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METHODS

How to visualize the innervation pattern in tendons: A methodical guide

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ABSTRACT

Background: Tendon pathologies are common and several data suggests that the peripheral nervous system is involved in this disorder. Immunohistochemistry (IHC) is one of the pillars to characterize nervous structures and their implication in the pathogenesis of chronic tendon pain. Most commonly, formalin-fixed, paraffin-embedded (FFPE) tendons are used for immunohistochemical characterization of the innervation. However, FFPE specimens exhibit major disadvantages: First, antigens (proteins) are masked and antigen retrieval is necessary to restore antigenicity. Second, FFPE specimens involve immunolabeling with enzyme-conjugated antibodies but this approach has limitations when multiple antigens are of interest simultaneously. Consequently, there is a demand in the orthopedic community for an alternative immunohistochemical approach to visualize tendon innervations.
Results: Here, we present a guide how to visualize tendon innervation. This guide couples paraformaldehyde fixation, cryo-embedding, immunofluorescence, and confocal laser scanning microscopy. We demonstrate the utility of our approach in the long head of the biceps tendon. For nerve fiber characterization, we used different neuronal markers including antibodies against neurofilament, protein gene product 9.5, calcitonin gene related peptide, and substance P. We show that it is possible to collect high quality, multicolor images of the innervation pattern of tendons. To map immunolabeled structures and the anatomical structures of the tendon fluorescence images and bright field images were merged.
Conclusion: For the orthopedic community our approach might be a convenient research tool to simultaneously utilize multiple neuronal markers on the same tissue section and to define with greater accuracy the heterogeneity of tendon innervation.

1. Introduction

Tendon pathologies are common in athletes as well as in recreational sportsmen and result in pain which in turn reduces mobility and quality of life. Pain transmitting and sympathetic nerve fibers have been identified in tendons and today, there is consensus that the peripheral nervous system plays an important role in tendinopathy. Immunohistochemistry (IHC) is a fundamental research tool to characterize nervous elements and their implication in the pathogenesis of chronic tendon pain. Most commonly, immunohistochemical characterization of tendon innervation has been performed in formalin-fixed, paraffin-embedded (FFPE) specimens (Alpantaki et al., 2005; Boesmueller et al., 2015; de Castro Pochini et al., 2011; Joseph et al., 2009; Schmalzl et al., 2019; Schubert et al., 2005; Singaraju et al., 2008; Tosounidis et al., 2013; Xu et al., 2011). However, a major disadvantage of FFPE specimens is that the tissue antigens (proteins) are masked and molecular markers (antibodies) cannot interact with the corresponding epitopes of the antigens. For that reason, antigen retrieval is required, a process by which antigens are unmasked and made accessible to antibodies. An additional unpleasant characteristic of FFPE tissue is its inherent autofluorescence (Shi et al., 2011) and immunolabeling commonly involves enzyme-conjugated antibodies which

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produce a colored deposit at the antibody binding site. This so-called enzymatic IHC is a perfect tool for antigen detection but is primarily used for detection of a single antigen in the same tissue sample (paraffin section). Simultaneous visualization of more than one antigen by different colors is technically laborious and less satisfactory because colors can overlap and one color can be obscured by the other one. As a consequence, multiple antigens are visualized sequentially on consecutive tissue sections. In conclusion: IHC in FFPE specimens requires antigen retrieval and multiple antigens are visualized sequentially on consecutive sections.

As an alternative to FFPE tissue, paraformaldehyde (PFA)-fixed, cryo-embedded tissue is widely used for immunohistochemical analyses. For several reasons, PFA-fixed, cryo-embedded tissue is much more qualified for IHC. First, PFA denatures protein less than formalin (Cinar et al., 2006). Second, cryo-embedding is a technique where the tissue is rapidly frozen and preserved close to its natural state whereas the whole process of paraffin embedding involves strong chemicals and embedding at high temperature which all together impairs the tissue antigenicity. Attempting to compensate for reduced antigenicity in FFPE tissue, IHC protocols have became more and more sophisticated but less convenient over the past years (Buchwalow et al., 2012). Third, PFA-fixed, cryoembedded tissue has low, if any, autofluorescence and antibodies with different fluorochrome can be used for simultaneous detection of multiple antigens.

