Video Article

Three-dimensional Imaging of Nociceptive Intraepidermal Nerve Fibers in Human Skin Biopsies

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Abstract

A punch biopsy of the skin is commonly used to quantify intraepidermal nerve fiber densities (IENFD) for the diagnosis of peripheral polyneuropathy [1,2]. At present, it is common practice to collect 3 mm skin biopsies from the distal leg (DL) and the proximal thigh (PT) for the evaluation of length-dependent polyneuropathies [3]. However, due to the multidirectional nature of IENFs, it is challenging to examine overlapping nerve structures through the analysis of two-dimensional (2D) imaging. Alternatively, three-dimensional (3D) imaging could provide a better solution for this dilemma.

In the current report, we present methods for applying 3D imaging to study painful neuropathy (PN). In order to identify IENFs, skin samples are processed for immunofluorescent analysis of protein gene product 9.5 (PGP), a pan neuronal marker. At present, it is standard practice to diagnose small fiber neuropathies using IENFD determined by PGP immunohistochemistry using brightfield microscopy [4]. In the current study, we applied double immunofluorescent analysis to identify total IENFD, using PGP, and nociceptive IENF, through the use of antibodies that recognize tropomyosin-receptor-kinase A (Trk A), the high affinity receptor for nerve growth factor [5]. The advantages of co-staining IENF with PGP and Trk A antibodies benefits the study of PN by clearly staining PGP-positive, nociceptive fibers. These fluorescent signals can be quantified to determine nociceptive IENFD and morphological changes of IENF associated with PN. The fluorescent images are acquired by confocal microscopy and processed for 3D analysis. 3D-imaging provides rotational abilities to further analyze morphological changes associated with PN. Taken together, fluorescent co-staining, confocal imaging, and 3D analysis clearly benefit the study of PN.

Video Link

The video component of this article can be found at https://www.jove.com/video/50331/

Introduction

At present, it is common practice for physicians to quantify intraepidermal nerve fiber densities, (IENFD) from skin punch biopsies, which can be used to diagnose small fiber neuropathies [3, 6-8]. Biopsies are taken from the distal leg (DL), 10 cm above the lateral malleolus, and the proximal thigh (PT), 20 cm below the anterior iliac spine [9]. All IENF are labeled using protein gene product 9.5 (PGP), a pan neuronal marker [10-12]. At present, it is standard practice to diagnose small fiber neuropathies using IENFD determined by PGP staining with brightfield microscopy [6]. Additionally, several research groups have used immunofluorescent protocols for PGP immunohistochemistry [7-9]. Small fiber neuropathy is commonly associated with neuropathic pain. In order to further understand the role of IENF essential for pain processing, we developed a technique to co-label total IENF with fibers that generate pain. Nociceptive IENF, specifically Aδ and C fibers, can be studied through the co-labeling of IENF with PGP and the nociceptive marker, tropomyosin-receptor-kinase A (Trk A) [5]. Trk A is the high affinity receptor for nerve growth factor that is essential for the development of nociception. The Trk A-positive nociceptive nerve fibers are peptidergic fibers that express substance P (SP) and calcitonin gene related peptide (CGRP). Previously, Lauria and colleagues applied the double-labeling technique to study PN, co-labeling PGP-positive IENF with a nociceptive marker [10]. In our previous study, we demonstrated that Trk A-positive IENF, but not Trk A-negative IENF, were upregulated in an animal model of painful diabetic neuropathy [5]. This co-labeling technique provides the ability to compare quantification of nociceptive IENFD to total IENFD and the ability to study morphological changes associated with PN. The capability to visualize nociceptive IENF and compare quantification of total IENFD to nociceptive IENFD could provide objective evidence for the presence of pain, and possibly insight into the severity of pain associated with PN. This technique is also applicable to skin of animal models. In comparison to previous studies, the current protocol describes methods for 3D image analysis, creating the opportunity to avoid errors that could occur in 2D image analysis.

