TOPICAL REVIEW

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Topical Review

Evaluation methods as quality control in the generation of decellularized peripheral nerve allografts

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Abstract

Nowadays, the high incidence of peripheral nerve injuries and the low success ratio of surgical treatments are driving research to the generation of novel alternatives to repair critical nerve defects. In this sense, tissue engineering has emerged as a possible alternative with special attention to decellularization techniques. Tissue decellularization offers the possibility to obtain a cell-free, natural extracellular matrix (ECM), characterized by an adequate 3D organization and proper molecular composition to repair different tissues or organs, including peripheral nerves. One major problem, however, is that there are no standard quality control methods to evaluate decellularized tissues. Therefore, in this review, a brief description of current strategies for peripheral nerve repair is given, followed by an overview of different decellularization methods used for peripheral nerves. Furthermore, we extensively discuss the available and currently used methods to demonstrate the success of tissue decellularization in terms of the cell removal, preservation of essential ECM molecules and maintenance or modification of biomechanical properties. Finally, orientative guidelines for the evaluation of decellularized peripheral nerve allografts are proposed.

Keywords: neural tissue engineering, decellularization techniques, allograft, histological techniques, biochemical methods, biomechanical evaluation

(Some figures may appear in colour only in the online journal)

Introduction

Basic histology of peripheral nerves

Peripheral nerves (PNs) are delicate organs, which form a complex network throughout the body that connects (through motor and sensory pathways) almost all tissues and organs with the central nervous system. They are composed of both a parenchyma and a stroma, the former being made up of highly specialized conductive units, the PN fibers (PNFs) and the latter consisting of three layers of specialized connective tissue as pictured in figure 1 [1].

The PNFs are responsible for the conduction of afferent and efferent nerve impulses between the central nervous system and peripheral targets [2]. Histologically, they are composed
of neuronal axons surrounded by Schwann cells (SCs) and an external basal lamina [1, 2]. Depending on the mechanism of interaction between SCs and axons, two types of PNFs can be distinguished (figure 1). When an SC surrounds a unique axonal segment, and elaborates a lipid-rich multilayer structure (the myelin sheath), a myelinated PNF is formed. These segments are separated by a myelin-free space, the node of Ranvier. In contrast, a single SC can interact with multiple thinner axons forming the unmyelinated PNFs [1, 3]. Most PNs contain a mixture of myelinated and unmyelinated PNFs. Therefore, in cross-sections of PNs, a heterogeneous group of PNFs with different diameters and myelin thickness can be seen.

The stroma of PNs is composed of a vascularized connective tissue, providing structure, mechanical strength and protection to the parenchyma [2, 4]. In addition, the stroma plays a key role in regulating the compartmentalization of PNs [1–3]. In cross-sections of PNs, it can be clearly observed that PNFs are embedded in a loose connective tissue called the endoneurium, which is further delineated by a specialized tissue, the perineurium, forming the PN fascicles. The number and diameter of these fascicles vary along PNs and they are surrounded by a variable amount of a typical connective tissue known as epineurium [1, 2, 4] (figure 1).

The cellular and extracellular composition of the stroma plays an important role in the normal function and regeneration of PNs. In the epineurium, the most abundant cells are fibroblasts, which produce the extracellular matrix (ECM), but a variable number of mast cells and adipocytes can be found as well [2]. The epineurial ECM is mainly composed of collagen type I, II and III and some elastic fibers [1, 4, 5]. The organization of these fibers allows some degree of torsion and limits the overdistension and tearing of PNs during exercise [4, 6]. The perineurium is a thin layer composed of flattened perineurial cells and ECM [1, 2]. The perineurial cells are organized in a variable number of concentric layers and each of these layers is surrounded by a double basal lamina, which is mainly composed of laminin, collagen type IV and fibronectin. Between the cellular layers, circumferentially, obliquely and longitudinally oriented collagen (types I and II) and elastic fibers can be found [2, 4–6]. Although the perineurium is thin compared to the other stromal layers, the complex composition and 3D organization of the ECM provides most of the resistance and protection to the endoneurial compartment. Indeed, the perineurium is the last structure to avoid irreversible mechanical failure of PNs [4, 7]. Finally, the endoneurium creates an adequate microenvironment for the normal function of PNFs and consists of different cell types and a loose ECM. Nearly 90% of the nuclei in the endoneurium corresponds to SCs, while the other 10% corresponds to fibroblasts (5%), mast cells, endoneurial macrophages and blood vessel-associated cells [2]. Endoneurial tubes are formed by collagen fibers and proteoglycans (PGs) and are composed of two sheaths around the PNFs. The outer sheath consists of thick and longitudinally oriented collagen fibers, while the inner sheath contains thin, circumferentially or obliquely oriented collagen fibers (types I, II, III, IV, V and VII) and PGs [2, 6].

Figure 1. Structural overview of PNs. (A) On the left, a schematic overview shows the three stromal layers of PNs: the endoneurium (En) containing the PNFs, the perineurium (P) and the epineurium (Ep). In the middle, the detail of a myelinated nerve fiber can be seen. The central axon (a) is surrounded by multiple myelin (M) layers produced by the SC and an external basal lamina (BL). On the right, the detail of unmyelinated nerve fibers can be seen. In this case, multiple axons are embedded in the cytoplasm of a single SC and no myelin is present. The basal lamina surrounds this complex as well. (B) Histological overview showing a cross-section of a PN stained with luxol fast blue. Myelin is clearly visible within the endoneurium. Several nerve fascicles can be distinguished, embedded in the epineurium. (C) Histological overview showing a cross-section of a PN stained with haematoxylin-eosin. The endoneurium contains both myelinated and unmyelinated nerve fibers and some blood vessels (BV) can be seen. The perineurium clearly delineates the nerve fascicle. The epineurium is built up of collagen and surrounds the nerve fascicles. Scale bar = 100 μm.
After structural damage of PNs, cells increase their number to promote tissue regeneration, especially cells from the endoneurium. Mast cells and endoneurial macrophages release several cytokines and neurotrophic factors to initiate the breakdown and removal of myelin and to activate the regenerative profile of SCs [8–10]. SCs proliferate, release neurotrophic factors, dedifferentiate and migrate from both nerve stumps, forming longitudinal cords called the bands of Büngner [1, 3, 11]. These cellular bands are surrounded by newly-formed basal lamina and ECM molecules and these structures act as guidance cues for axonal regeneration [1, 3, 12–14]. In addition, laminin and collagen fibers, which are produced during this process, play a key role in the promotion of the proliferation and migration of SCs [15, 16]. Finally, the neurotrophic factors released by SCs and endoneurial macrophages are crucial during this process, since they stimulate neurons to change their functional phenotype from a transmitting state to a regenerative state, which eventually promotes axonal regrowth [1, 3, 13, 15].