Only few studies have coupled PFA-fixation, cryo-embedding, and immunofluorescence to analyze tendon innervation (Ackermann et al., 2003; Ljung et al., 2004, 1999). Using single-color labeling, tendon innervation was visualized by epifluorescence microscopy (Ackermann et al., 2003; Ljung et al., 2004, 1999). Epifluorescence microscopy however, has limitations in resolution due to signals that come from below and above the focus plane. Consequently, there is demand in the orthopedic community for an optimized approach to visualize tendon innervations.

Over the past years, we have established a reliable protocol for molecular characterization of neuronal structures (Gesslbauer et al., 2017; Haider et al., 2015; Zimmermann et al., 2013). This protocol incorporates PFA fixation, cryo-embedding, immunofluorescence, and confocal laser scanning microscopy. In the present study, we tested the applicability of this guide for the characterization of nervous elements in the long head of the biceps tendon (LHBT). For immunolabeling, we used different neuronal markers including antibodies against neurofilament, protein gene product 9.5 (PGP9.5), calcitonin gene related peptide (CGRP), and substance P and performed multi-labeling experiments. Here, we demonstrate that our guide is a convenient research tool to generate high-quality, multicolored images of the innervation pattern of tendons which can easily be used for quantitative analyses.

2. Materials and methods

The LHBTs were harvested intraoperatively from patients suffering either from osteoarthritis (OA) or from a non-reconstructable head split fracture undergoing shoulder joint replacement. Eleven LHBTs were analyzed in this study. There were eight female and three male specimens and age ranged from 66 to 86 years. For our analyses, we used the most proximal part of the LHBTs. This study has been approved by the local ethics committee (No. 06/2017). A macroscopic view of the LHBT is provided in Fig. 1A.

2.1. Tissue preparation

Tendons were immersion-fixed for 24 h at 4 °C in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. After that, tissue was rinsed in phosphate buffered saline (PBS) at pH 7.4 and further processed for cryo-embedding.

2.2. Cryo-embedding

The procedure for cryo-embedding consists of four steps.

2.2.1. Cryo-protection

Following fixation in 4% paraformaldehyde and rinsing in PBS, the tissue was cryproptected in PBS containing graded concentrations of sucrose (10%, 25%, and 40%) which prevents crystallization and tissue destruction during subsequent thawing. In each solution, the tissue specimen stayed as long until it sank.

2.2.2. Intermedium

After cryo-protection, tissue was transferred into an intermedium. This intermedium consisted of PBS with 40% sucrose and embedding medium (Cryomatrix, Thermo Fisher). The mixing ratio of PBS-40% sucrose and embedding medium was 1:1. Depending on size, tissue remained in the intermedium up to five hours.

2.2.3. Embedding molds

Analog to paraffin-embedding, we used molds for cryo-embedding. Embedding molds had a cylindrical shape and were self-made. We used adhesive tape that was wrapped around a small vial. It is crucial that the sticky side of the tape faces away from the surface of the vial since the embedding mold is finally detached from the vial. For better orientation, Fig. 2A–C shows the individual steps how to prepare embedding molds.

2.2.4. Embedding

The floor of the embedding mold was covered with a thin layer of the embedding medium and then put into a −80 °C freezer until the embedding medium was frozen (Fig. 2D). The tendon was removed from the intermedium and aligned on the floor of the mold according to the desired orientation (Fig. 2E). Then the embedding mold was filled with the embedding medium until the tissue was covered entirely. Immediately after that, the embedding mold was moved into ice cold (−80 °C) 2-methyl butane until the embedding medium was utterly frozen (Fig. 2F). The embedding mold containing the frozen tendon was stored in a −80 °C freezer before further processing.

