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A reliable and easy-to-use 3D model for glioblastoma

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30 **Summary section:**
31

- 32 - An easy to use invasion assay for glioblastoma is described;
33 - The assay is suitable for glioblastoma stem-like cells;
34 - A macro in Fiji software is described for easy quantification of invasion, migration and
35 proliferation.
36

37
38 **Abstract:**
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40 Cell cultures in two dimensions (2D) do not correctly mimic in vivo tumor growth. To this
41 aim, three dimensional (3D) culture models, which include spheroid cultures, have been
42 developed. This is particularly true for neuro-oncology. Indeed, brain tumors have the
43 proclivity to invade the healthy brain environment. We describe herein a 3D glioblastoma
44 assay we developed in our laboratory, which is ideally suited to study the invasion process.
45 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 two dimensions (2D) represents disadvantages, as it does not mimic *in vivo* three-dimensional (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, it has also been shown that the non-physiological plastic surface triggers cell differentiation¹. Three-dimensional culture models have been developed to overcome these difficulties. They have the advantage of mimicking the multicellular architecture and heterogeneity of tumors², and thus could be considered to be a more relevant model for solid tumors³. The complex morphology of spheroids contributes to better evaluate drug penetrance and resistance⁴. The tumor heterogeneity in the spheroid impacts on the diffusion of oxygen and nutrients, and the response to pharmacological agents (**Figure 1A a-c**). Diffusion of oxygen is altered when 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 are taking place⁵. 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 studying invasion in a horizontal plane on a thin matrix layer or in a Boyden chamber assay⁹. Three-dimensional assays have been described with 3D spheroid cultures using classical glioblastoma cell lines¹⁰. More complex variants are represented by invasion of tumor spheroids on brain organoids in confrontation cultures¹¹. However, it is still important to develop an easy to use and reproducible assay that can be carried out in any laboratory. We wanted to setup an easy and reproducible assay that can be carried out in any laboratory. Furthermore, the quantification in this assay should be straightforward and only requires open-access online software. We have previously described our procedure to generate glioblastoma stem-like cells from patient samples (Guyon et al, in press). Briefly, tumor pieces are cut into small pieces and enzymatically digested. Single cells derived from the digestion are cultivated in neurobasal medium. After 4 to 7 days, spheroid structures form spontaneously. Upon intracranial implantation in mice models, they form tumors exhibiting a necrotic core surrounded by pseudo-palisading cells¹². This closely resembles to the characteristics found in GBM patients.

In this article, we describe our procedures to produce spheroids from 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 represents mammalian basal membrane required for cell attachment and migration. At the contrary, collagen type I, structural element of stroma, is the most common fibrillary extracellular matrix and is used in cell invasion assays. We characterize herein 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.

Protocols

1. Generation of uniform size tumor spheroids

Stem-like cells are cultured in neurobasal medium complemented with B27 supplement, heparin, FGF-2, penicillin and streptomycin, as described in previous articles¹². These cells are spontaneously growing in spheroids.

1. Wash tumor cells with Phosphate Buffered Saline (PBS; 5 mL) and incubate cells with accutase (0.5 – 1 mL) during 5 minutes at 37°C.
2. Wash with PBS (4 – 4.5 mL) and add complete growth medium (complete neurobasal medium, cNBM; 10 mL).
3. Count cells (here an automatic counting technique with Trypan blue and cell counting chamber slide is used).
4. To generate 100 spheroids with 10^4 cells per spheroid (number according to preferred size), mix 10^6 cells in 8 mL of NBM with 2 mL of methylcellulose 2%.
5. Transfer the suspension to sterile system container and with a multichannel pipette, dispense 100 μ L/well into a 96 well round bottom-plate.
6. Incubate the plate at 37°C, 5% CO₂ and 95% humidity.
7. Equal sized spheroids will form and can be used after 3 – 4 days.

2. 3D experiments

Proliferation

a. Preparation

1. Inhibitors (rotenone as in Figure 4A) and chemicals were suspended in 100 μ L of medium and added to 100 μ L of medium in each well (one spheroid per well).
2. Incubate the plate at 37°C, 5% CO₂ and 95% humidity.

b. Image acquisition and analyze

1. Take pictures with a video-microscope in brightfield to create series of conditions at T0 and the following time expected.
2. Use the software Fiji to analyze pictures from different way: manual or semi-automated.
 - Manual: draw a circle around the spheroid core with the freehand selection tool and measure the area of each spheroid.
 - Semi-automated: create the macro shown in **3. Fiji Macro** with //Core Area only.

