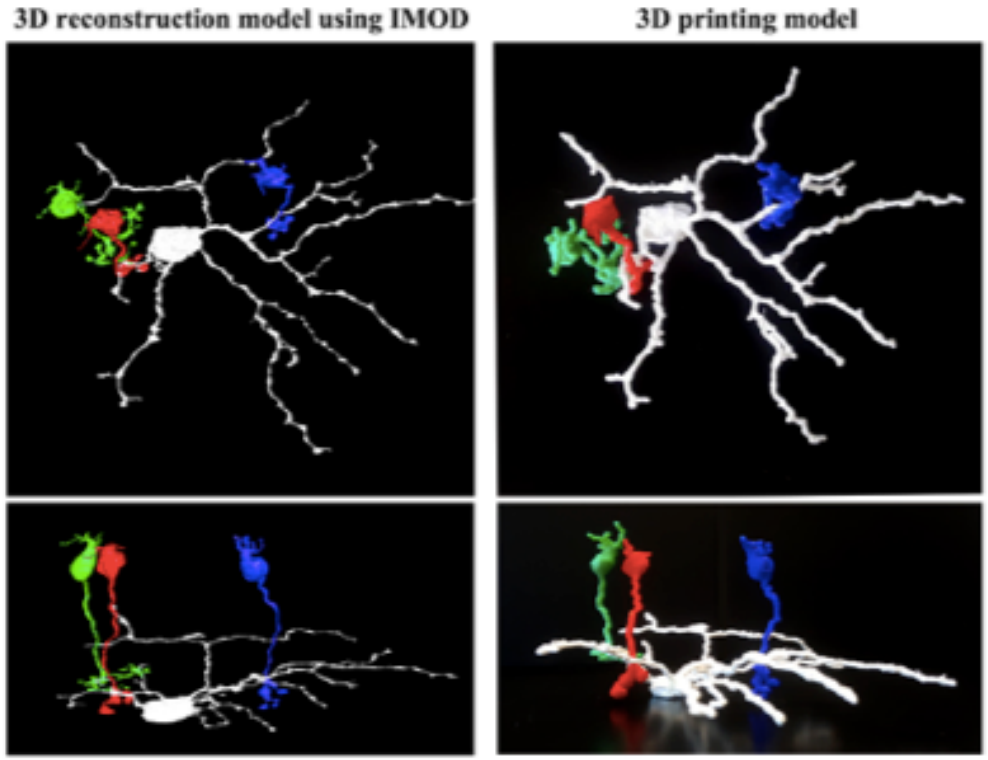
New visualization and analysis approaches using 3D electron microscopy and 3D printing technologies: Studies of optic glioma mouse model and retina microcircuit



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Introduction

- Serial block face scanning electron microscopy (SBEM) is a highly advanced technology that allows the acquisition of serially sectioned, imaged and digitally aligned electron microscopy data (Denk and Horstmann, 2004). There is a big benefit that can be obtained from the resulting 2D to 3D EM image stacks, but this presents a new challenge for scientists – how to computationally analyze and make the best use of the big datasets collected. One approach is to segment structures and features of interest in 3D. However, the software programs can appear overwhelming, tedious and not intuitive for those new to image analysis. There is a limited number of trials that provide a sufficient method for this type of 3D annotation. Thus, the challenges of 3D EM have now shifted from how to collect the 3D EM data in a quick and easy way to what to do with this big dataset (Perez, 2014).

- The methods of 3D EM data annotations can be classified into manual, semi-automated and automated methods. Despite the surplus of software options currently available, human-based manual annotations are necessary to obtain accurate reconstructions. The neuron that was manually traced is just one out of 10,000 neurons in a small section of a mouse's brain. With the manual segmentation method, it took more than a week to trace ten nuclei. The mathematical estimation showed that the manual segmentation of all nuclei in one SBEM dataset (~500,000 μm^3 volume size) would require 24/7 work for one year from three scientists. Furthermore, the reconstruction of all nuclei in the whole mouse brain (~500 mm^3) would require 24/7 work for one year from about 2.7 million scientists. Thus, the manual segmentation of organelles from SBEM image stacks represents a notable bottleneck to quantitative analyses and more advanced tools are needed to assist in measuring precise 3D structures of organelles within data sets that can include hundreds to thousands of whole cells.

- During my three year project, I introduced and tested three popular image analysis methods: manual (IMOD drawing tools), semi-automated (Interpolation) and autosegmented (Imodauto and ImageJ) segmentations for the qualitative and quantitative analysis of SBEM data. I provided the details of the data analysis processing and in doing so, I introduce to researchers the advantages and limitations of the software packages and tools. Also, I showed that these expedited approaches in 3D EM visualization and analysis reveal the intricate nature of dendritic branching and synaptic interactions in retinal neurons in a tangible model. Also, using automated segmentation, obvious alteration of the axon bundles of mouse optic nerve cancer model has addressed which never been addressed previously. Finally, the most remarkable aspect of my study is the combination of 3D EM technologies and 3D printing visualization in the synapse scale level. The 3D printed models of RGC and bipolar cells allow for the observation of whole-cell details including synaptic connectivity between two cell types. This is the first trial of the 3D printing visualization of the neuronal cell microcircuit at the EM level.

