Electron Microscopy in the Perspective of Modern Biology: Ultravision and Ultradimension

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Since its invention, the microscope has been a valuable tool in the development of science and technology. As telescope transforms our views of the universe; imaging vastness of cosmos, microscope helps us to depict intricate details of the cell and organism around us, resonating the saying "a picture is worth a thousand words". **E**lectron **m**icroscopy (EM), one of the most indispensable tools in modern biology, enables visualization of the finest of structures and that too; in higher resolution. Thus EM techniques make possible to illustrate the mechanisms of different physiological as well as pathological processes involved in the living systems. Determining the ultrastructures of significant molecules; viz. proteins, nanoparticles, intracellular organelles, individual viruses and macromolecular complexes in vivid detail, EM takes science one step closer in exploring the anomalies of various systems. **T**ransmission EM (TEM) having its resolving power in the range 3-10 Å in 2-dimension, plays a pivotal role in locating and characterizing intracellular nanoparticles, determining protein assemblies, detecting morphological changes at cellular and organelle level in normal or diseased condition. **S**canning EM (SEM); on the other hand, provides scientific mapping of surface details, in enhanced resolution at 30-100 Å in 3-dimension. With the advent of new technology in modern days, the varieties of EM e.g. **s**canning TEM (STEM), **h**igh-**r**esolution TEM (HRTEM), **l**ow-**v**oltage EM (LVEM) in addition to conventional TEM or SEM provide unprecedented visual evidences to give basic data a whole new dimension. Both technique and application, which are given the most detailed coverage in this chapter are the sample preparation in SEM and TEM, macromolecular localization and ultrastructural characterization and their importance for critical analyses of data. This chapter also endeavors to sketch the current and future uses of EM and with precise description of tissue architecture, cellular integrity, and detection of nanoparticles from a modern biological perspective.

Keywords Modern Biology; Ultrastructure; Electron microscopy; Transmission EM; Scanning EM

1. Introduction

Microscopy is an indispensable tool of science that captures important scientific observation. Microscopic images are essential in troubleshooting the quality control issues in research and development. It helps in the study of objects that are too small (i.e. 1-100 μm) to be examined by the unaided eye. The revolutionary moment goes back to the early 17th century, when Zacharias Jansen, a Dutch spectacle-maker made the first microscope by placing two lenses in a tube. Later on, in the same century, Anton van Leeuwenhoek developed the light microscope and observed many miniscule objects including blood cells, bacteria, and other structures for the first time. Then in 19th century, compound microscope containing an objective (placed close to the object to be magnified) and an eyepiece (placed closer to the eye) came into practice.

In 1924, Louis de Broglie documented that electrons could act as both particles and waves. He discovered the equation of wavelength of photon represented by $\mu = h/p = h/mv$, where $h = 6.626 \times 10^{-34}$ J.s is the Planck constant; p, m, and v stand for the momentum, mass, and speed of the electron [1]. When electrons are emitted into vacuum from a heated filament and accelerated through a potential difference of 50 kV, then the velocity of electron become $\approx 4.2 \times 10^6$ m/s having a wavelength (λ) of ≈ 0.17nm. Davisson and Germer in 1927 first observed that the wavelength of electrons matches with the atomic dimensions and due to slower velocity; electrons are diffracted from the regular array of atoms arranged at the surface of a crystal [2,3]. When the accelerating potential is raised above 50 kV, then the wavelength reduced inversely i.e. 0.005 nm or even lesser. As the wavelength decreases, energy increases and such high energy electrons have the power of penetrating ultrathin solid samples and also diffracted by atomic planes inside the material. In the same year, G.P. Thomson demonstrated transmission electron diffraction pattern through a thin specimen. It was then realized that if these transmitted electrons could be focused, the very short wavelength would allow the specimen to be imaged with a high resolution (1- 0.05 nm) than the light-optical microscope. Using this concept, German physicist Ernst Ruska and electrical engineer Max Knoll constructed the prototype EM in 1931 equipped with 400X magnification [4,5].

Biology today would have been incomplete without the intervention of EM. The unique ability of EM in biology is its power to delineate and integrate structural details down to molecular dimensions within the context of a complex living system. Thus, the fine structure of tissues, detailed architecture of individual cells and its organelles, the structure

of viruses etc. came into view that were all beyond the spectrum of light microscopy. When combined with molecular detection methods, EM can help localize proteins even in plasma membrane subdomains with ultrahigh resolution in the context of the cell. Modified EM technologies have recently been developed to assess the fine-structure identity of intracellular membrane-enclosed compartments. At the same time, the methods to localize molecules of interest within these compartments have been broadened and perfected.

