Swift Formation Of Optimal Single Spheroids Towards *In-Vitro* 3-Dimensional Tumour Models

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Abstract

Introduction: Monolayer cell cultures, while useful for basic *in vitro* studies, are not physiologically relevant. Spheroids, on the other hand provide a more complex 3-dimensional (3D) structure which more closely resemble *in vivo* tumour growth thereby allowing results obtained with spheroids relating to proliferation, cell death, differentiation, metabolism, and various anti-tumour therapies to be more predictive of *in vivo* outcomes.

Methods: The protocol herein presents a rapid and high throughput method for the generation of single spheroids whose applicability was demonstrated on various cancer cell lines including (U87 MG; SEBTA-027; SF188) brain cancer cells, (DU-145, TRAMP-C1) prostate cancer cells, and (BT-549, Py230) breast cancer in green coded 96-round bottom well plates.

Results: Homogeneous compact spheroid morphology was evidenced as early as 24 hours after following the protocol. By using confocal microscopy and IncuCyte live imaging, the proliferating cells were traced in the rim and the dead cells were found inside the core region of the spheroid. H&E staining of spheroid slices and Western blotting were utilised to investigate the tightness of the cell packaging by adhesion proteins. Carnosine was used as an example of treatment for U87 single spheroids.

Conclusions: This 5 step-protocol allows the rapid generation of spheroids, which will help towards reducing the number of tests performed on animals and encourage 3D modelling experiments from early-stage research.

Introduction

Glioblastoma multiforme (GBM) is the most malignant primary form of glioma with prognosis of around a year after diagnosis [1, 2]. The complete removal of GBM tumour is impossible to be achieved by surgery alone due to the complexity of the tumour cells' structure and their migration away from the bulk of the tumour to the neighbouring brain parenchyma [3]. Moreover, the tumours are frequently resistant to radiation and chemotherapy [4, 5]. Despite the availability of various anti-cancer treatments, the treatment of solid tumours remains challenging, this is due to the tumour tissues developing three-dimensional (3D) solid morphology with cell-cell and cell-matrix contacts. Due to the multicellular resistance, actual tumours are more resistant to therapy than the monolayers of cell cultures [6]. Single spheroids of tumour cells mimic the biological properties of micro-metastases and vessel distal regions of tumours by retaining the architectural, morphological, and physiological characteristics of the tumour. Thus, there is a serious need to look towards new effective therapeutic strategies to cure this terminal disease [7].

In vitro spheroid models bridge the complexity gap between monolayer and *in vivo* tumour growth because spheroids exhibit many of the tumour microenvironment features by the avascular region of tumour tissues which resist drug diffusion [8]. The limitation in the delivery of anticancer treatments to the far situated cancer cells from the tumour blood vessels occurs due to the major characteristics of solid tumours which include cell to cell adhesion, extracellular matrix, large distances between blood

vessels, and high interstitial fluid pressure. Thus, modelling the multicellular resistance of tumour cells may contribute to developing *in vitro* findings into effective clinical therapies [9] Whenever animal studies and expensive 3D modelling are unfeasible, administrating this 3D spheroids method will be able to initiate an *In vitro* mimic to the mouse model and towards assessing drug penetration [10].

We have previously demonstrated that carnosine can be safely used as a potential therapy for GBM [11]. The *in vitro* applications for spheroids are numerous for cancer cell lines. However, forming compact round spheroids in 3D was an obstacle [9]. To overcome this problem, generating single spheroids has been developed using various culture techniques [12]. However, many limitations remain regarding the standardisation of the spheroids' high throughput production, homogeneity, and expense of these 3D models [13–15]. Here, we describe a detailed cost-effective protocol for establishing an *in vitro* 3D single spheroid model which can be utilised to identify potential new therapeutic approaches. This practical technique is reliable and could be applied to investigate *in vitro* studies of various tumour targeting drugs, antibodies and immunoconjugates [16]. Here we describe the time of cell attachment when the cells stop shrinking and start growing as a spheroid by monitoring many parameters including: the diameter or bright field area over time, the presence of cell death, the fluorescence stains that enable the detection of the dead/ live cells, and we visualise the inner and outer area of the spheroids to examine the invasion. Eventually, we confirmed the tightness of the generated spheroids by the H & E histology test and the western blot protein expression.

