Imagen Biotech
See more than images

biostatus

DRAQ7™, a unique far-red viability dye used as a measurement of cell viability in patient-derived glioblastoma stem cells

Introduction

In flow cytometry and automated fluorescence microscopy (high content screening), a reliable estimation of cell viability is important because it is central to assays for apoptosis and *in vitro* toxicology. Likewise, it is often a useful measure for sample quality and for robust phenotypic analysis of clinical samples.

DRAQ7™ is a far-red fluorescing biomarker of cell viability. This water-soluble molecule selectively binds nuclear dsDNA with high affinity but is cell membrane impermeable. Accordingly, it only labels cells with compromised plasma membranes, indicative of cell death by primary or secondary necrosis.

DRAQ7™ has been robustly tested and exemplified in key cytometry publications (Akagi *et al.*, 2013b; Akagi *et al.*, 2013a; Wlodkowic *et al.*, 2013) for compatibility with multi-colour experiments and uniquely for long-term, real-time analysis. It has recently been utilised in imaging procedures to monitor cell viability in 2-D and 3-D spheroid/micro-tissue assays.

DRAQ7™ is optimally excited by orange/red wavelengths with two absorbance peaks at 599 nm & 644 nm. It emits in the far-red region of the electromagnetic spectrum (Emλ_{max} 694 nm).

High content screening (HCS) is limited by the narrow spectral space available and by the necessity to exploit very bright chromophores to label antibodies. Although brightness may be desirable for sensitivity, it increases the need for compensation between overlapping signals.

Traditionally, viability dyes such as propidium iodide (PI) have been employed to measure cell death. PI has a broad emission spectrum, occupying the red channels (555 & 594 nm). Therefore, bleeding into the green 488 channel can occur unless stringent filter sets are used. PI also binds RNA and is toxic to cells over a period of time.

In contrast, DRAQ7 has no bleed through, does not bind significant amounts of RNA and is not toxic in long-term culture. Therefore DRAQ7 is ideally suited for the measurement of cell death induced by chemotherapeutic drugs. Figure 1 shows an example of DRAQ7 staining in a 3D spheroid after treatment after Etoposide.

We have recently used DRAQ7 to measure cell death occurring in glioblastoma-derived stem cell lines in response to a library of 84 different chemotherapeutic agents.

Glioblastoma Multiforme (GBM) is a grade IV brain tumour and is a highly aggressive malignant cancer with a median survival time of 3 months, although with treatment survival can be increased to 1–2 years.

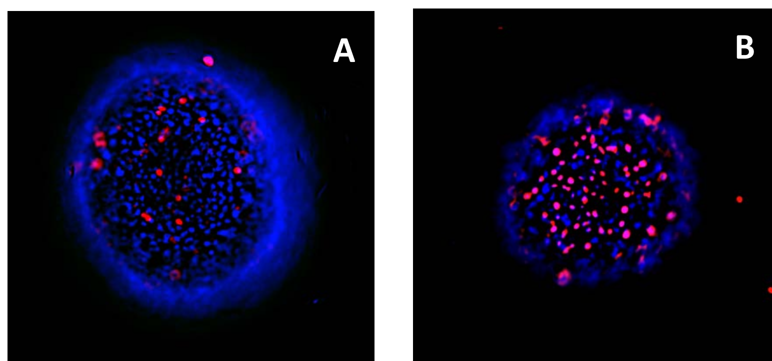

Figure 1: MCF7 cell spheroids stained with Hoechst 33342 (*blue fluorescence*) and DRAQ7 (*red fluorescence*). Untreated (*panel A*); treated for 72 hours with 1 µM Etoposide (*panel B*).

Established first line treatment (Stupp *et al.*, 2009) consists of maximally safe surgical debulking followed by fractionated radiotherapy and chemotherapy. For the last 15 years the treatment used in the majority of cases is x-irradiation combined with the chemotherapeutic agent Temozolomide (TMZ).

TMZ seems to work by providing some sensitization of the tumor cells to radiation[1]. However, given that relatively few chemotherapies have been explored in GBM, we wanted to test if a better drug could be utilized as a replacement for TMZ.

Cancer stem cells (CSC) have been documented in several types of cancer including GBM (Singh *et al.*, 2003). This subpopulation has been shown to recapitulate human tumours in xenografts as well as differentiate into cells of neuronal and astrocytic lineages. CSCs have been shown to be resistant to radiation and chemotherapeutics (Bao *et al.*, 2006) and they are thought to be responsible for disease recurrence. CSCs therefore represent a potentially critical target for therapeutics in GBM.

