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Development and Application of STEM for the Biological Sciences

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Abstract

The design of the scanning transmission electron microscope (STEM), as conceived originally by Crewe and coworkers, enables the highly efficient and flexible collection of different elastic and inelastic signals resulting from the interaction of a focused probe of incident electrons with a specimen. In the present paper we provide a brief review for how the STEM today can be applied towards a range of different problems in the biological sciences, emphasizing four main areas of application. (1) For three decades, the most widely used STEM technique has been the mass determination of proteins and other macromolecular assemblies. Such measurements can be performed at low electron dose by collecting the high-angle dark-field signal using an annular detector. STEM mass mapping has proven valuable for characterizing large protein assemblies such as filamentous proteins with a well-defined mass per length. (2) The annular dark-field signal can also be used to image ultrasmall, functionalized nanoparticles of heavy atoms for labeling specific aminoacid sequences in protein assemblies. (3) By acquiring electron energy loss spectra (EELS) at each pixel in a hyperspectral image, it is possible to map the distributions of specific bound elements like phosphorus, calcium and iron in isolated macromolecular assemblies or in compartments within sectioned cells. Near single atom sensitivity is feasible provided that the specimen can tolerate a very high incident electron dose. (4) Electron tomography is a new application of STEM that enables three-dimensional reconstruction of micrometer-thick sections of cells. In this technique a probe of small convergence angle gives a large depth of field throughout the thickness of the specimen while maintaining a probe diameter of < 2 nm; and the use of an on-axis bright-field detector reduces the effects of beam broadening and thus improves the spatial resolution compared to that attainable by STEM dark-field tomography.

Keywords

Scanning transmission electron microscopy (STEM); mass mapping; macromolecular assemblies; graphene; gold nanoparticles; electron energy loss spectroscopy (EELS); electron tomography; cellular structure

1. Introduction

From the outset Albert Crewe and his coworkers recognized the potential of their newly developed scanning transmission electron microscope (STEM) for applications to biological

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systems. In fact, the earliest paper from Crewe's Laboratory in 1970 describing the full design of a "scanning microscope with 5 Å resolution" was published in the Journal of Molecular Biology, and includes the use of STEM to study a variety of biological structures including viruses, bacteriophages, catalase crystals, and ferritin molecules [1]. It was quickly realized that the flexible configuration of detectors in the STEM not only enabled a very high collection efficiency for signals generated by interaction of the finely focused electron probe with the specimen but it also provided the means for obtaining quantitative information pixel by pixel. This is particularly clear in the case of the annular dark-field detector (ADF), which yields a signal that is proportional to the mass of the biological structure that is contained within a defined volume illuminated by the probe.

We shall begin our review by describing two types of applications of ADF-STEM. Firstly, ADF imaging can be used to determine the molecular masses of macromolecular assemblies, which has led to the vast majority of STEM applications in biology over the past 30 years [2,3]. Secondly, the strong electron elastic scattering produced by high atomic number elements, first exploited by Crewe and coworkers in the early 1970s to image single atoms [4], can be applied more broadly to visualize small heavy atom clusters that are used for labeling specific amino acid sequences of isolated protein assemblies or for localizing specific proteins in their cellular context [5–7].

Another technique originally developed for application to biological materials in Crewe's laboratory was electron energy loss spectroscopy (EELS). The initial work by Isaacson et al. in the early 1970s included acquisition of high resolution spectra of low-loss and core-edge fine structure from DNA bases and other compounds, as well as an assessment of how radiation damage degraded this information [8,9]. Subsequently, Isaacson and Johnson set out the formalism for determining detection limits for elemental nanoanalysis based on the cross sections for core edge excitations, and reached the prescient conclusion published in the first volume of Ultramicroscopy that single atom detection should be feasible [10]. The next section of this review summarizes the present capabilities and limitations of EELS nanoanalysis of biological specimens, as well as nanoanalysis by energy-dispersive x-ray spectroscopy (EDXS).

The capability today of carrying out high-performance STEM in transmission electron microscope columns has the potential to offer many more researchers the opportunity to apply STEM to biological systems. In this regard, amongst the biology community there has been rapidly growing interest in the use of electron tomography to image cellular ultrastructure in three dimensions. Recently, it has been demonstrated that STEM tomography provides some important advantages over conventional TEM tomography for imaging thicker sections of cells [11–14]. The final section of our review describes this new technique, which has considerable potential for imaging micrometer-sized volumes of complex cellular architecture at a spatial resolution of approximately 5 nanometers.

Thus the aim of the present paper is to demonstrate how the STEM has evolved into a flexible tool with capabilities for (1) determining the masses of individual protein molecules, (2) localizing specific sites on protein assemblies labeled with ultrasmall heavy-metal nanoparticles, (3) analyzing the elemental composition of macromolecular assemblies as well as subcellular organelles by EELS and EDXS, and (4) elucidating the 3D structure of large volumes of complex eukaryotic cells. Fig. 1 shows in schematic form the four principal modes of operation of the STEM microscope in connection with the types of applications discussed in this review. All these methods are complementary to TEM techniques, which can now be performed on the same instrument.

2. Mass measurement of macromolecular assemblies

STEM imaging in the ADF mode provides a unique means with which to determine the molecular mass of isolated protein assemblies [2, 15–19]. Importantly, because the mass of the protein is computed from an image, molecular mass can be directly correlated with molecular shape. Thus, it becomes feasible not only to measure the total mass of a complex and to determine its oligomeric state, i.e., tertiary and quaternary structure, assuming that the subunit mass is known, but also to measure the mass per area of planar assemblies of macromolecules or the mass per length (MPL) of filamentous structures. Additional advantages of STEM for mass determination include the wide range of masses that can be analyzed (from a few tens of kDa to hundreds of MDa), the need for only a very small amount of material on the order of micrograms, and the applicability to inhomogeneous populations of macromolecular assemblies providing a measure of variability in oligomeric state.

