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REVIEW

Clastogenic ROS and biophotonics in precancerous diagnosis

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BACKGROUND: Cancer is the leading cause of death worldwide. The application of biophotonics for diagnosing precancerous lesions is a major breakthrough in oncology and is associated with the expression of clastogenic bio-markers, such as reactive oxygen species (ROS), namely, superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, and lipid peroxidation products. These ROS are the major sources of ultra-weak biophotons emission; in addition, biophotons are emitted from other biomolecules, which are not associated with ROS. The precancerous phase is diagnosed on the basis of biophoton emission from biomarkers. The type of biophotons emitted depends on the structure of the clastogenic ROS.
METHODS: ROS-based emission of ultra-weak photons can be detected using charge coupled device (CCD) cameras and photomultiplier tubes. Furthermore, spectroscopic and microscopic analysis can yield more advanced and definite results.
RESULTS: The frequency and intensity of biophoton emission associated with each ROS provides information regarding the precancerous phase. Previous have attempted to show an association between precancerous growth and biophoton emission; however, their results were not conclusive. In this review, we have addressed multiple aspects of the molecular environment, especially light- matter interactions, to derive a successful theoretical relationship which may have the ability to diaganose the tumor at precancerous stage and to give the solutions of previous failures. This can be a major quantum leap toward precancerous diagnosis therapy.
CONCLUSION: Biophotonics provides an advanced framework, for easily diagnosing cancer at its preliminary stage. The relationship between biophotons, clastogenic factors, and biochemical reactions in the cellular microenvironment can be understood successfully. The advancement in precancerous diagnosis will improve human health worldwide. The versatility of biophotonics can be used further for novel applications in biology, biochemistry, chemistry and social fields.

Keywords biophotons, CCD camera, molecular environment, oncology, precancerous, photomultiplier, ROS

Introduction

Cancer, the leading cause of mortality worldwide, is characterized by evasion of normal cell cycle and aberrant proliferation as cells become insensitive to the cell cycle check points (Bozzone, 2007; Hanahan and Weinberg, 2011). Recent studies have identified hallmarks for defining the cancerous state, such as sustenance of proliferative signals, resistance to cell death, induction of angiogenesis, evasion of growth suppressors, cancer cell invasion and metastasis, and immortality (Bozzone, 2007; Cao, 2010; Coghlin and Murray, 2010). In addition, certain emerging hallmarks of cancer are deregulation of cellular energetics, avoidance of immune-mediated destruction, tumor-promoting inflammation, genome instability, and accumulation of mutations (Hanahan and Weinberg, 2011). At the molecular level, cancer is caused by lethal mutations in tumor suppressor genes and cell cycle regulatory genes that convert them into oncogenes (Bozzone, 2007; Cheng et al., 2008; Ciccia and Elledge, 2010).

The term "precancerous" defines the prevailing cellular environmental conditions before a cell becomes cancerous. In fact, the precancerous environment varies with cancer types. However, this review will specifically discuss the role of reactive oxygen species (ROS) in the molecular environment of cancer cells (Day et al., 1997; Bozzone, 2007; Dinh, 2010) and their utility in diagnosing ROS (superoxides, peroxides, singlet oxygen, peroxynitriles, excited carbonyls of biomolecules, hydrogen peroxide, and hydroxyl radicals)-associated precancerous tumors (Day et al., 1997; Klaunig et al., 1997; Lorch et al., 2002; Chiarugi, 2008; Liebel et al., 2012b) Although ROS production primarily occurs during chronic inflammatory diseases, they are also generated during

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precancerous environmental changes and stress (Emerit, 1994; Marnett, 2000a; Cooke et al., 2003; Valko et al., 2004; Emerit, 2007; Lindholm et al., 2010). Moreover, precancerous and inflammatory states can be distinguished on the basis of ROS-related oxidative damage and ROS physiology (Wiseman and Halliwell, 1996a; Van Wijk et al., 2008).

The role of ROS in cancer onset and progression has already been elaborated (Daniel et al., 1994; Deshpande et al., 2002; Ciccia and Elledge, 2010; Coghlin and Murray, 2010; Floryszak-Wieczorek et al., 2011; Ferrari and Quaresima, 2012). Essentially ROS act as clastogenic factors, which mutate nucleic acids and cause chromosomal breakage by activating nucleases (Emerit, 1994; Marnett, 2000a; Cooke et al., 2003; Valko et al., 2004; Emerit, 2007; Lindholm et al., 2010). ROS also affect proteinase function (Nelson and Melendez, 2004). Generally, specific quantities of intracellular ROS are produced for performing critical functions related to cell physiology, cell signaling, and metabolism (Chiarugi, 2003; Liu et al., 2003; Kamal and Komatsu, 2015). ROS is also produced during cellular energy production through the electron transport chain (ETC) (Davies, 2001; Inoue et al., 2003; Chiarugi, 2008; Liebel et al., 2012a; Tulah and Birch-Machin, 2013; Kamal and Komatsu, 2016). However, excess ROS are generated during stress conditions (such as inflammation), which in turn initiate the oxidative breakdown of biomolecules, such as lipids, proteins, and nucleic acids (Daniel et al., 1994; Emerit, 1994; Griendling and FitzGerald, 2003; Dotta et al., 2011). This increase in ROS production and other ROS-related reactions leads to the generation of certain harmful by-products (Davies, 2001; Ballardin et al., 2004; Wang and Wu, 2007). As clastogenic factors, ROS can mutate DNA (Feig et al., 1994; Emerit, 1994; Lindholm et al., 2010) at specific positions (C to A and G to A substitutions by pol-alpha, and C to A, G to A and C to T substitutions, as well as deletions of C, by pol-β).

DNA mutations can trigger tumor formation (Wiseman and Halliwell, 1996b; Ballardin et al., 2004). Therefore, those clastogenic factors that show an increase in their levels in the precancerous phase can be considered biomarkers for that phase (Marnett, 2000a; Dizdaroglu et al., 2002; Lindholm et al., 2010; Chen et al., 2012). The clastogenic biomarkers can be hydrogen peroxide, hydroxyl radicals, ozone, peroxynitriles, lipid peroxidation products, and malondialdehyde (MDA) (Feig et al., 1994; Davies, 2001; Wang and Wu, 2007). The determination of precancerous phase depends upon the light-matter interactions of these biomarkers, which are described primarily by the semi-classical theory of quantum mechanics (Saleh et al., 1992; Wang and Wu, 2007; Van Wijk et al., 2008; Kobayashi et al., 2009; Dinh, 2010). Light absorption, diffraction, and emission are different types of light-matter interactions. Since a single packet of electromagnetic waves (light) is called a photon, the light emitting from biological systems is termed biophoton. Different phenomena are involved in biophoton emission, such as ultra-weak biophoton emission, fluorescence, and chemiluminescence. These biophoton-related light emission phenomena are collectively studied as “Biophotonics”(Van Wijk et al., 2008; Kobayashi et al., 2009; Dinh, 2010).

Recent advanced studies demonstrate dominant involvement of ROS in ultra-weak biophoton emission during cancer onset and progression (Wiseman and Halliwell, 1996a; Takedaa et al., 1998). According to the semi-classical theory, ROS are electromagnetically excited species that emit biophotons of specific wavelengths. Evidences show the involvement of biomolecules and cellular structures such as DNA, cell membrane, and proteins (microtubules) in biophoton emission (Cohen and Popp, 2003; Deriu et al., 2010; Dotta et al., 2011). Interestingly, ROS generation renders these biomolecules and structures capable of biophoton emission. The concentrations of specific clastogenic factors (especially hydroxyl and hydrogen peroxide radicals, as these are involved in direct oxidative mutagenic DNA damage) and their relationship with specific biomolecule oxidation or damage can differentiate the precancerous stage of tumors from the normal stage (Wiseman and Halliwell, 1996a). Production of excess ROS is strongly associated with high intensity biophoton emission in the precancerous stage of cancer onset and progression (Cohen and Popp, 2003). Biomarkers other than ROS can also emit light in the form of fluorescence and chemiluminescence after absorbing light or upon application of specific luminescent wavelengths (Saleh et al., 1992; Wang and Wu, 2007; Van Wijk et al., 2008; Kobayashi et al., 2009; Dinh, 2010).

Several techniques have been developed to study biophoton emission from specific organs or cells. Ultra-weak biophoton emission can be studied using charge coupled cameras (CCD cameras) and photomultiplier tubes (Saar et al., 2010; Prasad and Pospisil, 2012; Suhalim et al., 2012). Fluorescence emissions and other types of biophoton emission can be recorded using spectroscopic and microscopic techniques such as vibrational spectroscopy, simple fluorescence microscopy, fluorescence resonance energy transfer imaging (FRET), and fluorescence lifetime imaging microscopy (FLIM) (Saleh et al., 1992; Wang and Wu, 2007; Saar et al., 2010).

Currently, precancerous and real-time diagnosis of tumor is a difficult task; therefore recovery from terminal phases of cancer is challenging. Although production of ROS and related biomarkers was investigated before the discovery of biophotons, application of biophotonics for studying these biomarkers was limited. Several studies have determined the relation between ROS, biophoton emission, and cancer development (Takedaa et al., 1998; Cohen and Popp, 2003); however, majority of them were unsuccessful because the physical aspects of intra and extra cellular environments were neglected and specific biochemical markers for confirmation were lacking. Second, sole dependence on biochemical techniques instead of physical examinations was another drawback (Sauermann et al., 1999; Van Wijk et al., 2006a;

Cifra et al., 2010). Currently, biophotonics has eased biomedical research on the micro and macro biochemical and biophysical environments of cells, which involves studies on different types of molecules and biochemical reactions participating in cancer onset (Gartel and Radhakrishnan, 2005; Burhans and Heintz, 2009). Therefore, emission of biophotons with respect ROS/ROS-related biomolecule production can be used to predict the probability of tumorigenic transformation. Analysis of the relationship between various types of mutation with specific ROS has enabled determination of the above-mentioned probability.

