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# Novel polyclonal antibodies as a useful tool for expression studies in amphioxus embryos

MATTEO BOZZO#, JIRI PERGNER#, ZBYNEK KOZMIK and IRYNA KOZMIKOVA*

*Laboratory of Transcriptional Regulation, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic,*

**ABSTRACT Cephalochordates, commonly called amphioxus or lancelets, are widely regarded as a useful proxy for the chordate ancestor. In recent decades, expression patterns of important developmental genes have been used extensively to infer homologies between cephalochordate and vertebrate embryos. Such comparisons provided important insight into cephalochordate biology and the origin of vertebrate traits. Most of the developmental expression data are collected using whole-mount *in situ* hybridization that allows the distributions of specific transcripts to be detected in fixed embryos. Here, we describe an experimental pipeline for production of small amounts of functional antibodies directed against amphioxus antigens for use in immunohistochemical labelling. In this pilot study, we generated antibodies against β-catenin and the transcription factors FoxA, Lhx1, Lhx3 and Pax6. We demonstrate the usefulness of antibodies by performing immunostainings on fixed specimens of *B. lanceolatum* and *B. floridae.* We anticipate that amphioxus-specific antibodies will provide a useful tool for high-resolution labelling of individual cells within the embryo and for determining the subcellular localization of the corresponding proteins.**

KEY WORDS: *Branchiostoma, amphioxus, antibody, expression pattern*

Cephalochordates, commonly called amphioxus or lancelets, are regarded as a key animal group for understanding the origin of vertebrates, and a useful proxy to the ancestral chordate condition. This position has recently been affirmed especially thanks to the access to genome sequence data (Holland *et al.*, 2008; Huang *et al.*, 2014; Putnam *et al.*, 2008), introduction of novel techniques (Acemel *et al.*, 2016; Kozmikova and Kozmik, 2015; Li *et al.*, 2017; Yue *et al.*, 2016), and establishment of amphioxus as a model species for evolutionary developmental studies (for review see Bertrand and Escriva (2011)). Cephalochordates include three genera, namely *Branchiostoma*, *Asymmetron* and *Epigonychtys.* The phylogenetic relationships within the extant amphioxus lineage were recently investigated providing divergence time estimates and suggesting a rather recent diversification (Igawa *et al.*, 2017). For example, the estimated divergence times among species within the *Branchiostoma* genus (22.6 +/- 2.3 Mya for *B.lanceolatum - B. floridae* split) are comparable to those among rodents belonging to Muridae family (such as mouse and rat). Close phylogenetic relationship is mirrored by a high degree of coding sequence identity (Holland *et al.*, 2008; Huang *et al.*, 2014; Putnam *et al.*, 2008; Yue *et al.*, 2014) and by evidence for stasis in developmental expression patterns (Somorjai *et al.*, 2008).

Expression patterns of important developmental genes have been used extensively to infer homologies between cephalochordate and vertebrate embryos. Such comparisons provided important insight into cephalochordate biology and the origin of vertebrate traits. Most of the expression data were collected using whole mount *in situ* hybridization (WMISH) that allows the distributions of specific transcripts to be detected in fixed embryos. A plausible alternative for analysis of gene expression is based on protein detection by immunohistochemical staining in sections or whole mount preparations. In principle, immunostaining is easy to perform, provided that there are antibodies which specifically recognize the antigen of interest. Unfortunately, the current antibody repertoire for non-model invertebrates such as amphioxus is

*Abbreviations used in this paper:* TH, tyrosine hydroxylase; WMISH, whole mount in situ hybridization.

***Address correspondence to:** Iryna Kozmikova. Institute of Molecular Genetics of the Czech Academy of Sciences, v.v.i., Videnska 1083, 14220, Czech Republic. Tel: +420 241062110. E-mail: kozmikova@img.cas.cz #Note: The indicated authors contributed equally to this work.

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rather limited. Both monoclonal and polyclonal antibodies can be made against a protein of interest. Polyclonal antibodies have the advantage of typically being of higher affinity, whereas the monoclonal antibodies have higher specificity since they recognize a single epitope. A clear disadvantage of polyclonal antibodies is the fact that they are a non-renewable resource. On the other hand, monoclonal antibodies are costly and are not within the reach of a typical lab working in the area of evolutionary developmental biology. Commercially available antibodies produced for vertebrate research can in principle be used if directed against a highly conserved epitope shared by vertebrate and amphioxus protein. Currently, only antibodies against acetylated tubulin are routinely used (Le Petillon *et al.*, 2017; Lu *et al.*, 2012; Soukup *et al.*, 2015; Vopalensky *et al.*, 2012). A fairly small number of antibodies (all polyclonal) has so far been generated that specifically recognize amphioxus proteins in developing embryos (Vopalensky *et al.*, 2012; Wu *et al.*, 2011).

