Immunoperoxidase and immunofluorescence technique for the identification of orexin and endocannabinoid receptors in the adult zebrafish
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Dear Prof. Werth,

Please find enclosed the manuscript entitled "Immunohistochemical identification of orexin and endocannabinoid receptors in the adult zebrafish at peripheral and central level" which we wish to submit for publication in JoVE.

This article describe the immunohistochemical protocols for the characterization and localization of orexin peptides, orexin receptor and endocannabinoid receptor expression in the gut and brain of normal and diet induced obesity (DIO) adult zebrafish, using immunoperixidase and double immunofluorescence methods. Specifically, we describe the best protocols to obtain optimal immunostaining signals.

We describe the methods of immunoperoxidase and immunofluorescence reporting the protocols for the preparation of all the reagents, fixation, paraffin-embedding and cryoprotection of zebrafish gut and brain and for the endogenous activities blocking step and background counterstaining. The complete set of parameters is obtained from previously IHC experiments, by which we have shown how the immunofluorescence can help to understand OXs, OX-2R and CB1R distribution, localization and conservation of expression in adult zebrafish tissues. The resulting images with high and specific signal intensity lead us to confirm zebrafish as a suitable animal model for the immunohistochemical study of distribution, localization and evolutionary conservation of specific biomarkers in physiological and pathological conditions. We recommend the protocols presented here for IHC experiments to detect general profiling of specific biomarkers in zebrafish.

For these reasons we believe this article to be of high potential interest for the readership of JoVE. We very much look forward to hearing your opinion regarding our article.

Sincerely,

Roberta Imperatore, PhD

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1 **TITLE:**
2 Identification of Orexin and Endocannabinoid Receptors in Adult Zebrafish Using
3 Immunoperoxidase and Immunofluorescence Methods
4
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22 **KEYWORDS:**
23 immunohistochemistry, immunoperoxidase, orexin receptor, endocannabinoid receptor,
24 zebrafish brain, zebrafish intestine
25

26 **SUMMARY:**
27 Presented here are protocols for immunohistochemical characterization and localization of
28 orexin peptide, orexin receptors, and endocannabinoid receptors in the gut and brains of
29 normal and diet-induced obesity (DIO) adult zebrafish models using immunoperixidase and
30 double immunofluorescence methods.
31

32 **ABSTRACT:**
33 Immunohistochemistry (IHC) is a highly sensitive and specific technique involved in the
34 detection of target antigens in tissue sections with labeled antibodies. It is a multistep process
35 in which the optimization of each step is crucial to obtain the optimum specific signal. Through
36 IHC, the distribution and localization of specific biomarkers can be detected, revealing
37 information on evolutionary conservation. Moreover, IHC allows for the understanding of
38 expression and distribution changes of biomarkers in pathological conditions, such as obesity.
39 IHC, mainly the immunofluorescence technique, can be used in adult zebrafish to detect the
40 organization and distribution of phylogenetically conserved molecules, but a standard IHC
41 protocol is not estasblished. Orexin and endocannabinoid are two highly conserved systems
42 involved in the control of food intake and obesity pathology. Reported here are protocols used
43 to obtain information about orexin peptide (OXA), orexin receptor (OX-2R), and cannabinoid
44 receptor (CB1R) localization and distribution in the gut and brain of normal and diet-induced

45 obese (DIO) adult zebrafish models. Also described are methods for immunoperoxidase and
46 double immunofluorescence, as well as preparation of reagents, fixation, paraffin-embedding,
47 and cryoprotection of zebrafish tissue and preparation for an endogenous activity-blocking step
48 and background counterstaining. The complete set of parameters is obtained from previous IHC
49 experiments, through which we have shown how immunofluorescence can help with the
50 understanding of OXs, OX-2R, and CB1R distribution, localization, and conservation of
51 expression in adult zebrafish tissues. The resulting images with highly specific signal intensity
52 led to the confirmation that zebrafish are suitable animal models for immunohistochemical
53 studies of distribution, localization, and evolutionary conservation of specific biomarkers in
54 physiological and pathological conditions. The protocols presented here are recommended for
55 IHC experiments in adult zebrafish.
56
57 **INTRODUCTION:**
58
59 Immunohistochemistry (IHC) is a well-established classic technique used to identify cellular or
60 tissue components (antigens) by antigen-antibody interaction[1,2]. It can be used to identify the
61 localization and distribution of target biomolecules within a tissue. IHC uses immunological and
62 chemical reactions to detect antigens in tissue sections[3]. The main markers used for the
63 visualization of antigen-antibody interactions include fluorescent dyes (immunofluorescence)
64 and enzyme-substrate color reactions (immunoperoxidase), both conjugated to antibodies[4].
65 Using microscopic observation is possible to determine the localization of labeled tissue, which
66 approximately corresponds to localization of the target antigen in the tissue.
67
68 Two methods exist for fluorescent or chromogenic reactions to detect protein: the direct
69 detection method, in which the specific primary antibody is directly labeled; and the indirect
70 detection method, in which the primary antibody is unconjugated while the secondary antibody
71 carries the label[5,6,7]. The indirect method has some advantages, which is mainly its signal
72 amplification. Moreover, unlike other molecular and cellular techniques, with
73 immunofluorescence, it is possible to visualize the distribution, localization, and coexpression of
74 two or more proteins differentially expressed within cells and tissues[7]. The choice of the
75 detection method used depends on experimental details.
76
77 To date, IHC is widely used in basic research as a powerful and essential tool to understanding
78 the distribution and localization of biomarkers and the general profiling of different proteins in
79 biological tissue from human to invertebrates[8-11]. The technique helps display a map of protein
80 expression in a large number of normal and altered animal organs and different tissue types,
81 showing possible down- or up-regulation of expression induced by physiological and
82 pathological changes. IHC is a highly sensitive technique that requires accuracy and the correct
83 choice of methods to obtain optimal results[12]. First of all, many different factors such as
84 fixation, cross-reactivity, antigen retrieval, and sensitivity of antibodies can lead to false positive
85 and false negative signals[13]. Selection of the antibodies is one of the most important steps in
86 IHC and depends on the antigen specificity and its affinity to the protein and species under
87 investigation[7].
88

89   Recently, we have optimized the IHC technique to detect members of orexin/hypocretin and
90   endocannabinoid systems in adult zebrafish tissue. We have focused mainly on fixation, tissue
91   embedding using two different approaches, sectioning and mounting (which can affect
92   resolution and detail during microscopic analysis), and blocking (to prevent false positives and
93   reduce background)[14]. Other important characteristics are the antibody specificity and
94   selectivity and reproducibility of individual IHC protocols. The key to providing antibody
95   specificity is the use of negative controls (including no primary antibodies or tissue that is
96   known to not express the target proteins) as well as positive controls (including tissue that is
97   known to express the target proteins)[15]. The selection of antibodies for IHC is made based on
98   their species-specificity (the likelihood with which they react with the antigen of interest) and
99   the antigen-antibody binding detection systems that is used[4-7]. In the case of
100 immunoperoxidase, the color of the reaction is determined by selection of the precipitating
101 chromogen, usually diaminobenzidine (brown)[16]. On the other hand, immunofluorescence
102 utilizes antibodies conjugated with a fluorophor to visualize protein expression in frozen tissue
103 sections and allows for easy analysis of multiple proteins with respect to the chromogenic
104 detection system[5,7].
105
106 In the immunoperoxidase technique, the secondary antibody is conjugated to biotin, a linker
107 molecule capable of recruiting a chromogenic reporter molecule [avidin-biotin complex (ABC)],
108 leading to amplification of the staining signal. With the ABC reporter method, the enzyme
109 peroxidase reacts with 3,3'-diaminobenzidine (DAB), producing an intensely brown-colored
110 staining where the enzyme binds to the secondary antibody, which can then be analyzed with
111 an ordinary light microscope. ABC staining, due to the high affinity of avidin for biotin, produces
112 a rapid and optimal reaction, with few secondary antibodies attached to the site of the primary
113 antibody reactivity. This chromogenic detection method allows for the densitometric analysis of
114 the signal, providing semi-quantitative data based on the correlation of brown signal levels with
115 protein expression levels[18].
116
117 With immunofluorescence techniques, simultaneous detection of multiple proteins is possible
118 due to the ability of different fluorochromes to emit light at unique wavelengths, but is
119 important to choose fluorochromes carefully to minimize spectral overlap[5]. Moreover, the use
120 of primary antibodies in different host species minimizes difficulties concerning cross-reactivity.
121 In this case, each species-specific secondary antibody recognizes only one type of primary
122 antibody. Fluorescent reporters are small organic molecules, including commercial derivatives,
123 such as Alexa Fluor dyes.
124
125 Many animal models are used to understand particular physiological and pathological
126 conditions. To date, it is established that many metabolic pathways are conserved over the
127 course of evolution. Therefore, IHC studies in model organisms such as zebrafish can provide
128 insight into the genesis and maintenance of pathological and non-pathological conditions[17]. It is
129 an aim of this report to illustrate IHC protocols that can be performed on adult zebrafish tissue
130 and used to obtain detailed images of the distribution and localization of OXA, OX-2R, and CB1R
131 at peripheral and central levels. Also reported are protocols for the application of two major
132 IHC indirect methods in peripheral and central tissues of adult zebrafish. Described is the

133 indirect method, which allows for signal amplification in cases where a secondary antibody is
134 conjugated to a fluorescent dye (immunofluorescence method) or enzyme reporter
135 (immunoperoxidase method). Both chromogenic and fluorescent detection methods possess
136 advantages and disadvantages. Reported in this protocol is the use of IHC, mainly
137 immunofluorescence, in adult zebrafish, an animal model widely used to study systems that are
138 evolutionary conserved across different physiological and pathological conditions.
139
140 **PROTOCOLS**
141
142 **1. Immunoperoxidase protocol**
143
144 NOTE: The zebrafish were obtained by Prof. Omid Safari (Department of Fisheries, Faculty of
145 Natural Resources and Environment, Ferdowsi University of Mashhad, Mashhad, Iran)[10].
146
147 **1.1. Tissue dissection**
148
149 1.1.1. Sacrifice the zebrafish by submersion in ice water (5 parts ice/1 part water, 4 °C); leave
150 them until cessation of all movement to ensure death by hypoxia.
151
152 1.1.2. Quickly remove the gut and brain with the following dissection method:
153
154 1.1.2.1. Dry the fish on a paper towel and place it sagittally on a dissecting mat, blocking the
155 ventral part of the eye socket and fleshy part of the tail.
156
157 1.1.2.2. For the intestine: cut the skin and carefully remove the skin and underlying muscle
158 from the side of the fish until the internal organs are visible. Remove the intestine from the
159 body cavity stretching it out.
160
161 1.1.2.3. For the brain: remove the head with a razor blade. Remove soft tissue from the ventral
162 side of the skull with forceps. Open the skull and remove the bone from the ventral side of the
163 brain. Remove the skin and skull bones from the dorsal side of the brain. Remove the brain.
164
165 **1.2. Tissue fixation**
166
167 1.2.1. Fix the dissecting tissues by immersion in fresh 4% paraformaldeyde (PFA) in phosphate
168 buffer (PB, pH 7.4, 4 °C) for 3 h at room temperature (RT).
169
170 1.2.1.1. Prepare 0.1 M PB by dissolving 1.755 g of $NaH_2PO_4H_2O$ and 4.575 g of Na_2HPO_4 in 450
171 mL of distilled water (dH_2O), and adjust to a pH of 7.4 with the necessary amount of NaOH 1N.
172
173 1.2.1.2. Prepare 4% PFA dissolving 4 g of PFA in 100 mL of 0.1 M PB (pH 7.4) by agitation on a
174 heat plate. When a temperature of 60 °C is reached, add 2-4 drops of NaOH 1N to obtain a clear
175 solution. Left PFA 4% at RT and control the pH . Adjust the pH to 7.4.
176

NOTE: Tissue fixation for more than 4–6 h may lead to overfixation, which masks antigens, limiting antibody-epitope binding. Time of fixation depends on tissue size.

