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Determination of quantitative distributions of heavy-metal stain in biological specimens by annular dark-field STEM

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Abstract

It is shown that dark-field images collected in the scanning transmission electron microscope (STEM) at two different camera lengths yield quantitative distributions of both the heavy and light atoms in a stained biological specimen. Quantitative analysis of the paired STEM images requires knowledge of the elastic scattering cross sections, which are calculated from the NIST Elastic-Scattering Cross-Section Database. The results reveal quantitative information about the distribution of fixative and stain within the biological matrix, and provide a basis for assessing detection limits for heavy metal clusters used to label intracellular proteins. In sectioned cells that have been stained only with osmium tetroxide, we find an average of 1.2 ± 0.1 Os atom per nm^3 , corresponding to an atomic ratio of Os:C atoms of approximately 0.02, which indicates that small heavy atom clusters of Undecagold and Nanogold can be detected in lightly stained specimens.

Keywords

Scanning transmission electron microscopy; STEM; elastic scattering; cross sections; electron tomography; gold clusters; immunolabeling; heavy-metal staining

1. Introduction

Most biological specimens imaged in the electron microscope are prepared by plastic embedding and sectioning along with the use of heavy metal fixatives and stains. Such standard preparation methods have been used for routine histology and cell biology for more than fifty years. Although the use of stained plastic sections does not provide such reliable high-resolution detail about the native structure of cells that is attainable by using cryo-electron microscopy of frozen hydrated preparations, plastic sections are much easier to prepare and can be obtained from almost any cell type. The conventional staining technique and cryo-preparation each has its advantages and disadvantages (Al-Amoudi et al., 2004a; Al-Amoudi et al., 2004b; Baumeister, 2005; Hsieh et al., 2002; Marko et al., 2007; McDonald, 1994; McIntosh et al., 2005; Steven and Aeby, 2003). In particular, conventionally stained plastic sections provide strong contrast for imaging subcellular organelles, and such specimens are relatively resistant to damage in the electron beam, unlike frozen hydrated specimens, which

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must be imaged at low electron dose ($\sim 10^3$ e/nm²). In addition, some of the advantages of cryo-preparation are retained using the technique of freeze-substitution, in which the water in a vitrified frozen specimen is substituted with a solvent at low temperature and the resulting dehydrated cells or tissues are embedded, sectioned and stained in the standard way. Indeed, freeze-substitution has been extensively used in combination with electron tomography (ET) for accurate 3D imaging of cellular ultrastructure at 5-10 nm resolution (McEwen and Marko, 2001; McIntosh, 2001; McIntosh et al., 2005)

Nevertheless, it is not generally possible to identify directly specific macromolecular complexes in 3D using ET (Baumeister, 2002; Baumeister, 2005; Koster et al., 1997; McIntosh, 2001; McIntosh et al., 2005). This is because the pattern of stain does not exactly match the shape of the macromolecules to which the heavy atoms bind. Furthermore, granularity of the stain interferes with the high-resolution information and does not enable shapes of individual macromolecules to be accurately defined. Identification of specific protein complexes in plastic sections is therefore generally achieved through labeling with colloidal gold particles of diameter around 10 nm or smaller gold clusters attached to antibody fragments (Hainfeld, 1996; Hainfeld and Furuya, 1992; Robinson et al., 2000). The use of small gold clusters of diameter approximately 1 nm provides an important advantage over the use of the larger colloidal gold since the smaller clusters can penetrate the cell more readily.

Plastic sections must be stained to create contrast between the cellular ultrastructure and the surrounding resin material. Too much stain, however, can obscure the signal originating from the small gold clusters that are used to label specific proteins within the cells. On the other hand, too little stain can result in loss of structural context for the nanoparticle-labeled proteins, even though the visibility of the small clusters themselves is enhanced. In the face of these drawbacks, Ziese et al. (2002) and Sousa et al. (2007) have shown that STEM tomography can be used to detect heavy atom clusters of approximately 1 nm diameter in 3D without resorting to silver enhancement. However, no systematic study has been undertaken to establish detectability of non-silver enhanced metal clusters as a function of specimen thickness and level of staining.

Despite the important role that heavy-metal staining plays in the determination of cellular ultrastructure in both two and three dimensions, little is actually known about the absolute numbers of stain atoms in any given volume of a biological specimen. The purpose of this study is therefore to develop a method for quantifying the density of heavy atoms within stained plastic sections. Such measurements can provide a basis for determining the detectability of heavy atom clusters used to label specific proteins in cells. Furthermore, they can also be used to estimate the number of osmium tetroxide fixative molecules that bind per unit area of membrane since this is expected to depend on the unsaturated carbon bonds in lipid components of the membrane.

One approach for quantifying the amounts of specific elements in biological specimens is electron energy-loss spectroscopy (EELS) (Egerton, 1996; Leapman and Andrews, 1992). EELS, however, is not well suited to quantifying heavy elements like osmium, lead and uranium. This is because the M edges at energy losses in the range 2000-2500 eV are too weak to detect, and the N edges at energy losses in the range 20-60 eV are superimposed on a strong background arising from excitations of valence electrons in the organic matrix (Egerton, 1996). X-ray microanalysis is better suited than EELS for identifying and quantifying heavy elements in a light matrix (Williams and Carter, 1996; Watanabe and Williams, 2006), where the typical detection limits are of the order of 10^{-4} atomic fraction for a single spectrum acquired in 100 s. However, acquisition times would be prohibitively large when attempting to record images containing 10^6 pixels from large specimen areas. (LeFurgey et al., 1992).