Here, we describe an experimental pipeline established for the production of small amounts of functional antibodies directed against amphioxus antigens. The procedure is based on cloning of cDNA fragments of the corresponding protein into an expression vector allowing production and hexa-histidine-tagged mediated purification of protein from *E. coli* lysates followed by immunization of mice to obtain polyclonal antibodies. Antibodies recognizing five *B. floridae* proteins (β-catenin, FoxA, Lhx1, Lhx3 and Pax6) were generated. We demonstrate the usefulness of antibodies generated in this way by performing immunostainings on fixed embryo specimens of *B. lanceolatum*, *B. floridae* and *A. lucayanum.*

## Results and Discussion

We set out a pilot project to develop antibodies against a panel of amphioxus regulatory proteins. Our main aim was to design and evaluate a generalized scheme of antibody production that could routinely be used in the lab for generation of functional antibodies at a reasonable cost. We opted primarily to create polyclonal antibodies due to the simplicity of production, modest cost and higher chance to cross-react with protein of interest in different amphioxus species. We chose mice as the host since they represent a cost-effective alternative to the more widely used rabbits. The amount of serum obtained is relatively small (200-400 μl per mouse) but appears to be sufficient to perform hundreds of immunostainings that are clearly enough to accomplish a focused study. It is of note that smaller amounts of purified antigen are necessary for immunization of mice compared to rabbits. Choice of mice as hosts for antibody production allows us to routinely immunize three mice with low animal cost. In the cases described in this study at least one functional antibody was obtained by immunizing three mice. However, if necessary, more mice can be immunized with minimal additional costs to increase the chance of obtaining a functional antibody and to overcome differences in immunological responses of individual mice. Although short synthetic peptides (15 to 20 amino acids) are often used for immunization, the functionality of the resulting antibodies is highly variable. Production and purification of fusion proteins encoding portions of an antigen is labor intensive. However, the use of longer peptide sequences from the antigen should in principle increase the chance of possessing an immunogenic epitope. When choosing *B. floridae* peptide sequences for immunization in our pilot study, we avoided sequences highly conserved among Metazoa, such as DNA-binding domains. In addition, in order to obtain antibody specific for a given protein, sequences with a high degree of identity among possible *B.floridae* paralogues were also omitted.

To generate antigens for immunization we expressed part of the selected proteins (ranging from 130 to 177 amino acids) in *E. coli* with hexa-histidine tags. After purification under denaturing conditions, the antigens were injected into mice for the generation of polyclonal antibodies. We standardized our procedure to

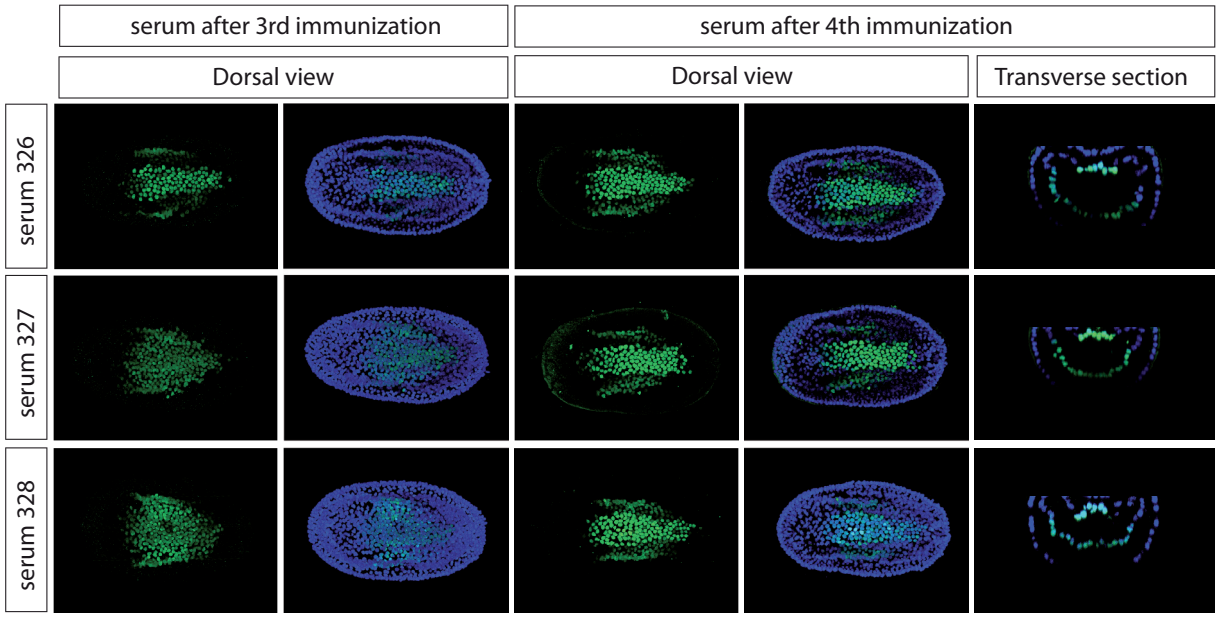



**Fig. 1. Immunostaining of FoxA in the neurula stage of amphioxus.** *Sera collected after 3rd and 4th immunizations of three individual mice were used for immunolabelling of FoxA (green signal). All three sera (mice 326 – 328) were able to specifically detect nuclear FoxA in the presumptive endoderm and notochord of the* B. lanceolatum *neurula. A transverse section through the embryo is shown in the right-most panel. Blue represents a nuclear DAPI signal.*

