CHAPTER FIVE

GraFix: Stabilization of Fragile Macromolecular Complexes for Single Particle Cryo-EM

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Contents

Abstract

Here, we review the GraFix (Gradient Fixation) method to purify and stabilize macromolecular complexes for single particle cryo-electron microscopy (cryo-EM). During GraFix, macromolecules undergo a weak, intramolecular chemical cross-linking while being purified by density gradient ultracentrifugation. GraFix-stabilized particles can be used directly for negative-stain cryo-EM or, after a brief buffer-exchange step, for unstained cryo-EM. This highly reproducible method has proved to dramatically reduce problems in heterogeneity due

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to particle dissociation during EM grid preparation. Additionally, there is often an appreciable increase in particles binding to the carbon support film. This and the fact that binding times can be drastically increased, with no apparent disruption of the native structures of the macromolecules, makes GraFix a method of choice when preparing low-abundance complexes for cryo-EM. The higher sample quality following GraFix purification is evident when examining raw images, which usually present a low background of fragmented particles, good particle dispersion, and high-contrast, well-defined particles. Setting up the GraFix method is straightforward, and the resulting improvement in sample homogeneity has been beneficial in successfully obtaining the 3D structures of numerous macromolecular complexes by cryo-EM in the past few years.

1. Introduction

The study of biological macromolecules at a molecular level has become a reality in the past decades. An important tool for this is high-resolution cryo-EM of single particles, which can be used to determine the three-dimensional (3D) structures of macromolecular complexes in their native forms. To prepare cryo-EM samples, purified complexes in solution are rapidly frozen, and the resulting vitrified complexes remain virtually artifact-free within their native buffer environments (Adrian *et al.*, 1990; Chapter 3, this volume**)**. However, since the density of proteins is only slightly greater than that of vitrified ice, the particles have low contrast against the background. The use of negative-stained cryo-EM can increase the particle contrast, yet staining also introduces a number of limitations. Thus, while negative-stain cryo-EM can be beneficial in certain circumstances, unstained cryo-EM is the method of choice. Sample preparation and image analysis of vitrified samples is technically challenging, and much effort has been made recently to develop new and improved methods for cryo-EM.

It is now routine to obtain resolutions of higher than 10 Å for particles that are well-suited for the technique, that is, particles that are mostly symmetrical, rigid, and conformationally homogenous (Schuette *et al.*, 2009; Wolf *et al.*, 2010; Zhang *et al.*, 2008, 2010a,b). Viral particles are ideal for this method, and 3D reconstructions of several have been determined at near-atomic resolution (3.7–4 Å) **(**Chapter 15, Vol. 482**)**. A recent breakthrough was made for a cryo-EM structure of the infectious subvirion particle (ISVP) of aquareovirus, which was resolved to 3.3 Å (Zhang *et al.*, 2010a,b). This resolution allowed *ab initio* model building for the final structure, in which a detailed protein structure could be distinguished. Reaching atomic-level resolution would allow cryo-EM to deliver as much structural information as X-ray crystallography yet with the

advantage that one could work with complexes that are available at only very low concentrations and/or are too large or difficult to crystallize.

However, there are still a large number of technical issues to be dealt with prior to obtaining subnanometer or better resolution levels for the majority of macromolecular complexes that do not fit into this description. One major issue is dealing with the conformational flexibility within complexes, which is often linked to the ability of a complex to take on various functional states. Thus, even though a sample may be chemically pure, it can contain numerous different conformations. Structure determination of such samples requires the computational separation of the data into subpopulations of images that represent all possible conformational states that are present in the given sample. In recent years, new image processing strategies have been developed that indeed allow such a computational "purification" of images (Sander *et al.*, 2006; Scheres *et al.*, 2005, 2007) **(**Chapter 13, Vol. 482; Chapter 10, Vol. 483**)**. While such attempts are currently used in the intermediate resolution regime, they will also become a prerequisite for high-resolution structure determination of dynamic macromolecules by single particle cryo-EM in the future.

In the light of this, heterogeneity that arises from sample degradation during purification is an additional complication that should be avoided to the greatest degree possible. However, the ability to undergo conformational changes also often infers a flexibility to the particles that makes them more labile when being processed. Additionally, *in vitro* buffer conditions can lead to destabilization of the complexes. Difficulties in distinguishing between the conformational isoforms, the rotational degree of freedom of a single complex isoform, and partially degraded particles, in the presence of high levels of background noise, can drastically limit the probability of obtaining a high-resolution 3D reconstruction.

