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Three-Dimensional (3D) in vitro cell culture protocols to enhance glioblastoma research

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LAB PROTOCOL

Three-Dimensional (3D) in vitro cell culture protocols to enhance glioblastoma research

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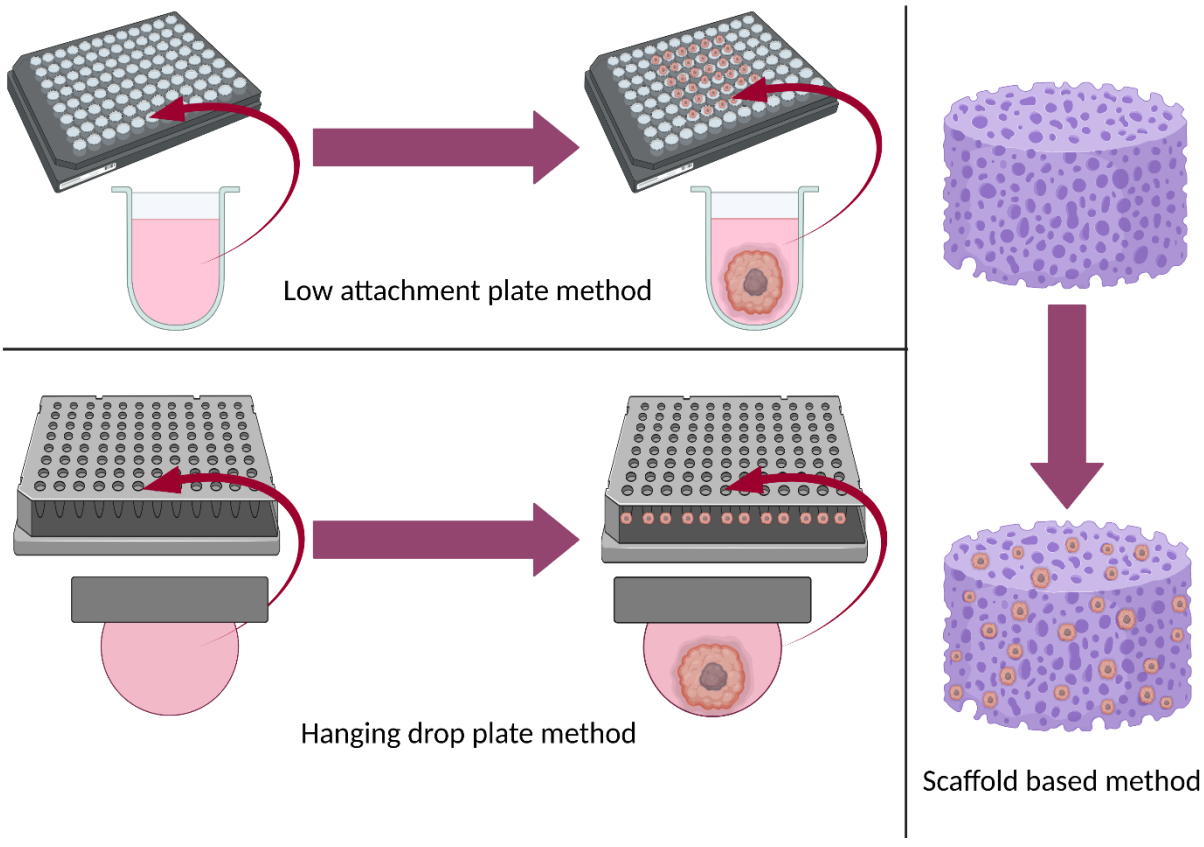
Abstract



3D cell culture models can help bridge the gap between *in vitro* cell cultures and *in vivo* responses by more accurately simulating the natural *in vivo* environment, shape, tissue stiffness, stressors, gradients and cellular response while avoiding the costs and ethical concerns associated with animal models. The inclusion of the third dimension in 3D cell culture influences the spatial organization of cell surface receptors that interact with other cells and imposes physical restrictions on cells in compared to 2D cell cultures. Spheroids' distinctive cyto-architecture mimics *in vivo* cellular structure, gene expression, metabolism, proliferation, oxygenation, nutrition absorption, waste excretion, and drug uptake while preserving cell–ECM connections and communication, hence influencing molecular processes and cellular phenotypes. This protocol describes the *in vitro* generation of tumourspheroids using the low attachment plate, hanging drop plate, and cellusponge natural scaffold based methods. The expected results from these protocols confirmed the ability of all these methods to create uniform tumourspheres.

Introduction

Two-dimensional (2D) cell culture models have become a cornerstone of biological research due to its ease of use, cheap cost, and repeatability, however *in vivo* tissue complexity can only be reached utilizing Three-dimensional (3D) cell culture (1). 3D cell cultures are an improved *in vitro* cell culture technology that uses an artificially produced microenvironment to grow cells in three dimensions. Cells in 3D cell culture contain natural cell-cell interactions as well as cell–extracellular matrix component interactions, allowing them to proliferate *in vitro* in a microenvironment that closely reflects *in vivo* settings (2, 3). 3D cell culture is vital in drug testing, because it is capable of replacing both 2D cell culture and animal trials. The initial step of traditional drug development begins with 2D cell culture, followed by animal studies and clinical trials; around 95% of possible preclinical trials in all therapeutic areas fail to result in effective human treatments. The primary reason for this is that original data from 2D cell culture-based testing was deceptive and mispredicted cellular responses, resulting in enormous loss of time and resources and, eventually, delaying the identification of viable treatments (4, 5). 3D cell cultures are a simplified reductionist concept. When compared to a whole animal, it is very transparent and straightforward to mimic complicated processes like as growth, invasiveness, and toxicity (6). Thus the 3D cell culture technology can be utilize to enhance the quality of laboratory experiments and minimizing overall expenditure. It will also be able to develop cancer models, for preclinical screening and monitoring, as well as novel *in vivo* cancer therapeutic research (5, 7-9).

