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Optical micro-tomography "OPenT" allows the study of large toadfish *Halobatrachus didactylus* embryos and larvae

Pedro M. Felix [a], Ania Gonçalves [b], Joana R. Vicente [c], Paulo J. Fonseca [d,e], M. Clara P. Amorim [c], José L. Costa [a,e], Gabriel G. Martins [b,d,*]

[a] MARE – Marine and Environmental Sciences Centre, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016, Lisboa, Portugal
[b] Instituto Gulbenkian de Ciência, R. Quinta Grande, 6, 2780-156 Oeiras, Portugal
[c] MARE – Marine and Environmental Sciences Centre, ISPA - Instituto Universitário, 1149-041 Lisbon, Portugal
[d] cE3c – Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
[e] Departamento de Biologia Animal, Centro de Biologia Ambiental, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

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ABSTRACT

Batrachoidids, which include midshipman and toadfish are less known among embryologists, but are common in other fields. They are characteristic for their acoustic communication, and develop hearing and sound production while young juveniles. They lay large benthic eggs (>5 mm) with a thick chorion and adhesive disk and slow development, which are particularly challenging for studying embryology. Here we took advantage of a classical tissue clearing technique and the OPenT open-source platform for optical tomography imaging, to image a series of embryos and larvae from 3 to 30 mm in length, which allowed detailed 3D anatomical reconstructions non-destructively. We documented some of the developmental stages (early and late in development) and the anatomy of the delicate stato-acoustic organs, swimming bladder and associated sonic muscles. Compared to other techniques accessible to developmental biology labs, OPenT provided advantages in terms of image quality, cost of operation and data throughput, allowing identification and quantitative morphometrics of organs in larvae, earlier and with higher accuracy than is possible with other imaging techniques.

1. Introduction

Fishes are the largest extant group of vertebrates and exhibit a tremendous diversity of features and adaptations (Nelson, 2006), including many homologous to vertebrate tetrapods (*e.g.* Bass et al., 2008). The study of embryonic development presents a unique opportunity to investigate those homologies. Most of what we known of fish embryology derives from work on the model organisms zebrafish and medaka (Kimmel et al. 1995; Iwamatsu, 2004), whose transparent and small embryos are easily studied with conventional microscopy. Fish embryos vary considerably both in size and shape (Richardson et al., 1997), with zebrafish and medaka falling at the small end of the spectrum. On the other end, larval stages (even of the two small species), are too large for conventional microscopy and are still studied resorting mostly to histological sectioning (*e.g.*, Sabaliauskas et al., 2006). (See Table 1.)

Morphomics and a rekindled interest in detailed anatomical studies have recently gained prominence in developmental biology, after mesoscopic imaging by "Optical Projection Tomography" or "Light-Sheet Microscopy", were introduced to embryology by Sharpe et al. (2002) and Huisken et al. (2004), respectively. Both techniques proved valuable to study embryos of model organisms, in ways that were not possible with conventional microscopy; for example, light-sheet microscopy is well suited for imaging the early development of live zebrafish and drosophila embryos (Huisken et al., 2004; Keller et al., 2008), and optical tomography for 3D imaging large embryos (Bryson-Richardson and Currie, 2004; Ruparelia et al., 2014). The open-source community has already provided "DIY" solutions based on hardware and software which, for the most part, are already familiar to developmental biologists (Pitrone et al., 2013; Gualda et al., 2013). A question some labs are facing is whether these techniques, in simpler open-source forms are "worth the trouble", in other words, whether they provide better results than those obtained with existing techniques, and effectively solve the difficulties of large non-model organisms.

Fishes from the Batrachoididae family, which include midshipman and toadfish, are less familiar to developmental biologists, but widely used in ecotoxicology and ethology (Caçador et al., 2012), in bioacoustics (Rice et al., 2011; Vasconcelos et al., 2012) and neurophysiology (Bass et al., 2008; Vasconcelos et al., 2011; Elemans et al., 2014). Batrachoidids lay large benthic eggs (>5 mm) with a thick chorion and adhesive disk, and the embryos develop rather slowly (Arora, 1948; Dovel, 1960; Britz and Toledo-Piza, 2012), making them less amenable for ontogenetic studies. They are characteristic for their acoustic communication, and although it is known that hearing and sound

* Corresponding author at: Instituto Gulbenkian de Ciência, R. Quinta Grande, 6, 2780-156 Oeiras, Portugal.
E-mail addresses: pmfelix@fc.ul.pt (P.M. Felix), gaby@igc.gulbenkian.pt (G.G. Martins).

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Table 1
Comparison of optical techniques used to image *H. didactylus* embryos.

