Laser-ultraviolet-A-induced ultraweak photon emission in mammalian cells

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Abstract. Photobiological research in the last 30 yr has shown the existence of ultraweak photon emission in biological tissue, which can be detected with sophisticated photomultiplier systems. Although the emission of this ultraweak radiation, often termed biophotons, is extremely low in mammalian cells, it can be efficiently increased by ultraviolet light. Most recently it was shown that UV-A (330 to 380 nm) releases such very weak cell radiation in differentiated human skin fibroblasts. Based on these findings, a new and powerful tool in the form of UV-A-laser-induced biophotonic emission of cultured cells was developed with the intention to detect biophysical changes between carcinogenic and normal cells. With suspension densities ranging from 1 to 8×10^6 cells/mL, it was evident that an increase of the UV-A-laser-light induced photon emission intensity could be observed in normal as well as melanoma cells. Using this new detection procedure of ultraweak light emission, photons in cell suspensions as low as 100 μL could be determined, which is a factor of 100 lower compared to previous procedures. Moreover, the detection procedure has been further refined by turning off the photomultiplier system electronically during irradiation leading to the first measurements of induced light emission in the cells after less than 10 μs instead of 150 ms, as reported in previous procedures. This improvement leads to measurements of light bursts up 10^7 photons/s instead of several hundred as found with classical designs. Overall, we find decreasing induction ratings between normal and melanoma cells as well as cancer-prone and melanoma cells. Therefore, it turns out that this highly sensitive and noninvasive device enables us to detect high levels of ultraweak photon emission following UV-A-laser-induced light stimulation within the cells, which enables future development of new biophysical strategies in cell research. [DOI: 10.1117/1.1899185]

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1 Introduction

Photons can be regarded as the information carriers of matter because they can be exchanged within atomic and molecular interactions. In the early 1920s, the Russian biologist Gurwitsch suggested that ultraweak photon emission transmit information in cells,[1] which has been refuted by Hollaender and Klaus[2] as reviewed before.[3] The presence of biological radiation was reexamined with the development of photomultiplier tubes in the mid-1950s by scientists from Italy.[4] In the 1970s, several pioneers, Quickenden and Que-Hee in Australia,[5] Ruth and Popp in Germany,[6] and Inaba et al. in Japan,[7] independently developed methods for ultraweak photon emission measurements in a variety of different cells by the use of an extremely low noise, highly sensitive photon counting system. This research showed spontaneous ultraweak photon emission, also called biophotonic radiation, in yeast, plant, and animals cells.[8–14] In a report published 12 yr ago, experiments of UV-irradiated skin fibroblasts were described and it was found that repair deficient xeroderma pigmentosum (XP) cells show an efficient increase of ultraweak photon emission in contrast to normal cells.[9] This important difference between normal and XP fibroblasts suggests that there is an effective intracellular mechanism of photon absorption in normal human cells. This mechanism is most probably coupled with delayed luminescence (DL), a phenomenon that was recently reviewed by Popp and Yan which described this ''longterm'' afterglow in biological systems (the intensity is significantly lower than the known fluorescence or phosphorescence) in terms of coherent states.[15] Furthermore, Popp et al. have proposed that nonclassical, so-called squeezed light, may play a

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role in biological systems.[16] Moreover, there is also evidence that DL is a sensitive indicator of the physiological state of cell systems. Experimental data obtained in this respect will open new application perspectives in environmental pollution and food quality control.[17,18] A theoretical model that correlates excitation and successive decay of coherent collective electron states in macromolecular structures as found in the cytoskeleton has been developed and theoretical prediction has been successfully compared to experimental data.[19,20] The importance of the cell nucleus for participating in ultraweak photon emission has been discovered by Devaraj et al. more than a decade ago,[21] a finding that was confirmed by our laboratory in the meantime.[22] We have further shown that in defined stages of the fibroblast differentiation system, which has been described by Bayreuther et al.[23] and Niggli et al.,[24] UV light elevates this ultraweak photon emission in mitomycin-C-induced postmitotic XP fibroblasts at least by a factor of 2 compared to mitotic XP cells.[9] Just recently, we reported for fibroblasts that the most important induction range for these very weak photon emissions is the UV-A range between[10] 330 to 380 nm, a finding confirming Albrecht-Buehler's observation of fluorescence increase in mammalian mitochondria after 365 nm excitation.[25] Based on all of these discoveries, we describe now a highly sensitive technique for UV-A-laser-induced ultraweak photon emission to evaluate differences between cancer and normal cells. The significant distinctions suggest that this new biological model system is an innovative and sophisticated approach to shed new insights on the importance of light in cell biology.

