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Three-dimensional locations of gold-labeled proteins in a whole mount eukaryotic cell obtained with 3 nm precision using aberration-corrected scanning transmission electron microscopy

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Abstract

Three-dimensional (3D) maps of proteins within the context of whole cells are important for investigating cellular function. However, 3D reconstructions of whole cells are challenging to obtain using conventional transmission electron microscopy (TEM). We describe a methodology to determine the 3D locations of proteins labeled with gold nanoparticles on whole eukaryotic cells. The epidermal growth factor receptors on COS7 cells were labeled with gold nanoparticles, and critical-point dried whole-mount cell samples were prepared. 3D focal series were obtained with aberration-corrected scanning transmission electron microscopy (STEM), without tilting the specimen. The axial resolution was improved with deconvolution. The vertical locations of the nanoparticles in a whole-mount cell were determined with a precision of 3 nm. From the analysis of the variation of the axial positions of the labels we concluded that the cellular surface was ruffled. To achieve sufficient stability of the sample under the electron beam irradiation during the recording of the focal series, the sample was carbon coated. A quantitative method was developed to analyze the stability of the ultrastructure after electron beam irradiation using TEM. The results of this study demonstrate the feasibility of using aberration-corrected STEM to study the 3D nanoparticle distribution in whole cells.

Keywords

whole cell; specific labels; gold nanoparticles; eukaryotic cell; critical-point drying; carbon coating; three-dimensional; specific labels; TEM; scanning transmission electron microscopy; STEM; 3D STEM

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1 Introduction

The biological processes responsible for cellular function are controlled by complex macromolecular systems, which are assembled from individual proteins and other biological components such as lipids and DNA. Accurate three dimensional (3D) maps of the spatial organization(s) of such bio-molecular assemblies within a whole cell provide a blueprint for investigating biological mechanisms (Lucic et al., 2005; Sali et al., 2003). Tomographic techniques using transmission electron microscopy (TEM) are typically employed to obtain nanometer-scale reconstructions of cellular structures in 3D (Pierson et al., 2009; Stahlberg and Walz, 2008); however high-resolution TEM tomography is limited to a maximum sample thicknesses of ~300 nm (Hohmann-Marriott et al., 2009), or a maximum of ~1 μm when using energy filtering techniques (Lucic et al., 2005). TEM imaging of a large structure, such as a whole cell, is challenging with tilt-series TEM tomography, primarily due to the increase in the effective sample thickness when tilting the stage. Thus, TEM imaging of whole eukaryotic cells is generally confined to the periphery, where the cellular material is thin enough to be contained within a single plastic section, or thin enough for reliable formation of vitrified ice for cryo-TEM preparation (Resch et al., 2010). One procedure to image the cytoskeleton of whole mount cells involves the extraction of the cellular membrane and cytosolic proteins (Resch et al., 2010). However, such procedure eliminates the possibility of imaging membrane or cytosolic structures, and thus cannot be considered for imaging an intact system.

Although long assumed to be too damaging for imaging biological structures (Aoyama et al., 2008), scanning transmission electron microscopy (STEM) has begun to gain acceptance as a technique for imaging thicker biological samples by both tomographic and focal-series techniques (Aoyama et al., 2008; De Jonge et al., 2007b; Hohmann-Marriott et al., 2009). Recently, our group introduced the use of aberration-corrected STEM to obtain 3D reconstructions of biological structures with nanometer resolution (de Jonge et al., 2010a; De Jonge et al., 2007b). Lateral scanning of the sample over consecutive focal depths avoids the difficulties associated with imaging a specimen at high tilt angles (Koning and Koster, 2009). Additionally, the acquisition time for focal-series STEM is faster than STEM tomography, because there is no need of realignment or adjustment of the microscope. Spherical aberration correction allows electron beam opening semi-angles (α) of up to 40 mrad, compared to conventional STEM with $\alpha \cong 10$ mrad, while maintaining a high lateral resolution. On account of the increased opening angle a depth of field of several nanometers is obtained (van Benthem et al., 2005). 3D information of a biological specimen is provided via the recording of a series of 2D images at different focus positions (Figure 1), a so-called focal series, and subsequent deconvolution (de Jonge et al., 2010a).

Here we present a method to prepare whole mount eukaryotic cells for TEM or STEM imaging. Existing protocols (Anderson, 1951; Glauert and Lewis, 1998; Nagata, 2000; Ris, 1985) were optimized so that critical-point dried, whole cells remained stable under the irradiation conditions of the STEM. We developed a quantitative method to analyze the sample stability in response to the electron dose using TEM imaging. The whole mount samples were imaged with aberration-corrected STEM, and several 3D focal series were obtained. The contrast mechanism of STEM using the annular dark field detector is especially suited to image heavy nanoparticles in a thick layer of a light material (de Jonge et al., 2010b). For this reason we have labeled the epidermal growth factor (EGF) receptor with gold nanoparticles. The datasets were analyzed for the 3D positions of the labels on the cell.

2 Materials and Methods

2.1 Cell Growth

Cells were grown directly on the electron microscopy sample supports, silicon microchips each with an electron transparent silicon nitride (SiN) window of an area of $250 \times 250 \mu\text{m}^2$, and 100 nm thickness (Protochips Inc, NC). The microchips were rinsed in ethanol for 2 minutes and then plasma cleaned (Harrick Plasma, NY) for 5 minutes. Next, the microchips were coated with poly-L-lysine (0.01%, Sigma, MO) to promote cell adherence to the SiN, by incubating them in solution for 5 minutes at room temperature. The microchips were then rinsed three times with HPLC-grade water (Fisher Scientific, PA) and placed, with the SiN surface facing upwards, into wells of a 96 well plate filled with Dulbecco's modified eagle medium (DMEM) (Invitrogen, CA) supplemented with 10% fetal bovine serum (Sigma, MO).