STEM mass measurement is performed on preparations of unstained and dehydrated macromolecular complexes adsorbed onto ultrathin (3 nm) layers of amorphous carbon. Rapid freezing and freeze-drying of the complexes provides the best structural preservation. Air-drying of TEM grids, while providing satisfactory results for large structures such as protein sheets and filaments, can cause some small oligomers to break apart due to the action of surface tension. Generally, an effective image resolution of 2–4 nm is attainable as limited by specimen preparation [2]. The absolute accuracy of STEM mass mapping depends on the purity and cleanness of the preparation, usually falling in the range of 5–10%. The precision of the measurements depends on the counting statistics and on the cleanness and uniformity of the carbon support film, which can produce random variations in background. Typically, a precision of a few percent is achievable in the case of large protein complexes, but for smaller complexes it can be much lower [3]. Significant improvements in the precision of the measurements and on the visibility of very small protein complexes (< 100 kDa) could be realized using a promising new substrate for electron microscopy consisting of a layer of graphene that is one carbon atom thick (see discussion below). Typical values of precision and accuracy obtained with STEM mass mapping, although not very high compared to those achievable with mass spectrometry [20], are sufficient to enable determination of the organization of protein subunits and the oligomeric state of a macromolecular complex. STEM mass mapping is therefore complementary to mass spectrometry, which provides high precision and accuracy but cannot be applied to large complexes or towards measurements of mass per area of planar structures and mass per length of filaments.

The basic principle of the ADF-STEM mass measurement technique relies on the proportionality between the dark-field STEM signal and the projected specimen mass at each image pixel. The constant of proportionality between image intensity and mass can be calculated from first principles taking into account such factors as the probe current, the collection angle subtended by the annular dark-field detector, the beam voltage, and the elastic and inelastic scattering cross sections for the atoms in the specimen [21]. However, a much simpler approach to mass determination, introduced by Wall et al., makes use of tobacco mosaic viruses (TMV) as an internal mass calibration standard (TMV has a MPL of 131 kDa/nm) [22].

Among its numerous applications, ADF-STEM mass mapping has been playing a most prominent role in the structural characterization of amyloid fibrils [23–28] and other filamentous structures such as DNA-protein complexes [29], collagen [30], muscle thick filaments [31], bacterial pili [32], intermediate filaments [33], etc. Amyloid fibrils in particular are implicated in a number of diseases including Alzheimer's disease, type 2

diabetes, Parkinson's disease, Huntington's disease, and transmissible spongiform encephalopathies (prion diseases). A hallmark feature of amyloid fibrils is a cross- β structure in which β strands are oriented perpendicular to the fibril long axis. These strands are spaced by about 0.47 nm and are held together noncovalently by interstrand hydrogen bonds approximately parallel to this axis. When combined with other structural techniques such as solid-state nuclear magnetic resonance (NMR) and cryo-electron microscopy, STEM MPL measurements on amyloid fibrils provide important constraints on molecular packing to assist in the generation of fibril structural models [23,26–28].

Only about 20 unrelated proteins involved in human disease are known to assemble into amyloid fibrils *in vivo*, among which is the β -amyloid peptide ($A\beta$) associated with Alzheimer's disease [34]. Fibrils formed by the full-length $A\beta$ peptide, its fragments or mutated variants have been extensively characterized by STEM under a range of different fibril growth conditions [28]. In one example, $A\beta$ (1-40) amyloid protofilaments with a periodically twisted morphology were analyzed by STEM yielding an average MPL value of 26 ± 2 kDa (Fig. 2A,B). This result indicated these fibrils were constructed from three $A\beta$ (1-40) molecules per cross- β repeat (0.47 nm), lending support to a threefold symmetric structure as suggested by NMR data [26]. In another example, amyloid fibrils of the HET-s prion protein were analyzed by STEM to test a structural model derived from NMR data [35]. In this model, each protein subunit, stacked in a β -solenoid conformation, spans two coils of the solenoid hence giving rise to a packing density of 0.5 of a subunit per 0.47 nm. STEM MPL analysis performed on HET-s (218–289) fibrils was indeed consistent with this proposed packing density (Fig. 2C–F). Moreover, identical results were obtained for HET-s (157–289) fibrils, indicating that the extra 60 residues appended to the N-terminus of the prion domain fragment HET-s (218–289) had no major influence on its axial packing density [25].

As mentioned above, the carbon film background intensity contributes a significant source of random noise to the mass measurements. Notably, this contribution of the background to measurement uncertainty could be greatly reduced by using novel one carbon atom-thick, atomically smooth sheets of graphene as specimen substrate [36–39]. In addition, graphene would significantly enhance the overall visibility (contrast) of protein complexes, particularly those with small masses in the tens of kDa range. To assess the limits of the technique it is useful to estimate the statistical precision associated with STEM mass measurement of protein assemblies that are adsorbed onto two types of substrates, namely a conventional 3 nm-thick film of amorphous carbon and a single sheet of graphene. The equations described next have been adapted from Engel [3].

The integrated ADF-STEM image intensity (in scattered electrons) associated with a globular protein complex of molecular mass M_p is given by

$$I_p = N_p \sigma_p D = \frac{M_p}{m} \sigma_p D \quad (1a)$$

The image intensity per unit length L for a filamentous protein assembly of molecular mass per length $(M/L)_p$ is given by

$$(I/L)_p = (N/L)_p \sigma_p D = \frac{(M/L)_p}{m} \sigma_p D \quad (1b)$$

where the integrated intensity for segment length L_{seg} is given as $I_p = (I/L)_p L_{seg}$.