Materials And Methods

Cell Culture

The human glioblastoma U87 MG-Red-FLuc (Bioware Brite, PerkinElmer, Waltham, Massachusetts, USA) was the authorised cell line in all experiments. These cells were incubated in Opti-MEM Reduced Serum Medium (Gibco™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) culture medium, supplemented with fetal bovine serum up to 10%. The antibiotic puromycin (Gibco®, Thermo Fisher Scientific Waltham, Massachusetts, USA) was added after the initial thaw at 2 µg/mL. The incubation was at 37°C in a humidified atmosphere containing 5% CO₂. Other cell lines were cultured in their specific media by following the same protocol outlines. The human Glioblastoma SEBTA-027 (Recurrent GBM cell line derived from the right parieto-occipital region of a 59-year-old female) and SF188 (GBM cell line derived from and 8-year-old male) were cultured in Gibco™ DMEM, high glucose, GlutaMAX™ Supplement, and 10% fetal calf serum (Gibco™, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Both cell lines were a generous gift from the University of Portsmouth, neuro-oncology group. The human Prostate cancer DU145, HTB-81™ (American Type Culture Collection ATCC, Virginia, USA) were cultured in Eagle's minimum essential medium modified to contain Earle's Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate (BioWhittaker® Medium EMEM Cell Culture Media, Lonza, Maryland, USA), and 10% fetal calf serum. The murine prostate cancer TRAMP-C1 (C57Bl/6 mice cells which are derived from prostate adenocarcinoma cells from TRAMP mice) were cultured in

Dulbecco's Modified Eagle Medium 4.5 g/L glucose w/L-Gln w/ sodium pyruvate (DMEM, Lonza, Maryland, USA), and 10% fetal calf serum. This cell line was provided by Matteo Bellone (University of Milan, Milan, Italy). The human breast cancer BT-549 are ductal carcinoma (American Type Culture Collection ATCC, Virginia, USA) were cultured in Corning RPMI 1640 Medium with L-Glutamine (Corning™ RPMI 1640 Medium, New York, USA), 10% fetal calf serum and 0.023U/mL insulin. The murine breast adenocarcinoma Py230 (American Type Culture Collection ATCC, Virginia, USA) was cultured in Corning Medium F-12K with L-glutamine (BioWhittaker® Medium F12K Medium, Lonza, Maryland, USA), and 10% fetal calf serum, 0.1% MITO + serum extender (Corning®, New York, USA) .

Generation Of Spheroids

A volume of 50 µl of Anti-Adherence Rinsing Solution (STEMCELL Technologies, Cambridge, UK) was added to each well of a clear green coded 96-well round base plate for suspension (Sarstedt, Nümbrecht, Germany). After 15 minutes all wells were washed with 50 µl of serum free media. U87 MG cells were grown as a monolayer then detached with Trypsin (Sigma-Aldrich, St. Louis, Missouri, USA) to generate a single-cell suspension. The cells were seeded (400 cells/well) in 100 µl full media. Directly, the spheroid formation was initiated by centrifuging the plates at 3700 RPM for 10 min. The plates were incubated under standard cell culture conditions at 37° C, 5% CO₂ in humidified incubators. The full media had been replaced each other day.

Localisation Of Cell Death And Proliferation Within Spheroids

U87 MG spheroids were generated from seeding 400 cells per well in 100 µl of media into a 96-well, round-bottom plate as explained previously. The spheroids were stained by IncuCyte® Cytotox red for counting dead cells (250 nM, Essen Bioscience). The spheroids were transferred to the IncuCyte S3 Live Cell Analysis System (Essen Bioscience Inc., Ann Arbor, Michigan, USA), images were snapped with 4× objective lenses in each well every hour inside an incubator over 10 days. The localisation of the red dead cells within the spheroids was assessed by phase-contrast images using the red channel to evaluate the real-time cell membrane integrity and cell death. The total phase and the red fluorescent areas with mask were quantified for different days using IncuCyte® spheroid analysis software (Version. 2020B, Essen Bioscience Inc., Ann Arbor, Michigan, USA).

Imaging And Microscopy Studies

The single spheroids were stained following the instructions of each kit. The dead cells were stained by IncuCyte® Cytotox red for counting dead cells (250 nM, Essen Bioscience) during the generation process. The cyanine nucleic acid dye permeated cells with compromised cell membranes. The green dye CFSE Cell Division Tracker Kit (BioLegend, San Diego, California, USA) and DAPI (Sigma-Aldrich, St. Louis,

Missouri, USA) were used to stain the live cells and the nucleus within before taking pictures. The morphology of the spheroids was assessed and recorded using the IncuCyte S3 Live Cell Analysis System and confocal laser scanning microscope (Leica, Wetzlar, Germany) by a 5× objective using the following settings: sequential scanning, ex/em: Mitotracker: 543/599 nm, Hoechst: 405/461 nm. The size of the spheroids was analysed using IncuCyte® spheroid analysis software (Version. 2020B, Essen Bioscience Inc., Ann Arbor, Michigan, USA), and ImageJ software (version. 1.44, National Institute of Mental Health, Bethesda, Maryland, USA).