Confluent COS7 cells (green monkey kidney fibroblast) were harvested from a 25 mL culture flask after detachment with trypsin and re-suspended in 5 mL of serum supplemented DMEM. A droplet of the suspended cell solution was added to each well, and cell adherence to the microchip was monitored using an inverted microscope (TS100 Nikon Instruments). The microchips were then transferred to new wells containing fresh media. Cells were grown in an incubator for four hours at 37°C in air with 5% CO₂. Thereafter they were rinsed with serum-free DMEM, and transferred to wells filled with serum-free DMEM, where they were incubated overnight at 37°C in air with 5% CO₂.

2.2 Receptor Labeling

EGF receptors were tagged using EGF conjugated to 10 nm gold nanoparticles (EGF-Au). Gold-streptavidin nanoparticles (KPL, MD) were coupled to biotin labeled EGF (Invitrogen, CA) using a previously described method (de Jonge et al., 2009). The cells were incubated with 5 nM EGF-Au solution for 5 minutes at room temperature, followed by rinsing and fixation.

2.3 Primary Fixation

COS7 cells were fixed directly on the electron microscopy support using 4% glutaraldehyde in phosphate buffered saline (PBS, Electron Microscopy Sciences, PA) (de Jonge et al., 2009). Samples were immersed at room temperature in fixative for periods of 15, 30, 45, or 60 minutes. After fixation, they were rinsed three times with PBS (5 minutes each) followed by three rinses with 0.1 M cacodylate buffer (Ted Pella, CA), 5 minutes each, to remove residual fixative.

2.4 Staining and Secondary Fixation

Fixed cells were stained with OsO₄ by immersing the samples in solutions of either 40 mM, or 40 nM OsO₄ (Ted Pella, CA) in 0.1 M cacodylate buffer at room temperature for one hour (Glauert and Lewis, 1998). Controls without OsO₄ were incubated in cacodylate buffer only. Samples prepared from different concentrations (0, 40 nM, and 40 mM) were processed separately from one another to prevent cross-contamination from OsO₄ vapor. After staining with OsO₄, the samples were rinsed three times with 0.1 M cacodylate buffer followed by three rinses with HPLC-grade water to remove salts.

2.5 Ethanol Dehydration of Samples

Cells were dehydrated with EM-grade ethanol (Ted Pella, CA). Microchips supporting fixed and stained cells were immersed sequentially in the following concentrations of ethanol:

25% for 5 minutes; 50% for 5 minutes; 70% for 5 minutes; 85% for 5 minutes; 95% for 5 minutes (twice); 100% ethanol for 10 minutes (three times) (Glauert and Lewis, 1998).

2.6 Critical Point Drying

Samples were critical point dried (EMS 850, Electron Microscopy Sciences, PA) (Bozzola and Russell, 1999). The loading of the microchips into the sample holder and into the chamber of the critical point dryer was done under ethanol to prevent air-drying of the samples. The chamber was sealed and cooled to 5°C. The ethanol in the chamber was replaced with liquid CO₂ via a series of fluid exchanges. Each fluid exchange consisted of a 1-minute flush of the chamber with liquid CO₂ followed by a five minutes immersion in CO₂ with no flow. The CO₂ fluid level was adjusted so that the sample holder remained submerged at all times. After the removal of the ethanol the chamber was heated to the critical point of CO₂ (31°C, 73 atm), and the chamber was depressurized over a period of 45 minutes. The dry samples were stored in a desiccator prior to imaging to prevent the absorption of water from the air (Bozzola and Russell, 1999).

2.7 Carbon Coating

Critical point dried whole mount cell samples were coated with a thin layer of amorphous carbon using a home built electron beam carbon evaporator. The carbon deposition rate was ~0.2 Å/s. The vacuum level at the onset of evaporation was 10⁻⁷ torr, which decreased to 10⁻⁴ torr upon carbon evaporation.

2.8 Electron Microscopy

TEM imaging was performed at 80 kV using a TEM (CM12, FEI, OR) equipped with a 2k × 2k CCD camera (Advanced Microscopy Techniques (AMT), MA). The electron dose was measured as follows: the diameter of the electron beam illuminating the phosphor screen was determined, and the current (I) collected on the phosphor screen was measured. The electron dose per unit time (D/t) was calculated via the CM12 software using the equation:

$$D/t = \frac{M^2 2.5 I D_{screen}^2}{e D_{meas}^2 A_{screen}}, \quad (1)$$

where, M is the magnification, 2.5 is a correction factor to account for backscatter electrons, D_{screen} is the diameter of the screen, e is the electron charge, D_{meas} is the measured diameter of illuminated region on the screen, and A_{screen} is the total area of the screen. After determination of the electron dose the specimen was inserted, whereby the diameter of the illuminated area on the phosphor screen was kept constant. The cumulative electron dose in an experiment was then determined from the irradiation time t.

3D focal series data sets were recorded with a 200 kV aberration-corrected STEM/TEM (2200 FS, JEOL). The corrector was aligned at 17.3 mrad opening semi-angle (α), and then a second round of correction was done at α = 26.5 mrad. Finally, α was set to 41 mrad. The corresponding probe current was I_p = 83 pA. Each image contained 512×512 pixels, and the pixel dwell time was τ = 8 μsec.

2.9 Image Alignment of TEM images

The individual TEM images of a series were arranged in order of increasing electron dose, merged into a single stack, and then aligned to correct for stage drift using the following procedure. Several individual objects that remained visible throughout the image series were used as alignment references. The positions of these objects were tracked for each frame in

the series using the ImageJ (Rasband, 1997–2005) plugin, MTrackJ (Meijering, 1996). Figure 2 shows an example of the observed drift of a sample containing COS7 cells coated with 10 nm of amorphous carbon. TEM images taken after electron doses of 10^3 and 10^6 e⁻/nm² were overlaid; the observed drift vectors are indicated by the arrows (Figure 2a). Stage drift presumably resulted in the translation of the objects between images. The coordinates (x, y) of several objects in Figure 2a were recorded for each frame in the image series (Figure 2b). The objects drifted by an average total of 229 nm. The direction and magnitude of the shift was approximately the same for all reference objects, which confirmed that the shift was a result of stage drift and not sample distortion. The images were then aligned using the average drift vector. The accuracy of the alignment was tested by re-plotting the coordinates of the same objects using the aligned image stack. Figure 2c shows that the reference objects did not exhibit notable shifts after alignment. The above procedure was used instead of cross-correlation (Midgley and Weyland, 2003), since we wanted to measure the drift of multiple reference objects simultaneously to be able to check for radiation damage.