The main benefits of employing 3D cell culture for drug discovery include greater cell-cell contact, ECM-cell interactions, varied rates of cellular proliferation, oxygen and nutrition availability,

physiological gradients for nutrients, waste, signalling factors and drugs, and the additional influence of stroma, all of which assist in replicating natural tissue distribution (1, 5, 10). It can also simulate drug resistance, cellular microenvironment activity, and the expression of intrinsic and genetic variables (7, 10). The declared goal of the European REACH legislation is "To ensure a high level of protection of human health and the environment from the effects of hazardous chemicals. It strives for a balance: to increase our understanding of the possible hazards of chemicals, while at the same time avoiding unnecessary testing on animals" (European Chemicals Agency, 2020). 3D cell cultures complement the 3Rs principles of animal research (Replacement, Reduction, and Refinement) and REACH regulations by reducing the number of animals used in testing, as well as saving time, money, and ethical issues (1, 10). Animal testing is both costly and time consuming. Furthermore, if the animal is in pain or under stress throughout the experiment, it may alter the biochemical, physiological, and metabolic processes, which might misrepresent the efficacy and adverse effects of drugs (6, 9, 10). 3D spheroid cultures can be readily established in many cell culture facilities using anchorage-independent or anchorage-dependent methods. Three methods that can be established in most cell culture facilities are outlined in detail here, low attachment plate method, hanging drop method and scaffold based method.

To produce spheroids, anchorage independent/scaffold-free approaches rely on non-adherent cell-to-cell aggregation. This anchorage independent category includes low attachment plate and hanging drop plate technologies (11). Low attachment plates are culture plates with an ultra-low attachment hydrophilic polymer covering that promotes cell aggregation to create spheroids (2, 12). Cell adhesion to the culture surface is often mediated by ECM proteins such as collagen-I and fibronectin. The hydrophilic polymer covering prevents protein adsorption to the culture vessel surface, reducing monolayer cell adherence. Finally, low attachment plates stimulate cell aggregation via cell-cell and cell-ECM interactions, while limiting ECM interactions with the plastic surface (5). The benefits of employing low adhesion plates are straightforward, simple, efficient spheroid formation improved repeatability, reproducibility and handling, suitable for multicellular spheroids / co-culture and the ability to grow a wide range of tumour cell types (3, 13, 14). The disadvantage is that it is time consuming, lack of uniformity between spheroids, coated plates are expensive, continuous passage culture and toxicity analysis is difficult, that the success rate in long term passage is low, and not suitable for migration/invasion assays (4, 9, 15-17). When considering hanging drop plates are open bottomless wells that encourage the development of droplets of media that offer room for the creation of spheroids by self-aggregation via gravity and surface tension (5, 18). Because there is no surface to connect to, cells develop inside a bubble of growth medium, and spheroids dangle in open bottomless wells, which are frequently contained at the bottom of the plate to adjust the cells'

ambient humidity (12). The well can typically accommodate up to 50µl of media while recommended drop volume is 10-20 µl (15) and the spheroid size is determined by the cell density (2). This cell suspension droplet can be held in place by surface tension, and following 3D spheroid creation, it may be dispensed by adding an extra drop of media to the well and the spheroid loaded to ~~an~~ another normal well plate (9). The hanging drop plate method produce smaller size spheroids compared to low attachment plate method. The advantages of adopting the hanging drop plate method are the ability to create uniform size spheroids, the low cost, the ease of handling, the suitability for co-culturing, short term culture, and high throughput testing (3). The fundamental disadvantage of this approach is that medium changes are difficult, different drug treatments at different time periods are impossible, it is not ideal for long-term culture, and it has a small culture volume (9, 15).

Anchorage-dependent techniques employ pre-designed porous membranes and polymeric fabric meshes known as "scaffolds," which can be made of natural or synthetic materials (16). This physical support can give structures ranging from basic mechanical to extracellular matrix-like structures (1). 3D spheroids can be created by seeding cells into the 3D scaffold matrices or by distributing cells in a liquid matrix followed by solidification and polymerization. Cells are immersed in extracellular components and can begin cell-cell and cell-matrix interactions, as well as provide physical support for cell growth, adhesion, and proliferation (1). Fibronectin, collagen, laminin, gelatin, cellulose, chitosan, glycosaminoglycans, fibroin, agarose, alginate, starch, and human decellularized ECM are some of the natural scaffolds (14, 15, 19, 20). The benefits of employing biological scaffolds are that they are extremely comparable to *in vivo* settings, that they can manage similar composition/elasticity/porosity to achieve better ECM presentation, and that they can be combined with appropriate growth factors. It can also increase biocompatibility and reduce toxicity. Disadvantages include the fact that it is an expensive, time-consuming, complicated procedure that is not ideal for large-scale manufacturing, that it is difficult to separate cells from scaffold for further investigations such as flow cytometry and confocal imaging (15). Polymers such as polyglycolic acid, polylactic acid, polyorthoesters and aliphatic polyesters such as polycaprolactone (PCL), polystyrene (PS), polycaprolactone (PCL), polyethylene oxide (PEO), and polyethylene glycol (PEG) can be used to make synthetic scaffolds (1, 14, 15). The advantages of using synthetic scaffold include the ability to control porosity, stiffness, elasticity, and permeability, higher versatility, reproducibility, enhanced workability, ease of use, and mechanical qualities of synthetic materials that can be adjusted according to the cell culture required, and also their chemical composition is well characterized (11, 15). The disadvantages include a lack of biodegradation, which may impair cellular function, inability to remove cells and perform cytotoxicity tests.

