Published in final edited form as:

J Struct Biol. 2017 March ; 197(3): 210–219. doi:10.1016/j.jsb.2016.11.001.

Real-time detection and single-pass minimization of TEM objective lens astigmatism

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Abstract

Minimization of the astigmatism of the objective lens is a critical daily instrument alignment task essential for high resolution TEM imaging. Fast and sensitive detection of astigmatism is needed to provide real-time feedback and adjust the stigmators to efficiently reduce astigmatism. Currently the method used by many microscopists is to visually examine the roundness of a diffractogram (Thon rings) and iteratively adjust the stigmators to make the Thon rings circular. This subjective method is limited by poor sensitivity and potentially biased by the astigmatism of human eyes. In this study, an s^2 power spectra based method, s^2 stigmator, was developed to allow fast and sensitive detection of the astigmatism in TEM live images. The “radar”-style display provides real-time feedback to guide the adjustment of the objective lens stigmators. Such unique capability allowed us to discover the mapping of the two stigmators to the astigmatism amplitude and angle, which led us to develop a single-pass tuning strategy capable of significantly quicker minimization of the objective lens astigmatism.

Keywords

Astigmatism correction; Objective lens stigmators; Real-time feedback; “Radar”-style display; Single-pass tuning strategy; cryo-EM

1. Introduction

Transmission electron microscopy (TEM) has become a powerful technique for structural characterization of a wide range of materials including macromolecular complexes at near-atomic resolutions. In order to obtain high-quality images, it is crucial to carefully align the microscope to reach optimal illumination lens and imaging lens conditions. Minimization of the astigmatism of the objective lens is a critical task of the daily microscope alignment.

The astigmatism of TEM objective lens is defined as the angular dependency of defocus, which is primarily represented by the two-fold astigmatism with elliptical Thon rings (Thon, 1971) in the power spectra of TEM images. The astigmatism is an essential component of the contrast transfer function (CTF) of TEM images. Many approaches have been proposed for the estimation of CTF parameters based on the power spectra of images (Fernando,

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2008; Huang et al., 2003; Jiang et al., 2012; Mallick et al., 2005; Mindell and Grigorieff, 2003; Sander et al., 2003; Sorzano et al., 2007; Vulović et al., 2012; Yang et al., 2009). The CTF parameters are typically determined by iterative fitting simulated power spectra to the experimental power spectra by varying the parameters in the CTF model. However, due to the iterative nature of current methods and their availability limited to offline batch processing on Linux computers, current CTF fitting methods are not used for providing real-time feedback to guide instrument alignment. As a result, current method widely used by many microscopists is to visually examine the roundness of Thon rings from live images and simultaneously adjust objective lens stigmators to make the Thon rings as circular as possible. However, the drawbacks of this subjective method have been well recognized (Saxton, 2000), such as its limited sensitivity for small astigmatism and potential bias caused by the astigmatism of human eyes. It is not uncommon that a user spends significant amount of time (e.g. 30 min) to iteratively adjust the two stigmator knobs just to convince himself/herself that the objective lens astigmatism is indeed minimized. Therefore, a quantitative method capable of fast and sensitive estimation of astigmatism is desirable for improved TEM instrument alignment by providing real-time feedbacks for the adjustment of objective lens stigmators to minimize the objective lens astigmatism.

In this work, we have developed such a method, *s²stigmator*, that uses a direct, closed-form solution and comprehensively takes advantage of Fourier transform theory to fast calculate the mean defocus, astigmatism amplitude and astigmatism angle. The method was implemented as a DigitalMicrograph script to allow fast and sensitive detection of the astigmatism of live images. It thus can provide real-time feedback and user-friendly “radar”-style display to help guide the adjustment of objective lens stigmators and efficiently correct the astigmatism of the objective lens.

2. Method

2.1. The *s²* power spectra based algorithm for astigmatism estimation

Fig. 1 shows an overview of the *s²stigmator* algorithm. Fig. 1A is a synthetic image in which each particle is generated by projecting a 3D density map of herpesvirus (EMD-3358) into an arbitrary orientation. A CTF is applied to this image with defocus 2000 nm, astigmatism amplitude 100 nm and astigmatism angle 30°. The astigmatism angle is defined as the angle of the maximum defocus direction according to the conventions defined by the Electron Microscopy eXchange (EMX) initiative (Marabini et al., 2016). This image is used here to represent a “live” image to help illustrate the algorithm as detailed in the following paragraphs.

