Journal of Visualized Experiments

Co-culture of glioblastoma stem-like cells on patterned neurons to study migration and cellular interactions

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62213R1
Full Title:	Co-culture of glioblastoma stem-like cells on patterned neurons to study migration and cellular interactions
Corresponding Author:	Thomas Daubon CNRS IBGC Bordeaux, Nouvelle Aquitaine FRANCE
Corresponding Author's Institution:	CNRS IBGC
Corresponding Author E-Mail:	thomas.daubon@u-bordeaux.fr
Order of Authors:	Joris Guyon
	Pierre-Olivier Strale
	Irati Romero-Garmendia
	Andreas Bikfalvi
	Vincent Studer
	Thomas Daubon
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Cancer Research
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bordeaux, FRANCE
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	

TITLE:

Co-culture of Glioblastoma Stem-like Cells on Patterned Neurons to Study Migration and Cellular Interactions

AUTHORS AND AFFILIATIONS:

Joris Guyon[1,#], Pierre-Olivier Strale[2,#], Irati Romero-Garmendia[3], Andreas Bikfalvi[1], Vincent Studer[2,*], Thomas Daubon[3,*]

#Co-first authors
*Co-last authors

[1]University Bordeaux, INSERM, LAMC, U1029, 33600, Pessac, France
[2]Joint Research Laboratory Alvéole/ UMR5297 CNRS, IINS, Bordeaux, France
[3]University Bordeaux, CNRS, IBGC, UMR5095, 33000, Bordeaux, France

Email addresses:

Joris Guyon (joris.guyon@u-bordeaux.fr)
Pierre-Olivier Strale (pierre-olivier.strale@u-bordeaux.fr)
Irati Romero-Garmendia (irati.romero@ibgc.cnrs.fr)
Andreas Bikfalvi (andreas.bikfalvi@u-bordeaux.fr)
Vincent Studer (vincent.studer@u-bordeaux.fr)

Corresponding author:

Thomas Daubon (thomas.daubon@u-bordeaux.fr)

KEYWORDS:

glioblastoma, patient-derived cell, spheroid, invasion, neurons, advanced in vitro models

SUMMARY:

Here, we present an easy-to-use co-culture assay to analyze glioblastoma (GBM) migration on patterned neurons. We developed a macro in FiJi software for easy quantification of GBM cell migration on neurons, and observed that neurons modify GBM cell invasive capacity.

ABSTRACT:

Glioblastomas (GBMs), grade IV malignant gliomas, are one of the deadliest types of human cancer because of their aggressive characteristics. Despite significant advances in the genetics of these tumors, how GBM cells invade the healthy brain parenchyma is not well understood. Notably, it has been shown that GBM cells invade the peritumoral space via different routes; the main interest of this paper is the route along white matter tracts (WMTs). The interactions of tumor cells with the peritumoral nervous cell components are not well characterized. Herein, a method has been described that evaluates the impact of neurons on GBM cell invasion. This paper presents an advanced co-culture in vitro assay that mimics WMT invasion by analyzing the migration of GBM stem-like cells on neurons. The behavior of GBM cells in the presence of neurons is monitored by using an automated tracking procedure with open-source and free-access software. This method is useful for many applications, in particular, for functional and mechanistic studies as well as for analyzing the effects of pharmacological agents that can block GBM cell migration on neurons.

INTRODUCTION:

Primary malignant gliomas, including GBMs, are devastating tumors, with a medium survival rate of 12 to 15 months reported for GBM patients. Current therapy relies on large tumor mass resection and chemotherapy coupled with radiotherapy, which only extends the survival rate by few months. Therapeutic failures are intimately related to poor drug delivery across the blood-brain barrier (BBB) and to invasive growth in perivascular spaces, meninges, and along WMTs[1]. Perivascular invasion, also called vascular co-option, is a well-studied process, and the molecular mechanisms are beginning to be elucidated; however, the process of GBM cell invasion along WMTs is not well understood. Tumor cells migrate into the healthy brain along Scherer's secondary structures[2]. Indeed, almost one century ago, Hans-Joachim Scherer described the invasive routes of GBM, which are now referred to as perineuronal satellitosis, perivascular satellitosis, subpial spread, and invasion along the WMT (**Figure 1A**).

