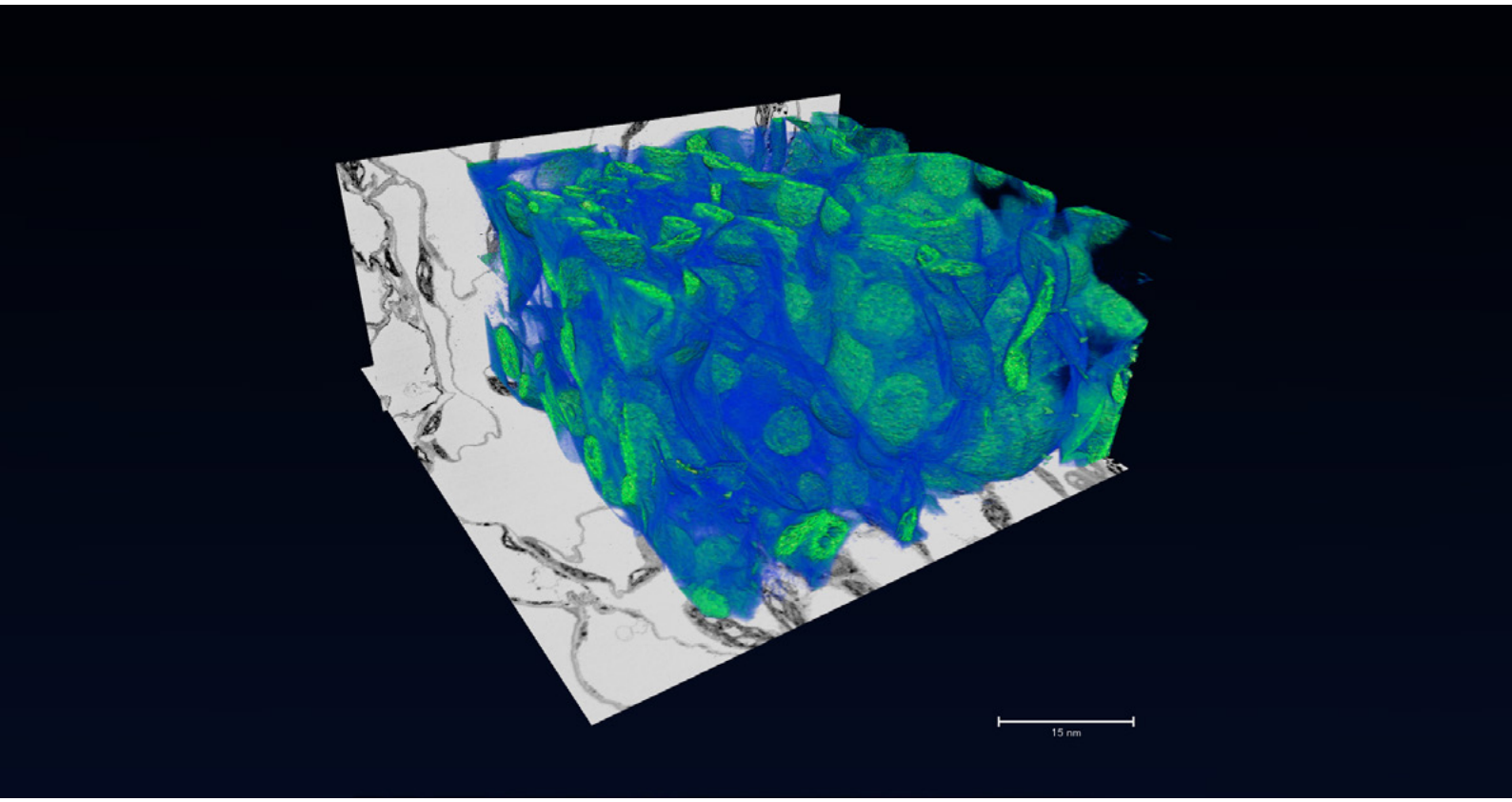
With ZEISS Focal Charge Compensation to high-quality 3D data sets

An easy way to high-quality serial block-face data sets without compromising charging effects and jittering