Strategies for PN repair

Due to the high distribution of PNs throughout the body, they are vulnerable to different kinds of traumatic injuries with an occurrence of 73% in patients with upper limb injury and with more than 200 000 PN repair procedures being performed annually in the United States [3, 17, 18]. In addition, different kinds of primary or metastatic neoplasms could affect PNs and their surgical resection creates a wide range of structural damage. After transection of PNs (complete or incomplete), the preferred method for repair is end-to-end neurorrhaphy or direct repair, but in the case of severe damage with loss of substance and creation of a critical nerve gap, tensionless repair is not possible. The current gold standard treatment for critical nerve gaps is the nerve autograft. However, this method is only effective in 50% of the cases and several well-known disadvantages, such as donor site morbidity, possible neuroma formation and limited availability of donor nerves have to be considered [3, 11]. Alternatively, nerve allografts can be used, having similar results to autografts, but with one major drawback that patients need immunosuppression for approximately 18 months [3, 11, 19]. To meet the need for an efficient alternative to repair critical nerve gaps, different strategies have been developed by tissue engineering. In this context, autologous alternatives such as the use of skeletal muscle tissue, veins and muscle-vein combinations [20–22], artificial nerve conduits (NCs) [15, 23–25] or decellularized PN allografts (DPNAs) [26–28] have been investigated for their potential use in PN repair. Skeletal muscle tissue has gained interest as a nerve substitute because of the presence of a properly oriented ECM and basal lamina, which can support the regeneration process of PNs. However, there is a risk of undesired dispersion of the regenerating axons [3, 20, 29]. Another option is the use of veins, which were used to avoid the misdirection of regenerating PNFs. Unfortunately, veins tend to collapse, especially in large nerve gaps, with the risk of nerve compression and unsuccessful recovery [3, 21]. More recently, the muscle-vein combined method was demonstrated to be more efficient than the use of skeletal muscle tissue or autologous veins separately [22, 30, 31]. In this method, a vein is filled with a longitudinally oriented segment of skeletal muscle tissue. The vein prevents axonal dispersion, whereas the ECM of the muscle tissue (especially the basal lamina) supports SC proliferation and migration and thus PN regeneration [3, 22, 30, 31]. Despite these advantages, this approach still requires the harvest of autologous tissues, which are not always available, and causes donor site morbidity and prolonged operation times.

Artificial NCs provide another alternative to nerve autografts for bridging of nerve gaps. They can be made from synthetic and/or natural biomaterials (for reviews see Carriel et al [3] and Daly et al [11]) and some of them have already been approved by the FDA [17]. NCs, which are available in different dimensions, have some advantages compared to other strategies. They limit or reduce stromal infiltration as well as misdirection of the regenerating tissue [3, 11, 13, 30]. This is especially important because it reduces scar and neuroma formation and episodes of neuropathic pain [11]. In addition, some NCs are porous, allowing some degree of exchange with the surrounding tissue (e.g. O₂ or CO₂), which is essential for cell function and survival. From a biomechanical point of view, most NCs have a slow or controlled degradation rate, especially synthetic-based NCs, rendering them mechanically stable during the complex process of PN regeneration [3, 11, 13, 30]. Initial studies with hollow NCs did not lead to satisfactory results (due to a lack of supportive intraluminal structures and the dilution of growth factors) and encouraged researchers to improve the efficacy through the development of more complex and functional structures. In this sense, several topographical cues were incorporated into NCs by means of filling them with acellular or cellular hydrogels [7, 15, 32–34] or nanofibers [35–37] or by introducing relevant ECM molecules such as laminin and/or collagen [37–39]. All these engineered strategies demonstrated significant improvements of PN regeneration and functional recovery compared to hollow NCs. However, at the moment, these strategies are limited to experimental approaches or are under preclinical research, whereas hollow NCs remain mainly restricted to the repair of sensory PNs affected by non-critical nerve gaps (<3 cm) [3, 11, 17, 40]. Nonetheless, NCs are excellent models for the evaluation of the cellular and molecular processes taking place during PN regeneration [23, 41, 42].

Another promising alternative is DPNAs (for extensive reviews see Johnson et al [43] and Isaacs et al [44]). Like NCs, DPNAs avoid the donor site morbidity associated with nerve autografts and the immunosuppressive treatment required for nerve allograft transplantation. In addition, DPNAs offer the advantage that they can be harvested from specific PNs, either motor, sensory or mixed PNs. Therefore, these DPNAs can provide a comparable 3D organization, number of fascicles or even dimensions for the PN that needs to be repaired. Moreover, previous studies showed superior results with DPNAs compared to silicone conduits [28] and the collagen-based NeuraGen® conduit [45]. It is thought that DPNAs are especially beneficial in longer defects, where NCs fail to support axonal regeneration. The key advantage is that DPNAs
provide a natural ECM to the regenerative microenvironment. This ECM is characterized by a correct orientation, distribution and heterogeneous molecular composition, where molecules such as laminin, collagens and fibronectin are essential to support and guide the cells involved in the complex process of PN regeneration [3, 46]. Nevertheless, DPNAs show variable experimental and even clinical results [28, 47]. This variable success could be related to an incomplete decellularization process of the tissues, an inadequate preservation of the 3D ECM structure, loss of essential ECM molecules to promote cell migration and proliferation and insufficient biomechanical properties to protect the regenerative microenvironment or bridge both nerve stumps. In this context, this review aims to discuss the different quality control methods currently available and used to evaluate the successfulness of an efficient decellularization of PNs.

Decellularization of allografts

Basic concepts of tissue decellularization

Tissue decellularization has gained interest in the past two decades to create natural scaffolds that can be used as alternatives for autografts and synthetic replacements. By using allo- or xenografts as a starting material, it is possible to have an off-the-shelf available scaffold with tissue-specific characteristics. The efficiency of the decellularization protocol will play an important role in the modulation of the host response. Since ECM molecules are highly conserved among species, immune reaction will mainly depend on the presence of cellular material [48]. Insufficient removal of cellular material can elicit an inflammatory response and can negatively influence or even terminate the regeneration process, possibly with graft rejection as a result [48–50]. Nevertheless, it is impossible to remove all cellular components without damaging the ultrastructure and ECM composition of a tissue to some degree. Therefore, criteria have been set by Crapo et al [51] to quantitatively determine limits for acceptable tissue decellularization. They propose the following guidelines focused on the removal of nuclear material: (i) <50 ng double-stranded DNA (dsDNA) per mg ECM weight; (ii) <200 bp DNA fragment length and (iii) lack of visible nuclear material in tissue sections stained with 4′,6′-diamidino-2-phenylindole (DAPI) or haematoxylin-eosin (HE). However, there are no guidelines related to the preservation of the ECM and/or the biomechanical properties of decellularized tissues.

To fulfill the above-mentioned criteria, several methods of tissue decellularization can be applied (for reviews see Crapo et al [51] and Gilbert [52]). In this regard, physical, chemical or biological agents can be used, as a single treatment or in combination. There are also different methods to apply the decellularizing agents such as perfusion or immersion. The choice of the decellularization method will depend greatly on the nature and molecular composition of the tissue. Several decellularization protocols have already been developed for organs such as the small intestine [53, 54], cornea [55], heart valves [56] and liver [57].

Decellularization of PN allografts

In recent decades, several methods have been developed to decellularize PNs to obtain DPNAs, as summarized in Table 1. However, the first attempts with physical methods were more focused on the reduction of the immunogenicity of PN allografts through degeneration, inactivation or rupture of the cells, rather than actually to decellularize them. PNs can be

<table>
<thead>
<tr>
<th>Processing technique</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction of immunogenicity</td>
<td>Induction of Wallerian degeneration</td>
<td>[46, 58–61]</td>
</tr>
<tr>
<td>– Predegeneration</td>
<td>Reduction of immunogenicity by progressive breakdown of cells</td>
<td>[19, 63–65]</td>
</tr>
<tr>
<td>– Cold preservation</td>
<td>Disruption of cell membrane due to the formation of intracellular ice crystals</td>
<td>[46, 60, 61, 63, 66, 67, 76, 80, 81]</td>
</tr>
<tr>
<td>– Freeze-thaw cycles</td>
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</tbody>
</table>

Chemical decellularization

Non-ionic detergents
- Triton X-100
  Disruption of lipid–lipid and lipid–protein interactions | [26, 49, 68–74] |

Ionic detergents
- Triton X-200
- Sodium deoxycholate
- Sodium dodecyl sulfate
  Solubilization of cell membranes and disruption of protein-protein interactions | [26, 68, 69, 74] |

Zwitterionic detergents
- Sulfobetaine-10 and -16
  Display properties of both ionic and non-ionic detergents | [27, 77] |

Hyertonic and hypotonic solutions
Lysis of cells by osmotic shock | [26, 27, 76, 78] |

Biological agents
- Nucleases
  Hydrolysis of phosphodiester bonds of nucleic acid sequences | [49, 69, 71, 76] |

- Chondroitinase ABC
  Removal of chondroitin sulfate GAGs | [66, 80, 81] |

- Protease inhibitors
  Inhibition of endogenous proteolytic enzymes | [71, 72, 74, 76] |
The thawing of PNs have also been extensively studied \[46, 60, 61, 63, 66, 67\]. Cells are irreversibly damaged with these methods, but they are still not removed from the ECM of the tissues and organs. The remaining cell debris will cause the activation of SCs, macrophages and other inflammatory cells once the graft is implanted, leading to degeneration of the allograft and subsequently a delay of tissue regeneration or even allograft rejection \[47\].