Some chemokines and their receptors, such as stromal cell-derived factor-1α (SDF1α) and C-X-C motif chemokine receptor 4 (CXCR4), but not vascular endothelial growth factor (VEGF), seem to be implicated in WMT invasion[3]. More recently, a transcellular NOTCH1–SOX2 axis has been shown to be an important pathway in WMT invasion of GBM cells[4]. The authors described how GBM stem-like cells invade the brain parenchyma on partially unmyelinated neurons, suggesting the destruction of myelin sheaths by GBM cells. A milestone was reached in 2019 when three articles were consecutively published in the journal, *Nature*, underlining the role of electrical activity in glioma development[5,6]. Seminal work by Monje and collaborators shed light on the central role of electric activity in the secretion of neuroligin-3, which promotes glioma development.

Winkler and collaborators described connections between GBM cells (microtubes) being crucial in invasive steps, and lately, interactions between GBM cells and neurons via newly described neuroglioma synapses. Those structures favor glutamatergic stimulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors located at the GBM cell membrane, which promotes tumor development and invasion. Tumor cell invasion is a central process in the dissemination of metastases or distant secondary foci, as observed in GBM patients. Several factors have been identified to be important in GBM invasion such as thrombospondin-1, a transforming growth factor beta (TGFβ-regulated matricellular protein, or the chemokine receptor CXCR3[7,8].

Here, a simplified biomimetic model has been described for studying GBM invasion, in which neurons are patterned on tracks of laminin, and GBM cells are seeded onto it, as single-cells or as spheroids (**Figure 1B**). The two experimental settings are aimed at recapitulating invasion on neurons, which is observed in GBM[9,10]. Such models have been developed in the past as aligned nanofiber biomaterials (core-shell electrospinning) that allow studying cell migration by modulating mechanical or chemical properties[11]. The co-culture model described in this article allows a better understanding of how GBM cells escape on neurons by defining new molecular pathways involved in this process.

PROTOCOL:

Informed written consent was obtained from all patients (from the Haukeland Hospital, Bergen, Norway, according to local ethics committee regulations). This protocol follows the

guidelines of Bordeaux University human and animal research ethics committees. Pregnant rats were housed and treated in the animal facility of Bordeaux University. Euthanasia of an E18-timed pregnant rat was performed by using CO_2. All animal procedures have been done according to the institutional guidelines and approved by the local ethics committee. All commercial products are referenced in the **Table of Materials**.

1. Preparation of the patterned slides

1.1. Substrate preparation for micropatterning

1.1.1. Treat 18 mm circular glass coverslips by air/plasma activation for 5 min. Place the coverslips in a closed chamber with 100 µL of (3-aminopropyl) triethoxysilane in a desiccator for 1 h.

1.1.2. Incubate with 100 mg/mL of poly (ethylene glycol)-succinimidyl valerate (molecular weight 5,000 (Peg-SVA)) in 10 mM carbonate buffer, pH > 8, for 1 h. Rinse extensively with ultrapure water, and dry under a chemical hood.

NOTE: At this stage, the sample can be stored at 4 °C in the dark for further use.

1.1.3. Add the photoinitiator, 4-benzoylbenzyl-trimethylammonium chloride (PLPP), at 14.7 mg/mL in phosphate-buffered saline (PBS).

NOTE: A concentrated form of PLPP, a PLPP gel, can also be used. It results in a shorter ultraviolet (UV) illumination time required to degrade the PEG brush (100 mJ/mm^2).

1.2. Photoinitiator gel deposition

1.2.1. Prepare a mixture of 3 µL of PLPP gel and 50 µL of absolute ethanol to deposit in the center of the slide. Place the sample under a chemical hood until complete evaporation of the absolute ethanol.

NOTE: At this stage, the sample can be stored at 4 °C in the dark for further use.

1.3. Glass slide micropatterning

1.3.1. Mount the coverslip in a Ludin chamber, and place it on the motorized stage of a microscope equipped with an auto-focus system.