1.3. Tissue embedding

1.3.1. Rinse the tissues 5x for 5 min each, by immersion in 0.1 M PB (pH 7.4).

1.3.2. Dehydrate the tissues by subsequent immersion in alcohol 70% (6 min), 80% (6 min), 95% (5 min), 95% (5 min), 100% (1 min), and 100% (1 min).

1.3.3. Clarify the tissues by immersion in 100% alcohol:xylene (1:1) for 10 min, then 2x in xylene for 5 min each.

1.3.4. Infiltrate the tissues with paraffin wax (56 °C) twice for 1 h each by direct immersion in paraffin.

1.3.5. Embed the tissues in paraffin blocks at room temperature (RT) and store at RT until sectioning.

1.4. Tissue cutting

1.4.1. Cut tissues into coronal or sagittal sections with 8 μm thickness with a microtome, and collect the sections in alternate series onto adhesive glass slides on the water and warmed at 38 °C.

1.4.2. Dry the slides with sections at 37 °C overnight.

1.5. Deparaffinization and rehydratation of tissue sections

1.5.1. Dewax the sections by immersion in xylene for 5 min followed by immersion in xylene for 3 min.

1.5.2. Rehydrate the sections by subsequent slides immersion in alcohol 100% II (1 min), 100% I (1 min), 95% (1 min), 75% (1 min), 50% (1 min), then place the slides in dH_2O (5 min).

NOTE: Completely dewax the sections before starting the reaction. If the sections still have traces of paraffin, perform an additional immersion in xylene for 5 minutes or more.

1.6. Antigen retrieval

1.6.1. Treat the sections with citrate buffer (0.01 M, pH 6.0) by immersing the slides in the solution and heating in a microwave oven for 5 min at maximal power.

1.6.2. Let the sections cool.

221

222 1.6.3. Repeat the cycle of 5 min in the microwave at maximal power to complete the antigen
223 retrieval.

224

225 1.6.3.1. Prepare citrate buffer (0.01 M, pH 6.0) by dissolving 2.10 g of citric acid in 100 mL of
226 dH_2O and 5.882 g of sodium citrate in 200 mL of dH_2O. Mix sodium citrate (0.01 M) with citric
227 acid (0.01 M) at a 1:4 ratio by volume and adjust the pH to 6 using HCl 0.1N.

228

229 **1.7. Blocking endogenous peroxidase**

230

231 1.7.1. Demarcate the tissue area on the slides with a solvent-resistant pen.

232

233 1.7.2. Rinse the sections 3 times, 5 min each, with tris-buffered saline solution (TBS) (0.1 M, pH
234 7.3).

235

236 1.7.2.1. Prepare 0.1 M TBS by dissolving 12.1 g of trizma base and 9 g of NaCl in 950 mL of dH_2O
237 and adjust to pH 7.3 with HCl 0.1N.

238

239 1.7.3. Block the endogenous peroxidase activity by slides immersion in a solution of 0.75% H_2O_2
240 for 5 min at RT.

241

242 1.7.3.1. Prepare the 0.75% H_2O_2 solution dissolving 5 mL of 30% H_2O_2 in 195 mL of dH_2O.

243

244 NOTE: Endogenous enzymes can react with IHC reagents and yield false positive results.
245 Moreover, highly vascularized tissues express many endogenous peroxidase, which can lead to
246 intense nonspecific staining and background levels. Treatment with 0.75% H_2O_2 quenches
247 endogenous peroxidase and significantly reduces the nonspecific background.

248

249 **1.8. Blocking of nonspecific binding sites and tissue permeabilization**

250

251 1.8.1. Incubate the tissue sections with the TBS/0.4% Triton (TBS-T) blocking buffer, containing
252 the primary antiserum (NRS), for 30 min at RT to block nonspecific binding sites.

253

254 1.8.1.1. Prepare 0.4% Triton by dissolving 0.4 mL of Triton X-100 in 100 mL of TBS.

255

256 1.8.1.2. Prepare the blocking buffer TBS-T solution by dissolving 1% normal serum in TBS
257 containing 0.4% Triton X-100 (1 mL of normal serum + 8.2 mL of TBS + 0.8 mL of 0.48 Triton X-
258 100).

259

260 NOTE: Select the species of the animal serum depending on the host of the secondary antibody
261 (e.g., when using a goat anti-rabbit secondary antibody, use normal goat serum).

262

263 **1.9. Tissue incubation with primary antibody**

264

265 1.9.1. Incubate the sections overnight in a humid box at RT with primary antibodies diluted in
266 TBS-T. Stain the sections with the following primary antibody:

267

268 1.9.1.1. Goat antibody against OX-2R diluted 1:200, which recognizes the C-terminal of the
269 protein.

270

271 1.9.1.2. Goat antibody against OX-A diluted 1:200 [orexin-A (C-19)], which recognizes the C-
272 terminal of the protein.

273

274 NOTE: Ensure that the primary antibody reacts with the biomolecule of interest. Define with
275 which epitope of the biomolecule the primary antibody reacts by using the gene alignment. For
276 instance, the OX-A epitope has been mapped between the aa 50–100 of human OX-A (O43612)
277 whereas the OX-2R epitope has been mapped near the last 50 aa at the C-terminal of human.

278

279 **1.10. Tissue incubation with secondary antibody**

280

281 1.10.1. Rinse the sections 3x for 5 min each, with 0.1 M TBS (pH 7.3).

282

283 1.10.2. Incubate the sections for 1.5 h at RT with biotinylated secondary antibody and rabbit
284 anti-goat immunoglobulin (H+L) conjugated with biotin, then dilute 1:100 in TBS-T.

285

286 NOTE: Test and find the different dilutions of both primary and secondary antibodies to find the
287 optimal dilution that allows reduction of the background.

288

289 **1.11. Peroxidase reaction**

290

291 1.11.1. Rinse the sections 3x for 5 min each, with 0.1 M TBS (pH 7.3).

292

293 1.11.2. Incubate the sections with avidin-biotin complex (ABC) for 1 h.

294

295 1.11.2.1. Prepare ABC solution adding two drops of component A followed by two drops of
296 component B in 5 mL of TBS.

297

298 1.11.3. Rinse the sections 3x for 5 min each, with 0.1 M TBS (pH 7.3).

299

300 1.11.4. Treat the sections with the chromogen substrate 3,3'-diaminobenzidine-4 (DAB).

301

302 1.11.4.1. Prepare DAB solution adding one drop (approximately 30 μL) of DAB chromogen
303 concentrate to 1 mL of DAB diluent, and mix well before use.

304

305 NOTE: Prepare ABC solution at least 30 min before use and keep the complex at 4 °C until use
306 to allow for stable avidin/biotin binding. Prepare the fresh DAB working solution, apply to the
307 tissue sections, and develop until the color is appropriate. When the chromogenic reaction
308 converts the epitope sites to a brown color and the intensity of the signal is appropriate for

imaging, proceed to the next step. Timing of DAB development may vary from a few seconds to 10 min. For OX-A and OX-2R 1, 2 min is enough. Handle DAB with care, as it is carcinogenic.

1.12. Tissue dehydratation and mounting

1.12.1. Rinse all the sections 3x for 5 min each, with 0.1 M TBS (pH 7.3) to stop the DAB reaction.

1.12.2. Dehydrate the sections by subsequent slides immersion in alcohol 50% (2 min), 75% (2 min), 95% (2 min), 100% I (2 min), 100% II (2 min).

1.12.3. Clarify the slices by immersion 2x in xylene 10 min each.

1.12.4. Mount the sections with DPX (dibutyl phthalate xylene) mountant for histology, adding two drops of mounting media to slides and topping slowly with coverslips.

NOTE: Since the immersion of tissue in increasing concentrations of alcohol during dehydration results in alcohol penetration of tissue and replacement of water with alcohol, determine a precise dehydration time and do not over dehydrate the tissues. Perform the immersion in xylene after dehydration to clarify the tissue and remove any excess or remaining alcohol.

1.13. Controls

1.13.1. Repeat sections 1.1–1.12, omitting the primary antibody and/or substituting the primary antibody or the secondary antibody IgG by TBS (negative controls).

1.13.2. Repeat sections 1.1–1.12 pre-absorbing each primary antibody with an excess of the relative peptide (100 mg of peptide/1 mL of diluted antiserum).

1.13.3. Repeat sections 1.1–1.12 using different tissue as positive control (e.g., mouse tissue).

NOTE: Prepare all buffers fresh, shortly before starting. Carefully remove the excess fluid at each step with a pipette or filter paper around the sections, always keeping the sections wet.

1.14. Image acquisition and analysis

1.14.1. Acquire digital images under constant light illumination and at the same magnification using a microscope equipped with a digital camera.

1.14.2. Perform a semi-quantitative analysis of staining intensity using imaging software (see **Table of Materials**).

1.14.2.1. Open the captured images, in .lif or .tiff file format, for evaluating indices of OX-A or OX2-R positivity.

1.14.2.2. Select the button under **Measure** and click on **Manual Counting**. Count the number of stained cell profiles directly from the screen by placing a mark onto positive cells by clicking the mouse.

1.14.2.3. Select a region of interest (ROI) area (3×10^3 μm^2) to quantify the immunosignal density (optical density, OD).

1.14.2.4. Determine the pixel with the highest intensity (high positive) and least intensity (negative) inside the analyzed image to assign the zero as the value of the background (i.e., a portion of tissue devoid of stained cells).

1.14.2.5. Assess the OD by working on a logarithmic scale of absorbance. In digital image analysis, the DAB pixel intensity ranges from the darkest (0 value) to lightest (255 value) shade.

1.14.2.6. Determine OD by plotting a histogram of the following: $log_{10}(255/I)$, where "I" is the pixel intensity value given by the program and determined by subtracting the background.

NOTE: The permanent and stable immunoperoxidase reaction can be analyzed under a bright-field microscope at any time. Perform the counting of labeled cells on alternate section to avoid double-counting of the same cell from adjacent sections.

2. Immunofluorescence protocol

2.1. Tissue dissection

2.1.1. Sacrifice and dissect the animals as described in section 1.1.

2.2. Tissue fixation

2.2.1. Fix the gut and brain by immersion for 3 h in 4% PFA at 4 °C.

2.2.1.1. Prepare PB and 4% PFA as in section 1.2.1.

NOTE: The time of fixation depends on tissue size. Tissue fixation for more than 4–6 h may lead to overfixation, which masks antigens and limits antibody-epitope binding.