Annular dark-field STEM imaging can also provide a means for the quantitative characterization of biological specimens. Previous work using quantitative ADF STEM imaging has been performed to determine the masses of biological molecules without resorting to calibration standards (Engel, 1978; Müller et al., 1992). More recently, we have also shown that ADF STEM imaging in a conventional 300 kV TEM can yield absolute mass determination of biological molecules (Sousa and Leapman, 2007). Another imaging mode in the STEM for quantitative measurements, developed in the 1980's, made use of combined elastic and inelastic electron scattering STEM measurements to determine distributions of the mean atomic number within a specimen (Bohrmann et al., 1993; Reichelt et al., 1985). This approach called Z-contrast is based on the principle that the total elastic scattering cross-section varies with the atomic number Z as approximately a $3/2$ power, whereas the total inelastic scattering varies approximately as a $1/2$ power, so that the ratio of the elastic to inelastic signals is proportional to Z (Colliex et al., 1984; Kellenberger et al., 1986). For example, Reichelt et al. (1985) explored the Z ratio-contrast technique to calculate the concentration of the biological components within unstained plastic sections. Ratio-contrast was also employed by Bohrmann et al. (1993) to quantify the DNA packing density within chromatin in unstained plastic sections. The Z-contrast technique has been largely abandoned in recent years because the older style EELS systems, incorporating photomultiplier tubes to detect the spectrum in series, have been replaced by EELS systems incorporating charge-coupled devices (CCDs) that detect the spectrum in parallel (Egerton, 1996). Although highly beneficial for spectroscopy, CCDs cannot be read out with sufficient speed to be used conveniently in Z-contrast imaging. An absolute quantification of the number of heavy atoms within a biological specimen would be in principle feasible with the Z-contrast ratio technique as it was implemented in the 1980's.

Here, we show that a closely related approach to the Z-contrast technique (Andree et al., 1985) can be also utilized to determine the number of heavy atoms in a biological sample. Specifically, we demonstrate that acquisition of two STEM images, each recorded with a distinct range of ADF scattering angles, allows us to determine the absolute number of heavy atoms as well as the number of light atoms in plastic embedded, sectioned cells that have been fixed and stained with heavy atoms.

2. Basis for the quantification of light and heavy atoms in plastic sections

For a sufficiently thin biological specimen containing heavy elements, the fraction $I(\beta)$ of the incident electron beam that is deflected onto the ADF detector can be approximated as

$$I(\beta) = I_{\text{inc}} N_l [\sigma_e(\beta)_l + \sigma_i(\beta)_l] + I_{\text{inc}} N_h [\sigma_e(\beta)_h + \sigma_i(\beta)_h] \quad (1)$$

Where, N_l is total number of light atoms (excluding hydrogen) in the organic matrix per unit area; N_h is the total number of heavy atoms per unit area; β is the collection semi-angle subtended by the ADF detector; I_{inc} is the number of incident electrons; $\sigma_e(\beta)_l$ and $\sigma_i(\beta)_l$ are, respectively, the average elastic and inelastic scattering cross sections characteristic of the light atoms in the organic matrix; and $\sigma_e(\beta)_h$ and $\sigma_i(\beta)_h$ are, respectively, the average elastic and inelastic scattering cross sections characteristic of the heavy atoms. In Eq. 1 we have assumed that the convergence angle of the incident beam is zero; later we will introduce the effect of a finite convergent beam into the calculations. Eq. 1 can be simplified by considering that elastic scattering events for heavy elements are much more frequent than inelastic events, particularly at large scattering angles. We thus neglect the term $\sigma_i(\beta)_h$ and rewrite Eq. 1 as

$$I(\beta) = I_{\text{inc}} N_l \sigma_t(\beta)_l + I_{\text{inc}} N_h \sigma_e(\beta)_h \quad (2)$$

where, for simplicity, the terms $\sigma_e(\beta)_l$ and $\sigma_i(\beta)_l$ were combined as

$$\sigma_e(\beta)_l + \sigma_i(\beta)_l = \sigma_l(\beta)_l \quad (3)$$

For a stained and osmium-fixed plastic-embedded cell the characteristic average elastic scattering cross section $\sigma_e(\beta)_h$ is given as an arithmetic mean of the cross sections of all the heavy atoms present in the specimen (typically Os, Pb and U):

$$\sigma_e(\beta)_h = \sum_j \frac{\sigma_e(\beta)_{h,j}}{n} \quad (4)$$

where $\sigma_e(\beta)_{h,j}$ is the elastic scattering cross section for a specific heavy element j and n is the total number of heavy-atom species in the specimen. We shall show that uncertainties in the determination of $\sigma_e(\beta)_h$ introduce only a small error in the calculation of the numbers of heavy and light atoms in the specimen.

$\sigma_e(\beta)_{h,j}$ in Eq. 4 is obtained by integrating the differential elastic scattering cross section over angles greater than the inner collection angle β_{inner} and less than the outer collection angle β_{outer} of the ADF detector.

$$\sigma_e(\beta)_{h,j} = 2\pi \int_{\beta_{inner}}^{\beta_{outer}} \frac{d\sigma_e(\theta)_{h,j}}{d\Omega} \sin(\theta) d\theta \quad (5)$$

where Ω is solid angle.

The characteristic average elastic scattering cross section $\sigma_e(\beta)_l$ is given as the weighted average of the cross sections of the light atoms that constitute the organic matrix:

$$\sigma_e(\beta)_l = \sum_j f_j \sigma_e(\beta)_{l,j} \quad (6)$$

where f_j and $\sigma_e(\beta)_{l,j}$ are, respectively, the atomic fraction and elastic scattering cross section of element j present in the organic matrix. To estimate the atomic fractions f_j of the light atoms in the specimen, we consider that a plastic embedded cell consists of approximately 30% protein and 70% resin (Bohrmann et al., 1993). We assume that a typical protein has an amino acid composition corresponding to the average of the Swiss Protein Database (Bairoch and Apweiler, 2000), which gives atomic concentrations (excluding hydrogen and sulfur) of 61% C, 19% N and 20% O. Finally, we assume the composition of the resin material to be 83% C and 17% O (Reichelt et al., 1985). Using the above numbers we arrive at an average composition for the organic matrix in a plastic-embedded cell of 76.4% C, 5.7% N and 17.9% O. Determination of $\sigma_e(\beta)_{l,j}$ for C, N and O is made using an equation analogous to Eq. 5.

In this study, theoretical values of the differential elastic scattering cross sections for each light

and heavy atom in the specimen, $\frac{d\sigma_e(\theta)_{l,j}}{d\Omega}$ and $\frac{d\sigma_e(\theta)_{h,j}}{d\Omega}$, respectively, were obtained from the NIST Electron Elastic-Scattering Cross-Section Database (Jablonski et al., 2003). This

database provides differential elastic scattering cross sections that are calculated from relativistic Dirac partial-wave analysis using atomic potentials derived from Dirac-Hartree-Fock electron densities.

