PeptiMatrix™
Next generation peptide hydrogels

APPLICATION NOTE

Building Better Brain Cell Models with PeptiMatrix™ hydrogels

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Introduction:

Fundamental limitations of 2D cell culture and animal models result in substandard matching to the human physiological environment, which has propelled the development of 3D *in vitro* cell culture systems that more accurately reflect the complexities of the human cerebral microenvironment to study brain function and disease [1]. In recent years it has become increasingly clear that numerous cell types and the networks of macromolecules comprising the extracellular matrix (ECM) that are organised in 3D, e.g. spheroids, organoids or on scaffolds, have an increased physiological relevance compared to 2D cell culture, specifically in drug discovery research. 3D *in vitro* models better mimic these conditions as they allow:

- Guided and spontaneous cell self-organisation
- Cell polarity is not forced
- Substrate stiffness can be matched to *in vivo* conditions
- Cell-cell and cell-matrix interactions occur in all dimensions
- Cell behaviour, such as spreading and migration, are more dependent on the matrix's properties

However, current 3D *in vitro* models typically still rely on animal-derived materials, such as those derived from Engelbreth-Holm-Swarm mouse sarcoma. These biomaterials have been shown to often be chemically ill-defined, have high batch variability, and do not accurately reflect human biological matrices *in vivo* [2].

The PeptiMatrix™ range of hydrogels are biocompatible 3D self-assembling peptide hydrogels (SAPHs) that are chemically defined and can therefore be tuned by altering their chemical composition and concentration to the desired application. As a fully synthetic SAPH, they are:

- Animal free: no animal products in product or used in manufacture
- Reproducible: limited batch variability and a strict batch testing protocol
- Customisable: adjustable peptide concentration to change gel stiffness, or addition of ECM components to mimic different tissue types
- Validated: verified across a wide variety of cell types
- Ready to use: no complex preparation required, simply combine your cells with the gel and plate out
- Cell-friendly: no ice required, cells can be encapsulated at 37 °C for a gentler, more physiological process
- Versatile: tested for compatibility with a range of standard end-point assays, with optimised protocols

The ability to alter these parameters allows each gel to be customised to match healthy and diseased tissues of the brain, influencing cellular behaviour to match *in vivo* conditions. The elastic modulus of neural tissue, a quantified measure of the material's resistance to deformation, has been reported to be in the range of 0.2-1 kPa, depending on the measurement technique used [3]. For this application note, the SAPH peptide concentrations were therefore chosen to ensure the hydrogel formed fell between this range to more accurately model the soft mechanical properties of brain tissue. Various brain cell lines of different disease states; atypical teratoid rhabdoid tumour (ATRT) cell line BT-16, neuroblastoma cell line SH-SY5Y, and glioblastoma cell line U87, were encapsulated

within different PeptiMatrix™ hydrogels at densities optimised to each cell type and grown to form 3D spheroids, then used for a variety of downstream analyses. This user guide details the specific conditions that allow this variety of brain cell lines to grow in PeptiMatrix™ hydrogels that promote cell survival and proliferation, as well as influence their cellular phenotype to become reflective of the tissue it is mimicking.

Methods:

Cell encapsulation

For each preparation, 1 mL of PeptiMatrix™ hydrogel (PeptiMatrix™) was placed into a 15 mL centrifuge tube and heated at 80 °C for 20 mins to ensure homogeneity. The hydrogel was then transferred to a 37 °C water bath whilst the cell suspension was being prepared. Cells were harvested, counted, and resuspended in cell culture medium [Dulbecco's Modified Eagle Medium (Gibco) + 10% FBS (BioSera) + 1% L-glutamine (Gibco)] at a concentration optimised for each cell line (see Table 1). To achieve a final volume of 1.25 mL, 250 µL of the cell suspension was added to 1 mL of hydrogel. The suspension was then thoroughly mixed by a combined action of reverse pipetting and gentle stirring. The gels were then incubated at 37 °C for 10 mins to allow gelation to occur. After incubation, 200 µL cell culture media was added on top of the gels and the plate was returned to the incubator. Cells were grown for 7 days, changing 180 µL of the media every other day, with slow and careful pipetting to not disturb or damage the gels.

