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Short communication

A novel immunohistochemical method for evaluation of antibody specificity and detection of labile targets in biological tissue

Michael Levin*

Cytokine Biology Department, The Forsyth Institute, and Department of Oral and Developmental Biology, Harvard School of Dental Medicine, 140 The Fenway, Boston, MA 02115, USA

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Abstract

Immunohistochemistry is a key tool for analyzing target molecule localization within tissues. However, accurate results require an antibody that can distinguish between similar compounds. We present a simple immunohistochemical method that can also be used to rapidly evaluate antibodies' specificities. We demonstrate this technique with serotonin, an extremely labile compound. Serotonin (5-hydroxytryptamine, 5HT) is an important neurotransmitter regulating normal cognition and several mental disorders, as well as tumor growth, cardiopathology, and embryogenesis. Immunohistochemical detection of serotonin is commonly used as a neuronal cell marker and to provide crucial information on serotonin's role as an embryonic morphogen. It is necessary to be able to distinguish serotonin from closely related molecules with significantly different biological activity. Using our method, we identify antibodies that are specific for serotonin and show that some commercial 5HT antibodies often used to identify serotonergic cells in published papers are not 5HT-specific. These data demonstrate the necessity of specifically testing antibodies (especially in areas of high clinical relevance such as 5HT). We also illustrate detection of serotonergic cells in embryonic tissue using our technique. This method offers a number of general advantages for testing specificity of antibodies to any biological molecule, and helps avoid false positives and negatives during immunohistochemistry.

Keywords: Serotonin; Immunohistochemistry; Melatonin; 5HT; Antibody specificity

* Tel.: +1-617-262-5200x403; fax: +1-617-456-7731.
E-mail address: mlevin@forsyth.org (M. Levin).

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1. Introduction

Immunohistochemistry is a key tool for the analysis of localization of target molecules within tissues, and is used routinely for almost every aspect of modern biomedical research. However, accurate results require an antibody that can distinguish between the intended molecule and related compounds. Technical ease of use, rapidity, and reliability usually determine the techniques utilized in academic or medical settings. Standard histological methods often suffer from the use of harsh solvents or high temperatures (which are particularly problematic for unstable targets). Most importantly, immunohistochemistry performed on real tissues offers no simple and direct way to compare the specificity of an antibody for related molecules. Here, we present a simple method for immunohistochemistry on tissue sections that can also be used to rapidly evaluate antibodies' specificity for the target of interest. We demonstrate this technique with serotonin, an extremely labile compound with high clinical relevance.

Since the 1950s, the indoleamine serotonin (5-hydroxytryptamine, 5HT) has become increasingly recognized to be a vasoactive substance and a neurotransmitter in the central nervous system that regulates psychodynamic function [1]. It has been linked to a variety of diverse medical conditions including tumor growth [2,3], irritable bowel syndrome [4], circadian rhythms [5], and cardiac disease [6]. Its crucial role in controlling neuronal activity has allowed it to be directly implicated in both normal cognition and a number of syndromes such as memory impairment [7], sleep disorders [8], migraine [9], schizophrenia [10], dementia [11], aggression [12], depression [13], and a number of other mood disorders. Because of this, the various elements of the serotonin pathway are extremely popular pharmaceutical targets for depression (viz. Prozac), gastric problems, seasonal and circadian syndromes, migraines, and many others. The top five leading CNS drug products in the world are serotonin modulators, totaling over US$11 billion in the year 2000 [14,15]. Thus, understanding the cellular mechanisms of serotonin signaling is of key importance to biomedicine. Many in vitro analyses of brain tissue use the presence of serotonin as a marker of neuronal subtypes, and this technique is likewise an integral part of studies other than those focussed on serotonin, by virtue of its value as a morphological and functional neuronal identity marker [16–23].

It is now appreciated that serotonin has another crucial role: control of pattern formation during early stages, prior to the appearance of neurons [24–30]. Serotonin is widely distributed in both the animal and the plant kingdoms and in addition to mammalian embryos [31], it is found in such diverse locations as mollusks, arthropods, fruits, and venoms [32]. 5HT has been detected in eggs and oocytes of both vertebrate and invertebrate embryos [33], and the serotonergic synapse has been suggested to be an evolutionary co-opting of far more primitive signaling events between embryonic blastomeres [34]. Thus, the understanding of non-neuronal serotonin signaling is a very important aspect of evolutionary developmental biology. However, the non-neuronal mechanisms of serotonergic signaling are still poorly understood, and detailed information on its localization in embryos will be required as part of the functional analysis.

