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TITLE:

Targeting Alpha Synuclein Aggregates in Cutaneous Peripheral Nerve Fibers by Free-Floating Immunofluorescence Assay

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KEYWORDS:

Skin biopsy, Peripheral nervous system, indirect Immunofluorescence, Free-floating, Protein gene product 9.5, alpha Synuclein, Phosphorylated alpha Synuclein, 5G4, protein aggregates

SHORT ABSTRACT:

Here, we present a protocol for a free-floating indirect immunofluorescence assay on skin biopsy sections that allows for the identification of disease specific conformation variants of alpha synuclein involved in Parkinson disease and multiple proteins of the peripheral nervous system.

LONG ABSTRACT:

To date, for most neurodegenerative diseases only a post-mortem histopathological definitive diagnosis is available. For Parkinson's disease (PD), the diagnosis still relies only on clinical signs of motor involvement that appear later on in the disease course, when most of the dopaminergic neurons are already lost. Hence, there is a strong need for a biomarker that can identify patients at the beginning of disease or at the risk of developing it. Over the last few years, skin biopsy has proved to be an excellent research and diagnostic tool for peripheral nerve diseases such as small fiber neuropathy. Interestingly, a small fiber neuropathy and alpha synuclein (αSyn) neural deposits have been shown by skin biopsy in PD patients. Indeed, skin biopsy has the great advantage of being an easily accessible, minimally invasive and painless procedure that allows the analysis of peripheral nervous tissue prone to the pathology. Moreover, the possibility of repeating the skin biopsy in the course of the follow-up of the same patient allows studying the longitudinal correlation with the disease progression. We set up a standardized reliable protocol

to investigate the presence of αSyn aggregates in skin nerve fibers of the PD patient. This protocol involves few short fixation steps, a cryotome sectioning and then a free-floating immunofluorescence double-staining with two specific antibodies: anti Protein Gene Product 9.5 (PGP9.5) to mark the cutaneous nerve fibers and anti 5G4 for detecting αSyn aggregates. It is a versatile, sensitive and easy to perform protocol that can also be applied for targeting other proteins of interest in skin nerves. The ability to mark αSyn aggregates is another step forward to the use of skin biopsy as a tool for establishing a pre-mortem histopathological diagnosis of PD.

INTRODUCTION:

Skin biopsy has acquired a great importance as the diagnostic and research tool in the field of neurological disorders[1]. Indeed, epidermis and dermis contain abundant somatic sensory nerve fibers (myelinated and unmyelinated), nociceptive free nerve endings, sensory receptors and autonomic innervation of sweat glands, vessels, sebaceous glands and muscle arrector pilorum[2].

In the mid-20th century, the setup for immunohistochemistry of PGP9.5 antibody allowed the evidence of an extensive innervation of human epidermis mammalian skin[3]. PGP9.5 is a carboxyl-terminal hydrolase equally distributed along axons of both the central and peripheral nervous system (PNS). The availability of this antibody allowed not only to clarify the morphology and anatomy of PNS in the skin but also implemented the study of diseases associated with it[3,4]. Skin biopsy contributed to defining a new clinical entity: the small fiber neuropathy. Several international groups demonstrated the association between the loss of intraepidermal nerve fibers and symptoms/signs of small fiber neuropathy[5] by skin biopsy analysis and provided standardized protocols for nerve morphometry as well as normative reference values to be used in the clinical practice[6,7,8].

Recently a large amount of evidence has shown that neurodegenerative diseases, characterized by misfolded protein accumulations in the central nervous system, are multi-system pathologies[9]. Indeed, PD is characterized by αSyn accumulation in the dopaminergic neuron of *substantia nigra*, but it has been demonstrated that αSyn and its pathological form, phosphorylated αSyn (P-αSyn), could be detected also in the peripheral tissues. Gastro-intestinal mucosa[10], salivary glands[11], skin autonomic fibers surrounding sweat glands and pilomotor muscles[12-14], show immunoreactivity to pathogenic forms of αSyn, in accordance with Braak hypothesis that intriguingly postulate that αSyn pathology may begin in PNS well in advance, before its accumulation in the brain[15]. Further, the presence of p-αSyn has been demonstrated in skin nerves of patients with REM Behavior Disorders that are considered prodromal PD[16,17] thus skin pathologic αSyn can be considered a promising early peripheral histopathological marker of synucleinopathy.

The association of small fiber neuropathy in PD has been demonstrated previously and it has been found that intraepidermal nerve fibers density reflects disease progression[18,19]. Hence, the skin biopsy is a useful tool for studying neurodegeneration in PD and for establishing a pre-mortem histopathological diagnosis of the disease. Indeed, skin biopsy has a great advantage of being an easily accessible and minimally invasive procedure, allowing the analysis of nervous

tissue prone to the pathology. Finally, the possibility of repeating the skin biopsy in the course of follow-up of the same patients allows studying the longitudinal correlation with disease progression.

In our laboratory, exploiting a double immuno-staining with PGP9.5 and the conformation-specific monoclonal 5G4 antibody, that recognizes disease specific forms of αSyn including small aggregates[20,21], we were able to show the presence of αSyn aggregates in skin nerves with a promising high diagnostic efficiency[19]. Immunofluorescence analysis of the skin biopsy in conformational diseases stands out as a promising source of biomarkers by combining both the detection of protein aggregates and the measure of the neurodegeneration in vivo. Hereafter, we illustrate an easy and versatile protocol on handling the skin biopsy and performing the free-floating immunofluorescence staining for detecting αSyn aggregates. Moreover, this protocol can be adapted for targeting any other protein of interest expressed in skin PNS.

The following study protocol has been used to evaluate the diagnostic utility of aggregated αSyn analysis in the PNS of PD by skin biopsy[19]. Inclusion criteria for PD were: a definite clinical diagnosis according to the UK Brain Bank diagnostic criteria, disease duration at least 3 years, no family history, and no major cognitive impairment or major dysautonomic symptoms in the history. Exclusion criteria were known causes of neuropathy (glycated hemoglobin, creatinine, vitamin B12, TSH, serum immunofixation, HIV, HCV, syphilis, and borreliosis). Each subject underwent to 3 mm-diameter skin biopsies at three anatomical sites (neck at C8 dermatomal level, thigh 10 cm above the knee, leg 10 cm above lateral malleolus) on the side, which was clinically more affected. In general, the following protocol is about handling the skin biopsy and performing the free-floating immunofluorescence staining and analysis. Hence it can be adapted and used for the detection of other proteins of interest in skin tissue.

