Serial sectioning and electron microscopy of large tissue volumes for 3D analysis and reconstruction: a case study of the calyx of Held

Brian K Hoffpauir[1,3], Brian A Pope[1,3], George A Spirou[1–3]

[1]Department of Otolaryngology, [2]Department of Physiology and Pharmacology, [3]Sensory Neuroscience Research Center, PO Box 9303, Health Sciences Center, One Medical Center Drive, West Virginia University School of Medicine, Morgantown, West Virginia 26506-9303, USA. Correspondence should be addressed to G.A.S. (gspirou@hsc.wvu.edu).

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Serial section electron microscopy is typically applied to investigation of small tissue volumes encompassing subcellular structures. However, in neurobiology, the need to relate subcellular structure to organization of neural circuits can require investigation of large tissue volumes at ultrastructural resolution. Analysis of ultrastructure and three-dimensional reconstruction of even one to a few cells is time consuming, and still does not generate the necessary numbers of observations to form well-grounded insights into biological principles. We describe an assemblage of existing computer-based methods and strategies for graphical analysis of large photographic montages to accomplish the study of multiple neurons through large tissue volumes. Sample preparation, data collection and subsequent analyses can be completed within 3–4 months. These methods generate extremely large data sets that can be mined in future studies of nervous system organization.

INTRODUCTION

Individual neurons influence and are influenced by the neural circuits in which they reside. It is increasingly clear that the analysis of events at a subcellular or cellular scale is best interpreted when placed in the setting of larger dimensions of neural interactions. Experimental techniques are typically designed for particular scales of analyses, and only with great effort are they applied to larger scales of tissue organization. A good example is recording from single units, which only through persistence generates data from a sufficiently large population of cells. Advantages in technology, such as arrays of recording electrodes[1,2], permit simultaneous recording of multiple single units, but challenges remain in collecting sufficient numbers of quality recordings and then manipulating and analyzing these large data sets.

Investigating the structural underpinnings of cell function also requires analytical approaches from subcellular to circuit-level scales. For example, knowledge of the convergence of particular classes of neural inputs and ultrastructural features of their synaptic connections is a key element to understanding synaptic integration and generation of activity patterns by the postsynaptic cell (for recent example, see ref. 3). These sets of information are typically approached individually by light and electron microscopic techniques, respectively. However, the most complete answers to these biological questions are achieved when the same piece of tissue is analyzed at both scales of resolution.

Serial section electron microscopy (EM) with three-dimensional (3D) reconstruction and quantitative analysis has been applied to multiple neural systems, usually focused on particular aspects of cellular components or organelles (see refs. 4–9 for examples). In certain instances, one or a few neurons have been reconstructed in their entirety (exclusive of the entire dendritic structure), involving dedicated analysis of larger tissue volumes (see refs. 10–14 for examples). These numbers are generally not sufficient, however, to draw conclusions about cell populations, nor do they incorporate enough neural geography to address more global issues of tissue organization such as branching and termination of innervating axons. A singular exception is the reconstruction of the entire *Caenorhabditis elegans* nervous system, an endeavor that required more than a decade of effort[15].

In the auditory system, several cell types that constitute circuitry important for sound localization, among other tasks, are innervated at their somata by small numbers of large nerve terminals[14,16–19]. The extreme example from these cell types is the principal neuron of the medial nucleus of the trapezoid body (MNTB). MNTB cells are located in the ventromedial portion of the brainstem and are innervated by a single large terminal called the calyx of Held[14,19–22]. We are interested in the early development of this terminal, including when mono-innervation of MNTB cells is established[23]. Given the tight packing of MNTB cells at early post-natal ages, light microscopic techniques did not provide sufficient resolution to address this issue. We therefore embarked on an extensive reconstruction of a large tissue volume using EM to count inputs onto cell bodies as a metric for competition and to describe structural features of these inputs.

Organization of the protocol

We implemented procedures that incorporate existing computer-based tools along with graphical strategies to work from large photomontages to reconstruct large tissue volumes. In the example described here, we studied a tissue volume of 6.3×10^5 μm^3, described by a 130×220 μm^2 window passed through a 22 μm tissue thickness. Given our goals at the outset, which combined questions about tissue organization (axon branching and input convergence) with identification of cellular features at high resolution, we decided upon a three-step analysis (green boxes in **Fig. 1**). The first step was a graphical reconstruction that involved constructing and analyzing large photomontages of multiple serial sections (75 montages, constituting every fourth serial section). This step of the analysis answered the fundamental questions we

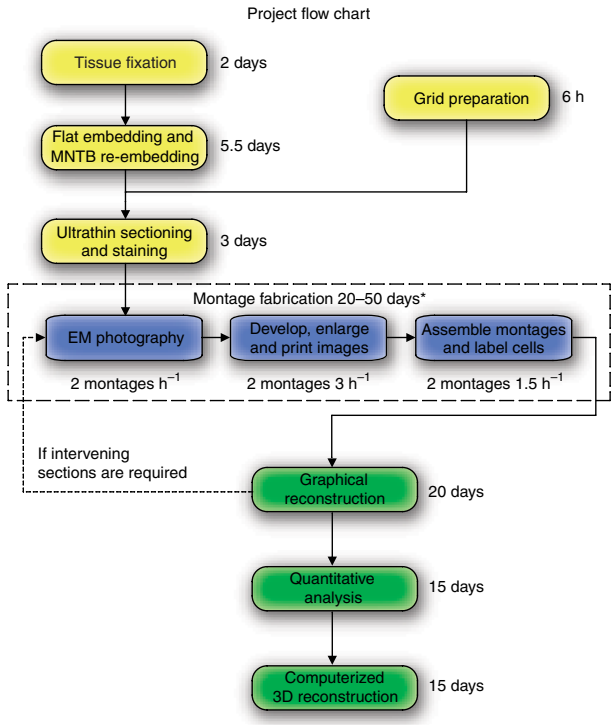

Figure 1 | Project flow chart showing estimated times for each step. Steps outlined in the vertical pathways are dependent on the completion of the preceding steps and therefore must be performed in order. Grid preparation must occur before ultrathin sectioning, but can be performed during any of the multi-day incubation periods that occur during the tissue preparation or embedding stages. Yellow indicates standard procedures that result in the preparation of tissue sections for EM; tutorials for these procedures are included in **Supplementary Box 1**. The horizontal steps (blue) must be performed in order, but can be completed by multiple personnel in an assembly line process to increase efficiency (20 vs 50 days). If multiple personnel are not available, we recommend that two montages be shot, printed and labeled each day. Taking the time to label the cells as the montages are produced will allow one to ascertain if a sufficient volume of tissue has been sampled, thus reducing the need to photograph any unnecessary sections. Graphical reconstruction (green) should be completed before quantitative analysis and the computerized 3D reconstruction, so that these latter steps are conducted most efficiently. Quantitative analysis and computerized 3D reconstruction can be performed concurrently. Note that the time estimates assume proficiency in all techniques and do not take into account any time required for training on software or equipment. The time estimates presented here assume that 20 cells will be completely analyzed and 4–6 cells will be reconstructed.

had posed regarding the innervation of MNTB cells during early development, such as the number of MNTB cells receiving single or multiple inputs, the trajectories of the presynaptic axons and qualitative estimates of terminal and cell sizes. In the second step of analysis, we sought to quantify parameters for which we had made qualitative estimates. Using the graphical analysis, we designed a sampling strategy to scan a limited number of prints (average of 12) of each cell and terminal into the computer for quantitative analysis. After completing this quantitative analysis, we explored the data to look for relationships among the quantified parameters, and therefore answered all of the questions we had posed at the outset of the study. Guided by the results of the graphical reconstruction and quantitative analysis, we selected a subset of MNTB cells (4/19) and their inputs for 3D computerized reconstruction and rendering. The 3D reconstructions were performed not only to illustrate structural features that were identifiable in sequential images but also to gain insights from viewing structures in 3D that were not apparent from inspecting a series of 2D images.