Hematoxylin and eosin H & E stain of spheroid slices

U87 MG spheroids were generated from 400 cells. Prior staining was cultivated for 1, 3, 5, 7, 10 days, washed once in PBS, fixed with 10% formalin (Merck, Darmstadt, Germany). The spheroids were washed in PBS and transferred to disposable biopsy Molds to encapsulate them with Eprelia™ HistoGel™ Specimen Processing Gel (Fisher-Scientific, UK). Each gel capsule was moved to a cassette and loaded inside a tissue processor (Excelsior AS, Thermo Scientific, Germany). The program was set to start with 6 times of alcohol, then 3 times of xylene, 3 times of wax. Each cycle time was 10 minutes. Then the capsules were embedded in paraffin using (Histostar, Thermo Scientific, Germany) with a temperature range of -3 °C to -12 °C. Microtome (Leica, RM2235) sections of 5 µm were placed on Super Frost glass slides (Menzel-Glaser, Thermo science, Germany) and allowed to dry for 2 hours at 37° C. The sections were deparaffinised by 2 changes of xylene for 5 min, rehydrated by 2 changes of 100% ethanol, followed by washing in 70% ethanol for 1 min. After a short single rinse in distilled water, the sections were stained for 20 min in Mayer's hematoxylin (Merck) and placed for 20 min under running tap water, then dipped in 1% Scott's Tap. The slides were observed under the microscope. If the nuclei were too blue, a quick dip in Acid alcohol followed by a tap water rinse. If it was not blue enough the staining was repeated from hematoxylin step. Sections were counterstained with eosin (Merck) for 2 min, rinse quickly in tap water, dehydrated by a dip in 70% ethanol, followed by 2 changes in 100% ethanol for 2 min each, and 2 changes of xylene for 2min. The slides were mounted in DPX (Sigma-Aldrich, Germany), and left to air dry for 2 hours. spheroid sections were assessed by bright field microscopy.

Immunoblotting Of Cell Membrane Proteins

A confluent between 70–80% U87 MG monolayer culture served as positive control. After 24 hours and 10 days, the spheroids were pooled from one 96-well round bottom plate. All spheroids were washed twice with ice-cold PBS, then lysed in 500 µl RIPA lysis buffer (50 mM Tris-HCl pH 8, 150 mM sodium chloride, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Triton x100, 1mM EDTA) containing protease inhibitor cocktail (Sigma). The lysates were vigorously vortexed and placed on ice every 10 minutes for a 30-minute period. After vortexing samples were centrifuged for 15 min at 15,000 rpm at a temperature of 4°C. The protein concentration was determined by a BCA protein assay (Sigma). A total of 50 µg protein extract was mixed with 5× Laemmli loading buffer (50% glycerol, 10% SDS, 0.25% bromophenol blue, 250 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol) resolved on a 10% SDS-PAGE gel and

a wet transfer was performed with 25 mM Tris, 192 mM glycine and 20% methanol for 90 minutes at 100 V onto a polyvinylidene fluoride (PVDF) membrane. Membranes were then blocked with a 5% milk in TBST solution and then probed with primary antibodies directed against β -actin (Sigma), vinculin (Abcam), vimentin and E cadherin (Cell Signaling Technologies). After incubation with primary antibodies, HRP-linked secondary antibodies (Cell Signaling Technologies) were used to detect bound primary antibodies in combination with Clarity Western ECL substrate (BioRad Laboratories).

Results And Discussion

To evaluate the homogeneity and the dynamic of the growing single-well spheroids, the increase in sizes was quantified by the IncuCyte spheroids software. Unlike aggregates, the spheroids were regular in shape, displayed a uniform round geometry, and exhibited a narrow size variation. The spheroids size was estimated using the mean confluence of the phase area for 4 spheroids. The cells were gathered over the first 24 hours which was displayed as a shrinkage in the detected area. However. After a day and 4 hours all wells showed homogeneous spherical single spheroid from U87 MG cell line. The diameter of the U87 MG spheroids formed from 400 cells per well ranged from $216 \pm 9 \mu\text{m}$ after 24 hours to $475 \pm 8 \mu\text{m}$ after 5 days, then $847 \pm 11 \mu\text{m}$ after 10 days. The homogeneity of the spheroids was reflected by the small standard deviation ranging of the mean (Fig. 1, a/ A).

