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Measuring the Autocorrelation Function of Nanoscale Three-Dimensional Density Distribution in Individual Cells Using Scanning Transmission Electron Microscopy, Atomic Force Microscopy, and a New Deconvolution Algorithm

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Abstract

Essentially all biological processes are highly dependent on the nanoscale architecture of the cellular components where these processes take place. Statistical measures, such as the autocorrelation function (ACF) of the three-dimensional (3D) mass–density distribution, are widely used to characterize cellular nanostructure. However, conventional methods of reconstruction of the deterministic 3D mass–density distribution, from which these statistical measures can be calculated, have been inadequate for thick biological structures, such as whole cells, due to the conflict between the need for nanoscale resolution and its inverse relationship with thickness after conventional tomographic reconstruction. To tackle the problem, we have developed a robust method to calculate the ACF of the 3D mass–density distribution without tomography. Assuming the biological mass distribution is isotropic, our method allows for accurate statistical characterization of the 3D mass–density distribution by ACF with two data sets: a single projection image by scanning transmission electron microscopy and a thickness map by atomic force microscopy. Here we present validation of the ACF reconstruction algorithm, as well as its application to calculate the statistics of the 3D distribution of mass–density in a region containing the nucleus of an entire mammalian cell. This method may provide important insights into architectural changes that accompany cellular processes.

Keywords

algorithm; STEM; AFM; 3D autocorrelation function; cellular mass-density distribution

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Introduction

Abnormal changes in chromatin ultrastructure are a hallmark of many pathological processes, including carcinogenesis, diseases of cell differentiation and apoptosis. One of the main statistical methods to characterize these alterations is the autocorrelation function (ACF) of the three-dimensional (3D) spatial distribution of nuclear mass–density (Mirny, 2011; Bancaud et al., 2012). In recent studies, a significant increase in correlation length and changes in the shape of chromatin mass–density ACF (Cherkezyan et al., 2014) have been reported in cancer as independent prognostic factors for cancer survival (Bedin et al., 2010) and detection (Metze, 2013). This difference in nanoscale ACF, in conjunction with advances in modeling light scattering (Rogers et al., 2014), allowed optimizing imaging modalities for early cancer screening and diagnosis (Subramanian et al., 2009; Damania et al., 2012; Yi et al., 2014; Radosevich et al., 2015).

Conventionally, ACF is calculated directly from the deterministic 3D mass–density distribution within a sample volume. To get the deterministic nanoscale 3D mass–density distribution, electron tomography (ET) using Z-contrast (ZC) is routinely conducted for thin biological samples (Midgley & Weyland, 2003; Yakushevska et al., 2007; Biskupek et al., 2010; Sousa & Leapman, 2012). However, as the sample thickness (volume) increases, the resolution of a reconstructed tomogram decreases rapidly, as dictated by the Crowther criterion (Baumeister et al., 1999). To achieve sub-10 nm resolution, an alternative method is to employ time consuming and experimentally challenging serial sectioning, and conduct ET on each slice (Choudhuri et al., 2014).

In this study, we developed a novel algorithm under the assumption of a statistically isotropic mass–density distribution in a biological sample in order to recover the ACF from a single projection image and a thickness map. The method enables measuring ACF without the need to know the 3D mass–density arrangement, and thus avoids the need for tomographic reconstruction. On one hand, the spatial resolution of the ACF obtained by this method is not limited by the Crowther’s criterion. On the other hand, the time required is significantly less than the thin-slice serial sectioning method for the entire cell nucleus with high axial resolution. The comparison between the conventional method and our method conceptually is shown in Figure 1. As a demonstration for this method, we implemented the algorithm using data obtained with scanning transmission electron microscopy (STEM) and atomic force microscopy (AFM), and successfully reconstructed the nuclear ACF of a human buccal cell.