The major task of high-resolution biological EM is to provide the structural information with which one can correlate its function; it is the only methodology to elucidate structures inside the complex biological systems. Specimen preparation and imaging techniques should therefore be directed towards preservation and imaging of the smallest significant details in order to fully exploit this unique feature of EM, and thereby complementing the progress of the techniques used in cell biology, biochemistry and molecular biology. The term "high resolution" in this context means "high information density". The smallest structural details that are correlated with the physiological state of the visualized biological system remains a query.

EM picture enables elucidating the changes occurring in cells under diseased and normal conditions. Whether be it any biological surface feature or any intracellular details, EM with its repertoire of modified forms provide all the intricacies in vivid detail. Transmission EM (TEM), with its conventional attributes, focuses in characterizing various nanoparticles, locating them in intracellular compartments. Structural protein assemblies and cellular integrity at cellular and organelle level in 2D ultra-high resolution. With high resolution TEM (HRTEM) it is even possible to image objects at an atomic scale (~0.05nm.) and acquire 3D images by electron crystallography [6, 7]. 3D images of subcellular macromolecules can also be achieved with Bright field TEM (BFTEM) and HRTEM by an advanced modification known as electron tomography (ET) [8]. Cryo-TEM (CTEM) bypasses the tedious sample fixation procedures and allows samples to be observed in their native conformations [9, 10]. By using a technique called cryo-electron tomography (CET) where a 3D reconstructed image of a sample can be created with this EM from tilted 2D images [11]. Additionally, Environmental TEM (ETEM), one of the newest members of the modified TEM family, enables atomic-resolution imaging and spectroscopic studies under dynamic operating conditions helping to decipher local electronic structure in nano scale structures to trace elemental contaminants etc.[12]. On the other hand, conventional scanning EM (SEM) enlightens all the information that any biological surface has to offer. Starting from cell surface pore formation due to chemical treatment to any surface topographical changes, SEM can elucidate all by letting the scanning electron beam of the machine to interact with the electrons of the atom that make up the surface of the sample [13]. Like the modified versions of TEM, SEM also has its own modified and improved forms. Environmental SEM (ESEM); for example; doesn't require the sample preparations i.e. gold coating, and thus, enabling the same precious samples to be utilized for other experiments. With the new age, more advanced, EMs like Scanning TEM (STEM) [14], Low-voltage EM (LVEM) that combines the principles of both the conventional EMs empower us to extract even more information from samples [15,16].

The purpose of this book chapter is to delineate the use of EM and to explain some of the important applications in the advancement of modern biology. The subsections deal with the important techniques, the underlying sample preparation and detailed information about any biological samples. Because EM is interdisciplinary, both in technique and application, the physical principles being discussed here involve not only biology but also aspects of other scientific fields. Perhaps a book on the applications of TEM and SEM will benefit the engineers and scientists who use these tools.

2. 2D & 3D tissue architecture

The tissue architecture in higher multicellular organisms signify the normal physiological or pathological state [17,18] Though light microscopy, till date, depicting the overall tissue arrangement, has been playing an unprecedented role in deciphering a diseased condition in comparison to normal [19,20] but with some limitations. EM, on the other hand, with its enormous resolution to illustrate every details of any tissue architecture. EM and modified EMs provide unparallel information in both two and three dimensions of any tissue sample with subtlety.

TEM is compared with a slide projector where parallel beam of light, produced by the condenser lens, passes through the object and focused as a distended image onto the screen by the objective lens which is equipped with CCD (charge-coupled device) camera. By replacing the visible light source with an electron beam; the maximum resolution of 0.2 nm. can be attained in TEM, while the resolution of light microscope is limited to ~200 nm. [21]. Thus, TEM provides a useful magnification of 1000000X, that is 1000 fold higher than that of a light microscope. The monochromatic beam of electrons generated either by a tungsten filament, LaB_6 filament or by a field emission gun, is accelerated through a potential difference (~ 20-120 kV for conventional TEM and ~ 320kV or more for HR-TEM) under vacuum condition through electromagnetic fields that act as lenses. These lenses focus the electron beam on the sample to be observed on a fluorescent screen. In a conventional TEM, spherical aberration of image resolution is overcome by improved lens manufacturing. Chromatic aberration can also be minimized by keeping the accelerating voltage stable and using ultrathin specimens. Astigmatism, another major limitation, can be corrected by using variable electromagnetic compensation coils [22].

