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Delayed luminescence as a tool for detecting oxidative stress in *Saccharomyces cerevisiae*

Qing Li*, Miao Tian*, Yang Liu, Peng Zheng, Jing Wang, Peng Gao, Chenhao Li, Bing Wang, Qing Tang, Xiaochun Zhang and Hong Wu

ENNOVA Institute of Life Science & Technology, ENN Group, Langfang, China

Abstract. Biophoton emission is a common life phenomenon, which is highly sensitive to the changes inside the biological system and the influence of the external environment. A lot of microscopic and equivocal information about the biological system and its weak response to the environment can be obtained through the detection and analysis of the biophoton emission. In this research, we studied the delayed luminescence (DL) of *Saccharomyces cerevisiae* cells treated with H_2O_2. The results showed that after H_2O_2 treatment the DL intensity of the yeast cells increased in a concentration- and time-dependent manner. The DL kinetics curves of yeast cells, treated with different concentration of H_2O_2 for 2 h or 3 mmol/l H_2O_2 for different time, well reflected the oxidative stress level of yeast cells. Furthermore, correlation analysis showed that there was a high positive correlation between DL and reactive oxygen species (ROS) under oxidative stress conditions. In conclusion, DL had the potential to be used as a tool to detect oxidative stress in yeast cells, and even to be developed as a new complementary medical method for health examination and disease diagnosis.

Key words: Delayed luminescence — *Saccharomyces cerevisiae* — Oxidative stress — ROS — H_2O_2

Introduction

All biological systems emit ultra-weak luminescence, commonly known as biophoton emission, which is also called low-level chemiluminescence or ultra-weak photon emission in some literature (Gu 2016). The spectral range of the biophoton is from 200 to 800 nm (Popp et al. 1994). The biological spontaneous ultra-weak photon emission (UPE) is a luminescent phenomenon which is present without any direct external stimulation or additionally applied external luminophores (Cifra et al. 2015). A light-induced method has been also used in order to intensify photonic signals by illuminating the samples and measuring its delayed luminescence (DL) (Kim et al. 2005). The photon number of DL (from a few up to 10^5–10^6 counts/s/cm^2) is much higher than that of UPE (from a few up to a few hundred counts/s/cm^2) and shows a regular attenuation over time (Bai et al. 2007).

Activated by light irradiance, the molecules in biological systems absorb the light energy and form many different electronic excited states. Molecules in high energy states are unstable and easily transit back to low energy states, and release the energy to form DL emission. At present, despite DL spectroscopy proved successful in several applications, the origin of DL from living cells was still under debate and many biochemical mechanisms were suggested. It was found that due to the interaction between various molecules of excited states, DL emission in biological systems followed the law of hyperbola.

With the development of relevant research, DL has been paid more and more attention by researchers all over the world. Using DL to investigate the structure and behaviour of water, the authors found that the DL emission related to the size of low-density water clusters (Grasso et al. 2018; Musumeci et al. 2012a). DL has been used to characterize humic acids in lake sediments (Mielnik and Asensio 2018),

* These authors contributed equally to this work.

Correspondence to: Hong Wu, ENNOVA Institute of Life Science & Technology, No.118, Huaxiang Road, Langfang 065001, Hebei, China
E-mail: wuhong@enn.cn

to measure the processing of Chinese herbal materials (Sun et al. 2018), and to study single-stranded nucleic acids with different base sequences (Liang et al. 2018). With the application of DL measurement in the study of human cells (Niggli et al. 2005, 2008), tissues (Lanzanò et al. 2007) and acupuncture points of human body (Lanzanò et al. 2008), researchers have proposed to use DL as a medical compensation method to monitor cell status, human health, disease and so on.