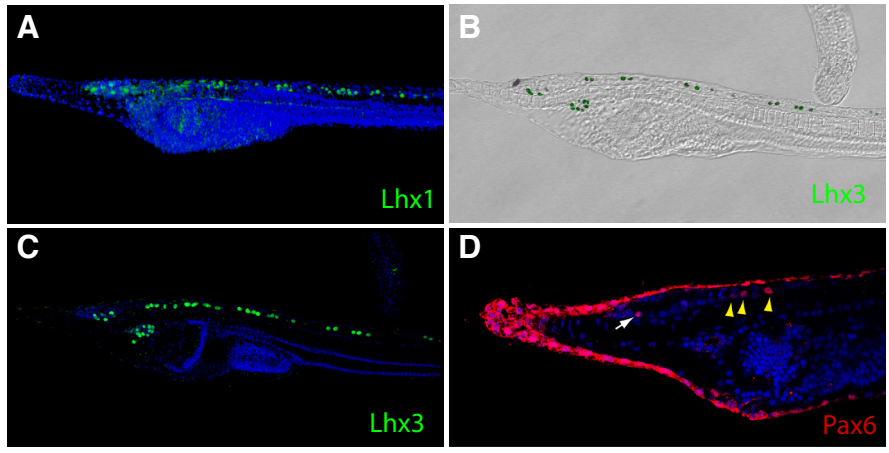



**Fig. 2. Immunostaining of Lhx1, Lhx3 and Pax6 in amphioxus larvae.** *Larvae of* B. lanceolatum *were immunostained with sera 249* **(A)**, *330* **(B,C)**, *and 925* **(D)**. *Lhx1* **(A)** *and Lhx3* **(B,C)** *are detected in cells of the dorsal nerve cord (green signal). In addition, nuclear signal for Lhx3 is present in the preoral pit* **(B)**. *A composite of a bright field and fluorescent image is shown in* **(B)**. **(D)** *The red nuclear signal represents Pax6 expression in the amphioxus primary motor centre (yellow arrowheads mark three pairs of Pax6-positive neurons) and in the frontal eye (white arrow). The conspicuous ectodermal signal represents unspecific labelling. Blue represents nuclear DAPI signal.*

routinely perform a series of four immunizations. A sample of the serum was taken from the tail vein after the 3rd immunization to monitor efficacy and specificity of the serum by immunostaining. Table 1 provides a summary of amphioxus-specific antibodies we produced. Antibodies recognizing five *B. floridae* proteins, namely FoxA, Lhx1, Lhx3, Pax6 and β-catenin, were generated in the current study. Below we describe each reagent that we produced and characterized by immunostaining of appropriate stages of amphioxus embryos and larvae.

The *FoxA* gene (alternative name *Hnf3*) encodes a forkhead transcription factor. Amphioxus has two *HNF3* class genes, named *AmHNF3-1* (*FoxAa*) and *AmHNF3-2* (*FoxAb*), with apparently identical expression pattern in the presumed organizer, endoderm, and notochord at the neurula stage (Shimeld, 1997). Molecular phylogenetic analysis revealed that these paralogues derive from an independent duplication in the cephalochordate lineage (Shimeld, 1997). Antibodies directed against FoxA were made by immunizing mice with a C-terminal part of *B. floridae* FoxAa. As shown in Fig.1, all three sera 326 - 328 were able to specifically detect nuclear FoxA in presumptive endoderm and notochord of *B. lanceolatum* neurula, in a pattern matching the one obtained by *in situ* hybridization (Shimeld, 1997; Terazawa and Satoh, 1997). Sera obtained after the 3rd immunization showed high specificity and good signal to noise ratio. However, a stronger signal was detected using sera 326-328 collected after the 4th immunization (Fig. 1). A similar trend regarding 3rd and 4th sera was observed for antibodies generated against other antigens as well.

Amphioxus *Lhx1* (*Lim1*) encodes a LIM-homeobox gene orthologous to vertebrate *Lhx1* and *Lhx5*. During amphioxus development, *Lhx1* is first detected in the ectoderm of the blastula (Langeland *et al*., 2006). Then, in the gastrula, in addition to the ectoderm *Lhx1* expression appears in the mesendoderm just within the dorsal lip

TABLE 1

**SUMMARY OF AMPHIOXUS-SPECIFIC ANTIBODIES DESCRIBED IN THIS STUDY**

| Gene | Antibody name (serum number) | Efficacy of the antibody in staining | Species tested | Fixation and storage | Comment | Reference |
|---|---|---|---|---|---|---|
| β-catenin | *097* | - | *Bl* | *a* | *Nonspecific staining of most nuclei.* | This study |
| β-catenin | *393* | ++ | *Bl* | *a, b* | *Functional on ethanol-stored samples.* | This study |
| β-catenin | *394* | ++ | *Bl* | *a* | | This study |
| FoxA | *326* | +++ | *Bl, Bf, Al* | *a* | | This study |
| FoxA | *327* | +++ | *Bl, Bf* | *a* | | This study |
| FoxA | *328* | ++ | *Bl, Bf* | *a* | | This study |
| Lhx1 | *249* | +++ | *Bl, Bf* | *a, b, c, d* | *Functional on ethanol-stored samples albeit with slightly worse signal/noise ratio.* | This study |
| Lhx1 | *250* | + | *Bl, Bf* | *a, b, c, d* | *Signal undetectable in ethanol-stored samples.* | This study |
| Lhx1 | *325* | ++ | *Bl, Bf* | *a, b, c, d* | *Diffused nonspecific staining of epidermal nuclei, together with specific signal in the neural tube. Nonspecific signal is dominant in ethanol-stored samples.* | This study |
| Lhx3 | *329* | ++ | *Bl, Bf* | *a* | | This study |
| Lhx3 | *330* | +++ | *Bl, Bf* | *a* | | This study |
| Lhx3 | *331* | + | *Bl, Bf* | *a* | | This study |
| Pax6 | *925* | ++ | *Bf, Bl, Al* | *a* | | This study |
| Pax6 | *926* | - | *Bf, Bl, Al* | *a* | | This study |
| Pax6 | *927* | + | *Bf, Bl, Al* | *a* | | This study |
| Otx | *72* | +++ | *Bf, Bl, Al* | *a* | | *Vopalensky et al., 2012* |
| Ops3 | *52* | + | *Bf, Bl* | *a* | *Non-functional for B. lanceolatum* | *Vopalensky et al., 2012* |

