Serial Block Face-Scanning Electron Microscopy: A Tool for Studying Embryonic Development at the Cell–Matrix Interface

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Studies of gene regulation, signaling pathways, and stem cell biology are contributing greatly to our understanding of early embryonic vertebrate development. However, much less is known about the events during the latter half of embryonic development, when tissues comprising mostly extracellular matrix (ECM) are formed. The matrix extends far beyond the boundaries of individual cells and is refractory to study by conventional biochemical and molecular techniques; thus major gaps exist in our knowledge of the formation and three-dimensional (3D) organization of the dense tissues that form the bulk of adult vertebrates. Serial block face-scanning electron microscopy (SBF-SEM) has the ability to image volumes of tissue containing numerous cells at a resolution sufficient to study the organization of the ECM. Furthermore, whereas light microscopy was once relatively straightforward and electron microscopy was performed in specialist laboratories, the tables are turned; SBF-SEM is relatively straightforward and is becoming routine in high-end resolution studies of embryonic structures *in vivo*. In this review, we discuss the emergence of SBF-SEM as a tool for studying embryonic vertebrate development.

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Key words: collagen; fibrils; extracellular matrix; electron microscopy; tendon

Imaging the Extracellular Matrix

The differentiation of individual cells into specific tissue types, and the formation of extracellular matrices (ECMs) that provide mechanical and chemical cues for cell survival and differentiation, underpin the development of shape and form of multicellular organisms. Indeed, cells would not be able to form cohesive tissues and organs without the support and signaling from a self-derived ECM. The deposition of an organized matrix can be seen as the key factor in the formation of vertebrate specific tissues during development (Huxley-Jones et al., 2009). In some tissues, such as tendon, the matrix acts primarily to transmit forces. In these tissues, the ECM can take up to 70–80% of the dry weight to resist forces of compression and tension (Kadler et al., 1996). In other tissues, the matrix has a physiological role, for example, in the kidney, the glomerular basement membrane filters solutes from the blood (Miner, 2011, 2012; Lennon et al., 2014). The ECM also acts as a major signaling resource, which is exemplified by mutations in fibrillin-1 that alter transforming growth factor-β signaling resulting in the Marfan syndrome (Ramirez and Rifkin, 2009). How cells make specialized ECMs that are essential for vertebrate life is unknown. This is partly because the matrix contains macromolecules that are insoluble or slowly turned over (e.g., 200 years for collagens in tendon; Heinemeier et al., 2013) and are therefore difficult to study by biochemical and genetic methods. Furthermore, the components of the ECM are assembled into millimeter-long fibrils (e.g., collagen), microfibrils (e.g., fibrillin), and planar networks (e.g., basement membranes), which extend far beyond the boundaries of individual cells and are difficult to study by light microscopy methods. Evidence from serial block face-scanning electron microscopy (SBF-SEM) shows that assembly and organization of the ECM occurs at, or very close to, the plasma membrane. SBF-SEM is emerging as the method of choice for obtaining detailed 3D visualizations of cells in the context of an organized ECM in vivo.

Studies of 3D Tissue Architecture—The Early Days

It has long been known that to understand tissue architecture it is necessary to examine the tissue in three dimensions. Early studies used the camera lucida to make hand-drawn representations of samples (e.g., see Stearns, 1940a, 1940b). Major advances occurred when the transmission electron microscope was invented, which led to approaches such as serial section reconstruction. Here, tissue samples are dehydrated and embedded in plastic resin, which allowed ultrathin sections (~50–100 nm) to be cut and imaged in the electron microscope. The researcher had to collect a ribbon of sections (Rieder, 1981), which was labor intensive and time-consuming. These sections would need to be stained with heavy metal salts to impart sufficient contrast in the electron microscope images. These approaches were technically challenging, because it was necessary to avoid contamination and loss or folding of

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sections. Even once the sample had been sectioned and the grids prepared and stained; care would still have to be taken to collect images of the same piece of tissue. The difficulties of working in three dimensions extended to methods for reconstructing the volumes in 3D. The first reconstructions were created by tracing cell structures onto thick sheets of card, or expanded polystyrene, and cutting these out to stack then together to form a physical model of the cellular structures (Reid, 1980). By the late 1980s, advances in computer sofware allowed David Birk and Robert Trelstad to use serial sectioning techniques to examine collagen fibril deposition in tendon and cornea (Birk and Trelstad, 1985, 1986). They were able to track individual collagen fibrils within a developing tendon bundle to discover that the fibrils intertwined within the fibril bundle, rather than remaining columnar (Birk et al., 1989). As computational power increased and software has improved, it has become easier to generate 3D models; however, the ability to cut thin sections of reproducible thickness and to carry the sections through staining and imaging remains in the domain of specialist laboratories.

