
<td>125</td>
</tr>
<tr>
<td>3.49</td>
<td>Euler angle distribution of tri-snRNP and U5</td>
<td>125</td>
</tr>
</tbody>
</table>
List of Tables

1.1 Protein and RNA composition of tri-snRNP ........................................ 7
2.1 Technical details of particle data sets ................................................. 37
3.1 Complete list of CCD camera settings .................................................. 43
3.2 B factors as measured with different microscopes ............................... 90
3.3 Amplitude-contrast proportions .............................................................. 90
List of Abbreviations

2D  two-dimensional
3D  three-dimensional
Å   Angstrom (1Å = 1\times10^{-10} m)
aa  Amino acid
ATP Adenosine triphosphate
CCC Cross-correlation coefficient
CCD Charge coupled device
CCF Cross-correlation function
Cs  Spherical aberration constant
CTF Contrast transfer function
DPR Differential phase residual
DTT Dithiothreitol
Eq. Equation
FEG Field emission gun
FFT Fast Fourier transform
Fig. Figure
FWHM Full width at half maximum
GTP Guanosine triphosphate
HAC Hierarchical ascendant classification
HSRU5 High-salt resistant U5 small ribonucleoprotein
I/O Input/Output
Magn. Magnification
m3G 2,2,7-trimethylguanosine
ML Maximum-likelihood
MRA Multi-reference alignment
MSA Multivariate statistical analysis
MTF modulation transfer function
NMR Nuclear magnetic resonance
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Number</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside triphosphate</td>
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<td>OD</td>
<td>Optical density</td>
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<tr>
<td>p.</td>
<td>Page</td>
</tr>
<tr>
<td>PhCTF</td>
<td>Phase contrast transfer function</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
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<tr>
<td>pre-mRNA</td>
<td>pre-messenger ribonucleic acid</td>
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<tr>
<td>RCT</td>
<td>Random conical tilt</td>
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<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SF3b</td>
<td>Splicing factor 3b</td>
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<td>snRNA</td>
<td>small nuclear ribonucleic acid</td>
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<tr>
<td>snRNP</td>
<td>small nuclear ribonucleoprotein</td>
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<td>SSNR</td>
<td>Spatial signal-to-noise ratio</td>
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<td>TBSV</td>
<td>Tomato bushy stunt virus</td>
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<tr>
<td>tri-snRNP</td>
<td>triple small nuclear ribonucleoprotein</td>
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<td>TMV</td>
<td>Tobacco mosaic virus</td>
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<tr>
<td>TPR</td>
<td>tetratricopeptide repeat</td>
</tr>
</tbody>
</table>
The 25S [U4/U6.U5] triple snRNP (tri-snRNP) particle is formed by its two stable subunits, the 20S U5 snRNP and the 13S [U4/U6] di-snRNP particle. It contains – besides the three snRNAs U4, U5 and U6 – a set of 30 distinct proteins. During the spliceosomal reaction cycle, both subunits of the tri-snRNP undergo dramatic conformational rearrangements. In isolated tri-snRNP, the U4 and U6 snRNAs are base-paired. Prior to spliceosomal catalysis, U4 snRNA together with various U4/U6-associated proteins dissociates, and the U6 snRNA base pairs with the U2 snRNA as well as with the 5' splice site of the pre-mRNA. Other protein splicing factors that do not interact with isolated tri-snRNP associate with the U5 snRNP to form the 35S U5 snRNP particle and remain stably bound even after catalysis. During various recycling events that occur after catalysis, the U5 snRNP is remodeled into a smaller complex, the [U4/U6] is reannealed and a new tri-snRNP assembled. Here, the morphological basis allowing these complexes to undergo such dynamic transitions was investigated by three-dimensional (3D) electron cryo-microscopy. 3D structures of the native human tri-snRNP as well as of its stable subunits, the 20S U5 snRNP and 13S U4/U6 snRNP, are presented. Both the [U4/U6] di-snRNP and the U5 snRNP particle have been localized within the fully assembled tri-snRNP. A significant domain flexibility of the U5 snRNP particle is demonstrated that sheds new light on the capability of the U5 snRNP to take part in remodeling events during spliceosomal catalysis and recycling.

The underlying methodical problem to solve the 3D structures of these macromolecules is that of de novo 3D structure determination of unknown, asymmetrical macromolecules using single-particle techniques. Available standard techniques exhibit certain restrictions in terms of sample quality. Thus, it has been impossible in the past to compute 3D structures of these particles with an accuracy, reproducibility and resolution high enough to enable meaningful biological interpretation of the structures. Therefore novel image-processing techniques had to be developed that (1) improve reliability, validity and reproducibility of the 3D
structures, and (2) improve the maximum resolution to be obtained. The advantages of CCD detectors compared to conventional film for de novo image-processing has been analyzed in detail, leading to an approximately 2-fold increase in image contrast and significantly more reliable initial phases compared to use of film. A 3D maximum-likelihood based weighted averaging procedure in combination with 3D multivariate statistics suitable for the analysis of large sets of 3D densities has been developed to facilitate the computation of an initial 3D model and to evaluate conformational heterogeneity of a given macromolecule. For improving the resolution of single-particle 3D structures, a new algorithm for the fast and accurate alignment of large sets of particle images was introduced, and a method to correct blurring in electron-microscopic images imposed by the contrast transfer function is presented.
1 Introduction

1.1 Pre-mRNA splicing

In eukaryotic gene expression, most genes are arranged in coding segments (exons) that are disrupted by intervening non-coding sequences (introns). After transcription, two transesterification reactions are necessary for the excision of an intron from the pre-mRNA and ligation of the exons. This reaction of pre-mRNA splicing occurs in the nucleus and is catalyzed by a large protein-RNA complex termed spliceosome. The small nuclear ribonucleoprotein (snRNP) particles U1, U2, [U4/U6], and U5 that sequentially bind to the pre-mRNA are the constitutive components of the spliceosome. The snRNP particles comprise the small nuclear RNAs (snRNAs), as well as stably associated proteins that are either common to all snRNP particles or characteristic of a given snRNP complex. In the spliceosome, several base pairing interactions occur between the snRNAs as well as between snRNAs and pre-mRNA. When the spliceosome is converted into a catalytically active complex, the RNA network formed by the snRNAs and the pre-mRNA is significantly remodeled emphasizing the highly dynamic nature of the spliceosome.

1.1.1 The splicing reaction

The splicing reaction takes place via two subsequent transesterification reactions (Burge et al. 1999). Besides the two splice sites at the 5' and 3' end of the intron, a conserved adenosine is involved in the reaction: in the first step, the 2' hydroxyl group of the conserved branchpoint adenosine nucleophilically attacks the 5' splice site leading to the formation of a free 3' hydroxyl group at the 3' end of the 5' exon and a branched intron intermediate termed lariat intermediate. Then, the free 3' hydroxyl group of the 5' exon nucleophilically attacks the 3' splice site resulting in the joining of both exons and the release of the intron lariat. No external source of energy is needed for this reaction, because in each step one phosphodiester
bond is broken and newly formed. However, NTPs are consumed in the course of spliceosome assembly and structural rearrangements leading to catalytic activation of the spliceosome (Staley and Guthrie 1998).

### 1.1.2 Structure of introns

Introns vary widely in length and exhibit a very low degree of conservation. However, characteristic nucleotide sequences can be found at the 5' and 3' splice sites as well as in the branch site region. The first two intronic nucleotides at the 5' splice site exhibit the sequence GU within the 5' consensus sequence AGGURAGU (where R denotes a purine). At the 3' splice site, the conserved dinucleotide AG is preceded by a pyrimidine. Close to the 3' splice site, the conserved branchpoint adenosine can be found as part of the short conserved sequence CURACU (where R denotes a purine). Between branchpoint adenosine and 3' splice site the polypyrimidine tract is located. The distance between branchpoint adenosine and 3' splice site ranges generally between 18 and 38 nucleotides (Hall and Padgett 1994).

The majority of introns is processed by the U2-dependent or major spliceosome. Additionally, a second, rare class of introns – that is recognized by an alternative spliceosome termed minor or U12-dependent spliceosome – exists in higher eukaryotes including plants, insects and human. In the minor spliceosome, the U12 snRNP which replaces the U2 snRNP and the U11 snRNP which replaces the U1
snRNP stably interact as a di-snRNP that recognizes these special introns. Both types of introns are compared in Fig. 1.2, p. 3.

1.1.3 The splicing cycle

Typical eukaryotic genes contain up to dozens of introns that have to be excised in a nucleotide-exact manner prior to translation. For each splicing reaction, a spliceosome forms out of preformed complexes and undergoes major structural rearrangements while performing the reaction. Upon completion, the spliceosomal components are recycled for a new round of splicing. In the stepwise assembly model, the snRNPs U1, U2 and the preformed [U4/U6.U5] tri-snRNP (compare section 1.1.4, p. 5) bind sequentially to pre-mRNA. Significantly, U1 and U2 snRNP have been shown to bind to pre-mRNA independent of the tri-snRNP, and the sequential assembly of the spliceosome can be observed by native gel electrophoresis in vitro (Konarska and Sharp 1986). Based upon observations in the yeast S. cerevisiae (Stevens et al. 2002) and human HeLa cells (Konarska and Sharp 1988) it is believed that all snRNP complexes may alternatively be preassembled prior to binding of pre-mRNA.

To date, a unified model explaining all possible aspects of spliceosome formation is not available. In the sequential model (Konarska and Sharp 1986; Konarska and Sharp 1987), the catalytically active spliceosomal complex C is preceded by various precursors (compare Fig. 1.3, p. 4):

1. U1 snRNP binds to the 5' splice site in an ATP-independent manner to form the spliceosomal complex E, and base pairing interactions are formed between the U1 snRNA and nucleotides +1 – +6 of the intron (Michaud and Reed 1991). The U2 snRNP was shown to be weakly associated with the E complex (Das et al. 2000). Additionally, the U5 snRNP-specific protein U5-220k (compare section 1.1.4, p. 5) has been shown to interact with the 5'
Figure 1.3: Sequential model of the cycle of pre-mRNA splicing. The catalytically active spliceosome is formed subsequently via the pre-spliceosomal complex E (U1 snRNP bound to pre-mRNA, not shown), complex A and complex B. The U1 snRNP thereby serves in the recognition of the 5′ splice site, whereas the U2 snRNP recognizes the branchpoint adenosine. Complex B is formed upon binding of the [U4/U6.U5] tri-snRNP to complex A. The catalytic activation is accompanied by far-reaching remodeling events on the RNA level as well as significant changes in the protein composition: U1 and U4 leave the complex, and proteins associated with Prp19p enter the spliceosome. A new U2/U6 RNA duplex is formed, and the U6 snRNA replaces the U1 snRNA at the 5′ splice site. The splicing reaction takes place via two transesterification steps (Complex C1 and C2). Then the processed mRNA is released, and the components undergo several recycling steps before a new round of splicing: The U5 snRNP dissociates from the posts spliceosome as a 35S particle and thus has to be reformed to the 20S particle present in the tri-snRNP. U4 and U6 have to be reannealed and the tri-snRNP has to be reassembled.

splice site independent of the interaction between U2 snRNP and branch site in an ATP-dependent manner (Maroney et al. 2000).

2. Formation of the pre-spliceosomal complex A is based upon stable binding of the U2 snRNP to the intronic branch site in the presence of ATP, thereby forming base pairing interactions between the U2 snRNA and a complementary sequence of the pre-mRNA at the branch point (Reed and Maniatis 1988; Wu and Manley 1989). The interaction of U2 snRNP with pre-mRNA is mediated by the heterodimeric splicing factor U2AF (Wu et al. 1999).

3. Upon binding of the [U4/U6.U5] tri-snRNP to complex A, complex B is formed (Konarska and Sharp 1987). Complex B then undergoes several con-
formational rearrangements for catalytic activation.

The U1 snRNP is replaced by the U6 snRNA which requires the disruption of the U1-5' splice site base pairing interaction (Konforti et al. 1993). The U4-U6 base pairing interactions are disrupted and the U4 snRNA as well as [U4/U6] di-snRNP associated proteins leave the spliceosome. New base pairing interactions between the U6 snRNA and the 5' splice site, between the U2 and U6 snRNA as well as a new internal U6 stem loop are formed (Wu and Manley 1991; Staley and Guthrie 1998) A new set of proteins associated with the splicing factor hPrp19p, but not associated with isolated [U4/U6.U5] tri-snRNP enters the spliceosomal complex B (Makarov et al. 2002) during these remodeling events to form the catalytically active complex B*.

4. The first step of the splicing reaction is carried out by the complex C1.

5. For the second step of splicing, a poorly defined transition of complex C1 to C2 occurs.

6. Finally the mature mRNA is released from the spliceosome. A postspliceosomal complex still contains the U2, U6 and U5 snRNAs. The U5 snRNP is thought to leave the postspliceosome in a large 35S form with the proteins of the prp19 CDC5 complex attached. To restore the [U4/U6.U5] tri-snRNP for a new round of splicing, the 35S U5 snRNP has to be converted into a 20S state. A new [U4 U6] di-snRNP has to reassemble, before U5 and [U4/U6] can again stably interact in the tri-snRNP (Raghunathan and Guthrie 1998; Bell et al. 2002).


In human and the yeast S. cerevisiae, U4, U5 and U6 small nuclear ribonucleoprotein (snRNP) particles are assembled in a [U4/U6.U5] triple snRNP (tri-snRNP) complex that constitutes the largest preformed assembly of early spliceosomes (Konarska and Sharp 1987; Black and Pinto 1989). Native U4/U6.U5 tri-snRNP complexes purified from HeLa nuclear extract sediment at ~25S and contain the U4, U5 and U6 small nuclear ribonucleic acids (snRNAs) as well as 30 distinct proteins that are conserved across species. In isolated tri-snRNP, U4 and U6 snRNA form a Y-shaped duplex enclosing two helices framing a stem/loop (compare Fig.
Figure 1.4: Structure of tri-snRNP snRNAs. On the left the [U4/U6] duplex is shown. The sites of base-pairing interactions between U6 and U2 snRNA in the catalytically active spliceosome are depicted in red. The U6 bases forming the U6 internal stem/loop are colored in green. On the right, the structure of the U5 snRNA is shown.

1.4, p. 6). Upon binding of the tri-snRNP to complex A, the spliceosomal complex B is formed.

Most of the 30 proteins found in the [U4/U6.U5] tri-snRNP are stably associated with either the 20S U5 snRNP or the 13S [U4/U6] di-snRNP alone. In human, the tri-snRNP proteins 220k, 200k, 116k, 102k, 100k, 52k, 40k, and 15k (compare Table 1.1) are also associated with purified 20S U5 snRNP (Bach et al. 1989). The proteins 90k, 60k, 20k, 61k, and 15.5k are associated with the [U4/U6] di-snRNP particle (Makarov et al. 2002). The set of the seven small Sm proteins (SmB/B', SmD3, SmD2, SmD1, SmE, SmF, and SmG) binds to the Sm binding site RAU_{4-6} GR that is common to all spliceosomal snRNAs except U6. From X-ray crystallographic studies it is known that Sm proteins consist of an N-terminal α-helix, and a five-stranded antiparallel β-sheet. The first three of the β-strands belong to the conserved Sm1 motif and the last two to the conserved Sm2 motif. Crystal structures of the D_{1}D_{2} and D_{3}B complex are available (Kambach et al. 1999). Based on electron-microscopic studies of the U1 snRNP particle the seven Sm proteins – the Sm core – form an asymmetric funnel-like structure of ~8 nm diameter (Stark et al. 2001). The U6 snRNA (and also the U6atac snRNA of the minor spliceosome) does not contain the Sm binding site. Instead, U6 binds the Sm-like LSM2 – LSM8 proteins, that facilitate formation of the U4/U6 duplex in vitro; electron microscopy has shown that the LSM proteins form ring like structures similar to the proteins of the Sm core (Achsel et al. 1999) As the U4 snRNA as well as the U5 snRNA are associated with an Sm core, in total two Sm cores and one Sm-like core are present in the tri-snRNP.
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Table 1.1: Protein and RNA composition of tri-snRNP and its sub-complexes, [U4/U6] di-snRNP and U5 snRNP. Note that a set of seven Sm proteins associates with the U4 as well as the U5 snRNA, so that these proteins occur twice in the tri-snRNP. Additionally, the yeast homologues and known structural motifs are given (second last and last column).

The proteins 110k, 65k and 27k are only found in the tri-snRNP and therefore classified as tri-snRNP specific. In human, the SR-related proteins 110k and 65k have been shown to be essential for the formation of mature spliceosomes. In contrast, immunodepletion of the 110k and 65k proteins did not affect stability of the tri-snRNP (Makarova et al. 2001).

Many proteins exhibit functions related to conformational changes: U5-100k and U5-200k are members of the DEXD/H box family of ATP-dependent RNA unwindases that are thought to drive the dramatic conformational rearrangements (Staley and Guthrie 1998). U5-100k (human orthologue of S. cerevisiae Prp28p) is implicated in displacing the U1 snRNA from the 5’ splice site (Staley and Guthrie 1999). U5-200k (Brr2p in yeast) is thought to facilitate U4/U6 unwinding (Laggerbauer et al. 1998). U5-116k (Snu114p in yeast), the sole GTPase among the spliceosomal proteins, plays a role in unwinding the U4/U6 duplex or more proba-
bly in controlling the action of Brr2p. Thus it may exhibit a similar function as its ribosomal homologue, the translocase EF-2 (Bartels et al. 2002). In yeast, Snu114p directly interacts with the U5 RNA 5' internal loop 1 close to the interaction site of Prp8p to this loop (Dix et al. 1998). The protein U5-220k (human orthologue of S. cerevisiae Prp8p) functions in the recognition of the 5' splice site, part of the polypyrimidine tract and the 3' splice site. Thus it contacts all important pre-mRNA sites involved in the splicing reaction. The interaction of U5-220k with the 5' splice site occurs within complex B. The human U5-220k is 62% identical to the yeast orthologue and thus besides the U6 snRNA — one of the most conserved components of the spliceosome across species (Teigelkamp et al. 1995; Siatecka et al. 1999; Luo et al. 1999; Reyes et al. 1999).

The U5-102k protein is essential for the integrity of the [U4/U6.U5] tri-snRNP and stably associated with purified 20S U5 snRNP particles. Sequence analysis revealed that the 102k protein contains up to 19 tetratrico peptide repeats (TPR). TPR is a 34 amino acid motif that forms a closely spaced helical arrays and is typically involved in multiple protein-protein interactions (Makarov et al. 2000). The 102k protein interacts with the [U4/U6] specific 61k protein and thereby forms a bridge between the two particles (Makarova et al. 2002). In addition, a stable interaction of the U5-specific proteins U5-220k, U5-200k, U5-116k, and U5-40k even in the absence of U5 snRNA has been reported (Achsel et al. 1998).

The U5-52k protein is the only protein found in the U5 snRNP particle, that is not integrated into the tri-snRNP. It has been shown to interact with the U5-102k and the U5-15k proteins and might thus function in tri-snRNP assembly (Laggerbauer et al. 2005). As shown by X-Ray crystallography, the U5-15k protein exhibits a thioredoxin-like fold and is required for splicing in S. cerevisiae. Interestingly, it is inaccessible to a 15k-specific antibody, so it might closely interact with other components of the U5 snRNP (Reuter et al. 1999). The important role of multiple protein interactions is also underlined by the fact that the U5 snRNP remains intact at high salt conditions up to chloride concentrations of 800 mM (Makarov et al. 2000). The so called "high salt resistant" U5 snRNP particle purified under high salt conditions contains all U5-specific proteins, only the U5-100k protein is missing (Laggerbauer et al. 1998). Structural data obtained by X-ray crystallography as well as protein interaction data are also available for components of the [U4·U6] di-snRNP: the U4/U6-specific 20k protein exhibits a cyclophilin fold with an eight-stranded antiparallel beta-barrel framed by two alpha-helices. The 20k protein belongs to a family of proteins exhibiting peptidyl-prolyl cis-trans isomerase (PPI)
activity and may therefore facilitate the isomerization of peptidyl-prolyl bonds. The 20k protein is part of a stable heterotrimeric complex consisting of the 20k, the 60k and the 90k protein and shows a highly specific binding site for the 60k protein. The 60k protein contains a C-terminal WD40 domain with seven WD40 repeats that is predicted to fold into a seven-bladed beta-propeller. The 60k protein is known to interact with both the 20k and the 90k protein in the 20/60/90k complex, whereas the 20k and the 90k do not stably interact with each other in the absence of the 60k protein. (Reidt et al. 2000; Reidt et al. 2003; Ingelfinger et al. 2003). The crystal structure of the U4/U6-specific 15.5 kD protein bound to the 5’ stem/loop has revealed a novel RNA binding domain (Vidovic et al. 2000). The 15.5k protein mediates the interaction of 61k and the 20/60/90k complex with the U4/U6 snRNA duplex, i.e. both 61k and 20/60/90k bind only to the U4/U6 snRNA duplex in presence of 15.5k. 61k contacts the 5’ portion of U4 snRNA, whereas the 20/60/90k complex requires stem II but not stem I of the U4/U6 duplex for binding. (Nottrott et al. 2002) Additionally, the 90k protein has been shown to cross link to a U6 oligonucleotide base-paired to U4 snRNA in the region of the stem II (Nottrott et al. 2002).


Many experimental findings underline the highly dynamic behavior of the [U4/U6.U5] tri-snRNP, in particular the U4/U6 duplex as well as the U5 snRNP portion of the tri-snRNP, during the spliceosomal reaction cycle. When the tri-snRNP is added to the spliceosomal complex A to form complex B, a number of RNA rearrangements occurs and changes in protein composition occur, and the tri-snRNP, despite its stability in the isolated form, disintegrates when the spliceosomal complex B is converted into an enzymatically active complex (Makarov et al. 2002): The U4:U6 duplex is unwound and the former U6 snRNA region of helix I binds to U2 snRNA. The U2 snRNA 5’ stem/loop is thereby disrupted and the U6 helix II region forms a new intramolecular stem/loop. Furthermore, a base pairing interaction between U6 snRNA and the 5’ splice site of the pre-mRNA replaces the initial U1 5’ splice site helix. U4 and U1 snRNA as well as various [U4 U6]-specific proteins (90k, 61k, 60k, 15.5k and 20k) which are not required for catalysis leave the spliceosome (Staley and Guthrie 1998; Makarov et al. 2002). A set of new splicing factors including hPrp19p and CDC5 joins the U5 snRNP portion and remains stably bound to the U5 snRNP even after disintegration of the tri-snRNP and completion of the splicing reaction. The U5 snRNA interacts with
exon sequences at the 5' and 3' splice sites during splicing. The conserved loop 1 of the U5 snRNA is hereby especially important for the second step (O'Keefe and Newman 1998).

Whereas U4 and U6 are thought to leave the spliceosome as single complexes that have to be reannealed with the help of the p110 protein before a new tri-snRNP can be assembled (Bell et al. 2002), the U5 snRNP dissociates from the post spliceosome as a 35S U5 snRNP particle. For a new integration into a tri-snRNP, the 35S U5 snRNP must undergo significant changes in protein composition, so that a new 20S U5 snRNP particle can be formed: prp19p and several prp19p-associated proteins have to dissociate from U5. The U5-100k, U5-102k, U5-15k and U5-52k which are not part of the 35S U5 have to reassociate with the U5 snRNP (Makarov et al. 2002). The U5-52k protein then mediates tri-snRNP assembly, but dissociates from the complex when a new tri-snRNP is formed (Laggerbauer et al. 2005).

Electron microscopic studies of the tri-snRNP and related particles

The tri-snRNP particle as well as its subunits U5 and [U4/U6] have been analyzed by conventional negative stain electron microscopy without using computer averaging procedures (Kastner et al. 1990; Kastner et al. 1991; Fabrizio et al. 1994). In case of the U5 snRNP isolated from human HeLa cells, an elongated structure with a small head and a large elongated body about twice the size of the head has been reported, and antibody labeling using antibodies directed against the 5' cap structure of the U5 snRNA has revealed that the 5' end of the U5 snRNA and thus the Sm core structure are most likely located within the main body of the U5 snRNP particle on the opposite site of the head domain.

In case of the [U4/U6] di-snRNP particle containing only the Sm proteins and the U4/U6 snRNA duplex, a structure consisting of two domains could be observed – the first domain appeared to be roundish and globular, whereas the second domain appeared significantly thinner and filamentous. In human, the [U4.U6.U5] tri-snRNP has been described as a triangular elongated particle, the main body of which closely resembled the U5 snRNP. An initial 3D structure of the pre-catalytic human spliceosomal complex B exhibited a triangular body connected to a flexible head domain. The general shape of the body was found to be similar to the shape of the [U4.U6.U5] tri-snRNP (Boehringer et al. 2004).
Theoretical molecular masses of the tri-snRNP and its components

Figure 1.5: Comparison of molecular weights of U5 snRNP, [U4/U6] di-snRNP, and [U4/U6.U5] tri-snRNP. For scaling of the weights, a 1:1 stoichiometry is assumed. The U5 snRNP complex contributes ~60% of the tri-snRNP’s molecular mass, whereas the [U4/U6] di-snRNP is expected to be much smaller (~30% of the mass of the tri-snRNP). The remaining 10% are contributed by the tri-snRNP specific proteins 110k, 65k and 27k that are not associated with one of the subunits alone. The molecular masses of the three snRNAs range from ~35 – 47 kD. Thus, RNA forms only ~7% of the total mass.

Expected molecular weight of the tri-snRNP and its subunits

In 3D electron cryo-microscopy, a 3D volume with individual grey values of the voxels is computed. A user-specified threshold is therefore needed to define which voxels represent density of the macromolecule and which only contribute to the background. For calculation of the threshold and proper 3D surface rendering of a new electron cryo-microscopic 3D structure, it is thus important to know the molecular weight of the particle. For the snRNP particles, the exact stoichiometry of the proteins has to date only been confirmed for the U1 snRNP by quantitative methods (Hochleitner et al. 2005). In this case a single copy of all proteins was found in the complex. In case of the [U4/U6.U5] tri-snRNP, it can be assumed that two sets of the Sm proteins are present that bind to the Sm site of the U4 snRNA and the U5 snRNA, respectively. However, a quantitative analysis has not been performed for the tri-snRNP, but from the even intensities in Coomassie-stained gels it can be concluded that the larger proteins should occur in a 1:1 stoichiome-
try. It should be noted that the two largest proteins exhibit a significantly higher molecular mass than would be suggested from their names: the U5-220k protein has a molecular mass of 274 kDa, the U5-200k has a molecular mass of 245 kDa. If a simple 1:1 stoichiometry for the [U4/U6.U5] tri-snRNP proteins (except the Sm proteins) is assumed, the theoretical molecular weight of the tri-snRNP is thus approximately 1.7 MDa. The calculated molecular mass of the U5 proportion of the tri-snRNP is about 1 MDa (60 % of the molecular mass of the tri-snRNP), and the mass of the [U4/U6] di-snRNP about 0.48 MDa (30 % of the molecular mass of the tri-snRNP). The remaining 10 % are contributed by the tri-snRNP specific proteins 110k, 65k and 27k. The snRNAs only form ~7 % of the mass of the tri-snRNP (compare Fig. 1.5, p. 11). In general, standard resolutions obtained for asymmetrical macromolecules by 3D electron cryo-microscopy are in the range of 15 – 35 Å. These resolutions are normally not sufficient to separate RNA and protein densities. Thus, it is important to note that observed global conformational heterogeneity, rearrangements of domains and domain flexibility of the snRNP particles most likely occurs at the level of proteins which make up for ~93 % of the visualized particle mass.

1.2 Electron Microscopy and Image Processing

1.2.1 Image formation in the transmission electron microscope (TEM)

The usage of electrons for microscopy is based on the discovery of the wave nature of electrons by Louis de Broglie 1924. As a general principle of imaging, the wavelength \( \lambda \) is a fundamental factor limiting the resolution of any optical device. The theoretical limit in resolution for typical TEM acceleration voltages of 100 – 200 kV lies in the range of a few picometers. However, due to other technical restrictions, the maximum resolution of a modern TEM is about 1 Å. The first transmission electron microscope was built by Ernst Ruska (1906 – 1988) in 1931.

1.2.2 Amplitude contrast and phase contrast

The two most important sources of contrast in the TEM are amplitude contrast and phase contrast. The high-resolution image information suitable for three-dimensional (3D) image processing is based upon phase contrast.
**Phase contrast:** Electrons scattered in the Coulomb field of the atomic nucleus undergo a phase shift of $\frac{\pi}{2}$ with respect to the undiffracted zero beam. Therefore the phase object can only be made visible when the diffracted wave is shifted by another $\frac{\pi}{2}$ (positive phase contrast) (compare Fig. 1.6, p. 14) or $-\frac{\pi}{2}$ (negative phase contrast). Whereas this transformation of a phase difference into visible amplitude contrast is achieved by a ring-like $\lambda/4$ (Zernike) phase plate in the phase contrast light microscope, an effect similar to that of light microscopic phase plates is reached in the electron lens by defocusing the objective lens (i.e. deviation of the sample from the Gaussian focus plane). The effect is further dependent on the given spherical aberration constant $C_s$ of the objective lens, as well as the acceleration voltage of the microscope. An optimum phase shift of the diffracted electron wave cannot be achieved for all diffraction angles (i.e. spatial frequencies) at the same time. The theoretical optimum defocus with maximum positive phase contrast is a very weak underfocus of $(C_s \lambda)^{1/2}$ that is termed “Scherzer focus” on behalf of its discoverer Otto Scherzer (1909 – 1982).

**Amplitude contrast:** The Amplitude contrast – in the case of scattering amplitude contrast – is caused by scattered electrons that hit the lens aperture and thereby diminish the intensity of the electron beam. In case of the absorption amplitude contrast, the electrons are absorbed in the sample itself. In thin samples as those used for 3D electron microscopy, only few electrons are absorbed and thus the amplitude contrast plays only a minor role. In this thesis, a method to measure the amplitude contrast proportion of a given image is presented (compare section 3.2.1, p. 74).

### 1.2.3 Phase contrast transfer function (PhCTF)

The spherical aberration of the objective lens can be described by a wave aberration function $W(\theta)$ as a function of the scattering angle $\theta$. The angular deviation of electrons with different scattering angles causes a path difference $\Delta s$. The wave aberration is a function of $\Delta s$ and $\lambda$; in the Scherzer formula, $\Delta s$ is expressed as a function of the spherical aberration $C_s$ of the objective lens and the defocus $\Delta z$ (Scherzer 1949):

\[
W(\theta) = \frac{2\pi}{\lambda} \Delta s
\]

\[
= \frac{\pi}{2\lambda} \left( C_s \theta^4 - 2\Delta z \theta^2 \right)
\]
If the proportionality of scattering angle $\theta$ and spatial frequency $q = \frac{\theta}{\lambda}$ is taken into account, the equation for the wave aberration $W(q)$ becomes:

$$W(q) = \frac{\pi}{2} \left( C_s \lambda^3 q^4 - 2\Delta z \lambda q^2 \right)$$

(1.3)

The PhCTF $B(q)$ is derived from this equation. The sign of $B(q)$ is chosen so that $B(q) > 0$ holds true for positive phase contrast:

$$B(q) = -2 \sin W(q) = -2 \sin \left[ \frac{\pi}{2} \left( C_s \lambda^3 q^4 - 2\Delta z \lambda q^2 \right) \right]$$

(1.4)

The PhCTF is zero at the origin and increases only slowly, so the very low spatial frequencies are not present in images modulated by the PhCTF. The PhCTF then reaches its first maximum and alternates between positive and negative phase contrast in the range of higher spatial frequencies. The higher the defocus, the closer to the origin can the first zero crossing be found. Thus a high defocus leads to better transmission of low spatial frequencies, but on the other hand also to a shorter continuous, positive frequency band and more alternations. The individual images of a typical data set for single-particle image processing normally contain a variety of different PhCTF parameters. When all images are combined in a single 3D volume without prior correction for negative frequency bands in Fourier space, the high-resolution information contained in the alternating parts of the PhCTF would totally be extinguished. For high-resolution 3D structure determination, the
Figure 1.7: Influence of the PhCTF on a given image point when the electron microscope is adjusted to underfocus values of 1 and 5 \( \mu m \), respectively. The figure illustrates the modulation of an individual point in an image (upper row) by the PhCTF (second column) as well as the Thon rings (Thon 1966) in the corresponding power spectra (third column). The PhCTF itself is plotted in the fourth column. As shown in the Plots, the PhCTF is zero at the origin and increases only slowly (i.e. almost no transmission of the very low spatial frequencies). It alternates between positive and negative contrast in the range of higher spatial frequencies. The pixel size of the image was assumed to be 3.2 \( \text{Å/pixel} \), the acceleration voltage set to 200 kV and the defocus set to 1 and 5\( \mu m \), respectively. The PhCTF causes a characteristic circular spreading of any point of the object over many image pixels and thus introduces a characteristic kind of blurring.