PROTOCOL:

The protocol has been approved by the Cantonal Ethics Committee and all enrolled subjects gave written informed consent to the study.

1. Skin biopsy collection

1.1. Let a qualified physician perform the skin biopsy in an appropriate clinical setting.

1.2. Choose the area to perform the skin biopsy and clean it with an alcohol swab.

1.3. Prepare the anesthetic solution with 1cc of lidocaine 2%.

1.4. With the needle parallel to the area, inject local anesthesia subcutaneously.

1.5. After checking the effect of anesthesia, take the 3 mm disposable punch and apply rotational and delicate downward pressure to rotate it down through the epidermis and dermis, until the subcutaneous fat is reached.

1.6. Withdraw the punch.

1.7. Use disposable forceps to gently pull the skin plug up.

1.8. Use scissors to cut out the base of the specimen at the level of the fatty tissue.

1.9. Place the specimen in a tube containing 10 mL of periodate-lysine-paraformaldehyde (PLP) fixative solution.

1.10. Disinfect the area and cover it with an adhesive bandage.

2. Tissue fixation and storage

2.1 Make fresh PLP fixative solution (see **Table 1**).

2.2 Immediately after the collection of the skin biopsy, submerge it in a tube containing 10 mL of PLP fixative solution and incubate it overnight (O/N) at 4 °C.

2.3 The day after, under the fume hood, remove the PLP fixative gently and in the same tube, wash the biopsy 3 times for 5 min with 5 mL of 0.1 M Sorensen's solution (**Table 1**).

2.4 Discard the Sorensen's solution and incubate the biopsy with 5 mL of cryo-protectant solution O/N at 4 °C.

2.5 Store the biopsy at 4 °C if the cut with a cryotome is performed within 1 week; store the biopsy at -20 °C if the cut is performed within 3 months; embed the biopsy in a cryomold (steps 3.2-3.4) and store it at -80 °C to conserve it for a longer period of time.

3. Tissue cut with a cryotome

3.1 Set the cryotome at -20 °C.

3.2 Take a cryomold and fill it up with the cryo-embedding medium. Avoid creating bubbles.

3.3 Using tweezers immerse the biopsy into the cryo-embedding medium with the longitudinal axis (epidermis - dermis) parallel to the bottom of the cryomold.

3.4 Snap freeze the sample with liquid nitrogen to obtain a solid cube of cryo-embedding medium containing the biopsy in the right orientation.

3.5 Put the sample in the cryostat and wait for 30 min to allow the biopsy to acclimatize.

3.6 Fix the sample on the cryostat and cut 50 µm sections.

3.7 With the help of a little brush, transfer the cryo-sections in a 96 well plate containing 200 µL of antifreeze solution in each well. One section per well.

3.8 Store at -20 °C.

NOTE: If the analyze the entire biopsy is not analyzed, cut it only partially. In this case, the sections must be stored at -20 °C and the remaining part of the biopsy at -80 °C to conserve it for a longer period of time.

4. Immunofluorescence staining

4.1 Fill a 96 well plate, with 100 µL of washing solution.

4.2 Transfer the sections to be analyzed from the storage plate to the new one containing the washing solution. 1 section per each well (4 sections per anatomical site, per patient: usually a total of 12 sections per patient).

4.3 Leave the section in the washing solution for 10 min at room temperature (RT). Transfer the section into another well containing the same solution and repeat the wash.

4.4 Move the sections into new wells containing 100 µL of Blocking solution and incubate for at least 90 min and for a maximum of 4 h at RT.

4.5 Dilute the primary antibodies anti-PGP9.5 (Rabbit polyclonal, 1:1000) and anti-5G4 (Mouse monoclonal, 1:400) in the working solution.

4.6 Transfer the sections into new wells containing 100 µL of the working solution of the primary antibodies and incubate them O/N at RT.

4.7 As step 4.3, wash the sections 2 times for at least 10 min at RT, with 100 µL of washing solution.

4.8 Dilute the secondary antibodies (conjugate with different fluorophores) Goat anti-Rabbit to detect PGP9.5 (1:700) and a Goat anti-Mouse to detect 5G4 (1:700) in the working solution.

4.9 Transfer the sections into new wells containing 100 µL of the working solution of the secondary antibodies for 90 min at RT. From this point, cover the 96 well plate with aluminum foil to avoid the bleaching of fluorophore conjugated with secondary antibody/ies.

4.10 As step 4.3, wash the sections 2 times for at least 10 min, with 100 µL of the washing solution.

4.11 Transfer the sections into new wells containing 100 µL of DAPI (diluted 1:1000 in PBS 1x) for 5 min at RT.

4.12 As step 4.3, wash the sections 2 times for at least 10 min, with 100 µL of the washing solution.

4.13 Mount the sections on a slide in the correct position avoiding misfolding.

4.14 Add a few drops of the mounting medium on the slide and cover with a coverslip.

4.15 Let slides dry O/N before using the confocal/fluorescence microscope.

4.16 Store the slides in an appropriate box at 4 °C avoiding the light exposure. If accurately stored, the signal will be visible for about 6 months.

NOTE: The transfer of a section from the 96 well plate containing the newly cut slices to the 96 well plate containing the washing solution (step 4.1) is performed using a small brush that helps to pick up the biopsy. After the transfer of the section into the first well, every time the slice needs to be incubated with a different solution, move it from well to well with the help of the brush.

5. Immunofluorescence Imaging

5.1. View sections under an inverted fluorescence microscope or a confocal microscope (20X, 40x or higher magnification, use successive frames of 2 µm increments on a Z-stack plan for best results).

5.2. Acquire images by a microscope connected camera

5.3. Use an adequate imaging software (i.e. ImageJ) to analyze positive signals in sections in terms of spatial distribution and intensity of the signal. To do this perform the steps mentioned below.