Protocol

Part A: Immunohistochemistry

Preparation of 96-well plate and prevention of background staining Punch skin biopsies are collected from human subjects and incubated for 12-24 hr in fixative solution (2% paraformaldehyde with 0.75 M L-Lysine solution (pH 7.4) and 0.05 mM sodium periodate) at 4 °C as previously described [8]. Samples are then cryoprotected in phosphate buffered saline (PBS) with 20% glycerol at 4 °C for up to 1 week, embedded in mounting media optimal cutting temperature (OCT), then sectioned into 50 µm thick sections on a cryostat. The protocol described below is designed for 8 skin sections, the maximum number of skin sections possible to undergo free-floating immunohistochemistry in one 96-well plate.

DAY 1:

1. Prevention of Non-specific Immunoreactivity in the Stratum Corneum

1. Label 96-well plate as shown in **Figure 1**.
2. Add 150 µl of Image-IT FX Signal (Image-IT), effective for blocking background staining [11,12], into each well in Row 1 of the 96-well plate.
3. Add 150 µl of 1X PBS into each well in Row 2 and 3 of the 96-well plate.
4. Acquire two 50 µm sections per patient: one section from the biopsy taken at DL, one section from the biopsy taken at PT. An inoculating loop (LeLoop) is used to transfer sections from one rinse to the next to avoid morphological damage. Gently transfer each 50 µm section, using an inoculating loop, into an individual well of Row 1, containing Image-IT. Incubate sections in Image-IT for 30 min at RT on a flat rocker.
5. Rinse sections twice with 1X PBS (Rows 2 and 3) for 10 min at RT. Place well plate on a flat rocker between rinses.

2. Preparation of 5% BSA Blocking Solution and 1% Rinsing Solution

1. While biopsy sections are incubating in Image-IT, prepare 5% blocking solution (5% BSA, 0.3% TX-100, 0.1 M PBS- Vortex solution until BSA completely dissolves).
2. Add 150 µl of 5% BSA blocking solution into each well of Row 4.
3. Using an inoculating loop, transfer sections into individual wells of 5% BSA blocking solution. Incubate sections in 5% BSA blocking solution for 1-2 hr at RT on a flat rocker.

3. Preparation of 1% BSA Rinsing Solution and Dilution of Primary Antibodies

1. While sections are incubating in 5% BSA blocking solution, prepare 1% rinsing solution (1% BSA, 0.3% TX-100, 0.1 M PBS- Vortex solution until BSA completely dissolves).
2. While sections are incubating in 5% BSA blocking solution, dilute primary antibodies in 1% rinsing solution.
    1. For eight sections (8 x 150 µl = 1,200 µl) a total of 1,200 µl is needed. The primary antibodies are made up in 1,500 µl (about 20% extra volume).
    2. Dilutions of the primary antibodies: PGP, 1:500; Trk A, 1:500 in 1% BSA rinsing solution.

4. Incubation of Sectioned Biopsies in Primary Antibody

1. Add 150 µl of diluted primary antibodies into the designated wells of the 96-well plate (Row 5).
2. Transfer sections from 5% blocking solution (Row 4) into the designated primary antibody wells (Row 5).
3. Seal the 96-well plate with parafilm and aluminum foil to avoid drying and light exposure.
4. Incubate the 96-well plate O/N at 4 °C on a flat rocker.

DAY 2:

5. Rinse Biopsies in 1% BSA Rinsing Solution

1. Add 150 µl of 1% BSA rinsing solution into each well in Row 6, 7, and 8.
2. Rinse sections three times with 1% BSA rinsing solution (Rows 6, 7, and 8) for 1 hr each time at RT. Cover well plate with aluminum foil and place on flat rocker between rinses.

6. Dilution of Secondary Antibodies

1. While sections are incubated in the last rinse of 1% BSA rinsing solution (Row 8), dilute secondary antibodies in 1% BSA rinsing solution:
    1. For eight sections (8 x 150 µl = 1,200 µl) a total of 1,200 µl is needed. The secondary antibodies are made up in 1,500 µl (about 20% extra volume).
    2. Dilutions of the secondary antibodies: for PGP (Alexa Fluor 488 donkey anti-rabbit, 1:250), for Trk A (Alexa Fluor 647 donkey anti-goat, 1:250) in 1% BSA rinsing solution.