To avoid the problems arising from sample heterogeneity when purifying macromolecular complexes, we have recently introduced the GraFix (from *Gra*dient *Fix*ation) protocol (Kastner *et al.*, 2008). In this protocol, macromolecular complexes are exposed to a low concentration of a chemical cross-linker during sedimentation by ultracentrifugation through a density gradient. We have determined that, following the mild fixation and purification with GraFix, samples display drastically reduced to no degradation, as well as an improved quality of both the individual particle characteristics and the particle dispersion in the raw images. We and others have now used GraFix to determine 3D structures of numerous macromolecule particles (Golas *et al.*, 2009; Herzog *et al.*, 2009). For particles that are present only at an extremely low abundance in cells, using GraFix has proved to be not only helpful for improving sample quality but also essential for obtaining enough particle images for 3D reconstruction (Golas *et al.*, 2009). I discuss the particulars of this method and how we have implemented it in our laboratory, in the following review.

2. Overview of the GraFix Procedure

The GraFix procedure combines purification by zonal ultracentrifugation with cross-linking through increasing exposure to a cross-linking reagent (see Fig. 5.1 for a schematic representation). Isolated particles can be directly used for negative-stain EM or for unstained cryo-EM following a buffer-exchange step (Fig. 5.1B). The GraFix procedure is highly

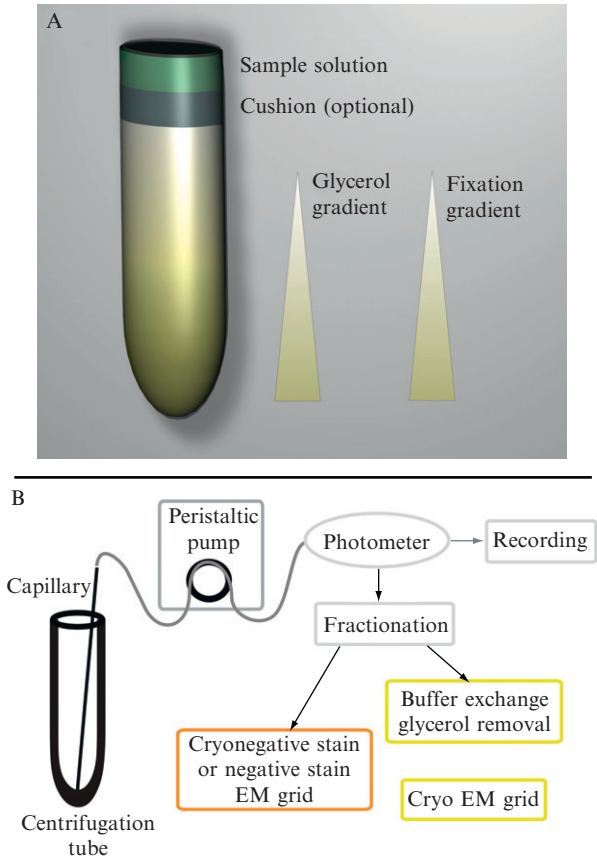

Figure 5.1 The GraFix method. (A) The centrifugation tube contains a gradient of both density (provided by glycerol) and cross-linking reagent, with an optional buffering cushion over the gradient. (B) Schematics of the GraFix method. Following ultracentrifugation, gradients are fractionated from bottom to top. Fractions can then be negatively stained or, following a one-step buffer exchange to remove the glycerol, plunge-frozen across EM grids.

reproducible, allowing for purification of a complex to proceed routinely after the initial setup. While we focus here mainly on using this procedure for the preparation of cryo-EM, it is important to point out that this is a universal procedure that could also benefit other structural analysis techniques that require the purification of high-quality, unbound complexes in their native state.