The term $\sigma_i(\beta)_l$ in Eq. 3 is given by

$$\sigma_i(\beta)_l = 2\pi \int_{\beta_{\text{inner}}}^{\beta_{\text{outer}}} \frac{d\sigma_i(\theta)_l}{d\Omega} \sin(\theta) d\theta \quad (7)$$

The differential inelastic scattering cross section characteristic of the light matrix, $\frac{d\sigma_i(\theta)_l}{d\Omega}$, is calculated according to the formulation described by Wall et al. (1974) for scattering angles smaller than the cut-off at θ_o , which is defined as $(\bar{E}/E_0)^{1/2}$ with $\bar{E}$ being the average energy loss of the electrons after traversing the sample and E_0 the energy of the incident electrons.

Above the cut-off angle θ_o , we adjust $\frac{d\sigma_i(\theta)_l}{d\Omega}$ to follow asymptotically the form θ^{-4} .

For an incident beam with a finite convergence angle α , the integrated differential elastic and inelastic scattering cross sections are modified to take into consideration the angular distribution of the incident beam. This is done by calculating the differential cross section's

dependence on convergence angle, $\frac{d\sigma(\theta)^*}{d\Omega}$, according to the convolution equation (Egerton, 1996):

$$\frac{d\sigma(\theta)^*}{d\Omega} = \frac{d\sigma(\theta)}{d\Omega} \otimes \frac{dD(\theta)}{d\Omega} \quad (8)$$

where $D(\theta) = 1$ for $\theta < \alpha$, and $D(\theta) = 0$ for $\theta \geq \alpha$. Taking into account the convergence angle of the illumination, Eq. 2 can then be rewritten as

$$I(\alpha, \beta) = I_{\text{inc}} N_l \sigma_i(\beta)_l' + I_{\text{inc}} N_h \sigma_e(\beta)_h' \quad (9)$$

Determining the number of incident electrons I_{inc} in Eq. 9 can be easily accomplished by acquiring an image of the defocused probe over a hole in the specimen with a CCD camera whose conversion efficiency had been previously calibrated. The final term to be defined in Eq. 9, $I(\alpha, \beta)$, which corresponds to the number of scattered electrons onto the ADF detector, could in principle be found by dividing the number of counts at each pixel in a STEM image, $C(\alpha, \beta)$, by the conversion efficiency ε of the ADF detector, i.e. $I(\alpha, \beta) = C(\alpha, \beta) / \varepsilon$. However, ε is not constant across the detector and therefore this simple relation cannot be applied to find $I(\alpha, \beta)$. The observation that conversion efficiency of the ADF detector is not constant with position has also been reported by Singhal et al. (1997). For now, however, we assume that ε is constant across the detector, and in the next section we return to the case of a non-uniform ε .

After experimentally measuring I_{inc} and $I(\alpha, \beta)$ and calculating $\sigma_e(\beta)_h'$, $\sigma_i(\beta)_l'$ and $\sigma_e(\beta)_h'$ as described above, we can determine the number of heavy atoms per unit area in a biological specimen from Eq. 9:

$$N_h = \frac{I(\alpha, \beta) - I_{inc} N_l \sigma_t(\beta)_l'}{I_{inc} \sigma_h(\beta)_h'} \quad (10)$$

Eq. 10, however, is a function of the total number of light atoms per unit area in the organic matrix, N_l . While N_l could be estimated if the thickness and density of the matrix were accurately known, an alternative approach is desirable, in which both N_l and N_h are calculated from experimental data. This is indeed possible if, instead of collecting a single ADF STEM image at a given detector inner semi-angle, two ADF STEM images are collected each at a different inner semi-angle. Thus, for an inner collection semi-angle β_1 Eq. 9 can be rewritten as

$$I(\alpha, \beta_1) = I_{inc} N_l \sigma_t(\beta_1)_l' + I_{inc} N_h \sigma_e(\beta_1)_h' \quad (11)$$

and for an inner semi-angle β_2 as

$$I(\alpha, \beta_2) = I_{inc} N_l \sigma_t(\beta_2)_l' + I_{inc} N_h \sigma_e(\beta_2)_h' \quad (12)$$

Eqs. 11 and 12 are simultaneous equations whose unknown variables are the total number of light and heavy atoms per unit area. Solving for these two variables gives the following two expressions for N_l and N_h :

$$N_l = \frac{I(\alpha, \beta_2) \sigma_e(\beta_1)_h' - I(\alpha, \beta_1) \sigma_e(\beta_2)_h'}{I_{inc} [\sigma_e(\beta_1)_h' \cdot \sigma_t(\beta_2)_h' - \sigma_e(\beta_2)_h' \cdot \sigma_t(\beta_1)_l']} \quad (13a)$$

$$N_h = \frac{I(\alpha, \beta_2) \sigma_t(\beta_1)_l' - I(\alpha, \beta_1) \sigma_t(\beta_2)_l'}{I_{inc} [\sigma_e(\beta_2)_h' \cdot \sigma_t(\beta_1)_l' - \sigma_e(\beta_1)_h' \cdot \sigma_t(\beta_2)_l']} \quad (13b)$$

3. Experimental

Specimen preparation

The cell that we choose to test our methods for determining the relative numbers of light and heavy atoms is the smallest known eukaryote, *Ostreococcus tauri*. *O. tauri* is a plant cell of diameter around 1-2 μm . Specimens were prepared by immersion in a cryopreservative solution consisting of 15% sucrose, and freezing the mixture in a BAL-TEC HPM-010 high-pressure freezing machine. The frozen blocks were processed at low temperature in a Leica EM-AFS freeze-substitution system using a solution of acetone containing 1.0% osmium tetroxide. After freeze-substitution for 3 days, the specimens were gradually warmed to room temperature and embedded in Epon-Araldite by graded exchange of the acetone. Specimens were polymerized in 100% resin by heating to 60°C for 2 days, sectioned to a nominal thickness of 30-40 nm with a Leica Ultracut E ultramicrotome, and mounted on 300 mesh Quantifoil grids. Some of the sections were stained with lead citrate for 5 min followed by staining with uranyl acetate for another 5 min. After each staining step the sections were washed nine times in three 50 ml volumes of de-ionized water.

