Chapter 11

# Luminescence Dating: Applications in Earth Sciences and Archaeology

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Additional information is available at the end of the chapter

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**Abstract**

Over the last 60 years, luminescence dating has developed into a robust chronometer for applications in earth sciences and archaeology. The technique is particularly useful for dating materials ranging in age from a few decades to around 100,000–150,000 years. In this chapter, following a brief outline of the historical development of the dating method, basic principles behind the technique are discussed. This is followed by a look at measurement equipment that is employed in determining age and its operation. Luminescence properties of minerals used in dating are then examined after which procedures used in age calculation are looked at. Sample collection methods are also reviewed, as well as types of materials that can be dated. Continuing refinements in both methodology and equipment promise to yield luminescence chronologies with improved accuracy and extended dating range in the future and these are briefly discussed.

**Keywords:** luminescence dating, thermoluminescence (TL), optically stimulated luminescence (OSL), paleodose, dose rate, earth sciences, archaeology

## 1. Introduction

Luminescence dating refers to age-dating methods that employ the phenomenon of luminescence to determine the amount of time that has elapsed since the occurrence of a given event. In this chapter, the application of luminescence techniques in dating geological and archaeological events is examined. Generally, the term luminescence dating is a collective reference to numerical age-dating methods that include thermoluminescence (TL) and optically stimulated luminescence (OSL) dating techniques. Other terms used to describe OSL include optical dating [1] and photon-stimulated luminescence dating or photoluminescence dating [2]. Luminescence dating methods are based on the ability of some dielectric and semiconducting

materials to absorb and store energy from environmental ionizing radiation. In earth sciences and archaeological applications, the dielectric materials are usually minerals such as feldspar and quartz. The materials are also sometimes referred to as dosimeters [3]. Environmental ionizing radiation for earth science and archaeological applications typically comes from radioactive elements within the immediate surroundings of the mineral grains as well as from cosmic radiation. When the minerals are stimulated, they release the stored energy in the form of light, from which the term luminescence is derived. Stimulation of energy release by heating is referred to as TL. When light is used, on the other hand, the technique is described as OSL. Measuring the amount of energy released in conjunction with a determination of the rate at which the energy was accumulated allows an age to be calculated, indicating time that has elapsed since the storage of energy began. Luminescence methods can generally be used to date materials that range in age from a few decades to about 100,000 years. However, ages of up to several hundred thousands of years have been reported in some studies [4]. Therefore, the method can be used for dating events of Late Pleistocene and Holocene age (ca. < 126 000 years). Radiocarbon dating is a technique that can also be used to date some materials of Late Pleistocene or Holocene age if they bear carbon. Hence, when dealing with materials that do not contain organic carbon, luminescence dating can serve as an optional chronometer to radiocarbon dating. Furthermore, the age range that can be dated using luminescence techniques is greater than that of radiocarbon dating, and this provides researchers with a viable alternative dating method.

This chapter presents a brief examination of how luminescence is used in earth sciences and archaeology to measure time. In both fields of study, the imperativeness of assigning a temporal scale to events and processes is an inherent aspect of the discipline. Hence, the role played by dating cannot be overstated. The chapter is not intended to be used as a practice manual. Rather, the aim is to provide a primer that acquaints scholars who may be familiar with the science of luminescence but are not accustomed to its application as a dating method. Thus, the chapter comprises eight sections. Following an introductory look at the development of luminescence dating in Section 1, principles of luminescence dating are examined in Section 2. In Section 3, basic luminescence measurement equipment and sample stimulation mechanisms during measurement are explored after which luminescence properties of some common minerals are examined. The determination of parameters used in the luminescence age equation is discussed in Section 5, and this is followed by a look at materials that can be dated with luminescence methods in Section 6. Section 7 looks at methods used in sample collection and preparation prior to the analysis. In Section 8, the chapter concludes with a look at future developments in luminescence dating.

### 1.1. Historical development of luminescence dating

The ability of some minerals to luminesce when stimulated is not a recent finding. The earliest recorded observation of the behavior in minerals has been attributed [5] to Robert Boyle, who in 1664 recounted his discovery [6] that a diamond that had been loaned to him could emit light when heated. Subsequently, Boyle would describe the phenomenon as 'self-shining' [7]. Throughout the eighteenth, nineteenth and early parts of the twentieth centuries a lot of activity went into examining the effects of TL such that by the middle of the twentieth century,

physicists were fairly familiar with the phenomenon. The focus of this section, however, is not on the history of the science of luminescence but rather on the history of the emergence of the application of luminescence as a dating technique for geological and archeological materials. That inception can be traced back to approximately seven decades ago, to a period during which experiments were being conducted into applications of TL [8, 9]. Zeller et al. [10] noted that research into applying TL to determine geological ages of rocks using minerals began at the University of Wisconsin around 1950. Progress in dating geological materials, however, was hampered by a limited understanding of the luminescence process in rocks and accurate ages were hard to come by [11]. It is in the dating of heated archaeological artifacts that progress was realized. In 1953, Daniels et al. [9] had proposed that TL from ancient pottery could be used to determine its antiquity [12]. The idea was premised on the recognition that heating the pottery to high temperatures (>500°C) during production had the effect of erasing any previously accumulated energy from constituent mineral grains. Subsequently, Kennedy and Knoff [13] provided some basic aspects of dating heated archaeological materials using TL, though the approach had not been tested on actual samples as yet. Around the same time, Grogler et al. [14] provided additional experimental details on how a dating experiment measuring TL of pottery could be conducted. It was in 1964 that Aitken et al. [12] designed a study that sought to determine the relationship between TL glow of an artifact and its archaeological age. Results demonstrated that the luminescence intensity emitted by the samples was linearly proportional to radiocarbon ages of sediments from which the samples were obtained. The positive results noted in these early studies gave impetus for further refinements in TL protocols, and throughout the rest of the 1960s and 1970s, the dating method gained a foothold in archaeological studies.

A major development in luminescence studies occurred when TL dating was extended to determining burial ages of unheated sediments. The evolution appears to have followed two parallel paths, one in the West and another in former Eastern Bloc countries. However, it seems there was minimal interaction between the two geographical regions, especially in the early stages. In the West, some of the earliest work includes a study [15] that looked at TL signals of deep-sea sediment that mostly comprised foraminiferal shells. The investigators [15] considered the signals to be from calcite and noticed that the TL intensity increased with depth. Later, another study [16] investigated a deep-sea sediment core that comprised predominantly siliceous plankton and reported results similar to those presented earlier [15]. Subsequent studies by Wintle and Huntley [17] provided additional TL data from deep-sea sediments that also showed increasing signal intensities with depth. However, it was later observed [17] that the TL signals from deep-sea cores actually came from detrital minerals that were mixed with the plankton. Significantly, it was also suggested [17] that what was being dated was the last time the ocean sediments had been exposed to sunlight. Though it was some time before researchers fully understood the zeroing mechanism for unheated sediment [18], that discovery was extremely important for sediment dating since it meant that the exposure of sediments to sunlight had the same zeroing effect of accumulated energy in sediment grains as did heating in pottery.

In former Eastern Bloc countries, the analysis of unheated terrestrial sediments using TL appears to have begun sometime during the 1960s. Early published reports from the former

Soviet Union include a study [19] that examined TL signals of Quaternary deposits. A few years later, Morozov [20] presented relative ages of Quaternary sediments from Ukraine that had been dated using TL methods. The study was mainly based on the recognition that luminescence signal intensities increased with depth, which was interpreted as commensurate with age. Morozov [20] also suggested that the signal was coming from quartz in the sediments. Shortly afterwards, Shelkoplyas [21] reported a range of Quaternary TL ages obtained from soils and loess deposits [18]. Throughout the 1970s, researchers in Eastern Bloc countries [22–29] as well in China [30] reported studies in which TL was used to date Quaternary deposits. The accuracy of some of these early ages, however, is questionable [18], not least because zeroing mechanisms were not well-understood at the time. In other studies, efforts were directed at understanding TL characteristics of dosimeters, especially quartz [31–33].

Around the time Wintle and Huntley [17] discovered that TL signals in their deep-sea cores were coming from detrital mineral grains mixed with the plankton, they became aware of the work by Eastern Bloc researchers who had worked extensively with terrestrial sediments. Ultimately, they realized that TL dating could be applied much more broadly to date Quaternary deposits [18]. These developments resulted in the landmark publications by Wintle and Huntley [17, 34, 35] in which TL dating of sediments was outlined. With increased research throughout the early 1980s, dating procedures improved as efforts were made to standardize procedures [18]. However, optimal conditions for solar resetting of sediments remained unclear to researchers and this hampered the accuracy of TL ages. Researchers who examined the problem include Huntley [36] who investigated solar resetting of sediments from various environments and proposed a method to address the issue of inadequate zeroing. By 1985, another monumental step in luminescence dating would be realized when

| Authors | Topic | Source |
|---|---|---|
| Murray and Olley (2002) | Luminescence dating of sedimentary quartz | [40] |
| Feathers (2003) | Luminescence dating in archaeology | [41] |
| Lian (2007) | Optically stimulated luminescence dating | [42] |
| Duller (2008) | Luminescence dating guidelines for archaeology | [43] |
| Fuchs and Owen (2008) | Luminescence dating of glacial sediments | [44] |
| Preusser et al. (2008) | Luminescence dating: principles and applications | [45] |
| Rittenour (2008) | Luminescence dating of fluvial deposits | [46] |
| Singhvi and Porat (2008) | Luminescence dating in geomorphology | [47] |
| Wintle (2008) | History of luminescence dating over the last 50 years | [39] |
| Wintle (2008) | Luminescence dating: history and prospects | [3] |
| Rhodes (2011) | Luminescence dating of sediments | [48] |
| Liritzis et al. (2013) | Luminescence dating in archaeology | [49] |

**Table 1.** Recent review studies examining luminescence dating and its applications.

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Huntley et al. [11] reported that a light source could be used to stimulate energy release from a dosimeter during measurement instead of heating as was used in TL. This led to the development of OSL dating which offered a number of advantages over TL methods when dating unheated sediments. With further equipment and methodological refinements, there was a burgeoning of OSL dating studies of Quaternary sediments throughout the 1990s that saw luminescence dating emerge as a robust dating technique. Over the last two decades, the technique has developed further [37–39], and today, it is the method of choice for dating detrital sediments of Late Pleistocene and Holocene age as well as previously heated archaeological artifacts. **Table 1** presents some recent review studies that have examined various aspects of luminescence dating and its applications.

## 2. Principles of luminescence dating

### 2.1. Luminescence behavior of dielectric materials used in dating

Many minerals such as quartz, feldspar, calcite and zircon are dielectric materials and, when subjected to ionizing radiation, they are able to store energy in their crystal lattices. In natural geological and archeological settings, the ionizing radiation emanates naturally from the immediate surroundings of the minerals. Cosmic radiation may also contribute a small component. If the minerals used in dating are stimulated, they release the energy by luminescing and, within certain constraints, the energy released is proportional to the stored energy.

### 2.2. Luminescence dating equation

In luminescence dating, the energy given out by the minerals or dosimeters following stimulation is measured using appropriate instrumentation. This energy is referred to as the paleodose [50]. In order to determine an age, the rate at which the energy was accumulated by the dosimeter, or the dose date, is also ascertained. The quotient of the paleodose and the dose rate, as indicated in Eq. (1), is the luminescence age.

$$Luminescence\,age = \frac{Paleodose}{Dose\,rate} \tag{1}$$

If the mineral grains were emptied of all previously accumulated energy prior to the latest energy storage episode, the age obtained will denote time that has elapsed since the start of that episode. Hence, both in geology and archaeology, the luminescence age simply connotes time that has passed since the occurrence of a specific energy zeroing event. In geology, this might be a geomorphic event that exposed sediment to sunlight. Zeroing by sunlight is also sometimes referred to as optical bleaching [3]. In pottery, zeroing would normally occur during a firing event associated with the manufacture.

### 2.3. Electron trapping mechanisms

Mechanisms by which minerals store energy in their crystal lattices as a result of ionizing radiation are complex [50–52]. However, it is thought that ionizing radiation drives mineral

crystals into a metastable state where electrons are displaced from their parent nuclei. The positions from which the electrons have been evicted act as holes. The electrons and holes then diffuse within the mineral crystals and become trapped separately at lattice defects. Examples of common defects include a negative ion missing from its lattice position, a negative ion positioned in an interstitial site or the presence of impurity atoms in the lattice through substitution [52]. Other more complex trap types exist [52]. **Figure 1** depicts an energy level diagram that is used to visualize the trapping mechanisms involved in luminescence in crystalline materials. The depth of the trap (T) below the conduction band, indicated by 'E' (**Figure 1**) is a reflection of the efficacy of a given trap. Stable traps are those that can withstand perturbations such as lattice vibrations that could dislodge the electrons from their traps. If the crystal lattice is stimulated using an appropriate mechanism, for example, by heating to an adequately high temperature or by exposure to an optical source with a suitable wavelength, trapped electrons will be evicted out of the traps. Once evicted, the electrons diffuse within the crystal lattice until they reach a site that is attractive to electrons. Such sites are referred to as recombination centers. Some recombination centers emit energy in the form of light when they capture electrons. Where stimulation is conducted by heating, the effect would be referred to as TL. When stimulation is by optical means, OSL will be obtained. The diffusion of evicted electrons to their recombination centers occurs fairly rapidly to the extent that the time between stimulation and recombination can be viewed as instantaneous. Effective recombination centers are usually those sites in the lattice where electrons are missing. These are the holes created when the materials are exposed to ionizing radiation (**Figure 1a**). The intensity of the luminescence that is obtained when a material is stimulated is proportional to the number of electrons that are trapped in the material which, in turn, is commensurate with the energy absorbed from the ionizing radiation [50, 51]. However, despite the energy

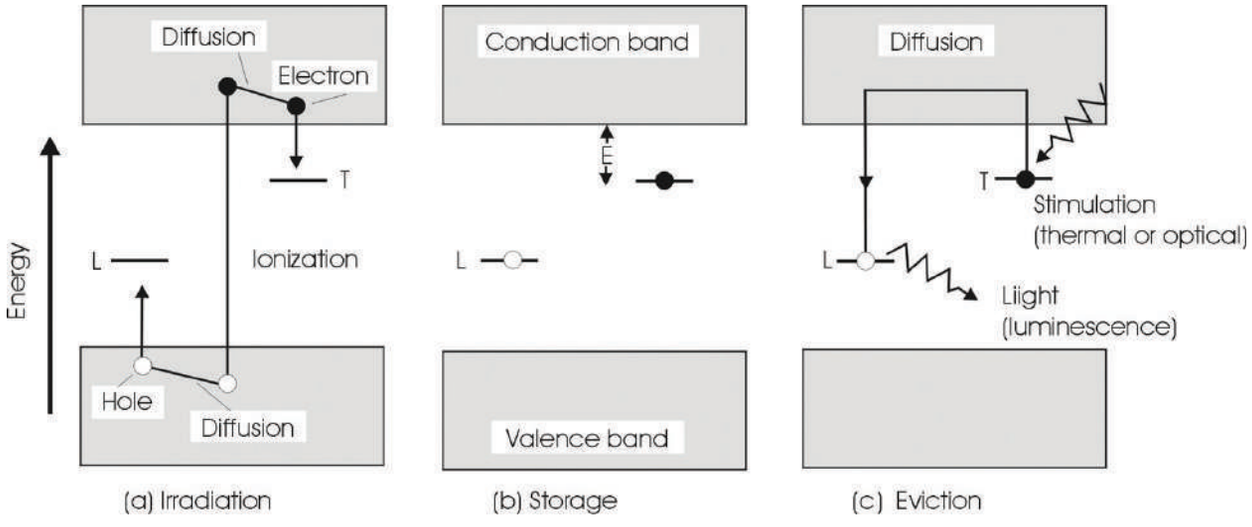


**Figure 1.** An energy level diagram that illustrates how ionizing radiation creates luminescence centers in crystal lattices (redrawn from Ref. [50]). (a) Following irradiation, electrons are expelled from their original sites leaving holes behind. Both electrons and holes diffuse within the lattice. (b) At appropriate sites, electrons are trapped while holes may become localized. (c) Thermal (TL) or optical (OSL) stimulation of the material results in electrons being evicted from the traps. Evicted electrons that reach luminescence centers result in light being emitted.

storage mechanism being the same for a given mineral, the sensitivity to radiation may vary greatly between samples, an aspect that has important implications for dating procedures as will be shown later.

Importantly, for dating purposes, the number of electron traps within any mineral lattice is finite. As a result, when minerals are exposed to ionizing radiation for an extended period, the traps become exhausted such that energy can no longer be stored efficiently. This effect is referred to as saturation. In dating, saturation determines the upper limit beyond which samples cannot be dated using luminescence techniques. Materials that are subjected to very high dose rates will have the number of traps exhausted more rapidly such that the specific age representing the upper age limit will depend on both the number of traps present as well as the dose rate.

### 2.4. Natural sources of ionizing radiation

In geological and archaeological dating applications, natural sources of ionizing radiation that contribute to the trapped energy in mineral grains include isotopes of uranium ($^{238}$U and $^{235}$U) and thorium ($^{232}$Th) decay chains, potassium ($^{40}$K) and rubidium ($^{87}$Rb). The elements are found in natural materials in very low concentrations (about 3–10 parts per million for uranium and thorium and less than 5% for potassium, where $^{40}$K is one part in 10,000). Despite the low concentrations, these radioactive isotopes collectively emit enough radiation to induce luminescence that is detectable for dating purposes. The radiation emitted includes alpha and beta particles as well as gamma radiation. Beta particles and gamma rays have penetration ranges of about 0.02 cm and 20 cm in earth materials, respectively, whereas alpha particles penetrate about 0.02 mm [51].

An additional though smaller radiation component received by earth materials comes from cosmic radiation. Cosmic rays from outer space consist of a soft and a hard component. On earth, surface substrate absorbs the soft component such that it cannot penetrate deeper than 50 cm. The hard component, however, largely comprising muons, penetrates deeper and is lightly ionizing. Hence, only the hard component is relevant to luminescence dating. On earth, the intensity of the hard component is also influenced by both latitude and altitude. Special formulae for evaluating cosmic ray contribution to dose rate have been developed for luminescence dating [53].

## 3. Basic luminescence measurement equipment and sample stimulation mechanisms

For dating studies, the primary aim of TL and OSL measurements is to determine the amount of energy that has been stored in the mineral grains of a given material since the start of the event that is being investigated. As outlined above, two main methods are used to stimulate energy release in luminescence studies. Heating allows TL to be measured, whereas stimulation using a light source is used for OSL measurements.

### 3.1. The luminescence reader

The basic layout of equipment used to measure luminescence in geological and archaeological dating is shown in **Figure 2**. Modern luminescence dating systems commonly possess both TL and OSL measurement capabilities [54, 55]. To conduct a measurement, samples are usually loaded on discs about 1 cm in diameter that sit on an appropriate sample holder in multiples. These are then introduced into the device, commonly referred to as a luminescence reader [44] and selectively moved into position for measurement. The luminescence signal from the sample is captured by a photon detector system [1] for example, photomultiplier tube (PMT) or charge-coupled device (CCD) camera after passing through optical filters. When conducting TL measurements, the filters exclude infrared signals from the heating but permit blue or violet emissions to pass through. In OSL measurements, wavelengths used for stimulation are rejected by the filters, whereas violet and near ultraviolet wavelengths are usually transmitted. Output from a TL measurement is distinctly different from that obtained using OSL stimulation (**Figure 2**).

### 3.2. Thermal stimulation

When using thermal stimulation, samples are heated at rates approaching 20˚C per second. On reaching a temperature that corresponds to the trap depth 'E' (**Figure 1**), usually characteristic

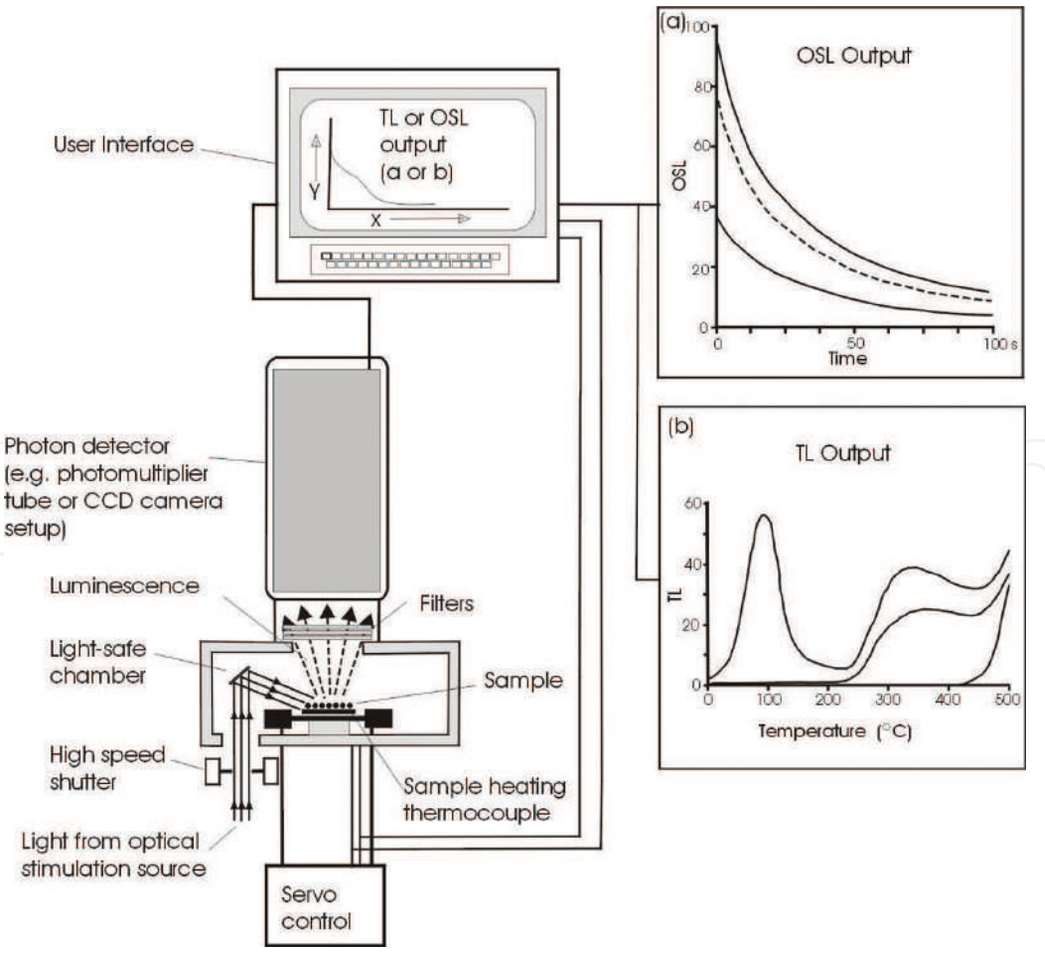


**Figure 2.** Main components of a luminescence reader that measures both TL and OSL signals (modified after Ref. [56]).

of a given trap type, trapped electrons are rapidly evicted [51] into the conduction band. The equation below gives the probability per second at temperature T that the energy provided is sufficient to evict a trapped electron at depth 'E' into the conduction band:

$$\rho(T) = s(T)\exp(-E/kT) \tag{2}$$

where $s(T)$ is a temperature dependent factor associated with the lattice vibrational frequency and change in entropy and $k$ is Boltzmann's constant [52]. Once evicted, electrons are free to be re-trapped at the same site, be trapped at a different site or get to a recombination site where luminescence occurs. The eviction temperature is depicted by a peak in emission on a plot of the luminescence signal versus temperature which is referred to as a TL glow-curve. If the heating continues, all the electron traps will be emptied. A glow-curve that is obtained after the first heating of a sample is given in **Figure 3**. Heating the sample again soon after the first heating will produce a different curve. The second curve corresponds to incandescence that is usually observed when any material is adequately heated to an elevated temperature. Hence, from this second heating, there will be no luminescence from trapped electrons that had accumulated from ionizing radiation since the last zeroing event.

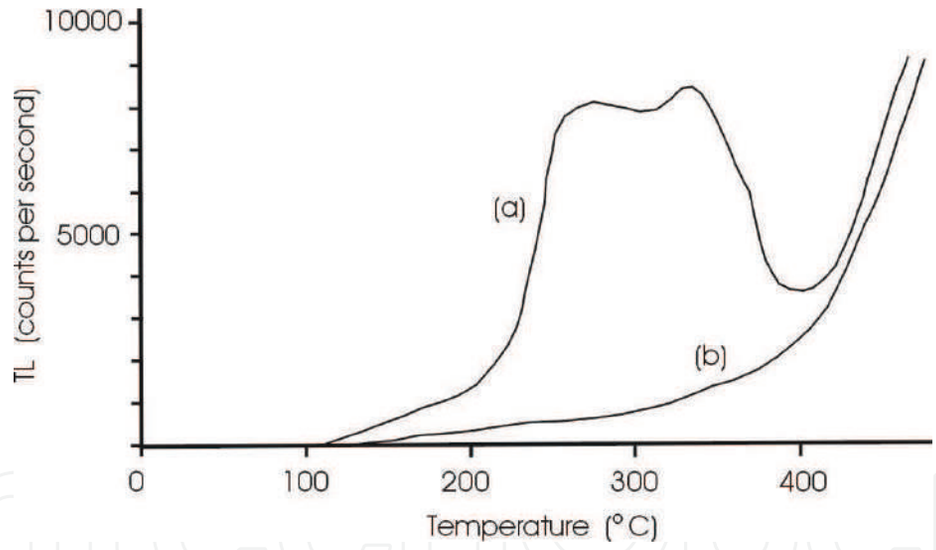


**Figure 3.** Illustration of glow-curves obtained following thermal stimulation. (a) A signal yielded after the first heating of a quartz sample with trapped electrons (paleodose). (b) A glow-curve from incandescence resulting from the second heating of the sample to a high temperature. It is important to note that incandescence is also realized above 400°C during the first heating (redrawn from Ref. [56]).

### 3.3. Optical stimulation

In optical stimulation, electrons are expelled from their traps using a source of a chosen wavelength. Commonly used sources in luminescence dating include blue, green or near-infrared wavelengths. The rate at which trapped electrons are evicted is influenced by the rate at which stimulating photons are emitted by the source as well as by the sensitivity of the trap types to optical stimulation. Generally, however, starting with a concentration of '$n$' trapped electrons

and if '*p*' is the probability for electrons to be evicted by an optical source to the conduction band per unit time, 'n' will change according to Eq. (3):

$$\frac{dn}{dt} = -np \tag{3}$$

assuming that no re-trapping of freed electron occurs. Resolving Eq. (3) shows that as traps are emptied, the concentration of trapped electrons decays exponentially as given in Eq. (4) [5].

$$n(t) = n_0 e^{-pt} \tag{4}$$

where $n_0$ is the concentration of trapped electrons prior to the stimulation. If all freed electrons reach recombination sites instantaneously, the luminescence intensity will be proportional to electrons being evicted from the traps. Thus from Eq. (3) and Eq. (4):

$$I_{OSl}(t) \alpha \left| \frac{dn}{dt} \right| = n_0 p e^{-pt} \tag{5}$$

The exponentially decaying emission curve obtained is referred to in OSL dating as a shine-down curve (**Figure 4**). With continued stimulation, a point is reached where all trapped electrons that are susceptible to optical stimulation are depleted. If all the photons released during stimulation are integrated, the total luminescence energy released by the mineral can be ascertained.

Factors that influence the sensitivity of a trap type to optical eviction include characteristics of the trap as well as the wavelength of the optical source. Generally, however, eviction rates are faster when shorter wavelengths are employed. Electron eviction from some traps could require more energy than that provided by an optical source. To circumvent that limitation,

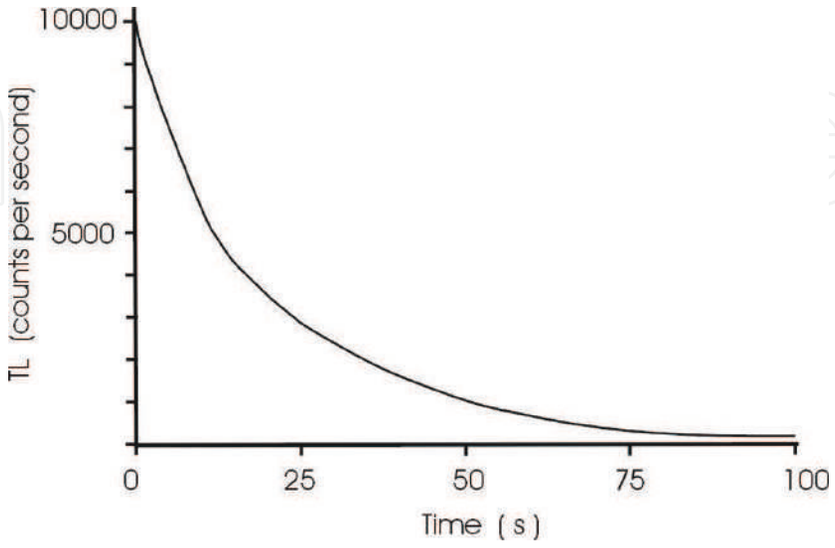


**Figure 4.** A shine-down curve obtained following the optical stimulation (OSL) of a hypothetical mineral sample for about 100 s (modified after Ref. [51]).

thermal assistance is used to attain the energy threshold required for eviction. This allows longer wavelengths that would not normally be employed for optical stimulation to be used in dating [50].

Generally, sources for optical stimulation are selected such that separation can be made between wavelengths of the source used for stimulation and those of signals emitted by the minerals being analyzed. That separation is usually aided by the use of optical filters. As an illustration, the main emissions for quartz and feldspar are in the near-ultraviolet (356 nm) and violet (410 nm) regions of the electromagnetic spectrum. Thus, filters that are employed when analyzing quartz and feldspars have windows in those respective regions but exclude wavelengths used for stimulation, for example, blue for quartz and near infrared for feldspar.

### 3.4. Advantages of OSL over TL

OSL dating has a number of inherent advantages compared with TL when analyzing sediments that have been zeroed by exposure to sunlight. Investigations have shown that solar bleaching of electron traps that are stimulated by TL proceeds more slowly than with traps that are sensitive to OSL [50]. In the study summarized in **Figure 5**, after 20 h of exposure to

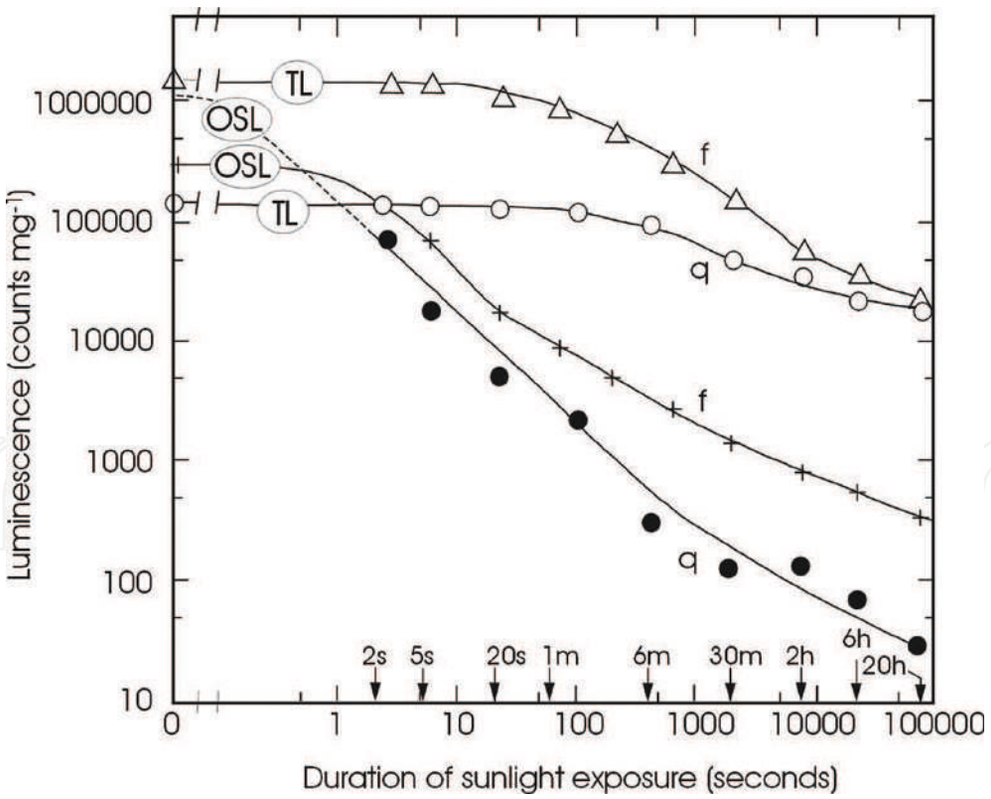


**Figure 5.** Bleaching rates of quartz (q) and feldspar (f) stimulated using TL and OSL (green light) conducted by Godfrey-Smith et al. [57] (redrawn from Ref. [50]). The slower bleaching curves are from TL signals. For quartz, TL measurement focused on a peak between 320 and 330°C whereas for feldspar, a peak at 310–320°C was analyzed. Detection of TL signals was through a window with a center at 400 nm (violet). For the OSL, both quartz and feldspar used a green laser and a detection window of 380 nm which is violet to near-UV [50].

sunlight, both quartz and feldspar were shown to have less than 0.1% of the signal originally present in the mineral remaining [57]. The TL signal that remained following the same period of bleaching, on the other hand, was a few orders of magnitude higher than the OSL signal. In practical terms, the slower bleaching of TL traps by solar energy means that higher residual signals will be found in unheated sediments analyzed using TL, to the extent that it is difficult to date very young samples using TL [11, 50]. Thus, OSL analysis is generally preferred for dating sediments that were not reset by heating [50]. For dating materials that have previously been zeroed by heating, however, such as archeological artifacts, TL remains an appropriate stimulation mechanism.

