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Observations of *in situ* deep-sea marine bioluminescence with a high-speed, high-resolution sCMOS camera

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ABSTRACT

Observing and measuring marine bioluminescence *in situ* presents unique challenges, characterized by the difficult task of approaching and imaging weakly illuminated bodies in a three-dimensional environment. To address this problem, a scientific complementary-metal-oxide-semiconductor (sCMOS) microscopy camera was outfitted for deep-sea imaging of marine bioluminescence. This system was deployed on multiple platforms (manned submersible, remotely operated vehicle, and towed body) in three oceanic regions (Western Tropical Pacific, Eastern Equatorial Pacific, and Northwestern Atlantic) to depths up to 2500 m. Using light stimulation, bioluminescent responses were recorded at high frame rates and in high resolution, offering unprecedented low-light imagery of deep-sea bioluminescence *in situ*. The kinematics of light production in several zooplankton groups was observed, and luminescent responses at different depths were quantified as intensity *vs.* time. These initial results signify a clear advancement in the bioluminescent imaging methods available for observation and experimentation in the deep-sea.

1. Introduction

Marine bioluminescence has generated increasing interest among the scientific community in the past several decades, spanning the disciplines of comparative biology (*e.g.* Haddock and Case, 1999), biochemistry (*e.g.* Prasher et al., 1992), physiology (*e.g.* Contag and Bachmann, 2002), neuroscience (*e.g.* Martin, 2008), population dynamics (*e.g.* Moline et al., 2009), and naval applications (*e.g.* Fucile, 2002). This research began with terrestrial fireflies (Green and McElroy, 1956) and later expanded to readily accessible marine organisms, most notably bacteria (Bassler et al., 1993; Surette et al., 1999), copepods (Campbell and Herring, 1990), and cnidarians (Prasher et al. 1985). Whereas almost all major phyla of deep-sea zooplankton exhibit bioluminescence (Haddock et al., 2010), with new discoveries reported annually, only a handful of studies have been published on *in situ* marine bioluminescence. As a result, the deep-sea offers vast potential for future multidisciplinary discoveries related to bioluminescence.

The greatest limitation to observing marine bioluminescence *in situ* is technology. As early as 1955, photomultiplier tubes (PMTs) were employed to quantitatively measure light in the ocean from a single point (Boden and Kampa, 1957), a method still used today (Adrián-Martínez et al., 2014; Johnsen et al., 2014; Craig et al., 2015). In the past three decades, a number of studies utilizing Intensified Silicon Intensifier Target (ISIT) cameras were published (Widder et al., 1989; Robison, 1992; Priede et al., 2006). ISIT cameras are very sensitive, but are limited in resolution and retain residual images between frames, offering visually-pleasing images but making them less than ideal quantitative imaging platforms. More recently, image-intensified and electron-multiplied CCD cameras have been employed to measure extremely low light sources in high resolution (*e.g.* Johnsen et al., 2012; Craig et al., 2015). Scientific complementary-metal-oxide-semiconductor (sCMOS) cameras offer high sensitivity, low noise, wide dynamic range, and high-resolution, high-speed capabilities

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without image amplification (Coates et al., 2009; Fowler et al., 2009).

We hypothesize that applying sCMOS technology *in situ* offers the ability to study the kinematics of luminescent organisms at an unprecedented resolution, and by pairing this camera with light-stimulation methods, it may be possible to quantify the luminescence of the midwater community in both spatial and temporal scales. Here we present the results of cross-platform deployment of a sCMOS camera *in situ* to depths up to 2500 m, utilizing light stimulation to elicit bioluminescent responses in a exploratory fashion.

2. Methods

A Hamamatsu Photonics ORCA-Flash4.0 V2 sCMOS camera was chosen for its high dynamic range (16-bit), high quantum efficiency (72%), 2048×2048 resolution, and high speed (up to 100 fps). All recordings in this study were conducted at 30 fps using a global shutter. The camera was fitted with a Navitar 17 mm *f*0.95 lens and integrated into a 2500 m-rated housing (Prevco) with a flat acrylic viewport (Fig. 1). The entire system was run on 24 VDC provided from surface power, with a nominal power draw of 100 W. Due to the high data output of the camera, a solid-state computer (Copperhead from VersaLogic, Tualatin, OR) with a 1 TB RAID0 SSD drive array was included in the housing along with a water-cooling pump and focus/aperture control. All internal components producing light, such as LEDs, were blacked out inside the housing. The recorded 16-bit intensity images were processed with Matlab v. R2012a. The camera power and focus was controlled by an RS232 serial connection. Remote access via Ethernet was used to control the embedded computer. Images were collected using software either from Stanford Photonics (Palo Alto, CA) or using custom software LabView GUIs. Raw data was uploaded from the embedded SSDs via Ethernet upon recovery of the instrument.