Materials and Methods

3D Mass–Density ACF Reconstruction Algorithm

The ACF of the 3D mass–density distribution $B'_{\rho}(\mathbf{r})$ bounded by the size of the sample is a convolution of the ACF of unbounded density spatial distribution $B_{\rho}(\mathbf{r})$ and ACF of the top-hat function $A_f(\mathbf{r})$.

$$B'_{\rho}(\mathbf{r}) = \int B_{\rho}(\mathbf{r}') A_f(\mathbf{r} - \mathbf{r}') d\mathbf{r}', \quad (1)$$

where $\mathbf{r} = (\mathbf{l}, z) = (x, y, z)$ is the radial distance. Note that

$A_f(\mathbf{r} - \mathbf{r}') = L\delta(\mathbf{l} - \mathbf{l}')T\left(\frac{z - z'}{L}\right)$, with constant L being the thickness of the sample (assuming the sample has uniform thickness) and $T(z)$ is a triangular function, equation (1) can be rewritten as equation (2).

$$B'_\rho(\mathbf{r}) = \frac{1}{L} \int B_\rho(\mathbf{l}, z') T\left(\frac{z - z'}{L}\right) dz', \quad (2)$$

Let $z = 0$, and replace z' with z , we get

$$B'_r(\mathbf{l}) = \frac{1}{L} \int B_r(\mathbf{r}) T\left(\frac{z}{L}\right) dz, \quad (3)$$

And the right hand side of equation (3) is exactly the equation for ACF of two-dimensional (2D) distribution of averaged density in the z dimension (projection), so $B'_\rho(\mathbf{l})$ is indeed the ACF of the density projection in 2D. Assuming the medium to be geometrically isotropic, we can reconstruct B_ρ from B'_ρ by solving equation (3) discretely

$$B_\rho = \frac{1}{2\Delta z} B'_\rho M^{-1}, \quad (4)$$

In equation (4), Δz is the step size of discretion, $B_{\rho,j} = B_\rho(\mathbf{i} \cdot \Delta z)$, $B'_\rho = B'_\rho(\mathbf{i} \cdot \Delta z)$, $i = 1, 2, \dots$,

$\frac{L}{\Delta z}$, is the discrete form of $B_\rho(\mathbf{r})$ and $B'_\rho(\mathbf{l})$, respectively.

$$M_{i,j} = r \cdot T\left(\sqrt{r_j^2 - l_i^2}/L\right) / \sqrt{r_j^2 - l_i^2} \text{ and } M_{i,j} = 0 \text{ for } i > j, r_i = j \cdot \Delta z, j = 1, 2, \dots, \frac{L}{\Delta z}, \text{ and } l_i = i \cdot \Delta z, i = 1, 2, \dots, \frac{L}{\Delta z}.$$

Numerical Validation of the Algorithm and Error Analysis for Sample with Nonuniform Thickness

Two assumptions were used in the derivation of the algorithm: (1) sample mass–density distribution is statically isotropic (2) sample thickness is uniform. We validated the algorithm numerically using a sample with an isotropic density distribution and a uniform thickness profile. However, for the second assumption, we found out that only small errors will be introduced if we use the mean thickness of the sample for L , given there is a moderate thickness variation, which is true for most biological samples. In other words, the uniform thickness assumption can be dropped while still maintaining the accuracy of reconstruction.

First, we generated two media to demonstrate an ideal sample for the algorithm and a sample where the algorithm would fail. To generate those two samples, one inhomogeneous

medium with random mass–density fluctuations was generated using MATLAB (MathWorks, Inc., Natick, MA, USA) and then processed differently (Cherkezyan et al., 2013). The dimension of the inhomogeneous sample is $6 \times 6 \times 6 \mu\text{m}^3$. The spatial correlation of the mass–density distribution is described by an exponential function:

$B_\rho(r) = \sigma_\rho^2 e^{-r/lc_\rho}$, where σ_ρ^2 is the variance and lc_ρ the correlation length. In the modeling, we used a σ_ρ of 0.25 g/cm^3 and a lc_ρ of 100 nm . As the length scale of ACF is much smaller than the dimension of the sample, even if we trimmed half of the sample, the mass–density distribution in the $6 \times 6 \times 3 \mu\text{m}^3$ volume is still isotropic enough (Figs. 2a–2c). This isotropic sample with uniform thickness is ideal for the algorithm. However, if we compressed the original sample along the z direction, the mass–density distribution in the compressed medium is no longer isotropic (Figs. 2b–2d), and the algorithm will fail in such sample.

Thus, we chose the isotropic medium (Fig. 2a) for numerical validation of the algorithm. We compared the ACF measured directly from this medium (Cherkezyan et al., 2014) with that reconstructed using our algorithm from the 2D projected image.

Finally, as real biological samples do not usually have uniform thickness, we need to estimate the error of the algorithm for samples without uniform thickness. We generated media similar to the one used in validation but with different thickness variations. We used the mean thickness of the medium in the reconstruction algorithm. The reconstructed ACF was compared with the measured ACF.

