Feasibility of using multiphoton excited tissue autofluorescence for *in vivo* human histopathology.

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Abstract: Rapid and direct imaging of microscopic tissue morphology and pathology can be achieved by multiphoton imaging of intrinsic tissue fluorophores and second harmonic signals. Engineering parameters for developing this technology for clinical applications include excitation levels and collection efficiencies required to obtain diagnostic quality images from different tissue types and whether these levels are mutagenic. Here we provide data on typical average powers required for high signal-to-noise *in vivo* tissue imaging and assess the risk potential of these irradiance levels using a mammalian cell gene mutation assay. Exposure times of ~16 milliseconds per cell to 760 nm, ~200 fs raster-scanned laser irradiation delivered through a 0.75 NA objective produced negligible mutagenicity at powers up to about 50 mW.

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1. Introduction

Endoscopes and laparoscopes play an important role in medical diagnostics by providing a means to visualize tissue morphology at remote internal sites in a minimally invasive fashion [1]. Laser scanning confocal reflection and fluorescence endoscopes have been developed that provide 3D cellular resolution in tissues [2,3]. Nonlinear excitation has the potential to improve the capability of these existing laser-scanning optical endoscopes and laparoscopes by enabling robust access to UV excitation regimes and nonlinear scattering. The concept of multiphoton endoscopy has attracted attention due to its ability to directly image tissue using

two-photon and three-photon excited autofluorescence and second harmonic signals [4] using nonlinear excitation in the 700 to 800 nm range. An instrument delivering femtosecond pulsed illumination in this wavelength range could provide high resolution imaging of tissue morphology without the use of contrast reagents. The information content extends beyond tissue morphology; for example, imaging based on intrinsic tissue emissions (ITE) such as NADH and flavin autofluorescence can provide clues about metabolic activity [5] and collagen second harmonic imaging can give a direct indication of changes in the extracellular matrix associated with disease [6].

There are a number of engineering challenges in building a functional multiphoton laser-scanning endoscope, such as ensuring efficient propagation of femtosecond pulses through optical fibers, miniaturizing scanning mechanisms and designing highly efficient collection of signal back through the fiber. Many of these aspects have been addressed and a number of advances reported [7], including fiber delivery of femtosecond pulses [8], miniature scanners [9] and the use of double clad fibers for more efficient signal collection [10]. Miniaturized fiber-delivered devices have been demonstrated either as research tools [11] or prototypes designed for future clinical applications [12]. Two-photon action cross-sections of intrinsic molecules are orders of magnitude lower than conventional fluorophores [4], and although a few of these instruments have been used to image collagen second harmonic generation (SHG) [13], imaging of weaker autofluorescence signals has not yet been demonstrated using a fiber-delivered and fiber-collected device.

Here we report two design constraints for multiphoton imaging and spectroscopy systems that would use intrinsic tissue emissions (ITE) for contrast. First, using a numerical aperture, wavelength, and pulsewidth well-suited for clinical ITE imaging, we measured the laser intensities required to acquire acceptable quality images from several epithelial tissue types. Second, we investigated levels of photodamage and DNA mutation as a function of laser power under similar imaging conditions as used in our tissue emission measurements. Studies of cell mutagenicity at the scanning intensities required to acquire useable diagnostic images are critical for determining design criteria for future multiphoton endoscopic and laparoscopic tools. In particular, it is not the lethality of focused nonlinear excitation, but rather the generation of *non-lethal* DNA mutations that could lead to an increased cancer risk and ultimately limit the possibility of using this mode of imaging for clinical applications. Current ANSI laser safety standards for femtosecond NIR pulses cover accidental exposures of the eye and skin to pulsed irradiation in terms of immediate damage, rather than a systematic planned exposure of tissue and any resulting long term effects. The research literature contains limited reports on the safety of NIR ultrashort laser pulses in humans, with the majority being related to the use of femtosecond laser pulses in the eye (e.g [14].) due to the increased use of femtosecond ablation in Lasik surgery. However, these reports focus on immediate damage and complications, rather than the long term effects. Here we present an initial assessment of the mutagenicity risks associated with the femtosecond laser irradiance levels necessary for multiphoton imaging of intrinsic tissue emissions.

