Expression of three novel opsins in the visual and nervous system of the box jellyfish *Tripedalia cystophora*

Anders Garm1, Jens-Erik Sværke1, Daniella Gurska1, and Todd Oakley2

1 Marine Biological section, University of Copenhagen, Denmark

2 Department of Biology, University of California Santa Barbara, USA

Corresponding author:

Anders Garm

Marine Biological Section

University of Copenhagen

Universitetsparken 4

2100 Copenhagen Ø

+45 51827004

[algarm@bio.ku.dk](mailto:algarm@bio.ku.dk)

Keywords: Photopigment, photoisomerase, Cubozoa, Cnidaria, vision, phototransduction

**Abstract**

**Introduction**

Photoreceptive organs, eyes and ocelli, have arisen many times in animal evolution and recent data suggest that this has even happened at least nine times within the cnidarian phylum alone (Picciani et al., 2018). Still, the existing data has shown that in most cases opsins are used as the photopigment initially harvesting the photons as a complex including a chromophore, which is most often retinal (Land and Nilsson, 2012). This has resulted in two major clades of opsins used as photopigments, rhabdomeric and ciliary opsins, with their expression following the morphology of the photoreceptors (Shichida and Matsuyama, 2009). Recently it has been shown that in rare cases another clade of opsins, xenopsins, are used as photopigments (Döring et al., 2020; Vöcking et al., 2017). Yet other opsins, Peropsins and RGR opsins, are not directly involved in light absorption but work as photoisomerases in vertebrate ciliary photoreceptors reactivating the opsin-retinal complex. In rhabdomeric photoceptors light absorption does not lead to full dissociation of retinal but to a metarhodopsin complex, which is reactivated through absorption of a second photon.

In cnidarians little experimental data are available about the details of the phototransduction, but molecular studies have found many of the putatively involved components (Koyanagi et al., 2008; Michaela et al., 2015; Picciani et al., 2018). Opsin genes have been identified in all examined cnidarian classes and especially in hydrozoans they are present in high numbers. In *Cladonema radiatum* 18 opsin genes were found but in Hydra vulgaris it was as many as 42 (Macias-Munez et al., 2019; Suga et al., 2008). These many opsins are expressed in basically all body parts but experimental evidence for their functional significance is lacking almost all cases. The known cnidarian opsins constitute three distinct clades called cniopsins, anthozoan opsins I and anthzoan opsins II, respectively (Vöcking et al., 2017). Interestingly, the three clades do not seem to be closely related to each other(Vöcking et al., 2017).

Within cnidarians cubomedusae, or box jellyfish, so far represent the only case of proper vision with image forming eyes, which have evolved independently of all other image forming eyes (Garm et al., 2011; Nilsson et al., 2005; Picciani et al., 2018). These image forming eyes, the upper and lower lens eyes, share many structural similarities with the vertebrate camera type eyes such as a hemisphere shaped retina made of ciliary photoreceptors, a single spherical lens at least partly focusing the light onto the retina and an adjustable pupil in the lower lens eye (Nilsson et al., 2005). Based on electrophysiological work, immunocytochemistry, micro spectrophotometometry, and *in situ* hybridization the two lens eyes also use opsins as photopigment (Bielecki et al., 2014; Ekström et al., 2008; Garm et al., 2007; Michaela et al., 2015; O'Connor et al., 2010). Even though the photoreceptors of all examined cubozoan photoreceptors are ciliary in structure they do not express ciliary opsins. Instead all their opsins belong to the clade cniopsins, so far only found in cnidarians . [Introduce the phylogeny of cnidops here].

Interestingly, all known species of cubomedusae have two eye types in addition to the lens eyes, the pit and slit eyes, where it still hasn’t been possible to identity the photopigments (Garm et al., 2008; Garm and Ekström, 2010). These eyes are structurally similar to the simple eyes found in many scyphomedusae and hydromedusae, and are putatively non-image forming, directional light meters. Still, no functional data exists from these eyes and the only evidence for them being photosensory comes from morphology.

