Improving the quality of electron tomography image volumes using pre-reconstruction filtering

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A R T I C L E   I N F O

Article history:
Received 29 February 2012
Received in revised form 16 May 2012
Accepted 25 May 2012
Available online 6 June 2012

Keywords:
Electron tomography
Denoising
Plasmodium
Non-linear anisotropic diffusion filtering

A B S T R A C T

Electron tomography produces highly magnified 3D image volumes useful for investigating the structure and function of cellular components. Image quality is degraded by multiple scattering events and quantum noise, which depend on the angle at which individual tilt projections are collected. We have adapted a biomedical imaging approach to improve image quality by enhancing individual tilt projections prior to volumetric reconstruction. Specifically, we have developed a family of non-linear anisotropic diffusion (NAD) filters parameterized by the tilt angle. We give a quantitative and qualitative evaluation of our pre-processing approach and the NAD filter. We show an improvement in the reconstructed volumes for tomograms generated from both plastic-embedded and cryo-stabilized samples of malaria parasite-infected erythrocytes.

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1. Introduction

Electron tomography is an important technique that is used to elucidate the 3D architecture of biological samples (Downing et al., 2007; Leis et al., 2008; Milne and Subramaniam, 2009; Vanhecke et al., 2011). The method involves generating sections of cells or tissues (usually 200 – 400 nm) and preparing the samples for electron microscopy (EM). Samples can be processed using typical EM protocols involving chemical fixation, plastic embedding, sectioning, and staining with heavy metals (Frey et al., 2006; Gan and Jensen, 2012; McIntosh et al., 2005). Alternatively samples can be snap frozen in vitreous ice, optionally sectioned and examined using a cryo-EM stage (Pierson et al., 2011; Tocheva et al., 2010). Ultrarapid freezing permits a marked improvement in the preservation of cellular structures in their native state; however unstained vitreous samples have rather low intrinsic contrast (Dubochet et al., 1988; McDonald and Auer, 2006). A third possibility is high pressure freezing followed by freeze-substitution and resin-embedding. This helps preserve cell structure but also permits the enhancement of sub-cellular features by contrasting with metal stains. Once the sections have been prepared they are examined in an electron microscope with a tiltable stage operating at moderate electron accelerating voltages (200–400 keV). Images of the sample are collected over a tilt range of at least 120°. The imaging dose is kept as low as possible (and for cryo-stabilized samples should not exceed 10,000 e⁻/nm²) in an effort to preserve the biological structures. The images are aligned, usually making use of colloidal gold fiducial particles that are deposited into the sample (Kremer et al., 1996; Mastronarde, 1997; Penczek et al., 1995). The final volume, or tomogram, is obtained through a computational volumetric reconstruction process (Herman, 2009) and processed using different segmentation tools (Ali et al., 2012; Kremer et al., 1996; Muncuoglu et al., 2012; Nguyen and Ji, 2008). The reconstructed volume is commonly post-processed to
improve the image by enhancing features of interest such as boundaries and regions of similar image density. This is done specifically to facilitate segmenting objects of interest.

The quality of the electron microcopy projection images is determined by factors that affect the mean free path of the electrons as they pass through the specimen. A major determinant is the thickness of the sample, a parameter that varies non-linearly as the section is tilted. For example the sample is approximately two times thicker at an angle of 60° and three times thicker at 70° (Grunewald et al., 2003; Lucic et al., 2005; Steven and Aeby, 2003). Thus, multiple scattering events increase markedly at higher tilt projection angles. This contributes to what is referred to as noise in the reconstructed tomogram (Frangakis and Hegerl, 2001). Where the appropriate hardware is available, energy filtering can be used to remove a significant portion of inelastically scattered electrons, which improves contrast and the signal-to-noise ratio (Mastronarde, 2005). Energy filters have been found to be valuable for improving imaging of both stained plastic sections (Bouwer et al., 2004; Han et al., 1996) and frozen-hydrated specimens (Grimm et al., 1997; Koster et al., 1997).

In cryo-electron tomography on cryo ultrathin sections (Al-Amoudi et al., 2004; Hoenger and Bouchet-Marquis, 2011; Hurbain and Sachse, 2011) or macromolecules embedded into thin layers of vitrified ice (Dudkina et al., 2011), multiple scattering is less of a problem, but quantum noise remains a problem due to insufficient sampling of the electron scattering from the specimen. Signal averaging is not possible due to the variability in the size and shape of cellular structures. Degradation of the signal can also arise due to instrument defects such as imperfections of the CCD cameras. The signal obtained at higher angles is particularly weak, and subject to the inherent granularity of Poisson counting statistics.

Noise-related artifacts are an important issue in electron tomography and can severely compromise the quality of the reconstructed volume (Baumeister et al., 1999). Improving the quality of the reconstructed tomogram would help elucidate important biological structures and facilitate further processing, such as segmentation and rendering. Indeed the impressive results obtained by electron crystallography (Fujiyoshi, 1998) and single particle analysis (Frank, 2002) are attributed to “noise” reduction strategies, that rely on image averaging (Bartesaghi and Subramaniam, 2009; Frangakis and Hegerl, 2001; Hegerl and Frangakis, 2006).

