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NEW FLUORESCENT PROTEINS FROM THE SEA

Thesis submitted for the degree of Doctor in Philosophy (Ph.D.) in
Life, Health and Chemical Sciences.

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September 2020

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ABSTRACT

The focus of this PhD project is to find a new fluorescent protein (FP) with spectral properties different from the ones already discovered, mainly in the red, far-red part of the spectrum. In this region, less light is scattered, absorbed and re-emitted by endogenous biomolecules, which makes it particularly interesting.

The green fluorescent protein (GFP) being the first FP isolated in the hydrozoa *Aequoria victoria*, many other GFPs were isolated in this clade as well as in Anthozoa, Arthropoda and Cephalochordata. Also, new FPs have been characterized in Vertebrata, namely UnaG and Sandercyanin, with features completely different from GFPs; UnaG being a fatty-acid-binding protein and Sandercyanin a lipocalin.

This work has been undertaken by several means; Long Term Ecological Research station (LTER-MareChiara) sampling in the gulf of Naples by assessing the epifluorescent and spectral properties of zooplanktons and thanks to bioinformatics tools exploring TARA database.

Many clades of zooplanktons have been examined throughout the PhD project with a focus on Annelid also with the sampling that was carried out on the seaside. The spectral properties of the zooplanktons were assessed scrupulously by confocal microscopy, as a test response to chlorophyll was able to rule out positive organisms and a test to UV excitation and visible light spectra allowed identifying species with red, far-red fluorescent emission patterns.

The exploration of available transcriptomes, genomes and TARA eukaryotic database by blastp search has been performed using different classes of reference proteins UnaG, Sandercyanin and 14 GFPs from four different groups. No similarity from the protein alignments were found with UnaG and Sandercyanin whereas the alignments resulted in the findings of GFP chromophores in 25 hits sequences from which we produced a phylogenetic tree.

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First, I would like to thank the Stazione Zoologica Anton Dohrn and the Open University for these three years of research. Many persons provided me with support to complete this thesis, and without their help this project would not have been possible.

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Particular thanks to my friends and lab colleagues for their brightness, support and warmth company. I want to thank particularly Dr Danila Voronov and Dr Elijah Lowe for their help during the thesis.

A very important and supportive person during the PhD thesis was my colleague, friend and roommate Dr Solenn Mordret. Her attentive listening, help and faith are qualities that I am and always will be very thankful to her. Without her support, I would have never been able to complete the PhD.

A big thank you to the MareChiara team for providing plankton samples every week, without which this work would not have been possible. I want also to stress the important contribution of our collaborator Giovanni Gragnaniello for his precious comments, availability, suggestions and assistance in microscopy.

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I wish to dedicate this work to my family especially to my mum for her love, patience and immense encouragement.

Finirò con la frase di Luciano De Crescenzo *“Napoli per me non è la città di Napoli, ma solo una componente dell’animo umano che so di poter trovare in tutte le persone. Che siano esse napoletane o no”*. Living in Naples is a unique, fulfilling, life changing experience that I will always remember.

GLOSSARY

Term	Definition
Aequorin	Calcium-activated photoprotein complex responsible for luminescence in the jellyfish <i>Aequorea victoria</i> .
Bioluminescence	Light generated by an enzymatic reaction (luciferase) within a living organism.
Carotenoids	Yellow, orange and red organic pigments produced by plants, algae, bacteria and fungi.
Chlorophyll	Green organic pigment present in plants and in cyanobacteria, which is responsible for light absorption during photosynthesis and dissipates its energy by emission as fluorescence radiation.
Chromatophore	Pigment-containing cell in the superficial skin tissue layer of an animal.
Exitance	Totality of light leaving the surface expressed in energy or photon flux units.
Fluorescence	Reflection of light at a longer-wavelength, in other words it is the absorption of light (excitation) followed by the release of a part of the absorbed energy at longer-wavelength (emission).
Fluorophore/Chromophore	Part of a molecule or chemical group composed of an atom or a group of atoms responsible for the colour emitted by a fluorescent protein.
Green fluorescent protein (GFP)	Protein able to emit green fluorescence in the presence of UV light discovered in <i>Aequorea victoria</i> .
Holoplankton	Group of organisms spending all stages of their life cycle as plankton drifting freely in the water.
Light absorption	Totality of light leaving the surface expressed in energy or photon flux units.
Light scattering	Phenomenon occurring when a ray of light strikes a surface and changes its direction
Meroplankton	Wide variety of planktonic organisms spending a portion of their lives in the benthic region of the ocean.
Mesozooplankton	Zooplankton measuring from 0.2 to 20 millimetres.
Photoactivatable fluorescent proteins (PAFPs)	Class of FP capable of acute changes in their spectral properties upon irradiation with light of a specific wavelength and intensity.
Photocyte	Gland specialized in the production of luminescent light.
Pigment	Coloured chemical substance found in animals or plants capable of changing colour after reflection and absorption of part of the visible light.

Polyphenism	Phenomenon where two or more distinct phenotypes are produced by the same genotype.
Quenching	Process of stopping a chemical or enzymatic reaction.
Reflectance	Fraction of photons reflected at each wavelength.
Sandercyanin	Lipocalin family protein, isolated from the freshwater fish <i>Sander vitreus</i> , able to bind to biliverdin IX α displaying blue colour naturally, or red fluorescence under UV radiation.
UnaG	Fatty acid binding protein (FABP), isolated from the marine eel <i>Anguilla japonica</i> able to bind endogenous bilirubin triggering green fluorescence.

LIST OF ABBREVIATIONS

ApoD: apolipoprotein D
ATP: Adenosine Triphosphate
avGFP: *Aequorea victoria* GFP
BLA: Biliverdin
BLAST: Basic Local Alignment Search Tool
BR: Bilirubin
FABP: Fatty Acid Binding Proteins
DNA: Deoxyribonucleic acid
eGFP: enhanced GFP
eUnaG: enhanced GFP
FMN: Flavin MonoNucleotide
FP: Fluorescent Protein
FSW: Fresh Sea Water
G2F: Globular fragment two
GFP: Green Fluorescent Protein
LTER: Long Term Ecological Research station
MC: MareChiara
MgCl₂: Magnesium Chloride
PAFP: Photoactivable FPs
NIR: Near InfraRed
ROI: Region Of Interest
ROS: Reactive Oxygen Species
UV: Ultra Violet
λ: Wavelength

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CHAPTER 1 - GENERAL INTRODUCTION

1. Light in the ocean, bioluminescence and fluorescence

In a clear ocean, blue light of wavelength around 480 nm predominates, although other biologically relevant shorter and longer wavelengths are present until around 75 meters (see Figure 1.1, middle panel). Only a small fraction of daylight penetrates the ocean's depth, becoming progressively dimmer before switching to a uniform blue spectrum light (470-490 nm). In fact, the orange-red light get absorbed by 15 meters and the ultraviolet light by 30 meters, see Figure 1.1 middle panel (Marshall, 2017). In coastal waters, freshwater creeks and lakes, other wavelengths of light may be absorbed because of the presence of sediments, phytoplankton and dissolved organic material. Therefore these waters appear more yellow-green, orange or brown in colour (Warrant and Johnsen, 2013) (see Figure 1.1, bottom panel).

Water has a profound effect on the transmission of light compared to the air. Water has a strong ability to absorb light, whereas associated organic particulate matter such as phytoplankton and dissolved materials have a high potential of scattering the light thus decreasing the intensity of downwelling irradiance with depth (Warrant and Johnsen, 2013).

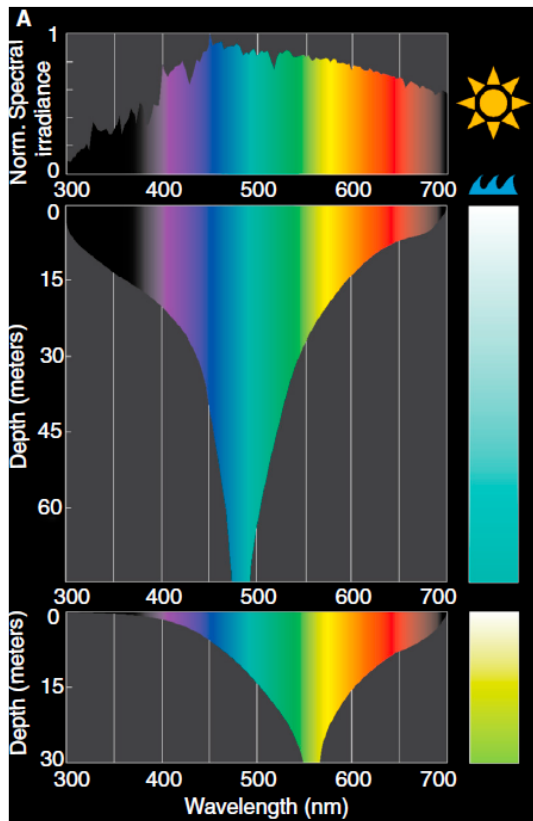


Figure 1.1: Light penetrance in the ocean (Marshall, 2017). Upper panel, coloured-in spectrum of light under an irradiance spectrum curve above water, full sunlight. Middle panel, attenuation of light with depth in clear oceanic water down to 75 m. Bottom panel, attenuation of light in green, chlorophyll and dissolved organic matter-laden coastal waters where maximal transmission is around 550 nm.

The two main light phenomena prevailing in the ocean are biofluorescence and bioluminescence. These two distinct processes can coexist, i.e in the jellyfish *Aequorea victoria* where both processes are present.

Bioluminescence is, along with the sun, one of the two major sources of light on the planet. In fact, it is the primary source of biogenic emission of light in the ocean from the epipelagic to the abyss zone, and from the poles to the equator, as over 80% of the light-producing genera are marine (Haddock. *et al.*, 2010; Johnsen, 2012). By definition, bioluminescence is the emission of visible light by an organism resulting from an endogenous chemical reaction consisting of the

oxidation of luciferin (Haddock et al., 2010). It is mediated by an enzyme, known as a “luciferase,” and generally requires energy via ATP. Instead, photoproteins - which are the primary substrates of the light-emitting reactions of various bioluminescent organisms - does not require luciferase enzyme activity (Shimomura, 1985). In fact, photoproteins in diverse phyla relay on Ca^{2+} or superoxide radicals and O_2 to trigger bioluminescence. In many fish and a few cephalopods, the light is produced by symbiotic bacteria, the animal can also produce it itself inside cells called “photocytes” (see glossary) (Johnsen, 2012). These cells are often part of complex organs that contain filters, mirrors, lenses, and other apparatus for controlling the colour and direction of the light.

Fluorescence, instead, is a phenomenon dependent on external light by which an organism absorbs it and emits it as a different wavelength with the remaining energy emitted as a photon. Natural fluorescence is a feature deriving not only from fluorescence emitting proteins within the organisms but also from organic pigments, such as chlorophyll, carotenoids, or rock minerals that are able to emit light at similar wavelengths (see glossary) (Johnsen, 2012). Chromophore is a key structure present in fluorescent protein responsible of the conversion of light to a given wavelength (see glossary).

The technical distinction between bioluminescence and fluorescence is sometimes as tricky as looking for a needle in a haystack. Indeed, some bioluminescent animals such as hydrozoans (medusa, siphonophores) and arthropods do use fluorescence to change the wavelengths of light that are emitted, but the fluorescence emission spectrum of a molecule can match its luminescence emission spectrum (Johnsen, 2012; Stabili *et al.*, 2008). Especially in aphotic habitats, biofluorescence and bioluminescence coexist and interact within the same organism, with the latter phenomenon acting as light energy source for biofluorescence. By virtue of this coexistence, photocytes often convert their naturally blue luminescent light into green by using GFP or, like in hydrozoans and arthropods, to enlarge the color range of emitted light (Lewis, 2012; Shagin *et al.*, 2004).

2. The discovery and isolation of GFP

The first fluorescent protein to be discovered was the green fluorescent protein (GFP) isolated from the hydrozoan medusa *A. victoria* (avGFP) and characterized for its ability to release fluorescent photons after absorbing electromagnetic energy (Shimomura *et al.*, 1962). This jellyfish is shaped like a hemispherical umbrella, measuring 7–10 cm in diameter and present both bioluminescent and biofluorescent organs, see Figure 1.2.

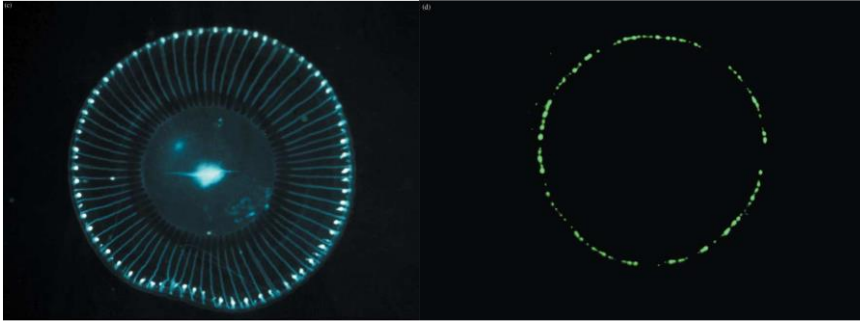


Figure 1.2: Top view of a specimen of *Aequorea victoria* in daylight at sea (left), bioluminescence and when stimulated in a darkroom (right), biofluorescence (Shimomura, 2009).

Nobel-awarded discoverers of GFP Osamu Shimomura, Martin Chalfie and Roger Y. Tsien identified aequorin as the luminescent protein responsible of storing energy and releasing it in presence of Ca^{2+} (Shimomura, 1979). During its purification, another protein present in small amount exhibited a bright green fluorescence. The biochemical characterization of aequorin showed that Ca^{2+} ion binding triggers the emission of blue light at 470 nm, in turn prompting an energy transfer to GFP which emits light at 508 nm (Figure 1.3) (Shimomura, 1979; Shimomura *et al.*, 1962).

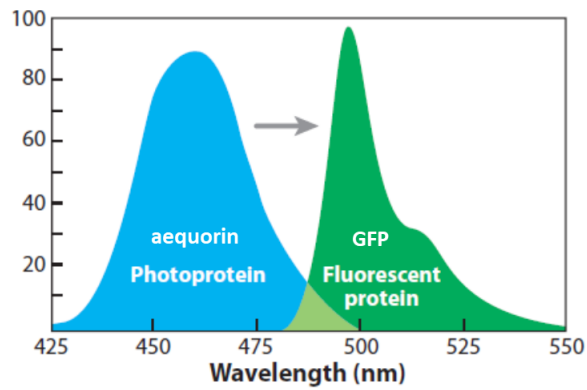


Figure 1.3: Light emission of the hydromedusa *Aequorea victoria*, the photoprotein aequorin and the fluorescent protein GFP (Haddock et al., 2010).

avGFP consists of a single polypeptide chain of 238 amino acids in length and does not require a cofactor or external substrates to function (Hink *et al.*, 2000). Therefore, this allows easily the monitoring of gene expression and protein tags in living organisms. The chromophore, located at the core of the protein, is responsible of the fluorescence. avGFP and GFPs are unique in the way that they genetically encode and catalyse their own chromophores without the assistance of cofactors or enzymes, as in bioluminescence (Hunt, 2013). The amino acids responsible of the autocatalyzation in avGFP are situated at positions 65–67 and are always corresponding to an XZG tripeptide, where X is a variable amino acid residue, Z is always an aromatic amino acid (tyrosine in naturally occurring proteins), and G corresponds to glycine (Dedecker *et al.*, 2013). The residue R96, has many role from stability and folding, to structural and electrostatic roles in chromophore maturation (Craggs, 2009). This process groups a series of protein folding, peptide cyclisation, equilibration and organization of the structure until the formation of a neutral chromophore (Craggs, 2009). Another important amino acid, E222 is involved in proton transfer reactions during the maturation process reaction (Sniegowski *et al.*, 2005) (see Figure 1.4).

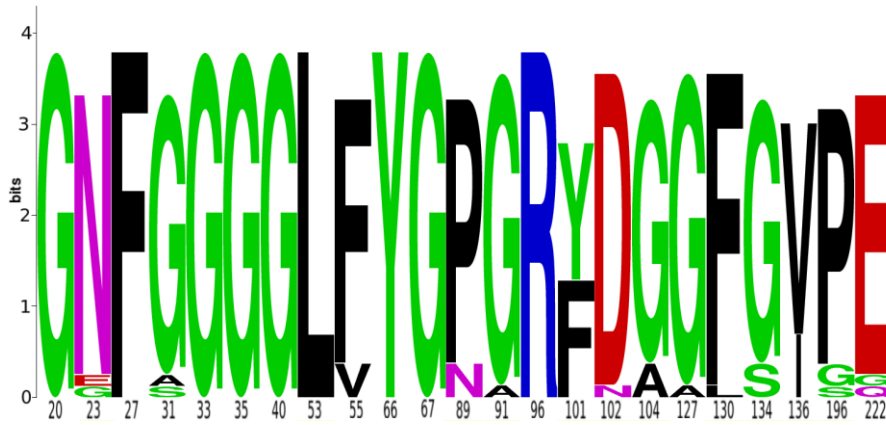


Figure 1.4a: A weblogo representation of amino acid diversity among the most conserved residues of the wild-type GFP structures (Li, 2013). Residues are numbered relative to avGFP. A high bit score (y-axis) on the logo plot reflects invariant residues.

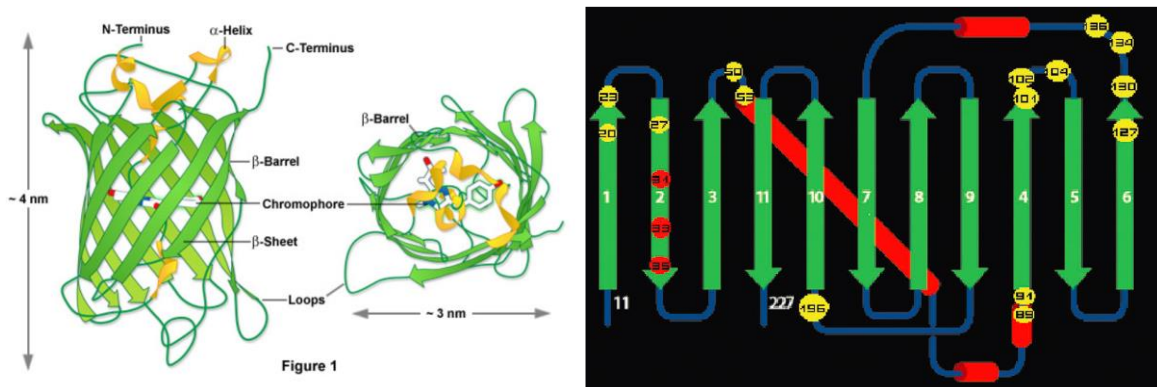


Figure 1.4b: The architecture of avGFP depicting the chromophore at the core of the crystal structure (left) and the location conserved amino-acids on β -sheet (Li et al., 2013).

3. GFP and fluorescence in the tree of life

3.1 GFP and G2F domain

Proteins sharing a GFP fold (β -barrel) constitutes a superfamily comprising two protein families, the first with GFPs and non-fluorescent chromoproteins characterized by the autocatalization of the chromophore from the GFP sequence and responsible of the colour of the protein (Chudakov *et al.*, 2010). The second family is composed of nidogens and fibulins that contains the G2F

fragment proteins ('globular fragment two') which are extracellular matrix proteins found in a variety of metazoans (Matz *et al.*, 2005; Shagin *et al.*, 2004). The β -barrels structures of nidogen G2 and avGFP are very similar (Hopf *et al.*, 2001), although the β -sheet of nidogen G2 does not form a chromophore and is neither coloured nor fluorescent (see Figure 1.5a).

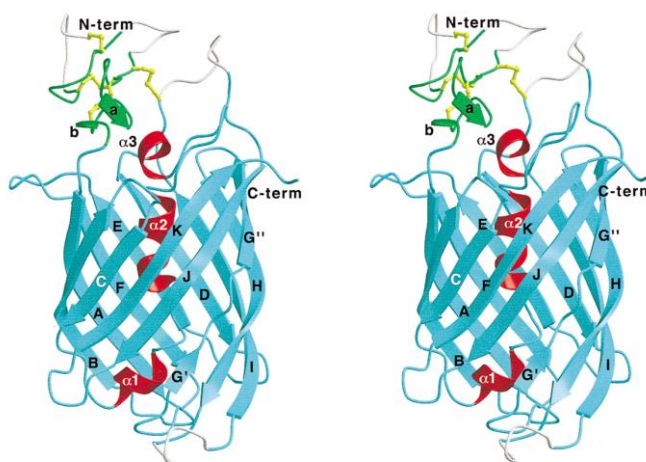


Figure 1.5a: The nidogen G2 structure. Stereo view of the structure with the EGF-like domain in green and the β -barrel domain in cyan. β -strands are labelled a–b in the EGF-like domain and A–K in the β -barrel domain. The α -helices 1–3 are in red and disulphide bridges in yellow (Hopf *et al.*, 2001).

Instead, the β -barrels share a common function, as several β -strands in G2Fs serves as a protein-binding module in the formation of the extracellular matrix during development (Shagin *et al.*, 2004) and in GFPs, they are found on the outside of the β -can, that is still free to bind other proteins (Hopf *et al.*, 2001; Wall *et al.*, 2000).

As stated in the previous paragraph, the fluorescence of GFP results from the autocatalytic conversion of the chromophore Ser65–Tyr66–Gly67 and the equivalent residues in nidogen G2, Ile460–Gly461–Gly462 are in helix α 2 (see red square on Figure 1.5b).

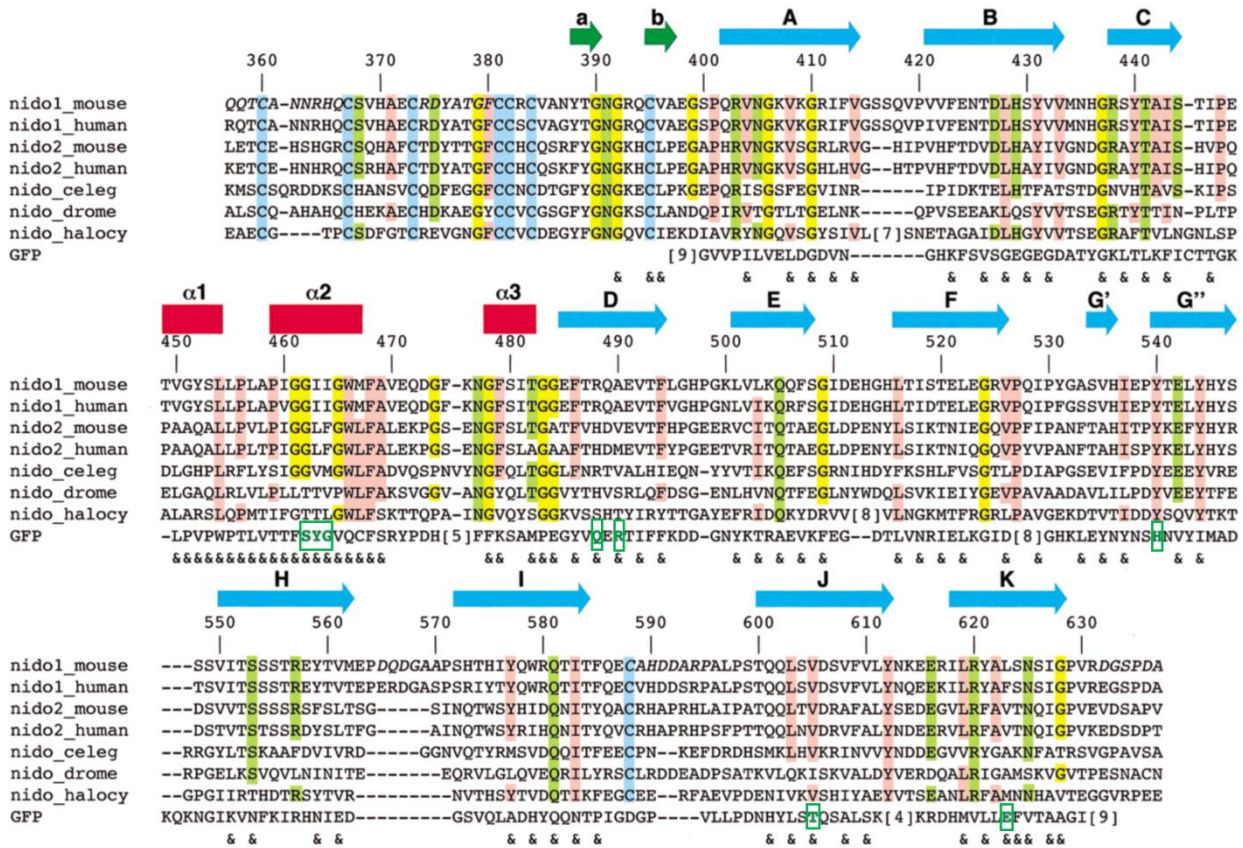


Figure 1.5b: Alignment of nidogen G2 sequences and *Aequorea victoria* GFP sequence. Mouse nidogen-1 (SwissProt P10493) and nidogen-2 (Q14112), Human nidogen-1 (P14543) and nidogen-2 (O88322), *Caenorhabditis elegans* nidogen (Q93791), *Drosophila melanogaster* nidogen (CG12908) and *Halocynthia roretzi* nidogen (S31213). In the GFP sequence, residues forming the chromophore (Ser65–Gly67) and those directly contacting it (Gln94, Arg96, His148, Thr203 and Asp222) are in green box (Hopf *et al.*, 2001).

Despite the sequence similarity of only 10%, some researchers believe that the nidogen G2 β -barrel and the GFP protein family derive from a common ancestor (Hopf *et al.*, 2001) while others argue the lack of distinct sequence similarity between them (Baumann *et al.*, 2008).

3.2 GFP in the tree of life

After the discovery of GFP in *A. victoria*, no other GFP were isolated from Cnidaria until the early 2000, where the first GFPs from Anthozoans (sea anemones and corals) were discovered. More than two decades ago, the DsRed protein was the first red fluorescent protein discovered in the non-bioluminescent reef coral genus *Discosoma* (Matz *et al.*, 1999). DsRed was widely distributed commercially until its chromophore underwent random and site-specific mutagenesis to convert it into at least seven green protein mutant variants (Baird *et al.*, 2000; Chudakov *et al.*, 2010). Thereafter, many GFP were isolated in anthozoans and in the early 2000, the first GFP were characterized in copepods (Arthropoda) from the species *Pontellina plumata*, *Labidocera aestiva*, and *Pontella meadi* (Shagin *et al.*, 2004). At that time, the first phylogeny distribution of GFP has been drawn to underline GFP and G2FP gene lineages and indicating colour diversification events (see Figure 1.6).

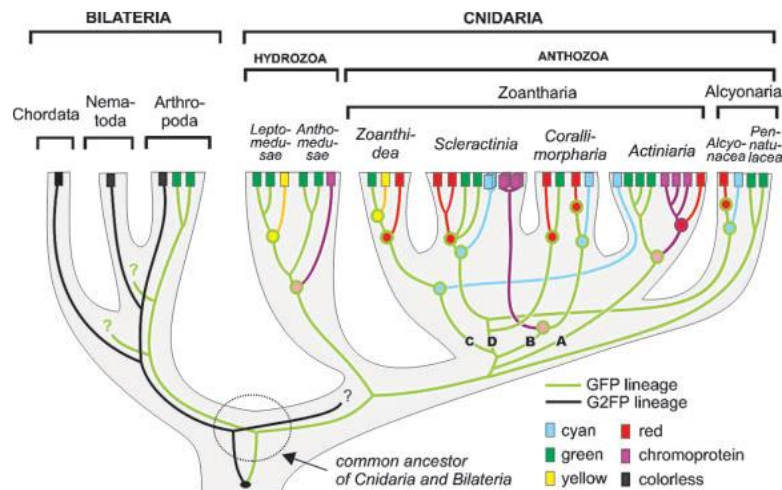


Figure 1.6: Phylogeny of GFP, overlaid upon the general taxonomy of the organisms to illustrate the extent of gene lineage sorting and inferred colour diversification events. The latter are shown as circles, with the border corresponding to the parent colour and the centre to the originating colour. Four major Anthozoan clades (A, B, C and D) are denoted. Gene lineages ending with question marks indicate that more extensive sampling of the genes in these taxa is required to draw a conclusion (Shagin *et al.*, 2004).

Since then, many GFP and other type of fluorescent proteins were identified, making this distribution outdated. In fact, in 2007 the first GFP was isolated in three amphioxus species (cephalochordate), namely *Branchiostoma floridae*, *Branchiostoma lanceolatum* and *Branchiostoma belcheri* (Deheyn *et al.*, 2007). Later two GFP encoding genes were found in the adult and larval species *Asymetron lucayanum* that are closely related to some of the Branchiostoma GFPs. Furthermore, the observation of tandem duplications events in both *B. floridae* and *B. belcheri* lead to an expansion of 13 GFP encoding genes.

Therefore, the genera *Asymmetron* and *Branchiostoma* share homologous GFP-encoding genes in which the acquisition in *Branchiostoma* was probably present in the ancestral cephalochordates.

Canonical GFP orthologues have evolved by speciation and is present exclusively in the phyla Cnidaria, Arthropoda and Chordata, suggesting the presence of GFP in the last common ancestor of all metazoans. Recently, GFP encoding genes have been found in transcriptomes of 30 ctenophores, which are relevant for their early divergent phylogenetic position (Francis *et al.*, 2016). Although initially one of them was described as a fluorescent protein (FP) (Haddock *et al.*, 2010), a deeper study revealed substitution in canonical residues involved in chromophore formation indicating that they are unlikely to possess any fluorescence features (Hunt *et al.*, 2012). Extensive searches in well-annotated transcriptomes and genomes of sponges, nematodes, annelids, molluscs, echinoderms, and hemichordates failed to find orthologs of canonical GFPs. This points to independent gene loss events occurred in the evolutionary history of several animal clades or, with less probability, they highly diverged from the ancestral gene. An alternative evolutionary scenario capable of explaining the scattered presence of GFPs in the animal tree implies the occurrence of independent events of horizontal gene transfer, probably through diet, a genomic mechanism that needs further investigation (Deheyn *et al.*, 2007). Two important evolutionary events seem to have taken place in the clade of chordates: the loss of GFP representatives in Olfactores (comprising tunicates and vertebrates) and, on the contrary, the extraordinary gene expansion recently detected in cephalochordates (Figure 1.6). In fact, 21

expressed GFPs have been identified in the amphioxus *B. lanceolatum*, although the significance of such extensive number of GFPs still needs functional clarification (Baumann *et al.*, 2008; Yue *et al.*, 2016). Therefore, GFPs seem to have a common bilaterian ancestor as supported by structural and sequence similarity from different phyla as reported in several phylogenetic studies (Alieva *et al.*, 2008; Labas *et al.*, 2002; Li *et al.*, 2009; Matz *et al.*, 2002).

Recently, new FP families have been characterized in vertebrates bearing different features in comparison with canonical GFP. For instance, a blue fluorescent protein named Sandercyanin has been isolated almost a decade ago in a fish and thereafter UnaG at the origin of green fluorescence in freshwater eels. These two proteins both need a cofactor for fluorescence to be activated, although they have different evolutionary origins. These exciting new discoveries raise the importance of having a new evolutionary version of fluorescence distribution in metazoans to point out the diversity of fluorescent sources in nature (Figure 1.7).

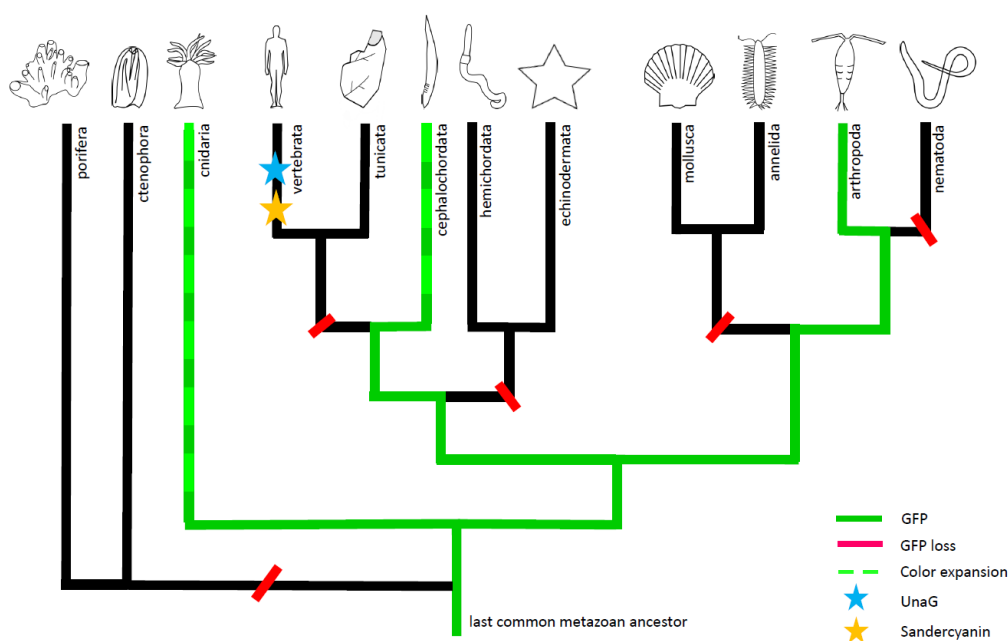


Figure 1.7: Distribution of fluorescent proteins in metazoan (Macel *et al.*, 2020). Canonical GFPs have been found in cnidarian, arthropods and cephalochordates, supporting the hypothesis of a common metazoan ancestral origin. Colour expansion is present in cnidarians and has been recently showed in cephalochordates as well. Furthermore, two other FPs, not related to GFP, have been characterized in vertebrates: UnaG and Sandercyanin.

4. Discovery of new fluorescent proteins

4.1 UnaG

The protein UnaG (unagi means eel in Japanese) has been isolated from the Japanese freshwater eel *Anguilla japonica*. This species is extensively used in aquaculture and is a historically dietary standard of Japanese cuisine. This fish travel thousands of kilometres into the ocean to breed in the Philippine Sea, and the larvae migrate back in freshwater habitats over several months (Tsukamoto, 2006). This long-distance migration cycle would require the eels to possess an efficient musculoskeletal apparatus, particularly to maintain the steady-state muscle homeostasis and anaerobic oxidative metabolism during continuous swimming. However, very little is known about the mechanisms of muscle metabolic physiology during travel in eels. An interesting idea emerged in 2009, where muscle fibres from unagi were reported to be fluorescent (see Figure 1.8A). The eels skeleton system physiology could be better understood with the identification of the molecular process behind the muscle fluorescence (Hayashi and Toda, 2009). A few years later, extensive search for the putative FP was conducted; UnaG was characterized as a considerable smaller size (~16 kDa) compared to GFP (26.9 kDa) and do not have intrinsic fluorescence (Kumagai *et al.*, 2013).

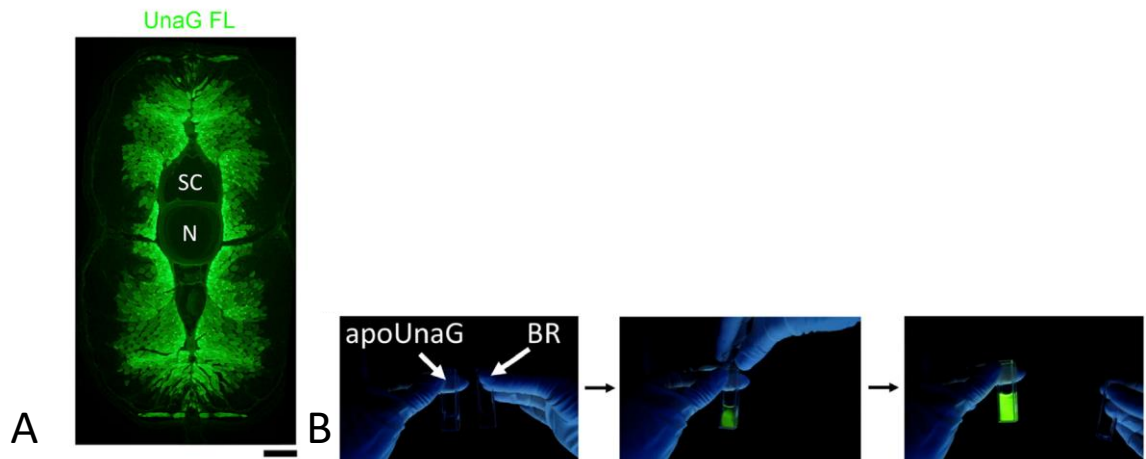


Figure 1.8: Bilirubin-Inducible Fluorescence of UnaG (Kumagai *et al.*, 2013). A) Fluorescence image of a transverse section of a formalin-fixed eel, UnaG expression in juvenile Japanese Eel. N, notochord; SC, spinal cord. Scale bars, 200 μ m. B) (fluorescence development) of holoUnaG from apoUnaG and bilirubin (BR).

To reconstitute the fluorescence development of the conjugated UnaG, researchers tested several serums of different animals until they focused on the most effective one, fetal bovine serum. After further fractions of the serum they were able to identify bilirubin as the cofactor responsible of the fluorescence (see Figure 1.8B). The crystallographic structure of UnaG demonstrated that bilirubin, the fluorogenic chromophore, does not require oxygen for its autocatalyzation unlike most GFP (see Figure 1.9). Bilirubin is a major lipophilic heme metabolite, a common marker for liver function and a key indicator of the widespread childhood diseases of jaundice and kernicterus (Kumagai *et al.*, 2013).

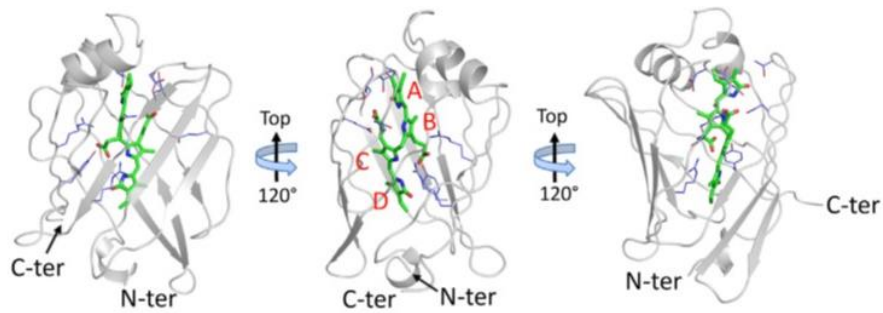


Figure 1.9: Crystal Structure of UnaG in Complex with Bilirubin (Kumagai *et al.*, 2013). Overall structure of holoUnaG viewed from three different angles. The protein secondary structure is shown in gray. Selected protein side chains are shown as sticks (with carbon atoms colored gray). N-ter, amino terminus; C-ter, carboxyl terminus.

These interesting properties make it the first protein whose fluorescence must be activated by a natural ligand. Spectral characterization of UnaG shows that it triggers bright green fluorescence (λ_{exc} 498nm, λ_{ems} 527nm) through the coupling of bilirubin with fluorescence greater than that of GFP. This protein does not autocatalytically create its chromophore from amino acid side chains and is oxygen dependent. The UnaG protein is part of the fatty-acid-binding protein family (FABP) with the highest homology (56%) to brain FABP (Kumagai *et al.*, 2013).

Among the many applications offered by UnaG compared to common GFPs, UnaG may shed light on the mechanistic functions of bilirubin in the body and can be used as a tool for the investigation of skeletal muscle physiology and metabolism during endurance exercise. Also, UnaG may shed light onto the mechanistic functions of bilirubin in the body and may thus have medical implications.

In parallel, two other fluorescent FABPs have been isolated from the false moray eel (*Kaupichthys hyoproroides*), originated from a gene duplication event (Gruber *et al.*, 2015) and named Chlopsid FP I and II. Fluorescent FABPs possess a unique tri-peptide sequence motif (Gly-Pro-Pro) that is

not present in non-fluorescent FABPs. In respect to UnaG, fluorescence spectroscopy of Chlopsid FP I and Chlopsid FP II exhibit blue shifted emission spectra.

4.2 Sandercyanin

The walleye (*Sander vitreus*) is a golden fish that inhabits the Northern American lakes. Some yellow and blue-pigmented cells have been reported, although their nature has not been reported. It is in 2005 that several walleye with blue mucus were analysed, from five lakes in central Quebec, Canada (Yu *et al.*, 2008) and a blue protein called Sandercyanin was isolated (Figure 1.10).

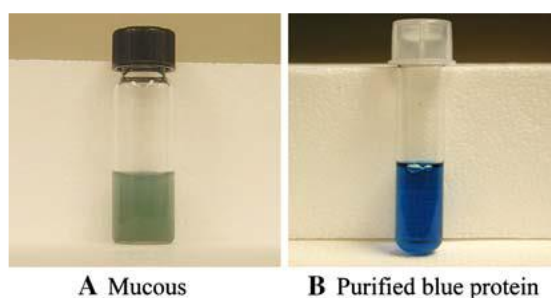


Figure 1.10: Mucous of blue walleye before and after purification (Yu *et al.*, 2008). (A) Mucus from blue walleye forms prior to purification. (B) Solution of purified blue protein obtained after hydroxyapatite chromatography.

Further complementary research showed that Sandercyanin is the smallest FP (18.6-kDa) with endogenous non-covalent ligand-inducible far-red fluorescence (Ghosh *et al.*, 2016). In fact, this protein has the largest spectral shifts observed to date, with blue absorption and far-red emission under UV radiation (λ_{exc} 375 nm, λ_{ems} 675 nm) (see Figure 1.11). Sandercyanin is a complex of a protein and the chromophore biliverdin IX α (BLA), a breakdown product of heme by UV radiation, which is non-covalently bound to the protein.

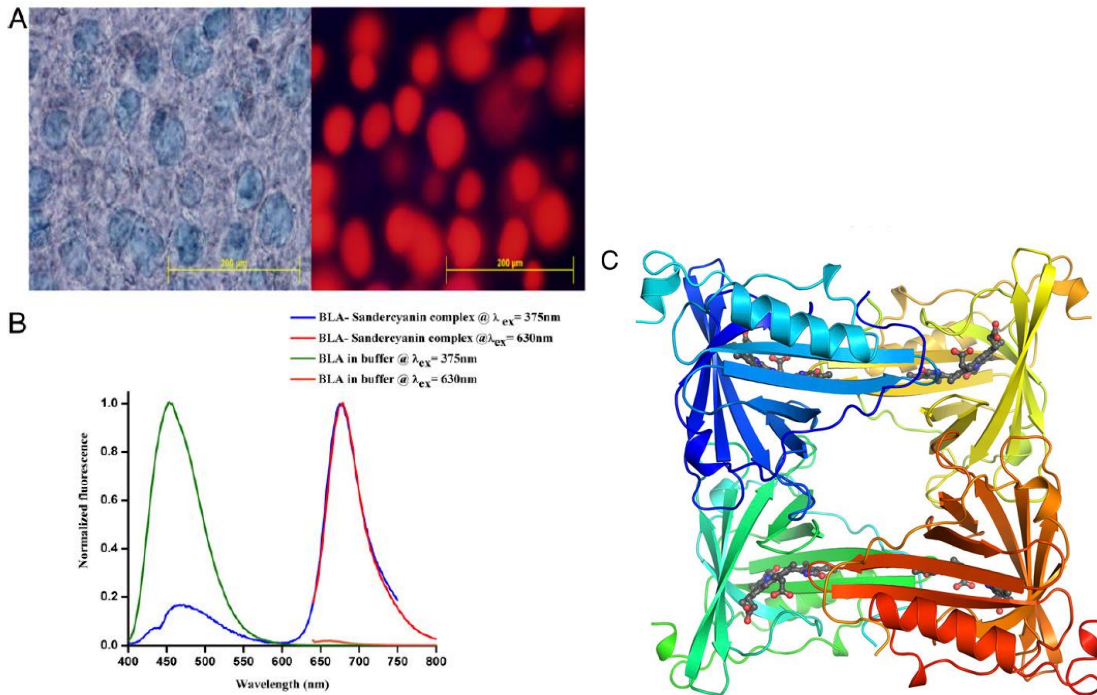


Figure 1.11: Structure and properties of the native Sandercyanin purified from walleye (Ghosh *et al.*, 2016) (A) Mucus from North American walleye (*Sander vitreus*) appear blue under bright field and shows intense red fluorescence on excitation at 375 nm. (B) Fluorescence spectra of BLA and native BLA–Sandercyanin complex on excitation at 375 or 630 nm. Unbound BLA (blue peak at 450 nm) appears as a small hump in the BLA-bound complex. (C) Cartoon representation of tetrameric structure of BLA-bound Sandercyanin. Each subunit of the tetramer binds to one BLA molecule (represented in ball-and-stick form).

The resulting protein sequence of Sandercyanin suggests that it belongs to the lipocalins family and show similarities with apolipoproteins of several species (zebrafish, guinea pig, mouse and human) (Yu *et al.*, 2008). So far, Sandercyanin is the first biliverdin-binding lipocalin showing fluorescence properties.

4.3 Fluorescence from metabolites

Scattered green fluorescence distribution has been denoted on the skin of elasmobranchs (Chordates), in both the family Scyliorhinidae, with *Cephaloscyllium ventriosum* (the swell shark) and *Scyliorhinus retifer* (the chain catshark). This high intensity fluorescence is located particularly in the lighter, beige-coloured areas, compared with the darker reticulated lines of the chain catshark or the dark spots of the swell shark (see Figure 1.12) (Gruber *et al.*, 2016).

A few years later, the explanatory chemical mechanism of biofluorescence behind these observations has been identified. High resolution metabolic structural studies of these two distinctly pigmented tissues were able to lead to the discovery small brominated tryptophan-kynurenine metabolic products (Park *et al.*, 2019).

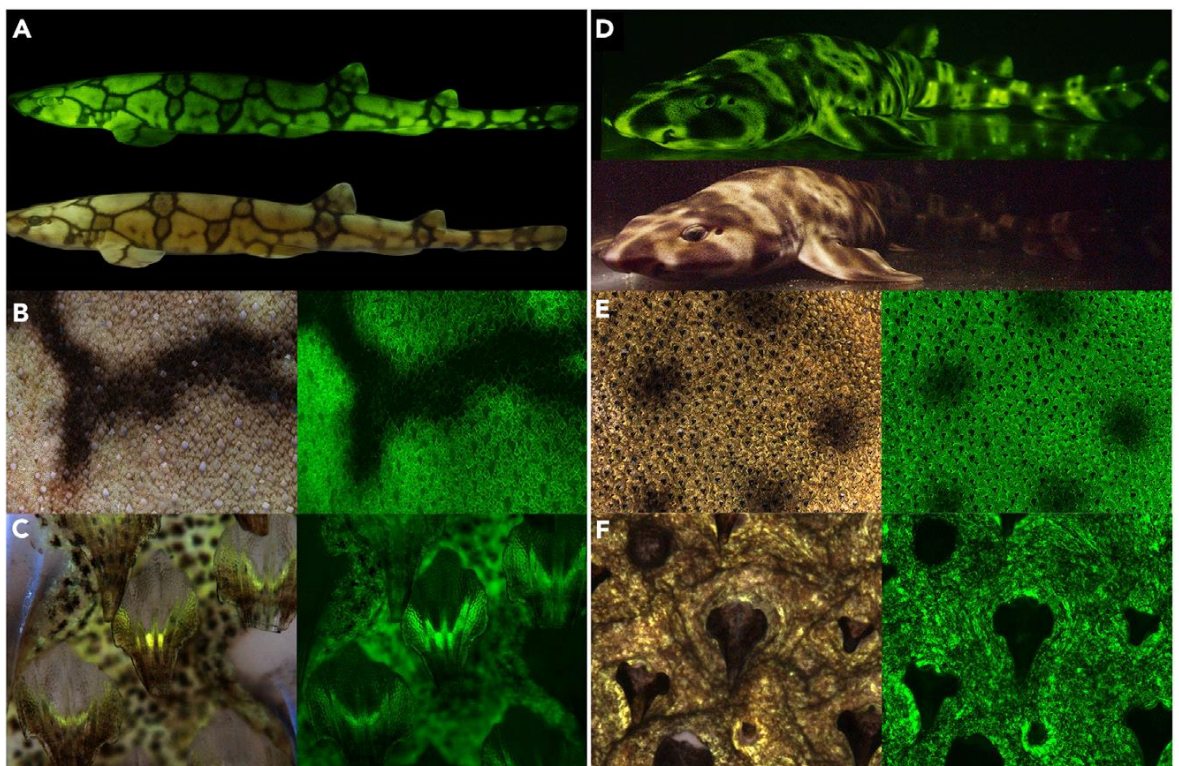


Figure 1.12: Fluorescent shark imaging (Park *et al.*, 2019) (A) *Scyliorhinus retifer* excited with 450–500 nm and imaged through 514nm LP, (B) cross section of skin with white light; (C) fluorescence emission, (D) *Cephaloscyllium ventriosum* excited with 450–500 nm and imaged through 514nm LP, (E) cross section of skin with white light, (F) fluorescence emission.

Since the identification of avGFP, other GFP and the recent findings of new FPs in vertebrates, many questions are raised about the function of fluorescence in metazoans and on the diversity of fluorescence sources in nature. What are the adaptive advantages conferred by fluorescence? While it is thought that not all fluorescence in nature is functionally relevant, few examples of its ecological role have been described. A number of hypotheses have been advanced to explain the roles of fluorescence, alone or in combination with luminescence. These include photoprotection from stem cells, photosynthesis enhancement, predation by prey lure or distraction, and protection against oxidative stress (Bou-Abdallah *et al.*, 2006; Haddock and Dunn, 2015; Meadows *et al.*, 2014; Salih *et al.*, 2000). A taxonomic approach to review advancements in the understanding of the ecological roles of fluorescence in marine organisms is presented.

5. Ecological role of fluorescence

GFPs and, in general, fluorescent pigments, act as a photoprotective system in case of excessive sunlight (Salih *et al.*, 2000). In fact, it was shown that UV_A and extreme photosynthetically active radiation trigger photo damage and photoinhibition in coral–dinoflagellate symbiosis that, in severe cases, may result in coral bleaching (Roth *et al.*, 2010; Salih *et al.*, 2000). In this context, GFPs histologically positioned above endosymbionts may function as an energy dispatcher through fluorescence and light scattering.

The sea anemone *Nematostella vectensis* was the first early metazoan whose genome was sequenced, and represents a powerful model system for evolutionary development biology (Putnam *et al.*, 2007). This species possesses 7 GFP genes of which only NvFP-7R codes for a red fluorescent protein that is functionally fluorescent. The transcriptional regulation of the NvFP-7R gene shows spatiotemporal complexity as well as unexpected capacity to sense positional information in the adult body plan (Ikmi and Gibson, 2010). In fact, bilateral symmetry of red fluorescent pattern is noticed at late development stage on polyps with twelve and sixteen

tentacles. This indicates that during tentacle formation the positional information is dictated by the body axes as well as the temporal process. Besides our knowledge of the functional significance of red fluorescence in *N. vectensis* (as well as in the vast majority of fluorescent organisms) is still based upon hypothetical reconstructions, the large toolkit of sophisticated approaches available for this species renders this small anthozoan a promising model for acquiring deeper insights on the role(s) of fluorescence.

The deep sea anemone *Cribrinopsis japonica* has its tentacles emitting green fluorescence, when excited by blue light, as a lure for prey attraction (Tsutsui *et al.*, 2016). Interestingly, the GFP isolated from this anemone (cjFP510) is more stable than other GFPs when subjected to high temperatures and repetitive freeze-thaw cycles; however it is unclear if this feature is due or not to an adaptation to low temperatures of the deep-sea habitat.

In the hydrozoan *A. victoria*, the response to superoxide radicals was investigated by looking at the protein structure of GFP. Superoxide radicals and Reactive Oxygen Species (ROS) are typically present in hyperoxic conditions that these organisms experience during the daytime due to the photosynthetic activity of algal symbionts. It was shown that GFP can quench (see glossary) these superoxide radicals without altering its fluorescence properties (Bou-Abdallah *et al.*, 2006) therefore providing protection from antioxidants.

In the hydrozoan jellyfish *Clytia hemisphaerica*, the intense green fluorescence observed in both endodermal and ectodermal cells of the mouth, stomach and gonads may have several functions, including UV protection of stem cells and maternal mitochondrial DNA from UV light. Each of the four GFPs (and the three aequorins) isolated in this species show life-cycle stage and tissue specificity, supporting the hypothesis that fluorescence has acquired multiple specialized roles depending on environmental (depth), physiological (life-cycle) or behavioural (spawning) conditions (Fourrage *et al.*, 2014).

The function of fluorescence in prey attraction has been assessed in a non-luminescent hydromedusa species, *Olindias formosus*, which possesses fluorescent and pigmented patches on

the end tips of its tentacles from early development of the polyp stage. In laboratory experiments under blue light conditions, these pigmented patches attract juvenile rockfish of the genus *Sebastes*, which would not respond in the absence of fluorescence (Haddock and Dunn, 2015). A similar mechanism has been observed in the siphonophore *Resomia ornicephala*, which possess fluorescent tentacles that attract and capture euphausiid shrimp (Pugh and Haddock, 2010). The siphonophore *Erenna sirena* is another example in nature of energy conversion from luminescence to fluorescence by creating yellow to red fishing lures on its tentacles, surrounded by a luminescent photophore (Haddock, 2005).

In the phylum Arthropoda, few copepod species possess luminescence, while few others, belonging to the Pontellidae and Aetideidae families, present the phenomenon of fluorescence, which is thought to serve as a mate perception and attraction signal and/or a camouflage mechanism (Hunt *et al.*, 2010; Shagin *et al.*, 2004). Interestingly, high brightness/stability and low cytotoxicity of copepod GFP proteins make these molecules particularly well-suited to a variety of molecular and biological applications (Neckles *et al.*, 2019).

Although neither stomatopod crustaceans nor mantis shrimps possess fluorescent proteins, many species have a very bright fluorescent coloration that is used in postural signalling to increase shrimp visibility when sensing a predator, for intra-species competition with other males, and in mate choice (Mazel, 2004).

Compared to terrestrial animals, marine organisms occupy a spectrally restricted visual environment, in which their eyes are adapted to different light conditions. In particular, a topic of interest in the evolutionary and ecology fields concerns how different types of visual systems developed specific spectral receptors and pigments (allowing them to detect fluorescence). Crustaceans like the mantis shrimp have developed a fascinating system of colour vision, based on at least eight primary spectral receptors ranging from 400 to 700 nm. In the species *Lysiosquilla glabriuscula* it was shown that at depths of 20, 30, and 40 m the fluorescence contributes 9, 11,

and 12%, respectively, of the photons that stimulate the shorter-wavelength receptor, and 15, 22, and 30%, respectively, of those stimulating the longer-wavelength receptor (Mazel, 2004).

However, to determine if enough energy is transferred in order to make a meaningful difference to the visual signal under natural lighting conditions, several optical factors such as the exitance and reflectance (see glossary) need to be calculated. In addition, fluorescence can only play a role in vision if it contributes to the total light leaving the surface and to a behavioural response, i.e., if the behaviour of the viewing organism is influenced by the presence or absence of fluorescence in the subject (Mazel, 2017). Another main issue that is still unresolved is whether fluorescence is bright enough to be seen under the appropriate light environment.

Studies on FPs in chordates provide deeper further information about their function when compared to invertebrates. In the cephalochordates *Branchiostoma floridae*, *Branchiostoma lanceolatum*, *Branchiostoma belcheri* and *Asymmetron lucayanum* different functions have been postulated such as an implication in antioxidant mechanisms by scavenging deleterious oxy-radicals, in photoprotection and attracting motile planktonic prey (Bomati *et al.*, 2014; Yue *et al.*, 2016). In cartilaginous and bony fish, such as catsharks and reef fish, fluorescence may have a role in communication, species recognition and camouflage (Gruber *et al.*, 2016) or it could be just a chemical by-product of skin composition.

In freshwater eels where UnaG was isolated, first assumptions of the function of fluorescence are emphasised on the binding of bilirubin to UnaG suggesting that it may regulate the dynamic transport of this metabolite and reducing cellular oxidative stress. In turn, this may confer to freshwater eels the ability to maintain oxidative muscle metabolism during long-distance migration (Kumagai *et al.*, 2013).

The presence of the blue FP Sandercyanin on the dorsal side of the fish mucus may be a protective mechanism, particularly in fish living in the northern latitude, where there is increased levels of UV radiation (Schaefer *et al.*, 2014). Sandercyanin being produced seasonally, especially in late summer, would protect the walleye from increased UV radiation by acting as a natural

sunscreen. In fact, the absorption of UV radiations can cause extent inner damage such as the production of free radicals, thus the antioxidant properties of Sanderycanin would dissipate this excess energy by emitting it in lower-energy red wavelength (Ghosh *et al.*, 2016). An alternate behavioural explanation of the advantage provided by the blue colour of Sandercyanin would be its plausible role in camouflaging and countershading in walleyes to avoid predation by another fish, the northern pike *Esox lucius* (Schaefer *et al.*, 2014).