In equations 1a and 1b, D is the electron dose in e/nm^2 ; N_P is the number of atoms in a globular protein complex, which is given by M_P/m where the mean atomic mass $m = 13.4$ Da assuming C:N:O = 0.56:0.17:0.27; $(N/L)_P$ is the number of atoms per unit length in a filamentous protein; and σ_P is the average integrated elastic scattering cross section per constituent atom of the protein assuming the previous atomic ratios and a given probe convergence angle and HAADF detector geometry. The value of σ_P can be calculated using differential elastic scattering cross sections from the database of the National Institute of Standards and Technology (NIST) [40]. For simplicity, in Equation 1a and 1b, we have disregarded the contribution of inelastic scattering to the HAADF STEM signal. For our microscope, an FEI Tecnai TF30 operating at 300 kV, we determined σ_P to be $1.48 \times 10^{-5} \text{ nm}^{-2}$.

The integrated image intensity (in scattered electrons) for the support film in the analyzed region of the globular protein is given as

$$I_C = N_C \sigma_C D = 2 \left(0.0138 M_P^{2/3} \rho_A \right) \sigma_C D \quad (2a)$$

The image intensity per unit length for the support film in the examined region of the filamentous protein is given by

$$(I/L)_C = (N/L)_C \sigma_C D = 2 \left(0.0394 (M/L)_P^{1/2} \rho_A \right) \sigma_C D \quad (2b)$$

where the integrated intensity for the support film along the filament segment L_{seg} is given as $I_C = (I/L)_C L_{seg}$

In equation 2a, $N_C = 2A\rho_A$ is the number of carbon atoms in the area $2A$ over which the protein signal is integrated, and $(N/L)_C = 2w\rho_A$ in equation 2b is the number of carbon atoms along the width $2w$ over which the signal from the filament is integrated. ρ_A is the number of atoms per unit area of the support film, where $\rho_A = 300 \text{ atoms}/\text{nm}^2$ for a 3 nm-thick film of amorphous carbon and $\rho_A = 38 \text{ atoms}/\text{nm}^2$ for graphene [41]. The factor 2 in equations 2a and 2b accounts for the fact that the protein signal should be integrated over an area that is typically twice that of its projected area to ensure that all the protein mass is included in the analysis. In these equations it is further assumed that the protein density is $820 \text{ Da}/\text{nm}^3$, in which case the projected area of the protein depends on its volume and can be written as a function of M according to the equation $A = 0.0138 M_P^{2/3}$ for a globular protein [3], while the width w of the analyzed area for a filamentous protein can be written as $w = A/L = 0.0394 (M/L)_P^{1/2}$. For our microscope operating at a beam voltage of 300 kV the elastic scattering cross section per carbon atom is $\sigma_C = 1.24 \times 10^{-5} \text{ nm}^{-2}$ (calculated from the NIST database).

The mass M of a protein complex can be measured after first subtracting the local background intensity I_C from the total integrated intensity I_T : $I_P = I_T - I_C$. The standard deviation S_P associated with I_P is then given by $S_P = (S_T^2 + S_C^2)^{1/2} = (I_T + I_C)^{1/2}$, and the percent statistical error $\%E$ in the mass determination of the protein can be finally written as $\%E = 100 S_P / I_P$. This analysis provides a lower bound for the statistical error associated with ADF-STEM mass determination of globular proteins (Table 1) and 50-nm length segments (L_{seg}) of filamentous proteins (Table 2). Actual measurement uncertainties can be larger due to impurities in the preparation, dissociation of some complexes, and local random fluctuations in substrate thickness in the case of amorphous carbon.

The ADF-STEM contrast associated with a protein complex can be defined as $C = I_P/I_C$. Thus, the corresponding increase in contrast when replacing amorphous carbon with graphene as specimen substrate is given by the ratio of the number of carbon atoms per unit area, namely $300/38 = 7.9$ (Tables 1 and 2).

Finally, we point out that since the time ADF-STEM mass mapping was first implemented by Engel and Wall in the late seventies, measurements have been mostly carried out using specialized, dedicated STEM instruments operating at 40–100 kV acceleration voltage. However, this technique is expected to work as well with widely available commercial TEM/STEM microscopes operating at 100–300 kV [21,30,42], bringing it to a wider community of biologists than has been previously possible. Furthermore, the ability to perform molecular mass measurement in higher voltage TEM/STEM instruments (e.g., 300 kV) opens up the possibility of applying this method to large structures such as whole organelles as well as polymer nanoparticles for nanomedicine applications, which otherwise might be too thick for imaging at lower voltages.

3. Visualization of ultrasmall heavy-metal clusters

Development of the STEM by Albert Crewe and colleagues was motivated by the prospect of visualizing single atoms of high atomic number elements supported on a lower atomic number matrix. This was indeed proved possible in 1970, in a milestone paper in Science [4]. Since then, the capability of ADF-STEM to image single heavy atoms has found innumerable applications in materials science (e.g., [43,44]). In biology however, the electron dose required to visualize single atoms exceeds by orders of magnitude the allowable dose for imaging most unstained biological macromolecules [45].