Our choice of methods was based on the analytical goals of each step as well as practical considerations, which were influenced by the necessary time and available resources required to accomplish each goal. For the graphical reconstruction, we chose to work with montages of photographic prints rather than digitized images displayed on a computer monitor (see discussion in Technical considerations below). This allowed us to efficiently view both small structures (e.g., synapses and vesicle clusters) and large structures (cell bodies and lengthy axon segments) at sufficient resolution on the same data set. We tracked the postsynaptic cells, identified synaptic contacts and determined the number of converging synaptic terminals onto each cell. These results were annotated directly on the montages, as has been performed previously for large-scale reconstructions[15]. We then cataloged key features in a spreadsheet and graphically reconstructed all of the cells in a series of tracings. For the subsequent quantitative analysis, the analyzed montages, rather than EM negatives, were scanned for measurements of somatic and terminal surface area. To accomplish this, we scanned portions of the montages, without completely disassembling them. Because the cellular processes had been identified and the relationships of all associated structures had been annotated directly on the prints, all pertinent structures could be efficiently quantified within the analysis program. The 3D reconstruction is time consuming, so care must be taken to select cells that are representative of the population. Because all overlapping images must be aligned precisely to place the resulting images in register, all images for the 3D reconstruction must be individually imported. Furthermore, placing the images into register also requires a more complete set of serial images (~70 per cell). Therefore, we scanned EM negatives for this purpose because we could digitize them more efficiently than prints (six negatives per scan rather than one print per scan), and did not have to spend time disassembling and reassembling all of the large photographic montages.

Technical considerations

Continual progress has been made to implement procedures for analysis of histological images in computerized systems (see refs. 24–27 for examples), but their relative efficiency decreases as the analyzed tissue volume increases owing to limitations in computer hardware and human interfaces to software. One challenge to computer-based systems stems from the large size of image files; for the montages we create, the file size would easily exceed 1 GB per montage. Low-magnification views of the entire montage, even using a dual-monitor computer system, do not provide useful resolution. As a result, to view the image at higher magnification, one navigates the montage using a narrow viewing window. Moving quickly among different regions of a single montage, or stepping through sequential montages requires shifting parts of the image or separate images in and out of computer memory (discussed in ref. 27). Panel array systems for displaying and searching large images are under development, but are currently specialized pieces of equipment that are not generally available or economical

to implement. We annotate the montages by outlining structures and writing labels and notes by hand, which is much quicker than manipulating cursors on a computer screen and does not lead to repetitive motion disorders associated with extensive use of a computer mouse. Human strategies for analysis are currently not well interfaced to the computer. The photographic montage permits rapid switching between low- and high-magnification views of the tissue (simply passing a magnifying glass over a structure gains additional resolution), so that detailed structure and global aspects of tissue organization can be investigated almost in parallel. One can also quickly move across a single montage among multiple cells and structures while rapidly switching magnification to search for consistencies in details of their innervation.

A common example of the challenges facing computerized systems to be user-friendly is the greater efficiency of reading reprints of scientific manuscripts held in the hand as opposed to scrolling through pdf files, even on a large computer monitor. The greater the requirement for cross-referencing and moving back and forth through the article, which increases with the size of the article and the resulting lessened ability of the reader to remember where figures and sections are located, the more complex are the human search strategies and, by contrast, the less efficient is the computerized version. Furthermore, it is much easier to highlight or write notes on a hardcopy than it is to select a tool and then highlight or fill in the text-box.

Image alignment between adjacent EM images is more challenging than working with confocal microscopy systems, where image planes are collected in register from the same piece of tissue. Small errors in alignment from frame to frame across the montage of one EM ultrathin section can lead to significant errors in linking small cellular profiles between montages. With an increasing number of images per montage and an increasing number of montages, it becomes essential to automate the alignment procedures. The individual photographic montages we use are quickly assembled using only local alignment of photographs, and we rely on human strategies to identify structures and remember them when tracing between sequential montages. Recent advancement[28,29] in using scanning EM to repeatedly image a tissue block face as it is sectioned[30] (serial block-face scanning EM) offers great promise for circumventing problems with aligning montaged images of tissue sections, even in tissue volumes that can exceed 10^6 μm^2. Further developments in electronics to achieve good signal to noise ratio and reduce image acquisition time for low accelerating voltages and improved digital camera resolution will permit this technique to approach the spatial resolution of transmission EM in the image plane.

Strategic considerations

Some strategic planning is required before embarking upon serial section analysis of large tissue volumes, and knowledge of the region and cells of interest is critical. The approximate dimensions of the cells should be known to determine the required tissue volume for study, which is influenced by the size of the photographed field and the packing density of the cells within a specific region. If cell density is such that one might consider imaging a larger field than we describe here, keep in mind the practical limitation of working with printed montages, which require a surface area twice the size of a single montage for assembly and analysis of the entire set.

Additional planning should set limits for analyses early in the study. The wealth of information contained in the montages can be overwhelming and distracting if specific goals for analysis are not established. Consider which cellular features should be studied for individual cells and determine whether or how far processes will be tracked from the cell body. Selected regions of the tissue volume may need to be studied at higher resolution than the montage photographs provide to reveal details of very small structures such as synaptic vesicles. In these cases, it will be necessary to re-photograph some portions of the montage at high magnification. We recommend obtaining these images, if possible, as the montage is generated. Alternatively, should the need arise for detailed quantitative analysis of smaller structures, separate images could be acquired from a limited tissue volume at very high magnification, using the large volume reconstruction to select a sampling location. The particular sampling intervals we have used (every fourth tissue section or about every 280 nm) are tailored for the dimensions of the calyx, its axon, the MNTB cell and small processes of these structures that we sought to identify. Analysis of structures having more complex branching geometry can also be performed, but may require slightly higher magnification images (and therefore covering a somewhat smaller tissue volume or constructing a larger montage) or different sampling of the serial EM sections.

We find that it is more efficient to first identify which cells are completely contained within the sampled tissue volume, and only then devote significant time and effort that are required to analyze only those cells. The complex 3D morphology of the MNTB cell somata and calyx-forming axons and nerve terminals can make this task difficult because multiple portions of both cells and calyces can be separated in the 2D montages. It is important to point out that the large montage permits development of various strategies to identify calyces and their axons. For example, we are able to identify features for tracking axons toward the calyx, such as noting that they often bend away from fiber fascicles in the vicinity of their termination.

If the goal is to reconstruct every cell contained within the tissue, then one might consider performing the quantitative analysis and 3D reconstruction in parallel. Note that the tracing operations for both procedures are often not the same, so the time savings may not be substantial. The strategy here would be to reconstruct local tissue volumes, thereby avoiding the difficulty of global alignment of images within and across montages. As serial block-face scanning EM moves from specialized development to a more general availability and if its current spatial resolution does not limit the investigation, then automated tracking algorithms can be extended and implemented that are capable of reconstructing multiple cells. Serial section electron tomography[31] at low magnification (G. Perkins & G. Spirou, personal communication) is subject to similar concerns, but offers a compromise in that alignment needs to be performed only at the interface between thick ($\sim$1 μm) tissue sections, because image planes within the tissue are generated in alignment.

We have attempted to highlight only the critical processes that were required for the reconstruction of calyces of Held and the postsynaptic MNTB cells. Even though reconstructing tissue volumes contained within the field of view of single electron micrographs (analyzing a stack of single micrographs) is itself considered to be quite time consuming, our intent is to reduce the intimidation factor for analysis of large volume 3D reconstructions (analyzing a stack of montages of micrographs), and to

increase the frequency for publication of such studies. Our methods can be implemented at any center that houses a transmission electron microscope. We should emphasize that proper tissue preparation and expertise in the use of an electron microscope are prerequisites for the successful acquisition of useful images. It is not our goal to provide detailed tutorials for tissue preparation for EM. We provide general tutorials for aspects of these topics in **Supplementary Box 1**, but refer the reader to refs. 32–36 for more expansive treatments of these subjects.

