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Two-photon irradiation of an intracellular singlet oxygen photosensitizer: Achieving localized sub-cellular excitation in spatially-resolved experiments

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Abstract

The response of a given cell to spatially-resolved sub-cellular irradiation of a singlet oxygen photosensitizer (protoporphyrin IX, PpIX) using a focused laser was assessed. In these experiments, incident light was scattered over a volume greater than that defined by the dimensions of the laser beam as a consequence of the inherent inhomogeneity of the cell. Upon irradiation at a wavelength readily absorbed by PpIX in a one-photon transition, this scattering of light eliminated any advantage accrued to the use of focused irradiation. However, upon irradiation at a longer wavelength where PpIX can only absorb light under non-linear two-photon conditions, meaningful intracellular resolution was achieved in the small spatial domain where the light intensity was high enough for absorption to occur.

Keywords: *Singlet oxygen, two-photon excitation, subcellular localization, protoporphyrin IX*

Abbreviations: *PpIX, Protoporphyrin IX; ALA, 5-aminolevulinic acid; ABM, artificial bath medium.*

Introduction

The scientific community has long been interested in elucidating the mechanisms of photodynamic cell death. In this process, reactive species formed as a consequence of light absorption by a specific molecule, the photosensitizer, can have an adverse effect when the photosensitizer is placed in or near the cell. Over the years, we have been particularly interested in the production of the lowest-energy excited state of molecular oxygen, singlet oxygen, $O_2(a^1\Delta_g)$, upon energy transfer from the photosensitizer to ground state oxygen, $O_2(X^3\Sigma_g^-)$ (Figure 1) [1]. It is well-established that singlet oxygen is the primary mediator of the photodynamic effect and can initiate both necrotic as well as apoptotic cell death [2–5].

Under normal conditions, photosensitizer excitation occurs as a consequence of light absorption in a linear one-photon process (Figure 1). For many photosensitizers, the one-photon molar absorption coefficient at the irradiation wavelength is sufficiently large that, even with a low irradiance, appreciable amounts of singlet oxygen can still be made. Photosensitizer excitation and singlet oxygen production can also occur as a consequence of light absorption in a non-linear two-photon process (Figure 1) [1,6–9]. Under these latter conditions, where the irradiation wavelength is long enough to preclude absorption in a one-photon process (i.e. there is no resonant transition), photosensitizer excitation will only occur when the irradiance is sufficiently large that two photons can be simultaneously absorbed (i.e. the pooled energy yields a resonant transition to populate a given state). One feature of light absorption in a two-photon process is that photosensitizer excitation and singlet

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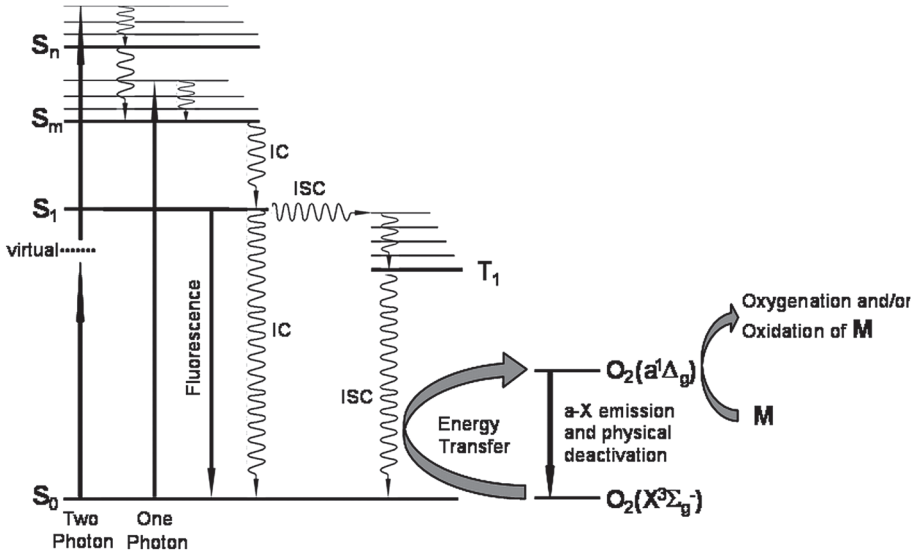

Figure 1. Diagram illustrating the processes of one- and two-photon excitation of a molecule, with the subsequent triplet-state sensitized production of singlet oxygen. It is implied that the oxygenation/oxidation of M plays a role in mechanisms of cell death. IC and ISC denote internal conversion and intersystem crossing, respectively. Depending on the photosensitizer, the simultaneous absorption of two photons may or may not populate the same state as that created upon the absorption of a single higher-energy photon [6,7].

oxygen production can be confined to spatial domains smaller than those in one-photon experiments [10]. (It is important to stress that, in our present context, we consider only the simultaneous absorption of two photons as illustrated in Figure 1, not the sequential absorption of two photons involving the intermediacy of a discrete electronic state with a finite lifetime. Aspects of the latter have likewise been examined with respect to photoinduced cell death [11,12].)

We have recently developed microscope-based techniques whereby singlet oxygen can be produced in, and subsequently detected from, a single cell [1,10,13,14]. The approach involves using a pulsed laser, focused with a microscope objective such that a beam with sub-cellular dimensions is used to irradiate an intracellular photosensitizer. Singlet oxygen can then be detected in a time-resolved experiment using the 1275 nm $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ phosphorescence as a probe. With this technique, we have demonstrated that the kinetic behaviour of singlet oxygen can reflect the inherent inhomogeneity of a cell; the lifetime, reactivity and, hence, diffusion distance of singlet oxygen depend on where in the cell it is made [15].

Over the years, data have been compiled to indicate that the site of singlet oxygen production within a cell influences the susceptibility of that cell to death [2,3,16–20]. Most of these conclusions have derived from experiments in which the sensitizer has been somewhat preferentially localized in a specific subcellular domain or organelle. For example, it is known that the production of singlet oxygen in or near the mitochondria can be particularly toxic. In light of our recent microscope-based singlet oxygen experiments, it is reasonable to now monitor cell viability under conditions in which focused laser light defines the intracellular domain where singlet oxygen is produced.

We first consider that the diffraction of light places a limit on the spatial resolution that can be easily obtained. Although a number of techniques are available by which one can obtain an excitation volume smaller than that defined by the diffraction limit [21–24], only one of these techniques has, to our knowledge, thus far been exploited in a distinct experiment related to singlet oxygen. In this case, evanescent wave irradiation achieved in a total internal reflection experiment was used to illuminate the bottom of a cell on a microscope cover slip (i.e. region in and near the plasma membrane) [25]. The data thus recorded showed that the light dose required for cell kill in the internal reflection experiment was significantly different from that in experiments where the incident light propagated through the cell.

Approaches more directly related to our microscope-based studies have also been used to study spatial aspects of one-photon photoinitiated cell death [26–29]. In one study [27], a continuous wave 630 nm laser beam was focused with a microscope objective to yield a ~ 500 nm diameter diffraction-limited spot in the image plane. This light was then used to irradiate cells containing the endogenously-produced photosensitizer protoporphyrin IX, PpIX (generated upon incubation of the cells with 5-aminolevulinic acid, ALA). It was shown that irradiation in a domain with a high concentration of mitochondria-localized PpIX (i.e. the perinuclear cytoplasm) was more effective with respect to cell kill than irradiation in a domain with a low concentration of PpIX (peripheral domains of the cytoplasm). Moreover, 630 nm irradiation into the nucleus was most effective at killing

the cells [27]. At a first glance, the latter is somewhat surprising given that (1) PpIX does not localize in the nucleus [30–32] and (2) there are no inherent chromophores in this spatial domain that efficiently absorb 630 nm light [33].