2 Experimental Arrangement

2.1 *Cell Culturing*

Normal skin fibroblasts GM 1717 (p 9) from a 40-yr-old male adult and cells from an XP patient of complementation group A (XPA), XP12BE (GM05509A; p 12; 17-yr-old female) as well as XP cells with low repair (25%; GM05293; p 15; 6-yr-old male) were obtained from the Human Genetic Mutant Cell Repository (Camden, New Jersey). Normal cells from a 40-yr-old male (CRL 1221; p 16-22), repair deficient XPA cells (CRL 1223; p 15; 10-yr-old female), Cloudman S91 mouse melanoma cells of clone m-3 (CCL 53.1; p 40), and human white melanoma cells from a 53-yr-old man (CRL 1585; p 5) were purchased from the American Type culture collection (Rockville, Maryland). Foreskin-derived normal human fibroblasts 3229 derived from a 1-day-old boy were a gift from Dr. Robert Zimmerman (Boston, Massachusetts) and used in passage 13 or 117. Normal cells were plated 1:2, XP cells 1:3, and melanoma cells 1:10. For the determination of ultraweak photon emission, cells were cultured in tissue culture plastic flasks (surface, 75 cm^2, Gibco Basel, Switzerland) in 15 ml Dulbecco's modified Eagle's medium (DMEM; Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum and 100 units (U) ml^{-1} penicillin-streptomycin, as previously described,[10,22] and grown for 3 to 7 days, depending on the cell type, until confluency. Cells were counted in a hemocytometer from Neubauer (Flow Laboratories, Baar, Switzerland) and cells were counted in triplicates ($\pm$10%). Cells were frozen in liquid nitrogen using the cryopreservation apparatus from Biotech Research Laboratories (Rockville, Maryland). Controlled gradual temperature reduction during cryopreservation was critical for the maintenance of cell life and viability. This apparatus preserves cells at the rate of 1°C/min when placed in a $-70\,°C$ freezer. After 5 h or overnight, frozen samples were transferred to the liquid nitrogen container. A special transport storage system for liquid nitrogen (Cryo Diffusion, Lery, France) was used to transfer the cells to Catania (Italy) to perform ultraweak photon emission measurements. For this analytical procedure, cells were prepared as follows: all cells were gently thawed, centrifuged at 1200 rpm, and the medium was discarded. The cells were washed two times in 5 mL phosphate buffered saline (PBS), centrifuged at 1200 rpm, and resuspended in 250 μL PBS. For ultraweak photon emission measurements, 100 μL of this ultimate cell suspension was used.

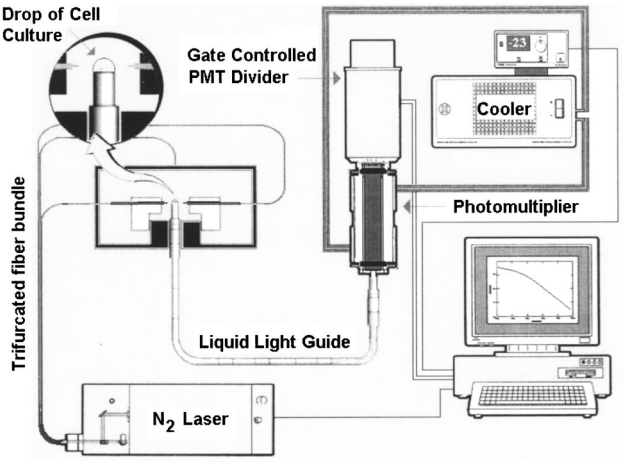

Fig. 1 Block diagram of the highly sensitive UV-A-laser-induced ultraweak photon emission detection system.