Image processing to count lit 'blobs' and calculate Intensity Units (IU) in each frame is described in the following pseudo-code:

for each sequential image in a recorded movie;

Subtract minimum image intensity calculated from entire movie (dark field; average value, 472 AU);

Calculate maximum image intensity;

Subtract reference image frame from current image (composite mean of 100 dark field images recorded with no bioluminescent sources);

High image pixel intensity thresholding to discount strobe-lit frames;

Further thresholding of low-light image, with threshold defined by the standard deviation of the image added to mean image intensity;

Morphological opening of thresholded image to isolate and count regions larger than 2 pixels in diameter;

end.

Field sites for this study were chosen based on collaborative opportunity with several research expeditions. All recordings were made at night to eliminate the incidence of weak ambient light from the surface. Bioluminescent responses were induced using strobe light stimulation following methods first described by Neshyba (1967). Limited mechanical stimulation was induced when bioluminescence animals randomly came into contact with the viewport.

Initial deployment of the camera system was aboard a Triton 3k3 submersible as part of a biodiversity exploration expedition to the Solomon Islands in September 2013 (Fig. 2C). The camera was mounted on a forward-facing instrumentation frame so that recorded video matched what observers inside the submersible sphere viewed in real-time. All lights inside and outside the submersible were either turned off or blacked out with opaque tape. Light stimulation was done using a handheld Nikon Speedlight SB-700 strobe, set to produce a sequence of 5 flashes at 2 Hz. Several dives were conducted at night to depths up to 973 m, with two notable dives taking place approximately 22 km SE of Gizo Island and 6 km N of Mborokua Island, respectively. At Mborokua Island, a vertical transect through the water column was conducted via submersible with a depth station every 100 m down to the seafloor at 900 m.

A second research cruise, to the New England shelf break, was conducted in November 2014 (Fig. 2B). The camera system was attached to a 1-ton towed body equipped with power switching and DSL internet to the surface, run through a standard 0.322″ coaxial oceanographic wire. The viewport was oriented sideways to limit transverse water movement against it. Light stimulation was accomplished using a Vivitar Thyristor strobe placed inside a borosilicate instrument sphere, set to produce a sequence of 5 flashes at 2 Hz. Two deployments were conducted at night to 900 m at Veatch Canyon and Atlantis Canyon.

A third deployment took place onboard the ROV HERCULES in the Galapagos Islands region in June/July 2015 (Fig. 2A). The camera was mounted vertically pointing downwards into open water. Light stimulation was conducted using a pair of Ocean Imaging Systems 3831 strobes mounted 3 m forward, pointed at an oblique 45° angle towards the camera, and set to produce a sequence of 2 flashes spaced by a system cycle time of 2 s. Recordings were made opportunistically in the water column and on the seafloor, including at a 'black smoker' hydrothermal vent and a lower-temperature vent community dominated by *Riftia* sp. tubeworms.

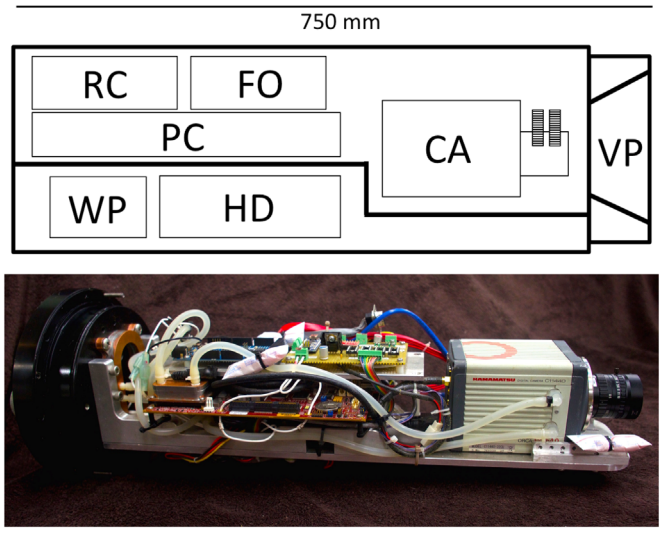

Fig. 1. (Top) Block diagram and internal assembly of subsea sCMOS camera system: RC=RS232 power/focus control; FO=fiber optic to Ethernet mux; PC=control computer; WP=cooling water pump; HD=RAID hard drive array; CA=camera head; VP=viewport. (Bottom) Internal view of camera system. The assembled subsea housing measures 75 cm long, 20 cm diameter, and has an approximate in-water weight of 18 kg.

