

A Reporter of UV Intensity Delivered to the Cytosol during Photolytic Uncaging

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ABSTRACT Photolytic uncaging of biologically-active molecules within cells is a powerful technique. However, the delivery of uncaging light into the cytosol can vary between cell types, individual cells of the same type, and different loci within an individual cell because of optical differences in absorbance and light-scattering properties of the cytoplasm. Here, we demonstrate a simple technique for monitoring the magnitude of cytosolic ultraviolet delivery during uncaging, which also leaves a quantitative and persistent record of this within the cells. The simple method shown here provides a much needed universal monitor of the delivery of ultraviolet light to molecules within the cytosol, providing a much needed parameter for the correct interpretation of uncaging experiments.

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Photolytic uncaging of compounds within the cytosol of cells is a powerful tool for cell biology, especially for investigating the signaling mechanism of cells (1). Photo-release of a biologically-active compound within an individual cell or a population of cells on demand has been used to explore the roles of Ca^{2+} , IP_3 , ATP, cAMP, and other molecules. The list of caged compounds is ever increasing as more reagents and chemistries are exploited (2). Some of these compounds can also be synthesized as cell permeant esters, which permits their loading into cell populations as it negates the need for microinjection into individual cells. The critical part of the photo-release technique is the delivery of light, typically ultraviolet (UV) light near 360 nm, into the cell cytoplasm. This causes photolysis of the light-sensitive bond and release of the caging moiety and unmasking of the required form of the compound in the cytosol. The amount of photolytically-generated compound in the cell thus depends on the amount of light (photon flux) which reaches the caged compound within the cytosol.

Attempts to quantify the efficacy of uncaging within the cell have been made by coupling a fluorescence change to the uncaging event (3). However, this is usually not possible and instead uncaging efficiency is estimated by extrapolating from experiments performed in droplets, in which the environment is controlled and defined, to the cytosol. As uncaging in a droplet allows an estimation of the relative photon flux from the uncaging illumination to be made, the percentage photolysis of caged compound within the cell can then be estimated by taking account of the uncaging parameters (1). However, optically, the cell is rarely as translucent to the uncaging illumination as is the experimental droplet. The optical properties of cytoplasm are complex with light scattering by small intracellular particles and granules that can attenuate the incident light entering the cell profoundly (4). In addition, there are a number of molecules

in the cytosol which absorb light at wavelengths necessary for uncaging. For example, with an extinction coefficient of $6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 339 nm (5), and a cytosolic concentration of about 1 mM (6), NADH alone will absorb >5% of the light passing through a cell 40- μm thick. When one considers that a number of other small molecules and proteins within the cell also absorb light in the UV region, the total absorbance by cell cytosol will be considerably higher. The problem for interpreting the effect of uncaging in different cells is exacerbated by cell-to-cell variation. Even in apparently homogeneous populations of cells, there is rarely uniformity of size, granularity, or biochemical parameters (7). The number of cytosolic light-scattering granules can also change during the cell cycle or after stimulation and NADH levels also change dramatically during cell activity (8). In cells which form pseudopodia, there can also be a large difference in light scattering between the pseudopod and the cell body. The organelle-free pseudopod can be optically translucent (4). Local uncaging in this region may generate more product than elsewhere in the cell.

The ability to monitor the relative cytosolic exposure to UV of the cytosol within individual cells would therefore facilitate the ability to interpret the effects of uncaging with response outcomes from individual cells.

We report here a simple approach which provides a monitor of the exposure of molecules within the cytosol of individual cells to UV illumination. The method can be used to monitor the relative extent of UV exposure of different cells in a population or to compare exposures of cells in different experiments. The method is especially useful as it records the extent

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of UV exposure with an innocuous but persistent fluorescent marker within cells. This may be especially useful in motile cell populations as the cells exposed to UV illumination carry a record of whether they were exposed and by how much. The future activity of cells can thus be charted even when the cells do not remain at the location of the original UV exposure.

This method relies on the UV-induced photo-oxidation of hydroethidine (also called dihydroethidium) (Fig. 1 a). There is a single report that UV exposure of hydroethidine (HE) generates a red fluorescent compound (9), but we are not aware that this reaction has previously been investigated or exploited. Although hydroethidine has an absorbance maximum at 345 nm (10), the UV light-induced reaction is unlikely to be the result of a direct photolytic event as we have found that it is dependent on molecular oxygen. Thus, it likely follows the pathway established for its reaction with superoxide (Fig. 1 a) leading to the generation of hydroxyethidium (11,12). The product of the reaction is similar to ethidium and becomes brightly fluorescent upon binding with DNA (12).