Sample Preparation

Cells from buccal mucosa (cheek cells) were collected using a Cytobrush (CooperSurgical Inc., Trumbull, CT, USA), suspended in $1 \text{ mL } 1\times \text{PBS}$ and spun for 5 min at $1,500 \text{ rpm}$ (accuSpin Micro17, Thermo Fisher Scientific Inc., Waltham, MA, USA). The supernatant was aspirated, and the pellet was resuspended in PBS. Droplets of $30 \mu\text{L}$ of the cell suspension were deposited on TEM grids in a moisture chamber for cell attachment. The chamber was kept in a cell incubator for 4 h ; then the sample was fixed with 2.5% glutaraldehyde and 2% formaldehyde in PBS for 20 min at room temperature. After fixation, the grids were rinsed in deionized (DI) water before dehydration in a series of graded ethanol. After dehydration, the samples were critical point dried (Tousimis Research Corp., Rockville, MD, USA) for STEM imaging.

Mass Projection Mapping Using STEM Z-Contrast Image

For samples with thickness smaller than electron mean free path, there is a simple linear relation between image contrast $I(x,y)$ in HAADF mode and projected mass $m(x,y)$ [equation (5)].

$$I(x,y) = AI_0 \langle \sigma_e \rangle m(x,y), \quad (5)$$

where I_0 is the intensity of the incident beam, $\langle \sigma_e \rangle$ the average elastic cross section, and A a constant associated with protein type and microscope settings. By using samples of similar

chemical composition with known thickness and density under the same microscope settings, we can calibrate the value of the prefactor A . For thick biological samples, this relation is affected by absorption and inelastic scattering. By selecting electrons scattered to sufficiently high angle, it is possible to screen out inelastically scattered electrons and maintain the linearity.

To find the rightful imaging condition, we used 5 μm polystyrene beads as our standard sample as opposed to tobacco mosaic virus. This is because both the chemical composition and the thickness of polystyrene beads are close to the whole cheek cell sample.

A STEM (Hitachi HD-2300, Hitachi Corp., Tokyo, Japan) was used for cell imaging, and all images were taken at 200 kV acceleration voltage with condenser aperture of 75 μm with probe current of about 0.7 nA. Polystyrene beads were imaged at different collection angles of the ADF detector as we changed the projection lens current from 1.065 to 1.10 mA. Conversion from projection lens current to detector collection angle can be found in the Supplementary Table 1. We then calculated the linear coefficient between the high angle annular dark field (HAADF) image contrast and projected mass of the polystyrene beads at each projection lens current (Fig. 3). Detailed experiments were described in Supplementary Material (Supplementary Fig. 1). On one hand, as we increased the collection angle of ADF detector, the influence of inelastic scattering was eliminated. On the other hand, the signal-to-noise ratio (SNR) gradually decreased as the collection angle increased.

We finally chose to use a projection current of 1.065 mA (inner semi collection angle 72 mrad, outer semi collection angle 380 mrad) to maintain good linearity and SNR. Images of the polystyrene bead and peripheral carbon/Formvar film background were taken with an exposure time of 35 s at 10 K magnification (pixel size: 10 nm). The cheek cell nucleus with surrounding cytoplasm and supporting film background were imaged under the same microscope settings for mass calibration. In addition, lower magnification images of the entire cheek cell were also captured with bright field (BF), Z-contrast, and secondary electron (SE) mode to assist alignment with AFM images.

Thickness Mapping Using AFM

A thickness distribution with comparable resolution with the STEM images was required to convert the mass projection to a density projection. To measure the thickness map, AFM was conducted on the same area of the cell.

In order to create a smooth and rigid substrate for AFM measurement, the grid was glued to a glass slide by epoxy (5-min epoxy, Devcon Corp., Danvers, MA, USA). The grid was placed on a region of epoxy much larger than itself and allowed to settle, ensuring the changes in cell height due to the curvature of the epoxy were minimal. To clean the sample surface, the grid was cleaned together with substrate in a plasma cleaner (PC 2000, South Bay Technology Inc., San Clemente, CA, USA). A 2.5 nm Pt film was coated on top of the sample by a high vacuum coater (Leica ACE600, Leica Microsystems, Vienna, Austria).

Sample thickness was determined at room temperature by peak force tapping mode using a Bruker Bioscope Catalyst AFM with silicon OTESPA-R AFM probes (Bruker AXS,

Madison, WI, USA). The low magnification SE and BF images were used to assist co-localization, as SE image provides topographic information of the cell and is more similar to the AFM topographic image, and BF image shows the exact location of the nucleus. The detailed registration steps can be found in Supplementary Material (Supplementary Fig. 2). A $6 \times 6 \mu\text{m}$ square was mapped with lateral resolution of 12 nm to cover the entire nucleus.