Non-linear anisotropic diffusion (NAD) is a generic, established statistical method (Perona and Malik, 1990; Weickert, 1999) that can be interpreted in terms of scale space theory (Koenderink, 1984; Lindeberg, 1992) and which can be used for volumetric denoising of reconstructed tomograms (Narasimha et al., 2008). Popular NAD filters for local enhancement are based on edge enhancing diffusion (EED) and coherence enhancing diffusion (CED) methods (Weickert, 1999). The successful application of NAD to noise reduction in X-ray computed tomography sinograms for medical imaging (Alrefaya et al., 2009; Demirkaya, 2001; Li et al., 2004; Wang et al., 2005; Zhou et al., 2007) suggests potential benefits from applying similar methods to EM tilt projections.

The first hybrid approach for the local enhancement of 3D features of electron tomography reconstructed volume was developed by Frangakis and Hegerl (2001). An enhanced image was obtained by the alternation of EED and CED filters triggered by a discrete switch. Some variations of the discrete switch approach have also been presented (Fernandez and Li, 2003). A continuous switch function that combines EED and CED was applied to computed tomography volumes (Mendrik et al., 2009). The switch did not, however, take into account the local gradient of the image at different tilt angles.

The noise in EM projection data varies with the tilt angle, decreasing the quality of the reconstructed electron tomogram. As a result, post-reconstruction correction might not be sufficient to obtain a clear, artifact-minimized volume for very noisy tomograms. The use of additional iterations of the post-reconstruction manipulation may result in cleaner images but at the price of lower correlation with the original dataset. Application of a filtering procedure to two-dimensional electron microscope images before they are assembled to form a three-dimensional tomographic representation provides a method for customizing the filtering process based on the quality of the projection image data at different angles.

A crucial question is when to stop the filtering process (i.e. the optimal number of iterations), so that the signal information is not misclassified or blurred out. Several objective stopping criteria have been proposed (Mrazek and Navara, 2003), but most of them are not suitable for cryo-electron tomography data (Fernandez and Li, 2003). The noise-estimate variance introduced by Fernandez and Li (2005) seems to provide statistical criteria that are best suited to electron tomography data. Unfortunately, a uniform region has to be manually selected by a user, which makes the outcome dependent on a subjective judgement regarding how the region is selected within a specific image.

Statistical analysis and manipulation of the individual tilt projection images can be used to optimize the reconstruction process. Statistical iterative reconstruction, rather than the classic filtered back-projection (FBP) with low-pass filtering, is one way to deal with the problem (den Dekker et al., 2005; Elbakri and Fessler, 2002, 2003; Whiting, 2002). Another possibility is to use statistics-driven processing (in general) and non-linear anisotropic diffusion (in particular) to obtain a noise-reduced sinogram that will satisfy the FBP reconstruction for the Radon transform (Alrefaya et al., 2009; Demirkaya, 2001; Li et al., 2004; Wang et al., 2005; Zhou et al., 2007). The benefits of the latter include a higher computational efficiency and more uniform spatial resolution in the reconstructed image. To our knowledge NAD has not previously been successfully applied to electron tomography tilt projections.

In this paper we present a method for applying an angle-dependent NAD filter (referred to as pre-NAD) to individual projection images before reconstruction. This involves scaling the information for each tilt image to allow for the effect of the altered geometry of the samples at higher angles. We use a stopping criterion for iterations in the pre-NAD filter based on masked variance difference and show that our pipeline can overcome problems associated with a lack of continuity of information observed with multiple 2D tilt projection denoising approaches (Frangakis and Hegerl, 2001). We show examples where the method is applied to EM data collected under sub-optimal imaging conditions. We have examined fixed and stained sections as well as cryo-stabilized sections.

2. Materials and methods

2.1. Electron tomography
+70°, using a 1 s exposure time. For test data we used both a lower exposure time (0.2 s) and a larger tilt interval (3°) to enhance noise and artifacts and degrade the signal.

2.2. Cryo EM methods

P. berghei sporozoites were isolated from midgut of a mosquito and cryo stabilized as described previously (Kudryashov et al., 2010). Tomography was performed on a CM300 microscope (FEI) operated at an accelerating voltage of 300 keV, equipped with Gatan Imaging Filter with a slit of 20 eV. Tilt series were acquired between −60° and +60° with 2° step with the total dose of electrons kept below 10,000 e-/nm², with objective lens under-focus of 12 μm. Tilt series alignment and reconstruction was performed using the IMOD package, aided by 10 nm gold beads (Kremer et al., 1996).

2.3. Image analysis

The test tilt projections were automatically aligned one-by-one to the ground truth projection. Following application of our filter to the tilt projection data, tomograms were reconstructed using Filtered Back Projection (FBP). No post-processing was applied.

For a visual assessment, we performed semi-automatic segmentation and 3D rendering. A threshold value was selected manually from each reconstructed tomogram using a noise-estimate variance criterion, after marking some of the relevant cellular features, as described by others (Fernandez and Li, 2005). Connected regions were identified automatically using connected component analysis (Ibanez et al., 2003). The automatic segmentation algorithm is described in Appendix I. The regions were labeled with different colors, and 3D models were generated and rendered (Yushkevich et al., 2006).

2.4. Simulation

A synthetic volume of dimensions 256 × 256 × 60 voxels was created in MATLAB (Mathworks® Natick, MA, USA).