In this review, the patterns of biophoton emission according to precancerous physiology and the biochemical reactions involving ROS and ROS by-products have been discussed with the aim of diagnosing precancerous lesions. This can be a quantum leap in an era where tumor-related changes can be predicted beforehand, enabling designing of correct therapies at the right time, which can be instrumental in saving innumerous lives.

The molecular basis and telltale signs of cancer

Recent studies show that biophotons play a crucial biophysical role in cell division regulation (Alipour, 2015). Several genes participate in this tight regulation, defects or mutations in which may trigger the onset of tumorigenesis (Bozzone, 2007; Cheng et al., 2008; Ciccia and Elledge, 2010). These genes are divided into different categories, of which seven are most important. First, genes encoding **signal molecules** that initiate cell division (Cheng et al., 2008) by inducing intracellular signaling cascades leading to the transcription of downstream effector genes (Bozzone, 2007). The signal molecules bind to the second category of **receptor molecules** either on the cell surface or inside the cell (cytoplasm) depending on the type of cell and signaling pathway (Coghlin and Murray, 2010; Hanahan and Weinberg, 2011). The third category includes genes encoding **signal transducers**, which transduce the information generated by binding of signaling molecules to the receptors to produce effector proteins (secondary messengers) that amplify and relay the information in a cascade (Bozzone, 2007; Coghlin and Murray, 2010; Hanahan and Weinberg, 2011). The fourth category comprises genes encoding **transcription factors** (Bozzone, 2007; Hanahan and Weinberg, 2011), which regulate gene expression and thereby cellular behavior in the context of the internal molecular environment, for example, decisions regarding whether a cell will divide or remain quiescent (Wiseman and Halliwell, 1996b; Cheng et al., 2008). The fifth category is composed of genes encoding **apoptotic proteins** that initiate and execute cell death (Ciccia and Elledge, 2010). The sixth category contains genes that **directly regulate cell division**. Finally, the seventh category comprises genes encoding **DNA damage repair proteins** (Ciccia and Elledge, 2010; Coghlin and Murray, 2010). Under normal conditions, all these groups work efficiently to regulate cell functioning and division, whereas in cancer cells, reciprocal functions of these groups cause uncontrolled division, proliferations, and other effects (Bozzone, 2007; Hanahan and Weinberg, 2011). This is caused by mutations in these molecules, which may convert normal cells into cancerous ones with certain hallmarks of cancer (Hanahan and Weinberg, 2011) (Fig. 1).

Precancerous chemical environment of ROS induces cancer:

The precancerous chemical environment of cells involves chemical and metabolic changes in cells prior to their transformation into the cancerous stage. Actually, the precancerous environment varies with cancer types such as lung cancer, brain tumors, breast cancer, pituitary carcinomas etc. (Wiseman and Halliwell, 1996b; Motohiro Takedaa et al., 1998). Similarly, the micro-environment of cancerous cells also varies with cell type (Beckman et al., 2000). ROS are associated with tumorigenesis under stress conditions (Emerit, 1994; Storz, 2005; Lau et al., 2008; Kobayashi et al., 2009) and play critical roles in tumor progression (Shukla et al., 2003). Hence, the concentration of ROS and its by-products (superoxides, peroxides, singlet oxygen, triplet oxygen, peroxynitriles (Lorch et al., 2002) excited carbonyls of biomolecules) is high in precancerous states and cancers known to be triggered by ROS (Feig et al., 1994; Davies, 2001; Emerit, 2007; Lindholm et al., 2010).

In normal cells, apoptosis is triggered by extended exposure and/or high levels of ROS (Chiarugi, 2003). A family of proteins is taken into the light for the blockage of cell cycle under abnormal conditions (Kops et al., 2002; Shukla et al., 2003; Kamal and Komatsu, 2016). In cycling cells, p21 is activated to counter oxidative stress and arrest cell cycle progression. Similarly, p27 production leads to G1 phase arrest. p53 and p21 induce dephosphorylation of retinoblastoma (RB) in the presence of oxidants in cycling cells (Vafa et al., 2002). Exposure to oxidants such as H_2O_2 or nitric oxide also results in dephosphorylation of RB, which is the freewheel of p53 or p21 (Gartel and Radhakrishnan, 2005; Burhans and Heintz, 2009). In either case, cells are arrested in the S-phase. Expression of p27 is partially controlled by the **Foxo** transcription factors that are well known for controlling genes involved in cell cycle progression, and metabolism and oxidative stress response (Kops et al., 2002). For example, mitogenic stimulation by the **PI3K/Akt** pathway sequesters **Foxo3a** in the cytoplasm, but in the absence of stimulation, Foxo3a localizes to the nucleus and upregulates genes for oxidative metabolism and cell cycle arrest, such as p27 (Dizdaroglu et al., 2002; Cooke et al., 2003; Valko et al., 2004). Under certain conditions, **Foxo3a** can directly increase BIM expression and promote apoptosis. Thus, Foxo3a enhances cell survival of cycling cells exposed to oxidative stress by enabling a stress-mediated survival cascade, but induces cell death under other conditions. Non-cycling cells such as neurons also acquire mechanisms to adapt to

oxidative stress, which involve **Foxo3a** (Kops et al., 2002). Glutathione (the intracellular concentration of which is in the mM range) is part of an important nonenzymatic antioxidative mechanism that detoxifies peroxides and regenerates a number of significant antioxidants (e.g. α-tocopherol and ascorbic acid). Reduced glutathione (GSH) is regenerated from its oxidized form (GSSH) by the action of a NADPH dependent reductase as shown below (Benhar et al., 2002; Nelson and Melendez, 2004; Storz, 2005; Valkoa et al., 2006; Halliwell and Gutteridge, 1985)(Eq. (1))(Held, 2015).

$$GSSH + NADPH + H^{+} \rightarrow 2GSH + NADP^{+} \quad (1)$$

ROS production from inflammatory diseases can trigger tumor development

Chronic inflammatory diseases are also linked to ROS production (Wiseman and Halliwell, 1996b; Marnett, 2000b; Valko et al., 2004). However, since short-term inflammation does not produce significant amounts of ROS (Sauermann et al., 1999), ROS sources leading to cancer need not be differentiated from inflammation (Valko et al., 2004). On the contrary, several studies have demonstrated that chronic inflammation is one of the major causes of cancer because of increased ROS production during inflammation (Daniel I. Feig et al., 1994; 1998). At normal ROS concentrations, one DNA base per 130 000 bases can be oxidized; however, owing to increased ROS production during inflammation, DNA oxidation levels are enhanced, which may lead to cancer. Studies showed that breast, liver, kidney, and lung cancers were strongly associated with inflammatory diseases (Wiseman and Halliwell, 1996b). In colon cancer, the predisposing sources of chronic inflammation include ulcerative colitis and infection with the parasite *Schistosoma japonicum* (Sauermann et al., 1999). Another study observed a strong relationship between the ROS-release stimulatory capacity of tumor promoters in inflammatory cells and their tumor-forming ability. In addition, inflammation indicators such as tumor necrosis factors and different cytokines have been identified in blood during tumor development (Feig et al., 1994; Klaunig et al., 1997; 1998).

Confirmatory tests for differentiating between ROS-mediated DNA damage in ordinary inflammatory disease and the precancerous environment is important as these will also act as biomarkers of the precancerous environment but not for inflammation. In addition, damage to nucleases and polymerases at specific ROS concentrations can be recorded to establish standards for determining precancerous conditions in further studies. The biomarkers associated with tumorigenesis are discussed under a separate heading below.

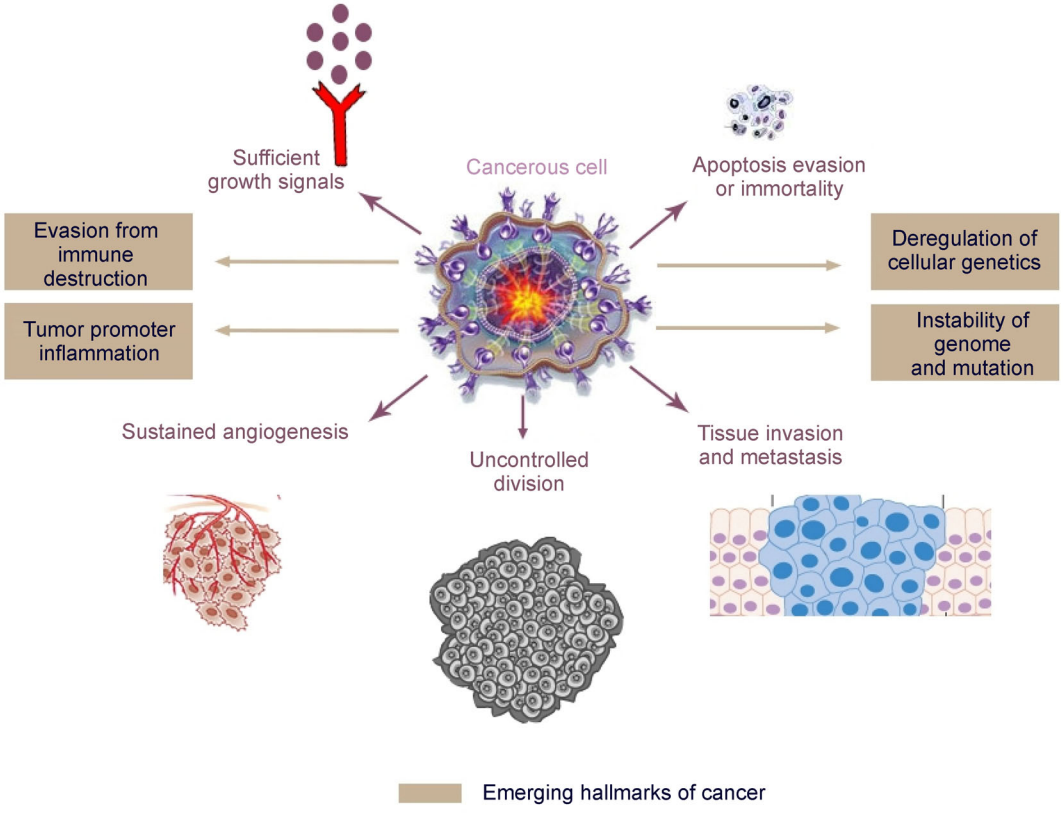


Figure 1 Major signs of cancer cells. The emerging hallmarks of cancerous cells can provide detailed information about specific stages of cancer.