Cell line	PeptiMatrix™ hydrogel	Cell suspension concentration prior to encapsulation (per mL)	Cell concentration in gel (per mL)
BT-16	PMCORE100	1.25 x 10^6	2.5 x 10^5
SH-SY5Y	PMCORE100	2.5 x 10^6	5 x 10^5
U87	PMCORE125 PMIKVAV150 PMRGD150 PMPLUS150	5 x 10^5	1 x 10^5

Table 1: Encapsulation conditions used for each brain cell line

Live cell imaging and detection

For a qualitative assessment of cell viability, gels were washed with Dulbecco's Phosphate Buffered Saline (DPBS; Gibco) and then incubated for 15 mins at room temperature in a solution of 40 µM ethidium homodimer and 20 µM calcein AM (Thermo Fisher Scientific) in DPBS. Images were then obtained using the EVOS Cell Imaging System (Thermo Fisher Scientific).

3D Cell Viability Assay

For a quantitative assessment of cell viability, gels were washed with DPBS (Gibco) and then incubated with 100 µL Cell Titer-Glo® 3D Reagent and mixed vigorously for 5 mins on a plate shaker to induce cell lysis. The plate was then incubated for a further 25 mins at room temperature to stabilise the luminescent signal. Luminescent signal was measured on a FLUOstar Omega plate reader (BMG Labtech) with luminescence probe (Emission lens gain = 2500, gain adjustment = 40%).

ICC staining

For assessment of spheroid shape and visualisation of the actin cytoskeleton, nuclei, and characteristic markers, gels were washed with DPBS (Gibco) and then incubated for 1 hour

in 4% Paraformaldehyde solution (Thermo Fisher Scientific). Gels were washed in DPBS and incubated with permeabilisation buffer [0.1% Triton-X 100 (Thermo Fisher Scientific) + 0.5% BSA (Sigma) in DPBS] for 1 hour. The plate was then incubated with primary antibody solution diluted in permeabilisation buffer overnight [α-SMA (Abcam), β-III-tubulin, (Abcam), MAP2 (Sigma)].

The plate was then incubated with 300 µM DAPI (Invitrogen), 300U Rhodamine Phalloidin (Invitrogen) at 1:250 dilution and appropriate secondary antibody [Alexa Fluor 488 goat anti-mouse IgG (Invitrogen), Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen)] at 1:250 dilution overnight. The plate was washed in DPBS then imaged on the Leica DMI400B Confocal SPE microscope with Leica LASX software.

RNA extraction and qPCR

Media was removed and 1 mL TRIzol® (Thermo Fisher Scientific) reagent per 1 x 10^7 cells was added to wells and left for 5 mins to allow dissociation of nucleoprotein complexes. Samples were transferred to nuclease free tubes and 0.2 mL chloroform/isoamyl alcohol was added per 1 mL TRIzol® and sample was shaken vigorously by hand for 10 secs. Samples were incubated on ice for 3 mins, then centrifuged for 15 mins at 12,000 x g at 4 °C. The top aqueous layer was transferred to a nuclease free tube, and an equal volume of 2-propanol was added and incubated overnight at -80 °C. Samples were centrifuged for 20 mins at 10,000 x g at 4 °C, then supernatant was discarded, RNA pellet was washed in 0.5 mL 75% ethanol, then resuspended in 20 µL nuclease free water.

RNA samples were quantified on a Nanodrop 2000c UV/IV Spectrophotometer, and equal concentrations were converted to cDNA using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions. qPCR plate was set up using 5 µL JumpStart™ Taq ReadyMix™ for Quantitative PCR (Sigma), 3.4 µL nuclease free water, 0.6 µL primer pairs (forward + reverse primers diluted 1:10) + 1 µL cDNA (diluted 1:5) per well. Plate was sealed and run on a ABI 7500 Fast Real-Time PCR System (96 wells), at 94 °C for 2 mins, then 40 x cycles: 94 °C 15 secs then 60 °C 1 min, then melt curve. Analysis was carried out by normalising results to 2x housekeeping genes.

Results:

Characterisation of BT-16 cells in PeptiMatrix™ hydrogels

ATRT cell line BT-16 cells were cultured in PMCORE100 for 7 days, where end point assays (LIVE/DEAD and ICC staining) were carried out (Figure 1). Brightfield images show cells growing in round spheroids from day 5 and LIVE/DEAD staining results show that the majority of the cells are viable at day 7. ICC staining shows expression of α-SMA, a marker frequently expressed in rhabdoid tumours [4], and β-III-tubulin, a neuronal marker expressed in some ATRT lines [5].