Goals of structural analysis

Inevitably, when studies of the brain at a particular level of organization are reported, questions arise regarding their greater significance (applicability to events at a larger scale) or underlying mechanisms (events at a smaller scale). Ideally, a combination of scales during data collection permits targeted analysis of these relationships. An important goal in neuroanatomy, we think, is to have efficient methods so that many studies of fixed tissue at the cellular to small circuit level of neural organization could be conducted by collecting serial images using EM, which could then lead toward establishing brain archives at ultrastructural resolution. These capabilities would complement the increasing application of techniques such as electron tomography to probe subcellular resolution, where the thickness of ultrathin sections becomes a limiting factor[37–41].

We have described protocols that complete a large-scale ultrastructural analysis within 3–4 months, including tissue preparation. Although a 3-month data collection and analysis time frame was permissible for the current study, it is too long for many applications. However, we envision the day when very large data sets can be surveyed, annotated and analyzed at the desired level of detail using computer-based systems. Improvements and cost reduction in hardware for wall-sized arrays of high-resolution monitors that can be annotated like a tablet using a hand-held stylus and computer systems with high memory and disk capacity that permit rapid movement of data between them will form the basis for these systems. Methods to semi-automate alignment within and between large montages or collect images in register must also be a feature of these systems. The characteristics that make working from large photomontages efficient, discussed in previous paragraphs, constitute the design principles for the hardware and software interfaces to these image analysis systems. Ultimately, implementation of computer-based systems is essential because the data set contains much more information than is used in any single study and should be archived in permanent form for future analysis in ways not envisioned by the experimenters who originally collected the data.

MATERIALS

REAGENTS

- Experimental animal: mice **! CAUTION** All animal experiments must comply with national regulations.
- 190 proof ethanol (Aaper)
- 200 proof ethanol (Aaper)
- Chloroform (Fisher Scientific)
- Glutaraldehyde—EM Grade (Polysciences)
- KCl (Sigma)
- Na_2HPO_4 (Sigma)
- $Na_2HPO_4 \cdot H_2O$ (Sigma)
- NaCl (Sigma)
- $NaHCO_3$ (Sigma)
- Osmium tetroxide 4% (wt/vol) solution (Polysciences)
- Paraformaldehyde (Sigma)
- Photoflo 200 (Eastman Kodak)
- Pioloform powder (Ted Pella)
- Poly/Bed 812 Embedding Kit (Polysciences) containing Poly/Bed 812, dodecenyl succinic anhydride, nadic methyl anhydride, DMP-30
- Professional D-19 Developer (Eastman Kodak)
- Professional Rapid Fixer w/ hardener (Eastman Kodak)
- Propylene oxide (Polysciences)
- Rapidoprint activator (Agfa)
- Rapidoprint fixer (Agfa)
- RO pure water
- Sodium borate (Sigma)
- Taab embedding resin (Marivac)
- Toluidine blue O (Sigma)
- Ultrostain 1 for EM stain, uranyl acetate (Leica)
- Ultrostain 2 for EM stain, lead citrate (Leica)
- Uranyl acetate (Polysciences)

EQUIPMENT

- 12-well plates (Fisher Scientific)
- Applicator sticks (Fisher Scientific)
- Dumont Biology Grade Self Closing Tweezers (Ted Pella)
- Embedding capsules; size 00 (Electron Microscopy Sciences)
- Glass strips for flat-embedding (6.4 × 25 × 400 mm glass knife strips, Electron Microscopy Sciences)
- Isotemp oven (Fisher Scientific)
- Jewelers saw (Structure Probe Inc.)
- Mylar 0.005 gauge 11 × 14″ sheets (Plastic Film Corp.)
- Paintbrush size 0 (Ted Pella Inc.)
- Pyrex specimen container (Fisher Scientific)
- Snap cap bottles (Fisher Scientific)
- Thermo orion sage syringe pump (VWR)
- Transfer pipette (Fisher Scientific)
- Copper Slot Grids; 1 × 2 mm slots with 3 mm outer diameter (Ted Pella)
- Custom eyelash probes: sharpen wooden applicator sticks and attach a single eyelash using nail polish
- Diamond knife for semithin sectioning (Ted Pella)
- Diamond knife for ultrathin sectioning (EM Corp.)
- Grid holders (Leica)
- Autoprocessor for film negatives (Energy Beam Sciences)
- EM Film 4489 (Eastman Kodak)
- Kodabrome II RC F3 glossy photographic paper 8 × 10″ (Eastman Kodak)
- Laborator negative enlarger (Durst)
- Negafile Glassine Envelopes for negatives (Electron Microscopy Sciences)
- Rapidoprint print processor (Agfa)
- Computer (must meet all software specifications; systems with large amount of RAM and high-end graphics card are recommended)
- High-resolution scanner for scanning reflective media and transparent negatives
- 3D-rendering software (such as Carrara, Eovia or 3DS Max, Autodesk)
- Excel (Microsoft)
- ImageJ software (freeware from http://rsb.info.nih.gov/ij/)
- Photoshop (Adobe)
- Reconstruct software (freeware by John Fiala: http://www.synapses.bu.edu/tools)
- Large flat surface for montage assembly; we recommend more than 2× the area required to assemble one montage
- Double stick tape (Scotch)
- Magnifying glass
- Tracing paper
- Single-edge razor blades (Electron Microscopy Sciences)
- Dissecting microscope
- RO water source (e.g., RO Diamondpure, Barnstead)
- Highlighting pens
- Vibrating microtome (Vibratome)
- Ultramicrotome, Ultracut UCT (Leica)
- Automated EM Grid Stainer (Leica)
- Electron Microscope, Jem-1010 (JEOL)

REAGENT SETUP

2 liters of 10× stock Ca^{2+}-free Ringer's variant 170 g NaCl, 4 g $NaHCO_3$, 5 g KCl, bring to 2 liters with RO pure H_2O.

1× Ca^{2+}-free Ringer's variant Add 25 ml 10× calcium-free Ringer's variant stock to 225 ml RO pure H_2O. The solution is filtered and then bubbled with 95%/5% O_2/CO_2 to bring the pH to 7.2. Before perfusion, the solution is warmed to 37 °C.

2 liters of 0.4 M sodium phosphate stock 17.9 g $Na_2HPO_4 \cdot H_2O$, 95.1 g Na_2HPO_4, bring to 2 liters with RO pure H_2O.

Fixative 2% paraformaldehyde (newly made, not from stock), 2.5% EM grade glutaraldehyde in 0.12 M $NaPO_4$, 5 g paraformaldehyde powder in 100 ml d-H_2O at 70 °C, 75 ml 0.4 M $NaPO_4$ stock, 25 ml EM grade glutaraldehyde, 50 ml RO pure H_2O. Filter, bring to pH 7.2 and warm solution to 37 °C.

1% osmium tetroxide in 0.12 M $NaPO_4$ Add 10 ml of 4% osmium tetroxide solution to 12 ml of 0.4 M $NaPO_4$; make up the volume to 40 ml with RO-H_2O.

2% uranyl acetate 1 g uranyl acetate in 50 ml RO-H_2O.

Ethanol solutions Dilute ethanol with RO-H_2O to obtain the various vol/vol concentrations. For example, a 30% solution contains 30 ml ethanol and 70 ml RO-H_2O.

Epon/Poly/bed 812 recipe for 100 g 25 g Poly/bed 812, 25 g Taab embedding resin, 30 g dodecenyl succinic anhydride, 20 g nadic methyl anhydride, 1.5 g DMP-30. Slowly mix together with wood applicator stick to avoid bubbles.

0.1% toludine blue O in 1% sodium borate Dissolve 5 g of sodium borate in 500 ml of RO-H_2O, then add 0.5 g of toluidine blue O powder and filter into container.