Ultimately, 3D models must have high-throughput application, easy and standardized culture protocols and analytic methodologies to get proper outcomes (1). In the present study, we used three different methods to construct ~~an~~ *in vitro* 3D glioblastoma and epidermoid tumourspheroid models to closely mimic the natural *in vivo* environment, shape, and cellular response. This is the first time that we are reporting all the three different approaches for successful U-251MG human glioblastoma astrocytoma tumoursphere development.

Materials and methods

The part of this protocols described in this peer-reviewed article is published on protocols.io, and is included for printing as S1, S2, S3 Files with this article.

Low attachment plate method - dx.doi.org/10.17504/protocols.io.bszmnf46

Hanging drop plate method - dx.doi.org/10.17504/protocols.io.btstnnen

Scaffold based method - dx.doi.org/10.17504/protocols.io.bszqnf5w

Image J analysis

Tumor spheroid formation was visually confirmed daily using an Optika XDS-2 trinocular inverse microscope equipped with a Camera ISH500, and their mean diameters were analysed using "ImageJ version 1.53.e" software (http://imagej.nih.gov/ij/). ImageJ is a free software that can be used for manually counting the cell numbers and calculating the cellular size (area/ diameter). The ImageJ program was calibrated (set scale) using an image obtained from the same microscope with a known scale before it was used to calculate the cell size (in diameter). Following the calibration, the pictures of the tumorspheres were opened in the program, and a line was drawn across the diameter to measure the tumorsphere's size. The diameters of the spheroids were measured at least three times to obtain the mean and standard deviation.

Growth Analysis at different incubations

U-251 MG tumourspheres growth were analysed during different incubations (ranging from 24 to 168h). Cells (Initial seeding density was 10000 cells/ml) were seeded in the above mentioned Nunclon™Sphera™96-well-low attachment plates. Fresh media were added every third day by replenishing old media in each well without disturbing the tumorspheroids. In Hanging drop plate method, 5000 cells/well were seeded in the HDP1096 Perfecta3D® 96-well Plate. While in scaffold based method, 5000k cells/ml were seeded in the hydroxipropylcellulose scaffold. The spheroid formation and growth were monitored daily by using an inverted phase-contrast microscope, and the sizes of the spheroids were measured as explained above for at least three independent experiments.

Growth analysis at different seeding densities

For growth analysis, varying numbers of U-251 MG cells (ranging from 2000 to 40 000 cells/ml) were seeded in the above mentioned Nunclon™ Sphera™ 96-well-low attachment plates for 96 hours. Fresh media were added every third day by replenishing old media in each well without disturbing the tumorspheroids. In Hanging drop plate method, U-251 MG cells (ranging from 1000 to 10000

cells/well) were seeded in the above mentioned HDP1096 Perfecta3D® 96-well Plate. While in scaffold based method, varying numbers of U-251 MG cells (ranging from $1x10^6$ to $6x10^6$ cells/ml) were seeded in the hydroxipropylcellulose scaffold. The spheroid formation was monitored after 96h by using an inverted phase-contrast microscope, and the sizes of the spheroids were measured as explained above for at least three independent experiments.

Spheroid cells health analysis

Spheroid cell health was analysed using Alamar Blue™ cell viability reagent (Thermo Fisher Scientific). After the post treatment incubation, tumourspheres were washed with sterile phosphate-buffered saline (PBS), and incubated for 3 h at 37°C with a 10% Alamar Blue™ solution (10). Fluorescence was measured using an excitation wavelength of 530 nm and an emission wavelength of 590 nm with a Varioskan Lux multi-plate reader (Thermo Scientific). The fluorescence signals were normalized by spheroid size (in diameter); a higher ratio indicates healthier spheroids. All experiments consisted of at least three independent tests.