ACF of 3D Mass–Density Reconstruction and Analysis

2D density projection, $\rho(x, y)$, was calculated by dividing the mass distribution with thickness distribution of the same region and resolution. To obtain the spatial fluctuations of density distribution $\rho(x, y)$, the mean value was subtracted from the original data. The ACF of the projected density fluctuation was calculated using Wiener–Khinchin theorem:

$$B'_\rho(x, y) = F^{-1} \left\{ |F[\rho_\Delta(x, y)]|^2 \right\} \quad (6)$$

where F and F^{-1} are the Fourier and inverse Fourier transform operators. Later, a polar coordinate rotational average of $B'_\rho(x, y)$ was calculated to get the one-dimensional spatial correlation $B'_\rho(r)$ as a function of separation r , and this r shares the same physical meaning as l in equations (2) to (4). In reconstructing 3D ACF $B_\rho(r)$, we selected a $2.5 \times 2.5 \mu\text{m}$ region with the least thickness variation while covering most of the nucleus. We calculated the mean thickness of this region, and used this value as L in the reconstruction algorithm [equation (4)].

To analyze the 3D ACF of the sample, we fit it to the Whittle–Matérn (WM) family of correlation functions (Rogers et al., 2014). WM is defined as the product of a power law and

a modified Bessel function of the second kind $\left(\frac{K_{D-3}}{2}\right)$ of the order $\frac{D-3}{2}$

$$B_\rho(r) = A_\rho \left(\frac{r}{\ln}\right)^{\frac{D-3}{2}} \frac{K_{D-3}}{2} \left(\frac{r}{\ln}\right), \quad (7)$$

In equation (7), A_ρ , $\ln$ and D are fitting parameters. In specific, A_ρ is the density fluctuation amplitude; $\ln$ correlation length indicating the characteristic length of heterogeneity; the dimension D controls the shape of ACF, such that $D \rightarrow \infty$ for Gaussian; $D = 4$ for exponential; $3 < D < 4$ for stretched exponential and $D < 3$ for power law. Particularly, when $D < 3$, the biological medium can be considered a mass fractal and D takes the special meaning of fractal dimension. In addition, when $D = 4$ (exponential), $\ln$ is equal to

correlation length (lc_ρ), which is defined as displacement at which $B_\rho(r)$ drops to $\frac{1}{e}$ of its value at origin. For nonexponential ACF, this lc_ρ is often used as a fast estimation of the spatial correlation.

Results and Discussion

Error Analysis

To validate the proposed algorithm, we generated an isotropic medium with uniform thicknesses (Fig. 2a), and calculated the 2D projection of the medium. We later compared ACF [$B_{\rho}(\mathbf{r})$] directly from the unlimited medium [the input $B_{\rho}(\mathbf{r})$ in generating the medium] with that reconstructed from 2D projection [reconstructed $B_{\rho}(\mathbf{r})$]. We found the reconstructed $B_{\rho}(\mathbf{r})$ matched well with the measured true $B_{\rho}(\mathbf{r})$, regardless of the shape of $B_{\rho}(\mathbf{r})$ (Fig. 4a). To ensure the accuracy of the measurement of $B_{\rho}(\mathbf{r})$, the pixel size should be much smaller than lc_{ρ} (e.g., 1/10 is sufficient) and lc_{ρ} should be much smaller than the lateral dimension (x, y) of the samples (again, 1/10 is sufficient).

Next we investigated the influence of thickness variance on the accuracy of the proposed algorithm to reconstruct the ACF. We generated an isotropic medium with known $B_{\rho}(\mathbf{r})$, and bounded the medium to form a sample with nonuniform thicknesses (Fig. 4b). $B_{\rho}(\mathbf{r})$ was reconstructed from the projection, and the mean thickness of the samples was used in the algorithm. This reconstructed $B_{\rho}(\mathbf{r})$ was compared with the input $B_{\rho}(\mathbf{r})$. For a range of mean thickness and thickness variance, we evaluated the errors of σ_{ρ}^2 and lc_{ρ} . Our results showed that for a 10% error tolerance, lc_{ρ} estimation is accurate through a range of mean thickness and thickness variance. σ_{ρ}^2 estimation is accurate except for very low thickness ($L < 2.5 \mu\text{m}$) with large thickness range ($L > 3.5 \mu\text{m}$). Generally the algorithm is more accurate at larger mean thickness and smaller thickness variations (Figs. 4c, 4d).

