Doctoral Thesis

ULTRA-WEAK PHOTON EMISSION AS A NON-INVASIVE METHOD FOR MONITORING OXIDATIVE RADICAL REACTION

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Declaration I

I hereby declare that the Ph.D. thesis is my original work and effort and that it has not been submitted anywhere for any award. I have written this thesis and where other information's have been used, they have been acknowledged in the section "References".

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Declaration II

I hereby declare that this thesis is from the student's own work and effort and all other source of information have been acknowledged in the section "References".

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List of Publications

The thesis is based on the following papers listed below. These papers are enclosed at the end of the thesis and have been referred in the text by the corresponding roman numerals.

I. **Prasad A**, Pospíšil P. Linoleic acid-induced ultra-weak photon emission from *Chlamydomonas reinhardtii* as a tool for monitoring of lipid peroxidation in the cell membranes. PloS One 2011 6 (7), e22345.

II. **Prasad A**, Pospíšil P. Two-dimensional imaging of spontaneous ultra-weak photon emisiion from the human skin: role of reactive oxygen species. Journal of Biophotonics 2011; 4 (11-12), 840-849.

III. **Prasad A**, Pospíšil P. Ultra-weak photon emission induced by visible light and ultraviolet A radiation via photoactivated skin chromophores: *in vivo* charge couple device imaging. Journal of Biomedical Optics 2012; 17 (8): 085004.

IV. **Prasad A**, Pospíšil P. Towards the two-dimensional imaging of spontaneous ultra-weak photon emission from microbial, plant and animal cells. Scientific Reports 2013; 3: 1211.

V. **Prasad A**, Pospíšil P. Photon source within the cell. Fields of the cell Eds. Cifra M and Fels D. (Review article)- Accepted for publication.

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2. Six Months (January 2008-June 2008) at Department of Biophysics, All India Institute of Medical Science, New Delhi, India under the guidance of Prof. Savita Yadav on the project titled "Molecular forms of prostate specific antigen- isolation, purification and characterization".

Workshop and trainings

1. Summer school (2010) at International Institute of Biophysics, Neuss, Germany.

2. Workshop (2008) on molecular biology techniques at J.C Bose Institute of life sciences, Bundelkhand University, Jhansi.

3. Field study (2005) on "Taxonomical identification of medicinal plants in north-east India".

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2. Organizer of 8th Ultra-weak photon emission workshop at Department of Biotechnology, Chemistry and Pharmacy, Siena, Italy (April 2013).

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1. International conference "Fields of the living cell", University of Basal, Switzerland, October (2012) on "Mechanism of ultra-weak photon emission".

2. 9th International Fröhlich's Symposium- electrodynamic activity of living cells, Institute of photonics and electronics, Prague, Czech Republic (2011) on "Ultra-weak photon emission from synchronized and non-synchronized yeast cells".

Seminar presentations (oral)

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2. Seminar at Department of Biotechnology, Chemistry and Pharmacy, Siena, Italy, October (2012) on "ROS mediated cell damage".

3. 5th ultra-weak photon emission research workshop at Department of biophysics, Palacký University, Czech Republic, October (2011) on "Ultra-weak photon emission and reactive oxygen species".

3. 3rd young biophoton scientist seminar at Meluna research Amersfoort, Netherlands, November (2010) on "Two-dimensional imaging of ultra-weak photon emission from human hand-role of ROS"

4. Seminar on Applied phycology and biotechnology at Department of botany, Palacky university, Czech Republic, September (2010) on "Involvement of lipid peroxidation in ultra-weak photon emission from green alga *Clamydomonas reanhardtii*".

5. 2nd young biophoton scientist seminar at Institute of photonics and electronics, Prague, Czech Republic (2009) on "Lipid peroxidation and ultra-weak photon emission".

Poster presentations

1. Recent developments in biotechnology at Bundelkhand University, India (2008) on "RNA splicing".

2. National conference on scope and application of microorganism in agriculture and environment at C.S.J.M. University, Kanpur, India (2007) on "Xenobiotic compounds".

Workshop and conference attended

1. Workshop- Plant response to UV radiation at Ostrava University, Czech Republic, October (2010)

2. Conference- free radical society of India, satellite meeting at All India institute of medical science, New Delhi, India (2008)

Publications

1. Prasad A., Pospíšil, P. (2013) Towards the two-dimensional imaging of ultra-weak photon emission from microbial, plants and humans*: Scientific Reports 3, 1211*

2. Prasad A., Pospíšil, P. (2012) Ultra-weak photon emission induced by visible light and UV A radiation via photoactivated skin chromophore: *in vivo* imaging by charge couple device*: Journal of Biomedical Optics 17, 085004*

3. Prasad A., Pospíšil, P. (2011) Two-dimensional imaging of Spontaneous ultra-weak photon emission from the human skin: role of reactive oxygen species. *Journal of Biophotonics 4, 840-849*

4. Prasad A., Pospíšil P. (2011) Linoleic acid-induced ultra-weak photon emission from *Chlamydomonas reinhardtii* as a tool for monitoring of lipid peroxidation in the cell membranes PLoS ONE 6(7): e22345. doi:10.1371/journal.pone.0022345.

5. Prasad A, Pospíšil P. Photon source within the cell. Fields of the cell Eds. Cifra M and Fels D. (Review article) - Accepted for publication.

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Abbreviations

AUC	area under the curve
CCD	charge coupled device
DPI	diphenyl iodonium
DTPA	diethylenetriamine penta-acetic acid
EMPO	2-ethoxycarbonyl-5-methyl-1-pyrroline N-oxide
EPR	electron paramagnetic resonance
$HO_2^{\bullet-}$	hydroperoxyl radical
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
$HO^{\bullet}$	hydroxyl radical
HPLC	high performance liquid chromatography
MDA	malonaldehyde
MGDG	monogalactosyldiacylglycerol
NADPH	nicotinamide adenine dinucleotide phosphate
NOS	nitric oxide synthase
$O_2^{\bullet-}$	superoxide anion radical
$^1O_2$	singlet oxygen
PMT	photomultiplier tube
POBN	α-phenyl N-tert-butylnitrone
$R^{\bullet}$	alkyl radical
R=O	ground state carbonyl
RNS	reactive nitrogen species
$RO^{\bullet}$	alkoxyl radical
ROH	hydroperoxide
$ROO^{\bullet}$	peroxyl radical
ROOOOR	tetroxide
$^3(R=O)^*$	triplet excited carbonyl
Sen	photosensitizer
$Sen^{\bullet-}$	photosensitizer anion radical

Sen*	singlet state of photosensitizer
SH	sulfhydryl group
SOD	superoxide dismutase
$Substrate^{\bullet+}$	substrate cation radical
TAP	tris-acetate-phosphate
$(TBA)_2$-MDA	thiobarbituric-malonaldehyde adduct
TBARS	thiobarbituric acid reactive substance
UV	ultraviolet
YPD	yeast peptide dextrose

Abstract

Oxidative stress is an imbalance between the systemic manifestation of the formation pro-oxidant and the capability of the organism to detoxify it via antioxidant. The reactive intermediates formed thus may cause irreversible damage to cell components via the oxidation of biomolecules. Reactive oxygen species (ROS) formed as a response to various abiotic and biotic stresses cause an oxidative damage of cellular component such are lipids, proteins and nucleic acids. The oxidation of biomolecules is initiated by radical ROS (superoxide anion, $O_2^{\bullet -}$; perhydroxyl, $HO_2^{\bullet -}$ and hydroxyl radicals, $HO^{\bullet}$), non-radical ROS (singlet oxygen, $^1O_2$; hydrogen peroxide, H_2O_2). The oxidation of biomolecules is initiated either by abstraction of a hydrogen atom or via cycloaddition of singlet oxygen ($^1O_2$) to the biomolecules forming varied reactive intermediates such as alkyl ($R^{\bullet}$), peroxyl ($ROO^{\bullet}$) or alkoxyl radicals ($RO^{\bullet}$) which finally through a series of reactions form tetraoxide (ROOR) and dioxetanes (ROOOR). These reactive intermediates upon decomposition leads to the formation of triplet excited carbonyl [^{3}(R=O)*] and $^1O_2$. Triplet excited carbonyls can either emit directly at the wavelength range of 400-500 nm or can transfer its excitation energy to chromophores. Singlet oxygen on the other hand is known to emit at the wavelength of 634 nm and 703 nm upon recombination referred to as dimol emission. The photon emission from the organism is known to be between 1-10^6 photons/s.cm^2 and thus its detection and characterization is intricate. Thus, low-noise photomultiplier tube (PMT) and highly-sensitive charge coupled device (CCD) are involved in the detection of ultra-weak photon emission by several authors during the past decades.

In our study with *Chlamydomonas reanhardtii*, a detailed account of the reaction mechanism on the peroxidation of the lipid in the cell membrane is presented. The lipid peroxidation was induced by addition of exogenous linoleic acid breaking the cellular integrity of the system. The involvement of ROS and the lipid peroxidation was illustrated utilizing ROS scavengers, removal of molecular oxygen and inhibitor of lipoxygenase, the key enzyme involved in the process. The reaction mechanism and the excited species formed was monitored utilizing low-noise PMT and highly sensitive CCD camera which is sensitive in the range of 185-730 nm and 200-1000 nm, respectively. Electronically excited species such as ^{3}(R=O)* is reported to be the primary excited

species formed during the lipid peroxidation, whereas chlorophylls are the final emitters of photons.

Reactive oxygen species have several deleterious consequences and is associated with premature skin ageing and skin cancer in human skin. In our study with human skin, we investigated the involvement of ROS in generation of ultra-weak photon emission using two-dimensional charge coupled device imaging. Evidences are provided on the qualitative and quantitative description of cell damage under the effect of topical application of different ROS (H_2O_2, $O_2^{\bullet -}$ and $HO^{\bullet}$) reflected by intensity of ultra-weak photon emission. The formation of different ROS in the chemical system was confirmed using EPR spectrometer. The generation of ultra-weak photon emission mediated by ROS was also studied under the influence of visible light and ultra-violet A radiation via photoactivated skin chromophores in human skin through photosensitization reaction (Type I and Type II).

In order to bring a detail analysis of measuring parameters such as accumulation time and binning factor in order to achieve minimum time required and best image quality, two-dimensional ultra-weak photon emission was also studied in microorganism (*Saccharomyces* sp.), plant (*Arabidopsis* sp.) and animal (human skin). Two dimensional ultra-weak photon emission can thus serve as a potential and a unique tool for monitoring the oxidative stress induced by various stress factors irrespective of its chemical or physical nature and thus can serve as a non-invasive methods for disease diagnosis and monitoring of physiological state of the organism.

Chapter 1

Introduction

1. Ultra-weak photon emission

All living organisms emit low-level of photons spontaneously without any excitation and hence differentiated from delayed luminescence known to occur by photoexcitation (Kim, 2005). During the past few decades, the biochemical reaction pathway has been studied to understand the mechanism and the species involved in the process. However, very little is known in relation to its biological and physiological role. Besides the biochemical theory of ultra-weak photon emission generation, the ultra-weak photon emission from DNA and later its coherence properties has also been studied by Fritz-Albert Popp (Popp, 1988). Biophoton emission, low-level chemiluminescence or autoluminescence are the terminologies also used alternatively to define ultra-weak photon emission (Lavorel, 1980; Wijk et al., 1988; Hideg et al., 1990; Kobayashi et al., 1997; Yan et al., 2003; Havaux et al., 2006; Rastogi and Pospíšil, 2010; Wijk et al., 2010; Cifra et al., 2010).

1.1. Experimental evidences on ultra-weak photon emission

1.1.1. Ultra-weak photon emission from non-photosynthetic organisms

Ultra-weak photon emission from cells and organelles

The ultra-weak photon emission in microorganism was first detected from the synchronized cells of *Candida utilis*. Ultraviolet (UV)-sensitive photomultiplier tube (PMT) was used for the detection of ultra-weak photon and the spectral range of photon was detected to be in the range of 250-380 nm. The source of ultra-weak photon was claimed to be originated from cell division [Konev 1966]. During the same period, Stauff and coworkers (1964), observed the luminescence dependency on molecular oxygen availability rather than the stage and the viability of the cells. In 1985, Quikendem and co-workers reported that the photon emission from *Saccharomyces cerevisiae* comprises of both UV and visible components. Luminescence from *Escherichia Coli* and *Klebsiella pneumoniae* was not observed (Roth and Kaeberle, 1980). However, when experiments were performed on *Listeria monocytogenes*, one major peak was observed during the exponential growth phase. Further investigation showed that superoxide dismutase (SOD) and catalase inhibited the photon emission while application of hydroxyl radical ($HO^{\bullet}$) brings about no change in the luminescence. These observations led to the understanding of the involvement of hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$) in photon emission.

The changes in ultra-weak photon emission have been employed to distinguish the state of the living cell. Normal and cancer cells have been demonstrated to differ in their ultra-weak photon emission which is referred as its biophoton signature (Kaliken et al., 2003). Two-dimensional ultra-weak photon emission has also been measured from mice transplanted with carcinoma cells showing strong photonic differences between the infected and healthy cell while the microscopic findings and intensity have been shown to reflect the viability of the tumor cells (Fig. 1). Plasma from hemodialysis patient show weak chemiluminescence and has been shown to occur in the blue region at the wavelength maximum of 430 nm (Agatsuma et al., 1992). Ultra-weak photon emission has also been detected in fibroblast cells, where it is demonstrated that under light irradiation, ultra-weak photon emission is induced in fibroblast. The ultra-weak photon emission however is lower in case of patients suffering from xeroderma pigmentosum, a recessive autosomal genetic disease under light induction. It was proposed that under the diseased condition, the fibroblast cells loose the capacity of photon storage in itself (Niggli, 2003).

