Chapter 4

Immunohisto- and Cytochemistry Analysis of Connexins

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Abstract

Immunohistochemistry (IHC) is a ubiquitous used technique to identify and analyze protein expression in the context of tissue and cell morphology. In the connexin research field, IHC is applied to identify the subcellular location of connexin proteins, as this can be directly linked to their functionality. The present chapter describes a protocol for fluorescent IHC to detect connexin proteins in tissues slices and cells, with slight modifications depending on the nature of biological sample, histological processing, and/or protein expression level. Basically, fluorescent IHC is a short, simple, and cost-effective technique, which allows the visualization of proteins based on fluorescent-labeled antibody–antigen recognition.

Key words Connexins, Antibody, Immunohistochemistry, Immunofluorescence, Immunocytochemistry, Microscopy, Morphology, Fluorophores, Localization

1 Introduction

Immunohistochemistry (IHC), or immunocytochemistry (ICC), is a commonly used technique to monitor protein expression and localization in tissue or in vitro cultures, respectively, using bright-field or fluorescent microscopy. Direct or indirect immunofluorescence is a powerful IHC-based technique that uses fluorescent-labeled antibodies to visualize protein expression while maintaining the composition, cellular characteristics, and structure of native tissue (Fig. 1) [1, 2]. Coon and colleagues were the first to describe the direct immunofluorescence technique using an antibody attached to a fluorescent dye, fluorescein isocyanate, to localize its respective antigen in a frozen tissue section [3, 4]. Subsequently, immunochemical methods based on peroxidase-labeled antibodies were introduced, allowing the development of new IHC, such as formalin-fixed paraffin-embedded (FFPE) tissues [5–9]. Currently, the use of antibodies to detect and localize individual or multiple

Mathieu Vinken and Scott R. Johnstone (eds.), *Gap Junction Protocols*, Methods in Molecular Biology, vol. 1437,
DOI 10.1007/978-1-4939-3664-9_4,

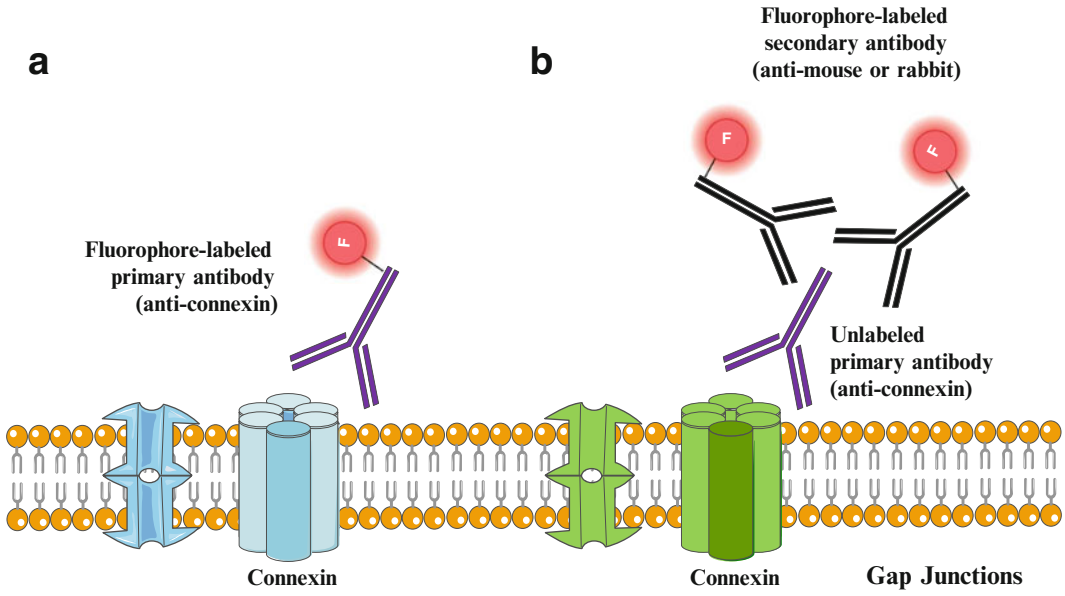

Fig. 1 *Direct and indirect immunofluorescence methods.* (**a**) Direct immunofluorescence method uses fluorophore-labeled primary antibody to bind directly to the connexin protein. This technique is rapid and quite specific. However, usually demands higher concentration of primary antibody and there are few options of antibodies conjugated directly to a fluorophore. (**b**) Indirect immunofluorescence method, or sandwich method, is a 2-step technique that uses an unlabeled primary antibody to bind the connexin protein, after which a fluorophore-labeled secondary antibody is used to detect the connexin antibody. This technique is more sensitive because more than one secondary antibody can bind to each primary antibody, which amplifies the fluorescence signal; however, this procedure has higher potential for cross-reactivity and immunostaining background, is more complicated and time consuming when compared to direct immunofluorescence

proteins in situ has developed into a powerful research tool in almost every field of biomedical research [10].