Research Paper: Previous Experiment

- Electron microscopes have been used for decades to image ultrastructural details of biology samples at high magnification. However, the resulting images are 2-dimensional. The improved resolution and amount of detail afforded by emerging EM techniques, such as serial block scanning electron microscopy (SBEM) (Denk and Horstmann, 2004), is enabling researchers to explore scientific questions related with to morphology and network connectivity that were previously impossible (Eisenstein, 2009). SBEM techniques couple with new staining protocols (Deerinck et al., 2010) and reveal cell boundaries, synapses, and many intracellular components, such as synaptic vesicles and mitochondria.
- Electron microscopes have been used for decades to image cellular details at high magnification, but its resulting images are 2-dimensional. The development of an electron microscope in which I can section tissue in situ means that I can now acquire stacks of images giving us details in 3-dimensions. However, the challenge with these large datasets is to reconstruct features of interest to form a 3D model. There are a number of computer programs that can be used to do this but they are not intuitive and can be overwhelming for researchers new to them. Here I explain the terminology used in the programs and provide a step-by-step guide with accompanying videos to help researchers get started with their image analysis and get the best from their data.
- Optic glioma is a common autosomal dominant disorder that affects 1 in 3,000 people worldwide (Friedman, 1999). One-third to one-half of these tumors cause clinical symptoms, including visual loss and hypothalamic dysfunction (Thiagalingam et al., 2004). In 2009, Kim et al., first reported the disoriented axonal projections and axon damage of the mouse optic glioma using EM and EM tomographic analyses (Kim et al., 2010). However, quantification of the axon loss or 3D visualization of axonal alteration has been limited by the lack of big 3D EM data set and analysis tools. They indirectly quantified the axon damage by measuring the number of RGCs and showed the evidences of the axonal alteration using 2D EM technique.
- Emerging 3D visualization techniques to study the microcircuit of neural network are very important to the field of neuroscience. In the retina, bipolar cells pass the visual information from photoreceptor cells to interneurons, which process the information for RGCs. RGCs are the only cell types whose axons convey visual information via the optic nerve to the lateral geniculate nucleus, and then through the optic radiation to the visual cortex (Sanes and Masland, 2015). The visualization of these structures and mapping of neural connectivity in retinal neurons with actual three-dimensional (3D) morphology are therefore critical challenges in retinal cell biology. The study of RGC intra-retinal circuitry has been limited by the lack of specific markers for retinal cells and RGC subtypes.
- Doctors McDougal and Shepherd showed that 3D printed cells can be readily examined, manipulated, and compared with other neurons to gain insight into both the biology and the reconstruction process (McDougal and Shepherd, 2015). I shared our printable models in a new database, 3DModelDB, and encourage others to do the same with cells that they generate using our code or other methods. To provide additional context, 3DModelDB provides a simulatable version of each cell, links to papers that use or describe it, and links to associated entries in other databases. They suggested that Connectomics techniques such as automated 3D electron microscopy (Kaynig et al., 2015) are a potentially important future source of high-resolution morphological data for microcircuits. My longer term goal is to move from showing multiple neurons together to be able to print out microcircuits to visualize how neurons interact via synapse in EM resolution.

Core Science & Purposes

1. To reveal axon degeneration and alteration of axon projection in mouse optic glioma SBEM data using automatic segmentation.

- Previous research showed some evidences of the disoriented axonal projections and axon damage of the mouse optic glioma using conventional EM analyses. However, quantification of the axon loss or 3D visualization of axonal alteration has been limited by the lack of big 3D EM data set and analysis tools. **In this project, the experimental SBEM dataset that fully covered the tumor area allowed for distinguishing the axon damage and alteration. Moreover, the automatic segmentation remarkably reduced the time spent on annotation and quantification of the large SBEM data and allowed reliable 3D visualization and quantification of the axon damage and alterations.**

2. To reveal retinal the neuron microcircuit between RGC and bipolar cells using alternative semi-automatic segmentation.

- Most of the SBEM dataset has variable micro organelles which cannot be addressed with present automatic segmentation tools. RGCs and bipolar cells have nuclei, dendrites and axons that showed various morphologies and manual tracing adapted to any dendrite shape and generated a very accurate 3D RGC model. However, the present SBEM volume contained thousands of neurons and it was impossible to extract all neurons with manual reconstruction. Thus, more advanced semi-automatic approaches are needed to annotate the retinal microcircuit. The interpolator plug-in of IMOD reduced the segmenting time by 5 to 6 times and showed a very similar mashed 3D volume with manual reconstruction. **In this project, an alternative way (IMOD interpolator segmentation) of approaching the reconstruction of retinal neurons with 3D EM data is tested and applied to show the interaction between RGC and bipolar cells via synapses.**

3. To test 3D Printing retinal neurons and their microcircuit with SBEM reconstructions and show new 3D visualization approaches for understanding the nature and role of these intricate ultrastructures and synaptic interactions.