Green and red fluorescence has been also observed in the sea turtles *Eretmochelys imbricata* and *Caretta caretta*. The origin of it is still unclear; if it comes from diet (corals, zooxanthelles) (Obura *et al.*, 2010) or is a by-product of chemical composition of the algae growing on their shells (Gruber and Sparks, 2015). In the case of the loose-jaw dragonfish *Malacosteus* sp., the animal emits luminescent light and far-red fluorescence that may be used in predation (Douglas *et al.*, 1998). Most of reef fish possess visual pigments ranging from UV to green wavelengths (Gerlach *et al.*, 2014; Losey *et al.*, 1999). Interestingly, red fluorescence has been observed in more than 180 species of marine fish (Sparks *et al.*, 2014), thus strongly suggesting its potential role in vision (Gerlach *et al.*, 2014).

Experiments conducted on the diurnal fish, *Cirrhilabrus solorensis* whose visual system is receptive to deep red fluorescent coloration, demonstrate that strong red fluorescence emission body pattern may affects male–male interaction (Warrant and Locket, 2004). A study on the spectral sensitivity of the goby *Eviota atriventris* has shown that this fish possesses long-wavelength visual pigments, making it physiologically sensitive to red fluorescent coloration (Michiels *et al.*, 2008). As further weight to this hypothesis, yellow intraocular filters have been found in reef fish, sharks, lizardfish, scorpionfish and flatfish, which could enable them to detect fluorescence (Heinermann, 1984). Nevertheless, despite several attempts, no sufficient experimental studies have clarified the functional and behavioural link between fluorescence and vision in organisms with complex visual systems (Gerlach *et al.*, 2014). Furthermore, it has been demonstrated that sharks and rays are capable of visualizing their own fluorescence, showing

sexually dimorphic fluorescent body patterns, which is suggestive of a communication/species recognition role for fluorescence (Gruber *et al.*, 2016). Finally, the brominated kynurenine compounds produced by the skin of catsharks could contribute to photo-protection from low-wavelength light (Sweet *et al.*, 2012).

It would be of great interest to characterize further the type of red fluorescent pigment involved and the biological characteristics of the photoreceptors.

6. Fluorescent proteins and biotechnology

After the cloning of the GFP gene (Prasher *et al.*, 1992) and *in vivo* demonstration that its ectopic expression in *Escherichia coli* and *Caenorhabditis elegans* induces fluorescence (Chalfie *et al.*, 1994), the interest and applications of GFP have continuously increased within the scientific community (see Figure 1.13). Originally used as a reporter gene for tracing proteins, organelles and cells, non-invasive GFP tagging has become a routine tool in scientific research for a variety of experimental approaches such as gene reporting, drug screening and labelling. In parallel to the discovery of new wild-type FPs, a hunt to develop new engineered FP mutants led to modifications of their chemical properties in order to broaden the horizon of potential applications in cell biology and biomedicine (Mocz, 2007).

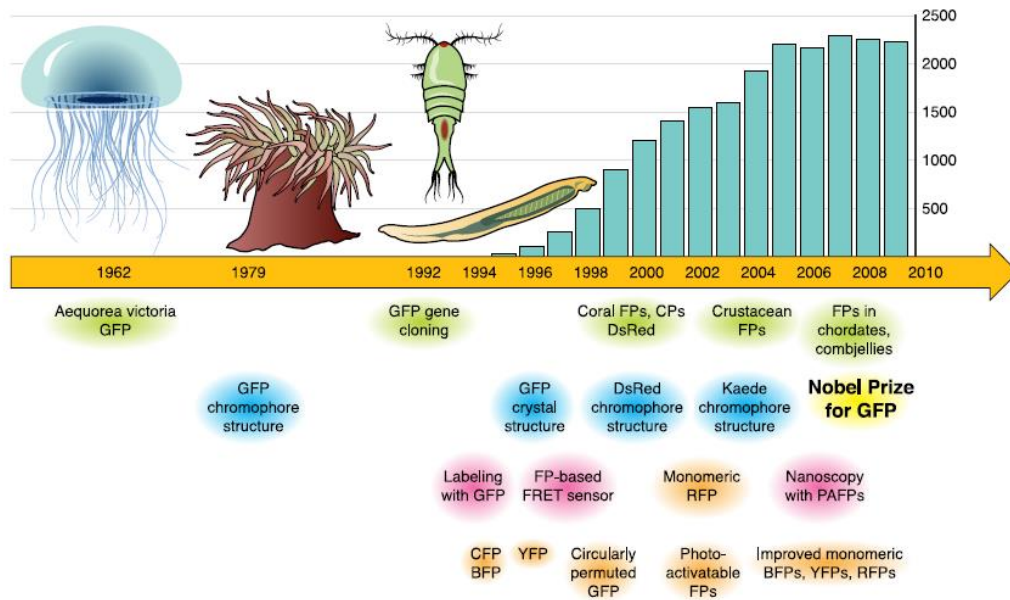


Figure 1.13: A timeline of major achievements in the field of FP-based tools (Chudakov *et al.*, 2010). Green colour below the text highlights basic studies of natural diversity of GFPs; blue, structural insights; orange, development of novel FP variants; magenta, appearance of FP-based technologies. Drawings of representative animals having FPs are shown above the timeline (from left to right: jellyfish, sea anemone, copepod, and lancelet). Columns above the timeline show the number of scientific articles per corresponding year that can be found searching PubMed with the term green fluorescent protein.

Continuously new FP are being developed with new biological properties such as the development of Gamillus from the jellyfish *Olindias Formosa*, a monomeric GFP, allows molecular tag suitable for imaging in acidic organelles through autophagy-mediated tracking to lysosomes. Unlike other reported monomeric GFPs, it has superior acid tolerance, better brightness, maturation speed and higher photostability (Shinoda *et al.*, 2018).

Also, Anthozoan FPs have been engineered to produce photoactivatable FPs (PAFPs), generating huge light induced spectral changes. Dendra, originally from octocoral *Dendronephthya* sp, was the first PAFP capable of photoconversion from green to red fluorescent states in response to either visible blue or UV-violet light (Gurskaya *et al.*, 2006). In addition to showing high

photostability, this PAFP is easily photoactivated by using ordinary 488-nm laser line. Similar to Dendra, Dronpa is a reversible bright green PAFP derived from the coral *Echinophyllia* sp. that shows interesting properties beyond its high brightness. Indeed, Dronpa can be switched on and off multiple times with high contrast and a minimal loss in fluorescence intensity (Ando, 2004; Drepper *et al.*, 2007).

Even the vertebrate FP UnaG has been modified to create a sensor, eUnaG, with increased fluorescence and thermostability that is used to measure drug transporter activity in live cells (Yeh *et al.*, 2017).

While FPs were characterized mainly in eukaryotes, interest in prokaryotes has raised in the last years as bacterial phytochromes (photoreceptors) have been engineered to create near infrared (NIR) FPs showing successfully results for deep tissue imaging in animals (Filonov *et al.*, 2011).

An interesting example in this group is a FP developed from a member of the same family of Sandercyanin derived from the cyanobacterium *Trichodesmium erythraeum*. The far-red Biliverdin-Binding FP (smURFP), possess an interesting feature being that its fluorescence is visible without exogenous biliverdin unlike Sandercyanin (Rodriguez *et al.*, 2016).

Finally, bacterial fluorescence has also been a template to counteract the oxygen dependence of GFPs. In this way, flavin mononucleotide (FMN)-based chromophores have been designed from *Bacillus subtilis* and *Pseudomonas putida*, requiring no oxygen as a cofactor. Therefore, the FMN based FP are very convenient for studying anaerobic biological systems (Drepper *et al.*, 2007).

7. Aims

This research project aims at identifying a new fluorescent protein from unicellular and multicellular planktonic organisms of the Gulf of Naples with “fluorescence characteristics” different from those already known i.e with emission spectrum in the red – far red. Also, I will exploit TARA data which is a worldwide consortium that has sampled plankton communities from

viruses to animals to coral reef ecosystems. I want to extend the search of new FP across the animal kingdom and to improve our evolutionary knowledge of fluorescence in the tree of life. To this end, each chapter dealt with these topics in the following way:

Chapter 2 explored the fluorescence response of various organisms of different phyla from MareChiara sampling. The coastal station Long Term Ecological Research – MareChiara (LTER-MC) located in the Gulf of Naples is the spot of weekly or biweekly sampling since 1984. In order to identify new genetically encoded fluorophores, sampling of marine organisms from coastal waters and offshore in the Gulf of Naples was carried out in different seasonal periods. Mixed samples, comprising plant and animal organisms, unicellular and multicellular organisms of the meso and micro-plankton, nekton and benthos were analyzed under a stereomicroscope for a first screening of biofluorescent organisms.

Thereafter, an experimental characterization through spectral analysis with white laser confocal microscopy was performed to characterize the fluorescence emission signature of zooplanktons and to assess the properties of potential fluorescent proteins. Particular efforts were made to identify proteins with fluorescent excitation and emission wavelengths different, even slightly, from already known fluorescent proteins (e.g., GFP, RFP).

Simultaneously, stereomicroscopy and confocal microscopy analysis were performed on polychaetes collected at the seafront of Stazione Zoologica Anton Dohrn, with the purpose to identify and isolate a new fluorescent protein.

Chapter 3 investigated *in silico* potential genes encoding new fluorescent proteins in TARA database using tree different fluorescent proteins as a reference: UnaG, Sandercyanin and GFPs from Cnidaria, Arthropoda and Chordata. A phylogenetic study of potential new GFP has been explored showing the evolutionary relationships among the potential hits in TARA data with the GFP reference protein sequences.

The material in this chapter forms the basis of paper Macel, M.-L. *et al.* (2020) 'Sea as a color palette: the ecology and evolution of fluorescence', *Zoological Letters*. BioMed Central, 6(1), p. 9. doi: 10.1186/s40851-020-00161-9. Bound at the end of the thesis in which I played a major role.

CHAPTER 2 - FLUORESCENT ZOOPLANKTON OF THE GULF OF NAPLES

1. MareChiara - role of plankton in ecosystem, biodiversity, marine invertebrates

The Mediterranean Sea is a reservoir for endemic species living in the coastal areas, compared with those living in the deep sea (Danovaro and Pusceddu, 2007). Among them, plankton occupy a central role in marine ecosystems as they are at the ground of food webs and catalyse biogeochemical cycles (D'Alelio, 2017). In particular, zooplankton occupy the intermediate trophic level between phytoplankton and fish (see Figure 2.1), where they serve both as trophic links between primary producers and higher trophic levels (Steinberg and Landry, 2017). Zooplankton are organisms drifting in the sea with insufficient locomotive assets to progress against currents (Lenz, 2000). Besides being important component of carbon and nutrient cycles, they are also responsible of transferring energy to deep water through the sinking of fecal pellets and carcasses (Henschke *et al.*, 2016; Stemmann *et al.*, 2000). Zooplankton constitute a phylogenetically and functionally diverse assemblage of protistan and metazoan, either permanently as holoplankton (e.g., copepods or arrow worms) or temporarily as meroplankton (larval stage) (Everett *et al.*, 2017).

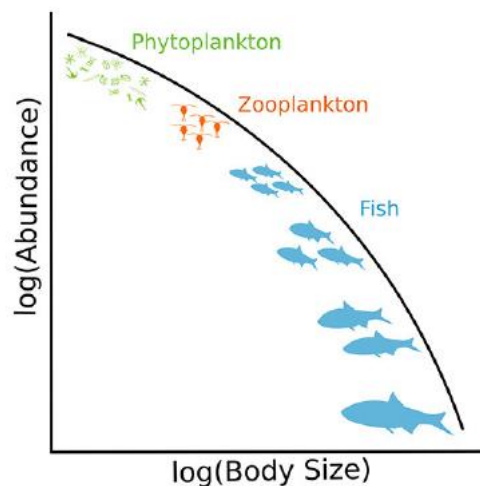


Figure 2.1.: Representation of Size-spectra model in the pelagic food web (Everett *et al.*, 2017)

Particularly in the open, most of epipelagic mesozooplankton is concentrated in the upper 100 m layer and decreases strongly below this depth (di Carlo *et al.*, 1984; Weikert and Trinkaus, 1990). These communities are highly diversified in terms of taxonomic composition, but small copepods (mostly ≤ 1 mm in total length) represent the major group in terms of both abundance and biomass (Siokou-Frangou *et al.*, 2010). Other crustaceans such as ostracods present a consistent distribution in different Mediterranean regions and contribute to total zooplankton distribution increasing gradually with depth (Angel, 1993). Gelatinous zooplankton also represents an important group in the pelagic communities as efficient filter feeders or predators. They are generally underestimated caused by nets during sampling, damaging or destroying their fragile bodies.

In the Gulf of Naples, the monitoring of plankton communities first started during the 19th century by Achille Costa. Thereafter, diverse parameters such as plankton diversity, zooplankton distribution or ecological studies have been assessed (Zingone *et al.*, 2019). Since 1984, data have been collected at a fixed station MareChiara (40° 48.5' N, 14° 15' E) either fortnightly (until 1991) or weekly (1995 to date) (Ribera d'Alcalà *et al.*, 2004). Since 2006, the MC time-series is part of the Italian, European and international network Long Term Ecological Research (LTER) (D'Alelio, 2017) and allows collecting biological, physical and chemical data at different depths. However, phytoplankton are analysed in surface waters and mesozooplankton are collected in the 0–50 m depth layer so as to gather information on the structure of coastal plankton communities, and their taxonomy based on microscopy and molecular analysis (Mazzocchi *et al.*, 2011). Within 36 years, more than 750 microalgal and 212 mesozooplankton taxa have been recorded (Zingone *et al.*, 2019). Despite the temperate latitude of the Mediterranean Sea, its subtropical characteristics may be linked with the observed population diversity. In the Gulf of Naples, the zooplankton population in winter is characterized by a high percentage of appendicularians and meroplankton in terms of biomass, underlining the role of phytoplankton blooms in this season that fill up the

zoobenthos. Instead, spring is the period of growth of the whole plankton component with the highest biomass of ciliates (Zingone *et al.*, 2019).

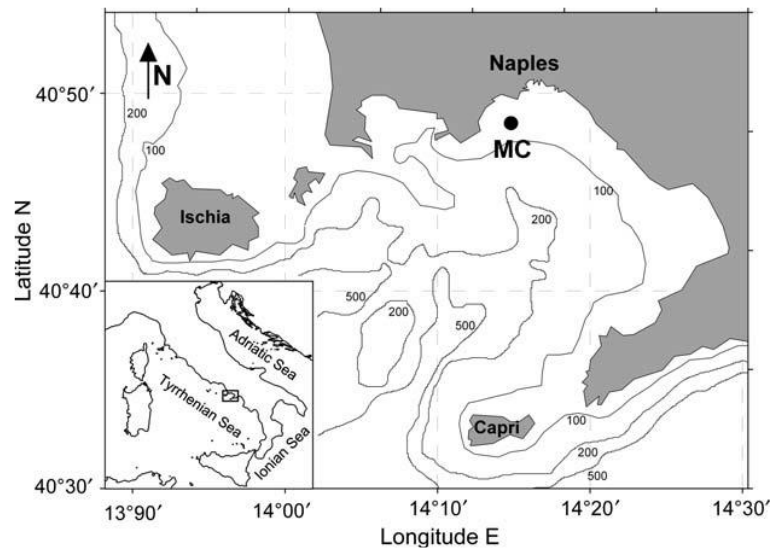


Figure 2.2: Map of the Gulf of Naples (Tyrrhenian Sea, Western Mediterranean) with the location of station MC, site of the LTER-MC time-series (Mazzocchi *et al.*, 2011).

2. Aims

The chase after new FPs with unique spectral, stable and bright characteristics is active since many years (see Figure 2.3). Much of FPs emit in the green and present a strong brightness compared to eGFP. Blue and red FPs, however, are not bright but are more stable. Nowadays, the direction is made towards FPs in the far red that are preferred for *in vivo* imaging since they avoid the natural green autofluorescence observed in plants and animals. In addition, the modification of already identified proteins emitting in the far-red region of the spectrum is conceivable and can be tested *in vivo*.

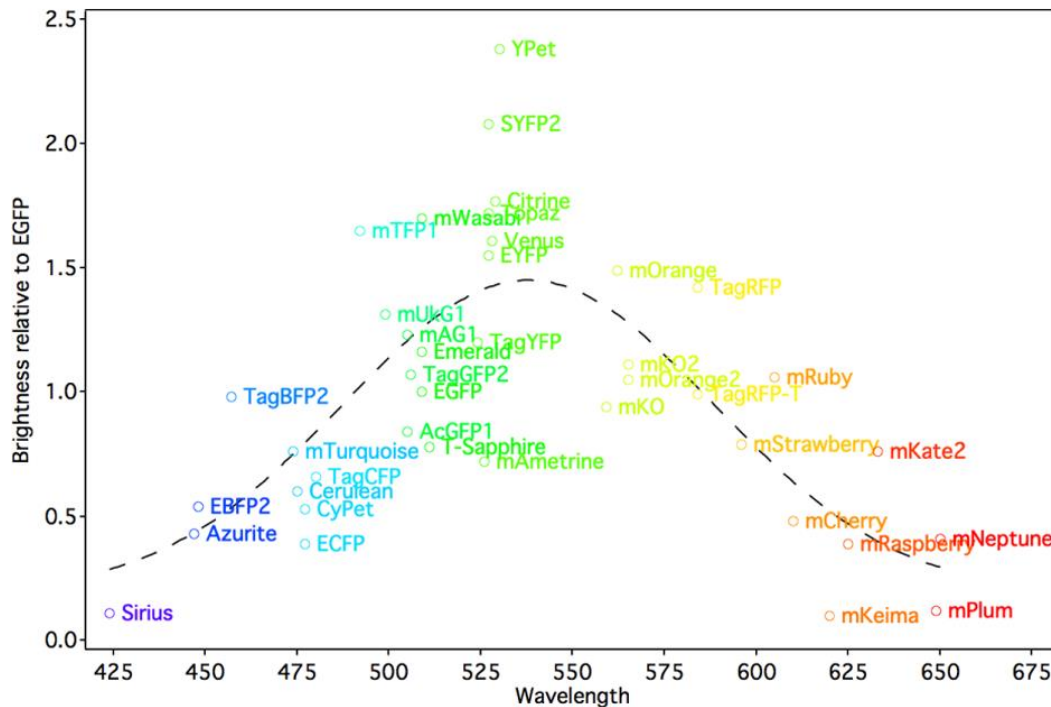


Figure 2.3: Popular FPs in terms of brightness as a function of emission wavelength (Dedecker *et al.*, 2013). The dashed line serves as a guide to highlight the general trend. The coloring of the tags approximates the appearance of the corresponding emission wavelength to the naked eye. The brightness of some fluorophores has been altered by very small amounts for clearer visual presentation.

The aim is the sampling of zooplanktons - which comprises holoplankton (e.g., copepods or arrow worms) and meroplankton (e.g., crab or fish larvae). Zooplanktons' sizes range from flagellates of about 20 μm to siphonophores up to 30 m long. Finally, they are capable of emitting light through biofluorescence and this property is an asset to identify new genetically encoded fluorophores to be used as biosensors for the *in vivo* study of molecules in basic research and biomedical approaches.

3. Methods

3.1 Sampling at MareChiara

Sampling of marine organisms from coastal waters at MareChiara station and offshore in the Gulf of Naples is carried out weekly throughout the year. Zooplankton is collected with a net mesh size of 40 μm diameter and at a distance of 50 m from the sea surface (see Figure 2.4).



Figure 2.4: The zooplankton net just before dropping in the sea.

Afterwards zooplanktons were poured in a 5 L glass jar and brought back to the lab to start the analysis. Heterogeneous samples comprising plant and animal organisms, unicellular and multicellular organisms of the meso and micro-plankton (cnidarians, copepods, ostracods, tunicates, ctenophors, protists), nekton (bony fishes, scyphozoans) and benthos (anthozoans, nudibranchs) were analyzed. For that, a 150 x 15 mm Petri dish is used to pour inside the planktons and visualize them under a standard stereomicroscope.

Then the organisms collected after the first screening were put one by one in a concave blank glass microscope slide with a few drops of 7% MgCl_2 to help cooling down the animal and a coverslip. In this way, the analysis under the epifluorescent apotome (either Zeiss Axioimager or

Zeiss apotome) allowed to capture samples in bright field and to assess the fluorescence under the GFP or rhodamin filters, followed by a first taxonomic identification of fluorescent positive organisms. See the examples below of several zooplanktons, A: Brightfield; B: GFP; C: Rhodamine; D: composite.

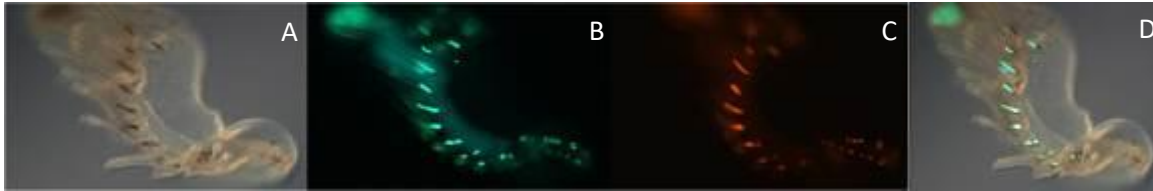


Figure 2.5: Actinotrocha larva, Phoronida observed at the microscope Zeiss Axio Imager (10X).

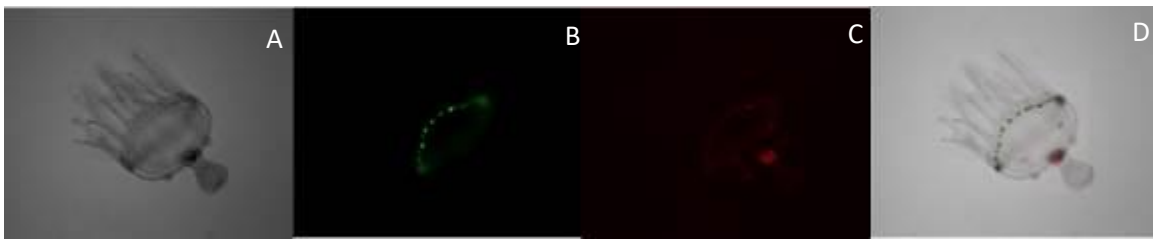


Figure 2.6: Hydrozoa, Obelia sp at the Zeiss Apotome (10X)

During the last year of the project, the stereomicroscope Leica 205 FA was used directly after collection of zooplankton sample as it is equipped with bright field and fluorescent filters (UV, GFP and rhodamine) using the same material as presented.

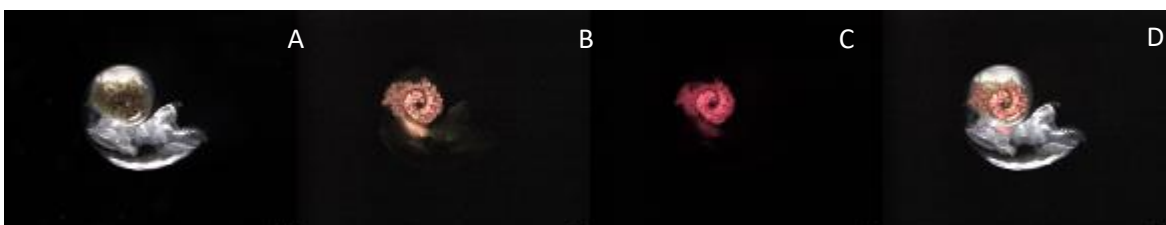


Figure 2.7: Gastropod larva at the Leica 205 FA

Selected samples after this first screening were analyzed by confocal microscopy to better define their “fluorescence characteristics” in the attempt to find new fluorescent proteins. The glass jar

containing zooplankton samples is conserved for further analysis by leaving it in a cold room (18°C) with a turbine engine at slow rotation. Finally, Iole Di Capua from the MOTaX service has helped in accomplishing the identification of zooplankton species and Dr Isabella D'Ambra helped in characterizing hydrozoans.

3.2 Sampling of annelids at the seaside

Two sessions have been organized on 22/10/2019 and 18/11/2019 in front of Stazione Zoologica Anton Dohrn under the supervision of Dr Luigi Musco. Samples were collected on rocks where algae were present and then put in a big jar with seawater. After, rocks were transferred in a large plate with low edges to start the dissociation of the rocks and algae. Samples of Nereididae, *Syllis prolifera*, *Alitta virens* and *Neanthes acuminata* were transferred in single jars covered with tights to allow respiration. Water was changed with filtered fresh seawater (FSW) using a 0.22 µm membrane filter, kept in a room at maximum 18°C and fed twice a week with fresh spinach, fragmented with a food mixer and suspended in FSW (see (Massa-Gallucci and Gambi, 2015)).

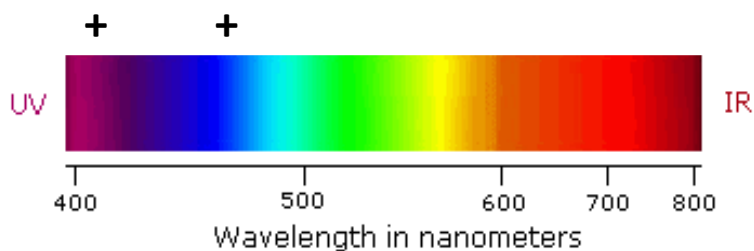
3.3 Microscopy analysis: epifluorescence and confocal microscopy assessment

This protocol has been designed in collaboration with Giovanni Gragnaniello from the microscopy service (RIMAR) from Stazione Zoologica Anton Dohrn.

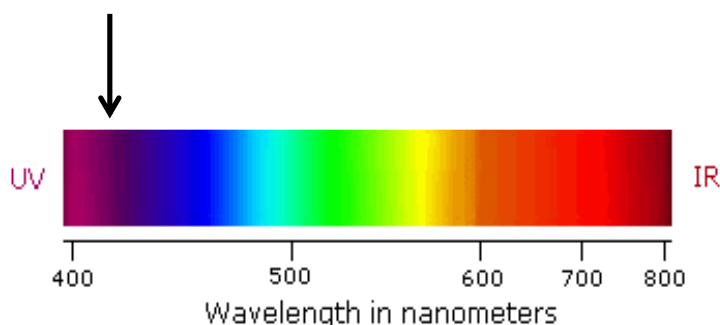
For precise wavelength assessment, samples from the first stereomicroscopy screening were next prepared in a 60 x 15 mm Petri dish and coverslip for the analysis at the confocal Leica TCS SP8X. This confocal microscope allows a more precise and complete analysis of the samples. Indeed, a second experimental characterization of the properties of the identified fluorescent proteins was done through spectral analysis both in excitation and in emission with the super resolution Leica confocal microscope.

All images have been done with the objective 10X as no 5X was available, for this reason it was sometimes not possible to make picture of the entire specimen. First, an RGB image is made to get a representative image of the organism from the blue, green and red channels. Then the

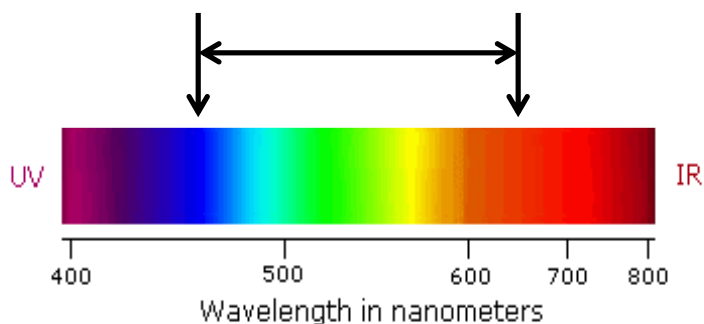
response to chlorophyll is assessed using specifically the laser UV line 405 nm (at power 20%) and 470 nm (power 50%). This will allow to rule out if organisms are positive to this pigment and if it is easily noticeable in their digestive apparatus.



The UV excitation response using laser line UV 405 nm (power 20%) is analyzed by screening the emission spectrum every 20 nm from 450 to 730 nm.



Finally, the laser line per continuum visible 490 nm (power 86%) allows to specifically excite wavelengths ranging from 470 nm to 670 nm (every 10 nm) and to analyze the response in the whole emission spectrum (every 20 nm) from 490 nm to 750 nm.



The computing analysis for the UV response and the visible light excitation assay are performed on the software MetaMorph® and on Excel.

3.4 Confocal spectra analysis

Each zooplankton species examined at the microscope Leica SP8X by UV excitation and dual screening was analysed on Metamorph which is a software allowing image analysis of fluorescence images.

- **UV excitation assay:**

This analysis consists of measuring the emission response of the sample of interest to a single excitation wavelength (λ_{exc} 405 nm). This is made on the software by drawing a region showing the strongest intensity. By measuring the average pixel intensity of this particular region, this allows to study further the average intensity response to this single UV excitation wavelength.

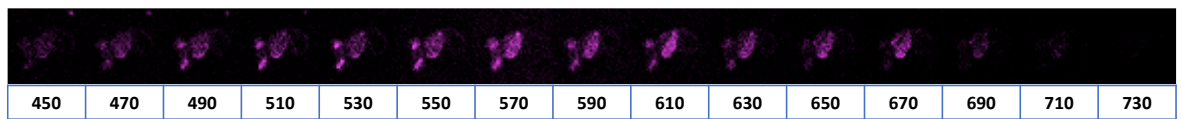


Figure 2.8: Example of a decomposition of a stack on Metamorph for an experiment done on a mollusc larva analysing UV response.

The emission response from 450 nm to 730 nm is done every 20 nm, which produces 15 images in total (Figure 2.8). Here the fluorescence emission starts to increase at 470 nm and it decreases at 690 nm. The information on the pixel average intensity 0 (black) to 255 (white) were collected on an excel file corresponding to the fluorescence emission response to the excitation wavelength 405 nm and is used to generate a graph depicting the average intensity versus emission wavelengths.

- **Visible light excitation assay:**

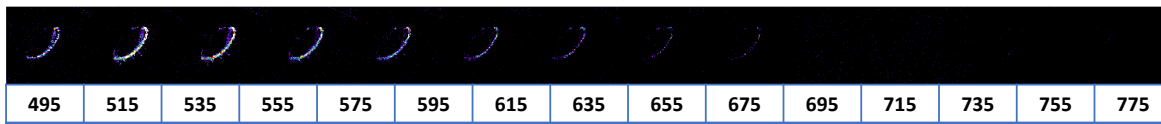


Figure 2.9: Example of a decomposition of a stack on Metamorph for an experiment done on *Hydroïdolina sp* at λ_{exc} 470 nm.

On Figure 2.9 we can notice on the images that the fluorescence emission intensity increases from the beginning at λ_{ems} 495 nm and starts to be very low at λ_{ems} 675 nm.

Overall, 15 images are produced for each λ_{exc} (from 470 nm to 670 nm) which in total represent 300 images. The stack of each λ_{exc} is analysed in the same way as for the UV assay by drawing an ellipse in the region of interest (ROI).

At the end, the values of the average intensity and the emission wavelengths for each stack are reported on excel (see following table). (The range of emission wavelengths has been slightly modified over the protocol experimentations and in the end, it was performed from 490 nm to 750 nm).

From these values, 21 graphs were drawn for each λ_{exc} (from 470 nm to 670 nm) which are all then put in a single graph representing the light intensity (Y axis) and the emission wavelength (X axis) for each excitation wavelength.

EMS	EKC 470nm	EKC 480nm	EKC 490nm	EKC 500nm	EKC 510nm	EKC 520nm	EKC 530nm	EKC 540nm	EKC 550nm	EKC 560nm	EKC 570nm	EKC 580nm	EKC 590nm	EKC 600nm	EKC 610nm	EKC 620nm	EKC 630nm	EKC 640nm	EKC 650nm	EKC 660nm	EKC 670nm	
490	15.144627																					
510	18.194951	18.4172345	17.2414																			
530	19.367069	19.5842917	18.52212	16.67588	14.530987																	
550	18.197252	18.1548923	17.3084	17.17205	17.909401	20.1044935	22.126313	54.570554	70.51966													
570	16.754568	18.4476511	20.44305	24.5351	31.109075	39.2429807	43.369256	39.2429807	43.369256													
590	15.844124	17.5111601	19.22567	23.00619	29.314826	37.161143	40.214263	50.409789	68.23378	73.73253	56.763725											
610	14.809524	15.4148041	16.11773	17.88192	20.948118	25.1655689	26.954857	32.596436	43.16774	47.08391	36.683554	22.136081	15.286062									
630	14.129257	14.5117399	14.9242	15.87001	17.842575	20.4495102	21.5445694	25.384506	32.60935	35.125	27.803756	18.494304	14.444444	13.361377	13.208391							
650	13.626452	13.9401352	13.95481	14.43398	15.461188	16.8305665	17.356136	19.331161	23.36343	25.14359	21.159533	15.988967	13.845691	13.204127	13.097338	13.120055	13.152027					
670	13.365441	13.4012224	13.48539	13.69752	14.130592	14.6636141	14.882161	15.780444	17.56094	18.27492	16.56855	14.343063	13.424114	13.133993	13.101105	13.109712	13.183972	13.077844	13.118475			
690	13.21458	13.2759819	13.30478	13.37082	13.538522	13.8677464	13.986473	14.471342	15.30812	15.70615	14.787978	13.694257	13.2544	13.123086	13.068783	13.112706	13.069658	13.095199	13.061779	13.04213	13.143189	
710	13.093257	13.070379	13.13345	13.15492	13.215281	13.3934412	13.453125	13.602145	13.97349	14.20542	13.751247	13.274456	13.172156	13.045191	13.132668	13.016883	13.105814	13.032807	13.028047	13.048934	13.020635	
730	13	13.0863787	13.08725	13.062	13.055085	13.1131949	13.108472	13.114937	13.25029	13.30339	13.174916	13.060074	13.029954	13.051799	13.060465	13	13	13.04797	13.038462	13	13.181818	
750	13	14.7666667	13	13	13	13	13	13	13.18531	13	13	13	13	13	13	13	13	13	13	13	13.613636	

Table 2.1: Average intensity values for each Excitation wavelength from 470 nm to 670 nm with Emission wavelengths from 490 nm to 750 nm.

Here below (Figure 2.10) is reported a positive result for the jellyfish *Hydroidolina* sp. Excitation wavelengths are represented only until 600 nm as no intensity was detected on Metamorph when measuring it in the (ROI).

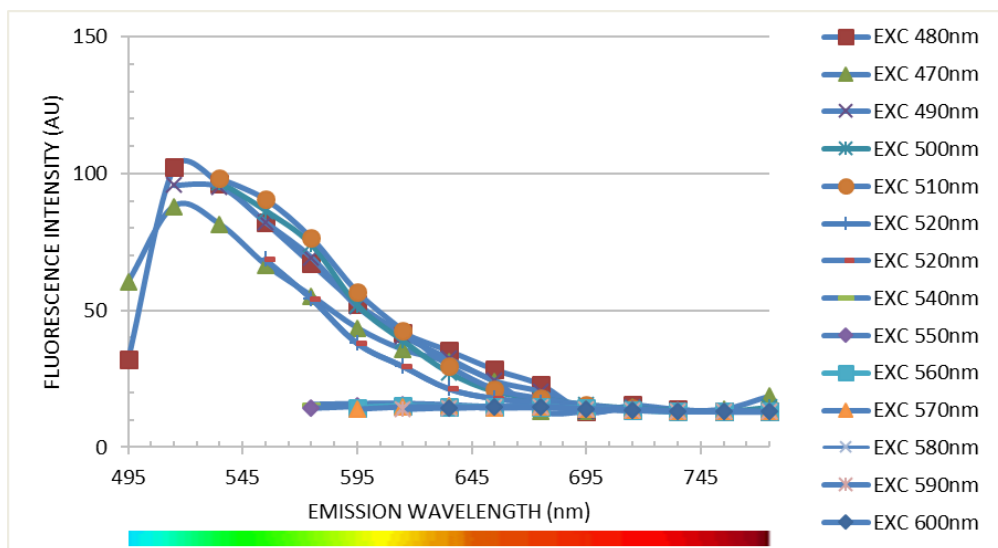


Figure 2.10: Visible light (470-670 nm) excitation response of *Hydroidolina* sp.

Finally, the highest peaks on the graphs for the UV and the visible light excitation tests were reported in a table; the emission wavelength peaks with the corresponding intensity were written for each species. The last column reports any matching fluorophores already engineered or to chlorophyll according to the emission wavelength. As stated in the introduction, natural fluorescence can derive from organic pigments, such as chlorophyll, which in zooplankton would come from their diet enriched in phytoplankton and bacterioplankton. Chlorophyll-a has an absorption maximum at around 430 nm, with fluorescence emission at 662 nm and 669 nm (see Figure 2.11).

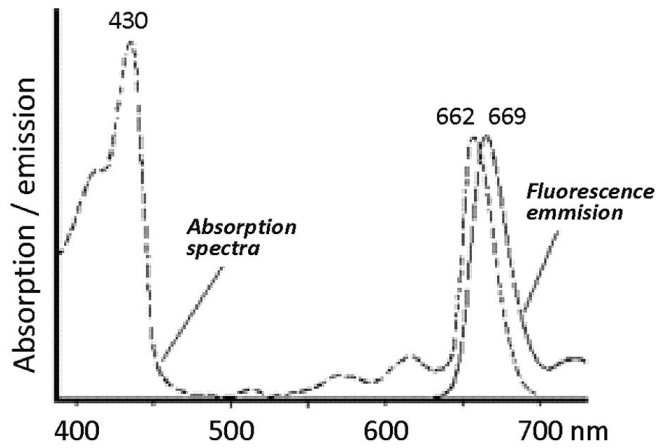


Figure 2.11: The absorption and emission spectra of chlorophyll-a (Poniedziałek *et al.*, 2017).

4. Results

The table of species collected from LTER-MareChiara sampling and from the seaside are organized in chronological order from 2017 to 2020 and can be found at the following link: (Table 2.2) <https://figshare.com/s/1dfd41b8f53b69620106>

4.1 Zooplankton light properties analysis

The epifluorescence analysis of zooplanktons from LTER-MareChiara and samples from the seaside allowed examining 70 organisms, corresponding to 46% of the total number of species analysed. I organized the pictures according to phyla on a powerpoint file where the specimens presenting the nicest fluorescent traits were shown. The link to the powerpoint can be found at this address: (Figure 2.12) <https://figshare.com/s/2b9678f673e72962af1f>

The pictures present several examples of fluorescence denoted in Cnidaria, Phoronida, Mollusca, Chaetognath, Annelida, Arthropoda, Echinodermata, Cephalochordata and Vertebrata.

On the *Aequorea* sp. and *Obelia* sp. we can notice a green fluorescent signal observed on the tentacular bulb of jellyfish which could originate from GFP (see slides 2 and 5 to 8). Jellyfish show also blue and red fluorescence on the stomach (slides 5, 6, 7), may be from algal diet.

Actinotroch larva on slide 12 shows nice green and red fluorescent patterns along their tentacles. The picture in bright field seems to indicate that it could come from pigmentation as we can observe a brown signal at the same location as the fluorescence.

Green fluorescence, certainly from chlorophyll autofluorescence is noticed in the digestive gland of Mollusca, both in gastropod larva (see slides 14 to 16) and pteropod (slide 17). Chaetognath, which is a predatory marine worm, has their hooks showing bright green fluorescence that could be due to the presence of pigments in them (slide 21). However, after observing other individuals throughout LTER-MareChiara sampling I did not observe this feature any longer.

Annelids present autofluorescence in the gut as it can be seen on slides 23 and 34, certainly from chlorophyll origin. Trocophores show interesting green fluorescent signals in their nerve cells that could be associated with a pigment, see slides 24 and 25. Alciopidae present several times green fluorescence around the eyes, probably due to pigmentation of the retina (see slides 27 to 31).

Annelids collected from the seaside namely *N. acuminata*, *S. prolifera* and *A. virens* were fluorescent. In fact, we can notice green fluorescence in the head of *N. acuminata* and both green and red fluorescence in the gut, see slide 39. *S. prolifera* shows bright green fluorescence on the chaete (slide 38) and *A. virens* (slide 40) presents both green and red fluorescence in the head. Although this characteristic being very interesting, it was not possible to analyse *A. virens* under the confocal microscope as the specimen was very big and only 10X objective is available at the Leica SP8X.

Copepods present both red and green fluorescence in their mouth and gut, due to the food content (slides 41 to 44 and 48). Larva of nauplio shows both green and red fluorescence in the labrum, which is a fold extending over the mouth (Dahms *et al.*, 2006), see slide 47. Decapods

instead seem to have fluorescence from pigments on their nerve cells along the abdomen and on the tail as they have brownish colour in bright field, see slide 50 to 53. Other crustaceans also have fluorescence in the stomach (slide 55 and 58 to 62) and in the head region (slide 52, 54, 56, 57). Holothuria larva present green and red fluorescence in their stomach from algal content and red fluorescence in the hyaline spheres, see slides 65 and 66.

Amphioxus show bright green fluorescence in the pharynx and the iliocolon, see slide 68, probably due to the expression of GFPs at these locations of the body as it is observed in the species *Branchiostoma floridae* under UV light (Deheyn *et al.*, 2007).

Finally, a fish embryo also showed green fluorescence particularly in the oil globules. This signal may come from autofluorescence of the lipids.

4.2 Fluorescence spectral analysis

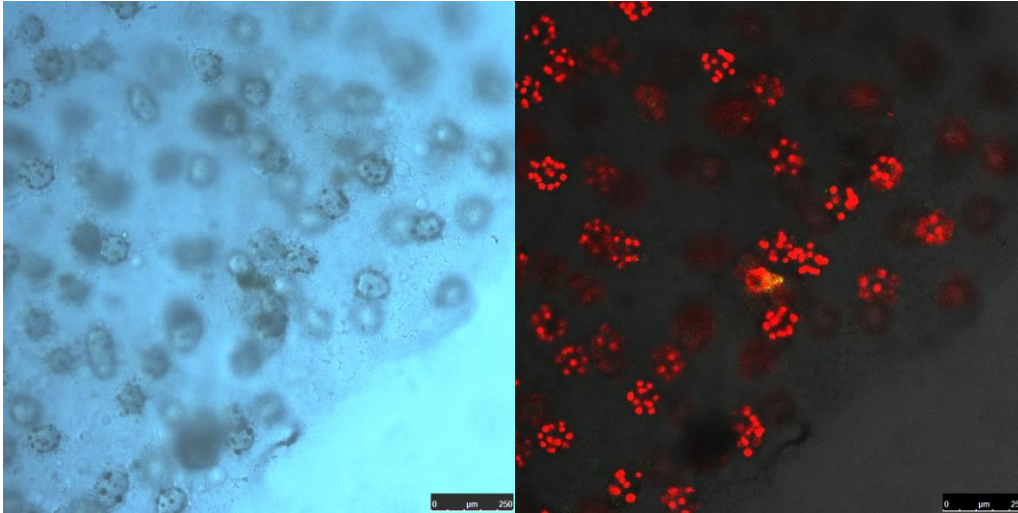
The following spectra represent confocal analysis assessing zooplankton species response to several tests. First, the excitation of the two lasers 405 nm and 470 nm allows appreciating if they have a similar emission pattern as the chlorophyll a molecule. Then, their fluorescent properties are evaluated by scanning the whole visible spectrum after UV excitation. Finally, their response to a visible light excitation every 10 nm is scanned every 20 nm.

The pictures and graph are compiled on a single page for a species in a given phylum, the number of specimens analysed during the whole MareChiara and polychaete sampling are summarized in the table below (Table 2.3).

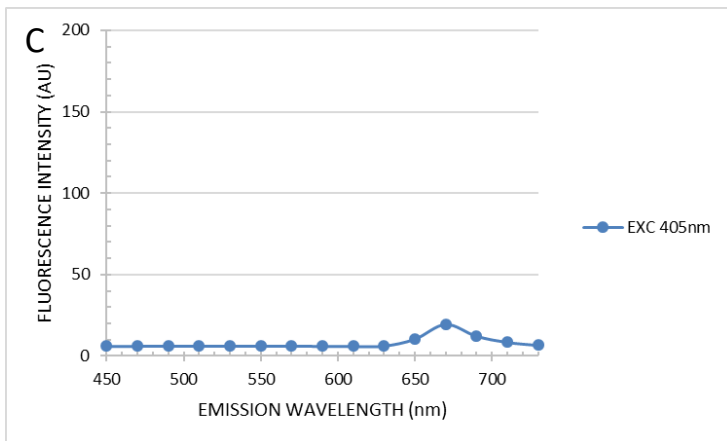
PHYLA	NUMBER OF SPECIMEN ANALYZED AT THE CONFOCAL MICROSCOPE	SPECIES/CLASS
Radiozoa	2	Sphaerozoum
Cnidaria	11	Aequorea <i>Cotylorhiza tuberculata</i> Obelia Cunina <i>Dimophyes arctica</i> Diphyidae
Phoronida	1	Phoronis
Mollusca	5	Veliger larva of gastropod Pteropod
Annelida	23	Polynoidae Opheliidae Serpulidae Alciopidae Spionidae Terebellidae Oweniidae Phyllodocidae <i>Syllis prolifera</i> <i>Neanthes acuminata</i> Polynoidae Nereididae
Arthropoda	5	Temora Corycaeus Oncaea Cirripedia Porcellanidae <i>Temora stylifera</i>
Chordata	7	<i>Thalia democratica</i> <i>Salpa fusiformis</i>
Vertebrata	1	

Table 2.3: Number of organisms analysed by confocal microscopy

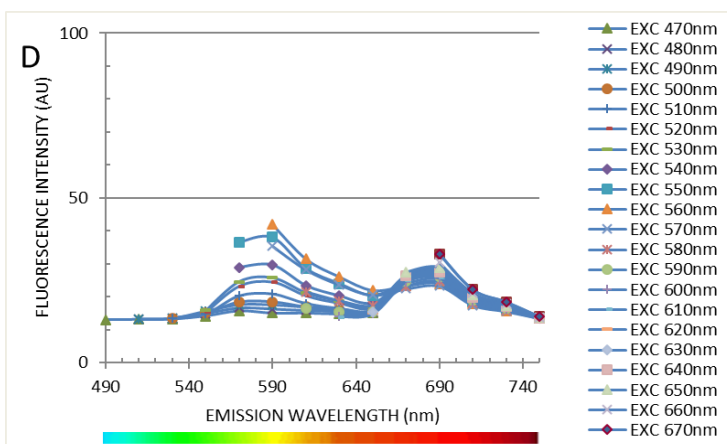
4.2.1 Radiozoa



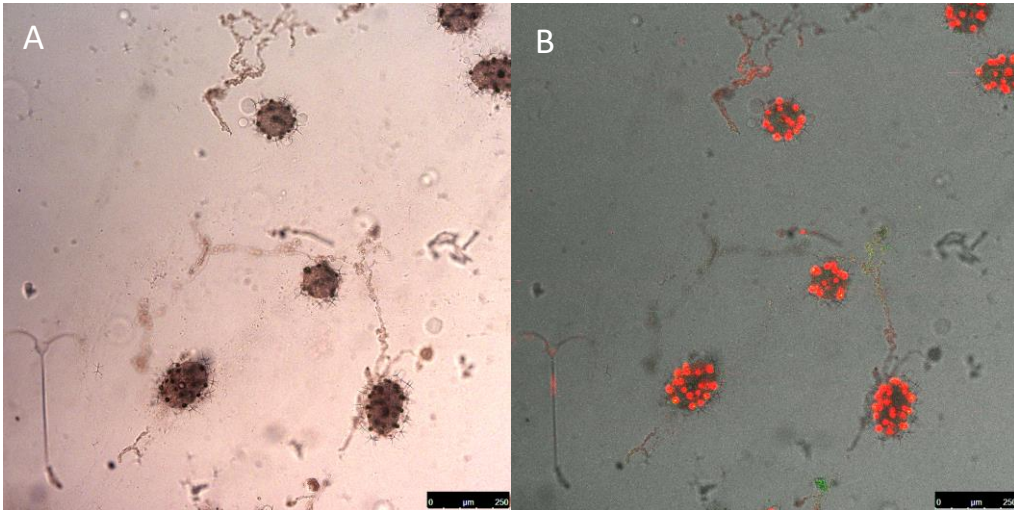
Excitation and Emission Spectra



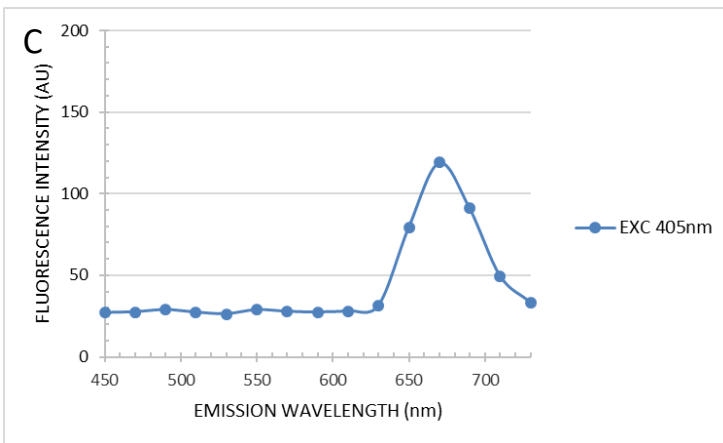
UV (405 nm)
excitation response



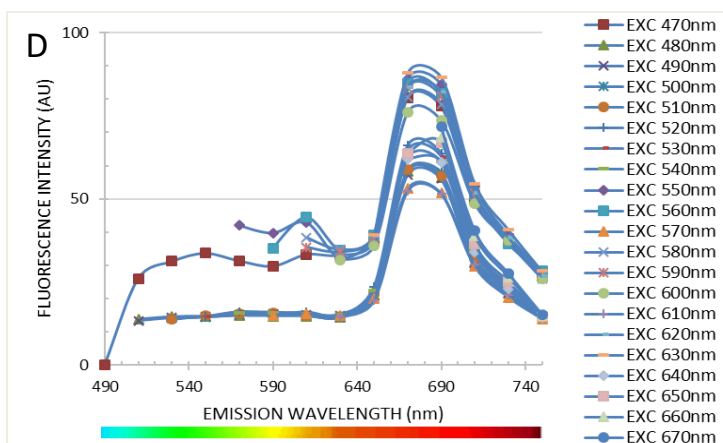
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



UV (405 nm)
excitation response



Visible light
(470-670 nm)
excitation response

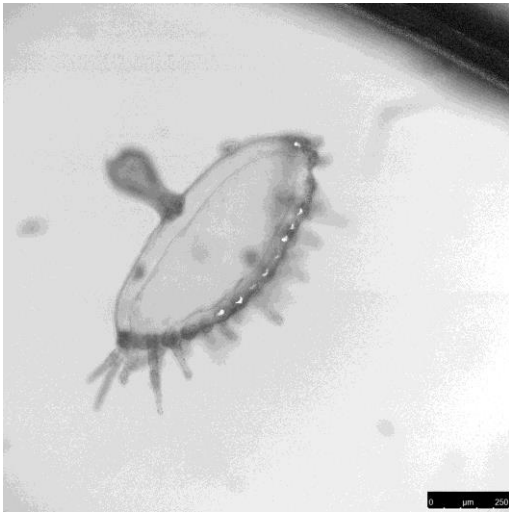
Comments:

Radiozoa were interesting species to investigate as they showed bright red fluorescence under the epifluorescent microscope and we also observed a strong red emission in response to UV excitation (Fig 2.14 C) at 670 nm which corresponds to the chlorophyll pattern. This is also visible on the test assessing the response to chlorophyll where there is a strong red signal (Fig 2.13 B and Fig 2.14 B). The response to the visible light excitation shows a very strong emission in the red part of the spectrum, especially for the second species (Fig 2.14 D), with intensity close to 100. Instead, the first sample shows emission with lower intensity and in the green part of the spectrum.

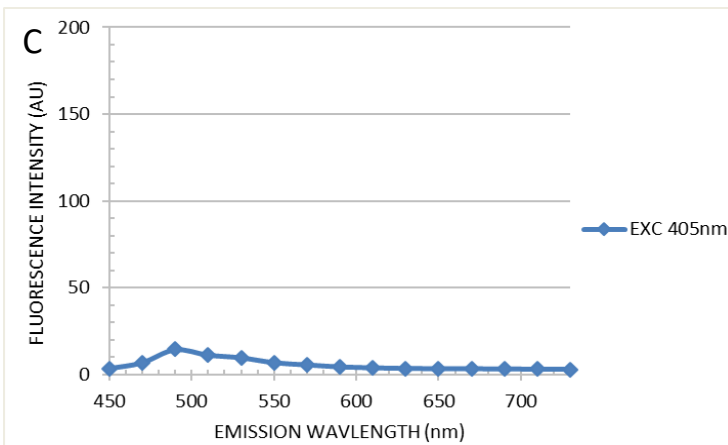
Radiozoans have been studied in the early twentieth century by light microscopy revealing numerous algal symbionts (Haeckel, 1887). Later, fine structure evidenced by transmission electron microscopy showed that polycystine radiolarians possess dinoflagellates (Anderson, 1976). The same research group clarified that the radiolarians and the microalgae form an ultramicrobial association in which the photosynthetically derived organic substances are transferred from the algae to the host radiolarians (Anderson, 1978).

In the early twenty first century, these algal symbionts within radiolarians were better characterized microscopically and under UV light. The chloroplasts of the algal symbionts are present in spherical cells measuring several micrometers in diameter and generally yellow-green under day light. Under UV irradiation, they emit autofluorescence and appear red (Takahashi *et al.*, 2003).