Gap junctions (GJs) are grouped in plaques at the plasma membrane surface of two adjacent cells and are composed of two juxtaposed connexons or hemichannels, each built up by six proteins named connexins [11]. At present, more than 20 connexin isotypes have been identified, which are expressed in a cell-specific way. Gap junction intercellular communication (GJIC) allows the direct flux of small and hydrophilic molecules, i.e., cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP3), and ions, through GJs channels [12–15]. GJs are dynamic and the half-life cycle of connexins is short (<5 h) [16]. Connexins are biosynthesized on endoplasmic reticulum membranes and delivery happens to the plasma membrane as oligomerized hexameric hemichannels (connexons) [17]. Regulation of connexin synthesis can occur on transcriptional, translational, and posttranslational levels, resulting in a downregulation or lack of connexin expression and GJIC. In disease, connexin proteins can be abnormally localized within the cytoplasm. The exact mechanisms are still unknown, but impaired trafficking of the connexins to the membrane and increased internalization and

degradation of connexons have been suggested [18–20]. It is known that alterations in the expression pattern and location of connexins are associated with potential oncogenesis and other chronic disorders, i.e., in liver and cardiac diseases [21–26]. In this regard, detection of aberrant subcellular location of connexin proteins is quite important to understand its role in pathological conditions.

In this chapter, fluorescent IHC-based protocols optimized to detect connexin proteins in cells or tissues slices will be outlined. Depending on the nature of biological sample, histological processing and/or protein expression level slight modifications are defined. The first step comprehends the adequate handling and fixation of cells or tissue specimens. The objective is to preserve tissue morphology and retain the antigenicity of the target proteins. To avoid loss during the procedure, cells or tissue sections should be placed on adhesive coating slides [1, 2]. For FFPE samples, tissue slides are deparaffinized with xylene and rehydrated in a series of ethanol solutions with decreasing concentrations. Afterward, the slides are subjected to heat-induced antigen retrieval (HIAR) in Tris–EDTA buffer (pH 9.0) or alternative method to reveal epitopes masked during the sample processing [27]. The background immunostaining caused by nonspecific antibody binding to endogenous Fc receptors or a combination of ionic and hydrophobic interactions should be blocked by bovine serum albumin (BSA), nonfat dry milk, gelatin, glycine, or normal serum from the species that the secondary antibody was raised in [28]. Incubation of monoclonal or polyclonal primary antibody is done for short (30–60 min, at 37 °C) or long time (overnight, at 4 °C) [1, 2]. Subsequently, the detection of connexins is performed using fluorescent-labeled secondary antibodies. This technique takes advantage of light emission with different spectral peaks against a dark background, with several options of fluorophores with different wavelengths of light emission (Table 1). The signal can be amplified by a tyramide signal amplification (TSA) method [28].

Table 1
List of most common dyes for immunofluorescence with their excitation and emission wavelength peaks

Color	Label (s)	Excitation (nm)	Emission (nm)
Blue	DAPI	365/50	450/65
Green	FITC; GFP; Alexa Fluor® 488	475/40	535/45
Yellow	TRITC; Alexa Fluor® 555, 546, 568; Cy3	546/12	585/40
Red	Alexa Fluor® 594; Propidium iodide; eFluor® 615; Texas Red	560/55	645/75
Near infrared	eFluor® 660; Alexa Fluor® 647; Cy5	620/60	700/75
Infrared	IRDye 800®	787	812