ZEISS

Seeing beyond

Authors: Dr. Anna Kremer, Peter Borghgraef, Dr. Saskia Lippens
VIB Bioimaging Core Ghent, Ghent, Belgium

Dr. Alexandra Elli
Carl Zeiss Microscopy GmbH, Jena, Germany

Date: September 2021

With the development of different 3D imaging methods, the area of scanning electron microscopy (SEM) has experienced a renaissance. Serial block-face SEM (SBF-SEM) enables the analysis of the ultrastructure of biological samples in 3D by cutting and imaging the sample alternatively using a SEM equipped with a diamond knife inside the SEM chamber. The popularity of this method has significantly risen over the last decade. Technical hurdles have been overcome and the solution became accepted in the community as one of the 3D imaging methods in electron microscopy in Life Sciences (Kremer et al., 2015, Smith & Starborg, 2018). With ZEISS Focal Charge Compensation, the technique is even more robust and can also be used for charge prone samples which was only possible at the expense of image quality before (Deerinck et al., 2018).

Serial block-face SEM in the context of Volume Electron Microscopy

Volume information from electron microscopy can be obtained in different ways. For serial section tomography (or array tomography (AT)), a resin-embedded sample is cut into ultrathin sections. The serial sections are imaged with an SEM (or TEM). The sequence of the sections defines the z-information of the subsequently computationally reconstructed 3D data set and the thickness of the sections determines the z-resolution of this data set. The thickness of the sections is typically between 40 – 100 nm.

As an alternative to sequentially cutting a tissue block into serial sections and following imaging, resin-embedded cells or tissues may be imaged in 3D directly within the SEM chamber in a fully automated workflow: the surface of a specimen block is repetitively cut away with images of the exposed block surface taken after each sectioning event (block-face imaging).
This can be done by using an ultramicrotome inside the SEM chamber (Gatan 3View® (Denk & Horstmann, 2004)) or a focused ion beam (FIB)-SEM that combines a FE-SEM with a focused ion beam for milling (Heymann et al., 2006).
While Gatan 3View® provides the fastest imaging of large 3D volumes with z-resolutions down to 15 nm, FIB-SEM is the best choice for higher z-resolutions down to 3 – 4 nm. Techniques such as FIB-SEM, SBF-SEM and AT open the world to structural information in 3D with highest resolution (Peddie & Collinson, 2014).

	FIB-SEM	Serial block-face-SEM with Gatan 3View®	Serial Section Tomography (Array Tomography)
Sample Type	resin block / Cryo	resin block	Serial sections on TEM grids, cover glasses or wafer
Sample Maintenance	consumed	consumed	maintained
z-resolution	3 nm	15 nm	40 nm
EM-Platform	Crossbeam	Sigma GeminiSEM	All ZEISS FE-SEMs

Table 1 *Overview of different 3D techniques*

Serial block-face Imaging with Focal Charge Compensation

As already mentioned, for SBF-SEM, the sample is repeatedly cut and imaged to build a 3D data set. The sample block itself can be in the larger micrometer range – so this technique is adaptable to a large variety of samples of different sizes. The cut thickness can go down to 15 nm and the images can go up to 32k by 24k pixels. Particularly using the Gemini technique of the ZEISS FE-SEMs in combination with Gatan 3View®, large volume data (~100 sections in 24hrs) can be acquired.

Samples for SBF-SEM can be prone to charging effects particularly when the samples are low in contrast or contain large regions of bare resin e.g. cell culture monolayers, highly vascularized tissues, or plant tissue. Charging effects compromise image quality, cause a low signal to noise ratio and result

in distortion. In the past, charging effects have been mitigated by using variable pressure (VP) SEM. The injected gas molecules in VP systems neutralize the charging, but this is at the expense of signal to noise and resolution.

Charging effects can now be prevented by using Focal Charge Compensation which can be combined with the 3View® system. A tiny capillary needle is precisely located above the sample and nitrogen is guided through this needle directly onto the block face surface while the chamber is maintained under high vacuum (Deerinck et al., 2018). This eliminates charging without degrading image quality. The needle retracts automatically during the cutting cycle, so the sectioning and imaging workflow is uninterrupted and high acquisition rates are maintained. In this way Focal Charge Compensation, enables easy imaging of the most charge-prone samples, without compromises in resolution.