The important feature of our method is that the reactant and product have very different water solubilities and, consequently, have different abilities to cross the cell membrane. The photo-reaction converts the cell permeant hydroethidine (soluble in dimethyl sulfoxide) into the membrane-impermeant, water-soluble, charged product, hydroxyethidium (Fig. 1 b). This change in ability to permeate the membrane is important because it means that photo-generated hydroxyethidium in the extracellular medium does not contaminate the signal as it cannot cross the membrane and gain access to nuclear DNA (Fig. 1 b). In contrast, intracellularly generated hydroxyethidium will have free access to the nuclear DNA. Nuclear fluorescence thus reports only the UV-induced reaction product which is generated within the membrane diffusion barrier, i.e., only within the cytosol (Fig. 1 B) and thus provides a monitor of UV exposure of molecules only within the cytosol.

We demonstrate here the UV light-induced photo-conversion of hydroethidine to hydroxyethidium within a number

of cell types with different nuclear shapes and with DNA of different degrees of condensation (Fig. 1 c). The increase in nuclear fluorescence within the cell after UV illumination confirms that a DNA-binding photo-product was generated. The intensity of the nuclear fluorescent signal is linearly related to the accumulated exposure and thus records the total number of UV photons of exposure (Fig. 2 a). In a given individual cell, the relationship between UV exposure and nuclear fluorescent signal remains constant as can be demonstrated by repeat UV exposures (Fig. 2 b). However, the effect of UV exposure varies between cell-types (Fig. 2 a). Since HE diffuses freely into the cells, its concentration is expected to be uniform. Furthermore, each cell has the same amount of DNA; therefore, the difference in the rate of rise of fluorescence is attributed to differences in the delivery of UV photons to the cytosol. These illustrate differences in optical properties of cytosol in different cell-types and different individual cells within a cell population (4). For HE to be a useful marker of UV exposure during uncaging, it is important that it does not interfere with the ability of the UV illumination to uncage. As HE has an absorbance maximum at 345 nm, it was possible that its absorbance would reduce the efficacy of the uncaging light. However, with an extinction coefficient of $9.75 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 345 nm (10), $20 \mu\text{M}$ cytosolic HE would absorb only 0.2% of the light in a cell $40\text{-}\mu\text{m}$ thick. It therefore adds little to the overall UV absorption of cytosol. This was shown experimentally in neutrophils loaded with caged IP₃, the uncaging of which elicited a classic Ca²⁺ signal (Fig. 2 c). In this system, we have previously found that a $\sim 3\text{--}7$ s exposure to our UV light system were required to elicit the Ca²⁺ signal (13). It was supposed that the generation of IP₃ within the cell was slow so that the delay time represented the time required for the concentration of IP₃ to reach a threshold for triggering the Ca²⁺ signal.

The explanation for the variable delay between cells (13,14) was less clear, but it was possible that it resulted artifactually from differences in the delivery of UV to

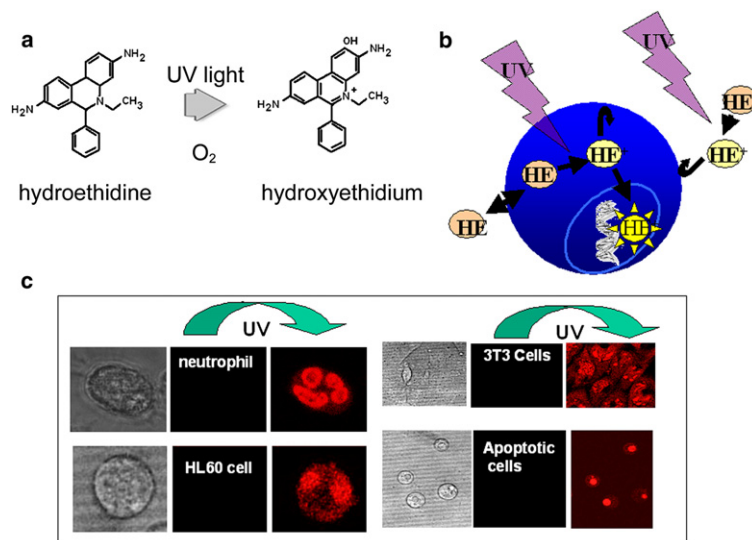


FIGURE 1 Monitoring UV light delivery to the cytosol using hydroethidine. (a) The photo-induced reaction of hydroethidine to generate hydroxyethidium in the presence of molecular oxygen. (b) The different water solubilities of hydroethidine and its photo-induced product allows it to act as a monitor for cytosolic UV exposure (c) Examples of the reaction in cells. The incubation medium for the cells contained hydroethidine (Sigma-Aldrich, Poole, UK, $20 \mu\text{M}$) and UV illumination was for 10 s delivered through a Leica (Leica Microsystems, Milton Keynes, UK) confocal microscope (RS2) objective (x 63 oil) using a 50W Hg arc lamp with filters (330/80 nm input filter; 430 DCLPO2 dichroic; Omega Optics, Brattleboro, VT). The fluorescent signal was detected using 543 nm excitation HeNe laser line and emission at 600–700 nm.

