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Three-Dimensional Reconstruction of Single Particles in Electron Microscopy

Image Processing

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Summary

Three-dimensional electron microscopy of single macromolecular assemblies has made large strides forward over the last decade. A large number of image processing techniques have been developed and many have found general distribution. For the proper usage of the wide range of available techniques, a clear concept of all processing steps is essential. This chapter provides step-by-step instruction for the three-dimensional reconstruction of an unknown macromolecule. Where possible, the limitations of the techniques are explained. The chapter attempts to be sufficiently general such so as not to adhere to a single image processing system. Described are alignment techniques for two and three dimensions, classification procedures, and the usage of three-dimensional reconstruction algorithms.

Key Words: Electron microscopy; image processing; three-dimensional reconstruction; Radon transform; image alignment; image classification; cross-correlation; single particles; random conical; angular refinement.

1. Introduction

The term "single particles" throughout this chapter is used in its traditional meaning and designates macromolecules without symmetry or only a low degree of symmetry. Specifically not included are techniques specialized in exploiting structural repeats such as those found in helical or icosahedral particles or two-dimensional crystals.

There are a large number of different reconstruction techniques, and within each technique, different algorithms are being used. However, all of these methods follow similar principles. Therefore, a detailed example of one technique opens the door to all other techniques. The most comprehensive book about image processing in electron microscopy is Frank ***(1)***.

From: *Methods in Molecular Biology, vol. 319: Cell Imaging Techniques: Methods and Protocols*
Edited by: D. J. Taatjes and B. T. Mossman © Humana Press Inc., Totowa, NJ

The theory of three-dimensional reconstruction algorithms is based on the projection theorem *(2–4)*, which states that the two-dimensional Fourier transform of the projection of a three-dimensional object is a central section through the three-dimensional Fourier transform of the object. For example, the two-dimensional Fourier transform of a projection provides the measurement of the Fourier coefficients in a plane that passes through the origin of the three-dimensional transform. The direction of this plane is the same as the direction of the projection relative to the three-dimensional object. Thus, by collecting projections from a variety of directions, eventually the complete three-dimensional Fourier transform of the object can be determined, and, in principle, the inverse Fourier transform of these data provides the three-dimensional structure. The reconstruction is rarely done by actual Fourier interpolation and inverse transforms, but a wide variety of algorithms exist that achieve a reconstruction from projections, each with its own set of advantages and disadvantages. The projection theorem exists not only for Fourier transforms but also for Radon transforms *(5,6)*. In recent years, several groups have taken advantage of the mathematical properties of Radon transforms and developed new alignment and reconstruction algorithms based on its theory *(7–11)*.

Three basic approaches are used to determine the structure of single particles, their main difference being the data collection strategy. These approaches are: tomography *(12–15)*, the random conical reconstruction technique *(16–18)*, and angular reconstitution *(7,19,20)*.

Tomography of single particles is based on the same principles as medical tomography *(21–40)*. A large number of images are collected from the same specimen area at different angles. A typical tilt series would start at –60° and proceed in 1° or 2° steps up to +60°. The limited tilt range of the microscope stage does not allow sampling of the full three-dimensional Fourier transform and a double wedge in the three-dimensional transform is undetermined. Remedies that alleviate this problem are the combination of two tilt series recorded with the tilt axis rotated by 90° between them *(41)* or the collection of a conical tilt series *(42,43)*. In a conical tilt series, the specimen is tilted by a fixed high angle, typically 60°, and then rotated within this tilted plane by small angular increments, recording an image for each position. This geometry allows measuring the three-dimensional Fourier transform with a better angular coverage than does a single axis tilt series, and only a double cone is undetermined. At 60°, this double cone encompasses 13.4% of the volume in Fourier space in contrast to the 33% missing for single axis tilting. The two tomographic approaches, single axis and conical tilting, allow the reconstruction of individual particles; however, the multiple exposures required can lead to extensive radiation damage, in many cases preventing a reconstruction at high resolution. These techniques have found, as their main application, the three-dimensional

reconstruction of cellular organelles like mitochondria. However, combined with averaging techniques, they can be extremely powerful also for the reconstruction of frozen hydrated particles.

Random conical reconstruction *(16–18)* and angular reconstitution *(7)* both overcome the problem of radiation damage through averaging over identical particles. The dataset for a random conical reconstruction consists of pairs of micrographs; one image in each pair is recorded at a high tilt angle of the specimen, typically 45° to 60°, and a second image of the same specimen area is recorded at 0°. If the specimen consists of identical particles attached in preferred in-plane orientations relative to the supporting carbon foil, then one tilt image provides a conical tilt series, yet with random azimuthal angles, in contrast to a series of images of one particle recorded in a conical geometry with equal angular increments. Every particle in the tilt image provides one view in a conical tilt series. Because the azimuthal angles are random, the technique is called the random conical reconstruction technique. The 0° image serves two purposes: one is the finding of the in-plane orientation of each particle, and the second purpose is to classify the particles into groups of identical projections to ensure that the reconstruction originates from projections of identical particles. Differences between the classes can have two major reasons. One reason is that the particles might have more than one orientation on the grid but are otherwise identical, the other is that the differences are caused by true differences among the molecules on the grid, a situation that is rather common. Which of the two is the reason for the class differences can only be decided after separate reconstructions for each class have been carried out. The images that are being used for the actual three-dimensional reconstruction are those extracted from the tilt image, and these particles have only been exposed once to the electron beam. Thus, the radiation damage can be kept to a minimum.

Angular reconstitution is based on the assumption that particles assume a wide variety of orientations on the specimen support. The images are again aligned and classified as is done for the 0° image in the random conical reconstruction technique. However, in angular reconstitution, the assumption is made that the differences between the classes are caused solely by differences in particle orientations. Under these conditions, the class averages can be uniquely related to each other and a projection angle relative to a common structure can be assigned to each particle projection. After a first low-resolution reconstruction, the classification is relaxed, and in the final step, each image is aligned to the common three-dimensional model.

The image processing in this chapter will focus on the reconstruction from a random conical dataset followed by a reference-based three-dimensional projection alignment, which uses the three-dimensional model calculated using random conical techniques as reference to align new projections in space. All

of the techniques used to analyze the 0° image are the same as those used in any two-dimensional study. A two-dimensional study should precede any three-dimensional reconstruction, because it can serve to get familiar with the gross structure of the molecules. A two-dimensional study allows a fast assessment of the quality of preparation and often the results influence the biochemistry of the project. Not rarely, in the first attempts to solve the structure of an unknown molecule, the researcher is faced with a specimen that shows a wide variety of shapes, even though all biochemical techniques indicate a highly pure preparation. A critical interpretation of both biochemical and structural results will lead, in most cases, to a preparation that shows a reasonable homogeneity. The extent of the homogeneity varies from specimen to specimen. Not only is the quality influenced by the original preparation but, obviously, also by the age of the preparation and, not to be neglected, by the dilution before being applied to the grid. It has been observed that the stability of a macromolecule can be highly dependent on its concentration *(44)*. The main reason for focusing on the random conical technique is the particle variability mentioned earlier. Random conical and tomographic techniques are currently the only techniques that, if used properly, do not lead to artificial three-dimensional structures when specimen homogeneity cannot be guaranteed. In fact, these techniques can be used to determine the variability of the sample.

The description of the processing is based on our experience using the image processing system SPIDER and the associated display program WEB *(45)*. However, other systems can be used if they have certain basic features implemented. Essential are an interactive display program for the selection of images from tilt-pairs, Fourier transforms and Fourier filters, correlation functions for translational and rotational alignments, and good image classification algorithms. For example, correspondence analysis followed by classification is one of the most powerful techniques and is available in most systems used in electron microscopy. It is also advantageous that the system has a good scripting language so that the different algorithms can be combined in a flexible manner. For most systems, you should be able to obtain example scripts from colleagues and these examples will give you a good start with your project. Even if scripting at first seems more tedious than if you had a single program that does everything, in the end it will give you the flexibility required to obtain the optimum results from your data.