Serial Block Face SEM—An Off-the-Shelf Tool for Large Volume Imaging

Serial block face imaging was first proposed in 1981 (Leighton, 1981). The basic principle sounds simple in that you build a miniature microtome that fits within a scanning electron microscope. The data collecting process consists of a near endless loop of cutting a slice off the top off the block, then imaging the region(s) on the block face, and saving these images. The cutting process is then repeated, allowing a stack of two-dimensional images to be collected (Fig. 1). The difficult part of the process is miniaturizing all of the components without losing any of the accuracy of the cutting process. Leighton's initial microtome design was successful, but the use was limited by the lack of variable pressure microscopes and the poor computational equipment of the day (discussed by Zankel et al., 2014). The advent of SEMs with environmental chambers capable of imaging uncoated samples allowed the development of a new miniature microtome (Denk and Horstmann, 2004), which was subsequently commercialized by Gatan as the 3view (Zankel et al., 2009). The rise in popularity of serial block face imaging can be seen in the number of papers using the technique in a growing range of fields from neuroscience (e.g., Briggman and Denk, 2006; Ellisman et al., 2011), through developing tissues (e.g., tendon [Kalson et al., 2011]), or parasites [Hughes et al., 2013], and into material sciences to examine alloys [Thompson et al., 2013] and polymers [Müllner et al., 2014]). We are now entering a time where SBF-SEM is seen as a complementary technique that will give new data to enhance a group's research rather than a novel, specialized, EM technique. Investigators are realizing the power of 3D EM to enhance their work, and this can also be seen in the increase in microscope manufacturers who are developing microscopes specifically to be sold for SBF-SEM use (Zeiss has a range of 3view microscopes, JEOL is working with Gatan to bring out a JEOL SEM with a 3view, and FEI has recently announced the production of their own, new microtome system). The initial delay in uptake after commercialization of the 3view microtome was in part due to a lack of understanding of how best to prepare samples for imaging within a scanning electron microscope.

SBF-SEM Imaging of a Developing Matrix

Using the serial block face SEM it is possible to image a larger volume of tissue faster and with greater ease than with standard serial section transmission electron microscopy (TEM). Figure 2 shows datasets from a series examining tendon development imaged by both the standard serial section method and using block face imaging. Collection of the serial section data involved many hours from a skilled electron microscope technician, while the SBFSEM dataset involved a similar sample preparation, but data collection was completely automated.

Preparing the sample for serial block face imaging is very similar to preparing the samples for sectioning and standard TEM. The sample needs to be embedded in a hard substance, for the microtome to be able to take slices down to a few tens of nanometers (Deerinck et al., 2010) The sample needs to be stained *en bloc*, to give contrast when sliced within the microscope. The quality of the "data is highly dependent on previous staining procedures and electron density of the target structures" (Lipke et al., 2014). It is possible to use this for specific applications by deliberately selecting staining regimes that understain some parts of the tissue and overstain others. These specific stains allow quicker data analysis, due to the enhanced site-specific contrast. The techniques have been used to good effect to examine the sarcoplasmic reticulum within heart muscles (Pinali et al., 2013) and glycosaminoglycan deposition within kidney glomeruli (Arkill et al., 2014). There are many metals that can be added to a sample, to increase the contrast within the samples (Tapia et al., 2012), but we have found that a good starting point is to use a variant of the osmium-thiocarbohydrazide-osmium stains (Seligman et al., 1966; Deerinck et al., 2010; Starborg et al., 2013). With a new sample, we would generally start with either the Deerinck stain using thiocarbohydrazide which enhances membrane staining within the sample (Deerinck et al., 2010) or our own stain which uses tannic acid to increase the staining of ECM proteins within the samples (Starborg et al., 2013).

Organelle Specific Stains

FINDING THE NEEDLE IN THE TISSUE HAYSTACK

The SBF-SEM technique is excellent at giving a broad overview of everything within a specific tissue, but one will

