GraFix: sample preparation for single-particle electron cryomicroscopy

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We developed a method, named GraFix, that considerably improves sample quality for structure determination by single-particle electron cryomicroscopy (cryo-EM). GraFix uses a glycerol gradient centrifugation step in which the complexes are centrifuged into an increasing concentration of a chemical fixation reagent to prevent aggregation and to stabilize individual macromolecules. The method can be used to prepare samples for negative-stain, cryo-negative-stain and, particularly, unstained cryo-EM.

Knowledge of the three-dimensional architecture of macromolecular machines is crucial for understanding living cells. Most molecular machines are dynamic, fragile multiprotein complexes[1] and are present at low copy number in the cell. Single-particle cryo-EM has been demonstrated to be a very powerful method for three-dimensional structure analysis of large macromolecular complexes at increasing resolution[2]. For macromolecules with a high level of internal symmetry, structure determination by cryo-EM has become routine at a resolution of $\leq$10 Å, and there are examples where resolution better than 6 Å has been obtained[3]. For asymmetric complexes, there are only a few examples where resolution of $\leq$10 Å has been achieved[4,5]. For asymmetric complexes, limitations in resolution can often be explained by structural heterogeneity (compositional and/or conformational) of purified macromolecular complexes[6,7], sample aggregation, buffer incompatibilities (for example, glycerol in samples for unstained cryo-EM) or anisotropic particle orientations.

Presently used sample preparation procedures, such as chromatography, dialysis and carbon film binding, involve exposure of the macromolecules to various surfaces. This can potentially lead to disruption or destabilization of the complex and may result in increased structural heterogeneity of the macromolecules on the electron microscopy specimen grid. However, reliable and high-resolution structure determination of a macromolecular complex by cryo-EM requires a structurally and compositionally homogeneous sample; this in turn requires advanced tools for sample handling. Here we present a universal sample preparation protocol that can be applied to macromolecular complexes as the final purification step to improve structure determination by single-particle cryo-EM. The highly reproducible procedure, named GraFix, combines the sedimentation of complexes in a density gradient (for example, glycerol, sucrose, trehalose) with weak chemical fixation (**Fig. 1** and **Supplementary Methods** online). This procedure leads to the formation of chemically stabilized monodispersed complexes that can be prepared for negative stain or unstained cryo-EM (**Fig. 2**).

Mild buffers in which the native structure of macromolecules is well preserved often promote aggregate formation, for instance, owing to low salt concentration. Thus, merely adding a chemical-fixation reagent directly to the purified macromolecular complex is not recommended because, first, the cross-linker may lead to artifacts or loss of material resulting from inter-particle fixation and aggregation, and second, there may be incompatibilities of buffer systems with chemical fixation. In such cases, the combination of centrifugation and fixation can be used as weak aggregates are dissolved during centrifugation because of the pressure acting on the molecules.

Chemical fixation reagents are widely used in electron microscopy[8,9], and it is known that the use of glutaraldehyde

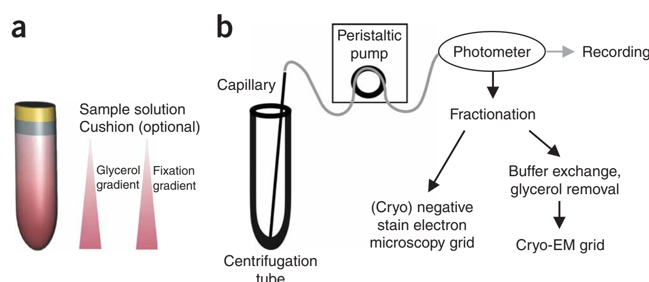

Figure 1 | Schematics of the GraFix setup. (**a**) The GraFix gradient is prepared such that the fixation reagent is added to the denser glycerol solution. (**b**) GraFix gradients are fractionated from bottom to top, then either used directly for negative-stained electron microscopy or purified away from glycerol for unstained cryo grid preparation.

