1. Introduction

It is broadly accepted that cancer is one of the most important medical challenges in the developed world. Cancer is the second most common cause of premature death in the USA after heart disease [1], and accounts for over $216.6 billion in direct and indirect costs per year [2]. One of the primary treatments, chemotherapy, is extremely taxing on the patient due to the toxicity and low selectivity of commonly-used anticancer agents. While cancer cells are preferentially killed, a number of other biochemical systems in the body are negatively impacted, leading to side-effects such as cognitive dysfunction, hair loss, and organ damage [3]. Photodynamic therapy (PDT) is a far more selective treatment option, wherein light is used to localize the antineoplastic effect and thereby avoid many of the downsides associated with more systemic treatment.

In PDT, a chromophore called a photosensitizer is illuminated with specific wavelengths of light in order to create reactive radical species that generate downstream effects, including apoptosis and necrosis in populations of cells. By controlling the administration of light to select regions of tissue, PDT can be carried out in just the tissue of interest, sparing critical nerves and organs, and preserving functionality where conventional surgery and chemotherapy would cause irreparable damage. In the ultimate limit, light administration could be confined purely to selected cells, avoiding the surrounding tissue entirely. In this case, the photodynamic agent need only be optimized for factors like quantum yield, clearance rate and dark (un-illuminated) toxicity. Such control would enable precise treatment of individual cells within intact tissue for pinpoint anti-cancer therapy, such as, for example, when tumor cells have extensively infiltrated sensitive organs such as the eye.

The method by which this light confinement is achieved in this paper is based on multiphoton absorption. This phenomenon, first predicted by Maria Göppert-Mayer in 1931 [4], occurs when a chromophore absorbs two or more photons in a very short space of time. These photons ordinarily would not possess enough energy to cause an electronic transition in the molecule, but the combined energy of two or more photons is sufficient to do so. In this manner, absorption only occurs in regions where the photon flux is sufficiently high to permit multiphoton absorption, such as at the focal...
point of a focused femtosecond pulsed laser. Elsewhere, the chances of two photons arriving at the same time is essentially negligible.

The use of multiphoton excitation for photodynamic therapy is not new; Fisher et al demonstrated two-photon excitation of some psoralen derivatives in 1997 [5], and in 1999, König, Riemann and Fischer described their work on two-photon excitation of photosensitizers using Verteporfin and 5-ALA [6]. Demonstration of two-photon absorption in Verteporfin and Photofrin in vivo was performed by Khurana et al in 2007 [7], and in the same year, two-photon occlusion of blood vessels in a chicken embryo was performed by Samkoe et al using Verteporfin [8]. Blood-vessel occlusion was demonstrated in mice by Collins et al, using a selection of different photosensitizers that were optimized for high two-photon cross-section [9]. The use of different novel photosensitizers was also pursued by Starkey et al, who argued that xenograft tumors could be treated using two-photon excitation from a regenerative amplifier focused through approximately 2 cm of tissue [10, 11].

Though this work is promising, the techniques used above all required the femtosecond laser focus to be scanned in the tissue, a serial process that is inherently slow. This paper describes a means of dramatically reducing the treatment time by exciting millions of points in parallel, using Temporal Focusing [12, 13]. The technique requires lasers with extremely high peak powers, but when the treatment time is dominated by the maximum power that can be tolerated in a tightly focused spot, temporal focusing can offer several orders of magnitude reduction in the time required to illuminate an area for either imaging or treatment.

Temporal focusing works by dispersing an ultrafast pulse using a grating or prism. According to the constraints of the time-bandwidth product, an ultrafast pulse must necessarily contain a broad bandwidth of different optical frequencies, which are separated from each other by the dispersive element. Once separated, the pulse duration of the separated components must be longer than the ultrafast pulse, due to the now-limited bandwidth of each pulse component. Since two-photon excitation is sensitive to the square of the light intensity [14], any two-photon effect is suppressed due to the longer effective pulse duration. Only once the pulse components are recombined using a lens (typically a microscope objective) does the ultrafast pulse reform, and even then, only at the focal plane of the microscope objective. Thus temporal focusing can project a 3D-resolved plane at the focal plane of a lens. As this excitation focal plane can be patterned, by patterning the light that strikes the grating, it is possible to individually illuminate several million diffraction-limited, 3D-resolved spots in parallel.

Our instrument is designed to project a temporal focusing plane approximately 700 μm × 700 μm onto a sample. Exposure times are on the order of 10 s to 30 s, which is approaching values that are clinically relevant. As an example, this exposure duration and illumination area could be suitable for the treatment of residual cancer cells in a resection margin.

