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Review Article

Challenges Confronted by Electron Microscopy in Microbe Detection

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Abstract

Electron microscopy has been contributing significantly to the study of microbes for biomedical, biological, and material science purposes in the world. Besides providing structural information, precise chemical analysis can be carried out by determining sample composition in thin films by Energy-Dispersive X-ray Spectroscopy (EDS) or Electron Energy Loss Spectroscopy (EELS), along with observing materials and specimens in the nano-level.

It can also help in formulating new technologies to aid humans in countless applications. At times, when focusing an electron beam on any surface, various elastic and inelastic interactions occur within the material. A couple of essential issues that occur with obtaining high-resolution Scanning Electron Microscope images of microbes are - a) to get adequate contrast, one need to condense the charging time for small organic particles such as bacteria and viruses that require magnifications greater than 1000x, thus, an appropriate conducting surface is essential; & b) biological specimens need to be first dehydrated from original condition, for the appropriate imaging performance for the Scanning electron Microscope.

The prominent limitation to date yet to be unraveled in electron microscopy is that none of them reveal the phylogenetic diversity of the microorganisms present in the study habitat. Unless detection schemes are developed allowing at least 3 orders of magnitude lower electron doses, the ability of electron microscopy to study life cell processes at the nanoscale, although very attractive, probably remains an idea concept.

This review summarizes the difficulty in microbe detection and numerous encounters faced by Electron Microscopy in doing so.

Keywords: Electron microscopy; Microbes; Energy-dispersive X-ray spectroscopy; Scanning electron microscopy; Transmission electron microscope

Introduction

In Electron Microscopy, scientists have already discovered the cellular morphology in the nanoscale resolution but haven't yet been able to provide adequate information on protein location. If it can provide the exact site of those proteins, we can unravel more insight into biology.

Also, microbes present special challenges in Electron Microscopy, with many of them prone to being damaged by the electron beam. The exposure to the electron beam leads to structural damage mostly due to the generation of reactive species, such as solvated electrons and H+ and OH− radicals, and the direct breakage of molecular bonds. They seldom contain light-sensitive elements (e.g., Lithium) or volatile components like organic molecules or Sulphur, which sublime under the electron beam. At times, when focusing an electron beam on any surface, various elastic and inelastic interactions occur within the material. During inelastic scattering, most of the energy that gets generated is dissipated as heat within the specimen, causing generation of local temperature rise, and indirectly disrupting the microbial homeostasis.

There are two fundamental reasons why living things can't survive in an electron microscope:

- The power of the electron beam that's directed at the sample.
- The vacuum inside the microscope.

Moreover, automated segmentation remains challenging in electron microscopy due to imaging modality disparities. [3] Also, an extensive sample preparation process and staining have led to a disparity in electron density while scanning regions of the specimen. This further surge the detection time for the microbe in hand. There's also the possibility of artifacts or noise getting introduced during specimen frame-up process. One approach to counter this, is to freeze the sample very quickly instead of fixing it. Providing the sample stays cold enough, this 'locks up' the water and prevents it from evaporating inside the microscope.

Electron microscopy has progressed ever since its invention, but to elucidate the dynamic morphology of various bacterial features, like flagella, fimbriae, and spores, the study of bacteriophages, etc., still constitutes an extensive detection technique. Detection sensitivity and virus identification can be augmented byincorporating another technique called immune electron microscopy. This functions

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through Ag – Ab interaction, where specific antiserum binds to a viral antigen of interestwhich then leads to the detection of the probable microbe in test.Further methodscomprise of enhancing Immune Electron Microscopy (IEM) that utilizes immune clumping, Solid-Phase Immune Electron Microscopy (SPIEM) and immuno-gold labeling.

The imminent use of this in disease-related biomarkers has paved way for rapid and direct detection of the microbe family. Yet infectious diseases still possess a serious threat to healthcare. Probable reasons constitute the incessant mutation of viruses,detection of new bacteria due to environment effluence, origination of more antibiotic-resistant pathogens, etc [4].

Extensive steps of specimen preparation methods have in the past limited the use of Scanning Electron Microscopes for routine microbiology. During its observation, drying usually led to collapse, shrinkage, and deformation of the specimen, even after preservation by chemical fixation. The intensity of electron beam incorporated within the microscope, proves to beintrinsically annihilating for the specimen, that leads to electron charge development. It also causes deformation due to absorption-based heating [5].

As per scientist Allan Mitchell, Microscopy Otago, "right from the word go, from the moment you collect your sample, you have to be thinking about preserving it in as close to the living state as possible".

Another disadvantage in EM is that, to attain a continuous uninterrupted signal, a detectable signal is essential, that can be translated into a pixel and later get transferred onto the next pixel. A major limitation comes from length scales, because with limited pixel size, it takes too long to achieve macroscopic length scales.

Again, if a wet specimen is placed within the microscope, operation under high vacuum conditions tends to dry out the specimen quickly. Both factors will compromise microscope performance and may reduce both contrast and resolution.

Image analysis in EM is particularly challenging because one of the greatest benefits of the electron microscope – the ability to capture many different classes of biological structure in a heterogenous sample in a single image – is at odds with current (human and software) capabilities to recognize and classify all structures in that information-dense image.

Critical point drying technique, permitted along with a conductive coating of biological specimens, led to reduced charge buildup, thus increasing the contrast, but the sample warped from cracking artifacts and shrinkage of up to 50%. Moreover, freeze-drying frequently caused disfigurement and disintegration due to ice formation. For visualization, one needs to generate an electrically conductive surface for Scanning Electron Microscopes, so biological specimens are coated using thin-film evaporation or sputtering of carbon or metal in a vacuum coater, which requires prior desiccation of the sample. This coating process can vagueminor ultrastructural details, disclosed within the thickness of the layer deposited [1].