In real world applications, the PhCTF is superposed by other contrast transfer function (CTF) parameters, namely the amplitude CTF (compare section 1.2.2, p. 12), exponential signal decay dependent on the spatial frequency (the so called "B-Factor") and two-fold astigmatism of the objective lens. A more detailed mathematical description is given in section 3.2.1, p. 74. The development of an automated procedure to identify and correct all these CTF parameters is part of the present thesis and described in the results chapter (compare section 3.2.1, p. 74).
1.2.4 Digital image processing for structure determination

The electron microscope produces two-dimensional (2D) projections out of 3D objects. If the ideal case of (i) a large depth of field together with (ii) a magnification factor independent of the position within the imaged object is assumed, the electron microscope produces geometrically correct "ideal" 2D projection images of any thin object. 3D image-processing aims at reversing this process and calculating the 3D structure of a macromolecule out of its 2D projection views. It in general encloses alignment of the particles that are randomly distributed on the micrograph, sorting with respect to angular orientation and back projection of the 2D electron-microscopic projection images into 3D space. Many algorithms actually work in reciprocal ("Fourier") space and rely on Fourier transformations of the data images. A detailed understanding of Fourier theory is of paramount importance for the proper application of the diverse image processing algorithms in the field of 3D single-particle reconstruction.

The Fourier transformation

Many important algorithms used in single-particle image processing take place in Fourier space. In analogy to the frequency as reciprocal unit of the time, the reciprocal length or spatial frequency is the unit of the Fourier space. During Fourier transformation, the signal of the input image data is decomposed into its underlying set of sine and cosine waves. In case of single-particle images, low spatial frequencies represent the general shape of a particle on a coarse level, whereas the high spatial frequencies define the fine structural details of a particle, like protein domains and secondary structural motifs. In digital Fourier analysis, the data are discrete, the basic unit being a single pixel. According to the sampling theorem (Nyquist 1928) a critical spatial frequency $f_c = \frac{1}{2}I$ (Nyquist frequency) exists that, within the interval $I$, band limits the maximum spatial frequency that may be represented. In particular, a sine curve represented by a sequence of single adjacent black and white pixels exhibits the critical Nyquist frequency. If, for example, a pixel of a data image corresponds to 3 Å on the specimen level, the high-resolution information content of that image is principally limited to 6 Å.

The Fourier transform of real space data is represented on the level of complex numbers; every complex number holds the value of phase and amplitude of a wave function belonging to a discrete frequency. A complex number $z$ can thus be shown as a function of its amplitude $a$ and frequency $\theta$ as follows:
\[ z = a [\cos(\theta) + i \cdot \sin(\theta)] \] (1.5)

However, it is known that the information content of a noisy electron microscopic image is largely determined by the reliability of its phases rather than its amplitudes. The real part of a complex number is described by a symmetrical cosine function and the imaginary part is described by an asymmetrical sine function. The Fourier transform, as opposed to pure sine or cosine transforms, can thus handle any given symmetry of the input data. As a main drawback for the application of Fourier transforms in single-particle image processing, the Fourier transform requires the input data to be periodic, i.e. in the 2D case the last, lower right pixel of a given image is treated as the direct neighbor of the first upper left pixel. However, this condition is approximated by the use of circular masks and image frames 30 – 50% larger than would be necessary to hold the largest particle diameter and circular disc-like masks around the normalized images to avoid “Fourier artifacts”.

The formula for the discrete (one-dimensional) Fourier transform \( H \) of data \( h \) of length \( n \) is given as:

\[ H(k) = \sum_{m=0}^{n-1} h(m)e^{-i2\pi km/n} \] (1.6)

If – like in electron microscopic images – the imaginary part of the input data is zero, the following symmetry occurs:

\[ H(n - k) = H^*(k) \] (1.7)

In analogy to the one-dimensional case, the 2D Fourier transform can be expressed as:

\[ H(j, k) = \sum_{m_x=0}^{n_x-1} \sum_{m_y=0}^{n_y-1} h(m_x, m_y)e^{(-i2\pi jm_x)/n_x}e^{(-i2\pi km_y)/n_y} \] (1.8)

with the following symmetry:

\[ H(j, k) = H^*(n_x - j, n_y - k) \] (1.9)

The power spectrum is defined as the squared amplitude of the Fourier transform. The computed power spectrum is equivalent to the optical diffraction pattern of the object. The power spectrum is visualized after a shift of the Fourier transform by a full period so that the low spatial frequencies are found near the center of the
power spectral image and the high spatial frequencies are represented at the edge like in a true diffraction pattern.

**Image acquisition on film and digitization**

Traditionally, black & white film based on silver halide technology are used for image acquisition in the electron microscope. Therefore, the micrographs have to be digitized using professional high-resolution (drum) scanners. Typical sampling intervals of these scanners are in the range of 4 – 20 µm. If the standard microscope magnification of 50,000x is used in combination with film, the resulting pixel size on the specimen level is thus 0.8 – 4 Å. The final high-resolution of a digital image acquired from a micrograph by drum scanners is, however, not only a function of the pixel size, but also of the modulation transfer function (MTF) of the scanner; the MTF describes an exponential decay of the original signal dependent on spatial frequency. The MTF normally reaches zero for spatial frequencies lower than that given as "optical resolution" in the data sheet of the scanner.

**Image acquisition on a CCD detector**

Nowadays, charge coupled device (CCD) detectors more and more replace black & white films in electron microscopy. As shown within this thesis, images from CCD exhibit, in contrast to film, certain characteristics with respect to single-particle image processing that have proven to contribute significantly to the correctness and accuracy of the final 3D structure (compare section 3.1.1, p. 40). CCD detectors for use in electron microscopes bypass the analog recording and digitization step. To date, a typical CCD detector measures 4 cm × 4 cm – 6 cm × 6 cm and is therefore significantly smaller compared to film (83 × 10.2 cm).

**Assessment of image quality**

In general, not all electron microscopic images exhibit enough signal in the range of high spatial frequencies to be suitable for the computation of a high-resolution 3D structure. Charging of the specimen during the exposure and thermical drift of the cryo specimen holder are the main sources of image distortions resulting in poor image quality. However, the electron microscopic images can be rated by visual inspection of their power spectra: the amorphous carbon support film (and to a certain extent the water surrounding the particles) deliver an unspecific signal that is modulated by the phase contrast transfer function (PhCTF) (compare section
1.2.3, p. 13). As a consequence of this CTF modulation, the characteristic Thon rings appear in the electron microscopic images. In the case of specimen drift or charging, the high-resolution signal is extinguished and the outer Thon rings disappear. A method to rate a complete image data set of many thousand particle images is given in this work (compare section 3.2.1, p. 74).

Selection of particles

For any macromolecule of to date unknown 2D and 3D structure it is recommended to manually select the individual macromolecule images on the computer monitor using software available for that purpose. Automated particle selection tools are nowadays also available that use a set of reference images to find similar views on a micrograph. These reference images are either manually selected from the micrograph or – in case a 3D structure of the particle is already available – computed from that structure. However, the operator must make sure that any automated selection algorithm is sensitive, i.e. does not miss important subsets of particles on a micrograph, and also specific, i.e. distinguishes between artifacts caused by contaminants (e.g. ice, frozen ethane) and true particles. After the coordinates of the particles on a micrograph have been established manually or automatically, small sub-windows are cut out of the original image and a data image file containing – in case large sets of micrographs are processed – up to more than 100,000 particle images is created for further image processing.

Band-pass filtering of the data images

If the data images are analyzed in Fourier space, very low spatial frequencies only represent properties of the image that are unrelated to the particle structure – e.g. local variations in carbon film, ice or stain thickness. In contrast, very high spatial frequencies often exhibit a spatial signal-to-noise ratio too low for initial image processing. Thus a 2D band-pass filter is applied to the data images to eliminate those parts of the image information that would otherwise interfere with the image processing.

2D alignment of the single-particle images

In 3D electron cryo-microscopy, the macromolecules are supposed to be randomly distributed on a carbon support film or in ice holes with respect to translation, rotation and angular orientation. Aim of any 2D alignment is the detection of a
correlation maximum of image and a given reference image as a function of translation and rotation of the image. In earlier days of single-particle image processing, a sequential algorithm using alternating rotational and translational searches was developed (Steinkilberg and Schramm 1980). It was applicable on comparatively slow computers. This alignment algorithm is called "direct alignment" via separate rotational and translational searches; it makes use of a rotational cross-correlation function to directly compute the rotation angle. In contrast, former algorithms relied on an indirect method using the auto correlation function as reviewed in (Frank 1996).

In direct alignment, the translational parameters leading to a correlation maximum of data image and reference image are computed upon 2D Fourier transformation (compare section 1.2.4, p. 16) of both images. After multiplication of the reference Fourier transform with the complex conjugated Fourier transform of the data image and reverse Fourier transformation of the result, the 2D cross-correlation function is obtained. A peak search then delivers the translational parameters as follows: the location of highest density in the 2D cross-correlation function is shifted away from the center by the same distance the data image is shifted with respect to the reference image. The algorithm for the rotational alignment of the data image to a reference image encloses the transformation of both image and reference in cylindrical (polar) coordinates. Consequently, the rotational alignment can be carried out like a translational one with only one degree of freedom. In contrast to the translational alignment, only 1D Fourier transforms of the images converted to cylindrical coordinates are calculated to yield a 1D rotational cross-correlation function. Again, a peak search within this function is necessary to find the rotational parameter resulting in a maximum of cross-correlation. In direct alignment, rotational and translational alignment are iteratively performed until a predefined termination condition like stability of parameters or total number of desired iterations is fulfilled. In digital single-particle image processing, normally a multi reference alignment (MRA) is carried out (Penczek et al. 1994): all data images are subsequently aligned with respect to all available reference images. Finally, the translational and rotational parameters leading to the highest correlation coefficient measured for a data image (with respect to all reference images) are applied to the particle image.

In contrast to separate alignments, the so-called "exhaustive alignment" methods are considerably slower (Joyeux and Penczek 2002). These algorithms evaluate all possible positions for a given molecular image within a defined range, and this
makes them computationally more costly than direct alignment. The "exhaustive alignment using resampling to polar coordinates" requires a transformation to polar coordinates for each possible centre of the particle in the pixel frame and scans directly all possible translational parameters without the need for 2D CCFs (compare section 1.2.4, p. 19). For each position a rotational cross-correlation is required to determine the possible rotation angle of the image. The input image to be aligned is shifted using the translational parameters that exhibit the highest rotational correlation coefficient. The rotational position is determined according to the 1D coordinate of the peak in the rotational correlation function.

In 2002, a comparative study of different alignment algorithms revealed that exhaustive alignment using resampling to polar coordinates is – albeit slower than traditional alignment algorithms by a factor of about 20 – 50 – superior to direct alignment in terms of accuracy, especially when the input images are very noisy like it is often the case in electron cryo-microscopy (Joyeux and Penczek 2002). Therefore, this algorithm was implemented and characterized in the present work. Furthermore, a technique to avoid time-consuming, but useless computations is presented to make the algorithm as fast as the traditional one (compare section 3.2.2, p. 91).

Multivariate statistical analysis of aligned data images

Within a large set of data images, many different projection angles occur simultaneously. Automated algorithms are therefore needed to separate the different views prior to further 3D image processing. Once identical views have been detected, they can be combined by averaging to get representations of the particle with significantly increased signal-to-noise ratio. In this thesis, hierarchical ascending classification (HAC) following multivariate statistical analysis (MSA) (Frank and van Heel 1982; van Heel 1984; Lebart et al. 1984) as implemented in the software package IMAGIC (van Heel et al. 1996) is used to split up the data set into many individual groups ("classes") of particle images, that ideally represent the different angular views of the macromolecule. After computation of averages from the class members, representations with significantly increased signal-to-noise ratio are retrieved, the so called "class averages". The data images with \( p \) pixels per image are hereby treated as vectors in a \( p \)-dimensional space. All these vectors then form a "cloud" in the \( p \)-dimensional hyper-space, and similar images are thought to be clustered close together.

To identify these clusters without the need for comparing all individual pixels of
all images (which would not be possible with respect to limited computer power), a dimensionality reduction is performed by application of MSA to compute the Eigenvectors (also called Eigenimages): An orthogonal coordinate system is iteratively rotated to describe the data “cloud” as accurate as possible. The first coordinate axis finally prompts into the direction of the highest variance of the data set, the next axis prompts into the direction of the second highest variance (orthogonal to the first one) and so on. A maximum of 69 Eigenvectors can be computed by the IMAGIC algorithm. After the coordinate system of Eigenvectors has become stable, all data images are projected onto the Eigenvectors. As a consequence, the dimensionality is drastically reduced to allow for fast classification of the images; on the other hand, about 95% of the variance of the original image data are preserved in the compressed representations of the images. The Eigenimages represent the main orthogonal sources of variance in the data set. Visual inspection of the Eigenimages by an experienced user is helpful to get information about the general properties of the data set with respect to signal-to-noise ratio and diversity of particle views.

For iterative computation of the Eigenvectors, the $n$ images, each consisting of $p$ pixels are summarized in a $n \times p$ matrix $X$. New values for the coordinate system as described above are retrieved by solving the equation:

$$X^tNXMU = U\Lambda$$  \hspace{1cm} (1.10)

$X_t$ is the transposed matrix of $X$. $U$ is the Eigenvector matrix. The matrix $\Lambda$ contains the Eigenvalues that are used to measure the amount of variance that is described by each Eigenvector. $M$ is a $p \times p$ and $N$ a $n \times n$ weighting matrix that define the metric. The Eigenvector matrix $U$ has to fulfill the orthonormalisation condition (Lebart et al. 1984)

$$U_tMU = I_p$$  \hspace{1cm} (1.11)

with $I_p$ being the $p$-dimensional unit matrix.

**Hierarchical ascending classification of the data images**

The classification method used in IMAGIC (van Heel et al. 1996) is the hierarchical ascending classification (HAC) with subsequent “moving elements refinement” (van Heel 1984). It is performed after treatment of the aligned data images by MSA and dimensionality reduction. The MSA algorithm as described above (compare section 1.2.4. p. 21) sorts the Eigenimages according to the corresponding Eigenvalues.
starting with the largest. If a typical particle data set is treated by MSA, only the first 20 – 40 Eigenimages contain information related to the particle whereas the higher Eigenimages only exhibit noise and can be discarded during classification. The data images can thus typically be reduced to a dimensionality of about 20 - 40, as opposed to 10^4 pixels of the original data images. Therefore HAC is not a computationally expensive task and even on a personal computer normally completed within a few minutes. The HAC algorithm aims at identifying clusters in the vector “cloud” of the input data. It is, however, not possible to detect the ideal number of clusters in a data set automatically. The desired number of classes is therefore specified by the operator and typically adjusted so that an average number of 10 – 30 members per class is retrieved. The algorithm starts with a number of classes equal to the total number of images, i.e. every image is positioned in its own class. Neighboring classes are joined as long as the desired number of classes is reached. In the subsequent moving elements refinement (van Heel 1984) every image has the possibility to leave its current class and move to another class that better fits its properties, to further lower the total intra class variance and to maximize the inter class variance.

**Averaging of the classified images**

All class member images are summed and the result is divided by the number of class members to normalize the class averages. By averaging of similar noisy images, the weak, but correlated signal contained in the single images is enhanced, whereas the random, non-correlated noise is substantially diminished.

**Iterative treatment of data sets by 2D alignment, MSA and classification**

To get stable 2D projection views of a given particle with enhanced signal-to-noise ratio, 2D MRA and MSA followed by classification and computation of class averages is sequentially performed and the class averages are then used as reference images for another round (“iteration”) of 2D MRA. The cycle is repeated until no further improvement with respect to the last result can be observed. After each round, the operator visually separates meaningful class averages that represent the typical views of the particle from nonsense average images and thus supports an iterative self organization of the data set.

When the data set contains a particle of to date unknown structure, a *de novo* analysis of the data set has to be performed. In this special case, no initial reference images from previous experiments are available to start the first round of 2D
alignment and classification. So called "reference-free" alignment schemes (Dube et al. 1993; Penczek et al. 1992) have been developed for startup of the procedure: Prior to the first alignment, a "blob" image is computed by averaging all unaligned particle images. As even the unaligned images are roughly centered after a manual or automated particle selection procedure (compare section 1.2.4, p. 19), the average of all images contains bright pixels at the center and dark pixels at the edge. This image has turned out to be an ideal initial reference image, as it (1) is virtually free of any detailed information that would bias the first alignment and (2) serves to center the images in a first alignment. In the first alignment, the rotational degree of freedom is set to zero and an exclusively translational alignment is carried out to restrict the effect of the first alignment to centering of the particles. Within large data sets of many thousand images, it is likely that small subgroups with similar projection angle as well as rotational orientation exist. Therefore, a first MSA followed by classification and class averaging of the centered particle images should be able to detect these subgroups that can then be averaged to form the first 'true' set of reference images. This "reference-free" alignment scheme is nowadays routinely applied to every new data set and has proven to be a robust, operator-independent procedure that reproducibly and stably reveals the main projection views of a macromolecule.

Definition of the Euler angles

A set of three Euler angles is necessary for each class average to describe the angular orientation of a given class average prior to the first 3D reconstruction. The $\alpha$ angle describes the rotation of the particle among its axis, the $\beta$ angle defines the latitude and the $\gamma$ angle the longitude (compare Fig. 1.8, p. 25). Only if all three Euler angles of an image are known, the image can be projected into 3D space.

Computation of the initial 3D model

When stable 2D class averages have been obtained after several rounds of 2D alignment and MSA, classification, several possibilities exist to find corresponding Euler angles for computation of an initial low-resolution 3D model. However, several situations require different computational approaches to establish the Euler angles:

1. A 3D structure of a very similar or even identical macromolecule is already available, and thus molecular replacement techniques may be used for the computation of the Euler angle, the most popular one being projection-matching (explained in more detail in section 1.2.4).
2. A startup 3D structure is not available. However, the macromolecule exhibits a high internal symmetry (e.g. icosahedral viruses). Then, automated procedures using angular reconstitution via symmetry related peak searches in sinogram correlation functions are available (van Heel 1987).

3. A startup 3D structure is not available (de novo techniques) and the macromolecule is thought to be asymmetric. If the angular distribution of the views is even, many different projections that clearly belong to the same 3D structure and do not represent structural heterogeneity of the data set can be retrieved by "reference-free" alignment (compare section 1.2.4, p. 23). In this case, angular reconstitution via sinogram correlation functions (see above) is applicable, but requires significantly more user interaction and experience compared to computation of highly symmetrical structures.

4. A startup 3D structure is not available and the macromolecule is thought to be asymmetric. The number of independent projection views is limited. In this case, angular reconstitution via sinogram correlation functions (see above) is often not successful. However, if the electron microscope allows for tilting of the specimen holder and each macromolecule has been recorded twice under two different tilt angles, the tilted images corresponding to the members of each class average can be used to compute a 3D structure via the random conical tilt (RCT) method (Radermacher et al. 1987) (see below).

**Angular reconstitution via sinogram correlation functions:** This method is based on the "common line theorem": Two different 2D projection views derived from a common 3D object exhibit at least one common 1D line projection. When
the angle between the common line projections is known, it can be used to compute the angular distance between the two projections. The resulting image holding all possible line projections of an image is called "sinogram". Sinograms of two images can be correlated line by line to yield a "sinogram correlation function". A peak search is performed within the sinogram correlation function and trigonometric functions are then used to compute the relative Euler angles (van Heel 1987).

**Random conical tilt reconstruction:** When tilted views of the particles have been recorded (by tilting the specimen holder) in addition to untitled images, RCT reconstructions (Radermacher et al. 1987) can be computed as follows: the untitled images are treated by 2D alignment followed by MSA/classification so that subgroups of identical projection angles are identified. Euler angles are assigned to each subgroup of the tilted representations of the particles using the available information of (1) tilt axis, (2) tilt angle, and (3) rotational alignment parameter of the corresponding untitled image. At least one complete tomographic series (i.e. \( \beta = 90^\circ \)) is required to allow isotropic a reconstruction of a 3D object. As the maximum specimen tilt angle is limited to \( \sim 60^\circ \), RCT reconstructions suffer from the so-called "missing cone phenomenon", and are therefore only poorly defined at Eulerian \( \beta \) angles of \( \sim 90^\circ \). A method to automatically analyze large data sets with up to several hundred class averages using RCT in combination with maximum-likelihood based 3D weighted averaging of individual RCTs is presented in this thesis (compare section 3.1.2, p. 59).

It is important to note that the maximum resolution obtained for tilted electron-microscopic images is much lower compared for untitled images due to technical reasons. Thus, once an RCT 3D structure has been obtained, the resolution is to be refined based on data sets of untitled particle images.

**Iterative refinements of resolution of the 3D structure**

Once a 3D structure is available, a projection algorithm is used to compute a set of new reference images with Euler angles evenly spaced over the unit sphere. In contrast to the use of class averages as reference images for the next round of 2D alignment, reference images computed from a 3D density can therefore serve to detect underrepresented projection views of the molecule. Furthermore, every reference image now contains the information of all class averages from which the 3D density has been computed and is thus significantly less noisy and more reliable than individual class averages. After 2D MRA and MSA-based classification have
been performed using the new reference images, Euler angles are assigned to the new class averages by angular reconstitution via sinogram correlation functions (van Heel 1987) and a new 3D density with improved resolution can be computed. This iterative cycle can be repeated until a stable 3D density is reached. The final resolution is determined by many parameters including optical quality of the data images, number of recorded particle images (Henderson 1995) and structural heterogeneity of the macromolecule.

In contrast to the standard refinement procedure implemented in IMAGIC (van Heel et al. 1996), a projection-matching procedure that omits the MSA-based classification and angular reconstitution of class averages can be applied (Penczek et al. 1994). Projection-matching takes advantage from the fact that the 2D MRA algorithm can be used for classification itself. For every reference image, “class” averages are computed by averaging all images that have shown the highest correlation coefficient to that reference during the MRA. Projection-matching is often capable to deliver higher resolutions compared to the combination of MSA and angular reconstitution via sinograms in case a reliable 3D model is available. The type of classification by MRA is also called “supervised” classification, as the classification result is a direct result of reference images used and particle views others than those supplied as reference images cannot appear. In contrast, MSA-based classification is termed “unsupervised” classification, as the MSA and HAC algorithm do not require any information about the reference images used for alignment of the data images. Thus MSA/HAC is especially useful when no reliable 3D structure has been retrieved and the suitability of a data set for 3D structure analysis has to be rated. Projection-matching is the preferred procedure when a reliable 3D structure should be improved in terms of resolution.

3D reconstruction via exact filtered back projection

For 3D reconstruction, it is required that the Euler angles are known for all 2D projection views. The reconstruction is performed in real space by “smearing” the projection views back into a 3D volume along a direction defined by the Euler angles. However, this operation in real space is equivalent to ordering the 2D Fourier transforms of the projection images as “central sections” according to their Euler angles in 3D Fourier space (compare Fig. 1.9, p. 28). As these central sections overlap in the region of low spatial frequencies, over-weighting of low spatial frequencies occurs. The method of “exact filtered back-projections” therefore computes individual filter functions for each projection image dependent on the total number of
Figure 1.9: 3D reconstruction (van Heel and Harauz 1986). A. In real space, 2D images are back projected ("smeared") in 3D space via an interpolation algorithm in the direction of their Euler angles. B. 3D reconstruction in Fourier space: The 2D Fourier transforms of the input projection images are placed in Fourier space so that each 2D transform corresponds to a section through the centre of the 3D Fourier space. Again, the angular position of each section is determined by the Euler angles of the projection.

projection images in the data set and relationship of the Euler angles within the data set (van Heel and Harauz 1986).

**Determination of the resolution by Fourier-Shell-Correlation**

The resolution of a 3D density is defined as the highest spatial frequency exhibiting structural information. However, different resolution criteria have been postulated. In the present work, the resolution is measured by analysis of the "Fourier shell correlation" (FSC) (van Heel and Harauz 1986): the data set is split into two subsets and two independent 3D structures are computed. The correlation of the two 3D structures is assessed in 3D Fourier space as a function of the shell radius $S$ (van Heel and Harauz 1986):

$$ FSC(S) = \frac{\sum_{R=S} F_1(R)F_2^*(R)}{(\sum_{R=S} |F_1(R)|^2 \sum_{R=S} |F_2(R)|^2)^{\frac{1}{2}}} $$  \hspace{1cm} (1.12)

$F_1$ is defined as the 3D Fourier transform of the first 3D structure, $F_2$ as the 3D Fourier transform of the second 3D structure. The * denotes the complex conjugated form of the Fourier transform. However, by no means does any positive value of the FSC at a given spatial frequency indicate that this spatial frequency lies within the range of resolution. In contrast, threshold criteria are needed that define a minimum positive FSC value to be reached. For asymmetrical objects, the fixed threshold value of 0.5 is a popular one (Beckmann et al. 1997), though exclu-
sively based on experience. Importantly, the $FSC_{0.5}$ criterion is characterized as a conservative one and it is thus unlikely that its application will lead to any overestimation of resolution. Based on information theory, the 0.143-criterion has been defined (Rosenthal and Henderson 2003). In contrast to fixed resolution criteria, the $3\sigma$-criterion individually defines a Gaussian significance level of $3\sigma$ as a function of the assumed particle symmetry. For application of the $3\sigma$-criterion, a threshold curve is defined that takes into account statistical properties of principally uncorrelated 3D noise: when two 3D volumes consisting of random voxels are correlated, they may nevertheless exhibit a certain degree of positive correlation. However, 99% of pairs of uncorrelated 3D volumes will not exhibit spatial correlations higher than those given by the $3\sigma$ curve. Using this criterion, the resolution is defined as the point of intersection between FSC and the threshold curve. Statistical threshold curves can easily be adjusted with respect to the particle symmetry assumed by the 3D reconstruction algorithm to compensate for effects of symmetrization.

1.3 Aim of the work

To date, the most promising method to investigate the 3D structures of large macromolecular assemblies, is the method of electron cryo-microscopy in combination with single-particle 3D image-processing. The work presented here aimed at solving the 3D structure of the human [U4/U6.U5] tri-snRNP structure its subunits, the [U4/U6] di-snRNP and the U5 snRNP particle leading to the localization of the subunits within the tri-snRNP. A better understanding of the 3D architecture of the particle can contribute to improve our understanding of the highly dynamic nature of the snRNP particles. Furthermore, an accurate 3D density of the tri-snRNP is needed for the interpretation of future 3D structures of complete spliceosomes, as the tri-snRNP forms the largest preformed macromolecular assembly of early spliceosomes. However, standard image-processing techniques had previously failed to reveal the 3D structure of the tri-snRNP. Thus, methodical improvements had to be established to (1) make new 3D structures of asymmetrical macromolecules computed $de novo$ more reliable, and (2) to improve the resolution that can be obtained. The results chapter is therefore divided in two parts; in the first part, the methodical developments mentioned above are described, whereas in the second part, the 3D structures obtained for the tri-snRNP and its subunits are presented.
2 Materials and Methods

2.1 Materials

2.1.1 Software

TVIPS EM-MENU software
Linocolor (drum scanner software)
IMAGIC-5 (single-particle analysis)
Amira Dev2.3
Program package “R” for Statistical calculations
Compaq Visual Fortran for Windows

Tietz Video System, Gauting, Germany
Heidelberger Druckmaschinen, Heidelberg, Germany
Image Science, Berlin, Germany
TGS Europe, Merignac Cedex, France
(R Development Core Team 2004)
Hewlett Packard, Böblingen, Germany

2.1.2 Chemicals

Acetone
Benzalconiumchloride
Ethanol
Ethyl acetate
Dithioerythrol (DTE)
Dithiothreitol (DTT)
Glycerol
HEPES
Magnesium chloride
Hydrofluoric acid
Millipore water
Phenylmethylsulfonylfuoride (PMSF)
Potassium chloride
Sodium chloride
Triafol

Merck, Darmstadt, Germany
Sigma, Seelze, Germany
Merck, Darmstadt, Germany
Fluka, Buchs, Switzerland
Sigma, Seelze, Germany
Roth, Karlsruhe, Germany
Merck, Darmstadt, Germany
Calbiochem, Läufelfingen, Germany
Merck, Darmstadt
Fluka, Buchs, Switzerland
Millipore GmbH, Schwalbach, Germany
Boehringer Mannheim, Germany
Merck, Darmstadt, Germany
Merck Darmstadt, Germany
Aldrich Chemical Company, Inc. Milwaukee, U.S.A.

Uranyl formate
Polysciences Inc., Warrington, PA
2.1.3 Laboratory materials

Carbon rods, highest grade
Copper EM grids, 3.05 mm, 300 mesh, square
EM negatives (8.3 cm × 10.2 cm)

Developer Kodak D19 for EM negatives
Ethane (liquid)
Filter paper 90 mm No. 4 and 5, Whatman, Dassel, Germany
Fixer Superfix for EM negatives
Glass slides (76 × 26 mm)

Mica, 75 × 25 mm
Nitrogen (liquid)
Teflon preparation block (volume: 25 μl – 200 μl)
Wetting agent for EM negatives

Ringsdorff Werke GmbH, Bonn, Germany
Plano, Wetzlar, Germany
Kodak Electron Image film SO-163, Kodak GmbH, Stuttgart, Germany
Kodak GmbH, Stuttgart, Germany
Messer, Sulzbach, Germany
Tetenal, Norderstedt, Germany
Gerhard Menzel GmbH + Co. KG, Braunschweig, Germany
Plano, Wetzlar, Germany
Messer, Sulzbach, Germany
manufactured by the precision engineering team, MPI Göttingen
Agepon, Agfa, Köln, Germany

2.1.4 Special Equipments

Cryo electron microscope CM200 FEG
Cryo electron microscope CM120
Camera boxes for 8.3 × 10.2 cm negatives
BioCam-0124 (slow scan 1k × 1k CCD camera) at CM200
TemCam F415 (slow scan 4k × 4k CCD camera) at CM200
TemCam F224HD (slow scan 2k × 2k CCD camera) at CM120
Room temperature EM specimen holder
Cryo EM specimen holder
Rotating drum scanner

Philips, Eindhoven, The Netherlands
Philips, Eindhoven, The Netherlands
Philips, Eindhoven, The Netherlands
Tietz Video System, Gauting, Germany
Tietz Video System, Gauting, Germany
Tietz Video System, Gauting, Germany
Philips, Eindhoven, The Netherlands
Gatan GmbH, München, Germany
Tango Scanner with Linocolor software, Heidelberger Druckmaschinen, Heidelberg, Germany
64 nodes (32 computers), 2 servers; Rittal GmbH and Co. KG, Wetzlar, Germany
Thermo Electron, Langenselbold, Germany
Thermo Electron, Langenselbold, Germany

Windows computer cluster
Sorvall Ultracentrifuge Discovery 90
Sorvall Rotor TLS-630 Surespin
2.1.5 Samples for methodological tests

- E. coli 70S ribosomes
- U1 snRNP particles
- Tobacco mosaic virus (TMV)
- Tomato bushy stunt virus (TBSV)

Prof. Dr. Marina Rodnina, Witten-Herdecke
Prof. Dr. Reinhard Lührmann and Dr. Holger Stark, Göttingen
Dr. Stephan Winter, Braunschweig
Dr. Stephan Winter, Braunschweig

2.2 Electron microscopy

2.2.1 Preparation of holey carbon copper grids

Clean glass slides are used to produce a holey triafol film as follows: the slides are pretreated in benzalconiumchlorid for 15 minutes, then rinsed with Millipore water. A slide is the placed on a cooled metal block for several seconds in order to condense water on its surface. Then, 1 ml triafol 0,5 % in ethyl acetate is allowed to flow down the slide and form a continuous film between the small drops of water on the slide. The holes in the film should are generally 1 – 2 μm in diameter. The triafol film is then incubated in 0.1 % hydrofluoric acid to separate it from the glass slide and floated in a beaker filled with water. Clean electron microscopic copper grids can then easily be placed on the film and lifted out of the beaker. Then a carbon vacuum evaporator is used to create a thin carbon film on top of the triafol support and the triafol is removed by incubating the coated grids in ethyl acetate so that the grids finally contain a single layer of holey carbon film.

