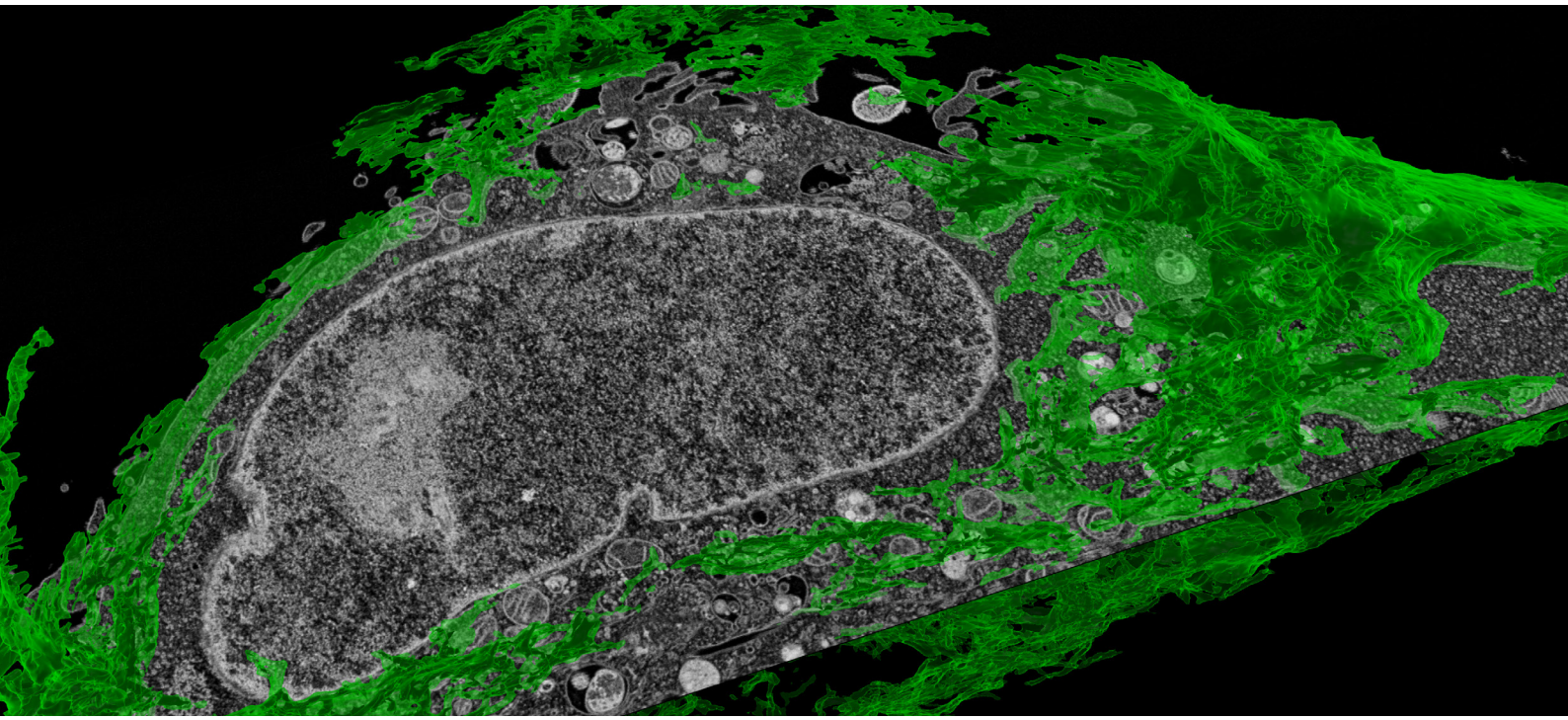
# Expanding Your Scanning Electron Microscopy (SEM) to 3-Dimensions and Beyond



*Dataset kindly provided by Anna Steyer and Yannick Schwab, EMBL Heidelberg, Germany*

ZEISS

Seeing beyond

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Scanning electron microscopy (SEM) is as a powerful and versatile technology for capturing high resolution information from a wide variety of life science specimens. Exploring samples using SEM provides a wealth of information about the specimen that can include structural, elemental, compositional and textural data. These data can be used alone, or in combination with microscopy data from other technologies, to reveal a comprehensive, more insightful representation of your experimental results.