1.3.2. Load images corresponding to the envisioned micropatterns into the software. Apply these parameters: replication 4 x 4 times, spacing of 200 µm, UV dose of 1,000 mJ/mm^2. After the automatic UV-illumination sequence, rinse the PLPP away with multiple PBS washes.

NOTE: If PLPP gel was used, remove it by extensive washes with deionized water, dry in a stream of N_2, and store at 4 °C.

1.3.3. Incubate with laminin (50 µg/mL in PBS) for 30 min. Wash extensively with PBS.

NOTE: A fluorescent solution of purified green fluorescent protein (GFP, 10 µg/mL in PBS) can be mixed with laminin to visualize the micropatterns by fluorescence microscopy.

2. Preparation of neurons and GBM cells for co-culture

2.1. Culture of embryonic rat hippocampal neurons

2.1.1. Dissect the hippocampus of embryonic (E18) rats, and transfer the tissue into a Hank's balanced salt solution (HBSS)/1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/penicillin-streptomycin solution in a 15 mL tube. Remove excess solution without drying the hippocampus.

2.1.2. Add 5 mL of trypsin-ethylenediamine tetraacetic acid (EDTA) supplemented with penicillin (10,000 units/mL)/streptomycin (10,000 µg/mL) and 1 mM HEPES, and incubate for 15 min at 37 °C. Wash 2x with the HBSS/HEPES/penicillin-streptomycin solution, and let the tissue remain in this solution for 2–3 min.

2.1.3. Dissociate the tissue using two flame-polished Pasteur pipettes, by pipetting up and down 10x with each tissue, taking care to minimize foaming. Count the cells, and evaluate the viability of the cell suspension. Plate the neurons on micropatterned coverslips as indicated below.

NOTE: Cell viability rate is 85–90% after extraction.

2.2. Cell culture for neurons on micropatterned coverslips

2.2.1. Rehydrate micropatterned glass slides with PBS, and incubate them in a solution of laminin (50 µg/mL) for 30 min.

2.2.2. Seed the hippocampal neurons obtained from E18 Sprague-Dawley rats directly over the micropatterned glass coverslip at a density of 50,000 cells per cm^2 in neurobasal medium (NBM) enriched with 3% horse serum. Place the micropatterned neurons in the incubator (37 °C, 5% CO_2) for 48 h.

NOTE: After ~6 h, primary hippocampal neurons can be seen adhering to the laminin micropatterns.

2.3. Co-culture of human GBM stem-like cells on neurons

NOTE: For this study, membrane GFP-positive and nuclear-tomato, patient-derived GBM cells were grown according to previous published protocols[10].

2.3.1. As the spheroid-shaped cells grow in suspension, centrifuge the suspension for 5 min at 200 × *g*. Wash the spheroids with 5 mL of PBS, and incubate the cells with 0.5 mL of the cell dissociation reagent (see the **Table of Materials**) for 5 min at 37 °C.

2.3.2. Add 4.5 mL of complete NBM (complemented with B27 supplement, heparin, fibroblast growth factor 2, penicillin, and streptomycin, as described previously[8]), and count the cells using an automatic counting technique.

2.3.3. Seed 1,000 GBM cells over the micropatterned neuronal culture in NBM enriched with 3% horse serum. Incubate the plate at 37 °C, 5% CO_2, and 95% humidity.

3. Live cell imaging

3.1. Immediately after GBM cell seeding, place the sample on the stage of an inverted microscope equipped with a thermostat chamber. Using the appropriate microscopy software plugin, acquire brightfield and fluorescence images every 2 min at 16 different positions.

3.2. Perform live-cell imaging on an inverted microscope equipped with a motorized stage for recording multiple positions by using a multidimensional acquisitions toolbox in the software. Acquire brightfield and epifluorescence GFP/Tomato images every minute over 12 h with a 20x objective in a temperature (37 °C) and gas-controlled (5% CO_2) environment.

