CHAPTER NINE

THE ORTHOGONAL TILT RECONSTRUCTION METHOD

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Abstract
Generating reliable initial models for novel asymmetric molecules, particularly heterogeneous ones, remains a major challenge in cryo-electron microscopy. Geometric reconstruction methods, relying on the ability to tilt the microscope stage to obtain two or more views of each molecule, are arguably the most robust for these types of samples as they generate independent reconstructions for each characteristic view obtained.

Random Conical Tilt (RCT) is the classic geometric reconstruction method. Pairs of images are collected at high tilt (around 50°) and 0°. The latter are used to sort the data into characteristic views of the molecule and the former are used for their reconstruction. RCT’s greatest strength is its ability to generate structures regardless of the number of orientations adopted by the sample on
the support. Its major drawback stems from the limited tilt of the microscope stage; this results in an incomplete sampling of the structure in Fourier space and artifacts in its real space representation. Orthogonal Tilt Reconstruction (OTR), a modification of this data collection strategy, results in fully sampled structures. It relies on collecting data at $-45^\circ$ and $+45^\circ$ and treating the tilt pairs as equivalent to the ideal $0^\circ/90^\circ$ that cannot be collected directly in the microscope. OTR requires a sample that adopts a large number of orientations on the support.

Here, the RCT and OTR methods are reviewed and their performances with a biological test sample are compared. The steps required to apply OTR are also discussed.

1. Introduction

Cryo-electron microscopy (cryo-EM) of single particles has made spectacular progress in recent years, with several projects reaching secondary structure and near-atomic resolution (Ludtke et al., 2008; Schuette et al., 2009; Seidelt et al., 2009; Yu et al., 2008; Zhang et al., 2008). Alongside this progress, invariably achieved with well-behaved or benchmark samples, we have seen a growing interest in the analysis of heterogeneity (biochemical and conformational) as a source of invaluable biological information that cryo-EM is uniquely well suited to address. The fact that several chapters in this volume are devoted to the subject is a testimony to its rising prominence.

A number of computational tools have been developed to sort out multiple species coexisting in a sample (see Spahn and Penczek, 2009; for a recent review). A common aspect of most of these approaches is the requirement for at least one initial model of the macromolecule in question. As a result, the challenge of obtaining initial models, particularly of potentially heterogeneous asymmetric molecules, has seen a revived interest.

There are currently four approaches to generating initial models from experimental data: Angular Reconstitution (Van Heel, 1987), Random Conical Tilt (RCT) (Radermacher et al., 1987), Orthogonal Tilt Reconstruction (OTR) (Leschziner and Nogales, 2006), and tomography. The first of these methods, Angular Reconstitution, is an analytical approach that can directly determine the relative orientations of different molecular views present in a sample without additional geometric information. The method is based directly on the Central Section theorem (described in Section 2.1), a consequence of which is that any two projections (images) of a given structure share a common central line in Fourier space (as well as a common one-dimensional projection in real space). While elegant, and extremely successful when applied to structures with high symmetry, Angular Reconstitution is less robust with asymmetric and/or heterogeneous samples.
The difficulty resides in the need for the user to distinguish between different views of the same species (as found in a homogeneous sample) and different views of different species (as found in a heterogeneous one). Without a priori structural information, this distinction becomes a serious challenge and mistakes may lead to incorrect reconstructions. Provided these problems can be overcome, and that a large number of views of the molecule are available in the sample, a major advantage of Angular Reconstitution is that it generates reconstructions that are fully sampled (the issue of sampling will be discussed further below).

Geometric methods are intrinsically more robust for two reasons. First, two (or more, in the case of tomography) views of each molecule are obtained experimentally and their spatial relationship is therefore known. Second, a reconstruction is generated for each characteristic view of the molecule(s) present in the sample. This removes the need to decide whether different views correspond to the same or different molecular species. Comparisons are postponed until after reconstructions have been generated.

Tomography, the one true “single particle” method, can generate three-dimensional (3D) reconstructions for each molecule present in the sample without the need for averaging. This is both its strength and its weakness: avoiding averaging means that each reconstruction is truly homogeneous but the price to be paid for this is the low signal-to-noise ratio of the reconstructions. The solution to this involves bringing averaging back in, this time at the level of the reconstructed volumes. Unlike Angular Reconstitution, tomography generates incompletely sampled reconstructions. This is a consequence of the limit in the extent to which samples can be tilted in the microscope during data collection (this is discussed further in Section 2.1).

The RCT method (Radermacher et al., 1987) was originally developed to reconstruct samples adopting preferred orientations on the support. While it remains the method of choice with samples taking one or a few orientations on the grid, our ability to collect and process ever larger data sets has made the method equally useful, and robust, with samples adopting a larger number of orientations. RCT’s main limitation is the same one faced by tomography: our inability to collect data with the sample tilted to 90°. As a result, the structure is incompletely sampled and suffers from artifacts (discussed in detail in Section 2.1). There are solutions to this problem but these are often not trivial and require the user to make judgments about the molecular identity of a number of different (noisy) reconstructions, a considerable challenge in the presence of heterogeneity.

The OTR method was developed recently (Leschziner and Nogales, 2006) to circumvent the limitations found with RCT. The method, which requires a sample that adopts a large number of orientations on the support, combines strengths of both RCT and Angular Reconstitution: like the former, it generates reconstructions for every characteristic view present in the sample regardless of how they relate to each other molecularly and,
like the latter, the reconstructions are fully sampled and thus free of artifacts. These two properties make OTR particularly amenable to automation, as user intervention is potentially unnecessary until after initial reconstructions have been refined to higher resolution. Techniques capable of this type of automation will become increasingly necessary as we focus our attention on complex samples containing multiple molecular species and conformations.

