A SYSTEMATIC APPROACH TO RECONSTRUCTING MICROCIRCUITRY BY ELECTRON MICROSCOPY OF SERIAL SECTIONS

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(Accepted June 25th, 1980)

Key words: serial electron microscopy — method — vision — microcircuit reconstruction

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1. INTRODUCTION

To analyze a particular region of the vertebrate brain will ultimately require a comprehensive account of its 'microcircuitry'. The detailed three-dimensional geometry of individual cells must be fully documented if one is to understand the electrical transformations performed by a neuron on its inputs. The detailed patterns of synaptic connections between adjacent neurons must also be fully documented if one is to understand how the neurons interact. Some features of such a complete description, e.g. the dendritic branching patterns, can be observed with the light microscope, but others, such as the location and nature of synaptic contacts, can only be seen with the electron microscope. The three-dimensional shape of a neuron and the relationship of this shape to synaptic inputs and outputs cannot, of course, be determined from single electron micrographs but must be obtained by large scale reconstruction from serial electron micrographs.

Although serial reconstruction has been employed almost since the invention of the electron microscope, routine reconstructions of long series in vertebrate material have been limited. This is in part because of the difficulty of preparing and photographing long series of fragile sections, and in part because of the difficulty of tracing many small profiles so commonly found in vertebrate material through hundreds of successive electron micrographs. However, with the introduction of computer assisted methods, pioneered by Levinthal, LoPresti, Macagno and Ware, large scale reconstruction has become a realistic possibility. Their approach has been to photograph a long series of conventional ultrathin sections and then to convert the sequence of photographs to a 'movie' by aligning successive negatives and rephotographing them on 35 mm film. The profiles of a neuron are identified in each movie frame, digitized, and finally displayed by computer as a reconstruction. Naturally, there are still a host of practical difficulties in preparing such long series as well as in the subsequent photography and analysis.

The present paper describes our own effort to develop a systematic routine for collecting and photographing serial sections as well as some refinements we have added to the analytical approach developed by Levinthal et al. The results of our method, illustrated with examples from cat retina and visual cortex, suggest that reconstruction of large numbers of adjacent vertebrate neurons is a practical goal.

2. PREPARING THE SERIES

2.1. Tissue preparation

All of our material is taken from animals perfused in the standard manner with
mixtures of glutaraldehyde and paraformaldehyde\textsuperscript{28,33}. Following refrigeration overnight in fixative, tissue is cut at 50–200 $\mu$m on a Vibratome. At this stage, if the material is experimental, sections may be reacted for horseradish peroxidase or selected sections may be taken for light microscope autoradiography. The remaining tissue following en bloc staining with uranyl acetate is osmicated (1%, 30 min), dehydrated in methanol, and embedded in epon.

2.2. \textit{Section thickness}

In the hope of reducing the number and fragility of the sections, we attempted to reconstruct from series of relatively thick sections (0.25–1.0 $\mu$m thick), photographed in the high voltage electron microscope (200–1000 kV). We found, as documented by Scott and Guillery\textsuperscript{36}, that although excellent pictures could be obtained, the problems of establishing continuity between fine processes included within the sections are formidable, even with stereo-pairs. Our thick section work was abandoned in favor of conventional thin sections of about 0.1 $\mu$m thick (pale gold to silver).

2.3. \textit{Series length}

Since the neurons with which we are working are for the most part radially symmetrical, there is a direct trade-off between photographing a small area in a long series and photographing a large area in a medium length series. Obviously the latter is more convenient. A series of 200 sections 100 nm thick (silver-gold) spans a depth of roughly 20 $\mu$m and contains major portions of some cells and fragments of many others that are substantial enough to be useful in identifying neuronal species. Furthermore, once some elements are identified with certainty, it is possible to identify some of the larger fragments with less than total reconstruction (see section 5).

2.4. \textit{Cutting, collecting and mounting}

When the neural processes to be analyzed are fine, of the same order as the section thickness, the series must be essentially perfect. That is, it must be complete, of uniform thickness, and without folds or dirt. Variations in section thickness are prevented by allowing the microtome to cut continuously and by minimizing temperature changes in the block caused by warmth of the operator's hands and the small breezes that accompany his movements. While the microtome cuts, ribbons must be detached and mounted, and the trough and manipulating instruments must be cleaned of the debris that accumulates during the cutting. To accomplish all these tasks we have modified a number of published systems\textsuperscript{1,15,27,37} and established a mini-assembly line using one operator to cut the sections and a second operator to mount them.