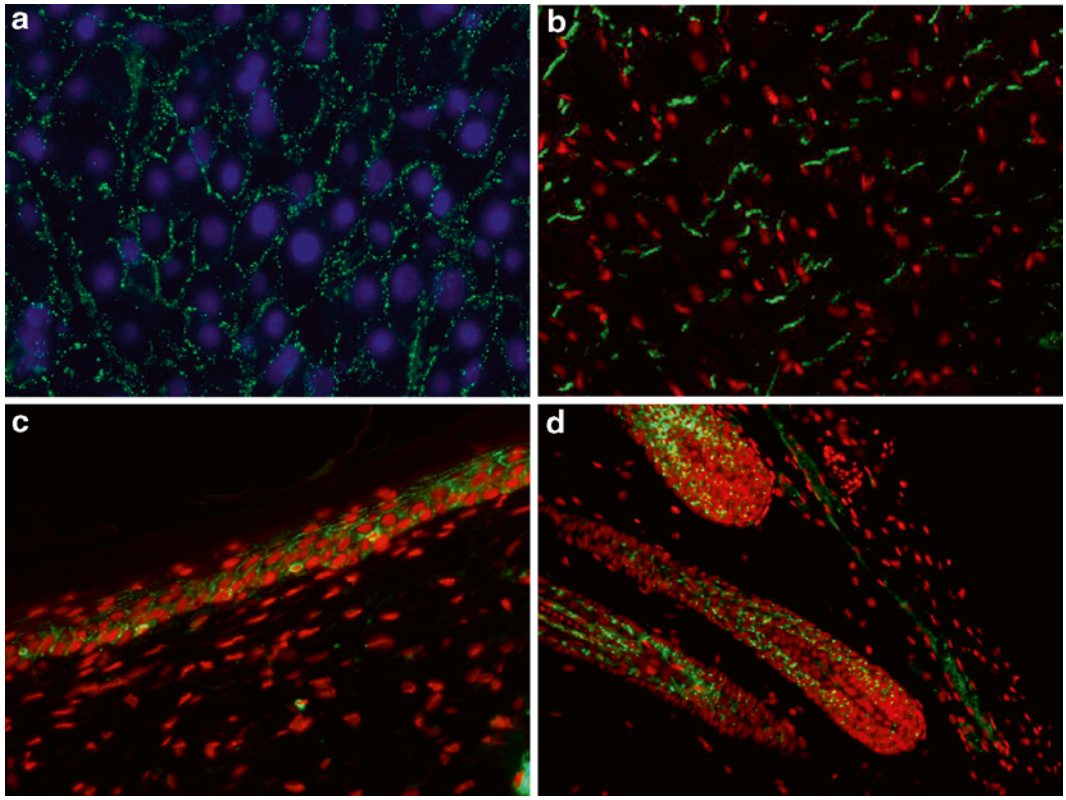

Fig. 2 *Detection of connexins (Cx) in frozen and paraffin-embedded tissue sections.* (**a**) Immunodetection of Cx32 in frozen sections of human liver tissue fixed in ice cold acetone, using 1/500 anti-Cx32 polyclonal antibody produced in rabbit (Sigma-Aldrich, USA), 1/200 Alexa Fluor® 488-conjugated secondary antibody polyclonal goat anti-rabbit IgG (H + L) (Life Technologies, USA), and 1/1000 DAPI (Life Technologies, USA) for nuclear counter stain (200×). (**b**) Cx43 detected in paraffin-embedded sections of mouse heart fixed in methacarn solution, using 1/500 anti-Cx43 polyclonal antibody produced in rabbit (Sigma-Aldrich, USA), 1/200 Alexa Fluor® 488-conjugated secondary antibody polyclonal goat anti-rabbit IgG (H+L) (Life Technologies, USA) and 1/1000 propidium iodide (Sigma-Aldrich, USA) for nuclear counter stain (400×). (**c, d**) Cx43 detected in paraffin-embedded sections of mouse skin epidermis and hair bulb using the tyramide signal amplification (TSA) method with 1/1000 anti-Cx43, polyclonal antibody produced in rabbit (Sigma-Aldrich, USA), 1/500 polyclonal goat anti-rabbit Immunoglobulins/HRP (Dako Cytomatic, USA), 1/100 tyramide working solution and 1/1000 propidium iodide (Sigma-Aldrich, USA) for nuclear counter stain (400×)

Finally, the slides are incubated with a DNA-fluorescent marker for the nuclei counterstain, mounted on antifade media to avoid photobleaching and then visualized under a fluorescent microscope with the appropriate filters (Fig. 2).

2 Materials

2.1 Frozen Tissue Sections

1. Optimal cutting temperature (OCT)-embedding medium (Leica Biosystems, Germany).
2. Cryomolds.
3. Liquid nitrogen.

4. Isopentane.
5. Cryostat.
6. Disposable blades and brushes.
7. Microscope adhesive glass slides (25 × 75 mm) (*see* **Note 1**).
8. Acetone, histological grade.

