Journal of Visualized Experiments

A 3D Spheroid Model for Glioblastoma

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60998R1
Full Title:	A 3D Spheroid Model for Glioblastoma
Section/Category:	JoVE Cancer Research
Keywords:	Glioblastoma tumor invasion metabolism acidosis 3D stem-like cells
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Additional Information:	
Question	**Response**
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TITLE:

A 3D Spheroid Model for Glioblastoma

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KEYWORDS:

glioblastoma, patient-derived cell, spheroid, invasion, migration, proliferation, in vitro models

SUMMARY:

Here, we describe an easy-to-use invasion assay for glioblastoma. This assay is suitable for glioblastoma stem-like cells. A Fiji macro for easy quantification of invasion, migration, and proliferation is also described.

ABSTRACT:

Two-dimensional (2D) cell cultures do not mimic in vivo tumor growth satisfactorily. Therefore, three-dimensional (3D) culture spheroid models were developed. These models may be particularly important in the field of neuro-oncology. Indeed, brain tumors have the tendency to invade the healthy brain environment. We describe herein an ideal 3D glioblastoma spheroid-based assay that we developed to study tumor invasion. We provide all technical details and analytical tools to successfully perform this assay.

INTRODUCTION:

In most studies using primary or commercially available cell lines, assays are performed on cells grown on plastic surfaces as monolayer cultures. Managing cell culture in 2D represents

disadvantages, as it does not mimic an in vivo 3D cell environment. In 2D cultures, the entire cell surface is directly in contact with the medium, altering cell growth and modifying drug availability. Furthermore, the nonphysiological plastic surface triggers cell differentiation[1]. Three-dimensional culture models have been developed to overcome these difficulties. They have the advantage of mimicking the multicellular architecture and heterogeneity of tumors[2], and thus could be considered to be a more relevant model for solid tumors[3]. The complex morphology of spheroids contributes to better evaluate drug penetrance and resistance[4]. The tumor heterogeneity in the spheroid impacts the diffusion of oxygen and nutrients, and the response to pharmacological agents (**Figure 1A**). Diffusion of oxygen is altered when the spheroid size reaches 300 µm, inducing a hypoxic environment in the center of the spheroid (**Figure 1A,C**). Metabolites are also less penetrating through the cell layers and compensating metabolic reactions take place[5]. When the diameter of the spheroid increases, necrotic cores can be observed, further mimicking characteristics found in many solid cancers, including the aggressive brain cancer glioblastoma (GBM)[6].

Several 2D or 3D invasion assays for glioblastoma have been reported in the literature[7,8]. Two-dimensional assays are mainly for studying invasion in a horizontal plane on a thin matrix layer or in a Boyden chamber assay[9]. Three-dimensional assays have been described with 3D spheroid cultures using classical glioblastoma cell lines[10]. More complex variants are represented by invasion of brain organoids by tumor spheroids in confrontation cultures[11]. However, it is still important to develop an easy-to-use and reproducible assay available to any laboratory. We have developed a protocol to generate glioblastoma stem-like cells from patient samples. The quantification of these assays is easily manageable and only requires open-access online software. Briefly, tumor pieces are cut into small pieces and enzymatically digested. Single cells derived from the digestion are cultivated in neurobasal medium. After 4–7 days, spheroid structures form spontaneously. Upon intracranial implantation in mice models, they form tumors exhibiting a necrotic core surrounded by pseudo-palisading cells[12]. This closely resembles the characteristics found in GBM patients.

In this article, we describe our protocol to produce spheroids from a determined number of cells to ensure reproducibility. Two complementary matrices can be used for this purpose: Matrigel and collagen type I. Matrigel is enriched in growth factors and mimics the mammalian basal membrane required for cell attachment and migration. On the other hand, collagen type I, a structural element of stroma, is the most common fibrillary extracellular matrix and is used in cell invasion assays. Herein, we illustrate our GBM spheroid model by performing migration and proliferation assays. Analysis was done not only at fixed time points but also by monitoring spheroid expansion and cell movement by live imaging. Furthermore, electron microscopy was done to visualize morphological details.

PROTOCOL:

Informed written consent was obtained from all patients from the Haukeland Hospital, Bergen, Norway, according to local ethics committee regulations. Our protocol follows the guidelines of our institution's human research ethics committee.

1. Generation of uniform size tumor spheroids

NOTE: Stem-like cells are cultured in neurobasal medium complemented with B27 supplement, heparin, FGF-2, penicillin, and streptomycin, as described in previous articles[12]. These cells spontaneously form spheroids in culture.

1.1. Wash the tumor cells with 5 mL phosphate-buffered saline (PBS) and incubate the cells with 0.5–1 mL dissociation enzyme (see **Table of Materials**) for 5 minutes at 37 °C.

1.2. Wash with 4–4.5 mL PBS and add 10 mL of the complete growth medium (complete neurobasal medium, cNBM).

1.3. Count the cells using an automatic counting technique with trypan blue and a cell counting chamber slide.

1.4. To generate 100 spheroids with 10^4 cells per spheroid (according to preferred size), mix 10^6 cells in 8 mL of NBM with 2 mL of 2% methylcellulose.

1.5. Transfer the suspension to a sterile system container and dispense 100 µL/well with a multichannel pipette into a 96 well round bottom plate.

1.6. Incubate the plate at 37 °C, 5% CO_2 and 95% humidity. Equal sized spheroids will form and can be used after 3–4 days.

2. Three-dimensional experiments

2.1. Proliferation

2.1.1. Preparation

2.1.1.1. Suspend inhibitors (e.g., rotenone as in **Figure 4A**) and chemicals in 100 µL of medium and add to the 100 µL of medium in each well (i.e., one spheroid per well).

