

Imaging Photosensitizer Distribution and Pharmacology using Multiphoton Microscopy

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ABSTRACT

Multiphoton microscopy is a powerful tool for imaging sub-cellular distribution of luminescent compounds present in living cells. We have used this tool to study the distribution and pharmacology of photosensitizers in tissue and tissue culture. Murine hepatoma tumor cells dosed with a photosensitizer were briefly photoactivated, then imaged for periods up to several hours. Using the photosensitizer Rose Bengal with green light activation, nearly immediate photolytic release of lysosomal enzymes resulted in catastrophic cell destruction within 5-30 minutes. The magnitude and rapidity of this response is markedly different than that observed with other photosensitizer agents, and is consistent with *in vivo* studies illustrating that Rose Bengal is capable of causing extremely rapid destruction of treated tumors.

Keywords: microscopy, imaging, multiphoton, MPE, femtosecond, photosensitizer

1. INTRODUCTION

Multiphoton excitation (MPE) has become a well accepted method for microscopic imaging of biological systems for a number of reasons: it causes minimal photo-bleaching and photo-damage of the sample; it affords greatly reduced interference from scatter in optically dense media; and it offers exceptional spatial selectivity, particularly along the optical axis.¹⁻³ Progress in the areas of ultrafast lasers, optical coatings, and system architecture over the past decade has transformed MPE microscopy from the realm of delicate laboratory prototype into a useful tool that can be routinely operated by users with minimal specialized training.

An area of particular promise for MPE microscopy is imaging of the distribution of luminescent pharmaceutical compounds in cells. For example, the ability to readily resolve sub-cellular membranes and organelles, coupled with the low photo-damage potential of MPE, facilitates detailed study of the distribution and pharmacology of photosensitizers in individual cells of tissue cultures and carefully prepared tissue specimens. This affords an ideal opportunity to elucidate mechanisms of drug action over prolonged, continuous observation (i.e., over a period of one or more hours). Thus, we report here recent work with the photosensitizer Rose Bengal,⁴⁻⁷ imaged in tissue specimen and tissue culture. Upon photoactivation with green light, the sub-cellular basis for the unique photosensitizing properties of the agent becomes evident.

2. METHODOLOGY

2.1 Microscopy

A titanium sapphire (Ti:S) laser (Coherent, Model 900F) mode-locked at ca. 80 MHz, with a pulse width of ca. 100 femtoseconds, was used to excite samples at 730-800 nm using an average power of ca. 5-20 mW. This light was directed to samples using a non-descanned inverted microscope (as described by Piston et al.⁸) that was constructed around a high-NA objective (such as the Olympus UPlanFL 100X / 1.3 N.A.). In addition, a cw laser (Coherent Verdi-V5-OEM, 532 nm)

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was used for photosensitizer activation; light from this second laser was delivered to the sample using a GRIN microlens fiberoptic diffuser (Pioneer Optics). Data acquisition and sample photoactivation were automated using proprietary system control software.

Reference micrographs were obtained using an Olympus BX60 microscope and transmitted light brightfield observation (halogen illumination). Specimens that were not H&E counterstained were illuminated with green light and the resultant fluorescence photographed using a BX-FLA reflected light fluorescence attachment and U-MWG fluorescence filter cube (510-550 nm excitation, >590 nm emission, Olympus), thus allowing Rose Bengal present in tissue to be readily imaged as orange fluorescence on an otherwise dark background. All photographs were made using Fujichrome 64-T slide film.

2.2 Sample Preparation

Samples were treated with a dilute aqueous formulation of Rose Bengal (i.e., using concentrations of 0.001% to 1%) prior to examination. For tissue samples, this comprised topical application or systemic delivery for times ranging from 5 minutes to 48 h prior to sample acquisition. Treated tissue specimens were then collected and processed by frozen sectioning. A portion of the resultant slides underwent H&E staining, while the remainder were processed without H&E counterstaining. This enabled cross-comparison of Rose Bengal distribution with standard histologic features.

Tissue cultures (murine hepatoma cells, Hepa 1-6, ATCC CRL-1830) were prepared on Nunc coverslip slides, and were treated with aqueous RB for ca. 30 min immediately prior to use. Treated cells were then washed with fresh growth media. Reference stains (i.e., LysoSensor Green, Ethidium Homodimer-1, Hoechst 33342) were added to the final growth media to enable observation of changes in sub-cellular structure via MPE microscopy.