Thus, the ability to localize serotonin within tissues and at the subcellular level is key to understanding its functional role in morphogenesis and brain function. As of the end of 2002, the Medline database contains approximately 2500 studies that have relied on

identification of serotonergic neurons for analysis of brain morphology and correlation with pathological brain states. This is most commonly done through immunohistochemistry—the identification of serotonin signal by binding a serotonin-specific antibody. A number of commercially available antibodies to serotonin exist, and are used in studies resulting in conclusions which have implications for medicine and future research directions. Importantly, almost all such studies rely on the manufacturers' claims of the antibody's specificity, and do not test this property directly.

Because of the high clinical and basic biological relevance of serotonin, it is crucial that the detection be specific. The serotonin pathway includes a number of biochemically related molecules (compare structures in Fig. 1); thus, false positives can easily arise if antibodies do not distinguish between serotonin and its metabolites. Because cells that contain serotonin precursor may not be synthesizing serotonin, and because serotonergic metabolites (such as melatonin) may enter cells via membrane transporters and gap junctions, the presence of serotonin-like molecules is no guarantee of serotonin signaling occurring within the cell. Such false positives can provide misleading data that could negatively impact further research in basic biology and clinical studies. False negatives can arise as well: if the immunohistochemical reaction is stopped due to the background (which may result from reactivity to 5HT-related molecules), serotonin itself may not be detected. Such problems can lead to the failure to find serotonin that may exist within cells but be masked by more abundant serotonergic molecules if a non-specific antibody is used.

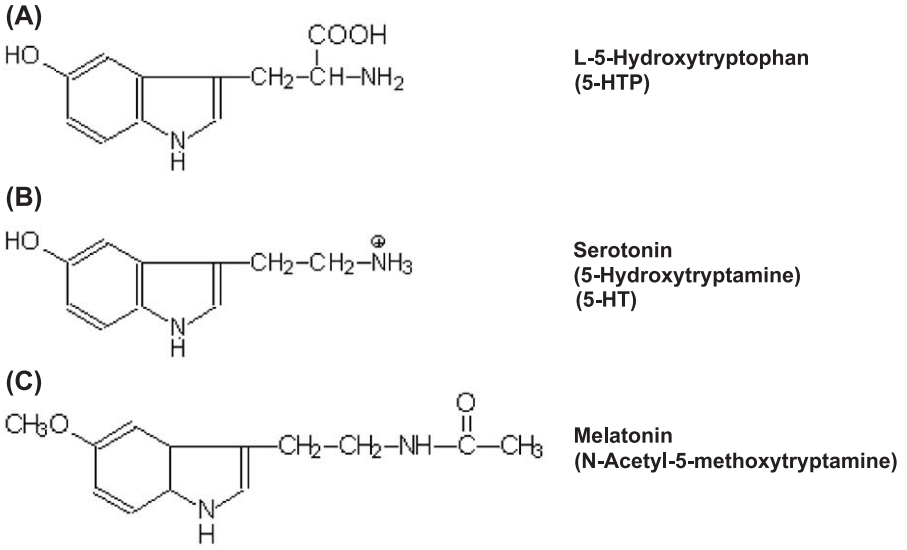


Fig. 1. Structures of serotonin and related molecules. Chemical structures of serotonin and metabolites, illustrating the difficulty antibodies face in discriminating among related molecules. The serotonin pathway includes molecules of similar biochemical structure, such as (A) 5-HTP—the serotonin precursor in synthesis via tryptophan hydroxylase, (B) serotonin itself, and (C) melatonin—created from serotonin by the action of the *N*-acetyltransferase enzyme. Each molecule has different biological activity; because the site of binding of antibodies are generally not known, it is important to determine whether any given antibody can distinguish among these targets.