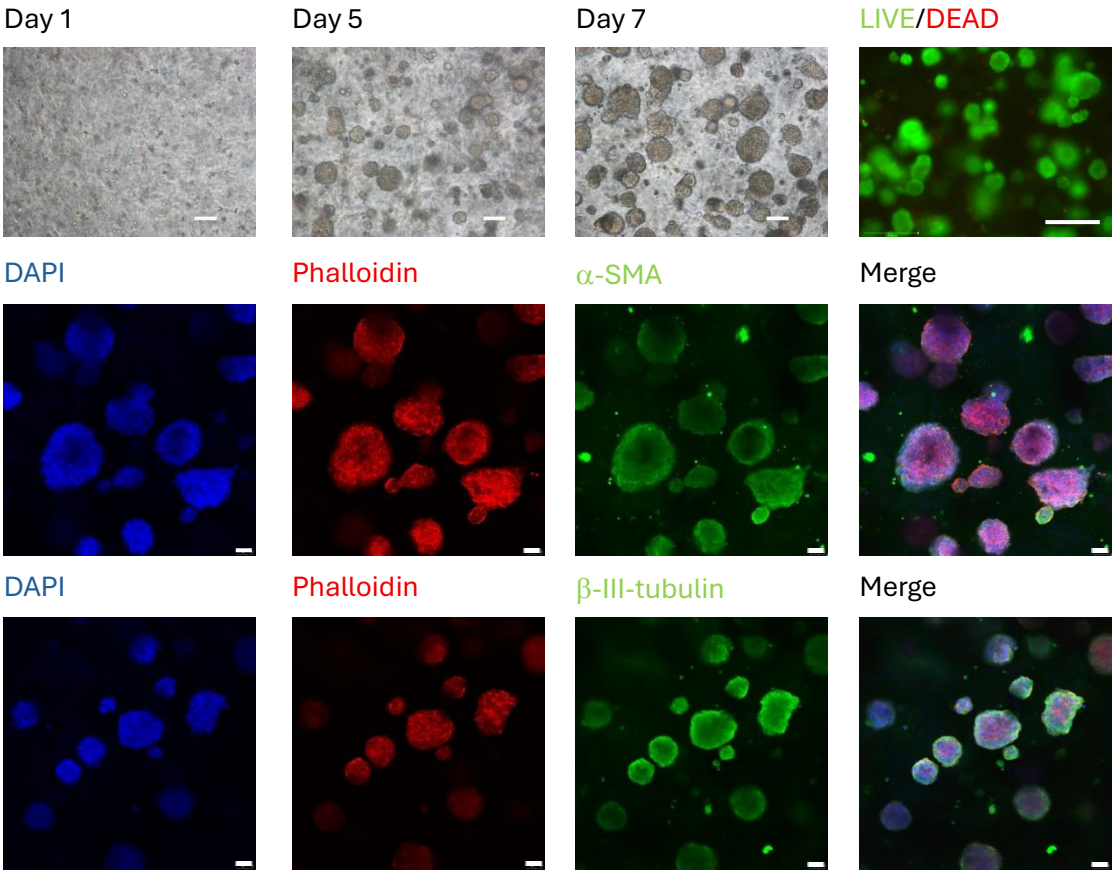


Figure 1: BT-16 ATRT cell line encapsulated in PeptiMatrix™ Core low stiffness hydrogel (PMCORE100) and grown for 7 days. Data shown is brightfield images (Scale = 100 µm), LIVE/DEAD assay (Scale = 275 µm), ICC staining (Scale = 50 µm) performed on cells on day 7.

Characterisation of SH-SY5Y cells in PeptiMatrix™ hydrogels

Neuroblastoma cell line SH-SY5Y (commonly used to model neuron-like cells) were cultured in PMCORE100 for 7 days, where end point assays (LIVE/DEAD and ICC staining) were carried out (Figure 2). Brightfield images show cells growing in round spheroids from day 5 and LIVE/DEAD staining results show that the majority of the cells are viable at day 7. ICC staining shows expression of mature neuronal markers frequently expressed in this cell line, MAP2 and β-III-tubulin [6]. In addition, when using a higher laser power in the red fluorescence channel during ICC, the outgrowth of small neurite-like protrusions became visible, a characteristic commonly measured in 2D and 3D *in vitro* neuronal models using SH-SY5Y cells [7].