2. Methods

2.1 Quantification of tissue fluorescence levels

Epithelial tissues were freshly excised and immediately imaged at 760 nm using a 27X/0.70 NA Olympus microprobe objective lens on an Olympus AX-70 upright microscope stand. The Olympus microprobe objective is 3.2 mm in diameter and 25 mm long with a 220 micron field of view (Fig. 1A). This specialized objective was used because its miniaturized optics can be taken as a reasonable estimate of the performance a future de novo designed multiphoton endoscope objective lens could exhibit. The laser scanning system was based around BioRad MRC-600 galvanometers with a custom acquisition system and a Spectra-Physics Tsunami Ti:S oscillator pumped by a 10W Millennia laser. Multiphoton imaging was carried out at

0.75 sec/frame with a 3 μ s pixel integration time. The pixel dwell time, defined as the spot size (0.4 μ m at 760 nm and 0.7 NA) divided by the line speed during the signal acquisition phase of the scan (176 mm/s) was 2.2 μ s. Epi-detected tissue autofluorescence and second harmonic signals were separated out by a 670 nm long pass dichroic, and then further separated into the two detection channels by a 405 nm long-pass dichroic. The reflected second harmonic signals at 380 nm were filtered using a 380/20 emission filter and detected by a blue sensitive bi-alkali photomultiplier tube (PMT) (R1924 Hamamatsu, Bridgewater, NJ). Tissue emissions from 400 to 580 nm were filtered using a BGG22 blue glass filter and collected by a second bi-alkali PMT. All filters and dichroics were from Chroma Technologies (Rockingham, VT).

To enable quantitative comparisons between images of different tissue types, pixel intensities (integrated PMT output) were converted to photons by a calibration based on shot noise, the dominant noise source with a PMT. For a constant flux of light at the detector, the mean pixel value taken from an image of uniform signal (e.g. a pool of fluorophore) will be given by γN where γ represents the system collection efficiency, PMT gain factor and other instrument parameters that scale the number of photons impinging on the detector (N) to the pixel value. For photon shot noise from a homogenous field the standard deviation will be $\gamma N^{1/2}$ and squaring the mean divided by the standard deviation yields the mean number of photons as a function of the pixel value at a given PMT gain setting. Our imaging systems use lab-built dual output preamps which have both high speed AC coupled pulse counting outputs (>1.5 GHz bandwidth) and DC coupled lower bandwidth outputs for integrating the PMT output at higher fluxes where pulse bunching makes photon counting impossible. Using these amplifiers, the pixel value to photon count conversion calibration factor was checked by direct photon counting at lower photon fluxes where accurate photon counting is possible (< ~10 MHz), and found to be accurate within a few percent. Images were analyzed using ImageJ and the photons/pixel values reported are based on the average pixel values from cytosolic regions of the epithelial cells.

2.2 Chinese hamster lung cell HPRT-mutation assay

V79 Chinese hamster lung cells were used as a mutagenicity test system [15]. The V79 Chinese hamster cell assay is based on the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene [16,17] which has been routinely used in studies of mammalian cell mutagenesis. HPRT is a purine salvage enzyme which converts pre-formed purine bases hypoxanthine and guanine to their respective nucleotides. A cell that sustains a mutation in this gene undergoes either a reduction or elimination of HPRT enzyme activity, and the cell must then use a de novo pathway for DNA synthesis. Culturing of cells in the presence of a toxic purine analogue such as 6-thioguanine, leads to the death of these cells that are HPRT⁺ positive (wild-type cells), allowing the selective enrichment and survival of cells that are HPRT⁻ negative (mutant cells). As a recessive X-linked gene, HPRT is frequently used to monitor mutagenic events in cells since only one gene must be mutated for an effect to be seen. Although mutations measured by reporter loci are the result of a number of complicated events involving a wide range of processes, the frequency of mutations as a function of mutagen (femtosecond excitation in this study) serves as a biomarker for mutagenic exposure and provides a means to quantify light-induced mutation rates.