In many studies, the DL characteristics of cancer cells were found significantly different from that of normal cells. For example, the distribution of DL spectrum of cultured normal cells and melanoma cells was significantly different, and the DL intensity of melanoma cells was at least three times higher than that of normal cells (Musumeci et al. 2005a, 2005b). There was a significant difference in the weight distribution of DL decay rate between normal and leukemic serum samples, which was used to distinguish the serum of leukemia patient from that of normal people (Chen et al. 2012). DL study of cancerous and normal tissues showed that both the adenocarcinoma and the squamous cell carcinoma emitted more photons after illuminations than the corresponding normal tissues, while the squamous cell carcinoma had more prominent character (Kim 2005). These studies showed the possibility of using DL to discriminate normal cells from cancer cells. In other researches, DL was used as a screening tool to assess a person's acute or chronic disease status as a whole. As an important fluid in human body, saliva was used as a useful indicator of various conditions of human body, and a spectral analysis system for whole saliva DL was developed (Jamaludin and Balakrishnan 2014). *In vivo* DL measurement of human skin showed the possible correlation between DL parameters and human ages (Lanzano et al. 2007; Musumeci et al. 2007).

H_2O_2 is a common oxidant with a tendency to attract extra electrons, leading to the formation of reactive oxygen species (ROS). Depending upon its biological context, ROS may act as a signaling agent, a toxic species, or a harmless intermediate that decomposes spontaneously (Sheng et al. 2014). Many researchers believed that ROS was the major sources of UPE (Pospíšil et al. 2012; Saeidfirozeh et al. 2018; Prasad et al. 2020). In some researches, DL was used to measure mitochondrial oxygen tension *in vivo* (Mik et al. 2006, 2008; Harms et al. 2011). Baran et al. (2010, 2012) evaluated the effects of menadione (MD), H_2O_2, and quercetin (QC) on DL of human leukemia Jurkat T cells under a variety of treatments and found a strong anti-correlation between apoptosis and DL on a specific time scale (0.1–1 ms after UV-excitation of the cell sample). MD, H_2O_2 and QC were potent inducers of apoptosis and DL inhibitors. However, in recent years it was reported that the ROS changed the chemical environments of mitochondrial complex I or III or be directly bound to them, resulting in longer lasting DL signals (Kim et al. 2017). The correlation between ROS and DL emission was supported by the big reduction in DL after addition of a ROS scavenger. Since the important role of ROS in cell growth and DL emission, extensive researches were required to clarify the relationship between intracellular ROS content and DL emission.

Yeast has been used as a model organism for its rapid growth, easy cultivation and feasible genetic modification. As a eukaryote, the yeast *S. cerevisiae* shares the complex internal cell structure of animals and human beings, and many important proteins of human were first discovered by studying their homologs in yeast. In a review, Slawinski had proposed that yeast cell cultures appeared to be a sensitive and versatile model system which could be used for further study of stress-induced UPE (Slawinski et al. 1992). Baran et al. (2009) investigated the relation between cell proliferation, DL, microtubule integrity and DNA damage in Hansen wild-type *Saccharomyces cerevisiae* yeast cells. In this paper, DL emission of yeast cells treated with H_2O_2 was measured, and the correlation between DL emission and ROS content was analysed. Finally, the possibility of using DL to characterize the state of intracellular oxidative stress was discussed.

Materials and Methods

Cell cultures

The industrial instant dry yeast *S. cerevisiae* (Angel Yeast CO. Ltd., China) was used in this study. The yeast cells were cultured to logarithmic phase in YPD medium (1% yeast extract, 2% tryptone, and 2% D-(+)-glucose) for each experiment. All reagents were purchased from Sigma-Aldrich (Steinheim, Germany). All glassware and medium were autoclaved at 121°C for 20 minutes.

The logarithmic cultured cells were harvested and resuspended in fresh YPD medium with a final concentration of 2×10^7 cells/ml. After addition of H_2O_2, the cells were cultured for specified time at room temperature (25 ± 0.5°C). Finally, cells were collected for detection of DL and intracellular ROS. Each experiment was performed three times.

DL measurement

The measurement of DL from cell cultures was performed using installed equipment with a single photon sensibility. The schematic diagram of the experimental setup was shown in Figure 1. The laser emitted 405 nm light, which passed through a lens with a focal length of 75 mm and irradiated the sample. The stimulated emission photons (DL) of the sample were condensed by a lens with a focal length of 5 mm, and collected into a single photon avalanche diode (SPAD) (SPCM-AQRH-16X, Excelitas Technology, USA) by fiber optic coupling (FOC). The circuit controller synchronized the laser and the single photon counter power supply with electrical signals, collected the SPAD counts and fed back to the PC.