3. DATA AND DISCUSSION

Use of various microscopies for analysis of the penetration pharmacokinetics of topically-applied RB is illustrated in Figures 1 - 4. Figure 1 shows an image of a fresh frozen section of skin that has been H&E counterstained, illustrating the major features of skin, principally the stratum corneum, epidermis, and dermis (progressing from left to right). Columnar epithelial cells are clearly visible at the base of the epidermis. Figure 2 shows the same sample without H&E counterstaining, and illustrates the distinct fluorescent signature of RB present throughout the epidermis. Figure 3 illustrates an MPE image of the H&E stained sample shown in Figure 1, while Figure 4 illustrates a similar MPE image of the non-H&E stained sample shown in Figure 2.

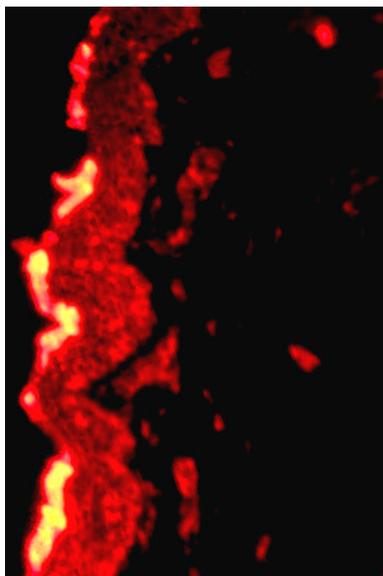
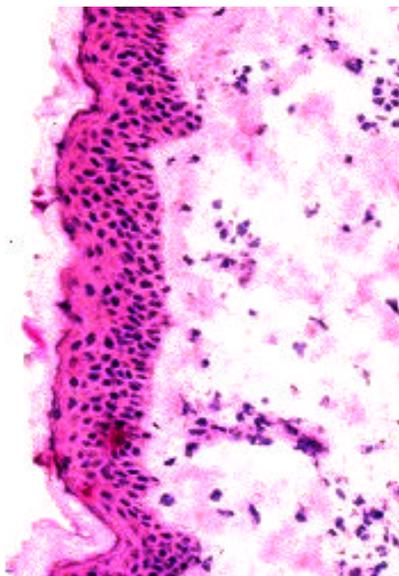


Figure 1 (far left). Brightfield transmitted light image of a fresh frozen section of skin. Sample was stained with H&E after frozen sectioning. Brightly stained keratinocytes of the epidermis are visible in the left third of image (due to uptake of eosin and hematoxylin).

Figure 2 (near left). Fluorescence micrograph of skin section. Localization of topically-applied RB is visible throughout the epidermis, with patches of highly stained stratum corneum visible at surface of the epidermis. Bright patches in the dermis appear to be artifacts from frozen sectioning.

