



Original article

IN TOTUM IMMUNOSTAINING: A HISTOLOGICAL ANALYSIS TOOL FOR SMALL DIMENSIONS BIOLOGICAL SAMPLES

TALITA SARAH MAZZONI^{a,b}, IRANI QUAGIO-GRASSIOTTO^{b*}

^aDepartment of Cell and Developmental Biology, Institute of Biomedical Sciences, Federal University of Alfnas (UNIFAL), Gabriel Monteiro da Silva 700, 37130-001 Alfnas-MG, Brazil

^bDepartment of Morphology, Institute of Biosciences of Botucatu, São Paulo State University (UNESP), Prof. Dr. Antonio Celso Wagner Zanin 250, 18618-689 Botucatu-SP, Brazil

ARTICLE INFO

Keywords:

antibody detection
histology
immunocytochemistry
immunofluorescence
immunolabeling
plastic resin

ABSTRACT

Immunostaining through immunohistochemical or immunofluorescence techniques has been widely used in recent years as a result of the increasing availability of antibodies. The identification of specific epitopes in biological tissue by antigen-antibody reactions, detected by a visual marker, that allows their identification under microscopy, provides more accurate diagnoses. Today, the most commonly used methods in immunohistochemistry refer to histological sections obtained from paraffin sections, cryostat or cell suspensions. However, these methodologies are not always suitable, especially for small samples. Thus, in order to perform immunostaining in difficult to manipulate biological tissues, in totum immunohistochemical processing was performed in embryos, larvae and total biological tissues of fishes. The development of this technique was adapted from proposed protocols for free-floating immunohistochemistry. The in totum biological samples immersed in different types of fixing solutions and washed in Sorensen Phosphate Buffer. Next, endogenous peroxidase was blocked. The antigen recovery was performed, followed by the nonspecific blockade and incubation with different primary antibodies. Subsequently, the in totum samples were incubated with different biotinylated secondary antibodies and stained with DAB-H2O2. The material was washed in distilled water and fixed again. Fluorescent secondary antibodies were also used in other similar samples, following the same initial protocol. The samples were submitted to historesin embedding and the histological sections (5µm thick) were obtained in microtome. DAB stained immunohistochemistry slides were counterstained with Hematoxylin. Samples fluorophore labeled were immunolabeling with DAPI. The immunostaining in both immunohistochemistry and immunofluorescence was maintained throughout historesin processing. The historesin embedding allows a better preservation of biological tissues and obtaining thinner histological sections than paraffin or frozen material. Consequently, the image resolution is potentially improved. In this regard, this work becomes an important tool to be used in immunohistochemical techniques, giving better results in tissues that are difficult to process in paraffin or freezing.

© Copyright 2010 BioMedSciDirect Publications IJBMR - ISSN: 0976:6685. All rights reserved.

1. Introduction

The immunostaining, through immunohistochemistry techniques [1], has been often used in the recent years, as a result of the increasing availability of antibodies. The development of monoclonal antibodies, which provided a huge source of highly specific reagents for the detection of various tissue or cellular antigens, and the advent of the antigen retrieval were significant facts in the evolution of immunohistochemistry [2]. Thus, the

possibility of identifying specific epitopes in biological tissue by means of an antigen- antibody reaction [1], detected by a visual labeling that allows their identification under microscopy has provided more reliable and accurate results and diagnoses. Currently, the most commonly used methods in immunohistochemistry refer to histological sections obtained from paraffin sections, cryostat, smears or cell suspensions [1,3,4]. However, these methodologies are not always appropriate, especially in small biological materials. In this regard, in order to perform immunostaining in difficult to manipulate biological tissues, in totum immunolocalization

* Corresponding Author: **IRANI QUAGIO-GRASSIOTTO**

e.mail: iraniqg@ibb.unesp.br
Tel.: +55-14-3880-0468

processing was performed in embryos, fish larvae and total biological tissues (fragments), followed by the evaluation of the positive response of the technique, after historesin embedded tissue.

Materials and Methods

The technique proposed here was adapted from protocols for free-floating immunohistochemistry, performed with thick tissue sections floating in solution [5].