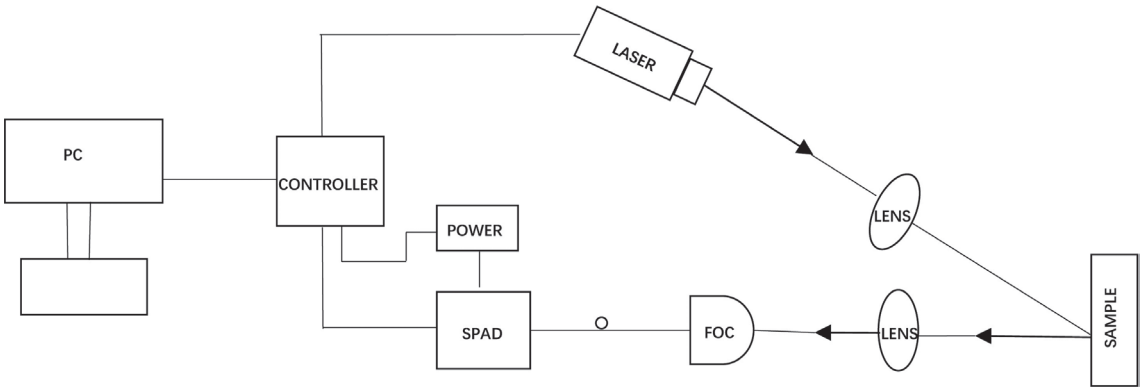

Figure 1. Scheme of the experimental setup used for delayed luminescence measurements. FOC, fiber optic coupling; PC, personal computer; SPAD, single photon avalanche diode.

To reduce the background signal, a quartz colorimetric cuvette with an optical diameter of 1 mm used for sample cell loading was fixed in a black circular holder with a 12 mm diameter hole on the bottom. The pulsed light travelled through the hole to the cell sample, and the resulting DL was received by FOC. The data acquisition was periodic, and the period is 50 ms, in each period, signals were initially collected after 0.035 ms of laser excitation. In an acquisition time of 20 ms, the controller collected data every 0.1 ms continuously for 200 cycles, i.e., photoemission was recorded between 0.035 and 20 ms after laser excitation. The same run was repeated 20 times to enhance the difference in DL emissions.

In order to prevent the possible photobleach and/or photodamage, the cell density and laser intensity were optimized. Our preliminary data indicated that DL had a linear relationship with cell density in $1.5–60\times10^8$ cells/ml, and a linear relationship with laser intensity in the range of 10–70 mW. Based on these results and previous research experience, we selected 3×10^9 cells/ml and 45 mW for the following experiment.

For DL measurement, the sample cells were collected by a rapid centrifugation, washed twice with PBS, and resuspended to 3×10^9 cells/ml. After the cell samples were loaded directly in the quartz colorimetric cuvette, the DL emission was measured immediately.

Kinetic curve analysis

The kinetic curve of DL intensity I(t) can be modelled by a hyperbolic function as: $I(t) = I_0/((1 + t/\tau)^{\beta}$, where I_0 is the initial DL intensity, τ and β are characteristic parameters related to the sample property (Gu 2016). The model was used for data acquisition and processing. The experimental data were in good agreement with the DL dynamics described by the formula. The fitting coefficient was more than 0.99.

Detection of ROS

After H_2O_2 treatment, 10^6 cells were harvested and washed twice with PBS. The washed cells were added with final concentration of 20 μM 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA) (Beyotime, Nantong, China) fluorescent probe, which can freely enter into cells and interact with intracellular ROS to produce highly fluorescent 2,7-dichlorodihydrofluorescin (DCF), and incubated at 30°C for 20 min in dark. The fluorescence was detected by Flow Cytometry (CytoFLEX, Beckman, China) at excitation wavelength of 488 nm and emission wavelength of 525 nm (FITC channel). For each sample, 10000 events were recorded and data were reported as mean fluorescence intensity.

