Autoluminescence imaging: a non-invasive tool for mapping oxidative stress

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Living organisms spontaneously emit faint light, and this autoluminescence is stimulated in response to many stresses. This phenomenon is attributable to the endogenous production of excited states during oxidative reactions, particularly during peroxidation of lipids, which generates light-emitting molecules such as triplet carbonyls and singlet oxygen. Using highly sensitive cameras, it is now possible to remotely image spontaneous luminescence with a good spatial resolution, providing a new non-invasive tool for mapping oxidative stress and lipid peroxidation in plants.

The types of luminescence of living organisms

Plants, animals and bacteria glow continuously. This spontaneous photon emission (SPE) occurs in the ultraviolet and visible range of wavelengths with a low intensity of $10^{-19}$ to $10^{-16}$ W cm$^{-2}$ [1]. This is about three orders of magnitude less than the luminescence arising from enzymatic reactions in fireflies, jellyfishes and some fishes in a transitory phenomenon commonly called bioluminescence [2]. It is different from the luminescence of chlorophyll, emitted by photosynthetic organisms following exposure to light, which results from recombination of positively charged carriers at the donor side of photosystem II and electrons on the acceptor side [3,4]. The chlorophyll signal decays rapidly in the dark within seconds to minutes. Here, we provide background information about SPE, with emphasis on the origin of the signal, and discuss in more detail imaging of SPE in plants in comparison with other available techniques.

Spontaneous photon emission, a signature of biological oxidation status

SPE, often called biophoton emission, is attributed to the endogenous production of excited states during oxidative metabolic reactions [5,6] and it is therefore closely related to the oxidation status of the organism. The major source of SPE is the slow spontaneous decomposition of lipid peroxides and endoperoxides, such as dioxetanes, which generates the light-emitting byproducts singlet oxygen and excited carbonyl groups [7]. The term ‘lipids’ is used here in a broad sense and is not restricted to fatty acids but also includes carotenoids or terpenoids, which can produce endoperoxides upon oxidation [8,9]. Singlet oxygen is produced in reactions between two lipid peroxy radicals, and the light emission peaks in the red (at 634 and 703 nm; Figure 1). By contrast, the bimolecular reaction of alkoxy radicals and the cleavage of dioxetanes lead to the formation of excited triplet carbonyls, which in turn decay and yield light in the range 350 to 480 nm (blue). Consistently, spectral analyses of the luminescence emission of oxidized lipids in vitro showed the expected peaks in the blue and red-near infrared domains [10]. However, in plants, triplet carbonyls can also transfer excitation energy to chlorophyll molecules [11] whose deactivation is associated with photon emission at around 730 nm (far-red light) [12,13].

Formation of activated carbonyls from lipid hydroperoxide decomposition is stimulated by heat [14], and this phenomenon is the basis of the luminescence burst observed at ~135 °C in plant leaves (Figure 2a) [3,4,15]. The amplitude of the luminescence measured at this temperature is two orders of magnitude higher than the room-temperature autoluminescence amplitude. Numerous thermoluminescence studies have validated the use of the luminescence measured at ~135 °C as an index of lipid peroxidation by correlating the signal amplitude with the extent of lipid peroxidation, which can be measured using a variety of biochemical methods [15–18]. The luminescence at ~135 °C has been used in plants to quantify lipid damage after oxidative stress [18–20] or to characterize the stress resistance of mutants [16,17,21]. Importantly, when the luminescence signal emitted by Arabidopsis leaves at 25 °C was plotted against the luminescence emission at 135 °C, a linear regression was obtained (Figure 2b), confirming that both signals reflect oxidative stress damage and supporting the hypothesis that they could have a common origin in plants [15].

Light produced during lipid peroxidation therefore constitutes an internal probe of oxidative stress that has many potential applications. Lipid peroxidation and oxidative stress have been measured by room-temperature SPE in a variety of samples, from whole organisms to isolated membranes or lipoproteins, and even in human breath (reviewed in Refs [1,15]). In many studies, the intensity of SPE correlated with other indexes of lipid peroxidative degradation, such as the level of conjugated dienes, the malondialdehyde content or the concentration of...
exogenously added lipid hydroperoxides [22–24]. This non-invasive technique is useful for real-time monitoring of rapid perturbations of the cellular oxidation state and is performed without an exogenous probe.

