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Methyl methacrylate embedding to study the morphology and immunohistochemistry of adult guinea pig and mouse cochleae

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HIGHLIGHTS

- Technovit 9100 New®, a low temperature embedding system was used on the inner ear.
- Preservation of the morphology and maintenance of the antigenicity was tested.
- The embedding system provided highly preserved morphology and immunogenicity.
- The embedding system allowed precise identification of specific cell types in the inner ear.

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ABSTRACT

Background: Histological analysis of the cochlea is required to understand the physiological and pathological processes in the inner ear. In the past, many embedding techniques have been tested in the cochlea to find an optimal protocol that gives both good morphological and immunohistochemical results. Resins provide high quality cochlear morphology with reduced immunogenicity due to the higher polymerization temperature.
New method: We used Technovit 9100 New®, a low temperature embedding system based on methyl methacrylate, on adult guinea pig and mouse cochleae to evaluate preservation of the morphology and maintenance of the antigenicity.
Results: Conventional toluidine blue staining, as well as immunohistochemical staining with a set of commonly used antibodies, showed highly preserved morphology and immunogenicity of decalcified adult guinea pig and mouse cochleae.
Comparison with existing method(s): We demonstrate both, well-preserved morphology and preservation of antigenicity, superior to other embedding techniques.
Conclusions: Our results showed that the Technovit 9100 New® embedding system provided highly preserved morphology and immunogenicity with our protocol in adult guinea pig and mouse cochleae.

1. Introduction

The cochlear anatomy is very delicate, and the fragile epithelium of the cochlear duct consists of cells of different sizes and shapes surrounded by fluid-filled spaces. The cochlea is embedded in the hardest bony capsule in vertebrates. High quality cochlear histological analysis is essential to comprehend cochlear physiology and pathophysiology. Standard light microscopy utilizing classic stains such as toluidine blue or hematoxylin-eosin on paraffin or resin sections is routinely used for morphological studies. These techniques provide homogeneous tissue blocks during embedding by complete infiltration of the media in the cochlear structure. Additional information on the distribution and localization of specific proteins can be gained using immunohistochemistry. The epitopes must be accessible to the antibodies for protein detection. Although many attempts have been made in the past to improve the

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Table 1
Contents of different solutions of the Technovit 9100 New® embedding system.

Name of solution	Base solution (BS)	Polymethylmethacrylate (PMMA) powder	Dibenzoylperoxide (hardener 1)	*N*,*N*,3,5-tetramethylaniline (hardener 2)	1-Decanthiol (regulator)
Pre-infiltration 2	Stabilized; 200 ml		1 g		
Pre-infiltration 3	Destabilized; 200 ml		1 g		
Infiltration	Destabilized; ad 250 ml	20 g	1 g		
Stock solution A	Destabilized; ad 500 ml	80 g	4 g		
Stock solution B	Destabilized; ad 50 ml			4 ml	2 ml

properties of the different media and embedding protocols, high-quality preservation of both the morphology and immunogenicity in the same tissue remains a challenge.

Paraffin embedding is widely used in morphological studies (Gillespie et al., 2003; Scheper et al., 2009), but the high melting temperature of paraffin causes heat-induced trauma that disrupts the morphology (Merchant et al., 2006). Celloidin (O'Malley et al., 2009) provides high-quality morphologically preserved sections. Application of this technique to immunohistology is limited due to the incomplete removal of celloidin from the tissue (Merchant et al., 2006).

For light or electron microscopy, resin embedding serves as an optimal technique (De Groot et al., 1987; Shepherd et al., 1993; Glueckert et al., 2008), providing highly preserved anatomical structures even in semi-thin sections. Unfortunately, there is limited use for immunohistology due to the high temperatures required for tissue polymerization (Ruddell, 1967). Low temperature polymerization embedding resin media, i.e., Lowicryl K4M, has been used on rat cochlea (Hirt et al., 2010). However, the lack of the complete removal of this resin results in limited applications for immunohistochemistry. To enhance the immunogenicity of samples embedded in paraffin or resin, retrieval techniques are widely used. High temperature antigen retrieval in different solutions (Shi et al., 2011; Yamashita and Okada, 2014) indeed leads to improved antigenicity, but it renders the protocol longer and more complicated, and it is disadvantageous for tissue preservation.