Image processing systems for electron microscopy include XMIPP, SUPRIM, MDPP, EMAN, IMAGIC, SPARK, and the EM-System. Information about most of the systems can be found on the em-outreach website http://em-outreach.sdsc.edu/community-codes/MicroscopySoftware.html. SPIDER and WEB are available from the Wadsworth Center (Albany, NY), SPARK is developed at the Dipartimento di Chimica Strutturale e Stereochimica

Inorganica (Milano, Italy), and XMIPP is available from the Biocomputing Unit at the Centre National de Biotechnologia CNB (Madrid, Spain). The EM-System, one of the oldest image processing systems developed for three-dimensional electron microscopy, is available from the Max-Planck-Institute for Biochemistry (Martinsried/Munich). An overview over many image processing systems can be found in a special issue of the *Journal of Structural Biology* (Vol. 116, 1996).

2. Random Conical Reconstruction

2.1. Image Recording and Negative Selection

1. Record pairs of micrographs, the first image with high tilt (~50° to 60°), and the second image of the same specimen area at 0° (see Chapter 19) (*see* **Note 1**).
2. Inspect the tilt-pairs visually. Make sure that the specimen area visible in the 0° image is the same as in the tilt image (*see* **Note 2**).
3. Inspect the negatives in the optical diffractometer. Thon rings should be visible out to the resolution that is intended. The complete area in the tilt image must be in underfocus. If not, the area in overfocus should be indicated (best with a felt-tip marker at the edge of the micrograph) such that it can be excluded during scanning. Alternatively, the overfocused area can be marked on a print copy of the negative and then excluded either during scanning or during particle picking. If a sufficiently large number of tilt-pairs is available, those that show overfocused areas should not be used.

2.2. Image Processing: Basic Considerations

Any image processing requires a reasonable choice of a wide range of parameters. Frequently used parameters are the values of low-pass and high-pass filters, the search ranges and increments, the number of classes in classification algorithms, and many others. Guidelines to the proper selection of these parameters will be given for every step; these can only be guidelines because every specimen will behave differently. It is worthwhile to spend some time at certain stages of the processing to determine the parameters for the best performance of the algorithm and fine-tune them to the data. Make sure that the image processing system that you use gives you access to these parameters where needed. Improper selection of parameters can lead to a reconstruction of lower quality than the data allow or, in the worst case, to wrong results.

Image processing of large datasets require a reasonable organization of the data such that it is possible to retrieve any step of the processing at any later step in the procedure. When a sample is analyzed for the first time, some steps might need multiple runs until satisfactory results are obtained and it is essential to be able to tell them apart. Image names might sound suggestive when you create them, but 1 mo later, they might not be clear anymore.

You will create different types of file during the processing of your data:

Image files are sometimes stored as series of images in one file; other times, each image has its own file. In most systems, the name of an image file consists of a name followed by a number that uniquely identifies the image within an image series.

Data files contain numbers, like coordinates, angles, magnifications, and so forth, which will be referred to as "document files" in accordance with the nomenclature used in SPIDER.

Printable files contain the descriptions and results of the processing, which will be referred to as "results files," also using the nomenclature of SPIDER.

Command files and scripts contain the sequences of image processing commands. Scripts can normally be reused for several applications without changes ("procedures" in SPIDER), whereas command files are used to run one specific job ("batch files" in SPIDER).

Other binary files that do not belong to any of the above categories are occasionally created mostly to store intermediate data.

In our experience, an efficient directory structure for random conical reconstruction is the following setup:

<main directory for the project> with the subdirectories.

<directory process> for storage of scripts (procedures, batch files), document files, results, and miscellaneous unique files.

<directories pair %%%%>, one directory for the scanned micrographs of each tilt pair. "%%%%" should be replaced by the last four digits of the micrograph number. Also the document files with particle coordinates and the scripts for particle windowing from each pair are stored here.

<directory not> for the storage of the complete series of all original windowed single-particle images from the 0° micrograph.

<directory tilt> for the storage of the complete series of all original windowed single-particle images from the tilt micrograph.

<directory ucent> for storage of the centered 0° images.

Directories <alia> <alib> <alic>,..., <ali(n)> for the storage of the 0° images after each rotational/translational alignment.

Directories <classi1> <classi2> <classi3>,..., <classi(n)> for each run of pattern recognition/classification, including support files, script files, and resulting images.

<directory tcent> for storage of the centered tilt images.

Directories <talia> <talib> <talic>,..., <tali(n)> for the storage of the tilt images after each alignment.

<directory threed> for storage of the three-dimensional volumes.

The basic idea of this structure is the following: Directory <process> is the directory where most of your processing is done, where your script/procedure, command files, and the results files are located. There might be some exceptions to this because the scripts you use may not have the flexibility built in to

read and deposit data in different directories. In this case, you might have to execute those in the directories where the data are located. In our application using SPIDER (5.0) in combination with the scripts that we have developed over the years, this is true for the windowing of the images from the original micrographs (reason: scripts), and correspondence analysis (reason: program structure). However, distributing scripts and command files over many directories should be kept to a minimum. Having all the scripts and command files in one directory will help to keep track of the order in which the processing was executed. Avoid using command files twice with different parameters; rather, create a separate copy for each run.

2.3. Image Digitization

1. Scan the negatives. Scan the negatives always in the same direction and orientation; for example, emulsion down, scanning starting at the side of the negative opposite to the number going from left to right. If you change any of these parameters, you might change the handedness of the final structure (*see* **Note 3**). The pixel size used should be smaller than one third of the final resolution expected. A factor of one fourth is better. For example, if the magnification in the microscope is ×60,000, a scanning pixel size of 21 μm will result in a pixel size equivalent to 3.5 Å, which would allow for a final resolution of about 11 Å (*see* **Note 4**).
2. Convert the images to the format of the image processing system that you are using (*see* **Note 5**).
3. Create smaller copies of the images by binning, typically a factor of 3. The reduction is needed for the selection of particles.

2.4. Particle Extraction

2.4.1. Particle Selection

1. Display the two images of a tilt-pair side by side, first the image of the untitled specimen and then the tilt image. (In the example here, we use WEB; if other systems are used, you have to find the equivalent operations.) Move the two images such that you see the same area in both. Select four to five particles (absolute minimum number of particles is three), clicking on the center of the particle first in the left image and then in the right image. Make sure that the particles are well separated and are not located along one line. These first particle coordinates will be used to calculate the geometrical relation between the two micrographs (*see* **Note 6**).
2. After the first five particles are selected, go to the menu of the particle picking program and let it determine the tilt angle first. This angle should be within 1° or 2° of the goniometer reading; if the value is systematically off for a series of tilt-pairs, the reason might be that the goniometer reading is not well calibrated. Next, determine the direction of the tilt axis. Enter the start value for the direction of the tilt axis within 40° accuracy. Make sure the tilt axis direction is approximately the same for all images recorded with the same experimental geometry. There is a

180° ambiguity in the fitting of the tilt axis direction. The wrong direction can lead to a mirrored structure (*see* **Note 7**).

3. After determining the geometry, calculate the positions of the particles in the tilt image from the location of the particles in the 0° negative. An overlay of the calculated positions in the tilt image should be within a few pixels of the selected position. If the deviations are large, redo the particle picking (*see* **Note 8**).
4 Once the geometry is determined, go back to selecting particles. Once you select a particle in the 0° image, the program is now capable of predicting the approximate location of the tilted counterpart. Check that the cursor points to the center of the tilt particle; if necessary, correct the location and continue selecting first the 0° then the tilt particles. Any time during this process you can go back to the menu and redetermine the geometry (*see* **Note 9**).
5. Select the particles from all of the tilt-pairs available. For each tilt-pair, the result of the particle picking will be one document file containing the center coordinates of the particles in the 0° image, one document file containing the center coordinates of the particles in the tilt image, and one document file containing data common to the tilt-pair, like the tilt angle and the direction of the tilt axis of the 0° image and the direction of the tilt axis in the tilt image.

2.4.2. Windowing of the 0° Images, Contrast Normalization, Tilt Axis Rotation

All three steps are done combined into one processing step.

Input data needed

1. The document files containing the 0° particle coordinates.
2. The document file containing the directions of the tilt axis in the tilt and the 0° micrograph.
3. The micrograph.

Input parameters required:

1. The micrograph number.
2. The dimensions for the images of each particle, which should be approximately twice the particle diameter.
3. The number of the first image that is extracted from the micrograph.
4. A radius for a circular mask, which should be sufficiently large to include the apparently largest particle completely. Allow additional space for particles not perfectly centered.
5. The name of the images, including the directory where they are to be stored.
6. The name of an output document file that will contain a copy of the image coordinates in the micrograph and the micrograph number for each particle.