The double membrane was simulated as a “Mexican hat” using the two 3D sinc functions:

\[
\sin(x)/x, \ x \in \mathbb{R}^3
\]

and

\[
10 + \sin(x)/x, \ x \in \mathbb{R}^3
\]

Projections of the synthetic volume were obtained at angles of −70° to +70°, with a 2° increment for each projection, using Xmipp (Sorzano et al., 2004). The final volumes were reconstructed and segmented. Reconstructions were performed using tomo3d (Agulliero and Fernandez, 2011). Segmentation were performed using a scale space approach, as explained in Appendix I. Rendering was performed using ITK-snap (Yushkevich et al., 2006).

2.5. Diffusion filtering

The grayscale intensity, I, of each image is regarded as a density that is redistributed by a conservative diffusive process. The equilibrium of intensity due to its inhomogeneity is determined by Fick’s law of diffusion, so the time-evolution of I is obtained by solving the diffusion equation:

\[
\frac{\partial I}{\partial t} = \nabla \cdot (D \nabla I)
\]

where D is the diffusion tensor. If it can be assumed that D is constant over the entire domain of I, the diffusion process is described as homogeneous and if it depends only on position it is described as homogeneous. Here, however, it is assumed that D depends on the local nature of the time evolution of I and the diffusion process that defines the filtering procedure is consequently described as non-linear, inhomogeneous and anisotropic.

The prototype non-linear diffusion filter (Malik and Perona, 1990) employed an isotropic model of the form \(D = g(\|\nabla I\|)I\), where I is the unit tensor of appropriate dimensionality and \(g(s^2) = (1 + s^2/\mu^2)^{-1}\) in which \(\mu\) is a real, positive parameter. The positive-definite character of D enables one to generalize this approach to the case of anisotropic non-linear filtering by writing, in two dimensions,

\[
D = \begin{bmatrix}
\nu_1^T & 0 \\
0 & \nu_2^T
\end{bmatrix}
\]

where \(\nu_1\) and \(\nu_2\) are orthonormal column vectors that specify the principal axes of the anisotropic diffusion tensor and \(\nu_1\) and \(\nu_2\) are scalar functions of the local image gradient. Generally, these orthonormal vectors are selected at each location in the image to coincide with the eigenvectors of \(J_0\) which is defined by the outer product of the intensity gradient,

\[
J_0 = \nabla I \cdot \nabla I^T.
\]

The components of the intensity gradient are approximated by the central difference formula. More generally, one may adopt eigenvectors derived from \(J_0 = J_0 - K_x\), which represents the convolution of \(J_0\) with a Gaussian function of width \(\sigma\). The solution of the matrix eigenvalue equation for \(J_0\) or \(J_0 - K_x\) determines two eigenvalues, \(\mu_1\) and \(\mu_2\), that may be used to quantify the local anisotropy of I, for which purpose we adopt the convention that \(\mu_1 \geq \mu_2\). The diagonalization of \(J_0\) generates a unitary rotation on an orthonormal basis of Cartesian unit vectors that may be used to align the diffusion tensor along the principal directions of intensity change. Having specified \(\nu_1\) and \(\nu_2\) which become, respectively, the major and minor axes of D, one then has the freedom to control the magnitude of the local diffusion characteristics by allocating real, non-negative numerical values to \(\nu_1\) and \(\nu_2\).

The magnitude of \(\sigma\) that is used to define the Gaussian convolution function \(K_x\), determines the spatial interval over which the structure tensor is calculated (Babaud et al., 1986; Koenderink, 1984; Nielsen et al., 1997). Small values of \(\sigma\) generates a narrow spatial Gaussian distribution which causes negligible smoothing of the image. Large values of \(\sigma\) smooth out noise but may obscure significant features, such as edges and discontinuities. The projected depth through the sample, which is assumed to be a thin slab, clearly depends on the tilt angle, \(\phi\) which is measured between the electron beam direction and a vector perpendicular to the slab. As a consequence, the smoothing parameter must depend on the tilt angle, for which purpose we have adopted the relation \(\sigma(\phi) = \sigma_0 \cos(\phi)\), where \(\sigma_0\) is the value of \(\sigma\) chosen in the case where the incident beam is perpendicular to the slab. In the limit \(\phi \to \pi/2\) the projected length traversed by the beam becomes large compared to the thickness of the slab and \(\sigma(\phi) \to 0\), indicating that no smoothing is to be performed and no small details are to be obscured by the convolution process. The decreasing magnitude of \(\sigma(\phi)\) for increasing \(\phi\) reduces, as a consequence, the spatial interval over which \(\nabla I\) must be calculated in the determination of \(J_0\).

The non-linear anisotropic diffusion (NAD) algorithm of Frangakis and Hegeri (2001) is a hybrid of the edge enhancing diffusion (EED) method, which enhances changes in image intensity, and the coherence enhancing diffusion (CED) method (Weickert, 1999), which enhances regions that exhibit correlations in image intensity. Here, the NAD approach is modified to incorporate \(\sigma\)-dependence in the determination of the principal axes of D for two-dimensional tilt projections. The hybrid NAD algorithm
employs the EED method if \( \mu_1 - \mu_2 \geq \mu_k \) for some user-defined threshold, \( \mu_k \), which controls the detection of edges in the image. If this inequality is not satisfied, the CED method is adopted. The modification of the EED and CED algorithms to include dependence on the tilt angle both require the calculation of the eigenvectors of \( J_{O\phi} \), which define the principal axes of \( D \) for a given \( \phi \).