## 4. Luminescence properties of some common minerals

Many minerals will luminesce when stimulated using an appropriate source following a period of exposure to ionizing radiation. However, not all such minerals are suitable for use in luminescence dating. Today, luminescence dating primarily employs quartz and feldspar. Zircon and calcite have been tried in some studies but both minerals are associated with a number of complications. As a result, they are not commonly used in luminescence dating at present. This section examines the luminescence properties of the four minerals. In the discussions below, natural dose refers to energy acquired from natural radiation sources by a mineral grain in its field setting. This is differentiated from an artificial dose that a sample would obtain when irradiated using an artificial source in a laboratory setting.

### 4.1. Quartz

Quartz is the most commonly used mineral in luminescence dating because it offers a number of advantages when contrasted with alternatives. Due to its resistance to both chemical weathering and mechanical abrasion, it is very stable at the earth's surface. As a result, it is one of the most abundant minerals in clastic depositional environments. Quartz's luminescence properties are also very stable. Additionally, it does not have an internal source of radiation as a major element of its composition. Thus, the ionizing radiation that quartz grains receive in nature is usually from an external source, which simplifies dose rate calculation procedures. Situations exist, however, where quartz grains may contain very low levels of uranium but these are rare [51].

#### *4.1.1. Quartz TL properties*

Quartz that has a natural dose displays TL peaks at 325˚C and 375˚C [58]. TL dating usually employs the 375˚C peak which is very stable and is thought to be the result of $AlO_4$ lattice impurities that serve as hole traps [59]. Under sunlight, the 325˚C peak bleaches much more rapidly than the 375˚C peak [60]. Quartz that has been irradiated artificially also shows a peak at 110˚C [**Figure 6**]. With regards to emissions, heating quartz above 300˚C shows a natural TL emission band around 460–480 nm (blue) and another in the region of 610–630 nm (orange). Laboratory irradiated quartz has a TL emission band below 300˚C in the region of 360–420 nm.

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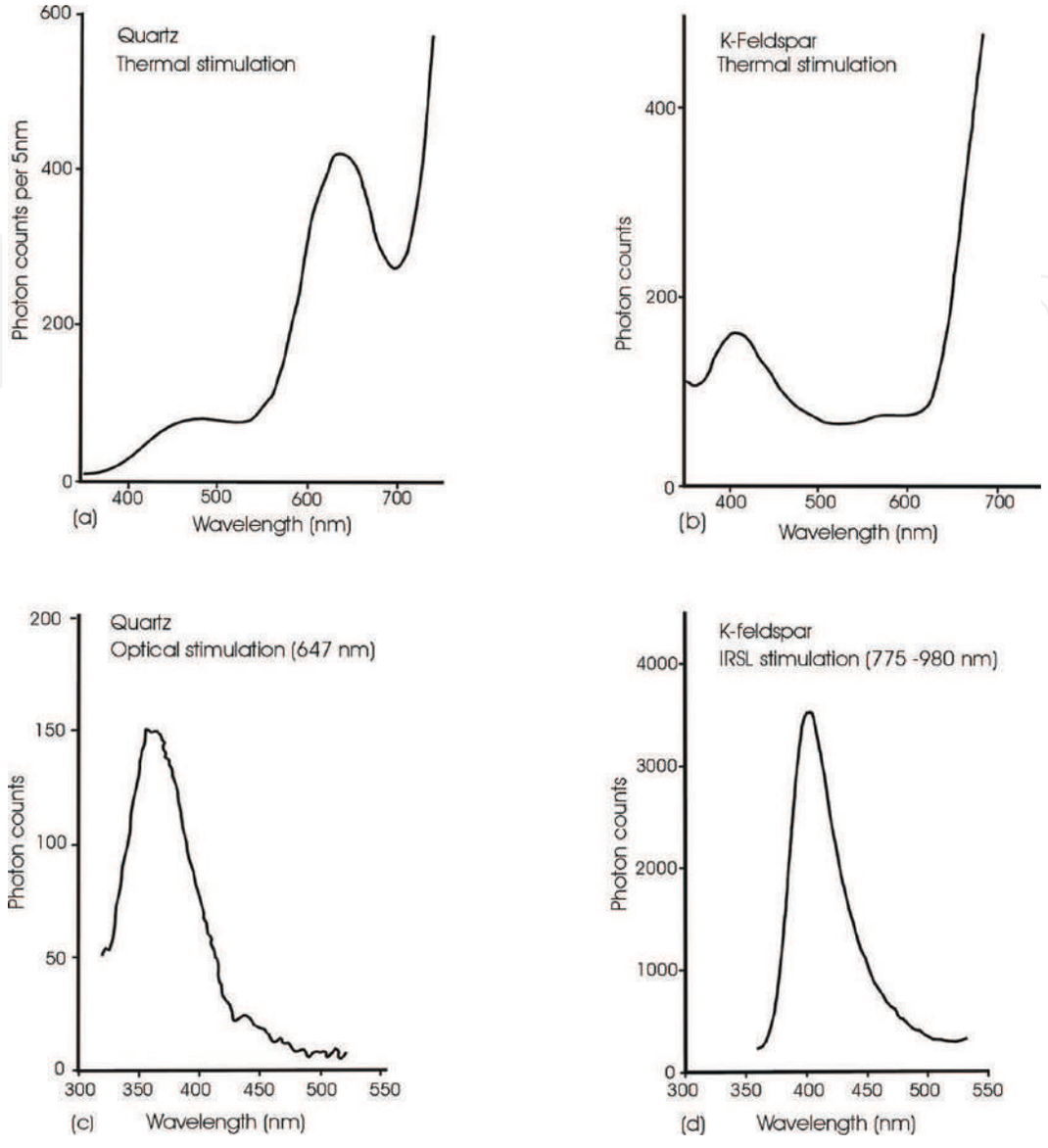


**Figure 6.** Examples of TL (a, b) and OSL (c, d) emission spectra (adapted with permission from Ref. [60]). For TL spectra, the sharp rise in emissions beyond 650 nm is largely from incandescence (rather than from electrons evicted from traps).

### *4.1.2. Quartz OSL properties*

Quartz has been shown to luminesce when stimulated by wavelengths from any part of the visible spectrum [60]. Most current OSL studies, however, prefer using blue light for stimulation because of the higher OSL intensities it yields [55]. Investigations have demonstrated that the OSL signal of quartz can consist of at least three or more components that are referred to as fast, medium and slow, based on their decay rates [61, 62]. To separate the components during stimulation, constantly increasing power is used to give linearly modulated OSL (LM-OSL) [63]. Most regular dating procedures, however, employ a constant power (continuous wave–CW) and are unable to resolve the components. Through the use of heat treatments or stimulation for limited times (to exclude the slower components), desired signals can be targeted

when using CW stimulation. Emission bands that are observed in quartz OSL are in the range of 360–420 nm (**Figure 6**), which corresponds to emissions that are seen in quartz TL at temperatures below 300°C [60] (**Figure 7**).

### 4.2. Feldspar

Feldspar is another widely used mineral in OSL dating. It constitutes about 60% of the earth's crust and even though it weathers more rapidly than quartz, it is also a very common mineral at the earth's surface. In terms of chemistry, feldspars are aluminosilicates that form solid solution series with potassium (K) calcium (Ca) and sodium (Na) as end members of a ternary system. Since potassium has an isotope that contributes ionizing radiation in luminescence dating, the potassium in K-feldspars has to be treated as a source of internal dose, in addition to dose contributions from sources external to the grains. As a result, when dating feldspars, it is necessary to separate K-feldspars from Ca and Na-feldspars and analyze them separately.

Compared with quartz, feldspar has a number of attractive luminescence features. First, feldspar emissions are generally brighter than those from quartz which produces stronger signals. This means that smaller doses can be measured during analysis. Second, the internal dose from potassium is not susceptible to external influences such as variations in pore water and this allows dose rates to be ascertained more accurately. Third, feldspar can be stimulated using infrared radiation which allows effective separation to be made between the stimulation source and emission wavelengths. The main drawback for feldspar, however, is its susceptibility to anomalous fading [64]. Anomalous fading occurs when trapped electrons reside in their traps for shorter periods than what would be predicted by physical models such that the luminescence intensity drops over time from the time of irradiation. Ultimately, the result of anomalous fading is that most feldspar grains yield equivalent doses that are slightly lower than they would in the absence of fading.

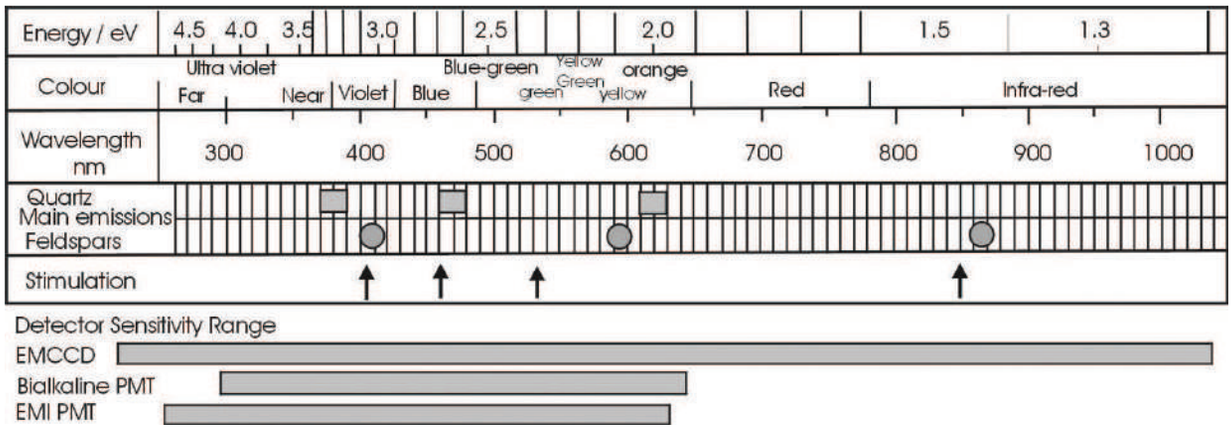


**Figure 7.** Main emission wavelengths for quartz and feldspars used in luminescence dating as well as wavelengths employed for stimulation. Sensitivity ranges for some detectors are also shown. Standard bialkaline photomultipliers (PMT) and EMI 9235QB PMTs do not detect emissions beyond 650 nm, whereas electron multiplying CCD (EMCCD) cameras can capture the whole range (modified after Ref. [54]).

Correction methods have been developed for dealing with anomalous fading when dating feldspars [65, 66].

*4.2.1. Feldspar TL properties*

Various studies have shown that K-feldspars extracted from sediments yield natural TL signals that display peaks at 280 and 330°C [60]. In terms of emission wavelengths, K-rich feldspars have been reported [67] to show maximums in the range of 390–440 nm (violet to blue). Conversely, emissions for some plagioclase feldspars have been reported to appear in the range of 550–560 nm (blue-green). Other studies, however, have intimated at a more complex emission pattern for feldspars [68].

*4.2.2. Feldspar OSL properties*

Optical stimulation of luminescence from feldspars has been investigated using visible light. Early studies employed lasers which included the 514.5 nm wavelength from argon and the 633 nm (red) wavelength from krypton. The emissions were then monitored at shorter wavelengths [1, 57] and shown to be centered around 400 nm [69]. The application of OSL stimulation in dating feldspars, however, has been relatively limited because near-infrared stimulation (discussed below) has been shown to be a more desirable approach. Nonetheless, a study [70] comparing green light stimulation (GLSL) of feldspars with infrared stimulated luminescence (IRSL) reported data suggesting that at 10°C, GLSL signals were more stable than IRSL signals. This would indicate that different trap types might be involved [50]. Apart from green and red stimulation, luminescence in feldspar has been demonstrated using a range of other wavelengths in the region spanning 380–1020 nm [71].

*4.2.3. Feldspar IRSL properties*

As mentioned above, wavelengths in the near infrared region (peaking around 880 nm) can also be used to induce luminescence in feldspars. Since this effect was first noticed [72], most research in optical dating of feldspars has focused on IRSL stimulation. The main advantage of using IRSL is that the rest of the visible spectrum can then be used for emission detection. Fine-grained sediments containing mixtures of both plagioclase and K-feldspars have also been demonstrated to display a major stimulation peak around 845 nm as well as a weaker one at 775 nm [73]. Today, most studies that use feldspar for optical dating employ light emitting diodes (LEDs) for stimulation that have emission peaks at around 880 ± 40 nm. LEDs are much cheaper than lasers and are widely available, making them a desirable alternative. Sedimentary K-feldspars stimulated using IRSL show a major natural emission peak at 410 nm (**Figure 6d**) and another minor peak in the range of 300–350 nm [74]. Some IRSL studies [75] have reported additional natural emission maxima for K-feldspars at 280, 560 and 700 nm. With plagioclase feldspar, an IRSL emission peak has been identified at 570 nm.

Feldspars stimulated using IRSL following the administration of a laboratory dose also exhibit an emission peak at 290 nm. That peak is not observed in feldspars that have a natural signal. When not required during dating, the peak can be removed by preheating the sample to an appropriate temperature.

### 4.3. Calcite

Thermally stimulated calcite has an emission maximum at 570 nm [60]. However, efforts to use the mineral in luminescence dating have been encumbered by the limited environmental occurrence of calcite. Calcite also tends to concentrate uranium in its lattice and this complicates dose rate calculations since isotopic disequilibrium of uranium has to be taken into account. Worth noting is that uranium disequilibrium dating can yield ages from calcite that are more reliable than those obtained using luminescence techniques. As a result, the incentive to employ luminescence methods in dating calcite has been small. It should be mentioned that some of the earliest, albeit unsuccessful, TL studies that tried to date rocks employed calcite [10]. Other attempts to use calcite in archaeological dating include a report by Ugumori and Ikeya [76].

### 4.4. Zircon

Zircon is an attractive dosimeter because it usually has a relatively high concentration of uranium. Consequently, the dose from the mineral grain's interior is far greater than that originating from outside. This yields a dose rate that is relatively constant since it is not susceptible to variations arising from external effects such as changes in water content or burial depth. An associated drawback, however, is that the uranium content of zircon varies between individual grains. Consequently, measurements for dose rate are made on single grains. Also notably, zircon crystal lattices often have natural inhomogeneities that make it difficult to make comparisons between artificial irradiation administered in the laboratory with natural doses originating from within the grain. As outlined in Section 5, such comparisons are the standard approach for determining the paleodose when dating quartz or feldspar. To address that problem, zircon dating uses a technique called autoregeneration. With autoregeneration, after the natural signal from the zircon grains is measured, the grains are stored for a few months to allow a new dose to accrue. Measuring the signal at the end of the storage period and comparing it to the natural signal obtained from the initial measurement allows a calibration to be made that yields an age of the natural signal.

Analysis of zircon using TL includes a study by Huntley et al. [67] and by Templer and Smith [77]. OSL studies using zircon include investigations by Smith [78].

## 5. Paleodose and dose rate determination

The age equation introduced in Section 1 (Eq. 1) shows that two parameters need to be determined before a luminescence age can be calculated: the paleodose and the dose rate. This section examines methods that are used to determine the two variables.

### 5.1. Paleodose determination

The start of the accumulation of the paleodose should typically coincide with a geomorphic or archeological event that emptied (or zeroed) any previously accumulated energy in the sample grains. The dose refers to the energy absorbed per kilogram of material, and it is

measured using units of gray (Gy), where 1 Gy = 1 J/kg. For materials that were previously zeroed by heating or firing, the start of the accumulation of the paleodose would correspond with the last time the material was heated to a temperature high enough to expel electrons from their traps. In the case of sediments that were zeroed by exposure to sunlight, the start of the accumulation of the paleodose would correspond to the last time that a sample was subjected to the bleaching effects of the sun for a period long enough to evict all trapped electrons. As indicated earlier, the natural signal refers to the luminescence signal yielded by a sample collected from the field. In order to determine the paleodose of a sample of unknown age, the natural signal is measured first after which the sample is irradiated artificially using a well-calibrated laboratory-based source. The signals from the artificial dose are then measured and compared with the natural dose signals in order to determine an artificial dose that gives a signal similar to that of the natural dose. This is referred to as the equivalent dose ($D_e$). Investigators use two main methods to determine $D_e$: the additive dose and the regenerative dose (or regeneration) methods [2, 50].

### *5.1.1. Additive dose method*

When determining luminescence ages using the additive dose method, a sample of unknown age is split into two sets of aliquots. The natural signal for one set is measured first after which the second set is irradiated with incremental doses using an artificial source and also measured. Plotting the artificial signals against the dose administered produces a dose–response curve depicting the luminescence signal against the laboratory dose (**Figure 8a**). The curve is also known as a growth curve. The natural signal is also plotted on the growth curve against zero dose (**Figure 8a**). Extrapolating the curve backwards until it intercepts the horizontal axis at zero signal intensity provides $D_e$ (**Figure 8a**) [51]. Growth curves are unique to each sample because luminescence sensitivity of mineral grains can vary from sample to sample. As a result, a new growth curve has to be constructed for each sample whose age is being determined. When dating materials that were zeroed by the sun using TL, the residual TL signal that is noted following solar bleaching would have to be taken into account when extrapolating the curve backwards. With both feldspar and quartz, the relationship between the luminescence signal and the laboratory radiation portrays a linear trend for low and moderate doses. At elevated doses, however, the growth curve plateaus, indicating that luminescence traps are getting exhausted, also referred to as saturation.

### *5.1.2. Regenerative dose method*

The procedure used in the regeneration method is similar to that employed in the additive dose method apart from that, before the laboratory dose is applied, the sample aliquots in the regeneration method are first zeroed to remove any previously acquired dose. Incremental doses are then applied to the zeroed aliquots and measured. The acquired signals are plotted against the administered dose to give a regenerative dose growth curve. To get the equivalent dose, the signal from the sample of unknown age is interpolated into the growth curve (**Figure 8b**) [51]. Hence, when constructing the growth curve, the laboratory irradiation doses are selected such that the signals they produce lie above and below the signal obtained from the natural dose.

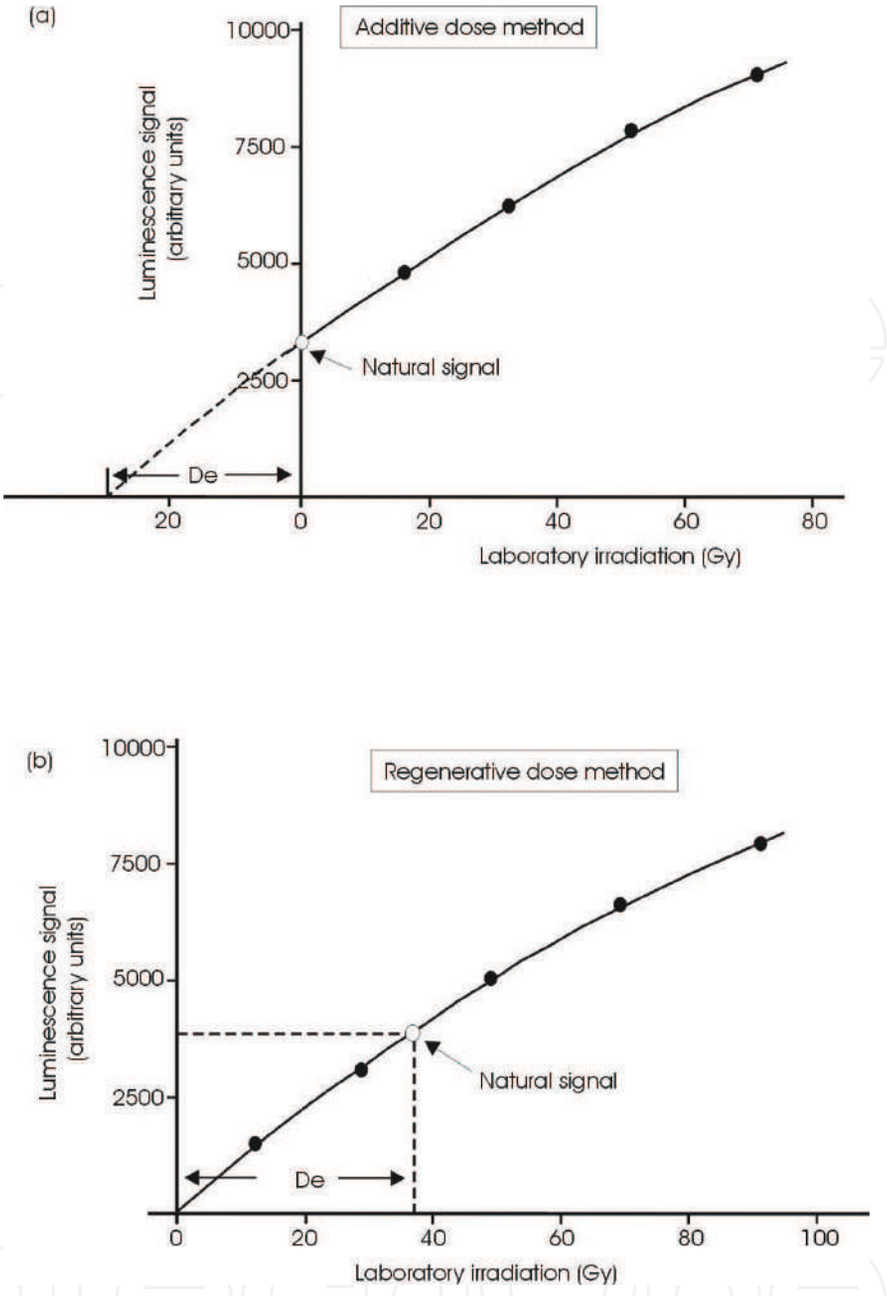


**Figure 8.** Main methods employed in determining the equivalent dose. (a) When using the additive dose method, incremental doses are administered on top of the natural dose and then measured. (b) With the regenerative dose approach, samples are first zeroed to remove any previously acquired dose and then given incremental doses, followed by measuring. Growth curves are unique to each sample being analyzed such that new measurements have to be made and a new curve constructed for every sample being dated.

### *5.1.3. Single aliquot methods versus multiple aliquot methods*

Both the additive dose and regenerative dose methods employed multiple aliquots when they were originally developed for TL dating. Later, when OSL dating emerged, the possibility of using single aliquots only was brought up [1]. However, the concept did not take hold initially and multiple aliquots were also adopted for OSL dating. Generally, the use of multiple aliquots assumes that all aliquots of a given sample behave similarly to the dose administered.

However, in reality, inter-aliquot variations occur for a range of reasons that include changes in sensitivity [3, 79]. Normalization is used in some cases to try and reduce the effects of the variations. Nonetheless, the effects cannot be eliminated entirely such that uncertainties are contributed to the calculated ages. Ultimately, there was an incentive to devise an approach that only employed a single aliquot.

### *5.1.4. Single-aliquot regenerative-dose (SAR) protocol*

When initially introduced for dating, single aliquot methods employed the additive dose method on feldspars [80, 81]. Quartz had not been used because sensitivity changes that it displays during repeat measurement cycles rendered single aliquot data unworkable. A few years later, however, following work carried out by Mejdhal and Bøtter-Jensen [82], a single aliquot regenerative (SAR) protocol for quartz that incorporated correction steps to address the changes in sensitivity was proposed. Subsequently, Murray and Wintle [37] presented an enhanced version of the SAR approach introduced by Murray and Roberts [83] in which a test dose was used to monitor sensitivity changes in the quartz. As initially proposed by Murray and Wintle [37], their SAR method entailed steps outlined in **Table 2**.

Minor modifications have been made to the SAR procedure presented in **Table 2** since its original inception [37, 38]. However, over the last two decades, the protocol has been widely adopted for routine dating of both sediments and heated materials using quartz and feldspar [84].

| Step | Treatment[a] | Observed signal |
|---|---|---|
| 1 | Irradiate sample with artificial dose, $D_i$ | – |
| 2 | Sample preheated (160–300°C) for 10s | – |
| 3 | Sample stimulated for 100 s at 125°C | $L_i$ |
| 4 | Sample irradiated with test dose, $D_t$ | – |
| 5 | Sample heated to 160°C | – |
| 6 | Sample stimulated for 100 s at 125°C[b] | $T_i$ |
| 7 | Return to step 1 and repeat sequence[c] | – |

$D_i$ is the dose that yields the signal $L_i$ while $D_t$ is the test dose that produces the signal $T_i$. These values are used to plot a curve of Li/Ti vs. the regeneration dose D.
[a]For the natural sample, $i = 0$ and $D_0 = 0$ Gy.
[b]Stimulation time varies with the intensity of the light used for stimulation.
[c]Subsequent modifications to steps above include an additional step to help attenuate recuperation.

**Table 2.** Sequence of steps in the SAR procedure as initially proposed by Murray and Wintle [37].

### *5.1.5. Single grain analysis*

SAR protocols have also been extended to determining paleodoses using individual mineral grains. The transition has been facilitated by the development of special equipment for loading and analyzing thousands of grains of sand size (<250 μm) relatively quickly [85]. The analysis of individual grains from the same sample is particularly useful for identifying differences in paleodose between grains [86]. Examples of cases where this may be expedient is

when studying sediments deposited by rivers (fluvial) where well-bleached grains might be mixed with partially bleached fractions [87].

### 5.2. Determination of the dose rate

As outlined above, once the paleodose has been ascertained, the dose rate needs to be evaluated before an age can be determined. In Section 1, it was mentioned that ionizing radiation responsible for the energy accumulation in mineral grains in natural settings emanates from uranium and thorium decay chains as well as from potassium and rubidium isotopes. Cosmic radiation also contributes a minor component. A number of methods can be used to evaluate the total contributions of all these components. Using the concentration approach, levels of uranium, potassium and rubidium in a given sample are quantified with the help of an analytical procedure such as atomic absorption spectroscopy (AAS), neutron activation analysis (NAA), flame photometric detection (FPD), X-ray fluorescence (XRF) and inductively coupled plasma spectroscopy (ICPS). Once the concentrations have been measured, dose rate is determined using special conversion tables prepared for the purpose [50]. For uranium and thorium, however, isotopic disequilibrium could render the measurements using these analytical techniques unreliable [50].

As opposed to measuring elemental concentrations "using methods outlined" above, direct measurements of the activities of specific radionuclides can be achieved using methods such as alpha or gamma ray spectrometry. Despite being costly, these methods can provide accurate measurements, including from uranium and thorium decay chains and in cases where disequilibrium exists. Nonetheless, lengthy measurement times may be necessary [50].

Alternatively, errors from isotopic disequilibrium in the uranium and thorium decay chains can be reduced by measuring the contributions of uranium and thorium using thick source alpha counting (TSAC) after which potassium is measured using an analytical technique such as AAS, FPM, XRF or ICPMS. Yet another approach to determine the dose rate is to use TSAC to determine the alpha particle contribution after which a beta particle counter is employed to determine the beta contribution. The gamma dose rate is best determined in the field whenever possible. Highly sensitive portable gamma-ray spectrometers that make such onsite measurements possible are now available [44].

Measurement of the dose rate can also be conducted in the field using synthetic dosimeters such as $\alpha$-$Al_2O_3$:C [44]. These are highly sensitive materials that are left in the field for a few weeks after which they are retrieved and analyzed.

The dose contribution from cosmic rays is usually minor. However, in settings where the radionuclide concentrations are low, the proportion from cosmic rays becomes significant. A methodology for calculating cosmic ray contribution to the luminescence dose rate was formulated by Prescott and Hutton [53].

Lastly, it is imperative to take the *in situ* moisture content of the material that is being dated into account when calculating the dose rate. This is because interstitial water absorbs part of the dose that should otherwise reach the dosimeter, with the attenuation of the dose rate intensifying as the moisture content increases.

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### 5.3. Lower and upper age limits in luminescence dating

At present, luminescence dating methods can be used to date samples that are as young as a few decades [88]. Dating using the single grain approach can produce young ages that are relatively precise. When dating such young samples, it is desirable to use mineral grains characterized by a high luminescence sensitivity and for the grains to have been completely bleached prior to the burial [39]. Minimizing thermal charge transfer during measurement also improves the accuracy of the results.

Maximum ages that can be obtained using luminescence dating methods are ultimately controlled by the fact that the population of electron traps within any given dosimeter is fixed. As

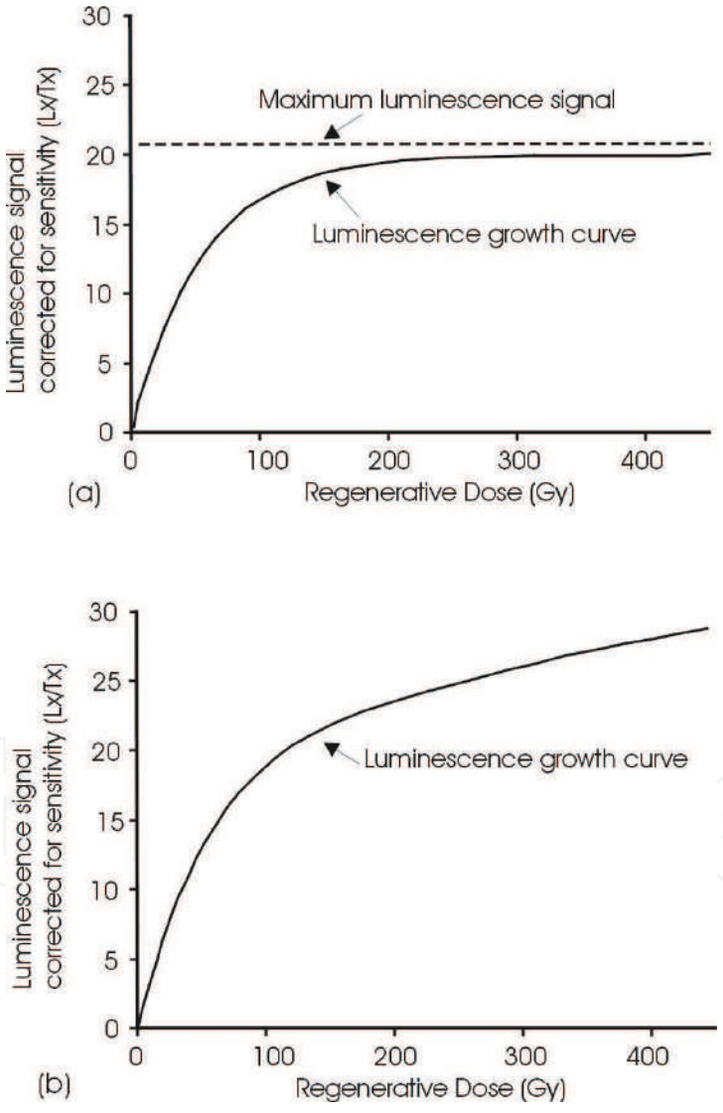


**Figure 9.** Examples of growth curves for quartz taken to saturation (redrawn after [3]). (a) Curve constructed using a saturating exponential function of the form $I(D) = I_0 (I - exp^{-D/D_0})$ where $I(D)$ is the luminescence signal produced by the dose D and $I_0$ is the maximum luminescence intensity produced. The parameter $D_0$ determines the shape of the curve. (b) Since curve (a) appeared to underestimate some older ages, the saturating function from (a) is combined with a linear function in (b) [3].

a result, the number electrons stored by trapping cannot increase indefinitely [50, 51, 56]. This is depicted in luminescence growth curves by a flattening of the signal obtained as the dose increases and is often expressed using a saturating exponential function. **Figure 9a** shows such a function expressing the fast component of a quartz signal. It shows that, once a certain dose is reached, the curve flattens. That dose is the upper limit above which the proportionality between the dose received and luminescence signal obtained breaks down. Quartz usually saturates with a dose of around 100–150 Gy. An approach used in some studies [4, 89] working with high doses is to model the growth curve by combining a linear function and a saturating function as shown in **Figure 9b** [3]. This approach has been used to report quartz ages in excess of 200 000–400 000 years. It should be noted that the limits of the ages that can be obtained are ultimately determined by the magnitude of the dose rate, with low dose rates giving higher age limits and vice versa. With feldspar, several studies have reported ages that exceed 100 000 years using IRSL [90]. Correction for anomalous fading of the feldspar at such high doses (>100–200 Gy) using standard methods [66], however, becomes problematic.