Lipid peroxidation, an important oxidative stress indicator leading to tumorigenesis

Lipid peroxidation is an important indicator of oxidative stress and is one of the most broadly used molecule for determining free radical formation (Feig et al., 1994). Unsaturated fatty acids such as those present in cellular membranes are common free radical targets. Typically, it involves a chain reaction, where a free radical reacts with a hydrogen moiety from an unsaturated carbon to produce water (Held, 2015; Muhammad Naveed et al., 2017). This reaction leaves an unpaired electron on the fatty acid, which is capable of sequestering oxygen, forming a peroxy radical (Liu et al., 2002; Lorch et al., 2002; Montillet et al., 2005; Birtic et al., 2011) (Fig. 2). Lipid peroxides are weak, and disintegrate readily to form a complex series of compounds, including reactive carbonyl compounds (Davies, 2001) such as MDA (Feig et al., 1994; Liu et al., 2002; Held, 2015). MDA, a reactive carbonyl compound, possesses both astringent and lethal mutagenic and carcinogenic properties (Valkoa et al., 2006) and reacts with DNA to form DNA adducts that contribute significantly to cancers associated with lifestyle and dietary factors (Day et al., 1999; Birtic et al., 2011). Lipid peroxidation also generates important and measurable biomarkers called F-2 isoprostanes (**Isop**) (Liu et al., 2002). Arachidonic acid acts as a precursor of these isoprostanes (Practico et al., 2002). These molecules are formed independent of enzyme-catalyzed reactions, and are generally stable, which is advantageous for quantifying cellular oxidative stress in vivo using different immunological assays (Practico et al., 2002; Kanofsky, 2011).

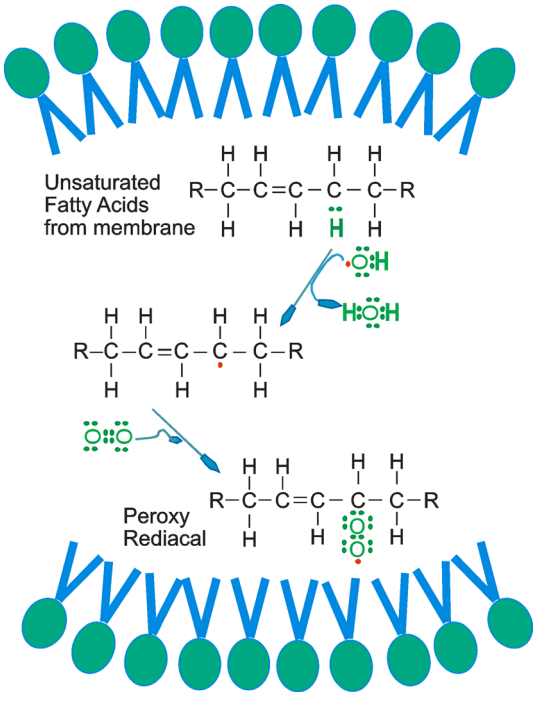


Figure 2 Oxidation of unsaturated fatty acids. The first step involves a hydroxyl radical attack with the removal of water, followed by peroxy radical formation after addition of diatomic oxygen.

ROS cause proteins and nucleic acid oxidation

ROS also oxidize other biomolecules such as DNA and proteins, and direct (by hydroxyl radical) or indirect (by hydrogen peroxide and peroxynitriles) ROS-mediated DNA oxidation results in mutations. These mutations may be lethal and may upregulate important proto-oncogenes, suppresser genes, or repair genes, leading to onset and progression of cancer (Feig et al., 1994; Lorch et al., 2002).

Protein oxidation (indirect mutation of DNA due to oxidation of nucleases and polymerases) fragments amino acid residues, disrupts formation of protein–protein cross linkages and promotes backbone oxidation, which eventually lead to loss of function (Kasprzak, 2002). These oxidized proteins affect intracellular pathways and are the main causative agents in the onset of different abnormal pathways leading to cancers and other disorders. Several ROS, e.g., $ONOO^{-}$and NO_2^{-}, can attack proteins and nitrate aromatic amino acid residues, thus, affecting the ability of these proteins to participate in signal transduction. Oxidative protein damage could also affect the activity of DNA repair enzymes (Wiseman and Halliwell, 1996b). Normally, the altered proteins are degraded readily in the cell by the proteolytic activity of different enzymes to protect the body from harmful and lethal pathological conditions that may result from the aberrant functioning of the proteolytic pathway (oxidized proteins accumulate inside cells and are a major source of serious pathological conditions) (Cooke et al., 2003). Various in vitro assays have been proposed to specifically detect biomarkers of oxidative protein damage. Nitrotyrosine (a biomarker of inflammation and NO) is a product of ROS-mediated tyrosine nitration (Kasprzak, 2002). Protein carbonyl (CO) may be considered as a biomarker of oxidative stress and is produced during oxidation of protein side chains (Emerit, 1994; 2007; Dotta et al., 2011).

Oxidative damage alters DNA bases, which when left unrepaired, subsequently lead to genetic defects. Since guanine oxidation is problematic, 8-hydroxy-deoxyguanosine has been traditionally used as a biomarker of oxidative DNA damage (Wiseman and Halliwell, 1996b; Beckman et al., 2000). ROS molecules are termed clastogenic factors as they are mutagenic and can cause chromosomal breakage (Emerit, 2007; Lindholm et al., 2010). Studies show that these factors can cause position-specific mutations in DNA (Kasprzak, 2002).

As a first step toward elucidation of the mechanisms of ROS-mediated mutagenesis in mammalian cells, the integrity of mammalian DNA polymerases alpha and beta on

oxidatively damaged DNA templates was examined. In these experiments, a duplex DNA with a single strand gap flanking the ***lacla*** target reporter gene was treated with FeSO4 plus H_2O or CuCI plus H_2O_2, followed by gap-filling DNA synthesis using the purified enzymes (Wiseman and Halliwell, 1996b). Mutants were identified phenotypically after transforming *Escherichia coli* with the synthesized DNA, and the mutations were found to be clustered in hot spot regions. Fe-catalyzed damage yielded high frequencies of C to A and G to A substitution by pol-a, and C to A, G to A, and C to T substitutions, as well as deletions of C by pol-beta. Cu-mediated damage yielded A to C and C to A substitutions, deletions of C by pol-a, and A to G, C to A, C to T, G to C, and G to T substitutions, and deletions of G by pol-beta (Wiseman and Halliwell, 1996b). As was observed in *E. coli*, the selective attachment of mutations secondary to DNA damage from the two sources is analogous, although Cu induces spare single base substitutions that may be rooted by Cu-oxygen complexes(Degan et al., 1995; Ciccia and Elledge, 2010).

Surprisingly, mutations occurred at different positions because the same template (i.e., damaged by incubating with the same system for ROS generation) was copied by different DNA polymerases. For example, Fe-mediated damage induces a variety of substitutions in place of C if pol-beta is used to copy the DNA, whereas only C to A substitutions emerge when pol-alpha is used (Kasprzak, 2002). Thus, pol-beta displays less specificity. In addition, certain abrasions or abrasions in certain sequence contexts may be mutagenic for one polymerase but not for others (Degan et al., 1995; Wiseman and Halliwell, 1996b; Ciccia and Elledge, 2010). For example, in the case of mutations secondary to Cu-mediated damage, substitutions inverse to G were identified only with DNA pol-beta. The sequence of M13mp2 *lacla* contains a small overlap for the two polymerases. When the same template is pretreated with Cu, only two mutable sites (positions 64 and 70) are available for both polymerases, whereas for templates treated with Fe only one site (position 103) is common to both polymerases. Interestingly, DNA polymerases play a crucial role in deciding the sites and types of mutations produced by ROS-mediated DNA damage (Degan et al., 1995; Wiseman and Halliwell, 1996b; Ciccia and Elledge, 2010). The various mechanisms involved in producing ROS-induced nucleic acid (DNA) mutations (Morgan, 2003) are summarized in Fig. 3.

Processes emitting biophotons in the precancerous micro-environment

Biophotons are electromagnetic waves emitted from biological environments by specific phenomena such as fluorescence, chemiluminescence (Chen et al., 2012), and ultra-weak biophoton emission. The main focus of the review is on ultra-weak biophoton emission, which occurs during ROS and ROS-related reactions such as peroxidation of biological molecules. The ultra-weak biophotons are emitted from both normal (Van Wijk et al., 2006b) and cancerous/precancerous cells and possess specific properties (Komatsu et al., 2014), which allows easy distinction between normal and cancerous/precancerous cells (Hossu et al., 2013; Cifra and Pospisil, 2014). Some of these properties include ultra-weak emission, spectrum range between 200 and 800 nm (Van Wijk et al., 2006b; Kobayashi et al., 2009), emission of few photons per second, high coherency, 10-times weaker intensity than regular daylight, variation in complexity among organisms, and ability to affect cell proliferation, differentiation, and morphogenesis (Hideg, 1993; Shen et al., 2000; Liu et al., 2003; Lozneanu and Sanduloviciu, 2008; Popp, 2009). Growing eggs stimulate the growth of other eggs of similar age. Nevertheless, biophotons from mature eggs can hinder and disrupt the growth of younger eggs at different stages of development. In some cases, biophotons from older eggs appear to terminate the growth of immature eggs (Shen et al., 2000; Kobayashi, 2003; Popp, 2009; Birtic et al., 2011).

