This research proposal was submitted to the “Researcher Project for Scientific Renewal” call from The Research Council of Norway in 2022

https://www.forskningsradet.no/en/call-for-proposals/2022/researcher-project-scientific-renewal/

The application was unsuccessful but received a fundable score (24/28 points). The research proposal and the feedback it received is published here for the benefit of future applicants.

This research is ongoing, so if you find it interesting and/or consider pursuing similar projects, please reach out via our lab website (www.marineneurolab.com) and cite this proposal in your work.

Deciphering bioluminescent communication in marine annelids

"Bioluminescence is one of the oldest and most prevalent languages on Earth – and one that is largely alien to us" [1]

Preamble: this application has been modified extensively to address the excellent evaluation received from the panel in 2021. This includes 1) more preliminary data and detailed descriptions of experiments to better demonstrate implementation and feasibility, 2) detailed discussions of risks and mitigation measures, 3) additional actions for communication and milestones/deliverables, 4) emphasis on my numerous leadership experiences, 5) publication of two major projects, 6) three expert collaborators (CVs included) and 7) improved outline of management strategy and structure.

1. Excellence

1.1 State of the art, knowledge needs and project objectives

"Life Below Water" is not only one of the UN's Sustainable Development Goals[2], it is also the origin of the most complex structure known to man: the brain. Yet, despite the strong evolutionary ties between the ocean and the nervous system, marine biology and neuroscience rarely intersect. This is due to a lack of precedence rather than a lack of pressing questions, one of which is the significance of one of the animal kingdom's most ancient communication forms – bioluminescence. The importance of this heat-free form of light to life on our planet is immense. A large variety of species across multiple habitats are capable of producing it, but most reside in the ocean[3]. Here, bioluminescence is the primary source of light at night and at depth, and is generated by 76% of pelagic organisms[4]. As the ocean's predominant communication signal, its assumed functions are diverse, ranging from predator defence to hunting and mating[5]. Colour is thought to play a role in mediating these functions, as a variety of hues are represented. These colours are, however, unevenly distributed among species. This, in turn, has led to the hypothesis that the rare yellow and red wavelengths facilitate private, intraspecies communication while the much more common blue and green also contribute to interspecies communication[5]. Whether or not this is the case remains unknown as **we still know very little about what bioluminescent signals mean and even less about the significance of colour** at both a behavioural and neuronal level**.**

This is because the majority of our interpretations regarding the use of bioluminescence is primarily based on studies of animal morphology, not behaviour, since *in situ* observations of individual bioluminescent signals are uncommon and experiments with live animals rare[6,7]. Recent technological developments that enable deep, underwater imaging with high-sensitivity cameras are improving opportunities for *in situ* observations, revealing the hidden diversity of bioluminescent species and signals, but still remain largely descriptive[8]. Behavioural experiments are rare partly due to the difficulty of obtaining and maintaining specimens in good condition as pelagic organisms are often fragile[9], but also because there is minimal precedence. Such studies are, however, the only way to decipher the true function of bioluminescence. Dinoflagellates, for example, have long been known to bioluminesce in response to mechanical disturbance. But controlled experiments were necessary to show that its function is to deter predation[10,11] by acting as a warning signal[12], that elicits high-speed swimming burst in grazing predators[13–15] and attracts secondary predators[12]. One other notable exception to the dearth of behavioural studies is the finding that an annelid from the Tomopteridae family, *Tomopteris helgolandica*, is able to distinguish between the colour of its own species and that of others: artificial light signals mimicking conspecifics seem to attract these animals, while signals mimicking another species is repelling[16], perhaps because they could indicate the presence of predators. In addition, similarities in the anatomical structure and neural control of the light emitting organs of the different species in this genus suggest that they may have derived from a common, recent origin[17]. Combined, these findings indicate a **potential for complex intra- and interspecies bioluminescent communication strategies and behaviours in related tomopterid organisms**.

The use of bioluminescence as an elaborate communication signal requires a high level of control over its onset, duration, and intensity. Such sophisticated modulation can be achieved if emission is subject to direct neural control, and this does seem to be the case for members of the Tomopteridae family. Not only are the bioluminescent light organs of these annelids innervated by nerve fibres[18,19], but emission depends on the activation of nicotinic cholinergic receptors and calcium channels, which is indicative of fast nervous system control[20]. Our knowledge of the tomopterids' nervous systems is, however, limited to descriptions

of their general anatomy. Hence, **to understand the neuronal underpinnings of bioluminescent communication it is essential to first characterise the underlying neuroanatomy** in detail.

This ground-breaking project will **determine how bioluminescence and its colours mediate inter- and intraspecies communication in marine annelids at a behavioural level and characterise the underlying neuroanatomy.** I will achieve this by using two species from the Tomopteris genus that are ideally suited for the following reasons: first, unlike many other gelatinous marine organisms, they are known to remain healthy under laboratory conditions for weeks after collection in the wild, and my own preliminary data shows that I can keep them alive for several months. This makes continuous behavioural observations and rigorous experimental protocols possible to implement. Second, the two species possess unique bioluminescent profiles: *Tomopteris helgolandica* emits yellow light, while the related *Tomopteris planktonis* uses the much more prevalent blue[17], which allows for comparisons of two potentially different communication strategies. Third, as predators they have a wide behavioural repertoire[16] that, in combination with their relatively large size (up to 6 cm in length), enable detailed behavioural readouts. Fourth, as they live their entire lives in the open ocean, they have evolved a convenient trait to conceal themselves from predators: they are wholly transparent at all life stages (**Figure 1**). This means it is possible to characterise the structure of their nervous systems both with traditional methods that require tissue sectioning (of which I have extensive experience) and with new, complementary cutting-edge optical techniques that enable visualisation of native and introduced fluorescence in intact animals. Fifth, because these species are thought to share a recent, common ancestor[17], an evolutionary perspective on the use of bioluminescent colours and the underlying neuroanatomy is attainable. This **unique combination of features, along with extensive behavioural experiments and neurobiological techniques** will allow me to fulfil two major objectives, pursued with a multidisciplinary approach:

Objective 1: Decipher the bioluminescent signals of two related annelid species
Objective 2: Characterise the neuroanatomy of both species

My extensive experience with systems neuroscience, animal behaviour, marine biology, and the methods that will be employed in this ambitious project puts me in a unique position to bridge these fields with innovative experiments to shed light on a major ecological phenomenon. This enables me to spearhead investigations into a novel research line with the overarching vision of **revealing the evolutionary, behavioural, and neuronal origins of bioluminescent communication**. Understanding how "Life Below Water" communicates will be imperative to minimise our increasing impact on this ecosystem that is the cradle of both life and the nervous system.