2.3. Tissue embedding

2.3.1. Rinse the tissues 3x for 5 min each, with 0.1 M PB (pH 7.4).

2.3.2. For the cryoprotection, transfer the tissues to 20% sucrose in PB (0.1 M, pH 7.4) and keep overnight at 4 °C. Then, transfer the tissues to 30% sucrose in 0.1 M PB (pH 7.4) and keep for an additional night at 4 °C.

397

398 2.3.3. Embed the tissues in a block of optimal cutting temperature (OCT) compound. To do this,
399 prepare a small dewar of liquid nitrogen, take aluminum foil, and cut it in half to create a pan.
400 The pan containing the tissues filled with the OCT compound has to be dipped in the liquid
401 nitrogen until the OCT compound is cooled. The frozen tissues can be stored at -80 °C until
402 sectioning.

403

404 **2.4. Tissue cutting**

405

406 2.4.1. Transfer the frozen tissue blocks to a cryostat at -20 °C and cut in coronal or sagittal
407 sections of 10 µm.

408

409 2.4.2. Collect the tissues in alternate serial sections onto adhesive glass slides suitable for
410 immunohistochemistry and store them at -20 °C until use.

411

412 NOTE: Store slides between -20 °C and 4 °C in a dark slide box or slide book.

413

414 **2.5. Blocking of nonspecific binding sites and tissue permeabilizzation**

415

416 2.5.1. Demarcate the tissue area on the slide with a solvent resistant pen.

417

418 2.5.2. Rinse the sections 3x for 5 min each, with 0.1 M PB (pH 7.4).

419

420 2.5.3. Incubate the sections with 1% normal donkey serum dissolved in the permeabilization
421 buffer PB-Triton X-100 0.3% (PB-T) for 30 min at RT to permeabilize the cell membrane and
422 block the nonspecific binding sites.

423

424 2.5.3.1. Prepare Triton X-100 0.3% dissolving 0.3 mL of Triton X-100 in 100 mL of 0.1 M PB (pH
425 7.4).

426

427 2.5.3.2. Prepare the blocking solution dissolving 1% normal donkey serum in PB containing 0.3%
428 TritonX-100.

429

430 NOTE: The animal species of the serum used in the permeabilization and blocking buffers are
431 dependent on the host of the secondary antibody.

432

433 **2.6. Incubation with mix of primary antibodies**

434

435 2.6.1. Rinse the sections 3x for 5 min each, with 0.1 M PB (pH 7.4).

436

437 2.6.1.1. Incubate the sections overnight in a humid box at RT with a mix of primary antibodies
438 diluted in PB-T. The following mixes of primary antibodies can be used: goat antibody against
439 OX-2R diluted 1:100/rabbit antibody against CB1R diluted 1:100, or goat antibody against OX-A
440 diluted 1:100/rabbit antibody against CB1R diluted 1:100.

2.7. Incubation with mix of secondary antibodies

2.7.1. Rinse the sections 3x for 5 min each, with 0.1 M PB (pH 7.4).

2.7.2. Incubate the sections for 2 h at RT with 1) a mix of donkey anti-rabbit Alexa Fluor 488-conjugated secondary antibody and donkey anti-goat Alexa Fluor 594-conjugated secondary antibody diluted 1:100 in PB-T; or 2) a mix of donkey anti-goat Alexa Fluor 488-conjugated secondary antibody and donkey anti-rabbit Alexa Fluor 594-conjugated secondary antibody diluted 1:100 in PB-T.

NOTE: Use secondary antibodies developed in the same animal host. The normal serum must belong to the same species of the secondary antibody (e.g., use secondary antibodies developed in donkey and a donkey normal serum). Dilute the primary and secondary antibodies with blocking buffer containing the detergent to increase cell permeabilization and reduce background.

2.8. Tissue mounting

2.8.1. Rinse the sections 3x for 5 min each, with 0.1 M PB (pH 7.4).

2.8.2. Counterstain the sections with nuclear dye DAPI (4',6-diamidino-2-phenylindole) prepared dissolving 1.5 µL of DAPI (1 mg/mL) in 3 mL of PB.

2.8.3. Coverslip slides with mounting medium (see **Table of Materials**). This aqueous mounting medium stabilizes the tissue sample and stains for long-term usage. Fluorescent samples can be stored in the dark at 4 °C. To prolong the life of fluorofores, use an antifed mounting medium.

NOTE: Choose a good mounting medium. One of the most important parameters of mounting agents is the refractive index (nD), which should be around 1.5, the refractive index of glass. The mounting medium used here can be used especially with specimen prepared for enzyme and lipid determinations (i.e., specimen that must not be dehydrated with an ascending series of alcohol).

2.9. Controls

2.9.1. Repeat sections 2.1–2.8, omitting the primary or secondary antibody, or substituting in the specific step the primary or secondary antisera with PB (negative control).

2.9.2. Repeat sections 2.1–2.8, pre-absorbing each primary antibody with an excess of the relative peptide (100 mg of peptide/1 mL of diluted antiserum).

2.9.3. Repeat sections 2.1–2.8 on slices of mouse brain (positive control).

485 NOTE: Prepare all the buffers fresh, shortly before starting. Remove the excess of fluid carefully
486 at each step with a pipette or filter paper around the sections, always keeping the section
487 humid.

488

489 **2.10. Image acquisition**

490

491 2.10.1. Use a confocal microscope equipped with an x-y-z motorized stage, digital camera, and
492 acquisition and image analysis software such as NIS-Elements C to observe and analyze the
493 immunostained sections. Acquire digital images using the 5-20-40x objectives.

494

495 2.10.2. Take the images of each section at low magnification (10x or 20x objective) in each of
496 the available channels to compose a low magnification montage, providing an overview of the
497 entire region to facilitate the localization and documentation of OX-2R/CB1R anatomical co-
498 expression. Normalize the fluorescence images to maximum contrast and overlay before the
499 analysis.

500

501 2.10.3. Collect serial Z-stacks of images throughout the area of interest. Acquire the images
502 through six focal planes (Z-step) with focal steps of 1–1.8 µm to cover the tissue volume in
503 which OX-2R/CB1R coexpression is visualized as yellow puncta. To do this collection for each
504 channel (red, green, blue) separately, by using a Z-motorized microscope.

505

506 2.10.4. Use an imaging deconvolution software to deconvolve images by application of ten
507 iterations and collapse the serial Z planes images into a single maximum projection image.

508

509 2.10.5. Adjust the micrographs for light and contrast using Adobe Photoshop 6.01 (Adobe
510 Systems, San Jose, CA).

511

512 NOTE: Perform the deconvolution step during observation to further decrease the background.
513 Limit the slide exposure to light to prevent photobleaching.

514

515 **2.11. Image analysis**

516

517 2.11.1.1. Perform quantitative analysis of OX-2R/CB1R coexpression on alternated 10 µm thick
518 sections (n = 5 animal per group) by covering all the region of interest of each animal.

519

520 2.11.1.2. Quantify the OX-2R/CB1R coexpression as number of yellow positive puncta with a
521 semiautomated system of image analysis. Imagins software can provide a higher level of detail,
522 with quantitative data regarding the regions of overlap between different fluorescent probes.

523

524 2.11.1.3. Quantify the puncta by using thresholding tools for signal intensity in the two
525 channels. Open the .liff, .tiff, or .jpg images file and select **Image–Threshold**. Select **Auto**
526 **Setting** or **Manual Method** and regulate **Threshold** until all the stained puncta are selected.
527 Analyze the distribution of the pixel intensities in an area of the image that does not contain
528 any immunolabelled objects to obtain the background threshold. Determine this background

529   individually for every image. The program then removes the background threshold by setting
530   the baseline of pixel intensities to the background value.

531

532   2.11.1.4. Select **Analyze–Measure** and choose the parameters to be measured (puncta
533   intensity-minimum, maximum and mean values; number/density of the immunolabelled
534   puncta).

535

536   2.11.1.5. Count the yellow puncta along the volume of the tissue in 4 Z-stacks for each section,
537   using the stacks immediately above or below to the best focus plane. Exclude the out-of-focus
538   regions from the analysis.

539

540   NOTE: Perform the counting of labeled cells on alternate section to avoid double-counting of
541   the same cells from adjacents sections.

542

543   **REPRESENTATIVE RESULTS:**
544   Representative data for the immunoperoxidase staining are shown in **Figure 1** and **Figure 2**.
545   Immunohistochemical analysis of OX-A and OX-2R distribution in the gut of adult zebrafish
546   showed different localization sites of OX-A and OX-2R and their increases in expression in the
547   intestinal cells of DIO zebrafish. An intense brown staining for OX-A was observed in the cells of
548   the medial and anterior intestine (**Figure 1A**, A_1). The immunoexpression of OX-A gave clear
549   signals in the different gut compartements, decreasing from the anterior toward the medial
550   intestine (**Figure 1B**, B_1). The negative control was used as a reference for the background and
551   to confirm the specificity of OX-A signal (**Figure 1E**). The prolonged exposure to the chromogen
552   DAB resulted in the increase of the background intensity (**Figure 1D**). Similar results were
553   observed for OX-2R immunoexpression in the intestine of DIO and control diet zebrafish (**Figure
554   2**). An increased OX-A signal in DIO adult zebrafish was accompanied by the overexpression of
555   OX-2R in others intestinal compartements (**Figure 2B**, B_1).

556

557   Using immunofluorescence, the data obtained by immunoperoxidase analysis were confirmed,
558   underlying the increase of OX-A and OX-2R expression in the gut of adult DIO zebrafish with
559   respect to the control (**Figure 3**). Moreover, using double immunofluorescence, it was possible
560   to obtain information about the expression of the endocannabinoid receptor CB1R and its co-
561   localization with OX-A or OX-2R in the gut and brain of control diet and DIO adult zebrafish. The
562   advantage of immunofluorescence is that it yields a more detailed signal with less provided
563   information about tissue morphology, compared to immunoperoxidase technique. Using the
564   immunofluorescence method, we have previously determined anatomical interactions between
565   OX-2R and CB1R in both the gut and brain[10].

566

567   The accurate analysis of immunofluorescent images showed the increase of OX-2R/CB1R co-
568   localization in the gut of DIO adult zebrafish compared to the control diet zebrafish (**Figure 3B**,
569   **C**). A similar situation was observed in different brain regions, such as the dorsal telencephalon,
570   hypothalamus (lateral, ventral, and dorsal zones), optic tectum, torus lateralis, and diffuse
571   nucleus of the inferior lobe (**Figure 4**). The negative (**Figure 5A,B**) and positive (mouse brain)

572 (**Figure 5C**) controls were used as references for the background and to confirm the specificity
573 of CB1R and OX-2R signals.

574

575 Moreover, by double immunostaining with OX-A/CB1R, it was observed in the orexinergic
576 neurons of the hypothalamus that there was an increase of co-localization accompanied by an
577 increase of OX-A fluorescent signal (**Figure 6**). These results show how double
578 immunofluorescence can help to identify physiologically conserved protein expression, co-
579 localization of target proteins, and their distribution and/or expression changes in different
580 pathological conditions.