The block, trimmed to a mesa roughly 200 $\times$ 750 $\mu$m, is cut on a Sorvall MT-2 microtome with a diamond knife. While the microtome cuts, the operator uses the edge of a 25 $\mu$m Teflon-coated wire to detach a ribbon of 7–10 sections, leaving one section attached to the knife. The free ribbon is lifted from the center of the trough.
with a loop made from 2 mil. beryllium copper (Fig. 1A) and transferred to the second operator for mounting. The first operator then cleans the loop by sonicating it briefly in acetone and prepares to receive the next ribbon. The trough is cleaned when necessary of accumulated debris by briefly raising the water level and drawing a piece of lens tissue over the water surface.

Fig. 1. A: lifting a ribbon from the trough. The loop is held by forceps clamped shut with an 'O' ring. The loop's inside diameter is longer and wider than the ribbon by about 30%. B: chuck for 3 mm grids. Micrometer is used to tighten the chuck. C: mounting a ribbon. The loop bearing a ribbon is lowered to within 0.5 mm of a single slot, formvar-coated grid. The grid chuck may be rotated and the loop may be moved laterally to align the ribbon within the grid slot. A micropipette mounted in a micromanipulator and attached to a vacuum pump slowly aspirates the water. Sections float gently to the formvar, aligned and without folds.
The second operator mounts the forceps bearing the loop on a micromanipulator by means of a magnet. Next, he or she positions the loop over a formvar-coated slot which is rigidly mounted in a small chuck that can be rotated about its center (Fig. 1B). Following adjustment so that the loop and grid are parallel and the ribbon correctly oriented, the loop is lowered until the drop of water bearing the ribbon contacts the formvar film (Fig. 1C). The water is gently aspirated through a vacuum micropipette (tip size 50-100 μm) introduced into the edge of the drop by means of a second micromanipulator. It is not necessary to stop the microtome at any stage of this procedure, thus ensuring a constant section thickness.

As the water is drawn off, the sections settle on the film in the correct orientation and without wrinkling. With this approach, two moderately practiced persons can easily cut and mount a series of 400 sections in a morning, without ever stopping the microtome.

2.5. Grids and formvar films

Conventional copper grids are rather flexible, and their bending in the grid chuck or during staining tended to damage the fragile formvar coating. We solved the problem by making a special rigid grid fabricated with full hard, 4 mil. beryllium copper*. The grids are cleaned before coating in formic acid followed by rinses in distilled water and acetone.

Our requirements for a large number of essentially perfect formvar-coated grids created a number of special problems. To ensure cleanliness the entire coating process takes place under a laminar flow hood. The formvar films are cast on microscope slides from 0.5-1.0% commercial formvar (Ladd). A slide is cleaned with 5% ammonia and a lint-free, linen cloth. Surgical rubber gloves are worn to prevent contamination by grease from fingers. The dry slide is coated by manually dipping it into the formvar. The film is allowed to dry, then fractured with a razor blade at the margins of the slide which is gently lowered at 45° into a pan of distilled water whose surface had just been cleaned by dragging hard lens paper across it. The formvar film floats onto the water’s surface, and the slot grids are dropped onto the film. The film, bearing the grids, is lifted from the water by touching to it a piece of hard lens tissue and placed in a petri dish to dry. Dried films with silver interference color seem to offer optimal stability, and do not require carbon-coating. Although they may be too thick for good contrast when sections are examined at accelerating voltages of 50-60 kV, they present no problem at 100-120 kV. This method is fairly standard, but a few improvements, such as the hood, cleaning the slides with ammonia and using rubber gloves, has increased the yield of acceptable grids by roughly 60%.

* Grids (Stevens Disk #1-EY00828-04 Be Cu Full Hard) and loops (Stevens Loop #1-EY0028-02 Be Cu Full Hard) are available from Dick Becker, Chem Fab Co., 300 N. Brod St., Doylestown, Pa. 18901, U.S.A.
2.6. Staining and storage

Grids bearing ribbons of sections are mounted edge-up in groups of 5 in a slotted plastic ring and stained in aqueous uranyl acetate (2%, 30 min), followed by lead citrate (0.1%, 1 min). The grids are allowed to dry before they are handled.

Whenever a grid is inserted or removed from the electron microscope, there is a risk of accidental loss. To minimize the risk we developed a special cassette into which each grid is placed after staining (Fig. 2). The cassette can be inserted into a JEOL 120B electron microscope in about 30 sec without further handling of the grid itself. Each cassette with its grid is stored in a large (000) gelatin capsule. The additional bulk of these cassettes over grids makes them much easier to handle and the reduced time required to insert and remove grids from the scope helps to keep the sections clean. When manufactured in quantities of 100 or more in a commercial tool shop, each cassette costs approximately U.S. $5.00. This added protection for the grids makes it quite easy to rephotograph an individual section or an entire series with minimal risk. We, for example, have one series of 150 sections which has been totally rephotographed more than 6 times, and it is still intact.