5.2.1. Open the file with ImageJ software.

5.2.2. Click on **Image > color > split channels** in order to analyze each color channel.

5.2.3. Click **Image > stack > Z project** to obtain a merge of multiple section acquisitions.

5.2.4. Click **Image > color > merge** channels to obtain a merge of different color channels of the same acquisition.

[Place **Table 1** here]

REPRESENTATIVE RESULTS:

Following the described procedure (**Figure 1**), we detected αSyn aggregates, labeled with 5G4 antibody, in dermal nerve fascicles innervating autonomic structures of PD patients. Morphology

of alpha-synuclein deposits appears as a dotted signal along the axons of dermal nerves (**Figure 2**). Indeed, exploiting this protocol in 19 PD patients and 17 controls in skin biopsies at three anatomical site (cervical, thigh and distal leg) we found that 5G4 had 81% of sensitivity and 86% of specificity respect to healthy controls and P-αSyn had 56% of sensitivity and 100% of specificity[19].

In particular, we found 5G4 and P-αSyn positive deposits mainly in nerves around sweat glands but also in muscle erector pili, small vessels, and subepidermal and dermal plexus, never in intraepidermal nerve fibers.

Generally, within the sweat gland's lumen, it is possible to observe a nonspecific signal, which could be misinterpreted as 5G4/ P-αSyn positivity. This type of signal is dotted, spherical and it is due most probably to intraluminal auto-fluorescent material, as we demonstrated in technical controls without primary and secondary antibodies (**Figure 3**). Co-localization with PGP9.5 that marks the nerve fibers, which morphologically are filamentous and elongated, helps to identify the correct signal. Therefore, the specificity of 5G4 signal is highly increased by a double immunostaining with an axonal marker (**Figure 4**).

An accurate fixation of the biopsy is a mandatory factor for the good quality of immunofluorescence staining, and for the reliable interpretation of the fluorescent signals. If the fixative is not correctly prepared or if an over-fixation has occurred, the result will be a high auto-fluorescence that will mask the signal of nerve fibers crossing the dermal-epidermal junction or innervate the main dermal structures (**Figure 5B, D, F**). In this case, the inability to visualize correctly the PGP9.5 positive fibers will make difficult to analyze correctly also 5G4.

Finally, this protocol can be used for the detection of any protein of interest, including among others αSyn, P-αSyn or tyrosine hydroxylase (TH) and vasoactive intestinal peptide (VIP) that mark respectively adrenergic and cholinergic subtype of autonomic innervation (**Figure 6**).

FIGURE AND TABLE LEGENDS:

Table 1. **Required solutions.** List of required solutions for the protocol and brief description of how to prepare it.

Figure 1. Schematic representation of the protocol. Graphical representation of critical steps in the fixation, cut and staining of skin sections for the visualization of αSyn aggregates in cutaneous peripheral nerve fibers.

Figure 2. Presence of 5G4 aggregates in dermal nervous fibers. Confocal merged Z-stack images of immunofluorescence with PGP9.5 (green) and 5G4 (red) of dermal nerves around sweat glands in PD patients (**A–C**) and healthy subject (**D-F**). This figure is modified from [19]. 3D visualization of the same sweat gland showed above from PD (**G**) and healthy (**H**) subjects. Indicated by white arrows, yellow colocalization of the two markers along axons. Scale bar 50 µm.

Figure 3. Auto-fluorescent signals: technical controls. Confocal merged Z-stack images of skin section stained with DAPI and without the primary antibody (**A-B**), without the secondary antibody (**C-D**), without primary and secondary antibodies (**E-F**). The images show the presence of non-specific dotted signals in sweat glands structures in all conditions. Scale bar 50 µm.

Figure 4. Consider nerve fibers morphology to recognize non-specific signal. Confocal merge Z-stack images of immunofluorescence with PGP9.5 (green) and 5G4 (red) of dermal nerves around sweat glands. White arrows indicate positive structures; asterisks indicate unspecific staining in non-neuronal structures. Scale bar 50 µm. This figure is modified from [19].

Figure 5. Incorrect fixation compromises the quality of immunofluorescence staining. Confocal merge Z-stack images of immunofluorescence with PGP9.5 (green) and DAPI (blue) of intra epidermal nerves fibers (**A-B**) and dermal nerves around sebaceous gland (**C-D**) and sweat glands (**E-F**). On the left result of correct fixation, on the right result of over fixation. Scale bar 50 µm.

Figure 6. Examples of other proteins detectable in skin nerves. Microscope images of dermal structures stained using a free-floating immunofluorescence assay. In red αSyn (**A**), p-αSyn (**B**), and TH (**C**), in green VIP (**D**). Scale bar 50 µm.

DISCUSSION:

We describe a free-floating immunofluorescence assay for skin biopsies for the diagnosis of PD: it exploits double immunostaining with anti-PGP9.5 antibody, a panaxonal marker, and anti-5G4, a conformation specific antibody that recognizes the aggregated form of αSyn.

The great advantages of skin biopsy for diagnostic purpose in PD and possibly in other protein conformational disorders are: 1) the direct access to nervous tissue prone to disease by a mildly invasive technique and thus with an expected better determination sensitivity for αSyn aggregates than biological fluids like blood or CSF; 2) the opportunity to detect and quantify epidermal and autonomic nerve fiber density as a measure of neurodegeneration; 3) the possibility of repeating skin biopsy in the course of follow-up of the same patients, in order to study longitudinally the correlation with disease progression.

The protocol proposed here is rapid, versatile and has few critical steps. First of all, tissue fixation has to be pursued correctly, otherwise a high auto-fluorescence will mask the specific signal and will prevent the correct analysis of the data. The paraformaldehyde in the fixative solution should be made fresh and pH of 7.4 should be accurately checked. It is extremely important to avoid the formation of formic acid that could damage biopsies. Before storage and cut, if the compactness of the biopsy suggests an incorrect fixation, the biopsy should be rinsed again with Sorensen's solution, and re-incubated with PLP solution O/N at 4 °C. Moreover, at the beginning or at the end of the immunofluorescence staining protocol, a few additional steps can be introduced to counteract auto-fluorescence. At the beginning of the protocol (between steps 4.1 and 4.2) a treatment with sodium borohydride solution (1 mg/mL in TBS; 3 times for 10 min), a treatment with hydrogen peroxide (3%; 15 min) or a treatment with glycine (0.1 M in TBS; 1h) can be performed. In alternative at the end of the staining (between steps 4.8 and 4.9), a treatment with

trypan blue (250 µg/mL in TBS; 20 min) or with Sudan Black (0.5% in 70% EtOH; 30 min) can be performed. However, in all cases, in addition to the reduction of auto-fluorescence, a reduction in the specific signal will also occur. Alternatively, biopsies incorrectly fixated can be used to perform classical bright field immunohistochemistry. In this case, however, the double immunostaining for colocalization of 5G4 and PGP9.5 will be more challenging and difficult to analyze.