This White Paper will highlight the advantages of using Focal Charge Compensation in combination with the in-situ ultramicrotome Gatan 3View® mounted in a ZEISS Field Emission Scanning Electron Microscope (FE-SEM).

Experimental procedure and results

Imaging biological samples using SBF-SEM in a high vacuum system, always results – to some degree – in electron charging of the sample block-face. The recent development of Focal Charge Compensation as a solution to avoid electron charging, opened up the possibility to use SBF-SEM imaging of less conductive samples (Deerinck et al., 2018).

Several samples that proved too difficult to image in high vacuum previously, were imaged using Focal Charge Compensation. The Focal Charge Compensation was installed on an FE-SEM (here: ZEISS Merlin) equipped with a Gatan 3View® 2XP, for SBF-SEM (Figure 1A).

Focal Charge Compensation was tested on 3 different categories of samples: 1) samples with space that is devoid of biological material (e.g. vacuoles), where the bare resin typically shows electron charging that hampers successful imaging, 2) small samples surrounded by bare resin, where the charging causes image distortions and drift during imaging runs, and 3) samples with low contrast that were initially prepared for transmission electron microscopy (TEM).

Mouse tissue samples were prepared for SBF-SEM using a standard OTO staining protocol (Deerinck et al., 2010, Steeland et al., 2018). *Arabidopsis thaliana* leaf was processed using an adapted protocol for plant tissue (Bhosale et al., 2018) and the samples prepared for TEM, went through a shorter staining protocol (Steeland et al., 2018). All samples were embedded in Spurr's resin. Next, samples were mounted on an aluminum pin, trimmed into a pyramid shape and coated with ~3 nm of platinum in a sputter coater. After loading and positioning the sample in the microscope, the Focal Charge Compensation needle was positioned to point towards the center of the pin with less than 1mm distance between the needle and the sample (Figure 1B). With the needle in position, the system was prepared for image acquisition and samples were first imaged at high vacuum and next with Focal Charge Compensation activated at imaging conditions of 1.6 kV and 100 pA.