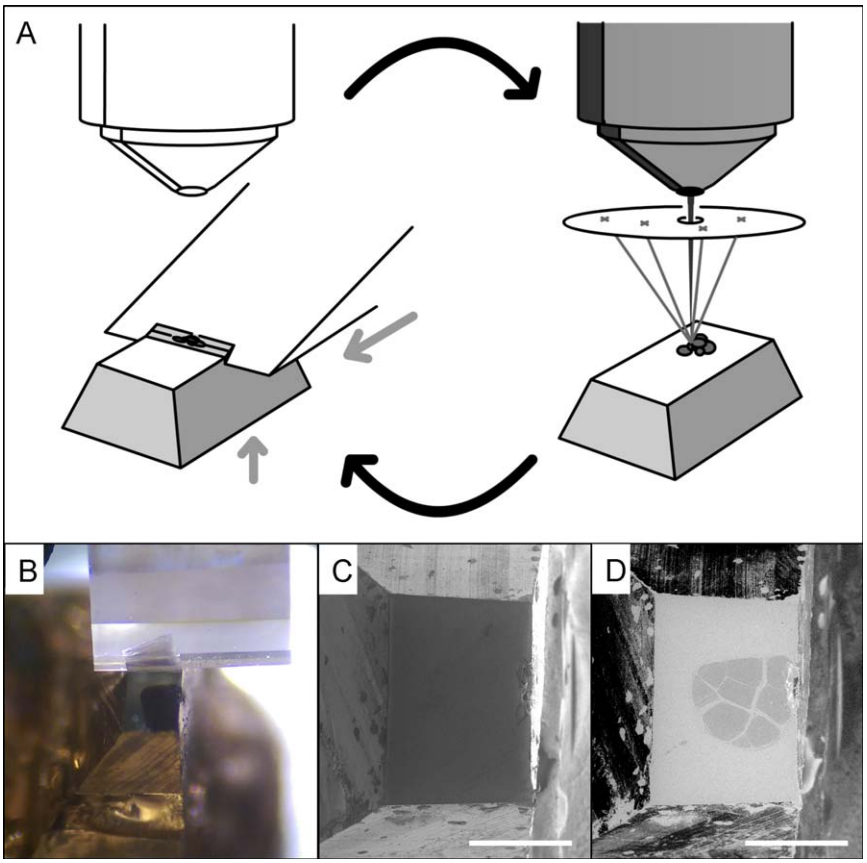

FIGURE 1. *The principles of SBF-SEM. (**A**): The SBF-SEM imaging routine consists of cycles of sectioning followed by imaging within the specimen chamber of a SEM. First a slice of a known thickness is taken with a diamond knife mounted within the chamber. The freshly sliced block face can then be imaged by scanning a beam of electrons across the surface of the block and collecting backscattered electrons using a detector mounted around the final aperture of the microscope. The process can be repeated to automatically collect a series of images with a known separation between images. (Image based on a figure in Arkill et al., 2014) (**B**): Gold-coated specimen viewed during mounting on the microtome. The diamond knife can be seen in the top of the image with a few compressed sections on the knife surface. The sample is coated with gold to help reduce charging artefacts. It is preferable to expose some of the stained sample surface for coating to give a path of conduction, but in this case the extracted tendon is surrounded by resin. (**C**, **D**): SEM images of the block surface. In (C), the block face has been imaged within the SEM using secondary electrons. The contrast is predominantly due to the topography, and so little of the sample can be seen, while the image in (D) was collected at the same using a back scattered electron detector. This image has been inverted to give a TEM like contrast. Atomic number is important for backscattered electron signal, so the gold coating gives a strong signal (seen as black in the images). It is also possible to localize the stain within the sample, which allows the individual fascicles to be seen within the block surface. (Scale bar for C and D is 500 μm.)*

often need to know the location of a specific protein, or organelle. It is possible to label proteins, using horseradish peroxidase (HRP), which can be used for localization in both light microscopy and EM. The Helsinki group has been using HRP staining to great effect to examine the dynamic restructuring of the endoplasmic reticulum using a combination of live cell imaging and SBF-SEM (Vihinen et al., 2013; Joensuu et al., 2014). Other groups are developing smaller genetically encoded tags that can be tracked in real time with a fluorescence microscope, but which generate an identifiable dark stain during preparation for block face imaging. A singlet oxygen generator, which is half the size of green fluorescent protein (GFP), has been successfully used to follow synaptic cell adhesion molecule transport in intact mouse brain (Shu et al., 2011) and a small ascorbate peroxidase has been used to track a mitochondrial protein in vitro (Martell et al., 2012). The development of site-specific tags will allow GFP-like discoveries on an EM scale. By labeling specific molecules, it will be possible to identify both the phenotypical change in tissue architecture and the localization of an aberrant protein. Colocalization with light microscopy techniques allows users to correlate information across a number of scales.