Step 1: Computation of the average 2D power spectra—In order to enhance the signal-to-noise ratio (SNR) and shorten the processing time in the subsequent steps, the 2D power spectra (Fig. 1B) is calculated using periodogram averaging method (Fernández et al., 1997; Zhu et al., 1997). We divided the image (Fig. 1A) into multiple small square patches, then applied the Fast Fourier Transformation (FFT) on each patch to obtain the individual 2D power spectra, incoherently summed all of them to obtain the average 2D power spectra, and finally performed log-transform on the power spectra to minimize the magnitude

difference from low to high resolution (i.e. center to edge). This method supports both square images (by most CCDs and Falcon direct electron detector) and rectangular images (by K2, DDD direct electron detectors).

Step 2: Conversion to 2D s^2 power spectra—After removing the intensity gradient by high pass filter, denoising by low pass filter, and truncating the 2D power spectra at a specific resolution (i.e. 5.3 Å in this example), 2D s^2 power spectra (Fig. 1C) is generated by resampling using bilinear interpolation to make the radii represent the square of spatial frequency s^2 instead of spatial frequency s (Jiang et al., 2012). Compared with the progressively increasing frequency of oscillations in regular 2D power spectra (Fig. 1B), 2D s^2 power spectra (Fig. 1C) consist of uniform oscillations from center to edge with equal spacing between adjacent Thon rings (Jiang et al., 2012). Furthermore, the Thon rings in s^2 power spectra appear more elliptic than those in regular power spectra when astigmatism exists in the image (Jiang et al., 2012).

Step 3: Generation of defocus-dependent ring from 2D s^2 power spectra—Due to the uniform oscillations in the 2D s^2 power spectra, a single ring (Fig. 1D) can be obtained after applying FFT on the 2D s^2 power spectra. The radius of the single ring along a specific direction is proportional to the CTF oscillation frequency or defocus in this direction since there is a positive linear relationship between the defocus and the frequency of oscillations in s^2 power spectra (Jiang et al., 2012). Consequently, the ellipticity of the single ring can be considered as a sensitive indicator to estimate the astigmatism in the image. The single ring is perfectly round for images without astigmatism. In contrast, it is elliptic for astigmatic images, suggesting angular variation of the defocus.

Step 4: Enlarging the ring with the 2nd type Fourier shifting—Note that smaller defocus corresponds to fewer Thon rings in the 2D s^2 power spectra, resulting in the single ring very close to the Fourier origin (i.e. center in Fig. 1D) with very coarse sampling by a small number of pixels. The coarse sampling inevitably causes difficulties in ring-shape determination and astigmatism estimation in subsequent steps. To overcome this drawback, we took advantage of the 2nd Fourier shift property (Eq. (1)) to move the single ring outward by the same amount in all directions (Fig. 1E) closer to the edge. The shifted/enlarged ring contains more pixels with finer sampling that will allow more accurate detection of the ellipticity of the ring.

The 2nd Fourier shift theorem (Tisdell, 2013a) is described in Eq. (1):

$$e^{j2\pi s_0 t} f(t) \leftrightarrow F(s-s_0) \quad \text{Eq. (1)}$$

where f represents the function in real space (e.g. 2D s^2 power spectra in Fig. 1C), F represents the Fourier transformed function (e.g. the shifted single ring in Fig. 1E), $\leftrightarrow$ represents Fourier transform, j is the imaginary unit, t and s represent the independent variables in real and Fourier space, respectively. The parameter s_0 represents the amount of shift in Fourier space. The amount of shift can be tailored to different images by first detecting the mean radius of the ring in the unshifted s^2 power spectra and then calculating

its distance to the target radius (e.g. 4/5 of the patch size). This 2nd Fourier shift theorem is closely related to the more commonly used Fourier shift theorem that relates shift in real space with phase changes in Fourier space (Tisdell, 2013b). However, the shift and phase changes occurred in opposite spaces for these two types of Fourier shift theorems.

In our algorithm, we multiplied the complex term ($e^{j2\pi s_0 t}$) to the 2D s^2 power spectra (Fig. 1C), then performed FFT and masked out the inner and outer areas, so that a shifted/enlarged single ring (Fig. 1E) sampled by more pixels can be obtained.