Microscope-based two-photon excitation of a photosensitizer has likewise been used to examine aspects of photoinitiated cell death [34–39]. However, to our knowledge, only one study has been performed under conditions where the unique attributes of two-photon excitation have been used to excite a photosensitizer with discrete sub-cellular spatial resolution. The key conclusion of this particular study by King and Oh [36] was that intracellular reactive oxygen species can indeed be created upon two-photon excitation of a photosensitizer.

With these previous studies in mind, we set out to examine in greater detail the effects of spatially-localized photosensitizer irradiation in a single cell. In the present report, we demonstrate that caution should be exercised when performing such focused laser experiments using a wavelength of incident light that is readily absorbed in a one-photon process. Rather, more meaningful and substantive intracellular spatial resolution is better achieved under non-linear two-photon excitation conditions where the light intensity high enough for absorption to occur is confined to a small spatial domain.

Materials and methods

Materials

5-Aminolevulinic acid hydrochloride, ALA (Fluka, Denmark), Alexa Fluor 568 Annexin V conjugate (Invitrogen, Denmark) and propidium iodide (Sigma, Denmark) were used as received. A physiologically compatible medium, our so-called Artificial Bath Medium, ABM, was prepared using 140 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 10 mM glucose and 10 mM HEPES (Sigma) [40]. The ABM pH was adjusted to 7.4 with NaOH and the osmolarity was adjusted to 310 mOsmol with sucrose. The Annexin binding buffer was prepared using 140 mM NaCl, 5 mM $CaCl_2$, 10 mM HEPES and the pH was adjusted to 7.4 with NaOH.

Cells

To maintain continuity with other work in our laboratories, both SH-SY5Y neuroblastoma and HeLa cell lines were used in this study. The preparation of samples followed the same procedure described previously [40]. Briefly, the cells were cultivated in 5% CO_2 at 37°C in Dulbecco's modified Eagles medium containing 10% foetal calf serum and antibiotics. Cells were plated when they reached ~ 80% confluence. Cells were washed in phosphate buffered saline and trypsinized (0.5% trypsin). When the cells had detached, they were centrifuged, washed twice with the cultivation medium, then re-suspended in the cultivation medium and plated onto poly-d-lysine coated cover slips. The cell density on the cover slip (~ 60–100 cells/mm^2) was chosen to (1) better facilitate relocation of a given irradiated cell after a period of incubation (e.g. neuroblastoma cells are known to readily migrate) and (2) preclude the influence of the so-called 'bystander effect' in which intercellular signalling influences viability [20]. Therefore, only isolated cells were studied.

Photodynamic treatment

Locally high intracellular concentrations of PpIX are readily achieved by incubating a cell with 5-aminolevulinic acid, ALA, which is a precursor in the biosynthesis of PpIX [41]. The final stages of this synthesis occur in the mitochondria and, as such, PpIX tends to first localize in this organelle [30–32]. PpIX as well as its immediate products of photo-oxidation sensitize the production of singlet oxygen [42,43] and this, in turn, is known to result in cell death [44–46].

Cells were incubated for 4 h in the dark in the cultivation medium containing 1 mM ALA. Preliminary manipulations of the cells on the microscope stage were performed using brightfield illumination with a 640 nm band pass filter (fwhm = 40 nm) to ensure that the PpIX generated from ALA did not absorb appreciable amounts of light. The corners of the cover slips served as a good reference point for repetitively locating a given cell and, therefore, cells in close proximity to the corners were chosen for study. During photodynamic treatment with the focused laser, the PpIX fluorescence was spectrally isolated using a 650 nm band pass filter (fwhm = 40 nm) and imaged with a CCD camera using the dynamic integration application in the associated software package (see below). The acquisition time used to collect this emission and thus create the images was the same as the laser exposure time. Experiments were performed using cells in air-saturated cultivation medium. After photodynamic treatment, the cells were incubated in the dark (5% CO_2, 37°C) prior to assessing cell viability.

Alexa Fluor 568 Annexin V apoptosis assay

A solution of the Alexa Fluor 568 Annexin V conjugate was prepared by adding 20 μL of the conjugate obtained from Invitrogen into 1 mL annexin binding buffer (see above). The cultivating medium was removed from the cells, the cells washed with ABM and 100 μL annexin solution was added. The cells were then incubated for 10 min in the dark at room temperature. Cells were then washed in ABM and

placed on the microscope stage. Fluorescent images were obtained using a steady state excitation lamp (Exfo, X-cite series 120) with a 535 nm band pass filter (fwhm = 20 nm, Thorlabs). A 600 nm band pass filter (fwhm = 40 nm, Thorlabs) was used to spectrally isolate the emission.

Propidium iodide staining

A solution of propidium iodide was added to the cells before actinic irradiation (final concentration = 2.3 μM) and the sample imaged using a 535 nm (fwhm = 20 nm) band pass filter for excitation and a 600 nm (fwhm = 40 nm) band pass filter for emission.

Optical system

The femtosecond laser system. Aspects of this system have been described previously [10,47]. Briefly, the output of a cw Nd:YVO_4 laser (Millenia, Spectra Physics, Santa Clara, CA, USA) was used to pump a Ti: sapphire laser (Tsunami, Spectra Physics). The Tsunami, operating at a repetition rate of 80 MHz, delivered tunable pulses over the spectral range of ~ 725–910 nm with a fwhm of ~ 80 fs [8,47]. This laser was used for the two-photon experiments.

For the one-photon experiments, the Tsunami output was amplified in a regenerative amplifier (Spectra Physics, Spitfire, pumped by a Spectra Physics Evolution Nd:YLF laser). The latter, operating with a repetition rate of 1 kHz, yielded tunable pulses over the spectral range 765–845 nm with a fwhm ~ 120 fs [48]. The Spitfire output was frequency-doubled using a β-Barium Borate (BBO) crystal. Before the BBO crystal, the power was adjusted using a Glan-Taylor polarizer. A long-pass filter placed after the BBO crystal removed excess non-doubled light. The power ultimately delivered to the sample was adjusted by angle-tuning the BBO crystal and with the use of neutral density filters.

Average light power was measured with a meter (Coherent, Head model No. P519Q and FieldMax-II controller).

The inverted microscope. The laser output was directed into an inverted microscope and focused (diameter of ~ 1 μm at the beam waist) using a water immersion 60× objective (Olympus LUMPLFL60XW/IR/0.90) [10,49]. A CCD camera (Evolution QEi controlled by Image-Pro software, Media Cybernetics, Bethesda, MD, USA) was attached to the microscope. The position of the laser spot on the sample stage was ascertained using the CCD camera to image (1) scattered/reflected light from a glass plate and/or (2) emission from a fluorescent dye. Bright field illumination was obtained with the microscope lamp (Olympus, TH4-200) through a bandpass filter (see above). A motorized stage (Prior, model: CS152DP) and controller (Prior, ProScan II) were used to move cells relative to the laser spot.

Results and discussion

Photo-induced cell death in a one-photon absorption process

Irradiation of PpIX in the peri-nuclear cytoplasm of SH-SY5Y neuroblastoma cells with a focused 420 nm laser beam indeed results in cell death as ascertained by the appearance of symptomatic morphological changes and a positive Annexin assay (Figure 2).

For all of the one-photon photosensitized experiments performed in the present study, the incident laser powers were below the threshold currently required for optical detection of the 1275 nm phosphorescence of singlet oxygen from a single cell [10,13]. Moreover, although the incident peak irradiance in our 120 fs pulses can be as high as 1×10^{11} W/cm^2 in these 420 nm experiments, our data do not appear to be influenced by a two-photon transition (i.e. 2 × 210 nm) that may occur in an inherent intracellular chromophore (e.g. DNA). This was confirmed upon irradiation of PpIX-free cells (*vide infra*) and, to a large extent, likely reflects the fact that the two-photon absorption cross-sections of the relevant inherent intracellular chromophores are quite small [50,51].