- 3D Printing neuron reconstructions and artificially generated neuronal morphologies in 3D provide new ways of understanding the nature and role of neurons. Due to the limitations of printer technology, the delicate morphologies of retinal neurons pose the main technological challenge to constructing these 3D printouts. Combination of 3D printing and the SBEM retinal microcircuit 3D model would allow a realistic observation instead of looking at microscopy images that are necessarily 2D projections of neurons. **In this project, the morphologies of retinal neurons, bipolar cells, and RGC are visualized using a 3D printer. This 3D visualization reveals the intricate nature of dendritic branching in retinal neurons in a tangible model. Also, a method for producing 3D printable neurons from the SBEM dataset of the retina is introduced.**

Materials and Procedures

- **Materials:**
  - **SBEM volume :mrc format electron micrograph image stack**
  - **Linux Desktop Computer**
  - **IMOD software**
  - **ImageJ software**
  - **Excel**

1-1. Segmentation of SBEM volume

- IMOD is a free set of image processing, modeling and display programs used for 3D reconstruction of EM serial sections and optical sections. The package contains tools for assembling and aligning data within multiple types and sizes of image stacks, viewing 3D data from any position, and modeling and display of the image files. IMOD was developed at the Boulder Laboratory for 3-D Electron Microscopy of Cells (Kremer et al., 1999). Once the terminal is opened, type “3dmod” to browse the image stack and model file.

1-2. Manual Segmentation using closed contour and drawing tools in IMOD

To start the manual segmentation, go to the Menu bar > Edit > Object > New. After naming the new object, the “closed contour” option is selected as the object type. Open the "Drawing Tools" under the “Special” menu will display the following tools:

- **Normal** - normal contour drawing behavior/ **Sculpt** – quicker contour drawing behavior/ **Join** – merges two contours or objects/ **Transform** - move, scale, rotate or stretch the currently-selected contour/ **Eraser** - deletes contours or points/ **Warp** - quickly correct bad regions of contour by warping/ **Curve** - draw a smooth contour by clicking a sequence of points and then end the contour by pressing [space] or connecting the end of the contour to the beginning; pressing [n] disconnects from the selected contour without adjustment/ **Measure** - allows you to draw a straight line between any two points and displays the distance between these points in the relevant units.

Materials and Procedures

1-3. Semi-automatic segmentation: Interpolator plugin - reducing the number of contours you have to draw

- Most images are hundreds of electron micrograph slices, so tracing every slice can be tedious. The IMOD interpolator accelerates segmentation by shape morphing between contours. The easiest type of interpolation is "Linear". Draw one contour several images above another contour and hit **[Enter]**. As long as the contours overlap in the XY plane, are within the specified number of "Z Bridge" slices, and originate from the same object, the interpolator will add a series of "interpolated contours" in between the contours. These will be shown as dotted contours and for further modification contours, hit [Enter]. This will make the dotted contour solid and refine the overall object shape.
- Measurement of meshed RGC reconstruction volumes were processed with the commend:

  imodinfo –c model_name.mod

1-4. Automatic segmentation: Combination imaging tools using by imodauto and Imagej

- Well aligned SBEM mrc stack converted to tiff image stack :

  mrc2tif –s image_name.mrc image_name.tif
- Open the tiff image stack using ImageJ (https://imagej.nih.gov) and adjust image brightness and contrast : Image>Adjust>Brightness/Contrast. To remove the noisy pixel of the images, filter the image stack with Gaussian Blur tool: Process>Filters> Gaussian Blur. When the image showed pretty much black and white 8 bit image, run binary image: Process>Binary>Make binary. Save the image stack with image_name_binary.tif extension and transfer to IMOD S/W and make sure the sequential image stack.
- Convert the image_name_binary.tif image stack to mrc image stack and alter the header information of the binary.mrc stack same as the original image_name.mrc stack.

  tif2mrc image_name_binary.tif image_name_binary.mrc → *alterheader image_name_binary.mrc* → input correct x,y,z pixel information → org → input correct original x,y,z location information

Materials and Procedures

- When you have corrected header informed binary mrc stack, run commend lines for the autosegmentation processing:

  imodauto –h 128 –u image_name_binary.mrc u image_name_binary.mod

  imodmesh -CTs -P 3 ....mod ....mod

  imodsortsurf -s ...mod ....mod

  imodmesh -CTs -P 3 ...mod ...mod

- Check the model with original mrc stack and remove the false positive objects and add false negative objects.

1-5. IMOD Model File Format Conversion for 3D Printing

- IMOD opens image files in the MRC format, which have the extension .rec or .mrc and represent a stack of 2D images in a stack of 2D images making up a 3D EM.
- Within IMOD, objects contain "Contours" and contours contain "Points". Meshing an object connect contour points together and a mesh is added to that object and each mesh can contain multiple surfaces. Save the meshed model with .mod extension after every few hours (or day) of annotation work ("model_name.mod"). Then, export the .mod file to .obj format file.

  imod2obj model_name.mod model_name.obj

Materials and Procedures

2. Processing meshed model files for 3D printing

2-1. Repair and orient models with Autodesk Meshmixer

- A model might require repair when it contains multiple overlapping contours that cause errors when the file is read by some slicing software. Imported OBJ file repaired, if there were problems with the mesh, a warning sign was displayed.
- Use ***Extras > Automatic Part Repair***, select ***Extended Repair***, and wait while the file is processed.
- Right-click on the model and select ***Export Part > As STL*** or use ***Project > Export Project as STL*** to save the repaired model.
- Optimal orientation of a model prior to slicing should be reduced the number of overhangs and therefore the number of supports required during the printing process. An optimally oriented model will print faster, use less material, and be less likely to fail during printing. More detail can be found in supplement 2.2.
- Select ***Analysis > Orientation***.
- Adjust the Strength Weight value to 100, Support Vol Weight value to 0, Support Area Weight to 0, and then update the model. This will rotate the model to minimize the number of overhangs. Accept the resulting orientation.
- Use ***File > Export*** and choose binary STL file from the dropdown menu. Save the file.