Expected results

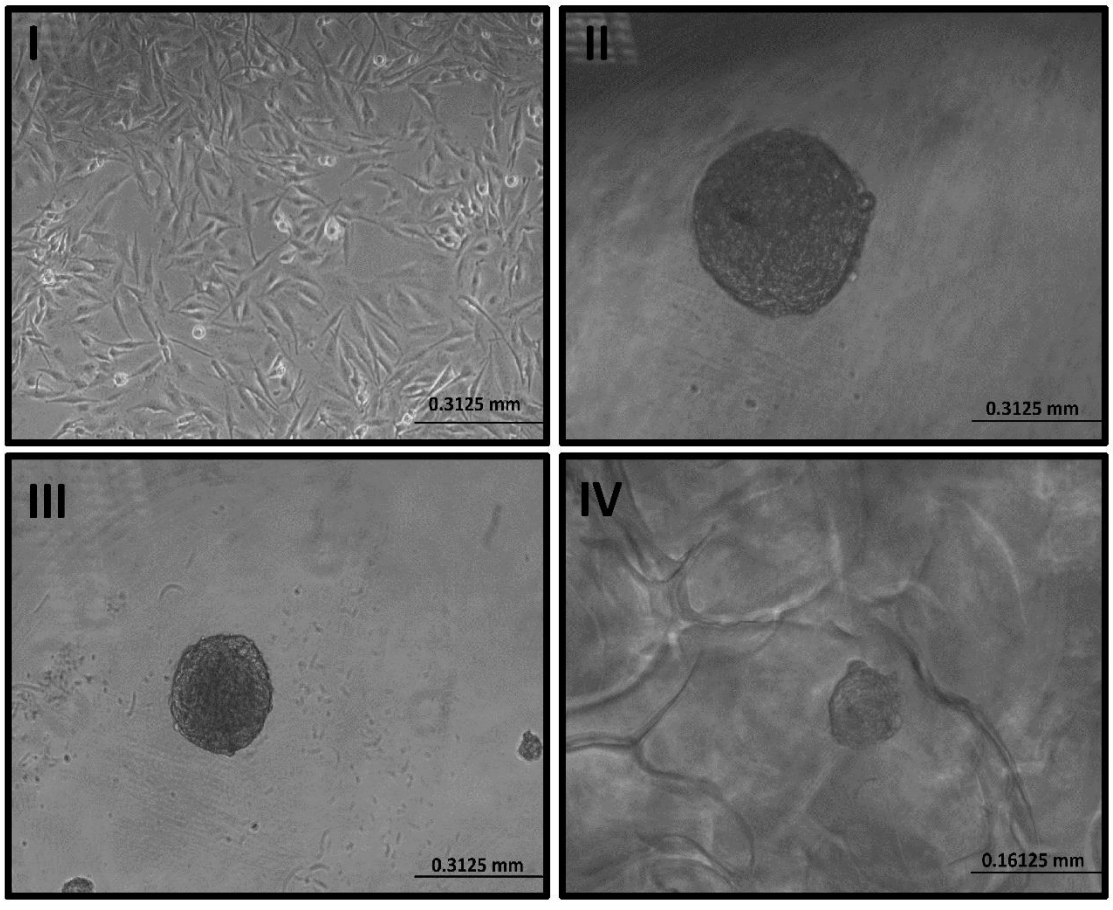


Figure 1 - Development of U-251MG human glioblastoma astrocytoma 3D *in vitro* cell culture model. I). Image of U-251MG 2D cells in T75 flask II) 3D tumoursphere constructed in low adhesion plate. III). 3D tumoursphere constructed in hanging drop plate. IV) 3D tumoursphere constructed in hydroxipropylcellulose scaffold. Tumour spheroid formation was visually confirmed using an Optika XDS-2 trinocular inverse microscope equipped with a Camera ISH500.

Three distinct approaches such as Low attachment plate (Figure 1-II), (S1), Hanging drop plate (Figure 1-III), (S2), and scaffold based methods (Figure 1-IV), (S3) were used to create 3D spheroids of U-251MG human glioblastoma astrocytoma. This facilitated 3D cell–cell and cell–ECM interactions and mirrored the diffusion-limited distribution of oxygen, nutrients, metabolites, and signaling molecules seen in the microenvironment of *in vivo* tumours. Most research to date has used 2D cell culture (Figure 1-I), which has limitations as experimental models to predict biological responses, as explained previously.

