Research Techniques Made Simple: Volume Scanning Electron Microscopy

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Volume scanning electron microscopy (VSEM) involves the serial sectioning and imaging of a sample using scanning electron microscopy (SEM), followed by segmentation and three-dimensional (3D) reconstruction using computer software packages to allow visualization of 3D structures. VSEM can reveal qualitative and quantitative properties of organelles and cells within tissues at nanoscale. The ability to visualize spatial relationships of structures of interest within and across cells in 3D space in particular sets VSEM apart from conventional SEM and transmission electron microscopy. Here, we provide an overview of VSEM platforms and image processing, highlighting characteristics that will aid selection of a method to address specific research questions in dermatological research.

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INTRODUCTION

Volume scanning electron microscopy (VSEM) represents an evolutionary step forward in scanning electron microscope (SEM) technology, enabling the three-dimensional (3D) visualization of single cells in biological samples (Ronchi et al., 2021). VSEM technologies acquire SEM images of serial sections of a biological sample in an automated, high-throughput manner, resulting in the generation of SEM image stacks (reviewed in [Titze and Genoud, 2016]). Using computational segmentation, structures of interest such as subcellular organelles (e.g., mitochondria) can be tracked and reconstructed across the image stack, allowing for 3D visualization (Liu et al., 2020). This 3D reconstruction can reveal size, shape, and spatial relationships of structures of interest not apparent in standard two-dimensional (2D) SEM, with the ability to also obtain qualitative and quantitative data. Mitochondria, for example, which typically appear as circular or oval objects in 2D, exist as complex branching structures in cells when visualized with VSEM (Liu et al., 2020) (Supplementary Videos S1 and S2). Recently, VSEM technologies have been used to study psoriasis (Lindberg et al., 2021), melanocytic disorders (Mizutani et al., 2021), and keratinocyte (KC) differentiation (Yamanishi et al., 2019), offering novel biological insights into a range of processes in skin health and disease.

SEM: FROM 2D TO 3D

VSEM builds on Ruska and Knoll's seminal Nobel prize winning work that led to the development of the first electron microscope (Knoll and Ruska, 1932). Electron microscopes use high-speed electron beams that are targeted at a suitably prepared sample section (stained with heavy metals, such as osmium and uranium) and generate an image from detected electrons that have interacted with the sample. Briefly, electrons are first generated from an electron source, such as a tungsten filament. A positively charged anode is then used to accelerate the electrons, which are focused as a beam by a series of electromagnetic lenses and apertures. The electron beam travels down a column under high vacuum, where the electrons interact with the sample section. Electron beam generation is similar in both the transmission electron microscope (TEM) and SEM, with the key differences relating to how the image is detected. In TEM, the electron beam passes through the sample, which creates a projection that is captured by a detector on the other side of the sample. Conversely, SEM uses a beam, focused to a point, to scan across the surface of a sample. Here, the detectors are on the same side of the sample as the electron beam, and an image is acquired from electrons bouncing back (referred to as backscatter) from the sample. Electrons from the beam interact with the sample and also generate secondary electrons (electrons originating from within the sample when exposed to the external beam). Both types of electrons are detected separately, and the intensity of either electron type determines the brightness of each scanned point in the image. SEM is recognized to be faster than TEM for large areas; however, this advantage comes at the cost of a lower image resolution. VSEM was developed to transform the process of serial imaging of TEM sections of a sample (serial section TEM) by automating sectioning and employing SEM imaging. There are three key VSEM platforms, with respective advantages (Table 1 and Figure 1): serial block

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Abbreviations: 2D, two-dimensional; 3D, three-dimensional; ATUM-SEM, automatic tape-collecting ultramicrotome combined with scanning electron microscopy; CNN, convolutional neural network; DL, deep learning; FIB-SEM, focused ion beam milling combined with scanning electron microscopy; KC, keratinocyte; ML, machine learning; SBF-SEM, serial block face sectioning combined with scanning electron microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TGN, trans-Golgi network; VSEM, volume scanning electron microscopy

SUMMARY POINTS

- Volume scanning electron microscopy (VSEM) allows for high-resolution imaging of biological features down to nanoscale.
- There are currently three main VSEM platforms: serial block face sectioning combined with scanning electron microscopy (SEM), focused ion beam milling combined with SEM (FIB-SEM), and automatic tape-collecting ultramicrotome combined with SEM.
- Each of the three platforms have respective advantages and should be chosen guided by the size of the structure of interest that is to be studied in the research question.
- Segmentation of structures of interest can be time consuming, and recently described machine and deep learning packages can help address this limitation.