3. Results

Over 200 15–30 s-length recordings were made at depths ranging from 30 m to 2500 m. Observed bioluminescence responses were most pronounced using repetitive flash stimuli; this was investigated on a submersible dive, when different flash characteristics were compared visually. At depths exhibiting a high

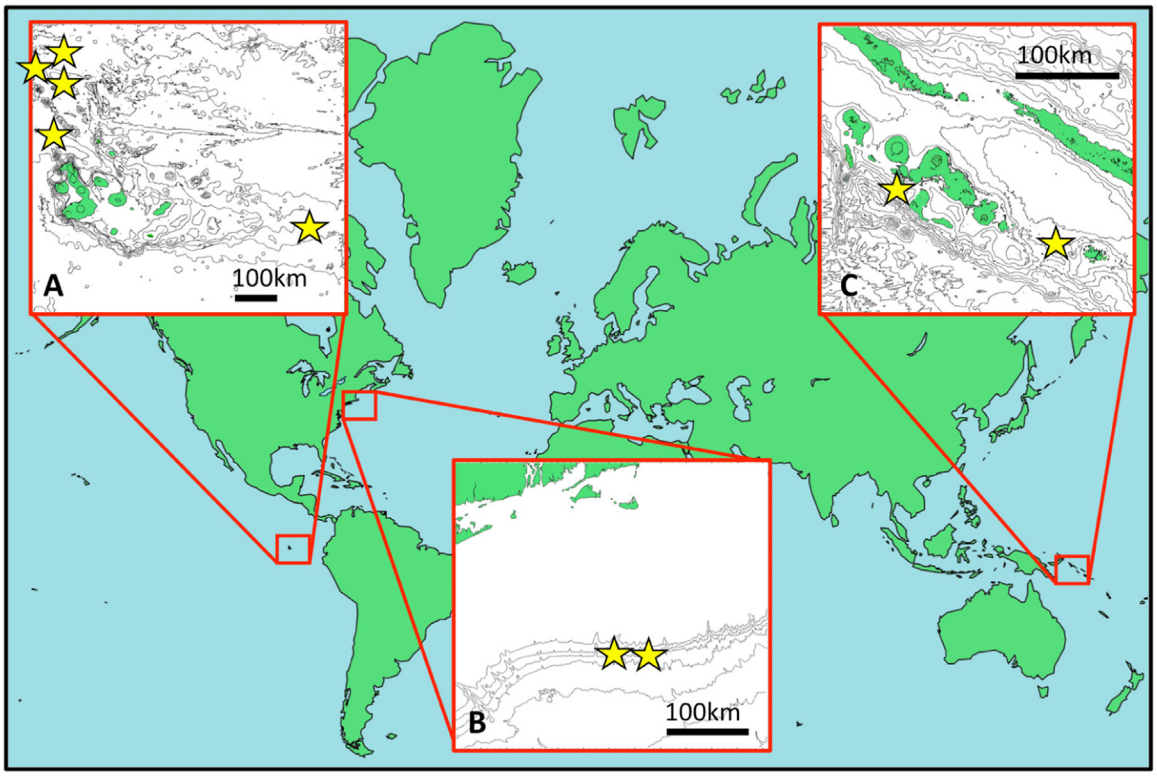

Fig. 2. Deployment sites (stars) for bioluminescent imaging in the Galapagos Islands (A), the New England shelf break (B) and the Solomon Islands (C). Bathymetry contours are spaced at 500 m.

bioluminescence response, human observers witnessed an omni-directional display that momentarily resembled a bright night sky (*i.e.* the "Milky Way"). In many cases, major zooplankton groups were recognized based on the shape and pattern of the emitted bioluminescence. Siphonophores, ctenophores, and fishes were among the organisms discernable by their light response in