In this article, I review the principles and applications of OTR. I begin with a more in-depth discussion of the geometry behind the method and its similarities and differences with RCT. I follow this with a description of the steps required to generate reconstructions using OTR. Throughout this description I focus mainly on those aspects unique to the application of OTR, mentioning (but not describing) those that are more general to all reconstruction approaches. I illustrate the different steps, whenever possible, using our current data. I conclude by comparing reconstructions of the same macromolecule obtained with RCT and OTR to highlight the properties of the two methods.

2. ORTHOGONAL TILT RECONSTRUCTION: PRINCIPLES AND APPLICATION

2.1. Random Conical Tilt and the “missing cone” problem

The RCT and OTR methods rely on the same geometric principle. If two views related by a known angle are available for each molecule in the sample, one set of images can be used to sort out the entire data set into characteristic views through alignment and classification and their “tilt mates,” having “fanned out” as a result of the alignment rotations, sample the 3D structure of the molecule (see Fig. 9.1). This sampling is a consequence of the Central Section Theorem, which states that the Fourier transform of a 2D projection of a 3D structure is equivalent to a central section through the 3D Fourier transform of that structure orthogonal to the projection direction (Frank, 1996). It follows that the closer the angle between the views used for alignment and reconstruction is to 90°, the more complete the sampling of the structure will be. A useful visual analogy is that of a coin being spun on a tabletop, where the coin corresponds to a projection and therefore a central section through the 3D Fourier transform of a molecule. Initially, as the coin starts to spin, it sweeps space fully. As it loses momentum and begins to tilt, the volume not being sampled by the coin grows. In this example, the position of the coin at a given point in time (its rotation around an axis perpendicular to the table) would be the result of aligning and classifying its “tilt mate” (some imaginary coin lying flat on the table) and the angle of the coin relative to the table at any point during the spin is the angle between the two views recorded from the sample.
The ideal situation is one where this angle is 90° (the beginning of the spin) as this is the only geometry that leads to a full sampling of the 3D Fourier Transform of the structure.

The RCT method, originally designed for samples adopting a preferred orientation on the support, involves the following basic steps (Radermacher et al., 1987):

1. Images are collected at 0° and at high tilt, typically in the 50–60° range (Fig. 9.1A and B). The tilted images, to be used for reconstruction, are

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Figure 9.1 Geometry of the Random Conical Tilt (RCT) and Orthogonal Tilt Reconstruction (OTR) methods. The basic steps in the RCT (top) and OTR (bottom) methods are illustrated in this figure. Throughout the figure, the green object represents a molecule; the orange rhomboid the support; blue arrows the direction of projection (imaging); and red arrows the in-plane rotations applied during alignment and classification. (A and A’): An image is collected with the sampled tilted, either to 50–60° (RCT) or 45° (OTR). (B and B’): A second image is recorded from the same area after the sample has been either returned to 0° (RCT) or tilted to −45° (i.e., 45° in the opposite direction) (OTR). The images collected in A and B (or A’ and B’) constitute a “tilt pair.” (C and C’): The 0° (RCT) or −45° (OTR) image from a second pair of images can be aligned to the first one if the images represent the same (but rotated) view of the molecule. The in-plane rotation applied to the image being aligned determines the new spatial location of its tilt mate (represented by the empty black frames). (D and D’): The two images are now members of the same “class” (i.e., they represent the same view and are aligned to each other). As indicated in the previous step, the alignment results in their tilt mates “fanning” out in a cone (RCT) or equator (OTR) around the molecule that gave rise to them. This step also illustrates one of the main differences between RCT and OTR: the two molecules giving rise to the two images in the “class” have the same orientation in RCT (although different in-plane rotations on the support) (D) while their orientations are entirely different in the case of OTR (D’). (E and E’): Once there are enough images in a class to fully sample the desired structure, it can be reconstructed. The orange truncated cone shown in E’ emphasizes the fact that every image in this arrangement has originated from a molecule adopting a different orientation on the support.
collected first to limit the dose to which they are exposed (Fig. 9.1A). The 0° images, used for alignment and classification, will have been exposed twice (Fig. 9.1B).

2. The 0° images are sorted into groups representing characteristic views of the molecule through cycles of alignment and classification (Fig. 9.1C and D). The in-plane rotation angles required to align images that show the same view determine the relative positions of the corresponding high-tilt images. The alignment and classification also generates the “class” files, indicating which images were grouped together.

3. The tilt geometry parameters must be determined. These are the tilt angle (e.g., 50° in Fig. 9.1A) and the tilt axis angle (the actual position of the tilt axis relative to the coordinate system). These are typically calculated by the software package used for extracting the tilt pairs from the micrographs.

4. The in-plane rotation angles (2) and tilt geometry parameters (3) are combined into a set of Euler angles describing the relative positions of the tilted images (Fig. 9.1E).

5. 3D Reconstructions are calculated for each “class” obtained in (2) (Fig. 9.1E). The “class” files are used to select the appropriate tilted images and their corresponding Euler angles for each reconstruction.