2.10 Stability Analysis Using Linescan Comparison

The following analysis was used to assess the sample stability in successive TEM images. The contrast of each image was inverted in ImageJ so that regions without cellular material had a corresponding signal intensity of zero. Changes to an ultrastructure as a result of electron beam irradiation were measured using linescans, measuring the signal $S(x)$ versus position x , at certain locations in the images. The $S(x)$ values of the linescan in the first image of a series served as the reference ($S_{ref}(x)$) against which the linescan profiles in subsequent images were normalized. The normalized difference in the signal intensity for each position in the linescan profile, $\Delta S_N(x)$, was calculated using:

$$\Delta S_N(x) = 1 - \frac{|S_{ref}(x) - S(x)|}{S_{ref}(x)} \quad (2)$$

The individual values of $\Delta S_N(x)$ were averaged in order to assign a single numerical value for the difference in the profile of the linescan (ΔS_N):

$$\Delta S_N = \frac{1}{x} \sum_x \Delta S_N(x) \quad (3)$$

The average stability of the sample or sample region was expressed as the mean ΔS_N of multiple linescans in a region, with the standard deviation providing a measure of the variance within a given sample.

2.11 Image processing of STEM images

The 3D STEM data sets were processed using the following image processing steps (de Jonge et al., 2010a). The noise in the image was reduced using a convolution filter (Kernel: 1,1,1; 1,5,1; 1,1,1) using the software Digital Micrograph (Gatan). The data was then automatically aligned slice-by-slice using the Autoquant software (Media cybernetics Inc.). The alignment algorithm used was particle alignment with respect to the nearest neighbor, with an alignment setting of 'no warp.' The maximal lateral drift was 70 nm. To increase the axial resolution, the 3D datasets were deconvolved using the Autoquant software (de Jonge et al., 2010a) via three cycles of deconvolution: (1) 50 iterations of adaptive deconvolution to estimate the object and the point spread function, (2) 50 iterations with fixed point spread function, and (3) 50 iterations of axial adaptive deconvolution.

3 Results

Whole mount cell samples were optimized for stability under electron beam irradiation, and their stability under electron beam irradiation was assessed using TEM. The following sample preparation steps were optimized: OsO₄ staining, primary fixation time, and carbon coating. After sample optimization, the stability was determined over an electron dose series of 10³ to 10⁶ e⁻/nm². Cellular samples, in which EGF receptors were labeled with 10 nm gold nanoparticles, were then imaged using aberration-corrected STEM. The acquired 3D data set was deconvolved and analyzed.

3.1 Stability Analysis

We developed a method to measure sample stability via linescan analysis. The method was tested on a sample containing ethanol dehydrated, critical point dried COS7 fibroblast cells grown on electron microscopy sample supports. Prior to dehydration the COS7 cells were fixed with glutaraldehyde for 15 minutes, stained with OsO₄ (40 nM), and the saline was removed by rinsing the samples in water. The analysis of sample stability was performed as follows. A reference image was recorded with an initial electron dose of 10² e⁻/nm². After the reference image was recorded, a second image was recorded after a cumulative electron dose of 10⁵ e⁻/nm² at the same position of the sample. We expected the thicker regions of the cells near the nucleus to be more sensitive to electron beam damage than the thin regions at the edge of the cells. For this reason, thick and thin cellular regions were analyzed independently. Two regions of different thickness within the same sample are shown in Figure 3. The first region, near the edge of the cell (Figure 3, a and b), remained stable during irradiation, as can be seen from the high degree of overlap of the linescans shown in Figure 3c. A thick region, near the nucleus envelope, is shown in Figure 3d and 3e. The two linescans across this region show several differences (Figure 3f). The largest changes in the profile occur at regions indicated by the arrows. The difference in the linescan profiles was quantified as the normalized change with respect to the first image in the series, and averaged for the pixels in a line scan (ΔS_N ; see methods section). The ΔS_N values for the linescans of Figures 3c and 3f are 0.90 and 0.89, respectively.

The overall sample stability was determined by analyzing several line scans and calculating the mean ΔS_N . For the thin cellular region the mean ΔS_N was 0.83 ± 0.02 (for a total of $n = 7$ averaged linescans), while the region near the nuclear envelope had a mean ΔS_N 0.80 ± 0.08 ($n = 14$). In addition to having a lower mean ΔS_N value, the standard deviation of the thick region was fourfold larger than for the thin cellular region, indicating that thick sample regions exhibited a larger variance in stability relative to thin regions. Thus, the stability measure, mean ΔS_N , is sensitive to a stability difference between two regions within a sample.

3.2 Osmium Tetroxide Staining

Staining with 1% OsO₄ (40 mM) is the standard in TEM sample preparation used to stabilize the lipid content and enhance contrast (Glauert and Lewis, 1998). We reasoned that the standard concentration of OsO₄ could possibly result in too much metal in the thicker regions of a whole cell sample, thus obstructing imaging in those regions. Therefore, staining with an ultra-dilute solution of OsO₄ (40 nM) was used. Cells were fixed for 45 minutes in 4% glutaraldehyde in PBS, and then incubated in 40 nM OsO₄ in cacodylate buffer for 1 hour. The samples were dehydrated with ethanol, critical point dried, and coated with 10 nm of carbon. For comparison we also tested the standard OsO₄ concentration of 40 mM (1%), and no OsO₄ at all, see Figure 4. Regions of darker contrast (as indicated by the arrow) were present in the unstained control sample (Figure 4a), but several organelles, such as the mitochondria, were not identifiable. The mitochondria are clearly visible in both the

OsO₄-fixed samples (Figure 4b, c). Yet, we did not observe a difference in contrast between the low and high concentrations of OsO₄, and, therefore, we chose the lower (40 nM) concentration for further the experiments described in this study.