Life science specimens pose unique challenges for SEM since they are prone to charging and often have very low contrast prior to staining. They are also extremely delicate and require low voltage acquisitions. Good image quality is dependent on achieving high contrast at low voltage while minimizing artifacts generated by charging. SEMs, such as ZEISS GeminiSEM, are uniquely suited for life science specimens due to their beam boosting technology which ensures optimal low voltage and high contrast performance. This makes ZEISS SEMs a solid electron microscopy platform for expanding ultra-resolution life science research into three dimensions and beyond with new technological advances.

Moving from 2D SEM images to large areas or 3D structural volumes provides valuable insights into biological structure that are unavailable in single sections. While historically it has been a challenging and tediously manual task to acquire large areas and/or 3D datasets using SEM, advances in data collection and detectors, as well as correlative technology and software are rapidly changing this. The opportunities now available for scaling acquisitions up to address a wide array of different scientific questions are exciting[1].

Before starting an experiment, one of the key decisions to be made is which technical approach is best suited for the specimen and scientific question. A description of several different technological approaches for structural explorations of life science specimens using large area and/or 3D volume EM is provided here.

## #1 Array tomography

**For resin embedded biological samples to reach 50-100nm axial resolution**

Serial thin sections of less than 100 nm thickness can be imaged using the SEM and the resulting images can be reconstructed to visualize cellular and subcellular structures in three dimensions. One such method of 3D reconstruction and visualization is array tomography. A fixed, heavy metal-stained, resin embedded specimen is physically sectioned with an ultramicrotome. The resulting thin sections are floated on the water surface of a diamond knife trough and collected to form an array on a solid substrate, such as a wafer, coverslip/ glass slide or TEM grid. In place of a standard ultramicrotome, an ATUMtome (automated tape collecting ultramicrotome) can be used to automatically collect hundreds of sequential sections from the water surface to a continuous ribbon of Kapton tape. After the sections are collected, the tape is mounted in strips to a silicon wafer or coated glass slide. Once the sections are placed on the appropriate substrate via the ultramicrotome, each section is imaged with the SEM. The resulting two-dimensional images of each section are precisely aligned resulting in coherent, three-dimensional images. Z- resolution is determined by section thickness, which is typically <100nm with this technology. Array tomography is well suited for smoothly capturing large tissue volumes with precise ultrastructural detail[2] (Figure 1).

