TomoAlign: A novel approach to correcting sample motion and 3D CTF in CryoET

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ABSTRACT
TomoAlign is a software package that integrates tools to mitigate two important resolution limiting factors in cryoET, namely the beam-induced sample motion and the contrast transfer function (CTF) of the microscope. The package is especially focused on cryoET of thick specimens where fiducial markers are required for accurate tilt-series alignment and sample motion estimation. TomoAlign models the beam-induced sample motion undergone during the tilt-series acquisition. The motion models are used to produce motion-corrected subtilt-series centered on the particles of interest. In addition, the defocus of each particle at each tilt image is determined and can be corrected, resulting in motion-corrected and CTF-corrected subtilt-series from which the subtomograms can be computed. Alternatively, the CTF information can be passed on so that CTF correction can be carried out entirely within external packages like Relion. TomoAlign serves as a versatile tool that can streamline the cryoET workflow from initial alignment of tilt-series to final subtomogram averaging during in situ structure determination.

1. Introduction

Electron cryotomography (cryoET) combined with subtomogram averaging allows visualization of the molecular architecture of cellular compartments in situ (Turk and Baumeister, 2020). Although structure determination at subnanometer, even near-atomic, resolution is possible in favourable cases, this is not the general case. The contrast transfer function (CTF) of the microscope and the beam-induced sample motion have been identified among the major resolution-limiting factors.

The CTF results in contrast reversals and signal attenuation at certain spatial frequency ranges (Fernandez, 2012). CTF correction is essential to recover high resolution structural information. In cryoET, this correction is hindered by the CTF variation along the electron beam direction, which involves a CTF gradient in tilted images and also through thick samples. For long, only strip-based CTF correction of tilted images was considered (Fernandez et al., 2006; Xiong et al., 2009). Recently, consideration of the full CTF variation through the thickness of the tilted sample (so-called 3D-CTF) has proven to be paramount to reach high resolution (Kunz and Frangakis, 2017; Tegunov et al., 2017), where the CTF is corrected during tomogram reconstruction. Other successful strategies include dealing with the 3D-CTF correction at the subtilt-series/subtomogram level (Bartesaghi et al., 2012; Bharat et al., 2015).

Several factors cause the sample motion and deformation during radiation of the cryo-sample (Naydenova et al., 2020). In cryo-ET, this smoothly-varying motion makes the acquired tilt images deviate from the basic assumption of the tomographic reconstruction, thereby deteriorating their mutual alignment and the quality of the resulting tomogram (Bharat et al., 2015). Determination and correction for the beam-induced local sample motion is therefore important for achieving higher resolution, as demonstrated by strategies working either at the tomographic reconstruction stage (Fernandez et al., 2018) or by refining the orientational parameters at subtilt-series level (Bartesaghi et al., 2012).

Currently, many advanced software packages for high resolution subtomogram averaging have adapted single-particle-like approaches to correcting 3D-CTF and the sample motion (Himes and Zhang, 2018; Chen et al., 2019; Song et al., 2020; Sanchez et al., 2020; Heymann, 2021; Tegunov et al., 2021). In these approaches, the 3D-CTF is determined and corrected on per-particle basis, and the motion is corrected by applying orientational refinement techniques based on the biological structures present in the images. These have brought striking resolution improvements. Nevertheless, these approaches might be suboptimal for

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thick samples or highly packed samples in their cellular milieu (250 – 300 nm in thickness, e.g. axonemes or centrioles (Li et al., 2019)) due to substantial overlap of features and low signal-to-noise ratio (SNR), especially at high tilts.

Here we introduce TomoAlign, a software for correcting both the 3D-CTF and the local sample motion. It is especially focused on the scenarios of cryoET of thick or crowded samples where accurate tilt-series alignment and motion correction require fiducial markers. TomoAlign is able to produce motion-compensated and 3D-CTF-corrected subtilt-series. It also provides an interface between IMOD (Kremer et al., 1996), the standard software for tilt-series alignment, and Relion for subtomogram averaging (Bharat et al., 2015).

