The world is round, and so are cells; and like our planet cells have a geometry in three dimensions. However, for many years this fact was often overlooked in electron microscopy because it was technically difficult to image cells in 3D. Now three new techniques (serial block face imaging, focused ion beam-SEM and array tomography) have emerged that bring the third dimension to imaging at the nanoscale.

Bringing 3D to EM

The development of transmission electron microscopy (TEM) in the 1930s was a technological breakthrough, and after 300 years of being limited by the wavelength of photons scientists finally went beyond the resolution barrier and entered the nanoworld of ultrastructure. For biologists however there were severe limitations to using the technique; it required fixed plastic embedded samples, the maximum sample size was small ($\approx 2 \text{ mm}^3$), and sections of cells or tissues had to be thin enough so that the electron beam could pass through (typically 70-90 nm). Although scanning electron microscopy (SEM) could image much larger samples, it could only resolve a few nm just under the surface of the specimen and intracellular ultrastructure could not be revealed. So although there was much to be learned from the use of TEM and SEM, one missing element was how sub-cellular organelles related to another in 3D space.

Over the 40 years following the development of TEM some brave scientists attempted to bring in the third dimension by serial section TEM, in which samples were manually sectioned and then each section was imaged and reconstructed from photographic prints [1]. While this approach worked it was both technically challenging and labor intensive. In the referenced study it took 6 years to partially reconstruct 6 cells. Once microscopy went digital this process was somewhat easier and it had the great advantage that it allowed for unlimited manipulation of the digital dataset and the reconstructed volume. Nevertheless, the process was still very demanding: reconstructing 450 x 350 x 52 $\mu$m of mouse brain containing approximately 1500 cell bodies required a custom built CCD camera array, a team of 9 microscopists, 400 hours of imaging time and 36 TB of storage space [2].
So although it could be performed 3D EM was rarely done.

A considerable boost came from solutions that would automate the slicing and imaging step, because this was the most time and resource consuming part of the whole workflow. Alan Kuzirian and Steve Leighton working at the Marine Biological Laboratory in Woods Hole developed an automated mini-microtome with a diamond knife that could be fitted in the chamber of an SEM [3] and used for serial block face imaging (SBFI). This was revolutionary in that the sectioning could occur directly in the microscope greatly simplifying the technical skills required. It used a SEM to produce a ‘TEM-like’ image of the embedded block face and the image could be limited to a very thin Z resolution by reducing the electron beam current. Because this development occurred in the very early days of digital image capture it was somewhat ahead of it’s time, and it was not until the early 2000’s that the technology was refined by Winfried Denk and later commercialized by Gatan (3View).

**Discovering Biological Tissues**

Another breakthrough in 3D EM came when a tool developed for nano-fabrication was employed to section biological tissues [4]. Using a focused Gallium ion beam integrated in the SEM chamber (FIB-SEM, also known as crossbeam systems), very precise milling of the sample block was achieved. Compared to the serial block face technique there is an improved z-resolution, because the ion beam was able to cut the sample in even thinner slices creating the possibility of collecting images with isotropic voxels (cubic pixels) in the nm range, which greatly increased the accuracy in the resulting reconstructions.

Finally using a variation on the serial section technique, Tapia and co-workers described array tomography, imaging serial sections using an FE-SEM to produce 3D data [5]. This technique has been further refined to include automated serial section collection [6] and multiple beam SEMs [7] which can collect data from up to
196 sections simultaneously. Figure 1 shows a diagram of how the three 3D SEM techniques work.

At the VIB Bioimaging Core in Ghent Belgium we are using volume EM [8,9] to collect 3D information from data sets for a broad range of biological questions. Being a core facility that provides expert imaging services with both light and electron microscopes, the sample variety and scientific questions are very diverse. From cells to tissues, mammals, plants, protozoa, bacteria, yeast and viruses; we never know what we will see next.

This diversity demands protocol optimization customized for each sample type. When a new sample type appears there is usually no “standard” staining and sample processing protocol that can be applied. Instead, depending upon the structures of interest and the nature of the sample a variation must be developed to yield the appropriate preservation and contrast in the SEM [10-12]. The nature of the instrumentation, especially the sensitivity of the detector employed, must be considered to produce an image of high quality. We have extensively tested different resins and formulations thereof, as well as fixation and stabilization techniques. The one thing we can definitively say is that no sample should be handled using a one-size-fits-all approach if you want to produce high quality results.

Since volume EM can encompass several techniques, choosing which is best for a given scientific question is important. For connectomics and other studies that require the acquisition of large volume samples, array tomography is the most efficient. For questions that can be addressed in a few samples with limited dimensions (no larger than 500 x 500 x 500 μm³) an SBFI system is ideal. However, the caveat with both these techniques is that the Z resolution is limited by the section thinness (and the scanning beam energy) which means that the acquired data will have lower resolution in Z than in X,Y. For questions where the maximum resolution in all three dimensions is necessary the FIB-SEM is the best choice. FIB-SEM though is limited in the size of the area that can be sectioned and imaged (usually less than 50 x 25 x 20 μm) so the region of interest must be smaller than that. Ideally all 3 technologies should be available so that a combination approach can be used. For example, where specific structures are being studied, SBFI can be used to incrementally section a large block of tissue or field of cells then when the event is detected the sample can be moved to the FIB-SEM for high-resolution imaging. We have used this combination approach in studies of cardiac intercalated discs (fig. 2)

The development of EM was a significant breakthrough in biological imaging. Volume EM, which is still under development, will add another layer of depth to the
The richness of information we can glean from cells at the nanoscale.

Authors
Christopher J. Guérin¹, Anna Kremer¹, Saskia Lippens¹, Jolanda van Hengel²

Affiliations
¹ VIB Bioimaging Core, Ghent, F.S.V.M. Building, Ghent, Belgium
² Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, University of Ghent, Belgium

Contact
Dr. Christopher J. Guérin
VIB Bioimaging Core
Ghent, Belgium
https://corefacilities.vib.be/bic

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