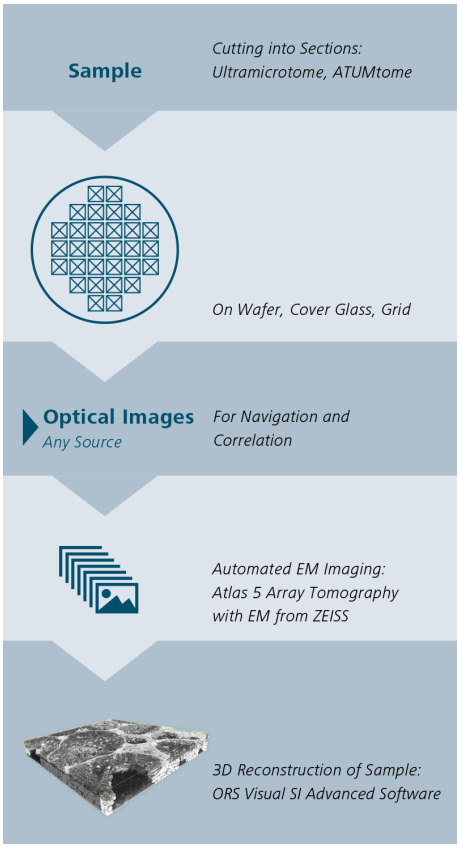


***Figure 1:*** *Typical array tomography workflow using a scanning electron microscope.*

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Array tomography is regularly performed using a Field Emission SEM (FE-SEM), such as ZEISS GeminiSEM or ZEISS Sigma, using a backscattered electron detector (BSD), such as ZEISS Sense BSD. Both methods allow for transmission electron microscope (TEM)-like imaging in the SEM. When the incident beam of the SEM interacts with the heavy metal-stained sample, backscattered electrons are produced. Areas on the sample composed of higher atomic numbered elements (such as osmium, uranyl acetate or lead) produce more backscattered electrons. EM images in life sciences typically show membranes as dark areas, whereas the cytoplasm, for example, is bright.

ZEISS Atlas 5 Array Tomography is a hardware/software tool used to automatically image serial sections down to nanometer resolution. As shown in Figure 1, once an overview image of the sample substrate, such as a wafer, is taken, the overview image is used as a navigation map inside of the electron microscope. The shape of the sections is determined and then the clone tool is used to define imaging sites for each section. Unlimited regions of interest can be defined over hundreds of sections using select, clone and trace tools. Lower resolution, overview images of each section are taken with the SEM and then selected areas are imaged with high resolution. This is automated for each subsequent section. The two-dimensional images of the individual sections are compiled and aligned into a three-dimensional volume (Figure 2).

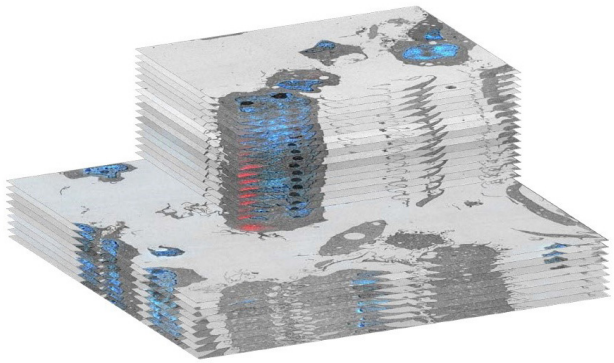

***Figure 2:*** *Assembly of a correlative 3D dataset. The dataset shows a Z-stack of macrophages expressing the protein huntington. Macrophages are used as a model system for the investigation of Huntington's disease. An overexpression of huntington results in an aggregation of the protein clearly visible within the Z-stack. An antibody was used against GFP-Huntington to locate plaques of huntington within the cells (Alexa Fluor 647, red). The nucleus is shown in blue (Hoechst). Courtesy of J. Caplan, E. Kmiec and S. Modla, University of Delaware, USA.*

## #2 Scanning transmission electron microscopy (STEM)

**For ultra-high resolution tomography from single sections**

STEM is another increasingly popular technique used to image thin, serial sections, producing two-dimensional images that are reconstructed into three-dimensional stacks. Like other serial section electron microscopy techniques, STEM allows for nanoscale lateral resolution, which is important for many ultrastructural biological studies such as neuronal morphology. Many of the subcellular structures, such as microtubules and endosomes, require very high resolution to be accurately identified, making acquisition of serial sections using the transmission of electrons though the specimen essential for ultra-high resolution studies. In contrast to TEM, STEM imaging has the benefit of increased single image field of view while maintaining the image quality necessary for high-resolution volume imaging.

The sample is prepared in a similar fashion as it would be for transmission electron microscopy (TEM) studies or array tomography studies using a backscattered electron detector in the SEM. The sample is heavy metal stained, embedded in epoxy or acrylic resin and sequentially sectioned to less than 100 nm thickness with an ultramicrotome. The serial sections are then placed on TEM grids and mounted on a specialized TEM grid holder adapted for the SEM stage. The STEM detector, which is available on the ZEISS GeminiSEM, ZEISS Sigma and ZEISS Crossbeam, is mounted on the underside of the specimen, and used to collect the electrons with enough energy to pass through the specimen, also referred to as transmitted electrons. ZEISS Atlas hardware/software is used to automatically move the stage to acquire SEM images section-to-section and grid-to-grid for each TEM grid after the region of interest is marked on each section. Atlas can also be used to create mosaics for large imaging fields. For more information see the following white paper.