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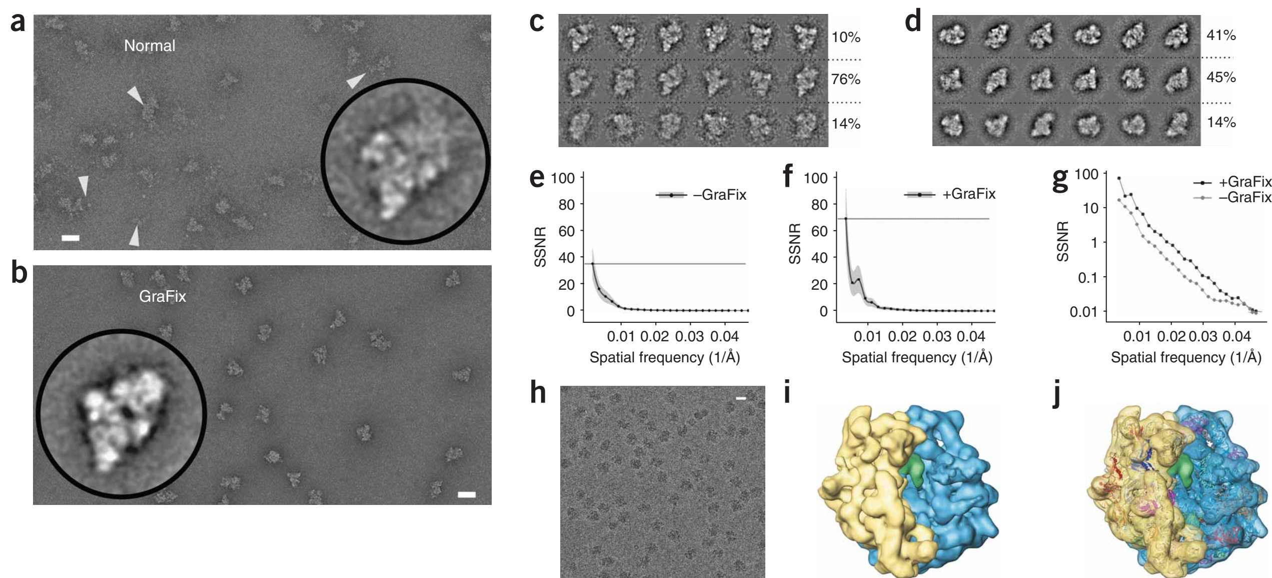

Figure 2 | Effect of GraFix-based sample preparation on B complex spliceosomes and the 70S ribosome. (**a**,**b**) Uranyl formate–stained electron microscopic raw image of spliceosomes prepared by a conventional glycerol gradient (**a**) or GraFix (**b**). Scale bars, 40 nm. Arrowheads, smaller broken parts and flexible elements. Insets, similarly oriented spliceosomal class average. (**c**,**d**) Class averages obtained from a set of 5,000 raw images of non-GraFix-prepared (**c**) or GraFix-prepared (**d**) samples. The average number of class members is 15 images. Class averages were sorted vertically with respect to contrast and structural definition. GraFix treatment (**d**) generates computed class averages with much improved contrast (top and middle; 86% of images), as compared to samples prepared by the conventional method (**c**), where only ~10% of class averages (top) show relatively well defined structural features. (**e**,**f**) Quantitative analysis of signal quality in class averages of non-GraFix-prepared (**e**) and GraFix-prepared (**f**) spliceosomes (gray shading, s.d.). (**g**) Logarithmic representation of data in **e** and **f**. (**h**) Unstained cryo-EM raw image of the 70S *E. coli* ribosome initiation complex after GraFix preparation and buffer exchange. (**i**) Three-dimensional reconstruction of the ribosomal 70S initiation complex at ~15 Å resolution. Blue, 50S subunit. Yellow, 30S subunit. Green, P-site tRNA. (**j**) Fit of the 70S *E. coli* X-ray structure into the electron microscopy density map.