2.1. Chemical fixation of complexes during GraFix

2.1.1. Promotion of intramolecular cross-linking

Stabilization of large, fragile complexes by chemical cross-linking is a way to avoid disruption of complexes during the preparatory steps of the samples for cryo-EM. During cross-linking, covalent bonds are formed between the functional groups of the cross-linking reagent and those of the macromolecule, increasing the rigidity of the complex. However, the direct addition of chemical cross-linkers to the purified complexes is not usually a viable option for several reasons. Most purified macromolecular complexes are purified under buffer conditions that often promote weak aggregation of the complexes, so that the direct cross-linking of the complexes can result in intermolecular fixation, thereby increasing sample heterogeneity. Additionally, intermolecular cross-linked complexes are more likely to aggregate and precipitate out of the solution, leading to loss of sample material. The GraFix procedure avoids this problem through the increased pressure acting on the macromolecules as a result of the centrifugal force. This force is usually sufficient to disrupt weak aggregations, so that macromolecules are exposed to the chemical cross-linking reagent as individual complexes. Thus, the vast majority of the complexes will only undergo intramolecular, but not intermolecular, cross-linking. Obviously, if the concentration of the sample is too high, intermolecular cross-linking will occur. We have been able to avoid intermolecular cross-linking completely by applying less than 180 pmol of sample on a single GraFix tube (as tested for the ribosome). When using quantities higher than this, dimer cross-linking was observed. However, in general, amounts of sample higher than 180 pmol are not necessary when purifying samples for cryo-EM, since highly concentrated samples have to be subsequently diluted prior to preparing the EM grids. In any case, high sample concentrations are normally not obtainable for most macromolecules following commonly used biochemical purification procedures. For sample concentrations that are well-suited for EM grid preparation (e.g., that do not need to be diluted after GraFix), there is little danger that intermolecular cross-linking will occur.

2.1.2. Cross-linking in a compatible buffer

A further advantage of cross-linking during GraFix is that the buffer in which the cross-linking occurs be selected for compatibility with the cross-linker. Reactivity of buffer components with the cross-linking reagent is an important problem when cross-linking samples directly. For example, primary amino groups, such as those found in the TRIS buffer, are reactive with the commonly used aldehyde cross-linking agents. Reactive agents may also have been introduced during a previous purification step, such as by purifying a complex from an immunoaffinity column with peptides, which adds a significant amount of primary amine containing amino acids to the final buffer. This source of contamination is especially relevant due to the recent advances in purifying complexes by affinity-selection. The problem with the presence of cross-reactive agents in the sample buffer is by-passed by the GraFix method, by adding the cross-linking reagent to a density gradient rather than to the sample directly. In this way, the buffering environment of the macromolecular complex is completely exchanged prior to contact with the cross-linking reagent, without loss of sample due to an extra buffer-exchange step. An important point here is that there is a gradient not only of density but also of the cross-linking reagent, since the cross-linker is added only to the heavier, bottom solution and not to the lighter, top solution. The concentration of the cross-linking reagent at the top of the gradient is extremely low, and this is usually sufficient to prevent sample buffer artifacts during cross-linking. An additional (but not essential) precaution can be taken by replacing the top-most layer of the gradient with a cushion without a cross-linker reagent prior to applying the sample (Fig. 5.1A); in this manner, the sample buffer will never come in direct contact with the cross-linking reagent in the presence of the macromolecular complex, completely avoiding any chances of artifacts due to buffer reactivity with the cross-linking reagent. Thus, the composition of the original sample buffer has no impact on the cross-linking reaction.

2.1.3. Weak cross-linking with no apparent structural artifacts

Chemical cross-linking has always had a somewhat bad reputation of generating artifacts, mainly in more traditional cell biological EM applications (Hayat, 1986). Importantly, we were able to demonstrate that cross-linking with glutaraldehyde did not lead to any visible artifacts using the GraFix method up to the $\sim$12 Å resolution level. This may be due in part to the fact that the cross-linking conditions that we have established using glutaraldehyde lead to a weak cross-linking, in which not all lysines within each particle are chemically modified. Nonetheless, as previous reports have determined that glutaraldehyde may lead to artifacts during cross-linking, it is important to note that other cross-linkers, such as formaldehyde and

acroleine, can also be used. It is still unclear whether, and to which extent, cross-linking by GraFix will limit the obtainable resolution due to potential perturbations in the native structure that could only be visualized at higher resolution. However, we have established that GraFix does not interfere with sample integrity at intermediate resolutions of up to 12 Å, a resolution which can be extremely useful for determining reliable initial structures.

2.2. Purification over density gradients

In addition to providing a compatible environment for chemical cross-linking of macromolecular complexes, GraFix can also introduce a convenient purification step that may make it possible to eliminate previous steps of the purification. Density gradient centrifugation is a powerful technique for separating complexes based on their molecular masses, and the range of separation can be determined by selecting the density range. It should be noted that glycerol can be substituted for other sugars to create a density in the gradient, if required. To date, we have successfully tested sucrose, trehalose, and arabinose.