Mass and Thickness Mapping

The STEM images of human cheek cell sample (Fig. 5) were captured under ZC to obtain mass projection. The intensity of gray scale HAADF images is proportional to local mass. The cell images showed clear micro-ridges surface features for cheek cells (typical for squamous epithelial cells). The mass content was low in the upper left corner of the cheek cell in both HAADF and BF images. The significant uneven mass distribution indicated extraction of cellular material, possibly occurred during the serial ethanol dehydration process. For the polystyrene beads, the linear coefficient between HAADF image intensity and mass projection was calculated to be 0.99. This again proved the validity of using HAADF for mass mapping even for thick biological sample.

The AFM images of the selected $6 \times 6 \mu\text{m}$ region with the cell nucleus, the background, and their relative position are shown in Figure 6. The mean thickness difference between (Fig. 6a) and (Fig. 6b) of the overlapping area is $4.97 \mu\text{m}$. Adding to the relative thickness map, we calculated the absolute thickness of a region over the nucleus. Based on the AFM measurement, we learned that part of this specific cheek cell could be as thick as $11 \mu\text{m}$. We also noticed that the nucleus region was not the thickest. In general, the nucleus region is supposed to be thicker than the cytoplasm region for cheek cells. This observation indicated that the membrane was folded during sample preparation.

ACF of 3D Mass–Density Reconstruction and Analysis

We interpolated and scaled the thickness map so that the pixel size is the same as the mass map. As the membrane of the cell was continuous, this interpolation should give a fairly accurate result after scaling. By dividing the mass over thickness and area of pixel, we got the density projection map $\rho(x,y)$ (Fig. 7a). The solid content (wt%) can be calculated directly by dividing $\rho(x,y)$ by the density of condensed dry protein (1.25 g/cm³). The average solid content in the cytoplasm and nucleus is about 30 and 50%, respectively. The high solid content is consistent with the outmost layer of cheek cells (Barer, 1957).

Furthermore, most optical techniques characterize the mass–density distribution of biological samples with the refractive index (RI) distribution as the variance (Cherkezyan et al., 2013; Zhang et al., 2016), which can be calculated $\rho(x,y)$ by the Gladstone–Dale relation:

$$n(x,y)=n_0+\alpha\rho(x,y), \quad (8)$$

In the equation, n_0 is 1.33, the RI of water, and α the specific refraction increment of the intracellular solids with a chemistry independent value of ~0.17 mL/g (Barer & Tkaczyk, 1954; Davies et al., 1954). The RI of the sample ranged from 1.4 to 1.45.

With $\rho(x,y)$, we calculated $B'_\rho(l)$, the ACF of the fluctuation of the mass–density, and reconstructed $B\rho(r)$ using the deconvolution algorithm described before (Figs. 7b, 7c). In the reconstruction, a $2.5 \times 2.5 \mu\text{m}$ window over the nucleus that has the least thickness variation was used for a more accurate result. The error was estimated by numerical experiments with the same surface profile. In this specific case, the mean thickness was $7.55 \mu\text{m}$ and the root-mean-square (RMS) variance was $0.22 \mu\text{m}$, ensuring σ_ρ^2 and lc_ρ errors of less than 8%. As expected, $B_\rho(0)$, the variance of the 3D mass–density distribution, is much larger than $B'_\rho(0)$, the variance of the projected mass–density distribution. This is because the averaging process along the z -axis suppressed the fluctuation in the distribution.

To characterize the shape of $B_\rho(r)$, we fitted the experimentally obtained ACF to Whittle–Matern family of analytical functional forms. Our results revealed a characteristic length of heterogeneity $\ln$ of 984 nm, and D_ρ of 2.95, indicating a fractal mass–density distribution. The finding that the ACF is indicative of a mass fractal behavior ($D_\rho < 3$) is biologically significant, as it provided the experimental confirmation of a prior hypothesis of a mass fractal architecture of chromatin (Bancaud et al., 2009; Bedin et al., 2010). The correlation length of mass–density (lc_ρ) is 100 nm and the standard deviation (σ_ρ) is 0.24 g/cm³. Since RI is linear to mass–density, ACF of RI [$B_n(r)$] has the same correlation length (lc_n) and dimension (D_n) with $B_\rho(r)$. The standard deviation of RI (σ_n) for this specific cheek cell was estimated as 0.04 by using $\sigma_n = \sigma_\rho \alpha$, which falls into the reported value range (0.04–0.1) (Schmitt & Kumar, 1998). However, a more accurate statistical measure of the cheek cell mass–density or RI distribution requires further samples.