Abbreviiations: +++, easily detected; ++, detected; +, detectable; -, not detected; Bf, Branchiostoma floridae, Bl, Branchiostoma lanceolatum, Al, Asymmetron lucayanum.

*a* Embryos or larvae fixed 15 minutes on ice and stored in 100% methanol; *b* Embryos or larvae fixed ON 4°C and stored in 100% methanol; *c* Embryos or larvae fixed 15 minutes on ice and stored in 70% ethanol; *d* Embryos or larvae fixed ON 4°C and stored in 70% ethanol.

of the blastopore. By the mid-neurula stage *Lhx1* is expressed in the anterior part of the central nervous system, in the hindgut, in Hatschek's right diverticulum, and in the wall of the first somite on the left side (Langeland *et al.*, 2006). At the larval stage, Lhx1 expression remains in lateral and ventral cells along the anterior third of the dorsal nerve cord, in Hatschek's nephridium, in the wall of the rostral coelom, in the epidermis of the upper lip, and in mesoderm cells near the opening of the second gill slit (Langeland *et al.*, 2006). Antibodies directed against amphioxus Lhx1 were made by immunizing mice with a C-terminal part of *B. floridae* protein. We tested three anti-Lhx1 antibodies (sera 249, 250 and 324) on amphioxus larvae and obtained nuclear staining in cells of the dorsal nerve cord (Fig. 2A, Supplementary Fig. S1A), matching previously described expression domain of the gene (Langeland *et al.*, 2006). Notable differences were observed for the three sera, with the best signal/noise ratio obtained for serum 249 (Supplementary Fig. S1A, Table S1).

The amphioxus *Lhx3* gene encodes a LIM-homeobox gene. Its expression was studied in *B. belcheri* by WMISH (Wang *et al.*, 2002). Expression of *Bblhx3* first appeared in the vegetal and future dorsal side of the gastrula and became restricted to the endoderm during gastrulation. At the neurula and early larva stage, *Bblhx3* was expressed in the developing neural tube, the notochord and preoral pit. We generated three antibodies (sera 329, 330, and 331) directed against amphioxus *Lhx3* by immunizing mice with a C-terminal part of the *B. floridae* protein. All three antibodies detected Lhx3 in dorsally located cells of the nerve cord and in the preoral pit of *B. lanceolatum* larvae (Fig. 2 B,C, Supplementary Fig. S1B), i.e. in areas characteristic for the expression pattern of amphioxus *Lhx3* (Wang *et al.*, 2002). As in the case of the Lhx1 antibody, notable differences were, however, observed for the three sera with the best signal/noise ratio obtained for serum 330 (Supplementary Fig. S1B, Table S1).

*Pax6* encodes a paired and homeobox gene which constitutes a member of the Pax-Six-Eya-Dach network in amphioxus (Kozmik *et al.*, 2007). *Pax6* is expressed in the anterior ectoderm from the early neurula stage until the early larval stages (Glardon *et al.*, 1998). Expression is also detectable in Hatschek's left diverticulum as it forms the preoral ciliated pit. Regional expression in the anterior neural plate of early embryos continues later in the cerebral vesicle, most conspicuously in the lamellar body, in some cells of the frontal eye and in the primary motor center (Glardon *et al.*, 1998; Vopalensky *et al.*, 2012). We have previously generated antibodies specifically recognizing *B. floridae* Pax6 by immunizing rabbits with a C-terminal part of the corresponding protein (Vopalensky *et al.*, 2012). Here, we used the same antigen to immunize mice in order to produce mouse polyclonal antibodies (sera 925, 926 and 927). Serum 925 was the most efficient one in labelling Row 1 cells of the frontal eye and cells of the primary motor center whereas serum 926 did not yield a positive signal (Fig. 2D, Supplementary Fig. S1C). In addition to specific labeling of individual neurons all Pax6 sera produced a very conspicuous (unspecific) ectodermal signal (Fig. 2D; see also Figs. 4C, 5B).