In totum biological materials were fixed in 10% Formaldehyde, Bouin's solution or 4% Paraformaldehyde for 5 to 12h, depending on the sample size. After this time, the samples remained in 70% alcohol until the immunostaining technique, according to traditional immunohistochemistry protocols.

The biological materials used were: embryos, larvae, juveniles and gonads of several fish species, namely, *Amatitlania nigrofasciata* (Cichliformes), *Amphiprion ocellaris* (Perciformes), *Cyprinus carpio* (Cypriniformes), *Corydoras* sp (Siluriformes) *Danio rerio* (Cypriniformes), *Chaetodon striatus* (Perciformes), *Gymnocorymbus ternetzi* (Characiformes), *Gymnotus* sp (Gymnotiformes), *Poecilia reticulata* (Cyprinodontiformes), *Synbranchus marmoratus* (Synbranchiformes), *Steindachneridion melanodermatum* (Siluriformes) and *Tanichthys albonubes* (Cypriniformes).

The selected samples were prepared for immunolabeling, according to the following protocol:

Immunohistochemistry

- 1) Washing in Phosphate-buffered saline (PBS; pH 7.3) or the buffer used to prepare the fixative solution + 1% permeabilizing agents (detergent to disrupt proteins of cells, such as Triton™ X-100 or Tween 20®) for 1h;
- 2) Washing in Tris-buffered saline (TBS; pH 7.6) for 20min (4x5min);
- 3) Transfer of samples to eppendorfs or microtube (0.5mL) to avoid reagent waste;
- 4) After washing, the samples were treated for antigen retrieval in 0.01 M citrate buffer (pH 6.0) in steam cooker for 15min;
- 5) The samples were treated with 3% hydrogen peroxide in Tris-buffered saline (TBS; pH 7.6) for 15min, to block endogenous peroxidase;
- 6) Thereafter, samples were washed in TBS and submitted to a protein blocker (1% nonfat, powdered milk in TBS) for 15min;
- 7) Subsequently, the material was incubated with various types of primary antibodies* (200µm per sample) for 1h30min at room temperature (regulated by air conditioning - 20°C);

8) The samples were rinsed in TBS (2 x 5min) and incubated with MR HRP-Polymer (MACH4 Universal HRP Polymer Kit®) or another type of secondary antibody as Novostain Super ABC Kit - universal (Novocastra - NCL) (50 - 100µm per sample) for 40min at room temperature;

9) Peroxidase activity was revealed using 0.1% 3'.3'-diaminobenzidine (DAB) in TBS (pH 7.6) containing 0.03% hydrogen peroxide (around 300µm per sample for 5-7min, depending on sample size). For the negative control, primary antibody was replaced with TBS in some samples;

10) Finally, the samples were rinsed in distilled water to end the reaction.

*Primary antibodies used: Anti-mouse monoclonal PCNA antibody (Novocastra Laboratories, Newcastle, UK) diluted 1:300; Anti-mouse monoclonal Anti-KI-67 (MIB-1) (SC-101861, Santa Cruz Biotechnology, USA) diluted 1:100; Anti-mouse monoclonal Anti-3β-HSD (SC- 100466, Santa Cruz Biotechnology, USA), diluted 1:100; Anti-fish polyclonal Anti-VASA (IM-0338, Rhea Biotech, BR) diluted 1:300, Anti-zebrafish polyclonal P450 Aromatase (CYP19A1A) antibody (AP55401SU-N, Acris Antibodies) diluted 1:300; Anti-rabbit polyclonal Anti-MMP14 (ab53712, ABCam) diluted 1:50; Anti-rabbit polyclonal Anti-MMP2 (ab37150, ABCam) diluted 1:50; Anti-mouse polyclonal Anti-MMP9 (ab38898, ABCam) diluted 1:50; Anti-zebrafish polyclonal Anti-CD34 (Imuny, BR) diluted 1:100; Anti-mouse monoclonal Anti-Vimentin (ab8978, ABCam) diluted 1:100; Anti-mouse polyclonal Anti- Vitellogenin (IM-0129, Rhea Biotech, BR) diluted 1:100.