Acquisition of STEM images

STEM images were acquired with a Tecnai TF30 transmission electron microscope (FEI, Hillsboro, OR, USA) operating at an acceleration voltage of 300 kV and equipped with a Shottky field-emission gun. This microscope is fitted with a Model 3000 in-column HAADF detector (Fischione, Export, PA, USA) situated after the projection-lens system and above the viewing screen. The HAADF detector incorporates a single crystal yttrium aluminum perovskite scintillator optically coupled to a photomultiplier tube. Selected specimen regions from both unstained and stained osmium-fixed *O. tauri* were first pre-exposed to a broad electron beam in TEM mode in order to stabilize shrinkage of the plastic section. STEM images were then collected from these regions with an electron dose per pixel of 2.5×10^4 e/nm². The image dimensions were 2048×2048 pixels and the pixel size 0.87 nm. The convergence semi-angle of the illumination was 12 mrad. For each selected specimen region two STEM images were acquired, one with an ADF detector inner semi-angle of 16 mrad, and another with a detector inner semi-angle of 36 mrad. The outer semi-angle of the ADF detector was five times the inner angle. Control over the collection angles was achieved by varying the magnification of the diffraction pattern (camera length).

Clearly, the value of the small inner detector semi-angle, β_1 , must be necessarily higher than the convergence semi-angle of the illumination, α (in our case $\beta_1 = 16$ mrad). For a fixed value of β_1 , the semi-angle β_2 should be made large enough so that the difference in the scattering cross-sections between light and heavy atoms is amplified. If β_1 and β_2 are chosen to be too close together (e.g. 16 and 25 mrad), then precision in determining N_l and N_h is reduced, as is evident from Eq. 13. From simulations, we have found that 16 and 36 mrad are reasonable choices for the angles β_1 and β_2 , although β_2 could in principle be made as large as possible.

STEM tomography

The same specimen regions of *O. tauri* selected for 2D STEM imaging were also used to acquire STEM tomographic tilt series. Prior to image collection, 10-nm fiducial markers were deposited on both sides of the sections. The tilt series were acquired from -60° to $+60^\circ$ with steps of 2° , with a pixel size of 0.87 nm, with an integral electron dose per pixel of 4×10^4 e/nm², and with a fixed ADF detector inner semi-angle of 16 mrad. Data were collected by means of the FEI STEM tomography software. To perform the 3D reconstruction, tilt series were first aligned with the IMOD computer program (Kremer et al., 1996) using gold fiducial markers and reconstructed, also in IMOD, by weighted back-projection (Radermacher, 2006). The reconstructed tomograms were then used to determine the thickness of the selected specimen regions. Finally, an additional STEM tomographic tilt series was acquired from a stained osmium-fixed *O. tauri* cell. The parameters used to acquire the tomographic tilt series were identical to those above except the pixel size of the images was 0.43 nm and the integral dose was 1.7×10^5 e/nm². Alignment of the tilt series was done using fiducial markers in IMOD and tomographic reconstruction was performed using the simultaneous iterative reconstruction tomography (SIRT) algorithm (Gilbert, 1972; Herman, 1980) implemented in our lab (Aronova et al., 2007). After alignment and prior to reconstruction, the fiducial markers were removed from each tilt image to eliminate the presence of ghost streaks in the tomogram (Sousa et al., 2007). The SIRT-reconstructed tomogram was then used to estimate the distribution of stain atoms in 3D.

Accounting for the conversion efficiency of the ADF STEM detector

In order to take into account the non-uniform conversion efficiency of the Fischione ADF STEM detector in the calculation of N_l and N_h from Eq. 13, we implement a scheme similar to the one described by Singhal et al. (1997). First, using the diffraction shift coils, the incident beam is shifted directly onto the inner edge of the ADF detector and scanned across a hole in the specimen to acquire an image. During this procedure the intensity of the beam must be

small enough to avoid saturation of the ADF detector. Then, a positive (zero-current) offset is subtracted from the STEM image intensity, and the resulting average number of counts that is accumulated per pixel is divided by the number of electrons in the incident beam:

$$\varepsilon_{\text{inner}} = (I - I_{\text{offset}}) / I_{\text{inc}}. \quad (14)$$

where, $\varepsilon_{\text{inner}}$ is the conversion efficiency of the ADF detector at its inner edge, I is the average image intensity per pixel in the STEM image, I_{offset} is the offset signal, and I_{inc} is the number of electrons in the incident beam.

Next, the undiffracted beam is shifted across the ADF detector from its inner edge to its outer edge, while scanning the beam over a hole and acquiring a STEM image. Fig. 1 shows a line profile across the STEM image obtained with this procedure, after subtracting an intensity offset. In Fig. 1, the number of counts at the inner edge of the ADF detector was normalized to 1 and the x coordinate was scaled to range from 16 to 80 mrad. It is observed that the normalized conversion efficiency of the ADF detector, $\varepsilon_{\text{norm}}(\theta)$, increases abruptly at the detector's inner radius, after which it increases slowly up to the detector's outer radius. The functional form of $\varepsilon_{\text{norm}}(\theta)$ is used in the calculation of the ADF detector conversion efficiency – corrected for the detector's non-uniformity – according to:

$$\varepsilon_{\text{corr}} = \varepsilon_{\text{inner}} \cdot 2\pi \int_{\beta_{\text{inner}}}^{\beta_{\text{outer}}} \varepsilon_{\text{norm}}(\theta) \frac{d\sigma(\theta)}{d\Omega} \sin(\theta) d\theta \quad (15)$$

The number of scattered electrons impinging on the ADF detector, $I(\alpha, \beta)$, is then found from the measured number of counts $C(\alpha, \beta)$ using the relation

$$I(\alpha, \beta) = C(\alpha, \beta) / \varepsilon_{\text{corr}}. \quad (16)$$

In Eq. 15, we use a differential elastic scattering cross section $\frac{d\sigma(\theta)}{d\Omega}$ characteristic of the heavy atoms in our specimens since they make a larger contribution to the ADF signal than do the light atoms.

Although the conversion efficiency of the ADF STEM detector depends on the specific settings of the detector contrast, the normalized functional form of the conversion efficiency, $\varepsilon_{\text{norm}}(\theta)$, is essentially independent of the specific contrast values. Thus, $\varepsilon_{\text{inner}}$ is the only parameter that must be effectively recalculated whenever different values of detector contrast are selected.