Moreover, an initial pre-requisite in all electron microscopy operation, is the sample preparation prior to being loaded on the microscope in vacuum environment. Biological specimens are generally composed of nonconductive, thermally sensitive, fragile material, which if not stabilized, results in specimen damage and imaging artifacts. Practices incorporated for this, relies on both sample type and analysis mode, inclusive of freeze-fracture, freeze-etching and sputter coating techniques [6].

The couple aforementioned procedures coincide with histological specimen preparation steps like cryofixation, dehydration, embedding, sectioning and staining. This requires dedicated training and any errors during sample manipulation or preparation, it may result in artifacts or inadvertent changes to the local arrangement of the specimen. The use of various chemical fixations, dehydrators, and embedment also proves to be dangerous for microbe samples [5].

The tungsten filament of electron gun generates energy in the form of heat, i.e., above 2700^{0}C when in function, this further leads to immediate temperature rise within the specimen sample. Also, the electron velocity due to accelerating voltage can range between 1.5 X 10^{8} m/sec to 2.3 X 10^{8} m/sec, which is more than three-quarters the speed of light, leading to generation of heat in the vicinity and within the layers of specimen. Heat kills microbes by altering their membranes and denaturing proteins. The Thermal Death Point (TDP) of a microorganism is the lowest temperature at which all microbes are killed in a 10-minute exposure. When electrons impinge on the specimen, it leads to emission of X-rays which disrupts the specimen's elemental composition due to the irreversible breakage of molecular bonds and generation of reactive chemical species. Ionizing radiation of high intensity can penetrate into the cell, where it alters molecular structures and damages cell components [7].

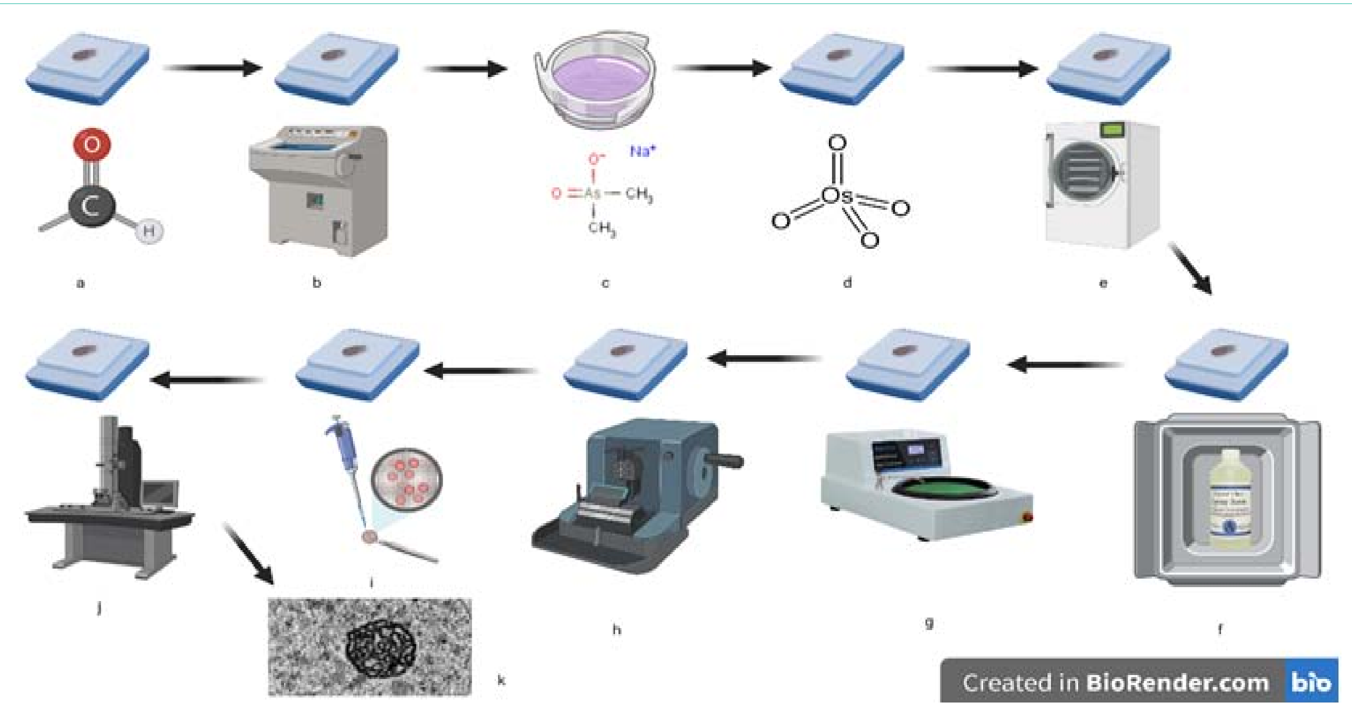
Another major issue is with enrichment of microbes, which is done at suitable conditions for growth and must be sustained over long periods since samples require pre-processing before Electron Microscope identification. Developing a method to analyze microbes is especially challenging due to the inherent complexity of matrix composition, the heterogeneous distribution of low numbers of polysaccharides, stress encountered by the microorganisms during different conditions disrupt the microbe homeostasis.

Since, the entire operation requires quantum high vacuum environment, these conditions used (approximately up to 10^{-7} Pa) is another limiting factor for the survival of biological specimens. These conditions cause rapid evaporation of water from the organisms, resulting in their collapse and cell death.