Limitations

- Heavy metal staining employed in VSEM prevents the use of fluorescent imaging of the same sample; however, this is being overcome using cryo-FIB-SEM.
- Some features (e.g., mitochondria) still require manual segmentation to refine automatic segmentation.

face sectioning combined with SEM (SBF-SEM), focused ion beam milling combined with SEM (FIB-SEM), and automatic tape-collecting ultramicrotome combined with SEM (ATUM-SEM).

VSEM

All three VSEM platforms typically require impregnation or staining of the sample with heavy metals, because these provide electron-dense elements that are differentially absorbed by structures in the sample, necessary to provide contrast between structures within the detected image. Tissue preparation involves fixation of the tissue sample, heavy metal staining of the sample, dehydration, resin infiltration, and embedding (Figure 1a). After embedding, the sample is located by hand trimming excess resin, mounted on a block or aluminum pin, and subjected to serial sectioning. Alternative preparatory techniques such as high-pressure freezing may also be used, termed cryo-FIB-SEM, obviating the need for heavy metal staining but requiring additional equipment to keep the sample at very low temperatures (Ronchi et al., 2021).

In SBF-SEM (Figure 1b), the sample is under vacuum, and SEM scans and images the block face of the sample. An internal ultramicrotome within the vacuum chamber then removes a section of tissue (section thickness, ~30–70 nm) and the next SEM image is acquired; this process repeats for a user-specified number of iterations resulting in a z-stack of typically several hundred image slices. SBF-SEM offers the most consistent imaging across the longest continuous z-depth of the three platforms; however, there is only one opportunity to image a section given the intrinsic destructive nature of this technique. FIB-SEM uses a focused gallium ion beam to remove layers of the sample after each SEM imaging pass. FIB-SEM acquires very thin physical sections at 3–5 nm (Titze and Genoud, 2016), allowing for high-resolution data capture. The depth of tissue that can be imaged with FIB-SEM runs to less than 1 mm (Xu et al., 2017) owing to technical limitations of the gallium ion beam technology used and imaging drift. ATUM-SEM differs from the previous two techniques in that the sectioning of the sample is performed outside the microscope at atmospheric pressure. The technique uses a conveyor belt to automatically collect ultrathin sections cut on an ultramicrotome, allowing the collection of thousands of sections on a tape, which is then manually cut and mounted onto 100-mm silicon wafers. Wafers can then be imaged in a standard SEM, multiple SEMs simultaneously, or a multibeam SEM (Eberle et al., 2015). ATUM-SEM can cut the widest sections (3–4 mm) and is nondestructive, so sections can be reimaged. However, sections can be lost and subject to X-Y plane distortion, which makes subsequent alignment of the images acquired more challenging than those acquired with SBF-SEM or FIB-SEM.

Table 1. Comparison of VSEM Platforms

Technical Specification	FIB-SEM	SBF-SEM	ATUM-SEM
Minimum isotropic voxel size (nm)	3 × 3 × 3	15 × 15 × 15	30 × 30 × 30 (Titze and Genoud, 2016)
Ideal feature size range[1] (diameter)	100 nm–200 μm	1,000 nm–500 μm	1,000 nm–200 μm
Destructive sectioning?	Yes	Yes	No
Max. cutting/milling depth (Z)	Limited to ion gun range (manufacturer dependent), system reliability	700 μm	200 μm (ATUMtome attached to a PowerTome PC ultramicrotome)
Max. block face width	100 μm	1.5 mm	Diamond knife width (3–4 mm)[2]
Data capture rate[3] voxels/sec	74,074	222,222	171,468

Abbreviations: ATUM-SEM, automatic tape-collecting ultramicrotome combined with scanning electron microscopy; FIB-SEM, focused ion beam milling combined with scanning electron microscopy; Max., maximum; SBF-SEM, serial block face sectioning combined with scanning electron microscopy; VSEM, volume scanning electron microscopy.