7. Incubation of Sectioned Biopsies in Secondary Antibody

1. Add 150 µl of diluted secondary antibodies into their designated wells of the 96-well plate (Row 9).
2. Transfer sections from 1% BSA blocking solution (Row 8) into secondary antibody well (Row 9).
3. Seal the 96-well plate with parafilm and aluminum foil to avoid drying and light exposure.
4. Incubate sections in the designated secondary antibodies O/N at 4 °C on a flat rocker.

8. Rinse Biopsies in 1% BSA Rinsing Solution

1. Add 150 µl of 1% BSA rinsing solution into each well in Row 10, 11, and 12.
2. Rinse sections three times with 1% BSA rinsing solution (Rows 10, 11, and 12) for 1 hr each time at RT. Cover well plate with aluminum foil and place on a flat rocker between rinses.

9. Preparation of Microscope Slides and Mounting Sectioned Biopsies

1. While biopsy sections are incubating in the last rinse of 1% BSA rinsing solution (Row 12), prepare microscope slides.
2. Place 50 µl of 1% BSA rinsing solution on one slide at a time.
3. Remove sections from 1% BSA rinsing solution (Row 12), and place it in the 50 µl drop of 1% BSA rinsing solution on the designated microscope slide. After optimizing the position of the section, remove excess 1% BSA rinsing solution with a bulbed glass pipette, taking precautions to avoid touching the specimen.

NOTE: Make sure section is not folded over; the specimen should be flat against the surface of the microscope slide.

4. Place 1 drop of Prolong Gold antifade mounting reagent with DAPI near the biopsy on the microscope slide. Take a 22x22 mm microscope glass coverslip and gently place it over biopsy and a drop of Prolong Gold antifade reagent with DAPI drop. Repeat for each section.
5. Let microscope slides dry O/N at RT in dark.

NOTE: Remove any air bubbles using a pipette tip. Wipe away excess Prolong Gold antifade reagent.

Part B: Confocal Imaging

10. Confocal Imaging

1. Image fluorescence signals using an Olympus FluoView 500 laser scanning confocal microscope with a 40X oil-immersion (1.3 NA) objective and zoom two times with FluoView version 5.0 software.
2. Use Alexa Fluor 488 and Alexa Fluor 647 to excite the 543-nm HeNe green laser and the 633-nm HeNe Red laser, respectively. The Alexa Fluor 488 signal is represented by a green look up table (LUT) and the Alexa Fluor 647 by a red LUT.
3. Set the confocal apertures for each detector at 400 µm to enhance signals. Sequential scans should be taken at a resolution of 1,024 x 1,024 to maximize signal separation.
4. Capture three-dimensional z-series using 1.2 µm z-step intervals (based on the optimal scan unit calculated by the FluoView software) with Kalman averaging (two frames).

Part C: Three-dimensional Visualization and Animation

11. Three-dimensional (3D) Visualization and Animation

1. Open Fluoview files in Imaris x64 software (version 7.3, Bitplane AG) to visualize the 3D image sets.
2. Adjust display as needed to enhance contrast of the nerve specific signal.
3. Create a surface to visualize the dermal-epidermal boundary. Use the Contour tool in draw mode to draw the boundary.
4. Assign a semi-transparent surface to the boundary in order to see the underlying fluorescent signals.
5. Capture still images of the 3D fluorescent signals to create Snapshot images of the 3D movie.
6. Use the Animation feature to create a 3D movie. The images can be rotated 360 degrees several times to show each signal separately and merged (**Figures 2, 3, and 4**). Set animation to create movies consisting of 200 frames animated at 15 frames per second. Save each movie as a raw AVI file.
7. Compress the final AVI movie with VirtualDub software (version 1.9.4).