To study the mechanisms associated with biophoton emission in the precancerous environment, the fundamentals of light-matter interactions must be understood. The semi-classical theory of quantum physics is the main theory underlying biophoton emission. Light-matter interactions, which mainly depend on the optical properties of the material (i.e., how the material interacts with light) and the characteristics of the light source, include a wide range of phenomena such as absorption of electromagnetic waves, light scattering, refraction, and reflection of light from biological molecular systems (Saleh et al., 1992).

Two fundamental approaches can explain light–matter interactions: (i) the **classical harmonic oscillator model** and (ii) the **semi classical mechanical model**. According to the classical model, when a material is illuminated by an incident harmonic light wave (i.e; an EM wave), its internal electric charge distribution is disturbed. More specifically, the charges experience a time-varying force which is proportional to the strength of the oscillating E field in the EM wave. For instance, in polar molecules, the dipoles tend to align with the E-field orientation, while, in non-polar molecules, the surrounding electron cloud is adversely affected. Thus, a dipole is created in alignment with the field. In both cases, the medium is polarized and the resultant dipoles in both cases are formed because of electronic polarization (Tennenbaum, 1998-1999). Similar processes can also be adapted to atoms in molecules. Atoms or ions can be set into vibration by the incoming E field, and vibrations can exist in different states such as stretching, rotating, bending, etc. Since nuclei are larger than the electron clouds, the vibrational behavior is different from the aforementioned electronic contribution. Such effects are termed molecular polarization (Bischof, 2005).

In general, polarization P, which is proportional to the input E field, is expressed as

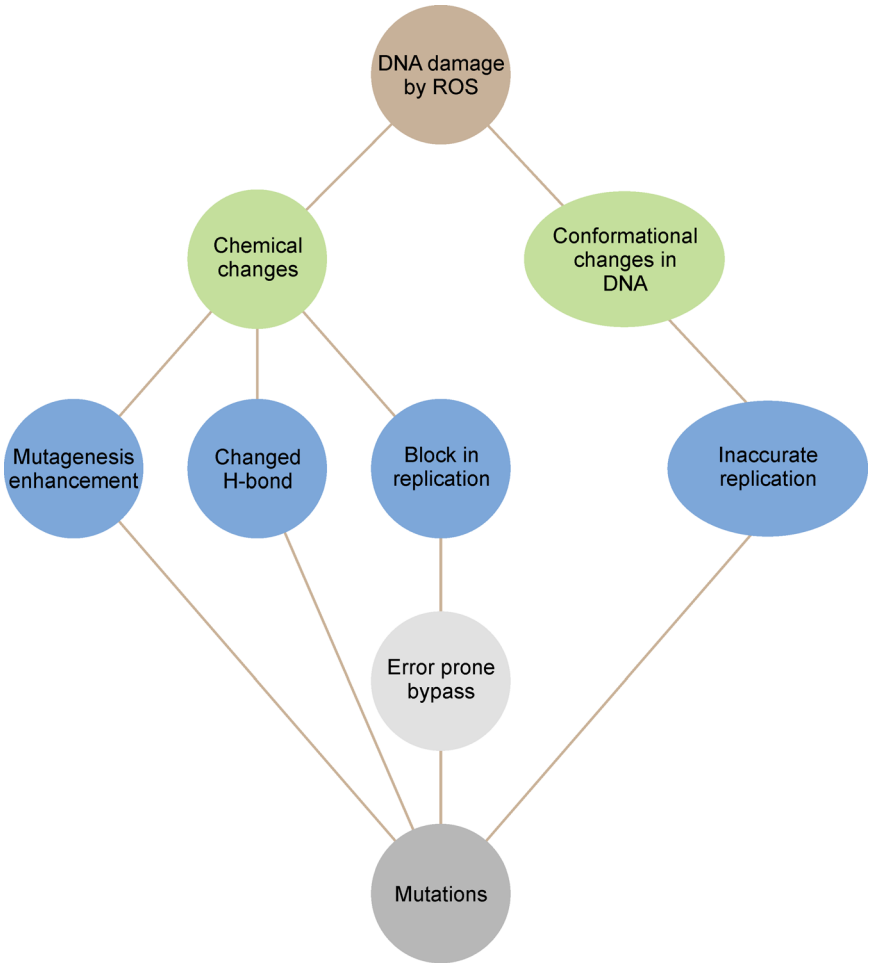


Figure 3 Different ROS-dependent pathways that mutate DNA.

$$P = \varepsilon_0 \chi E$$

where, ε_0 is the permittivity of the free space, χ is electric susceptibility, which characterizes material response to the incoming E field, and χ is directly related to the refractive index of the material N by

$$N = 1 + \chi$$

Therefore, refractive index is an indicator of a material's response to light waves. Light–matter interaction at the atomic/molecular level is best explained by quantum mechanics, as it deals with energy exchange between photons and atoms/molecules (Tennenbaum, 1998-1999). Quantum mechanics generally consists of the following physical concepts:

(i) Atoms, the fundamental (indivisible) units of matter, possess a generic structure consisting of a positively charged nucleus surrounded by orbiting electrons. This model was proposed by Niels Bohr in 1913 (Fig. 4A).

(ii) The energies of the orbiting electrons in an atom or a molecule can only exist in discrete or quantized values, which are depicted by an energy diagram (Fig. 4B).

(iii) Matter can display characteristics of both particles and waves—an intriguing property called wave–particle duality, similar to light waves versus photons. This is apparent for microscopic or quantum particles, e.g., electrons, protons, and photons.

(iv) It is impossible to simultaneously and accurately determine the position and momentum of a particle. Similarly, the absolute energy value of a particle at any instant cannot be determined with certainty. Therefore, a probabilistic description of particle properties (e.g., energy and position) is crucial. This is the premise of the famous Heisenberg uncertainty principle—a core concept of quantum mechanics (Dinh, 2010; Kai, 2012).

The wave nature of particles is best explained using the probability-based interpretations of quantum mechanics. For example, an electron in an atom at different quantized electron energy levels can display different modes of the wave function. In fact, these wave functions can radically be the achievable solution of the Schrodinger equation, "a central equation in quantum mechanics to describe the behaviors of any quantum systems (e.g., atoms, molecules)." The physical meaning of the wave function, interpreted by

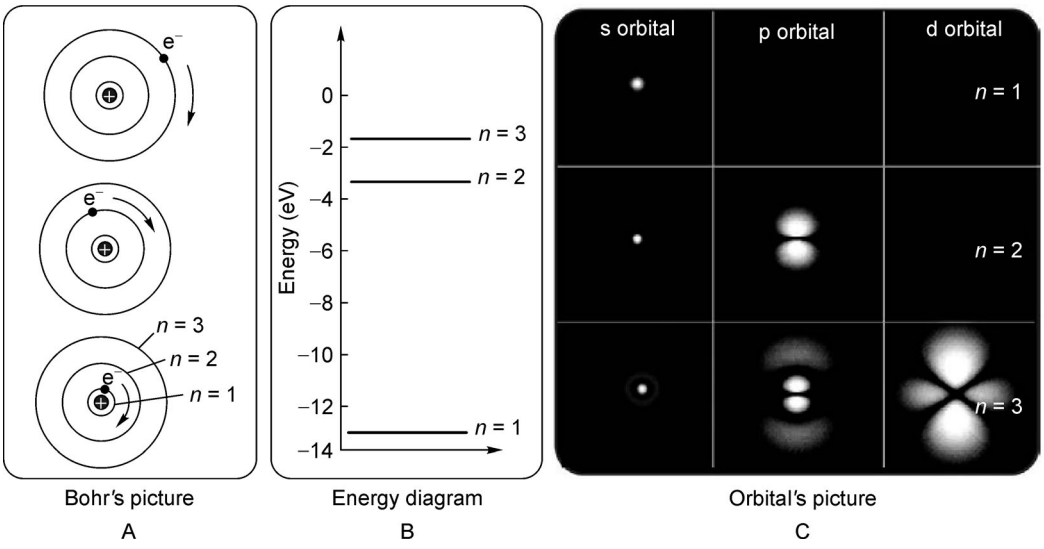


Figure 4 (A) Atomic structure of a hydrogen atom described by Bohr's model. The electron (e^-) can travel in a discrete set of circular orbits around the positively charged nucleus. The electron can gain or lose its energy to occupy different orbits (n = 1, 2, 3 ...) depending upon its energy. The more energetic the electron, the more distant it is from the nucleus. (B) Energy diagram of the hydrogen atom. (C) The orbitals of the electron of the hydrogen atom. Note that energy level n = 1 has only one type of orbital (called s orbital). In contrast, higher energy levels have more than one orbital with the same energy. For example, s and p orbitals for n = 2; s, p, and d orbitals for n = 3). This can be explained by the fact that there is more than one possible solution (wave function) to the Schrodinger equation for $n > 2$ (source (Tsia, 2015).

Max Born, complements the probability distribution of finding an electron in space. In chemistry, this distribution is mostly described as electron orbitals (Fig. 4c), spaces where the probability of finding electrons of a particular energy level (also named as energy state) in an atom is high and can be obtained by solving the Schrodinger equation. The electron can only change its energy to the allowed quantized levels, and each level is linked to one or more wave functions, which implies that the electron orbitals change accordingly (Atkins and Paula, 2002; L, 2008; Rastogi and Pospisil, 2011; Prasad and Pospisil, 2012). The relationships between the electron energy levels and the linked wave functions (or orbitals) are shown in Fig. 4. However, a comparative analysis reveals that both theories are identical.