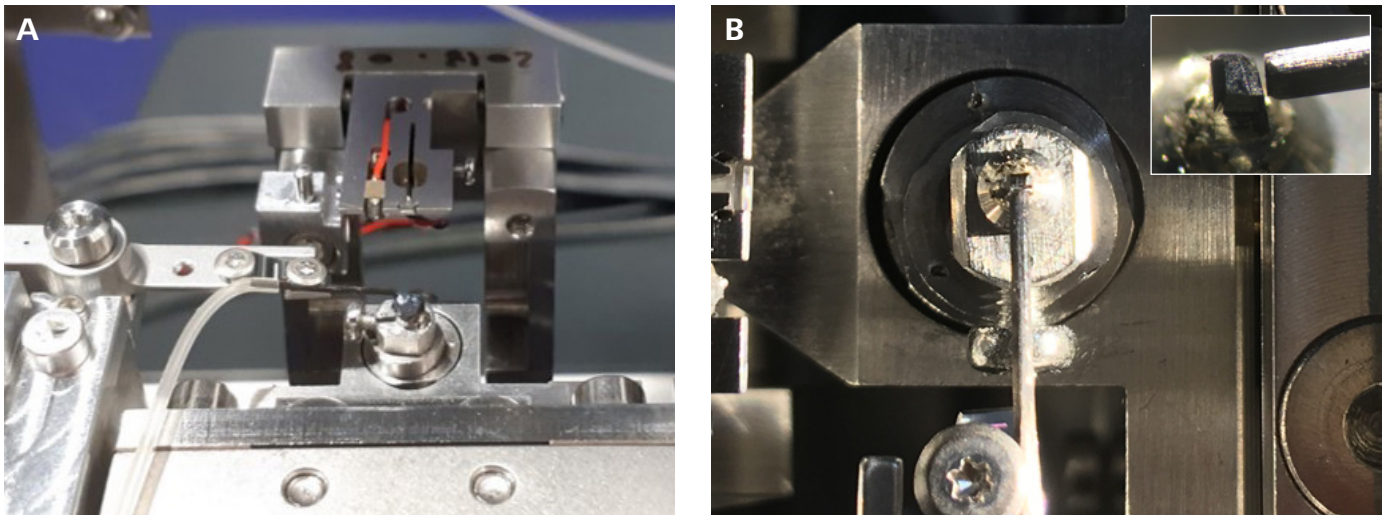


Figure 1 *ZEISS Focal Charge Compensation mounted on the Gatan 3View® system.*

A: Focal Charge Compensation is attached to the 3View® system in such a way that the needle can be positioned close to the sample but is moved away from the sample with the movement of the knife.

B: Position of the Focal Charge Compensation needle with respect to the sample-block. The inset image shows a close up of the distance between needle and block-face. This distance should not exceed 1 mm (Deerinck et al., 2018).

When applying Focal Charge Compensation, nitrogen levels were increased gradually, allowing the ion getter pump (IGP) of the microscope to adjust to the new vacuum conditions. For this experiment, samples were standardly imaged using 85 % opening of the valve, which resulted in a chamber pressure of 2.4×10^{-3} mbar. In comparison, runs at high vacuum were performed at a chamber pressure of ~5.0×10^{-7} mbar.

Focal Charge Compensation was first tested on mouse lung tissue, where the alveoli, usually filled with air, were now areas of bare Spurr's resin and *Arabidopsis thaliana* leaf, where vacuoles, water storage cells and veins account for bare resin in EM preparations. Imaging at high vacuum resulted in images that were almost completely obscured by charging effects and uncontrolled drift of the sample (Figure 2A, C). As already shown by Deerinck et al. (Deerinck et al., 2018), using Focal Charge Compensation, lung tissue could be imaged without the occurrence of charging artifacts (Figure 2B) and the quality of SBF-SEM imaging runs was greatly improved. Even though some charging lingered in the bare resin areas (Figure 2B), this had no effect on the imaging and sectioning for SBF-SEM and drift was minimal. The same was true for the leaf samples (Figure 2D), which were successfully imaged using Focal Charge Compensation, and after registration the resulting data sets could be used for volume rendering, showing the distribution of chloroplasts in the leaf (Figure 2E).

One of the advantages of SBF-SEM is that not the section but the block-face is imaged. In addition to eliminating the labor-intensive process of serial sectioning, this also ensures that a region of interest (ROI) is always positioned in the exact same position, as the block-face is mounted and does not move or rotate. However, due to the electron charging there are small movements (jumps) in x, y, called "jitter" by Deerinck et al. (Deerinck et al., 2018), and in worse cases charging effects can cause severe drifts between images during an SBF-SEM acquisition run. These drifts can be adjusted after image acquisition in a process called registration, where individual images are shifted in x and y directions to align the data in 3D. However, image distortions caused by electron charging makes this process significantly more difficult and, in some cases, impossible as the features used for aligning are warped between subsequent images.

Previous SBF-SEM runs on mouse choroid plexus were successful (Steeland et al., 2018, Brkic et al., 2015), but electron charging of the bare resin surrounding these small structures resulted in extensive image distortions and jitter in SBF-SEM runs. Additionally, image distortions were completely eliminated, and jitter was decreased. When processing the data sets imaged at high vacuum and with Focal Charge Compensation, the shifts in

