Glioblastoma-Neuroblastoma co-cultured multicellular spheroid model for anti-cancer drug screening

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ABSTRACT

Preclinical drug screening often relies on 2D mono-cultures and animal models, which fail to replicate the complexity of the human brain tumor microenvironment. This study presents a novel 3D co-culture spheroid model combining glioblastoma (U87-MG) and neuroblastoma (SH-SY5Y) cells to better mimic the cellular and molecular dynamics of brain tumors. Using the liquid overlay technique, reproducible and scalable spheroids were developed and characterized for growth, morphology, and drug sensitivity to temozolomide (TMZ), an FDA-approved brain cancer drug. Our findings demonstrate that glioblastoma spheroids thrive under hypoxic conditions and show significant resistance to TMZ, maintaining ~88% viability even at 1000 µM. In contrast, neuroblastoma spheroids exhibit marked sensitivity, with viability reducing to ~67% at the same concentration. Co-cultured spheroids displayed intermediate drug sensitivity (~68% viability), highlighting the influence of neuroblastoma cells on glioblastoma's drug response. Notably, neuroblastoma cells in the co-culture model exhibited neurite-like morphologies, suggesting differentiation and enhanced tumor-tumor interactions. This model addresses key limitations of existing preclinical systems by bridging the gap between simplistic 2D cultures and time-intensive organoids. It provides a biologically relevant and time-efficient platform for investigating tumor-stroma interactions, drug responses, and the role of neuron-glia dynamics in glioblastoma progression. Therefore, this co-culture spheroid system represents a critical step forward in developing more effective therapeutic strategies for brain tumors.

Keywords: Spheroids, Glioblastoma, Neuroblastoma, Temozolomide, 3D Tumor models.

1 INTRODUCTION

Cancer remains a leading cause of illness and death worldwide, with approximately 20 million new cases and nearly 10 million deaths reported in 2022 alone. Advances in diagnosis and treatment have improved the outcomes for some cancers, but significant challenges persist, particularly for aggressive forms such as brain cancer. The current projections suggest that by 2050, there will be approximately 56% increase in incidences and 65% increase in mortality for brain and related cancers[1]. Among these, glioblastoma (GBM) represents the most common and lethal primary brain tumor, accounting for 45-50% of all gliomas. Despite aggressive multimodal treatment involving surgery, radiation, and chemotherapy, the median survival for glioblastoma patients is only 12-15 months[2], with a five-year survival rate of less than 10%[3,4]. This prognosis is attributed to glioblastoma's highly invasive nature, genomic heterogeneity, and resistance to conventional therapies.

The interactions between neurons and glial cells are fundamental to maintaining brain function and are intricately involved in both the progression and resistance mechanisms of brain tumors. Glioblastoma exploits the surrounding tumor microenvironment (TME), which includes astrocytes, neurons, and other glial components, to promote its invasiveness and chemoresistance. The dynamic communication between these cells, mediated through signaling molecules and metabolic exchange, plays a pivotal role in glioblastoma's adaptive capabilities[5,6]. Understanding these interactions is essential for developing more effective therapeutic strategies for brain cancer.

Preclinical studies investigating tumor biology and anti-cancer drug screening have majorly relied on two-dimensional (2D) cell cultures (~66% [7]) and animal models. While 2D cultures are simple, cost-effective, and reproducible, they lack the structural and functional complexity of in vivo environments, failing to accurately replicate cell-cell and cell-matrix interactions. Animal models provide a more comprehensive microenvironment, but their use is limited by ethical concerns, high variability, and species-specific differences, particularly in the brain[8]. For example, human astrocytes are larger, more complex, and exhibit unique functional traits compared to their rodent counterparts, raises concerns regarding the relevance of findings from animal studies[9].

Three-dimensional (3D) multicellular tumor spheroids (MCTS) have emerged as a promising alternative, closely replicating the in vivo architecture and microenvironment of

tumors. Unlike 2D models, spheroids provide gradients of oxygen, nutrients, and waste, simulating the spatial and functional heterogeneity observed in tumors. This enables a more realistic evaluation of tumor growth, invasion, and drug responses[10]. However, most existing spheroid models focus on mono-cultures, which do not account for the critical role of neuron-glia interactions in brain tumor progression. Although organoids offer advanced modeling capabilities, they require extended maturation periods, often several months, and fail to maintain precise neuronal-glial ratios, limiting their utility in rapid preclinical testing[11].

To address these gaps, this study introduces a novel 3D co-culture spheroid model combining glioblastoma (U87-MG) and neuroblastoma (SH-SY5Y) cells. This model aims to mimic the bi-directional communication and interdependence observed in the brain TME, incorporating both neuronal and tumor components to create a more biologically relevant system. The liquid overlay technique was employed to generate reproducible and scalable spheroids, ensuring ease of handling and reduced variability[12]. By studying the growth dynamics, morphology, and drug response of these co-cultured spheroids, this work seeks to provide insights into the interplay between glioblastoma and neuroblastoma cells under 3D conditions.

A key objective of this research is to evaluate the efficacy of temozolomide (TMZ), an FDA-approved chemotherapy agent, within this co-cultured spheroid system. The co-culture model offers a unique opportunity to study how neuronal cells influence glioblastoma's drug sensitivity. By comparing the drug responses of glioblastoma mono-cultures, neuroblastoma mono-cultures, and co-cultured spheroids, the study provides a comprehensive understanding of drug efficacy in a more representative brain tumor model.

This research bridges the gap between simplistic 2D models and complex organoid systems, offering a time-efficient and biologically relevant platform for studying brain tumor biology and testing potential therapeutics. Beyond glioblastoma, this 3D co-culture spheroid model has the potential to advance preclinical research in other neurodegenerative and neurodevelopmental disorders, where neuron-glia interactions play a significant role in disease pathology. By integrating the strengths of spheroid-based models with the complexity of co-cultures, this work contributes to the development of innovative approaches for addressing critical challenges in brain cancer research.

2 METHODOLOGY

2.1 CELL CULTURE AND FORMATION OF SPHEROIDS

The glioblastoma and neuroblastoma cells were provided by Prof. James Curtin (Technical University Dublin) and Prof. Dennis Barry (Trinity College Dublin) respectively. The cells were cultured in a DMEM media supplemented with 1% penicillin-streptomycin, 1% L-Glutamine (Merck Life Sciences) and 10% Fetal Bovine Serum (Cytiva HyClone). The Phosphate Buffer Saline (PBS) and Trypsin needed for the washing and detachment ofcells were also obtained from Merck life sciences. The cells were maintained at 37˚C and5% CO_2.

Initially, Flat-bottomed 96 well plates (SPL Life Sciences) were coated with 1% agarose solution (Fisher Scientific). Then, cell suspensions with cell densities of 5000, 10000and 15000 per well were prepared in 200µL medium for U87-MG and SH-SY5Y. Equalamount (100µL each) of U87-MG and SH-SY5Y cells were used to develop the co-cultured spheroidmodel. The spheroids were grown for 14 days. Media was changed on the 4th,7th and 10th day after imaging. While changing media, only 100 µL of the old spent media was removed so as not to perturb the spheroids and dissociate them. Then new 100µL mediawas added gently on the walls of the well to minimize the stress on the spheroids. Phase contrast microscope images of the spheroids were taken each day using a Keyence BZ-X810 Microscope.

2.2 LIVE DEAD ASSAY

Live Dead Assay, a qualitative way of assessing the cell viability of spheroids was performed on 7th, 10th and 14th day. Briefly, 150 µL of media was removed from each well, washed with PBS and were added with 10µL of Propidium Iodide (PI) followed by Fluorescein Diacetate (FDA) dissolved in PBS and Acetone respectively. The concentration of dyes was kept constant at 0.5mg/mL. After an incubation period of 10min, the spheroids were washed twice with PBS and taken directly for fluorescence imaging using a Keyence BZ-X810 Microscope.

