Sources and Remedies of Phototoxicity in Live Cell Microscopy

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During the past decade there has been remarkable progress in understanding the behavior and function of biological cells. Progress was accelerated by the development of microscopic imaging techniques and fluorescent dyes that allowed investigators to visualize dynamic processes within subcellular compartments in heterogenous populations of living cells. These capabilities led to exciting new discoveries in cellular and molecular studies of a wide variety of cell types.

Efforts to study living cells under microscopic conditions are not without problems, however. The most vexing problem is phototoxicity caused by either illumination alone (endogenous toxicity) or illumination of fluorescent dyes loaded into cells (exogenous toxicity). In this report I provide an overview of these general types of toxicity as well as describe recent results that may shed light on how to reduce them.

A. Exogenous Toxicity

A century ago Raab noted that solutions containing the dye acridine were more toxic to cells when presented in the presence of light. This discovery was intensely studied over the next 30 years[1], especially in Tappeiner's laboratory in Munich. Many phototoxic compounds were identified, most notably fluorescent dyes (*e.g.*, fluorescein, eosine), leading Tappeiner to incorrectly surmise that this "photodynamic action" was a unique property of fluorescent compounds. Other investigators demonstrated that phototoxicity could be induced by using non-fluorescent chemicals (*e.g.*, methylene blue) or in the absence of exogenous compounds altogether (see following part B). The only common requirement was the presence of molecular oxygen (dioxygen) which distinguished this type of toxicity from the direct effects of UV light.

Tappeiner's theory was not altogether off the mark. Photochemical studies revealed that molecules with conjugated double bonds are highly efficient at absorbing photons, and brightly fluorescent molecules contain many such structures. Furthermore, in the presence of dioxygen, photons catalyze the oxidation of double bonds generating byproducts that include peroxides, superoxide ions and singlet oxygen molecules. Each of these byproducts is capable of initiating further oxidative reactions inducing chain reactions that propagate the initial effect[2]. In short, illuminating cells in the presence of exogenous sensitizers can cause a wide variety of molecular changes that are either repaired by the cell or result in irreparable damage.

During live cell microscopy, induction of phototoxicity is sometimes obvious, as with loss of cell motility or some other physiological response. More often, phototoxicity goes unnoticed either because the experiment is too short to detect it or because it doesn't interfere directly with the response under study. An example of exogenous toxicity is shown in figure 1. The cell was stained with a mitochondrial-specific dye and illuminated for 1 second every 10 minutes over a 50 minute period. Even this minimal amount of exposure was sufficient to cause drastic changes in mitochondrial morphology and cell viability 19 hours later. Of course, not all dyes display such dramatic effects.

Recognition that light-sensitive compounds generate toxic byproducts has resulted in at least one constructive outcome. It led to the development of "photodynamic therapies" in which unwanted cells and tissues are intentionally destroyed by illuminating them in the presence of exogenous sensitizers[3]. While such thera-

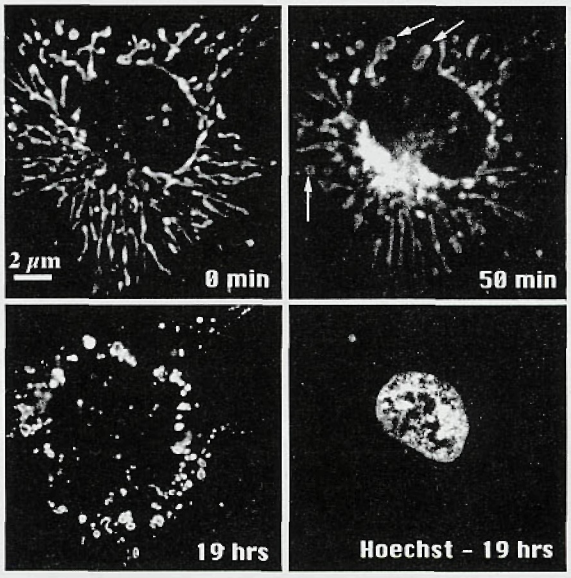

Figure 1. Monkey epithelial (CV1) cell loaded with 1 μM MitoTracker Red (Molecular Probes), washed in saline, and illuminated for 1 sec every 10 min at room temperature (40x Nikon Fluor objective, 535 ± 25 nm ex filter, 585 LP em filter). Mitochondria were clearly visible in the initial image as long, cylindrical worms (0 min). After 6 exposures (50 min), some mitochondria were swollen (arrows), the dye had clustered around the nucleus, and there was diffuse background fluorescence within the cell indicative of loss of dye from the mitochondria. After 18 hrs at 37 C (19 hrs), the mitochondria were uniformly reduced to small spheres, and the nucleus stained with Hoechst 33258 (Molecular Probes, 380 ± 5 nm ex filter, no em filter) indicative of DNA fragmentation (apoptosis). These changes were not present in unilluminated cells.

pies have been effective for treating skin diseases and many kinds of tumors, there are adverse side effects when the light reaches normal cells that have accumulated the photosensitzer. Nevertheless, photodynamic therapy is a provocative reminder that one person's bane may be another's salvation.

B. Endogenous Toxicity

Light has direct effects on cells, and the most well-understood effects are those caused by UV[4]. Among these, the most serious is DNA damage due to direct absorption of photons at 200 - 350 nm[5]. Such damage occurs in the absence of oxygen at irradiation doses far less than those used in microscopy. Fortunately, there are cellular mechanisms that can repair the more common types of DNA damage[6]. With the high intensities used in light microscopy, such damage may not be so easily repaired.