Historesin Embedding

After immunohistochemistry, the samples must be refixed to avoid losing antibody labeling. Thus, the material were fixed by immersion in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2) or 10% formaldehyde for at least 24h. After, the samples were dehydrated in ethanol and embedded in Historesin (Leica HistoResin®), following the manufacturer's instructions. Then, histological sections were:

- 1) Cut** at 5µm with Leica® 2145 Rotary Microtome, equipped with glass-knife;
- 2) Counterstained with Harris Hematoxylin for 3min;
- 3) Washed with running water for 3min;
- 4) Dehydrated in the oven at least 12h;
- 5) Diaphanized with xylol and mounted with Permount™ Mounting Medium or Entellan® non-aqueous mounting medium;
- 6) The slices were documented using a computerized image analyzer (Leica LAS Interactive Measurements).

**The material must be serial sections cut in order to find the desired portion.

Immunofluorescence

The same procedure used for immunohistochemistry was applied to immunofluorescence, until the incubation with the primary antibody. After that, the samples were incubated with different types of fluorophore-conjugated secondary antibody*** diluted in TBS (1%) for 40min and washed in TBS for 10min (2 x 5min).

These materials must be refixed to avoid losing antibody labeling. Thus, they were fixed by immersion in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2) or 10% formaldehyde for at least 24h. After, the samples were embedded in HistoResin (Leica HistoResin®). However, although not recommended by the manufacturer, alcohol was exchanged for distilled water to avoid autoimmunofluorescence. Then, histological sections were:

- 1) Cut at 5µm with Leica® 2145 Rotary Microtome, equipped with glass-knife;
- 2) Mounted with Fluoroshield Mounting Medium with DAPI (Sigma-Aldrich®);
- 3) The slides were kept in the refrigerator (4°C) and protected from light until reading;
- 4) The slices were documented using a fluorescence microscope (BX61 Olympus). Some in totum samples were immersed in DAPI solution (diamidino-2-phenylindole) for 40min. In this case, after microtomy, the slides should be mounted with aqueous mounting medium only.

***Fluorescent secondary antibodies (fluorophore-conjugated secondary antibody) used: Goat Anti-Mouse IgG (Alexa Fluor & 488 ABCam); Donkey Anti- Rabbit IgG (AlexaFluor & reg594 ABCam); Anti-rabbit IgG (FITC - Sigma-Aldrich).

After photographic documentation, some immunofluorescence slides were dismantled and stained with Hematoxylin-Eosin (HE) for general visualization of the tissue.

Some specific protein detection kits have also been tested on in totum material too. The kits tested were for the investigation of the cell death by apoptosis. DNA fragmentation is one of first changes to occur in apoptotic cells, that was detected by the Terminal deoxynucleotidyl transferase (TdT) - mediated dUTP Nick end Labeling (TUNEL) assay.

Immunohistochemistry (TUNEL)

In totum samples were:

- 1) Washed in Sorensen's phosphate buffer (0.1 M, pH 7.2) for 1h;
- 2) Rinsed in TBS (20 mM Tris, pH 7.6, 140 mM NaCl);
- 3) Permeabilized by incubation in Proteinase K (2 mg/mL in 10 mM Tris pH 8.0);

- 4) Incubated with TdT labeling reaction mix and with TdT enzyme;
- 5) Further procedures were conducted in accordance with the manufacturer's instructions (TdT-FragEL™ DNA Fragmentation Detection Kit, CalbiochemVR, Merck KGaA, Darmstadt, Germany). The 3,30-diaminobenzidine (DAB) substrate was used for chromagen development;
- 6) The samples, taken in totum, were fixed by immersion in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2) for at least 24 hr and embedded in histoResin (LeicaHistoResin);
- 7) Cross sections (5µm) were counterstained with hematoxylin or fast green and mounted.