The extraction proceeds as follows:

1. Read micrograph.
2. Read direction of tilt axis.

3. Loop over the coordinates in the document file.
4. Read coordinates x,y.
5. Extract image at point x,y from the micrograph.
6. Rotate image so that the tilt axis is parallel to the y-axis.
7. Mask image, using a circular mask.
8. Determine image average $\bar{I}$ outside of this mask.
9. Normalize the (nonmasked) image using the equation $I_c = (I\text{-}\bar{I})/\bar{I}$, where I_c is the corrected image value, I is the original value of each image pixel, and $\bar{I}$ is the average of the image outside the circular mask.
10. End of loop (*see* **Notes 10** and **11**).

2.5. Image Alignment

2.5.1. Image Centration

Most image alignment algorithms require a good estimate for the rotation center of each particle. One of the most useful centration algorithms creates a rotationally averaged, centered template and uses this as a reference for translational alignment.

2.5.1.1. Create a Centered, Rotationally Averaged Reference

1. Add all of the 0° particle images, without any alignment (*see* **Note 12**).
2. Create an image of a disk with the approximate diameter of the averaged particles; if uncertain, make the disk slightly smaller.
3. Strongly low-pass filter this image to about half the disk diameter.
4. Cross-correlate this disk with the average image and determine the maximum of the cross-correlation function.
5. Shift the average image by the negative value of the location of the maximum relative to the correlation function's origin (*see* **Note 13**).
6. Rotationally average the centered particle average.
7. Apply a high-pass filter and a low-pass filter to the rotational average. (High pass is equivalent to one particle diameter; low-pass to 3 nm). (*See* **Note 14**.)

The result is the reference that is used for the centration of the image series.

2.5.1.2. Centration

1. Loop over all particles
2. Cross-correlate the particle with the reference.
3. Determine the location of the maximum of the cross-correlation function.
4. Shift the image by the coordinates of the cross-correlation peak. (Whether this shift has to be negative or positive depends on the program and how it was used. In SPIDER, operation CC, if the image is the first file and the reference is the second file, the shift must be negative.)
5. End of loop.

First, try the centration with about 50 particles; inspect the centered particles. If the centration is apparently unsuccessful, adjust the high-pass and low-pass

filters and try again. Check how the cross-correlation function looks. If it looks too noisy, then a stronger low-pass filter will help. If there is a peak visible in the center, yet the function is lighter toward its corners, then a stronger high-pass filter of the reference may help (*see* **Note 15**). If your particles are elongated or L-shaped, this centration procedure might not perform quite satisfactory. As a centration reference, try using a low-pass filtered disk with the approximate size of the particle instead of the rotational average.

2.5.2. Rotational and Translational Alignment

The goal of the rotational and translational alignment is to position all particle images in the center of each image in identical orientations. In most image processing systems, this alignment is carried out by a sequence of rotational followed by translational alignments *(46–48)*. An alternative algorithm for simultaneous translational/rotational alignment, currently only available as an extension to SPIDER V. 5.0, will also be explained *(10,49)*. These algorithms will become available as stand-alone programs in the future.

At the start of the alignment, a reference is created. (For reference-free alignment, see **Subheading 2.5.2.2.**)

2.5.2.1. Selected Reference-Based Alignment

1. From the set of centered images, visually select a typical particle as first reference, or use the average created by reference-free alignment (*see* **Subheading 2.5.2.2.**).
2. Mask the reference image using a circular mask that contains the complete particle. This radius can now be smaller than the radius used when windowing the particles.
3. Apply a low-pass filter and a high-pass filter, the high-pass filter corresponding to the particle diameter and the low-pass filter corresponding to 3 nm.
4. Loop over all of the images.
5. Calculate the rotational cross-correlation.
6. Determine the maximum of the rotational cross-correlation function.
7. Rotate the image by the negative angle found in the rotational correlation. (The direction of the rotation again depends on the specific program used and the order of the input images.)
8. Calculate the translational cross-correlation between the resulting image and the reference.
9. Determine the location of cross-correlation maximum.
10. Shift the image by the negative of the location of the cross-correlation peak. (The direction of the shift depends on the program used and the order of the input images.)
11. End of loop.

For rotational alignment, the particle image is transformed into some form of polar coordinates, resulting in an image with coordinates r and φ. A one-dimensional cross-correlation function along the φ-coordinate is calculated

and the location of the correlation maximum determines the relative rotation between the reference and each particle. In some systems, a subset of radial coordinates can be selected for this alignment. It is worthwhile to carefully select the best radii. The periphery of the particle, for example, has the most sensitive rotational information, the center has almost none, and the image outside the particle only adds noise to the correlation. This alignment should also be run first only over a few particles (~50) and it should be ascertained that the alignment is working successfully. In most cases, this can easily be done by visual inspection of the aligned images. The parameters that might need fine-tuning are the values of the radii used for rotational alignment and the low- and high-pass filters used for the reference. If the cross-correlation functions are too noisy, a stronger low-pass filter will help; if the translational cross-correlation function shows a clear peak near the center, yet very bright areas near the edges, a stronger high-pass filter might be required.

2.5.2.2. Reference-Free Alignment

The first reference in the above example was selected visually. A more objective reference can be found using the following procedure ***(50)***.

1. Loop over the image series.
2. Select the first, third, fifth, and so on. (i.e., every second) image in the series as reference. Mask, low-pass filter and high-pass filter them (same criteria as for the reference in the reference-based alignment).
3. Align the next image in the series to this reference using the rotational/translational alignment (*see* **Subheading 2.5.2.1.**, **steps 5–10**).
4. Average the aligned image and the image used as reference.
5. End of loop.

The result is a series of averages from two images each. Apply the same procedure to the new image series.

6. Repeat until only one image is left. This is the image average obtained by reference-free alignment.

2.5.2.3. Simultaneous Rotational/Translational Alignment

1. Select a reference, apply mask, low-pass filter, and high-pass filter as in **step 2** of **Subheading 2.5.2.2**.
2. Calculate the two-dimensional Radon transform of the reference (coordinates p and φ).
3. Calculate the Fourier transform of the Radon transform along the radial coordinate p.
4. Loop over all of the images.
5. Calculate the two-dimensional Radon transform of every image.
6. Calculate the Fourier transform along p of all the Radon transforms of the images.
7. End of loop.

8. Calculate the three-dimensional (x,y,φ) cross-correlation functions between the reference transform and the transform of every image n and determine the locations of the maxima of these functions $(x_m,y_m,\varphi_m)_n$. All of this is done within one program. The output is a list of angles and shift vectors.
9. Loop over all original images.
10. Shift each image n by $+x_{mn}$, $+y_{mn}$.
11. Rotate the resulting image by $+\varphi_{mn}$.
12. End of loop.

The simultaneous rotational/translational alignment can also be used in the reference-free alignment. In many cases, even the precentering step can be skipped. If this is done, however, the average of the aligned images might not be in the image center, and if this is required, the average image needs to be centered separately. In general, the simultaneous translational/rotational alignment gives better results than an iteration of translational followed by rotational alignments.

2.6. Pattern Recognition and Image Classification

In most cases, the particles seen in a micrograph do not have the same apparent shape. To obtain a high-resolution average, the images must be classified into groups of images that show the same motif.

There are two distinct approaches to classification and which is best depends on the specimen and the intended result. The two major options that exist are a neural network approach using self-organizing maps for classification *(51)* or correspondence analysis *(52,53)* followed by a classification algorithm. The major difference between the two approaches is that self-organizing maps allow for an analysis of differences between the complete images, whereas correspondence analysis followed by classification allows focusing on specific changing features. For most projects, both types of technique should be applied and then it should be decided which of the approaches provides the best separation of the essential variations in the specimen.