In essence, with both feldspar and quartz dating, there are maximum dose limits above which reliable ages cannot be produced as a result of electron trap exhaustion.

## 6. Sample collection and preparation for analysis

Preceding sections explored the basics of luminescence dating. A subject that now needs to be addressed is the nature of materials on which luminescence dating methods can be applied. Before that can be looked into, however, it is pertinent to examine the topic of sample grain size, since protocols employed in luminescence analysis are contingent upon the granulometry of the material being analyzed.

### 6.1. Sample grain size

There are generally two broad mineral grain size ranges that are employed in luminescence dating: coarse grains and fine grains. When using the coarse grain method, sand-sized particles in the diameter range 90–250 μm are extracted and analyzed. Grains in this size range normally receive ionizing radiation from alpha and beta particles as well as gamma and cosmic rays. Because of their size, alpha particles would only be able to penetrate the mineral grain's exterior 25 μm. When working with coarse grains, the outer rim that is affected by the alpha particles is removed by etching using hydrofluoric acid (HF). For this reason, coarse grain luminescence dating is sometimes described as inclusion dating [58, 91, 92]. When dating pottery using quartz inclusions, grains in the size range 90–125 μm are commonly selected. In sediment dating, the usual practice is to extract grain sizes that represent the modal size. Thus, for eolian dunes, grains in the size range 150–180 μm are often used [3]. Feldspar inclusion dating employs procedures similar to those used for quartz dating, including etching to remove the outer rim penetrated by alpha particles.

An important aspect of inclusion dating is that, since parts of the grain affected by alpha particles are removed prior to the performing luminescence measurements, only the contributions of

beta, gamma and cosmic rays are taken into account when determining the dose rate. If Eq. (**1**) is modified to take those analytical considerations into account, the age equation becomes:

$$Luminescence\ Age = \frac{Paleodose}{0.90D_{\beta} + D_{\gamma} + D_{C}} \tag{6}$$

where $D_{\beta}$, $D_{\gamma}$ and $D_{c}$ are the doses from beta, gamma and cosmic ray contributions, respectively [50, 51]. If the dose rates are given as annual values, the equation yields the age in calendar years. The beta dose rate is factored by 0.9 as a correction for the grain size and the etching that disposes of the outer rim which also received a beta dose [50, 51].

Worth noting is that, on rare occasions, quartz may contain some uranium and thorium within its lattice. In such cases, Eq. (**6**) may need to be modified if coarse grains are used. Similarly, when dating feldspar using coarse grains, potassium-40 isotopes in K-feldspar constitute an additional source of beta particles that has to be factored into the dose rate calculation. As a result, feldspars are often separated into K-feldspar and low-potassium fractions that are then analyzed separately.

For fine grains, mineral grains in the size range 4–11 μm are usually extracted and mounted for analysis. When calculating the dose rate for such mineral grains, since their diameters are such that alpha particles can penetrate them entirely, contribution from alpha particles has to be included in the dose rate. Consequently, the age equation becomes:

$$Luminescence\ Age = \frac{Paleodose}{\mathrm{k}D_{\alpha} + D_{\beta} + D_{\gamma} + D_{C}} \tag{7}$$

where $D_{\alpha}$, $D_{\beta}$, $D_{\gamma}$ and $D_{c}$ are the doses from alpha, beta, gamma and cosmic ray contributions, respectively.

Mineral grains in the diameter range 33–63 μm have been dated in other studies [93]. Because the size range is intermediate between coarse grain and fine grain categories, the analysis is sometimes referred to as middle-grain dating [50]. Etching with HF to remove the outer rim of the grains is not usually employed with middle-grain dating. Thus, the alpha particle contribution is also included when calculating the dose rate.

## 6.2. Sample collection and preparation

### *6.2.1. Sample collection*

As outlined earlier, exposure of luminescence dating dosimeters to sunlight results in the zeroing of any previously acquired dose. Thus, a basic constraint when collecting samples is that they should be shielded from light until the time of analysis. Accordingly, when dealing with archaeological artifacts, sampling usually entails using a power drill in safe-light conditions to retrieve a representative sample from the interior of the artifact. When collecting sediments from their natural settings, on the other hand, sampling could be conducted at night [50]. However, working in the dark is inconvenient and chances of accidentally exposing the sample to light are also greater. Hence, night-time sampling is not the most popular

approach. More often than not, sampling is conducted during the day. In cases where sediments have a firm consistency, it may be possible to cut out a block of sediment and transport it to the lab where the sample for dating is extracted from the block's interior. A more commonly used approach is to insert an opaque cylindrical pipe into a targeted geological unit. Both ends of the pipe are capped once it has been retrieved. At the laboratory a sample is analysis is removed from the center of the pipe. Samples for luminescence analysis can also be collected at depth by drilling vertically using sampling modules specially designed to avoid exposure of the sample to sunlight [94].

*6.2.2. Sample preparation prior to the measurement*

Collected samples have to be pretreated before luminescence analysis can be conducted. There are a number of standard procedures that have been developed for pretreating samples, depending on whether the grains targeted for analysis are fine or coarse. Analysis of coarse grains is usually performed on pure separates of either quartz or feldspar. Fine grains, however, are often analyzed as polymineralic fractions. The choice of what mineral or grain size to analyze will generally depend on the sample type. For instance, when dating loess, which is a silt-sized deposit, it is more appropriate to extract fine grains whereas using coarse grains would be more suitable for dating wind deposited dune sands. Where sediments have equal proportions of fine and coarse components, it is possible to validate results by dating both fractions.

In coarse grain dating, sample pretreatment entails first removing carbonates and organic material using dilute hydrochloric acid (HCl) and hydrogen peroxide ($H_2O_2$), respectively. Pure separates of quartz or feldspar are then extracted from the sample using a heavy liquid such as sodium polytungstate solution. Finally, the separates are mounted on discs (about 10 mm in diameter) before being analyzed. More details on sample extraction are given in [95].

When extracting fine grains for analysis, carbonates and organic materials are also first eliminated using dilute HCl and $H_2O_2$, respectively. To avoid flocculation of the grains, dilute sodium oxalate is added. After thorough mixing, a sedimentation column is then used to extract grains of the desired size range (usually 4–11 μm). If no further separation is required, the sample will be ready for mounting on discs. Occasionally, however, some studies do extract pure quartz from fine-grained samples. This is achieved by digesting feldspar from the polymineralic sample using fluorosilisic acid [96]. Additional details on separating and mounting fine grains can be found in [97].

## 7. On what materials can luminescence dating be applied?

Materials of geological or archaeological origin that are dated using luminescence methods have to satisfy a number of characteristics before they can be successfully analyzed. First, the material must be composed of a substance that behaves as a dosimeter. Second, the luminescence energy contained in the material must have been zeroed at some point that now serves as the starting point for counting time. Third, energy storage in the materials should not have reached saturation at the time of analysis, otherwise stored energy would cease to be

an accurate measure of time that elapsed. Within the context of these requirements, materials from which ages can be obtained using luminescence methods can be classified into two main categories: substances whose accrued energy has been reset by heating and sediment grains of sand and silt size whose previously acquired energy has been zeroed by exposure to sunlight while being transported.

### 7.1. Heated materials

The heating process employed in pottery production commonly fires earthenware to temperatures in excess of 800°C. Such elevated heat effectively evicts trapped electrons in constituent materials, providing a starting event for a luminescence age. As a result, archeological materials such as ancient pottery, bricks, terracotta figures and tiles are artifacts whose ages can be ascertained using luminescence methods, especially TL. This is because the artifacts commonly contain mineral grains such as quartz and feldspar. If the quartz or feldspar grains from the artifacts are extracted and dated using the inclusion dating approach (coarse grain technique), ages can be obtained. Alternatively, the fine-grained components can be extracted from the same materials and polymineralic fractions dated. Fine-grained quartz separates can also be isolated and dated similarly. It is thought that humans began making pottery about 30,000 years ago, during the Neolithic period [98]. By 10,000 years ago, the skill had spread globally [51]. Thus, most ancient pottery artifacts should be amenable to dating using luminescence methods.

In addition to pottery, other materials of archaeological context that could experience episodes of heating include flint and any other stones that may have been heated to high temperatures by fires associated with human settlements. These include residual rock chips from the production of tools by ancient humans that may have been heated either intentionally or otherwise. Dating any of these artifacts using luminescence techniques provides a timeline that approximates the age of the occupation of the archaeological site by humans [99].

Geological materials that have undergone heating to high temperatures at any point during the last about 100,000 years should also be datable using TL methods if they contain appropriate dosimeters. Materials that could fall into this category include sediments that border volcanic eruptions, as well as products of the volcanic eruptions themselves including lava and ash. In all instances, dating the materials using luminescence methods allows the age of the eruption to be approximated [100].

A final category of substances that can be dated using luminescence methods after being zeroed by elevated temperatures are materials associated with meteorite impacts. Such impacts create thermal shocks that erase any previously acquired dose in mineral grains contained in proximal geological structures, providing a convenient bleaching mechanism for dating the impact using luminescence methods [101].

### 7.2. Dating of sediments reset by sunlight

Granular sediments that contain dosimeters that have been bleached of all previously acquired energy by exposure to sunlight before being buried form an important class of materials that

can be dated using luminescence methods. Stemming from studies [36] that showed that the exposure of some minerals to sunlight could evict electrons from traps in mineral lattices, luminescence methods have been successfully used to date sediment from a range of environments. While TL was used in the beginning, the subsequent development of OSL techniques was nurtured by the dating of this category of materials. The types of sediments that fall into this category include sediments transported by wind (eolian sediments), water-lain sediments, sediments associated with glaciers, as well as sediment related to earthquakes. The section below examines each of these sediment classes. In each case, luminescence ages denote time that has passed since the sediments were last zeroed by sunlight. Similarly, the ages correspond to time that has passed since the sediment was last shielded from sunlight (or buried) by overlying geological units.

*7.2.1. Eolian deposits*

Of all classes of geological materials that can be dated using luminescence methods, wind deposited (eolian) sediments are the most ideal. This is because the sediment grains are transported at the earth's surface or in mid-air, affording them adequate exposure to the sun to cause complete zeroing of any dose the sediment may have [50]. Consequently, the largest share of luminescence ages that have been published to date are from deposits of eolian origin. Eolian deposits generally fall into two main categories: sand-sized and silt-sized grains. Sands comprise sediment grains in the range 63–250 μm. The wind transports such particles via a process called saltation [102], which is a sequence of low altitude jumps, less than a foot from the surface of the depositional bed. Such grains usually form dunes. Silt-sized grains comprise particles in the size range 2–63 μm. These are transported in suspension and typically stay airborne for long periods of time [102]. In either case, eolian sands and silt grains are usually well-zeroed by the time they are buried by other sediments.

Eolian dune sediments have been dated in multiple studies [103] using quartz or feldspar extracts (mostly employing blue OSL and IRSL for stimulation, respectively) and produced ages that have been compared with radiocarbon ages from related sediments. In all cases, the results have validated luminescence dating as a competent dating method that provides reliable ages. Since eolian dune deposits are often treated as indicators of dry conditions, chronologies from eolian sands have been used to reconstruct environmental changes from the past [104–107]. Published ages span the last ca. 100,000 years. Hence, the method has emerged as an indispensable tool for the study of Late Pleistocene and Holocene paleoenvironments.

In addition to studying environmental change in inland deserts, luminescence chronologies from coarse-grained eolian sands have also been used to investigate depositional chronologies of coastal dune deposits [108]. Results from such studies have been used to develop temporal frameworks for processes in coastal environments, including sea level change.

Silt-sized eolian sediments can form extensive sequences of deposits referred to as loess [102]. Fine grain dating has been used to provide depositional chronologies of loess in numerous studies [107]. As in coarse grain dating, the chronologies have invariably been used to reconstruct paleoenvironments from the late Quaternary period. Luminescence analysis of fine grains

can be conducted by targeting feldspar in polymineral fractions using IRSL. Alternatively, if quartz fines are extracted as outlined above, blue OSL is employed for stimulation [109].

### *7.2.2. Water-lain deposits*

Coarse as well as fine-grained sediments that have been deposited by water have also been investigated using luminescence methods. However, studies have shown that sediments transported by rivers (fluvial deposits) are not always well-zeroed at deposition [46, 110]. As a result, single grain dating methods have been used to identify well-zeroed grains from such settings [46, 110]. Useful information on modern and ancient depositional rates of river systems have been acquired through luminescence studies of fluvial sediments [110]. Influences of tectonic and climatic effects on river systems have also been investigated using luminescence chronologies [110]. Other applications of luminescence ages from fluvial deposits include paleoseismic and archaeological studies. Additional information on dating fluvial deposits using luminescence methods can be found in [46, 110].

### *7.2.3. Sediments of glacial origin*

As with water-lain deposits in fluvial settings, the greatest concern with deposits from glacial environments is the degree of bleaching that sediments experience prior to the burial. Sediments with the highest probability of being well-zeroed are deposits that meltwaters transport away from the glacier as outwash [44, 111]. Hence, chances for complete zeroing increases with distance from the glacier's front [111–113]. Nonetheless, even with outwash deposits, studies have shown that sediments are often partially zeroed [112]. As with fluvial deposits, single grain dating methods can be used to identify well-bleached grains in such instances. Some investigators have tried to identify signal components that bleach more rapidly for use when dating partially bleached glacial deposits [111]. More detailed discussions on luminescence dating of glaciofluvial deposits are found in [44, 111].

### *7.2.4. Earthquake-related studies*

Tsunami episodes generated by earthquakes often displace sandy deposits from beach areas, depositing them further inland in tidal marshes, bogs and lakes that rim ocean margins. Afterwards, as part of the coastal sequence, other deposits that can include peat or mud may be emplaced atop the displaced sands, shielding them from sunlight. If the sands are dated using luminescence methods, the chronology they yield can be used to approximate recurrence rates of the tsunamis and, by extrapolation, of the earthquakes that cause the tsunamis. Such information would be critical when evaluating environmental hazards within the region [50]. Worth noting, however, is that tsunami events are rapid and often do not provide time for adequate bleaching of the sediments they mobilize. As a result, investigators target sediments that were exposed at the surface before the tsunami event and hence were already zeroed [50, 114]. Other deposits associated with earthquakes on which luminescence dating has been applied are sediments that occur on horizontal surfaces that have experienced differential vertical movement through earthquake-related faulting [49]. Sediments on the uplifted surfaces could be correlated by dating them using luminescence methods.

## 8. Current and future trends in luminescence dating

Luminescence dating has evolved into a full-fledged and robust chronometer over the last six decades. Concurrent with and driving this development has been the establishment of numerous luminescence dating laboratories throughout the world. To foster interaction between scholars, the Luminescence and Electron Spin Resonance Dating (LED) Conference is held triennially to examine a range of topics that encompass luminescence dating. In the years between the LED conferences, regional and national meetings are also held, including the UK Luminescence Dating Conference, the German Luminescence and ESR Conference or the North American New World Luminescence Dating Workshop. Topics explored at the meetings include fundamental research into luminescence dating as well as applications of luminescence chronologies in environmental, geological and archaeological sciences. Improvements in instrumentation for luminescence dating also constitute an important element at the meetings.

Current topics in fundamental research include investigations that aim to improve knowledge on luminescence characteristics of dosimeters such as feldspar and quartz. Ultimately, this should lead to the production of ages that are more accurate as well as the extension of maximum and minimum age limits that can be obtained using luminescence methods. Such efforts include studies that aim to understand the behavior of quartz at high doses [115, 116]. The information should lead to the formulation of protocols that extend the upper limit of ages obtainable using quartz [116].

Research on feldspar has included efforts to identify IRSL signals that are less inclined to experience anomalous fading [66]. Employing such signals would allow older ages to be determined using feldspar than are currently obtainable [117, 118]. Some studies have demonstrated that IRSL signals that are acquired by stimulating feldspar at a low temperature (for instance, 50˚C) after which another measurement is immediately conducted at a higher temperature (for instance, 290˚C), have a lower fading rate than those obtained using standard protocols [118]. This experimental procedure, termed post-IR IRSL [118] has yielded encouraging results that could potentially raise the upper limit of ages that can be obtained using current methods [118].

Improvements in instrumentation are also continuously being realized and advances include the development of luminescence readers equipped with broader spectral ranges for emission detection [54]. The recent development of portable OSL measurement systems that can be used to conduct rapid on-site measurements is also worth mentioning [119]. Compared to regular OSL readers, the portable systems have limited functions. However, if they were to be equipped with add-ons such as external X-ray sources for sample irradiation, practitioners would be faced with expanded options for signal collection.

Today, luminescence dating is a dynamic field of research that is set to continue witnessing ground-breaking developments in years to come. As both laboratory procedures and analytical equipment are improved, ages should be reported with higher precision and accuracy.

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## References

[1] Huntley DJ, Godfrey-Smith DI, Thewalt MLW. Optical dating of sediments. Nature. 1985;313:105–101.

[2] Aitken MJ. Luminescence dating: a guide for non-specialists. Archaeometry. 1989;31:147–159.

[3] Wintle AG. Luminescence dating: where it has been and where it is going. Boreas. 2008;37:471–482.

[4] Murray A, Buylaert JP, Henriksen M, Svendsen, JI, Mangerud, J. Testing the reliability of quartz OSL ages beyond the Eemian. Radiation Measurements. 2008;43:776–780.

[5] Yukihara EG, McKeever SWS. Optically Stimulated Luminescence: Fundamentals and Applications. Chichester: Wiley; 2011. 378 p.

[6] Boyle R Experiments and Considerations Upon Colors and Observations on a Diamond that Shines in the Dark. 1st ed. London: Henry Herringham; 1664.

[7] Hunter MCW. The Boyle Papers: Understanding the Manuscripts of Robert Boyle. 1st ed. Aldershot: Ashgate Publishing; 2007. 688 p.

[8] Daniels F, Boyd CA, Saunders DF. Thermoluminescence as a research tool. Abstracts of Papers presented at the 1949 Meeting. Science. 1949;109:440.

[9] Daniels F, Boyd CA, Saunders DF. Themoluminescence as a research tool. Science. 1953;117:343–349.

[10] Zeller EJ, Wray JL, Daniels F. Factors in age determination of carbonate sediments by thermoluminescence Bulletin of the American Association of Petroleum Geologists. 1957;41:121–129.

[11] McDougal DJ, editor. Themoluminescence of Geological Materials. New York: Academic Press; 1968.

[12] Aitken MJ, Tite MS, Reid J. Thermoluminescence dating of ancient ceramics. Nature. 1964;202:1032–1033.

[13] Kennedy GC, Knopff L. Dating by thermoluminescence. Archaeology. 1960;13:147–148.

[14] Grogler N, Houtermans FG, Stauffer H. About the dating of ceramics and bricks using thermoluminescence. Helvetica Physica Acta. 1960;(33):595–596.

[15] Bothner MH, Johnson NM. Natural thermoluminescent dosimetry of late Pleistocene perlagic sediments. Journal of Geophysical Research. 1969;74:5531–5338.

[16] Huntley DJ, Johnson HP. Thermoluminescence as a potential means for dating siliceous ocean sediments. Canadian Journal of Earth Sciences. 1976;13:593–596.

[17] Wintle AG, Huntley DJ. Thermoluminescence dating of a deep-sea sediment core. Nature. 1979;279:710–712.

[18] Wintle AG, Huntley DJ. Thermoluminescence dating of sediments. Quaternary Science Reviews. 1982;1:31–53.

[19] Shelkoplyas VN, Morozov GV. Some results of an investigation of Quaternary deposits by the thermoluminescence method. In: Materials on the Quaternary period of the Ukraine (for the VIIth International Quaternary Association Congress); 1965; Kiev: Nuakova Dumka; pp. 83–90.

[20] Morozov GV. The relative dating of Quaternary Ukranian sediments by the thermoluminescence method. In: Unpublished paper presented at the 8th International Quaternary Association Congress; Paris. Washington DC: US Geological Survey Library cat no. 208M8280.167; 1968.

[21] Shelkoplyas VN. Dating of the Quaternary deposits by means of thermoluminescence . In: Chronology of the Glacial Age. Leningrad: Geographic Society of the USSR, Pleistocene Commission; 1971. pp. 155–160.

[22] Shelkoplyas VN, Il'ichev VA, Svitoch AA. Thermoluminescence dating of recent sediments of the Ob Plateau and Gorray Altai. Doklady Academii Nauk SSSR. 1973;**212**:935–937.

[23] Dobredeyev OP, Il'ichev VA. Age of loess and fossil soils of the Russian Plain and their correlation with glacial events of the Pleistocene. Dolkady Akademii Nauk SSSR. 1974;**214**:401–403.

[24] Faustov SS, Il'ichev VA, Bol'shakov VA. Paleomagnetic and thermoluminescence analyses of the Likhvin section. Dolkady Akademii Nauk SSSR. 1974;**214**:1160–1162.

[25] Shelkoplyas VN. The age determination of loess deposits. In: Zubakov VA, editor. Geokronologiya SSSR 3. Leningrad;1974. p. 31–35.

[26] Hutt GI, Raukas AV. Perspectives for using the thermoluminescence method for determining the age of Quaternary deposits. Bulletin of the Commission for the Study of the Quaternary Period. 1977;**47**:77–83.

[27] Troitsky L, Punning JM, Hutt G, Rajamae R. Pleistocene glaciation chronology of Spitsbergen. Boreas. 1979;**8**:401–407.

[28] Borsy Z, Flerservalvi J, Szabo PP. Thermoluminescence dating of several layers of loess sequences at Paks Mende (Hungary). Acta Geologica of the Academy of Science, Hungary. 1979;**22**:451–459.

[29] Linder L, Proszyriski M. Geochronology of the Pleistocene deposits exposed at Washock, northern part of the Holy Cross Mtns. Acta Geologica Polonica. 1979;**29**:121–132.

[30] Li JL, Paie JX, Wang ZZ, Lu YC. A preliminary study of both the thermoluminescence of quartz powder in loess and the age determination of the loess layers. Kexue Tongbau. 1977;**22**:498–502.

[31] Hütt GI, Smirnov AV. Dosimetric properties of natural quartz and its possibilities in thermoluminescent dating of geological objects. Izvestiya Akademii Nauk SSSR, Seriya Fizichskaya. 1977;**41**:1367–1369.

[32] Hütt G, Vares K, Smirnov A. Thermoluminescent and dosimetric properties of quartz from Quaternary deposits. Academii Nauk ESSR, Khimiia-Geologia. 1977;**26**:275–283.

[33] Hütt G, Smirnov A, Tale I. On the application of thermoluminescence of natural quartz to the study of geochronology of sedimentary deposits. PACT. 1978;**3**:262–373.

[34] Wintle AG, Huntley DJ. Thermoluminescence dating of sediments. PACT. 1979;**3**:374–380.

[35] Wintle AG, Huntley DJ. Thermoluminescence dating of ocean sediments. Canadian Journal of Earth Science. 1980;**17**:348–690.

[36] Huntley DJ. On the zeroing of the thermoluminescence of sediments. Physics and Chemistry of Minerals. 1985;**12**:122–127.

[37] Murray AS, Wintle AG. Luminescence dating of quartz using an improved single-aliquot regenerative protocol. Radiation Measurements. 2000;**32**:57–73.

[38] Murray AS, Wintle AG. The single aliquot regenerative dose protocol: potential improvements in reliability. Radiation Measurements. 2003;**37**:377–381.

[39] Wintle AG. Fifty years of luminescence dating. Archaeometry. 2008;**50**:276–312.

[40] Murray AS, Olley JM. Precis**io**n and accuracy in the optically stimulated luminescence dating of sedimentar**y** quartz: a status review. Geochronometria. 2002;**21**:1–16.

[41] Feathers JK. Use of luminescence dating in archaeology. Measurement Science and Technology. 2003;**14**:1493–1509.

[42] Lian OB. Optically-stimulated luminescence. In: Elias S, editor. Encyclopedia of Quaternary Science. 1st ed. Amsterdam: Elsevier Press; 2007. pp. 1480–1491.

[43] Duller GAT. Luminescence Dating: Guidelines on using Luminescence Dating in Archaeology. 1st ed. Swindon: English Heritage; 2008. 44 p.

[44] Fuchs M, Owen L. Luminescence dating of glacial and associated sediments: a review, recommendations and future directions. Boreas. 2008;**37**:636–659.

[45] Preusser F, Degering D, Fuchs M, Hilgers A, Kadereit A, Klasen N et al. Luminescence dating: basics, methods and applications. Eiszeitalter und Gegenwart Quaternary Science Journal. 2008;**57**(1–2):95–149.

[46] Rittenour TM. Luminescence dating of fluvial deposits: applications to geomorphic, palaeoseismic, and archaeological research. Boreas. 2008;**37**:613–635.

[47] Singhvi AK, Porat N. Impact of luminescence dating on geomorphological and palaeoclimate research in drylands. Boreas. 2008;**37**:536–558.

[48] Rhodes EJ. Luminescence dating of sediment over the past 200,000 years. Annual Reviews in Earth and Planetary Sciences. 2011;**39**:461–488.

[49] Liritzis I, Singhvi AK, Feathers JK, Wagner GA, Kadereit A, Zacharias N et al. Luminescence dating in archaeology, anthropology, and geoarchaeology. 1st ed. Berlin: Springer; 2013. 70 p.

[50] Aitken MJ. An Introduction to Optical Dating. 1st ed. Oxford: Oxford University Press; 1998. 267 p.

[51] Aitken MJ. Thermoluminescence Dating. 1st ed. London. Academic Press; 1985. 359 p.

[52] McKeever SWS, Moscovitch M, Townsend PD. Thermoluminescence Dosimetry: Properties and Uses. 1st ed. Ashford: Unclear Publishing Technology; 1995. 304 p.

[53] Prescott JR, Hutton JT. Cosmic ray contributions to dose rates for luminescence and ESR dating; large depths and long-term time variations. Radiation Measurements. 1994;**23**:497–500.

[54] Richter D, Richter A, Dornich K. Lexsyg–a new system for luminescence research. Geochronometria. 2011;**20**:220–228.

[55] Bøtter-Jensen L. Luminescence techniques: instrumentation and methods. Radiation Measurements. 1997;**17**:749–768.

[56] Stokes S. Luminescence dating applications in geomorphological research. Geomorphology. 1999;**59**:153–171.

[57] Godfrey-Smith DI, Huntley DJ, Chen WH. Optical dating studies of quartz and feldspar sediment extracts. Quaternary Science Reviews. 1988;**7**:373–380

[58] Fleming SJ. Thermoluminescence dating: refinement of the quartz inclusion method. Archaeometry. 1970;**12**:133–145.

[59] McKeever SWS. Thermolum**i**nescence of Solids. 1st ed. Cambridge: Cambridge University Press; 1985. 390 p.

[60] Wintle AG. Luminescence dating: **l**aboratory procedures and protocols. Radiation Measurements. 1997;**27**:769–817.

[61] Bailey RM, Smith BW, Rhodes EJ. Partial bleaching and the decay form characteristics of quartz OSL. Radiation Measurements. 1997;**27**:124–136

[62] Jain M, Murrary AS, Bøtter-Jensen L. Characterisation of blue-light stimulated luminescence components in different quartz samples: implications for dose measurement. Radiation Measurements. 2003;**37**:441–449.

[63] Bulur E. An alternative technique for optically stimulated luminescence (OSL) experiment. Radiation Measurements 1996; **26**: 701–709

[64] Wintle AG. Anomalous fading of thermoluminescence in mineral samples. Nature. 1973;**245**:143–144.

[65] Lamothe M, Auclair M. A solution to anomalous fading and age shortfalls in optical dating of feldspar minerals. Earth and Planetary Science Letters. 1999;**171**:319–323.

[66] Huntley DJ, Lamothe M. Ubiquity of anomalous fading in feldspars and the measurements and correction of it in optical dating. Canadian Journal of Earth Sciences. 2001;**38**:1093–1106.

[67] Huntley DJ, Godfrey-Smith DI, Thewalt MLW, Berger GW. Thermoluminescence spectra of some mineral samples relevant to thermoluminescence dating. Journal of Luminescence. 1988;**39**:123–136.

[68] Krbetschek MR, Gotze J, Dietrich A, Trautmann T. Spectral information from minerals. Radiation Measurements. 1997;**27**:495–748.

[69] Godfrey-Smith DI, Huntley DJ, Chen WH. Optical dating studies of quartz and feldspar sediment extracts. Quaternary Science Reviews. 1988;**7**:373–380.

[70] Li SH, Tso MYW. Lifetime determination of OSL signals from potassium feldspar. Radiation Measurements. 1997;**27**:119–121.

[71] Bøtter-Jensen L, Dull**er** GAT, Poolton NRJ. Excitation and emission spectrometry of stimulated luminescence from quartz and feldspars. Radiation Measurement. 1994;**23**:613–616.

[72] Hutt G, Jack I, Tchonka J. Optical dating: K–feldspars optical response stimulation spectrum. Quaternary Science Reviews. 1988;**7**:381–386.

[73] Barnett SM, Baillif IK. Infrared stimulation spectra of sediment containing feldspar. Radiation Measurements. 1997;**27**:237–242.

[74] Huntley DJ, Godfrey-Smith DI, Haskell EH. Light-induced emission spectra from some quartz and feldspars. Nuclear Tracks and Radiation Measurements. 1991;**18**:127–131.

[75] Krbetscheck MR, Rieser U, Stloz W. Optical dating: some dosimetric properties of natural feldspars. Radiation Measurements. 1996;**27**:107–117.

[76] Ugumori T, Ikeya M. Luminescence of $CaCO_3$ under $N_2$ laser excitation and application to archaeological dating. Japanese Journal of Applied Physics. 1980;**19**:459–465.

[77] Templer RH, Smith BW. Auto-regenerative TL dating with zircon from fired materials. Nuclear Tracks and Radiation Measurements. 1988;**14**:329–332.

[78] Smith BW. Zircons from sediments: a combined OSL and TL auto-regenerative dating technique. Quaternary Science Reviews. Quaternary Science Reviews. 1988;**7**:401–406.

[79] Pietsch TJ, Olley JM, Nanson GC. Fluvial transport as a natural luminescence sensitizer of quartz. Quaternary Geochronology. 2008;**3**:365–376.

