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Physical aspects of delayed luminescence in *Acetabularia acetabulum*

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Abstract. Delayed photo-induced luminescence has been studied in *Acetabularia acetabulum* individuals. The number of photons re-emitted when the wavelength, the intensity, or the duration of the incident radiation were varied are reported for 10 individuals. Reproducibility of the measurements in the same and in different individuals was good. The association between the incident radiation and photon re-emission was found to be non-linear. The relationship between the quantities involved in the phenomenon is discussed and a phenomenological equation describing the relationship is presented.
Key words. Bioluminescence; biophysical techniques; biooptics.

The increased sensitivity of photometric techniques has objectively proved the existence of a ubiquitous low-intensity luminescence, both spontaneous and photo-induced, emitted by all living systems. This radiation ranges at least from the UV to the near infrared section of the electromagnetic spectrum, with an intensity of the order of 10 up to 10^5 photons/cm^2/sec.

To date, the mechanism of this phenomenon is not well known, and two completely different hypotheses have been proposed in order to interpret it: i) a biochemical hypothesis, and ii) a biophysical one.

The biochemical hypothesis[1] states that the phenomenon originates essentially from spontaneous chemiluminescence ('imperfection theory'); accordingly the emission is associated with biological oxidation or radical reactions and has no function in the mechanism of regulation of cellular activities. The biophysical hypothesis[2,3] asserts that spontaneous and photoinduced luminescence, also referred to as 'biophoton emission', originates from a delocalized coherent electromagnetic field within the tissue, where the main source is thought to be DNA, and plays a role as a regulator of biological processes like cell growth, cell division and death, photosynthesis and carcinogenesis. In this scheme, biophotons act as information carriers in intra- and intercellular communication[4].

The experimental results collected thus far do not discriminate between the two hypotheses, so more complete data is needed.

A promising approach[3] to the study of low-intensity luminescence seems to be the analysis of the response of biological systems to photon excitation, namely delayed luminescence, to gain new information[5] on the processes responsible for the phenomenon, and to determine the significant parameters for describing the luminescence in biological systems.

Following previous studies[5], this paper reports a systematic experimental investigation on the yield of the long-term delayed luminescence (i.e. the total number of counted photons stored in a preset time by the detection apparatus) emitted by a simple living organism: the unicellular alga *Acetabularia acetabulum*. The aim is to examine the relationship between the macroscopic quantities involved in the phenomenon. Observed correlations could be used to define a simple model in order to explain the mechanism of the long-term delayed luminescence.

Materials and methods

Acetabularia acetabulum (L.)[7] is a unicellular alga whose dimensions may vary considerably (up to a few centimeters long); it has a basic structure comprised of a tube-like axis, called the stalk, a more or less branched rhizoid and, in the advanced growth stage, a reproductive cap. *Acetabularia* has the advantage of being a simple organism. It can be easily grown in the laboratory, so that it is possible to obtain several samples with uniform characteristics.

The experimental setup for measuring the photons emitted from biological systems has been described in a previous paper[6]. It consists of a steel dark chamber where the samples to be analyzed can be maintained at a constant temperature. This was set at 20 ± 0.1 °C in the present experiment, i.e. within the range of the normal living conditions of *Acetabularia*.

The radiation emitted from the sample was detected by a photomultiplier Thorn Emi 9558 QA, cooled down to -30 °C to decrease the dark current. The photocathode diameter was 50 mm and it was placed 15.5 cm from the sample holder. The spectral sensitivity of the instrumentation ranged from 200 nm to 850 nm.

The exciting radiation sources used were circular arrays of high intensity LEDs. Three different types of LED

were used (Oshino OLUY 153, OLUG 153, OLUR 153, SUR 150B) with different emission wavelengths; 565 nm (25 nm HWHM), 585 nm (35 nm HWHM) and 660 nm (25 nm HWHM) respectively. The LEDs were fed current from a stabilized power supply (Hewlett-Packard 6216 A).

The area covered by the light was greater than the sample holder, and the non-uniformity of its intensity within the sample holder was less than 5%. The flux of light impinging on the sample was measured by a Laser Pico-Watt Digital Power Meter (Newport Corporation mod. 835) equipped with a Silicon Photosensor (mod. 818-SL); in the measurements performed the flux ranged from $0.8 \cdot 10^{12}$ up to $1.08 \cdot 10^{16}$ photons/cm^2/sec.