3.3 Glutaraldehyde Fixation Time

The length of the primary fixation was varied to determine if the fixation time influenced the sample stability. COS7 cells were fixed at room temperature in 4% glutaraldehyde for 15, 30, 45, or 60 minutes. The fixed cells were stained for one hour with 40 nM OsO₄, rinsed, then dehydrated with ethanol and critical point dried. For each condition 10 linescans were analyzed. The mean ΔS_N values are shown in Figure 5. There was no significant difference amongst the four fixation times examined, and, therefore, subsequent experiments utilized a standard fixation time of 45 minutes in 4% glutaraldehyde (Bozzola and Russell, 1999; Glauert and Lewis, 1998).

3.4 Influence of Carbon coating on Stability

Additional samples were prepared to test if coating the samples with a layer of amorphous carbon improved the sample stability. A continuous layer of carbon provides electrical conductance, and mechanical stability. Samples were fixed for 45 minutes with 4% glutaraldehyde in PBS followed by OsO₄ staining for 1 hour (40 nM). Following ethanol dehydration and critical point drying, the samples were coated with 10, 20, or 50 nm of amorphous carbon, and then imaged using TEM (Figure 6). In an area near the cell grains of deposited material are visible on the SiN window surface, for the samples coated with either a 20, or a 50 nm layer of carbon. The average sizes of these grains were 6 ± 1 nm, and 20 ± 2 nm, for the 20 and 50 nm layers, respectively. For the sample coated with 10 nm of carbon the deposited material did not exhibit distinguishable grains.

3.5 Stability Analysis of Carbon Coated Samples

The carbon-coated samples were analyzed for stability, and compared with an uncoated control sample. For each sample, a TEM reference image was recorded using an electron dose was 10^2 e⁻/nm². The same region was then recorded after a receiving a cumulative electron dose of 10^5 e⁻/nm². Linescans throughout both thin and thick cellular regions were analyzed to measure the difference in stability between the coated and uncoated samples. Figure 7 compares the stability of samples with, and without, a coating of amorphous carbon. TEM images of the uncoated control sample (Figure 7a) shows a loss of cellular material near the region indicated by the arrow in after irradiation (Figure 7b). However, TEM images of samples coated with a 10 nm layer of amorphous carbon (before: Figure 7c, after: Figure 7d) show no visible structural change upon electron beam irradiation. A comparison of the mean ΔS_N values for both thick and thin regions samples coated with different carbon thicknesses is shown in Figure 7e. The control sample (no carbon) was the least stable, with a mean ΔS_N of 0.85 ± 0.02 , while each of the carbon coated samples had a mean ΔS_N of 0.93 ± 0.02 or above. Thus, the application of the carbon coating significantly improved the stability, but only a small difference was found between the tested coating thicknesses.

3.6 Stability Range: Electron Dose

In order to determine the dose resistance of the optimized samples coated with 10 nm carbon to high electron doses, we tested the following series of electron doses: 10^3 , 10^4 , 10^5 , and 10^6 e⁻/nm². The mean ΔS_N for both thick and thin cellular regions at each electron dose are shown in Figure 8. Thick regions remained stable for each dose in the series with values of mean ΔS_N remaining at 0.93 or higher, with a standard deviation of less than $\pm 3\%$ for all data points. Thin and thick sample regions were of the same stability within the standard

deviation. From these results we conclude that the samples should also withstand the electron dose needed for the recording of a focal-series dataset with STEM with a dose of up to $10^6 \text{ e}^-/\text{nm}^2$, assuming there would not be a large difference in radiation damage between TEM and STEM. The stability data was obtained using an accelerating voltage of 80 kV, optimized for achieving the largest contrast in the TEM on the biological specimen. The 3D STEM focal series were recorded at 200 kV optimized for the highest possible resolution. Typically, an electron beam of 80 kV causes larger radiation damage than a beam of 200 kV (Dykstra and Reuss, 2003), on account of the lower cross section for inelastic scattering at 200 kV. It is thus reasonable to assume that the stability data obtained at 80 kV also applies to STEM imaging at 200 kV.

3.7 3D STEM Imaging of Nanoparticles on Whole Cells

Samples of whole mount COS7 cells were prepared with gold-tagged EGF receptors localized at the cell surface. The cells were fixed, stained, critical point dried, and coated with 10 nm of amorphous carbon. 3D focal series were recorded over several cellular regions at a magnification of 100,000 and a probe semi angle of 41 mrad, with an aberration-corrected STEM. The sample was first pre-irradiated with an electron dose of $1 \times 10^5 \text{ e}^-/\text{nm}^2$ to reduce contamination and sample shrinkage during imaging. The first dataset was collected at the edge of the cell. It contained $N = 500$ images, acquired with focus steps of $dz = 2.5 \text{ nm}$, and a pixel size of $s = 2.8 \text{ nm}$ (magnification of 100k). The image series was acquired such that sufficient focal positions above and below the specimen were obtained to allow deconvolution (de Jonge et al., 2007a). The total imaging time was 30 minutes. Since the divergence of the electron beam over the entire vertical range of the focal series was much smaller than the image size, the average electron dose during the focal series acquisition can be approximated by:

$$D = \frac{I_p \tau N}{es^2}, \quad (4)$$

with electron charge e . It followed that $D = 2.6 \times 10^5 \text{ e}^-/\text{nm}^2$ for the settings used in this experiment. The cumulative dose the sample was irradiated with at this location was thus $3.6 \times 10^5 \text{ e}^-/\text{nm}^2$.