EM Doesn't have to be Daunting

FINDING YOUR WAY ON AN EM SCALE

The benefit of electron microscopy is that one can image structures with greater precision than other imaging

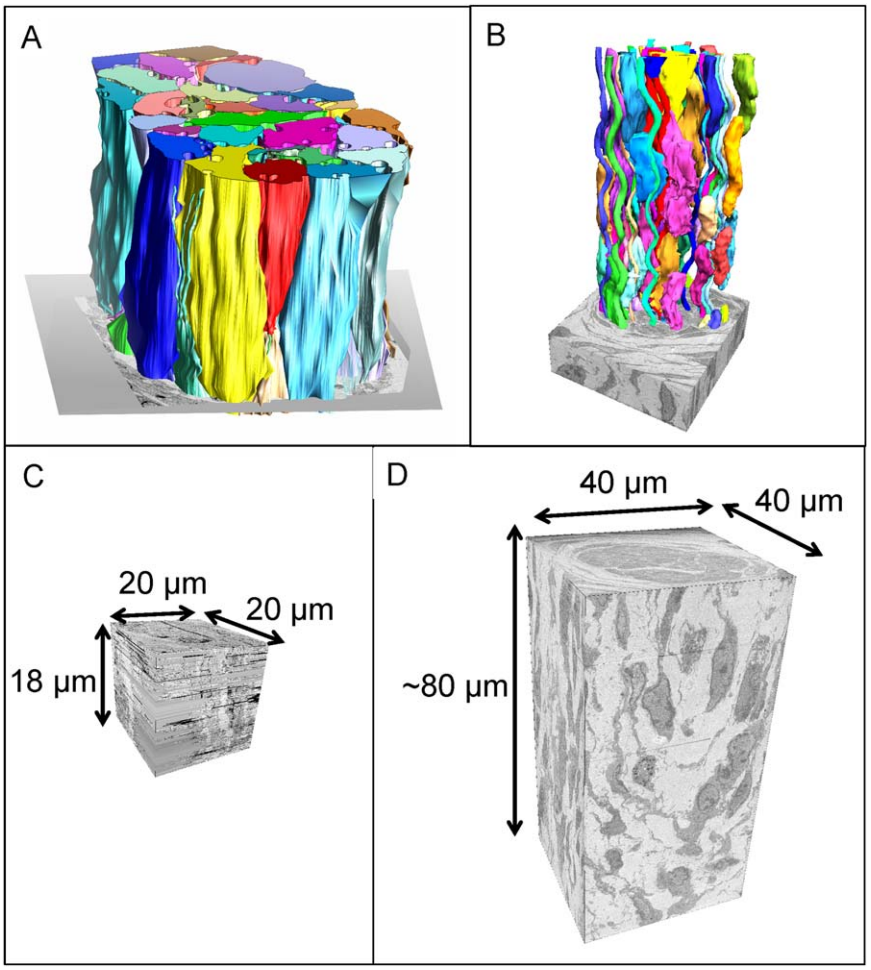

FIGURE 2. *SBF-SEM imaging can be used to image large volumes with great spatial fidelity. (***A***): Demonstrates a reconstruction from serial sections through an E15.5d mouse tail tendon showing the close packed cells aligning to form channels in which collagen fibrils are deposited (from Richardson et al., 2007). (***B***): A similar reconstruction from an SBF-SEM volume (from Kalson et al., 2013). Here slices were taken through a newborn tendon and modeled to examine the cellular organization. It is possible to see an overall wavy structure within the volume that would have been lost if the sections had been aligned to form a stack based on aligning similar features within adjacent sections as is done with traditional serial sectioning. (***C***, ***D***): The external surfaces of the volumes used to generate the images in (A) and (B). The cubes demonstrate the difference in volume between the two data collection methods. The serial section stack (C) consisted of 245 nm × 70 nm sections, while the SBFSEM stack shown (D) included 795 nm × 100 nm images. The maximum volume collated by serial sectioning is limited by the technical skills and patience of the operator collecting and imaging the sections, while the SBF-SEM volumes are usually limited by the time on the machine.*

techniques. The down side is that the data can be very rich, as everything that stains is seen, rather than targeted molecules. The data volumes are also, typically very small, compared with those from a light microscope and so more care needs to be taken to make sure that the correct region of the sample is imaged. Fortunately, there are resources available that can help with both decoding of the EM section images and with localizing the data within a bigger volume. The creation of a number of good development atlases has helped with preparing samples, as morphological cues can be used when trimming the samples (e.g., mouse http://www.emouseatlas.org/emap/home.html, zebrafish http://zfatlas.psu.edu/progress.php, or an example of a more specific tendon atlas of the mouse forelimb; Watson et al., 2009). The standard microtome can be used to quickly cut larger volumes and periodically test the location, based on what can be seen on the block face. It can help to add markers that can be identified while trimming. One approach is to mount the samples in thin volumes of resin and to use the contrast that is left in the sample to identify gross morphological markers using a standard light microscope. Using a laser it is then possible to physically damage the resin next to the region of interest, so that it can be quickly identified when the sample is transferred to a microtome for trimming (Kolotuev, 2014). Others are marking the sample during

light microscope imaging before fixation and embedding has occurred, effectively forming "landing lights" within the sample to guide your approach during trimming (Bishop et al., 2011; Maco et al., 2014). With care it is possible to pinpoint a region close to the specific region of interest. It is always possible to use the serial block face technique at lower resolution to image the volume before the region of interest, to find the specific start point for high-resolution imaging. Once the data have been collected it is possible to learn more by examining the slice images. If those who are not experienced electron microscopists, some of the online resources that catalog typical EM images of cells and organelle are valuable (e.g., http://www.uni-mainz.de/FB/Medizin/Anatomie/workshop/EM/EMAtlas.html or cell centered database http://ccdb.ucsd.edu/CCDBWebSite/index.html; Martone et al., 2003, 2008).