Recently, a detailed account of ultra-weak photon emission mediated via ROS from radish root has been demonstrated (Rastogi and Pospíšil, 2010). It has been concluded that ROS-mediated biomolecule oxidation is responsible for the photon emission which is in support of the work published on soybean embryos claiming role of ROS during the seed imbibition and high generation of ultra-weak photons (Boveris et al.,1984). The ultra-weak photon emission from soybean seed has been found to emit both in the UV and visible range of the spectrum (Kobayashi et al., 1997).

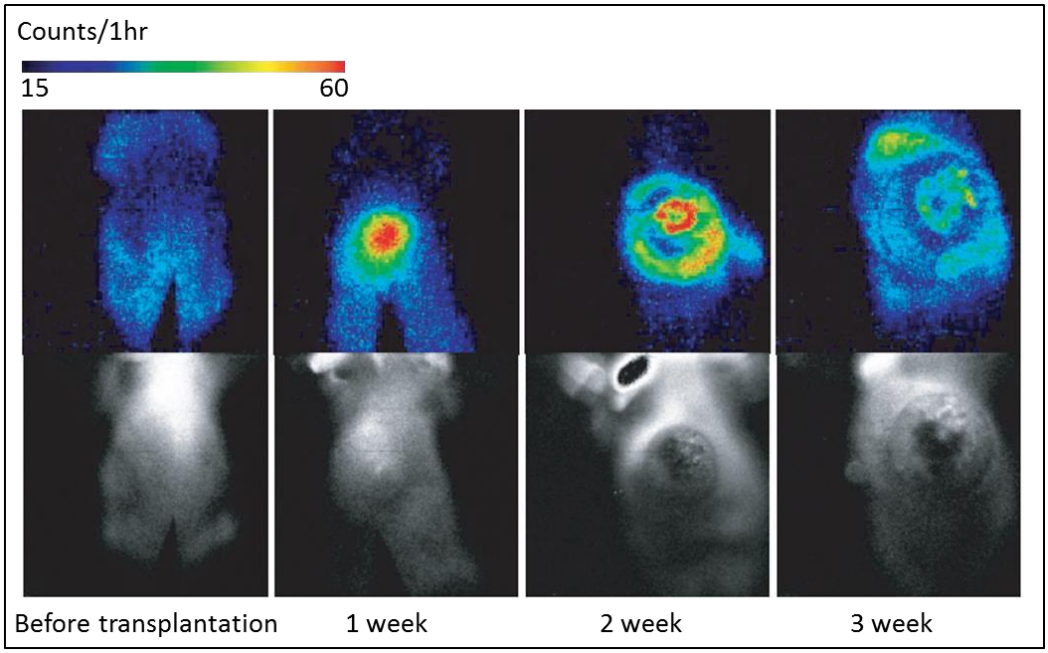


Fig. 1*:* Ultra-weak photon emission from mice transplanted with carcinoma cells. Two-dimensional ultra-weak photon emission was measured at the regular interval from 1-3 weeks (adapted from Takeda et al., 2004).

Ultra-weak photon emission from humans

The ultra-weak photon emission from humans has always been an area of great interest among researcher and thus the experimental evidences are large as compared to plant and animals. Among the pioneers in human ultra-weak photon detection are Cohen and Popp (1997), who performed measurements on spontaneous and delayed ultra-weak photon emission stating a periodicity of emission. The periodicity, however, was found disturbed in the case of diseased condition such as neurological disorders. Considering ultra-weak photon emission as an important tool in diagnosis and to pave its way in health screening, the spectra of photon emission was studied from human fingertips and various other cellular systems. Later, ultra-weak photon emission from humans was found in the spectral range of 500-700 nm (Kobayashi et al., 2001). Using one- and two- dimensional ultra-weak photon emission studies, evidences have been provided on the anatomic distribution of photon emission and fluctuation between human subjects. Distribution pattern shows that abdomen region emitted lowest number of photons, increasing gradually from face till the head where spectral distribution was found to dominate in the spectral region of 470-570 nm (Wijk et al., 2007). Ultra-weak photon emission from human and porcine skin was also measured and found to correspond under the effect of UVA-irradiation (Fig. 2). The photon emission was found to be highest in the wavelength range of 500-550 nm (Hagens et al., 2008).

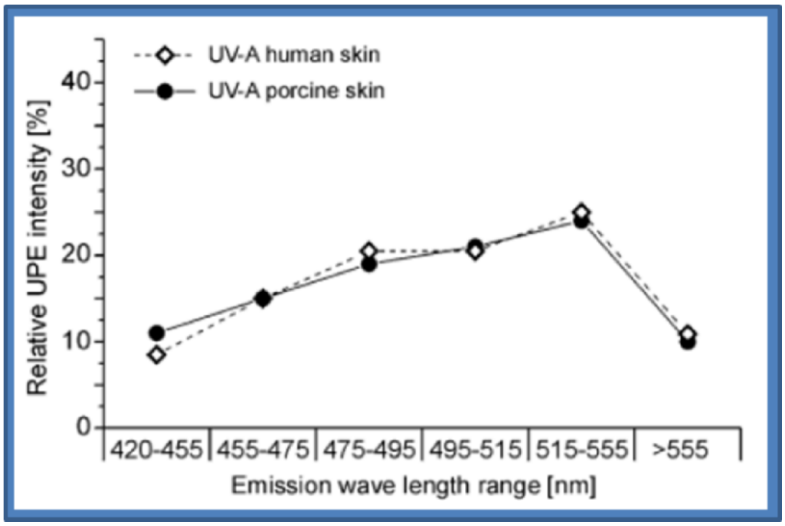


Fig. 2*:* Spectral distribution of ultra-weak photon emission from human and porcine skin under UVA-exposure. Ultra-weak photon emission was measured immediately after the UVA exposure (adapted from Hagens et al., 2008).

Recently, experimental evidences are provided on the effect of various antioxidants investigated under spontaneous and UV-A induced ultra-weak photon emission (Jain et al., 2010). The topical application of antioxidants was found to suppress the intensity of ultraweak photon emission and can be considered via the suppression of ROS-mediated biomolecule oxidation (Rastogi and Pospíšil, 2011).

1.1.2. Ultra-weak photon emission from photosynthetic organism

The reports on spontaneous and induced ultra-weak photon emission from photosynthetic organisms are limited as compared to its evidences in animal cell.

Microscopic organism and cell organelles

Dark adapted spinach chloroplast has been demonstrated to emit in the red region of the spectrum several hour after the excitation (Inaba, 1988). The leaves and isolated spinach chloroplast have been shown to emit low light after a long term of dark adaptation claiming it to be an intrinsic property of the organelles and has been found to be dependent on temperature. The reactions resulting in ultra-weak photon emission are suggested to be associated with slow back flow of electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to molecular oxygen via the plastoquinone (Hideg et al., 1990).

Plants

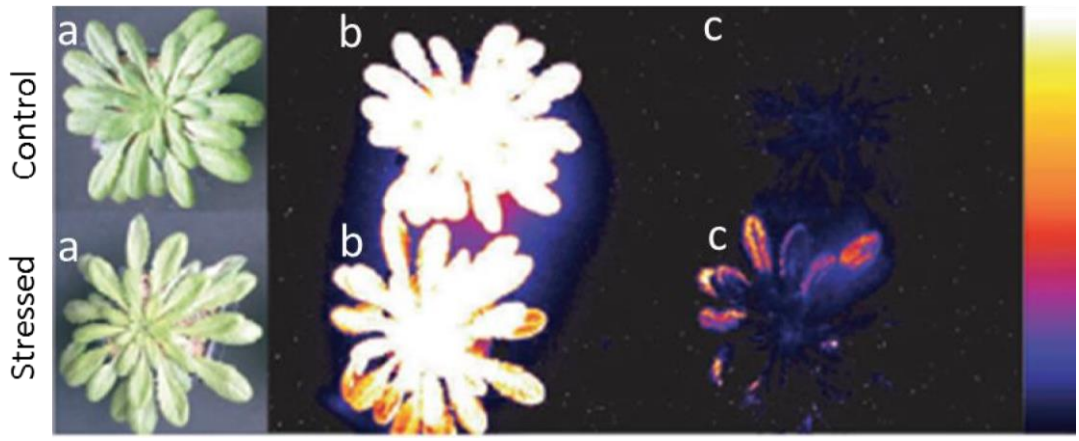


Figure 3*:* Spontaneous photon emission from Arabidopsis plant. Photograph of control (a) and stressed (a) Arabidopsis plant. Stress was induced by exposure to high light at low temperature. Control (b) and stressed (b)/ Control (b) and stressed (b) are autoluminescence image taken immediately/2 hours after transfer to dark chamber (adapted from Birtic et al., 2011).

Enhancement in spontaneous photon emission under high light stress from *Arabidopsis thaliana* have been shown by Birtic and co-workers, 2011 (Fig. 3). Mechanical wounding in *Arabidopsis thaliana* leaves has been shown to enhance the two-dimensional ultra-weak photon emission (Henry et al., 2004). It was proposed that lipid peroxidation which is known to be mediated by ROS is responsible factor. The formation of secondary end product of lipid peroxidation in photoresistant wild-type and double mutant *Arabidopsis thaliana* was studied and led to the conclusion that lipid peroxidation is involved in ultraweak photon emission.

In contrary, Bennett et al., (2005) have completely ruled out the prospect of ultraweak photon emission from ROS mediated process in the case of pathogenic infections. He claimed ultra-weak photon generation via reactive nitrogen species (RNS) and hypersensitive reaction through gene-for-gene interaction. Mansfield et al., (2005) demonstrated a correlation between the hypersensitive reaction forming RNS proceeding via lipid peroxidation thereby leading to ultra-weak photon emission. Leaves infected with fungi *Pseudomonas syringae* and role of RNS related gene-to-gene mediated hypersensitive cells death in the ultra-weak photon emission was demonstrated. The role of ROS, however, was not completely ruled out.

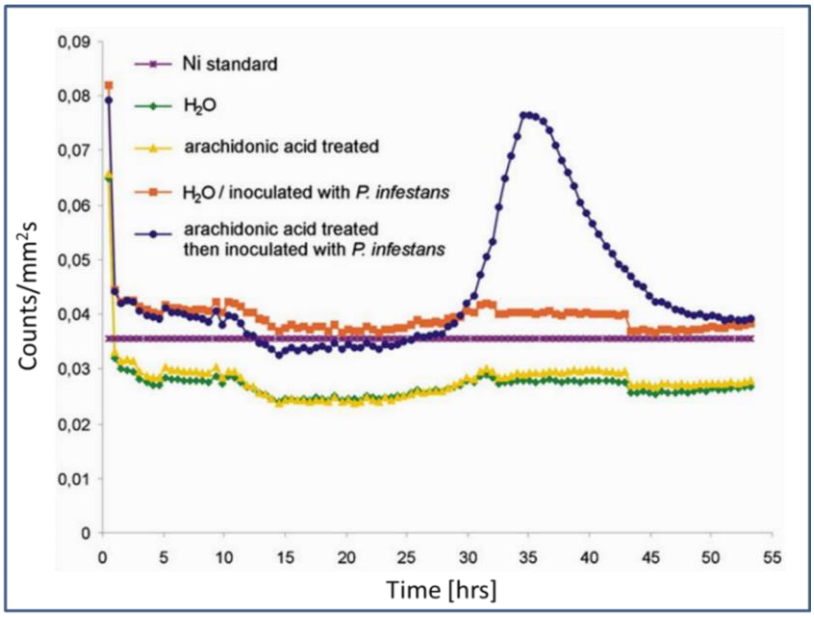


Figure 4*:* The effect of *Phytophthora infestans* on ultra-weak photon emission from potato leaves elicited by arachidonic acid (adapted from Floryszak et al., 2011).

It has also been reported that photon emissions is affected by environmental stress factor and also by the disease response under the pathogen attack (Yoshinaga et al., 2006; Floryszak et al., 2011). Potato leaves infected with *Phytophthora infestans* have been demonstrated to emit high level of ultra-weak photon emission than the non-infected leaves (Fig. 4). Ultraweak photon emission can be measured in living cells non-destructively and thus it can serve as a unique tool for analyzing the physiological changes in plants. The role of ROS, however, is not being completely understood and thus to give an insight, the formation of ROS and the oxidation leading to the formation of electronically excited species have to be discussed in the following chapters.

Chapter 2

ROS and ultra-weak photon emission

2.1 Generation of ROS and ultra-weak photon emission

Reactive oxygen species are generated inside the living cells via two processes- energy transfer and electron transport. The energy transfer involves the transfer of excitation energy from triplet state of excited photosensitizer to molecular oxygen while electron transport is transfer of electron from one species to the other. The major organelles contributing to the generation of ROS are mitochondria and chloroplast (Halliwell and Gutteridge, 2007). Besides, there are various other endogenous and exogenous sources contributing to the generation of ROS. A schematic representation of all the possible source of ROS generation in living cells and organism is shown in Fig. 5.