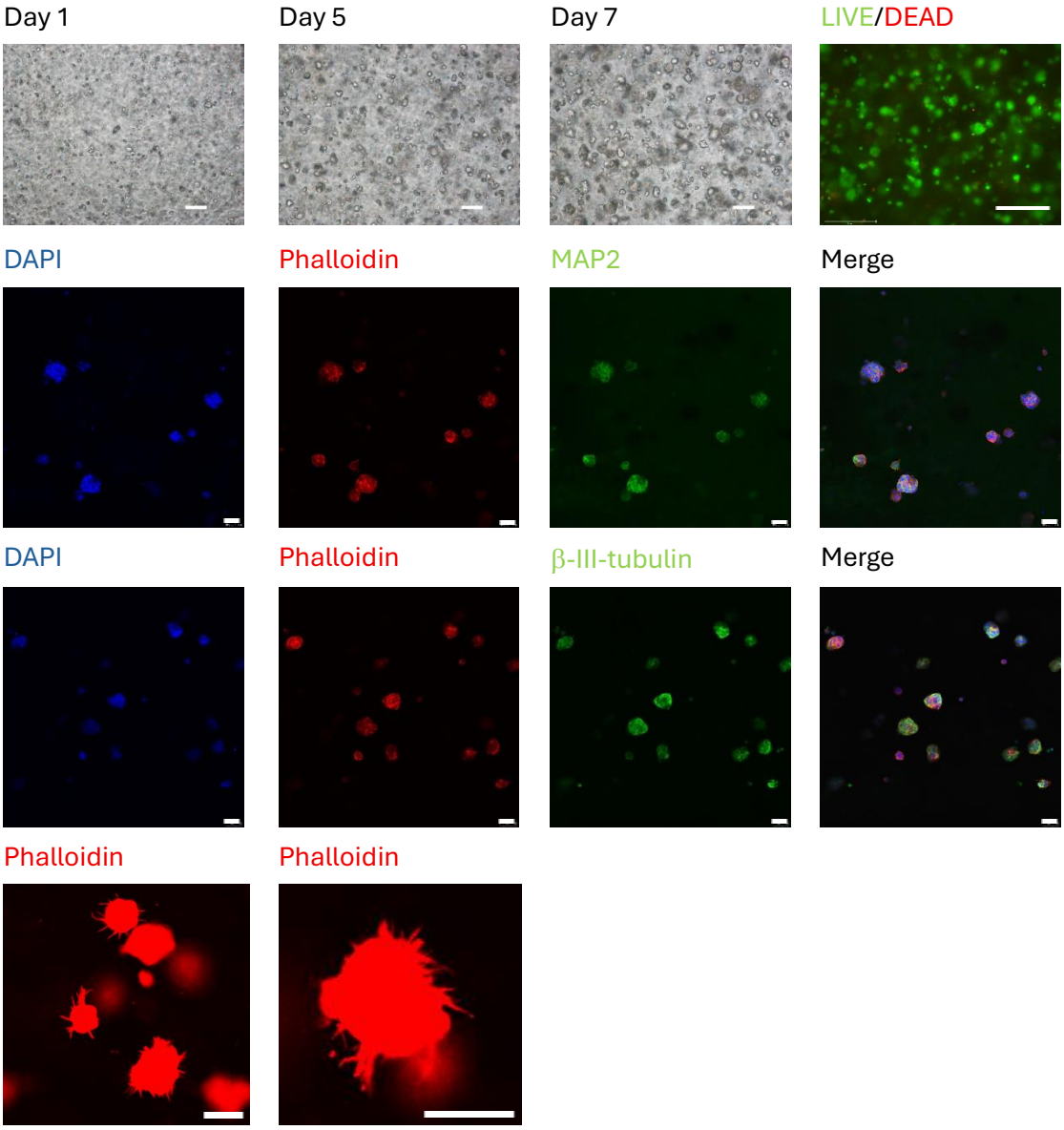


Figure 2: Neuroblastoma SH-SY5Y cell line encapsulated in PeptiMatrix™ Core low stiffness hydrogel (PMCORE100) and grown for 7 days. Data shown is brightfield images (Scale = 100 µm), LIVE/DEAD assay (Scale = 275 µm), ICC staining (Scale = 50 µm) performed on cells on day 7.