Step 5: Representation of the shifted single ring in polar coordinates—The shifted single ring (Fig. 1E) is transformed into polar coordinates (Fig. 1F) using bilinear interpolation. In Fig. 1F, the horizontal dimension represents the radius of the shifted single ring and the vertical dimension represents angles from 0 to 2π .

Step 6: Direct, closed-form solution of the mean defocus, astigmatism amplitude and angle—We detected the peak position (radius of the shifted single ring) in each row (i.e. each direction) of the polar representation (Fig. 1F) and displayed them in Fig. 1G. Since the peak position corresponds to the defocus in a specific direction, we can convert the peak position to defocus according to Eq. (2):

$$\begin{aligned} ds &= 1.0 / (apix \times b) \\ r_{\max} &= \text{int}(1.0 / res / ds + 0.5) \\ s_{\max} &= r_{\max} \times ds \\ \lambda &= 12.2639 / \sqrt{V \times 10^3 + V^2} \times 0.97845 \\ f &= (p - \tau) / (s_{\max}^2 \times \lambda \times 10) + f_{s4} \\ &= r / (s_{\max}^2 \times \lambda \times 10) + f_{s4} \end{aligned} \quad \text{Eq. (2)}$$

where $apix$ is the sampling ($\text{\AA}/\text{pixel}$) of the original image (Fig. 1A); b is the size of the small square patch in periodogram averaging (see Step 1); res is the specific resolution at which the 2D power spectra is truncated (i.e. $5.3 \text{ \AA}$ in this example, see Step 2); λ is the electron wavelength; V is the accelerating voltage (kV) of TEM; f is the defocus; p is the peak position (pixels) of each row in Fig. 1F, τ is the amount of Fourier shifting (pixels) in Fig. 1E and r is the radius of the single ring along one direction in Fig. 1D. f_{s4} is a small adjustment factor to compensate the contribution of the s^4 term in the CTF model (see Supplementary Text). In Fig. 1G, the X-axis indicates the angles from 0 to 2π and Y-axis indicates the defocus (nm) after conversion from peak positions.

The wavy curve shown in Fig. 1G represents the angular distribution of the defoci. The average would equate the mean defocus. The apparent oscillations would indicate noticeable defocus variations and thus astigmatism in the image. To obtain the astigmatism amplitude and angle, we performed FFT of this 1D curve. Fig. 1H shows the amplitude of the first ten elements in the FFT. It is well-established that the twofold astigmatism is the primary astigmatism of objective lens, which is consistent with the dominant second element in Fig. 1H. To demonstrate the second element is sufficient to depict the curve in Fig. 1G, we only kept the second element in the complex array and zeroed all other complex numbers, then performed inverse FFT and produced a smooth 2-cycle sine wave (red curve in Fig. 1G). As

can be seen in Fig. 1G, there is a good agreement between the curve of measured defoci (greenish region) and the 2-fold astigmatism curve (red curve). Thus, we can directly estimate the astigmatism amplitude and angle from the amplitude and phase of the second complex number, respectively.

In addition, the astigmatism angle derived from the phase of the second complex number is marked in Fig. 1B–E by red arrows. It is noted that the major axis direction of the single ring (red arrows in Fig. 1D and E) is the maximum defocus direction since the distance to the center here is linearly proportional to the defocus. This direction corresponds to the minor axis direction in the Thon rings (red arrows in Fig. 1B and C) along which the defocus is the largest and the Thon rings oscillate fastest.

Step 7: Final “radar”-style display to provide real-time feedback for the correction of astigmatism—Fig. 1I and J are the final plots of our script that serve as a visual guide for the adjustment of objective lens stigmators. As shown in Fig. 1I, the intensity of pixels along the major and minor directions of the shifted single ring are plotted in blue and red, respectively. According to Eq. (2), these 2 peaks are converted to defocus in the major and minor directions and the difference between them is the astigmatism amplitude. Defocus, astigmatism amplitude and astigmatism angle are displayed at the top of Fig. 1I and all of them closely match the set values of the simulated image. When astigmatism is reduced, these two peaks will move closer and overlap at astigmatism-free conditions.

The radar plot in Fig. 1J provides a big picture for the correction of astigmatism. The black dot in Fig. 1J indicates the current astigmatism with the distance to the center proportional to the astigmatism amplitude and the angle from +X axis equal to the astigmatism angle. Based on this plot, it is intuitive to recognize the astigmatism amplitude and angle and the trajectory of astigmatism in response to the tuning of objective lens stigmators. When astigmatism is perfectly corrected, the black dot will move to the center of the plot.