Frozen sections are used as a gold standard in immunohistology. Although many studies have attempted to improve tissue preservation (Coleman et al., 2009), the quality of cryosections usually remains inferior to the quality resulting from the above-mentioned embedding techniques, mainly due to freezing artifacts and crystal formation (Whitlon et al., 2001).

Technovit 9100 New® is a resin embedding system that contains methyl methacrylate and catalysators, i.e., *N*, *N*-dimethylaniline and benzoyl peroxide. This combination allows the medium to polymerize at low temperatures, that is, between −8 °C and −20 °C, which results in highly preserved integrity of the anatomy as well as immunoreactivity of bone sections (Yang et al., 2003).

In our study, the applicability of the Technovit 9100 New® embedding system was tested. Adult guinea pig and mouse cochleae were chosen to show the reliability of the method in parallel on species widely used in the field of hearing research. We demonstrate well-preserved morphology with conventional toluidine blue staining and preservation of antigenicity in immunohistologically stained sections using a set of cochlear cell type-specific antibodies without retrieval.

2. Materials and methods

2.1. Animals

Seven adult guinea pig (BFA bunt; 8–34 weeks old) and 6 mouse (NMRI; 8 weeks old) cochleae were used. All animal procedures were approved by the authorities of the Regional Council (Regierungspräsidium) of Tübingen (Reference no. HN 2/11 and 2012.11.06).

2.2. Tissue preparation

For guinea pigs, general anesthesia with a combination of fentanyl citrate (0.025 mg/kg; Fentadon, Eurovet Animal Health), midazolam (0.2 mg/kg; Midazolam-Ratiopharm, Ratiopharm) and medetomidine hydrochloride (Sedator, 1 mg/kg; Eurovet Animal Health) was induced. In the guinea pigs, a 0.5 ml solution of embutramide, mebezonium iodide and tetracaine hydrochloride (T61; Intervet Deutschland GmbH, Unterschleißheim, Germany) was injected intracardially. The chest was opened, and perfusion with 4% phosphate buffered formaldehyde (Roti-Histofix, Roth, Karlsruhe, Germany) was performed through the opened left ventricle with a pressure of 120–140 mm of mercury controlled by a manual manometer. Mice were suffocated with carbon dioxide (CO_2) and decapitated. The skull was quickly opened along the sagittal suture, and the complete bony labyrinth capsule was removed.

In both species, to open the perilymphatic space of the cochlea, the apex was gently opened with a 27 G needle, stapes and the round window membrane were removed with forceps and the cochlea was perfused with cold 4% formaldehyde (Roti-Histofix) through the round window and postfixed for 2 h at 4 °C. The cochleae were then stored in 1% Roti-Histofix at 4 °C overnight. The temporal bones of guinea pigs were then thinned with a diamond burr. Decalcification (Sigma-Aldrich, St. Louis, MO, USA; pH: 7.4) of the mouse and guinea pig cochleae with 0.2 M EDTA was performed for 3 days and 4–6 days, respectively.

2.3. Embedding procedure

Technovit 9100 New® base solution (regarded as stabilized base solution) was destabilized using a chromatography column filled with 50 g of Al_2O_3 (Sigma-Aldrich, St. Louis, MO, USA). Solutions for the phases of pre-infiltration were prepared according to the guidelines of the manufacturer (Sigma-Aldrich). One gram of dibenzoylperoxide (hardener 1) was added to 200 ml of stabilized and destabilized base solution for the pre-infiltration 2 and pre-infiltration 3 solutions, respectively. Infiltration solution and stock solution A for polymerization (Table 1) were prepared in two steps as described by Steiniger et al. (2013). For the infiltration solution, 20 mg of polymethylmethacrylate powder (PMMA powder) was diluted in 160 ml of destabilized base solution. As the powder did not dissolve easily, stirring for 3 h was necessary to obtain a homogenous solution. One gram of hardener 1 was then added, and base solution was added until the total volume reached 250 ml; the solution was then stirred for 1 h. For stock solution A for polymerization, the same procedure was performed with 80 mg PMMA powder in 300 ml of destabilized base solution, and then 4 g of hardener 1 was added. Base solution was added until the total volume reached 500 ml. For polymerization stock solution B, 4 ml of *N*,*N*,3,5-trimethylaniline (hardener 2) and 2 ml of 1-decanthiol (regulator) were diluted in a destabilized base solution until the