2.3 DRUG TREATMENT

The spheroids were treated with Temoside® formulation obtained from Cipla Ltd, India at a logarithmic range to know the efficacy range of the drug in spheroid models developed. The concentration was based on the amount of active pharmaceutical ingredient – Temozolomide. The final drug concentration was maintained at 10,100 and 1000µM.

ATP Assay:

On the 7th day of spheroid growth, 150µL spent medium was removed from the 200µL media in each well and 50µL of Drug dissolved media was added. After 24h, the spheroids were added with 100 µL of Cell-Titer Glo 3D®, incubated at room temperature for 30min. The luminescence values were obtained using BioTek Synergy H1 multimode plate reader.

Live Dead Assay:

On the 7th day of spheroid growth, 100µL spent medium was removed from the 200µL media in each well and 100µL of Drug dissolved media was added. On Day 8, the spent media was removed and washed with PBS. Incubated with PI and FDA and imaged.

2.4 STATISTICS

All image measurements were made using ImageJ. The circularity obtained from ImageJ followed the formulae,

$$Circularity = 4\pi\left(\frac{Area}{Perimeter^2}\right)$$

All statistical analyses in this work were performed using either Python or GraphPad Prism software version 10.2.0 (GraphPad Software, Inc., La Jolla, California, USA). Repeated measures Two-way ANOVA statistic method with post-hoc Tukey test was used. $P < 0.05$ was considered significant ($n = 3$).

3 RESULTS

3.1 SPHEROID GROWTH

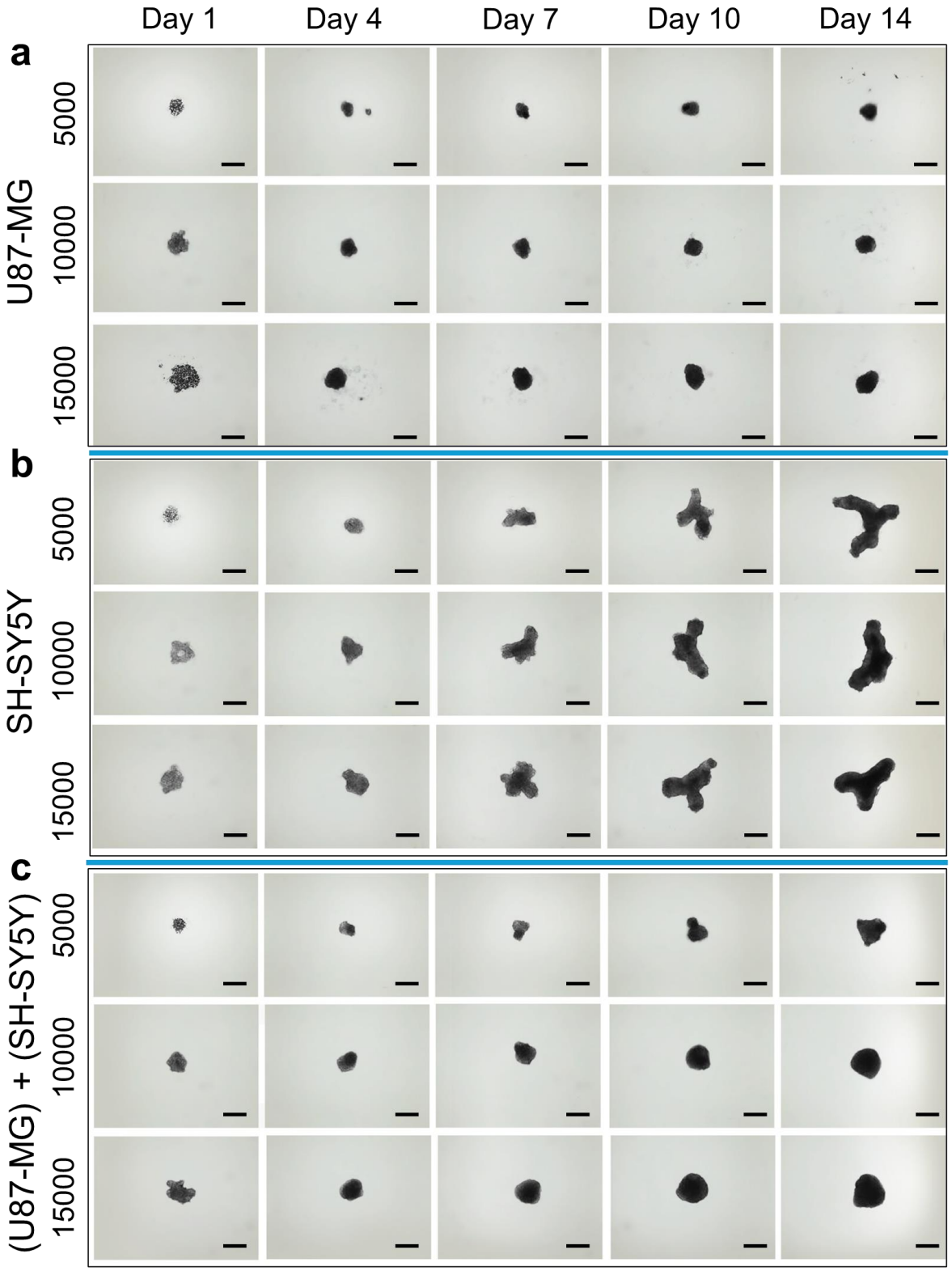


Figure 1. Spheroid growth over 14 days with cell seeding densities of 5000,10000 and 15000 cells: U87-MG (a), SH-SY5Y (b) and co-cultured spheroid (U87-MG)+(SH-SY5Y)) (c). Scale 500µm. (Images processed using Adobe Photoshop. Raw images in supplementary)

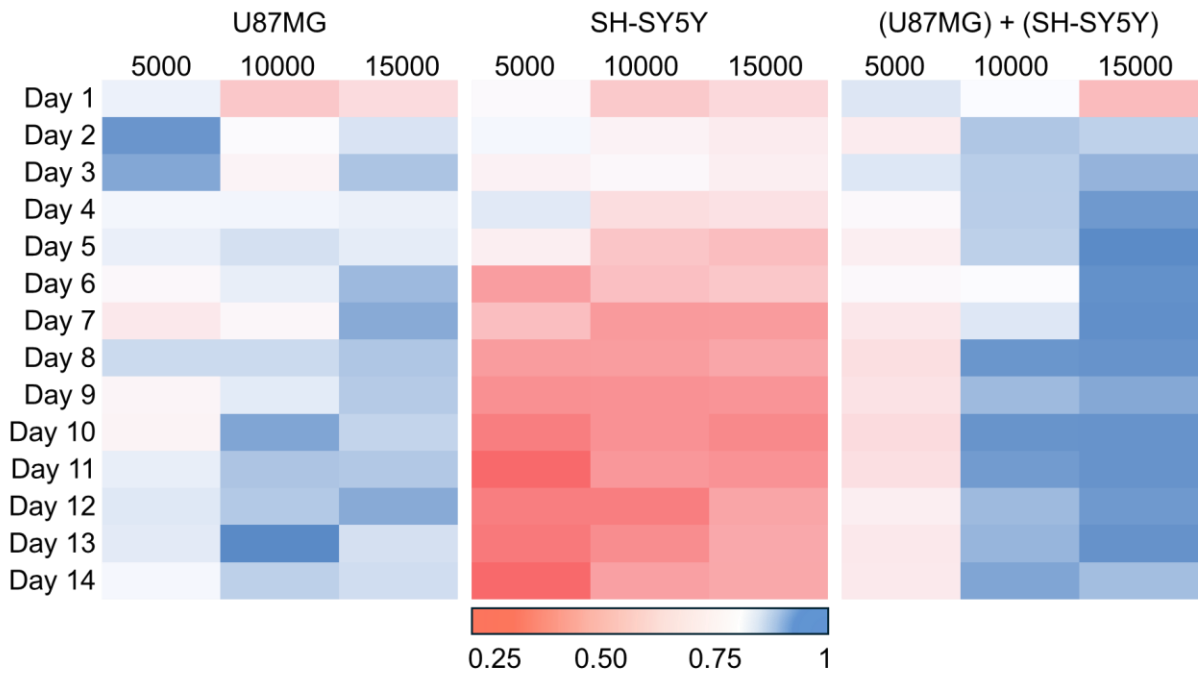


Figure 2. Circularity of Spheroids with cell seeding densities of 5000,10000 and 15000 cells: U87-MG (a), SH-SY5Y (b) and co-cultured spheroid (U87-MG)+(SH-SY5Y)) (c).