2.2. Implementation

The algorithm described here has been implemented as a standalone DigitalMicrograph script *s²stigmator.s*, allowing immediate processing of live images acquired by the computer controlling the microscope. The script was tested on DigitalMicrograph version 1.85 on our CM200 and version 2.32 on our Titan Krios microscopes. Fig 1B–J and similar plots in other figures were generated by this DigitalMicrograph script. An offline version of our algorithm has also been written as *s2ctf.py*, a Python script for batch determination of the CTF parameters of images post acquisition. Both scripts will be available on our web site (<http://jiang.bio.purdue.edu>).

2.3. Test datasets

Our method was first validated by simulated images generated using EMAN2 library functions (Tang et al., 2007). Images were synthesized by projecting a herpesvirus density map (EMD-3358) into arbitrary orientations. A CTF function was applied to each synthetic image with a specific setting of defocus, astigmatism amplitude and angle.

Furthermore, four published experimental datasets (EMPIAR-10038, EMPIAR-10031, EMPIAR-10028 and EMPIAR-10002) of experimental datasets were downloaded from EMPIAR (Iudin et al., 2016) to test the performance of our method using these real micrographs. The estimated defocus, astigmatism amplitude and angle values were cross-validated with CTFFIND3 method (Mindell and Grigorieff, 2003).

Finally, the real-time feedback was tested using live images of carbon film obtained on our CM200 at 200 kV and FEI Titan Krios at 300 kV, and recorded on a Gatan CCD and K2 Summit camera, respectively. In order to test the dose sensitivity of our script, 10 movies (22 frames per movie) were collected with a dose rate of 9 electron/pixel/s (eps) and 0.5 s exposure time for each frame using the Titan Krios microscope and K2 camera. Subsequently, motion correction (Li et al., 2013) was performed on different numbers (e.g. the first two frames, the first four frames, etc) of frames in each movie to generate 11 groups of images with different total dose. These images were analyzed using our method and the root-mean-square deviation (RMSD) of the results in each dose group was calculated to find the appropriate dose range for our method. In addition, videos were recorded on CM200 and Titan Krios to demonstrate the performance of our method and illustrate the recommended strategy of astigmatism correction. The anisotropic magnification distortion was corrected from the live images according to the previously measured parameters (Yu et al., 2016).

3. Results

3.1. Validation of s^2 stigmator method using simulated images

To substantiate the performance of our s^2 stigmator method, we first tested four simulated images obtained as described in Section 2.3 with different combinations of CTF parameters. Fig. 2 displays the results for the simulated images with different values of defocus, astigmatism amplitude and angle. The first row (Fig. 2A–D) contains images of the single ring before Fourier shifting. It can be seen that the size of the ring increases as defocus increases, however, it is very hard to visually examine the ellipticity. It is worth pointing out that Fourier shifting (Step 4 in Section 2.1) is essential for the detection of ellipticity, especially at small defocus (Fig. 2A). The second (Fig. 2E–H) and third (Fig. 2I–L) rows are the standard plots of our script. Apparently, the offsets between the red and blue peaks (Fig. 2E–H) becomes larger with increased astigmatism. The black dots (Fig. 2I–L) also become more distant to the origin in the radar plots. Both types of plots accurately reflect the defocus, astigmatism amplitude and angle of the corresponding images. The astigmatism angles are marked using red arrows in Fig. 2A–D.

Next, we tested our method on more simulated images with varying defocus (500 nm–4000 nm), astigmatism amplitude (20 nm–120 nm) and angle (5° – 175°). The tight clustering along the diagonal lines in Fig. 3 confirms a good consistency between the determined defocus (Fig. 3A), astigmatism amplitude (Fig. 3B), astigmatism angle (Fig. 3C) and their true values used in simulations, respectively. Hence, all these tests with simulated images fully support the accuracy and reliability of our method.