The EED and CED algorithms differ primarily in their specifications of \( \lambda_1 \) and \( \lambda_2 \), which are used to construct \( D \) to smooth two-dimensional images obtained at fixed \( \phi \). For the EED algorithm, these parameters are defined by,

\[
\lambda_{E1} = \begin{cases} 
1 & \text{if } G(\sigma_x) = 0 \\
1 - \exp[-c(G(\sigma_x))^2] & \text{if } G(\sigma_x) > 0
\end{cases}
\]

\[
\lambda_{E2} = 1
\]

where \( G(\sigma_x) = \nabla l_{\sigma_x} \cdot \nabla l_{\sigma_x} \), \( C = 3.31488 \) is a threshold parameter adopted from Mendrik et al. (2009) and \( W = \lambda_k^2 \), where \( \lambda_k = 30 \) is a user-specified parameter defined by Weichert (1996) and Weichert (1999). For the CED algorithm, the corresponding parameters are chosen to be,

\[
\lambda_{C1}(\phi) = \lambda
\]

\[
\lambda_{C2}(\phi) = \begin{cases} 
1 & \text{if } \mu_2 = 0 \\
(1 - \lambda) \exp\left(\frac{\log(2\lambda^2)}{\log(2)}\right) & \text{otherwise}
\end{cases}
\]

where \( \lambda_c \) is another user-defined parameter whose value is typically set to \( \lambda_c = 30 \), \( \lambda = 0.001 \),

\[
K(\phi) = \frac{\mu_1(\phi)}{\lambda + \mu_2(\phi)}
\]

and the parameters \( \mu_1(\phi) \) and \( \mu_2(\phi) \) are the eigenvalues of \( J_{O\phi} \).

A straightforward generalization of the hybrid NAD approach to arbitrary tilt angles would swap discontinuously between the EED and CED algorithms based solely on the value of \( \mu_1(\phi) - \mu_2(\phi) \). Instead, we have developed a continuous switch between EED and CED based on Mendrik’s parameterization of the diagonal terms in the diffusion tensor,

\[
\lambda_i(\phi) = (1 - \varepsilon(\phi))\lambda_{Ei}(\phi) + \varepsilon(\phi)\lambda_{Ci}(\phi),
\]

where \( i = 1, 2 \). The tilt-dependent parameter, \( \varepsilon(\phi) \), is defined by,

\[
\varepsilon(\phi) = \frac{e}{e + \exp\left[\frac{-(\lambda - \lambda_{0})(\lambda(\phi)})}{\lambda(\phi)}\right]}
\]

where

\[\zeta(\phi) = 4\log(1 + \lambda_1(\phi) - \lambda_2(\phi)) + 4\log\left(1 + \lambda + \frac{\lambda_1(\phi)}{\lambda + \lambda_2(\phi)}\right),\]

\( \zeta(\phi) \) is the mean value of \( \zeta(\phi) \) taken over all pixels for tilt angle \( \phi \) and \( \lambda \) is a small, real, positive regulating parameter. The limit \( \zeta(\phi) = 0 \) corresponds to the presence of an edge and the EED algorithm is favored in the choice of \( \varepsilon(\phi) \). Otherwise, a progressively larger contribution from the CED algorithm in included in our tilt-dependent hybrid approach. Finally, we have constructed a tilt-parameterized representation of the diffusion tensor,

\[
\mathbf{D}(\phi) = \begin{bmatrix} 
\mathbf{v}_1(\phi) & 0 \\
\mathbf{v}_2(\phi) & \lambda_2(\phi)
\end{bmatrix} \begin{bmatrix} 
\lambda_1(\phi) & 0 \\
0 & \lambda_2(\phi)
\end{bmatrix} \begin{bmatrix} 
\mathbf{v}_1(\phi) & \mathbf{v}_2(\phi)
\end{bmatrix}.
\]

The specification of the principal axes of \( \mathbf{D}(\phi) \), \( \mathbf{v}_1(\phi) \) and \( \mathbf{v}_2(\phi) \), defines two independent, orthogonal directions of diffusion with magnitudes \( \lambda_1(\phi) \) and \( \lambda_2(\phi) \), respectively. Both the directions and magnitudes are parameterized with respect to the tilt angle, and the magnitudes are further parameterized to accommodate a continuous transition between regions in which there are edges and regions in which the image is smooth.

In our approach, anisotropic filtering is applied using this continuously parameterized hybrid approach to each tilt projection image before tomographic reconstruction is performed. Features in each tilt projection may be difficult to identify, however, because of the dependence of the electron path-length through the sample as a function of \( \phi \). As a consequence, anisotropic filtering of the tilt images is performed iteratively until either (1) self-consistency has been obtained in the reconstructed image or (2) a predetermined number of iterations has been reached.