The U87-MG spheroids maintained a compact spherical shape in allthree different cell densities. The 5k cell group images displayed (Figure 1a) the ability of cells tohave cell-cell communication. It can be noticed that two smaller spheroids formed apart from themain spheroid, moving towards (See supplementary Figure S1a for Day 1 to 14 images) the main spheroid and getting fused with them. Similar cell-cell communication was observed on 10k group as well, on the initial 3 days (See supplementaryFigure S1b for Day 1 to 14 images), after which they merged to form a single spheroid. The 14thday image implies only the formation of a dense hypoxic region. This holds true as the size ofthe spheroids with 5k and 10k cells for different sets remained at around ~400µm and ~500µmrespectively at the end of 14 days. Literature studies suggested that only spheroids of size above ~500µm show necrotic core[13]. Necrotic core was visible in the 15k group on the last couple of days in the 14-day period. And rightfully, their sizes were above ~500µm at about ~600µm. The U87-MG glioblastoma spheroids have been consistent in their growth showing slow and gradual linearity in terms of increase in their area (Figure 1a).

For the neuroblastoma spheroids formed from SH-SY5Y cell line, all three spheroid groups followed an irregular but identical elongated pattern. The growth morphology observed is close to that of neurite protrusions in neurons following a pyramidal neuronal architecture which strongly branches out with a central core. During the initial days, they developed the core

and around the 7th day (earlier in case of highest density 15k cells) they started to branch out in different directions. In the case of 15k cells, even when they protruded in anotherdirection on Day 11, they fused back (Day 12) to form the tripolar pyramidal neuronal structure (See supplementary Figure S2c). All three cell densities started showing hypoxic regions from Day 9. The hypoxic regions were majorly seen in the node points from where the neurite like protrusions began. On Day 14, evident necrotic regions were observed in 10k and 15k cell groups (Figure 1b) in the same hypoxic region found on Day 9 clearly depicting hypoxia leads to necrosis in neuroblastoma. It is important to note the initial couple of days of growth in 10k cells density group, they had formed into a dough nut shaped spheroid. This was also faintly visiblein 15k group of glioblastoma spheroids on the 1st day. In our trial studies testing different cell densities and types as well, this pattern of growth was seen. Hence, we hypothesize that all cells once cultured usually form a doughnut-like pattern irrespective of the cell type and move inward towards center and then grow outward to form the spheroids.

Co-culture spheroids of U87-MG and SH-SY5Y grew at a ratio of 1:1 cell density. The spheroids grew larger than their glioma counterparts. However, they maintained spherical morphology as displayed by glioma spheroids. The 5k group alone slightly deviatedfrom the spherical morphology. This could be reasoned for astrocytes to influence their behavior, there might be a minimum threshold of cells required. Unlike the neuroblastoma spheroids which showed strong necrotic core and glioma spheroids which didn't show any necrosis, the co-culture took a middle stand with a small necrotic core compared to neuroblastoma spheroids. This can be attributed to their sizes as they remained consistently above 600µm for all cell densities. But the spherical morphology and area being closer to thatof U87-MG spheroids could be due to the higher proliferation rate of U87-MG cells found in 2D compared toSH-SY5Y, being seen in 3D environment as well [14].

The aspect ratio (Major axis over Minor axis) results obtained from ImageJ state that values between 0 to 1 to be Oblate spheroids while values above 1 to be Prolate spheroids. And from the results we obtained, all our spheroids were prolate, meaning that our spheroids are ellipses rotated along their major axis[15].

Circularity (Figure 2), measured in a scale of 0 to 1 with 0 being irregular and 1 being highly circular in shape, we were able to identify a pattern in the 5000 initial cell density group in all spheroid categories. Over the 14 days of growth, they all have moved away from their initial circularity due to proliferation[16]. It was also observed that the circularity increased with

an increase in initial cell density in all spheroid categories. Also, the higher the initial cell density, the earlier the co-cultured spheroids moved towards forming a circular morphology. The circularity of U87MG lied between 0.7 and 0.9 with one outlier value on either end of the value spectrum. For SH-SY5Y, majority of the spheroids showed circularity less than 0.7. In case of co-cultured spheroids, the circularity range increased consistently in each cell group. Most spheroids were above 0.7 but less than 0.8 in 5000 cells group followed by 0.8 to 0.9 in 10000 cells group and stayed around 0.9 in 15000 cells group. Circularity results from 15000 group in co-cultured category suggests the influence of cell-cell communication as they are the most circular spheroids in the study.

In this work, we have calculated the area of spheroids not the volume and circularity instead of sphericity, mainly due to irregular shape of neuroblastoma which on visual observations showed longer length and width (see also in Figure 1b) but the depth was comparatively thinner, and the z-levels ofprotrusions were different. This way of extrapolating to volume or sphericity might not reflect the actual morphology and might lead to misinterpretation of data.

This growth morphologies of spheroids, may it be spherical shape in glioblastoma andco-cultured spheroid or the neuronal elongations seen in neuroblastoma spheroids indicate the inherent behavior of cells having strong implications on a tissue level. Thus, thesemorphological understandings are of key importance as they give a sense of direction tounderstanding different spheroid's growth mechanism helping in design better 3D models reflecting the *in vivo* environment.