4. Image analysis

NOTE: Using Fiji, two-dimensional (2D) images stack were semi-automatically preprocessed or processed by using a handmade and user-friendly tool (available at this address: https://github.com/Guyon-J/Coculture_Gliomas-Neurons/blob/main/README.md), which is written in IJ1 macro language (**Figure 2A**). The automated workflow and procedures are summarized in **Figure 2B**.

4.1. Neuronal network analysis (**Figure 2Bi**)

4.1.1. Select one image of the stack. Right-click on the **Network** tool to open the corresponding **Options** dialog box and adjust the settings (e.g., **Threshold** = Triangle, Li, Huang…, **Gaussian Blur**, and **Median** filters = 1, 2, 3…) to produce a precise segmentation of images. Then, click on **OK**.

4.1.2. Left-click on the **Network** tool to automatically activate the following procedure.

4.1.2.1. Duplicate the selected image, and **split** it into three color channels (Red–Grey–Green).

4.1.2.2. Select the grey channel (brightfield), and perform **contrast stretch enhancement** (CSE) to enhance the separation between different areas. Use the **Sobel edge detector** (SED) to perform the 2D signal processing convolution operation already grouped under the **Find Edge** command.

4.1.2.3. For **double-filtering** (F), apply a Gaussian blur and median filter to reduce noise and smooth the object signal. **Convert to Mask** (CM) by executing adapted threshold algorithms to obtain a binary picture (BIN-grey) with black pixels (cell area) and white pixels

(background). **Skeletonize** (Sk) the cell area into a simple network (NET), and **filter particles** (EP) in a NET image by removing small, non-networked particles.

4.1.2.4. For red and green channels (nucleus and membrane), perform **double-filtering**, **convert to Mask** using the adapted thresholding method, and allow BIN-green to determine cell morphology with the **Analyze Particles**… command.

4.1.2.5. Merge all channels using their region of interest (ROI) with the **OR** (combine) operator, and readjust their initial color into a simple RGB image.

4.2. Single-cell motility analysis (**Figure 2Bii**)

4.2.1. Right-click on the **Single Cell Tracking** tool to open the corresponding **Options** dialog box, and adjust the settings (e.g., **Trail type** = Nucleus or Membrane, **Threshold** = Triangle, Li, Huang…, **Z projection** = Max intensity, Sum Slices…, **Gaussian Blur** and **Median** filters = 1, 2, 3…) to produce a precise segmentation of images. Then, click on **OK**.

4.2.2. Left-click on the **Single Cell Tracking** tool to automatically activate the following procedure.

4.2.2.1. Remove the grey channel. Apply a **Z projection** on the stack, which will generate an image corresponding to an image stack according to the time (T-stack). **Double-filter** and **convert to Mask** the trails left by cells. Remove small particles to the BIN-red/green image.

4.2.2.2. By using ROI, select each contour of the cell trace, and check the box **Skip edge detection** in the **Options** dialog box to skip this preprocessing step for subsequent steps.

4.2.2.3. **Isolate** the red channel (**Trail type** = Nucleus) on the original stack. Select one ROI and remove the outer area. **Double-filter** all images and **convert to Mask** (BIN-red). Determine the centroid X/Y position of each binarized nucleus.

4.2.3. Using an already published macro for spreadsheet software[12], calculate the mean square displacement, directionality ratio, and average speed for this cell.

4.3. Multiple-cell tracking analysis (**Figure 2Biii**)

4.3.1. Right-click on the **Tracking** tool to open the corresponding **Options** dialog box and adjust the settings (e.g., **Threshold** = Triangle, Li, Huang…, **Gaussian Blur** and **Median** filters = 1, 2, 3…) to produce a precise segmentation of images. Then, click on **OK**.

4.3.2. Left-click on the **Tracking** tool to automatically activate the following procedure.

4.3.2.1. Remove the grey channel.

4.3.2.2. **Split** the red and green channels, **double-filter**, and **convert to Mask**.

4.3.2.3. Merge the channels using **Image Calculator…** command with the **AND** operator, leaving only the nucleus signal found in the membranes.

NOTE: Several plugins in Fiji can be used to determine the X/Y position of several cells in this binary preprocessed image at the same time (see the **Table of Materials**).

