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Embedding of Large Specimens in Glycol Methacrylate: Prerequisites for Multi-Signal Detection and High-Resolution Imaging

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ABSTRACT Acrylic resin mixtures are commonly used to study microscopic sections of biological specimens, giving the advantage of good morphological preservation. Existing embedding protocols, however, are suitable for tissue blocks, not exceeding 1 mm in thickness. We have developed a protocol to embed larger specimens (up to 2 cm^3) in Technovit 8100. This medium allowed us to perform classic histological (trichrome), silver, as well as immunohistochemical staining, needed for multi-signal detection at high-resolution imaging to reconstruct a three-dimensional interpretation of a serially sectioned muscle. The technique was applied to reconstruct the semitendinosus muscle of a fetal pig, 44 days post conception, featuring connective tissue, intramuscular nerves, blood vessels, and muscle fibre types. For the reconstruction, a technique was used that enabled us to insert high-resolution images of histological details into low-resolution images of the entire muscle. *Microsc. Res. Tech. 66:25–30, 2005.*

INTRODUCTION

Acrylic resin mixtures are now widely used as embedding media to obtain microscopic tissue sections of biological specimens. Resin-embedding media such as methacrylates are found to preserve morphology far better than the more classic methods like paraffin, celloidin, or frozen sections (Gerrits et al., 1987, 1991; Hanstede and Gerrits, 1983; Iwadare et al., 1984). Paraffin embedding, for example, causes modification of tissue components, especially proteins, thereby deactivating several enzymes and causing loss of antigenicity (Gerrits and Horobin, 1996). Furthermore, it leads to considerable tissue shrinkage, introducing errors in three-dimensional reconstructions, and it is not very suitable for sectioning tissue components of different hardnesses. In contrast, the water miscible resin 2-hydroxyethyl methacrylate or glycol methacrylate (GMA) was shown to be particularly suitable for enzyme histochemistry (Higuchi et al., 1979) and immunohistochemistry (Craig et al., 1979; Hoshino and Kobayashi, 1972). The commercially available GMA Technovit 8100 was found to be appropriate (Gerrits and Horobin, 1996) because of the possibility to control polymerization at low temperatures. However, according to the manufacturer's protocol, the tissue blocks to be processed can be maximally 1-mm thick; a suitable commercial GMA mixture to embed larger blocks is not yet available (Gerrits and Horobin, 1996). Polymerization of Technovit 8100 should be performed in special hermetically sealed embedding molds because the polymerization process is extremely sensitive to oxygen or any other radical scavenger that could terminate the polymerization.

Our purpose was to image the spatial relationships between the growing muscle fibers, nerve fibers, and connective tissue in a fetal muscle with the help of a three-dimensional reconstruction of serial sections. Specific contrast of the muscle fibers and nerves was required to allow analysis of the details of the sections. The chosen embedding medium must allow application of specific contrast agents, for general and silver staining. Furthermore, antigenicity must be preserved for immunohistochemical staining. To achieve a reliable reconstruction, the medium should not shrink or deform during polymerization and must be hard enough to allow sectioning of bone and prevent serious deformation of the tissue during sectioning. In this report, we describe a procedure for the application of Technovit 8100 to study the fetal porcine semitendinosus muscle. The procedure was adapted to allow embedding of large tissue blocks such as the fetal hind limb, which had a volume of about $2 \times 2 \times 0.5$ cm. The technique overcomes complications with infiltration and provides a means to embed tissues that do not fit into embedding molds that are specifically designed for plastic embedding.

To study the micro-anatomy, the images of the tissue under study should resolve the required detail such as fine-meshed axons and different myosin heavy chain (MyHC) isoform expression by the myotubes. This is only possible at higher magnifications, i.e., at least with a 10× objective. However, at this magnification the overview of the muscle and its surrounding

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structures is lost. Traditional and existing computer-aided methods of three-dimensional reconstruction have so far not solved this problem as they stack the images of the sections of the entire specimen, obtained at a suitable low magnification. We applied the solution of Verbeek and Boon (2002): large view high-resolution images are obtained by combining multiple adjacent images of the section. The combination is realized by stitching the adjacent image tiles to one large image. This procedure is part of a semi-automated image acquisition process. To reconstruct the third dimension, we had to cut serial sections throughout the entire hind limb.