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may result in formation of artifacts[10]. To test the consequences of mild GraFix fixation of macromolecules we determined the structure of GraFix-prepared *Escherichia coli* 70S ribosomes and compared it with the X-ray structure[11]. We used ~15,000 images to compute a three-dimensional structure. At ~15 Å resolution, we detected no major structural differences comparing the GraFix three-dimensional structure of the 70S ribosome (unstained cryo preparation) and the X-ray structure (**Fig. 2h–j** and **Supplementary Data** online). GraFix is thus unlikely to cause artifacts that can be attributed to chemical fixation at intermediate resolution. We expect that glutaraldehyde or formaldehyde treatment might allow higher-resolution structure determination as it has been shown that high-resolution X-ray structures can be obtained in the presence of chemical fixation reagents[12,13].

We tested GraFix on various fragile complexes with low cellular copy number such as spliceosomes (**Fig. 2a–g**), anaphase promoting complex/cyclosome (APC/C; **Fig. 3**), small nuclear ribonucleoproteins (snRNPs) and kinetoplastid protozoan RNA-editing complexes (**Supplementary Fig. 1** online). In no instance were there indications of artificial structural distortions due to the presence of fixation reagents. We generally observed a considerable reduction of aggregation and fewer smaller (degraded) particles, as exemplified here for the spliceosome. Particle images of the non-GraFix-prepared HeLa spliceosomal B complex are heterogeneous whereas the corresponding GraFix fractions show homogenous particles with increased image contrast (**Fig. 2a–d**). The improvement in quality of electron microscopy raw images of GraFix-prepared samples becomes even more pronounced during statistical image analysis. After alignment, multivariate statistical analysis and classification, the number of good-quality class averages can be increased about fivefold for GraFix-prepared spliceosomal B complexes (**Fig. 2c,d**). Furthermore, the signal in the class averages is considerably higher as a result of increased structural homogeneity.

For quantitative comparison of class average quality we measured the mean spectral signal-to-noise ratio[14] of all class averages, which revealed an increase in signal quality by a factor of 2–5 for the GraFix-prepared spliceosomes over a broad range of spatial frequencies (**Fig. 2e–g**). Whereas GraFix considerably reduced the sample heterogeneity caused by particle degradation, conformational heterogeneity imposed by domain motions and flexibility cannot be entirely avoided[15]. Consequently, conformational heterogeneity has still to be taken into account during image processing, at levels that may differ for different macromolecular complexes. However, GraFix may also contribute to a reduction of conformational heterogeneity by trapping mobile elements in defined positions, no longer allowing any continuous domain movements.

We also tested GraFix on human and *Xenopus laevis* APC/C[7] (**Fig. 3a**). The APC/C is a large molecular machine that acts as a ubiquitin ligase during cell-cycle regulation. APC/C binds in preferred orientations to the carbon support film, which makes structure determination challenging. GraFix-prepared APC/C revealed many more 'views' (**Supplementary Fig. 2** online), which is extremely valuable during image processing and instrumental to computing a three-dimensional structure with improved

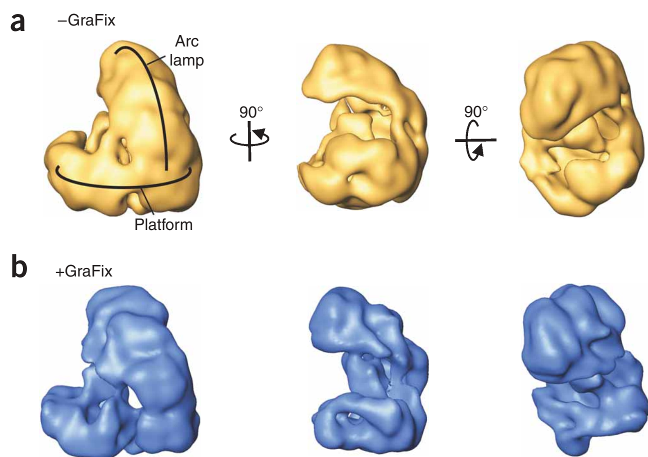

Figure 3 | Improved structure of GraFix-prepared APC/C. (**a**,**b**) The structure of non-GraFix-prepared human APC/C at a resolution of ∼25 Å (ref. 7) (**a**) and of GraFix-prepared human APC/C at a resolution of ∼21 Å (**b**).