A previous study on the eyes of the cubomedusae *Chiropsella bronzei*, hinted at other parts of phototransduction in Cubomedusae (O'Connor et al., 2010). Bleaching the photoreceptors in this species did not lead to the formation of a metarhodopsin, thus indicating that reactivation of the retinal-opsin complex happens through a photoisomerase as in vertebrate ciliary photoreceptors (Shichida and Matsuyama, 2009). The nature of this potential photoisomerase remains unknown, though.

In this study, we raised antibodies against three novel opsins recently discovered in transcriptomic data from the Caribbean cubozoan, *Tripedalia cystophora* (Nielsen et al., 2019). We used these antibodies to examine the expression pattern in polyps and medusae of *T. cystophora* but also in the hydromedusa, *Sarsia tubulosa*, and in the eye carrying rhopalia of the scyphomedusae *Aurelia aurita* and *Cassiopea xamachana*. Not surprisingly because of the distinctiveness of cnidarian opsins, antibodies raised to T. cystophora opsins were only found to be expressed in the medusae of *T. cystophora* and the expression patterns allowed us suggest specific functions for each of them.

**Materials and Methods**

Animal cultures

Polyps and juvenile (bell diameter (BD): 1.5 – 2.5 mm), sub-adult (BD: 3 – 5 mm) and adult (BD: 6-8 mm) medusae of *T. cystophora,* Conant 1897, were obtained from cultures at the Marine Biological Section, University of Copenhagen. They were cultured in 250 l tanks with recycled seawater on a 10:14 light-dark-cycle, a temperature of 28 - 29 ºC and a salinity of 36 – 37 psu. The cultures were fed Selco enriched *Artemia salina* nauplii daily.

Medusae of *Sarsia tubolosa,* Lesson 1843, *Aurelia aurita,* L. 1758, and *Cassiopea xamachana,* Péron & Lesueur 1809, were likewise obtained from cultures at University of Copenhagen. They were cultured in 150 l tanks with recycled sea water on a 10:14 light-dark-cycle. The temperature and salinity were kept 5-6 ºC and 24-26 psu for *S. tubolosa*, 10-11 ºC and 24-26 psu for *A. aurita*, and 24-25 ºC and 33-34 psu for *C. xamachana*. All cultures were fed Selco enriched *Artemia salina* nauplii daily.

Custom made antibodies against opsins

The opsins investigated in this paper were originally identified from transcriptomics of juvenile and adult medusae of *T. cystophora* (Nielsen et al., 2019). Specific antibodies against three novel opsin were produced in rabbits (Genosphere, Paris, France) using the following three amino-acid sequences:

*Opsin 1*: RPEQTSVSAPTTQAVTAANA

*Opsin 2*: ASGVQPEKENTNTVETTREP

*Opsin 3*: GLDESEIMPTEGQEPDGQPEIT

Immunostaining procedure

Each of the three antibodies were used for immune-stain in 3 polyps, and 12 juvenile, 4 sub-adult and 2 adult medusae of *T. cystophora* (Fig 1A). The adult medusae were dissected into quarters prior to the staining and their rhopalia were cut off and stained on their own (Fig. 1B). Each quarter contained a pedalium with tentacles and a pair of gonads. Medusae were starved for 24 hours prior to fixation/staining to prevent interference from their gut content. The polyps were likewise starved but for three days also to maximize their tentacle extension. To prevent the polyps from retracting their tentacles prior to fixation, they were anesthetized in 3.5% MgCl2 in seawater for 60 minutes prior to fixation. The polyps were kept in a dish containing approximately 20 ml seawater and then 20 ml of 7% MgCl2 were slowly (over 20 min) titrated into the dish.

The three opsin anti-bodies were additionally tested on 3 medusae of *S. tubolosa* (BD: 2 mm), 3 rhopalial niches of *A. aurita* (BD: 3 – 5 cm) and 3 rhopalial niches of *C. xamachana* (BD: 2 – 4 cm) each.