Invasion

a. Preparation

1. Prepare the collagen matrix in a tube on ice with type I collagen at 1 mg/mL final concentration, 1X PBS, $0.023 \times V_{\text{collagen}}$ NaOH 1M and sterile H₂O. Incubate the solution on ice for 30 min;
2. Collect spheroids of the round-bottom well plate in 500 μ L tubes and wash twice with 200 μ L 1X PBS;
3. Pipette carefully spheroids in 100 μ L of the collagen matrix and insert in the center of a well of normal 96 well plates;
4. Incubate the collagen gel for 30 min at 37°C and then add cNBM on top of the gel. Inhibitors or activators (HCl as shown in Figure 4B, 4C) can be added at this step to the medium.

b. Image acquisition and analyze

1. Take pictures sequentially with a videomicroscope in brightfield mode 24 hours after collagen inclusion.
2. Use Fiji software to analyze pictures either manually or semi-automatic.
 - **Manually:** just 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.
 - **Semi-automatic:** create the macro as indicated in **3. Fiji Macro** to determine the invasive area.

Migration

a. Preparation

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. Transfer spheroids in 50 μ L of cNBM from the round-bottom well plate to the 6-well plate;
3. Incubate the plate at 37°C and wait 30 min for spheroids to adhere;
4. After 24 hours of incubation, stain with 10 ng/ml of Hoechst and incubate 30 min at 37°C.

b. Image acquisition and analysis

1. Images are obtained using a videomicroscope (Nikon, Eclipse Ti) in brightfield. Laser 405 is used for visualization of Hoechst staining.
2. Use Fiji software to analyze pictures and run the macro as indicated in **3.Fiji Macro**.

Important points

- Touching the bottom of the well or completely removing the supernatant damage the spheroids.
- for collagen type I gel handling:
 - Keep the gel on ice to avoid collagen polymerization ;
 - Adding an acidic component is not advised (change of pH will affect the compactness of the gel) ;
 - Cells must be included rapidly into the collagen to prevent cell death and the degradation of the gel.

3. Fiji Macro

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:

1. Open the macro window: Plugins > Macros > Interactive Interpreter;
2. Copy and paste the following adapted purple loop, keep purple sentences and add the green sentences of interest;
3. Adjust the parameters in red for a specific quantification and run the macro (Macros > Run Macro or Ctrl+R) to analyze the entire series;
4. Check and, if necessary, adapt the Region Of Interest (ROI).

4. Electron microscopy of spheroid

Fixation step:

- Collect the spheroid with a cut tip and put it in a 1.5 ml tube;
- Wash once with 0.1 M Phosphate Buffer (PB);
- Fix the spheroid overnight at 4°C in 2% glutaraldehyde/2% paraformaldehyde (PFA) in PB 0.1 M;
- Replace the fixation solution by a solution of 1% PFA in PB 0.1 M followed by sample preparation.

Sample preparation:

- Transfer the spheroids into a strainer and put it in a glass backer in order to avoid spheroid damages;
- Wash 3 times carefully with 0.1 M PB;
- Incubate with Osmium for 2 hours in the dark (Osmium 4% which must be diluted in 0.1% PB 0.1M buffer);
- Wash 3 times carefully with 0.1 M PB;
- Dehydrate as in the following steps:
 - Ethanol 50%: 1 time for 10 minutes
 - Ethanol 70%: 1 time for 10 minutes
 - Ethanol 90%: 2 times for 15 minutes
 - 100% ethanol: 2 times for 20 minutes
 - Acetone: 2 times for 30 minutes
- Include samples in a 50/50 mixture of Acetone/Resin for 2 h; during this step, prepare EPON resin (Embed-812: 11.25 g; DDSA: 9 g; NMA: 4.5 g);
- Discard the Acetone / Resin mixture; replace them by freshly prepared resin;
- Incubate overnight;
- Include the spheroids in resin into a mold.