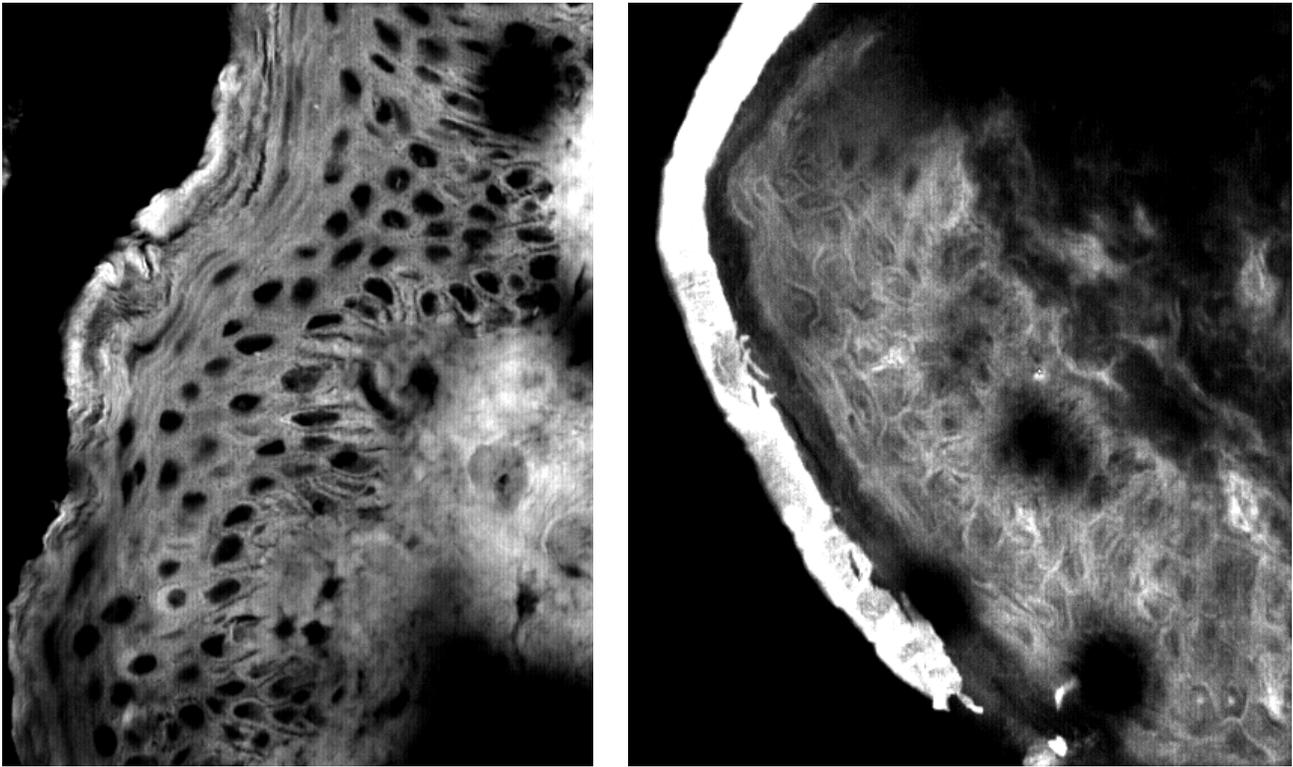


Figure 3 (above left). MPE image of a fresh frozen section of skin (stained with H&E after frozen sectioning). The fluorescence emission of eosin is visible throughout the epidermis, with some areas of dermis also exhibiting staining. This pattern is comparable to that seen in Figure 1. Note that hematoxylin staining is not visible in the MPE image, and that no significant nuclear staining is apparent from the eosin signature. Moreover, apparent staining of membranes in the columnar epithelium is visible at the base of the epidermis.

Figure 4 (above right). MPE image of skin section following topical application of RB. As is evident with the single-photon fluorescence micrograph (Figure 2), RB is localized within the epidermis, with patches of high concentration in the stratum corneum. Sub-cellular localization within cell membranes and exclusion from nuclei is evident.

Comparison of these images demonstrates the valuable insight into drug pharmacokinetics made possible through MPE imaging of tissues. The use of many photosensitizers, such as RB, that exhibit some intrinsic luminescence, allows drug distribution to be directly imaged with MPE microscopy.

Imaging of systemically applied RB in a mouse with an implanted hepatoma tumor is illustrated in Figure 5. Here, a portion of a subcutaneously implanted tumor is shown, along with overlying normal tissue (i.e., skin and underlying tissues). Distribution of drug in the normal tissue is primarily extracellular, which is consistent with that observed in normal skin tissue following topical application (Figure 4). In contrast, RB is located intracellularly in tumor cells. A more detailed image of the tumor (Figure 6) shows evidence of compartmentalization of RB within individual tumor cells.

Imaging of hepatoma cells in tissue culture (Figures 7 and 8) yields results that are also consistent with these observations, showing that RB is internalized within tumor cells and excluded from the nucleus. Concentration of RB within internal structures of the cell is also evident.