2.2.2 Preparation of continuous carbon films for specimen adsorption

Continuous carbon film without holes is needed for adsorption of the particles. For practically all larger snRNP particles, the affinity of the macromolecules to the carbon film is very high and low particle concentrations (10 – 50 μg/ml protein) are sufficient for visualization of the sample. For preparation of thin continuous carbon films, freshly cleaved mica is coated with carbon in a carbon vacuum evaporator. To achieve an even distribution of carbon, the carbon vapor must not directly hit the mica. Therefore the direct vapor has to be blocked by a piece of metal and only the carbon reflected by glass slides is allowed to reach the mica. The carbon films with the support layer of mica are stored at a dry place and are cut into small pieces prior to floating them on the sample solution.
2.2.3 Preparation of electron microscopic samples for negative stain electron microscopy

All specimens were prepared according to a published protocol (Valentine et al. 1968; Tischendorf et al. 1974; Kastner 1998): a carbon film evaporated onto freshly cleaved mica was floated on the surface of the solution allowing adsorption of virus over 2 minutes. The carbon film was then transferred to a second well filled with 2% uranyl formate solution and incubated for 2 minutes. Subsequently, the carbon film with adsorbed particles was attached to a 400 mesh copper grid on which a carbon film containing holes of ~1 μm diameter has been mounted (compare section 2.2.1, p. 32). Finally, another carbon film floated onto a second solution of uranyl formate was used to form a “sandwich”, enclosing the specimen in a layer of staining solution between two carbon films. The specimens were stored under dry conditions until image acquisition at room temperature (in case of TMV, TBSV, U4/U6 di-snRNP, SF3b, 70S E. coli ribosomes, initial U5 snRNP and [U4/U6.U5] tri-snRNP samples) or frozen in liquid nitrogen until image acquisition at liquid nitrogen temperature (in case of the U5 snRNP particle at low salt conditions). As the stain layer is thought to be hydrated, the level of hydration of the stained particles is conserved even in the ultra-high vacuum of the electron microscope when the sample is frozen. Furthermore, the susceptibility of the particles to radiation damage is reduced when the sample is cooled to liquid nitrogen temperatures during image acquisition. This reduced radiation damage facilitates higher resolutions to be obtained in the final 3D reconstruction (Golas et al. 2003).

Importantly, the “sandwich” technique even works for samples of very low concentration (e.g. <10 μg/ml protein) and often leads to successful visualization of macromolecules when native cryo or single layer negative stain techniques fail. Dependent on size, shape and flexibility of the particle, the “sandwich” technique normally leads to flattening of the particle of about 30% (Frank 1996). However, many examples indicate that the flattening effect the “sandwich” method does not affect the suitability of the preparation method for high-resolution 3D structure determination (Golas et al. 2003; Golas et al. 2005). The procedure is illustrated in Fig. 2.1.

2.2.4 Preparation of particles in vitrified ice

Preparation of the particles in vitrified buffer without the need for heavy metal staining solutions (“native cryo”) (Adrian et al. 1984) was performed for the
Figure 2.1: Illustration of the “sandwich” technique. (A) A carbon film on a support mica is floated onto the surface of the particle solution to allow adsorption of particles. (B) Carbon film decorated with particles. (C,D) The carbon film is floated onto a stain solution of 2 % uranyl formate and fished out of the solution using a copper grid on which a perforated carbon film has been mounted before. (E) A second carbon film is floated onto another solution of stain. The grid is brought underneath the second carbon film so that a “sandwich” consisting of the two carbon films with the particles embedded in layer of uranyl formate is formed.

[U4/U6.U5] tri-snRNP: the particles were adsorbed to a carbon film that was then attached to a copper grid on which a perforated carbon film had been mounted before. Excess buffer was removed from the grid using filter paper. The grid was rapidly frozen in liquid ethane and stored in liquid nitrogen until image acquisition. This method is considered the gold standard of electron cryo-microscopy, however, it requires higher concentrations than the “sandwich” method and is restricted to a limited set of buffer conditions.

2.2.5 Image acquisition

For image acquisition on film, the electron microscopic black & white film Kodak SO-163 (8.3 × 10.2 cm) based on a conventional silver halide emulsion layer was used. The electron microscope was operated at 50,000× magnification, so that optical density of ~1 OD could be achieved for an electron dose of ~20 e⁻/Å². The film was developed in Kodak D19 for 12 minutes at room temperature. The micrographs were digitized on a professional drum scanner (Tango, Heidelberger Druckmaschinen) equipped with photo multiplier at sampling intervals of 0.8 – 4
μm.

For image acquisition on CCD detector, a variety of different settings were characterized in terms of applicability for de novo single-particle image processing (compare section 3.1.1, p. 40). 5×5 – 6×6 series of small overlapping images (“small spot scanning”) were recorded to allow for later stitching of the small individual images. For detailed illustration and discussion of this technique, compare section 4.1, 128, as well as Fig. 3.12, 58.

2.3 Purification of snRNPs

All U snRNP samples were prepared from HeLa nuclear extracts. HeLa cells were fractionated into cytoplasm and nuclei (Zieve and Penman 1981), then splicing extracts were prepared from the nuclei (Dignam et al. 1983). A mixture of all U snRNP particles (“total snRNPs”) containing snRNAs with m3G cap were obtained by α-m3G-immunoaffinity chromatography using the monoclonal antibody H-20 (Bochnig et al. 1987). This mixed U snRNP sample was then further separated on a 36 ml 10 – 30 % glycerol gradient (20 mM HEPES, 150 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.5 mM PMSF, pH 7.8) using 6 ml of mixed snRNP solution on a Sorvall TLS-630 rotor (17 hours at 27000 rounds per minute).

25S and 20S Fractions containing tri-snRNP and U5 snRNP particles, respectively, were directly used for further preparation of the electron-microscopic as described below. Preparation of 13S [U4/U6] di-snRNPs was performed from 25S fractions by reconstitution in rhodanide buffer as described below.

2.3.1 [U4/U6.U5] tri-snRNP

For “sandwich” negative stain preparations, fresh as well as previously frozen 25S glycerol gradient fraction of “total snRNPs” (compare 2.3 were incubated with 0.1 % glutaraldehyde for three hours. Then, a continuous carbon film was floated on the surface of the sample for 2 min and a “sandwich” grid was prepared as described (section 2.2.3). I am grateful to Dr. E. Makarov for preparation of various tri-snRNP samples for negative stain analysis, as well as for the analysis of RNA and protein composition.

For native cryo preparations, a 25S fraction, that had been freshly prepared from fresh HeLa cells and nuclear extract, was incubated with 0.1 % glutaraldehyde overnight. Then, the buffer was exchanged using identical buffer conditions without glycerol by a spin column. A continuous carbon film was floated on the glycerol-
free particle solution for 2 minutes. The carbon film was attached to a perforated carbon grid (for preparation, compare section 2.2.1) and rapidly frozen in liquid ethane as described in section 2.2.4. Biochemical analysis of the tri-snRNP samples for native cryo imaging was performed by Dr. C. L. Will, whose contribution should be acknowledged here.

### 2.3.2 U5 snRNP

All electron-microscopic grids containing U5 snRNP particles under low salt as well as under high salt conditions were prepared for electron microscopy by the "sandwich" negative stain method. In case of the low salt U5 snRNP, a frozen 20S fraction of "total snRNPs" (compare 2.3 separated on a 10 – 30 % glycerol gradient was thawed and incubated with 0.1 % glutaraldehyde for three hours. Electron-microscopic grids were prepared as described (compare section 2.2.3, p. 33) and frozen in liquid nitrogen until image acquisition under cryo conditions. For recording of tilt pairs, grids were stored under dry conditions until image acquisition at room temperature.

High salt resistant U5 was retrieved upon addition of sodium chloride to the 20S U5 fraction so that a final chloride concentration of 800 mM was reached. The sample (400 µl) was then loaded on a 10 – 30 % glycerol gradient containing 20 mM HEPES, 800 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTE, and 0.5 mM PMSF at pH 7.8. After ultracentrifugation, the 20S fraction was incubated with 0.1 % glutaraldehyde for three hours. Biochemical analysis of the high salt resistant U5 snRNP reproducibly revealed that the U5-100k protein is missing under these conditions (kindly performed by Dr. E. Makarov). Then, electron-microscopic grids were prepared by the "sandwich" method as described. Biochemical purification and characterization of high-salt resistant U5 snRNP particles was performed by Dr. E. Makarov.

### 2.3.3 U4/U6 di-snRNP

Electron-microscopic grids containing the [U4/U6] di-snRNP particles were prepared for electron microscopy by the "sandwich" negative stain method. Biochemical purification and analysis of [U4/U6] di-snRNP particles was performed by Dr. E. Makarov (Makarov et al. manuscript in preparation). 25S tri-snRNP glycerol gradient fractions as described above were pelleted by ultracentrifugation, resuspended in rhodanide buffer (20mM HEPES, 200mM NaSCN, 1.5mM MgCl₂, 0.5mM
<table>
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<th>Recorded on...</th>
<th>Original pixel size [Å/pixel]</th>
<th>Pixel size for calculations [Å/pixel]</th>
<th>Number of Particles</th>
<th>tilt pairs</th>
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<td>TemCam F415, 4× binned</td>
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<td>BioCam-0124, 2× binned</td>
<td>2.9</td>
<td>7.3</td>
<td>1569</td>
<td>(0°,45°)</td>
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<tr>
<td>U5 snRNP</td>
<td>Cryo-negative stain</td>
<td>50000×</td>
<td>Kodak SO-163</td>
<td>2.1</td>
<td>6.3</td>
<td>19303</td>
<td>-</td>
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<tr>
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<td>Negative stain</td>
<td>122000×</td>
<td>TemCam F415, 2× binned</td>
<td>2.5</td>
<td>5.7</td>
<td>3866</td>
<td>(0°,45°)</td>
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<tr>
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<td>Negative stain</td>
<td>165000×</td>
<td>BioCam-0124, 2× binned</td>
<td>2.9</td>
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<td>TemCam F415, 2× binned</td>
<td>2.5</td>
<td>5.8</td>
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<td>TemCam F415, 2× binned</td>
<td>2.5</td>
<td>3.7</td>
<td>124147</td>
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<td>TMV 70S U1 snRNP</td>
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<td></td>
<td>described in detail in section 3.2.1 and 3.1.2</td>
<td></td>
<td></td>
<td></td>
<td>described in detail in section 3.2.1</td>
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Table 2.1: Technical details of particle data sets. For each sample investigated here, the preparation method and electron-microscopic image acquisition conditions are stated. Particle images were recorded either on CCD detector or on conventional film. All images were binned to lower Nyquist frequencies prior to calculation accelerate the computations and lower the influence of the high-resolution noise contained in the electron-microscopic images.

DTT) and applied on a 5 – 20 % glycerol gradient (20mM HEPES, 150mM Nacl, 1.5mM MgCl, Sorvall TH-660 rotor). The 13S fraction containing the [U4/U6] di-snRNP particles was then incubated with 0.1 % glutaraldehyde for three hours. Then, a continuous carbon film was floated on top of the solution for 30 minutes to allow adsorbance of the particles and a “sandwich” grid was prepared as described (section 2.2.3). The grids were stored under dry conditions until image acquisition at room temperature.

2.4 Electron microscopy and image processing

Image data sets were recorded as summarized in Table 2.1. For de novo 3D reconstruction of the U5 snRNP at low salt and high salt conditions, of the [U4/U6]
di-snRNP and the [U4/U6.U5] tri-snRNP, negative stain preparation of the particles was performed as described above and tilt pairs were recorded on CCD camera by the small spot scanning technique (compare section 2.2.5, p. 34). 2D de novo analysis of the particle images was performed using 4 – 6 rounds of the “reference-free” alignment scheme as described in section 1.2.4, p. 23 in combination with exhaustive MRA via resampling to polar coordinates as described later in section 3.2.2, p. 91. In case of the [U4/U6] di-snRNP, 380 MSA-based class averages were computed from 3866 untilted images, and 118 class averages were manually selected for further processing. In case of the U5 snRNP particle at low salt conditions, 75 MSA-based class averages were computed from 1569 untilted images and 56 class averages were manually selected for further processing. In case of the U5 snRNP particle at high salt conditions, 350 MSA-based class averages were computed from 5973 untilted images and 84 class averages were selected for further processing. For the [U4/U6.U5] tri-snRNP particle, 180 MSA-based class averages have been computed from 2607 untilted particle images, and 46 class averages were selected for further processing. Additionally, 6232 particle images of tri-snRNP from a frozen 25S glycerol gradient fraction have been recorded. 300 MSA-based class averages were computed from this data set and 83 class averages selected for further processing. While the initial low-resolution 3D structure looked nearly identical to the 3D structure of the fresh tri-snRNP, conformational heterogeneity with respect to the head domain could be observed as shown in the results chapter in Fig. 3.46, p. 123. Then, RCT 3D reconstructions (compare section 1.2.4, p. 26) corresponding to the selected class averages have been computed and combined by the maximum-likelihood based 3D weighted averaging procedure described in the results chapter (compare section 3.1.2, p. 59). For the [U4/U6.U5] tri-snRNP particle, the [U4/U6] di-snRNP particle and the U5 snRNP particle at low salt conditions, initial 3D models of the macromolecules could be computed by the RCT/ML method. In case of the [U4/U6.U5] tri-snRNP and U5 snRNP, these models were directly used for refinement of the 3D structure based on the untilted images. The untilted images were therefore corrected for defocus and astigmatism using the method described later (compare section 3.2.1, p. 74), and standard projection-matching as described (section 1.2.4) was applied. The resolution was measured by computation of the FSC as implemented in the IMAGIC software (van Heel et al. 1996). In case of the [U4/U6] di-snRNP 3D MSA (as described in section 3.1.2, p. 59) revealed a major flexibility of the two main domains with respect to each other. Accordingly, refinement of resolution using the complete
data set via projection-matching was not successful. Instead, the results of the 3D MSA analysis were used for further interpretation of the [U4/U6.U5] tri-snRNP structure (compare section 3.3.7, p. 121). When the U5 snRNP particle at high salt conditions was analyzed by RCT/ML, the structural flexibility of the macromolecule turned out to be even higher than that of the [U4/U6] di-snRNP, so that only an elongated body with a maximum dimension of 25 nm, but without any structural details was retrieved by RCT/ML analysis. In contrast, the different conformations could be separated by 3D MSA analysis. To localize the U5 snRNP and the [U4/U6] di-snRNP within the tri-snRNP, rigid body fitting was performed as described in the results chapter using the 3D visualization software Amira.

In case of the [U4/U6.U5] tri-snRNP visualized under native cryo conditions, 124,147 data images were selected and aligned against the structure obtained from the negative stain data set described above. MSA-based class averages were computed and the class averages were compared to the negative stain class averages. It was ensured that the particle had the same electron-microscopic 2D appearance with and without use of negative stain before further refinement of the native cryo data set. Then, projection-matching was applied to refine the resolution to ~24 Å. For the refinement, only 61,734 particle images were used: 33% of the particle images were excluded due to low maximum cross-correlation coefficients in the MRA, and 33% of the images were excluded due to histograms of the corrimas indicative of multiple peaks as described (explained in detail in section 3.2.2, p. 91; in Fig. 3.32 on p. 106, examples of corrimas corresponding to included and excluded tri-snRNP particle images are shown). The final number of references used in the MRA was 10248 (angular separation: 2°). A further increase in number of reference images (ung 41,000 images, angular separation: 1°) did not improve the resolution further. For all data sets, the sampling intervals used for the computations are summarized in Table 2.1.
3 Results

3.1 Developments for improvement of reliability of de novo 3D structures

3.1.1 Advantages of a CCD detector for 3D de novo structure determination of single particles

Aim of the work

In de novo 3D structure determination, the reliability of any 3D structure computed from two-dimensional class averages is strongly dependent on the correctness of the first two-dimensional steps. Such class averages normally exhibit structure information up to ~20 Å. Thus, to obtain a reliable 3D structure, the information content of raw images in the resolution range where initial 2D analysis takes place is of great importance. Tests to compare the performance of CCD detectors with photographic film are required - not only in the range of very high resolution (where film is usually better than CCD), but also at low and intermediate spatial frequencies. Traditionally, conventional silver halide film has been favored for single-particle data collection, as the procedure is faster and allows the recording of large numbers of particle images in a short time. CCD detectors, in contrast, have the intrinsic disadvantage of low readout speed, coupled with small pixel numbers that limit the speed and efficiency of data collection. So far, CCD detectors have widely been used to record high-resolution electron diffraction patterns of two-dimensional protein crystals (Bullough and Henderson 1999; Subramaniam et al. 1999), and their applicability for single-particle 3D work (Booth et al. 2004; Stewart et al. 2000; Zhang et al. 2003) and for high tensions up to 400 kV (Brink and Chiu 1994; Downing and Hendrickson 1999) has been demonstrated. Currently, CCD cameras are equipped with very efficient polycrystalline phosphor scintillators and cooled to approximately -30°C, to lower the readout noise. As a
consequence, CCD detectors offer a higher SNR at low resolutions than film does (Booth et al. 2004). Therefore, the use of CCD detectors may nevertheless be worth the extra effort when the image quality of conventional film is inadequate because of low image contrast. Routinely, the signal decay (Saad et al. 2001) is determined from power spectra of the electron-microscopic images as a first estimate of the quality of signal transmission in terms of a B factor. This means that the amplitude information is used to estimate the quality of signal transmission. However, for computerized image-processing, the phase information is of paramount importance, and yet it is not possible to describe the quality of image phases at a certain spatial frequency from the B factor without further experimental evidence (Sherman and Chiu 1997). From practical experience, image alignment together with MSA-based classification is indeed more reliable when a CCD camera is used instead of film (Golas et al. 2005). Another advantage of the CCD camera is the smaller number of particle images needed to obtain first meaningful results. The aim of this work was thus to find an explanation for this practical observation, and to discover in which situations images recorded on a CCD camera are superior for the initial image-processing in single-particle electron cryomicroscopy. Two different approaches were applied to test the reliability of image phase transmission of a 4k $\times$ 4k CCD camera and film. First of all, the reliability in image phase transmission was measured by recording series of images from identical areas on a carbon support layer on photographic film or with a CCD camera. After pairwise alignments within each image series, the phase similarity was measured by means of the differential phase residual (DPR) (Frank et al. 1981). The signal from the amorphous sample allowed determination of phase transmission reliability and spectral signal-to-noise ratio (SSNR) in a manner that was independent of radiation damage, but took account of the influence of image alignment. Secondly, images of negatively stained tobacco mosaic virus (TMV) were recorded, and the first steps of single-particle analysis were performed. In this case, the ability of the computer averaging procedure to recover the characteristic helical features in the range of 11.5-23 Å was determined from the power spectra.

As a consequence of these experimental data, all initial 3D models of snRNP particles were computed from CCD images.

**Procedure and findings**

**Specimen preparation:** For specimen preparation of TMV, a solution of TMV in a buffer containing 200 $\mu$g/ml virus, 20 mM HEPES and 150 mM NaCl was placed
in a Teflon well. Specimen preparation of TMV was carried out using the "sand-
wich" procedure (compare section 2.2.3, p. 33). For comparable measurements of
signal transmission independent of radiation damage, a single carbon film mounted
on a copper grid was imaged directly.

**Electron microscopy:** Images were taken on a 200 kV transmission electron mi-
roscope (TEM) equipped with a field emission gun and constantly operated at
200 kV keeping the sample at room temperature. A 4k × 4k 16 bit CCD camera
(TemCam-F415) with 15 μm pixel size and polycrystalline phosphor scintillator
cooled to -25°C was used. The post-magnification factor of the CCD camera was
found to be 1.37-1.4 fold with respect to calibration using the TMV specimens.
Camera settings included 1×, 2× and 4× binning of the pixels to yield effective
pixel side lengths of 15 μm, 30 μm and 60 μm. The mean number of counts per
pixel was adjusted to an electron dose of about 20 e−/ Å² for a single exposure,
and ranged from about 2,800 to about 19,000 counts dependent on magnification
and CCD pixel binning. For a complete list of settings applied, see Table 3.1.
For comparison with film, images were recorded on Kodak SO-163 film at a mag-
nification of 50,000× and processed as described in the methods chapter (section
2.2.5) The micrographs were digitized at the maximum optical resolution of 6350
dpi, and then coarsened by two-fold binning, leading to a sampling interval of 1.6
Å/pixel. For the CCD camera, TMV images were recorded with slight overlap of
≈15% so that a continuous rectangular area of ≈1 μm² could be obtained by precise
cross-correlation-based stitching of the single images using the TVIPS EM-MENU
software. All images of carbon film and TMV were taken at defoci of about -1 μm.

**Measuring the reliability of phase transmission by using images of carbon
support film:** Fifty images of the same area of carbon film were recorded on film
and on CCD for each setting of the CCD camera. Owing to minor drift of the
sample holder, the images within each series had to be aligned by using the "direct
alignment" algorithm (compare section 1.2.4, p. 19). For all alignment operations,
the images were slightly high-pass filtered and normalized. The images were used to
compute the differential phase residual (DPR), spatial signal-to-noise ratio (SSNR)
and power-spectral signal decay as follows: To measure the DPR between two con-
secutively recorded images, images of 800 × 800 pixels were used. Image no. n+1 of
the series was aligned against image no. n for n = 1 - 49 to yield 49 aligned pairs of
carbon film images. By using the Imagic "Fouring" routine, the DPR between the
Table 3.1: Complete list of CCD camera settings, stating effective magnification, pixel binning of the CCD camera, mean number of counts used to achieve an electron dosage of $\sim 20e^-/\text{Å}^2$, and maximum imageable area on the specimen level. Last column: resolution at which the Kodak SO-163 film transmitted image phases are better than the CCD camera when the predicted minimal DPR curves of film and CCD camera were compared.

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* Assuming a magnification of 50,000× and a film area of 9 × 12 cm or 1.8 $\mu$m × 2.4 $\mu$m at the specimen size. If the CCD images are recorded with a slight overlap to allow for later stitching, the number will be higher dependent on the proportion of redundantly recorded areas.

Two single images of each pair was computed. Finally, all results for $n = 1..49$ were averaged leading to an almost negligible standard deviation of the mean ($< 1^\circ$).

Pixel frames smaller than 800 × 800 pixels (e.g. 256 × 256) were also used to verify that the results were independent of the size of the carbon film area; only the 'smoothness' of the DPR curves was reduced at high spatial frequencies, when smaller frames were applied. For further analysis and interpretation, the minima of DPR (at the positions of the CTF$^2$ maxima) were connected by interpolation (see below). For calculation of SSNR, a procedure identical to that of TMV image-processing (see below) was used: eight consecutively recorded images of carbon film representing an identical area of about 470 × 470 Å were aligned against one of the eight images that served as reference. Thereafter the eight aligned images were averaged. The SSNR of the eight images with respect to the average was computed as described previously (Unser et al. 1987). To verify the statistical significance of the results, this procedure was also performed for six independent sets of eight images. The power-spectral signal decay of the images was determined from the rotationally averaged power spectrum of one representative image of size 1024 × 1024 pixels. A fitting procedure similar to that described in section 3.2.1, p. 74 was applied to the rotationally averaged power spectrum in order to determine simultaneously the defocus, an exponential signal decay function connecting the maxima,
and a noise function connecting the minima. In addition, these parameters were also estimated by applying a quasi-Newton-based fitting routine (R Development Core Team 2004). No relevant differences between the two methods were found. Finally, the power-spectral signal decay in the important resolution range from 1/20 Å⁻¹ to 1/10 Å⁻¹ was calculated from the fit of the envelope functions.

**Image-processing of TMV:** For TMV image-processing, TMV fibres were selected manually from the images so that about 200-300 sub-windows of 470 × 470 Å were obtained. Images of TMV were corrected for defocus and astigmatism as described in section 3.2.1, p. 74, thereby verifying a defocus of about -1 µm. All TMV images were normalized to a mean of zero and a standard deviation of 10, and were band-pass filtered. For band-pass filtering, a constant lower cut-off of 1/180 Å⁻¹ was applied, while the upper cut-off was adjusted to the Nyquist frequency of the image. Two or three rounds of alignment via polar coordinates (compare section 1.2.4, p. 19) and HAC based on MSA were performed using on average 10 members per class average (compare section 1.2.4, p. 22). For the first alignment, a randomly selected single image was used as reference, and then the class average, where the typical repetitive motifs of TMV were most clearly visible, was selected as reference. After the result was stable, (1) the average of 8 images belonging to the same MSA class and (2) the average of 20 aligned images possessing highest cross-correlation coefficient with the last reference image were computed. In both cases, the power spectrum was analyzed for helical features in the range 11.5-23 Å. To investigate the effect of computational pixel binning (coarsening), some images taken at high sampling rates were Fourier-transformed and only the central part up to the desired Nyquist frequency was reverse-transformed. The coarsened images thus obtained were subjected to the alignment and MSA procedures as described above.

**Phase transmission reliability using images of carbon support film:** The complete analysis for film and for one of the camera settings tested (279,000 magnification, 4 binning) at a constant electron dosage of about 20 e⁻/Å² is shown in Fig. 3.1.

As expected, the average differential phase residual function between two subsequently recorded images is minimal (i.e. best phase transmission) at those spatial frequencies where the CTF² function reaches a maximum. The minima of the DPR, at least in the spatial frequency region of 0.05 - 0.15 Å⁻¹, can be connected by an approximately straight line (Fig. 3.1A).
Figure 3.1: Analysis of DPR, SSNR and signal decay: comparison between film (left) and one of the CCD camera settings tested (right). (A) Average differential phase residual (DPR) between two consecutively recorded images of amorphous carbon film at a defocus of 1 μm and a voltage of 200 kV for Kodak SO-163 film at 50,000x magnification (left panel) and the CCD detector at 279,000× magnification with 4× binned pixels (right panel). The DPR follows the CTF and is lowest at the maxima of the CTF. Therefore, minima of the DPR are connected by a dashed line to allow the interpolation of the defocus-independent minimal predicted DPR values, which are characteristic for the settings used. (B) SSNR of eight aligned and averaged images of carbon film. Again, as the function follows the CTF, the maxima have been connected to allow linear interpolation of the defocus-independent maximum values of SSNR obtainable at the settings used, CCD detector settings as in (A). (C) Power-spectral signal decay (arbitrary density values) of a representative single image obtained by using film (left panel) and CCD detector (right panel), using the same settings as in (A). The rotationally averaged signal is shown in grey. An exponential function connecting the maxima is shown in blue to indicate the defocus-independent signal decay, and a noise function connecting the minima of the power-spectral signal is shown in red.
Figure 3.2: Dependence of predicted minimum DPR and predicted maximum SSNR on the defocus. (A) Interpolation curves connecting the minima of DPR at three different defoci of -0.3 \(\mu m\) (green), -1.6 \(\mu m\) (blue) and -3 \(\mu m\) (red). The location of the DPR minima is shown by correspondingly colored dots. The three curves are very close together, indicating that slope and position of the curves are mainly independent of defocus, but are characteristic of the camera settings used. (B) Linearly interpolated maxima of the SSNR for the three different defoci as described in (A). The dots indicate the position of the SSNR maxima. Again, the maximum predicted SSNR shows only a minor dependence on the defocus (slightly better values at around 15 Å for the lower defoci).

As plotted in Fig. 3.2A, the slope and position of that line are practically independent of the defocus in a range from -0.3 \(\mu m\) up to -3 \(\mu m\), but are highly characteristic of the camera settings used. Therefore, the maximum achievable consistency of phases can be predicted by interpolating the measured minima of the DPR curve, referred to herein as the 'predicted minimal DPR'. At 279,000× magnification and 4-fold binning, the predicted minimal DPR of the CCD was better than that of film at resolutions lower than 7.3 Å. In case of the SSNR functions (Fig. 3.1B), the maxima of SSNR were connected by linear interpolation similar to the analysis of the DPR minima, and the predicted maximum SSNR was extracted from the interpolation curves as a function of spatial frequency. Again, the exponential decay of the predicted maximum SSNR shows only a minor dependence on the level of defocus chosen (Fig. 3.2B). The predicted maximum SSNR is slightly better at \(\sim 15\) Å, if the images are taken closer to focus. The maximum SSNR (of 8 averaged images, see above) at \(\sim 25\) Å of the CCD camera was about twice as high as that of film, whereas photographic film revealed a better SSNR at high spatial frequencies beyond 1/10 Å\(^{-1}\) (Fig. 3.1B). As shown in Fig. 3.1C, the CCD camera and film have very similar spatial signal decay. In contrast, the function of the unconvoluted background noise has – at lower spatial frequencies – considerably higher values relative to the signal for film than for the CCD camera. A comparison of all the different camera settings used with film is made in Fig. 3.3A. In summary, the predicted minimal DPR of the CCD camera at 20 Å was better than that of film under all conditions tested (by a difference of 7.5°-10.6° for
4× binning of the camera, a difference of 5.2°-8.2° for 2× binning and a difference of 2.0°-6.9° for no binning). At 15 Å, the predicted minimal DPR was lower for the CCD detector compared with film for all 4-fold binned settings of the CCD camera (difference: 4.8°-8.5°) and the 2-fold binned ones at magnifications above 161,000 (difference: 5.7°-6.3°). In a comparison at the 10 Å level, the CCD camera performed better than film when 4 binning was used in combination with magnifications of ≥ 214,000 (difference: 3.5°-7°) or 2× binning and a magnification of 214,000 (difference: 2.9°). At resolutions around 7 Å, only the combination of 4-fold binning together with the highest tested magnification of 343,000× led to a better transmission of phases in comparison with film. The spatial frequencies where the predicted minimal DPR of film crosses the curves obtained for the CCD camera are listed in Table 3.1, p. 43. For most of the settings, the CCD camera exhibited a significantly better performance at 20 Å and 15 Å, while – even with the highest possible magnification of the electron microscope together with 4-fold binning – photographic film retained the phase information better than the CCD camera at resolutions beyond 6 Å. The situation is very similar for the SSNR. The SSNR measured at the first contrast maximum of the CTF at approximately 25 Å was higher than that of film for all camera settings tested (Fig. 3.3B); for 4× binning and 2× binning combined with magnifications higher than 161,000× the maximum SSNR at 25 Å was 80-120 % higher. At 20 and 15 Å, the combination of at least 2-fold binning together with magnifications ≥ 161,000 led to a significantly improved signal compared with film. In contrast, the CCD camera exhibited lower SNRs than film at resolutions better than 15 Å under most of the conditions tested. Only at the highest tested magnification of 343,000× and 4-fold binning of the CCD camera were the values for the SSNR comparable to those of film up to 7 Å (Fig. 3.3B).