The fact that the tilted images in RCT are not collected at 90° (impossible to do in the microscope) means that they sample Fourier space incompletely (a spinning coin that is tilting does not sweep space fully). The volume that fails to be sampled is known as the “Missing Cone” due to its shape in Fourier space. In the coin analogy, the region of space not swept by the coin once it begins to tilt has a conical shape as well (remember that the coin represents a central section through the 3D Fourier transform of the molecule). The missing data are an important challenge in RCT as they lead to artifacts in the final reconstruction (Fig. 9.2). Typically, a loss of internal detail and an elongation in a direction parallel to the incident electron beam are observed (Fig. 9.2B). The standard method for addressing this problem is to combine independent reconstructions corresponding to molecules having adopted different orientations on the support. Because the missing data are a function of the orientation of the molecules relative to the electron beam, two reconstructions representing different orientations will be missing information in different regions of Fourier space. In order to fully fill each other’s Missing Cones, the two reconstructions must be related by a “tilt” angle (around an axis perpendicular to the electron beam) equal to or larger than \([90 - \Theta] \times 2\), where \(\Theta\) is the tilt angle used during data collection (e.g., if the tilted data were collected at 50° the two reconstructions must be related by 80°). This solution, while easy in principle, can be far from trivial in practice. The deformations resulting from the Missing Cone, combined with the noisy nature of the individual reconstructions can
make it difficult to determine whether any two of them truly represent the same molecular species and can therefore be merged. This can be further complicated by the presence of heterogeneity (biochemical and/or conformational) in the sample (Fig. 9.3). An additional challenge arises from the fact that cross-correlation coefficients are typically used to score the goodness of the match between any two reconstructions during the rotational search used for their alignment. Because one is looking for volumes that will fill each other’s missing data, these will by definition have the least amount of overlap in Fourier space, resulting in a lower cross-correlation coefficient. The desired answers can be, and often are, found but the process requires a significant level of expertise and interaction with the data.

The OTR method bypasses the Missing Cone problem entirely by taking advantage of the realization that obtaining the ideal 90° (orthogonal) relationship between the two views of the sample does not require that one of them be collected at 0°. Since it is only the angle between the views we
are interested in, a 90° angle can easily be achieved by collecting data at −45° and +45° (Fig. 9.1A–E). Any combination resulting in 90° will work but the ±45° option is often the most practical (discussed in Section 2.4).

A fundamental difference between RCT and OTR lies in the images used for alignment and classification (see Fig. 9.1). The alignments applied to the particles during this step consist of X, Y shifts and an in-plane rotation (about an axis perpendicular to the image plane). In RCT, where 0° images are used at this stage, these rotations also correspond to rotations of the molecules about an axis perpendicular to the support (Fig. 9.1B and C). Therefore, all particles within a given class come from molecules that adopted the same orientation on the support, differing only on their trivial in-plane rotation (Fig. 9.1E). Consequently, a 3D reconstruction (albeit with a Missing Cone) can be obtained from a sample showing even a single orientation, a powerful feature of RCT. In OTR, where the images being aligned and classified come from a tilted sample (e.g., −45° images), the in-plane rotations applied during alignment do not correspond to rotations of the molecules about an axis perpendicular to the support. This is due to the

Figure 9.3 Incomplete sampling is particularly challenging in the presence of heterogeneity. This figure illustrates the difficulties in solving the “Missing Cone” problem by merging reconstructions when heterogeneity is present in the sample. (A) The hypothetical sample contains two conformations of the hand, “closed” and “open.” (B) Two reconstructions, for hands adopting two different orientations relative to the electron beam (arrow on top), were generated for each conformation. It can be seen that the “Missing Cone” results in structures with the same orientation being more similar to each other than those representing the same conformation. (C) The fully sampled reconstructions of the same orientations for the two conformations are shown.
fact that the axes for the in-plane rotation of the molecule on the support and for the rotational alignment of images are no longer parallel (Fig. 9.1B' and C'). Therefore, every particle in a class with a unique in-plane rotation angle has a different orientation on the support (Fig. 9.1E'). Hence one of the most important requirements for OTR: the sample must adopt a large number of orientations. In principle, a molecule adopting orientations that correspond to a precession with a 45° angle would be sufficient to obtain a fully sampled (i.e., no Missing Cone) reconstruction. I discuss below an approach to determining whether a given sample appears to satisfy this requirement and is therefore amenable to OTR. While preferred orientations may often be a limitation with negatively stained samples, we expect OTR to be far more generally applicable with cryo-EM samples.

Another important difference between OTR and RCT stems from the fact that OTR deals exclusively with tilted images and, as outlined above, particles assigned to a given class represent molecules with different orientations on the support. A consequence of this is that the −45° and +45° particles are interchangeable. If we use the −45° set for alignment and classification, the +45° particles are effectively their +90° tilt mates. Conversely, if the +45° particles are used to generate the classes, the −45° particles become their −90° tilt mates. It follows that there is no need to use only one half of the data for alignment and classification, saving the other half for reconstruction. Although this is the situation we simulated when we first introduced the method (Leschziner and Nogales, 2006), we have since begun pooling the +45° and −45° particles for alignment and classification, treating them as a single data set (Leschziner et al., 2007). The data presented here to illustrate different aspects of OTR have been treated in this way as well. The only potential disadvantage to this approach is that the images coming from the second tilt have been subjected to two exposures, the reason behind the fact that tilted images are collected first in RCT (Radermacher et al., 1987). While this will result in some loss of resolution, this is likely beyond what is expected from initial models and is far outweighed by the advantage of doubling the size of the data set.

2.2. The test sample: The yeast exosome

When we first introduced the OTR method, we illustrated its features using synthetic data (Leschziner and Nogales, 2006). While this approach allowed us to assess the performance of the technique quantitatively by comparing our results with the known structure that gave rise to the synthetic data (Leschziner and Nogales, 2006), it bypassed many of the difficulties associated with real samples. Subsequently, we applied OTR to the reconstruction of the ATP-dependent chromatin remodeling complex RSC from the yeast S. cerevisiae in negative stain (Leschziner et al., 2007). However, a few single-particle electron microscopy reconstructions of this complex have
been published to date and they disagree with each other (Asturias et al., 2002; Chaban et al., 2008; Leschziner et al., 2007; Skiniotis et al., 2007). The moderate resolution of all these reconstructions and the absence of high-resolution structures of any significant components of the RSC complex make it difficult, if not impossible, to validate any of them at this point. We were therefore still interested in testing the performance of OTR with a biological sample of known structure.