3. Slicing the 3D model and printing

- In the last step, the STL file was loaded into Ultimaker Cura software which is called “slicer” because it converts .stl files into slices that printers can sequentially produce to build 3D objects. Several options were modified, but in every case, changes were necessary only to fit the characteristics of the 3D printer. After checking the position of the object on the production plane, the user needs to switch from *view mode* to *layers* in order to check the absence of gaps in the building procedure. After this semiautomatic elaboration, the object information was exported as a G-CODE file and loaded into the printer by using a Secure Digital (SD) card. The 3D copy was then printed with the same commercially available printer used to manufacture the test objects.

Observations and Results

1. Automatic segmentation addressed axon degeneration and alteration of axon projection in mouse optic glioma SBEM data.

Previous research showed evidence of the disoriented axonal projections and axon damage of the mouse optic glioma using conventional EM analyses. However, quantification of the axon loss or 3D visualization of axonal alteration has been limited by the lack of big 3D EM data set and analysis tools. First, the experimental SBEM dataset that fully covered the tumor area and corresponding area of the wild type (WT) optic nerve (ON) allowed for an abundance of information that can be obtained from the resulting 2D axon bundle image stacks (Fig 1A-a). However, manual segmentation of each axon from the SBEM image stacks represents a notable bottleneck, and more advanced tools are needed to assist precise 3D ultrastructures of axon bundles within data. Thus, three SBEM volume analysis from each WT and tumor ON reconstructions using auto-segmentation were tested and compared in axon diameter and volume (Fig 1). Since IMOD uses the MRC image format, each SBEM MRC stack has been converted to TIF format image stack in order to create a binary image stack using ImageJ S/W, an open source image processing program designed for scientific multidimensional images (https://imagej.nih.gov/ij/). For making an optimal binary image, the brightness of the TIF stack should be adjusted and contrasted and processed with the Gaussian blur filter. Electron micrographs of the axon bundle in the optic nerve had highly contrasting images. Thus, the outcome of the binary image stack was pretty successful compared to the other ultrastructures (Fig 1A-b). The converted MRC binary stack from the ImageJ TIF binary stack was processed with imodauto programs to detect the boundary of each axoplasm and to create the contours around all axoplasms in the SBEM volumes (Fig 1A-c). Imodmesh was used to create mesh data from closed contours. Each contour was connected to contours directly above and below by an algorithm that minimizes the total area of the triangles used to connect the contours. Contours contained totally within other contours are considered to represent inside surfaces and will be connected to the outer surface where appropriate (Fig 1A-d). However, meshed surfaces were recognized as only one object that was not appropriate to analyze each individual axon's information. Thus, using the imodsortsurf program, lumped axon surfaces have been sorted to each individual surface (Fig 1A-e). The imodinfo program computed the axon diameters (Fig 1B) and the axon volumes (Fig 1C). Quantification through the combination of two methods, 3D EM (SBEM) and automatic segmentation, remarkably reduced the data annotation time (data not shown). The time spent with manual segmentation for about a hundred axon was about 40 hours. However, automatic segmentation time spent was 30 minutes to an hour, depending on the SBEM data quality. Auto-segmentation analysis clearly addressed the alteration and degeneration of the axon in the optic glioma. In the glioma model, each axon diameter was smaller than a WT axon and the volume of the axon was significantly reduced (Fig 1 B,C)

Observations and Results

2. Revealing the retinal neuron microcircuit between RGC and bipolar cells using alternative semi-automatic segmentation.

