Alignment of cryo-EM movies of individual particles by global optimization of image translations

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Abstract

Direct detector device (DDD) cameras have revolutionized single particle electron cryomicroscopy of protein complexes. In addition to increasing the detective quantum efficiency with which images can be recorded, acquisition of DDD movies during exposures allows for correction of movement of the specimen, due both to instabilities in the specimen stage of the microscope and electron beam-induced movement. Unlike specimen stage drift, beam-induced movement is not always homogeneous within an image. Local correlation in the trajectories of nearby particles suggests that beam-induced motion is due to deformation of the ice layer. Algorithms have already been described that can correct movement of entire frames or large regions of frames at exposures of 2-3 $e^{-}/\text{pixel/frame}$. Other algorithms allow individual particles in small regions of frames to be aligned, but require rolling averages to be calculated from frames and fit linear trajectories for particles. Here we describe an algorithm that allows for individual $<1$ MDa particle images to be aligned without frame averaging when imaged with 2.5 $e^{-}/\text{pixel/frame}$ and without requiring particle trajectories in movies to be linear. The algorithm maximizes the overall correlation of the shifted frames with the sum of the shifted frames. The optimum in this single objective function is found efficiently by making use of analytically calculated derivatives of the function. Two additional measures are proposed to smooth estimates of particle trajectories. First, rapid changes in particle positions between frames are penalized. Second, weighted averaging of nearby trajectories ensures local correlation in trajectories. DDD movies of the \textit{Saccharomyces cerevisiae} V-ATPase are used to demonstrate that the algorithm is able to produce physically reasonable trajectories for a 900 kDa membrane protein complex.

1 Introduction

The use of the CMOS technology in direct detector device (DDD) cameras for electron cryomicroscopy (cryo-EM) has enabled the acquisition of exposure series ‘movies’. Movies of radiation sensitive specimens revealed that beam-induced motion blurs images [1, 2, 3]. DDD movies are typically acquired with exposures of 1 to 3 $e^{-}/\AA^2/frame$ on the specimen, which corresponds to 2 to 5 $e^{-}/\text{pixel/frame}$ on the detector, depending on microscope magnification. These low exposures result in low signal-to-noise ratios (SNRs) in individual movie frames. Optimal extraction of high-resolution information from images of single particles requires alignment of movie frames, a process which is complicated by the low SNR. Fig. 1A shows the average of a 30 frame movie acquired with 2.5 $e^{-}/\text{pixel/frame}$, corresponding to 1.2 $e^{-}/\AA^2/frame$ on the specimen at 200 kV with a K2 Summit DDD (Gatan Inc). A few representative particles are circled in red. Fig. 1B shows a single frame from the movie, illustrating the low SNR of the frames. Frame alignment is complicated further by the presence of fixed pattern noise in images from errors in sensor gain normalization. Significant progress in image analysis has already been enabled by programs to perform rigid body translationally alignment of entire field-of-view movie frames (currently 4000 $\times$ 4000 pixels for most cameras). A method introduced by Li and colleagues [4] down weights high spatial frequencies in images to suppress artifacts from fixed pattern noise before calculating pairwise cross-correlation functions between movie frames. The optimal frame displacement values from the cross-correlation functions are used to create a system of over-determined linear equations. Matrix algebra is then used to determined the frame-to-frame translations that best fit the data in a least squares sense. This least squares whole frame alignment method has allowed high-resolution structures to be
The amount of phase change is given by \( F_{\phi} \), where \( \phi \) is the phase shift. The phase shifted Fourier component is given by \( F_{\phi} \). The effect of translation on the Fourier transform of a movie frame is described by the vector \( \vec{t} \). Here we aim to identify the translations \( \vec{t} \) that best bring the frames into alignment. In order to produce a robust and computationally efficient method for correcting the effects of beam-induced motion in small regions in images, or on individual small (<1 MDa) particles, we propose an objective function based on the correlation of the Fourier transforms of individual frames with the sum of all frames. A well-established iterative optimization algorithm that makes use of partial derivatives of the objective function is then used to find the desired translation values. Once optimized, this objective function gives frame-to-frame trajectories for images of individual particles that show strong local correlation. We show that smoothing of trajectories for individual particles can be used to identify and correct beam-induced particle movement. These approaches were implemented in a new program, alignparts_lmbfgs.