2.1.1.2. Incubate the plate at 37 °C, 5% CO_2, and 95% humidity.

2.1.2. Image acquisition and analysis

2.1.2.1. Take pictures with a video microscope in brightfield to create a series of conditions at T_0 and the following times expected.

2.1.2.2. Use Fiji to analyze pictures either manually or in a semiautomated manner. To do so manually, draw a circle around the spheroid core with the freehand selection tool and measure

the area of each spheroid. To analyze the images in a semiautomated manner, use the macro shown in **Suppl. Document 1** with //Core Area only.

2.2. Invasion

2.2.1. Preparation

2.2.1.1. Prepare the collagen matrix in a tube on ice with type I collagen at 1 mg/mL final concentration, 1x PBS, $0.023 \times V_{collagen}$, 1 M sodium hydroxide, and sterile H_2O. Incubate the solution on ice for 30 min.

2.2.1.2. Collect spheroids from the round bottom well plate in 500 µL tubes and wash 2x with 200 µL 1x PBS.

2.2.1.3. Pipette the spheroids carefully into 100 µL of the collagen matrix and insert in the center of a well in normal 96 well plates.

2.2.1.4. Incubate the collagen gel for 30 min at 37 °C and then add cNBM on top of the gel. Inhibitors or activators (e.g., hydrogen chloride as shown in **Figure 4B**, **4C**) can be added to the medium at this step.

2.2.2. Image acquisition and analysis

2.2.2.1. Take pictures sequentially with a video microscope in brightfield mode 24 h after collagen inclusion.

2.2.2.2. Use Fiji to analyze pictures either manually or in a semiautomated manner. To do so manually, draw around the core and the total area of the spheroid with the freehand selection tool and measure the invasive area of each spheroid by subtract total area with core area. To analyze the images in a semiautomated manner, use the macro as indicated in **Suppl. Document 1** to determine the invasive area.

2.3. Migration

2.3.1. Preparation

2.3.1.1. Coat a 6 well plate with Matrigel (0.2 mg/mL) in NBM for 30 min at 37 °C, then remove the Matrigel and add 2 mL of cNBM.

2.3.1.2. Transfer spheroids into 50 µL of cNBM from the round bottom well plate to the 6 well plate.

2.3.1.3. Incubate the plate at 37 °C and wait 30 min for the spheroids to adhere.

2.3.1.4. After 24 h of incubation, stain with 10 ng/mL of Hoechst and incubate 30 min at 37 °C.

2.3.2. Image acquisition and analysis

2.3.2.1. Obtain images using a video microscope in brightfield. A 405 nm laser is used for visualization of Hoechst staining.

2.3.2.2. Use Fiji software to analyze pictures and run the macro as indicated in **Suppl. Document 1**.

NOTE: Touching the bottom of the well or completely removing the supernatant damages the spheroids. For collagen type I gel handling, keep the gel on ice to avoid collagen polymerization, do not add an acidic component because the change in pH will affect the compactness of the gel, and pipette cells rapidly into the collagen to prevent cell death and the degradation of the gel.

3. Fiji Macro

NOTE: Fiji is an image analysis program developed in the public domain that allows the development of macros to speed up image analysis. Manual analysis is also possible, but this is a slow process and may introduce biases. Images can be imported by drag-and-drop in the software and quantified with the ROI Manager Tools plugin. The procedure used in this study is described below:

3.1. Open the macro window: **Plugins** | **Macros** | **Interactive Interpreter**.

3.2. Copy and paste the following adapted purple loop. Keep the purple sentences and add the green sentences of interest (**Supplementary Document 1**).

3.3. To analyze the entire series, adjust the parameters in red for a specific quantification and run the macro using **Macros** | **Run Macro** or pressing **Ctrl+R**.

3.4. Check and, if necessary, manually adapt the region of interest (ROI).

4. Electron microscopy of spheroids

NOTE: Most of the following steps must be done in a chemical hood.

4.1. Fixation step

4.1.1. Collect the spheroid with a cut pipette tip, put it in a 1.5 mL tube, and wash 1x with 0.1 M phosphate buffer (PB).

4.1.2. Fix the spheroid overnight at 4 °C in 2% glutaraldehyde/2% paraformaldehyde (PFA) in 0.1 M PB.

4.1.3. Replace the fixation solution with a solution of 1% PFA in 0.1 M PB followed by sample preparation.

4.2. Sample preparation

4.2.1. Transfer the spheroids into a strainer and put them in a glass beaker in order to avoid spheroid damage.

4.2.2. Carefully wash 3x with 0.1 M PB.

4.2.3. Incubate with osmium for 2 h in the dark. Dilute osmium to 4% in 1% 0.1 M PB buffer.

4.2.4. Carefully wash 3x with 0.1 M PB.

4.2.4.1. Dehydrate as follows: soak in 50% ethanol for 10 min, 70% ethanol for 10 min, 2x 90% ethanol for 15 min, 2x 100% ethanol for 20 min, and 2x acetone for 30 min.

4.2.5. Incubate the samples in a 50/50 mixture of acetone/resin for 2 h. During this step, prepare the EPON resin (Embed-812: 11.25 g; DDSA: 9 g; NMA: 4.5 g).

4.2.6. Discard the acetone/resin mixture, replace with freshly prepared resin and incubate overnight.

4.2.7. Replace the resin by a new one and incubate between 2–6 h.

4.2.8. Add the spheroids in resin into a mold at 60 °C for 48–72 h.

REPRESENTATIVE RESULTS:

Spheroids were prepared as described in the protocols section and observations were made regarding migration, invasion, proliferation, and microscopy. To measure hypoxia in distinct areas of the spherical structure, carboxic anhydrase IX staining was used for determining hypoxic activity (**Figure 1A–C**). More CAIX-positive cells were observed in the spheroid center (**Figure 1A–C**). Hypoxic cells located in the spheroid core tend to be more glycolytic than the surrounding ones. Mitochondria can be imaged for further analyses as shown by electron microscopy (**Figure 1Ba–1Bb**). Spheroids composed of 2.5 x 10^3, 5 x 10^3, 10^4, or 2 x 10^4 cells exhibit a spheroid diameter of about 350, 400, 500, or 650 µm respectively (Error! Reference source not found.**A**). Spheroids may be used within 4 days after starting the experiment (**Figure 2B**). The quantification of each assay (i.e., proliferation, invasion, migration) is shown in **Error! Reference source not found.**. Fiji macros were developed to quantify proliferation, invasion, or migration (**Figure 3** and **Suppl. Document 1**).