General biophoton properties assist in accurately determining results

The general properties of biophotons emitted from organisms can assist in understanding the root causes of several biological processes and obtain accurate results. Some of these properties are: (1) the bio-photon emission concentration from bio-tissues is generally weak (approximately 10–100 000 photons per 1 cm^2) and vary significantly among organisms. Complex organisms produce biophotons of higher intensity (Pang, 1995). For example, biophoton emission from human beings is one of the strongest. Additionally, live tissues in different areas of the body emit biophotons of different intensities. Chi et al. demonstrated that different parts on the body surface show varying biophoton emissions at room temperature. For example, the fingers generated biophotons of the strongest intensity, followed by the palms, forehead, cheeks, forearms, upper arms, chest, and abdomen. These intensities were determined using an optical multi-channel analyzer (OMA) and single-photon counter (Pang, 2012). (2) The frequencies and wavelengths of emitted biophotons are widely distributed from the infrared (IR) to gamma radiation spectrum (Pang, 1995). (3) The intensity of biophoton emission is sensitive to body temperature and is closely linked to the growth period of live body or cells. Generally, the emission intensity increases with temperature and decreases with age. Biophoton emission from cells is highest in the G-phase of cell division, and increases till cell death, followed by a decrease (Van Wijk et al., 2006a; Pang, 2012). (4) Biophoton emission is nonlinear and coherent (Pang, 1995). (5) The live body is in a non-equilibrium state when biophotons are emitted. The distribution of the emitted bio-photons fulfills a statistical rule and exhibits an oscillatory feature (Pang, 2012). (6) Biophoton emission increases with stress. (7) Biophoton emission intensisty and incoherency increases with tumor cell density, whereas the reverse is observed for normal cells. Furthermore, biophoton emission becomes more distinct with increase in tumor malignancy Cohen and Popp, 2003).

Ultra-weak biophoton emission from ROS induces precancerous phase

Biophotons are emitted from certain specific regions of the spectrum regions. This can be elucidate by several examples

(Lozneanu and Sanduloviciu, 2008): triplet excited carbonyl emits near ultraviolet A (UVA) and blue–green areas (350–550 nm), whereas singlet and triplet excited pigments emit in the green–red (550–750 nm) and near-red-IR (750–1000 nm) areas, respectively, and singlet oxygen emit in the red (634 and 703 nm) and near-infrared (NIR) (1270 nm) areas (Alarcon et al., 2007; Pospisil et al., 2014).

The formation of these electronically excited species is mainly due to oxidative stress or oxidation of biomolecules (Alarcon et al., 2007; Pospisil et al., 2014). Here, the basic mechanisms involved in the formation of ROS (electronically excited species) by peroxidation of biomolecules and conformational changes of biomolecules have been elucidated.

Role of lipid peroxidation in ultra-weak photon emission

Studies show that oxidation of linoleic acid by soybean lipoxygenase leads to the production of $^1O_2$ which is identified by $^1O_2$ mono-molecule photon emission at 1270 nm. It is speculated that $^1O_2$ is produced by recombination of ROO via Russell's mechanism (PR, 2010). Certain lines of evidence show that decomposition of lipid ROOH by metal ions, peroxynitrite, chloroperoxide, and hypochlorous acid is a promising source of $^1O_2$ (Fig. 5A. In this reaction, the ROOH group is oxidized to ROO, which leads to the formation of highly unstable ROOOOR. The consecutive ROOOOR disintegration is responsible for the formation of organic hydroxide, ground carbonyl, and $^1O_2$. Evidence shows that approximately 10% $^1O_2$ is produced via Russell's mechanism. The oxidation of several lipids (monogalactosyldiacylglycerol, linoleic acid, and β-carotene) either by $^1O_2$, generated by illumination in the affirmation of methylene blue or HO produced by Fenton's reagent increased the ultra-weak photon emission. Based on the correlation between ultra-weak photon emission from linoleic acid and *Arabidopsis* post-$^1O_2$ induction, researchers hypothesized that oxidized lipids are the main sources of ultra-weak photon emission in *Arabidopsis*. The relation between the photon emission from the lipid ROOH/horseradish peroxidase and EPR spin-trapping experiments revealed that ROOR is generated by the cyclization of ROO instead of through cyclo-addition of $^1O_2$ (Winkler et al., 2009; Birtic et al., 2011; Prasad and Pospisil, 2012).

The fact that photon emission was restrained by the addition of $^1O_2$ quencher (β-carotene) and increased by $^1O_2$ enhancer (1, 4-diazabicyclo[2,2,2]-octane) shows that $^1O_2$ actively participates in photon emission. It was proposed that the breakdown of ROOH by metalloproteins generates ROO, the recombination of which results in the formation of the highly unstable ROOOOR that disintegrates into organic hydroxide, ground carbonyl, and $^1O_2$ (Ogilby, 2010; Prasad and Pospisil, 2012) (Fig. 5B Reports show that increasing the concentration of H_2O_2 in an aqueous fetal bovine serum albumin solution lured ultra-weak photon emission and formation of protein carbonyl compound in a concentration-dependent manner.

Reaction between Fenton's reagent and fetal bovine serum results in significant photon emission. Experimental data show that the addition of H_2O_2 to the isolated amino acid

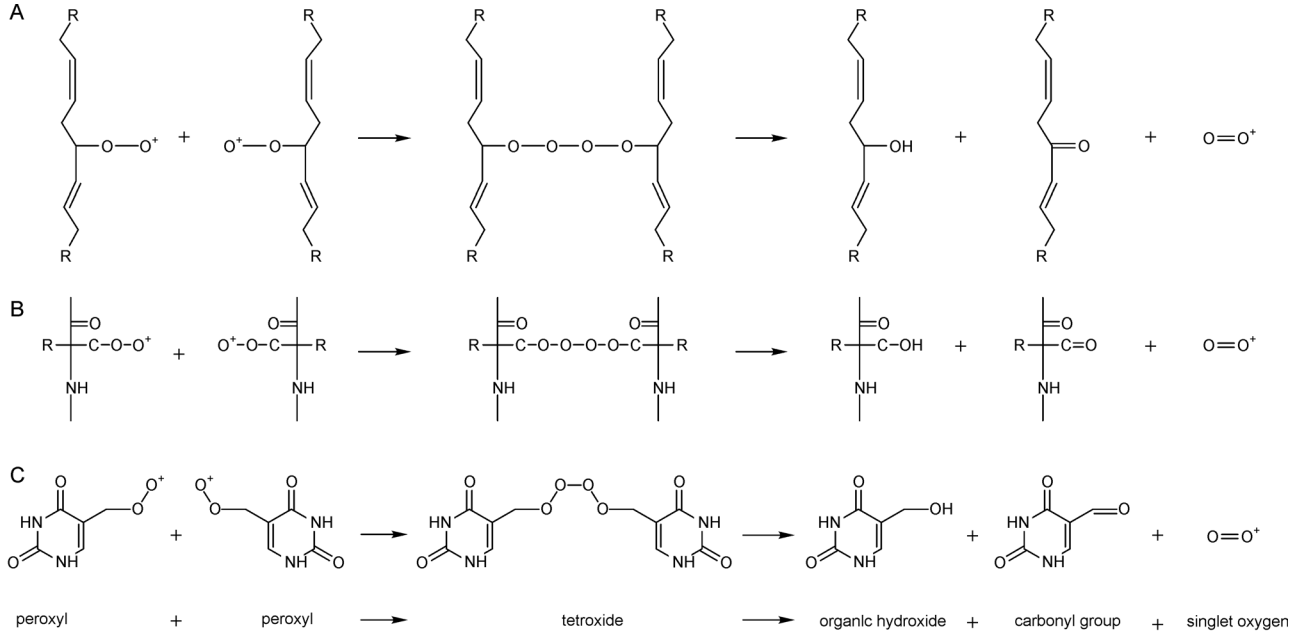


Figure 5 Formation of singlet oxygen by combination of two peroxyl radicals in lipids (A), proteins (B), and DNA (C). The combination of two peroxyl radicals (ROO) results in the formation of the highly unstable tetroxide (ROOOOR). Subsequent decomposition of ROOOOR leads to the formation of organic hydroxide (ROH), ground carbonyl (R = O), and singlet oxygen ($^1O_2$).

induced photon emission solely from Cys, although the addition of HO (generated by the Fenton's reagent) caused trumpet photon emission from Phe, Trp, His, and Cys. Two phases were observed in the ultra-weak photon emission. The first phase originates from the reaction of unstable ROO and RO, whereas the second phase shows breakdown of more stable protein ROOH. The ultra-weak photon emission was observed after $^1O_2$-mediated protein oxidation. The measurement of certain peptides containing Trp, His, or Tyr showed that photon emission was actively dependent on the peptide bonds of the compound. The dipeptide Trp-Ala revealed a three magnitude higher photon emission than any other combination of amino acids. Surprisingly, there was no difference in the magnitude of oxidation of all dipeptides, highlighting that the increase in ultra-weak photon emission is due to structural changes in the dipeptide induced by the formation of the peptide bond with the amino group of the Trp moiety (Kobayashi, 2003; Prasad and Pospisil, 2011; Cifra and Pospisil, 2014).

Role of proteins in ultra-weak photon emission

Studies show that protein molecules can both emanate and absorb biophotons with wavelengths of < 3 μm and 5–7 μm, which is consistent with the energy level transitions of the excitons (Pang, 2012).

Role of nucleic acid oxidation in ultra-weak photon emission

A significant enhancement in the emission of ultra-weak photons was observed after adding the copper/ascorbate/H_2O_2 system to guanine, whereas no significant effect on the ultra-weak photon emission of other nitrogenous bases of nucleic acid was observed. These observations showed that guanine is exclusively responsible for ultra-weak photon emission. Furthermore, ultra-weak photon emission increases in the following order: base (guanine) < nucleoside (guanine + ribose or deoxyribose) < nucleotide (guanine + ribose or deoxyribose + phosphate). Therefore, in addition to guanine, oxidation of ribose and deoxyribose also actively participate in the emission of ultra-weak photons. As phosphate oxidation unlikely contributes toward ultra-weak photon emission, it was proposed to be a causative agent for structural changes that promoted ribose or deoxyribose oxidation (Feig et al., 1994; Kobayashi, 2003; Rastogi and Pospisil, 2010; 2011). In addition, the HO (sodium benzoate) and O_2 (SOD) antioxidants moderately effect ultra-weak photon emission, whereas the $^1O_2$ antioxidant (sodium azide) abolishes photon emission. Considering these observations, we suggest that the oxidation of deoxyguanosine monophosphate by $^1O_2$ produced via Russell's mechanism leads to the production of ROOR and breakdown into 3(R = O)* (Cao, 2010; Kai, 2012).