[1]Limited by knife width and feasibility, less practical with increasing object size.

[2]Sectioning becomes less reliable with increasing block widths.

[3]Adapted from Titze and Genoud (2016) (voxels in a 20 × 20 × 20 μm volume per time taken in seconds).

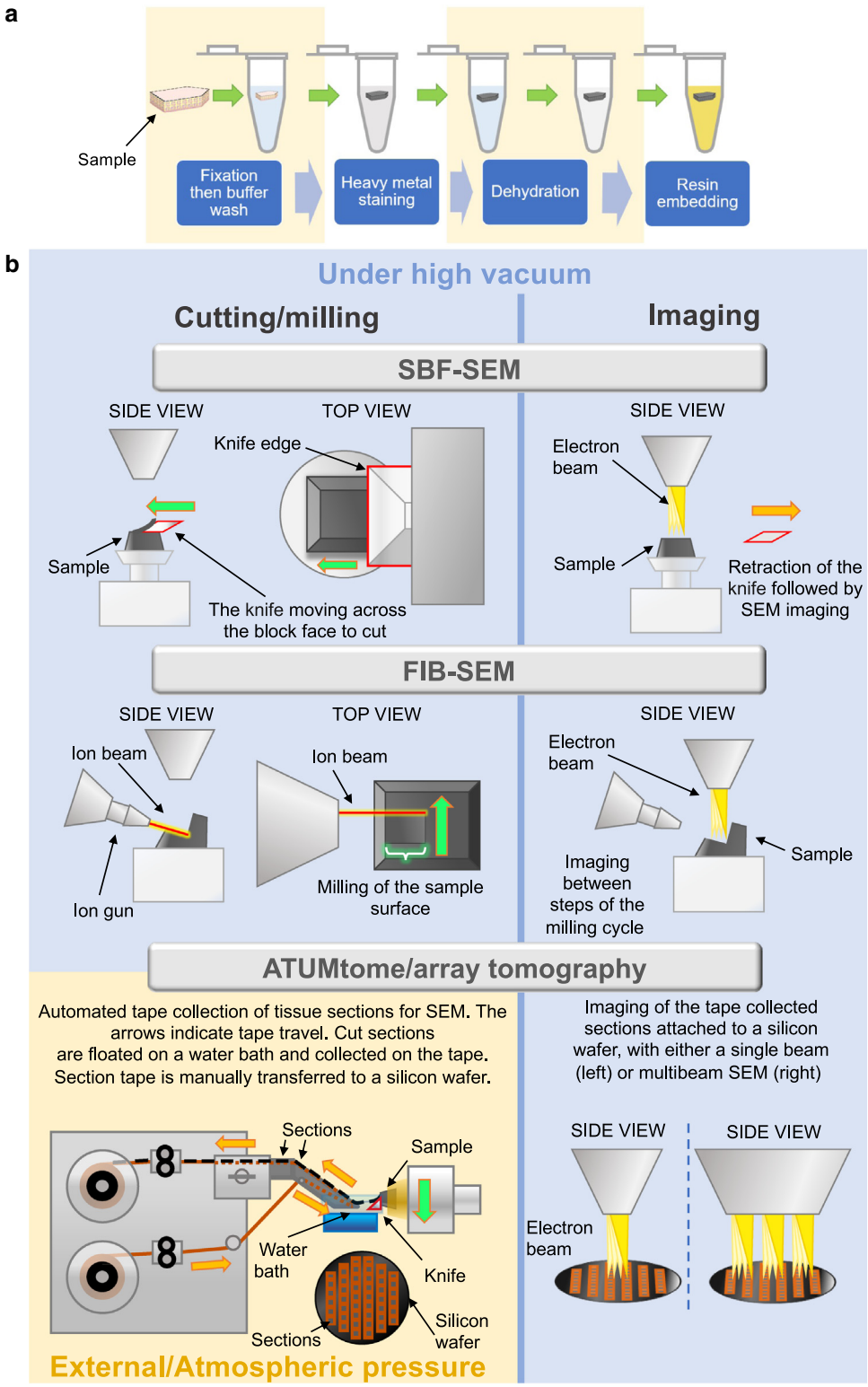

Figure 1. An overview of VSEM platforms. (**a**) Typical sample preparation involving fixation with glutaraldehyde, heavy metal staining, dehydration, and resin embedding. (**b**) Sectioning/milling and imaging processes used in VSEM platforms. FIB-SEM, focused ion beam milling combined with scanning electron microscopy; SBF-SEM, serial block face sectioning combined with scanning electron microscopy; SEM, scanning electron microscopy; VSEM, volume scanning electron microscopy.