The increase of spheroid core reflected the stimulation of cell proliferation (**Figure 4A**). Upon inhibition by rotenone, an established inhibitor of complex I of the mitochondrial respiratory

chain, the vast majority of ATP production in the mitochondria was compromised. As a consequence, proliferation was reduced by 20% after 72 h (**Figure 4A**). Invasion of collagen type I was calculated by the subtraction of the total area from the core area. Acidic treatment enhanced invasion over a period of 24 h (**Error! Reference source not found.B**). Furthermore, we found that hydrogen chloride treatment reduced the migratory area of the spheroids by 1.5 fold compared to control (**Error! Reference source not found.C**). Spheroid dynamics was studied by live imaging. Spheroids had high internal dynamics and moved quickly (**Movie 1** and tracking analysis in **Figure 4D**).

FIGURE LEGENDS:

Figure 1: The spheroid is a relevant model to mimic solid tumors. (**A**) Spheroid as a round 3D structure with different areas. (**a**) A brightfield picture of a P3 spheroid shows a round appearance with a dense central area. Scale = 100 µm. (**b**) Schematic representation adapted from Hirschhaeuser et al.[6] shows the O_2, CO_2, metabolite, and catabolite gradients in the spheroid. (**c**) Left panel: confocal picture of a spheroid stained with DAPI (blue) and with antibodies against carboxic anhydrase IX (green). Right panel: quantification of the fluorescence from the dashed area. Scale = 100 µm. (**B**) Electron microscopy images with delineated mitochondria (dashed lines). Large mitochondria are seen in the quiescentarea while they are smaller in the proliferation area. Scale = 250 nm.

Figure 2: Overall spheroid preparation steps. (**A**) Generation of human P3 glioblastoma spheroids. Representative images are on the left panel and corresponding proliferation analysis on the right panel. At 24 h, the P3 cells formed dense spheroids. The initial number of cells determined the size of the spheroids. Scale bar = 250 µm. (**B**) Schematic illustration of the easy protocols for studying proliferation, invasion, or migration. Spheroids under various conditions: (**a**) In serum-free medium for tumor growth, (**b**) In collagen matrix for facilitating single cell invasion, and (**c**) On Matrigel coating for cell migration.

Figure 3: Quantification of in vitro assays with Fiji software. Representation of the regions of interest (ROI) obtained using Fiji. The core area is represented in red and the total area, which contains the core area, in yellow. The invasive area corresponds to the subtraction of the total area from the core area. (**A**) Proliferation assay. (**B**) Invasion assay in collagen gel in brightfield acquisitions. (**C**) Migration assay on Matrigel coating by fluorescence acquisition. Nuclei were stained with DAPI, in blue.

Figure 4: Glioblastoma P3 spheroid in proliferation, invasion, or migration assays. (**A**) Proliferation assay. Left panel: Representative pictures with DMSO as control or with 20 µM of rotenone (respiratory chain complex I inhibitor) at time 0 or 72 h. Right panel: Spheroid area quantification represented as dashed lines in the images. Scale = 250 µm. (**B**) Invasion assay in collagen matrix. Left panel: Representative pictures with or without 20 mM hydrogen chloride, at time 0 or 24 h. Right panel: Quantification of invasive areas. Scale = 100 µm. (**C**) Migration assay on Matrigel coating. Left panel: Representative images in brightfield mode at time 0 or 24 h. Magnified areas are represented in the bottom panels. Right panel: Quantification of migratory areas. Scale = 250 µm. (**D**) Z-stack representation of the spheroid (40 µm step) in (**a**) spheroid

dynamic tracked over 18 h (image with 3 h interval) in (**b**). Cells stably expressed nuclear mCherry (orange) and cytoplasmic GFP (blue). Scale = 100 µm.

Movie 1: P3 spheroid dynamic recorded over 18 h (imaged every 30 min). Scale bar = 100 µm. The movie represents a merged Z-stack over time with a Z-step of 5 µm for an approximate total volume of 150 µm. Cells were infected with lentivirus to express NLS:mCherry (nuclei are orange) and with cytoplasmic GFP (blue color).

Supplementary document 1: Fiji macro for analyzing invasion, proliferation, and migration of 3D spheroid.

DISCUSSION:

Tumor spheroid assays are well adapted to study tumor characteristics including proliferation, invasion, and migration, as well as cell death and drug response. Cancer cells invade the 3D matrix forming an invasive microtumor, as seen in **Figure 4B,C**. During the invasive process, matrix metalloproteinases (MMP) digest matrices surrounding tumor cells[13], and MMP inhibitors (e.g., GM6001 or Rebimastat) may impair cell invasion but not migration[14]. Migration and invasion involve overlapping but separate molecular events[15], which can be studied in our spheroid assay. To do so, specific signaling pathways can be targeted either at the genetic level or through pharmacological inhibition.