Tissue samples meant for TEM analysis are collected in a cacodylate buffer, then treated with a mixture of glutaraldehyde and paraformaldehyde fixative solution. The fixed samples are cut into small rectangular cube shaped pieces (~1 mm^3) and osmificated subsequently. The samples are then dehydrated by a series of graduated alcohol or acetone solutions and then finally infiltrated and embedded in semi-hard, EM grade, epoxy resin mixture. The embedded tissue sample blocks thus finally produced, should then be cut into ultrathin sections (~70nm.thickness) and mounted on uncoated grids (usually copper or nickel)of 300 or 400 mesh, to be observed under TEM. Since the biological specimen consisted of carbon, oxygen, hydrogen etc which are less electron dense elements, thus produced micrographs are of moderate contrast. In order to overcome this problem, heavy metal salt (uranyl acetate, lead citrate, phosphotangstic acid, ammonium molybdate etc.) staining methods are employed to elucidate better the ultrastructural details of the sample under observation [23-28]. The arrangement of cells, their polarization status, the connective tissues networks within various tissue samples etc.in 2D can be vividly examined by TEM. It is even possible to focus on specific locations of interest, e.g. basal lamina, membrane consistencies, capillary endothelial layer to study organizational pattern of cells in a particular tissue layer and thus to acquire knowledge regarding intricate tissue structure and integrity. TEM enables to find out histopathological abnormalities that occur in various diseased conditions namely; neoplastic condition and viral infections [6]. The ultrastructural information of connective (interstitial) cells supporting the pleural mesothelium is precious. Telocytes have been identified with TEM by their characteristic prolongations named telopodes (invisible by light microscopes) which also help to establish specialized cell-to-cell junctional complexes. ET has revealed the complex junctional structures and tight junctions connecting these pleural telocytes, indicating their participation in long distance homo- or hetero-cellular communication[8].TEM also revealed close vicinity of telopodes with nerve endings, capillaries, satellite cells and myocytes, suggesting a telocytes role in intercellular signaling (*via* exosomal pathway). Immunolabelling provided additional information regarding their molecular markers i.e. their capability of c-kit, caveolin-1 and secretory VEGF expression [29].The localization and resorption of elastic fibers in the fibroblasts of lamina propria of monkey gingiva were examined by transmission EM to enlighten the phenomena of the remodeling of gingival connective tissue that may be involved in facilitating the tissue adaptation to physical requirements during mastication [30]. With the help of HRTEM, it was also possible locate their intracellular number as well as their polyhedron shape [7].

SEM produces images of a sample by scanning it with an electron beam in a raster scan pattern. These bombarded electrons interact with that of the constituent atoms that make up the sample and producing informative signals (secondary electrons, back-scattered electrons etc.) that are represented as high resolution <1nm. images. SEM can provide 3D information about surface topography, crystalline structure, and electrical conductivity of any surface etc. and with the aid of Energy-dispersive X-ray spectroscopy (EDX), it is even possible to know about chemical composition and electrical behavior of a tissue sample surface; cut in ~1 micrometer slice [10].

SEM allows us to correlate between different anatomical structures and their corresponding physiological function. Sample preparation techniques are less destructive for the cell in SEM than that for TEM. Therefore, it is better to use SEM for examining bulky and sensitive samples that under harsh conditions of chemical treatment or drying, easily lose their 3D conformation.

Tissue samples for SEM are initially fixed in 2.5% glutaraldehyde solution for overnight followed by washing with phosphate buffer and water. The tissues are then osmificated with 2% osmium tetroxide and again washed with water. The tissues are then dehydrated in a series of increasing concentration of ethanol followed by critical point drying. The samples are then mounted on a stub with silver or graphite paste. For imaging by conventional SEM, specimens to be examined must be electrically conductive, at least at the surface. In order to that, the specimens are sputtered with gold or palladium alloy after mounting on a stub. This sputtering or shadow casting technique not only increases the contrast of the obtained image, but also provides a 3D representation of surface characteristics of sample under examination [31].

The image in a SEM is completely electronically produced; it can be subjected to refined analysis and exploitation using modern digital techniques, such as contrast enhancement, inversion (black turns into white etc.), filtering, blending of images from various detectors, subtraction of the image from one detector from that produced by a different detector and image analysis.

SEM provides information regarding shape, size and height of cells or fibres in the tissue organization, surface textures, projections or depressions etc. The typical ridged structure of the myofibrils for bovine, pig and human was illustrated employing SEM. Moreover, by determining consistencies in tissue samples, it is possible to detect different incorporated crystals among the cellular arrangements. Presence of crystallized salts such as carbonate salts in the cardiac tissues just before the sacrifice was established using SEM [32]. SEM was reportedly used in evaluating the cytotoxic effects of various commonly used topical ocular preparations, drugs, vehicles, and preservatives on the corneal surface [33]. Specimens that are temperature sensitive, Cryo-SEM or low temperature SEM can be employed to decipher 3D information of cryogenically fixed specimens. These cryo-fixed specimens can further be cryo-fractured under vacuum to reveal their internal structures! These cryo-fixation methods viz. freeze-fracturing, freeze-etch or freeze-and-break methods are particularly useful in gathering 3D surface information about lipid membranes and their embedded proteins [34,35]. For the specimen that cannot be fixed and subsequently processed, Environmental SEM (ESEM) or low voltage SEM (LV-SEM) are employed to image these nonconducting specimens. ESEM operates by

placing the specimen (unfixed but appropriately cut) in a relatively high-pressure chamber under vacuum condition to provide an amplification of the secondary electron signal. On the other hand, LV-SEM is typically employed in an FEG-SEM where FEG produces high primary electron beam which provides enhanced brightness even at low accelerating potentials (~ 0.3-4 kV) [12,13].