1.2 Research questions and hypotheses, theoretical approach and methodology

The two objectives will be addressed through four work packages (WPs):

WP1: Identify naturalistic stimuli associated with bioluminescent emission
WP2: Establish whether distinct properties of bioluminescent signals elicit different behaviours
WP3: Determine if *T. helgolandica* and *T. planktonis* can communicate with each other
WP4: Characterise the neuroanatomy of *T. helgolandica* and *T. planktonis*

Together, these WPs will allow me to decipher the bioluminescent communication of two related species and characterise the underlying neuroanatomy. This will be achieved by identifying naturalistic stimuli that evoke bioluminescent emission (WP1), whether distinct properties of bioluminescent signals elicit different behaviours (WP2), and if these two species react to the signals emitted by the other species. If so, I will determine if they understand each other or if the meaning of the signal was lost in the evolutionary mediated colour conversion (WP3). Finally, I will characterise and compare the nervous systems of both species to discover the underlying neuroanatomy of bioluminescent communication (WP4). To succeed, I will use a wide range of methods, from specimen collection in the wild to behavioural analyses and histological techniques as described below.

General methodology

For all WPs, *T. helgolandica* and *T. planktonis* specimens (**Figure 1A**) will be collected using plankton nets in the Trondheim fjord at approximately 300 meters depth, where both species are frequently found during the day: each of my net deployments typically yields about three *T. helgolandicas* and one *T. planktonis*. After collection and identification (in addition to their bioluminescent colour, the two species can be distinguished by size, number of parapodia and presence of a tail[17]), they will be kept in aquariums in dedicated facilities

at NTNU SeaLab. Here, fresh seawater is pumped up from the Trondheim fjord and is carefully checked and controlled by resident experts to provide a constant supply of high-quality seawater. I will keep the specimens in the dark at approximately 10°C and exchange the water every few days. From my preliminary experiments, **I have found them to remain healthy under these conditions at NTNU SeaLab for up to 3 months**. I will continue to optimise these holding conditions with expert advice from my project partner Dag Altin at NTNU SeaLab (see section 3.1). All behavioural experiments (WPs 1-3) will be performed in the dark by video monitoring individuals under infrared light. A 2D version of this method has already been successfully established in my lab (**Figure 1B, Figure 2**), inspired by a previous study[16]. Bioluminescent emission will be monitored in parallel with a separate, high-sensitivity camera and the two video feeds synchronised with Bonsai[21]. My collaborator Prof. Jérôme Mallefet at UCLouvain has extensive experience with bioluminescent organisms and will provide specialist advice for acquiring images of bioluminescence. To track the behaviour of one or more individuals, I will employ recently developed deep-learning algorithms at the forefront of the field, such as DeepLabCut[22] and TRex[23], which enable marker-less tracking of individuals at high spatial resolution. As the setups develop, I will progress to 3D monitoring and tracking in collaboration with Assoc. Prof. Benjamin Dunn at the Department of Mathematical Sciences at NTNU (see section 3.1). This will enable detailed quantification of behaviour in more naturalistic, spacious environments. Additional WP-specific methods are described in their respective sections below.

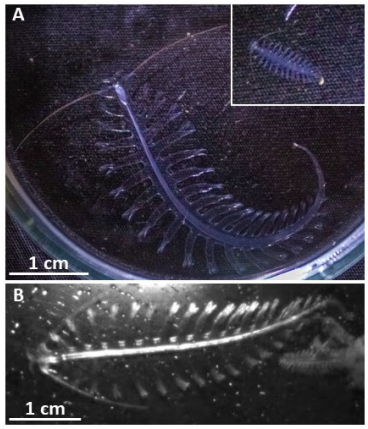

Figure 1: *T. helgolandica* and *T. planktonis*
A) *T. helgolandica* and *T. planktonis* (insert, to scale) photographed under white light.
B) Single frame from video recording of *T. helgolandica* in darkness illuminated with infrared light. Bioluminescence was observed but not captured on camera.

WP1: Identify naturalistic stimuli associated with bioluminescent emission

Background and hypotheses: while bioluminescence can be induced by artificial means in tomopterids (e.g., strong chemical/electrical stimulation[17] or rough handling[16]), spontaneous bioluminescence is rarely observed in single-housed *T. helgolandica* specimens[16]. These findings suggest that naturally occurring emission is likely associated with physiologically relevant stimuli, such as the presence of predators, prey, or conspecifics. The rare, yellow colour used by *T. helgolandica* further suggests it might be using bioluminescence for intraspecies communication. **I therefore hypothesise that *T. helgolandica* will be more likely to emit these signals when in the company of conspecifics compared to when they are alone. In contrast, I hypothesise that the more common colour of *T. planktonis* is more likely to be elicited during (interspecies) prey or predator interactions.**

Methodology: to determine which stimuli are associated with bioluminescent emission in two related tomopterid species, I will perform three sets of experiments on both the yellow emitter *T. helgolandica* and the blue emitter *T. planktonis* while monitoring both behaviour and bioluminescence. **First, single animals will be exposed to artificial stimuli simulating potential predators**. Replicating a previous study on *T. helgolandica*[16] and extending it to *T. planktonis*, I will first use plastic pipettes to simulate potential predators, by creating indirect water disturbances at various locations and distances from each individual as well as provide direct mechanical stimulation on their bodies. Expanding on this established method, I will then use surface transducers to create more quantifiable, localised vibrations in their environment on different substrates, including the aquarium wall and through dummy predators (submerged fish models). **Second, the same individuals will be exposed to live prey**. It has not yet been conclusively demonstrated what tomopterids feed on[24], but larger individuals are known to readily eat smaller *Tomopteris* specimens and oocytes if offered[25]. They are also thought to eat other zooplankton. I will therefore use smaller zooplankton found in the same net samples (an indication that they occupy the same environment as our specimens) and readily available copepods at several, defined, developmental stages (cultured and provided by my collaborator Dag Altin at NTNU SeaLab). Prey will be introduced in increasing numbers to see if abundance affects bioluminescent emission. **Third, single animals will be exposed to members of their own species**. One common purpose for intraspecies communication is the identification of suitable mates, in which case maturation stage and availability of potential partners are likely to matter. I will therefore introduce individuals to 1) conspecifics of various maturation stages and 2) increasing numbers of maturation-matched conspecifics. I will determine maturation stage by the number of parapodia, as this

number increases with age[25].