2.2 Paraffin-Embedded Tissue Sections

1. Methacarn solution. Methanol:chloroform:glacial acetic (60:30:10 v/v). This solution can be stored for 3 months at 4 °C.
2. 10 % neutral buffered Formalin: 4 g NaH_2PO_4, 6.5 g Na_2HPO_4, 100 mL formaldehyde 37–40 % (w/v) (*see* **Note 2**), 900 mL distilled water. Mix and adjust for pH 6.8. This solution can be stored at room temperature.
3. Xylene, histological grade (*see* **Note 3**).
4. Xylene: 100 % Ethanol (1:1, v/v).
5. Ethanol, anhydrous denatured, histological grade (70, 95 and 100 % EtOH). Prepare the EtOH solutions in distillated water (v/v).
6. Fume hood.
7. Microtome, blades, and thermostatic water bath.
8. Microscope adhesive glass slides (25 × 75 mm).
9. Oven (55–65 °C).
10. Tris-ethylenediaminetetraacetic acid (EDTA) buffer: 10 mM Tris Base, 1 mM EDTA Solution, 0.05 % Tween® 20 in ultrapure water. Adjust to pH 9.0. This solution can be stored at room temperature for 3 months or at 4 °C for longer storage.
11. Electronic pressure cook (*see* **Note 4**).

2.3 In Vitro Cultures

1. Cell line or primary cells.
2. Cell culture medium and supplements.
3. Thermostatic water bath (37 °C).
4. Laminar flow cabinet.
5. CO_2 humidified incubator (37 °C ± 1 °C, 90 % ± 5 % humidity, 5 % ± 1 % CO_2).
6. Tissue culture plates (6-, 12-, or 24-well plates) or chamber culture slides (*see* **Note 5**).
7. Sterile glass coverslips (*see* **Note 6**).
8. Acetone, histological grade.

2.4 General Procedure for Fluorescent IHC

1. Vessels with slide rack and plastic slide holders.
2. Humidified chamber (*see* **Note 7**).
3. Shaker.
4. Refrigerator or refrigerated incubator (2–8 °C).

5. Wash buffer: 10× Phosphate-buffered saline (PBS) with Tween® 20. 70 mM Na_2HPO_4, 30 mM NaH_2PO_4, 1.37 M NaCl, 0.5% Tween® 20 in distilled water (*see* **Note 8**). Adjust to pH 7.2–7.4 (*see* **Note 9**). To prepare 1 L of 1× PBS: add 100 mL of 10× PBS with Tween® 20–900 mL of distilled water. Mix and adjust pH if necessary. This solution can be stored up to 6 months at room temperature.
6. Blocking buffer: 5% (m/v) nonfat dry milk powder in 1× PBS (*see* **Note 10**). Prepare *ex tempore*.
7. Buffer for antibody dilution: 1% BSA (Sigma-Aldrich, USA) and 0.1% Sodium Azide diluted in 1× PBS without Tween® 20. This solution can be stored for 3 months at 4 °C (*see* **Note 11**).
8. Appropriate monoclonal or polyclonal primary antibodies (*see* **Note 12**).
9. Isotype IgG or preimmune sera (*see* **Note 13**).
10. Alexa Fluor® 488 goat anti-mouse or Alexa Fluor® 594 goat anti-rabbit as appropriate secondary antibody (Life Technologies, USA). Other labels can also be used (i.e., FITC or Texas Red) (Table 1).
11. Nuclear dyes: propidium iodide (PI) (Sigma-Aldrich, USA) diluted in 1× PBS without Tween® 20 (1 μg/mL) or 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, USA) diluted in 1× PBS without Tween® 20 (10 μg/mL).
12. Aqueous antifading mounting medium (Vector Laboratories, USA).
13. Glass coverslips.
14. Nail polish.
15. Fluorescence microscope with the appropriated filters (Table 1).

2.5 Tyramide Signal Amplification (TSA)

1. TSA Fluorescein Kit (Perkin Elmer, USA).
2. Blocking buffer: 0.1 M Tris–HCl (pH 7.5), 0.15 M NaCl, 0.5% Blocking Reagent. Prepare according to the manufacturer's recommendations (Perkin Elmer, USA).
3. Fluorophore tyramide stock and working solution. Prepare according to the manufacturer's recommendations (Perkin Elmer, USA).
4. Polyclonal Goat Anti-Rabbit or Mouse Immunoglobulins/Horseradish peroxidase (HRP) (Dako Cytomatic, USA).

3 Methods

3.1 Fluorescent IHC in Frozen Tissue Sections

1. Snap freeze fresh tissue in liquid nitrogen or isopentane precooled in liquid nitrogen (*see* **Note 14**). Store flash frozen tissue at –80 °C.