### Methods and Results

#### 2.1 Choice of objective function

Based on the observation that averages of unaligned particle frames appear blurred, we propose that a reasonable alignment for each region of the frame that contains a particle is the alignment that makes the sum of all of the frames best agree with each of the frames. Accordingly, we propose an objective function that maximizes the sum of the correlations of the Fourier transform of each shifted frame with the sum of the Fourier transforms of the shifted frames. Prior to analysis, we apply a temperature factor in Fourier space with the form \( \exp\left(-\frac{k^2}{B}\right) \) to prevent fixed pattern noise from dominating the analysis. The effect of translation on the Fourier transform of a movie frame is a phase change, \( \phi_{jz} \), in each Fourier component of the frame, written \( F_{jz} \) for the \( j^{th} \) Fourier component of frame \( z \). The phase shifted Fourier component is given by \( F_{jz}(\cos \phi_{jz} + i \sin \phi_{jz}) \) or \( F_{jz}S_{jz} \) where \( S_{jz} = (\cos \phi_{jz} + i \sin \phi_{jz}) \). The amount of phase change is given by

\[
\phi_{jz} = k_x(j) \cdot x_z + k_y(j) \cdot y_z \cdot \frac{2\pi}{N}
\]

where \( N \) is the extent in pixels in both the \( x \) and \( y \) direction of the \( N \times N \) image, and \( k_x(j) \) and \( k_y(j) \) are the distance of the \( j^{th} \) Fourier component from the origin in the \( k_x \) and \( k_y \) directions, respectively. As described above, \( -x_z \) and \( -y_z \) are the difference in particle position between frame \( z \) and frame 1 in the \( x \) and \( y \) directions, respectively. The Fourier transform of a sum is equal to the sum of Fourier transforms. Consequently, the sum of the \( j^{th} \) Fourier components from all of the shifted frames of a movie with \( Z \) frames is given by \( \sum_{z=1}^{Z} F_{jz}S_{jz} \). The unnormalized...
correlation between two Fourier transforms, $F_1$ and $F_2$, is given by $F_1 \cdot F_2^*$ where $*$ denotes the complex conjugate. For the correlation between the sum image and the individual frame, these values must be summed for the $J$ Fourier components in a resolution band $\tilde{k}(j) \in [r_{\min}, r_{\max}]$. It is only necessary to consider two times the real part of the expression for the correlation, because the Fourier transforms of real functions, such as images, are Hermitian, so $\vec{k} \vec{J}(j)$. For the correlation between the sum image and the individual frame, these values must be summed for the objective function in equation 2. By providing equations 2, 3, and 4 for lm-bfgs optimization, values of $x_z$ and $y_z$ were obtained for movies of V-ATPase particles in ice. Fig. 2A shows the calculated trajectories from optimization.

2.2 Partial derivatives of the objective function

Numerous algorithms exist for optimizing objective functions. Optimization problems can benefit greatly from the ability to analytically determine partial derivatives, or gradients, of the objective function with respect to all variables. The derivatives of $S_{jz}$ and $S_{jz}^*$ with respect to $x_a$, the shift in the $x$ direction for the $a^{th}$ frame, are

$$\frac{\partial S_{jz}}{\partial x_a} = (-\sin \phi_{jz} + i \cos \phi_{jz}) \frac{\partial \phi_{jz}}{\partial x_a} = i S_{jz} \frac{\partial \phi_{jz}}{\partial x_a}$$

and

$$\frac{\partial S_{jz}^*}{\partial x_a} = (-\sin \phi_{jz} - i \cos \phi_{jz}) \frac{\partial \phi_{jz}}{\partial x_a} = -i S_{jz}^* \frac{\partial \phi_{jz}}{\partial x_a}$$