In all three sets of experiments the different stimulus types will be presented on different days to avoid interaction effects. Each repetition (10 per animal) of a single stimulus will be preceded by a 2-minute baseline period serving as an internal no-stimulus control. The time between repetitions will be adjusted according to the time it takes for behaviour to return to baseline. All instances of bioluminescent emission (both stimulus-associated and spontaneous) will be analysed for their timing relative to the start of stimulus presentation, their duration and maximum intensity, and compared across stimulus- and baseline periods.

Expected outcomes and impact**:** the experiments in WP1 will yield a comprehensive overview of behavioural and bioluminescent responses to a large variety of physiologically relevant stimuli for two tomopterid species. By comparing responses across species, I will be able to collect the first evidence for whether the evolutionary change in colour has influenced the conditions under which bioluminescence is used. As one of the first experiments of its kind, it will pave the way for similar studies in other bioluminescent species.

Risks and their management: **low risk**. A working setup for monitoring behaviour is already established in my lab (**Figure 1B, Figure 2**) and I have observed bioluminescent emission from tomopterids. Acquiring video footage of bioluminescence is, however, not trivial as it requires sophisticated cameras. I have allocated time specifically for testing a variety of high-sensitivity cameras (on loan from suppliers) to find the best one for my experiments. I will also receive expert advice from my collaborator Prof. Jérôme Mallefet who has extensive experience imaging bioluminescent species, including tomopterids, to ensure swift progress.

WP2: Establish whether distinct properties of bioluminescent signals elicit different behaviours

Background and hypotheses: the properties of bioluminescent signals, such as their kinetics, patterns, and intensity, can contain information. Ostracod crustaceans, for example, use bright, long, high-frequency signals and dim, short, uniform pulses, all blue, to differentiate between two phases of their courtship display[26]. Further, the number of bioluminescent flashes emitted by artificial dinoflagellate predators modulates ostracod swimming behaviour[15]. The yellow-emitter *T. helgolandica* is also capable of producing different bioluminescent patterns, a "glow" and "flash" response[20], and they respond differently to these patterns if presented in blue but not if presented in yellow[16]. These findings indicate that the properties of interspecies bioluminescent signals are relevant for behaviour, but the significance of the distinct intraspecies signals remains a mystery. A reason for this may be that the "glow" and "flash" signals are less informative individually than when presented in sequences incorporating both, as observed *in situ*[27]. **I therefore hypothesise that real versus scrambled bioluminescent signals will elicit distinct behavioural responses in both *T. helgolandica* and the related *T. planktonis*.**

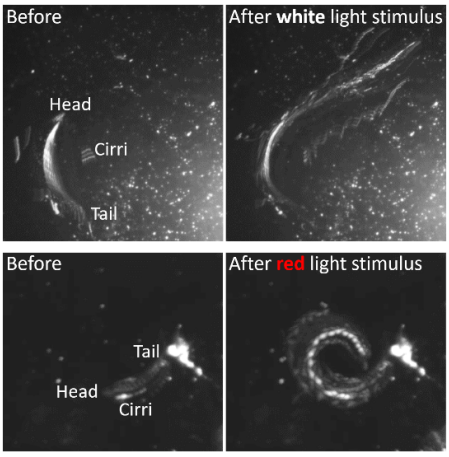

Figure 2: Response of *T. helgolandica* to light Maximum intensity projections across 3 seconds immediately before (left) and after (right) a white (top) or red (bottom) light stimulus was presented, showing a sudden change in behavioural activity levels.

Methodology: to determine if distinct signal properties elicit different behaviours, I will use miniature light emitting diodes (LEDs) with emission spectra resembling those of my blue and yellow-emitting tomopterids to simulate the bioluminescence of each species. Preliminary data from my lab shows that tomopterids respond to such LED light (**Figure 2**), in line with previous results[14,16]. Inspired by recent work by my collaborator Prof. Jérôme Mallefet[16], who will also advise these experiments, the LEDs will be arranged in spatial patterns mimicking the light organ distribution of real tomopterids. I will present individual animals to two types of light patterns that **1) mimic naturally occurring bioluminescent signals** (as observed in WP1 and the published literature[16,20,27,28]) and **2) are scrambled versions of those patterns** (different inter-flash intervals, durations, intensities, and patterns, varied independently of each other). The different stimulus types will be presented 10 times each in randomised interleaved trials across several days, each preceded by a 2-minute baseline period serving both as an internal no-stimulus control and to avoid potential habituation/sensitisation effects. I will monitor any change that may occur in velocity, acceleration, body undulation, tortuosity[29], and movement direction in response to each stimulus as compared to baseline.

Expected outcomes and impact**:** experiments from WP2 will result in detailed readouts of behavioural responses to distinct signal properties in two tomopterid species. In addition we expect to determine if tomopterids bioluminesce in response to light signals, of which there is currently conflicting and limited

evidence[16,28]. This WP will hence elucidate which properties of bioluminescent signals are behaviourally the most important to marine annelids, which will aid our interpretation of the function of similar signals in other bioluminescent species considerably.

Risks and their management: **low risk**. Given that I already have a working setup, preliminary data, and a collaborator who has performed such experiments before, the risks associated with the execution of this WP are minimal. However, although behavioural responses to artificial light signals have been observed both by me (**Figure 2**) and others[14,16], responses to specific signal properties may be subtle and hence challenging to distinguish[16]. To ensure that any behavioural effect is not lost to 'noise', I will employ cutting-edge software that enables detailed tracking, including the orientation of specimens relative to the light stimulus, as they may be insensitive to stimuli from certain egocentric directions. Similarly, I will pay close attention to any habituation/sensitisation effects that may influence the results and increase the trial intervals accordingly.