581

582 **FIGURE LEGENDS:**

583

584 **Figure 1: Orexin immunolocalization in the intestines of DIO vs. control diet adult zebrafish.**
585 (**A**) OX-A immunoreactivity in the cells of the medial intestine of control diet zebrafish. (A$_1$) OX-
586 A immunoreactivity in the cells of the medial intestine of DIO zebrafish. (**B**) OX-A
587 immunoreactivity in the cells of the anterior intestine of control diet zebrafish. (B$_1$) OX-A
588 immunoreactivity in the cells of the anterior intestine of DIO zebrafish. (A$_1$, B$_1$) An increase of
589 OX-A positive cells in different tissue compartement of the medial and anterior intestine of DIO
590 zebrafish. (**C**) OX-A immunoreactivity in the cells of the anterior intestine of DIO zebrafish. (**D**)
591 immunoperoxidase reaction for OX-A after a prolonged exposure to DAB. (**E**) Negative control.
592 Scale bar: 50 μm (A, A$_1$, B, B$_1$); 100 μm (C, D, E).

593

594 **Figure 2: Orexin 2 receptor immunolocalization in the intestine of DIO vs. control diet adult**
595 **zebrafish.** (**A**) OX-2R immunoreactivity in the cells of the medial intestine of control diet
596 zebrafish. (A$_1$) OX-2R immunoreactivity in the cells of the medial intestine of DIO zebrafish. (**B**)
597 OX-2R immunoreactivity in the cells of the anterior intestine of control diet zebrafish. (B$_1$) OX-
598 2R immunoreactivity in the cells of the anterior intestine of DIO zebrafish. (A$_1$, B$_1$) An increase
599 of OX-2R positive cells in different tissue compartement of the medial and anterior intestine of
600 DIO zebrafish. Scale bar: 50 μm.

601

602 **Figure 3: Distribution of OX-A (green)/CB1R (red) and OX-2R (red)/CB1R (green) and their co-**
603 **localization with OX-2R/CB1R (yellow) in the intestines of DIO vs. control diet adult zebrafish.**
604 (**A**) OX-A/CB1R co-expression in the intestine of control diet adult zebrafish. (**B**) OX-2R/CB1R co-
605 expression within the intestine of control diet zebrafish. (**C**) OX-2R/CB1R co-expression within
606 the intestine of DIO adult zebrafish. An increase of OX-2R/CB1R co-localization (yellow dots) in
607 the intestine of DIO zebrafish. Scale bar: 25 μm.

608

609 **Figure 4: Distribution of OX-2R (red) and CB1R (green) and their co-localization with OX-**
610 **2R/CB1R (yellow) in the brain coronal sections of DIO vs. control diet adult zebrafish.** (**A**) OX-
611 2R/CB1R co-expression in the telencephalon of the control diet zebrafish. (A$_1$) OX-2R/CB1R co-
612 expression in the telencephalon of DIO zebrafish. (**B**) OX-2R/CB1R co-expression within the
613 lateral, ventral and dorsal zone of hypothalamus, optic tectum, torus lateralis, diffuse nucleus
614 of the inferior lobe of control diet zebrafish (B1) OX-2R/CB1R co-expression within the lateral,
615 ventral, and dorsal zones of the hypothalamus, optic tectum, torus lateralis, and diffuse nucleus

616   of the inferior lobe of DIO zebrafish. (**C**) Higher magnification of the optic tectum showing the
617   co-expression of OX-2R/CB1R (yellow) in the control diet zebrafish. (**C₁**) Higher magnification of
618   the optic tectum showing the co-expression of OX-2R/CB1R (yellow) in the DIO zebrafish. (**D**) A
619   particular of the optic tectum showing the distribution and co-expression of OX-2R/CB1R
620   (yellow) in the DIO zebrafish. DAPI (blue) was used to counterstain nuclei. CP: central posterior
621   thalamic nucleus; Dd: dorsal; Dc: central; Dl: lateral; Dm: medial; Dp: posterior part of the
622   dorsal telencephalon; Hd: dorsal zone of the periventricular hypothalamus; Hv: ventral zone of
623   the periventricular hypothalamus; LH: lateral part of the hypothalamus; PGl: lateral and PGm:
624   medial preglomerular nuclei; PGZ: periventricular gray zone of the optic tectum; TeO: optic
625   tectum; TL: torus longitudinalis; Vd: dorsal part of the ventral telencephalon. Scale bar: 50 μm
626   (A, A₁, C, C₁); 250 μm (B and B₁); 25 μm (D).
627
628   **Figure 5: OX-2R and CB1R protein expression and specificity in adult brain of zebrafish and**
629   **mouse hippocampus.** (**A**) Negative control of OX-2R by pre-absorption with the relative
630   peptide. (**B**) Negative control of CB1R by pre-absorption with the relative peptide. (**C**) Positive
631   control of OX-2R/CB1R in hippocampus of mouse. PGZ: periventricular grey zone of the optic
632   tectum; TeO: optic tectum. Scale bar: 100 μm (A, B); 250 μm (C).
633
634   **Figure 6: Distribution of OX-A (green) and CB1R (red) and their co-localization with OX-**
635   **A/CB1R (yellow) in the hypothalamic coronal sections of DIO vs. control diet adult zebrafish.**
636   (**A**) OX-A/CB1R co-expression in the hypothalamus of control diet zebrafish (A₁) Higher
637   magnification showing the OX-A/CB1R co-expression within the lateral hypothalamus. Detail of
638   OX-A/CB1R co-expression showing a putative adjacent localization of OX-A/CB1R or co-
639   localization and overlap of OX-A/CB1R in the same cells. (**B**) OX-A/CB1R co-expression in the
640   Hypothalamus of DIO zebrafish (B₁) Higher magnification showing the increased OX-A/CB1R co-
641   expression within the lateral hypothalamus. Detail of OX-A/CB1R co-expression showing a
642   putative adjacent localization of OX-A/CB1R or co-localization and overlap of OX-A/CB1R in the
643   same cells. LH: lateral part of the hypothalamus. Scale bar: 50 μm (A, B); 25 μm (A₁, B₁).
644
645   **DISCUSSION:**
646
647   **Sample preparation**
648   Sample preparation is the first critical step in IHC. A reliable protocol allows for maintenance of
649   cell morphology, tissue architecture, and antigenicity. This step requires correct tissue
650   collection, fixation, and sectioning[22,23]. The purpose of fixation is to preserve tissue and reduce
651   the action of tissue enzymes or microorganisms. In particular, the fixation step preserves
652   cellular components and biomolecules, prevents autolysis and shifting of cell constituents (such
653   as antigens and enzymes), stabilizes cellular materials against aversive effects of the following
654   procedures, and facilitates immunostaining[4,7,24].
655
656   Before sectioning, the tissue is prepared and preserved through paraffin embedding
657   (immunoperoxidase method) or cryopreserved (freezing in cryomedia, in multiple
658   immunofluorescence methods). The preservation method is associated with the type of
659   fixation[7]. After fixation and preservation, the tissues are sliced by a microtome if embedded in

660 paraffin, or by a cryostat if embedded in a cryomedia. Tissues are typically sliced at a thickness
661 range of 8–10 μm and mounted on slides. For immunoperoxidase staining, the sample may
662 require additional steps to unmask the epitopes for antibody binding, including
663 deparaffinization and antigen retrieveal[25,26]. It should be kept in mind that overfixation can
664 cause epitope masking, while underfixation can cause little to no positive signal with heavy
665 edge staining.
666
667 **Blocking and background reduction**
668 In the immunoperoxidase method, the high affinity of avidin for biotin is likely responsible for
669 the rapid production of background staining. Moreover, since the avidin-biotin reaction is
670 irreversible, the background cannot be removed[18,27]. During IHC, high background can also be
671 produced by nonspecific binding to endogenous tissue biotines. Hydrophobic and ionic
672 interactions (such as those produced by collagen and other connective tissues such as
673 epithelium and adipocytes), as well as endogenous enzyme activity, are major causes of
674 background staining. Endogenous biotin or enzymes and hydrophobic binding must be
675 minimized prior to antibody staining, which can be achieved by the addition of a detergent,
676 such as Triton X-100, in the blocking buffers[28].
677
678 The use of 0.3%–0.4% Triton X-100 in the blocking buffers also allows for full permeabilization
679 of the antibodies into tissue sections. Moreover, although antibodies are preferentially specific
680 for one epitope, partial binding to sites on nonspecific proteins is possible, leading to high
681 background staining[29]. The nonspecific bindings can mask the signal of the target antigen[30]. To
682 reduce nonspecific background staining, samples should be incubated with a buffer that blocks
683 nonspecific reactive sites. Common blocking buffers include normal serum or bovine serum
684 albumin[30]. The species of the blocking serum should be the same as the host of the secondary
685 antibody. It is recommended to determine the best incubation time. Concentration of the
686 normal serum in the blocking buffer is another important determination[31]. Furthermore, to
687 eliminate the background staining, it is crucial to use the optimal dilution of the primary and
688 secondary antibodies. Thus, incubation time must be chosen carefully, in addition to the
689 temperature (i.e., increase the time if performing at 4 °C, decrease the time if at RT) and
690 detection system.
691
692 **Antibody choice**
693 The selection of antibodies for IHC staining is important and can affect experimental outcome[32].
694 To ensure that the antibody will respond appropriately, the epitope recognized by it must be
695 considered. Understanding the target protein and its function, tissue and subcellular
696 localization, and whether it undergoes post-translational modifications can help to determine
697 the choice of antibody. Another important step is the testing of different concentrations of
698 primary/secondary antibody to keep the background and aspecificity at a minimum level
699 compatible with a specific signal. Moreover, it is important to check species reactivity to
700 confirm primary and secondary antibody compatibility and the capability of the primary
701 antibody to recognize the antigen target in its native conformation. Another important step for
702 primary antibody choice is gene alignment. This ensures that the primary antibody reacts with
703 the biomolecule of interest and provides information about which epitope is recognized by the