2. Technical features

2.1. Overall workflow

The general workflow in TomoAlign is composed of three steps depicted in Fig. 1. First, TomoAlign implements a fiducial-based tilt-series alignment strategy that includes estimation of the beam-induced sample motion. Second, the motion model can be applied during tomographic reconstruction to yield motion-compensated tomograms. To reduce processing time and disk usage, this tomogram is usually calculated in binned size (typically 4x or 6x) and no CTF correction is considered. The tomogram is then analyzed, particles of interest selected and their coordinates recorded. Once the particles of interest are identified, the last step in TomoAlign consists of extracting the subtilt-series associated to the selected particles. TomoAlign uses the motion model determined in the first step to provide motion-compensated subtilt-series. In addition, the information about per-tilt CTF allows it to find out the per-particle per-tilt CTF, based on their coordinates and the acquisition geometry, to generate the CTF-corrected subtilt-series. Alternatively, the package will record this 3D-CTF information in a metadata file and supply it to other software packages along with non-CTF-corrected subtilt-series. The output subtilt-series are then subjected to tomographic reconstruction with external programs to produce subtomograms for subsequent subtomogram averaging. The details of these steps are further described in the following sections.

TomoAlign interfaces directly with IMOD, from which it receives the fiducial model and the tilt-series, in the form of either the original or aligned stack, in MRC format. The reconstructed tomograms are in MRC format. For the last step, any common software in the field for particle picking and CTF determination can be used to provide TomoAlign with the particle coordinates and per-tilt defocus, respectively, as simple text files. TomoAlign also interfaces directly with Relion by writing the output subtilt-series in MRC format and metadata files containing the alignment and CTF parameters in .star format. Reconstruction of subtomograms can be done preferably with the Fourier inversion method using Relion, but other methods and programs can be used instead, for instance Weighted Back Projection (WBP) using Tomo3D (Agulleiro and Fernandez, 2011). The motion-corrected CTF-corrected subtomograms could also be used as an input for any other subtomogram averaging software.

TomoAlign consists of Unix-like command-line programs (Fig. 1). This facilitates its integration into scripts or higher-level wrapping software packages (Sharov et al., 2020). TomoAlign programs include parallelization of the most computationally expensive procedures, namely tomographic reconstruction and CTF correction.

2.2. Sample motion in TomoAlign

In TomoAlign, the tilt-series alignment and sample motion estimation rely on fiducial markers. It assumes that the movement of the fiducials is in close proximity to the movement of the biological sample. The procedure consists in a two-step process that is transparent to the user. The first step applies a standard alignment, i.e. without consideration of the motion, to determine the usual parameters for each image in the tilt-series (shift, rotation, magnification, tilt) and the 3D coordinates of the fiducials representing the stationary sample (Fig. 2, black dots). The IMOD tilt-series alignment can be imported for this first step, either by providing the IMOD alignment parameters or the IMOD-aligned stack. The second step in the TomoAlign alignment then consists in the determination of the sample motion model $S'(x)$ that accounts for the residuals, i.e. discrepancies between the experimental fiducial positions and their coordinates. TomoAlign models the sample motion.

Fig. 1. Block diagram of the procedures involved in TomoAlign. The major components in TomoAlign are in the highlighted boxes, including the name of specific programs. White boxes denote external software that provides input to TomoAlign or receives its output. First, the program tomalign models the sample motion. Next, the program tomorec is run in two rounds. It firstly computes a tomogram from which the particles of interest are selected. The second round extracts the subtilt-series.
in the acquired images and the motion-free positions expected from the standard alignment (Fig. 2, red arrows).

The motion model $S'(x)$ represents the movement undergone by any 3D point of the sample, $x = (x, y, z)$, during the acquisition of the $i$-th image. This is the movement observed at the image plane $(u, v)$. It represents the 2D motion perpendicular to the electron beam direction: $S'(x) = (S'_x(x), S'_y(x))$ (Fig. 2).