Characterisation of U87 cells in PeptiMatrix™ hydrogels (laminin + IKVAV functionalisation)

Glioblastoma cell line U87 cells were cultured in medium stiffness hydrogel PMCORE125, as glioblastomas are known to have a stiffer microenvironment compared to the surrounding brain tissue [8]. U87 cells were also cultured in PMCORE125 with a 10% functionalisation of animal-free laminin-111 (final concentration 10 µg/mL). Laminin-111 is a crucial basement membrane protein involved in maintenance of the blood brain barrier (BBB) that is also the main component of animal derived basement membrane matrix products commonly used in *in vitro* research [9]. U87 cells were also cultured in PMIKVAV150, a hydrogel stiffness matched to PMCORE125 that has an additional -IKVAV

amino acid motif attached to the peptide sequence, found in the α1 chain of laminin that plays a role in cellular processes, such as adhesion, neurite outgrowth and angiogenesis [10]. Brightfield images show cells growing in spheroids from day 5 and LIVE/DEAD staining results show that the majority of the cells are viable at day 7 (Figure 3). CellTiter-Glo® 3D assay results show a significant increase in cell viability for cells grown in PMIKVAV compared to PMCORE functionalised with laminin-111 (p=0.0425). qPCR results comparing PMCORE to PMIKVAV show a significant increase in N-Cadherin transcript expression (p=0.0046) but no significant difference in Nestin (p=0.1586) or Vimentin (p=0.1154), all markers commonly used in U87 characterisation [11].