Fig. 3. Low power (750 x) electron micrograph of the cat retina. G, ganglion cell layer; INL, inner nuclear layer (containing amacrine, bipolar, and horizontal cells); IPL, innerplexiform layer. This micrograph covers an area of 170 \times 100 \mu m. To cover the same area with 20\% overlap at a magnification of 1300 \times (see box) requires approximately 4 negatives; 16 negatives are necessary at a magnification of 2500 \times (see box), and more than 50 negatives at a magnification of 6500 \times. (Reduced 25\% in publication procedure.)
Fig. 4. Electron micrographs of a rod spherule taken at different original magnifications (see numbers at upper left of each photo) and enlarged photographically to the same final magnification (6500 ×). Insets at lower left of each photo are photographic enlargements to the same final magnification of the synaptic ribbon (see box at center of each photo). The figure demonstrates that adequate resolution can be obtained from micrographs taken at about 1300 × or higher.
3. ELECTRON MICROSCOPY PHOTOGRAPHY

3.1. Magnification

The goal at this stage is to photograph as large an area as possible with a minimum number of pictures. At the same time, adequate resolution must be maintained in each negative to permit tracing of the finest processes and to identify synaptic contacts. The lowest magnification commonly used for such purposes in neuroanatomy is about 6500×. At this magnification one negative covers an area of about 10 × 15 μm (Fig. 3), roughly the area occupied by the soma of a small neuron. In contrast, the area occupied in Fig. 3 by a dozen ganglion cell bodies and the inner plexiform layer containing their dendrites is about 170 × 100 μm. To cover this area at 6500×, allowing for 20% overlap at the edge of each negative, would require about 50 negatives. The same area can be covered at 750× with a single negative (Fig. 3), but at this magnification too much detail is lost.

Fig. 4 indicates how the usable resolution of an electron microscope varies with the original magnification. The same structure (a rod spherule from cat retina) was photographed at 4 different magnifications and enlarged photographically to the same final magnification. The photograph taken originally at 1275× is similar in resolution to that taken at 6500×. The print taken originally at 750× is surprisingly good, but the inset shows a clear loss of resolution. Thus, we have found that for most routine work negatives taken at about 1300× provide enough resolution to identify membrane boundaries and synaptic contacts. To cover the area of Fig. 3 at this magnification requires about 4 negatives.

3.2. Microscope conditions

It is essential to minimize contamination of the sections while they are in the microscope. Therefore, we invariably use a cold trap filled with liquid nitrogen. We also use the highest accelerating voltage available on our microscope (120 kV). Since fewer electrons are inelastically scattered at higher voltages, there is less energy loss, resulting in reduced rates of sublimation and heating of the tissue. These reductions lead to less total distortion and a reduced likelihood of movement. High accelerating voltages have the disadvantage of reducing image contrast since the deflection angle of the elastically scattered electrons is reduced, but adequate contrast can be obtained by holding the size of the objective aperture to the minimum required for a full view of the field. Furthermore, the contrast can be enhanced when the 35 mm movie is made, as described below.

Since much of the useful information in biological material is, in effect, contained in the 'phase' of the electron beam rather than in its amplitude, maximum contrast is in a plane below the images' focal plane in the so-called Scherzer focus plane. Therefore, in photographing a series we bring the image to true focus using a 'wobbler' device and back off several steps until a strongly underfocused image is obtained. The best contrast must be determined empirically for each magnification and we have found is not necessarily the same for all material.
4. ANALYSIS AND RECONSTRUCTION

We have developed several methods for reconstructing neurons from the serial electron micrographs. The computer system is the most rapid and systematic; however, it is also expensive and time-consuming to build. While this system was being constructed, we adopted a simple manual method for reconstructing from conventional 8 × 10 inch prints.

4.1. Manual reconstruction

A print (final magnification 3250 ×) is illuminated from below by a light box and viewed through a dissecting microscope. A jig, such as a cartoonist might use, is loaded with 5 sheets of acetate ('sheet protectors' for 3-hole notebooks) and laid over the print (Fig. 5). On the uppermost acetate sheet one traces the outline of the neuron under reconstruction and also (in a different color) the outlines of 3–4 small dendritic or axonal cross-sections to be used as fiducial marks. The jig is laid on the next print in the series and aligned by superimposing as closely as possible the fiducial marks from the first print onto their corresponding profiles in the second. The first acetate sheet is folded back and the fiducial and neuronal outlines traced on the second sheet. The choice of profiles on the second sheet may be checked for accuracy by returning the first sheet to its original position on the jig. When the second tracing is complete, the first acetate sheet is removed, and the cycle is repeated with a third print.

Fig. 5. The cartoon jig and acetate sheets used for manual reconstruction. See text for details.
As the original fiducial marks disappear from the series or change from transverse to oblique profiles, new ones are chosen.