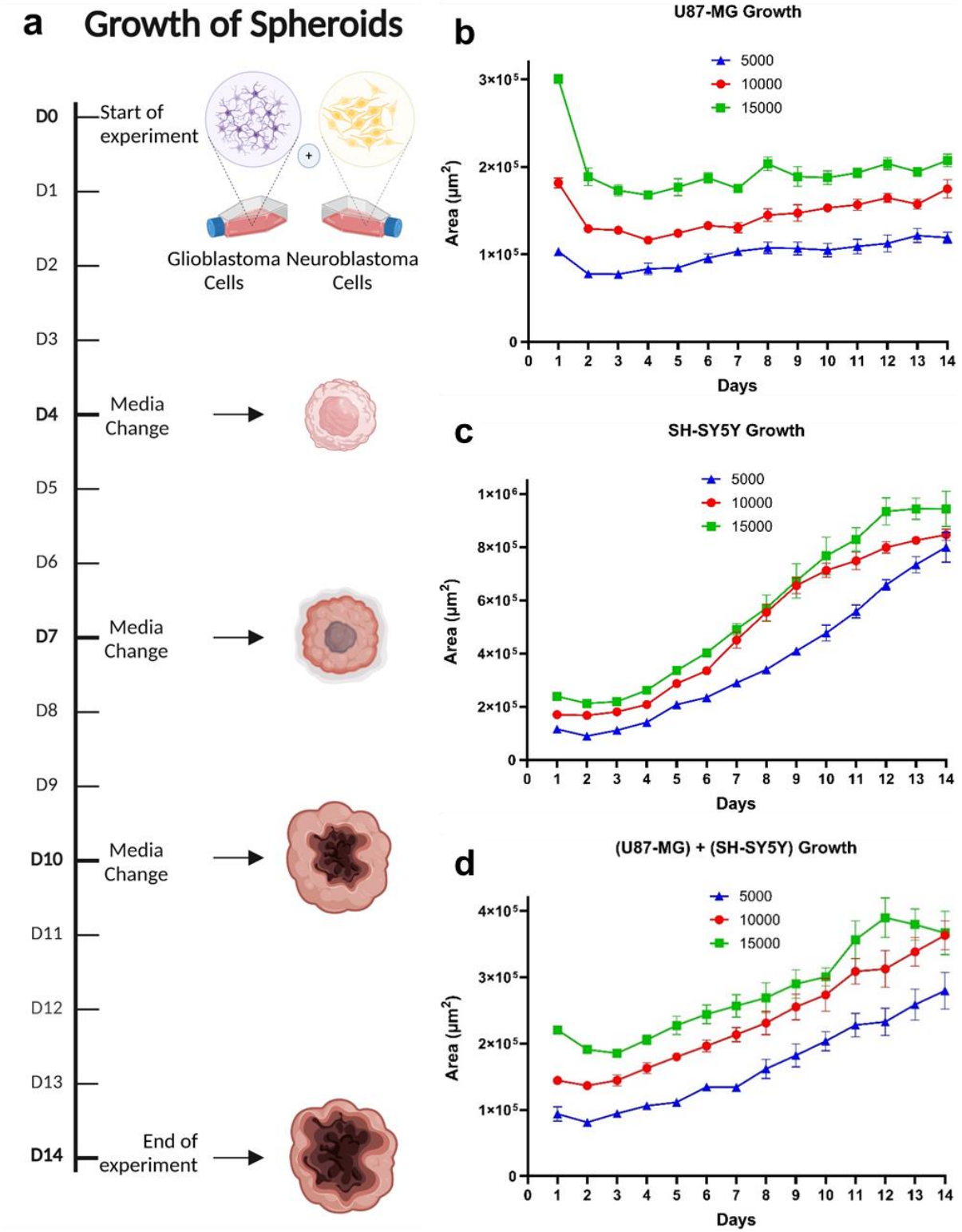


Figure 3. Growth Rate of Spheroids: Schematic (a) [Created with Biorender.com], U87-MG (b), SH-SY5Y (c), (U87-MG) +(SH-SY5Y) co-cultured spheroids (d)

The growth of spheroids over a period of 2 weeks (14 days) has revealed thatspheroid growth rate is dependent on cell type. (Figure 3) Still, all the groups discussed had the same pattern of growth irrespective of the type and took about 4 days to form a spheroid. After the

initial 4 days, they started growing steadily. The increase in area of U87-MG here was slow compared to SH-SY5Y. The area increases on the co-cultured spheroids remained in between but tending to a slower rate as that of U87-MG. This was quiteunexpected as U87-MG has a faster proliferation rate than SH-SY5Y in 2D cell culture.This could be due to irregularity in the shape of SH-SY5Y where the depth was less formed and well contained but loosely aggregated spheroids unlike the U87-MG, which formed tightly packed spheroids. The growth rate of co-culture being higher than but close toU87-MG demonstrates the dominant behavior of glioma at the same time represents theactivity of neuronal cells. Some data points on the (Figure 3a) suddenly sees a spike in area, it is attributed to the media replenishment. The larger area on the 8th day in U87-MG could be because of the media replenishment was done on 7th day which in turn led to a faster proliferation of theproliferation zone. This again falls back to the growth pattern expected on 9th day shows compaction of the spheroids is a continuous process visibly seen whenever mitosis overtakes the rate of necrosis. The growth after 10th day in SH-SY5Yand co-culture groups started moving towards attaining a state of saturation. This saturation happens based on size rather than being time dependent or cell density. ThusU87-MG also would reach the stage, but the timeline required is expected to be longer.This said, based on the cell type a minimum cell density is needed for them to reach saturation. This seems to be lacking with U87-MG cells with an initial cultured cell density of 5000 cells.