To benefit from a thoroughly tested commercially available embedding kit, we improved the method of infiltration and polymerization of large tissue blocks. The resulting tissue sections could be stained with classical (trichrome and silver) methods and immunohistochemically with antibodies recognizing different muscle fibre types; they allowed accurate 3-D reconstruction.

MATERIALS AND METHODS

Fixation and Preparation of the Specimen

Hind limbs still attached to the hemisected pelvic girdle were obtained from fetal porcine specimen at 44 days of gestation and fixed by immersion in phosphate buffered 4% formaldehyde for 24 hours at 4°C. Thereafter tissues were stored in 70% alcohol, at 4°C. The hind limb was prepared trimming away the excess of pelvic tissue, resulting in a tissue block of about $2 \times 2 \times 0.5$ cm. To protect the tissue during handling and to facilitate orientation, the specimen was embedded in 2% low melting point agarose, melting point at 65.5°C, gel point at 24–28°C (Sigma-Aldrich, St. Louis, MO) in PBS. All sides were covered with agar, after solidification excess was trimmed away.

Dehydration

The agar-covered specimen was dehydrated gently shaking through graded acetone solutions at 4°C, starting with 70% overnight or longer, 80% for 2 hours, and 3 changes of 100% 2 hours each. Dehydration times were prolonged in cases of larger tissue blocks.

Infiltration

Specimens were infiltrated and embedded using commercially available GMA Technovit 8100 (Heraeus Kulzer GmbH, Werheim, Germany). All infiltration and embedding solutions were de-aerated by application of vacuum and kept as air-free as possible at 4°C. The infiltration fluid was prepared as described by the protocol of the manufacturer, that is 0.6 g peroxide lucidol CH_{50} added to 100 ml base-liquid Technovit 8100, di-hydroxyethylmethacrylate with a hydroxyether as plasticizer. The specimen, which floats initially, was infiltrated for 2 up to 4 days while gently shaking, interrupted 2 to 3 times by de-aeration for 2–3 hours at 0–4°C (on crushed ice). Periods of infiltration were prolonged in cases of larger tissue blocks. The infiltration fluid was refreshed two or more times (depending on the size of the specimen) until the specimen and surrounding agarose had become completely transparent and the specimen had sunken.

Embedding

The specimen was positioned in a for-general-purpose polyethylene-embedding mold (Peel-A-Way by Polysciences Europe, Eppelheim, Germany) placed on crushed ice. The embedding fluid was prepared according to the protocol of the manufacturer that is 1 ml "hardener II" thoroughly mixed with 30 ml infiltration fluid, this time keeping the de-aerated solutions at room temperature. The solution was gently poured into the embedding mold until the specimen was generously covered thereby avoiding the introduction of air (bubbles). Air-contact was prevented by applying a 3–5-mm-thick layer of de-aerated paraffin oil (Paraffinum liquidum, 68-81 mPa.s by Brocacef bv, Maarssen, The Netherlands). The block was left to polymerize for at least 24 hours, at 4°C (on crushed ice and in the refrigerator). Thereafter the paraffin oil was poured off. If the polymerized block was still sticky on the outside, it was stored to solidify at 37°C for 24 hours or longer.

Cutting

After polymerization, the GMA block was mounted onto a Perspex base with cyano acrylate–based glue, which could be clamped into the microtome. Using a motorized base sledge microtome with a retracting (300 μm) K-knife (Jung Polycut E), 5- to 10-μm-thick sections were cut and stretched on distilled water, mounted on uncoated glass slides, and dried at 40°C. Cutting throughout the total hind limb, 10-μm sections were collected, as follows: the first three sections were collected for three different series, the next seven discarded, the three next collected, and so on. The three series, each with inter-section distances of 90 μm, were subjected to different staining procedures.

Staining Procedures

Series 1 was subjected to cartilage staining with Alcian blue for 1 hour followed by trichrome staining with Celestine blue for 10 minutes, Mayer's hematoxylin (Mayer, 1903) for 10 minutes, and Van Gieson's stain for 1 hour. This was expected to stain nuclei dark blue, cartilage light blue, cytoplasm yellow, and collagen red.

Series 2 was subjected to silver nitrate staining procedure for nerves, a variation on the method of Sevier and Munger (1965).