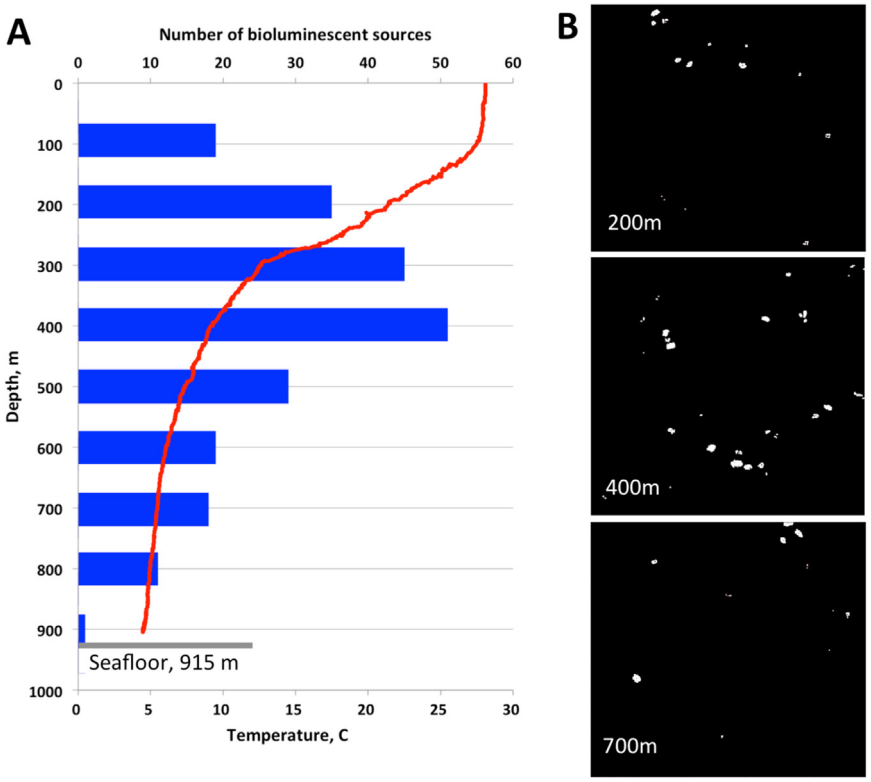

Fig. 3. A) Maximum counts of bioluminescent sources, or 'blobs' following light stimulation at 100 m-spaced depth levels at a station located 6 km N of Mborokua Island, Solomon Islands at approx. 22:00 – 23:30 local time. B) Representative images of maximum bioluminescent response at three depth stations shown in (A).

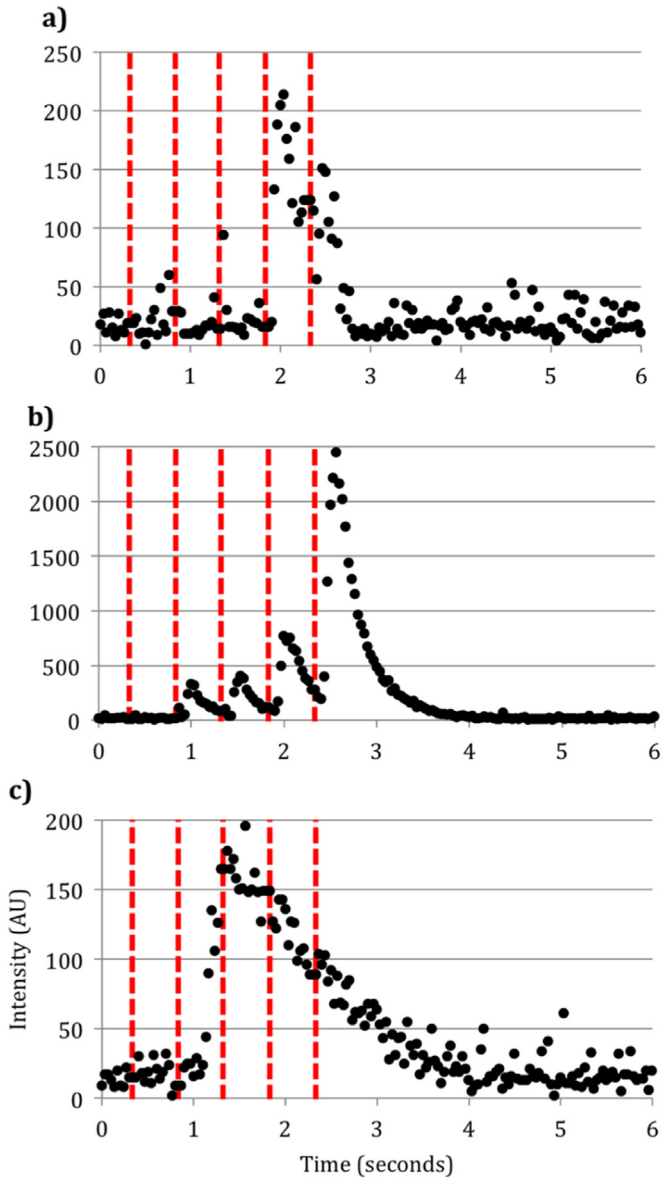

Fig. 4. Maximum image intensities, reported in arbitrary units (AU) *vs*. time, for whole image light-stimulated responses at a) 100 m, b) 200 m, and c) 400 m depths recorded from a manned submersible in the Solomon Islands in September 2013. Vertical dashed lines indicate strobe events.

otherwise complete blackness. Notably, light-stimulated bioluminescence responses on the seafloor were almost non-existent, and therefore layers of dense zooplankton populations in the water column were targeted for subsequent recordings.