3. Ultradimension of cellular integrity

Determination of cellular structure by EM is straightforward, however it has some drawbacks. One is high sensitivity of cytoplasmic and membrane proteins towards electron damage, and the other is the loss of 3D orientation that generally occurs when the specimen's natural aqueous environment is dehydrated at high vacuum.

In addition, reallocation of proteins through loss of cellular integrity during sample preparation is a potential flaw of EM [36]. Moreover, immuno–EM technique exists for the intricate localization of even multiple proteins inside the cell on a regular basis. Promising novel approaches circumvent chemical fixation using highpressure freezing, freeze-substitution and freeze-fracturing and combinations of these with immunolabelling. The best available approach so far to immobilize and preserve cellular architecture is probably cryofixation. High-pressure freezing is the only way to freeze biological samples of 10–200-μm thickness without generating artifacts as a result of ice-crystal formation.

ESEM, which bypasses the needs of above sample processing, has shown significant promise in effectively quantitating parasite–host adhesion index, carried out on *G.duodenali.* Instead, it is difficult to estimate by conventional SEM methods since the adhesion might lost in crucial sample processing steps [37].

Different types of cells belonging to different origin; can be examined under EM. For example, varied shape from round to spindle of fibroepithelial cells can be examined under SEM. Additionally, the nucleus as well as nucleoli can be observed prominently. Frequently the cytoplasm shows many intracellular fibrils, sometimes in parallel bundles and occasionally associated with; what we consider to be; junctional complexes. Furthermore, neuronal cells (l0 X 30 μm. in size) can be analyzed with numerous vesicles of various sizes erupting from the surface [38]. The ultrastructural characteristics of nasal mucosa revealed squamous epithelium showing keratinization in large areas through SEM. Additionally, desquamation of the horny cells of the nasal mucosa epithelium can be distinguished from the above in the nasal mucosa. At higher magnifications, these abnormalities were found to be the result of desquamation of the horny cells [39]. Another aspect of examining tissue architecture is to compare between two different species or between tissue appearances of two different tissues. For instance, labrum had a coarser appearance with thick collagen fibres oriented generally perpendicular to the bone contours, cartilage had a smoother cross-sectional surface and the columnar structure of the thin collagen fibrils running perpendicular to the bone contour was discernible [40].

Not only the cells, but also the integrity and dimension of the intracellular organelles can be investigated through EM and special fixation techniques [41,42]. Also, EM has been frequently used to study the morphology of bacterial cells or cell compartments in cultures and isolated samples. Under TEM, intact bacteria with an intact cytoplasmic membrane, damaged bacteria with shredded membrane and empty bacteria without any plasma inside the cell wall were seen in a culture of marine bacteria. Simultaneously with the assessment of the intracellular integrity, the morphology and size of other structures, such as slime layer and capsule can also be investigated through EM [43].

EM has provided valuable information on the organization of the Golgi complex, the polarized distribution of proteins on the plasma membrane, insights into the essential structure of mitochondria [44]. The first ultrastructural study of mitochondria was done by George Palade in 1953 [45] and recently by Koshiba *et. al*. in 2004 [46], characterized the structural basis of mitochondrial fusion [47]. Moreover, EM study has enabled to confirm mitochondrial involvement in the pathomechanism of certain neurodegenerative diseases. In such diseases, alteration of the mitochondrial membrane and its integrity becomes clearly visible under EM [48].

Several approaches have already contributed to the knowledge of the relative topography of ribosomal components, the absolute location of the ribosomal proteins has only recently emerged absolutely from EM of antibody-labeled 30S and 50S subunits . By immuno EM, proteins S4, S5, S13, and S14 were mapped on the surface of the 30S subunit and preliminary data on the location of proteins S6, Sil, S12, and S19 have also been reported [49-52]. In 1964, Inouye *et. al*. first described different forms of brain ribosomes, stability of which depended on the Mg^{++} concentration of the medium using EM. A 80S particle could dissociate to form a 60S particle and a 40S particle [53].