Our collaborator, Dr Brian Bigger (University of Manchester), has derived 10 patient-specific GBM cell lines with phenotypic characteristics of neural stem cells. Furthermore, they can recapitulate these tumours in an orthotopic xenograft mouse model. We studied 8 of these lines and tested each one with a library of 84 FDA approved currently used chemotherapy drugs.

Methods

Cells were cultured in a B27 supplemented media that has been demonstrated to be selective for cancer stem cells. In this media, cells grew in suspension and formed large spheroids. The addition of laminin to cultures caused the cells to adhere and grow as a monolayer. In this way we were able to culture and screen cells in both 3D and 2D formats. According to the community, and numerous companies promoting specialized 3D culture plates, the 3D format should be more physiological.

Clinically, GBM is normally treated with x-irradiation. Therefore we tested all 84 anticancer drugs as monotherapies or in combination with x-irradiation (5GY). The questions we wanted to answer were:

1. Do cells respond differently in 2D to their response in 3D?
2. Do any chemotherapies not normally used to treat GBM significantly enhance irradiation-induced cancer cell death?

Eight patient-derived glioblastoma cell lines were studied in this project. Duplicate 384 well plates +/- laminin were used and one of each was treated with 5GY of x-rays (deemed the optimal dose from previous work).

Cells were plated in 384-well plates and dosed with a Hamilton Star robot. In the 2D format, cells were incubated for 72 hours whereas in 3D format they were incubated for 96 hours. DRAQ7 was added to the samples 1 hour before the endpoint and Hoechst 33342 was also then added 30 minutes prior to running the plates on the Arrayscan. Hoechst was used to label nuclei as it enters cells irrespective of their membrane integrity.

[1] http://en.wikipedia.org/wiki/Glioblastoma_multiforme

For 2D analysis, image acquisition was at 5X magnification and a single field of view was captured for each well. Figure 2 shows GS1 cells treated with 100 nM Staurosporine for 72 hrs. Image Analysis was carried out using the compartmental analysis algorithm supplied with the software. This was adjusted to identify nuclei and segment them into single cells using Hoechst staining captured in channel 1. Channel 2 was used to capture the DRAQ7 fluorescent signal and the algorithm set up to classify cells that are positive for this fluorophore – expressed as the percentage of dead cells. Three-dimensional image analysis was also carried out using the compartmental analysis algorithm. In these experiments, the algorithm was configured to identify spheroids instead of single cells by turning off secondary segmentation. This resulted in single ROIs for cells that were packed together in a spheroidal structure. As with the 2D work Hoechst and DRAQ7 staining were captured in channels 1 and 2 respectively. However, in contrast to the 2D work, the algorithm measured the total DRAQ7 fluorescent intensity coming from the spheroid rather than identify individual cells that were positive stained by the fluorophore. This modification was necessary because spheroids can consist of over a 1000 cells and it is only possible to observe the cells on the outside of the structure. It was not possible to use the confocal module on the Arrayscan because the spinning disc resulted in too much signal reduction. However, even if it was technically possible to use confocal microscopy, the Hoechst stain itself does not penetrate large spheroids so central z-sections would not provide enough valid cell information to make this approach a viable option. Another drawback of using confocality is the need for a z-stack. This can slow the run by up to ten times making large screens very impractical.

