UC Santa Barbara
UC Santa Barbara Previously Published Works

Title

Whole-mount fluorescent in situ hybridization staining of the colonial tunicate Botryllus schlosseri

Permalink

https://escholarship.org/uc/item/0sq064mj

Journal

Genesis, 53(1)

ISSN

1526-954X

Authors

Langenbacher, Adam D
Rodriguez, Delany
Maio, Alessandro
et al.

Publication Date

2015

DOI

10.1002/dvg.22820

Peer reviewed

Whole-mount fluorescent *in situ* hybridization staining of the colonial tunicate *Botryllus schlosseri*

Adam D. Langenbacher, **Delany Rodriguez**, **Alessandro Di Maio**, and **Anthony W. De Tomaso**

Department of Molecular, Cellular and Developmental Biology, University of California Santa Barbara, Santa Barbara, California, 93106 USA

Abstract

Botryllus schlosseri is a colonial ascidian with characteristics that make it an attractive model for studying immunology, stem cell biology, evolutionary biology, and regeneration. Transcriptome sequencing and the recent completion of a draft genome sequence for *B. schlosseri* have revealed a large number of genes, both with and without vertebrate homologs, but analyzing the spatial and temporal expression of these genes *in situ* has remained a challenge. Here we report a robust protocol for *in situ* hybridization that enables the simultaneous detection of multiple transcripts in whole adult *B. schlosseri* using Tyramide Signal Amplification in conjunction with digoxigenin- and dinitrophenol-labeled RNA probes. Using this protocol we have identified a number of genes that can serve as markers for developing and mature structures in *B. schlosseri*, permitting analysis of phenotypes induced in loss-of-function experiments.

Keywords

gene expression; ascidian; tyramide signal amplification; stem cells; allorecognition

Introduction

Botryllus schlosseri is a colonial marine chordate in the subphylum Tunicata, a group of invertebrates now thought to be the closest living relatives of the vertebrates (Delsuc *et al.*, 2006). Each week, the adult body of *B. schlosseri*, known as a zooid, undergoes apoptosis and is replaced in an asexual budding process (Lauzon *et al.*, 1992; Manni *et al.*, 2007). The development of a bud in *B. schlosseri* requires two weeks, and as such, three simultaneous generations exist in a colony at all times (0-1 week old secondary buds, 1-2 week old primary buds, and 2-3 week old adult zooids). Up to four secondary buds may arise as thickenings of the epithelium of a primary bud, which then expand in size and pinch off to form hollow ellipsoids. Subsequent organogenesis and growth results in new bodies that are physically and genetically indistinguishable from the previous generation (Berrill, 1941).

Correspondence: Anthony W. De Tomaso Department of MCD Biology, UC Santa Barbara, Santa Barbara, CA 93106-9610 805-895-7276 detomaso@lifesci.ucsb.edu.

Individuals in a *B. schlosseri* colony share a common extracorporeal circulatory system that terminates at the colony's periphery in specialized structures called ampullae. When the ampullae from two colonies come into contact, a histocompatibility reaction dependent on a single locus results in one of two outcomes: (1) the vasculatures of these colonies fuse and circulating cells are freely exchanged or (2) fusion does not occur and inflammatory lesions may form between the colonies (Cima *et al.*, 2006; De Tomaso *et al.*, 2005; De Tomaso and Weissman, 2003; Rinkevich and Weissman, 1992b). Numerous studies have documented roles for putative receptors and ligands in the fusion/histocompatibility locus (the *fuhc*), and suggest that *B. schlosseri* allorecognition represents a protochordate "missing-self" immune response that is genetically encoded (McKitrick *et al.*, 2011; Nyholm *et al.*, 2006; Voskoboynik *et al.*, 2013b).

Transfer of circulating cells between two fused *B. schlosseri* colonies produces a state of chimerism that may ultimately result in the complete replacement of the germline or specific somatic tissues of one colony (the "loser") by that of the other (the "winner") (Rinkevich and Weissman, 1992a; Sabbadin and Zaniolo, 1979; Stoner and Weissman, 1996). This phenomenon, known as parasitism, can be replicated by manually transplanting a FACS-isolated population of cells high in aldehyde dehydrogenase activity, a biomarker for stem cells in vertebrates (Kastan *et al.*, 1990; Laird *et al.*, 2005). Winner and loser phenotypes are hierarchical and heritable, suggesting that the ability of one colony to successfully parasitize another is likely to be genetically encoded (Stoner *et al.*, 1999). The properties of parasitism and allorecognition make *B. schlosseri* an excellent model for addressing questions in the fields of immunology, stem cell biology, regeneration, and evolutionary biology.