The dynamic local effects that occur, could have been caused by either or a combination of structural rearrangement after radiation damage, movements of the sample preparation fluid, for example, when gas bubbles are created, or simply by Brownian motion.

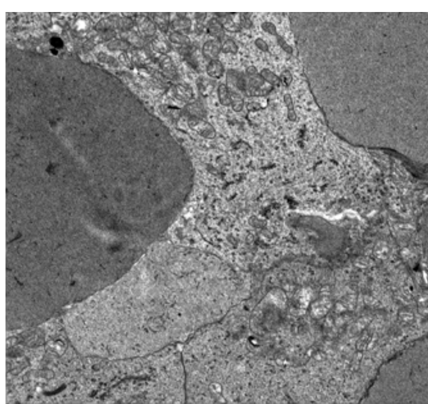
The limited speed of current scanning systems is a major disadvantage. Due to their limited acquisition rate, microscope operators are forced to balance the dimensions of the imaged area against the resolution needed to ascertain the specified level of detail. Also, the incapability to present phylogenetic or genetic information regarding microbes has been a significant limitation of the Scanning Electron Microscopes in environmental microbiology [1].

In electron microscopy, electrons are diffracted by the sample and refocused by electromagnetic lenses to produce an image. Unfortunately, biological tissue doesn't diffract many electrons.

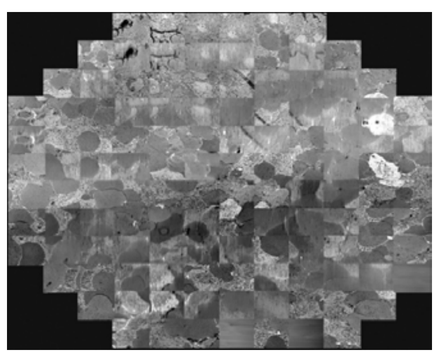


Sample Preparation Steps in TEM before scanning.

Figure 1: (a) Chemical Fixation (Glutaraldehyde) & (b) Cryofixation (N or He), (c) Rinsing (using sodium cacodylate buffer), (d)Secondary Fixation (using osmium tetroxide), (e)Dehydration (using ethanol or org. solvent), (f) Infiltration (by epoxy resin), Embedding & Polymerization, (g) Polishing (by ultrafine abrasive), (h) Cutting & Sectioning (by Microtome), (i) Staining (using heavy metals like uranium, lead, or tungsten), (j) Specimen scanning through TEM & (k) Final image under microscope.



Troubleshooting and accepted protocols.
Sample movement problem.
Cycling between a capture where there is a subtle amount of movement in the sample, vs. one where there is no movement, and the image is sharp.



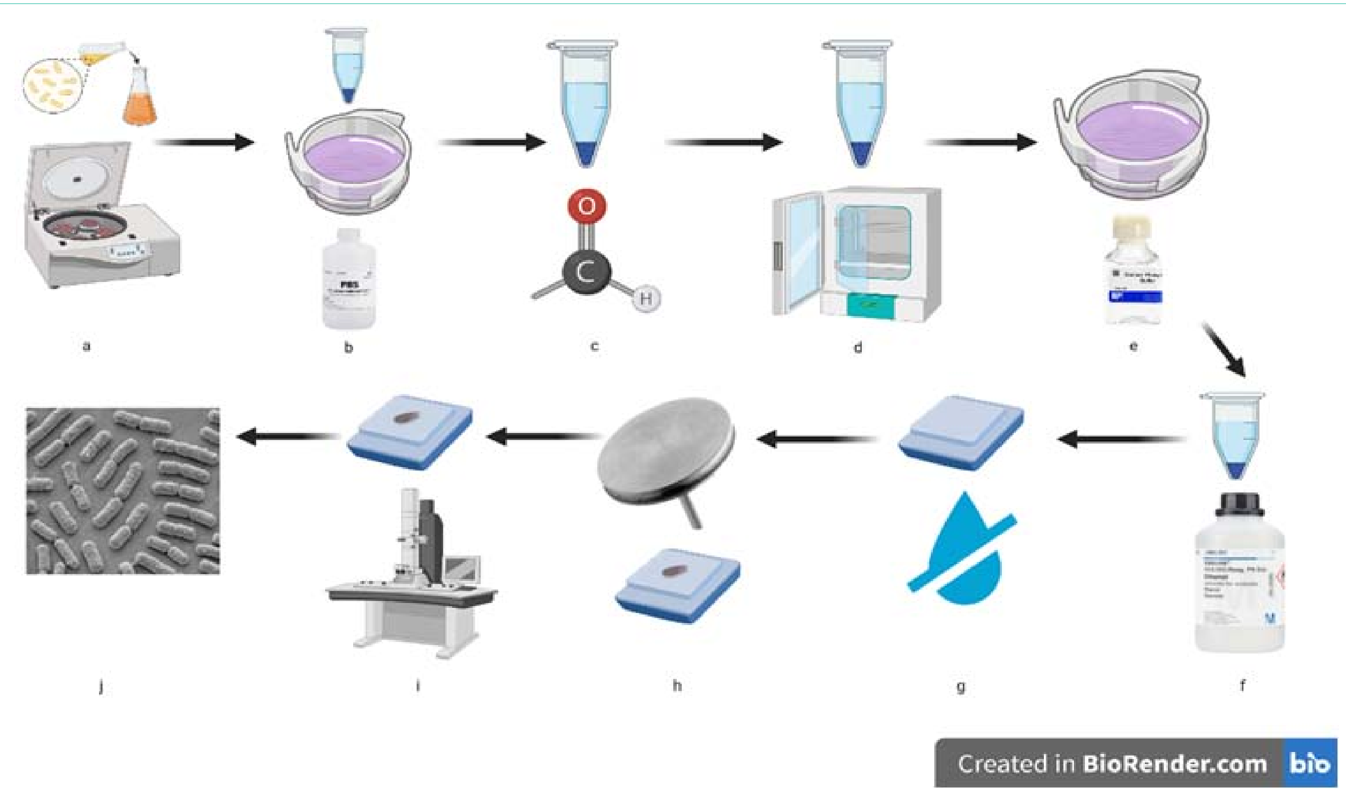
Incorrect optimization
Due to issues during image reconstruction, enhancement and improvisation, the resulting image has limited resolution
Source - JONES BLOG and P. Scientist, "Troubleshooting Transmission Electron Microscopes," 2019. https://prometheus.med.utah.edu/~bwjones/2019/11/troubleshooting-transmission-electron-microscopes/.