2.2 *Delayed Luminescence Measurements in Mammalian Cells after UVA Laser Induction*

The delayed luminescence or light-induced ultraweak photon emission has been measured using[26] the device ARETUSA, developed at the Southern National Laboratory in Catania. This measuring system consists of a cooled single-photon-count photomultiplier (PMT), a highly efficient optical system able to collect the emitted light from the sample and to transmit it to the PMT, a high-intensity pulsed nitrogen laser with pulses at 337 nm, an electronic device able to deactivate the PMT during the laser pulses and an electronic setup able to coordinate the measurement procedure and to collect, analyze, and elaborate the signals from the PMT. A block diagram of such a system is shown in Fig. 1.

We used the Hamamatsu R1878 PMT. It is a multialkali photocathode having a spectral response from 300 to 850 nm with a maximum response at 420 nm. The effective area is small (only 4 mm in diameter) and therefore a high optical efficiency of the setup is required. However, under these conditions, the background noise is lowered to 0.01 compared to the 2 in. area of PMTs, which we described previously.[9,17] To further reduce the noise, the PMT was cooled to $-20\,°C$, using an original forced circulation cooling system (ethanol as liquid), which is placed in direct contact with the lateral surface of the PMT, as shown in Fig. 1. We placed 100 μL of cell suspension directly, without using any cuvette, as depicted in

Table 1 Comparision of the classical DL procedure with the new design.

XPA Fibroblasts (cells/mL)	Classical Design (counts)	New Device (million counts)
10^6	766±20	3.4±0.04

Initial white light induced ultraweak photon emission after 150 ms (dwell time 50 ms) in repair, deficient XPA monolayer fibroblasts (GM5509) compared to initial values following UV-A laser induction in the same cell strain after 8.5 μs (dwell time 1 μs). Medium is subtracted.

the same Fig. 1, on the upper quartz termination of a liquid light guide NT 53-691 (Edmund Optics, New Jersey), which shows a transmission coefficient greater than 50% in the range of 300 to 650 nm.

As the light source, we used a pulsed nitrogen laser (Laser Photonics LN203C, Lake Mary, Florida) characterized by a wavelength of 337.1 nm, a pulse width of 5 ns, and an energy of about 100 μJ/pulse (±3% standard deviation). The laser was used in a single pulse mode. In short, the sample was illuminated by a single pulse and immediately afterward the DL was measured. Every measurement required about 2 s (the operation frequency of the laser was 0.5 Hz, which is roughly two orders of magnitude less than its maximum operation frequency). To receive a uniform illumination of the measured samples (a hemisphere having a diameter of approximately 5 mm) we used a lighting system based on a trifurcated quartz fiber. The three terminations surround the sample at a constant distance with an angle of 120 deg to each other (see Fig. 1). Under these conditions almost all of the light emitted by the laser source (as controlled by an optical power meter) reached the sample with a very high power per unitary volume (about 6×10^{11} W/m^3).

One of the main problems in ultraweak photon emission measurements is the necessity to protect the PMT during the illumination time. This action was performed until now by electromechanical shutters whose operational times are[9,27] of the order of 150 ms. In the system presented here, an electronic gate was developed that was controlled by a digital signal. This device is able to establish on all dynodes the same tension as a normal voltage divider circuit, when the digital signal has a lower level, while it inverts the sign of the voltage between the cathode and the first dynode and between the third and the second dynode if the digital signal has its higher level. Using this protocol, it enables us to start the photon counting only a few microseconds after the laser pulses have reached the sample.

The measurement process is performed and controlled by a microcomputer boosted through an Ortec multichannel scaler (MCS) plug-in card characterized by dwell times ranging from 100 ns to 1300 s, a memory length of 65.536 channels, and input counting rates up to 150 MHz. The signals comings from the PMT are first processed by an octal discriminator (Lecroy 6408B) and then the transistor-transistor logic output (TTL) is sent to the MCS for acquisition.