Recordings of bioluminescence along a vertical cast in the water column illustrate the system's potential to measure light-stimulated bioluminescence as an assay of zooplankton population density (Fig. 3). Spatially, total bioluminescence exhibited an increasing trend with depth to a maximum at 400 m, followed by a steady decrease towards the seafloor. Typical bioluminescent responses at each depth were characterized by a sharp increase following the flash stimulation, rising to a maximum within 1 s followed by a complete decline within ~4 s (Fig. 4). Fig. 4A demonstrates a relatively short-lived bioluminescent response event of approximately 0.5 s that occurred after the fourth strobe event, while the response in Fig. 4B shows a discrete response after each strobe with increasing intensity. Fig. 4C is characteristic of many light-stimulated responses observed in this study, with a delay in luminescence of ~1 s following an initial strobe event and a gradual decline thereafter. In some cases, the decline in luminescence follows an exponential pattern (Fig. 4B) and in others a more linear regression was observed (Fig. 4A and C). Low levels of light measured before stimulus (< 50 AU) reflect a background signal on the image sensor itself.

Full-frame image sequences recorded at 30 fps demonstrate the system's ability to track light production on free-swimming pelagic animals (Fig. 5), offering the potential to gain new insight into the pathways that mediate bioluminescence in multicellular organisms. Two recordings of medusa show a swift response from swimming bell to tentacles, indicating the use of eyespots to sense light and a sequential reaction that dissipates quickly. In a spectacular video of a *Tomopteris* sp. pelagic polychaete, (Supplementary material), the organism displayed an elongated, > 2.5 s luminescent reaction initiating in the head and traveling outward to the parapodia. Several recordings of bioluminescence in *Cestum veneris*, the 'venus girdle' ctenophore, illustrate light traveling through the animal in a single wave (not shown). Many other patterns were observed, but are not presented due to the absence of positive identification of the organism.

4. Discussion

The three different deployment setups described in this paper allow for a comparison of methodology and recommendations for future research. The manned submersible was by far the most stable platform for observing midwater bioluminescence, and offered the unique opportunity to witness bioluminescent responses with the human eye. Phylum-level identification of several bioluminescent animals was possible, an effort that may be improved upon through repetitive application of this method. The use of a towed body with low-bandwidth telemetry produced poor results that were largely unusable, mostly due to the unstable nature of this setup. The use of a heave-compensated winch would likely absolve this issue, at least in moderate seas. ROV-based observations worked very well due to the stable nature of the decoupled platform and the high-bandwidth telemetry offered by the system. However, in both the manned submersible and ROV, the need to switch off all internal and external lighting hindered all other concurrent observations. While all of the deployment methods used in this study are viable for further development efforts, the authors envision using an autonomous vehicle to conduct light-stimulated bioluminescence surveys in high spatial and temporal resolution.

The video-based vertical profile of bioluminescence presented in Fig. 3 matches previous measurements of deep-sea zooplankton distribution (Banse, 1964; Weikert, 1982; Sameoto, 1986; Kosobokova and Hirche, 2000), roughly characterized by a maximum at 300 – 500 m followed by an exponential decline beyond depths > 1000 m. Our observed maxima of bioluminescence at 400 m may also represent a vertically migrating population of zooplankton as this dive was conducted in the early evening, just after sunset. Vertically migrating populations of major planktonic groups are known to rise from depths exceeding 700 m in the daytime to near-surface waters at night (Ringelberg, 2009); repeated vertical casts using this method over a 24-h period might produce results similar to that shown using net tows (Nishikawa and Tsuda, 2001), video plankton recorders (Ashjian et al. 2001), and acoustic analysis (Kringel et al. 2003). One other study has observed the vertical migration of plankton using bioluminescence as an indicator (Widder et al. 1992), and Gillibrand et al. (2007) report an extensive bioluminescent layer at ~1500 m occurring seasonally in the NE Atlantic Ocean. Depressed bioluminescence