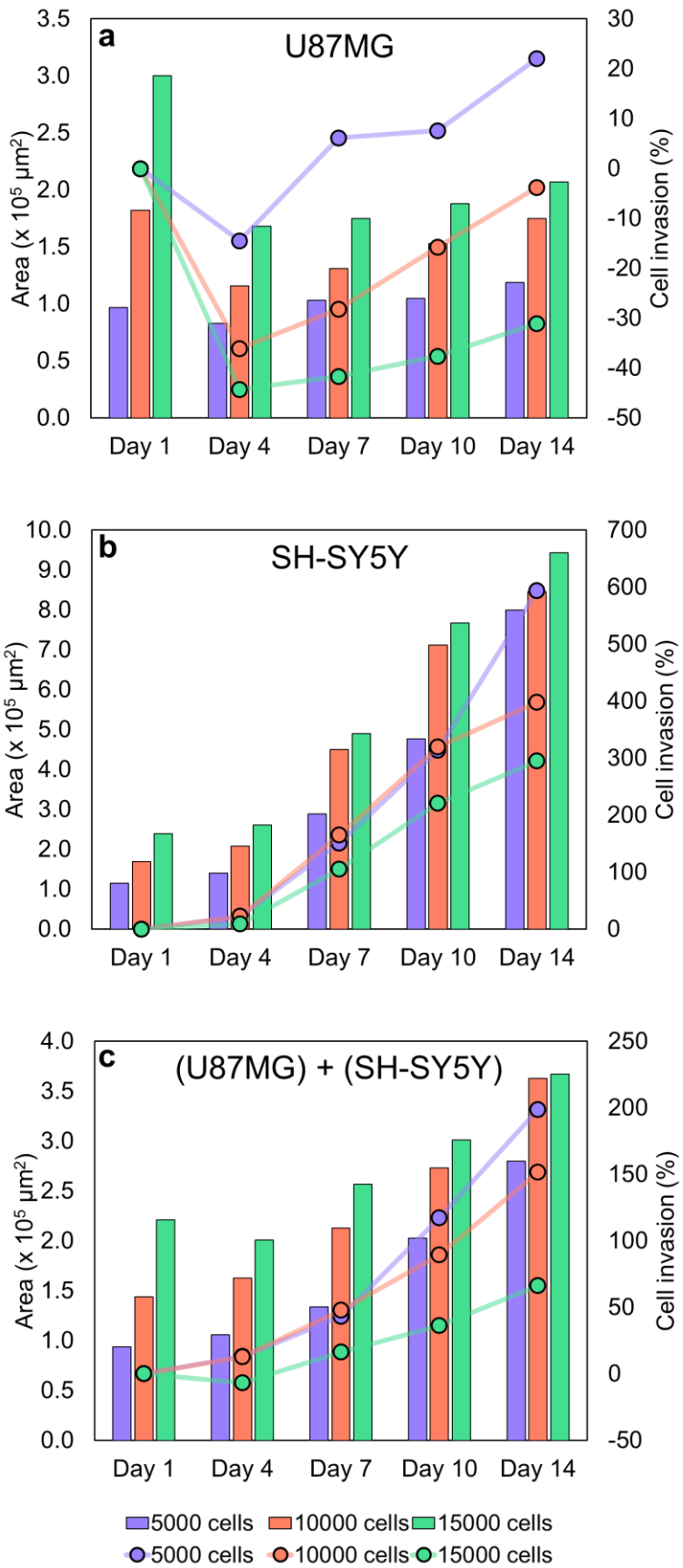
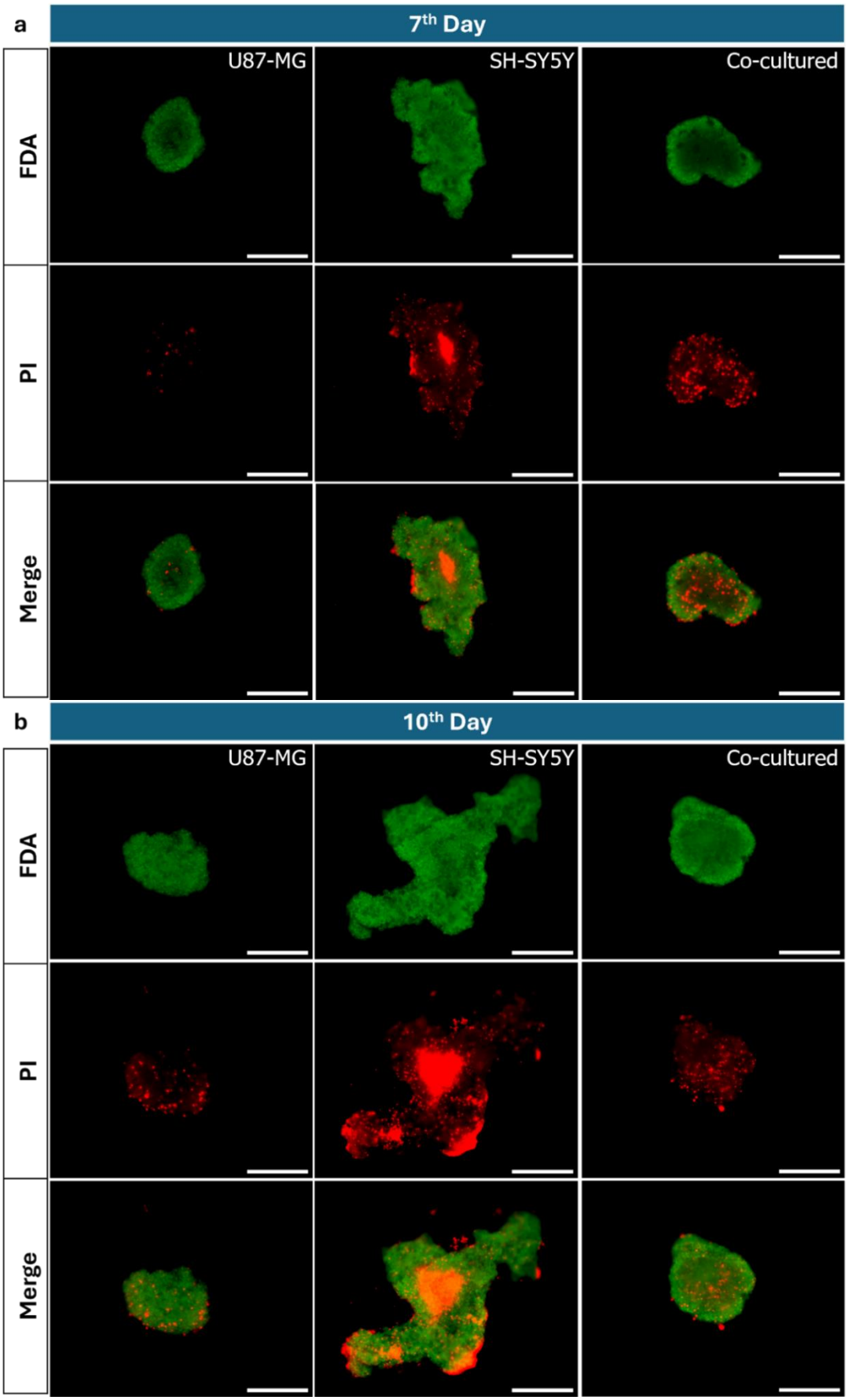


Figure 4. Spheroid Invasion rate: U87-MG (a), SH-SY5Y (b), (U87-MG)+(SH-SY5Y) co-cultured spheroids (c)

Irrespective of the spheroid group, the less the cell density higher was the invasion.The rate of invasion as well, was faster in less dense groups (Figure 4). This is because, in groups with smaller initial cell density there is more nutrient and oxygenper capita. Likewise, all cells regardless of their type and density undergo compactionbetween Day 1 and 4 (Supplementary Figure S2). In the co-cultured 3D model, approximately ~66% increase in invasion area was observed in 15k cells while ~150% and ~200% growth was noted in 10k and 5k cells respectively, indicating exponential growth. Over time, it was noticed that higher density cell groups moved towards a size limiting phase sooner compared to other low-density groups. For neuroblastoma, it was ~300%, ~400% and ~600% for 15k,10k and 5k cells. But this invasion wasn't consistent. Between days 4 and 7, 15k cell's invasion grew by 10 times while 10k and 5k cells grew only by 6 times. But later within Days 7 to 10, they doubled for all cell densities. In the final 10th to 14th day, 15k and 10k grew only by 50% whereas 5k still maintained the rate and doubled in invasion area (See Supplementary Figure S4a,b,c for each of 14 day invasion rate). This clearly shows that high dense cells grow at high rates initially but slow down later and the opposite stands true for less dense cells. The glioblastoma group were loosely aggregated till 4 days (Supplementary Figure S1) for all cell densities and started invasion only after. The 15k cells saw an insignificant growth of about 10% over 14 days. 5k and 10k cells showed 40% increased invasion area. This growth pattern, although not significant to the levels of neuroblastoma and co-cultured spheroids, holds true with high cell density groups invading slowly than the less dense ones.

3.2 LIVE DEAD ASSAY


a
7th Day
U87-MG
SH-SY5Y
Co-cultured
FDA
PI
Merge
b
10th Day
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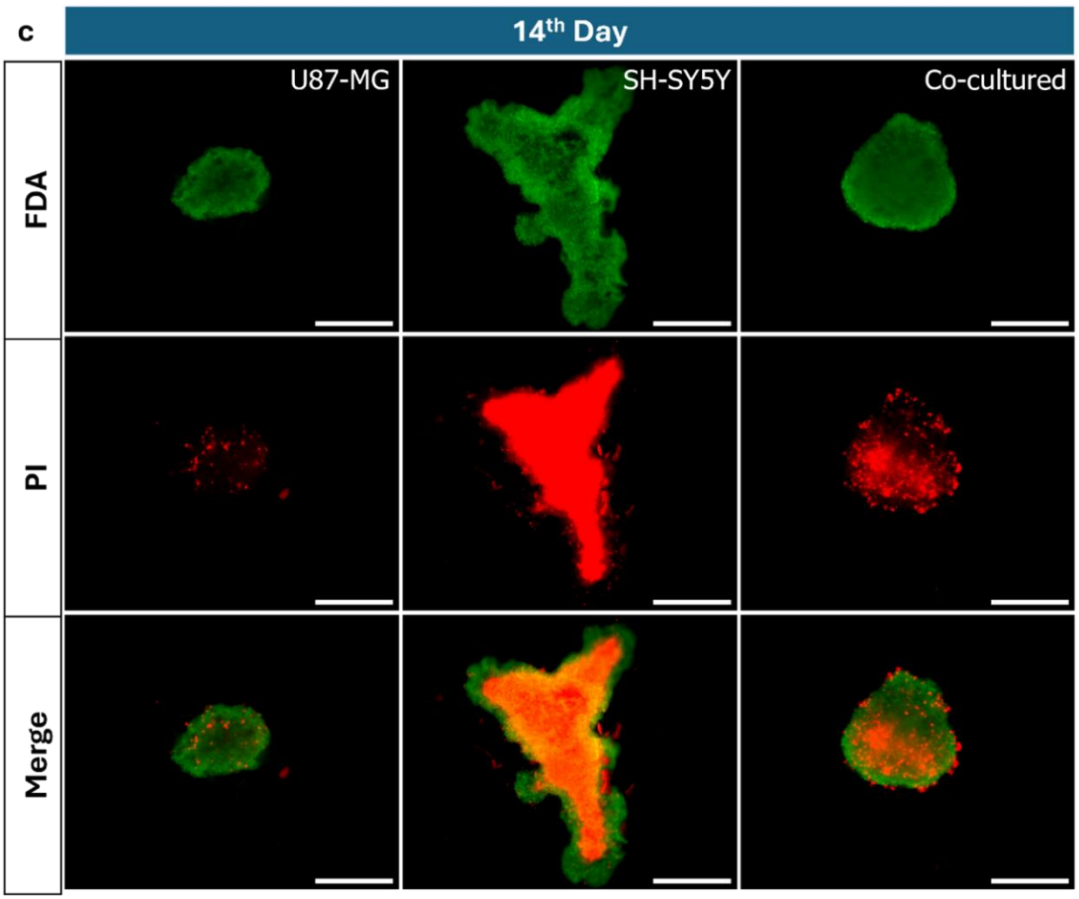