2. Embed flash-frozen tissue in OCT compound in cryomolds. This can be stored at −80 °C.
3. Cut 5–10 μm thick sections in the cryostat and mount on microscope adhesive glass slides (*see* **Note 15**).
4. Store slides at −80 °C for long-term storage or at −20 °C for few weeks. For best results, use them immediately.
5. Before staining, fix the slides in ice cold acetone for 10–20 min at −20 °C (*see* **Note 16**).
6. Wash slides three times for 5 min with wash buffer on a shaker.
7. Incubate slides with monoclonal or polyclonal primary antibody diluted in antibody dilution buffer for 1 h at 37 °C in a humidified chamber (*see* **Note 17**). Use the appropriate negative and positive controls (*see* **Note 18**).
8. Wash slides three times for 5 min with wash buffer on a shaker.
9. Incubate slides with a secondary antibody Alexa Fluor® 488 goat anti-mouse or Alexa Fluor® 594 goat anti-rabbit, diluted in antibody dilution buffer (10 μg/mL), for 1 h at room temperature in a humidified chamber. From this point, the procedures should be done in the dark to avoid photobleaching.
10. Wash three times for 5 min with wash buffer on a shaker.
11. Perform the nuclear counter stain with PI for 15 min or with DAPI for 5 min at room temperature (*see* **Note 19**).
12. Wash three times for 5 min with wash buffer on a shaker.
13. Mount coverslip with a drop of antifading mounting medium.
14. Seal coverslip with nail polish (*see* **Note 20**).
15. Store in dark at 4 °C.

3.2 Fluorescent IHC in Paraffin-Embedded Tissue Sections

1. Cut the tissue specimens in small fragments (*see* **Note 21**) and immerse in methacarn fixative solution for 12 h (*see* **Note 22**) or in 10% neutral buffered formalin for 18–24 h (*see* **Note 23**).
2. Embedded fixed samples in paraffin wax (Table 2).
3. Cut 3–5 μm tick sections using a rotary microtome and float the sections in 40–45 °C thermostatic water bath.
4. Place the sections on microscope adhesive glass slides within the water bath and remove the water excess.
5. Dry slices in an incubator at 56–60 °C for 2 h (*see* **Note 24**).
6. Store slides at 4 °C for short period and at −20 °C or −80 °C for long-term usage (*see* **Note 25**).
7. Before proceeding with the staining protocol, the slides should be deparaffined in xylene (*see* **Note 26**) and rehydrated in ethanol (Table 3).
8. Place slides in plastic slide holders and fill with Tris–EDTA buffer.

Table 2
Tissue processing for *paraffin wax embedding*

Solution	Incubation/time
70% Ethanol	Two changes[a], 1 h each
80% Ethanol	One change, 1 h
95% Ethanol	One change, 1 h
100% Ethanol	Three changes[a], 1.5 h each
Xylene	Three changes[a], 1.5 h each
Paraffin wax (58–60 °C)	Two changes[a], 2 h each

[a]Use different solutions for each change

Table 3
Deparaffin and rehydration processing of paraffin-embedded sections

Solution	Incubation/time
Xylene	Two changes[a], 30 min each
Xylene:100% Etanol (1:1)	One change, 30 min
100% Ethanol	Two changes[a], 5 min each
95% Etanol	One change, 5 min
70% Etanol	One change, 5 min
Running tap water to rinse	10 min
Distillated water on a shaker	10 min

[a]Use different solutions for each change

9. Place holders in an electric pressure cooker and add at least 1.5 L of distillate water to pressure cooker chamber.
10. Set target temperature and heating time according to the manufacturer's instructions, normally boil for 30 s to 5 min and allow the pressure cooker to cool for 20 min prior to opening (*see* **Note 27**).
11. Immediately after, rinse the slides in running tap water for 10 min
12. Wash slides in distillate water for 10 min on a shaker.
13. Place slides in wash buffer for 5 min on a shaker.
14. If the signal of a specific connexin needs to be amplified, perform Tyramide signal amplification (*see* **Note 28**).
15. Incubate slides with primary antibody diluted in antibody dilution buffer (*see* **Note 17**) overnight at 4 °C in a humidified

chamber (*see* **Note 29**). Use the appropriate negative and positive controls (*see* **Note 18**).