Immunofluorescence (TUNEL)

In totum samples were:

- 1) Washed in Sorensen's phosphate buffer (0.1 M, pH 7.2) for 1h;
- 2) Rinsed in TBS (20 mM Tris, pH 7.6, 140 mM NaCl);
- 3) Further procedures were conducted in accordance with the manufacturer's instructions of the In Situ Cell Death Detection Kit, Fluorescein – Roche;
- 4) Permeabilized by incubation in Proteinase K;
- 5) Incubated with dUTP digoxigenin-conjugated nucleotides with Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT) for 1h at 37°C;
- 6) Washing in PBS to remove unincorporated fluorescein-12-dUTP;
- 7) In totum samples were fixed by immersion in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2) for at least 24 hr and embedded in histoResin (LeicaHistoResin);
- 8) Cross sections (5µm) were mounted with Fluoroshield Mounting Medium with DAPI (Sigma-Aldrich®);
- 9) The slides were kept in the refrigerator (4°C) and protected from light until reading.

All steps of the techniques showed here (immunohistochemistry and immunofluorescence) were done on a shaking table.

After immunostaining and before starting processing material to histoResin embedding, the samples were analyzed in totum to verify their immunolabeling.

The protocol steps were summarized and can be appreciated in the Figure 1.

Results

Macroscopy

After the ending of the immunostaining, in totum materials were observed under stereomicroscope (Fig. 2) and fluorescence microscopy (Fig. 3), showing positive responses to both immunohistochemistry (Fig. 2) and immunofluorescence (Fig. 3).

Just as commonly an edge effect can be observed in traditional histological sections of immunohistochemistry, this can also be found in in totum material, depending on the time the material is exposed under DAB (Fig. 2). As a consequence, there is a nonspecific labeling. However, this can be easily corrected by preliminary testing of each material, as observed in the gonads of *Amatitlania nigrofasciata* and *Tanichthys albonubes*, which were immunolabeling for 3β-HSD (Fig. 2A), CYP19A1A (Fig. 2B) and PCNA (Fig. 2C).

The embryo of *Danio rerio* (Fig. 3A), in totum immunolabeling with fluorophore showed nuclei stained by DAPI making and specific proteins (VASA protein) could be detected (Fig. 3B-D) in the primordial germ cells.

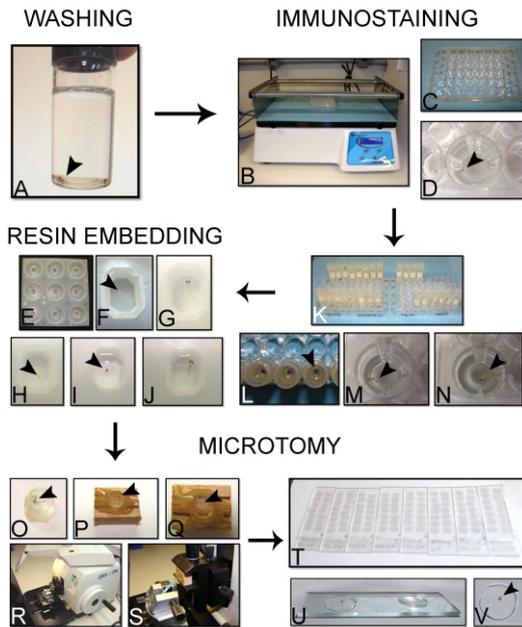


Figure 1. The scheme summarizes the steps taken in the proposed protocol. A) Samples wash. B) Immunostaining performed on the shaking table. C-D) Detail of the in totum samples inside the microtubes. K-N) Ending of the immunostaining. Note the staining of the samples that received DAB for immunohistochemistry (L and M) and fluorophore for immunofluorescence (N). O-Q) Histoiresin embedding. Note the staining of the samples that received DAB for immunohistochemistry (O and P) and fluorophore for immunofluorescence (Q). R-S) Samples being cut in microtome equipped with glass knife. T) Serial sections of the samples labeling by immunohistochemistry, after hematoxylin staining. U-V) Samples labeling by immunofluorescence, after mounting with DAPI. V) Detail of U showing a sample without apparent staining. Biological samples are pointed by arrowhead.