Series 3 was analyzed immunohistochemically using antibodies directed against myosin heavy chain isoforms. Briefly, sections were soaked in PBS; incubated in 0.05% v/v Trypsin for 30 minutes at RT, rinsed with PBS, blocked in TENG-T (100 mM Tris, 50 mM EDTA, 1.5 M NaCl, 2.5% gelatin, 0.5% v/v Tween 20) with 2% w/v albumin bovine fraction V (Boom bv, Meppel, The Netherlands); this was followed by rinsing the sections in PBS and incubating with primary monoclonal antibodies (e.g., fast myosine heavy chain, monoclonal, raised in mouse [Sigma], dilution 1:500 in PBS, with 1% w/v albumin bovine fraction V) for 18 hours; the first hour was at 37°C and the remaining time at RT. Next, the sections were rinsed in PBS, incubated with biotinylated horse anti-mouse polyclonal antibody (1:100 in PBS), and visualized with the ABC peroxidase staining method using the Elite kit (Vector Labs;

Burlingame, CA), as described elsewhere (Eizema et al., 2003).

3-D Image Acquisition and 3-D Reconstruction

The 3-dimensional reconstruction was created out of the stacked sections of series 1 and 2. In the acquired images, both detail and overview were preserved and the original alignment restored. A dedicated acquisition system (Van Raaij et al., 1999; Verbeek and Boon, 2002) was used to acquire the images. Data acquisition took place with a 10× (NA 0.3) lens objective, which sufficiently resolved histological detail. Of each section, an 8 × 8 matrix of 64 images was acquired, covering the entire section, which were combined into one composite image, 5,850 × 4,300 pixels in size. In these composite images, the contours of the following structures were marked manually: the femur, the outer border of the semitendinosus muscle, the internal tendineous inscription of the muscle, and the visible blood vessels and nerves. These contours are called annotations to the total image.

The composite section images were aligned on the basis of the continuity of the structures and the image stack was used in a software program for annotation of anatomical structures: TDR-3Dbase (see http://bio-imaging.liacs.nl) (Verbeek et al., 1995; Verbeek, 2000). The 3-D reconstruction emerges as the contours of chosen structures are drawn in the images. In the software, a mechanism is incorporated to convert from one graphical representation to the other; in this manner, contour stacks as well as volumetric and surface reconstructions can be displayed on the fly. The depicted reconstruction (Fig. 1) was based on 4,493 contours. The structures used in the reconstruction are indicated in the figure.

RESULTS

After polymerization, the block appeared slightly yellowish and became more yellow after exposure to daylight. Further solidification at 37°C resulted in a hardened block, including the area that had been exposed to paraffin oil. However, in particular on the side that had been exposed to paraffin oil an approximately 1-mm-thick surface layer remained rubbery. This layer may be similar to the rubbery layer that has been described for Technovit 7100, which makes it possible to produce ribbons of sections (Gerrits et al., 1991).

The block was cut into 5- or 10-μm sections with a D-knife. The cutting properties of the resin block were good, despite the fact that the embedded tissue was of mixed texture, including muscle, cartilage, and even mineralized bone. The sections of 10 μm could be handled and stretched on distilled water more easily then those of 5 μm. The latter tended to stick to forceps when picked up from the knife and twisted when put to float and stretch on the water surface. Application of these sections on glass is more difficult. However, when a small amount of water was held underneath the section on the glass, further flattening occurred when heat was applied to dry the section. The sections containing partly tissue and partly solid resin stretched without problems. This could be observed from the alignment of the large compiled images (built up out of 64 images) that were used to create the 3-D reconstruction (Fig. 1).

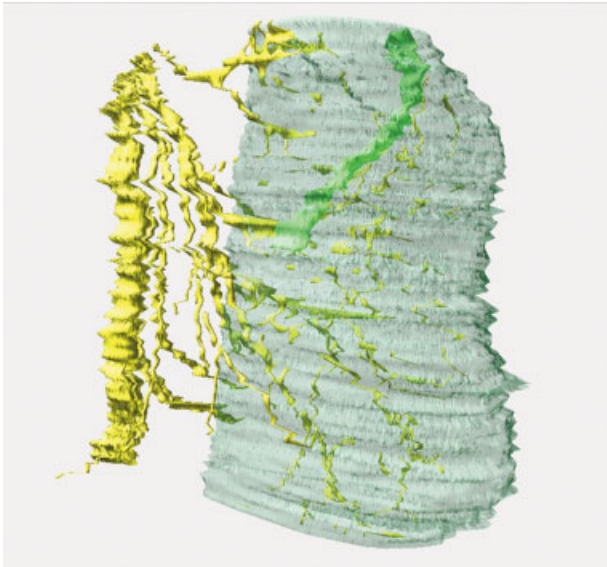

Fig. 1. Lateral view of a three-dimensional reconstruction of the porcine semitendinosus muscle. Structures indicated: the muscular branches of the tibial nerve (yellow); m. semitendinosus (transparent green); tendineous inscription (green).