[80] Duller GAT. Equivalent dose determination using single aliquots. Nuclear Tracks and Radiation Measurements. 1991;**18**:371–378.

[81] Galloway RB. Equivalent dose determination using only one sample: alternative analysis of data obtained from infrared stimulated luminescence of feldspars. Radiation Measurements. 1996;**23**:103–106.

[82] Mejdhal V, Bøtter-Jensen L. Luminescence dating of archaeological materials using a new technique based on single aliquot measurements. Quaternary Science Reviews (Quaternary Geochronology). 1994;**13**:551–554.

[83] Murray AS, Roberts RG. Measurement of the equivalent dose in quartz using a regenerative-dose single aliquot protocol. Radiation Measurements. 1998;**29**:503–515.

[84] Wintle AG, Murray AS. A review of quartz optically stimulated luminescence characteristics and their relevance in single-aliquot regeneration dating protocols. Radiation Measurements. 2006;**41**:369–391.

[85] Duller GAT, Bøtter-Jensen L, Koshiek P, Murray AS. A high sensitivity optically stimulated luminescence scanning system for measurement of single sand-sized grains. Radiation Protection Dosimetry. 1999;84:325–330.

[86] Olley JM, Roberts RG, Yoshida, Bowler JM. Single-grain optical dating of grave infill associated with human burials at Lake Mungo, Australia. Quaternary Science Reviews. 2006;**25**:2469–2474.

[87] Olley JM, Catcheon GG, Roberts RG. The origin of dose distribution in fluvial sediments, and the prospect of dating single grains from fluvial deposits using optically stimulated luminescence. Radiation Measurements 1999; 30:207-217.

[88] Ballarini M, Wallinga J, Murray AS, van Heteren S, Oost AP, Bos AJJ et al Optical dating of young coastal dunes on a decadal time scale. Quaternary Science Reviews. 2003;**22**:1011–1017.

[89] Pawley SM, Bailey RM, Rose J, Moorlok BSP, Hamblin RJO, Booth SJ et al Age limits on Middle Pleistocene glacial sediments from OSL dating, north Norfolk, UK. Quaternary Science Reviews. 2008;**27**:1363–1377.

[90] Buylaert JP, Murray AS, Huot S. Optical dating of an Eemian site in northern Russia using K-feldspar. Radiation Measurements. 2008;**43**:505–512.

[91] Ichikawa Y. Dating of ancient ceramics by thermoluminescence. Bulletin of the Institute of Chemical Research, Kyoto University. 1965;**43**:1–6.

[92] Mejdahl V. TL dating based on feldspars. Nuclear Tracks and Radiation Measurements. 1985;10:133-136.

[93] Stevens T, Armitage SJ, Lu H, Thomas DSG. Examining the potential of high–resolution OSL dating of Chinese loess. Quaternary Geochronology. 2007;2:15-22.

[94] Munyikwa K, Telfer M, Baker I, Knight C. Core drilling of Quaternary sediments for luminescence dating using the Dormer DrillmiteTM. Ancient TL. 2011;29:15-22.

[95] Stokes S. Optical dating of young sediments using quartz. Quaternary Science Reviews (Quaternary Geochronology). 1992;11:153-159.

[96] Rees-Jones J. Dating young sediments using fine grained quartz. Ancient TL. 1995;13:9-14.

[97] Munyikwa K. Luminescence Chronology. In: Mörner NA, editor. Geochronology: Methods and Case Studies. 1st ed. Rijeka: Intech; 2014. pp. 31–71.

[98] Vandiver PB, Soffer O, Klima B, Svoboda J. The origins of ceramic technology at Dolni Vestonice, Czecholosvakia. Science. 1989;**246**:1002–1008.

[99] Mejdahl V. Feldspar inclusion dating of ceramics and burnt stones. PACT. 1983;**9**:351–364.

[100] Berger GW, Huntley DJ. Dating volcanic ash by thermoluminescence. PACT. 1983; **9**:581–592.

[101] Sutton SR. TL measurements on shock-metamorphosed sandstone and dolomite from Meteor Crater, Arizona: Pt. 1 shock dependence of TL properties. Journal of Geophysical Research. 1985;**9**:3683–3689.

[102] Pye K, Tsoar H. Aeolian Sand and Sand Dunes. 2nd ed. Heidelberg: Springer. 2009. 458 p.

[103] Stokes S, Gaylord DR. Optical dating of Holocene dune sands in the Ferris dune field, Wyoming. Quaternary Research. 1993;**39**:274–281.

[104] Munyikwa K. Synchrony of southern hemisphere late Pleistocene arid episodes: a review of luminescence chronologies from arid aeolian landscapes south of the Equator. Quaternary Science Reviews. 2005;**24**:2555–2583.

[105] Wolfe SA, Huntley DJ, Ollerhead J. Relict Late Wisconsinan dune fields of the Northern Great Plains. Canada. Geographie Physique et Quaternaire. 2004;**58**:323–336.

[106] Munyikwa K, Feathers J, Rittenour T, Shrimpton H. Constraining the Late Wisconsinan retreat of the Laurentide Ice Sheet from western Canada using luminescence ages from postglacial eolian dunes. Quaternary Geochronology. 2011;**6**:407–422.

[107] Frechen M. Luminescence dating of loessic sediments from the Loess Plateau, China. Geologische Rundschau. 1999;**87**:675–684.

[108] Munyikwa K, Choi JH, Choi KH, Byun JM, Kim JW, Park K. Coastal dune luminescence chronologies indicating a mid-Holocene highstand along the east coast of the Yellow Sea. Journal of Coastal Research. 2008;**24**:92–103.

[109] Lai Z, Fan A. Examining quartz OSL age underestimation for loess samples from Luochuan in the Chinese loess plateau. Geochronometria. 2013;**41**:57–64.

[110] Cunningham AC, Wallinga J. Realizing the potential of fluvial archives using robust OSL chronologies. Quaternary Geochronology. 2012;**12**:98–106.

[111] Thrasher IM, Mauz B, Chiverrell RC, Lang A. Luminescence dating of glaciofluvial deposits: a review. Earth Sciences Reviews. 2009;**97**:133–146.

[112] Rhodes EJ, Bailey RM. The effect of thermal transfer on the zeroing of the luminescence of quartz from recent glaciofluvial sediments. Quaternary Geochronology. 1997; **16**:291–298.

[113] Munyikwa K, Brown S, Kitabwala Z. Delineating stratigraphic breaks at the bases of postglacial dunes in central Alberta, Canada using a portable OSL reader. Earth Surface Processes and Landforms. 2012;**37**:1063–1614.

[114] Huntley DJ, Clague JJ. Optical dating of tsunami-laid sands. Quaternary Research. 1996;**46**:127–140.

[115] Lowick SE, Preusser F. Investigating age underestimation in the high dose region of optically stimulated luminescence using fine grain quartz. Quaternary Geochronology. 2011;**6**:33–41.

[116] Timar-Gabor A, Constanin D, Buylaert JP. Fundamental investigations of natural and laboratory generated SAR dose response curves for quartz OSL in the high dose range. Radiation Measurements. 2015;**81**:150–156.

[117] Thompsen KJ, Murray AS, Jain M, Bøtter-Jensen L. Laboratory fading rates of various luminescence signals from feldspar-rich sediment extracts. Radiation Measurements. 2008;**49**:1474–1486.

[118] Buylaert JP, Yeo EY, Thiel C, Yi S, Stevens T, Thompson W, Frechen M, et al A detailed post-IR IRSL chronology for the last interglacial soil at the Jingbian loess site (northern China) Quaternary Geochronology. 2015;**30**:194–199.

[119] Sanderson DCW, Murphy S. Using simple portable measurements and laboratory characterisation to help understand complex and heterogeneous sediment sequences for luminescence dating. Quaternary Geochronology. 2010;**5**:299–305.

Chapter 12

# Bioluminescent Fishes and their Eyes

José Paitio, Yuichi Oba and
Victor Benno Meyer-Rochow

Additional information is available at the end of the chapter

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**Abstract**

What shaped the evolution of vision in fish more than anything else is the need to see, be it to avoid obstacles or find shelters, and recognize conspecifics, predators and prey. However, for vision to be effective, sufficient light has to be available. While there is no shortage of light in shallow water depths, at least during the day, the situation for species occurring at greater depths is a different one: they live in an environment where sunlight does not reach, but which nevertheless, is not totally devoid of light. Numerous marine organisms, including fishes of at least 46 families, possess the ability to 'bioluminesce', i.e. they can produce biological light. This chapter focuses on the interaction between bioluminescence and specific photoreceptor adaptations in fishes to detect the biological light.

**Keywords:** bioluminescence, light, sea, eye, vision, fish

## 1. Introduction

Bioluminescence is rare, but widespread. This seemingly contradictory statement refers to the fact that there is almost no animal phylum that does not have at least a few bioluminescent species, even if the vast majority are non-luminescent. Freshwater contains the smallest number of bioluminescent species; terrestrial environments possess a slightly greater number, but the oceans are inhabited by a wide variety of bioluminescent creatures and the vertebrate class of Pisces represents one of the best examples of this [1]. When one thinks of the evolutionary plasticity of fishes and the fact that most of the ocean is dark, it is not surprising that these animals show a high degree of remarkable luminous features. In fact, fishes present the most diverse and complex examples of bioluminescent adaptations in the world [2].

In an environment where organic light is used for different biological purposes [1, 3], light perception may be a determinant factor for the survival and success of the species involved [4]. The eyes of all animals on the planet are adapted to the light regimes of the habitat they are meant to function in [5] and bioluminescent fishes are no exception. The visual capacities of oceanic fishes are adapted not only to the spectral properties and intensity of downwelling sunlight at depths the fish inhabit, but also to the bioluminescence in their environment [6]. Vision and bioluminescence (at least in fishes) are likely to share a common evolutionary history, which is reflected in the bioluminescent fishes' ontogeny, behaviour and ecology.

## 2. Taxonomy of luminous fishes

While preparing this chapter, the authors found records of bioluminescence for around 1500 species of fish, but most likely this number is too low as some records of luminescent species may still be missing. The tally of luminescent fishes represents about 8% of the approximately 20,000 known species [7]. At least 43 families of 11 orders of bony fishes and 3 families of 1 order of sharks are luminous (**Table 1**). There are families with no luminescent representatives and there are families in which the luminescent species are more abundant than in some other families, which is often a reflection of the habitat that these species inhabit (see next section of this chapter).

| **Order (total number of families)** | **Family** | **Biolumine scent genus** | | **Functions[a]** |
|---|---|---|---|---|
| | | **Ratio[b]** | **Genus** | |
| **Cartilaginous fishes** | | | | |
| Squaliformes (7) | Dalatiidae | 7/7 | All [8] | L [8], CI [8] |
| | Etmopteridae | 5/5 | All [8] | M, CI, S[c] [92], A [67] |
| | Somniosidae | 1/5 | *Zameus squamulosus* [9] | ? |
| **Bony fishes** | | | | |
| Anguilliformes (16) | Congridae | 1/30 | *Lumiconger arafura* [16] | ? |
| Aulopiformes (16) | Chlorophthalmidae | 1/2 | *Chlorophthalmus* [2] | S [60] |
| | Evermannellidae | 2/3 | *Cocorella atrata* [2], *Odontostomops normalops* [11] | ? |
| | Paralepididae | 2/12 | *Lestidium, Lestroplepis* [23] | CI [4] |
| | Scopelarchidae | 2/3 | *Benthalbella, Scopelarchoides* [95] | R [95], CI [4] |
| Batrachoidiformes (1) | Batrachoididae | 1/22 | *Porichthys* [21] | M [4], L [21], CI, I [33], A [2] |

| Order (total number of families) | Family | Biolumine scent genus | | Functions[a] |
|---|---|---|---|---|
| | | Ratio[b] | Genus | |
| Beryciformes (7) | Anomalopidae | 6/6 | All [12] | M, L [33], S, I, Br [62] |
| | Monocentridae | 2/2 | All [26] | L, I [53] |
| | Trachichthyidae | 1/18 | *Aulotrachiththys* [2] | CI [21] |
| Clupeiformes (7) | Engraulidae | 1/17 | *Coilia dussumieri* [15] | CI [33] |
| Gadiformes (10) | Macrouridae | 14/23 | *Cetonurus, Coelorinchus, Haplomacrurus*[d], *Hymenocephalus* [23], *Kumba* [24], *Lepidorynchus* [23], *Lucigadus* [24], *Malacocephalus, Nezumia, Odontomacrurus, Sphagemacrurus* [23], *Spicomacrurus* [24], *Trachonurus, Ventrifossa* [23] | S [60], I [30] |
| | Merlucciidae | | *Steindachneria argentea* [24] | ? |
| | Moridae | 5/11 | *Gadella* [23], *Physiculus* [23], *Salilota* [24], *Tripterophycis* [23] | ? |
| Lophiiformes (18) (Suborder Ceratioidei) | Centrophrynidae | 1/1 | All [14] | M, L, I [14], Sa, Ss [41] |
| | Ceratiidae | 2/2 | All[14] | |
| | Diceratiidae | 2/2 | All [14] | |
| | Gigantactinidae | 1/2 | *Gigantactis* [14] | |
| Lophiiformes (18) (Suborder Ceratioidei) | Himantolophidae | 1/1 | All [14] | |
| | Linophrynidae | 5/5 | All [14] | |
| | Melanocetidae | 1/1 | All [14] | M, L, I [14], Sa, Ss [41] |
| | Oneirodidae | 16/16 | All [14] | |
| | Thaumatichthyidae | 2/2 | All [14] | |
| (non-Ceratioidei) | Ogcocephalidae | 1/10 | *Dibranchus atlanticus* [17] | ? |
| Myctophiformes (2) | Myctophidae | 34/34 | All [11] | R [32], M [4], S [59], CI [2], I [1], Sa [2] |
| | Neoscopelidae | 1/1 | *Neoscopelus* [15] | CI [11], L [20] |
| Osmeriformes (14) | Alepocephalidae | 4/19 | *Microphotolepis, Photostylus, Rouleina, Xenodermichthys* [23] | CI [57], I [30] |

| Order (total number of families) | Family | Biolumine scent genus | | Functions[a] |
|---|---|---|---|---|
| | | Ratio[b] | Genus | |
| Perciformes (164) | Microstomidae | 1/3 | *Nansenia* [100] | ? |
| | Opisthoproctidae | 3/8 | *Opisthoproctus, Rhynchohyalus, Winteria* [23] | CI [21] |
| | Platytroctidae | 13/13 | All [13] | R, CI, Ss [13] |
| | Acropomatidae | 2/8 | *Acropoma* [23], *Synagrops* [17] | CI [21] |
| | Apogonidae | 5/32 | *Apogon, Archamia* [23], *Jaydia* [18], *Rhabdamia, Siphamia* [23] | M, L, CI [51], I [33] |
| | Chiasmodontidae | 1/4 | *Pseudoscopelus* [23] | CI [21] |
| | Epigonidae | 3/7 | *Epigonus, Florenciella, Rosenblattia* [23] | ?, Ss [2] |
| | Howellidae | 1/3 | *Howella* [23] | ? |
| | Leiognathidae | 7/7 | All [18] | R, M, L, S, CI, I, Sa [53] |
| | Pempheridae | 2/2 | *Pempheris, Parapriacanthus* [23] | CI [33] |
| | Scianidae | 3/65 | *Collichthys* [23], *Larimichthys, Sonorolux* [106] | CI [33] |
| Saccopharyngiformes (4) | Eurypharyngidae | 1/1 | All [2] | ? |
| | Saccopharyngidae | 1/1 | All [22] | L [15] |
| Stomiiformes (4) | Gonostomatidae | 8/8 | All [22] | CI [11] |
| | Phosichthyidae | 7/7 | All [22] | ? |
| | Sternoptychidae | 10/10 | All [22] | CI [2] |
| | Stomiidae | 27/27 | All [22] | R [2], M [46], L [4], S [46], CI [2], I [48], A [46], Sa, Br [4] |

[a]R—recognition; M—mate; L—lure; S—school; CI—counterillumination; I—illumination; A—aposematism; Br—blink and run; Sa—startle predators; Ss—smokescreen; ?—unreported functions in the references.
[b]Ratio, the number of the genera containing luminous species/the total number of the genera in the family; All, all the species are luminous.
[c]Etmopterids also seem to use its photophore patterns to coordinate hunting in schools [8].
[d]Bioluminescence in *Haplomacrurus* is only stated by Herring [23] and no other references found to contradict or agree with this information. The taxonomy used in this table is in accordance with the WoRMS Editorial Board, 2016 [7]. The taxonomic data used in this table are included and available online in the website of the Living Light List Project (http://www3.chubu.ac.jp/faculty/oba_yuichi/Living%20Light%20List/).

**Table 1.** Bioluminescent families and genera of fishes and respective suggested ecological functions.

In cartilaginous fishes, bioluminescence is not as common as in bony fishes. Only three families of luminous sharks are known, Dalatiidae, Etmopteridae and Somniosidae [8, 9]. Luminescence in rays and skates as well as in Holocephali seems non-existent or has not been confirmed. In the luminescent sharks, numerous small light organs are predominantly present in the ventral region of the body, but lower densities can even reach the dorsal areas in dalatiid species [10]. Luminous sharks, like the majority of fish species, possess external light organs with intrinsic light production, which are denominated as photophores [8, 11].

In bony fishes, bioluminescence reaches its zenith in terms of complexity and diversity of light organs and their corresponding biological and ecological functions. It is common to find whole families of bioluminescent species, such as the Myctophidae [11], Leiognathidae, Anomalopidae [12], Platytroctidae [13] and several families of anglerfishes [14]. In such cases, the patterns or shapes of the light organs are generally species-specific and can even be used for taxonomic purposes. Nevertheless, the most common scenario is that only some genera of fishes are bioluminescent as, for example, in families of the order Perciformes with very few luminescent species or in Macrouridae with considerably more luminescent species [2]. An uncommon scenario is when there is only a single luminous species known in an entire order as is the case with *Coilia dussumieri* in the Clupeiformes [15] and *Lumiconger arafura* in the order Anguilliformes [16, 17]. Equally uncommon is it to find only one luminescent species within a family as for the merluciid *Steindachneria argentea* [2] and the ogcocephalid *Dibranchus atlanticus* [17]. On the other hand, we have the order Stomiiformes, in which all of its species produce their own light [1].

Generally speaking, the origin of the light emissions do not vary much within families, being either bacterial or intrinsic. Exceptions exist in the Apogonidae where only *Siphamia* species are known to have bacterial symbionts whereas the remainder of the luminescent species employ intrinsic bioluminescence with *Cypridina* luciferin [18]. A rare case is observed in anglerfishes of the families Lynophrinidae and Centrophrynidae which have an intrinsic bioluminescent barbel in addition to the symbiotic luminous lure of the first ray of the dorsal fin that projects from the dorsum of the anglerfishes and is known as the esca [14]. The location of the light organs is also generally similar within a family, but exceptions exist, namely in families where all the species are luminous. In Myctophidae, for example, only members of the genus *Diaphus* have head photophores and unlike fishes of some other genera do not possess caudal glands. Another example is that of luminous barbels, present in some species of Stomiidae [15]. Indirect bioluminescence is produced by internal luminous organs that depend on transparent tissues underneath to emit diffuse light from the ventral part of the body. Although this method is more common in the shallow-water species like those of the families Leiognathidae and Apogonidae [19], it can also be found in some fishes of greater depths as from the families of Opisthoproctidae [12] and Evermannellidae [2].

### 2.1. Dubious species

Misidentifications, confusion of non-luminous tissues and luminous bacteria on damaged specimens [20] are common sources of errors that led to reports of the luminous species, which do not exist or are not luminescent at all. Furthermore, unusual phylogenetic and habitat

placements in records for certain species claimed to be luminous, apparently backed up with dubious references, are suspicious vis-à-vis the phylogeny and habitat preferences of the accepted luminous species and therefore cast doubt on the validity of some records. Another problematic issue are contradictory statements, when some authors report a species to be luminescent and others contradict such statements. As the authors of this chapter did not have the chance to observe and confirm the status of luminosity of all the species mentioned, they decided to be neutral and include in the chapter cases that could be dubious.

The batoid *Benthobatis moresbyi* [20] is one of the most doubtful cases of bioluminescence reported in fishes. Sharks of the genus *Somniosus* were erroneously categorized as bioluminescent [2]. The megamouth shark possesses reflective tissue along its upper jaw, which can be used to attract its prey through reflecting the bioluminescence of other animals [1].

In bony fishes, reports of luminescent members of opisthoproctids, *Dolichopteryx, Bathylychnops* and *Bathylagus* have been shown to be erroneous [2]. Some trachichthyid genera like *Hoplostethus* [21] and *Sorosichthys* [22] may have luminous members, but there is no convincing proof of their bioluminescence. In macrourids, the genera *Coryphaenoides, Mesobius, Pseudocetonurus, Macrourus* and *Idiolophorhynchus andriashevi* are stated as luminous by some, but not all investigators [21, 23, 24]. *Antimora* is another uncertain case among otherwise luminescent morids [2, 23].

Apart from the well-known anglerfishes, two unrelated species of shallower water lophiiformes, *Antennarius hispidus* and *Chaunax* [20, 25] are described as luminous based on a single reference. There are also some species of ogcocephalids, which have dubiously been reported as luminous [21, 23]. Bioluminescence in cetomimids has been stated by reference [21], but other authors disagree [26]. Reference [21] consider luminescence in the species of the families Halosauridae and Brotulidae and the gempylidae genus *Ruvettus* as dubious. The uncertainty of bioluminescence in *Kasidoron* and *Bathylagus* has been discussed [2] and possible luminescence in *Derichthys serpentines* has been muted [27] but definitive proof is lacking. The silaginid *Sillago bassensis* was found with bioluminescent bacteria [28] probably attached to its skin; this might also have been the explanation for the dorsal luminescence of a flying fish reported in Japan [29].

## 3. Habitat dispersal of luminous fishes

Light penetrates the sea and is then subjected to scattering by air bubbles and water molecules [3], phytoplankton and zooplankton as well as suspended particles [30]. This essentially imposes limits on the use of vision in the aquatic environment [31]. Major light attenuation occurs in the epipelagic zone where the planktonic biomass, dissolved nutrients and floating debris 'filter' most of the light, reducing its intensity and altering its spectral composition. However, the angle of this light is almost completely vertical apart from very near the surface [32]. Not many bioluminescent fishes inhabit the epipelagic zone (**Figure 1**) and in those that are present, photoreceptive structures and visually induced behaviours seem less complex than that encountered in species inhabiting deeper habitats [33]. This seems likely to be related

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to two main factors. Primarily, at these shallow depths light intensities, especially during the day, are too high for luminescence [4, 31] to be useful for ecological purposes without investing a considerable energy [32]. Secondly, if a very strong light were to be produced, it would readily be spotted by keen eyes of the abundant photopic predators around [32, 33]. The bioluminescent epipelagic species are mainly active at night [34] and use internal counterillumination to deter and avoid dusk-active piscivorous predators [19, 33]. Some exceptions are the headlights of anomalopid and monocentrid species [12, 35] and fishes with photophores, like the batrachoidid *Porichthys*.

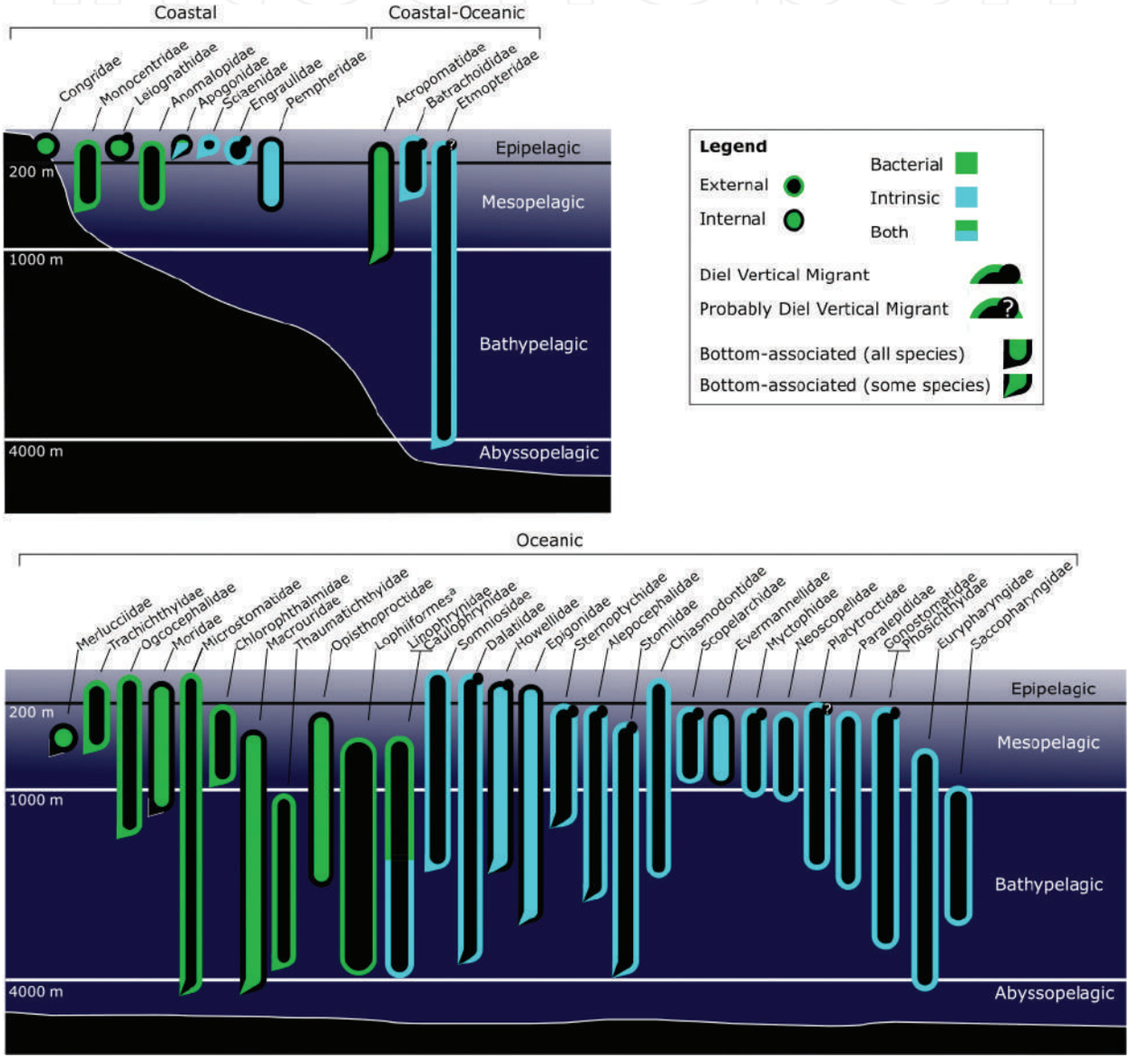


**Figure 1.** Schematic representation of the spatial distribution of bioluminescent fish families and some ecological details.[a] Lophiiformes families other than Thaumatichthyidae, Linophrynidae, Caulophrynidae and Ogcocephalidae. The data presented only refer to species that are known as bioluminescent. The order of disposition within the coastal, coastal-oceanic and oceanic categories is ecological and not spatial. References used for spatial distribution and diel vertical migrations [10, 13, 15, 26, 40, 65, 79, 95–99] and for bioluminescence [2, 9, 16, 21, 24, 47, 48, 50, 58, 60, 67, 100].

Only dim light from the short wavelength spectra of the blue–green component of sunlight (but no moonlight) reaches the mesopelagic zone [30]. In contrast to the epipelagic zone, the mesopelagic zone is characterized by a uniformity of light in all lateral directions and the highest intensity from above [4, 30]. This is the oceanic 'bioluminescent hotspot' where more than 80% of the species present are bioluminescent [32, 36] and biological light reaches its greatest splendour in diversity and complexity of structures and purposes [37]. In terms of fish biomass, it is the mesopelagic zone that stands out [38]. Myctophids are one of the families of teleosts with a very high number of species, representing the second most abundant taxon of mesopelagic fishes. In terms of sheer numbers, the gonostomatid genera *Vinciguerria* and *Cyclothone* represent the most abundant fauna on the planet [11]. The mesopelagic zone is also the transference zone from an extended origin of light to a point source. The downwelling sunlight is the primary source of light in the upper mesopelagic zone (200–610 m depth) while bioluminescence takes over as the main source of light in the lower mesopelagic zone (610–1000 m depth). Under the essentially monochromatic scotopic light, the use of bioluminescence is far more advantageous for camouflage and vision, be it in the case of predators or prey, than at depths more brightly illuminated by a fuller spectrum of light. In the depths where silhouette distinction is of visual importance and it comes as no surprise that most of the bioluminescent fish species inhabit this realm. The colorations of the fish at greater depths are a reflection of the different light environment. In the upper mesopelagic silver bodies acting as mirrors and assisting in camouflaging are common [30]. The animals inhabiting the lower mesopelagic and bathypelagic tend to have darker bodies, preventing reflections of bioluminescence in these deeper realms [4, 21].

At 1000 m depth, sunlight is no longer strong enough to allow fishes to see in and that depth by definition corresponds to the beginning of the bathypelagic zone [30]. The latter is the aphotic zone where no sunlight is available [30] and complete homogeneity in terms of the light environment occurs in all directions [4, 31]. Here, light from above does not possess much relevance for the impoverished faunal assemblages present and the only source of photic stimulation, in contrast to that of the mesopelagic zone, is bioluminescence [3, 30]. Consequently, there is a drop in the number of bioluminescent species and individuals from that in the mesopelagic zone [2]. Even deeper living abyssal fishes, phylogenetically related to bathypelagic or mesopelagic luminous species, are not bioluminescent (e.g. macrourids) [4, 12].

It is not surprising that the majority of all bioluminescent fishes are oceanic and pelagic [33], as there are no structures blocking the path of the light in this environment, it seems to be optimal for the realization of many of the roles bioluminescence can ultimately be involved in [30, 37]. On the other hand, some benthopelagic species that are not known as vertical migrators possess ventral luminescence (e.g. morid species). These are puzzling cases of bioluminescence and its possible role [2], because emitting ventral light close to the bottom does not fulfil the purpose of camouflage. In fact, it may cause a reflection by the substrate and expose the fish [21]; however, there may be advantages too, which have not been looked into like, for instance, diverting attention and misguiding potential predators to the reflection rather than its originator. Coastal and benthic fishes more often employ bacterial luminescence while the pelagic species possess mostly intrinsic bioluminescence [2, 33, 39].

Diel vertical migrations are common among mesopelagic fishes [40] but are also known from some species of epipelagic [4, 41] and bathypelagic fish [4, 21]. These migrations do not always involve all taxa present [15] or genders in the same population and may vary geographically and seasonally. Generally speaking, the diel vertical migrant approaches surface waters at night to feed on zooplankton or other migrants when diurnally hunting visual predators are less active. The vertical movements of these individuals provide an important source of carbon (and other nutrients) to the deeper layers of the ocean [4].

## 4. Bioluminescence purposes in fishes

### 4.1. Light organs and control

Despite the extensive diversity of luminous organs in fishes, the basic structure is rather uniform [37] irrespective of whether we deal with photocytes or bacterial symbionts (**Figure 2**). The photogenic mass is situated at the centre and is associated with an external lens. An internal reflector is enclosed by a pigmented layer. Innervation of blood capillaries is also a common feature [2, 37]. Bacterial light organs usually possess an opening to exterior allowing the release of excess bacterial cells to the environment [42]. Photophores, with the exception of those in neoscopelids, are closed [2, 4].

The majority of the bioluminescent fishes have luminous tissues capable of generating light by themselves, but others maintain a culture of luminous symbiotic bacteria in their light organs [4, 39, 43]. The light organs of the species with symbiotic bacteria possess ducts in which they culture extracellular luminous bacteria [39]. In most cases, these bacteria are species like *Photobacterium*, but *Aliivibrio fischeri* and possibly *Vibrio harveyi* can also be found as symbionts in light organs of some luminous fishes [42]. Intrinsically luminescent fish possess photocytes with *Cypridina* luciferin and coelenterazine. *Cypridina* luciferin is only used by coastal fishes of the families Batrachoididae, Pempheridae and Apogonidae while coelenterazine has been suggested to occur in Stomiiformes, Myctophiformes and Platytroctidae [44]. The remainder of intrinsically bioluminescent fishes may belong to coelenterazine or unknown luciferin types.