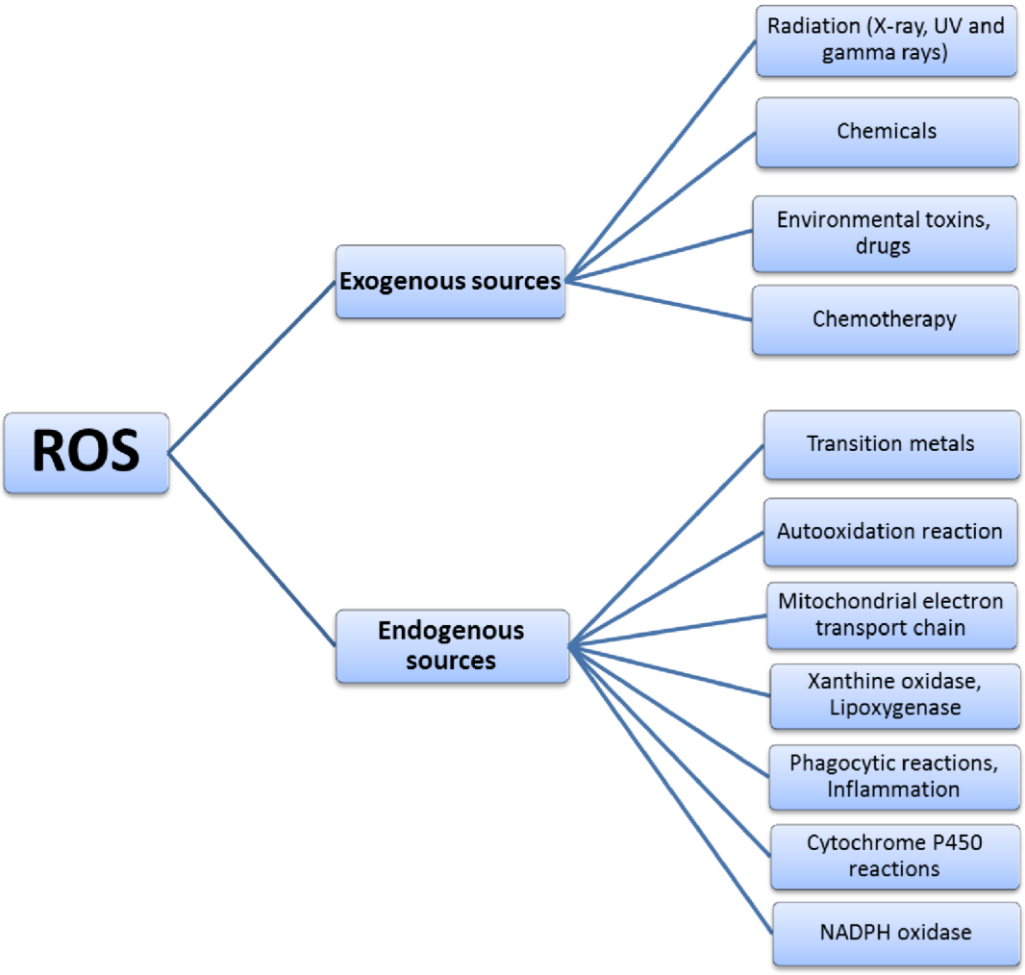


Fig. 5: Schematic representation which depicts the endogenous and exogenous sources of ROS inside the living cells.

2.1.1 Superoxide anion radical and ultra-weak photon emission

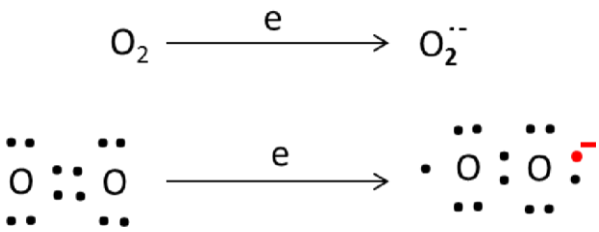


Fig. 6: Formation of $O_2^{\bullet-}$ from molecular oxygen. The anionic unpaired electron is shown in red.

Superoxide anion radical is formed either by one-electron reduction of molecular oxygen or one-electron oxidation of H_2O_2. Both the processes are catalyzed by the various types of membranous or cytoplasmic enzymes. Superoxide anion radical is formed by one-electron reduction of molecular oxygen by complex I and the complex III in the mitochondria, by NADPH oxidase during the respiratory burst in the phagocytic cells and by xanthine oxidase in the cytoplasm of the cell. Superoxide anion radical is also formed by one-electron oxidation of H_2O_2 catalyzed by flavin oxidases in peroxisomes (Muller et al., 2004, Zalba et al., 2007, Till et al., 1989, del Rio et al., 1990). The redox potential of the redox couple $O_2^{\bullet-}/H_2O_2$ is $E_0^{'}$=0.94 V, pH 7 and thus the oxidation of H_2O_2 to $O_2^{\bullet-}$ is feasible only by highly oxidizing redox component.

Besides NADPH oxidase and xanthine oxidase, $O_2^{\bullet-}$ is known to be produced by peroxidases (phagocyte myeloperoxidase, thyroid peroxidase) in plant, bacteria and some animal tissues; nitric oxide synthase (NOS) in mammalian cells; tryptophan dioxygenase and aldehyde oxidase in liver cells of animals (Halliwell and Gutteridge, 2007). The reactivity of $O_2^{\bullet-}$ is relatively lower than other ROS and thus $O_2^{\bullet-}$ does not react with all biological molecules, but it consequently forms highly reactive $HO^{\bullet}$ upon reduction of H_2O_2. The protonated form of $O_2^{\bullet-}$, the hydroperoxyl radical ($HO_2^{\bullet}$) on the contrary is more reactive due to its uncharged nature which makes is readily permeable to the membrane and bears high redox potential (E_m=$HO_2^{\bullet}$/ H_2O_2 1.06 V, pH 7) (Halliwell and Gutteridge, 2007).

The role of $O_2^{\bullet-}$ in the oxidation of biomolecules and formation of electronically excited species has been reported in human leukocytes in the presence of xanthine/xanthine oxidase system (Rosen and Klebanoff, 1976). Ultra-weak photon emissions have been shown to be enhanced upon the addition of myristic acid to polymorphonuclear leukocytes. The

process was found to be correlated with the oxygen uptake and hence the authors proposed the involvement of ultra-weak photon emission with $O_2^{\bullet-}$ production (Kakinuma et al., 1979).

Recently, Kageyama et al., (2006) have studied the effect of *N*acetylchitooligosaccharide, a β-1,4-linked *N*-acetylglucosamine polymer which is a component of fungal cell wall as a elicitor in plants. *N*-acetylchitooligosaccharide is known to generate ROS in rice besides its role in expression of defense genes and cytoplasmic acidification. The addition of N-acetylchitohexose to the rice cells leads to a linear increase in ultra-weak photon emission. The addition of diphenyl iodonium (DPI), an NADPH oxidase inhibitor highly suppressed the ultra-weak photon emission leading to the conclusion that $O_2^{\bullet-}$ is involved in the *N*-acetylchitooligosaccharide mediated ultra-weak photon emission.

The exogenous application of antioxidants on ultra-weak photon emission was performed. It has been reported that exogenous application of SOD partially suppressed myristic acid-induced ultra-weak photon emission (Kakinuma et al., 1979). Similarly, it has also been shown that addition of exogenous SOD to spinach chloroplast and to cotyledons of etiolated seedlings pronouncedly suppressed ultra-weak photon emission (Hideg and Inaba, 1991; Mathew et al., 1992). The exogenous topical application of different antioxidant of $O_2^{\bullet-}$ viz. ascorbate, glutathione, coenzyme Q10 and α-tocopherol have also been shown to considerably suppress ultra-weak photon emission (Rastogi and Pospíšil, 2011)

2.1.2 Hydrogen peroxide and ultra-weak photon emission

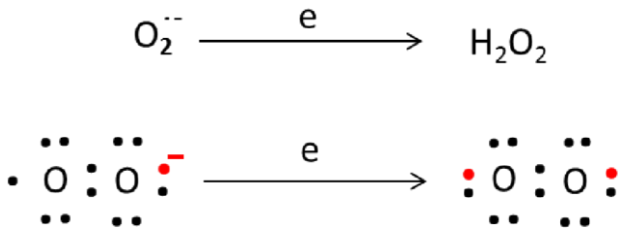


Fig. 7: Formation of H_2O_2 from $O_2^{\bullet-}$. The additional gained electrons are shown in red.

Hydrogen peroxide is formed either by one-electron reduction of $O_2^{\bullet-}$ or by two-electron reduction of molecular oxygen. In mitochondria and cytoplasm, it is formed by one-electron reduction of $O_2^{\bullet-}$ catalyzed by SOD (Turrens, 2003). Two-electron reduction of molecular oxygen occurs in mitochondria and peroxisomes in which specific substrates are oxidized by the various types of oxidases in the mitochondria (monoamine oxidases) and peroxisomes

(urate oxidase, L-alpha-hydroxy acid oxidase, polyamine oxidase, oxalate oxidase and fatty acyl-CoA oxidase) (Scheme I).

Hydrogen peroxide at the low concentration promotes cell proliferation and higher concentration (10-100 µM) causes senescence and apoptosis. Hydrogen peroxide is a weak oxidizing or reducing agent while it can play role in inactivation of certain enzymes usually by the oxidation of sulfhydryl (–SH) group essential for catalysis (Halliwell and Gutteridge, 2007). The oxidation of biomolecules by H_2O_2 is indirectly via the formation of highly reactive $HO^{\bullet}$ which is formed upon the diffusion of H_2O_2 across the cell membrane and reacting with iron or copper. Hydrogen peroxide has been also known to enhance the production of $O_2^{\bullet-}$ via activation of NADPH oxidase. It can further also form hypochlorous acid (HOCl) catalyzed by myeloperoxidase in the presence of chlorine ion. Hypochlorous acid bears the ability to oxidize and is a powerful two-electron oxidizing agent (Halliwell and Gutteridge, 2007).

The role of H_2O_2 in the oxidation of biomolecules and ultra-weak photon emission have been reported in intact soybean seedling where the addition of exogenous H_2O_2 enhanced considerably the level of ultra-weak photon emission (Suzuki et al., 1991). Different phases of photon emission were observed. The fast phase of which was proposed to occur due to the direct oxidation of the biomolecules while the slow phase was suggested to be from the decomposition of H_2O_2 which subsequently created an oxidizing environment. It has also been reported in case of unsaturated fatty acid that excited species leading to ultraweak photon emission are formed as a result of lipid peroxidation initiated by H_2O_2 (Cadenas, 1980; Cadenas et al., 1984). During the hypoxia condition in brain, there is higher production of H_2O_2 and it has been correlated with an increase in ultra-weak photon emission indirectly proving the involvement of H_2O_2 in ultra-weak photon emission. Recently, it has also been shown that the addition of exogenous H_2O_2 to kidney (MDCK cells) resulted in an increase in ultra-weak photon emission (Cheun et al., 2007). Direct evidence on the protein oxidation and ultra-weak photon emission was presented by Khabiri et al., (2008). It was shown that addition of H_2O_2 to bovine serum albumin induced linearly correlated with ultraweak photon emission. The photon emission induced by H_2O_2 from amino acids were also tested which showed high photon emission from Phe, Trp, His and Cys while Lys and Thr showed a comparatively lower

photon emission. The spectral range of photon emission measured showed the formation of protein carbonyl under H_2O_2 treatment (Khabiri et al., 2008).

Recent report on application of exogenous H_2O_2 has been provided on radish root cells which showed an enhancement in ultra-weak photon emission in a concentration dependent manner (Rastogi and Pospíšil, 2010). The authors demonstrated that ascorbate, which is known to scavenge $HO^{\bullet}$ suppressed ultra-weak photon emission thereby concluding that $HO^{\bullet}$ which is formed by reduction of H_2O_2 by intrinsic metals is involved in the phenomenon. In view of the fact that H_2O_2 is less reactive and bears low capability to oxidize biomolecules, the potential deleterious effect is mainly via the subsequent formation of $HO^{\bullet}$.

Besides the damage of lipid and proteins, there are also reports on the H_2O_2 induced DNA damage and mainly via the modification of guanine bases. Exogenous addition of the Phen-Cu^{2+}/ascorbate/ H_2O_2 to the DNA molecules was found to linearly increase the intensity of the photon emission (Cao W).

2.1.3 Hydroxyl radical and ultra-weak photon emission

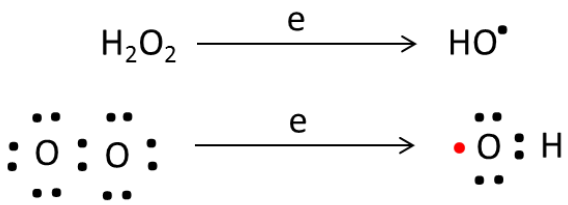


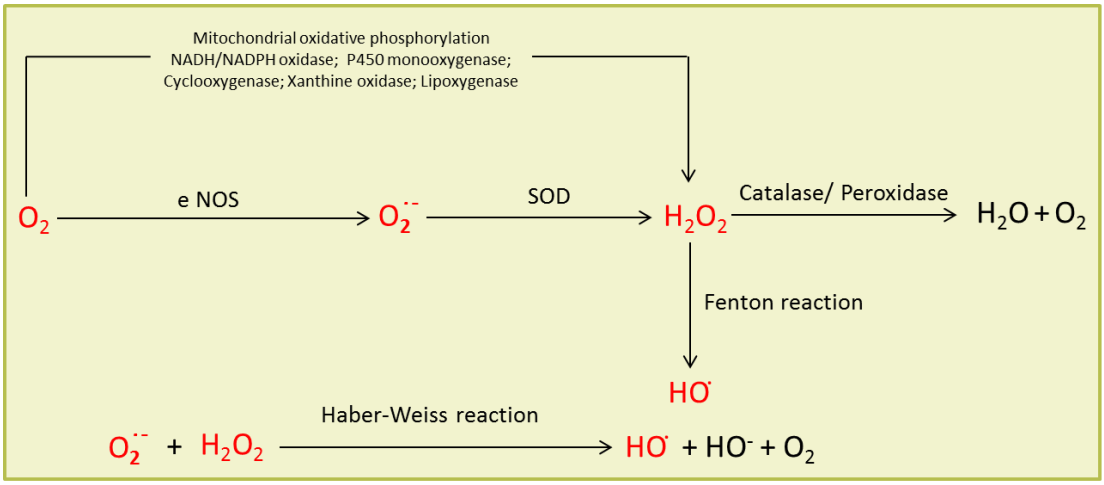
Fig. 8: Formation of $HO^{\bullet}$ from H_2O_2. The unpaired electron is shown in red.

