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

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23
24 **Keywords:** glioblastoma, patient-derived cell, spheroid, invasion, **migration, proliferation**, *in*
25 *vitro* models.

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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:**

39

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.

46 **Introduction**

47 In most studies using primary or commercially available cell lines, assays are performed on
48 cells grown on plastic surfaces as monolayer cultures. Managing cell culture in two
49 dimensions (2D) represents disadvantages, as it does not mimic *in vivo* three-dimensional
50 (3D) cell environment. In 2D cultures, the entire cell surface is directly in contact with the
51 medium, altering cell growth and modifying drug availability. Furthermore, it has also been
52 shown that the non-physiological plastic surface triggers cell differentiation¹. Three-
53 dimensional culture models have been developed to overcome these difficulties. They have
54 the advantage of mimicking the multicellular architecture and heterogeneity of tumors²,
55 and thus could be considered to be a more relevant model for solid tumors³. The complex
56 morphology of spheroids contributes to better evaluate drug penetrance and resistance⁴.
57 The tumor heterogeneity in the spheroid impacts on the diffusion of oxygen and nutrients,
58 and the response to pharmacological agents (**Figure 1A a-c**). Diffusion of oxygen is altered
59 when spheroid size reaches 300 µm, inducing a hypoxic environment in the center of the
60 spheroid (**Figure 1A-c**). Metabolites are also less penetrating through the cell layers and
61 compensating metabolic reactions are taking place⁵. When the diameter of the spheroid
62 increases, necrotic cores can be observed, further mimicking characteristics found in many
63 solid cancers, including the aggressive brain cancer glioblastoma (GBM)⁶.

64 Several 2D or 3D invasion assays for glioblastoma have been reported in the literature^{7,8}.
65 Two-dimensional assays are mainly studying invasion in a horizontal plane on a thin matrix
66 layer or in a Boyden chamber assay⁹. Three-dimensional assays have been described with
67 3D spheroid cultures using classical glioblastoma cell lines¹⁰. More complex variants are
68 represented by invasion of tumor spheroids on brain organoids in confrontation cultures¹¹.
69 However, it is still important to develop an easy to use and reproducible assay that can be
70 carried out in any laboratory. We wanted to setup an easy and reproducible assay that can
71 be carried out in any laboratory. Furthermore, the quantification in this assay should be
72 straightforward and only requires open-access online software. We have previously
73 described our procedure to generate glioblastoma stem-like cells from patient samples
74 (Guyon et al, in press). Briefly, tumor pieces are cut into small pieces and enzymatically
75 digested. Single cells derived from the digestion are cultivated in neurobasal medium. After
76 4 to 7 days, spheroid structures form spontaneously. Upon intracranial implantation in mice
77 models, they form tumors exhibiting a necrotic core surrounded by pseudo-palisading
78 cells¹². This closely resembles to the characteristics found in GBM patients.

79 In this article, we describe our procedures to produce spheroids from determined number of
80 cells to ensure reproducibility. Two complementary matrices can be used for this purpose,
81 Matrigel and collagen type I. Matrigel is enriched in growth factors and represents
82 mammalian basal membrane required for cell attachment and migration. At the contrary,
83 collagen type I, structural element of stroma, is the most common fibrillary extracellular
84 matrix and is used in cell invasion assays. We characterize herein our GBM spheroid model
85 by performing migration and proliferation assays. Analysis was done not only at fixed time
86 points but also by monitoring spheroid expansion and cell movement by live-imaging.
87 Furthermore, electron microscopy was done to visualize morphological details.

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89

90 **Protocols**

91 **1. Generation of uniform size tumor spheroids**

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

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

107

108 **2. 3D experiments**

109 **Proliferation**

110 a. Preparation

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

114

115 b. Image acquisition and analyze

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

123

124 **Invasion**

125 a. Preparation

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

136

137 b. Image acquisition and analyze

- 138 1. Take pictures sequentially with a videomicroscope in brightfield mode 24 hours after
139 collagen inclusion.
- 140 2. Use Fiji software to analyze pictures either manually or semi-automatic.
- 141 - **Manually:** just draw around the core and the total area of the spheroid with the
142 freehand selection tool and measure the invasive area of each spheroid by
143 subtract total area with core area.
- 144 - **Semi-automatic:** create the macro as indicated in **3. Fiji Macro** to determine the
145 invasive area.