2.3. Cryo-sectioning

For cryo-sectioning, the adhesive tape of the mold was removed. Then the frozen block was allowed to equilibrate for 30 min in the cryostat chamber. Sections of 10 μm thickness were cut with a cryostat (Leica CM1950, Wetzlar, Germany). The optimal temperature for cryostat sectioning of tendons was −20 °C. For sectioning, we used disposable microtome blades R35 (Feather, Köln, Germany). To evaluate the microscopic anatomy of tendons, cryosections were stained with hematoxylin and eosin. An overview image shows that the tendon histology exhibited superior quality (Fig. 1B).

2.4. Immunofluorescence

For immunolabeling the following antibodies were used: Chicken anti-neurofilament (Merck Millipore, Cat# AB5539, RRID:AB_177520; 1:2000), rabbit anti-PGP9.5 (Merck Millipore, Cat# NE1013, RRID:AB_2210632, 1:300), rabbit anti-CGRP (Merck Millipore, Cat# AB15360, RRID:AB_672958), and mouse anti-substance P (Abcam, Cat# AB14184, RRID:AB_300971, 1:500). Anti-neurofilament and anti-PGP9.5 are general markers for nerve fibers and were used to visualize the general innervation of

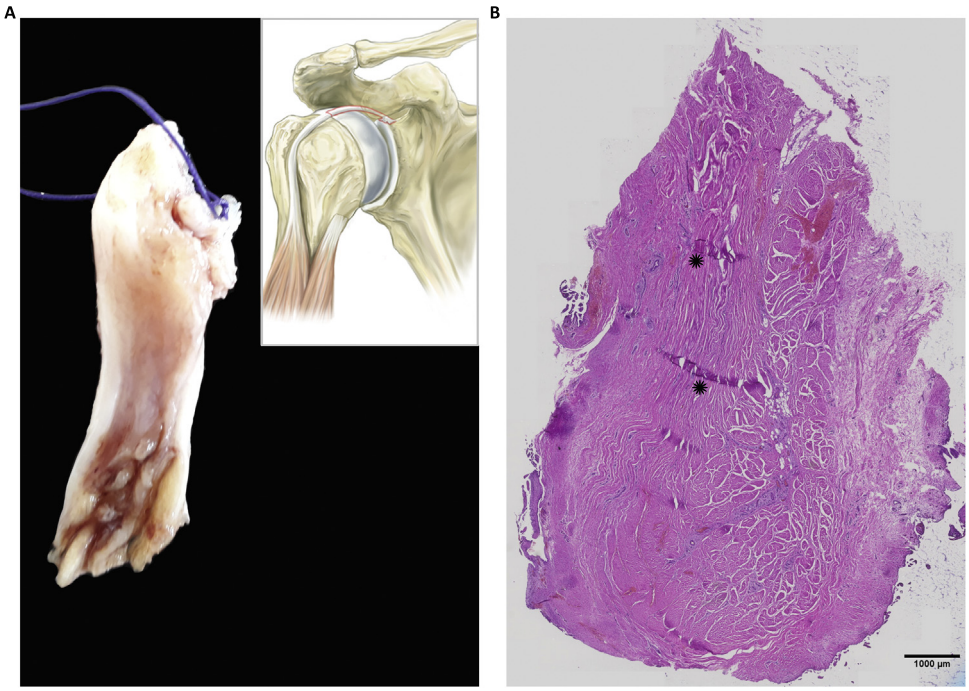

Fig. 1. Macroscopic and microscopic view of the LHBT.
(A) Showing the proximal portion of the LHBT. The purple thread indicates the anterior aspect of the LHBT. Schematic drawing (inset) shows the LHBT in its anatomical context. The proximal part of the LHBT is outlined in red color. (B) Cryo-section stained with hematoxylin and eosin showing that the tendon exhibits superior histological structure. Minor folding artifacts (asterisks) were rarely seen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the LHBT irrespective of the nerval quality. Anti-CGRP and anti-substance P are selective markers for nociceptive nerves. To demonstrate the utility of our protocol for the molecular detection of innervation of tendons, we performed two double-labeling experiments and one triple-labeling experiment. Double-labeling included incubation 1) with antibodies against neurofilament and PGP9.5 and 2) with antibodies against neurofilament and CGRP. For triple-labeling, incubation with antibodies against neurofilament, CGRP, and substance P was performed.