Cell response to light dose in a one-photon absorption process

Using spatially-localized irradiation, as shown in Figure 2, experiments were performed on neuroblastoma cells to quantify the effect of light dose on cell death. Cells were incubated with ALA and the resultant intracellular PpIX formed was irradiated at 420 nm over a fixed time period of 2 min. For these experiments, light dose was altered by changing the incident laser power. Attempts were not made to quantify the number of incident photons actually absorbed by a given cell (e.g. by integrating the PpIX fluorescence intensity). Rather, we assumed that, if cells were incubated with ALA under identical conditions, roughly the same amount of PpIX would be formed in each cell. Although we have independently shown that this assumption has its limits [31], useful results are nevertheless obtained from this study. Data were recorded under conditions where the focal spot of the laser was localized in the cytoplasm and, independently, in the nucleus. After irradiation, the cell was incubated in the dark for 4 h before the cell's condition was assessed.

Cell response to irradiation was assessed using simple visual examination of cell morphology as well as the Annexin labelling for apoptosis and three response categories were defined accordingly: (1) the

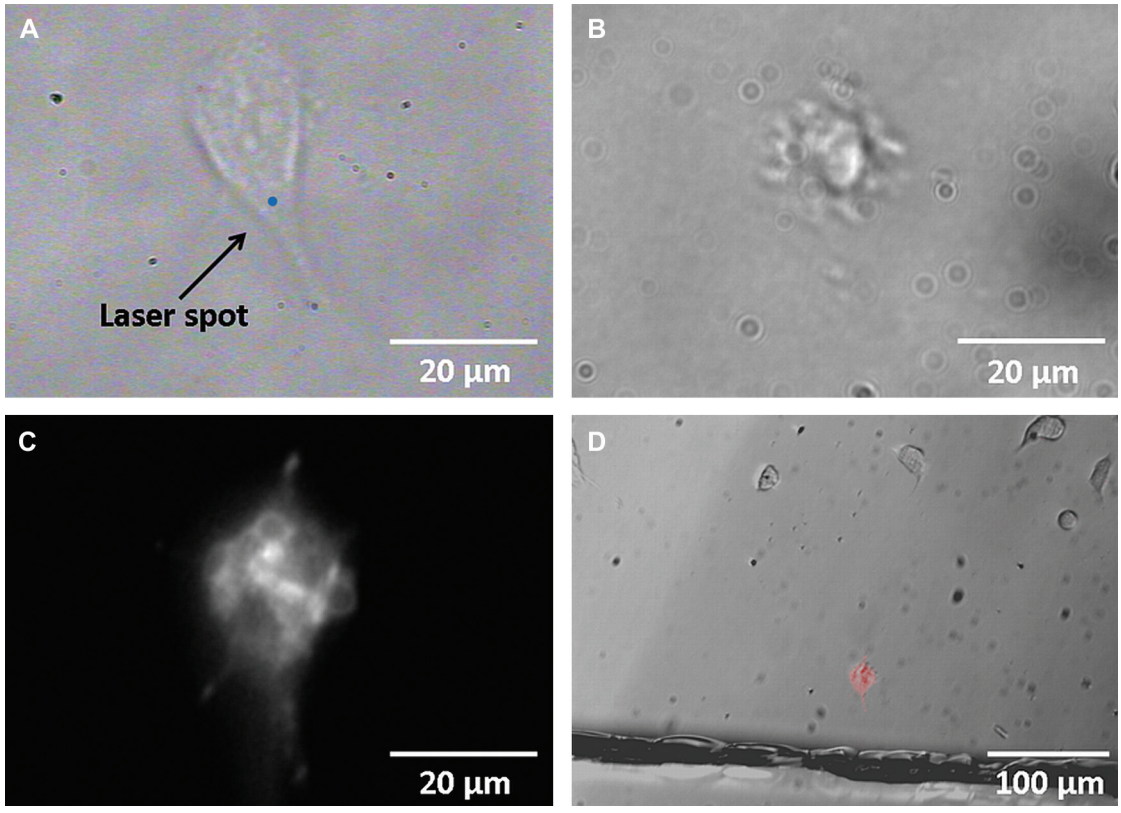

Figure 2. (A) Brightfield image of a SH-SY5Y neuroblastoma cell. The dot shows the position in the peri-nuclear cytoplasm of localized irradiation using the focused laser (420 nm for 2 min at 0.07 µW). After 4 h incubation in the dark, the cell showed (B) classic morphological features of cell death and (C) a positive Annexin response. (D) An overlay of a 20 × brightfield image and a 20 × Annexin fluorescence image (red) shows that only the irradiated cell yielded a positive Annexin response.

cell appeared unaffected, (2) the cell was perturbed such as to yield a positive Annexin response and (3) the perturbation was sufficient that the cell detached from the cover slip during post-irradiation incubation. The results are shown in Figure 3.

We first consider data recorded upon irradiation into the cell cytoplasm (Figure 3A). At low incident power (0.021 µW), irradiation of PpIX appeared not to perturb the cells. As the incident power was increased, however, the fraction of unaffected cells dropped markedly, reaching 0% upon 0.2 µW irradiation. Of the cells that were affected by irradiation, the fraction that detached from the cover slip increased dramatically with an increase in incident power. Nevertheless, of the cells that remained on the cover slip, there was clear evidence of a positive Annexin response, indicating early stage apoptosis. Control experiments performed on cells that had not been incubated with ALA indicate that, even at the highest incident powers used, a large fraction of the cells are unaffected by light. Thus, PpIX absorption is required for the effect.

A less extensive dose–response study was performed upon 420 nm irradiation through the nucleus of neuroblastoma cells incubated with ALA (Figure 3B). The data show that, at low incident powers, irradiation into the nucleus is not as effective as irradiation into the peri-nuclear cytoplasm. However, at higher incident powers, it is clear that one can likewise initiate cell death upon irradiation through the nucleus. This observation is consistent with those of Liang et al. [27], who also showed that spatially-selective irradiation through the nucleus of a PpIX-containing cell was effective in killing the cell. Indeed, Berns et al. found that irradiation into the nucleus was more effective at killing a cell than irradiation into the surrounding peri-nuclear cytoplasm, a result that is arguably mirrored in our limited data set (i.e. see results for the incident power of 0.105 µW).

At first glance, these latter observations may seem odd given that PpIX preferentially localizes in the mitochondria [31,32]; there should be only a small amount of PpIX in the nucleus or in the membranes associated with the nucleus [30]. In considering possible explanations for the data of Liang et al. [27], as well as our own results shown in Figure 3B, one must first be clear about the logistics of sample irradiation in these single cell experiments.