Measurements consisted of illuminating an individual *Acetabularia* and counting the number of photons re-emitted from the sample after the light source had been switched off. During the illumination a light shutter, above which the photomultiplier was fastened, was closed in order to prevent the preillumination of the photomultiplier. After the light source had been switched off, the shutter was opened by an electromagnetic actuator. Owing to the resulting time lag, the photon counting started 100 msec after the source had been switched off. The illumination time varied from 1 to 1000 sec. The counting of photons emitted by the sample, after each illumination, was stored by a 4096 channel scaler; a dwelling time of 100 msec was chosen in order to measure the decay dynamics as well. No spectral analysis of the emitted photons was performed.

In the detection time of 400 sec more than 95% of the total re-emission from the *Acetabularia* could be collected. In addition, it was found that a time interval of 1800 sec between two successive illuminations was sufficient to enable the emission from the *Acetabularia* to return to the value prior to the perturbation, within the margin of experimental error.

The measurements were performed on 10 different individuals of *A. acetabulum* in the growth phase, in which it is characterized by a long stalk (2–3 cm long and 0.1–0.2 mm diameter) without any reproductive cap. The algae were maintained in daylight, at room temperature in sterile artificial sea water. During measurements a single individual was placed in a plastic Petri cuvette (50 mm diameter) filled with 10 cc of the same artificial sea water. In order to reduce the environmental influence, and to avoid residual luminescence from the materials, each sample, i.e. each individual in a cuvette filled with artificial sea water, was kept in the dark room for 12 h before starting the measurements.

The yield of photons emitted from the cuvette filled with artificial sea water, but without *Acetabularia*, was measured under the same experimental conditions for each set of measurements; the background values obtained, averaged over five runs, have been subtracted from the measurements taken for each sample.

Results and discussion

The reproducibility of the measurements was tested by comparing several runs and different individuals. Measurements of the counting yield values, under the same excitation conditions, were repeated up to four times for every biological individual. On average, the ratio between the standard deviation and the mean value of each set of measurements was 4% for measurements repeated on the same sample within 8 h, while it increased up to 13% if the set included measurements made on different days during a week, i.e. in the time required to perform a complete set of measurements on a single individual, including different illumination fluxes and durations.

Figure 1 shows, for a single individual, the counting yield as a function of the incident photon flux for three exciting light wavelengths. It appears that the sample response is independent of the wavelength of the incident radiation. This result suggests that the re-emitted radiation is not directly related to absorption by *Acetabularia* pigments. In fact, data from the literature[7] show that in the absorption spectrum of pigment extracts of green *Acetabularia* cells there are peaks at 433 and 663 nm, while a weak structured trend is present between the two peaks. By taking into account the emission spectrum of the LED sources used for illumination, we can define an 'effective spectral absorption' of the *Acetabularia* pigments. This is obtained by multiplying, for each wavelength of the spectrum, the optical density of *Acetabularia* pigments[7] by the emissivity of the LED source at that wavelength. Figure 2 reports the values obtained: obviously, the areas of the peaks are proportional to the number of photons absorbed in each wavelength range. If the only mechanism involved in producing the re-emitted radiation was related to pigments, one would expect a much more intense emission after illumination with red LEDs than with green

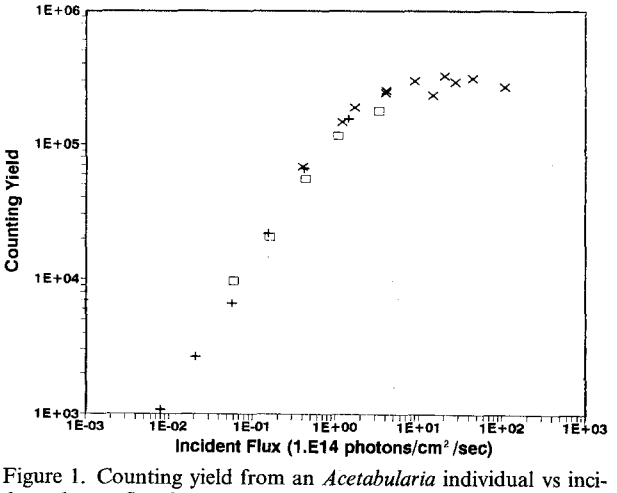


Figure 1. Counting yield from an *Acetabularia* individual vs incident photon flux for an illumination interval of 5 sec: (+) green light (565 nm); (□) yellow light (585 nm); (×) red light (660 nm).