Our current test sample is the yeast Rrp44-exosome complex, an RNA-processing assembly from *S. cerevisiae*. We chose this 398 kDa macromolecule for several reasons: (1) The structure of both the core exosome and its complex with the Rrp44 subunit have been solved by the RCT method (Wang et al., 2007); (2) A high-resolution structure of the yeast core exosome is available (Liu et al., 2006); and (3) We had access to the same grid used for the determination of the RCT structure of the exosome (Wang et al., 2007) (courtesy of Hongwei Wang, Yale University), thus eliminating sample preparation as a variable when comparing reconstructions obtained with the OTR and RCT methods.

I use data from our current work on the exosome to illustrate the different steps along a reconstruction using OTR. I focus on those aspects that are unique to OTR, bypassing those that are general to any reconstruction process.

### 2.3. Sample preparation

Although we are currently working on implementing OTR with cryo-EM samples, we have so far tested it only on negatively stained samples. I therefore focus exclusively on the latter.

The main goals when preparing negatively stained samples to be reconstructed by OTR are (1) to minimize flattening and (2) to maximize stain thickness. While these may be desirable properties for any sample, they are particularly important in OTR because their absence (i.e., flattened, thinly stained samples) manifests itself most severely in data collected from a tilted sample.

As is discussed below, it seems possible to detect and eliminate particles affected by flattening during alignment and classification of tilted data. However, if a sample is severely affected by flattening, it becomes equivalent to one with preferred orientations and OTR is no longer applicable (discussed in Section 2.9). Similarly, samples embedded in a shallow layer of stain will exhibit stain pooling on one side when imaged at a tilt (Fig. 9.4C and D). Because the location of the stain pool is a function of the tilt geometry, particles with different orientations on the support that would otherwise give identical projections on the support that would otherwise give identical projections become different due to the staining. This will negatively affect alignment and classification.
Figure 9.4 Example of a pair of micrographs collected at $-45^\circ$ and $+45^\circ$ and the effect of stain thickness. (A and B) A representative pair of micrographs of the yeast Rrp44-exosome complex collected at $-45^\circ$ (A) and $+45^\circ$ (B) for OTR processing. The images were recorded using automated OTR data collection as implemented in Leginon (Yoshioka et al., 2007) at NRAMM, The Scripps Research Institute. The insets show enlarged versions of corresponding small areas in the two micrographs (indicated by the stippled squares) to highlight the absence of stain pooling around the particles, one of the criteria we use to select micrographs for further processing. The fact that particles are not elongated along the tilt axis (running vertically on the page) is also an indication that the sample is not severely flattened. Due to the fact that the two micrographs were collected at the same absolute angle (but different sign), the positions of the tilt mates are virtually identical. (C and D) This tilt pair illustrates the effect of stain thickness on the appearance of “pooling” around particles. The same region was cut out from micrographs collected at $0^\circ$ (C) and $55^\circ$ (D). The sample imaged contains both yeast dynein motor heads (white arrows) and a much larger yeast ribosome (black arrow). Due to their size difference and the fact that the dynein motor head (ring-shaped) tends to lie flat on the grid, dynein molecules are fully embedded in the stain while a stain meniscus surrounds the ribosome. This incomplete embedding results in the ribosome showing asymmetric “pooling” of stain in the tilted micrograph (see darker rim on the left side in D). This artifact is not seen for either the dynein molecules in D or the exosome molecules in A and B.
We are able to routinely satisfy these requirements using a staining protocol based on the deep staining described by Ohi et al. (2004). Briefly, our protocol consists of the following steps:

1. Approximately, 50–70 μL of freshly prepared 2% uranyl formate is drawn into a pipette tip, followed by a small air gap and then about 5 μL of sample.
2. A freshly glow-discharged grid (home made holey carbon or Quantifoil® with a layer of thin continuous carbon) held by tweezers is set up in a stand so it is positioned at a tilt.
3. The sample is applied to the grid in a continuous motion; the grid’s tilt helps in draining the stain as it is applied immediately after the sample.
4. The grid is rinsed for 10 s (without blotting) in four consecutive drops of about 70 μL of 2% uranyl formate.
5. The grid is allowed to sit in stain for another minute, either on a drop or with a droplet of stain on it.
6. If the “sandwich” method is to be used, a small square of thin carbon is floated on a pool of 2% uranyl formate in a well and picked up from underneath with the grid (again, without blotting it before going into the stain pool).
7. Whether sandwiching has been used or not, the grid is now carefully blotted from the side, stopping while a thin layer of liquid is still clearly visible on the grid.
8. The grid is allowed to air-dry.

We have found that the “rapid” stain (i.e., applying the sample and the stain, separated by an air gap, in one continuous motion) results in particles that are both more homogeneous and display a larger number of orientations. Homogeneity also seems to be improved by blotting only once at the end of the staining. We often include a small percentage of trehalose (3–6%) in our samples, as this seems to increase the depth of the stain as well. For a detailed discussion on staining protocols, see Ohi et al. (2004).

We use two criteria to determine, visually, whether a sample appears to satisfy the flattening and stain depth requirements. We collect a few tilted images at ±45° and look for shadows around the particles (indicating thin staining) or a general elongated appearance in the direction of the tilt axis (indicating flattening). Only samples that show neither are used for data collection. We use the same criteria to select micrographs after data collection. Figure 9.4 shows an example of a ±45° tilt pair with the desired characteristics: deep staining and no evidence of severe flattening.

2.4. Data collection

In RCT and OTR, two images must be collected from each field of molecules at two different angles in order to provide the necessary information for 3D reconstruction. In OTR, the goal is to obtain pairs of images
related by a 90° angle. Although many combinations for the first and second tilt angles can satisfy this requirement, there are certain advantages to using +45° and −45°. First, the overlap in the number of particles that are present in both micrographs is maximized, as the image compression due to the tilt is the same in both. Second, this geometry keeps the tilt angle for both micrographs in a pair to a minimum, helping to lessen the effect that stain-related artifacts may have during alignment and classification when negatively stained samples are used (discussed below). Third, because all particles come from micrographs having the same tilt angle, the effects of stain and tilt on their appearance will also be similar; the particles coming from the −45° and +45° micrographs can be aligned and classified together without the risk of tilt angle-related artifacts driving the process.