3.2. Cross-validation with experimental images

Our algorithm was also cross-validated using experimental datasets. Fig. 4 shows the results of four images, one from each experimental datasets, EMPIAR-10038 (the first column, Fig. 4A, E, I), EMPIAR-10031 (the second column, Fig. 4B, F, J), EMPIAR-10028 (the third column, Fig. 4C, G, K) and EMPIAR-10002 (the fourth column, Fig. 4D, H, L). The astigmatism angles are also marked in the figures of single rings (Fig. 4A–D) using red arrows. As can be seen from Fig. 4B–D, the elliptical shape is not obvious due to the relatively small size of the single ring even though the astigmatism is large (>200 nm in Fig. 4B), emphasizing the importance of Fourier shifting in the detection of ellipticity. Due to the varying combinations of *apix*, box size (*b* in Eq. (2)) and the specific resolution (*res* in Eq. (2)) at which the 2D power spectra is truncated, the radius of the single ring is widely variable. The Fourier shifting is able to deal with the variability of the ring size by shifting/enlarging the radius large enough (such as Fig. 1E) to reliably measure the ellipticity. The distance between the two peaks (Fig. 4E–H) clearly indicates the amount of astigmatism. These results were then cross-validated using CTFFIND3 of which the results are shown as red dots in the radar plots (Fig. 4I–L). The superimposition of black and red dots indicates a good agreement between the results independently derived from these two approaches.

Based on the above results, our method is able to reliably determine the astigmatism and defocus of both simulated and experimental images. To further corroborate this conclusion, we used our method and the CTFFIND3 method to test larger number of experiment images (i.e. the four experimental datasets listed in Section 2.3, 100 micrographs in each). In Fig. 5A, there is an excellent agreement between these two methods in the estimation of defocus. The results of astigmatism amplitude and angle are also clustered along the diagonal lines in Fig. 5B and C although with small spread. Considering the challenges in estimating astigmatism and the large discrepancies among the different CTF fitting methods found by the CTF Challenge (Marabini et al., 2015), such small spread shown in Fig. 6B and C can be considered excellent agreement. As these two astigmatism determination methods are dramatically different in theory, overall consistent results shown in Fig. 5 suggest that the results by both methods are correct although the ground truth is unknown.

3.3. Performance tests with live images

For a comprehensive evaluation of the real-time feedback of our *s²stigmator* method, we tested our script with live images acquired as described in Section 2.3. We first analyzed the reproducibility (RMSD of astigmatism amplitude and angle) of our method with images acquired at different doses. As shown in Fig. 6A, it is evident that the RMSD is quickly reduced as the dose increases. The RMSD decreases to less than 1 nm when the dose is higher than $10 \text{ e}/\text{\AA}^2$, a dose level that is smaller than the dose used by typical single particle cryo-EM images. Fig. 6B–D display the distribution of results from 10 measurements for total dose of $5.2 \text{ e}/\text{\AA}^2$, $10.3 \text{ e}/\text{\AA}^2$ and $56.9 \text{ e}/\text{\AA}^2$, respectively, which correspond to the first, second and last points in Fig. 6A. It is apparent that the points are tightly clustered in Fig. 6C and D, demonstrating that the measurement error (<1 nm) of our method can be essentially ignored for doses larger than $10 \text{ e}/\text{\AA}^2$.

3.4. Single-pass tuning strategy for adjusting objective lens stigmators

The excellent reproducibility of our method for live images allowed us to use its radar plot (Fig. 1J, Fig. 2I–L, Fig. 4I–L, Fig. 6B–D) as a visual guide for real-time minimization of objective lens astigmatism by adjusting the two objective lens stigmators (i.e. M-X and M-Y knobs). By observing how the points move in the radar plot after adjustments of these two stigmators, we quickly realized that the two stigmators independently controls the astigmatism amplitude (by M-X) and angle (by M-Y), instead of the X or Y components of the astigmatism as one would infer from the labels of the two knobs. This observation has led us to understand the relationship of the objective lens astigmatism and the correction field generated by the stigmators (Fig. 7A), and how the stigmators can be controlled to optimally compensate the astigmatism (Fig. 7B, C).

In the vector diagram (Fig. 7A), the residual astigmatism of the objective lens is represented by a solid vector. The correction field generated by the objective stigmators is represented by a dashed vector. The M-Y stigmator would simply rotate the dashed vector while the M-X stigmator would change the length of the dashed vector. The objective lens astigmatism would be perfectly corrected when the dashed vector is adjusted to have the same length but in opposition direction to the solid vector (Fig. 7C). The challenge is thus how to arrive at the optimal correction state.