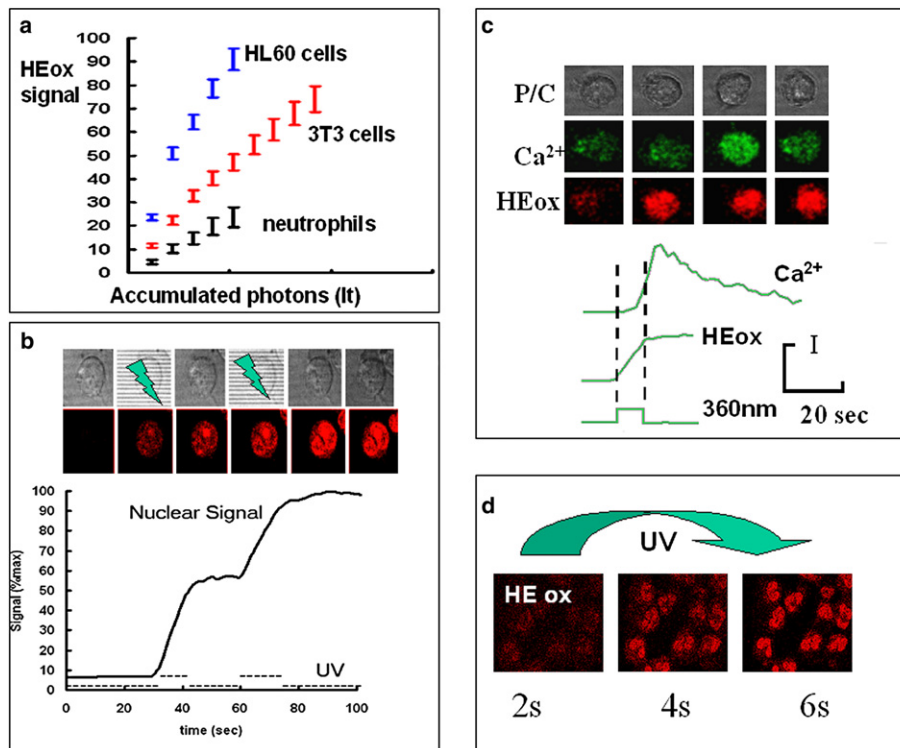


FIGURE 2 Use of hydroethidine during uncaging (a) The graph shows the relationship between nuclear fluorescence (ex. 543 nm; em 600–700 nm) resulting from HE oxidation and the accumulated photons delivered (intensity \times time) for the cell types shown in Fig. 1 C (HL60-blue, 3T3 - red, neutrophil - black) where the bars show the data range for each cell type. (b) A typical two-step exposure experiment demonstrating the repeatability and linearity of HE oxidation within an individual HL60 cell. Each pulse of UV light produced a similar magnitude of nuclear fluorescence at a constant rate of rise. (c) Uncaging of cytosolic IP₃ in a neutrophil loaded with both fluo4 (Invitrogen, Paisley, UK) as a Ca²⁺ probe and caged-IP₃ from its IP₃-PM ester (Alexis-Biochem, Nottingham, UK).

The HE oxidation response begins at the start of UV light illumination, but the Ca²⁺ signal (in response to cytosolic IP₃) begins several seconds later. The data shows the phase contrast image of the cell (P/C), the fluo4 signal (Ca²⁺) and the fluorescence from photo-oxidation of HE (HEox). (d) The

uniformity of UV exposure of cytosol in neutrophils within a cell population. HE oxidation in all neutrophils exposed to UV respond synchronously.

individual cells. Using the UV light monitor, however, it was found that the variable time delays did not result from variations in the optical properties of individual cells as the rate of HE oxidation on UV exposure was synchronous in individual cells within a microscopic field (Fig. 2 d). Instead, the delay may originate within the signaling mechanism within the cell.

The simple method outlined here therefore provides a universal monitor of the delivery of UV light to molecules within the cytosol, providing a much-needed ingredient for the correct interpretation of uncaging experiments.

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