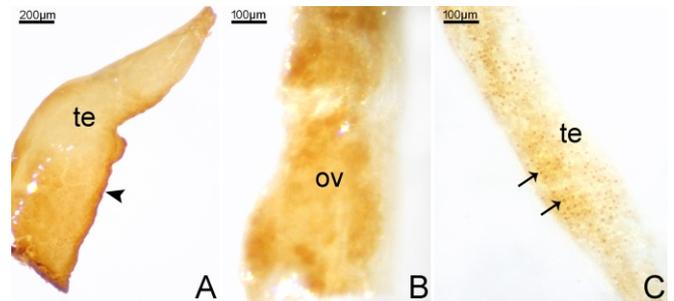


Figure 2. Macroscopic view of the gonads after the ending of the in totum immunohistochemistry. A) Testis (te) of *Amatitlania nigrofasciata*, labeling by 3β-HSD. Note the edge effect (arrowhead). B) Ovary (ov) of *Tanichthys albonubes*, labeling by CYP19A1A. C) Testis (te) of *Tanichthys albonubes* labeling by PCNA (arrow). Note specific marking and absence of edge effect.

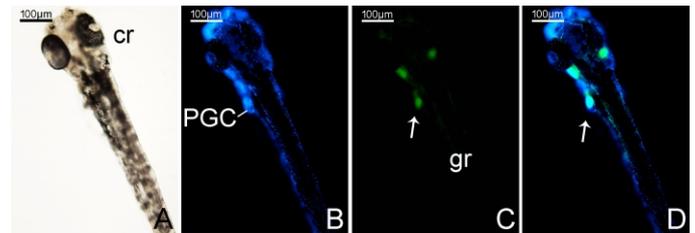


Figure 3. Embryo of *Danio rerio*. A) General view after the ending of the in totum immunofluorescence. B-D) In totum immunolabeling with fluorophore showed nuclei stained by DAPI making (B and D) and VASA protein (C and D) (arrow) in the primordial germ cells (PGC).

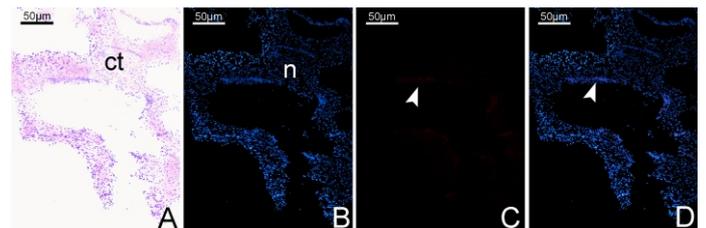


Figure 4. Connective tissue (ct) of the gonad of *Synbranchus marmoratus*. A) General view of the tissue stained with HE. B-D) Cross section of the samples by immunofluorescence. Note the nuclei (n) labeling by DAPI (B and D) and the negative response (arrowhead) to VASA (C and D). The fluorescence was not altered by the histoiresin processing.

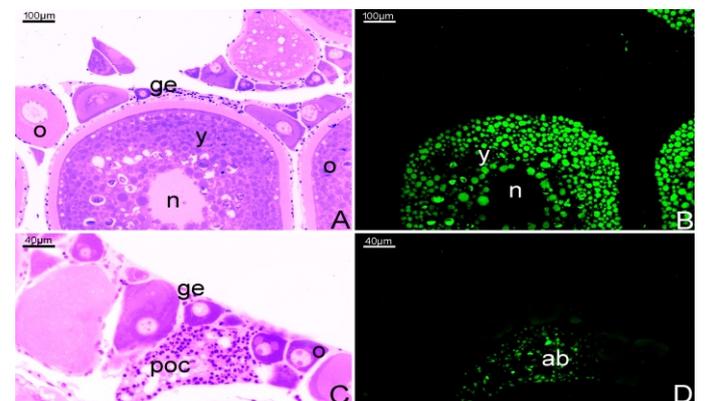


Figure 5. Cross sections of ovaries of *Chaetodon striatus*, showing oocytes (o) and the germinal epithelium (ge). A) General view of the ovary stained with HE. B) Immunofluorescence for vitellogenin, detected within yolk granules (y). C) General view of the ovary stained with HE. D) Apoptotic bodies (ab) labeling by TUNEL, detecting cell death in post-ovulatory complexes (poc). Nucleus (n).