EM data showed that golgi consists of parallel lamellae, vacuoles and small granules. The cisternae that comprise the golgi complex are thin in their central portions but expanded at their periphery. The parallel arrays or stacks of cisternae are usually curved so that a convex (forming) face is distinguishable from the concave (maturing or secretory) face [54]. In freeze-substitution fixation method, specimens for TEM and SEM were prepared simultaneously. Under SEM, the golgi stack appeared to consist of smooth membranes and small vesicles were present beside it [55].

4. Exploring macromolecular structures

Apart from structural characterization of proteins or small synthetic peptide in solution, EM also provides structural insight of various biological macromolecules. It is possible to characterize critical structural assembly process that eventually give rise to their functional forms. TEM is widely used for protein or peptide structural studies and their supramolecular assembly [56]. TEM examinations showed three typical morphologies (film, bicontinuous and fibril) of homotrimer assembly of a new type of collagen mimetic peptide, (PKG)n (POG)2n (DOG)n , with charged-domain ends which self-assembled into collagen-like triple helices homotrimers. The collagen-like homotrimers underwent higher level of self-assembly via static electrical interaction between positive and negative domains. The mechanism of assembly process was proposed based on TEM observations, which depicted that the film was formed in the initial stage and gradually transformed to bicontinuous or fibril morphology to improve stability of the assemblies or decrease surface energy [57]. With the aid of EDX, TEM can also help in identification of elemental composition and chemical bonding among them. For characterization of peptide by TEM, a single drop of peptide solution is added to the coated grid, followed by heavy metal salt staining to increase resolution of images [58,59]. TEM is particularly useful in documenting supramolecular assembly process of microtubules, ribosomes etc. and the mechanism of action of drugs on them. In addition, high resolution TEM (HR-TEM) allows examination of peptide crystallographic structures. For peptides that are particularly sensitive to harsh conditions such as drying, fixing and staining, their characterization and high contrast imaging is carried out by cryo-TEM (CTEM) and energy-filtered TEM (EF-TEM) that bypasses these conventional needs [8,9].

For high resolution images of thick samples, SEM imaging is usually favoured over TEM imaging. To carryout SEM, peptide solutions are dropped onto a glass coverslip, air-dried and nanocoated with Au or Pd. Peptides reaching ~ 80 nm. in size can be observed by this method. High-resolution scanning EM (HRSEM) is being used increasingly to gain new insights into three-dimensional organization of biological structure, macromolecular complexes and interactions of cellular components as well as isolated cell organelles. Modern SEMs e.g. E-SEM (environmental SEM) and cryo-SEM (cryogenic SEM), combined with adequate sample preparation can now provide resolution comparable with that achieved using TEMs, i.e. down to 2–5 nm. for biological material. The versatility of the instrument and new sample preparation techniques have allowed detailed analysis of chromosomes, cytoskeletal components, virus and other biological material that has not been possible with TEM [60]. HR-SEM and E-SEM provides vivid morphological three-dimensional and very high resolution images that can even identify the flexible regions in individual fibers, that was invisible in conventional SEM. For imaging non-conducting samples E-SEM seems to be a better choice, because E-SEM sample is located in a chamber with high pressure rather than vacuum. A combination of Scanning Transmission EM (STEM) configuration in Environmental Scanning EM (ESEM) provides a new approach for the characterization of the 3D structure of materials, as it optimizes a compromise between the resolution level of a few tens of nm. and the large tomogram size due to the high thickness of transparency. The method is well adapted for non-conductive samples and exhibits good contrast even for materials with low atomic number [11]. Cryo-SEM involves snap-freezing of sample in solution form and provides a precise idea about the supramolecular structures of the peptides. High-resolution cryo-SEM is even able to depict freeze-dried and tungsten-coated actin filaments in which actin subunits are directly visible. Thanks to Cryo-field emission SEM (Cryo-FESEM) it is possible to view inner nuclear membrane and even image and measure nuclear pore diameters! [60]. Visualization of supramolecular structures in encapsulated form such as that in liposomes can also be achieved by quickfreeze/ deep-etch (QFDE) technique. In this technique replicas are produced by etching from the fractured surface of a rapidly frozen sample, providing analysis of exclusive regions which otherwise would have hidden in ice [9].