One of the hallmarks of canonical Wnt signaling activation is the nuclear accumulation of β-catenin, which interacts with Tcf/Lef family members to activate transcription of target genes. Thus, monitoring the distribution of nuclear β-catenin in the cells of the embryo permits the detection of regions in which the canonical Wnt signaling is active. Indeed, antibody labeling with immunohistochemical detection of β-catenin in *B. floridae* and *B. belcheri* embryos has previously been performed (Holland *et al.*, 2005; Yasui *et al.*, 2002). Surprisingly, the two studies demonstrated inconsistent differences in the distribution of nuclear β-catenin during early development in these two species. This could be due to the use of distinct antibody reagents derived against β-catenin of vertebrates (chicken and human) (Yasui *et al.*, 2002) or sea urchin (Holland *et al.*, 2005). We generated three antibodies (sera 097, 393, and 394) directed against amphioxus β-catenin by immunizing mice with a C-terminal part of *B. floridae* protein. To check the specificity of individual sera and to validate previously used commercially available anti-human β-catenin antibody (Yasui *et al.*, 2002) we performed double

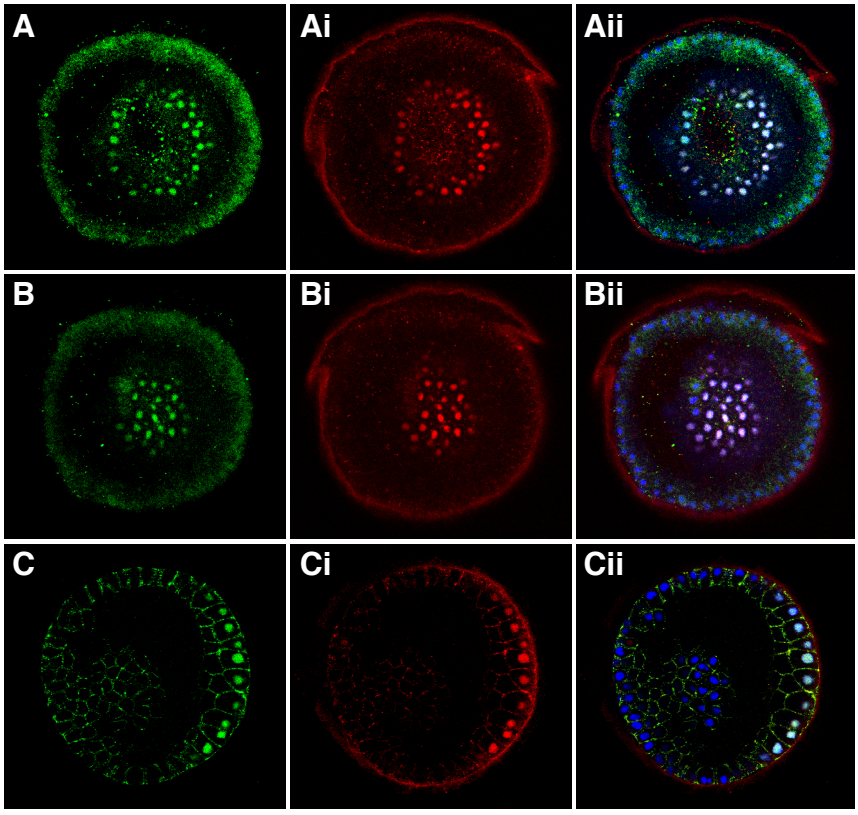
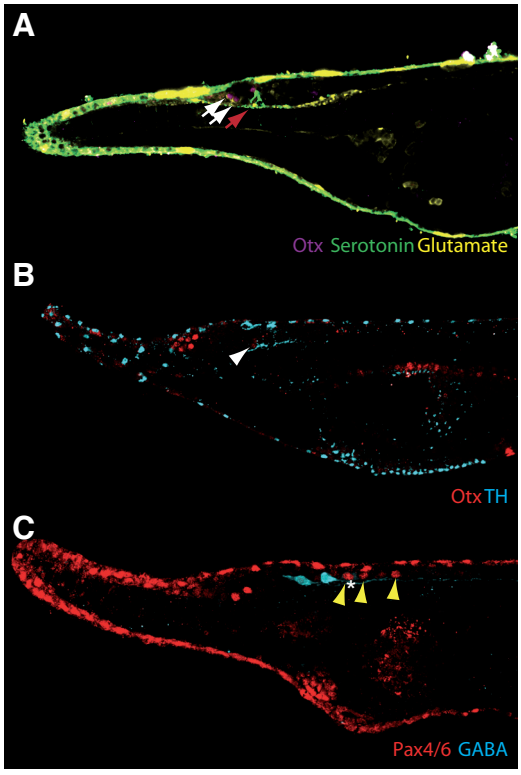



**Fig. 3. Double immunostaining of β-catenin with amphioxus-specific and commercially available cross-reacting antibodies. (A,B,C)** *The signal of amphioxus-specific β-catenin antibody;* **(Ai, Bi, Ci)** *the signal of commercial anti-human β-catenin antibody (Sigma C2206).* **(Aii, Bii, Cii)** *Overlapping signals of DAPI (blue), amphioxus-specific (green signal) and commercial anti-human antibody (red signal).* **(A-Aii, B-Bii)** *Two z-stacks at different positions of the same embryo at mid-gastrula stage; optical z-sectioning was taken from a blastopore view.* **(A-Bii)** *β-catenin is detected in the mesendoderm.* **(C-Cii)** *A z-stack of the embryo at the late blastula stage. The embryo in* **(A-Bii)** *was stained with serum 393, whereas the embryo in* **(C-Cii)** *was with serum 394.*