Representative Results

We applied the current protocol to study the morphology of IENF in PT and DL skin biopsies from patients with PN. The skin, from three subjects, was collected at the University of Utah to demonstrate the pathomorphology associated with PN. The subjects include: Case 1: a 51-year-old male with a history of PN of type 2 diabetes (duration: 14 months; pain score: 51); Case 2: a 56-year-old male with a history of PN of type 2 diabetes (duration: 108 months; pain score: 47); and Case 3: a 66-year-old male with a history of PN of type 2 diabetes (duration: 42 months; pain score: 46). Neurological history and examination, including quantitative sensory testing and nerve conduction studies, were performed to assess peripheral neuropathy. Pain scores were determined based on the 0-100 visual analog pain scale.

Using this protocol, we are able to study the 3D structure of IENFs in human skin (**Figure 2**). In **Figure 2**, PGP-positive IENFs were identified by a green signal, and Trk A-positive IENFs were labeled by a red signal. All Trk A-positive IENFs are also positive for PGP; the overlapping

signals appear yellow. In addition to the ability to examine IENFs, these methods can also be used to study axonal swellings in IENFs (**Figure 3**). 3D imaging of axonal swellings, presented in **Figure 3**, demonstrates that these swellings are globular structures distributed along IENFs. In addition, these methods can be used to study branching in IENFs (**Figure 4**). 3D images of branching presented in **Figure 4** demonstrate that morphological changes take place with the presence of PN. As previously described, the dermal-epidermal border was determined based on morphology and orientation of nerves and the pixel intensity difference between dermis and epidermis [8]. The blue tracing, depicted in **Figures 2, 3, and 4**, indicates the dermal-epidermal junction.

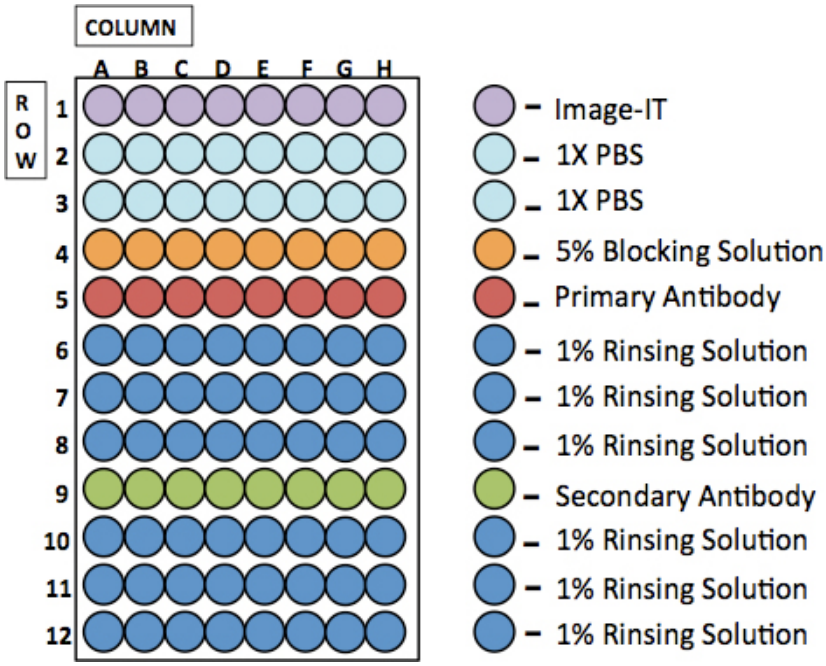

Figure 1. Schematic diagram representing the setup for a 96-well plate for skin biopsy immunohistochemistry.

Figure 2. Three-dimensional (3D) images of IENF. Representative 3D image of (A) PGP-positive IENF, labeled green, representing total IENF (first 360 ° rotation) and (B) Trk A-positive IENF, labeled red, representing nociceptive IENF (second 360 ° rotation), and (C) a merged image demonstrating PGP-positive, nociceptive IENF (third 360 ° rotation) in a skin sample from Case 1. Bar = 20 µm. Click here to view movie.