Indeed, the same problems are central to the study of many other types of biological molecules, especially those which are not proteins (such as other neurotransmitters and a plethora of small signaling compounds). In the course of our lab's efforts to understand the function of serotonin during very early embryogenesis, we developed a simple technique for detection of serotonin within tissues, and for testing the specificity of antibodies for a range of targets. We specifically sought to avoid harsh treatments (such as the high temperatures and solvents used for paraffin embedding), since serotonin and many other signaling molecules are fairly unstable, and we wished to retain the native tissue morphology of embryonic structures. Our method allows the direct comparison of the specificity of any antibody for a variety of molecules within the same section, thus providing an internal control for other variables arising during immunohistochemistry. It offers a number of benefits over traditional methods, and can be used to provide semi-quantitative analyses of target content within tissue. To illustrate its use, we apply this technique to assessing several commercially available antibodies for specificity to serotonin, and demonstrate detection of serotonergic cells in frog embryos.

2. Materials and methods

2.1. Embedding and sectioning

The embedding medium consists of: 22.5 ml 10 × phosphate-buffered saline (PBS), 1.1 g of Gelatin (Sigma Type A, #G1890), and 225 ml dH_2O. The mixture is stirred with constant heating for 1 h at 60°, approximately 1 h, then cooled down to room temperature 67.5 g of bovine albumin (Sigma #A3912) is added, and the mixture is again stirred at room temperature until it is completely dissolved. The beaker is covered and stored at 4 °C until the next day. It is then heated up to 37 °C in a water bath, aliquoted into 40 ml samples, and stored at − 20 °C. The stocks can be thawed at 37 °C when needed, and the unused portion can be re-frozen and reused as many times as needed.

To embed embryos or tissue, 1.5 ml of thawed embedding mix is put on ice in a plastic mold for 10 min. It does not solidify (the cold does not impair subsequent steps), but chilling is necessary to slow down the reaction and allow thorough mixing. 105 μl of glutaraldehyde (Sigma, #C5H8O2) is added with a plastic pipette and mixed thoroughly, trying not to introduce bubbles (which can be removed from the top surface). It solidifies in 5 min. If the material to be embedded is contained in an aqueous medium (such as many embryonic samples that are stored in PBS), it should first be placed into a sample of embedding mix to equilibrate for 5 min. This is not necessary for dry samples. It is then placed on top of the solidified block and oriented as desired under a dissecting microscope. As much as possible of the liquid (in the case of equilibrated samples) is removed with a P200 Pipetteman. Another ice-chilled 1.5 ml aliquot of embedding mix is combined with glutaraldehyde, and quickly but gently poured over the sample to be embedded. About 20 s is available for final spatial adjustment of the sample(s) before the block solidifies; the movements are made under a dissecting microscope (on low magnification) using a P200 Pipetteman. Within 10 min, the sample becomes thoroughly embedded in a solid block of embedding mix. If the secondary antibodies to be used are fluorescent (not enzymatic), one

modification should be made to avoid autofluorescence: instead of glutaraldehyde, 210 μl of formaldehyde is mixed in with the aliquot of embedding mix, and the sample is left still for 4 h (formaldehyde fixes slower than glutaraldehyde, but exhibits no autofluorescence).

The plastic mold is then cut away and the sample trimmed to be about 0.7 cm wide with a razor blade. The trimming can be done in any manner which orients the sample to the correct cutting plane. The trimmed blocks can be stored in an airtight container with a wet paper towel (to prevent dehydration) at 4 °C for several weeks without loss of signal.

For testing antibody specificity, sections are made by vibratome: blocks are attached to metal chucks by any brand of super-glue. The chucks are then used in a Leica VT1000S vibratome (or similar device). They are cut in water at thickness of 20–50 μm, speed 2, frequency 3. Each section is collected by forceps into a scintillation vial containing PBS + 0.01% sodium azide. Because the azide inhibits bacterial and mold growth, the sections can be stored this way for several weeks at 4 °C without loss of signal. For immunohistochemistry on real tissues, vibratome sections work very well, but if extremely thin sections are desired, the blocks can be chilled and cut using a conventional microtome. After cutting, the material is easily removed from the metal chucks using a razor blade, leaving a smooth surface and allowing the chucks to be reused indefinitely.