All the material was initially fixed in 0.1M phosphate buffered saline (PBS) with 4% paraformaldehyde and 5% sucrose for an hour on a rocking table at room temperature. Afterwards the tissue samples were washed 4 x 2 times with PBS buffer with triton X and bovine serum albumin (0.1M PBS with 0.1% TritonX and 0.5% BSA) after 0, 5, 15 and 60 min.

After the last wash the primary antibodies were added at a concentration off 1:1000 diluted in 0.1 M PBS with 0.1% TritonX and 0.5% BSA. The incubation with the primary antibodies lasted for 72 h at 5 ºC in darkness followed by a second series of washes with the same time schedule as the first series. Next, the secondary antibody was added at a concentration of 1:1000 diluted in 0.1M PBS with 0.1% TritonX and 0.5% BSA. This incubation lasted 24 h at 5 ºC in darkness. After the 24 hours the material was washed with pure 0.1M PBS following the schedule above and mounted on glass slides. The material was mounted in glycol using cover slips with wax “feet”, sealed with nail polish and stored at 5 ºC in darkness until scanned.

Controls preparations

To verify that the obtained staining with the three custom made anti-bodies was specific, negative controls and pre-absorption preparations were made. The pre-absorption experiments followed the general staining protocol described above with the exception that the primary antibody was mixed with the corresponding peptide for 60 min before it was included in the protocol. The peptide was diluted 1:500 by weight in 0.1M PBS. Three juvenile *T. custophora* medusae were used for each of the three antibody pre-absorption tests.

The negative control experiments were also following the general staining protocol with the exception that the primary antibodies were excluded from the protocol. The material used for the negative control experiments were three juvenile *T. custophora* medusae, three juvenile medusae of *S., tubulosa*, three rhopalial niches from *A. aurita,* and two rhopalial niches from *C. xamachana* for each of the three opsin antibodies*.*

Confocal laser scanning microscopy

The preparations were all scanned on a Leica SP2 microscope using 10X, 40X or 63X oil immersion objectives. The scan depth varied between 5 and 70 µm with a z-resolution between 0.2 and 1 µm. Both single slides and maximum projections from the scans are used in the illustrations, which were prepared in Corel Graphics Suit 2020. A standard argon-laser was used for both fluorescent and transmission scans.

Opsin phylogeny

**Results**

Opsin 1

The antibodies against opsin 1 gave a clear result in *T. cystophora*. There was no stain in the polyps but all stages of medusae displayed a bright stain in both slit eyes (SE) and no other places (Fig. 2). The stain was located inside the pigment screen in the area matching the outer segments of the photoreceptors (Fig. 2C-E). The retina was evenly stained indicating that antibodies had attached to all outer segments (Fig. 2C).

Opsin 2

The anti-bodies against opsin 2 also gave a clear result in *T. cystophora*. Like for the antibody against opsin 1 it stained the outer segments of the photoreceptors, but in all four eye types (Fig. 3). The stain against opsin 2 was not as strong as for opsin 1, especially the slit eyes were weakly stained (Fig. 3B,D,I). Interestingly, the staining pattern in the outer segments differed when compared to opsin 1 since the stain is not homogeneous but appears as lines. In the upper lens eye (ULE) the lines are 10-30 µm long and 2-4 µm wide in the adults and 5-15 µm long and 2-4 µm wide in the in juveniles. Most of the lines in ULE are more or less straight and oriented like the outer segments (Fig. 3G). In the lower lens eye (LLE) which has the thickest screening pigment the stain is harder see. Most are seen through the lens of LLE and appears as dots (Fig. 3F,G). The dots are 3-4 µm wide in both adults and juveniles. In the pit eyes (PE) the lines seem to have a random orientation crossing each other several time (Fig. 3H). The lines are hard to decipher in juvenile PE but in adults they are more easily seen and are approx. 0.5 µm wide. In the SE the stain is uneven with the lateral part of the retina being more stained (Fig. 3D). The lines are also randomly oriented here and 5-10 µm long and approx. 0.5 µm wide in the adult SE.