Cell cultures were maintained in phenol red-free Dulbecco's medium supplemented with 10% fetal bovine serum and buffered with 25 mM Hepes to maintain the cellular pH in air atmosphere during irradiation. 10⁵ V79 cells were seeded into 7 mm wells in cover-slip bottom culture dishes, and incubated overnight. Using a lab-built multiphoton microscope system consisting of a Zeiss Axiovert 35 inverted microscope stand, BioRad MRC-600 galvanometers, a custom built acquisition system and a Spectra-Physics Mai Tai HP Ti:S laser, the cells were illuminated by raster scanning a ~0.75 x 0.5 mm area of the well for 12 scans and then moving the stage in XY using an translation stage (ASI, Eugene, OR) and

tiling macro until the entire 7 mm well was covered. Excitation at 760 nm (~200 fs pulsewidth in the sample plane with a repetition rate of 80 MHz) was delivered through a 20x/0.75 NA Zeiss Fluor air immersion objective lens. The pulse width was measured by interferometric autocorrelation after the objective. Epi-collected V79 cell autofluorescence in the 400-580 nm range was collected during the irradiation scans through a 670LP dichroic and BGG22 blue glass filter (Chroma Technologies, Rockingham, VT). A Pockels cell modulator (model 350-80LABK, Conoptics, Danbury CT) synchronized to the laser scanner was used to blank the laser beam during fly-back so that the specimen was illuminated only while pixel data was being collected. Each field of view received 1.68 seconds of illumination per frame for a total of ~20 seconds (12 passes). In these experiments the pixel dwell time was 3.2 μ s.

Immediately following irradiation, the cells were replated and resuspended in growth medium and incubated at 37°C and 5% CO₂. Negative control cells were either kept at room temperature or in a 37°C incubator for a period equivalent to the total illumination time. After irradiation, cells were grown in standard medium for 7 days (expression period). At this time the plating efficiency (PE) of each experimental group was determined by seeding 200 cells per plate in standard growth medium. The plating efficiency is defined as the proportion of cells that attach and grow relative to the number of cells originally plated. Mutation induction for each group was determined by seeding 2×10^5 cells into each of 5 plates containing growth medium with 40 μ M 6-thioguanine (6-TG) (Sigma Chemical, St. Louis MO). Following 11-14 days of growth, resulting mutant colonies were stained with Giemsa and counted. Cytotoxicity was determined by measuring the relative cloning efficiency or survival of the cell cultures after mutagenic treatment. The Mutation Frequency (MF) is defined as the number of mutants per 10^6 cells and was calculated from the ratio of mutant colonies to the total number of cells plated, and corrected for the plating efficiency.

3. Results

3.1 Quantifying epithelial tissue fluorescence levels

To better understand the levels of excitation and emission collection efficiencies required for ITE imaging we carried out quantitative tissue autofluorescence imaging using optics analogous to what we envision in future microendoscope or laparoscope optics (0.7 NA Olympus microprobe lens with a 3.2 mm diameter tip – Fig. 1A). Several types of mouse epithelial tissues (colon, small intestine, bladder and ovary) were imaged. Based on our previous experience with ITE imaging of both human and rodents tissues [4,18,19], these brightness comparisons between mouse tissue types map directly to human tissues. Although intrinsic emission signals were found to be spectrally similar (broad blue emission peaked around 460 - 480 nm, data not shown), dramatic differences in absolute brightness were observed. A quantitative comparison of the autofluorescence levels under identical imaging parameters of three epithelial tissues from different organs is shown in Fig. 1B-H. For the plot in Fig. 1B, pixel values taken from the cytosolic regions of the epithelial cells were converted to average number of photons per pixel as described in the methods section. Curves were fit to αP^2 where P is the average power measured at the specimen and α is a comparable metric that represents tissue brightness. Colon epithelium was determined to be about 36 times more fluorescent under two-photon excitation than urinary bladder epithelial tissue, while ovarian epithelium was ~10 times brighter than bladder. Intrinsic fluorescence varied as P^2 at lower intensities for all tissues. Differences observed in the power levels at which saturation is reached (indicated by deviation from P^2) suggest that different species of intrinsic fluorophores may be involved, since saturation is a function of the average two-photon absorption cross-section and extent of photobleaching. Bladder autofluorescence increased as the square of the excitation power throughout the measurement range, while signal saturation was observed in colon tissue at powers above ~50 mW.