Based on this understanding, we designed a single-pass tuning strategy (Fig. 7D). *Initial state.* The yellow star indicates the initial state in which the two vectors have an arbitrary relationship (length and angle) as shown Fig. 7A. *Step 1.* The angular knob (M-Y) is continuously adjusted to find the optimal angle. In the radar plot, the points representing the residual astigmatism should exhibit an arc-like trajectory as represented by the black markers (black circles and black triangle) and the long black arrow in Fig. 7D. The position in the arc closest to the origin (black triangle in Fig. 7D) will be the optimal M-Y setting that makes the dashed vector in opposite direction of the solid vector (Fig. 7B). This effect of angular knob adjustment can be explained using vectors in Fig. 7B in which the dashed vector is continuously rotated until it has sampled the optimal angle (i.e. opposite to the solid angle). *Step 2.* The angular knob is turned backward to bring the point back to the optimal point (black triangle). This part of trajectory can be represented by the short black arrow in Fig. 7D. This will bring the dashed vector in opposite direction of the solid vector again (Fig. 7B). *Step 3.* The amplitude knob (M-X) is continuously adjusted. In this process, the resulted points in the radar plot should directly move to the origin along radial direction as shown by red markers (red squares and red triangle) and red arrow in Fig. 7D. In this step, turning M-X knob will simply change the length of the dashed vector until its length is equal to that of the solid vector (Fig. 7B, C). The red triangle located at the origin of Fig. 7D means that the astigmatism of objective lens has now been completely corrected (Fig. 7C).

3.5. Validation of the single-pass tuning strategy

To validate the strategy illustrated in Fig. 7, we have tested it on our CM200 and Titan Krios microscope using live images. Fig. 8 and Fig. S2 show screenshots of the process collected from Titan Krios (Fig. 8, S2A) and CM200 (Fig. S2B, C) in which the darkest circle is the current point and the gray circles are historical points. To make the sequence of the points

more obvious, the greyness level of the circles is varied to make more recent ones darker. It is noted that the real-time trajectory in Fig. 8 perfectly matches the theoretical prediction in Fig. 7, thus confirming the validity and feasibility of the proposed strategy for correction of the astigmatism of the objective lens. In addition to the screenshot shown in Fig. 8 and S2, we have also recorded videos (Videos S1, S2) that include the entire tuning process to demonstrate the strategy in its fullest details.

To further validate the single-pass tuning strategy, we tested our method with different initial astigmatism values by using different initial stigmator values. Fig. S3 displays 8 screenshots of the entire trajectories acquired from CM200 microscope. The first row (Fig. S3A–D) are the trajectories with different initial astigmatism values in the first quadrant of the radar plot. It is noted that the direction of the straight trace segment does not change with varying initial astigmatism amplitudes and the angles are all around 62° (green dash lines in Fig. S3A–D). This can be explained by the vector diagram shown in Fig. 7: the dashed vector (Fig. 7B) controlled by the stigmators at optimal correction angle is always opposite to the solid vector (Fig. 7B) representing the residual astigmatism of the objective lens, which is independent of the stigmator values. The plots in the second row (Fig. S3E–H) also have a similar property as the first row, however, the angle of the straight trace segment is around 152° (green dash lines in Fig. S3E–H), orthogonal to that in the first row. The 90° change of the orientation is due to the over-correction of the astigmatism which causes the switch of the major and minor axis in the power spectra (Fig. 1B–C) and the single ring (Fig. 1D).

4. Discussion

In this paper, we have introduced a new method, s^2 stigmator, for real-time detection of astigmatism in live images and established a reliable single-pass strategy for users to correct the astigmatism and improve image quality during TEM operation. Systematic tests have demonstrated that this method works accurately and robustly with both synthetic and experimental images. Owing to the closed-form algorithm without the need of iterative search of multiple parameters, the method is inherently fast and it takes only a few seconds on a desktop computer to measure the astigmatism of one image even as a script in DigitalMicrograph software. The speed can be significantly enhanced by converting to compiled code and using GPU acceleration in the future.

The s^2 power spectra provides the essential basis for our fast closed-form algorithm. Due to the uniform oscillation property of s^2 power spectra, the problem of determining the defocus along all directions is drastically simplified to measuring the radii of a single ring (Fig. 1D). This is in stark contrast to existing methods, such as dividing the 2D power spectra into sectors for angular rotational average (Huang et al., 2003; Yang et al., 2009), applying edge detection method to approximate the ellipticity of the Thon rings (Mallick et al., 2005), studying the mathematical relationship between the radial averages of TEM images with and without astigmatism (Fernando, 2008), and performing a fully 2D power spectra optimization to fit CTF model (Mindell and Grigorieff, 2003; Sorzano et al., 2007). The single ring not only completely maintains the information of ellipticity but also becomes much easier and faster to evaluate.