TomoAlign provides two different models for the sample motion, polynomials and splines (Fernandez et al., 2018, 2019). Their trivariate forms (i.e. dependent on X, Y and Z axes of the sample space) model the complex variation of motion throughout the depth of thick samples (i.e. along Z) (Fernandez et al., 2018).

After tilt-series alignment and motion modelling, TomoAlign can compute motion-corrected tomograms with WBP (Fig. 1). In this process, the sample motion model $S'(x)$ is used to determine accurately the location of any voxel projected in the acquired tilt-series images. In practice, tomograms are computed in binned size for the sake of speed and using smoothing filters to enhance the contrast and facilitate their visualization and particle picking (Fig. 1).

Once the particles of interest are identified, the next step in TomoAlign is extracting subtilt-series in unbinned format. Similar to tomographic reconstruction, TomoAlign makes use of the sample motion model $S'(x)$ to track precisely any position in the unbinned tomogram throughout the acquired tilt-series. For a given particle at the position $p$ in the tomogram, the motion model $S'(p)$ (Fig. 2) provides the deviation with respect to its expected location in the $i$-th image according to the standard tilt-series alignment (that is, only considering rotation, magnification, tilt and shifts). This allows precise extraction of unbinned motion-corrected subtilt-series from raw or aligned tilt-series for all selected particles (Fig. 2).

2.3. 3D-CTF correction in TomoAlign

TomoAlign determines the 3D-CTF of each selected particle based on its coordinates in the tomogram, the acquisition geometry and the average defocus estimated for each image in the tilt-series (i.e. per-tilt) (Fig. 1). The estimation of the average per-tilt defocus is conducted by any external program (e.g. IMOD, CTFIND4, TomoCTF (Xiong et al., 2009; Rohou and Grigorieff, 2015; Fernandez et al., 2006)) and the results are input to TomoAlign (Fig. 1). Given a particle with coordinates $p = (p_x, p_y, p_z)$ with respect to the center of the tomogram, its defocus in the $i$-th image is determined as $D'(p) = p_x \sin(\theta_i) + p_y \cos(\theta_i) + D'(\alpha)$ (Fig. 2). Here $\theta_i$ represents the tilt angle at the $i$-th image and $D'(\alpha)$ is the average defocus estimated for that image, assuming it corresponding to the center of the tomogram $\alpha$, and the tilt axis is assumed to run along the Y axis of the images/tomogram, as usual. This procedure allows estimation of the 3D-CTF for all selected particles.

In TomoAlign, CTF correction can be carried out in several ways. First, TomoAlign can combine subtilt-series extraction with CTF correction to produce motion-corrected, CTF-corrected subtilt-series. In cryoET, relatively high defocus values are often used for thick specimens, which results in significant signal delocalization (Downing and Glaeser, 2008). For CTF correction, TomoAlign applies a large box size different from that used for output subtilt-series extraction, with both sizes tunable by the user (Fig. 2). This allows better CTF correction since most of the delocalized, especially high frequency, signal can be recovered and also CTF aliasing problems are avoided (Rosenblad and Henderson, 2003; Mortya et al., 2020; Tegunov et al., 2021). TomoAlign applies CTF correction based on standard phase flipping to restore the contrast throughout the spatial frequency. Another option is CTF multiplication, which additionally attenuates information affected by the CTF (Downing and Glaeser, 2008). After CTF correction, the subtilt-series are cropped to the user-defined final box size (Fig. 2). This strategy enables computation of CTF-corrected subtomograms from these subtilt-series, which can be later supplied to any external subtomogram averaging software (Fig. 1).