We have collected OTR data both manually and automatically. The data used for our reconstruction of the ATP-dependent chromatin remodeling complex RSC were collected manually (Leschziner et al., 2007). The data for the Rrp44-exosome reconstruction shown here were collected at NRAMM (The Scripps Research Institute) in an automated manner using the OTR option in the Leginon software package (Yoshioka et al., 2007).

For manual data collection, we have found it easier to collect all the images from a square at a given tilt before moving to the second tilt and collecting their tilt mates. This minimizes drift due to constant tilting of the goniometer and reduces data collection time. Our strategy typically involves the following steps: (1) Select good squares at very low magnification and store their coordinates, (2) Go to the first square and tilt (e.g., to −45°), (3) Take an image at a magnification that includes the entire square, (4) Print the image and mark the target holes, (5) Collect the full magnification images from all the holes, (6) Tilt to the second angle (+45°), and (7) Collect the tilt mates. Because one must be able to identify the target holes during data collection, we have found it convenient to use homemade holey carbon grids (with a continuous carbon support) as they provide unique and easily identifiable patterns.

For a thorough description of automated data collection with RCT and OTR geometries using the Leginon software package, see Yoshioka et al. (2007). The Rrp44-exosome data shown in this article were collected from samples that had been prepared on Quantifoil® grids.

2.5. Selection of particles

Of the few programs available for the selection of particles from micrograph tilt pairs (Frank et al., 1996; Scheres et al., 2008; Voss et al., 2009) only TiltPicker (Voss et al., 2009) is capable of handling both RCT and OTR geometries. Due to the underlying assumption of RCT geometry, the others cannot properly process OTR data. Whereas small deviations from the 0° assumed for the untitled micrograph in RCT result in negligible
compression of the image in the direction perpendicular to the tilt axis, this
effect is far more severe with 45° images. (For example, an image collected
at an actual 5° shows a ~0.4% compression relative to a 0° image, whereas
one collected at 50° is compressed by ~9.1% relative to the 45° image.)

We have semiautomated the picking of particle pairs from OTR micro-
graphs: the particles from one micrograph in each pair are selected manually
and their corresponding tilt mates are obtained automatically from the
second micrograph. TiltPicker (Voss et al., 2009) is now capable of extract-
ing OTR tilt pairs in a fully automated way but we have not tested it with
our data yet. We typically do the initial selection from one micrograph
(either the +45° or −45°) using EMAN’s Boxer (Tang et al., 2007), either
in interactive or semiautomated (Autoboxer) mode, but we have used
SPIDER’s WEB (Frank et al., 1996) as well. The coordinates from the
initial selection are then used to automatically find their tilt mates using a
series of SPIDER scripts that perform the following steps:

1. An initial search (performed with the operation AP SH) over in-plane
rotations (tilt axis angles) and “tilts”—manifested as cosine stretches or
compressions of the micrographs—finds the transformation that results
in the best match between the two micrographs (+45° or −45°) in a tilt
pair. These transformations are useful only in the context of these scripts,
as they do not represent a real geometric relationship between the two
micrographs. The parameters describing the tilt geometry must be
obtained separately (see Section 2.7).

2. The transformations are used to determine the overlap between the two
micrographs in a tilt pair (i.e., what particles are present in both micro-
graphs); particles that were originally selected but would not have mates
are ignored in subsequent processing.

3. The coordinates of the originally selected particles are used to window
out relatively large areas—several times the size of the box that will be
used for processing—centered on each particle.

4. The transformation calculated in (1) is used to generate a “guess” for
where the tilt mate of the particle windowed out in (3) will be located.

5. An area of the same size as the one in (3) is windowed out centered on
the “guess” coordinates calculated in (4).

6. A cross-correlation is calculated between the two windows and the
position of the peak is used to refine the initial guess and provide the
final tilt mate coordinates. These coordinates are used to window out the
tilt mates.

We have tested these scripts with a few different data sets and the success
rate (measured as the fraction of tilt mates that are both correct and centered
in their windows) is around 90%. (Scripts are available upon request.)
Does the sample adopt enough orientations to satisfy OTR requirements?

As discussed in Section 2.1, one of the main requirements for the application of the OTR method is the adoption by the sample of a large number of orientations on the support. Although it is simple to determine with alignment and classification whether a sample adopts one or a few preferred orientations, it is not possible to unambiguously establish that enough orientations are present without a priori knowledge about the structure.

We have addressed this limitation using the approach illustrated in Fig. 9.5. The basic idea behind this approach is that a sample adopting random orientations on the support will present the same overall set of views regardless of whether or not it is tilted during data collection. Therefore, the set of class averages obtained from untilted data should be matched by that obtained from tilted data. This ideal scenario is unlikely to ever be true: random orientations are seldom, if ever, observed and the lower quality of tilted data (due to staining artifacts or increased thickness in the stain) will affect the class averages. However, one can still expect to observe a good match between class averages obtained from tilted and untilted data, provided the sample adopts a large number of orientations. Conversely, failure to observe such a match can be taken as an indication that the sample is not amenable to OTR.

The untilted data required for this test are almost always available at the beginning of a project when $0^\circ$ images are collected for an initial characterization of the sample. Once the $0^\circ$ and $\pm 45^\circ$ particles have been separately aligned and classified an alignment is performed between the two sets of class averages to find the best-matching pairs. Although we usually judge the quality of the matches visually (Fig. 9.5), more quantitative comparisons
between class averages (such as Fourier Ring Correlation (FRC); Harauz and van Heel, 1986) could be used.