The acetate sheets are 3 mil. thick and correspond at the magnification used for sections up to 23 nm thick. Since the original thin sections used are actually about 100 nm thick, a fairly good three-dimensional representation of the neuron can be achieved by interleaving 3 blank sheets of acetate between successive tracings. The final stack of tracings can fairly easily be converted to a two-dimensional representation by an illustrator (see Fig. 14).

4.2. **Computer reconstruction**

4.2.1. **Movie production**

The first step in computer reconstruction is to produce a 'movie' by aligning successive negatives and rephotographing them to produce positives on 35 mm film. Our method is based on that developed by Levinthal, LoPresti, Macagno and Ware\textsuperscript{20,21,26,42} and employs the apparatus shown in Figs. 6 and 7. The first two negatives in a series are viewed simultaneously through an 'image combiner'. The second negative is translated and rotated into rough correspondence with the first while both are alternately illuminated by strobe lights. There is a strong illusion of image movement when the negatives are out of line, and the illusion progressively weakens as perfect alignment is approached.
The double negative carrier slides on linear ball bearings to an adjacent optical bench where either negative can be fixed (by a solenoid-operated pin) in the optical path of a full frame 35 mm camera (Automax). Following the alignment of negatives one and two, the first is photographed and replaced in the carrier by negative three. Negatives two and three are then aligned, negative two photographed and replaced by negative four, and so on. As noted by Macagno et al.\textsuperscript{28}, it is not possible to bring all portions of two successive negatives simultaneously into perfect correspondence. If the centers are aligned, the corners will be out, and vice versa. These errors result from the distortions introduced in the original sections by cutting, heating in the electron beam, and a host of other factors. They are corrected for at a later stage.

The 35 mm copying process reduces the original 8 $\times$ 10 cm negative to a 2.5 $\times$ 3.5 cm positive. Space must also be allowed on this format for the frame-to-frame alignment corrections just described; therefore, the final reduction is about 3.5:1. With
Fig. 9. Enlargements of two 35 mm copies made from the same 1275 × E.M. negative. Pictures on the left were enlarged from a 35 mm copy made using a diffuse light source in place of a condensing lens (Fig. 8). Pictures on the right were enlarged from a 35 mm copy made using the quasi-coherent illumination system (Fig. 10). Top, 80 ×; bottom, 600 ×.
such a large reduction, the fine details that one tries so hard to preserve in the original negative are easily lost. Consequently, the resolution and overall quality of this copying system are critical. One of the most important features of the system, we found, is the copy lens. After testing several, we chose the Nikon APO EL 210 mm which produces an image that is virtually diffraction limited over the entire area of the negative. As one would predict, when the lens $f/\#$ is reduced from its maximum of $f/5.6$ the resolution of the system decreases.

The illumination system is another major determinant of resolution and contrast in the final image. A diffuse light source (a light box) is inexpensive and convenient, but produced unsatisfactory results. A laser-generated coherent light source is also relatively inexpensive and easy to construct, but speckling of the image and narrow range of available wavelengths make it unsatisfactory. After some experimentation we found that the quasi-coherent illumination provided by a 100 W mercury arc lamp with a 436 nm interference filter and a 200 $\mu$m pin-hole (Fig. 8) produced the same high contrast of a laser but without speckling and yielded 35 mm copies far superior to the diffuse system. The dramatic difference between this quasi-coherent source and a conventional diffuse source is illustrated in Figure 9.

We estimate that our original electron microscope (E.M.) negative has a usable resolution of 90 lines/mm. The copy system illustrated in Fig. 9 reduces this image 3.5 times and has an image plane resolution of 224 lines/mm. Thus, the final 35 mm copy retains about 64 lines/mm or 70% of the information contained in the original E.M. negative. This copy is actually superior to a typical 20 $\times$ 25 cm enlargement on standard print paper, which retains at best about 50 lines/mm, or 56% of the resolution of the original negative.

The correct exposures, using this illumination system, cannot be easily calculated from measurements on a conventional, diffuse-source densitometer. Negative densities must be measured instead with a densitometer whose optics approximate that of our quasi-coherent copy system. We obtained satisfactory results with a Macbeth projection densitometer ($f/\# 4.0$) by adding a blue filter.

The 35 mm film and developer are also important determinants of the information content of the final movie. We tried several films and developers and found one particular combination that consistently produces excellent copies: high contrast Kodak microfilm (SO115 or AHU 2460) on a 7 mil. estar base and Kodak HC-110 developer. The estar base resists tearing and increases the useful life of the movie in the film transport. Although D-19 is the recommended developer for both films, it produces very high contrast with an extremely narrow dynamic range. With HC-110 in dilutions A to F, one can match the resolution achieved with D-19 while varying the contrast and dynamic range. Dilution $A$ produces high contrast with a narrow dynamic range while dilution $F$ produces low contrast with a wide dynamic range. We found dilution $D$ the best compromise for routine work.