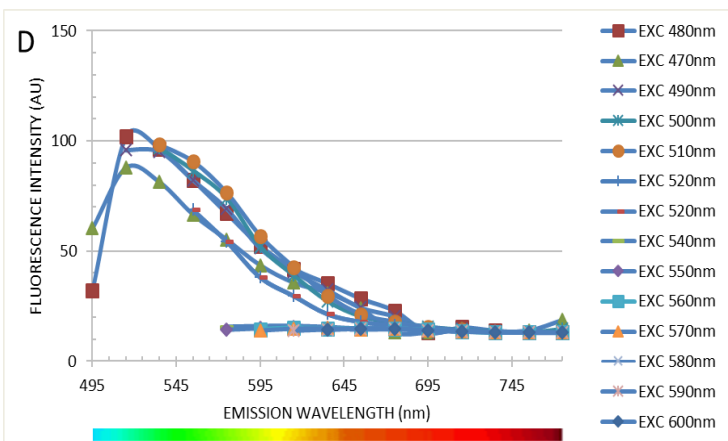
4.2.2 Cnidaria



Excitation and Emission Spectra



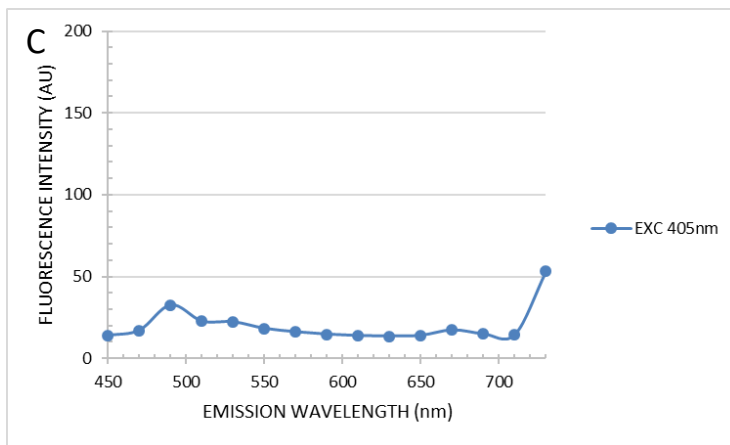
UV (405 nm)
excitation response



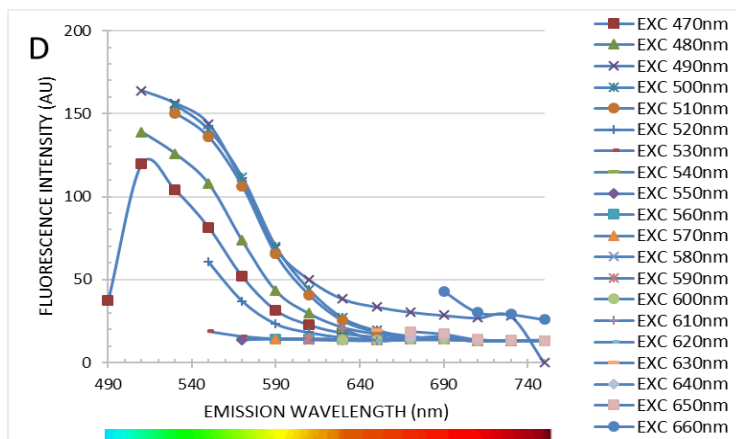
Visible light
(470-670 nm)
excitation response



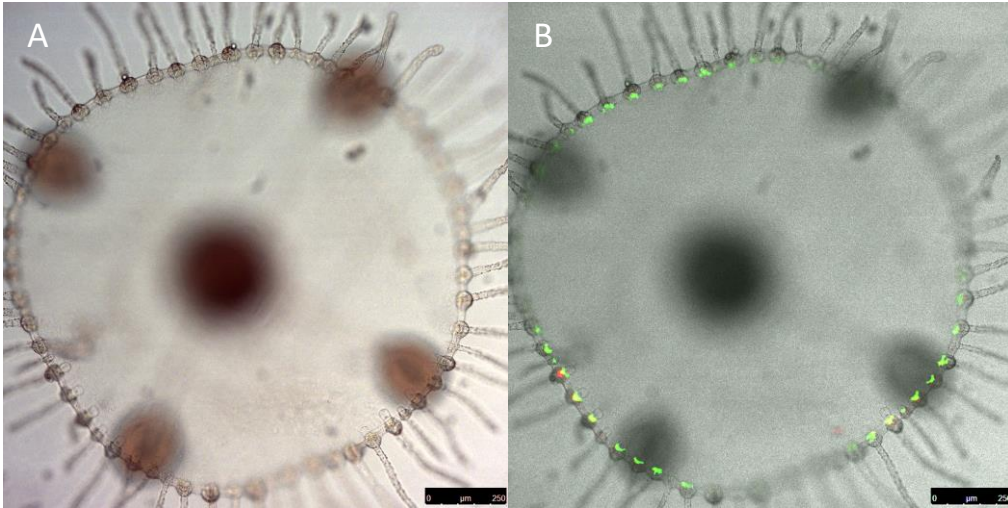
Excitation and Emission Spectra



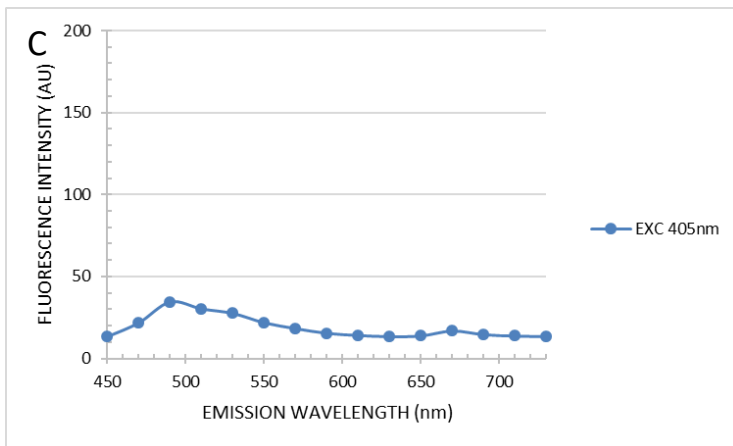
UV (405 nm)
excitation response



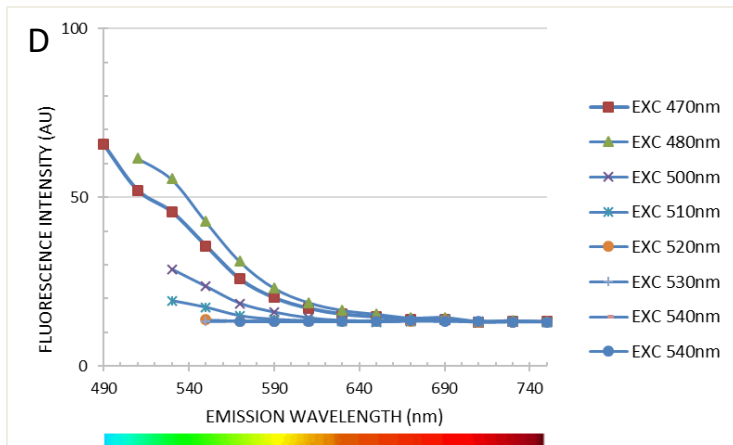
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



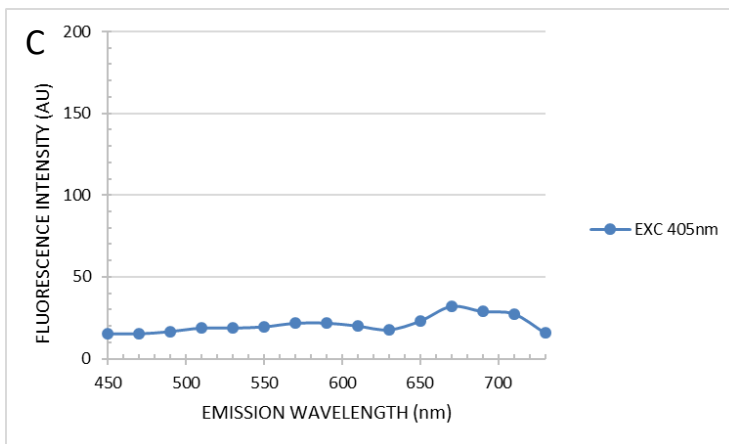
UV (405 nm)
excitation response



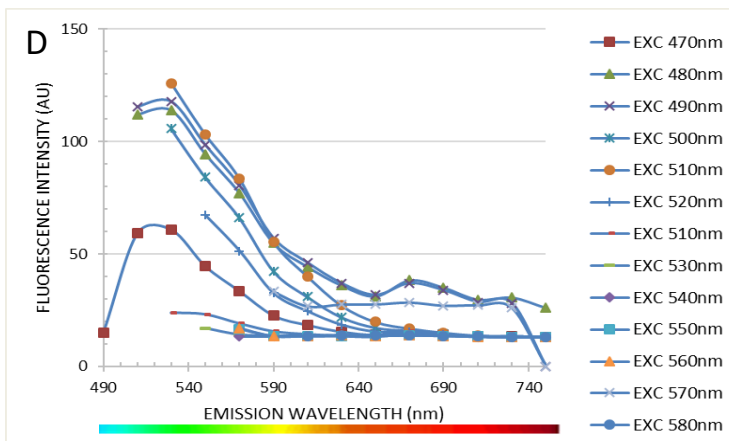
Visible light
(470-670 nm)
excitation response



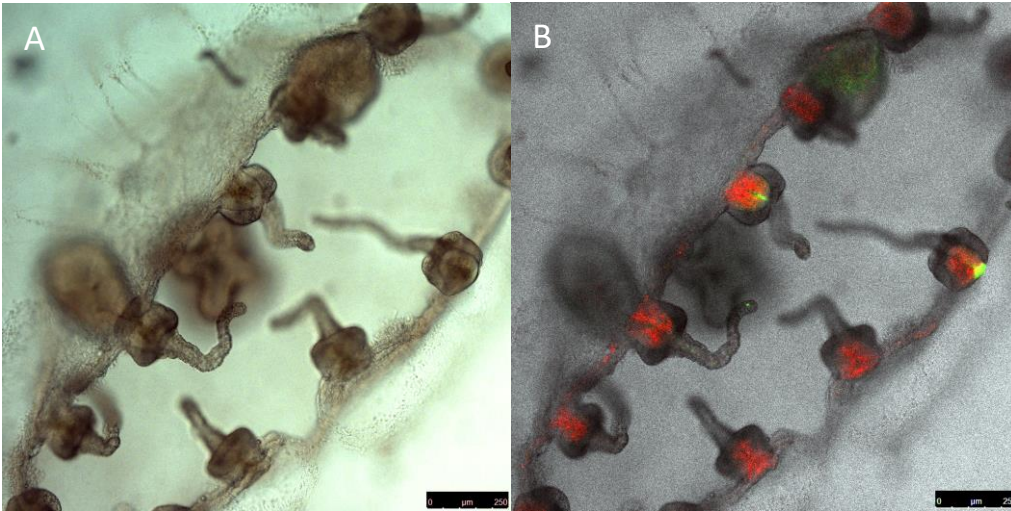
Excitation and Emission Spectra



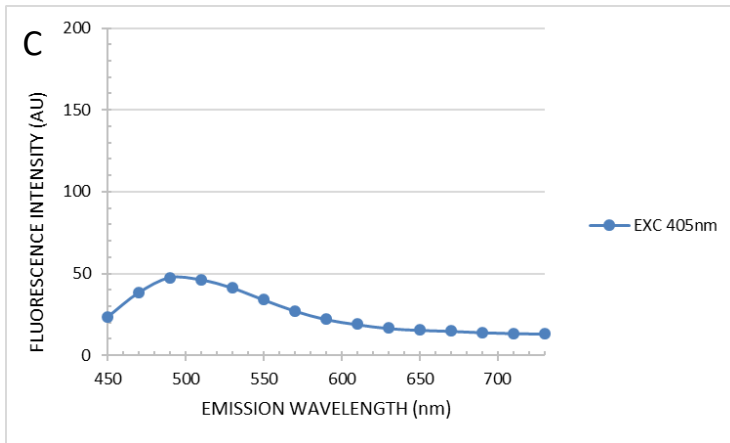
UV (405 nm)
excitation response



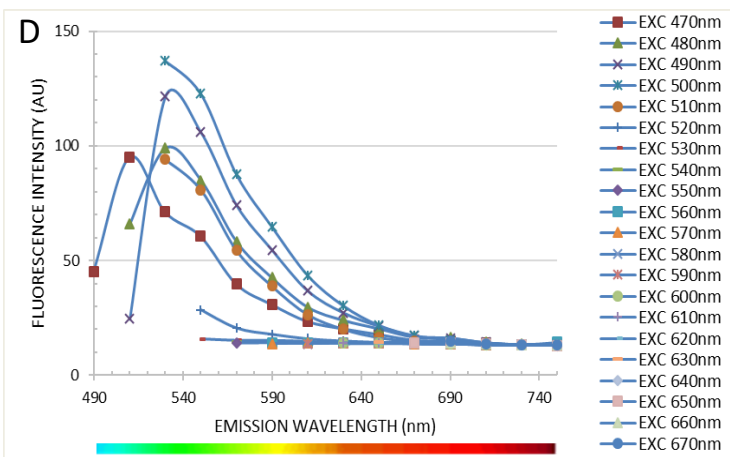
Visible light
(470-670 nm)
excitation response



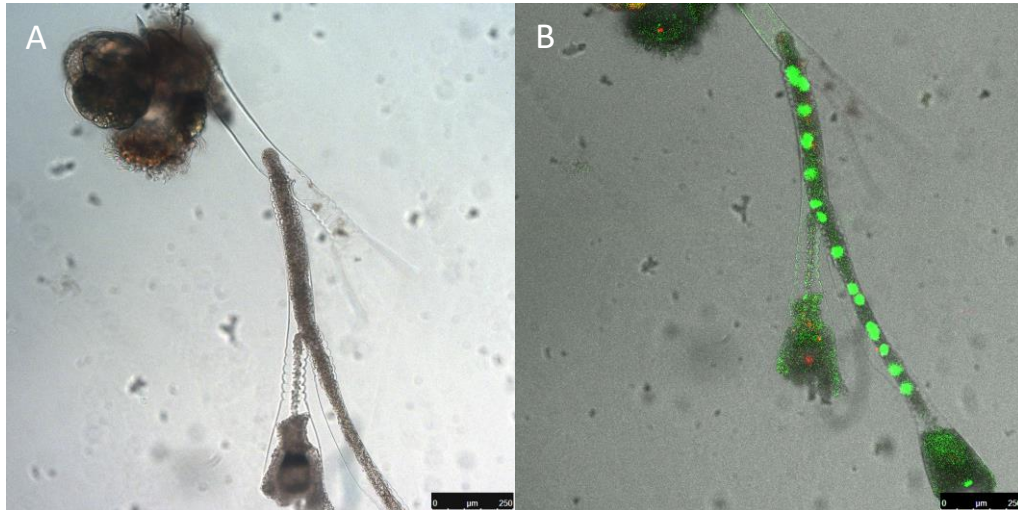
Excitation and Emission Spectra



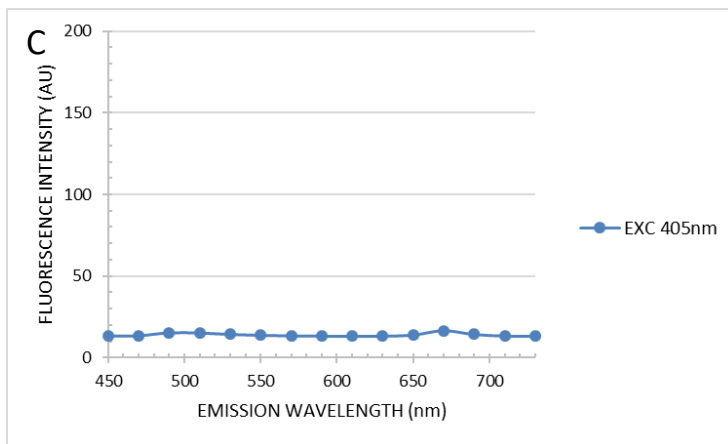
UV (405 nm)
excitation response



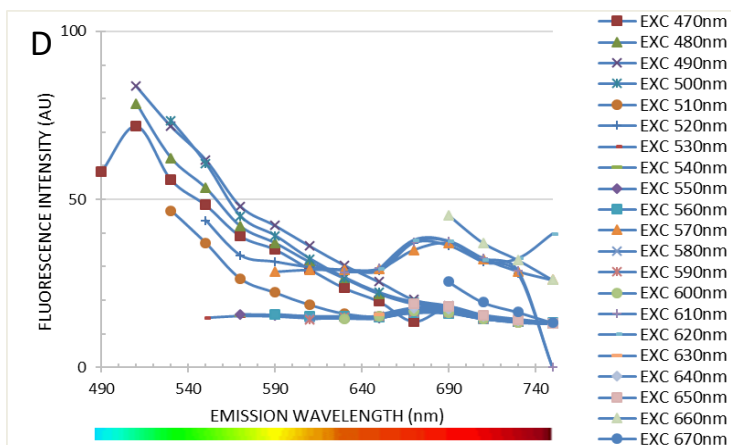
Visible light
(470-670 nm)
excitation response



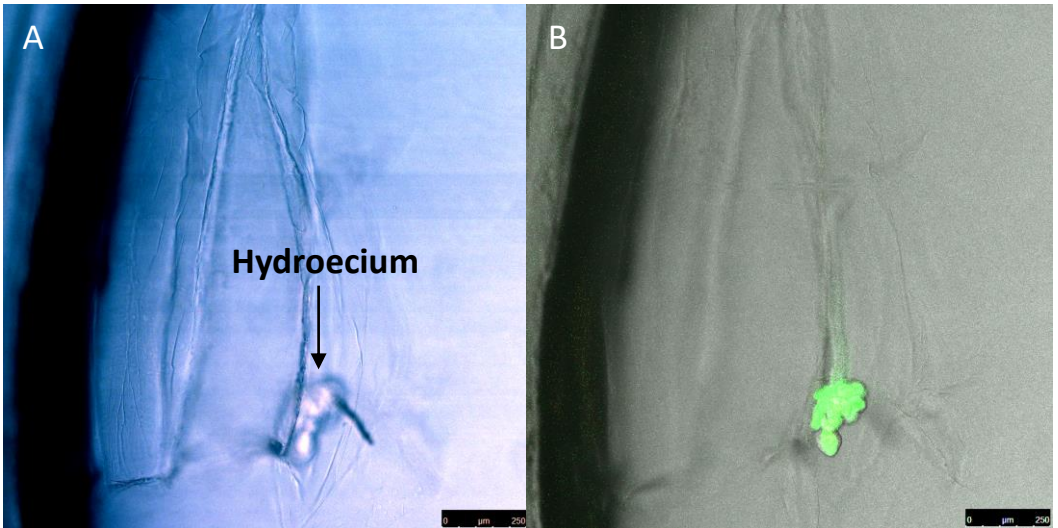
Excitation and Emission Spectra



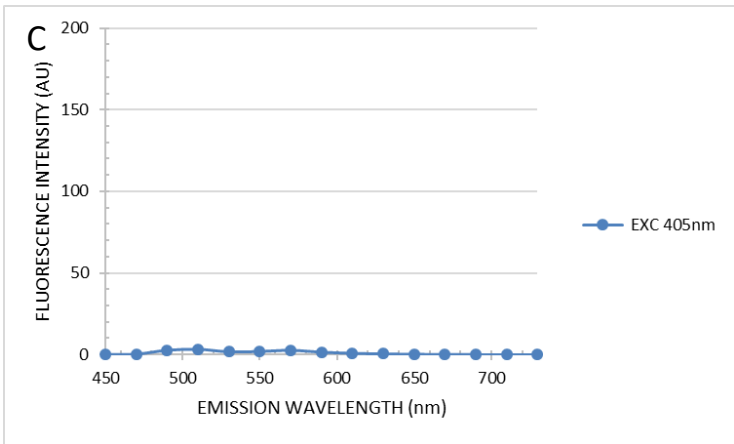
UV (405 nm)
excitation response



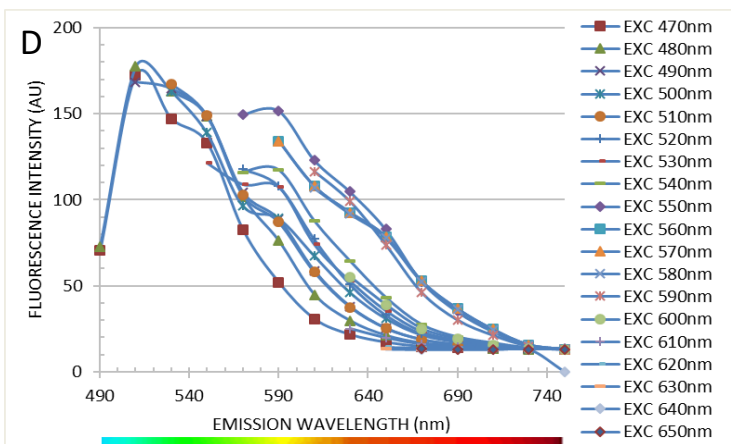
Visible light
(470-670 nm)
excitation response



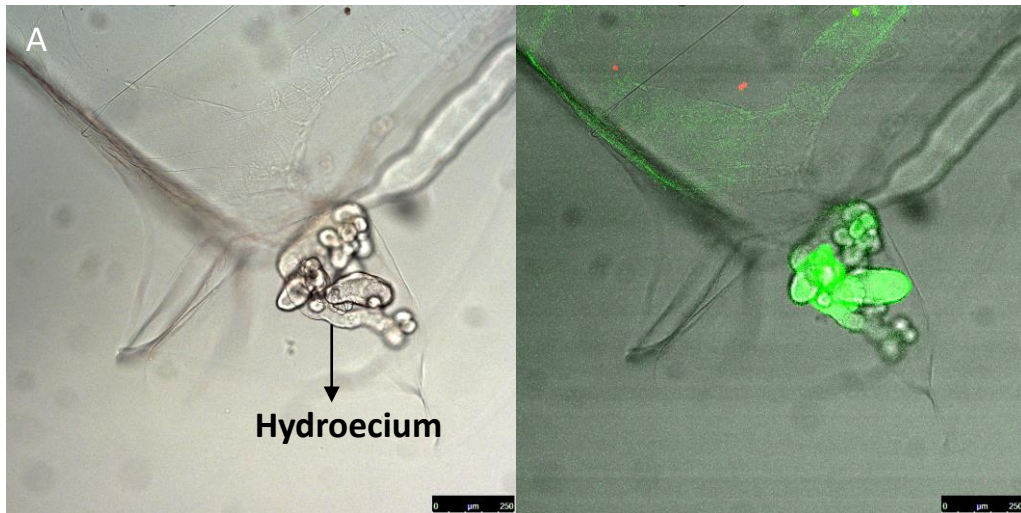
Excitation and Emission Spectra



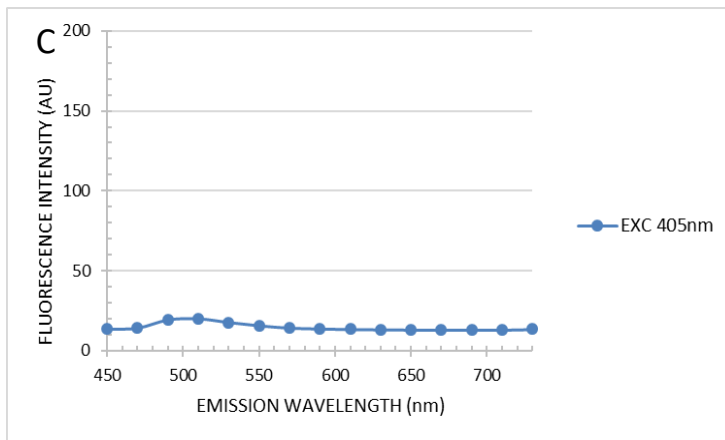
UV (405 nm)
excitation response



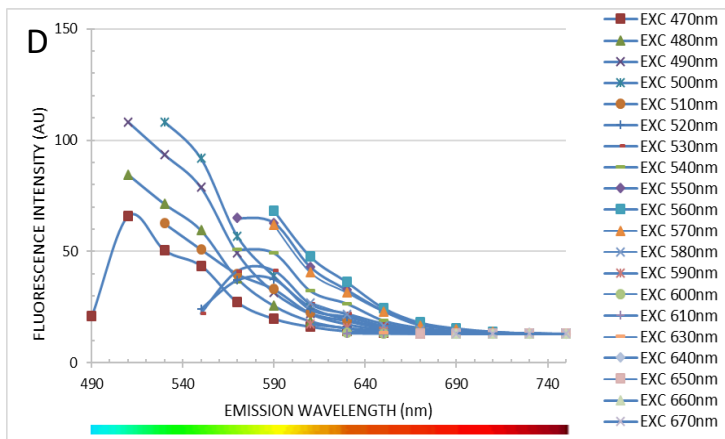
Visible light
(470-670 nm)
excitation response



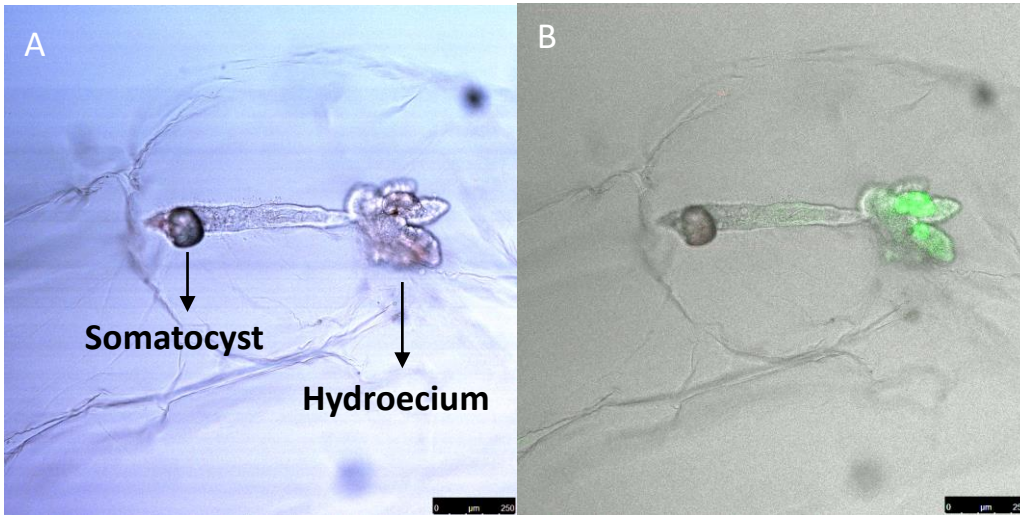
Excitation and Emission Spectra



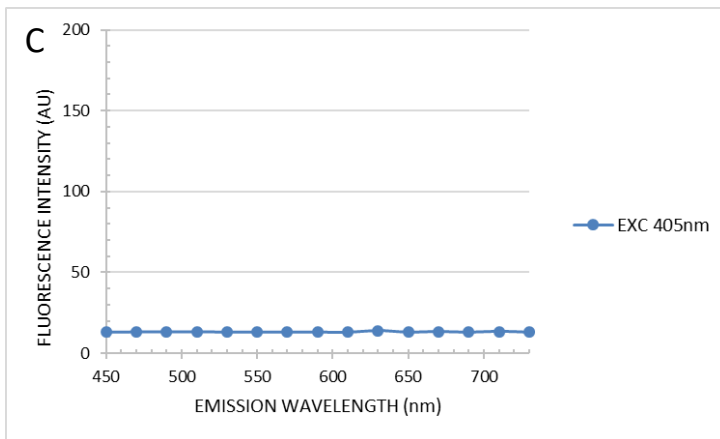
UV (405 nm)
excitation response



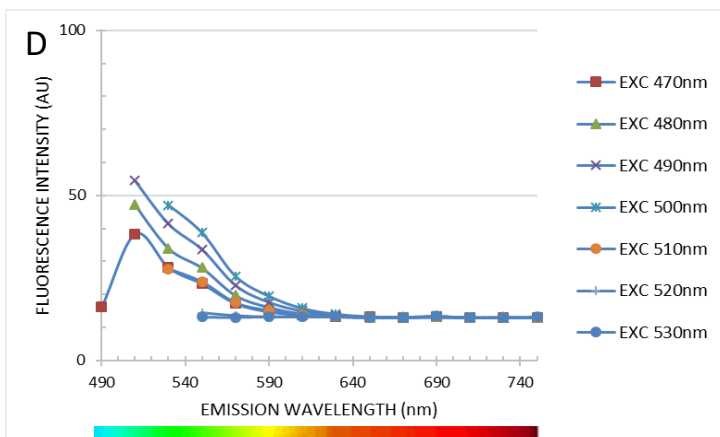
Visible light
(470-670 nm)
excitation response



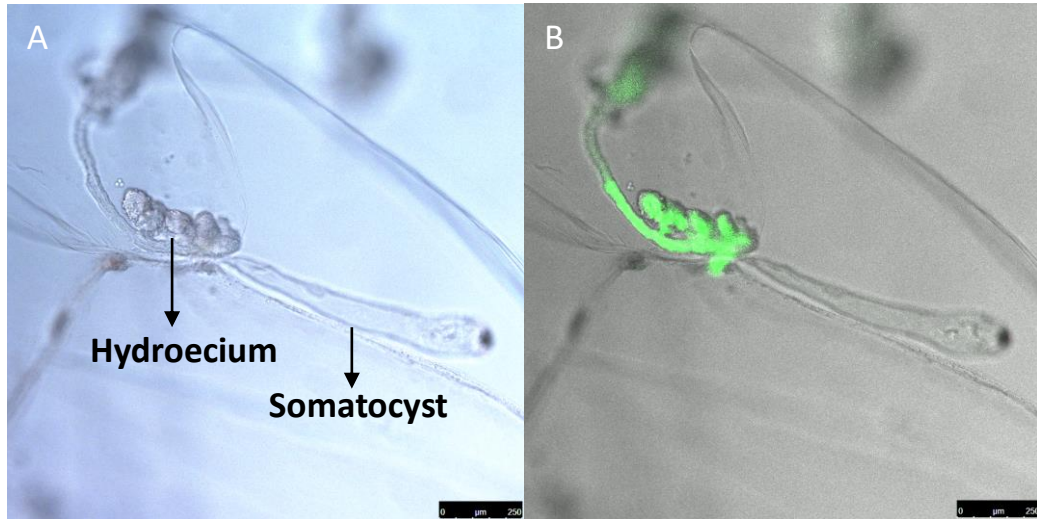
Excitation and Emission Spectra



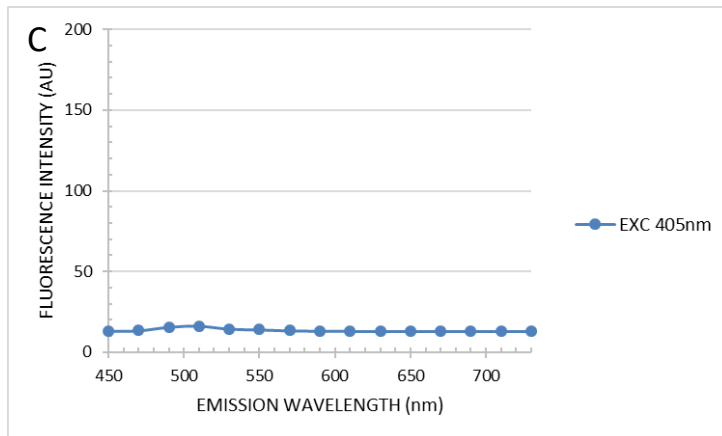
UV (405 nm)
excitation response



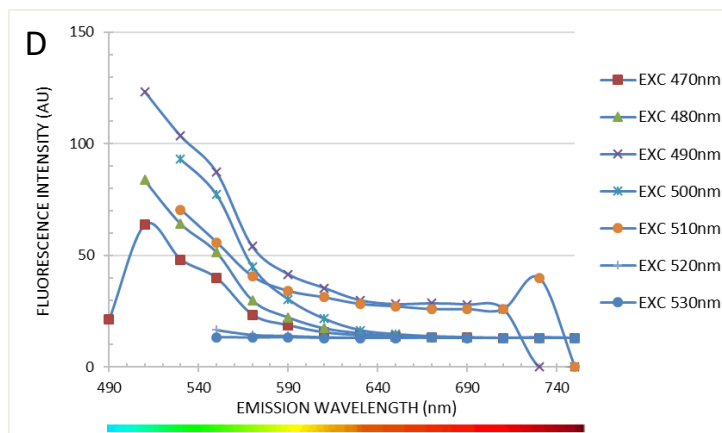
Visible light
(470-670 nm)
excitation response



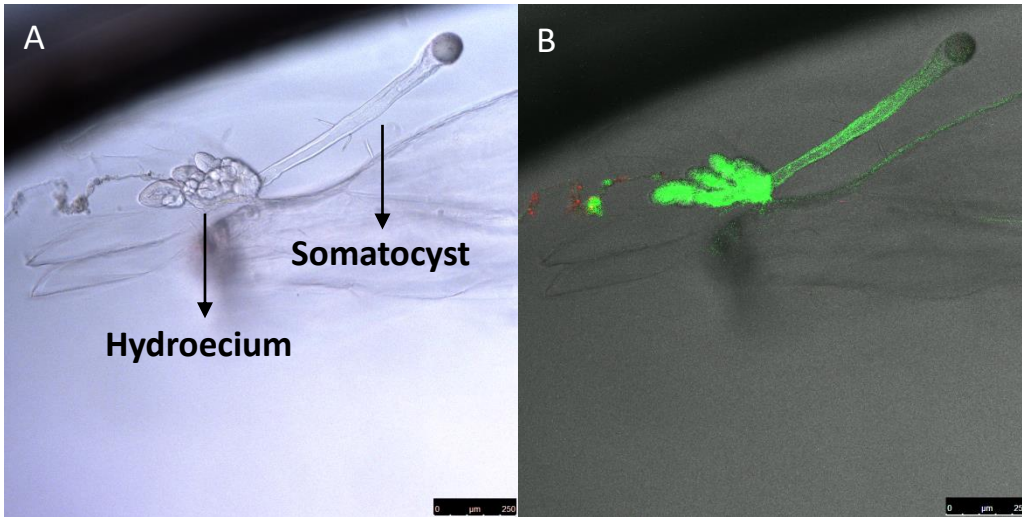
Excitation and Emission Spectra



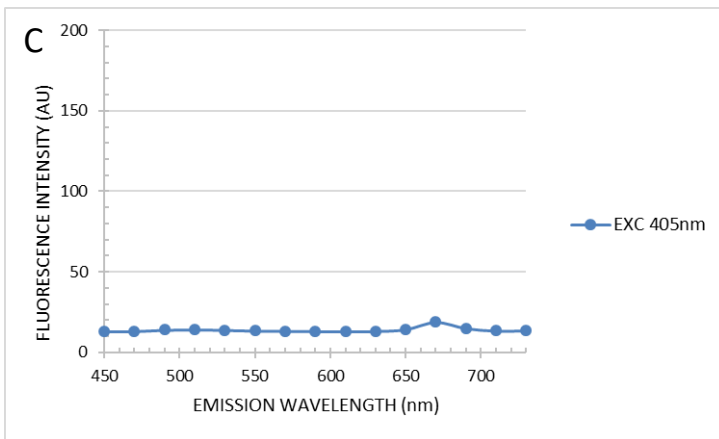
UV (405 nm)
excitation response



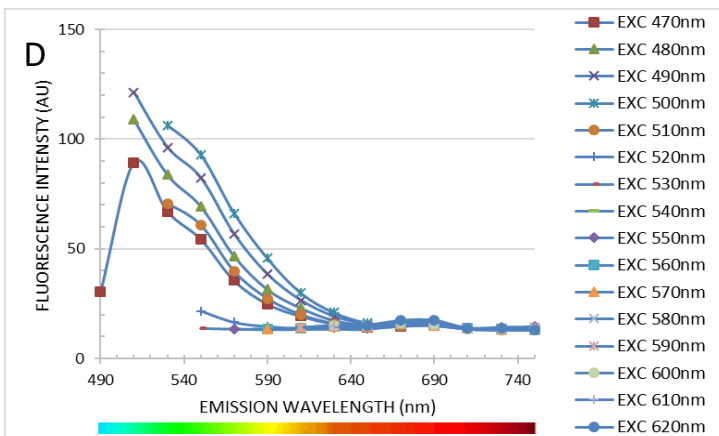
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



UV (405 nm)
excitation response



Visible light
(470-670 nm)
excitation response

Comments:

Many species of hydrozoans have been studied in the attempt to find a fluorescent protein with new characteristics, especially in siphonophores where no FP have been characterized yet.

Interestingly, most of all hydrozoans display fluorescent patterns similar to GFP (λ_{exc} 488 nm - λ_{ems} 510 nm) with excitation between 470 and 490 nm and emission between 510 and 530 nm with high intensity. Overall, we notice a very low signal in response to the UV light excitation.

In jellyfish, on Figures 2.15 and 2.16, no response test to chlorophyll have been performed as at this point of the study, the protocol was still in implement. In Figures 2.15 D to 2.19 D, we observe from the visible light excitation test a high intensity emission between 510 nm and 530 nm with excitation around 470 nm and 510 nm. Only the hydrozoan on Figure 2.19 C shows an intensity at around 50 A.U with green emission.

In the scyphozoa, Figure 2.20 B might correspond to zooxanthellae responding to laser excitation and showing bright green light. In fact, it is known that zooxanthellae are single-celled dinoflagellates, able to live in symbiosis with jellyfish. Figure 2.20 D is consistent with this pattern as we denote a high green emission signal after assessing the visible light excitation test. Excitation wavelength from 470 nm to 490 nm shows a strong emission at 510 nm with intensity between 70 and 85 A.U.

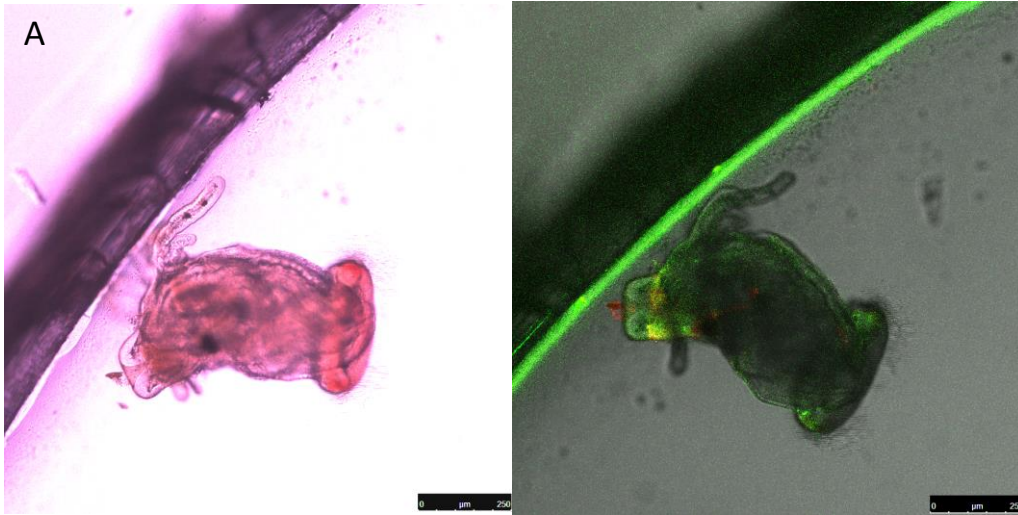
In siphonophores, the test assessing chlorophyll exhibit mainly a response in the green, see Figures 2.21 B to 2.25 B. This bright green light is located mainly in the hydroecium and in the somatocyst. It has been described that somatocyst represent a prominent extension of the gastrovascular system that runs anteriorly from the external pedicular canal at the point it reaches the hydroecial wall (Haddock *et al.*, 2005). Therefore, these two structures may be the location of algal content and to this extent correspond to chlorophyll. All siphonophores show a high green emission pattern after testing the visible light excitation response, with excitation

wavelengths ranging from 470 nm to 490 nm and emission at 510 nm, see Figures 2.21 D to 2.25

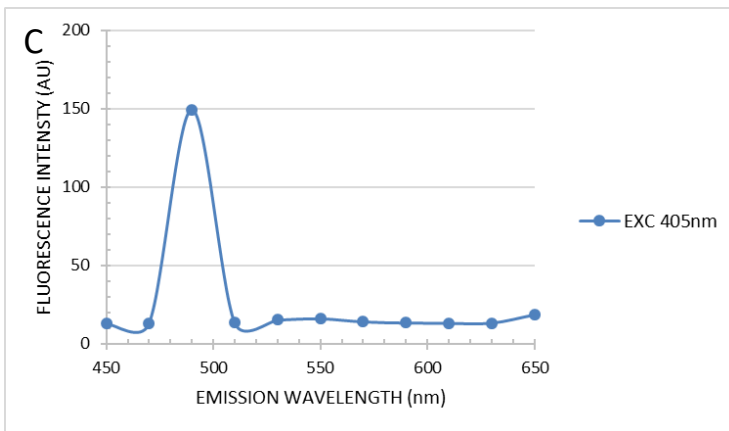
D.

As most bioluminescent emission spectra are blue (λ_{ems} 475 nm), it could be possible that the fluorescent emission pattern observed in siphonophores originate from a FP.

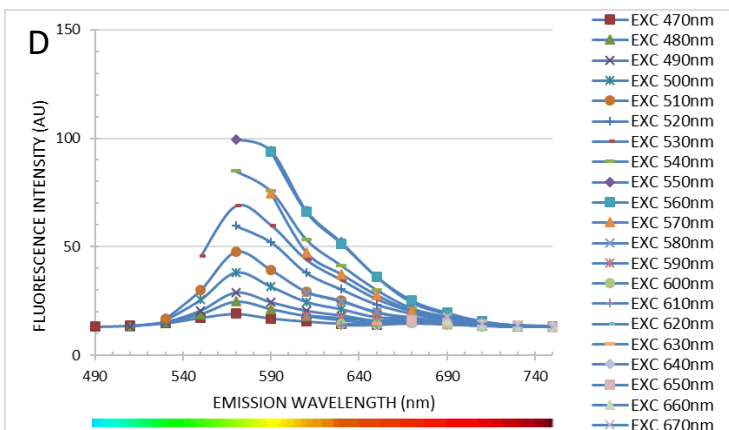
4.2.3 Phoronida



Excitation and Emission Spectra



UV (405 nm)
excitation response



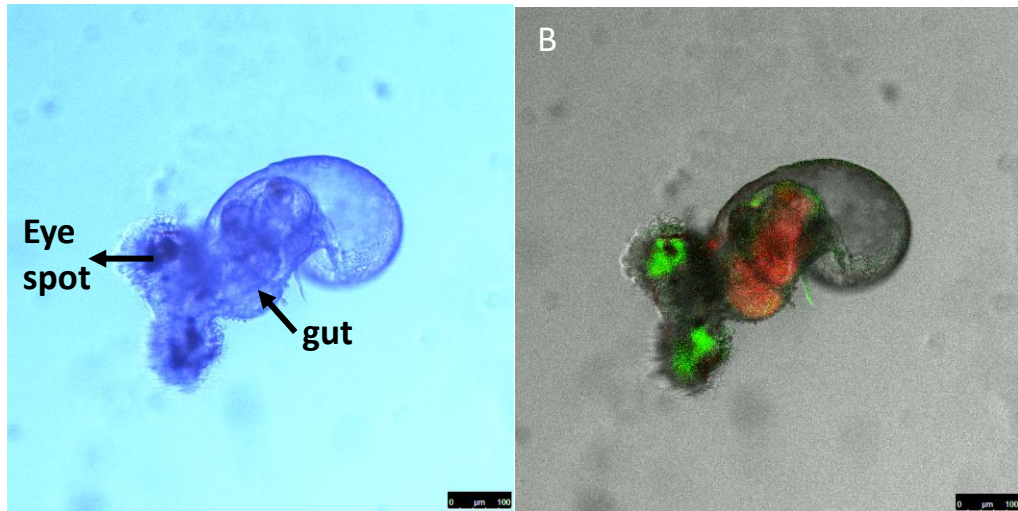
Visible light
(470-670 nm)
excitation response

Comments:

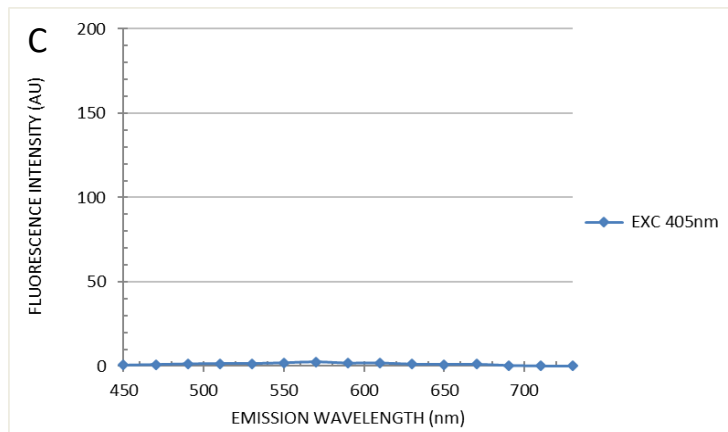
Being not very abundant in the zooplankton sampling, only one specimen of Phoronida has been analysed at the confocal microscope to assess its fluorescent characteristics. This species presents a high peak emission at 490 nm in response to UV excitation, see Figure 2.26 C. We can notice an excitation mainly in the green part of the visible spectrum with a rather high emission in the yellow spectrum. However, it is important to underline that when the experiment was performed the animal was next to the edge of the Petri dish. Therefore, the fluorescence may originate from a reflection of the laser on this surface, see Figure 2.26 B.

4.2.4 Mollusca

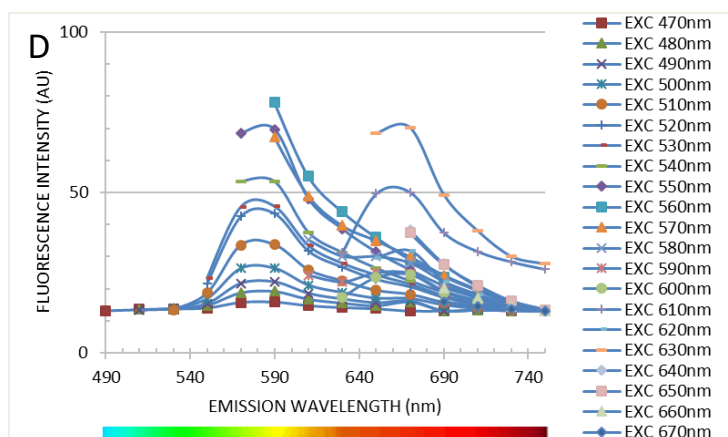
Figure 2.27 Mollusca, Veliger larva of a Gastropod 07/06/2018



Excitation and Emission Spectra

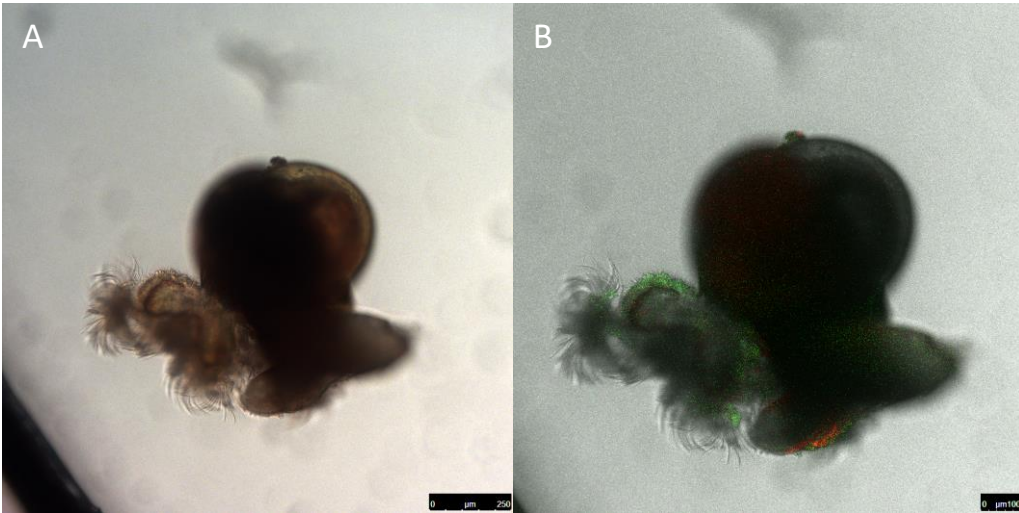


UV (405 nm)
excitation response

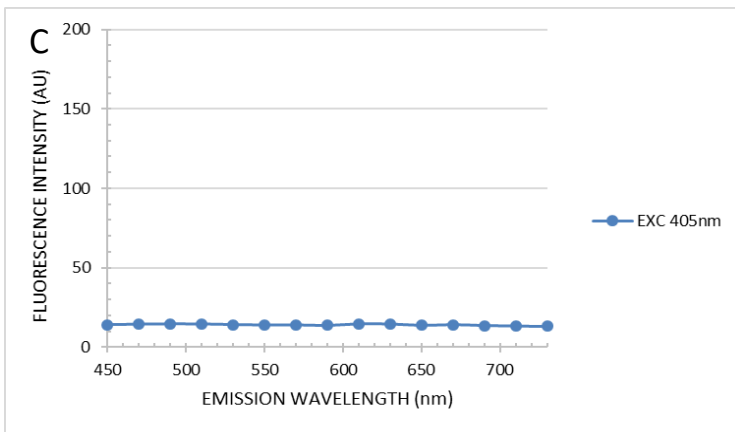


Visible light
(470-670 nm)
excitation response

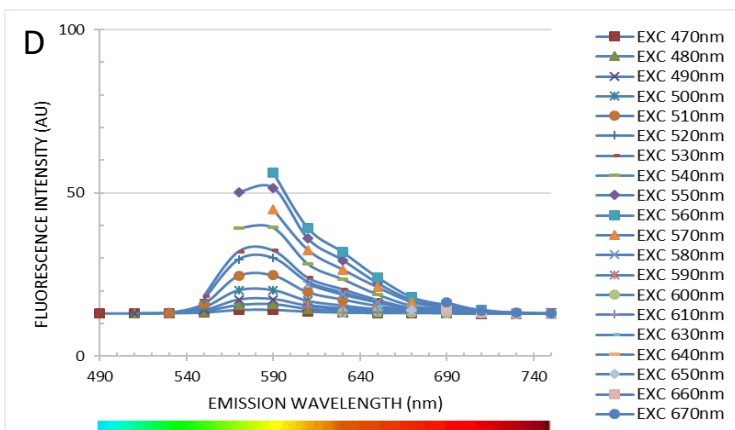
Figure 2.28 Mollusca, Veliger larva of a Gastropod 29/05/2018



Excitation and Emission Spectra

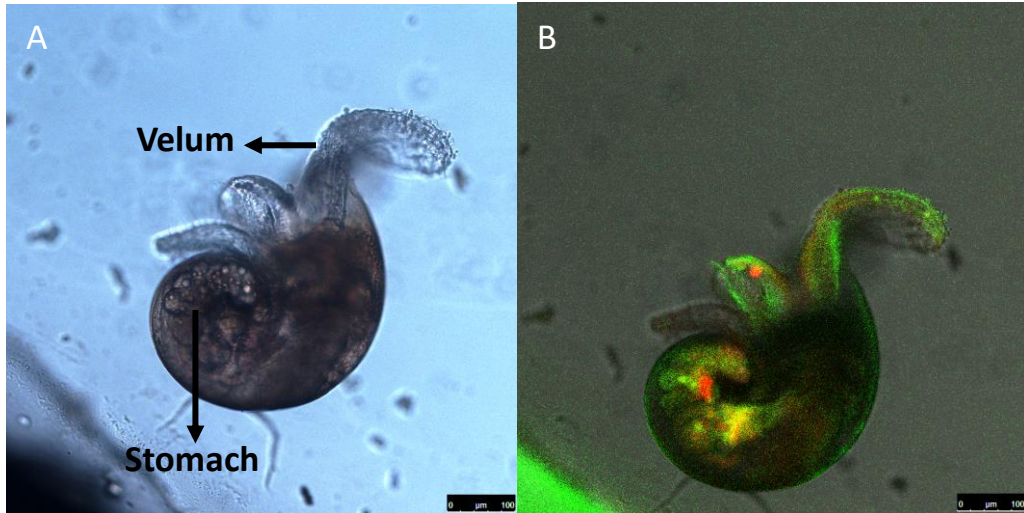


UV (405 nm)
excitation response

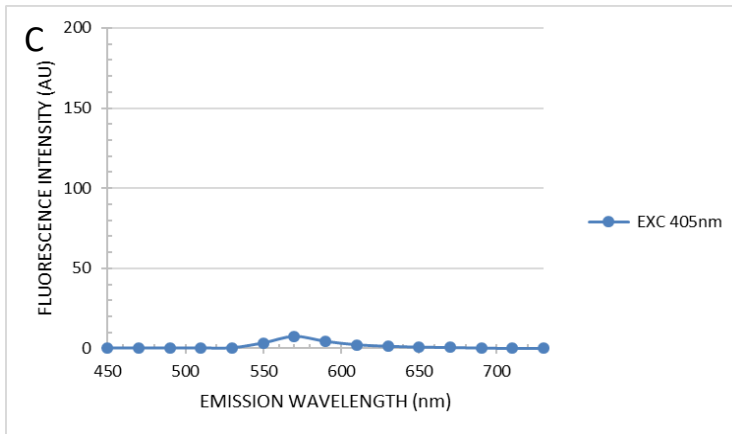


Visible light
(470-670 nm)
excitation response

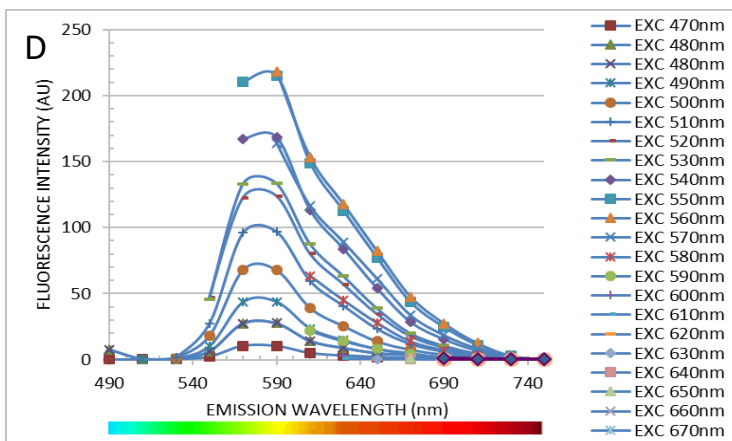
Figure 2.29 Mollusca, Veliger larva of a Gastropod 06/07/2018



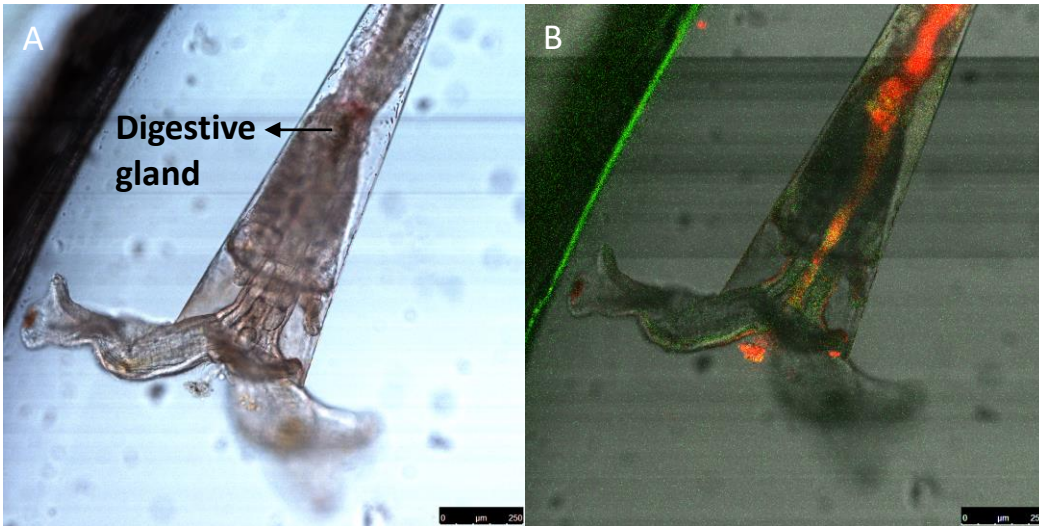
Excitation and Emission Spectra



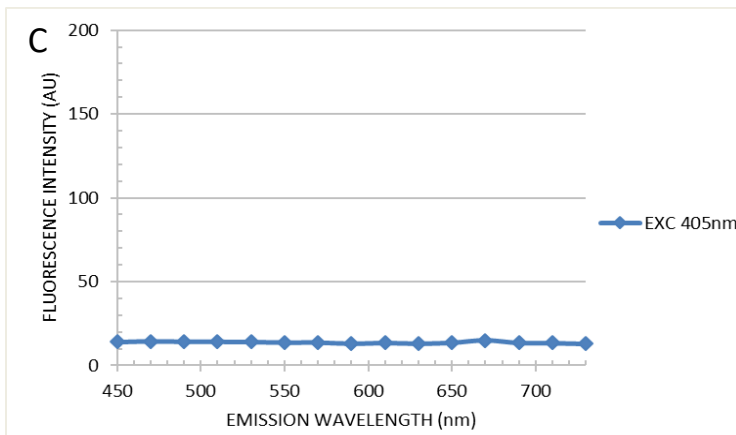
UV (405 nm)
excitation response



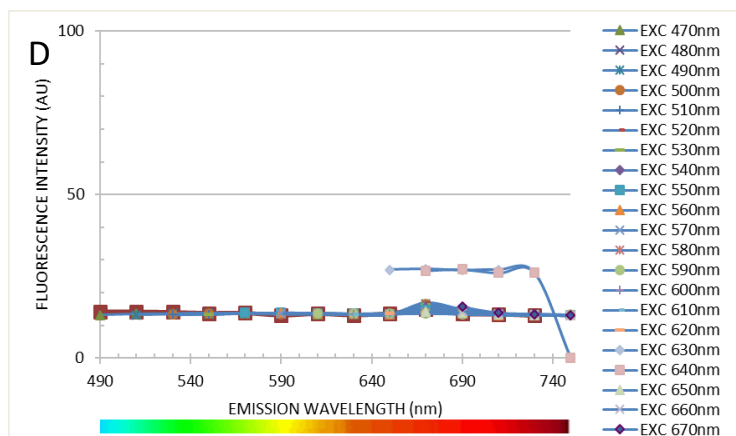
Visible light
(470-670 nm)
excitation response



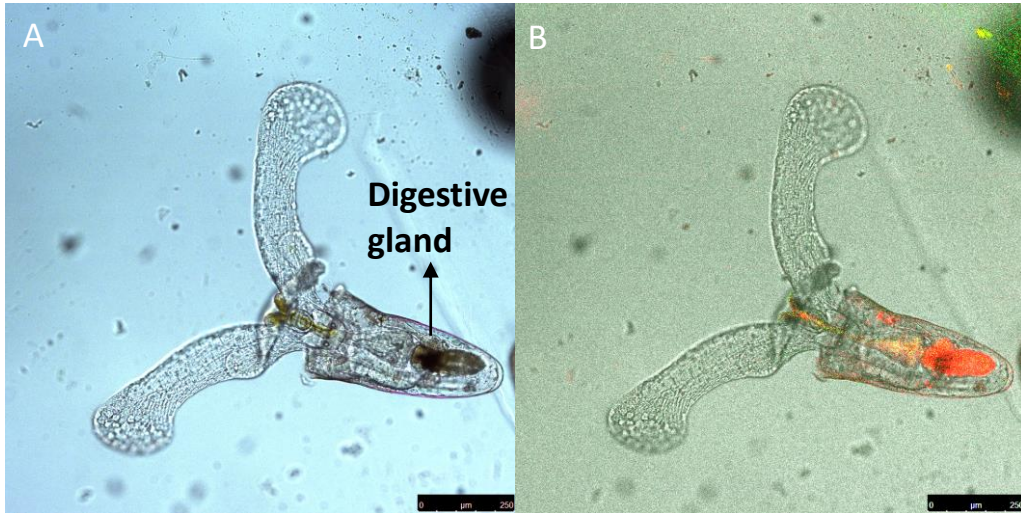
Excitation and Emission Spectra



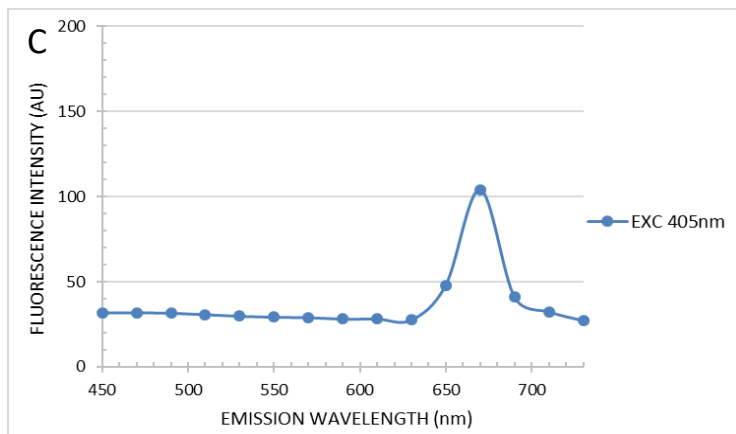
UV (405 nm)
excitation response



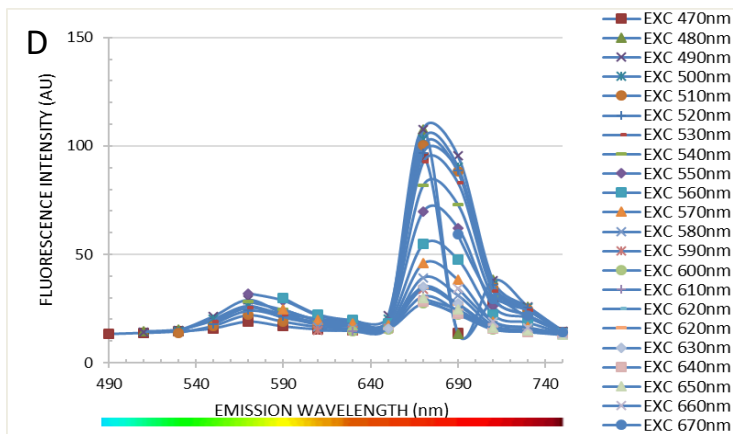
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



UV (405 nm)
excitation response



Visible light
(470-670 nm)
excitation response

Comments:

Several molluscs have been investigated at the confocal microscope, mainly larva of gastropod and pteropod.