TEM is most widely trusted for detailed nanoparticle analysis (size, shape, morphology, crystallinity etc.) where sample in the solution phase is examined by the droplet method. In this method, a small drop (~5 microlitre) of a relatively concentrated solution of the sample specimen is deposited onto the matte side of the coated (usually with a thin layer of amorphous holey carbon) grids and this thin coating provides the necessary support for the minute molecules under observation. The solution to be used should not be excessively concentrated since that might lead to sample overlapping and thus restricting electron beam to pass through and also overburdening of the supporting sheet which subsequently can lead to tearing of the supporting film following sample drying. Wet samples are strictly avoided since that might lead to vacuum column contamination and destabilize the vacuum column. These dried samples are then taken for TEM analysis. High resolution TEM (HRTEM) provides direct determination of the conformation, topology and crystallinity of nanoparticles and crystal lattice including the lattice spacing of nanocrystals. By using other materials such as carbon nanotubes (CNT) as sample supporting film, it is even possible to measure the dimensions of amorphous shells around the core crystalline structures that have lesser electron density than the thin amorphous carbon film support. A special attachment, namely EDX, enables to reconfirm as well distinguish the composition of nanoparticles from the surrounding supporting film and contaminants [61,62]. Traceable size measurements of nanoparticles are accomplished by means of a calibrated SEM operated in transmission mode (TSEM). It individually determines the boundary, size of every particle and particle size distribution based on modelled TSEM signals obtained by Monte Carlo simulations. Nanoparticles of three different material classes (gold, silica,

latex) with sizes ranging from about 5 to 60 nm. were determined by this technique. Its measuring capabilities can be expanded with 95% confidence interval to a mean diameter in the range of 1 to 3 nm. [63].

TEM also plays a huge role in assessing the viral morphological traits which can significantly aid in viral identification, characterization and subsequent classification. Some viruses have projections on their capsid which can be confirmatically characterized and their dimensions determined by SEM. These invaluable, detailed information obtained from SEM can in turn facilitate anti-viral drug designing in future [64-68]. EM is widely used in research and diagnostics purposes of fluid biological specimen (e.g. urine, CSF, tears, tissue fluids, spleen etc.) suspected for viral infection. For TEM study, a viral solution typically having a concentration in order of approx. 10^5-10^6 viral particles/ ml. are used for analysis by the droplet method, described earlier [69]. The viral infected cells can also be identified by Cryo-FESEM which can directly detect budding capsids on the nuclear membranes [60]. Moreover, heavy metal salt staining can be done to increase the contrast but selection of stain is one of the crucial factors since some stains (such as phosphotungstic acid) may lead to degradation of viral entities. Immunolabeling technique can also be employed to specifically identify the whole mature virions. This can be done by adsorping them onto the grids by treating them with primary antibody followed by secondary gold tagged-antibodies. It can also be negatively stained to increase the contrast.