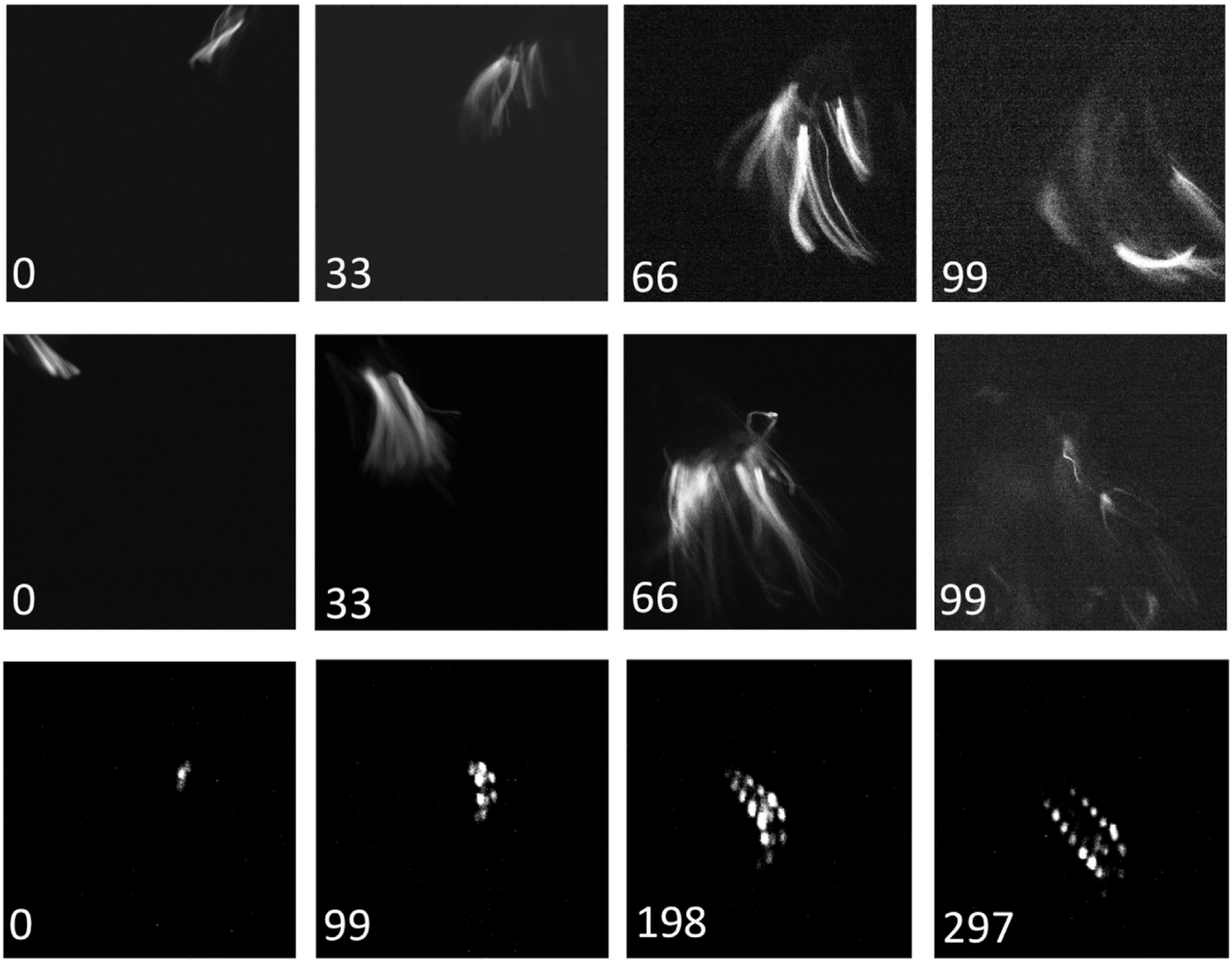

Fig. 5. Sample image sequences of *in situ* bioluminescence responses recorded at 30 fps. Time between frames is indicated in milliseconds in the lower left of each image. Top and middle row: tentacles from a hydromedusa show a distinct wave of bioluminescence traveling from the bell outwards and dissipating completely within 0.2 s, recorded at 700 m and 900 m depth (respectively) off the New England shelf. Bottom row: a *Tomopteris* sp. pelagic worm at 850 m in the Galapagos Islands, which exhibited a bright response for a total time of ~2.5 s.

responses approaching the seafloor corroborate similar observations in the Bahamas (Johnsen et al. 2012) and on the Mid-Atlantic Ridge (Craig et al. 2015).

In strict terms, these data represent the light-responsive bioluminescence community that exists in the water column. Because of the widespread prevalence of bioluminescence in deep-sea midwater species (with the exception of one major phylum, Tunicate), we therefore assert that the number of bioluminescent sources may be a proxy of total living biomass, at least among animals that are large enough to be imaged by the camera. The same line of comparison has been drawn with instruments using physical stimulation to elicit a bioluminescence response (*e.g.* Widder, 2002; Herren et al., 2005; Heger et al., 2008; Craig et al., 2015).