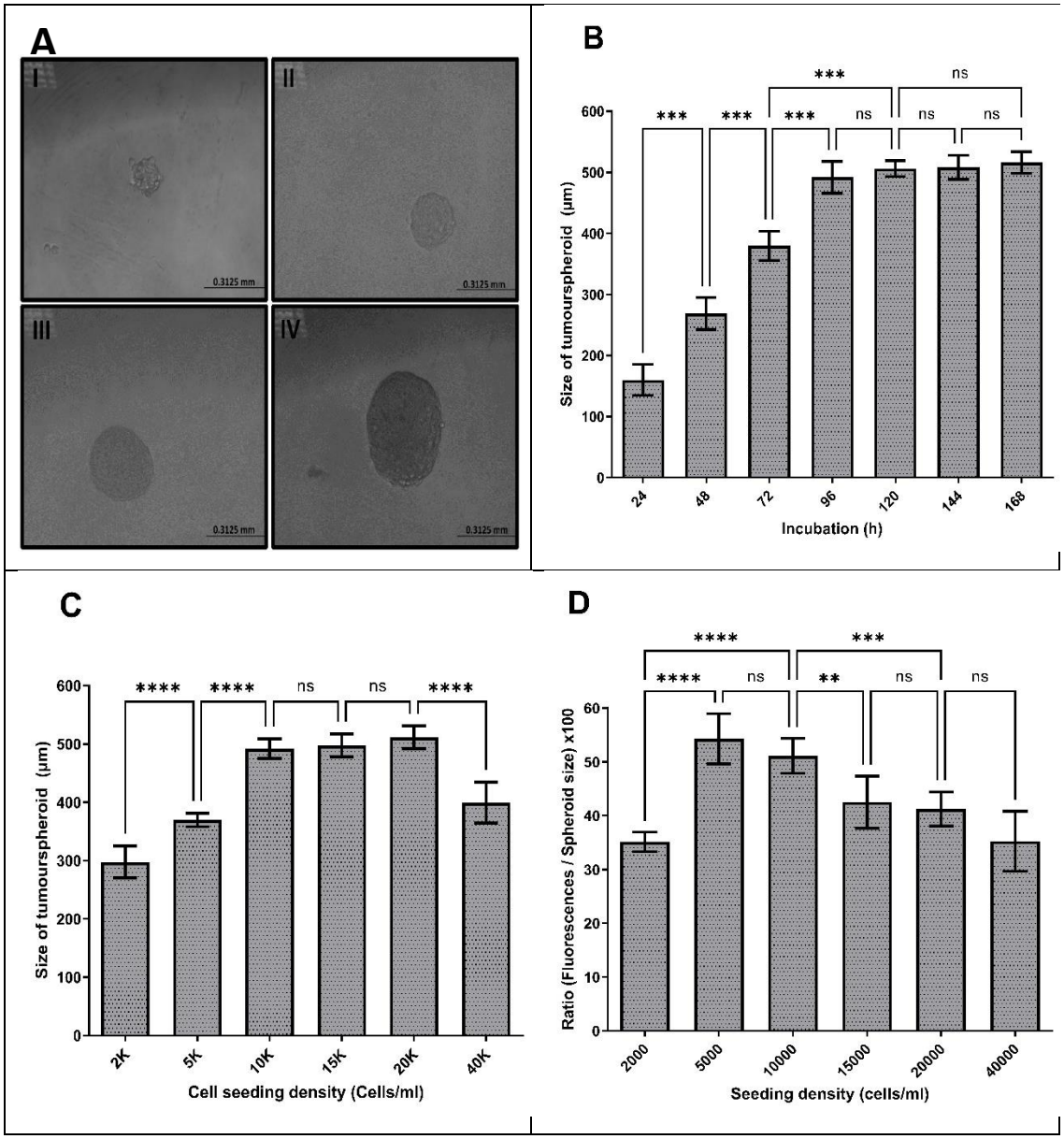


Figure 2: Development of U-251 MG human glioblastoma astrocytoma 3D *in vitro* cell culture models using low attachment plate method. A) U-251MG tumourspheroids formation, I) Tumourspheres formation after 24 h incubation, II) after 48 h incubation, III) after 72 h of incubation, IV) after 96h of incubation. B) U-251 MG tumoursphere growth (diameter in µm) analysis during different incubations. C) U-251 MG Growth analysis after 96h incubation (diameter in µm) at increasing seeding density. The mean of the diameter was used to plot the values on columns and analysed using one-way ANOVA with Tukey's post-test (ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$). D) U-251 MG Spheroid cell health analysed and a higher ratio indicates healthier spheroids. The mean of the [(fluorescence / spheroid size) x 100] was used to plot the values on columns and analysed using one-way ANOVA with Tukey's post-test (ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$).

The U-251MG human glioblastoma astrocytoma spheroid formation and growth were monitored daily by using an inverted phase-contrast microscope, and their mean diameters were analysed using "ImageJ version1.53.e" software for at least three independent experiments. U-251MG tumoursphere growth during low attachment plate method was found to be significantly increased with the incubation time, the size ranging from 135µm, 229 µm, 323 µm and 461 µm for 24 to 96 h incubation respectively (Figure 2A). The optimum U251 MG tumourspheroids formation was observed within 96h of incubation for the 10000 cells/ml initial seeding density. One-way analysis of variance (ANOVA) demonstrated that there is a significant difference in tumoursphere diameter during 24 -96h incubation, while, there was no significant difference during 96h to 168h incubation (Figure 2 B). It was also observed that exponential growth (Log) was achieved within the initial 4 days of growth, after which the growth curve became stationary.

For growth analysis, varying numbers of U-251 MG cells (ranging from 2000 to 40 000 cells/ml) were seeded in the Nunclon™ Sphera™ 96-well-low attachment plates as explained above. The largest U-251 MG tumourspheres were observed with 10 000, 15 000, and 20 000 cells/ml initial seeding densities after 96h incubation. One-way ANOVA demonstrated that there is a significant difference in tumoursphere diameter between each initial seeding densities as shown in Figure 1C. However, there was no significant difference between diameters in 10 000, 15 000, and 20 000 cells/ml seeding densities.

U251-MG cell health analysed after 96h incubation using Alamar Blue™ cell viability reagent as explained above and the fluorescence signals were normalized by spheroid size (diameter in µm). A higher ratio suggests that the spheroids are healthier. During U251-MG growth confirmed that 5000 and 10 000 cells/ml initial seeding densities were having highest spheroids cell health. One-way

ANOVA confirmed that there was no significant difference in tumoursphere health during 5000 and 10 000 cells/ml.