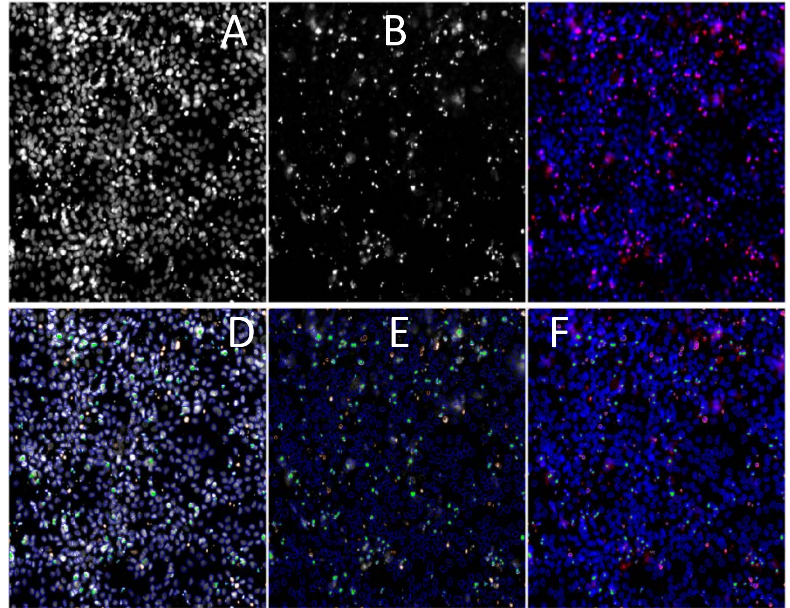

Figure 2: The effect of 100 nM Staurosporine treatment on GS1 cells after 72 hours. Hoechst 33342 and DRAQ7 staining is shown in panel A and panel B respectively and the colour composite is shown in panel C. Panels D to F are the equivalent images with the Arrayscan algorithm analysis displayed. Region of interest (ROIs) identified by the compartmental analysis algorithm are shown in blue with rejected objects shown in orange. Green objects (panel E) represents areas that the algorithm has identified as DRAQ7 positive.

In 3D mode, image acquisition was performed using a 5X objective and 4 fields of view were captured for each well. Figure 3 shows one of these fields of view from GS1 cells exposed to Staurosporine for 96 hours.

Results

There are two important requirements for a chemotherapy to have clinical benefit. The first is that the anti-cancer drug must be cytotoxic to the cancer being treated. The second is that the dose at which drug kills the cancer must be low enough that it creates a viable therapeutic window whereby the drug is differentially targeting the patient's cancer while leaving the patient's normal cells relatively unharmed. Thus the potency of a chemotherapy against a particular cancer is also a critical measure. In order to measure a drug's potency, the drug must be tested at several different doses. Therefore not only was each chemotherapy tested in 2D and 3D

mode as a monotherapy or in combination with x-radiation, but we also tested each compound at 5 different doses. The response of one cell line (GS1) to the standard of care therapy (TMZ) is shown as a monotherapy and with x irradation in 2D and 3D formats (Figure 4). This particular example is of interest because there was a very different response when cells were treated in 3D versus 2D mode. In order to get a more global view of the complete dataset, we employed heatmap summaries where each dose response curve was collapsed into a single value for each drug tested by taking the average value over the dose response range. This mathematical summary captures drugs that are more potent since the average reading across the dose response curve will be higher for compounds that have low dose responses (Figure 5).

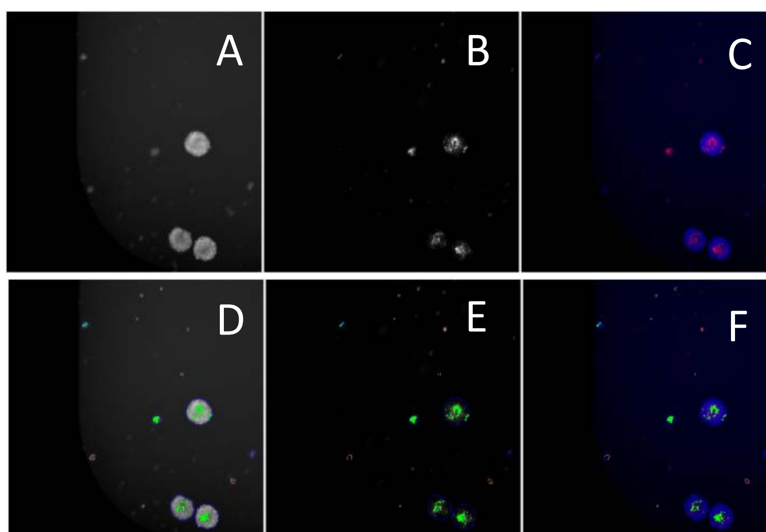

Figure 3: The effect of 100nM Staurosporine treatment on GS1 spheroids after 96 hours. Hoechst and DRAQ7 staining is shown in panel A and panel B respectively and the colour composite is shown in panel C. Panels D to F are the equivalent images with the Arrayscan algorithm analysis displayed. Region of interest (ROIs) identified by the compartmental analysis algorithm are shown in blue with rejected objects shown in orange. Green objects (panel E) represents areas that the algorithm has identified have a DRAQ7 intensity that is above the background threshold.

In reference to the plate maps in figure 5 the following observations were made:

- Glioblastoma lines did not respond to the majority of drugs tested.
- Where responses were recorded, different patients responded differently.
- Some drugs appeared to work across a wider range of patient lines and these are not standard therapies.
- There was a surprisingly good correlation between 2D and 3D for drugs that were acting across the majority of cell lines. However, when a drug was only working in one or two cell lines often the 2D and 3D data were divergent (TMZ being a prime example).
- Two drugs not currently used in GBM, enhanced IR effects on 3 of the cell lines. Critically this was only evident in the 3D spheroid cell growth. 2D did not show these differences.

