Image processing experiments for computer-based three-dimensional reconstruction of neurones from electron micrographs from serial ultrathin sections

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Summary
This study examined an image processing technique that uses a computer to reconstruct a three-dimensional image of neurones from electron micrographs of serial ultrathin sections. The major problems involved were: (a) a distortion of features in electron micrographs; (b) a significant change of cross-section features of neurones in electron micrographs of neighbouring sections; and (c) disagreement between the electron microscopic section face and the coordinate plane desired for the reconstruction. Electron micrographs of a retinal bipolar cell stained with a biotinylated tracer were used. We corrected the distortion of features by means of a warp, a widely used algorithm in morphing image processing. The change of features between neighbouring electron micrographs was minimized by filling the gaps with an interpolated image produced by a dissolve, another algorithm in morphing, as well as the warp. The distortion of the three-dimensional reconstructed image made by piling up features was corrected by making the image with a wire frame model. Furthermore, in order to estimate a closed contour of features, an active contour model, Snakes, was applied to the electron microscope features. Snakes successfully detected the contour of the target feature, but in some electron microscope images broke into the target feature.

Introduction
The physiological function of neurones is closely related to their morphological appearance, which can be examined by a light microscope or an electron microscope. As the processes of neurones extend in various directions, it is important to characterize the morphology of neurones in three dimensions. Both light and electron microscopes, however, provide only a two-dimensional objective picture of neurones by focusing at a certain level in the tissue or by applying an electron beam to a sectioned tissue.

Confocal scanning microscopes and deconvolution methods that show a three-dimensional (3D) image have been developed and are now in wide use in the field of biology. The confocal microscope is one of the most promising advances in optical microscopy this century (Lichtman, 1994). In the area of electron microscopy, the 3D reconstruction of relatively large features, such as thick processes and the somata of neurones, from thick section electron micrographs, has been achieved, manually or by means of computer (Kolb, 1979; Macagno et al., 1979; Stevens et al., 1980; Smith, 1987; Strettoi et al., 1990), without unusual technical difficulty. However, because serial ultrathin sectioning at the electron microscopic level presents some special difficulties that can normally be ignored when studying thick serial sections or when studying only individual sections (see below), 3-D reconstruction from serial ultrathin section electron micrographs is not a commonly used procedure.

In the computer-based reconstruction from electron micrographs of serial ultrathin sections, we confronted the following three major problems. First, there is a non-homogeneous distortion of features in the electron micrograph due to a localized expansion and shrinkage of thin sections. Such distortions present changing images of the neurone's morphological size and shrinkage of thin sections. Such distortions present changing images of the neurone's morphological size and shrinkage of thin sections.
sections enhances the electron beam–caused distortion of section. The often unavoidable variation in section thickness during cutting sections also causes distortions. Second, cross-section features of neurones are sometimes significantly changed between neighbouring electron micrographs. This occurs because the shapes of fine neural processes are sometimes altered considerably within the thickness of the section. Also, in the course of the process from cutting the tissue block to observing with the electron microscope some thin sections are often damaged or lost. As a result, an artificial shape is created in the reconstructed image. Third, thin sections usually cut some neurones obliquely because it is technically difficult to cut the tissue at a particular chosen angle. Stacking pictures from such sections distorts the reconstructed neurones. These problems can be minimized technically. For example, the electron beam–induced distortion can be reduced by transferring tissues on Formvar-coated and carbon-stabilized grids or by using a low dose method, but in practice the distortion can never be avoided. It should also be noted that in order to get serial sections, the process from sectioning a tissue block to the electron microscope observation should be simple as possible. Studies reported so far usually reconstruct the 3-D features from serial electron micrographs without examining the problems mentioned above, and the reconstructed features are often ragged and artificial.

This study aimed to resolve these three problems by using an appropriate image-processing technique that allowed us to achieve a 3-D reconstruction by means of a computer. The neurone preparation used in this study was stained intracellularly with a biotinylated tracer, but some methods presented in this study may be useful for tissues without intracellular staining. This study also examined an application of an active contour model, Snakes (Kass et al., 1988), to detect the outlines of the stained neural processes.