A pigmented layer of cells surrounds the light organs internally to absorb stray light [2]. Below the light organ, there usually is a layer of cells with a reflecting material that redirects the light towards the lens [45]. Some species lack reflectors [20, 21], and in others, the reflectors may not be present in all types of luminous tissues, as stomiids [46]. In fishes with indirect bioluminescence, the reflector is located above the ventral diffusive tissues (see **Figure 3**) instead of being inside the light organ [47]. Reflectors affect the emission spectra in some fishes, as in alepocephalids, and myctophids that lack pigmented lenses in front of the light organ [48, 49]. When the light produced by the photogenic chamber is emitted to the outside of the photophores, it passes through the lens [45], whose pigments filter the emitted light and adjust its spectrum to match that of the downwelling light [3, 48]. Some fishes like the monocentrids lack lenses. In the case of *Cleidopus gloriamaris*, a red-orange skin tissue external to the light organ alters the spectrum of the light emission [12].

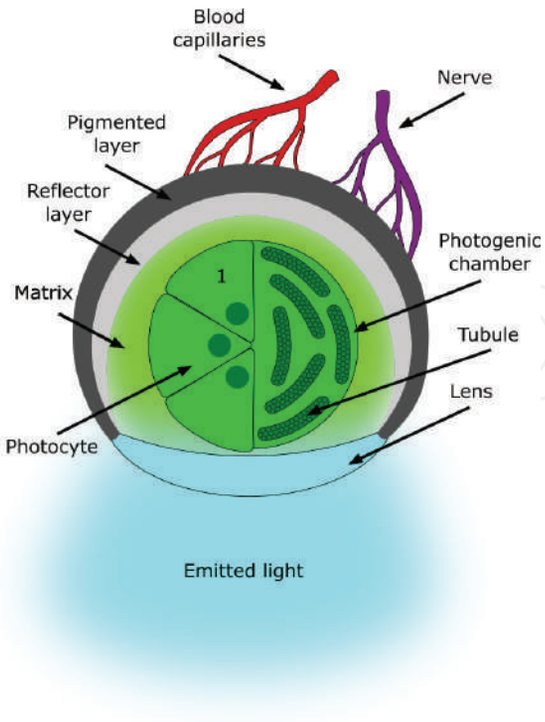


**Figure 2.** Schematic representation of the general structure of light organs in fishes. External side with light emission and internal side with blood capillaries and nerve supply. Matrix represents the space between the photogenic chamber and other structures. The right half of the photogenic chamber represents an intrinsic light organ with photocytes. The left half of the photogenic chamber represents a symbiotic light organ with transverse section of tubules filled with luminous bacteria.

Fishes with luminous symbiotic bacteria emit light continuously, but may have some control over the light by chromatophores [34] and nutrient supply to the bacteria [34, 43, 45]. Shutters are tissue patches with chromatophores that can partially or totally cover the light organs and are under muscular control [41, 50]. These shutters are different across genera of the same family and may occlude internal organs as in leiognathids and *Siphamia* or may be restricted to external organs as in anomalopids [41, 50, 51]. It is suggested that anglerfishes possess a similar mechanism to control the light emission from the escae [41]. Skin chromophores can be used to control light emissions in bioluminescent fishes with internal bacterial symbiotic organs (leiognathids [50], pempherids [47], acropomatids [19], evermannelids [2]) or externally (Gadiformes [52], Trachichtidae [12]), and bacterial symbionts in the lower jaw organs as in monocentrids [34]. There are some exceptions like *Opisthoproctus*, which may regulate light with ventral scale movements [2] and thaumatichthyids [41] and *Cleidopus gloriamaris* [12] that have light organs inside the mouth closing it to block the light emission. Leiognathids use branchiostegals to control their light emissions [53], and according to the observations of one of the authors (Yuichi Oba), the pigmented pelvic fins of *Chlorophthalmus* may serve the same purpose. The regulation of bacterial luciferase by oxygen and ion supply from the blood has been stated as a possibility, but there are no clear conclusions on this [39]. Observations by Meyer-Rochow [43] on *Anomalops katoptron* have shown that their bacterial light organs become successively dimmer when the fish starves.

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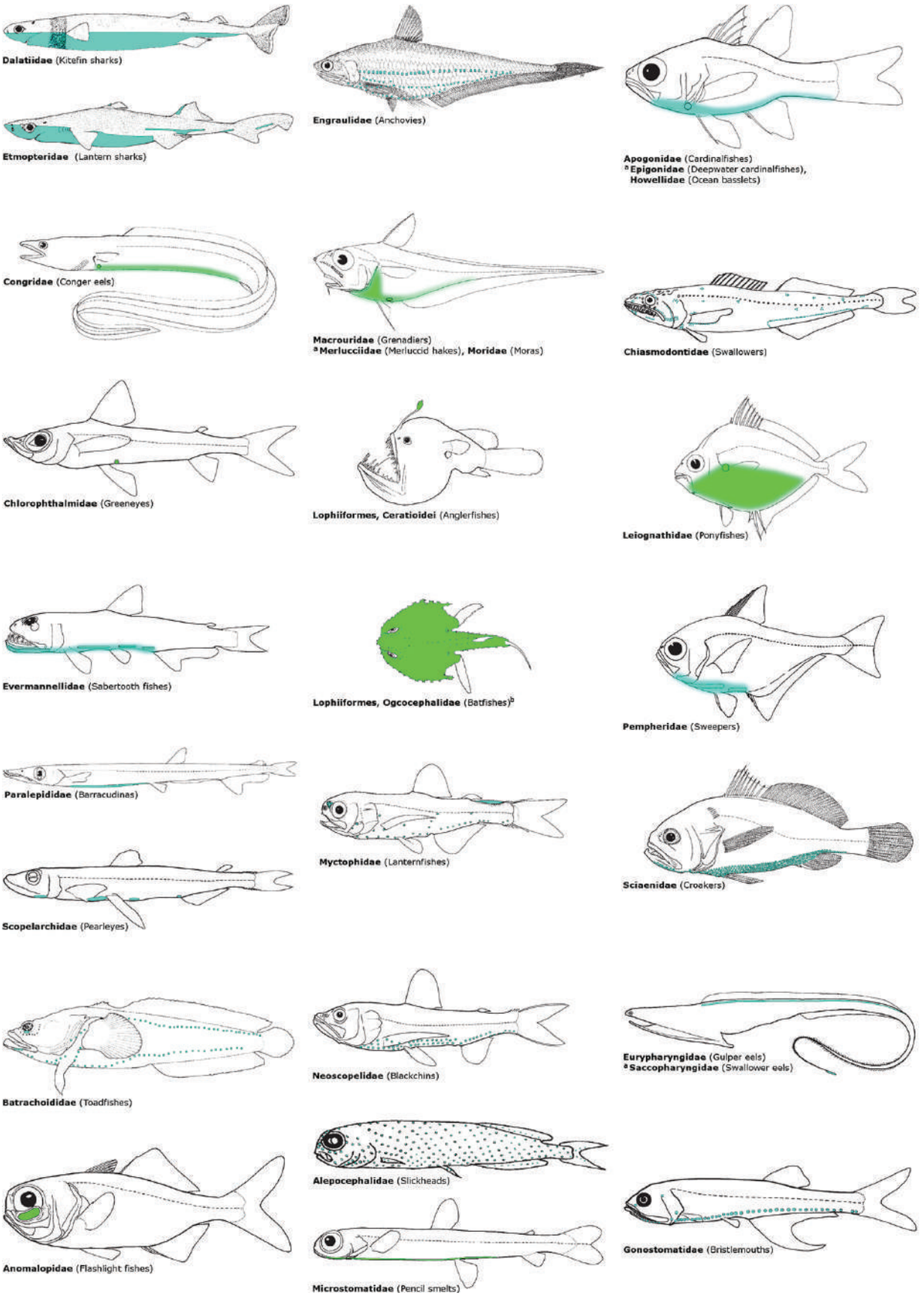

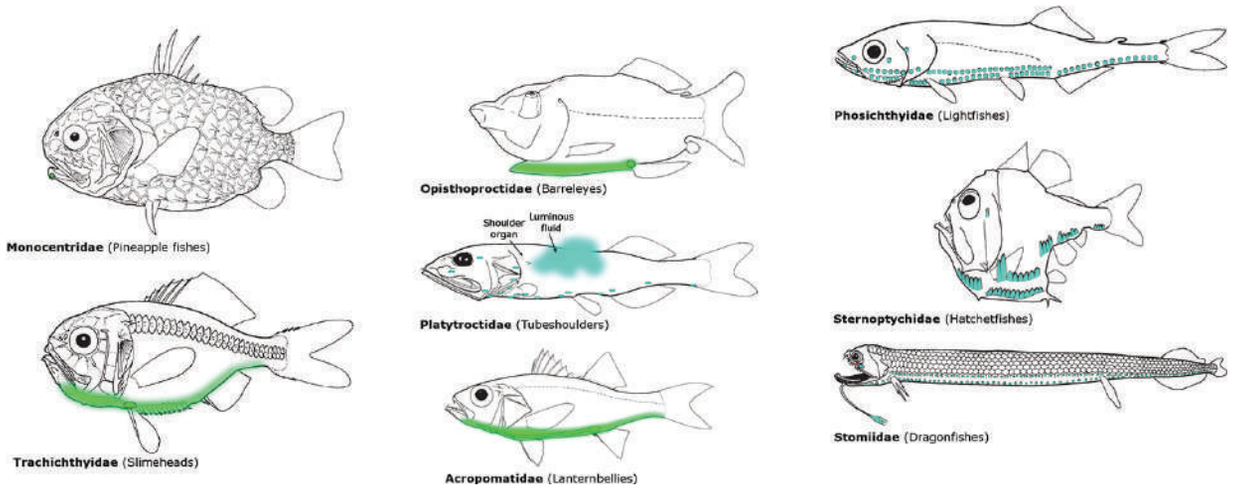


**Figure 3.** Light organs in fishes per family. Intrinsic bioluminescence in blue and symbiotic in green. The light organs are represented in coloured bands or circles delimited by a black outline. Blur coloured forms represent internal bioluminescence through diffusive tissues. [a]Used in cases that bioluminescent emissions and organs are very similar between families of the same order.[b] Crane [101] also found luminescence in skin samples of the ventral surface of the fish. Images are representative for the families and are not on scale. Same references used for bioluminescence in **Figure 1** plus [10, 13, 15, 95]. Source of drawings: Food and Agriculture Organization of the United Nations [15, 26, 96, 102–105]. Reproduced with permission.

Neural light emission control of intrinsic bioluminescence occurs in sharks [54], Stomiiformes [46, 55], myctophids, *Porichthys* [55] as well as alepocephalids [49] and proceeds through neurons in scopelarchids [56] and other coastal fishes besides *Porichthys* [33]. Neurons may be able to individually regulate light outputs of single photophores as in myctophids [55]. However, blood supply has also been suggested to be involved, at least in the control of the linophrynid's intrinsically luminous barbels [41].

Exceptions are platytroctids, which excrete luminous liquids; a process that invokes muscular control [2, 41]. In some anglerfishes, the liquid may not actually be secreted by the Escae, but from small symbiotic luminous bulbs (the so-called caruncles) that in the family Ceratiidae are dorso-posteriorly positioned from the escae [14].

Luminous organs are predominantly ventral [21, 37] (**Figure 3**) but some light tissues can be dorsal, as in the escae of anglerfishes and photophores in dalatiid sharks. Some fishes have buccal light organs like in the apogonids *Siphamia* spp. [18], the myctophid genus *Neoscopelus* [20] and thaumatichthyids and *Cleidopus*. Light organs are present in or on the heads of some fish especially deep-sea species like alepocephalids [57], platytroctids [13], myctophids, stomiids, and the chiasmodontid *Pseudoscopelus* [58], but also the shallow-water anomalopids and monocentrids [12]. Apart from these organs, there are other types of luminous tissues in fishes. Besides the 'normal' photophores, i.e. primary photophores, some species also possess secondary photophores that are smaller and of less complex structure [57]. These small photophores are then, rarely with some degree of preference, distributed all over the body of the fish, cf. platytroctids, alepocephalids, myctophids and stomiids [13, 15, 57]. Myctophids possess caudal glands that usually present sexual dimorphism [2] and glandular light organs can also be found in the Stomiiformes [2, 46] and dalatiid species [8]. Luminous tissue with a

similar structure to photophores can be found in the body of myctophids [2] and stomiids [46] and barbels, while luminous mucus clinging to teeth was seen in some stomiids [20] and anglerfishes [2]. Some studies suggest the existence of bioluminescent skin in *Himantolophus azurlucens* and *Cryptopsaras couesii* [41].

The intensity of light emission in fishes can vary individually depending on the type of organ, depth and function (see further). Nevertheless, it seems that intensity is higher in coastal and shallow rather than mesopelagic, deep water species. Light spectra of the emitted lights usually peak in the blue-green, but are to some extent variable mostly in relation to depth. Coastal and epipelagic fishes produce light principally in the green and meso- and bathypelagic in the blue [3, 4, 33]. A rare case is the red light emission of some stomiids. Regarding angular distribution, bioluminescence starts to exceed downwelling light in the lower mesopelagic zone. At that depth, luminescence signals change from a ventral direction to a point-light scenario with bioluminescence emitted in all directions. The angle of the light emitted by an individual depends on the position of the light organ and its reflector in the body of the fish [2, 48], on the kind of transmission tissue involved and the body position of the light-emitting fish in the water column [37, 50]. Some shallow-water fishes like anomalopids and leiognathids can control the angle of the emitted lights [41, 50]. The light emitted also depends on the trophic level and nutritive state of the fish [43] and whether the light-emitting individual is looking at a conspecific side by side in a school, or, for example, prey above or a predator below.

### 4.2. Functions of light organs and likely roles of the emitted lights

A bioluminescent signal is defined by its intensity, spectral peak(s), temporal and spatial characteristics [3]. To what extent the degree of polarization, if any, plays a role has never been examined. The same luminous tissue may serve multiple kinds of functions, and the locations of the light organs are generally related to the roles the emitted lights are expected to play. The light generated can be used by a fish to communicate with its congeners, attract prey or avoid and startle predators. The range of functions of biological light is less complex in the coastal species than that in the deep water species, but even in the former, the emitted light has a variety of different biological roles.

#### *4.2.1. Intraspecific communication*

Fishes use light signals for intraspecific recognition, schooling and mating. Some fishes possess species-specific structures that purely on account of their placement and position may also assist specific recognition, as the photophore patterns in myctophids apparently do [59]. As aggregations of bioluminescent fishes help confusing predators, light emission may also assist school-forming species of etmopterids [10, 54], chlorophthalmids and macrourids [60].

A role in reproduction seems to be the part of the bioluminescence repertoire of at least some luminescent fishes including leiognathids, with their complex system of controlled light emission, and stomids [2, 3]. Light organs are sexually dimorphic in some species of *Gazza*,

*Secutor* and *Leiognathus*, and courtship behaviours using light signals have been observed in these fishes [61].

*4.2.2. Interspecific communication*

Some fishes use luminous organs located in or on the head to illuminate their surroundings, in search of prey and to detect predators. The bright headlights of anomalopids illuminate their surroundings, help them to avoid predators [62] and allow them to spot zooplankton [35]. Luminescence may be used for illumination in alepocephalids and macrourids in search of prey on the seafloor [30].

Many fish seem to attract their prey with bioluminescent lures. The escae of anglerfishes have been suggested to mimic faecal pellets with luminous bacteria [32]. Monocentrids have been assumed to attract prey with their light organs [53], but the cephalic photophores of *Diaphus* may be used to stun preys with bright flashes [1]. *Malacosteus niger* has small greenish light-emitting spots near its mouth and larger red light-emitting patches below its eyes. It is believed that the small greenish lights attract prey and the red light, invisible to most deep water organisms, is used by the fish to visually detect the approach of the unsuspecting prey in order to seize it when sufficiently close [2, 30, 48, 63].

Bioluminescent fishes can also use their lights in various ways to distract predators and avoid to be seen by them. Counterillumination is widespread and particularly common in the mesopelagic species, which emit light ventrally to match the downwelling light from above. In that way these often strongly laterally compressed species become camouflaged and almost invisible as silhouettes to the eyes of a predator below [37, 50]. This notion of the use of bioluminescence is strongly supported by biological and ecological facts. Photophores tend to be larger in the species of mesopelagic fishes occurring at shallower depths [2, 4], but the intensity of the ventral light emission from some fishes may change according to the light they find themselves in as from leiognathids [50] and myctophids [64]. Extraocular photophores in Stomiiformes, myctophids [2] and sharks [65] are located in optimal positions for comparisons of the downwelling sunlight with their own light in order to adjust it, a scenario that has also been suggested for apogonids [51]. Counterillumination appears to be of importance to the vast majority of luminous shallow water [33, 35] and mesopelagic teleosts [4, 21, 57] and sharks [66]. Whether the control of the matching luminescence involves eyes and ocular feedback or whether the light organs and cells within them are able to independently and directly regulate photic output of the light organ are questions yet to be answered.

Aposematism is a likely reason for the bioluminescent dorsal spines in etmopterids [67]. Aposematism is one of the proposed functions for bioluminescence in Porichthys as these species possess venomous dorsal spines [2]. When a possible predator is close by and ready to attack, other methods have to be used for luminous fishes to escape. One possible strategy for prey to evade a predator's attack is to emit a brief and blindingly bright flash of light right before changing its swimming direction. This has been suggested for the post-orbital photophores of anomalopids [62] and stomiids [4]. As most of the predators of these fishes have eyes adapted to dim light, very bright flashes may have a temporary effect of rendering them incapable of vision [32, 33]. In addition to the species already mentioned,

this survival strategy also seems to be employed in cases of the brief and bright flashes of leiognathids [53], the cephalic photophores in *Diaphus* [1] and the caudal organs of various myctophids [2]. A very similar defence has been proposed in cases in which a bioluminescent liquid is secreted into the water, acting as a luminous smokescreen and confusing the predator while the prey escapes [4]. Luminous liquids or mucoid secretions are produced in the 'shoulder organ' of platytroctids [57], the gill slits of the epigonid *Florenciella* [2], the anal gland of the macrourid *Malacocephalus* [21] and the escae and caruncles of some anglerfishes [21, 41].

*4.2.3. Unreported functions*

For the bioluminescence in several families of fish, no particular reason for the biological light is known (**Table 1**). Nevertheless, most of these unreported cases deserve some attention. As for the majority of other pelagic species, counterillumination is probably the most important function in Somniosidae, Evermannelidae, Microstomatidae, Phosichthyidae, Epigonidae and Howellidae as well as in some other families of the same order. Eurypharyngidae may use their luminescence as a lure to attract food items as with members of Saccopharyngidae. The fact that bioluminescence characteristics exhibit considerable similarities within the families of the order Gadiformes may be used as evidence that functions suggested for Macrouridae can apply equally to Moridae and Merlucciidae.

## 5. Vision in bioluminescent fishes

Light enters the eye through the cornea (**Figure 4**), passes the pupil and is refracted by the lens and focussed on the retina. Photons are absorbed by pigments in the photoreceptor cells, transformed into synaptic signals, the latter being transmitted through interneurons to the retinal ganglion cells [30, 68, 69]. The axons of these cells are grouped together and form the optic nerve which passes the visual information to the optic areas in the brain [70]. The basic structure of the eye in fish conforms to that of other vertebrates, but there are features that differ, depending on the life style of the fish involved (predator or prey) and on the photic environment [3], i.e. essentially the epipelagic and mesopelagic zones, in which the fish eyes are supposed to operate [30]. The most obvious differences in the structure and function of the fish eye accompany differences in the light intensities prevailing along the depth gradient of the oceanic zones. The majority of the bioluminescent species inhabit deeper waters and their vision is adapted to dim light conditions [30]. The luminescent crepuscular or nocturnal epipelagic fishes [33, 34, 39] have photoreceptors that, too, are mainly adapted to function under low light conditions [68, 70, 71].

### 5.1. Eye (structure, size and position)

There are no bioluminescent fishes with degenerated eyes and all possess binocular vision [3]. Epipelagic [4, 33] and mesopelagic fishes [30] inhabit environments that can be reached by sunlight and they usually possess large laterally positioned eyes. In contrast, the aphotic

bathypelagic species often have smaller eyes [4, 30]. The majority of the bioluminescent fishes have typical single lens, camera-type eyes, but some mesopelagic fishes possess tubular eyes. The latter are cylindrical eyes located at the top of the head and on account of their position allow the fish to distinguish silhouettes against downwelling sunlight. In these eyes, their binocular overlap provides high quantum capture, and accessory retinae and additional visual structures allow them to also detect a certain amount of light coming from lateral and ventral regions [68].

### 5.2. Lens, pupils and gaps

Fish lenses present little spherical aberration and a gradient of refractive index with the highest value in the centre guarantees that the focus of the light is sharp [4]. Lens transparency helps sensitivity [3], but lens pigments may influence the colour of the light reaching the retina [36]. Large pupils corresponding to wide apertures allow the admission of more photons, increasing

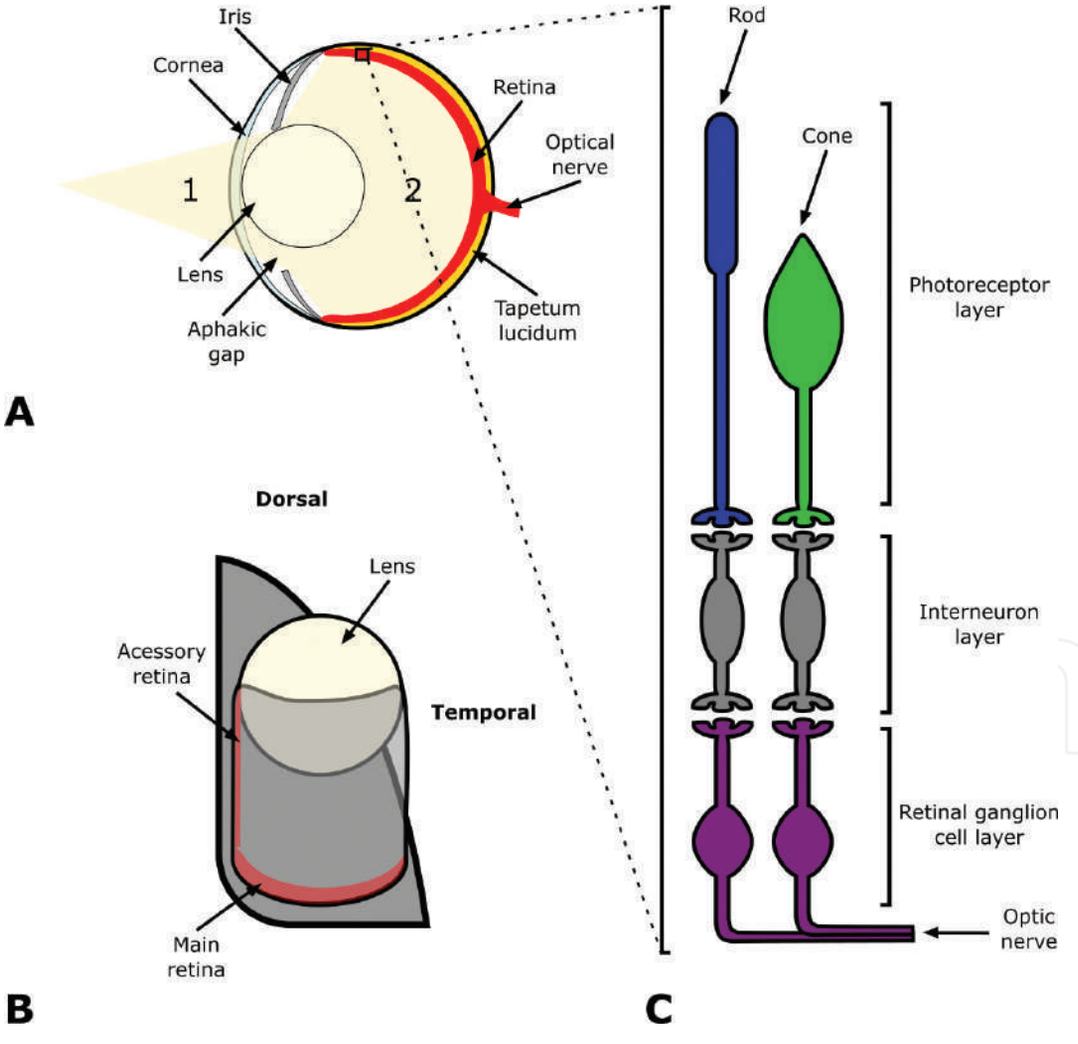


**Figure 4.** Schematic representations of the eye structure in fishes. (A) Camera-eye representing light entering the eye (1) and refracted by the lens focused on the retina (2). (B) Front-view of a left tubular eye in the head of a fish. (C) Basic retinal structure by cell layers.

visual sensitivity [30]. Aphakic gaps can enhance sensitivity by allowing light capture from specific oblique areas of the visual field [3, 4]. Such gaps are more commonly rostrally placed and extend binocular frontal vision in the lateral eyes. Light passing through gaps may get focussed in specialized retinal areas [30, 31, 72].

### 5.3. Tapeta

Animals that live in dim environments often possess *tapeta lucida*, which consist of a reflective layer positioned behind the photoreceptors acting as a mirror [30, 71]. This layer reflects the photons that are not captured by the photoreceptors, sending them back to the photoreceptors [68] and doubling quantum capture success; thus improving the sensitivity of the eye [31]. The spectral reflection peak of the tapetal regions in some fish species is thought to coincide with the absorption peaks of the visual pigments in the photoreceptors of the fish, but in some cases specific tapetal areas reflect spectrally different light thereby fulfilling specific visual tasks [36, 72].

### 5.4. Retina

Photoreceptor cells are responsible for the perception of light and their anatomical and ultrastructural make up, organization, dimensions, density, and distribution, and visual pigment is what has to match the behavioural visual tasks required of them by their owners in the specific photic habitats [69]. Like other vertebrates, fishes have two anatomically distinguishable types of photoreceptors. Cones, of which physiologically different two, three or more kinds with non-identical peak spectral sensitivities may be present, perceive different colours under conditions of bright light. Contrary to cones, rods are monochromatic photoreceptors, but provide the fish with high sensitive vision under low levels of light [36]. Cones occur in some bioluminescent fishes but are often reduced in total number and/or size even in the shallow-water species [60, 68, 70, 71, 73, 74]. Deep-sea fishes have mostly pure rod retinas of high cell density, for there is little need for them to be able to distinguish colours. For them the priority is to possess high sensitivity in a blue-light dominated nearly monochromatic dim, almost dark environment. High sensitivity of the rods is achieved by longer and wider cells. Sensitivity is also enhanced when rods are arranged in groups or are arranged in multi-layers called banks [30].

The differences in function between rods and cones are due to different kinds of photopigments in the visual cells. Rods possess either rhodopsin, a visual pigment with maximum absorbance at short wavelength (chemically a retinaldehyde, retinal 1 or vitamin $A_1$-based compound) or porphyropsin (i.e. chemically a 3,4-dehydroretinal, retinal 2 or vitamin $A_2$-based compound) [36]. Deep-sea fishes usually have high concentrations of rhodopsin giving the fish broad sensitivity to blue-green wavelengths [69]. However, some deep-sea fish have rod types containing different wavelength-sensitive rhodopsins or in addition may also have porphyropsins, the latter with longer-wavelength peak absorbances. The variety of receptor types and the development of banked retinas to increase absolute sensitivity can provide deep-water fish with the potential to make use of a wide range of photic stimuli [36, 75].

The density of retinal ganglion cells and, in particular, the ratios between receptors to ganglion cells define visual acuity and sensitivity. A high ratio of rods to retinal ganglion cells is indicative of high absolute sensitivity, while a low ratio would favour acuity. Most of the deep-water fishes possess unspecialized retinae and retinal cell ratios typical of sensitivity improvement [30, 31]. However, even so slight regional enhancements of acuity may be found in some species [68, 76].

Retinal specializations are regions of concentric increases in visual cell densities that enhance the visual sensitivity (rods) or acuity (retinal ganglion cells). These specializations seem to be related to visual behavioural tasks [72, 76]. In bioluminescent fishes, *areae centralis* and foveas can usually be identified as areas with higher visual acuity than the rest of the retina [30, 68], but it is possible that such specialized retinal regions change during the lifetime of a fish [77].

## 6. Evolution of bioluminescence and vision

The eyes of bioluminescent fishes evolved as adaptations to a dim light environment, in which bioluminescence provided important visual stimuli to perceive predators, prey items and mates. The fact that the bioluminescence in these species shares optical properties with the light pervading the environment the fish inhabit indicates the role selective pressures must have played [4]. Nevertheless, one cannot discount the idea that light emission and reception together represent a mutual and progressive adaptation [3].

In fishes, the adaptation of rhodopsin pigments to dim-light vision occurred in 12 separate occasions, suggesting that dim-light vision was a subject to considerable adaptive evolution [5]. In deep-sea fishes, these pigments were already shifted and adapted to the same visual environment as today in the Early Triassic. This shift was an adaptation to maximize the quantum capture of the dim sunlight and the biologically generated light in the deeper water of the sea. Along the way, convergent evolution seemed to have occurred with regard to rhodopsins of deep-sea fishes for adaptation to specific light regimes. This would explain the variation of wavelength sensitivities in these fishes, some adapted to specific bioluminescence peaks [75, 78]. Diversity in the pigmentation of the lenses of deep-water fishes suggests that it too has been the subject of separate evolutionary events a number of times [36]. Much later than vision, and assumed to have happened from the Early Cretaceous to the Late Cenozoic, bioluminescence appears to have evolved in teleosts [17]. A total of 27 independent occurrences are regarded to have left representatives in the marine teleosts assemblage, appearing first in the Stomiiformes [17]. Bioluminescence in the three closely related families containing luminescent species of sharks is also thought to have evolved from a common ancestor during the Cretaceous [9, 10, 17]. Bioluminescent patterns could have contributed to speciation in fishes [9, 11].

### 6.1. Bioluminescence evolution

An exposure to different light regimes powerfully influences the type of bioluminescence characteristics in the species of different habitats. This can express itself through different

spectral emission peaks and intensities of the biologically generated lights matching the photic environment of a species' habitat. The angle and direction of the emitted light are also subject to evolutionary pressures as most of luminescence glows ventrally in epipelagic and mesopelagic zones [2, 21, 33], where traces of sunlight from above are still discernible. No light from above, on the other hand, reaches the bathypelagic zone where the bioluminescent species exhibit a reduction in the number and sizes of photophores [2]. A good example of this tendency is the bathypelagic myctophids of the genus *Taaningichthys*, in which only the deepest living species, *T. paurolychnus* [79], lacks photophores (but still possesses caudal glands) [15].

### *6.1.1. Functions*

Bioluminescence plays major roles in the ecology of the luminescent fish species. The evolutionary history of bioluminescence in fishes shows that the ability to generate light arose dozens of times in unrelated clades [17] and yet, the basic structure of the light organs is nearly the same in all cases, independent of substrate, position and functions of the organ [20, 37]: counterillumination seems an excellent example. Fishes developed different ways to hide their silhouette from predators, using their ventral light emission. Some species make use of rows of photophores for that purpose, whereas others use indirect luminescence, but each case arose repeatedly and independently during evolution [17, 21].