2. Methods

2.1. Microscope

The temporal focusing system was constructed around a microscope body (Zeiss, Axiovert S100 TV) with a 20x, 1.0 NA immersion objective lens (Zeiss, 421452-9880-000) mounted on a piezoelectric objective stage (Piezosystem Jena MIPOS 500 SG). The fluorescence port was modified with a tube lens (164.5 mm focal length, Zeiss, 425308-0000-000) such that the image plane lay approximately 30 mm beyond the back of the microscope. A 50 mm square 1200 lines mm⁻¹ grating (Richardson Gratings, 53006BK02-540R) was in turn imaged onto this intermediate plane, using a 1.75 × beam reducer, consisting of 25 mm diameter 100 mm and 175 mm focal length lenses (Thorlabs, LA1050-B and LA1399-B) in a 4f configuration. A regenerative (or chirped-pulse) amplifier (Coherent, Legend Elite, seeded by a Mantis oscillator) produced 130 fs pulses at 10 kHz, with a pulse energy of 0.65 mJ per pulse; power was reduced when required using a half wave plate and polarizing beamsplitter. The amplifier works by picking a single pulse from the oscillator, stretching it in time using a grating, then amplifying the pulse by several orders of magnitude in a titanium sapphire crystal located in an optical cavity. The resulting amplified pulse is then compressed back to its original pulse duration using a grating compressor.

The pulses were magnified in the vertical axis by 5 × using a Galilean cylindrical telescope consisting of a –15 mm focal length lens (Thorlabs, LK1006L1-B) and 75 mm focal length lens (Edmund Optics, 69–762), and projected onto the grating at an angle, such that the –1 order diffracted beam propagated along the optical axis of the beam reducer. The magnification ratio was chosen in order that the image on the grating was approximately circular. Total light throughput from the amplifier to the sample was about 10%; in order to lower the treatment duration as much as possible, light exposure was always performed at 550 mW average power at the sample, since this was the maximum power that could be reliably be achieved at the sample on a day-to-day basis.

The bottom port of the microscope was connected to a rotating filter holder with two filters (Semrock, FF03-525/50-25 and Semrock, FF01-630/92-25). This in turn was connected to an EMCCD (Andor, iXon 885 K) to image fluorescence from the sample. An illustration of the system can be found in figure 1.

2.2. Samples and treatment protocol

Samples consisted of OVCAR-5 ovarian cancer cells (Fox Chase Cancer Institute) either grown in 2D on a collagen-coated 35 mm glass-bottomed dish (MatTek, P35GCOL-1.5-14-C) or grown in 3D model tumor nodules [15]. Cells were cultured in T75 flasks (VWR, BD353136) in RPMI 1640 (VWR, 45000–396) with 10% fetal bovine serum (VWR, 26410-111) and 1% penicillin / streptomycin (VWR 45000–652). Medium was changed every two days and cells were passaged every four days. To construct the 2D samples, 4 × 10⁴ cells were transferred into a 35 mm glass-bottomed
dish along with 1 ml of cell-culture medium. The cells were allowed to grow, with the medium exchanged after two days. The samples were treated on day four. To construct 3D samples, the same collagen-coated dishes were covered with 100μl of Matrigel (BD Biosciences, Growth Factor Reduced 356230) and allowed to cure at 37 °C in an incubator for 30 min. Approximately 18 600 cells in 200μl of cell culture medium were then transferred to the Matrigel surface and allowed to incubate for at least another hour to promote adhesion. Afterwards, an additional 2 ml of complete medium supplemented with 2% Matrigel was added to the dishes. Every two days the medium was exchanged for 1 ml of medium with 2% Matrigel. After 6 d, the samples had grown to approximately 100μm in diameter, and could be treated.