Alternatively, TomoAlign will generate the subtilt-series without CTF correction and produce the metadata in Relion .star format that contains the alignment and the 3D-CTF parameters for all particles. This metadata, along with the reconstructed non-CTF-corrected subtomograms, will allow subtomogram averaging and CTF correction to be conducted entirely within Relion (Fig. 1). In this way, the user can take advantage of the Relion CTF model for full CTF correction (both amplitude and phase) (Bharat et al., 2015).
3. Implementation details

3.1. Program tomoalign

The sample motion observed at the image plane \((u, v)\) during the acquisition of the \(i\)-th image is modelled with two components \((S^i(x) = (S^i_x(x), S^i_y(x)))\) (Fig. 2). Each of those components is a polynomial or an interpolation spline. Here we describe details of the implementation of their trivariate forms (i.e. dependent on \(X, Y\) and \(Z\) axes of the sample space) to model the motion across the 3D space.

TomoAlign uses quadratics polynomials by default because they can appropriately model the doming motion (Naydenova et al., 2020), though any polynomial degree can be used. As a result, the polynomials have 10 terms, with coefficients \(P_{pol}\), and are of the form:

\[
P(x, y, z) = P_{000} + P_{010} x + P_{100} y + P_{001} z + P_{101} xz + P_{011} yz + P_{002} z^2
\]

Therefore, polynomial motion modelling involves two quadratic polynomials per image (that is, \(S^i_x(x) = P_x^i(x, y, z)\) and \(S^i_y(x) = P_y^i(x, y, z)\)), which amounts to 20 parameters per image. Their determination is formulated as a non-linear least-square problem to minimize the sum of squared residuals at the fiducials, and it is solved by quasi-Newton optimization implemented using routines from Numerical Recipes (Press et al., 2002). Sufficient number of fiducials are required (usually \(2 \times 3 \times \text{the number of parameters}\)) to ensure their reliable determination. Further details of this optimization are available in Fernandez et al. (2018).

TomoAlign can also model the motion with interpolation splines, namely thin-plate splines (TPS). Here the residuals from the standard alignment are assumed to represent estimates of the sample motion at the location of the fiducials, and these splines allow smooth interpolation of these estimates across the entire 3D space. These splines have the form:

\[
\text{TPS}(x, y, z) = a_{00} + a_{01} x + a_{10} y + a_{20} z + \sum_{j=1}^{N_f} w_j \sqrt{(x_j - x)^2 + (y_j - y)^2 + (z_j - z)^2}
\]

where \(N_f\) denotes the number of fiducials and \((x_j, y_j, z_j)\), with \(j = 1 \ldots N_f\), represents the 3D coordinates of the fiducials. They comprise a global affine transformation (with coefficients \(a\)) and a non-rigid one (the sum term with coefficients \(w\)). There are two splines per image (that is, \(S^i_x(x) = \text{TPS}_x^i(x, y, z)\) and \(S^i_y(x) = \text{TPS}_y^i(x, y, z)\)), so a total of \(2 \times (N_f + 4)\) parameters are involved. The parameters of each spline are determined by solving a linear system of equations established from the interpolation conditions (i.e. at the location of the fiducials the interpolants must give the original residuals). Therefore, there are two linear equation systems per image that are easily solved by matrix inversion, implemented using routines from Numerical Recipes (Press et al., 2002). For a detailed description, the reader is referred to Fernandez et al. (2019).

As an output, the program tomoalign produces a file with the alignment parameters. For each image in the tilt-series, the standard description, the reader is referred to Fernandez et al. (2019). As an output, the program tomoalign produces a file with the alignment parameters. For each image in the tilt-series, the standard description, the reader is referred to Fernandez et al. (2019). For each image, tomographic parameters \((u, v)\) are written. Therefore, most of memory demanded by tomorec is to keep two buffers of \(T\times S\times H\times W\) pixels in memory. The program proceeds by processing slabs of \(T\) slices perpendicularly to the tilt axis, which are reconstructed in parallel by \(T\) compute threads using the algorithm sketched above. An additional thread is concurrently writing to disk the slab of \(T\) slices that were reconstructed previously. This strategy is implemented by a sliding window that sweeps across the slices of the tomogram. It is composed of two buffers of \(T\) slices that are used interchangeably for computing and for writing. Therefore, most of memory demanded by tomocrec is to keep the entire tilt-series and \(2 \times T\) slices of the tomogram using single precision floating point number. This amount of memory is small enough for modern computers equipped with at least 4–8 GB of RAM memory. Tomocrec automatically detects the number of cores available in the computer to set \(T\), though the user can force manually a particular configuration.