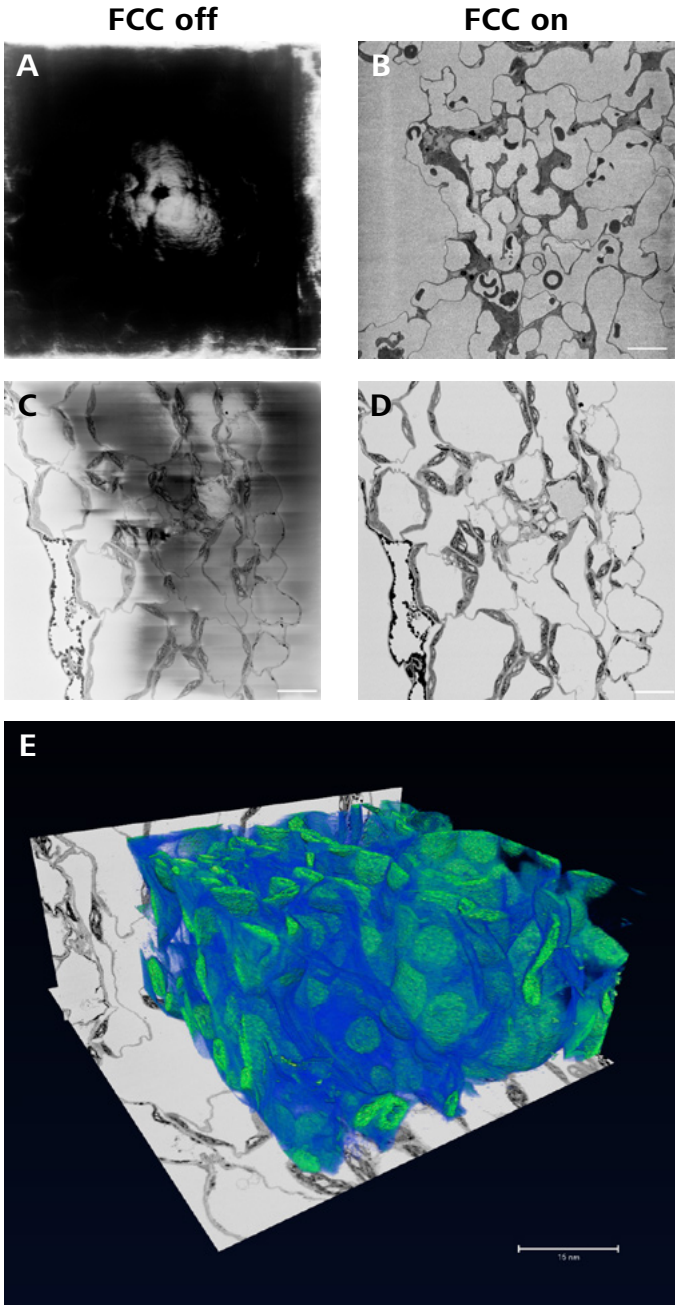


Figure 2 *Imaging of mouse lung tissue and* Arabidopsis Thaliana *leaf with large areas of bare resin using Focal Charge Compensation (Scale bars A, B, C, D = 10 µm, E = 15 µm)*

A, C: Mouse lung tissue (A) and Arabidopsis thaliana *leaf (C), both samples containing areas devoid of biological material resulting in large areas of bare resin could not be imaged at high vacuum despite the extensive SBF-SEM sample processing.*

B, D: Activating Focal Charge Compensation results in mitigation of charging and improved image quality; mouse lung tissue (B); Arabidopsis thaliana *leaf (D). SBF-SEM imaging runs were successfully performed resulting in three-dimensional data sets.*

E: An example of volume rendering from leaf, shows cellular membranes (blue) and chloroplasts (green) in relation to ortho-slices of the data set.