If one uses the analogy of viewing the cell as a 'fried egg', then the incident laser beam propagates through the cell as shown in Figure 4. Upon positioning the focused laser beam to intersect the nucleus, domains of cytoplasm above and below the nucleus will also be irradiated. Moreover, these cytoplasm domains

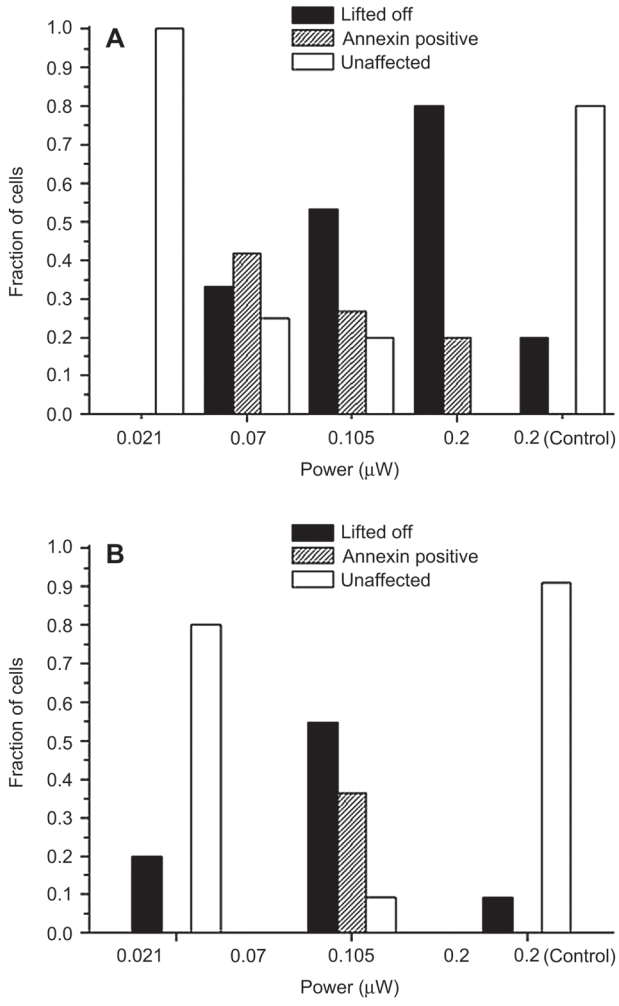

Figure 3. The average incident laser power is shown. Response of SH-SY5Y neuroblastoma cells containing PpIX to one-photon excitation at 420 nm. The irradiation period was 2 min. Focused irradiation into (A) the cytoplasm and (B) the nucleus. Cells that had not been incubated with ALA were used as the control. At a given laser power, the response of ~ 10 cells was assessed.

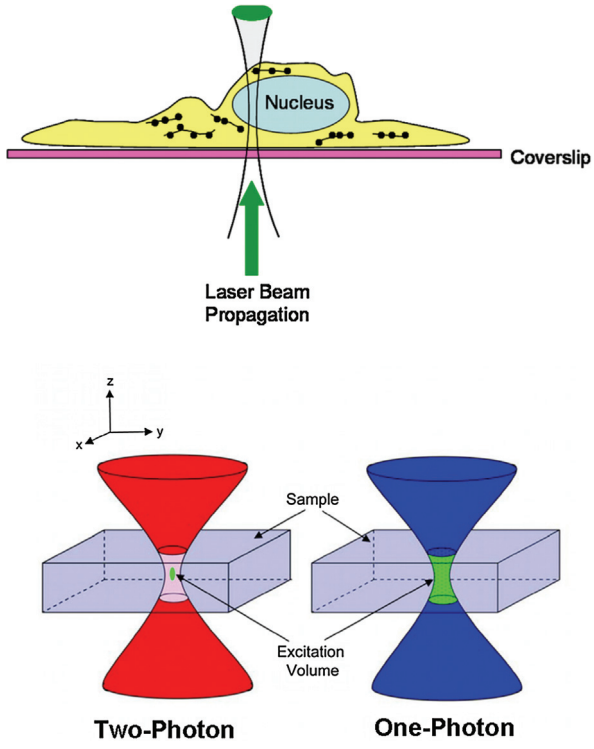

Figure 4. (top) Illustration of the propagation of the focused laser beam through the cell. The short lines with attached dots are meant to illustrate mitochondria associated with cytoskeletal proteins. (bottom) Illustrations of the excitation volumes, coloured in green, in one- and two-photon experiments. Given the optics used in our experiments, the ellipsoidal excitation volume upon 800 nm irradiation in a two-photon experiment has a diameter at the beam waist (i.e. x–y plane) of ~1 μm and an axial length (i.e. z direction) of ~4 μm [10]. Thus, in a single cell experiment, it is expected that two-photon excitation will occur along a large fraction of the path of beam propagation through the cell.

will likely contain some PpIX-containing mitochondria that would, in turn, produce singlet oxygen (mitochondria tend to be more densely located in the peri-nuclear cytoplasm [31,52]). It is now fairly-well established that the lifetime of singlet oxygen in a cell can be as long as ~ 2–3 μs [1]. In turn, this means that the distance travelled by singlet oxygen over the period of ~ 10 μs (i.e. ~ 4–5 lifetimes) can be as great as ~ 155 nm [53]. Thus, any singlet oxygen produced immediately outside the nucleus could easily migrate into the nucleus. Indeed, it has already been established that intracellular singlet oxygen can diffuse across the cell membrane into the extra-cellular medium [49,54]. Alternatively, PpIX and/or its photoproducts could diffuse out of the mitochondria and into the nucleus and thereby sensitize the production of singlet oxygen *in situ*. As already mentioned, however, the available evidence indicates that PpIX does not localize to a great extent in the nucleus [30].

Although the points mentioned above may indeed play a role upon irradiation into the nucleus, it is still difficult to accommodate the observation of Liang et al. [27] that the most effective cell kill arises upon selective nuclear irradiation; particularly in light of independent observations that irradiation of a singlet oxygen sensitizer that specifically localizes in the nucleus is not as effective in killing cells as irradiation of mitochondria-localized PpIX [20]. The answer to this apparent conundrum becomes clear when imaging PpIX fluorescence upon focused irradiation of a single cell.

Sensitizer fluorescence in a one-photon absorption process

In creating images based on PpIX fluorescence, it is important to recognize that the fluorescence quantum yield of PpIX may depend on its intracellular location (i.e. Φ_f can change by a factor of ~ 4–8 depending on whether the immediate environment contains proteins, for example) [55]. As will become apparent shortly, however, even if our data are influenced in