Statistical analysis

The DL curve was smoothened by using software AcqBiophoton developed by Dr. Xiaochun Zhang. The I_0 was expressed as mean ± standard deviation, differences between the means were analysed by using an ANOVA (SPSS version 25; IBM Corp., Armonk, NY), and $p < 0.05$ indicated a statistically significant difference. The correlation between the I_0 and ROS was analysed using Pearson correlation coefficient (Microsoft Excel 2016).

Results

Effects of H_2O_2 concentration on DL emission

A series of H_2O_2 concentrations (ranging from tens of μmol/l to tens of mmol/l) were conducted to evaluate the effect on yeast cell growth. We found that H_2O_2 at concentrations under 1 mmol/l had no effect on cell growth, whereas at concentrations higher than 5 mmol/l cause oxidation of

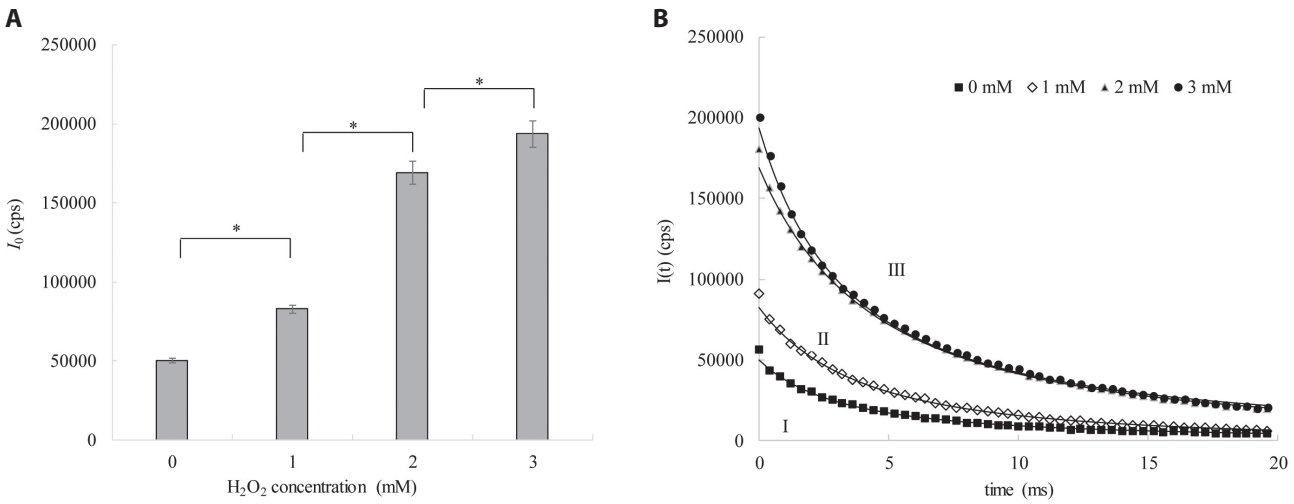

Figure 2. Initial delayed luminescence (DL) intensity I_0 (**A**) and kinetics curves of DL emission (**B**) from *S. cerevisiae* cells treated with 0, 1, 2, and 3 mmol/l H_2O_2 for 2 h. Each curve was obtained by fitting the data to the equation mentioned in the text. The dynamic curves of group I, II, III revealed the DL relaxation trends of cell treated with 0 mmol/l, 1 mmol/l, and 2 and 3 mmol/l H_2O_2, respectively. * $p < 0.05$ represented a significant difference.

biomolecules leading to reactive intermediate formation and finally to cell death. In order to detecting the oxidative stress appropriately, the H_2O_2 concentration of 1, 2 and 3 mmol/l were conducted in this study, i.e., yeast cells were treated with 1, 2 and 3 mmol/l H_2O_2 for 2 h and the DL emission was detected. The results showed that H_2O_2 significantly affected the DL emission of yeast cells (Fig. 2). The DL initial intensity I_0 of the untreated cells was 49964 cps (counts *per* second), and that of 1, 2, and 3 mmol/l treated cells was 82541, 169080 and 193521 cps, respectively (Fig. 2A). The results showed that DL of yeast cells was obviously affected by H_2O_2, and the emission of DL increased with the H_2O_2 concentration. The DL kinetic curves of the treated yeast cells were different from that of the untreated cells, i.e., the higher the H_2O_2 concentration, the greater the change of DL dynamic curve (Fig. 2B). According to their dynamic curve patterns, the cells could be classified into three groups. The group I was untreated cells, the group II was 1 mmol/l treated cells, and the group III was 2 and 3 mmol/l treated cells. The dynamic curves of cells treated with 2 and 3 mmol/l H_2O_2 were different before 2 ms, though later on they tended to be the same. Our results indicated that the I_0 and dynamic curve changed regularly with the concentration of H_2O_2.