Hydroxyl radical is formed by the one-electron reduction of H_2O_2 or by the homolytic fission of the O-O bond in H_2O_2. Water under high ionizing radiation such as γ-rays also form $HO^{\bullet}$ (Halliwell and Gutteridge, 2007).

Hydroxyl radical can be generated by the reaction of metal ions such as Fe^{2+}, Mn^{2+} or Cu^{+} which is known as the fenton reaction (Scheme I). Iron, in the biological system is stored in the form of ubiquitous protein ferritin which acts as a pool for iron source. The iron in animal cells are present are also present in the form of hemosiderin, transferrin and lactoferrin which can lead to fenton reaction in the presence of H_2O_2 (Treman et al., 1992; Halliwell and Gutteridge, 2007). The superoxide-driven Fenton reaction is the main source of $HO^{\bullet}$ in

biological system and is referred to as the Haber-Weiss reaction. Hydroxyl radical can also be formed upon reaction of HOCl with $O_2^{\bullet-}$ (Halliwell and Gutteridge, 2007).

Hydroxyl radicals are highly reactive and its roles in the oxidation of biomolecules and ultra-weak photon emission have been studied extensively, however, its mechanism behind is not completely understood. The addition of Fenton reagent to the isolated amino acids has been shown to increase the ultra-weak photon emission (Khabiri et al., 2008).The addition of exogenous ferric iron (Fe^{2+}) and iron-containing pigments (haematin) to etiolated seedlings of *Cicer arietinum* lead to an enhanced ultra-weak photon emission and it is evident that one-electron reduction of H_2O_2 by transition metal is involved in ultra-weak photon emission. It has also been shown that topical application of oligomeric proanthocyanidins, which is a scavenger of $HO^{\bullet}$, considerably suppressed UVA-induced ultra-weak photon emission (Wijk et al., 2010). Evidences on $HO^{\bullet}$ as the main cause of cell damage and cell injury have been provided in U937 cell culture. It has been reported that exogenous application of $HO^{\bullet}$ leads to severe cell damage measured utilizing reverse phase high performance liquid chromatography (HPLC) where end products of lipid and protein oxidation were determined to correlate with the high intensity of observed ultra-weak photon emission (Rác, 2010). Since the life time of $HO^{\bullet}$ is short, it readily reacts with polyunsaturated fatty acid and amino acid.



Scheme I: Generation of ROS showing the enzymatic and non-enzymatic pathways involved in the formation of different oxygen radicals.

2.1.4 Singlet oxygen and ultra-weak photon emission

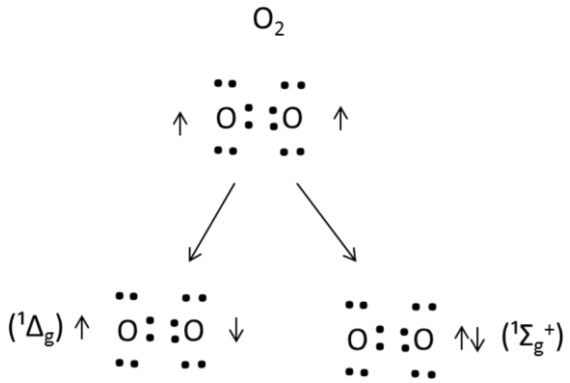
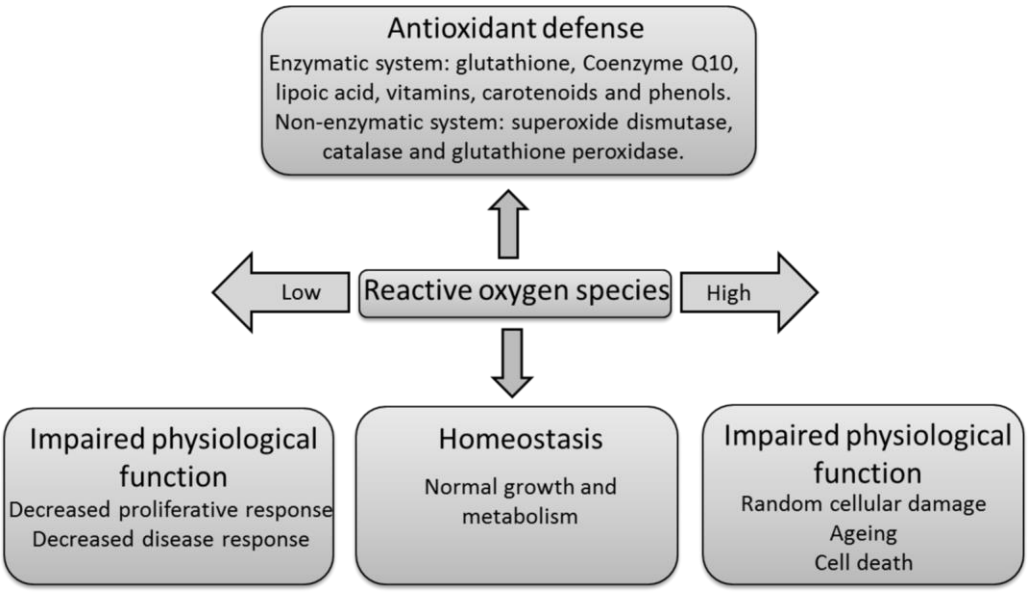


Fig. 9: Formation of $^1O_2$ from molecular oxygen. The $^1O_2$ can either form first singlet excited state ($^1\Delta_g$) or second singlet excited state ($^1\Sigma_g^+$).

Singlet oxygen ($^1O_2$) is formed by the triplet-singlet energy transfer. The excitation energy is transferred from the triplet chromophores to molecular oxygen. The absorption of UV or visible light by various types of chromophores (tetrapyrroles, flavin, pyridine nucleotide, melanin, urocanic acid, pterins) leads to the formation of singlet excited state known to overcome to the triplet excited state by intersystem crossing (DeRosa and Crutchley, 2002). The excitation energy transfer from triplet chromophores to molecular oxygen occurs either to the first singlet excited state ($^1\Delta_g$) or the second singlet excited state ($^1\Sigma_g^+$). The $^1\Sigma_g^+$ state readily decays to $^1\Delta_g$ and thus the high-excited state is rarely considered in the biological system. The detailed mechanism of photosensitization reaction comprising of type I and type II are described in Chapter 3.

Singlet oxygen is reactive and its roles in the oxidation of biomolecules and ultra-weak photon emission have been studied. Ultra-weak photon emission have been shown to be emitted from sub-mitochondrial particle treated with hydroperoxides and have been claimed to originate via the formation of $^1O_2$. Specific quenchers of $^1O_2$ were used to conclude that $^1O_2$ acts as the final emitter (Cadenas et al., 1980 a,b,c). Singlet oxygen produced by methylene blue upon light illumination was found to oxidize linoleic acid and lysozyme (Birtic et al., 2011). The ultra-weak photon emission from monogalactosyldiacylglycerol (MGDG) and linolenic acid has also been demonstrated under the action of $^1O_2$. There are reports on the oxidation of lipid and proteins by $^1O_2$. The main target are however proteins in which side chains including His, Cys, Met, Trp and Tyr are oxidized and targeted (Wright et al., 2000; Wright et al., 2002). However, a correlation has not been established yet.

2.2 ROS scavenging and cell damage



Both non-enzymatic and the enzymatic antioxidant defense lines have been developed by the organism to prevent the deleterious action of ROS. The non-enzymatic antioxidant defense line involves cellular antioxidants (glutathione, Coenzyme Q10 and lipoic acid), vitamins (ascorbic acid and α-tocopherol), carotenoids (α- and β-carotene, lutein and β-cryptoxanthin) and phenols (phenolic acids, coumarins, flavons and flavonols). The enzymatic antioxidant defense line comprises of SOD, superoxide reductase, catalase and glutathione peroxidase.

Besides the enzymatic and non-enzymatic antioxidant defense system, there are other modes by which the oxidative damage is minimized in cells. Their exist mitochondrial uncoupling proteins which minimizes the availability of pro-oxidants such as metal ions or replacement of sensitive molecules with resistant molecules against ROS as observed in case of *E. coli* (Halliwell, 2007).

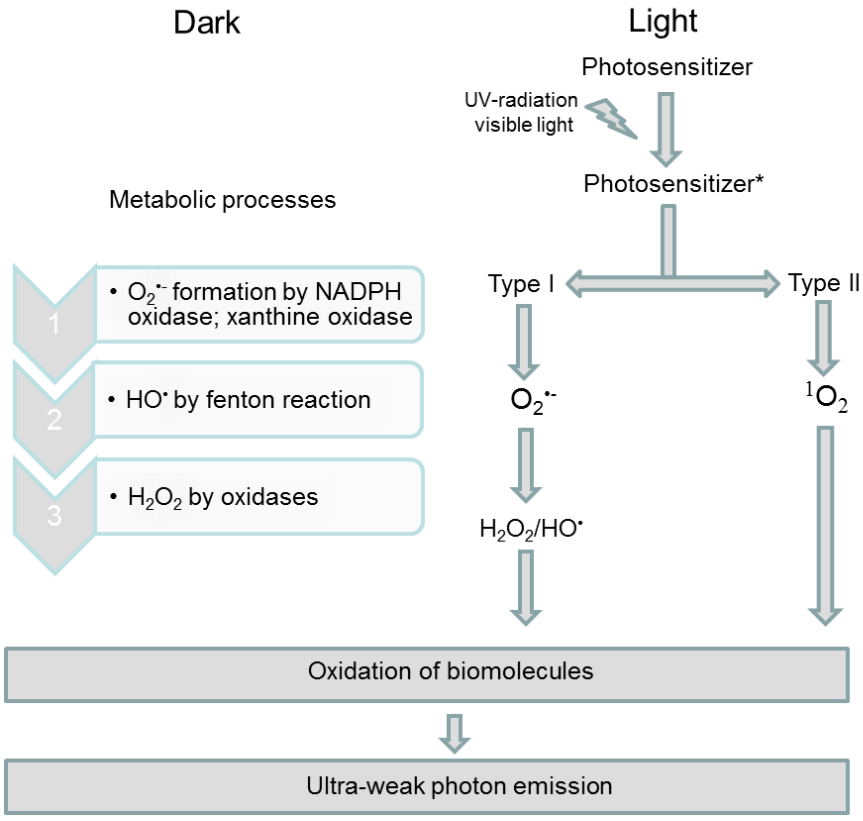
The consequence of oxidative stress can include the activation and upregulation of the cellular defense response which can completely or partially prevent against the cellular damage. Reactive oxygen species can cause potential cell damage. The uncontrolled production of which can lead the oxidation of biomolecules such as lipids, proteins and nucleic acid leading to apoptosis, necrosis or cell death.

Chapter 3

Mechanism of ultra-weak photon emission

3. Mechanistic aspect on the formation of electronically excited states and ultra-weak photon emission

The ROS which leads to the oxidation of biomolecules generating electronically excited states can occur via two pathways (Scheme II). The ROS can be generated via oxidative metabolic processes occurring inside the cell, herewith referred as the dark condition. In the presence of UV radiation or visible light, ROS are generated via photosensitization reaction hereby referred as light condition. The pathway of ROS generation differs in the case of dark and light condition in living cells, but the route for generation of electronically excited species remains the same once different ROS are formed.



Scheme II: Generation of electronically excited state in dark and light condition. In dark, the ROS are spontaneously formed during the metabolic processes via dedicated enzymes while in light condition (visible light or UV-irradiation), the ROS are formed via photosensitization reaction.

3.1 Electronically excited species formation under dark condition

Oxidative metabolism generates various kinds of ROS (chapter 2) via dedicated enzymes and under stress conditions. The oxidation mediated by different ROS has been described as follow:

3.1.1 Initiation

Cycloaddition of singlet oxygen

The reaction of $^1O_2$ with biomolecules leads to the formation of dioxetane (ROOR) via the addition of oxygen atoms (Mascio et al., 1992). The ROOR decomposes to triplet excited carbonyls [^{3}(R=O)*] with the release of organic hydroxides (ROH) as secondary by-products (Mascio et al., 1992; Timmins et al., 1997).

Hydrogen abstraction

The hydrogen abstraction can be mediated either by non-enzymatic or enzymatic reaction pathway. The non-enzymatic reaction pathway is mediated through the abstraction of hydrogen atom from lipid or protein by the free oxygen radical leading to the formation of alkyl radical ($R^{\bullet}$). The enzymatic reaction pathway, however, is catalyzed by the enzyme lipoxygenase which is initiated by the abstraction of the hydrogen atom from carbon by the ferric non-heme iron of the enzyme (Fe^{3+}–OH) to generate $R^{\bullet}$. The active site of the enzyme is reduced to the ferrous non-heme iron (Fe^{2+}–OH_2) (Brash et al., 1999; Maccarrone et al., 2001; Halliwell and Gutteridge, 2007).