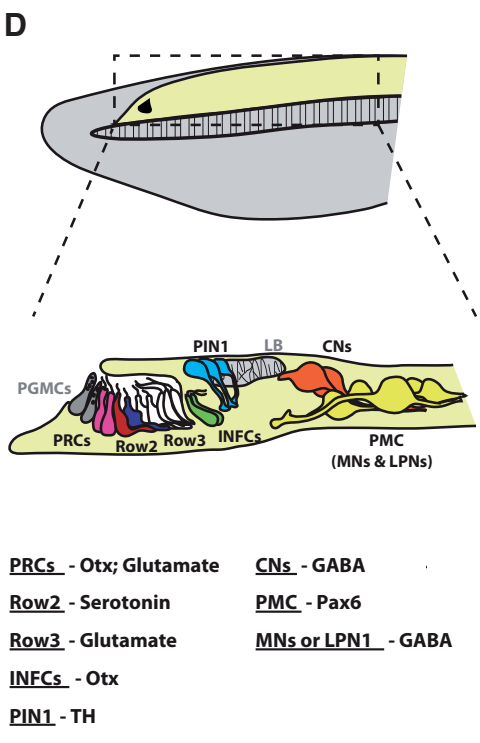




**Fig. 4. High-resolution detection of specific neurons in the cerebral vesicle by double immunostaining with amphioxus-specific and commercial antibodies.** *Amphioxus-specific antibodies were used to localize neuronal populations of interest and commercial antibodies were used to localize specific neuronal populations in the cerebral vesicle of* B. lanceolatum *larvae.* **(A)** *Co-staining of* B. lanceolatum *larvae with mouse polyclonal antibody generated against amphioxus Otx and commercial polyclonal antibodies against neurotransmitters serotonin (raised in goat) and glutamate (raised in rabbit). White arrows mark Row1 cells of the frontal eye (Otx positive). The red arrow points to Row2 cells (serotonin positive). Glutamate serves as a neurotransmitter in Row1 cells and Row3 cells (positive signal posterior to Row2 cells).* **(B)** *Otx was used to mark infundibular cells (white arrowhead). Projections of tyrosine hydroxylase (TH)-positive neurons can be traced from the dorsolateral region of the cerebral vesicle to infundibular organ.* **(C)** *Yellow arrowheads mark the Pax6-positive three pairs of neurons in the amphioxus primary motor centre. The first pair of neurons is GABA positive. Note, that two other pairs of GABA-positive neurons (the so-called commissural neurons) were stained more anteriorly. The conspicuous ectodermal signal represents unspecific labelling.* **(D)** *Schematic diagram of the anterior part of amphioxus neural tube indicating labelling of individual neuronal populations in cerebral vesicle. Cells populations stained in A – C are highlighted by colors and described by black letters. Other important landmarks are depicted and described in grey. PGMCs – pigment cells of the frontal eye; PRCs – photoreceptors cells of the frontal eye; Row2 – so called "Row2 cells" of the frontal eye (potential visual projecting interneurons); Row3 – so called "Row3 cells" of the frontal eye (potential projecting interneurons); INFCs – infundibular cells (possible homolog of vertebrate balance organ); PIN1 - type 1 parainfundibular neurons; LB – lamellar body (possible homolog of vertebrate pineal gland); CNs – commissural neurons (possibly involved in regulation of amphioxus larval movement); PMC – primary motor center; MNs – motoneurons; LPNs – large paired neurons. Antigen positivity shown in this study is highlighted for each of the neuronal populations. More data are necessary to decipher the identity of Pax6 positive motoneurons in PMC. Scheme adapted after Lacalli (2008).*

immunostainings (Fig. 3, Supplementary Fig. S1D). Two of the three sera (serum 393 and serum 394) labeled nuclear β-catenin at the blastula and mid-gastrula stage in the same pattern as the commercial anti-human β-catenin antibody. At mid-gastrula stage, both commercial and amphioxus-specific antibodies detected nuclear β-catenin throughout the mesendoderm (Fig. 3 A-Bii). At late blastula stage double immunostaining revealed asymmetrical distribution of nuclear β-catenin (Fig. 3 C-Cii). The immunostaining with one of the sera (serum 097) did not show any specific pattern and, in contrast to the commercially available antibody, which labeled β-catenin throughout the mesendoderm at the mid-gastrula stage, detected nuclear β-catenin in all nuclei of the embryo (Supplementary Fig. S1D). It is of note that we observed a variable degree of staining for β-catenin to adherens junctions among individual embryos. This was the case for amphioxus-specific sera 393 and 394, as well as for commercial anti-human β-catenin antibody (Fig. 3, Supplementary Fig. S1D, and data not shown). The reason for inconsistent detection of β-catenin in adherens junctions is currently unclear. Combined, we not only generated functional antibody reagents against amphioxus β-catenin, but also validated the use of cross-reacting antibody originally developed against the human protein.

Next, we tested the usefulness of the newly generated homemade antibodies for high-resolution mapping of individual cells in the amphioxus larva. We performed double immunolabelling using mouse polyclonal antibody generated in this study and a commercial antibody made in another host to obtain single-cell resolution of specific neuronal populations in the cerebral vesicle (e.g. Row1 cells, primary motor centre, infundibular cells). This approach allowed a more precise characterization of individual neurons within the cerebral vesicle with respect to their anatomical position (Fig. 4), a task which is otherwise very difficult to perform. For example, to obtain similar results Lacalli and Candiani (2017) had to deduce their conclusion from a combination of data from *in situ* hybridization experiments and detailed electron microscopy analysis (analyses had to be, however, performed on larvae from different batches). By using the approach presented here, one can localize individual cell types within a single larva and easily obtain the information about their relative positions.