Contrary to ADF-STEM imaging of single heavy atoms, STEM imaging of ultrasmall clusters of heavy atoms affords the necessary contrast and SNR ratio for visualization at electron doses that are compatible with biological specimens. In one important application, ultrasmall clusters are used for site-specific labeling of supramolecular assemblies to identify precisely the location of specific subunits within these assemblies [46]. Two of the most widely used clusters are Undecagold and Nanogold, which contain 11 and approximately 67 gold atoms, and have a total diameters (including the associated organic shell) of approximately 2 and 2.6 nm, respectively [5,6]. When taking into account the size of the antibody fragment attached to these clusters, the effective probe resolution (i.e., the distance between particle center and antigen) is around 5–7 nm [5,6]. We emphasize that Undecagold and Nanogold are both too small to be easily detected by conventional TEM in most types of specimens. Thus, imaging gold-labeled protein complexes by TEM requires bigger colloidal gold nanoparticles whose associated probe resolution is much poorer (approximately 20 nm) [5].

The study presented in Fig. 3A–D illustrates the capability of the STEM in detecting Nanogold clusters in negatively stained preparations of macromolecular complexes [47]. Nanogold was covalently attached to the amyloid β protein ($A\beta$), and this conjugate incubated with eukaryotic proteasomes to form a proteasome- $A\beta$ -Au complex. Analysis of both end-on and side-on views of the proteasome- $A\beta$ -Au complex was consistent with the Nanogold labels (i.e., the $A\beta$) being inside the proteasome along its axial channel. In another study, the binding stoichiometry of the mushroom poison phalloidin (PHD) to F-actin was investigated by imaging Undecagold-tagged PHD molecules attached along F-actin filaments [48]. Dark-field STEM images of unstained and freeze-dried F-actin revealed individual Undecagold particles spaced by 5.5 nm consistent with a 1:1 PHD:actin binding stoichiometry (Fig. 3E). Furthermore, STEM MPL measurements on PHD- and

Undecagold-PHD-stabilized F-actin produced results again in excellent agreement with a 1:1 binding stoichiometry (Fig. 3F).

Compared to bigger colloidal gold nanoparticles, ultrasmall immuno-gold clusters also allow more efficient labeling of specific intracellular proteins in lightly fixed and permeabilized cells [49]. Electron tomography of the cells after plastic embedding, sectioning and staining can then provide the 3D distribution of the labeled proteins within the context of the surrounding cellular ultrastructure [50]. In studies of this type, the gold clusters have had to undergo a step of silver enhancement to become visible by conventional TEM tomography [49–51]. This step often leads to heterogeneous particle sizes, causes coalescence of nearby ultrasmall clusters into a single large particle, and generates bigger particles that obscure the neighboring cellular ultrastructure, factors that compromise the original probe resolution of the unenhanced ultrasmall clusters.

ADF-STEM tomography provides an attractive approach with which to visualize ultrasmall clusters in 3D without the need for silver enhancement [52–55]. Detection of the clusters by STEM, however, is limited to lightly stained preparations (e.g., only osmium fixed) and sections thinner than 100 nm [52–54]. To overcome these restrictions effectively would entail the development of ultrasmall nanoparticles containing higher numbers of atoms. In this regard, we have synthesized 144-atom gold clusters (Au_{144}) having a core diameter of about 2 nm in order to test the feasibility of visualizing these larger nanoparticles in osmium-fixed and stained plastic sections thicker than 100 nm. Synthesis of Au_{144} was accomplished following a procedure outlined by Ackerson et al. [56,57]. ADF-STEM images of Nanogold and Au_{144} particles adsorbed onto ultrathin carbon films show a tight distribution in size and intensity for both nanoparticles (Fig. 4A,B). Using Nanogold as a calibration standard, the number of gold atoms in Au_{144} was confirmed to be close to the expected value of 144 (inset). To evaluate the visibility of Au_{144} in 3D by STEM tomography, these nanoparticles were adsorbed onto the top surface of a stained plastic section of *Chlamydomonas reinhardtii*. First, the sections were pre-irradiated by exposure to a broad electron beam to stabilize the structure prior to collection of tomographic data. An ADF-STEM tilt series of the specimen was then acquired with a pixel size of 0.43 nm and processed with the IMOD software package [58]. The thickness of the resulting tomogram was found to be approximately 140 nm. Slices across the middle and top surface of the STEM tomogram are shown in Fig. 4C,D, where Au_{144} nanoparticles are visible in the slice across the top sample surface. This result indicates that it would be feasible to visualize in 3D ultrasmall gold clusters containing about 150 atoms in stained plastic sections that are thicker than 100 nm. It remains to be determined what gold nanoparticle size (e.g., 67 atoms, 144 atoms, or larger) would be optimum for immunolabeling applications in conjunction with electron tomography. Specifically, in addition to the visibility and detectability of the nanoparticles in 3D by tomography, other important factors to consider would also include ease of preparation and purification, solution stability, total particle diameter (core + shell), ease of conjugation with antibodies, etc.

Finally, we point out that besides facilitating visualization of ultrasmall clusters in 3D, STEM tomography has been suggested to afford higher contrast and SNR for 3D imaging of cell ultrastructure in comparison with conventional TEM tomography [59]. In this regard, an interesting example of the application of STEM tomography in the life sciences has been recently reported by Noda et al. [60]. In their work, 200 nm thick sections of Influenza A virus-infected cells were analyzed by STEM to help elucidate the 3D structure of ribonucleoprotein complexes within progeny virions.

4. Biological nanoanalysis

In STEM the capability of collecting multiple signals originating from the interaction of the incident electron probe with atoms in the specimen enables point-by-point compositional analysis [61–62]. Electrons that are scattered inelastically by the specimen carry quantitative information about the numbers of atoms of specific elements. This information is acquired by means of a magnetic prism spectrometer that disperses the electrons across a detector consisting of a scintillator coupled to a sensitive CCD camera, and a series of magnetic lenses focuses the spectrum on the detector and controls the dispersion [63,64]. The resulting electron energy loss spectrum (EELS) enables quantitative analysis of metal and low atomic number atoms contained within macromolecular complexes or subcellular compartments after extrapolation and subtraction of the spectral background underlying core edges. The advantage of STEM-EELS over energy-filtered imaging in the TEM is that compositional images are obtained by reading out multichannel (e.g., 1024 channel) EELS data at each pixel while the probe is rastered over the specimen. This enables weak spectral features to be detected and quantified, whereas in EFTEM imaging it is typically only possible to collect images at a few selected energy losses.