704 antibody in a specific animal model. Gene alignment also provides the possibility of choosing
705 antibodies capable of recognizing epitopes that are evolutionary conserved.
706
707 **Controls**
708 To clarify specificity of the antibodies, an important aspect is performance of the controls,
709 which allows detection of specific staining. The controls include: i) a tissue known to express
710 the antigen as a positive control; ii) a tissue known not to express the antigen as a negative
711 control; iii) the omission of the primary antibody or absorption of the primary antibody with a
712 specific peptide to confirm that the secondary antibody does not crossreact with other tissue
713 components[14,33].
714
715 In IHC techniques, several steps can cause problems prior to achieving the final staining. Strong
716 background staining and nonspecific target antigen staining can be caused by endogenous
717 biotin or primary/secondary antibody cross-reactivity and poor enzyme activity or primary
718 antibody potency, respectively. Background staining and nonspecific binding can be prevented
719 by blocking the endogenous enzymes prior to the incubation with the primary antibody. Using
720 normal serum is the best way to block nonspecific interactions[30]. The choice of the blocking
721 buffer depends on the method of detection used. Moreover, a tissue known to lack the
722 expression of the target antigen as negative control can act as the reference to determine the
723 amount of background staining[34]. The deposition of chromogenic or fluorescent signal in the
724 negative control confirms the presence of nonspecific staining. Furthermore, insufficient
725 blocking time blocking leads to a high background, while excessive blocking leads to a low
726 signal[35].
727
728 In immunoperoxidase staining, the presence of endogenous peroxidase in the tissue is another
729 cause of brown background deposition. The treatment with H_2O_2 prior the incubation with the
730 primary antibody blocks the endogenous peroxidase[36,37]. On the contrary, in
731 immunofluorescence, fixation plays a key role in the generation of autofluorescence. To avoid
732 autofluorescence, the best fixation method, time of fixation, and preparation of tissues must be
733 carefully chosen. Another critical step of IHC is validation of the primary antibody. An antibody
734 is considered valid if it produces a consistent and specific staining pattern in a particular tissue
735 or cell/subcellular components and if pre-absorption of the primary antibody with a specific
736 peptide does not yield staining[38]. In immunofluorescence, nonspecific binding shows similar
737 fluorescent intensity under three color fluorescence detectors, while the signal is variable since
738 different fluorescent conjugated secondary antibodies are used. These aspects of IHC tissue
739 preparation and antibody staining must be addressed to overcome staining issues.
740
741 IHC, although a relatively simple technique, presents some limitations and depend on many
742 factors[39]. One of the crucial points is formalin fixation, which can alter the expression of post-
743 translation modified proteins. On the other hand, formalin-fixed paraffin-embedded tissues can
744 be stored for long-term at room temperature, whereas frozen tissues can only be stored for up
745 to one year at -80 °C. Moreover, frozen tissues can be damaged by the formation of ice crystals,
746 which can affect the subcellular details altering IHC staining[40,41]. Regarding our studies, the
747 most difficult aspect was finding specific primary antibodies against zebrafish molecules.

748 Although zebrafish have been recognized as a valid animal model with a highly conserved
749 degree of structure, very few antibodies have been developed that can recognize specific
750 proteins and other molecules in zebrafish. To overcome these limitations, it is important to
751 validate antibody specificity by western blotting analysis and gene alignment.
752
753 Growing interest in IHC methods has led to the development of highly specific immunostaining
754 that can help investigative studies[42]. IHC is being used with increasing frequency to identify the
755 presence of specific molecular markers and their changes across different pathologies. The two
756 approaches illustrated here, immunoperoxidase and immunofluorescence, have respective
757 advantages and disadvantages[43]. Paraffin-embedded tissue, used in the immunoperoxidase
758 technique, can allow for high resolution of cells and tissue and reveal details about the
759 distribution and amount of target proteins[44,45]. However, paraffin-embedded tissues are not
760 suitable for immunofluorescence, since paraffin can mask the antigenicity and lead to
761 nonspecific fluorescence[46]. On the other hand, cryosection of PFA-fixed tissue preserves
762 endogenous antigenicity and lead to a decrease in nonspecific fluorescence. Even if the
763 technical quality of the cryosections are much lower than that of paraffin-embedded tissue,
764 they can yield valid results using the IHC technique[47].
765
766 Moreover, while paraffin embedding better preserves morphological details, cryopreservation
767 better preserves enzyme and antigen expression, leading to more detailed immunostaining.
768 The immunofluorescence technique also allows the contemporary detection of two or three
769 different biomolecules, revealing possible interactions, as illustrated in our previous work for
770 orexin and endocannabinoid systems[10]. Among the techniques used to detect the distribution
771 and levels of specific biomolecules, IHC allows not only the determination of specific
772 morphological expression and distribution of molecules and proteins, but also the possibility to
773 perform quantitative analysis.
774
775 Using immunofluorescence, the codistribution and coexpression of biomolecules can also be
776 further understood, as well as possible interactions and their changes in cases of different
777 pathologies[48]. Confocal microscopy, during the last 20 years, has often been used to study the
778 cellular and subcellular distribution of numerous proteins in the mammalian brain.
779 Fluorescence microscopy also allows the visualization (using fluorophores or fluorescent dyes)
780 of specific structures of interest, such as proteins, organelles, and other biological matter; such
781 signals can be used in both fixed and living biological systems to image specific subcellular
782 structures[49]. The potential modulatory function of biomolecules in specific cell compartments
783 can be explored via the visualization of their expression patterns, whereas the role of protein
784 signaling within the tissues may be uncovered via the neuroanatomical distribution of
785 metabolic enzyme expression or receptors.
786
787 The presented technical work introduces IHC approaches, mainly immunofluorescence, to
788 studying two highly conserved systems, orexin and endocannabinoid, in an adult zebrafish
789 model. In particular, immunofluorescence methods can be used to determine the distribution,
790 relative amount, and anatomical interactions of specific proteins in target tissues.
791 Cryopreservation of PFA-fixed tissues better preserves highly sensitive proteins susceptible to

792 rapid deterioration. Moreover, cryopreservation is thought to better preserve antigen and
793 antigenicity, and it allows for studying of post-translation modified proteins and DNA.
794
795 Even if frozen tissues (compared to paraffin-embedded sections) are thicker, which hampers
796 the ability to observe tissue morphology in detail, confocal microscopy allows for sample
797 visualization in great detail and enhances imaging capabilities. Moreover, immunofluorescence
798 can be used for quantitative analysis of staining intensity, and densitometric analysis of the
799 signal provides quantitative data. This allows for determining correlations of fluorochrom signal
800 levels with protein expression levels by looking at areas of co-localization. This work describes
801 and illustrates protocols that can be used to study evolutionary conservation of important
802 proteins in the adult zebrafish. The use of IHC, mainly immunofluorescence, in adult zebrafish
803 can help highlight the usefulness of this animal model in studying the morphological expression
804 and distribution of highly conserved biomolecules, as well as reveal possible alterations across
805 different pathological conditions correlating with human physiopathology.
806

813 **REFERENCES**
814

815 1. Brandtzaeg, P. The increasing power of immunohistochemistry and
816 immunocytochemistry. *Journal of Immunological Methods*. 216, 49–67 (1998).
817 2. Haines, D. M., West, K. H. Immunohistochemistry: forging the links between
818 immunology and pathology. *Veterinary Immunology and Immunopathology*. 108, 151–6
819 (2005).
820 3. Onul, A. et al. Application of immunohistochemical staining to detect antigen
821 destruction as a measure of tissue damage. *Journal of Histochemistry & Cytochemistry*.
822 **60** (9), 683-93. doi: 10.1369/0022155412452146 (2012).
823 4. Ramos-Vara, J. A. Technical aspects of immunohistochemistry. *Veterinary Pathology*. **42**
824 (4), 405–426 (2005).
825 5. Coons, A. H., Kaplan, M. H. Localization of antigen in tissue cells, II: improvements in a
826 method for the detection of antigen by means of fluorescent antibody. *The Journal of*
827 *Experimental Medicine*. **91** (1), 1–13. 67. (1950).
828 6. Coons, A. H., Leduc, E. H., Connolly, J. M. Studies on antibody production, I: a method
829 for the histochemical demonstration of specific antibody and its application to a study of
830 the hyperimmune rabbit. *The Journal of Experimental Medicine*. **102** (1), 49–60 (1955).
831 7. Ramos-Vara, J. A., Miller, M. A. When tissue antigens and antibodies get along: revisiting
832 the technical aspects of immunohistochemistry--the red, brown, and blue technique.
833 *Veterinary Pathology*. **51** (1), 42-87 doi: 10.1177/0300985813505879 (2014).

834   8. Duraiyan, J., Govindarajan, R., Kaliyappan, K., Palanisamy, M. Applications of
835   immunohistochemistry. *Journal of Pharmacy and Bioallied Sciences.* (Suppl 2), S307-9
836   doi: 10.4103/0975-7406.100281 (2012).
837   9. Al-Hussinee, L. et al. Immunohistochemistry and pathology of multiple Great Lakes fish
838   from mortality events associated with viral hemorrhagic septicemia virus type IVb.
839   *Diseases of Aquatic Organisms.* **93** (2), 117-27. doi: 10.3354/dao02285 (2011).
840 10. Imperatore, R. et al. Overlapping Distribution of Orexin and Endocannabinoid Receptors
841   and Their Functional Interaction in the Brain of Adult Zebrafish. *Frontiers in
842   Neuroanatomy.* **12**, 62, doi: 10.3389/fnana.2018.00062 (2018).
843 11. Concas, A. et al. Immunochemical Localization of GABA$_A$ Receptor Subunits in the
844   Freshwater Polyp Hydra vulgaris (Cnidaria, Hydrozoa). *Neurochemical Research.* **41** (11),
845   2914-2922 (2016).
846 12. Mania, M. et al. Expression and distribution of leptin and its receptors in the digestive
847   tract of DIO (diet-induced obese) zebrafish. *Annals of Anatomy.* **212**, 37-47, doi:
848   10.1016/j.aanat.2017.03.005 (2017).
849 13. Matos, L. L., Trufelli, D. C., de Matos, M. G., da Silva Pinhal, M. A. Immunohistochemistry
850   as an important tool in biomarkers detection and clinical practice. *Biomarker Insights*. **5**,
851   9-20 (2010).
852 14. Holmseth, S. et al. Specificity controls for immunocytochemistry: the antigen
853   preadsorption test can lead to inaccurate assessment of antibody specificity. *Journal of
854   Histochemistry & Cytochemistry.* **60** (3), 174-87, doi: 10.1369/0022155411434828,
855   (2012).
856 15. Burry, R. W. Controls for immunocytochemistry: an update. *Journal of Histochemistry &
857   Cytochemistry*. **59** (1), 6-12, doi: 10.1369/jhc.2010.956920. Review. (2011).
858 16. Hsu, S. M., Soban, E. Color modification of diaminobenzidine (DAB) precipitation by
859   metallic ions and its application for double immunohistochemistry. *Journal of
860   Histochemistry & Cytochemistry*. **30** (10), 1079-82 (1982).
861 17. Seth, A., Stemple, D. L., Barroso, I. The emerging use of zebrafish to model metabolic
862   disease. *Disease Models & Mechanisms.* **6** (5), 1080-8, doi: 10.1242/dmm.011346.
863   Review. (2013).
864 18. Hsu, S. M., Raine, L., Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in
865   immunoperoxidase techniques: a comparison between ABC and unlabeled antibody
866   (PAP) procedures. *Journal of Histochemistry & Cytochemistry*. **29** (4), 577–580 (1981).
867 19. Cristino, L. et al. Obesity-driven synaptic remodeling affects endocannabinoid control of
868   orexinergic neurons. *Proceedings of the National Academy of Sciences of the United
869   States of America.* **110** (24), E2229-38, doi: 10.1073/pnas.1219485110. (2013).
870 20. Imperatore, R. et al. Genetic deletion of monoacylglycerol lipase leads to impaired
871   cannabinoid receptor CB$_1$R signaling and anxiety-like behavior. *Journal of
872   Neurochemistry.* **135** (4), 799-813, doi: 10.1111/jnc.13267. (2015).
873 21. Laperchia, C. et al. The excitatory/inhibitory input to orexin/hypocretin neuron soma
874   undergoes day/night reorganization. *Brain Structure and Function.* **222** (8), 3847-3859,
875   doi: 10.1007/s00429-017-1466-3. (2017).
876 22. Eltoum, I., Fredenburgh, J., Grizzle, W. E. Advanced concepts in fixation: 1. Effects of
877   fixation on immunohistochemistry, reversibility of fixation and recovery of proteins,

878 nucleic acids, and other molecules from fixed and processed tissues. 2. Developmental
879 methods of fixation. *Journal of Histotechnology.* **24** (3), 201–210 (2001).