Methods

Sources of data

The 3-D reconstruction used dendritic trees and the primary processes from somata of five bipolar cells of black bass (large-mouthed black bass, Micropterus salmoides) retina stained with biocytin, and some cone photoreceptors connected to the bipolar cell dendritic trees (Umino et al., 1994). Bipolar cells exhibit a centre-surround antagonistic receptive field, and thus play an important role in the initial processing of the contrast information in the visual pathway (Werblin & Dowling, 1969; Kaneko, 1970; also, see inset of Fig. 1). After a biotinylated tracer, biocytin, was injected into the bipolar cells via a glass microelectrode, the stained bipolar cells were examined in a flat-mount view with a light microscope (dark features in Fig. 1A). Because neighbouring bipolar cells are coupled via gap junctions (Umino et al., 1994), the biocytin injected into one bipolar cell labelled 2 in Fig. 1A diffused intracellularly to surrounding bipolar cells so that the injected cell and surrounding cells were stained with biocytin. Next, in order to obtain an approximate location for the stained bipolar cell processes, the whole-mount view of stained cells at various focus levels was traced on a transparent sheet (dotted lines in Fig. 1B). The retina was then embedded in epoxy resin and a block containing stained dendritic trees was trimmed with a razor to a size covering one mesh hole (283 × 283 μm); serial thin sections were then cut radially from the inner nuclear layer to the photoreceptor cell layer (299 sections, thickness = 80 nm). The dendritic tree region enclosed by a rectangle in the bipolar cell illustration (inset of Fig. 1) was cut serially; thus, only this region of five bipolar cells (see A) was provided for the 3-D reconstruction. Thin sections were photographed at a magnification of 1500× (nine montages for each thin section) with a Hitachi H700 electron microscope. In Fig. 1B, the biocytin-stained cell process that appeared in the micrograph was coloured black, as shown by the filled areas in the dotted illustration of the light microscope whole-mount view of stained cell processes. Although the position of each stained cell process was distorted in the electron micrographs (see above), the position was corrected manually with the aid of a flat-mount view in a light microscope (dotted lines). Figure 1C shows an electron micrograph of the area enclosed by a rectangle in B. Some cross-sections of stained bipolar cells are indicated by arrows in C. A 3-D contour plot of the dendritic trees of five retinal bipolar cells reconstructed manually was reported in Fig. 9 in Umino et al. (1994).

Software and hardware

The electron micrographs were digitized with a flat-bed scanner (255 brightness levels, 600 pixels per inch) (GT9500, Epson Co.) and were provided for image processing experiments. In these experiments, the image resolution was sometimes reduced with a minimal change of features to reduce the computation time.

A morphing algorithm was written in the program language Visual Basic. Algorithms for other image processing and 3-D reconstruction were written in the programming language C. The 3-D reconstruction image of bipolar cells as well as cones (terminals) were displayed on the CRT monitor by AVS (Application Visualization System, KGT Inc., Tokyo), a software for 3-D images. An AVS station Vistra 800a (KGT Inc.), a workstation, and a personal computer system were used.

Based on the present study, the computer reconstruction of neurones from ultrathin section electron micrographs would be carried out by the following steps: (1) entering data from electron micrographs into the computer, (2) correcting the
distortion of features in electron microscope images stored in the computer, (3) filling the gaps with an interpolated image when features between neighbouring sections are significantly changed, (4) detecting the continuous contour of the feature of interest, (5) examining the connection patterns of features of interest, (6) making a wire-frame model with a geometric rendering and a surface rendering, and (7) correcting the distortion of the reconstructed model.

Fig. 1. Retinal bipolar cells provided for 3-D reconstruction. (A) Biocytin coupling of bipolar cells in the teleost retina. A flat mount view from photoreceptor side. Biocytin was injected to the bipolar cell labelled 2 via a glass microelectrode. Biocytin diffused to surrounding bipolar cells through gap junctions. Bipolar cells provided for the computer-based reconstruction are numbered 1–5. Focused at the level of bipolar cell dendrites. (adapted from Umino et al., 1994). (B) An illustration of bipolar cells stained with biocytin (dotted pattern), and the cross-section of stained bipolar cells appearing in the electron micrograph of one thin section (black areas). The dotted pattern was obtained by examining the whole-mount view of stained bipolar cells at various focus levels with a light microscope. The dotted pattern was illustrated on a transparent sheet. In order to correct the local distortion of features in the electron micrograph (see text), each cross-section of staining bipolar cells in the electron micrograph was superposed manually so that all cross-sections coloured black were almost at the real position indicated by the dotted pattern. The number of bipolar cells was indicated on the dotted illustration of its somata (1–5). Thin solid lines indicate the outline of cone pedicles in one electron micrograph (some pedicles are indicated by arrows); because the thin section was cut obliquely to the level of cone pedicle (see text), a limited number of cone pedicles in a specific region appeared in one electron micrograph. (C) An electron micrograph. Arrows indicate cross-sections of bipolar cells stained darkly with biocytin. This is an image of the area enclosed by a rectangle in B. Scale bars: 10 µm (A, B), 2 µm (C). Inset: a vertical view of a bipolar cell. A rectangle indicates the dendritic tree region where the 3-D reconstruction was carried out from their serial thin sections (299 horizontal sections, 80 nm). Note, that although the inset illustrates one bipolar cell, the dendritic trees of five bipolar cells (see A) were provided for the 3-D reconstruction.