**Imaging of spontaneous photon emission**

A currently emerging and promising application of SPE is the imaging of oxidative stress in intact tissues. Early attempts at autoluminescence imaging in the 1990s used

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**Figure 1.** Chemiluminescence associated with lipid peroxidation. The emission of light is related to the production of excited carbonyl compounds and singlet oxygen (1O2) that occurs during lipid peroxide degradation. Self-reaction of lipid peroxy radicals (the Russel reaction) gives a tetroxide intermediate, which decomposes either into a triplet state carbonyl compound (reaction 1a; the triplet state is indicated by a star) or into singlet oxygen (reaction 1b). Self-reaction of alkoxy radicals produces excited carbonyl compounds in a redox reaction (reaction 2). The singlet oxygen that is produced during lipid peroxidation (such as reaction 1b) reacts with unsaturated compounds, leading to the production of dioxetanes, which yield triplet state carbonyl compounds upon decomposition (reaction 3). Upon relaxation, the excited carbonyl compounds emit light in the blue region (380–460 nm; reaction 4a) whereas bimolecular emission of singlet oxygen occurs in the red region (634 and 703 nm; reaction 4b). Abbreviation: \( hv \), energy of a single photon. Adapted from Refs [6,7].

**Figure 2.** Luminescence of Arabidopsis leaves and its variation with temperature. (a) High-temperature luminescence emission from Arabidopsis leaves exposed to photo-oxidative stress. The curves are of wild type (Columbia; 1) and a \( \text{vte1 npq1} \) double mutant deficient in the carotenoid zeaxanthin and in vitamin E (2) [17]. Plants were exposed for 3 days to high light stress (1200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), 8 h photoperiod) at low temperature (8 °C). The luminescence band peaking at around 135 °C is attributed to light-emitting molecules produced during lipid peroxidation [15]. (b) Correlation between the luminescence emission at 25–30 °C (commonly used for imaging) and the amplitude of the luminescence band at 135 °C. The results shown were obtained in a series of experiments on a range of Arabidopsis mutants exposed to various stress conditions.
single photon counting imaging techniques that demonstrated the feasibility of this approach. However, most of the images obtained with these systems had low spatial resolution. Nevertheless, SPE from the brain of a rat was visualized and the imaged signal correlated with cerebral energy metabolism and oxidative stress [25]. SPE in plant tissues infected with a pathogen, injured mechanically or exposed to drought stress were also visualized with this technique [26–28]. These pioneering studies generated interest in SPE and prompted researchers to use alternative technologies to improve the imaging of this signal.

Recent studies on plants, animals and humans have shown that autoluminescence imaging can be improved using highly sensitive charge-coupled device (CCD) cameras. Cooling of the sensor, using peltier devices (thermo-electric modules) or liquid nitrogen, is required for reducing thermal noise and enabling measurement of faint light by signal integration. Using this approach, SPE from mouse tumors and from the human body were recently imaged [29,30]. In plant science, the first application of CCD to SPE imaging was made using germinating seedlings [31]. High-resolution images of SPE from detached leaves were also reported [32]. So far, the best images of plant autoluminescence allowing quantitative mapping have been obtained by Michel Flor-Henry and co-workers [13], who used a cooled CCD detector coupled to optical fibers on which detached leaves were placed. This configuration enables maximal capture of light and high sensitivity, but it is applicable to flat samples only (e.g. detached leaves). Wound-induced luminescence of leaves was imaged with a good spatial resolution (~222 000 pixels per image). Although the origin of the signal was not investigated in detail in this study, lipid peroxidation products are probable candidates [7,33]. Because wound-induced photon emission is quenched by sodium azide and amplified by deuterium oxide [34], singlet oxygen is likely to be involved in this light emission.