To show the performance of tomocrec, we measured the processing time for example reconstruction problems: tomograms of size \(1024 \times 1024 \times 256\) and \(2048 \times 2048 \times 512\) from tilt-series of 61 images with 15
fiducials, using trivariate polynomial and spline motion models. Suppl. Table S1 presents the actual times in seconds for different configurations of compute threads (1, 2, 4, 8, 16). Fig. 3B shows the speedup factors. The parallel implementation shows good scalability that decays as a function of threads and achieves speedups around 1.95, 3.6, 6.5 and 12 for 2, 4, 8 and 16 threads, respectively. These results indicate that motion-corrected tomograms in binned size can be computed in reasonable time even in modest computers equipped with processors of 2 or 4 cores.

In the last step of TomoAlign (Fig. 1), tomorec receives the particle coordinates and the per-tilt defocus as text files and then proceeds as described in Section 2.3 and in Fig. 2. The particles are processed sequentially, each one passing through motion-aware subtilt-series extraction, CTF correction, cropping and writing to disk. CTF correction is parallelized by distributing the subtilt-series of a particle among the threads, but the global performance is limited by the remaining sequential operations. Nonetheless, the processing time is in the order of seconds, as shown in Suppl. Table S2, and scales linearly as a function of the number of particles.

4. Illustrative results

We show the performance of TomoAlign with two datasets. We first used a dataset of T20S proteasomes from *Thermoplasma acidophilum*. This is a relatively small-sized sample in thin ice, not representative of thick samples in cryoET studies. Even so, this dataset serves as a proof-of-concept because the specimen is very well characterized, its atomic resolution structure is available for comparison and is an ideal sample for benchmark. Secondly, we used a dataset of flagella axoneme isolated from *Tetrahymena thermophila* from which segments of axoneme
doublets at 16-nm periodicity were extracted. This is a thick specimen (250 nm) where the acquired raw images show substantial overlap of similar features and fiducial markers are required for alignment. It thus represents a case of in situ structural studies by cryoET which TomoAlign is aiming at. Table 1 summarizes the details and results for both test datasets.

Both datasets were processed following the workflow illustrated in Fig. 1. Fiducial-based standard tilt-series alignment was carried out in IMOD (Kremer et al., 1996). Motion-aware alignment was then applied with bivariate polynomial surfaces and trivariate splines for the proteasomes and axoneme doublets, respectively. These configurations were set based on their thickness and the number of fiducials available, as described in Section 3. For comparison, the standard alignment (i.e. assuming no sample motion) was also included in this study. Motion-aware tomographic reconstruction then followed, and was carried out in 4x or 6x-binned size. Particles were then picked from the binned tomograms. Particle picking in the proteasome case was done by template-matching using Spider (Frank et al., 1996) and the results were manually revised. For the axoneme case, initial segments were manually annotated by marking the center of the nine doublets and estimating the direction of the axonemes in the tomograms. The positions of subsequent segments at 16-nm periodicity were automatically computed with Spider. Total 3928 proteasomes and 9540 axoneme doublet segments were selected. Per-tilt CTFs were determined for all tilt-series with TomoCTF (Fernandez et al., 2006). Coordinates of the selected particles and per-tilt CTF values were provided to TomoAlign to extract motion-corrected subtilt-series associated to all particles. In the axoneme case, TomoAlign was run in 2x-binned size (pixel size 5.3 Å) at this stage. The subtilt-series were CTF-corrected based on phase-flipping using a squared box size of 512 pixels, which were finally cropped to a tighter size of 120 and 140 pixels for the proteasomes and axoneme doublets, respectively. In addition, non-CTF-corrected subtilt-series were extracted along with the CTF parameters so that full CTF correction, including both amplitude and phase, could be carried out inside Relion. Subtomograms were reconstructed by Fourier inversion with Relion or WBP with Tomo3D. In all tests, subtomogram averaging was conducted with the standard workflow in Relion, including the weights to account for the accumulated radiation and the thickness of the tilted sample (Bharat et al., 2015; Bharat and Scheres, 2016). In this workflow, the 3D auto-refine procedure in Relion is applied, whereby the data is divided into 4x or 6x-binned size of 120 and 140 pixels for the proteasomes and axoneme doublets, respectively. These configurations are applied in Relion. The resolution of the final map was assessed against an external high-resolution single particle cryoEM map in the proteasome case. The resolution of the axoneme doublet was estimated by the FSC from independent half-maps due to the unavailability of a comparable external cryoEM map. For the resolution assessment, a loose mask was used and its effect was accounted for (Chen et al., 2013).