146

147 **Migration**

148 a. Preparation

- 149 1. Coat a 6-well plate with Matrigel (0.2 mg/mL) in NBM for 30 min at 37°C, then
150 remove the Matrigel and add 2 mL of cNBM;
- 151 2. Transfer spheroids in 50 μ L of cNBM from the round-bottom well plate to the 6-well
152 plate;
- 153 3. Incubate the plate at 37°C and wait 30 min for spheroids to adhere;
- 154 4. After 24 hours of incubation, stain with 10 ng/ml of Hoechst and incubate 30 min at
155 37°C.

156

157 b. Image acquisition and analysis

- 158 1. Images are obtained using a videomicroscope (Nikon, Eclipse Ti) in brightfield. Laser
159 405 is used for visualization of Hoechst staining.
- 160 2. Use Fiji software to analyze pictures and run the macro as indicated in **3.Fiji Macro**.

161

162 **Important points**

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

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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**

216

217 staining was used for determining hypoxic activity (**Figure 1A-c**). More CAIX-positive cells
218 were observed in the spheroid center (**Figure 1A-c**). Hypoxic cells located in the spheroid
219 core tend to be more glycolytic than the surrounding ones. Mitochondria can be imaged for
220 further analyses as shown by electron microscopy (**Figure 1B a-b**). Spheroids composed of
221 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
222 μm respectively (Erreur ! Source du renvoi introuvable.**A**). Spheroids may be used within 4
223 days after starting the experiment (**Figure 2B**). The quantification of each assay
224 (proliferation; invasion; migration) is shown in Erreur ! Source du renvoi introuvable.. Fiji
225 macros were developed to quantify proliferation, invasion or migration (**Figure 3**).
226 The increase of spheroid core reflects stimulation of cell proliferation (**Figure 4A**). Upon
227 inhibition by rotenone, an established inhibitor of complex I of the mitochondrial respiratory
228 chain, the vast majority of ATP production in the mitochondria was compromised. As a
229 consequence, proliferation was reduced by 20 % after 72 h (**Figure 4A**). Invasion collagen
230 type I was calculated by the subtraction of the total area from the core area. Acidic
231 treatment enhanced invasion over a period of 24h (Erreur ! Source du renvoi introuvable.**B**).
232 Furthermore, we found that HCl treatment reduced the migratory area of the spheroids by
233 1.5 fold compared to control (Erreur ! Source du renvoi introuvable.**C**). Spheroid dynamics
234 was studied by live imaging. Spheroids have a high internal dynamic and moved in a fast
235 motion (**movie 1** and tracking analysis in **Figure 4D**).

236

237 **Discussion**

238 Tumor spheroid assays are well adapted to study tumor characteristics including
239 proliferation, invasion and migration, as well as cell death and drug response. Cancer cells
240 invade the 3D matrix forming an invasive “micro-tumor”, as seen in **Figure 4B and 4C**. During
241 the invasive process, matrix metalloproteinases (MMP) digest matrices surrounding tumor
242 cells¹³, and MMP inhibitors (such as GM6001 or Rebimastat) may impair cell invasion but
243 not migration¹⁴. Migration and invasion involve overlapping but also separate molecular
244 events¹⁵, which can be studied in our spheroid assay. To this aim, specific signaling
245 pathways can be targeted either at the genetic level, or through pharmacological inhibition.

246 Glioblastomas are known to extensively invade the surrounding tissues by different
247 processes (co-option, white matter tract invasion, interstitial invasion)¹⁶. We have recently
248 described two novel mechanisms of glioblastoma invasion^{9,12,17}. In particular, we have
249 studied the matricellular, thrombospondin-1 (TSP1), and shown that it involved in tumor cell
250 invasion, through the activation of CD47 in tumor cells¹². Furthermore, using a proteomic
251 approach, we have discovered unexpected role of PLP1 and DNM1 in GBM invasion¹⁷. In
252 these studies, 3D invasion assays were successfully used with or without pharmacological
253 blockade of TSP1, PLP1 or DNM1. Besides pharmacological blockade, we have also shown in
254 this article that acid treatment with HCl impacts on invasion in the 3D assay. It is known that
255 tumor acidosis activates a number of signaling pathways, including metabolic pathways
256 (glycolysis), growth factors as TGF β , and inhibits immune response⁵.