Glioblastomas are known to extensively invade the surrounding tissues by different processes (e.g., co-option, white matter tract invasion, interstitial invasion)[16]. We recently described two novel mechanisms of glioblastoma invasion[9,12,17]. In particular, we studied matricellular thrombospondin-1 (TSP1) and showed that it is involved in tumor cell invasion through the activation of CD47 in tumor cells[12]. Furthermore, using a proteomic approach, we discovered the unexpected role of PLP1 and DNM1 in GBM invasion[17]. In these studies, 3D invasion assays were successfully used with or without pharmacological obstruction of TSP1, PLP1, or DNM1. Besides pharmacological obstruction, we also showed that acid treatment with hydrogen chloride impacts the invasion in the 3D assay. It is known that tumor acidosis activates a number of signaling pathways, including metabolic pathways (glycolysis), growth factors as TGFβ, and inhibits the immune response[5].

Three-dimensional cultures provide a more physiological relevant environment than 2D cultures, and many molecular and metabolic parameters may be differently regulated. Thus, pharmacological modulation may have a different impact. Consequently, besides standard immunohistology, metabolic events can also be studied in 3D culture using probes such as 2-DG-IR. To corroborate these findings, the electron transport chain complex I inhibitor may also be used in this context.

Additionally, the 3D culture system is also well-suited to the study of dynamic processes using live imaging under basal conditions or in the presence of stimuli or pharmacological cues.

The following critical steps should be considered when carrying out the procedures described in this article: 1) The spheroid diameter should not exceed 400 µm in order to avoid necrosis; 2) The

quantification of invasion using Fiji software must be carefully calibrated and performed as indicated in the detailed description of the procedure.; 3) The gel stiffness must be suitable to hold the spheroid in a stable configuration; 4) The pH value must be controlled, as a very acidic pH will impede the invasion process.

One limitation of the spheroid system described in this article is the lack of the complete tumor microenvironment. We acknowledge that the matrix used does not fully represent the stroma found in glioblastoma. However, collagens are part of the brain matrix and we wanted to develop a ready- and easy-to-use assay that can be used in any laboratory. Nevertheless, future experiments may also include additional matrix components as well as cellular elements, including stromal and immune cells. Another level of complexity is the inclusion of neuronal components, but these experiments must be carefully calibrated and designed.

In conclusion, we believe that our spheroid 3D system and the analytical tools we provide in this article may be useful for investigators, especially in studying brain tumor development.

ACKNOWLEDGMENTS

This work was supported by Transcan 2017, ARC 2017, Ligue Contre le Cancer (Comité de la Gironde et de la Charente-Maritime). Joris Guyon is a recipient of fellowship from the Toulouse University Hospital (CHU Toulouse).

DISCLOSURES:

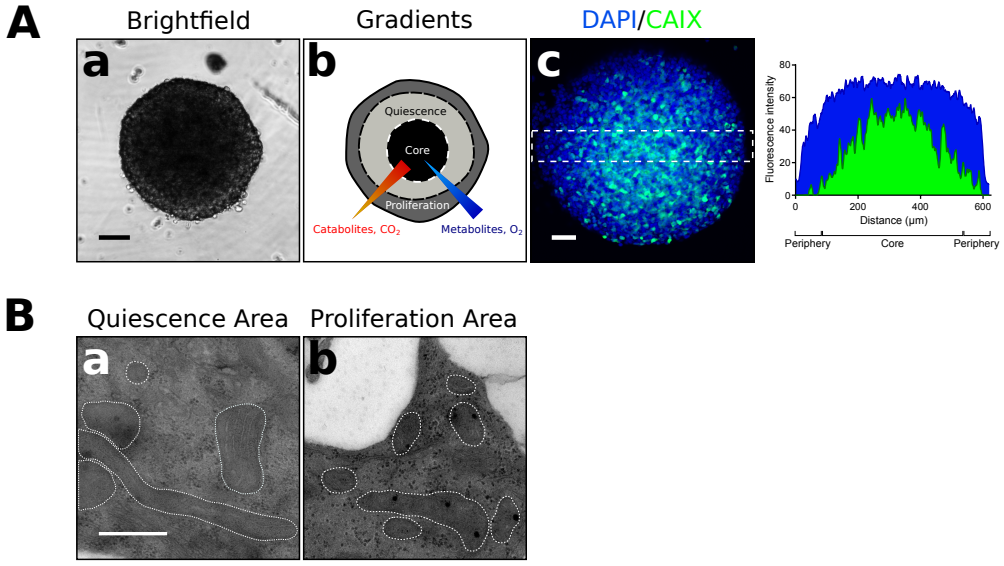
The authors declare that they have no competing financial interests.

REFERENCES:

1. Pelissier, F. A. et al. Age-related dysfunction in mechanotransduction impairs differentiation of human mammary epithelial progenitors. *Cell Reports.* **7**, 1926–1939 (2014).
Ishiguro, T. et al. Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications. *Cancer Science.* **108**, 283–289 (2017).
2. Sutherland, R. M. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science.* **240**, 177–184 (1988).
3. Desoize, B., Jardillier, J. Multicellular resistance: a paradigm for clinical resistance? *Critical Reviews in Oncology/Hematology.* **36**, 193–207 (2000).
4. Corbet, C., Feron, O. Tumour acidosis: from the passenger to the driver's seat. *Nature Reviews Cancer.* **17**, 577–593 (2017).
5. Hirschhaeuser, F. et al. Multicellular tumor spheroids: an underestimated tool is catching up again. *Journal of Biotechnology.* **148**, 3–15 (2010).
6. Berens, E. B., Holy, J. M., Riegel, A. T., Wellstein, A. A Cancer Cell Spheroid Assay to Assess Invasion in a 3D Setting. *Journal of Visualized Experiments.* (105), e53409 (2015).
7. Cavaco, A. C. M., Eble, J. A. A 3D Spheroid Model as a More Physiological System for Cancer-Associated Fibroblasts Differentiation and Invasion In Vitro Studies. *Journal of Visualized Experiments*. (150), e60122 (2019).
8. Boye, K. et al. The role of CXCR3/LRP1 cross-talk in the invasion of primary brain tumors. *Nature Communications.* **8**, 1571 (2017).