Before labeling, frozen sections were dried at room temperature for 30 min. Then unspecific binding was blocked by incubation for two hours in 10% normal goat serum in PBS containing 0.1% Triton (PBS-T). Thereafter, sections were incubated for 48 h with the primary antibodies, washed with PBS-T, and incubated for four hours with the secondary antibodies. We used Alexa Flour conjugated secondary antibodies at a concentration of 1:500. Finally, the tissue was rinsed again in PBS-T and mounted in fluorescence mounting medium (Dako, Carpinteria, CA 93013 USA).

Fluorescent-labeled sections were qualitatively analyzed with a confocal laser scanning microscope [CLSM (Olympus FV3000, Olympus Europa SE & Co. KG, Hamburg, Germany)]. For CLSM analyses, a series of virtual sections of 0.9 µm thickness were cut through the structures of interest. Each section was photo-documented with a 1024×1024 pixel resolution, and 3D projections were rendered using Image J (NHI, Bethesda, MA, USA) software. Double-colored images were generated using lasers with excitation wavelength 488 and 568 nm and triple-colored images using an additional laser with excitation wavelength 633 nm. Additionally, bright filed images were recorded in the CLSM using a transmitted light detector to show morphological details of the tendons. Fluorescence images and bright field images were merged. This technique allowed us to map immunolabeling and the anatomical structure of the tendon.

For negative controls, primary antibodies were omitted, and secondary antibodies used alone. In all these cases, the omission of the primary antibody resulted in a complete lack of immunostaining.

3. Results

For molecular detection of tendon innervation, we used two general neuronal markers (anti-neurofilament and anti-PGP9.5) and two selective markers for nociceptive nerve fibers (anti-CGRP and anti-substance P). To map immunolabeling and the anatomical structure of the tendon, fluorescence and bright field images were merged.

To visualize the overall tendon innervation, we performed double-labeling with anti-neurofilament and anti-PGP9.5. In all LHBTs, we observed axons that were positive for neurofilament as well as PGP9.5. There was almost complete overlap of neurofilament and PGP9.5 thus, confirming the specificity of the antibodies (Fig. 3A–A' and B–B"). In most cases, several axons grouped in smaller or larger nerve fascicles which accompanied blood vessels (Fig. 3A'). Occasionally, single axons were observed. Double-labeling with anti-neurofilament and anti-CGRP exhibited that many neurofilament-positive axons expressed CGRP as well (Fig. 4A–A"). However, other neurofilament-positive axons were CGRP-negative indicating that these axons are not involved in nociception. Triple-labeling, combined anti-neurofilament with anti-CGRP and anti-substance P. Findings showed that neurofilament co-localized with substance P (Fig. 4B) and CGRP (Fig. 4B'). Additionally, we observed co-localization of substance P and CGRP (Fig. 4B"). In conclusion, this triple-labeling experiments confirmed that CGRP/substance P was along with axons and that CGRP and substance P were co-expressed in the same axon (Fig. 4B"').

4. Discussion

Tendon pathologies are common in athletes as well as in recreational sportsmen and involve the peripheral nervous system.