2.2. Immunohistochemistry

The sections can be processed directly in vials, using trap-vacuum aspiration to replace solution between washes. This avoids having to mount section on slides, and makes all slide-handling equipment unnecessary, as well as avoiding problems with section loss, staining on slides, over-drying, etc. We have found this method to be much more convenient than working with slides. The secondary antibody can be coupled for either fluorescent or enzymatic detection (alkaline-phosphatase or horse-radish peroxidase), and multiple proteins can be detected in the same section if different secondaries are used. The conditions can easily be modified for tissues and samples of various properties; the data described in this paper was obtained using the following protocol.

PBSTB = 1 × PBS + 0.1% Tween-20 + 2 mg/ml BSA. Sections are blocked in PBSTB + 10% goat serum for 1 h. Primary antibody at optimal concentration (usually 1:1000 dilution for monoclonal) is added in PBSTB + 10% goat serum, and incubated with slow shaking overnight at 4 °C. They are then washed 6 × in PBSTB (1 h each wash), blocked again for 1 h, and the appropriate secondary antibody (anti-rabbit for example) coupled to alkaline phosphatase (Jackson Biolabs) is added at 1:1500 dilution in PBSTB + 10% goat serum. The sections are incubated with slow shaking overnight at 4 °C, and then washed 6 × in PBSTB (1 h each wash); one wash with alkaline-phosphatase chromogenic solution (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.5% Tween 20, 5 mM levamisole) is followed by development in the dark using BCIP and NBT chromogens (75 mg/ml in 70% dimethylformamide of NitroBlue Tetrazolium stock used at 1 μl/ml, and 50 mg/ml in water of 5-bromo-4-chloro-indolyl-phosphate sodium salt stock used at 3.5 μl/ml). When color is as dark as possible on low background (usually 2 h), the reaction is stopped by a wash in PBST pH 4.5, and post-fixed in 4% paraformaldehyde + 0.5% glutaraldehyde for 2 h at room temperature. It should be noted that the incubation and wash times indicated give optimal results (good signal, low background) for difficult

antibodies or tissues. If using strong monoclonals or affinity-purified polyclonals, the times can be shortened considerably. In most cases, incubation in primary antibody for 2 h at room temperature, followed by 30 min washes (and an identical reduction for the secondary incubation and washes) are sufficient.

3. Results and discussion

The preceding protocol describes the procedure for embedding, sectioning, and processing tissue samples, which can be fixed using any desired fixative. This protocol works well for whole frog and chick embryos, as well as other material such as adult tissues, flatworms, zebrafish, and a number of other sources. We used this technique to test antibodies' specificity for the serotonin pathway, seeking to locate an antibody that gives the strongest possible signal for serotonin (to avoid false negatives), and at the same time does not give background to the embedding medium nor cross-reactivity to related molecules (false positives). We were also interested in evaluating the specificity of commercial serotonin antibodies commonly used in the published literature.

Gelatin/albumin blocks (containing no tissue) were made as described above (illustrated in Fig. 2), and equal concentrations (10 mg/ml stock, diluted as 126 μl into a block of 1.8 ml) of pure serotonin, 5HTP (the immediate precursor of serotonin), or melatonin (a metabolite of serotonin) were mixed into each block prior to solidification with glutaraldehyde. The blocks were hand-trimmed with a scalpel blade into a distinct geometric shape (circle, square, or triangle), placed next to each other on top of another solidified albumin/gelatin block, and then flooded with fresh medium + glutaraldehyde. They were then sectioned by vibratome, and alternating sections were placed in one of three vials. Each of the vials was then processed for immunohistochemistry using a different commercially available primary serotonin antibody (with alkaline-phosphatase secondary detection). The sections were photographed using a digital camera and no differential image processing was applied to the shapes within any section. This allowed us to directly compare, in the same section (and thus under identical conditions of immunohistochemistry and detection), the degree of affinity of each antibody for each molecule.