Figure 5. Live Dead results - Day 7 (a), Day 10 (b), Day 14 (c). Scale 500 µm

The live-dead assay works by the interaction dyes with the cellular componentsto represent live cells and dead cells with different fluorescence. In this case, the dyesused were Propidium Iodide (PI) and FDA (Fluorescein diacetate). PI permeates the dead cells and intercalates with their DNA. These cells, when excited with a particular wavelength result in red fluorescent emissionindicating dead cells. On the other hand, though FDA does not have inherent fluorescent potential but when permeating live cells, the esterases in FDA are converted to Fluorescein, a green,fluorescent molecule.

Over the course of 14 days (Figure 5), the viability of U87-MG spheroids remained high whileevidently SH-SY5Y spheroids viability decreased significantly. This could be attributed to the ability of U87-MG to grow well in hypoxia conditions[17] and there is no necrotic core formed yet as their sizes are still around 500µm. Studieshave reported that spheroids are more quiescent and hypoxic in this size range[13]. Also,hypoxia itself can create a necrotic core, and this tendency is seen in neuroblastomaspheroids. Right from Day 7, they have a necrotic core, and we can clearly see the size ofthe necrotic core increasing. By Day 14, almost the entire central region of the neuroblastoma spheroid has become necrotic. This indicates that, even in co-culturedspheroids, the neuroblastoma cells could be the ones which are necrotic.

3.3 DRUG TREATMENT

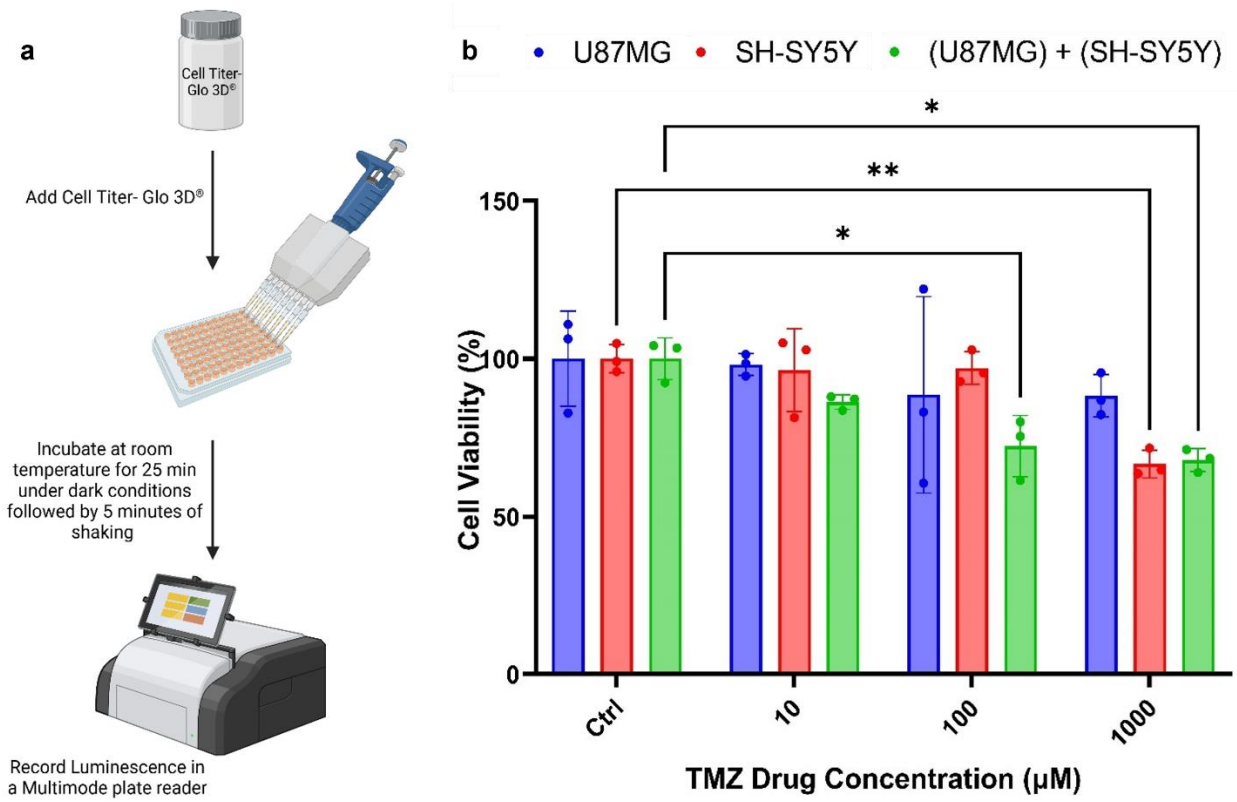
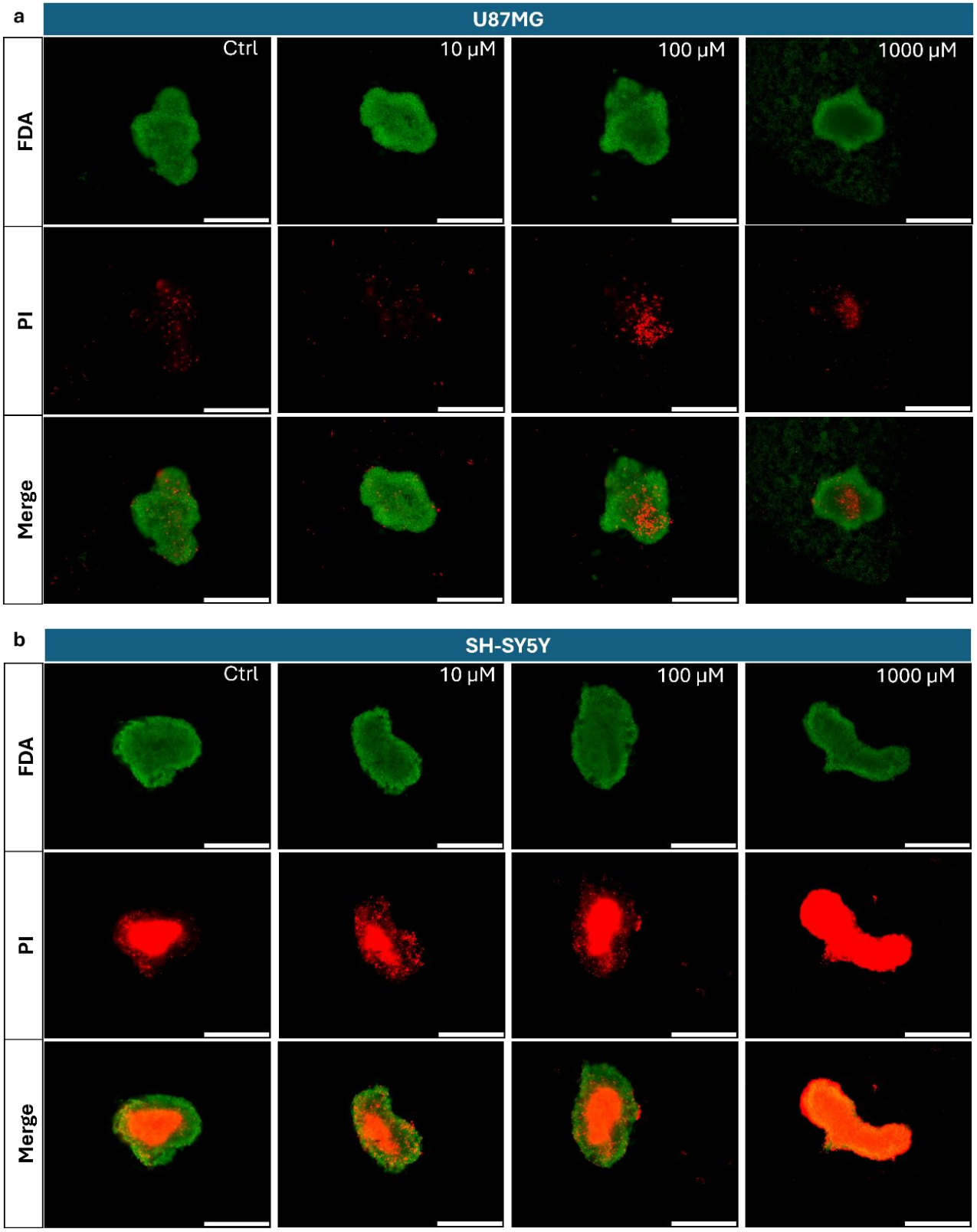