WP3: Determine if *T. helgolandica* and *T. planktonis* can communicate with each other

Background and hypotheses: because the vast majority of bioluminescence in the ocean is blue or green, the visual system of most species is also typically centred around these wavelengths[5]. The yellow-emitting *T. helgolandica* seems to be an exception, however, as it is able to discriminate between blue and yellow signals[16]. This suggests a capacity for interspecies (in addition to intraspecies) communication, including sensitivity to the blue signals emitted by *T. planktonis*. **For the first time**, **my preliminary data further shows that red light also elicits behavioural responses (Figure 2), indicating that *T. helgolandica* can detect a variety of light stimuli**. Whether the same is true for *T. planktonis* is unknown, but a recent study suggests that yellow bioluminescence might have evolved from blue[17]. If so, the blue-emitter *T. planktonis* may be unable to discriminate between blue and yellow wavelengths because it represents an evolutionary stage in which yellow sensitivity has not yet developed. **I therefore hypothesise that signals emitted by *T. planktonis* results in behavioural and/or bioluminescent responses from *T. helgolandica*, but not vice versa.**

Methodology: to determine if these two species respond differently to intra- vs. interspecies signals, I will perform two sets of experiments. **First, individuals will be exposed to artificial bioluminescent signals that only vary in colour** (using blue, and yellow-emitting LEDs mimicking the temporal signatures of each species' naturally occurring bioluminescence). Keeping all other signal properties identical, these experiments will otherwise be performed as in WP2. **Second, individuals will be introduced to members of the other species**. These experiments will be a continuation of the third set described in WP1 (where single animals are introduced to conspecifics), enabling a comparison of behaviours and interactions between individuals of the same (WP1) versus another tomopterid species (this WP). The experiments will therefore be performed in the same manner, including individuals of all maturation stages as it remains unknown how the developmental trajectories between the two species compare.

Expected outcomes and impact: results from WP3 will provide a comprehensive overview of behavioural responses to both artificial and actual members of another, related species for two *Tomopteris* annelids. These findings will determine if the two species can communicate with each other despite their differing bioluminescent colours and **provide important evidence for (or against) the hypothesis that yellow light signals mainly facilitate intraspecies interactions while blue light also contributes to interspecies communication**. The use of two related species also means that these findings will have a significant impact on theories concerning the evolutionary origin of bioluminescent colours.

Risks and their management: **low risk.** This WP shares the same risks as WP1 and WP2 as the experiments are similar. Given the low risk of those WPs and the measures described, I am confident that I will also be able to carry out the experiments in WP3 successfully.

WP4: Characterise the neuroanatomy of *T. helgolandica* and *T. planktonis*

Background and aims: the bioluminescent signals emitted by *T. helgolandica* have been shown to be under neural control[20], but our current knowledge of the tomopterid nervous system is very limited. Apart from some early morphological work describing their embryonic development[25] and eyes[30], there are studies from the 1800s that show that their parapodia and light organs are innervated by nerve fibres[18,19]. More recently, it has been shown that their brain consists of a central serotonergic neuropil surrounded by neuronal somata[31]. WP4 will benefit from my extensive experience with neuroscience methods in general, and with histological and imaging techniques in particular, to characterise the neuroanatomy of both *T. helgolandica* and *T. planktonis* in detail. This will enable me to **1) determine if these species also differ on the neuroanatomical level, and 2) provide an essential foundation for future work aiming to reveal the neuronal underpinnings of bioluminescent communication.**

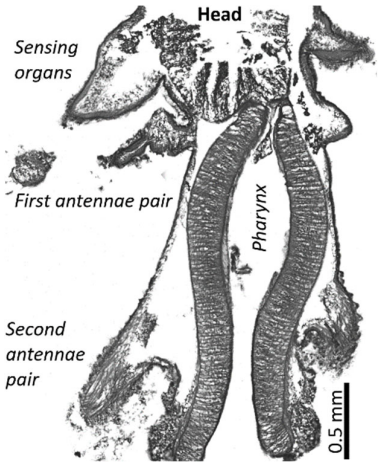

Figure 3: Horizontal section of *T. helgolandica*
20 µm section I stained with cresyl violet to visualise general anatomy.

Methodology: to achieve my aims, I will combine both traditional and cutting-edge techniques. **First, I will employ traditional methods with which I am an expert** and that I and others have found to work well in tomopterids. Here, individual specimens of both species will be preserved with a standard formalin-based fixation protocol and thin (10-40 µm) sections of their entire bodies made. These sections will be stained using histochemical and immunofluorescent methods (cresyl violet (preliminary data in **Figure 3**), DAPI[31] and NeuN) to enable gross characterisation of their nervous system layout. In addition, primary antibodies against specific neurotransmitter systems (serotonin, glutamate and GABA) combined with fluorescent secondary antibodies will be used to determine neuronal identities and their anatomical distribution. Brightfield (for non-fluorescent labels), epifluorescence (for fluorescent labels), darkfield (in conjunction with epifluorescence to visualise non-fluorescent structures), and confocal (to check for colocalization of labels) microscopy will be used to visualise anatomical features. **Second, I will use cutting-edge methods that enable neuroanatomy to be characterised in intact animals**. By using a newly described protocol that shows improved preservation of gelatinous organisms[32], the reduction in transparency associated with traditional fixation methods can be avoided. This protocol is also compatible with immunohistochemistry[32], which in combination with the exceptional transparency of tomopterids makes examinations with light-sheet microscopy ideal. Using the same molecular markers, this approach will complement the more traditional method, avoiding tissue distortions that can occur with sectioning and enable a **detailed characterisation of neuroanatomy in intact tomopterids for the first time.**

Expected outcomes and impact: the experiments in WP4 will provide a detailed neuroanatomical characterisation of two marine annelids using cellular and molecular markers. The obtained findings will elucidate to what extent these two related species differ on a neuronal level and provide important insights into the evolution of neurotransmitter systems. Further, they will **pioneer a new research line into the neuroscience of bioluminescence**. As such, the results obtained here will provide an essential foundation for future projects that aim to uncover the neurophysiological underpinnings of bioluminescent communication.