Before and after the recording of a focal-series dataset we also took an image with the cell mostly in focus, to test for possible radiation damage. We analyzed the stability of the sample by comparing these two STEM images. Figure 9 shows selected areas of 256×270 pixel of STEM images recorded before (Figure 9a) and after (Figure 9b) the focal series acquisition. From these images it can be seen that the ultrastructure was mostly preserved after the 3D imaging. The only visible change was a shift along the fold-like feature highlighted by the dotted line at arrow #1. Also the positions of gold nanoparticles were conserved, as shown, for example, at the arrow #2. Linescans were taken along the dashed lines in Figure 9a and b, and their profiles compared in Figure 9c. The linescan analysis demonstrates a good structural stability as indicated by the overlap of the two profiles. The nanoparticle at arrow #2 is also visible in the linescans. The region with less stability clearly shows at arrow #1. Since the less stable region at arrow #1 was surrounded by overlapping regions in the linescan, we suggest that the sample distortion was a movement of a cellular structure, possibly a membrane protrusion. Further analysis of the images via linescans resulted in a mean ΔS_N value of 0.90 ± 0.03 ($n = 21$) in most section of the images Figure 9a and b, except for the region along the dashed line, where the mean ΔS_N was 0.82 ± 0.09 ($n=11$). The specimen thus remained mostly stable during the recording of a focal series with STEM.

Figure 10a shows an image obtained from a projection of the focal series at the same coordinates as Figure 9. The focal series was projected in axial direction on the lateral plane as the sum of the individual slices to obtain the image (using ImageJ). This dataset was recorded in a cellular region close to the edge of a cell, where several filaments are visible. A total of 26 gold nanoparticles can be distinguished as bright spots. These are homogeneously dispersed, as expected for the short period of incubation (Lidke et al., 2004). Because 3D STEM is a wide field imaging methodology each image contains also out-of-focus information of the adjacent slices, and the axial resolution is limited. To optimize the axial information in the 3D data set we have applied adaptive deconvolution (de Jonge et al., 2010a). To enhance the processing speed the first 100, and the last 100 images were removed from the focal series as these slices contained only out-of-focus information, beyond that which was necessary for deconvolution. Figure 10b depicts a projection on the lateral plane of the deconvolved data at the same position as Figure 10a. The labels are visible with more contrast. Some of the information of the ultrastructure was lost while the deconvolution functions optimal for objects with a strong contrast.

The increase in axial resolution due to deconvolution was evaluated with axial linescans over nanoparticles, as exemplified in Figure 10c for nanoparticles #1 and #3 (indicated in Figure 10b). The average full width half maximum (FWHM) of the axial intensity peaks (signal intensity versus axial position) of 10 nanoparticles was 340 ± 75 nm. The deconvolution procedure reduced the noise and increased the axial resolution, as shown in Figure 10d. After deconvolution, the average FWHM over the intensity peaks in the axial linescans of 10 nanoparticles was 99 ± 28 nm. The precision at which a position of a single nanoparticle can be determined is much higher than the resolution, as long as the nanoparticles are well separated. The axial positions of the 26 gold nanoparticles were determined from the maximum in the axial intensity profile, see Table 1. The precision was found to be ± 3 nm, which corresponded to the focal step size. The precision of the position determination in lateral direction follows directly from the pixel size of 2.8 nm, and was ± 3 nm. The two labels, #1 and #3, shown in Figure 10d, were located at vertical positions of 260 and 282 nm, respectively. The positions of the gold nanoparticles range from 260 ± 3 nm (label #1) to 483 ± 3 nm (label #26). A histogram of the distribution of the axial locations (Figure 10e) shows that the largest number of nanoparticles is located at a depth of 400–425 nm. The cells face towards the point of beam entry, and a smaller axial value resembles a vertical position closer to the surface of the SiN membrane. Thus the smaller the axial position the farther the nanoparticle is relative to the SiN support. Thus we can conclude that the topography of the cell surface is inhomogeneous.

A 3D dataset was also recorded at a thicker cellular region, near the nuclear envelope (indicated the dashed line), see Figure 11. This dataset was recorded with the following microscope settings, $N = 400$ images, $dz = 5$ nm, and $s = 4.6$ nm (magnification of 100k), resulting in a total dose after pre-irradiation of $D = 1.8 \times 10^5$ e⁻/nm². All images were used in the deconvolution. The mean ΔS_N was 0.91 ± 0.03 ($n = 25$), indicating that also this cellular region was stable during the recording of the focal series. Prior to deconvolution (Figure 11a), the gold nanoparticles are not visible within the increased signal from the cellular material compared to imaging at the edge of the cell (Figure 10). After deconvolution the nanoparticles were visible (Figure 11b). A total of 9 gold labels were observed, and their axial positions are shown in Table 2. Nanoparticles # 2 and #6 were separated by an axial distance of 730 nm. Thus the cellular surface curved from the region near the nucleus towards the nuclear region causing its surface to rise by 730 nm. Thus the thickness of the cell must have been greater than 730 nm.

Obtaining 3D information with nanometer precision from cellular specimens of this thickness is very difficult with conventional TEM tomography, even with energy filtering

(Lucic et al., 2005). We have successfully applied 3D STEM to the imaging of a whole-mount cellular sample, and detected the vertical locations of the labels with high precision (3 nm). In our sample we found the labels to be mainly at the cellular surface, but several labels were probably located just below the surface, or within membrane regions of high curvature. It is important to notice that the STEM detected electrons transmitted through the entire sample. It does not matter for the STEM if the labels are located at the surface, or within the cell, as long as the labels are not so deep in the sample that beam blurring prevents their imaging, which does not happen until at least 2 μm depth depending on the density of the specimen (de Jonge et al., 2010b).