9. Dejeans, N. et al. Autocrine control of glioma cells adhesion and migration through IRE1alpha-mediated cleavage of SPARC mRNA. *Journal of Cell Science.* **125**, 4278–4287 (2012).
10. Golembieski, W. A., Ge, S., Nelson, K., Mikkelsen, T., Rempel, S. A. Increased SPARC expression promotes U87 glioblastoma invasion in vitro. *International Journal of Developmental Neuroscience.* **17**, 463–472 (1999).
11. Daubon, T. et al. Deciphering the complex role of thrombospondin-1 in glioblastoma development. *Nature Communications.* **10**, 1146 (2019).
12. Friedl, P., Wolf, K. Tube travel: the role of proteases in individual and collective cancer cell invasion. *Cancer Research.* **68**, 7247–7249 (2008).
13. Das, A., Monteiro, M., Barai, A., Kumar, S., Sen, S. MMP proteolytic activity regulates cancer invasiveness by modulating integrins. *Scientific Reports.* **7**, 14219 (2017).
14. Schaeffer, D., Somarelli, J. A., Hanna, G., Palmer, G. M., Garcia-Blanco, M. A. Cellular migration and invasion uncoupled: increased migration is not an inexorable consequence of epithelial-to-mesenchymal transition. *Molecular and Cellular Biology.* **34**, 3486–3499 (2014).
15. de Gooijer, M. C., Guillen Navarro, M., Bernards, R., Wurdinger, T., van Tellingen, O. An Experimenter's Guide to Glioblastoma Invasion Pathways. *Trends in Molecular Medicine.* **24**, 763–780 (2018).
16. Daubon T., et al. The invasive proteome of glioblastoma revealed by laser-capture microdissection. *Neuro-Oncology Advances*. **1** (1), vdz029 (2019).