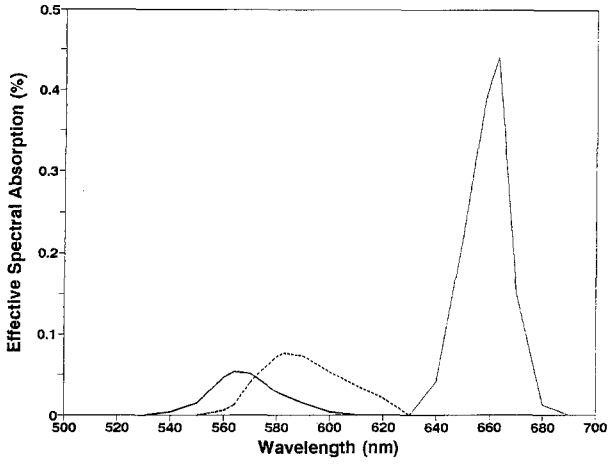
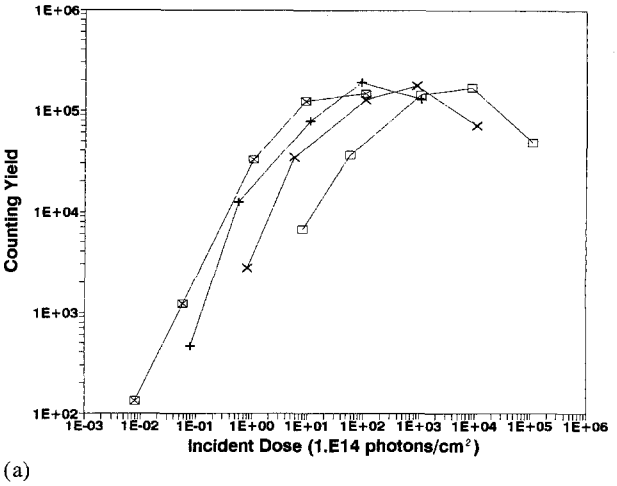
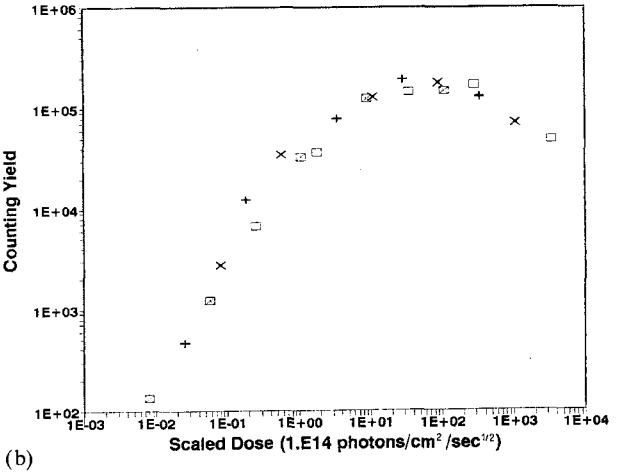


Figure 2. Effective spectral absorption of the pigments of *Acetabularia* (i.e. the spectral absorption of pigments times the spectral emission curve of sources) for the sources used: (marked solid line) green light (565 nm); (marked dashed line) yellow light (585 nm); (solid line) red light (660 nm).



(a)



(b)

Figure 3. *a* Counting yield of an *Acetabularia* individual vs incident photon dose with different times of illumination: (⊠) 1 sec; (+) 10 sec; (×) 100 sec; (□) 1000 sec. *b* Effect of rescaling on the counting yield curves of figure 3a. Here counting yield is plotted against incident photon flux times the square root of the illumination time: (⊠) 1 sec; (+) 10 sec; (×) 100 sec; (□) 1000 sec illumination time.

or yellow ones, for a fixed incident photon flux. Figure 1 shows that this does not occur.

Experimental results have shown that the counting yield, as a function of the incident luminous flux, has the same trend for different *Acetabularia* individuals, and it has been possible to evaluate a normalization factor for each sample, weighted over all the experimental points referring to the same individual. Such an empirical normalization factor, which ranges between 0.6 and 1.7, takes into account the biological differences between samples such as size, age and so on; its use allows the results that refer to different individuals to be compared.

Figure 3a shows the total counting yield values for an *Acetabularia* individual as a function of the incident photon dose, i.e. the incident photon flux times the illumination time; the solid lines connect experimental points obtained with the same illumination time. The plot covers an incident photon flux range of five orders and a time-interval range of three orders. It appears that the *Acetabularia* response does not exhibit a simple dependence either on the incident dose, or separately on incident photon flux or illumination time.

A procedure was found empirically which makes it possible to combine the curves of figure 3a into a single one. This is done by plotting the number of re-emitted photons against the "scaled incident dose", which is the incident photon flux multiplied by the square root of the illumination time. As an example, figure 3b reports the same y-values as in figure 3a as a function of the new x-coordinates calculated as described above; for clarity, the same symbols have been used in the two figures.