Recent transcriptomic (Rodriguez *et al.*, submitted) and genomic (Voskoboynik *et al.*, 2013a) analyses of *B. schlosseri* have produced a treasure trove of genetic data and permitted the identification of a large number of *B. schlosseri* homologs of significant vertebrate genes. Notably, examination of the draft genome of *B. schlosseri* has revealed homologs of genes known to be critical for the development and function of the heart, eye, and immune system, some of which also play a role in human disease (Voskoboynik *et al.*, 2013a). Given the natural suitability of *B. schlosseri* for addressing questions involving immunology and stem cell biology, forward and reverse genetic approaches based on this new genetic data will undoubtedly lead to important advances in these fields.

The ability to analyze spatial and temporal gene expression patterns is a critical tool required for any model organism. In this manuscript, we describe a robust *in situ* hybridization protocol for examining gene expression in *B. schlosseri* and use this protocol to analyze the expression patterns of several genes that are useful markers for developing and mature structures.

Results and Discussion

Current methods for *in situ* hybridization analysis in *B. schlosseri* are hampered by high levels of background staining in extracorporeal tissues (tunic and vasculature) and have frequently relied on sectioning to clearly reveal staining in tissues of interest (Brown *et al.*, 2009; De Tomaso *et al.*, 2005; Nyholm *et al.*, 2006; Rinkevich *et al.*, 2012; Rinkevich *et al.*,

2013; Rosner *et al.*, 2013). While effective for ascertaining the expression patterns of some transcripts, these methods have not been able to provide a global view of the expression domains of more than one transcript simultaneously. We sought to develop a protocol without these limitations that could be used to reproducibly analyze gene expression in whole adult *B. schlosseri* at all developmental stages.

B. schlosseri are highly pigmented, and pigment cells are a major source of autofluorescent background signal in fixed samples. Different color morphs of *B. schlosseri* also exist, ranging from blue-black to orange-brown. We found that treatment of formaldehyde-fixed blue-black specimens with 6% hydrogen peroxide in methanol under bright light was able to nearly eliminate autofluorescence within 24 hours. Orange specimens were much more resistant to bleaching and were therefore not used in this study. Our *in situ* hybridization protocol utilizes several reagents designed to increase signal strength. First, we included a ribonuclease inhibitor in all solutions used prior to signal development. Second, we used Denhardt's solution, which contains macromolecular crowding agents, in our hybridization solution since it provided a roughly two-fold increase in signal intensity (data not shown). Lastly, we used the highly sensitive Tyramide Signal Amplification method to produce strong fluorescent signals with minimal noise. Non-specific binding of antibodies to the tunic is an unavoidable problem in *B. schlosseri*, but detection of gene expression by fluorescent means circumvents this problem by allowing confocal imaging of samples. Furthermore, it permits the quantification of cell numbers via fluorescent nuclear counterstains. Our whole-mount fluorescent *in situ* hybridization (FISH) procedure, including alternative steps for detecting multiple transcripts simultaneously, is summarized in Figure 1.

In order to test the power of our technique and identify genes that could potentially be used as markers for different structures in *B. schlosseri* zooids and buds, we examined gene expression patterns in the *Ciona intestinalis* Adult In situ hybridization Database (http://bioinfo.s.chiba-u.jp/ciaid/) and rationalized that the *B. schlosseri* homologs of these genes may be expressed in similar tissues. We chose two genes for further analysis, *Na+/K+ ATPase alpha 3* (*atp1a3*) and *von Willebrand factor like 2* (*vwfl2*), because of their expression in the stomach and endostyle, respectively. We performed a translated BLAST (tblastn) of the *Ciona* protein sequences for these genes to the *B. schlosseri* transcriptome (Octopus Bioinformatics Server; http://octopus.obs-vlfr.fr/public/botryllus/blast_botryllus.php) and identified one putative homolog of *atp1a3* (E-value = 0.0) and one putative homolog of *vwfl2* (E-value = 1.0×10^{-45}). We then cloned fragments of the *B. schlosseri* homologs of *atp1a3* and *vwfl2*, generated antisense riboprobes, and examined their expression patterns in whole *B. schlosseri* using our FISH protocol. Our protocol yielded strong and specific staining patterns for both of these genes, and as we predicted, their expression domains mirrored those of their *Ciona* homologs. Expression of *atp1a3* was present in an accessory organ to the stomach, the pyloric caecum (Colton, 1910), of both zooids (Figure 2a) and primary buds (Figure 2b), as well as tissues inside of and adjacent to the developing endostyle in primary buds (Figure 2b). Signal for *vwfl2* was detected in the ventral glandular region of the endostyle of both zooids (Figure 2c) and developing primary buds (data not shown) suggesting that this region resembles Zone 2 of the *Ciona* endostyle

both morphologically and with respect to gene expression (Sasaki *et al.*, 2003). We also visualized both *atp1a3* and *vwfl2* expression simultaneously using sequential antibody detections and fluorescent depositions and did not observe any inappropriate colocalization of signal (Figure 2d), indicating that our FISH protocol can be used to reliably analyze multiple gene expression patterns simultaneously.