All immunostainings were initially performed on specimens fixed in 4% PFA for 15-45 min on ice (alternatively at 4ºC overnight). Fixed samples were subsequently transferred to 100% methanol and stored at -20ºC until use. For selected sera, we also tested the efficacy of immunostainings on embryos and larvae prepared

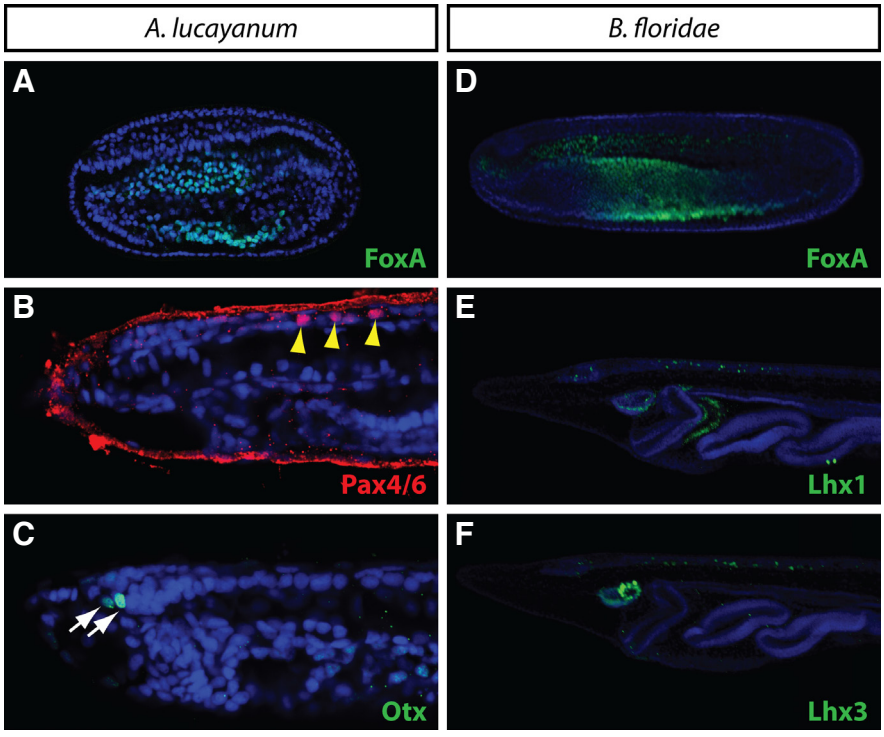



**Fig. 5. Immunostaining of B. floridae and A. lucayanum specimens**. *Embryos and larvae of* A. lucayanum **(A-C)** *and* B. floridae **(D-F)** *were immunostained with FoxA serum 326* **(A,D)**, *Pax6 serum 925* **(B)**, *Otx serum 72* **(C)**, *Lhx1 serum 249* **(E)** *and Lhx3 serum 330* **(F)**. *The conspicuous ectodermal signal in (B) represents unspecific labelling.*

for *in situ* hybridization (fixed in 4% PFA at 4ºC overnight and stored in 70% ethanol). We found that some polyclonal antibodies are functional on such specimens making the logistic of embryo harvest simpler – the same sample can in principle be used for *in situ* hybridization and immunostaining (see Table 1).

We presume that an apparently high success rate of obtaining functional antibodies against amphioxus proteins by the procedure described here is largely due to sequence divergence of selected peptides between amphioxus and mice and choice of long peptide antigens. At the time of commencing the project, the complete genome sequence of *B. floridae* was available. Hence the antigens used in this study are of *B. floridae* origin but due to the high degree of sequence identity among different amphioxus species, we expected that most of the antibodies would allow cross-species detection. In general, interspecies cross-reactivity for home-made polyclonal antibodies was indeed observed (Table 1). All antibodies generated against *B. floridae* antigens, except for one, appear to be functional in detecting the corresponding orthologous protein in the European lancelet (*B. lanceolatum*). Some of the antibodies were even successfully validated in the more distantly related *A. lucayanum* (Fig. 5). However, in the case of Ops3 no positive immunostaining was observed in species other than the one from which the epitope was derived from, even though 45 out of 67 amino acids of the epitope were conserved (Supplementary Fig. S2). Combined, our data suggest that high degree of sequence identity typical for transcription factors and other regulatory proteins increases the likelihood of producing an antibody cross-reacting with proteins of different amphioxus species. We anticipate that in the near future the access to genomes of individual lancelet species will allow cross-species homology analyses and a more rational choice of antigenic peptides with the aim of generating universal anti-amphioxus antibodies even for faster evolving genes, such as opsins.

## Conclusions

We developed novel mouse polyclonal antibodies for use in immunostainings on amphioxus embryos. Our pilot study shows the feasibility of the experimental procedure that reliably yields functional antibodies at modest cost. Such antibodies can be used for elucidating embryonic gene expression at a single-cell resolution, for co-labelling using other antibodies or for investigating the sub-cellular localization of endogenous proteins in the developing embryo. Immunostaining can potentially be combined with WMISH (Lu *et al.*, 2012) or transgenic fluorescent reporter proteins (Kozmikova and Kozmik, 2015), allowing double labeling of cell types or of embryological structures of interest. Polyclonal antibodies often allow detection of the same protein in different amphioxus species, such as *B. floridae*, *B. lanceolatum* and *A. lucayanum*. In summary, we anticipate that a panel of antibodies such as those generated by us here and by others in the future will represent a useful toolkit in amphioxus research.