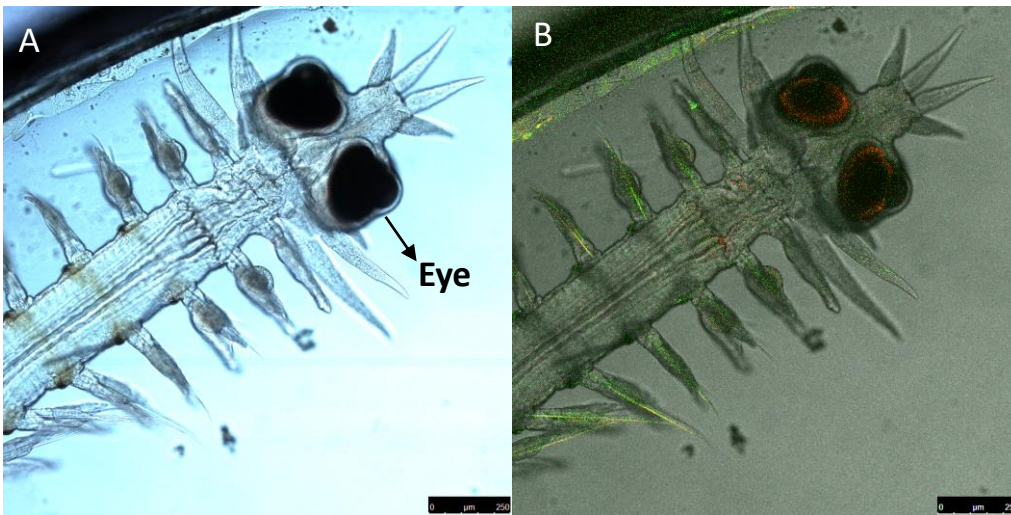
The test in response to UV excitation shows only a high emission peak at 670 nm for the pteropod of Figure 2.31 C, which corresponds to the chlorophyll spectra. This is also confirmed by the red fluorescence in this animal (Fig 2.31 B) located in the digestive gland, most probably due to algae.

The other pteropod also presents fluorescence in the digestive gland and stomach (Fig 2.30 B).

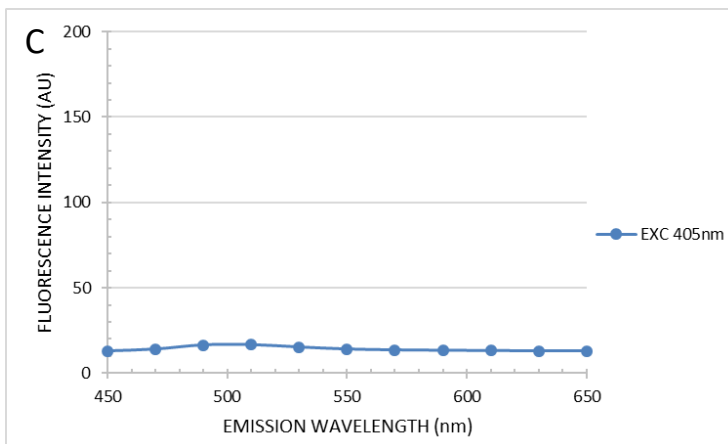
Only the pteropod of Fig 2.31 D shows a high red emission pattern with an intensity of 100 A.U. However, the one on Fig 2.30 D is not exploitable as we cannot distinguish a particular signal on the graph.

The test to the visible light excitation response in veligers (Fig 2.27 D to 2.29 D) show a signal in the green-yellow part of the spectrum mainly, with intensities from 50 to 210 A.U and excitation wavelengths of 550 nm and 560 nm.

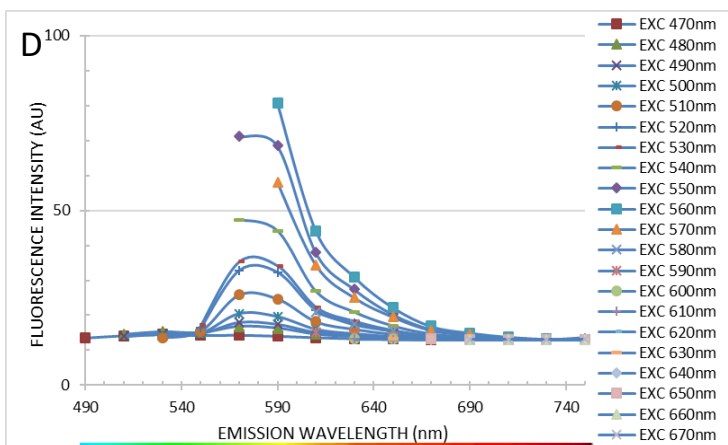
4.2.5 Annelida



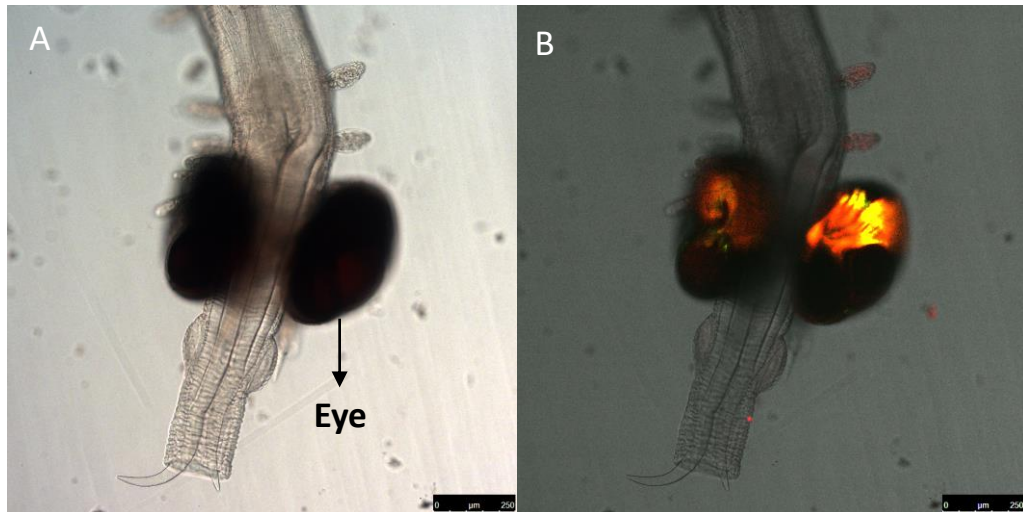
Excitation and Emission Spectra



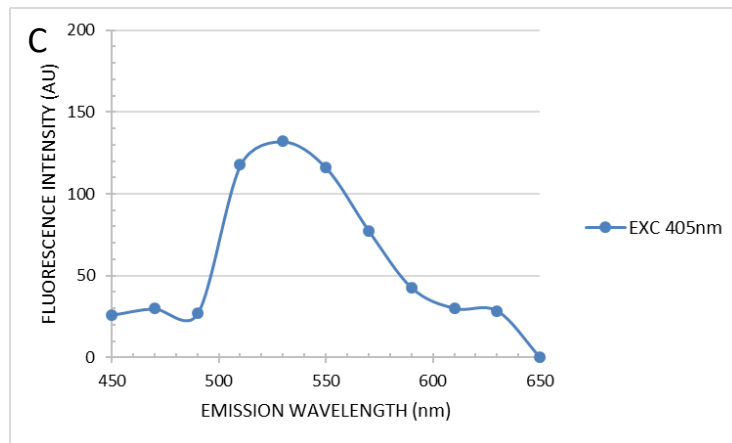
UV (405 nm)
excitation response



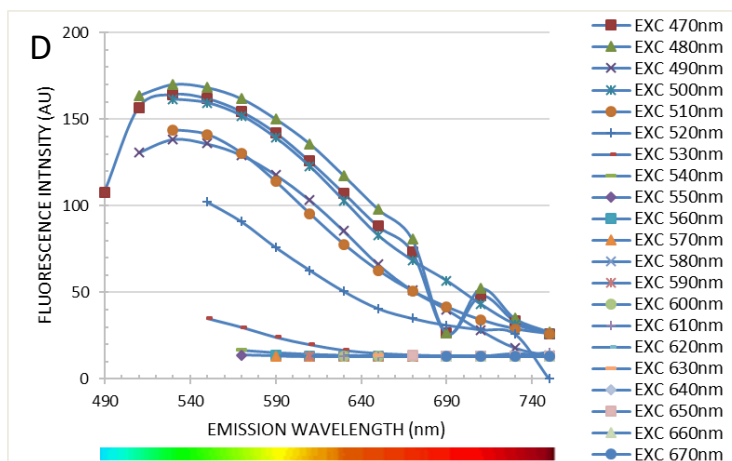
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



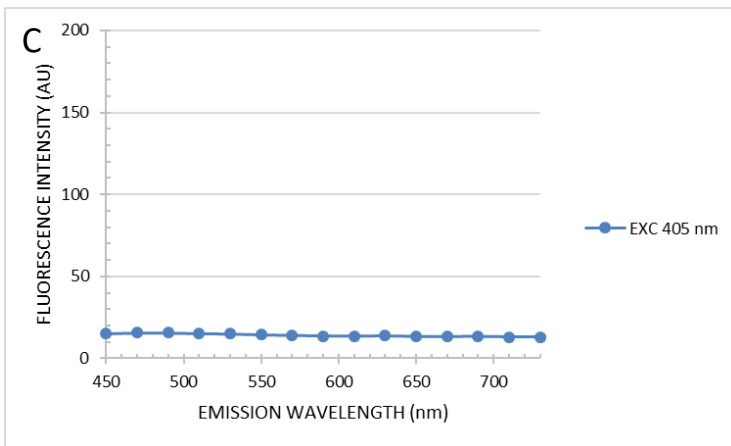
UV (405 nm)
excitation response



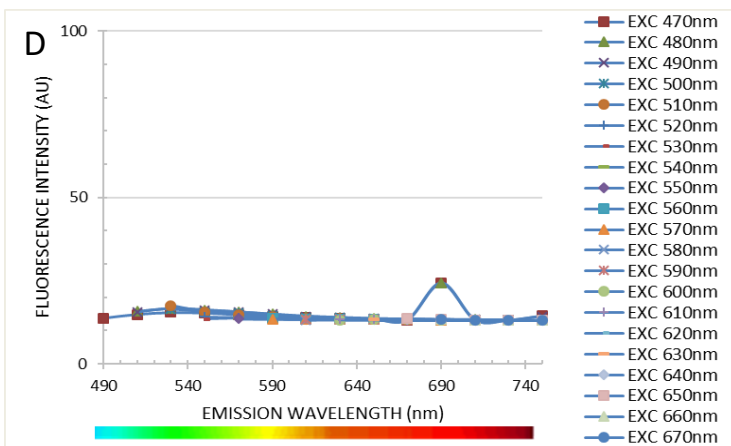
Visible light
(470-670 nm)
excitation response



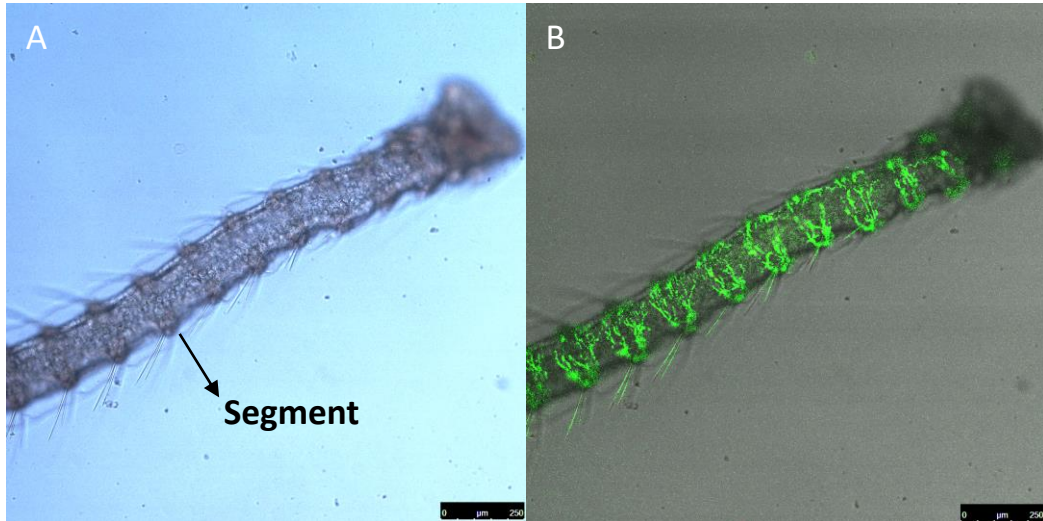
Excitation and Emission Spectra



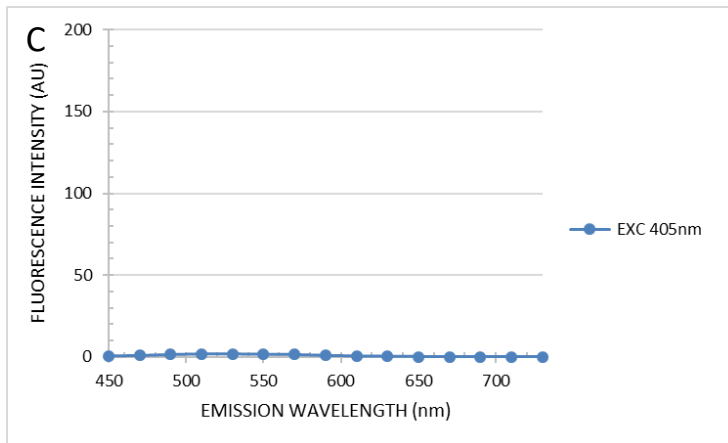
UV (405 nm)
excitation response



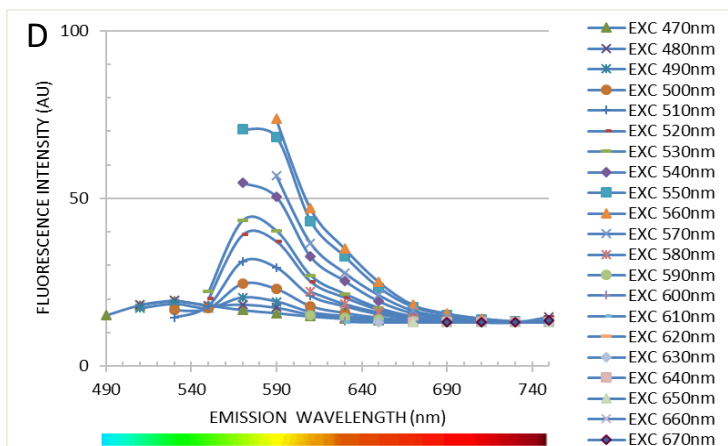
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



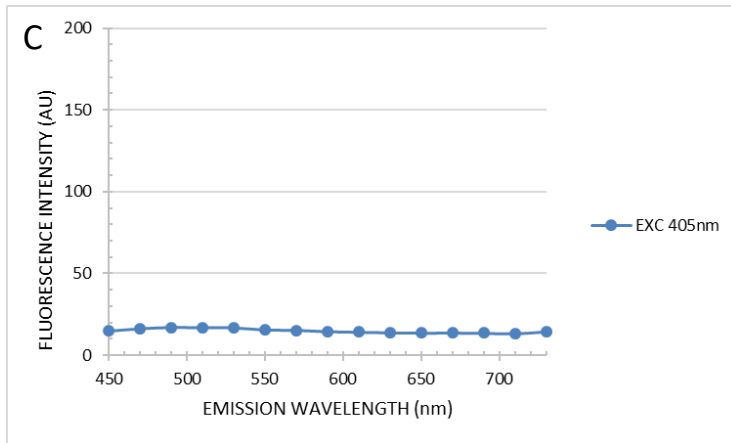
UV (405 nm)
excitation response



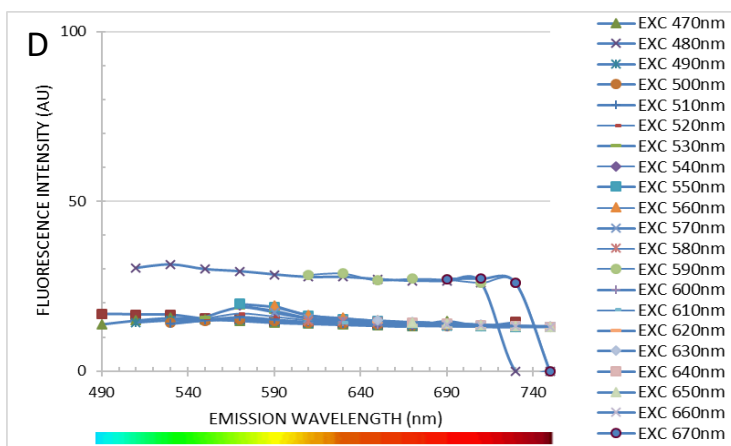
Visible light
(470-670 nm)
excitation response



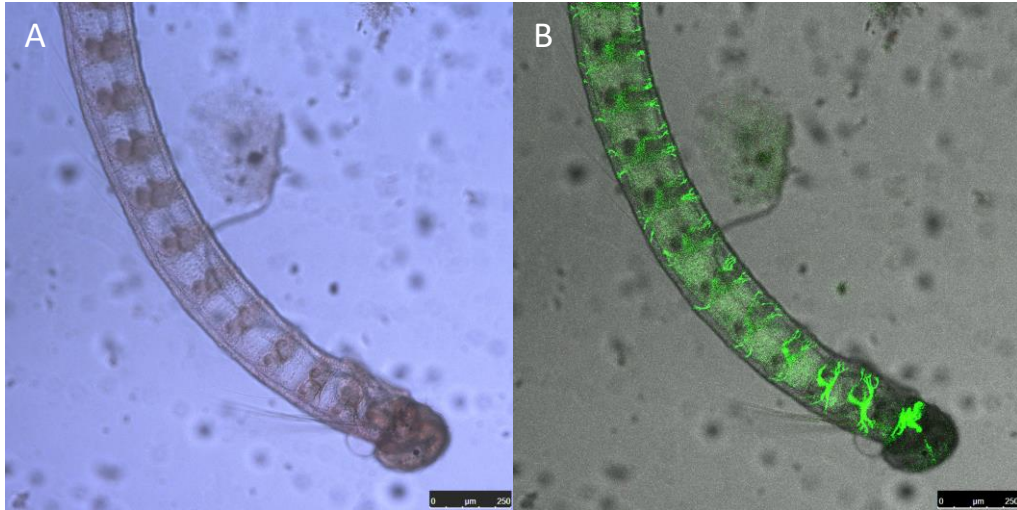
Excitation and Emission Spectra



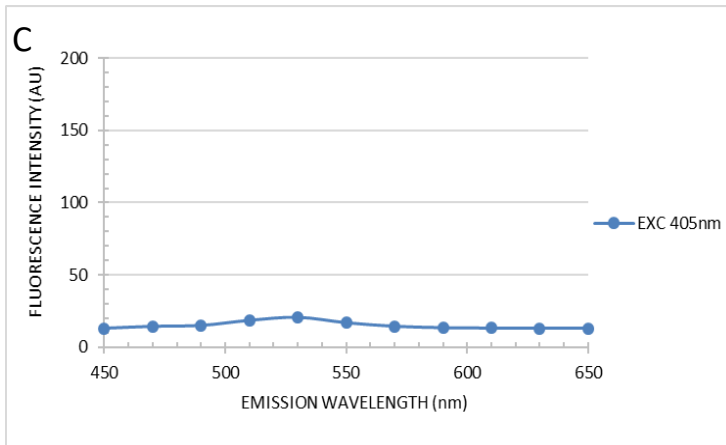
UV (405 nm)
excitation response



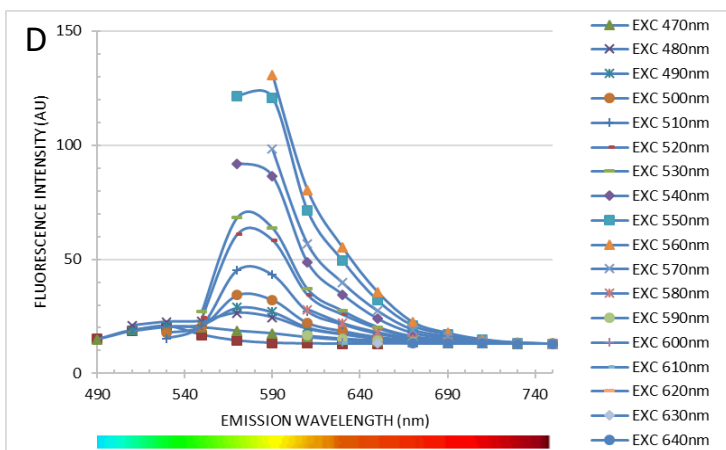
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



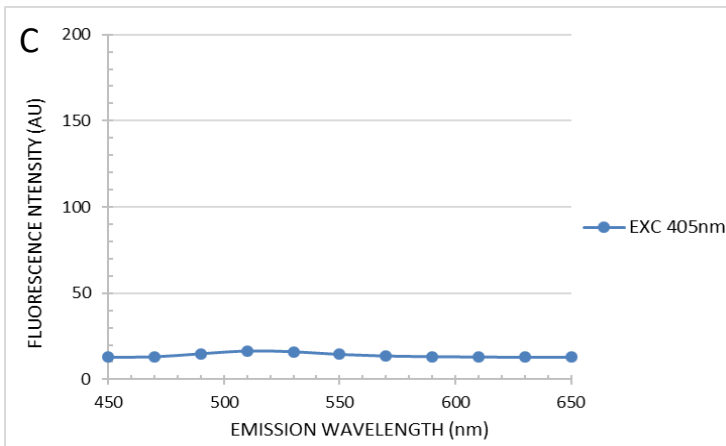
UV (405 nm)
excitation response



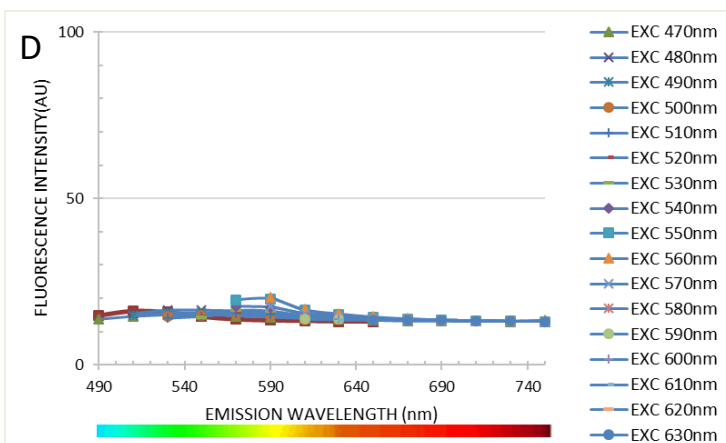
Visible light
(470-670 nm)
excitation response



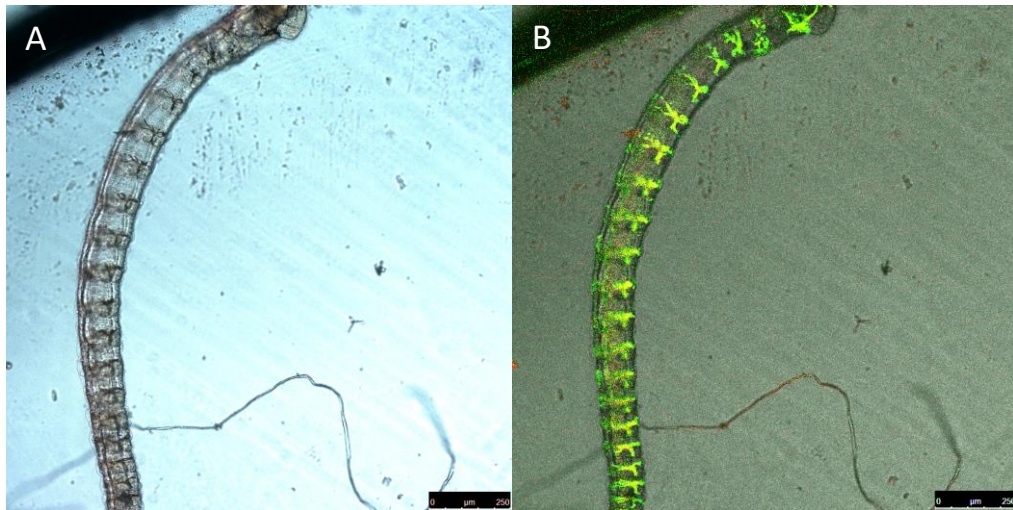
Excitation and Emission Spectra



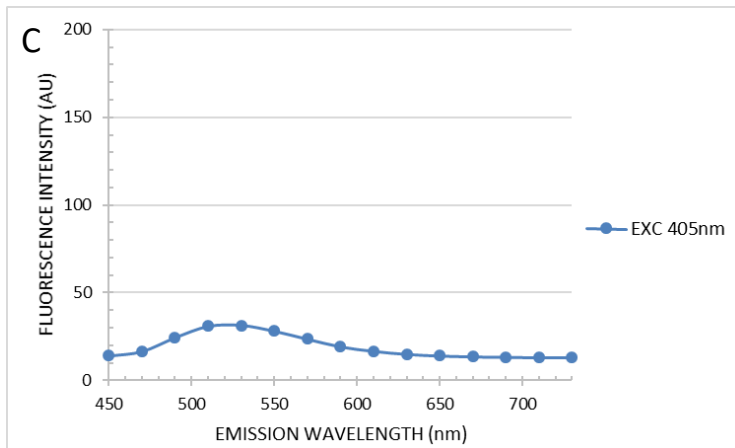
UV (405 nm)
excitation response



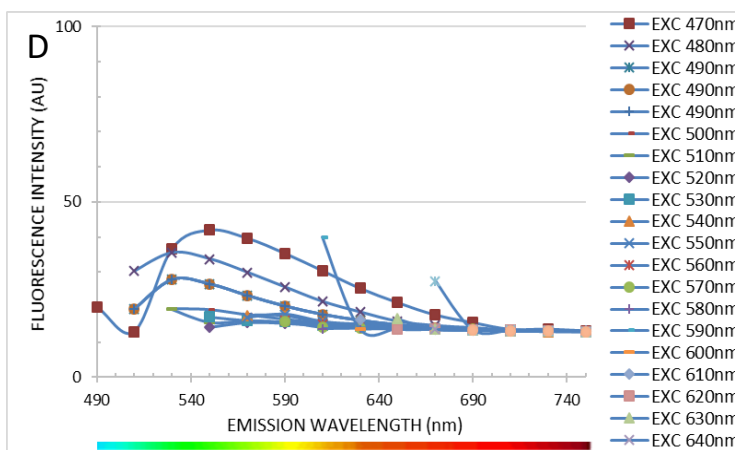
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



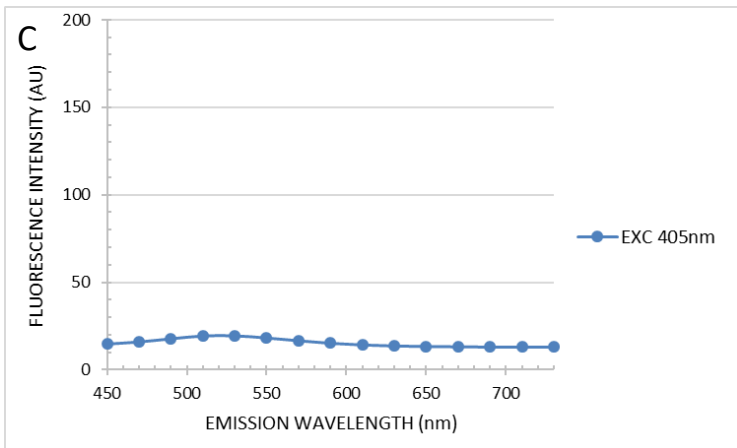
UV (405 nm)
excitation response



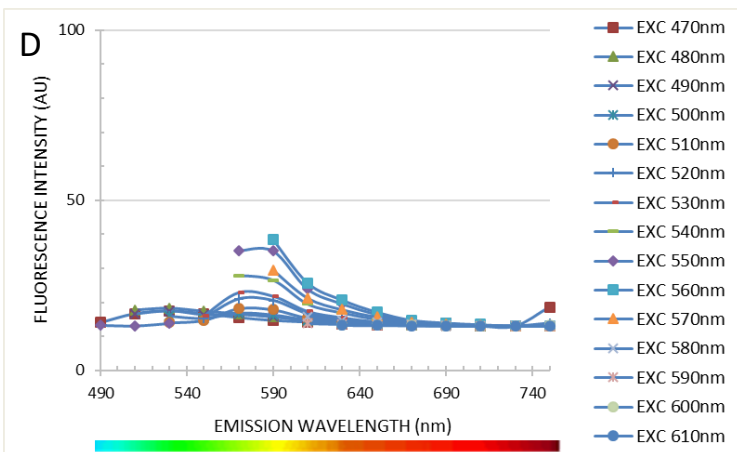
Visible light
(470-670 nm)
excitation response



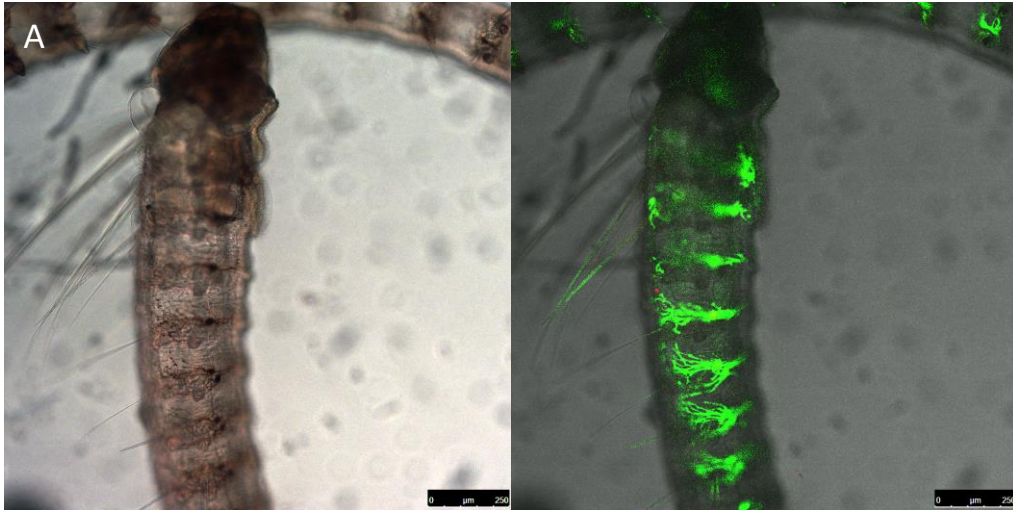
Excitation and Emission Spectra



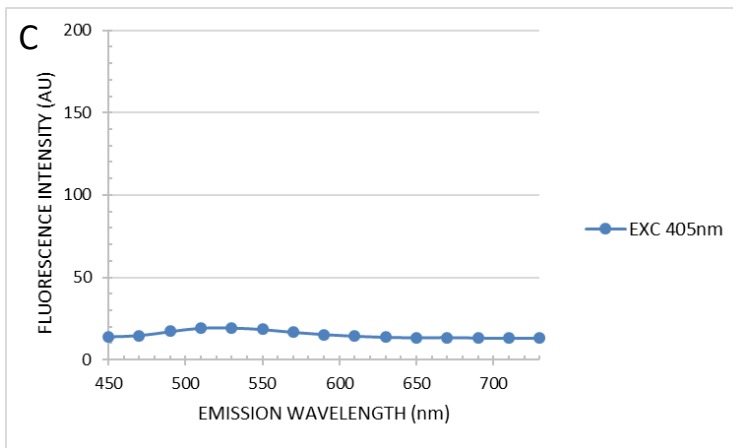
UV (405 nm)
excitation response



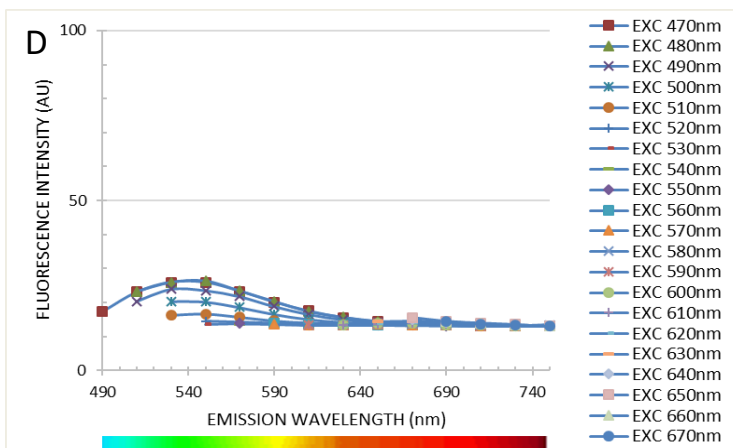
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



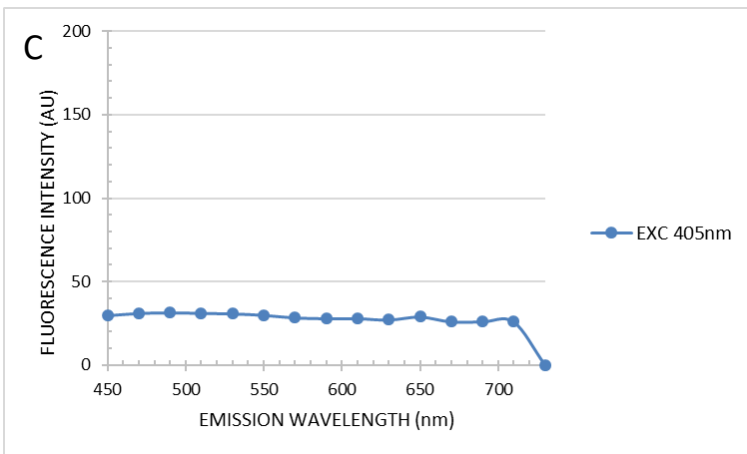
UV (405 nm)
excitation response



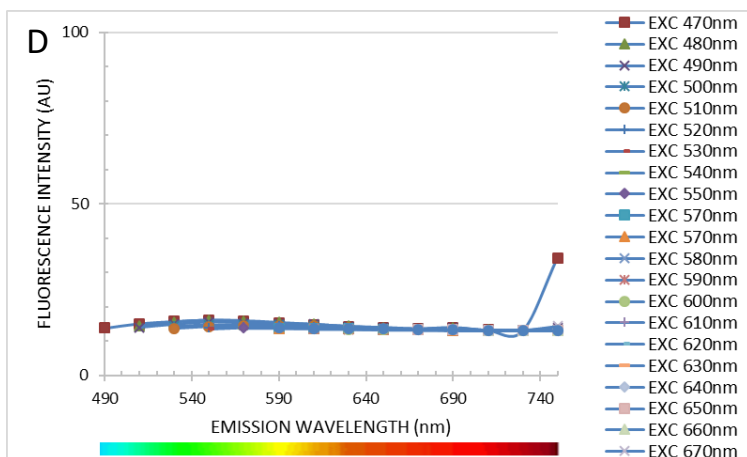
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



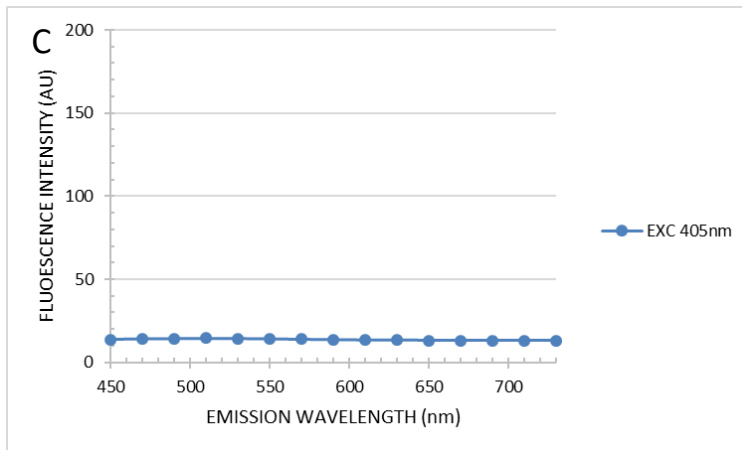
UV (405 nm)
excitation response



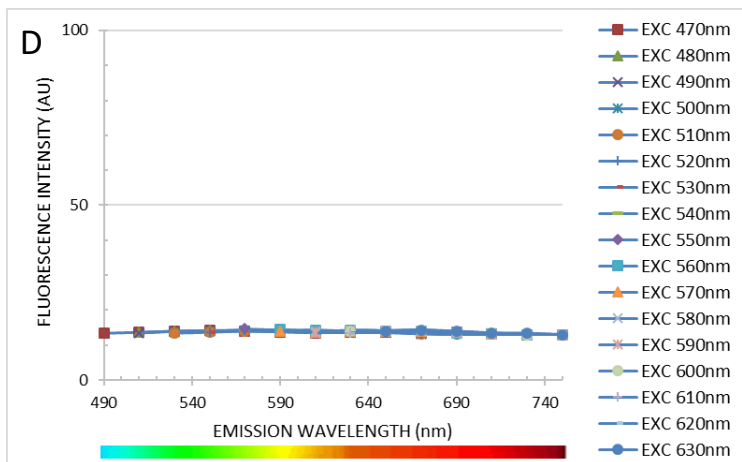
Visible light
(470-670 nm)
excitation response



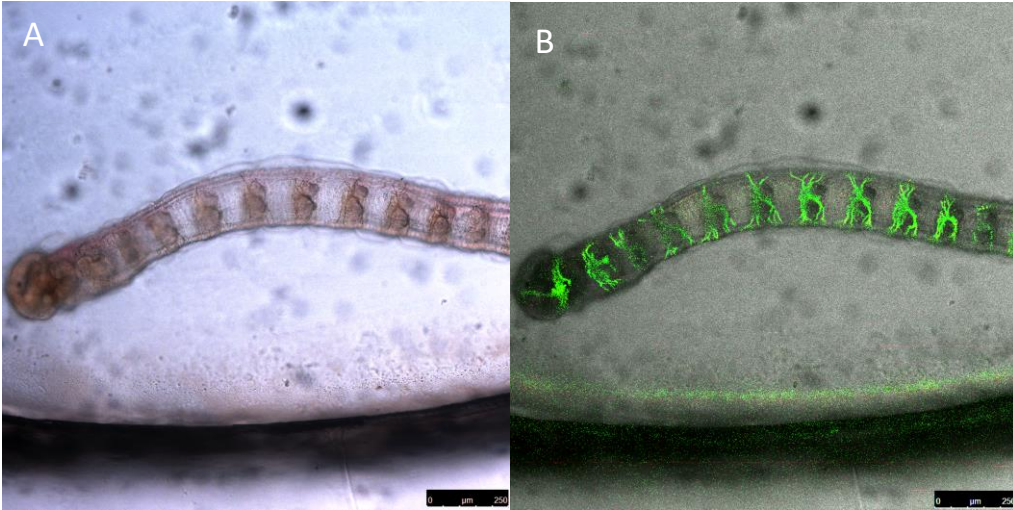
Excitation and Emission Spectra



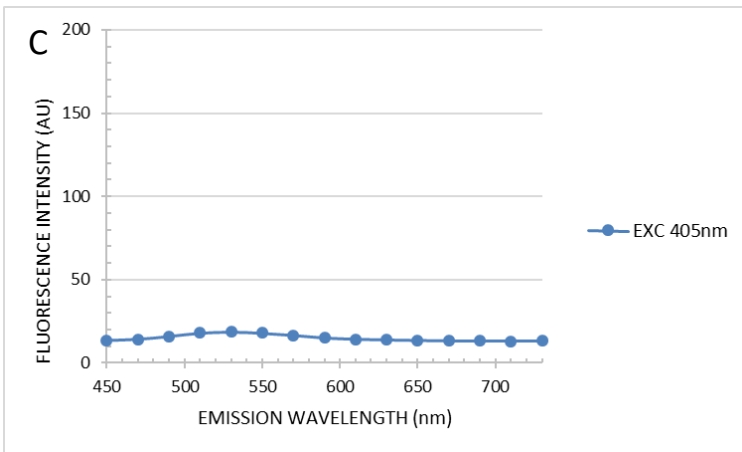
UV (405 nm)
excitation response



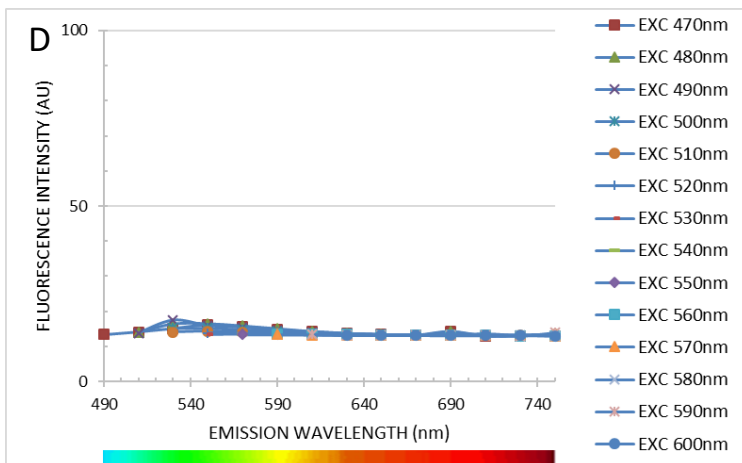
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



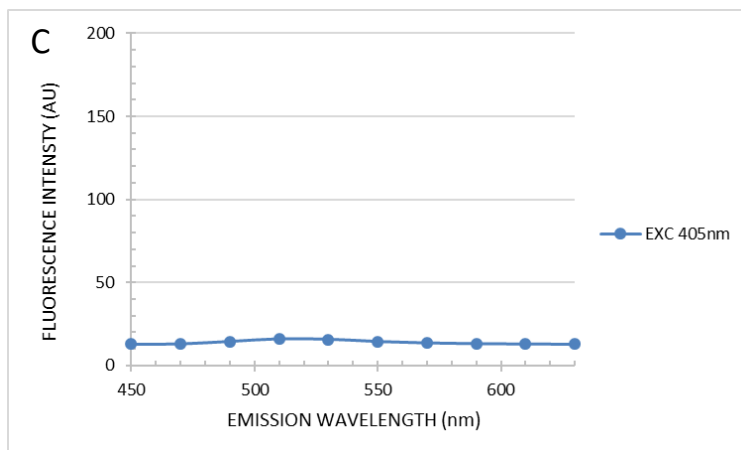
UV (405 nm)
excitation response



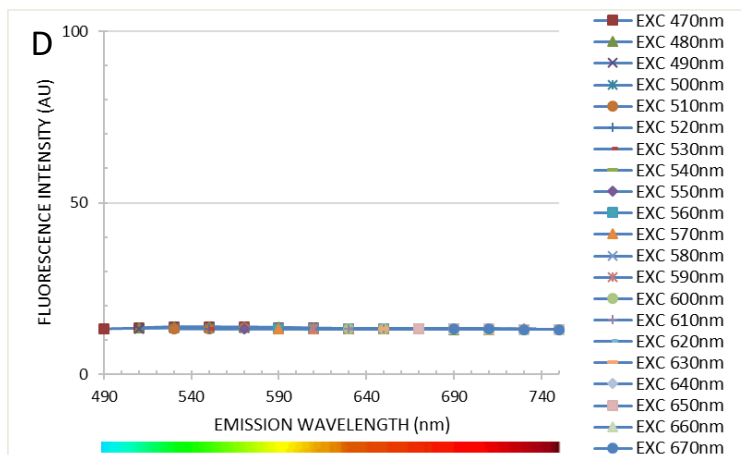
Visible light
(470-670 nm)
excitation response



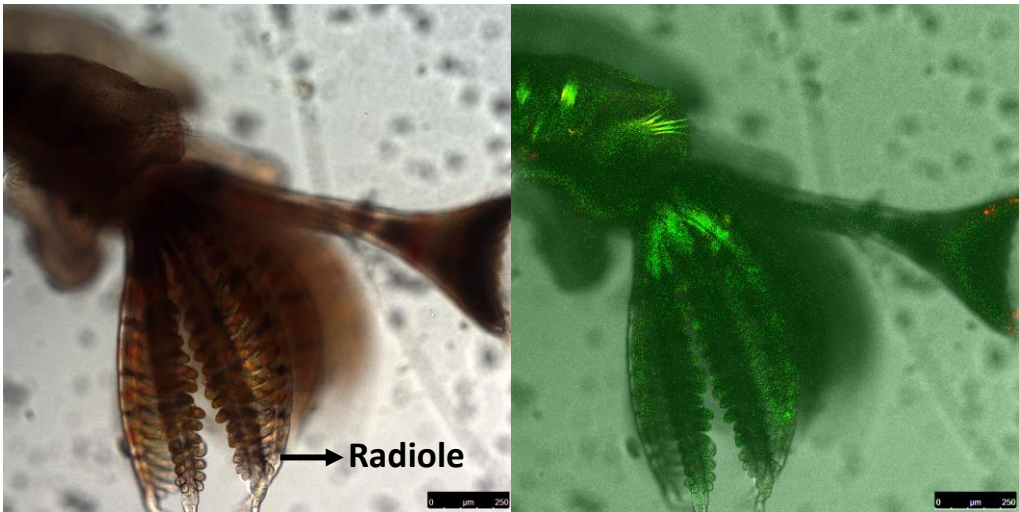
Excitation and Emission Spectra



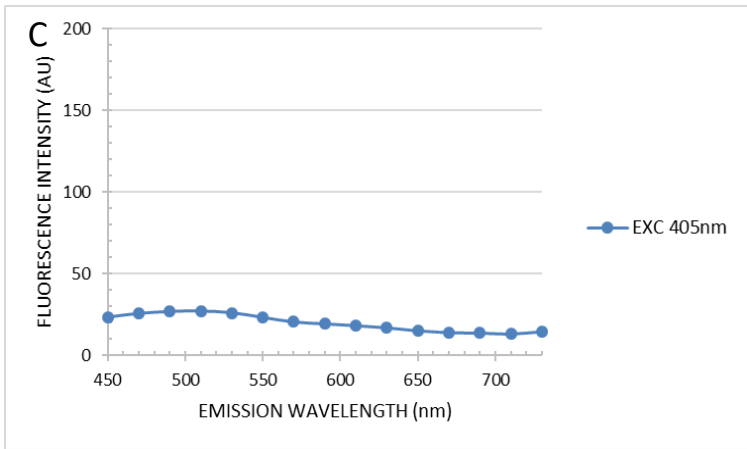
UV (405 nm)
excitation response



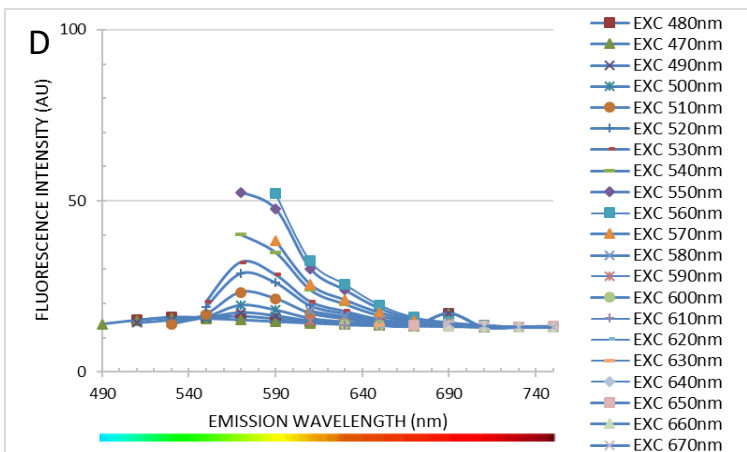
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



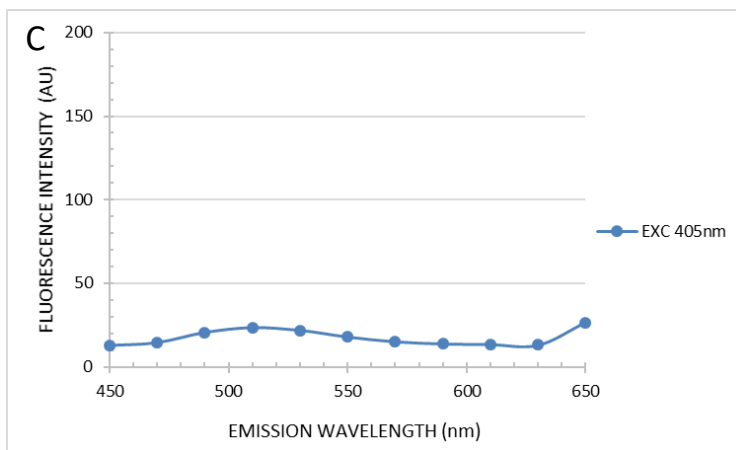
UV (405 nm)
excitation response



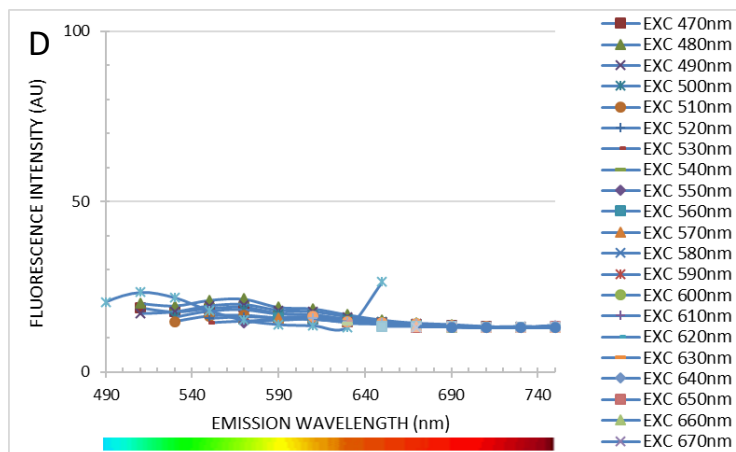
Visible light
(470-670 nm)
excitation response



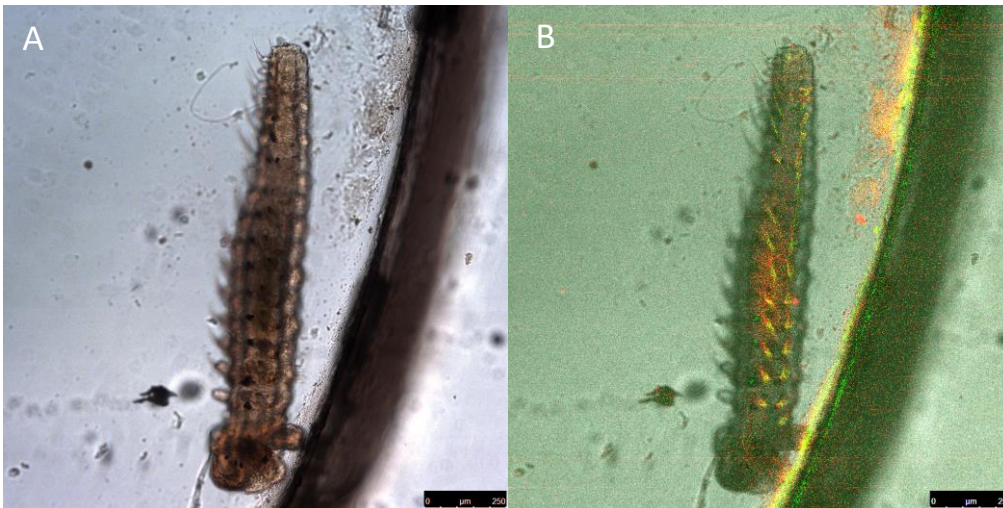
Excitation and Emission Spectra



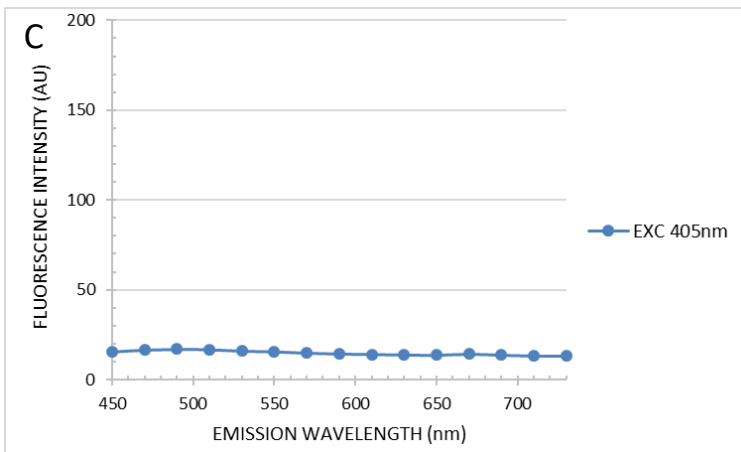
UV (405 nm)
excitation response



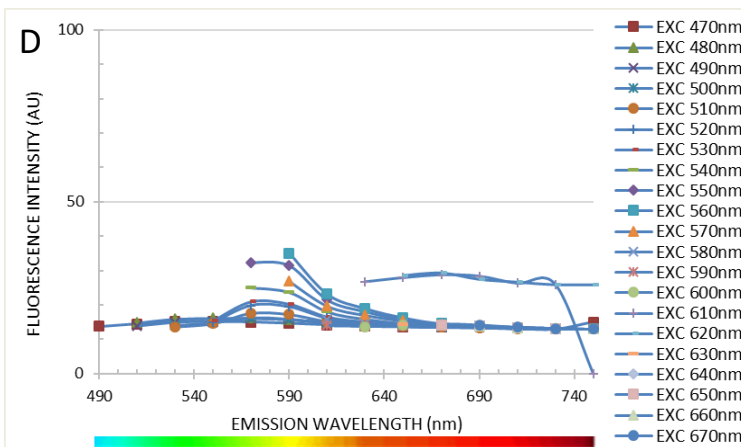
Visible light
(470-670 nm)
excitation response



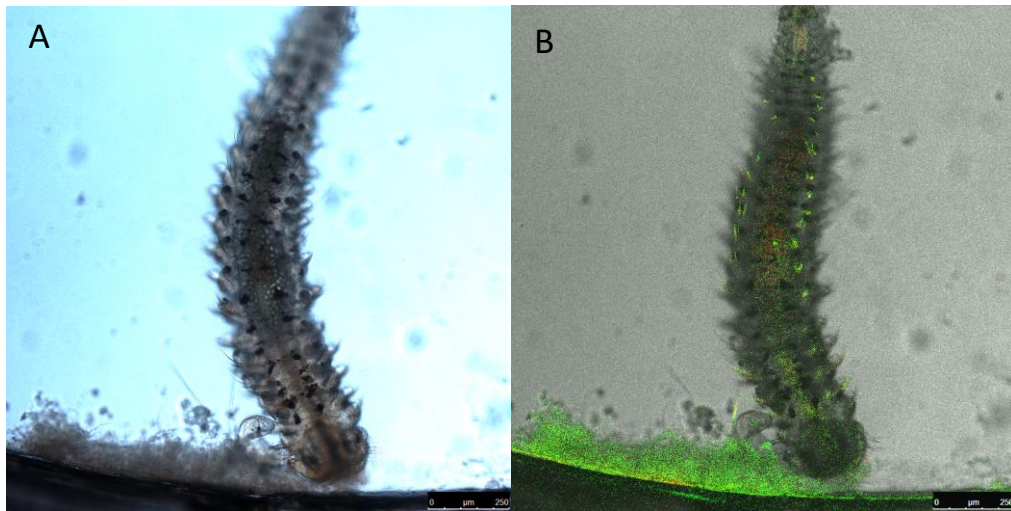
Excitation and Emission Spectra



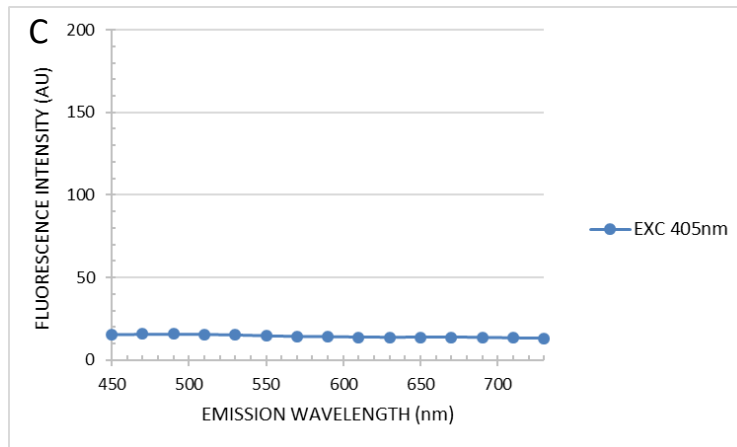
UV (405 nm)
excitation response



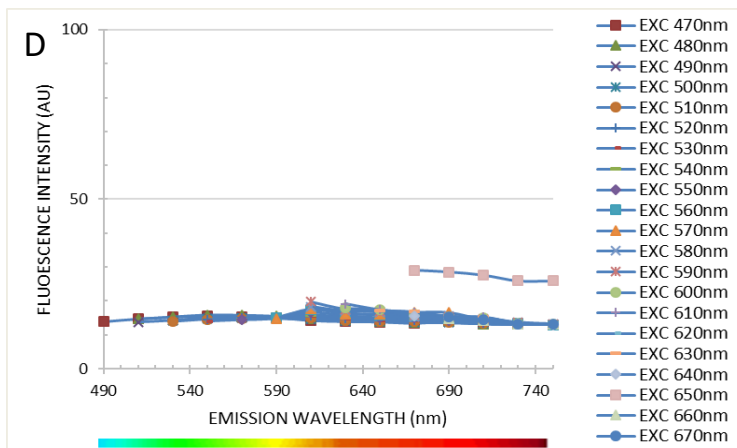
Visible light
(470-670 nm)
excitation response