Treatment with Verteporfin (Selleck Chemicals S1786) was performed in the following manner: Verteporfin powder was dissolved in dimethyl sulfoxide (Sigma Aldrich, 276855-100ML) to yield a 50 mg ml−1 stock solution. This solution was then frozen at −20 °C until required. During treatment, the stock solution was diluted down to the required concentration using cell culture medium, to yield a working solution. The working solution was then mixed using a vortex mixer to encourage homogeneous mixing, and placed in a sonicator for one hour to further dissolve the Verteporfin. After an hour, the working solution was placed in a water bath at 37 °C for 15 min. The medium was removed from the 35 mm dishes and replaced with 1 ml of working solution per dish. The cells were then incubated for 2 h in the case of 2D samples, and 4 h in the case of 3D samples. After incubation, the working solution was replaced with 1 ml of fresh, pre-warmed medium and the dishes returned to the incubator. One at a time, the dishes were removed, and a piece of transparent tape affixed to the bottom of the dish to serve as a position marker. The microscope was then used to position the illumination plane relative to the marker, as quickly as possible so as to minimize unwanted exposure of the Verteporfin to light. The samples could be accurately de-focused using the objective translation piezo-stage, and exposure duration was controlled using an automated shutter (Vincent Associates, VS255SZM1R3), which was, in turn, controlled using a digital I/O box (National Instruments USB-6009) and a program that was custom-written in LabVIEW 2011 (National Instruments). After several exposures were carried out at different positions in each sample (translating the sample significantly between exposures to avoid overlap) the dish was returned to the incubator.

2.3. Performance quantification

After approximately 8–10 h, the dishes were removed and treated with Calcein AM and Ethidium Homodimer-1 (LIVE/DEAD stain, Molecular Probes L-3224) in pre-warmed phosphate-buffered saline (PBS) before being returned to the incubator. After a 45 min incubation period, the dishes were imaged using a conventional fluorescence microscope (Olympus IX-71, with Chroma Technology 11001v2 filter set and a Thorlabs DCC1645C camera).

Performance was quantified by automated image analysis. As the illumination spot was approximately Gaussian in shape, cells in the center of a given illumination field-of-view were over-exposed relative to those at the edges. As such, the diameter of the region of dead cells would provide an indirect metric of the degree of cell-killing. Simple cell-counting, where the number of dead (red) cells is measured versus the number of viable (green) cells was found to be inadequate for this task; 2D cell cultures had a range of surface densities, and apoptotic cells would regularly detach from the glass surface. Instead, a method was devised wherein the image was first segmented, using a Canny edge filter [16] and a watershed algorithm [17], to determine the center of all the cells present. A circle was then placed such that, to the greatest extent possible, red cells were placed inside the circle and green cells placed outside. This was performed using an optimization routine written in Matlab 2011b (Mathworks). The mathematical basis for the circle placement was similar to that used to determine the soft boundary in a support vector machine [18]; every cell that was on the wrong side of the boundary contributed to the error metric proportional to the square of the distance of the cell to the boundary. Minimizing this error metric resulted in the ‘best’ placement of the circular boundary; i.e. at the location where there was a 50% probability of a cell being alive or dead. The radius of the circle was then taken as a measure of the extent of cell-killing. An illustration of this process can be found in figure 2.
3. Results and discussion

One simplistic approach to modelling PDT-induced cell death is to treat illumination light dose as a time series of accumulated damage that, once a threshold is crossed, leads to death. In this experiment, damage can assumed to be cumulative and is proportional to both Verteporfin concentration and total exposure duration (for a given peak power, repetition rate and wavelength). For a very simple Gaussian damage profile, derived from the incident Gaussian light intensity, and assuming Verteporfin concentration is uniform across the sample:

\[ D = A e^{-\frac{x^2}{B^2}} \]

where \( D \) is the damage, \( A \) is the value of the peak of the Gaussian function, \( B \) is a width parameter equal to twice the variance of the Gaussian function, and \( x \) is the radial distance from the peak. By rearrangement, it is possible to determine a radius \( x_T \) at which the damage \( D \) is at a threshold value \( D_T \):

\[ x_T = \sqrt{-B \ln(D_T / A)} = \sqrt{B \ln(D_T / A)} \]

Since changes in exposure duration and concentration are expected, to a first approximation, to linearly affect the value of \( A \), and since \( D_T \) and \( B \) are constant for a given experiment, the radius \( x_T \) is expected to vary as the square root of the logarithm of the exposure duration, Verteporfin concentration or incident light intensity.

3.1. Dose–response

Several comparisons were made in order to determine the optimal conditions for treatment. First of all, the effect of changing the exposure time was investigated. The 50% cell death radius was plotted as a function of exposure duration (see figure 3).
The data are broadly consistent with the fit function, despite the inaccuracy inherent in estimating the 50% cell death radius using image analysis and the simplifications inherent in the fit function itself. The data suggest that, above approximately 15 s of exposure, increases in exposure do not increase the treatable area by a large degree. Therefore, optimal exposures should be around 10 s to 20 s, if treatment speed is a concern.

