UV Laser Stimulation of Ca\textsuperscript{2+} Transients in Aggressive Glioblastoma Brain Cancer Cells*

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Abstract—Glioblastoma (GBM) is a lethal astrocytoma being the most common highest-grade adult brain cancer. GBM tumours are highly invasive and display rapid growth to surrounding areas of the brain. Despite treatment, diagnosed patients continue to have poor prognosis with average survival time of 8 months. Calcium (Ca\textsuperscript{2+}) is a main communication channel used in GBM and its understanding holds the potential to unlock new approaches to treatment. The aim of this work is to provide a first step to accurately evoking Ca\textsuperscript{2+} transients in GBM cells using single UV nanosecond laser pulses \textit{in vitro} such that this communication pathway can be more reliably studied from the single-cell to the network level.

I. INTRODUCTION

Grade IV Glioblastoma (GBM) is the most lethal and prevalent form of adult brain cancer [1], [2]. Standard treatment involves major surgical resection of the tumour with subsequent treatments of radiotherapy and chemotherapy [3], [4]. Despite such invasive treatments, recurrence is frequent [5] leaving patients with a very poor prognosis of 6-15 months.

Due to the inertia in new treatment advances and the rapid re-proliferation and colonization of GBM in the brain after treatment, there is an urgent need to re-investigate the basic science of GBM communication to understand how to slow down or stop GBM’s growth and aggressive invasion.

GBM is an astrocytoma and thus displays similarities with astrocytes, a type of glial cell [6], [7]. Hence, GBM cells, like astrocytes, are believed to predominantly communicate through calcium (Ca\textsuperscript{2+}) ion signaling [8]–[10]. Furthermore, Ca\textsuperscript{2+} signaling is involved in a variety of cellular functions such as control of cell proliferation, cell size, cell migration, intercellular coordination [11], [12], and executing apoptosis [13], [14].

Laser stimulation is a technique that allows for the non-invasive spatially and temporally precise, quantized photon-delivery of energy to stimulate Ca\textsuperscript{2+} elevations in non-electrical cells, such as astrocytes and other glial phenotypes. Ultraviolet (UV) and infrared (IR) lasers are the most commonly used and have been used to stimulate a variety of cell types including HeLa [15], rodent and human astrocytes [16]–[20]. Laser work with GBM, has primarily been concerned with the ablation of the tumour mass [21], [22] rather than the stimulation of GBM cells in order to investigate the basic science of Ca\textsuperscript{2+} communication.

The motivation of our group, is to understand brain cell communication for both healthy and diseased states from the single-cell level to network level. So far we have achieved this for healthy brain cells, both human neurons [23], [24] and astrocytes [25]–[27] and have evoked Ca\textsuperscript{2+} in astrocytes with UV nanosecond laser pulse stimulation [28], [29]. The aim of this work, is to extend this concept to GBM brain cancer and to demonstrate, for the first time, how single UV nanosecond laser pulses, can be effectively employed to stimulate Ca\textsuperscript{2+} transients in GBM cells in a spatially and temporally precise manner which is the first step to exploring controlled Ca\textsuperscript{2+} communication in GBM in a reliable and repeatable manner.

II. METHODS

A. Cell Culture

In this research, we employed a single patient-derived GBM cell-line, FPW1, using the described methods of Stringer et al [30]. Ethics approval to use these cells for this work was granted on 21/03/2021 through the Auckland Health Research Ethics Committee, The University of Auckland, New Zealand (Ref. AH22121). The cell-line was the most aggressive of GBM phenotypes, namely the mesenchymal subtype of GBM. GBM cells were cultured at a low-passage (<p1.5) and grown under serum-free conditions. GBM cells were cultured on Matrigel (Corning, Cat# 354234) coated T75 flasks with StemPro media (Gibco, Cat# A1050901). Media was changed every two days or as required and the cells were passaged 1:4 when 80%...
confluency was achieved. Passaging was performed by rinsing the cell culture T75 flasks with PBS before the cells were detached by the addition of Accutase (Sigma-Aldrich, Cat# A6964). A mixture of PBS and Trypsin Inhibitor (Gibco, Cat# R007100) was then used to collect the detached cells the cell suspension was then centrifuged at 200 g for 5 min. For the laser experiment cells were seeded onto Matrigel coated 35 mm Petri dishes at a density of 100,000 cells per Petri dish. Incubation was performed at 37°C and 5% CO₂. The typical morphology of a single GBM cell is shown in Fig. 1a as well as a typical network of cells, Fig. 1b.