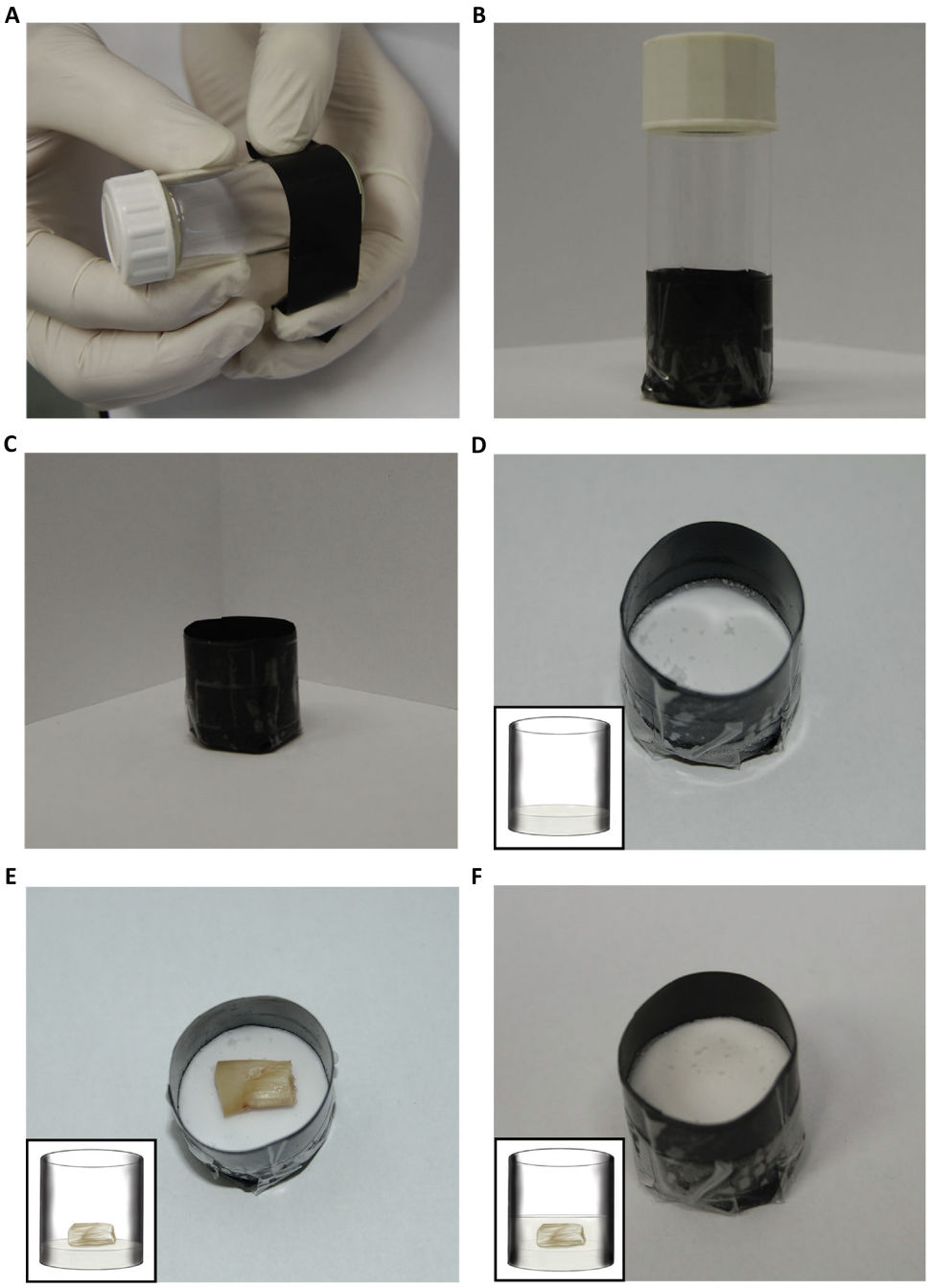

Fig. 2. Showing the sequential steps for the preparation of a mold for cryo-embedding and the embedding procedure.
(A–C) Preparation of a mold. (A) An adhesive tape is wrapped around the glass vial with the sticky side facing away from the surface. Showing the completed mold (B) and the mold following detachment from the glass vial (C). (D–F) Sequential steps of cryo-embedding. For better orientation a schema (inset) is provided which allows an interior view of the mold. (D) The floor of the embedding mold is covered with a thin layer of frozen embedding medium. (E) A piece of tendon is placed on the frozen embedding medium. (F) Showing the embedding mold after the tendon was covered with embedding medium following freezing in −80 °C methyl butane.