Figure 1



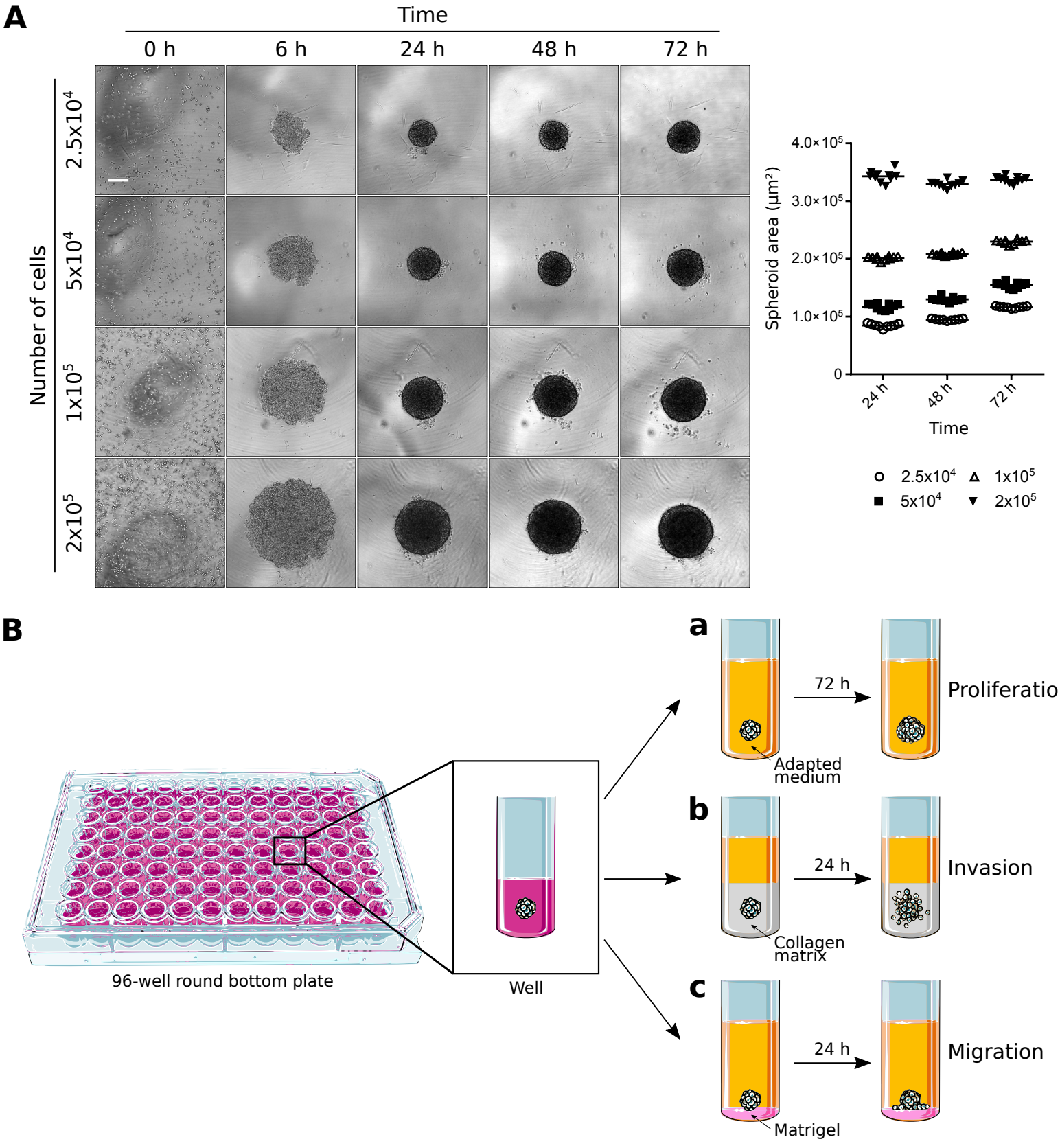


Figure 3

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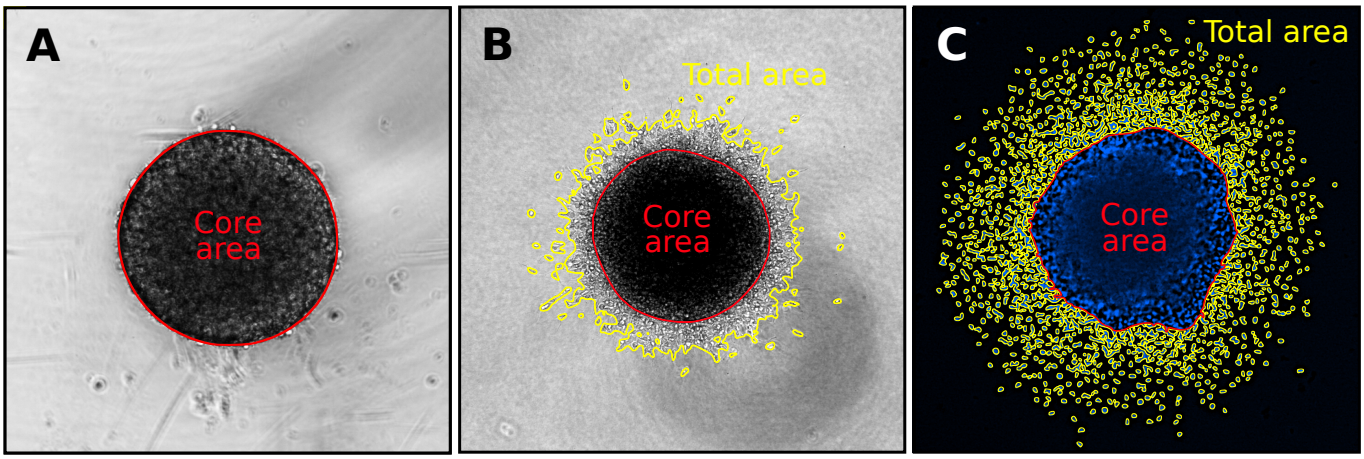
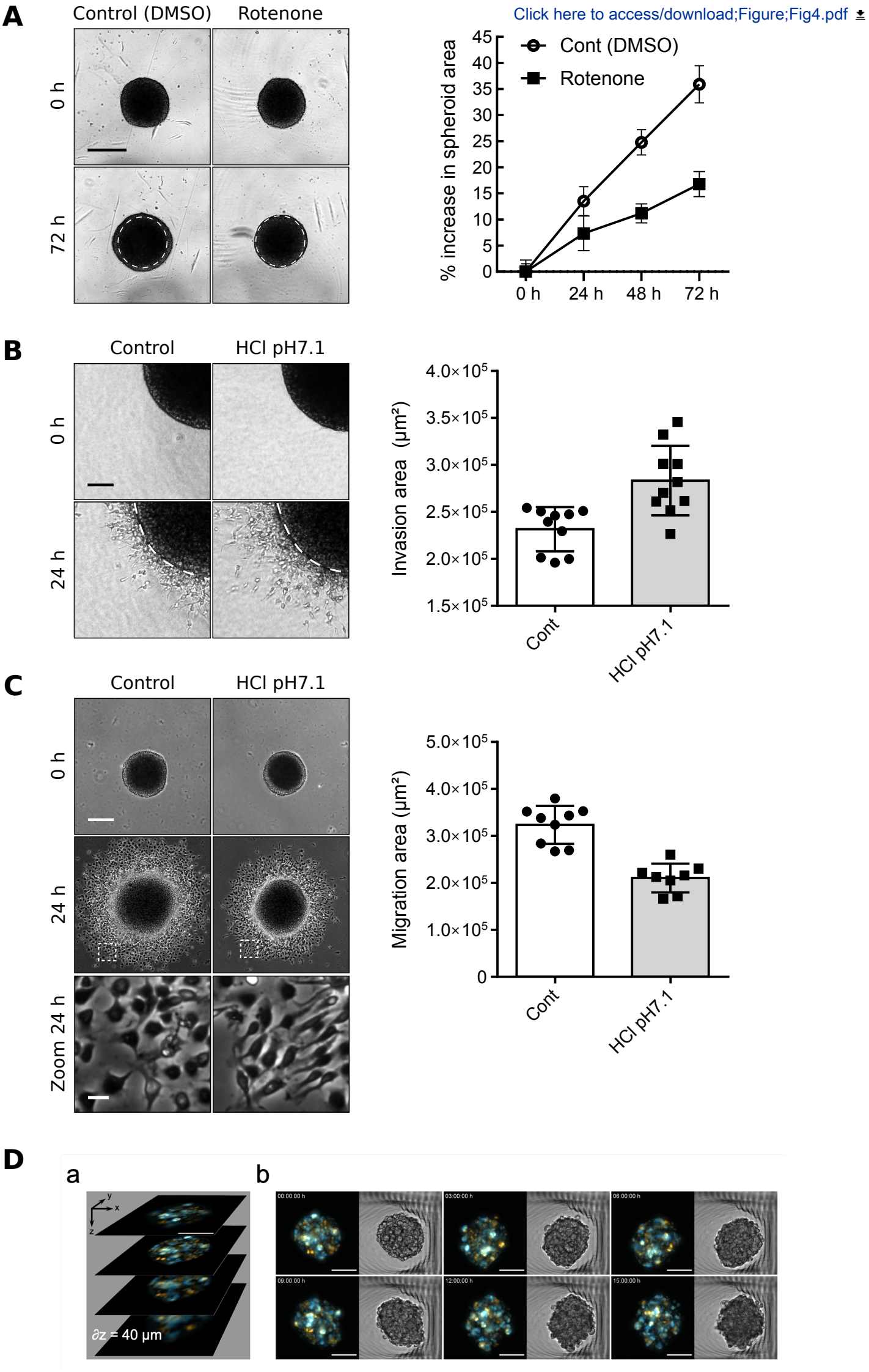