PROCEDURE

Animal perfusion ● TIMING approximately 1 h

1| Anesthetize animals on ice (post-natal days 0–5) and perfuse them transcardially first with filtered Ca^{2+}-free Ringer's variant and then with fixative. Both solutions should be warmed to 37 °C, and 8–10 ml of each is perfused at a rate of 0.75–1 ml min^{-1}. After perfusion, cover the head of the animal with a plastic bag containing flaked ice for 30 min so that the fixation stabilizes while the brain maintains its proper shape within the skull.

! CAUTION All animal experiments must comply with national regulations.

Postfixation ● TIMING approximately 2 days

2| Decapitate the animal, dissect the whole brain from the skull and drop it into a vial containing fixative for 2 days at 4 °C. Shaking is not required.

■ **PAUSE POINT** Brains can be kept in fixative up to 2 weeks.

Tissue sectioning and flat-embedding ● TIMING approximately 3.5 days

3| Dissect the brain until a single piece of tissue containing the region of interest is isolated (in this case, brainstem, pons and midbrain; in small animals, we preserve the cerebellum). Using a no. 10 scalpel blade, make a fiducial mark by removing a narrow V-shaped piece of tissue from the dorsal surface on the right side just lateral to the midline, running the rostro-caudal length of the fourth ventricle (**Fig. 2**). Alternatively, for small brains such as those of rodents, trim the cerebellum so that left and right sides of the brain can be distinguished. Section tissue coronally at 200 µm in thickness using a vibratome, and collect slices in order into 12-well plates filled with 0.12 M $NaPO_4$, one section per well.

4| Flat-embed the tissue sections in resin. These procedures and other standard procedures for tissue preparation are outlined in **Supplementary Box 1**.

■ **PAUSE POINT** Embedded sections can be stored at room temperature (20–25 °C) for years.

? TROUBLESHOOTING

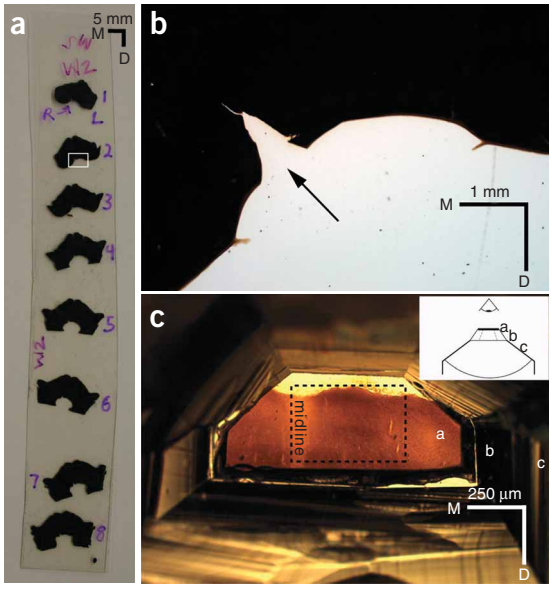

Figure 2 | Flat embedding and re-embedding of tissue for ultrathin sectioning. (**a**) Sections through cat brainstem embedded in mylar after fixation, vibratome sectioning and treatment with uranyl acetate and osmium tetroxide. Sections are numbered from caudal to rostral. As sections are cut, they are distributed sequentially into eight wells; so each well contains every eighth section from caudal brainstem to the midbrain. We show cat brainstem to demonstrate the effectiveness of techniques for flat-embedding large tissue sections. Neonatal mouse brainstem measures less than 5 mm side to side, and is relatively easy to keep flat during the embedding process. (**b**) A notch (arrow) is cut along the dorsal surface of the tissue before sectioning so that the left and the right sides of the brain can be identified for proper ordering of sections. **b** is a high-magnification view of the region outlined by a white box on section 2, panel **a**. In small rodent brains, the tissue can be trimmed on one side to maintain left–right orientation. (**c**) Top-down view of the face of the tissue block from neonatal mouse after trimming for ultrathin sectioning. The face is trimmed to a house shape, and the sloping sides of the resin block extend downward from each edge of the face. The taller part of the block (top to bottom in the picture) contains the midline, MNTB and a portion of the lateral superior olivary complex (regions contained within dotted line). Inset shows a lateral view of the embedding capsule, trimmed around the tissue, which is viewed on edge (thick line) at the top of the block. The sloping sides of the resin block are represented by dashed lines, indicating that they are located on the far side of the block. M, medial; D, dorsal.

Identifying the MNTB and re-embedding the tissue ● TIMING approximately 2.5 days

5| The embedded sections can be viewed under a compound microscope under low power at high light intensity. Identify the region of interest, which in this case is the MNTB.

▲ **CRITICAL STEP** Carefully cut the MNTB from the larger tissue sections using a single-edge razor blade under a dissecting microscope. Include the MNTB, neighboring cell groups and tissue extending to the ventral brain surface and at least 200 μm contralateral to the midline.

6| Re-embed and trim the sections as outlined in **Supplementary Box 1**.

■ **PAUSE POINT** Embedded sections can be stored at room temperature for years.

Ultrathin sectioning and staining ● TIMING approximately 3 days

7| Prepare slotted grids as outlined in **Supplementary Box 2**.

? TROUBLESHOOTING

8| Begin sectioning at 70 nm. We adjust the block angle to orient the face of the trimmed block parallel to the knife motion. This adjustment is typically within ±2 °. We achieve good results with the diamond knife clearance angle set to 4 °, although this value can be optimized for the particular resin and desired section size. The sections of appropriate thickness have a silver sheen.

9| Cut 21 sections and pause to collect 20 sections onto slotted grids (**Supplementary Box 2**), leaving one section on the edge of the knife. Note that each section should adhere to the previous section as they are cut, forming one continuous ribbon.

10| Repeat Step 9, cutting 20 sections each time, until the appropriate number of sections have been collected. The section left on the knife-edge facilitates formation of the next ribbon of sections. The knife-edge is backed away from the tissue during section collection, then advanced again to cut the next group of sections. As a result, the first section in each group is thinner than the others, averaging one-half the desired thickness. By estimating the approximate thickness from the color of the section[42], the resultant error in quantifying terminal and cell size is $<1.5\%$ (assuming 30% error in color estimate[43] applied to 1/20 tissue sections).

▲ **CRITICAL STEP** Determine what depth of tissue should be sectioned to include a sufficient number of complete cells for analyses. We suggest cutting enough sections to encompass at least 1.5× the longest diameter of the target neuron. We cut 306 sections (~22 μm tissue depth) to study the neonatal MNTB.

▲ **CRITICAL STEP** Care should be taken to keep sections as centered and straight as possible on the slot of the grid to facilitate consistent alignment of photographs from grid to grid (**Supplementary Box 2**).

? TROUBLESHOOTING

11| Stain grids using Leica EM Stain automated grid stainer. This is the only commercial device that we are aware of and it is capable of staining 25 grids at one time. We use program no. 1, which wets the sections in RO water for 8 min and exposes the tissue to uranyl acetate (1% wt/vol; Ultrostain 1) for 30 min and lead citrate (3% wt/vol; Ultrostain 2) for 10 min, with each stain followed by RO water rinses of 5 min. Grids can be stained manually[32–34] if one does not have access to an automated grid stainer. One must take great care with manual staining because the grids are fragile and extra handling increases the chance of damaging them. We have found the automated grid stainer to perform well in this regard and to provide greater consistency and better overall quality of staining. Steps 1–11 are indicated by yellow ovals in the flow chart of **Figure 1**.

EM photography ● TIMING approximately 20–50 days

12| Begin by photographing a section collected from the middle of the tissue series.

▲ **CRITICAL STEP** This first section will be used to better define the borders of the MNTB and to identify a region of interest for subsequent photography. A section from the middle of the tissue series is selected because the cells contained within it are most likely to be fully contained within the series of serial sections. The region of interest should be tracked and photographed in both forward and backward directions from this beginning point to ensure that the same area on each tissue section is being examined.