It has been demonstrated that if the probe size is reduced to a 1-nm diameter, STEM-EELS can provide near single atom detection of biologically important elements such as calcium and iron [65]. By considering the inelastic cross sections for core-edge excitation as well as the cross sections for the EELS background corresponding to the tails of lower energy core-edges, it is possible to write an approximate expression for the minimum detectable number of atoms p_X of element X

$$p_X \approx \frac{(S/\xi)d^2}{\sigma_X(\Delta,\beta)} \sqrt{\frac{hn_B\sigma_B(\Delta,\beta)}{[\eta I_0\tau/e]}} \quad (3)$$

where d is the probe size; h is a constant in the range 3–10 that depends on the shape of the background underlying the core edge; η is the detective quantum efficiency of the detector; I_0 is the incident probe current; τ is the integration time; e is the electron charge; (S/ξ) is the required signal-to-noise ratio, where S is the signal and ξ is the statistical noise; $\sigma_X(\Delta,\beta)$ is the inelastic cross section for the core excitation of element X for collection semi-angle β and integrated over energy window Δ ; $\sigma_B(\Delta,\beta)$ is the inelastic cross section for the background in the same spectral energy window originating from matrix atoms B ; and n_B is the number of matrix atoms per unit area [66].

From equation 3 we find that it is feasible to detect single metal atoms bound to macromolecules that are supported on thin carbon films [65]. It is predicted that single atoms of iron are detectable with $S/\xi=3$, and single atoms of calcium with $S/\xi=7$, for a 1-nm diameter probe, a beam voltage of 100 kV, and total incident charge of 0.4 nC. These predictions have been confirmed by experimental measurements, but this requires an extremely high electron dose of $\sim 10^{10}$ electrons per nm^2 .

There are several schemes for applying microanalysis to biological systems. In the first scheme macromolecules or cellular structures are initially imaged at low dose using the elastic ADF-STEM signal; and then EELS is used to analyze those same structures destructively at high dose analysis to determine the number of bound atoms of a specific element [67]. Such an approach has been used for example to study the phosphorylation state of intermediate filament proteins [68]. Second, if the specimen contains many similar regions, the EELS data can be recorded at lower dose and summed to increase the net signal-to-noise ratio. Such an approach has been employed in the measurement of calcium

concentrations in specific subcellular compartments in freeze-dried cryosections of biological tissues [69]. Third, when we consider the detection of a single atom, we can see from Equation 3 that the required probe current is proportional to the fourth power of the probe size, and the dose is inversely proportional to the square of the probe size. It should therefore be feasible to operate with a 100 times lower electron dose for single atom detection in an aberration-corrected STEM capable of providing a probe size of 0.1 nm, compared with an uncorrected STEM only capable of delivering the required current into a probe of size 1 nm. Such an approach has been applied to STEM-EELS mapping of single atoms in inorganic specimens, but not yet to biological specimens [70]. Finally, the conditions for high dose can be relaxed when STEM-EELS is applied at the cellular level to detect larger numbers of atoms, under conditions for which larger pixel sizes can be used. When EELS requires very high electron doses, particularly for the analysis of isolated macromolecular assemblies supported on thin carbon films, it is preferable to operate at lower accelerating voltages. At higher voltages, light atoms in the specimen can be directly sputtered from the surface by high-angle elastic scattering events involving momentum transfer to the nuclei. Below a certain threshold energy of the incident electrons (e.g., 60 to 80 keV) insufficient momentum is imparted to the nuclei to break simultaneously all the chemical bonds holding the atoms in place [71]. However, detection limits for EELS analysis are severely degraded when the specimen is thicker than 0.5 to 1.0 inelastic mean free paths (approximately 100 nm at 100 keV incident energy for a plastic section), so that in practice it is not always possible to decrease the accelerating voltage.

An example of the application of STEM-EELS is to image the iron cores of the storage protein, ferritin, in mouse brain [72]. Iron is an important element for the function of the nervous system since it is involved in critical processes including myelination of axons and neurotransmitter synthesis. High levels of iron are neurotoxic so that the iron regulatory protein (IRP) is present to maintain iron homeostasis. To determine how IRP influences the expression and distribution of ferritin, STEM-EELS analysis was performed on thin sections of mouse cerebellar tissue from mice in which the IRP gene had been knocked out. Measurements were made using a VG Microscopes HB501 STEM equipped with a cold field-emission source operating at a beam voltage of 100 kV and providing a 1-nm diameter probe. Hyperspectral images were recorded with a Gatan Enfina electron spectrometer, equipped with a cooled CCD detector. Specimens were cooled to -160°C to minimize mass loss of the tissue sections and to avoid polymerization of diffusible contaminants on the specimen surfaces. Specimen drift was compensated by cross correlating dark-field STEM images during acquisition of the hyperspectral images, and iron was quantified from the resulting spectrum-image data as previously described [72]. Dark-field STEM images (Fig. 5A) revealed electron-dense particles in oligodendrocytes surrounding the myelinated axons. EELS data extracted from 6-nm sized regions centered on the particles showed a strong Fe $L_{2,3}$ core edge peak (Fig. 5B), whereas no iron peak was found from just outside those regions (Fig. 5C). After processing the hyperspectral images at each pixel by fitting the pre-edge intensity to an inverse power of the energy loss, extrapolating into the post-edge energy window and subtracting, clusters of iron particles are evident (Fig. 5F). The pre-edge and post-edge images are shown in Figs. 5D and 5E, respectively. Quantitative analysis of the intensities in the resulting iron maps (Fig. 5G) indicated that the particles contained between 1,000 and 3,000 Fe atoms, which is consistent with the expected complement of iron in the cores of ferritin molecules [73,74].