The synchronization of the process is managed by MCS. Its start-out TTL signal is split in three identical signals, and each of them is addressed to a gate and delay generator (Ortec GG 8000) to temporarily protect the PMT during the sample irradiation. After a delay time of about 6 μs, the inhibition stops and the acquisition can begin.

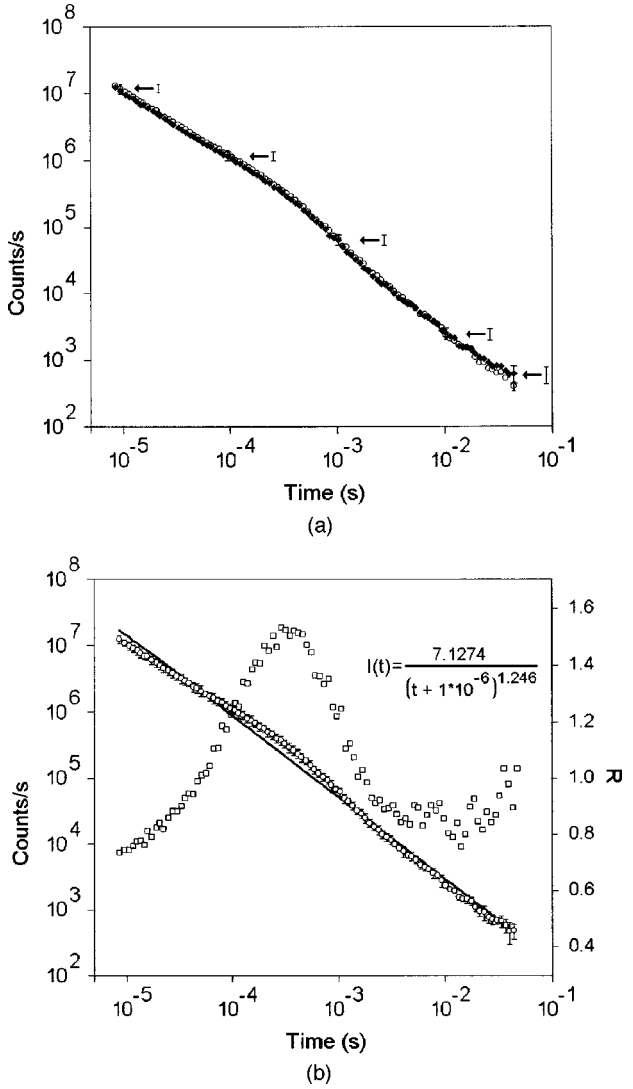

Fig. 2 Delayed luminescence of Cloudman S91 mouse melanoma cells of 4×10^6 mouse melanoma cells after preillumination with 337-nm UV-A laser light. Error bars shown by arrows represent standard deviations of three fully independent experiments. Each experiment represents an average of 100 determinations in the same cells. Standard deviations are less than 5% up to 1 ms. Background values obtained with medium are not subtracted. (a) (■) Experiment 1 and (○) experiment 2. (b) Experimental data (○) compared to hyberbolic decay (line). The first value after 8.5 μs represents 11.4×10^6 counts; the last value after 62.1 ms corresponds to 931 counts. To outline the oscillations, the ratio *R* between the data trend and the hyperbolic fitting is calculated (□).

3 Experimental Results

3.1 *Delayed Luminescence of S91 Melanoma Cells after UVA-Laser Irradiation*

Table 1 shows a comparision between our classical design and our new setup. As depicted in this table, the improvement factor for the detection of ultraweak photon emission in XPA fibroblasts using the classical design[9,10] compared to the new