- To resolve the ultrastructure of RGC and their neurites, the horizontal retinal section from SBEM data was collected using a Zeiss Merlin scanning electron microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with a Gatan 3View. Automatically-repeated slicing of the horizontal mouse retina specimen and acquisition of images made it possible to collect 973 slice images (Fig 2A) and align the image stack to reconstruct neuron morphology (Fig 2B). Because mice lack a fovea or visual streak, all retinal cell types including RGCs are mostly equally distributed across the retina. The horizontal retinal SBEM volume was 125 x 125 x 58 um (volume = 906,250 um^3), spanning the outer half of the ganglion cell layer (GCL) to the inner edge of the photoreceptor layer (PRL). Representative low magnification electron micrographs of the SBEM image stack showed the locations of the target RGC soma (Fig 2C) and dendrites (Fig 2F). In the higher magnification micrographs, ultrastructural features of the RGC soma (Fig 2D and E) and dendrites (Fig 2G and H) displayed the well-preserved micro-organelles, including the mitochondria and endoplasmic reticulum.
- To build the appropriate 3D morphology of the RGC, 3D model reconstruction was performed from serial micrographs of the SBEM data (Fig 3 A-F) using IMOD segmentation tools. Using manual segmentation, plasma membranes of the RGC were annotated in each serial image and accumulated contours were presented in the IMOD model view (Fig 3). A total of 3,950 contours were manually segmented (Fig 3 G) for the whole 3D RGC model, and a series of unconnected contours were meshed into a surface model of the RGC (Fig 3 F). The meshed RGC clearly formed a branched dendritic network, and 3D reconstruction of those segmented RGC allowed for the identification of the cell type. Based on the morphology of the reconstruction, the RGC is a bistratified retinal ganglion cell whose dendrites stratify with both ON and OFF sublamina of the inner plexiform layer (Fig 3 G, H). Manual segmentation of the RGCs from the SBEM image stacks represents a notable bottleneck, and more advanced tools are needed to assist precise three-dimensional structures of neurons within data. Thus, four RGC reconstructions using manual or interpolation methods were compared in total contour numbers, total segmenting hours spent and total meshed volume. First, four randomly selected RGCs were segmented manually using the normal, sculpt, and join tools in the drawing tool plug-in of IMOD. Time spent using the manual segmentation was about 130 to 170 hours per RGCs (Fig 3 M). The majority of the RGC showed various dendrites. Tracing the plasma membrane of the dendrites took more time and effort using manual reconstruction. However, manual tracing adapted to any shaped dendrites and generated a very accurate 3D RGC model. An obstacle is that present SBEM volume contains thousands of neurons and it is impossible to extract all neurons with manual reconstruction. The interpolator plug-in of IMOD reduced the segmenting time by 5 to 6 times (Fig 3 M) and showed a very similar mashed 3D volume with manual reconstruction. The total volume of the four RGCs didn't show a significant morphology difference between the manual segmentation and the interpolation (Fig 3 N). Volumes inside the mesh of RGC 1-4 using manual segmentation were 794.17 μm^3, 832.04 μm^3, 1333.72 μm^3 and 1333.72 μm^3. Volumes of RGC 1-4 using the interpolator were 787.64 μm^3, 877.95 μm^3, 1497.43 μm^3 and 1299.34 μm^3.
- As a cell type of the retina, bipolar cells exist between the photoreceptors and the RGCs as presynaptic partners of the RGCs. In figure 3, a total 435 contours were segmented for the whole 3D bipolar cell model and 376 contours were built by automatic interpolation segmentation (Fig 4D). A series of unconnected contours were meshed into a surface model of bipolar cell (Fig 4F) and has a conventional synapse with the reconstructed RGC. Based on morphology of the reconstruction, the bipolar cell made direct connections with the RGCs in the ON-sublamina (Sanes and Masland, 2015).

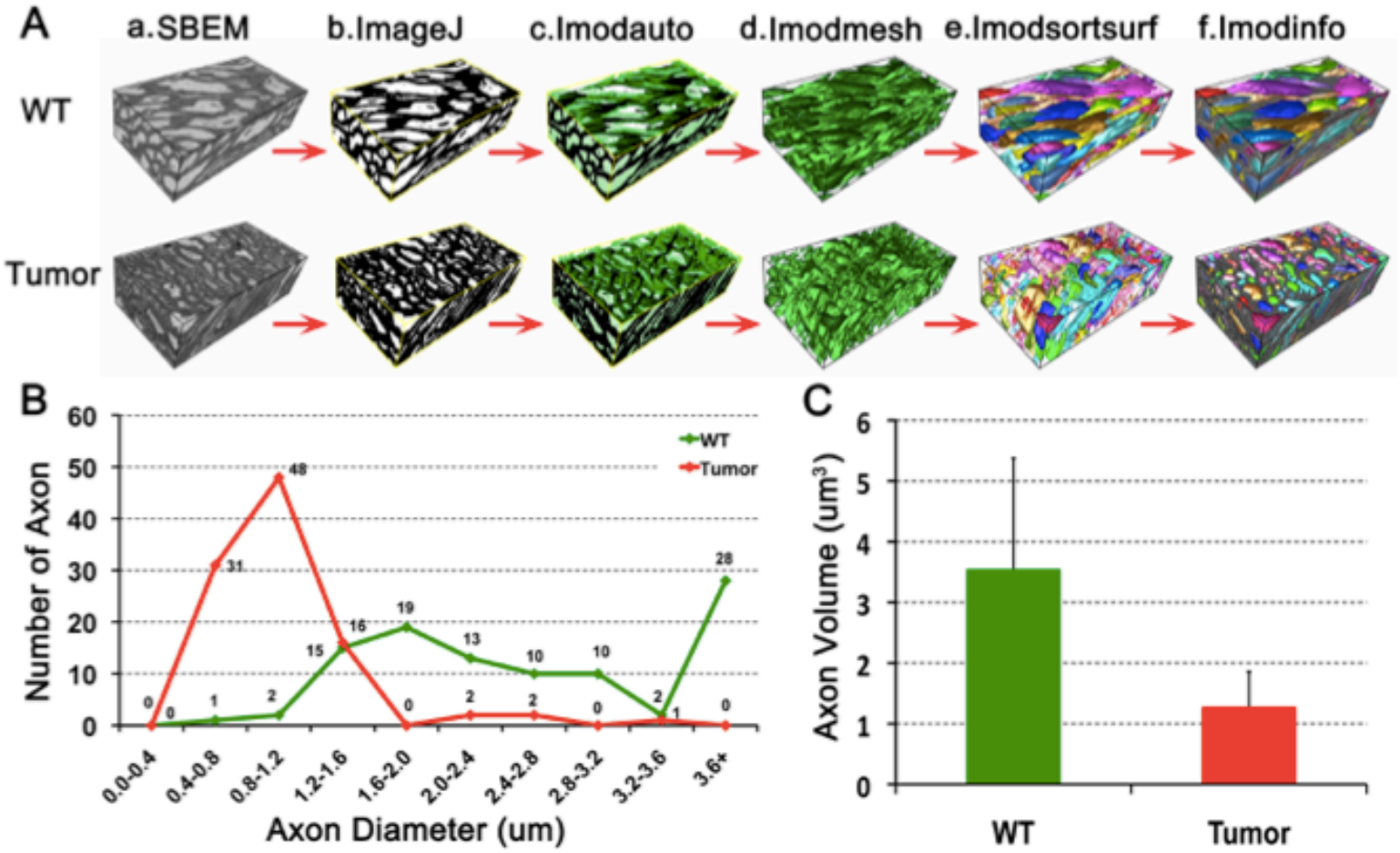
Observations and Results

3. Testing 3D Printing retinal neurons and their microcircuit with SBEM reconstructions and showing new 3D visualization approaches for understanding the nature and role of these intricate ultrastructures and synaptic interactions.