Figure 1C shows an image of the colon surface taken at 20 mW and consisting of ~26 photons collected per 3 μ s pixel integration time in the cell cytoplasm. Reasonable signal-to-noise (S/N) images could be collected at powers as low as 12 mW (8.6 photons/pixel). This is in agreement with results reported in Rogart et al. [18] from the human GI tract where high contrast intrinsic fluorescence multiphoton images of colon, small intestine, stomach and esophagus were acquired at average powers less than 20 mW. Gastrointestinal tissue is highly autofluorescent and 3D data can be obtained by sectioning down through the colonic surface to base of the crypts (Fig. 1E and Fig. 1G). Imaging depth in this case was limited by the short working distance of the microprobe objective lens (WD = 200 microns). Other tissues such as bladder epithelia exhibit much lower levels of intrinsic fluorescence (Fig. 1D), and although high quality images can be obtained (Fig. 1F and [20]), significantly higher laser intensities are required. We found ovarian tissue to typically be of intermediate brightness, but still in the range that high quality autofluorescence images can be obtained at moderate laser powers, as was true in human ovarian tissue as well [19].

3.2 Effects of irradiation treatment on cellular fluorescence

Figure 2A shows a typical field of V79 cells used in the irradiation/mutation assays imaged by cellular intrinsic fluorescence at 760 nm. During the course of the illumination phase of the experiment, cellular autofluorescence levels were measured and provide insight into the complexity of the photobleaching and photodamage mechanisms as a function of femtosecond laser power. All cells exhibited a decrease in cellular autofluorescence by the end of irradiation period (Fig. 2B). The excitation pulsewidth after the objective (20X/0.75 Zeiss Fluor) was measured to be ~200 fs and the pulse energies ranged between 0.25 to 1.9 nJ. In terms of light dose per cell, the total irradiation scan time per cell in our experimental protocol was ~16 ms (12 scans of 1.33 ms illumination time on an average 20 μ m diameter cell at ~1 second intervals). The 760 nm total dose ranged from 0.32 to 2.4 mJ/cell delivered as 1.3×10^6 pulses per cell. However, it is clearly the nonlinear nature of the femtosecond illumination that is the primary cause of photobleaching, cell damage and mutagenicity, but this aspect is more difficult to quantify since the nonlinear absorptivities, concentrations and species of the cellular chromophores involved are generally unknown. In terms of instantaneous irradiance reached at the center of focal volume at the peak of the pulse, the cells were exposed to peak irradiances ranging from 3.4×10^{15} to 2.5×10^{16} W/m². To determine the relationship between the laser power and the photobleaching rate of the intrinsic fluorophores, a first approximation would be that the decrease in autofluorescence is governed by two- and three-photon (n = 2 or 3) dependent first-order photobleaching:

$$F(t) \propto N_{nb} + N_b \exp[-\beta t]; \quad \beta = a_2 P^2 + a_3 P^3 \text{ and } a_n = \phi_{b,n} \sigma_n \eta_n \int_V p s f^n dV \quad (1)$$

where N_{nb} represents some “unbleachable” fluorescent species, N_b the bleachable species, and the constants a_n contain the first-order photobleaching quantum yield ($\phi_{b,n}$), the absorption cross-section (σ_n), a factor that equates average power in mW to the irradiance at the focus (η_n), times the volume integral of the illumination point spread function raised to nth power.

Fig. 1. Comparison of epithelial layer 2P fluorescence at 760 nm. A. Olympus microprobe lens. B. Power dependence of mouse colon, ovarian and bladder epithelial layers. Solid lines: αP^2 fit (over data points that exhibit P^2 dependence). Photons/pixel values are averages of cytosolic signal. C. Colon surface image acquired at 20mW (~26 photons/pixel). D. Bladder epithelia image acquired at 24mW (1-2 photons/pixel). A standard histogram equalization algorithm was applied to D to adjust contrast. E. Merged autofluorescence and collagen SHG images of colon at 40 μ m deep (40 mW, 37 photons/pixel). F. Merged image of bladder taken at 120 mW (20 photons/pixel). G. Colon at 80 μ m deep (50 mW and 47 photons/pixel). H. Ovarian surface epithelia images at 40 mW (24 photons/pixel). All images were acquired with 760 nm delivered through a 0.7NA 27x microprobe objective lens; emission collected from 420 to 520 nm. 384 x 384 pixels; pixel time: 3 μ s (~0.7 sec/frame; 0.44s illumination time) with no frame averaging, a 20 μ m dia. cell is irradiated for ~4 ms total. All scale bars are 40 μ m.