3.8 Axial Distribution of Labels

An important question is the cause of the variation in the axial positions of the gold nanoparticle labels; was this caused by (1) passive diffusion through the membrane (2) internalization of the EGF receptor or (3) vertical inhomogeneity of the cellular surface? Diffusion of the nanoparticles through the membrane of cells is unlikely to occur in glutaraldehyde fixed cells without first permeabilizing them with a detergent, such as saponin or tritonX (Jamur and Oliver, 2009). Also, endocytosis of the EGF labeled gold nanoparticles is unlikely to occur during the five-minute incubation at room temperature (de Jonge et al., 2009; Dukes et al., 2010; Lidke et al., 2004). Thus the difference in axial position in Figure 9 is presumably due to the topography of the cellular membrane. Because EGF is internalized via endocytosis of clathrin coated pits (Willingham and Pastan, 1982), one possibility is that some of the nanoparticles are localized within clathrin enriched buds, or invaginations within the cellular surface that occur during the first step of vesicle formation. Although internalization of the EGF ligand-receptor complex occurs within minutes of binding at 37°C, the process of endocytosis is much slower at room temperature (Schlessinger et al., 1978; Schreiber et al., 1983). Furthermore, clusters of gold nanoparticles, which form prior to vesicle formation are absent, which suggests that the difference in axial height is not due to pit formation (Leamon et al., 1993; McKanna et al., 1979; Schreiber et al., 1983; Willingham and Pastan, 1982).

A second possibility is that the difference in axial height is due to ruffling of the cellular membrane (Jackowski et al., 1990). Ruffling is the formations of transient, fold-like protrusions of the membrane, particularly near the edge of the cell. Stimulation of COS7 cells, as well as other cell lines, with EGF is well known to induce ruffling of the cellular membrane 1–2 minutes after binding of EGF (Brunk et al., 1976; Kurokawa and Matsuda, 2005; Ridley, 1994). The cellular ultrastructure observed in Figure 9 is similar to the long fold-like structure of membrane ruffles observed by others using SEM (Chinkers et al., 1979; Jackowski et al., 1990; Ridley, 1994). Two pairs of adjacent nanoparticles are located on such a fold, for example, the structures: #5 and # 21, and #2 and #19. Interestingly, the individual nanoparticles, which comprise each pair, are separated by an axial distance greater than 100 nm despite their lateral proximity. The difference in axial position of these nanoparticle pairs is likely due to the slope of the membrane ruffle on which they are bound. Thus, we conclude that the difference in axial heights among the gold nanoparticles is a result of the surface topography of the membrane, specifically membrane ruffles, rather than internalization of the receptor ligand complex.

The presence of membrane ruffling may also account for the localized instability of the sample described in Figures 9. Because the ruffle structure protrudes out, away from the cellular surface, it is capable of being deflected by the electron beam. Thus the shift in the linescan observed in Figure 9c may have resulted from a change in position, such as folding, of the membrane ruffle structure, while the main cellular body was stable.

In Figure 11, the dashed line indicates the edge of the nuclear envelope. The two nanoparticles located on the nucleus, # 1 and #2, have an axial position relative to the remaining nanoparticles of at most, 730 nm. This vertical difference can be explained by the vertical protrusion of the nuclear envelope. The vertical positions of nanoparticles near the nuclear envelope differ by a maximum of 185 nm, which indicates both a high degree of membrane surface roughness near the nucleus, and bending of the membrane towards the nucleus.

4 Conclusions

A procedure was developed for stabilizing whole mount eukaryotic cell samples grown on SiN windows. The key step was the coating of the critical point dried cells with amorphous carbon. The stability of the samples was measured quantitatively by analyzing linescans obtained from TEM images. The samples remained stable during the acquisition of a 3D focal series using aberration-corrected STEM with an electron dose of $3.6 \times 10^5 \text{ e}^-/\text{nm}^2$. The 3D datasets highlighted different cellular regions containing gold nanoparticles tagging the EGF receptor. The gold nanoparticles were resolved on the surface of the whole cell at a range of vertical positions, and an axial precision of $\pm 3 \text{ nm}$ was achieved after deconvolution. The positions of the nanoparticles in lateral direction were also determined with a precision of $\pm 3 \text{ nm}$. From analysis of the variation of the axial positions of the labels we concluded that the cellular surface was ruffled.

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Abbreviations

TEM	transmission electron microscopy
STEM	scanning transmission electron microscopy
3D	three dimensional
EGF	epidermal growth factor receptor
DMEM	Dulbecco's modified eagle medium
SiN	silicon nitride

References

- Anderson TF. Techniques for preservation of three dimensional structure in preparing specimens for the electron microscope. *Trans NY Acad Sci.* 1951; 13:130–134.
- Aoyama K, Takagi T, Hirase A, Miyazawa A. STEM tomography for thick biological specimens. *Ultramicroscopy.* 2008; 109:70–80. [PubMed: 18977089]
- Bozzola, JJ.; Russell, LD. *Electron Microscopy Principles and Techniques for Biologists.* Jones and Barlett Publishers; Boston: 1999.
- Brunk U, Schellens J, Westermarck B. Influence of epidermal growth factor (EGF) on ruffling activity, pinocytosis and proliferation of cultivated human glia cells. *Exp Cell Res.* 1976; 103:295–302. [PubMed: 1001364]
- Chinkers M, McKanna JA, Cohen S. Rapid induction of morphological-changes in human carcinoma-cells A-431 by epidermal growth-factor. *J Cell Biol.* 1979; 83:260–265. [PubMed: 315943]