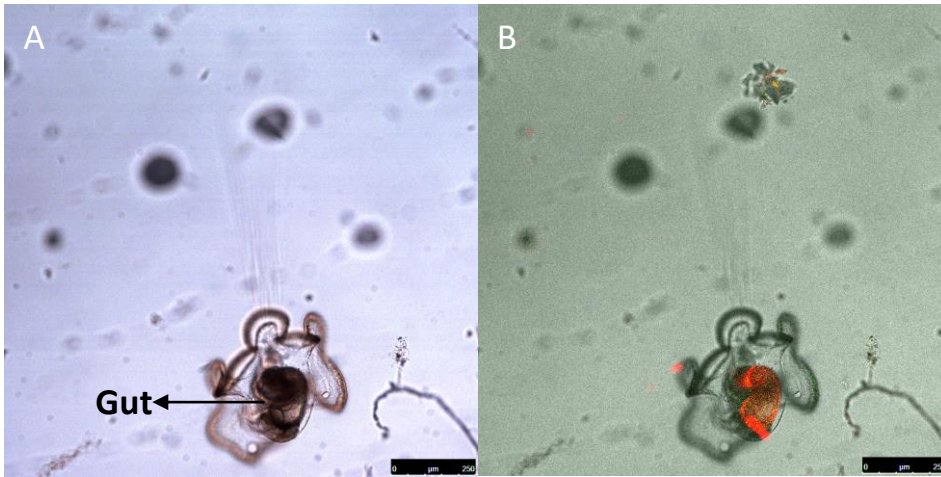
Excitation and Emission Spectra



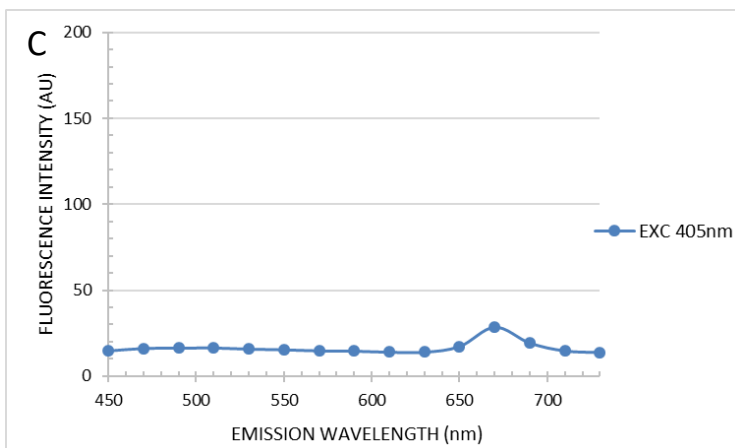
UV (405 nm)
excitation response



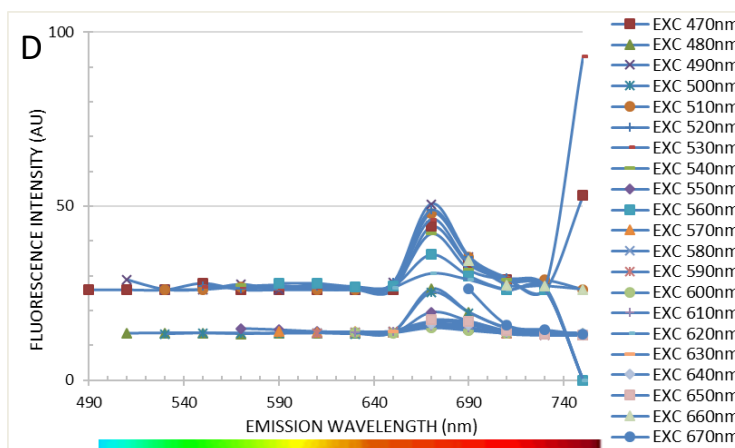
Visible light
(470-670 nm)
excitation response



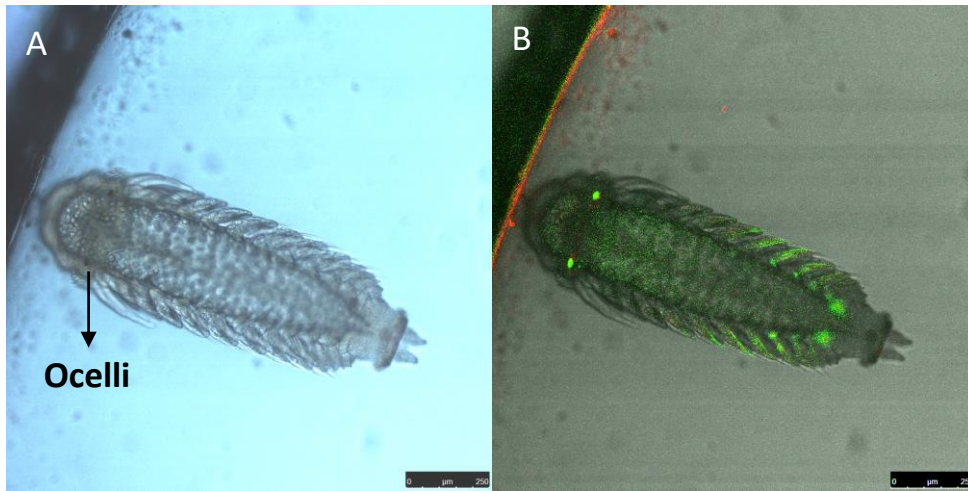
Excitation and Emission Spectra



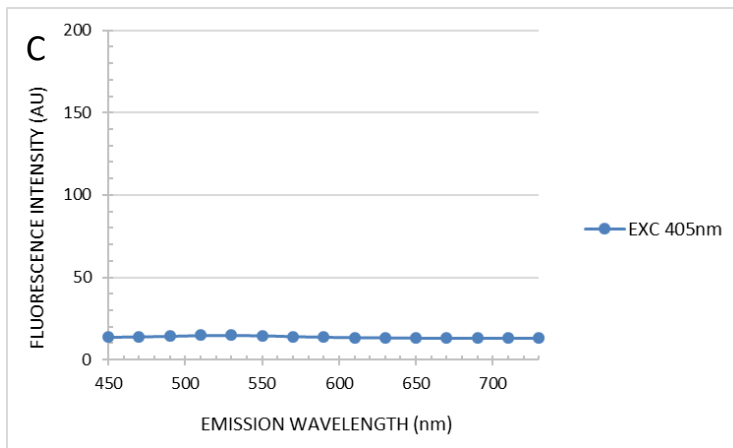
UV (405 nm)
excitation response



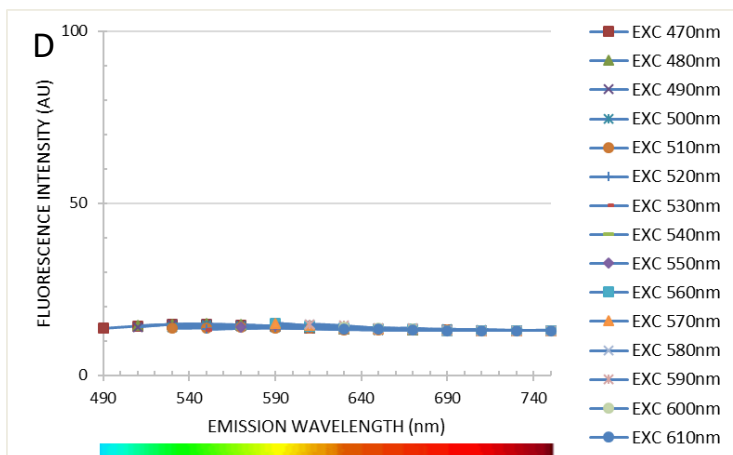
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



UV (405 nm)
excitation response



Visible light
(470-670 nm)
excitation response

Comments:

Polychaetes could also be an interesting phylum to investigate, as no FP has been isolated from this group yet, only bioluminescence (see discussion). Many polychaete species have been investigated as they were showing interesting features at the epifluorescent microscope.

Alciopidae:

The two Alciopidae species on Figure 2.32 B and 2.33 B show orange-red fluorescence in the eye and one present a strong response to the UV excitation with strong emission in the green (140 A.U) at 530 nm (Fig 2.33 B). The response to the chlorophyll test on Figure 2.34 B does not show any signal, as well as the test to UV excitation and to the visible light excitation.

The visible light test shows moderate yellow emission for the polychaete on Figure 2.32 D and strong emission (160 A.U) in the green to the orange part of the spectrum for the polychaete on Figure 2.33 D. This is slightly different as what we observe on the test to the response of chlorophyll where we notice mostly a red signal.

I became interested in this species as we could denote a bright red florescent signal in the eye of the animal. This species was a candidate for further molecular biology investigations, however, I haven't been able to extract enough RNA and perform sequencing.

Opheliidae:

We can notice a bright green fluorescence signal on the nerve segments of the animals on almost all tests presenting the response to chlorophyll, only Figure 2.43 B and 2.45 B do not show this pattern.

The UV excitation test shows very low intensity for all animals with no distinctive spectral features.

The visible light excitation test presents a modest green-yellow emission response (70 A.U) with excitation wavelengths at 550 nm and 560 nm (see Fig 2.35 D). We observe the same but stronger

pattern (130 A.U) for the animal on Figure 2.37 D. Figure 2.39 D shows a modest green emission response (50 A.U) with excitation at 470 nm. Also, Figure 2.40 D presents the same pattern with excitation at 560 nm whereas Figure 2.41 D shows a very low green-yellow response (20 A.U) with no distinguishable excitation wavelengths pattern.

Finally, Figure 2.36 D, 2.38 D and 2.42 D to 2.45 D do not show any response to the visible light excitation test. In particular, the slow increase in fluorescence intensity on Figure 2.42D may be due to an artefact of the laser.

Serpulidae:

The serpulidae shows green fluorescence on its radiole, in direction to the tube (see Fig 2.46 B). The response to the UV test does not show any pattern (see Fig 2.46 C) and the test to the visible light excitation presents a mild green-yellow emission (50 A.U) with excitation at 550-560 nm.

Spionidae:

The three species do not show a high response to the chlorophyll test. The response to the UV excitation is also very low. Finally, for the visible light excitation test, Figure 2.48 D shows a slight green-yellow emission with excitation at 550-560 nm with an intensity around 35 A.U. The two other species do not present any distinct pattern.

Oweniidae:

We observe a bright red fluorescence signal in the gut (see Fig 2.50 B) which could come from algal diet. This is also noticeable after the UV excitation test where there is a slight red signal at 670 nm (30 A.U). The response to the visible light excitation test presents a mild response in the red emission of the spectrum at around 670 nm.

Phyllodocidae:

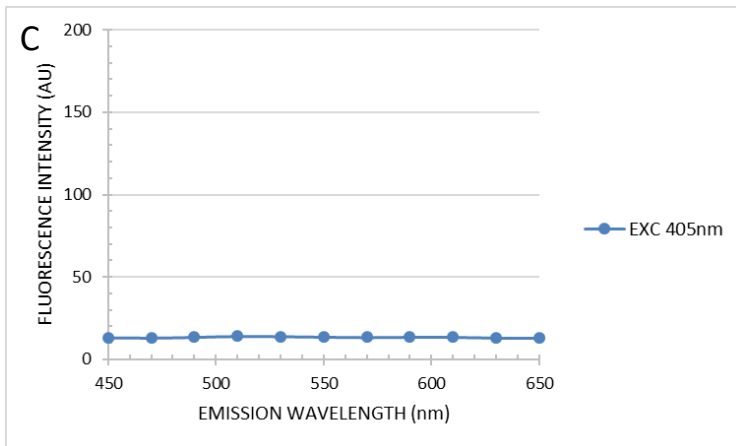
Bright green fluorescence is observed in the ocelli of the animal (Fig 2.51 B). An ocellus is known to be a simple eye, found in many invertebrates, consisting of a number of sensory cells and often

a single lens. This green pattern is however not noticed after the UV excitation response and the visible light excitation test (Fig 2.51 C and D).

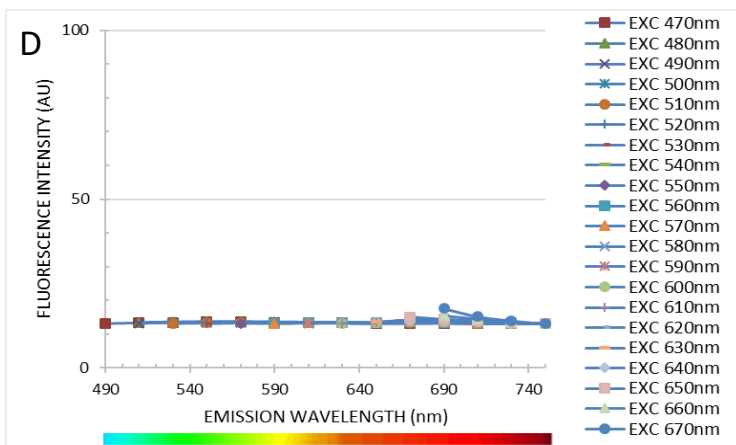
4.2.6 Annelida – seaside sampling



Excitation and Emission Spectra

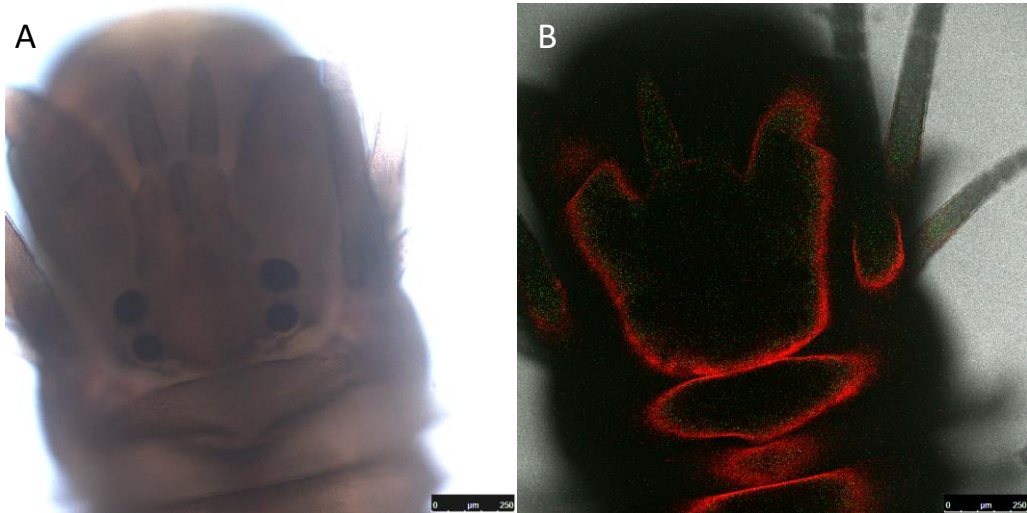


UV (405 nm)
excitation response

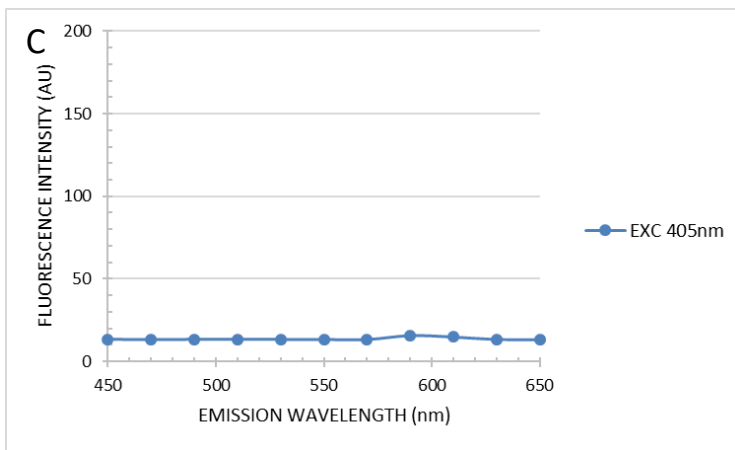


Visible light
(470-670 nm)
excitation response

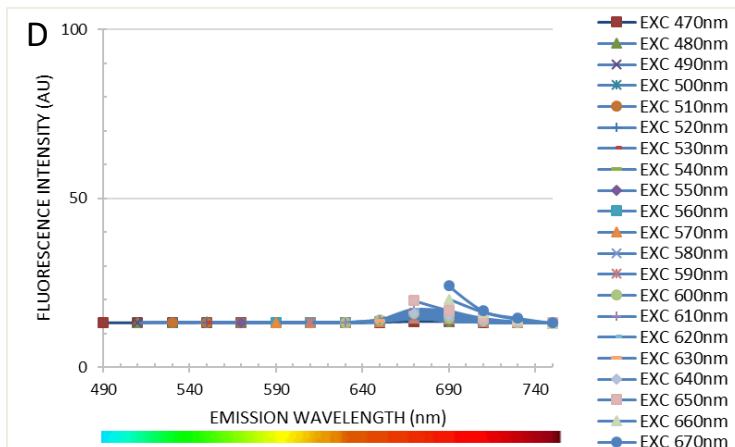
Figure 2.53 Annelida, *Neanthes acuminata* (head) 23/10/2019



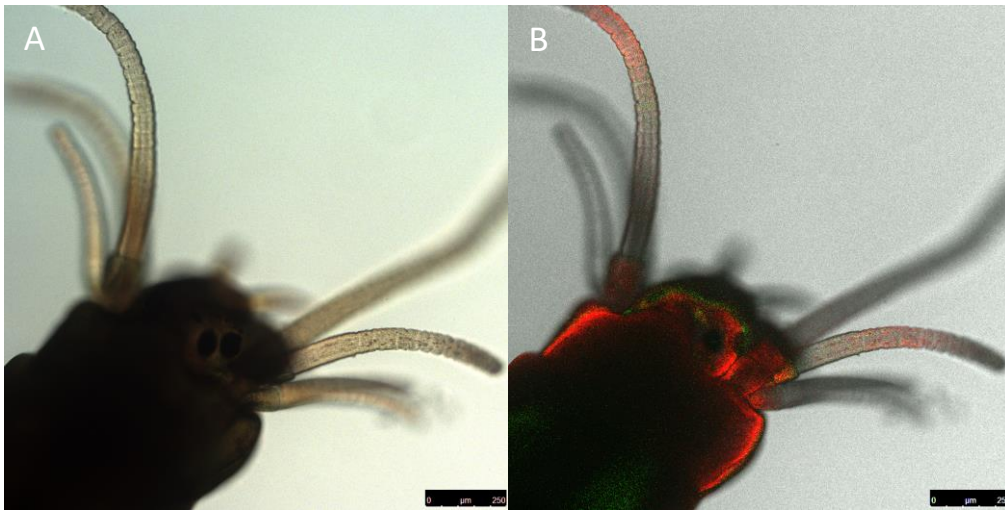
Excitation and Emission Spectra



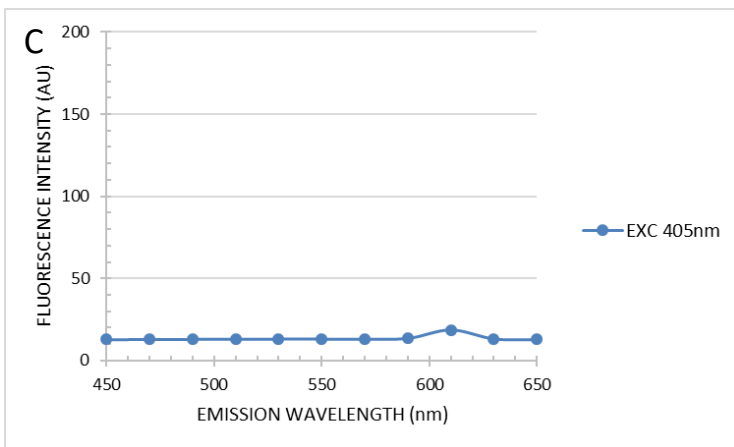
UV (405 nm)
excitation response



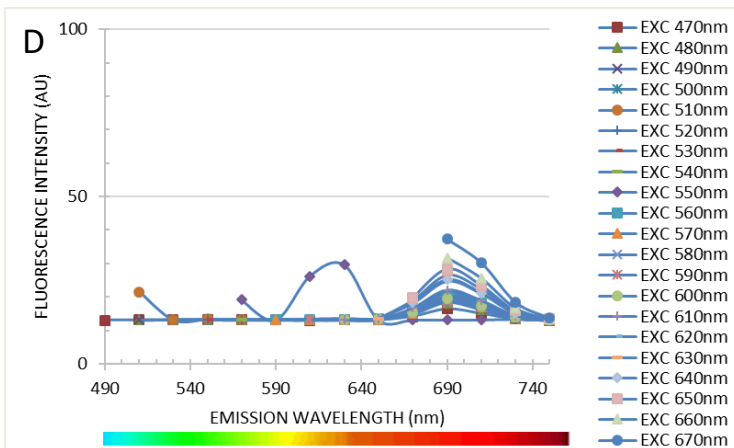
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



UV (405 nm)
excitation response



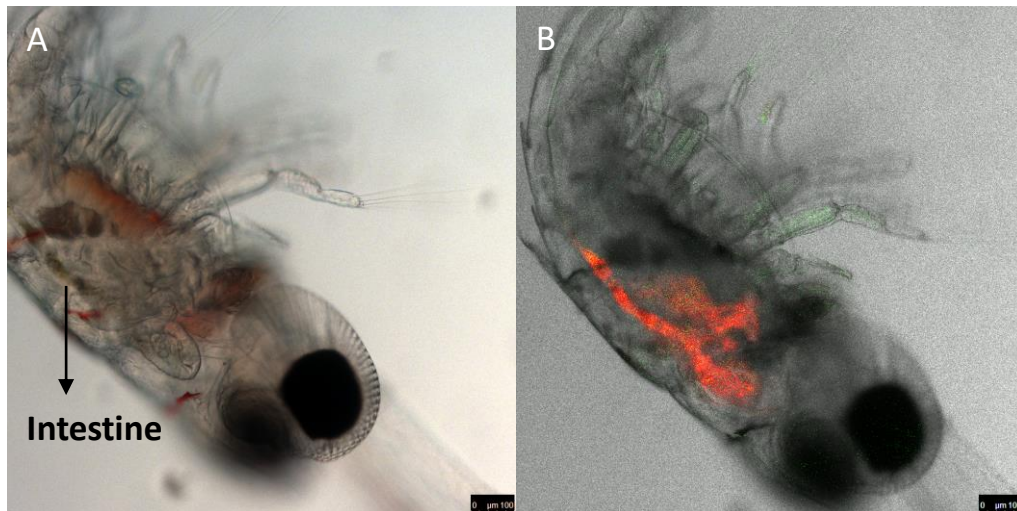
Visible light
(470-670 nm)
excitation response

Comments:

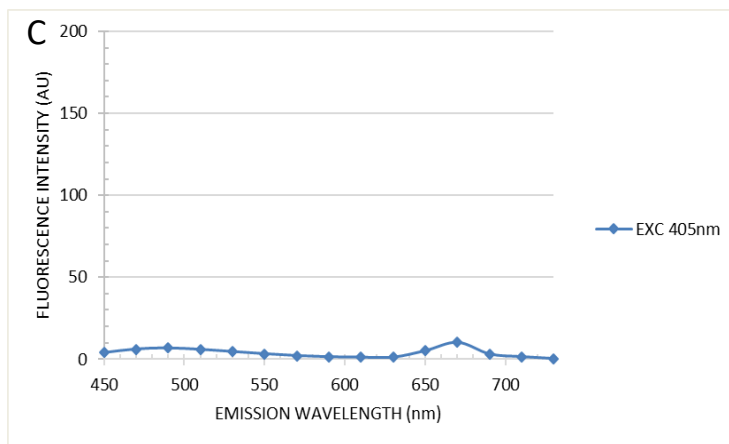
The annelid *Syllis prolifera* do not show any fluorescence in response to the chlorophyll test (Figure 2.52 B) whereas we notice red fluorescence in the heads of both *Neanthes acuminata* and Nereididae (Figures 2.53 B and 2.54 B). As both of the animals were large and fluorescence was seen on the head we decided to focus on this part of the body for the confocal analysis.

N. acuminata has a very low red emission in response to the visible light excitation spectra (Figure 2.53 D) and Nereididae shows a response at 690 nm at around 50 A.U with excitation at 670 nm (Figure 2.54 D).

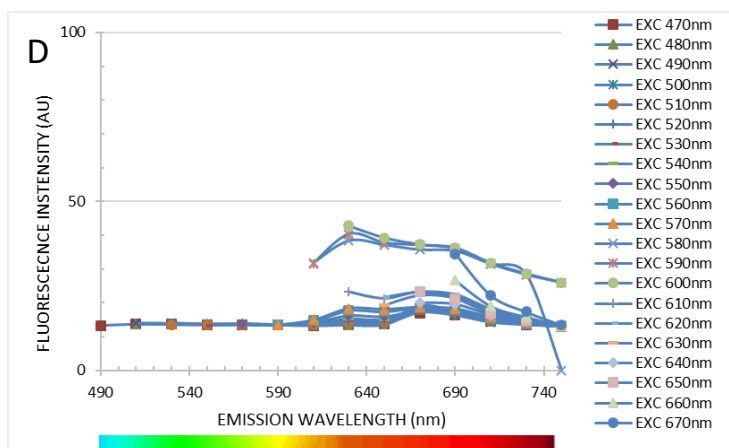
4.2.7 Arthropoda



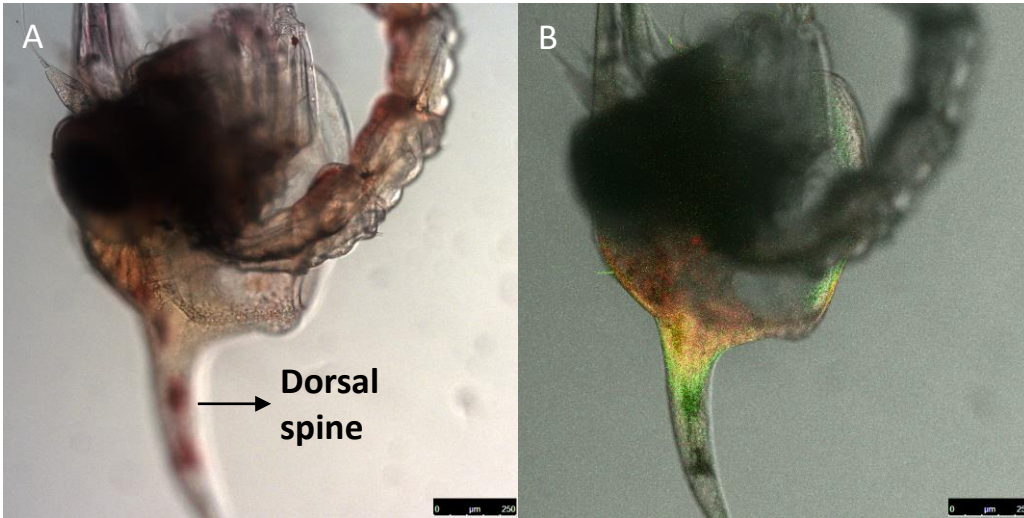
Excitation and Emission Spectra



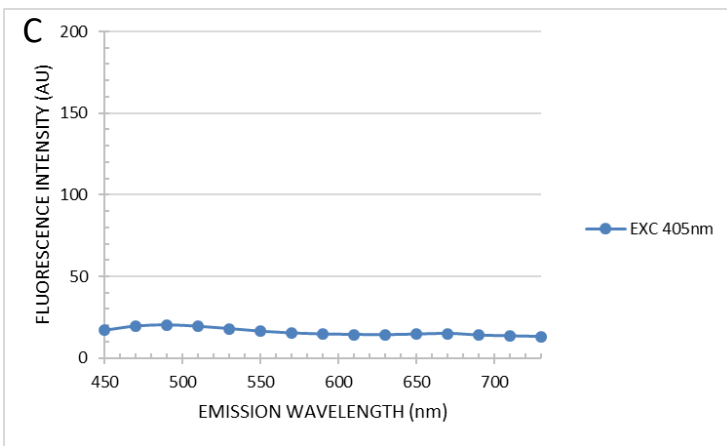
UV (405 nm)
excitation response



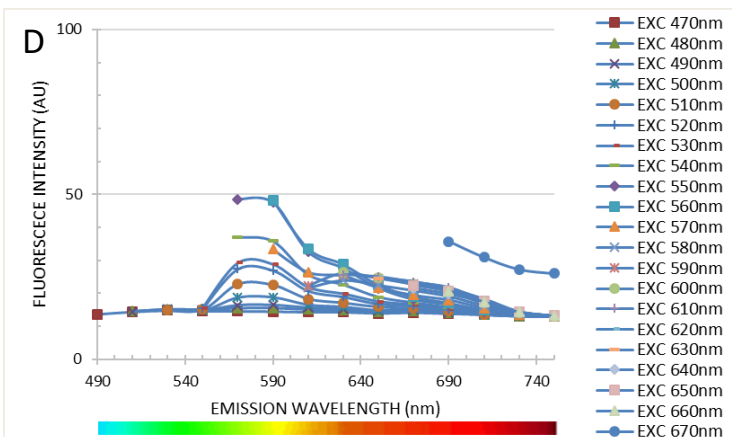
Visible light
(470-670 nm)
excitation response



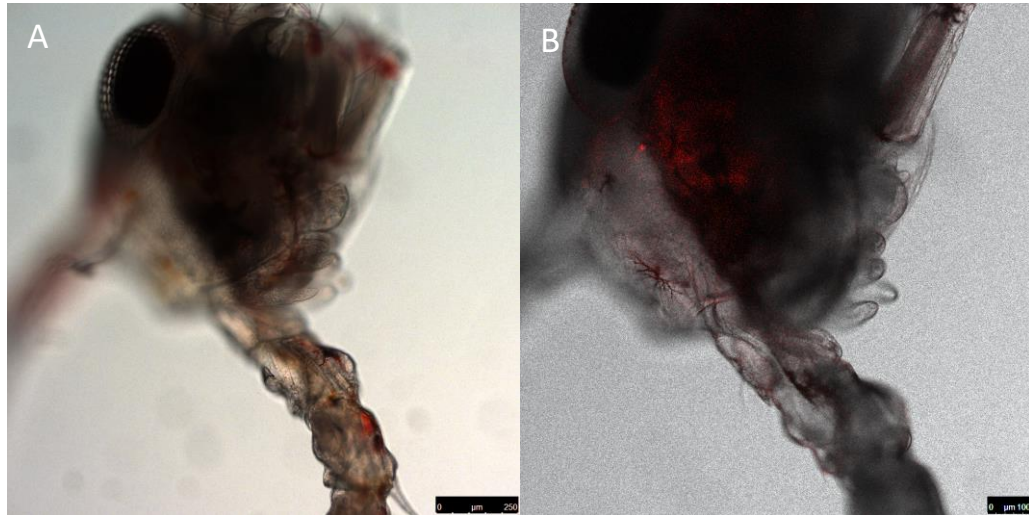
Excitation and Emission Spectra



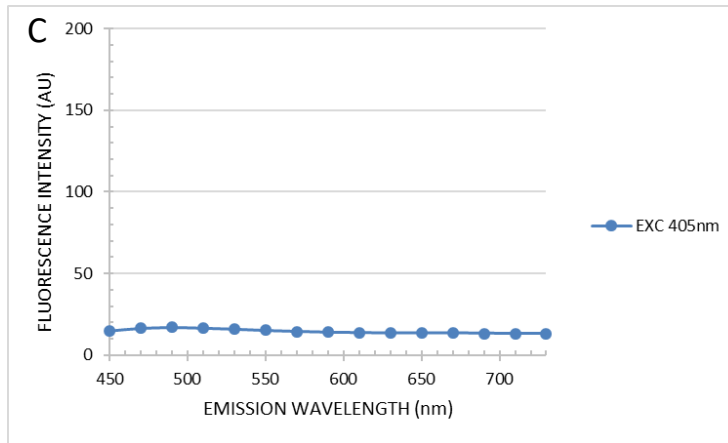
UV (405 nm)
excitation response



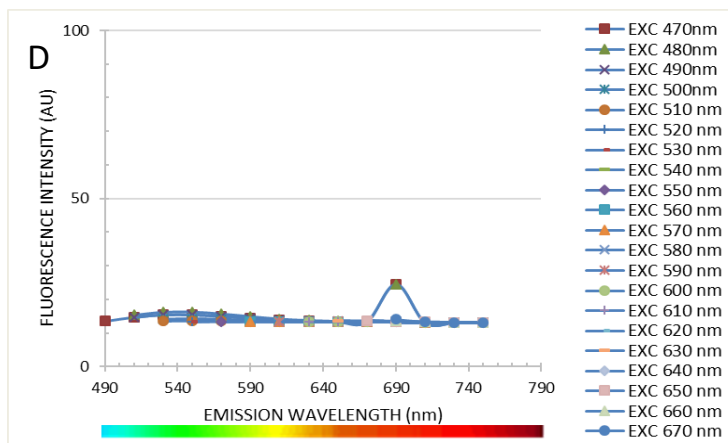
Visible light
(470-670 nm)
Excitation response



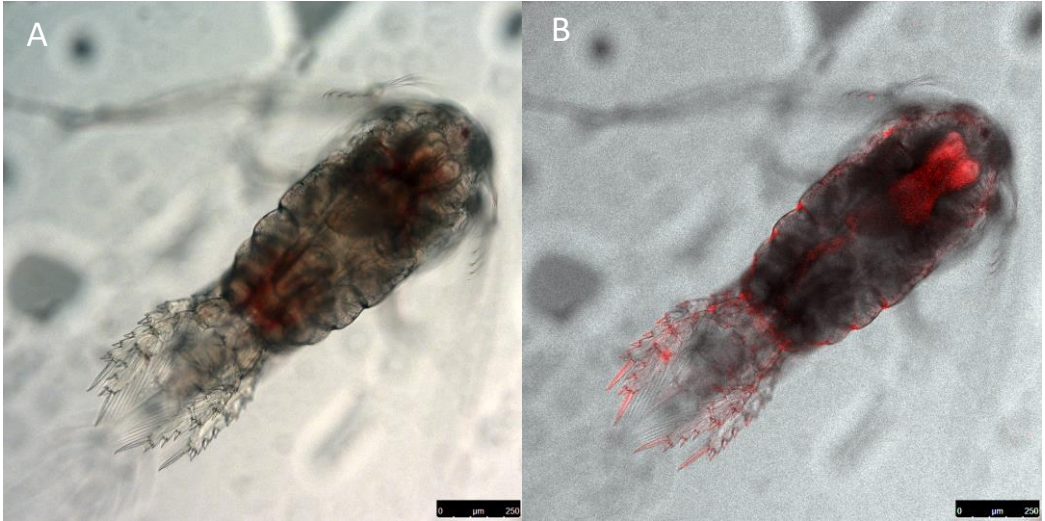
Excitation and Emission Spectra



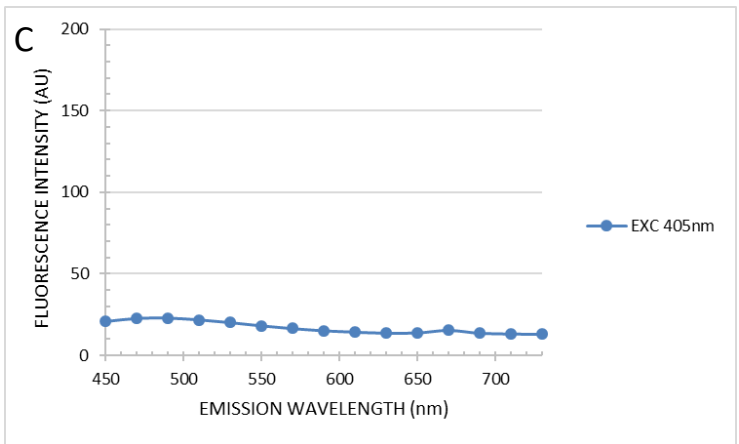
UV (405 nm)
excitation response



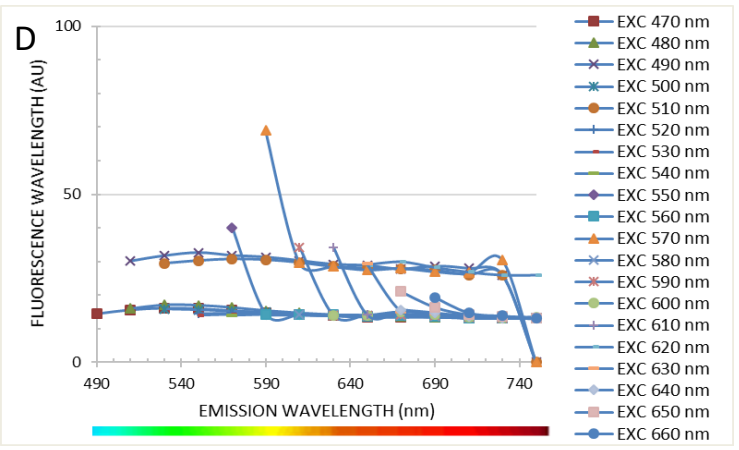
Visible light
(470-670 nm)
Excitation response



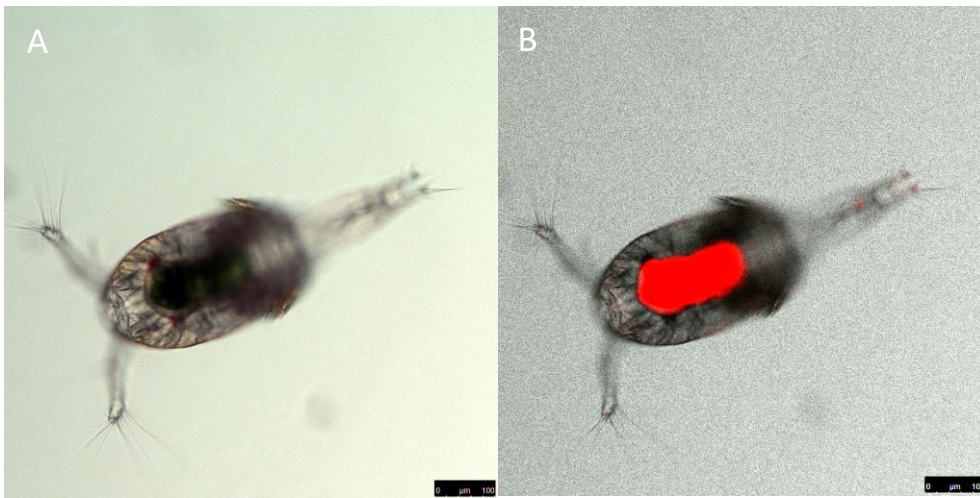
Excitation and Emission Spectra



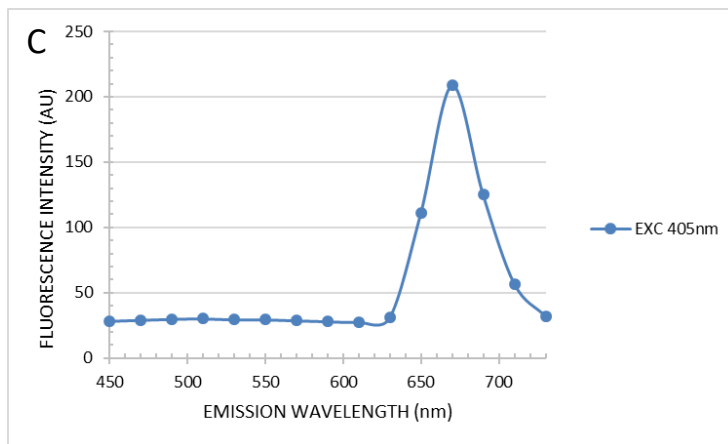
UV (405 nm)
excitation response



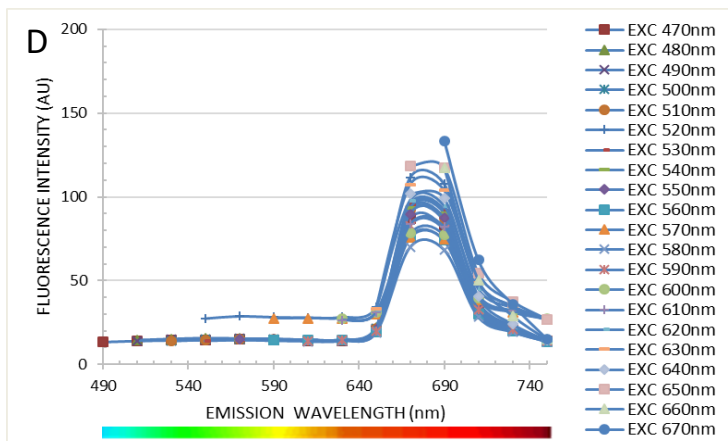
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



UV (405 nm)
excitation response



Visible light
(470-670 nm)
excitation response

Comments:

A few years ago, several GFPs have been characterized from Copepods (*Pontella mimocerami* and *Labidocera aestiva*) therefore we explored if other fluorescent patterns mainly in the red part of the spectrum were present. The experiments show a light fluorescent emission intensity mainly in the green part that are similar to some already characterized fluorophores.

Decapods:

We observe a response to chlorophyll mainly in the intestine that could be due to the algal diet (see Figure 2.55 B). Chlorophyll is also present on the dorsal spine of the decapod (see Figure 2.56 B) and no response to the UV excitation test is detected for all decapods.

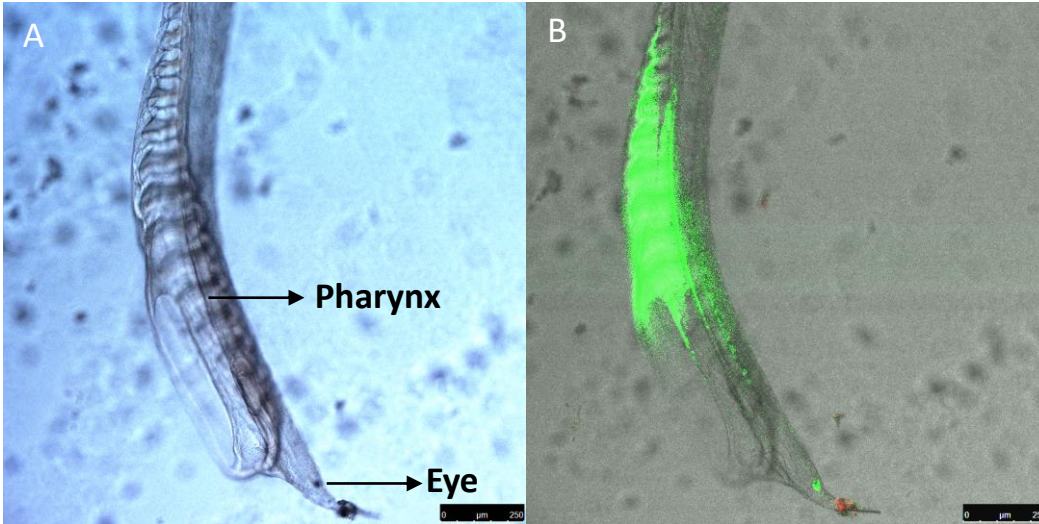
The response to the visible light excitation test shows a mild red-far red emission (630 nm to 740 nm), with excitation from 580 nm to 600 nm, see Figure 2.55 D. The brachyura on Figure 2.56 D presents a moderate emission in the green-yellow part of the spectrum (570 nm to 590 nm) with excitation at 550-560 nm. Finally, the brachyura on Figure 2.57 D shows a slight red emission at 690 nm with excitation at 470-480 nm.

Copepods:

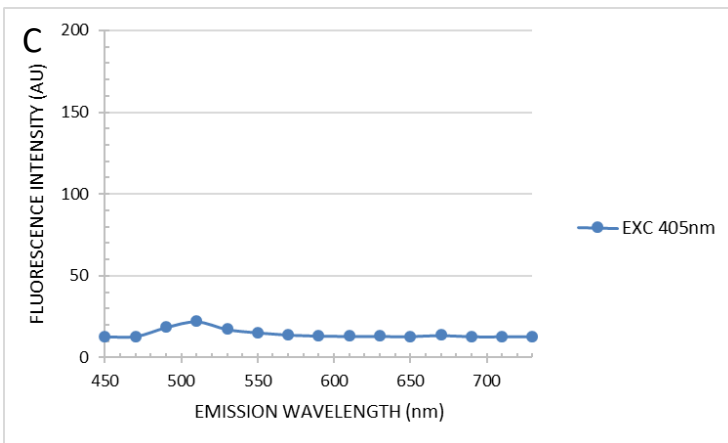
For both copepods we observe a red fluorescent signal in the gut, *Temora stylifera* does not show a response to UV whereas the species *Oncaea* presents a high red emission pattern at 670 nm (200 A.U), characteristic of chlorophyll (see Figure 2.59 C).

The visible light excitation test exhibits a scattered pattern on Figure 2.58 D, the emission pattern is more distinguishable with the species *Oncaea* where we discern an intense red emission response at 670 nm to 690 nm around 130 A.U (see Figure 2.56 D).

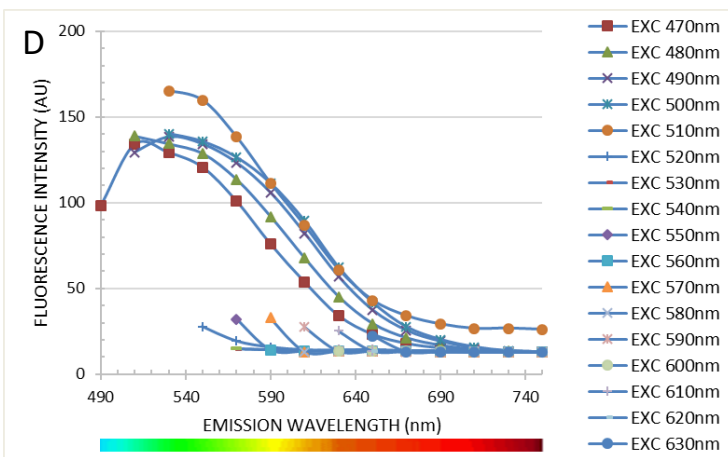
4.2.8 Chordata



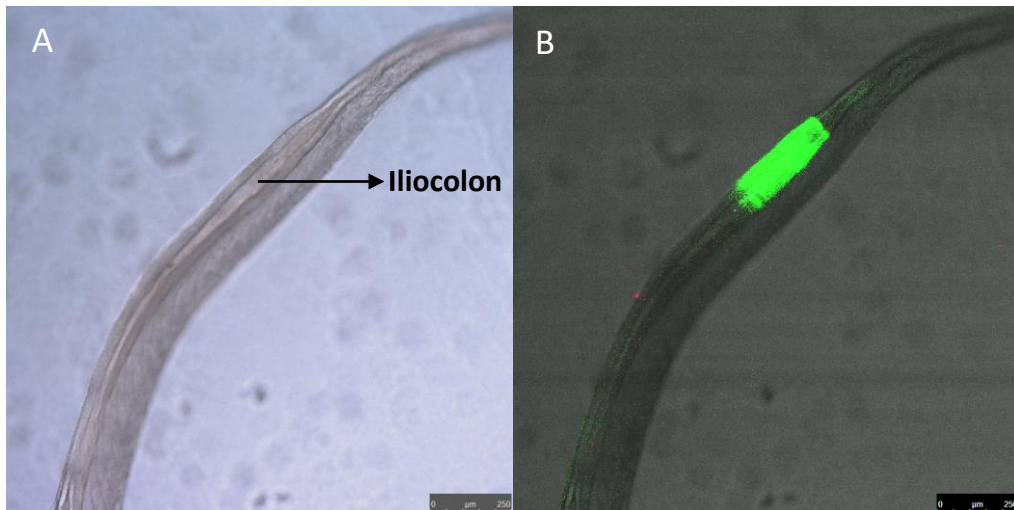
Excitation and Emission Spectra



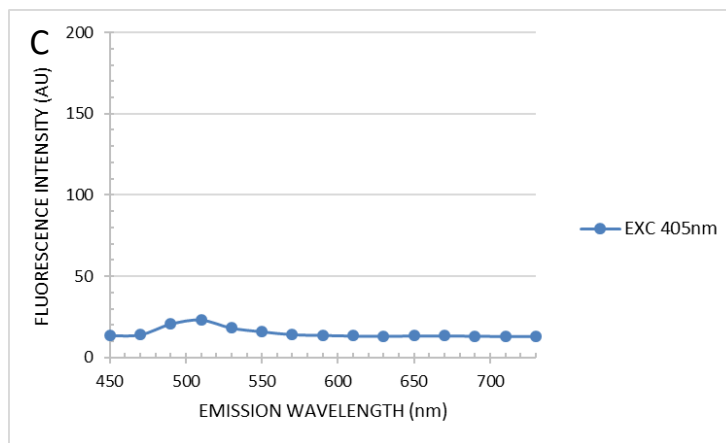
UV (405 nm)
excitation response



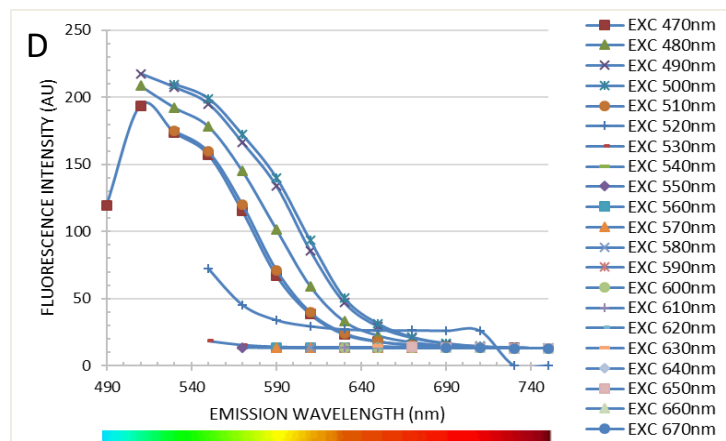
Visible light
(470-670 nm)
excitation response



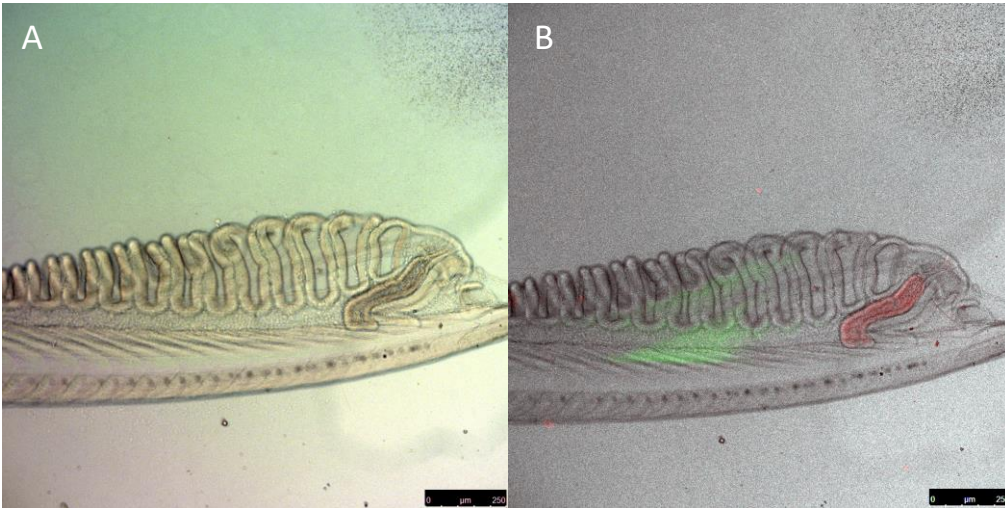
Excitation and Emission Spectra



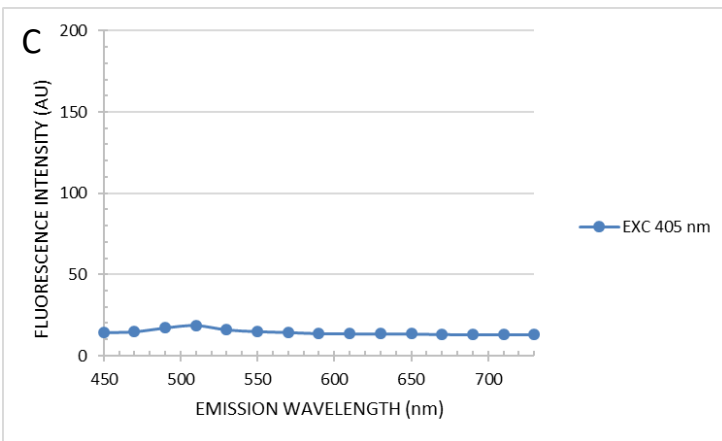
UV (405 nm)
excitation response



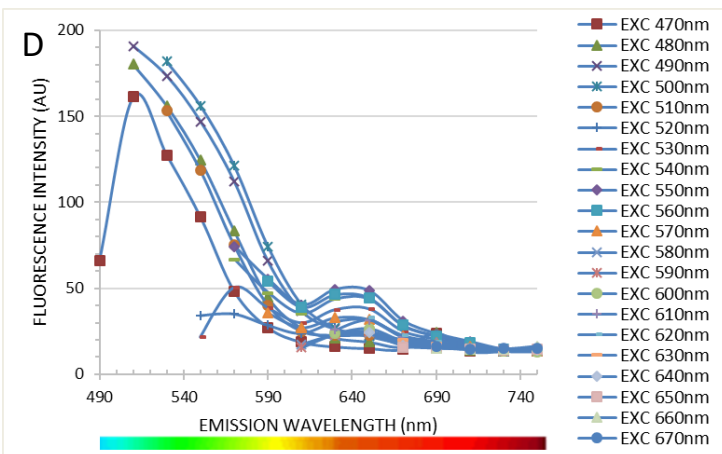
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



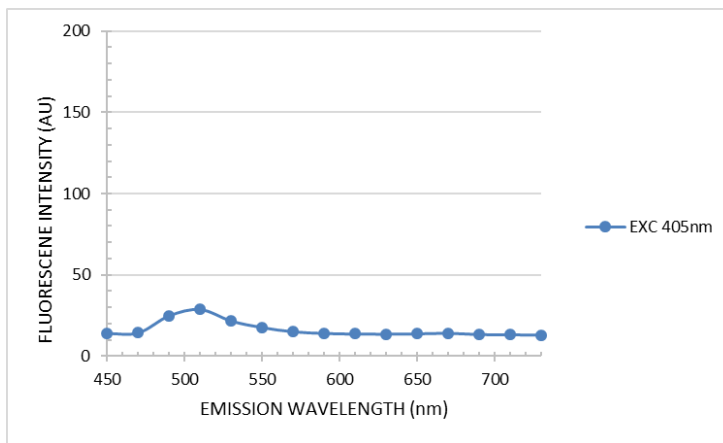
UV (405 nm)
excitation response



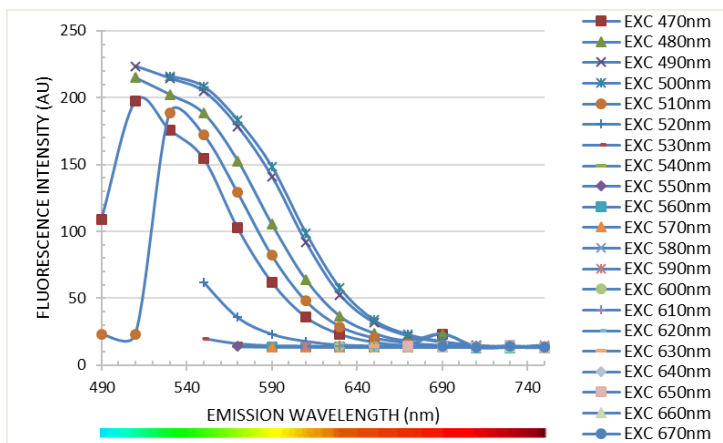
Visible light
(470-670 nm)
excitation response