Electron-dense stains (made of Lead or Uranium are used) or advanced EM techniques are often necessary to visualize biological ultrastructure.

Biological samples are non-conductive, creating issues when bombarded by a negatively charged electron beam. The samples may get unstable and drift, blurring the image. Or they could create distortions in the signal due to the accumulation of negative charges on the sample. Heavy metal staining ameliorates this, like conductive coating is applied to the sample to dissipate the charge which further hinders the novel biological property of specimen [8].

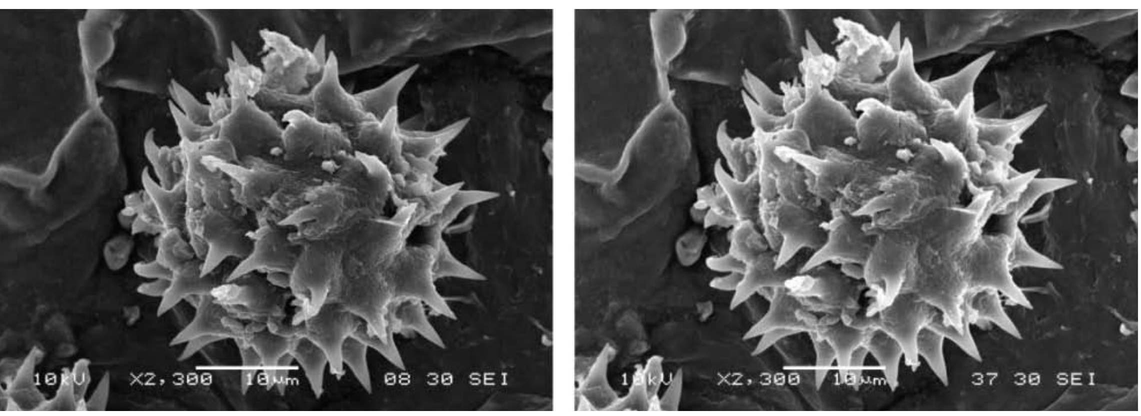
Another drawback is the negative staining of Transmission Electron Microscope, where there is the requirement of adequate concentration of bacterial cells or virus particles for detection since these get adsorbed to a thin support film. Thus, microbes are cultured at a high rate and later concentrated by centrifugation. This is practically unfeasible when samples are acquired from infected patients. Thus, electron microscopy has been disregarded due to this declined test sensitivity for numerous micro-organisms investigations [9].

We must also consider that; the image resolution of an SEM is at least an order of magnitude poorer than that of a TEM. However,



Sample Preparation steps in SEM before scanning

Figure 2: (a) Centrifugation of microbial broth culture at specific rpm, (b) Pellet rinsing with PBS solution (various concentrations), (c) Fixation of pellet with aldehydes (glutaraldehyde), (d) Pellet incubation at 37°C followed by freeze fracture, (e) Pellet rinsing with Sodium Phosphate buffer, (f) Pellet cleaning by various Ethanol concentrations (30%, 50%, 70%, 80%, 90% and 100%), (g) Critical point dry of sample specimen, (h) Acquirement of Aluminum SEM stub, and then fixing with adhesive tape of microbe sample over it,(i) Specimen scanning through SEM& (j) Final image under microscope.



Troubleshooting and accepted protocols
Pollen, 8 mm WD 100μm Aperture
Pollen, 37 mm WD 100μm Aperture
Source -K. Hagglund, "Tips for Improving Image Quality Using the SEM," *›MODERN MICROSCOPY*

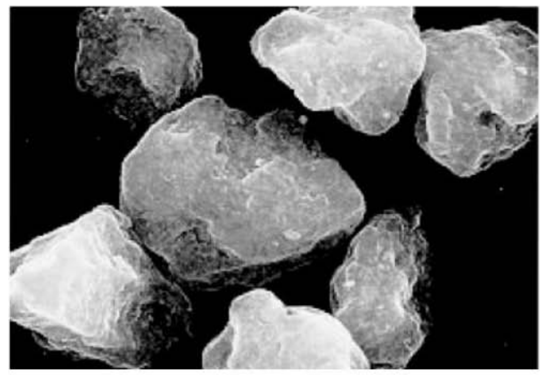
because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimeters in size and (depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample.

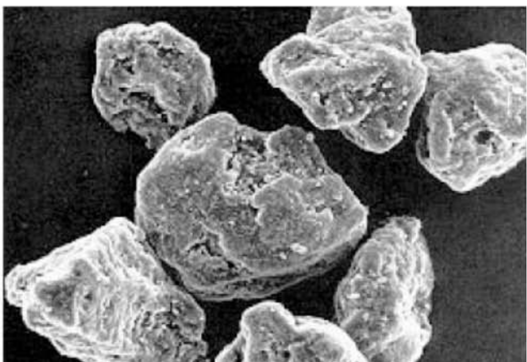
Now let's ascertain how both the types of electron microscopes regress the microbe detection process.