In Figure 2, the results of the staining procedures are shown. The classical trichrome staining (Fig. 2A) and silver nitrate staining (Fig. 2B) gave the morphological information needed to create the 3-D reconstruction; these structures were traced and served as annotations. In Figure 2A, one can discern muscle tissue (yellowish) surrounded by the endomysium (red). Within the muscle, a fascicle-like organization of the muscle already can be distinguished with the collagenic perimysium (red) surrounding the myotubuli. Relatively large nerves and blood vessels can also be found lying in between muscles. The femur, with cartilage (light blue) or already mineralized bone (greenish blue) is surrounded by the periostium (deep red). In Figure 2B, silver nitrate staining reveals the large nerves (black) but also the more fine meshed nerves lying between the fascicles (not distinguishable at the presented magnification).

The third series was used to stain MyHC isoforms using specific monoclonal antibodies. In the fetal tissue, only the fast isoform of MyHC could be detected (Fig. 2C). The minimal amount of certain MyHC isoforms in fetal tissue in combination with plastic embedding, limited further detection. This was not due to the choice of embedding material because when adult semitendinosus tissue was embedded with Technovit 8100 according to our protocol, clear staining of the fast and slow isoforms was found (Fig. 2D and E).

An image of the computer reconstruction (Fig. 1) shows that despite the differences in staining intensities between the two series and the interruption of maximal 80 μm between succeeding sections, implicating a considerable lack in information, the alignment of the structures was reasonable. The reconstruction shows a lateral view of the major part of the m. semitendinosus (transparent green) with ingrowing muscular branches of the tibial nerve. Already at this stage (44 days of gestation), the major divisions of the