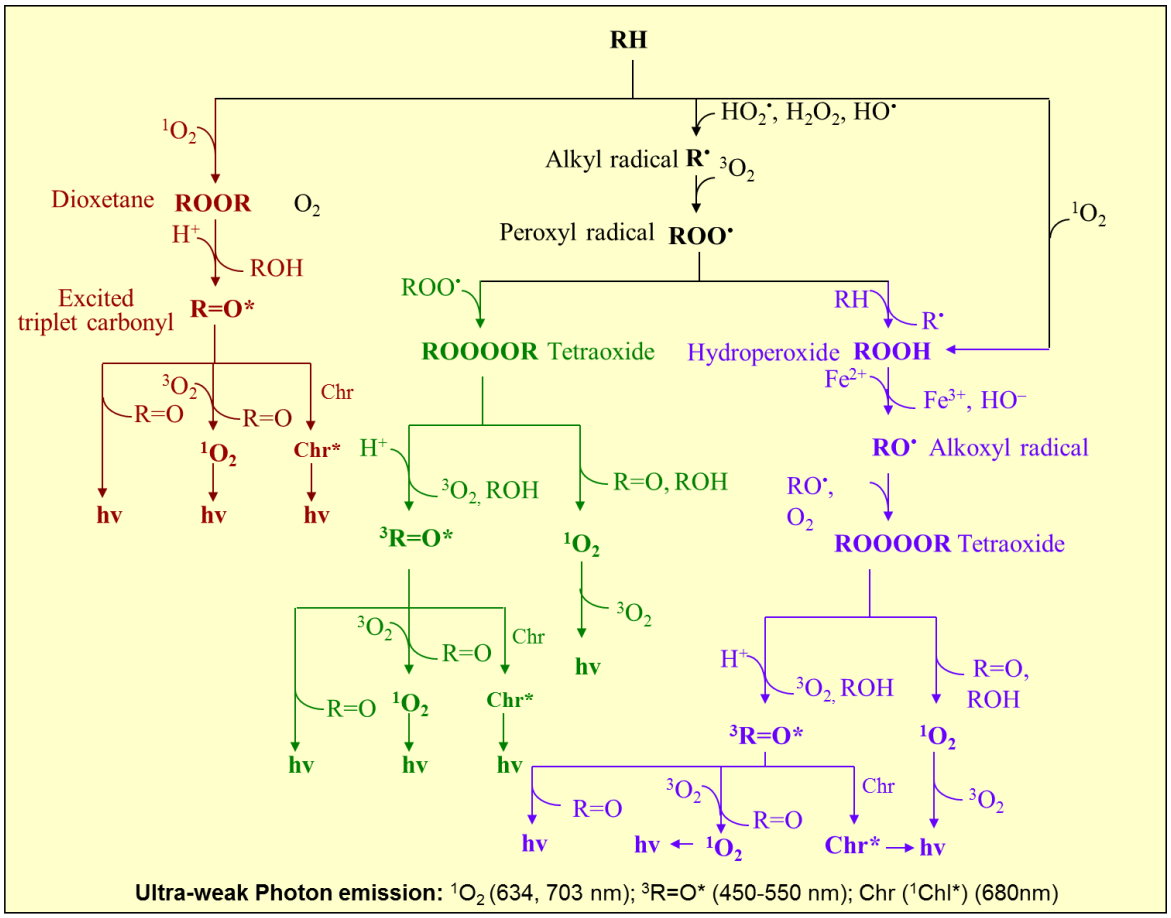
3.1.2 Self recombination of organic radicals

Self recombination of peroxyl radicals

The self-reaction of $ROO^{\bullet}$ leads to the formation of tetroxide (ROOOOR) or ROOR which are reactive intermediates. The peroxyl radical ($ROO^{\bullet}$) can also react with ROOH which in presence of metal ions such as Fe^{2+} can readily form alkoxyl radical ($RO^{\bullet}$) (Dean et al., 1997; Federova et al., 2007).

Self recombination of alkoxyl radical

The recombination of $RO^{\bullet}$, in the presence of molecular oxygen leads to the formation of ROOOOR which either can decompose to form ^{3}(R=O)* or can form $^1O_2$ and ground state of carbonyl (R=O).



Scheme III: Mechanism of ultra-weak photon emission

3.1.3 Excitation energy transfer to chromophores and photon emission

The ^{3}(R=O)* can either directly emit photons in the wavelength range of 400-500 nm with the formation of R=O. The other pathway for photon emission is via transfer of excitation energy to molecular oxygen or to chromophores (Bohne et al., 1986; Wondrak et al., 2006). The excitation energy transfer to molecular oxygen leads to the formation of $^1O_2$ which emits at 634 nm or 703 nm via dimol emission. Besides this, the excitation energy can also be transferred to chromophores such as chlorophyll molecules, melanin, urocanic acid, porphyrin, bilirubin, flavins and pterins.

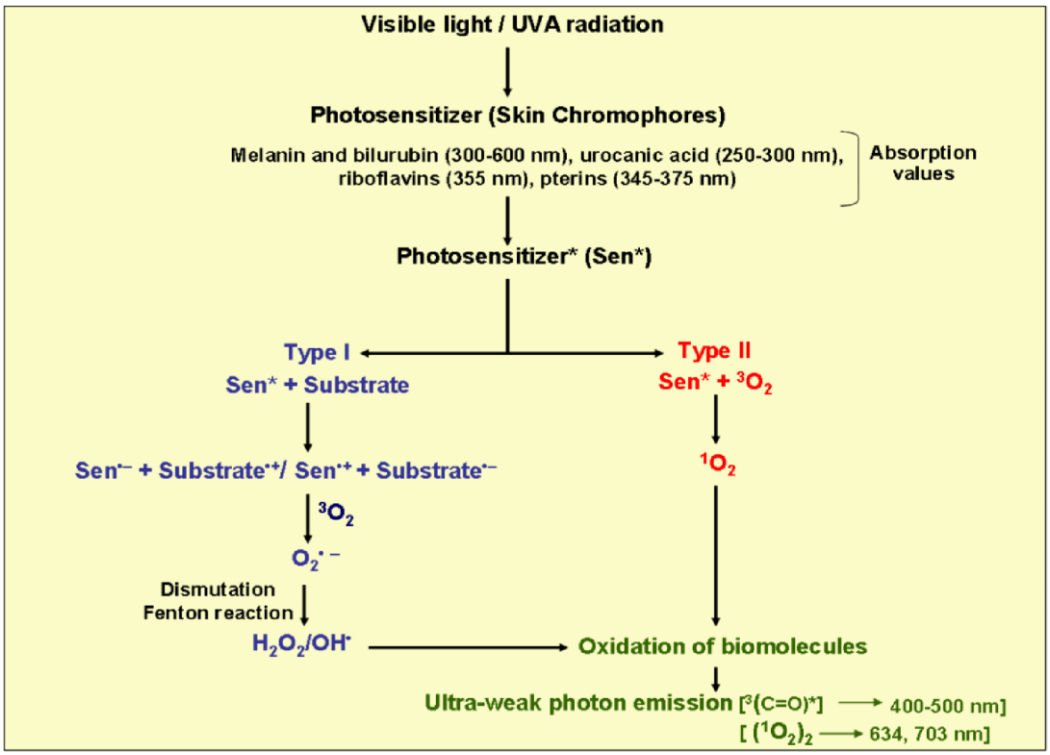
3.2 Electronically excited species formation under UV radiation and visible light

Endogenous chromophores in plant, animals and humans such as porphyrins, bilirubins, melanins, pterins and urocanic acid act as photosensitizers. The photosensitizers (Sen) bear the capability to absorb photons and get excited to their singlet state. The singlet state of

photosensitizer (Sen*) than via intersystem crossing forms the triplet excited state. The excited photosensitizer undergoes either electron transport (Type I) forming $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$ or energy transfer forming $^1O_2$ (Type II) (Wondrak et al., 2006; Kruft and Greer, 2011; Buettner) (Article III)

Electron transport in Type I reaction leads to the formation of $O_2^{\bullet-}$ via formation of photosensitizer anion radical ($Sen^{\bullet-}$) and a substrate cation radical ($Substrate^{\bullet+}$) or vice versa (Buettner ; Kanofsky, 2011). The dismutation of $O_2^{\bullet-}$ than can lead to the formation of H_2O_2 which subsequently forms $HO^{\bullet}$ via Fenton reaction or other metals catalyzed reactions (Buettner; Kanofsky, 2011; Winterbourn, 1995; Coudray, 1998; Lloyd, 1998). Type II reaction proceeds via transfer of energy from the excited photosensitizer to molecular oxygen thus forming $^1O_2$.

The oxidation of biomolecules leading to formation of electronically excited species and ultra-weak photon emission proceeds through the same pathway as described in the previous section irrespective of the pathway for the formation of ROS (light or dark) and hence collective mechanism is described in section 3.1.



Scheme IV: Mechanism of ultra-weak photon emission via photosensitization reaction showing Type I and Type II reaction mechanism leading to formation of ROS and ultra-weak photon emission (Article III).

Chapter 4

Material and methodology

4. Materials and methodology

The chapter describes the materials and methods used in the experiments which are relevant to the main focus of the study. For more specific details, please refer to the respective publications (I to V).

4.1 Biological material

Yeast cells

Yeast cells *Saccharomyces cerevisiae* (Cold sensitive β-tubulin mutant *tub2-401*, strain CUY67 Mata *tub2-401 ura3-52 ade2-101*) were cultured on yeast peptone dextrose (YPD) medium containing agar. The yeast cells were cultivated overnight in an incubator at a constant temperature of 28 °C and were studied during the growth phase.

Chlamydomonas reinhardtii

Green algae, *Chlamydomonas reinhardtii* (wild type: CC-002) was obtained from the Chlamydomonas Genetic Center (Duke University, Durham, NC, USA). The cells were grown in a continuous white light (100 µmol photons m^{-2} s^{-1}). Tris-Acetate-Phosphate (TAP) medium in which acetate represents the main carbon source was used for cultivation of the cells utilizing multi-position magnetic stirrer RT 5 power **(**IKA Werke GmbH, Staufen**,** Germany**)** to obtain constant CO_2 concentration the medium. The cells were studied at a concentration of approximately 7×10^7 cells ml^{-1}.

Arabidopsis plant

The *Arabidopsis thaliana* ecotype Columbia (Col-0) seeds were sown in the pots filled with peat substrate (Klasman, Potground H). The pots were kept under controlled conditions at 25°C (constant temperature) and 60% relative humidity for 6 weeks under photoperiod of 18h light/6-h dark cycle with a white light at illumination intensity of 100 µmol photons m^{-2} s^{-1}.

Human skin

The study on human skin was performed in compliance with the ethical principles stated in the Declaration of Helsinki and its revisions. All experiments were performed on the author's

hand and no other subjects were involved in the study. During the course of measurement on the human subject, the use of any kind of cosmetics was avoided.

4.2 Chemicals

The chemicals purchased were of analytical grade or highest purity available from Sigma-Aldrich GmbH (Steinheim, Germany) and BDH chemicals (England). The spin trap, 5(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide (EMPO) was purchased from Alexis biochemical (Lausen, Swithzerlands).

4.3 Instrumentations

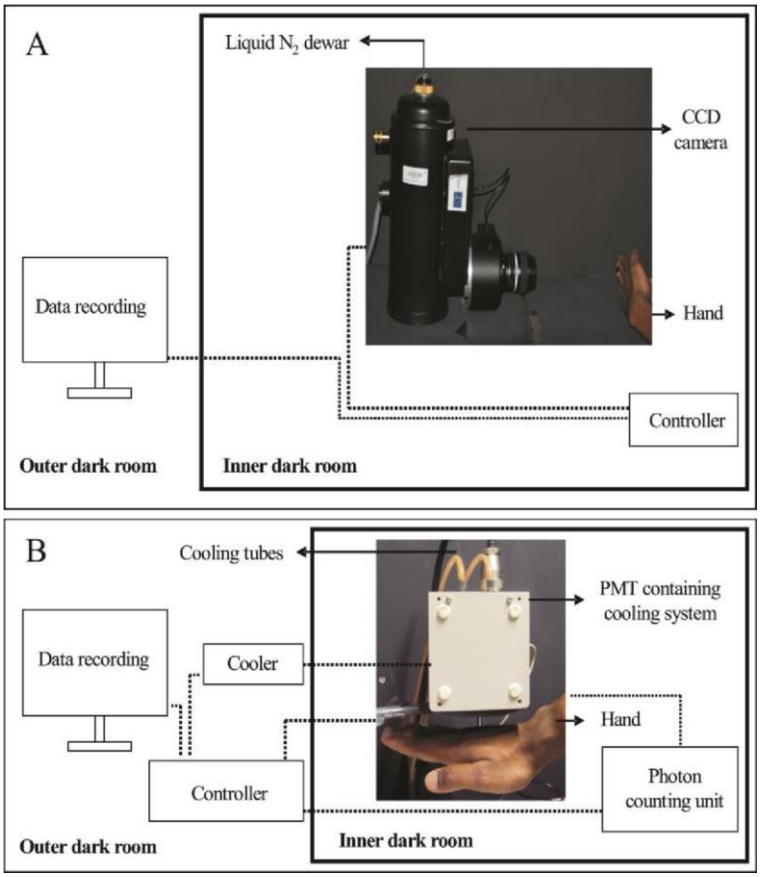
- Charge couple device (CCD) camera VersArray 1300B (Princeton instruments, Trenton, NJ, USA) comprising of metal oxide semiconductor was used for the twodimensional spatial and temporal imaging of ultra-weak photon emission.
- Photomultiplier tube R7518P and photon counting unit C9744 (Hamamatsu Photonics K.K., Iwata city, Japan) were employed to measure one-dimensional ultra-weak photon emission.
- Electron paramagnetic resonance (EPR) spectroscopy for detection of free oxygen radicals was performed using EPR spectrometer MiniScope MS200 (Magnettech GmbH, Berlin, Germany).
- Spectrophotometer Unicam UV 550 (ThermoSpectronic, Cambridge, UK) was used for quantification of thiobarbituric acid reactive substance (TBARS).
- Visible light and UVA exposure was achieved utilizing Philips 60W/ 240V light source and Philips UVA CLEO SWIFT lamp (Philips Electronics Ltd, Guildford, UK), respectively. The intensity of exposure was measured using Spectral Radiometer; LI-COR LI-1800 (LI-COR Biosciences, St. John's Innovation, Cambridge, UK).

4.4 Methods

4.4.1 Ultra-weak photon emission

The CCD camera and the PMT system were utilized for detection of ultra-weak photon emission. Both CCD and PMT were placed in a black painted inner dark room with a dimension of 3 m x 1.5 m x 2.5 m, whereas the control unit was monitored from the outer dark

room. The door in the inner dark room was protected with a black screen to limit any external light. The data recording computer was installed in the outer dark room (Scheme V).