Varying patterns of response to individual stimulus events may represent the diversity of the bioluminescent community itself, a corollary also drawn by Dominjon et al. (2012) and Craig et al. (2015). We envision developing a library of bioluminescent response profiles based on intensity vs. time and shape, paired with animal identification, which could allow for automated classification based solely on light production measured passively using a camera system. Applying such a method spatially and temporally would address a major technical challenge in deep-sea biological oceanography that has resulted in a dearth of zooplankton distribution data (Wiebe and Benfield, 2003; Webb et al., 2010; Sutton, 2013). It may also produce the resolution needed to resolve zooplankton population patchiness at critical time and space scales (Benoit-Bird et al., 2013; Benoit-Bird and McManus, 2014).

The utilization of light stimulation to produce a bioluminescent response offers a unique advantage over mechanical, electrical and chemical stimulation techniques. In dinoflagellates, mechanical stimulation results in the deformation of cell membranes, initiating a cascade of reactions within the unicellular organism (Latz and Rohr, 1999; Cussatlegras and Gal, 2005). Larger animals, such as gelatinous zooplankton and fishes, may also exhibit a bioluminescent response based on the location and magnitude of the physical and/or electrical disturbance (*e.g.* Barnes and Case, 1974; Herring and Widder, 2004). Chemical stimulation is typically achieved by injecting a solution into laboratory aquaria, resulting in a widespread stimulus unlikely to be encountered in a natural setting. As such, luminescent responses in relation to an organism's neural/sensory network are poorly understood. Light stimulation induces bioluminescence with no mechanical or chemical invasion, and may be the least intrusive method for eliciting a 'natural' response from animals *in situ*.

The high-speed global shutter capability of the sCMOS camera offers a new window into the kinematics of bioluminescence. Our observation of the *Tomopteris* sp. polychaete supports the conclusions of Gouveneaux and Mallefet (2013) who conducted laboratory experiments on *T. helgolandica* and witnessed neural

Table 1
Reference list of studies observing bioluminescence *in situ*.

	Sensor	Citation	Depth (m)	Observation platform
Mechanical stimulation	Human observer	Beebe et al. (1934)[a]	0–660	Bathyscaphe
		Galt et al. (1985)	Surface water	SCUBA diver
	Photomultiplier tube	Boden and Kampa (1957)	0–300	Vertical profiler
		Hardy and Kay (1964)	62	Vertical profiler
		Bradner et al. (1987)	4300	Vertical profiler
		Webster et al. (1991)	4500	Vertical profiler
		Amram et al. (2000)	~2350	Moored array
		Widder et al. (1999)	0–250	Vertical profiler
		Herren et al. (2005)	0–30	Muiltplatform
		Moline et al. (2009)	0–40	AUV
		Tamburini et al. (2013)[a]	~2350	Moored array
		Johnsen et al. (2014)	20	Vertical profiler
	ISIT camera	Widder et al. (1989)	0–600	Submersible
		Robison (1992)	n/a	Submersible
		Widder and Johnsen (2000)	0–200	Submersible
		Widder et al. (2005)	600	ROV, benthic lander
		Priede et al. (2006)	0–4000	Vertical profiler
		Gillibrand et al. (2007)	0–4800	Vertical profiler
		Heger et al. (2008)	0–3000	Vertical profiler
		Craig et al. (2010)	0–5000	Vertical profiler
	I^2CCD (image-intensified CCD)	Craig et al. (2011)	1500–2700	Benthic lander, ROV
		Craig et al. (2015)	0–2500	Vertical profiler
		Johnsen et al. (2012)[a]	1000	Submersible
	ebCMOS camera (electro-bombarded CMOS)	Dominjon et al. (2012)	2500	Moored array
Light stimulation	Human observer	Lapota et al. (1986)	0–600	Submersible
	Photomultiplier tube	Neshyba (1967)	700	Profiler
	sCMOS camera	This study	0–2500	Submersible, ROV and vertical profiler

[a] These studies did not actively stimulate bioluminescence. Their results may result from naturally occurring mechanical stimulation (*i.e.* currents causing collisions) or may be from animals actively producing bioluminescence with no external stimulation.

control of bioluminescence under contrived conditions. In several recordings of siphonophores, most of the organism becomes luminescent at the same time, which correlates with previous work on a siphonophore species that exhibited no coordinated response to mechanical stimulation (Freeman, 1987). Without a size scale, velocities cannot be strictly calculated, but the image sequences presented here illustrate the potential of the system for future research in this area. Several methods could be employed to resolve scale such as utilizing stereo cameras (*e.g.*, Rife and Rock, 2001), scaling lasers, or simply constraining the volume being imaged to minimize the depth of field. The stereo method is particularly attractive, since it would allow volume-pixel regions or 'voxels' to be constrained and characterized.