More recently, interest has shifted towards chemical decellularization methods, which result in more efficient removal of cellular material (figure 2). Depending on the type of detergent, the impact on the ECM of the tissue can be different. The non-ionic detergent Triton X-100 is one of the detergents that has been used for decellularization of PN allografts \[26, 49, 68–74\]. Triton X-100 was described to cause minimal damage to the tissue structure and it acts by mainly disrupting lipid–lipid and lipid–protein interactions rather than protein–protein interactions \[75\]. In combination with sodium deoxycholate (SDC), it was shown that the cellular content of rat PN allografts was efficiently removed and regeneration of axons was demonstrated after transplantation, but functional recovery was not reported \[26\]. Another research group combined Triton-X100 with SDC and sodium dodecylsulfate (SDS), resulting in functional reinnervation of rat hind limb muscles across 2 cm nerve gaps, but not 4 cm nerve gaps \[74\].

SDS and other ionic detergents are excellent in removing both cytoplasmic and nuclear material, but have the tendency to disturb the native architecture of the tissue by disrupting protein–protein interactions \[75\]. Indeed, a recent study on porcine tibial and pereoneal nerves showed a decrease of glycosaminoglycan (GAG) content after decellularization with SDS, without the use of any enzyme that is known to reduce GAG content such as Chondroitinase ABC (ChABC) \[76\]. Furthermore, Wakimura et al \[70\] investigated the regeneration potential of rat allografts decellularized with a combination of SDS and Triton X-100. After 24 weeks, no significant differences in histology or electrophysiology were observed between the decellularized allografts and the autografts. These results should be interpreted with care, however, since the high regeneration capability of rat PNs \(3 \text{mm d}^{-1}\) might not allow us to distinguish significant differences between experimental groups after 24 weeks \[47\].

Zwitterionic detergents have properties of both non-ionic and ionic detergents. They are less efficient in the removal of cellular content than ionic detergents, but on the other hand they better preserve the ultrastructure of tissues. This was clearly demonstrated in the study of Hudson et al \[27\], where 12 different detergents were compared. Eventually, they found that the combination of the ionic detergent Triton X-200 with the zwitterionic detergents sulfobetaine-10 and -16 led to superior results in terms of cell removal and maintenance of nerve architecture. When implanted in a 1 cm nerve gap, their optimized DPNA performed as well as the isograft after \(84 \text{d}\) of regeneration and outperformed the allografts decellularized by the protocol of Sondell et al \[26\] and by freeze-thawing \[77\]. However, it is important to mention that Triton X-200 was discontinued, making it unavailable for further investigation in this field.

Many decellularization protocols include hypo- and hypertonic solutions to cause an osmotic shock to the cells and facilitate removal of cellular material from tissues by dissociating DNA from proteins \[51\]. Hypo- and hypertonic solutions alone are generally not sufficient to obtain completely cell-free scaffolds. Interestingly, Ishida et al \[78\] claim to have found a simple decellularization protocol by just submerging PN allografts in 1 M sodium chloride solution for 24 h followed by 7 d in phosphate-buffered saline. However, their conclusions of having created a cell-free PN allograft are solely based on HE staining and were not confirmed by more accurate and specific methods such as DAPI staining, DNA quantification or other methods for assessing cell removal.

Finally, biological agents form a third class of decellularization agents. Nucleases such as DNase and RNase can

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**Figure 2.** Impact of chemical decellularization on rat PNs. After decellularization with Triton X-100 and SDC, no more visible nuclei can be seen in both cross-sections (left) and longitudinal sections (right) of PNs stained with haematoxylin-eosin. The epineurium and perineurium are well-preserved, while the endoneurium is slightly affected by the decellularization and appears disorganized. Scale bar = 100 µm.
fragment nucleic acid sequences by hydrolyzing the phosphodiester bonds [51, 79]. In this way, DNA and RNA are specifically degraded and solubilized, favoring the removal of these molecules from tissues. Several researchers reported good outcomes with PN allografts decellularized with Triton X-100 [49, 69, 71] or SDS [76] in combination with nucleases.

During decellularization, different proteases can be released from ruptured cells, causing damage to the surrounding tissue structure. To minimize this damage, protease inhibitors such as Aprotinin and Leupeptin are sometimes added to the decellularization protocol [71, 76]. Another protease inhibitor, the ethylenediaminetetraacetic acid (EDTA), can also be used to interfere with cell attachment due to its chelating properties [72, 74]. Other enzymes can be used to remove specific target molecules in tissues and ChABC is an enzyme of particular interest for PN regeneration. It can remove chondroitin sulfate GAGs, which are normally upregulated after nerve injury. One subtype, chondroitin 6-sulphate, forms a molecular complex with laminin, inhibiting the axon-guiding function of the basal lamina and consequently compromising nerve regeneration [62]. Interestingly, ChABC-treated PN allografts enhanced PN regeneration in rats compared to freeze-thawed nerve allografts [80, 81], even over a 4 cm nerve gap [66]. Moreover, ChABC treatment is also an important step in the decellularization protocol of the commercially available Avance® nerve graft. This is the only commercially available DPNA and the first clinical studies show promising results [82–84]. Nevertheless, despite years of research, regeneration through decellularized PN allografts over long distances remains challenging and it is not clear yet which decellularization method yields the best balance between removal of cells, preservation of the 3D nerve structure and maintenance of adequate biomechanical properties. Unfortunately, standard evaluation methods are lacking, making it difficult to thoroughly compare the different decellularization methods available. Many DPNAs are immediately tested in animals, without assessing the effectiveness or the impact of the decellularizing agents on the histological structure, molecular composition and biomechanical properties of the DPNAs first. Therefore, different in vitro evaluation methods will be discussed in the next section and some guidelines for the evaluation of DPNAs will be suggested.

**Evaluation methods for decellularized PN allografts**

After decellularization of any tissue or organ, it is necessary to confirm that a cell-free scaffold has been obtained with maintenance of the native 3D tissue architecture, without severely affecting the composition of the ECM and with comparable biomechanical properties as the native tissue. Therefore, DPNAs should be subjected to both qualitative and quantitative evaluation methods, including histological, ultrastructural, biochemical and biomechanical analysis.