- Reconstructed 3D neuron model files were exported to OBJ or STL 3D model files and processed for the 3D printing. A model may require repair when it contains multiple overlapping pieces with intersecting contours. However, RGC and bipolar cell models did have overlapping contours that caused errors when the file was read by some slicing software, as intersecting regions can be interpreted as the exterior of the model (Fig 5). Using Autodesk
- Meshmixer, optimal orientation of a model prior to slicing will reduce the number of overhangs and therefore the number of supports required during the printing process (Fig 5 A,D). An optimally-oriented model will print faster and use less material, as well as have less chance of failing during printing. For the RGC model orientation, the original meshed IMOD model showed that the RGC soma was located at the bottom and the dendrites branched towards the top. However, the 3D printing outcome resulted in difficulty of removing the supporting materials (data not shown). Thus, 180 rotation of the model using the Ultimaker Cura reduced the number of supports and successfully produced the 3D printing RGC (Fig 5 B,C). For the bipolar cell model orientation, the bipolar cell axon was relatively long and thin. Thus, if the meshed cell were printed in the original orientation, the bond between layers would be weak and easily break. 90-degree rotation of the original model orientation produced a more strongly-bonded model (Fig 5 D-F).
- Mapping synaptic connections between neurons are essential to understanding the neuronal circuits that facilitate the flow and processing of information in the brain. However, the ability to resolve a complete circuit is hindered by the necessity to process large tissue volumes and by the resolution that can be achieved with available microscopy techniques. SBEM provides the ultrastructural resolution necessary to visualize and assess single synapses and the ability to automate the collection of serial sections of large volumes of tissue (Denk and Horstmann, 2004). In the retina SBEM data, synaptic interaction between RGC and bipolar cell was addressed in Fig 6. Post-synaptic RGC dendrite (white) received synaptic inputs from a presynaptic partner, bipolar cell axon terminal (red) which included neurotransmitter vesicles (yellow) and mitochondria (Fig 6 E and F). This synaptic interaction successfully displayed in IMOD 3D reconstruction model (Fig 6 A and B) and 3D printing model (Fig 6 C and D).
- The Workflow of the 3D printing visualization of neuron models is illustrated in the Figure 7.

Data and Graph Summary

Figure 1. Workflow of the automatic segmentation with WT and optic glioma SBEM dataset and quantification results of axon diameter measurement and volume.