Whereas the core-edge spectrum provides information about elemental composition, the low-loss fine structure originating from excitations of valence electrons and transitions between bound molecular states can provide information about a specimen's chemical composition. Examples of this technique have been to map chemical components in cryosections of frozen hydrated cells, e.g., to obtain water distributions in different

subcellular compartments of hepatocytes [75]. The technique has also been applied to map the distributions of water in layers of epidermis [76] as well as in polymeric biomaterials [77]. Since the organic and frozen aqueous components of cells are highly sensitive to beam damage, the spatial resolution of low-loss EELS mapping is limited to approximately 10–50 nm.

Despite detailed early work by Isaacson and coworkers in Crewe's laboratory to characterize the carbon K-edges of DNA bases and other compounds [8], it has proven even more difficult to exploit the energy loss near-edge fine structure (ELNES) for biological applications due to the low excitation cross sections and the high required dose. Nevertheless, recently van Schooneveld et al. have been able to analyze core-edge EELS fine structure acquired from hybrid organic-inorganic nanoparticles consisting of lipid-coated silica shells with quantum dot cores, which have potential applications for medical imaging and therapy [78]. Although these authors found that the carbon K-edge fine structure was degraded at doses of around 10^7 e/nm², they were able to detect features attributable to carbon-oxygen sigma bonds that are retained in the lipid coating after exposure to the electron probe.

Whereas EELS has high sensitivity for detecting light atoms, as well as certain other elements such as phosphorous, calcium and iron, it is not as sensitive for other biologically important elements such as potassium, sodium, magnesium and zinc. STEM-based energy-dispersive x-ray spectroscopy (EDXS) was developed extensively in the 1980s for use in biology by Somlyo et al. [79,80]. This enables detection of most ions and other bound elements found in cells and tissues. To preserve the distributions of diffusible elements, EDXS is generally applied to freeze-dried cryosections of rapidly frozen cells. The relatively low background underlying the x-ray emission peaks in the energy-dispersive x-ray spectrum provides detection limit of around 10 millimoles/kg (or 100 parts per million) in a single 100-second spectrum; and a sensitivity of 10 parts per million can be achieved by averaging spectra from many areas. Recent improvements in instrumentation are likely to make STEM-EDXS analysis more sensitive for the application to biological specimens. In particular, the available solid angle has increased by almost an order of magnitude through the addition of multiple silicon-drifted detectors (SDDs), which can provide a solid angle up to 1 steradian, and a count rate of order 10^5 per second [81]. This new capability has the potential to give STEM-EDXS near single atom sensitivity.

5. Three-dimensional imaging of micrometer-thick sections

Electron tomography (ET) in the TEM is a well-established technique for generating detailed 3D views of cellular ultrastructure at nanoscale (3–10 nm) spatial resolution [82–84]. For 3D ultrastructural studies of large eukaryotic cells and tissues, most work is carried out on samples prepared by rapid freezing/freeze substitution, heavy metal staining and sectioning in an ultramicrotome. The characteristic sample thickness for a 300 kV microscope falls in the range of 100 to 400 nm, with thinner sections providing the best level of ultrastructural detail at the cost of a reduced sample volume. For thicker sections (e.g., 1 μ m) however, the computed 3D volumes are blurred on account of the electrons that undergo multiple inelastic scattering events and are focused into different imaging planes by the objective lens [85].

For decades, it has been well known that the STEM is capable of imaging plastic sections that are much thicker than those suitable for TEM, an attribute that can be mainly explained by the absence of imaging lenses after the specimen in the STEM [86–89]. Nevertheless, only recently has STEM become more broadly utilized for 3D imaging of thick specimens in both biological and materials science applications [11–14, 90–94].

A few studies have established that STEM tomographic reconstructions from micrometer-thick sections present adequate resolution and signal-to-noise ratio to allow visualization of characteristic subcellular membrane architecture [11–14]. This is illustrated in Fig. 6B, which shows a STEM tomographic slice from a preparation of pancreatic islet beta cells microtomed to a thickness of 1 μm . The corresponding dual-axis STEM tomogram was acquired with a pixel size of 4 nm and computed using the IMOD software package [58]. For comparison, a two-dimensional STEM projection image of the same region is shown in Fig. 6A.

The tilt series used to generate the STEM tomogram in Fig. 6B was recorded under a special electron-optical configuration of the STEM microscope suitable for imaging of thick sections. First, the convergence semi-angle of the incident probe was adjusted to 1.6 mrad to decrease the geometrical spreading of the probe and thus keep the entire thick section in focus along its depth [12,13,91,92,95]. Second, an axial bright-field (BF) detector of about 10 mrad outer semi-angle was used instead of a standard ADF detector of large inner semi-angle to collect the transmitted electrons. The BF detector geometry was found to reduce blurring of sample features towards the bottom surface (i.e., the beam exit surface) of thick and stained biological specimens [12,96]. This advantage is realized because the component of the transmitted electron probe that is blurred by multiple scattering towards the bottom surface also exits the specimen with higher average scattering angle and is therefore largely excluded by the axial bright-field detector [96].

