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Robust image alignment for cryogenic transmission electron microscopy

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ABSTRACT

Cryo-electron microscopy recently experienced great improvements in structure resolution due to direct electron detectors with improved contrast and fast read-out leading to single electron counting. High frames rates enabled dose fractionation, where a long exposure is broken into a movie, permitting specimen drift to be registered and corrected. The typical approach for image registration, with high shot noise and low contrast, is multi-reference (MR) cross-correlation. Here we present the software package *Zorro*, which provides robust drift correction for dose fractionation by use of an intensity-normalized cross-correlation and logistic noise model to weight each cross-correlation in the MR model and filter each cross-correlation optimally. Frames are reliably registered by *Zorro* with low dose and defocus. Methods to evaluate performance are presented, by use of independently-evaluated even- and odd-frame stacks by trajectory comparison and Fourier ring correlation. Alignment of tiled sub-frames is also introduced, and demonstrated on an example dataset. Zorro source code is available at github.com/CINA/zorro.

1. Introduction

In the transmission electron microscope (TEM) image quality is often limited by the signal-to-noise ratio (SNR) arising from limited source brightness and exposure time rather than the ultimate instrument resolution. The TEM is highly sensitive to the quiescence of its environment, such as drift and harmonic disturbances, that restrict the range of reasonable exposure times. A popular approach to manage image drift is dose fractionation, where a long exposure is split into many frames, the drift of each frame is registered (typically by cross-correlation techniques), and the resulting aligned image stack is averaged (Campbell et al., 2012). Conventional approaches with a phase cross-correlation (PXC) (Kuglin and Hines, 1975) or the normalized cross-correlation (NXC) algorithm (Lewis, 1995), require a template, also known as a reference, against which all other frames (known as bases) are registered. However, in images with a poor SNR, typically around or below 1.0 electron per pixel, template-matching often fails to accurately register image motion. The introduction of direct detection devices (DDDs) such as the Gatan K2 (Gatan Inc., Pleasanton, CA), Direct Electron DE-16 (Direct Electron LP, San Diego, CA), and FEI Falcon-2 (FEI Company, Hillsboro, OR) (Ruskin et al., 2013; McMullan et al., 2014) greatly improved the detector quantum efficiency (DQE) over scintillator-coupled, slow-scan charge-coupled device (CCD) detectors. DDDs allow recording frames with minimal dark noise, so that the sum of a multitude of dose-fractionation exposures does not show significantly more noise than one accumulated high-dose exposure would have contained.

The laboratory of Y. Cheng (University of California at San Francisco) introduced a very successful multi-reference correlation strategy for dose-fractionated movies implemented as the *MotionCorr* algorithm (Li et al., 2013a). *MotionCorr* uses a low-pass filtered phase correlation, to suppress high-frequency artifacts, paired with a multi-reference model where every image in the movie stack is correlated to every other image. The multi-reference approach results in an over-determined set of equations that are subjected to a minimization of the root-mean-square error (i.e. 'least-squares') to solve the specimen trajectory. In addition, *MotionCorr* performs subpixel peak location by Fourier interpolation (Guizar-Sicairos et al., 2008). However, the phase correlation is sensitive to correlated noise introduced by detector artifacts

Abbreviations: CCD, charged coupled device; CDF, cumulative distribution function; CTF, contrast transfer function; Cryo-EM,, cryogenic electron microscopy; Cryo-TEM,, cryogenic transmission electron microscopy; DDD, direct (electron) detection device; DQE, detector quantum efficiency; FoM, figure of merit; FRC, Fourier ring correlation; FSC, Fourier shell correlation; MNXC, masked, (intensity) normalized cross-correlation; MTF, modulation-transfer function; NPS, noise power spectrum; NXC, (intensity) normalized cross-correlation; PXC, phase cross-correlation; SNR, signal-to-noise ratio; STEM,, scanning transmission electron microscope; TEM, transmission electron microscope.

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(i.e. fixed-pattern noise) and variation in image background-intensity. The Fourier phase correlation also makes the intrinsic assumption that the object is periodic, which introduces additional artifacts from discontinuous boundaries.

Therefore an algorithm with a noise model that handles corrected noise should improve the robustness of dose fractionation frame registration. Another potential avenue for improved performance is successful alignment of image stacks with less dose per frame, such that ice contrast is not lost, and less defocus, where there is less delocalization of intensity. The defocus condition with the least delocalization of information, i.e. where the spatial damping envelop of the CTF is broadest, is known as the Lichte defocus (Lichte, 1991). The Lichte defocus is typically in the range of 500–800 nm for cryo-pole piece TEMs. Higher magnification mitigates the impact of detector DQE and aliasing that reduces information content for spatial frequencies near Nyquist.

The sensitivity of the phase correlation to artifacts led to the development of the Normalized Fast Cross-Correlation algorithm developed by Industrial Light and Magic (Lewis, 1995), which remains the standard method for template matching. This algorithm is computed in real-space and requires that the template be smaller than the base image, therefore making it computationally expensive and difficult to employ with large images. It is important to distinguish between intensity-normalization, where the shared background between the base and template is removed, and normalizing the mean and standard deviation such that the correlation falls within the range $[0, 1]$. The intensity-normalization deconvolves correlated (or fixed-pattern) noise shared between frames, which is a common in electron microscopy detectors.