The self-consistency criterion is based on the weighted variance of the reconstructed image compared to the measured values. In order to control the amount of smoothing that is applied to the image, pixels are excluded from the calculation of the variance if they are likely to correspond to edges, which are identified if the inequality \( \zeta(\phi) < \zeta(\phi) \) is satisfied. A binary mask \( f_\phi \) is constructed for each pixel, \( i \). If the pixel is judged to contain an edge, then \( f_\phi = 0 \); otherwise, \( f_\phi = 1 \). The masked variance difference, \( \delta \), is defined to be

\[
\delta = \text{var}[f_\phi(I(\phi) - f_\phi)]
\]

where \( f_\phi \) is the value of the image at the pixel labeled \( i \) after \( t \) iterations and \( f_\phi \) represents the corresponding measured values. The mask restores the conventional definition of the variance to include all values of \( i \) in the image. The iterative refinement of the tilt images proceeds until \( \delta < \delta_{\text{stop}} \), where \( \delta_{\text{stop}} \) is specified by the user in a manner similar to that described by other groups (Fernandez and Li, 2005). The maximum noise variance, \( \delta_{\text{stop}} \), was estimated from one of the highest tilt angle projections, by the following method. The pre-NAD filter is iterated until the relative difference, \( (\delta_{\text{stop}} - \delta)/\delta_{\text{stop}} \), is 0.05 or less, and then \( \delta_{\text{stop}} \) is defined to be \( \delta_{\text{stop}} \).

This procedure results in a variation in the number of iterations used for each projection. We found that the number of iterations was approximately linearly correlated with the cosine of the tilt angle.

3. Results

Electron tomograms are generated from projection images collected at different tilt angles. The apparent thickness of the sample varies non-linearly as the section is tilted with resultant variations in the mean electron path and anticipated degradation in the quality of the images at high tilt angles. When a tomogram is generated from a data set that includes images with a low signal-to-noise ratio, faithful isosurface representation and volume rendering may be problematic.

In an effort to improve the quality of electron tomography data we have designed an approach that uses NAD filtering before volume reconstruction. For a simple visual impression the approach was applied to a 2D test image (photo of Marie Curie), which was degraded by addition of Gaussian noise (Fig. 1A). The degraded image was subjected to isotropic and median filters, or to pre-NAD processing. The masked variance difference increases with increasing iterations (Fig. 1B).

We have assessed the usefulness of our approach using a simulated model. A synthetic volume was created using MATLAB and consists of two regions, one simulating a double membrane and the other the cell cytoplasm. Electron density values for the two regions were randomly picked in the ranges 600–850 and 750–900, respectively (Fig. 2A). Projections of the synthetic volume were simulated at angles of −70° to +70° (Sorzano et al., 2004) and the final volumes were reconstructed (Agulleiro and Fernandez, 2011) and segmented (see Appendix 1). As shown in Fig. 2B, very little structural information is retrieved from the unprocessed data (first column). A traditional post-reconstruction NAD approach (second
column) improves the analysis but our pre-NAD approach (third column) or a combination of traditional approach with our pre-NAD (fourth column), shows a marked improvement. For this comparison the number of iterations used for the post-reconstruction NAD approach was chosen to be equivalent to the average number of iterations used for the pre-NAD approach.

It is likely that reconstructions using the unprocessed and traditional NAD approaches are more sensitive to missing wedge artifacts than our pre-NAD approach. It is also possible that our non-linear pre-filtering approach, which reduces noise and enhances edges, could enhance some boundaries and suppress others, creating some anisotropy in the reconstruction.

In an effort to assess the success of the different approaches we have computed the distance between the obtained and the expected segmentation data using Hausdorff symmetric distance, measured in pixels (Gerig et al., 2001). For the unprocessed data we obtained 149, for the traditional NAD processed data, 103, while for the pre-NAD processed data, 82. A slightly better result (78) was obtained from application of the pre-NAD filter before reconstruction and traditional NAD after reconstruction.

We have also assessed the usefulness of our approach using transmission EM images of *P. falciparum*-infected RBCs collected under different conditions. To generate these data parasite-infected RBCs were harvested, fixed and prepared for embedding. Sections (200 nm) were stained with uranyl acetate and lead citrate, then layered with gold fiducials as described previously (Abu Bakar et al., 2010; Hanssen et al., 2008). A high quality "ground truth" data set was collected by recording tilt images between $-70^\circ$ and $+70^\circ$ at $1.5^\circ$ intervals, using a 1 s exposure time. The data were aligned using IMOD and binned by a factor of 3. Some of the projection images at different tilt angles are presented (Fig. 3i). An additional tilt series of the same specimen was acquired at a lower exposure time (0.2 s) (Fig. 3ii). The degradation of the data and the appearance of instrument noise are evident. Application of the pre-NAD filtering algorithm significantly improved the quality of the data (compare Fig. 3i–iii).

The tilt images for the high quality data set were aligned with IMOD and a tomogram was reconstructed using FBP (Fig. 4A). In a virtual section from the tomogram, a pair of electron-dense rhoptry organelles (marked R1 and R2) is observed at the apical end of