Sample data applying this method to three commercially available antibodies are shown in Fig. 3. It was found that antibody A (Fig. 3A) and antibody B (Fig. 3B) differed in the level of background and affinity for 5HT but clearly bound serotonin with greater affinity than either melatonin or 5HTP (the square is far darker than the other two shapes). To quantify this difference, we applied a simple histogram algorithm (Fig. 3D) to a grayscale version of each panel (selecting a 1024 pixel^2 region in the center of each shape). Higher grayscale values indicate darker stain, while lighter stain is mapped to lower numbers. For panel B, the mean gray value for the circle was 167.86, and that of the triangle was 182.97 (standard deviations of 4.04 and 3.55, respectively). In contrast, that of the square (which contained serotonin) was 30.78 (standard deviation 8.8). These measurements confirm the easily observable qualitative difference between the stain created by the secondary antibody reaction, and thus the higher affinity of this antibody for serotonin as compared to similar molecules. Importantly, antibody C (Fig. 3C) (widely used in the mammalian neuron literature) recognized the precursor (5HTP, the circle) slightly better than 5HT, but

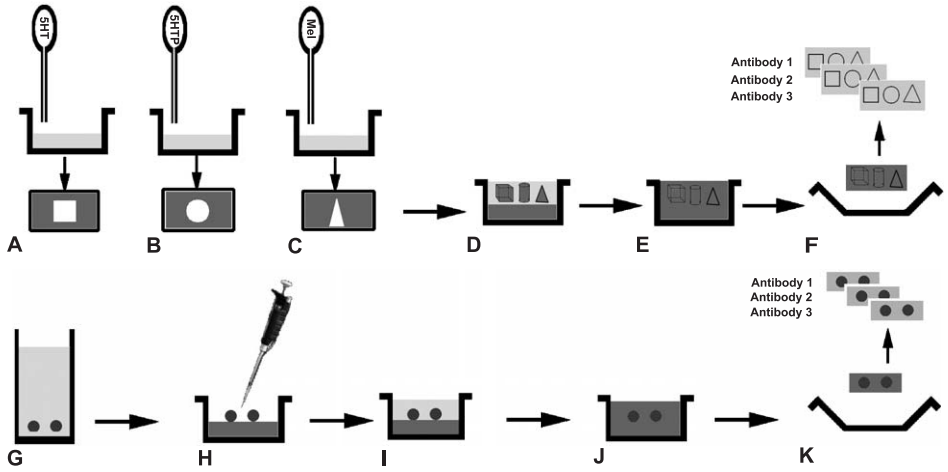


Fig. 2. Schematic of embedding process. (A–C) To compare the avidity of antibodies for different compounds, equivalent concentrations of each was mixed with embedding mix, solidified by glutaraldehyde, and cut into a distinctive shape. (D) The shapes were placed next to each other on a solidified block of embedding mix, and flooded with new embedding mix + glutaraldehyde. (E) The mix solidified, resulting in one solid block comprising shapes each of which contains a different compound. (F) The block is then removed from the plastic mold, trimmed, and sectioned; alternate sections are processed with different antibodies to test discrimination. (G) Embedding of real samples using this method begins with a quick equilibration of the material in embedding mix. (H) Samples are then placed and oriented on a solidified block of embedding mix, and extra liquid removed carefully. (I) They are then flooded with embedding mix + glutaraldehyde. (J) The block solidifies within 5 min. (K) The block is then removed from the mold, trimmed, and sectioned for immunohistochemistry; alternating sections can be processed using different antibodies.

did not discriminate among the molecules well. The gray values for the triangle, square, and circle of panel C were 149.85, 141.29, and 130.32, respectively (standard deviations 3.66, 3.81, 3.73).

Our results indicate that antibody B is most specific for serotonin, and that some commercially available antibodies which are listed as serotonin-specific do not in fact distinguish serotonin from related molecules. Our data suggest that findings derived from this antibody may need to be re-evaluated, to the extent that the 5HT signal may have been due to serotonin precursor or melatonin instead. Our findings likewise indicate caution for future studies, and point to the necessity for testing the antibodies to ensure that their specificity is acceptable for a given application. However, knowing the lack of specificity, antibody C is a useful reagent when one is interested in simultaneously detecting a broad spectrum of serotonergic molecules within a cell or tissue.