2.7. Determination of tilt geometry parameters

As I discussed in Section 2.5, it is not possible to obtain the tilt geometry parameters directly from particle-picking software. The parameters we obtain for the transformation between the two micrographs in a tilt pair as part of our automated particle picking are simply a tool to make this automation possible but they do not bear any relationship with the real tilt geometry. The same is true for TiltPicker, which automates the extraction of the molecular images but does not calculate the tilt geometry parameters for OTR data (Voss et al., 2009).

We obtain the tilt angle, tilt axis angle (the position of the tilt axis relative to the coordinate system), and defocus (at the center) for each micrograph using the program CTFTILT (Mindell and Grigorieff, 2003). An additional advantage of determining the parameters using CTFTILT is that the program outputs both degree and sign of the tilt, preventing bookkeeping errors if the user has switched the order in which the two tilts are collected. Of course, this is not an issue when automated data collection is used (as was the case for the exosome data shown here, obtained using Leginon) as the database stores this information.

We store the output from CTFTILT in a SPIDER-format file to be used in subsequent processing.

Although we have not implemented it, one could use the output from CTFTILT to constrain the search over tilt angles and tilt axis angles used to find the tilt mates in our semiautomated particle picking (see Section 2.5)

2.8. CTF correction

Images collected from a tilted sample have, by definition, a defocus gradient. This is quite significant at 45°; an image collected at a magnification of 50,000 on a 4 × 4 k CCD camera will have a defocus gradient of 1.23 μm. This is clearly more severe on film or larger CCD cameras. Since OTR relies on the alignment and classification of tilted data, it is to some extent affected by the wide range of defoci present in the data. In order to minimize this effect, we always correct the CTF of our particles by multiplying by the calculated CTF.

Our CTF correction is done particle-by-particle using the defocus value and tilt geometry parameters obtained from CTFTILT (Mindell and Grigorieff, 2003). The coordinates of each particle are used to calculate the defocus at that particular location and that defocus is then used to generate the estimated CTF for correction.
2.9. Alignment and classification

Alignment and classification of OTR data are similar to those used with other reconstruction methods. The data used in this article were aligned and classified using multivariate statistical analysis and hierarchical ascendant classification as implemented in the IMAGIC software suite (van Heel et al., 1996). There are two aspects specific to OTR: (1) the utilization of alignment information to detect and eliminate classes that are likely to be enriched in flattened particles and/or particles with preferred orientations and (2) the fact that the $-45^\circ$ and $+45^\circ$ data can be combined rather than be treated as two separate sets used for alignment/classification and reconstruction (discussed in Section 2.1).

As I discussed in Section 2.1, OTR requires samples that adopt a large number of orientations on the support. This was illustrated in Fig. 9.1E', where each image shown in the equator surrounding the structure arises from a molecule adopting a different orientation on the grid. If the sample adopted one or a limited number of orientations, all the equatorial images used for reconstruction would be located at a single point on this equator, on a line perpendicular to the tilt axis (the left image shown in Fig. 9.1B'). This situation can result from a sample adopting preferred orientations (Fig. 9.6C) and/or from flattening of the sample (Fig. 9.6B). Even with samples where flattening has been minimized and the number of orientations maximized (see Section 2.3 above), one is likely to see certain orientation bias and a distribution in the severity of flattening in different parts of the sample. Whenever these factors result in unique species, a situation like that in Fig. 9.1B' will arise. The noisy nature of the data, however, will lead to the equatorial images spreading out around the line perpendicular to the tilt axis (i.e., not all images will coincide exactly with that shown in Fig. 9.1B' but will rather lie in its vicinity). This unusual distribution is immediately apparent in a plot of the in-plane rotation angles from alignment and classification (Fig. 9.6D) and we have observed these biased angular distributions with both real (Leschziner et al., 2007) and synthetic data (Leschziner and Nogales, unpublished). We take advantage of such plots to detect and remove classes that are likely to contain preferred orientations and/or flattened particles. Regardless of their origin, these classes would not lead to useful reconstructions given their incomplete sampling of Fourier space. Whether this potential selection against flattened particles is responsible for the fact that OTR reconstructions appear relatively unaffected by flattening even when generated from the same grid that gave rise to apparently flattened RCT reconstructions (see Section 2.11 below and Fig. 9.7) is something we have not established yet.
Figure 9.6  Flattening and preferred orientations manifest themselves in tilted images. In panels A–C, a molecule (the exosome) is first shown looking down towards the support ("top view," top row) and then tilted 90° ("side view," middle row). The bottom row shows the resulting projection in an orientation equivalent to that of the "top view." (A) Flattening is mostly not apparent when images are collected at 0°. The molecule on the right has been flattened by 1/3 along the Z-axis (parallel to the projection direction), an effect that is not seen in the projection. (B) This situation changes when images are collected from tilted samples. In the absence of flattening, the same projection can be obtained from molecules adopting different orientations on the support (first two columns); this is what makes OTR possible. If flattening occurs, however, the two projections are now different because the direction of flattening and projection are no longer parallel (last two columns). Therefore, flattening that was mostly unnoticed in an untilted sample (A) becomes obvious in a tilted one (B). (C) Samples with preferred orientations are not amenable to reconstruction by OTR. In an untilted sample, the random in-plane rotations of the molecules on the support result in trivial in-plane rotations between projections that are otherwise identical (first two columns). Upon tilting, however, an in-plane rotation of the molecule results in projections that are no longer the same (second two columns). It can be seen in the last two columns in C that the projections shown can be obtained with one and only one orientation of the molecule on the support. It therefore becomes impossible to obtain a class containing a full set of equatorial views of the molecule as shown in Fig. 9.1E. A comparison of panels B and C shows that flattening is, in a sense, a particular case of preferred orientation in that flattening turns each orientation into a unique species. (D) Representative plots of in-plane rotation angles from alignment and classification showing classes with random (top) and biased (bottom) distributions of angles. Each line in the plots represents the in-plane rotation angle (from multireference alignment) for a given particle in a class. Panels B and C show why flattening and/or preferred orientations will give rise to biased distributions of in-plane rotation angles.
Once alignment and classification have been completed, the final in-plane rotations obtained from the alignment must be combined with the tilt geometry parameters (see Section 2.7) to generate the Euler angular file for reconstruction. Particular attention must be paid to any conversions required to account for the different conventions adopted by the programs used during data processing. In our case, we must combine rotation angles from alignment with IMAGIC (van Heel et al., 1996) with tilt geometry parameters obtained with CTFTILT (Mindell and Grigorieff, 2003) to generate an Euler angular file that can be used for reconstruction in SPIDER (Frank et al., 1996). The following formulas are used to calculate the final Euler angles (Φ, Θ, Ψ), which should be written out in that order in the angular file to be used for reconstruction with the SPIDER commands BP RP or BP 32F:

\[
\begin{align*}
\phi & = RCT \left( Y + 90^\circ \right) \\
\theta & = RCT OTR \\
\psi & = Merged (A) \\
\end{align*}
\]
\[ \Phi = \gamma_{\text{untilted}} - 90^\circ - \text{MRA} \]
\[ \Theta = \Theta_{\text{tilted}} - \Theta_{\text{untilted}} \]
\[ \Psi = 90^\circ - \gamma_{\text{tilted}} \]

where \( \gamma_{\text{untilted}} \) is the tilt axis angle (i.e., the in-plane rotation of the actual tilt axis relative to the coordinate system) obtained from CTFTILT for the “untilted” micrograph; “MRA” is the in-plane rotation obtained from alignment and classification; \( \Theta_{\text{tilted}} \) and \( \Theta_{\text{untilted}} \) are the tilt angles from the “tilted” and “untilted” micrographs, respectively, from CTFTILT and \( \gamma_{\text{tilted}} \) is the tilt axis angle from CTFTILT for the “tilted” micrograph. The “90°” terms correct for the different conventions used by SPIDER and CTFTILT. The “MRA” angle is subtracted in this case to calculate \( \Phi \) to account for the different rotation conventions used by SPIDER and IMAGIC. If the alignment and classification were performed in SPIDER (or any other package with the same convention—a positive angle being a counterclockwise in-plane rotation), the “MRA” angle would be added instead.

It is important to keep in mind that in OTR, unlike RCT, “tilted” and “untilted” are relative terms. We use “untilted” to refer to the particles in a class and “tilted” to refer to their tilt mates, which are used for reconstruction. Because the +45° and −45° particles can all be used for alignment and classification (see Section 2.1), every particle will get to play both roles. For example, if a −45° particle A is assigned to class X and its 45° tilt mate B is used for reconstruction, we would say that A is “untilted” and B is “tilted”. B is the tilt mate of A with an effective tilt angle (\( \Theta \)) of 90°. At the same time, particle B will have been assigned to a different class (Y) with A now being its tilt mate. In this context, B is the “untilted” particle and A is its “tilted” mate with a tilt angle (\( \Theta \)) of −90° (note the reversal in sign when the two particles switch roles).

Once the Euler angular file has been created, reconstruction of OTR single-class volumes proceeds in the same manner used for RCT volumes. The main difference, introduced in Section 2.1, lies in the fact that in OTR the same set of particles used for alignment and classification can be used for reconstruction, while in RCT one switches from the untilted to the tilted set of particles. As a result of this, a typical OTR class would consist of particles coming from both +45° and −45° micrographs and the tilt (\( \Theta \)) angles used for reconstruction of that class volume would contain values around both +90° and −90°.

We perform all data processing following alignment and classification using SPIDER (Frank et al., 1996). Class volumes are generated by back-projection (using the commands BP RP or BP 32F). The translational parameters of the particles used for reconstruction are iteratively refined using projection matching.
2.11. Comparison between OTR and RCT reconstructions

In order to illustrate the different properties of OTR and RCT reconstructions, I will end with a direct comparison of volumes generated from Rrp44-exosome data with both methods. As I indicated in Section 2.2, the OTR data presented here were obtained from the same EM grid Wang et al. (2007) used for their RCT reconstruction of the yeast exosome, thus removing sample preparation as a potential source of variability. The main differences between the two data sets are the following: (1) While both were collected at 120 kV, the OTR data were collected on a Tecnai F20 microscope (NRMM, The Scripps Research Institute) and the RCT data set on a Tecnai G2 (LaB6) instrument (Wang et al., 2007), (2) The RCT data were collected on film (Wang et al., 2007) and the OTR data on a 4 × 4 k CCD camera, (3) The OTR data set is significantly larger (12,692 tilt pairs vs. 3,872 tilt pairs for the RCT reconstruction), as would usually be the case due to the requirement for a large number of orientations. Data processing was otherwise comparable, with IMAGIC (van Heel et al., 1996) used for alignment and classification and SPIDER (Frank et al., 1996) for reconstruction and refinement in both cases.

The comparison presented here is between the two RCT reconstructions reported by Wang et al. (2007) and two of our best initial reconstructions. We selected these with a combination of visual inspection and by looking at the match between the 0° projection of the volume and the class average giving rise to it as well as the match between a set of pseudo-evenly spaced projections of the volume and the experimental class averages. It should be noted that the two RCT volumes are the result of merging either six or four different class volumes, while the OTR reconstructions correspond to single classes (see Figs. 9.7 and 9.8). This is a more relevant comparison than looking at single-class RCT reconstructions because the merging of volumes (to try to solve the Missing Cone problem) is an integral part of RCT. Conversely, we have not attempted to merge OTR reconstructions because the ability to use single-class volumes as initial models for refinement is one of the strengths of the method. Partly as a consequence of the difference in the type of volume used for the comparison—merged versus single-class—the RCT reconstructions contain a larger number of particles: they were generated from 701 (volume A in Figs. 9.7 and 9.8) and 633 (volume B in Figs. 9.7 and 9.8) particles, while the OTR ones are the result of 222 (volume A in Figs. 9.7 and 9.8) and 240 (volume B in Figs. 9.7 and 9.8) particles.