Given the successful visualization of *atp1a3* and *vwfl2* expression, we attempted to identify additional markers for tissues and developing structures in *B. schlosseri*. By BLAST analysis, we identified homologs of *Ciona fibroblast growth factor 11/12/13/14* (*fgf11-14*; E-value = 8.0×10^{-79}), human *par-6 family cell polarity regulator alpha* (*par6*; E-value = 2.0×10^{-68}), human *runt-related transcription factor 1* (*runx*; E-value = 5.0×10^{-73}) and human *histone 3* (*h3*; E-value = 3.0×10^{-87}). We detected expression of *fgf11-14* in a subset of cells in the developing cerebral ganglia of stage C1 primary buds (Figure 3a) (Lauzon *et al.*, 2002). Signal for *par6* was present in all epithelial cells of the primary (Figure 3b) and secondary buds (Figure 3c). Expression of *runx* was restricted to the developing secondary buds. At stage A2, *runx* was expressed throughout the secondary bud (Figure 3d), but by stage B1, expression of *runx* was restricted to the junction between the secondary and primary bud (Figure 3e). Following morphogenesis of the double-vesicle, expression of *runx* was no longer detected in the secondary bud (Figure 3f). These data indicate that our FISH protocol is capable of visualizing developmentally relevant genes expressed by tissues in both the primary and secondary buds.

Previous studies have detected expression of *histone 3* during S-phase in mitotic cells (Gown *et al.*, 1996; Muskhelishvili *et al.*, 2003). We observed expression of *B. schlosseri h3* in a non-ubiquitous manner in both primary and secondary buds (Figure 4a-c), suggesting that this gene may be marking those cells that are actively dividing. Interestingly, we also found expression of *h3* in individual cells on the ventral side of the zooid (Figure 4d), in the vicinity of a putative hematopoietic and germline niche known as the "cell islands" (Rinkevich *et al.*, 2013).

Our FISH protocol also reliably detected gene expression in germline and germline support cells. The *B. schlosseri vasa* gene has been previously described (Brown *et al.*, 2009; Rosner *et al.*, 2009), and we identified a homolog of *B. primigenus* Piwi (*piwi*; E-value = 8.0×10^{-151}). We found expression of these germline markers in clusters of primordial germ cells associated with the secondary and primary buds (Figure 5a-b). A novel TGFβ ligand and marker for germline support cells, *tgfβ-f*, was also identified during our analysis of the *B. schlosseri* transcriptome. Expression of *tgfβ-f* was present in the primitive follicle cells of juveniles (Figure 5c) as well as the follicle cells surrounding maturing oocytes and testes of primary buds (Figure 5d). Using our two-color FISH protocol, we could simultaneously visualize germline tissues and their support cells in non-fertile (Figure 5e) and fertile (Figure 5f) animals.

Taken together, these data show that our FISH protocol is capable of detecting gene expression throughout the asexual reproduction cycle in a wide array of tissues (zooid, primary bud, secondary bud, and germline). Compared to previous techniques used in *B. schlosseri*, this protocol provides a strong and specific signal without the need to first embed

and section specimens. Combination of this technique with antisense knockdown approaches, pharmaceutical inhibitions, and microsurgical manipulations in the future will greatly increase our understanding of the molecular mechanisms underlying *B. schlosseri* development and physiology.

Materials and Methods

Animals

B. schlosseri colonies were raised on glass slides in a mariculture system with circulating 0.5 μm filtered seawater at 18-20 °C. Animals were fed daily with live algae. Developmental staging of animals was performed by examination of primary and secondary buds under a dissecting microscope (Lauzon *et al.*, 2002).

Cloning of genes used for in situ hybridization analysis

B. schlosseri homologs of genes of interest were identified by tblastn searches of the *B. schlosseri* transcriptome (http://octopus.obs-vlfr.fr/public/botryllus/blast_botryllus.php) using human or *Ciona* (when available) protein sequences. If possible, primer pairs were designed to amplify a 1000 bp fragment of each transcript.

Total RNA was isolated from *B. schlosseri* colonies with the NucleoSpin RNA II kit (MN, 740955) and SuperScript II Reverse Transcriptase (Life Technologies, 18064-014) was used to synthesize cDNA primed by random primers (Life Technologies, 48190-011). PCR was performed with Advantage cDNA Polymerase (Clontech, 639105) and products were cloned into the pGEM-T Easy vector (Promega, A1360).