16. Wash slides three times for 5 min with wash buffer on a shaker.
17. Incubate slides with a secondary antibody Alexa Fluor® 488 goat anti-mouse or Alexa Fluor® 594 goat anti-rabbit (10 μg/mL), diluted in antibody dilution buffer, for 1 h at room temperature in a humidified chamber. From this point, the procedures should be done in the dark to avoid photobleaching.
18. Wash slides three times for 5 min with wash buffer on a shaker.
19. Perform the nuclear counter stain with PI for 15 min or with DAPI for 5 min at room temperature (*see* **Note 19**).
20. Wash slides three times for 5 min with wash buffer on a shaker.
21. Mount coverslip with a drop of antifading mounting medium.
22. Seal coverslip with nail polish (*see* **Note 20**).
23. Store in dark at 4 °C.

3.3 Fluorescent Immunocytochemistry

1. Grow cultured cells on sterile glass coverslips or alternatively culture cells in 1 to 8-chamber glass slides (*see* **Note 30**).
2. Aspirate the supernatant from each chamber/well and rinse the cells three times for 5 min with ice cold wash buffer on a shaker.
3. Fix cells using ice cold acetone for 10 min at −20 °C. When removing acetone make sure the cells are not allowed to dry out (*see* **Note 31**).
4. Wash cells three times for 5 min with wash buffer.
5. Incubate cells with 5% skim milk diluted in PBS for 30 min at room temperature.
6. Wash cells three times for 5 min with wash buffer.
7. Incubate cells with primary antibody diluted in antibody dilution buffer for 1 h at 37 °C or overnight at 4 °C in a humidified chamber (*see* **Note 17**). Use the appropriate negative and positive controls (*see* **Note 18**).
8. Wash three times for 5 min with wash buffer on a shaker.
9. Incubate slides with a secondary antibody Alexa Fluor® 488 goat anti-mouse or Alexa Fluor® 594 goat anti-rabbit (10 μg/mL), diluted in antibody dilution buffer, for 1 h at room temperature in a humidified chamber. From this point, the procedures should be done in the dark to avoid photobleaching.
10. Wash three times for 5 min with wash buffer on a shaker.
11. Perform the nuclear counter stain with PI for 15 min or with DAPI for 5 min at room temperature (*see* **Note 19**).
12. Wash slides three times for 5 min with wash buffer on a shaker.

13. Mount coverslip with a drop of antifading mounting medium on a microscope glass.
14. Seal coverslip with nail polish (*see* **Note 20**).
15. Store in dark at 4 °C.

3.4 Tyramide Signal Amplification (TSA) Method

1. Quench endogenous peroxidase activity by incubating slides in 6% H_2O_2 in 1× PBS for 30 min in dark (*see* **Note 32**).
2. Wash slides three times for 5 min with wash buffer on a shaker.
3. Block slides for 30 min in blocking buffer at room temperature.
4. Incubate slides with primary antibody diluted in blocking buffer overnight at 4 °C in a humidified chamber (*see* **Note 17**). Use the appropriate negative and positive controls (*see* **Note 18**).
5. Wash slides three times for 5 min with wash buffer on a shaker.
6. Incubate slides with a polyclonal goat anti-rabbit or mouse immunoglobulins/HRP, diluted in blocking buffer (1:500) for 30 min at room temperature.
7. Wash slides three times for 5 min with wash buffer on a shaker.
8. Incubate in tyramide working solution (1:100) for 10 min at room temperature. From this point, the procedures should be done in the dark to avoid photobleaching.
9. Wash slides three times for 5 min with wash buffer on a shaker.
10. Perform the nuclear counter stain with PI for 15 min or with DAPI for 5 min at room temperature (*see* **Note 19**).
11. Wash slides three times for 5 min with wash buffer on a shaker.
12. Mount coverslip with a drop of antifading mounting medium.
13. Seal coverslip with nail polish (*see* **Note 20**).
14. Store in dark at 4 °C.

4 Notes

1. Use of an adhesive coating, such as poly-l-lysine, charging, or silanization, can improve the tissue adherence and reduce damages in fragile tissues (i.e., dermis skin layer or adipose tissue) or to avoid tissue loss during the immunostaining procedure. This is strongly recommended when enzymatic or heat-induced antigen retrieval methods are applied in frozen or paraffin-embedded tissue slides [1, 2].
2. Formalin is formaldehyde gas dissolved in water and reaches saturation at 37–40% formaldehyde (this solution is considered as 100% formalin). In this context, 10% formalin actually represents 10% of a 37–40% stock solution; this means that the actual amount of dissolved formaldehyde in the 10% formalin is therefore only 3.7–4.0%. As formaldehyde is a severe eye and

skin irritant with known carcinogenic and corrosive potential, always work in well-ventilated area and wear goggles, gloves, and lab coat [28, 29].