Scheme V*:* Schematic illustration of the experimental setup for detection of two-dimensional (A) and one dimensional (B) ultra-weak photon emission. Two-dimensional ultra-weak photon emission was detected by CCD camera positioned horizontally. One dimensional ultra-weak photon emission was detected by PMT positioned vertically. The distance between the sample and PMT window varied based on the sample measured [Fig. 1, Article II].

4.4.1.1 Two-dimensional ultra-weak photon emission imaging

Highly-sensitive CCD camera with a spectral range of 200–1000 nm was employed for the two-dimensional photon imaging. The spectral sensitivity was restricted to 350–1000 nm by the lenses. 50 mm focal distance objective lens (F mount Nikkor 50-mm, f:1.2, Nikon) was used to enhance the light collecting efficiency. The CCD unit was cooled down to -110 °C using liquid nitrogen to reduce the dark count. The following parameters were used during the measurements: scan rate, 100 kHz; gain, 2; image format, 1340 × 1300 pixels and varying accumulation time. Data correction was made by subtracting the background signal prior to

each measurement. Improvement of signal-to-noise ratio was accomplished using the binning mode with the varying binning factor, which resulted in the different image formats (670 × 650 and 335 × 325).

4.4.1.2 One-dimensional ultra-weak photon emission

Low-noise PMT sensitive in the spectral range of 185-730 nm was employed to measure onedimensional photon emission. To reduce the thermal electrons, thermoelectric cooler C9143 (Hamamatsu Photonics, K.K., Iwata city, Japan) was used to cool the PMT down to – 30 °C. The dark count was adjusted to approximately 1.5 counts s^{-1} at -1150 mV and further to minimize the background light noise to 0.5 counts s^{-1}, PMT was kept vertically. During the measurements with the different sample, varied distances in cm were kept between the subject and the PMT window (refer material and methods, Articles I-V).

4.4.2 Electron paramagnetic resonance (EPR) spin-trapping spectroscopy

The detection of $O_2^{\bullet-}$ was accomplished by spin trap EMPO (Alexis biochemicals, Lausen, Switzerland) (Zhang et al. 2000). Xanthine was supplemented in the reaction mixture containing 25 mM EMPO, 100 µM diethylenetriamine penta-acetic acid (DTPA) and xanthine oxidase in phosphate buffer (pH 7.2). For the detection of $HO^{\bullet}$, α-(4-pyridyl-1oxide**)**-N-tert-butylnitrone (POBN)/ethanol spin-trapping system (Sigma) was used (Pou et al. 1984). Hydroxyl radical was generated in the reaction mixture containing 10 mM POBN, 170 mM ethanol and $FeSO_4$ upon addition of H_2O_2. The EPR spin-trapping spectra were recorded using EPR spectrometer MiniScope MS200 (Magnettech GmbH, Germany) at microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1} (refer material and methods, Article II).

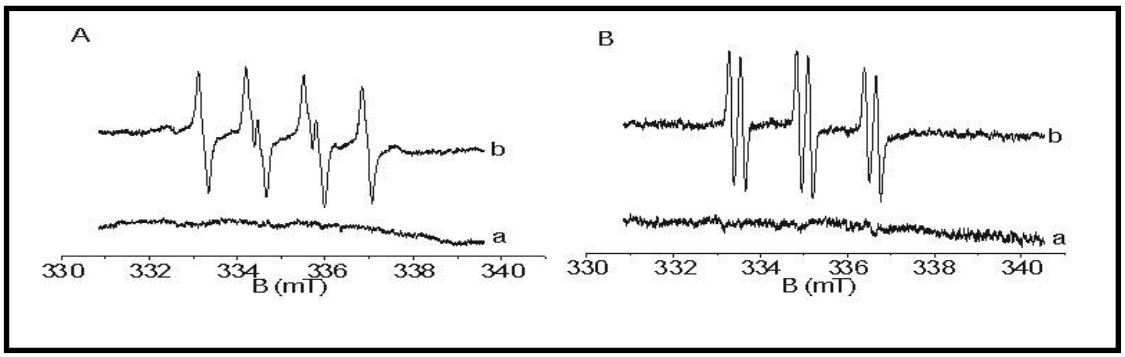


Figure 10*:* Detection of $O_2^{\bullet-}$ (A) and $HO^{\bullet}$ (B) by EPR spin-trapping spectroscopy in the absence (a) and presence (b) of reaction mixture forming ROS (Fig. 5, Article II).

4.4.3 Determination of Thiobarbituric acid reactive substance

The end product of lipid peroxidation under the exogenous application of linoleic acid was estimated by measuring the formation of TBARS with minor modifications (Halliwell and Chirico, 1993). Thiobarbituric acid reactive substance formed was measured by the change in A_{532} (in nm) utilizing Spectrophotometer Unicam UV 550 (ThermoSpectronic, Cambridge, UK). The amount of thiobarbituric-malonaldehyde [$(TBA)_2$-MDA] adduct was determined using of an molar extinction coefficient of 1.54×10^5 M^{-1} cm^{-1} (Halliwell and Gutteridge, 2007).

4.4.4 Light exposure

4.4.4.1 Visible light exposure

Philips 60W/ 240V (Philips Electronics Ltd, Guildford, UK) light source was used for exposure of the dorsal and the palmar side of the hand. The spectral range and intensity was measured employing Spectral Radiometer; LI-COR LI-1800 (LI-COR Biosciences, St. John's Innovation, Cambridge, UK). The power density on the skin surface of the hand was 14.8 W m^{-2} (refer material and methods, Article III).

4.4.4.2 UVA radiation exposure

Philips UVA CLEO SWIFT lamp was used for exposure of the dorsal and the palmar side of the hand. The spectral range and intensity was measured by employing Spectral Radiometer;

LI-COR LI-1800 (LI-COR Biosciences, St. John's Innovation, Cambridge, UK)**.** The power density on the skin surface of the hand was 30.6 W m^{-2} (refer material and methods, Article III).

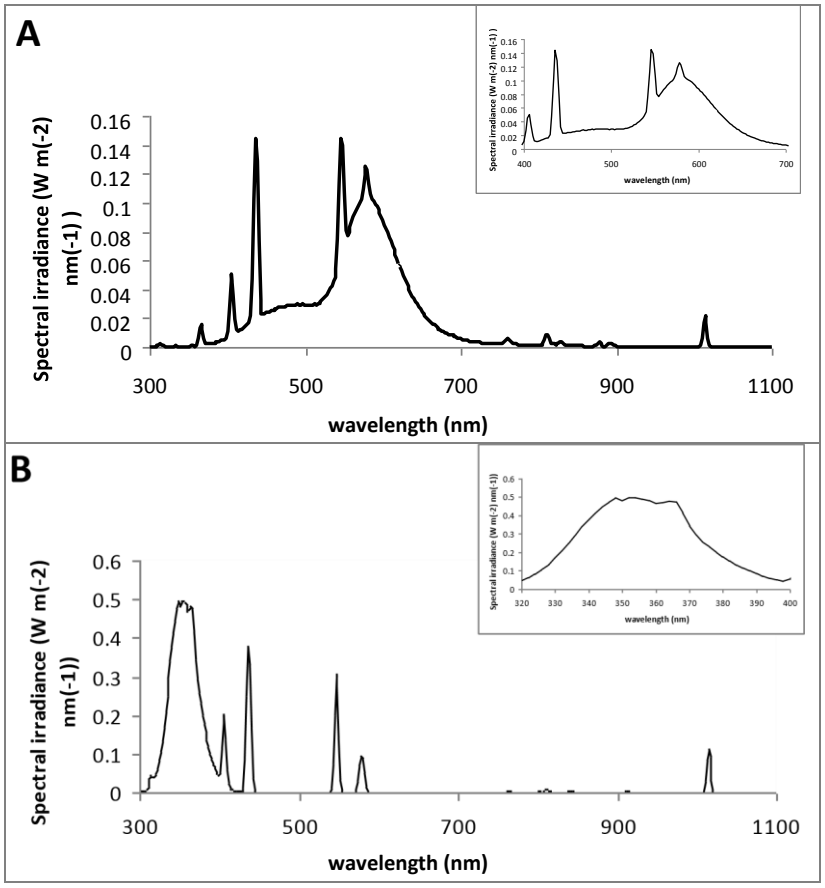


Figure 11*:* Spectra of visible light (A) and UVA radiation (B) source was measured by employing Spectral Radiometer; LI-COR LI-1800 (LI-COR Biosciences, St. John's Innovation, Cambridge, UK).

4.4.5 Data analysis

The experimental data from CCD camera and PMT were corrected by subtracting the background noise of the instrument and noise, respectively (refer material and methods, Article II). Area under the curve (AUC) was calculated by the integration of signals over the decay time using Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Origin 8.5.1 (OriginLab Corporation, Northampton, USA) was used for processing of results and preparation of graphs.

Chapter 5

Results and discussion

5. Results and Discussion

The current section is a summary of our results, its significance and application to biological problems.

5.1 Lipid peroxidation and ultra-weak photon emission (Article I)

The current study deals with the involvement of lipid peroxidation in generation of ultraweak photon emission. The study comprised of measurement of spontaneous and induced ultra-weak photon emission from *Chlamydomonas reanhardtii* cells, a unicellular flagellate green alga. To decipher the role of lipid peroxidation in the phenomenon, several approaches such as addition of ROS scavengers, removal of molecular oxygen and inhibitors of enzyme catalyzing the process were used. For analysis of spectral region of ultra-weak photon emission, sets of band pass and interference filters were also utilized.

5.1.1 Spontaneous and linoleic acid-induced ultra-weak photon emission

Spontaneous and linoleic acid induced ultra-weak photon emission from intact and disrupted *Chlamydomonas reanhardtii* cells were studied using one-dimensional ultra-weak photon emission and two-dimensional ultra-weak photon imaging. The spontaneous ultra-weak photon emission was detected in intact cells while it was found to be comparatively higher in disrupted cells. To study the involvement of lipid peroxidation in ultra-weak photon emission, linoleic acid was exogenously applied. When linoleic acid was added to the intact cells, an enhancement in the ultra-weak photon emission was observed [Fig. 12C; Figure 13 (Left panel, A, trace b)]. Similarly, the addition of linoleic acid to the disrupted cells caused a significant increase in the two- ultra-weak photon emission [Fig. 12D; Figure 13 (Left panel, B, trace b). Based on these observations, it is concluded that the oxidation of linoleic acid results in the enhancement in ultra-weak photon emission.

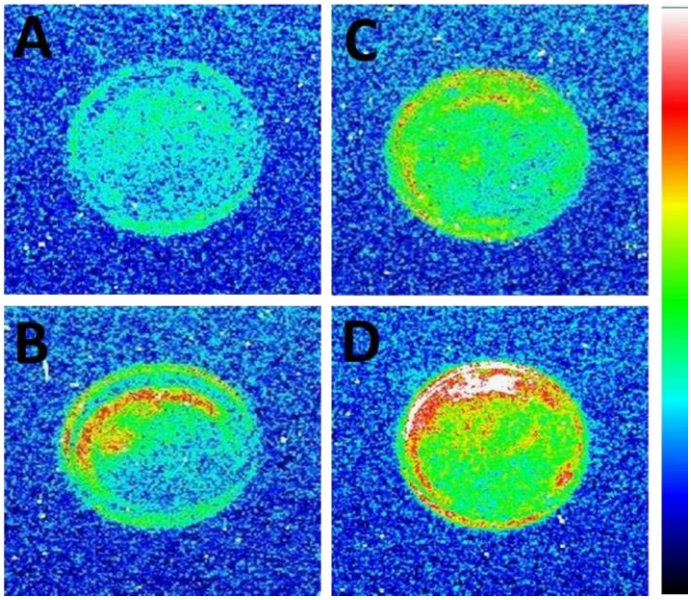


Figure 12: Two-dimensional ultra-weak photon emission imaging of the intact (A, C) and the disrupted (B, D) cells measured in the absence (A, B) and the presence (C, D) of linoleic acid. Ultra-weak photon emission imaging was measured using a highly sensitive CCD camera with an integration time of 30 min.

5.1.2 Effect of scavenger, inhibitor and molecular oxygen

5.1.2.1 Role of hydroxyl radical in lipid peroxidation

Lipid peroxidation is known to be initiated by the abstraction of H-atom from the fatty acid. For instance in linoleic acid, two unsaturated bonds are present between C9-C10 and C12-C13 carbons. The process is known to be initiated by the $HO^{\bullet}$ and to confirm its involvement in lipid peroxidation and ultra-weak photon emission, linoleic acid was added in the presence of mannitol, a $HO^{\bullet}$ scavenger. The observation clearly indicates the suppression of ultraweak photon emission and lead to the conclusion that $HO^{\bullet}$ is involved in the ultra-weak photon emission [Figure 13 (Right panel, A, trace b)].

5.1.2.2 Inactivation of lipoxygenase

Lipoxygenase is an iron containing enzyme that catalyzes the deoxygenation of fatty acids. To understand the mechanism of lipid peroxidation and ultra-weak photon emission in details, the effect of catechol, an inhibitor of lipoxygenase, on ultra-weak photon emission was studied. The addition of catechol to the disrupted cells resulted in the significant suppression in the ultra-weak photon emission [Figure 13 (Right Panel, B, trace b)]. These observations reveal that the oxidation of linoleic acid by intrinsic lipoxygenase is involved in the ultra-weak photon emission.