Figure 4



Movie 1

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Video or Animated Figure

Movie1.avi

Table of Materials

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Name	Company	Catalog number
96 well round-bottom plate	Falcon	08-772-212
Accutase	Gibco	A11105-01
B27	Gibco	12587
Basic Fibroblast Growth Factor	Peprotech	100-18B
Countess Cell Counting Chamber Slides	Invitrogen	C10283
DPBS 10X	Pan Biotech	P04-53-500
Fiji software	ImageJ	
Flask 75 cm^2	Falcon	10497302
Matrigel	Corning	354230
Methylcellulose	Sigma	M0512
NBM	Gibco	21103-049
Neurobasal medium	Gibco	21103049
Penicillin - Streptomycin	Gibco	15140-122
Trypan blue 0.4%	ThermoFisher	T10282
Type I Collagen	Corning	354236

Comments
Stored at 4 °C, sphere dissociation enzyme
Stored at -20 °C, defrost before use
Stored at -20 °C, defrost before use
Stored at 4 °C
Used to analyze pictures
Stored at -20 °C, diluted to a final concentration of 0.2 mg/mL in cold NBM
Diluted in NBM for a 2% final concentration
Stored at 4 °C
Stored at 4 °C
Stored at 4 °C
Used to cell counting
Stored at 4 °C

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JoVe revised article 60998_R1_111119

Dear Benjamin,

Please find attached our revised manuscript re-entitled "A reliable and easy-to-use 3D model for glioblastoma" and the response to reviewers in the next pages.

Sincerely yours,

Dr. Thomas Daubon, PhD

Pr. Andreas Bikfalvi, MD PhD

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1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Reply: this was highlighted with a grey back-color.

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
Reply: This was checked.

Specific Protocol steps:
1. Glucose flux analysis, 1: 'As described above'-which steps exactly?
Reply: this was removed from the text.

2. Fiji Macro: It may be best to include the macro itself as a supplemental file.
Reply: the macro was inserted in a supplemental file.

Figures:
1. Figure 2: Please include a space between numbers and units; e.g., ’24 h’ instead of ‘24h’ (see also Figure 4). Please also use ‘x’ for scientific notation instead of ‘.’.
Reply: We apologize for these mistakes. This was corrected in all Figures.

2. Please include a legend for Movie 1.
Reply: This was added in the text.

Discussion:
1. Please also include discussion of critical steps of the procedure and limitations here.
Reply: This was added to discussion.

Acknowledgment and Disclosures:
1. Please include a Disclosures section, providing information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
Reply: This was added in the text.

References:
1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. **Source**. *Volume* (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
2. Please do not abbreviate journal titles.

Table of Materials:
1. Please remove trademark (™) and registered (®) symbols from the Table of Materials.
2. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the manuscript entitled: "A 3D spheroid model for studying glioblastoma development", Guyon et.al., describe a 3D culture protocol for human glioblastoma spheres that can be used for in vitro migration and invasion studies. The protocol is well written and explains the necessary steps in detail. I have two minor concerns:

Minor Concerns:
1. In Figure 4D, what is the staining? The authors should describe exactly what they are showing.
Reply: Cells were infected with lentivirus for expressing NLS:mCherry (nucleus are in orange) and with cytoplasmic-GFP (blue color). Details were added into the text and in the legends.

2. In Figure 1B, the authors are showing representative EM images of quiescence and proliferative zones of glioma spheres. Can they provide some detail on why they are showing the mitochondria and is the share of the mitochondria indicative of the cellular state or something else?

Reply: The EM images were shown to demonstrate cellular structures can be evidenced such as mitochondria which non-uniformly distributed in the core or the peripheral areas of these spheroids. This would indicate the oxidative state of the cells. The precise ultrastructure of the mitochondria is also indicative for this state.

Reviewer #2:
Manuscript Summary:
The manuscript "A 3D spheroid model for studying glioblastoma development" by Guyon et al presented a protocol to form a glioblastoma and characterize the invasion of the microtumor. In general, the authors introduced an interesting method for the microtumor fabrication. However, a number of issues should be clarified in the protocol.

Major Concerns:
1. The type of tumor cells used in the presented study should be introduced.
Reply: We used a patient-derived glioblastoma cell line, which exhibit a mesenchymal phenotype (Wang J et al, 2005; Bougnaud et al, 2016 ; Daubon et al, 2019).

2. In page 6, before the tumor cells are washed, the authors should clarify how the cells were cultured.
Reply: This information was added to the text in page 6.

3. Inhibitors and chemicals in page 6 should be clarified.
Reply: We have now added the sentences indicating potential inhibitors or chemicals which could be used in this assay.

4. Inhibitors or activators in page 7 should be clarified.
Reply: We have now added the sentences indicating potential inhibitors or activators which could be used in this assay.

5. What does cNBM stand for?
Reply: This stands for complete NeuroBasal Medium, as indicated in the text.

6. It is not clear how the recorded video was analyzed. The authors should clarify.
Reply: This was added into the movie and Figure 4 legends.

Reviewer #3:
Manuscript Summary:
The authors present methods for 3D spheroid modeling of glioblastoma invasion using collagen and Matrigel matrices. 2D culture systems lack many elements of the 3D microenvironment that dictate disease progression, and the authors report on an in vitro assay to observe more realistic tumor dynamics. However, there are some major and minor concerns regarding the manuscript content and discussion, as outlined below:

Major concerns:
1. The abstract is severely lacking in description of the context of this work and rationale, specifically with regards to why this platform is necessary for GBM culture specifically, considering how important invasion is to GBM tumor progression.