Effects of H_2O_2 treatment time on DL emission

3 mmol/l H_2O_2 was used to investigate the effect of treatment time on DL emission. I_0 of the untreated cells was 43588 cps, and that of the 30, 60, 120 and 240 min-treated cells were 54627, 97143, 222430 and 262764 cps, respectively, i.e., the longer of H_2O_2 treatment, the higher the DL emission intensity (Fig. 3A). According to the DL kinetic curves, the cells were classified into three groups (Fig. 3B). The group i was cells of untreated and cells treated for 30 min. The I_0 of cells for 30 min treatment was higher than that of the untreated cells, but there was no significant difference in the relaxation trend of DL kinetic curves. The group ii was 60 min-treated cells, and the group iii was 120 and 240 min-treated cells. These results showed that the I_0 and dynamic curve changed with the treatment time.

Relationship between DL emission and oxidative stress

In order to illustrate the relationship between DL emission and oxidative stress, we used ROS to quantify the degree of oxidative stress in cells treated with 1, 2 and 3 mmol/l H_2O_2 for 2 h. The results showed the content of ROS in cells increased with the added concentration of H_2O_2, and the relationship between the intracellular ROS and exogenous H_2O_2 concentration was a S shape non-linear curve (Fig. 4A). When the concentration of the added H_2O_2 was low, i.e., 0–1 mmol/l H_2O_2, the intracellular ROS content increased slowly, which was consistent with common doctrine when the cells encounter with oxidative stress conditions. At low concentration of the ROS, as the antioxidative system, including anti-oxidant and anti-oxidase, was still working normally, it inhibited the ROS content to increase. When 1–2 mmol/l of exogenous H_2O_2 was added, the intracellular ROS content increased rapidly, which indicated ROS defence system did not match up the increase of the ROS and collapsed. With the further increase of the H_2O_2 concentration, the intracellular ROS increased slowly with the exogenous