4.2.2. The computer reconstruction system

The computer system uses as its input the ‘roughly’ aligned movie described above. The movie is mounted in a precision film transport which permits aligned,
frame-by-frame movement in both the forward and reverse directions (Figs. 10 and 11). It is illuminated from below by a diffuse source and viewed from above through a zoom lens and a high resolution video camera and monitor (Cohu). The zoom lens magnification can be varied from 1 to 6× and the video system has a fixed magnification of 14 ×. The final magnification of the system is, therefore, 14–84 times. Thus, material photographed in the electron microscope at 1300 × and reduced 3.5 × in the 35 mm movie, can be viewed at final magnifications between 5,200 and 32,000 ×.

4.2.3. **Microalignment**

One finds, on attempting to follow a fine process at high magnification, that despite the rough alignment during movie making the profile jumps about from frame to frame. This 'jitter' or displacement is caused by regional misalignments which arise, as noted earlier, from intrinsic distortions in the original material and E.M. negatives. The jitter makes it impossible to track fine processes. Since these distortions are local, and show considerable variation within the frame of a single E.M. negative, absolute fiducial marks are of no use. For example, we found, when the movie is aligned using an external fiducial such as the edge of the mesa, that even the processes immediately adjacent to this absolute reference become misaligned in only a few frames. This misalignment seemed, on the average, to increase in proportion to the distance from the accurately aligned edge (Fig. 12), but was neither systematic nor predictable for any given frame. Thus, local distortions cannot be corrected by the rough alignment step during movie making nor can they be corrected by using absolute, externally imposed fiducial marks. Yet, the local distortions must be corrected if small processes are to be followed. We concluded that the correction must be accomplished by a frame-by-
frame 'microalignment' step performed individually for each process using local fiducials. The apparatus described below was designed to make this crucial step as convenient as possible.

The film transport (Mekel) is mounted on an X–Y stage controlled by two stepping motors that allow a microcomputer (Zilog Z80) to rapidly control the absolute position of the stage. A digital pen (Summagraphics Bit Pad) serves as an input to the computer allowing full control by the user of the stage position. A high resolution video memory (PEP 500 Princeton Electronics Products) can store one complete video frame and a video switcher (Cohu) makes it possible to rapidly alternate between frames to compare the stored image and a 'live' image of the next frame. Finally, a digital graphics device (Lexidata 3400) also under control of the Z80, is superimposed upon the image of the stored and live video images which are displayed on a high resolution monitor (Conrac). All of these devices are controlled by means of the Z80 microprocessor (see Fig. 10).

Fig. 11. Photograph of the film transport, X–Y stage, and high resolution video camera with zoom lens.
Fig. 12. Average alignment error as a function of distance from an absolute and perfectly aligned fiducial (the trimmed edge of the mesa on the epon block). Distance is in arbitrary units corresponding to one step of a stepping motor (0.22 μm on the original micrograph). Numbers in parentheses represent the maximum error for a given position.

Microalignment is accomplished by bringing the process of interest to the center of the screen and advancing to the next frame. In the instant before the film advances, the video memory automatically stores frame one. Frame two appears on the screen but because of the local distortions the process and all surrounding processes are usually shifted. The user may remove this displacement by placing the Z80 in 'correction' mode. The video switcher rapidly alternates between the stored frame and the live frame at a frequency of 4 Hz. Meanwhile, the user moves the X–Y stage (using the digital pen) so that the two frames are well aligned for the profiles surrounding the one of interest ('local fiducials'). This correction is stored, the film is advanced to frame three and the same procedure is repeated.

As these successive corrections are made, the process of interest may drift away from the center of the screen because of a true biological displacement (i.e., its three-dimensional structure). When this happens, the user need only change to 'biological coordinates' by taking the computer out of correction mode and move the stage so that the process reappears in the center of the screen. This gives the Z-80 a new set of biological coordinates and a new presumed center for the process. At the end of this microalignment procedure, the Z-80 contains a string of these biological coordinates indicating the approximate center of the process and an associated set of corrections. We call this string of points and corrections the 'process vector'. Advancing under control of the process vector makes it possible to follow the process of interest from one frame to another without jitter.