Risks and their management: **low to moderate risk**. The risks associated with the traditional approach are very low given my extensive experience with all the listed techniques and my preliminary data (**Figure 3**). In addition, previous studies show that common molecular markers and staining protocols frequently used in other animals can also be applied to tomopterids[31,33]. Some of the more sophisticated immunohistochemical approaches may need to be adapted to work with the new fixation method, but given my substantial experience with histological processing methods and the availability of detailed protocols[32], I am confident that these obstacles will be overcome. Should it prove to be too time consuming, however, then I will pursue the traditional approach in more detail instead (adding additional molecular markers to further differentiate neuronal subtypes). Light-sheet microscopy carries minimal risks as it is a widely used technique in systems neuroscience, a commercial system is in current use at the Kavli Institute for Systems Neuroscience (KISN), and I have over a decade worth of personal experience with a wide variety of microscopy techniques. Further, the most troublesome step in preparing samples for light-sheet imaging, tissue clearing, is of no consequence since our samples are already transparent. Should light-sheet imaging still prove problematic, then the transparency and small size of our samples means confocal and 2-photon microscopy are valid alternatives. State-of-the-art microscopes of both kinds are also readily available at KISN, and I am an expert user of both.

Summary
The results obtained under WPs 1-3 will address my first objective by providing some of the first experimental insights into how bioluminescent signals are used, how members of the same and a closely related species respond to them, as well as the significance of colour in bioluminescent communication. WP4 addresses my second objective by providing, for the first time, a detailed neuroanatomical characterisation of two related marine annelid species. The findings will have extensive implications for our understanding of bioluminescent signals and set an important and timely precedent for experiments in other species. This will be crucial to determine if my findings on bioluminescent communication and neurobiology generalise, and to increase the much-needed research effort into an ecosystem on which we are having an unprecedented impact.

General risks and their management

In addition to WP-specific risks and their management (detailed above), I have also considered more general risks. First, I need to collect animals to perform the proposed experiments. While there are variations in the abundance of *T. helgolandica* in the Trondheim fjord throughout the year, they are never entirely absent[34]. We also typically find both species in the same net samples, and with a unique and immediate vicinity to collection sites (each field trip totals 5-6 hrs), the risk of not gathering enough specimens is minimised. Second, behavioural experiments with wild, aquarium-housed animals means that consistent, long-term maintenance is important. With the dedicated facilities at NTNU SeaLab and their excellent infrastructure enabling a continuous supply of high-quality seawater, I am now able to keep specimens alive for up to 3 months. While this is more than sufficient for all planned experiments, I continually strive to further extend this timeframe in close collaboration with my collaborator, head engineer Dag Altin, to enable unprecedented opportunities for longitudinal observations of individual tomopterids. Third, I aim to track individuals in 3D, which is a technically demanding task. With expert support from my collaborator Assoc. Prof. Benjamin Dunn, the risk of not achieving this within a reasonable timeframe is reduced, but should it prove too time consuming then my current, functional 2D setup is still sufficient for collecting all the data I need. Fourth, there is limited precedence for behavioural experiments with bioluminescent organisms. However, my preliminary data (**Figure 1, Figure 2**), and one previous study[16] using *T. helgolandica*, clearly demonstrate the feasibility of performing behavioural experiments with tomopterids. In addition, my collaborator Prof. Jérôme Mallefet has extensive experience with bioluminescent organisms, including tomopterids, further minimising procedural risks. Fifth, with minimal methodological risks for completing the WPs, the main risks become conceptual: the behavioural responses observed in the lab may not be identical to those in the wild, leading to inconclusive results. I will minimise this risk by replicating the conditions observed in the wild as closely as possible, including fresh seawater collected from similar depths at which these annelids are found (part of NTNU SeaLab infrastructure) and using spacious aquariums with moderate currents to support their pelagic nature. Similarly, although *T. helgolandica* does not appear to display seasonal variations in maturation[25], the differences in relative abundance throughout the year might result in an alteration of bioluminescent signals, especially if they are used for intraspecies communication. I have therefore allocated time to perform the experiments in WP1 four times throughout one year to capture any seasonal variations that may exist. If such effects are found to be present, then the timing of remaining WPs will be adjusted accordingly, and these novel findings will be explored in separate project applications. Finally, although related, the individual WPs can be performed independently and do not fully depend on each other for success, further minimising the overall risk of the project.

Ethics

As this proposal aims to decipher bioluminescent communication, the use of animals is necessary. However, the marine annelids in this proposal are zooplankton and all experiments involve either non-invasive or terminal procedures. Ethical concerns regarding animal experiments are therefore minimal. Nonetheless, I will firmly comply with the "Three Rs" of humane experimental technique by using a simple animal model (replacement), the lowest number of animals possible by using individuals in several experiments (reduction), and optimise housing and handling conditions to minimise distress and enhance animal welfare (refinement).

1.3 Novelty and ambition

Behavioural studies involving bioluminescence are extremely rare because emitting organisms are often fragile and there is minimal precedence. This ground-breaking project will overcome these challenges by using relatively robust species whose suitability for the planned experiments has been demonstrated by my preliminary data (**Figure 1B, Figure 2**) and a previous study by my collaborator Prof. Jérôme Mallefet [16]. I will thereby gain direct and unprecedented insights into how bioluminescence is used that **go beyond morphological and physiological inferences**. Further, this project will **bridge two rarely overlapping fields**, marine biology and neuroscience. Hence, this constitutes a truly interdisciplinary research project that will shed light on a major ecological phenomenon and determine the roles that bioluminescence and colour plays in mediating inter- and intraspecies communication. In combination with neuroanatomical characterisations, I will **spearhead novel research lines that will reveal the evolutionary, behavioural, and neuronal origins of bioluminescent communication**. Finally, this project will **provide the essential foundation for my independent research career**. My ambition is to build on the proposed behavioural and neurobiological studies to **unravel the underlying neurophysiology of bioluminescent communication**: by leveraging my

extensive experience with state-of-the-art imaging methods, my future aim is to perform non-invasive, functional imaging of the tomopterid nervous system. This will allow me to reach my overarching ambition of taking my principal field, neuroscience, into the future by going back to where it all started: the ocean.