The same trend can be recognized for the set of data for any of the *Acetabularia* individuals examined. Figure 4 reports the results of the whole set of measurements including 10 illumination-time intervals, 19 values of incident photon flux, and 10 *Acetabularia* individuals. In this plot, the results that refer to different individuals have been normalized by means of the normalization factor described above, which takes into account the differences among different individuals.

Figure 4 shows that all the measured counting yields follow a simple and unique curve if they are plotted against the scaled incident dose; the curve is independent of the particular sample considered and of the irradiation conditions, including wavelength. In this plot the counting yield increases up to a broad maximum and then decreases.

Experimental points were fitted to a quadratic form of log(y) on log(x), where x denotes the scaled incident dose as described above and y the counting yield. No varying weights for the variable $z = \log(y)$ were consid-

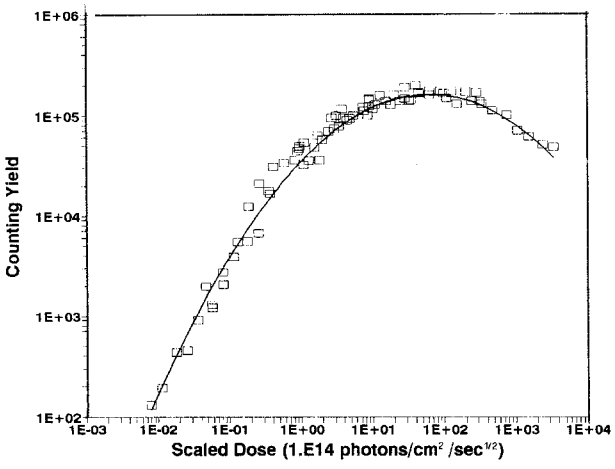


Figure 4. Normalized counting yield values for 10 *Acetabularia* individuals, plotted vs the scaled incident dose, i.e. the incident photon flux multiplied by the square root of the illumination time. Experimental points refer to different values both of the incident flux and the illumination time intervals. (□) Experimental points; (solid line) fit according to eq. (1).

ered in the least-square fit: in fact, due to the propagation of errors, the uncertainties of the z-values are equal to the percent error on the y-values, which can be assumed to be constant under our experimental conditions, and is equal to 13%, as stated above. A look at figure 4 shows that such an error is comparable with the dimension of the symbol used for the experimental point, so it is not reported.

The fit is quite good: a coefficient of determination of 0.985 has been obtained, with a residual mean square of 0.05.

Starting from the estimation of the parameters of the regression, the coordinates of the maximum point of the theoretical 2nd-order curve were determined. If x_m and y_m denote the values of scaled incident dose and counting yield corresponding to such a maximum point, it is easy to show that the relation between y and x can be written in the form:

$$y = y_m(x/x_m)^{-c \cdot \ln(x/x_m)}$$

where:

$$x_m = (58 \pm 7) \cdot 10^{14} \text{ photons/cm}^2/\text{sec}^{1/2},$$

$$y_m = (1.8 \pm 0.2) \cdot 10^5 \text{ counts},$$

$$c = 0.094 \pm 0.002.$$

The standard errors of estimates of these parameters were estimated according to the propagation of errors.

Conclusions

Results of measurements show that photoemission after illumination is a highly reproducible process in *Acetabularia*, both in the same and in different individuals.

It is worth noting the following two aspects: 1) the counting yield of re-emitted photons reaches a maximum at a relatively low figure of the incident dose (about 10^{16}–10^{17} photons/cm^2) and then it decreases (see fig. 3a); 2) despite the absorption spectrum of the *Acetabularia* pigments, the counting yield is completely independent of the incident wavelength.

It seems very difficult to explain these experimental results by using a theoretical model which attributes delayed luminescence to the random decay of molecular levels.

On the contrary, the decreasing counting of the yield after the maximum, and the collapse of all counting yield curves into a single one by rescaling (in terms of incident flux times the square root of illumination time) suggests the presence of cooperative phenomena and the possibility of explaining delayed luminescence within the framework of non-linear optical theory.

The above hypothesis is also supported by experimental results previously published on re-emission decay curves; in all the cases examined it was found that decay curves cannot be fitted by a simple exponential decay but follow a hyperbolic trend[5].

At present, no model exists that can explain the experimental results reported in this paper. The phenomenological equation obtained, which connects the input parameters, i.e. the photon flux and the illumination time, with the output parameter of the phenomenon, i.e. the counting yield, could provide a starting point for sketching a simple model which would help to explain the mechanism of delayed luminescence.

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