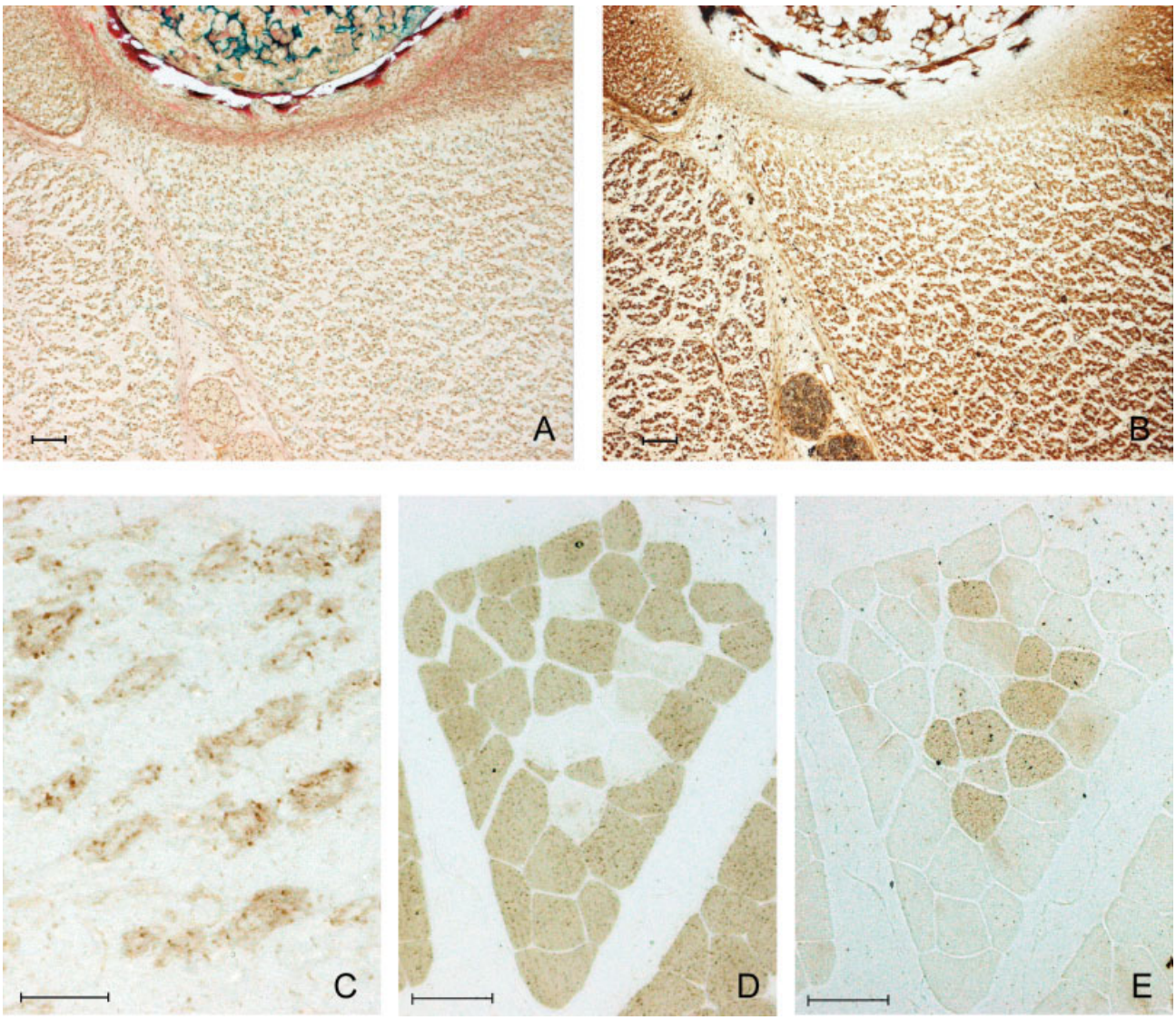

Fig. 2. Light microscopic images of T-8100 sections. **A,B:** Sections of fetal porcine hind limb (44 days of gestation) that received classical trichrome staining (A) or silver nitrate staining (B). **C:** Section of a fetal porcine hind limb (44 days after gestation) immunohistochemically stained with an antibody against fast-MyHC. **D,E:** Sections of adult m. semitendinosus tissue immunohistochemically stained with antibodies against fast MyHC (D) or slow MyHC (E). Scale bar (A, B, D, E) = 100 μm; (C) = 25 μm.

nerves are restricted to domains within the proximal and distal compartments separated by a tendineous inscription (green).

DISCUSSION

The embedding of large specimens into Technovit 7100 has been developed in neuroscience research (Quester et al., 2002). However, to perform immunoanalysis of myosin heavy chains, the use of Technovit 8100 seemed to be inescapable as this resin can be subjected to controlled polymerization at low temperatures. Furthermore, it shows little cross-linking to tissue proteins and concomitant loss of antigenicity. The GMA contains minimal amounts of hydroquinone (200–300 ppm), therefore, only relatively small amounts of initiator and accelerator are needed. This is beneficial for the homogeneous progress of polymerization and results in a stable GMA plastic. Moreover, only a small % of cross linker (ethylene-glycol-di-methacrylate) needs to be added, allowing stabilization of the plastic without a strong increase in the molecular density. A high molecular density hampers the penetration of antibodies into the tissue and subsequent binding to their antigens. However, Technovit 8100 is sensitive to oxygen or other radical scavengers that will terminate the polymerization process. This, in combination with a large volume of the specimen, complicates infiltration and polymerization.

Handling of the Specimen

In Technovit 7100, the specimen can be oriented during the first 30 minutes of the polymerization process

when the polymerizing solution becomes gelatinoid. As polymerization of Technovit 8100 is extremely sensitive to oxygen, the polymerizing solution must be sealed air-free with paraffin oil; it must also be kept refrigerated. Clearly, one can not benefit from the gelatinoid phase to orient the specimen. We, therefore, covered the specimen in low melting point agarose, which would not only protect the fragile tissue of the fetus, but also made it possible to position the specimen before the gelatinoid phase. Low melting point agarose was used at a temperature (lukewarm but above the gel point of 24–28°C) to preserve antigenicity and the agarose did not interfere with dehydration, infiltration, polymerization, or sectioning of the incorporated specimen. During dehydration in acetone, the agarose becomes opaque but turns transparent again when impregnation is completed.