### Table 2. Overview of histological techniques for evaluation of cell content in decellularized PN allografts.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nuclear stainings</strong></td>
<td></td>
</tr>
<tr>
<td>Haematoxylin-eosin</td>
<td>Detection of cell nuclei and general morphology</td>
</tr>
<tr>
<td>Feulgen method</td>
<td>DNA (red)</td>
</tr>
<tr>
<td>Methyl green-pyronin</td>
<td>DNA (blue-green), RNA (pink-red)</td>
</tr>
<tr>
<td>DAPI or Hoechst</td>
<td>Nuclear material (blue fluorescence)</td>
</tr>
<tr>
<td><strong>Myelin stainings</strong></td>
<td></td>
</tr>
<tr>
<td>Luxol fast blue</td>
<td>Myelin (blue)</td>
</tr>
<tr>
<td>MCOLL method</td>
<td>Myelin (blue), collagen (red)</td>
</tr>
<tr>
<td>Osmium tetroxide</td>
<td>Myelin (black)</td>
</tr>
<tr>
<td>Oil red O</td>
<td>Myelin (red) in frozen sections</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>Myelin (black) in frozen sections</td>
</tr>
<tr>
<td>Toluidine Blue</td>
<td>Myelin (blue) in semithin sections</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td></td>
</tr>
<tr>
<td>P0: protein 0</td>
<td>PMP22: peripheral myelin protein</td>
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<tr>
<td></td>
<td>MBP: myelin basic protein</td>
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<tr>
<td><strong>Axonal stainings</strong></td>
<td></td>
</tr>
<tr>
<td>Bodian’s silver method</td>
<td>Neurofilament polypeptides in nerve fibers</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Neurofilament: light, medium and heavy chains of the neuronal and axonal cytoskeleton</td>
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<tr>
<td></td>
<td>β-III tubulin: cytoskeleton of axons</td>
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<tr>
<td><strong>Specific cell stainings</strong></td>
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<tr>
<td>Toluidine Blue</td>
<td>Mast cell granules (metachromatic reaction)</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>S-100: SCs</td>
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<tr>
<td></td>
<td>Vimentin: fibroblasts, macrophages and SCs</td>
</tr>
<tr>
<td></td>
<td>CD34, CD117, CD203c: mast cells</td>
</tr>
<tr>
<td></td>
<td>CD68, CD163: endoneurial macrophages</td>
</tr>
</tbody>
</table>

**Histology**

Histology is considered one of the most reliable quality control methods in PN tissue engineering [1, 85] and in tissue decellularization [79]. Histological analysis can be performed on either frozen sections or formalin-fixed and paraffin-embedded tissues, the latter being most commonly used. Depending on the histological technique, it is possible to determine the degree of cell removal, the tissue microarchitecture and/or the composition of the ECM after tissue decellularization. An overview of possible histological techniques for the evaluation of DPNAs is given in tables 2 and 3.

Routine staining with HE can be used to determine the presence of cells and to give a general overview of the tissue architecture. However, HE staining is not an accurate and specific method to demonstrate complete decellularization of tissues or to evaluate the ECM structure and composition [86]. Currently, most authors are evaluating decellularization by the identification of nuclei or their remnants by specific staining of DNA. In this context, fluorescent dyes such as DAPI or
Table 3. Overview of histological techniques for evaluation of structural components in decellularized PN allografts.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibrillar ECM components</strong></td>
<td></td>
</tr>
<tr>
<td>Picrosirius</td>
<td>Collagen (red)</td>
</tr>
<tr>
<td></td>
<td>Polarized microscopy (red, orange and green)</td>
</tr>
<tr>
<td>Masson trichrome</td>
<td>Collagen (blue or green), muscle fibers (red), nuclei (dark brown-black), cytoplasm (pink)</td>
</tr>
<tr>
<td>MCOLL</td>
<td>Myelin (blue), collagen (red)</td>
</tr>
<tr>
<td></td>
<td>Polarized microscopy (red, orange and green)</td>
</tr>
<tr>
<td>Gömöri silver staining</td>
<td>Reticular fibers (black precipitate)</td>
</tr>
<tr>
<td>Periodic acid-Schiff</td>
<td>Reticular fibers (pink)</td>
</tr>
<tr>
<td>Orcein</td>
<td>Elastic fibers (brown)</td>
</tr>
<tr>
<td>Verhoeff’s</td>
<td>Elastic fibers (black), collagen fibers (red), cytoplasm (yellow)</td>
</tr>
<tr>
<td>Aldehyde-fuchsinne</td>
<td>Elastic fibers (violet)</td>
</tr>
<tr>
<td><strong>Immunohistochemistry</strong></td>
<td>Collagen types I, II, III and V: connective tissue layers</td>
</tr>
<tr>
<td></td>
<td>Collagen type IV: basal lamina of nerve fibers, perineurium and blood vessels</td>
</tr>
<tr>
<td><strong>Non-fibrillar ECM components</strong></td>
<td></td>
</tr>
<tr>
<td>Alcian Blue</td>
<td>Acid proteoglycans (blue)</td>
</tr>
<tr>
<td>Toluidine Blue</td>
<td>Sulfated proteoglycans (metachromatic reaction)</td>
</tr>
<tr>
<td><strong>Immunohistochemistry</strong></td>
<td>Chondroitin 6-sulfate: endoneurium</td>
</tr>
<tr>
<td></td>
<td>Chondroitin 4-sulfate: epineurium</td>
</tr>
<tr>
<td></td>
<td>Lammin: basal lamina of nerve fibers, perineurium and blood vessels</td>
</tr>
<tr>
<td></td>
<td>Fibronectin: basal lamina and connective tissue</td>
</tr>
</tbody>
</table>

Hoechst, which strongly bind to A-T rich regions of DNA, can be used in frozen and paraffin-embedded material [87]. DAPI staining is frequently used as contrast in immunofluorescence techniques [23, 85], as quality control in tissue decellularization [53, 55, 56] and to determine cell content in tissue engineering [7]. However, despite the higher specificity compared to HE staining, DAPI or Hoechst staining are almost never used by researchers to confirm the absence of cellular material in DPNAs. In addition, there are also some classic histochemical methods for specific staining of DNA and RNA in light microscopy, such as the Feulgen and methyl green-pyronin methods [87], but they have not yet been used as quality control methods in tissue engineering.

It is important to consider that a negative reaction for DAPI staining does not automatically mean that the tissue has been completely decellularized, because cytoplasmic remnants from different kinds of cells could still be present in the ECM of decellularized tissues. Curiously, to the best of our knowledge, there is only one article in which cytoplasmic remnants were analyzed in DPNAs [27]. The most abundant cells in PNs are the SCs (representing nearly 90% of cells in histological cross-sections), which can be immunohistochemically identified by various antibodies. In this sense, SCs express molecules such as vimentin, CD56, CD57, CD146, NCAM, GFAP, O4 and p75, but are most commonly identified by S-100) [2, 88–91]. Fibroblasts are present in the three PNs' stromal layers, representing nearly 5% of endoneurial cells in histological cross-sections [2]. The fibroblast cytoskeleton is mainly composed of vimentin, which is a useful marker for their immunohistochemical identification, but vimentin is also an important intermediate filament of SCs and other mesenchymal-derived cells [2]. Mast cells are normal components of the epi- and endoneurium of PNs and they can be identified by several immunohistochemical markers (CD34, CD117, CD203c) or more easily through histochemical staining of their granules with Toluidine Blue [87]. Endoneurial macrophages are also present, albeit in a small percentage, and are positive for vimentin, CD68 and CD163 [2].

As mentioned before, SCs elaborate complex myelin sheaths around individual axons, forming the myelinated PNFs. Myelin sheath composition differs between central and peripheral nervous systems, but in both cases are composed of approximately 70%–75% of a highly heterogeneous group of lipids, while the other 25% is represented by proteins, proteolipids and lipoproteins [92–94]. Some of these components are resistant to organic solvents, allowing identification with histochemical methods such as luxol fast blue (LFB), the MCOLL method (which contains LFB) or osmium tetroxide [41, 85, 94–97]. In addition, myelin can be easily stained by using Oil red O and Sudan black B histochemical methods in cryosections [95, 98]. Another accurate alternative is the use of resin-embedded semithin sections stained with Toluidine Blue [85, 99]. This method allows the evaluation of most myelinated axons, which are sharply delimited due to post-fixation and staining with OsO4. Furthermore, the high morphological quality obtained with this method makes it suitable to perform different kinds of morphometric analyses. For this reason, it is considered a gold standard technique in PN histology [85, 99].