13| Load and secure grid into specimen holder with the sections facing up. Some electron microscopes may allow you to rotate the image of the sections, but if the grids are seated in the specimen holder to ensure a consistent orientation of sections, it will not be necessary to rely on this feature. Maintaining a consistent orientation of sections ensures montages of equal size and shape can be easily stacked and analyzed while turning through them like pages in a book (**Fig. 3**).

? TROUBLESHOOTING

14| Load specimen holder into the electron microscope.

! **CAUTION** Individuals should be well trained to operate the electron microscope.

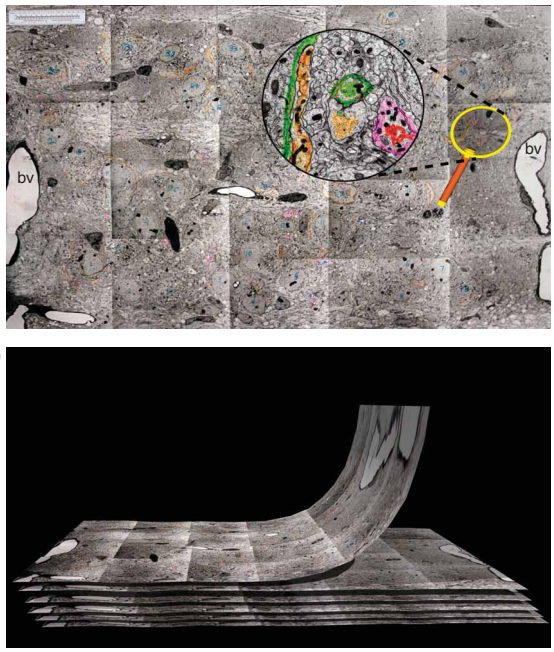

Figure 3 | EM montages of ultrathin sections. (**a**) An assembled and analyzed EM montage. This montage is comprised of 20 prints (five columns of four prints) and measures ~26 × 44″. The actual size of the photographed area on tissue section was ~130 × 220 μm^2. Cells were numbered (blue ink) and calyces were highlighted in orange as the montages were assembled. These marked prints were scanned for quantitative analysis of somatic surface and calyx apposition area, which was our measure of calyx size. Calyx-forming axons were highlighted in pink; these were identified during the graphical analysis. We initially used the large blood vessels (bv) on the right and left sides for photographic landmarks; note that other landmarks were required as these blood vessels were not present throughout all of the tissue sections. A 150 mm (~6″) ruler has been placed at the top left corner for reference. (**b**) An illustration of a stack of montages. To track the cells through the montages, we typically flip through these stacks as turning the pages of a book. Consistent alignment of structures permits a rapid global assessment of their spatial relationship to other structures and how they change through the tissue depth.

15| Photograph the initial section at a low magnification (×800–×1,000) to complete a montage of the entire section. ▲ **CRITICAL STEP** Begin photographing on the ventral edge of the tissue, just slightly lateral to the midline, contralateral to the MNTB; the midline will be included in this initial montage. Photograph a column (ventral to dorsal) of images by moving dorsally in a straight line and slightly (~10%) overlapping each picture to facilitate the montage assembly (below). Then move laterally through the midline toward the MNTB, maintaining a slight overlap of the previously photographed column, and photograph another column, this time from the dorsal edge to the ventral edge. Repeat this zigzag pattern as necessary until the entire MNTB, much of the superior olive lateral to the MNTB, including the medial superior olive, and ventral reticular formation overlying the MNTB have been photographed.

16| Develop film negatives using automatic film processor and store each negative in a glassine envelope. Label each envelope with the negative number. If images are captured using a digital camera, then high-resolution glossy prints should be generated from digital images for assembly of montages. We prefer the resolution of photographic film for these analyses.

17| Project images with an enlarger to fit onto a sheet of 8 × 10 glossy photographic paper.

18| Print enlarged images with a print processor.
▲ **CRITICAL STEP** Write the negative number on the back of each print for reference.

BOX 1 | ASSEMBLING MONTAGES (30–45 MIN FOR EACH MONTAGE)

1. Lay out all of the prints from a section on a large flat surface and match overlapping edges. Keep the EM labels containing the date, grid number, section designation and magnification to the left.
2. Use a poster cutter to trim the borders, including the data labels, from all of the prints except those that will form the left column of the montage. Take care to leave enough overlap to allow proper alignment for taping the prints. For those prints that will form the left column of the montage, trim only the top, right and bottom borders of the prints so that the label data.
3. Tape all of the prints together using double-sided tape. Place the tape at the extreme corners of the underlying prints. This allows one to easily peel up and look under the top prints to inspect areas of interest that may fall on the edges. The completed low-magnification montage described in Steps 15–21 measured 32 × 70 inches, whereas the completed montage of the region of interest, which corresponded to an area of tissue measuring ~130 × 220 μm, was comprised of 20 prints with final dimensions of ~26 × 44 inches.
▲ **CRITICAL STEP** Montages must be well assembled and sturdy enough to withstand the frequent turning, as pages in a book, that occurs while tracking cells and axons during the analytical stages of the process (**Fig. 3b**).
4. Label each montage on both ends with the grid and section designations. Try to label each montage in approximately the same location. This allows one to flip through what will be a large stack of montages and easily find specific sections.
5. Clearly label every cell with an identifying number on each montage with a permanent marker (**Figs. 3a** and **4b**). Avoid writing over critical areas. The nucleus, when present, is often an ideal area to label each cell. The cells that are present in the montage of the middle section are the most likely cells to be contained within the sampled tissue. Therefore, we suggest that you begin labeling cells from the middle montages and work continuously in one direction and then the other.
6. After all montages have been assembled and the cells have been identified, determine which cells are completely contained within sections and target these for subsequent analyses.

19| Assemble a montage of this section by following Steps 1–3 of **Box 1** (blue ovals in **Fig. 1**). Note that this montage will be quite large. Our initial low-magnification montages typically measure ~3′ × 6′.

20| Study this first montage carefully; it will be your first viewing of such a large expanse of tissue, under the particular experimental conditions (in our case developmental age), at ultrastructural resolution. Associate landmarks (cell types and locations, fiber tracts, blood vessels) with your previous studies of this tissue using other techniques. Identify the cell group of interest (in this case, the MNTB) and select the region of tissue for study, which could be a subset of the cell group. Use large surrounding structures (i.e., blood vessels, section edges) for photographic landmarks (see **Fig. 3a**). Although the area of this region will vary, we chose an area measuring 130 × 220 μm in the medial half of the MNTB, which contained 20 complete cells and significant portions of an additional 32 cells.

21| Determine what interval between serial sections should be sampled.
▲ **CRITICAL STEP** For our purposes, photographing every fourth section (280 nm) was sufficient to follow the large diameter calyx-forming axons (~1 μm) and terminals that we wanted to trace through the section. The diameter of calyx-forming axons was measured from the large montage. This sampling interval may differ with the specific application. If smaller caliber axons are studied, it is necessary to photograph sections at smaller intervals.

22| Increase the magnification of the electron microscope to ×2,000 and create a new montage of the identified region of interest using the landmarks as a guide. Note that these landmarks, such as blood vessels, are not consistent throughout the sections, so some attention will be required to ensure that the entire region of interest is included in all of the montages.
▲ **CRITICAL STEP** The appropriate magnification should be determined by shooting the same test field at different magnifications. We determined that ×2,000 was the minimum magnification necessary to identify and confirm synaptic contacts onto the MNTB cells. This was accomplished by examining two montages of the same field that were shot at ×2,000 and ×2,500. We operate our JEOL 1010 electron microscope at 80 kV, and check microscope alignment using the manufacturer's recommended standard procedures to obtain crisp photographic images. Although the ×2,500 images were easier to inspect, we chose to use the lower magnification images because the assembled montages of higher magnification were significantly larger and would require more photographic supplies and time for processing. Our requirement, which was achieved at ×2,000, was to identify postsynaptic densities and presynaptic vesicles as evidence for functional contacts between cells. Given the costs in time and money, practical considerations such as these should be considered on a case-by-case basis.