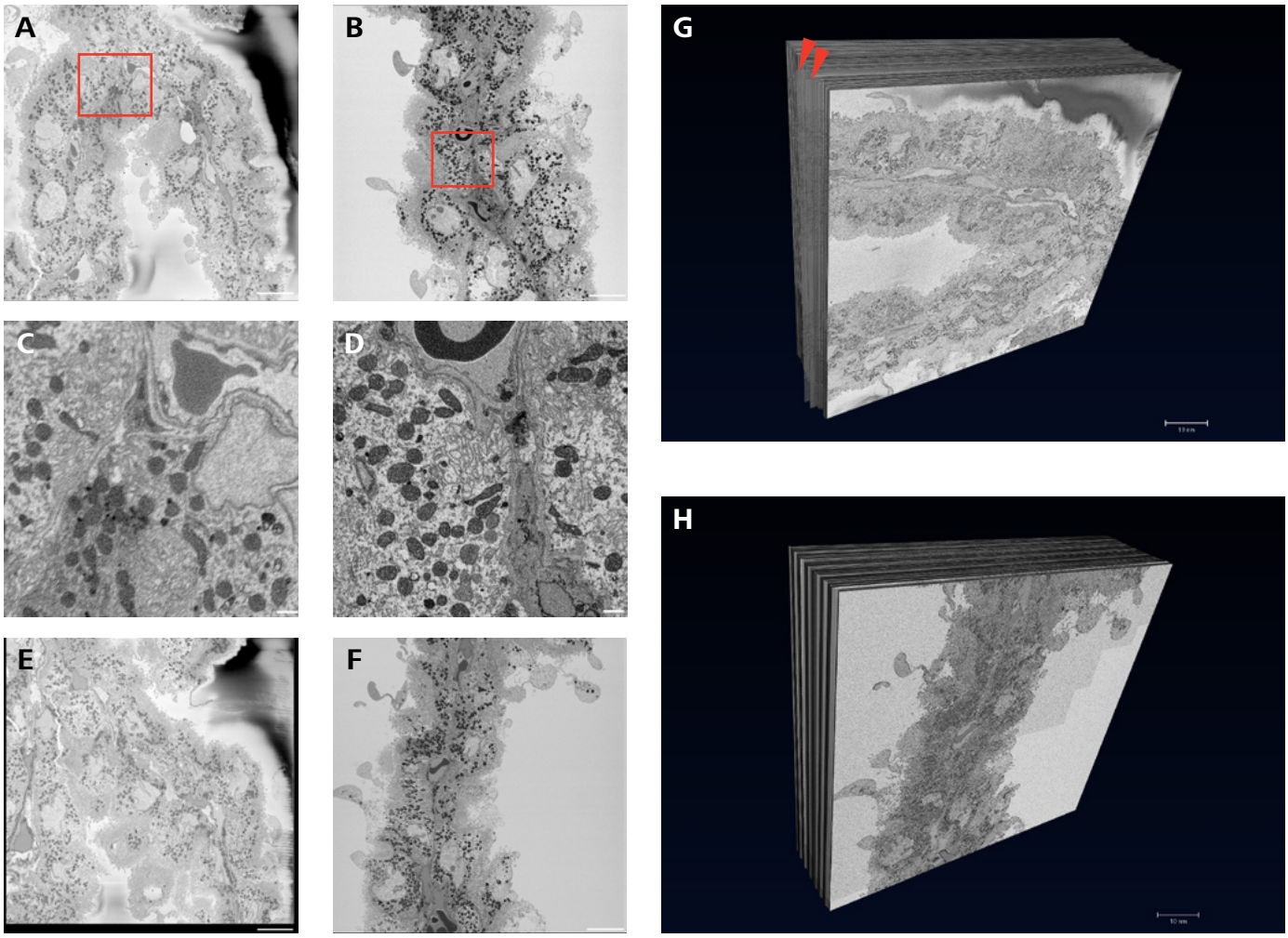


Figure 3 *Imaging of choroid plexus (small structures of epithelial cells enveloping capillaries and connective tissue) highly surrounded by bare resin (Scale bars A, B, E, F = 10 µm, C, D = 1 µm)*

A, B: Although possible to image at high vacuum (A), the activation of Focal Charge Compensation resulted in improved image quality meaning reduction of noise and improved contrast (B).

C, D: Enlarged areas of the red boxes in A and B emphasizing the improvement of image quality when Focal Charge Compensation is used.

E, F: Single images of the high vacuum (E) and Focal Charge Compensation data sets (F) after registration. The maximum shift is represented by the black border surrounding the images.