2.2.1. Glycerol removal prior to unstained cryo-EM

Direct processing of GraFix-purified complexes is possible when the samples are used for negative-stain or cryonegative stain EM (Chapter 6, this volume). However, high concentrations of glycerol (such as the 15–25% used in GraFix) interfere with the high-contrast image formation of macromolecules embedded in vitrified ice. Thus, prior to using the GraFix-purified particles for unstained cryo-EM, the glycerol in the buffer has to be removed. This can be done in a simple, one-step procedure using a buffer-exchange column. It is important to note that buffer-exchange columns cannot normally (e.g., in the absence of particle fixation) be included in the purification scheme, since unfixed complexes are easily damaged during this procedure. However, GraFix-stabilized complexes are usually not affected. This step allows a rapid removal of almost all the glycerol in the buffer, and the samples are then suitable for direct analysis by unstained cryo-EM.

2.3. Reduction of sample heterogeneity

The reduction of sample heterogeneity due to particle disintegration can be dramatically improved when handling GraFix-stabilized complexes during grid preparation. This is exemplified by images of the spliceosomal B complex, a macromolecular complex formed by three snRNAs and more than 100 proteins that sediments at 40S. The spliceosomal B complex is too labile to be subjected to any type of column purification but can be successfully purified over a glycerol gradient. When complexes were

purified in the absence of chemical cross-linking and used directly for negative-staining cryo-EM, the resulting raw images were of poor quality, displaying heterogeneous particles, a high background of particle fragments, and poor particle distribution (Fig. 5.2A, top). The low quality of the raw image is reflected in the class averages following statistical analysis: only ~10% of the images could be ordered into class averages that display well-defined structures (Fig. 5.2A, top row of the bottom panel). In contrast, purification with the GraFix method stabilized the spliceosomal B complexes sufficiently to almost completely prevent their disruption during grid preparation. The raw images of the GraFix particles show clearer outlines and improved fine structural features, and the number of smaller, broken particles was significantly reduced. This improvement in image quality can drastically increase the number of good quality class averages that can be obtained. For instance, following image alignment, multivariate statistical analysis, and classification, there was an approximately fivefold increase in the number of high-quality class averages for the spliceosomal B complex prepared by GraFix as compared to that prepared by gradients alone. Since the individual particles displayed a higher degree of structural homogeneity, these class averages also had a higher signal.

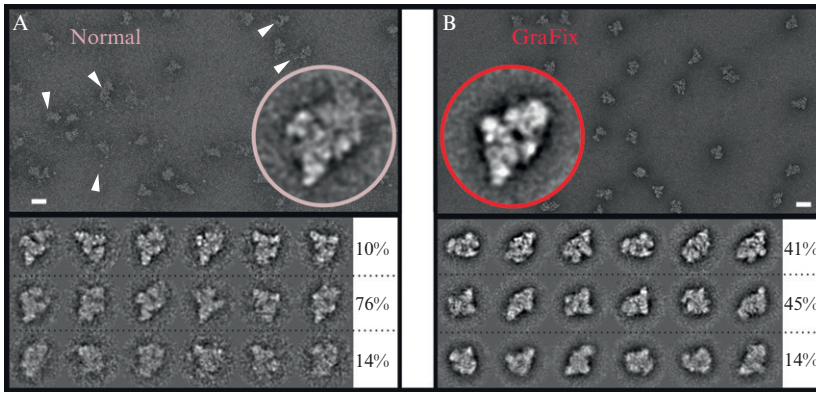

Figure 5.2 Negative-stain cryo-EM images of spliceosomal B complexes with or without GraFix. (A, B) Electron microscopic raw image of uranyl formate-stained spliceosomes prepared by a conventional glycerol gradient (A) or GraFix (B). Scale bars, 40 nm. Arrowheads, smaller broken parts and flexible elements. Insets, similarly oriented spliceosomal class average. Class averages obtained from a set of 5000 raw images of non-GraFix-prepared (A) or GraFix-prepared (B) samples are shown in the bottom panels. The average number of class members is 15 images. Class averages were sorted with respect to contrast and structural definition. GraFix treatment (B) generates computed class averages with much improved contrast (top and middle; 86% of images), as compared to samples prepared by the conventional method (A), where only 10% of class averages (top) show relatively well-defined structural features.