4. Results and Discussion

Quantification of Nanogold onto a 20 nm-thick carbon film

Here we use a specimen of Nanogold particles deposited onto a 20 nm-thick carbon film to test whether the proposed method gives a reasonable value for the gold content of a Nanogold particle, which is expected to be ~ 67 atoms (Hainfeld and Powell, 2000). To calculate the number of gold atoms, N_{Au} , we applied Eq. 13 to the integrated number of counts within several Nanogold particles (within 7×7 windows, pixel size = 0.3 nm) from two ADF STEM images recorded with different collection angles. N_{Au} was found by multiplying N_{h} obtained from Eq. 13 by the pixel area of the images (0.09 nm^2). We also determined the number of gold atoms

within several 7×7 pixel windows located in regions of pure carbon. The results are shown as a histogram in Fig. 2. We can observe from Fig. 2 the presence of two peaks, one centered between 60 and 70 atoms – corresponding to the calculated number of gold atoms within Nanogold – and another centered between 0 and 10 atoms – obtained from regions of pure carbon. The second peak has an associated average number of gold atoms of 63 ± 13 , in good agreement with the known number of gold atoms within Nanogold particles (~ 67 atoms). The average number of gold atoms obtained within regions of background was found as 6 ± 6 atoms. The standard deviation in the number of gold atoms in the background (6) gives an indication of the noise level in these measurements.

Using Eq. 13, we also determined the number of carbon atoms within the same 2.1×2.1 nm² regions for which N_{Au} was calculated. Our results gave a total number of 7600 carbon atoms associated with regions of pure carbon, and 8250 carbon atoms within regions containing Nanogold particles. Using TMV as a mass calibration standard, we found that 2.1×2.1 nm² regions of pure carbon have approximately 6500 carbon atoms. However, due to the oxygen and nitrogen content of TMV, we estimate that its average elastic scattering cross section is approximately 1.2 times higher than that of pure carbon (calculated for the specific experimental parameters that we used). Thus, we find that using TMV as a calibration standard gives a final number of ~ 7800 carbon atoms within the 2.1×2.1 nm² regions, which is in good agreement with the values we found using Eq. 13.

Quantification of osmium in plastic-embedded sections

We apply the method outlined in this paper to determine the total numbers of light atoms (excluding hydrogen) and heavy osmium atoms within unstained osmium-fixed plastic-embedded sections of *O. tauri*. An ADF STEM image of *O. tauri* (Fig. 3) shows that fixation with osmium is sufficient to reveal some level of structural detail. In particular, the thylakoid stacks of the chloroplast, and the cell nucleus are easily visible. To determine N_l and N_h , we first acquired two ADF STEM images each with a distinct detector inner semi-angle (Fig. 4). The number of counts $C(\alpha, \beta)$ at each pixel in Figs. 4a and 4b were converted to $I(\alpha, \beta)$ according to Eq. 16 and used in Eq. 13 to generate maps of the distribution of light and heavy atoms (Fig. 5). It is observed in Fig. 5a that there is minimal contrast from the light atoms in *O. tauri*. On the other hand, the distribution of heavy atoms within *O. tauri* exhibits much stronger contrast (Fig. 5b). These observations are explained by essentially all of the observed contrast in plastic-embedded biological specimens being due to heavy atoms that are introduced as fixatives and staining agents. We attribute the residual contrast in the number of light atoms within the thylakoid stacks (Fig. 5a), among other things, to a subpixel misalignment between the two ADF STEM images in Fig. 4.

We can obtain a quantitative measure of the number of light and heavy atoms per nm² in Fig. 5 by taking line profiles across any region of interest. For example, Figs. 6a and 6b show the distribution of light and heavy atoms along a 40 nm-wide line profile across the chloroplast. A line profile across the same region in the ADF STEM image acquired at 36 mrad detector inner semi-angle (Fig. 4b) is also shown in Fig. 4c. The average number of light and heavy atoms/nm² within the thylakoid stacks in the chloroplast from Fig. 6 is 2213 ± 91 atoms/nm² and 38 ± 3.6 ($\pm$ s.d) atoms/nm², respectively. Fig. 6b also reveals that a residual number of about 5 osmium atoms/nm² is associated with regions of the section containing pure resin. We observe that the oscillations in the line profile in Fig. 6b follow approximately the same pattern as those in Fig. 6c, which is consistent with the contrast in Fig. 4b being mostly due to the presence of osmium.

We observe that the noise in the line profile in Fig. 6b is higher than in Fig. 6c. This is expected since the calculation of N_h involves taking the difference (weighted by the appropriate cross sections) between two images (Eq. 13). Furthermore, misregistration between the two original

images will also increase the noise in the calculated atomic distributions. To reduce noise in the processed images, the original ADF STEM images could be binned and/or pre-processed with a low-pass filter, at the expense of decreasing resolution.

The measured value of ~ 2200 light atoms/nm² (excluding hydrogen) within the thylakoid stacks in *O. tauri* enables us to estimate the specimen thickness. Assuming a density for the plastic section of 1.25 g/cm³ (Reichelt et al., 1985) and an average atomic mass of 13 g/mol for the light atoms excluding hydrogen (according to the assumed matrix composition) gives an estimated local thickness value of 38 nm. We also measured the thickness of the same thylakoid stacks shown in Fig. 4 from a reconstructed STEM tomographic tilt series. This was accomplished by measuring the distance between 10 nm fiducial gold markers deposited on both sides of the section. The thickness measurement of 32 nm obtained from the reconstructed tomogram was consistent with the estimated value of 38 nm obtained from the scattering measurements. The small discrepancy between calculated and measured thickness values could be partly due to an increased density of the plastic section with shrinkage. Interestingly, the measured thickness in the regions of pure plastic was considerably smaller than that of the regions containing cellular material. Thus, a thickness of 22 nm was obtained from the reconstructed tomogram in agreement with a value of 20 nm calculated from the measured local number of light atoms/nm² (Fig. 6a). The fact that regions of pure plastic are thinner than regions containing cellular material explains the darker contrast outside of the boundaries of *O. tauri* in the image displaying the distribution of light atoms (Fig. 5a).