Transmission Electron Microscope

Transmission Electron Microscope is regarded as irrelevant for live specimen identification due to initial dehydration and embedding steps before sample loading, thus imaging is performed under a non-native environment.

A couple of necessities before initiating the Transmission Electron





30 kV Accelerating Voltage 5 kV Accelerating Voltage

Source: C. Scheu and W. D. Kaplan, "Introduction to Scanning Electron Microscopy," *In-Situ Electron Microsc. Appl. Physics, Chem. Mater. Sci.*, pp. 1–37, 2019, doi: 10.1002/9783527652167.ch1.

Table 1: Characteristic variations between Transmission Electron Microscope and Scanning Electron Microscope [24].

Property	M	TEM
Fundamental design	e^- detector e^- sample	e^- sample e^- detector
Image Processing	Creates an image by detecting reflected or knocked-off electrons	Uses transmitted electrons (electrons that are passing through the sample) to create an image
Image Analysis	Provides information on the sample's surface and its composition. Topographical	Offers information on the inner structure of the sample, such as crystal structure, morphology at the atomic scale, elemental composition, and stress state information. Internal
Choice for experiment	Suitable to acquire insight on the surface of the sample, like roughness or contamination detection	Suitable to acquire insight on the crystal structure of the sample, or interstitial structural defects or impurities
Image dimensions	Provide a 3D image of the surface of the sample	Images are 2D projections of the sample
Sample preparation	Requires little effort with minimal steps and can be directly imaged by mounting them on an Al/Si/Au stub.	Complex and tedious procedure that only trained and experienced users can perform with multiple preparation stages.
Acceleration Voltages	1-30 kV. Less damage to specimens	60-300 kV. Immediate specimen micro-environment disrupted
Field of view	Large.	Limited.
Sample Restriction	Least	Stringent
Depth of Field	High	Moderate
Sample Size	Can be thick. Not much constrained	Extremely thin. ≤150 mm
Optimal Spatial Resolution	~0.5nm	<50 pm
Nanoparticle application	Least appropriate	Largest throughput. Most desirable for rapid characterization.
Sample Processing	Fast rate of analysis	Relatively slower rate
Specimen Mounting	Al/Si stubs	Thin films on Cu grids
Magnification	~1-2 million X	>50 million X

Microscope include:

First, the accelerating voltage has to be adequate such that the beam of electrons is able to traverse the specimen partially without being fully absorbed [10].

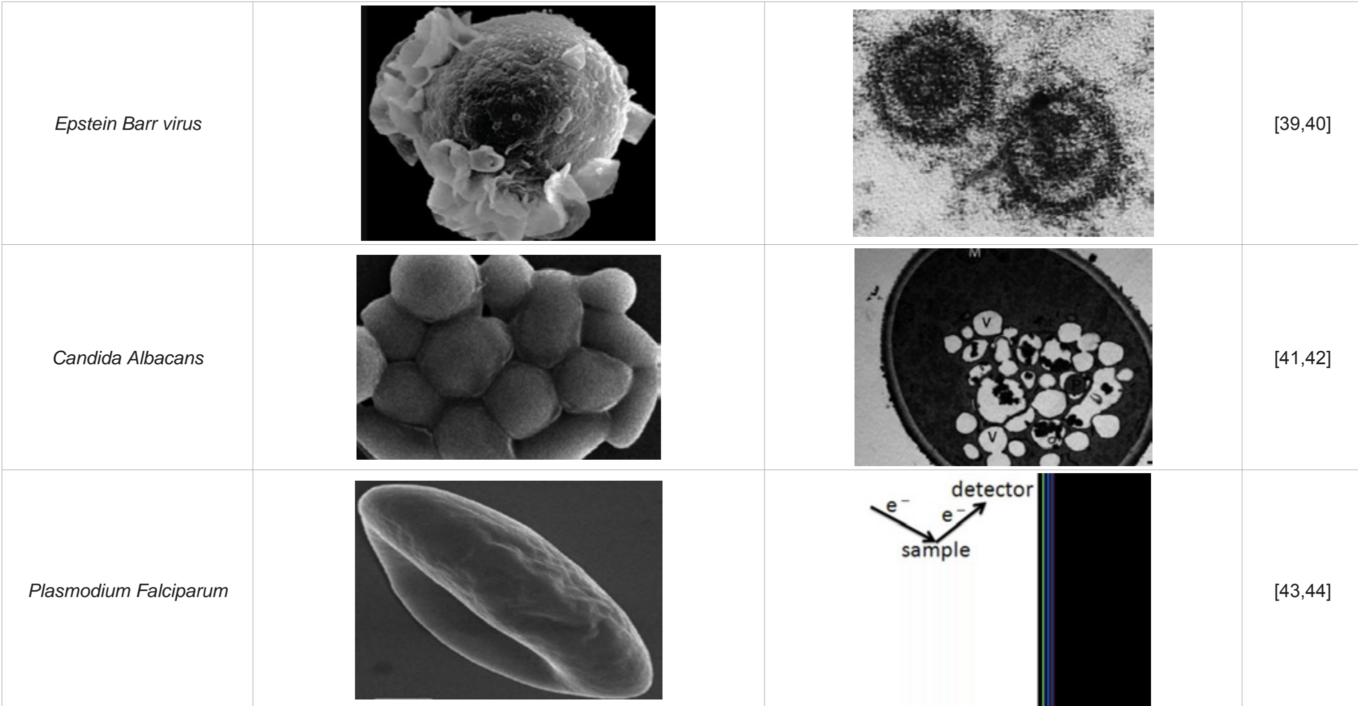
Secondly, in response to this prerequisite, the samples must also be trimmed, in the range of 100 nm in thickness before loading. Biological samples chiefly comprise Carbon, Hydrogen, Oxygen, Nitrogen and these possess low atomic numbers. Due to this, micro-organisms don't participate in electron absorption and can thus reconcile to thicker samples. The prominent disadvantage with these samples is that they'll dissipate away from the point of perforation which affects sample thickness dimensions, leading to subsequent image shrinkage and consecutively reducing uniform microscope transmission [11].