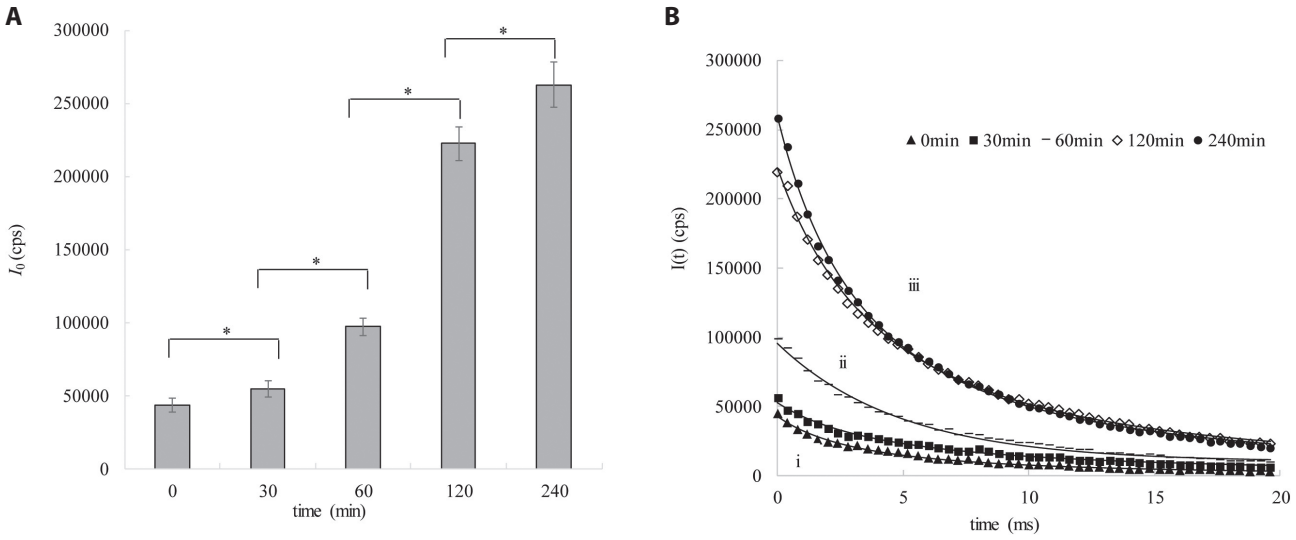

Figure 3. Initial delayed luminescence (DL) intensity I_0 (**A**) and kinetics curves of DL emission (**B**) from *S. cerevisiae* cells treated with 3 mmol/l H_2O_2 for 10, 30, 60, 120, and 240 min. Each curve was obtained by fitting the data to the equation mentioned in the text. The dynamic curves of group i, ii, iii revealed the DL relaxation trends of cell treated with 3 mmol/l H_2O_2 for 0 and 30 min, 60 min, and 120 and 240 min, respectively. * $p < 0.05$ represented a significant difference.

H_2O_2 and gradually get saturated. There were many possible reasons for these results, including (1) oxidative damage caused ROS leakage or (2) cell damage caused explosion of various reductant which scavenged or neutralized the ROS.

As the results showed in Figure 2A, the relationship between the DL initial intensity I_0 and exogenous H_2O_2 concentration was like that between the intracellular ROS and exogenous H_2O_2 (Fig. 4A), which was divided in three response phases, i.e., i: homeostasis, ii: rapid increase, and iii: saturation. This result was consistent with the DL dynamic curves (Fig. 2B), where DL was divided in three groups accordingly, meaning that DL emission curve contained a verity of information including the intracellular state of ROS contents and the oxidative stress. The fundamental DL emission at the initial time indicated that in living cells there were many sources of DL, including the original intracellular ROS, NADH, PpIX etc., as reported in literature (Ince et al. 2016). With addition of more H_2O_2, I_0 started to increase rapidly and then slowly, similar