This process of correcting frame to frame misalignments by means of 'local fiducials' assumes that displacements shared by all the processes and their internal organelles within a local region must represent non-biological distortion. Our microalignment procedure simply removes these correlated displacements while retaining the largely random biological displacements of the neuropil. Providing that the microalignment for a process is initiated from an already aligned process, and that this assumption is correct, the reconstruction should approximate the actual shape of the original cell. If, however, a cell or a process is aligned from a non-microaligned point,
Fig. 13. Computer reconstruction (stereo pair) from digitized profiles of the same cell shown in Fig. 14. A: 'hidden lines' in this display are not suppressed. B: same cell but with 'hidden lines' removed.
Fig. 14. A: illustrator's rendering of computer reconstruction shown in Fig. 13. B: branch schematic of a ganglion cell generated from its 'process vector' in 150 serial sections. Each line represents a dendritic branch numbered arbitrarily for identification. Dotted lines join inputs derived from the same presynaptic neuron.

the entire reconstruction will be displaced from other cells or processes by a fixed or unknown amount. In other words, microalignment can correct only for relative distortion within a cell; absolute distortions and the absolute X, Y, Z location of a neuron in a block of tissue will always be unknown.

4.2.4. Identifying synapses and tracing the cell

When microalignment has been performed for all the processes connected to the same cell, the neuronal process vector describes a stick figure or skeleton of the entire neuron which may in turn be used as an aid for identifying synapses and tracing the cell. The investigator replays the movie under control of the process vector, noting synaptic inputs and outputs that involve the cell of interest. These synaptic contacts are marked with one of 15 possible 'flags', each of which has a predefined meaning and is displayed as an arrow on the final reconstruction (see Fig. 15).

The final step is to digitize each member of the sequence of microaligned profiles. The process vector is replayed while the outline of each profile is traced with the cursor, the Z80 providing a continuous update of the traced outline on the digital display. The digitized outlines are sent by high speed interface to a PDP 11/34 computer.

4.2.5. Displaying the traced data

We have experimented with three simple display strategies similar to those described by Macagno et al., all of which are within the computational abilities of a minicomputer.

The first and simplest display is a schematic or 'branch graph' of the cell's structure based upon the process vector (Fig. 14B). This method is derived from graph
Fig. 16. Reconstructions of 14 ganglion cells from electron micrographs of 150 serial sections. Somas fell by diameter and surface area into three classes (a, β, γ). Alpha cells had sparsely branched ('radiate') dendrites while dendrites of beta cells branched repeatedly ('bushy'). Dendrites of alpha and beta cells on left reached outer third of IPL (sublamina a); dendrites of alpha and beta on right were confined to the inner IPL (sublamina b). Synaptic contacts on cells of both classes were confined to sublamina of the IPL containing cell's major arborization. Gamma cells on left bore synaptic contacts primarily on dendrites; cells on right had somatic as well as dendritic contacts. Data were collected with the help of B. McGuire. (From ref. 39).

Fig. 15. Cone bipolar reconstructed by computer from 100 serial sections and rotated about its long axis. Rotation is clockwise if viewed from above; read left to right and top to bottom. Rotation and translation statistics at left margin correspond to successive views in upper row; statistics at right margin correspond to successive views in lower row. Data were collected by B. A. McGuire.
theory and represents a nodal analysis of the cell. The cell's full, three-dimensional organization is not represented by this method, but the lengths of the dendrites, the branch points (nodes) and the positions of synaptic inputs are represented accurately. The diameters of dendritic branches, as well as their surface areas, cross-sectional area and volumes, are easily calculated from the digitized data. With this information and the branch graph the cell may be structurally characterized by a simple matrix. By translating each point in this matrix into a series of current loops (based on assumptions about axial resistance, membrane resistance and capacitance), one may create a passive electrical equivalent for the reconstructed neuron.

A second display strategy is to represent the neuron's three dimensions by stacking the digitized profiles. This is the simplest possible representation of the three dimensions that can be carried out on a very small computer with limited memory (see Fig. 13A), but for cells with complex geometries and many synaptic contacts it proved unacceptable. The third method uses the data base of the second but removes 'hidden lines' using algorithms similar to those described by Veen and Peachey and Newman and Sproull. When the cell is displayed as a stereo-pair, with the hidden lines removed, a clear impression is obtained of its three-dimensional geometry (Fig. 13B).

A fourth display strategy is simply to redraw a computer reconstruction (Figs. 16–18) with perspective and shading (Fig. 14A). A skilled draftsman can produce such a version from the computer drawing or from the manually produced stack of acetate sheets in about an hour. This display is the one most easily compared with drawings of Golgi or horseradish peroxidase filled cells.

5. NEUROBIOLOGICAL RESULTS

We have used the system so far to answer 4 different kinds of questions regarding microcircuitry of the cat visual system. The results are summarized briefly here to illustrate the uses of the approach and to reassure that such effort has its rewards.