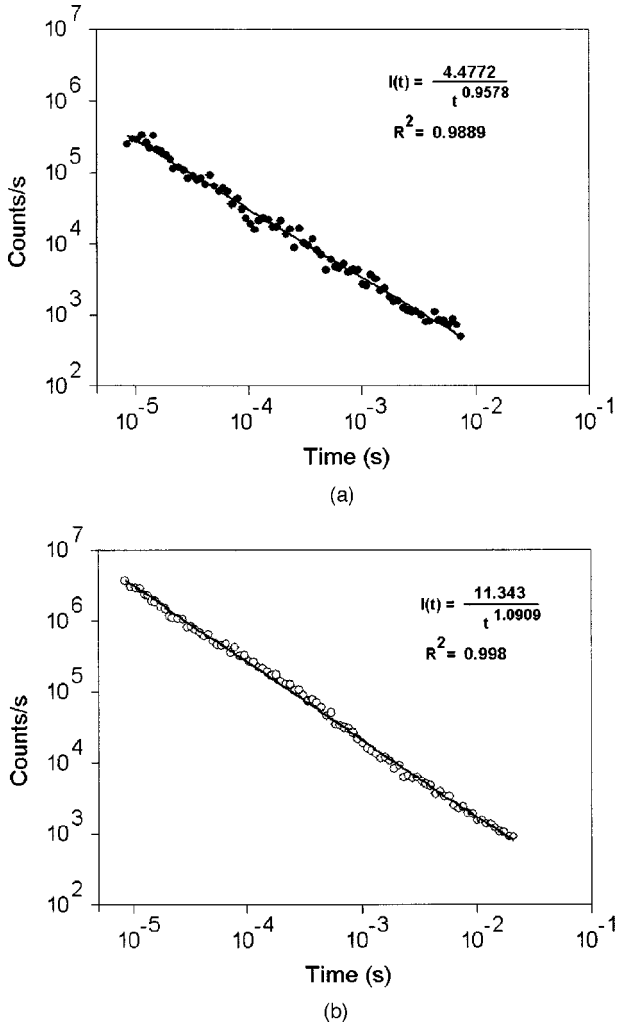

Fig. 3 Ultraweak photon emission dynamics of 1×10^6 mouse melanoma cells (b) and PBS (a) after irradiation with 337-nm UV-A laser light. Points represent the average of 100 determinations. Standard deviations are less than 5%. (a) Experimental data (●) compared to hyberbolic decay (line) in PBS. First value after 8.5 μs represents 2.9×10^5 counts. The calculated regression coefficient is 0.989. (b) Experimental data (○) compared to hyberbolic decay (line) in S91 cells. First value after 8.5 μs represents 3.8×10^6 counts. The calculated regression coefficient is 0.999.

UV-A laser induction procedure is roughly 10^4. Note that our classical device uses a mechanically based closing shutter system in the time range higher than 100 ms and cell quantities up to 10 ml. Our new detection procedure can measure ultraweak photon emission in cell quantities as low as 100 μL, which is a factor of 100 lower compared to the previous procedure (10 mL). Therefore, the improvement is increased by a factor of up to 10^6.

Figure 2(a) shows two fully independent experiments for emission of delayed luminescence following UV-A laser induction at 337 nm in Cloudman S91 mouse melanoma cells at a cell density of 4 million cells/mL. It is evident that our new technique enables us to determine this light-induced ultraweak photon emission in a very short time interval of less than 10 μs, leading to the observation of a light burst of more than 10^7 photons.

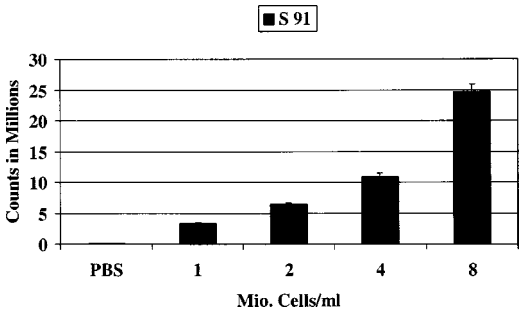

Fig. 4 Initial rates of ultraweak photon emission of Cloudman S91 mouse melanoma after preillumination with UV-A laser light in nanoseconds at suspension densities ranging from 0 to 8×10^6 mouse melanoma cells. Mean values with standard deviations of three independent experiments are given. One experimental point included in the graph represents the average of 100 determination with standard deviation less than 5%.