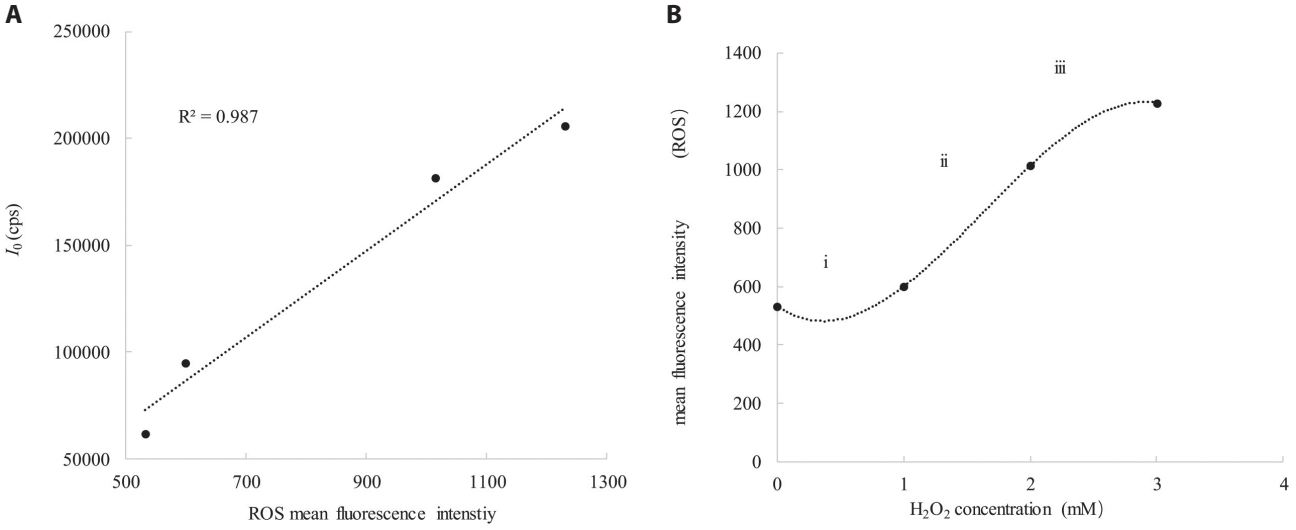

Figure 4. Intracellular ROS (**A**) of cells treated with 0, 1, 2, and 3 mmol/l H_2O_2 for 2 h, and correlation analyses between ROS and DL (**B**). Response phases i, ii, iii represented the homeostasis, rapid increase, and saturation stage of intracellular ROS. R^2, Pearson correlation coefficient.

to that of intracellular ROS, disclosing the intrinsic and close correlation between the ROS and DL emission.

The correlation between the intracellular ROS and initial DL intensity I_0 was further analysed (Fig. 4B), and the results showed DL intensity I_0 was positively correlated with intracellular ROS (R^2 = 0.987). The intracellular ROS was not equal to exogenous oxidative stress conditions, but it represented the intracellular oxidative stress, an important state character for living cells. The results also implied that when cells suffered from oxidative stress, the intracellular ROS was the major source of DL emission.

Discussion

Many evidences showed that ROS was the main source of UPE. When ROS are produced during the metabolic processes, the ultra-weak photons are emitted spontaneously by the relaxation of electronically excited species, which formed during the oxidative metabolic processes. For example, UPE enhanced at the injured site where ROS formed of wounding in *Arabidopsis* leaves (Prasad et al. 2020). Exogenous oxidants cause oxidative stress in cells, produce a large amount of ROS and increase UPE emission, e. g. H_2O_2 treatment increases chemiluminescence intensity of *A. thaliana* seeds in a dose-dependent manner (Saeidfirozeh et al. 2018). Our study showed that UPE intensity of yeast cells increased in a concentration-dependent manner after H_2O_2 treatment too (data not shown).

DL represents the long-term scale luminescence after exposure to pulsed light or UV radiation. Activated by light irradiance, the molecules in biological systems absorb the light energy and form many different electronic excited states. Molecules in high energy states are unstable and easily transit back to low energy states, and release the energy to form DL emission. It is generally believed that the DL hyperbolic relaxation of biological systems is a characteristic active response of an ergodic coherent state (Popp and Yan 2002). Until now, the origin of DL is still a controversial issue, but it is generally believed that in biological systems DL may arise from a variety of possible reactions and sources, such as direct emitters like flavins, carbonyl derivatives and aromatic compounds, molecular oxygen and its species, the DNA, as well as collective molecular interactions, e.g. triplet-triplet annihilation, collective hydrolysis, or the cytoskeleton (Baran et al. 2009). Baran et al. (2013) suggested that DL was mainly produced within the mitochondrial electron transfer system at the level of complex I. Investigation of the relation between cell proliferation, DL, microtubule integrity and DNA damage indicated that DL was correlated with the activation of some molecular species involved in cellular repair after irradiation (Baran et al. 2009). The comparison of the features of DL emission from a unicellular alga *Acetabularia acetabulum* and from some solid state systems showed that DL was related to the collective molecular interactions (Scordino et al. 2000). Since the biological system is composed of tens of thousands of chemical substances and biological macromolecules, and a large number of biological and chemical reactions occur in the biological system all the time, it was particularly difficult to determine the source of DL. As UPE associated with the production of ROS, we speculated that the increase in DL emission of yeast cell under oxidative stress condition was also from the superoxide anion radical, hydrogen peroxide, hydroxyl radical, singlet oxygen, and other kinds of ROS that from the oxidation of lipids, proteins, nucleic acids and so on.