In general, although not comparable in quality to electron tomograms obtained from the thinnest sections (100 nm), STEM tomograms of thick sections present a level of ultrastructural detail similar to that which can be observed by conventional ET on sections of intermediate thickness (300–400 nm). The spatial resolution and SNR are sufficient in micrometer-thick STEM reconstructions to visualize identifiable ultrastructural features and to surface-render these in considerable detail (Fig. 6C). It is anticipated that the increased range of specimen thickness accessible to STEM tomography will be useful in the analysis of more complex and larger cellular structures, for example to visualize fine details of 3D neuronal morphology across several microns of brain tissue.

Recently, STEM has also been used to image fully hydrated eukaryotic cells loaded into a microfluidic chamber in a specially built specimen holder [97–98]. When cells are labeled with fluorescent dyes, they can be first visualized by optical microscopy and then specific structures correlated with images obtained by dark-field STEM imaging. And when structures of interest, such as membrane proteins, are labeled with gold nanoparticles, they can be imaged by STEM by focusing in the appropriate plane, even in specimens as thick as 5 μm [90, 94]. Such an approach has potential for application to materials as well as biological systems.

6. Conclusion

Forty years after the development of the STEM by Albert Crewe and colleagues, the technique has matured to the point where it can be applied almost routinely to biological structures, and the new generation of (S)TEMs has made the technique more generally available. Mass mapping based on imaging with the annular dark-field signal, as originally developed by Wall [17] and by Engel [15], has been STEM's most widespread application. This technique enables the analysis of large and heterogeneous protein complexes, and is particularly useful for analyzing filamentous proteins of indefinite total mass but with well defined mass-per-length. The annular dark-field signal also enables detection of specific sites within large protein complexes by using labels consisting of functionalized heavy atom clusters. Information about the presence of bound chemical elements is also attainable using

electron energy loss hyperspectral imaging. It has been demonstrated that near single atom sensitivity is achievable for elements like calcium and iron. The great versatility of the STEM for application to biology, which was appreciated at the time of the instrument's conception, is still evident today in promising new techniques for determining the three-dimensional structure of cells. In particular, axial bright-field STEM tomography offers the exciting prospect of being able to visualize 3D structures within micrometer-thick sections at a spatial resolution of a few nanometers.

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Highlights

1. We review, with a historical perspective, current applications of STEM in the biological sciences.
2. The most widely used application of biological STEM is mass determination of proteins.
3. Dark-field STEM enables localization of ultrasmall bionanoparticles containing heavy atoms.
4. STEM-EELS hyperspectral imaging enables elemental mapping of subcellular compartments.
5. Axial bright-field STEM tomography provides 3D ultrastructure in micrometer thick sections.

Figure 1.

Five principal modes of operation of the STEM microscope for applications in biology. (1) In the annular dark-field (ADF) imaging mode, elastically scattered electrons are collected by an ADF detector and the resulting image intensities used to determine the molecular masses of unstained macromolecular assemblies. This mode also allows the visualization of ultrasmall gold clusters that are used to label specific aminoacid sequences of isolated protein assemblies. (2) In the electron energy-loss spectrum (EELS) mode, acquisition of EEL spectra at each pixel in a hyperspectral image makes it possible to map the distributions of specific bound elements like P, Ca and Fe in isolated macromolecular assemblies or in compartments within sectioned cells. (3) In the energy-dispersive x-ray spectroscopy (EDXS) mode, acquisition of EDX spectra at each pixel makes it possible to map the distributions of important elements like Na, Mg, and K as well as other heavy elements. In operating mode (1), cooling the specimen down to LN₂ temperature using a cryo specimen holder minimizes beam-induced mass loss, while in modes (2) and (3) it minimizes the build-up of contamination thus allowing the use of high electron doses needed for improved detection sensitivity. (4) In the ADF tomographic imaging mode, ADF STEM images are acquired over a tilt series to compute a tomogram, from which it is feasible to determine the 3D distribution of nanogold-labeled intracellular proteins within the context of the surrounding 3D cellular ultrastructure. (5) In the bright-field (BF) tomographic imaging mode, transmitted electrons recorded by an axial BF detector are collected over a tilt series to generate tomograms from micrometer-thick stained specimens. The convergence angle of the illumination, shown as the darker gray incident beam, is reduced to maximize depth of field.

Figure 2.

STEM mass measurement of amyloid fibrils formed by the Alzheimer's β -amyloid peptide ($A\beta$) and the HET-s prion protein. (A) Annular dark-field STEM image of $A\beta$ (1-40) fibrils, with tobacco mosaic virus (TMV) included for mass calibration. (B) Mass-per-length (MPL) histogram indicating that fibrils are constructed from three $A\beta$ (1-40) molecules per cross- β repeat (0.47 nm), lending support to a threefold symmetric structure. Adapted from Paravastu et al., 2008 [26]. (C–F) STEM images of HET-s (218–289) (C) and HET-s (157–289) (E) with corresponding MPL distributions (D,F). Values of MPL are consistent with a fibril packing density of 0.5 of a subunit per 0.47 nm. The brighter straight rods are TMV particles. Scale bar, 100 nm. Adapted from Sen et al., 2007 [25], with permission from The American Society for Biochemistry and Molecular Biology.

Figure 3.