A subsequent experiment characterized the dependence on Verteporfin concentration (see figure 4). Experiments were performed using 10 s and 20 s exposure durations. Results were consistent from experiment to experiment, with no cell killing observed for 10 s exposure and 3.75 µg ml⁻¹ Verteporfin concentration, which is consistent with the line of best fit. The intercept along the concentration axis lies at approximately 5 µg ml⁻¹, indicating that there should be no observable cell killing at 3.75 µg ml⁻¹, which was observed for all three tested samples.

3.2. Patterning

Once suitable dose parameters had been established, attempts were made to characterize the axial resolution of the system, since this ultimately limits the accuracy with which cells could be killed. 2D cell cultures were treated with Verteporfin and the microscope intentionally defocused using a piezoelectric objective stage. The optical full-width half-maximum of the instrument, measured by translating a thin film of fluorescent dye through the focal plane and measuring the fluorescent intensity change for each pixel in the image, is 19 µm. The temporal focusing axial resolution can be expressed as the square-root of a Lorentz-Cauchy function [19], therefore the effect of defocusing the image is to scale the amplitude A by a Lorentzian scale factor with a previously-measured width parameter z₀:

\[ x_{y} = B \ln \left( D_{y} \cdot A \cdot \frac{1}{\sqrt{1 + \left( \frac{\varepsilon - z_{0}}{\varepsilon} \right)^{2}}} \right) \]

where \( z \) is the defocus distance and \( z_{0} \) is the previously-measured origin. The results in figure 5 show that for short exposures, cell death due to treatment can be completely eliminated within 25–30 µm of the focal plane; larger exposure values require a larger defocus before cell death due to treatment is eliminated. Care should also be taken in analyzing these results, as at the concentrations used, Verteporfin, while having a very low dark toxicity, is known to inhibit autophagy [20]. Therefore in environments with increased cellular stress, the dark toxicity may change.

In order to demonstrate that the temporal focusing plane could be patterned, a mask was constructed by cutting holes out of aluminium foil and placed at the intermediate image plane. This pattern was then projected onto the sample. An example is given in figure 6; a mask with the MIT logo was projected onto the sample. Verteporfin concentration was 30 µg ml⁻¹ and exposure duration was 30 s. The increased duration was necessary in order to maintain the patterning fidelity at the edge of the mask, since, on account of the Gaussian intensity profile, the edges became under-exposed at shorter exposures. A version of the instrument with a uniform intensity profile in the sample plane is in development; this will eliminate the need to over-expose the center of the image in order to achieve patterning at the edges.

The final tests to be performed were to demonstrate that cell killing could be performed in 3D; killing a layer of cells at the center of a nodule while leaving the cells above and below the treatment plane intact. OVCAR-5 nodules were grown to a diameter of approximately 100 µm and then treated with Verteporfin. 3D-resolved Live/Dead stained examples of temporal focusing PDT are shown in figure 7; the images show the XZ projection of a focal stack taken along the Z axis (top to bottom in the case of the illustrated figures) using the same temporal focusing system as for treatment. The focal stack was ‘resliced’, in order to change from an XY stack to an XZ stack, and then the sum of all the pixel intensities was taken along the Y axis. The focal
stacks that were used to create this data are available as supplementary information.

The resulting images demonstrate the axial sectioning capabilities of the instrument, and may also reflect the previously-known difficulty in getting Verteporfin to penetrate dense tumor nodules [15]; the edges of the tumor nodules are often very well treated, but the center is under-exposed. In addition, the Bystander effect [21, 22] will cause the apparent width of the dead layer to be larger than expected, and any subsequent movement or growth of the live cells will cause infiltration of live cells into the dead layer. The actual resolution of the system is therefore likely to be better than illustrated, and will improve even further if combined with a drug possessing better tumor-penetration characteristics.

4. Conclusion

In conclusion, 3D-resolved photodynamic therapy has been demonstrated in both 2D and 3D cell culture models. Patterning resolution is on the order of a few cells, and illumination times are less than 30 s. Work is ongoing to improve the system and reduce exposure times; moving from a Gaussian spot to a top-hat beam is expected to provide considerable improvement in axial resolution, as well as more efficiently using the power from the laser. Different drugs are also being tested in order to reduce treatment times either by increasing the nodule penetration, the two photon cross-section, or the phototoxicity. Improving the two-photon cross-section is expected to be particularly effective, as the cross-section of current photosensitizers is poor; Verteporfin has a cross-section of approximately 50 GM (Göppert-Mayer units, 1 GM = 10⁻⁵⁰ cm⁴ s per photon), while potential alternatives in the literature have cross-sections of 17 000 GM and higher [9]. Assuming the chromophores are not being saturated and that the phototoxicity of the higher cross-section photosensitizers is comparable to Verteporfin, this implies a reduction in exposure time by a factor of 340, to approximately 50 ms, or similar reduction in the required photosensitizer dose. Once treatment times are reduced below approximately 1 s, it is expected that this method will be clinically applicable, being able to selectively kill cells with extremely high precision during oncological surgery.