Figure 6. Schematic for Cell Titer Glo 3D® Assay (a) [Created with Biorender.com], Viability of the spheroids on Day 8 (after 24h DT on Day 7) (b).

The CellTiter Glo-3D assay (Figure 6a) quantifies the ATP activity of the viable cells in luminescent form. The luciferase enzyme promotes the oxidation of luciferin in the presenceof ATP generating luminescence. After 24h drug incubation, the cell viability results (Figure 6b) asquantified with luminescence shows that U87MG spheroids were resistant to TMZ at all concentrations with minimal change. Even after 1000µM treatment, the cell viability remained around 88% while in case of SH-SY5Y spheroids and co-cultured spheroids, the % viabilities went down to ~67% and ~68% respectively at the same concentration level showing significant cancer reduction. Also, the viability results show that SH-SY5Y spheroids are sensitive only after 100uM concentration whereas the co-cultured spheroids are vulnerable to TMZ treatment at all the concentrations treated. The viability of co-cultured spheroids, decreased from 86% to 72% to 68% at concentrations 10, 100 and 1000µM. This clearly shows the effect of interplay between U87MG spheroids and SH-SY5Y spheroids when cultured together as compared to individual U87MG spheroids where they were resistant to the TMZ treatment and individual SH-SY5Y spheroids were more sensitive. And on co-culture, the spheroids are sensitive to treatment even at 10µM and consistently going down in % viability. The individual

U87MG and SH-SY5Y groups are not sensitive to treatment at 10µM maintaining viabilities around ~97%. At 100µM U87MG was sensitive while SH-SY5Y still remained resistant. Then suddenly a significant decrease in viability was observed in SH-SY5Y spheroids at 1000µM.



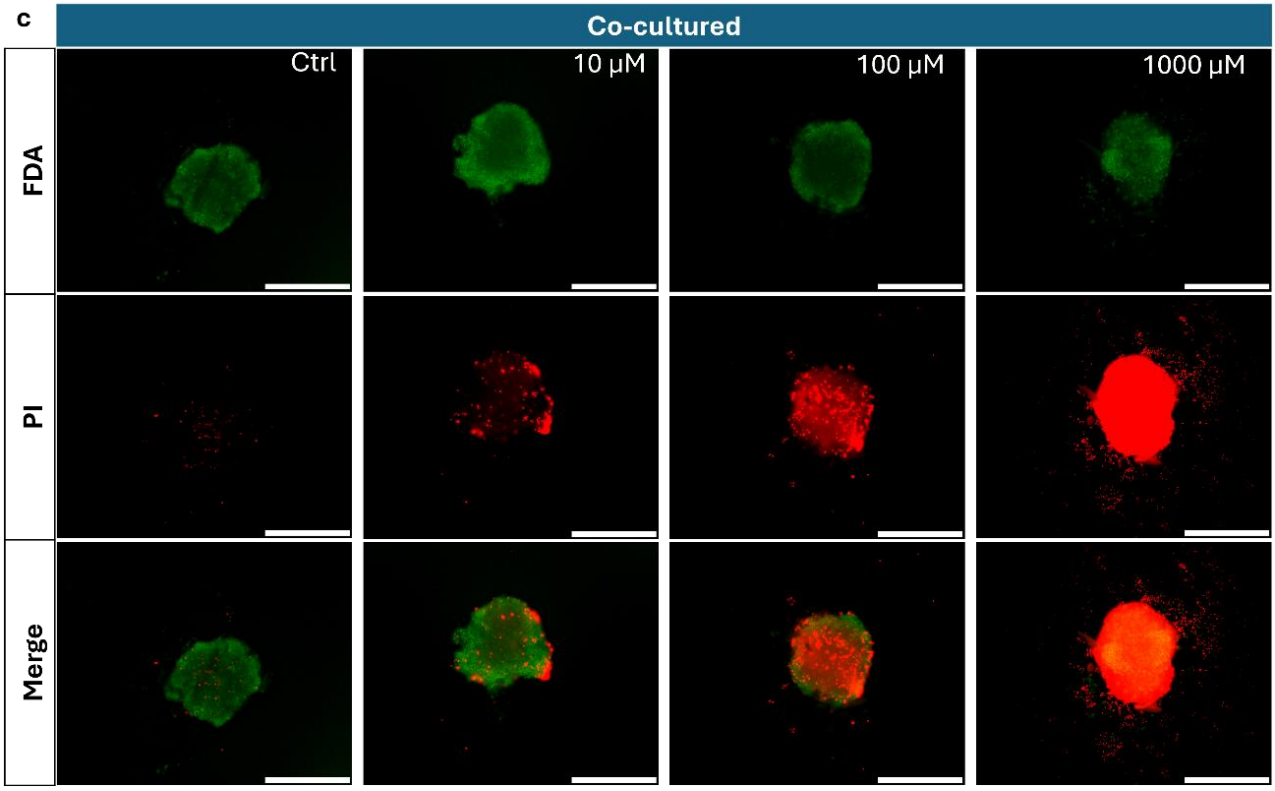


Figure 7. Live Dead Assay on the spheroids on Day 8 (after 24h DT on Day 7)