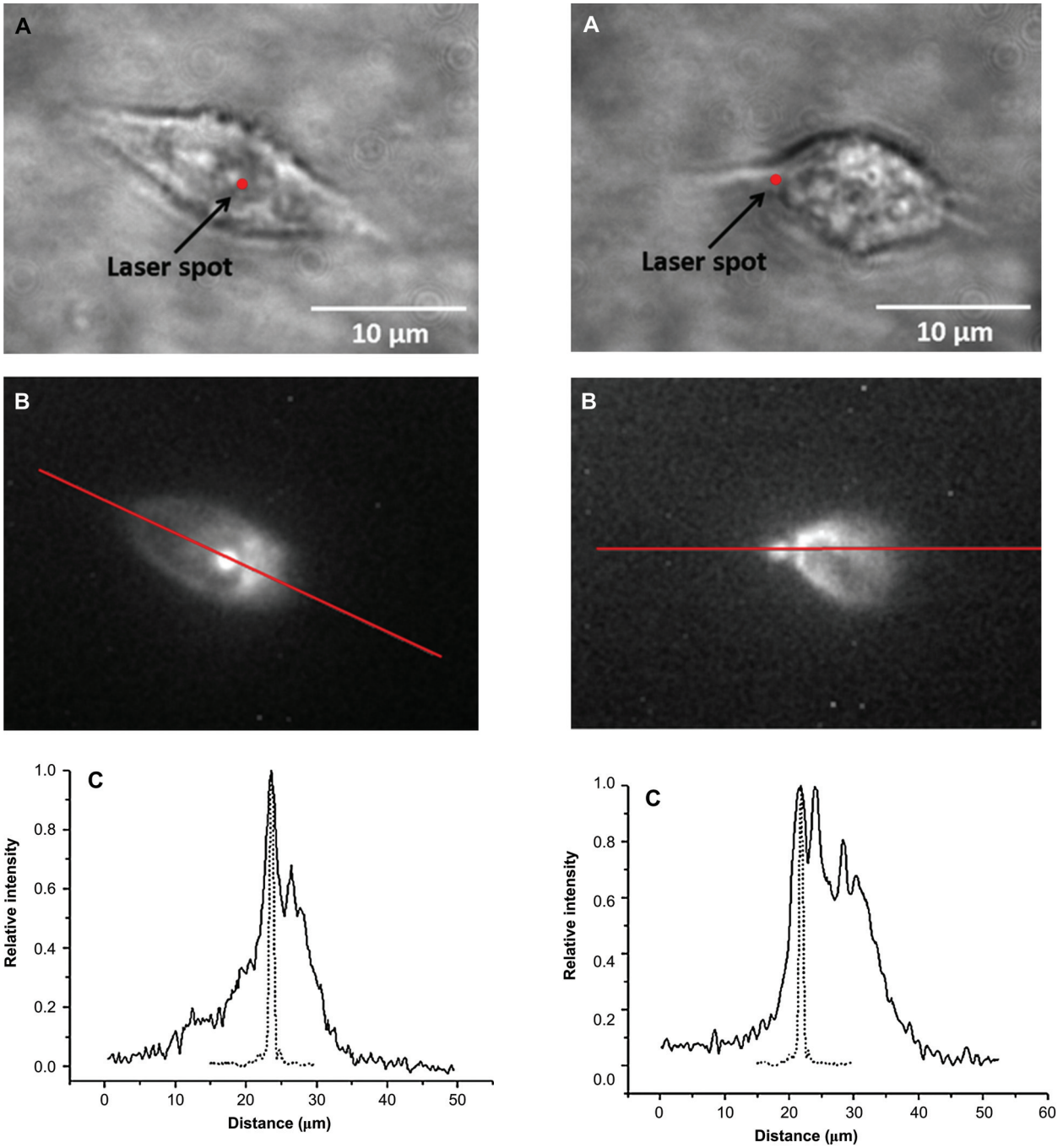

Figure 5. (A) Brightfield image of a SH-SY5Y neuroblastoma cell irradiated in the nucleus with a focused laser beam; 420 nm at ~ 0.40 µW for 1 min. The red dot approximates the cross-sectional area irradiated. (B) Image of PpIX fluorescence obtained upon integration over the period of irradiation. A line has been drawn through the cell to indicate the spatial domain from which an intensity profile (C) was obtained. The dotted line in (C) shows the intensity profile of the laser beam where it intersects the cell. Analogous data were recorded upon focused 420 nm irradiation of a HeLa cell.

Figure 6. (A) Brightfield image of a SH-SY5Y neuroblastoma cell irradiated in the cytoplasm with a focused laser beam; 420 nm at ~0.40 µW for 1 min. The dot approximates the cross-sectional area irradiated. (B) Image of PpIX fluorescence obtained upon integration over the period of irradiation. A line has been drawn through the cell to indicate the spatial domain from which an intensity profile (C) was obtained. The dotted line in (C) shows the intensity profile of the laser beam where it intersects the cell. Analogous data were recorded upon focused 420 nm irradiation of a HeLa cell.

this way, we can still draw substantive conclusions from the results obtained.

PpIX fluorescence-based images of a cell recorded upon focused irradiation into the nucleus and cytoplasm are shown in Figures 5 and 6, respectively. As shown in these figures, the cross-sectional intensity profile of the focused laser beam corresponds to a single peak [10].

Several features are clearly visible from the PpIX fluorescence data obtained. First, in both the nucleus and cytoplasm experiments, there is a distinct domain

of high fluorescence intensity that corresponds to the width of the laser beam, as expected. Secondly, a relatively intense signal is recorded from the irradiated domain when the focused laser is positioned in the nucleus. This second observation could reflect several things: (a) there are indeed PpIX-containing domains of cytoplasm above and/or below the nucleus, as illustrated in Figure 4, (b) some PpIX and/or its photoproducts has migrated out of the mitochondria into the nucleus and/or (c) some fraction of the 420 nm excitation light is transmitted through the 650 nm bandpass filter used to isolate PpIX fluorescence. With respect to the latter point, however, control experiments performed on cells to which ALA had not been added only showed a very weak signal. Thirdly, and most importantly, the PpIX fluorescence observed is not confined to the spatial domain irradiated. Rather, fluorescence is observed from a large fraction of the cell upon irradiation in either the nucleus or cytoplasm. This last observation clearly indicates that, despite focused irradiation, the intracellular spatial selectivity obtained in these one-photon experiments is very poor.

The PpIX fluorescence intensity profiles shown in Figures 5 and 6 are consistent with what one would see if an appreciable amount of the incident light was scattered upon interaction with the cell [56,57]. Indeed, this observation is entirely expected. The cell is a very inhomogeneous entity consisting of domains with a different density and different refractive index, many of which are on the same size scale as the wavelength of light used to excite PpIX.

Some preliminary conclusions can be readily drawn on the basis of the data presented thus far. In particular, it is now more easily seen how focused laser irradiation into the nucleus of a PpIX-containing cell could, in fact, result in very efficient killing of the cell. Although some singlet oxygen will indeed be generated upon PpIX irradiation in the direct path of the focused laser beam, light scattering by the nucleus will deliver photons to a comparatively larger neighbouring spatial domain. Because mitochondria tend to be more densely located in the peri-nuclear cytoplasm [31,52], it stands to reason that one could, in fact, have a larger PpIX excitation cross-section due to scattered light from the nucleus as opposed to direct focused excitation into the cytoplasm itself. Thus, the observations of Liang et al. [27] can be readily explained using this argument of light scattering.

It is expected that the extent of light scattering, and hence absorption by the photosensitizer, will not only vary greatly from cell to cell, but will depend on (1) the intracellular position irradiated, (2) which stage in the cell division cycle one encounters at the time of the experiment and (3) whether or not a given cell is already responding to a given perturbation and, as such, may have altered the density of intracellular domains [31,58].

Spatially-resolved irradiation under conditions of two-photon light absorption

Given the fact that the cell inherently scatters light, a more reasonable approach to achieve spatially-resolved irradiation is to exploit the features of a non-linear two-photon absorption process. Specifically, we would like to exploit the fact that scattered light will be less intense than light within the beam waist of the focused laser and, as such, the scattered light may not exceed the threshold necessary for non-linear two-photon absorption.

Irradiation of PpIX at 800 nm will not populate an excited state in a one-photon transition; the lowest energy excited state of PpIX corresponds to a one-photon transition with a band onset at ~ 700 nm. However, PpIX does absorb light in a two-photon process, admittedly with a comparatively small absorption cross-section (2.0×10^{-50} cm^4 s/photon at 790 nm) [59]. Thus, upon irradiation of PpIX at 800 nm using a sufficiently intense laser beam, light absorption will occur as a consequence of two-photon absorption only where the incident photon flux is sufficiently large (i.e. within or very close to the beam waist of the focused laser), and lower intensity scattered light should not be absorbed at all.

The PpIX fluorescence data obtained are consistent with this hypothesis and indicate that spatially-resolved excitation can indeed be achieved using a focused laser under two-photon conditions (Figure 7). Specifically, the cross-sectional intensity profile obtained from the PpIX fluorescence image shows a distinct localized population of PpIX excited states. In the ideal case, one would confirm that the fluorescence intensity obtained from this image scales quadratically with the incident photon flux. Unfortunately, this latter experiment is difficult to perform given (a) the integration times currently required to obtain an acceptable PpIX fluorescence signal-to-noise level (i.e. 10–15 min) and (b) the dynamic events that occur over this integration period (e.g. PpIX photobleaching, PpIX relocation in the cell, cell movement in the laser beam). Rather, we must be content in knowing that, at 800 nm, PpIX excitation can only occur via a two-photon process (a phenomenon that has been confirmed in bulk solution phase experiments).