An example of different structures employed in counterillumination can be seen in shallow-water species. In that environment, all the fishes use an indirect form of bioluminescence for camouflage with the exception of *Porichthys*, sciaenids and *C. dussumieri* that have photophores for that purpose [2, 33]. Different photogenic origins (i.e. symbiotic and intrinsic) may have led to the evolution of counterillumination, and some excellent examples are those that involve indirect bioluminescence. Some shallow (e.g. pempherids) and deep-water (e.g. *Cocorella atrata*) fishes use intrinsic light organs for indirect ventral bioluminescence, but the majority of species with this kind of light emission are employing symbiotic bacteria [2, 19, 39]. This divergence of photogenic origins can also be observed within apogonids [2]. Ventral luminescence in diel vertical migrants must also have come under selective pressures to conceal the silhouettes of the migrants from predators, when ascending into shallower and more illuminated waters [21, 37]. It is also conceivable that light signals in interspecific schools of diel vertical migrants play a role in keeping individuals together [3]. Although we focussed on counterillumination, there are other less well studied examples of structures of photogenic as well as non-photogenic origins serving identical functions in bioluminescent fishes. What immediately comes to mind are the symbiotic lures in anomalopids [53] and ceratiids [14] and the intrinsic barbels of stomiids [4] and photophores in sharks [8].

### *6.1.2. Acquisition*

Whether we deal with cases of intrinsic or symbiotic bioluminescence in fishes, the acquisition of the bioluminescence must have appeared at some point in the evolutionary history. It is still not clear how and when this happened, but based on some widely accepted suggestions the process behind it is thought to have involved the higher availability of luminous bacteria in coastal areas as a cause for the widespread use of symbiotic bacteria in the bioluminescence

of fishes inhabiting seashore-near habitats [80]. Similar kinds of constraints could have acted upon the *Cypridina* luciferin containing species of intrinsic luminous fish. These fishes are coastal species [33, 44] and the ostracods, which produce *Cypridina* luciferin, *Vargula* and *Cypridina* are benthic species and seem to inhabit mainly coastal and shallow waters [81–83]. It is well established that fishes like apogonids, pempherids and *Porichthys* have obtained the *Cypridina* luciferin from ostracods they ingested [18, 47, 84]. As there are no records that fish can synthesize coelenterazine, dietary acquisition seems a plausible scenario. This suggestion is supported by the widespread occurrence of coelenterazine also in luminous mid-water fishes and the fact that this substance is most abundant in the tissues of the digestive tracts of these fishes [85].

On the other hand, we have the symbiotic luminous fishes, in which light-producing bacteria are responsible for the bioluminescent capacity of the host. In return, the host provides protection, a place to grow, nutrition and oxygen to the bacteria [42]. This symbiotic relation could have started with facultative luminous bacteria in the gut of fishes (e.g. leiognathids), evolving into the obligatory symbiosis we now witness in anglerfishes and anomalopids [39, 41]. Hosts are ecologically dependent on the bacterial light and 'host family-bacterial specificity' seems to be the trend [42]. However, contrary to what had been believed for decades, nowadays coevolution between facultative symbiont and the host fish species is regarded as an unlikely scenario [41] as different fish host the same bacterial species and different species of bacteria can be co-symbionts of fish species. Moreover, evolutionary divergence in the bacterial species is unrelated to that of the host species [86]. Nevertheless, the absence of non-luminous bacteria in the organs indicates that some selection must have taken place [42] and associations could have arisen (or been bolstered) through environmental factors like the depth that the metamorphosing larvae inhabit. Support for this notion comes from *Photobacterium leiognathi* and *Photobacterium mandapamensis* found in organs of fishes that inhabit warmer and shallower waters and *Photobacterium kishitanii* that is found in deeper and colder waters [39, 42, 86].

### 6.2. Visual adaptation to bioluminescence

Vision in fishes evolved primarily as an adaptation to light intensity changes to extended or point sources in the near and far field of vision and secondarily to species-specific ecological needs [30, 31]. Bioluminescence has, to a large extent, affected the evolution of the optical system in luminescent fishes, which is obvious when one compares the eyes of fishes inhabiting the mesopelagic and bathypelagic realms. In the mesopelagic zone, animals use counterillumination to match the dim background light [36] and under such conditions fish evolved certain adaptations like tubular eyes [68], yellow lenses and retinae with banked receptor cells. In terms of their function, such eyes had to be sensitive, able to distinguish small spectral discrepancies from background light and to detect camouflaged prey [36]. For bathypelagic fishes, on the other hand, the only light sources available to them would have been of bioluminescent origins, restricting the range of their visual adaptational possibilities [31]. The bathypelagic species therefore tend to have larger aphakic gaps than mesopelagic species even within the same genus [3, 68]. As food is scarce at these greater depths, fishes living there

evolved less energetically expensive small eyes [4]. Nevertheless, these fishes do possess eyes with visual acuities capable of distinguishing point-source bioluminescence. Moreover, the eyes of the bathypelagic bioluminescent species show overall fewer regressed features than the eyes of the non-luminescent bathypelagic species do, indicating that the former rely more on vision than the latter. There are even some bottom-dwelling deep-sea fishes that have severely degenerated eyes ,which are likely to detect no more than the presence or absence of bioluminescence of perhaps invertebrates in the seabed [31].

The types of visual pigments [5] and the balance between visual acuity and sensitivity are the results of millennia of selective pressures stemming from the diverse light regimes and visual tasks fishes had to contend with [4]. Visual pigments evolved primarily to light from the sun, perhaps to guide specimens to darker regions and shelters away from the light or to seek illuminated areas, but in deep-sea fishes photopigments seem to be much more attuned to bioluminescence spectra [4, 6, 30, 36]. The eyes of most of the deep-water species have high concentrations of rhodopsin giving them a broad sensitivity to the blue-green range of the spectrum [69], comprising wavelengths that seem ideally suitable for perceiving bioluminescence emissions of prey, predators and conspecifics [6]. Potential colour vision in some deep-sea fishes may be an adaptation to identify species by differences in their bioluminescence hues [69, 75].

## 7. Ontogeny of bioluminescence and vision

### 7.1. Metamorphosis of eye and luminous tissues

During larval metamorphosis in fishes, the eyes change in shape, size, structure and even location [27]. The best examples among bioluminescent species are the tubular eyes, which are formed from laterally placed camera-type eyes in the larvae that become tubular in shape and migrate dorsally during metamorphosis. The lens remains unchanged but the retina suffers many alterations [87]. Retinae in larvae of the deep-water species, whether or not luminescent, are poorly specialized and vision, generally, is much less developed than in adults [88]. Almost all fish larvae have pure-cone retinae that are totally or partially substituted by rods during metamorphosis. The only exceptions in bioluminescent fish larvae are Saccopharyngiformes and *Lumiconger arafura*, in which pure rod retinae are the rule. In retinae with rod multilayers, the number of banks increases throughout development [68, 87, 88].

Photophores are formed during metamorphosis of a fish [22, 27, 87] and some are then already functional [2]. The luminous organs of symbiotic fishes also develop during larval metamorphosis [39]. This is the case of other luminous tissues too, as for example, the caruncles of ceratiids [41] and the caudal glands of myctophids [27]. Furthermore, the barbels of stomiids seem to arise during metamorphosis [27] and continue to elaborate throughout the fish's development [4].

The acquisition of the photogenic substrate in fishes is not well known. The luminous organs of symbiotic fishes start to develop prior to their being colonized by bacteria. Colonization

occurs during metamorphosis and it seems that each new generation of fish needs to acquire its symbiont afresh from the environment. The larvae of the shallow-water species are spatially close to adults, which suggests that bacteria may be released from the adult light organ and transferred to the young [39]. This does not seem to apply to the deep-sea species as the larvae are planktonic and hundreds of metres away from the adults. In these cases, it was suggested that larvae are infected when they migrate to coastal areas where luminous bacteria are more abundant [42, 80]. For ceratioids, the secretory cells that are common only in young fish may liberate pheromones to attract the right bacterial symbiotic species [41]. In intrinsic species, maternal transference of *Cypridina* luciferin was observed in *Porichthys* [84]. The same mechanism was suggested to apply to other teleosts and etmopterid sharks [89, 90].

### 7.2. Ecology and ontogeny: vision and bioluminescence in young fishes

Most symbiotic and intrinsically bioluminescent fishes have epipelagic larvae that will inhabit mid- or deep-waters as adults [4, 12, 15, 22, 26, 39]. During this ontogenic vertical migration to deeper waters, the visual habitat changes to an increasingly dim and monochromatic light scenario. These changes in the light environment require the modifications of the fish's photoreceptive as well as luminescent structures (described above) to coincide with the larvae undergoing metamorphosis. The migration to deeper waters and the metamorphic changes previous to the habitat change are believed to be completed in a relatively short time [87]. The development of rods and the changes in photoreceptor arrangements prepare the metamorphosing larvae to a life as a juvenile or adult in an environment where superior visual sensitivity is paramount. The transformations provide the young fish with visual capabilities for recognizing conspecifics, forming schools, engaging in predation and tracking and evading predator advances. In multi-layered retinae, a greater development of banks appears to coincide with the greater depths juvenile fish seek to spend their lives in as adults. In shark species, a metamorphosis similar to that seen in teleost is not present and the ontogenesis of photogenic organs or eyes in bioluminescent sharks has not been studied in much detail [68, 87].

In teleosts, the development of luminous organs is also affected by the changes in the photic environment and the behaviour of the young fishes. In some Stomiiformes and myctophids, the early development of cephalic photophores suggests that they are used to lure crustacean prey at night [22]. The more common scenario is that in the species whose larvae occur in dimly illuminated waters, photophores develop on the body first [2, 22, 91] and then gradually become functional as the migration to deeper water proceeds [22]. Larvae tend to develop photophores earlier in the species that inhabit deeper water as adults. For juveniles, light emission changes during growth and maturation [22]; ventral luminescence develops rapidly during that phase of the life of many teleosts [2, 4, 12, 52]. Ventral photophores also appear to be more developed in juvenile etmopterid sharks that inhabit shallower depths than the adults [8]. Considered together, these observations highlight the importance of counterillumination in young fishes while they migrate to deeper waters [2, 22].

Not only do photophores and light organs change in relation to a fish's developmental stage, the role or roles bioluminescence is to play in the life of a fish may also change with age. This

has been suggested for etmopterid sharks as ventral patterns of photophores used for mating and schooling are more developed in adults [8, 92]. Photophores of young platytroctids are horizontally placed on the body and are probably used to illuminate prey, while distracting predators. These photophores, however, do acquire more vertical positions as the ontogenic development proceeds [13] and this very likely implies a similar explanation to that seen in scopelarchids in which luminescence is limited to the adults [2]. In both cases, the use of the ventral lights only in adults is strong evidence for its function as counterillumination devices to help camouflage silhouettes of larger adult bodies.

## 8. Visual ecology

Considering bioluminescence as a means for communication and vision may help us understand its origins in fishes. Luminous species exhibit adaptations for a wide range of visual tasks [30] as light organs are multi-purpose and in most cases their predators and preys are also bioluminescent [2, 30]. Most bioluminescent signals are of rather low intensity, but bioluminescent fishes frequently possess rod-dominated retinae, conferring to them high visual absolute sensitivity [68, 70, 71] allowing them to perceive even very dim lights.

### 8.1. Bioluminescence detection

The optical characteristics, i.e. 'quality and quantity' of the light emitted by the vast majority of mid and deep-water luminescent species of fish relate to the downwelling light of their habitats. Vision accompanies this tendency. All of the luminescent species, whether shallow or deep-water fishes, possess eyes with at least some visual overlap, i.e. binocular vision, and that enhances sensitivity to and detection of distant point sources in dimly lit environments [68]. Bioluminescent fishes, moreover, frequently exhibit large pupils, which help them to detect bioluminescent flashes against a wider background [30]. The visual pigments present in the eyes of deep-sea bioluminescent fishes seem to be perfectly adapted to the bioluminescence spectra they encounter [2, 6, 30, 36].

Additionally to these more general adaptations, bioluminescent fishes evolved numerous visual particularities in order to improve detection of bioluminescence signals. Large eyes in epipelagic and in particular mesopelagic species admit greater amounts of photons, banked photoreceptors further improve the photon yield and efficient tapeta permit an at least two-fold photon catch. Such adaptations are critically important if sensitivity improvement in order to use bioluminescence is the goal. Different spatial relation of aphakic gaps, retinal specializations and tapeta are particularly well developed among the myctophid species [72] and luminous sharks [65]. Most deep-sea fishes can detect bioluminescence signals up to 30 m away [6, 48]. Some are even able to see bioluminescence up to 51 m as in bathypelagic fishes [21]. Bioluminescent bathypelagic fishes possess small eyes with wide pupils, rostral aphakic gaps and acute foveas. Although these eyes are less sensitive than those of the mesopelagic species, their resolving power is better and they seem perfectly adapted to spot point sources of biological lights in their environment. Contrary to non-luminescent bathy-

pelagic fishes, the eyes of the luminous species are not widely separated, aiding them in distances estimation [31, 68].

### 8.2. Intraspecific communication

In many bioluminescent fish species, their luminescence seems to play a role in communication between conspecifics. The eyes of these species are by necessity optimally adapted to their own bioluminescence. In shallow-water species, anomalopids seem to have eyes and retinae adapted to their own lights [74] and the visual pigments of *Porichthys* are also a good match to the emission spectrum in this species [73].

In deeper species, the eyes of *Chlorophthalmus*, with yellow tainted lenses and a specialized retina with some cones seem specifically adapted to their own blue-green light emissions [60]. Myctophids also possess visual pigments adapted to the spectra of their own light emissions [6]. Most male anglerfishes possess relatively large laterally positioned eyes with aphakic gaps [14] and long rods for heightened absolute sensitivity, adaptations that may be linked to the use of the female's esca to attract the attention of a male [3]. The genera *Malacosteus*, *Photostomias* and *Aristostomias* possess sub-ocular photophores, which emit red-light used as a 'private waveband'. These species evolved long-wavelength pigments in their rods and possessed tapeta and lenses that allow them to see their own red light [36].

Dalatiid sharks do not school, contrary to etmopterids [10]. In bioluminescent sharks, only the etmopterids seem to use their light emissions for intraspecific purposes, specifically the lateral patterns of the photophores [8, 92]. These patterns are more nasal and temporal in *Etmopterus lucifer* and seem to be detected by specific sensitive and acute zones in the nasal and temporal areas of the retina of this species [65].

### 8.3. Hunting

The use of bioluminescence in the context of food procurement in luminescent fishes is common. Photic lures to attract prey are one of the better known examples of luminous hunting devices. In order to be seen by and attract prey, the light tissue of the predator needs to be brighter than the background and has to be located in a visible place of the predator body, preferably the head and near the mouth [21]. Indeed, the most luminous structures of fishes that are likely to act as lures are easily spotted and emit an intense light, as the escae of anglerfishes [41], the barbels of stomiids [4] and the head organs of anomalopids [33], monocentrids [12] and *Diaphus* [1]. Most of their prey organisms possess highly sensitive eyes [30], so that the perception by them of the lights emitted by the photic lures is not an issue.

Some luminous fishes use their light to illuminate their surroundings, helping them in the search and seizure of prey, as suggested for anomalopids [35, 62] and *Diaphus* [1]. The same is possible for the benthopelagic deep-sea fishes, as alepocephalids and macrourids, that have uncommonly well-developed eyes, which might serve them to search for prey with their ventrally aimed luminescence [31].

It is common for bioluminescent fishes to prey on other luminous organisms. A good indication of this is the acquisition of *Cypridina* luciferin by fish through the consumption of luminous

ostracods [18, 33] and the high amount of coelenterazine in the digestive tissues of bioluminescent fishes [85]. The eyes of these predators, exhibit characteristics that help them perceive the bioluminescence of their prey. The highly sensitive retinae of fishes like myctophids, ensures that light from prey reaching them over relative long distances, i.e. several metres, is registered [72, 76]. Luminous sharks have retinal specializations, aphakic gaps and tapeta that also seem to be used for the detection of luminescent prey. In the specific case of the dalatiid *Squaliolus aliae*, the shortwave sensitive pigments in its retina seem to be more adapted to prey emitting blue-luminescence [65].

The fact that the prey of luminous fishes is frequently bioluminescent can create problems for the predator as most prey may employ counterillumination for concealment [4]. Bioluminescent fishes therefore appear to have evolved visual mechanisms allowing them to distinguish light emissions of prey from the downwelling surrounding lights. Most of the predators seeking to capture fish that employ counterillumination have large eyes that can disrupt the prey's counterillumination at close range [32]. Other species have evolved tubular eyes which possess acute retinal areas [30] perfect to detect at greater distances even silhouettes camouflaged through counterillumination [21, 46] and diverticula and accessory retinae to detect lateral luminescent stimuli [3, 31]. Some predators use yellow eye lenses to cut-off blue spectra, and to recognize the minimal green mismatches revealing the luminous silhouettes of prey [36]. Aphakic gaps 'lined' with retinal specializations of photoreceptor and ganglion cells as in myctophids may further help detecting counterilluminated prey [72, 76]. The enhancement of binocular resolution by foveas in deep-sea fish may also aid in breaking luminescent camouflages [68]. Different visual pigments and banks in rods that potentiate colour vision in deep-sea fishes are probably involved in breaking counterillumination [36, 75] and the translucent skin area above the eyes in etmopterid sharks may filter the spectra of luminescent prey, thereby compromising the camouflage of the latter [65].

### 8.4. Predator avoidance

One of the major functions of bioluminescence is defence [4, 33]. It is therefore not surprising to see such a vast number of different self-protective mechanisms in luminescent fishes. The trick is to avoid being seen by predators and in the case that has already happened, to deceive the predator and 'to go on the run'. The method of defence as well as vision of the predator and prey is always involved. We already stated the possibility for the predators to be luminous and that the eyes of bioluminescent fishes serving as prey are sufficiently sensitive to detect the predator. Curiously, except for the purpose of camouflage, smaller species tend to produce faster and shorter flashes than the larger predators [93]. This seems a strategy to reduce the risk to be accepted by a predator. A predator cannot afford the effort and risk of checking out every single flash it encounters, so that the predator would probably use the size and duration of a flash it encounters in deciding whether pursuit is worthwhile and the emitter of the luminescence is large or small [32].

The spectrum of a luminescent species' light used in counterillumination is not always a perfect match of the downwelling light [30]. This is not a problem for the deep-sea species as most of their predators do not possess colour vision [4, 48] and intensity rather than spectral matching

is most important and apparently within the capability of most species that employ counter-illumination concealment [4, 30, 32, 64]. On the other hand, epipelagic predators of bioluminescent fish have eyes more capable than those of deeper waters [94] to disrupt counterillumination [33]. The majority of coastal luminescent fishes exhibit internal light organs providing them a kind of disruptive luminescence that is more effective in turbid and dynamic near-shore waters than the uniform glow [33, 50] of deep-water species that live in more homogeneous and optically transparent photic environment [4].

Since both nocturnal predators of shallow-water bioluminescent fishes [94] and deep water predators [30] possess highly sensitive eyes, luminescent 'smokescreens' and very bright, brief flashes can temporarily confuse or even blind a predator and allow potential prey to flee [33].

Bioluminescence is not always straightforward and what seems to be an advantage can become a disadvantage under certain conditions. The 'private waveband' of the red light in stomiids seems an outstanding evolutionary trait for a predator, but some prey like scopelarchids [69], gonostomatids [75] and myctophids [6] have retinae with additional long-wave sensitive photopigments enabling the potential prey to see the stomiids red light. Fishes with 'head lamps' to illuminate the surroundings may be coastal and nocturnal or midwater inhabitants. Their bright emissions may be an advantage to pick up prey, but they are also a disadvantage as predators can easily spot these fishes and be attracted to them [12, 32, 33]. Nevertheless, the risks of that happening appear to be reduced as these species feed in schools from which predators usually find it more difficult to focus on an individual fish and seize it [12, 33, 94]. Moreover, the luminescent anomalopid also can rapidly and repeatedly occlude their lights [35, 41, 43].

## 9. Final remarks

### 9.1. Methodology

To understand the inter-relationship between vision and bioluminescence in luminescent species, detailed information on eyes and light organs of these animals is paramount. Spectral properties and intensities of the emitted lights, development, organization, structure and function of photophores as well as photoreceptors have to be studied and behavioural correlates need to be identified. None of that is easy; accessibility is difficult, measurements of the light emissions in luminescent fishes can be a tricky undertaking (especially for deep-sea teleosts and sharks) and when caught, these animals are more than often moribund and in most cases have to be stimulated to glow. This is possible using electricity and/or certain chemicals [34, 55]. The intensity and spectra of such induced light emissions can be measured using a spectrometer and a photo detector, but the data may not exactly reveal their properties in the way the fish use their luminescence in their natural environment. The angle of the light emissions depends on the light organ's position on the body and the internal angle of the organ, both analysable to a fair degree of accuracy using histological techniques [46]. Chemical analyses of the chemical compounds involved in the generation of the biological light would help understand the underlying mechanism involved, but control of the light emissions,

whether direct by the photophores themselves, through nervous signals or hormonal mediators requires living, healthy specimens.

Anatomical details of the eye, cell topography, photoreceptor cell types, cell ultrastructures and distributions as well as certain visual parameters can be gleaned from histological investigations, but while functional parameters like sensitivity, acuity, angular acceptance, etc. can to some extent be deduced from the anatomical details, electrophysiological techniques or the use of microspectrophotometry must be the methods of choice when it comes to functional questions. Spectral sensitivity peaks of the visual pigments obtained spectrophotometrically or electrophysiologically ought to be connected with chemical analyses of the photopigments involved and opsin gene analyses [36]. Chemical and optical studies like refractive indices, focal lengths, etc. on the dioptric structures of the fish eye ought to complement the other investigations so that predictions can be made on the way a fish uses its eyes.

Evolutional studies of vision receive support from investigations on the mutagenesis of the visual pigments [5] and genetics of nuclear and mitochondrial gene fragments can also be used in studies on phylogenetic relationships between species [17]. Often lacking first-hand direct observational evidence of vision/luminescence interactions in the natural environment, the next best approach would involve realistic analyses of the underwater transmission of bioluminescence in specific cases and ecological modelling, based on the information available [48].

### 9.2. Future prospects

Communication by light is a subject of considerable interest to ethologists and ecologists, chemists, geneticists, anatomists, physiologist, opticians and vision researchers. Biotechnologists, too, become increasingly interested in bioluminescence generally and communication by light in particular. For the comparative zoologist it is important to realize that behaviour exhibited by individual luminescent fish in captivity may not be the same as that in the fish's natural environment. This is even more important to keep in mind when interpreting the induced light emissions obtained from nearly dead deep-sea species. Moreover, vision and bioluminescence are only part of a bigger picture. There is evidence that pineal photoreceptors support light perception and are even involved in light output regulation in deep-sea fish [64]. Therefore in analyses of the ecological role, i.e. overall biological significance that bioluminescence plays, we must not ignore the fact that other sense organs like, for instance the lateral line system, semicircular canals, otoliths, chemo, electro and magnetoreceptors, etc. may also be involved [4].

Regarding the eco-ethological roles of the relationship between bioluminescence and vision, some questions have been answered but many more remain to be solved. Remotely operated underwater vehicles (ROVs) can be expected to help in this task and should provide new *in vivo* insights into the various eco-ethological roles of bioluminescence while advances in genetic techniques can be expected to shed further light onto the phylogeny and evolution of these 'brightest creatures of the animal world'.

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## References

[1] Haddock SHD, Moline MA, Case JF. Bioluminescence in the sea. Annual Review of Marine Science. 2010;2:443–493. DOI: 10.1146/annurev-marine-120308-081028.

[2] Herring PJ, Morin JG. Bioluminescence in fishes. In: Herring PJ, editor. Bioluminescence in Action. London: Academic Press; 1978. pp.273–329.

[3] Nicol JA. Bioluminescence and vision. In: Herring PJ, editor. Bioluminescence in Action. London: Academic Press; 1978. pp. 367–408.

[4] Herring P. The Biology of the Deep Ocean. New York: Oxford University Press Inc.; 2002. 314 p.

[5] Yokoyama S. Evolution of dim-light and color vision pigments. The Annual Review of Genomics and Human Genetics. 2008;9:259–282. DOI: 10.1146/annurev.genom.9.081307.164228.

[6] Turner JR, Whit EM, Collins MA, Partridge JC, Douglas RH. Vision in lanternfish (Myctophidae): Adaptations for viewing bioluminescence in the deep-sea. Deep-Sea Research I. 2009;25:1003–1017.DOI: 10.1016/j.dsr.2009.01.007.

[7] WoRMS Editoral Board. World Register of Marine Species [Internet]. [Updated: 2016]. Available from: www.marinespecies.org [Accessed: 20/06/2016].

[8] Claes JM, Mallefet J. Bioluminescence of sharks: First synthesis. In: Meyer-Rochow VB, editor. Bioluminescence in Focus - A Collection of Illuminating Essays. Kerala: Research Signpost; 2009. p. 51–65.

[9] Straube N, Li C, Claes JM, Corrigan S, Naylor GJP. Molecular phylogeny of Squaliformes and first occurrence of bioluminescence in sharks. BioMed Central Evolutionary Biology. 2015;15:162. DOI: 10.1186/s12862-015-0446-6.

[10] Reif W-E. Function of scales and photophores in mesopelagic luminescent sharks. Acta Zoologica. 1985;66(2):111–118.

[11] Davis MP, Holcroft NI, Wiley EO, Sparks JS, Smith WL. Species-specific bioluminescence facilitates speciation in the deep sea. Marine Biology. 2014;161:1140–1148. DOI: 10.1007/s00227-014-2406x.

[12] Herring PJ. Aspects of the bioluminescence of fishes. In: Barnes H, Barnes M, editors. Oceanography and Marine Biology. An Annual Review. Volume 20. Aberdeen: Aberdeen University Press; 1982. pp. 472–541.

[13] Matsui T, Rosenblatt RH. Review of the deep-sea fish family Platytroctidae (Pisces: Salmoniformes). Bulletin of the Scripps Institution of Oceanography. 1987;19:1–159.

[14] Pietsch TW. Oceanic Anglerfishes. Extraordinary Diversity in the Deep Sea. Berkeley: University of California Press; 2009. 557 p.

[15] Carpenter KE, Niem VH, editors. FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Pacific. Volume 3: Batoid Fishes, Chimaeras and Bony Fishes Part 1 (Elopidae to Linophrynidae). Rome: Food and Agriculture Organization of the United Nations; 1999. pp.1407–2068

[16] Castle PHJ, Paxton JR. A new genus of luminescent eel (Pisces: Congridae) from the Arafura Sea, Northern Australia. Copeia. 1984;1:72–81.

[17] Davis MP, Sparks JS, Smith WL. Repeated and widespread evolution of bioluminescence in marine fishes. PLoS One. 2016;11(6):e0155154. DOI: 10.1381/journal.pone.0155154.

[18] Thacker CE, Roje DM. Phylogeny of cardinalfishes (Teleostei: Gobiiformes: Apogonidae). Molecular Phylogenetics and Evolution. 2009;52:735–746. DOI: 10.1016/j.ympev.2009.05.017.

[19] Haneda Y. Luminous organs of fish which emit light indirectly. Pacific Science. 1950;4:214–227.

[20] Harvey EN. Pisces. In: Harvey EN, editor. Bioluminescence. New York: Academic Press; 1952. pp. 494–553.

[21] McAllister DE. The significance of ventral bioluminescence in fishes. Journal of the Fisheries Research Board of Canada. 1967;24(3):538–554.

[22] Suntsov AV, Widder EA, Sutton TT. Bioluminescence. In: Finn RN, Kapoor BG, editors. Fish Larval Physiology. Enfield: Science Publishers; 2008. pp. 51–88.

[23] Herring P. Systematic distribution of bioluminescence in living organisms. Journal of Bioluminescence and Chemiluminescence. 1987;1:147–163.

[24] Cohen DM, Inada T, Iwamoto T, Scialabba N. FAO Species Catalogue. Gadiform Fishes of the World (Order Gadiformes). An Annotated and Illustrated Catalogue of Cods, Hakes, Grenadiers and Other Gadiform Fishes Known to Date. FAO Fisheries Synopsis. Number 125. Volume 10. Rome: Food and Agriculture Organization of the United Nations; 1990. 442 p.

[25] Ramaiah N, Chandramohan D. Occurrence of Photobacterium leiognathi, as the bait organ symbiont in frogfish Antennarius hispidus. Indian Journal of Marine Sciences. 1991;21:210–211.

[26] Carpenter KE, Niem VH, editors. FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Pacific. Volume 4: Bony Fishes Part 2 (Mugilidae to Carangidae). Rome: Food and Agriculture Organization of the United Nations; 1999. pp. 2069–2790 .

[27] Moser HG, editor. The Early Stages of Fishes in the California Current Region. Atlas number 33. La Jolla: California Cooperative Oceanic Fishery Investigations; 1996. 1505 p.

[28] McKay RJ. FAO Species Catalogue. Sillaginid Fishes of the World. (Family Sillaginidae). An Annotated and Illustrated Catalogue of Sillago, Smelt or Indo-Pacific Whiting Species Known to Date. FAO Fisheries Synopsis. Number 125. Volume 14. Rome: Food and Agriculture Organization of the United Nations; 1992. 87 p.

[29] Terao A. Photophore in flying fish. Zoological Magazine. 1950;13(40):59–60.

[30] Warrant EJ, Locket NA. Vision in the deep sea. Biological Reviews. 2004;79:671–712. DOI: 10.1017/S1464793103006420.

[31] Warrant EJ, Collin SP, Locket NA. Eye design and vision in deep-sea fishes. In: Collin SP, Marshall NJ, editors. Sensory Processing in Aquatic Environments. New York: Springer-Verlag; 2003. pp. 303–320.

[32] Young RE. Oceanic bioluminescence: An overview of general functions. Bulletin of Marine Science. 1983;33(4):829–846.

[33] Morin JG. Coastal bioluminescence: Patterns and functions. Bulletin of Marine Science. 1983;33(4):787–817.

[34] Harvey, EN. The luminous organs of fishes. In: Brown ME, editor. The Physiology of Fishes. Volume II. Behaviour. New York: Academic Press; 1957. pp. 346–366.

[35] Meyer-Rochow VB. Some observations on spawning and fecundity in the luminescent fish *Photoblepharon palpebratus*. Marine Biology. 1976;37:325–328.

[36] Douglas RH, Partridge JC, Marshall NJ. The eyes of deep-sea fish I: Lens pigmentation, tapeta and visual pigments. Progress in Retinal and Eye Research. 1998;17(4):597–636.

[37] Clarke WD. Function of bioluminescence in mesopelagic organisms. Nature. 1963;198:1244–1246.

[38] Sutton TT. Vertical ecology of the pelagic ocean: Classical patterns and new perspectives. Journal of Fish Biology. 2013;83:1508–1527. DOI: 10.1111/jfb.12263.

[39] Haygood MG. Light organ symbioses in fishes. Critical Reviews in Microbiology. 1993;19(4):191–216.

[40] Salvanes AGV, Kristoffersen JB. Mesopelagic fishes. In: Steele JH, Thorpe SA, Turekian KK, editors. Encyclopedia of Ocean Sciences. Volume 3. 2nd ed. San Diego: Academic Press; 2001. pp. 748–754.

[41] Karplus I. The associations between fishes and luminescent bacteria. In: Karplus I, editor. Symbiosis in Fishes: The Biology of Interspecific Partnerships. New Jersey: John Wiley and Sons; 2014. pp. 7–57.

[42] Urbanczyk H, Ast JC, Dunlap PV. Phylogeny, genomics, and symbiosis of Photobacterium. Federation of European Microbiological Societies. 2011;35(2):324–342. DOI: 10.1111/j.1574-6976.2010.00250.x.

[43] Meyer-Rochow VB. Loss of bioluminescence in *Anomalops katoptron* due to starvation. Experientia. 1976;32:1175–1176.

[44] Thompson EM, Rees J-F. Origins of luciferins: Ecology of bioluminescence in marine fishes. In: Hochachka PW, Mommsen TP, editors. Biochemistry and Molecular Biology of Fishes. Amsterdam: Elsevier Science; 1995. pp. 435–466.

[45] Cavallaro M, Mammola CL, Verdiglione R. Structural and ultrastructural comparison of photophores of two species of deep-sea fishes: *Argyropelecus hemigymnus* and *Maurolicus muelleri*. Journal of Fish Biology. 2004;64:1552–1567. DOI: 10.1111/j.905-8649.2004.00410.x.