A comprehensive review of published literature presenting *in situ* observations of bioluminescence is given in Table 1. While the human eye can still outperform the best digital cameras in terms of sensitivity, dynamic range and resolution, and PMTs have long been able to sense single photons, the inability to record imagery is an obvious drawback to using these methods. The electron-bombarded CMOS camera used by Dominjon et al. (2012), while likely the most sensitive instrument ever used to record bioluminescence *in situ*, does not collect focused imagery and is therefore categorized as similar to a PMT device. The defining feature of sCMOS technology is achieving high quantum efficiency (QE) and low readout noise without electron multiplication or image intensification. This distinction makes direct comparison of sCMOS cameras to electron multiplier CCD cameras (EMCCD) and image intensified CCD cameras (I^2CCD) not straightforward, and best quantified by empirical studies (*e.g.* Fullerton et al., 2012; Beier and Ibey, 2014). While contemporary EMCCD cameras still report higher quantum efficiencies (*e.g.* Andor iXon 2015 series at $>90\%$ QE) compared to the most advanced sCMOS cameras (*e.g.* Hamamatsu ORCA-Flash4.0 2015 model at $>82\%$ QE), the electron multiplication process can dramatically reduce the effective QE of EMCCD cameras (Long et al., 2012). Given the upward trajectory in sCMOS QE performance combined with their characteristic high speed and resolution capability, sCMOS cameras have the potential to become the benchmark of bioluminescence imaging technology.

Regardless of the imaging sensor employed, most *in situ* bioluminescence research has relied exclusively on mechanical stimulation to elicit a response. For example, Herren et al. (2005), Moline et al. (2009), and Johnsen et al. (2014) used essentially the same excitation chamber/PMT instrument; Widder et al. (1989), Widder and Johnsen (2000), Widder et al. (2005), and Johnsen et al. (2012) used similar 'splat-screen' approaches; and Priede et al. (2006), Heger et al. (2008), Craig et al. (2010, 2011, 2015) use the 'splat-screen' method for their vertical profiling work. By far the most comprehensive investigation into light-stimulated bioluminescence was conducted by Neshyba (1967), who measured responses using PMTs. Notably, Buskey and Swift (1985) performed a series of light-stimulation experiments on copepods and euphausiids, but these were conducted in a laboratory environment. Lapota et al. (1986) conducted a vertical dive in a manned submersible similar to that in this study, but bioluminescence was not recorded beyond written observation. Therefore, we believe these data presented herein represent the only published imagery of the phenomenon *in situ*.

5. Conclusions

Marine bioluminescence research has resulted in transformative applications to a wide range of medical, military and industrial disciplines (*e.g.* Shimomura et al., 1962), and the potential for important new discoveries is high. The diversity of light-producing organisms is remarkable, and observed in every ocean from shallow to deep seas. Despite the ubiquity of bioluminescence among marine taxa, the phenomenon is categorically underexplored and limited by the technology available to observe it *in situ*. To address this, an sCMOS camera was deployed on multiple oceanographic platforms in three different oceanic regions to explore bioluminescence *in situ*. Using light stimulation to elicit responses, counts

of bioluminescent sources were compared against water depth, demonstrating a potential new method to assess midwater zooplankton populations. High-resolution, quantitative measurements of light intensity responses were observed to vary greatly among depth zones and individual organisms. The kinematics of bioluminescence was imaged among several animals at a high frame rate and resolution, presenting new visualizations of how marine animals produce light. While this study demonstrates the utility of light stimulation for eliciting bioluminescent responses, it also highlights the current lack of scientific knowledge on the subject; further work is needed to identify which organisms respond to light stimulation and varying light intensities, patterns, and wavelengths. Future work should focus on pairing spatial and temporal observations of light-stimulated bioluminescence with traditional-method biomass assessments, in a true effort to estimate total living biomass. Advanced control software will lead towards support onboard autonomous vehicles and other self-powered platforms, and may allow for bioluminescent surveys to be conducted in high spatial and temporal resolution. We conclude that sCMOS cameras paired with light stimulation can offer a wide range of new applications in bioluminescence research.

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Appendix. Supporting material

Supplementary material associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr.2016.02.012.

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