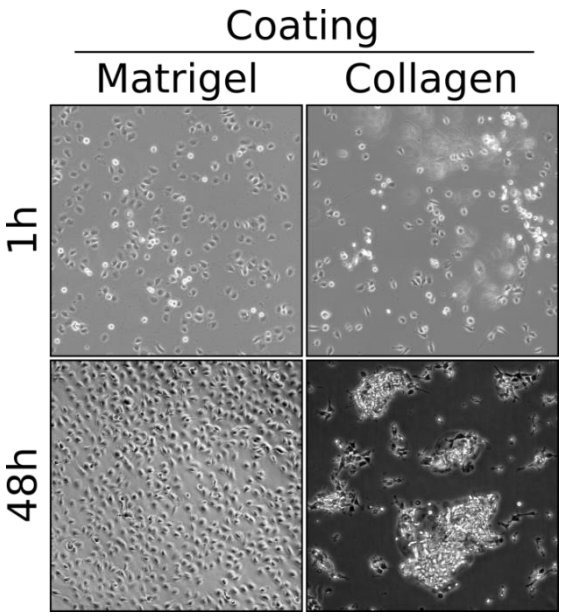
Reply: We have entirely revised the abstract and provide a rationale for the development of this assay.

2. The introduction is missing a review of other 3D invasion assays and why the authors' specific method is different or advantageous compared to other methods. A lot of sentences within lines 33-51 are also redundant and can be condensed. Ideally, more information on GBM modeling of tumor dynamics is necessary.
Reply: We have discussed other existing assays in the introduction section, as the reviewer requested.

3. The authors post that their first figure proves a difference in diffusion of oxygen and the presence of a hypoxic center, but there is not a robust characterization of hypoxia in these spheroids. A quantification of IR-labeled glycolysis across the spheroid bulk might be included here.
Reply: We have replaced the entire figure of a spheroid stained with calcein or IR-labeled 2-DG by a spheroid section depicting the internal cellular organization, and a staining with anti-carboxic anhydrase IX, as hypoxic marker.

4. The authors lack addressing in the Discussion section the contradictory nature of using matrices that do not mimic composition of brain tissue in GBM invasion assays.
Reply: We acknowledge that the matrix we are using does not fully represent the stroma found in glioblastoma. However, collagens are part of the brain matrix and we wanted to develop a ready- and easy-to-use assay which can run in any laboratory.

5. The proliferation experiment does not accurately represent 3D proliferation without also embedding it into a 3D matrix after spheroid formation.
Reply: Spheroid expansion is an indirect indicator of tumor cell proliferation, and this was measured in Figure 4A.
The authors also do not address why the difference in using collagen for invasion but Matrigel for migration.
Reply: We agree with the reviewer on this point. However, if the assay was performed on collagen I-coated plates, cells will not only migrate but also assemble into clusters (**image below**). This does not occur when this assay is performed on Matrigel (Figure 4C).



6. The images used for quantification are 2D representations of area, which seems a limited way to measure 3D tumor invasion, can they show quantifications of tumor expansion in terms of 3D volume using their z-stack images as shown in Figure 4d?
Reply: The pictures were taken in brightfield and not from fluorescence images. The quantification of the invasive area was done the median section of the spheroid, and we have validated the reproducibility of this approach in using knock-down for specific genes (***Daubon et al, 2019 Nat Comm***) or pharmacological inhibition (***Daubon et al, 2019 Neuro-Oncology Advances***). Z-stack does not add anything more to the quantification.

7. Discussion line 264: more description of the "micro-tumor" would be appreciated and discussed in the context of the assay.
Reply: Instead of micro-tumor, we now wrote invasive micro-tumor to underline the specific property studied in this assay. In the subsequent sentences, this was more discussed in detail.

8. More discussion on the limitations of this assay is missing, in addition to a lack of discussion on where this assay fits with the current standard of spheroid culture/invasion assays already used.
Reply: We wanted to setup an easy and reproducible assay that can be carried out in any laboratory. The theoretical limitation is, of course, that the quantification of invasion in the 3D structure is performed on the median section. However, we have validated the reproducibility of this approach in using knock-down for specific genes or pharmacological inhibition. Therefore, we consider our approach as sound.

When compared to similar assays published in JoVe (Berens et al, 2015 ; Vinci et al, 2015 ; Cavaco et al, 2019), our assay has the following advantages:
- our approach characterized specifically the invasive properties of stem-like cells derived from patients;
- we have developed a macro using FiJi software that allows rapid and easy quantification, and no specific equipment or other software is needed.

Minor concerns:
1. The title is misleading- spheroids are already formed when they are embedded and cultured, and the imaging/measurements are described for invasion, therefore invasion is a more apt description than "development."
Reply: We modified the title which is now “a reliable and easy-to-use 3D model for glioblastoma.”

2. Abstract line 29- definition of the acronym for GBM is missing.
Reply: The abstract was completely modified. By consequence, this is not appearing in the text.

3. Line 249- Sentence "Quantification of each assay is shown in Figure" seems unfinished.
Reply: This was modified in the text.

4. Line 256-257- Figure references are incomplete.
Reply: This was modified in the text.

5. Figure legend for Figure 1c and 1d are in incorrect order.
Reply: The Figure was modified, and the corresponding legend is in a correct order.

6. Discussion line 277: the transition to discussing the HCl acid treatment was abrupt and lacking the context of acid treatment and why that would stimulate invasion in your model.
Reply: This was completed into the discussion as “It is known that tumor acidosis activates a number of signaling pathways, including metabolic pathways (glycolysis), growth factors as TGFβ, and inhibits immune response”.

7. Line 278: "physiological environment" sounds awkward, is that supposed to read "physiologically relevant environment"?
Reply: we have changed this sentence.

8. The figure legends are lacking in description, particularly figures 2 and 3.
Reply: We have now described into details the figures.

9. There are other grammatical and typographical errors in this manuscript, please edit scrupulously.
Reply: this has been done.