2.3.1. Structural heterogeneity

The question arises as to whether macromolecular complexes that have been chemically stabilized have been locked into a specific conformation, thereby reducing the conformational heterogeneity. This could be advantageous in simplifying the complexity of sorting the particles presented in the raw images. Our observations so far suggest that, while this may occur, it is a minor effect, which is perhaps not a bad thing. Aside from the technical difficulties that arise, dealing with various conformations of a macromolecular complex within the same conditions can be extremely informative. Often, the assumed conformations parallel the functional conformations, so that comparison of their structures can be key in understanding how the complex functions at a molecular level. We and others have recently observed this for the ribosome (Fischer *et al.*, 2010) working with a highly dynamic preparation of ribosomes in various stages of translocation (that had not been treated with GraFix). While the initial resolution was limited to $\sim$20 Å, computational sorting allowed us to obtain a subnanometer resolution for certain subpopulations of the data set. The level of computational sorting was also sufficient to observe the molecular dynamics of the ribosome (i.e., the movement of tRNAs through the ribosome and the correlated motions within the ribosome itself). While dealing with structural heterogeneity currently represents one of the major resolution-limiting factors for 3D structure determination, it will make cryo-EM a powerful tool for analyzing molecular functions of macromolecular complexes once methods become routine to separate mixed population of images by computational image processing.

2.4. Advantages of using GraFix particles during carbon film binding

2.4.1. Increased particle binding

We have observed that GraFix purification of macromolecular complexes can often increase their binding to the carbon support film. Importantly, because of their chemical stabilization, GraFix-purified complexes can also be allowed to adsorb onto carbon film for extended periods of time without sacrificing the quality of the complexes. The combination of an improved binding over a longer time can lead to a significant increase in the number of bound particles. This may be able to compensate for any particle loss that may have occurred during the gradient purification. Importantly, this also increases the chances of being able to acquire enough images from particles that are present only in low-copy numbers in cells and cannot be easily purified at higher concentrations. As an extreme example, images of the *Trypanosoma brucei* kinetoplastid RNA editing complex following purification with GraFix are shown (Golas *et al.*, 2009). After a normal adsorption

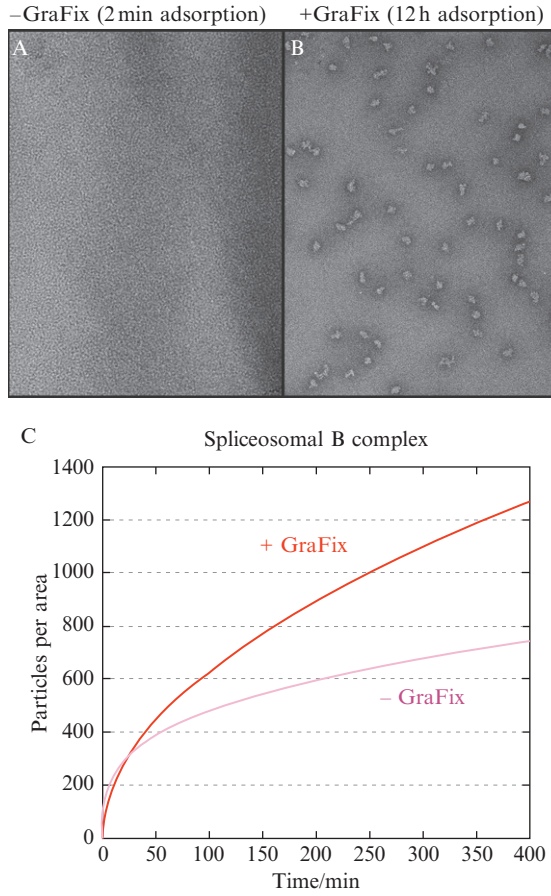

Figure 5.3 Enhanced GraFix particle binding to carbon support film. (A, B) Negative-stain cryo-EM image of the *T. brucei* kinetoplastid RNA editing complex after GraFix purification. After 2 min of adsorption time, no particles were detected on the grids (A). Extending the adsorption time up to 12 h led to good quality images with an acceptable particle distribution that allows further single particle analysis (B). (C) Binding rates of spliceosomal B complexes either treated with GraFix (red) or purified over a glycerol gradient in the absence of cross-linking (pink). No difference in particle concentration was observed after 2 min, but there was an increase of approximately twofold after ~6 h of adsorption. There were no visible signs of particle disintegration even at the longest time points for the GraFix-stabilized particles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this chapter.)

time of 2 min, no particles were visible in the raw image (Fig. 5.3, top left panel). In contrast, when the GraFix particles were allowed to adsorb for 12 h, a large number of good quality particles were distributed throughout

the image (Fig. 5.3, top right panel). Additionally, as shown by comparing the particle binding of the spliceosomal B complex, there was a higher binding density over time of particles treated with GraFix as compared to nontreated particles (Fig. 5.3, bottom panel). The ability to increase particle binding during grid preparation is important as it could play a critical role in determining 3D structures of low-abundance complexes that can only be purified in extremely low concentrations.