[46] O'Day WT. Luminescent silhouetting in stomiatoid fishes. Contributions in Science from the Natural History Museum of Los Angeles. 1973;246:1–8.

[47] Haneda Y, Johnson FH. The photogenic organs of *Parapriacanthus beryciformes* franz and other fish with the indirect type of luminescent system. Journal of Morphology. 1962;110(2):187–198.

[48] Denton EJ, Herring PJ, Widder EA, Latz MF, Case JF. The roles of filters in the photophores of oceanic animals and their relation to vision in the oceanic environment. Proceedings of the Royal Society of London B: Biological Sciences. 1985;225:63–97.

[49] Best ACG, Bone Q. On the integument and photophores of the alepocephalid fishes Xenodermichthys and Photostylus. Journal of the Marine Biological Association of the United Kingdom. 1976;56:227–236.

[50] McFall-Ngai M, Morin J. Camouflage by disruptive illumination in leiognathids, a family of shallow-water, bioluminescent fishes. Journal of Experimental Biology. 1991;156:119–138.

[51] Dunlap PV, Nakamura M. Functional morphology of the luminescence system of Siphamia versicolor (Perciformes: Apogonidae), a bacterially luminous coral reef fish. Journal of Morphology. 2011;272:897–909. DOI: 10.1002/jmor.10956.

[52] Haneda Y. The luminescence of some deep-sea fishes of the families Gadidae and Macrouridae. Pacific Science. 1951;5(4):372–378.

[53] McFall-Ngai MJ, Dunlap PV. Three new modes of luminescence in the leiognathid fish Gazza minuta: Discrete projected luminescence, ventral body flash, and buccal luminescence. Marine Biology. 1983;73:227–238.

[54] Claes JM, Mallefet J. Comparative control of luminescence in sharks: New insights from the slendertail lanternshark (*Etmopterus molleri*). Journal of Experimental Marine Biology and Ecology. 2015;467:87–94. DOI: 10.1016/j.jembe.2015.03.008.

[55] Baguet F. Excitation and control of isolated photophores of luminous fishes. Progression in Neurobiology. 1975;5(2):97–125.

[56] Case JF, Strause LG. Neurally controlled luminescent systems. In: Herrin PJ, editor. Bioluminescence in Action. London: Academic Press; 1978. pp. 331–366.

[57] Sazonov YI. Morphology and significance of the luminous organs in alepocephaloid fishes. Biosystematics and Ecology Series. 1996;11:156–163.

[58] Tatsuta N, Imamura H, Nakaya K, Kawai T, Abe T, Sakaoka K, Takagi S, Yabe M. Taxonomy of mesopelagic fishes collected around the Ogasawara islands by the T/S Oshoro-Maru. Memoirs of the Faculty of Fisheries Sciences, Hokkaido University. 2014;56(1):1–64.

[59] Nicol JA. Spectral composition of the light of the lanternfish *Myctophum punctatum*. Journal of Marine Biological Association of the United Kingdom. 1960;40:27–32.

[60] Somiya, H. Bacterial bioluminescence in chlorophthalmid deep-sea fish: a possible interrelation between the light organ and the eyes. Experientia. 1977;33:906–909.

[61] McFall-Ngai MJ, Dunlap PV. External and internal sexual dimorphism in leiognathid fishes: Morphological evidence for sex-specific bioluminescent signaling. Journal of Morphology. 1984;182:71–83.

[62] Morin JG, Harrington A, Nealson K, Badlwin TO, Hastings JW. Light for all reasons: Versatility in behavioural repertoire of the flashlight fish. Science. 1975;190:74–76.

[63] Meyer-Rochow VB. Light of my life – Messages in the dark. Biologist. 2001;48(4):163–167.

[64] Young RE, Roper CFE, Walters JF. Eyes and extraocular photoreception in midwater cephalopods and fishes: Their roles in detecting downwelling light for counterillumination. Marine Biology. 1979;51:381–390.

[65] Claes JM, Partridge JC, Hart NS, Garza-Gisholt E, Ho H-C, Mallefet J, Collin SP. Photon hunting in the twilight zone: Visual features of mesopelagic bioluminescent sharks. PLoS One. 2014;9(8):e104213. DOI: 10.1381/journal.pone.0104213.

[66] Claes JM, Ho H-C, Mallefet J. Control of luminescence from pygmy sharks (*Squaliolus aliae*) photophores. The Journal of Experimental Biology. 2012;215:1691–1699. DOI: 10.1242/jeb.066704.

[67] Claes JM, Dean MN, Nilsson D-E, Hart NS, Mallefet J. A deepwater fish with "light-sabers" – a dorsal spine-associated luminescence in a counterilluminating lanternshark. Scientific Reports. 2013;3:1308. DOI: 10.1039/srep01308.

[68] Wagner H-J, Fröhlich E, Negishi K, Colin SP. The eyes of deep-sea fish II. Functional morphology of the retina. Progress in Retinal and Eye Research. 1998;4:637–685.

[69] Douglas RH, Hunt DM, Bowmaker JK. Spectral sensitivity tuning in the deep-sea. In: Collin SP, Marshall NJ, editors. Sensory Processing in Aquatic Environments. New York: Springer-Verlag; 2003. pp. 323–342.

[70] Ali MA. Retinas of Fishes: An Atlas. Berlin: Springer-Verlag; 1976. 284 p. DOI: 10.1007/978-3-642-66435-9.

[71] Warrant E. Vision in the dimmest habitats on earth. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural and Behavioural Physiology. 2004;190:765–789. DOI: 10.1007/s00359-004-0546-z.

[72] de Busserolles F, Fitzpatrick JL, Marshall NJ, Collin SP. The influence of photoreceptor size and distribution on optical sensitivity in the eyes of lanternfishes (Myctophidae). PLoS One. 2014;9(6):e99957. DOI: 10.1381/journal.pone.0099957.

[73] Fernandez HR, Tsuji FI. Photopigment and spectral sensitivity in the bioluminescent fish *Porichthys notatus*. Marine Biology. 1976;34:101–107.

[74] Meyer-Rochow VB, Barburina V, Smirnov S. Histological observations on the eye of the two luminescent fishes *Photoblepharon palpebratus* (Boddaert) and *Anomalops katoptron* (Blkr). Zoologischer Anzeiger – A Journal of Comparative Zoology. 1982;209(1/2):65–72.

[75] Douglas RH, Partridge JC. On the visual pigments of deep-sea fish. Journal of Fish Biology. 1997;50:68–85.

[76] de Busserolles F, Marshal NJ, Collin SP. Retinal ganglion cell distribution and spatial resolving power in deep-sea lanternfishes (Myctophidae). Brain, Behaviour and Evolution. 2014;84(4):262–276. DOI: 10.1159/000365960.

[77] Miyazaki T, Iwami T, Meyer-Rochow VB . The position of the retinal area centralis changes with age in *Champsocephalus gunnari* (Channichthyidae), a predatory fish from coastal Antarctica. Polar Biology. 2011;34:1117–1123. DOI: 10.1007/s00300-011-0969-2.

[78] Hunt DM, Dulai KS, Partridge JC, Cottrill P, Bowmaker JK. The molecular basis for spectral tuning of rod visual pigments in the deep-sea fish. The Journal of Experimental Biology. 2001;204:3333–3344.

[79] Froese R, Pauly D. Fishbase [Internet]. [Updated: 2015]. Available from: fishbase.org [Accessed: 15/06/2016]

[80] Dunlap PV, Davis KM, Tomiyama S, Fujino M, Fukui A. Developmental and microbiological analysis of the inception of bioluminescent symbiosis in the marine fish *Nuchequula nuchalis* (Perciformes: Leiognathidae). Applied and Environmental Microbiology. 2008;74(24):7471–7481. DOI: 10.1128/AEM.01619-08.

[81] Tsuji FI, Lynch III RV, Haneda Y. Studies on the bioluminescence of the marine ostracod crustacean Cypridina serrata. Biological Bulletin. 1970;139(2):386–401.

[82] Cohen AC, Morin JG. Six new luminescent ostracodes of the genus Vargula (Myodocopida: Cypridinidae) from the San Blas region of Panama. Journal of Crustacean Biology. 1989;9(2):297–340.

[83] Morin JG. Firefleas of the sea: Luminescent signaling in marine ostracode crustaceans. The Florida Entomologist. 1986;69(1):105–121.

[84] Mensinger AF, Case JF. Bioluminescence maintenance in juvenile *Porichthys notatus*. Biological Bulletin. 1991;181:181–188.

[85] Shimomura O, Inoue S, Johnson FH, Haneda Y. Widespread occurrence of coelenterazine in marine bioluminescence. Comparative Biochemistry and Physiology – Part B. Biochemistry & Molecular Biology. 1980;65:135–138.

[86] Dunlap PV, Ast JC, Kimura S, Fukui A, Yoshino T, Endo H. Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. Cladistics. 2007;23:507–532. DOI: 10.1111/j.1096-0031.2007.00157.x.

[87] Evans BI, Fernald RD. Metamorphosis and fish vision. Journal of Neurobiology. 1990;21(7):1038–1052.

[88] Evans BI, Browman HI. Variation in the development of the fish retina. American Fisheries Society Symposium. 2004;10:146–166.

[89] Claes JM, Mallefet J. Early development of bioluminescence suggests camouflage by counter-illumination in the velvet belly lantern shark *Etmopterus spinax* (Squaloidea: Etmopteridae). Journal of Fish Biology. 2008;73:1337–1350. DOI: 10.1111/j.1095-8649.2008.02006.x.

[90] Mallefet J, Shimomura O. Presence of coelenterazine in mesopelagic fishes from the Strait of Messina. Marine Biology. 1995;124:381–385.

[91] Badcock J, Larcombe RA. The sequence of photophore development in *Xenodermichthys copei*. Journal of the Marine Biological Association of the United Kingdom. 1980;60:277–294.

[92] Claes JM, Mallefet J. Ontogeny of photophores pattern in the velvet belly lantern shark, Etmopterus spinax. Zoology. 2009;112:433–441. DOI: 10.1016/j.zool.2009.02.003.

[93] Mensinger AF, Case JF. Luminescent properties of deep-sea fish. Journal of Experimental Marine Biology and Ecology. 1990;144:1–15.

[94] Munz FW, McFarland WN. Evolutionary adaptations of fishes to the photic environment. In: Crescitelli F, editor. Handbook of Sensory Physiology, Volume VII/5.The Visual System in Vertebrates. Berlin: Springer-Verlag; 1977. pp. 193–274. DOI: 10.1007/978-3-642-66468-7

[95] Merret NR, Badcock J, Herring PJ. The status of Benthalbella infans (Pisces: Myctophoidei), its development, bioluminescence, general biology and distribution in the eastern North Atlantic. Journal of Zoology, London. 1973;170:1–48.

[96] Carpenter KE, Niem VH, editors. FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Pacific. Volume 5: Bony Fishes Part 3 (Menidae to Pomacentridae). Rome: Food and Agriculture Organization of the United Nations; 2001. pp. 2791–3390 .

[97] Fernandez I, Devaraj M. Dynamics of the gold-spotted grenadier anchovy (*Coilia dussumieri*) stock along the northwest coast of India. Indian Journal of Fisheries. 1996;43(1):27–39.

[98] Roe HSJ, Badcock J. The diel migrations and distributions within a mesopelagic community in the North East Atlantic. 5. Vertical migrations and feeding of fish. Progress in Oceanography. 1984;13:399–424.

[99] Badcock J. The vertical distribution of mesopelagic fishes collected on the SOND Cruise. Journal of the Marine Biological Association in the United Kingdom. 1970;50:1001–1044.

[100] Poulsen JY. A new species of pencil smelt *Nansenia boreacrassicauda* (Microstomatidae, Argentiniformes) from the North Atlantic Ocean. Zootaxa. 2015;4030(3):517–532. DOI: 10.11646/zootaxa.4020.3.6.

[101] Crane Jr JM. Bioluminescence in the batfish *Dibranchus atlanticus*. Copeia. 1968;2:410–411.

[102] Carpenter KE, Niem VH, editors. FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Pacific. Volume 2: Cephalopods, crustaceans, holothurians and sharks. Rome: Food and Agriculture Organization of the United Nations; 1998. pp. 687–1406.

[103] Carpenter KE, Niem VH, editors. FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Pacific. Volume 6: Bony

Fishes Part 4 (Labridae to Latimeriidae), Estuarine Crocodiles, Sea Turtles, Sea Snakes and Marine Mammals. Rome: Food and Agriculture Organization of the United Nations; 2001. pp. 3391–4218 p.

[104] Carpenter KE, editor. FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Atlantic. Volume 2: Bony Fishes Part 1 (Acipenseridae to Grammatidae). Rome: Food and Agriculture Organization of the United Nation and American Society of Ichthyologists and Herpetologists. Special Publication Number 5; 2002. pp. 601–1374.

[105] Carpenter KE, editor. FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Atlantic. Volume 3: Bony Fishes Part 2 (Opistognathidae to Molidae), sea turtles and marine mammals. Rome: Food and Agriculture Organization of the United Nation and American Society of Ichthyologists and Herpetologists. Special Publication Number 5; 2002. pp. 1375–2127 .

[106] Sasaki K. Phylogeny of the family Sciaenidae, with notes on its zoogeography (Teleostei, Perciformes). Memoirs of the Faculty of Fisheries Hokkaido University. 1989;36(1–2):1–138.

Chapter 13

# Bioluminescence Microscopy: Design and Applications

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Additional information is available at the end of the chapter

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**Abstract**

Bioluminescence imaging by microscopy is performed using an ultra-low-light imaging camera. Although imaging devices such as sensor and camera have been greatly improved over time, such improvements have not been attained commercially which are available for microscopes now. We previously optimized the optical system of a microscope for bioluminescence imaging using a short-focal-length imaging lens and evaluated this system with a conventional color charge-coupled device camera. Here, we describe the concept of bioluminescence microscope design using a short-focal-length imaging lens and some representative applications, including intracellular calcium imaging, imaging of clock gene promoter assays, and three-dimensional reconstruction of *Drosophila* larva. This system facilitates the acquisition of bioluminescence images of single live cells using luciferase, which is similar to fluorescence microscopy using a fluorescent protein.

**Keywords:** bioluminescence microscopy, short-focal-length imaging lens, single live-cell analysis, three-dimensional imaging

## 1. Introduction

Aequorin is a calcium-specific light-emitting protein extracted from the jelly fish *Aequorea* [1]. Additionally, aequorin-injected eggs of the medaka (*Oryzias latipes*), a fresh water fish, showed a dramatic increase in free calcium during fertilization, as determined by measuring light from the eggs using a photomultiplier tube [2]. Notably, bioluminescence microscopy with an image intensifying system using a vidicon camera was performed in 1978 to show the spatial distri-

bution of the free calcium in the egg [3]. This system revealed a spreading wave of high free calcium (calcium wave) during fertilization from the animal pole, as discussed with the fertilization wave of cortical changes in eggs observed by light microscopy [4]. Although the potential of low-light imaging has been recognized in physiology and developmental biology, this technique was not commonly used at that time due to a lack of commercially available instrumentation. Later, advances in detector and digital imaging processing systems facilitated the commercial production of appropriate instrumentation and made it possible for low-light imaging to be carried out using a silicon-intensifier target (SIT) tube camera or a high-sensitivity cooled charge-coupled device (CCD) camera.

Since the cloning of firefly luciferase in the late 1980s, luciferase has been used as a reporter enzyme to assay the activity of a particular gene promoter using the photon-counting luminometer method [5–7]. Additionally, bioluminescence microscopy of promoter activity in single cells has been performed using ultra-low-light imaging cameras, such as liquid nitrogen-cooled CCD cameras, photon-counting CCD cameras, or image-intensifying CCD cameras [8–16]. However, temporal and spatial resolution was not enough for the observation of cellular biological events and for the detection of single cells compared with that of conventional CCD cameras. Therefore, satisfactory analysis has not been achieved at the single-cell level by bioluminescence microscopy.

Recently, electron-multiplying CCD (EM-CCD) camera, which yields higher sensitivity and image quality, was commercially released and subsequently used for bioluminescence microscopy [17–19]. Although the image sensor of ultra-low-light imaging cameras has been greatly improved over time, such improvements have not been made commercially available for microscopes.

In our previous studies, we optimized an optical system using a short-focal-length imaging lens for bioluminescence microscopy and performed bioluminescence imaging of single live cells expressing the luciferase gene using a conventional CCD camera [20, 21]. This system is commercially available now and has been widely used for gene expression analysis in chronobiology [22–28], neurobiology [29, 30], developmental biology [31], medical research [32–35], signal transduction analysis [36–38], molecular interaction [39–41], and radiation biology [42, 43]. Accordingly, in this study, we describe the concept of bioluminescence microscopy adopting a short-focal-length imaging lens and present several representative applications, including a three-dimensional analysis, to demonstrate the advantages of the short-focal-length imaging lens system.

## 2. Microscope design

Bioluminescence microscopy is based on the detection of light emitted by living cells expressing a luciferase gene or other luminescence-related gene. Conventional microscopes are inefficient at transmitting light from the sample to the detector, necessitating long exposure times. We designed a new type of microscope for ultra-low-light imaging based on modifications to the imaging lens, vignetting, and effective field area.

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### 2.1. Imaging lens

**Figure 1** shows a diagram of an inverted microscope equipped with an infinity-corrected optical system. Light from a sample is collected by an objective lens (OB), and the sample image is created by an imaging lens (tube lens; IM) on a CCD chip. Generally, the degree of brightness (I) of an image is directly proportional to the square of the numerical aperture (NA) of the OB and is inversely proportional to the square of magnification (M) of the image, namely as $I \propto (NA/M)^2$. Therefore, a higher NA and lower M yield much brighter images. However, it is difficult to obtain both conditions. Because higher NA OB yields higher M (shorter focal length), or lower M OB yields lower NA. Thus, high NA and low M are mutual trade-offs. On the other hand, the value of NA/M is the same as the NA of the IM, geometrically denoted as NA′. Therefore, a microscope with a high NA′ (short-focal-length imaging lens) makes it possible to achieve a higher NA and lower M without further improvement of the objective lens. This was the concept on which we based the design of the bioluminescence microscope [20, 21].

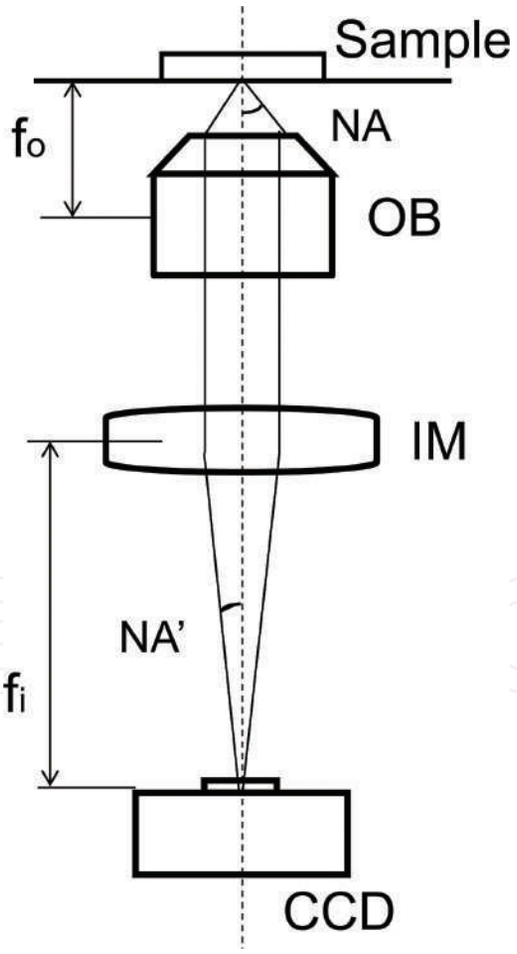


**Figure 1.** A diagram of an inverted microscope with an infinity-corrected optical system. OB, objective lens; IM, imaging lens; NA, numerical aperture of the objective lens; NA′, numerical aperture of the imaging lens; $F_o$, focal distance of the objective lens; $F_i$, focal distance of the imaging lens.

**Figure 2** shows one example of the I condition to capture bioluminescence images of live cells using a microscope. The X-axis indicates the I value of the *in vivo* macro-imaging system, OV100 (Olympus, Tokyo, Japan), using a 0.8× objective lens with an NA varying from 0.05 to 0.25 (I = 0.004–0.098). HeLa cells transiently expressing the Luc+ luciferase gene (pGL3 control vector; Promega, Madison, WI, USA) in Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) containing 1 mM D-luciferin, potassium salt (Promega) at room temperature were imaged using a CCD camera (ST-7; SBIG, Ottawa, Canada) for astronomical imaging. The exposure time was 1 min, and the cooling temperature was −20°C. The Y-axis indicates the normalized luminescence intensity of the entire area of the image captured (**Figure 2A**). As shown in the graph (**Figure 2B**), luminescence images could be captured at I values of greater than 0.02, although the M was lower for single-cell imaging. Therefore, we designed an IM to achieve an I value of 0.02 with a higher M using a conventional OB.

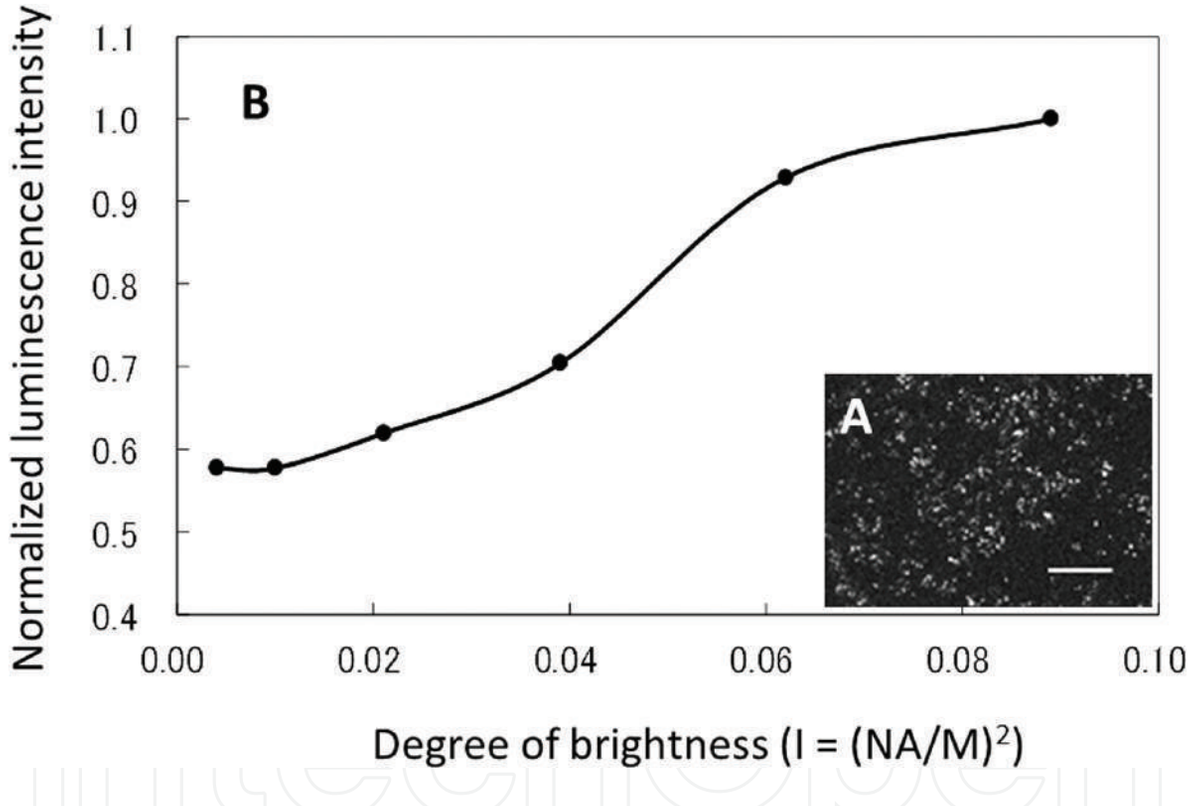


**Figure 2.** The I condition to capture bioluminescence images of HeLa cells using an OV100 *in vivo* macro-imaging system with a 0.8× objective lens and an NA ranging from 0.05 to 0.25 (I = 0.004 to 0.098). (A) HeLa cells expressing the Luc+ luciferase gene in HBSS containing 1 mM D-luciferin. Scale bars, 1000 μm and (B) normalized luminescence intensity of the image (A) against degree of brightness I.

### 2.2. Vignetting

**Figure 3** shows diagrams of light passing from an object in an infinity-corrected optical system. In this system, light from object runs parallel between the OB and IM (**Figure 3A** and **B**). Therefore, this system is suitable for several observations because several optical elements (such as mirror units for fluorescence observations or polarizing filters) can be inserted

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between the OB and IM without light-pass correction for image formation. However, light from peripheral vision (ray "a") is vignetted by the imaging lens, when the distance between the OB and IM becomes longer (**Figure 3C**). Therefore, vignetting can be avoided by shortening the distance between the OB and IM.

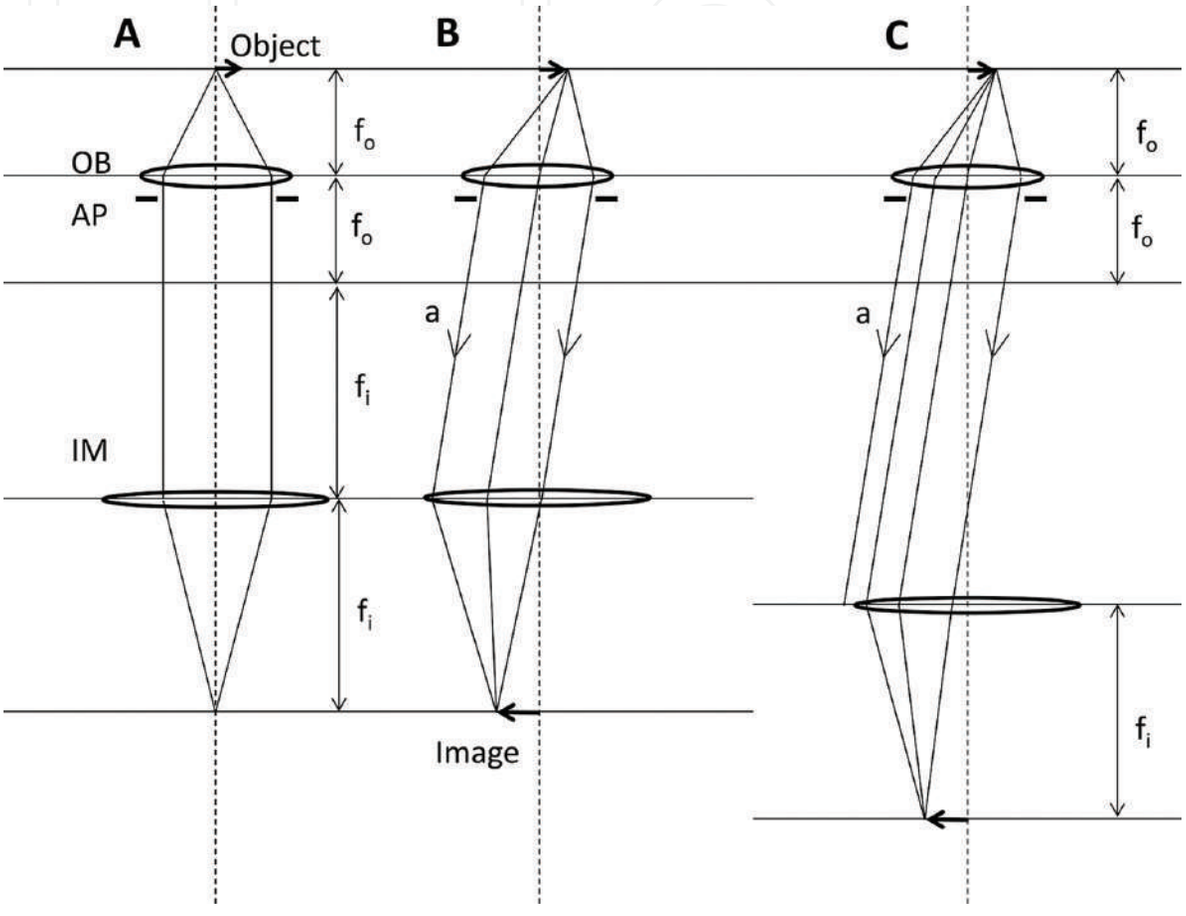


**Figure 3.** Diagrams of light passing from an object in an infinity-corrected optical system, illustrating the vignetting of light from peripheral vision by the imaging lens. (A) Light passing through the central axis. Light flux was restricted by the aperture of the objective lens; (B) light passing through peripheral vision; and (C) light passing through peripheral vision when the distance between the objective and the imaging lens was greater than that in B. Light from peripheral vision (ray "a") was vignetted by the imaging lens. OB, objective lens; AP, aperture; IM, imaging lens; $f_o$, focal distance of the objective lens; $f_i$, focal distance of the imaging lens.

### 2.3. Effective field area

**Figure 4** shows an effective field diagram on a CCD chip. Generally, the area of the CCD chip is smaller than the effective field area because peripheral vision is affected by several optical aberrations. Images on the CCD chip only show part of the light collected by the OB. If all light is collected on the CCD chip as an image by reducing the magnification using an intermediate tube lens or modified IM, the light intensity of each pixel becomes greater. Thus, image quality is sacrificed to obtain brighter images.

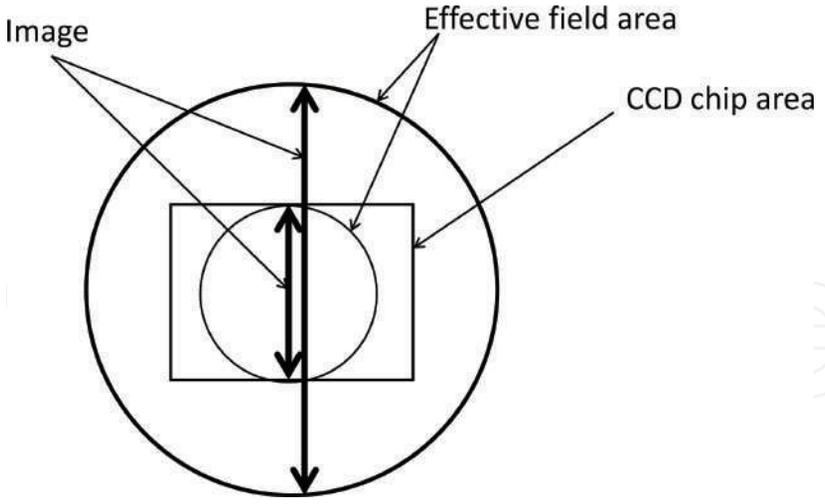


**Figure 4.** An effective field diagram on a CCD chip. Generally, the area of a CCD chip is smaller than the effective field area, and an image on a CCD chip is only some of light collected by the objective lens. If all light is collected on the CCD chip as an image by reducing the magnification using an intermediate tube lens or modified imaging lens, the light intensity of a pixel of the CCD chip increases.

### 2.4. The bioluminescence microscope, LV200

**Figure 5** shows the inverted bioluminescence microscope used in our studies (Luminoview LV200; Olympus, Tokyo, Japan). A halogen lamp was used as the source of transmitted bright-field light. The light was directed to a sample through a condenser lens with a glass fiber. A short focal-length imaging lens (f = 36 mm, NA = 0.2) was customized based on the condition of I to capture dim bioluminescence images in this system. Normal OBs are available for observation. Using the IM, total magnification was reduced to one-fifth of the magnification of the OB because the focal distance of the IM is fixed (180 mm; Olympus) in a conventional microscope body. The distance between the OB and IM was set at 17 mm to avoid vignetting. A stage-top incubator with temperature and $CO_2$ gas controllers (MI-IBC-IF; Tokai Hit Co., Shizuoka, Japan) was added to the sample stage. The observation area was covered with a dark box [21, 44].