2.6.1. Image Classification With Self-Organizing Maps (Available in XMIPP)

If you used a different image processing system for the rest of the processing, you have to make changes to comply with the syntax and file formats of XMIPP. If you used SPIDER previously, then the images do not need to be converted. The only file that needs to be created is the selection file that XMIPP requires. In the first column, this file contains the list of file names and, separated by a space, the number 1 or 0 in the next column. All images having a 1 in the second column will be processed. A detailed description of the complete

procedure can be found in the XMIPP manual, including an example script that only requires the change of a few parameters:

1. The circular mask being used, which depends on the size of the particle.
2. The number of nodes displayed in the self-organizing map. This number should be large enough so that the final map contains nodes with no members. The best value requires test runs.
3. The radius of nodes that will be influenced by one new image in the calculation of the neural network. Typically, five nodes for the first iterations and later reduced to three nodes.
4. The number of iterations.
5. The image size used for the representation of the self-organizing map.

As for correspondence analysis, it can be advantageous to low-pass filter the images before using them in the neural network algorithm. This reduces the influence of high-frequency noise.

From the neural network map, node images can be directly selected and used as references for multireference alignment. These images can be found in the same directory in which the program was run. The name of the images depends on the input names; the number is the counter shown in the square in the neural network map. In newer versions of the program, the node images might have a step between the inside and outside of the mask used in the algorithm. In this case, remask the node image and specify the image average as the background before using them as references for a multireference alignment (*see* **Subheading 2.7.**).

2.6.2. Correspondence Analysis (Available in SPIDER, SUPRIM, IMAGIC, SPARK, EM)

The example follows the implementation in SPIDER and SUPRIM.

1. Low-pass filter all of the aligned images, typically to 3 nm or lower. If needed, also apply a high-pass filter, typically corresponding to the particle diameter.
2. Create a sharp mask that closely matches your particle, yet includes all possible variations. If in doubt, a circular mask of sufficiently large diameter will work.
3. Create the input matrix for correspondence analysis from your file series.
4. Run Correspondence analysis typically with 8–12 factors. The result is a coordinate file, in which each image is represented by the specified number of factors. Identical images have identical coordinates; the coordinates of very different images point to very different locations. For all of the following explanations, we assume that eight factors have been specified.
5. Analysis of the factor space. The eight-dimensional space can be analyzed in each possible combination of two dimensions. These two-dimensional representations are called maps, usually labeled by the factors they represent (e.g., “map factor 2 vs 5”).

The maps can be represented as printable maps on which each image is identified with a number. Image points far apart indicate images with large differences in the features described by the two factors represented. Points close to each other represent very similar images relative to the feature represented by the two factors.

Each factor can be represented as an image, showing the feature that is changing in the direction of this factor. Each image can be reconstituted from the image that is in the origin of the factor space plus the factor images added with a weight corresponding to the coordinates of the image. If the reconstitution is done without the image representing the origin, then the reconstituted image shows the difference between the image at the origin and the image at a specific coordinate. These images are called differential reconstituted images. The features that are described by each factor can be shown as an image by reconstituting an artificial image with all factor coordinates except one set to zero. For example, the images at point (0,1,0,0,0,0,0,0) and (0,-1,0,0,0,0,0,0) show the feature described by factor 2 in its two extremes, missing on one side of factor 2 and present on the opposite side. Correspondence analysis allows the calculation of these images.

In SPIDER, the command for reconstituting images requires as input the factor(s) that should be used for reconstitution and the number of the image that should be reconstituted. The program looks up the coordinates of the specified image and uses these coordinates to calculate either the reconstituted or the differential reconstituted image. If the image numbers provided for the calculation of the reconstituted images is not within the range of the numbers present in the input file series (i.e., no coordinates can be looked up for this image), the program asks for the input of the coordinates. Entering 1 or –1 for the specified factors results in the creation of the extreme images, representing the feature change described by the specified factor(s).

6. Visualization of factor space. A visual representation of the two-dimensional maps can also be created from the file containing the image coordinates in factor space. For each visual map, one factor vs another, an empty large image is created and divided into typically 10×10 fields. One axis is assigned to the first factor represented; the other axis is assigned to the second. The coordinates of each image can be extracted from the coordinate file, and for each field, an average of all images whose coordinates fall into the same field can be calculated. This average is then inserted in the position of this field into the large originally empty image. The result is an image of the two-dimensional factor map that shows how the images change from one end of the map to the other. In SPIDER, there exists a single command for this operation ("ca vis").

In addition to the visual representation of the factor maps, differential factor images can be reconstituted. As a first step in an eight-factor analysis, seven maps should be created: map factor 1 vs 2, 2 vs 3, 3 vs 4, 4 vs 5, 5 vs 6, 6 vs 7, and 7 vs 8. Both the visual maps and a gallery of the reconstituted factor images should be printed or displayed side by side on the screen. An examination of all these images can be extremely helpful for a better understanding of the variations in the specimen. For example, in negatively stained specimens, one of the lower factors often shows, on one side, the particle very bright, and on the other side, rather dark, but with no significant differences in other features. We often interpret this

factor as a variability of stain thickness, which is not important for understanding the particles themselves. Other factors might describe a distinctly moving feature.

7. Classification. Correspondence analysis provides a set of points forming "clouds" in the eight-dimensional factor space. These points can be sorted into classes. It should be noted, however, that in a dataset of electron microscopical images, distinct classes can rarely be observed. In most cases, changes in the images are continuous (e.g., a flexible arm, a continuous rotation of the particle, etc.). Thus, the purpose of classification is, in most cases, to sort the images into groups of similar images, and the transitions can be fluent. There are many different classification algorithms. One of the best algorithms is the method of classification with moving centers developed by Diday *(54)*, followed by a hierarchical ascendant classification. This algorithm is applied to the results of correspondence analysis.

The implementation of this algorithm in SPIDER allows the following parameters to be specified:

1. Factor numbers: 1, 3, 5, 7, and 8. (These should be those factors that, according to the inspection of the visual maps, are the most meaningful. For example, a stain-depth-describing factor should be omitted).
2. Number of starting classes (seeds) (typically three to five; the final classification can result in many more classes).
3. Number of iterations: between 5 and 100.
4. Number of repeats: typically 5.
5. Random or specific choice of seeds: random. (Seeds for starting the class formation can either be specified as specific coordinates, specific images, or created by a random generator.)

The algorithm in this example uses three to five points that will be randomly picked within the eight-dimensional space. The distances of each image to all seed coordinates are calculated and the image is assigned to the closest seed. In the next step, the centers of gravity of each group of images are calculated and used as new seeds. The distances of each image to the new seeds are determined and the images are reassigned. This process is iterated between 5 and 100 times, depending on the iteration parameter specified, each time starting with new seeds. After the iteration, final classes are formed and the classification reflects which particle images were combined most often in the same class and how often they were reclassified. The final number of classes can be more than 100. The program in SPIDER allows for a maximum of 99 classes, and all of the particles that do not fit into the 99 classes are assigned to class 100. The parameters have to be chosen such that the total number of classes is less than 100. More iterations result in fewer classes.

After this classification, the classes are arranged into a tree using a hierarchical ascendant classification algorithm. The tree should be interpreted as in any other hierarchical classification and similar classes can be merged by specifying

a threshold for the class differences (i.e., a level for cutting the classification tree). How many classes are appropriate strongly depends on the specimen.

2.7. Multireference Alignment

For the following, we assume that classes have been obtained and the particles in each class are added to form an average image.

In the early steps of alignment, only one reference is available and all images are aligned such that they match best the reference. Having class averages allows a classification with more references and the combination of alignment and classification.

1. Recalculate the class averages, and for each average, use the same number of particle images. Calculating the averages from the same number of particles reduces the effect that the alignment/class assignment depends on the signal-to-noise ratio.
2. Align the references toward each other, choosing one of the class averages as the main reference. In this way, the new classes will not be based on different in-plane orientations.
3. Mask, low-pass, and high-pass filter all references, using the same parameters as earlier.
4. Align all images relative to all references and assign them to the reference with the highest cross-correlation coefficient. Use the alignment parameters (x,y-shift, rotation) that were found relative to this reference and shift and rotate each image.
5. Repeat either the self-organizing map or correspondence analysis combined with classification. The number of classes can be different from before.
6. Repeat the multireference alignment.
7. Repeat the complete process (classification/multireference alignment) a minimum of three times or until the class memberships of the particles are stable.