Another critical step is the insertion of a skin biopsy into the cryomold (step 3.3). The orientation of the biopsy towards the cutting blade is crucial to obtain slices in which epidermis and dermis are both present. The longitudinal axis (epidermis-dermis) has to be parallel to the bottom of the cryomold, so that the side of the biopsy, not the top or the bottom, is facing the operator. The choice of slices thickness, 50 µm, is in accordance with European guidelines for the use of skin biopsy as a diagnostic tool[7]. Moreover, for a αSyn aggregates detection, 50 µm thickness allows with greater probability to have within the section more dermal structures, where pathological deposit are mainly found in PD. Due to the high thickness, to be sure that the whole section is dyed, it is not recommended to place the sections on the slide for staining, but instead, a free floating staining is mandatory. For this procedure, the major difficulty is to transfer the sections from one well to another using a small brush without damaging the sections. It is recommended to insert the tip of the brush below the biopsy that floats in the liquid, allowing the biopsy to settle on the tip of the brush, gently carry the biopsy from one well to another, immerse the biopsy in the new solution and remove the brush making sure that no biopsy residue is left on the brush. It is important that the operator acquire manual skills with practice.

In conclusion, this is a short and easy protocol for the optimal handling and fluorescent immunostaining of skin biopsy. The advantage of this protocol in respect to previous studies is the use of 5G4 antibody, allowing the detection of αSyn small aggregates for the first time in dermal autonomic nerve fibers of PD patients. It can be potentially used for diagnostic purposes in different types of neurodegenerative disorders involving PNS, especially at early phases, when potential cure can be most effective. Limitations of the method is that sensitivity of P-αSyn and specificity of 5G4 are still suboptimal but a combination of different markers in future studies could certainly improve the diagnostic yield of skin biopsy in PD.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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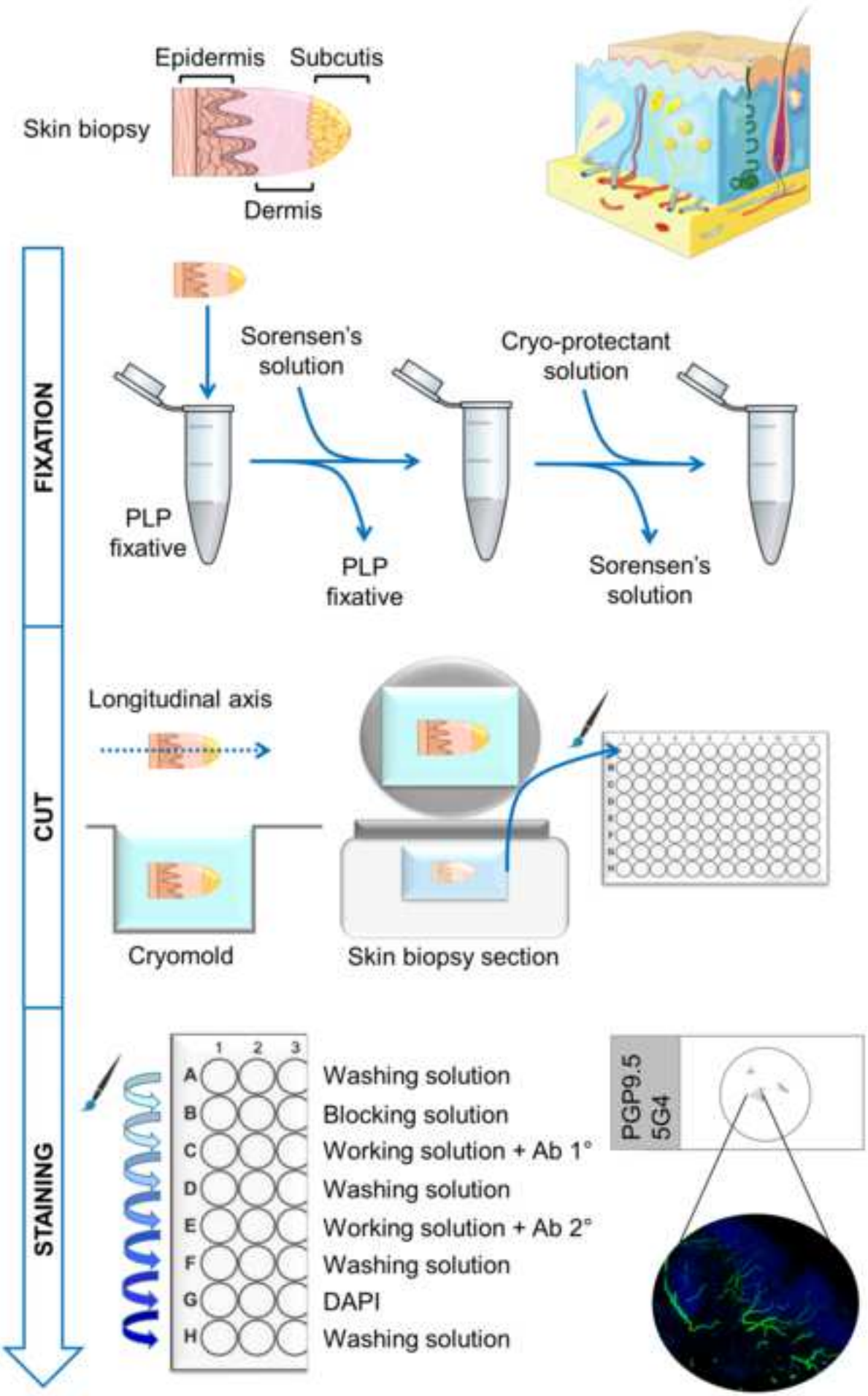