Consequently, there is need to characterize nervous elements in tendons with high accuracy. Here, we provide a reliable guide for visualization of tendon innervation by coupling PFA-fixation, cryo-embedding, immunofluorescence, and confocal laser scanning microscopy. Following this guide, we have clearly demonstrated that it is possible to obtain high quality, multicolored fluorescence images of the tendon innervation. By merging fluorescence images and bright field images, immunolabeled structures were mapped with the anatomical structure of the tendon.

Most commonly, immunohistochemical examination of tendon innervation has been performed in FFPE specimens although formalin fixation and paraffin embedding reduce the tissue antigenicity (Alpantaki et al., 2005; Boesmueller et al., 2015; de Castro Pochini et al., 2011; Joseph et al., 2009; Schmalzl et al., 2019; Schubert et al., 2005; Singaraju et al., 2008; Tosounidis et al., 2013; Xu et al., 2011). To restore tissue antigenicity, antigen retrieval was mandatory in FFPE tendons. In tendons of the present study, this step could be skipped prior to immuno-

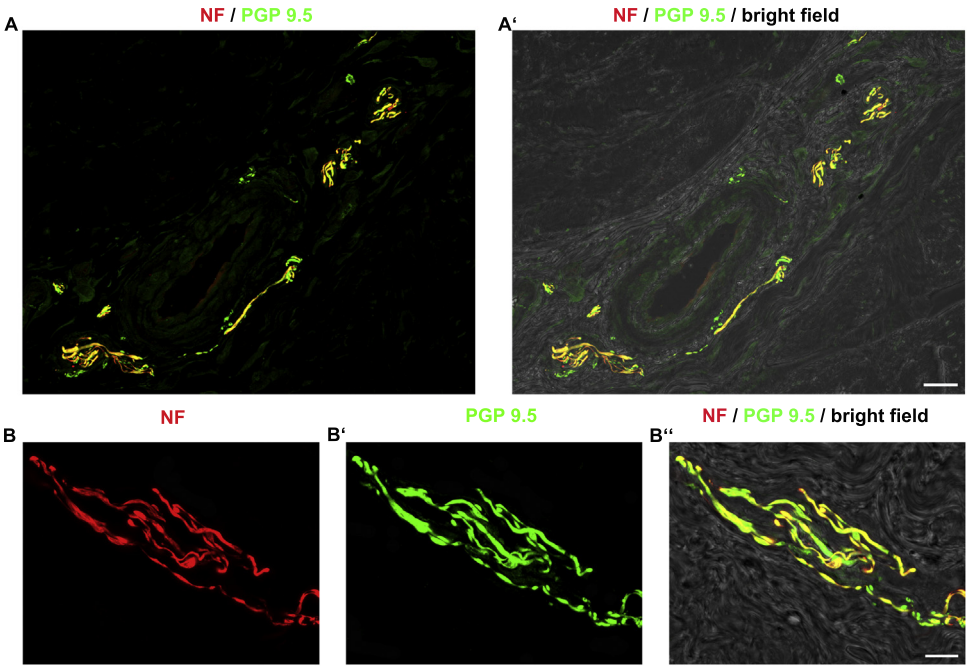

Fig. 3. CLSM images showing nerve fibers double-labeled with anti-neurofilament (NF) and anti PGP9.5 In addition to fluorescence images corresponding bright field images were generated which show structural details of the tendon.
(A) Overlay of neurofilament (NF, red) and PGP9.5 (green) stain, showing co-localization of neurofilament and PGP9.5 resulting in yellow color mixture. A', The fluorescence image (A) and the corresponding bright field image are merged**.** The bright field image shows the anatomical structure of blood vessels surrounded by immunolabeled axons. B–B", High magnification image. Axons exhibiting neurofilament immunoreactivity (B) and PGP9.5 immunoreactivity (B'). B", Fluorescence images and the corresponding bright field image are merged. The image shows co-localization of neurofilament and PGP 9.5 resulting in yellow color mixture. Collagen fibers of the tendon are visible around the axons. Scale bars, A' 20 μm and B" 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