G, H: Volume rendering of the data set imaged at high vacuum (G) clearly shows shifts of individual sections (red arrowheads) representing jitter and drift, while these shifts are reduced in the data set imaged with Focal Charge Compensation (H).

x and y needed for alignment of the data reflect this reduction in jitter: at high vacuum the maximum shift per section was 1.3 µm in the x direction and 3.4 µm in the y direction and with Focal Charge Compensation this was reduced to 0.84 µm in x and 0.74 µm in y, respectively. This was measured on two data sets of 300 sections of 70 nm taken from the same sample, first imaged at high vacuum and again imaged after Focal Charge Compensation was switched on. This reduction is also apparent when looking at single sections of the registered data sets (Figure 3E, F) and when creating a volume rendering of the registered data (Figure 3G, H), where the sections that were shifted in x or y stand out clearly in the high vacuum data set (Figure 3E, G), while in the rendering of the registered data acquired with Focal Charge Compensation no individual sections can be distinguished (Figure 3F, H). Again, this shows that the use of Focal Charge Compensation is a significant improvement for SBF-SEM imaging and data reconstruction.

Sample preparation for SBF-SEM aims to maximize the conductivity of samples and is based on en-bloc staining with a combination of different heavy metals (Deerinck et al., 2010). Traditionally, samples prepared for TEM are only treated with Osmium and Uranyl Acetate en-bloc (Steeland et al., 2018), which means these samples are significantly less contrasted compared to SBF-SEM prepared samples. In a high vacuum SBF-SEM system it is therefore difficult to image samples that

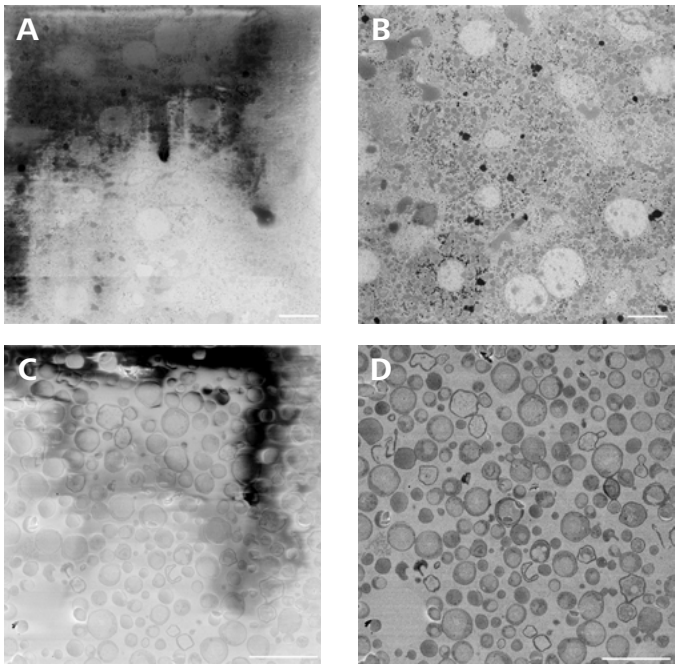


Figure 4 *SBF-SEM of samples prepared for TEM (Scalebars = 10 µm)*

A, B: Brain tissue stained with OsO4 only imaged at high vacuum (A) and with Focal Charge Compensation (B).

C, D: Yeast cells prepared for EM by HPF and FS could be imaged using Focal Charge Compensation (D), while at high vacuum noisy images were distorted by charging (C).

were prepared for TEM. This is even the case for dense tissues like brain where conductivity is higher due to very little open spaces with bare resin (Figure 4A). Activating Focal Charge Compensation when imaging these samples improves the image quality (Figure 4B) and allows for recuperating TEM prepared samples for SBF-SEM. Additionally, samples processed by high pressure freezing (HPF) and subsequent freeze substitution (FS) contain even less contrast, resulting in bad signal-to-noise and charging in the SBF-SEM (Figure 4C), which can be remedied by switching on Focal Charge Compensation (Figure 4D).

Conclusion

SBF-SEM enabled scientists to add three-dimensional information to the ultrastructural detail of electron microscopy. Although the method provides automated sectioning and imaging, eliminating the need for time consuming manual serial sectioning and alignment of the resulting data set, working under (high) vacuum can be a disadvantage as biological samples need to be embedded in resin for imaging and sectioning. By eliminating electron charging of the block-face, Focal Charge Compensation increased the range of samples that can be imaged with SBF-SEM significantly (Deerinck et al., 2018). Using Focal Charge Compensation, we were able to image samples with large areas of bare resin, small samples surrounded by bare resin and samples with low contrast.