Synthesis of antisense RNA probes

Constructs were linearized and approximately 1 μg of template was used in an *in vitro* transcription reaction with SP6 or T7 RNA polymerase (Roche, 10810274001, 10881767001). For digoxigenin labeling of probes, DIG RNA labeling mix (Roche, 11277073910) was added to the reaction. Dinitrophenol labeling was accomplished by adding dinitrophenol-11-UTP (Perkin Elmer, NEL555001EA), UTP, CTP, GTP, and ATP (Roche, 11277057001) to the reaction at final concentrations of 0.35 mM, 0.65 mM, 1 mM, 1 mM, and 1 mM, respectively. RNases were inhibited with Protector RNase Inhibitor (Roche, 03335399001). The transcription reaction was incubated at 37 °C for 2 hours and then RNase-free DNase I (Roche, 04716728001) was added to remove the plasmid template. Probes were then precipitated twice with LiCl/ethanol to maximize removal of unincorporated labeled nucleotides (Streit and Stern, 2001).

Antisense probes used in this study include *atp1a3*, *fgf11-14*, *h3*, *par6*, *piwi*, *runx*, *tgfβ-f*, *vasa*, and *vwfl2*. Primer sequences used for amplifying these genes are listed in Table 1.

Whole-mount in situ hybridization

B. schlosseri were anesthetized with 0.02% Tricaine (TCI, T0941) in seawater for 10 minutes, and then fixed with 4% formaldehyde in 0.5 M NaCl, 0.1 M MOPS pH 7.5 for 3 hours at room temperature with agitation. We detected an improvement in signal when an

RNase inhibitor (Sigma, R7397-30ML) was added to all solutions from fixation to post-antibody washes inclusive. Fixed samples were then dehydrated with methanol and bleached overnight in 6% H_2O_2 in methanol under direct light. Once all pigment was eliminated, samples were rehydrated stepwise into PBST and permeabilized by treatment with 10 μg/ml proteinase K (Roche, 03115879001) in PBST for 30 minutes at room temperature. After permeabilization, a postfixation step was performed with 4% formaldehyde in PBST for 20 minutes at room temperature. Samples were then washed with PBST and incubated in hybridization buffer (65% formamide, 5X SSC, 1X Denhardt's solution, 0.1% Tween-20, 5 mg/ml torula yeast RNA, 50 μg/ml heparin) without probe for 4-6 hours at 58-65 °C, followed by overnight incubation with digoxigenin-labeled riboprobes diluted in hybridization buffer at 58-65 °C. For simultaneous detection of two transcripts, samples were also incubated with dinitrophenol-labeled riboprobes.

Following hybridization, unbound probes were washed away by a series of low-salt washes and samples were incubated in blocking solution (PBST, 5% heat-inactivated horse serum, 2 mg/ml bovine serum albumin) for 4 hours at room temperature with agitation. HRP-conjugated anti-digoxigenin antibody (Roche, 11207733910) diluted 1:1000 in blocking solution was then added and the samples were incubated overnight at 4 °C with agitation. After incubation with antibody, the samples were washed extensively in PBST + 2 mg/ml bovine serum albumin and transferred to PBS. Digoxigenin-labeled probes were then detected by fluorophore deposition using the TSA Plus System (Perkin Elmer, NEL753001KT).

If detection of a second transcript was needed, the HRP-conjugated anti-digoxigenin antibody was inactivated by incubating samples in PBS + 2% H_2O_2 + 0.1% Triton-X100 for 1 hour at room temperature with agitation. Following washing in PBST, dinitrophenol-labeled probes were detected with an HRP-conjugated anti-dinitrophenol antibody (Perkin Elmer, FP1129) diluted 1:200 in blocking solution. Samples were washed extensively in PBST + 2 mg/ml bovine serum albumin to remove unbound antibody, and probes were detected with a second fluorophore deposition using the TSA Plus System. Lastly, samples were stained with DAPI (Life Technologies, D1306) and flat-mounted with VECTASHIELD (Vector Labs, H-1000). Imaging of labeled samples was performed using an Olympus FLV1000S Spectral Laser Scanning Confocal.

A detailed step-by-step protocol is provided in the Supplementary Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

Berrill NJ. The Development of the Bud in Botryllus. Biological Bulletin. 1941; 80:169–184.

Brown FD, Tiozzo S, Roux MM, Ishizuka K, Swalla BJ, De Tomaso AW. Early lineage specification of long-lived germline precursors in the colonial ascidian Botryllus schlosseri. Development. 2009; 136:3485–3494. [PubMed: 19783737]

Cima F, Sabbadin A, Zaniolo G, Ballarin L. Colony specificity and chemotaxis in the compound ascidian Botryllus schlosseri. Comp Biochem Physiol A Mol Integr Physiol. 2006; 145:376–382. [PubMed: 16962802]

Colton HS. The "Pyloric Gland" of the Ascidian Botryllus: An Organ of Excretion? Biological Bulletin. 1910; 19:35–54.