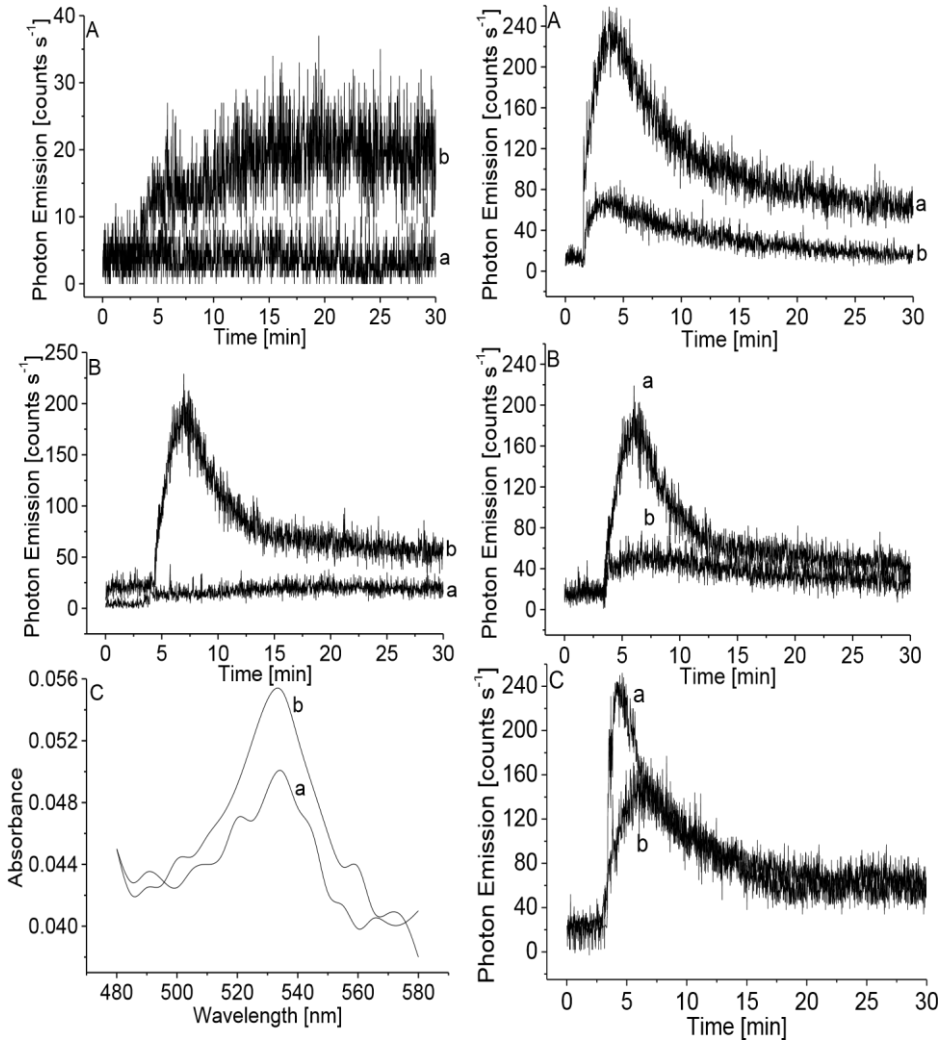


Figure 13: Left panel, Effect of exogenous addition of linoleic acid on the ultra-weak photon emission from the intact (A) and the disrupted cells (B), Absorption difference spectra of the $(TBA)_2$-MDA adduct measured in the cells in the intact (trace a) and the disrupted cells (trace b) (C). Absorption difference spectrum represents the absorption spectrum of the $(TBA)_2$-MDA adduct obtained before and after the addition of linoleic acid. **Right panel**: Effect of mannitol (A), catechol (B) and molecular oxygen (C) on the ultra-weak photon emission from the disrupted cells measured in the presence of linoleic acid. Ultra-weak photon emission was observed in the absence (trace a) and the presence of mannitol, catechol and removal of molecular oxygen (trace b).

5.1.2.3 Role of molecular oxygen in lipid peroxidation

To test the involvement of molecular oxygen in the lipid peroxidation and ultra-weak photon emission, the photon emission was measured after elimination of molecular oxygen using glucose/glucose oxidase enzyme system. Upon removal of molecular oxygen from the cell system, the ultra-weak photon emission was considerably suppressed which shows that

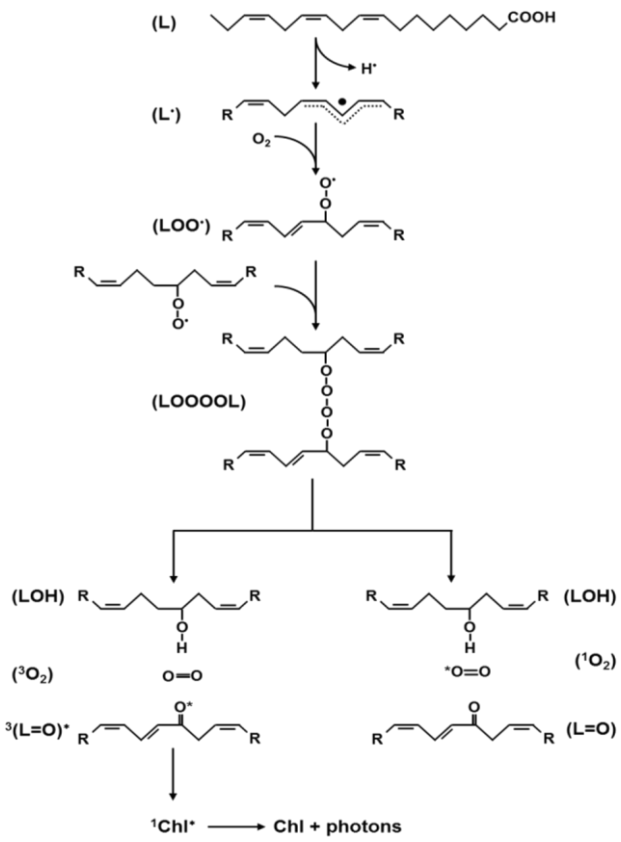
molecular oxygen participate in the ultra-weak photon emission [Figure 13 (Right panel, C, trace b)].

5.1.2.4 Determination of lipid peroxidation product

The decomposition of lipid peroxides results in the formation of MDA, which reacts with thiobarbituric acid forming $(TBA)_2$-MDA adduct**.** We performed spectroscopic detection of TBARS to monitor the formation of lipid hydroperoxides in the both intact and disrupted cells (Pryor and Castle, 1984; Halliwell and Gutteridge, 2007). The absorption spectrum of $(TBA)_2$-MDA adduct obtained in the disrupted cells cells [Figure 13 (Left panel C, trace b)] was found to be higher as compared to the control [Figure 13 (Left panel C, trace a)]. Moreover, it is evident from the absorption spectra that the enhancement in absorbance at 532nm observed in the disrupted as compared to the intact cells led to the conclusion that accumulation of MDA is higher in case of disrupted cells.

5.1.3 Mechanism of lipid peroxidation and formation of electronically excited species

The oxidation of linoleic acid results in the formation of $L^{\bullet}$ and in the presence of molecular oxygen forms $LOO^{\bullet}$. Series of non-enzymatic and enzymatic reactions finally leads to the formation of electronically excited species. The excited species formed in case of *Chlamydomonas reanhardtii* cells was found to be from $^3(R=O)^*$, in which excitation energy was transferred to chlorophyll molecules (Scheme VI). The spectral evaluation shows that the ultra-weak photon emission is mainly contributed by the chlorophyll molecules and partially by singlet oxygen dimol emission (Article I).



Scheme VI: Mechanism of generation of electronically excited species by oxidation of polyunsaturated fatty acids.

5.2 Role of ROS in ultra-weak photon emission from human skin (Article II)

5.2.1 Imaging of spontaneous ultra-weak photon emission from human skin

Two- and one-dimensional spontaneous and ROS-induced photon emission from human body parts and hands were measured. The spontaneous imaging of photon emission was measured from the dorsal and the palmar side of the human hand where a high emission was observed from the palmar side of the hand (Fig. 3, Article II). The observation that photon emission from the dorsal side is higher reveals that oxidative metabolic process is responsible for ultra-weak photon emission. Besides the hand, photon emission measured from different parts of the body shows the clearly evident contour of the human body. A clear difference in photon emission was observed from the different parts of the body (Fig. 2, Article II). The photon emission from the face and the forehead was larger compared to the eye socket, where almost no photon emission was observed.

5.2.2 Two-dimensional imaging and one-dimensional emission of ROS-induced ultra-weak photons from human skin

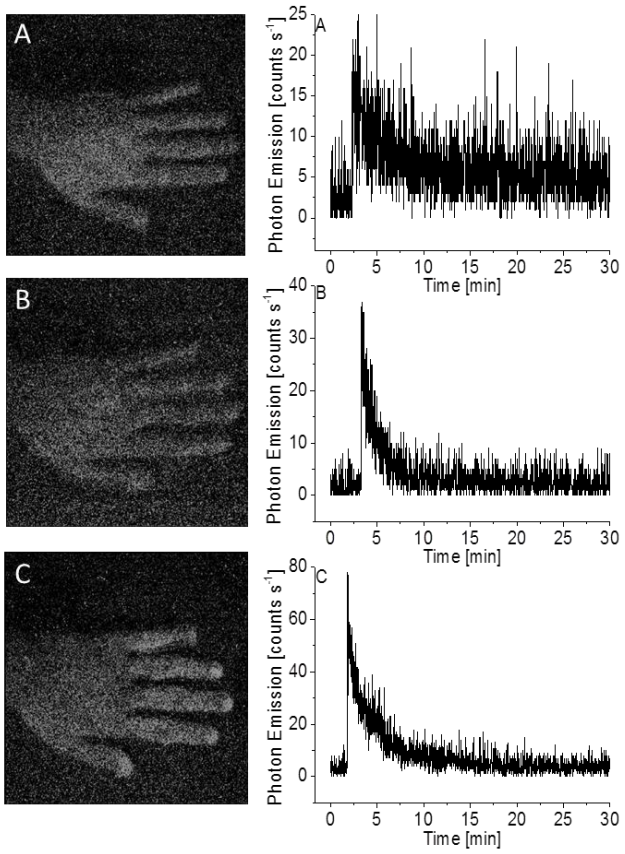


Figure 14: Left panel: Two-dimensional imaging of the ROS-induced ultra-weak photon emission from the dorsal side of the hand. Superoxide anion radical (A), hydrogen peroxide (B) and hydroxyl radical (C) -induced ultra-weak photon emission measured with an integration time of 30 min. **Right panel**: One-dimensional ROSinduced ultra-weak photon emission from the dorsal side of the hand. Superoxide anion radical (A), hydrogen peroxide (B) and hydroxyl radical (C) -induced ultra-weak photon emission measured with an integration time of 30 min.

To test the involvement of ROS in the ultra-weak photon emission, the effect of exogenous ROS ($O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$) on the one- and two-dimensional ultra-weak photon emission was analyzed on the dorsal side of the hand. Superoxide anion radical was generated by the application of xanthine oxidase and xanthine. When xanthine/xanthine oxidase system was topically applied on the dorsal side of the hand, an enhancement in two- and one- dimensional ultra-weak photon emission was observed (Fig. 14, Left panel, A; right panel, A). Similarly, the topical application of H_2O_2 on the dorsal side of the hand resulted in an increase in the

two- and one-dimensional ultra-weak photon emission (Fig. 14, Left panel, B; right panel, B). When $HO^{\bullet}$ generated by Fenton reagent system was applied on the dorsal side of the hand, an increase in the two- and one-dimensional ultra-weak photon emission was observed (Fig. 14, Left panel, C; right panel, C). Based on these observations, it is concluded that the topical application of ROS on the human skin results in an enhancement in ultraweak photon emission. To determine whether subsequent mixing of xanthine oxidase and xanthine forms $O_2^{\bullet-}$ and H_2O_2 with transition metals forms $HO^{\bullet}$, EPR spin-trapping technique was used (refer chapter 4, Fig. 10).

5.3 Photosensitization in human skin (Article III)

The current study deals with the role of visible light and UVA radiation in the generation of ROS in the human skin. The generation of ROS is claimed to be originated via protoactivated skin chromophores via photosensitization reactions. A comparative analysis on the dorsal and the palmar side was performed utilizing one- and two-dimensional ultra-weak photon emission.

5.3.1 Visible light and oxidative stress

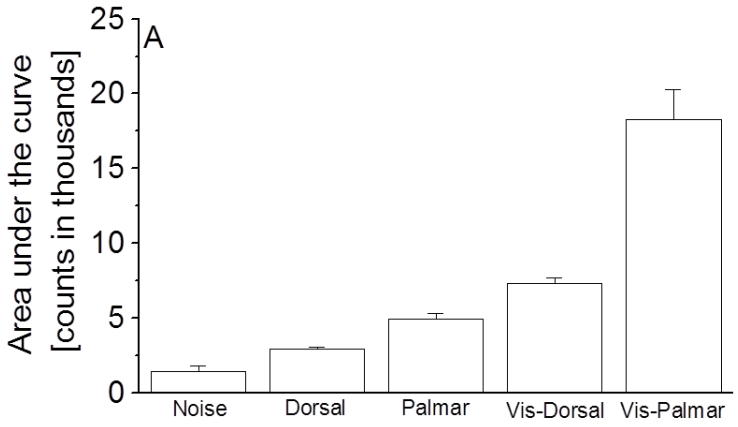


Figure 15*:* Efficacy of the visible light on ultra-weak photon emission from the dorsal and the palmar side of the hand. The Y-axis represents the area under the curve (counts in thousands) obtained with the visible light exposure. The presented data are expressed as the mean value and the standard deviation of at least three measurements (mean ± SD, n=3).