Under this assumption, which neglects biological responses to oxidative stress, one might expect bleaching to scale as P^2 at lower powers and P^3 at the highest intensities, however, a plot of the bleaching rate constants versus laser power shows a more complex response (Fig. 2C). At low powers there is negligible net loss of fluorescence due to bleaching which may be a result of cellular repair mechanisms or an increased NADH production in response to oxidative stress. At higher intensities the oxidative stress response becomes overwhelmed and bleaching then begins to follow a more typical power law.

Fig. 2. Mutation assay and autofluorescence bleaching during irradiation. A. Typical field of V79 cells used in the mutation assay imaged via cellular autofluorescence. B. Loss of cellular autofluorescence during illumination as a function of laser power. Solid lines are fits to a simple model assuming an exponential decrease (see text). C. Exponential bleaching constant (β) as a function of laser power (solid line is cubic-spline of data to help visualize the bleaching response).

3.3 Cell viability and mutation frequency (MF) levels

The cytotoxic effect of laser irradiation treatment was evident during the first 24 hours after the illumination period and after the 7-day expression period. The survival rates of the irradiated cells measured 1 day and 7 days after illumination (Fig. 3A) showed significant differences (two-tailed P value = 0.0301 at the 0.05 level). The cell count from the control plates were used as the 100% reference in gauging illumination cytotoxicity and cell survival rates. Although there was an overall decrease in survival rate with laser power, some cells continued to propagate normally during the expression period. Explanations for this observation include the possibility that a fraction of the cells missed the irradiation beam during our illumination protocol, cells in certain phases of the cell cycle are immune, or that potentially lethal cellular damage arising from the laser excitation can be repaired in some cases. We believe the latter two explanations are most likely since our XY stage tiling procedure was designed to cover all of the cells. A fraction of these surviving cells, however, have incurred non-lethal mutations as indicated by the HPRT assay results.

The background or spontaneous HPRT MF (mutants per 10^6 cells) observed in control cells was 0.35 ± 0.24 . Figure 3B shows the averaged results of 5 independent experiments relating laser power levels to the observed mutation frequency. Multiphoton scanning for 20 s at 760 nm at laser powers less than ~25 mW produced no significant increase in the observed MF over background, while scanning at powers between 50 and 100 mW produced a 2 - 4 fold increase in MF over the control value. An 11-fold increase in mutation frequency (MF = 3.9 ± 0.72) was found for cells exposed to 150 mW excitation for 20 seconds, indicating a highly significant mutagenic response at the single gene (HPRT) level. The power related increase in MF suggests that high laser power intensities will readily induce localized genetic lesions in V79 mammalian cells.

Fig. 3. Cell survival and mutation frequency as a function of femtosecond laser illumination. A. Cell death after irradiation (plating efficiency) as a function of laser power. B. Increase in Mutation Frequency (MF) (defined as mutants per 10^6 cells) normalized to the control MF value (0.35 ± 0.24 , $n = 5$) as a function of laser power. Dashed line is a fit to aP^2 ; solid line to $aP^2 + bP^3$. Plotted values are mean $\pm$ SEM, $n = 5$ for all points.

4. Discussion

Multiphoton autofluorescence imaging is usually carried out with laser illumination in the 700-800 nm range since the majority of the biological molecules that generate multiphoton intrinsic fluorescence signals absorb maximally at these wavelengths [4]. Nonlinear excitation in this wavelength range can be quite damaging, although still significantly less damaging than ultraviolet (UV) excitation [21]. However for clinical diagnostic imaging, light induced cell death is not the critical parameter – epithelial cells in many tissues turn over at rapid rates, (e.g. ~daily in the colon [22]), so cell death resulting from diagnostic imaging is expected to be of little concern. The true danger lies in non-lethal photo-induced DNA mutations, which may lead to cancerous lesions. In terms of two-photon excitation, the 700-800 nm wavelength range corresponding to long-wave UV light is known to induce genomic instability and delayed mutations in mammalian cells [23]. Pulsed light in ~500 nm range induces mutations in mammalian cells as a result of two-photon absorption of DNA [24], and femtosecond pulses at 750 nm have been shown to produce three-photon damage to DNA [25].