De Tomaso AW, Nyholm SV, Palmeri KJ, Ishizuka KJ, Ludington WB, Mitchel K, Weissman IL. Isolation and characterization of a protochordate histocompatibility locus. Nature. 2005; 438:454–459. [PubMed: 16306984]

De Tomaso AW, Weissman IL. Initial characterization of a protochordate histocompatibility locus. Immunogenetics. 2003; 55:480–490. [PubMed: 14520503]

Delsuc F, Brinkmann H, Chourrout D, Philippe H. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature. 2006; 439:965–968. [PubMed: 16495997]

Gown AM, Jiang JJ, Matles H, Skelly M, Goodpaster T, Cass L, Reshatof M, Spaulding D, Coltrera MD. Validation of the S-phase specificity of histone (H3) in situ hybridization in normal and malignant cells. J Histochem Cytochem. 1996; 44:221–226. [PubMed: 8648081]

Kastan MB, Schlaffer E, Russo JE, Colvin OM, Civin CI, Hilton J. Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. Blood. 1990; 75:1947–1950. [PubMed: 2337669]

Laird DJ, De Tomaso AW, Weissman IL. Stem cells are units of natural selection in a colonial ascidian. Cell. 2005; 123:1351–1360. [PubMed: 16377573]

Lauzon RJ, Ishizuka KJ, Weissman IL. A cyclical, developmentally-regulated death phenomenon in a colonial urochordate. Dev Dyn. 1992; 194:71–83. [PubMed: 1421521]

Lauzon RJ, Ishizuka KJ, Weissman IL. Cyclical generation and degeneration of organs in a colonial urochordate involves crosstalk between old and new: a model for development and regeneration. Dev Biol. 2002; 249:333–348. [PubMed: 12221010]

Manni L, Zaniolo G, Cima F, Burighel P, Ballarin L. Botryllus schlosseri: a model ascidian for the study of asexual reproduction. Dev Dyn. 2007; 236:335–352. [PubMed: 17191252]

McKitrick TR, Muscat CC, Pierce JD, Bhattacharya D, De Tomaso AW. Allorecognition in a basal chordate consists of independent activating and inhibitory pathways. Immunity. 2011; 34:616–626. [PubMed: 21497115]

Muskhelishvili L, Latendresse JR, Kodell RL, Henderson EB. Evaluation of cell proliferation in rat tissues with BrdU, PCNA, Ki-67(MIB-5) immunohistochemistry and in situ hybridization for histone mRNA. J Histochem Cytochem. 2003; 51:1681–1688. [PubMed: 14623936]

Nyholm SV, Passegue E, Ludington WB, Voskoboynik A, Mitchel K, Weissman IL, De Tomaso AW. fester, A candidate allorecognition receptor from a primitive chordate. Immunity. 2006; 25:163–173. [PubMed: 16860765]

Rinkevich B, Douek J, Rabinowitz C, Paz G. The candidate Fu/HC gene in Botryllusschlosseri (Urochordata) and ascidians' historecognition--an oxymoron? Dev Comp Immunol. 2012; 36:718–727. [PubMed: 22085780]

Rinkevich B, Weissman IL. Allogeneic resorption in colonial protochordates: consequences of nonself recognition. Dev Comp Immunol. 1992a; 16:275–286. [PubMed: 1505688]

Rinkevich B, Weissman IL. Incidents of rejection and indifference in Fu/HC incompatible protochordate colonies. J Exp Zool. 1992b; 263:105–111. [PubMed: 1645117]

Rinkevich Y, Voskoboynik A, Rosner A, Rabinowitz C, Paz G, Oren M, Douek J, Alfassi G, Moiseeva E, Ishizuka KJ, Palmeri KJ, Weissman IL, Rinkevich B. Repeated, long-term cycling of putative stem cells between niches in a basal chordate. Dev Cell. 2013; 24:76–88. [PubMed: 23260626]

Rosner A, Moiseeva E, Rabinowitz C, Rinkevich B. Germ lineage properties in the urochordate Botryllus schlosseri - from markers to temporal niches. Dev Biol. 2013; 384:356–374. [PubMed: 24120376]

Rosner A, Moiseeva E, Rinkevich Y, Lapidot Z, Rinkevich B. Vasa and the germ line lineage in a colonial urochordate. Dev Biol. 2009; 331:113–128. [PubMed: 19406116]

Sabbadin A, Zaniolo G. Sexual Differentiation and Germ-Cell Transfer in the Colonial Ascidian Botryllus-Schlosseri. Journal of Experimental Zoology. 1979; 207:289–304.