In Fig. 3.4, the decay in the power-spectrum signal for the CCD camera is compared with the corresponding result for film. The resolution range important for standard single-particle analysis was analyzed by computing a signal decay factor when going from 20 Å to 10 Å. The best value (i.e., the one with the lowest decay factor) can be obtained with film where signal in the power spectrum going from 20 Å to 10 Å drops by a factor of only 3.6. In contrast, this factor is higher in case of the CCD camera and increases with decrease in binning and magnification of the electron microscope (3.8-6.7× for 4× binning of the camera, 4.5-10.9× for 2× binning of the camera and 8.9-14.4× for 1× binning of the camera; see Fig. 3.4).
Figure 3.3: Summary of DPR and SSNR for all CCD detector settings. (A) Histogram of all values measured for the predicted minimum DPR (compare Fig. 3.1, p. 45) for 20 Å (blue), 15 Å (green), 10 Å (orange), and 7 Å (dark yellow). The correspondingly colored dotted lines indicate the values obtained for Kodak SO-163 film. For a magnification of 161,000× and 4× binning of the CCD detector, a 7 Å value cannot be obtained (asterisk). (B) Histogram of all values measured for the SSNR of eight aligned and averaged images of carbon film. Red, SSNR at the first maximum (25-27 Å); blue, SSNR at 20 Å; green, SSNR at 15 Å; orange, SSNR at 10 Å; and dark yellow, SSNR at 7 Å. Again, the dotted lines show the values obtained for Kodak SO-163 film.
Figure 3.4: Spatial frequency dependent signal decay for all CCD detector settings. The power-spectral signal decay was obtained from the rotationally averaged power spectrum of a representative image. To allow easy comparison of the resolution range of 10-20 Å, the signal decay from 20 to 10 Å was calculated and plotted as a histogram. The lowest signal decay can be obtained with film (3.6×, left bar and dotted line), while with the CCD camera a comparable factor of 3.8× can only be found for a magnification of 343,000×, and 4× binning; at all other settings the spatial signal decay is considerably higher compared to film.

Analysis of TMV images: For all the ten settings of the CCD camera and also for film, TMV was imaged and analyzed by standard averaging procedures (compare section 1.2.4, p. 23). When a power spectrum was calculated from the average, a characteristic X-like arrangement of spots in the resolution range of 18 Å (outermost spots) to 23 Å (innermost spots) could be seen. In every figure, the power spectra computed from normalized images are shown with identical interactive grey values of 0-500 density units to make the level of background noise and the signal comparable. In Fig. 3.5, the results for Kodak SO-163 film are shown; the results for CCD camera follow in Figs. 3.6-3.8 (for 1×, 2× and 4× binning, respectively).

In general, the visibility of single TM virus is better when the magnification (and thus the Nyquist frequency) is low. The same holds true for the class averages with \( n = 8 \) members, although in every class average the 23 Å spacings of TMV become clearly visible. The power-spectral signal pattern is visible best in the power spectra of the class averages (\( n = 8 \)) for lower magnifications and \( \geq 2\times \) pixel binning of the camera. In the power spectra of the class averages (\( n = 8 \)), the background noise increases with finer sampling of the images (e.g., compare the top row with the bottom row in Fig. 3.7). Whereas these power spectra of the class averages (\( n = 8 \)) mainly differ in the amount of noise present in the 15-25 Å range, the
3.1 Developments for improvement of reliability of *de novo* 3D structures

![Kodak SO-163 Film, 50kx](image)

**Figure 3.5:** Analysis of TMV images recorded on Kodak SO-163 film at 200kV, 50,000× magnification and a sampling interval of 1.6 Å/pixel. From left to right: single aligned image, MSA-based class average with eight members, power spectrum of that class average, power spectrum of an average image from 20 aligned images.

Direct averages of 20 aligned images are virtually noise-free, although they differ in the visibility and intensity of the outer (~18 Å) spots. In the power spectra of the alignment averages (*n* = 20), the complete (18 - 23 Å) helical motif can be seen not only on film (as expected), but also for 2× binning of the CCD pixels and magnifications ≥ 122,000, or 4× binning of the camera and magnifications ≥ 214,000, whereas spots in the 23 Å range are visible for all the settings tested. When the power spectra of the 20 directly averaged images were displayed with a narrower range of grey values ranging from 0-150 (not shown), weak spots at 11.5 Å became visible for no binning and a magnification of 91,000, 2× binning and magnifications of 91,000-161,000 and 4× binning and magnifications of ≥ 279,000 as well as for film. Only at 4× binning and a magnification of 343,000× were relatively strong spots at 11.5 Å visible.

**Interference of sampling interval and cross-correlation-based alignment:**

The apparently increasing low-resolution noise in the range of about 20 Å, obtained in those cases when finer sampling was applied, does not agree with the fact that the SSNR increases with increasing magnification. However, the cross-correlation-based alignment clearly fails to make full use of the high SNR in the low-resolution range, when the images are highly oversampled (i.e. Nyquist frequencies around 1/3 Å⁻¹). The reason may be found in the application of the standard cross-correlation coefficient: this implies that all spatial frequencies over the whole range of resolution contribute equally to the alignment, leading to competition between the low-frequency signal and the high-frequency noise. This behavior of the alignment is demonstrated in Fig. 3.9: In Fig. 3.9A the CCD camera is used with 2× binning of the pixels and a very fine sampling (Nyquist frequency 1/3.1 Å⁻¹), while in Fig. 3.9B the Nyquist frequency is only 1/7 Å⁻¹, owing to a lower magnification...
Figure 3.6: TMV images recorded on CCD detector (no pixel binning; pixel size: 15 μm). The mean number of counts has been adjusted to the same electron dose as applied for the film exposure of approximately 20e^-/Å² (compare Fig. 3.5, p. 50). First row: 70,000× magnification, 2.3 Å/pixel at the specimen level; second row: 91,000× magnification, 1.75 Å/pixel at the specimen level. Columns from left to right: single aligned image, MSA-based class average with eight members, power spectrum of that class average, and power spectrum of an average image from 20 aligned images.

of the electron microscope. Although the SNR around 20 Å should be higher at the settings used to produce Fig. 3.9A (compare results obtained with carbon film, right column), the power-spectral spots can - in contrast to the results obtained with a lower magnification (Fig. 3.9B) - hardly be distinguished from noise. When the images from Fig. 3.9A are computationally coarsened to a Nyquist frequency of 1/7 Å⁻¹, the alignment and averaging procedure work well and a clear, noise-free power-spectral signal can be obtained (Fig. 3.7C). The image quality is even better than in Fig. 3.9B, as predicted by the DPR and SSNR curves obtained with carbon film.

Accuracy of the measurements of DPR, SSNR and B-Factor: The accuracy of the measurements shown here was evaluated by determining the 95%-confidence intervals of the curves. In Fig. 3.10, p. 56, the statistical analysis is shown for CCD detector, 279,000× magnification, 4× binned pixels. The DPR curves (compare Fig. 3.1, p. 45) were averages from 49 curves obtained after pairwise alignment of single carbon film images. In Fig. 3.10A, the 95%-confidence interval for the blue DPR curve is shown as red and green curve, respectively. For the SSNR curve - calculated from data sets of eight aligned carbon film images (compare Fig. 3.1, p. 45) - the 95%-confidence interval was plotted showing the 2σ-Interval after repeating the experiment six times (in Fig. 3.10B). Again, the standard error curves
Figure 3.7: TMV images recorded on CCD detector (2 × pixel binning; pixel size: 30 μm). The mean number of counts has been adjusted to the same electron dose as applied for the film exposure, i.e., about 20e⁻/Å² (compare Fig. 3.5, p. 50). First row, 91,000× magnification, 3.5 Å/pixel at the specimen level; second row, 122,000× magnification, 2.6 Å/pixel at the specimen level; and third row, 161,000×, 2.0 Å/pixel; last row, 214,000×, 1.5 Å/pixel. Columns from left to right: single aligned image, MSA-based class average with eight members, power spectrum of that class average, and power spectrum of an average image from 20 aligned images.
Figure 3.8: TMV images recorded on CCD detector (4 × pixel binning; pixel size: 60 μm). The mean number of counts has been adjusted to the same electron dose as applied for the film exposure, i.e., about 20e−/Å² (compare Fig. 3.5, p. 50). First row, 161,000× magnification, 4 Å/pixel at the specimen level; second row, 214,000× magnification, 3.1 Å/pixel at the specimen level; and third row, 279,000×, 2.3 Å/pixel; last row, 343,000×, 1.8 Å/pixel. Columns from left to right: single aligned image, MSA-based class average with eight members, power spectrum of that class average, and power spectrum of an average image from 20 aligned images.
Figure 3.9: Influence of the sampling interval on the performance of alignment and classification. (A) Class average of eight images of TMV recorded at 1.5 Å/pixel as in Fig. 3.7, fourth row. The power spectrum of the class average shows clearly visible background noise at around 20 Å, although the SSNR at this resolution should be very high (right column, results obtained from eight images of carbon film that have been treated identically to the TMV images). (B) Class average of eight images of TMV recorded at 3.5 Å/pixel as in Fig. 3.7, first row. The power spectrum shows a low level of background noise, the first maximum of the SSNR is about 50% lower, too. (C) Results obtained with the images shown in (A), but binned in Fourier space by a factor of 2.3 so that the Nyquist frequency becomes identical to that in (B) and oversampling is reduced. In contrast to (A), the alignment and averaging algorithms are able to amplify the helical signal in the 23-18 Å range. The high SSNR is retained, leading to a better visual image quality compared with (B).

In conclusion, this experiment underlines the importance of the sampling interval for 2D alignment: In de novo image-processing – when particle details in the range of ~20 Å are to be revealed, coarse intervals of 3 – 5 Å/pixel are to be preferred, as otherwise the alignment is influenced by high spatial frequency noise and fails to enhance the low-resolution particle characteristics.
are hardly distinguishable from the data curve (with the exception of the spatial frequencies close to the origin of the FFT, where the CTF approaches zero). With respect to the B-Factor, the bar plot in Fig. 3.10C shows the 95%-confidence interval of $n = 50$ consecutively recorded images of the same area of carbon film. As expected the standard deviation is very low. Even with a single image a high statistical significance can be reached after rotational averaging of its power spectrum. To test for systematic differences between various fitting algorithms, the spatial signal decay and noise curves were fitted (1) using sequential fitting of the CTF parameters as presented in this work (compare section 3.2.1, p. 74), and (2) using a quasi-Newton optimizer algorithm (R Development Core Team 2004). Again, the results are nearly identical, so that it is not assumed that the fitting algorithm introduces significant systematic errors.

**High-resolution capabilities of film compared to CCD:** In addition to demonstrating that the CCD detector produces superior results in the range of low and intermediate spatial frequencies, the high-resolution capabilities in the range of 2 - 4 Å were evaluated using a combined carbon/gold test specimen (Agar Scientific) that delivers diffraction spots at 3.4 Å (carbon), 2 Å (gold) and 1.4 Å (gold). On film (Fig. 3.11A), strong diffraction spots at 3.4 Å and weak spots at 2 Å are visible. On the TemCam F-415 CCD detector at 214,000× magnification, 2× binning of pixels (pixel size at the specimen level: 1.4 Å/pixel), the 3.4 Å spots are much weaker compared to film, higher-resolutions (e.g. the 2 Å spots) are not captured due to the Nyquist limit of 2.8 Å (compare Fig. 3.11B). Thus, for molecular resolutions, e.g. in electron crystallography, film is still superior to CCD.

In contrast, the higher contrast and better phases in the range of low to intermediate resolutions (20-40 Å) can be decisive for the success of the initial 2D de novo image-processing.

**Considerations for practical work:** An important disadvantage of CCD pixel binning is that the imageable area becomes very small (~2000 × 2000 Å), especially when high magnifications are applied at the same time. The situation becomes even worse at higher defoci, where image information is delocalized: if a defocus of 20,000 Å, a high tension of 200 kV and a spherical aberration constant of 2.0 mm are assumed, then the 10 Å information of a given point is distributed around a circle of ~50 Å radius, while the 5 Å information is spread over an area of ~100 Å radius. So a globular particle 300 Å in diameter would need a circular area of at least 500 Å to be recorded, in order to allow a complete defocus correction
Figure 3.10: Accuracy of the measurements of DPR, SSNR and B-Factor for CCD detector, 279,000× magnification, 4× binned pixels, 200kV. (A) The DPR curves (compare Fig. 3.1, p. 45) are averages from 49 curves obtained after pairwise alignment of single carbon film images (blue curve). The 95%-confidence interval is shown as red and green curve, respectively. Note that these error curves mark the 2σ-Interval. As the standard deviation of the mean decreases with \( n^{-\frac{1}{2}} \), \( n = 49 \) should be sufficient to ensure reliability of the data. The SSNR was calculated from data sets of eight aligned carbon film images (compare Fig. 3.1, p. 45). In (B), the 95%-confidence interval was plotted according to the 2σ-Interval obtained after repeating the experiment six times. Again, the standard error curves are hardly distinguishable from the data curve (with the exception of the spatial frequencies close to the origin of the FFT, where the CTF approaches zero). (C) With respect to the B-Factor, the bar plot shows the 95%-confidence interval of \( n = 50 \) consecutively recorded images of the same area of carbon film. As expected the standard deviation is very low. Even with a single image a high statistical significance can be reached after rotational averaging of its power spectrum. (D) To test for systematic differences between various fitting algorithms, the spatial signal decay and noise curves were fitted (1) using sequential fitting of the CTF parameters as presented in this work (compare section 3.2.1, p. 74), and (2) a quasi-Newton optimizer algorithm (R Development Core Team 2004). Again, the results are nearly identical, and it is therefore unlikely that the fitting algorithm introduces significant systematic errors.
Figure 3.11: High-resolution capabilities of film compared to CCD. Test specimen: combined carbon/gold specimen (Agar Scientific) that causes diffraction spots at 3.4 Å (carbon), 2 Å (gold) and 1.4 Å (gold). (A) Kodak SO-163 film, magnification 50,000×, digitized at 0.8 Å/pixel. On film, strong diffraction spots at 3.4 Å and weak spots at 2 Å are visible. (B) TemCam F-415 CCD detector at 214,000× magnification, 2× binning of pixels, pixel size at the specimen level 1.4 Å/pixel. The 3.4 Å spots are much weaker compared to film, higher resolutions are not captured due to the Nyquist limit of 2.8 Å. Thus for molecular resolutions, e.g. in electron crystallography, film is still superior to CCD.
Figure 3.12: Cross-correlation based stitching of overlapping CCD images. Optimum recording conditions for the CCD are characterized by high magnifications of the microscope together with pixel binning. At these settings sometimes only less than 10 molecules can be recorded on one digital image. All CCD images in the present thesis were recorded with slight overlap and stitched to yield continuous areas of about $2 \times 2 \mu m$. In the example of TBSV shown here, more than 200 single images have been stitched based on 2D cross-correlation functions as implemented in IMAGIC.

(compare section 3.2.1, p. 74). If the particle is too close to the edge of the image, it is therefore to be excluded, and the useful chip length is reduced by about 10%. On the other hand, CTF effects do not argue against the use of particles close to the edges in the initial, low-resolution 2D and 3D analyses. In practice, the simplest method to record large continuous areas is a variant of small-spot scanning over adjacent areas using the electron microscope’s image and beam shift (Sherman et al. 1996). The CCD control software EM-MENU allows images to be recorded with slight overlap in both the x and the y direction and then to be stitched by a translational 2D cross-correlation algorithm (compare Fig. 3.12, p. 58). As a main advantage, manual or (semi-) automated particle selection is faster with only a few large images than with many small ones. As a main disadvantage, small overlap zones with doubled electron dosage occur, owing to the overlap and to the fact that a circular beam has to illuminate a square CCD detector up to the edges of the detector. Furthermore, the use of overlapping image parts is not applicable to native cryo-specimens in ice holes, as the signal of the carbon film is required to determine the exact relative image positions.
3.1.2 Development and characterization of a 3D maximum-likelihood based weighted averaging algorithm for 3D startup model computation

Aim of the work

The aim of this thesis was the determination and characterization of the 3D structures of the snRNP particles [U4/U6.U5] tri-snRNP, [U4/U6] di-snRNP and U5 snRNP. However, several methods to retrieve a first 3D model by standard algorithms as, for example, angular reconstitution as described in section 1.2.4 failed due to structural heterogeneity of the samples and limitations in angular diversity of the projection views obtained. Thus, a reliable, robust and fast procedure for creation of an initial 3D model was needed that was able to (1) deal with structural heterogeneity and (2) to accurately and reproducibly predict underrepresented or missing projection views. The approach presented here combines the use of “reference-free” 2D alignments followed by RCT 3D reconstructions with a ML-based 3D weighted averaging scheme for fast computation of highly reproducible 3D models at low resolution that then be used for further refinements of resolution using available standard methods. Additionally, 3D MSA is performed on the 3D reconstructions to reveal structural heterogeneity of the respective macromolecule. The algorithm was tested using specimens with known 3D structure (the splicing factor SF3b as well as 70S E. coli ribosomes).

General idea of the 3D maximum-likelihood based weighted averaging algorithm

2D de novo procedures that combine alignment and MSA, but do not need reference images from a known structure, are today routinely used in the field (compare section 1.2.4. p. 23). These "reference-free" alignment schemes can deservedly be characterized as extremely robust and reliable algorithms that are capable of reproducibly revealing the main 2D views in a data set. However, in case of the data sets presented in this thesis (compare section 3.3, p. 108) it was often impossible to judge whether different class averages belong to a common 3D structure or merely represent structural heterogeneity of a data set. Differences between individual class averages computed from a set of typically thousands of particle images can basically result from two sources: (1) desired angular diversity of the molecule due to random orientation. (2) undesired structural heterogeneity on the 3D level due to partially missing ligands or as a consequence of different functional states with
respect to subunit flexibility. Whereas angular diversity is essential for the computation of a 3D structure, structural heterogeneity is one of the most time-consuming obstacles during the computational process and the main source of erroneous results. A set of three Euler angles (compare section 1.2.4, p. 24) must be assigned to each class average to describe the angular orientation of a given class average prior to the first 3D reconstruction. If the Euler angles have to be established for the first time for a to date unknown macromolecule, severe errors may occur: First, different class averages may misleadingly be interpreted as different angular views of a common structure, although they are caused by true 3D structural heterogeneity and must thus not be mixed in a single 3D reconstruction. Second, the angular distance of two given projection views is overestimated: this error introduces pseudo-symmetries and is tempting as it produces a more even 3D density with less smear by improving the angular uniformity. At third, the angular coverage of the initial set of class averages may be insufficient so that a distorted 3D density results that is not capable of predicting the currently missing views correctly; thus the small proportion of underrepresented views in the data set will not be detected and not be assigned to correct Euler angles in the next round of multi-reference alignment. However, for every 3D density used as a reference in a subsequent alignment of the particle images, it is mandatory that the general shape, dimensions and characteristic architectural features of the macromolecule are portrayed correctly, as standard 3D alignment and averaging procedures are susceptible to reproducing errors contained in the reference, leading to poor quality of the final 3D reconstruction. The use of random conical tilt (RCT) reconstructions (Radermacher et al. 1987) for 3D startup model computation in this context has a clear advantage over pure zero-tilt approaches, as at least two of the three Euler angles are directly derived from the tilt axis and specimen tilt angle which significantly narrows down the possible degrees of freedom. Importantly, raw RCT reconstructions only contain the tilted images of particles whose zero-tilt images have been grouped together by, for example, MSA-based classification. MSA-based classification algorithms in practice produce optimum results when not more than $\sim 10 - 30$ images are grouped together. Otherwise, for large numbers of particles per class the amount of detail is often reduced due to mixing of heterogeneous image subsets; for large numbers of images per class even visually many ‘wrong’ particle images can be found among the class members despite a more noise-free appearance of the average. A data set of $\sim 10,000$ images will thus lead to 300 - 1000 class averages, several of which may be considered useful for further processing with respect to signal-to-noise ratio and
visibility of high-resolution image details. When tilt pairs have been recorded in
the electron microscope, every MSA-based class average can be "converted" to a 3D
RCT reconstruction consisting of only 10 - 30 tilted images whose zero tilt counter-
parts belong to the same class. So up to many hundreds of 3D reconstructions are
obtained, that on the one hand side are very noisy and suffer from the missing cone
phenomenon, but on the other hand have to be regarded as correct representations
of the 3D structures underlying the class averages. When such 3D reconstructions
are strongly low-pass filtered and converted to rendered surface representations,
differences between the densities often become noticeable, but it is not possible to
differentiate between biologically meaningful structural heterogeneity and simple
effects of noise in the range of low spatial frequencies, particularly when higher
resolution 3D structures are not yet available for comparison. Furthermore, it is
difficult to manually screen up to several hundreds of 3D volumes that would result
from very large data sets. In this context, a very powerful technique to combine
noisy and initially misaligned representations of images was given on the 2D level in
(Sigworth 1998). In that study, an iterative maximum likelihood (ML) alignment
procedure capable of recovering the true underlying information of a noisy data set
was presented that did not suffer from bias introduced by imperfect intermediate
reference images. As in standard alignments, cross correlation coefficients (CCCs)
for all possible alignment parameters, i.e. translations and rotations of the particle
images, were computed, but - unlike in standard alignment procedures - projected
onto an exponential function to yield individual weighting factors for each set of
transformations. Whereas in traditional alignment methods only those alignment
parameters are considered for averaging that belong to the highest CCC (compare
section 1.2.4. p. 19), ML alignments compute a weighted integral over all possi-
ble transformations of the particle images and take into account the probability
density functions of the transformation parameters. Significantly, it is possible to
apply an 'information-free', i.e. bias-free image (e.g. random density values) as the
first startup reference in a ML alignment, and the algorithm iteratively converges
to the true underlying structure while at the same time describing the probability
densities of the translational alignment parameters and the noise contained in the
data set better and better. According to (Sigworth 1998), the weighting factor w
belonging to a set of alignment parameters is given by

\[ w = c \cdot e^{ccc/s^2} \]  

(3.1)

where ccc is the CCC measured, s is a parameter dependent on the standard
deviation of the noise in the data set and \( c \) is an independent constant. In the work presented here, a similar algorithm for alignment and weighted averaging was implemented on the 3D level, so that 3D Gaussian noise could be used as the first startup reference for a de novo 3D alignment and sets of up to several hundred 3D densities could be iteratively aligned and combined by weighted averaging. In addition to 3D ML alignments, 3D MSA followed by HAC (van Heel 1984) was subsequently performed to reveal structural heterogeneity on a 3D level. A summarizing flow chart is given in Fig. 3.13, p. 63.

Procedure and Findings

Test specimens and electron microscopy: The splicing factor SF3b (Golas et al. 2003) as well as 70S \textit{E. coli} ribosomes were used as test specimens. For both macromolecular complexes, the 3D structure is known for comparison. For electron microscopy, both specimens were embedded in a layer of uranyl formate stain according to the “sandwich” method (compare section 2.2.3, p. 33). Image acquisition was performed at a defocus of \( \sim 1.4 - 2.4 \mu m \) on the CM200FEG 200 kV transmission electron microscope (TEM) operated at 200kV equipped with a field emission gun with the specimen at room temperature.

In case of SF3b, 45° tilted and untilted images were taken on Kodak SO-163 film at a magnification of 50,000\( \times \), developed and digitized at 2,540 dpi as described (compare section 2.2.5, p. 34). After 2\( \times \) coarsening of the images, the final pixel size was 4 Å/pixel. In case of \textit{E. coli} ribosomes, the 4kx4k CCD camera “TemCam-F415” was used at 2\( \times \) binning of the pixels and 122,000\( \times \) magnification. For further characterization of this condition, see also section 3.1.1, p. 40 and table 3.1, p. 43. Again, images of particles were recorded at a tilt angle of 45° and untilted. After 2\( \times \) coarsening, the sampling interval used for further computation was 4.9 Å/pixel. 7,784 SF3b tilt pairs and 8,483 ribosomal particles, respectively, and 17,808 untilted SF3b images and 9,884 untilted 70S particles were interactively selected and corrected for defocus as described in this thesis (compare section 3.2.1, p. 74).

2D image processing: Particle images of SF3b and 70S ribosomes were subjected to 5 – 8 rounds of iterative exhaustive multi-reference alignment via polar coordinates (compare section 1.2.4, p. 19), followed by MSA and HAC (compare section 1.2.4, p. 22). The final average number of images per class was 20 in the case of SF3b and 30 in the case of the ribosome. 62 - 250 class averages of SF3b
Tilt pairs (3,000 - 20,000)

zero-tilt images

tilt images

"reference-free" alignment, Euler angle γ

MSA/classification

100-2,000 class averages (10-30 class members)

visual evaluation

50-500 selected class averages

~5-50 3D class averages

weighted 3D average

ML-based weighted averaging

6 - 15 iterations

initial 3D reference:
Gaussian noise

50-500 RCT
3D reconstructions

3D alignment

3D reference:
final weighted 3D average

3D alignment

3D MSA/classification

Figure 3.13: Flow chart describing the ML-based 3D weighted averaging procedure for de novo single-particle 3D image processing. A data set containing 3,000 - 20,000 image tilt pairs (e.g. particles recorded under 45° tilt angle and without tilt of the specimen holder) is initially analysed by the well-established "reference-free" alignment scheme (Dube et al. 1993) followed by MSA/HAC (compare section 1.2.4, p. 21) so that stable class averages are obtained. The classification information in combination with the rotational alignment parameter of the images, tilt axis and tilt angle enables the computation of one RCT 3D reconstruction (Radermacher et al. 1987) per selected class average. The resulting set of 50-500 RCT 3D reconstructions is then subjected to iterative rounds of 3D alignment and ML-based weighted averaging as described in the text. As initial 3D reference, 3D Gaussian is chosen comparable to the 2D case (compare Fig. 3.14, p. 66). The stable weighted 3D average then represents the 'consensus' 3D model resulting from evaluating the whole data set at once. It can further be used as 3D reference in a 3D alignment of all input 3D densities. Then, 3D MSA/classification is performed to form 3D class averages to separate structural sub-populations of particle with respect to conformational heterogeneity or ligand binding state.
and 133 class averages of ribosomes were selected for further 3D image processing. To test the algorithm with simulated and real world data on a 2D level – synthetic test images of 70S ribosomes were created as described in this thesis (compare Fig. 3.27, p. 98). The quotient of variance of the signal and variance of Gaussian noise was set to 0.06 (i.e. hardly visible). In addition, 771 untitled images from the 70S ribosome data mentioned above representing the main side view were extracted from the data set and used for testing the algorithm on a 2D level.

**3D image processing and implementation of 3D ML alignment:** RCT 3D reconstructions (Radermacher et al. 1987) corresponding to each selected class average were computed as described in section 1.2.4, p. 24. Specimen tilt angle, specimen tilt axis, and the rotational angle from the final multi-reference alignment were combined to a set of three Euler angles. 3D reconstructions were computed and iteratively refined by several rounds of parallel alignment to the intermediate 2D forward projections corresponding to the input images. In addition, mass centering of the 3D densities was performed. A 3D alignment procedure taking an arbitrary number of individual 3D densities and one 3D reference as input was implemented on a PC cluster (compare Fig. 3.28, p. 101). The degree of angular spacing with respect to longitude and latitude Euler angles could be specified by the user, the Euler angle $\alpha$ determining the rotation about the main particle axis (compare section 1.2.4, p. 24) was determined by 3D alignment via polar coordinates as in the 2D case described in section 1.2.4, with the only difference that the 1D polar correlation functions had to be computed from all 2D sections of the 3D volume and summed prior to peak search. All CCCs identified from local maxima of the resulting polar cross-correlation functions where stored with their respective Euler angles. Direct neighbors of local peaks were consciously discarded to avoid 'bystander' effects. To improve execution speed, the alignment was restricted to the rotational case, as the input 3D densities turned out to be sufficiently mass centered prior to the alignment and weighted averaging procedure described here. For weighted averaging, equation 3.1 (Sigworth 1998) was used to convert the normalized CCCs into individual weighting factors as follows: the well-proven concept of user-specified numbers of images per average established in MSA-based classification (compare section 1.2.4, p. 21) was applied to the weighted averaging procedure presented here. Therefore the constant parameter $c$ of equation 3.1 was adjusted so that the maximum CCC ($\alpha = \max$) resulted in a constant weight of 1. The desired sum of all weights ($\sum w$) specified by the user was obtained by iteratively refining parameter $s$ (see equation 3.1) and parameter $c$ treated as a variable dependent
on $s$ and $cc_{\text{max}}$. A lower weight limit could also be specified (typically 0.0005) to avoid time consuming 3D averaging computations for weights that would hardly contribute to the final result. As the averaging algorithm should be able to deal with low $\sum w$ without being inappropriately influenced by a single input 3D density, the concept of blocking was introduced: for low $\sum w$ the influence of the input 3D density with the highest CCC on the overall appearance of the average is likely to be relatively high. Therefore the number identifying this input density was stored and excluded from averaging in the next cycle to make sure that the final result was not only a consequence of a random event at the beginning of the iteration cycle. About ten rounds of iterative 3D alignment and weighted averaging were performed. As first reference, a 3D volume filled with Gaussian noise computed from random numbers (Prime Modulus Multiplicative Linear Congruential Generator from the Compaq Fortran software-development package) was used. The procedure was also implemented for the 2D case to establish the concept using well characterized data sets with known class averages as described above.

The algorithm was tested on the 2D level using real world as well as synthetic image data to improve the understanding of ML-based weighted averaging. Data sets of $\sim 750$ 70S ribosomal particle images were applied to 10 iterative rounds of alignment and weighted averaging as shown in Fig. 3.14, p. 66. As first reference, a Gaussian noise image created from random numbers was chosen. A typical computer-generated noisy data image is shown in Fig. 3.14A, upper row (labeled "single"). The noise-free counterpart (lower row, labeled "target") served - after each round of alignment - as ideal target density to determine CCCs of any possible weighted average with respect to $\sum w$ in the range of $w = 1...1000$. The curves showing the CCC as a function of $\sum w$ are plotted in Fig. 3.14A, lower row for the synthetic data and in Fig. 3.14B, lower row, for the real data. The maximum CCC was determined from the curve and the weighted average with $\sum w$ corresponding to the maximum CCC was taken as reference image for the next round of alignment (For the synthetic data and the first round of alignment, the maximum of the CCC as a function of $\sum w$ was a local one with $\sum w = 22$, then the CCC curve was monotonically increasing with higher $\sum w$.) The same iterative procedure was applied to $\sim 750$ real world ribosome images of a common angular view taken from the data set (Fig. 3.14B). However, the visual SNR was considerably higher for the electron-microscopic images than for the generated test data (shown in Fig. 3.14A). In case of the real data, the noise-free appearance of the image data was estimated by the sum of all aligned images (see Fig. 3.14B, last column, lower row,
Figure 3.14:
Characterization of ML-based weighted averaging for 2D images using synthetical and real world data images.