3. Xylene is an aromatic hydrocarbon and is potentially dangerous due to its volatility and inflammability capacities. Acute and chronic exposure to xylene is associated with nonspecific clinical signals related to the central nervous system. It must be used in a fume hood to avoid occupational hazard [30].
4. Heat-induced antigen retrieval by microwave radiation may lead to inconsistent IHC results. Because pressure cookers or vegetable steamers induce uniform and gentle heat, they are currently used in most laboratories.
5. Choice of slide design is often dictated by the experiment. Some slides have four wells, others have eight some are from glass, and others are from plastic. Glass is recommended as the slide becomes more versatile, acetone can be used as a fixative and slides can be dehydrated in ethanol and cleared in xylene. However, some cell types need a plastic surface to grow with a good cell confluence, i.e., rat primary hepatocytes.
6. 12 mm circular glass coverslips fit in 24-well plates. These coverslips may be autoclaved in a petri dish. Most cells adhere well to glass. Cells that do not adhere may require treatment of the glass with an adhesive coating, such as poly-l-lysine, silane, or other attachment factor.
7. The humidified chamber can be either the incubator itself or a container with wet paper towels. This procedure is important to prevent evaporation and drying of the tissue sections during the incubation time as this may cause nonspecific antibody binding and therefore high background staining.
8. The use of Tween® 20 in the wash buffer helps to reduce surface tension, allowing reagents to cover the whole tissue section with ease. It is also believed to dissolve Fc receptors, therefore reducing nonspecific binding.
9. Do not adjust pH using concentrated HCl or NaOH. If necessary, use a separate 0.2 M solution of either the monobasic or dibasic sodium phosphate (depending on how you need to adjust the pH) and add accordingly.
10. When preparing blocking buffer, make sure that all milk powder is dissolved by stirring the solution for about 20 min. It may be necessary to heat the solution slightly to fully dissolve nonfat dry milk. This solution can be used up to 5 days when stored at 4 °C. It is recommended to mix before use. Alternative block solutions are 1 % gelatin or 10 % serum from the species from which the secondary antibody was raised in.
11. Do not use this buffer to dilute HRP conjugated antibody as the sodium azide is inhibitor of HRP.

12. Monoclonal antibodies have high affinity and specificity to a single epitope, which can be directly affected by structural changes in the proteins during the fixation and histological processing. On the other hand, polyclonal antibodies are more stable and can recognize multiple epitopes; this last one represents the main reason why this kind of antibody is more frequently used for IHC. However, polyclonal antibodies can induce higher background immunostaining and nonspecific bindings should be efficiently blocked [1, 2, 27].
13. The isotype IgG control should be applied when working with monoclonal primary antibodies as a negative control test. The slides are incubated with a nonimmune immunoglobulin of the same isotype and concentration as the primary monoclonal antibody. For polyclonal antibodies, the slides should be incubated with preimmune sera. In addition, cells and tissue from genetically engineered animals, as well as siRNA, with PCR confirmation, can be used as positive and negative controls [31].
14. Snap freezing is the process by which samples reach ultralow temperatures very rapidly using nitrogen liquid or isopentane cooled in nitrogen liquid. In contrast to the slow freezing, this method reduces the ice crystal formation which causes distortion in cell morphology and, consequently, a low-quality cryosection. Take serious care using nitrogen liquid and use all equipments for individual security.
15. Frozen tissue sections give much better antigen preservation than paraffin-embedded tissue sections. However, the morphological details and resolution are usually reduced. The temperature in the cryostat must be correct for the specimen being cut. The microtome and the antiroll plate must be correctly adjusted and operated. The cutting blade must be sharp [32].
16. Air-dried frozen tissues are not recommended for IHC; it is better to choose and test different fixative for each kind of protein [28]. In our experience, tissue slices fixated with acetone show satisfactory results for connexins immunostaining.
17. The primary antibody should be diluted to the manufacturer's recommendations or to a previously optimized dilution. A plethora of companies have primary connexin antibodies available, including Sigma Aldrich, Abcam, Thermo Fischer, Santa Cruz, EMD Millipore, Genetex, ProteinTech, Alomone, Biocompare, Life Technologies, and Merck Millipore. It is recommended to test different concentrations of the primary antibody and keep the concentration of the secondary antibody low to avoid nonspecific binding (Fig. 2). Most antibodies will be used in IHC at a concentration between 0.5 and 10 μg/mL [1, 2, 28].
18. Besides the negative control with isotype IgG or preimmune sera, it is strongly recommended to include a positive control

to ensure that the antibody is performing as expected, i.e., liver and heart cells or tissue for Cx32 and Cx43 immunostaining, respectively.