Sasaki A, Miyamoto Y, Satou Y, Satoh N, Ogasawara M. Novel endostyle-specific genes in the ascidian Ciona intestinalis. Zoolog Sci. 2003; 20:1025–1030. [PubMed: 12951410]

Stoner DS, Rinkevich B, Weissman IL. Heritable germ and somatic cell lineage competitions in chimeric colonial protochordates. Proc Natl Acad Sci U S A. 1999; 96:9148–9153. [PubMed: 10430910]

Stoner DS, Weissman IL. Somatic and germ cell parasitism in a colonial ascidian: possible role for a highly polymorphic allorecognition system. Proc Natl Acad Sci U S A. 1996; 93:15254–15259. [PubMed: 8986797]

Streit A, Stern CD. Combined whole-mount in situ hybridization and immunohistochemistry in avian embryos. Methods. 2001; 23:339–344. [PubMed: 11316435]

Voskoboynik A, Neff NF, Sahoo D, Newman AM, Pushkarev D, Koh W, Passarelli B, Fan HC, Mantalas GL, Palmeri KJ, Ishizuka KJ, Gissi C, Griggio F, Ben-Shlomo R, Corey DM, Penland L, White RA, Weissman IL, Quake SR. The genome sequence of the colonial chordate, Botryllus schlosseri. Elife. 2013a; 2:e00569. [PubMed: 23840927]

Voskoboynik A, Newman AM, Corey DM, Sahoo D, Pushkarev D, Neff NF, Passarelli B, Koh W, Ishizuka KJ, Palmeri KJ, Dimov IK, Keasar C, Fan HC, Mantalas GL, Sinha R, Penland L, Quake SR, Weissman IL. Identification of a colonial chordate histocompatibility gene. Science. 2013b; 341:384–387. [PubMed: 23888037]
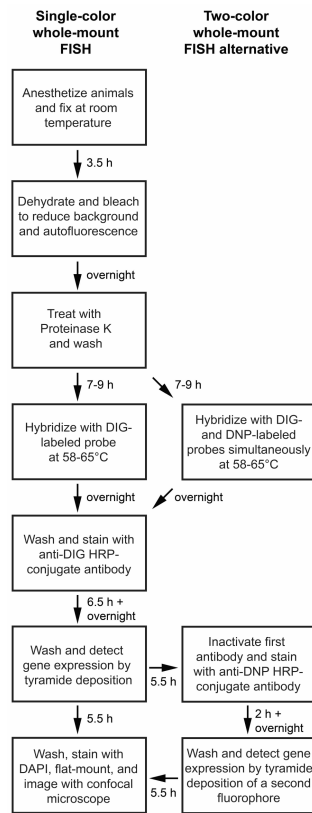
Figure 1. Flowchart of single and double whole-mount fluorescent *in situ* hybridization (FISH) protocols