4.3.3. Using a previously described macro[12], calculate the trajectory plot, mean square displacement, directionality ratio, and average speed for these cells.

4.4. Spheroid migration on the neural mat (**Figure 2Biv**)

4.4.1. Right-click on the **Migration** tool to open the corresponding **Options** dialog box and adjust the settings (e.g., **Threshold** = Triangle, Li, Huang…, **Gaussian Blur** and **Median** filters = 1, 2, 3…) to produce a precise segmentation of images. Then, click on **OK**.

4.4.2. Left-click on the **Migration** tool to automatically activate the following procedure.

4.4.2.1. Remove the red channel.

4.4.2.2. **Split** the green and grey channels.

4.4.2.3. For the grey channel, **draw** manually the contour of the neuronal mat, and measure its area.

4.4.2.4. For the green channel, **double-filter** the stack and **convert to Mask**. Remove the area outside the pattern (BIN), and determine the binarized cell area for each image.

NOTE: Parameters described above are calibrated by changing their values by left-clicking on the icon of interest. This processing can be done manually. However, for a large number of images (approximately a hundred per acquisitions), channels (generally 3 channels), and processing steps, an automated or semi-automated tool would be preferable.

REPRESENTATIVE RESULTS:

Patterned neurons co-cultured with fluorescent GBM cells were prepared as described in the protocol section, and tracking experiments were performed. GBM cells quickly modified their shape while migrating on the neurons (**Figure 1B: panel 6** and **Video 1**). Cells migrated along the neuronal extensions, in a random motion (**Video 1**). Fluorescent GBM cells and non-fluorescent neurons can be easily distinguished, and this allowed the tracking of cell movements by using the FiJi macro, as described in the protocol section (**Figure 2**). Fiji is an open license software that facilitates image processing and analysis. Manual procedures that are relatively time-consuming for the analysis of numerous images can be automatized in a macro. Images are imported by drag-and-drop procedure, processed, and quantified, generating data which are available using an ROI manager.

When deposited in the **Fiji.app | macros | toolsets** folder, the **Gliomas-Neurons** tool was available in the **More Tools** menu and displayed several icons (**Figure 2A**). A workflow of the image processing, obtained by clicking on the icons, is illustrated in **Figure 2B (i–iv)**. Cell shape

can be analyzed on the neural network (**Figure 2B, i**). Several parameters from cell tracking can be obtained for one (**Figure 2B, ii**) or several cells (**Figure 2B, iii**) by using two distinct image processes. The speed of recovery by spheroid GBM cells on the neurons can be also analyzed (**Figure 2B, iv**). Cells seeded onto neurons displayed an elongated shape with multiple protrusions following neuron tracts (**Figure 3A, i**), but had a round shape when cultured directly on laminin (**Figure 3A, ii**). Cells cultured on neurons efficiently modified their shape, although they were not elongated when cultured on laminin (**Figure 3A, iii–v**). Thin protrusions, sometimes linking two cells, were seen in cells co-cultured with neurons at later stages (**Video 1**).

The migratory capacity of GBM cells seeded on neurons was compared to cells directly seeded on laminin. Cells seeded on neurons had greater migratory capacities than on laminin alone (**Figure 3B, i,ii**). Random movement of P3 cells was detected in both conditions, with greater distance for P3 on the neurons, as shown in the trajectory plot (**Figure 3B, i,ii**). Cell motility was quantitatively estimated by the mean square displacement (MSD), and its log representation was fitted with a linear function[13] (**Figure 3B, iii**). Directionality and average speed were also calculated for both conditions (**Figure 3B, iv,v**). Cell migration of P3 spheroids was also followed by detecting the fluorescent area over time on the neurons and compared with migration on laminin alone (**Figure 3C, i,ii** and **Video 2)**. Half of the pattern was covered with GBM cells after 500 min in a linear profile; however, spheroids did not adhere to the laminin pattern (**Figure 3D, iii** and **Video 2**).