Data Analysis: "Organelle Specific Stain: Finding the needle in the tissue haystack"

Once the sample has been loaded into the SBF-SEM, it is possible to collect large numbers of images (1000 is not uncommon) corresponding to approximately 100 μm z-depth (along the cutting axis) when 100 nm is removed with each cycle of the microtome. What one does with these images will depend on the biological question asked. The large amounts of data collected can pose a new, but exciting, problem for the inexperienced user. An issue that we have found is that many investigators are not used to working with and discussing 3D datasets. The vocabulary chosen to describe the data has to be altered, so terms like "region of interest" are used as opposed to "area of interest." It can also be confusing to examine the raw data, as we have grown up looking at whole objects from the outside, rather than slices through the object. For example a looping blood vessel within a volume will appear as two separate blood vessels in many slices, but only a few slices will show that the two cross-sections are part of the same vessel. When specific questions are asked about the locations of features and how their size, shape, and overall structure changes from sample to sample (for example through a developmental series), a method has to be used to extract that information from the raw data. The process of defining specific regions within a 3D volume is known as segmentation. The simplest method of generating a model is to use a form of thresholding or isosurfacing (modeling examples are shown in Fig. 3). There are a number of software packages that will provide methods for the selection of a particular voxel density or to select all voxels above/below a certain density. Free packages include IMOD (Kremer et al., 1996), which is designed for use with tomographic data but can be adapted for use with serial section stacks, and Fiji (Schindelin et al., 2012), which has plugins for 3D data analysis. The software takes advantage of the fact that we know the exact position of every voxel in the volume; by switching them on, or off, in a reconstruction based on the image pixel intensity, it is possible to generate a 3D reconstruction of the objects in the volume (Fig. 3c, d). Other software allows the investigator to color the volumes based on the pixel intensity, for example, giving the nucleus a dark blue color and other features in red to get an idea of the overall organization within the volume (Fig. 3f). An issue with this form of analyses is that the pixel intensity might not be sufficient to separate close, but different structures within a volume. The simplest models will only give a visual representation of the data and little information will be gained about volumes, size, and spacing, as the software will have no way of distinguishing between separate features. With suitably stained datasets it can be possible to model the structures and generate measurable volumes based on binarised image data (Pinali et al., 2013). In general, some form of manual manipulation will be needed, to make sense of the data. Using tools within the available software, it is possible to trace features, to define that the voxels within the selected boundary are all part of the same structure (Fig. 3b, e). Manually tracing structures can be a laborious task, but the advantage of manual segmentation is that one can accurately define the volumes of interest by putting in as much time as is needed, to understand what is "going on" in the volume. The fact that the investigator has to sit down and work through the image slices means that he/she will start to learn more about how the structures are interacting and also start to see smaller interactions that might not have been expected and could have been missed in the overall reconstruction.

Advances in Data Analysis

The development of tools to make segmentation easier is bringing a whole new method of segmentation into existence. The idea of crowdscourcing using semiskilled or unskilled users to generate the volumes that a single skilled user would take months or years to complete. We have some experience of training final year undergraduate students in the use of IMOD with a graphics tablet and allowing them to trace the structures within a developing tendon. On a small scale this has allowed us to model some interesting structures that might otherwise have remained hidden within the raw data. Groups within the connectomics field are taking this approach much further, testing whether they can use crowdsourcing techniques to segment their datasets (Marx, 2012). The main issue with crowdsourcing is that the cohort that chooses to participate in the analysis will often have little experience in EM, and so methods have to be found to break the data analysis into simple questions. Groups have experimented by turning the segmentation into a game where you color in different neurons (Kim et al., 2014) or a simple question