Discussion

This work set out to identify drugs that could provide an alternative to standard GBM therapy. Using patient derived cancer stem cells, we explored whether there was a personalized response to drugs as well as an ability to enhance the effect of irradiation therapy. The ability to grow 3D spheroids has clearly gathered pace as a method that has more clinical significance than 2D cultures growing on plastic.

Cell death is of central importance in our analysis. It is difficult to use antibody staining techniques in non-adherent conditions and so we are limited to cell dyes. Permeability dyes which enter dying cells such as propidium iodide all have significant drawbacks. They are either transient in there detection, can give 'dirty'

signals or be toxic to the cells. This means that often only a single fixed time point is possible. Time courses require multiple spheroids to be fixed at each point. The result is a decrease in reliance as spheroid sizes can vary from well to well in some instances, but also cost becomes a significant factor.

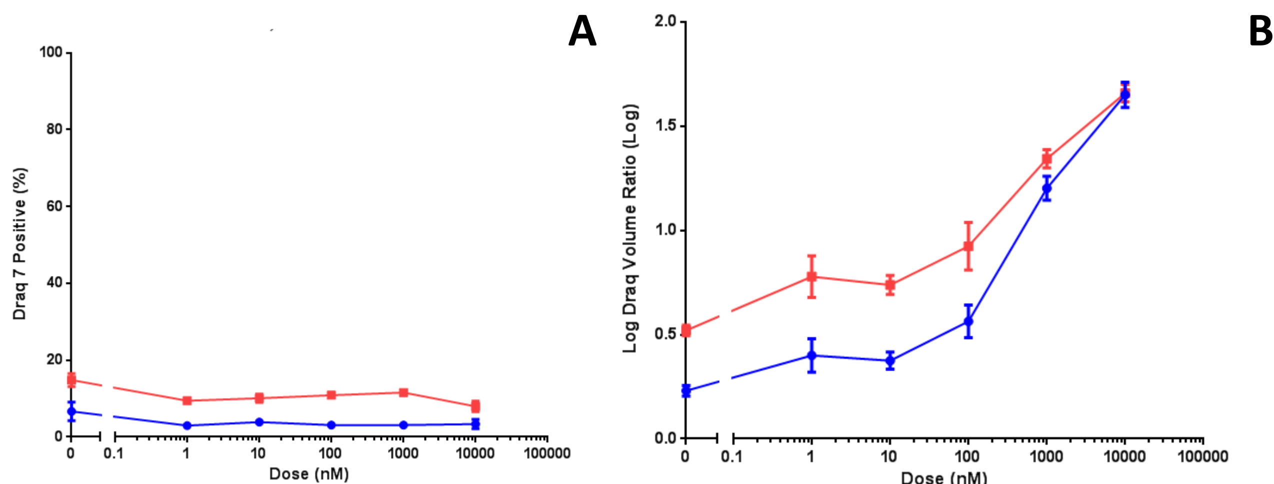

Figure 4: The effect of TMZ treatment on the patient derived glioblastoma cell line GS1. Cells were treated for 96 hours with varying doses of TMZ (abscissa) either in combination with 5 GY x-irradiation (*red symbols*) or as a monotherapy (*blue symbols*) and cell death was measured as the number of cell positive for DRAQ7 in 2D (*panel A*) or as the Log of the total DRAQ7 fluorescence measured in the spheroid normalised by the volume of the spheroid. The volume of the spheroid was approximated from the area measurement by raising the area by a factor of 1.5 (ie Area^3/2). It was found that this correction gave tighter confidence intervals than normalising against the area alone. Data points represent the Mean±SEM of 4 plate replicates.

DRAQ7™ is a fluorescent probe that has ideal properties for enumeration of dead cells with nuclear DNA specificity, intact cell membrane impermeability, far-red fluorescence and good signal:noise ratio. It can also be used in long-term, real-time analysis.

We used DRAQ7 for both the 2D assays and 3D assays. The analysis was performed on a cell-by-cell basis in 2D, but on a spheroid basis in 3D. Percentage dead cells could this be calculated in 2D whereas in 3D we measured total intensity in the spheroid as well as the spheroid sizes. Drugs had a general effect of causing spheroids to shrink as the intensity of DRAQ7 increased.

This study demonstrates that in this particular case, 3D was arguably superior to 2D when it came to identifying drug effects more accurately. Our 3D dataset supports the current hypothesis that screening in this format is likely to yield more hits that are also more clinically relevant.