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

263 Additionally, the 3D culture system is also adapted to study dynamic processed using live
264 imaging and this under basal conditions or in the presence of stimuli or pharmacological
265 cues.

266 The following critical steps should be considered when carrying out the procedures
267 described in this article. The spheroid size should not exceed diameter of 400 μm to avoid
268 necrosis. The quantification of invasion using Fiji software must be carefully calibrated and
269 performed as indicated in the detailed description of the procedure. The gel stiffness must
270 be appropriate not to hold the spheroid in a stable configuration. Furthermore, the pH value
271 must be controlled since too acidic pH will impede the invasion process.

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

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

282

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287

288 **DISCLOSURES**

289 The authors declare that they have no competing financial interests.

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334

335 **Figure legends:**

336

337 **Figure 1: Spheroid is a relevant model to mimic solid tumors.**

338 **(A)** Spheroid as a round 3D structure with different areas. **(a)** A brightfield picture of a P3
339 spheroid showed a round appearance with a dense central area. Scale = 100 μm . **(b)**
340 Schematic representation adapted from (Hirschhaeuser et al., 2010) showed the O_2 , CO_2 ,
341 metabolite, and catabolite gradients in the spheroid. **(c)** Left panel: confocal picture of a
342 spheroid stained with DAPI (blue) and with antibodies against carboxic anhydrase IX (green).
343 Right panel: quantification of the fluorescence from the dashed area. Scale = 100 μm .
344 **(B)** Electron microscopy images with delineated mitochondria (dashed lines). Large
345 mitochondria are found in the quiescent area while they are smaller in the proliferation
346 area. Scale = 250 nm.

347

348 **Figure 2: Overall spheroid preparation steps.**

349 **(A)** Generation of human P3 glioblastoma spheroids. Representative images are on the left
350 panel and corresponding proliferation was analyzed on the right panel. At 24 h, the P3 cells
351 formed dense spheroids. The initial number of cells determines the size of the spheroids.
352 Scale bar = 250 μm . **(B)** Schematic illustration of the easy-to-use experiments for studying
353 proliferation, invasion, or migration. Spheroids under various conditions: **(a)** in serum-free
354 medium for tumor growth, **(b)** in collagen matrix for facilitating single cell invasion, and **(c)**
355 on Matrigel coating for cell migration.

356

357 **Figure 3: Quantification of *in vitro* assays with Fiji software.**

358 Representation of the regions of interest (ROI) obtained by using Fiji's software. Core area is
359 represented in red and total area, which contains core area, in yellow. Invasive area
360 corresponds to the subtraction of the total area by the core area. **(A)** Proliferation assay, **(B)**
361 invasion assay in collagen gel in brightfield acquisitions. **(C)** Migration assay on Matrigel
362 coating from fluorescence acquisition (nucleus were stained with DAPI, in blue).

363

364 **Figure 4: Glioblastoma P3 spheroid in proliferation, invasion or migration assays**

365 **(A)** Proliferation assay. Left panel: representative pictures with DMSO as control or with 10
366 μM of rotenone (respiratory chain complex I inhibitor) at time 0 or 72 hours. Right panel:
367 spheroid area quantification (represented as dashed lines in the images). Scale 250 μm .
368 **(B)** Invasion assay in collagen matrix. Left panel: representative pictures with or without XX
369 μM HCl, at time 0 or 24 h. Right panel: quantification of invasive areas. Scale 100 μm .
370 **(C)** Migration assay on Matrigel coating. Left panel: representative images in brightfield
371 mode at time 0 or 24 h. Magnified areas are represented in the bottom panels. Right panel:
372 quantification of migratory areas. Scale 250 μm .
373 **(D)** Z-stack representation of the spheroid (40 μm step) in **(a)**. Spheroid dynamic tracked
374 over 18 hours (image with 3 h interval) in **(b)**. Blue cells were on top position and orange
375 cells deeper in the spheroid. Scale = 100 μm .

376

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

381