To the best of our knowledge, our method is the first to employ the 2nd Fourier shifting theorem (Eq. (1)) in TEM image processing, although the 1st Fourier shifting theory ($f(t-t_0) \leftrightarrow e^{-j2\pi s t_0} F(s)$) has already been commonly used in the cryo-EM field. The application of Fourier shifting effectively overcomes the limitation of coarse sampling caused by small single ring at small defocus, and notably improves the accuracy of ellipticity detection. Although the size of the single ring is enlarged after Fourier shifting, the angular variation of the radius is unchanged due to the constant shift in all directions.

The single ring representation of the angular distribution of the defoci also allows the use of 1D FFT to directly compute the amplitude and phase of a sine-like curve (red curve in Fig. 1G), which correspond to the astigmatism amplitude and angle of the twofold objective lens astigmatism. The accurate direct solution of the astigmatism amplitude and angle not only eliminates the need to explicitly search for major/minor axes of the defocus ring but also avoids the errors due to noise (Fig. 1G) in assigning the radii (i.e. defocus) and angles of the major/minor axes.

Most importantly, our script provides real-time radar-style visual feedback and quantitative values for the correction of objective lens astigmatism. Compared to current approach relying on visual examination of the roundness of Thon rings, our new method considerably enhances the sensitivity in ellipticity determination, and simultaneously prevents the bias and subjective results from operators. Furthermore, we have established an efficient single-pass tuning strategy (Fig. 7), which allows the users to rationally and sequentially adjust, instead of blindly playing with, the two stigmator knobs to correct the objective lens astigmatism. Combined with our results of real-time tests (Fig. 8, S2, and Videos S1, S2), we have also gained insights to the underlying principle of astigmatism correction (Fig. 7).

Currently, most microscopists correct astigmatism at very high magnification and very small defocus, which is necessary due to the limited sensitivity of current approach relying on visual examination of the roundness of Thon rings. However, the imaging condition that astigmatism is corrected is drastically different from the conditions, magnification and defocus, used for data collection. In contrast, our method is capable of guiding users to correct astigmatism at any magnification and any defocus, and it thus allows the users to correct astigmatism at the same magnification and defocus level used for final imaging. We think that this is not only more convenient but also likely to further reduce the residual astigmatism in the final images.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by NIH grant (1R01AI111095). We thank the Purdue Cryo-EM Facility (<http://cryoem.bio.purdue.edu>) for the use of the Titan Krios and CM200 microscopes. We thank Steve Wilson for assistance in recording the videos.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jsb.2016.11.001>.

Fig. 1.

An overview of the s^2 stigmator algorithm for astigmatism determination and correction. (A) A synthetic image used for algorithm demonstration, defocus = 2000 nm, astigmatism amplitude = 100 nm, astigmatism angle = 30°, pixel size = 1 Å, B-factor = 150 Å², amplitude contrast = 0.1 and image size 2048 × 2048 pixels. (B) Regular 2D power spectra. (C) 2D s^2 power spectra truncated to 5.3 Å resolution at the edge. (D) A single ring generated by applying FFT to (C). (E) A shifted/enlarged single ring after performing the 2nd type Fourier shifting of (D). (F) Unwrapped ring in (E). (G) Data curve (greenish region) of the bright wave in (F) and its 2 Hz Fourier component curve (red curve). (H) FFT of data curve (greenish region). (I) Peak plot in final output. The two curves display the pixel values of rows in (F) with largest (blue) and smallest (red) defocus. (J) Radar plot in final output. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Comparison of results of four representative simulated images with varying defocus, astigmatism amplitudes and angles. Columns from left to right are results for synthetic images with defocus 1000 nm, astigmatism amplitude 20 nm and angle 0° (A, E, I); defocus 2000 nm, astigmatism amplitude 40 nm and angle 30° (B, F, J); defocus 3000 nm, astigmatism amplitude 60 nm and angle 60° (C, G, K), and defocus 4000 nm, astigmatism amplitude 80 nm and angle 120° (D, H, L), respectively. For all simulated images, voltage = 300 kV, $C_s = 2$ mm, pixel size = 1 Å, B-factor = 150 Å^2 , amplitude contrast = 0.1 and image size is 2048×2048 pixels. The high resolution limit is 5.3 Å for 2D s^2 power spectra. The first row (A–D) illustrates the images of the single rings before Fourier shifting, the second (E–H) and third row (I–L) are the standard outputs, representing peak plots and radar plots, respectively.