## Materials and Methods

### *Generation of antibodies*

For overexpression of protein fragments, the pET system (Novagen) was used. Selected coding sequences were cloned into the pET42a(+) vector to create proteins containing 6xHis-GST fused to the protein fragment of interest. Amino acid sequences of antigens are shown in Supplementary Table 1. Expression vectors were introduced into the bacterial production strain of bacteria BL21 (DE3) RIPL (Stratagene). A total volume of 500 ml fresh LB medium without antibiotics was inoculated with an overnight culture grown in LB medium supplemented with 12.5 μg/ml chloramphenicol and 30 μg/ml kanamycin. Bacteria were grown at 37°C at 200 RPM until $OD_{600}$ reached 0.6, then induced by 0.5 mM IPTG for 3 hours. Cells were harvested at 6000 x g for 20 minutes and the pellet stored at -80°C until further processing. The pellet was resuspended in Lysis buffer (6M guanidine hydrochloride, 0.1M $NaH_2PO_4$, 0.01M Tris.Cl, pH 8.0, supplemented with fresh β-mercaptoethanol to a final concentration of 20 mM). The suspension was sonicated 6 x 20 s and incubated for 3 hours at room temperature. The resulting lysate was centrifuged at 10,000 x g for 10 minutes and the supernatant mixed with Ni-NTA agarose beads (Qiagen) previously equilibrated with Urea buffer (8 M urea, 20 mM Tris.Cl, 50 mM $NaH_2PO_4$, 100 mM NaCl, pH 8.0, supplemented with fresh β-mercaptoethanol to a final concentration of 20 mM). The suspension was incubated on a rotating platform overnight at room temperature. The beads with bound proteins were washed two times with 40 ml Urea buffer and loaded onto a disposable chromatographic column (Bio-Rad). The column was washed with Urea buffer with decreasing pH (8.0 – 6.8) and His-tagged protein was eluted by Urea buffer at pH 4.2 into several 1 ml aliquots. After elution, pH was immediately increased to 7.5 with 1 M Tris.Cl, pH 8. Protein concentration was estimated using Protein Assay Reagent (Bio-Rad). Three mice of the B10A-H2xBALB/CJ strain were immunized four times in monthly intervals with 30 μg purified protein mixed

with Freund's Adjuvant (Sigma). An aliquot of serum was collected ten days after the 3rd and 4th immunization.

***Animal collection and immunohistochemistry***

*B. lanceolatum* adults were collected in Argeles-sur-Mer (France), transported to Institute of Molecular Genetics (Prague, Czech Republic) and preserved in the lab in a day/night cycle of 14h/10h until spawning, which was induced by a shift in temperature (Fuentes *et al.*, 2007). Adults of Florida amphioxus (*B. floridae*) were collected from Old Tampa Bay, Florida, during the summer breeding season. Adults were induced to spawn by electrostimulation as described (Yu and Holland, 2009). Ripe adults of *A. lucayanum* were collected in Bimini and allowed to spawn naturally in the laboratory (Holland and Holland, 2010). Embryos were raised in the laboratory on site. Embryos for immunohistochemistry were fixed with 4% PFA/MOPS (0.1 M 3-(N-morpholino)propanesulfonic acid, 2 mM MgSO4, 1 mM EGTA, 0.5M NaCl, pH 7.5) for 15 minutes on ice and stored in 100% methanol. Some specimens were fixed with 4% PFA/MOPS overnight at 4°C and stored in 70% EtOH. Specimens were transferred to 1× PBS 0.1% Tween-20 (PBT) through 70% and 30% methanol in PBS. Following four 15-minute washes in PBT, samples were blocked in blocking solution (10% BSA in PBT) for (at least) 1 h and incubated with primary antibodies overnight at 4°C. Amphioxus-specific antibodies were diluted 1:200 to 1:500 in blocking solution. The following commercial primary antibodies were used: β-catenin (Sigma C2206, rabbit polyclonal, dilution 1:500), GABA (Sigma A2052, rabbit polyclonal, 1:500), tyrosine hydroxylase (TH, Abcam ab112, rabbit polyclonal, 1:1000), glutamate (Sigma G6642, rabbit polyclonal, 1:500), serotonin (Abcam ab66047, goat polyclonal, 1:1000). On the following day, samples were washed five times in PBT (20 minutes each wash) and were incubated with secondary antibodies and 1 μg/mL DAPI for 3 hours at room temperature. Alexa Fluor 488 or 594 anti-mouse, anti-rabbit or anti-goat were used as secondary antibody at 1:500 dilution. For confocal microscopy, the samples were mounted in VECTASHIELD (Vector Laboratories, Inc.) using three layers of Scotch tape as spacers between the slide and the coverslip. The confocal images were taken using a Leica SP5 or SP8 confocal microscope and were processed (brightness and contrast) with FIJI image analysis software. Images were further processed (rotation) and assembled in tables with Adobe Photoshop CS4.

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