Personalized medicine is being used more widely as it becomes clear that every individual has a cancer specific to them and not a more generalised classification. By testing 8 different patient derived lines it was found each had a unique 'fingerprint' of drug responses. In fact the two 'hits' we identified did not appear in all the patient lines. To be able to determine which patient will respond to these two drugs would be paramount to any clinical trial and guide future practices on treatment of glioblastoma.

A phenotypic approach using high content screening has the ability to provide this information, however we have also employed exome sequencing to determine if there is also a genetically identifiable link.

Reference List

Akagi, J., Kordon, M., Zhao, H., et al. (2013a). Real-time cell viability assays using a new anthracycline derivative DRAQ7(R). *Cytometry A* **83**, 227-234.

Akagi, J., Skommer, J., Matuszek, et al. (2013b). Multivariate analysis of apoptotic markers versus cell cycle phase in living human cancer cells by microfluidic cytometry. *Proc.SPIE* **8615**.

Bao, S., Wu, Q., McLendon, R. E., et al. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**, 756-760.

Singh, S. K., Clarke, I. D., Terasaki, M., et al. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res.* **63**, 5821-5828.

Stupp, R., Hegi, M. E., Mason, W. P., et al. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* **10**, 459-466.

Wlodkowic, D., Akagi, J., Dobrucki, J., et al. (2013). Kinetic viability assays using DRAQ7 probe. *Curr.Protoc.Cytom.* **Chapter 9**, Unit

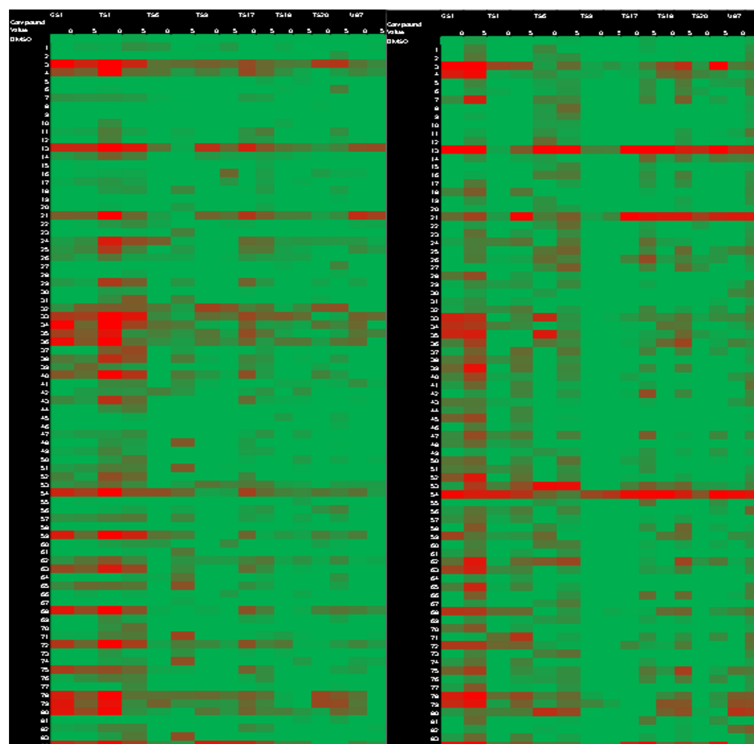

Figure 5: Heat map summarizing the result from a selection of compounds (*rows*) tested against 7 glioblastoma stem cell lines (columns top line) derived directly from patients. Each compound was tested either on its own or in combination with 5 GY x-irradiation (columns second line 0 on left, 5 on right). The assay was conducted on 3D spheroids and cell death was calculated using a combination of spheroid area and cell membrane integrity loss. The top death value was coded red with all values below the 50 percentile coded green. All other values were coded with mathematically interpolated colours between green and red such that the colour distance in the pixel values from green to red was directly proportional to the percentile position of that value within the complete data set. Each value represents the average death response for the compound across a 5 point log dose response curve (1, 10, 100, 1000, 10000 nM). This type of analysis is mathematically very similar to an "Area Under Curve" measurement and captures the potency as well as the efficacy of the compound as more potent compound will have more doses with higher than average spheroid death values.

BioStatus is very grateful to Imagen-Biotech for sharing this research data. For further details on their work visit:
www.imagen-biotech.com

If you would like to know more about DRAQ7™ or any other BioStatus product get in touch:-
email: enquiry@biostatus.com
phone: +44 1509 558163
website: www.biostatus.com

BioStatus products are for research use only.
BioStatus products are the subject of several international patents. DRAQ7™ is a trademark of BioStatus Limited.

BioStatus Limited

56A Charnwood Rd, Shepshed, Leicestershire LE12 9NP UK
Company registered in England and Wales. No. 3079239

An ISO9001:2008 registered company