	Advantages	Disadvantages
Stereoscope	–Allows "fresh"/live embryos (*i.e.*, not fixed) –Fast screening –Fluorescence not required –Natural colour images –Inexpensive and widely available	–No 3D imaging –Limited internal anatomy –Low contrast/resolution
Confocal (micro & macro)	–3D imaging at cell resolution –High-contrast/resolution –Widely available	–Requires embryo fixation + clearing –Requires 3D stitching of whole embryos –Impractical to image larvae whole –Anisometric resolution; especially limiting in "macro" variant –Limited imaging in depth, even with macro confocal –Expensive to buy and operate
Optical μTomography (OPenT)	–3D imaging of larvae (3–30 mm) –3D in fluorescence & brightfield modes –Isometric resolution –High contrast images –Fast screening of whole anatomy (even considering image processing steps) –Inexpensive to build and operate	–Requires embryo fixation + clearing –Limited resolution in early stages (before organogenesis) –Requires more experience with image processing –Commercially unavailable (has to be custom built, *e.g.*, OPenT) — as of 2015

production develop early (Vasconcelos and Ladich, 2008; Alderks and Sisneros, 2011; Vasconcelos et al., 2015) the details on the ontogeny of the associated anatomical structures remain largely unknown. Those structures are too minute and delicate for micro dissection, and yet too large and deep inside the larvae to be accessible by conventional microscopy; furthermore, since some of the structures are cavities (*e.g.*, the contents of the otic capsule) they cannot be properly dissected out, and are best studied intact *in toto*. Having obtained a collection of *Halobatrachus didactylus* (the Lusitanian toadfish) embryos at several stages with sizes ranging from 3 to 30 mm in length, we took advantage of a classical tissue clearing technique and a custom-built "OPenT" - optical tomography scanner, based on the OpenSpinMicroscopy platform (Gualda et al., 2013), to gain insight into the anatomy and development of the stato-acoustic organs, swimming bladder and associated sonic muscles, and highlight the potential of optical tomography as a prime tool for developmental biologists.

2. Results & discussion

The *H. didactylus* embryos were first visible only between 10–12 days post-fertilization (dpf) as a 2.8–2.9 mm long streak with an engorged rostral end. After the second week, embryos reached >3 mm in length, and showed a neural tube, otic and optic vesicles, pectoral fin buds and overt segmentation of paraxial mesoderm (Fig. 1C), with 15–20 somites; none of the major organ systems were yet recognizable at this stage. As a way of comparison, this was merely equivalent to a zebrafish 17 h post-fertilization (Kimmel et al., 1995). Up to this stage, the embryos were too small and positioned far from the centre of the large yolk mass, to allow optimal imaging with optical tomography. They could be imaged with confocal microscopy (Fig. 1), but that required excising the embryo from the yolk sac and acquiring Z-stacks in multiple adjacent fields (followed by 3D-image stitching) using a 10× objective, otherwise the embryo's natural curvature did not provide access to the limited working distance of high-quality objectives; confocal imaging with low NA (*e.g.* 4× magnification) objectives did not provide images properly resolved in depth.

After the first two weeks, and throughout organogenesis, embryos could no longer be imaged with confocal microscopy and only OPenT provided images of the whole embryo and its internal anatomical details (Figs. 1, 2), with magnifications of 0.3–3× at the detector plane, a range of magnifications not available on conventional confocal microscopy setups. Large-scale (*i.e.*, low magnification) laser-scanning imaging with a "macro confocal" allowed imaging of a lateral field-of-view closer to that of OPenT, but with significantly limited axial field-of-view and resolution, when imaging 30 dpf embryos. Though the lateral resolution was high (at the embryo surface), the 3D dataset was highly anisotropic, showing low axial resolution and light penetration when compared to images obtained with OPenT (not shown).

After the first four weeks of development, all *H. didactylus* embryos had hatched and most organ systems were already visible. These larvae were developmentally equivalent to a 60 h pec-fin stage zebrafish (Kimmel et al., 1995), though almost 3× larger. Our observations of external anatomy were similar to those described for the oyster toadfish *Opsanus tau* by Tracy (1959) and Dovel (1960). Our use of OPenT allowed the reconstruction and analysis of both the external and internal 3D anatomy *in situ* of *H. didactylus*, without the need to dissect or section embryos or larvae, up until free-living forms 3 months old (>20 mm long; Fig. 1), often allowing identification of organs earlier than was detected using a dissecting stereoscope. This demonstrates that OPenT is a useful technique to study large embryos and larvae, allowing detailed morphological studies up to late stages, covering the full mesoscopic range.

Measurements obtained from 3D datasets, allowed us to follow the embryo's natural curvature and determine the full length of the body and head. The body grew progressively from the second week onward at a pace of 0.147 mm/day (~6 μm/h), slowing down at the end of the second month (Fig. 2C). This rate of growth is comparable to that previously reported for *O. tau* (Tracy, 1959), and considerably slower than that reported for *Danio rerio* which grows at a rate of 125 μm/h during embryogenesis and at 20 μm/h during larvagenesis (Kimmel et al., 1995). By the end of the second month, the yolk mass had been consumed (Fig. 1) and larvae begun feeding and swimming freely. Interestingly, the head of *H. didactylus* (measured as the distance from tip of mouth to level of pectoral fin bud) grew constantly throughout organogenesis and larvagenesis (Fig. 2C), which contrasts with zebrafish and medaka whose heads' length practically does not increase during organogenesis and early larvagenesis (as per figures in Kimmel et al., 1995; Iwamatsu, 2004). A disproportionately large head is a feature of most vertebrate embryos and early larvae, and is lost during larvagenesis as the body lengthens at a fast pace. Our observations suggest that the disproportionately large head patent in juvenile and adult Batrachoidids, may be a neotenic trait, instead of a morphological characteristic that develops secondarily.

Because of the interest of *H. didactylus* as a model organism for studies of communication we then focused our observations on the anatomical details of the stato-acoustic organs. We found that the semicircular canals (SCCs) + sacullae and swim bladder were well visible before the end of the first month (Fig. 2). Using OPenT 3D reconstructions we were able to identify and precisely measure these structures earlier than was possible using a stereoscope and dissection of fresh larvae. Before the