Also, it routinely provides a black-to-white scale, two-dimensional images as resultant.

Consecutively, this microscope requires a constant inflow of 50-400 kilo-volts to generate the electromagnetic field and maintain

Table 2: Contrasting images of various microbes under SEM & TEM.

Microbe	SEM	TEM	Resource
E. Coli			[25,26]
Sars-Cov-2			[27,28]
Fungal Hyphae			[29,30]
Staphylococcus Aureus			[31,32]
Streptococcus Pyrogens			[33,34]
Bordetella Pertussis			[35,36]
Mycobacterium Tuberculosis			[37,38]



Epstein Barr virus			[39,40]
Candida Albacans			[41,42]
Plasmodium Falciparum		detector e⁻ e⁻ sample	[43,44]

the same, which isn't considered a chief pre-requisite while working in wet labs. Depending on the accelerating voltage, electrons from the electron beam can interact with electrons in the atoms of the sample. This addition further involves the implementation of design modifications and construction to accommodate and run securely. Transmission Electron Microscope analysis is extremely sensitive to vibrations and static electro-magnetic artifacts, hence they are performed in isolated areas with ambient conditions, where they are not exposed to external artifacts or unintended interruptions.

The absorbed radiation instilled within the specimens during TEM, is equivalent to the radioactivity estimated from a hydrogen bomb. The sample constituents on getting exposed to extreme energetic cathode rays endure disintegration, alteration of chemical bonds, or other structural modifications [12].

In TEM, sample analysis is limited to materials that are electron transparent.

Transmission Electron Microscope functions similar to how we observe 2D images of 3D specimens, which are observed as part of the transmission spectrum often referred to as projection limitation. A basic characteristic of this constraint is that the images, diffraction patterns, and the spectral information obtained through microscopy are quantified by measuring the thickness of the specimen only. This signifies devoid of depth sensitivity in a single Transmission Electron Microscope image, which owes to difficulty in image visualization and interpretation [13].

TEM remains the main technique which makes it possible to study biological systems owing to its near-atomic-level resolution. Moreover, TEM gives opportunities to visualize an interesting target with surrounding structure, when unlabeled surroundings still remain hidden at fluorescence sample.

In TEM, most recently, new hybrid techniques have been developed for difficult-to-fix organisms and antigens or labile and anoxia-sensitive tissues.

Protocol to Observe Damaged Cell Membranes

A fast way to see damaged membrane in TEM is to do negative staining:

- Initially, the sample has to adhere at the surface of a coated EM grid (formvar for example) then contrast with uranyl acetate before drying.

- Next, we can see the morphology of bacteria and locate blobs at the surface of bacteria indicating pores and leak of cytoplasm.

- Bottomline is to make the sample adhere at the surface of glass coverslip, and contrast with osmium tetroxide (optional), dehydrate with ethanol and then dry with a critical point dryer or with hexamethyldisilasane.

For dehydration, acetone or acetonitrile can be used instead of ethanol.

Bacteriophage Sample Analysis

Sample should be pure and isolated bacteriophages, otherwise, we will see contaminants present in your sample. For any disparities, it's recommended to prepare a fresh saturated aq. UA stain and filter it before use. Also, we must look for uniformly stained dark circles under TEM (not look for dark granular particles). Operate TEM at 80kV, which is standard for biological sample analysis. Another suggestion is to check the spot size to work on, because the image may seem out of focus, usually for viruses, it is convenient to work with a smaller spot size [15].

Scanning Electron Microscope

Identification with Scanning Electron Microscope is a major expenditure, since specimens undergo coating with vaporized gold or palladium ions. This is done in order to generate secondary emitted electrons from the coating which is necessary for this type of microscope detection.

Hence, while working with this kind of microscope, specimens have to be both surfaces electrically conductive and electrically grounded to avert the accumulation of static charge. When an incident electron beam of the Electron Microscope strikes a sample, many different signals originate from momentary layers of the specimen structure. The layers of emission of the different signals are not constant and relyon the accelerating voltage of the electron beam and the sample constituents. The topography thus generated of the micro-organism must retain a distinct resolution for 3D visualization and image acquiring [16].

Also, the entire set-up of atop stacked electron lenses called as electron column of the scanning electron microscope is flanged to a high vacuum pump which is maintained continuously. Similarly, the electron source generator is too enclosed inside a superior chamber to encompass a vacuum and defend it against contamination, vibrations, and noise. Scanning Electron Microscopes are only limited to solid, inorganic samples which are small enough to fit inside the vacuum chamber, with the ability to endure moderate vacuum pressure. Reduced vacuum levels will shorten the life of the electron emission source. Also, budgeting in designing the vacuum system will prove to be expensive if filaments need constant replacement [17].

Most SEMs use a solid-state x-ray detector (EDS), and while these detectors are very fast and easy to utilize, they have relatively poor energy resolution and sensitivity to elements present in low abundances [18].