23| Assemble and label this montage as outlined in **Box 1**.

24| Working in one direction from this middle section, repeat the necessary steps to create montages of the remaining sections (photographed at ×2,000) at regular intervals until all of the cells identified in the middle section are completely contained in the series. Then work in the other direction from the middle section to create the remaining montages. By proceeding methodically and efficiently, exposure to and possible damage by the electron beam are minimized.
▲ **CRITICAL STEP** If one is using the small folds technique[43,44] to calibrate section thickness, then identify small folds in the tissue as the montage is being photographed. Increase magnification to ×10,000 and photograph the fold.
? TROUBLESHOOTING

Graphical reconstruction: tracking cells and counting inputs ● TIMING approximately 20 days
25| Create a spreadsheet to document which sections contain each of the structures of interest for each cell. **Supplementary Figure 1** shows three cells that were tracked through the montages. Note that our spreadsheets typically span multiple pages in both the horizontal and vertical directions. After the data have been tabulated, we suggest printing the spreadsheet and taping it together to provide a convenient summary of the analysis.

26| Track the cells and identify presynaptic contacts through the montages. **Box 2** outlines our strategy for this process.

27| Obtain images of any required intervening sections. For example, if large structures extend fine processes that should be tracked, then a 280 nm section interval may not be sufficient. In this case, one need not assemble an entire montage of the intervening section, but only the region of interest.
▲ **CRITICAL STEP** The grids are fragile, and repeated use can result in damage that will prevent future analyses of all the sections on an individual grid. Therefore, if any intervening images are required, we recommend photographing the entire area of interest on all remaining sections on individual grids. Note that it may not be necessary to print and assemble all of these montages, but this precaution will ensure that, should any of these data be necessary, they will be accessible.
? TROUBLESHOOTING

BOX 2 | SUGGESTIONS FOR TRACKING FEATURES AND ANALYZING CONVERGENCE THROUGH SERIAL EM MONTAGES

Tracking features through montages

1. Make multiple passes through the montage for each cell, but confine the focus of each pass to a single feature of one cell. Keep the aim of the initial analyses simple to allow one to become well acquainted with the targeted cell and surrounding neuropil before trying to discern finer details. For example, the presence of the soma, nuclei and axosomatic contacts is more easily tracked and should be noted in the first passes. Then, axons of what are perceived to be multiple inputs or fine processes of calyces could be tracked in later passes to confirm that they are not branches from the same parent axon.
2. Establish that suspected synaptic inputs form synapses by confirming the presence of synaptic vesicles and postsynaptic densities. Note that we often observe large diameter axons and other cellular features directly apposing the postsynaptic membrane without forming synapses.
3. Lightly highlight the outline of structures of interest, but take care not to obscure key organelles such as synaptic vesicles (**Figs. 3** and **5**). We use pastel highlighters that deposit see-through coloration onto the prints. The same color codes can also be applied to the spreadsheet data to facilitate "quick glance" analyses (see **Supplementary Fig. 1**).
4. Track calyx-forming axons back to their fiber fascicle of origin in the trapezoid body.
5. Next, follow presynaptic axons of small inputs to determine their origin.
6. Record the features present in each section in the spreadsheet.

Documenting anatomical features through montages with graphical reconstructions

1. Trace or sketch each cell and all synaptic contacts at regular intervals (every 3rd–5th montage). Cells can be traced onto transparencies; however, we find that large sheets of tracing paper are sufficient (**Supplementary Fig. 1**), allow more cells per page and are easily photocopied and stored. This process will take about 20 min per cell.
2. Indicate which sections contain the presynaptic axon for each input.
3. Clearly indicate when the origin of individual presynaptic terminals cannot be determined.
4. Clearly indicate whether isolated synaptic contacts comprise the same presynaptic terminal, especially if the connection occurs within a section that is not traced.
5. Note that it is possible that additional intervening sections will be required to track fine processes.

28| If necessary, assemble montages of intervening sections, label the structures of interest, perform necessary analyses and insert the intervening montages into the stack.

29| Make a final pass through the montages and document the findings for each cell by tracing its cell body and presynaptic terminals on about every 3rd–5th montage (**Supplementary Fig. 1; Box 2**). This series of 2D tracings graphically describes all of the cells in 3D and, together with the spreadsheet, documents the number of large nerve terminals contacting each cell. After completing this step, one will know the number of cells that factor into the analysis and have in hand good estimates for the number of cells contacted by large nerve terminals.

▲ **CRITICAL STEP** At the conclusion of this analysis, fundamental questions of tissue organization, such as the number of inputs converging onto single neurons, are answered. Furthermore, the relative size of nerve terminals or other cellular structures of interest has been determined by making the tracings. This information guides the quantitative analysis of structural parameters and provides a rough check for the numbers that emerge from that analysis. For example, we use our knowledge of the extent of tissue sections that contain particular cells and terminals to develop a systematic random sample for application of Cavalieri's principle for surface area measurements (described in the next step).

? TROUBLESHOOTING

Quantitative image analysis: measuring surface areas and sizes ● TIMING approximately 15 days

30| Import images of the cells to be analyzed by scanning the portions of the printed and analyzed montages at regular intervals, as outlined in **Box 3**. Use the same photographs that were traced rapidly in Step 29. Scanning the 12 prints per cell for this analysis takes about 30 min, for a total of 9.5 h in our study of 19 cells in the MNTB.

31| Using ImageJ (http://rsb.info.nih.gov/ij/), measure the perimeter of each cell and the length of cell membrane apposed to the presynaptic terminal in each image. Use the online documentation for specific guidelines regarding the use of this software. **Box 3** also contains some general suggestions for making these measurements.

32| Using the Cavalieri principle[45], calculate the total surface areas of the cells and the surface area of the synaptic inputs that are apposed to the soma, by multiplying the individual perimeters or lengths, respectively, by the distance separating the scanned sections. Methods to calibrate tissue thickness are summarized briefly in **Box 3**. At the end of this step, all of the analyses are complete.

BOX 3 | SUGGESTIONS FOR IMPORTING IMAGES AND MAKING QUANTITATIVE MEASUREMENTS

Although scanning the bulky montages can be tedious, this method offers the advantage of importing images that have been highlighted and labeled. That way, judgments about the identity of a particular piece of tissue and whether it should be measured do not interfere with rapid data collection. We also find that scanning prints that comprise the montages can be carried out at a significantly lower resolution (150–300 dpi) than that required for scanning the negatives (1,200 dpi). Calibration of section thickness is made using the method of small folds[43,44], whereby the thickness of a single fold is assumed to be twice the thickness of the tissue section. We have also calibrated tissue thickness using the method of cylindrical diameters, whereby the number of tissue sections required to pass tangentially through a cylindrical object, such as a mitochondrion or axon, is counted and multiplied by the tissue thickness to equal the diameter of the structure in the plane of section[5]. Distance in the plane of the tissue section is determined using a calibration grid provided by the electron microscope manufacturer. Calibration grids can also be purchased from electron microscopy vendors.

Importing images

1. Select the appropriate interval to scan about 12 images per cell and terminal. Use the printed spreadsheet to easily make this determination for each cell.
2. Determine the minimum resolution required to clearly discern the membranes of the somata and presynaptic terminals. We suggest trying 150–300 dpi. For calibration purposes, scan all of the montages at this resolution.
3. Scan only the portions of the montages that contain the cells that will be analyzed. Save the images as JPEG images and include the cell numbers that are contained within each image in the file name.
4. If targeted cells fall on the edge of certain prints, it is not critical that the overlapping prints be precisely aligned, as long as all portions of the entire perimeter of the cell can be seen in the image (see below).
5. Scan the scale bar from one print for calibration.