Curiously, despite the advantages offered by this technique, it has only been used once before as a quality control for DPNAs [63]. Finally, the 25% of proteins present in myelin, such as P0 (myelin protein 0), PMP22 (peripheral myelin protein 22) and MBP (myelin basic protein) can be used for specific immunohistochemical identification of the myelin sheath [2, 41, 93]. The characteristic organic solvent-resistance of myelin is an advantage for histochemistry, but a disadvantage for the decellularization of PNs. It is a challenge to completely remove the myelin and SC cytoplasm from PNs while preserving the basal lamina, which is essential for regeneration. In addition, remaining myelin is described to have an inhibitory effect on axonal regeneration [14]. Therefore, complete removal of myelin and SCs should be controlled when evaluating DPNAs.

An often-overlooked component when evaluating DPNAs are the axons. This small functional unit should be completely removed during decellularization to avoid immune reaction after transplantation and to make the basal lamina accessible for axonal sprouting. The cytoskeleton of axons is mainly composed of neurotubules and neurofilaments, which can be easily detected with immunohistochemistry of β-III tubulin and neurofilament, respectively [2, 85]. In addition, some classic
metal reduction techniques such as Bodian’s silver method could be used [100].

Once all the cellular material is removed, it is important to evaluate the structural integrity of DPNAs. The ECM of PNs is mainly composed of fibers (collagen, reticular and elastic) and non-fibrillar components (PGs and glycoproteins). These molecules are present in different proportions throughout the stromal layers of PNs, being important cues from a regenerative and biomechanical point of view [1, 2, 4]. The fibril-forming collagens type I and III build up the epineurium, while the network-forming collagen type IV is localized in the basal lamina of blood vessels and PN fibers [16, 101]. Collagen type V is described as a ‘minor’ fibril forming collagen, but is abundant in PNs and might play a role in myelination [16, 102]. In addition, reticular fibers are mainly composed of collagen type III, but also contain collagen type V and several glycoproteins [103]. These fibers are associated with the basal lamina and are commonly identified by using metal reduction techniques such as the Gömöri silver staining or the Periodic Acid-Schiff (PAS) histochemical methods [2, 53, 87]. Furthermore, in order to have an overview of the fibrillar organization and distribution in decellularized tissues, the fibril-forming collagens can easily be demonstrated by trichrome staining, Picrosirius staining or by the integrated histochemical MCOLL method (that contains LFB, Picosirius and Haematoxylin), which simultaneously stains myelin and fibrillar collagens, and also provides an adequate contrast to evaluate the morphology [41, 87, 94]. Moreover, Sirius Red-based stainings increase the natural birefringence of collagen fibers, allowing an accurate evaluation of the 3D organization of these fibers in both native and decellularized tissues by using polarized light microscopy [53, 55, 56, 86, 94]. Finally, specific types of collagens can also be identified by immunohistochemistry (table 3).

Elastic fibers can be found in the perineurium of PNs. They are responsible for the visco-elastic properties of PNs and consist of about 90% of the protein elastin and 10% of glycoproteins. Elastic fibers can be histochemically detected by methods such as Orcein, aldehyde-fuchsin or Verhoeff’s staining (which also stains collagen) [87]. High preservation of these fibers has already been demonstrated in, for example, cardiac tissue after chemical decellularization [56]. However, elastic fibers are in general poorly evaluated in PNs. Therefore, the impact of decellularization on the preservation, structure and 3D organization of these fibers remains unknown in DPNAs.

The biggest group of non-fibrillar ECM components is represented by the PGs. They are composed of a core protein with several GAGs attached, which have either carboxylic or sulfated side chains. Sulfated PGs are generally identified with cationic dyes such as Alcian Blue and Toluidine Blue [87]. However, distinct subtypes of PGs can be targeted with antibodies, giving more specific information about their distribution and preservation after decellularization. In particular, chondroitin sulfate-rich PGs are interesting to evaluate in DPNAs. As mentioned earlier, it can be beneficial to remove chondroitin 6-sulfate during the decellularization process to overcome its negative influence on PN regeneration.

Chondroitin 4-sulfate-rich PGs, on the other hand, are localized in the epineurium and play a role in the inhibition of aberrant axonal growth [62].

Glycoproteins are represented by a heterogeneous group of ECM molecules (mainly composed of globular proteins associated with oligosaccharides), which play crucial roles in cell adhesion, migration and proliferation [104]. In PNs, the most important glycoprotein from a structural, functional and regenerative point of view is laminin. This essential component of the basal lamina of PNFs promotes the proliferation and migration of SCs during regeneration and guides newly-formed axons [16, 105]. In this sense, preservation of laminin is an important issue in the generation of DPNAs. Fibronectin is another glycoprotein present in the basal lamina and in the connective tissue. This molecule plays a similar role to laminin, supporting different cell functions during regeneration [106]. Although there are histochemical methods for the staining of glycoproteins (such as PAS), most of them are unspecific and, therefore, immunohistochemistry is the best option to specifically identify different types of laminin and fibronectin [87, 106].

**Ultrastructure**

In addition to conventional light microscopy, electron microscopy can be used to evaluate the ultrastructure of DPNAs. Valuable information about the structural integrity and general morphology of DPNAs can be obtained with scanning electron microscopy (SEM). SEM analysis of DPNAs reveals the characteristic porous pattern of the scaffold generated. This method will allow us to clearly identify all the stromal layers. In addition, the successful removal of cells and especially the PNFs (axons, SCs and myelin) will leave small cavities. The endoneurium will be characterized by the presence of well-defined or partially preserved endoneurial tubes, which are essential cues for PN regeneration. Furthermore, interconnected and randomly oriented vascular structures can also be evident (figure 3) [69, 70, 78].

Ultrathin sections of resin-embedded tissue can also be used for the assessment of the ultrastructure of DPNAs with transmission electron microscopy. The main advantage of this technique is that, due to the post-fixation with OsO₄, the myelin sheath and the remnants (after decellularization) are sharply delineated, which is not visible with routine histological techniques [2]. Furthermore, the preservation of the basal lamina and the organization of the collagen fibrils can be evaluated in cross-sections [63].

Another interesting but less frequently used technique for visualization and 3D reconstruction of the microstructural pattern of tissues is microcomputed tomography (microCT). Usually, microCT is only used for the study of hard tissues such as bone, since these are dense enough to scatter the x-rays and thus create grayscale images [107]. However, with an appropriate staining agent, it is possible to visualize the structural pattern of soft tissues as well. Iodine-based staining agents are most commonly used for this purpose compared to osmium or phosphotungstic acid [108, 109]. One of the advantages of microCT is that it allows the evaluation of...
While histology is only a semi-quantitative technique, ECM components, myelin and DNA content can be quantified through a variety of biochemical assays. A first and simple method is to separate the proteins extracted from native and decellularized nerve tissue with gel electrophoresis and to compare the resulting bands with a reference ladder, which contains a mixture of proteins with a predefined molecular weight. The most commonly used type of electrophoresis is SDS-PAGE and it has already been used to quantify collagen and myelin-associated proteins in DPNAs [26, 27]. The bands of collagen type I alpha chains are typically found around 130–140kDa, but dimeric (270kDa) and trimeric (400kDa) forms can be found as well. The major glycoprotein of myelin in PNs, protein P0, has a molecular weight of 30kDa [115]. To visualize all proteins in the gel after separation, staining with Coomassie Brilliant Blue R-250 can be performed [116]. In this way, quantitative differences for a certain protein between native and decellularized tissues can be detected. Furthermore, gel electrophoresis can also be used to determine the length of any remaining DNA fragments after decellularization. As mentioned before, DNA fragments should be shorter than 200bp to avoid an undesired immunological response in vivo [51].