Opsin 3

The antibodies against opsin 3 did not provide as strong a staining pattern in *T. cystophora* as the two other antibodies. Weak stain was seen in two areas and only in the medusa stage. The one area is along the midline of the gonads in the area where the radial nerve is running (Fig. 4A,B). The stain was not found along the entire midline but appeared as approx. 20 elongated dots between 3 and 10 µm long. The other area is in the tentacles in between the cnidocytes (Fig. 4C,D). The stain again appears as dotted lines but here with dots smaller than 1 µm.

Control staining

The preabsorption tests with the anti-body against opsin 1 removed all staining of the SE (Fig. 5A, D). Similarly, the preabsorption tests with the antibody against opsin 2 also removed all staining in the rhopalia (Fig. 5B,E). The weak staining obtained with the antibody against opsin 3 was not seen in the tentacles (Fig. 5C, F) or in the gonads (not shown) in the preabsorption tests. Auto-fluorescent cnidocysts were seen in all three preabsorption tests (Fig 5).

The negative control staining without the primary antibodies displayed no stain in the rhopalia but several auto-fluorescent cnidocysts were again seen (Fig. 5G,H).

Stain in *A. aurita*, *C. xamachana* and *S. tubulosa*

None of the three opsin anti-bodies provided any specific stain in *A. aurita*, *C. xamachana* and *S. tubulosa* (Fig. 5). In *S. tubulosa* epithelial cells in the middle part of the manubrium did fluoresce in all three but this was auto-fluorescence. The same area also has fluorescence in the negative controls (not shown). Further, like for *T. cystophora* most of the cnidocysts were auto-fluorescent.

Opsin phylogeny

**Discussion**

We have examined the expression pattern of three novel opsin found in transcriptomic data from the box jellyfish *T. cystophora*. Using custom made antibodies against the three opsins we found that opsin 1 is highly expressed in the photoreceptor outer segments in the slit eyes, opsin 2 is expressed in the outer segments of the photoreceptors in all eyes, and opsin 3 is weakly expressed between the cnidocytes of the tentacles and along the midline of the gonads. The phylogenetic analysis places the three opsins….

Visual pigment of the slit eyes

Potential photoisomerase

Light guided control of gamet release/maturation and cnidocyte discharge

**Acknowledgements**

The authors appreciates the help maintaining the cultures offered by the members of Sensory Biology Group, University of Copenhagen. AG acknowledges the financial support from the Danish Research Council (DFF), grant#, and TO acknowledges grant # from XX.

**Figure legends**

**Figure 1.** The box jellyfish *T. cystophora*. A) Adult medusa of T. cystophora high lighting the paired gonads (Go) and tentacles (Te). B) Close up of a rhopalium showing the four eye types: upper and lower lens eye (ULE, LLE), slit eyes (SE) and pit eyes (PE). Cr = crystal, L = lens.

**Figure 2.** Expression of opsin 1 in *T. cystophora*. **A)** Overlay of stain with anti-body against opsin 1 (green) and transmission showing a rhopalium and parts of bell rim of a juvenile medusa. The retina of the slit eye (SE) is brightly stained. **B)** The area in A only showing the immune-stain. Note the complete absence of stain outside the retina of SE. **C)** Close up of SE from A. The entire retina is rather evenly stained. **D)** A juvenile rhopalium seen frontally showing identical staining in the two slit eyes. **E)** Close-up of left SE in D. Note that the stain is restricted to the photoreceptor outer segments and nor the cell bodies (encircles by broken white line) or the lens-like cells (LLC) are stained. F) Staining of the retina in an adult SE. LLE = lower lens eye, PE = pit eye, ULE = upper lens eye.