To evaluate the performance of LV200, bioluminescence images of U2OS cell lines stably expressing CBG99, CBR, and Luc2 beetle luciferase (Promega) were captured using an UPlanFLN 40× oil objective lens (NA = 1.30, I = 0.026) and DP70 color CCD camera (Olympus). Cells were cultured on 35-mm glass-bottomed dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum and 1 mM beetle D-luciferin at 37°C with 5% $CO_2$. Binning of the CCD camera was 1 × 1 (1360 × 1024 pixels), International Organization for Standardization gain was 1600, and exposure time was 2 min. **Figure 6A** shows the bioluminescence images of cells expressing CBG99, CBR, and Luc2, captured within 2 min using a conventional color CCD camera as green, red, and orange color, respectively. Notably, bioluminescence images could not be captured under the same conditions (stable cell lines, OB, CCD camera, and exposure time) using a conventional inverted microscope (IX70; Olympus; NA = 1.30, I = 0.001), although a 10-min exposure time was required to obtain images

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for the beetle luciferase-expressing cell line (**Figure 6B**) [21]. Despite the use of blank image subtraction, 10 min is the upper limit of exposure time for the DP70 color CCD camera due to intense background evaluation [21]. Thus, bioluminescence images of cells expressing the luciferase gene can be captured using an LV200 microscope with a 40× OB and color CCD camera. In this case, the M of the image was reduced by a power of 8 owing to the short-focal-length IM, and the I value was 0.026. To equalize the I value between the LV200 and IX70 microscopes, a low M and high NA OB (e.g., 8×, NA 1.3) is required for IX70. However, an OB with such a high NA cannot be purchased commercially.


**Figure 5.** Bioluminescence microscope, LV200. A stage-top incubator with temperature and $CO_2$ gas controllers was added to the sample stage. The observation area was covered with a dark box.

To show the spatial resolution of the bioluminescence images acquired using LV200, organelle-targeted images were captured using an UPlanFLN 100× oil objective lens (Olympus) and ImagEM EM-CCD camera (C9100-13; Hamamatsu Photonics, Shizuoka, Japan). The binning of the EM-CCD camera was 1 × 1 (512 × 512 pixels), EM-gain was 1024, and exposure time was 300 ms to 1 s. NanoLuc luciferase (Promega), which is 150-fold brighter than beetle luciferase [37], was used as a tag for organelle localization, similar to a fluorescent protein. Before substrate addition (12.5 μM furimazine; Promega), cells were washed with culture medium three times.

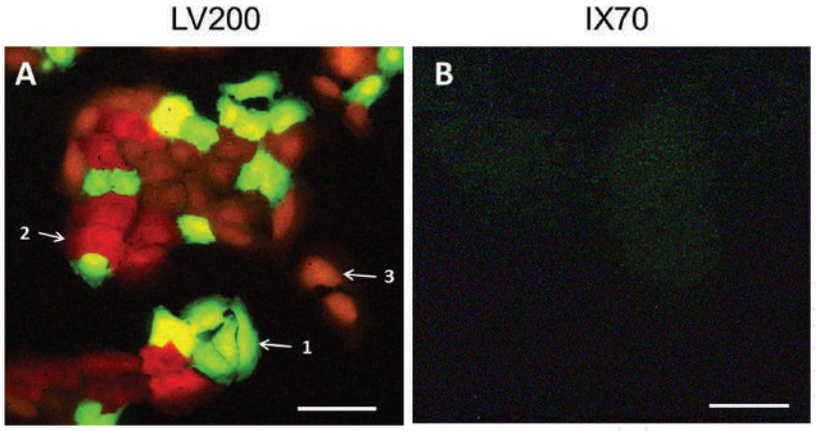


**Figure 6.** Bioluminescence images of U2OS cells expressing beetle luciferase CBG99 (arrow 1), CBR (arrow 2), and Luc2 (arrow 3) at 37°C captured by LV200 and IX70 microscopes with a UPlanFLN 40× oil objective lens and DP70 color CCD camera. The exposure times were 2 and 10 min for LV200 (M = 8, I = 0.026) and IX70 (M = 40, I = 0.001), respectively. D-Luciferin, 1 mM. Scale bars, 100 μm (A) and 20 μm (B). This figure was quoted and modified from Ref. [21] with Wiley's open access terms and conditions.

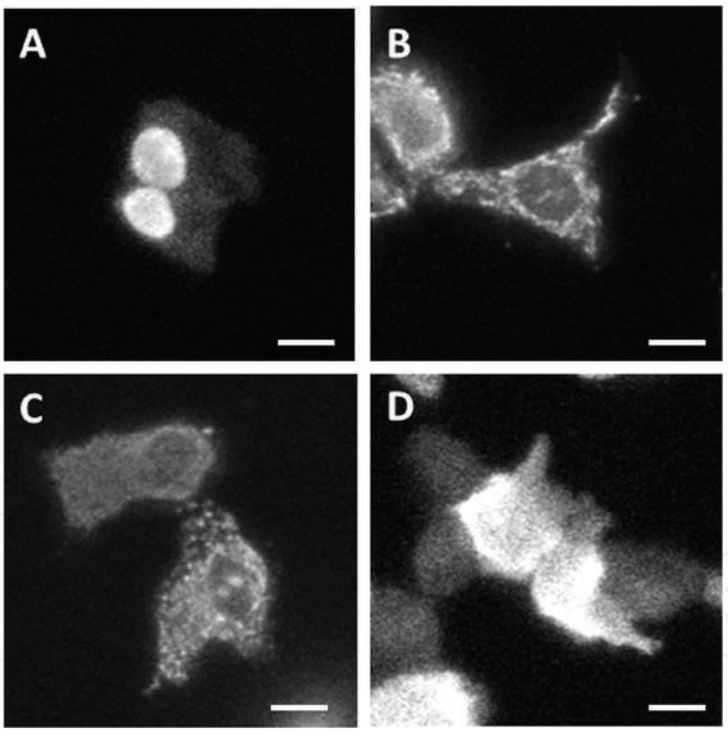


**Figure 7.** Bioluminescence images of NanoLuc fused with NLS (A), CoxVIII (B), calreticulin (C), and no targeting sequence (D) in U2OS cells at 37°C. Images were captured using an LV200 microscope with an UPlanFLN 100× oil objective lens and an ImagEM EM-CCD camera. Exposure time, 300 ms (A, D), 500 ms (B), and 1 s (C). Furimazine, 12.5 μM. Scale bars, 20 μm. This figure was quoted from Ref. [21] with Wiley's open access terms and conditions.

**Figure 7** shows bioluminescence images of NanoLuc fused with nuclear localization sequence (NLS) (**Figure 7A**), mitochondrial targeting sequence (subunit VIII of human cytochrome C oxidase, CoxVIII) (**Figure 7B**), endoplasmic reticulum resident protein, calreticulin

with KDEL retrieval sequence (**Figure 7C**), or no targeting sequence (**Figure 7D**) in U2OS cells [21]. The NanoLuc-NLS accumulated in the nucleus of the cell, and the CoxVIII-NanoLuc and calreticulin-NanoLuc-KDEL appeared in a meshwork pattern in the cytoplasm. Thus, the nucleus and cytoplasm were discriminated clearly, and mitochondria and endoplasmic reticulum were recognized in the cytoplasm.

## 3. Applications

As examples of bioluminescence microscopy using our system (LV200), we introduce three applications: (1) calcium imaging of single cells, (2) imaging of clock gene promoter assays, and (3) three-dimensional imaging of *Drosophila* larva.

### 3.1. Intracellular $Ca^{2+}$ imaging using obelin

Obelin is a calcium-specific bioluminescent protein similar to aequorin; using obelin, intracellular calcium was imaged by ATP and ionomycine (A23187) stimulation for calcium release from intracellular membranes (mitochondria and endoplasmic reticulum) and inflow from outside of the cell, respectively. The apoobelin gene [45] was inserted into the mammalian expression vector, pCDNA3.1 (Invitrogen), and transfected into HeLa cells. HeLa cells transiently expressing apoobelin were incubated in DMEM containing 60 μM coelenterazine (Promega) for 4 h to reconstitute obelin. The cells were stimulated with 500 μM ATP, and bioluminescence images were captured using an LV200 microscope with an UplanApo 20× OB (NA = 0.70; Olympus) and an iXon EM-CCD camera (DU-897l; Andor Technology, Belfast, UK). Binning of the CCD was 2 × 2, EM gain was maximum, and the exposure time was 25 s with a 30-s interval. The cells were restimulated by 10 mM ionomycine at 20 min after ATP stimulation.

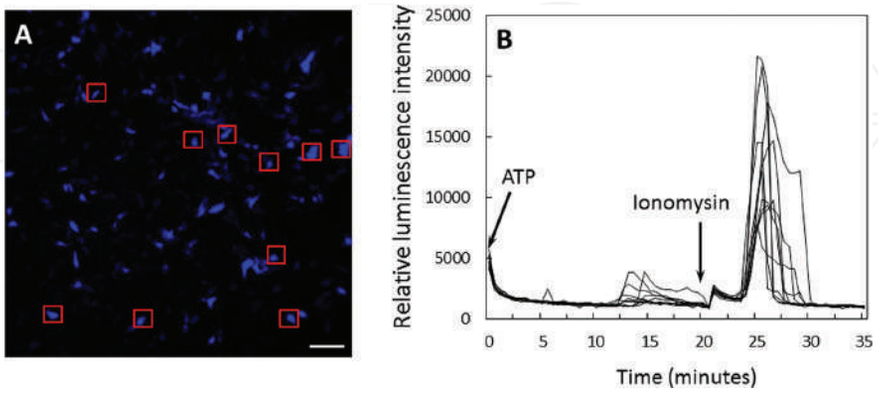


**Figure 8.** Bioluminescence image of intracellular calcium levels in HeLa cells using the photoprotein obelin (A). Time course of light intensity in single cells (B). Images were captured using an LV200 microscope with an UPlanApo 20× objective lens and an ImagEM EM-CCD camera. Cells were stimulated with 500 μM ATP and 1 mM ionomycine. Coelenterazine, 60 μM. Exposure time, 25 s. Scale bar, 200 μm.

**Figure 8A** shows pseudocolor-coded bioluminescence images of intracellular calcium in HeLa cells at 8 min after ionomysin stimulation. **Figure 8B** shows a time course of the intracellular calcium response for 10 single cells using time-laps image analysis software TiLIA [46]. Calcium responses in each cell varied temporally, were broad in intensity at around 15 min after ATP stimulation, and were uniform and greater in intensity after ionomysin stimulation [47, 48]. Using this imaging system, ATP-induced calcium oscillation in HEK-293 cells was confirmed using a bioluminescent calcium sensor constructed by aequorin and GFP with 1 s exposure time using a bioluminescence resonance energy transfer (BRET) system [36].

### 3.2. Imaging of clock gene promoter assays

The circadian rhythm is monitored by measuring the promoter activity of clock genes from individual cells as a cellular clock. However, it is impossible to resolve whether loss of circadian rhythm following stimulation is caused by dis-periodicity or dis-synchronicity in individual cells using a luminometer because the luminometer captures total luminescence from the cell population. Bioluminescence microscopy can provide clear single-cell analyses of promoter activity.

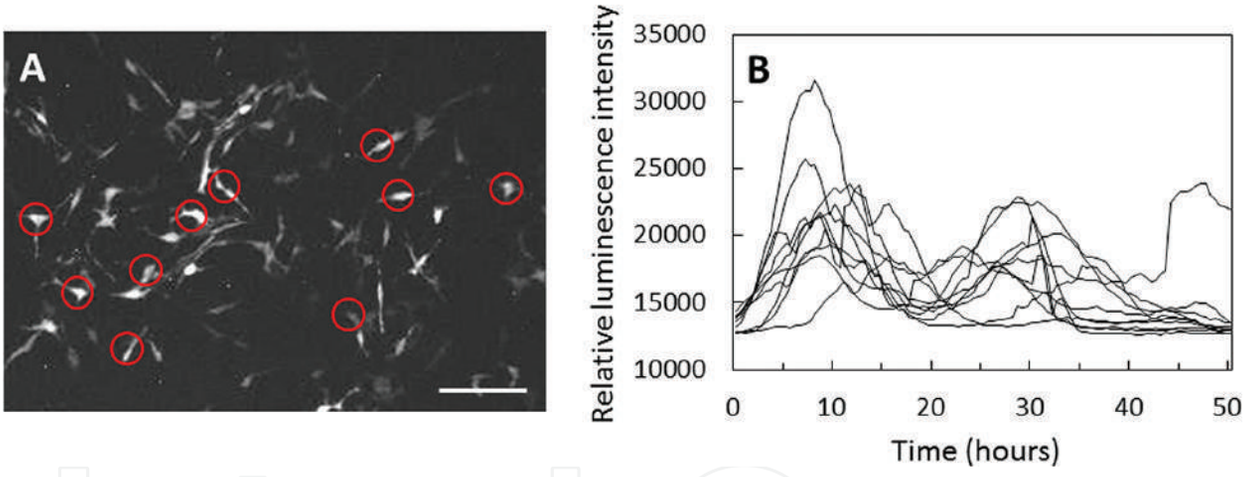


**Figure 9.** Bioluminescence image of *Per2* gene expression in NIH 3T3 cells using a *Luc*+ luciferase promoter vector (A). Time course of light intensity of the single cells (B). Images were captured using an LV200 microscope with an UPlanApo 20× objective lens and a DP30 CCD camera. Cells were stimulated with 1 mM dexamethasone. D-Luciferin, 500 μM. Exposure time, 5 min. Scale bar, 200 μm.

**Figure 9** shows an example of imaging of *Per2* clock gene promoter activity in cultured cells. The promoter region of the *Per2* clock gene in mice was inserted into the luciferase promoter vector, pGL3 (Promega), and the vector was transfected into NIH3T3 cells. Cells were cultured in $CO_2$-independent DMEM (Invitrogen) containing 500 μM D-luciferin. Bioluminescence images were captured using an LV200 microscope with an UPlanApo 20× OB (NA = 0.70) and DP30BW CCD camera (Olympus) at 37°C. The binning of the CCD was 1 × 1 (1024 × 1024 pixels), the cooling temperature of the sensor chip was 5°C, and the exposure time was 5 min with 30-min interval for 28 h. As shown in **Figure 9A**, bioluminescence images of single cells expressing the *Per2* gene were captured clearly using a conventional CCD camera. **Figure 9B**

shows a time course of *Per2* promoter activity in 10 cells selected appropriately for 48 h using TiLIA [46]; this time course allowed us to analyze synchronicity among cells [49]. Using this imaging system, Ukai et al. [23] produced photoresponsive mammalian cells by introducing the photoreceptor melanopsin and monitored the effects of photoperturbation on the state of the cellular clock. They observed that a critical light pulse drove cellular clocks into singularity behavior and proved that loss of the circadian rhythm of a cellular clock may be caused by dis-synchronization of individual cells underlying singularity behavior by single-cell analysis.

### 3.3. Three-dimensional imaging of *Drosophila larva*

Because our bioluminescence microscope system utilizes a short-focal-length imaging lens, the magnification is lower and the focal depth is shallower than those of conventional microscopy systems using the same OB. Therefore, depth of field is also shallower. This is convenient for three-dimensional image reconstruction by light sectioning.

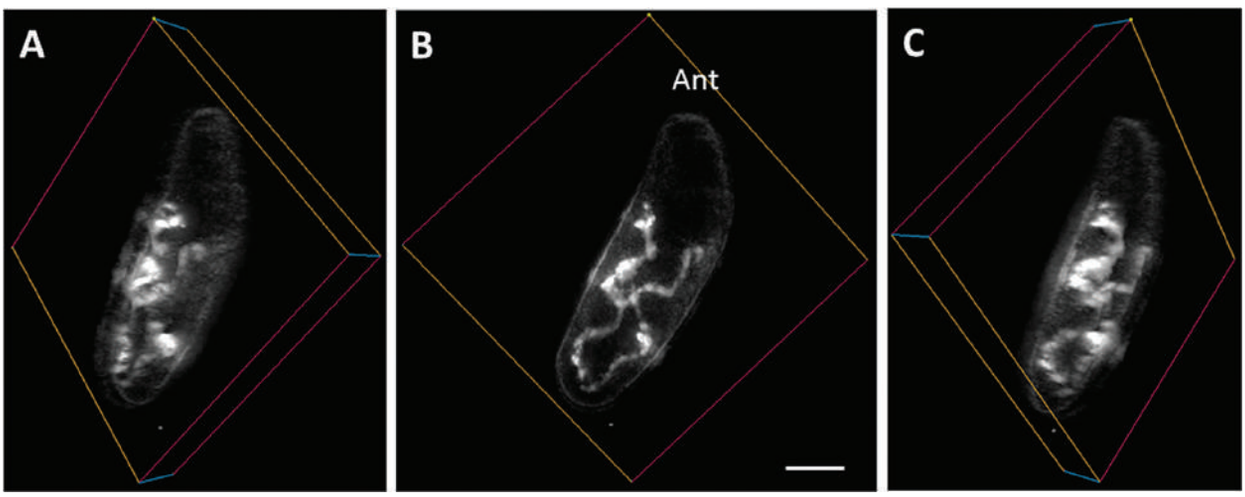


**Figure 10.** Three-dimensional bioluminescence image of armadillo promoter activity of insular larva for the transgenic *Drosophila melanogaster* reconstructs from 11 sectionalized images (100 μm depth). Expression of *armadillo* was observed in the midgut from a tissue depth of 100 μm. The larva was immersed in 3 mM D-luciferin for 5 min before image acquisition. A and C, slant view. B, front view. Scale bar, 100 μm. Ant, anterior part of the body.

Accordingly, we constructed transgenic *Drosophila melanogaster* carrying an armadillo (a member of the segment polarity gene) promoter and luciferase fusion gene [31]. **Figure 10** shows three-dimensional images of armadillo promoter activity from insular larva of transgenic flies reconstructed from 11 sectionalized images (front and slant views). The larva was immersed in 3 mM D-luciferin for 5 min before image acquisition. Bioluminescence images of the anesthetized larva with cold treatment were captured using an LV200 microscope with an UPlanFLN 60× OB (NA = 0.90) and iXon EM-CCD camera. The binning of the CCD was 1 × 1; EM gain was maximum; and exposure time was 30 s. Eleven sectionalized images were obtained with 10-μm steps from top to bottom (0–100 μm) of the larva. After deconvolution, three-dimensional images were reconstructed using CelSens Dimension image analysis software (Olympus). As shown in the **Figure 10**, the expression of the *armadillo* was observed

in the midgut from a tissue depth of 100 μm, although it needed clearing treatment of kidney tissue for imaging of 100–200 mm depth by confocal fluorescence microscopy [50].

## 4. Conclusion

In this study, we presented the concept of bioluminescence microscopy using a short-focal-length IM. This system facilitates the acquisition of bioluminescence images of single live cells using luciferase, similar to fluorescence microscopy using a fluorescent protein, although M is lower than that of conventional microscopy. Furthermore, this method is applicable for studies of cellular activity at the single cell level, including analyses of signal transduction, gene expression, and embryogenesis.

As bioluminescence microscopy requires no excitation light, it leads to substantive differences from fluorescence microscopy. Bioluminescence observation lacks the phototoxicity and background autofluorescence problems associated with fluorescence observation and permits the long-term, non-lethal observation of living cells such as embryonic stem cells, iPS cells, and embryos. **Table 1** summarized the substantive differences between the fluorescence and bioluminescence microscopy. Thus, bioluminescence microscopy is a powerful tool in cellular biology and complements fluorescence microscopy.

| | **Fluorescence** | **Bioluminescence** |
|---|---|---|
| Excitation energy | Photon | Chemical reaction |
| Auto-fluorescence | Affected | None |
| Phototoxicity | Affected | None |
| Long-term observation | Acceptable | Excellent |
| Observation of photosensitive cell | Acceptable | Excellent |
| Brightness of image | Excellent | Acceptable |
| Spatiotemporal resolution | Excellent | Acceptable |

**Table 1.** Substantive differences between fluorescence and bioluminescence microscopy.

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## References

[1] Shimomura O, Johnson FH, Saiga Y. Extraction, purification and properties of aequorin, a bioluminescent protein from hydromedusan *Aequorea*. J. Cell. Comp. Physiol. 1962; 59:223–239.

[2] Ridgway EB, Gilkey JC, Jaffe LF. Free calcium increases explosively in activating medaka eggs. Proc. Nat. Acad. Sci. U. S. A. 1977; 74:623–627.

[3] Gilkey JC, Jaffe LF, Ridgway EB, Reynolds GT. A free calcium wave traverses the activating egg of the medaka, *Oryzias latipes*. J. Cell Biol. 1978; 76:448–466.

[4] Yamamoto T. Physiology of fertilization in fish eggs. Int. Rev. Cytol. 1961; 12:361–405.

[5] de Wet JR, Wood KV, DeLuca M, Helinski DR, Subramani S. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell Biol. 1987; 7:725–735.

[6] Brasier AR, Tate JE, Habener JF. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. BioTechniques. 1989; 7:1116–1122.

[7] Alam J, Cook JL. Reporter genes: application to the study of mammalian gene transcription. Anal. Biochem. 1990; 188:245–254.

[8] Frawley LS, Faught WJ, Nicholson J, Moomaw B. Real time measurement of gene expression in living endocrine cells. Endocrinology. 1994; 135:468–471.

[9] Thompson EM, Adenot P, Tsuji FI, Renard JP. Real time imaging of transcriptional activity in live mouse preimplantation embryos using a secreted luciferase. Proc. Natl. Acad. Sci. U. S. A. 1995; 92:1317–1321.

[10] White MRH, Masuko M, Amet L, Elliott G, Braddock M, Kingsman AJ, Kingsman SM. Real-time analysis of the transcriptional regulation of HIV and hCMV promoters in single mammalian cells. J. Cell Sci. 1995; 108:441–455.

[11] Castaño JP, Kineman RD, Frawley LS. Dynamic monitoring and quantification of gene expression in single, living cells: a molecular basis for secretory cell heterogeneity. Mol. Endocrinol. 1965; 10:599–605.

[12] Kennedy HJ, Viollet B, Rafiq I, Kahn A, Rutter GA. Upstream stimulatory factor-2 (USF2) activity is required for glucose stimulation of L-pyruvate kinase promoter activity in single living islet β-cells. J. Biol. Chem. 1997; 272:20636–20640.

[13] Takasuka N, White MRH, Wood CD, Robertson WR, Davis JRE. Dynamic changes in prolactin promoter activation in individual living lactotrophic cells. Endocrinology. 1998; 139:1361–1368.

[14] Maire E, Lelièvre E, Brau D, Lyons A, Woodward M, Fafeur V, Vandenbunder B. Development of an ultralow–light–level luminescence image analysis system for dynamic measurements of transcriptional activity in living and migrating cells. Anal. Biochem. 2000; 280:118–127.

[15] Welsh DK, Yoo SH, Liu AC, Takahashi JS, Kay SA. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr. Biol. 2004; 14:2289–2295.

[16] Masamizu Y, Ohtsuka T, Takashima Y, Nagahara H, Takenaka Y, Yoshikawa K, Okamura H, Kageyama R. Real-time imaging of the somite segmentation clock: revelation of unstable oscillators in the individual presomitic mesoderm cells. Proc. Natl. Acad. Sci. U. S. A. 2006; 103:1313–1318.

[17] Hoshino H, Nakajima Y, Ohmiya Y. Luciferase-YFP fusion tag with enhanced emission for single-cell luminescence imaging. Nat. Methods. 2007; 4:637–639.

[18] Kwon HJ, Enomoto T, Shimogawara M, Yasuda K, Nakajima Y, Ohmiya Y. Bioluminescence imaging of dual gene expression at the single-cell level. BioTechniques. 2010; 48:460–462.

[19] Suzuki T, Kondo C, Kanamori T, Inouye S. Video rate bioluminescence imaging of secretory proteins in living cells: localization, secretory frequency, and quantification. Anal. Biochem. 2011; 415:182–189.

[20] Suzuki H, Dosaka S, Ohashi-Hatta Y, Sugiyama T. Luminescence microscope for reporter assay of single live cells. In: Proceedings of the 14th International Symposium on Bioluminescence and Chemiluminescence Chemistry, Biology, and Applications; 15–19 Oct 2006; San Deago. Singapore: World Scientific; 2007. pp. 53–56.

[21] Ogoh K, Akiyoshi R, May-Maw-Thet, Sugiyama T, Dosaka S, Hatta-Ohashi Y, Suzuki H. Bioluminescence microscopy using a short focal-length imaging lens. J. Microsc. 2014; 253:191–197.

[22] Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, Welsh DK, Kay SA, Ueda HR, Hogenesch JB. Feedback repression is required for mammalian circadian clock function. Nat. Genet. 2006; 38:312–319.

[23] Ukai H, Kobayashi TJ, Nagano M, Masumoto K, Sujino M, Kondo T, Yagita K, Shigeyoshi Y, Ueda HR. Melanopsin-dependent photo-perturbation reveals desynchronization underlying the singularity of mammalian circadian clocks. Nat. Cell Biol. 2007; 9:1327–1334.

[24] Akashi M, Hayasaka N, Yamazaki S, Node K. Mitogen-activated protein kinase is a functional component of the autonomous circadian system in the suprachiasmatic nucleus. J. Neurosci. 2008; 28:4619–4623.

[25] Dibner C, Sage D, Unser M, Bauer C, d'Eysmond T, Naef F, Schibler U. Circadian gene expression is resilient to large fluctuations in overall transcription rates. EMBO J. 2008; 28:123–134.

[26] Fukuda H, Tokuda I, Hashimoto S, Hayasaka N. Quantitative analysis of phase wave of gene expression in the mammalian central circadian clock network. PLoS One. 2011; 6:e23568, 1–8.

[27] Yagita K, Horie K, Koinuma S, Nakamura W, Yamanaka I, Urasaki A, Shigeyoshi Y, Kawakami K, Shimada S, Takeda J, Uchiyama Y. Development of the circadian oscillator during differentiation of mouse embryonic stem cells in vitro. Proc. Natl. Acad. Sci. U. S. A. 2010; 107:3846–3851.

[28] Edwards MD, Brancaccio M, Chesham JE, Maywood ES, Hastings MH. Rhythmic expression of cryptochrome induces the circadian clock of arrhythmic suprachiasmatic nuclei through arginine vasopressin signaling. Proc. Natl. Acad. Sci. U. S. A. 2016; 113:2732–2737.

[29] Asai S, Takamura K, Suzuki H, Setou M. Single-cell imaging of *c-fos* expression in rat primary hippocampal cells using a luminescence microscope. Neurosci. Lett. 2008; 434:289–292.

[30] Chang E, Pohling C, Natarajan A, Witney TH, Kaur J, Xu L, et al. AshwaMAX and Withaferin A inhibits gliomas in cellular and murine orthotopic models. J. Neurooncol. 2016; 126:253–264.

[31] Akiyoshi R, Kaneuch T, Aigaki T, Suzuki H. Bioluminescence imaging to track real-time armadillo promoter activity in live *Drosophila* embryos. Anal. Bioanal. Chem. 2014; 406:5703–5713.

[32] Sramek C, Mackanos M, Spitler R, Leung LS, Nomoto H, Contag CH, Palanker D. 2011. Non-damaging retinal phototherapy: dynamic range of heat shock protein expression. Invest. Opthalmol. Vis. Sci. 2011; 52:1780–1787.

[33] Horibe T, Torisawa A, Akiyoshi R, Hatta-Ohashi Y, Suzuki H, Kawakami K. Transfection efficiency of normal and cancer cell lines and monitoring of

promoter activity by single-cell bioluminescence imaging. Luminescence. 2014; 29:96–100.

[34] Horibe T, Torisawa A, Kurihara R, Akiyoshi R, Hatta-Ohashi Y, Suzuki H, Kawakami K. Monitoring Bip promoter activation during cancer cell growth by bioluminescence imaging at the single-cell level. Integr. Cancer Sci. Therap. 2015; 2:291–299.

[35] Kikuchi O, Ohashi S, Horibe T, Kohno M, Nakai Y, Miyamoto S, Chiba T, Muto M, Kawakami K. Novel EGFR-targeted strategy with hybrid peptide against oesophageal squamous cell carcinoma. Sci. Rep. 2016; 6:22452, 1–12.

[36] Rogers KL, Martin JR, Renaud O, Karplus E, Nicola MA, Nguyen M, Picaud S, Shorte SL, Brûlet P. Electron-multiplying charge-coupled detector-based bioluminescence recording of single cell $Ca^{2+}$. J. Biomed. Optics. 2008; 13:031211, 1–10.

[37] Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, Otto P, Zimmerman K, Vidugiris G, Machleidt T, Robers MB, Benink HA, Eggers CT, Slater MR, Meisenheimer PL, Klaubert DH, Fan F, Encell LP, Wood KV. Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. ACS Chem. Biol. 2012; 7:1848–1857.

[38] Sugiyama T, Suzuki H, Takahashi T. Light-induced rapid $Ca^{2+}$ response and MAPK phosphorylation in the cells heterologously expressing human OPN5. Sci. Rep. 2014; 4:5352, 1–10.

[39] Binkowski B, Fan F, Wood K. Engineered luciferases for molecular sensing in living cells. Curr. Opin. Biotechnol. 2009; 20:14–18.

[40] Cosby N. Enabling kinetic studies of GPCRs. Live-cell, non-lytic biosensor for GPCR profiling. Screening. 2009; 4:28–29.

[41] Compan V, Pierredon S, Vanderperre B, Krznar P, Marchiq I, Zamboni N, Pouyssegur J, Martinou JC. Monitoring mitochondrial pyruvate carrier activity in real time using a BRET-based biosensor: investigation of the Warburg effect. Mol Cell. 2015; 59:491–501.

[42] Pratx G, Chen K, Sun C, Martin L, Carpenter CM, Olcott PD, Xing L. Radioluminescence microscopy: measuring the heterogeneous uptake of radiotracers in single living cells. PLoS One. 2012; 7:e46285, 1–9.

[43] Pratx G, Chen K, Sun C, Axente M, Sasportas L, Carpenter C, Xing L. High-resolution radioluminescence microscopy of 18F-FDG uptake by reconstructing the β-ionization track. J. Nucl. Med. 2013; 54:1841–1846.

[44] Goda K, Hatta-Ohashi Y, Akiyoshi R, Sugiyama T, Sakai I, Takahashi T, Suzuki H. Combining fluorescence and bioluminescence microscopy. Microsc. Res. Tech. 2015; 78:715–722.

[45] Marcova SV, Vysotski ES, Blinks JR, Burakova LP, Wang BC, Lee J. Obelin from the bioluminescent marine hydroid *Obelia geniculate*: cloning, expression, and comparison

of some properties with those of other $Ca^{2+}$-regulated photoproteins. Biochemistry. 2002; 41:2227–2236.

[46] Konno J, Hatta-Ohashi Y, Akiyoshi R, Thancharoen A, Silalom S, Sakchoowong W, Yiu V, Ohba N, Suzuki H. TiLIA: a software package for image analysis of firefly flash patterns. Ecol. Evol. 2016; 6:3026–3031.

[47] May-May-Thet, Sugiyama T, Suzuki H. Bioluminescence imaging of intracellular calcium dynamics by the photoprotein obelin. In: Proceedings of the 15th International Symposium on Bioluminescence and Chemiluminescence Light Emission: Biology and Scientific Applications; 13–17 May 2008; China. Singapore: World Scientific; 2009. pp. 359–362.

[48] Olympus. Intracellular calcium imaging using photoprotein, obelin. 2009. Available from: http://bioimaging.jp/appli/007/pdf/app_lv200.pdf [Accessed: 2016-06-06]

[49] Olympus. Imaging of clock gene promoter assay of culture cells. 2009. Available from: http://bioimaging.jp/appli/004/pdf/app_lv200.pdf [Accessed: 2016-06-06]

[50] Clendenon SG, Young PA, Ferkowicz M, Phillips C, Dunn KW. Deep tissue fluorescent imaging in scattering specimens using confocal microscopy. 2011; Microsc. Microanal. 17:614–617.

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