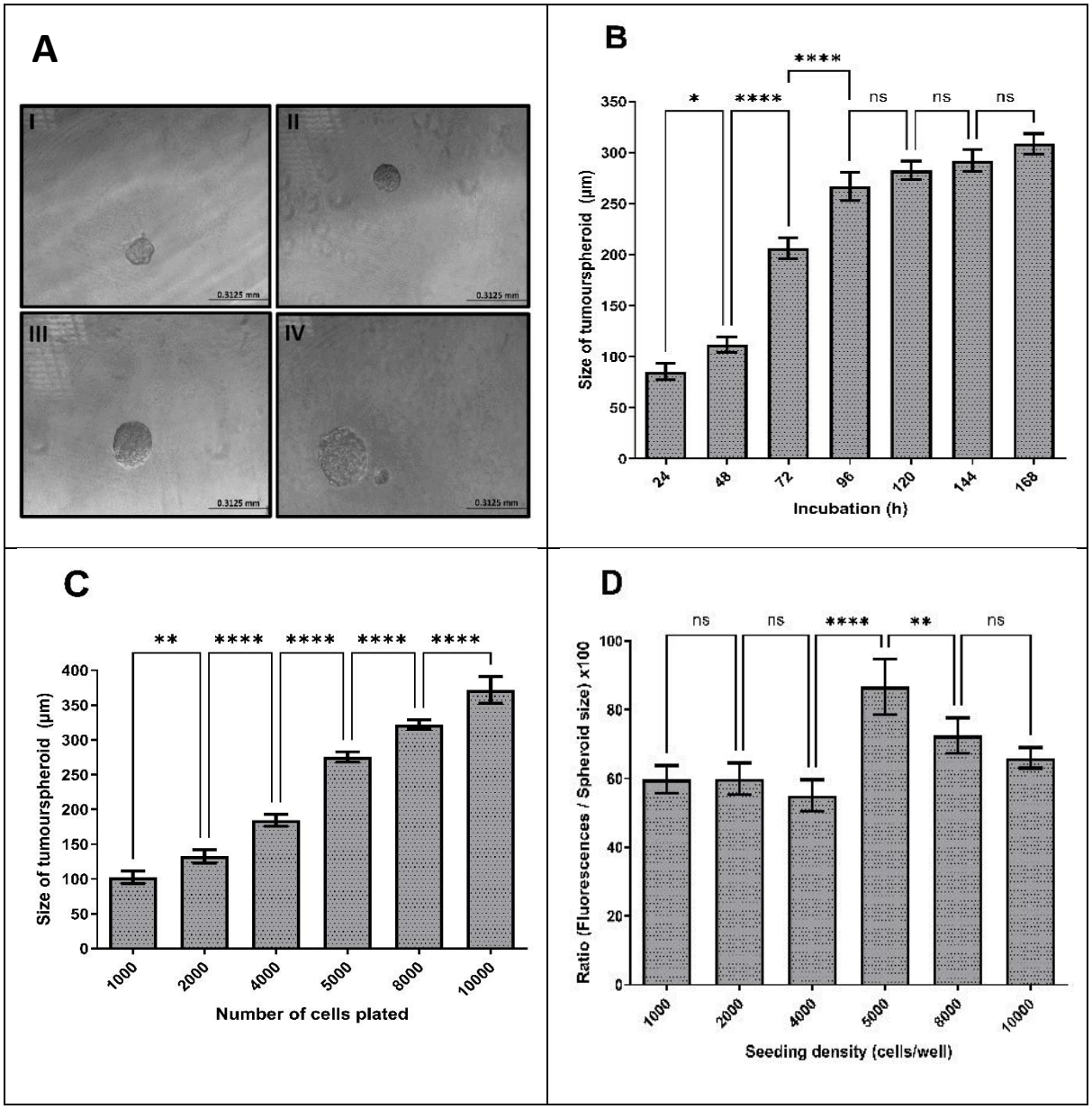


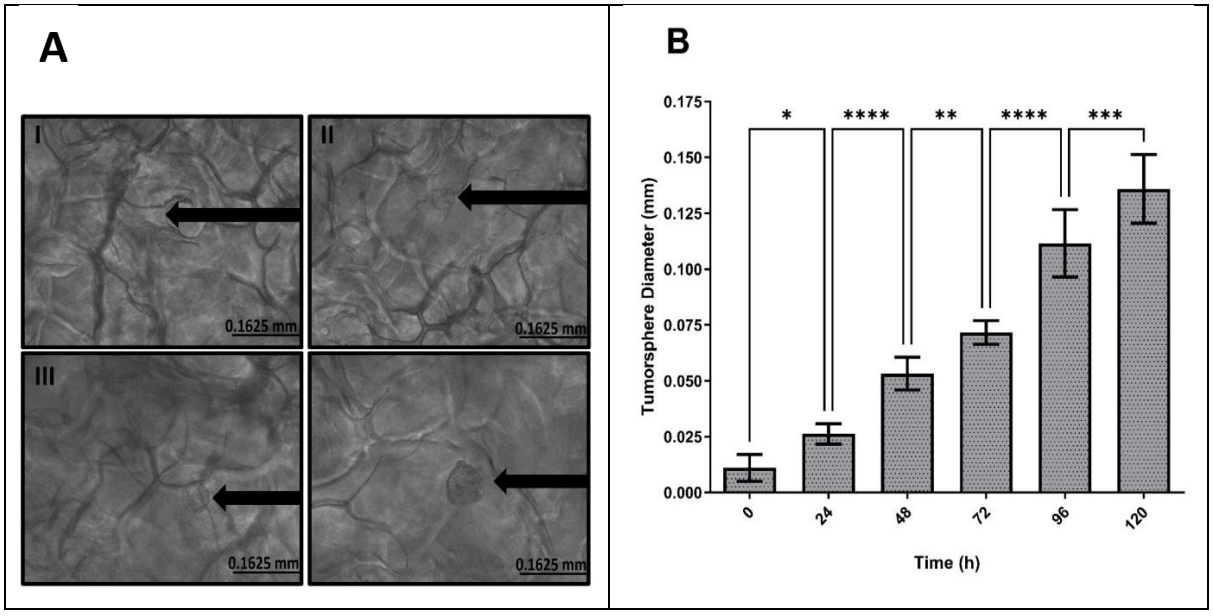
Figure 3: Development of U-251 MG human glioblastoma astrocytoma 3D *in vitro* cell culture model using hanging drop plate method. A) U-251MG tumourspheroids formation, I) Tumourspheres formation after 24 h incubation, II) after 48 h incubation, III) after 72 h of incubation, IV) after 96h of incubation. B) Tumoursphere growth (diameter in µm) analysis during different incubations. C) Growth analysis after 96h incubation (diameter in µm) at increasing seeding density. The mean of the diameter was used to plot the values on columns and analysed using one-way ANOVA with Tukey's post-test (ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$). D) Spheroid cell health analysed and a higher ratio indicates healthier spheroids. The mean of the [(fluorescence / spheroid size) x 100] was used to plot the values on columns and analysed using

one-way ANOVA with Tukey's post-test (ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$).