The live dead assay performed over TMZ treated spheroids reveal that the co-cultured spheroids at higher concentrations were able to kill the tumor. But when the SH-SY5Y spheroids are observed, is where the clarity that the tumor death on co-cultured spheroids is realized to be due to the influence of neuroblastoma and not due to glioblastoma. Though the live cells remain significantly high in glioma (Figure 7a), compared to Day 7 before drug treatment, the area on Day 8 after 24h drug treatment showed a decrease in size (Supplementary Figure S5, S6a). In individual neuroblastoma spheroids (Figure 7b), increase in concentrations has led to cell death in the spheroid core which can be attributed to the necrotic core being susceptible to the drug easily. The areas observed there indicate a linear downward trend in proliferation with increasing concentration (Supplementary Figure S5, S6b). At 1000 µM the area remains almost the same both before and after drug treatment. For other concentrations and control, the amount of increase in area has decreased. In case of co-cultured spheroids, for control and 10µM there was a non-significant increase in area of spheroid and at 100 and 1000µM there was a non-significant decrease (Supplementary Figure S5, S6c). In live dead of co-cultured spheroids, cell death has started from the periphery and moves towards the center with the increase in concentration as observed in (Figure 6c) at 10µM and 100µM. In glioma spheroids at lower concentrations, the drug only disintegrated the cell debris from the outer

proliferation zone. But at 1000µM, spheroids have dispersed as cellular aggregates but still they remained viable. For neuroblastoma spheroids, from Fig 4 we can see that they have a central necrotic core on Day 7 unlike the glioma and co-cultured spheroids. A similar pattern has been noticed even here in the control on Day 8. Lower concentrations (10µM and 100µM) on neuroblastoma spheroids, maintained cell viability closer to that of control but a logarithmic increase to 1000µM strongly decreased the cell viability. The live dead results of co-cultured spheroids indicated their high sensitivity to TMZ as cell viability decreased in linear manner with increase in dosage. Similar to glioma spheroids, at 1000µM co-cultured spheroids also showed a strong disaggregation of cells but here they stained red indicating cell death. Overall, these results exactly reflect the quantitative cell viability (ATP Assay) results of our work.

4 DISCUSSION

Many groups have developed mathematical models to understand and predict the growth kinetics of spheroids[18–22]. The results of our work as well, aligned well with their findings. All three zones of the cellular spheroid, namely proliferative outer zone, quiescent intermediary zone and necrotic core zone were visible in all our three typesof spheroids. The spheroids (Fig 2.2.2) clearly saw a contractile phase for the initial 4 days[23].

During the initial phase of spheroid formation, cell-cell adhesion forces dominate, pulling cells together into a compact cluster, causing the spheroid to shrink. As cells begin to proliferate, the increased cell concentration within the spheroid generates pressure that counteracts the adhesion forces. This balance prevents further shrinking and leads to steady growth. This phase continues until necrosis occurs, where the interplay between cell division (mitosis) and cell death (necrosis) determines the growth pattern[22] . The transient structure of a spheroid does not depend on its initial size, and the final structure can be independent of the seeding density[24]. So, the growth after an initial 4 days followed a sigmoidal curve pattern with exponential growth between 4th and 7th day while 7th to 10th day saw a transient slow growth phase indicating movement to a saturation phase (10th to 14th day).

In the initial days when diameter was small, necrosis was not observed but with increase in size necrosis region was formed and grew larger. A linear relationship was observed in viable cell numbers and spheroid diameter. This was notably seen through the live dead results on Day 7,10,14 where Glioblastoma and Co-cultured spheroids which had a saturated size values maintained their cell viability while Neuroblastoma which increased in size showed increased

cell death[25]. As per Aria and group, spheroids of glioblastoma and non-small cell lung cancer (NSCLC) were extremely compact, making it difficult to assess the amount of free space between neighbouring nuclei. They also displayed signs of cell crowding, which could have led to changes in cell size near the core. On the other hand, neuroblastoma spheroids maintained measurable and constant free space throughout the model, which was consistent with findings from their simulated low-density spheroids and our results[26].

Spheroids with sizes below 100µm and within 4 days of growth generallydoes not have any defined zone[17]. Similarly, only spheroids above 500µm showcase a necrotic zone which however might differ based on cell line. In our case, some spheroidsabove 500µm showed necrotic zone. But clearly with the increase in size, the probability of anecrotic zone and the necrotic zone's area increased. This is becausethe diffusion limit of oxygen is 150-200µm from the outer layer[13]. Furthermore, the hypoxia-inducible factor (HIF)-1α is stabilized at around 80µm inward from the proliferative zone. This suggests that all necrotic cores are hypoxic but not the other way around[20]. In U87-MG spheroids, a necrotic core was observed based on the grayscaleintensity, but live dead results showed them completely viable demonstrating they werehypoxic. Similar results were obtained by Jingyun and team with glioma spheroids[27]. SH-SY5Y had a necrotic core as early as 7th day and U87-MG did not even have on14th day. This suggests that necrosis occurs in a size dependent manner in spheroidsthan time dependent.

When the circularity of the spheroid was high, even diffusion was observed, the growth rate was low, and the response to the drug was high. This was not true to our case where Glioblastoma whose circularity was better than that of neuroblastoma was resistant to drug. At the same time, the co-cultured spheroids which also had good circularity showed a strong sensitivity to the drug. So, we find that it depends on the cell type and drug and cannot be generalized[28].

Furthermore, we found that it is important to choose the right cell viability assay. For 3D tumor models it is advised to prefer ATP based assays rather than colorimetric assays like MTT, Resazurin etc. The colorimetric assays suffer from issues like level of reagent penetration which varies based on spheroid size, density and heterogeneity of cells[29,30]. This was observed in our experiments as well on trials with MTT.

Previous works on monolayered cultures of U87 and SH-SY5Y suggests IC50 values between 100 -150µM on treatment with TMZ [31–33] indicating clear differences from our work

with 3D models where 1000µM was not enough to bring down the viability to 50% in all groups. The monolayered cells henceforth misrepresent the actual TME without a holistic view whereas various other studies with drug treatment of temozolomide (TMZ) in glioblastoma spheroids have shown similar results to that of ours. While U87-MG spheroids with sizes between 150-350µm responded to 100µM, the spheroids above 400µm did not. All our spheroids were above 400µm, at 100µM there was not significant decrease in viability of any of our spheroid groups. Concentrations above 200µM have resulted in spheroid disaggregation in a 24h study while concentrations lesser than that ablated the spheroid surface[12]. As our studies were at logarithmic concentrations, the spheroid disaggregation pattern was observed at 1000µM and only in glioma and co-cultured spheroid groups. This mechanism of action of the drug and spheroid response has been reported by Anna et al[34]. TMZ showed a dose- dependent inhibition of cell growth in all groups but significant effects were seen only in neuroblastoma and co-cultured spheroids. Temozolomide has been reported to be more toxic to neurons than glial cells, strongly validating our results[22].

5 CONCLUSION

In this work we have come up with a co-cultured 3D tumor disease model to screen brain cancer drugs. We have focused mainly on morphological and visual cues of spheroid models developed and their control groups to know the efficacy of the drug.This simple straight forward methodology can be widely opted for drug screening. Following this, we were able to identify that glioblastoma spheroids thrive under hypoxic conditions and show significant resistance to TMZ while neuroblastoma and co-cultured spheroids exhibited marked sensitivity. The sensitivity on co-cultured spheroids can be ascribed to the influence of neuroblastoma over glioblastoma. Our further work, however, will be towards learning the biochemical cues of the model through proteomic and transcriptomic studies as it will help in designing and developing targeted drugs for Brain cancer meanwhile enhancing the understanding on the disease. This co-culture model can also be used to study epilepsy, other neurodegenerative diseases and neurodevelopmental disorders as in many cases, strong correlations between brain cancer and other neural issues have been reported.

Data statement

All data presented in this manuscript is available from the corresponding author on request.

Author Contributions

Conceptualization – NT, SM. Data Curation – NT, SM. Formal Analysis – SM, AM. Funding Acquisition – NT. Methodology – SM. Project Administration – SM, NT. Resources – NT. Software – SM, AM. Supervision – NT. Validation – SM. Visualization – SM. Writing/Original Draft Preparation – SM. Writing/Review & Editing – SM, NT.

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Author Declarations

Conflict of interest

The authors have no conflicts to disclose.

Ethics approval

Ethics approval is not required.

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