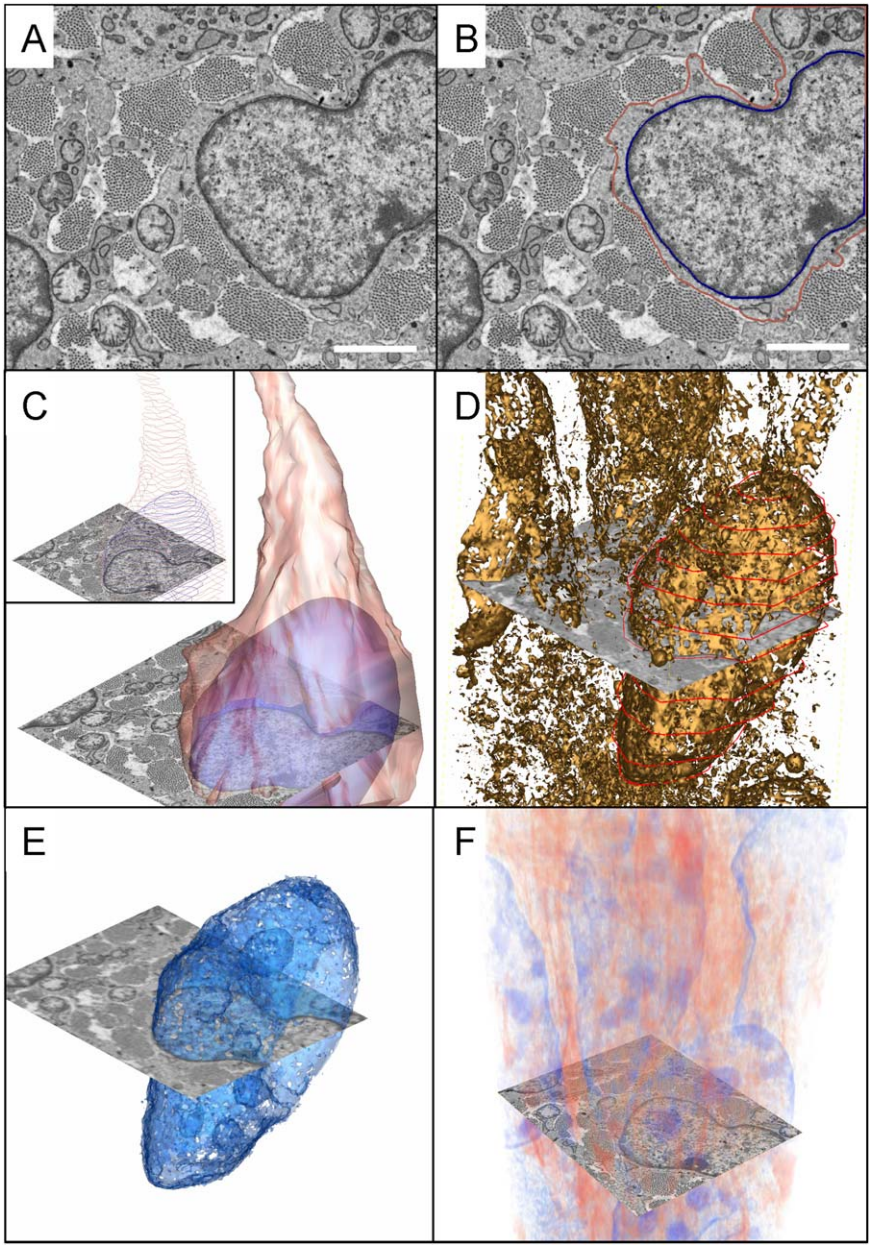

FIGURE 3. *Three methods of model generation from SBF-SEM data. To demonstrate a few basic methods of modeling data within an SBFSEM volume, we have cut out a small volume of an E17.5d mouse-tail tendon dataset. (***A***): A slice from the middle of the test volume. The image shows a tenocyte surrounded by organized bundles of collagen fibrils that have been sliced transverse to their long axis. The nucleus of the cell can be seen as the large, well-stained, organelle to the left of the image. (***B***): Many of the methods of data analysis will involve some manual effort to segment the data. Here the outline of the main tenocyte has been traced in brown, while the nuclear envelope has been manually traced in blue (tracing has used IMOD [Kremer et al., 1996], but other software uses a similar principle). (***C***): Demonstrates how manually tracing structures over many sections can be used to generate a 3D reconstruction of the structures of interest. Here the cell and nuclear envelope shown in (B) are traced over multiple slices (insert). As the voxel size is known the software is able to place each contour in 3D space. By covering the contours in a semitransparent "skin" it is possible to visualize the overall shape of the tenocyte within this volume. (***D***): It is possible to get the software to connect regions of a similar density together to form an isosurface image of the data. Here we have used IMOD to isosurface the full test volume. (***E***): By restricting the isosurface to the region around the cell nucleus (using a very crude model of the nucleus shown as red lines in D) It is possible to select a surface that shows the location of the dark stain within the nucleus. The figure shows the semitransparent nuclear isosurface, which demonstrates the overall nuclear volume and the presence of several densely stained nucleoli within the nucleus. (***F***): Using a similar principle it is possible to give each voxel a color and transparency based on the original grayscale value of the imaged pixels. Here we have used Amira (FEI Visualization Sciences Group, Bordeaux) to select values that color the nucleus and nucleoli blue, with matrix and cellular components are shown in red. It is possible to generate complex coloring procedures to identify cellular components, but in data rich volumes it can become confusing with multiple overlapping features, so this coloring procedure works best for high contrast samples, or for examining small subvolumes of data. Scale bars for A and B are 2.5 μm. The same slice data is shown in all images to demonstrate the relative scale.*

of whether two points are within the same object (Giuly et al., 2013). The errors in these approaches are high, but by collecting enough data from different users you can average out the errors to give a good approximation.