(A) 771 generated 70S ribosome projections of SNR ≈ 0.06 were applied to 10 iterative rounds of alignment/weighted averaging. In the last column, a typical data image (upper row, labeled "single") and the noise-free counterpart (lower row, labeled “target”) is depicted. After each round of alignment, CCCs of weighted averages to the target are shown as a function of the sum of weights (Σ w). For Σ w (x-axis), a range up to 1,000 is plotted in the diagrams below each average. The maxima are highlighted by a red dot and labeled with the corresponding CCC. The Σ w and s belonging to the maximum CCC are shown below each diagram. The average representing the Σ w for the maximum CCC (shown in the upper row) is used as a reference for the next round of alignment. Convergence to the underlying structure occurs in parallel with a continuous increase of Σ w. After ∼6 rounds of alignment, a stable plateau of CCC = ∼0.91 is reached. (B) Iterative alignment and weighted averaging of 771 real 70S projection images. A typical noisy data image (labeled "single") is shown in the upper row, last column. Here, the noise-free appearance of the density is estimated by the unweighted sum of all aligned images (lower row, last column, labeled “target”). Again, a Gaussian noise image is chosen as first reference and 10 rounds of alignment/weighted averaging are performed. As in (A), after each round of alignment the Σ w belonging to the maximum CCC to the target is identified and used to compute the next reference average image (each shown in the upper row). As with test images, convergence to a CCC of ∼0.96 is bound to an increase of Σ w from ∼100 to ∼450. (C) Alignment/weighted averaging applied to the real 70S data set as in B. Again, a Gaussian noise image served as first reference. Unlike in B, Σ w is not computed (and thus the iterative procedure is not driven in a specific direction by comparison with any 'ideal' reference image), but user-specified starting with an arbitrary low value of 2. Σ w is manually increased on a pseudo-exponential scale to values 10 – 20 times lower than the optimum computed in (B). Here, Σ w stays in a range that has traditionally been applied in unweighted MSA-based averaging schemes. In the last column of (C), the upper black curve illustrates the course of the 'ideal' Σ w, whereas the blue curves denotes the user-specified curve. Significantly, the high-resolution image details are emphasized much clearer after ten rounds of alignment/weighted averaging in (C), no. 10 than in (B), no. 10, despite a slightly noisier appearance.
labeled “target”) to again enable the fastest possible convergence of the algorithm. By computing the parameters $c$ and $s$ (compare Eq. 3.1) from measurements of CCCs to an “ideal” target image, one could argue that the algorithm would be driven into a specific direction and could get caught within another local maximum of likelihood when different weights were applied, especially when it is kept in mind that maximum likelihood methods only identify local maxima of likelihood. Therefore, the computations shown in Fig. 3.14A and 3.14B were repeated using a variety of different $\sum w$, numbers of particle images and SNRs and it could be made sure that – except for very small particle image statistics combined with very low SNRs – the convergence to the underlying structure was not critically dependent on these variables, thereby underlining the robustness of the method. However, by estimating parameter $s$ from the CCC curves, “ideal” values leading to the fastest possible convergence could be established that should closely resemble those found by an ideal predictive algorithm for re-estimating the parameters contributing to the weighting function. However, the observed convergence speed – the main architectural features of the particle were already established after $\sim 6 \to 10$ rounds of alignment/weighted averaging – was in good agreement with a recent application of ML theory for 2D multi-reference alignments (Scheres et al. 2005). Importantly, convergence of the procedure went in parallel with a continuous increase of $\sum w$ (from $\sim 20$ to $\sim 600$ in case of synthetic data, from $\sim 100$ to $\sim 500$ in case of real image data). This behavior adds to the understanding of ML-based alignments: in the first rounds of alignment, the transformation parameters are imprecise and only averaging of a very few images can lead to a better SNR without blurring effects: when the CCCs are projected onto an exponential function using the ‘ideal’ parameter $s$, the second highest CCC is found relatively far away from the highest and so on. The better the average gets in further rounds of alignment, the closer can the CCC values move together on their weighting function, allowing more and more images to significantly contribute to the average. However, the $\sum w$ values of $\sim 100$ seemed relatively high compared to those applied in unweighted MSA-based averaging schemes. In traditional MSA-based averaging, the best level of high resolution detail is normally achieved for averaging of $10 \to 30$ images (i.e. $\sum w = 10 \to 30$, each individual weight being 1 in the absence of a weighting function). In contrast, when inappropriately large MSA-based classes are formed high resolution details disappear despite a more noise-free appearance of the resulting average because of a stronger influence of structural heterogeneity. Thus, in Fig. 1C $\sum w$ was manually increased on a pseudo-exponential scale to values $10 \to 20\times$
3.1 Developments for improvement of reliability of *de novo* 3D structures

Figure 3.15: Application of the 3D ML algorithm to a set of 133 70S ribosomal RCT 3D densities. This test served to demonstrate the general applicability of the 3D weighted averaging (ML) algorithm.

Eight rounds of iterative 3D alignment and weighted averaging were performed similar to the 2D case shown in Fig. 3.14C. A volume filled with random Gaussian noise was used as first reference (upper row). The input 3D densities were gradually bandpass filtered after each round of alignment as described in the text. The first $\sum w$ was 1.8, the final $w$ was 12.0. In (A), the RCT 3D densities were computed without cosine stretching of the tilted images in (B) RCT 3D densities were computed after cosine stretching (Frank 1996) perpendicular to the tilt axis to compensate for the flattening effect of the sample embedded between two carbon films. However, cosine stretching did not improve the final result.

lower than the optimum computed in Fig. 3.14B, so that $\sum w$ stays in a range that is traditionally used for unweighted MSA-based averaging schemes. Significantly, the high-resolution image details of the final average (Fig. 3.14C, upper row, 10th column) are much clearer than in Fig. 3.14B. In conclusion, underestimation of the noise parameter $s$ is useful to better compensate for blurring effects occurring in heterogeneous data sets.

When applied to the sets of 3D RCT reconstructions of SF3b and 70S *E. coli*
results (see Fig. 3.15 and Fig. 3.16), final $\sum w$ of $\sim 10 - 20$ were used, as higher values did not improve the result, but could lead to a significant contribution of low-quality input data and blurring. However, when the amount of structural details in the 3D averages improved, stronger high-pass filters were applied to the 3D densities. For SF3b the $\sum w$ and corresponding lower cut-off values with respect to the Nyquist frequency were $1.7 - 8.0$ for $\sum w$ and $0.01 - 0.3$ for the lower band filter cut-off. For the ribosome data, the $\sum w$ and corresponding lower cut-off values with respect to the Nyquist frequency were $1.8 - 12.0$ for $\sum w$ and $0.01 - 0.2$ for the lower band filter cut-off.

For the ribosomal image data, the procedure was performed with and without cosine stretched particle images to compensate for particle flattening perpendicular to the tilt axis. However, for a tilt angle of $45^\circ$, stretching resulted in inferior results (see Fig. 3.15B). The algorithm was applied to the protein complex SF3b (see Fig. 3.16), as its small size of only $\sim 450$ kDa (Will et al. 2002), roundish appearance and even angular distribution on the carbon support film makes its structure difficult to solve for classical RCT. The similarity of the pure zero-tilt angular reconstruction approach (Golas et al. 2003) and the RCT/ML based approach - apart from differences in resolution - is striking: in Fig. 3.16, architectural features observable in both structures are highlighted. To align the RCT/ML 3D structure of SF3b to the angular reconstruction structure (Golas et al. 2003), exclusively the prominent features of the outer wall were used. Significantly, the protein density assigned to the RRM-containing protein p14 can be found at the same position in the inner cavity of the particle including its connecting bridges as predicted from the angular reconstruction structure (Golas et al. 2003). The computation time for each data set on a PC cluster containing 64 nodes was short enough to accomplish the necessary iterations in less than one day. When a stable RCT/ML result was reached, the sets of 3D reconstructions were conventionally aligned against that density and MSA followed by HAC was applied to the 3D volumes. This technique is called "3D MSA" within this thesis as opposed to the standard method of applying the MSA algorithm to 2D projection views of a particle. 3D class averages containing on average 4 3D RCT volumes were computed. Then, the strongly low-pass filtered 3D structure of SF3b obtained by angular reconstruction, the RCT/ML result and four 3D MSA-based class averages were selected as 3D startup references for an alignment of untilted 2D MSA-based class averages computed in a "reference-free" alignment (compare section 1.2.4, p. 23), resulting in 6 reconstructions with clear differences in the inner part of SF3b (Fig. 3.174C-G). The range of angles measured
for p14 bound to the outer wall was \(~58 - 96°\) compared to \(60°\) for the angular reconstitution model (Fig. 3.17A, B). A multi-reference alignment of the complete particle image set using all 3D structures as competitive references revealed a proportion of more than 50% to be in a 50-70° position in contrast to minor groups of perpendicular to obtuse angles (Fig. 3.17H). Importantly, this movement of SF3b p14 relative to the outer wall has been predicted to occur upon integration of SF3b into the minor spliceosomal U11/U12 di-snRNP particle (Golas et al. 2005). So it is likely that a certain degree of structural heterogeneity of that part of SF3b already exists in solution prior to any integration event. When 3D MSA was applied to the ribosome data set, which was known to be heterogeneous with respect to ligands binding at the factor binding site (Fig. 3.17I-J), indeed variations in density could be observed at the factor binding site (A more detailed analysis of these is under investigation and given in another Ph.D. thesis).

Both ML-based 3D weighted averaging and 3D MSA are applied to data sets of the [U4/U6.U5] tri-snRNP, the [U4/U6] di-snRNP and the U5 snRNP for computation of an initial low-resolution model as well as for analysis of structural heterogeneity as described later in this chapter in section 3.3, p. 108.
Figure 3.16: Application of the algorithm to the SF3b data set containing 62 3D densities. This experiment aimed at independently confirming the SF3b 3D structure (Golas et al. 2003) and was conducted in collaboration with Mrs. M. M. Golas. The computation of a SF3b 3D structure at high resolution and description of the structure was part of her Ph.D. thesis. (A) Iterative convergence of the weighted averaging procedure. The first user-specified $\sum w$ was 1.7, the final $\sum w$ was 8.0, with gradual band-pass filtering as described in the text. (B) First column: high-resolution zero-tilt SF3b structure from (Golas et al. 2003) computed by angular reconstitution depicted in blue (upper row, full surface view; lower row, section revealing the inner cavity with a prominent density assigned to the RRM motif of the protein p14). Second column: RCT/ML weighted average after 11 iterations (the corresponding density is shown in (A), last row). Prominent features (protrusions, holes, inner cavity) recognizable in both types of reconstructions are identically labeled in both structures.
Figure 3.17: Application of the RCT/ML algorithm followed by 3D MSA using test specimens of different particle size. These tests were conducted in collaboration with Mrs. M.M. Golas.

(A)-(H) Result of 3D MSA of the SF3b data set containing 250 3D reconstructions of SF3b. 80 3D classes were calculated after MSA and HAC resulting in an average number of 3.1 3D densities per class average.

(A) View of the zero-tilt reconstruction computed by angular reconstitution (Golas et al. 2003). The angle between the protein p14 and the outer wall is 60°. (B) Projection-matching structure from untilted data set using the structure published in (Golas et al. 2003) as first 3D reference, band-pass filtered to ~40 Å. (C)-(G) Structural heterogeneity of SF3b with respect to the relative position of p14 as revealed by 3D MSA. Five class averages have been selected as 3D startup references for an alignment of untilted 2D MSA-based class averages, resulting in 6 reconstructions with clear differences in the inner part of SF3b. The range of angles measured for p14 bound to the outer wall is 58 - 96°. (H) Histogram of binding angles of p14 to the outer wall of SF3b derived from a competitive multi-reference alignment of the untilted image data to the reference 3D densities shown in (B)-(G). The largest proportion is found in the conformation tilted by 51 - 70°. Perpendicular and obtuse positions are found in roughly 20% of the images, respectively.

(I)-(J) Analysis of a 70S E. coli ribosome sample that had previously been characterized as being heterogeneous with respect to ligands binding at the factor binding site. 3D MSA-based class averages (after application of the RCT/ML technique) containing on average 10 individual 3D reconstructions were computed from a data set of 133 3D densities. The view at the subunit interface reveals different sub-populations occurring in the sample. A more detailed description of this sample is part of another thesis.
3.2 Developments for improvement of resolution and speed

Whereas the previous section was devoted to the de novo computation of initial 3D models of particles the 3D appearance of which is not known to date, the current section deals with methods to be applied when a 3D structure is already available for further computations. Once a valid and reproducible 3D model of a macromolecule is available, this model can be used as initial 3D model for the analysis of larger data sets of the same particle and thus be refined to a better resolution. The standard technique to perform this task is projection-matching of the particle images with respect to reference images computed from the 3D structure (compare section 1.2.4, p. 26). In the past, the resolution limit of refinement procedures was limited by the speed and accuracy of the alignment of the image data. If, for example, 50,000 images were to be accurately aligned in a multi-reference alignment with $2 - 4^\circ$ angular separation of the reference images, every data image had to be evaluated with respect to $\sim 2500 \rightarrow 10000$ reference images. However, it turned out that recent improvements of the computer hardware, in particular speed of the processors, were not sufficient to perform this enormous computational task in a short time. For refinement of the [U4/U6.U5] tri-snRNP structure (compare section 3.3, p. 108), $\sim 66,000 \rightarrow \sim 121,000$ images were aligned against reference image set of up to 41,000 images, and this procedure had to be repeated several times to find optimum parameters for band-pass filtering of the data images. The computation speed sufficient for these calculation was achieved by using the corrim-based MRA algorithm described in this chapter (compare section 3.2.2, p. 91).

In addition, resolutions higher than the first zero crossing of the CTF can only be achieved if the CTF parameters are accurately determined and the frequency bands exhibiting negative contrast are flipped to avoid extinction of high-resolution information in the 3D structure (compare section 1.2.3, p. 13). A procedure to accurately determine defocus, amplitude contrast proportion, astigmatism automatically for large data sets as those used for the [U4/U6.U5] tri-snRNP and the U5 snRNP particle is also given within this chapter (compare section 3.2.1, p. 74). This algorithm also serves to recognize images of low quality due to drift of the specimen holder during exposure or charging of the specimen (compare section 1.2.4, p. 18). The techniques presented here have also served to improve the resolution of the 3D structure of the splicing factor 3B (SF3b) (Golas et al. 2003) to $\sim 7-10$ Å.
3.2 Developments for improvement of resolution and speed

3.2.1 Development and characterization of an automatic procedure for CTF correction

Aim of the work

The periodic change of sign of the contrast transfer function (CTF) reviewed in section 3.2.1 has to be considered in high-resolution single-particle 3D reconstructions. In summary, the appearance of the CTF is a consequence of the necessary deviation from the Gaussian focal plane into the underfocus to achieve phase-contrast electron microscopy (Scherzer 1949). Optimal phase contrast cannot be achieved for all diffraction angles and space frequencies simultaneously. In particular, although it is true that a high degree of defocusing (several times the Scherzer focus) leads to an early increase in CTF and thus to selective emphasis of the lower spatial frequencies, allowing the particles to appear with enhanced contrast, a strong defocus has the attendant drawback of producing a very rapid sign change of the CTF in the region of high-resolution image information. The [U4/U6,U5] tri-snRNP data set shown in 3.3, p. 108 has been imaged using relatively high defoci of $\sim 6 \mu m$. On the one hand side, the image contrast was thereby improved because of the relative abundance of low spatial frequencies which largely determine the contrast, on the other hand the first zero crossing of the CTF occurred at around 40 Å resolution, so this resolution would have been the limit without CTF correction. Electron microscopic images taken at underfocus exhibit a characteristic kind of blurring introduced by the CTF: modulation by the CTF causes points in the object plane to appear not as points, but as concentric, overlapping rings. The rings are a consequence of the phase reversals in Fourier space that occur with increasing rapidity at higher spatial frequencies. Almost all the calculations of 3D structures solved recently by electron-microscopic methods have therefore included a deconvolution step intended to free the 3D density from the influence of the CTF and thus to raise the attainable resolution beyond the first zero-crossing of the CTF. In this context one can distinguish between, on the one hand, the method based upon pairs of recordings of the same field with different defoci (Toyoshima and Unwin 1988; Frank and Penczek 1995; Conway and Steven 1999) and, on the other hand, methods that are aimed at extracting the CTF parameters from the power spectra of individual micrographs. There exist various possibilities to improve the visibility of poorly visible phase reversals in power spectra (also called Thon rings: (Thon 1966)). These methods include averaging over large negative regions together with rotational averaging (Zhu et al. 1997), summing of power spectra to enhance the
signal-to-noise ratio (Zhou et al. 1996) and periodogram averaging from mutually overlapping regions (Fernandez et al. 1997). Various mathematical methods have been applied successfully to the extraction of the CTF parameters from Thon-ring images. These include least-squares fitting (Zhu et al. 1997), graphical user interfaces (Zhou et al. 1996; Ludtke et al. 1999) and the fitting used here, which is based upon the coefficient of correlation between the estimated power spectral density and theoretical, 2D CTF functions (Tani et al. 1996; Radermacher et al. 2001).

In contrast to previous approaches, the method described here allows the precise correction of defocus and astigmatism of large data-sets of several thousand single particles and has, in addition to a high degree of automation, the advantage that the exact position of the particle in the micrograph does not need to be known. It is based upon the diffuse signal of the supporting amorphous carbon film onto which the particles are adsorbed, and it employs the MSA classification (compare section 1.2.4, p. 21) implemented in the software package IMAGIC (van Heel et al. 1996).

Physical foundations:

The contrast in the representation of biological particles can be described with good approximation by the phase contrast transfer function (PhCTF, compare 1.2.3, p. 13). However, especially in the region of low and medium spatial frequencies, a shift in the zero crossings of the measured CTF relative to the theoretical PhCTF can be observed. This has its origin in the absorption amplitude contrast (compare section 1.2.2, p. 12). A good approximation of the combined effect of phase and amplitude contrast in the generation of images is achieved - on the assumption of an amplitude-contrast proportion that is independent of the spatial frequency and without taking into account different kind of atoms in the sample (Erickson and Klug 1971; Toyoshima et al. 1993) - by adding a cosine term to equation 1.4, p. 14. If $F$ is the amplitude-contrast proportion, then the combined CTF $H(q)$ is obtained:

$$H(q) = -2 \cdot [(1 - F) \cdot \sin W(q) + F \cdot \cos W(q)]$$  \hspace{1cm} (3.2)

However, under realistic imaging conditions a simple, radially symmetric CTF cannot be assumed, since in practice astigmatism cannot be completely corrected for experimentally. Two-fold astigmatism leads to a superposition of two CTFs with two (different) defocus values $\Delta z_x$ and $\Delta z_y$ along the two orthogonal principal axes.
Rotational averaging of power spectra, which in many cases is a first step in the determination of the CTF parameters, prevents the determination of the astigmatism parameters. However, if the signal-to-noise ratio is obtained from averaging similar power spectra applying MSA and subsequent classification, then the astigmatism information is preserved. The astigmatism parameters can then be determined and corrected for. A further factor that must be taken into consideration is the spatial-frequency-dependent reduction in signal strength; this has several causes and is summarized in the so-called experimental B factor. Contributions to this exponential decay in the CTF maxima come from aberrations in the microscope, the incoherence of the electron source (Frank 1973; Wade and Frank 1977), the instability of the sample holder in the microscope, the modulation transfer function (MTF) of the film material used and the MTF of the scanner used (Saad et al. 2001). The influence of various resolution-limiting factors can be summarized as an exponential factor $e^{-Bq^2}$, where B is the B factor (Glaeser and Downing 1992; Saad et al. 2001). Furthermore, an additive frequency-dependent background noise term $S(q)$ that is not convoluted by the CTF must be considered; this arises from various sources such as noise in the optical media and non-elastic scattering of electrons. The background noise can be described by the exponential decay of the absolute positions of the CTF$^2$ minima and the noise function can be approximated by a Gaussian profile with three unknown parameters (Zhu et al. 1997). In contrast to the exponential B factor that describes the amplitude decay of the maxima in the CTF$^2$, the background noise function explains the exponential decay of the values of the CTF$^2$ minima in the power spectrum. Since the method to be described employs a correlation-based fitting that is not influenced by the absolute values in the power spectra and the theoretical reference-CTFs, the rotational average of a Gaussian profile with two unknown variables $(a, c)$ gives a good representation of the non-convoluted background noise to improve the correlation based fitting procedure:

$$S(q) = a \cdot e^{-q/c^2}$$  \hspace{1cm} (3.3)

Thus, all in all, the contrast to be expected is expressed by:

$$H(q_{xy}) = -2 \cdot e^{-Bq^2} \left[ (1 - F) \cdot \sin W(q_{xy}) + F \cdot \cos W(q_{xy}) \right] + S(q)$$  \hspace{1cm} (3.4)

The CTF correction method presented here aims at accurately determining the parameters B, F and $q_{xy}$ as well as the unconvoluted background noise parameters.
for correction of image blur caused by these parameters. Importantly, it enables an individual correction of particle images without assuming a constant CTF over the whole micrograph.

**Procedure and Findings**

**Preparation and measurement:** To test the algorithm, two different samples (70S ribosomes and U1 snRNPs) were prepared by three different methods: native cryo-preparation in holes (compare section 2.2.4, p. 33), native cryo-preparation on carbon film (compare section 2.2.4, p. 33), and cryo-negative-staining (compare section 2.2.3, p. 33). The samples were examined in three different Philips electron microscopes: SOPHIE, CM200FEG, and CM120. SOPHIE is a microscope operated at liquid helium temperature (∼3K) (Zemlin et al. 1994). Philips CM200FEG and Philips CM120 are operated at liquid nitrogen temperature (∼80K). SOHPIE and CM200FEG are equipped with a field emission gun (FEG) which delivers – in contrast to conventional electron sources – a highly coherent electron beam and thus lower spatial signal decay. The electron lenses, high tensions and specimen holders were: CM200FEG (Cs = 2.0 mm), operated at 200 kV with a Gatan cryo-holder; CM120 (Cs = 6.3 mm) with a LaB₆ cathode operated at 120 kV with a Gatan cryo-holder; and SOPHIE, a modified Philips CM20FEG, equipped with a helium-cooled superconducting objective lens (Cs = 1.35 mm) (Zemlin et al. 1994) and operated at 200 kV. The preparations were frozen in liquid nitrogen and images were obtained in the electron microscope under low-dose cryo-conditions using electron dosages of 15-20 electrons/Å². After selection of particles, the particle images were extracted from the digitized negative, and the CTF parameters were corrected locally, at the level of single particles. To obtain high-resolution information in the corrected images, it was necessary on account of the point spreading to use a sufficiently large window when the single-particle images were being extracted for defocus correction; according to the contrast transfer theory, the point spreading (compare section 1.2.3, p. 13) and thus the required size of the pixel window generally depends upon the defocus value and was in this case about twice the size of the maximum particle diameter. To test the applicability of the method for "native cryo" preparations (compare section 2.2.4, p. 33) and to compare amplitude-contrast values between cryo-negative-stain samples and native cryo conditions (Adrian et al. 1984), three different additional "native cryo" samples were prepared. First of all, U1 snRNP particles in vitreous ice (Stark et al. 2001) were prepared by shock-freezing in liquid ethane as described (compare section 2.2.4, p. 33). The concentration of U1
snRNP was high enough to cover the entire ice hole with a lawn of protein-RNA particles, so that a signal of adequate strength for iterative analysis was obtained. Secondly, native 70S ribosomes were prepared in vitreous ice on a carbon support film and the images thus obtained were used for comparative measurement of the amplitude-contrast proportion. Thirdly, native 70S ribosomes were also prepared in vitreous ice on a holey carbon grid. The different samples were imaged in various microscopes to test the applicability of our method for a wide range of different sample preparation techniques and to allow comparison of the B factors measured.

Classification of power spectra according to CTF parameters: For correction, data sets of selected single particles with typically several thousand individual images are used. MSA is carried out on the power spectra of these images (compare Fig. 3.18, p. 81). The prerequisite for optimal results is a conventional high-pass filtering of the images with an inverse Gaussian profile for the computation of the power spectra, in order to suppress the large signal amplitude at the centre of the power spectrum and at the same time to raise the relative signal strength in the regions of rapid CTF sign change. In the digitization of the micrographs, very small sampling increments are generally used, in order to exploit the scanner's resolution as far as possible. Thus, the signal in the power spectra approaches zero long before the Nyquist frequency is reached. The procedure described here is greatly accelerated by storing only the central regions of the computed power spectra and using these to perform the MSA. In the MSA, 10-20 Eigenimages are computed and a classification (HAC) of the 2D power spectrum is carried out, so that some 20-50 individual power spectra with similar CTF parameters are placed in each class. If a data set contains astigmatic images, then a characteristic phenomenon can be observed, as shown in the example: some Eigenimages display characteristic displaced semi-circles (see Eigenimage no. 8 in Fig. 3.2.1A, p. 80).

Iterative determination of the CTF parameters: The class averages of power spectra thus obtained from individual molecular images, with almost identical CTF parameters, are used to determine iteratively the fundamental parameters defocus, amplitude-contrast proportion, astigmatism, B factor and the non-convoluted background noise. The parameters defocus, amplitude contrast and astigmatism are then used for correction of the single images already classified according to the similarities found in their power spectra. The basis of the iterative procedure is the computation of theoretical power spectra over a presumed interval and the calculation of the correlation coefficient with the measured power spectra, as already
described (Tani et al. 1996; Radermacher et al. 2001). Various orders for iterative fitting of the parameters have been tried out on theoretical 2D power spectra with various defocus and astigmatism values, the goal being to find a strictly converging algorithm. In the procedure described here, one parameter at a time is varied, and the value of this parameter corresponding to the correlation maximum found is stored and used in subsequent fitting until a value that correlates more closely with the class average is found. Because of the point symmetry of power spectra, it is possible to accelerate this process by conducting the calculations on one half of the image only. In principle, systematic errors can prevent the convergence of the iterative fitting to correlation maxima. These errors include especially the neglect of all effects that lead to intensity gradients between the centre and the edge, such as the B factor and, to a lesser extent, non-convoluted background noise. The procedure shown in Fig. 3.18 therefore begins with preliminary estimation of the B factor by fitting to decreasing 2D exponential functions with varying full width at half maximum. There follows a first search for the defocus at intervals of 50 Å in a region defined by the user. The correlation of the Thon ring images with theoretical CTF references can – according to signal strength – lead to periodical secondary maxima, as is observed in interference phenomena. The amplitude of these secondary maxima is lowered by initial high-pass filtering of the class average, so that an unambiguous determination of the principal maximum becomes possible. The course of the correlation coefficient as a function of the defocus used to calculate the reference CTF, with a typical peak in the height of the defocus sought, is shown in Fig. 3.21. At high two-fold astigmatism values, even correlation curves with two principal peaks separated by a trough can arise; these express different defocus values in the two principal directions. In this case, the local minimum between these maxima is automatically chosen as a good first estimate of the underlying defocus. To determine the direction of the astigmatism it is not necessary to know its exact magnitude. For the first iteration we assume an astigmatism of 50 Å and vary the direction in steps of 6°. The direction of the astigmatism thus found is then used to find the magnitude of the astigmatism. All parameters are then refined sequentially, as shown in Fig. 3.18, whereby both the step size is reduced and the search region is systematically restricted, in order to accelerate the search; if the data inserted by the user were incorrect, then the search region can also be moved automatically by the software. In the generation of the theoretical CTF functions the high-pass filter, with which the single-particle images were pre-treated, is also taken into account (compare Fig. 3.22, p. 86). Once all the parameters have reached
stable values, the individual images, which were classified into corresponding class averages, are corrected. The success of the iteration can immediately be judged, as at the end of processing each class average, the program stores an image with the measured and (opposite to it) the fitted CTF; in this image, the Thon rings should fit one another. A gallery of individual power spectra, class averages and the fitted rings at various defocus and astigmatism values is shown in Fig. 3.2.1. The correlation coefficients obtained are, dependent on the number of single images and the signal strength in the data set, usually in the region of 60-95%.

The general applicability and precision of the CTF parameter-determination method were evaluated by using computer-generated test power spectral images with varying signal-to-noise ratios and B factors as shown in Fig. 3.20, p. 83. The high B factors used in the test make high-resolution Thon rings invisible. However, it turned out that the accuracy of the CTF parameter determination was not strongly dependent on the visibility of high-resolution Thon rings. The method also appeared quite robust with respect to variations of the SNR in the range of 10-0.1, with relative errors in the range 0.2-1.5% for the defocus determination and in the range of 5-10% for the astigmatism determination.

**Accuracy of the method:** The general strategy for finding local CTF parameters rests upon the retrospective estimation of the local power-spectral density, by means of which the square of the CTF can be made visible. The CTF parameters at a position on the image are analysed by Fourier-transforming extracted images of individual macromolecules and squaring the result. The smaller the pixel frame used, the more accurately it represents local conditions. At the same time, the signal-to-noise ratio at the level of the individual power spectrum becomes increasingly poor, and the local FFT\(^2\) results in a correspondingly worsening estimate of the underlying power-spectral density. For this reason, in procedures for defocus correction that are based upon the analysis of local power spectra, the attainable accuracy should be correlated with the size of the pixel frame: the larger the pixel frame, the better the signal-to-noise ratio in the power spectra - that is, the visibility of the Thon rings - becomes. However, if very large image excerpts or even the entire micrograph are used, then local differences in the CTF - arising, for example, through tilting of the sample - cannot be taken into account. Generally, the same is true for "native" cryo-preparations (compare section 2.2.4. p. 33) of individual macromolecules (here, ribosomes) embedded in vitreous ice in the holes on a perforated carbon film. In such cases, the signal in the power spectra is very weak, so that MSA analysis and classification of these power spectra usually fail. How-
Figure 3.18: Flow-chart for defocus correction. High-pass-filtered and excised power spectrums are subjected to multivariate statistical analysis (MSA), whereby the properties of the Thon rings are obtained as principal components of the data set. The class averages obtained from the classification with enhanced signal-to-noise ratios are used in the iterative determination of the parameters defocus, astigmatism, B factor, amplitude-contrast proportion and a noise function on the basis of the correlation coefficient with theoretical CTF functions. The parameters defocus and astigmatism, found in this way, are then used for correction of the single-particle images in each class.
Figure 3.19: Multivariate statistical analysis (MSA) of power spectra. (A) Twelve Eigenimages from the MSA of a typical data set of power spectra (size of the particle images, 1024 × 1024 pixels, corresponding to approximately 80 × 80nm in the sample; size of power spectra, 256 × 256 pixel). (B) Left, individual power spectrum; centre, class average of a class containing 50 images; right, visualization of the fit. Each picture on the right contains the result in the left-hand half and the class average in the right-hand half. On a PC with AMD MP1800+ CPU running under Windows 2000, a typical calculation of the CTF parameters in the 256 × 256-pixel frame takes about 3 min for each spectrum to be corrected. (C) As in (B) in a case where astigmatism appeared. The class average shown represents 57 images. (D) As in (B) with drift of the sample cryo-holder and directionally truncated Thon rings. The class average shown represents 42 images.
Figure 3.20: Assessment of accuracy of defocus and astigmatism as a function of power spectral SNR and B-Factor. The signal-to-noise ratio (SNR) of computed images (CTF convolution of central single white image point plus additive noise) and their corresponding power spectra was modified in the range of SNR 10-0.1. Visually, such test power spectra with defined defocus and astigmatism result in images with Thon rings ranging from clearly visible to barely visible at all. Power spectra representative for some of the SNRs and B-Factors used for this text were directly included in the plots. The power spectra were analysed by our software to determine the mean and standard deviation of the defocus and astigmatism depending on the SNR of the power spectra. As can be seen from the 3σ interval in the plots, the parameters for defocus and astigmatism were accurately found by the program, even in the case of very low signal-to-noise ratios. (A) B-factor-dependent accuracy of defocus determination. The B-factor was varied in the range of 0-100 Å². The defocus of the calculated test power spectrum was 10,000 Å and the value for the astigmatism was set to 200 Å. The image size used for calculations was 200 × 200 pixel at 1.6 Å/pixel. (B) B-factor-dependent accuracy of astigmatism determination. The B-factor was varied in the range of 0-100 Å². The defocus of the calculated test power spectrum was 10,000 Å² and the value for the astigmatism was set to 200 Å. The image size used for calculations was 200 × 200 pixel at 1.6 Å/pixel. (C) SNR-dependent accuracy of defocus determination. The SNR was varied in the range of 10-0.1. The defocus of the calculated test power spectrum was 10,000 Å and the value for the astigmatism was set to 200 Å. Again, the image size used for calculations was 200 × 200 pixel at 1.6 Å/pixel. (D) SNR-dependent accuracy of astigmatism determination. The SNR was varied in the range of 10-0.1. The defocus of the calculated test power spectrum was 10,000 Å and the value for the astigmatism was set to 200 Å. Again, the image size used for calculations was 200 × 200 pixel at 1.6 Å/pixel.
ever, the method still works in some special cases where either the concentration of molecules observed in the ice holes (simulated here by the lawn of U1 snRNP) is very high and/or macromolecular complexes with high molecular weight – such as icosahedral viruses – are imaged. In all other cases, the method cannot be used to determine the "local" CTF parameters of each macromolecule. Instead, however, the automatic fitting procedure can be applied to the average of overlapping power spectra from an entire micrograph, and the resulting "global" CTF parameters can then be applied to correct all molecular images of a single micrograph using the same CTF values. The accuracy of this global CTF parameter determination can be much improved by prior calibration of the zero-tilt position of the holder in the electron microscope (see below). Our method can be used to determine this zero tilt when it is applied to images that give an additional carbon-film signal.