4.1 Comparison to UV-mediated mutagenicity levels

The mechanisms involved in mutation induction by focused femtosecond irradiation are not fully understood. Assuming analogies to single photon mediated DNA damage and chromosomal mutation, two-photon induced damage at 760 nm would be similar to the effects of UVA radiation (315-400 nm) where damage appears to be mediated through reactive oxygen species (ROS) generation [26–28] and by excitation of pyrimidine dimers and pyrimidine (6,4) pyrimidone photoproducts [29]. Shorter UV wavelengths (UVB – 290 to 320 nm and UVC – 100 to 290 nm) cause direct damage to DNA. Three-photon excitation at 760 nm is expected to excite UVC absorbing bands so that three-photon mutagenicity most likely resembles UVC damage. In either case, light-induced damage can result in DNA base pair substitutions, large and small deletions, inversions or somatic recombinations between non homologous chromosomes [30].

Assuming that mutation induction is a simple first-order process and any nonlinearity in response is due to photon order of the excitation, our observed mutation frequency appears to be primarily a two-photon process at the lower powers, but requires a power cubed term for the highest intensities used in the experiment (Fig. 3B). However data from many UV-induced mutation experiments indicate nonlinear responses with continuous wave UV irradiation as well, indicating that higher-order photochemistry and biological responses to damage also

play a role in CW damage mechanisms. There are numerous published reports using the HPRT assay in V79 and other cell lines to investigate UV induced mutations. A direct comparison of our results with published reports using UV light is difficult to make due to the nonlinear, spatially localized intensity dependence and unknown nonlinear absorption cross-sections and absorbing species. However, a rough comparison based on the average dose and peak instantaneous irradiance can be made. The majority of the published UV-induced mutation investigations examine mutagenicity caused by added photosensitizers and UV light, but provide + UV control values (no photosensitizer) for comparison. These data indicate that UVA illumination doses as high as 50kJ/m^2 do not produce an increase in mutation frequency above the background rate [31–33]. The work by Li and Rossman [34] has the most relevant data for comparison since the same cell line was used and they report mutation frequencies for all three classes of UV light. Without attempting to convert the energy doses of 760 nm pulsed light to a theoretical two and three photon excitation equivalent dose (for example, based on estimated number of excitations per second), a comparison of our results to the reported results from ref [34] is shown in Table 1.

Table 1. Reported HPRT mutation frequencies in V79 cells under 760 nm femtosecond irradiation doses compared to UV illumination

UV illumination induced mutagenicity results from ref 34						Multiphoton mutagenicity results from this work (0.75 NA, 760 nm)		
UVA Dose ($\text{nJ}/\mu\text{m}^2$)	MF Increase	UVB Dose ($\text{nJ}/\mu\text{m}^2$)	MF Increase	UVC Dose ($\text{nJ}/\mu\text{m}^2$)	MF Increase	760 nm Dose ($\text{nJ}/\mu\text{m}^2$)	Instantaneous Irradiance $\text{nJ}/(\mu\text{m}^2 \text{ s})$	MF Increase
110	10×	0.4	63×	0.005	86×	4.9×10^6	1.7×10^{13}	4×
220	20×	0.8	328×	0.015	433×	7.4×10^6	2.5×10^{13}	11×

In Table 1 UV induced mutation frequencies were taken from control data shown in ref [34]; UV dose values were converted to $\text{nJ}/\mu\text{m}^2$ from kJ/m^2 and increase in the mutation frequency (mutants per 10^6 cells) is reported normalized to the –UV background values reported in the publication. Multiphoton data points shown for comparison are the 100 and 150 mW values from Fig. 3B. Normalization to area used to calculate the 760 nm dose is based on the integrated area of the 760 nm lateral point spread function ($0.325 \mu\text{m}^2$) at the focus (rather the integrated area of the PSF^2 or PSF^3). Instantaneous peak irradiance was calculated based on an 80 MHz laser repetition rate, 200 fs pulsewidth, assumption of a sech^2 pulse shape and an area of $0.325 \mu\text{m}^2$. Multiphoton induced increases in MF shown are relative to our background mutation rate of 0.34 per 10^6 cells.