In the cells that were irradiated in this way, morphological signs of cell death became apparent after a short incubation period (Figure 7D). This is consistent with the expectation that singlet oxygen is indeed produced upon two-photon excitation of PpIX [38,59]. Although the 1275 nm phosphorescence of singlet oxygen has been detected upon focused-laser two-photon excitation of photosensitizers in bulk solutions, to our knowledge it has yet to be detected in a two-photon experiment performed on a single cell [10]. Among other things, this reflects the low yield of

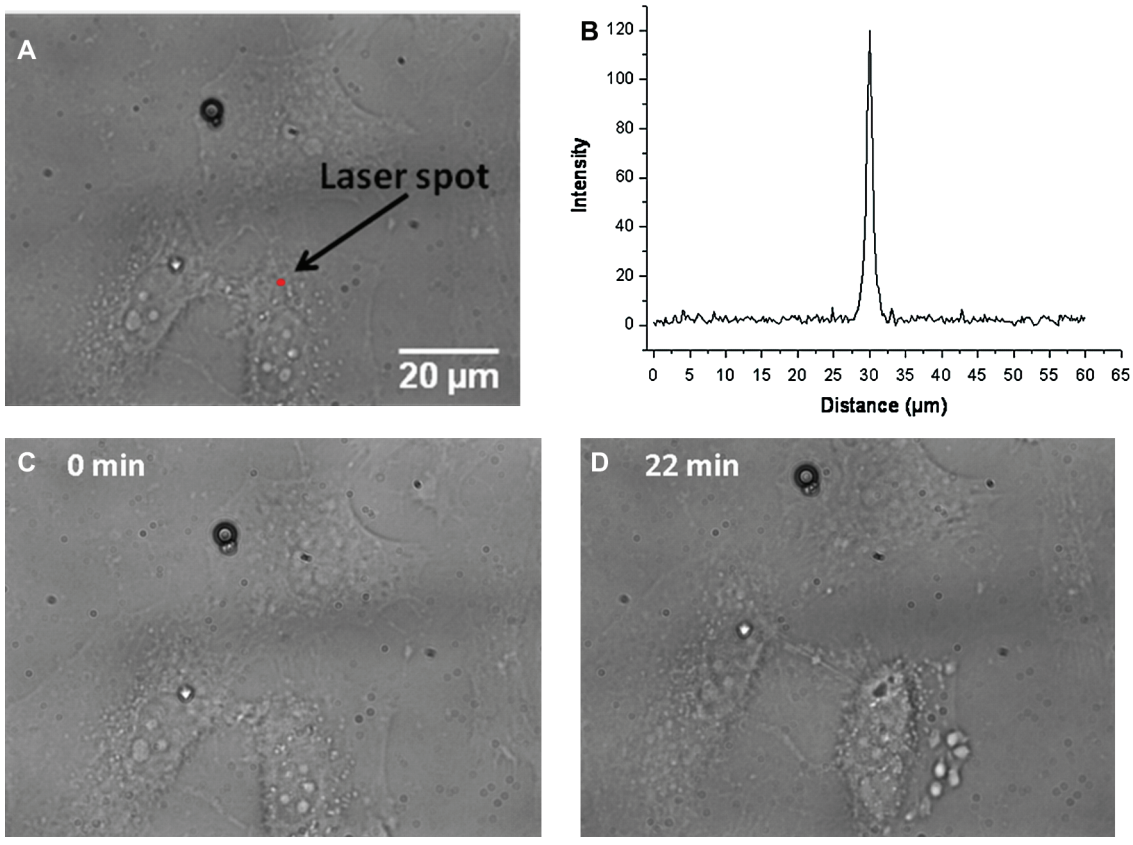

Figure 7. (A) Brightfield image of HeLa cells that had been incubated with ALA. The cell on the lower right was irradiated with a pulsed laser under conditions suitable for two-photon absorption by PpIX (2.2 mW, 800 nm, 15 min exposure). The focused laser beam was positioned in the cytoplasm (red dot). (B) Cross-sectional intensity profile obtained from the fluorescence image of the sample. Data were collected over the 15 min exposure period using a 650 nm bandpass filter with fwhm of 40 nm and were independent of the direction through the sample that was examined. (C) Immediately after the irradiation period, the cell showed no apparent morphological changes that we assign to cell death. (D) After an incubation period of 22 min in the dark, vacuoles characteristic of a stage in cell death appeared.

photosensitizer excited state production in a two-photon process as opposed to a one-photon process.

For the cell shown in Figure 7, it is also interesting to note that, during the post-irradiation 22 min incubation period, a dark spot appeared in this particular cell at the point where the laser was focused. At present, we have no explanation for this isolated observation.

Cell response to light location in a two-photon absorption process

Corresponding two-photon experiments were performed in which the nucleus of a PpIX-containing HeLa cell was irradiated (Figure 8). Under these conditions, a spatially-confined PpIX fluorescence signal was likewise recorded. Although, as outlined elsewhere in this paper, we want to be cautious in ascribing meaning to the intensity of this signal, it was nevertheless clear that fluorescence signals recorded upon irradiation into the nucleus under these two-photon conditions were less intense than those observed upon irradiation into the cell cytoplasm (Figure 7). This observation is consistent with (1) the model proposed in Figure 4 in which the number of PpIX-containing mitochondria is much smaller in this volume element of the cell and (2) the expectation that there is little, if any, PpIX in the nucleus itself.

Although the data in Figure 8 show that PpIX excited states are indeed produced upon 800 nm irradiation into the spatial domain of the nucleus, we were not able to observe signs of cell death after 38 min of incubation in the dark. In the least, this result is consistent with the crude dosimetry recorded in the cytoplasm and nucleus experiments, respectively (i.e. a larger number of PpIX excited states were produced in the cytoplasm experiment where cell death occurred).

Corresponding experiments with 800 nm irradiation were performed on cells that had not been incubated with ALA. Morphological signs we assign to cell death were likewise not observed upon irradiation, either in the cytoplasm or in the nucleus (Figure 9).

Can PpIX fluorescence-based dosimetry predict cell response upon two-photon excitation?

The data shown in Figures 7 and 8 suggest that, upon focused two-photon excitation of an intracellular