2.8. Three-Dimensional Reconstruction

2.8.1. Centration of the Tilt Images

All of the images of the tilted specimen can be centered without a separation of classes. There are two basic schemes for centering tilt images. Scheme 1 is most applicable for thin specimens, like standard negatively stained samples; scheme 2 is appropriate for thicker specimens, like frozen hydrated or deep-stain specimens. For thin specimens, the object can, in approximation, be considered as being a two-dimensional sheet, and stretching of the tilt image creates the same two-dimensional pattern as can be seen in a projection of the specimen without tilt *(55)*. As the specimen becomes thicker, the two patterns match less and less.

2.8.1.1. Scheme 1 (Thin Specimens)

1. Create a loop over all the images in the series.
2. Take one aligned image from the untilted series; read the tilt angle and the in-plane rotation.

3. Using the in-plane rotation angle, rotate the image back to its original orientation.
4. Take the corresponding tilt image.
5. Using the tilt angle, interpolate the image up in the *x*-direction to a dimension which is $n_{x0}/\cos(\theta)$, where n_{x0} is the original *x*-dimension and θ is the tilt angle.
6. Cut the image back to its original size.
7. Cross-correlate the last version of the 0° image and the last version of the tilt image.
8. Low-pass and high-pass filter the cross-correlation function.
9. Determine the location of the maximum of the cross-correlation function.
10. Multiply the *x*-direction of the peak coordinate by cos(θ).
11. Apply the shift to the original tilt image.
12. Continue with the next image in the series.
13. End of loop

2.8.1.2. Scheme 2 (Thick Specimens)

1. Read the reference that was used for the centration of the 0° images, either an image of a low-pass-filtered disk or the rotationally averaged image average.
2. Interpolate down in the *x*-direction by cos(θ).
3. Pad back to its original size; this is your reference for centration.
4. Loop over all tilt images.
5. Cross-correlate reference and tilt image.
6. Determine the location of the cross-correlation maximum.
7. Shift the tilt image using the coordinates of the cross-correlation maximum.
8. Continue with the next image.
9. End of loop

2.8.2. Assignment of Angles

1. Loop over all tilt images.
2. Read the angles that belong to this image from the document file created during the 0° alignment.
3. Enter the angles into the header of the image.
4. End of loop over images (*see* **Note 16**).

2.8.3. Class Separation

From the document file created by the last classification of the 0° image, create a separate document file for each class. For standard application in SPIDER, each document file contains as a line identifier (key) the image number followed in the second column by a counter of how many values will follow, and in the third column the value 1 if the image belongs to the class or the value 0 if it does not.

The next step depends on the reconstruction algorithm used. There exist many reconstruction algorithms, each with its own advantages and disadvantages. Here, we give two examples: reconstruction by weighted back-projection and reconstruction by a two-step Radon inversion algorithm. These algorithms

are linear algorithms. They do not require any *a priori* information, yet they also do not allow including *a priori* information during the reconstruction process. *A priori* information, however, can be applied in a separate step. The advantage of first using a linear algorithm without any *a priori* information is that the results represent solely the information contained in the data. *A priori* information applied in a separate step should, if correct, only improve the resolution and not create any features that were not present in a reconstruction from measured data only.

2.8.4. Three-Dimensional Reconstruction Per Class

2.8.4.1. Weighted Back-projection ***(17,42,56,57)***

1. Apply the weighting function to all the projections in one class (this is usually a single command).
2. Sum the weighted projections into a volume.

2.8.4.2. Two-Step Radon Inversion ***(8)***

1. Calculate the two-dimensional Radon transform of each projection.
2. Sum all two-dimensional transforms into a three-dimensional transform.
3. Invert the three-dimensional transform.

2.8.5. Projection Onto Convex Sets

Projection onto convex sets (POCS) allows the imposition of *a priori* data onto the reconstruction ***(58–60)***. The *a priori* information can be used to fill in data into the missing cone and reduce the artifacts originating from it. It is imperative that the *a priori* information be correct; otherwise artifacts could be created. One of the safest *a priori* information in three-dimensional electron microscopy is the limit in space of the three-dimensional object.

2.8.5.1. POCS by Applying a Mask in Real Space (Similar to Solvent Flattening)

1. Create a mask of the particle. Make sure the mask is sufficiently large. The *a priori* information imposed is the following: The molecular structure is confined to the inside of the mask, whereas outside the mask, no structure is present. If this mask is applied to negatively stained specimens, make sure to include the stain halo into the mask. Empty space is present only outside the stain shell. Create the mask following the same procedure used to create masks in two dimensions, by a sequence of low-pass filtrations and thresholding operations on the volume.
2. Calculate a three-dimensional Fourier transform of the reconstruction (without a low-pass filter).
3. Loop over typically 10 iterations.
4. Multiply the volume with the mask.

5. Fourier transform the masked volume.
6. Replace the data outside the missing cone with the data from the Fourier transform of the original volume.
7. Inverse Fourier transform the volume to obtain the new volume in real space.
8. End of loop.

2.8.5.2. POCS as Part of the Radon Inversion Algorithm *(11)*

Radon transforms have to comply with certain mathematical rules. Within the suite of programs for three-dimensional Radon inversion, a filter can be applied that enforces that the experimental three-dimensional Radon transform complies with these rules. The experimental transform normally does not comply because of contradictions within the data arising either from noise, the missing cone, or both. The mathematical rules can be imposed in one single step using, as a parameter, a generous estimate of the largest object dimension. This filter is available as a single command in SPARK and in a modified version of SPIDER (5.0).

2.8.6. Refinement of Projection Alignment

2.8.6.1. Two Types of Methods Exist for Refinement of the Alignment of Projections

Mathematically both are equivalent. One is the realignment by a combination of reprojection of the volume followed by two-dimensional alignments (called reprojection methods). The other is the refinement of the projection directions directly by correlation of the projection transform in the three-dimensional Radon or Fourier space. Here, we describe the refinement of projection in Radon/Fourier space ***(8,61).***

1. Low-pass filter the volume to the measured resolution.
2. Calculate the three-dimensional Radon transform of the volume.
3. Calculate the one-dimensional Fourier transform of the three-dimensional Radon transform; apply any further high- and low-pass filters needed to optimize the reference.
4. Loop over all projections.
5. Calculate the two-dimensional Radon transform of the projection.
6. Calculate the one-dimensional Fourier transform of the two-dimensional Radon transform.
7. Correlate the two-dimensional Fourier-Radon transform of the projection with the three-dimensional Fourier-Radon transform of the volume. Input parameters for this correlation are as follows:
    a. Specify if this is a total search (i.e., over the complete angular range) or a refinement search over a limited angular range around the current position of the projection. Here, answer “subsearch” for refinement.

   b. Specify the angular range for each Euler angle. The suggested range for a first refinement is ± 30° in 5° increments for all three angles.
   c. Specify the range of translational refinement. In the first round, a range of five pixels is appropriate.
8. End of loop over projections.

The result of this process is a document file containing the values of the cross-correlation maximum of each projection and the corresponding angle and translational offset.

2.8.6.2. Correct the Position and Orientation of the Projections

1. Loop over all projections.
2. Shift all the original real space projections by the translational offset found in the refinement.
3. Update the header of each projection with the new Euler angles.
4. Recalculate the two-dimensional Radon transforms.
5. Add the two-dimensional Radon transforms into an empty three-dimensional Radon transform.
6. Invert the three-dimensional Radon transform to obtain the new volume.

For a first three-dimensional reconstruction from a single random conical series, one round of refinement should suffice. Additional iterations are most appropriate after several volumes have been merged.

2.8.6.3. Iteration of the Refinement Process

The refinement process should be iterated. As the iterations proceed, the angular search range and the angular increment should be reduced. The translational search can be limited to fewer pixels, because the translation should be well defined by now. Always leave one pixel translational alignment. As the alignments proceed, the low-pass filter of the reference volume should be relaxed. The best filter is to a resolution about 15% lower than the measured resolution of the volume (*see* **Note 17**).

2.8.6.4. A Variant to the Projection Alignment With Signal-to-Noise Ratio Correction

This procedure requires a well-filled three-dimensional Radon transform.

1. Calculate the three-dimensional Radon transform from a set of aligned images; do not apply either a nontomographic noise reduction filter or a Radon inversion.
2. Calculate the one-directional Fourier transform of this three-dimensional radon transform.
3. Radon transform and Fourier transform the projections.
4. If it was used in the calculation of the three-dimensional transform, subtract the Fourier/Radon transform of the current projection from the three-dimensional transform.