Fig. 3.

Validation tests using simulated images with varying defocuses, astigmatism amplitudes and angles. The ranges of defocus, astigmatism amplitude and angle are 500 nm ~ 4000 nm, 20 nm ~ 120 nm, and 5° ~ 175° , respectively. For all simulated images, voltage = 300 kV, $C_s = 2$ mm, pixel size = $1 \text{ \AA}$, B-factor = $150 \text{ \AA}^2$, amplitude contrast = 0.1 and image size is 2048×2048 pixels. The high resolution limit ranges from $4 \text{ \AA}$ to $5.5 \text{ \AA}$ for 2D s^2 power spectra. The measured defocus (Y-axis in A), astigmatism amplitude (Y-axis in B) and astigmatism angle (Y-axis in C) are compared with the corresponding ground truth values (X-axis in A–C).

Fig. 4.

Results for four representative experimental micrographs with varying defoci, astigmatism amplitudes and angles. Columns from left to right are results for images downloaded from EMPIAR-10038 (A, E, I), EMPIAR-10031 (B, F, J), EMPIAR-10028 (C, G, K), and EMPIAR-10002 (D, H, L), respectively. In the top row (A–D), the red arrows indicate the direction of the major axis (i.e. largest defocus) found by s^2 stigmator. In the radar plots of the last row, black dots represent the results of s^2 stigmator and red dots representing the results of CTFFIND3 were manually added to the radar plots for comparison. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Cross validations of $s^2stigmator$ and CTFFIND3 method using experimental images. Shown are the estimated defocus (A), astigmatism amplitude (B) and astigmatism angle (C) of experimental micrographs. 100 micrographs in each of the four datasets, EMPIAR-10038, EMPIAR-10031, EMPIAR-10028 and EMPIAR-10002, were used for the tests.

Fig. 6. Reproducibility tests with images of different electron doses. (A) The RMSD of results for 10 images at each of the tested dose levels. (B–D) The distribution of the 10 results for total dose of 5.2 $\text{e}/\text{\AA}^2$ (B), 10.3 $\text{e}/\text{\AA}^2$ (C), and 56.9 $\text{e}/\text{\AA}^2$ (D), respectively. The corresponding RMSD of these three distributions are shown as the first, second and last point in (A).

Fig. 7.

A new single-pass strategy of astigmatism correction using s^2 stigmator. (A–C) Vector diagrams to illustrate the principle of correction of astigmatism by the two objective lens stigmators. (A) Initial state. The correction vector (dashed) is at an arbitrary state (length and direction) relative to the residual astigmatism vector (solid). (B) Angle search. The angle for the correction vector (controlled by M-Y knob) is optimal when it is at the opposite direction of the astigmatism vector. (C) Amplitude minimization. The astigmatism is corrected when the length of the correction vector (controlled by M-X knob) is changed to the same length of the astigmatism vector. (D) A marked radar plot showing the expected trajectory of data points representing the astigmatism in the process of astigmatism correction using our new tuning strategy. The yellow star suggests the initial state (A) before correction. The black markers forming an arc-like trajectory (black arrows) indicate the change of data points if only the angular knob (M-Y) is turned (as explained in B). To find the optimal angle (shown as the black triangle) that results in minimal astigmatism in the black trajectory, the M-Y angle knob needs to cover sufficiently wide range to clearly reveal a data point (black triangle) in the arc that is closest to the origin. Starting from the black triangle (i.e. optimal M-Y setting), only the M-X amplitude knob is adjusted, which will result a series of red markers following a straight path represented by the red arrow. The red triangle located at the origin of the radar plot implies the complete elimination of objective lens astigmatism. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. Screenshot of the radar plot by *s²stigmator* running on Titan Krios microscope. The current point is marked by a black circle and all gray circles exhibit the trajectory of previous data points in the process of astigmatism correction. The grayness level of the circles is progressively reduced for data points further in the history of the correction process. A red circle (overlapped with the current point here) is used to indicate the currently best point with minimum astigmatism. Additional screenshots from Titan Krios and CM200 microscopes are shown in Fig. S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)