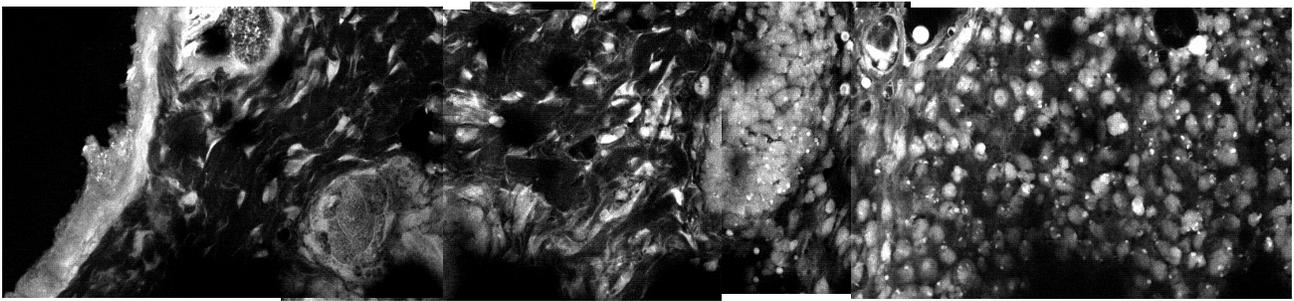


Figure 5. Composite MPE image showing a cross-section of murine tissue spanning the transition from normal skin (leftmost edge of image) to a subcutaneously implanted hepatoma tumor (area at right hand third of image). The sample was prepared by fresh frozen sectioning following systemic administration of RB.

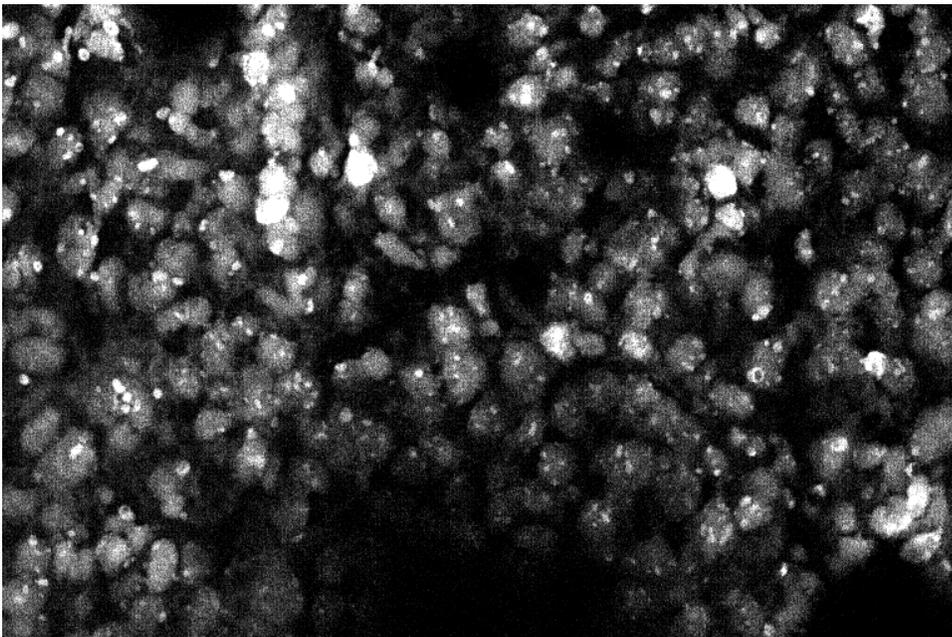


Figure 6. Detailed view of murine hepatoma tumor illustrated in Figure 5. RB localization within tumor cells, especially within sub-cellular organelles, is evident.

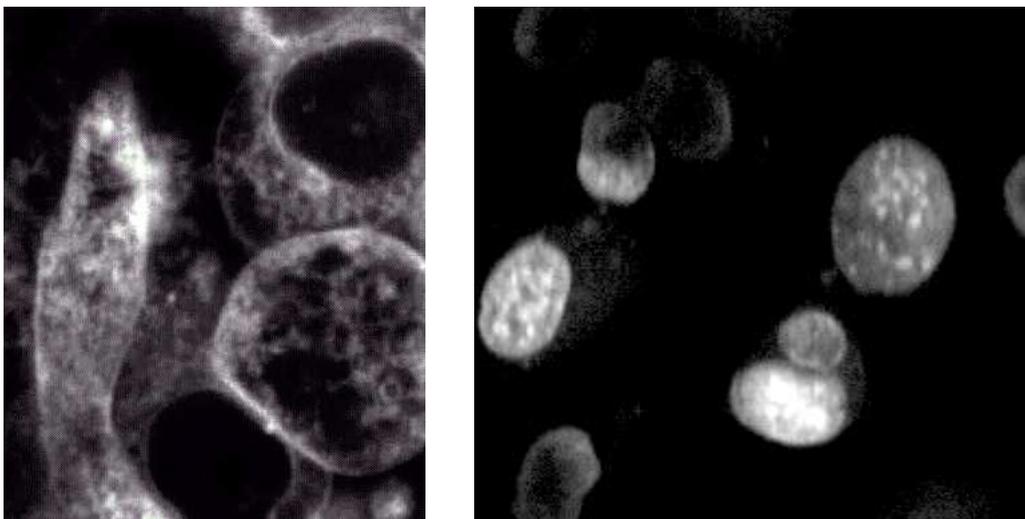


Figure 7 (above left). MPE image of hepatoma cells (grown in tissue culture) following treatment with aqueous RB. Internalization of the agent and exclusion from the nucleus is evident.