VSEM IMAGE PROCESSING AND ANALYSIS

To obtain 3D images, serial section images must undergo postprocessing and segmentation before 3D visualization can be performed (Figure 2). Postprocessing (Figure 2a) can include brightness/contrast adjustments, contrast normalization across slices, alignment (also referred to as image registration), and application of filters to improve image quality. The physical section thickness is required when the stack is virtually assembled, because software packages need to know how far apart image slices are to determine the estimation of the voxel size, a 3D image building block analogous to 2D pixels. FIB-SEM can deliver isotropic voxels where all three dimensions are equal, for example, 5 nm × 5 nm × 5 nm (x, y, and slice

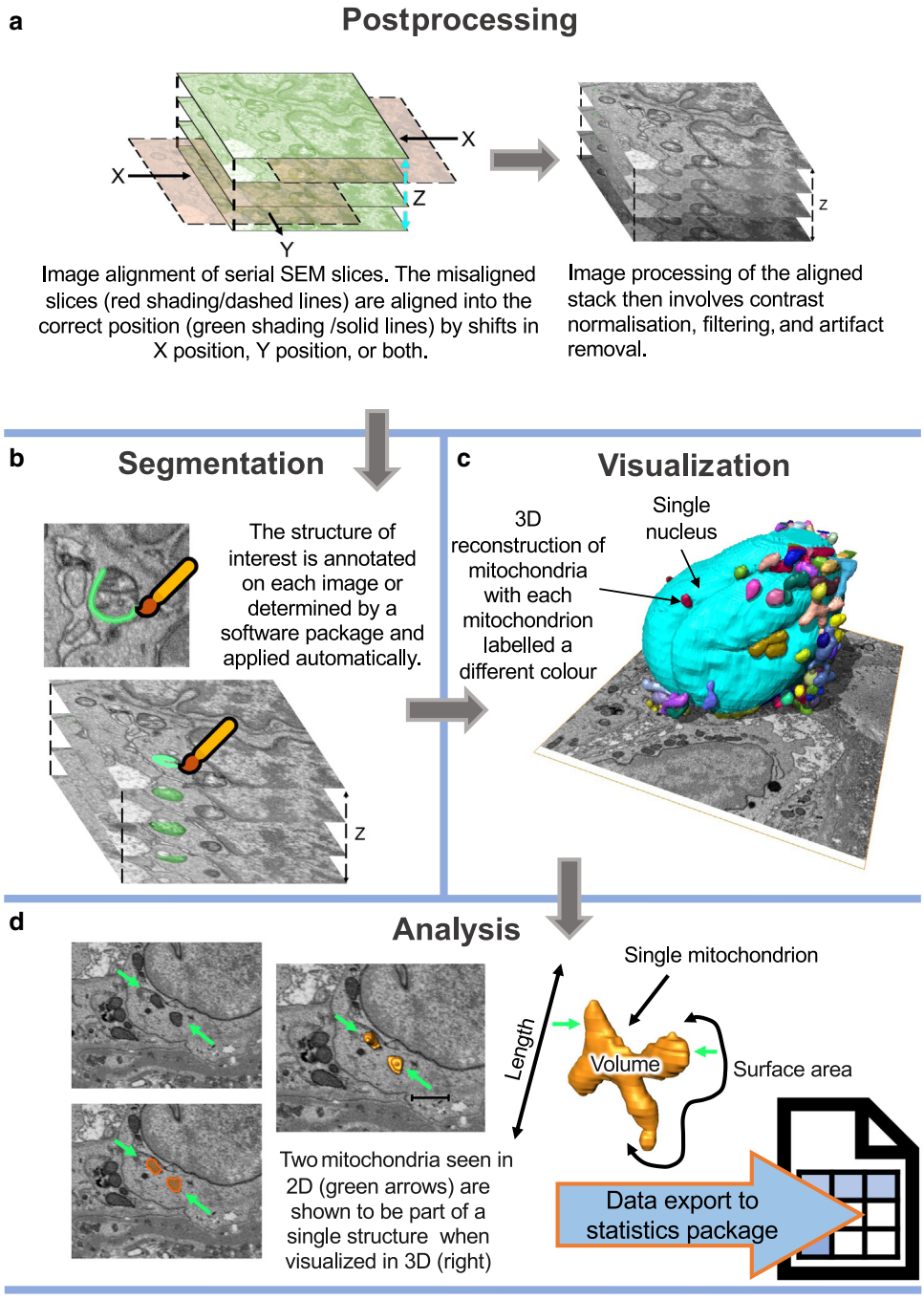

Figure 2. Postprocessing and image analysis of VSEM data. (**a**) Postprocessing of images is used to correct image shifts, contrast, and artifacts or to apply filters to enhance features such as edges. (**b**) Segmentation, where the microscopist annotates structures of interest. (**c**) 3D visualization of the segmented structures is achieved using surface rendering, allowing for qualitative and quantitative analysis. (**d**) Measurement of physical properties of surface-rendered structures of interest, including size, shape characteristics, and spatial relationships, is feasible, followed by tabulation for each identified structure and data export for statistical analysis. 2D, two-dimensional; 3D, three-dimensional; SEM, scanning electron microscopy; VSEM, volume scanning electron microscopy.

thickness z), whereas SBF-SEM and ATUM-SEM routinely deliver anisotropic data (e.g., 6 nm × 6 nm × 30 nm). Alignment, which is largely automated, is then performed and involves aligning each image to the adjacent image above and below in a stack, which may contain several hundred images. Segmentation (Figure 2b), the process of annotating specific features of interest, has to be performed on each image slice in the stack and can