Data and Graph Summary

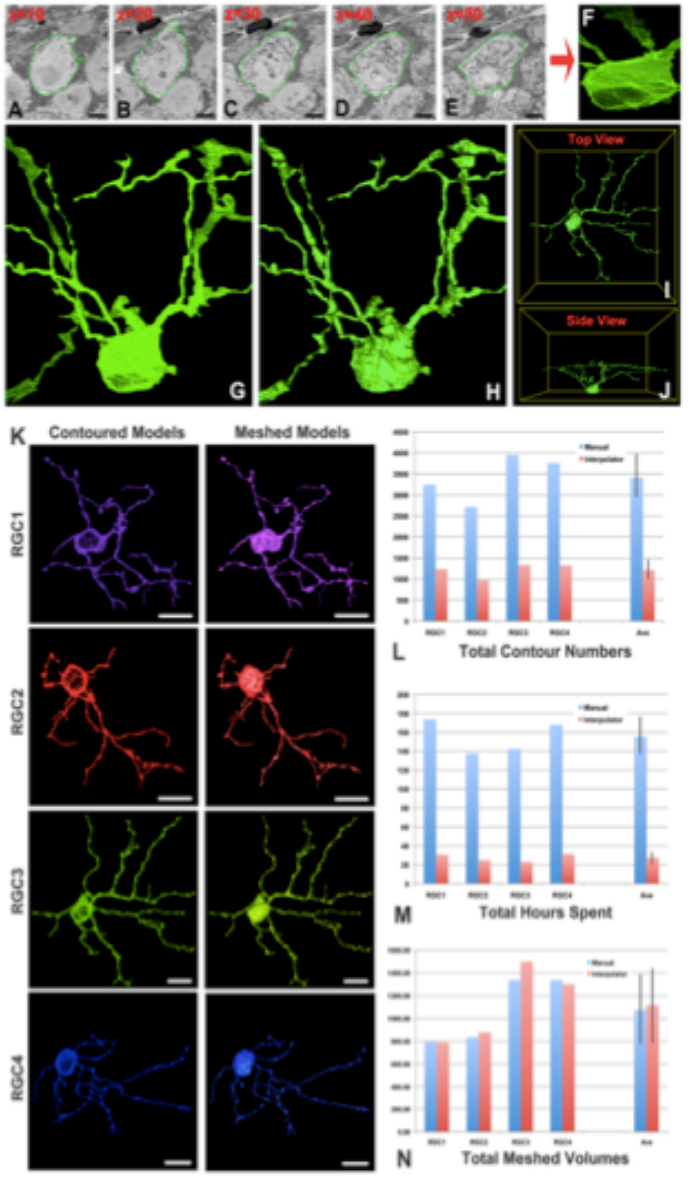


Figure 2. Semi-auto segmentation using IMOD drawing tools and interpolator plug-in in sequential Z series image slices. Green contours annotated plasma membranes of RGC soma (A-E). Consecutive contours showed 3D RGC soma morphology in 3D model view (F). Total 3,950 RGC membranes annotated whole RGC (G) and rendered into one RGC surface (H). Top (I) and side view (J) the bistratified retinal ganglion cell. Four RGC reconstructions to compare the manual or interpolated segmentation methods (K). Total contour number analysis (L). Total hours spent measurement (M). Total meshed Volume (N). Scale bars = 5 µm (A-E), 20 µm (K).

Data and Graph Summary

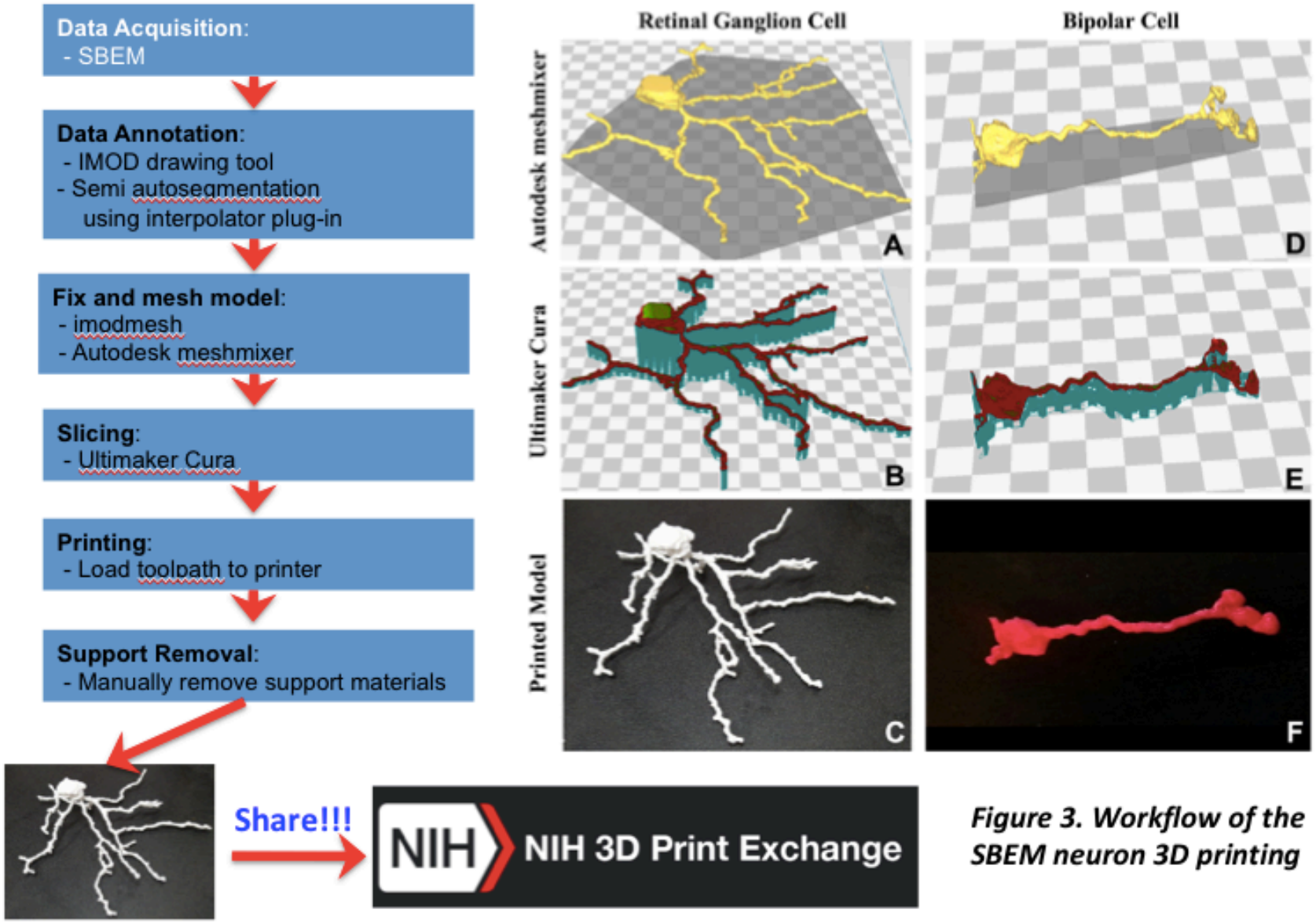


Figure 3. Workflow of the SBEM neuron 3D printing

Conclusion and Recommendations

- Solving the mysteries of the brain requires powerful technologies as well as manual man power. First, I have presented a method for automated tracing of myelinated axons in serial stacks of optic nerve images. After creating binary image stack from the 3D electron micrograph MRC stack, automatic segmention time spend was 30 min to an hour to annotate about hundreds axon segmentation which corresponds to a tremendous reduction of the manual annotation load. Kim et al., showed evidences of the disoriented axonal projections and axon damage of the mouse optic glioma using conventional EM analyses. However, quantification of the axon loss or 3D visualization of axonl alteration has been limited by the lack of big 3D EM data set and analysis tools. Figure 1 showed well organized workflow of the automatic segmentation process. Quantification through out combination two methods, 3D EM (SBEM) and automatic segmentation, remarkably reduced the data annotation time. Automatic segmention reduced annotation time spend about 60 times faster than manual segmentation. Auto-segmentation analysis clearly addressed that the alteration and degeneration of the axon in the optic glioma. In the glioma model, each axon diameter was smaller than WT axon and, the volume of the axon was significantly reduced.