B. UV Nanosecond Laser Stimulation & Ca²⁺ Live Cell Imaging

Fluo-4 (Invitrogen, Cat# F14201) was used to visualize the change in internal Ca²⁺ concentration. Cells were loaded with Fluo-4 (consisting of 1 mL of StemPro media containing 1.5 µL of 1 mM Fluo-4) for 20 min in an incubator. Once loaded the cells were rinsed three times with imaging media, imaging media was Fluorobrite (Gibco, Cat# A1896701) supplemented with 1% Glutamax (Gibco, Cat# 35050061). 4 mL of imaging media was then added to the Petri dish and the cells left to incubate for a further 15 min before imaging proceeded.

Imaging was performed on an Olympus BX53 upright microscope with a customized incubation system using a 20× or 40× water immersion lens. The 40× lens was used to interrogate single cells with a laser pulse and the 20× lens was used to observe the Ca²⁺ network response (as it provided a wider field of view). Integrated with the microscope was a MicroPoint Laser Illumination and Ablation System (Andor Technology) which was used to stimulate the cells. The laser system comprised of a steerable UV pulsed nitrogen tunable dye laser with a wavelength of 340 nm, producing 3 ns 200 µJ pulses.

Ca²⁺ imaging and laser stimulation were performed simultaneously. Images were captured using an Andor Clara-C cooled CCD camera. A initial protocol was defined to capture a baseline of 50 images after which laser stimulation occurred and then the fluorescent response was recorded. The exposure time was set 250 ms and a GFP filter was used to capture the fluorescent changes of the Fluo-4 due to changes in internal Ca²⁺ of the imaged cells. In addition, brightfield images were captured before and after the protocol to assess cell viability.

C. Image Processing and Analysis

Individual cells were identified as regions of interest (ROI) using the opensource software (ImageJ). The mean fluorescence of each ROI across all captured images was calculated and exported to the MATLAB (2021b, The MathWorks Inc., Natick, Ma, USA) programming environment. The mean fluorescence was then filtered and baseline subtracted, and normalized using the method described by [31] with a processed signal was referred to as ΔF/F₀. Where F₀ is the mean fluorescence of the ROI for each image and ΔF is the change in fluorescence over a preceding baseline window.

III. RESULTS

A. Effect of Laser Pulse Power on Cell Viability and Ca²⁺ Response after Laser Stimulation

In [29], we have previously investigated the relationships between laser pulse power and the viability of the cells after laser stimulation and the probability that a single pulse elicits a single Ca²⁺ response. For GBM cells we observed that a single laser pulse with a pulse energy of 14.7 µJ resulted in 95% of targeted cells viable and had a 80% probability of eliciting a single Ca²⁺ response in the target cell. Thus, we determined that a pulse energy of 14.7 µJ was the most reliable pulse energy to stimulate the cell while not ablating the target cell. Hence, all subsequent results were obtained using a pulse energy of 14.7 µJ.

B. Temporal Profile of Laser Induced Ca²⁺ Transient in GBM Cells

When successfully stimulated with a UV ns laser pulse GBM cells were observed to display a Ca²⁺ transient that has a profile with characteristics similar to other Ca²⁺ excitable cells such as astrocytes [29]. Examples of typical Ca²⁺ transients stimulated by a laser pulse are shown in (Figs. 1c,1e, and 1g). The laser pulse was delivered at 12.5 s (red square) and produced a transient that lasted ~100 s. (Figs. 1d, 1f and 1h) show the corresponding fluorescent images and the location of the laser pulse (red square) relative to the stimulated GBM cell. The fluorescent images show the spatial characteristics of the Ca²⁺ transient where the Ca²⁺ increase is initially localized to the area of the laser pulse before spreading throughout the cell.