labeling as PFA-fixed, cryo-embedded tissue retains superior molecular quality. Additionally, our PFA-fixed, cryo-embedded LHBTs exhibited negligible autofluorescence which allowed us to perform multicolor immunolabeling and to visualize multiple tissue antigens (neuropeptides), simultaneously. Specifically, by double-labeling with anti-neurofilament/anti-PGP9.5, we observed co-localization of both neuronal markers. Double-labeling with anti-neurofilament/anti-CGRP exhibited co-localization of neurofilament/CGRP in many axons whereas other neurofilament-positive axons lacked CGRP-immunoreactivity. These findings indicate that besides nociceptive axons, axons with other functions contribute to tendon innervation. By triple-labeling, we confirmed co-expression of CGRP/substance P along with neuronal elements.

In FFPE tendons, enzyme-conjugated antibodies have been used for nerve fiber characterization. Enzyme conjugated antibodies accumulate stain at every antibody binding site of the primary antibody and by signal amplification the staining sensitivity can be increased. Immunofluorescence stains in our sections were not faint but exhibited high levels of contrast between color signal and background thereby confirming excessive sensitivity. Enzyme-conjugated antibodies are ideal for detection of a single antigen but less qualified for simultaneous detection of multiple antigens. As a consequence, previous visualization of multiple neuropeptides has been performed sequentially on consecutive tissue sections of FFPE tendons (Alpantaki et al., 2005; Boesmueller et al., 2015; Joseph et al., 2009; Singaraju et al., 2008; Tosounidis et al., 2013; Xu et al., 2011). Sequential antigen visualization is, however, time-consuming and co-localization of neuronal markers can only be assessed by matching adjacent sections which is less conclusive and interpretation errors can occur. This possibly happened in a previous study of the LHBT (Alpantaki et al., 2005). Specifically, following sequential immunolabeling Alpantaki et al. (2005) reported that all neurofilament-positive axons were CGRP positive as well which is however not the case as demonstrated by simultaneous double-labeling with anti-neurofilament/anti-CGRP in the present study.

Analog to our approach, few studies used PFA-fixed, cryo-embedded tendons to analyze tendon innervation (Ackermann et al., 2003; Ljung et al., 2004, 1999). However, different to us, innervation was imaged by epifluorescence microscopy. In the present study we used confocal laser scanning microscopy which has major advantages over epifluorescence microscopy. First, optical resolutions and image contrast is increased and second, antibody co-localization can be visualized with high fidelity since out-of-focus background is eliminated. Thus, our guide coupling PFA-fixation, cryo-embedding, immunofluorescence, and confocal laser scanning microscopy represents a novel, optimized approach for visualization of tendon innervation. Since our digital slide images visualized individual axons with high contrast and accuracy, they can easily be used for computational quantitative analyses. Moreover, multicolored images allow simultaneous quantification of axons with different qualities on the same slide which in turn provides more precise neuronal profiling of tendon samples.

5. Conclusion

This study presents a methodical approach how to visualize different nervous qualities in tendons and generate high quality, multicolored images. This guide might be a convenient research tool for the orthopedic community to depict the heterogeneity of tendon innervation with greater accuracy and to perform simultaneously quantitative analyses of more than one neuronal marker. We also want to highlight that our strategy is not only useful to visualize innervation, but can also be used for detection of other tendon molecules.