Figure 8 (above right). MPE image of hepatoma cells following staining with Hoechst 33342 nuclear stain. Clear demarcation of the nucleus is evident upon comparison with the RB-stained image (Figure 7).

Hepatoma cells pre-treated with RB (i.e., comparable to those illustrated in Figure 7), then briefly illuminated with light at 532 nm to photoactivate the incorporated RB, are illustrated in Figures 9-12. These cells (grown in tissue culture) were counter-stained prior to imaging using LysoSensor Green (LSG, which stains intact lysosomes and is visible in the blue detector channel) and Ethidium Homodimer-1 (ED-1, which is excluded from intact nuclei, but becomes visible in the red detector channel if nuclear integrity is lost); both vital stains were present throughout the experiment, and do not appear to be cytotoxic or cause photosensitization.

In the first set of images (Figure 9A and 9B, corresponding to the red and blue detector channels, respectively, obtained immediately prior to illumination with green light), intracellular staining with RB is apparent with no evidence of nuclear staining by either RB or ED-1 (red channel, Figure 9A); the blue channel (Figure 9B) exhibits a distinctive pattern of lysosomal staining with LSG. Immediately post-illumination (i.e., after 30 s illumination with 532 nm light at an intensity of ca. 250 mW/cm²), some photobleaching of intracellular RB is evident in the red channel (Figure 10A), while the blue channel (Figure 10B) shows marked loss of lysosomal integrity; however no nuclear changes are noted at this point. However, within 5 minutes of photoactivation, two cells exhibit leakage of ED-1 into their nuclei (Figure 11A); further loss of lysosomal integrity is apparent in the blue channel (Figure 11B). At 50 minutes post-photoactivation (Figure 12A and 12B), all cells exhibit marked change of morphology, while the remaining cells exhibit loss of nuclear integrity and advent of large intracellular voids. Thus, these data suggest that photoactivation of Rose Bengal precipitates nearly immediate release of lysosomal enzymes, thereby triggering rapid, catastrophic cell destruction. The magnitude and rapidity of this photolytic response is markedly different than that observed with other photosensitizers (which typically elicit apoptosis or necrosis over a period of several hours or more), and is consistent with *in vivo* studies illustrating that Rose Bengal triggers extremely rapid destruction of treated tumors.

This series of images demonstrates that MPE microscopy can afford high-resolution, non-destructive data useful for elucidation of the pharmacodynamics of a photosensitizer over prolonged periods of time. Similar results can be expected for other classes of pharmaceuticals, allowing the sub-cellular basis of therapeutic activity to be assessed. Note that the illustrated images are from a continuous series, obtained every 30 s, that encompass a total observation time of more than 1 h. Thus, high-resolution animation of such images is possible, further increasing the available information content.

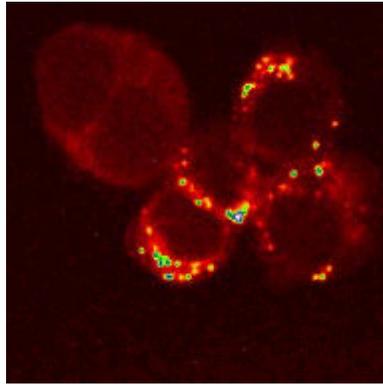
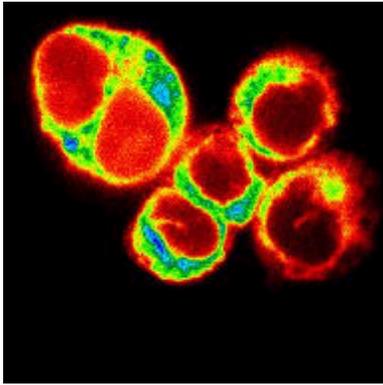


Figure 9A (far left) and **9B** (left) illustrate red and blue detector channel images, respectively, of hepatoma cells cultured on coverslip slides. These MPE images were obtained immediately prior to photoactivation with green light. Intracellular staining with RB is apparent in the red channel (9A), while the blue channel (9B) exhibits a distinctive pattern of lysosomal staining with LSG.