The reactions that lead to the formation of thymine ROOH is mediated by the absorption of hydrogen from the methyl group of thymine. Ultra-weak photons were emitted upon addition of metal ions (Ce^{4+}, Fe^{2+}, and Cu^{2+}) or hypochlorous acid to thymine ROOH. Thymine ROOH subsequently undergoes oxidation to thymine ROO when redox active compounds are exposed (Kai, 2012). Then, recombination of two thymine ROO generates the unstable ROOOOR, which disintegrates to $^1O_2$ through Russell's mechanism (Fig. 5C. The restraining of ultra-weak photon emission by the $^1O_2$ antioxidant, sodium azide, indicates the involvement of $^1O_2$ in photon emission. The emission of ultra-weak photons from $^1O_2$-treated DNA (generated from the H_2O_2 + OCl system) highlighted emission of these photons from nucleic acids. Ultra-weak photon emission was blocked by lycopene, β-carotene, vitamin C, and vitamin E; nonetheless, mannitol, SOD, and NaN_3 did not affect the ultra-weak photon emission (Brizhik, 2008; Kobayashi et al., 2009; Rastogi and Pospisil, 2011; 2013; Cifra and Pospisil, 2014).

Biophoton emission is related to ROS production

Several studies have investigated the origin of biophoton emission, including the involvement of cell membrane, microtubules, DNA, and mitochondria. Biophotons were associated with ROS production irrespective of their origin (Watts et al., 1995; Cifra et al., 2010; Deriu et al., 2010; Rahnama et al., 2011).

According to several studies, change in cell membrane potential is associated with biophoton emission. The change in potential is mainly caused by influx or out flux of Ca^{2+} ions (Dotta et al., 2011). In the brain, cortico-thalamic ionic movement causes biophoton emission due to change in the electromagnetic field (Kataoka et al., 2001). The Ca^{2+} flux is controlled to some extent by ROS production, which can initiate several biochemical signaling pathways including MAPK and ERK1/2 pathways (1998). Ultra-weak biophoton emission from neural tissue depends on neuronal membrane depolarization and Ca^{2+} entry into cells Wright et al., 1979; Rahnama et al., 2011).

DNA conformational changes and the natural tendency of nucleotides to be excited after ROS-mediated DNA oxidation are the other sources of biophoton emission (Cohen and Popp, 2003; Klotter, 2010). Excimers and exciplexes of DNA molecules are relatively stable than the parent molecules and can absorb energy to re-emit in another wavelength (Anwijk, 2001). In addition, ROS signaling pathways are involved in the activation and inactivation of proteins involved in inducing conformational changes in DNA (Pang, 1995; Popp, 2009).

Conformational changes in the cytoskeleton of cellular

structures, especially microtubules, is yet another mechanism of biophoton emission (Kataoka et al., 2001; Alarcon et al., 2007). The energy of mitochondrial biophoton emission or those of other absorbed light induce conformational changes in microtubules (Cifra et al., 2010; Deriu et al., 2010). Tubulin dimer is an intrinsically fluorescent molecule mainly because of the presence of eight tryptophan residues. It is well known that the absorption (280 nm) and fluorescence (335 nm) wavelength (and intensity) of tryptophan are dependent on tubulin conformation (Pang, 2012). Furthermore, microtubule polymerization is sensitive to UV and blue light, and mitochondria are known to be sources of biophotons corresponding to these wavelengths, which provides an immediate logical connection between these two processes (Deriu et al., 2010).

The involvement of mitochondria in biophoton emission is critical (Tulah and Birch-Machin, 2013). Different ionic and/or radical forms of oxidized/reduced flavins thatare important components of the respiratory chain also exhibit strong and broad absorption bands as well as emission in broad spectral range from near-UV to near-IR (Kamal and Komatsu, 2015). Most importantly, ROS produced by the respiratory chain (Inoue et al., 2003) are excited species that can emit biophoton and produce other excited species, which emit biophotons on relaxation (Vladimirov and Proskurnina, 2009) as discussed in the Biomolecule peroxidation section. Studies suggest that biophotons are products of redox reactions of free radicals. This hypothesis is supported by the correlation between biophoton counts and doses of reactive oxygen species (Tulah and Birch-Machin, 2013).

Comparison of biophoton emission from cancerous and normal cells

Popp et al. observed that biophoton emissions from healthy humans display rhythmic patterns (Kobayashi et al., 2009). He also observed that the coherence, intensity, and rhythmic patterns of the emissions varied with diseases (Cohen and Popp, 2003). People with multiple sclerosis, for example, absorb more light and their photon emissions display more ordered than those of people with other diseases. Biophoton emissions from cancer patients lack coherence and do not follow natural rhythmic patterns. In addition, tumors emit high amounts of photons (Chen et al., 2012), an average of 300 [or-] 90 photons/cm per minute compared to normal tissues that emit an average of 22 [or-] 6 photons/cm per minute. Popp and colleagues discovered that surface tumors and tumors excised during surgery respond to remedies with changes in photon emissions. Most anticancer treatments have no effect on the high emission rate of tumors. However, when tumors respond to nontoxic remedies with decreased emissions, the emitting agent is likely to improve the patient's condition and may even provide a cure (Pang, 1995; Klotter, 2010).

Changes in the emission intensities of ultra-weak biophotons during proliferation of human carcinoma cell culture (TE9 cell line) were detected using a highly sensitive and low noise measurement apparatus coupled with a flow culture system. In the sampling period of 93 h, the biophoton emission intensity from the culture followed a similar course as that of the growth curve. Spectral analysis of the biophoton emission from the cell culture demonstrated a significant peak at around 530 nm. Results suggest that the emission intensity mainly depends on the cell population (Takedaa et al., 1998). Bustamante et al. observed differences in the emission intensities of different proliferative phases of a malignant melanoma cell culture; however, their measurements were performed using harvested cell suspension and not under physiologic conditions (Takedaa et al., 1998).

Biophoton emission has also been detected and analyzed in humans. Human biophoton emissions demonstrate spatio-temporal differences and are linked to ROS production and oxidative stress. Cohen and Popp have conducted long-term systematic research of biophoton emission from the hands and forehead in vivo using a moveable, hanging photomultiplier. The intensity of biophoton emission was closely related with time, recording procedures, and temperature (Sauermann et al., 1999; Tafur et al., 2010).

Biophoton emission from hands suggest that emitted photons are generated from both the skin surface and interior sources. Spectral analysis documents major spontaneous emission at 470–570 nm, indicating a specific electron-excited state. Excited carbonyl groups, perhaps generated by reactions between excited singlet oxygen molecules and unsaturated lipids, may be responsible for this emission. Furthermore, biophoton emission is altered in the presence of antioxidants. Several studies have confirmed that human biophoton emission is oxygen-dependent and is reduced in hypoxic conditions. The summary of overall biophotonic emission from four anatomical sites with respect to wavelength and intensity is presented in Graph 1 (Van Wijk et al., 2006a; Tafur et al., 2010).

Biomarkers of ROS-associated reactions are used to further confirm precancerous environment

Biomarkers of DNA damage can be used for predicting precancerous conditions (Emerit, 2007). Biomarkers include oxidized DNA bases either from nuclear or mitochondrial DNA. $O_2^{\cdot -}$ and H_2O_2 do not react directly with DNA bases. $OH^{\cdot}$ generates multiple products from all four DNA bases, which appears to be a diagnostic "fingerprint" of $OH^{\cdot}$ attack (Wiseman and Halliwell, 1996b). Fortunately, nuclear and mitochondrial DNA can be differentiated by determining the type of product, i.e., 8-hydroxyguanosine (8-OHG) from nuclear DNA and 8-hydroxydeoxyguanosine (8-OHdG) from mitochondrial DNA (1998; Marnett, 2000b). The unusual 3′

and 5′ ends (i.e., non 3′–OH, non 5′-PO_4) are generated depending upon the site of radical attack, which can also be termed as indicators of alterations (mutations) in DNA (Marnett, 2000b), showing that higher concentrations of ROS leads to cancer (Feig et al., 1994; Wiseman and Halliwell, 1996b; Emerit, 2007).

In mammals, the expression of several genes are increased during stress response (Day et al., 1997). Mammalian genetic libraries provide access to sequences that can be mutated after DNA damage. Transcript levels increase after H_2O_2 treatment, which were then cross hybridized with probes from the constructed libraries to identify the mechanisms of oxidative stress-induced DNA repair enzymes in mammals e.g. redoxyendonucleases and glutathione peroxidase (Lorch et al., 2002; Chiarugi, 2008), heme oxygenase, DT diaphorase or quinone reductase, and a protein-tyrosine phosphatase. Peroxide treatment increases the expression of these enzymes. Temporary adaptation to H_2O_2 also increases adapt66 levels, a MAFG nuclear transcription factor/oncogene homolog (Deshpande et al., 2002). During H_2O_2 adaptation, adapt73 levels are also highly upregulated in Hela cells. Last but not the least, adapt78 levels are dramatically increased during the adaptation of Hela cells to H_2O_2. High adapt78 levels are of considerable importance as it is a Down's syndrome critical region homolog (Wiseman and Halliwell, 1996b; Davies, 2001).

Thymidine phosphorylase is associated with breast carcinomas and is generally involved in the conversion of thymidine to 2-deoxy-D-ribose-1-phosphate, that in turn favors the generation of oxygen radicals (Feig et al., 1994; Lorch et al., 2002). Other ROS-mediated signaling pathways and bio-molecular reactions such as activation of tyrosine kinase receptors during cell division, and secondary messengers of pathways that are specifically involved in uncontrolled division, proliferation, and tumor development (MAPK/ERK1,2 pathway) also regulate the precancerous environment.(1998) Several ROS, e.g. $ONOO^-$and NO_2^-, attack proteins and nitrate the aromatic amino acid residues, which possibly affect their signal transduction ability (Wiseman and Halliwell, 1996b). Furthermore, physical properties of cellular structures, such as refractive index (the refractive indexes of both mitochondria and microtubules are higher than that of the surrounding cytoplasm (Deriu et al., 2010)), density, composition, and diffraction of biophotons through these materials should be considered as recent studies have demonstrated changes in physical properties during tumor development.