As shown in Fig. 2 for S91 cells at a cell density of 4 million/mL these two experiments are almost identical from 8.5 μs to 62 ms. Figure 2(b) confirms a further remarkable feature of DL, as calculated by the ratio R between the data trend and the hyperbolic fitting and previously described:[15,28] the fact that cells display sinusoidal oscillations around the decay function. Note, however, that this phenomenological observation has not received any full theoretical explanation until now.

Because several laboratories described in cells following induction with white light ultraweak photon emission curves as a hyperbolic decay function,[11,12,15,17,22] we tested additionally this observation with 1×10^6 cell/mL in comparison to the control of PBS, as depicted in Fig. 3. While the cells show a high linear regression coefficient of 0.999 [see Fig. 3(b)], almost ideal to 1, the control value of PBS was with 0.989 slightly lower, as shown in Fig. 3(a).

The DL in PBS shown in Fig. 3(a) is due to a contribution from the quartz window on the inner light guide, which could unfortunately not be excluded. Therefore, when a drop of PBS, was placed on the top of the light guide, a significantly lower light signal, compared to cell suspension, was measured, as shown in Fig. 3(a). Nevertheless, because no DL was shown from the liquid itself using the classical design,[27] this DL of PBS is clearly due to some contribution of the quartz window of the light guide because the drop acts as a hemispherical lens deviating the laser light on the top of the fiber.

A linear increase with higher cell densities was found in this mouse melanoma cell system for the first measured value after 8.5 μs in the density range from 1 to 8 million cells/mL, as described in Fig. 4. The control value of PBS in the range of 10^5 photons was very low.

3.2 *UVA-Laser-Induced Ultraweak Photon Emission of Normal, Cancer-Prone, and Melanoma Cells as a Biophysical Marker of Cell Stage*

Figure 5 depicts DL following UV-A laser induction in low passages (13) and high passages (117) of foreskin derived human skin fibroblasts 3229. Similar results were obtained with normal skin fibroblasts CRL 1221 (data not shown). As shown in Table 2, there is a significant tendency that normal cells can be induced more significantly by the UV-A laser

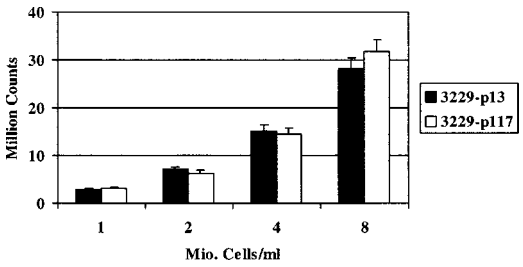

Fig. 5 Initial rates of DL from normal young skin fibroblasts 3229. Comparison of low passage number 13 with high p 117 after preillumination with UV-A laser light in nanoseconds at suspension densities ranging from 1 to 8×10^6 cells. Mean values *n* with standard deviation of three independent experiments are given. One experimental point included in the graph represents the average of 100 determinations with standard deviation less than 8%.

system than cells originating from melanoma. To exclude the influence of melanin on ultraweak photon emission as shown before in S91 melanoma cells,[22] white melanoma cells from a 53-yr-old donor were additionally used. A similar observation of reduced DL for melanoma cells was found when cancer-prone XP cells were used, as shown in the same Table 2.

4 Discussion

An improved method was developed for the DL determination induced by a UV-A nitrogen laser system in mammalian cells. Two main factors contribute to the increased sensitivity and reliability of this procedure: (1) the cell quantity used was reduced to a density as low as 100 μl before it was exposed to UV-A laser light and (2) electronic closing of the PMT system was used and UV-A laser pulses in nanoseconds were chosen for the irradiation conditions. As a consequence of these changes, the first time interval for measuring DL is as low as 8.5 μs, leading to light intensities in the range of more than 10^7 photons/s at cell densities up to 8 million cells/mL or 8×10^5 cells per experiment. The improvement factor for the detection of ultraweak photon emission in cells relative to earlier procedures[8–12,15,18] is as high as 10^6, as presented in Table 1. Note that the previously described devices used mechanically closing shutter systems for PMT protection in the time range higher than 100 ms and cell quantities up to 10 mL.