As computational power is increasing and image analysis concepts are developing, it seems obvious to try to use the computers themselves to speed up the process of data segmentation. Driven by the connectomic field, there are now a number of computational concepts that are being tested and continually improved. The problem with discussing software strategies is that they are often out of date by the time they are published. Currently there is no forerunner among the computer software. The data can be so complex that even the better algorithms are still producing a large number of false positives (e.g., 13% was reported for a ridge detection method Knowles-Barley et al., 2011). Therefore, it appears that segmentation needs to be seen as an iterative process. A round of computation can be followed by proofreading to check the accuracy of the model. Proofreading tools are being developed to simplify the process of examining a model of a large detailed volume; these will become useful tools for examining large SBF-SEM datasets (Jurrus et al., 2013; Haehn et al., 2014).

Users should not be put off by the current state of analysis software. Most users will be able to manually segment their own data using current free or commercial software packages. The analysis of smaller datasets can give amazing insight and detail that is not possible with 2D EM or light microscopy. When becoming more ambitious the investigator will need to think like Winfred Denk, who developed the first 3view machine: "My current thinking is that we'll just produce a large volume. . . Then, that will embarrass the analysis people into coming up with something." (Marx, 2013).

What should we Expect to see?

The ability to collect volumes of data with high spatial resolution in three dimensions is opening up opportunities to study cell–matrix interactions over dimensions only dreamed of previously. Within our own research field it should soon be possible to trace entire collagen fibrils within a fibrous tissue and to identify every interaction that a particular fibril undergoes (Fig. 4). In this way, we can accurately define the interactions that allow force transfer across long distances and to understand how a cell senses those forces. We can examine how intimately the cells interact with the matrix, and whether cells are interacting with matrix molecules within a single axis, or across multiple potential force directions.

Alternative Imaging Approaches

Of course, serial block face imaging is by no means the only method for imaging large volumes at higher resolution. While it currently offers the best mix of speed, ease of use, and resolution, there are many complementary techniques that could be used to collect comparable datasets. High-resolution volumes of data can be generated to examine cell–matrix interactions using electron tomography to visualize the 3D organization of structures within a slice of tissue. By tilting the sample within the microscope, it is possible to collect a series of projection images that may be used to reconstruct the volume within the original section (for more information on the principles of electron tomography principles see Frank, 2006). It is possible to collect serial thick sections and recombine the resultant tomograms into a stack comparable with those collected by standard serial section reconstruction (an example volume generated by stacking 60 serial thick (each 300 nm in thickness) section tomograms is shown in Kalson et al., 2013). Electron tomography allows larger volumes to be imaged with fewer problems of folded sections, lost sections, or staining issues, but the process still relies on good technical skills to collect the sections and computational skills to compute the data and recombine to form the overall volume.

Several new methods have been developed to try and speed up the data collection process. A few groups are experimenting with speeding up the collection of sections, using either a modification of the microtome process (e.g., by collecting sections from multiple blocks on a ribbon [e.g., Hayworth et al., 2006] or by collecting sections on a silicon film and automatically imaging these sections within a SEM [e.g., Horstmann et al., 2012]).

There is also a technique analogous to the SBF-SEM, where a specialized imaging machine contains two columns: a standard SEM column and a focused ion beam. The ion beam accelerates ions (often gallium) down the column onto the sample. The ions are dense and the impact on the sample can cause parts of the sample to sputter away (rather like sand blasting, but on a much smaller scale). Most of the dual beam manufacturers now have software that allows the ion beam to be used to mill away precise slices of the sample, while the electron beam is then used to collect a back-scattered electron image of the stain in the newly exposed sample surface (e.g., Knott et al., 2008). The advantage of ion beam milling is that very precise section thickness can be taken (down to 5 nm when accurately detecting the beam edge; Boergens and Denk, 2013), but the current disadvantage is that the milling is time consuming, and so it is difficult to image large volumes (Knott et al., 2008).