Cross-correlation coefficient

The similarity of features was examined by calculating a cross-correlation coefficient between them. In order to minimize data outside the feature of interest, cross-correlation values were calculated for the region of the feature-orientated bounding box, which was set to the rectangular region including the feature of interest. Where necessary, histogram equalization was conducted to minimize the difference in grey levels between the two images.

Results

Correction of the distortion of features in the electron micrographs

When aligning features obtained from sequential electron micrographs of ultrathin sections, we found that corresponding neural features changed from section to section. This change of features mainly reflected a real change of biological structure. However, it was also due to a distortion of the material caused by the method of sectioning because the change was irregular when the feature was followed from section to section, and also because some corresponding features in neighbouring sections did not overlap. Because such distortion of features is known to occur when cutting a tissue block, the distorted features were corrected with the aid of the light microscope view of the tissue in whole mount view by referring to their position in micrographs of ultrathin sections, we found that corresponding neural features changed from section to section. This change of features mainly reflected a real change of biological structure. However, it was also due to a distortion of the material caused by the method of sectioning because the change was irregular when the feature was followed from section to section, and also because some corresponding features in neighbouring sections did not overlap. Because such distortion of features is known to occur when cutting a tissue block, the distorted features were corrected with the aid of the light microscope view of the tissue in whole mount view by referring to their position in micrographs of ultrathin sections, we found that corresponding neural features changed from section to section. This change of features mainly reflected a real change of biological structure. However, it was also due to a distortion of the material caused by the method of sectioning because the change was irregular when the feature was followed from section to section, and also because some corresponding features in neighbouring sections did not overlap. Because such distortion of features is known to occur when cutting a tissue block, the distorted features were corrected with the aid of the light microscope view of the tissue in whole mount view by referring to their position in

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cell processes must be closed. Here, we tried to apply an active contour model, a Snakes (Kass et al., 1988; Araki et al., 1995), to a stained bipolar cell dendritic process (Appendix 1).

An example of the application of a Snakes model for detecting the contour of one stained cell process is shown in Fig. 5A. In the image, the central black region is a stained feature of a bipolar cell process and was a target feature for Snakes. In the Snakes image processing an initial contour indicated by a white circle was roughly drawn in the image displayed on the CRT monitor by means of a pointing device (top-left image in Fig. 5A indicated by an iteration time of ‘0’). Snakes is an energy-minimizing spline guided by external constraint forces and influenced by image forces that pull it toward features such as lines and edges. As a result of minimizing an energy function, the contour initially drawn around the stained bipolar cell process gradually shrank to detect the outline of the target feature (a white line in the images indicated by the iteration time 0–80 in Fig. 5A); in the processing of Snakes, the contour served to impose a piecewise smoothness constraint as well as to space contour points evenly with a particular average distance between adjacent contour points. After 100 iterations, the external constraint forces and the image forces were in a state of equilibrium so that the model reasonably detected the outline of the stained features. Snakes was also applied to the interpolated image, which had two stained features obtained by morphing (Fig. 5B). As expected, the initial single contour split into two contours by means of cutting at crossing points (at an iteration of 90), so that Snakes automatically extracted two objects. In this case, however, the contour line broke into the target features at the regions indicated by white arrows; this occurred because the outline of the target feature was blurred and the intracellular organs (not stained) were very close to the cell membrane in these regions (see Discussion).

**Correction of the distortion of features using a contour detected by Snakes**

When the size and shape of features in the electron
microscopic image are very close to those of the corresponding feature in the light microscopic image, the positional distortion of features was corrected using a feature contour as follows.