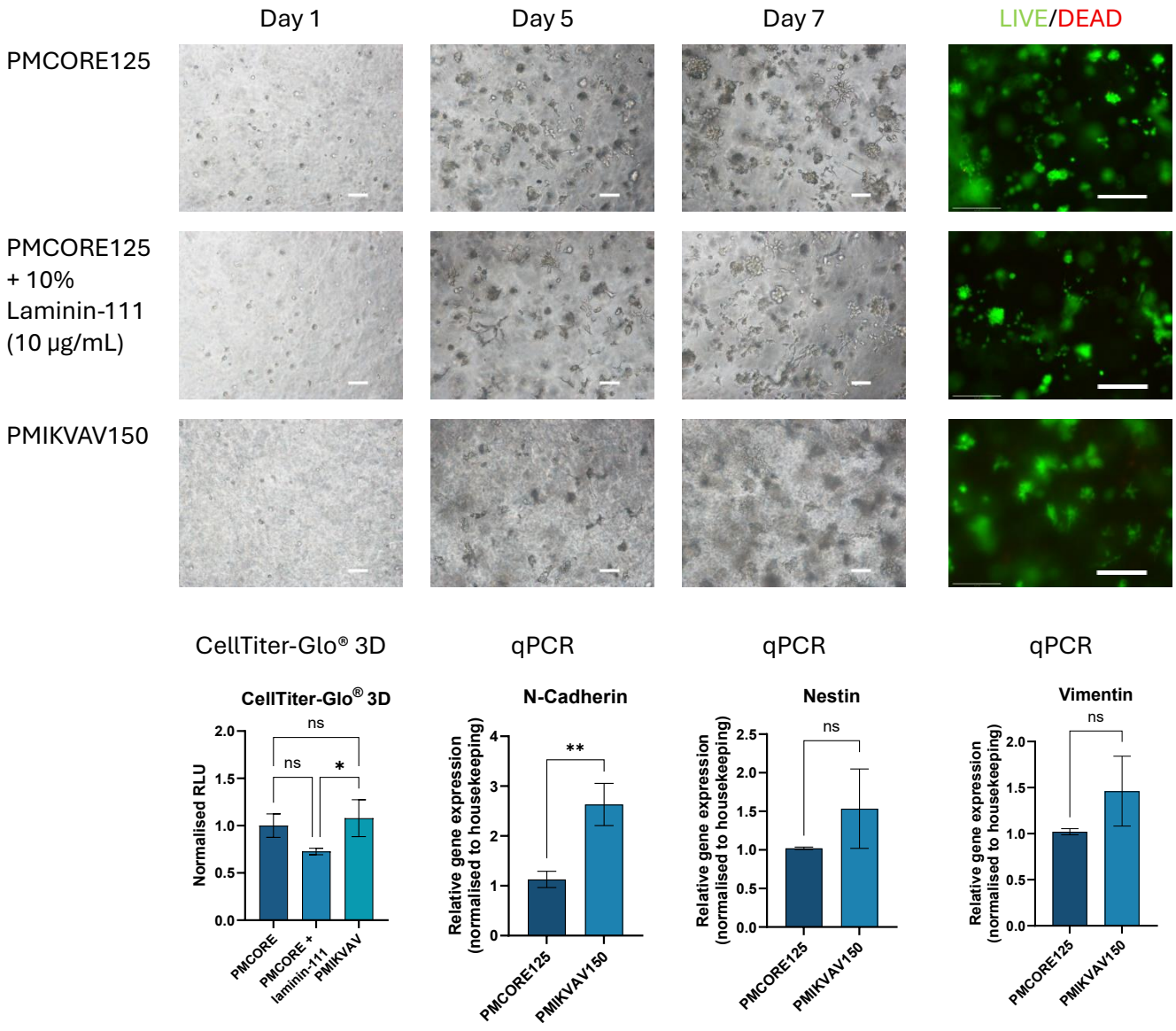


Figure 3: U87 glioblastoma cell line encapsulated in PeptiMatrix™ Core medium stiffness hydrogels (PMCORE125, PMIKVAV150 or PMCORE125 + 10% laminin-111) and grown for 7 days. Data shown is brightfield images (Scale = 100 µm), LIVE/DEAD assay (Scale = 275 µm), CellTiter-Glo® 3D assay performed on day 7 (ANOVA, N=3), and qPCR from RNA extracted from cells on day 7 (unpaired t-test, N=3).

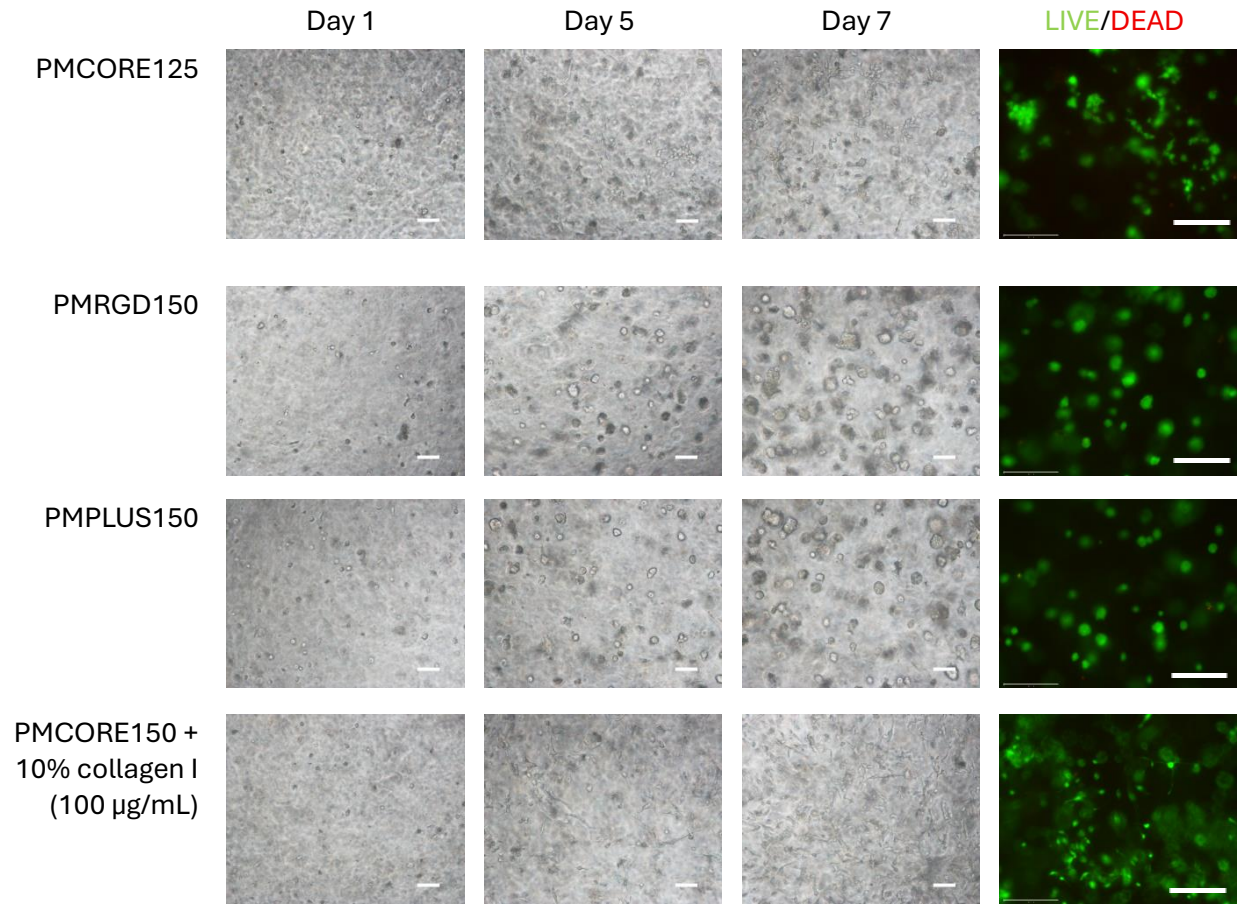
Characterisation of U87 cells in PeptiMatrix™ hydrogels (collagen/fibronectin + RGD/GFOGER functionalisation)