The future instrumentation is looking to increase the speed with which data can be acquired. Zeiss has developed a system with 61 parallel beams that can image multiple regions of the block surface and collect data from these regions at the same time (discussed in Marx, 2013). This will allow data to be collected at higher speeds, putting more strain on the data processing side of the equation. For now we are content to "chip away" at our

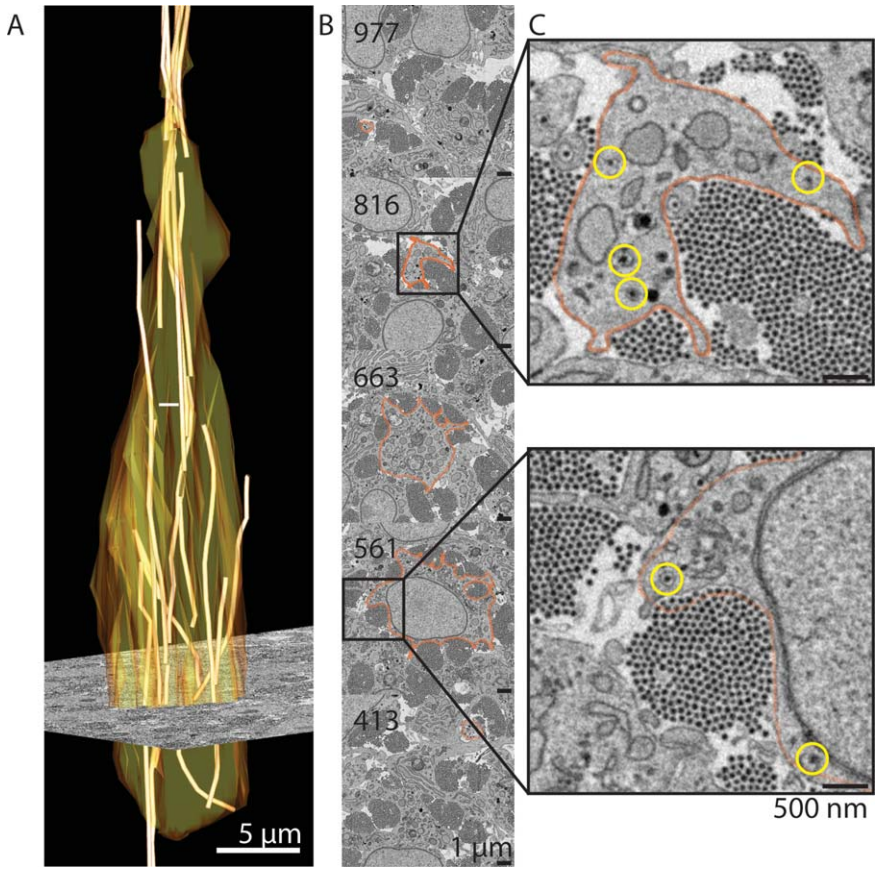

FIGURE 4. *SBF-SEM gives enough resolution to allow fine structures to be traced through the volume. Here entire collagen fibrils (30–40 nm diameter) have been tracked to plot cell–matrix interactions (figure taken from Kalson et al., 2013). The images are generated from volume of 30 µm × 30 µm × 100 µm of 14-day embryonic chick tendon. (***A***): 3D reconstruction of an entire tenocyte showing the location of collagen fibrils that are associated with the plasma-membrane. (***B***, ***C***): Demonstrate how the individual cell and collagen fibril have been modeled in the sections. Individual fibrils have been tracked through multiple sections to examine the close association of some fibrils with the cell membrane. The numbers shown in (B) show the section number of the particular image. (C): A higher magnification view of sections 816 and 561 demonstrating how internal collagen fibrils can be seen in the cross-sectional images.*

samples and generate volumes that provide extra insight into our model systems.

Conclusions

SBF-SEM is a new tool in the developmental biologist's kit-bag that fills an important resolution gap between TEM and multiphoton fluorescence microscopy. TEM (especially in cryo tomography mode) provides high resolution imaging that is capable of identifying multiprotein complexes in three-dimensions (Lucic et al., 2008). However, samples are frozen, need to be thin (a few microns at most), the TEM instruments are expensive, and the computational and noise reduction methods are not routine. Multiphoton fluorescence microscopy allows imaging of living tissue (to ~100 µm deep) and can be combined with the use of fluorescent proteins. However, the resolution is insufficient (at the present time) to visualize, for example, transport vesicles or extracellular matrix assemblies. This is where SBF-SEM fills the gap; it can image relatively large volumes of tissue at a resolution in-between that of TEM and multiphoton microscopy. Extra benefits can be obtained by combining SBF-SEM and multiphoton microscopy. Research groups are imaging fluorescent proteins in living tissue using multiphoton microscopy, marking the tissue with "landing lines" and taking the tissue to SBF-SEM. In conclusion, exciting developments are on the horizon with correlative 3D–3D microscopy, in which the best of 3D multiphoton microscopy is combined with the best of 3D SBF-SEM.

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