Times listed next to arrows are the length of time needed to complete the step described in the preceding text box. A single-color FISH experiment requires approximately four days to complete, and a two-color FISH experiment requires approximately one day more.
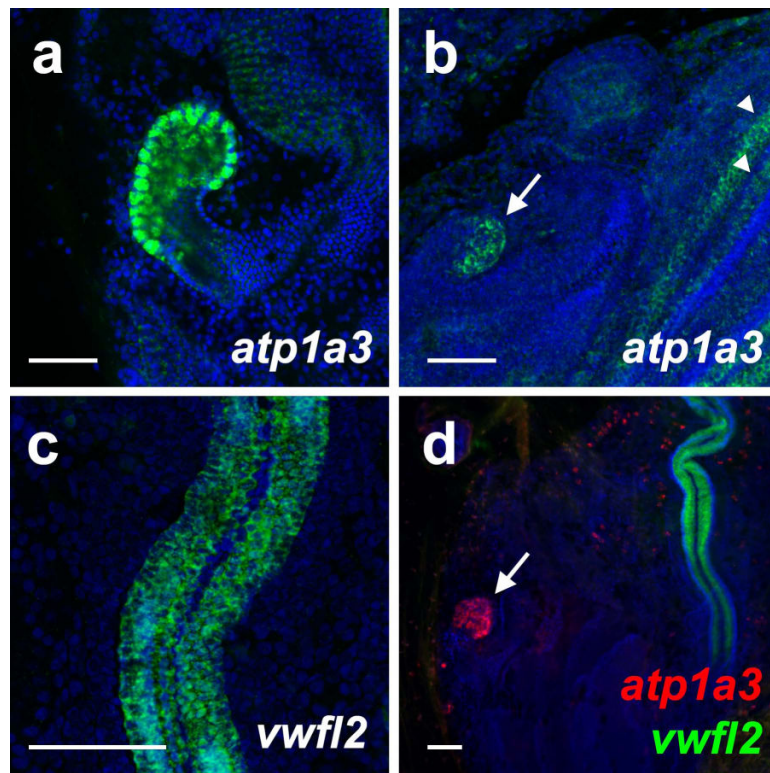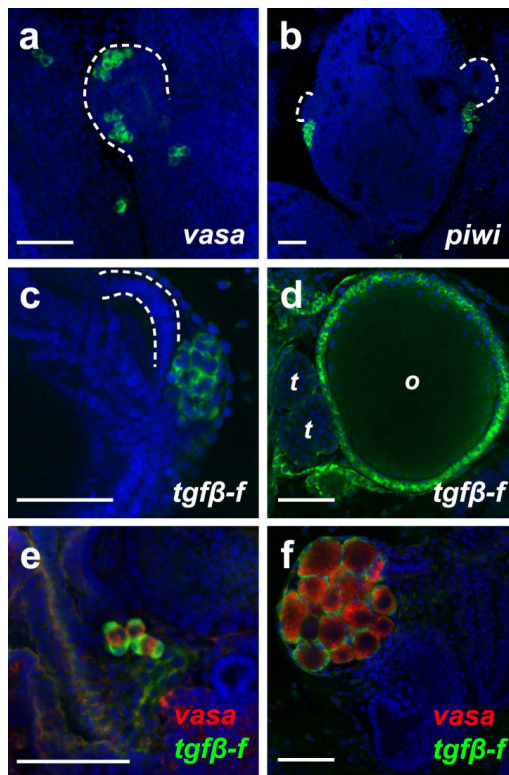
Figure 2. Expression patterns of markers for developing and adult structures in *B. schlosseri*
(a-b) *atp1a3* is expressed by cells of the pyloric caecum in the digestive system of the adult zooid (a) and the primary bud at stage C1 (b, arrow). *atp1a3* expression is also present in part of the developing endostyle and adjacent tissues in primary buds at stage C1 (b, arrowheads indicate expression visible on the left side of the endostyle). (c) *vwfl2* expression is present in the ventral portion of the endostyle of the zooid. (d) *atp1a3* (red, arrow) and *vwfl2* (green) can be simultaneously visualized in adult zooids by two-color FISH. *atp1a3* expression is also visible in individual non-epithelial cells of unknown identity that are associated with the adult zooid. Scale bars indicate 50 μm.
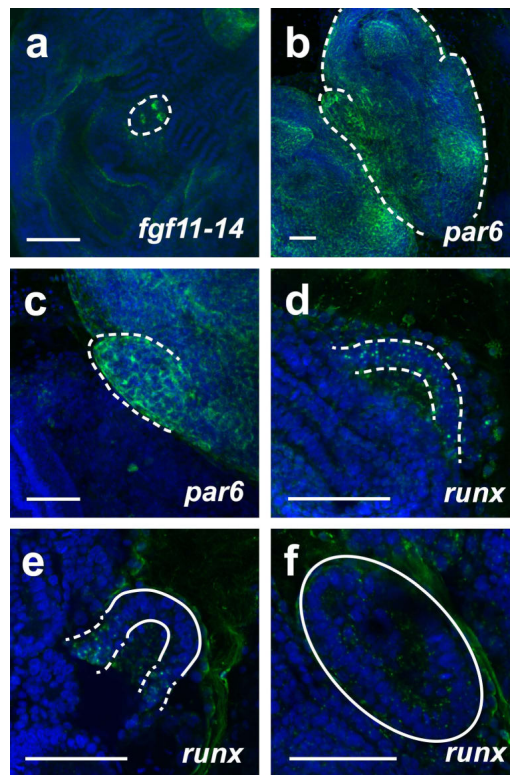
Figure 3. Expression patterns of markers for developing structures in *B. schlosseri*
(a) *fgf11-14* is expressed by a subset of cells in the developing cerebral ganglion (dashed line) of the primary bud at stage C1. (b-c) *par6* expression is present in epithelial cells of the primary (b) and secondary (b and c) buds. Primary and secondary buds in panel b and secondary bud in panel c are demarcated by dashed lines. (d-f) *runx* is expressed in a stage-specific manner. At stage A2, expression of *runx* is present throughout the secondary bud (d, dashed lines). At stage B1, *runx* expression is restricted to the boundary between the primary and secondary buds (e, dashed lines). By stage C1, *runx* is no longer expressed by cells of the secondary bud (f). Faint staining inside the secondary buds at stages A2, B1, and C1 is indicative of trapping of reagents, and is present in a region devoid of cells (d-f). Background staining is also evident outside of the demarcated areas in panels d-f as a result of non-specific binding of reagents to the tunic and the surface of the primary and secondary buds. Solid lines in panels e and f define the portions of the secondary bud where *runx* expression is not detected. Scale bars indicate 50 μm.
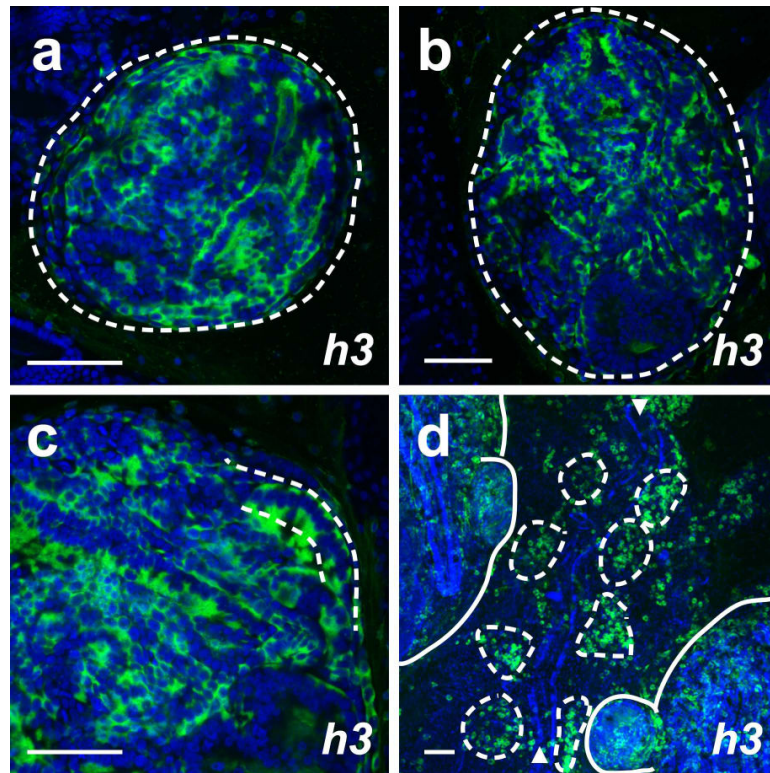
Figure 4. Expression pattern of marker for mitotically active cells in *B. schlosseri*
(a-c) *h3* is expressed by a portion of the cells in the primary and secondary bud at stages A1 (a) and A2 (b). The majority of cells in the secondary bud at stage A2 strongly express *h3* (c). Primary buds in panels a and b and the secondary bud in panel c are demarcated by dashed lines. (d) *h3* expression is also present in clusters of blood cells known as "cell islands" on the ventral side of the zooid at stage C1 (surrounded by dashed lines) on both sides of the endostyle (marked by arrowheads). Other non-epithelial cells of unknown identity outside of these clusters also exhibit *h3* expression. The primary and secondary buds in panel d are demarcated by solid lines. Scale bars indicate 50 μm.