Since gel electrophoresis of proteins lacks specificity, it is recommended to also perform Western blotting, which allows identification of specific proteins through the use of specific antibodies. In this regard, it is necessary to transfer the separated proteins first from the gel to a nitrocellulose or polyvinylidene difluoride membrane [117–119]. After a blocking step, any protein of interest for which a commercially available antibody exists, can be labeled. Finally, depending on the type of secondary antibody, the labeled proteins can be detected by various visualization methods. Most commonly, colorimetric detection is used, whereby enzymes such as alkaline phosphatase or horseradish peroxidase convert a substrate to a colored precipitate. Alternatively, chemiluminescence or fluorescence detection can be used [117–119]. In the case of DPNAs, Western blotting has already been used to evaluate the preservation of essential ECM molecules. In a study of Kvist et al., the presence of laminin and MBP in PNs decellularized with the methods described by Hudson and Sondell was evaluated [120]. The main advantage offered by Western blotting is that it can be correlated to histological analysis and can be an accurate semi-quantitative complement to structural analyses. However, Western blotting cannot be considered as the main or unique quality control method for the assessment of tissue decellularization. Despite the high specificity and valuable, semi-quantitative results obtained with this method, the results only demonstrate the presence or absence of certain proteins after tissue decellularization.

In recent years, several colorimetric and fluorescence-based assays have been developed for more accurate...
measurement of ECM molecules such as collagen or GAGs and DNA in tissues. Colorimetric assays are based on the formation of a colorimetric product of which the optical density can be measured through spectrophotometry. In relation to collagens, hydroxyproline is one of the most characteristic amino acids of these ECM molecules and serves, therefore, as an indicator of collagen content in tissues. Hydroxyproline can be set free by alkaline hydrolysis of the tissue and can subsequently be oxidized by Chloramine-T. After the addition of p-dimethylaminobenzaldehyde, a chromophore is formed that can be measured at a wavelength of 550 nm [121]. In the case of sulfated GAGs (sGAGs), they form part of different kinds of PGs, they can be easily detected in tissues that have first been digested with papaain. After complexation with 1,9-dimethylmethylene blue, the metachromatic shift in the absorbance maximum of the dye can be measured [122]. On the other hand, assays for DNA quantification make use of fluorescent dyes. The intercalation of a dye such as PicoGreen with dsDNA results in a fluorescent signal proportional to the amount of dsDNA present in the tissue [123]. Comparison of the obtained values with a standard curve makes it possible to determine the exact amount of dsDNA in tissue samples and thus, to confirm if the decellularized tissue meets the criterion of Crapo et al of less than 50 ng dsDNA per mg ECM dry weight [51]. Alternatively, purified DNA can also be easily quantified by spectrophotometry with devices such as NanoDrop. However, after decellularization, the DNA content is often too low for accurate measurement.

Nowadays, many researchers have already employed biochemical assays (such as the Hydroxyproline Assay Kit from Sigma-Aldrich, the Total Collagen Assay Kit from QuickZyme Biosciences or the Blyscan sGAG Assay from Biocolor) to evaluate decellularized tissues [56, 124–127]. When using such assays, it is important to normalize the resulting values in a proper manner. To avoid possible differences in water content between samples, it is recommended to normalize these values with respect to the tissue dry weight. Despite the availability of these methods, however, only a few researchers used biochemical assays to complement histological findings and to quantitatively demonstrate the DNA and ECM content in DPNAs. In a study by Sridharan et al, a decrease of more than 50% of hydroxyproline content was found after decellularization with Triton X-100, SDC and nucleases, while Picrosirius staining was histochemically confirmed with Picrosirius staining. In relation to the sGAGs, they found a decrease of 33% compared to native control nerves, despite the comparable intensity of Alcian Blue staining. These results indicate that care should be taken when drawing conclusions about the impact of a decellularization protocol on the cell content and ECM based on histology alone. Finally, biochemical assays can give valuable additional information to complement overall histological findings and specifically provide quantitative parameters related to the cell content and/or the preservation of ECM molecules.

**Biomechanical evaluation**

Biomechanical properties are a key factor in many tissue-engineering applications, including PN repair. The stroma of PNs not only provides structural organization and protection to the parenchyma but supports the elongation, sliding and compression of PNs during movement and exercise, especially for PNs located near the joints [4]. It has already been demonstrated several times that a decellularization process can have an impact on the structure and ECM composition of decellularized organs [53–56]. In this context, it is important to characterize DPNAs from a biomechanical point of view, in order to determine the impact of the decellularization process on the overall biomechanical properties of these substitutes. Therefore, these analyses will demonstrate if DPNAs have comparable biomechanical properties to native nerves.

Nowadays, it is possible to determine the overall biomechanical properties of any tissue or engineered construct through the application of different forces under specific conditions. In this context, samples can be subjected to rheological, compression and tensile tests [128, 129].

Rheology offers the possibility to characterize samples under shear stress, which can be conducted by using stationary and/or oscillatory regimens. Stationary rheological characterization is used to determine the rigidity modulus ($G_r$), whereas the rheological analysis carried out under dynamic state will provide the elastic and viscous modules ($G’$ and $G’’$, respectively) [130, 131]. Rheology provides a complete characterization of the biomechanical properties of different kinds of structures, but it is important to take into account the following technical parameters: (i) all analyses should be performed under controlled-stress modulus; (ii) samples should have a discoid shape with sufficient diameter to favor the contact between the samples and the plates of the rheometer (between 2–3 cm), since with a higher contact surface more reliable results are obtained; (iii) the thickness of the samples should be uniform and not too thick, in order to avoid undesired or irregular results; (iv) the temperature and humidity should be constant for all samples during analysis [129, 130]. Rheology has already been successfully used in tissue engineering, especially for the characterization of bio-artificial tissues and hydrogel-based scaffolds [128–130, 132–134]. However, from a methodological point of view, it would be difficult to perform a rheological analysis on native and decellularized PNs, due to their limited diameter. Moreover, anatomical variations between different individuals and different nerves would raise additional challenges.
Table 4. Selected overview of tensile testing of native PNs.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Species</th>
<th>Nerve type</th>
<th>Strain rate</th>
<th>Young’s modulus</th>
<th>Ultimate stress</th>
<th>Ultimate strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>[49]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>N/A</td>
<td>10.36 ± 1.12 MPa</td>
<td>4.78 ± 0.71 MPa</td>
<td>0.47 ± 0.10 mm mm⁻¹</td>
</tr>
<tr>
<td>[69]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>2% strain s⁻¹</td>
<td>21.23 ± 5.36 MPa</td>
<td>±5 MPa</td>
<td>42.4 ± 4.1 %</td>
</tr>
<tr>
<td>[139]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>1 mm min⁻¹</td>
<td>19.33 ± 2.00 MPa</td>
<td>7.14 ± 1.50 MPa</td>
<td>0.49 ± 0.09 mm mm⁻¹</td>
</tr>
<tr>
<td>[140]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>1% strain s⁻¹</td>
<td>580 ± 150 kPa</td>
<td>2720 ± 970 kPa</td>
<td>0.81 ± 0.11 mm mm⁻¹</td>
</tr>
<tr>
<td>[141]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>1% strain s⁻¹</td>
<td>4964 ± 1014 kPa</td>
<td>2834 ± 297 kPa</td>
<td>0.76 ± 0.11 mm mm⁻¹</td>
</tr>
<tr>
<td>[76]</td>
<td>Porcine</td>
<td>Tibial nerve</td>
<td>0.1 mm s⁻¹</td>
<td>7.43 ± 0.53 MPa</td>
<td>0.87 ± 0.09 MPa</td>
<td>0.16 ± 0.01 mm mm⁻¹</td>
</tr>
<tr>
<td>[76]</td>
<td>Porcine</td>
<td>Peroneal nerve</td>
<td>0.1 mm s⁻¹</td>
<td>7.75 ± 0.56 MPa</td>
<td>1.23 ± 0.06 MPa</td>
<td>0.23 ± 0.04 mm mm⁻¹</td>
</tr>
<tr>
<td>[145]</td>
<td>Rabbit</td>
<td>Tibial nerve</td>
<td>10 mm min⁻¹</td>
<td>11.7 ± 0.7 MPa</td>
<td>N/A</td>
<td>38.5 ± 2.0 %</td>
</tr>
<tr>
<td>[146]</td>
<td>Rabbit</td>
<td>Brachial plexus</td>
<td>10 mm min⁻¹</td>
<td>28.5 ± 1.8 MPa</td>
<td>6.9 ± 0.39 MPa</td>
<td>24.0 ± 1.1 %</td>
</tr>
<tr>
<td>[147]</td>
<td>Mouse</td>
<td>Sciatic nerve</td>
<td>1.22 mm s⁻¹</td>
<td>7.0 MPa</td>
<td>3.2 MPa</td>
<td>0.43 mm mm⁻¹</td>
</tr>
</tbody>
</table>