Annular dark-field (ADF) STEM imaging of ultrasmall gold clusters in both negatively stained and unstained preparations. (A) STEM image of negatively stained proteasomes that had been incubated with the amyloid β protein covalently attached to Nanogold clusters ($A\beta$ -Au). Arrows point to complexes containing $A\beta$ -Au (bright dots). (B,C) Collection of end-on (B) and side-on (C) views of proteasome- $A\beta$ -Au complexes. (D) Model for the locations of different $A\beta$ -Au obtained from several images. Results are consistent with the $A\beta$ -Au being inside the proteasome along its axial channel. Scale bar, 10 nm. Reproduced from Gregory et al., 1997 [47], with permission from The American Society for Biochemistry and Molecular Biology. (E) From left to right: (i) ADF STEM image of negatively stained phalloidin (PHD)-stabilized F-actin filament; (ii) same as previous, but unstained; (iii) STEM image of unstained Undecagold-PHD-stabilized F-actin filament; (iv) same as previous image but with contrast adjusted to reveal the highest image intensities associated with Undecagold clusters. Single gold clusters appear spaced by 5.5 nm consistent with a 1:1 PHD:actin binding stoichiometry. Scale bar, 20 nm. (F) Histograms of mass-per-length (MPL) data from PHD- and Undecagold-PHD-stabilized F-actin filaments (histograms ii and iii, respectively). The STEM MPL of 19.8 kDa/nm for Undecagold-PHD-stabilized F-actin agrees with the expected value of 19.3 kDa/nm for a 1:1 binding stoichiometry between Undecagold-PHD and F-actin. Adapted from Steinmetz et al., 1998 [48], with permission from Elsevier.

Figure 4.

Visibility of ultrasmall gold clusters in 3D by STEM tomography. (A–B) ADF STEM projection images of Nanogold (A) and Au144 (B) adsorbed onto an ultrathin carbon film. Inset: histogram of number of atoms for Au144 showing good agreement with expected value of 144. (C) Tomographic slice (contrast reversed) from a specimen of plastic-embedded and stained *C. reinhardtii* taken across the middle of ADF STEM tomogram. (D) Tomographic slice across the top surface of STEM tomogram. The higher number of atoms of Au144 in relation to Nanogold (144 vs. 67) allows visualization of the Au144 clusters in stained sections that are thicker than 100 nm (140 nm as measured from the tomogram).

Figure 5.

Identification of ferritin molecules by STEM-EELS. (A) Dark field image (contrast reversed) from a thin tissue section of mouse brain; scale bar, 100 nm. (B and C) EELS from 6-nm diameter regions containing an electron dense particle (B), and a region immediately adjacent to the particle (C). Arrows in B and C indicate the energy of the Fe $L_{2,3}$ edge excitation. (D–F) Iron mapping of the region marked with a box in A. The Fe $L_{2,3}$ pre-edge image (D) and the post-edge image (E) were obtained by integrating the spectral intensity over 10-eV energy windows. The iron map (F) was obtained by extrapolating the pre-edge intensity using an inverse power law into the region of the Fe $L_{2,3}$ edge and then subtracting. (G) Iron map obtained from a different region for estimating the number of iron atoms in each particle. The iron signal from each particle was integrated over the boxed regions; scale bar, 20 nm. Reproduced from Zhang et al., 2005 [72].

Figure 6.

Axial bright-field STEM tomography on 1 μm -thick section of pancreatic beta cells. (A) STEM projection image. (B) Tomographic slices through xy and xz planes of tomogram (top and bottom panels, respectively). (C) 3D model from similar 1 μm -thick region of pancreatic beta cells. Insulin granules are rendered blue; Golgi complex, yellow; mitochondria, green; plasma membrane, purple. Scale bars, 1 μm .

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Table 1

Calculated contrast, C , and percent statistical error, $\%E$, associated with STEM mass measurement of globular protein complexes in a 300 kV microscope, where $C = I_p/I_C$ and $\%E = 100(S_p/I_p)$. Estimated values suggest that the use of graphene as specimen substrate would enable more precise STEM mass measurements on protein complexes of intermediate sizes (e.g., 100 kDa) and STEM mass analysis of complexes that are too small (< 50 kDa) for reliable measurements using traditional substrates.

Mass (Da)	Contrast		Statistical error (%)			
			$D = 1000 \text{ e/nm}^2$		$D = 3000 \text{ e/nm}^2$	
	3 nm film	Graphene	3 nm film	Graphene	3 nm film	Graphene
10,000	0.24	1.92	91	43	52	25
20,000	0.31	2.41	58	29	34	16
30,000	0.35	2.76	45	23	26	13
40,000	0.39	3.04	37	19	21	11
50,000	0.42	3.28	32	17	19	10
75,000	0.48	3.75	25	14	14	8
100,000	0.52	4.13	21	12	12	7
250,000	0.71	5.60	12	7	7	4
500,000	0.89	7.06	8	5	4	3
1,000,000	1.13	8.89	5	3	3	2

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Calculated contrast, C , and percent statistical error, $\%E$, associated with STEM mass measurement of 50-nm segments of filamentous proteins in a 300 kV microscope, where $C = I_P/I_C$ and $\%E = 100(S_P/I_P)$. As for the analysis of globular proteins in Table 1, estimated values suggest that the use of graphene as specimen substrate would enable more precise STEM mass measurements of mass per length.

Table 2

Mass per length (Da/nm)	Contrast		Statistical error in 50-nm segments of filaments (%)					
			$D = 1000 \text{ e/nm}^2$			$D = 3000 \text{ e/nm}^2$		
	3 nm film	Graphene	3 nm film	Graphene	3 nm film	3 nm film	Graphene	Graphene
1,000	0.12	0.94	41	19	24	24	11	11
2,000	0.17	1.33	25	13	14	14	7	7
3,000	0.21	1.63	19	10	11	11	6	6
4,000	0.24	1.88	15	8	9	9	5	5
5,000	0.27	2.10	13	7	8	8	4	4
10,000	0.38	2.98	8	5	5	5	3	3
20,000	0.53	4.21	5	3	3	3	2	2
30,000	0.65	5.16	4	3	2	2	2	2
50,000	0.84	6.66	3	2	2	2	1	1
100,000	1.19	9.41	2	1	1	1	1	1