**Figure 3.** Expression of opsin 2 in *T. cystophora*. **A)** Overlay between transmission and anti-body stain of a juvenile rhopalium. Note that the retina of all eyes are stained. **B)** The area in A only showing the immune-stain. The slit eyes (SE) are only weakly stained (arrow). **C)** Close-up of pit eye (PE) from A. The stain is only seen inside the pigment screen (arrowhead) which is the outer segments of the photorecptors. **D)** Close-up of SE from A. The stain is again only seen in the outer segments (arrowhead) of the photorecptors inside the pigment screen. **E)** Close-up of the upper lens eye (ULE) from A. Note that the stain in the outer segments are not homogeneous but rather appear as individual lines. **F)** Close-up of the lower lens eye (LLE) from A. **G)** Immuno-stain of a juvenile rhopalium clearly showing the staining pattern as lines following the orientation of the segments in the ULE (insert, arrowhead). **H)** Stain in an adult PE also showing the expression of opsin 2 as individual lines (arrows). **I)** The immuno-stain is also weak in the adult SE (outlines by broken white line) and appears as randomly oriented lines (arrows). **J)** The immune-stain in the adult ULE is similar to the juvenile ULE (compare with G) except for the number of stained lines (arrows) being higher.

**Figure 4.** Expression of opsin 3 in *T. cystophora*. **A)** Punctuated staining is seen along the midline of the immature gonade (Go, framed by broken white line) in the area where the radial canal and redial nerve are also situated. **B)** Close up of framed area in A showing the punctuated staining (arrows). **C)** Lines of punctuated staining is also found between the autofluorescent nematocyst (Nc) on the tentacles (Te). **D)** Close up of framed area in C showing lines of punctuated staining (arrows) between the Nc. Note that the larger nematocysts are not autofluorescent (asterisks).

**Figure 5.** Control staining in *T. cystophora*. **A, D)** Overlay between transmission and staining and staining alone from preabsorption test with opsin 1 anti-bodies. Note the complete absence of staining in the rhopalium including the slit eye (Se). Broken white line in D encircles the rhopalium. Asterisks indicate autofluorescent nematocysts. **B, E)** Overlay between transmission and staining and staining alone from preabsorption test with opsin 2 anti-bodies. Note the complete absence of staining in any of the four eye types on the rhopalium (Outlined by broken white line in E). Asterisks indicate autofluorescent nematocysts. **C, F)** Overlay between transmission and staining and staining alone from preabsorption test with opsin 3 anti-bodies. Note the absence of punctuated staining between the autofluorescent nematocyst on the tentacle (Te) (insert). **G, H)** Negative control staining of juvenile medusa. Note the lack of staining in the rhopalium (Rho). Broken white line in H encircles the rhopalium. The only fluorescence comes from the autofluorecent nematocysts on the Te. LLE = lower lens eye, PE = pit eye, ULE = upper lens eye.

**Figure 6.** Staining with opsin 1 anti-body in other medusae. **A, D)** Overlay between transmission and staining and staining alone of a rhopalium (Rho) of *Aurelia aurita*. No staining is seen including in the aboral ocellus (Aoc) and the oral ocellus (Ooc). **B, E)** Overlay between transmission and staining and staining alone of a rhopalium of *Cassiopea xamachana*. No staining is seen including ocellus (Oc). **C)** Overlay between transmission and staining in the tentacular bulb of *Sarsia tubolosa*. No staining is seen including ocellus (Oc). F) A huge number of autofluorescent cells are found on the middle part of the manubrium (Ma, outlined by broken white line) of *S. tubolosa*. Cr = crystal, EsD = endosymbiontic dinoflagellates, Te = tentacle.

**Figure 7.** Phylogenetic analysis of the three opsins.

Reference list

**Bielecki, J., Zaharoff, A., Leung, N., Garm, A. and Oakley, T. H.** (2014). The cubozoan visual system utilizes several opsins. *PLoS ONE* **9**, 1-9.

**Döring, C. C., Kumer, S., Tumu, S. C., Kourtesis, I. and Hausen, H.** (2020). The visual pigment xenopsin is widespread in protostome eyes and impacts the view on eye evolution. *eLife* **9:e55193**, DOI: 10.7554/eLife.55193.