be achieved with several software packages (Table 2). For example, to track mitochondria through an aligned stack, each mitochondrion has to be identified and annotated on each image slice, a task that is labor intensive to perform manually but considered the gold standard when performed by an expert microscopist (Spiers et al., 2021). Semiautomated segmentation techniques can be effective in selected applications, for example, where the structure of interest has a significantly different pixel intensity from surrounding structures. Thresholding segmentation selects all pixels in the structure of interest in the image slice within a defined intensity range and, although quick, is prone to erroneous segmentation. Watershed segmentation is a region-based semiautomated technique, requiring limited user input to annotate structures of interest. The watershed algorithm requires the user to select a seed point within a structure of interest and denote background with a separate marker. This seed area is then grown until

Table 2. Comparison of Image Processing and Visualization Packages

Software Package	Description	Open Source or Commercial	Postprocessing	Segmentation	3D Visualization	3D Analysis
Amira (Thermo Fisher Scientific, Waltham, MA)	High-end 3D bioimage analysis software	Commercial	Yes	Manual to machine learning	Extensive tools including animation	Yes
Fiji Imagej.net	General bioimage analysis software	Open source	Yes	Manual to machine learning	Yes	Yes
Ilastik (Berg et al., 2019)	Bioimage analysis software with a selection of machine learning tools	Open source	Limited to steps relevant to each algorithm	Extensive machine learning	Limited to 3D preview of segmentations	No
Imaris (Oxford Instruments, Abingdon, United Kingdom)	High-end 3D bioimage analysis software	Commercial	Yes	Limited	Extensive tools including animation	Yes
Microscope image browser mib.helsinki.fi	General bioimage analysis software	Open source	Yes	Manual to machine learning	Yes	Yes

Abbreviations: 2D, two-dimensional; 3D, three-dimensional.