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resolution (by ∼4 Å) and quality (**Fig. 3b**). Practically all structural features are better defined in the APC/C three-dimensional structure obtained after GraFix treatment, including a reduction of the pseudo-symmetric appearance of this macromolecular complex. This can be attributed to the higher image quality as well as to the increase in projection angle diversity.

Macromolecules prepared by GraFix exhibit improved binding behavior to the carbon support film used for electron microscopy grid preparation. Instead of using standard adsorption times of 1–2 min, extended adsorption times (several hours at 4 °C) can be applied productively with GraFix-prepared samples (**Supplementary Fig. 3** online). After 6 h the number of intact particles bound to the grid increased 10–15-fold whereas the increase of particles for non-GraFix–prepared samples was only 6–8-fold. Notably, for non-GraFix-prepared samples such long adsorption times are not generally recommended because nonstabilized macromolecules may disintegrate structurally during the extended adsorption. The dilution effect due to the additional gradient centrifugation step of the GraFix procedure can thus be compensated for by increasing particle adsorption time. The most dramatic effect observed so far was for the *Trypanosoma brucei* RNA editing complex (**Supplementary Fig. 1c**,**d**). We detected practically no particles on the grid, applying the standard adsorption time of 1.5 min. However, an adsorption time of 1.5–12 h did result in a sufficient particle distribution. Use of GraFix with longer adsorption times can even lead to a net increase in particle concentration on the grid in spite of the additional purification and dilution step. An even larger increase in particle density can be expected if GraFix is used as a replacement of a typical purification step rather than an extension to the standard protocol. For very low-abundance macromolecules[1] this may be envisaged as a prerequisite for structure determination by single-particle cryo-EM.

In cryo-EM, high-contrast image formation of macromolecules embedded in vitrified ice requires glycerol concentrations to be low. Typical glycerol concentrations obtained after GraFix (15–25%) are not compatible with unstained cryo-EM and will invariably result in poor image contrast, rendering the macromolecules almost invisible in the electron micrographs. To make GraFix-prepared samples accessible to unstained cryo-EM analysis, we used buffer exchange spin columns to remove glycerol from the fixed sample in a single centrifugation step (**Fig. 1b** and **Supplementary Methods**). Such spin columns usually do not work well for unfixed macromolecular complexes because they can result in damage of the complexes. In contrast, GraFix-prepared samples are stabilized after chemical fixation, and glycerol can thus be efficiently removed by buffer exchange with spin columns to a final concentration below the detection limit as measured by the refractive index (data not shown). This results in samples highly suitable for unstained cryo-EM (**Fig. 2h–j** and **Supplementary Methods**).

Many affinity selection–based methods have been developed in recent years and have allowed the purification of hundreds of different cellular complexes[1]. Application of the GraFix method should lead to a considerable increase in the number of successful three-dimensional structure determinations of fragile and flexible complexes. For such complexes, the GraFix method can enable also the successful use of other technologies, such as scanning transmission electron microscopy for molecular mass determination. Furthermore, in spite of chemical fixation, the protein composition of macromolecules can be analyzed by mass spectrometry using matrix-assisted laser desorption/ionization–tandem time-of-flight analysis (H.U., manuscript in preparation). Other methods for measuring structural parameters of macromolecular complexes, such as light scattering or X-ray diffraction, should also benefit from the GraFix technique, in particular if complex stability is necessary over a longer measurement time.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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