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References

[1] Heron M 2013 Deaths: Leading Causes for 2010 (National Vital Statistics Reports vol 62)
[2] American Cancer Society 2014 Cancer Facts and Figures (Atlanta: American Cancer Society)
[3] Partridge A H, Burstein H J and Winer E P 2001 Side effects of chemotherapy and combined chemohormonal therapy in women with early-stage breast cancer JNCI Monogr. 2001 135–42
[4] Göppert-Mayer M 1931 Über Elementarakte mit zwei Quantensprüngen Ann. Phys. 401 273–94
[5] Fisher W G, Partridge W P, Dees C and Wachter E A 1997 Simultaneous two-photon activation of type-I photodynamic therapy agents. Photochem. Photobiol. 66 141–55
[6] Koenig K, Riemann I and Fischer P 1999 Photodynamic therapy by nonresonant two-photon excitation Proc. SPIE 3592 43–9
[7] Khurana M, Collins H A, Karotki A, Anderson H L, Cramb D T and Wilson B C 2007 Quantitative in vitro demonstration of two-photon photodynamic therapy using photofrin and visudyne. Photochem. Photobiol. 83 1441–8

[8] Samkoe K S, Clancy A A, Karotki A, Wilson B C and Cramb D T 2007 Complete blood vessel occlusion in the chick chorioallantoic membrane using two-photon excitation photodynamic therapy: implications for treatment of wet age-related macular degeneration. J. Biomed. Opt. 12 034025

[9] Collins H A et al 2008 Blood-vessel closure using photosensitizers engineered for two-photon excitation Nat. Photon. 2 420–4

[10] Starkey J R, Rebane A K, Drobizhev M A, Meng F, Gong A, Elliott A, McInerney K and Spangler C W 2008 New two-photon activated photodynamic therapy sensitizers induce xenograft tumor regressions after near-IR laser treatment through the body of the host mouse Clin. Cancer Res. 14 6564–73

[11] Starkey J R, Pascucci E M, Drobizhev M A, Elliott A and Rebane A K 2013 Vascular targeting to the SST2 receptor improves the therapeutic response to near-IR two-photon activated PDT for deep-tissue cancer treatment. Biochim. Biophys. Acta 1830 4594–603

[12] Zhu G, van Howe J, Durst M, Zipfel W and Xu C 2005 Simultaneous spatial and temporal focusing of femtosecond pulses. Opt. Express 13 2153–9

[13] Oron D, Tal E and Silberberg Y 2005 Scanningless depth-resolved microscopy. Opt. Express 13 1468–76

[14] Svoboda K and Yasuda R 2006 Principles of two-photon excitation microscopy and its applications to neuroscience. Neuron 50 823–39

[15] Evans C L, Abu-Yousif A O, Park Y J, Klein O J, Celli J P, Rizvi I, Zheng X and Hasan T 2011 Killing hypoxic cell populations in a 3D tumor model with ErNBS-PDT PLoS One 6 e23434

[16] Canny J 1986 A computational approach to edge detection IEEE Trans. Pattern Anal. Mach. Intell. PAMI-8 679–98

[17] Beucher S and Lantuejoul C 1979 Use of watersheds in contour detection Proc. Int. Workshop on Image Processing pp 2.1–2.12

[18] Cortes C and Vapnik V 1995 Support-vector networks Mach. Learn. 20 273–97

[19] Dana H and Shoham S 2011 Numerical evaluation of temporal focusing characteristics in transparent and scattering media. Opt. Express 19 4937–48

[20] Donohue E, Tovey A, Vogl A W, Arns S, Sternberg E, Young R N and Roberge M 2011 Inhibition of autophagosome formation by the benzoporphyrin derivative verteporfin. J. Biol. Chem. 286 7290–300

[21] Oleinick N L, Morris R L and Belichenko I 2002 The role of apoptosis in response to photodynamic therapy: what, where, why, and how. Photochem. Photobiol. Sci. 1 1–21

[22] Mothersill C and Seymour C 2001 Radiation-induced bystander effects: past history and future directions Radiat. Res. 155 759–67