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**Fig. 1.** Analysis of noise reduction by NAD filtering using masked variance difference. (A) A test image "Marie Curie" (193 × 241 pixels) (a) was degraded with Gaussian noise (standard deviation = 25) (b), and the degraded image was subjected to isotropic and median filters (c and d) or to pre-NAD-processing for 2–8 iterations; $k_c = k_a = k_h = 30$, $\sigma = 1$, $\alpha = 0.001$, maximum noise variance = 0.053. (B) Monotonically increasing behavior of masked variance difference at different numbers of iterations.
Fig. 2. Application of pre-NAD filtering to a test object. (A) A synthetic volume was created simulating a double membrane in the cell cytoplasm. (B) Projections of the synthetic volume were generated with pre or post filtering and the final volumes were reconstructed and segmented. Row (i) shows a view of the volume, row (ii) the volume with segmentation superimposed, and row (iii) the 3D rendering of the segmented simulated membrane. The parameters used for post-NAD were $k = 30$, 6 iterations, and $\sigma = 0.5$. The parameters used for pre-NAD were maximum noise variance ($\lambda_{\text{stop}}$) = 0.7, maximum number of iterations = 15, $\lambda_{\text{c}} = \lambda_{\text{s}} = 30$. The number of iterations (from 3 to 15, average of 6 (standard deviation 2.5)) varied in an approximately linear fashion with the projection angle, with a strong correlation between the number of iterations and the cosine of the tilt angle (Pearson's correlation values of $r = 0.63$, $p = 5 \times 10^{-6}$). The parameters for the automatic segmentation were $n = 5$, $\sigma = 1.0$, $t = 1/(n-1) = 0.25$.

Fig. 3. Application of pre-NAD filtering to electron micrograph projection images collected at different angles. EM transmission projection images collected at angles from $-70^\circ$ to $+70^\circ$ of a stained fixed section (200 nm) of a P. falciparum-infected RBC. A high exposure “ground truth” data set (i; 1 s exposure) and a low exposure “test” data set (ii; 0.2 s) were collected. The low exposure data set was filtered using the pre-NAD algorithm (iii).
the daughter merozoite (M), which is developing within a mother parasite. The tilt projection images for the lower exposure data set were automatically aligned to the equivalent ground truth data set. Due to the extremely low quality of the test data and the different grayscale values, the normalized mutual-information distance metric (Pluim et al., 2003) was preferred over both cross-correlation and manual alignment. We applied our filter to the poor quality tilt projections before the data were reconstructed using FBP. There is no substantial difference whether reconstructions were performed using the FBP method (shown) or the Simultaneous Iterative Reconstruction Technique (SIRT) method (not shown). The improvement in the quality of the data upon application of the pre-NAD filtering algorithms is evident from visual examination of the reconstructed tomograms. A low electron-density region between the rhoptries (c), a region at the edge of rhoptry 1 (d) and region within rhoptry 2 (e) are indicated. Low and high magnification views of the reconstructed volumes that have been semi-automatically segmented and rendered. Automatic segmentation was achieved as described in Appendix I.

An analysis of the intensity variations across the tomogram in the region of the line (Row iii) shows the improvement with the different filtering methods. The ground truth profile (column A) clearly shows the boundary between the two rhoptry organelles (R1 and R2) as an electron lucent region (high gray values) indicated by the aqua arrow (c) that separates two electron-dense regions (lower gray values). Each of the filtered low exposure reconstructions (columns C–E) shows a clear electron-dense edge (low gray values) at the boundary of rhoptry R1 (i.e. under the red arrow), which is less well defined in the unfiltered reconstruction (B). For example, the unfiltered tomogram contains a feature (marked with red arrow (d)) that is not present in the ground truth tomogram. This feature is partially removed by post-filtering (C) and more effectively removed by pre-filtering (D and E). The pre-NAD filtered reconstructions (D and E) also more faithfully return the more homogenous staining profile of the adjacent rhoptry (marked with green arrows (e)) than the post-NAD filtered reconstruction (C).

We performed semi-automatic segmentation and 3D rendering of the data (Fig. 4iv and v). Connected regions were automatically identified using scale space analysis and Otsu thresholding (Yushkevich et al., 2006). In the reconstruction from the high quality data, a number of features are detected (Fig. 4A-iv and v). A very similar set of features was detected when the pre-NAD filtering algorithm was the applied to the low quality data set (Fig. 4D and E-iv, v). By contrast, when the lower quality data was reconstructed without pre-processing only very minimal cellular information is retrieved (Fig. 4B-iv and v). The improvement obtained using pre-NAD filtering that is apparent by visual inspection is confirmed by computing the distance between the obtained and the expected segmentation results.

**Fig. 4.** Improvement in electron tomogram reconstruction following application of pre-NAD filtering to a limited angle data set. A high exposure “ground truth” data set (A; 1.0 s exposure, 1.5° interval) and a low exposure “test” data set (B; 0.2 s exposure, 3° interval) were collected. Reconstructions were performed using the FBP approach using tomo3d (Agulleiro and Fernandez, 2011). Low (i) and higher (ii) magnification images are presented. Scale bars: 200 nm. The low exposure data set was filtered using the post-reconstruction NAD filter of Frangakis (C) or the pre-NAD algorithm (D), or both pre- and post-NAD filtering (E). The RBC cytoplasm (RBC), two rhoptry organelles (R1 and R2), the merozoite apical end (M), and the peripheries of two nuclei (n) are marked. The parameters used for post-NAD were $k = 30$, 6 iterations, and $\sigma = 4.1$ nm. The pre-NAD filter parameters were: $\lambda_u = \lambda_e = \lambda_h = 30$, $\sigma = 4.1$ nm, maximum noise variance ($\sigma_{\text{stop}}$) = 0.08, maximum number of iterations = 10, $\alpha = 0.001$. The number of iterations varied in an approximately linear fashion with the projection angle from 3 to 10 with an average of 6 iterations (standard deviation 1.1). All the aligned tilt projections have been down-sampled (bin = 4). The graphs (iii) show analyses of the intensity variations across the data in the region of the line. A low electron-density region between the rhoptries (c), a region at the edge of rhoptry 1 (d) and region within rhoptry 2 (e) are indicated. Low and high magnification views of the reconstructed volumes that have been semi-automatically segmented and rendered. Automatic segmentation was achieved as described in Appendix I.
data using Hausdorff symmetric distance. For the unprocessed data we obtained 122, for the traditional NAD processed data, 63, for the pre-NAD processed, 48, and for the combination of traditional NAD and pre-NAD, 49.