Measuring dimensions using ImageJ

1. Create a spreadsheet in Excel to keep track of the measurements that are made.
2. Use the straight line tool to measure the scanned scale bars and to calibrate the software.
3. Measure the perimeter of somata using the line segment tool. For those cells that are contained in multiple prints or negatives, sum the measurements of non-overlapping regions from each of the individual images. Choose distinct landmarks to define the beginning and end points of the measurements in each image.
4. Measure the length of the membrane of each cell that is in direct contact with presynaptic terminals. We use this measurement to quantify the fraction of the somatic surface that is directly apposed to the presynaptic terminal. This method, although not precise, is likely to be a better indicator of synaptic strength than the total surface area of the entire synaptic input.

Computerized 3D reconstructions of cells from serial EM ● TIMING approximately 15 days

33| Using identification of singly and doubly innervated cells from the graphical analysis and measurement of cell and terminal sizes from the quantitative analyses, select representative subjects for 3D reconstruction. Computerized 3D reconstruction is time consuming, in part because we align the reconstruction using every montage (average of about 70 images per cell). As a result, we performed this procedure on only 4/19 cells. If possible, select a group of neighboring cells that are contained in 2–3 prints from each montage and which exemplify the range of structural variation that was observed. The need to scan fewer images per montage will minimize the work and time required to import the images for reconstruction. Catalog which EM negatives contain these cells for each montage and scan them into the computer. We scan EM negatives rather than build upon the 12 prints per cell already scanned as part of the quantitative analysis (Steps 30–32) because it is faster. We can scan six negatives versus one print during a single scan, and need not spend time disassembling and reassembling the montages.

34| Scan the EM negatives at the minimum resolution necessary for tracing outlines of the desired features. The scanner speed and the selected resolution will determine the speed of this process. We found that the negatives had to be scanned at 1,200 dpi to reconstruct the calyces of Held and the MNTB cells. We suggest taking the time to determine the lowest resolution that is sufficient to suit specific needs. Save all of the images into the same folder for the reconstruction.

35| If portions of some cells are contained in multiple images, stitch the images together to create digital montages. There are multiple ways to do this, but we find the automated photomerge function in Adobe Photoshop to be most efficient (**Fig. 4a**). Always check the results of each photomerge for accuracy. This operation can also be performed within the Reconstruct software[26].

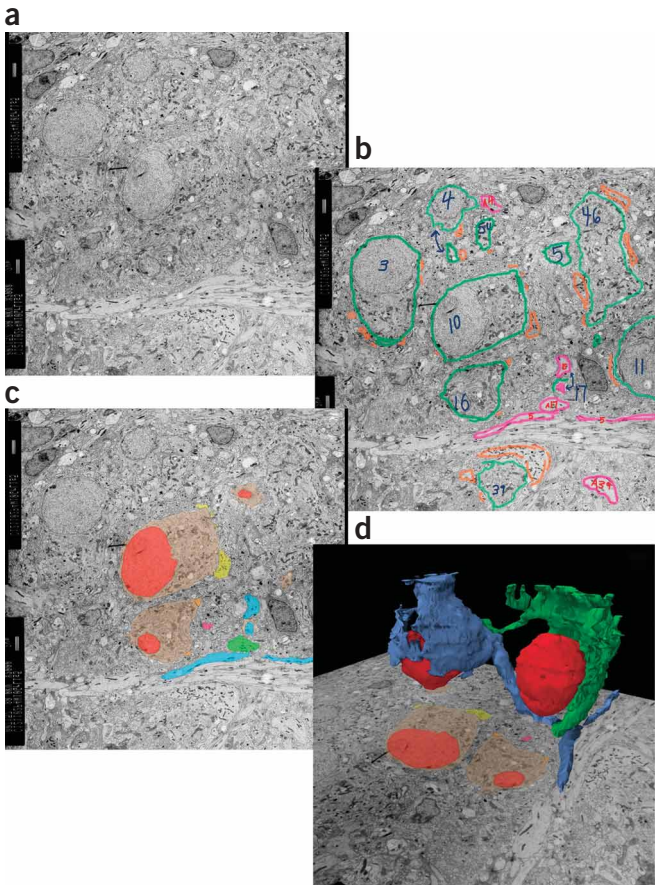

Figure 4 | Required steps for reconstruction of cells from serial EM. (**a**) A montage of two scanned negatives (converted to positive images) that have been assembled using Adobe Photoshop. Scale bars (2 μm) are contained in the data labels on the left of each negative. (**b**) An example showing how the cells in this field were labeled and marked during the graphical analysis. Individual cell numbers are written inside the nucleus in blue ink. Cell bodies have been outlined in green for identification, but we typically only numbered and did not outline them on the montage. Synaptic inputs were highlighted in orange after the presence of synaptic vesicles and postsynaptic densities was confirmed. Axons (outlined in pink and labeled with red ink) were tracked throughout the stack of montages. Note that the highlighters do not obscure the underlying features. (**c**) Four cells and their synaptic inputs were chosen for reconstruction. The cell bodies (light brown) and nuclei (red) of three of the cells are contained in this section and have been colored as they are coded in the computerized 3D reconstruction. Individual synaptic inputs and their axons were traced with different colors (blue, green and yellow). This image is representative of the strategy used to trace and reconstruct cells within the Reconstruct software. (**d**) Two reconstructed calyces of Held and the postsynaptic nuclei of their target neurons. The EM montage shown in **a**–**c** has been rotated and aligned with the reconstructed cells. These features were reconstructed using Reconstruct. A 3D scene was rendered and exported as a .dxf file. This panel was then created and rendered using Carara 4.

36| Reconstruct the MNTB cells and the presynaptic calyces of Held using the Reconstruct software (**Fig. 4**). Refer to the software help file for specific guidance regarding the software. **Box 4** contains suggestions that highlight our strategy for reconstructing cells with this software.
? TROUBLESHOOTING

37| Create a 3D scene of the reconstructed structures using Reconstruct. Export the scene as a .dxf file. This file format preserves the labels associated with each reconstructed feature.
? TROUBLESHOOTING

38| Import the .dxf file into a 3D graphics program such as Carrara or 3DS Max to render a final production quality image (**Fig. 4d**) or animated movie (**Supplementary Movie**) of your 3D reconstruction. Refer to the software documentation for specific help with rendering 3D scenes. The required time for this step depends on the complexity of the scene and the user's experience with the software. Also note that animations can require several hours for complete rendering, although user input is usually not required after the rendering process begins.
? TROUBLESHOOTING

39| Inspect the rendered images and make final observations from the data (**Fig. 5**).

● **TIMING**
Steps 1–6, tissue preparation, 8 days
Steps 7–11, grid preparation, ultrathin sectioning, 3 days
Steps 12–24, EM photography, developing, printing and montage assembly, 20–50 days (two laboratory personnel will complete this step in 20 days)
Steps 25–29, graphical reconstruction, 20 days; ~6–10 h per cell
Steps 30–32, quantitative analysis, 15 days; ~2–3 h per cell
Steps 33–39, computerized reconstruction, 15 days; 3–4 days per cell

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

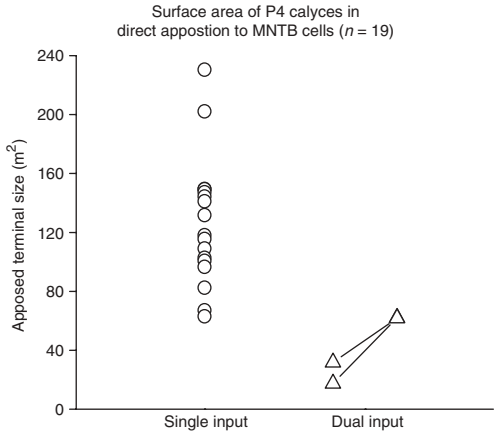

Figure 5 | Quantitative measures of calyces of Held in P4 mice. The surface area of calyces in direct contact with the postsynaptic MNTB cell was determined for 19 reconstructed cells. MNTB cells (n=17) contacted by one terminal are shown on the left. The two cells contacted by two inputs are shown on the right. These data indicated that multiply innervated MNTB neurons were contacted by the smallest terminals in the sample. (Adapted from ref. 23.)