## #3 Serial block face scanning electron microscopy (SBF-SEM)

**For resin embedded biological samples to reach <25nm axial resolution**

Both array tomography and STEM rely on the generation of serial thin sections by an ultramicrotome which are subsequently transferred to the SEM for imaging. With serial block face SEM

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(SBF-SEM), the ultramicrotome is physically located inside the SEM chamber. The SEM images the top of the resin embedded specimen and then the ultramicrotome slices off the top of the specimen. This is repeated many times to generate a 3D dataset (Figure 3).

As with array tomography and STEM, SBF-SEM is an increasingly popular approach to generating high resolution 3D datasets as it offers flexible slice thickness down to ~15nm, and a relatively large field of view (example shown in Figure 4). SBF-SEM brings the added convenience of not having to slice the sample outside the SEM.

To ensure the best image quality, sample preparation and staining are crucial. All samples imaged using SBF-SEM must be embedded in resin. One of the most used sample preparation protocols that has fine tuned each step can be found here.

Very often, resin embedded specimens can be challenging to image with SEM due to sample charging. This is most apparent in highly vascularized tissues or samples with a high proportion of resin which is normally non-conductive; images can show artifacts, degraded image quality and distortion. For SBF-SEM this is a particular challenge because imaging is entirely automatic and optimizing imaging conditions for each single image to minimize charging is unfeasible. Nitrogen gas can help to reduce these artifacts and significantly increase image quality. The National Center for Microscopy and Imaging Research (NCMIR, USA) collaborated with ZEISS to develop a solution to reduce charging when using SBF-SEM called Focal Charge Compensation[4] (Figure 5).

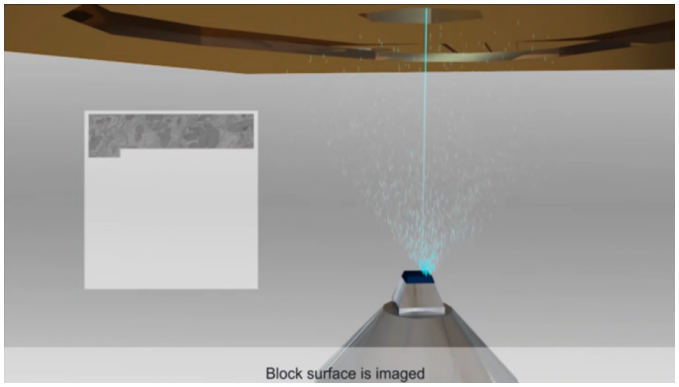


***Figure 3:*** *Schematic video showing how SBF-SEM generates a series of 2D images from the specimen block that are subsequently rendered into the 3D volume*

Focal Charge Compensation (FCC) works by using a tiny capillary needle to guide nitrogen gas directly onto the sample surface. The needle retracts automatically during the cutting cycle so as not to interfere with image acquisition and the resulting increase in image quality throughout the whole specimen volume is striking (Figure 5). FCC is available for ZEISS Gemini SEM and ZEISS Sigma FE-SEM when fitted with a SBF-SEM solution.

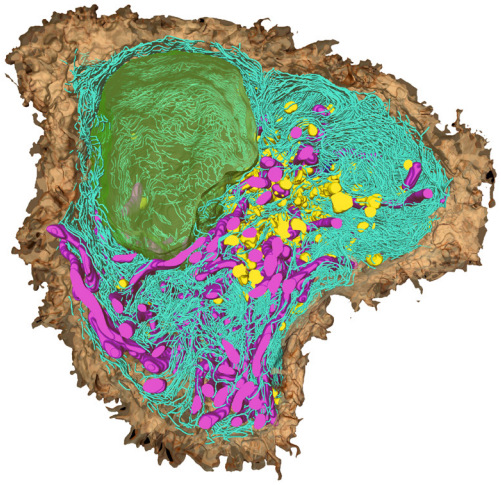

***Figure 4:*** *Dendritic cell imaged in 3D using SBF-SEM. The matrix of internal cellular structure can be clearly visualized and segmented, providing many opportunities for structural interrogation. Courtesy of Dr. Peter Munro and Hannah Armer, UCL - Institute of Ophthalmology, United Kingdom*