Figure 2 confirms with UV-A-laser induction the previous finding[15,28] that biological systems after white light induction display hyperbolic relaxation with sinusoidal oscillations around the decay function. Note that this relaxation cannot be assigned to the usual optical transition of isolated or triplet states as found, for example, for the picosecond fluorescence decay time of calf thymus DNA reported by Ge and Georghiou,[29] but are actually due to collective excitation within the cells under study. This follows from the rather long decay time, lasting at least several hundred milliseconds, as shown in Fig. 2.

As shown in Fig. 3 with correlation coefficients calculated by linear regression of the mean, there is a better accordance of UV-A-laser-light-induced emitted photons with the hyperbolic decay law in cells shown in Fig. 3(b) than with PBS [Fig. 3(a)] and can be interpreted in terms of coherent states.[15,30]

It is noteworthy that our dilution experiments shown in Figs. 4 and 5 exhibit an increase of the laser-UV-A-induced ultraweak photon emission intensity with higher cell density for both the carcinogenic and normal cells. In fact, this quite perfect linear relationship of the signal with higher cell densities can be easily explained with the increase by the number of emitters within the cell cultures. In this respect, we previously found that after white light induction, S91 Cloudman melanoma cells show increasing ultraweak photon emission up to 200 million cells.[22] As far as we know, DL studies with normal cells in PBS were not described in the literature until now. All the DL experiments with mammalian cells performed to date were done in a culture medium, which is known to contain photosensitizers.[9–12]

We previously found that the UV-induced ornithine decarboxylase response decreases with age and can therefore be used as a marker of aging.[31,32] As aging parameter DL measurements show no significance in contrast to the previously cited method, while comparing melanoma with normal cells as shown in Table 2 indicates that UV-A-induced DL can serve as a marker of carcinogenesis. Note that for this testing, well-defined fibroblastic cells[10,24,31–33] as well as melanomic cells were used.[20,34,35] Nevertheless, further investigations are necessary to confirm this observation.

In conclusion, a new highly sensitive method using UV-A-laser-induced DL in mammalian cells was presented and our

Table 2 Significance of UV-A-induced ultraweak photon emission in cells from normal and cancer-prone donors compared to mammalian melanomas, respectively.

Cultured Cells (cells/mL)	DL Normal[a] (million counts)	Significance[b]	DL Melanoma[c] (million counts)	DL Cancer-Prone[d] (million counts)	Significance[e]
2×10^6	8.2 ± 1.5 ($n=4$)	$P=0.08$; none	6.1 ± 1.5 ($n=2$)	10.0 ± 2.15 ($n=3$)	$P=0.05$; yes
4×10^6	16.3 ± 0.47 ($n=5$)	$P=0.0001$; yes	11.5 ± 0.47 ($n=2$)	19.4 ± 2.9 ($n=3$)	$P=0.02$; yes
8×10^6	30.0 ± 2.8 ($n=4$)	$P=0.04$; yes	25.1 ± 0.55 ($n=2$)	31.4 ± 3.9 ($n=3$)	$P=0.06$; none

[a] Mean values of four to five independent experiments with cell strains 3229, CRL 1221, and GM1717 with standard deviations given and *n* represents number of total independent experiments with 100 repetitions per experimental determination.

[b] Student *t* test for paired data used for calculation of significance between normal and melanoma cells.

[c] Mean values of two independent experiments with melanoma cells (mouse melanoma 53.1; human melanoma (CRL 1585) with standard deviations given and for *n* see note a.

[d] Mean values of three independent experiments with cell strains CRL1223, GM 5509, and GM 05239 with standard deviations given and for *n* see note a.

[e] Student *t* test for paired data used for calculation of significance between cancer-prone and melanoma cells.

results showed evidence that this photobiophysical model system is a new and powerful noninvasive tool to determine DL changes within cells that can be used for the development of new strategies in future research.

Acknowledgments

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