- Despite its peripheral location, the retina of the eye is actually part of the central nervous system (CNS). Consistent with its status as a full-fledged part of the CNS, the retina comprises complex neural circuitry that converts the graded electrical activity of photoreceptors into action potentials that travel to the brain via axons in the optic nerve. Although it has the same types of functional elements and neurotransmitters found in other parts of the central nervous system, the retina comprises only a few classes of neurons, and these are arranged in a manner that has been less difficult to unravel than the circuits in other areas of the brain. In the retina, bipolar cells pass the visual information from photoreceptor cells to interneurons, which process it to the RGCs. RGCs are the only cell types that have axons that convey visual information via the optic nerve to the lateral geniculate nucleus, and then through the optic radiation to the visual cortex. There are around 12 types of bipolar cells in the inner nuclear layer and 30 types of RGCs that contain the most elaborate spine-free dendritic arbors in the inner plexiform layer (Sanes and Masland, 2015). Visualization of these structures and mapping of neural connectivity in retinal neurons with actual 3D morphology are crucial to consider in retinal cell biology. There has been significant progress in the visualization and study of the morphologies of retinal neurons. Advances in instrumentation for light and electron microscopy and modern computer technology allow researchers to trace neurons in 3D to quantify the morphology (Kaynig et al., 2015). The study of RGC intra-retinal circuitry has been limited by the lack of specific markers for retinal cells and RGC subtypes. However, the experimental SBEM dataset that fully covered inner retinal layers allowed for distinguishing the RGCs with the location of the RGC axons of the nerve fiber layer (Data not shown). Moreover, the SBEM dataset allowed for the annotation of the details of RGCs and their intra-retinal partner, bipolar cells. Manual segmentation of RGC from SBEM image stacks represents a notable bottleneck, and more advanced tools are needed to assist precise three-dimensional structures of neurons within data. Thus, one of the semi-automatic approaches for the retinal neuron reconstruction tested and confirmed to be advantageous in drawing plug-ins, “Interpolator”, allows smart interpolation of contours across multiple slices via special interpolation interface**.** Manual and interpolation segmentation in randomly-picked RGCs revealed that the manual segmentation method is the most accurate yet takes more time than the interpolator. The interpolator resulted in a dramatic reduction in runtime and mostly-accurate 3D models.

Conclusion and Recommendations

- Recently, the NIH launched the NIH 3D Print Exchange (https://3dprint.nih.gov), a public website that enables users to share, download and edit 3D print files related to health and science. Researchers are now able to turn virtual models into physical objects, aiding in planning complicated, life-saving surgeries or teaching. They're also able to digitally visualize objects and systems that were never before possible, helping power research discussions and science communication (McDougal and Shepherd, 2015, Da Veiga Beltrame, 2017). However, printing out microcircuits to visualize how neurons interact in synapse level (nanometer scale) is still unrevealed. IMOD is an image processing program used for 3D reconstruction and modeling of microscopy images with a special emphasis on EM data and, allowing microscopists to segment structures of interest by drawing contours around them using a mouse or other input device (Kremer et al., 1999). Such contours are sorted into objects that can be individually meshed, displayed, and morphologically quantified. IMOD uses the MRC image format, which provides the advantage of adding all micrographs from a series into one file as a 3D stack. Thus, it was a critical tool in addressing the morphologies in whole neurons in micrometer scale to the synaptic interaction in nanometer scale. My expedited approach of IMOD segmentation utilizes semi-automatic drawing tools for rapid tracing and refinement of cell boundaries and 3D reconstruction of segmented RGCs and their pre-synaptic partners for the morphological identification of specific cell types involved in RGC circuitry.

- The most remarkable aspect of my study is the combination of 3D EM technologies and 3D printing visualization in the synapse scale level. The 3D printed models of RGC and bipolar cells allow for the observation of whole-cell details including synaptic connectivity between two cell types.

- In summary, automatic segmentation with 3D EM dataset is a strong tool to diagnos illnesses of the optic glioma 3D EM which is relatively high contrast dataset. This technique should be extended to segment myelinated axon boundaries in brain images, where the axons do not follow the same direction and the staining is not limited to myelin sheaths. IMOD Skip interpolator segmentation use in 3D EM data is another advanced tool to accelerate the reconstruction of low contrast dataset. Type of retinal neurons and their synaptic interaction has addressed in shorten time. Thus, these tools fill a critical need by allowing for the quantitative analysis of volumetric EM datasets at a nanoscale. The combination of the optimized annotation technique with 3D EM datasets and 3D printing can deserve the high-resolution morphological data for microcircuits. Future goals are to aim to elucidate multiple types of RGCs and bipolar cells in the SBEM dataset and eventually print out the whole retina connectome to visualize neuronal interaction and morphology.

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