5. Cross-correlate projection transform to the reference transform. In this procedure, specify the signal-to-noise ratio of a single projection. The alignment program uses this value to calculate the signal-to-noise ratio of every line in the three-dimensional reference and corrects the cross-correlation value accordingly (*see* **Note 18.**)

2.9. Resolution Measurement

The most common technique for the determination of the resolution of a three-dimensional reconstruction is through the calculation of the Fourier shell correlation ***(62,63)***. A prerequisite is to calculate two independent volumes of the same structure to carry out a comparison.

2.9.1. Calculation of the Fourier Shell Correlation

1. Split the projection set into two half-sets, assigning the first projection of the series to set 1, the second to set 2, the third again to set 1, the fourth in the series to set 2, and so forth. (Because the series of projections might not be numbered continuously, a split between even- and odd-numbered projection files might not split the dataset into two equal halves.)
2. Calculate two volumes.
3. Calculate a three-dimensional mask.
4. Apply a strong low-pass filter to the mask; use a cutoff mask of four to five Fourier units. The mask must be very smooth to minimize its influence on the resolution measurement. The low-pass-filter radius must be substantially lower than the expected resolution value. It is acceptable if the smoothed mask also reaches up to the edge of the image or shows ripples originating from the low-pass filter.
5. Multiply each volume with the mask.
6. Fourier transform both volumes.
7. Calculate the Fourier shell correlation function.

2.9.2. Determination of the Resolution

The resolution value determined from the Fourier shell correlation curve depends on the criterion used. Currently, there are a large number of different criteria used simultaneously and any resolution value should include the criterion that was used in its determination.

The criteria are listed below. Criteria 1–3 are based on a comparison with a predicted noise correlation function. In this case, a theoretical function is calculated that shows the Fourier ring correlation that would be obtained from volumes containing pure noise. This curve starts at 1 and falls off with the square of the radius. It is $1/\sqrt{n}$, where n is the number of points compared within a shell (*see* **Note 19**).

Criterion 1: Intersection of volume shell correlation with twice the noise correlation function (extremely optimistic criterion, essentially shows the resolution that

would be possible to obtain if sufficient data were available to calculate a noise-free reconstruction).
Criterion 2: Intersection of the volume shell correlation with three times the noise correlation function (still a quite optimistic criterion).
Criterion 3: Intersection with five times the noise correlation function This criterion has been shown in specific cases to be equivalent to the 45° phase residual criterion *(64,65)*.
Criterion 4: Intersection with the value 0.5. This criterion is conservative for asymmetrical particles and has been shown to correspond to a signal-to-noise ratio of 1 in each of the two volumes. Thus, the signal-to-noise ratio of the complete volume should be 1.4 $(\sqrt{2})$.

If symmetry was enforced, then the criteria based on a comparison with the noise correlation functions should be used (criteria 1–3), where the noise correlation is multiplied additionally with the square root of the enforced symmetry.

2.9.3. Interpretation of the Fourier Shell Correlation Curve

The Fourier shell correlation curve not only provides the information for a specific resolution value but also contains information about the quality of the dataset. If the curve is very steep (i.e., the difference in resolution when the different criteria are applied is minimal), then the information in the data is exhausted. Higher resolution can then only be achieved from better data. If the curve is very flat, and thus the difference between the different criteria is large, then increasing the size of the dataset should lead to a higher resolution—in the best case, to the resolution predicted by twice the noise correlation criterion.

2.10. Merging of Volumes With Different Orientations

After classification of the 0° images, volumes have been calculated separately for each class. If you apply POCS, the following steps will be easier to do. First, inspect the volumes and decide if they show the same molecule viewed from different angles. Mainly check that all subunits in a multisubunit complex are present. Please note that some differences might be caused by the missing cone in a reconstruction from a conical dataset.

Before attempting to merge volumes, do the following. First, low-pass filter each volume to its resolution; second, compare the volumes and decide which ones show the same structure. Merge only those volumes that show the same structure.

The merging process proceeds in several steps *(44)*. The volumes are first aligned relative to each other. Make sure that the angles are realistic. Volumes with small angular differences are merged first; volumes with large angular differences are merged later. If the volumes originate from a random conical tilt series with a tilt angle of, for example, 45°, then there is not sufficient overlap

in the data to reliably align a volume that is tilted by a large angle relative to the reference. Applying the merging procedure as described, the missing cone will be filled in steps and will not compromise the alignment.

2.10.1. Alignment of Volumes

1. Select one volume as reference and apply low- and high-pass filters.
2. Calculate the three-dimensional Radon transform of the reference volume.
3. Calculate the one-directional Fourier transform of the three-dimensional Radon transform (Fourier/Radon transform).
4. Calculate the 0° projections of all other volumes that should be merged.
5. Calculate the two-dimensional Radon transforms of the 0° projections.
6. Calculate their one-directional Fourier transforms.
7. Align each two-dimensional Fourier-Radon transform of the 0° projections to the three-dimensional Fourier-Radon transform of the reference volume using a full-range search and a 12°–15° increment.
8. Iteratively refine the alignment using a search range of approx ±1.5 times the last increment and, accordingly, smaller search increments. A 3° increment is sufficient for the last refinement.

2.10.2. Merging

Start with the volumes whose 0° projection shows the smaller out-of-plane orientations (angle θ). Ignore all volumes whose orientation deviates by more than the tilt angle of the tilt-pair used for the random conical reconstruction. If all volumes show a larger angle than this angle, chose a new reference and repeat the alignment process. For example, if the tilt angle for data collection was 50°, merge only volumes tilted up to about 30°, to be safe.

1. Choose the projection set of the first volume that is to be merged.
2. Update the projection angles to the new orientation. Your system should have a command for doing this. In SPIDER, the command is "vo ceul" with option external. Enter the Euler angles of the projection first and the rotation angles of the volume second.
3. Calculate a reconstruction from the updated projections by themselves.
4. Check visually that the new volume now has the same orientation as the reference. If the new volume is blurred and it was not before, the updating of the angles went wrong. If it is rotated in the wrong orientation, then either the alignment did not work or the volume rotation angles were entered either in the wrong order or with the wrong sign, or both.
5. Chose the projection set of the next volume, update angles, reconstruct, and compare.
6. Do **steps 1–4** for all the volumes that are to be merged.
7. Combine the projection sets of all volumes that show the correct orientation into one series.
8. Calculate a reconstruction from this combined series.
9. Run a translational/rotational refinement of this projection series; the same as for a single set (*see* **Subheading 2.8.6.**).

10. Use the combined volume as a reference and repeat the alignment of the 0° projection from those volumes that were not included in the combined structure. If the angular range is filled now and no significant missing cone is left, then all datasets, even those with larger angles, can be merged now.
11. Always follow the merging with angular refinements.

3. Three-Dimensional Projection Alignment

If it was possible to combine all datasets, because all of them showed the same three-dimensional structure, then the reconstruction can be improved by adding data from single micrographs. In many cases, 0° micrographs might be appropriate if the particle shows a good distribution of orientations; otherwise, tilt images should be collected to obtain a sufficiently complete angular coverage.

If more than one conformation of the particle was present, then the safest continuation of the reconstruction process is adding more data from random conical tilt-pairs. Techniques for separating different conformations or different particles by comparison to three-dimensional volumes are only in their very first stages of development and are not reliable yet. (*See* **Note 20**.)

The projection alignment *(8)* described here requires a three-dimensional reference volume. This volume, however, should always be available, because reconstructions and classifications as described earlier are a prerequisite for establishing the structural homogeneity of the sample.

1. First, select and extract the series of new images from micrographs.
2. Calculate the three-dimensional Radon transform of the reference volume.
3. Calculate the one-directional Fourier transform of the reference Radon transform (three-dimensional Fourier/Radon transform).
4. Loop over all images.
5. Calculate the two-dimensional Radon transform of the image.
6. Calculate the one-directional Fourier transform of the image (two-dimensional Fourier/Radon transform).
7. Correlate the two-dimensional Fourier Radon transform of the image with the three-dimensional Fourier-Radon transform of the reference. Use a complete search in all angles, starting with an angular increment of 15°–20° and translation range of five pixels (if you picked the particles rather precisely, otherwise increase to a range of seven pixels). Check in the output data file that the cross-correlation maximum is not at the end of any search range; otherwise, increase the range.
8. Translate image.
9. Enter the new angles into the image header.
10. End of the loop over images.
11. Calculate a three-dimensional volume from the aligned images.
12. Check if the volume is correct.
13. Refine projection directions as described previously.