Table 1 presents the reduced alignment residual (in pixels) thanks to the consideration of motion. In the axoneme case in particular, the spline motion model cancels out the residual entirely. This is an inherent attribute of this model (Fernandez et al., 2019). For a more objective analysis of the motion model, especially for sample locations away from the fiducials, we calculated the residual by using the Leave-One-Out (LOO) cross-validation residual. Specifically, the motion model is fitted using all fiducials except one. The residual of the excluded fiducial is then calculated. By repeating this process for all fiducials and averaging the collected residual values, the LOO residual is obtained (Kukulski et al., 2011; Fernandez et al., 2018). In the proteasome case, the LOO residual is able to reach below 1.0 pixel owing to the large number of fiducials that ensures robust motion models for the entire field of view. In the axoneme case, the LOO residual is similar to that from the standard alignment. This indicates that the motion models are sufficiently good and maintain the quality of the original tomogram in areas not covered by fiducials. Suppl. Fig. S1 shows an example of tomogram from flagella, where the improvement thanks to the compensation for the motion is apparent.

The resolution of the proteasome subtomogram averages was assessed by an external high-resolution cryoEM map (Grant and Gregoriiff, 2015) (EMD-6464) (Suppl. Fig. S2). Fig. 4(top) shows that the standard tilt-series alignment gave a structure around 10 Å resolution whereas consideration of the motion substantially improved the structure up to 7.3 Å. These results were obtained with 3D-CTF phase flipping directly in TomoAlign. In order to correct both amplitudes and phases, full 3D-CTF correction was applied within Relion. This further pushed forward the resolution to 7.1 Å with an overall improvement of the FSC curve at all ranges. The FSC curves from independent half-maps presented similar improvements (Suppl. Fig. S3). All these results show a significant boost compared to the previous 9 Å structure, where the strip-based CTF correction and standard subtomogram averaging methods were used on the same dataset (Fernandez et al., 2018).

The subtomogram averages of the axoneme doublets were assessed based on the FSC curves obtained from two independent halves of data (Fig. 4(bottom)). While the standard alignment yielded a structure at 14.3 Å resolution, the motion compensation in TomoAlign with phase-flipping improved the resolution further to 12.5 Å. During the acquisition, all tilt-series were acquired with a relatively narrow nominal defocus range (3 μm underfocus). This results in noticeable dips in the FSC curves at certain frequencies that match CTF zeros (Fig. 4(bottom)). Full 3D-CTF correction with Relion manages to restore the amplitudes and to significantly attenuate the FSC dips. Moreover, the resolution is slightly improved to 12.3 Å.

To further corroborate this resolution level, we compared our best axoneme doublet average with a recent high-resolution cryoEM map from the same organism (Ichikawa et al., 2019) (EMD-20602). The two maps are not directly comparable in terms of FSC because they represent average axoneme substructures at different resolutions, which restricts us to a visual comparison. Fig. 5 shows there is remarkable similarity between the two maps, particularly in the details in the microtubule protofilaments and the microtubule inner proteins (MIPs) with 16 nm periodicity, thereby confirming the resolution reported by the FSC curves from independent half-maps (Fig. 4).