23. Mueller, C. et al. One-step preservation of phosphoproteins and tissue morphology at room temperature for diagnostic and research specimens. *Public Library of Science One.* **6** (8), e23780, doi: 10.1371/journal.pone. (2011).

24. Howat, W. J., Wilson, B. A. Tissue fixation and the effect of molecular fixatives on downstream staining procedures. *Methods.* **70** (1), 12-9, doi: 10.1016/j.ymeth.2014.01.022. (2014).

25. Dupre,´ M. P., Courtade-Saidi, M. Immunocytochemistry as an adjunct to diagnostic cytology. *Annales de Pathologie.* **32** (6), 433–437 (2012).

26. Shi, S. R., Liu, C., Taylor, C. R. Standardization of Immunohistochemistry for Formalin-fixed, Paraffin-embedded Tissue Sections Based on the Antigen Retrieval Technique: From Experiments to Hypothesis. *Journal of Histochemistry & Cytochemistry.* **39**, 741–8 (2006).

27. Giorno R. A comparison of two immunoperoxidase staining methods based on the avidin-biotin interaction. *Diagnostic Immunology.* **2** (3), 161-6 (1984)

28. Ramos-Vara, J. A., Saeteele, J. Immunohistochemistry. In: Howard GC, Kaser MR, eds. Making and Using Antibodies: A Practical Handbook. *Boca Raton, FL: CRC Press*. 273–314 (2007).

29. Jamur, M. C., Oliver, C. Permeabilization of cell membranes. *Methods in Molecular Biology*. **588**, 63-6, doi: 10.1007/978-1-59745-324-0_9 (2010)

30. Buchwalow, I., Somoloiva, V., Boecker, W., Tiemann, M. Nonspecific binding of antibodies in immunohistochemistry: fallacies and facts. *Scientific Reports*. **1**, 28 (2011).

31. Ansorg, A., Bornkessel, K., Witte, O. W., Urbach, A. Immunohistochemistry and multiple labeling with antibodies from the same host species to study adult hippocampal neurogenesis. *Journal of Visualized Experiments.* (98), doi: 10.3791/52551 (2015).

32. Kalyuzhny, A. E. The dark side of the immunohistochemical moon: industry. *Journal of Histochemistry & Cytochemistry.* **57** (12), 1099–1101 (2009).

33. Hewitt, S. M., Baskin, D. G., Frevert, C. W., Stahl, W. L., Rosa-Molinar, E. Controls for immunohistochemistry: the Histochemical Society's standards of practice for validation of immunohistochemical assays. *Endocrinology.* **155** (3), 676-87, doi: 10.1210/en.2013-1971 (2014).

34. Ivell, R., Teerds, K., Hoffman, G. E. Proper application of antibodies for immunohistochemical detection: antibody crimes and how to prevent them. *Journal of Histochemistry & Cytochemistry.* **62** (10), 693-7, doi: 10.1369/0022155414545224 (2014).

35. Stradleigh, T. W., Ishida, A. T. Fixation strategies for retinal immunohistochemistry. *Progress in Retinal and Eye Research.* **48,** 181-202, doi: 10.1016/j.preteyeres.2015.04.001 (2015).

36. Boi, G., Scalia, C. R., Gendusa, R., Ronchi, S., Cattoretti, G. Disaccharides Protect Antigens from Drying-Induced Damage in Routinely Processed Tissue Sections. *Journal of Histochemistry & Cytochemistry.* **64** (1), 18-31, doi: 10.1369/0022155415616162 (2016).