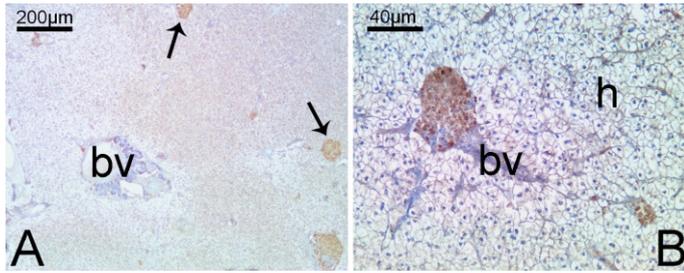


Figure 6. Cross sections of liver of *Steindachneridion melanodermatum*. Immunohistochemistry. A) Immunostained regions by Vitellogenin (arrow). B) Detail of A, showing labeled hepatocytes clusters (h). Blood vessel (bv).

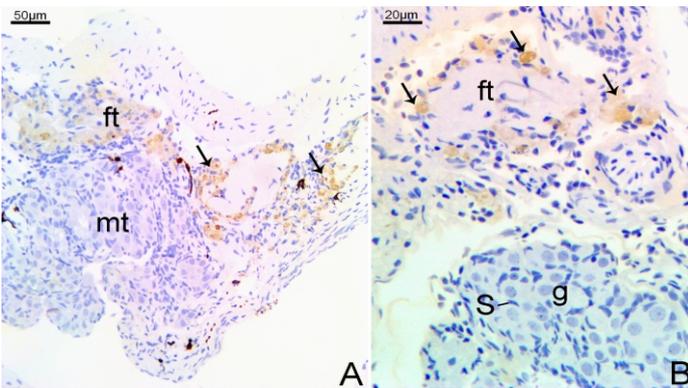


Figure 7. Cross sections of gonad of *Synbranchus marmoratus*, formed by female (ft) and male tissue (mt). Immunohistochemistry. A) Immunostained regions by TUNEL (arrow). B) Detail of A, showing labeled cell death (arrow) in female gonadal tissue. Note that there is no apoptotic cells detected in the male tissue, formed by spermatozoa (s) and Sertoli cells (S).

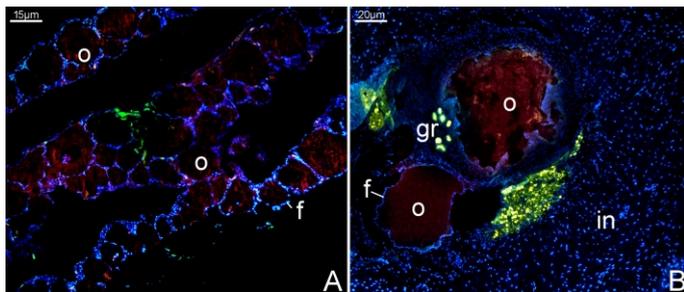


Figure 8. Cross sections of gonad of *Synbranchus marmoratus*, formed by female tissue. Immunofluorescence. A-B) In this portion of the female gonadal tissue, the oocytes are labeling by MMP-2 metalloproteinases (in red). Follicle cells (f), interstitial cells (in) and granulocytes showed positive response for MMP-9 metalloproteinases (in green). The nuclei (in blue) were labeling with DAPI.

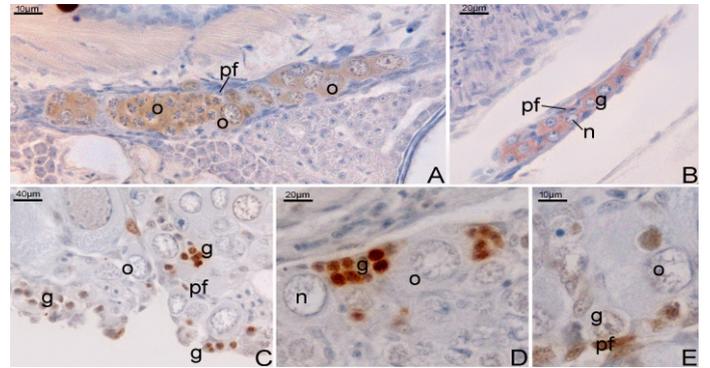


Figure 9. Longitudinal sections of gonads of fish. Immunohistochemistry. A) Oocytes (o) in the ovary of *Tanichthys albonubes* labeled by CYP19A1A. Prefollicle cells (pf) were not labeled. B) Oogonia (g) in the ovary of *Amatitlania nigrofasciata* labeled by VASA. Prefollicle cells (pf) were not labeled. C-E) Proliferating cells of oogonia (g) and follicle cells (pf) detected by Ki-67 in ovaries of *Tanichthys albonubes*. Oocytes (o) were not labeled. Nucleus (n).