Another possibility is to subject tissues or biomaterials to tensile and compressions tests by using an electromechanical material testing instrument [7, 129]. For compression tests, samples should be subjected to unconfinement compression at a constant strain rate until the sample fails completely. Therefore, this test is used to determine the ultimate compressive strength and compressive elastic modulus, the latter resulting from the slope of the stress–strain curve under a stationary regimen [129]. Similar to rheological analysis, samples should have a wide and homogeneous diameter with a controlled thickness. Although few reports about the strength of PNs under compression are available [135], it is necessary to consider the technical limitations raised by these organs (limited diameter and anatomical variations). In addition, and to the best of our knowledge, compression testing has not yet been applied to DPNAs.

Tensile testing is the most common biomechanical evaluation method for PNs and implies the uniaxial elongation of the nerve until failure. The cylindrical/tubular shape of PNs allows for alignment of the samples along the direction of the tension. A constant distance should be left between the clamps (known as the gauge length) and a constant strain rate should be applied. By measuring load and deformation during the tensile test, a stress–strain curve can be plotted (figure 4).

![Figure 4. Tensile testing of PNs. (A) Typical stress–strain curve obtained after tensile testing of a native PN. Different regions can be distinguished: (i) the toe region; (ii) the linear region and (iii) the failure region. (B) Macroscopic view of a native PN during strain in the linear region. (C) Macroscopic view of a native PN during failure, showing the epineurium that weakly connects both nerve stumps. Images are courtesy of Dr Giuseppe Scionti (Universitat Politècnica de Catalunya, Spain).](image-url)
Table 5. Selected overview of tensile testing of decellularized PN allografts.

<table>
<thead>
<tr>
<th>Ref</th>
<th>Species</th>
<th>Nerve type</th>
<th>Decellularization</th>
<th>Strain rate</th>
<th>Young’s modulus</th>
<th>Ultimate stress</th>
<th>Ultimate strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>[49]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>Triton X-100 and SDC</td>
<td>N/A</td>
<td>9.91 ± 0.42 MPa</td>
<td>4.74 ± 1.03 MPa</td>
<td>0.48 ± 0.12 mm mm⁻¹</td>
</tr>
<tr>
<td>[49]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>Triton X-100, DNase and RNase</td>
<td>N/A</td>
<td>9.27 ± 1.71 MPa</td>
<td>4.38 ± 0.56 MPa</td>
<td>0.49 ± 0.13 mm mm⁻¹</td>
</tr>
<tr>
<td>[49]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>Hypotonic buffer, freeze-thaw cycle, pancreatin, DNase and RNase</td>
<td>N/A</td>
<td>10.09 ± 1.10 MPa</td>
<td>4.79 ± 0.74 MPa</td>
<td>0.48 ± 0.10 mm mm⁻¹</td>
</tr>
<tr>
<td>[69]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>Triton X-100, SDC, DNase and RNase</td>
<td>2% strain s⁻¹</td>
<td>46.03 ± 15.65 MPa</td>
<td>±12.5 MPa</td>
<td>43.2 ± 8.7%</td>
</tr>
<tr>
<td>[139]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>Freeze-thaw cycles</td>
<td>1 mm min⁻¹</td>
<td>26.70 ± 5.22 MPa</td>
<td>8.20 ± 1.25 MPa</td>
<td>0.44 ± 0.10 mm mm⁻¹</td>
</tr>
<tr>
<td>[139]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>SB-10, SB-16 and Triton X-200</td>
<td>1 mm min⁻¹</td>
<td>24.10 ± 7.41 MPa</td>
<td>6.95 ± 2.33 MPa</td>
<td>0.45 ± 0.11 mm mm⁻¹</td>
</tr>
<tr>
<td>[140]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>Glycerol, SDC, SDS and Triton X-100</td>
<td>1% strain s⁻¹</td>
<td>576 ± 160 kPa</td>
<td>1400 ± 290 kPa</td>
<td>0.48 ± 0.12 mm mm⁻¹</td>
</tr>
<tr>
<td>[141]</td>
<td>Rat</td>
<td>Sciatic nerve</td>
<td>Triton X-100 and SDC</td>
<td>1% strain s⁻¹</td>
<td>5654 ± 1710 kPa</td>
<td>1622 ± 63 kPa</td>
<td>0.54 ± 0.13 mm mm⁻¹</td>
</tr>
<tr>
<td>[76]</td>
<td>Porcine</td>
<td>Peroneal nerve</td>
<td>Freeze-thaw cycle, hypotonic buffer, SDS, Aprotinin, EDTA, Benzonase and hypertonic buffer</td>
<td>0.1 mm s⁻¹</td>
<td>8.03 ± 0.81 MPa</td>
<td>1.81 ± 0.30 MPa</td>
<td>0.43 ± 0.10 mm mm⁻¹</td>
</tr>
<tr>
<td>[76]</td>
<td>Porcine</td>
<td>Tibial nerve</td>
<td>Freeze-thaw cycle, hypotonic buffer, SDS, Aprotinin, EDTA, Benzonase and hypertonic buffer</td>
<td>0.1 mm s⁻¹</td>
<td>8.45 ± 0.36 MPa</td>
<td>2.69 ± 0.51 MPa</td>
<td>0.36 ± 0.09 mm mm⁻¹</td>
</tr>
</tbody>
</table>
and to set the gauge length for all experiments at 10 mm.

In this regard, it is advisable to minimize technical variation by always employing the same strain rate of 1% strain s⁻¹ and to set the gauge length for all experiments at 10 mm.

The many variations in nerve type and size and experimental set-up make it difficult to directly compare the biomechanical properties of different DPNAs. Therefore, it is advisable to correlate the values obtained for DPNAs to an equal number of native nerves evaluated under the same conditions by using the same technical parameters. Previous studies have already demonstrated that, depending on the detergents used for the decellularization of PN allografts, different outcomes can be obtained (table 5). Borschel et al. [140] found a decrease in tensile strength after decellularization with glycerol, SDC, SDS and Triton X-100, while the stiffness remained comparable to the native control nerve. Others reported an increase in stiffness after decellularization with either decreased [141], unchanged [69, 139] or increased [76] tensile strength. Interestingly, Wang et al. [49] did not find any differences between native and decellularized PNs from rats. All these studies demonstrate the importance of evaluating the impact of tissue decellularization on the biomechanical properties of PNs. In this context, the organization of collagen fibers after decellularization plays an important role in the resulting biomechanical properties of the DPNAs [79, 142]. Disruption of the collagen network can lead to increased fiber mobility and subsequently to increased stiffness [143, 144]. Moreover, it is plausible that the loss or preservation of elastic fibers, involved in tissue elasticity, and PGs, involved in tissue hydration, will have an influence on the biomechanical properties of DPNAs as well. However, up until now, little information has been available about this correlation.