The typical profile of a Ca²⁺ transient displayed a rapid increase in ΔF/F₀ (<10 s) followed by a much slower recovery (>100 s). Fig. 1c shows the average Ca²⁺ transient across different cells stimulated with a single laser pulse at a pulse energy of 14.7 µJ (N = 15).
Figure 1. GBM cell morphology and the profiles of Ca$^{2+}$ transients for three different GBM cells. (a) Fluorescent image of a single GBM cell displays a long thin elliptical morphology. (b) Fluorescent image of a network of GBM cells with a variety of morphologies. (c), (e), and (g) Ca$^{2+}$ transients from three different GBM cells. All Ca$^{2+}$ transients display similar dynamics with a fast initial increase followed by a slower recover. (d), (f), and (h) are the corresponding time-series fluorescent images for (c), (e), and (g) respectively. All scale bars are 50 µm and red squares indicate time and locations of laser pulses.

C. Intercellular Ensemble Ca$^{2+}$ Transients Induced from a Single UV-Laser Stimulated GBM Cell

Finally, for network communication to be studied, it was necessary to determine if a single UV laser pulse stimulated GBM cell could activate ensemble intercellular Ca$^{2+}$ transients in a network of GBM cells. Fig. 2a and Fig. 2b, demonstrate instances of how a single-UV laser stimulated GBM cell could then go on to evoke subsequent intercellular Ca$^{2+}$ transients in a GBM network in vitro. As observed in Fig. 2a a single cell was stimulated (Fig. 2a at 1.5 s) from which a wave of Ca$^{2+}$ moves along its process to the neighboring cells (Fig. 2a at 7 s). This cell can then be observed activating second neighbors of the original targeted cell (Fig. 2a at 22 s and 50 s respectively). A larger field of view of a different network is shown in Fig. 2b. In this example the wave of stimulation is initiated at the targeted cell (Fig. 2b at 8 s) before progressing outwards until most of the cells within the field of view have been stimulated (Fig. 2b at 30 s) before the majority of the cells recover (Fig. 2b at 120 s).

Figure 2. Network communication is elicited by a single laser stimulating pulse. (a) shows a close up of a Ca$^{2+}$ transient being transmitted to neighboring cells. 1.5 s after laser pulse stimulation, the targeted cell has been stimulated. By 7 s, the stimulus has been transmitted to two close neighboring cells. At 22 s and 50 s, more distant cells have been stimulated. (b) shows a wider field of a different network also displaying transmission of the initial laser pulse stimulus. At 4 s the targeted cell as well as its nearest neighbors have been stimulated the stimulation then moves in a wave like fashion away from the targeted cell. By 30 s, most cells in the field of view have been stimulated and by 120 s, almost all cells have recovered. All scale bars are 50 µm.

IV. DISCUSSION & CONCLUSION

Glioblastoma (GBM) is the most lethal, adult brain cancer leaving diagnosed patients with an average life expectancy of 8 months. Calcium (Ca$^{2+}$) is a main communication channel in GBM and its understanding holds the potential to unlock new approaches to treatment. The aim of this work was to provide a first step to accurately evoking Ca$^{2+}$ transients in GBM cells using single UV nanosecond laser pulses in vitro such that this communication pathway can be more reliably studied from the single-cell to the network level.
In this pilot work, we observed that UV nanosecond laser pulses with a pulse energy of 14.7 µJ were sufficient to provide a response rate of eliciting Ca²⁺ transients in 80% of targeted GBM cells and resulted 95% of cells remaining viable. This is a slightly higher pulse energy than that which we have previously reported to be appropriate for healthy human astrocytes (13 µJ) [29] from which this astrocytoma is derived. However the response rate and the viability after stimulation observed in human astrocytes, 85% and 93% respectively, are similar to those we observe here [29].

Furthermore, we demonstrated how a single UV laser pulse activated GBM cell was then able to continue to naturally activate a wider network of GBM cells.

In conclusion, we demonstrate, for the first time, how GBM brain cancer cells can be stimulated in a spatially and temporally precise manner using single UV nanosecond laser pulses of 14.7 µJ to activate wider network Ca²⁺ activity. We see this as a first step to aid in a better understanding of the basic science of GBM Ca²⁺ communication in a reliable and repeatable manner from the single-cell to network level which holds the potential to unlock new approaches to treatment.

REFERENCES