To investigate the defocus variance between neighboring locations on micrographs with carbon support film as a function of pixel size, a micrograph measuring 8.3 × 10.2 cm was taken (50,000-fold enlargement, digitization with 4 μm, i.e., 0.8 Å per pixel at the level of the sample) and about 21,000 points were distributed on it at a uniform spacing of 128 pixels. Overlapping image areas with variously sized pixel frames were excised. Pixel frames of sizes 256 × 256, 384 × 384, 512 × 512, 640 × 640 and 800 × 800 pixels were used. The sizes of the power spectra after excision of the central region were 80 × 80, 128 × 128, 160 × 160, 200 × 200 and 256 × 256 pixels, each of which corresponded to a critical Nyquist frequency of about 5 Å.

The method described here was applied to this data set and the dependence of the defocus found upon the position on the negative was represented in a 3D plot (Fig. 3.24). The thickness of the layer of neighboring measurement points was used as a measure of the accuracy in determining the defocus attainable with a given pixel frame. While small image excerpts of 256 × 256 pixels (20 × 20 nm at sample level) cause high error scatters of more than 900 Å, a substantial reduction in the scatter of neighboring measurement points is possible with larger excerpts; for the largest pixel frames examined here (800 × 800 pixels; 64 × 64 nm at sample level) this lay below 200 Å. The cryo-negative-stain preparation of 70S ribosomes in a sandwich of ribosome-stain-water mixture between two carbon films used in the experiments has an estimated thickness of approximately 350 Å (2×50 Å carbon plus 250 Å ribosomes). The maximum accuracy of the defocus determination of 200 Å leads to the conclusion that the program detects an average defocus between the upper and lower carbon film layers. The defocus value determined for the particle thus lies somewhere in the central region of the sandwich and therefore
Figure 3.21: Detection of defocus via correlation measurements between predicted and measured power spectra. Correlation coefficient between theoretically calculated power spectra in the range of 6000 – 15,000 Å and the measured power spectrum of a class average with a defocus of approximately 11,000 Å. The defocus sought is revealed by a clear maximum, implying that the best possible correspondence between the class average and the power-spectral density reference is found at this point.

Intersects a plane through the molecule. Remarkably, the narrow scattering of the results allows the mounting of the preparation, including tilt of the sample holder and local distortion, to be visualized directly. In Fig. 3.24E an overall tilt of about 3° is seen, and this is ascribed to inaccurate calibration of the zero position of the holder. Furthermore, a bending of the foremost, left-hand edge can be seen. Especially with preparations on carbon film, bends and folds of this kind can be expected, because of the manipulation of the material with tweezers. For the sample-holder we use, the difference in focus that arises through the tilt of the sample is ~1000 Å and is thus very significant for the CTF correction in high-resolution investigations. Alternatively, the tilt angle of the holder can be measured and reduced to zero by suitable calibration. However, a slight tilt can also be desirable for high-resolution 3-D structure determination, as in this way the defocus multiplicity in the data set is raised and the zero-crossings in the CTF can thus be compensated for more easily.

Dependence of the B factor upon the conditions of measurement: The consideration of the B factor in the iterative calculation of CTF parameters in the present work is especially important because - as has been shown by tests with theoretically calculated power spectra - it allows a continuous convergence of the algorithm without the appearance of systematic errors due to deviation of
Figure 3.22: Quality of a theoretical CTF including amplitude contrast, B-Factor and unconvoluted noise fitted to the power spectrum of a high-pass filtered data image. The rotationally averaged power spectral density (arbitrary grey values) was plotted as a function of the spatial frequency. Dashed line: 1D plot of a rotationally averaged class average. Continuous line: 1D plot of the theoretical CTF found for this class average. There is good correspondence between the calculated and measured functions with respect to the location of the zero-crossings of the CTF as well as to the estimation of the unconvoluted background noise. The first maximum has a smaller amplitude than the following maxima, in consequence of the high-pass filtering of the single-particle images.

the measured power spectra near the Nyquist frequency in the theoretical CTF references. A similar effect could also arise through the use of a weighting function in the calculation of the correlation coefficient that takes less full account of the high-frequency regions in the power spectra (Tani et al. 1996). However, accurate knowledge of the experimental B factor (compare Fig. 3.23, p. 87) can in several ways be useful for the computation of the 3D structure of the particle: a correction of the B factor at the level of the individual particle, the class averages or the 3D density is necessary for the visualization of highly-resolved structural information (Böttcher et al. 1997). However, correction of the B factor immediately after particle selection, before the actual structure determination, seems in the author's opinion to be disadvantageous, for two reasons. Firstly, the B factor correction at the level of individual particles may lead to a signal-to-noise ratio that is too low for many of the algorithms in use in image-processing, resulting in extremely poor particle visibility and a very low image contrast, on account of the exponential increase in high-frequency noise at high spatial frequencies. Secondly, an upper frequency limit is necessary for B factor correction of single-particle images, after
Figure 3.23: Microscope-dependent signal decay as a function of spatial frequency using $B$ factors calculated from cryo-negative-stain micrographs of 70S ribosomes ins 2% uranyl formate (sandwich method). Values of $B$ factors obtained were: In the Philips CM120 microscope, 80 Å$^2$; in the Philips CM200-FEG, 28 Å$^2$; in the helium-cooled, superconducting objective lens of the microscope SOPHIE, approximately 17 Å$^2$.

which the correction can no longer be applied, as excessive noise amplification must be avoided. This frequency limit should ideally correspond to the resolution to be attained in the 3D reconstruction and is therefore not known a priori. A $B$ factor correction at the single-particle level can thus only be meaningfully carried out after a stable level of resolution has been reached, corresponding to the highest resolution attainable with the data set. Furthermore, the knowledge of local $B$ factors in the micrograph can be utilized in estimating the image quality as a function of the position of a particle (Gao et al. 200). The $B$ factors of a typical micrograph are plotted as a function of position in Fig. 3.25. A wedge-shaped zone with increased $B$ factors is seen; this may be due to local charging effects during flood beam microscopy. It was not the aim of this study to measure systematically $B$ factors under many different conditions using the methods described here. Nevertheless, we describe some significant results obtained on negatively-contrasted preparations under cryo-conditions (see also Table 3.2) and at typical defocus values between 0.5 and 1 μm, where the $B$ factor can be regarded as being largely independent of the defocus (Saad et al. 2001). The strong dependence of the $B$ factor upon the coherence of the electron source is known (Zemlin et al. 1994; Hewat and Neumann 2002). With the Philips CM200 electron microscope (Twin-Lens, Cs = 2.0 mm) and FEG-cathode, we measured a $B$ factor of approximately 56 Å$^2$.
Figure 3.24: Measurement of the accuracy to be expected in the defocus method dependent on size of the pixel window used for calculation of power spectra. Measured defocus values of closely adjacent points are shown in a 3D plot as a function of $x, y$-position in the micrograph. As enlarging the pixel frame results in an improved estimate of the underlying power-spectral density, the scatter of the measured defocus values of the points in the micrograph decreases correspondingly. (A) 256 × 256-pixel sections of the micrograph (20 nm × 20 nm on the scale of the sample). (B) 384 × 384-pixel sections (30 nm × 30 nm) with a scatter of 600 Å. (C) 512 × 512-pixel sections (40 nm × 40 nm). (D) 640 × 640-pixel sections (51 nm × 51 nm). (E) 800 × 800-pixel sections (64 nm × 64 nm). In (A) the scatter is more than 900 Å, and the tilt of the sample can hardly be seen. With the largest pixel frame used here, 800 × 800 pixels, the scatter resulting from the defocus values of closely adjacent neighbors is approximately 200 Å. Besides the deviation of the cryo-holder (about 3°) from the zero position, the foremost left-hand edge indicates an additional bending of the sample leading to an overall measured defocus gradient of about 1,000 Å in the micrograph.
in typical micrographs. A comparable preparation of 70S ribosomes on a single carbon film without stain gave a B factor of about 85 Å². The smaller value appears to be due to the use of stain together with the double carbon film. Another significant quantity is the ratio of the B factors obtained with a conventional liquid nitrogen-cooled electron microscope and those obtained with the liquid helium-cooled electron microscope SOPHIE (34 Å²), which was found to be about 1.6 when the techniques described were used. This significant difference shows that the use of helium-cooled microscopes may come to play a substantial part in improving the resolution of 3D structures (Fig. 3.23).

**Dependence of the amplitude-contrast proportion upon the preparation method:** The amplitude-contrast proportion leads to a shift of the Thon rings (especially the more central ones) in the direction of low spatial frequencies and thus to unambiguous changes compared with pure PhCTF. The mean amplitude-contrast proportions found under three different sets of preparation conditions are shown in Table 3.3. For a native cryo preparation of RNA-protein particles (human U1 snRNP particles) without stain or support film we obtained an amplitude-contrast proportion of approximately 6%. For a native preparation of 70S ribosomes on
carbon film we found an amplitude-contrast proportion of 9%. The amplitude-contrast proportion for the cryo-negative-stain preparation was, however, 14%, and thus about twice as large as in native cryo preparations (see Table 3.3). The values found here are in good agreement with values determined by other methods (Toyoshima and Unwin 1988; Toyoshima et al. 1993; Zhu et al. 1997).

<table>
<thead>
<tr>
<th>Microscope</th>
<th>electron source</th>
<th>acceleration voltage [kV]</th>
<th>$B$ factor (Å$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM 120</td>
<td>LaB$_6$</td>
<td>120</td>
<td>160±5</td>
</tr>
<tr>
<td>CM 200</td>
<td>FEG</td>
<td>200</td>
<td>56±2</td>
</tr>
<tr>
<td>SOPHIE</td>
<td>FEG</td>
<td>200</td>
<td>34±1</td>
</tr>
</tbody>
</table>

Table 3.2: B factors (cryo-negative-stain, double carbon film) as measured with different microscopes.

<table>
<thead>
<tr>
<th>Preparation method</th>
<th>Amplitude-contrast proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryo-negative-staining (sandwich)</td>
<td>14 ±3</td>
</tr>
<tr>
<td>Native cryo-preparation on carbon film</td>
<td>9 ±4</td>
</tr>
<tr>
<td>Native cryo-preparation in vitreous ice</td>
<td>6 ±2</td>
</tr>
</tbody>
</table>

Table 3.3: Amplitude-contrast proportions (Philips CM200, FEG-cathode) as measured for different preparation methods.
3.2.2 Development of corrimg-based exhaustive multi-reference alignment via polar coordinates and comparison to direct alignment

Aim of the work

Single-particle electron cryomicroscopy of asymmetrical macromolecular complexes is increasingly recognized as being powerful enough for structure determination at very high resolution (Henderson 1995; Jensen 2001; van Heel et al. 2000) However, the number of individual molecular images needed is large, and computational image processing is very time-consuming. It is still unclear how many images will be needed for structure determination at close to atomic resolution. However, it is obvious that in the presence of structural heterogeneity with respect to conformation and functional states, very high particle statistics are needed. In the present work, more than 66,000 images of [U4/U6,U5] tri-snRNP were selected to reach a resolution level of 19-24 Å. Various theoretical estimates have been made of the number of images that might be needed to reach true molecular resolution (Henderson 1995; Jensen 2001). By extrapolating from the current experience, one can expect that up to several hundred thousand images showing the particle (without internal symmetry) in the same conformational state may be needed for single-particle structure determination at molecular resolution. At least five parameters (two translational and three Euler angles) are required for a complete description of the individual images, and these have to be known for each image before a 3D reconstruction can be computed (compare section 1.2.4, p. 24). The need to determine these parameters is a consequence of the random orientation of the individual imaged particles and the variable displacement of the centre of the particle when it is picked out of the micrograph field. In the commonly used projection-matching technique, all these parameters are determined and refined by several iterations of a multi-reference alignment procedure (Penczek et al. 1994). The reference images used are projections of a preliminary 3D density map, evenly distributed over the unit sphere. Each raw image is shifted and rotated with respect to its best-matching reference image. In addition, the Euler angles of this best-matching reference are assigned to the aligned image. The aligned images are then used to compute a new 3D reconstruction, from which a refined set of references can be calculated (compare section 1.2.4, p. 26). It is to be expected that, in the course of several alignment iterations, the noise of the reference images will lessen, and improved accuracy of the alignment parameters will ultimately be obtained. The
multi-reference alignment of thousands, or even hundreds of thousands, of particles is the most time-consuming step in the image-processing procedure. Aside from large numbers of images, a fine angular spacing of the reference images may be needed for high-resolution work. A given multi-reference alignment using an angular spacing of $3^\circ$ between the reference images may thus utilize, e.g., $\sim 250,000$ raw images (each measuring $128 \times 128$ pixels) that have to be aligned against a set of $\sim 4500$ reference images. On the Compaq Alpha EV6 500 MHz computer which was commonly in use for MRA computations when the author first faced the problems of limited computer power in 1999, equipped with four CPUs and a standard installation of the IMAGIC-5 software package (van Heel et al. 1996), a single iteration would have taken about two years. Today, standard personal computers are much faster than the famous EV6. Nevertheless, it is obvious from this estimate that increasing computational power must be combined with improvements in software if such computationally demanding tasks are to be made feasible on a reasonable time scale.

**General idea for reduction of alignment computations**

An increase in computational efficiency has so far been achieved by the use of algorithms for parallel processors on so-called "computer farms" (van Heel et al. 2000). Here, the approach of "corrim-based alignment", that greatly increases the speed of iterative alignments, is presented. Furthermore, its implementation on a PC cluster consisting of up to 68 AMD processors is described. In the present study, two different algorithms for the alignment of single image-reference pairs were applied: "direct alignment" by separate translational/ rotational searches and exhaustive alignment using resampling to polar coordinates (compare section 1.2.4, p. 19).

In both multi-reference alignment procedures described here, Pearson's real-space correlation coefficients are computed for all image-reference pairs after completion of each individual alignment operation. These correlation coefficients are required to compare the results of the alignments of a single image against all references. The highest correlation coefficient determines the best-matching reference for any given particle image. The resulting alignment parameters are used to assign the shifts and Euler angles to the particle image. In the course of several alignment iterations, it is obviously desirable to avoid unnecessary time-consuming alignment calculations that involve reference images exhibiting reproducibly low correlation coefficients. For any given individual particle image, this can be achieved by re-
stricting the iterative alignments to those references with known high correlation coefficients only. Under realistic conditions, a refined alignment restricted to the best matching reference of any given particle image only, is not sufficient. Therefore, when performing a first multi-reference alignment, we record the correlation values of each molecular image to the entire set of references. The set of correlation values of any given raw image is used to calculate an interpolated cross-correlation image, a so-called "corrim". The idea of corrims was first developed in the author's "Diplom" thesis in 2000, and is characterized here with respect to accuracy and implemented for different alignment algorithms.

In the corrim-based refinement iteration of MRA, alignment operations have to be computed with respect to those references in regions of high correlation coefficients only. The fraction of references to be omitted is adjusted by a threshold correlation value specified by the user. The performance and accuracy of corrim-based alignment were tested for both of the alignment algorithm types named above.

**Procedure and findings**

In the extension of the MRA algorithm presented here, the calculated correlation coefficients between all pairs of reference images and raw images are stored in a normal iteration of the multi-reference alignment, and their values are then represented by a spline interpolation as a 2D function of the Euler angles in a diagram, the ordinate of which represents the $\beta$ angles and the abscissa the $\gamma$ angles. The Euler angles were denoted according to Fig. 1.8, p. 25. The grey value at any point of such a cross-correlation image (corrim) can be interpreted as a measure of the probability with which a particle image can be assigned to a reference projection corresponding to the Euler angle defined by this point. The grey level thus determines whether or not the alignment to a specific reference image is computed in the subsequent MRAs. For this purpose a threshold value for the corrim is defined. Only when the threshold value has been exceeded is the alignment of the raw image and the corresponding reference image computed; otherwise, the program moves on to the next reference image. The threshold value is defined in terms of the mean and standard deviation of the grey values in the corrim, leading to a normalization of grey values. Prior to the simulations shown here, a sensible threshold parameter was determined empirically in multi-reference alignments of real data sets (the splicing factor SF3b (Golas et al. 2003)). It turned out that a conservative threshold of $\sim1.2$ times the standard deviation above the mean density of the corrim led to the computation of practically identical 3D densities compared
to a standard MRA. By omission of alignment operations that are not needed, a significant reduction in processor time was achieved.

The multi-reference alignment presented here does not strongly depend on the kind of interpolation used in the calculation of the corrims. However, spline interpolations in this case are suitable to give the user the possibility to choose the size of the corrims freely and independently of the underlying angular distance of the reference images. The spline condition used here for interpolation of correlation values, which fulfils the conditions of a monotonic first and a continuous second derivative, is given by (Press et al. 1997):

\[
\frac{x_j - x_{j-1}}{6} y_{j-1}'' + \frac{x_{j+1} - x_{j-1}}{3} y_j'' + \frac{x_{j+1} - x_j}{6} y_{j+1}'' = \frac{y_{j+1} - y_j}{x_{j+1} - x_j} - \frac{y_j - y_{j-1}}{x_j - x_{j-1}}
\] (3.5)

The 2-D interpolation in the corrims is achieved by performing two successive steps of 1D interpolation that are orthogonal to one another. The first of these interpolations takes place between the projection angles at constant \( \beta \) and variable \( \gamma \), that is, along a latitude line of the unit sphere. The second interpolation is then carried out in the longitudinal direction and always involves the correlation values measured for the two poles of the unit sphere as starting and finishing point, respectively. For \( n \) points \((x_j, y_j)\) to be interpolated (where \( j = 1, ..., n \)), Eq. 3.5 leads to a system of \( n - 2 \) equations with \( n \) unknown variables, so that two additional conditions must be fulfilled for a unique solution to exist. For the longitudinal direction of interpolation, unambiguous definition of the system of equations is achieved by arbitrarily setting \( y_1'' \) and \( y_n'' \) (the second derivatives of the spline curve measured at \( \beta = 0^\circ \) and \( \beta = 180^\circ \) to zero (the so-called natural cubic spline). In the case of the cyclic spline interpolation used along a latitude line the two necessary additional conditions are given by taking account of the immediate neighborhood of the first and last points of measurement:

\[
\frac{x_1 - x_n}{6} y_n'' + \frac{x_2 - x_n}{3} y_1'' + \frac{x_2 - x_1}{6} y_2'' = \frac{y_2 - y_1}{x_2 - x_1} - \frac{y_1 - y_n}{x_1 - x_n}
\] (3.6)

\[
\frac{x_n - x_{n-1}}{6} y_{n-1}'' + \frac{x_1 - x_{n-1}}{3} y_n'' + \frac{x_1 - x_n}{6} y_1'' = \frac{y_1 - y_n}{x_1 - x_n} - \frac{y_n - y_{n-1}}{x_n - x_{n-1}}
\] (3.7)

Taking these additional conditions into account, the system of equations can be represented as a cyclic tridiagonal matrix with two additional boundary values \((\alpha, \beta)\) which can be solved efficiently by existing algorithms (Press et al. 1997):
\[
\begin{pmatrix}
  b_1 & c_1 & 0 & \ldots & \beta \\
  a_2 & b_2 & c_2 & \ldots & \\
  \vdots & \vdots & \vdots & \ddots & \\
  \alpha & \ldots & 0 & a_n & b_n \\
\end{pmatrix}
\begin{pmatrix}
  x_1 \\
  x_2 \\
  \vdots \\
  x_n \\
\end{pmatrix}
= 
\begin{pmatrix}
  r_1 \\
  r_2 \\
  \vdots \\
  r_n \\
\end{pmatrix}
\]

(3.8)

The result of the cyclic interpretation is a corrim, the left and right edges of which merge seamlessly into one another (Fig. 3.26, p. 97). In the interpretation of the corrims, a distortion in the vicinity of the poles of the unit sphere can be discerned when the surface of the unit sphere is projected onto the plane. However, this distortion does not impede their function. Furthermore, the natural tendency of spline interpolations to oscillate, which can sometimes be seen in the vicinity of lighter regions, has not been found to have any disturbing influence upon the practical application in multi-reference alignment.

**Preparation of the test data:** The goal of this study was to demonstrate the use of the method by applying it to a set of constructed test images that were as similar as possible to experimental electron-micrographic data. For this purpose, PDB data for the bacterial ribosomal subunits (50S, PDB entry 1FFK (Ban et al. 2000); 30S, entry 1FJG (Carter et al. 2000) and the [EF-Tu.aa-tRNA.GTP] ternary complex (PDB entry 1MJ1 (Stark et al. 2002)) were used to model a ribosome, and this was transformed into a 3D density map by using the software package IMAGIC (van Heel et al. 1996). To define the spectral signal decay, first, amplitudes in Fourier space were normalized by multiplication with the corresponding inverse rotational average, so that a signal strength independent of spatial frequency was obtained and the high spatial frequencies could be reduced by a Gaussian filter in an exactly defined manner. The model measured 256×256×256 pixels (1 pixel corresponding to 1.6 Å), whereas the alignment tests were carried out in (binned) 128×128-pixel windows. The pixel window was chosen such that the particle's largest diameter made up about two-thirds of the diameter of the window. After Gaussian filtering of the amplitudes, the 3D model had a signal that had decreased by a factor of 1/e at 12.8 Å and was set smoothly to zero at 4.5 Å (0.7 times the Nyquist frequency), as otherwise reconstruction artifacts of the 3D algorithm used may appear in the region of very high spatial frequencies. To generate a model that includes the dark edges that are seen both around computed 3D reconstructions and around 2D class averages, a heavily low-pass-filtered version of the 3D model was added to the unfiltered model: the tests described below were performed both with and without
this additional component of low spatial frequencies, in order to investigate to what extent the results are influenced by their presence. The 3D structure thus obtained was first projected with Euler angles that had been obtained by a random-number generator (Prime Modulus Multiplicative Linear Congruential Generator from the Compaq Fortran software-development package) to simulate projection images in the EM with random angular distribution. These projection images were then coarsened by averaging of 4-pixel fields, so that the 256×256-pixel images could be binned to a 128×128-pixel frame, simultaneously altering the critical Nyquist frequency to 1/6.4 Å⁻¹. The projection images generated by the software IMAGIC were then temporarily padded with zeros in a 256×256-pixel frame, so that there would be sufficient room for the broadening of points when convoluted with a CTF function based on a defocus of 10 000 Å, an assumed acceleration voltage of 200 kV and a spherical aberration of the electron lens of 2.0 mm. Finally, to simulate defocus-corrected particle images, the images were subjected to defocus correction, so that images with positive phases over the entire frequency interval and zero crossings corresponding to the defocus were received. The images were then rotated and displaced randomly, to simulate the random distribution of the particles on the micrograph. The imposed rotation angles were in the range of 0-360° and the imposed radial displacements were up to 12.8 pixels (i.e., ~15% of the greatest particle diameter). To simulate various SNR values, a random-number generator was used to produce white Gaussian noise with a bell-shaped grey-value distribution, calculated as described (Ross 1997); the noise was added to the particle images. Taking into account the fact that most of the noise in the EM field is convoluted by a CTF, calculated noise images were overlaid with a CTF with the same parameters as for the test-image production. Thereafter, a low-pass filter was applied to the noise images, to simulate the - normally frequency-dependent - signal decay. The filter was chosen such that at 8 Å - i.e., later than for the projected images - decrease to 1/e was reached, whereby at the same time the decrease in the spectral SNR expected in practice was also taken into account. Both the calculated particle images and the noise images were normalized in respect of their variance, so that defined SNR were obtained (Fig. 3.27): the SNR was defined as the quotient of the variance of the noise-free image and the variance of the additive noise (Frank and Al-Ali 1975). By tests with experimental noise obtained from images of a carbon film in the EM, it was confirmed that there were no significant differences between the (more easily varied) calculated noise and the experimental noise. The size of the test-image data sets to be aligned lay
between 500 and 5000 images and was chosen, on the basis of preliminary tests, so as to be large enough to yield highly reproducible results, with negligible scatter, when applied with various randomly-chosen parameter values and various noise values. Reference images needed for all tests described in this paper were likewise calculated from the 3D density maps generated by projection using the software IMAGIC-5 (see above). The angular separation between the reference data sets was chosen to be between 30° and 4°, so that reference image sets contained between 46 images (angular separation 30°) and ~2500 images (separation 4°).

Conduct of the tests: The test images to be aligned, and the reference images, were generated as described above and a multi-reference alignment was carried out, so three Euler projection angles and two translational parameters were given to each simulated raw image by assigning it to the best-fitting reference image. The evaluation of these data was intended to answer the question of how the overall alignment error, resulting from the sum of individual errors, influenced the 3D reconstruction. Back projections of single points (see below) in 3D space were performed and the point-spread function resulting from the overall error was evaluated by a method similar to that of (Jensen 2001). Alignment errors can arise (i) through a deviation of the angle, assigned in the alignment, between the reference
image and the aligned image, and (ii) through errors in the translation and rotation parameters found; the effects of these two factors influence each other. Therefore, a 3D density map (128×128×128 pixel) was generated with a single image point that laid 40 pixels from the centre of the 3D model (about 130 Å at the level of the constructed model – i.e., at the edge of the particle, where errors in the rotational parameters should have their greatest effect). This 3D model was projected in all projection directions that had originally been assigned to the test image data set. These “point projections” were then displaced/rotated by amounts corresponding to the respective differences between the imposed translational/ rotational parameters and those found in the alignment. Furthermore, the Euler angles found for the test images in the multi-reference alignment were assigned to the corresponding “point projections”. Afterwards, the set of displaced point projections was back-projected into 3D space. The combination of all alignment errors caused a blurring of the point in the 3D density. To analyze this blurring, 3D rotational averaging around the expected position was performed. By fitting the average grey values thus obtained to a decaying Gaussian function, the magnitude of the grey value at the origin voxel and the half-width of the signal decay were determined. The magnitude of the grey value was then compared with the highest possible grey
value obtainable under ideal alignment conditions (usually found at SNRs greater than 0.3). It should be noted that the projection images of single points used for determining the envelope functions were set to be noise-free, so that any dependence of the envelope function on the number of particle images in the data set was avoided. In practice, the number of particle images that go into the calculation of a 3D structure is naturally of great importance (Henderson 1995). In consequence, the simulation experiments described here led to envelope functions that reflected the precision and significance of the results independently of the number of particle images used; that is, a larger number of particles in the simulation merely had the effect of reducing the scatter in the results.

The influence of the corrims-based refinement on the computing time for multi-reference alignment: The speed with which an algorithm can be implemented on a given computer system depends upon many factors, and bottle-necks can arise at various points – for example, the transfer of data from the mass storage medium into the main memory of the computer (I/O time) or, especially with parallel processing, the transfer of data through the computer network. The option presented here for rapid refinement of 3D models by multi-reference alignment makes very high demands on the computer, and it can only convey an advantage in terms of time if the system is able to transport the necessary data – such as reference- and particle images – in and out of the computers used in a time less than the time required for processing. Furthermore, it is theoretically possible that a refinement’s advantage in terms of speed can shrink if the processor time required for the individual alignment of an image against a reference drops below a certain threshold. In such a case, a further reduction in the number of computing operations carried out would reduce the load on the processor but not the computing time. The attainment of a system-specific limit of this kind is, for the algorithms investigated, especially probable for alignment by separate searches, as this alignment is in itself much more compact than the exhaustive methods. To prevent the appearance of such effects with the use of corrims in alignment by separate searches, in the present work all available programming strategies were adopted to eliminate bottlenecks. So far, computational efficiency was enhanced mainly by establishing a memory buffer, which provided a high number of references preventing interruptions by I/O operations (van Heel et al. 2000). In addition, a similar buffer was set up for subsets of the particle images. These buffers led to more efficient data-package sizes to be transported across the network (100 Mbit) and decreased the frequency of transmissions, so that “data queues” were avoided. To
perform the 2D and 1D Fourier transformations, the FFTW algorithm (Friso and Johnson 1998) was used, and routines for the translation/rotation of images and for the calculation of polar coordinates were optimized for the type of processor (AMD Athlon). For parallel computation on many processors, the software library NT-MPICH (http://www.lfbs.rwth-aachen.de/mp-mpich) was used. As shown in Fig. 3.28, the alignment by separate searches – which, when used with refinement by corrim, represents the most unfavorable case with respect to I/O and network resources – proceeded at a rate that rose approximately linearly with the number of processors (up to 65 processors). For the refinements shown here, with a reasonable corrim size of 64×64 pixels, an approximately 8-fold increase in speed over conventional alignment was reproducibly obtained.

Characteristic appearance of corrim in dependence upon the SNR: Sharp peaks of correlation can be obtained with ideal (simulated) particle images and high signal-to-noise-ratios (compare Fig. 3.29, p. 102). In real EM data sets, multiple correlation peaks may occur. This can have many reasons, including conformational inhomogeneity of the sample, local variation in quality of the exposure (Gao et al. 200), differences in the amount of irradiation damage from image to image, low SNR values depending on the size of the imaged particles, characteristics of the surrounding medium (salt concentration, carbohydrates) and other factors. The characteristic change in appearance of the corrim with decreasing SNR is shown in Fig. 3.29. In the alignment using resampling to polar coordinates, the transition from a single, clearly visible peak to multiple peaks occurs at lower SNR than in direct alignment. It can differ from one image to another. At SNR values below 0.04 (that is, for images in which the particles are scarcely visible; see Fig. 3.27) multiple peaks occur regularly. None the less, the simulation experiments shown here demonstrate that the alignment still functions well enough to produce meaningful results. The threshold for the transition to random alignment results thus lies at significantly lower SNR values (~0.004-0.007) than does the threshold for the transition from unambiguous correlation peaks to multiple peaks.

Characterization of the multi-reference alignment by signal strength and the half-width of a reconstructed voxel: As expected, the accuracy of the alignment parameter determination was dependent on the SNR. Interestingly, a decrease in SNR primarily reduced the maximum grey value of a reconstructed point, while the half-width did not depend strongly on the SNR (Figs. 3.30A and 3.30B, p. 104). An increase in the number of reference images improved the signal strength
Figure 3.28: Evaluation of corrims-based MRA on a computer cluster with 68 nodes. (A) Illustrative view of the PC-based computer cluster with 68 nodes used for this test as well as for all 3D structure computations. (B) Scalability of the refinement described here in the parallel version on 1-64 processors (AMD Athlon MP 2000+, Windows 2000 Prof., MPI-Software: NT-MPICH). Refinement of 20,000 test images measuring 128×128 pixels against 1650 reference images using corrims of 64×64 pixels and the fast direct alignment by separate rotational and translational searches. Four of the processors of the type described performed the alignment in 5 h 57 min, while 64 processors required 23 min 30 s for the task. The reciprocal computing time in min⁻¹ is plotted against the number of processors. In this example (which puts a high load on the network and I/O processes), the speed is almost proportional to the number of processors used. Only above 55 processors can a slight downward deviation be detected, which, however, appears not to be related to the increasing load upon the system, but rather to the initial distribution of reference images among the in-core buffers of the processors. C. Illustrative view inside a water-cooled client PC containing two processors.

similarly to an increase in SNR (Figs. 3.30C and 3.30D, p. 104). Furthermore, a relatively narrow transition was observed from (in principle) correct functioning of the alignment to random-like results. This transition appears to be specific for the algorithm used; it occurs at lower SNR values, and more abruptly, for alignment by polar coordinates (0.004) than for alignment by separate searches (0.007-0.01, see Fig. 3.31A, p. 105). As described above, test images with various proportions of low spatial frequencies were generated, thus simulating the situation frequently encountered in practice, e.g. as engendered by the use of different microscopes. It emerged that the result shown in Fig. 3.31A was in principle independent of the ex-
Figure 3.29: Appearance of the corrimas as a function of SNR. (A) Appearance of the corrimas as a function of SNR, as obtained in direct alignment by separate rotational and translational searches. A characteristic transition from unique to multiple correlation peaks can be observed with decreasing SNR. The individual SNR value where this transition occurs may differ from one image to another. At SNR values below 0.05, multiple peaks occur regularly. (B) The same as (A), but obtained in alignment by polar coordinates. Note that the transition from an alignment that in principle is still correct to an alignment that is largely random takes place at lower SNR values than does the transition from unambiguous correlation peaks to multiple peaks.
act form of the spectral SNR curves. The appearance of a sharp boundary between
conditions leading to correct and arbitrary alignment parameters confirms the re-
results of a recent study in two dimensions (Joyeux and Penczek 2002) and shows
that its findings are equally applicable to multi-reference alignment and demon-
strable in 3D space. In all the simulation experiments described here (Figs. 3.30
and 3.31), the measured signal strength obtained was found to be the parameter
that reacted most sensitively to changes in the alignment parameters such as SNR
or the angular spacing of the reference images. It is clear that the signal strength,
as measured, represents a complex function of the exactness of the alignment and
the ratio between (in principle) correctly aligned and randomly misaligned images.
In image-processing this signal strength would usually correspond to a subjective
term such as “brilliance” or “intensity”; for the refinement of a 3D model to the
highest possible resolution it is more important that, with growing signal strength,
an increasingly significant signal appears above the level of random noise, which
leads ultimately to convergence of the structure model and, in turn, of the orien-
tation parameters. The considerable increase in speed introduced by the corrim
based alignment makes it possible to enhance the signal intensity by using larger
data and reference sets, without incurring the disadvantage of longer computation
time. Thus, a relatively small data set of approximately 180 reference images (an-
gular separation \(\sim15^\circ\)) can be used to calculate the corrims and then, on the basis
of this alignment, larger reference sets can be used. As Figs. 3.31B and 3.31C
show, the signal strength measured over a wide range of SNR is comparable with a
conventional alignment against a larger reference set (\(6^\circ, \sim1100\) reference images),
whereby in the alignment with separate searches (using a threshold of 1.2 times
the standard deviation above the mean density of the corrim) some 70 – 90 % of
the signal of a conventional alignment with 1100 reference images is reached, while
with alignment using polar coordinates practically identical signal strengths (i.e.,
\(\sim100\%) are reached over the entire range of SNR investigated.