19. Both the excitation and emission wavelengths are specific characteristics for each fluorophore; however, they may show overlapping fluorescence emission wavelengths among different fluorescent labels and nuclear dyes, i.e., Alexa Fluor® 594 is visually similar in color with the PI nuclear dye (Table 1).
20. Sealing the coverslips with nail polish prevents drying and movement under microscopy.
21. To enhance penetration of the fixative during immersion fixation, it is recommended that tissues be no thicker than 10 mm. For complete fixation, the ideal volume of formaldehyde solution compared to tissue volume should be in the ratio of 1:25 (w/v), with a minimum ratio of 1:10 (w/v) [29].
22. Methacarn is a non-cross-linking protein-precipitating fixative that was shown to maintain tissue morphology and usually give superior immunohistochemical results than aldehyde-based cross-linking fixatives, because antigenicity is usually maintained intact [33]. In our experience, methacarn is the best option of fixative solution to immunostaining connexins in paraffin-embedded tissues.
23. Prolonged fixation in formalin (more than 24 h) results in gradual loss of the antigenicity, which will require heat-induced antigen retrieval methods to unmask the epitopes and allow the antigen–antibody binding. It is very important to optimize fixation conditions since underfixation or overfixation may reduce or abolish tissue antigenicity [29].
24. This also will allow a better slice adherence in the slides and remove the paraffin excess from the tissue section. Do not allow higher temperature and time incubation, as this may reduce or abolish tissue antigenicity.
25. Storage of unstained tissue sections longer than 2 months can promote loss of antigenicity by oxidation, protein degradation by high temperature, or other unknown factors. Then, it is recommended to use fresh cut sections or storage of the unstained sections paraffin coated in vacuum-sealed desiccators and cold temperature (at 4 °C, −20 °C or −80 °C) [10].
26. Due to the potential for autofluorescence with FFPE tissues, the first xylene can be used at 56–60 °C for 30 min. Additionally, the deparaffinized section can be incubated with 1 mmol/L glycine in 1× PBS for 30 min at room temperature and, after washes, with 1 mg/mL $NaBH_4$ in 1× PBS to further reduce autofluorescence in FFPE slides [34].
27. Pretreatment with these solutions may induce a dramatic enhancement of immunoreactivity. However, they also have

the potential to affect tissue morphology. The optimal method of antigen retrieval must be determined experimentally, using different incubation time, temperatures, and alternative options of buffer, i.e., citrate buffer (pH 6.0) [27]. Proteolytic enzymes, i.e., Proteinase K and Trypsin, also can be tested in FFPE slides. The concentration of enzyme and incubation time will depend on type of tissue and fixation but is usually 0.05–0.1 % for 5–30 min at 37 °C [2].

28. The TSA method is based on the ability of HRP to catalyze in the presence of hydrogen peroxide the oxidation of the phenol moiety of labeled tyramide onto protein surrounding the HRP. This allows an increase in the detection of an antigenic site up to 100-fold compared to the conventional indirect method, with no loss in resolution [1, 2]. In this context, the TSA detection method is recommended to identify connexins with low expression levels.
29. Overnight incubation allows the use of lower titer of antibodies and reduces nonspecific background staining.
30. Grow the cells at a concentration that will allow the cells to spread out without growing on top of each other (around 70–80 % of confluence).
31. If the target protein is localized intracellularly, it is very important to permeabilize the cells. Acetone or methanol-fixed samples do not require permeabilization. If cells were fixed in 4 % paraformaldehyde or alternative fixative, permeabilize cells in acetone or 100 % ethanol for 10 min at −20 °C. Alternatively, use 100 μM Digitonin or 0.5 % Saponin diluted in 1× PBS for 10 min at room temperature. Cell permeabilization with Triton X-100 is not recommended for membrane-associated antigens (i.e., connexins) since it destroys membranes.
32. The blockage of endogenous peroxidase is necessary as the activation and covalent binding of TSA reagent is catalyzed by peroxidase. The incubation period can range from 10 to 60 min, depending on the endogenous activity of peroxidase in each kind of tissue.

Acknowledgements

This work was financially supported by the University Hospital of the Vrije Universiteit Brussel-Belgium (Willy Gepts Fonds UZ-VUB), the Fund for Scientific Research-Flanders (FWO grants G009514N and G010214N), the European Research Council (ERC Starting Grant 335476), the University of São Paulo-Brazil and the São Paulo Research Foundation (FAPESP SPEC grant 2013/50420-6).

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