- de Jonge N, Peckys DB, Kremers GJ, Piston DW. Electron microscopy of whole cells in liquid with nanometer resolution. *Proc Natl Acad Sci*. 2009; 106:2159–2164. [PubMed: 19164524]
- de Jonge N, Sougrat R, Northan BM, Pennycook SJ. Three-dimensional scanning transmission electron microscopy of biological specimens. *Microsc Microanal*. 2010a; 16:54–63. [PubMed: 20082729]
- de Jonge, N.; Sougrat, R.; Peckys, DB.; Lupini, AR.; Pennycook, SJ. *Nanotechnology in Biology and Medicine: Methods, Devices, and Applications*. 2007a. Three-Dimensional Aberration-Corrected Scanning Transmission Electron Microscopy for Biology.
- de Jonge N, Poirier-Demers N, Demers H, Peckys DB, Drouin D. Nanometer-resolution electron microscopy through micrometers-thick water layers. *Ultramicroscopy*. 2010b; 110:1114–1119. [PubMed: 20542380]
- Dukes MJ, Peckys DB, de Jonge N. Correlative fluorescence microscopy and scanning transmission electron microscopy of quantum-dot-labeled proteins in whole cells in liquid. *ACS Nano*. 2010; 4:4110–6. [PubMed: 20550177]
- Dykstra, MJ.; Reuss, LE. *Biological Electron Microscopy: Theory, Techniques, and Troubleshooting*. 2. Springer; 2003.
- Glauert, AM.; Lewis, PR. *Biological specimen preparation for transmission electron microscopy*. Portland press; London: 1998.
- Hohmann-Marriott MF, Sousa AA, Azari AA, Glushakova S, Zhang G, Zimmerberg J, Leapman RD. Nanoscale 3D cellular imaging by axial scanning transmission electron tomography. *Nat Methods*. 2009; 6:729–31. [PubMed: 19718033]
- Jackowski MM, Swift LL, Cohen S, McKanna JA. Morphologic changes in human carcinoma-cells (A-431) stimulated by epidermal growth-factor - effect of cholesterol and low-density lipoproteins on the ruffling response. *J Cell Physiol*. 1990; 142:458–468. [PubMed: 2312611]
- Jamur MC, Oliver C. Permeabilization of Cell Membranes. *Methods Mol Biol*. 2010; 588:63–66. [PubMed: 20012820]
- Koning RI, Koster AJ. Cryo-electron tomography in biology and medicine. *Annals of Anatomy - Anatomischer Anzeiger*. 2009; 191:427–445.
- Kurokawa K, Matsuda M. Localized RhoA Activation as a Requirement for the Induction of Membrane Ruffling. *Mol Biol Cell*. 2005; 16:4294–4303. [PubMed: 15987744]
- Leamon CP, Pastan I, Low PS. Cytotoxicity of Folate-Pseudomonas Exotoxin Conjugates toward Tumor-Cells - Contribution of Translocation Domain. *J Biol Chem*. 1993; 268:24847–24854. [PubMed: 8227046]
- Lidke DS, Nagy P, Heintzmann R, Arndt-Jovin DJ, Post JN, Grecco HE, Jares-Erijman EA, Jovin TM. Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. *Nat Biotechnol*. 2004; 22:198–203. [PubMed: 14704683]
- Lucic V, Foerster F, Baumeister W. Structural studies by electron tomography: from cells to molecules. *Annu Rev Biochem*. 2005; 74:833–865. [PubMed: 15952904]
- McKanna JA, Haigler HT, Cohen S. Hormone Receptor Topology and Dynamics: Morphological Analysis Using Ferritin-Labeled Epidermal Growth Factor. *Proc Natl Acad Sci*. 1979; 76:5689–5693. [PubMed: 230489]
- Meijering, E. ImageJ Plugin. 1996. MTrackJ.
- Midgley PA, Weyland M. 3D electron microscopy in the physical sciences: the development of Z-contrast and EFTEM tomography. *Ultramicroscopy*. 2003; 96:413–431. [PubMed: 12871805]
- Nagata T. Three-dimensional high voltage electron microscopy of thick biological specimens. *Micron*. 2000; 32:387–404. [PubMed: 11070359]
- Pierson J, Sani M, Tomova C, Godsave S, Peters PJ. Toward visualization of nanomachines in their native cellular environment. *Histochem Cell Biol*. 2009; 132:253–62. [PubMed: 19649648]
- Rasband, WS. ImageJ. U. S. National Institute of Health; Bethesda, Maryland, USA: 1997–2005.
- Resch, GP.; Urban, E.; Jacob, S. The Actin Cytoskeleton in Whole Mount Preparations and Sections. In: Thomas, M-R., editor. *Methods in Cell Biology*. Academic Press; 2010. p. 529-564.
- Ridley AJ. Membrane ruffling and signal transduction. *BioEssays*. 1994; 16:321–327. [PubMed: 8024541]

- Ris H. The cytoplasmic filament system in critical-point dried whole mounts and plastic-embedded sections. *J Cell Biol.* 1985; 100:1474–1487. [PubMed: 4039327]
- Sali A, Glaeser R, Earnest T, Baumeister W. From words to literature in structural proteomics. *Nature.* 2003; 422:216–225. [PubMed: 12634795]
- Schlessinger J, Shechter Y, Willingham MC, Pastan I. Direct Visualization of Binding, Aggregation, and Internalization of Insulin and Epidermal Growth Factor on Living Fibroblastic Cells. *Proc Natl Acad Sci.* 1978; 75:2659–2663. [PubMed: 307249]
- Schreiber AB, Libermann TA, Lax I, Yarden Y, Schlessinger J. Biological role of epidermal growth factor-receptor clustering - investigation with monoclonal anti-receptor antibodies. *J Biol Chem.* 1983; 258:846–853. [PubMed: 6296087]
- Stahlberg H, Walz T. Molecular electron microscopy: state of the art and current challenges. *ACS Chem Biol.* 2008; 3:268–281. [PubMed: 18484707]
- van Benthem K, Lupini AR, Kim M, Baik HS, Doh SJ, Lee JH, Oxley MP, Findlay SD, Allen LJ, Pennycook SJ. Three-Dimensional Imaging of Individual Hafnium Atoms Inside a Semiconductor Device. *Appl Phys Lett.* 2005; 87:034104–1–3.
- Willingham MC I, Pastan H. Transit of epidermal growth factor through coated pits of the Golgi system. *J Cell Biol.* 1982; 94:207–212. [PubMed: 6288737]

Fig. 1.

Image acquisition using aberration-corrected three-dimensional (3D) scanning transmission electron microscopy (STEM). The electron beam is scanned in the lateral directions (x, y) at the focal plane (dashed line) across the cellular sample to record an image. Subsequently, the focus Δz is changed, and a second image is recorded. This process is repeated to obtain a 3D focal series. The cells are grown on a silicon nitride support window (solid line). Strong contrast is obtained on gold nanoparticles used as specific protein labels.