The importance of 3D-CTF and large box sizes in CTF correction was demonstrated previously in high resolution studies by cryoET (e.g. Turonova et al., 2017) and cryoEM (e.g. Moriya et al., 2020),

### Table 1
Test datasets. Details and summary of results.

<table>
<thead>
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<th></th>
<th>Proteasome</th>
<th>Axoneme doublet</th>
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<td>12.3</td>
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</table>
respectively. To evaluate their contribution on the proteasome and axoneme datasets, we conducted additional experiments. First, the same workflow was applied using standard phase-flipping strip-based 2D-CTF correction of the tilt-series (Suppl. Fig. S4). Second, the small box size of the output subtilt-series was used for 3D-CTF correction (Suppl. Fig. S5). Subtle (proteasome) or no (axoneme) effects were observed because the resolution in these datasets may still be too modest.

5. Discussion and conclusion

TomoAlign integrates tools to deal with two major resolution-limiting problems in cryoET, namely the CTF and the beam-induced sample motion. The package is especially focused on cryoET studies of thick specimens (around 250–300 nm) where accurate tilt-series alignment and motion correction require fiducial markers.
TomoAlign is a versatile package that provides several options to correct for the motion and 3D-CTF, which are tunable at the user’s discretion. Furthermore, the package is designed to link between the standard software for cryoET tilt-series alignment (IMOD) and software for subtomogram averaging (Relion).

TomoAlign consolidates several strategies to deal with the CTF that have been used previously in isolated or partial form, such as calculation of the per-particle 3D-CTF (Bartesaghi et al., 2012), extraction of subtilt-series (Bartesaghi et al., 2012; Chen et al., 2019), their CTF-correction (Chen et al., 2019) and computation of subtomograms from subtilt-series (Pfeffer et al., 2015). One feature in TomoAlign is the use of a larger box size, different from that used in subtilt-series extraction, for CTF correction of subtilt-series so as to accommodate to the signal delocalization caused by the CTF (Downing and Glaeser, 2008), though the benefits will depend on the actual defocus value. This strategy for CTF correction turns out to be another practical implementation of the 3D-CTF correction originally established by Jensen and Kornberg (2000). Alternatively, TomoAlign can provide the information required to take full advantage of the CTF correction inside Relion (Bharat et al., 2015).

TomoAlign has a unique approach to estimate the sample motion in the 3D tomogram space from the fiducials by using inherently smooth models (quadratic polynomials or splines). These models ensure smoothly-varying motion estimates across the entire tomogram (Fernandez et al., 2019). This type of smoothness constraints has also proved to be of paramount importance in image-based approaches for motion correction (Tegunov et al., 2021).

One unique feature in TomoAlign is the application of the sample motion model to produce motion-corrected subtilt-series. From them, subtomograms can be computed directly by means of sophisticated methods aiming at high-resolution preservation, such as Fourier inversion. Working with subtilt-series also speeds up the entire process, avoids the generation of huge tomograms and alleviates the potential overwhelming of the I/O (input/output) system.

TomoAlign makes possible the reconstruction of motion-corrected and 3D-CTF-corrected subtomograms from the corresponding subtilt-series. These subtomograms can be fed into classic subtomogram averaging packages, thus allowing these packages to benefit from motion-correction and 3D-CTF correction mechanisms while keeping their workflow intact.

The package, documentation and example datasets can be obtained at the following website http://tiny.cc/tomoalign. The best subtomogram averages obtained from the proteasome and the axoneme doublet have been deposited in EMDB (EMD-13183 and EMD-13184, respectively).

CRediT authorship contribution statement

Conceptualization: JF and SL. Investigation: JF and SL. Methodology: JF and SL. Software: JF. Validation: JF and SL. Writing: JF and SL.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jsb.2021.107778.

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