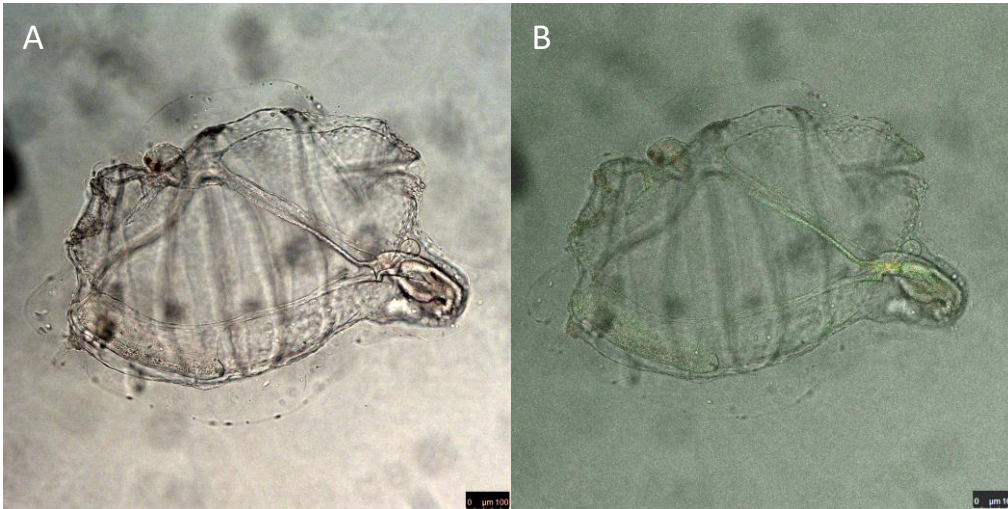
Excitation and Emission Spectra



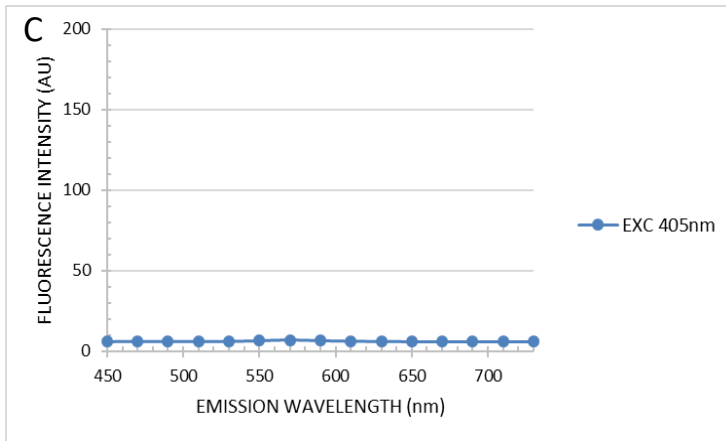
UV (405 nm)
excitation response



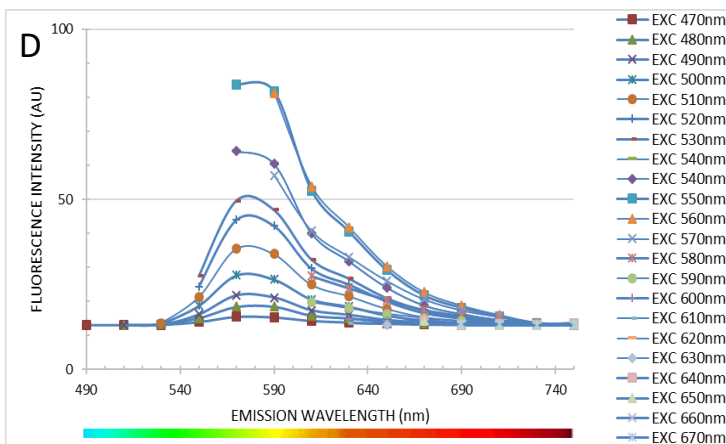
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra

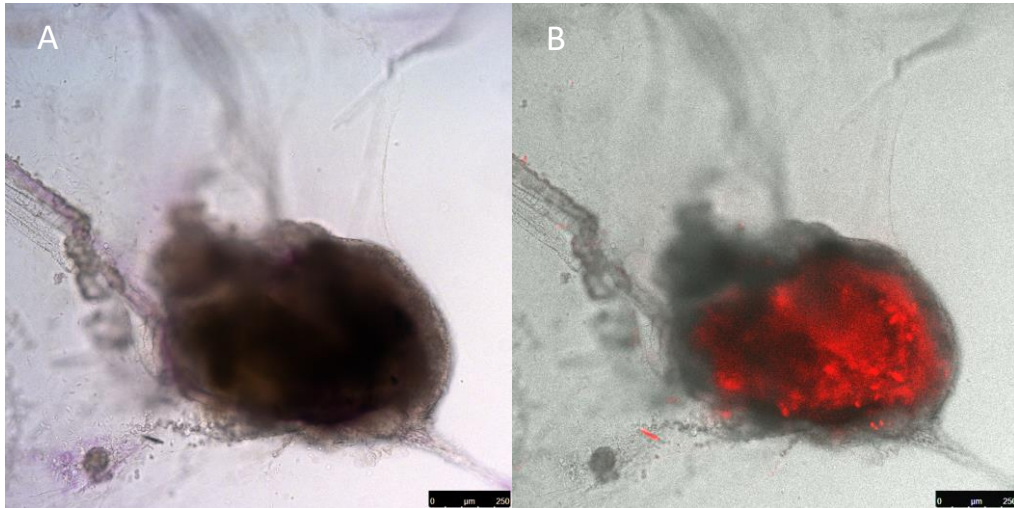


UV (405 nm)
excitation response

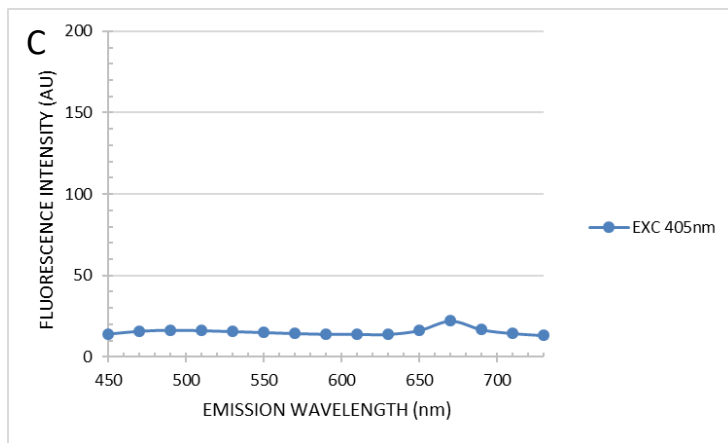


Visible light
(470-670 nm)
excitation response

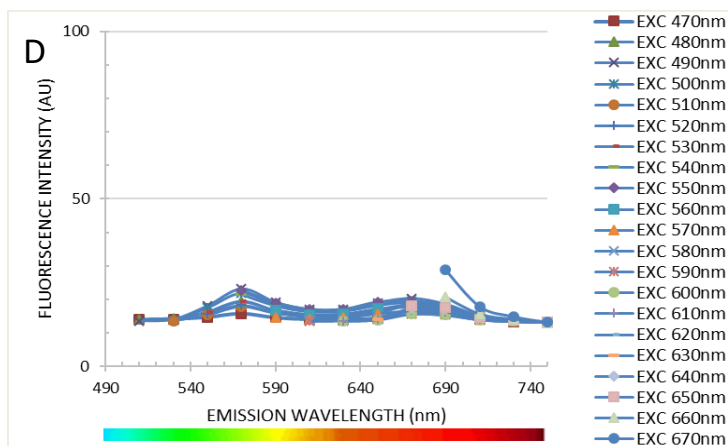
Figure 2.65 Tunicata, *Thalia democratica* (stomach) 25/06/2020



Excitation and Emission Spectra

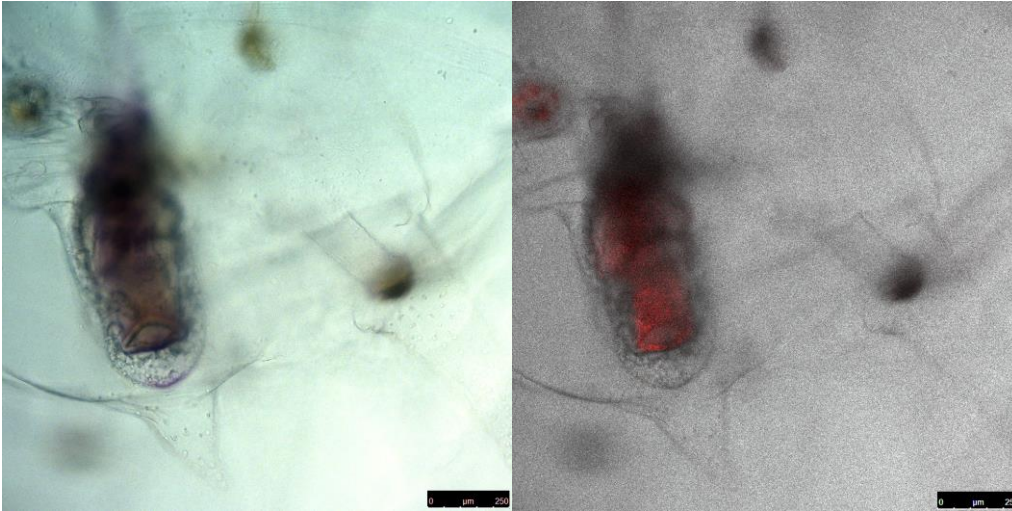


UV (405 nm)
excitation response

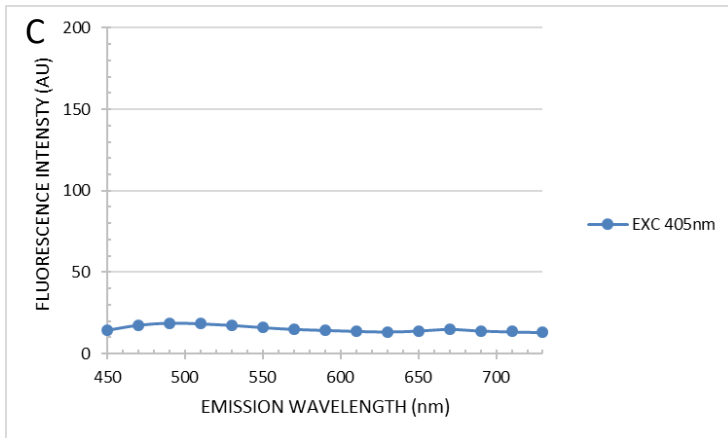


Visible light
(470-670 nm)
excitation response

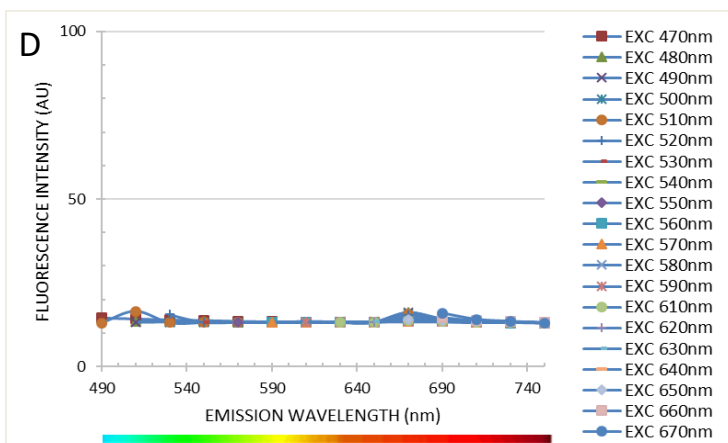
Figure 2.66 Tunicata, *Thalia democratica* (stomach) 07/07/2020



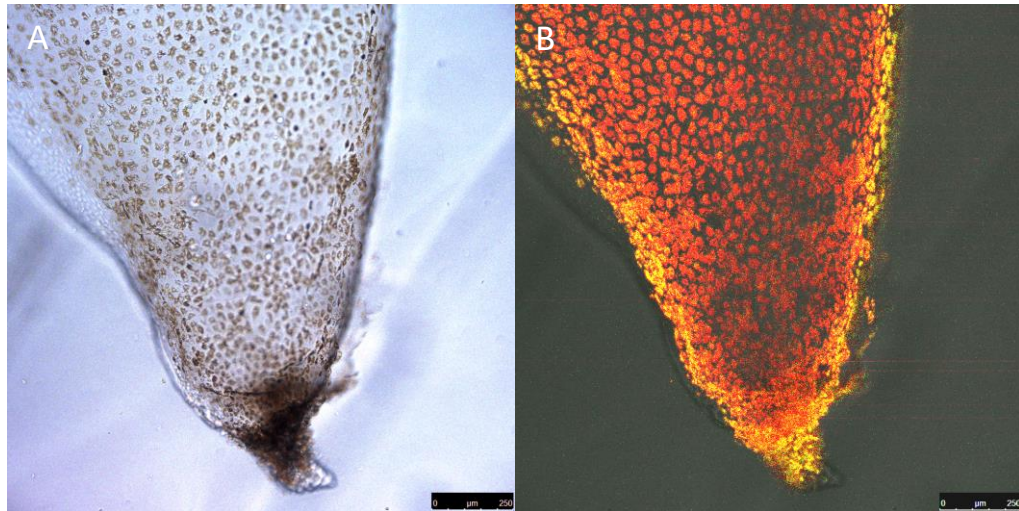
Excitation and Emission Spectra



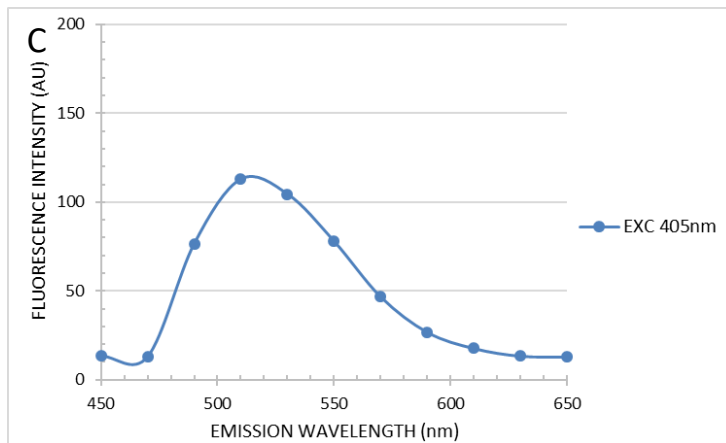
UV (405 nm)
excitation response



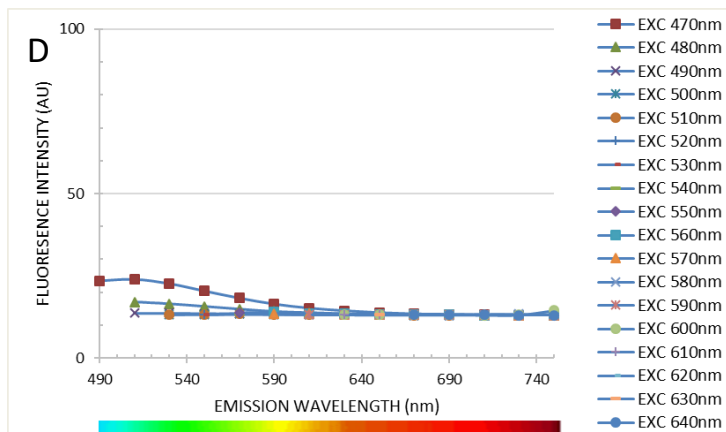
Visible light
(470-670 nm)
excitation response



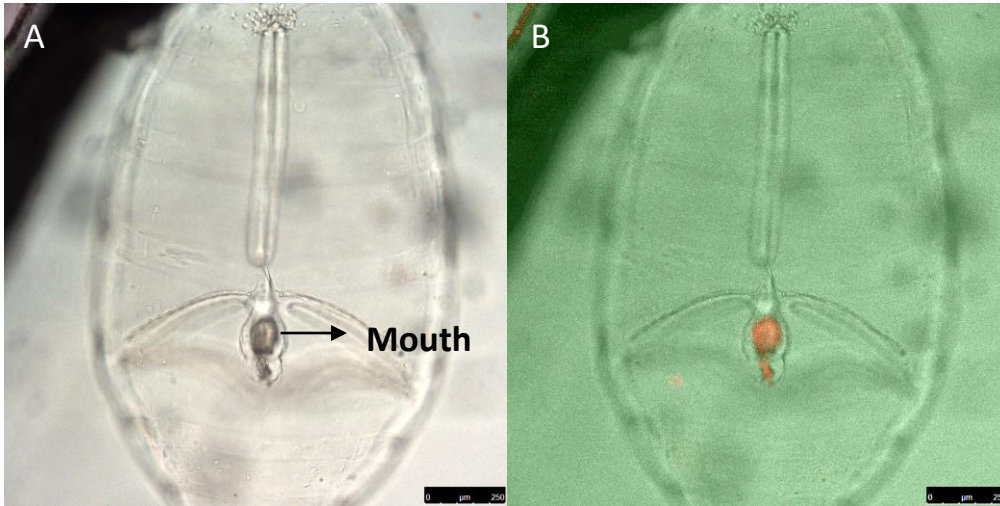
Excitation and Emission Spectra



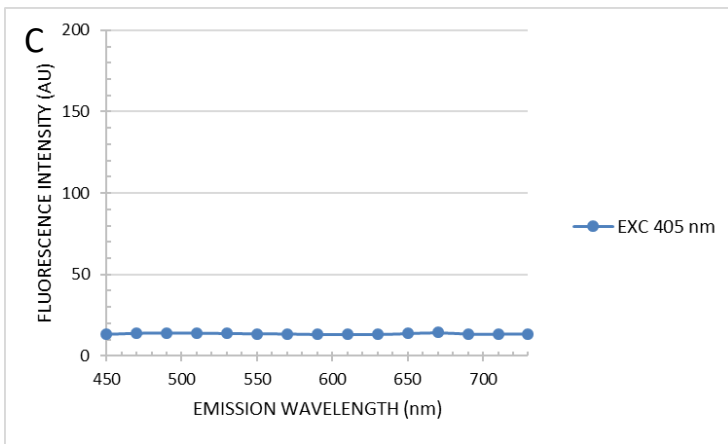
UV (405 nm)
excitation response



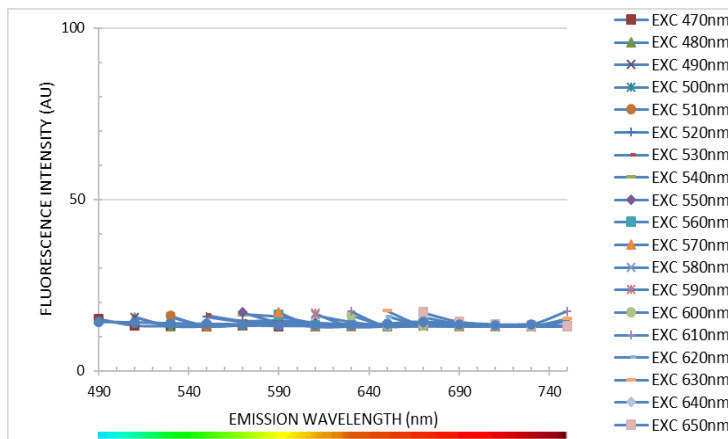
Visible light
(470-670 nm)
excitation response



Excitation and Emission Spectra



UV (405 nm)
excitation response



Visible light
(470-670 nm)
excitation response

Comments:

Several GFP from the species *Branchiostoma floridae* have been characterized a few years ago in the ovaries, eggs and in the mouth, encoding the largest known family of GFPs in a single organism. It was interesting to assess where fluorescence was present in the animal and if new fluorescent pattern could be denoted.

The first animal shows a bright green fluorescent signal in the iliocolon, pharynx and eye (see Figures 2.60 B and 2.61 B) whereas the other animal has a faint signal in the same organs. This response to chlorophyll is quite high for the first animal although it may not come from this pigment as GFP from this organism have been already characterized with green emission. The two lancelets do not present a response to the UV excitation test (see Figures 2.58 C and 2.59 C).

The test to the visible light excitation shows a green-yellow emission with a very high intensity in the two species (until 250 A.U), with excitation wavelengths ranging from 470 nm to 510 nm.

Tunicate have also been investigated by confocal microscopy as no fluorescent proteins were isolated from this clade.

Thalia democratica:

Red fluorescence can be observed in the stomach in two species (see Figures 2.65 and 2.66 B) but only a slight green signal is visible in the species on Figure 2.64 B. All species present no distinct response to the UV excitation test.

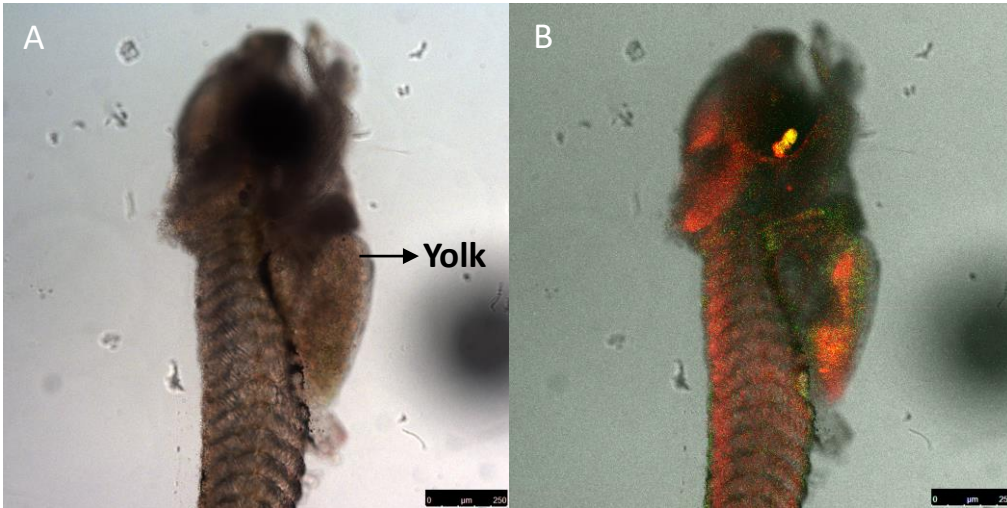
The visible light excitation test on Figure 2.64 D shows a strong emission signal in the green-yellow part of the spectrum with intensity until 80 A.U. and excitation wavelengths around 550-560 nm.

The species presented on Figure 2.65 D present a low intensity (30 A.U) in the green and red part of the spectrum and the last doesn't have any emission response (see Figure 2.66 D).

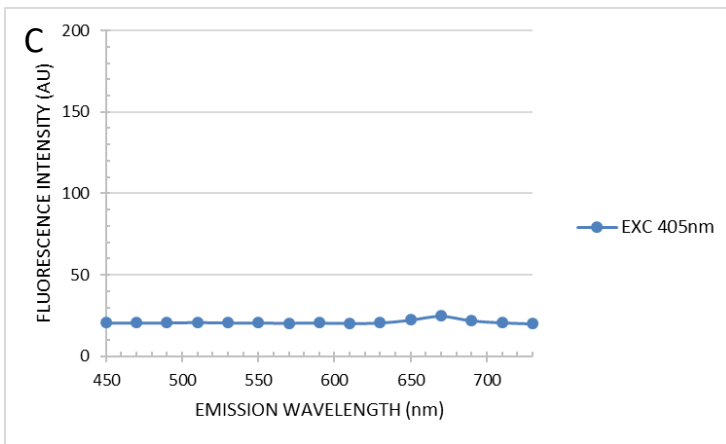
Salpa fusiformis shows an orange-red fluorescent signal and a strong green emission in response to UV excitation (Figure 2.67 C) with a peak at 510 nm. The test to the visible light excitation do not present a clear pattern.

Finally the doliolid on Figure 2.68 B has red fluorescence in its mouth but no emission pattern after both UV excitation and visible light excitation tests (Figures 2.68 C and 2.68 D).

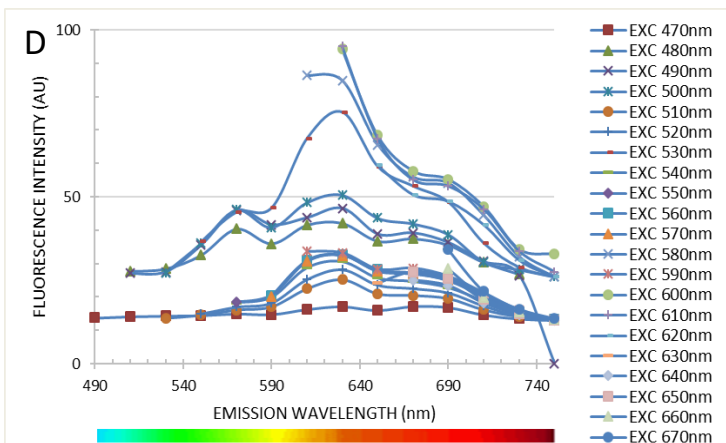
4.2.9 Vertebrata



Excitation and Emission Spectra



UV (405 nm)
excitation response



Visible light
(470-670 nm)
excitation response

Comments:

This fish larva presents orange fluorescence in the yolk and near the eye, although it wasn't possible to have a clear focus in this region so it may be dirt. Also, it doesn't have any response to the UV excitation, whereas a strong intensity (around 100 A.U) is noticed after the visible light excitation test mainly in the orange part of the spectrum.

4.3 Excitation and emission spectra in comparison to chlorophyll and already characterized fluorophores

In this part, I will compare our results by listing the UV response emission of the peaks as well as the response to the visible light excitation test, with either chlorophyll, as it is the most common pigment in zooplankton and with already known fluorophores. This will give us an idea of which excitation and emission spectra could be interesting to exploit and in which phyla.

As fluorescent proteins have been characterized in Cnidaria, Arthropoda and Cephalochordata, many other clades should be explored in the attempt to find new fluorescent proteins with red-far red emission.

4.3.1. UV response

UV response			
Species and figure	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
Radiozoa (Fig 2.13)	-	-	-
Radiozoa (Fig 2.14)	670	100	chlorophyll
Hydroidolina (Fig 2.15)	-	-	-
Cunina sp. (Fig 2.16)	-	-	-
Obelia sp. (Fig 2.17)	490	40	-
Hydrozoa (Fig 2.18)	-	-	-
Hydrozoa (Fig 2.19)	-	-	-
Scyphozoa (Fig 2.20)	-	-	-
Siphonophorae (Fig 2.21)	-	-	-
Siphonophorae (Fig 2.22)	-	-	-
Siphonophorae (Fig 2.23)	-	-	-
Diphyidae (Fig 2.24)	-	-	-
Diphyidae (Fig 2.25)	-	-	-
Phoronida (Fig 2.26)	490	150	-
Veliger larva (Fig 2.27)	-	-	-
Veliger larva (Fig 2.28)	-	-	-
Veliger larva (Fig 2.29)	-	-	-
Pteropod (Fig 2.30)	-	-	-
Pteropod (Fig 2.31)	470	100	-
Alciopidae (Fig 2.32)	-	-	-
Alciopidae (Fig 2.33)	-	-	-
Alciopidae (Fig 2.34)	-	-	-
Opheliidae (Fig 2.35)	-	-	-
Opheliidae (Fig 2.36)	-	-	-
Opheliidae (Fig 2.37)	510	40	-

Opheliidae (Fig 2.38)	-	-	-
Opheliidae (Fig 2.39)	-	-	-
Opheliidae (Fig 2.40)	-	-	-
Opheliidae (Fig 2.41)	-	-	-
Opheliidae (Fig 2.42)	-	-	-
Opheliidae (Fig 2.43)	-	-	-
Opheliidae (Fig 2.44)	-	-	-
Opheliidae (Fig 2.45)	-	-	-
Serpulidae (Fig 2.46)	-	-	-
Spionidae (Fig 2.47)	-	-	-
Spionidae (Fig 2.48)	670	50	chlorophyll
Spionidae (Fig 2.49)	-	-	-
Oweniidae (Fig 2.50)	-	-	-
Phyllodocidae (Fig 2.51)	-	-	-
<i>Syllis prolifera</i> (Fig 2.52)	-	-	-
<i>Neanthes acuminata</i> (Fig 2.53)	-	-	-
Nereididae (Fig 2.54)	-	-	-
Decapod larva (Fig 2.55)	-	-	-
Decapod larva (Fig 2.56)	-	-	-
Decapod larva (Fig 2.57)	-	-	-
<i>Temora stylifera</i> (Fig 2.58)	-	-	-
Oncaea (Fig 2.59)	670	210	chlorophyll
Amphioxus, part 1 (Fig 2.60)	510	30	-
Amphioxus, part 2 (Fig 2.61)	510	30	-
Amphioxus, part 1 (Fig 2.62)	-	-	-
Amphioxus, part 2 (Fig 2.63)	510	30	-
<i>Thalia democratica</i> (Fig 2.64)	-	-	-
<i>Thalia democratica</i> (Fig 2.65)	670	30	chlorophyll
<i>Salpa fusiformis</i> (Fig 2.66)	-	-	-
Doliolidae (Fig 2.67)	-	-	-
Doliolidae (Fig 2.68)	-	-	-
Larva Osteichthyes (Fig 2.69)	-	-	-

Table 2.4: UV test excitation assay of all specimens analysed. Figure numbers, emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns.

The UV response analysis is interesting to explore as very few fluorescent proteins have been identified so far with excitation in the UV. This test was able to show that some species possess chlorophyll (i.e. Radiozoa, Spionidae, Oncaea and *Thalia democratica*), while some others present

an emission in the green part of the spectrum. The intensity of the response is quite variable, from around 30 A.U in Amphioxus and Tunicates to 210 A.U in copepod.

Although most of the organisms tested did not present any response to the UV excitation, it may be better to use more excitation wavelengths as it has been performed with the visible light excitation spectra. The UV test nevertheless can be used to rule out positive organisms to chlorophyll.

4.3.2. Visible light test

- **Radiozoa:**

• Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	intensity (0-200)	Fluorophore/chlorophyll
Radiozoa (Fig 2.13)	550 to 570	590	40	-
Radiozoa (Fig 2.14)	550 - 630	670	90	-
	550 - 630	690	90	-

Table 2.5: Visible light test of Radiozoa. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns.

Radiozoa were interesting species to investigate as they showed bright red fluorescence under the epifluorescent microscope and by confocal microscopy with bright red emission response to the UV light in the red wavelengths, corresponding to chlorophyll (see figure 2.14). Also, the visible light test showed a high response in the red part of the spectrum to both green and red excitation wavelengths. However, these excitation and emission wavelengths do not directly correspond to chlorophyll spectra as the absorption should be more around 430 nm.

- **Cnidaria:**

Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
Hydroidolina (Fig 2.15)	470 to 490	515	100	Superfolder GFP
Cunina sp. (Fig 2.16)	470 to 490	510	120 to 160	Superfolder GFP
Obelia sp. (Fig 2.17)	470 - 480	490-510	60 to 65	mMiCy
Hydrozoa (Fig 2.18)	480-490	510 to 530	115	Superfolder GFP, mEmerald, mWasabi
	510	540	130	-
Hydrozoa (Fig 2.19)	470 to 500	510 to 530	100 to 140	Superfolder GFP
Scyphozoa (Fig 2.20)	470 to 490	510	70 to 85	Superfolder GFP
Siphonophorae (Fig 2.21)	480 - 490	510	100	Superfolder GFP
	500	530	100	-
Siphonophorae (Fig 2.22)	490	510	110	Superfolder GFP
	500	530	110	-
Siphonophorae (Fig 2.23)	490	510	55	Superfolder GFP
Diphyidae (Fig 2.24)	480 - 490	510	90 to 120	Superfolder GFP
	500	530	80	-
Diphyidae (Fig 2.25)	480 - 490	510	110	Superfolder GFP
	500	530	110	-

Table 2.6: Visible light test of Cnidaria. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns. (Superfolder GFP and mEmerald are derived from the hydrozoa *Aequorea victoria*. mWasabi is derived from the Anthozoa *Clavularia* sp. mMiCy1 is derived from the Anthozoa *Acropora* sp.) (see Appendices A1.1, A1.2, A1.3, A1.4)

Many species of hydrozoans have been studied in the attempt to find a fluorescent protein with new characteristics, especially in siphonophores where no FP have been characterized yet. Interestingly, most of all hydrozoans display fluorescent patterns similar to GFP (λ_{exc} 488 nm - λ_{ems} 510 nm) with excitation between 470 and 490 nm and emission between 510 and 530 nm with high intensity. As most bioluminescent emission spectra are blue (λ_{ems} 475 nm), it could be possible that the fluorescent emission pattern observed in siphonophores originate from a FP.

- **Phoronida:**

Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
Phoronida (Fig 2.26)	540 - 550	570	80 to 100	mOrange2, mKO2
	560	590	90	mRuby3

Table 2.7: Visible light test of Phoronida. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns. (The three fluorophores all originate from Anthozoa. mOrange2 is derived from *Discosoma* sp., mKO2 from *Verrillofungia concinna* and mRuby3 from *Entacmaea quadricolor*). (see Appendices A1.5, A1.6, A1.7).

Being not a very abundant species, only one specimen of Phoronida has been analysed at the confocal microscope to assess its fluorescent characteristics. We can notice an excitation mainly in the green part of the visible spectrum with a rather high emission in the yellow spectrum. Several fluorophores possess spectral characteristics similar to those measured at the confocal microscope, namely mOrange2 (λ_{exc} 549 nm, λ_{ems} 565 nm), mKO2 (λ_{exc} 551 nm, λ_{ems} 565 nm) and mRuby3 (λ_{exc} 558 nm, λ_{ems} 592 nm). However, it is important to underline that when the experiment was performed the animal was next to the edge of the Petri dish. Therefore, the fluorescence may originate from a reflection of the laser on this surface, see Figure 2.26.

- **Mollusca:**

Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
Veliger larva of gastropod (Fig 2.27)	540 - 550	570	170 to 210	mOrange2, mKO2
	540 to 560	590	170 to 220	mRuby3
Veliger larva of	550	570	70	-

gastropod (Fig 2.28)	550 - 560 - 570	590	70 - 80	mRuby3
	630	650	70	-
	630	670	70	-
Veliger larva of gastropod (Fig 2.29)	560	590	55	mRuby3
	550	570 - 580	50	-
Pteropod (Fig 2.30)	-	-	-	-
Pteropod (Fig 2.31)	490 - 510	690	100	chlorophyll

Table 2.8: Visible light test of Mollusca. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns.

Several molluscs have been investigated at the confocal microscope where they show mainly high intensity response in the yellow-orange and red part of the visible spectrum, located mainly in the gut. Some of their spectral properties correspond to already identified fluorophores (mRuby3, mKO2 ad mOrange2). Finally, in Pteropod (Figure 2.31) we observe a high signal in the red part of the spectrum with green excitation mainly, which originates from chlorophyll.

- **Annelida:**

Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
Alciopidae (Fig 2.32)	550 - 560	590	60 to 80	mRuby3
Alciopidae (Fig 2.33)	470 - 480	690	20	chlorophyll
Alciopidae (Fig 2.34)	-	-	-	-
Opheliidae (Fig 2.35)	540 - 550	570	50 to 70	mOrange2, mKO2
Opheliidae (Fig 2.36)	550 - 560	590	70	mRuby3
	550 to 570	590	100 to 130	mRuby3
Opheliidae (Fig 2.37)	540 - 550	570	90 to 120	mOrange2, mKO2
	470	550	40	-
Opheliidae (Fig 2.38)	-	-	-	-
Opheliidae (Fig 2.39)	470	550	40	-
Opheliidae (Fig 2.40)	560	590	40	-
Opheliidae (Fig 2.41)	-	-	-	-

Opheliidae (Fig 2.42)	-	-	-	-
Opheliidae (Fig 2.43)	-	-	-	-
Opheliidae (Fig 2.44)	-	-	-	-
Opheliidae (Fig 2.45)	-	-	-	-
Serpulidae (Fig 2.46)	550	570	50	mOrange2, mKO2
Serpulidae (Fig 2.47)	560	590	50	-
	-	-	-	-
Spionidae (Fig 2.48)	560	590	40	mRuby3
Spionidae (Fig 2.49)	-	-	-	-
Oweniidae (Fig 2.50)	490 - 510	670	50	chlorophyll
Phyllodoceidae (Fig 2.51)	-	-	-	-
<i>Syllis prolifera</i> (Fig 2.52)	-	-	-	-
<i>Neanthes acuminata</i> (Fig 2.53)	-	-	-	-
Nereididae (Fig 2.54)	670	690	50	-

Table 2.9: Visible light test of Annelida. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns.

Many polychaete species have been investigated as they were showing interesting features at the epifluorescent microscope, especially the species Alciopidae showing green fluorescence in the eye. However, the analysis at the confocal microscope shows a different fluorescent pattern where the eyes appear red instead of green (see Figures 32 to 34).

Polychaetes could also be an interesting clade to investigate, as no FP has been isolated from this class yet, only bioluminescence (see discussion). Here we can observe emission mainly in the yellow part of the spectrum with rather small intensity. Only the species Opheliidae presented high intensity with excitation in the green and emission in the orange.

Several excitation and emission spectra from our study have similarities with some already characterized fluorophores, however polychaetes could be interesting to study as they have interesting orange-red emission fluorescent patterns.

- **Arthropoda:**

Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
Decapod larva (Fig 2.55)	600	630	40	-
Decapod larva (Fig 2.56)	550	570	50	mOrange2, mKO2
	570	590	50	-
Decapod larva (Fig 2.57)	-	-	-	-
<i>Temora stylifera</i> (Fig 2.58)	550-570	570-590	40-70	-
<i>Oncaea</i> (Fig 2.59)	670	690	-	-

Table 2.10: Visible light test of Arthropoda. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns.

A few years ago, several GFPs have been characterized from Copepods (*Pontellidae* and *Labidocera aestival*), therefore we explored if other fluorescent patterns mainly in the red part of the spectrum were present. The experiments show a light fluorescent emission intensity mainly in the green part that are similar to some already characterized fluorophores and the red signal could originate from chlorophyll as is it located in the gut.

- **Cephalochordata:**

Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
Amphioxus, part 1 (Fig 2.60)	480 to 510	530	140 to 170	Superfolder GFP, mEmerald mWasabi
Amphioxus, part 2 (Fig 2.61)	470 to 490	510	190 to 220	Superfolder GFP, mEmerald mWasabi
Amphioxus, part 1 (Fig 2.62)	470 to 490	510	160-180-190	Superfolder GFP, mEmerald mWasabi
	490-500	530	180	-
Amphioxus, part 2 (Fig 2.63)	470 to 490	510	200 to 220	Superfolder GFP, mEmerald

Table 2.11: Visible light test of Cephalochordata. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns.

Several GFP from the species *Branchiostoma floridae* have been characterized in the ovaries, eggs and in the mouth a few years ago, encoding the largest known family of GFPs in a single organism. It was interesting to assess where fluorescence was present in the animal and if new fluorescent patterns could be denoted. We noticed a high fluorescence intensity mainly in the green part of the spectrum close to already characterized fluorophores such as mEmerald, Superfolder GFP and mWasabi. GFPs derived from *Branchiostoma floridae* have green emission: bfoGFPa1 (λ_{exc} 500 nm, λ_{ems} 512 nm), bfoGFPc1 (λ_{exc} 493 nm, λ_{ems} 521 nm), LanFP1 (λ_{exc} 500 nm, λ_{ems} 510 nm) and LanFP2 (λ_{exc} 500 nm, λ_{ems} 516 nm). Those isolated from *Branchiostoma lanceolatum* have green, yellow and red emission: dLanYFP (λ_{exc} 513 nm, λ_{ems} 524 nm), laGFP (λ_{exc} 502 nm, λ_{ems} 511 nm), lanRFP (λ_{exc} 521 nm, λ_{ems} 592 nm), mNeonGreen (λ_{exc} 506 nm, λ_{ems} 517 nm).

- **Tunicata:**

Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
<i>Thalia democratica</i> (Fig 2.64)	550	570	80	mOrange2, mKO2
	550 - 560	590	80	mRuby3
<i>Thalia democratica</i> (Fig 2.65)	670	690	30	-
<i>Thalia democratica</i> (Fig 2.66)	-	-	-	-
<i>Salpa fusiformis</i> (Fig 2.67)	-	-	-	-
Doliolidae (Fig 2.68)	-	-	-	-

Table 2.12: Visible light test of Tunicata. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns.

Tunicate have also been investigated by confocal microscopy as no fluorescent proteins were isolated from this clade. Two specimens of *Thalia democratica* present a fluorescent emission in response to the visible light excitation test, mainly in the green and red part of the spectrum. *Salpa fusiformis* and a doliolid did not show any interesting pattern.

- **Vertebrata:**

Visible light				
Species and figure	λ_{exc} (nm)	λ_{ems} (nm)	Intensity (0-200)	Fluorophore/chlorophyll
Larva	580 - 610	630	80 to 90	-
Osteichthyes (Fig 2.69)	580	610	90	FusionRed

Table 2.13: Visible light test of Vertebrata. Figure numbers, excitation and emission wavelengths with corresponding peak intensities are reported as well as analogous fluorophores or chlorophyll patterns. (FusionRed derives from the anthozoa *Entacmaea quadricolor*) see A1.8

We can notice a moderate red fluorescent signal in this larva, which is similar to the FusionRed protein.

5. Discussion

The use of a brand-new protocol to perform the confocal microscopy analysis has allowed the measurement of fluorescence in numerous phyla of the Gulf of Naples so as to isolate a new fluorescent protein. This technique can rule out which class of zooplanktons are fluorescent and those that are not. From it, we can draw a few conclusions if an organism is fluorescent or if it comes from chlorophyll pigmentation.

First, we have been analysing the protozoa Radiozoa, presenting bright red fluorescent signal at the confocal microscope. Further reading suggests that this species possess chloroplasts, therefore the signal observed is not from a fluorescent protein.

Another attempt to find a new FP was to observe Cnidarians and especially Siphonophores that are in high proportion in the Mediterranean sea (Siokou-Frangou *et al.*, 2010). They showed an intense green fluorescent pattern in the hydroecium, a sac containing oil droplets. It is not sure if this signal is due to chlorophyll so it could come from a fluorescent protein with green emission. So far, no FP has been identified in this type of Hydrozoa so it could be of interest.

Then, the small phylum of filter feeders called Phoronida has been examined too, the confocal microscopy experiment nevertheless was not conclusive as the specimen was next to the edge of the dish. While observing a specimen at the epifluorescent microscope, an interesting fluorescent pattern was noticed on the tentacles, with a both green and red fluorescent signal. This species is not abundant in the gulf of Naples, but another laser confocal test could be practiced to have a better picture of its fluorescence signal.

The mollusc taxon presents a dual picture, in fact green fluorescent signal from the confocal microscopy analysis can be observed in the eye of veliger and this could originate from a fluorescent protein. However, numerous red fluorescent signals have been characterized by confocal microscopy in the gut or digestive gland and those patterns have also been noticed by

epifluorescence. Therefore, it would be important to analyse more veliger of gastropod so as to know if the green signal in the eye observed again.

The phylum with the most interesting features is most likely Annelida. Many organisms of different genus have been observed and important fluorescent signals have been noticed either at epifluorescent and confocal microscopy assessment. In fact, the segments of the genus Opheliidae appear green fluorescent at the confocal microscope and this is also confirmed in some specimens by laser scanning analysis where green fluorescence emission is noticed in the visible light excitation response. Furthermore, two of the polychaete species collected at the seafront present red fluorescence in the head which is observed at the epifluorescent microscope and confirmed with the visible light laser assessment. The inconvenient of this evaluation was the size of the specimens, as only one organism was possible to analyse at the confocal microscope. Smaller specimens of these two species of polychaetes could nonetheless be examined. Alciopidae having a red pigmented eye is so far the species with the most exciting signal. The observations at the epifluorescent microscope and the plots at the confocal microscope could lead in the direction towards the identification of a fluorescent pigment or fluorescent protein.

In Arthropoda, a few observations of green fluorescence were noticed in the nerve cells along the tail and the abdomen, which could originate from a pigment. Other fluorescent patterns are noticed in the gut, which is due to algal content, this was also confirmed by analysis at the confocal microscope.

Finally, the phylum Chordata has been assessed and an organism of interest would be *Salpa fusiformis*, presenting a strong UV response at its pinnacle in the orange/red part of the visible spectrum.

These findings enrich the knowledge of the existence of fluorescence in the sea, particularly in the Gulf of Naples. In fact, it is the first time that such a broad study examining fluorescence in zooplanktons has been performed. The distinction between autofluorescence from chlorophyll and fluorescence probably originating from a pigment or protein has led us to highlight one

particular phylum, Annelida, that could be investigated at the molecular level in the future. Also, these results highlight the specific role of fluorescence in UV protection or in food ingestion. In this sense, multiple fluorescent signals are observed in the tentacle of phoronid, jellyfish and along the body of crustaceans where fluorescence could protect these cells from UV light. Another interesting insight is the presence of fluorescence in both the pelagic and benthic species of the Mediterranean Sea.

Assessing the spectral properties of zooplankton is not an easy task as I routinely encounter technical issues. Zooplankton specimens after sampling are often associated with dirt that can be difficult to remove as it can damage the organism. In fact, it is omnipresent in the marine environment and sticks onto the species often masking hairs, chaetae and many other features that are crucial for taxonomy and fluorescence observation.

A study using UV illumination to counteract this issue noticed a positive effect as it makes body features such as exoskeleton become more clearly visible. However, they observed that green light failed to make it “light up”, and in that case dirt continued to remain visible (Koken and Grall, 2011). In our study, using UV light did not help to better visualize zooplankton features, it was easier to look at them with white light and then switch to either green or red-light filters.

Also, the confocal microscopy analysis used during the project is quite advantageous and interesting to use as we have been able to examine the spectral properties of many clades and perform a dual excitation/emission study.

A very interesting technique developed on TARA samples would have been interesting to use for our project for better taxonomic and evolutionary studies. Environmental high content fluorescence microscopy (e-HCFM), allows 3D-fluorescence imaging and classification for high throughput analysis of microbial eukaryotes over a broad taxonomic range (Colin *et al.*, 2017). The technique has been used on 72 plankton samples, specimens were automatically imaged and over 330,000 organisms were classified. This technique could have been interesting to be employed on MareChiara zooplankton samples as this technique can simultaneously identify eukaryotic

organisms acquired at a single focal plane and measure auto-fluorescence from photosynthetic pigments such as chlorophyll (Hense *et al.*, 2008). Therefore, I would have collected more information and differentiate better the organisms from their chlorophyll response and have a more detailed taxonomic analysis. From this data, it could also be interesting to organize an evolutionary scenario for marine invertebrates to evaluate which organisms possess fluorescence. The main advantage of e-HCFM is the use of 3D fluorescence microscopy in an automated and quantitative manner that can be applied to samples from any aquatic environment and be adapted for other habitats.

5.1 Autofluorescence and fluorescence linked with pigmentation.

Epifluorescence microscopy and examination of autofluorescence allows the visualization of plankton so as to identify taxon-specific fluorescent signals and to determine the sources for the isolation of new fluorescent proteins. As mentioned in the introduction, natural fluorescence in marine samples in particular, caused by endogenous origins (chlorophyll, lipofuscin, collagen, elastin, NADH, riboflavins and flavin coenzymes), is a universal and well-recognized problem (Braley *et al.*, 2018). Therefore, to isolate a fluorescent protein it is important to take in consideration all these features.

For example, there is a huge diversity of autofluorescent planktonic species such as in Radiolaria, the most diverse group of planktonic hosts with eukaryotic microalgal symbionts (Pierella Karlusich *et al.*, 2020). Green autofluorescence was also detected in the gut of the larval mollusc *Tridacna noae* (Braley *et al.*, 2018), as well as in many hydrozoan species displaying fluorescence in their polyps and/or medusa stages (Maggioni *et al.*, 2020).

Specific compounds like chemical derivatives of the organic substance coumarin seem to be at the origin of fluorescence observed in the cuticle of arthropods (e.g., spiders, scorpions) (Andrews *et al.*, 2007; Stachel *et al.*, 1999). Many planktonic organisms observed in MareChiara sampling present fluorescence linked with pigments (i.e crustaceans, phoronida). A recent study described fluorescent chromatophores in all life stages of the daggerblade grass shrimp *Palaemonetes pugio*

(Phelps, 2013). In fact, fluorescent chromatophores were the only pigmented cells present during embryonic and early larval development. To minimize exposure to UV radiation, crustaceans accumulate protective pigments such as carotenoids or melanin and practice escape behaviour (Dahms and Lee, 2010; Hansson, 2004; Johnsen, 2001; Ribeiro Gouveia *et al.*, 2004). It is also hypothesized that chromatophores contains fluorescent pteridine, one of the most common fluorescent pigments. It has already been described in the chromatophores of a number of diverse chordates such as amphibians or zebrafish (see Table 2.14) (Guyader and Jesuthasan, 2002; Ortiz and Williams-Ashman, 1963). It is often the case that GFP are not associated with distinct chromatophores (Baumann *et al.*, 2008; Hunt *et al.*, 2010) but with pigmented cells of some corals (Salih *et al.*, 2000; Schlichter *et al.*, 1994).

Pigment name	Pigment type	Phylum/Organism	λ_{Exc} (nm)	λ_{Emi} (nm)
Crustacyanin	Carotenoid	Arthropoda Malacostraca	530	580
β -carboline	Tryptophan derivative	Arthropoda Aracnida	360–370	445–490
Psittacofulvin	Non-carotenoid	Chordata Aves	N/A	N/A
Spheniscins	Pterins-like	Chordata Aves	UV	N/A
Sepiapterin	Pteridin	Chordata Actinopterygii	UV	450–490
Porphyrin	Porphyrin	Mollusca Gastropod	UV	625
Hyloin	Dihydroisoquinolinone	Chordata Amphibia	390–430	450–470
Betaxanthins	Betalains	Plantae caryophyllales	463–474	509–512

Table 2.14: Pigments participating in the process of fluorescence (Macel *et al.*, 2020).

As stated in the results paragraph on section 4.2.5, the annelid Alciopidae presented a bright red fluorescent signal in the eye. It is interesting to investigate the nature of this red fluorescent signal by examining the composition of their eyes. It has been shown that the eyes of Alciopidae prevail the highest organisation of the visual organs among polychaetes as they rival the camera-type eyes of vertebrates and cephalopods in structural complexity (Hermans and Eakin, 1974).

The adult eyes are even reported to be capable of adapting themselves to variations in light (Tampi, 1949), which is an interesting concept while studying fluorescence and other sources of light in the ocean. The exceptional development of the eyes, which in some species occupy almost

the whole head, suggests that the alciopids detect their prey mainly by sight. The lens, which is red in life and presumably acts as a light-filter, lies behind a thickened corneal area (Dales, 1954).

The eyes anatomy of the Alciopidae *Vanadis tagensis* have been examined extensively, they appear as a darkly pigmented spheroid, with a large transparent cornea protruding from its antero-lateral surface (Hermans and Eakin, 1974).

5.2 Coexistence of fluorescence and bioluminescence in organisms

Bioluminescence, the first primary source of light in the ocean can be difficult to distinguish from fluorescence if both coexist in the same species. In fact, bioluminescent compounds found in photophores for example may also be autofluorescent, and thus can often be visualized through their fluorescence under short-wavelength or UV illumination. An example of it are the lures of the siphonophore *Erenna* sp. where young tentacles are bioluminescent and mature ones are characterized by red fluorescent signals (Haddock, 2005).

Another concern with the distinction between fluorescence and bioluminescence is that their emission spectrum can match, as the same molecules are participating in the excitation-emission process. It happens with natural materials that can be fluorescent under UV-blue light such as chitin and calcium phosphate. A study conducted in hydroid species observed clear fluorescence it on the external side of the chitinous exoskeleton under blue light excitation (Stabili *et al.*, 2008). It was shown that fluorescence is due to the development of a bacterial biofilm as when the species were treated with antibiotics fluorescence disappeared.

Therefore, these examples underline the complexity to discriminate if the measured light emission is autofluorescence from chlorophyll, fluorescence, bioluminescence or combination of both phenomena.

Most emission spectra of bioluminescence are blue, centred on the wavelength that travels farthest through seawater ($\lambda_{\text{max}} \sim 475 \text{ nm}$), as this light phenomenon has evolved in the pelagic zone (Widder, 2010). Whereas green, the second most common colour in bioluminescence, is mostly found in benthic and shallow coastal species.

Not all marine fauna will bear bioluminescence, as we can notice on Figure 2.70, among 13 taxonomic groups, most all of them are bioluminescent or likely to be. Pteropoda, Thaliacea and Rhizaria are non-bioluminescent whereas Chaetognatha are unlikely to be and Crustacean are undefined. This diagram allowed me to understand better which phenomenon is behind the spectral traits that we observed in our experiment and to have a better idea if it is bioluminescence or autofluorescence from chlorophyll.

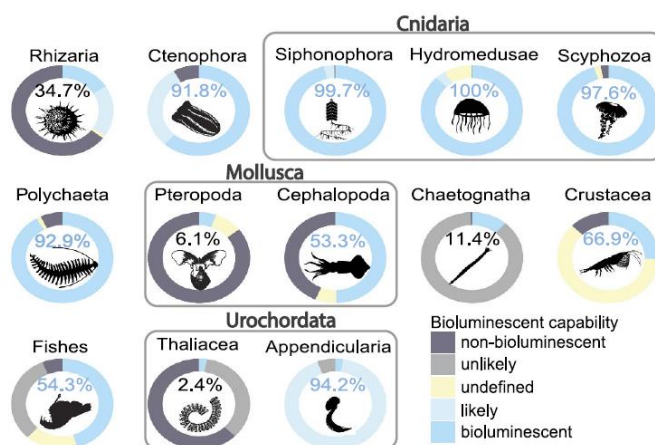


Figure 2.70: Bioluminescence capability over the main observed taxa (Martini and Haddock, 2017). The percentages only represent the probably bioluminescent organisms relative to the sum of probably bioluminescent and probably non-bioluminescent ones.

Many samples of Annelids have been explored at the confocal microscopy from LTER-MareChiara samples and the seaside collection. It is interesting to explore what could be at the origin of light emission in this taxon. Here I will discuss especially about bioluminescence in Annelida as several studies investigated the phenomenon that might be in relation to the observations at the confocal microscope.

5.3 Bioluminescence in polychaetes

Luminescence is present in polychaetes of many families such as Tomopteridae, Syllidae, Chaetopteridae, Cirratuliformia, Terebelliformia, Polynoidae (Nicol, 1953), see Figure 2.71. Compared to other taxa such as in Cnidaria where bioluminescence is essentially blue in colour, Annelid present a wide array of bioluminescent colours—including yellow light, which is very rare among marine taxa. The diversity of emitted colours implies that light production in this phylum might be involved in a variety of functions (Verdes and Gruber, 2017).

As we can notice on the tree, bioluminescence has evolved independently in several lineages (Haddock. *et al.*, 2010) with almost 100 luminous species distributed in thirteen families.

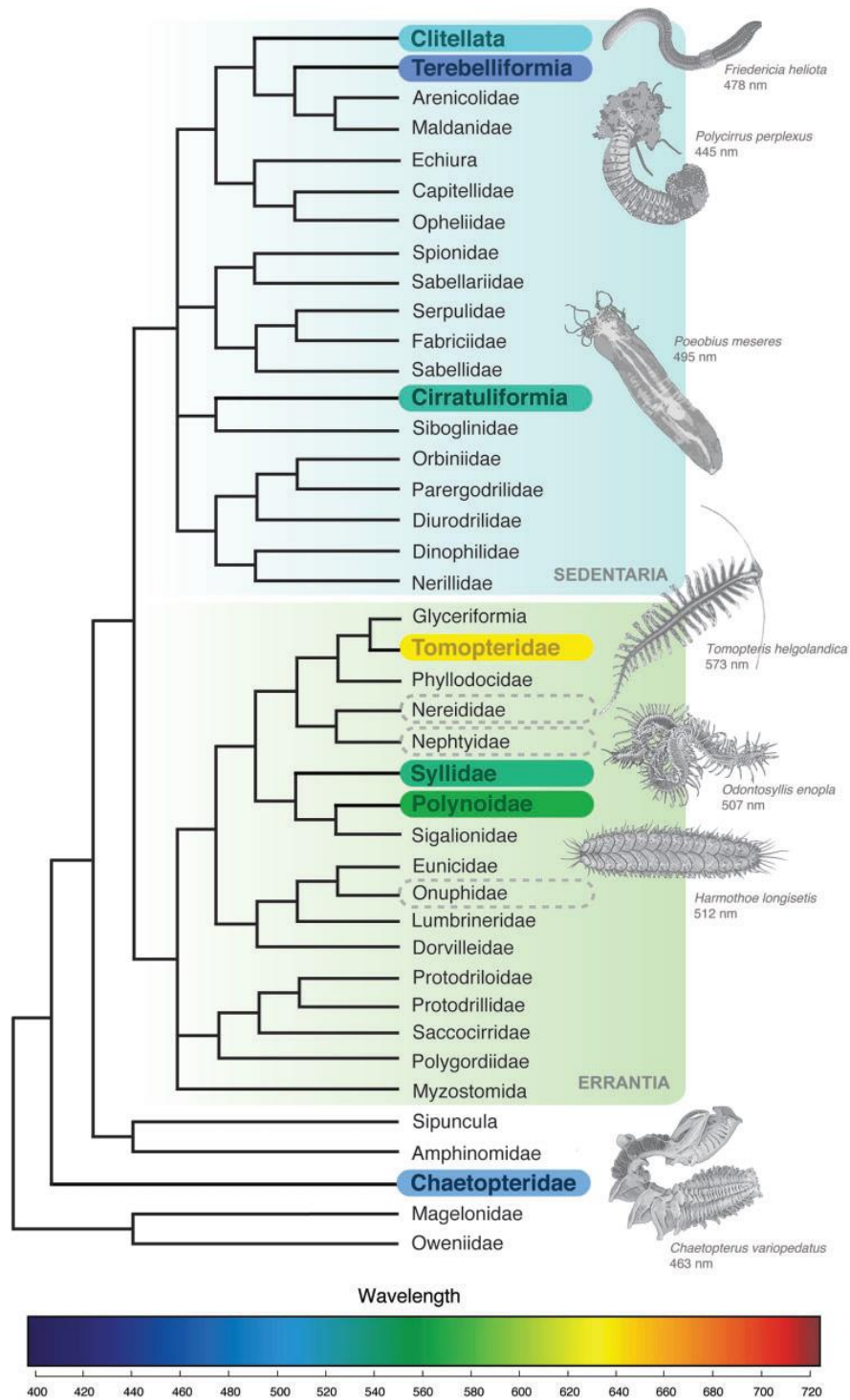


Figure 2.71: Distribution and spectral diversity of bioluminescence in the phylum Annelida (Verdes and Gruber, 2017). Lineages surrounded by ovals include bioluminescent species. Colour of the oval indicates the bioluminescence emission maxima of a representative species; the corresponding wavelength, species name, and a schematic representation are shown to the right. Ovals with a dashed-line contour indicate lineages with dubious reports of bioluminescent species.