5.1. Reconstruction of retinal ganglion cells

At least 5 major classes of ganglion cells have been identified in the cat retina by single unit recordings and by Golgi impregnation. Little was known about the distribution of synaptic contacts on these cells, not even such basic information as whether the contacts are on the somas, primary dendritic shafts, or on the fine dendritic branches. Little was also known about the biophysically relevant parameters for each cell type, such as soma surface area. From a series of 150 sections we have partially reconstructed the somas of 24 adjacent ganglion cells and, for 14 of these, significant portions of their dendritic trees (Fig. 16).

The cells were partitioned into three distinct groups by the surface areas, volumes and diameters of their somas. In agreement with the observations of Boycott and Wassle, the alpha cells (large somas) had large dendrites that remained either low in sublamina b of the inner plexiform layer (near the ganglion cells) or high in sublamina a (near the amacrine cells). A critical finding provided by reconstruction was that the synaptic contacts on each cell were restricted mainly to the sublamina of the inner-
Fig. 17: Top: an electron microscope autoradiogram showing localization of [3H]GABA. Heavily labeled, pale neuron (IP) was determined by reconstruction to be an 'interplexiform' neuron. GA, moderately labeled GABA amacrines (darker cytoplasm); BP, bipolar; M, Muller cell (moderately labeled); IPL, inner plexiform layer. Scale, 5 μm. Bottom: partial reconstruction from serial electron microscope autoradiograms of interplexiform cells presynaptic to rod bipolars (filled circles), cone bipolars (open circles), and amacrines (dotted circle). Triangles, interplexiform cell postsynaptic to amacrines. OPL, outer plexiform layer; INL, inner nuclear layer. (From ref. 29).

plexiform layer containing the cell's major arborization. This same laminar segregation of synaptic inputs was found for the beta cells (medium somas) but was not as clear for the gamma cells (small somas).

5.2. Reconstruction and electrical modeling of amacrine cells

Amacrine cells with varied dendritic geometries have also been reconstructed. Among these processes is a type with large, varicose expansions connected by narrow
necks at intervals of 4–9 μm. Each varicosity contains a mitochondrion and makes reciprocal synaptic contact with a rod bipolar. By calculating the surface area and volumes of these varicosities and making a few assumptions about membrane resistance and axial resistivity, we were able to create a passive electrical model. The model suggests, assuming the processes to be non-spiking, that the varicosities help to electrically isolate local circuits within a single dendrite. A single varicosity, or perhaps a short string of them, may serve as an independent, local input and output circuit.

5.3. Identification of GABA-labeled neurons in the amacrine layer

Certain neurons in the amacrine layer of the retina are known to accumulate exogenous [³H]GABA. Which types of amacrine are involved is unknown, however, because the fine processes whose morphology and distribution characterize the neurons cannot be recognized in autoradiograms of single sections. Following intravitreal injection of [³H]GABA and subsequent fixation, we prepared a series of 210 autoradiograms for electron microscopy. Heavily labeled neurons reconstructed from these autoradiograms were found to send presynaptic processes into both the outer and inner plexiform layers where they contact bipolar neurons (Fig. 17). Based upon their three-dimensional morphology and synaptic connections, we con-

Fig. 18. Thirty-two adjacent neurons reconstructed from layer IVab. (From ref. 11).
cluded that the heavily labeled neurons belong to a specific class of retinal neuron, the 'interplexiform' cell®.

5.4. Reconstruction from layer IV of striate cortex

That the lateral geniculate nucleus innervates layer IV of the striate cortex has long been known. It has proved difficult, however, to determine by indirect methods which types of neurons in layer IV receive the inputs and in what specific patterns the input is distributed. We have obtained a partial answer to these questions by reconstructing a patch (15 × 150 × 200 μm) of layer IVab from a cat that had survived 4 days following a lesion of its lateral geniculate®. Thirty-three adjacent neurons were partially reconstructed from a series of 150 sections (Fig. 18). The neurons were placed in 7 classes based on the differences in size, dendritic branching, and pattern of normal synaptic input. Degenerating geniculate terminals were identified on the soma or dendrites of 6 of the 7 cell classes. The distribution of geniculate terminals was distinctive for several of the classes. Thus, pyramidal cells with somas on the layer III–IV border received geniculate input only on their basilar dendrites (Fig. 19), while the large stellate cell that characterizes layer IVab received rich geniculate input on the soma, primary, secondary, and tertiary dendritic branches.
6. DISCUSSION

We have indicated by means of a few examples that reconstruction of neurons from serial sections photographed at low magnification in the electron microscope provides a powerful and direct method for answering questions about microcircuitry in the mammalian central nervous system. In principle, at least in favorable sites such as the retina, the approach might ultimately provide a comprehensive circuit diagram. This level of detail can be approached once the basic synaptic patterns have been worked out for a group of adjacent neurons. For example, one might start from the identified synaptic contacts on ganglion cells (Fig. 16), trace them back to their bipolars and amacrines of origin, find the inputs to these cells, and so on. The approach also provides detailed quantitative information about branching patterns, surface areas, and volumes of neuronal processes. Such information has made it possible, using the methods developed by Rall and co-workers to construct passive models of neurons whose patterns of synaptic input are also known.