If leaf autoluminescence does reflect lipid oxidative damage, mutants known to be sensitive to oxidative stress should emit more photons than do wild types. This is shown in Figure 3, in which SPE from whole Arabidopsis plants was imaged using a liquid nitrogen-cooled CCD camera similar to that described in Ref. [32]. The plants were pre-adapted to darkness for ~2 h to allow photosynthesis-related chlorophyll luminescence to decay [13]. Two photosensitive mutants, the vtc2 single mutant deficient in ascorbate and the vtc2 npq1 double mutant deficient in both ascorbate and zeaxanthin [21], were compared with the photoreistant wild type after high light stress. Both mutants showed a much higher stimulation of SPE after photostress than did the wild type (Figure 3a), and SPE of the mutants was proportional to their relative sensitivity to oxidative stress [21]. Mature Arabidopsis leaves are well known to be less tolerant to photo-oxidative stress than young leaves at the center of the rosette, and their SPE reflected this difference. Furthermore, the SPE intensity of mature leaves was related to the concentration of malondialdehyde, a biochemical marker of lipid peroxidation (Figure 3c). Another Arabidopsis double mutant, vte1 npq1, deficient in two major antioxidants of the plastid membranes (vitamin E and zeaxanthin), is highly sensitive to high light, with leaves suffering extensive lipid peroxidation, which was measured by various methods [17]. The sensitivity of the vte1 npq1 double mutant to lipid peroxidative damage can be readily monitored by autoluminescence imaging (Figure 4a): stimulation of SPE occurs much more rapidly and more intensively in mutant leaves compared with wild-type leaves. Importantly, stimulated SPE from vte1 npq1 double mutant leaves was detected after 6 h in high light, before symptoms of oxidative damage were visible on the leaves (>26 h).

A field that has much to gain from this new imaging method is phytopathology. For example, infiltration of a tobacco leaf with the elicitor cryptogein in the dark induced
hypersensitive programmed cell death [35] and an enhancement of SPE [19] that can be quantified by CCD imaging, as shown in Figure 4b. Increased SPE was detected 18 h after infiltration, matching the time course of the massive enzymatic lipid hydroperoxide accumulation [35]. Recently, Mark Bennett and co-workers [36] showed bursts of light over the Arabidopsis leaf surface induced during the hypersensitive response to a pathogen. It seems that this photon emission is different to the SPE associated with lipid peroxidation because it was transient, beginning around 2 h after inoculation and lasted only 2–3 h. This transient light signal, which presumably reflects some signaling phenomena related to nitric oxide in the plant defense response [36], also has great potential for applications in phytopathological studies.

Because SPE imaging can be performed on intact plants and does not require an exogenously added probe or sample preparation (except adaptation to darkness), it is expected to provide realistic mapping of lipid peroxidative damage and oxidative stress in plants. This new approach is complementary to more direct measures of reactive oxygen species such as fluorescence imaging using fluorescent probes sensitive to singlet oxygen and/or reduced forms of oxygen [16,37–39]. Fluorescence imaging is useful because it provides information on specific reactive oxygen species and their sites of production in tissues. However, fluorescence imaging of reactive oxygen species requires pre-infiltration of plant tissues with an exogenous probe and it is therefore usually restricted to cells or organs (e.g. detached leaves). A new approach based on redox-sensitive green fluorescent proteins (GFPs) has recently emerged to monitor non-invasively redox changes in living cells [40–42]. Redox sensing is achieved by introducing cysteine residues close to the chromophore – their oxidation causes changes in the GFP fluorescence characteristics. Although application of this technique to imaging in plants is not yet fully documented [43], redox-sensitive GFPs are promising tools for visualizing the oxidation of specific proteins or organelles. However, this approach is technically more complex than SPE imaging because it requires genetic transformation of the biological material.

Concluding remarks
Autoluminescence imaging is likely to become an important tool in stress physiology to measure the oxidation status of plants non-invasively. Oxidative stress imaged by SPE is useful not only for quantification purposes, but also for the early detection of oxidative stress before damage becomes visible. SPE can be measured at different integration levels, from small populations of organisms to cells or organs (e.g. detached leaves). Because of its high sensitivity and easy use, SPE imaging should enable the rapid screening of mutants, including algae, cyanobacteria and vascular plants, with variable tolerance to oxidative stress. Even though the sensitive equipment required for acquiring SPE images is still relatively expensive, we predict that this new imaging approach will successfully complement the assortment of functional imaging tools available to plant biologists [44].

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