Visible light is also toxic to cells. Tappeiner recognized this, but Earle was the first to systematically study it. He reported that green light was more toxic than red light, and that the effect was more pronounced in the presence of red blood cells, thereby implicating an endogenous sensitizer in the latter[7]. Years passed before other studies demonstrated that visible light was toxic even in the absence of red blood cells. Parshad and colleagues demonstrated that violet-blue (400 - 490 nm) light was especially toxic and capable of causing genetic damage as well as induction of H_2O_2 production[8,9]. Since H_2O_2 is toxic to cells, it seemed plausible that light-induced H_2O_2 production was a central element in phototoxicity.

Recently, my colleagues and I studied a source of light-induced H_2O_2 production in cells[10]. We found that H_2O_2 was produced when light activated flavin-containing oxidases within peroxisomes and mitochondria. An example is shown in Figure 2A in which blue light was used to stimulate H_2O_2 production in peroxisomes detected using a H_2O_2-sensitive fluorophore. (Note: The same light that stimulated the oxidases also excited the fluorophore.) The wavelength sensitivity of the response was consistent with the involvement of flavins (Figure 2B), and cells overexpressing flavin-containing oxidases produced more light-induced H2O2 than control cells. This and additional experiments led us to propose the model diagrammed in Figure 2C. With sustained stimulation, H_2O_2 production exceeded the local catalase activity allowing H_2O_2 to infiltrate the cytoplasm. The latter could provide a route by which to cause damage in other intracellular compartments.

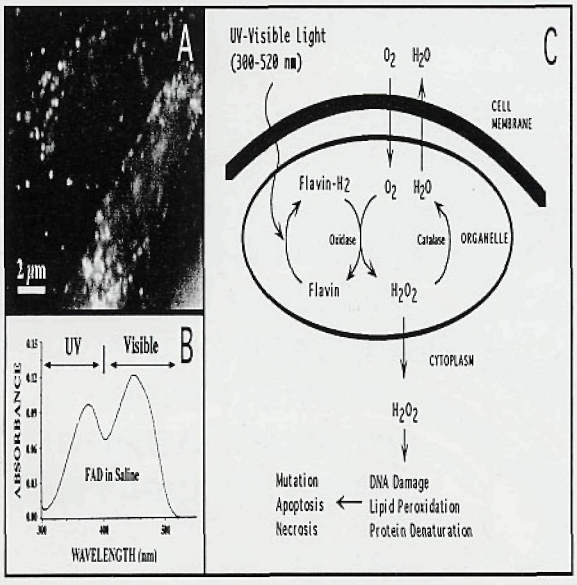

Figure 2. A. Fluorescent response of two CV1 cells loaded with a dye (6-carboxy-2',7'-dichlorodihydrofluorescein diacetate di-acetoxymethyl ester, Molecular Probes) used to detect H_2O_2 production. Upon illumination with blue light (40x Nikon Fluor objective, 490 ± 5 nm ex filter, 520 nm LP em filter), fluorescence was induced in subcellular peroxisomes. The cytoplasmic response was quenched by pre-incubating cells in vitamin C. **B.** Absorption spectrum of flavin adenine dinucleotide (FAD, 10^{-6} M) in saline. **C.** Diagram depicting a flavin-based mechanism underlying production of H_2O_2 and toxicity in response to light between 350-520 nm.

In addition to flavins, there are other endogenous photosensitizers that may also contribute to phototoxicity, *e.g.*, porphyrins, NADPH and tryptophan. Yet another type of phototoxicity is damage caused by illumination with high intensity lasers. Such effects have been found using visible[11] and infrared lasers[12] and highlight the need for caution when using these sources.

C. Remedies

While it is clear that phototoxicity is a serious problem in live cell microscopy, there are ways to reduce it. The most obvious include minimizing the intensity and duration of illumination and using long wavelength filters whenever possible, preferably 580-650 nm (yellow-red). Another approach is to switch from direct visual inspection of cells to the use of solid-state cameras for monitoring cell behavior. Not only are solid-state cameras more sensitive than human photoreceptors to longer wavelengths (peak sensitivity 700-800 nm), their range of sensitivity can be increased using video contrast enhancement. The only drawback to this approach is that visual acuity is slightly degraded at longer wavelengths, although this can be minimized by filtering out wavelengths greater than 700 nm. Another remedy is to bathe cells and tissues in antioxidants, e.g., vitamin C. This water soluble compound can be added to growth media in millimolar concentrations

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to combat light-induced, as well as metabolic-induced, production of reactive oxygen molecules[8,10].

A most remarkable technical advance in live cell microscopy has been the development of multiphoton microscopy. This technique allows excitation of UV-visible fluorophores using high-frequency, pulsed infrared lasers[13]. Near simultaneous absorption of two or more infrared photons is required to excite the fluorophore, and the low probability of such events ensures that it occurs primarily within the focal volume of the objective (ca. 1 μm^3). Infrared lasers are used to minimize or eliminate absorbance by endogenous photosensitizers[10,14], and scanning of samples minimizes stimulation of exogenous photosensitizers. This technique has been used to perform long-term imaging studies of developing embryos without loss of viability[15]. The main drawback is the high cost of the instrument which can exceed $250K even when attached to an existing confocal microscope. As competition among commercial suppliers grows, the cost should decrease although it may be several years before a stand-alone system is offered in the same price range as confocal microscopes. In the meantime, custom-built systems have been developed in many labs, and there is a list server for scientists wishing to learn more about this technique (mplsm-users@its.caltech.edu). ■

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