921 37. Curran, R. C., Gregory, J. Demonstration of immunoglobulin in cryostat and paraffin
922 sections of human tonsil by immunofluorescence and immunoperoxidase techniques.
923 Effects of processing on immunohistochemical performance of tissues and on the use of
924 proteolytic enzymes to unmask antigens in sections. *Journal of Clinical Pathology.* **31**
925 (10), 974-83 (1978).
926 38. O'Hurley, G. et al. Garbage in, garbage out: a critical evaluation of strategies used for
927 validation of immunohistochemical biomarkers. *Molecular Oncology*. **8** (4), 783-98, doi:
928 10.1016/j.molonc.2014.03.008 (2014).
929 39. Matos, L. L., Trufelli, D. C., de Matos, M. G., da Silva Pinhal, M. A. Immunohistochemistry
930 as an important tool in biomarkers detection and clinical practice. *Biomarker Insights.* **5**,
931 9-20 (2010).
932 40. Mayersbach, H. V. Principles and limitations of immunohistochemical methods. *Journal*
933 *of Royal Microscopical Society.* **87** (2), 295-308 (1967).
934 41. Shi, S. R., Liu, C., Taylor, C. R. Standardization of immunohistochemistry for formalin-
935 fixed, paraffin-embedded tissue sections based on the antigen-retrieval technique: from
936 experiments to hypothesis. *Journal of Histochemistry & Cytochemistry.* **55** (2), 105-9
937 (2007).
938 42. Dixon, A. R., et al. Recent developments in multiplexing techniques for
939 immunohistochemistry. *Expert Review of Molecular Diagnostics.* **15** (9), 1171-86, doi:
940 10.1586/14737159.2015.1069182. Review (2015).
941 43. Mölne, J., Breimer, M. E., Svalander, C. T. Immunoperoxidase versus
942 immunofluorescence in the assessment of human renal biopsies. *American Journal of*
943 *Kidney Diseases*. **45** (4), 674-83 (2005).
944 44. Gerdes, M. J. et al. Highly multiplexed single-cell analysis of formalin-fixed, paraffin-
945 embedded cancer tissue. *Proceedings of the National Academy of Sciences of the United*
946 *States of America.* **110** (29), 11982-7, doi: 10.1073/pnas.1300136110 (2013).
947 45. Zhang, P., Lehmann, B. D., Shyr, Y., Guo, Y. The Utilization of Formalin Fixed-Paraffin-
948 Embedded Specimens in High Throughput Genomic Studies. *International Journal of*
949 *Genomics*. 1926304, doi: 10.1155/2017/1926304 (2017).
950 46. Xie, R. et al. Factors influencing the degradation of archival formalin-fixed paraffin-
951 embedded tissue sections*. Journal of Histochemistry & Cytochemistry.* **59** (4), 356-65.
952 doi: 10.1369/0022155411398488 (2011).
953 47. Webster, J. D., Miller, M. A., Dusold, D., Ramos-Vara, J. Effects of prolonged formalin
954 fixation on diagnostic immunohistochemistry in domestic animals. *Journal of*
955 *Histochemistry & Cytochemistry.* **57** (8), 753-61, doi: 10.1369/jhc.2009.953877 (2009).
956 48. Drummen, G. P. Fluorescent probes and fluorescence (microscopy) techniques--
957 illuminating biological and biomedical research. *Molecules.* **17** (12), 14067-90, doi:
958 10.3390/molecules171214067 (2012).
959 49. Marks, K.M., Nolan, G.P. Chemical labeling strategies for cell biology. *Nature Methods.* **3**
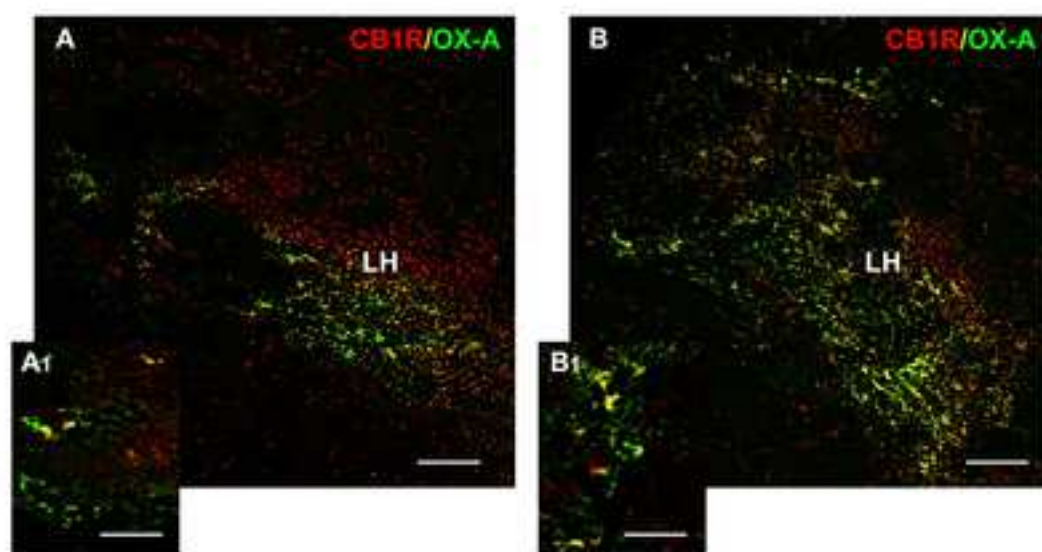960 (8), 591-6. Review. (2006).
961
962
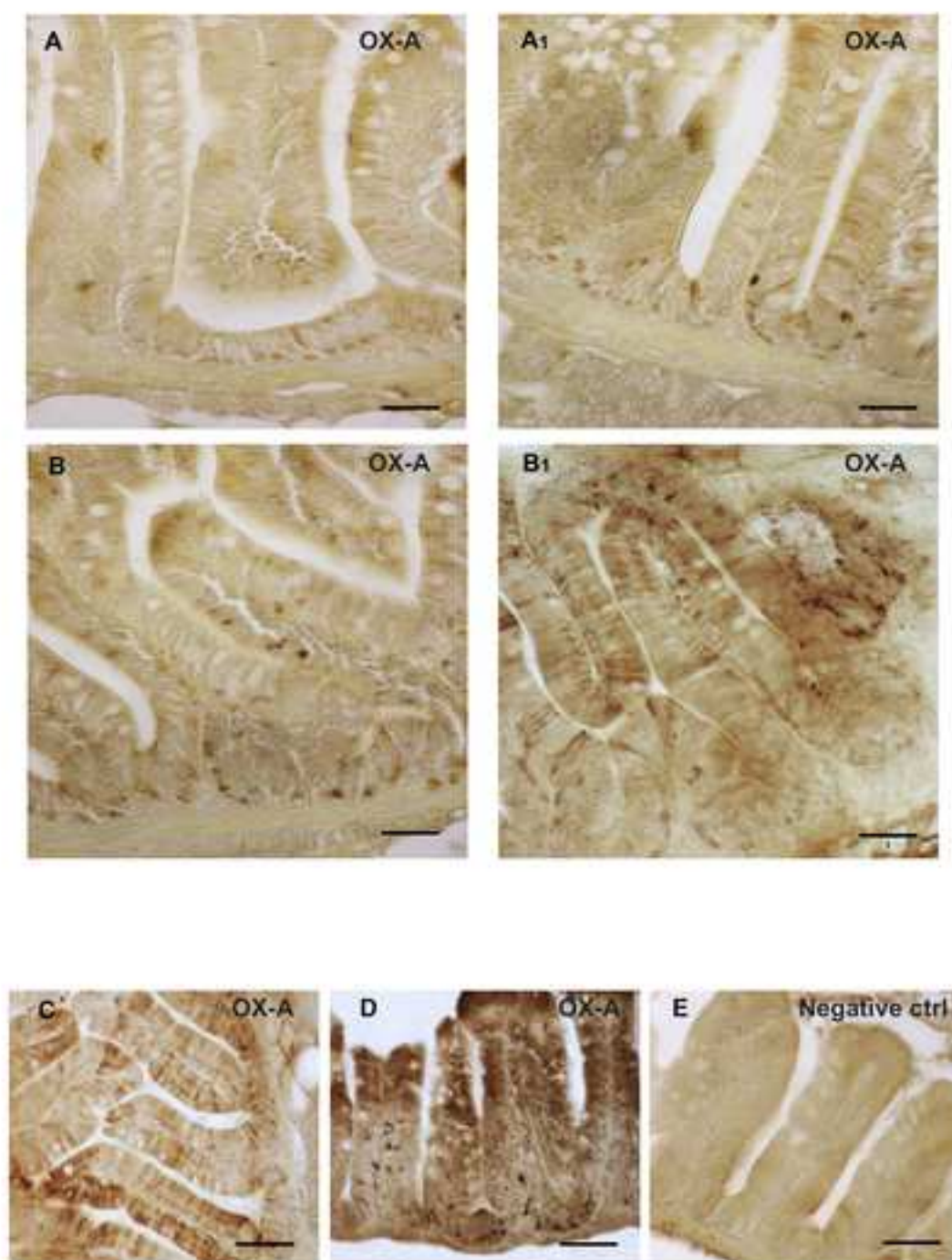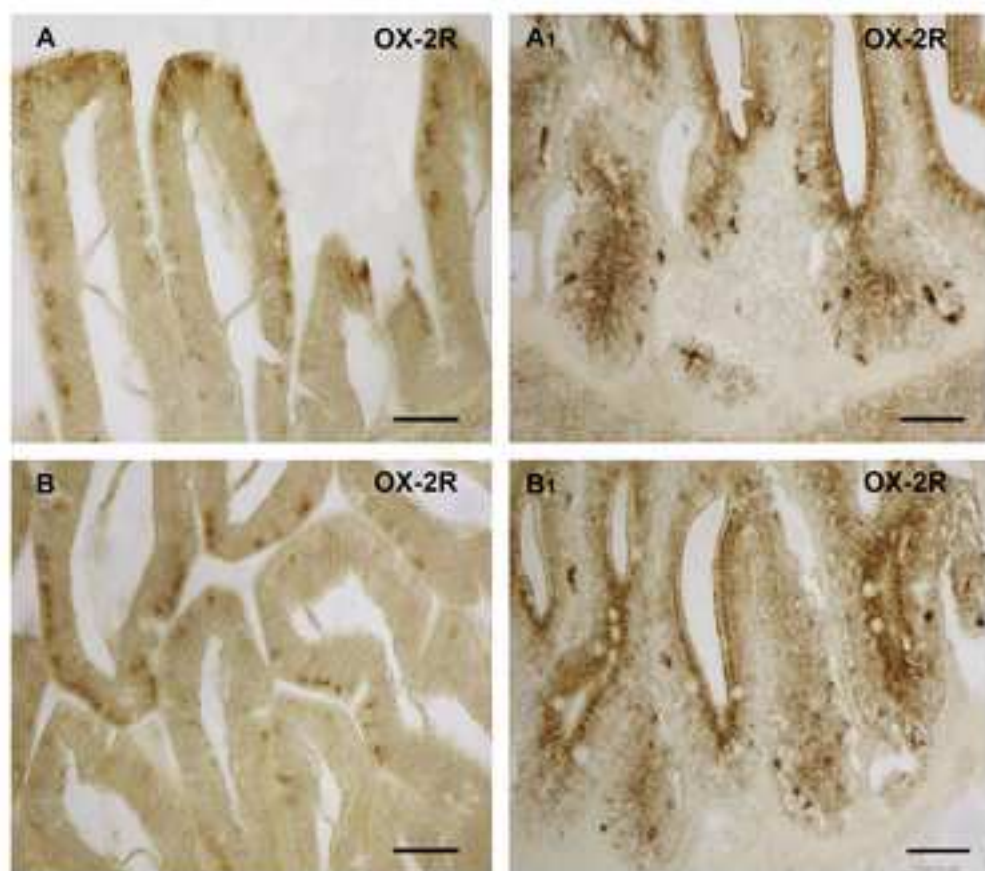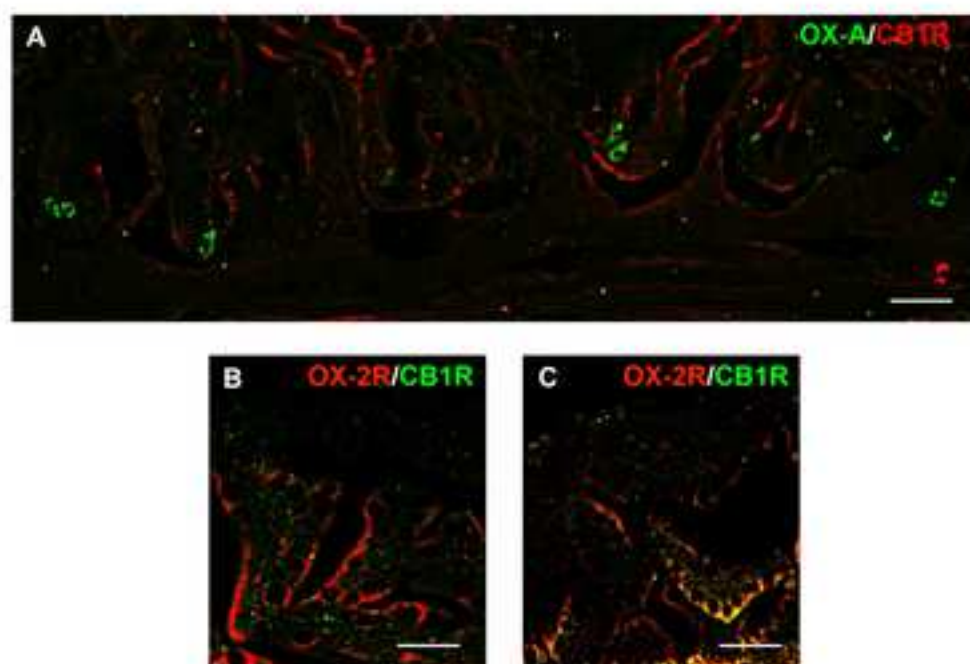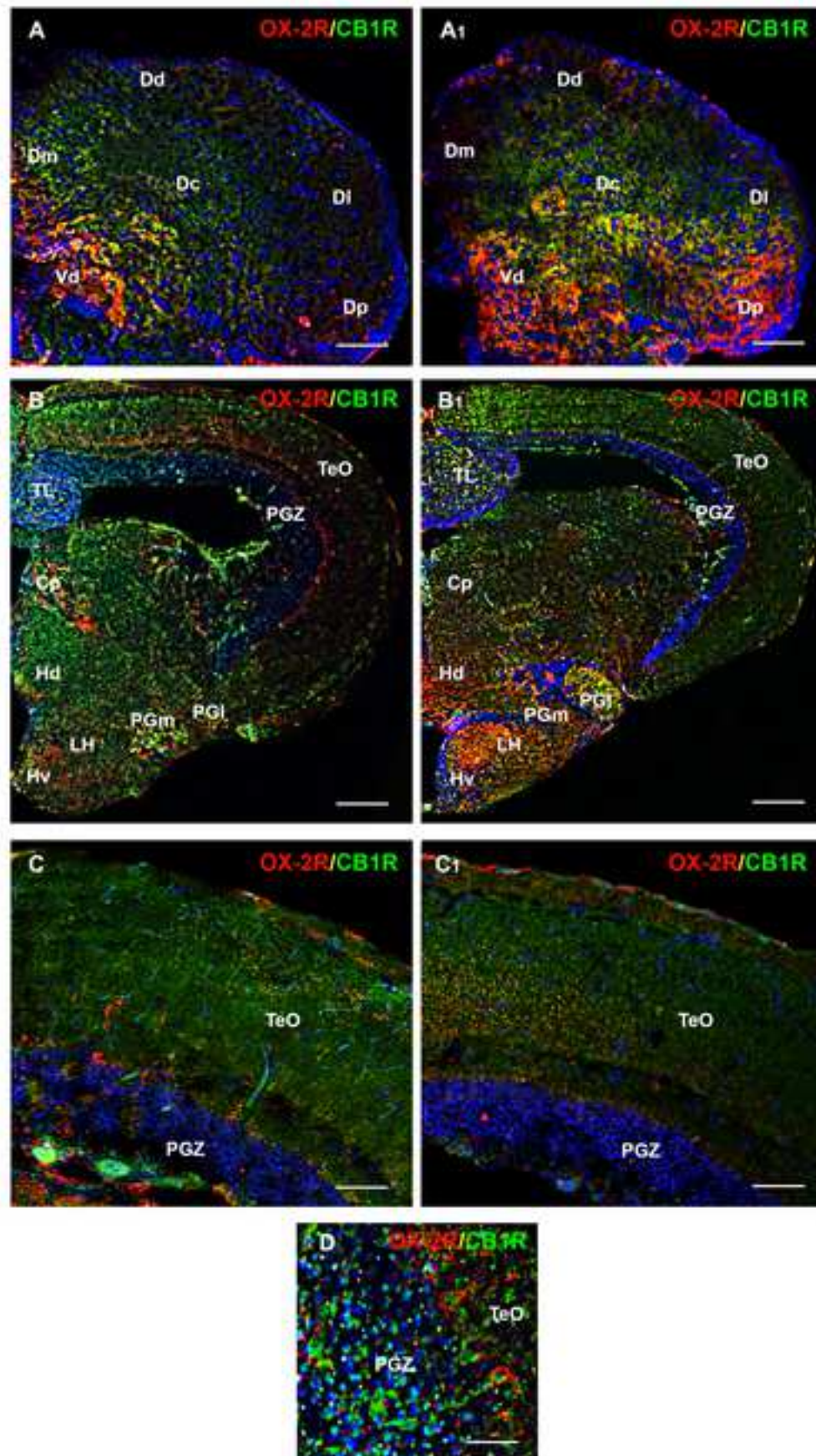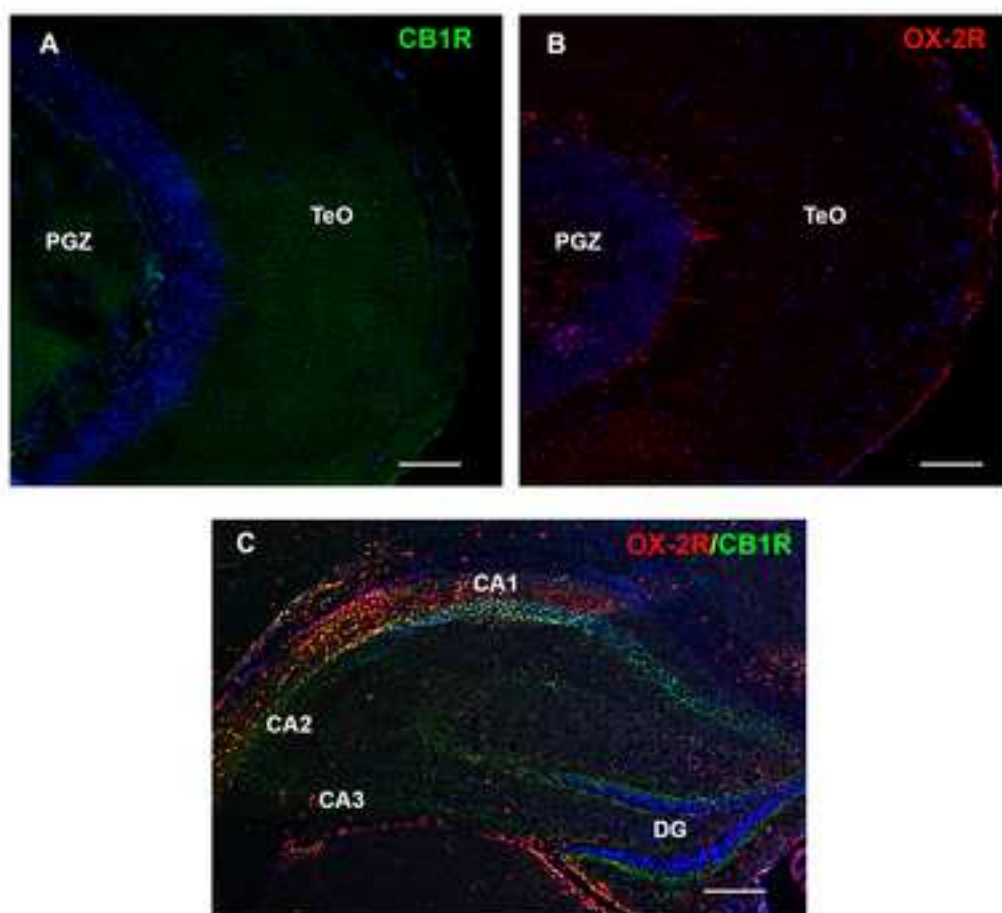
Name of Material/ Equipment	Company
Anti CB1	Abcam
Anti OX-2R	Santa Cruz
Anti-OXA	Santa Cruz
Aquatex	Merck
Biotinylated rabbit anti-goat	Vector Lab
citric acid	Sigma Aldrich
Confocal microscope	Nikon
Cryostat	Leica Biosystem
DAPI	Sigma Aldrich
Digital Camera	Leica Biosystem
Digital Camera for confocal microscope	Nikon
Donkey anti goat Alexa fluor 488-conjugated secondary antibodies	Thermo Fisher
Donkey anti goat Alexa fluor 594-conjugated secondary antibodies	Thermo Fisher
Donkey anti rabbit Alexa fluor 488-conjugated secondary antibodies	Thermo Fisher
Donkey anti rabbit Alexa fluor 594-conjugated secondary antibodies	Thermo Fisher
Ethanol absolute	VWR
Frozen section compound	Leica Biosystem
H2O2	Sigma Aldrich
HCl	VWR
ImmPACT DAB	Vector lab
Microscope	Leica Biosystem
Microtome	Leica Biosystem
Na2HPO4	Sigma Aldrich
NaCl	Sigma Aldrich
NaH2PO4H2O	Sigma Aldrich
NaOH	Sigma Aldrich
Normal Donkey Serum	Sigma Aldrich
Normal Rabbit Serum	Vector lab
paraffin wax	Carlo Erba
Paraphormaldeyde	Sigma Aldrich
sodium citrate dihydrate	Sigma Aldrich
Triton X-100	Fluka Analytical