Polymerization

The polymerization solution was prepared according to the manufacturer's protocol, after thorough deaeration of the solutions. It proved to be of importance that the solutions used were at room temperature. Below 18°C, the polymerization process did not initiate well. Subsequently, when placed on crushed ice positioned in the refrigerator, the polymerization did not proceed well and solution remained fluid and turned orange due to formation of stained reaction-products. The commercial T8100 kit provided by the manufacturer plastic-seals to air-free shelter the polymerizing solution. However, these seals fit onto the commercially available embedding molds that were too small to carry our specimen. Moreover, the polymerizing solution is still in contact with air at the borders of the seal and air can easily collect in bubbles underneath it. Paraffin oil appeared to be a good alternative. The liquid floats on top, excludes all air, and it does not mingle with the polymerizing solution. Moreover, molds of any size can be used.

Cutting, Mounting, and Staining

After polymerization, water-miscible resins remain relatively elastic. The hardness of T8100 is, e.g., 40% lower than T7100 (Gerrits et al., 1991). Because of its size and elasticity and the concomitant possibility of distortion by sectioning, the block should be clamped firmly into the microtome. This, however, will provoke deformation. We, therefore, mounted the GMA block onto a Perspex basis, which instead was clamped into the microtome. In addition, a motor-driven microtome with retraction device is advised. Retraction of the block during the back stroke is said to be crucial to avoid damage and scratches on the block surface by the knife (Gerrits and Horobin, 1996). This would give artifacts, which become visible after staining.

Using a K knife, 5- to 10-μm thick sections were cut and stretched on distilled water. However, often, 5 μm and also thinner sections curled after sectioning and unfolded incompletely when put on the water surface. They did not stretch optimally due to tiny air bubbles clinging into the folds. Only by manipulation with small paintbrushes on the bottom of the bath could the sections be completely unfolded and mounted onto the uncoated glass, and this procedure may have introduced changes in size and shape of the sections. Furthermore, the temperature at which the sections are mounted is of influence on the size of the mounted sections (Gerrits et al., 1987). It is, therefore, of importance to standardize the procedure.

3-D Acquisition and 3-D Reconstruction

When judging the result, a 3-D reconstruction of a fetal m. semitendinosus, which had a real length of 5.5 mm, we acknowledge 3 sources of distortion in the followed procedure: (1) section deformation, (2) multi-signal staining, and (3) section distance.

1. Section deformation can have its origin in several steps of the protocol: cutting, mounting, and staining. As Technovit 8100 is relatively soft (see above), cutting will indent the plastic as well as the tissue. Stretching and mounting the sections on glass may cause further deformation and should be standardized. Finally, when staining, sections are dehydrated and re-hydrated and exposed to acid environments, changing the size and shape of the section. From the reconstruction, we have estimated that a compression of about 10% occurs in the direction of sectioning.
2. The multi-signal staining and detection permitted more precise annotation in the 3-D modeling phase as described in Materials and Methods. In the alignment phase, this posed a problem, as it was difficult to align section images of such different density content due to differences in color intensities between trichrome and silver stains. Structures with a high special contrast in both section images were chosen in the alignment procedure. Empirically, this has proven the right choice. The alignment was somewhat hampered by the distance between the section images (see also 3).
3. For reasons of manageability, we have chosen not to process all sections to section images. This slightly complicated the 3-D reconstruction. The distance between succeeding sections was 10 or 80 μm. The latter is a relatively large gap when structures such as axons had to be reconstructed. However, we were not hampered in the interpretation of the result. In future work, a careful consideration on the number of sections will have to be made in advance. This helps to prevent a number of other problems in the processing phase.

Overall, at high resolution mild distortions were found. Minor misalignment can be mostly attributed to the multi-signal setup; that is, the subsequent images had different density content and, therefore, it was difficult to compare them directly. At the same time, high resolution was required to obtain the detail in the annotations. Once the annotations are all found, for a 3-D image one may switch to lower resolution in the visualization phase. This down-sampling is required to be able to display the very large dataset on a regular computer display. The immediate effect is that the apparent misalignments are no longer dominant in the visualization as we now look at a different scale. This result of this effect can be seen in Figure 1.

In conclusion, we have optimized a protocol to infiltrate and polymerize large ($2 \times 2 \times 0.5$ cm) specimens in Technovit 8100 GMA. The atmospheric air

sensitivity of the polymerization process complicated the infiltration and polymerization of large objects. We have shown that this protocol can be used in a very complex experimental setup providing excellent morphology, which can be simplified via a high-resolution 3-D image to a comprehensive 3-D model.

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