Proteins have been used to measure oxidative base damage (Dizdaroglu et al., 2002). One of the methods uses the ability of endonuclease III to cause breaks in DNA specifically at sites of base damage; these breaks are then further assessed by single-cell gel electrophoresis. Normal human lymphocytes contain numerous endonuclease III-sensitive sites per cell in vitro. In contrast, HeLa cell lines do not harbor endonuclease III-sensitive sites, which may indicate low oxidative damage or more effective repair processes (Wiseman and Halliwell, 1996b).

Biomarkers can be analyzed using gel electrophoresis, simple spectroscopic techniques, as well as complex FRET, FLIM, and other in vivo spectroscopy and fluorescence-based techniques.

Techniques to measure ultra-weak photon emission, bioluminescence, chemiluminescence, and ordinary photon emission from precancerous environment

Emission of ultra-weak photons, quantification of biomarkers, and prediction of pre-cancerous/cancerous phase require effective techniques (Creath and Schwartz, 2004; Tafur et al., 2010; Guo, 2013) Enhancer compounds such as luminol and lucigenin are used in chemiluminescence-based studies because of potential variability and low intensity of native chemiluminescence. Luminol and lucigenin react with ROS to form complexes such as 3-amino-phthalate and N-methyl-acridone. The excited electrons in these complexes shift to their ground state with the emission of energy as light (luminescence), which can be detected by photomultipliers. The sensitivities of luminol and lucigenin are different. Luminol detects H_2O_2, OH, hypochlorite, peroxynitrite, and lipid peroxy radicals, whereas lucigenin is particularly sensitive to the superoxide radical (Larason et al., 1998; Ferraro et al., 2011).

1- CCD CAMERAS

The spectral sensitivity of the CCD camera ranges from 200 to 1000 mm. The spectral sensitivity of the CCD camera is limited to only the visible range of the spectra because of the usage of lenses (Tafur et al., 2010). Thermal electrons in CCD camera may elevate the temperature above that of the red signal. To circumvent this problem, the CCD camera is cooled down to − 110°C with liquid nitrogen. These CCD cameras generate 2-D images of biomarkers and emit photons (Gartel and Radhakrishnan, 2005; Prasad and Pospisil, 2011; 2012; Rastogi and Pospisil, 2013). The basic characteristics of this apparatus are shown in Fig. 6.

2- PHOTOMULTIPLIER

Low noise photomultiplier is a chief photo-electric device that counts the number of photons emitted from a specific zone by generating a quantifiable electric current. The amount of current generated is directly proportional to the magnitude of biophoton emission (Tafur et al., 2010). These photomultipliers are primarily used for one dimensional (1-D) imaging (Gartel and Radhakrishnan, 2005; Saar et al., 2010; Prasad and Pospisil, 2011; 2012; Rastogi and Pospisil, 2013) and are extremely sensitive detectors of light in the UV, visible, and

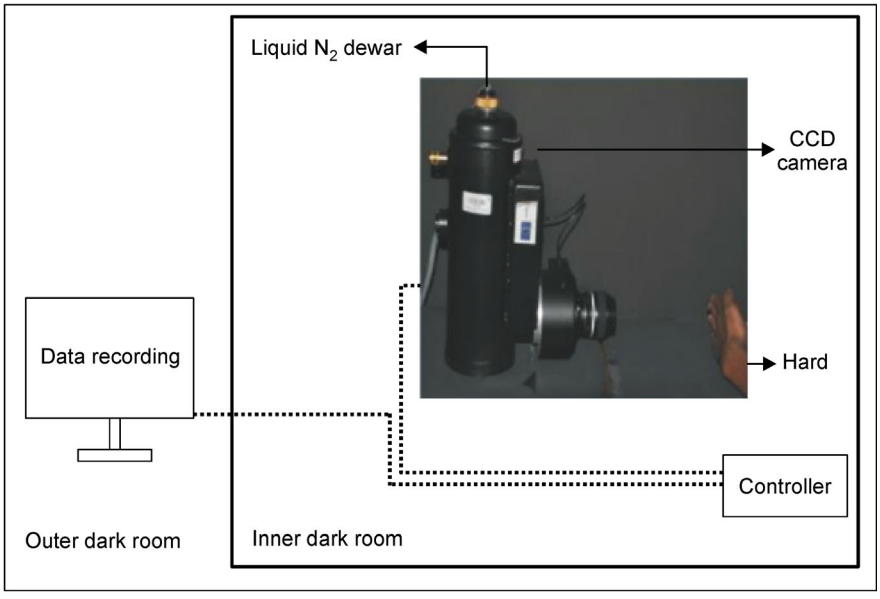


Figure 6 Basic apparatus for measuring ultra-weak biophoton emission from a precancerous micro-environment using CCD cameras, which produce a two-dimensional image. (Source: Rastogi and Pospisil, 2013)

NIR ranges of the electromagnetic spectrum (Pho, 2008) A diagrammatic representation of photomultipliers is shown in Fig. 7.

3- FLUORESCENCE MICROSCOPY

Fluorescence microscopy can be used for specifically observing the interactions of fluorophores with different biological molecules such as DNA, proteins, and lipids (Mason, 1999). Several fluorophores that bind to specific nucleotides of DNA have been identified (Savage, 2006). Mutations in DNA can be identified using this technique. Table 2 shows the exogenous fluorophores used for detecting DNA (Hoechest 33342, $2Cl^-$ DAPI), along with their wavelengths of one-photon excitation, the resulting emission maxima, and convenient light sources for excitation (Tennenbaum, 1998-1999; Solli et al., 2008; Ferraro et al., 2011; Ferrari and Quaresima, 2012; Suhalim et al., 2012; Maitland and Wang, 2013).

Spectroscopic techniques for the determination and quantification of biomarkers produced in precancerous environment

Vibrational spectroscopy

Vibrational spectroscopy comprises IR spectroscopy and Raman spectroscopy (Owrutsky et al., 1995).

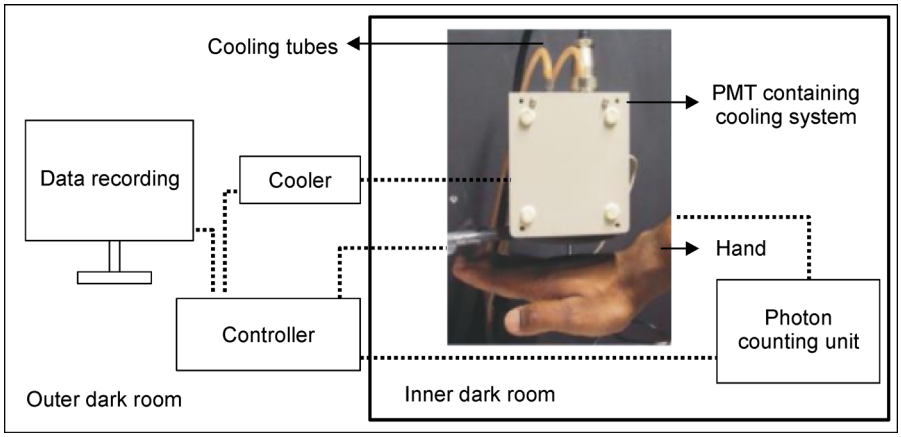


Figure 7 Instrumentation of a photomultiplier for obtaining one-dimensional image of ultra-weak biophoton emission. (Source: Rastogi and Pospisil, 2013)

IR spectroscopy (routinely now in the form of FT–IR spectroscopy) and Raman spectroscopy are used as complementary techniques to provide information on various vibrational transitions or vibrational bands (Saleh et al., 1992), which provide detailed fingerprint of different bonds, functional groups, and conformations of molecules, biopolymers, and microorganisms (Saleh et al., 1992; Tennenbaum, 1998-1999; Chalmers and Griffiths, 2002; Ferrari and Quaresima, 2012). The fundamental principle of vibrational spectroscopy is that it assesses the stretching or bending vibrations of molecules when they absorb photons of specific energy (Saleh et al., 1992). The basic principle of IR spectroscopy (an example of vibrational spectroscopy) is shown in Fig. 8.

Each molecule (ROS and by-products of ROS-mediated reactions) produced in the precancerous cellular microenvironment is characterized by different structure and biomarkers of the precancerous stage such as ROS species, MDA, F-2 prostanes, nitrotyrosine, and 8-hydroxy-deoxyguanosine (Wiseman and Halliwell, 1996b), which can be determined using vibrational spectroscopy techniques. This technique is also useful for the determination and quantification of other molecules such as oxidants and antioxidants. Vibrational spectroscopy calculations are evaluated using specific values of vibrational frequencies that vary with compounds (shown in Table 1) and their relationship with the biomarkers of the precancerous stage (Chalmers and Griffiths, 2002; Norppa et al., 2006). Following is a formula based on Hook's law for determining the relationship between IR absorption of photons of specific energy by the bond between two specific atoms and the vibrational frequencies of different compounds:

$$\tilde{V} = \frac{1}{2\pi c\{\mathrm{f}/[(m_a \cdot m_b)\cdot m_b] + m_b\}^{1/2}}$$

where $\tilde{V}$ = wave number (called frequency in spectrophotometry) in cm^{-1};

m_a, m_b = masses of first and second atoms in grams;

c = velocity of light in $cm \cdot s^{-1}$;

f = force constant of bond in $g \cdot s^{-2}$. The approximate values of force constant of the single, double, and triple bonds are 5, 10, 15 × $10^5 g \cdot s^{-2}$ respectively.