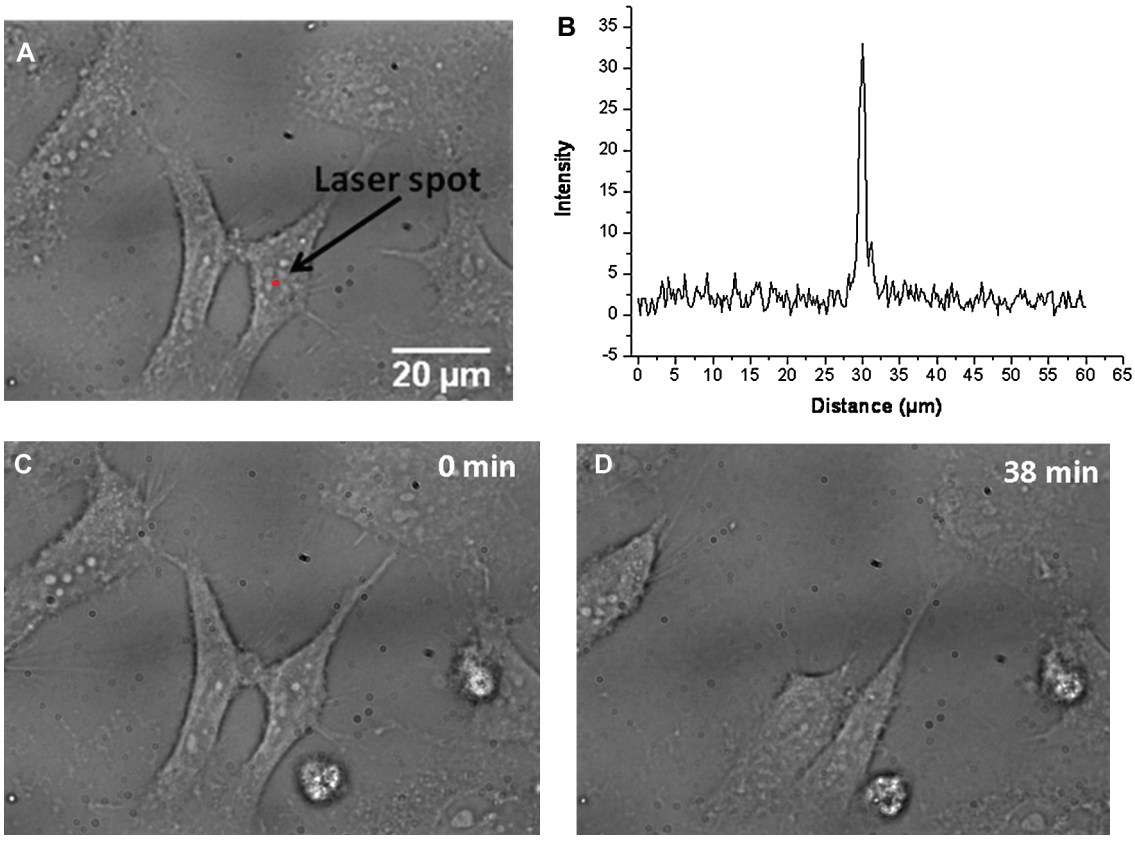

Figure 8. (A) Brightfield image of HeLa cells that had been incubated with ALA. The cell on the right was irradiated with a pulsed laser under conditions suitable for two-photon absorption by PpIX (2.2 mW, 800 nm, 15 min exposure). The focused laser beam was positioned in the nucleus (red dot). (B) Cross-sectional intensity profile obtained from the fluorescence image of the sample. Data were collected over the 15 min exposure period using a 650 nm bandpass filter with fwhm of 40 nm and were independent of the direction through the sample that was examined. In contrast to the data shown in Figure 7, this cell showed no apparent morphological changes that we assign to cell death (C) immediately after the irradiation period as well as (D) after an incubation period of 38 min.

singlet oxygen photosensitizer, where the effects of light scattering are removed, one might be able to obtain a meaningful correlation between the amount of light absorbed and the response of the cell. In turn, one could then examine cell response as a function of the specific intracellular location irradiated. Thus, we set out to perform experiments in which cell response was assessed as a function of the number of excited state photosensitizer molecules produced.

To quantify the effective light dose (i.e. amount of light absorbed by the sensitizer), we used the fluorescence intensity of PpIX as a probe. We have already alluded to potential problems associated with this approach. Indeed, others have likewise pointed to limitations in using PpIX fluorescence as a measure of the light dose upon irradiation of a PpIX-containing system [60,61]. Nevertheless, for our single cell experiments it is certainly the easiest way to start, and useful data are still obtained (*vide infra*). In an attempt to minimize spatially-dependent variations in the PpIX fluorescence intensity, however, experiments were performed by irradiating in approximately the same peri-nuclear domain of a given cell.

In the experiment outlined in Figure 7, we observed signs of cell death upon localized two-photon excitation of PpIX in the peri-nuclear cytoplasm. However, in repeating these experiments, we were unable to establish a reproducible correlation between the effective light dose and the cell response. This is illustrated in Figure 10 for an experiment performed under the same conditions as the experiment shown in Figure 7. In particular, the PpIX fluorescence data show that the cell in Figure 10 likewise absorbed an appreciable amount of light. Nevertheless, unlike the experiment in Figure 7, we were not able to find morphological evidence of a perturbed cell. To be more definitive, we also performed a propidium iodide assay. In the latter, if the cell membrane is compromised, propidium iodide will enter the cell and, upon complexation with DNA in the nucleus, render a fluorescent signal at ~ 600 nm. As seen in Figure 10F, 600 nm fluorescence was not observed, which supports our assertion that, in this particular experiment, the cell was apparently not perturbed.

Thus, on the basis of these experiments, the response of a single cell can apparently not yet be predicted solely on the basis of the light dose absorbed in a two-photon PpIX-sensitized experiment with spatially-localized excitation.

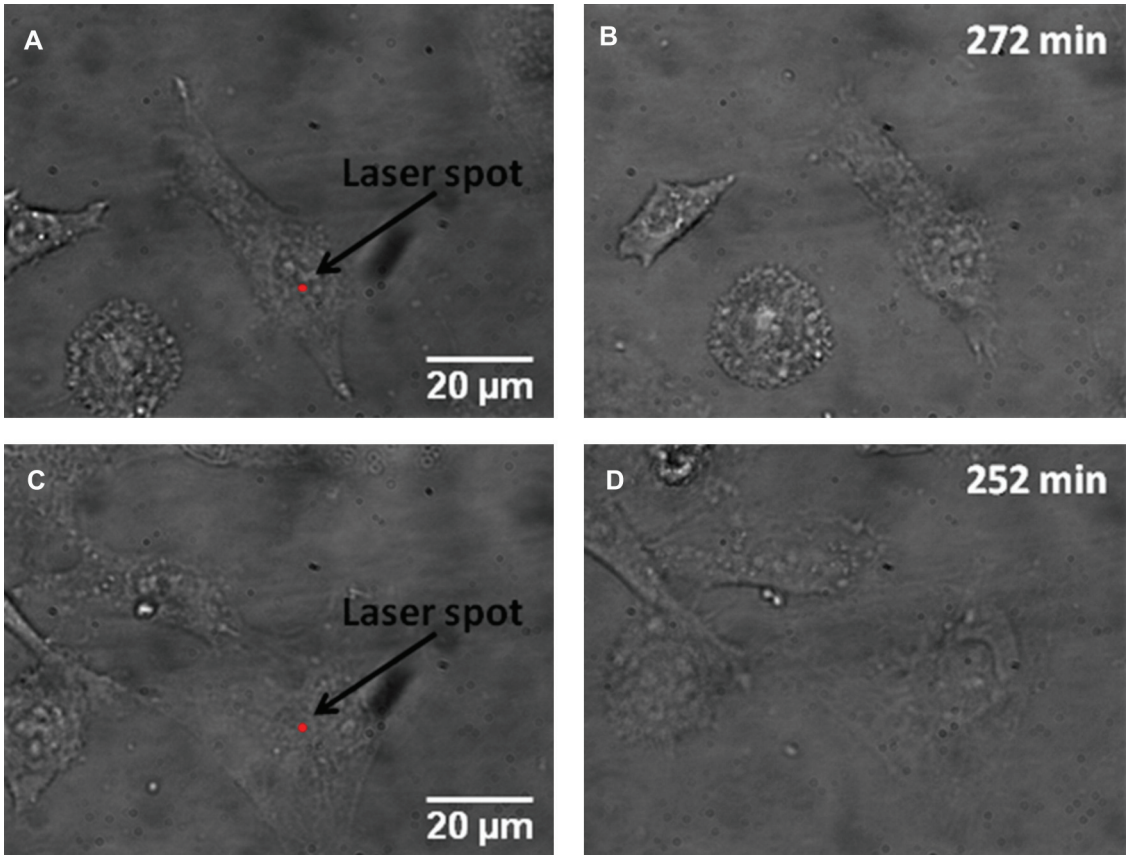

Figure 9. Brightfield images of HeLa cells that had not been incubated with ALA. (A) The cell was irradiated at 800 nm (2.2 mW for 15 min) in the nucleus. The red dot shows the approximate location and area irradiated. (B) Image of the same cell after 272 min incubation in the dark. (C) The cell was irradiated at 800 nm (2.2 mW for 15 min) in the peri-nuclear cytoplasm. The red dot shows the approximate location and area irradiated. (D) Image of the same cell after 252 min incubation in the dark.

Why the failure to predict cell response upon spatially-localized two-photon excitation?

We must now consider why there is a lack of correlation between the apparent number of photons absorbed by PpIX and the propensity for cell death in these spatially localized two-photon experiments. Throughout this discussion it is important to recognize that, in these two-photon experiments, we have truly succeeded in creating an intracellular volume of excited state photosensitizer that is comparatively small (Figures 4 and 7–10).