2.4.2. Increased isotropic particle orientation on film

Many macromolecular complexes tend to bind to the carbon support film with only a few preferential orientations. This binding bias reduces the angles from which the particle is viewed, leading to nonisotropic sampling of information in 3D space. Following chemical fixation, the charge distribution of the complex changes. We have observed that this can sometimes lead to a more isotropic particle orientation when binding on carbon film, making it possible to obtain more particle views. This is beneficial not only for the initial structure determination phase, where a large number of different views make the initial model-more reliable, but also for higher resolution structure determination with isotropic resolution.

2.5. Coanalysis of proteins within the cross-linked particles

Once cross-linking of the particles has occurred, it is no longer possible to directly analyze the composition of the complex by gel electrophoresis, since the cross-linking is irreversible and the proteins no longer dissociate following heat or detergent treatment. However, it is still possible to analyze the protein composition by running a parallel gradient without a cross-linking reagent which may then be used for SDS gel electrophoresis. Ideally, of course, one would like to analyze the GraFix-stabilized fraction directly. This could be done by using a reversible cross-linker, such as paraformaldehyde, in which case the cross-linking could be reverted prior to running the sample on an SDS gel. Another option that we are currently testing is to apply the chemically stabilized complexes to mass spectrometry analysis. Initial studies on several complexes indicate that it is indeed possible to study the protein composition of glutaraldehyde-cross-linked complexes by a combination of trypsin digestion and mass spectrometry (Richter *et al.*, 2010). Since trypsin requires accessible lysines (that have not been cross-linked) to enzymatically cleave the protein, it is important to note that this is possible following GraFix only because the cross-linking is weak and not all lysines have been modified.

3. Methods

3.1. Guidelines for determining centrifugation parameters

Determining how to set up a centrifugation gradient depends on the sedimentation value of a complex. In most cases, however, the *S*-value is not known, and often the exact size of the entire complex is unknown. To overcome this, we use a simulation program that predicts the centrifugation of a complex based on a rough estimate of its *S*-value. Based on this, we have compiled guidelines for determining the gradient conditions (Table 5.1). Use of these guidelines has proved very effective, which can avoid trial-and-error when setting up gradient conditions for the first time, reducing loss of sample and time.

3.2. Preparing a continuous density gradient

Gradients are created by mixing two solutions with a low and a high density and are obtained by adding glycerol, sucrose, or another carbohydrate in appropriate concentrations. Table 5.1 shows the density values for the gradient based on the molecular mass. Thus, for a macromolecular complex of 850 kDa, the top solution should contain 10% glycerol, and the bottom one, 30%. A successful gradient centrifugation will allow the complex-to-be-purified to move about two-thirds of the way down the gradient. This assures that the complex has been completely removed from smaller contaminants (such as found in the original buffer) yet is not too close to the bottom, where it could be contaminated with sediments.

Table 5.1 Ultracentrifugation guidelines for GraFix, based on a selection of various complexes

Molecular mass (kDa)	Gradient	RPM	Time
125	5–20%	40,000	18
450	10–30%	50,000	16
700	10–30%	33,000	16
850	10–30%	33,000	18
1500	10–40%	37,000	14
3600	15–45%	22,500	14

A rough estimate of the centrifugation conditions, based on the approximate molecular mass of the complex, is given. Gradient: the percentage of glycerol (or other sugar) to use in the top and bottom gradient solutions; RPM: the speed of the ultracentrifugation; and time: hours of centrifugation.

3.2.1. Solution preparation

Two buffer solutions should be prepared with the appropriate densities for creating the gradient (see Table 5.1). The cross-linker, such as glutaraldehyde, should be added only to the denser solution, at a concentration of 0.05–0.2% (v/v). Other cross-linkers can also be used. The buffers should not contain any primary amino groups (such as in TRIS). For instance, to prepare a typical 10–30% glycerol gradient, the top buffer would contain HEPES 50 m*M*, pH 7.5, $\leq$100 m*M* salt (as appropriate for the complex), and 10% (v/v) glycerol, while the bottom buffer would contain HEPES 50 m*M*, pH 7.5, $\leq$100 m*M* salt, 30% (v/v) glycerol, and 0.15% glutaraldehyde. Buffers should be filtered through a 0.3-μm filter prior to use.