In comparison to the intensity-normalized algorithm, the correlation coefficient of the phase correlation varies with both raw image intensity, and the shape of the low-pass (B-factor) filter applied, which renders a general comparison of correlation-coefficients difficult. Alternatives to the phase-correlation have been proposed within the cryo-TEM community. Stewart and Grigorieff introduced a correlation where the Fourier constituent frequencies are known, the correlation can be Wiener filtered by an associated noise model, popularized in the *Frealign* software package (Stewart and Grigorieff, 2004). Roseman introduced a Fourier-space correlation with a single mask for particle picking (Roseman, 2003).

The Industrial Light and Magic intensity-normalized cross-correlation was generalized to the Fourier domain by Padfield for ultrasound images and photography (Padfield, 2012). Padfield further introduced the application of two of separate binary masks for both the template and base images in real-space, known as a masked (intensity-) normalized cross-correlation (MNXC). The effect of the masks is subtracted from the final correlation map, thereby limiting the pixels that contribute to the correlation coefficient. For problems that can be effectively segmented the masks can be used to exclude feature-free pixel region such as vacuum that contribute noise but not signal to the alignment.

Other approaches have appeared in the cryo-TEM literature since *MotionCorr* was published. Bartesaghi et al. use the cumulative average of prior frames as the reference (Bartesaghi et al., 2014). *UnBlur* (Grant and Grigorieff, 2015) utilizes an alternative registration philosophy. In *UnBlur,* the shifts are refined over several iterations against the sum reduced by the current frame until the solution converges. The initial estimate of *UnBlur* is the unregistered stack average. *UnBlur* Fourier crops the data to a $[1024, 1024]$ pixel frame, so only low-frequency information is permitted in the solution, and by default a strong low-pass B-filter is applied. *UnBlur* uses a phase correlation algorithm where the central-cross artifact is removed, to reduce the amount of correlated noise on the X and Y axes. *UnBlur* also applies the heuristic of spline-smoothing the measured trajectory over each iteration. The heuristic assumption in this case is that the drift trajectory is similar to a smoothed spline, which is probably true for charge-driven drift and probably not for finer Brownian motion. Version 2.1 of *MotionCorr* (released by Li on 23-Nov-2013, and not be confused with the forthcoming *MotionCor 2* from the Agard laboratory), uses a similar technique whereby a cumulative average of the prior-frames' Fourier spectra serves as the reference. Rubinstein and Brubaker use a functional minimization applied with the Fourier-space coherence function, where shifts are applied with a phase wedge (Rubinstein and Brubaker, 2014). Trajectory smoothing is also applied by deweighting large shifts within the gradient function used by the minimization function.

A refinement procedure intended for sub-frame registration was also implemented in *Zorro*, and it does not improve alignment performance on complete stacks.

Progress has also been made towards local (non-rigid) micrograph registration. Abrishami used an optical flow approach that assumes local motion must have smooth, continuous derivatives (Abrishami et al., 2015). *Relion* is capable of registering non-rigid particle motion by linear fits to a moving average of the region around a particle (Scheres, 2014). Using the trajectory smoothing approach, Rubinstein has shown that sub-megadalton particles in cryo-EM images can be aligned using non-linear trajectories (Rubinstein and Brubaker, 2014). Here we introduce a refinement step called *subZorro*, intended to be used on sub-frame oversampled tiles, which on tilted images of a vitrified 2D membrane protein crystal shows improvements in coherence.

The approach here merges the MNXC algorithm with the *MotionCorr* approaches of multi-reference correlation and low-pass filtering, or weighting, of frequencies contained in the cross-correlation. Additionally, the least-squares solution of the set of shifts in *MotionCorr* has been extended by moving to a global minimization of the weighted error, using the Basin-hopping algorithm (Wales and Doye, 1997). With the normalized cross-correlation, correlation values are consistent and a figure of merit (FoM) is devised to assess the statistical significance of each cross-correlation. Logistic function weights based on this FoM are applied to each equation in the minimization of the global error. The logistic function is fit to the cumulative distribution of cross-correlation peak statistical significance. This step is critical for cryo-TEM, because as shown in Section 2.3 the correlation scores drop rapidly to insignificance as frames become separated by larger time/dose steps.

To summarize the manuscript, Section 2.1 discusses the theoretical background for image drift in TEM and Section 2.2 compares the cross-correlation algorithms used to register drift. Section 2.3 introduces the noise model and justification. In Section 3, the performance of *Zorro* is analyzed for a number of challenging test cases together with *MotionCorr* 2.1 and *UnBlur*. Additional results are found in the online Supplemental material. Last, in Appendix 1, a brief on the usage of *Zorro* and its pipeline GUI *Automator* is provided. The software described herein has been released as the *Zorro* package on Github under the MIT license (at: www.github.com/C-CINA/zorro). All results were acquired at C-CINA, Universität Basel, on a Titan Krios operated at 300 keV, equipped with a Gatan Quantum-LS image filter and a K2 Summit DDD.

2. Materials and methods

The general principle of dose fractionation is that if a long exposure can be broken into many frames, then the drift can be registered, such that the movie stack can be aligned and summed. Registration is typically performed via cross-correlation. Here the fundamental nature of specimen drift in the TEM is examined, and a comparison between the PXC and MNXC algorithms made.

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