Fig. 2. Stage drift over a transmission electron microscopy (TEM) image series. (a) Overlay of two TEM images after irradiation doses of $10^3 \text{ e}^-/\text{nm}^2$ and $10^6 \text{ e}^-/\text{nm}^2$. Drift vectors are shown as arrows. (b) Coordinates of the reference objects in the TEM image series. The stage drifted approximately 229 nm. (c) Reference object coordinates after image alignment by lateral shifts only.

Fig. 3.

Linescan analysis of TEM images of critically point dried COS7 cells during irradiation series. (a) TEM image of a thin cellular region recorded with an electron dose of $10^2 \text{ e}^-/\text{nm}^2$. (b) TEM image of the same region after receiving a cumulative electron dose of $10^5 \text{ e}^-/\text{nm}^2$. (c) Signal versus position $S(x)$ of the linescan indicated by the dashed white line shown in images (a) and (b). Both profiles exhibit a high degree of correlation, with only a minimal decrease in signal intensity at the higher electron dose. (d) TEM image of a thick region in the same sample recorded with an electron dose of $10^2 \text{ e}^-/\text{nm}^2$. (e) TEM image of the same region shown in (d) after receiving a cumulative electron dose of $10^5 \text{ e}^-/\text{nm}^2$. (f) Profile of the linescan indicated by the dashed white line shown in images (d) and (e). The changes in the profile resemble structural changes corresponding to the two regions in the indicated by arrows in the TEM images.

Fig. 4.

Comparison of OsO₄ staining conditions in TEM images. (a) No OsO₄. (b) 40 nM OsO₄. (c) 40 mM OsO₄. Cellular features in the unstained control (a), such as the mitochondrial envelope, were indistinct in comparison to the samples treated with OsO₄ (b and c). However, a clear advantage in contrast or ultrastructure preservation was not observed between the samples stained with 40 nM (b) or 40 mM of OsO₄ (c).

Fig. 5.

The sample stability measure, mean ΔS_N , as function of the glutaraldehyde fixation time. TEM images were recorded after the samples had received an electron dose of $10^2 \text{ e}^-/\text{nm}^2$ and $10^5 \text{ e}^-/\text{nm}^2$, and analyzed for stability.

Fig. 6. TEM images of carbon coated critical point dried cells. (a) 10 nm carbon coating. (b) 20 nm carbon coating. (c) 50 nm carbon coating. Grain-like particles indicated by the arrows measured were observed for both the 20, and the 50 nm layer, but were not visible for 10 nm of carbon.

Fig. 7.

Evaluation of the sample stability obtained with carbon coating. (a) TEM image of a control (no carbon), recorded with an electron dose was $10^2 \text{ e}^-/\text{nm}^2$. (b) TEM image of the same region of the control sample after cumulative irradiation of $10^5 \text{ e}^-/\text{nm}^2$. The arrows indicate a region that underwent distortion. (c) TEM image a sample coated with 10 nm of carbon and recorded with an electron dose of $10^2 \text{ e}^-/\text{nm}^2$. (d) TEM image of the same region of the carbon coated sample after irradiation with a dose of $10^5 \text{ e}^-/\text{nm}^2$, demonstrating the high degree of structural preservation. (e) Stability measure, mean ΔS_N , of samples coated with different thicknesses of carbon. The stability was tested for two different cellular regions.

Fig. 8.

The sample stability, measured with ΔS_N , as function of the electron dose, separated by cellular region.

Fig. 9.

Sample stability during the recording of a STEM focal series of COS7 cells. (a) STEM image recorded prior to the acquisition of the focal series. The cellular structure is visible in grey. The round spots indicate gold nanoparticles. (b) Same region as shown in (a), images after the recording of the focal series. The cumulative electron dose was $3.6 \cdot 10^5 \text{ e}^-/\text{nm}^2$. (c) Intensity profiles of the linescan shown as dashed line in (a) and (b). The arrows indicate the regions in the STEM images indicated by #1 and #2. The dotted line in (a) indicates a structural feature that underwent a shift as observed in the profile in (c) at arrow #1.

Fig. 10.

STEM focal series of COS7 cells. The epidermal growth factor (EGF) receptor at the cellular surface with labeled with gold nanoparticles conjugated to EGF. (a) Projection of the images of the focal series on the lateral plane, showing both the ultrastructure and the nanoparticles. (b) The dataset was deconvolved to increase the axial resolution obtained on the nanoparticles. The image resembles the projection of the focal series on the lateral plane. (c) Axial profiles (intensity versus vertical position) over adjacent nanoparticles (#1 and #3) before deconvolution. (d) The plot profiles of the same linescan after deconvolution. (e) Histogram of the vertical locations of the gold nanoparticles with axial increments of 25 nm. The axial region that contained the largest number of gold nanoparticles was between 400 nm and 425 nm.

Fig. 11. STEM focal series of a COS7 cell recorded in a thick cellular region at the edge of the nuclear envelope (indicated by dashed line). (a) Projection of the focal series on the lateral plane. (b) Projection of the deconvolved dataset. A total of 9 gold nanoparticles is visible.

Axial Locations of Gold Nanoparticles at the edge of the cell (Figure 9). The precision in these numbers is ± 3 nm.

Table 1

Axial Locations of Gold Nanoparticles at the edge of the cell (Figure 9). The prec

Particle	Axial Position (nm)	Particle	Axial Position (nm)	Particle	Axial Position (nm)
1	260	11	385	21	443
2	265	12	393	22	450
3	282.5	13	395	23	468
4	313	14	400	24	468
5	318	15	405	25	470
6	330	16	408	26	483
7	338	17	415		
8	350	18	415		
9	353	19	423		
10	358	20	425		

Table II

Axial Locations of Gold Nanoparticles at the Nuclear Envelope (Figure 10). The precision in these numbers is ± 5 nm

Particle	Axial Position (nm)
1	1230
2	1335
3	760
4	800
5	645
6	605
7	650
8	665
9	790