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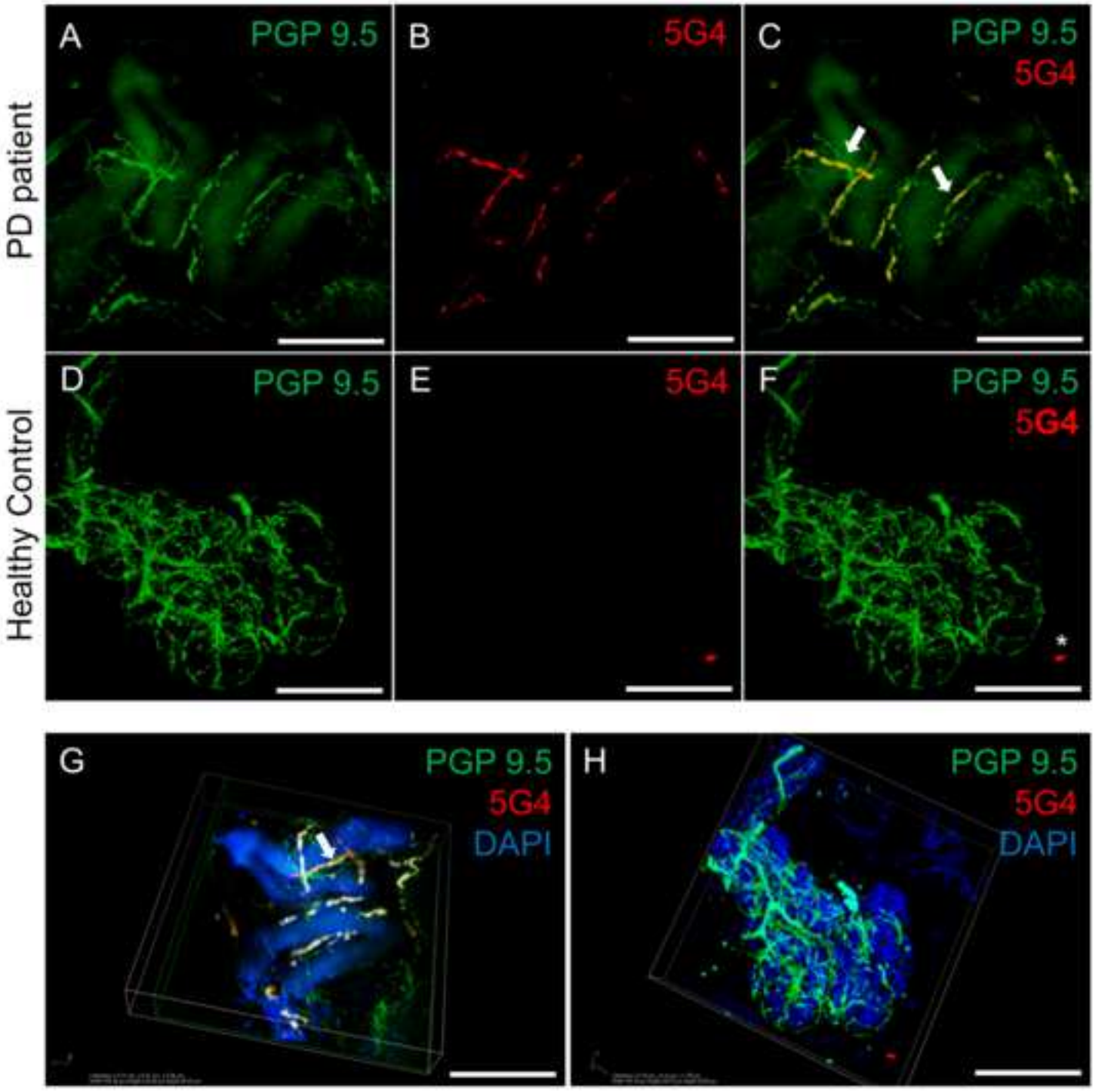

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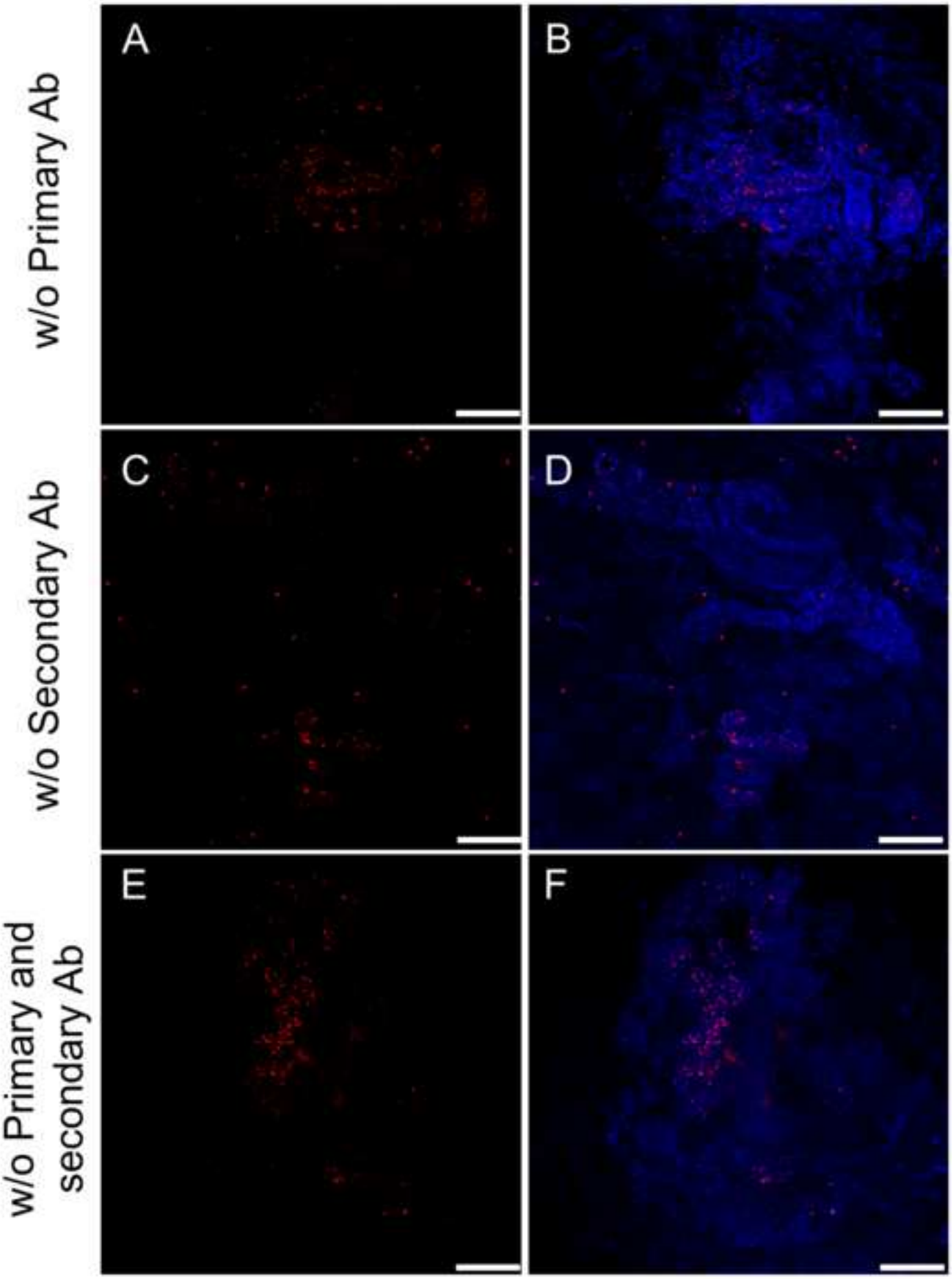