- **Syllidae:**

The green emission pattern seen under UV light by *Syllis prolifera* might be bioluminescence as the first record of a bioluminescent *Odontosyllis* was likely by Christopher Columbus in 1492 when the Santa Maria was approaching the Bahamas (Crawshay, 1935). Later on, the bioluminescent systems of *Odontosyllis enopla* and *Odontosyllis phosphorea* have been briefly investigated and a luciferin from *O. enopla* has been partially purified (Shimomura *et al.*, 1963) whereas the bioluminescent system of *O. phosphorea* uses a photoprotein as a substrate, instead of a luciferin-luciferase reaction (Deheyn and Latz, 2009).

Overall, light production in polychaetes is very diverse and can exhibit a wide range of emission wavelengths from 445 nm to 573 nm. Bioluminescence in this clade evolves independently in several lineages with almost 100 luminous species distributed in thirteen families (Verdes and Gruber, 2017).

- **Polynoidae:**

Polynoidae, also called scale worms are found worldwide from the tropics to the Antarctic (Hartman, 1978) and Arctic Seas where they are present from the seashore, deep waters (Pettibone, 1989) to abyssal depths (Hartman and Fauchald, 1971). The bioluminescence originates from a membrane photoprotein called polynoidin that reacts specifically to the presence of superoxide anions (Bassot and Nicolas, 1995).

An interesting study investigating the spectral properties of the tubercle of a White Sea polynoid worm *Lepidonotus squamatus*, was shown to emit green and orange fluorescence under UV and blue light excitation (Plyushcheva *et al.*, 2017). The spectra of their tubercles have been measured with a method similar to the one we used by confocal microscopy, by using eight different lasers for excitation from the violet (405 nm) to the red (632 nm) (Plyuscheva *et al.*, 2014). Using different excitation wavelengths allowed to perform a quantitative analysis of the observed

spectral changes. Thereafter, this analysis could assess the contributions of different chromophores, only if the excitation energy was sufficiently large to excite it.

**CHAPTER 3 - FLUORESCENT PROTEIN MINING IN GENOMIC
AND TRANSCRIPTOMIC DATABASES**

1. Significance of TARA project and computational analysis of already available transcriptomes and genomes

To better characterize microscopic planktons, the TARA Oceans consortium initiated in September 2009 and was led by a group of French scientists aimed at combining several disciplines (ecology, systems biology, and oceanography) to study plankton in their environmental context (Zhang and Ning, 2015). The purpose of the expedition was to investigate the planktonic world, including viruses, bacteria, protists, and zooplankton diversity at global scale (Karsenti *et al.*, 2011).

Three major studies were conducted, first to examine the ocean microbial function and community structure (Bork *et al.*, 2015), second to study the ocean virome (Brum *et al.*, 2015) and third to interpret ocean plankton interactome (Lima-Mendez *et al.*, 2015). By means of sampling a wide variety of planktonic organisms (from viruses to fish larvae) from the ocean's surface (0–200 m) and mesopelagic zone (200–1,000 m) at a global scale, TARA Oceans surveyed 210 ecosystems in 20 biogeographic areas, collecting over 35,000 samples of seawater and plankton (Pesant *et al.*, 2015). This project took advantage of omics approaches of sequencing technologies, allowing high-throughput analyses of DNA, RNA, proteins, and metabolites (Planes *et al.*, 2019). This conferred to TARA Oceans the generation of a tremendous amount of environmental sequencing data, from metabarcoding (metaB), metagenomic (metaG) and metatranscriptomic (metaT) data sets as well as single-cell genomes (Sunagawa *et al.*, 2020). Therefore, the use of modern sequencing combining state-of-the-art imaging technologies, TARA Oceans is at the cutting edge of marine science and bioinformatics compared to other genomic and transcriptomics tools (Karsenti *et al.*, 2011). In fact, TARA Oceans gather the access to genomic content of organisms that are not available in culture (including zooplankton species).

Even though several collections of reference marine eukaryote organisms' sequences have been created, derived from databases of cultured organisms such as Aniseed (Brozovic *et al.*, 2018), Echinobase (Cary *et al.*, 2018) or Ensembl Genomes (Yates *et al.*, 2020), they do not compete with

the dataset generated by TARA Oceans. It represents a limited amount of representative species in the open ocean, including zooplanktons, that depict only a small fraction of the natural biological diversity (Vargas *et al.*, 2015). TARA dataset also confers a holistic approach to investigate organisms as genes-to-ecosystem models (Guidi *et al.*, 2016) and encourage scientists to explore the biodiversity and functioning of our oceans.

2. Aims

The objectives of this study are first to perform a bioinformatics and molecular evolution analysis to identify by sequence homology potential new GFPs by screening available genomes and transcriptomes of known planktonic species. Second, the use of TARA ocean database will be carried out to investigate sequence homology in the attempt to identify a new GFP or a new FP. Blastp of different reference proteins were realized in the eukaryotic database using Sandercyanin, UnaG and 14 GFP representatives from three different clades. Alignments of protein sequences and molecular evolution studies will allow the identification of potential new GFPs and FPs.

3. Methods

3.1 Search of potential new GFPs in genomes and transcriptomes of planktonic species.

The transcriptomes of several eukaryote species where no GFP have been identified yet have been surveyed in the attempt to find potential new GFP sequences. Particularly, 14 GFP

sequences from 4 different clades are reported in Table 3.1 that have been used for blastp analysis on the genomes of tunicates (<https://www.aniseed.cnrs.fr/aniseed/species/>), echinoderms (<https://www.echinobase.org/entry/>) and species of sponges, ctenophores, nematodes, annelids and molluscs (<https://metazoa.ensembl.org/index.html>).

Protein ID	Accession number	Taxonomy, (class, subclass, order)
FP512	AAQ11989.1	<i>Cerianthus membranaceus</i> Anthozoa, Ceriantharia, Spirularia
Green fluorescent protein	AAK97633.1	<i>Montastraea cavernosa</i> Anthozoa, Hexacorallia, Scleractinia
Green fluorescent protein	AAM10626.2	<i>Heteractis crista</i> Anthozoa, Hexacorallia, Actiniaria
asFP499	AAG41205.1	<i>Anemonia sulcata</i> Anthozoa, Hexacorallia, Actiniaria
Green fluorescent protein	AAK83923.1	<i>Orbicella faveolata</i> Anthozoa, Hexacorallia, Scleractinia
Cyan fluorescent protein C1	AAU04450.2	<i>Pocillopora damicornis</i> Anthozoa, Hexacorallia, Scleractinia
Green fluorescent protein	AAN41637.1	<i>Aequorea coerulescens</i> Hydrozoa, Hydroidolina, Leptothecata
Green fluorescent protein	AAL33915.1	<i>Aequorea macrodactyla</i> Hydrozoa, Hydroidolina, Leptothecata
Green fluorescent protein	AAA27721.1	<i>Aequorea victoria</i> Hydrozoa, Hydroidolina, Leptothecata
Green fluorescent protein	AAQ01185.1	<i>Labidocera aestiva</i> Hexanauplia, Copepoda, Calanoida
Green fluorescent protein 1	AAQ01186.1	<i>Pontella meadi</i> Hexanauplia, Copepoda, Calanoida
green fluorescent protein	BAE78442.1	<i>Chiridius poppei</i> Hexanauplia, Copepoda, Calanoida
bIFP-Y1	ACA48230.1	<i>Branchiostoma lanceolatum</i> Leptocardii, Branchiostoma
green fluorescent protein	ABO61190.1	<i>Branchiostoma floridae</i> Leptocardii, Branchiostoma

Table 3.1: List of reference proteins used for the protein alignment: GFPs from Anthozoa, Hydrozoa, Hexanauplia and Cephalochordates.



Figure 3.1 : Alignment of the 14 reference GFPs and their chromophores highlighted.

The reference GFP proteins all possess a XYG chromophore motif, with the first amino acid that is different. We observe a diversity among Anthozoans, whereas Hydrozoans and Arthropoda possess the same first amino acid (see Figure 3.1). In this respect, the chromophore variations observed in Anthozoans is at the origin of GFP colour diversity in corals.

The genomes of 15 tunicates species are covered on Aniseed, 3 species of echinoderms on Echinobase, Ensembl Metazoa allows a blastp search in the genome of the Porifera *Amphimedon queenslandica*, of the ctenophore *Mnemiopsis leidyi*, 11 species of Nematoda, 2 species of Annelida, 4 of Mollusca. The blastp search has been realized with 14 GFP references proteins in Ensembl Metazoa and Echinobase while in Aniseed a tblastn analysis was done. The blastp search was always the standard method used for good annotated genomes and tblastn was performed specifically on the website Aniseed as the blastp option isn't available.

3.2 TARA ocean database analysis

In collaboration with the bioinformatics core of Stazione Zoologica Anton Dohrn (BIOINFORMA) the sequences in Fasta format obtained by blastp analysis for UnaG and Sandercynin, were extracted from TARA eukaryotic assemblies. The prediction of the encoded proteins was obtained by using TransDecoder software v 5.3.0. The coding sequences were identified with the software based on: 1) a minimum length Open Reading Frame (100 bp) by default to minimize the number

of false positives); 2) an internal score system. The functional annotation of the analyzed sequences was performed with the software InterProScan (version 5.33) (Jones *et al.*, 2014), a comprehensive tool able to assign functions or signatures by querying 16 different biological databases. We obtained for both UnaG and Sander cyanin sequence hits called “MATOU” (Marine Atlas of TARA Oceans Unigenes) which is a catalogue obtained from the assembly of 16.5 terabases of plankton metatranscriptome (cDNA sequences), representing 441 TARA Oceans samples (Villar *et al.*, 2018). Our colleague Dr Elijah Lowe performed the search of GFP related proteins in TARA eukaryotic database.

3.2.1 UnaG

UnaG is a new fluorescent protein characterized in eels, which is part of the fatty acid-binding protein (FABP) family and triggers bright green fluorescence with the coupling of bilirubin. The GPP motif in UnaG has been reported to be crucial in the fluorescence process as mutation of asparagine-57 to an alanine preceding the GPP motif leads to a decrease of fluorescence intensity (Kumagai *et al.*, 2013). Also, two residues, Lys (K) and Arg (R) are conserved in UnaG and in the eels *Anguilla australis*, *Anguilla mossambica* and *Anguilla bicolor bicolor* but the nature of their function is yet unknown.

Five representative reference proteins have been used to proceed with the alignment, one human brain FABP, UnaG and four other reported UnaG from *Anguilla australis*, *Anguilla mossambica* and *Anguilla bicolor bicolor* that are reported in the following table.

Protein ID on NCBI	Accession number	Assignment	Taxonomy, (class, subclass, order)
bilirubin binding protein, UnaG	BAN57322.1	UnaG	<i>Anguilla japonica</i> (Actinopterygii, Anguilliformes)
green fluorescent protein	BAU98067.1	UnaG 1	<i>Anguilla australis</i> (Actinopterygii, Anguilliformes)
green fluorescent protein	BAP76197.1	UnaG 2	<i>Anguilla mossambica</i> (Actinopterygii, Anguilliformes)

green fluorescent protein	BAU98068.1	UnaG 3	<i>Anguilla bicolor bicolor</i> (Actinopterygii, Anguilliformes)
FABP7	CAG33338.1	FABP7	<i>Homo sapiens</i> (Mammalia, Theria, Primates)

Table 3.2: List of reference proteins used for the protein alignment: UnaG, fluorescent FABPs and human FABP7.

3.2.2 Sandercyanin

As stated in the general introduction, Sandercyanin has homology to the lipocalins from different species, especially from the apolipoprotein D from *Larimichthys crocea*. The three peptides of Sandercyanin, A, B, and C, are located at positions 24–53, 88–105, and 111–139, respectively.

Peptide A (30 amino acids): PGRCPKPAVQEDFDAARYLGVVYDIQRLPN

Peptide B (18 amino acids): SQIGSAIAEDPSEPAKLQ

Peptide C (29 amino acids): NAAPVPYCVLLTDYDNYALVYSCINLGAS

Interestingly, the peptides have a sequence similarity of 73% to an unannotated protein in the genome of the green spotted pufferfish, *Tetraodon nigroviridis* (Yu *et al.*, 2008). To date no motif has been identified in Sandercyanin responsible for the fluorescence. However, mutation of Phe-55 to alanine abolished BLA binding, suggesting that this aromatic stacking interaction plays a crucial role in binding (Ghosh *et al.*, 2016). To perform the alignment, 3 Apolipoproteins D from the mouse, human and *Larimichthys crocea* were chosen as well as the Sandercyanin from *Tetraodon nigroviridis*, see Table 3.3.

Protein ID on NCBI	Accession number	Assignment	Taxonomy, (class, subclass, order)
Apolipoprotein D precursor	NP_031496	Apolipoprotein D	<i>Mus musculus</i> (Mammalia, Theria, Rodentia)
Apolipoprotein D precursor	NP_001638	Apolipoprotein D	<i>Homo sapiens</i> (Mammalia, Theria, Primates)
Apolipoprotein D	XP_010738283	Apolipoprotein D	<i>Larimichthys crocea</i> (Actinopterygii, Perciformes)
unnamed protein product, partial	CAF98955	Sandercyanin	<i>Tetraodon nigroviridis</i> (Actinopterygii, Tetraodontiformes)
Chain A, Sandercyanin Fluorescent Protein	5F1E_A	Sandercyanin	<i>Sander vitreus</i> (Actinopterygii, Tetraodontiformes)

Table 3.3: List of reference proteins used for the protein alignment: Sandercyanin, Apolipoproteins and unnamed protein product.

3.2.3 GFP

The 14 GFP representatives (9 from Cnidaria, 3 from Arthropoda and 2 from Chordata) used for the genomic and transcriptomics analysis have been chosen as known references in the analysis (see Table 3.1).

After the alignment of the MATOU hits with the reference protein, a maximum likelihood (ML) bootstrap analysis was conducted using MEGA X software (Kumar et al., 2018), with 100 bootstraps replicates. The amino-acid substitution model used was Jones-Taylor Thornton with uniform rates among sites and to optimize ML, the nearest-neighbor-interchange method was performed with no branch swap filter.

4. Results

4.1 Genomics and transcriptomics analysis

The searches of potential new GFPs in the genomes of sponges, nematodes, annelids, molluscs, echinoderms, and ctenophores did not identify any sequences with homology to GFP and did not possess the XZG motif corresponding to the chromophore, the core of the sequences. The chances of finding orthologs of canonical GFPs in other planktonic phyla as it has been demonstrated a few years ago in amphioxus, is quite low. This could be due to the small number of genomes available online as Blast is possible in only two species of Annelida, 4 of Mollusca, one of Ctenophores and one of Porifera. With the continuous development and growth in genomics and transcriptomics, it is nevertheless plausible that GFPs will be discovered in new available planktonic phyla genomes.

4.2 UnaG

I obtained a total of 693 MATOU hits and 466 sequences were annotated corresponding to Fatty acid-binding protein (FABP) signature, the excel file with the annotations is available at <https://figshare.com/s/0e75c98c0b1d59decdcd> (Table 3.4). After aligning the MATOU hits proteins with MUSCLE software, different isoforms predicted from the same protein were present, generally p1, p2 and p3. As the annotation is the same for all the MATOU sequences, I decided to keep the p1 (normally the longest) and then looking for the specific motifs GPP and the two residues K47 and G86 in the MATOU sequences. Many sequences from the MATOU hits list have been deleted as many presented duplicates. From these sequences, 10 representative sequences from the list were chosen for the final alignment realized on Jalview with the reference proteins (Figure 3.2). Blind blastp which is a search against the whole protein database without searching exclusively in a species has been realized on the 10 MATOU sequences in NCBI resulted 100% as fatty acid binding protein.

Subject ID	% Identity	E-value
MATOU-v1_79140502	53,96	8,00E-40
MATOU-v1_23330938	54,01	1,00E-39
MATOU-v1_60079799	54,74	1,00E-39
MATOU-v1_49093138	54,01	8,00E-39
MATOU-v1_28366541	53,24	1,00E-38
MATOU-v1_103936938	52,55	1,00E-38
MATOU-v1_41122356	53,28	2,00E-38
MATOU-v1_105439838	52,52	2,00E-38
MATOU-v1_92365456	52,55	3,00E-38
MATOU-v1_92388662	53,28	3,00E-38
MATOU-v1_100121203	53,28	4,00E-38

```

100          110          120          130          140          150
MATOU-v1_79140502.p1  DVDQ - - - - - LVSVHG - HRGF AVS VI SGGF VELL SQFEGD - - - - - LCVFEG
MATOU-v1_23330938.p1  I LSK - DGDKVV I KTQS - - - TFKNTE I SFKLGEEFDEATADDRN - CKSTVTMEG
MATOU-v1_60079799.p1  I I SQ - EGDKVV I RTQS - - - TFKNTE I SFKLGEEFDETTADDRN - CKSTVTLEG
MATOU-v1_49093138.p1  DVDQ - - - - - LVSVHG - HRGF AVS VI SGGF VELL SQFEGD - - - - - LCVFEG
MATOU-v1_28366541.p1  I I GQ - EGDKVV I RTQS - - - TFKNTE I SFKLGEEFDETTADDRN - CKSTVTMEG
MATOU-v1_41122356.p1  I I SQ - EGDKVV V RTQS - - - TFKNTE I SFKLGEEFDETTADDRN - CKSTVTMEG
MATOU-v1_105439838.p1  I I SQ - EGDKVV I RTQS - - - TFKNTE I SFTLGEEFDEATADDRN - CKSTVTMDG
MATOU-v1_92365456.p1  I I SK - EGDKVV I KTQS - - - TFKNTE I SFKLGEEFDESTADDRN - CKSTVTMEG
MATOU-v1_92388662.p1  V I SQ - DGDKVV V KTQS - - - TFKNTE I SAKLGEEFDETTADERN - VKS I LTMEG
MATOU-v1_100121203.p1  V I SQ - DGDKVV V KTQS - - - TFKNTE I SAKLGEEFDETTADERN - VKS L L TMEG
BAP76197.1_Anguilla_mossambica_UnaG2  Y I S Q K D R D K M T V K I E N G P P T F L D T E V K F K L G E E F D E F P S D R R K G V R S V V N L V G
BAU98067.1_Anguilla_australis_UnaG1  Y I S Q K D G D K M T V K I E N G P P T F L D T E V K F K L G E E F D E F P S D R R K G V R S V V N L V G
BAU98068.1_Anguilla_bicolor_UnaG3  Y I S Q K D G D K M T V K I E N G P P T F L D T E V K F K L G E E F D E F P S D R R K G V R S V V N L V G
BAN57322.1_Anguilla_japonica_UnaG  Y I S Q K D G D K M T V K I E N G P P T F L D T Q V K F K L G E E F D E F P S D R R K G V K S V V N L V G
CAG33338.1_Homo_sapiens_FABP7  I I S Q - EGDKVV I RTLS - - - TFKNTE I SFQLGEEFDETTADDRN - CKSVVSLDG

```

Figure 3.2: Percentage of identity and e-value of the 10 MATOU-hits and alignment of UnaG, fluorescent FABPs and human FABP7 against 10 MATOU hits.

The values of percentage identity are $\pm 50\%$ for all the hits. The alignment of UnaG, Anguilla FPs with the outgroup protein human FABP7 and the 10 MATOU hits present few similarities. Particularly, in terms of important residues, the GPP motif responsible for the fluorescence is not present in the MATOU hits, so these FABP sequences may not have the potential to be fluorescent as well as the two residues K and G that are absent.

4.3 Sandercyanin

707 hits were obtained from the blastp search and 496 sequences have been annotated, the link is available at this address <https://figshare.com/s/a88fb9d27c983abd2069> (Table 3.5). In this way, I looked up at GeneOntology, the source of information on the functions of genes. The ID GO

GO:0031409 “Invertebrate colouration protein signature” is the closest annotation that could be an indicator for possible fluorescence.

Alignment of Sandercyanin with ApoD from zebrafish, guinea pig, mouse and human showed that some residues are unique to Sandercyanin (Yu et al., 2008); in the peptide A, amino acids AA (position 15-16) and QRL (position 26 to 28) and in peptide B amino acid AE (position 8 and 9). To complete the alignment I chose 10 representative sequences, proceeded by a blind blastp and a blastp against *Sander vitreus* in NCBI. Blind blastp of each sequence resulted in ApoD, while blastp against *S. vitreus* resulted in Sandercyanin. I present the alignments with peptide A and B of Sandercyanin where the motifs AA, QRL and AE are present respectively as no specific residue are present in peptide C.