TABLE 1 | Troubleshooting table.

Problem	Reason	Solution
Step 4 (**Supplementary Box 1**), tissue does not remain flat during flat embedding	Tissue section is too large and curls at the edges	Use six-well plates instead of 12-well plates. Cut 1,000 μm nylon mesh to fit in the wells. Place the mesh over the sections. Use rubber washers that fit snuggly in the wells to hold the mesh in place. Keep the mesh and stoppers in place through the alcohol dehydration. The procedure based on ref. 46
Step 7 (**Supplementary Box 2**), transparent film does not detach from the slide during grid preparation	Film may not be completely etched	Ensure that the film has been completely etched along the sides and bottom
	The slide was not properly fogged	Try to place the slide into water immediately after it has been fogged
	The angle of entry into the water was incorrect	Experience is required to learn the best speed and angle to ensure that the film floats off the slide
Step 10, sections do not separate easily or tear when separated	Problems with the resin, which may not be of appropriate hardness	Carefully measure all of the components when preparing the resin
	The top of the section is not significantly shorter than the bottom	Trim the top of the sections to be shorter than the bottom, but not too short, or sections will not stay adhered to one another
	Inexperience with ultramicrotomy techniques	Practice with resin blocks that contain nonessential tissue. Ultrathin sections are extremely delicate. Sections are easily torn or punctured. It will take a fair amount of time to become skilled enough to manipulate sections consistently
Step 13, sections in EM viewfinder are not properly aligned or appear to be rotated	The sections have a different alignment than on the previous grid	Remove the grid holder from the EM and rotate the grid position
Step 24, critical feature is obscured by precipitate on the section or tissue defect	This problem can occur even under optimal staining conditions	Photograph adjacent section
	Grid stainer tubing may not be clean	Photograph adjacent section
	Staining solutions may be old	Flush tubing on grid stainer according to the manufacturer's procedures and replace staining solutions
Step 27, grid pops during photography	A small puncture may be induced in the pioloform during handling, which enlarges when heated in the electron beam	Plan on photographing the entire area of interest for all sections that are contained on the grid when acquiring intervening sections to minimize handling of individual grids
	Beam is too intense	Quickly identify tissue landmarks and focus image. Then reduce beam intensity for photography
Step 29, the origin of small inputs could not be determined	The linkage occurs outside of the sectioned tissue or the axon terminals were too fine and could not be reliably tracked through the sections	Note when small terminals cannot be tracked to their source in the traced data records. Determine if the unlinked contact reaches the size threshold to be considered a calyx. If so, include the result as a possible example of multiple large converging inputs. Distinguish these inputs from the larger calyces using different colors in reconstructions
Step 36, reconstructed structures appear to be leaning in one direction or portions of the cell are misaligned	The objects that were used for the alignment were not running perpendicular to the section plane.	Establish new landmarks for placing the images into register. Change landmarks for each alignment
Step 37, Reconstructed structures have a bumpy and uneven texture	The interval used for the computerized reconstruction was too large	Trace the structures in the intervening sections.
	The fine protrusions of the cell membrane can change significantly from one section to the next.	Smooth the objects within the 3D graphics program

TABLE 1 | Troubleshooting table (continued).

Problem	Reason	Solution
Step 38, objects in 3D scenes are not colored appropriately when rendered in 3D graphics programs	Filtered (colored) lights were used to illuminate the scene	Use white lights
	The appearance of underlying colored solids was altered (filtered) by colored transparent objects	Choose color combinations carefully. Consider using tan, gray or very light colors for external transparent features (somata)
	An object with a reflective surface is reflecting a nearby colored structure	Decrease the reflectivity associated with that structure

ANTICIPATED RESULTS

The goal of this process is to acquire a large data set that describes an atypically large number of cells. Each step in our data analysis revealed important information about calyx formation during early development. The graphical reconstruction resolved an important question about the prevalence of multi-innervation, and therefore competition, by revealing that most MNTB neurons (17/19) were mono-innervated when many young calyces formed at P4. The quantitative analysis revealed that nerve terminals varied in size by a factor of 4×, from 60 to 230 μm^2. Quantitative analysis also revealed that the terminals that dually innervated MNTB cells were the smallest terminals in this sample (**Fig. 5**). The computerized 3D reconstruction revealed, in a more definitive manner, the impressions that were gained from the graphical reconstruction. Calyces appeared to grow from the location at which the axon first approached the MNTB cell (**Supplementary Movie**), as is evident from previous light microscopic studies of the developing MNTB[21,47,48]. The advantage of performing reconstructions from EM images is that all cellular elements and their fine processes can be identified, and not only those that are labeled by staining procedures. For example, the axon, which innervates the lower cell in the **Supplementary Movie,** wraps around 1/2 the circumference of an

BOX 4 | RECONSTRUCTING CELLS IN 3D FROM SERIAL EM SECTIONS

The purpose of this box is only to provide general suggestions regarding the reconstruction of cells using currently available software. Note that there are several different software packages that are suitable for 3D reconstruction from serial EM images.

1. Import each negative or pre-assembled mini-montage as a domain of a section into the Reconstruct software. Assign the true section number to each section; this will allow you to insert additional intervening sections should you choose to do so. We recommend that the images from each photographed montage (every fourth section) be imported. Our experience indicates that importing every fourth section (each photographed montage) is sufficient for proper alignment of sections. However, we find that it is extremely difficult to place the sections into register if we skip any of the photographed montages.
2. Enter the corresponding spacing between each section. If each imported image corresponds to every fourth section (70 nm thick), then this dimension should be 0.28 μm.
3. If necessary, align individual domains within each section to create montages of individual sections (not necessary if images were stitched together using Adobe Photoshop (Step 35 of PROCEDURE)).
4. Place all of the sections into register. We typically use at least five non-collinear points of reference for this alignment and frequently change reference points as we work through the series of images. We find that mitochondria or small, clearly identifiable cellular processes such as axons that run perpendicular to the section plane work well for this purpose. We can predict which of these features are most likely running perpendicular to the section plane based on their appearance. For example, mitochondria that are round in shape with crisp edges are likely running perpendicular through two images, whereas elongated or oval-shaped mitochondria with blurry edges are more than likely running at a transverse angle. We emphasize that this step requires some patience, but experience will increase the efficiency and accuracy of the alignment.
5. After the images have been imported and properly aligned, begin tracing the outline of the structures to be reconstructed.
6. Develop a color-coding strategy. It may be beneficial to have all nuclei of the same color, while having synaptic inputs with distinct colors. Although the color schemes can be altered at any point, establishing the rules early in the process will increase the efficiency and save time later. Also note that the choice of color is not critical at this stage, because the colors, textures and appearance of each feature will have to be edited within a 3D graphics program before final rendering (**Fig. 4**).
7. Use descriptive object labels that can be easily grouped and identified. This will be beneficial when working with the reconstruct software as well as other 3D graphics program that you might use to create production quality images for publication.
8. Although it might be necessary to import every montage for proper alignment, it may not be necessary to trace the features of interest in each imported section. We suggest that one begin tracing features in every other section and trace intervening sections only when necessary, such as when tracing fine processes that may only be contained in one or two of the scanned montages.
9. Routinely render objects within the Reconstruct software as you go in order to check the results and determine if certain features should be traced in intervening sections.

adjacent cell and, although in direct apposition to that cell, does not form functional contacts. Subsequent reconstructions of the same tissue regions could highlight other features, such as the relationship of axon trajectories and terminals to glial cells, without the need to generate new histological specimens.

Note: Supplementary information is available via the HTML version of this article.

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