4. Achievement of High Resolution

The resolution of the three-dimensional reconstruction depends on the size of the dataset and on the quality of the images. With careful alignment of the microscope and under the proper imaging conditions (*see* Chap. 19), it is possible to obtain large datasets of frozen hydrated particles reaching to resolutions of 0.7–0.8 nm even when using a microscope equipped only with a LaB_6 filament. However, high resolution requires the correction of the transfer function.

Images of single particles are recorded at a defocus that allows their visual recognition in the micrograph. For 0° images, typical defoci are in the range 1.5–3.5 µm. The center of a tilt image is typically at about 1.5 µm, so that the complete micrograph is in underfocus. The values given are for an accelerating voltage of 100 kV. Visibility of single particles at higher voltages requires a larger defocus.

4.1. Correction of the Transfer Function

Images collected at 100 kV with a defocus of 1.5 µm show the first 0 of the transfer function at approximately 3 nm. To obtain high-resolution reconstructions, the phase changes of the transfer function must be corrected ***(66,67)***. Some image processing systems contain closed programs for the determination of the parameters of the transfer function; others contain elements that can be combined in a script to perform the same task. A prerequisite for the fitting procedure is a program that can calculate the power spectrum for any combination of defocus, astigmatism, and strong scattering (amplitude) contrast ***(68)***, with and without envelope function. For correction of the transfer function in tilt images, additionally a program is required that can calculate the parameters of a plane in space in any direction, usually done by a least squares fit. In addition, all other typical image processing tools (Fourier transform, correlations, mask, filter, value comparisons, etc.) must be available. Fitting and correction follows the methods described in **ref.** ***44***.

4.1.1. Fitting of the Transfer Function to 0° Images

4.1.1.1. Calculate the Averaged Periodogram of the Complete Micrograph

1. Select a window size (typically 256 × 256 pixels).
2. Window frames from the micrograph starting in the upper left corner and moving by half the window dimension. Continue the next row, moving down by half a window dimension. From this process, exclude areas where the micrograph is obviously "bad", like areas with large nontransparent contamination. Calculate the power spectrum of each window and average all of the power spectra. This is the averaged periodogram.
3. High-pass filter the periodogram with a filter radius corresponding to approximately 100 pixels. (This is done instead of a background subtraction). The parameter might have to be adapted for the best convergence of the procedure.

4. Mask out the center of the periodogram using a mask whose radius is approximately the distance of the first maximum of the transfer function from the origin.

4.1.1.2. Fit the Values of Defocus and Astigmatism

To begin, assume an amplitude contrast parameter of 0.1 for frozen hydrated images and 0.2 for stained images. Assume a value of 0 for the astigmatism.

Iterate over the defocus value. Select a value that is within 1 μm of the estimated defocus. (The defocus value that you were aiming for when you collected the images is a good starting value.) Perform a search in 10-nm steps, over a range that is such that the first minimum of the theoretical transfer function cannot fall into the second minimum of the experimental function and vice versa. Experimentally determine the parameters for the theoretical envelope function that matches the falloff of the transfer function in the periodogram. If unsure, use an envelope that shows less falloff than experimentally observed. Chose this parameter such that the falloff is small enough to fit to all micrographs that are being corrected.

Defocus:

1. Start a loop over defocus values.
2. Calculate the theoretical transfer function. Apply the same high-pass filter that had been applied to the periodogram.
3. Cross-correlate the periodogram and the theoretical transfer function. Use a normalized cross-correlation program (perfect fit =1, less than perfect fit <1).
4. In a table, store the iteration step, cross-correlation coefficient, the defocus value, the astigmatism value, astigmatism direction, the amplitude contrast parameter, and the micrograph number.
5. Check if the cross-correlation value is higher than any previous value. If yes, store all of the parameters, including the iteration step.
6. Continue with the next defocus value.
7. End of loop.
8. Write the parameters that resulted in the highest cross-correlation at the end of the table (document file).
9. Calculate the theoretical transfer function that belongs to the highest cross-correlation.

Astigmatism:

10. Loop over the astigmatism values; use defocus values determined previously.
11. Starting with 0 nm, go in steps of 1–10 nm up to a value slightly larger than expected.
12. Loop over the direction of the astigmatism. Steps of 10° from 0° to 170° are usually sufficient.
13. Calculate the theoretical transfer function. Apply the same high-pass filter as had been applied to the periodogram.

14. Cross-correlate the periodogram and the theoretical transfer function. Use a normalized cross-correlation program.
15. In a table, store the iteration step, cross-correlation coefficient, the defocus value, the astigmatism value, the amplitude contrast parameter, and the micrograph number.
16. Check if the cross-correlation value is higher than any previous value. If yes, store all of the parameters, including the iteration step.
17. Continue with the next direction of the astigmatism.
18. Continue with the next value of the astigmatism.
19. End of the loop over astigmatism.
20. Calculate the theoretical transfer function with the parameters that yielded the highest cross-correlation.

Refinement:

21. With these values, start a second refinement of the defocus followed by astigmatism correction. A repeat of three times is usually sufficient.

To check the performance of the fit, display the periodogram with your image display program; to its side and within the same window, show the theoretical transfer function. Display the theoretical transfer function underneath the periodogram also. If you use WEB, from the edit menu select the option bar with a length of approximately twice the periodogram dimension. Position the bar in the minima of the periodogram. This bar then also should cross the minima of the theoretical transfer function. Do the same procedure with vertical bars. If the minima in all of them coincide, the defocus and astigmatism values are correct. If the minima fit well only at low radii and there is an increasing discrepancy at higher resolutions, then the value for the amplitude contrast is incorrect. Run the same procedure again with an outer iteration over amplitude contrast values, or if you have a good guess, run the procedure a few times with different specified values. The amplitude contrast values should only need to be fitted once for the same type of specimen and can be used for all other images from the same specimen without fitting.

4.1.2. Correction of the Transfer Function in 0° Images

1. Calculate the theoretical transfer function with the fitted parameters. This function will have values between +1 and –1. Do not apply an envelope function. Modify the transfer function such that it has a value of constant –1 at radii below the first maximum and does not start at 0. Some programs have this option built in. Otherwise, you will have to mask the center of the Fourier transform and replace the circle below the first maximum with –1. This works only if the astigmatism is not too large.
2. Loop over the images of each micrograph and multiply the Fourier transform of each image with the transfer function.

This procedure is equivalent to flipping the signs in each band with a smooth function.

4.1.3. Fitting of the Transfer Function to Tilt Images

The fitting of the transfer function to tilt images follows a procedure similar to the fitting of the function to 0° images.

1. Split the micrograph into a number of quadrants, typically 3×4.
2. For each quadrant, determine the transfer function parameters in the same way as was done for the 0° micrographs.
3. For each quadrant, collect the values of astigmatism, astigmatism direction, defocus, and amplitude contrast together with the center coordinate of the quadrant.
4. Average the astigmatism values for all quadrants. If some values (most of the time those of the corner quadrants) deviate strongly, leave them out of the average.
5. Using the *x,y*-coordinates of the quadrants and the defocus as the *z*-coordinate, fit a plane to this set of values. Save the parameters of the equation of this plane.

4.2. Correction of the Transfer Function in the Tilt Images

1. For each single image, look up the micrograph number and the coordinates from where the particle was picked.
2. Use the particle coordinates and the equation of the plane determined in the fitting procedure to calculate the defocus at the location of this particle.
3. Using this defocus value and the other transfer function parameters, calculate the theoretical transfer function at this position in the image, again without envelope and, as above, with a value of constant –1 at radii lower that the radius of the first maximum.
4. Multiply the Fourier transform of the particle image with this modified theoretical transfer function.

The corrections can be applied either to the aligned images or to the images at any stage in the processing. It is recommended to apply the corrections either to the first windowed and contrast normalized images or to the images after the alignment. If the correction is applied at an intermediate step, there is a greater chance that the correction might get lost when some of the processing steps are repeated.