After an appropriate antibody has been identified, this method can be used to easily detect signal in tissue. To illustrate this, we chose to assay the presence of serotonergic neurons in the optic nerve, since a number of published studies using standard immunohistological methods have reported serotonin presence in neurons of the optic nerve in a variety of species [35–44]. Tailbud stages of *Xenopus* embryos were fixed overnight in 4% formaldehyde, and embedded as described above. They were then sectioned perpen-

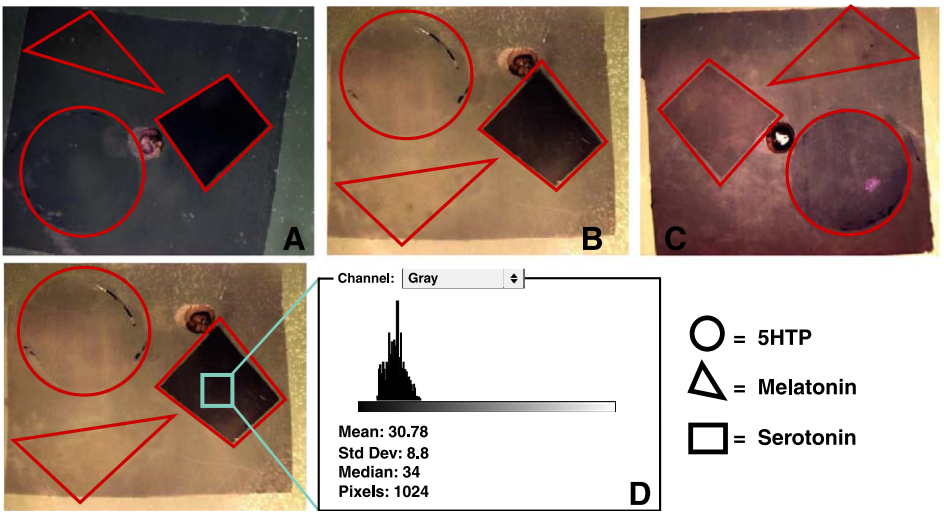
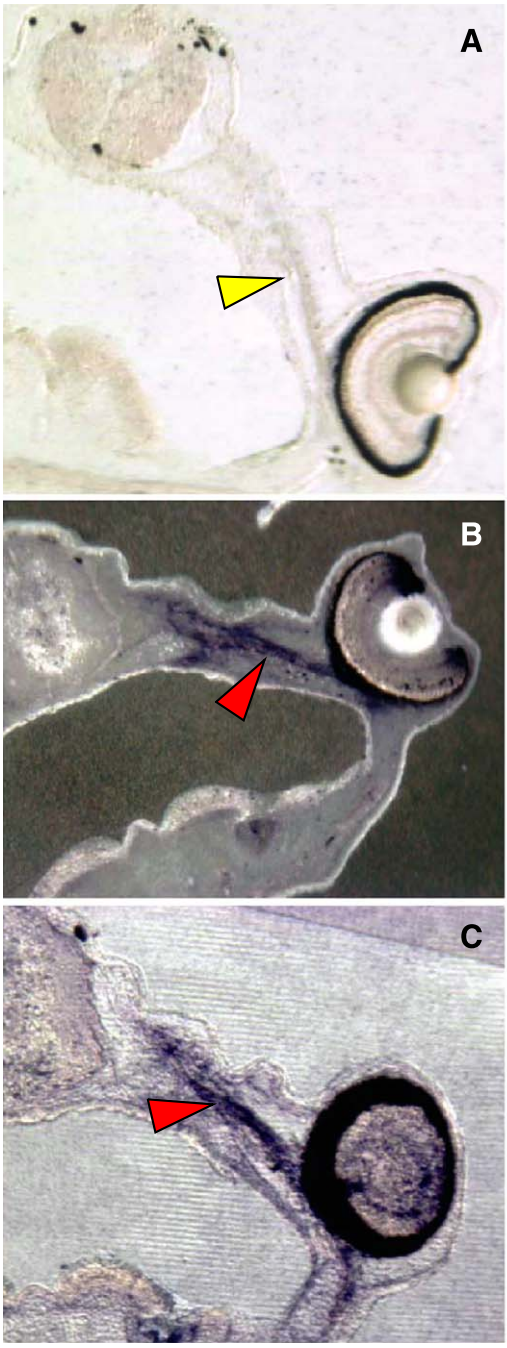