2. Impact

2.1 Potential for academic impact of the research project

This pioneering project will determine how bioluminescence and its colours mediate inter- and intraspecies communication through a range of sophisticated behavioural paradigms. Additionally, it will lead to important insights into the underlying neuroanatomy, which will lay the foundation for future neurophysiological work. While previous morphological and physiological studies of bioluminescence are valuable, they remain largely descriptive and cannot tell us how or why bioluminescence is used. Knowing that humans produce sound does not tell us what those sounds are for, let alone if they are important. **The ambitious behavioural experiments in this project (WPs 1-3) will be among the first in the field and help us finally decipher what bioluminescent signals mean and their importance for the organisms themselves**. In addition, by comparing two related annelid species, *T. helgolandica* and *T. planktonis*, this project will significantly contribute to our understanding of the evolution of both behaviour and that of the nervous system (WP4). By characterising the neuroanatomy of these species, **this project will also pave the way for a future application to the European Research Council (ERC) with a goal to establish, for the first time, *in vivo* imaging of neural function in non-transgenic gelatinous animals**. Using state-of-the-art techniques, my cross-disciplinary approach will advance both the fields of marine biology and neuroscience, providing novel insights into the behavioural significance of bioluminescence on the one hand, and its supporting neuroanatomical elements on the other. Further, this novel, interdisciplinary research line will have extensive implications for our understanding of nervous function in all species, whose nervous systems first evolved from the same habitat in which these marine annelids still live.

Measures for monitoring successful project impact include completion of 1) each WP, 2) the activities described in section 2.3 below, and 3) the additional milestones and deliverables listed in **Figure 4**.

2.3 Measures for communication and exploitation

Project results will be presented and discussed in several ways. This will include internal, departmental seminars and both general and more field specific national and international conferences. General conferences allow for my research to be communicated to a large and interdisciplinary scientific audience, while field specific ones enable more detailed discussions and insights. Specifically, I plan to present at the Society for Neuroscience (SfN) meeting, the Living Light (LL) meeting, the International Symposium on Bioluminescence and Chemiluminescence (ISBC), the International Congress on Neuroethology (ICN) and the Norwegian Research School in Neuroscience (NRSN) meeting (see **Figure 4** for anticipated calendar, subject to change with official release of conference dates). Other conferences of interest that I will consider on a case-by-case basis include the Federation of European Neuroscience Societies Forum, the European Marine Biology Symposium, the Deep-Sea Biology Symposium and the Association for the Study of Animal Behaviour meetings. I will make a data management plan at the beginning of the project period that complies with the FAIR principles for scientific data management: data, code and analysis will be made available in open repositories such as EBRAINS, the lab's own GitHub repository, and Figshare, and results will be published in open access articles. **I aim to publish 4 primary research manuscripts, all in non-profit journals such as eLife, Biology Open and PLOS journals to ensure the science is widely accessible and as a result has the largest possible impact**. I also plan to publish a review summarising the current state and outlook within the field of bioluminescent communication at the end of the project period. Press releases and the KISN annual report will also serve to highlight work and specific findings from my lab, and to communicate them to the general public. I will further communicate and disseminate my lab's work in an accessible manner to non-academic audiences through online platforms and physical events – efforts that have already resulted in the award of a major science communication prize for a piece I wrote about my bioluminescence research. Major outreach efforts will take place towards the end of each project year (**Figure 4**). This may include writing articles for Gemini and forskersonen.no, presenting during Forskningsdagene and The European Researchers' Night, and arranging workshops and lab tours associated with Brain Awareness Week and the UN Decade of Ocean Science initiatives. Last, but not least, my lab website and dedicated Twitter account is regularly updated and

will continue to be used to announce and describe my lab's activities to all audiences throughout the project period. All activities will be aided by the extensive and dedicated communication team at KISN.

3. Implementation

3.1 Project manager and project group

Project manager

My educational background and most of my research have been within the field of neuroscience. Inspired by notable neuroscientists such as Kandel, Hodgkin and Huxley, I firmly believe that marine life holds the key to many unanswered questions about the brain. Ever since the start of my undergraduate studies, my dedication to research has led me to seek out and participate in a large variety of research activities. More recently, this included volunteering at a shark conservancy that helped me familiarise myself with research methods in marine biology. I have also actively developed my leadership skills through courses and the many responsibilities I have been entrusted with (including being a nominated and elected representative for all temporary researchers at KISN). My aim has been to gain the knowledge, skills and experiences necessary to establish and lead my own research group and to take my principal field, neuroscience, into the future by going back to where it all began: the ocean.

After training in international world-leading research institutes with top scientists within the field of neuroscience and having mastered several cutting-edge techniques, I finally started making my dream a reality in 2019. Utilising the project management and leadership skills I have gained through several roles (see CV), most recently by leading a hugely ambitious 5-year research project with several team members to completion[35], I started contacting experts within marine biology, researched candidate organisms and unresolved questions in the field, and initiated national and international collaborations. With this foundation in place and the generosity of my collaborators, I began the first experiments with the organisms of my choice in August 2020 and at the same time launched the Marine Neuroscience Laboratory (MNL). Since then, **I have secured two equipment grants** for the lab, allowing me to rent and purchase essential services and equipment, **won a major science communication award** for my writing on bioluminescence research, gathered **considerable experience with behavioural experiments in tomopterid species** and received **several invitations to speak about this work**. In addition, **I actively strive to make science more open, fair, and sustainable** by leading by example: I use open-source research software (including switching to Python from Matlab), publish my code and major funding applications, publicly share the ups and downs of my research, and aim to only publish in open-access, non-profit journals. It is worth noting that **all my work with the MNL is done in my spare time and at my own initiative, while simultaneously employed and working full-time on my separate postdoctoral projects**, providing further testimony to my passion for research, project management experience and abilities. I also have **first-hand experience starting pioneering projects from scratch and completing them**: my main postdoctoral work has been to establish a method to identify the monosynaptic inputs to individual, birthdate defined, neurons in deep brain areas *in vivo*[35]. This had never been achieved before.