A major disadvantage of the method as we have presented it is that it may fail to yield the same detailed picture of the entire dendritic tree for single isolated cells that can be obtained by Golgi impregnations or intracellular injection of a label. Even this limitation, however, may be overcome by reconstruction of selected prelabeled cells as well as their unlabeled neighbors.

6.1. Computer vs manual reconstruction

It is legitimate to ask, since the computer system is expensive and time consuming to build, what advantages it offers over manual methods. The first advantage is in speed: to reconstruct manually each of the neurons illustrated in Fig. 16 required, on the average, 3 days. Using the computer system, we generated a process vector for one of these cells (Figs. 13 and 14), including all its synaptic inputs, in about a day. The actual reconstruction (digitizing the profiles) required an additional 4–5 h. This total time may increase or decrease depending upon the complexity of the neuron. The time saving offered by the computer system is significant when large scale reconstruction is contemplated.

The second advantage of the computer system over the manual method is that it permits rapid, jitter-free replay and comparison of successive frames. This rapid review creates a sense of continuity and depth that cannot be reproduced with the manual method and gives one a very high degree of confidence in the validity of a reconstruction. In some cases, particularly with very fine dendrites, it would have been difficult or impossible to follow the process without this rapid replay. The computer system also makes it convenient to return to the original data for rechecking observations. For example, by returning to the original movie, one can locate and redisplay any of the synaptic contacts on the branch schematic (Fig. 14) in about 30 sec. The convenience of re-access to the original data also makes detailed cross-checking of observations between two observers a simple matter.

The decisive advantage of computer over manual reconstruction lies in the possi-
bilities for manipulating and analyzing the data once it is gathered. When a reconstruction is in the form of a stack of acetate sheets, little can be done with it beyond reducing it to a two-dimensional drawing and counting the synaptic contacts. Once the digitized outlines of a neuron are stored in the computer, however, the possibilities for manipulation are substantial. Any view of the neuron can be obtained automatically (Fig. 16). One can also estimate biophysically relevant data such as the lengths, surface areas, cross-sectional areas and volumes of dendrites and the relative positions of all inputs and outputs. Electrical equivalents of complete neurons may be generated from these data with little increment in effort.

In summary, the advantages of performing reconstructions with a computer-based system grow rapidly with the scale on which reconstruction is performed, and a computer system becomes essential if one hopes to quantify the reconstructions. The system we describe, though not inexpensive, is not more than about a third of the cost of a high resolution electron microscope. As an adjunct to the electron microscope in investigating neuronal microcircuitry, it seems quite promising.

7. SUMMARY

To observe certain quantitative features of neuronal geometry and microcircuitry, it is necessary to reconstruct neurons from electron micrographs of serial, ultrathin sections. We describe here an approach to preparing, photographing, and analyzing moderately long series (100-500 sections). A series is prepared using an assembly line approach: one operator cuts while a second mounts ribbons of sections using various mechanical aids. Photographs are taken in the electron microscope at low magnification and high accelerating voltage. Sequential negatives are aligned using an image combiner and copied, using quasi-coherent illumination, onto 35 mm film. The resulting 'movie' is mounted on a precision film transport mounted on an X-Y stage controlled by stepping motors. The movie is viewed through a high resolution video system while a video storage device and switching system permit rapid alternation between frames for comparisons. The profiles of a process in successive frames are 'microaligned' by small adjustments of the transport's X-Y position. The absolute X-Y biological coordinates for each frame and the correction necessary to bring it into alignment are stored in a Z80 microprocessor as a process vector. When the movie is re-examined with the stepping motors under control of the computer, the microaligned process shows almost no frame-to-frame jitter. The process vector may be used to generate a 'branch schematic' of the neuron. The microaligned profiles can also be digitized and displayed as a reconstruction using a PDP 11/34 computer. Uses of the approach are presented with examples from the cat retina and visual cortex.

ACKNOWLEDGMENTS

We are particularly indebted to Fred Letterio and Ed Shalna for their superb machining. We thank Mitch Fujita, Pete Lombardo, and Rob Smith for their excellent electronic support, John and Betty Woolsey for artistic support and Barbara McGuire
for her interest and help and for providing data included in Figs. 15 and 16. We thank Mrs. J. Trogadis for help in preparing the manuscript. This work was supported by N.I.H. Grants EY01832, EY00828, EY01583, and a Research Career Development Award (EYO0080) to Peter Sterling.

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