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Author(s): ROBERTA IMPERATORE, LIVIA D'ANGELO, PAOLO DE GIROLAMO, LUIGIA CRISTINO, MARINA PAOLUCCI

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Author(s): ROBERTA IMPERATORE, LIVIA D'ANGELO, PAOLO DE GIROLAMO, LUIGIA CRISTINO, MARINA PAOLUCCI

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Dear Dr. Phillip Steindel

Thank you so much for your very helpful and constructive comments, which we indeed appreciated. Enclosed you will find the revised manuscript JoVE59308R1 "Immunoperoxidase and immunofluorescence technique for the identification of orexin and endocannabinoid receptors in the adult zebrafish,". We would like to submit the revised manuscript for publication in *JoVE*. All comments were addressed point by point in a below. All the changes are track in yellow within the manuscript. We added another file with the requested 2.75 pages of the Protocol that identify the essential steps of the protocol for the video. We look forward to hearing your comments.

Best regards,

Dr. Roberta Imperatore

Department of Sciences and Technologies (DST)

University of Sannio, Benevento, Italy

Editorial   **comments:**

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (formatted as in the attached manuscript, including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

R1. We have provided 2.75 pages of rilevant steps for the protocols.

2. There are some errors and unclear parts in the manuscript-see notes in the attached manuscript, and please proofread again.

R2. We have checked the note errors and we have follow all the commenst reported in the manuscript

3. There is still some commercial language (mainly Leica products; also Nikon); please remove and ensure such equipment is in the Table of Materials.

R3. We have removed all the commercial language and introduced them in the Table of Materials.

PROTOCOLS

1. Immunofluorescence protocol

1.1. Tissue cutting

1.1.1. Transfer the frozen tissue blocks to a cryostat at −20 °C and cut in coronal or sagittal-sections of 10 μm.

1.1.2. Collect the tissues in alternate serial sections onto adhesive glass slides suitable for immunohistochemistry and store them at −20 °C until use.

Note: Store slides between -20°C and 4°C in a dark slide box or slide book.

1.2. Blocking of non specific binding sites and tissue permeabilizzation

1.2.1. Demarcate the tissue area on the slide with a solvent resistant pen.

1.2.2. Rinse the sections 3 times, 5 min each, with PB 0.1M pH7.4.

1.2.3. Incubate the sections with 1% normal donkey serum dissolved in the permeabilization buffer PB-Triton X-100 0.3% (PB-T) for 30 minutes at RT to permeabilize the cell membrane and to block the nonspecific binding sites.

1.2.3.1. Prepare Triton X-100 0.3% dissolving 0.3 mL Triton X-100 in 100 mL of PB 0.1M, pH7.4.

1.2.3.2. Prepare the blocking solution dissolving 1% normal donkey serum in PB containing 0.3% TritonX-100.

Note: The animal specie of the serum used in the permeabilization and blocking buffers are dependent on the host of the secondary antibody.

1.3. Incubation with mix of primary antibodies

1.3.1. Rinse the sections 3 times, 5 min each, with PB 0.1M, pH7.4.

1.3.2. Incubate the sections overnight in a humid box at RT with a mix of primary antibodies diluted in PB-T. The following mix of primary antibodies can be used:

1.3.2.1. goat antibody against OX-2R diluted 1:100/rabbit antibody against CB1R diluted 1:100.

1.3.2.2. goat antibody against OX-A diluted 1:100/rabbit antibody against CB1R diluted 1:100.

1.4. Incubation with mix of secondary antibodies

1.4.1. Rinse the sections 3 times, 5 min each, with PB 0.1M, pH7.4.

1.4.2. Incubate the sections for 2 hrs at RT with a mix of donkey anti rabbit Alexa fluor 488-conjugated secondary antibody/donkey anti goat Alexa fluor 594-conjugated secondary antibody diluted 1:100 in PB-T or a mix of donkey anti goat Alexa fluor 488-conjugated secondary antibody/donkey anti rabbit Alexa fluor 594-conjugated secondary antibody diluted 1:100 in PB-T.

Notes: Use secondary antibodies developed in the same animal host and the normal serum must belong to the same specie of the secondaru antibody (e.g. use secondary antibodies developed in donkey and a donkey normal serum).
Dilute the primary and secondary antibodies with blocking buffer containing the detergent to increase cell permeabilization and reduce the background.

1.5. Tissue mounting

1.5.1. Rinse the sections 3 times, 5 min each, with PB 0.1M, pH7.4.

1.5.2. Counterstain the sections with the common nuclear dye DAPI (4',6-diamidino-2-phenylindole) prepared dissolving 1.5µl of DAPI (1mg/mL) in 3 mL of PB.

1.5.3. Coverslip slides with the Microscopy Aquatex mounting medium. This aqueous mounting medium stabilizes the tissue sample and stains for long-term usage. Fluorescence samples can be stored in the dark at 4 °C. To prolong the life of fluorofores use an antifed mounting medium.

Note: Chose a good mounting medium. One of the most important parameters of mounting agents is the refractive index (nD) which should be around 1.5, the refractive index of glass. Aquatex®, an aqueous mounting medium, can be used especially with specimens prepared for enzyme and lipid determinations i.e. specimens that must not be dehydrated with an ascending series of alcohol.

1.6. Controls

1.6.1. Repeat all the steps omitting the primary or secondary antibody or substituting, in the specific step, the primary or secondary antisera with PB (negative control).

1.6.2. Repeat all the steps pre-absorbing each primary antibody with an excess of the relative peptide (100 mg of peptide/mL of diluted antiserum).

1.6.3. Repeat all the steps on slices of mouse brain (positive control).

General Notes: Prepare all the buffers freshly shortly before starting.
Remove the excess of fluid carefully at each step with a pipette, or filter paper around the sections, but keeping always the section humid.

1.7. Image acquisition

1.7.1. Use a confocal microscope equipped with x-y-z motorized stage, a digital camera and the acquisition and Image analysis software like NIS-Elements C to observe and analyze the immunostained sections. Acquire digital images using the 5-20-40x objectives.

1.7.2. Take the images of each section at low magnification (10X or 20X/objective) in each of the available channels in order to compose a low magnification montage providing an overview of the entire region to facilitate the localization and documentation of OX-2R/CB1R anatomical co-expression. Normalize the fluorescence images to maximal contrast and overlaid before the analysis.

1.7.3. Collect serial Z-stacks of images throughout the area of interest. Acquire the images through 6 focal planes (Z-step) with focal steps of 1-1.8 μm to cover the tissue volume in which OX-2R/CB1R coexpression is visualized as yellow puncta. To do this collection for each channel (red, green, blue) separately, by using a Z-motorized microscope.

1.7.4. Use an imaging deconvolution software to deconvolve images by application of ten iterations and collapse the serial Z planes images into a single maximum projection image.

1.7.5. Adjust micrographs for light and contrast using Adobe Photoshop 6.01 (Adobe Systems, San Jose, CA).

Notes: Performe the deconvolution step during observation to further decrease the background. Limit the slide exposure to light to prevent photobleaching.