Figure 5. Expression patterns of markers for germline and germline-associated cells in *B. schlosseri*

(a) *vasa* is expressed by clusters of germ cells associated with the secondary bud (dashed line) and just posterior to the secondary bud at stage B2. (b) At stage B1, *piwi*-positive germ cells are present posterior to the secondary buds (dashed lines). (c-d) *tgfβ-f* expression is detected in clusters of primitive follicle cells posterior to the secondary bud (dashed lines) at stage A2 in non-fertile juveniles (c) and in follicle cells surrounding the maturing oocytes (*o*) and testes (*t*) in the primary buds of fertile animals (d). (e-f) Two-color FISH can be used to simultaneously visualize *vasa* expression (red) and *tgfβ-f* expression (green) in non-fertile (e) and fertile (f) animals. Scale bars indicate 50 μm.

Table 1

Summary of *B. schlosseri* sequences and primer pairs used for *in situ* hybridization analysis.

Transcript name	Accession number	Forward (F) and Reverse (R) Primers (5′ to 3′)	Size of amplified fragment (bp)
atp1a3	KJ747407	F: GACCCTGACCGCGAAACGTATG R: CGAGGTCCTGGCGAACACAATC	1,051
fgf11-14	KJ747408	F: CAGCAGCTCAAGGGTGTGAAGAC R: CCTCTTCTTCACCTCTCCCGAACC	492
h3	KJ747409	F: ATGGCGAGAACAAAGCAAACCGC R: CTGGCGAGCTGGATGTCCTTC	386
par6	KJ747410	F: TCCGTGAATAGGATCAGTGTAGGTGAT R: CTTACAAAATGGACAAGCAGTCTTTGG	212
piwi	KJ747411	F: CGTGTGGATGGAATTGAGTGGGATATG R: CAATTTTTGTGGCGACGCTCATAAC	865
runx	KJ747412	F: CTACCCAACACATTCATACCACGC R: CGTTAGCGGAGTCTTCTCTCTTCAC	1,049
tgfβ-f	KJ747413	F: CATGGATTCTTGCAGGAGAG R: GTTACCGAACTTTCTGACCC	735
vasa	KJ747414	F: AGGCACTATGATTCAGCCTGTG R: ATCATAATCACCCGTCTCGCG	976
vwfl2	KJ747415	F: CATCCGCTTGCAGAGACGTTG R: GGACAGACCGACTCATCACATTG	985