Figure 6A shows a light microscope image of the stained bipolar cell dendritic process. The tissue was cut close to the dendritic process (approx. 5 μm from the surface of the tissue block), so that despite the high magnification the process feature would be in better focus. Figure 6B shows an electron microscope image at the corresponding location: the feature is the same as the stained process indicated by an arrow in the left middle of Fig. 2A. The dark area in the centre of the image is the stained bipolar cell dendritic process. The dendritic process in Fig. 6B was distorted (from the corresponding darkly stained dendritic process in the light microscope image of Fig. 6A); if the electron microscope image were not distorted, the dendritic process in image B would lie on top of the dendritic process of the light microscope image A.

In electron microscope images the cross-sections of cell membranes appeared in numerous positions (Fig. 6B), and such cross-section features often disturbed the detection of contours by Snakes. The thickness of cell membranes is constant (approx. 9 nm). Therefore, we detected the cross-section of cell membranes using a line detector, a Kasvand operator, and then calculated the brightness value of pixels on the line by interpolating linearly between neighbouring pixel values. The membranes of neighbouring cells are often apposed closely. The total membrane thickness there is approximately 30 nm. Such cross-sections were removed by enlarging the filter size of the line detect operator. An alternative method was to filter the image twice with the normal filter size of the line detector and interpolating. The first filtering of the apposed membrane feature produced two parallel lines whose width is almost the same as the thickness of one membrane; the second filtering removed these parallel lines. The cell membrane detected for the image in Fig. 6B is shown in Fig. 6C (white lines). Subsequent interpolation removed the cross-section features of the cell membrane with a minimal effect on the stained dendritic process feature (Fig. 6D).

As the neurones of interest were darkly stained in the present study, the cell membrane of these neurones was usually not affected by the filtering using a line detector. Intracellular organs were not stained in our preparations, and appeared as a bright region in the electron microscope image. When the intracellular organ was apposed to the cell membrane, the thickness of membrane there was almost 30 nm. Such a cross-section feature was also detected and removed by the method described above of a line detector and interpolating. Thus, although filtering using a line detector effectively removed membranes of unstained cells.

Fig. 4. Morphing experiments for producing an interpolated image. The image labelled 1 was a source electron micrograph (as in Fig. 3A), the image labelled 6 was a target electron micrograph (as in Fig. 3C) and the image sequence 2–5 was produced by morphing. 299 × 180 pixels. Scale bar: 5 μm.

in the electron microscope image, the filtering rarely removed the membrane of the cell of interest.

The Snakes model applied to the filtered image successfully detected the contour of the dendritic process and is shown as a white line in Fig. 6D. Next, the contour image obtained by Snakes was matched to the corresponding feature in the light microscope image using a cross-correlation function between the two images (Fig. 6E).
Lastly, the dendritic process feature in the original electron microscope image (Fig. 6B) was warped using the contour in Fig. 6D as an original control line and the corresponding contour in Fig. 6E as a target control line. The calculated warping image was shown in Fig. 6F. The cross-correlation coefficient value was 0.88 between the image shown in Fig. 6F and the corresponding region of the image in Fig. 2C, while the value was 0.64 between images Fig. 6B and F. Thus, although control lines were determined by different methods in the image processing of Figs 2 and 6, the warped image gave a similar result.

Reconstruction of 3-D image

Five bipolar cells were reconstructed from the outlines of stained features. Because of the limitation of memory and the reduction of the computation time for image processing and for displaying the reconstructed image, 80 out of 299