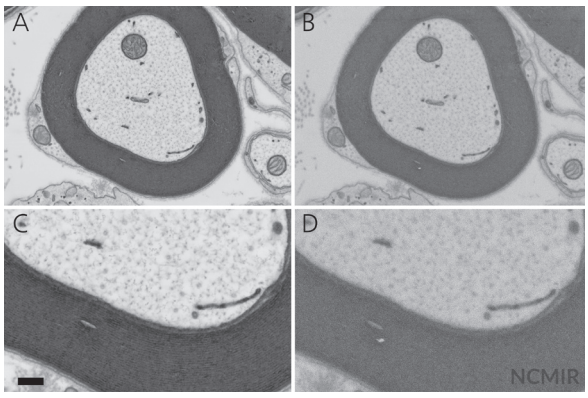


***Figure 5:*** *Cross section of a rat spinal cord imaged using the ZEISS Gemini SEM and SBF-SEM both with (A and C) and without (B and D) Focal Charge Compensation (FCC). The significant improvement in image quality can be seen with the use of FCC. Courtesy of NCMIR, USA.*

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### #4 Focused ion beam SEM (FIB-SEM)

**For vitrified samples or resin embedded biological specimens to reach <5nm resolution in Z**

A FIB-SEM generates a 3D dataset by imaging the surface of the sample with an electron beam and then removing a section of the sample with the focused ion beam (FIB). The new surface is then imaged and so on, through the whole sample. This approach can be used for targeted sectioning of biological samples – either cryo preserved (vitrified) or conventional (embedded in resin) or when very high axial resolution, such as 5 nm, is desired (for example Figure 6).

FIB-SEM can generate higher resolution 3D datasets than those created with an ultramicrotome since the surface is removed using a highly focused beam of charged particles rather than a physical diamond knife. However, the field of view when working with FIB-SEM is smaller. The solution of choice for each experiment greatly depends on the required resolution and field of view as well as the required volume.

For maximum efficiency, image capture of the sample surface and stripping another part of the surface with the ion beam should be performed simultaneously. This removes the need to wait for surface removal to take place prior to image capture and significantly increases productivity and throughput. High efficiency combined with excellent image quality at low voltages is critical for high data quality for life science specimens.

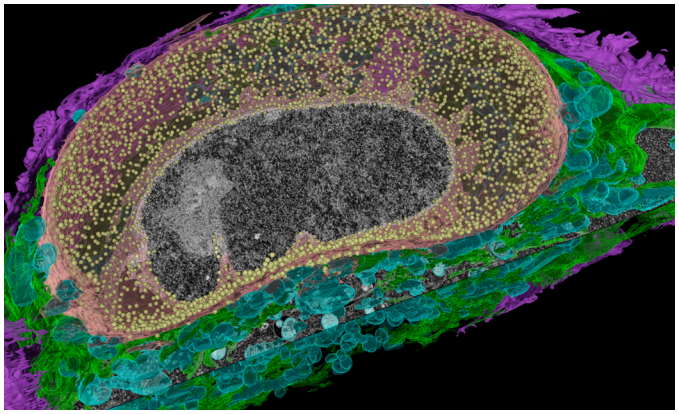

***Figure 6:*** *3D reconstruction of a HeLa cell imaged with the ZEISS Crossbeam 550 FIB-SEM. Automated segmentation and visualization of cellular components achieved with an **APEER**-trained deep learning model in **arivis Vision4D**. Courtesy of Anna Steyer and Yannick Schwab, EMBL Heidelberg, Germany.*