Generally, in SEM, samples must be processed to dryness. Bulk, granular, and powdered materials may not require processing. Biological specimens will typically be treated with protein and lipid cross-linking reagents, such as glutaraldehyde (GA) and osmium tetroxide (OsO4) respectively, dehydrated with an organic solvent, such as ethanol (ETOH) or acetone, and critical-point dried through carbon dioxide. In recent years, silicon chips have become popular steb mounting substrates for SEM imaging of widely varied types of samples [19].

The effect of vacuum on microbes corresponds to water desorption capacity. Extremely maintained water vapor pressure can lead to inactivation and dormancy of the sample in hand. Hence, the vacuum inactivated cells (indicated as loss of colony-forming ability) get compromised. The Vacuum alsodisrupts the normal cellular elongation, phage production or inhibits the respiration of the microbes. The cellular membrane of the microbe becomes permeable due to vacuum exposure, which can further lead to the release of intracellular contents [20].

SEM is also limited when used to image non-conductive samples and color images, or when used to take measurements involving height. Although, sputter coating with an additional thin layer (~10 nm) of a conductive material, such as gold, silver, platinum or chromium, can help remove charging effects and produce better quality images. However, the drawback of the coating is, it removes the atomic number-contrast and elemental composition analysis.

Depth of field can be increased by choosing the largest available f-stop (smallest aperture) which increases the focal range, thus ensuring that distant points within the landscape are in sharp focus. Similarly, smaller apertures and longer working distances both increase depth of field in the SEM.

Increasing the depth of field in an image for acquiring better quality can be done by: -

a) Escalating the distance of working magnification

b) Reducing objective lens aperture size

c) Culminating both the interventions

Choosing the right accelerating voltage is critical for obtaining a good clear image however the most suitable voltage level depends will depend on mostly on the type of material being examined. In the above image, we notice at low accelerating voltage, surface structure appears clearer.

Preparation of Cell Suspensions

#1. Adherence of Cells to Poly-L-Lysine-Coated Slides - Candida albicans yeast cells attached to the surface of a poly-L-lysine coated microscope slide.

- To adhere cells to a substrate, transfer several milliliters of culture to a microscope slide coated with poly-L-lysine and allow the cells to settle at room temperature for 1 hr.

- If cells do not adhere adequately using the poly-L-lysine procedure, then the organo-silane procedure should be used.

- Protect the slide from evaporation.

- Tip the slide to drain the culture liquid, leaving some cells adhering to the surface of the slide.

- Use a pipet to very gently flow 2.5% glutaraldehyde in phosphate buffer over the slide.

- Allow the slide to stand undisturbed for 1 h at room temperature.

- Drain the glutaraldehyde from the slide and rinse three times in phosphate buffer, each for 5 min.

- Replace the distilled water with 1% osmium tetroxide in either phosphate buffer or distilled water.

- Rinse in distilled water and dehydrate the specimen using a graded ethanol series consisting of 25%, 50%, 75%, and two x 100% each for 10 min.

- Transfer the slide, still submerged in absolute ethanol, to the critical point drying apparatus complete the drying of the specimen.

- Affix the slide to a specimen stub and apply a heavy metal coating using either a sputter coater or vacuum evaporator and finally observe under SEM.

#2. Cultured cells on an Agar Surface - Aspergillus fungi grown

on agar medium.

- Place excised agar pieces in a Petri dish or other vessel containing 2.5% glutaraldehyde in phosphate buffer to submerge the cells. Keep the cell layer uppermost.

- After 30 min fixation at room temperature, rinse the specimen in three changes of phosphate buffer for 5 min each.

- Post-fix the cells 1 h at room temperature in 1% osmium tetroxide, phosphate buffered or in distilled water.

- Rinse three times in distilled water for 5 min each. After the last rinse, transfer the fragile specimens into holders, such as the microporous holders for subsequent steps and finally observe under SEM [23].

Future Prospects

Electron microscopy must be considered to be entitled as the supreme "catch-all" detection technique, providing us with an "open view" for complicated measurements. It needs to get inducted as a novel or emerging pathogen investigating platform of which there isn't any prior knowledge of the category or the agent present. Electron microscope must compose of a model for the open, highly integrated and data-driven architecture, that needs to address future challenges in the field such as energy storage, quantum information science, and materials design. In terms of analyzing multiple representative samples and integrating large volumes of specimen data from high-speed detectors, present technology contend [45].

The big caveat is that, improvising greater resolution comes with even variant ambient temperature conditions. Most lab studies performed is generally available only near room temperature. But temperature sensitive microbes like thermophilic bacteria and fungi, viruses, etc., requires conditions different from room temperature. There are significant issues in reaching those types of resolutions if we go to very low temperatures or very high temperatures. Thus, high-resolution-low-temperature-regulative electron microscope is a thing of future to advance on manipulating in less than a degree of Kelvin. Going cold would be one way to try to slow down processes to examine materials dynamically that are too fast to capture at room temperature. It'll also enable to retrieve a better picture and precise measurements and enable atomic resolution imaging, in an environment that is important for many advanced technologies, like quantum computing.

An approachto enhancing the ability of a Scanning Electron Microscope can be to design and position an appropriate detector beneath the thin specimen, that will assemble the electrons that got transmitted through the sample.If lasers of the Electron Microscope can manipulate the energy and phase of electrons it will open up new potential applications and horizons in the microbial detection field [46].

Any instrument should possess the ability to image a sample in its "native state" instead of first being desiccated, coated in gold, and held under a high vacuum. If water vapor is used as the imaging gas, the sample chamber becomes a suitable environment for stabilizing liquid-containing specimens and performing in situ experiments — for example, a sample can be studied because it is put through cycles of wetting and drying.