U-251MG tumoursphere growth during hanging drop plate method was shown to be considerably enhanced with the incubation time, with size ranging from 105µm, 139 µm, 208 µm and 269 µm for 24 to 96 h incubation respectively (Figure 3A). The optimum tumourspheroids formation attained after 96h incubation by achieving a size range 251-285 µm for the 5000 cells/well initial seeding density. One-way ANOVA indicated that there is a significant difference in tumoursphere diameter during 48 - 96h incubation, while, there was no significant difference during 96h to 168h incubation (Figure 3B).

For growth analysis, varying numbers of U-251 MG cells (ranging from 1000 to 10 000 cells/well) were seeded in the HDP1096 Perfecta3D® 96-Well Hanging Drop Plates and the mean sizes were computed after 96h of incubation. The largest U-251 MG tumourspheres were observed with 10 000 cells/well initial seeding densities after 96h incubation. As illustrated in Figure 3C, one-way ANOVA revealed a significant difference in tumoursphere diameter between each initial seeding density.

During U251-MG spheroids cell health investigation, it was established that the initial seeding density of 5000 cells/well had the best spheroids cell health. The substantial difference in 4000 to 5000 cells/well and 5000 to 8000 cells/well was verified by one-way ANOVA, however there was no significant difference in tumoursphere health at the other seeding densities (Figure 3D).



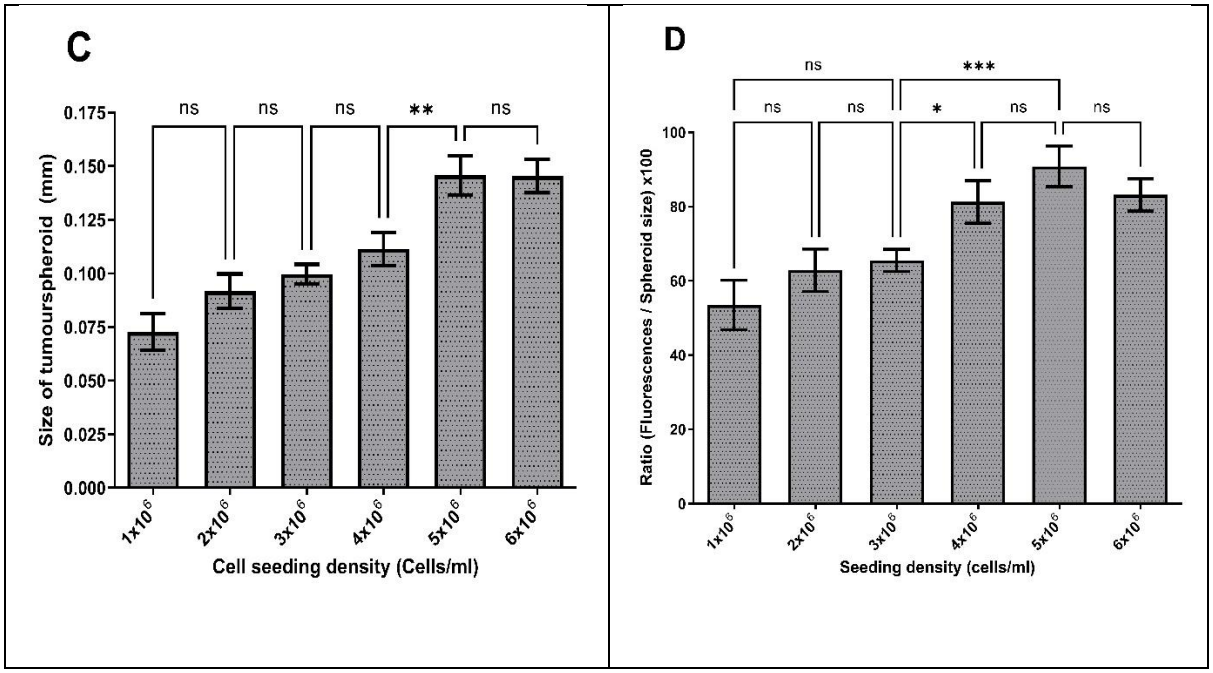


Figure 4: Development of U-251 MG human glioblastoma astrocytoma 3D *in vitro* cell culture model using Cellusponge 3D scaffolds. A) U-251MG tumourspheroids formation, I) Tumourspheres formation after 24 h incubation, II) after 48 h incubation, III) after 72 h of incubation, IV) after 96h of incubation. B) Tumoursphere growth (diameter in mm) analysis during different incubations and all the data points were statistically significant. C) U-251 MG Growth analysis after 120h incubation (diameter in mm) at increasing seeding density. The mean of the diameter was used to plot the values on columns and analysed using one-way ANOVA with Tukey's post-test. (ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$). D) U-251 MG Spheroid cell health analysed and a higher ratio indicates healthier spheroids. The mean of the [(fluorescence / spheroid size) x 100] was used to plot the values on columns and analysed using one-way ANOVA with Tukey's post-test (ns, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$).

U-251MG tumoursphere growth in hydroxipropylcellulose 3D scaffold was shown to be considerably enhanced with incubation time, with sizes ranging from 22µm, 49µm, 70µm, and 110 µm for 24 to 96 h incubation, respectively (Figure 4A). The largest tumourspheroids formation attained after 120h incubation by achieving a size range 0.110-0.156 mm for the 5000k cells/ml initial seeding density. One-way ANOVA indicated that there is a significant difference in tumoursphere diameter during throughout the incubation (Figure 4B).