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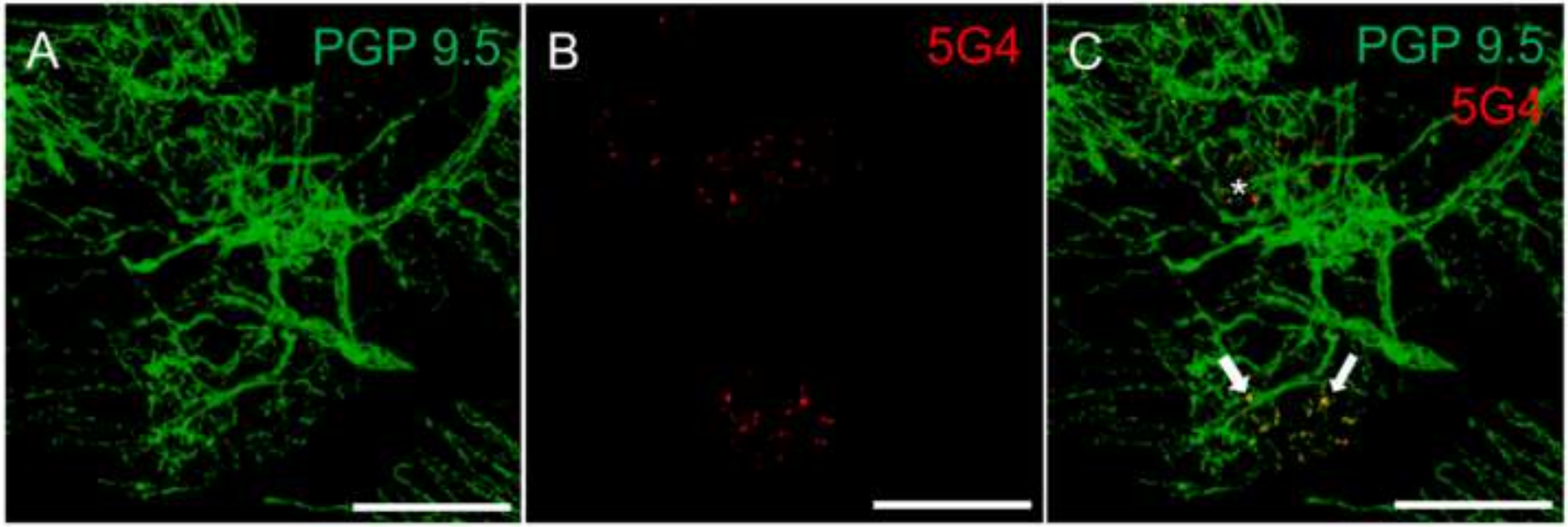