Funding from the Researcher Project for Scientific Renewal will allow me to take my dream one step further by allowing me to 1) continue to build the foundation upon which my research plan and future funding opportunities (e.g., from the ERC) rely, 2) elevate my lab from spare-time ambition to full-time endeavour, and 3) answer important, pressing questions in the field. Combined, these elements will help me to continue to establish myself as an independent researcher that bridges and leads both marine biology and neuroscience into novel, fruitful directions.

Project group

Apart from my personal full-time commitment to the project, a PhD student (in-kind donation from NTNU), a Postdoctoral Fellow and three collaborators whose expertise complement my own will contribute to it. As I have experience with all the techniques employed, I will directly supervise and train both the PhD student and Postdoctoral Fellow to perform the proposed experiments with me. The collaborators will provide expert advice at key stages of the project (see **Figure 4** for timeline): Prof. Jérôme Mallefet at UCLouvain has extensive experience with bioluminescent organisms, including performing behavioural experiments on tomopterids. He will provide insights when optimising the setups for bioluminescence imaging and designing artificial light stimuli for WPs 2-3. Head engineer Dag Altin at NTNU SeaLab is an expert on the culturing and maintenance of zooplankton. He will guide my efforts to further improve housing conditions for the animals and provide live prey for WP1. Assoc. Prof. Benjamin Dunn at the Department of Mathematical Sciences at

NTNU is an expert in (neural) data analysis and has considerable experience with building setups that enable 3D monitoring of behaviour. He will help extend my current 2D setup for WPs 1-3 once data collection for WP1 is well underway. Both the PhD student and the Postdoctoral Fellow will be recruited based on the interests and skills they bring to the lab, such as experience with behavioural experiments and analysis of complex (behavioural) data, histological methods, and/or marine invertebrates. Their positions will be advertised widely through my and my collaborators' networks to ensure they reach a wide and diverse audience, and the hiring process will be designed to reduce subconscious bias (e.g., by blinding applications in the initial round). My support of these team members will be tailored to their personal and career-related needs, to ensure that they receive the training and experience necessary to perform the project tasks at hand, as well as prepare them for the career step they would like to pursue next.

Network

In addition to the project group directly involved in the project, I will make use of my continuously expanding network of collaborators and supporters for guidance and assistance whenever necessary. This network currently includes, among others, additional experts on bioluminescence as well as experts on the collection, maintenance, and culturing of marine invertebrates. Further, I both seek and provide support in dedicated global online communities, including the NeuroMethods (>8000 members), FuturePI (>4500 members), and Deep-Sea Biology Society (>900 members) Slack communities.

Summary

I am confident that I have the leadership, technical and experimental expertise to carry out this pioneering project successfully, especially given the team of experts I have assembled to support and assist me.

3.2 Project organisation and management

Work plan and strategy

This project will last four years and consists of four complementary WPs (**Figure 4)**. Communication and dissemination measures outside the writing and publication of research manuscripts will take place throughout, with major outreach activities planned for the end of each project year (e.g., producing larger articles/videos/presentations and participating in events, see section 2.3 and **Figure 4**). In the first six months, I will acquire the necessary additional equipment and reagents, build setups, and recruit team members. A Postdoctoral Fellow will be recruited first to assist with the creation of analysis pipelines and data collection for WP1. A PhD student will start after one year and immediately be involved with data collection for WP1. Both will be trained by me and as the project progresses contribute to all WPs to ensure they gain a wide range of experience while considering their individual areas of expertise (e.g., the Postdoctoral Fellow will likely contribute more to analysis in the beginning unless the PhD student already knows a programming language). The building and optimisation of setups for WPs 2-3 is expected to be faster as they share commonalities with WP1 and all team members will be sufficiently trained by then. I will begin WP4 once data collection for WP1 is well underway, and train and involve my lab members when behavioural experiments are less time consuming (i.e., in between field trips). My collaborators will contribute as described in each WP-section and section 3.1, at the times indicated in **Figure 4**.

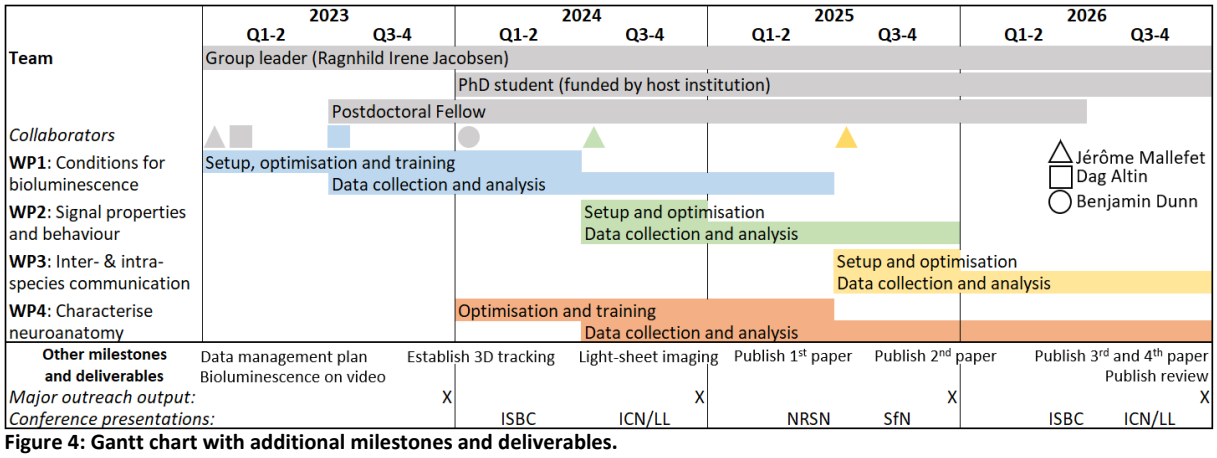

Figure 4: Gantt chart with additional milestones and deliverables.

See section 2.3 for descriptions of outreach and conference activities. X: timing of *major* outreach activity. Shapes: general (grey) and WP-specific (WP colour) contributions. ISBC: Int. Symposium on Bioluminescence and Chemiluminescence, ICN: Int. Congress on Neuroethology, LL: Living Light, NRSN: Norwegian Research School in Neuroscience, SfN: Society for Neuroscience.