Fig. 3. Testing antibodies' specificity to serotonin, melatonin, and 5HT precursor. Comparative immunohistochemistry of serotonin pathway molecules. Blocks were made containing pure 5HTP, melatonin, and serotonin. Each one was trimmed into a distinctive shape and embedded together in a new block (outlined in red in the figure). A frog embryo was embedded (the small circle in the middle) to make the sections easier to see in the vials when changing solutions. The new block was sectioned, and the sections were processed for immunohistochemistry using three different, commonly used, commercially available antibodies (panels A–C). The sections were photographed with a digital camera and the darkness levels of each shape were quantified using a histogram algorithm (sample shown in panel D).

dicular to the anterior–posterior axis, and the sections were processed for immunohistochemistry using the best antibody to serotonin (antibody B) and an alkaline phosphatase-coupled secondary. The results are shown in Fig. 4. Control sections (receiving no primary antibody) are extremely clean and show no signal (Fig. 4A). In contrast, serotonergic neurons can be easily seen in the optic nerve (Fig. 4B,C) in sections incubated with antibody B. The results using this method are in good agreement with the results of other immunohistological methods; moreover, we have used this technique to detect serotonin in a variety of tissues and stages in frog, chick, and flatworm tissues (data not shown).

The gelatin/albumin protocol is an improvement on traditional histology for a number of reasons. The tissue is never exposed to harsh solvents, alcohols, or high temperatures (all of which can contribute to false negatives by degrading serotonin and other molecules

Fig. 4. Detection of serotonin in embryonic tissues. *Xenopus* laevis embryos at st. 43 were fixed, embedded, sectioned through the head perpendicular to the anterior–posterior axis, and processed for immunohistochemistry as described in the Materials and methods using an alkaline phosphatase-coupled secondary antibody. (A) Sections processed without primary antibody exhibit no signal. In contrast, sections incubated with an anti-serotonin primary antibody exhibit clear signal in the optic nerve, viewed in darkfield (B) or bright-field (C) microscopy on a Nikon SMZ-1500 dissecting microscope. Red arrowheads point to blue signal indicating presence of serotonin. Yellow arrowhead indicates absence of signal in the same location in control sections.


A
Control (no primary)
B
5HT antibody (darkfield)
C
5HT antibody (brightfield)

of interest). This method is faster and less work-intensive than most common histology protocols because no transfer through alcohol or organic solvents, clearing or de-paraffinizing is needed. Once all of the reagents are assembled, a vial of sections can be produced (including embedding) in a little over 1 h. The orientation of tissue samples in the block is easy because the method takes place at room temperature, and the medium is transparent (unlike paraffin). Hard tissues can be embedded as easily as soft ones, since the medium never needs to infiltrate the sample—glutaraldehyde holds it inside the block; the fact that the tissue is not impregnated with any exogenous medium also helps to guard against epitope masking or distortion of tissue morphology. Finally, the glutaraldehyde cross-linking of sample to block makes for very sturdy sections (with none of the sample detachment or curling problems seen in cryosections)—the sections can be lifted up by a corner with forceps and dropped into vials of PBS (no mounting on slides is required). The immunohistochemistry is done directly in vials, allowing volumes of liquid to be quickly changed out by aspiration. When the detection is finished, sections can be observed directly in petri dishes of PBS or mounted onto slides for permanent storage.

Even if a lab stays with conventional histology methods for analysis of tissues, this method allows rapid testing of new antibodies, or characterizing existing antibodies against many novel targets, as shown in the case of serotonin. It can be used to assess antibodies' specificity for many different biological molecules besides serotonin, and to test reactivity against closely related compounds. The method is easily adapted to alternate methods of detection (fluorescent, HRP enzyme, etc.) by simply varying the secondary antibody and detection reaction. It avoids the problems of finding suitable positive controls in real tissue because the pure target molecules are provided at known concentrations. It may thus also be used for semi-quantitative estimates of target concentration in tissue, by comparing darkness of stain in the sample (under identical chromogenic development time) to the color values of blocks containing known amounts of target compounds. By providing an easy way to perform immunohistochemistry in vials (with no slide-dipping equipment needed) and at room temperature, this method can increase the reliability of findings drawn from immunohistological analysis.

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