it reaches a change in pixel intensity that it recognizes as a border calculated on intensity of the seed and background markers. Some software programs, such as Microscopy Image Browser (http://mib.helsinki.fi/) and Ilastik (Berg et al., 2019), include watershed segmentation options that can be applied per slice or across multiple slices, where the structure of interest is treated as a volume. Magic wand segmentation, a further semiautomated method, expands a user-selected region within a structure of interest and defines boundaries based on neighboring pixel data. Magic wand semiautomated segmentation has been used to segment lamellar granules in KCs (Yamanishi et al., 2019). Fully automated segmentation using machine learning (ML) and deep learning (DL) methods has been recently described and shows promise to reduce the time taken for high-quality segmentation (Liu et al., 2020). ML has been applied to psoriatic skin samples for the purpose of segmenting nuclei, where the pixel classification tool Ilastik was used (Lindberg et al., 2021). Here, supervised ML is performed on user-defined manually segmented training data with the assumption that the training data with ground truth annotations reflect the range of pixel variability within the structure of interest in the dataset. The ML package may require extensive training before it can be deployed, limiting its usefulness in small-scale experiments. Recently, DL packages such as DeepMIB (Belevich and Jokitalo, 2021) allow for training of convolutional neural networks (CNNs) on 2D or 3D SEM data. Training for DL also requires preparation of manually segmented datasets to define structures of interest; however, it can leverage prior CNN training resources (e.g., U-Net) such that fewer training image slices are required before deployment. Computational hardware requirements for different methods of segmentation may also be a factor in selection (Titze and Genoud, 2016). Irrespective of the method of segmentation used, quality control assessments by a trained microscopist are an important part of VSEM analysis pipelines, because the quality of the segmentation will impact on downstream data analysis.

Once segmentation of the structure of interest is achieved throughout the stack of image slices, 3D visualization (Figure 2c) permits study of the complex relationships between biological structures. Surface rendering provides a visible 3D shape to the segmented structures. Surface-rendered 3D images can be explored, allowing the user to virtually fly around structures of interest, with the option to enhance the onscreen experience using virtual reality headsets. Qualitative observations of the data that can be made include shape, connectivity, and spatial relationships between objects. Quantitative analysis (Figure 2d) of VSEM delivers geometric data (length, surface area, volume), shape metrics (sphericity, solidity, elongation), counts, clustering, contact (between objects), and distance (between objects). Assumptions made when volumetric analysis is performed include a consistent section thickness and lack of distortion of a section by stretch. Methods for estimating the extent of stretch and variation in z-thickness have been developed using nonparametric Bayesian regression of image statistics, allowing for correction of such artifactual changes (Ambegoda et al., 2020[1]).

APPLICATIONS OF VSEM IN DERMATOLOGY RESEARCH

FIB-SEM has been used to study the morphology of the trans-Golgi network (TGN) and lamellar granule structure during KC differentiation in human skin samples (Yamanishi et al., 2019). The lamellar granules are recognized to transport lipids, proteins, and protein inhibitors from the TGN to the extracellular space. Yamanishi et al. (2019) studied sections of human skin and demonstrated detailed imaging of 3D lamellar granule structure within cells, demonstrating transition from discrete vesicles to a reticular network as KCs underwent differentiation. This change was seen from the first to the second layer of cells in the stratum granulosum. Changes in lamellar granules across cellular strata corresponded with

[1] Ambegoda TD, Martel JNP, Adamcik J, Cook M, Hahnloser RHR. Estimation of Z-thickness and XY-anisotropy of electron microscopy images using Gaussian processes. arXiv 2020.

MULTIPLE CHOICE QUESTIONS

1. Which volume scanning electron microscopy (VSEM) technique has the smallest physical voxel size?
   A. Serial block face sectioning combined with scanning electron microscopy (SBF-SEM).
   B. Focused ion beam milling combined with scanning electron microscopy (FIB-SEM).
   C. Automatic tape-collecting ultramicrotome combined with scanning electron microscopy (ATUM-SEM).
2. Which of these statements about VSEM is true?
   A. Sample sections are destroyed in all VSEM techniques.
   B. VSEM is faster than serial section transmission electron microscopy (TEM).
   C. VSEM is slower than serial section TEM.
   D. VSEM cannot be applied to skin samples.
3. What does FIB-SEM use to cut a sample?
   A. A laser.
   B. A diamond knife.
   C. A lathe.
   D. A gallium ion beam.
4. Which of these is true about SBF-SEM?
   A. It can image larger whole samples than FIB-SEM.
   B. Sections are kept after cutting.
   C. It can achieve isotropic voxel sizes of $3 \times 3 \times 3$ nm (x,y,z).
   D. Sectioning is performed at atmospheric pressure.
5. Which of these is true of ATUM-SEM?
   A. Collection of sections occurs under vacuum.
   B. Sections are lost after cutting.
   C. Sections are kept after cutting.
   D. The combined process of sectioning and imaging is fully automatic.