Figure 9.7 shows a few views of surface renderings of the two RCT and two OTR reconstructions along with the corresponding views of the final refined reconstruction from Wang et al. (2007). This figure illustrates the fact that single-class OTR reconstructions display more of the features observed in the refined structure than even the RCT merged reconstructions. Most
In this figure, we have used FRCs to compare projections from the RCT and OTR reconstructions with the corresponding projection from the refined exosome structure (EMDB entry EMD-1438) (Wang et al., 2007). This measure can be taken as an indication of the amount of information about the final structure already present in the initial reconstructions. All four initial models (the same ones introduced in Fig. 9.7) were aligned to the refined structure (shown in grey). (A and B) projections were calculated at $0^\circ$ and $90^\circ$ ($0^\circ$ being the direction of the class average that gave rise to the volume and $90^\circ$ being directions orthogonal to it). The volumes in (B) are shown in the orientation corresponding to the $0^\circ$ projection. FRCs were calculated between the $0^\circ$ and $90^\circ$ projections and the corresponding ones from the refined structure; a few representative plots are shown in (C). The FRC plots are color-coded following the coloring of the volumes in (B) (and Fig. 9.7). I have indicated, below each plot, the resolution (in Å) corresponding to 0.5 FRC for the better-performing RCT and OTR reconstruction in each case. The RCT and OTR volumes were low-pass filtered to 20 Å and displayed using Chimera (Pettersen et al., 2004).
strikingly, the OTR reconstructions do not show any sign of flattening despite the fact that all the data originated from the same grid. As I mentioned in Section 2.9, we expect that alignment and classification of tilted data and the removal of classes that show a biased distribution of rotation angles (see Fig. 9.6) could lead to reconstructions that are enriched in particles unaffected by flattening. Additionally, since an OTR class is comprised of projections coming from particles adopting different orientations on the support, it is possible that flattening is “averaged out” to some extent in the reconstructions. Whatever its origin, this relatively more faithful representation of the structure’s dimensions appears to be a feature of OTR volumes.

Although surface renderings provide some information in terms of comparing reconstructions, it is the projections of the initial volumes that are most important, as these will be used for refinement to higher resolution. The best initial models will contain the highest amount of information relative to the final structure and will yield good representations of the structure (i.e., references) in all possible projection directions. As a result of the geometry of the Missing Cone, projections generated from RCT reconstructions in a direction perpendicular to the beam axis (orthogonal to the class average that gave rise to the volume) would be most affected by artifacts. On the other hand, the $0^\circ$ projection, corresponding to a section in Fourier space that is fully sampled, should be of a high quality. OTR volumes, because of their fully sampled nature, should give rise to projections of equal quality regardless of their direction. To determine whether these predictions held for the exosome reconstructions, we measured FRC (Harauz and van Heel, 1986) between projections generated from the four initial reconstructions (both OTR and RCT) against the corresponding projection from the refined exosome structure (Wang et al., 2007) (EMDB entry EMD-1438). We generated projections in the $0^\circ$ direction (corresponding to the class average) as well as a number of $90^\circ$ directions (Fig. 9.8A and B). Figure 9.8C also shows a few representative plots of the FRCs along with an indication of the “resolution” of the projections (defined as the frequency at which the correlation between a projection from an RCT or OTR volume and the corresponding one from the refined exosome structure reached 0.5) (see Chapter 3).

As can be seen in Fig. 9.8C (first plot) and even visually in Fig. 9.8B ($0^\circ$ projection), RCT outperforms OTR in the direction of the class average giving rise to the reconstruction. We had observed the same phenomenon with synthetic data and had attributed it to the absence of a defocus gradient in the $0^\circ$ data used for alignment and classification of RCT data. Additional factors with experimental data that would further favor the alignment and classification of RCT data are the staining and data collection artifacts associated with tilted images.

All other directions, however, show either very minor differences among the different projections (plots 4 and 5 in Fig. 9.8C) or significantly higher resolution in the OTR projections (plots 1 and 7 in Fig. 9.8C).
We are yet to find a 90° projection of an RCT volume that significantly outperforms its OTR equivalent. It should be emphasized that the RCT volumes contain approximately three times more particles than the OTR ones. Furthermore, the FRGs were calculated using projections from the refined exosome structure ([Wang et al., 2007], which should, if anything, favor the merged RCT volume A, which was used as its initial reference. Our OTR single-class volumes can be refined, by projection matching, to structures very similar to that published by Wang et al. (2007) (not shown).

3. Conclusion

I have presented here an overview of the main steps required to generate reconstructions using the OTR method. All the steps can be performed using tools available in the field and even data collection, arguably the most challenging aspect of the method, has been automated (as shown by the data presented here and discussed in further detail in Chapter 15 of vol. 483).

A comparison of reconstructions obtained from the same sample using the RCT and OTR methods shows the ability of the latter to generate fully sampled, artifact-free initial models. This makes OTR particularly useful for novel asymmetric samples with potential heterogeneity where geometric methods are required but the merging of volumes to solve the Missing Cone problem poses a serious challenge. OTR volumes, due to their full sampling, can be directly refined to higher resolution without the need for user intervention.

ACKNOWLEDGMENTS

I thank Hongwei Wang for generously providing us with the sample grid used for his exosome reconstructions, for sharing data necessary for our comparisons, and for discussions on the differences among the reconstructions. I thank NRAMM, where the data shown here were collected and, in particular, Craig Yoshioka and Neil Voss for their help during data collection. I also thank Preethi Chandramouli for generating most of the data presented in this review. AL is supported by a fellowship from the Alfred P. Sloan Foundation.

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