As it is clear from Fig. 4, the average number of 38 ± 3.6 osmium atoms/nm² within *O. tauri* is sufficient to outline structural detail in the cell. Fig. 7 shows a magnified view of a thylakoid stack from the raw ADF STEM image in Fig. 4b as well as a line profile across this stack. The dotted lines in Fig. 7 match specific features in the line profile to osmium-rich regions across the thylakoid stack. We can observe from Fig. 7 a maximum number of 48 osmium atoms/nm² within the thylakoid membranes. It can be also seen from Fig. 7 that the highest contrast between a thylakoid membrane and its immediate vicinity stems from a variation in only ~ 14 atoms of osmium/nm². The quantitative line profile across the thylakoid membranes also enables us to make a rough estimate of the number of osmium atoms bound per unit area of membrane. For this, we assume that a thylakoid membrane is a thin sheet 5-nm wide perpendicular to the xy plane of the specimen. Furthermore, we assume that the membrane extends all the way along the z axis of the specimen, i.e. all the way from the top to the bottom of the section. From Fig. 7, we obtain 48 atoms of osmium/nm² (in the xy plane) within a single thylakoid membrane. To calculate the number of osmium atoms per unit area of this membrane (in the xz plane), we multiply the 48 atoms of osmium/nm² by the width of the membrane (5 nm) and divide the result by the thickness of the section (~ 40 nm before shrinkage). We therefore arrive at a value of 6 atoms of osmium per square nanometer of membrane.

It is important to consider the average number of osmium atoms per unit volume, since this plays a direct role on the detectability of ultrasmall gold labels in 3D. For the specimen that we consider here, there are 38 osmium atoms/nm² and a specimen thickness of ~ 32 nm gives rise to approximately 1.2 osmium atoms/nm³. This rather small number of atoms per unit volume should be adequate to detect small labels such as Nanogold that contain ~ 67 atoms of gold in a sphere of 1.4 nm in diameter. It is conceivable, however, that the distribution of osmium atoms in 3D might not be uniform and that small granules could be formed during fixation and/or during electron irradiation that are of the size of Nanogold. We will address the formation of granules of heavy atoms later when we analyze the case of stained osmium-fixed *O. tauri*. Nevertheless, we believe that the average concentration of 1.2 osmium atoms/nm³ is small enough to detect nanometer diameter heavy atom labels, and even some coalescence of dispersed osmium atoms during irradiation could still allow detection of the nanoscale labels.

Quantification of osmium, lead, and uranium in plastic-embedded sections

We now consider the total number of light and heavy atoms (N_l and N_h , respectively) within osmium-fixed sections of *O. tauri*, which have also been stained with uranium and lead salts. The ADF STEM image in Fig. 8 reveals that the addition of stain has increased significantly the contrast of cellular structure, compared with the contrast observed in sections that have been only osmicated (Fig. 3). In particular, the thylakoid stacks appear better delineated; part of the Golgi complex becomes visible; and ribosomes are evident throughout the cell. To determine the number of light and heavy atoms from a specified region within *O. tauri*, we acquired two ADF STEM images with distinct detector inner semi-angles (Fig. 9). The number of counts $C(\alpha, \beta)$ at each pixel in both these images were converted to $I(\alpha, \beta)$ according to Eq. 16 and used in Eq. 13 to generate maps of the distribution of light and heavy atoms (Fig. 10). As in the unstained osmium-fixed *O. tauri*, contrast originating from the distribution of light atoms is minimal in the stained preparation (Fig. 10a). Conversely, Fig. 10b shows strong contrast originating from the distribution of heavy atoms.

20-nm-wide line profiles displaying the number of light and heavy atoms across the thylakoid stacks in Figs. 10a and 10b are shown in Figs. 11a and 11b, respectively. A line profile across the corresponding region in the ADF STEM image of Fig. 9b is also shown in Fig. 11c. Clearly, the oscillations in the line profile in Fig. 11b are almost identical to those in Fig. 11c. The average number of light and heavy atoms/nm² within the thylakoid stacks can be found from Fig. 11 and is given as 2056 ± 250 ($\pm$ s.d.) atoms/nm² and 109 ± 19 atoms/nm², respectively. Based on the average number of light atoms per unit area and on the density of Epon, the thickness of the specimen was estimated as 36 nm. This was close to a thickness value of 31 nm obtained from a reconstructed STEM tomogram of the same cell. The average amount of 109 ± 19 heavy atoms/nm² gives rise to thylakoid stacks with very high contrast (Fig. 9). In order to look closer at the thylakoid membranes and at the number of heavy atoms associated with them, we took a magnified view of two thylakoid stacks from Fig. 9 and traced a line profile across them (Fig. 12). The dotted lines in Fig. 12 match specific features in the line profile to stain-rich regions across the thylakoid stacks. Fig. 12 reveals a maximum number of 134 stain atoms/nm² within the thylakoid membranes. We can also observe from Fig. 12 that the highest and lowest contrast between a thylakoid membrane and its immediate vicinity stem from variations in 40 and 10 stain atoms/nm², respectively.

As we have already indicated, it is useful to estimate the number of stain atoms per unit volume since these atoms limit the visibility of ultrasmall gold labels in 3D reconstructions. From the ~ 110 stain atoms/nm² within *O. tauri* and from the specimen thickness of ~ 31 nm, we arrive at approximately 3.6 stain atoms/nm³. Although the atoms of stain are on average relatively sparsely distributed within the specimen volume, we shall show that the atoms of stain can coalesce locally into clusters that are commensurate in size to the gold nanoparticle labels.

3D quantification of osmium, lead and uranium

In the above 2D analysis, two STEM images with distinct ADF collection angles were used in the calculations of N_l and N_h . Having determined the number of light atoms and having shown them to be distributed approximately homogeneously within *O. tauri*, we now determine the 3D distribution of heavy atoms from a single STEM tomogram acquired with a detector inner semi-angle of 16 mrad. Fig. 13a shows an image of a stained osmium-fixed *O. tauri* cell. We acquired a STEM tomographic tilt series from this cell and performed reconstruction with SIRT. As demonstrated by Aronova *et al.* (2007), reconstruction with SIRT enables generation of tomograms whose projected voxel intensities match the pixel intensities of the original tilt images. Quantitative information is therefore retained within such tomograms.