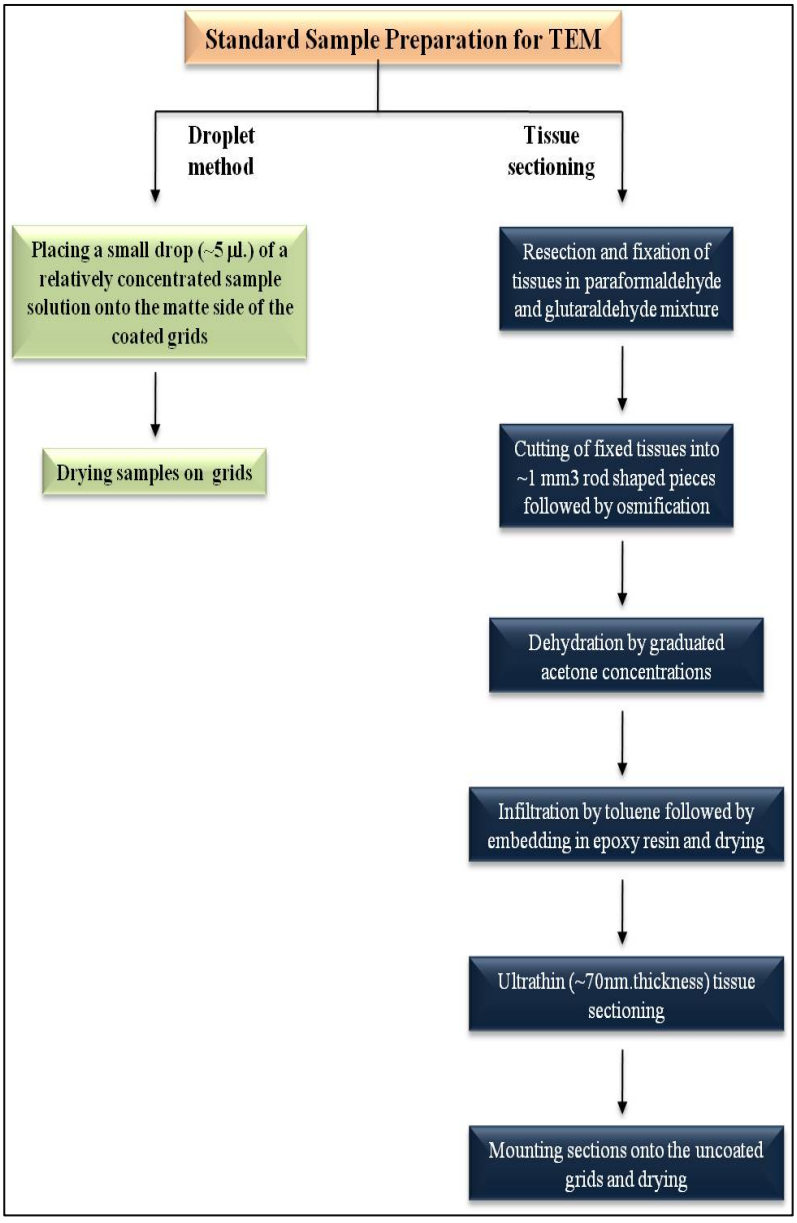

Fig.1 Standard sample preparation technique for TEM (as described in the text).

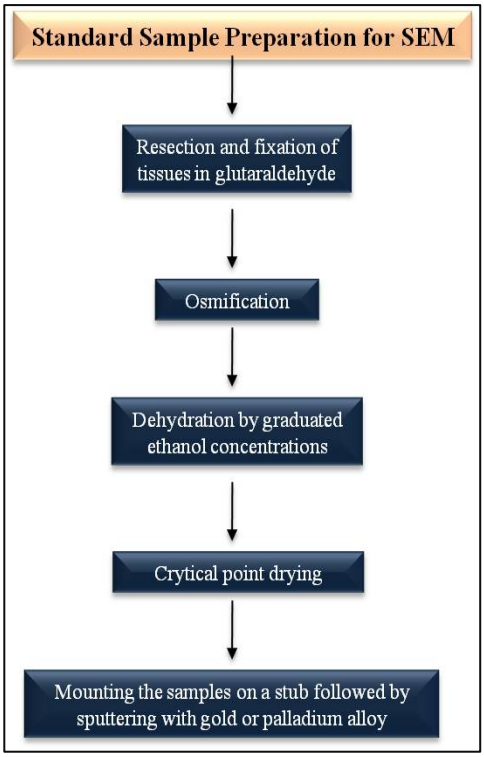

Fig 2 Standard sample preparation technique for SEM (as described in the text).

5. Expression profiling of macromolecules

Ultrastructural imaging techniques serve an increasingly essential role in the rigorous characterization of engineered tissues and biomaterials [70]. Detection and quantitation of proteins, virus and nanoparticles inside or at the surface provide crucial information about any biological events and even, sometimes help to decipher the cause of any biological condition of any macromolecule [71]. Here, we illustrate some of the applications where EM find its most frequent uses. The distribution of metallic nanoparticles in tissue samples can be easily carried out with the help of TEM [72]. Tissue samples obtained from pretreated experimental models are processed accordingly (as mentioned previously) and can be subjected to TEM analysis. The tissue samples from various locations can be screened for the presence of metallic nanoparticles (Gold, Silver etc.) which can be detected in tissues as darker, electron dense dots against a relatively light background [73,74]. Whether the nanoparticles are distributed as individual entities or they remain aggregated together in clusters, all this can be determined with the help of EM [75]. Even the no. of nanoparticles participating in the cluster can also be determined. Amidst numerous nanoscale components present in the tissue, whether the observed and suspected electron dense dots are actually the nanoparticles of interest, can be identified by an attachment system called Energy dispersive X-ray analysis (EDX). EDX generates characteristic spectral peaks corresponding to the constituent elements that helps us to identify the actual nanoscale structures that correspond to the metallic nanoparticles [76,77].

Detection, identification, quantitation and even tracking for localization of proteins of interest or ultrastructural antigens in intracellular or cell surface of cell and tissue samples by EM (TEM and SEM) can be achieved by the technique known as Immunogold labeling [78,79]. Where TEM allows identification of concerned protein in intracellular regions in 2D [80], SEM on the other hand, provides all the information needed to detect and trace a protein entity on the cellular or tissue surface [81]. For this process, thin sample sections are cut and incubated with specific primary antibody, specifically designed to bind to the protein of interest. Then, colloidal gold conjugated secondary antibodies or protein A or protein G or streptavidin is employed that binds preferentially to the IgG Fc domains of the primary antibodies. Gold, being electron dense molecule, scatters more electrons relative to the surrounding sample and appears as darker spots. Using this technique two or more proteins can be identified simultaneously by using different sized gold conjugates [82, 83].

TEM help to identify the viral entity in biological tissue samples directly by deciphering the presence and their characteristic features, viz. size, shape of their capsid etc [84,85]. To enable the electron beam to penetrate the sample; thin sections of cells and tissues, suspected to be infected by virus, are used. The localization of mature virions or their proteins and composition of viral inclusions inside the cell and tissue sections are demonstrated by viral antibodies and gold-labeled secondary antibodies (Immunolabeling). With the help of ultrasmall gold probes and silver enhancement techniques it is possible to detect the presence as well as their site of activity in thin sections [86,87]. In addition,

specially engineered antibodies can be used to distinguish between native and altered viral proteins, thus providing an idea about the functions of various components.