Representative Results

Spheroids were prepared as described in the Protocols section and the following experiments were carried out: migration, invasion, proliferation, and microscopy imaging. To measure **hypoxia** in distinct areas of the spherical structure, the **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 1B a-b**). Spheroids composed of 2.5×10^3 , 5×10^3 , 10^4 or 2×10^4 cells exhibit a spheroid diameter of about 350, 400, 500 or 650 μm respectively (Erreur ! Source du renvoi introuvable.**A**). Spheroids may be used within 4 days after starting the experiment (**Figure 2B**). The quantification of each assay (proliferation; invasion; migration) is shown in Erreur ! Source du renvoi introuvable.. Fiji macros were developed to quantify proliferation, invasion or migration (**Figure 3**). The increase of spheroid core reflects 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 collagen type I was calculated by the subtraction of the total area from the core area. Acidic treatment enhanced invasion over a period of 24h (Erreur ! Source du renvoi introuvable.**B**). Furthermore, we found that HCl treatment reduced the migratory area of the spheroids by 1.5 fold compared to control (Erreur ! Source du renvoi introuvable.**C**). Spheroid dynamics was studied by live imaging. Spheroids have a high internal dynamic and moved in a fast motion (**movie 1** and tracking analysis in **Figure 4D**).

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 “micro-tumor”, as seen in **Figure 4B and 4C**. During the invasive process, matrix metalloproteinases (MMP) digest matrices surrounding tumor cells¹³, and MMP inhibitors (such as GM6001 or Rebimastat) may impair cell invasion but not migration¹⁴. Migration and invasion involve overlapping but also separate molecular events¹⁵, which can be studied in our spheroid assay. To this aim, 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 (co-option, white matter tract invasion, interstitial invasion)¹⁶. We have recently described two novel mechanisms of glioblastoma invasion^{9,12,17}. In particular, we have studied the matricellular, thrombospondin-1 (TSP1), and shown that it involved in tumor cell invasion, through the activation of CD47 in tumor cells¹². Furthermore, using a proteomic approach, we have discovered unexpected role of PLP1 and DNM1 in GBM invasion¹⁷. In these studies, 3D invasion assays were successfully used with or without pharmacological blockade of TSP1, PLP1 or DNM1. Besides pharmacological blockade, we have also shown in this article that acid treatment with HCl impacts on invasion in the 3D assay. It is known that tumor acidosis activates a number of signaling pathways, including metabolic pathways (glycolysis), growth factors as TGF β , and inhibits immune response⁵.

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

Additionally, the 3D culture system is also adapted to study dynamic processes using live imaging and this 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. The spheroid size should not exceed diameter of 400 μm to avoid necrosis. The quantification of invasion using Fiji software must be carefully calibrated and performed as indicated in the detailed description of the procedure. The gel stiffness must be appropriate not to hold the spheroid in a stable configuration. Furthermore, the pH value must be controlled since too acidic pH will impede the invasion process.

One limitation of spheroid system we described in this article is the lack of the complete tumor microenvironment. 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. 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.

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DISCLOSURES

The authors declare that they have no competing financial interests.

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Figure legends:

Figure 1: 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 showed a round appearance with a dense central area. Scale = 100 μm . **(b)** Schematic representation adapted from (Hirschhaeuser et al., 2010) showed 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 found in the quiescent area 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 was analyzed on the right panel. At 24 h, the P3 cells formed dense spheroids. The initial number of cells determines the size of the spheroids. Scale bar = 250 μm . **(B)** Schematic illustration of the easy-to-use experiments 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 by using Fiji's software. Core area is represented in red and total area, which contains core area, in yellow. Invasive area corresponds to the subtraction of the total area by the core area. **(A)** Proliferation assay, **(B)** invasion assay in collagen gel in brightfield acquisitions. **(C)** Migration assay on Matrigel coating from fluorescence acquisition (nucleus 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 10 μM of rotenone (respiratory chain complex I inhibitor) at time 0 or 72 hours. **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 XX μM HCl, 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 hours (image with 3 h interval) in **(b)**. Blue cells were on top position and orange cells deeper in the spheroid. Scale = 100 μm .

Movie 1: P3 spheroid dynamic was recorded over 18 hours (images steps every 30 min). Scale bar = 100 μm . The movie represents a merged Z-stack over time (z-step of 5 μm for an approximate total volume of 150 μm). Colors represent the position of the cells in the spheroid (blue cells are the top ones and orange cells are deeper in the spheroid).