1. An obvious and indeed likely factor that influences our observations is that, for these single cell experiments, we have simply not accumulated enough data to render the results statistically meaningful. It is well-known that for a given perturbation, two cells can respond in totally different ways. Such behaviour can reflect, for example, the position of each cell in its respective cycle of division or that one cell may have experienced a slightly different environment during growth. The ability to record the requisite amount of statistically meaningful data from such single cell experiments will require some substantive changes in the experimental methodology (i.e. tools that facilitate recording data from hundreds of cells).
2. If it is assumed that the apoptotic cascade is a result of a reaction between singlet oxygen or a secondary reactive species derived from singlet oxygen and a *specific* intracellular target, then the probability of this reaction might not only scale with the volume in which singlet oxygen is produced, but it might also scale with where in the cell that volume is located. In some cases, the focused laser might just be in the 'wrong' place and the singlet oxygen thus produced would not be as effective. If true, this conclusion would have many significant ramifications. As such, this hypothesis certainly justifies further work in this area.
3. Our data may simply reflect the limitations associated with using PpIX fluorescence intensity as a dosimeter [60,61]. However, given the data in Figures 7 and 10, and considering that (a) the fluorescence intensities observed are similar, (b) PpIX photoproducts also sensitize the production of singlet oxygen [42,43] and (c) the experiments were performed under identical laboratory conditions, it seems reasonable that

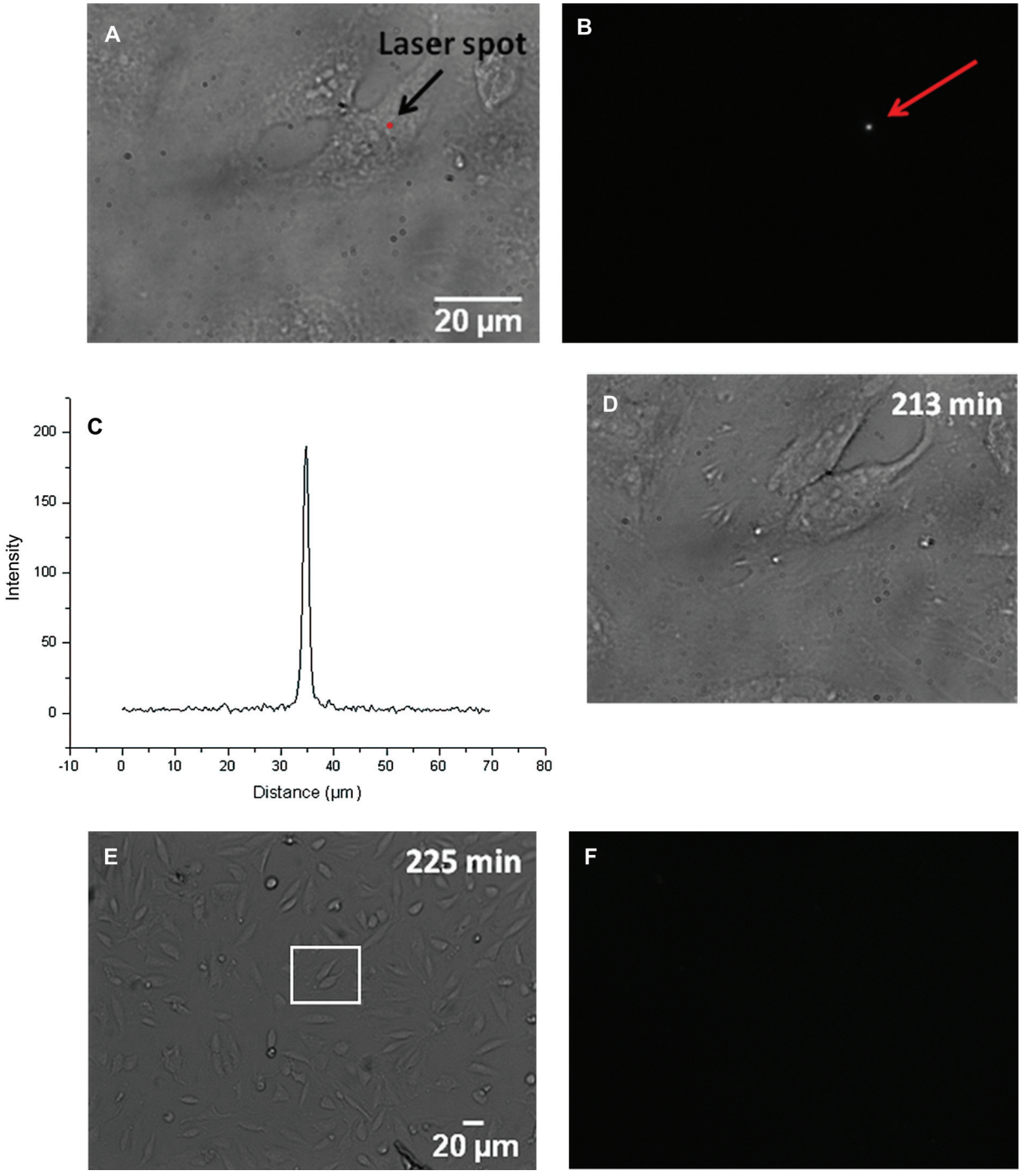

Figure 10. (A) Brightfield image of HeLa cells that had been incubated with ALA. The cell on the right was irradiated with a pulsed laser under conditions suitable for two-photon absorption by PpIX (2.2 mW, 800 nm, 15 min exposure). The focused laser beam was positioned in the peri-nuclear cytoplasm (red dot). (B) PpIX fluorescence image accumulated over the irradiation period using the dynamic integration application in Image-Pro. Irrespective of the direction chosen, the intensity profile along the line through the cell yielded a localized PpIX fluorescence profile (C). (D) The irradiated cell appeared to show no adverse morphological effects 213 min after light exposure. (E) Lower magnification image of the cells shown in (A) and (D) (white square) and the corresponding fluorescence image that shows the lack of propidium iodide incorporation into the cells (F).

our data, rather, reflect the effects of points 1 and 2 above.

In light of our discussion thus far, it is indeed expected that, upon focused one-photon excitation of an intracellular sensitizer, one could see a reasonably good correlation between the propensity for cell death and either (a) the light dose or (b) the general location irradiated (i.e. our data in Figure 3 or the data of Liang et al. [27–29]). As we have established, this is simply a consequence of the fact that, in one-photon experiments, one simply does not irradiate

with true sub-cellular spatial resolution; light is spread throughout the cell which, in turn, contributes to the 'averaging' of the response data.

Conclusion

Upon irradiation of a cell with a focused laser beam, light scattering plays an important role. As a consequence of this light scattering, a comparatively large intracellular spatial domain is irradiated. Because light absorption in a one-photon process does not have an intensity threshold, irradiation of an intracellular photosensitizer with a focused laser beam at a wavelength suitable for one-photon absorption does not impart a high degree of spatial resolution (i.e. scattered light is readily absorbed). On the other hand, given the intensity threshold required for light absorption in a two-photon process, photosensitizer irradiation at a wavelength suitable for two-photon excitation ensures that a comparatively small and localized intracellular volume of excited state photosensitizer will be produced. Our data obtained upon localized two-photon excitation are consistent with the fact that there is indeed a spatial component in the response of a cell to the photosensitized production of singlet oxygen.

These experiments based on spatially-resolved irradiation should complement independent studies where a given photosensitizer is localized in a given intracellular domain by chemical means (e.g. covalently attached to a specific intracellular protein) [1]. The results obtained should further elucidate the spatially-dependent components of mechanisms by which singlet oxygen can initiate apoptotic cell death.

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