**Ekström, P., Garm, A., Pålsson, J., Vihtlec, T. and Nilsson, D. E.** (2008). Immunohistochemical evidence for several photosystems in box jellyfish using opsin-antibodies. *Cell and Tissue Research* **333**, 115-124.

**Garm, A., Anderson, F. and Nilsson, D. E.** (2008). Unique structure and optics of the lesser eyes of the box jellyfish *Tripedalia cystophora*. *Vision Research* **48**, 1061-1073.

**Garm, A., Coates, M. M., Seymour, J., Gad, R. and Nilsson, D. E.** (2007). The lens eyes of the box jellyfish *Tripedalia cystophora* and *Chiropsalmus sp.* are slow and color-blind. *Journal of Comparative Physiology A* **193**, 547-557.

**Garm, A. and Ekström, P.** (2010). Evidence for multiple photosystems in jellyfish. *International Review of Cell and Molecular Biology* **280**, 41-78.

**Garm, A., Oskarsson, M. and Nilsson, D. E.** (2011). Box jellyfish use terrestrial visual cues for navigation. *Current Biology* **21**, 798-803.

**Koyanagi, M., Takano, K., Tsukamoto, H., Ohtsu, K., Tokunaga, F. and Terakita, A.** (2008). Jellyfish vision starts with cAMP signaling mediated by opsin-Gs cascade. *Proceedings of the National Academy of Sciences U. S. A* **105**, 15576-15580.

**Land, M. F. and Nilsson, D. E.** (2012). Animal eyes. Oxford: Oxford University Press.

**Macias-Munez, A., Murad, R. and Mortazavi, A.** (2019). Molecular evolution and expression of opsin genes in *Hydra vulgaris*. *BMC genomics* **20**, 1-19.

**Michaela, L., Pergner, J., Kozmikova, I., Fabian, P., Pombinho, A. R., Strnad, H., Paces, J., Vlcek, C., Bartunek, P. and Kozmik, Z.** (2015). Cubozoan genome illuminates functional diversification of opsins and photoreceptor evolution. *Scientific Reports* **5**, 1-18.

**Nielsen, S. K. D., Koch, T. L., Wiisbye, S., Grimmelikhuijzen, C. J. P. and Garm, A.** (2019). Neuropeptide expression in the box jellyfish Tripedalia cystophora—New insights into the complexity of a “simple” nervous system. *Journal of Comparative Neurology* **529**, 2865-2882.

**Nilsson, D. E., Gislén, L., Coates, M. M., Skogh, C. and Garm, A.** (2005). Advanced optics in a jellyfish eye. *Nature* **435**, 201-205.

**O'Connor, M., Garm, A., Hart, N. S., Nilsson, D. E., Ekström, P., Skogh, C. and Marshall, J. N.** (2010). Visual pigments of the box jellyfish species *Chiropsella bronzie*. *Philosophical Transactions of the Royal Society London* **277**, 1843-1848.

**Picciani, N., Kerlin, J. R., Sierra, N., Swafford, A. J. M., Ramirez, M. D., Roberts, N. G., Cannon, J. T., Daly, M. and Oakley, T. H.** (2018). Prolific Origination of Eyes in Cnidaria With Co-Option of Non-Visual Opsins. *Current Biology* **28**, 2413-2419.

**Shichida, Y. and Matsuyama, T.** (2009). Evolution of opsins and phototransduction. *Philosophical Transactions of the Royal Society London* **364**, 2881-2895.

**Suga, H., Schmid, V. and Gehring, W. J.** (2008). Evolution and functional diversity of jellyfish opsins. *Current Biology* **18**, 51-55.

**Vöcking, O., Kourtesis, I., Tumu, S. C. and Hausen, H.** (2017). Co-expression of xenopsin and rhabdomeric opsin in photoreceptors bearing microvilli and cilia. *eLife* **6:e23435**.