3.2.2. Gradient formation

To form the gradient, the different-density solutions are layered in a 4.4-ml centrifuge tube (such as polyclear tubes, #S7010, Science Services), by first adding 2.1 ml of the less dense (top) solution to the tube. Next, 2.1 ml of the heavier (bottom) solution is drawn into a syringe with a blunt-end stainless steel needle (such as a Hamilton syringe). The end of the needle is placed at the bottom of the tube at a slight angle, and the solution is slowly expelled, so that the lighter solution is displaced upward. This must be done carefully to avoid disturbing the interface as much as possible; the interface should form a sharp line when finished. Tubes are then closed with a BioComp cap.

To form a continuous density gradient, tubes are placed into a specialized gradient mixer (such as the Gradient Master 107, BioComp Instruments) and rotated briefly, following the manufacturer's recommendations for determining the parameters (time[s]/angle/speed). Gradients should be prepared in advance and allowed to settle for an hour at 4 °C prior to centrifugation.

3.2.3. Adding a buffering cushion

As mentioned earlier, a buffering cushion can be used on the top of the gradient to completely avoid contact between the original sample buffer and the cross-linking reagent (Fig. 5.1). This prevents any reactivity of original buffer components (such as TRIS, or peptides) with the glutaraldehyde-cross-linking reagent. This is not usually necessary due to the extremely low concentration of the cross-linking reagent at the top of the gradient (we usually do not use this cushion for our gradients).

The cushion should contain the same buffer solution as the top solution, but with a slightly lower density; thus, if the top solution contains 10% glycerol, the cushion should contain 7% glycerol. To add a 200-μl buffering cushion, that amount plus any amount of the sample over 200 μl should be

removed from the top of the gradient (e.g., if the sample will be 500 μl, remove the 200 μl for the cushion plus 100 μl for the sample; see in the following paragraphs). Carefully add the cushion to the top of the gradient and let the gradient settle at 4 °C as normal.

3.3. Sample concentrations

Since most macromolecular complexes can only be purified in small quantities, it is important to maintain a small gradient volume to keep the sample concentration within adequate ranges in the final gradient fractions. We usually use an ~4-ml gradient. The sample to be loaded should ideally be between 10 and 80 pmol of complex in a maximum volume of 400 μl (we routinely load 200 μl or less). The amount of the macromolecular complex that can be loaded onto the gradient can be increased, but we recommend loading less than 180 pmol onto a 4-ml gradient, as we observed artifacts due to intermolecular cross-linking when loading amounts higher than this. While it is preferable to have a minimum of 10 pmol of complex, this is not always feasible when handling low-abundance particles. As mentioned earlier, the apparent concentration of extremely low-concentration samples can be increased during the grid preparation, by increasing the binding times to the carbon support film. This can help overcome the problem of starting with low sample quantities in some cases. For example, only about 1 pmol of the RNA editing complex was loaded onto a GraFix gradient, yet we were able to obtain good quality raw images (Fig. 5.3).

When setting up the centrifugation, it might be useful to have an internal control of the sample under the same conditions except without cross-linker, centrifuged in parallel. This control reveals whether the fixation has changed the sedimentation of the macromolecular complex. An advantage of performing this control is that it provides aliquots for which the protein composition of the complex can be analyzed by SDS-PAGE, since the complex has not been cross-linked. However, this control is not essential (and we do not perform it once the centrifugal conditions have been determined).

3.3.1. Loading the sample

After removing the cap, there will be enough space at the top of the gradient to load up to 200 μl of sample. Since it is not important to completely fill the tube, sample volumes less than 200 μl can also be loaded. However, if the sample is larger than 200 μl, an amount of the gradient that is equivalent to the extra volume should be removed (e.g., if the sample is 300 μl, remove 100 μl prior to loading). After loading, tubes within the buckets need to be balanced prior to carefully placing them into the rotor that has been precooled to 4 °C.

3.4. Centrifugation

Ultracentrifugation is carried out at 4 °C in swing-out rotors, such as SW60 rotors (Beckmann) or TH-660 rotors (Kendor Laboratory). Note that, although both of these rotors use the same tubes, there are minor differences between centrifugation in the two rotors that can result in a shift of 1–2 fractions. Depending on the mass of the complex, centrifugation times are between 14 and 18 h with speeds of 22,500–40,000 rpm (Table 5.1).

3.5. Fractionation

Following centrifugation, the gradients are fractionated (at 4 °C) from the bottom, in order to minimize contamination with material from the top of the gradient. We fractionate using a capillary to pump the gradient out from bottom to top, taking fractions of five drops (corresponding to ~175 μl). Fractionation can also be performed by removing the fractions through the bottom of the tube, such as by using the Brandel Isco tube piercer (Isco, Inc., Lincoln, USA). The optical density of each fraction is measured with a photometer during fractionation (see Fig. 5.1). If required, the glutaraldehyde within the fraction can be neutralized by adding glycine to a final concentration of 80 m*M* (note that this has not been necessary for us).