For growth analysis, varying numbers of U-251 MG (ranging from $1x10^6$ to $6x10^6$ cells/ml) were seeded in the hydroxipropylcellulose 3D scaffolds. Fresh media were added every third day by replenishing old media in each well without disturbing the scaffolds and the mean sizes were calculated after 120h of incubation. The largest tumourspheres were detected with $5x10^6$ and $6x10^6$ cells/ml initial seeding

densities after 120h incubation. One-way ANOVA verified that there is a significant difference in tumoursphere diameter between $4x10^6$ and $5x10^6$ seeding densities as shown in Figure 4C.

U251-MG spheroids cell health analysed after 120h incubation as explained above, confirmed that $5x10^6$ cells/ml initial seeding density was having highest spheroids cell health. One-way ANOVA confirmed that there was no significant difference in tumoursphere health during $4x10^6$, $5x10^6$ and $6x10^6$ cells/ml. While there was a significant difference between $3x10^6$ and $4x10^6$ densities as shown in Figure 4D.

Supporting information

S1 File. U-251MG Spheroid generation using low attachment plate method protocol
Also available on protocols.io.
https://www.protocols.io/view/u-251mg-spheroid-generation-using-low-attachment-p-bszmnf46.pdf

S2 File. U-251MG Spheroid Generation Using Hanging Drop Method Protocol
Also available on protocols.io.
https://www.protocols.io/view/u-251mg-spheroid-generation-using-hanging-drop-met-btstnnen.pdf

S3 File. U-251MG Spheroid generation using a scaffold based method protocol
Also available on protocols.io.
https://www.protocols.io/view/u-251mg-spheroid-generation-using-a-scaffold-based-bszqnf5w.pdf

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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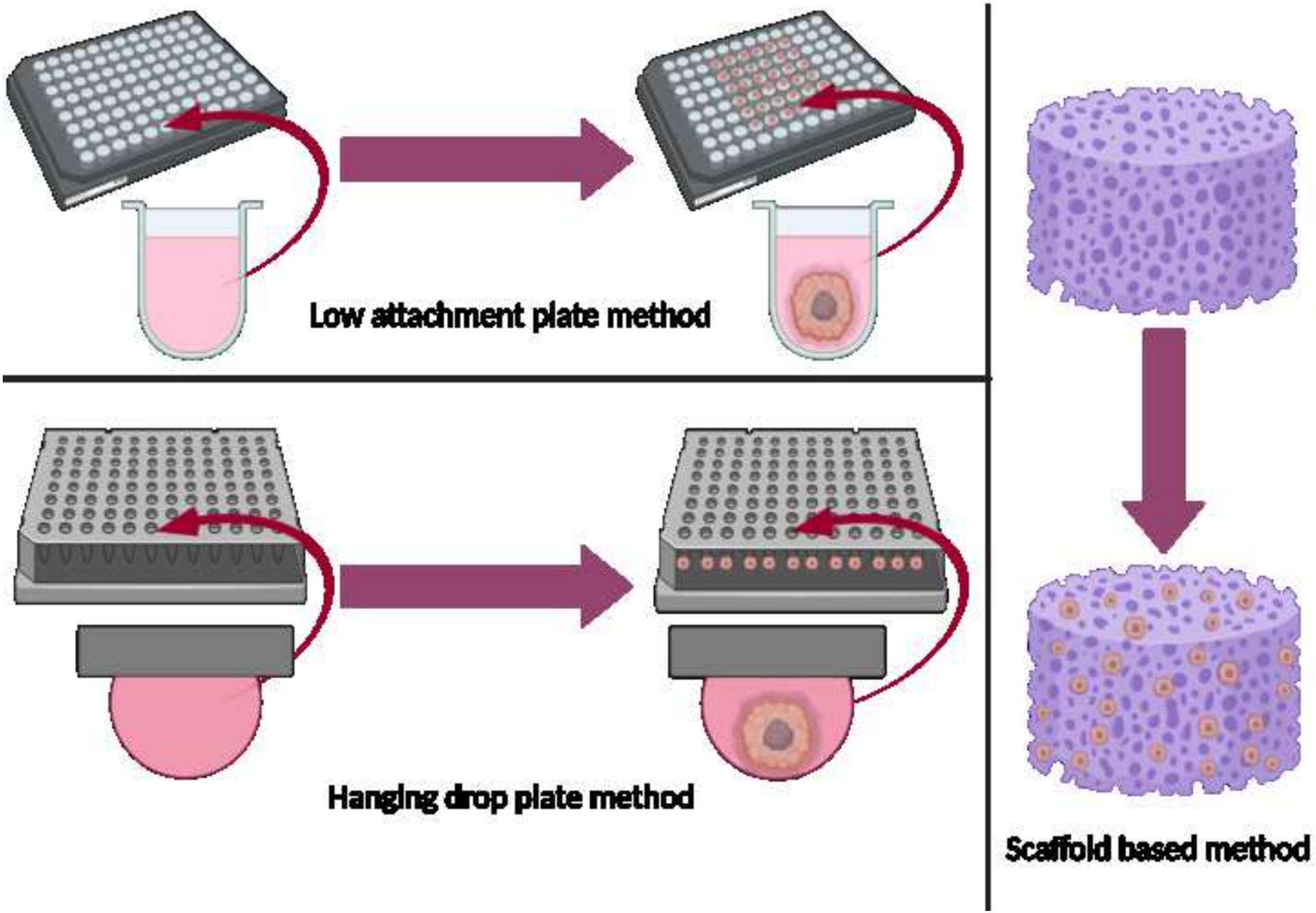
Graphical Abstract



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