4.3. Achieving High Resolution

1. Using the corrected images, recalculate a three-dimensional reconstruction.
2. Carry out an angular and translational refinement as described in **Subheading 2.8.6.**
3. High-pass filter the last reconstruction using a value of approximately one-third of the particle diameter.
4. Low-pass filter the volume to a resolution about 15% lower than the last measured resolution.
5. Repeat the angular refinement, starting with an angular range of approx 15° and increments of about 3° and reduce the range stepwise down to 5° and an increment of 1°. Iterate until stable.

6. Check the cross-correlation coefficients. Remove those particles from the reconstruction that result in the lowest 5% of the cross-correlation. By removing the worst particles, you will observe an improvement in resolution.
7. Run one more iteration of the refinement.

5. Notes

1. Typically, one can collect about 200 particles from each tilt-pair. For a medium-resolution structure (a particle with a 30-nm diameter at a resolution of slightly better than 3 nm), 5000 particles are usually sufficient. For heterogeneous samples, the number of particles should be such that all major conformations are represented by approx 1000 images. The same number applies for a first three-dimensional inspection of a homogeneous sample. Thus, between 5 and 25 tilt-pairs suffice for a medium-resolution reconstruction.
2. The tilt image shows a larger area than the 0° image. If the match is only partial, decide if you want to process only parts of the micrographs or if you want to leave out the micrograph.
3. Check at least once your complete experimental setup, the direction of the tilt axis, the direction of the tilt angle that you call positive, and the scanning geometry. At least once, go through all of the steps and make sure that you obtain the correct handedness in the final result. This can be done in a Gedankenexperiment, but if you are not quite sure, take a well-known molecule and go through the complete reconstruction process to make sure that you obtain the correct handedness. Once you have done this, do not change any of the geometric parameters without rechecking the handedness.
4. In theory, a pixel size of 3.5 Å is sufficient for a reconstruction with a resolution of about 7 Å; in practice, because of possible interpolation errors, at best a resolution of 11–14 Å can be expected. Make sure that the data you obtain from the scanner are optical densities (maybe with a scaling factor) and that no other correction curve has been applied. Only the optical density of the film is proportional to the number of electrons recorded. If your scanner provides transparencies, convert them to optical densities. Make sure that in addition to the proper resolution, your scanner has a sufficiently large dynamic range. Quantization of densities is an additional source of noise and should be kept to a minimum. A 14- to 16-bit transparency range can be expected from most modern scanners and should suffice.
5. We have used SPIDER (version 5.0) and WEB (the non-Java version), but many other systems are available and should be able to carry out most of the calculations. The most important differences can be found in the interactive programs for particle picking, in the reconstruction algorithms — the last step of processing — and in the pattern recognition algorithms used for classification. You might consider using more than one system. We regularly use both SPIDER and XMIPP.
6. If you cannot identify five particles uniquely in both images, you can change the areas that you are comparing. In extreme cases, it is helpful to identify the first particles in photographic prints of the negatives.
7. The direction of this axis can be slightly different in the 0° image and the tilted image if the negatives were not perfectly aligned in the scanner. Thus, two angles

are determined. Because the micrographs are projections and the coordinates that are collected during particle picking lie in a plane, the direction of the tilt axis is ambiguous by 180°. Once the first direction has been determined, make sure that it is the same in all tilt-pairs, by entering an appropriate start value. Otherwise, you might obtain a mixture, and from one data portion, you would get the enantiomorphous structure to the one you will get from the other portion. They cannot be combined without correcting all of the angles.

8. The particle picking program displays information at the bottom of the display screen. During the fitting of the tilt angle, a list of points is presented that might be mismatched. For the determination, of the tilt angle, the program forms all possible triangles with all possible combinations of points in the 0° image and in the tilt image. The area of each triangle should be smaller in the tilt image than in the 0° image by the cosine of the tilt angle. If the triangle area in the tilt image is larger than in the 0° image, the program issues a warning that one of the points might be wrong. A mismatch most often occurs when the three points used for forming a triangle are positioned along a line. In this case, the area in the 0° image is close to zero and a minor inaccuracy in picking the particle position in the tilt image creates a conflict. The parameter "minimal area for tilt angle determination" protects against too many conflicts. Triangles that have a smaller area than the minimal area specified will not be used in the determination of the tilt angle. If warnings appear at the bottom of the screen, check the mismatched points. If these points seem correct, increase the value for the minimum area.
9. As more coordinates are available, the angles will become more accurate. With about 50 particles, the values are within less than 0.5° of the true values. For negatively stained specimens, the tilt angle is usually within 1° of the goniometer reading. Images from frozen hydrated specimens might have larger deviations. The algorithm for the determination of the tilt angle assumes that the 0° image is truly at 0°. If the supporting carbon is warped, this condition is not fulfilled. However, the tilt angle can be refined at a later stage in the reconstruction. For frozen hydrated specimens, deviations up to 15° from the goniometer reading are acceptable. If the difference is larger, the tilt-pair should be discarded from the random conical dataset.
10. The basic rotation of the images such that the tilt axis direction is parallel to one axis of the images eliminates the need to keep track of this direction for every image. It simplifies the alignment and makes it easier to add further data to a dataset.
11. There are a number of different image normalization procedures, all of them being approximations to the true purpose of normalization. The simple formula in **step 9** is based on the assumption that there is empty space around each particle and that the only effect that needs to be corrected for is the exposure of the micrograph. There exist normalizations based on the image variance; however, if analyzed closer, these are dependent on the resolution and on the imaging conditions, having the tendency to assign lower weights to higher-resolution images and vice versa. If the micrograph is very evenly illuminated, then instead of separate values

of the image average for each particle, one single average value for the whole micrograph can be used. For all methods that are based on Radon transforms, it is essential that the background surrounding a particle has an average of zero.

12. Because the particles were selected approximately centered, but not aligned, this creates a blurred average image. This image will be approximately centered; however, it might have an offset resulting from a systematic offset during the selection procedure.
13. The direction of the shift varies and depends on the correlation program used. In SPIDER (5.0), the direction of the shift is negative if the image is entered first and the reference image is entered as the second image in the cross-correlation program.
14. The low-pass filter is intended to reduce the effect of noise and, as a rule of thumb, should be to about 3 nm resolution. The high-pass filter should approximately correspond to the diameter of the particle.
15. The cross-correlation is carried out through a multiplication of the Fourier transforms of the image and the reference. Thus, if the reference is low-pass filtered, so will the cross-correlation function, because all Fourier components that are 0 in the reference will also be 0 in the cross-correlation function.
16. For certain reconstruction algorithms, the angle might have to be stored in document files separate from the image series.
17. Do not apply many consecutive shifts to one image. Every image shift uses an interpolation that might reduce the resolution of the image. Instead, add up all of the shifts and apply then to the starting image. Rotations of the tilt images that are used for the reconstruction are only applied as a parameter in the image header for use by the reconstruction algorithm and, therefore, multiple rotational alignments do not deteriorate the image.
18. If the reference volume is calculated from a real space volume or if the reference Radon transform has been filtered, a prediction of the signal-to-noise ratio of every single line in the transform can no longer be calculated. In this case, you can avoid the problem of the varying signal-to-noise ratio by calculating the volume from a subset of projections that are evenly distributed in the angular space. Which of the two approaches performs better might need to be tried for different situations.
19. The comparison with the noise correlation curve assumes that all points in the compared Fourier transforms are statistically independent. This, in general, is not the case. The Fourier transform is convoluted with the shape function of the molecule, thus creating dependencies. A simple example might illustrate the problem. When the shell thickness is increased from one to two pixels, then the corresponding noise correlation curve is lowered by a factor of 2 because now, at the same Fourier radius, four times as many points are being compared. The Fourier shell correlation function of the volumes, however, does not change significantly.
20. There are a few techniques that can be considered for separating different particles. If the volumes obtained for different classes of particles show a good angular coverage when reconstructed from 0° projections only, then it might be

possible to just collect additional 0° images, coclassify them with the original dataset, and separate them into the same classes. This could best be achieved by using correspondence analysis and coprojecting the new images as inactive images within the old classification. However, coprojection, even though it is one of the great advantages of correspondence analysis vs other techniques, is not implemented in all systems. The technique should be available in SUPRIM and, possibly, in SPARK.

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