Using these simplifications, the derivative of the objective function in equation 2 with respect to $x_a$ is

$$\frac{\partial O(\Theta)}{\partial x_a} = -Re \sum_{j=1}^{J} \sum_{z=1}^{Z} \left[ F_{jz}^* S_{jz}^* \sum_{z'=1}^{Z} F_{jz'} S_{jz'} - F_{jz}^* S_{jz}^* \sum_{z'=1}^{Z} F_{jz'} S_{jz'} \right]$$

Noting that $\frac{\partial \phi_{jz}}{\partial x_a} = 0$ when $a \neq z$ and $\frac{\partial \phi_{jz}}{\partial x_a} = 2\pi \kappa_a(j)/N$ when $a = z$, the expression simplifies further:

$$\frac{\partial O(\Theta)}{\partial x_a} = -Re \sum_{j=1}^{J} \sum_{z=1}^{Z} \left[ i F_{jz}^* S_{jz}^* F_{ja} S_{ja} \frac{2\pi \kappa_a(j)}{N} \right] - \left[ i F_{ja} S_{ja} \frac{2\pi \kappa_a(j)}{N} \sum_{z=1}^{Z} F_{jz} S_{jz} \right]$$

$$= -Re \sum_{j=1}^{J} \sum_{z=1}^{Z} \left[ \frac{2\pi \kappa_a(j)}{N} \right] \left[ F_{ja} S_{ja} \sum_{z=1}^{Z} F_{jz}^* S_{jz} - F_{ja} S_{ja}^* \sum_{z=1}^{Z} F_{jz} S_{jz} \right]$$

Similarly, the partial derivative of equation 2 with respect to $y_a$ is

$$\frac{\partial O(\Theta)}{\partial y_a} = -Re \sum_{j=1}^{J} \frac{2\pi \kappa_a(j)}{N} \left[ F_{ja} S_{ja} \sum_{z=1}^{Z} F_{jz}^* S_{jz} - F_{ja} S_{ja}^* \sum_{z=1}^{Z} F_{jz} S_{jz} \right]$$

We elected to use the limited memory Broyden-Fletcher-Goldfarb-Shanno (lm-bfgs) algorithm [10] to optimize the objective function in equation 2. By providing equations 2, 3, and 4 for lm-bfgs optimization, values of $x_z$ and $y_z$ were obtained for movies of V-ATPase particles in ice. Fig. 2A shows the calculated trajectories from optimization.
of 200 regions of 320 × 320 pixels in each frame. These 200 image regions were selected by template matching from
the image in Fig. 1A, and contain a mixture of usable particle images and other image features. The trajectories
show local correlation, even though at this stage in the analysis individual particle trajectories are not provided with
any information about the trajectories of nearby particles, except for any overlap in the 320 × 320 pixel boxes. Close
inspection of the trajectories in two regions of the micrograph (Fig. 2Bi and ii) reveals noise in the trajectories of
individual particles obtained by the optimization method.

2.3 Smoothing

Although encouraging, the noise seen in trajectories of particles in Fig. 2Bi and ii suggests that the optimization
does not show the true trajectories of individual particle images. One obvious approach to reducing noise in a
trajectory is to calculate the trajectory from a larger portion of the image, thereby increasing the signal available
for calculating the objective function. Unfortunately, as the size of the box used for determining particle positions
increases, particles must progressively be excluded that fall too close to the edge of the image. Increasing box sizes
also results in almost identical trajectories for nearby particles that may mask the local variation in movement that
this technique aims to recover. Better noise removal can be achieved by using two reasonable assumptions that
are neglected in the analysis presented in Fig. 2. The first assumption is that trajectories are unlikely to have
sudden changes in direction, although the possibility of these changes cannot be eliminated. The second assumption
is that nearby particle trajectories are correlated. Enforcing these two conditions can be used to ‘smooth’ particle
trajectories to remove noise.