Glioblastoma cell line U87 cells were cultured in medium stiffness hydrogel PMCORE125, as described above, and were also cultured in stiffness matched PMCORE150 with a 10% functionalisation collagen I (final concentration 100 µg/mL). Collagen I is an ECM component that is expressed at low levels in healthy brain tissue, but has increased deposition within aggressive glioblastoma tumour microenvironments [12]. U87 cells were also cultured in PMPLUS150, a hydrogel stiffness matched to PMCORE125 that has additional amino acid motifs -RGD and -GFOGER attached to the peptide sequence that is designed to mimic collagen, by providing the α1β1, α2β1, α5β1, and αv-class integrin binding domains of Col I & IV. Cells were also cultured in PMRGD150, containing only the -RGD motif, designed for modelling tissues rich in fibronectin, another ECM component with increased deposition in glioblastoma tissues [12]. Brightfield images show cells growing in spheroids from day 5 and LIVE/DEAD staining results show that the majority of the cells are viable at day 7 (Figure 4). CellTiter-Glo® 3D assay results show a significant increase in cell viability for cells grown in PMRGD compared to PMCORE functionalised with collagen I ($p=0.0356$).

Conclusions:

- 3x different cell lines used in a variety of *in vitro* models of various disease states of the brain are able to grow in PeptiMatrix™ hydrogels for at least 7 days and display phenotypic characteristics and markers of the tissue they are designed to model.
- A variety of end point assays are compatible with the gel to characterise these cell lines, including LIVE/DEAD imaging, Cell Titer-Glo® 3D cell viability assay, ICC staining and RNA extraction for qPCR analysis, producing consistent results between both biological and technical repeats.
- Functionalisation of the hydrogel with synthetic amino acid motifs present in common ECM components result in comparable or in some cases increased viability compared to the CORE unfunctionalised gel, or the CORE gel functionalised with selected matrix proteins.

In conclusion, these hydrogels are highly reproducible and their ability to be chemically modified opens opportunities for controlling changes to ECM modelling that could prove beneficial for various applications in 3D *in vitro* cell culture systems, including development of cancer therapeutic opportunities [13].



CellTiter-Glo® 3D

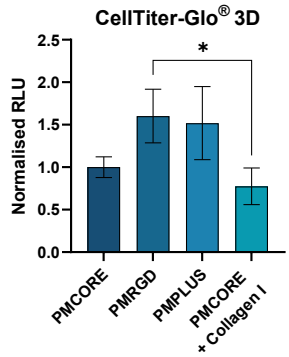


Figure 4: U87 glioblastoma cell line encapsulated in PeptiMatrix™ Core medium stiffness hydrogels (PMCORE125, PMRGD150, PMPLUS150 or PMCORE150 + 10% collagen I) and grown for 7 days. Data shown is brightfield images (Scale = 100 µm), LIVE/DEAD assay (Scale = 275 µm), and CellTiter Glo® 3D assay performed on day 7 (ANOVA, N=3).

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