As a further test we examined electron tomography data collected from a low contrast sample (Fig. 5). *P. falciparum*-infected erythrocytes in the early stages of schizogony (cell division) were fixed and sectioned (200 nm) and stained with uranyl acetate but no lead citrate. A series of tilt projections was collected and tomograms were constructed with and without pre-NAD filtering. In a virtual section from the unprocessed tomogram (Fig. 5A; detail in C) cellular features are difficult to distinguish. The contrast is improved in the virtual section from the tomogram generated after anisotropic smoothing of the tilt projections (Fig. 5B and D). In the data from the improved tomogram (Fig. 5D), electron dense rhoptries (R) are evident near the apical end of one of the developing daughter cells. The uppermost rhoptry organelle is very difficult to discern in the unprocessed data but readily distinguished in the processed data. Similarly, an apical prominence (a) is hard to discern in the unprocessed data but more evident in the processed data. The improvement is also evident in the intensity variations across the tomograms in the region of the line (Fig. 5E). We performed a fully automatic segmentation and 3D rendering (see Appendix I) of the data (Fig. 5F). A number of important features are identified.

Unstained vitreous sections imaged by cryo EM have low intrinsic contrast, and could benefit from the application of the pre-NAD filtering algorithm. We generated a data set for an entire unsectioned ~400 nm thick *P. berghei* sporozoite that was shock-frozen and prepared for cryo EM (Kudryashev et al., 2010) (Fig. 6). These data were collected with electron filtering but similar results were obtained for data collected without electron filtering (not shown). Tomograms were generated without (Fig. 6A and C) and with (Fig. 6B and D) application of the filtering algorithm prior to

![Fig. 5. Application of pre-NAD filtering to poor contrast EM data. A fixed section of a *P. falciparum*-infected erythrocyte was subjected to a staining with uranyl acetate alone. A tilt series was collected and tomograms were constructed without (A and C) and with (B, and D) pre-NAD filtering. Scale bar: 300 nm. Parameters: $\lambda_e = \lambda_c = \lambda_h = 30$, $\sigma = 13.7$ nm, maximum noise variance ($\delta_{\text{stop}}$) = 0.0078, maximum number of iterations = 20, $\alpha = 0.001$. The RBC membrane (RBC), the parasite surface (P), hemoglobin crystals (Hz), the electron dense rhoptries (R) and an apical prominence (a) are marked. The intensity profiles of the yellow lines in (C) and (D) are depicted in (E) (top and bottom panels). (F) Automatic segmentation was achieved as described in Appendix I.](image-url)
generation of the tomograms. Analysis of individual sections from the 3D reconstructions reveals a substantive improvement in the quality of the data. This is evident in the variation in the gray levels along lines through the data sets (Fig. 6E). Features in the reconstructed tomogram were identified using fully automatic segmentation and 3D rendering (Fig. 6F–H). The surface of the parasite was readily detected (red), along with the underlying inner membrane complex (blue). We observed two layers of microtubules (pink). These features are more difficult to detect in the raw tomogram data. For example, the red and green arrows in (E) point to the RBC membrane and the second row of microtubules; these features are very difficult to distinguish without pre-NAD filtering.

4. Discussion

Diffusion filtering is often used to smooth or restore noisy images. The formulation of such partial differential equation filtering schemes is based on the observation that an image locally...
resembles a fluid flow pattern that may be recognized as a significant feature, as it might be either a boundary or a homogeneous region of the image. The target image is, in effect, regularized and modeled as a physical, diffusive system that evolves from an initial state specified by the original noisy image into a succession of smoother states. This evolution may be regarded as a scale space, in which the original image is embedded into a hierarchy of images in which detail is visible on variable length scales. An extension of this approach was proposed (Perona and Malik, 1990), in which a non-linear diffusion filtering scheme utilized information about the gradients of the evolving images to control their diffusion characteristics and to detect and enhance edges.

NAD filters have been in use for some years for post-reconstruction denoising of tomograms. There is, however, a potential problem with this approach, when applied to electron tomograms, which are generated from a series of projection images at different tilt angles. In these data the signal to noise ratio varies with the tilt angle, and the data at very high angles (>70°) cannot be collected, resulting in the so-called “missing wedge” of information. Furthermore, the reconstructed electron tomogram is potentially affected by a range of reconstruction artifacts. The application of post-reconstruction NAD filtering may exacerbate these artifacts. Here, we extend the general NAD filtering approach by applying the diffusive filtering procedure to two-dimensional electron microscope images before they are assembled to form a three-dimensional tomographic representation.