The irradiation of the dorsal side and the palmar of the hand to visible light bear the potential to generate oxidative stress in the human skin. The ultra-weak photon emission detected after the exposure of human hand to visible light was found to be higher than the spontaneous

emission (Fig 2&4, Article III). The chromophores such as melanins and bilirubin which are present in the skin are known to absorb in the wavelength range of 300-600 nm. However, these pigments are capable of initiating photosensitization reaction which leads to the formation of ROS via Type I and Type II photosensitization reaction.

5.3.2 UVA radiation and oxidative stress

The photon emission observed from the UVA-irradiated dorsal and the palmar side of the hand was found to be higher than the unexposed sides and visible light exposed surface of the hand indicating that UVA generates higher oxidative stress in the human skin. The reason being that other chromophores, besides melanin and bilirubin such as urocanic acid (250300nm), porphyrins (320-400 nm), flavins (345-375 nm) and pterins (345-375 nm) are found to absorb mainly in the UV region of the spectrum. Thus, the photosensitization reaction can be proposed to be responsible for higher emission of ultra-weak photon emission via type I and type II reactions. The large amount and varied chromophores absorbing in the UV region is believed to be the responsible factor for higher level of ultra-weak photon emission.

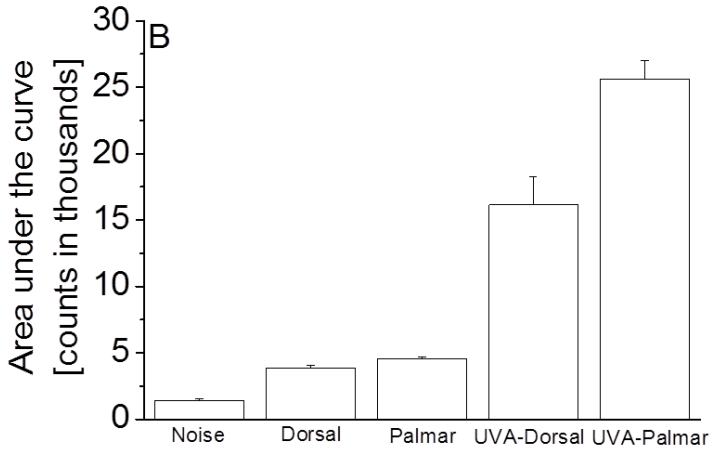


Figure 16*:* Efficacy of the UVA irradiation on ultra-weak photon emission from the dorsal and the palmar side of the hand. The Y-axis represents the area under the curve (counts in thousands) obtained on the visible light exposure. The presented data are expressed as the mean value and the standard deviation of at least three measurements (mean ± SD, n=3).

5.4 Charge couple device imaging in microbial, plant and medical research (Article IV)

This work provides a detailed analysis of measuring parameters such as accumulation time and binning with the aim to achieve good quality images of two-dimensional ultra-weak photon emission.

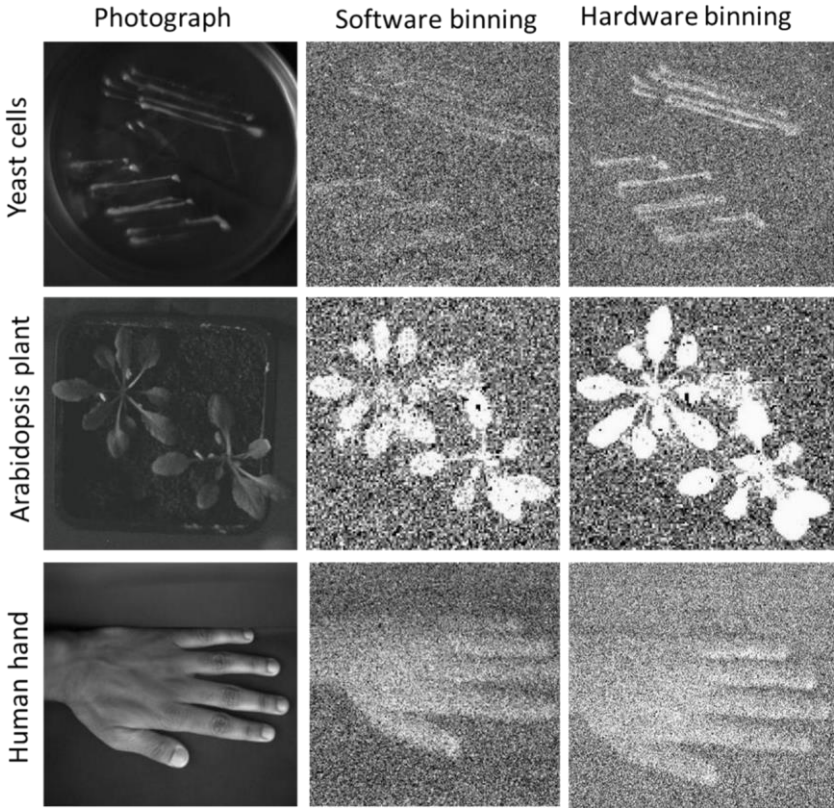


Figure 17: Two-dimensional imaging of spontaneous ultra-weak photon emission from *Saccharomyces cerevisiae* (A), *Arabidopsis thaliana* (B) and human hand (C) measured with software and hardware binning mode of 4×4 and accumulation time of 30 min.

5.4.1 Ultra-weak photon emission imaging from microorganism

The two-dimensional imaging of ultra-weak photon emission was measured in the microorganism for the first time with evaluation of different measuring parameters in order to achieve two-dimensional images of ultra-weak photon emission.

The detailed analysis shows that with an accumulation time of 30 min with application of no binning mode, ultra-weak photon emission from *S. cerevisiae* cells is not detectable (Fig. 1, Article IV). For obtaining an image of good quality in microbes, a minimum accumulation time of 30 min with binning mode with a binning factor of 4×4 is required (Figure 17). The low photon emission is microorganism is suggested to be because of lower cell density on the petriplate and low pigment content.

5.4.2 Ultra-weak photon emission imaging from plants

Two-dimensional ultra-weak photon emission was measured from *Arabidopsis thaliana* with varied accumulation time and binning mode. The ultra-weak photon emission with an accumulation time of 30 min was found to be enough for visualization of the ultra-weak photon emission (Fig.3, Article IV). With the application of software and hardware binning mode, the intensity of image was considerably enhanced. Based on the evaluation, it was concluded that for two-dimensional imaging of ultra-weak photon from plants, a minimum accumulation time of 10 min with a binning factor of 4×4 is required (Fig. 4, Article IV) while 30 min of accumulation time with 4×4 binning provided an high intensity photon emission image (Fig. 17). The cell density is higher in plants than that of microorganism (in culture plate) and this could a possible reason of high emission in plants. The pigments in plants such as chlorophyll are known to be the absolute emitters of ultra-weak photons upon receiving excitation energy from triplet excited carbonyls formed as a product of oxidative metabolic processes. The emission is also contributed by singlet oxygen dimol emission.

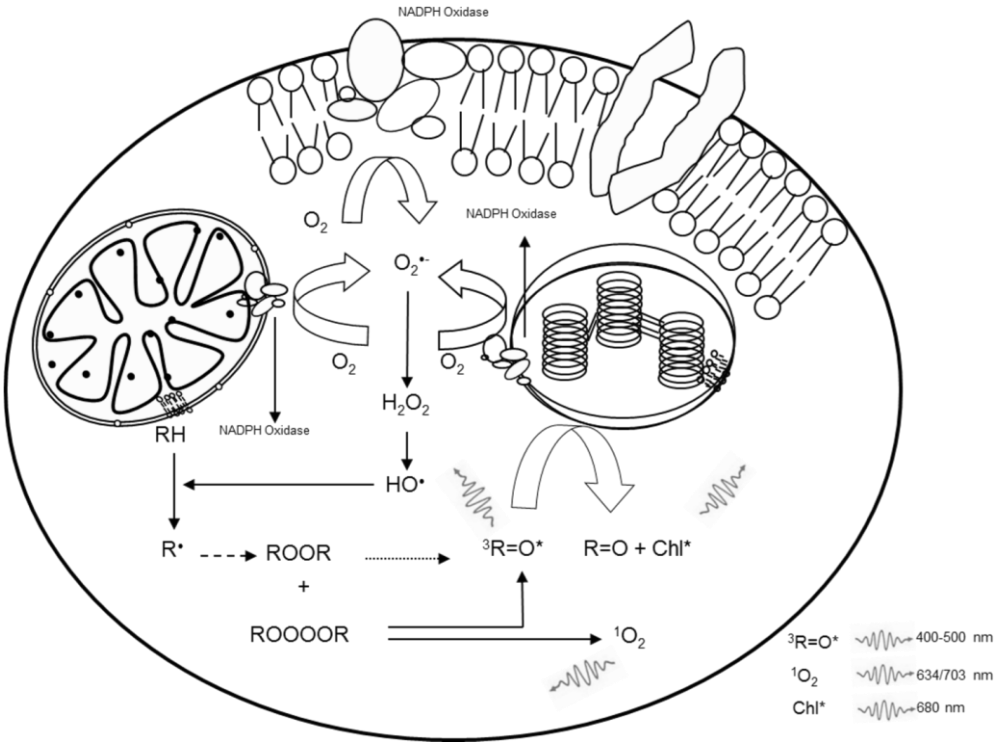
5.4.3 Ultra-weak photon emission imaging from human hand

Two-dimensional imaging of ultra-weak photon emission was measured from the dorsal side of the human hand with varied accumulation time and binning mode. The image obtained shows that accumulation time of 30 min is required to generate an ultra-weak photon emission image of human hand (Fig.5, Article IV). Upon application of hardware and software binning mode, a good quality image can be obtained at an accumulation time of 20 min with a binning factor of 4×4 (Fig.6, Article IV) while 30 min of accumulation time with 4×4 binning provided an comparatively high intensity photon emission image (Fig. 17). The predominant pigments in the human skin such as melanin and bilirubin have been demonstrated to absorb in both visible and UV region of the spectrum and it is proposed that emission from human hand originates from the $^3(R=O)^*$, $^1O_2$ and singlet excited melanin and bilirubin.

5.5 Photon source within the cell (Article V)-Review article

The current review attempts to focus on the current knowledge and advancement on the involvement of ROS-induced oxidation of biomolecules in the biological systems. Reactive oxygen species are known to oxidize electron-rich organic molecules including lipids, proteins and nucleic acids. The oxidation of lipids initiated by radical ROS ($O_2^{\bullet-}$, $HO_2^{\bullet-}$and $HO^{\bullet}$), non-

radical ROS ($^1O_2$, H_2O_2) or by enzymatic reaction pathway (lipoxygenase) is known to form $LOO^{\bullet}$ and further, combination and recombination via Russell mechanism is known to form reactive intermediates. The reactive intermediates decomposes to the triplet $^3(R=O)^*$ and $^1O_2$. The review attempt to highlight the research on involvement of ROS in the formation of excited molecules thereby leading to ultra-weak photon emission. The source of ultra-weak photon has been discussed with emphasis particularly on microbes, plants and animals.



Scheme VII: A model showing the involvement of reactive oxygen species (ROS) in ultra-weak photon emission in different organelles of the cell. Superoxide anion radical ($O_2^{\bullet-}$) is produced via membrane-bound enzyme complex NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) embedded within the plasma membranes and membranes of various organelles such as mitochondria and chloroplast. The dismutation of $O_2^{\bullet-}$ is accompanied by the formation of hydrogen peroxide (H_2O_2) and further hydroxyl radical ($HO^{\bullet}$) via fenton reaction. The hydroxyl radical being highly reactive has the capability to damage all types of macromolecules such as lipids, proteins and nucleic acids. The oxidation of the biomolecules is accompanied by the formation of reactive intermediates such as dioxetanes (ROOR) and tetraoxide (ROOOOR) which further upon decomposition generates electronically excited species.

Chapter 6

Conclusion

Following conclusions are made on the basis of obtained experimental results (Articles I-V):

1. In photosynthetic organisms, oxidation of lipid molecules by ROS results in the ultra-weak photon emission. Electronically excited species such as $^3(R=O)^*$ are the candidates for the primary excited species formed during the lipid peroxidation, whereas chlorophylls are established as final emitters of ultra-weak photon emission. Our results indicate that the ultraweak photon emission can be used as a non-invasive method for the detection of lipid peroxidation in the cell membranes or organism as a whole.
2. In non-photosynthetic organism, the role of ROS and its degree of damage was analyzed. Based on this, it is concluded that ROS in the order $H_2O_2 < O_2^{\bullet-} < HO^{\bullet}$ bears the potential to oxidize the biomolecules. The data obtained shows that ultra-weak photon emission is a potential method for analysis of physiological and pathological state of the organism. Besides, it can be applied in assessment of metabolic processes in humans which reflect the state of the organism. Therefore, it can also be applied in clinical studies viz. diagnosis and rather pre-diagnosis of ailments like skin cancers. The extreme advantage of the technique is that it is non-invasive and without any probes.
3. The quantitative analysis of ultra-weak photon emission generated by visible light and UVA radiation on the human hand provided information on the degree of oxidative damage in the human skin including the viable epidermis and dermis. This suggest that, the application of ultra-weak photon emission for monitoring the effect of UVA stress on the human skin is highly important for the development of effective photoprotective agents in the human skin against UVA radiation and determination of dose of light utilized in photodynamic therapy.
4. Analysis of measuring parameters provided a detailed account of standard condition to be used for two-dimensional spontaneous ultra-weak photon imaging in microbes, plants and animals.
5. The ultra-weak photon emission can be used as a non-destructive tool in *in-vivo* studies and can be of great importance in quality control in food industry. The use of ultra-weak photon emission as a non-invasive diagnostic tool for monitoring of biomolecules oxidation helps to better understand the mechanistic insights into the response of organism to the numerous abiotic and biotic stresses. Information on the kinetics of ultra-weak photon emission enables to follow the temporal characteristics of the response to the environmental stress factors in all kind of living system.

Chapter 7

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Chapter 8

Publications

Chapter 9

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