Fig. 6. Correction of the distorted features in an electron micrograph with the aide of Snakes. (A) A light micrograph of a stained bipolar cell dendritic process (dark feature). The tissue was cut close to the dendrite so that the blurring of the dark-stained process was minimized. Subsequent electron microscopic observation showed that the distance from the process to the surface of plastic block is approx. 5 µm. (B) An electron microscope image of the section taken at the same location as image A. The central dark feature in the image is the stained bipolar cell dendritic process. Note that the intracellular organs were not stained and thus appeared as bright areas in the process. The stained dendritic process in the electron microscope image was distorted from its light microscope image in A. The stained dendritic process is the same as the stained process indicated by the arrow in the left middle of Fig. 2A. (C) A cross-section of cell membranes calculated using a line detector. Cross-sections, whose width is represented as one pixel in the image, were detected using a line detector of 5 x 5 Kasvand operator and are indicated by white lines. One pixel is approx. 27 µm, which is larger than the thickness value 9 µm of membrane. The blur in the cross-section was caused during the process of taking a real biological feature on the electron microscope film to the digitization of electron micrographs. Original image from B. (D) The cell-membrane-removed electron microscope image and a contour superimposed on the stained dendritic process. The brightness value of pixels on the cross-section detected (C) was calculated by interpolating linearly between neighbouring pixel brightness values. Next, Snakes was applied to the processed image, and the resultant contour of the stained dendritic process is shown by a white line. The Snakes parameters were as follows: \( W_{\text{norm}} = 0.1; W_{\text{area}} = 3; W_{\text{dist}} = 1; W_{\text{edge}} = 0.1; W_{\text{intens}} = 2 \). (E) Matching of the contour image with the dendritic process in the light microscope image. The contour in the electron micrograph (C) is the original image, and was shifted to the target feature of dendritic process in the light microscope image (dark feature in D) by using a cross-correlation function of the two images. (F) A feature of the stained dendritic process as processed by image warping. The electron microscope image, B, is the original image. The original control line was a contour in D, and the target control line was a contour in E. 250 x 250 pixels. Scale bars: 2 µm.
sections were provided for reconstruction. The number of bipolar cell processes that appeared in each electron micrograph was usually more than one (see Fig. 1B), and was \( \approx 1500 \) in total in electron micrographs of 80 sections (for the process of reconstruction, see Appendix 2).

Figure 7 shows 3-D views of five bipolar cells reconstructed from serial thin sections. Figure 7A is a reconstruction of five bipolar cells viewed from the photoreceptor side; a vertical view of bipolar cell reconstruction from the point indicated by a large arrow in A is shown in B. Primary processes from five bipolar cell somata (not illustrated) travel upward and make dendritic trees. A part of some Landolt club processes that extends distally from the outer plexiform layer was not reconstructed (an upper part of cell processes from the level indicated by arrows in Fig. 7B; see also Fig. 9 of Umino et al., 1994).

Although bipolar cells should make their dendritic trees at the same level in the intact retina, the reconstructed view was distorted as indicated by a dashed line in B. This distortion was caused by cutting the thin section obliquely to the plane desired for the 3-D reconstruction (see Methods). Our 3-D model was based on a wire frame model; consequently, such a distortion was easily corrected on the CRT display by operating a pointing device, as shown in Fig. 8: the processed image was stored in the computer. In images A and B, the bipolar cell labelled 5, and red- and green-sensitive cone photoreceptor terminals were reconstructed. The corresponding wire frame model of the bipolar cell of B is shown in C. Figure 9 shows some views of the reconstructed bipolar cells and cone terminals. As shown, although bipolar cell dendritic trees have a complex morphology, the reconstructed 3-D feature is close to the real cells. As mentioned above, with this computer system the rotation, zooming and animation of the reconstructed image were easily manipulated on the CRT display by operating a pointing device. Furthermore, 3-D features of neurones displayed on the monitor were seen as stereo pairs by using a stereoviewing 3-D system (CrystalEYES2, StereoGraphics Inc., CA).

**Discussion**

In the present study, we show that a morphing algorithm works to give a satisfactory correction of the distortion. As shown here, image morphing using appropriate control lines aligned the features in the electron microscope image to the corresponding feature in the light microscope image. The validity of the warped features depends on the resolution of the light microscope image. Morphing is a linear algorithm, and thus the calculation time is small. For example, for an image of \( 1020 \times 640 \) pixels the calculation time was less than 30 s with a personal computer.

In order to reconstruct a 3-D image, spatial coordinates must be given to the features of interest. In this study, the value of the X-Y coordinates of features was determined by referring to the light micrograph of a whole-mount view, while the Z coordinate value was from the thickness of sections multiplied by the number of sections. When a neurone of interest is not stained, because the positional relationship may be maintained among features in the neighbouring neural processes, the features of interest can be followed by aligning from that relationship as a clue (Stevens et al., 1980; Smith, 1987).