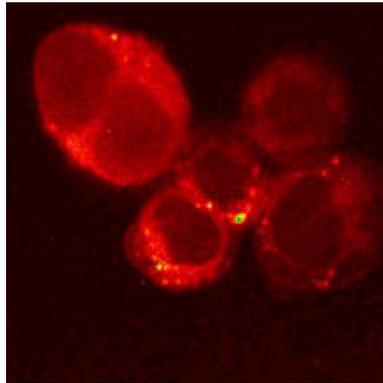
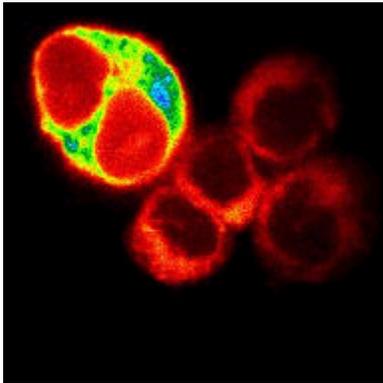


Figure 10A (far left) and **10B** (left) illustrate the cells immediately post-illumination. Some photo-bleaching of RB is evident in the red channel (10A), while the blue channel (10B) shows significant loss of lysosomal integrity.

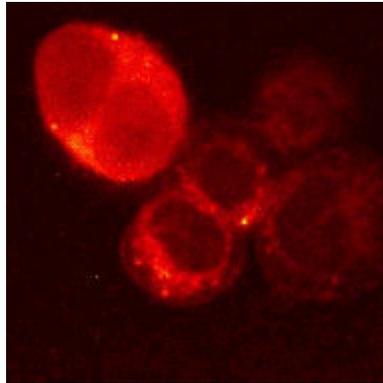
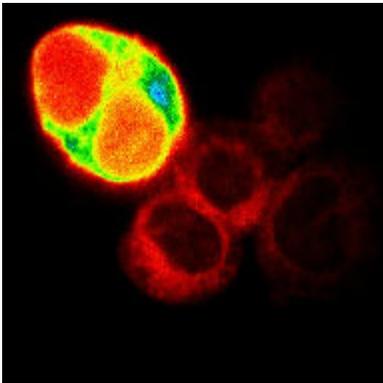


Figure 11A (far left) and **11B** (left) illustrate the cells ca. 5 minutes after photoactivation. Two cells in upper left quadrant (11A) exhibit leakage of ED-1 into their nuclei; further loss of lysosomal integrity is apparent in the blue channel (11B).

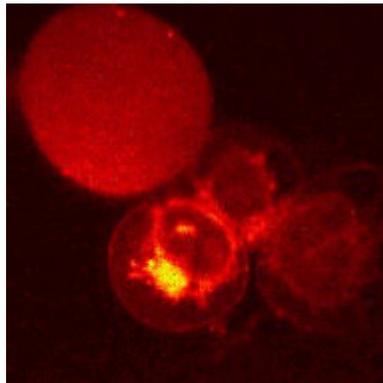
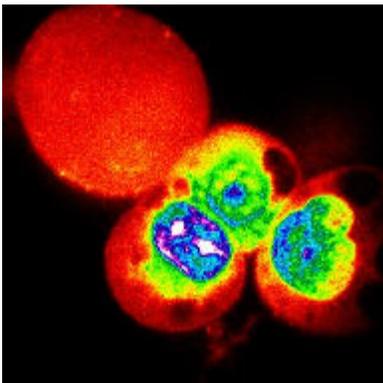


Figure 12A (far left) and **12B** (left) illustrate cells ca. 50 minutes after photoactivation. All cells exhibit marked change of morphology, loss of nuclear integrity and formation of large intracellular voids.

4. CONCLUSIONS

Multiphoton microscopy enables prolonged, quasi-continuous imaging of the sub-cellular distribution of luminescent compounds present in living cells. The unique distribution and pharmacology of Rose Bengal provides a powerful demonstration of the value of MPE imaging for such investigation. Comparison of tumor tissue specimens and tissue cultures confirms the validity of the latter as a model for the former, showing that tissue cultures may be used to accurately and conveniently gauge pharmaceutical mechanism.

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