Using the red coded 96 well plate resulted in a complete failure in the production of single spheroids with or without washing the wells by the anti-adherence solution. Without using the washing solution, the cells started to adhere at the bottom of the plate. Even with washing the wells, the images showed an incomplete circle shape. Thus, the cells were not able to gather properly during the centrifugation (Fig. 1, a/ B & C). The IncuCyte results in Fig. 1/ b show the ability of the U87 MG cells to form a single spheroid after around 24 h. The critical step in the protocol is the use of the anti-adherence washing solution which consists of an amphipathic component to prevent cell adhesion [17]. We found that using the green coded 96 well round bottom plates promoted the formation of homogenous spheroids instead of cell aggregates. The method was applied horizontally and vertically in the same plate to monitor the consistency and reproducibility in all locations. The IncuCyte live cells system showed the 3D structure of the spheroids which was tightened after the same period in all seeded wells. All spheroids were grown consistently over a period of 12 days.

The spheroid morphology in IncuCyte images confirmed the overall transformation from forming aggregates to generating tight morphology of single spheroids where the dead cells (in red) were localised in the centre. To trace the localisation of the dead and live cells, an IncuCyte images were taken using the green channel filter to show the live CFSE stained cells surrounding the Cytotox red colour taken up by dead cells at the core of the spheroid (Fig. 2, a/ A-D). This method has the advantage of assessing the mobility of the tumour cells by applying the invasion study of the U87 MG cells from the surface of the spheroid. The invasion mask can be added, and the area of the dead cells can be subtracted from the whole spheroid over time (Fig. 2, a/ E). The confocal microscopic images for the spheroid morphology

showed the live cells (green with blue nucleus) proliferated outwards and the volume of the single spheroid increased significantly from day 5 (Fig. 2/ b).

To investigate the tightness of the cell packaging in the single spheroid, histological sections were examined for spheroids which were grown for 1, 3, 5, 7, 10 days in culture. H & E is the combination of hematoxylin which stains cell nuclei with a purplish-blue colour, while eosin stains the extracellular matrix and cytoplasm pink. Figure 3/ a shows an example of H & E stain from the centre and the rim section of a spheroid at day 7. This evidenced that the density of U87 MG cells was high in the core region, whereas the daughter cells around the rim gathered to tighten and increase the spheroid size. The figure displayed an H & E stain of the middle section of the spheroid which encompassed the complete tight structure of the spheroid from core to rim. The rim of the spheroid consisted of evenly layers of packed cells toward the centre despite the death of the cells. During spheroid formation, a small proportion of cells did not integrate inside the sphere and lost cell-cell adhesion properties. The reason for this separation is the gravity-sedimentation [18]. However, adhesion and tight junction proteins are the principal factors involved in turning cell aggregates into spheroids. Western blotting experiment revealed a down regulation of E-cadherin and vimentin in spheroid cultures compared to cells grown as a monolayer (Fig 3/ b). These findings imply that these spheroids adopt a highly invasive mesenchymal phenotype as opposed to an epithelial phenotype [19]. This epithelial to mesenchymal transition (EMT) has previously been identified in spheroids formed using the CAL33 head and neck squamous cell carcinoma [20]. These EMT changes are indicative of a cancer stem cell phenotype; a population known to be highly treatment resistant and responsible for tumour recurrence [21]. As a result of these findings, the proposed technique represented an excellent *in vitro* single spheroid model for testing potential therapies as a preliminary platform before moving to *in vivo* mouse model.

In this project, we have generated single-well spheroids from seven different murine and human tumour cell lines (brain, prostate, and breast cancer) by starting from same cell density per well (Fig. 4/ a). To optimise the mimicking of different stages of avascular tumour regions, the size of the spheroids can be adjusted by seeding different cell numbers prior to centrifugation. The treatment application which targets primarily viable cells at the rim area of the spheroids while the core region should be small but large spheroids will harbour a big centre with a necrotic area [22]. The resulted single spheroids could be transferred by ease of mechanical access to any plate or cell culture vessel for further investigations or analysis. Cell viability in the spheroid might be significantly affected differently by the 3D microenvironment of multicellular contacts such as the expression of tight junction molecules, which establish a delay in the initial shrinking of the spheroid size. On average the spheroids reach a stable symmetrical size with smooth surfaces after 24 to 48 hours the growth starts. However, some cell lines require a longer time to adjust before growing such as DU145 cells which need around 5 days to produce firm spheroid. In this project we demonstrated the benefit of using the spheroid model by utilising carnosine in a sustained release designed experiment. We showed the inhibition of the single spheroids growth by applying different concentrations from carnosine and comparing them with the untreated spheroid. Carnosine was added every other day for 3 days to mimic the sustained release therapy. The change in morphology of the single spheroids was monitored using the IncuCyte spheroids software for a