Because the accuracy of the X-Y coordinates of the reconstructed image is limited by the resolution of the light microscope, it could be argued that a feature reconstructed from serial electron microscope images is similar to one obtained using a 3-D light microscope, such as a confocal microscope or a deconvolution microscope, especially for large organs and thick processes. For fine organs and processes, however, a 3-D reconstruction from electron micrographs has several advantages over a 3-D light microscope. First, in a 3-D light microscope, a 3-D feature is presented as an accumulation of (bright) particles. However, the number of particles is very small for fine organ features, making it difficult to reconstruct ‘continuous’ 3-D features. Second, an electron microscope image shows the features of a neurone’s organelles in sharp focus, while their image in a light microscope is significantly blurred because the size of fine organelles is often smaller than the resolution of a light microscope. The Z-coordinate values of images reconstructed from electron microscope images are determined from the section thickness, \( 80 \text{ nm} \) in the present study, a value much smaller than the resolution of a light microscope. In practice, sharp boundaries of features on the X-Y plane and a high resolution Z-coordinate value in electron microscopy contribute to reconstructing a 3-D feature.

In comparison with light micrographs, the fine structure of the cell in electron micrographs is well resolved. Superimposing the outlines of cell features and intracellular organs in a reconstructed 3-D feature could enhance our ability to predict neuronal function from its morphology. In this regard, the ability to correct electron microscope images using a warp algorithm rather than the outlines of features is important. Organs outside the organ of interest in the warped image, such as processes from other cells, can also be reconstructed, but because the validity of the coordinate values of organs in the warped image would decrease with increasing distance from these organs to the organ of interest, the construction should be conducted only for nearby organs.

The diameter of the fine processes of neurones is often less than \( 200 \text{ nm} \), and thus the shape of these processes is significantly changed within the \( \approx 100 \text{ nm} \) thickness of the electron microscopic section. Furthermore, in the process of making serial thin sections, some sections are usually damaged or lost. As a result, the shape of the cross-section
Fig. 7. Three-dimensional reconstruction of five bipolar cells. (A) A view of five bipolar cell dendritic processes from the photoreceptor side. For the number of bipolar cells, see Fig. 1A. Scale bar: 20 μm. (B) A vertical view of five bipolar cells. Cells were viewed from the large arrow in A (see explanation below). Photoreceptor side is upward (see Figs 8 and 9). Numbers under the five main cell processes indicate the corresponding bipolar cells. Thin sections were cut obliquely; thus, the reconstructed bipolar cells were morphologically distorted, as indicated by a dashed line. In this view the dashed line should be a horizontal line if the thin sections were cut ideally. The distal parts of the cell processes from the level indicated by arrows were not reconstructed (see text). Note that vertical and horizontal scales are different. For the region of reconstruction, see the inset of Fig. 1. Scale bars: 5 μm (vertical) and 20 μm (horizontal).
of neural processes from the serial electron micrographs used for the reconstruction is sometimes significantly changed. As the reconstruction of 3-D objects from the contour of these features causes artefacts, the surface of the reconstructed image is a major concern. Various surface interpolation algorithms have been proposed (Chen et al., 1990). Although these interpolation algorithms worked well when the feature of interest was located almost in the same position in two consecutive electron micrographs, the algorithms usually generated unsatisfactory results when the feature, of large concave shape, in neighbouring electron microscopic sections does not overlap (not shown). When a branched cell process was involved in the feature, the result was even more unsatisfactory. In contrast to these interpolation algorithms, the processed image of the morphing algorithm was satisfactory, as shown in Fig. 4. The problem of using the morphing algorithm was the relative blurring of the processed image. To obtain a sharp image, image matching techniques may be useful (Gerlot & Bizais, 1988; Maguire et al., 1991; Goshtasby et al., 1992). A major problem in using image matching is the selection of the right transformation function for the mapping of one image into another. That is, if the selected transformation does not truly model the geometric difference between the feature images, an overall accurate match will not be obtained. When the image matching technique of Goshtasby et al. (1992) was applied to the electron microscope image of Fig. 3A and C, the processed image was less close to the original image of Fig. 3B (not shown, correlation coefficient = 0.68) when compared with the morphing image of Fig. 4 (correlation coefficient = 0.86). Furthermore, parameters used in the image matching experiments strongly depended on the image, and it usually took a lot of time to find the appropriate parameters for the particular image. Thus, the usefulness of image matching techniques between electron microscope images was limited.