Research infrastructure and management structure

The multidisciplinary nature of this project means that it will take place in two locations (within the same city) that are ideally suited for the planned experiments. Behavioural experiments (WPs 1-3) will be performed at SeaLab in the Faculty for Natural Sciences (NTNU): SeaLab has state-of-the-art facilities and technical expertise specifically tailored for marine biology research ranging from algae to fish. This includes a continuously monitored and quality-controlled seawater supply, pumped up from the fjord in which our species of choice live, and specialised climate-controlled rooms for housing a variety of organisms and conducting behavioural experiments. Neuroanatomical characterisations (WP4) will be performed at the Kavli Institute for Systems Neuroscience (KISN) in the Faculty of Medicine and Health Sciences (NTNU): KISN houses world-class core facilities and expertise for the advanced processing and imaging of neural tissue. In addition to modern equipment for sectioning and performing immunohistochemistry, an impressively large suite of sophisticated microscopes is available through NORBRAIN. This includes a new light-sheet microscope, with dedicated technical support. An affiliation with both places has already been well established through my postdoctoral work at KISN and independent collaboration with NTNU SeaLab.

The team will be managed by me, the project manager and group leader Ragnhild Irene Jacobsen. I will directly supervise the PhD student and Postdoctoral Fellow. This core, 3-person team will be supported by my collaborators Prof. Jérôme Mallefet, Assoc. Prof. Benjamin Dunn and head engineer at NTNU SeaLab Dag Altin, in addition to the technical and administrative units that are available at KISN and SeaLab.

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Summary of marks

Criterion	Mark
Excellence – potential for advancing the state-of-the-art	6
Excellence – quality of R&D activities	6
Impact	6
Implementation	6

Criteria

Excellence – potential for advancing the state-of-the-art

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The extent to which the proposed work is ambitious, novel, and goes beyond the state-of-the-art
• Scientific creativity and originality.
• Novelty and boldness of hypotheses or research questions.
• Potential for development of new knowledge beyond the current state-of-the-art, including significant theoretical, methodological, experimental or empirical advancement.

The panel much appreciated the importance of gaining insight into a topic that is relevant for more than 70% of marine animals. The panel appreciated the boldness of the approach to develop a new laboratory accessible model system. There is clearly the potential for development of new knowledge beyond the current state-of-the-art, although there might be the question for the future how this could be developed beyond a descriptive characterization. In this context the exact choice of the selected annelid species was discussed. It was not entirely clear, if there could be better suitable animal models (especially consider accessibility and potential future functional approaches). Furthermore, the approaches taken by the applicant could be better embedded into the scientific literature existing on bioluminescence in cephalopods.

Selected mark : 6 - Excellent
The proposal successfully addresses all relevant aspects of the criterion. Only minor shortcomings are present.

Excellence – quality of R&D activities

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The quality of the proposed R&D activities
• Quality of the research questions, hypotheses and project objectives, and the extent to which they are clearly and adequately specified.
• Credibility and appropriateness of the theoretical approach, research design and use of scientific methods. Appropriate consideration of interdisciplinary approaches.
• The extent to which appropriate consideration has been given to ethical issues, safety issues, gender dimension in research content, and use of stakeholder/user knowledge if appropriate.

The overall questions and approaches were well justified and clearly exhibit a noteworthy interdisciplinary component, spanning from behavioral neuroscience to behavioral ecology, photobiology and cell biology to engineering. The neuroanatomical work package may not provide the expected resolution to reach functional conclusions. Some of the neurotransmitter antibodies (GABA, Glu) may not work or would label very broadly the neuropil. It is not clear how cellular-level detail would be achieved with the proposed markers. Furthermore, how the neuroanatomy might help to inform the other work packages could be better explained.

Selected mark : 6 - Excellent
The proposal successfully addresses all relevant aspects of the criterion. Only minor shortcomings are present.

Impact

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Potential impact of the proposed research
• Potential for academic impact:
The extent to which the planned outputs of the project address important present and/or future scientific challenges.
• Potential for societal impact (if addressed by the applicant):
The extent to which the planned outputs of the project address UN Sustainable Development Goals or other important present and/or future societal challenges.
• The extent to which the potential impacts are clearly formulated and plausible.

Communication and exploitation
• Quality and scope of communication and engagement activities with different target audiences, including relevant stakeholders/users.

Given the widespread occurrence of bioluminescence in the oceans and the obvious lack of knowledge on its role and mechanisms underlying the processing in animal nervous systems, it is clear that the work will have a significant academic impact. However, the precisely expected impact could be described in more detail. Furthermore, the mid- to long-term impact would be more obvious, if there was a strategy communicated how (and which) technical developments might be realistically achievable with these annelid model systems.

Selected mark : 6 - Excellent
The proposal successfully addresses all relevant aspects of the criterion. Only minor shortcomings are present.

Implementation

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The quality of the project manager and project group
• The extent to which the project manager has relevant expertise and experience, and demonstrated ability to perform high-quality research (as appropriate to the career stage).
• The degree of complementarity of the participants and the extent to which the project group has the necessary expertise needed to undertake the research effectively.

The quality of the project organisation and management
• Effectiveness of the project organisation, including the extent to which resources assigned to work packages are aligned with project objectives and deliverables.
• Appropriateness of the allocation of tasks, ensuring that all participants have a valid role and adequate resources in the project to fulfil that role.
• Appropriateness of the proposed management structures and governance.

It was positively noted that the leading applicant has a very solid background in mammalian neuroscience and now aims at moving with this expertise into a novel and relevant biological field. The listed collaboration partners are very well chosen to complement potential expertise gaps of the main applicant. The technological expertise and animal facilities, including the infrastructure to collect and keep the animals are available and of very high level. This makes the proposal overall convincing.
The planned roles for the PhD student and postdoc and how they will interact with each other and the collaborators could be improved. More clarity there would have helped to assess whether the requested level of funding is appropriate.

Selected mark : 6 - Excellent
The proposal successfully addresses all relevant aspects of the criterion. Only minor shortcomings are present.