The results in Table 1 indicate that UV excitation is orders of magnitude more mutagenic than 760 nm light, as would be expected. At the highest average power used in our experiments (150 mW) after ~16 ms of irradiation per cell, the mutation rate was about equal to what has been reported for $110 \text{ kJ}/\text{m}^2$ of 365 nm CW light.

4.2. Relevance to clinical multiphoton instrument design

Taken together, the data presented here provide an assessment of the feasibility of using nonlinear excitation of intrinsic tissue species for clinical diagnostic and intraoperative imaging. A critical limit to the femtosecond laser irradiance that could be used for diagnostic imaging in humans is ultimately dictated by the long term danger of mutagenicity. Depending on the tissue type being targeted, laser intensities required to generate the same level of tissue fluorescence can vary by an order of magnitude or more. Colon and ovary are particularly autofluorescent and may be feasible targets for nonlinear clinical imaging tools [18,19]; however, potentially dangerously high laser powers may be necessary for high quality morphometric and metabolic imaging in other tissues. A clear indication of average powers and dwell times that should be avoided is presented. It is important to note that in the tissue brightness experiments, we used a single scan that lasted for 0.7s during which the tissue was

illuminated for 0.44s (beam is blanked during raster scan turn around and line fly-back). Based on an average cell diameter of 20 μm , each cell received about ~4 ms of irradiation which is 1/4 of the illumination time per cell that was used in the mutagenicity experiments. For tissues that are brightly autofluorescent, the dose required would be well below levels we found to be mutagenic, assuming collection efficiencies similar to our microscope with the 0.7 NA Olympus microprobe lens. Fiber based microendoscope systems designed to collect emissions back through the delivery fiber or through auxiliary fiber optics may have much lower collection efficiencies. This problem has been addressed by several groups, for example, fiber endoscopes based on dual clad microstructured fibers have been shown to have as much as a 40 fold increase over similar designs using a single mode fiber [35]. However, values for the emission that can be coupled back into the fiber for detection is often not reported. We have made measurements of the fraction of emission delivered back through a fiber delivery/collection system using a bench top test system that allows for quantification of signal at both ends of the delivery/collection fiber (by means of a removable dichroic directly after the focusing lens). When using a large core (25 μm) photonic crystal fiber capable of delivering ~100 fs pulses [8], we have measured a 20% collection efficiency back through the fiber relative to the total fluorescence collected by the delivery objective lens. Although this could be optimized further by improved coupling optics, through-fiber collection efficiency of 20% implies acquiring colonic epithelium images of the same quality shown in Fig. 1C would require an average power of about 45 mW at the sample. Interpolating from the mutagenicity data shown in Fig. 3B, this would be of relatively low risk - especially since the light dose per cell used to acquire the images in Figs. 1C-H was only 1/4 of the dose per cell used in the mutagenicity assay protocol. Fiber endoscopic imaging of tissue types requiring much higher powers to obtain useable diagnostic images may indicate limited feasibility due to the danger of potentially carcinogenic mutations occurring. For example, the image of bladder epithelium in Fig. 1E required 120 mW of 760 nm excitation to collect. Scaled up to counteract the reduced collection efficiency expected in a fiber-based system, the same image would require >200 mW to generate, resulting in significant numbers of mutated cells.

5. Summary

In vivo high-resolution optical biopsies based on multiphoton excited intrinsic signals would have many advantages. These include optical sectioning, direct imaging of collagen structures and acquisition of metabolic information in addition to morphometric parameters such as cell and nuclear size. All signatures can be obtained without the use of added fluorophores. The data presented in this work illustrates that the tissue type is important to consider due to dramatic differences in intrinsic tissue brightness, and we provide estimates of the possible risks that may be associated with this type of imaging due to illumination-induced mutations. These results are relevant to the design of future multiphoton-based imaging tools targeted for clinical diagnostic purposes.

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