It might be argued that the traditional edge detectors, such as the Laplacian filter (for example, see Pratt, 1991) or the Laplacian of Gaussian edge detection operator (Marr & Hildreth, 1980), can be used for electron microscope features. If the entire surface of the process were perpendicular to the cutting plane, the cell process outlines would be sharp in the electron micrograph and would be detected by the traditional operator. Usually, however, cell processes are cut at various angles because they project in different directions in the tissue, and because serial sections are cut at a constant angle. As a result, the angle of certain regions on the surface of a cell process to the cutting plane is usually different from that of other regions on the surface of the same process cell. Consequently, the scale of the sharpness of the outline of the cell process varies depending on the position on the outline in an electron microscope image, even for one cell process. If a traditional edge-detection operator is applied to such features the estimated outline would be discontinuous, although in order to reconstruct a 3-D profile of neurones the outline of their processes should be continuous. Thus, for features in electron micrographs it is necessary to enclose the feature having a varying scale of sharpness in its outline with a continuous line.

It took a relatively large computation time to trace the outlines of features with the Snakes model. For example, it took 2–3 h of computation time for the experiments shown in Fig. 5 when the image (400×360 pixels) was processed by using a workstation computer system. A decrease in computational time was achieved by drawing the initial contour near the stained feature of interest (by using a
pointing device in our system). In this regard, Fukuoka et al. (1998) proposed a method for determining an initial contour in Snakes by using a skeleton function determined from the image. Their method is reported to detect a complex feature in a noisy image. If the feature of interest is clearly recognized, a modified Snakes model applied to the processed image with an edge-detective filter is effective (Hashimoto et al., 1994). For example, the model estimated the continuous contour of features within 30 s, which was much faster than the time required for the original Snakes model. In addition, an improved computer system would dramatically lower the computational time. Thus, the problem with computational time for the Snakes model might be easily resolved.

The following problem would be more difficult in the Snakes. As indicated by white arrows in Fig. 5B, the outline estimated by Snakes sometimes failed to detect the real outline of features. After a tracer, biocytin, was injected, the intracellular space was darkly stained, while the intracellular organs such as endoplasmic reticulum were not stained. As a result, the picture of the stained cell was composed of dark and light regions. Furthermore, although the cause is not known, the cell membrane of the biocytin stained cell process was sometimes not clearly recognized (for example, see Fig. 12 of Umino et al., 1994). As a result, in the image processing experiments shown in Fig. 5B, Snakes moved through the blurred membrane feature and the white intracellular organs, and reached inside the

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**Fig. 9.** Three-dimensional reconstruction of one bipolar cell and cone photoreceptor terminals. (A) Bipolar cell main process and dendritic trees, and cone terminals in the vicinity of dendritic trees. A view from the proximal side at an angle. (B) A bipolar cell and cone terminals. A view from the photoreceptor side. (C) A bipolar cell main process and dendritic trees. A view from the proximal side at an angle. (D) A bipolar cell main process and dendritic trees. A view from the photoreceptor side.
stained cell process. Without a knowledge of electron microscopy and the structure of the retina it would be hard to know whether Snakes successfully detected the membrane surface of the neurone. Whether the estimated outline represents the real outline of the feature can only be determined by a researcher who is familiar with the electron microscope feature of interest. Consequently, at present, the involvement of researchers in tracing the outline of the features is a practical necessity.

The deformable 3-D active surface model may offer an interesting approach for reconstructing 3-D features directly (Cohen & Cohen, 1993; Zhang & Braun, 1997). However, as mentioned above, as the detection of object boundaries by a simpler 2-D active model sometimes met with failure and because neuronal dendritic processes show very complex features (such as branches and long tube-like processes, etc.), the number of organs which can be targets of the 3-D model is limited, especially in neural science.

Neurones exhibit various morphological features. In order to correlate these features with the function of neurones, various types of morphological information, such as the length and diameter of their processes as well as the positions and sizes of synapses, should be examined in three dimensions. For this purpose, it is apparent that the 3-D reconstruction of neurones at the electron microscope level by computer is very important. Another important method for correlating the morphological features with the physiological function is to construct a compartmental model from the 3-D reconstruction of neurones and apply an electrical logical function is to construct a compartmental model from the particular region of the model (Smith, 1992; Shishido & Umino, 1997; Yumamoto et al., 1998).

Appendix 1

Snakes (Kass et al., 1988) is an active contour model that involves giving an initial contour for the target in the image, then deforming contours to detect object outlines by minimizing an energy function.