In the pre-experiment, we studied the effect of different concentrations of H_2O_2 on yeast. The results showed that 1, 2, and 3 mmol/l H_2O_2 caused different degrees of oxidative stress of yeast (data not shown). Therefore, these concentrations were selected for further research in this study. After 0, 1, 2 and 3 mmol/l H_2O_2 treatment for 2 h, the DL initial intensity I_0 of the yeast cells stimulated by 405 nm laser increased as the H_2O_2 concentration increased (Fig. 2A). Meanwhile, the DL emission stimulated by 532 nm laser of yeast cells treated with H_2O_2 also showed that I_0 increased in a H_2O_2 concentration-dependent manner (data not shown). The higher concentration of H_2O_2 was added, the increase of the DL emission observed, which was consistent with UPE in many researches (Saeidfirozeh et al. 2018; Prasad et al. 2020). Different concentrations of H_2O_2 causes different levels of oxidative stress in cells, and the higher the concentration, the higher level of oxidative stress (Fig. 4). This result supported our proposal that the accumulated ROS was the main cause of the increase in DL emission under oxidative stress condition. 1 mmol/l H_2O_2 induced mild oxidative stress, and the kinetic curve shifted upward within 15 ms after laser stimulation compared with that of the untreated cells which had no oxidative stress caused by exogenous oxidants, and then the DL intensity relaxed to the same level as that of the untreated cells after 15 ms (Fig. 2B). The DL intensity of the 2 and 3 mmol/l H_2O_2-treated cells tended to be the same after 2 ms, but could not relaxed to the level of that of the untreated cells. It maintained a high level until the end of the measurement. DL emission continuously increased with time period after H_2O_2 added (Fig. 3A) indicates that the antioxidant ability gradually weakened and the cells were in a higher oxidative stress with the treatment time prolonged. With the increase of the treatment time, I_0 increased slowly at the beginning, the dynamics curve changed more dramatically in 60 min, and gradually became saturated after 240 min (Fig. 3B). The 120 and 240 min-treated cells tended to be the same after 2 ms indicated that cells were gradually approaching oxidative saturation, which was consistent with the research of Rastogi (Rastogi and Pospíšil 2010). Farthermore, correlation analysis showed that ROS production was highly correlated

with I_0 (Fig. 4), indicating that cells with high oxidative stress emitted more DL and the dynamic curve changed more.

To date, many studies have shown that DL measurements provided information about the biological processes, cellular states, and even certain changes in human health. For instance, DL analysis was used to monitor programmed cell death (Scordino et al. 2014), to identify unknown homeopathic remedies (Lenger et al. 2014), to discriminate between normal and tumour cells in a quick and non-invasive way (Grasso et al. 2020), to monitor cell status and cell cycle progression, to evaluate the pro-apoptotic capacity of certain treatments, to analyse the function of mitochondria at the level of MRC Complex I (Baran et al. 2013; Cifra et al. 2015; Grasso et al. 2020), and so on. In this work it was revealed that DL emission was positively correlated with intracellular ROS in *S. cerevisiae* cells. Although UPE is also linked to oxidative stress, the intensity of UPE is only about $10–10^3$ photon/ (cm^2·s), which is difficult to measure accurately and interfered easily by the environment. DL has spectral emission from optical range to near infrared, and its intensity is significantly higher than UPE, so DL could be used as a tool to detect oxidative stress.

Since oxidative stress cause many diseases and health problems, it was important to find a rapid, simple and non-invasive technique to detect the oxidative stress for clinical diagnosis and treatment. Usually, intracellular oxidative stress is associated with increased production of ROS or a significant decrease in the oxidative defence systems, including superoxide dismutase, catalase, and glutathione peroxidase. While low concentration of ROS served as a signal in cell metabolism regulation, intense oxidative stresses triggered cells apoptosis or even caused cells necrosis. Oxidative stress was suspected to be involved in neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, depression, autism, and multiple sclerosis. Oxidative stress was also linked to the most common human diseases such as cardiovascular disease, and the age-related development of cancer.

In conclusion, we found that in *S. cerevisiae* cells, the emission intensity and kinetics of DL changed in concentration- and time-dependent manner after H_2O_2 treatment, and there was a high positive correlation between DL and ROS. This study provided a new potential method for detecting oxidative stress in cells, which could be used as a complementary medical detection method. So far, there was little research in this research area and we will continue to work on human cells and animal models.

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Conflict of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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