Figure 3. Three-dimensional (3D) image of axonal swellings in IENF of PN. Representative 3D image of axonal swellings (arrowheads) along IENFs, labeled by PGP with a green signal, and within the subdermal plexus in a skin sample from Case 2. Bar = 10 µm. Click here to view movie.

Figure 4. Three-dimensional (3D) image of axonal branching in IENF of PN. Representative 3D image of axonal branching (arrowheads) along IENFs, labeled by PGP with a green signal, in a skin sample from Case 3. Bar = 20 µm. Click here to view movie.

Components of 5% BSA Blocking Solution:	**Amount Needed for 12.5 ml**
1X PBS	8.125 ml
0.1% Triton X-100 (TX-100) [Final Concentration: 0.03%]	3.75 ml
Bovine Serum Albumin (BSA)	0.625 g
TOTAL	12.5 ml

Table 1. 5% BSA Blocking Solution.

Components of 1% Rinsing Solution:	**Amount Needed for 12 ml**
1X PBS	8.625 ml
0.1% TX-100 [Final Concentration: 0.03%]	3.25 ml
Bovine Serum Albumin (BSA)	0.125 g
TOTAL	12 ml

Table 2. 1% BSA Blocking Solution.

Discussion

Measurement of IENFD has been widely used to determine the degree of peripheral neuropathies [13,14]. At present, the most commonly used protocol only measures the densities of nerve fibers that penetrate the basement membrane of the epidermis; it does not take into consideration axonal branching and/or morphological changes of the nerves. In addition, current IENFD analysis has not been shown to correlate IENFD with the presence of pain in PN [15].

We previously reported that increased numbers of nociceptive IENFD are associated with pain behaviors in db/db mice, a mouse model of type 2 diabetes [5]. In that report, we first applied a double immunofluorescent protocol to study nociceptive IENF. In the current study, we expand our protocol to human skin from patients with PN. The double immunofluorescent analysis provides measurements of both total IENF and nociceptive IENF, the subset of IENFs that mediate pain. These nociceptive IENF are mostly positive for Trk A and other nociceptive neuropeptides [5]. Similar double immunofluorescent analysis for painful neuropathies has been described by Lauria and colleagues [10]. They reported reduced IENFD for transient receptor potential cation channel subfamily V member 1 (TRPV1) in patients with painful neuropathies. The current protocol could provide an alternative approach for measurement of nociceptive IENFD based on our animal data [5].

The current protocol offers a 3D approach for studying IENF structure. In combination with double immunofluorecent analysis, the current protocol provides methods to examine the morphology of nociceptive IENFs. Previous studies have not reported an association between densities or branching of nociceptive IENF with PN. Here, we demonstrate a protocol for studying axonal branching and swelling in IENFs of human skin. Our 3D analysis suggests that this method could be used to study the morphological changes of IENF in PN. In addition, 3D imaging could improve the current protocols of IENFD measurement by avoiding errors that are associated with reduced visualization of overlapping structures associated with 2D-imaging.

Axonal swelling is defined as an enlargement of an axonal portion with a diameter greater than two times that of the original axonal caliber [16]. This structure contains fragments of axonal cytoskeleton and components of axonal transport. Axonal swelling is considered an early feature of axonal neuropathy [6,16,17]. However, 3D analysis of axonal swelling structures has not been reported in the literature. As depicted in **Figure 3**, 3D imaging could provide important insight for axonal swelling associated with PN.

It is well characterized that axonal branching accompanies the recovery from nerve injury [18]. However, the available methodology for quantifying axonal branching is still limited based on 2D imaging. As described in **Figure 4**, axonal branching could be complicated by the tortuous nature of the regenerating nerves. Consequently, the branching points could be obscured by nearby nerve fibers to affect the accuracy of quantification. The 3D imaging analysis of our protocol provides improved visualization for details of the branching nerves.

Disclosures

No conflicts of interest to declare.

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