A 4-pixel slice (1.72 nm) from the reconstructed tomogram of *O. tauri* is displayed in Fig. 13b. In order to estimate the 3D distribution of heavy atoms within the cell, we first integrate the number of counts within 200 randomly distributed regions in the reconstructed tomogram. By selecting regions containing 4^3 voxels, i.e. 5 nm^3 volumes, we obtain the histogram in Fig. 14a. According to the results of the previous section, our plastic-embedded sections of stained osmium-fixed *O. tauri* contain ~ 2050 and 110 light and heavy atoms/ nm^2 , respectively. These values indicate that 70% of the STEM signal for an ADF inner angle of 16 mrad is due exclusively to the presence of the heavy atoms. Thus, we multiply the total integrated counts within the 5 nm^3 regions by 0.7 to obtain the integrated counts originating from the heavy atoms. Finally, we rescale the resulting histogram of counts so that the mean of the distribution corresponds to the average number of heavy atoms within the 5 nm^3 volumes. For a pixel size of 0.43 nm containing 110 stain atoms/ nm^2 , and a specimen thickness of 31 nm (Fig. 13c), there are approximately 18 heavy atoms per 4^3 -voxel region (5 nm^3), as shown by the histogram in Fig. 14b. The histogram indicates that a small heavy-metal cluster containing a sufficiently high number of atoms should be distinguishable from the background stain. For example, it should be possible to visualize in the presence of stain a Nanogold cluster containing around 67 atoms.

A magnified view of the reconstructed tomogram (Fig. 13d), however, reveals that the distribution of heavy-metal stain within *O. tauri* is not uniform throughout. In particular, in Fig. 13d we observe the coalescence of stain atoms to form granules of a size commensurate with that of the Nanogold cluster (Hainfeld et al., 1994). We have integrated the number of counts within 100 different 5 nm^3 regions containing such granules and, using the same approach as above, obtained a histogram of the total number of stain atoms within the granules. Fig. 14c shows that the stain granules contain an average of 42 and as many as 52 atoms. Clearly, the coalescence of heavy atoms of stain suggests that small gold clusters like Nanogold would be difficult to distinguish. To avoid development of stain granularity it would be necessary to collect data under low dose conditions. However, this would be difficult to achieve because relatively high electron doses are needed to image small heavy-metal clusters in the presence of stain with sufficient signal-to-noise ratio. It might be preferable to avoid the use of uranium, and possibly lead, and to rely exclusively on osmium as a contrast-enhancing agent of cellular ultrastructure. It might also be feasible to employ lower atomic number stains based on vanadium and molybdenum.

It is important to point out that the atomic density of gold clusters (e.g. Nanogold) is typically higher than what we have measured for the stain. Namely, we have measured a maximum of ~ 50 atoms within 5 nm^3 volumes (4^3 voxels), whereas Nanogold contains 67 atoms within $\sim 1.5 \text{ nm}^3$. In principle, therefore, it should be possible to distinguish Nanogold from coalesced stain by comparing the numbers of gold and stain atoms within volumes smaller than 5 nm^3 . In a real labeling experiment, however, the possibility of incorrectly assigning stain granules as being gold clusters would lead to erroneous conclusions about the specific location of the protein which is being labeled. We therefore have adopted above a more conservative approach and compared the numbers of gold and stain atoms within 4^3 voxels which are approximately of the same diameter of Nanogold (1.4 nm).

Accuracy of measurements

In the calculations of N_l and N_h we have used an average elastic cross section for the organic matrix assuming an average matrix atomic composition of $76.4\% \text{ C}$, $5.7\% \text{ N}$ and $17.9\% \text{ O}$. However, since we did not record our data under low dose conditions, the true matrix composition might have significantly deviated from the one we used. We checked the effect of different matrix compositions on the calculation of N_l and N_h for stained osmium-fixed *O. tauri*. For instance, assuming a matrix composition of $60\% \text{ C}$ - $40\% \text{ O}$ changed the calculated

N_l and N_h by less than 5%. The same result was obtained when a matrix composition of 90% C-10% O was assumed. From these results we conclude that a reasonably accurate determination of N_l and N_h is possible even though the composition of the organic matrix might be only approximately known. We also checked the effect of different elastic scattering cross sections for the stain, $\sigma_e(\beta)_h$, on the determination of N_l and N_h . In our analysis $\sigma_e(\beta)_h$ was calculated from an arithmetic mean of the individual cross sections for Os, Pb and U. Using cross sections for pure Os and U atoms changed the original calculated N_l and N_h values by only 10% at most. We thus conclude that estimating $\sigma_e(\beta)_h$ with an arithmetic mean of the heavy atoms in the specimen suffices for a reasonably accurate determination of N_l and N_h .

In Fig. 1 we showed a graph displaying the normalized conversion efficiency of the ADF detector with position. The non-uniform conversion efficiency of the detector was taken into account in the calculations of N_l and N_h according to Eq. 15. Clearly, it would be desirable to have ADF STEM detectors with uniform conversion efficiency. In that case, the ADF STEM image intensities could be readily recalibrated in terms of number of collected electrons, as is possible for images acquired with CCD cameras. Such a capability combined with an automatic measurement of the incident beam current would significantly simplify the use of the STEM microscope for quantitative measurements and would potentially enable more accurate data to be obtained.

Finally, situating an ADF STEM detector after an energy filter provides an attractive configuration for the types of quantitative measurements that we have described. The idea of performing energy-filtered ADF STEM has been proposed by a number of authors, e.g., in the context of eliminating thickness fluctuations in organic matrices by ratio-contrast imaging (Egerton, 1982; Haider, 1989). In the context of the present work, removing the contribution of inelastically scattered electrons from ADF STEM images would simplify the calculations since only elastic scattering cross sections would be needed. Furthermore, filtering out inelastically scattered electrons would enable a more accurate determination of the number of atoms per unit area since no mixed elastic-inelastic scattering events would contribute to the ADF signal. In any case, a simple quantitative analysis of ADF STEM images would still need to be confined to thin specimens to avoid a significant contribution of double elastic scattering events to the STEM signal.