For example, the C-H bond vibration frequency of 3032 cm^{-1} changes to 1108 cm^{-1} when H is replaced with O in the peroxy radical, forming a C-O bond (Fig. 2).

As described earlier, vibrations are of two types. The stretching vibrations require more energy compared to bending vibrations, and therefore absorb photons of higher energy (Larason et al., 1998). Generally, the C-H stretching and bending vibrations exist at around 3000 cm^{-1} and 1400 cm^{-1}, respectively. In addition, the finger print (region between 625 cm^{-1} and 1600 cm^{-1} that provides information on unique characteristics of compounds) and functional group regions (region between 1600 cm^{-1} and 4000 cm^{-1} that provides information on functional group structure) of the IR spectrum have noticeable differences, which makes structure determination easier.

Simple fluorescence microscopy, FRET, and FLIM can also be used to identify bioluminescence and the structure of biomarkers involved in precancerous environment determination (Saleh et al., 1992; Wang and Wu, 2007; Saar et al., 2010).

Reasons for the failure of previous studies and tips for future success

Previous studies were unable to detect the precancerous stage as they did not use modern biophotonic techniques such as in vivo spectrophotometry and fluorescence microscopy (Tafur et al., 2010; Pang, 2012). In addition, these studies focused primarily on ROS generation and breakdown, and the mechanisms underlying biophoton emission from ROS (using classical and semi-classical models). Furthermore, most studies focused only on one type of biomolecule such as DNA, proteins, or lipids. Finally, these studies did not investigate the molecular and subcellular origin of biophoton emission and ROS production. The ROS types were not correlated with biochemical reactions or the physical properties of the cellular environment (Alarcon et al., 2007; Rastogi and Pospisil, 2011; Pang, 2012; Pospisil et al., 2014).

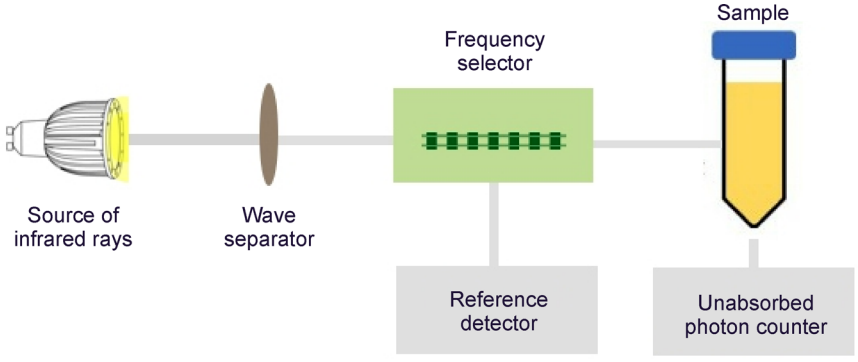
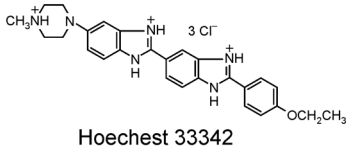
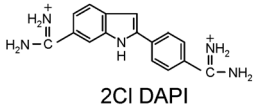


Figure 8 Basic instrumentation (principle) of infrared spectroscopy.

Table 1 Fluorescence dyes such as Hoechest 33342 and 2Cl-DAPI can be used to determine ROS-induced position-specific or non-specific mutations in DNA.

Bond	Vibrational motion	Functional group	Frequency (cm^{-1})
O-H	Strech	Alcohol, Phenols	3640-3200
N-H	Strech	Amines, Amides	3400-3250
O-H	Strech	Carboxylic Acids	3300-2500
C-H	Strech	Alkynes (−C≡C−H)	3330-3270
C-H	Strech	Aromtics	3100-3000
C-H	Strech	Alkanes	3000-2850
C-H	Strech	Aldehydes (H−C=O)	2830-2695
S-H	Strech	Thiols	2600-2550
C≡N	Strech	Nitriles	2260-2210
−C≡C−	Strech	Alkynes	2260-2100
C=O	Strech	Carbonyles, Esters, Ketones	1760-1665
−C=C−	Strech	Alkanes	1680-1640
N-H	Bend	Primary amines	1650-1680
C-C	Strech (in - ring)	Aromatics	1600-1400
N-O	Asymmetric stretch	Nitro compound	1550-1475
C-H	Bend	Alkanes	1470-1450
C-H	Rock	Alkanes	1370-1350
N-O	Symmetric stretch	Nitro Compound	1360-1290
C-N	Strech	Aromtic amines	1335-1250
C-O	Strech	carboxylic acids, esters, ethers	1320-1000
C-N	Strech	Aliphatic amines	1250-1020
=C−H	Bend	Akenes	1000-650
O-H	Bend	Carboxylic acids	950-910
N-H	Wag	Primary, Secondary amines	910-665
C-Cl	Strech	Alkylhalides	850-550
C-H	Rock	Alkanes	725-720
C-S	Stretch	Disulfides	705-570
S-S	Stretch	Disulfides, Aryldisulfides	620-430

Table 2 Determination of biomarkers on the basis of vibrational frequencies of functional types.

Flourophore	Excitation/Emission	Excitation sources	Applications
 Hoechest 33342	355/465	Ar-UV ion Laser (351 nm), Hg lamp Tisapphire laser (TPE)	A-Tsequences of DNA labbeling
 2Cl DAPI	372/456	Ar-UV ion Laser (364 nm), Hg lamp Tisapphire laser (TPE)	A-Tsequences of DNA labbeling

Light-matter interactions and the physical properties of intra and extracellular environments, such as density, shape, and refractive indices of organelles, cellular fluids, and membranes should be considered for identifying biophotons associated with ROS production and obtaining accurate results by determining the interactions of the emitted biophotons with their intra and extracellular environments. In addition, the origin of biophotons (from biomolecules and organelles), type of ROS (related to direct or indirect DNA damage), ROS biomarkers of oxidation or damage, physiology of ROS and their biochemical signaling pathways, and the use of modern physical techniques alongside conventional biochemical techniques should be considered for obtaining the best possible results.

Future perspectives

Disease detector

The techniques described above can be used to detect majority of genetic, metabolic, or physiologic disorders. Biophotonics is a diverse and interdisciplinary field related to medicine, genetics, molecular biology, bioinformatics, optics, acoustics, ecology, and other biological, physical, and chemical sciences. Therefore, this versatile combination of sciences may lead to the invention of a "disease detector," which will be based on the principle of metal detectors that use a combination of acoustic, photonics, electrical, and biological sciences. This detector will inspect the nature and severity of diseases, and other physiologic changes caused by the diseases within seconds simply by exposing it to or touching it with the body, or body secretions and fluids. Recently, artificial intelligence-based devices that can inspect diseases by evaluating the programmed genetic material (not biophotons) in silico and generate prescriptions have been manufactured.

Terrorism inspector

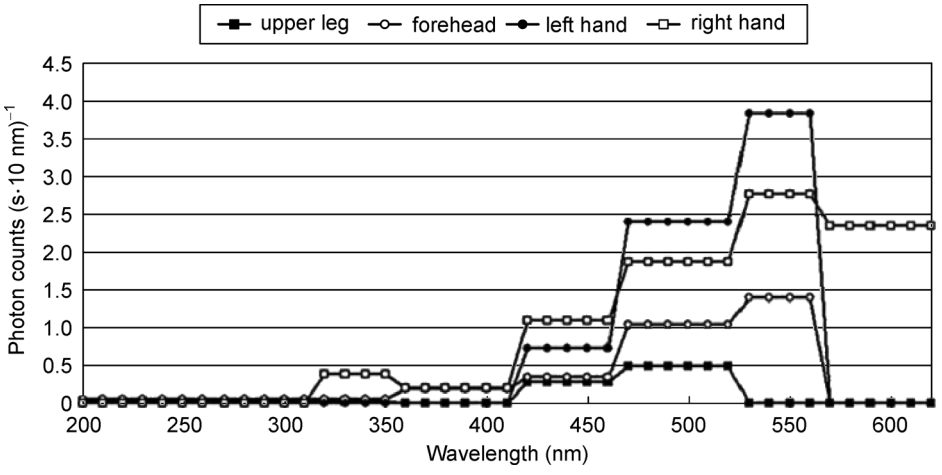
This involves creation of an instrument that will capture the biophoton pattern (aura) emitting from the human body. The aura is almost the reflection of human thinking, as shown by Popp and coworkers under specific psychological conditions. Therefore, the aura capturing technique can be used to identify the nature of human thinking; based on the emitted aura, biophotonic poisoning can be used for brainwashing terrorists. Several studies have captured and classified the aura according to surface shape, intensity, color, and time of existence, and correlated them with human habits and thought.

Biophotonic therapy and biophotonic poisoning

Several studies provide strong evidence regarding the relationship between biophoton signaling (ultra-weak photon emission and bioluminescence) and cell physiology. Biophotonic poisoning can be used to understand the relationship between different types of medications and herbal medicines (which change biophoton emission) with change in thought process. The other application involves hypnotism, which also uses electromagnetic biophotons to hack brains or deliver messages. Therefore, biophotonic poisoning can be used to change the physiology of thinking.

Conclusion

Cancer is the leading cause of the deaths worldwide and correct diagnosis of the precancerous stage is necessary to reduce mortality associated with this disease. Biophotonics provides an advanced framework, for easily diagnosing cancer at its preliminary stage. The relationship between biophotons, clastogenic factors, and biochemical reactions in the cellular microenvironment can be understood successfully owing to the multi-faceted nature of biophotons (physical and structural organization, biochemical reactions, electromagnetic interaction). Different analytical tools such as CCD cameras, photomultiplier tubes, and spectroscopic techniques can be used for precancerous diagnosis using biophotonics. The advancement in precancerous diagnosis will improve human health worldwide. The versatility of biophotonics can be used further for novel applications in biology, biochemistry, and chemistry.



Graph 1: Biophoton emission in four anatomical sites with respect to wavelength and intensity of biophotons. The legends above the graph explain the meaning of each line (source: Tafur et al., 2010)

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