2.3.1 Second order smoothing

The assumption that true particle trajectories are unlikely to undergo sudden and dramatic changes in direction
can be enforced by penalizing changes in $\partial x_z / \partial z$ and $\partial y_z / \partial z$. If $\partial x_z / \partial z$ and $\partial y_z / \partial z$ are constant ($\partial^2 x_z / \partial z^2$ and
$\partial^2 y_z / \partial z^2$ are 0), the expected value for $(\vec{t}_z - \vec{t}_{z-1})$ is $(\vec{t}_{z-1} - \vec{t}_{z-2})$. Deviation from this expected linear trajectory can
be penalized by an amount $\lambda ((\vec{t}_z - \vec{t}_{z-1}) - [\vec{t}_{z-1} - \vec{t}_{z-2}])^2$. The overall penalty imposed on the objective function
to encourage smoothness is then given by

$$P(\Theta) = \sum_{z=3}^{Z} \lambda \left[ (x_z - 2x_{z-1} + x_{z-2})^2 + (y_z - 2y_{z-1} + y_{z-2})^2 \right]$$

(5)

where $\lambda$ is a user selected weighting parameter. This penalty is known as second order smoothing because it penalizes
finite difference approximations of the second derivatives of $x_z$ and $y_z$ with respect to $z$, $\partial^2 x_z / \partial z^2$ and $\partial^2 y_z / \partial z^2$.
The penalty function described in equation 5 is added to the objective function in equation 2 to obtain the overall
objective function that is optimized. The contribution to the penalty function in equation 5 from shifting of the $a^{th}$
frame when $a \in [3, Z-2]$ is

$$\lambda((\vec{t}_a - 2\vec{t}_{a-1} + \vec{t}_{a-2})^2 + (\vec{t}_{a+1} - 2\vec{t}_a + \vec{t}_{a-1})^2 + (\vec{t}_{a+2} - 2\vec{t}_{a+1} + \vec{t}_a)^2)$$

and consequently the first derivative of equation 5 with respect to $x_a$ is given by

$$\frac{\partial P(\Theta)}{\partial x_a} = \begin{cases} 
2\lambda (x_a - 2x_{a+1} + x_{a+2}), & a = 1, \\
2\lambda (-2x_{a-1} + 5x_a - 4x_{a+1} + x_{a+2}), & a = 2, \\
2\lambda (x_{a-2} - 4x_{a-1} + 6x_a - 4x_{a+1} + x_{a+2}), & a \in [3, Z-2]. \\
2\lambda (x_{a-2} - 4x_{a-1} + 5x_a - 2x_{a+1}), & a = Z-1, \\
2\lambda (x_{a-2} - 2x_{a-1} + x_a), & a = Z.
\end{cases}$$

(6)

and similarly

$$\frac{\partial P(\Theta)}{\partial y_a} = \begin{cases} 
2\lambda (y_a - 2y_{a+1} + y_{a+2}), & a = 1, \\
2\lambda (-2y_{a-1} + 5y_a - 4y_{a+1} + y_{a+2}), & a = 2, \\
2\lambda (y_{a-2} - 4y_{a-1} + 6y_a - 4y_{a+1} + y_{a+2}), & a \in [3, Z-2], \\
2\lambda (y_{a-2} - 4y_{a-1} + 5y_a - 2y_{a+1}), & a = Z-1, \\
2\lambda (y_{a-2} - 2y_{a-1} + y_a), & a = Z.
\end{cases}$$

(7)

The derivative of the smoothed objective function is therefore the sum of the values from equation 3 and 6 for the
derivative with respect to $x_a$, and the sum of the values from equation 4 and 7 for the derivative with respect to $y_a$.
Fig. 3A shows the effect of increasing values of the user set parameter $\lambda$ for two regions on opposite sides of
the micrograph (Fig. 3Ai and ii). With \( \lambda = 0 \), the trajectories are noisy, as seen in Fig. 2. With \( \lambda = 1 \times 10^5 \) a significant amount of noise has been removed from the trajectories. Note also that nearby trajectories appear to be correlated even though this condition has not been enforced. With \( \lambda = 1 \times 10^{10} \), an excessively large number, trajectories have been forced to become linear. Forcing trajectories to be linear is equivalent to fitting a single drift rate for each particle in the movie.