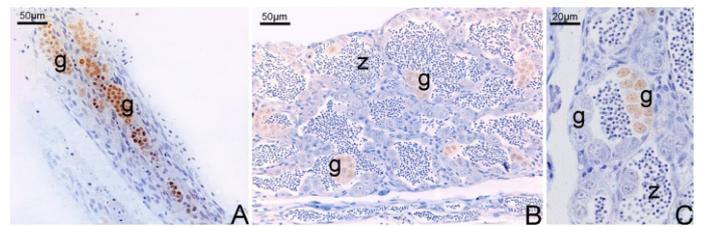


Figure 10. Longitudinal sections of gonads of fish. Immunohistochemistry. A) Spermatozoa (g) in the testis of *Amatitlania nigrofasciata* labeled by Ki-67. B-C) Spermatozoa (g) in the testis of *Tanichthys albonubes* labeled by PCNA. C) Detail of B, showing the proliferation of some spermatozoa. Spermatozoa (z) were not labeled.

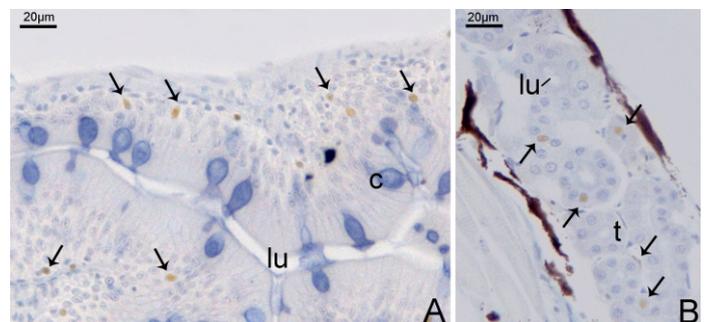


Figure 11. Longitudinal sections of *Cyprinus carpio* larvae. Immunohistochemistry. A-B) Some cells from gut (A) and kidney (B) were detected by PCNA (arrow). Goblet cell (c), lumen (lu), renal tubule (t).

Microscopy

All materials tested showed immunostaining after sections. Even immunofluorescence labeling was preserved after the historesin embedding and microtomy.

The fluorescence was not altered by the historesin processing. The negative response to VASA (protein characteristic of germ cells) in the connective tissue of the gonad of *Synbranchus marmoratus* confirms that there was no false-positive (Fig. 4).

In contrast, there was a positive response when the protein is specific for the antibody used, such as in the detection of vitellogenin (Fig. 5A,B) in oocytes of *Chaetodon striatus*. Cell death (by TUNEL) was also detected in post-ovulatory complexes (Fig. 5C, D) in their ovaries after the spawning period. Both proteins were also detected in other samples by immunohistochemistry. Vitellogenin was labeled in hepatocytes clusters (Fig. 6) in the liver of adults of *Steindachneridion melanodermatum* females.

Apoptotic cells (Fig. 7), detected by TUNEL, were also immunostaining in gonads of *Synbranchus marmoratus*, a hermaphrodite fish, during the sex reversion of the gonadal tissue (period in which there is cell death of the female gonad). In this phase, intense tissue remodeling of the gonad occurs and matrix metalloproteinases become active, can be detected by immunofluorescence (Fig. 8). Since proteins are specific, double labeling can be performed on the same tissue (Fig. 8). Other proteins could be detected by immunohistochemistry, all of which showed specific response. Oocytes in the ovary of *Tanichthys albonubes* and oogonia in the gonadal tissue of *Amatitlania nigrofasciata* were labeled for gonadal aromatase - CYP19A1A (Fig. 9A) and VASA protein (Fig. 9B), respectively. Proliferating cells (oogonia and follicle cells) were detected by KI-67 in ovaries of *Tanichthys albonubes* (Fig. 9C-E). Male germ cells in cell proliferation were also detected in the testes of the same fish species. In *Amatitlania nigrofasciata*, spermatogonia were immunostaining by KI-67 (Fig. 10A), while the cell proliferation was identified by PCNA in *Tanichthys albonubes* (Fig. 10B, C).