Specimens can also be heated, cooled, stretched, compressed, and otherwise manipulated, when in association with a variety of inert gases appropriate to the microbial environment. Deep learning-based super-resolution with Artificial Intelligence can also be incorporated due to its robustness and it's being apractical tool. This can analytically increase the resolution in Scanning Electron Microscopes. Without appropriate operation and management in EM, it results in various artifacts like multivariate noises, sensing anomaly, scan contortion, specimen-on-slide movement, and cathode rays carring. These can be eradicated through complex algorithms designed through Artificial Intelligence and Machine Learning.

Adopting spatial frequency analysis method, scientists can process images that are supplied with frequency spectra along with coordinating higher resolution SEM images constituting identical perspective. This technique assists in attaining higher resolution SEM images rapidly, concurrently reducing both electrons charging and sample damage [47].

Alternative imaging techniques like ptychography also can be accustomed that will further intensify the resolution in SEM.

Another major intervention in improving could be, supplementing the electrons into a better amalgamated probe, i.e., circumventing them through an array of detectors and filters to alter their phase. This imperative additional data induced in the electron beam, will aid proficient scientists to determine more about the sample's properties. "The pursuit should be to acquire more accurate information from the striking electron, with least characteristics variation."

Cryogenic electron microscopy technique relies on frozen samples embodiment in a glass type ice and tested by an electron beam. This could be a revolutionary approach if we could pre-determine the optimum temperature suitable for the microbe to incubate and remain active for a lengthy time, without sliding into the lag phase. But here the samples should be unstained, hence, this method generates reduced contrast images. Moreover, cryo-sectioning of delicate tissues is a physically endured process.

Algorithmic augmentations incorporated into SEM images include de-noising and de-convolution to reduce the spatial distortion in the image. Imaging Technologies like UHD and 3D if integrated into electron microscopes may fuel the demand for the same commercially.

The establishment of serial block-face SEM (SBEM) which employs image blocks of ultra-thin tissue removed using an ultramicrotome and later incorporated into the microscope. This technology produces 3D images with a greater resolution with precision.

The progression of FIB-SEM (Focused Ion beam Scanning Electron Microscopes) signifies another major advancement in SEM technology. In FIB-SEM, a focused beam of ions replaces the utilization of a beam of electrons. Here, the ion beam is used to remove ultrathin layers of tissue, allowing the reconstruction of z-stacks. The major advantage of the FIB-SEM method is that it can produce resolutions of up to a couple of nanometers, enabling the visualization of the intracellular environment in high resolution.

A recent novel technique was developed to observe live hydrated specimens using a low-energy electron beam, a rapid vacuum system,

and minimal specimen preparations. First, the biological samples were anesthetized via refrigeration and mounted on a stub using denatured alcohol and graphite. Next, they were observed using SEM with a fast pump-down vacuum system at 5 kV. It took approximately 1 min to reach 6.5 × 10−3 Pa. After SEM observations, a few specimens were still alive and returned to the field [48].

Another property one can value-add is to obtain high-resolution information about the structure of nanomaterials with co-localized elemental mapping in samples of nanometer dimensions.

Ultimate objective is to manipulate materials' behavior, i.e., initially from intra-atomic dynamicity progression to nanoscale structural dismantling and finally electronic phase segregation, that incorporates pump-probe-based rapid detection method.

Most challenging of all is to improve the spatial resolution of electron microscopes. Although, it has got revolutionized and enhanced by the development of optical aberration detectors for image-forming and probe-forming lenses, but still is inaccurate and ambiguous.

One can introduce liquid cells in the design blocks of the microscope, that'd help in isolating the liquid from the high vacuum environment. Liquid cell TEM will not only preserves the liquid state of the specimens inside the TEM vacuum but also allow the in-situ observation of the biological process without pre-dehydration of the sample [49].

There is an urgent necessity to fill the void that exists between forthcoming sophisticated technologies with the advances of research, understanding the concrete application of newly developed methods must be within the standard pipeline of routine analysis.

Conclusion

The advancements on electron microscope in recent years has been strictly directed with the desire to improve the spatial resolution of electron microscopes. Electron Microscope is still designated as the spearhead discovery in virus identification, predominantly in circumstances where pathogens are unknown or unanticipated. This has been regarded as a valuable technique in the supervision for monitoring emerging diseases. Also, due to environment effluence and other lab practices, the emergence of novel antibiotic drug resistant bacteria is on the rise ever since, along with highly mutating viruses. All of these are posing detrimental effects on the longevity of human generation and requires rapid detection and identification. A recent study estimated that approx. 1400 pathogenic agents are the etiological factors in majority of human diseases. Microbe like bacteria alone is the indispensable source of over 350 million cases of infectious diseases.

Scanning Transmission Electron Microscopy (STEM), a foundation in the study of chemical and materials systems, will stand to assist significantly from Artificial Intelligence-driven automation. Scanning electron microscopy renders bacteria as three-dimensional objects in various environments close to their natural appearance provided that the nature of the specimens is rigorously respected. But, existing hurdles like ambiguous instrument control, along-with operational and interpretable feature detection, reminds us that fully automated microscopy is still a thing of future [50]. Overall, TEM offers unparalleled detail but can only be used on a limited range of specimens and tends to be more demanding than SEM.

Moreover, an Electron microscopy practical training program costs in the range of 20,000 INR to 50,000 INR per individual, owing to understanding its sophistication and rigorous sample preparation techniques.

Thus, we are familiarized now that Transmission electron microscopes cannot be used to image living cells because of the high energy electrons will destroy the samples. Scientists have hypothecated an idea to overcome this limitation. They feel that by introducing a quantum mechanical measurement tech with the exclusive feature of admitting electrons to sense the material remotely, damage can be averted since the electrons would never directly hit the sample object but scan through the same [47].

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