2.3.2 Local averaging for smoothing

Local correlation of nearby particle trajectories without the use of an increased box size can be achieved by weighted averaging after trajectories are calculated. In this approach, ‘raw trajectories’ are determined for individual particles with or without second order smoothing. Once raw trajectories are determined, locally averaged trajectories are calculated according to

\[
t_n = \frac{\sum_{m=1}^{M} w_{mn} \hat{r}_{nz}}{\sum_{m=1}^{M} w_{nm}}
\]

where \( t_n \) is the smoothed displacement vector for the \( n^{th} \) particle in the \( z^{th} \) frame and \( \hat{r}_{nz} \) is the original displacement vector for the \( m^{th} \) particle in the \( z^{th} \) frame. The weight \( w_{mn} \) is given by

\[
w_{mn} = \exp \left( -\frac{d_{mn}^2}{2\sigma^2} \right)
\]

where \( d_{mn} \) is the distance between the \( m^{th} \) and \( n^{th} \) particles and \( \sigma \) is a user set parameter that determines the extent to which the smoothing is applied. This Gaussian weighting is equivalent to the local averaging proposed used for fitting linear trajectories in Relion [9]. Because of the Gaussian form of equation 9, 95% of the weight for a particle trajectory will come from the trajectories within \( 2\sigma \) pixels of that particle. Fig. 3B shows the effect of increasing the \( \sigma \) parameter for two sets of nearby trajectories (Fig. 3Bi and ii), without the use of second order smoothing. With \( \sigma = 0 \), the trajectories are noisy, as seen in Fig. 2. With \( \sigma = 500 \) a significant amount of noise has been removed from the trajectories, even though smoothness has not been enforced. With \( \sigma = 5000 \), an excessively large number, trajectories on opposite sides of the micrograph from each other have been forced to be similar. In this situation, depending on the number of particles selected in the micrograph, the method becomes a nearly rigid frame alignment. Ideal smoothing of particle trajectories comes from combining the two approaches described above. Fig. 4A shows trajectories with \( \lambda = 1 \times 10^5 \) and \( \sigma = 500 \). As can be seen in two enlarged regions from opposite sides of the micrograph (Fig. 4Bi and ii), individual particle trajectories appear smooth with strong local correlation but significant variation from one edge of the micrograph to the other.

3 Discussion

For full frame alignment, the least squares algorithm proposed by Li and colleagues possesses a significant advantage over the approach described here, in that the frame translations are highly over-determined: a movie consisting of \( Z \) frames will provide \( (Z - 1) \times 2 \) equations that can be used to determine the \( Z - 1 \) frame translations needed to correct motion [4]. Consequently, the least squares method will likely outperform the global optimization approach in situations where whole frames are aligned. However, while the least squares method correlates low SNR frames with other low SNR frames, the global optimization approach correlates low SNR frames with the relatively high SNR sum of frames. Consequently, the global optimization method is able to work with image boxes at least as small as the 320 \( \times \) 320 pixel boxes used here. The global approach should behave similarly to an non-global iterative approach where frames are averaged and individual frames are subsequently aligned to the average. Special care must be taken in the non-global iterative approach to ensure that the \( a^{th} \) frame is not aligned to an average where the \( a^{th} \) frame has been included at a fixed position, which could bias the alignment of the \( a^{th} \) frame. In the global optimization approach presented here, the average always includes the \( a^{th} \) frame with the translations for the \( a^{th} \) frame that are being tested. Also, in the global optimization approach changing the translations for the \( a^{th} \) frame instantaneously affects the correlation of all other frames with the sum image, while with the non-global iterative approach it does not, possibly making the identification of a global optimum less robust. The non-global iterative approach will also almost certainly be slower than the global optimization algorithm at finding the optimum alignment of frames. The global optimization approach benefits from being able to incorporate the second order smoothness constraint directly into the objective function. Both algorithms could become trapped in a local alignment minimum. However, the form of equation 2 suggests that the problem is convex and global solutions will usually be found. The Relion procedure [9] integrates estimation of particle trajectories with projection matching from a reference map of the protein complex.
Both procedures attempt to regularize particle trajectories: Relion by using a running average of particle frames and fitting of a linear trajectory, the global optimization approach by introducing the second order smoothness constraint. The Relion approach has the potential advantage that projections from a refined 3D map will possess stronger signal than the sum of all frames used as a reference in the global optimization approach. The potential disadvantage of the Relion approach relative to the global optimization approach is that errors in contrast transfer function (CTF) estimation, structured noise in images from sample contamination or ice contamination, differing conformation of the protein particle in the image and map, and any other sources of inaccuracy in projection matching could affect the accuracy of trajectory estimation. Compared to the procedure introduced in Relion, the global optimization approach will also be much less computationally expensive.