5. Conclusions

We have shown that by collecting dark-field STEM images at two different camera lengths it is possible to determine the numbers of heavy and light atoms in a stained biological plastic section. The method requires values for the elastic scattering cross sections, which are calculated from the NIST Elastic-Scattering Cross-Section Database for the two different ranges of scattering angles. Analysis of the STEM data provides quantitative information about the distribution of stain within a biological specimen, and will be important for testing the feasibility of detecting heavy metal clusters like Nanogold and Undecagold that are attached to antibody fragments for use in labeling intracellular proteins. For example, in sectioned cells that have been stained only with osmium tetroxide, we find an average of 1.2 ± 0.1 Os atom per nm^3 , corresponding to an atomic ratio of Os:C atoms of approximately 0.02, which indicates that small heavy atom clusters should be detectable. The STEM analysis might also provide insight into the chemistry that determines the binding of heavy atom stains and fixatives to molecules within cells. Quantitative STEM imaging could also be applied to soft non-biological materials such as polymers that are stained with heavy atoms, in order to learn about the binding of heavy atoms to different phases within the light atom matrix.

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Fig.1.

Non-uniform conversion efficiency of the ADF STEM detector as a function of position. The conversion efficiency was normalized to unity at the inner edge of the ADF detector and the x coordinate was transformed to an angular coordinate ranging from 16 to 80 mrad. The normalized conversion efficiency increases abruptly at the inner edge of the detector, beyond which it increases slowly until its outer edge is reached.

Fig. 2.

Histogram displaying the number of gold atoms within Nanogold particles (second peak) and within regions of pure carbon (first peak) calculated using Eq. 13. The calculated average number of gold atoms within Nanogold, 63 atoms, is in good agreement with the known value of 67.

Fig. 3. ADF STEM image of unstained osmium-fixed *O. tauri*. Fixation with osmium is sufficient to reveal some level of structural detail. Evident in the image are thylakoid stacks in the chloroplast (*c*), the nucleus (*n*), and nuclear membrane (arrow). Cell is located within hole in Quantifoil grid. Scale bar = 200 nm.

Fig. 4. ADF STEM images of unstained osmium-fixed *O. tauri* acquired with detector inner semi-angles of (a) 16 mrad and (b) 36 mrad. Profile across the line in (b) is displayed in Fig. 6c. Scale bar = 100 nm.

Fig. 5. Distributions of (a) light and (b) heavy atoms/nm² within *O. tauri* obtained from Eq. 13. Contrast in the distribution of light atoms in *O. tauri* is minimal, whereas contrast in the distribution of osmium atoms is significantly stronger. Line profiles along the lines in (a) and (b) are displayed in Figs. 6a and 6b, respectively. Scale bar = 100 nm.

Fig. 6.

Line profiles displaying distribution of (a) light and (b) heavy atoms across the thylakoid stacks in Figs. 5a and 5b, respectively. (c) Line profile across the same stacks in the ADF STEM in Fig. 4b. From the line profiles, the average number of light and osmium atoms/nm² within the thylakoid stacks is given as 2213 ± 91 atoms/nm² and 38.1 ± 3.6 atoms/nm², respectively.

Within regions of the plastic section containing pure resin, the average number of light and osmium atoms/nm² is calculated as 1160 ± 50 atoms/nm² and 5 ± 2 atoms/nm², respectively. Both values of light atoms/nm² yield estimated thickness values which are consistent with direct thickness measurements from STEM tomographic reconstructions.

Fig. 7. Magnified view of a thylakoid stack from Fig. 4b and line profile across this stack. Dotted lines match specific features in the line profile to osmium-rich regions across the thylakoid stack. Line profile shows a maximum number of 48 osmium atoms/nm² within the membrane stacks. Scale bar = 20 nm.

Fig. 8. ADF STEM image of osmium-fixed *O. tauri* stained with lead citrate and uranyl acetate. Addition of stain has significantly increased the overall contrast of cellular structure compared with the unstained cell in Fig. 3. Evident in the image are thylakoid stacks in chloroplast (c), Golgi apparatus (g), nucleus (n), nuclear membrane (arrow), and ribosomes (r). Scale bar = 200 nm.

Fig. 9. ADF STEM images of stained osmium-fixed *O. tauri* acquired with detector inner semi-angles of (a) 16 mrad and (b) 36 mrad. Profile across the line in (b) is displayed in Fig. 11c. Scale bar = 100 nm.

Fig. 10. Distributions of (a) light and (b) heavy atoms within *O. tauri* obtained from Eq. 13. Profiles along the lines in (a) and (b) are displayed in Figs. 11a and 11b, respectively. Scale bar = 100 nm

Fig. 11.

Line profiles displaying the distribution of (a) light and (b) heavy atoms across the thylakoid stacks in Figs. 10a and 10b, respectively. (c) Line profile across the same stacks in the ADF STEM in Fig. 9b. From the line profiles, the average numbers of light and osmium, lead and uranium atoms/nm² within the thylakoid stacks is given as 2056 ± 250 atoms/nm² and 109 ± 19 atoms/nm², respectively. Numbers of light atoms/nm² yield a thickness consistent with direct measurement from STEM tomographic reconstructions.

Fig. 12. Magnified view of two thylakoid stacks from Fig. 9b and line profile across the stacks. Dotted lines match specific features in the line profile to stain-rich regions across the thylakoid stacks. Line profile shows a maximum number of 134 stain atoms/nm² within the membrane stacks. Scale bar = 20 nm.

Fig. 13.

(a) ADF STEM image of osmium-fixed *O. tauri* stained with lead citrate and uranyl acetate. STEM tomographic tilt series was acquired from this cell and reconstructed using SIRT algorithm. (b) 4-pixel slice across reconstructed tomogram reveals thylakoid stacks in chloroplast (c), ribosomes (r), and nuclear membrane (arrows). (c) Side view of reconstructed tomogram showing thickness of specimen ~ 30 nm. (d) Magnified view of a 4-pixel slice in tomogram showing stain granules. Scale bars: (a) = 200 nm; (b) = 100 nm; (c) = 30 nm; (d) = 10 nm.

Fig. 14.

(a) Histogram of integrated number of counts within 200 randomly distributed regions in reconstructed tomogram. Size of regions was taken as 4^3 voxels, i.e. $(4 \times 0.43 \text{ nm})^3$ volumes. The lateral size of these regions ($4 \times 0.43 = 1.7 \text{ nm}$) matches approximately the diameter of a Nanogold cluster (1.4 nm). (b) Histogram of total number of heavy atoms within 4^3 voxel-regions. (c) Histogram of total number of heavy atoms within 100 different 4^3 voxel-regions containing stain granules such as those observed in Fig. 13d.