In addition to these labeling found, when in totum marking is performed on an entire animal, for example in a larvae or juvenile fish, all organs may show immunostaining. For example, in serial sections of *Cyprinus carpio* larvae, cells proliferating from gut (Fig. 11A) and kidney (Fig. 11B) were identified.

Discussion

Immunohistochemistry occupies a prominent position in the laboratory today, constituting an indispensable complementary technique in the solving certain differential diagnosis problems formulated in slides stained with routine basic staining - Hematoxylin-Eosin [6].

However, it is not always possible to obtain good results from histological sections of paraffin or frozen processed materials, depending on the biological material worked. This difficulty is mainly due to the size of the material, which cannot always be dissected, nor preserved in paraffin sections. This happens, for example, with small organs from embryonic or larval stages, which often are a size smaller than 1mm. In this respect, in totum immunostaining is an excellent solution, as it remains preserved throughout historesin processing, allowing its positive visualization in histological sections.

In totum immunostaining described here showed results extremely similar to those found in traditional immunolabeling, and can also be applied to confocal microscopy. The historesin embedding allows better preservation of biological tissues and obtaining thinner histological sections and, consequently, a potentially improved image capture resolution [7,8]. Plastic resins for routine histology are hydrophobic and do not allow the immunolabeling technique to be performed directly [9], since their polymers prevent the passage of the antibodies, constituted a physical barriers. Although there are some types of hydrophilic resins that allow immunostaining, especially for transmission electron microscopy [9], they are not always readily available on the market, depending on the country of origin in question.

Moreover, its processing protocol is not so simple, often requiring polymerization in special low temperature chambers with ultra violet light [10]. In addition, the cost of these hydrophilic resins is quite high, complicating the research in certain situations.

Conclusion

This protocol becomes an important tool to be used in immunohistochemical techniques, giving better results in tissues that are difficult to process in paraffin or freezing, through simpler and more accessible techniques.

References

- [1]. Jackson P, Blythe D. Immunohistochemical techniques. In: Theory and practice of immunohistological techniques. Bancroft JD, Gamble M (eds). Elsevier, 2008, pp 433-472.
- [2]. Bodey B. The significance of immunohistochemistry in the diagnosis and therapy of neoplasms. *Expert Opin Biol Ther*. 2002; 2(4):371-393.
- [3]. O'Leary TJ. Standardization in immunohistochemistry. *Appl Immunohistochem Molec Morphol*. 2001; 9(1):3-8.
- [4]. Kabiraj A, Gupta J, Khaitan T, Bhattacharya PT. Principle and techniques of immunohistochemistry - a review. *Int J Biol Med Res*. 2015; 6(3):5204-5210.
- [5]. Bachman J. Immunohistochemistry on Freely Floating Fixed Tissue Sections. *Methods in enzymology*. Academic Press, 2013, pp 207-215.
- [6]. Hsi ED. A practical approach for evaluating new antibodies in the clinical immunohistochemistry laboratory. *Arch Pathol Lab. Med*. 2001; 125(2):289-94.
- [7]. Sims B. A simple method of preparing 1-2 micron sections of large tissue blocks using glycol methacrylate. *J Microsc*. 1974; 101:223-227.
- [8]. Mazzoni TS, Quagio-Grassiotto I. In totum deparaffinization of biological samples and re-embedded in historesin for better diagnostic. Under review.
- [9]. Souza W. Técnicas de Microscopia Eletrônica Aplicadas às Ciências Biológicas, 3th ed. Rio de Janeiro-RJ, Editora da Sociedade Brasileira de Microscopia e Microanálise, 2007, 357p.
- [10]. Hayat MA. Immunogold-silver staining: principles, methods, and applications. CRC Press, 1995, 304p.