The two different smoothing approaches, second order smoothing and local weighted averaging of trajectories, have different advantages and uses. Second order smoothing is independent of particle density in images. If images contain few particles, their trajectories should be smoothed by increasing the second order smoothing parameter $\lambda$. Local averaging of trajectories will have little effect for particles that are far apart but can be applied effectively where there are many particles or other image features that can be aligned. A value of $\sigma$ should be chosen that reflects how quickly trajectories change across the image. For this situation, the amount of second order smoothing can be decreased somewhat. In the future, trajectory smoothing could benefit from a physical model of what is causing the apparent translations of particles. Basing the allowed particle trajectories on a physical model would limit particles to reasonable trajectories, decreasing the effects of noise in the the final estimates of trajectories. At higher resolution, or for larger particles, it could be useful to interpret trajectories to include the rotations of particles in the ice layer.

In addition to drift and beam-induced movement, another problem that can occur during exposure series movies is that specimens are increasingly radiation damaged in each frame. It was proposed previously that exposure series movies from DDDs could be used to account for radiation damage that occurs during imaging [11]. In this approach, each frame of the movie is filtered to limit its contribution to the average only to spatial frequencies that still contain information after radiation damage, based on measurements of the optimal total exposure for each spatial frequency. Exposure weighting is easily included in the program that implements particle drift correction, and indeed was included in the program alignparts_lmbfsgs.

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### 5 Author contributions

JLR conceived of the Fourier space objective function, wrote the programs, characterized their performance, and wrote the manuscript. MAB suggested the use of the gradient-based lm-bfgs algorithm, proposed the second order smoothing, and made other critical suggestions for the programs and the manuscript.

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Figure 1: Example micrograph. **A,** The average of 30 frames after least squares alignment of whole frames for an exposure series movie of the *Saccharomyces cerevisiae* V-ATPase in ice. The exposure used 2.5 e⁻/pixel/frame and each pixel corresponds to 1.45 × 1.45 Å. The *S. cerevisiae* V-ATPase has a molecular weight of 900 kDa. Several example complexes are circled in red. **B,** An individual frame from the movie shows the low SNR in frames.
Figure 2: **Raw trajectories from lm-bfgs optimization of the objective function.** A, A plot of individual particle trajectories in image regions that are $320 \times 320$ pixels. Each line in the plot indicates the trajectory of a single particle from frame 1 (black) to frame 30 (blue), exaggerated by a factor of 5. As can be seen from the plot, there is local correlation of particle trajectories. B, Inspection of individual particle trajectories from two regions of the micrograph (i and ii) reveals that there is significant noise in the trajectories.
Figure 3: Effects of two approaches for smoothing particle trajectories. **Ai** and **ii**, Plots of trajectories in two small regions of the same micrograph in Fig. 2 show unsmoothed trajectories (green line), second-order smoothed with trajectories with sufficient smoothing $\lambda = 5 \times 10^5$ (black line), and second-order smoothed trajectories with excessive smoothing $\lambda = 1 \times 10^{10}$ (red). With excessive smoothing, trajectories have been forced to have a single linear drift rate. **Bi** and **ii**, Plots of trajectories in two small regions of the same micrograph showing unsmoothed trajectories (green line), locally averaged trajectories with a reasonable weighting $\sigma = 500$ (black line), and locally averaged trajectories with excessive weighting $\sigma = 5000$ (red). With excessive local averaging, trajectories in different parts of the micrograph have been forced to be identical.
Figure 4: **Combined smoothing approaches.** A, Particle trajectories for the whole micrograph and for two regions on opposite sides of the image (Bi and ii). By applying a combination of local averaging ($\sigma = 500$) and second-order smoothing ($\lambda = 1 \times 10^4$) individual particle trajectories display local uniformity but global variation and avoid sudden direction changes for trajectories.