period of 3 days which proved the penetration of carnosine to the live cells in the single spheroids. The series of carnosine concentrations reflected the critical amount to hinder the viability of the spheroid which was above 100 mM (Fig. 4/ b).

Conclusion

Monolayer cultured tumour cells exhibit less resistance to therapeutic interventions than *in vivo* cells. Developing a 3D model that represents the tumour microenvironment is important, especially when trying to bridge the gap between *in vitro* and *in vivo* tumour models. This protocol produced uniform single spheroids without any additives. Consistently, this method demonstrated reproducible high throughput U87 MG spheroids formation within a reasonable timeframe and cost. The spheroids exhibited the ability of being solid and allowed the penetration of the carnosine treatment, simultaneously. Examination of the U87-MG-Red-Fluc spheroids also revealed that these spheroids mimic tumour morphology, with a necrotic core of dead cells being visible much like the necrotic core often observed in GBM tumour histology. Optimising different cell lines will provide the research workers with an easily accessible and 20 folds cheaper than the average models which depend on using the ultra-low adherent plates for *in vitro* investigating.

Declarations

Author Contributions: K.H. performed the experiments and wrote the first draft. J.R.D.P. performed the Western blot experiment and contributed to the final manuscript. S.E.B.M. contributed to the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical statement: The authors declare that no animal or human tissue were used in this study.

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Figures

Figure 1

IncuCyte S3 live cell system (4x) Live spheroids images analysis shows **(a) A.** the proliferation curves of the confluence ratio of U87 single spheroid for four spheroids shrunk to the half size after 24 hours then grew over time. The images show the negative effect of using different type of plate. **B.** The instant adherence of the cells when using different plate without washing. **C.** The cells gathered when the anti-adherence solution. However, a crack in the middle appeared after the centrifugation directly. **(b)** the proliferation curves of the confluence ratio of U87 single spheroid upon using the protocol steps. The first triplicate failed to convert the aggregates to spheroids without using the washing solution. However, following the protocol showed consistent reproducibility for the single spheroids on three triplicates 2, 3, and 4. The images show the difference between the shape of the aggregates and the successful shape of the spheroid.

Figure 2

(a) Confocal images show the distribution of the green live cells with the blue nucleus which are located close to the rim of the spheroid in day 5 and 10. **A & C.** The 5- and 10- days spheroid images consequently across the centre show the dark shade where the dead cells are existed. **B & D.** The 5- and 10-days spheroid images consequently across the rim show the tightness of the cells surrounding the spheroid from the edges. (b) IncuCyte S3 live cell system (4x) Live spheroids images after 10 days. **A.** The phase image shows the confluence of the live cells around the spheroid distributed uniformly. **B.** The phase with the red channel filter image shows the localisation of the dead cells in the centre of the spheroid. **C.** The phase with the green channel filter image shows the 3D localisation of the live cells around the spheroid. **D.** The overlaps of the red and green filter show the 3D shape of the cells dead/ live in the same spheroid. **E.** The masks of the whole and invasion of the cells which could be used to monitor the effect of any applied treatment over time and convert it to a curve using the IncuCyte spheroid invasion software.

Figure 3

(a) Cell tightness and interaction analysis of U87 MG spheroids. H & E stain of spheroid slices from **A**, the core area, and **B**, top rim area of 7 days spheroid generated from 400 cells. **(b)** Expression of **A**, E-cadherin and Beta Actin. **B**, Vinculin and vimentin in U87MG cells grown either in a monolayer, or as spheroids derived from 400 cells for 24 hours and 10 days.

Figure 4

(a) IncuCyte S3 live cell system (4 $\times$) Live spheroids images show the potential of using the protocol to obtain single spheroids from various cell lines by the mean size and standard deviation for 6 individual samples from each cell line **(b)** IncuCyte images show the effect of the sustained treatment of carnosine on U87 single spheroids. The spheroid tightness was affected with carnosine > 100 mM. Scale bar 400 μm .