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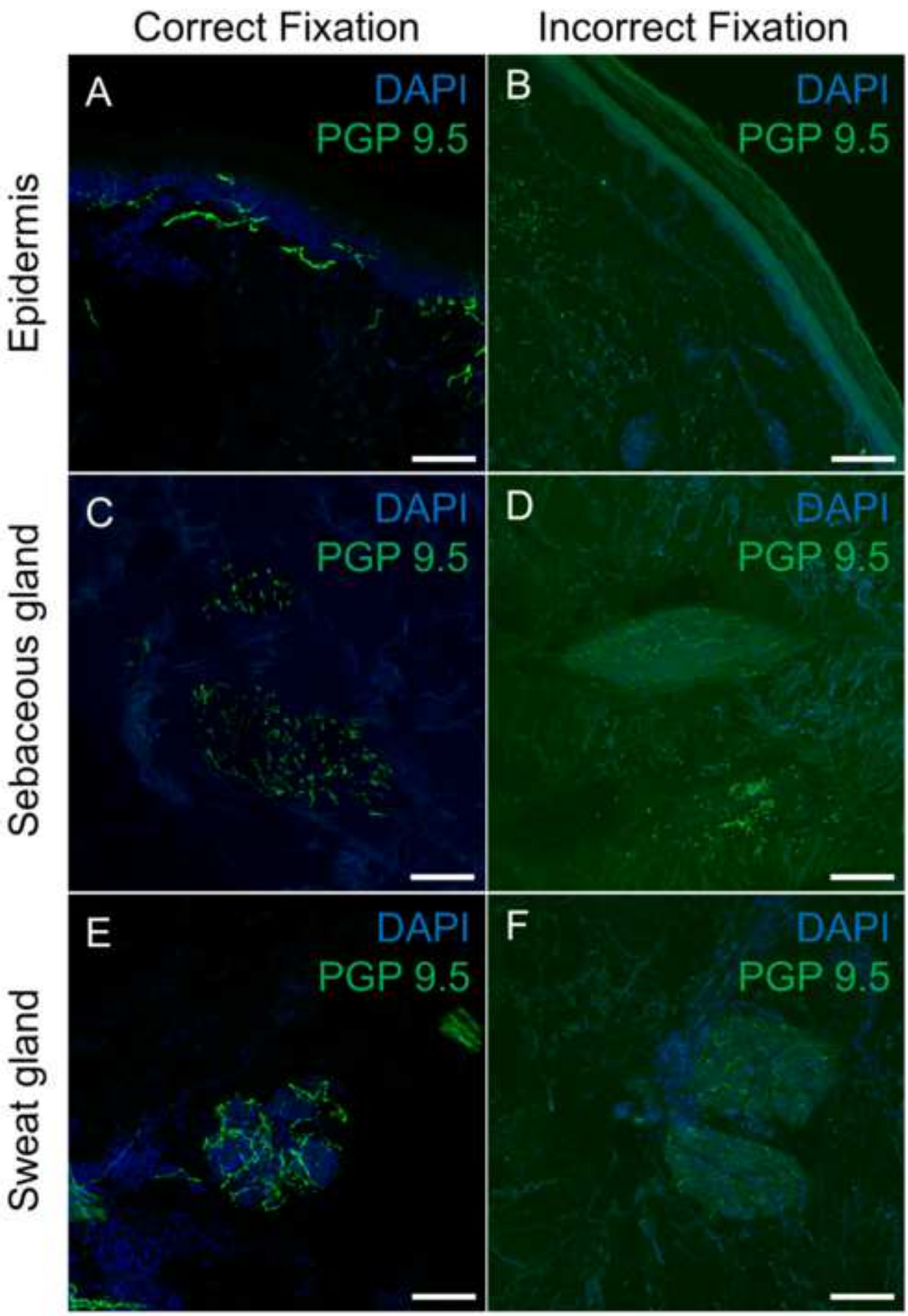

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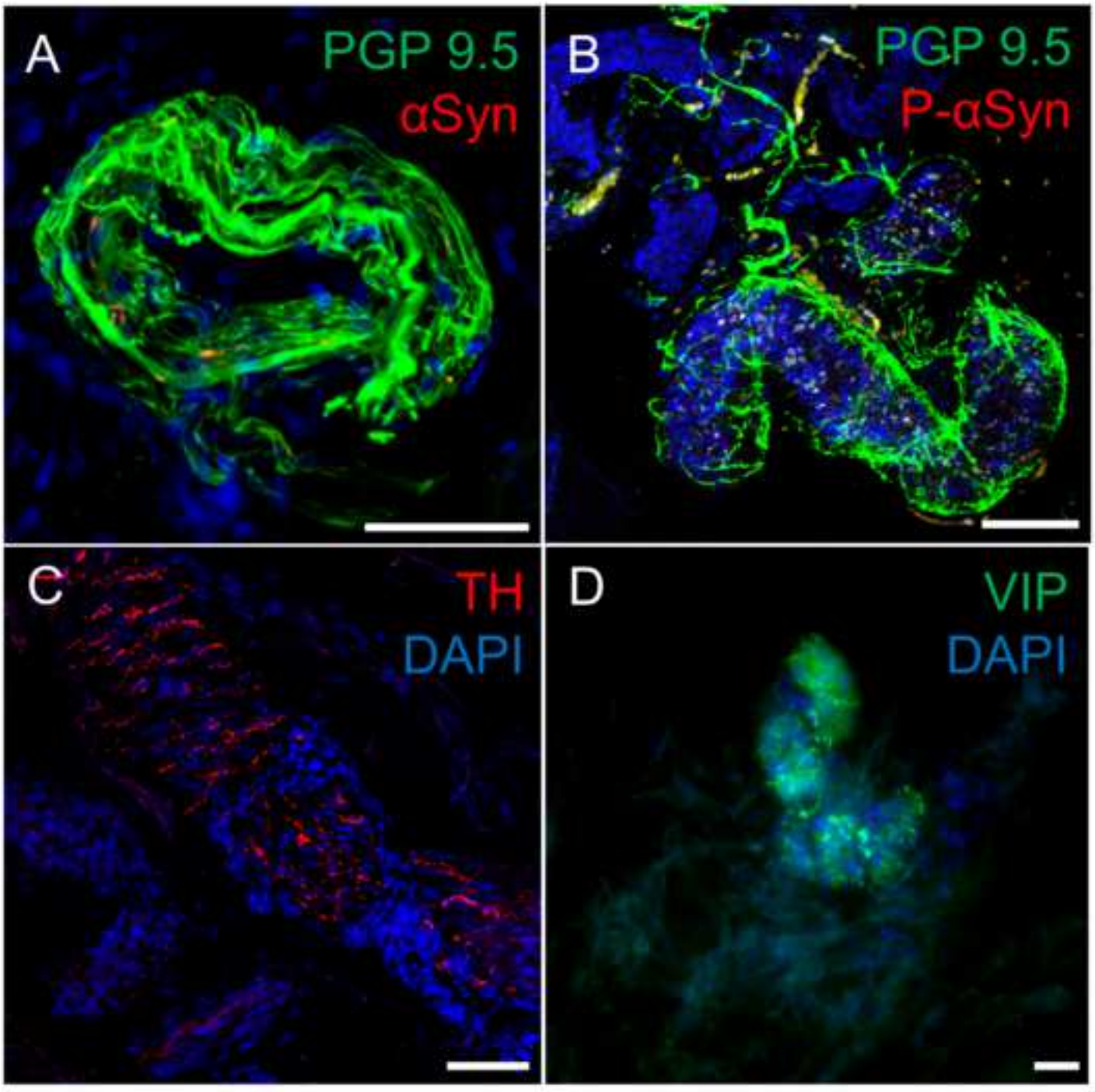