Besides simply detecting the presence of viruses or characterizing them in clinical specimens, the EM study also reveals information regarding the virus host-specificity and their mechanism of action. Cryo-EM and tomography have been used to decipher 3D information of non-chemically fixed viral structures [88]. Even various pleomorphisms among enveloped viruses and their relation with subcellular organelles, cellular receptors etc. can be elucidated by this technique. SEM, on the other hand, provides all the topographical information regarding the infected cells [89]. It provides detailed information about cell surface attachments, budding processes employed by some kinds of viruses, cell surface changes occurring due to the viral infections which can altogether provide invaluable information which may lead to better antiviral targeted drug designing.

6. Conclusion and future directions

An electron microscope's capacity to offer high resolution image over large areas and volumes of tissues or cells is very useful in discriminating associations among biological systems across huge differences in spatial scale. EM is being used today in research laboratories around the world to investigate the molecular mechanisms of disease and to envisage the 3D architecture of tissues and cells, conformation of protein structures, nanoparticles, individual viruses and macromolecular complexes in biological milieu [90]. EM as a technique, alternative to genomics or proteomics approach, provides information of biological organization [91].

Demand of EM was rapidly growing in the sixties, when early biochemical methods were insufficient to understand the properties as well as the morphology of the large variety of cellular proteins [92]. The major intricacies of intracellular architecture were being drawn on that era. Then Immunocytochemistry came into the scene, which was certainly a bridge between ultrastructure and molecular composition of many cellular macromolecules and organelles [93]. Scientists then developed several good immuno–EM methods which helped to elucidate the localization of molecules within their cellular micro-environment at such a level of resolution. To make it more useful and sophisticated, scientists have modified EM in several ways. For example, TEM is modified to a convergent beam camera and shadow microscope, with two specimens located in different planes [94] or the vacuum system of a SEM has been modified in such a way that it has enabled hydrated specimens to be placed inside the specimen chamber of the instrument and to be surrounded by pressurized water vapour [95]. The incorporations of monochromators in commercial TEMs have improved the energy resolution to 0.1 eV [96].

In addition to the conventional EM, CryoEM can be applied to those complexes that are not suitable for X-ray crystallography because of their size, instability or the fact that they are available only in insufficient amounts. The resolution of cryoEM allows the relative positions and orientations of individual components of a macromolecular complex to be determined to within a few angströms. For example, recently, single-particle cryoEM of the ribosome-bound class I release factor RF2 indicated that binding of RF2 to a stop codon in the decoding centre of the 30S ribosomal subunit induces a conformational change in RF2 that allows it to interact simultaneously with a stop codon and the peptidyl-transferase centre of the 50S ribosomal subunit [97]. The rapidly growing number of human diseases that are identified as defect in intracellular membrane-trafficking shows how protein function is directly associated to intracellular localization and emphasizes the significance of protein-trafficking pathways. Protein-trafficking events crucially depend on membranes but remarkably little is known about the *in situ* intracellular distribution of lipids. EM, perhaps, is the only practice that can merge sensitive protein-detection methods with detailed information on the ultrastructure of intracellular compartments. Routine fixation procedures immobilize lipids insufficiently and cause their extraction and redistribution. The best results so far were obtained with chemically fixed and freeze-substituted cells. This combined resolution power has given EM an essential position in defining intracellular protein distribution patterns and predicts a continuing and ever increasing demand for EM in cell biology [91,93,98].

EM images are the best possible visual proofs of chemical and biological samples, though it needs tedious sample preparation techniques [98]. The most burning issues facing electron microscopists are how to gain existing technology and expertise and to build up infrastructure– as soon as possible [99]. Apart from cell biology, EM should be exploited with its diverse types in every corner of life sciences. EM in cell biology is still as fascinating and imaginative as thirty years ago. It has evolved even into ultramodern technologies like molecular topography. Indeed, the application and development in cryo-EM field is already expanding fast and powerfully [100]. The high resolution structural data obtained from cryo-EM are absolutely reliable and convincing [101]. It also complements X-ray crystallography or it can often be used to extract additional information [90]. However if the structures cannot be obtained from X-ray crystallography, EM is preferred because electrons are much more appropriate as imaging particles due to their interaction with atoms [101]. Moreover, during the last decade, constant technological developments have gradually augmented the resolution of the EM to somewhat below 1 Angstrom. The need of the hour is to search potential solutions to popularize EM. So this is a wake-up call to those molecular and cell biologists who need EM.

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