**Use of corrims for assessment of image quality:** Visually one can distinguish
on the one hand side corrims with unambiguous correlation peaks and on the other
hand side a more or less “random” progress of correlation with many peaks of
nearly the same height. As shown here, multiple peaks occur as a result of very
low SNRs. To the author’s experience, multiple peaks are also often observed
when the corresponding images exhibit useless content such as ice crystals, dirt,
disintegrated or aggregated particles. In these cases, the data image information is
not reflected by any of the reference images, thus no distinct peak can be observed.
Figure 3.30: Behavior of multi reference alignment via direct alignments and via polar coordinate alignments. The overall error after 3D reconstruction is made visible by superposing the measured individual errors upon projections of a single point (the 3D model of this individual point was reconstructed as described in the text). (A) Influence of the SNR when alignment by separate searches is performed (5000 images, 286 reference images separated by 12°). The signal in the reconstruction of a single point obtained at SNR = 1 was arbitrarily assigned a signal strength of 1.0. The half-width in voxels, after rotational averaging about the expected point position, is shown in each case directly to the left of the maximum of the curve. The abrupt transition from a significant to a random-like result at an SNR of about 0.007 is illustrated by the 3D reconstructions of the reconstructed test point, shown in cross-section on the right. (B) As in (A), but with alignment via polar coordinates. The transition from a significant to a random result is seen here at an SNR of 0.004. (C) Influence of the size of the reference data set with alignment by separate searches (1500 images; SNR = 0.04). Between 128 and 2582 reference images were used in MRA, corresponding to angular separations from 18° to 4°. Both the sharpness of the reconstructed image point — measured by the half-width of the rotationally averaged grey values — and, in particular, the signal strength that was obtained in alignment against reference images with an angular separation of 4° was arbitrarily set to 1.0. (D) As in (C), but with alignment performed by resampling to polar coordinates (1000 images; SNR = 0.04). The FWHM of the reconstructed test point is significantly smaller in case of alignment via polar coordinates compared to conventional direct alignment. This results underlines the suitability of alignments via polar coordinates for high-resolution work.
Figure 3.31: Accuracy of fast corrim-based MRA for small Euler angle refinements (angular separation 6°). A. The greatest signal strength obtained in (A) and (B) as a function of the SNR in direct alignment by separate rotational and translational searches and in exhaustive alignment via polar coordinates. Both curves show plateaus over a wide range of SNR and collapse at low SNR values of zero; there is a region of low signal in which alignment by polar coordinates produces a significantly higher signal than that by separate searches. (B) Use of corrim alignment by separate searches. First, an alignment with 194 reference images (angular separation 15°) was performed. Corrims were calculated from the correlation coefficients obtained, and the signal strengths were plotted as in (A) (lower hatched line). These then served to restrict the set of reference images in a subsequent refinement step with 1146 reference images (angular separation 6°) so that an 8-fold acceleration of the computation was achieved in comparison with conventional alignment (signal strength: upper hatched line). The unbroken line shows the results of a conventional alignment in which the test images are oriented with respect to all 1146 reference images without use of the refinement option via corrim. The closeness of the two uppermost curves shows that the refinement option has worked well, giving a result only marginally worse than that of a conventional alignment with 1146 reference (6°). The greatest signal strength that can be attained by conventional alignment against 1146 reference images was arbitrarily set to 1.0. (C) As in (B), but with alignment by polar coordinates. The comparison of the two uppermost curves shows that there is practically no difference between the conventional alignment and the refinement by corrim.

When automatic particle selection software is used, it is often unavoidable that a certain percentage of the data images will this kind of useless information and not contribute any particle information. The most commonly applied technique for exclusion of these images is based on the maximum cross-correlation coefficient measured in the MRA alone: a certain percentage of images is excluded from the calculation of the 3D structure based on a user-defined correlation threshold. Alternatively, the grey value histogram of the corrim can be analyzed. Images with corrim whose histograms contain high numbers of bright pixels (and low numbers of dark pixels) are indicative for occurrence of multiple peaks, can be excluded (compare Fig. 3.32, p. 106). For the ~124,000 images of tri-snRNP prepared under native cryo conditions (compare section 3.3, p. 108) 33 % (specified by the user) of the images were excluded from 3D structure refinement, as the corresponding corrim histograms were most indicative of multiple correlation peaks.
Figure 3.32: Corrims for assessment of image quality. (A,B) Corrims were computed for \(~124,000\) images of tri-snRNP prepared under native cryo conditions (compare section 3.3, p. 108) after alignment against 178 reference images separated by \(15^\circ\). Images corresponding to corrims with unambiguous correlation peaks shown in (A) were selected for further image processing, whereas those exhibiting multiple random correlation peaks (B) were excluded. (C,D) A further set of corrims with unambiguous correlation peaks as opposed to corrims with multiple random peaks for illustrative purposes. The corresponding images are icosahedral viruses (TBSV); therefore the 60-fold symmetry had to be considered for computation of the corrims, and even the unambiguous peaks occur multiple times.

**Capability of polar coordinate alignment to compensate for effects of erroneous intermediate reference images:** The more precise performance of exhaustive alignment using resampling to polar coordinates raised the question whether this type of alignment was able to compensate the bias from typical errors contained in intermediate reference images like false “blobs”. As shown in Fig. 3.33, p. 107, 200 noisy test images were generated based on a typical projection view of the 70S *E. coli* ribosome. However, for alignment of the images not the underlying noise-free projection view as shown in Fig. 3.33A was chosen, but an almost identical reference image with a false “blob” added at the outer portion of the 30S ribosomal subunit. When the results after averaging all images are compared for direct alignment (Figs. 3.33D-F) and alignment via polar coordinates (Figs. 3.33G-I), the direct alignment was obviously more susceptible to reproducing the false reference image than the alignment via polar coordinate. In consequence, alignment via polar coordinates was exclusively used for alignment of the data sets ([U4, U6, U5] tri-snRNP, [U4, U6] di-snRNP, U5 snRNP) in this study.
Figure 3.33: Consequences of errors contained in reference images. (A) Ideal computed projection image generated from crystallographic ribosomal data as described above (compare Fig. 3.27, p. 98). (B) Reference image corresponding to the projection view shown in A. However, a false “blob” has been added at the outer portion of the 30S ribosomal subunit. (C) Noisy data image. A data set of 200 noisy particle images was generated from the image shown in (A) by addition of white Gaussian noise followed by a common band-pass filter. The SNRs were 1/8 – 1/15, i.e. barely visible particles like in many critical real world applications. (D-F) Averages of the 200 images after exhaustive alignment via polar coordinates to the reference shown in (B). Almost no bias was introduced by the error contained in the reference image. (G-I). Averages of the 200 images after conventional direct alignment. Alignment errors have occurred leading to a clearly visible reproduction of the false “blob”. Thus, exhaustive alignment via polar coordinates can be expected to be less susceptible to errors contained in intermediate reference images during the refinement of image alignment parameters.
3.3 Structural analysis of the tri-snRNP and its subunits

3.3.1 2D electron-microscopic analysis of the snRNPs [U4/U6.U5], [U4/U6] and U5

The work presented here aims at establishing reliable 3D models for the tri-snRNP particle [U4/U6.U5] as well as its subunits, the [U4/U6] di-snRNP and the U5 snRNP at different salt concentrations. The first important step in de novo image processing was the characterization and description of the main 2D projection views of the particles. Therefore, all particles were initially prepared using the “sandwich” negative stain method in combination with uranyl formate stain, as this preparation method delivers the highest particle contrast of all methods that were evaluated for snRNP particles. Particularly, preparation methods using just one carbon film or other stain solutions did not produce sufficient contrast for further image processing and reliable characterization of the typical projection views. Additionally, the risk of aggregation of the particles and the concentration needed for visualization of the
particles was the lowest observed among all preparation methods.
All particle images were taken using a CCD detector at settings that have been
tested with respect to suitability for de novo analysis (compare section 3.1.1, p. 40):
combinations of magnification of the electron microscope and pixel binning were
chosen that yielded about twice the contrast (in the important range of 20 – 30
Å resolution) than to conventional black & white film (for a summary of all tech-
nical data with respect to image acquisition, compare Table 2.1 on p. 37). A few
thousand particle images were selected manually from the raw images and under-
went the "reference-free" alignment procedure described in section 1.2.4. Typical
micrographs of the preparations as well as the main, reproducible 2D projection
views are shown in Fig. 3.34. As depicted in Fig. 3.34A, the tri-snRNP shows
a characteristic triangular appearance with a maximum dimension of ~300 Å. In
the upper left part of each class average, a characteristic domain ("head" domain)
separated by from the main body by a cleft can be observed. The maximum di-
diameter of the particle perpendicular to the main axis is ~200 Å in the region of
the "head" domain. The U5 snRNP particle under low salt conditions (Fig. 3.34B)
is significantly smaller with a maximum dimension of about 280 Å and exhibits a
more roundish appearance with a characteristic globular protrusion at the bottom
that is similar in size and shape to the bottom part of the tri-snRNP shown in
Fig. 3.34A. In the upper part of the U5 snRNP particle, dark pixel density values
are observed that seem to separate the particle into a "head" domain and a much
larger "body". When the U5 snRNP particle is visualized at high salt conditions
Fig. (3.34C), particles of similar maximum dimensions can be observed. However,
much less high-resolution details are visible in the class averages of U5 under high
salt conditions compared to U5 under low salt conditions as a first indication for
a higher degree of structural heterogeneity in the sample. Es expected from the
protein composition, the [U4/U6] di-snRNP is much smaller than U5 and the tri-
nsnRNP (Fig. 3.34D). Its maximum dimension is approximately 190 Å. In both class
averages, two domains of continuous density are visible that are connected by thin
bridge densities. The larger domain of the two (in the left upper part of the class
averages) exhibits an accumulation of stain in the centre, whereas the smaller do-
main (lower right part of the class averages) appears to be more compact and does
not show fine structural details.
3.3 Structural analysis of the tri-snRNP and its subunits

Figure 3.35: 3D appearance of the [U4/U6.U5] tri-snRNP, [U4/U6] di-snRNP and U5 snRNP based upon RCT/ML. (A) [U4/U6.U5] tri-snRNP 3D structure obtained after 10 rounds of iterative alignment/weighted averaging using 46 selected MSA-based class averages with \( \sim 15 \) members per class. The data set for computation of the 3D RCT densities comprised 2607 image pairs. (B) U5 snRNP 3D structure obtained after 10 rounds of iterative alignment/weighted averaging using 56 selected MSA-based class averages with \( \sim 21 \) members per class. The data set for computation of the 3D RCT densities comprised 1569 image pairs. (C) [U4/U6] di-snRNP 3D structure obtained after 10 rounds of iterative alignment/weighted averaging using 118 selected MSA-based class averages with \( \sim 11 \) members per class. The data set for computation of the 3D RCT densities comprised 3866 image pairs. The scale bar corresponds to 10 nm.

3.3.2 Computation of initial 3D models via the RCT/ML method

For all samples shown in Fig. 3.34, sets of typical projection views were established by the “reference-free” alignment scheme including multiple MRA and MSA followed by HAC and computation of class averages. All data sets were exclusively aligned by the exhaustive alignment via resampling to polar coordinates, as it could be shown that this algorithm is less susceptible to artifacts contained in intermediate references than the standard “direct alignment” (compare Fig. 3.33, p. 107).

For the [U4/U6] di-snRNP, 380 MSA-based class averages were computed from 3866 untilted images, and 118 class averages were manually selected for further processing. In case of the U5 snRNP particle at low salt conditions, 75 MSA-based class averages were computed from 1569 untilted images and 56 class averages
were manually selected for further processing. In case of the U5 snRNP particle at high salt conditions, 350 MSA-based class averages were computed from 5973 untitled images and 84 class averages were selected for further processing. For the [U4/ U6. U5] tri-snRNP particle, 180 MSA-based class averages have been computed from 2607 untitled particle images, and 46 class averages were selected for further processing. Additionally, 6232 particle images of tri-snRNP from a frozen 25S glycerol gradient fraction were recorded. All selected class averages of each particle were then “converted” into 3D RCT reconstructions as described (section 1.2.4 and iteratively combined by the maximum-likelihood based 3D weighted averaging procedure described in section 3.1.2. For the [U4/ U6. U5] tri-snRNP particle, the [U4/ U6] di-snRNP particle and the U5 snRNP particle at low salt conditions, the main conformation of the respective particle was revealed, while nonsense RCT densities were weighted down. In case of the U5 snRNP particle at high salt conditions, no RCT/ML 3D model with distinct structural features could be obtained as many conformations with global differences in particle shape occurred in the data set with similar probability. Therefore, U5 snRNP particles were analysed by 3D MSA in addition to RCT/ML (compare section 3.1.2, p. 59) as described below (section 3.3.4). The initial 3D models thereby independently obtained are shown in Fig. 3.35. These models were then used as startup reference for refinement of the 3D structure using the untitled images.


The initial model of [U4-U6. U5] tri-snRNP obtained by RCT/ML was used for refinement of the structure via projection-matching (compare section 1.2.4, p. 26) – first, based on the untitled negative stain images which were corrected for defocus and astigmatism as described in section 3.2.1 on p. 74. It was then possible to obtain images of the [U4-U6. U5] tri-snRNP under native cryo conditions attached to a continuous carbon film without the use of stain. This data set served as an independent control that the heavy metal ions did not cause any artifacts. Significantly, no matter whether the particles were stained, the same triangular views with a maximum dimension of 300 Å could be obtained; a typical micrograph with selected tri-snRNP particles and a representative result of the SDS-PAGE analysis of the proteins is shown in Fig. 3.36. To increase the resolution, about 100 CCD images obtained by cross-correlation based stitching from sets of 6×6 slightly overlapping CCD images were recorded and ~124,000 particle images were selected
3.3 Structural analysis of the tri-snRNP and its subunits

Figure 3.36: [U4/U6.U5] tri-snRNP as visualized under native cryo conditions. Left panel, silver stained SDS-PAGE analysis of proteins; right panel, typical micrograph of the sample with manually selected particles in vitrified ice. The scale bar corresponds to 30 nm.

Figure 3.37: Resolution of tri-snRNP 3D reconstruction. The FSC is shown in the left panel (blue curve). The FSC\(_{0.5}\) threshold criterion is 24 Å. Using the alternative FSC\(_{0.143}\) criterion, a resolution of 21 Å is determined, whereas the intersection of 3\(\sigma\) threshold curve (denoted in grey) and the FSC curve can be found at 19 Å. On the right, the main projection views obtained by projection-matching and the corresponding reprojections from the 3D structure are depicted. The scale bar corresponds to 30 nm.

based on cross-correlation coefficient to a small set of 2D reference images computed from the 3D structure. To obtain the highest image contrast possible (in combination with high particle numbers), the CCD detector was used in combina-
tion with 2× binning as described (compare section 3.1.1, p. 40), and the objective lens was adjusted to high defoci of 2 – 9 μm, so that the information content of the images in the range of 20 – 50 Å was as high as possible. All particle images then underwent correction for defocus and astigmatism as described (compare section 3.2.1, p. 74). Images representing cryo artifacts like frozen ethane or aggregated particles were excluded using very strict cross-correlation based limits: 33 % of the particle images were excluded from further rounds of refinement based on a low cross-correlation coefficient in the MRA, and another 33 % of the particle images were excluded based on the occurrence of multiple peaks in the corrims as shown in Fig. 3.32. As these two characteristics overlap, around 50 % of the images were excluded so that ~61,000 images were used for refinement of the 3D structure:

Projection-matching was performed using exhaustive corrims-based MRA via resampling to polar coordinates as described (compare section 3.2.2, p. 91). Due to the high speed of the improved algorithm in combination with the high computer power obtained from the parallel computer farm (64 nodes) it was possible to perform refinement loops with reference image data sets of up to ~41,000 reference images (1° angular separation). However, the resolution reached a stable level, when ~10,000 reference images (2° angular separation) were used. The resolution was measured via FSC (compare section 1.2.4, p. 28). According to the FSC0.5 criterion (shown in Fig. 3.37, the final resolution was 24 Å. Typical surface views of the final 3D reconstruction of the [U4/U6.U5] tri-snuRNP from the six main directions are shown in Fig. 3.38. Compared to the initial 3D model, the general shape and architectural features are identical, only the visibility of high-resolution details has significantly improved: the particle exhibits a characteristic triangular appearance from all sides including the top and bottom views. Certain characteristic structural features reminiscent of the those found in 2D projection views are also visible: a ring-like head domain can be seen in Fig. 3.38A, and a cleft that extents into an inner cavity can be observed in Fig. 3.38B. At the bottom, the a globular foot domain contacts the main body and is mainly responsible for the elongated, triangular appearance of the particle. On the top (Fig. 3.38E), a characteristic bridge density connects the ring-like head domain to a more compact density resulting in a V-shaped, triangular appearance of the top view. From the bottom (Fig. 3.38F), the thin connections of ring-like head domain and body can be observed.
3.3 Structural analysis of the tri-snRNP and its subunits

Figure 3.38: 3D structure of [U4/U6.U5] tri-snRNP. Shown are views from the six main directions. The scale bar corresponds to 50 Å. The maximum dimension of the tri-snRNP is 300 Å. The particle exhibits a characteristic triangular appearance from all sides including the top and bottom views. Certain characteristic structural features reminiscent of those found in 2D projection views are visible: A ring-like head domain, (A), (B) a cleft/inner cavity between head and main body, (C,D) At the bottom, the a globular foot domain contacts the main body and is mainly responsible for the elongated, triangular appearance of the particle. On the top (E), a characteristic bridge density connects the ring-like head domain to a more compact density resulting in a V-shaped, triangular appearance of the top view. From the bottom (F), the thin connections of ring-like head domain and body can be observed.

3.3.4 3D structural analysis of human native U5 snRNPs

Human native U5 snRNPs under low-salt conditions

As in the case of the [U4/U6.U5] tri-snRNP, the initial model of the U5 snRNP particle obtained by RCT/ML (compare Fig. 3.35, p. 110) was used for refinement of the structure via projection-matching (compare section 1.2.4, p. 26). A data set comprising ~19,300 negative stain images was recorded under cryo conditions. For this purpose, a “sandwich” negative stain grid containing U5 particles was air-dried for 15 minutes after preparation and frozen in liquid nitrogen until image acquisition using a cryo-specimen holder. The images were corrected for defocus
and astigmatism as described in section 3.2.1. Again, the resolution was measured by computing the FSC (compare section 1.2.4, p. 28). According to the FSC$_{0.5}$ criterion (shown in Fig. 3.39), the final resolution was 32 Å. Typical surface views of the final 3D reconstruction of the [U4/U6.U5] tri-snRNP from the six main directions are shown in Fig. 3.40. Owing to the lower resolution of 32 Å compared to 24 Å obtained for the [U4/U6.U5] tri-snRNP, much less high-resolution details are visible. The maximum particle length is approximately 280 Å. Similar to the [U4/U6.U5] tri-snRNP, a ring-like head domain can be observed, that is separated from the main body of the particle by a characteristic cleft. A globular foot domain contacts the main body and is responsible for the elongated appearance of the particle. A network of prominent densities (labeled “back” in C) is responsible for the roundish, convex-concave shape of the particle.

**Human native U5 snRNPs under high-salt conditions**

For the U5 snRNP particle under high salt conditions, the 3D RCT/ML analysis revealed an elongated compact density without characteristic structural details, indicated that many conformations of the particle with significant differences on a global level were present in the data set in approximately equal proportions. In this case, the iterative weighted averaging procedure only had the effect of aligning
3.3 Structural analysis of the tri-snRNP and its subunits

Figure 3.40: 3D structure of the U5 snRNP. Shown are views from the six main directions. The scale bar corresponds to 50 Å. The maximum dimension is of the U5 snRNP is ~280 Å. Similar to the [U4/U6,U5] tri-snRNP, a ring-like head domain can be observed, that is separated from the main body of the particle by a characteristic cleft (A,B). A globular foot domain contacts the main body and is responsible for the elongated appearance of the particle (A-D,F). A network of prominent densities (labeled “back” in C) is responsible for the roundish, convex-concave shape of the particle.

the 85 individual RCT 3D structures as a prerequisite to perform 3D MSA analysis as described in section 3.1.2, p. 59. By using HAC (compare section 1.2.4, p. 22), 15 3D class averages containing on average 5.7 3D RCT densities were obtained. Significantly, various conformational states of the U5 particle could be observed. Figure 3.41 shows the wide variety of angles between head domain and main body of the U5 that occurs under high salt conditions. Additionally, the characteristic “back” densities described for the low salt particle are only incompletely observed, leading to a slimmer appearance. When compared to the low salt particle, the U5 snRNP under low salt conditions seems to be in the most bended conformation, so that the flexibility of the head domain mainly causes a further stretching of the elongated particle.
Figure 3.41: 3D MSA analysis of high-salt resistant U5 snRNP. Five representative 3D class averages of high-salt resistant U5 snRNP are depicted in blue. The 3D structure of U5 snRNP under low salt conditions is also shown (last column, colored in yellow). Various conformational states of the head domain with respect to the main body can be observed. In the upper row, the five structures are viewed from the side. In the lower row, the corresponding views from the top are shown. The scale bar corresponds to 5 nm.

3.3.5 3D structural analysis of human native [U4/U6] di-snRNPs

For the [U4/U6] di-snRNP, a representative 3D model could be obtained by the RCT/ML method (compare Fig. 3.35, p. 110). This initial model exhibited a bipartite structure with two globular domains connected by a thin bridge density. As with the [U4/U6.U5] tri-snRNP and the U5 snRNP particle, this low-resolution 3D structure was used as initial reference for a MSA-based and projection-matching refinement procedure of the untitled ~3800 particle images as described in section 1.2.4. However, no improvement of resolution was possible. In particular, projection-matching only had the effect of introducing contortions into the density without improving the recognizability of fine structural details. As this adverse disadvantageous effect is in practice mainly a result of structural heterogeneity of the sample, 3D MSA as described in section 3.1.2 was applied to a set of 118 selected 3D RCT densities, and 15 3D class averages containing ~10 RCT densities per average were computed. Five representative classes are shown in Fig. 3.42. All 3D structures share a common global architecture with two domains (one large and one small domain) connected by a thin bridge. However, as shown by 3D MSA, the relative position of the two domains with respect to each other appears to be variable within the data set: the 3D structures in the fourth and fifth column of Fig. 3.42 appear more compact than the those in column one to three. Furthermore, some 3D structures exhibit small additional variable domains.
3.3 Structural analysis of the tri-snRNP and its subunits

Figure 3.42: 3D MSA analysis of [U4/U6] di-snRNP. Five representative 3D class averages of [U4/U6] di-snRNP are depicted in yellow. In the upper row, the five 3D structures are shown from the site. In the lower row, the corresponding top views can be found. The scale bar corresponds to 5 nm. All 3D structures share a general architecture with to domains connected by a thin bridge. However, as shown by 3D MSA, the relative position of the two domains is variable within the data set. The 3D structures in the fourth and fifth column appear more compact than the those in column one to three. Some 3D structures exhibit small additional variable domains.

3.3.6 Rigid body fitting of U5 snRNP into [U4/U6.U5] tri-snRNP

At a level of resolution of 20 – 30 Å – as obtained for U5 snRNP and the [U4/U6.U5] tri-snRNP particle – molecular details like secondary structural motifs are not yet visible. Therefore proteins cannot be visualized directly. However, the complete U5 density was identified in the tri-snRNP by “rigid body” fitting of the U5 density into the tri-snRNP density. As shown in Fig. 3.43, certain landmarks could be taken into account at two levels of resolution (the low resolution of ~40 – 50 Å obtained by RCT/ML, and the currently highest possible resolution of ~24 Å for the tri-snRNP and ~32 Å for the U5 snRNP particle). In both particles, an elongated body with a thin, globular “foot” domain represents the main proportion of continuously connected densities. In U5 snRNP as well as [U4/U6.U5] tri-snRNP, the body forms approximately 70 – 80 % of the structure. A “head” domain is connected to the body via thin bridging densities, and a characteristic cleft between these domains is visible.

These global similarities – head and body domains as well as the direction of the particle axis, and the globular foot domain – were considered for rigid body fitting of U5 into the [U4/U6.U5] tri-snRNP. Owing to the flexibility of the U5 “head” domain, the head domain and body domain were separately fitted into the tri-snRNP, as shown in Fig. 3.44, p. 120. In Fig. 3.44A, the head domain of U5 (depicted in blue) alone has been fitted into the ring-like head domain of the tri-snRNP (brown,
Figure 3.43: Main features of U5 snRNP and [U4/U6.U5] tri-snRNP. In both particles, "body" denotes the main proportion of continuously connected densities. In U5 snRNP as well as [U4/U6.U5] tri-snRNP, the body is elongated and forms approximately two thirds of the structure. A "head" domain is connected to the "body" via thin bridging densities, and a characteristic cleft between these domains is visible. To allow comparison of the structures at different resolutions, the low-resolution RCT/ML result (~40-50 Å) is shown as well as zero-tilt result: In (A), two side views are depicted at ~24 Å resolution. In (B), two corresponding side views of the U5 snRNP are shown at ~32 Å resolution. The scale bar corresponds to 10 nm.

transparent), in Fig. 3.44B, only the body of U5 (shown in green) has been docked into the tri-snRNP. Both domains show strikingly good correspondence, leading to the full visualization of U5 within the tri-snRNP. However, the fit reveals two different conformations of the U5 snRNP alone and integrated into the tri-snRNP, respectively. In Fig. 3.44C, the 3D structure of isolated U5 purified as a 20S particle from nuclear extract is shown using the same colors as in (A) and (B). In Fig. 3.44D, the relative position of the head domain to the body of the U5 snRNP particle as obtained in the fit shown in A and B is depicted. Thus, Fig. 3.44D illustrates the conformation of U5 snRNP integrated into the tri-snRNP. The appearance of the particle is more elongated owing to a combined turn and tilt of the U5 head domain with respect to the body. Significantly, the flexibility of the U5 head domain causing a more stretched shaped has been predicted from the 3D structures of the high salt resistant U5 particle (compare Fig. 3.41, p. 117). Thus, part of the isolated U5 snRNP particle can be found in a conformation similar to that of the integrated particle under high-salt conditions.
Figure 3.44: Rigid body fitting of the U5 snRNP head and body domains reveal a conformational change of U5 snRNP upon integration. Here, the body domain of U5 snRNP (green) and the head domain of U5 snRNP (blue) were docked into the [U4/U6.U5] tri-snRNP (brown, transparent). The fit reveals two different conformations of the U5 snRNP alone and integrated into the tri-snRNP, respectively (for details, see text). In each row, the four principal side views of the particles are shown: (A) Fit of the U5 snRNP head domain (blue) into the tri-snRNP. (B) Fit of the U5 snRNP body domain (green) into the tri-snRNP. (C) U5 snRNP alone, with the head domain colored in blue and the body domain colored in green. (D) U5 snRNP in the conformation observed within the tri-snRNP. Compared to (C), a tilt upwards accompanied by a slight turn of the head domain has occurred, leading to a more elongated appearance of the U5 snRNP similar to the conformation observed for the high-salt resistant U5 snRNP. The scale bar corresponds to 10 nm.
Conformational flexibility of U5 snRNP head domain

The movement of the U5 snRNP head domain as predicted from rigid body fitting of isolated U5 snRNP into the [U4/U6.U5] tri-snRNP density is illustrated in more detail in Fig. 3.45. In (A,B), the characteristic side view of isolated U5 snRNP under low-salt conditions is shown using the same colors as in Fig. 3.44 (blue, head domain; green, body). Upon integration into the [U4/U6.U5] tri-snRNP, the conformational change encloses a combined tilt/turn of the head domain with respect to the body (C,D). In (E,F) the U5 snRNP portion of the tri-snRNP (based on rigid body fitting, compare Fig. 3.43) is shown for comparison. Except for clear differences in resolution between tri-snRNP and isolated U5 snRNP as expected from the FSC analysis, there is a good agreement between the two densities. In summary, the U5 snRNP exhibits a more stretched shape when integrated into the tri-snRNP compared to the isolated conformation.

The flexibility of the head domain has also been observed on a 2D level by comparison of tri-snRNP negative stain class averages as shown in Fig. 3.46, p. 123: In Fig. 3.46 a data set containing 6232 negative stain particle images has been analysed de novo on a 2D level by reference-free alignment (compare section 1.2.4, p. 23) followed by MSA, HAC and computation of class averages. The particle images have been grouped into 300 class averages so that on average 21 particle images contribute to one class averages. Six representative class averages are depicted in the Figure. All class averages exhibit the same appearance of the elongated body. The cleft separating head and body can clearly be visualized in all class averages. However, the most obvious variances in density distribution is observed in the region of the head domain. The angle between the U5 head density and the main axis of the body varies significantly. These results support the results obtained for the U5 snRNP under high salt conditions (compare Fig. 3.41, p. 117) and provide further support for the flexibility of the U5 head domain. The conformational heterogeneity of the tri-snRNP head domain occurred predominantly in samples that had previously been frozen. Thus, for computation of the native cryo 3D structure, fresh material that had never been frozen before was used exclusively.