Due to the decrease in charging while using Focal Charge Compensation, image quality is improved and drift, jitter and image distortions are reduced. These last effects eliminate the need for extensive registration of the resulting data sets. Although the time to set-up a run increases slightly, due to the conditioning of the IGP, the advantages of using Focal Charge Compensation weigh up against this small increase in time, as practically all samples can now be imaged at high resolution, in 3D and the resulting data sets need less processing before 3D reconstruction and volume rendering.

Overall, Focal Charge Compensation allows to image more diverse samples in three dimensions without charging and at high resolution.

Bibliography

[1] Bhosale, R., Boudolf, V., Cuevas, F., Lu, R., Eekhout, T., Hu, Z., Van Isterdael, G., Lambert, G. M., Xu, F., Nowack, M. K., Smith, R. S., Vercauteren, I., De Rycke, R., Storme, V., Beeckman, T., Larkin, J. C., Kremer, A., Hofte, H., Galbraith, D. W., Kumpf, R. P., Maere, S. & De Veylder, L. (2018) A Spatiotemporal DNA Endoploidy Map of the Arabidopsis Root Reveals Roles for the Endocycle in Root Development and Stress Adaptation. Plant Cell, 30, 2330-2351.

[2] Brkic, M., Balusu, S., Van Wonterghem, E., Gorle, N., Benilova, I., Kremer, A., Van Hove, I., Moons, L., De Strooper, B., Kanazir, S., Libert, C. & Vandenbroucke, R. E. (2015) Amyloid beta Oligomers Disrupt Blood-CSF Barrier Integrity by Activating Matrix Metalloproteinases. J Neurosci, 35, 12766-12778.

[3] Deerinck, T. J., Bushong, E., Thor, A. & Ellisman, M. H. (2010) NCMIR methods for 3D EM: A new protocol for preparation of biological specimens for serial block-face SEM. Microscopy, 6-8.

[4] Deerinck, T. J., Shone, T. M., Bushong, E. A., Ramachandra, R., Peltier, S. T. & Ellisman, M. H. (2018) High-performance serial block-face SEM of nonconductive biological samples enabled by focal gas injection-based charge compensation. J Microsc, 270, 142-149.

[5] Denk, W. & Horstmann, H. (2004) Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. PLoS Biol, 2, e329.

[6] Heymann, J. A., Hayles, M., Gestmann, I., Giannuzzi, L. A., Lich, B. & Subramaniam, S. (2006) Site-specific 3D imaging of cells and tissues with a dual beam microscope. J Struct Biol, 155, 63-73.

[7] Kremer, A., Lippens, S., Bartunkova, S., Asselbergh, B., Blanpain, C., Fendrych, M., Goossens, A., Holt, M., Janssens, S., Krols, M., Larsimont, J. C., Mc Guire, C., Nowack, M. K., Saelens, X., Schertel, A., Schepens, B., Slezak, M., Timmerman, V., Theunis, C., R, V. A. N. B., Visser, Y. & Guerin, C. J. (2015) Developing 3D SEM in a broad biological context. J Microsc, 259, 80-96.

[8] Peddie, C. J. & Collinson, L. M. (2014) Exploring the third dimension: volume electron microscopy comes of age. Micron, 61, 9-19.

[9] Smith, D. & Starborg, T. (2018) Serial block face scanning electron microscopy in cell biology: Applications and technology. Tissue Cell.

[10] Steeland, S., Gorle, N., Vandendriessche, C., Balusu, S., Brkic, M., Van Cauwenberghe, C., Van Imschoot, G., Van Wonterghem, E., De Rycke, R., Kremer, A., Lippens, S., Stopa, E., Johanson, C. E., Libert, C. & Vandenbroucke, R. E. (2018) Counteracting the effects of TNF receptor-1 has therapeutic potential in Alzheimer's disease. EMBO Mol Med, 10.

Carl Zeiss Microscopy GmbH
07745 Jena, Germany
microscopy@zeiss.com
www.zeiss.com/3view

EN_41_013_253 | CZ 09-2021 | Design, scope of delivery and technical progress subject to change without notice.