Evaluation of scaffold cytotoxicity and biocompatibility

In general, it is well-accepted that a decellularized tissue or organ is one of the most biocompatible and non-cytotoxic scaffolds available for a wide range of tissue-engineering applications [48, 52, 79]. However, the tissues and organs subjected to decellularization are exposed to different kinds of chemical, biological and/or physical agents that could have a variable impact on the biocompatibility of these scaffolds. In this context, the main risk associated with this process could be related to an inadequate cleaning of chemical residues from the decellularized scaffolds [148]. Furthermore, it is important to demonstrate if these scaffolds will support cell functions and allow an adequate recellularization, e.g. if DPNAs support proliferation and migration of SCs [76].

Nowadays, it is possible to determine the cytotoxicity or biocompatibility of decellularized tissues by using different morphofunctional and biochemical techniques [128, 130, 134]. In this regard, an easy way to assess the biocompatibility of DPNAs is to culture cells on the surface of the DPNA. From this simple experiment, several morphological and/or biochemical parameters can be obtained. To demonstrate cytotoxicity, an easy and accurate method is to quantify the amount of DNA, which is released from the damaged or dead cells into the medium. This method, although simple, is considered feasible for the demonstration of irreversible cell-membrane damage, which could be associated with the action of residual detergents [130, 134]. Furthermore, the metabolic activity of the cells can be quantitatively determined through a colorimetric reaction resulting from the enzymatic activity of the cells by using one of the commercially available kits, such as MTT assay, WST-1 reagent or Prestoblue® [128, 130, 149–151]. Alternatively, the number or proportion of viable and dead cells can be assessed with a calcine/ethidium bromide fluorescent staining, which is commercially available as Live/Dead®. Next to the determination of the proportion of viable cells (green fluorescence) and dead cells (red fluorescence), this staining also allows for the evaluation of morphological features of the cells [130, 149–152].

Finally, DPNAs with cells seeded on top can be processed for a wide range of histochemical and immunohistochemical analyses. These methods will provide clear information concerning the distribution and/or viability of the cells after seeding. In this context, DAPI staining could be a useful tool to determine the number of cells over time. However, it is important to mention that biocompatible scaffolds will support cell proliferation with an increase of cellularity over time, but it is possible that cell numbers will decrease again after some time in culture due to contact inhibition or a decrease of viability and functionality in culture [152]. Furthermore, HE or DAPI staining can be used for the evaluation of the distribution of the cells and can confirm if the cells were able to migrate through the decellularized scaffold [7, 153]. In addition, specific antibodies against cell proliferation-related proteins (such as Ki-67 and PCNA) could be used to determine the cell proliferation index, which is a feasible indicator of cell viability and functionality in vitro [7].

Guidelines for the evaluation of decellularized PN allografts

From the sections above, it is clear that a plethora of evaluation methods is available for the characterization of DPNAs. However, many of them serve the same purpose. In this regard, it is important to select those methods that enable a thorough evaluation in the most efficient manner. For this reason, and based on the literature, it seems logical to perform the characterization of DPNAs with focus on the following parameters: (i) demonstration of the removal of cells and their remnants; (ii) confirmation of the preservation or removal of essential ECM (such as collagens, basal lamina and GAGs); (iii) determination of the structural and biomechanical properties and (iv) determination of the ex vivo or in vivo biocompatibility.

For the first part, determining if the tissue is cell free, minimal criteria were already proposed by Crapo et al. [51]. They focus on nuclear material as the main reason for adverse host reactions. We believe, however, that cytoplasmic remnants
can significantly contribute to this reaction as well. Therefore, specifically for DPNAs, we propose an extension of the criteria as follows:

For nuclear material, DPNAs should have:
- < 50 ng dsDNA per mg ECM dry weight.
- < 200 bp DNA fragment length.
- A lack of visible nuclear material in tissue sections stained with DAPI and HE.

For cytoplasmic remnants, DPNAs should be:
- Negative for S-100/β and/or vimentin immunostaining, which recognize SCs and fibroblasts.
- Negative for structural axonal proteins such as neurofilament and/or β-III tubulin.
- Negative for myelin histochemical or immunohistochemical stainings such as LFB, MCOLL or MBP.

For the second and third part, analyzing the preservation and/or removal of ECM components, there are no criteria or guidelines available so far. The quality of a DPNA will mainly depend on the relation between the nerve architecture and the biomechanical properties. Therefore, we propose that the following analyses should be included in the evaluation of DPNAs:

• Histological analysis of collagens, PGs and elastic fibers with the appropriate histochemical methods (respectively Picrosirius, Alcian Blue and Orcein) and specific immunostaining for collagen type I and laminin to demonstrate the preservation and adequate distribution of the main ECM components, which play an important role in the PN regeneration process.
• Analysis of the ultrastructure with SEM to demonstrate the preservation and organization of all stromal layers, especially the endoneurium, which should be characterized by the presence of empty, well-preserved endoneurial tubes. Removal of myelin and other cell remnants can also be evaluated.
• Quantitative analysis of collagen and GAG content with biochemical assays, expressing the total number of these components relative to the ECM dry weight and ideally, complementing the histological findings.
• Analysis of biomechanical properties under tensile forces to demonstrate that decellularization did not significantly affect these properties, while employing a strain rate of 1% strain s⁻¹ and a gauge length of 10 mm to obtain the Young’s modulus, ultimate stress and ultimate strain.

With these guidelines, it should be possible to have more standardized criteria for the characterization of DPNAs, enabling easier comparison of decellularization protocols and possibly increasing the quality of further in vivo experiments in this field. However, after thorough evaluation of a DPNA, there are still some steps to take before it can be safely used in animals and especially in humans. As mentioned before, when using chemical, biological and/or physical agents, it is advisable to determine if all toxic residues were properly washed away, by performing cytotoxicity assays. Furthermore, the appropriate sterilization method should be selected in such a manner that it removes any possible contamination without affecting the ECM and biomechanical properties of the DPNA. This issue is not further discussed here, as it is beyond the scope of this review.

Conclusions

Within the field of PN regeneration, there is a need for alternatives for the use of autografts. DPNAs offer a valuable, off-the-shelf available substitute for damaged nerves, providing the regenerating microenvironment with a natural 3D nerve architecture and topographical cues due to the preservation of essential ECM components. Many decellularization protocols, mainly detergent-based methods, have already been developed, but comparison is difficult because of a lack of standard evaluation methods. When assessing the impact of decellularizing agents on PN allografts, the degree of cell removal is an important aspect to evaluate. Both histology and biochemistry should be included to verify that there is no longer cellular material present that could elicit an immune response. Furthermore, the structural integrity, preservation of essential ECM components (such as collagens and endoneurial tubes) and biomechanical properties of DPNAs should be demonstrated with an adequate correlation between histological analysis of specific ECM components, ultrastructural evaluation, quantification of ECM components through biochemistry and/or molecular biology and determination of biomechanical properties. Based on our findings, we conclude in this review that it is necessary to improve quality control methods for DPNAs, since there are no well-defined criteria in this field so far. For this reason, we hypothesize that by employing similar evaluation methods for each decellularization protocol, a more comprehensive comparison can be obtained and the DPNAs holding the most promise for future in vivo experiments can be selected.

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