For localization of the [U4 U6] di-snRNP, the rigid body fit of U5 snRNP was taken into account and the tri-snRNP density was divided into a “U5 snRNP portion”
3.3 Structural analysis of the tri-snRNP and its subunits

Figure 3.45: Conformational flexibility of U5 snRNP head domain. In (A,B), the characteristic side view of isolated U5 snRNP under low-salt conditions is shown (blue, head domain; green, body). Upon integration into the [U4/U6.U5] tri-snRNP, a conformational change is made possible by a combined tilt/turn of the head domain with respect to the body (C,D). In (E,F) the U5 snRNP portion of the tri-snRNP (based on rigid body fitting, compare 3.43) is shown for comparison. Except for clear differences in resolution between tri-snRNP and isolated U5 snRNP, there is a good agreement between the two densities. The observed flexibility of the head domain of U5 snRNP is further supported by 3D MSA analysis of the U5 snRNP under high-salt conditions (compare Fig. 3.41, p. 117). The scale bar corresponds to 5 nm.

and a "Non-U5 snRNP portion" as shown in Fig. 3.47A and 3.47BB. Here, the U5 snRNP portion was colored in dark brown, whereas the Non-U5 snRNP portion was colored in yellow. In Fig. 3.47C and 3.47D, the Non-U5 snRNP part has been cut out of the complete structure for better visualization. As in the case of isolated [U4/U6] di-snRNP, the Non-U5 snRNP portion exhibited a bipartite structure with a large and a small domain connected by a bridge. When the Non-U5 snRNP density is compared to a representative 3D structure of isolated [U4/U6] in Fig. 3.47E and 3.47F (compare Fig. 3.42, p. 118), the striking similarity of size and shape becomes visible. However, slightly more density is visible in the Non-U5 snRNP portion compared to isolated [U4/U6] around the bridge. This finding is in good agreement with the fact that part of the Non-U5 snRNP portion of the tri-
snRNP if formed by the three tri-snRNP specific proteins (compare Fig. 1.5, p. 11). It should also be noted that for the current level of resolution obtained, the border between U5 snRNP portion and Non-U5 snRNP portion can only be estimated but not exactly be determined as would be possible for sub-molecular resolutions of 7–10 Å. Furthermore it has to be take into account that some very flexible densities might be wiped out in the 3D reconstruction and thus be only partially visible. So it is currently not possible to exactly predict the exact position of the tri-snRNP specific proteins due to technical limitations. In Fig. 3.47G, the resulting rigid body fit of isolated [U4/U6] di-snRNP into the [U4/U6.U5] tri-snRNP has been shown. To facilitate orientation, the flexible ring-like U5 head domain already identified has been labeled. As expected from the comparisons shown in Fig. 3.47C-F, there is good correspondence of the masses.

Based upon the complete localization of both subunits, the the U5 snRNP portion and Non-U5 snRNP portion, within the [U4/U6.U5] tri-snRNP, the morphology of the contacts can be described in detail: As shown in Fig. 3.48, the two subunits are clearly separated in the upper part of the tri-snRNP by the cleft described

Figure 3.46: Conformational flexibility of U5 snRNP head domain within the tri-snRNP. Six selected MSA-based 2D class averages of [U4/U6.U5] tri-snRNP (from a frozen 25S glycerol gradient fraction) from a data set consisting of 6232 images (compare section 2.1, p. 37) that have been grouped into 300 class averages are shown, together with contour images of the upper densities of tri-snRNP. All class averages exhibit the same appearance of the elongated body. The cleft separating head and body can clearly be visualized in all class averages. However, the most obvious variances in density distribution can be observed in the region of the head domain. The angle between the U5 head density and the main axis of the body varies significantly.
3.3 Structural analysis of the tri-snRNP and its subunits

Figure 3.47: Localization of [U4/U6] di-snRNP in the [U4/U6.U5] tri-snRNP. (A,B) Based upon the localization of U5 snRNP (compare Fig. 3.43, p. 119), the tri-snRNP 3D structure was colored (brown, density assigned to U5 snRNP; yellow, density not assigned to U5 snRNP). (C,D) The proportion not assigned ("Non-U5 snRNP density") was then cut out of the tri-snRNP structure for comparison with [U4/U6] di-snRNP alone. (E,F) A representative 3D RCT class average in the same orientation as the Non-U5 snRNP density. In both cases, two domains (the left one slightly larger than the one on the right) can be observed. In case of isolated [U4/U6] di-snRNP, only a very thin connection between the domains can be visualized. However, additional masses appear at this connection site that could represent parts of the tri-snRNP specific proteins. These proteins (27k, 110k, 65k; \(\sim\)175 kDa) are not present in the isolated subunits. (G) Rigid body fit of the isolated [U4/U6] di-snRNP structure shown in (E,F) into the tri-snRNP (brown, transparent). The scale bar corresponds to 5 nm.

earlier, and only a single, very prominent contact (labeled "head contact") can be observed. In the lower part of the tri-snRNP, a complex network of interactions occurs (labeled "network of contacts") and it is at the level of resolution obtained – not possible to exactly visualize the border between the two subunits.

3.3.8 Euler angle distribution of projection views

Both the U5 snRNP as well as the [U4/U6.U5] tri-snRNP particle show preferred angular orientations of the projection views. When typical data sets were classified by MSA, often more than 70% of the projection views obtained exhibited nearly identical orientations. To investigate the binding characteristics with respect to the carbon film, the Euler angle distribution of the particle images were analysed based on the angles obtained from the final round of projection-matching. As shown in Fig. 3.49, 178 reference image Euler angles (angular separation: \(15^\circ\)) evenly spaced over the unit sphere were considered and for every position, all particles exhibiting an Euler angle most similar to that position were counted. The 178 numbers obtained were grouped into 10 linear quantiles and plotted as a function of the \(\beta\)
Figure 3.48: Sites of contact between Non-U5 snRNP proportion and U5 snRNP proportion in the [U4/U6.U5] tri-snRNP. In the upper part of the tri-snRNP, both parts are clearly separated by a characteristic cleft, and form only a single contact at the head domain of the U5 snRNP (labeled “head contact”). In the lower part, a complex network of interactions occurs (labeled “network of contacts”). The scale bar corresponds to 5 nm.

Figure 3.49: Euler angle distribution of [U4/U6.U5] tri-snRNP and U5. The numbers of particles assigned to the 178 reference images evenly separated on the unit sphere by 15° has been counted for the U5 snRNP data set (~19,300 images) and the tri-snRNP data set (~61,000 images) and grouped into 10 quantiles. In each plot, large blue dots denote a preferred angular orientation of the particle, whereas especially the small yellow dots show the underrepresented areas of projection views.

... and γ Euler angle (small yellow dots: few particle images assigned to the angle, large blue dots: many particle images assigned to that angle). For the [U4/U6.U5] tri-snRNP, three preferred orientations are visible (at a β angle of ~110° and a γ angle of ~100°, a β angle of ~140° and a γ angle of ~100°, and a β angle of ~30-90° and a γ angle of ~80°) In case of the U5 snRNP, a continuous are in the range of ~90-160° for the β angle and ~+170-120° for the γ angle can be observed. Additionally, a second preferred orientation occurs for a β angle of ~50° and a γ angle of ~50°. Note that the orientation of the particles within the coordinate system is only dependent on the orientation of the 3D structure based upon the RCT/ML procedure. So the two coordinate systems are not aligned...
according to the rigid body fitting. In addition to structural heterogeneity of the sample, preferred angular orientations are another main factor limiting the maximum attainable resolution of a 3D structure.
4 Discussion

The aim of this work was the 3D structure determination of an important spliceosomal component, the [U4/U6.U5] tri-snRNP and its stable subunits, [U4/U6] di-snRNP and the U5 snRNP. To date, the most promising method to perform this task is 3D electron cryo-microscopy in combination with single-particle 3D image-processing. The general underlying problem is that of de novo 3D image processing in case (1) no symmetry of the macromolecule can be assumed, and (2) reliable 3D data for comparative validation, e.g. from X-ray crystallography or NMR are not yet available for such large and dynamic macromolecules. At the beginning of this work, a general road map towards reliable 3D structures in this situation was not established, and still many open questions remain to be solved. However, it is believed that the obstacles that currently limit accuracy and resolution in 3D electron cryomicroscopy, are no fundamental issues (Henderson 1995). Importantly, these issues have to be solved for a steadily growing class of newly discovered macromolecular complexes suitable for single-particle 3D electron cryomicroscopy: more and more proteins are revealed to be members of large macromolecular complexes suitable for 3D electron cryo-microscopy (Aloy et al. 2004; Forler et al. 2003; Gavin et al. 2002; Jurica and Moore 2003).

Many biologically highly relevant macromolecules in the cell – like the snRNP particles investigated here – can only be found in small copy numbers, making current biochemical purification procedures difficult and usually not efficient enough to produce complexes in the amount and homogeneity needed for structural studies by other well-established methods like NMR or X-ray crystallography.

As a consequence, there is an increasing demand for 3D structure determination of these complexes by single-particle electron cryomicroscopy. Single-particle cryo-EM structures of large macromolecules can add significantly to our understanding of the architecture, dynamics and function of macromolecular complexes even at intermediate levels of resolution. However, one of the major present difficulties in single particle cryo-EM is thus not the refinement of an available structure to obtain
higher resolution, but rather the structure determination \textit{de novo} of a molecule.

### 4.1 Importance of proper recording conditions in \textit{de novo} single-particle structure determination

It is important to start \textit{de novo} structure determination with images that are of the best quality obtainable. It is known from the phase problem of X-ray crystallography (Ficner 1998) that the image phases contribute substantially to the total image information. Whether the use of images recorded on a CCD camera helps in improving the image phases in the resolution range important for \textit{de novo} structure determination was therefore tested in this part of the work. As it is shown by analysis of a CCD detector in comparison to standard black & white film, the quality of the image phases of low (\(\sim 20\ \text{Å}\)) spatial frequencies can be improved by about 8° as estimated from analyzing images of amorphous carbon film when a CCD detector is used instead of film. The fact that phases of CCD images are more reliable adds significantly to the understanding of the better performance of CCD-recorded images in \textit{de novo} single-particle analysis. On the basis of the results of the comparison between digital and analogue image recording, the use of a CCD detector instead of conventional photographic film can clearly be recommended for making initial image-processing in single particle analysis more reliable. The results obtained in the tests show that the CCD camera is superior in all relevant aspects, and they confirm the practical experience that CCD images are better for \textit{de novo} structure determination.

A possible explanation for better image phases of CCD detectors compared to film may be due to the thickness variations in the emulsion over large regions of the film. Such variations are automatically eliminated by the application of flatfielding combined with dark field subtraction in case of the CCD camera. The higher consistency of image phases is associated with higher SSNR of CCD detectors at low resolutions in comparison with film. Recently reported results on the SNR of CCD cameras in comparison with film (Booth et al. 2004) are in good agreement with our results. In addition, the present work also takes into account the influence of image-processing, by applying alignment algorithms to the image data. The continuous signal obtained from the carbon film alone makes such films ideal specimens for the kind of tests performed here (Zhang et al. 2003). The scattering
properties of amorphous carbon are similar to those of biological specimens, the alignment of carbon-film images always works perfectly, and the signal is equally distributed over the total bandwidth of the recording device. Therefore, the various results obtained by operating the camera in different modes can be used to determine the optimum settings for image-recording in single-particle cryo-EM. A more routine method to test the performance of a recording device is the determination of the modulation transfer function (MTF) from averages of Fourier-transformed electron-noise images (de Ruijter and Weiss 1992). The MTF has the value one at the base frequency of the device, whereas for all other spatial frequencies up to the Nyquist frequency the relative signal strength is plotted. This approach is especially suitable for the evaluation of the higher-resolution information, whereas differences close to the origin of the MTF are not accentuated. A similar situation is encountered in connection with the B factors (compare Fig. 3.4, p. 49): in fact, the data presented here for spatial decay appear to imply that the film is superior to all settings of the CCD camera. However, the direct measurement of phase consistency at low-to-intermediate resolutions, using real-world specimens, provides an excellent explanation for the superior quality of images recorded on a CCD detector. When the performances of different media are being discussed, the interference of the recording device and microscope settings should also be addressed: a large area of the recording device such as a film measuring $8.3 \times 10.2$ cm also requires a large diameter of the electron beam in the image plane, leading to longer exposure times and possibly a stronger influence of unavoidable specimen movement. If the brightness of the beam is adjusted by opening the condensor apertures, the advantage of a shorter exposure time is unfortunately cancelled out by the lower coherence of the beam and, thus, higher spatial signal decay. With film, this problem can be overcome by the traditional small-spot scanning procedure (Bullough and Henderson 1987; Downing 1991). In contrast, the full area of the much smaller CCD chips has to be exposed in practice to avoid time-consuming reading of dead areas. Two-fold binning of the CCD pixels (in the present study leading to an effective pixel length of $30 \mu m$, twice the $15 \mu m$ for the unbinned settings) leads to a combination of three effects: (1) 4 fold increased light sensitivity, allowing shorter exposure times and maximum beam coherence, (2) approximately 4-fold increased readout speed and (3) lower internal readout noise. These effects are of course even stronger for $4 \times$ binning, when in total 16 pixels are connected to one "super-pixel" of a side length of $60 \mu m$. The combination of these three effects, in particular the extraordinarily high light sensitivity of CCD detectors,
is also responsible for the dramatic improvement of image quality when non-FEG microscopes are used.

The application of the different camera settings to TMV reveals the important influence of the sampling interval. The low-frequency spectrum of an image is known to be important to direct the alignment of the higher spatial frequencies (Shaikh et al. 2003). However, as an intrinsic feature of cross-correlation based algorithms, a large amount of high-frequency noise will misdirect cross-correlation-based alignments to a certain extent and lead to poorer alignment of lower spatial frequencies. For best results in the low- to medium (15-25 Å) resolution range, it is therefore useful to record "oversampled" CCD images and then to coarsen the images to Nyquist frequencies of around 7 Å (see Fig. 3.9). As expected, and as further demonstrated by the carbon-film experiments, the high SNR of the lower spatial frequencies is unaltered by this procedure. Moreover, interpolation is not necessary, as upon Fourier transformation of the image only the central part of the transform can be reverse-transformed to yield any desired sampling interval. A more sophisticated way to avoid the influence of high-frequency noise is the use of a spatial weighting scheme to assess the similarity of particle and reference images. The weakness of traditional cross-correlation-based alignment procedures to deal with high-frequency noise, and the applicability of weighted correlation coefficients, have recently been reviewed in detail (Stewart and Grigorieff 2004). Overall, the results obtained with improper sampling intervals illustrate that the advantages of higher image quality can easily disappear if over-weighting of the high-frequency components of the images occurs during the initial calculations. These results demonstrate the excellent qualities of images recorded with CCD detectors when the low-to-medium resolution signal (10-25 Å) is to be recovered from the superposed noise in the initial steps of image-processing. This feature is helpful in de novo structure determination of single particles and in other low-contrast situations, e.g. when small protein complexes are imaged and computer processing of the images is difficult. The use of CCD cameras for single-particle structure determination is therefore recommended in the initial steps of the analysis in such situations. In contrast, the current generation of 4k × 4k CCD cameras does not perform better than conventional silver halide film in situations where molecular resolutions beyond 7 Å are to be reached. However, the performance of the CCD detector depends strongly on the settings of the electron microscope and of the detector itself. Binning of the CCD pixels thus leads to larger effective pixel sizes, with higher SNRs and better transmission of image phases. while the
high sensitivity of the CCD camera enables the use of small condenser apertures for maximum coherence of the electron beam. Because for these settings the imageable area becomes very small, the images should be recorded with overlapping small-spot scanning and then mounted as shown in Fig. 3.12, p. 58 for a sample of tomato bushy stunt virus (TBSV). Consequently, all particle images for initial image-processing (compare section 3.3, p. 108) were recorded on CCD with slight overlap and then stitched prior to further processing.

4.2 Advantages of ML-based 3D weighted averaging for computation of initial 3D models and analysis of structural heterogeneity

For the 3D structures of the [U4/U6.U5] tri-snRNP and its subunits, the [U4/U6] di-snRNP and the U5 snRNP particle, standard angular reconstitution techniques via sinograms (compare section 1.2.4, p. 24) for de novo computation of 3D models were not successful for two reasons: (1) the angular distribution on the carbon support film and thus the diversity of projections obtained by 2D MSA and classification was limited to only a few projections that exhibited very similar triangular shapes. Angular reconstitution was not able to clearly separate these triangles and to reproducibly assign correct Euler angles to these views. (2) In addition, structural heterogeneity occurred with respect to the U5 snRNP head domain, and standard angular reconstitution exhibits only very limited capabilities to take heterogeneity into account, as in the standard angular reconstitution algorithm the user has to select class averages that belong to the same 3D model and not to different conformations of this model. In case of the [U4/U6.U5] tri-snRNP and its subunits, nothing was known about the 3D appearance of these macromolecules and thus a manual pre-selection of class-averages with respect to different conformations was not possible. In contrast, an independent approach for 3D startup model creation in single-particle electron cryo-microscopy is presented here that has been shown to deliver reproducible results even in the presence of structural heterogeneity. Aside from its application for the snRNP particles shown here, its application is generally intended for solving 3D structures of to date unknown or biochemically heterogeneous macromolecules, as well as for 'high-throughput' approaches: as the procedure works very fast, communication between the structural
biologist and the protein biochemist who purifies the sample is significantly facilitated, and thus the biochemical purification procedure can be optimized efficiently. Hereby, the independent 3D de novo analyses of many data sets, when yet no molecular replacement techniques are applicable, were in the past often the bottleneck that significantly retarded the work and complicated efficient discussions about the purification protocol of the particle. In contrast, the main advantage of the method presented here lies in speed, reproducibility and operator-independence even with heterogeneous and low-quality data sets. Therefore specific problems concerning a biochemical purification procedure can be detected in a short time and efficiently optimized.

4.3 Influence of CTF evaluation and correction and on the quality of 3D reconstructions

In the CTF correction method shown here (compare section 3.2.1, p. 74) the general strategy for finding local CTF parameters rests upon the retrospective analysis of the local power-spectral density. It allows automatic detection and correction of all CTF parameters (defocus, amplitude contrast portion, astigmatism, B Factor) when the particles are adsorbed on a carbon film and Thon rings are visible. The method was thus applicable for all particles investigated in the thesis and proved to be useful to extend the resolution of a 3D structure beyond the limit given by the first zero-crossing of the CTF (e.g. \( \sim 40 \, \text{Å} \) in case of the [U4/U6.U5] tri-snRNP). In the determination of the exact CTF parameters at the position of the imaged particle, it should not necessarily be assumed that the CTF is constant in the entire negative. Rather, the individual parameters may vary owing to a tilt of the sample holder, to local charging or to distortion of the sample. For this reason, MSA (compare section 1.2.4, p. 21) is ideally suited not only to the identification of different views of individual particle images, but also to the analysis of the power spectra, especially as power spectra are in themselves optimally centered. The classification implemented in IMAGIC (HAC) (van Heel 1984) is able to classify power spectra into categories of very similar CTF parameter values. Surprisingly, this allows not only the defocus, but also other parameters, in particular astigmatism, to be distinguished without the need for rotational averaging. Under the right conditions this even becomes visible in the MSA in characteristic Eigenimages. Averaging of the power spectra grouped into the same classes leads to class averages with Thon rings that, owing to the summation of signals, have become
clearly visible. These can be estimated, on the basis of their power spectra, with much greater accuracy than the FFT² of the individual particle images. The class averages can thus be used for accurate determination of CTF parameters. The multivariate statistical analysis finds - as shown in the Eigenimages - mainly the Thon rings as principal properties of the data set. It may therefore be taken that the subsequent classification is really based upon CTF parameters and not upon structure factors. Furthermore, the averaging of many power spectra reduces the contribution of individual particles to the signal in the FFT². Qualitatively poor images, characterized by charging or drift effects, are also identified, as even power spectra with directionally truncated Thon rings are reliably classified by the MSA procedure. In case of the [U4/U6.U5] tri-snRNP data set, about 5% of the images were excluded due to low quality power spectra.

4.4 Influence of the type of alignment algorithm on the quality of 3D reconstructions

The number of particle images needed to attain a desired resolution cannot be generally stated, as it is influenced by the symmetry and the molecular weight of the particle under investigation and also by the homogeneity, individual image quality and randomness of the angular distribution. However, if the homogeneity of the sample limits the resolution, even an infinite data set might not exhibit a resolution higher than a certain limit imposed by disadvantageous sample properties. The simulation experiments presented here (compare section 3.2.2, p. 91) indicate that the use of smaller angles between the reference images in the multi-reference alignment raises the quality of the calculated 3D structure, and this improvement may be relevant for the success of the iterative refinement. On the basis of simulated data, the present study indicates that higher numbers of reference images, i.e. smaller angular spacings, may have an influence on the convergence of the MRA algorithm similar to that of high SNR values of the raw images. The method of corrims-based alignment presented here facilitates the use of large data image sets as well as of small angular separations of the reference images by improving the speed of computation: If a conventional refinement of the alignment parameters is carried out, the speed improves by a factor of eight in the subsequent MRAs. Additionally, when the sampling interval (Å per pixel) of the particle images is reduced in the refinement iterations, the use of corrims can lead to an acceleration of sometimes more than ~40-fold: a transition to a version of the particle images
with a higher Nyquist frequency causes – through the enlargement of the image size – a decrease in the alignment speed in rough proportion to the square of the enlargement. The previously determined alignment information (contained in the corrims) can directly be used for the data set with enlarged pixel window and thus conveys a very large advantage in terms of speed. Furthermore, an additional effect with respect to accuracy of alignments after decreased sampling interval: it is known that with decreasing size of the sampling interval, the statistical significance of the correlation peak decreases (Frank 1982) and the alignment becomes susceptible to high-resolution noise contained in the data images (compare Fig. 3.9, p. 54). On the other hand, large sampling intervals result in more significant correlation peaks. Using corrims, the cross-correlation information determined from an alignment using large sampling intervals can be applied to subsequent rounds of the iterative refinement independent of the actual sampling interval. Thus, corrim-based alignment may have a favorable influence upon the quality of the final result when increasingly smaller sampling intervals are used.

When comparing the performance of traditional “direct alignment” to alignment via polar coordinates, it turned out that the accuracy of alignment via polar coordinates in the presence of noise was significantly higher, whereas the susceptibility to errors contained in the reference images was lower (compare Figs. 3.31A and 3.33). Thus, for calculation of the 3D structures presented in this work, alignment via polar coordinates was exclusively used, although this algorithm is 20-50× slower than “direct alignment”. Therefore alignment via polar coordinates was combined with the corrims presented here to refine the resolution.


For all macromolecules investigated here, independent 3D structures were calculated. In case of the [U4/U6.U5] tri-snRNP, a resolution of ~24 Å was obtained from a large data set of more than 60,000 particle images recorded under native cryo conditions. The 3D structure of the U5 snRNP particle was calculated from more than 19,000 negative stain images recorded under cryo conditions. The [U4 U6] di-snRNP and high salt resistant U5 snRNP could only be solved at lower resolutions of about 50 Å due to far-reaching global flexibility and structural heterogeneity of these macromolecules.

Several points support the correctness of structures and rigid body fitting: all 3D
structures were obtained independently, and no tri-snRNP densities were used as initial references to assign Euler angles to the data sets of the [U4/U6] di-snRNP and the U5 snRNP (so called molecular replacement). Instead, de novo reference-free alignment was applied to all data sets to independently obtain stable and reproducible 2D class averages, and the accuracy of the MSA-based classification and quality of the class averages was further improved by using images from a CCD detector. The CCD detector was operated at conditions that delivered about twice the signal in the spatial frequency range important for de novo analysis compared to standard film. Then RCT was used do directly determine the Euler angles and an extensively tested 3D weighted averaging procedure was applied to combine similar, but initially misaligned 3D RCT densities. Structural heterogeneity of the sample was assessed using 3D MSA. For refinement of resolution, the particle images were corrected for all parameters of the CTF and an exhaustive alignment via resampling to polar coordinates was used, as this algorithm has been shown to produce outstanding results in terms of accuracy in the presence of noise. Furthermore, the algorithm was preferred as it was less susceptible to errors contained in intermediate reference images compared to standard algorithms.

Finally, the complete U5 snRNP portion could be localized in the tri-snRNP based on similarities of characteristic features (shape of the body, cleft, head domain), and thus the 3D appearance of the [U4/U6] di-snRNP (~490 kDa) together with three tri-snRNP specific proteins (~175 kDa) – the Non-U5 snRNP portion – could be predicted from the tri-snRNP and U5 snRNP 3D structures. Significantly, the 3D structure independently obtained for the [U4/U6] di-snRNP exhibited the same shape and architectural features as the Non-U5 snRNP portion of the tri-snRNP, except being slightly smaller. Small differences in size of the Non-U5 snRNP portion compared to isolated [U4/U6] di-snRNP are expected, as the tri-snRNP specific proteins are missing in isolated [U4/U6] di-snRNP. However, due to the limited resolution of the structures, the exact border between [U4/U6] di-snRNP, tri-snRNP specific proteins and U5 snRNP can of course not be accurately determined. Thus, 3D structures of higher resolution are required to describe the interface between the subunits in more detail.

The contacts observed between the [U4/U6] di-snRNP and the U5 snRNP within the tri-snRNP were very characteristic. In the upper part of the tri-snRNP, both subunits are clearly separated by a characteristic cleft, and only one prominent contact is visible between the ring-like head domain of the U5 portion and the bridge densities of the [U4/U6] portion. As the interaction of the [U4/U6] 61k
protein to the U5-102k protein has been described to be important for tri-snRNP stability (Makarova et al. 2002), the contact presented here is one of the candidate morphological correlates for the biochemically described contact between 61k and 102k. Of course, these two proteins could also be located within the complex network of interactions observed in the lower part of the tri-snRNP. In summary, the [U4/U6] di-snRNP was shown to consist of a large and a small globular domain; in addition, some variable small domains could be observed in the 3D MSA class averages. This architecture corresponds well to the known protein-protein and protein-RNA interactions: the 15.5 kD protein has been shown to bind to the 5' stem/loop (Vidovic et al. 2000) – i.e. in proximity of the U6 Lsm core – and to mediate the interaction of 61k and the 20/60/90k complex with the U4/U6 snRNA duplex (Nottrott et al. 2002) Additionally, the 90k protein has been shown to cross-link to a U6 oligonucleotide base-paired to U4 snRNA in the region of the stem II (Nottrott et al. 2002). So it is likely that the larger domain contains the U6 Lsm core as well as most of the [U4/U6]-specific proteins. In contrast, the U4 Sm core is located at the 3' end of the U4 snRNA and may thus form the second, small domain. Furthermore, it is possible that the 5' end of the U6 snRNA that does not interact with the U4 snRNA forms an additional small domain.

A characteristic flexibility of the U5 snRNP particle has been predicted by separate rigid body fitting of the U5 snRNP ring-like head domain and the main body of the macromolecule into the tri-snRNP. Characteristically, the U5 snRNP appeared to be more elongated and stretched upon integration into the tri-snRNP, and this rearrangement was primarily caused by a combined tilt/turn of the head domain with respect to the body. Significantly, this movement of the head domain is possible without dramatic conformational remodeling steps in the densities connecting head and body, as these densities exhibit a rather thin and filamentous appearance that is likely to be flexible enough to accommodate the observed tilt/turn of the head domain. Importantly, these results were corroborated by (1) observation of the same degree of flexibility in the high salt resistant U5 particle, and (2) visualization of structural heterogeneity of the tri-snRNP head domain on the level of 2D class averages independently obtained from negative stain images and a sample that had previously been frozen. The U5 snRNP particle at high salt conditions seems to oscillate between a totally stretched conformation and a bended conformation also observed under low salt conditions. Thus, interactions within the U5 snRNP molecule that are needed to maintain a certain position of the head with respect to the body, are likely to be lessened at high salt conditions. So a move-
ment of the head domain that would otherwise only be observed upon integration of the U5 snRNP into the tri-snRNP, can be visualized under high salt conditions in isolated U5 snRNP, and it can therefore be speculated that an assembly factor that destabilizes the U5 head domain is needed to accomplish the integration of U5 into the tri-snRNP at physiological salt concentrations. However, a protein shown to be involved in tri-snRNP assembly is the U5-52k protein, that is present in U5 at both salt concentrations (Laggerbauer et al. 2005).

As the only difference in protein composition between U5 at low and at high salt concentrations, the U5-100k (hPrp28p) was missing in all high salt resistant samples of the U5 particle. Correspondingly, the main structural difference between the various conformation of the high salt resistant U5 and the U5 3D structure at low salt concentrations was a thinner and slimmer appearance of the main body, with less density observed at the characteristic "roundish back" of the U5 snRNP particle (compare Fig. 3.40 C,D for description of the back). So, either the 100k protein may stabilize the back region, or it may be located itself in this region of the U5 snRNP particle. According to the 3D structures shown here, the U5 back domain contacts the [U4/U6] di-snRNP in the region of the larger [U4/U6] di-snRNP domain that has been discussed to contain the U6 snRNA. As the U5-100k is implicated in displacing the U1 snRNA from the 5' splice site (Staley and Guthrie 1999) in favor of the U6 snRNA during catalytic activation of the spliceosomal complex B, a location of the 100k protein in this back region of the U5 would be in good agreement with its presumed function in splicing.

Furthermore, the observed large-scale flexibility of the U5 snRNP particle sheds some new light on the role of proteins in the various remodeling events during the splicing reaction and the recycling steps of its components, as ~93% of the visualized U5 mass should correspond to protein rather than RNA density: the U5 snRNP particle is known to reside within the 25S tri-snRNP as a 20S particle. Upon catalytic activation of the spliceosome prior to the first reaction step, a new set of proteins including hPrp19p and CDC5 (Makarov et al. 2002) is added to the U5 snRNP, in parallel with far-reaching remodeling events of the [U4/U6] di-snRNP that totally disintegrates. Significantly these new proteins appear to remain in proximity to the U5 snRNP during both steps of splicing, so that a 35S U5 particle leaves the spliceosome upon completion of the splicing reaction. This 35S U5 snRNP particle has then to be remodeled into the 20S form before a new tri-snRNP can be assembled. It is furthermore likely that the U5 snRNP is involved in other remodeling steps of the spliceosome as well: the large U5-220k protein
(hPrp8) is thought to function in the alignment of the exon ends as a prerequisite for the precise splicing reaction (Teigelkamp et al. 1995). However, it is not known whether two separate catalytic centers are formed for the two reaction steps, or the reactants of the second step are brought to the common catalytic center after the first step. In any case, highly dynamic molecules will be needed to accomplish this task. The flexibility and dynamics of the U5 snRNP particle observed here adds to the understanding of the role of U5 during splicing and makes it likely that a dynamic, flexible network of proteins is the morphological basis for the proper function of U5 in the various steps of spliceosome assembly, catalysis and recycling.

4.6 Outlook

3D structures of the [U4/U6.U5] tri-snRNP and its subunits have been obtained at an intermediate level of resolution that allows many structural details to be morphologically described without the possibility to directly localize individual proteins or snRNA molecules. However, a further improvement of resolution – e.g. by using alternative biochemical purification procedures to obtain more homogeneous data sets or by further improvements of the image-processing software – seems to be important, and even a moderate increase of resolution to $\sim$10–15 Å will be extremely helpful to better understand the architecture of the tri-snRNP: several proteins contain known structural domains that are likely to become visible at that resolution, for example, the TPR repeats (Makarov et al. 2000) of the 102k protein. It is to date not known which level of resolution is needed to clearly visualize the Sm and Lsm core domains. Isolated Sm core domains form characteristic rings $\sim$8 nm in diameter. In larger snRNPs, however, the snRNAs have to be considered as well as many other proteins in close proximity. Additionally, resolutions of single-particle 3D structures are mostly too low to visualize the $\beta$-strands of the Sm proteins directly, and it is further unknown, whether or not the snRNA is threaded through the central pore of the Sm core.

The structure presented here will definitely be helpful in explaining future 3D structures of the fully assembled spliceosome and localizing the U5 snRNP portion within spliceosomal complexes. A first low-resolution 3D structure is available for the pre-catalytic complex B (Boehringer et al. 2004). The structure reveals an elongated, triangular main body approximately 300 Å in diameter that is attached to a flexible domain, leading to a maximum diameter of the whole spliceosome of $\sim$350-370 Å. It is thus very likely that the triangular body of the spliceosomal com-
plex B mainly represents the tri-snRNP. However, further 3D structural results of the spliceosome are needed to exactly locate the tri-snRNP within the spliceosome and to accurately characterize the interface site of tri-snRNP and pre-spliceosomal complex A.


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Appendix

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Erklärung

Hiermit erkläre ich an Eides Statt, daß die Dissertation mit dem Titel


selbständig und ohne unerlaubte Hilfe angefertigt wurde.

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Erklärung

Hiermit erkläre ich, daß ich mich an keiner anderen Universität um einen Doktorgrad beworben habe.

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