Araki et al. (1995) incorporated an area term $E_{\text{area}}$ for the energy function. That is, when a contour is composed of discrete points $v_i (x_i, y_i) \ (i = 1, 2, \cdots, n)$, the energy function $E_{\text{snakes}}$ in their model is defined as the sum of several energy terms:

$$E_{\text{snakes}} = \int \left( E_{\text{area}} (v_i) + E_{\text{spline}} (v_i) + E_{\text{dist}} (v_i) + E_{\text{edge}} (v_i) + E_{\text{intens}} (v_i) \right) \, dv_i$$

(1)

$$E_{\text{area}} (v_i) = \frac{1}{2} \sum W_{\text{area}} (x_i (y_{i+1} - y_i) - (x_{i+1} - x_i) y_i),$$

$$E_{\text{spline}} (v_i) = \frac{1}{2} \sum \left( W_{sp1} |v_j - v_{i+1}|^2 + W_{sp2} |v_{i+1} - 2v_i + v_{i-1}|^2 \right),$$

$$E_{\text{dist}} (v_i) = \frac{1}{2} \sum W_{\text{dist}} |v_i - v_{i-1}|^2,$$

$$E_{\text{edge}} (v_i) = -\frac{1}{2} \sum W_{\text{edge}} |\nabla I(v_i)|^2,$$

$$E_{\text{intens}} (v_i) = \sum W_{\text{intens}} I(v_i).$$

$E_{\text{snakes}}$ represents the first- and second-order constraints and serves to impose a piecewise smoothness constraint (Kass et al., 1988). $E_{\text{dist}}$ spaces contour points evenly with an average distance, $d_{\text{av}}$ between adjacent contour points (Williams & Shah, 1990). $E_{\text{edge}}$ means edge potential defined by a spatial gradient of intensity $I(v_i)$ (Kass et al., 1988). $E_{\text{intens}}$ is the intensity of a pixel in the image (Kass et al., 1988). $W_{sp1}$, $W_{sp2}$, $W_{\text{area}}$, $W_{\text{dist}}$, $W_{\text{edge}}$ and $W_{\text{intens}}$ are coefficients. Each contour point $v_i (x_i (t), y_i (t))$ is moved to minimize $E_{\text{snakes}}$ so that an actual contour of the target can be detected. Note, $i = 0, 1, 2, \cdots, n$, denotes the number of iterations. $v_i (x_i (t), y_i (t))$ is an initial contour and $v_0$ and $v_1$ are equal to $v_n$ and $v_{n+1}$, respectively. Araki et al. (1995) provided an algorithm in which an initial single contour iteratively splits into multiple contours by cutting at crossing points, so that the model can automatically extract plural objects. When the image includes small noise(s) as well as large target(s), the model extracts the large target alone because the contour whose area term is small can be eliminated in the splitting process of the algorithm.

Appendix 2

In order to reconstruct a 3-D model, nine programs, written in C language, were developed. After a scanner captured the illustrated image, the following steps were carried out. First, pixel data from the stained features were transferred to a byte-string (program 1). Next, with the use of program 2, the outline of cell processes in the form of a closed curve was determined in each electron micrograph and was stored as a text file. Program 3 was used to calculate the parameters of features related to their spatial profile, such as an area and $X-Y-Z$ coordinates; Z-value was given from the thickness of sections. Program 4 was then used to investigate the type of connections of cell processes, such as continuity and branching, between adjacent sections. The continuity of stained cell processes was based mainly on the assumption that the position of cell processes and the shape of their contour do not change very rapidly from section to section (Nakamura et al., 1990). The propriety of continuity and branching was examined immediately by using a simple reconstruction program written for a personal computer; this program, developed at our laboratory, transferred each cross-section of a cell process to a circle of fixed area, and it displayed a simplified 3-D reconstruction image on the CRT monitor. Programs 5 and 6 were used to modify the centre of gravity and size of cell processes in each electron micrograph so that the outline of cell processes could be
changed smoothly in serial sections. In program 7, the modification was also affected by the connection pattern of cell processes. Program 8 examined the correspondence of the digitized data of feature outlines in neighbouring sections and then calculated a square patch and its X–Y–Z coordinates from these data. Finally, program 9 converted the format of patch data to a geometry data format so that the 3-D reconstruction of bipolar cells as well as cone terminals were displayed on the CRT monitor by AVS, a software for 3-D reconstruction.

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