The Gemini column used in the ZEISS Crossbeam FIB-SEM combines simultaneous acquisition and milling as well as beam booster technology for excellent image quality at low voltages (Figure 7)

Another key consideration when milling the specimen using FIB-SEM is that the structure of interest may be irregular in shape and may not be aligned with the microscope stage. In this case, small adjustments need to be made to the acquisition of each image to ensure that the structure of interest remains in the volume that is captured. If done manually, this process requires significant input and time from the FIB-SEM operator; it is much more convenient to use software driven acquisition routines. ZEISS Atlas 3D provides advanced sample tracking and predictive drift correction to ensure that the structures of interest are captured throughout the 3D volume (Figure 8).

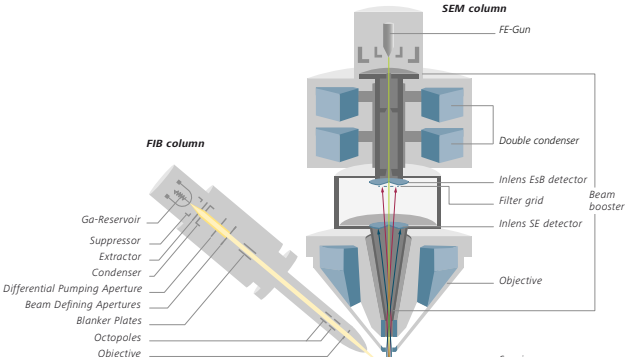


***Figure 7:*** *The SEM and FIB columns are differentially angled relative to the specimen to enable simultaneous milling of the surface and image acquisition. Beam booster technology ensures high image quality, even at low voltage, which is particularly important for life science specimens.*

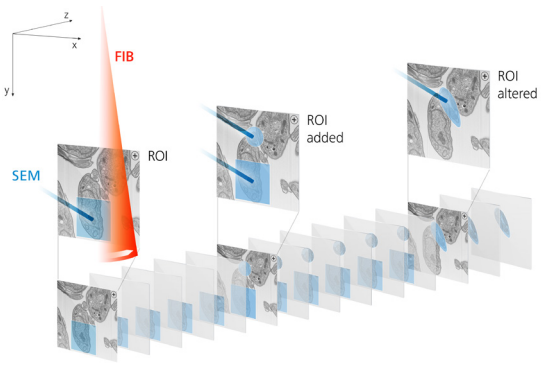


***Figure 8:*** *ZEISS Atlas 3D enables automatic tracking of the structure to be captured throughout the full 3D volume of the FIB-SEM dataset. Manual correction of the same variables is challenging, time consuming and often results in data runs with significantly reduced volume acquisitions as a result.*

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### #5 Large area EM acquisition

**For high resolution acquisition of samples up to centimeters in size**

For applications that demand very large acquisition areas, on the scale of centimeters, the limitations of a single electron beam cause a significant bottleneck in terms of specimen throughput. For example, brain specimens are relatively large biological samples for microscopy and for applications such as Connectomics, where a high-resolution map of the brain is the goal, the acquisition speed of a single beam is simply too slow to enable any meaningful progress in a mapping project.

One solution to this challenge is to capture several adjacent regions of the specimen using a tiling approach in the SEM. This is shown in Figure 9 where the larger specimen area is segregated into smaller acquisition regions for comprehensive coverage of the specimen at high resolution. The ease with which this tiling acquisition can be set up, implemented and the resulting images stitched together makes this routine straightforward and feasible, even for those who are not experienced SEM users.

To further increase the speed of image capture beyond a single region at a time, the acquisition can be parallelized by using multiple electron beams and detectors at once. This parallelization has an enormous impact on total experimental time such that you can achieve the imaging volume in 4 weeks that would otherwise take many years using a single beam SEM!