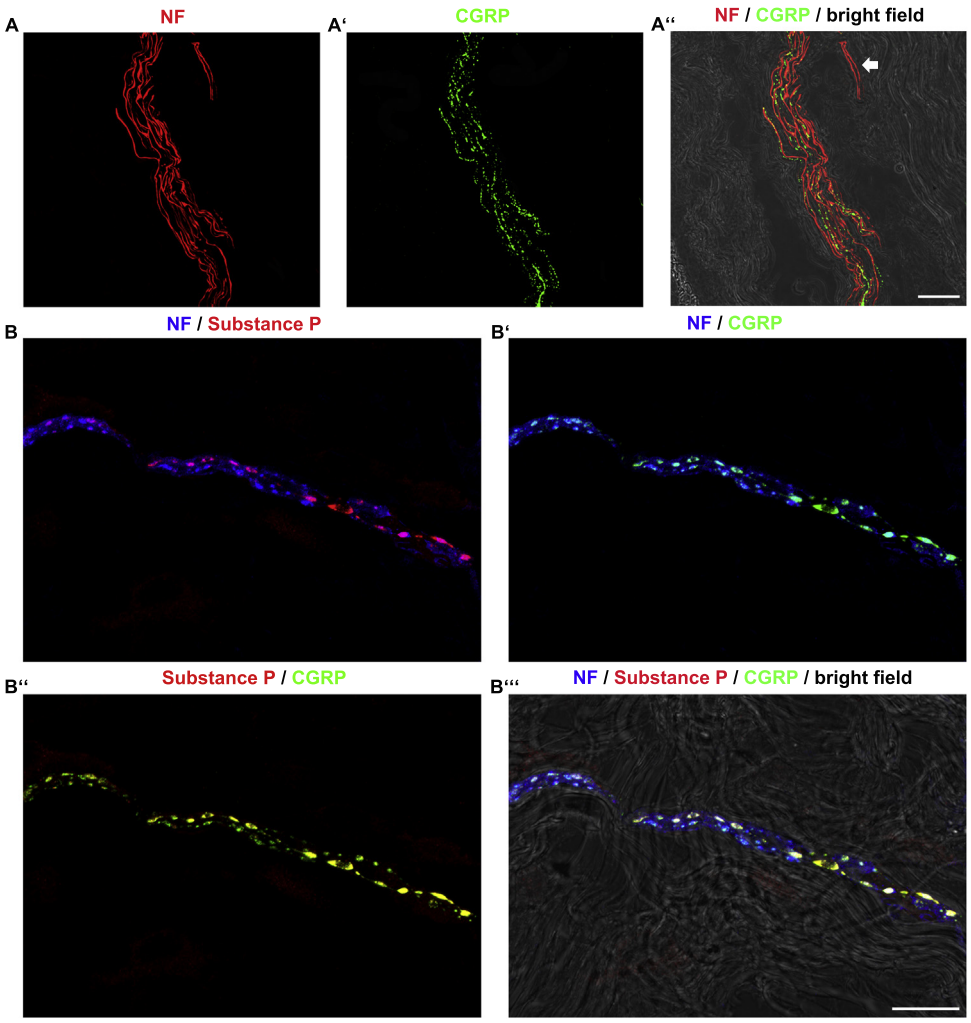

Fig. 4. CLSM images showing double-labeled and triple-labeled nerve fibers. In addition to fluorescence images corresponding bright field images were generated which show structural details of the tendon.
A–A", Double-labeling with anti-neurofilament and anti-CGRP showing axons that exhibit neurofilament (NF) immunoreactivity (A) and CGRP immunoreactivity (A'). A", Fluorescence images and the corresponding bright field image are merged. Besides neurofilament/CGRP-positive axons, other neurofilament-positive axons lack CGRP (arrow). Collagen fibers of the tendon surround the axons. B–B"', Triple-labeling with anti-neurofilament, anti-substance P, and anti-CGRP. (B) Showing co-localization of neurofilament (NF) and substance P and B' co-localization of neurofilament and CGRP. B", Showing co-localization of substance P and CGRP. B"', Merged fluorescence and bright field images showing triple co-localization of neurofilament, substance P, and CGRP. Axons are surrounded by collagen fibers. Scale bars, A" 25 μm and B"' 20 μm.

Authors contribution

SB, RM, and RB designed the research. SB, RM, BG, JS, DB and RB performed research and analyzed data. RB wrote the manuscript. All authors performed a critical revision of the manuscript.

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Ethical statement

This study has been approved by the local ethics committee (No. 06/2017).

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