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Antifreeze solution (store at 4 °C for up to 6 months)
Blocking solution (prepare at the moment)
Cryo-protectant (store at 4 °C for up to 6 months)
Disodium hydrogen phosphate solution (store at RT up to 6 months)
Lysine solution (store at 4 °C for up to 3 weeks)
Paraformaldehyde (PFA) 8% (prepare under fume hood and store at 4 °C for up to 1 month)
Phosphate buffer 2x (store at 4 °C for up to 6 months)
PLP fixative solution (prepare at the moment, under fume hood)
Sodium Dihydrogen Phosphate Monohydrate (store at RT up to 6 months)
Sorensen's solution (store at 4 °C for up to 1 month)
Washing solution (prepare at the moment)
Working solution (prepare at the moment)

30% Glycerol 30% Ethylene glycol 30% dH_2O 10% 2x Phosphate buffer
4% Normal Goat Serum 1% Triton X-100 in Washing solution
20% Glycerol 80% Sorensen's solution
0.45M Disodium hydrogen phosphate (Na2HPO4) in dH2O Filter in a sterile bottle
50% of 0.3M L-Lysine monohydrochloride solution 50% of 0.1M Sorensen’s solution pH7.6 pH 7.4, filter in a sterile bottle
2.6M PFA in dH2O (to 55°C_do not exceed 60 °C to avoid formic acid formation) filter in a sterile bottle
6% Monosodium phosphate (NaH2PO4) solution 45% Disodium phosphate (Na2HPO4) solution in dH_2O
25% Paraformaldehyde 8% 0.01M Sodium (meta)periodate 75% Lysine solution
0.52M Sodium Dihydrogen Phosphate Monohydrate (NaH2PO4*H2O) in dH2O Filter in a sterile bottle.
2.5% Monosodium phosphate solution 18.7% Disodium phosphate solution pH7.6, in dH2O
0.25M Trizma base 0.26M NaCl pH7.6, in dH2O
50% Blocking solution 50% Washing solution

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Name of Material/ Equipment	Company
5G4 (anti human αSyneclein 5G4)	Analytik Jena Roboscreen
AlexaFluor 488 Goat anti Rabbit IgG	Invitrogen
AlexaFluor 594 Goat anti Mouse IgG	Invitrogen
Disodium hydrogen phosphate solution	Merk Millipore
Ethylene Glycol	Sigma-Aldrich
Glycerol	Sigma-Aldrich
L-Lysine monohydrochloride	Sigma-Aldrich
Paraformaldehyde	Aldrich Chemistry
PGP9.5	Abcam
Sodium Chloride	Sigma
Sodium Dihydrogen Phosphate Monohydrate	Merck Millipore
Sodium (meta)periodate	Sigma-Aldrich
Trizma Base	Sigma
Tryton X-100	Sigma-Aldrich
Vectashield	Vector Laboratories

Catalog Number	Comments/Description
847-0102004001	Mouse monoclonal
1971418	2mg/ml
1922849	2mg/ml
106586	
324558	
G7757	
L5626	
441244	
ab15503	Rabbit polyclonal
S3014	
106346	
S1878	
T1503	
X100	
H-1000	Mounting medium

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Author(s):	Elena Vacchi, Sandra Pinton, Alain Kaelin-Lang, Giorgia Melli

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Institution:	Neurocenter of Southern Switzerland
Title:	Targeting alpha Synuclein aggregates in cutaneous peripheral nerve fibers by free-floating immunofluorescence assay.
Signature:	Giorgia Melli
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- Line 27-37: This section is not required. Please expand during the first-time use and delete this part.

We had followed the guidelines of the journal defining all abbreviations during the first-time use and then using the acronym. Although, we had created this section following reviewer 2 suggestion: in the first comment asked for a list of abbreviation. Now we have removed the section.

- Line 69, citation. We added citation 1

- Line 79, citation. We added citations 3 and 4

- Line 87, citation. We added citation 9

- Line 116, Highlighted section should not be more than 2.75 pages including headings and spacing. Please check and edit.

The highlighted section is less than 2.75 pages.

- Line 121, The protocol needs to be stand alone in itself. Please add details to this section 1.

We added more details.

- Line 123, From whom – what kind of patients/controls, any age or sex specific bias? Which part of the body is the biopsy taken from? How many per person? Please list all details here.

Before the protocol we added a brief section in which we answer all this questions.

- Line 148, “Is this correct”?

We corrected the mistake.

- Line 164, Where are the sections stored?

We had explained how to store the section at point 3.8 and in the following note.

- Line 166, Added for clarity please check.

We re-wrote the phrase.

- Line 171, “Why so?”

We re-wrote the phrase.

- Line 176, “Isn’t this same as 3.7”?

We clarify the concept, re-writing the first steps.

- Line 179, “Do you transfer the sections from one plate to another or aspirate the liquid and add the second round to the same plate?”

We had explained this point in the note, however we re-wrote some passages to clarify the concept.

- Line 188, “Added this here, please check. Please bring out this kind of clarity throughout.”

We re-wrote the phrase.

- Line 217, “Please reword”.

We change “catch” with “pick up”

- Line 227, Analyze for what and how. Please provide all the button clicks, graphical user interface, etc. We need discrete experimental steps. For example, Click “Open”. Use the xxx tool and count the number of cells stained. Then Click “Analyze”

We added few steps.

- Line 236, Include this in the protocol somewhere.

We added this details at the beginning of the protocol.

- Line 240, Reworded.

We changed the phrase.

- Line 240, Please provide a Table to show how all of the patients used for the study were classified as PD? What does G and H represent?

We provide this information at the beginning of the protocol. We explained figure 2G and 2H.

- Line 304, “Please include limitation of the protocol The significance with respect to existing methods Any future applications of the technique

We included this information in the last paragraph of discussion:
“The advantage of this protocol in respect to previous studies is thee use of 5G4 antibody, allowing the detection of αSyn small aggregates for the first time in dermal autonomic nerve fibers of PD patients. It can be potentially used for diagnostic purposes in different types of neurodegenerative disorders involving PNS especially at early phases when potential cure can be mostly effective. Limitations of the method is that sensitivity of P-αSyn and specificity of 5G4 in identifying PD are still suboptimal but a combination of different pathological markers in future studies could certainly improve the diagnostic yield of skin biopsy in PD.”

- Line 344 “??”

We changed “furtherly” with “moreover”.

- Line 352 “??”

We changed “in alternative” with “alternatively”.

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