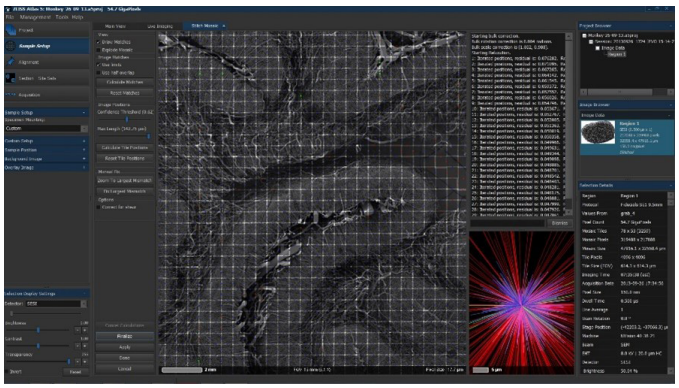

***Figure 9:*** *Atlas 3D software enables the straightforward capture of a very large field of view of the monkey brain specimen using many separate, much smaller images captured at high resolution using the SEM.*

The technology works by a finely tuned detection path collecting a large yield of secondary electrons for imaging. Each beam carries out a synchronized scanning routine at one sample position, resulting in a single sub-image. The electron beams are arranged in a hexagonal pattern and the full image is formed by merging all image tiles (see Figure 10). The ZEISS MultiSEM uses exactly this approach to generate up to 91 images simultaneously with 91 independent electron beams. Using this instrument an area of 1 $cm^2$ can be imaged at 4 nm pixel size in less than 3 hours!

This extremely powerful technology is straightforward to run but one of the biggest challenges to consider is management of the vast volumes of data that are generated. To reach meaningful insights it is necessary at minimum to run a parallel computer setup for fast data recording and when it comes to the subsequent processing and analysis, national computing infrastructure is often considered. For more information, please see the technical note here.

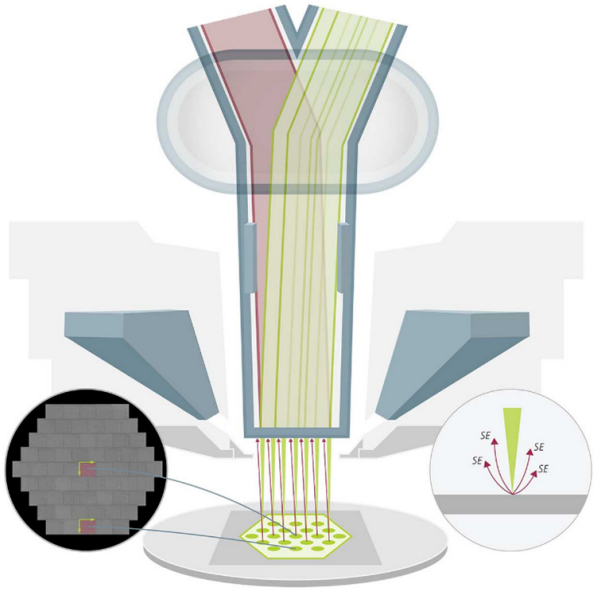


***Figure 10:*** *Parallelizing electron beam acquisition significantly increases the speed of acquisition to allow larger fields to be captured and a vast increase in imaging acquisition and throughput. Scaling up acquisition in this way unlocks possibilities for experiments that were simply too large to consider using just a single beam due to the unfeasibly long timelines that would have been needed for acquisition.*

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**Summary**

The number of methods now available for upscaling electron microscope acquisitions to larger areas and/or three dimensions is unlocking exciting new possibilities in life science research. The relative ease of implementation of these technologies is also helping to assist their widespread uptake into research institutions globally. Improvements in data handling and image analysis approaches are successfully producing quantifiable, reproducible information from the resulting data, leading to experimental success.
For some research groups, such as the Connectomics community, the formidable power for scaling up electron microscopy acquisitions has driven widespread adoption of these approaches[5, 6, 7]. Many examples using specimens other than neural tissue are also being published as the value of these approaches is appreciated[3, 8, 9].

With such a range of technologies available, it can be difficult to decide which tool or approach is the most appropriate for each sample type and application. **Contact us** to discuss your project in detail and to establish which path would be the best solution for addressing your research question.

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