Subject ID	% Identity	E-value
MATOU-v1_60983947	60.87	2,00E-36
MATOU-v1_57768694	50.79	4,00E-36
MATOU-v1_68883296	41.98	8,00E-33
MATOU-v1_50579543	39.13	8,00E-33
MATOU-v1_33241546	41.14	2,00E-31
MATOU-v1_95281794	38.37	3,00E-31
MATOU-v1_47602351	37.35	1,00E-29
MATOU-v1_28834765	38.18	3,00E-29
MATOU-v1_16356197	37.91	3,00E-29
MATOU-v1_31439698	41.25	4,00E-29

```

                                     60          70          80          90
-----
MATOU-v1_60983947.p1                - - - - - PAVQQNFDPTRYMGKWYEIQKLNNMFQRG
MATOU-v1_57768694.p1                IGG - CPKTVT VKQDFDVTKY LGTWYEIEKIPNTFQSG
MATOU-v1_68883296.p1                FGG - CSKKVTVKQNFVDVKS YMGTWY EYGSIPNTFQSG
MATOU-v1_50579543.p1                LGA - CP - KVTVDNLDVSKY LGDWYEIQKFRFLIEAG
MATOU-v1_33241546.p1                PGS - CK - KVETKKDFDLSKYMGRWFGIEKYPNSFAKG
MATOU-v1_95281794.p1                FFSKCP - EVKTKENFDLSKYLGRWYEIEKYPAWFEKG
MATOU-v1_47602351.p1                FFSKCP - EVKTKENFDLSKYLGRWYEIEKYPNWFEKG
MATOU-v1_28834765.p1                FGG - CSRKVTVKQNFVDVAKYMGTWY EYGSIPNTFQKG
MATOU-v1_16356197.p1                FGG - CSKRVTVKDNFDVEKYMGTWLEYGKIPNWFQKD
MATOU-v1_31439698.p1                LGK - CP - SPPVQENFDVKKYLGRWYEIEKIPASFEKG
NP_031496.2_Mus_musculus_Apolipoprotein_D
NP_001638.1_Homo_sapiens_Apolipoprotein_D
XP_010738283.3_Larimichthys_crocea_Apolipoprotein_D
oab|5F1E|A_Sander_vitreus_Sandercyanin
CAF98955.1_Tetraodon_nigroviridis_Sandercyanin
PGR - CP - RPAVQPEFDAAARYLGTWYDIQRLPHTFQKG
PGR - CP - KPAVQEDFDAARYLGVWYDIQRLPNKFQKG
PGR - CP - RPAVQQEFDAAARYLGTWYEIQRLPHRFQTG
-----
                                     Peptide A

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	130	140	150	160
MATOU-v1_60983947.p1	T I S G S A W S E D P S E	- - P A K L L V S F F E N S P P	- - - A P Y W	
MATOU-v1_57768694.p1	T A S G T A R V V D P S E	- - P A K L E V W F F E N Q P A	- - - G P Y W	
MATOU-v1_68883296.p1	S F I G E A K V M D Q A E	- - P A K L G V R F Y K F Q P W	- - - G P Y W	
MATOU-v1_50579543.p1	D I T G E A K V L D A S E	- - P A K L G V R F F S L Q P F	- - - G P Y W	
MATOU-v1_33241546.p1	V A T G D A V P A D P S V	- - P A K L L V E F - N G S P R	- - - V P Y W	
MATOU-v1_95281794.p1	F A I G S A R - Q D P S S S I A S H L Q V R F S D Y Q P W I L S A P Y F			
MATOU-v1_47602351.p1	S I I G I A R - Q D P S S S I A S H L Q V S F S K W Q P W - - - D Q Y W			
MATOU-v1_28834765.p1	Y V I G E A R - Q D P S S S I A S H L Q V R F S K W Q P W - - - G Q Y W			
MATOU-v1_16356197.p1	D I T G E A K V L D E S E	- - P A K L G V R F F S L Q P Y	- - - G P Y W	
MATOU-v1_31439698.p1	G T K G E A E V V G - P E	- - A A K L G V R F S K A Q P V	- - - P G P Y W	
NP_031496.2_Mus_musculus_Apolipoprotein_D	Q V K G E A K Q S N V S E	- - P A K L E V Q F F P L M P P	- - - A P Y W	
NP_001638.1_Homo_sapiens_Apolipoprotein_D	Q I E G E A T P V N L T E	- - P A K L E V K F S W F M P S	- - - A P Y W	
XP_010738283.3_Larimichthys_crocea_Apolipoprotein_D	T I S G S A M A K D P T E	- - P A K L L V S F F E N S P P	- - - A P Y W	
odb 5F1E A_Sander_vitreus_Sandercyanin	S V I G S A I A E D P C E	- - P A K L Q F - F H E N A A P	- - - V P Y W	
CAF98955.1_Tetraodon_nigroviridis_Sandercyanin	S I S G T A V A E D P S E	- - P A K L A V S F Y E N S P P	- - - A P Y W	

A E
 Peptide B

Figure 3.3: Percentage of identity and e-value of the 10 MATOU-hits and alignment of three ApoD, the unnamed peptide from *T.nigroviridis* against 20 MATOU HITS. Peptide A (up) and Peptide B (down) of Sandercyanin FP.

The values of percentage identity are diverse for all hits which is between 37% to 60%. The alignment realized with the first 10 sequences from the annotation file present very few similarities between the MATOU hits and the protein of interest. In fact, important residues in peptide A, AA and QRL are not present in the alignment as well as the motif AE in the peptide B.

4.4 GFP

The search of hits in TARA data using 14 GFPs protein references resulted in 1145 nucleotide sequences, which were translated as 3075 proteins of the six frames with the longest ORF. An e value of 1e-6 was chosen, then to refine the list of TARA proteins, a blastp have been proceeded with the GFP representative and 57 hits were selected.

The functional annotation of the analyzed GFP hits sequences was performed on the software Blast2go with the tool InterPro, the annotation file can found at this address <https://figshare.com/s/e15f092f6475d32a3c7d> (Table 3.6). From the 57 sequences, 3 of them were not annotated and were discarded from the analysis. Also, a sequence showing these annotations was excluded from the study (protein tyrosine kinase activity, protein binding, ATP binding, protein phosphorylation). All the other annotations were “bioluminescence, generation

of precursor metabolites and energy”, which are features that I am interested in. Alignments with the reference proteins have been done on Jalview software, from which 14 sequences were discarded as they didn’t align with the reference protein and didn’t possess a chromophore XYG. Finally, protein duplicates were removed for the phylogeny analysis (ORF 13877732|g.63, ORF 28576028|g.255, ORF 42632168|g.489, ORF 508721|g.1, ORF 55773605|g.745, ORF 61752073|g.777, ORF 65945761|g.881, ORF 65945762|g.858, ORF 88844756|g.1095, ORF 88856543|g.1119, ORF 25144380|g.262, ORF 92348321|g.1176). Then I looked for the crucial motifs Y66-G67, R96 and E222 (positions for *A. victoria*) which refined the list with 25 sequences. Single blastp of the 25 sequences have been realized on NCBI to assess if the hits protein have similarities to known GFPs (see Table 3.7).

	360	370	380
1497_ORF_108940964	CPMSWAVLAPT	FAYGYKVVAKYP	
34_ORF_11003060	CPMSWAVLAPT	FAYGYKVFAYKYP	
31_ORF_13877732	CPLSWELLHPTL	GYGYRVFGQYP	
152_ORF_20927374	MPMSWAALAST	LYGMKCF AHFP	
206_ORF_2177130	LPMAWGVLAPT	FAYGMKLYAQYP	
261_ORF_25144380	LPMSWKALCHVL	QYGGP AFGLYP	
257_ORF_28576028	LPISWAALAST	FQYGFKLFAKTS	
424_ORF_30786909	CPMAWGVLAPT	FAYGMKVYAKYP	
550_ORF_41431829	CPMSWGVLAPT	FAYGMKVYAKYP	
490_ORF_42632168	LPISWAALAST	FQYGFKLFAKTS	
545_ORF_44754025	CPMAWGVLAPT	FAYGFKCYAKYP	
358_ORF_46919897	CPMSWDALHPT	FQYGFVFGHYP	
4_ORF_508721	LPLSWAAFAS	FQYGYKVF AKTN	
743_ORF_55773605	LPISWAALAST	FQYGYKVF AKTS	
778_ORF_61752073	LPMSWI ALSTT	FQYGFKVFGR TS	
384_ORF_65945761	LPMAWAPLMCY	FQYGFKVFVKS A	
356_ORF_65945762	LPMAWAPLMCY	FQYGFKVFVKS A	
1081_ORF_81931046	CPISWAVLAPT	FAYGYKCYAKYP	
1094_ORF_88844756	CPI AWPALGGT	LYGFKVF TQQP	
1118_ORF_88856543	CPI AWPALGGT	LYGFKVF TQQP	
1308_ORF_90021400	CPMSWAVLAPT	FAYGYKVFAYKYP	
1325_ORF_92331593	CPMAWGVLAPT	FAYGMKVYAKYP	
1177_ORF_92348321	LPISWAALAST	FQYGFKLFAKTS	
1324_ORF_92944772	CPMAWGVLAPT	FAYGFKCYAKYP	
1340_ORF_93021459	CPMAWGVLAPT	FAYGMKVYAKYP	
4AG41205_Aneponia_sulcata_green_fluorescent_protein_asFP499	LPFAFDILSHAF	QYGIKVFAYKYP	
4AM10626_2_Heteractis_crispa_green_fluorescent_protein	LPFAFDILSHAF	RYGNKVFAYKYP	
4AQ11989_Cerianthus_membranaceus_green_fluorescent_protein_FP512	LPFSYDILTMGF	QYGFRAF TKYP	
4AK97633_Montastraea_cavernosa_green_fluorescent_protein	LPFAFDILTTVF	DYGNRVFAYKYP	
4AK83923_Orbicella_faveolata_green_fluorescent_protein	LPFAFDILTTVF	DYGNRVFAYKYP	
4AU04450_2_Pocillopora_damicornis_cyan_fluorescent_protein_C1	LPFSFDILSSAF	KYGNRCFTKYP	
4AL33915_Aequorea_macrodactyla_green_fluorescent_protein	LPVPWPTLVTT	LYGILCFARYP	
4AN41637_Aequorea_coerulescens_green_fluorescent_protein	LPVPWPTLVTT	LYGVQCF SRYP	
4AA27721_Aequorea_victoria_green_fluorescent_protein	LPVPWPTLVTT	LYGVQCF SRYP	
4AQ01186_Pontella_meadii_green_fluorescent_protein_1	ISFSPYLLSHIL	LYGYHYHFATFP	
3AE78442_Chiridius_poppei_green_fluorescent_protein	LAFSPFLLSHCM	GYGFYHFASFP	
4AQ01185_Labidocera_aestiva_green_fluorescent_protein	LSFSPYLLSHIM	GYGFYHYATFP	
4BO61190_Branchiostoma_floridae_green_fluorescent_protein	LPCSPLLIGPNL	LYGFYQYLPFP	
4CA48230_Branchiostoma_lanceolatum_green_fluorescent_protein_bIFP-Y1	LQFSPWILVPI	LYGFYQYLPFP	
4CA48238_Branchiostoma_lanceolatum_red_fluorescent_protein_bIFP-R1	LSFSPWILVPI	LYGFYQYLPFP	

Figure 3.4a: Alignment of 14 GFP reference proteins against the 25 MATOU sequences underlining the chromophore motif in the red square.

	410	420
1497_ORF_108940964	PE	GYTQRRRLTRF
34_ORF_11003060	PN	GYTQRRRLTRF
51_ORF_13877732	PE	GYTQDRTRTRF
152_ORF_20927374	PE	GYTQERVTRF
206_ORF_2177130	PQ	GYNQRRVTRF
261_ORF_25144380	PE	GFTIDRTVRF
257_ORF_28576028	HK	AGGYTMTRRLRF
424_ORF_30786909	PK	GYTQRRRLTRF
550_ORF_41431829	PL	GYTQRRRLTRF
490_ORF_42632168	YK	AGGYTMTRRLRF
545_ORF_44754025	PNKPNGCYP	SGYSQRRITKF
558_ORF_46919897	PE	GYTQRRRLTRF
4_ORF_508721	ME	AGGYTMTRRLRF
743_ORF_55773605	VQ	GGGYTMTRRLRF
778_ORF_61752073	YN	AGGYAMDRRLRF
384_ORF_65945761	YK	SGGYTMVRKLR
356_ORF_65945762	YK	SGGYTMVRKLR
1081_ORF_81931046	PN	GYTQRRRLTRF
1094_ORF_88844756	PQ	GYTQERTYEF
1118_ORF_88856543	PE	GYTQERTYEF
1308_ORF_90021400	PN	GYTQRRRLTRF
1325_ORF_92331593	PK	GYTQRRRLTRF
1177_ORF_92348321	YK	AGGYTMTRRLRF
1324_ORF_92944772	PN	GGCYSAGYSQRRITKF
1340_ORF_93021459	PL	GYTQRRRLTRF
4AG41205_Anemonia_sulcata_green_fluorescent_protein_asFP499	PG	GFSWERVSTY
4AM10626.2_Heteractis_crispa_green_fluorescent_protein	PE	GFTWERSVSNY
4AQ11989_Cerianthus_membranaceus_green_fluorescent_protein_FP512	PE	AFQWNRRIEF
4AK97633_Montastraea_cavernosa_green_fluorescent_protein	PE	GYFWERSMTY
4AK83923_Orbicella_faveolata_green_fluorescent_protein	PE	GYFWERSMTY
4AU04450.2_Pocillopora_damicornis_cyan_fluorescent_protein_C1	PA	GMSFERTFTF
4AL33915_Aequorea_macroductyla_green_fluorescent_protein	PE	GYIQERTIFF
4AN41637_Aequorea_coerulescens_green_fluorescent_protein	PE	GYIQERTIFF
4AA27721_Aequorea_victoria_green_fluorescent_protein	PE	GYVQERTIFF
4AQ01186_Pontella_meadii_green_fluorescent_protein_1	KN	GGYNSVTRTERY
4AE78442_Chiridius_poppei_green_fluorescent_protein	TN	GGYTNTRKEIY
4AQ01185_Labidocera_aestiva_green_fluorescent_protein	KN	GGYTNTRTERY
4BO61190_Branchiostoma_floridae_green_fluorescent_protein	TD	GGYQVHRVFKF
4CA48230_Branchiostoma_lanceolatum_green_fluorescent_protein_bIFP-Y1	ND	GGYQVHRTMQF
4CA48238_Branchiostoma_lanceolatum_red_fluorescent_protein_bIFP-R1	HD	GGYVMHRTMQF

Figure 3.4b: Alignment of 14 GFP reference proteins against the 25 MATOU sequences underlining the Arginine residue (R).

	450	↓	460
1497_ORF_108940964	EN	-	PEDKRDHVCQDE INQHFSF
84_ORF_11003060	EN	-	PTDEREHVCQDE INQHFSF
61_ORF_13877732	QN	-	SNEKKDHI IQDELNLFCD
152_ORF_20927374	NI	-	DDDSEHV IMEEMMQ - - -
206_ORF_2177130	KG	-	PEDPTDHI CQDE INQYFHF
261_ORF_25144380	QD	-	SETRDHI VQRE INTARPA
257_ORF_28576028	GNK	-	CYQEETHVNNNNCW - - -
424_ORF_30786909	KN	-	EGEKRDHRLQDE INQVFSF
550_ORF_41431829	QN	-	EGEKRDHRLQDE INQHFF
490_ORF_42632168	GNK	-	CYQEETHI DTNNCW - - -
545_ORF_44754025	KN	-	PNEKRDHVLQDE INVQFSF
658_ORF_46919897	KN	-	PLEKKDHI VQDE INKYEF
4_ORF_508721	GNK	-	CYQEETHVNQNNCW - - -
743_ORF_55773605	GNK	-	CDQEETHI TNNNTW - - -
778_ORF_61752073	GRK	-	CYQEEI HNI TNNCW - - -
884_ORF_65945761	GNS	-	CYQEAYHI ARKAT - - -
856_ORF_65945762	GNS	-	CYQEAYHI ARKAT - - -
1081_ORF_81931046	EN	-	PDDKRDHVCQDE INQYFSF
1094_ORF_88844756	NS	-	EETRDHI VQHEVLKG - - -
1118_ORF_88856543	TS	-	DETRDHI VQNE I LQG - - -
1308_ORF_90021400	EN	-	PTDEREHVCQDE INQHFSF
1325_ORF_92331593	KN	-	EGEKRDHRLQDE INQVFSF
1177_ORF_92348321	GNK	-	CYQEETHI NKNNCW - - -
1324_ORF_92944772	KN	-	PNEKRDHVLQDE INVQFSF
1340_ORF_93021459	QN	-	EGEKRDHRLQDE INQHFF
AAG41205_Anonomia_sulcata_green_fluorescent_protein_asFP499	QS	-	DDWKT VEQHEVVASYSQV
AAM10626.2_Heteractis_crispa_green_fluorescent_protein	QS	-	DDEKT IEQHENVRAS YFND
AAQ11989_Cerianthus_membranaceus_green_fluorescent_protein_FP512	KE	-	PGFKMVEQAEYATAHVCDL
AAK97633_Montastraea_cavernosa_green_fluorescent_protein	QD	-	KDYNKVKLYEHAEA - XXGL
AAK83923_Orbicella_faveolata_green_fluorescent_protein	QD	-	KDYNKVKLYEHAEA - HSGL
AAU04450.2_Pocillopora_damicornis_cyan_fluorescent_protein_C1	QD	-	I EDKVV LLEETAVAHVNPL
AAL33915_Aequorea_macroductyla_green_fluorescent_protein	GSKDRNETRDHVMVLEFFSACGH		
AAN41637_Aequorea_coerulescens_green_fluorescent_protein	GSKDPNEKRDHMI YFEFV TAAAI		
AAA27721_Aequorea_victoria_green_fluorescent_protein	GSKDPNEKRDHMLLEFV TAAAI		
AAQ01186_Pontella_meadii_green_fluorescent_protein_1	QNEVA IVEYQNVFKT - PTAF A - -		
BAE78442_Chiridius_poppei_green_fluorescent_protein	KENLAMVEYQQVFNSAPRDM - - -		
AAQ01185_Labidocera_aestiva_green_fluorescent_protein	QSDVGI VEQHVFKT - PTAF A - -		
ABO61190_Branchiostoma_floridae_green_fluorescent_protein	KTEITLSEKVKAFIDTV - - - - -		
ACA48230_Branchiostoma_lanceolatum_green_fluorescent_protein_bIFP-Y1	KTELNFKEQWQTA FSDVM - - - - -		
ACA48238_Branchiostoma_lanceolatum_red_fluorescent_protein_bIFP-R1	KTELNFKQWQKAFQDIV - - - - -		

Figure 3.4c: Alignment of 14 GFP reference proteins against the 25 MATOU sequences underlining the Glutamic Acid residue (E) present in some hits.

Interestingly, all the hits sequences found in TARA possess a chromophore that is representative of GFP proteins. Three different ones were present: AYG, GYG and QYG, aligned with the chromophores from the GFPs reference proteins (Figure 3.4a). Also, they all possess the conserved residue Arg96 important in the stability, folding and maturation of the chromophore, here in position 426 (Figure 3.4b) (Sniegowski *et al.*, 2005). Another conserved amino acid in GFP,

the glutamic acid, crucial in the maturation process of the chromophore in Aequorea GFPs is present in the alignment at position E627 in a few hits sequences (Figure 3.4c).

Studying more carefully at the chromophore motifs, AYG has been denoted in a GFP of the amphioxus *Branchiostoma belcheri* (Yue *et al.*, 2016), while QYG is found in the anemone *Montastrea cavernosa* and the engineered modified protein KillerRed (Tsien, 1998).

GYG is a chromophore found in several taxa as amphioxus, copepods and the anemone *Discosoma* sp at the origin of the red fluorescent protein, Dsred (Pletnev *et al.*, 2013; Shaner *et al.*, 2004). Almost all the amphioxus GFP-like proteins have a GYG motif, except one recent mutation in the two alleles of both GFPb1 and GFPb2 genes of *Branchiostoma lanceolatum*, containing a GYA triplet, being the first chromophore with a non-XXG motif (Bomati *et al.*, 2009).

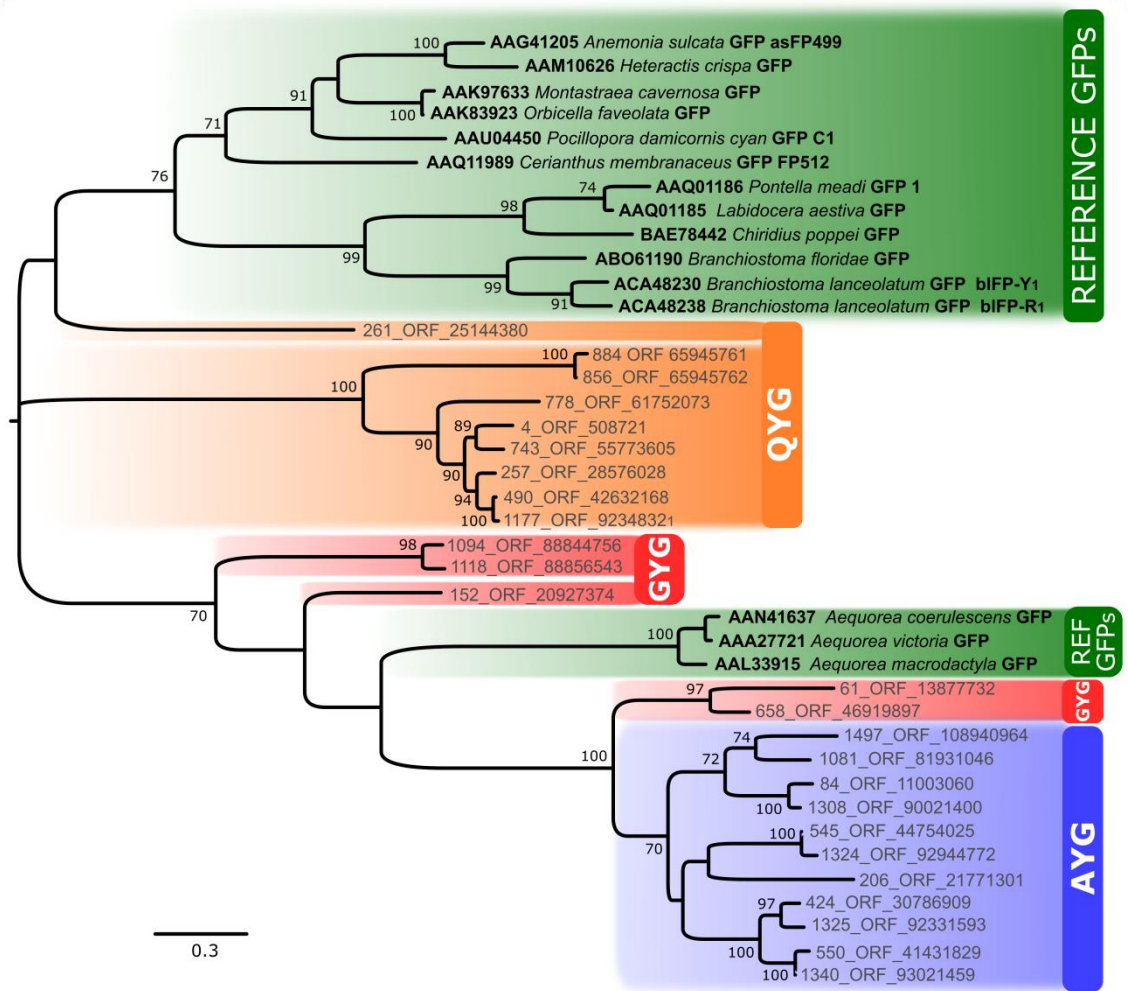


Figure 3.5: Phylogenetic tree of GFPs reference proteins and hits from TARA data. 3 different chromophores from the alignment are shown with colour code (Bootstrap > 70% are shown).

We can denote three different groups corresponding to proteins bearing different chromophores namely QYG, GYG and AYG where many divergent events are observed that may be due to mutations from a common ancestor. We observe that the AYG and GYG clades at the bottom of the tree share a common ancestor with the reference GFP proteins. Instead, the other part of the clade GYG does not share a common ancestor and is therefore paraphyletic. We can hypothesize that these two groups may be hydrozoans from the genus *Aequorea* and that the clade GYG originates earlier than AYG. It is impossible to predict from the protein sequence the spectrum profile, nevertheless different residues in key positions could certainly give rise to a different

emission spectrum. The paraphyletic GYG clade has well supported branches with Bootstrap values of 98 and 97, only the protein 152_20927374 is excluded. This is also the case for the whole clade AYG where the expansion originates from a non-supported branch.

The QYG clade is polyphyletic meaning that is equally related, one sequence shares a common ancestor with the reference GFPs at the top of the tree. The clade with the chromophore QYG is well supported, only the protein 261_25144380 has a low bootstrap value.

GFP HITS	PROTEIN SIMILARITY (blastp)	% IDENTITY	e-value	QUERY COVER (%)	ACCESSION NUMBER
1497_ORF_108940964	DGFP [Abylopsis eschscholtzii]	51.12	8,00E-92	86	AEP13895.1
84_ORF_11003060	DGFP [Abylopsis eschscholtzii]	50.97	8,00E-81	82	AEP13895.1
61_ORF_13877732	DGFP [Abylopsis eschscholtzii]	62.77	0	96	AEP13895.1
152_ORF_20927374	green fluorescent protein 2 [Anthomedusae sp. SL-2003]	34.70	9,00E-44	68	AAR85351.1
206_ORF_2177130	DGFP [Abylopsis eschscholtzii]	48.89	2,00E-81	89	AEP13895.1
261_ORF_25144380	non-fluorescent GFP-like chromoprotein [Anthomedusae sp. SL-2003]	62.61	2,00E-105	95	AAR85352.1
257_ORF_28576028	fluorescent protein 5 [Olindias formosus]	31.65	8,00E-50	83	AXX69743.1
424_ORF_30786909	DGFP [Abylopsis eschscholtzii]	46.37	4,00E-78	93	AEP13895.1
550_ORF_41431829	DGFP [Abylopsis eschscholtzii]	46.74	4,00E-75	94	AEP13895.1
490_ORF_42632168	fluorescent protein 5 [Olindias formosus]	32.49	6,00E-43	92	AXX69743.1
545_ORF_44754025	DGFP [Abylopsis eschscholtzii]	47.26	3,00E-77	94	AEP13895.1
658_ORF_46919897	DGFP [Abylopsis eschscholtzii]	95.65	0	100	AEP13895.1
4_ORF_508721	fluorescent protein 5 [Olindias formosus]	32.74	1,00E-52	88	AXX69743.1
743_ORF_55773605	fluorescent protein 5 [Olindias formosus]	29.58	2,00E-49	94	AXX69743.1
778_ORF_61752073	ember [Anthoathecata sp. MH-2011]	29.22	9,00E-44	85	AEP13896.1
884_ORF_65945761	fluorescent protein 5 [Olindias formosus]	27.74	6,00E-26	97	AXX69743.1
856_ORF_65945762	fluorescent protein 5 [Olindias formosus]	28.61	3,00E-35	78	AXX69743.1
1081_ORF_81931046	DGFP [Abylopsis eschscholtzii]	52.50	6,00E-78	89	AEP13895.1
1094_ORF_88844756	DGFP [Abylopsis eschscholtzii]	31.96	2,00E-78	97	AEP13895.1
1118_ORF_88856543	DGFP [Abylopsis eschscholtzii]	32.02	2,00E-47	99	AEP13895.1
1308_ORF_90021400	DGFP [Abylopsis eschscholtzii]	52.65	1,00E-85	88	AEP13895.1
1325_ORF_92331593	DGFP [Abylopsis eschscholtzii]	47.40	1,00E-78	93	AEP13895.1
1177_ORF_92348321	fluorescent protein 5 [Olindias formosus]	31.47	8,00E-51	85	AXX69743.1
1324_ORF_92944772	DGFP [Abylopsis eschscholtzii]	47.24	9,00E-77	93	AEP13895.1
1340_ORF_93021459	DGFP [Abylopsis eschscholtzii]	42.81	2,00E-74	94	AEP13895.1

Table 3.7: GFPs hits with corresponding blastp result on NCBI.

We can observe that the hits obtained in our study have similarities with already identified GFPs and are all from the class Hydrozoa. This could be interesting to further clone one of these hits obtained to study their spectral characteristics.

5. Discussion

This blind search and unbiased approach looking for a new fluorescent protein has been executed using sequences from the whole ocean. After blasting the TARA eukaryotic database, it would have been interesting to know from which phyla and at which station these hits come from, however with the enormous eukaryotic data present in TARA, it is impossible to have this information.

From this molecular evolutionary study, we can conclude that the hits found are interesting as they bear structural features found in GFP (chromophore motif) and may be functional. For this, an experimental work carrying out cloning and expression of these sequences in cells would have permitted to investigate if these proteins are fluorescent *in-vitro*.

In terms of depth and size of samples, TARA ocean sampled organisms ranging from 0,02 μm up to 680 μm , in depth until 1000 m (Sunagawa *et al.*, 2020). In the present study, no similar sequences to UnaG and Sander cyanin FPs were found in TARA eukaryotic data, which could be explained by the size of the animals in which these proteins originate. In fact, TARA ocean has sampled mainly small species therefore, it is dubious to get data on eels and fish. This also implies the fact that TARA ocean samples were mainly done in pelagic areas where *Sander vitreus* is unlikely present.

5.1 Evolutionary biology at the origin of GFPs colour diversity

A few studies have been carried out in Anthozoans and Cephalochordates to investigate the diversity of chromophores and the evolutionary history of natural GFPs colour in these clades. The structure of the chromophore reveals a specific amino acid sequence, however the precise molecular event during its maturation is still discussed (Chudakov *et al.*, 2010). Several studies have shown a complex organizational process in RFP chromophore synthesis in comparison to green and cyan FP (Wall *et al.*, 2000; Yarbrough *et al.*, 2001). The most commonly found spectral

patterns in Anthozoans are cyan, green, red, and photoconvertible green-to-red FPs, as well as purple-blue non-fluorescent chromoproteins (Labas *et al.*, 2002).

The evolutionary scenarios leading to the GFP colour diversity have been extensively studied in Anthozoans. In fact, GFP can constitute up to 14% of the total soluble proteins in their expressing tissue (Leutenegger *et al.*, 2007; Oswald *et al.*, 2007; Smith *et al.*, 2013).

In *Montastrea cavernosa* and related reef-building corals the role of point mutation has been unveiled, responsible for the generation of new colour phenotypes which is the consequence of adaptive evolution (Field *et al.*, 2006). Another study underlines that the colours of *M. cavernosa* GFPs, cyan, green, and red, are due to phenotypic plasticity (polyphenism) (Kelmanson and Matz, 2003). This phenomenon first implies that the colour trait is determined by the effect of the environment. In fact, it is postulated that multi-coloured FPs evolved as part of a mechanism regulating the relationships between the coral and its algal endosymbionts (zooxanthellae) (Field *et al.*, 2006). Second, polyphenism suggests that the same genome coding for GFP-like proteins gives rise to different colour phenotypes of GFPs (Kelmanson and Matz, 2003).

Additionally, a study investigating the multitude of FP in the reef-building coral *Montipora* sp. showed that the diversity results of gene duplication events and alternative splicing (Takahashi-Kariyazono *et al.*, 2015). The analysis resulted with at least four monGFP genetic loci in the genome of *Montipora* sp., without changes in emitted fluorescence even though the RNA splicing of variants from the first exon lead to different translated protein sequences (Takahashi-Kariyazono *et al.*, 2015). The similarity in phenotypes may be explained by homogenization necessary for an increasing amount of the protein due to various light stresses.

Finally, a single specimen of the coral *Acropora millepora* was shown to express three different cyan and green FPs, three spectrally distinct purple-blue chromoproteins and one red FP (amilFP597) (D'Angelo *et al.*, 2008; Smith *et al.*, 2013). Particularly, it was demonstrated that the protein expression of amilFP597 increases with light intensity. This significant expression is due to

photoprotection of these FPs, reducing photo damage of zooxanthellae in intense light stress situation (Gittins *et al.*, 2015).

All these studies show examples of species having symbionts, however the phenomenon of multiplicity can also be observed in nonsymbiotic species such as in *Corynactis californica*, having six closely related GFP-like coding genes encoding proteins that fluoresce across the green-to-red visible spectrum (Schnitzler *et al.*, 2008). The difference between non-symbiotic and symbiotic species may be explained that FPs in *Corynactis californica* evolved for reasons other than light regulation, and symbiotic species got this function later.

In comparison to cnidarian FP, amphioxus FPs are evolutionarily distant and share only 20% sequence identity (Pletnev *et al.*, 2013), while they are 30–40% identical to copepod GFPs (Baumann *et al.*, 2008). As it has been shown in Anthozoans, reconstruction of estimated ancestral genes demonstrated that green fluorescence is an ancestral state, whereas RFP originated independently in different animal groups. The RFP isolated in *Branchiostoma lanceolatum* a few years ago bears a novel chemical structure, a GYG chromophore that has not been observed in GFP from Cnidaria. It is an interesting discovery as it is the only known RFP outside the Cnidaria phylum.

It was shown that the expansion of GFP-encoding genes denoted recently in the genus *Branchiostoma* is due mainly to tandem duplications. Also in the same study, the authors underline that *Asymmetron* and *Branchiostoma* share clearly homologous GFP-encoding genes and postulate that they were probably present in the ancestral cephalochordates but it is not clear yet via in which evolutionary manner this transfer has been realized (Bomati *et al.*, 2009).

Our findings in TARA data indicate that there may be new GFPs from hydrozoans origin, however further experiments are needed to know if these sequences express fluorescence *in vitro* as their percentage identity is low and they may not possess a XZG chromophore.

CHAPTER 4 – GENERAL DISCUSSION AND PERSPECTIVES

The present project on fluorescence in the sea has allowed to explore many aspects that has not been investigated in other research projects on fluorescence. As a first survey, I have been able to assess fluorescence in the Gulf of Naples (Italy), sampling specimens from many marine phyla thanks to the MareChiara project. Furthermore, the fluorescent analysis has been performed on a cutting-edge instrument, a confocal microscope equipped with high performance lasers. This equipment allowed me to run accurate analysis, notably by measuring the response to the UV and visible light. This is the first time that the UV-visible light spectra have been scanned so precisely in terms of both excitation and emission wavelengths. This generated quantitative fluorescent measurements of the most represented planktonic communities of the Gulf of Naples. Investigating fluorescence in planktons allowed me to rule out species with intrinsic chlorophyll and explore fluorescence linked with pigmentation. I was able to explore in which phyla fluorescence was present as it is an important factor to take in consideration in an evolutionary point of view. For example, fluorescence is likely not present in Mollusk as demonstrated by the epifluorescence and confocal fluorescent assessment as well as the bioinformatics search in the genome. The fluorescent signal observed on gastropod and the bivalve larva may originate from chlorophyll in their stomach. In Arthropod, several FPs have been already isolated from copepod, all emitting green fluorescence. It could have been interesting to investigate this species so as to find a FP with red emission, however, the red fluorescent pattern seems mainly situated in the gut of the organisms, therefore with chlorophyll origin. The other group of Arthropod, crustaceans, bear beautiful green fluorescence mainly along the abdomen, in-between the segments, which could be linked to a pigment in their nerve cells helping with motility. Many FPs have been isolated in lancelets, cephalochordates, with fluorescence from green to orange emission but none with red emission. The fluorescence is noticed mainly in the primordial eye, pharynx, iliocolon (gut) and in the ovaries (Yue *et al.*, 2016).

From the present study, it is not sure if fluorescence observed in polychaetes comes from chlorophyll or a pigment. The idea of investigating a potential FP in the polychaete species

Alciopidae has not been conclusive as very few specimens were found in the sample. Despite the low abundance of Alciopidae in MareChiara sampling, it would be interesting to carefully investigate the fluorescent properties of this species and to understand the origin of the red fluorescent patterns in their eyes in the attempt to identify a red FP. This would be the first FP described in a polychaete and would enlarge our knowledge of fluorescence in the tree of life. Holoplanktonic polychaetes are common in marine zooplankton, although they are not highly important in terms of abundance (Bilbao *et al.*, 2008), especially in Alciopidae as it is reported in several studies performed in South America. In fact, they represented 4 species on 236 holoplanktonic polychaete specimens examined in Venezuela (Márquez-Rojas *et al.*, 2013). In Mexico, 24 holoplanktonic polychaete species belonging to five families were identified and Alciopidae represented 6.6% (Fernández-Álamo and Sanvicente-Añorve, 2005). Seven species of Alciopidae were collected during five oceanographic cruises off the western Caribbean Sea (Jiménez-cueto *et al.*, 2008). Finally, a study conducted in Otranto concluded that Alciopidae ranked second in density and represented 23.8% of the polychaete community (Guglielmo *et al.*, 2019). As a comparison in copepods, researchers used 300 specimens of total RNA preparation to isolate a FP (Masuda *et al.*, 2006), it is therefore important to consider the abundance of a given organism to isolate a FP.

Finally, this present thesis emphasizes the importance of the location of fluorescence in the organism. By inquiring the anatomy of planktons, I was able to reject organisms with fluorescence in their stomach/gut while other anatomical parts could give us information on why fluorescence is present at this spot. For example, fluorescence located on the tentacle (locophore) of Phoronida may have a link with the feeding as it surrounds the mouth. The most striking example is the eye of the polychaete Alciopidae with red fluorescence, that seems to be linked with a pigment as they appear dark brownish in bright field.

Some technical points of the fluorescent evaluation will be discussed, from the epifluorescent to the confocal analysis, as it is a long and challenging process. In fact, only a few specimens can be analysed after receiving the sample and time is a limiting factor since an hour at least is needed at the confocal microscope to realize the whole spectra investigation (from the RGB image to the visible light excitation test). To counteract the time restriction, I attempted several times to preserve the samples by placing it in a cold room with an agitator to analyze it the next day but it wasn't successful. This trial would have been an opportunity to test if chlorophyll content in the gut or other fluorescent patterns in the organisms would still be present the day after sampling.

Another technical cue to consider is the autofluorescence artefact caused by dirt on specimens as discussed in Chapter 2. If it is present on very small organisms, it is delicate to take it out and therefore can damage the tissue of the species. During all the assessments, attentive care was made to preserve the entire organism or to remove the dirt.

Furthermore, to estimate the fluorescent patterns of zooplanktons, the use of 7% $MgCl_2$ was preferably chosen rather than other chemical fixatives as it has been reported they can cause autofluorescence (Heaney *et al.*, 2011). Finally, another practical point to consider is the size of species that were sometimes too large to be entirely analysed at the confocal microscope. It is the case particularly for Annelid specimens taken at the seaside and many other species from MareChiara sampling. All the experiments at the confocal were realized with the 10X objective and it was not possible to perform with another one.

Overall, the confocal microscopy assessment is a unique tool to study fluorescent patterns in zooplanktons and rule out positive organisms to chlorophyll, but it shows a few disadvantages. Many species show discrepancies in the spectral analysis especially in the response to the visible light test and the inconsistency of MareChiara samplings over the years is also to take in consideration, which makes it difficult to draw an overview of fluorescence for each phylum. Also, the response to the UV excitation has been successful for very few species thus it may not be

used in further experiments. Nevertheless, the visible light spectral assay allows a precise assessment of excitation and emission patterns at a particular site or of the whole species.

The second part of the subject has proved the possibility of finding new chromophores using TARA Ocean project. It is the first time that TARA data have been exploited with the purpose to find a FP. It could be interesting in the future to exploit more attentively the results of GFP hits to know from which species they come when this will be possible. With this information, it could be interesting to dress a panel of geographical, depth and species data. This would allow us to envisage a more specific sampling to isolate cDNA for a putative new FP.

Regarding the other FPs, UnaG and Sandercyanin being conjugated with other proteins to trigger fluorescence, the difficulty to find new protein of this type in TARA data is conceivable. The annotations giving crucial information about the biochemical and physiological functions of the proteins, the hits found in TARA for UnaG and Sandercyanin may not be related with fluorescence but more to their endogenous ligand.

As perspective ideas of finding new fluorescent proteins, the use of other types of fluorescent technologies is a perceivable argument. For example, to measure red fluorescence emitted from chlorophyll pigments contained in phytoplankton and other organisms scientists have used underwater fluorometers illuminating the water with blue light (435–480 nm) (Tanaka *et al.*, 2019). This technique could be applied on zooplankton to differentiate chlorophyll autofluorescence from fluorescence generated by a GFP or a FP. Furthermore, a recent new technology appeared called underwater planar laser imaging fluorometer (PLIF), which is coupled with a zooplankton-imaging device. PLIF technology can detect the subsurface chlorophyll maximum (SCM) and fluorescent particle maximum (FPM) while the camera system allows good estimations of concentrations of crustacean and gelatinous zooplankton groups (Briseño-Avena *et al.*, 2020). This could be of great utility to assess fine scale fluorescence distribution in a given geographical area and determine which zooplankton groups are fluorescent. With this information in hands, it will be easier to target the area of investigation (pelagic or benthic) as

well as the phyla. Another advantage of PLIF is that it can also be applied in the lab as researchers have been able to acquire measure of the chlorophyll content in the gut of a Copepod without the need to kill it (Karaköylü *et al.*, 2009). Complementary to this technique it could be interesting to pair it with the advanced laser fluorometry system where narrow band excitation lasers are used at wavelengths of 408 and 532 nm and measures nearly continuous emission in the range of 308–808 nm (Jaffe *et al.*, 2013).

To better characterize fluorescent patterns on the sea floor as well as on the water column to discover a new FP, it is crucial to determine the nature and distribution of it, both in terms of geography and taxonomy. An imaging system has been used on coral reef surface to perform automated classification. This technology has the potential to use multi-wavelength fluorescence imaging generated by the Fluorescence Imaging Laser Line Scanner (FILLS). The FILLS technology possesses four detector channels (blue, green, orange and red), produces high-resolution multispectral fluorescence images (Mazel *et al.*, 2003) and could be used on other seafloor and taxa.

A very interesting recent study has investigated the distribution and quantification of bioluminescence in the marine habitat of the Monterey Bay area of central California, USA. Researchers have been able to dress profiles of the diversity of bioluminescence between pelagic and benthic ecosystems. 621 dives using remotely operated vehicle (ROV) for 25 years has allowed scientists to observe 369,326 pelagic and 154,275 epibenthic species of 29 taxa (Martini *et al.*, 2019). The proportion of the bioluminescent capability of several taxa is plotted according to the sea environment (see Figure 4.1). Very few species in the benthic area are bioluminescent which could be explained by technical constraints of ROV i.e., organism's sensitivity to the light and sound generated by the vehicle and rare sampling opportunities of deep-sea habitats.

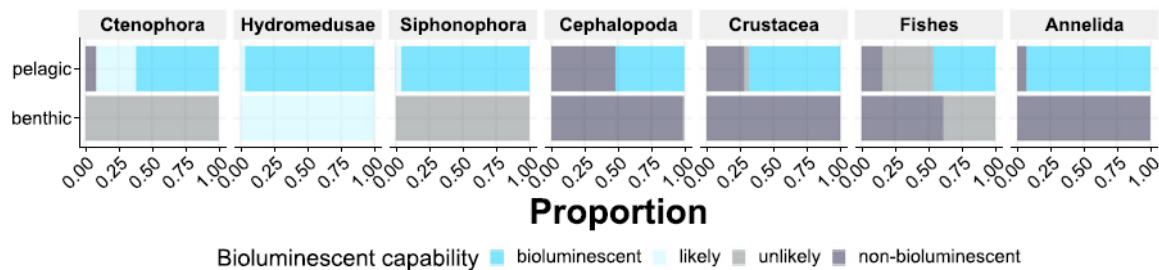


Figure 4.1: Distribution of bioluminescence capability among individuals in taxa present in both benthic and pelagic ecosystems. Numbers of observations are indicated as $N = (\text{numbers for benthic})/N = (\text{number for pelagic})$. Cephalopoda ($N = 985/N = 4,993$), Ctenophora ($N = 234/N = 28,268$), Hydromedusae ($N = 4,077/N = 64,500$), Siphonophora ($N = 3/N = 54,703$), Crustacea ($N = 8,345/N = 40,608$), Annelida ($N = 2,401/N = 35,409$), Fishes ($N = 28,995/N = 16,156$). (Martini *et al.*, 2019).

The data generated by this analysis are of great interest for researchers studying light phenomena in the sea and it would be remarkable to apply it for fluorescence. Both bioluminescence and fluorescence are widely spread phenomena in the sea and it is crucial to map and understand their role in the marine environment as they play a key role in animals through predator-prey relationships, camouflage, species recognition etc. As this study points out, it is essential to map further the benthic area where fluorescent organisms may also be present. It is however a difficult environment characterized with low visibility, energy limitation and different optical, physical, and biogeochemical properties than the mesopelagic area. With more studies and advanced technologies such as ROV applied to fluorescence, it would be possible to assess its distribution in the water column from pelagic to benthic areas in diverse phyla and to get more knowledge on the ecological functions of fluorescence in the sea. This would also allow scientists to target which zones and species may present fluorescence to isolate a new fluorescent protein. At a molecular level to identify a new FP such as UnaG and Sandercyanin, these proteins require cofactors to be fluorescent and do not bear a chromophore from the same nature as GFP family. Therefore, this may be a concern in the chemical characterization of the FP. However, new FP can

be identified in subspecies from the same genus, as it has been done in the genus *Anguilla*. Thanks to RNA extraction and transcriptomics analysis, the genome of another species of eel can be realized as well as a search of FABP in the genome. If this leads to some hits, an alignment followed by a phylogenetic tree would be done in order to know the extent of relation between these proteins, if homologies and crucial motifs are observed. The identification of any orthologous genes coding for UnaG would allow their synthesis and cloning in a vector to monitor protein expression. If the protein concentration is satisfying, the fluorescent pattern of this new FP can be followed *in vitro* in a cell model.

Finally, independent gene loss events occurred in the evolutionary history of several animal clades. In this regard, we have hypothesized that several genes were lost or that the ancestral GFPs have been changing, thus they cannot be recognized. On one hand, if they are lost, we have enough tools to recognize divergent genes (i.e blast, alignments, phylogenetic tree). On the other hand, if they are different, the answer can be to clone and follow the expression of the protein and notice any fluorescent pattern.

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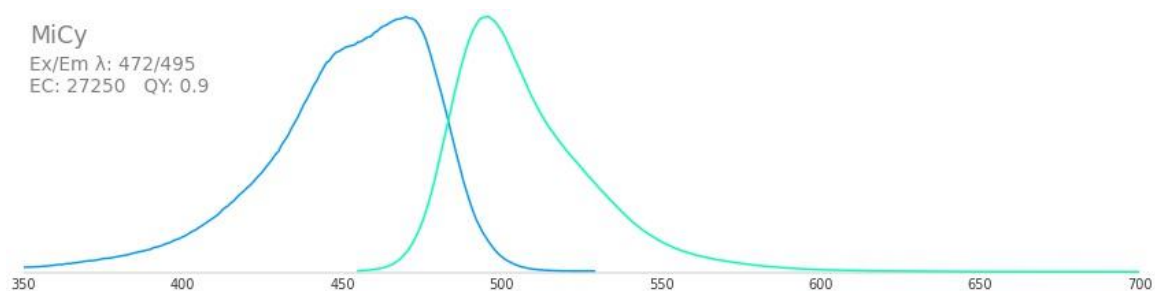
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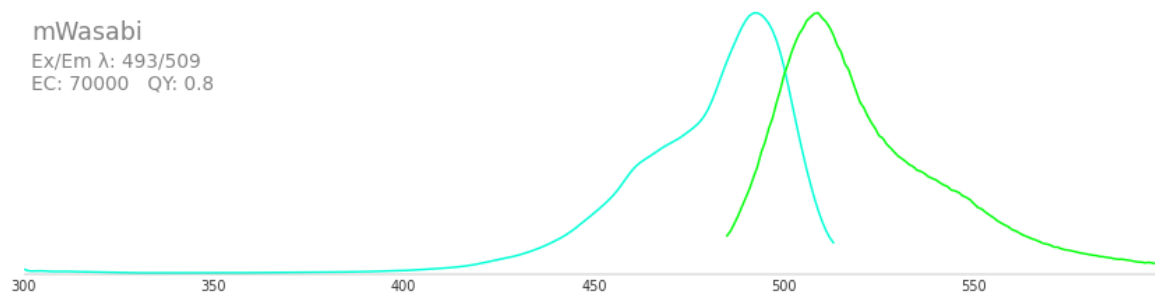
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APPENDICES

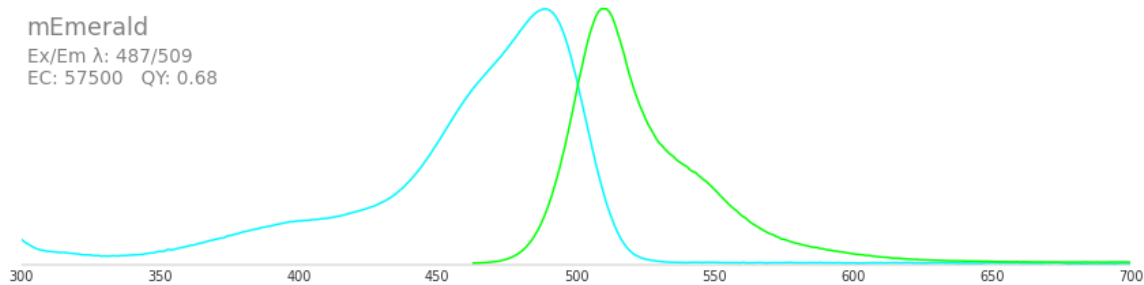
Appendix 1: Fluorescent properties of several fluorophores mentioned in Chapter 2 from the website FPbase (Lambert, 2019)



A1.1: Fluorescent spectrum of the fluorophore MiCy

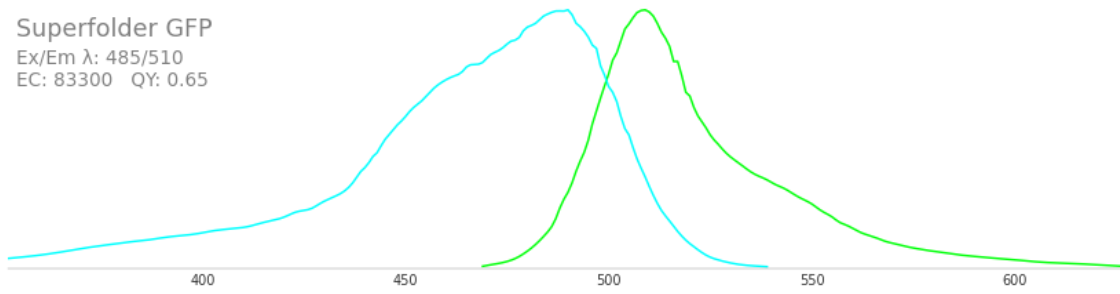


A1.2: Fluorescent spectrum of the fluorophore mWasabi



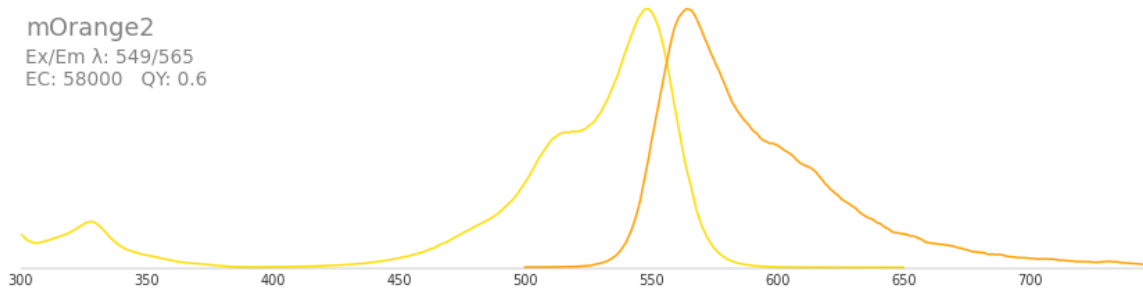
A1.3: Fluorescent spectrum of the fluorophore mEmerald

Superfolder GFP
Ex/Em λ : 485/510
EC: 83300 QY: 0.65



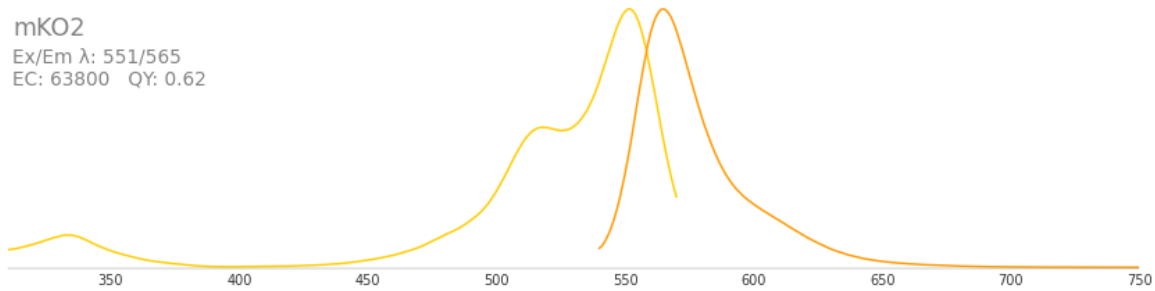
A1.4: Fluorescent spectrum of the fluorophore Superfolder GFP

mOrange2
Ex/Em λ : 549/565
EC: 58000 QY: 0.6



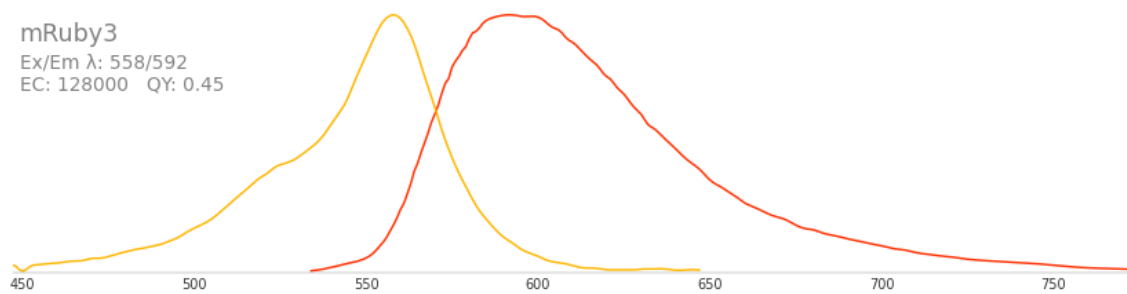
A1.5: Fluorescent spectrum of the fluorophore mOrange2

mKO2
Ex/Em λ : 551/565
EC: 63800 QY: 0.62

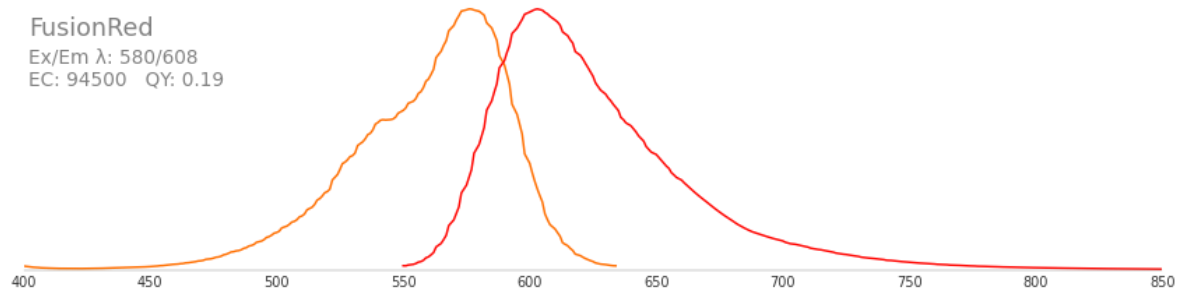


A1.6: Fluorescent spectrum of the fluorophore mKO2

mRuby3
Ex/Em λ : 558/592
EC: 128000 QY: 0.45



A1.7: Fluorescent spectrum of the fluorophore mRuby3




A1.8: Fluorescent spectrum of the fluorophore FusionRed

REVIEW

Open Access

Sea as a color palette: the ecology and evolution of fluorescence



Marie-Lyne Macel, Filomena Ristoratore, Annamaria Locascio, Antonietta Spagnuolo, Paolo Sordino* and Salvatore D'Aniello 

Abstract

Fluorescence and luminescence are widespread optical phenomena exhibited by organisms living in terrestrial and aquatic environments. While many underlying mechanistic features have been identified and characterized at the molecular and cellular levels, much less is known about the ecology and evolution of these forms of bioluminescence. In this review, we summarize recent findings in the evolutionary history and ecological functions of fluorescent proteins (FP) and pigments. Evidence for green fluorescent protein (GFP) orthologs in cephalochordates and non-GFP fluorescent proteins in vertebrates suggests unexplored evolutionary scenarios that favor multiple independent origins of fluorescence across metazoan lineages. Several context-dependent behavioral and physiological roles have been attributed to fluorescent proteins, ranging from communication and predation to UV protection. However, rigorous functional and mechanistic studies are needed to shed light on the ecological functions and control mechanisms of fluorescence.

Keywords: Fluorescence, fluorescent proteins, Tree of life, Function, Metazoan, Evolution

Background

The emission of light by living organisms relies on two primary mechanisms; natural luminescence, based on endogenous chemical reactions, and fluorescence, in which absorbed light is converted into a longer wavelength. The first observations of luminescence were made almost a century ago, when several species of hydromedusae—e.g., *Aequorea forskalea*, *Mitrocoma cellularia*, *Phialidium gregarium*, *Stomatoca atra*, and *Sarsia rosaria*—were illuminated with UV light [1, 2]. Later studies of the luminescent properties of the hydrozoan medusa *Aequorea victoria* led to the isolation of aequorin, a chemiluminescent protein that emits blue light (reviewed in [3]). The green fluorescent protein (GFP) was identified as a by-product of aequorin, and was shown to release fluorescent photons after absorbing electromagnetic energy [4] (see glossary in Table 1).

The discoverers of GFP showed that calcium ion binding triggers the emission of blue light from aequorin at 470 nm, in turn prompting an energy transfer to GFP, which emits light at a longer wavelength, giving off green fluorescence at 508 nm [3, 4].

Green fluorescent protein consists of a single polypeptide chain of 238 amino acids in length, and does not require a cofactor [5]. The chromophore, the structural feature of GFP responsible for color emission, is formed by the autocatalytic cyclization of the tripeptide 65-SYG-67 [3]. Members of the GFP family constitute a distinct protein class, all of which share similar structures [6]. After the cloning of the GFP gene [7] and the in vivo demonstration that its recombinant expression in *Escherichia coli* and *Caenorhabditis elegans* induces fluorescence [8], interest in and applications of GFP have continued to increase within the scientific community. Originally used as a reporter gene for tracing proteins, organelles, and cells, non-invasive GFP tagging has become a routine tool in scientific research for a variety of

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Table 1 Glossary

Term	Definition
Aequorin	Calcium-activated photoprotein complex responsible for luminescence in the jellyfish <i>Aequorea victoria</i>
Carotenoids	Yellow, orange, and red organic pigments produced by plants, algae, bacteria and fungi
Chlorophyll	Green organic pigment present in plants and in cyanobacteria, which is responsible for light absorption during photosynthesis and dissipates its energy by emission as fluorescence radiation
Chromatophore	Pigment-containing cell in the superficial skin tissue layer of an animal
Exitance	Totality of light leaving the surface expressed in energy or photon flux units
Fluorescence	Emission of light at a longer wavelength, in other words it is the absorption of shorter-wavelength light (excitation) followed by the release of a part of the absorbed energy at a longer wavelength (emission)
Fluorophore/ Chromophore	Part of a molecule or chemical group composed of an atom or a group of atoms responsible for the color emitted by a fluorescent protein
Green fluorescent protein (GFP)	Protein able to emit green fluorescence in the presence of short-wavelength light discovered in <i>Aequorea victoria</i>
Light absorption	Phenomenon occurring when a ray of light strikes a surface. The energy from the light (photons) is transferred to the surface material
Light scattering	Phenomenon occurring when a ray of light strikes a surface and changes its direction
Luminescence	Light generated by an enzymatic reaction (luciferase) within a living organism
Phosphorescence	Type of photoluminescence related to fluorescence displaying gradual light emission over a long period of time
Photophore	Gland or organ specialized in the production of luminescent light
Photoactivatable fluorescent proteins (PAFPs)	Class of FP capable of acute changes in their spectral properties upon irradiation with light of a specific wavelength and intensity
Pigment	Colored chemical substance found in animals or plants capable of changing color after reflection and absorption of certain wavelengths of visible light
Quenching Reflectance	Process of stopping a chemical or enzymatic reaction Fraction of photons reflected at each wavelength
Sandercyanin	Lipocalin family protein, isolated from a freshwater fish, able to bind to biliverdin IXa displaying blue color naturally, or red fluorescence under UV radiation
UnaG	Fatty acid binding protein (FABP), isolated from marine eels, able to bind endogenous bilirubin triggering green fluorescence

Technical terms defined to clarify concepts in the field of natural fluorescence

experimental approaches, such as gene reporting, drug screening, and labeling. In parallel to the discovery of new wild-type fluorescent proteins (FPs), the hunt to engineer novel FP mutants has led to modifications of their chemical properties in an effort to broaden their potential applications in cell biology and biomedicine [9]. Beyond the biotechnological revolution prompted by the discovery of FPs, no mechanistic explanation has been proposed for the presence of fluorescence in nature. Recent findings relating to new FPs have prompted investigations in novel research directions, such as evolutionary ecology, as little is known about the eco-physiological role of fluorescence in nature. In the present review, we present and discuss several perspectives, such as the phylogenetic distribution of fluorescence in nature, the expansion of FPs in the tree of life, pigment-generated fluorescence, and the ecological functions of fluorescence in aquatic and terrestrial environments.

Differences between marine fluorescence and luminescence

In the sea, the sources of light energy are sunlight, moonlight, and luminescence. Only a small fraction of daylight

penetrates the ocean's depths, becoming progressively dimmer before resolving to a uniform blue spectrum (470–490 nm) light. Orange-red light penetrates only to a depth of 15 m and ultraviolet light to 30 m [10]. Bioluminescence is the emission of visible light by an organism resulting from luciferin oxidation under the control of luciferase. Instead, photoproteins, which are the primary substrates of the light-emitting reactions of various bioluminescent organisms in diverse phyla, do not require luciferase enzyme activity [11], but instead rely on Ca^{2+} or superoxide radicals and O_2 to trigger bioluminescence. This mechanism is the primary source of biogenic emission of light in the ocean from the epipelagic to the abyssal zone, in regions from the poles to the equator [12]. For many marine species, the primary visual stimulus comes from biologically generated light rather than from sunlight. Given its widespread distribution, bioluminescence is clearly a predominant form of communication in the sea, with important effects on diurnal vertical migration, predator–prey interactions, and the flow of material through the food web [12].

Biofluorescence is a phenomenon dependent on external light, in which a fluorophore converts absorbed short-

wavelength light to a longer wavelength. In other words, incident light is re-emitted at a longer, less energetic wavelength, therefore with low energy conversion efficiency. Natural fluorescence may derive not only from fluorescence-emitting proteins, but also from organic pigments, such as chlorophyll, carotenoids, flavonoids, pterins, or minerals, such as zinc, strontium, aluminium, selenium, and cadmium, that are able to emit light at similar wavelengths (Table 2 and Fig. 1) [33]. In terrestrial

animals, specific compounds, such as chemical derivatives of the organic substance coumarin, seem to be at the origin of fluorescence observed in the cuticles of some arthropods (e.g., spiders and scorpions) [26, 34]. These biotic and abiotic substances may also be phosphorescent under UV light, a fluorescence-like physical process characterized by a longer emission time course. Especially in aphotic habitats, fluorescence and luminescence may co-exist and interact within the same organism, in which the

Table 2 Natural and photoactivable fluorescent proteins and pigments

Gene ortholog	Protein class	Phylum/Organism	λ_{Exc} (nm)	λ_{Emi} (nm)	Reference
GFP	GFP	Cnidaria, Hydrozoa	395	510	[13]
DsRed	GFP	Cnidaria, Anthozoa	558	583	[14]
cFP484	GFP	Cnidaria, Anthozoa	456	484	[14]
zFP538	GFP	Cnidaria, Anthozoa	528	538	[14]
ZsGreen	GFP	Cnidaria, Anthozoa	496	506	[14]
asulCP	GFP	Cnidaria, Anthozoa	572	595	[15]
cgigGFP	GFP	Cnidaria, Anthozoa	399	496	[15]
hcriGFP	GFP	Cnidaria, Anthozoa	405	500	[15]
dis3GFP	GFP	Cnidaria, Anthozoa	503	512	[15]
dendFP	GFP	Cnidaria, Anthozoa	492–557	508–575	[15]
mcavGFP	GFP	Cnidaria, Anthozoa	506	516	[15]
mcavRFP	GFP	Cnidaria, Anthozoa	508	580	[15]
rflGFP	GFP	Cnidaria, Anthozoa	508	518	[15]
rflRFP	GFP	Cnidaria, Anthozoa	566	574	[15]
scubGFP1	GFP	Cnidaria, Anthozoa	497	506	[15]
zoan2RFP	GFP	Cnidaria, Anthozoa	552	576	[15]
asCP562	GFP	Cnidaria, Anthozoa	562	595	[16]
Kaede	PAFP	Cnidaria, Anthozoa	508–572	518–580	[17]
Dendra	PAFP	Cnidaria, Anthozoa	488–556	505–575	[18]
Dronpa	PAFP	Cnidaria, Anthozoa	503	518	[19]
pmeaGFP1	GFP	Arthropoda, Copepoda	489	504	[20]
GFPa1	GFP	Chordata, Cephalochordata	497	516	[21]
UnaG	Fatty Acid binding	Vertebrata, Teleostea	500	527	[22]
Sandercyanin	Lipocalin	Vertebrata, Teleostea	375	630	[23]
SmURFP	Phycobiliprotein	Cyanobacteria	642	670	[24]
Pigment name	Pigment type	Phylum/Organism	λ_{Exc} (nm)	λ_{Emi} (nm)	Reference
Crustacyanin	Carotenoid	Arthropoda Malacostraca	530	580	[25]
β -carboline	Tryptophan derivative	Arthropoda Aracnida	360–370	445–490	[26]
Psittacofulvin	Non-carotenoid	Chordata Aves	N/A	N/A	[27]
Spheniscins	Pterins-like	Chordata Aves	UV	N/A	[28]
Sepiapterin	Pteridin	Chordata Actinopterygii	UV	450–490	[29]
Porphyrin	Porphyrin	Mollusca Gastropod	UV	625	[30]
Hylolin	Dihydroisoquinolinone	Chordata Amphibia	390–430	450–470	[31]
Betaxanthins	Betalains	Plantae Caryophyllales	463–474	509–512	[32]

Fluorescent molecules, their taxonomic distribution, excitation/emission wavelengths, and the original scientific reference. Fluorescent proteins belong to three different classes: GFPs, fatty acid binding proteins, and lipocalins. Various pigments can also participate in the process of fluorescence

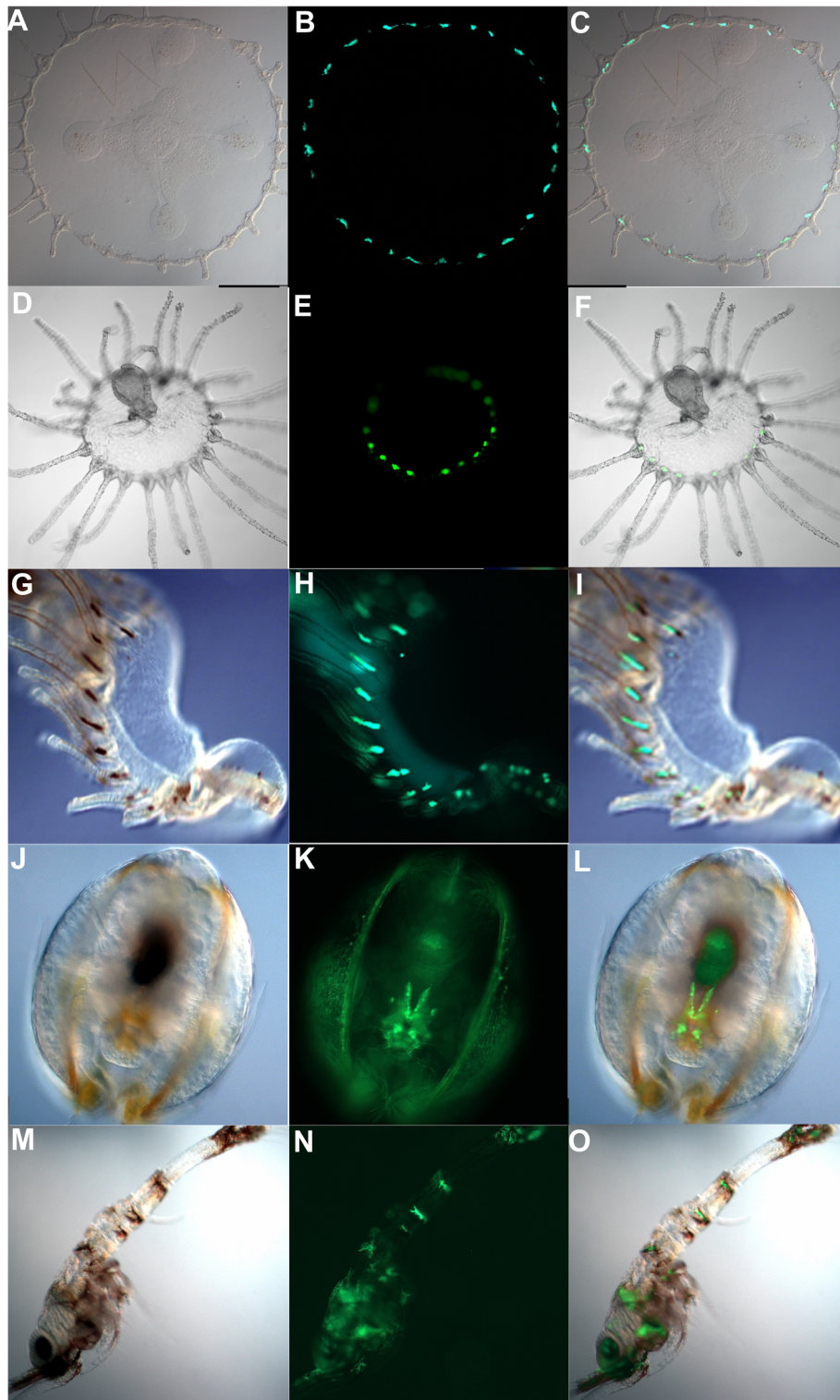


Fig. 1 Samples of GFP in cnidarians and pigment generated by fluorescent organisms from the Gulf of Naples (Italy). **a-f**: Cnidarian hydrozoans, *Clytia hemisphaerica* **a-c** and *Obelia* sp. **d-f**; **g-i**: Phoronida, actinotroch larva of unknown species; **j-o**: Arthropoda, unknown ostracod species **j-l** and unknown crustacean species **m-o**

latter acts as light energy source for fluorescence since some luminescent compounds (e.g., those in dinoflagellates) may also be autofluorescent [35]. By virtue of this coexistence, photophores (luminescent organs) often convert their naturally blue luminescent light into green light by using GFP [13, 20]. This is also the case of chromatophores, which are dermal cells that mediate color changes in vertebrates (see glossary in Table 1). In fish, for example, these cells are specialized in the synthesis and storage of light-absorbing pigments [36].

Fluorescent proteins in the tree of life

The evolutionary origin of FP genes in metazoans remains subject to debate. Canonical GFP orthologs have been identified only in the phyla Cnidaria, Arthropoda, and Chordata, suggesting the presence of GFP in the last common ancestor of all metazoans (Fig. 1). Recently, GFP-like genes have been found in transcriptomes of 30 ctenophores, which is relevant to their early divergent phylogenetic position [37]. Although one of them was initially described as a fluorescent protein [38], a deeper study indicated that fluorescence in the ctenophore was

not intrinsic, but originated from a siphonophore it had consumed [37].

For the present review, we performed an extensive search for well-annotated transcriptomes and genomes of sponges, nematodes, annelids, molluscs, echinoderms, and hemichordates, but did not identify any orthologs of canonical GFPs. This suggests that independent gene loss events occurred in the evolutionary history of several animal clades. An alternative phylogenetic scenario capable of explaining the scattered phylogenetic distribution of GFPs would involve independent horizontal gene transfer events, probably through diet; this possibility requires further investigation [39]. Two important evolutionary events appear to have occurred chordate clade: the loss of GFP representatives in Olfactores (comprising tunicates and vertebrates) and, in contrast, extraordinary gene expansion recently detected in cephalochordates (Fig. 2). In fact, 21 expressed GFPs have been identified in the amphioxus *Branchiostoma lanceolatum*, although the significance of this extensive number of GFPs requires further functional clarification [6, 40].

Recently, new FP families have been characterized in vertebrates bearing different features in comparison with

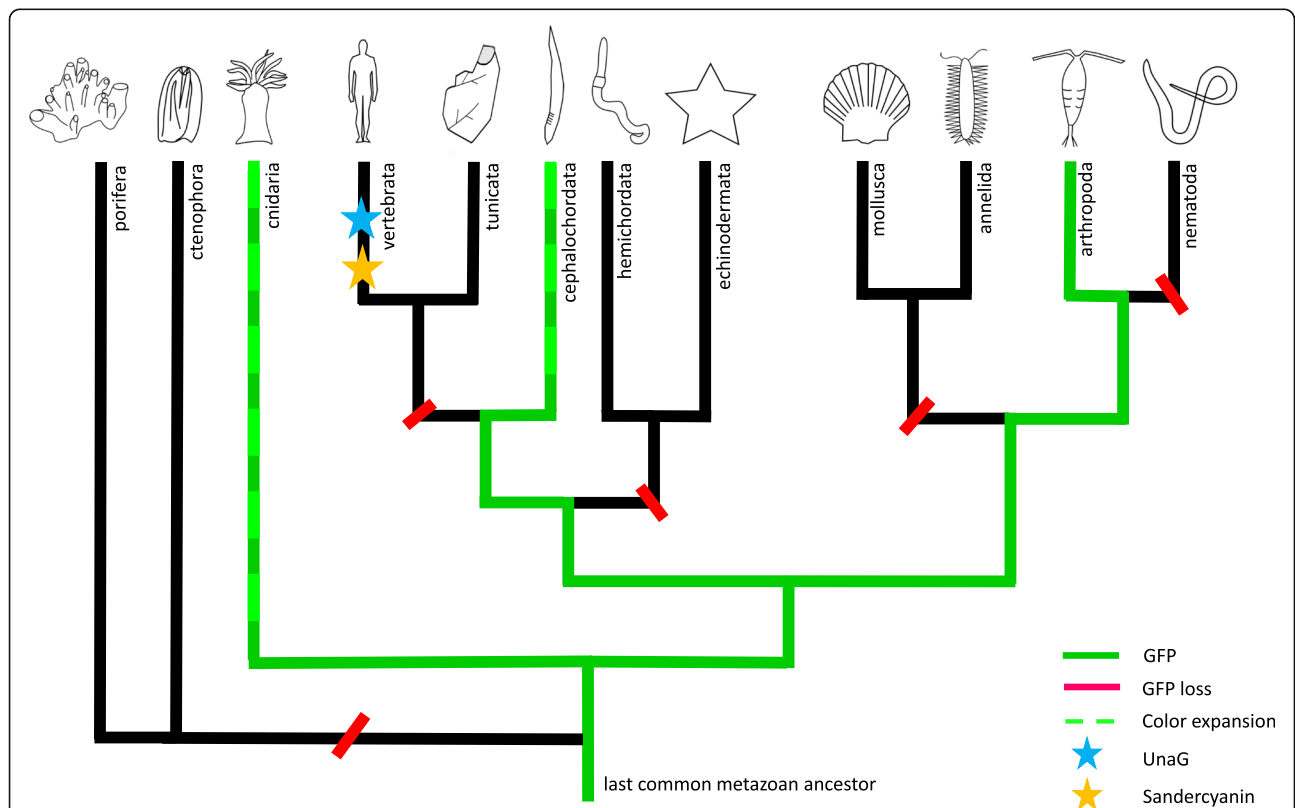


Fig. 2 Distribution of fluorescent proteins in metazoan. Canonical GFPs have been found in cnidarian, arthropods and cephalochordates, supporting the hypothesis of a common metazoan ancestral origin. Color expansion is present in cnidarians and has been recently showed in cephalochordates as well. Furthermore, two other FPs, not related to GFP, have been characterized in vertebrates: UnaG and Sandercyanin

canonical GFP proteins. For instance, a blue fluorescent protein named Sandercyanin was first isolated almost a decade ago from the freshwater walleye (*Sander vitreus*), a fish found in the lakes of North America (Fig. 1). This is the first FP described with blue absorption and far-red emission under UV radiation [23]. Furthermore, a non-GFP green fluorescent protein belonging to the fatty-acid-binding protein family (FABP) was isolated from the muscles of the freshwater eel *Anguilla japonica* (Fig. 2) [22]. This protein, named UnaG (*unagi* is the Japanese name for this species of eel), triggers bright green fluorescence through coupling with bilirubin [22]. Two novel brightly fluorescent FABP proteins originating from a gene duplication event have also been characterized in the false moray eel (*Kaupichthys hyoproroides*) [41]. Since this cryptic eel occupies a nearly monochromatic marine environment predominated by blue wavelengths, further analyses are needed to determine the ecological function of this green emission [41].

Finally, fluorescence has been recently identified in two catshark species, *Cephaloscyllium ventriosum* (swell shark) and *Scyliorhinus rotifer* (chain catshark), in which light emission from the skin is essentially due to bromokynurenin yellow metabolites. This discovery raises new questions about the diversity of fluorescence sources in nature and the ecological roles in vertebrates [42].

A wide range of color-emitting GFPs characterize the phylum Cnidaria, in particular in anthozoans (sea anemones and corals). More than two decades ago, the DsRed protein was discovered in non-bioluminescent reef coral species of the genus *Discosoma* [14]. Chromophore synthesis, responsible of the color of the protein, is a molecular process that requires genomic stability, as any mutation disrupting the autocatalytic reaction in DsRed would convert it into green protein [43]. Indeed, at least seven different mutant variants of DsRed emitting in the green range have been generated by random and site-specific mutagenesis events [44, 45].

Anthozoan FPs have been engineered to produce photoactivable FPs (PAFPs) generating huge light-induced spectral changes. Dendra, originally from octocoral *Dendronephthya* sp., was the first PAFP shown to be capable of photoconversion from green to red fluorescent states in response to either visible blue or UV-violet light [18]. In addition to its high photostability, this PAFP is easily photoactivated by ordinary 488-nm laser light. Similar to Dendra, Dronpa is a reversible bright green PAFP derived from the coral *Echinophyllia* sp. that shows interesting properties beyond its extreme brightness, as it can be switched on and off repeatedly with high contrast and a minimal loss in fluorescence intensity [19, 46].

While FPs have been characterized mainly in eukaryotes, interest in prokaryote orthologs has increased in

the last years. A far-red Biliverdin-Binding FP (smURFP) was developed from a member of the same family as Sandercyanin derived from the cyanobacterium *Trichodesmium erythraeum*. Unlike Sandercyanin, smURFP fluorescence is visible without exogenous biliverdin, and is the brightest far-red/near-infrared FP created to date [24]. Bacterial fluorescence has also been a template for generating non-oxygen-dependent FPs. Flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs), unlike GFPs, do not require oxygen as a cofactor to synthesize the FMN chromophore, which makes these FPs very convenient for studying anaerobic biological systems [46].

Ecological functions of fluorescence

Compared to terrestrial animals, marine organisms occupy a spectrally limited visual environment, in which their eyes are adapted to different light conditions. In particular, a topic of interest in the evolutionary and ecology fields concerns how different types of visual systems developed specific spectral receptors and pigments (allowing them to detect fluorescence). Crustaceans such as the mantis shrimp have developed a fascinating system of color vision, based on at least eight primary spectral receptors ranging from 400 to 700 nm. In the species *Lysiosquilla glabriuscula* it has been shown that, at depths of 20, 30, and 40 m, fluorescence contributes 9, 11, and 12% of the photons that stimulate the shorter-wavelength receptor, and 15, 22, and 30% of those stimulating the longer-wavelength receptor, respectively [47].

However, to determine whether sufficient energy is transferred in order to make a meaningful difference to the visual signal under natural lighting conditions, several optical factors such as exitance and reflectance (see glossary, Table 1) need to be calculated. In addition, fluorescence can only play a role in vision if it contributes to the total light leaving the surface and to a behavioral response; i.e., if the behavior of the viewing organism is influenced by the presence or absence of fluorescence in the subject [48]. Another key unresolved issue is whether fluorescence is sufficiently bright to be visible against the background light environment.

What are the adaptive advantages conferred by fluorescence? While it is thought that not all biofluorescence is functionally relevant, few examples of its ecological role have been described. A number of hypotheses have been advanced to explain the roles of fluorescence, alone or in combination with luminescence. These include photoprotection for stem cells, photosynthesis enhancement, predation by prey lure or distraction, and protection against oxidative stress. Below, we use a taxonomic approach to review advances in the understanding of the ecological roles of fluorescence in marine organisms.

Green fluorescent proteins and, in general, fluorescent pigments, act as a photoprotective system against damage from sunlight [49]. It has been shown that UV_A and extreme photosynthetically active radiation (PAR) trigger photodamage and photoinhibition in coral-dinoflagellate symbiosis that, in severe cases, may result in coral bleaching [49, 50]. In this context, FPs histologically positioned above endosymbionts may function as an energy dispatcher through fluorescence and light scattering. In the hydrozoan *A. victoria*, the response to superoxide radicals was investigated by examining the protein structure of GFP. Superoxide radicals and reactive oxygen species are typically present in the hyperoxic conditions that these organisms experience during the daytime due to the photosynthetic activity of algal symbionts. It has been shown that GFP can quench (see glossary, Table 1) these superoxide radicals without altering its fluorescence properties [51], thereby providing protection from antioxidants.

Marine organisms

It is fascinating how in the deep sea, the largest habitat on earth, marine organisms can live in constant darkness without access to high-energy blue light [52], using instead luminescence as the predominant light-signaling phenomenon. It is even more fascinating that, as a consequence of the production of blue luminescent light in this habitat, fluorescence acts as an energy-collecting device that enhances photosynthesis in cnidarians [53].

The tentacles of the deep-sea anemone *Cribrinopsis japonica* emit green fluorescence, when excited by blue light, potentially as a lure for prey attraction [54]. Interestingly, the GFP isolated from this anemone is more stable than other GFPs; however, it is unclear whether this results from adaptation to its deep-sea habitat. The sea anemone *Nematostella vectensis* was the first early metazoan whose genome was sequenced, and represents a powerful model system for evolutionary development biology [55]. This species possesses seven GFP genes, of which only *nvfp-7r*, which codes for a red fluorescent protein (NvFP-7R), is functionally fluorescent. The transcriptional regulation of the *nvfp-7r* gene shows spatio-temporal complexity as well as the unexpected capacity to respond to positional information in the adult body plan [56]. Despite the current knowledge of the functional significance of red fluorescence in *N. vectensis* (as well as in the vast majority of fluorescent organisms), it is nonetheless based upon hypothetical reconstructions. The large toolkit of sophisticated approaches available for this species renders this small anthozoan a promising model for the acquisition of deeper insights into the role(s) of fluorescence.

The function of fluorescence in prey attraction has been assessed in a non-luminescent hydromedusa species,

Olindias formosus, which possesses fluorescent and pigmented patches on the end tips of its tentacles from early development of the polyp stage. In laboratory experiments under blue light conditions, these pigmented patches attract juvenile rockfish of the genus *Sebastes*, which do not respond in the absence of fluorescence [57]. A similar mechanism has been observed in the siphonophore *Resomia ornicephala*, which possesses fluorescent tentacles that attract and capture euphausiid shrimp [58].

In the hydrozoan jellyfish *Clytia hemisphaerica*, the intense green fluorescence observed in the endodermal and ectodermal cells of the mouth, stomach, and gonads may have several functions, including protection of stem cells and maternal mitochondrial DNA from UV light. Each of the four GFPs (and the three aequorins) isolated in this species show life-cycle stage and tissue specificity, supporting the hypothesis that fluorescence has acquired multiple specialized roles in response to environmental (depth), physiological (life-cycle) or behavioral (spawning) conditions [59]. The siphonophore *Erenna sirena* is another example in nature of energy conversion from luminescence to fluorescence by creating yellow to red fishing lures (583–680 nm) on its tentacles surrounded by a luminescent photophore [60].

In the phylum Arthropoda, few copepod species exhibit luminescence, while several others, belonging to the Pontellidae and Aetideidae families, exhibit biofluorescence, which is thought to serve as a mate perception and attraction signal and/or a camouflage mechanism [20, 61]. Interestingly, the high brightness and stability and low cytotoxicity of copepod GFP proteins make these molecules particularly well-suited to a variety of molecular and biological applications [62].

Although neither stomatopod crustaceans nor mantis shrimps possess fluorescent proteins, many species display a very bright fluorescent coloration that is used in postural signaling to increase shrimp visibility when sensing a predator, for intra-species competition with other males, and in mate choice [47].

Studies on FPs in chordates provide further information about their function when compared to invertebrates. In the cephalochordates *Branchiostoma floridae*, *Branchiostoma lanceolatum*, *Branchiostoma belcheri* and *Asymmetron lucayanum*, an expansion of GFPs has been reported in the genome [6, 21, 63]. Different functions have been postulated, such as playing a role in antioxidant mechanisms by scavenging deleterious oxy-radicals, in photoprotection and attracting motile planktonic prey [6, 21]. In cartilaginous and bony fish, such as catsharks and reef fish, fluorescence may function in communication, species recognition, and camouflage [64] or it may simply be a chemical by-product of skin composition.

Green and red fluorescence have also been observed in the sea turtles *Eretmochelys imbricata* and *Caretta*

caretta. Whether these originate from diet (corals, zooxanthelles) [65], or as a by-product of the chemical composition of algae growing on their shells [66] remains unclear. It is also uncertain what role fluorescence might play in a sea turtle. In the case of the loose-jaw dragonfish *Malacosteus* sp., the animal emits luminescent light and far-red fluorescence, which may be used in predation [67]. Most reef fish possess visual pigments ranging from UV to green wavelengths [68, 69]. Interestingly, red fluorescence has been observed in more than 180 species of marine fish [52], strongly suggesting its potential role in vision [69].

Experiments conducted on the diurnal fish *Cirrhilabrus solorensis*, whose visual system is receptive to deep red fluorescent coloration, have demonstrated that its strong red fluorescence emission body pattern affects male–male interaction [70]. A study on the spectral sensitivity of the goby *Eviota atriventris* revealed that this fish possesses long-wavelength visual pigments, making it physiologically sensitive to red fluorescent coloration [71]. Lending further weight to this hypothesis, yellow intraocular filters have been found in reef fish, sharks, lizardfish, scorpionfish, and flatfish, which could enable them to detect fluorescence [72]. It has also been demonstrated that sharks and rays are capable of visualizing their own fluorescence, showing sexually dimorphic fluorescent body patterns, which is suggestive of a function in communication or species-recognition role [64].

Nevertheless, despite several attempts, no sufficient experimental studies have clarified the functional and behavioral link between fluorescence and vision in organisms with complex visual systems [69].

Terrestrial organisms

Fluorescence produced by fluorescent metabolic chemical by-products is also observed in many terrestrial organisms. Several recent studies have suggested ecological and behavioral roles similar to those highlighted in marine organisms. In amphibians, the tree frog *Hypsiboas punctatus* emits hyloins, fluorescent compounds secreted from the lymph and gland nodes [31]. This suggests that fluorescence is part of the integumentary pigment system in this amphibian, representing a novel extra-chromatophore source of coloration. In low-light conditions, frog fluorescence accounts for 18–29% of the total emerging light comprising fluoresced and reflected photons. This confers greater brightness to *H. punctatus* and matches the sensitivity of night vision in this clade.

Another interesting example is represented by butterfly wings, which possess an intrinsic controlled system that is remarkably similar to recent LED technology, utilizing a photon crystal-like structure capable of producing directed fluorescence [73].

Behavioral experiments performed in arthropods and chordates underline the potential role of fluorescence in

communication. In fact, the fluorescent plumage in the parrot *Melopsittacus undulate* was shown to have behavioral implications in mate selection, rather than in social communication [74]. In fact, female and male parrots exhibit significant preferences for fluorescent birds of the opposite sex [75].

The jumping spider *Cosmophasis umbratica* has been shown to interact differently in the presence of UV reflectance or UV-induced fluorescence while testing sex-specific courtship signaling [76]. Males present UV-reflective patches of scales on the face and body that are shown during conspecific posturing [77]. These patches are lacking in females, which instead have palps with a UV-excited bright green fluorescence that are absent in males. During the experiment, female spiders made a postural response to male courtship under full-light spectrum, while they did not respond or turned away without UV. Similarly, males ignored non-fluorescing females. The courtship responses of the spiders were an effect of sexual coloration instead of behavioral changes. To determine this, the behavioral responses of individuals of one sex under full-spectral light were compared when the partner of the opposite sex was illuminated with UV-deficient light. Most UV-irradiated male spiders that courted fluorescent females failed to do so when the female lacked fluorescence even though her response was the same as under normal light.

Desert scorpions exhibit blue/green fluorescence under UV light in laboratory conditions, although this phenomenon does not manifest in natural daylight conditions. Beta-carboline, a tryptophan derivative molecule is responsible for the fluorescence in the cuticle of scorpions (Table 2) [26]. Although it has been hypothesized that fluorescence in scorpions may serve as a prey lure, it is clear that the formation of this substance on the cuticle of this animal serves no function [78].

Finally, it has been recently shown that tubercles protruding from the skull of chameleons reveal fluorescence upon short-wavelength UV-irradiation; this may play a role in species recognition [79]. Emission signals corresponding to deep blue are reasonably rare in tropical forests; this form of biofluorescence thus appears to be a distinct signal against the green vegetation background reflectance [79].

Fluorescence plays roles in terrestrial animals as well as plants, such as the carnivorous *Nepenthes*, *Sarracenia*, and *Dionaea*. Preliminary studies have quantitatively measured fluorescence in flowers in several species and concluded that its relevance for communication is negligible [80]. However, more recently, blue fluorescence emission at the catch sites of these plants was detected, suggesting that it could play a role in attracting arthropod prey compared to non-illuminated plants [81]. In the yellow flowers of the plant *Portulaca grandiflora*, the pigment betaxanthin is at the origin of green emission

when the flower is excited by blue light (Table 2) [32]. The flower exhibits natural yellow coloration; its brightness may be increased by this fluorescent pigment at a particular wavelength, making the flower more visible to pollinators.

Conclusion and perspectives

Both fluorescence and luminescence are prevalent optical processes present in nature and crucial for species communication and predator–prey interactions, and may co-exist or cooperate in many species, such as deep-sea animals. The discovery of GFP in 1962 in the cnidarian jellyfish *A. victoria* and the subsequent characterization of numerous GFPs in several taxa have prompted research on biotechnological applications. More recently, orthologs of GFP have been identified in arthropods and chordates; nevertheless, the evolutionary and ecological significance of fluorescence requires substantial further study.

Recently, the exciting discovery of novel types of fluorescent proteins in vertebrates (i.e. UnaG and Sandercyanin) has led to novel evolutionary insights, given that until recently only GFPs and GFP-like proteins were thought to support fluorescence. The identification of yellow fluorescent metabolites in sharks has also opened new avenues of inquiry into their roles in central nervous system function, photoprotection, and resilience to microbial infections. For example, genome editing of fluorescent proteins in a living model organism such as *Clytia hemisphaerica* may be highly informative in order to assess its biological function. Although its roles in communication, predation, and camouflage in several taxa are widely accepted in the scientific community, evidence for and interpretation of additional functions require stronger scientific support. It will also be crucial to conduct further functional studies of fluorescence in both terrestrial and marine species to assess whether the emission of fluorescence is quantitatively significant in natural environments, which is necessarily different from that under laboratory conditions.

Finally, our understanding of the role of fluorescence in animal vision is in its early stages, as seen from a few recent studies in reef and deep-sea fish. Further research is also needed to clarify the anatomy of the visual apparatus, which we did not examine in this review, and the molecular toolkits involved in color, contrast, and fluorescence detection, in order to shed light on these unresolved questions.

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Authors' contributions

M.M. and D'A.S. conceived the review project. M.M., P.S. and D'A.S. wrote the manuscript. All the authors edited the manuscript and approved the final version. D'A.S. supervised the project.

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