We have applied a scale space approach to compensate for the increasing amount of information per pixel in each tilt projection, which is linked to the increased mean free path length of electrons within the sample at different tilt angles. This permits better distinction between boundary and homogeneous regions, allowing accurate denoising of each tilt projection before reconstruction, thus allowing better visualization and rendering of the reconstructed tomogram.

Tomographic reconstruction processes usually assume a linear relationship between the projection image intensity values and the object density values. We have instead assumed that the diffusion tensor depends on the local nature of the time evolution of the tilt projection image; the diffusion process that defines the filtering procedure is consequently described as non-linear, inhomogeneous and anisotropic. We have shown that the careful treatment of the non-linearities in each tilt projection greatly facilitates the subsequent tomographic reconstruction of the volume using the NAD approach.

There is a potential problem due to superimposition of features in the projection images, which might exacerbate the employment of a pre-filtering approach. We have attempted to ameliorate this effect by selecting the parameters that control the diffusion process using a continuous discriminator for edges and homogeneous regions without anticipating what the structures should look like, resulting in the so-called “missing wedge” of information. Furthermore, the reconstructed electron tomogram is potentially affected by a range of reconstruction artifacts. The application of post-reconstruction NAD filtering may exacerbate these artifacts. Here, we extend the general NAD filtering approach by applying the diffusive filtering procedure to two-dimensional electron microscope images before they are assembled to form a three-dimensional tomographic representation.

In our approach the masked variance difference value, δ, is defined by the user in an approach similar to that employed by other groups (Fernandez and Li, 2005). This gives the user the flexibility to vary the process depending of specimen characteristics through the use of a mask function to exclude the likely positions of edges from the smoothing procedure. The termination of the iterations of the algorithm is ensured by its monotonically increasing behavior and by setting a maximum number of iterations. Further work to develop an automatic process for detecting the variance difference threshold may be desirable.

In summary, we provide a method for noise reduction with signal preservation by denoising 2D projection images before reconstructing 3D images. It is faster than post-reconstruction processing, and while all filtering approaches have the potential to introduce artifacts, the pre-filtering approach is designed to minimize artifacts due to contribution of the degraded signal from high tilt data in the reconstructed volume. We have applied the method to EM images of malaria parasite-infected RBCs. We find that features can be retrieved from data collected under sub-optimal imaging conditions (i.e. low-dose or low-staining or thick specimens). The method provides improvements in tomogram quality for both fixed, stained sections and cryo-stabilized sections (with or without energy filtering). Filtering techniques are used to optimize data obtained from a range of X-ray- and electron-based imaging methods. Pre-reconstruction filtering techniques may also prove useful in other tomographic imaging applications.

Acknowledgments

The authors acknowledge support from the Australian Research Council and the Australian National Health and Medical Research Council. We thank Kenneth Downing, Lawrence Berkeley Laboratories, CA, USA and Carlos Oscar Sorzano, Centro Nacional Biotecnología, Madrid, Spain, and Bernard Heymann, National Institutes of Health, MD, USA, and David Mastronarde, University of Colorado, CO, USA, and Ruben Dilanian, University of Melbourne, Australia, for helpful discussions. We thank Freddy Frischknecht and Marek Cyrklaff (University of Heidelberg, Germany) and Wolfgang Baumeister (Max Planck for Biochemistry, Munich, Germany) for support in cryo data acquisition. The photo of Marie Curie is from the nobelprize.org website.

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.2012.05.019.

References


Improving the quality of electron tomography image volumes using pre-reconstruction filtering

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Journal of Structural Biology

Appendix I

Automatic segmentation method for pre-NAD reconstructed volumes

An automatic segmentation algorithm was developed to provide a method for retrieving features in the reconstructed volumes. This provides an objective means of assessing the improvement obtained by application of the different filters.

A 3D image $I: X \rightarrow Y$ where $X \subseteq R^3$ and $Y \subseteq R$, can be partitioned in $n$ parts. This means obtaining from $I$ an image $K: X \rightarrow Y_1 \cup \ldots \cup Y_n$ where $Y_k \subseteq R$, $k = 1..n$, $1 \ Y_i=\emptyset$ and $\bigcup_{i=1..n} Y_i = Y$.

A common way of partitioning images using a set of global threshold values that can be automatically retrieved using inter-class variance minimization is described by others (Otsu, 1979; Sezgin and Sankur, 2004; Liao et al, 2001).

Let $S_i$ (with $i = 0,1, 2, .., n$) be an ordered set of $n+1$ boundary values of the image $I$, with $S_0 = \min(I)$, $S_n = \max(I)$, and $S_k$ ($k=1, 2, .., n-1$) the $n$-l threshold values. Let be $T: X \rightarrow [0,1]$ that associates each voxel, $x$, of the image $I$ with the value $i/n$. The value $i/n$ is in the interval [0,1] and spaces the $n$ partitions evenly over the interval. Define $L: X \rightarrow [0,1]$ as the image associated with the function $T$. Call $L_{\sigma}$ the regularized image at the scale $\sigma$ (Babaud et al., 1986, Nielsen et al., 1997, Koenderink, 1984).

The final segmented image is obtained by thresholding $L$ with a user-define value $t \in (0,1)$ which aims to distinguish relevant structures from the background. The thresholded image then
undergoes connected component analysis (Ibanez et al., 2003) in preparation for rendering (Yushkevich et al., 2006).

References