As for other errors in ACF calculated by the algorithm, the validity of isotropic assumption needs to be taken into account. In general, within the length scale of chromatin fractal structure, the mass–density distribution is considered to be isotropic. However, the potential anisotropy of the mass–density distribution of other components in the nucleus of the cheek cell may cause errors in the ACF reconstruction. In addition, the tested cheek cell sample was processed intensely before imaging, artifacts may occur during sample preparation, such as cellular content extraction, volume shrinkage, and collapsing, etc. Another possible experimental error comes from AFM measurement. The mechanical movement of the probe may introduce slight offset between real displacement and desired value.

Summary and Conclusions

In this work, we have developed an analytic solution to deconvolute the ACF of the 3D mass–density distribution from the ACF of its 2D projection, assuming the distribution is geometrically isotropic and the sample thickness is uniform. We also estimated the error of the algorithm for samples with thickness variations. We discovered that, for samples with mean thickness $>2.5 \mu\text{m}$ and thickness variation $<3.5 \mu\text{m}$, the error of the algorithm was smaller than 10%. In implementing the algorithm to a region of cheek cell containing the nucleus, we calculated the distribution of the mass–density projection from the STEM mass projection and AFM thickness map, the ACF of the 2D mass–density projection, and further reconstructed the ACF of the 3D mass–density distribution with small error.

We believe with better sample preservation method (high-pressure freezing, freeze-drying, etc.) and an updated Bioscope, the ACF reconstruction will give a more accurate statistical characterization of the nanostructure in biological samples in their native state. The nanostructure, in turn, may provide valuable information for understanding many cellular biological processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Comparison between work flows calculating autocorrelation function (ACF) using the conventional method and the method in this paper. As only one high angle annular dark field (HAADF) image is required in the proposed method, the experimental time and beam damage will be greatly reduced. The resolution of ACF from the new method is not constrained by Crowther's criterion. In addition to assuming the sample is isotropic, the proposed method also assumes the sample thickness is uniform within the region of interests. However, the error introduced by this assumption is not significant for most biological samples. 2D, two-dimensional; 3D, three dimensional; AFM, atomic force microscopy, HAADF: high angle annular dark field.

Figure 2. Statistically isotropic and anisotropic medium. **a:** An isotropic sample with (c) autocorrelation function (ACF) along all direction identical. **b:** An anisotropic sample with (d) ACF different along z - and x -axis.

Figure 3. Relationship between intensity and mass in HAADF images as a function of the projection lens current. **a:** Linear coefficient of intensity and mass as we change the projection lens current, the selected projection lens current is 1.065 mA when considering linearity and signal to noise ratio. **b–f:** HAADF image of a polystyrene bead recorded at 10 K magnification from larger projection lens current to smaller projection lens current. Scale bar is 1 μm .

Figure 4.

Numerical validation of the reconstruction of three-dimensional (3D) density distribution properties from a two-dimensional mass projection for isotropic sample: (a) the comparison between measured true 3D autocorrelation function (ACF) and reconstructed 3D ACF from projected image; (b) isotropic medium with nonuniform thickness; (c) error $\sigma_n = \sigma_n \cdot a$ of plotted versus mean thickness and thickness variance, and (d) error of lc versus mean thickness and thickness variance.

Figure 5. Scanning transmission electron microscopy images of the same cheek cell. **a:** HAADF image of the entire cell. **b:** Nucleus region of the same cell. Scale bar: **(a)** 20 μm , **(b)** 3 μm .

Figure 6. Regional thickness distribution measured by atomic force microscopy (AFM). **a:** AFM measurement on a large area as reference height. **b:** AFM measurement performed on a smaller region enclosing the nucleus, with lateral resolution of 11 nm to reveal the fine topographic detail. **c:** Relative position of the two images. Scale bar: **(a)** 3 μm, **(b)** 50 nm, **(c)** 10 μm.

Figure 7. Cheek cell mass-density distribution. **a:** Two-dimensional mass-density projection. **b:** autocorrelation function (ACF) of (a) and (c) reconstructed three-dimensional mass-density ACF of (b). Scale bar: (a) $1 \mu\text{m}$.