increased volume and branching of the TGN, and lamellar granules were demonstrated in the intercellular space. The detailed structural data contribute to the understanding of membrane trafficking of epidermal cells and will inform the development of models to refine understanding of how specific cargoes are handled by the TGN and sorted to lamellar granules in KCs. FIB-SEM was also used to study psoriatic skin (Lindberg et al., 2021), demonstrating increased nuclear size and nuclear membrane pore features specific to lesional psoriatic KCs compared with perilesional skin. FIB-SEM (Mizutani et al., 2021) and SBF-SEM (Noh et al., 2019) have been used to study the interaction of melanocytes and KCs with the aim of further understanding the mechanisms by which melanosome transfer occurs from melanocytes to KCs in senile lentigo, melasma, and seborrheic keratosis.

CONCLUSIONS AND EMERGING DEVELOPMENTS

VSEM platforms have their respective advantages and ideally should be chosen based on the biological question to be addressed. The applications to dermatological research are broad, and in addition to tissue sections, cultured cells and organoids may be studied (Buskin et al., 2018). VSEM is continually being advanced, with recent modifications to techniques such as cryo-FIB-SEM allowing both visualization of fluorescent reporter proteins in labeled cell populations and FIB-SEM on the same sample, opening up new avenues for research. High-quality segmentation is a bottleneck in VSEM data processing that may soon be eased with continued advances in ML and DL, bringing benefits to end users. Recently, aggregated crowdsourced segmentation of the nuclear envelope was used to train a CNN to segment nuclei, utilizing large numbers of citizen scientists to perform manual segmentation (Spiers et al., 2021). In biology, correlative study of structure and function has long provided novel insights, and VSEM is well placed to advance understanding of ultrastructure in relation to biological function in dermatological research.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Conceptualization: RL, DHS, NR; Data Curation: RL, DHS, NR; Supervision: DHS, NR; Writing - Original Draft Preparation: RL, DHS, NR; Writing - Review and Editing: RL, DHS, NR

SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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DETAILED ANSWERS

1. **Which volume scanning electron microscopy (VSEM) technique has the smallest physical voxel size?**

CORRECT ANSWER: B. Focused ion beam milling combined with scanning electron microscopy (FIB-SEM).

Physical sectioning with an ultramicrotome is limited to sections in the order of tens of nanometers, but focused ion beam milling surpasses this.

2. **Which of these statements about VSEM is true?**

CORRECT ANSWER: B. VSEM is faster than serial section transmission electron microscopy (TEM).

VSEM is faster than serial section TEM because it automates the process of sectioning and imaging and also removes the need to manually collect the serial sections that then must also be imaged in order, which is a laborious and time-consuming step.

3. **What does FIB-SEM use to cut a sample?**

CORRECT ANSWER: D. A Gallium ion beam.

The beam used in focused ion beam milling is not a laser (beam of photons) but a beam of ions, which have mass, allowing the beam to ablate (mill) the sample in 5-nm layers.

4. **Which of these is true about SBF-SEM?**

CORRECT ANSWER: A. It can image larger whole samples than FIB-SEM.

Continuous imaging runs with FIB-SEM of whole samples is limited to a depth of hundreds of microns (commonly <300 μm) and 100-μm width (Titze and Genoud, 2016). Serial block face sectioning combined with scanning electron microscopy (SBF-SEM) can routinely image samples greater than 600−700 μm in depth and with a width of up to 1.5 mm.

5. **Which of these is true of ATUM-SEM?**

CORRECT ANSWER: C. Sections are kept after cutting.

The sections are collected on tape in automatic tape-collecting ultramicrotome combined with scanning electron microscopy (ATUM-SEM) and can be reimaged and even restained. It is worth noting that unlike FIB-SEM and SBF-SEM, the whole process of ATUM-SEM is not automatic, because the sections must be manually transferred to the microscope for imaging.