3.6. Buffer exchange prior to unstained cryo-EM

If the gradient fraction sample is used for unstained cryo-EM, the glycerol must be removed prior to grid preparation. This is performed in a single step using a buffer-exchange column, such as the PD MINITRAP G-25 (GE Healthcare, following the manufacturer's protocol). This is a simple and quick procedure that is carried out by loading the fraction onto a prewashed microcolumn at 4 °C and, after a brief washing, eluting the complexes in a compatible buffer. Importantly, for many macromolecular complexes, it is only possible to use exchange columns if the complexes have been purified with GraFix due to the stability of the complexes afforded by cross-linking; most nonstabilized complexes will not survive this method intact. Note also that we prefer gravity-flow columns to spin columns, as they are less harsh on the complexes.

3.7. Sample preparation for EM grids

3.7.1. Placing carbon film onto samples

Following purification either from the gradient (for negative staining) or from the buffer-exchange columns (for cryo), the samples can be processed for grid preparation as normal. In our laboratory, we use thin (~10 nm)

Figure 5.4 Placement of carbon film over a sample. A homemade plastic block with holes drilled into it, each with a holding capacity of about 25 μl, is precooled on ice, and 30 μl of a sample is loaded into one hole. A thin carbon film is floated off a mica support by holding the mica with forceps and placing it into the sample at a slight angle. Once the carbon film is released, the mica is removed, and macromolecular complexes are allowed to adsorb for the appropriate time.

carbon films that have been coated onto a piece of mica and dried using a carbon vacuum evaporator system (Boc Edwards GmbH). To adsorb the complexes onto the carbon film, we first place the sample ($\sim$30 μl) into a hole drilled into a homemade black plastic block (made from polyoxymethylene) that had been previously placed on ice to cool (Fig. 5.4). Next, we cut a piece of the carbon-coated mica (about 2 mm $\times$ 2 mm) and carefully place this at an angle into the sample, with the mica side facing down. Contact with the sample releases the carbon from the mica. This method has the advantage that the carbon film side that is bound by the macromolecular complexes has never been exposed to the air. Using this method of introducing the carbon film to the sample has shown to be highly reproducible in our hands to produce a film with low background noise.

3.7.2. Adsorption times

While we still use a short adsorption time of a 1–2-min for samples with an acceptable concentration of macromolecular complexes, the adsorption time can be greatly extended for lower concentration samples. We have found that adsorption times of up to 24 h still give high-quality particle images (see, e.g., the 12-h adsorption for the RNA editing complex; Fig. 5.3). When we are working with very low-concentration samples, we usually allow the binding to occur overnight ($\sim$12 h). When using longer adsorption times, we cover the plastic block (containing the sample and the carbon film) with a lid sealed with an O-ring, to reduce sample evaporation. This is placed for the necessary time at 4 °C.

3.7.3. Grid preparation

After the allotted adsorption time, the carbon film is picked up using a holey carbon grid. Negative-stain sample preparation differs only by the additional incubation with the heavy metal salt solution used for staining. For cryogrid preparation, an additional 3–4 μl of sample is placed over the carbon film prior to vitrification, to facilitate blotting of excess solution, and the films are plunged into liquid ethane. Grids can be stored in liquid nitrogen until they are used for cryo-EM.

4. Conclusions

Currently, the field of cryo-EM is experiencing an exciting expansion, following advances in methodology and instrumentation that has allowed it to deliver near-atomic resolution 3D structures of several well-behaved macromolecular complexes. The GraFix protocol expands the type of specimens that can be analyzed to include molecules that are more difficult to handle due to internal asymmetry, flexibility, and low concentrations of the complex following purification. It is important to note, however, that GraFix cannot be used to ''repair'' broken macromolecular complexes that were damaged already during sample purification. Rather, GraFix can only be helpful in stabilizing intact molecules. Optimization of biochemical tools and methods used for complex purification is still of utmost importance in single particle cryo-EM, and special care needs to be taken to determine the optimal buffer conditions in order to maintain the complex integrity to the greatest possible extent during purification. When starting with intact macromolecular complexes, it is reasonable to expect resolutions of 10 Å and better at the present for complexes purified at the end stage with GraFix. We also expect near-atomic resolution structure determination to be a viable goal in the foreseeable future for GraFix-treated macromolecular complexes.

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