FIGURE LEGENDS:

Figure 1: Experimental setup of glioblastoma cells migrating on patterned neurons.
(**A**) Representation of glioblastoma cells invading the contralateral hemisphere through the corpus callosum. (**B**) Experimental setup. In step 1, the plate surface is coated with an antifouling PEG layer. The photoinitiator is added in step 2, covering the entire coating. In step 3, a UV widefield image is projected through the objective of the microscope, which locally activates the photoinitiator molecules. The activated photoinitiator locally cleaves PEG molecules and allows the subsequent adsorption of laminin. In step 5, neurons are seeded and adhere on the laminin arrays. P3 (GFP/Tomato$_{nuclear}$) glioblastoma cells are then deposited on the neuronal pattern, and images are acquired (step 6). Abbreviations: PEG = polyethylene glycol; UV = ultraviolet; GFP = green fluorescent protein.

Figure 2: Fiji tool presentation and analysis workflow.
(**A**) The tool called **Gliomas-Neurons** is available in the **More Tools** menu when the macro is added to **FiJi.app folder | macros | toolsets** (left panel). It is composed of several action tools described below (right panel). (**B**) **i.** Network tool: image processing, which is used to draw the neural network and glioma cells in a simplified representation. **ii.** Single-cell tracking tool: image processing, which is used to draw and select the cell movement area for analyzing the displacement of single cells. **iii.** Tracking tool: image preprocessing steps for the use of pre-installed Fiji tracking plugins. **iv.** Relative migration tool: image processing, which is used to determine the relative cell migration on a manually selected pattern. Some parameters can be calibrated with a left click on the tool button. Abbreviations: CSE = Contrast Stretch Enhancement; SED = Sobel Edge Detector; F = double Filtering; CM = Convert to Mask; Sk =

Skeletonize; EP = Elimination of particles; OR (combine) = Union operator on selected images ROIs; AND = Conjunction operator on selected images.

Figure 3: Comparison of P3 cells or spheroids on patterned neurons vs. laminin coating.
(**A**) Shape descriptors of dissociated P3 GFP/Tomato$_{nuclear}$ cells on neurons or on laminin. Example of processed networks of P3 cells on **i**. patterned neurons or on **ii**. laminin coating. Scale bar = 50 µm. **iii.** Average cell area on patterned neurons or on laminin. **iv.** Formfactor is the ratio of the circumference to the area normalized to a circle, providing parameters on cell elongation and cell branching. **v.** Aspect ratio is the ratio of the major axis to the minor axis of the cell. In **iii**, **iv**, and **v**, 15 cells were analyzed per field; 4 independent patterns; data are represented as mean ± S.E.M. (**B**) Tracking analysis of dissociated P3 cells. One representative chart plot of cells on (**i**) patterned neurons and (**ii**) on laminin coating. **iii.** MSD of P3 cells migrating on neurons or on laminin coating. X/Y values are in logarithmic scales. **iv.** Directionality ratio. **v.** Average cell speed migration. In **iii**, **iv**, and **v**, 15 cells were analyzed per field; 4 independent patterns; data are represented as mean ± S.E.M. (**C**) Migration analysis of P3 GFP spheroids. Representative images at different time points of P3 spheroids on (**i**) patterned neurons or on (**ii**) laminin coating. Scale bar = 100 µm. **iii.** Spheroid migration represented by the pattern confluency; 4 independent patterns; data are represented as mean ± S.E.M. Abbreviations: GFP = green fluorescent protein; S.E.M. = standard error of the mean; MSD = Mean Square Displacement.

Video 1: P3 cells on neurons or laminin recorded over 8 h (imaged every 5 min).
The video shows P3 single-cell migration on neurons (left) and on laminin coating (right). Cells expressed green fluorescent protein (GFP, green color) and nuclear Tomato (red color). Bar = 50 µm.

Video 2: P3 spheroids on neurons or laminin recorded over 8 h (imaged every 5 min).
The video shows P3 spheroid migration on neurons (left) and on laminin coating (right). Cells expressed green fluorescent protein (GFP, green color). Bar = 100 µm.

DISCUSSION:
Glioblastomas extensively invade the parenchyma by using different modes: co-option of surrounding blood vessels, interstitial invasion, or invasion on WMTs[17]. This latter mode is not well characterized in the literature because of the difficulty in finding suitable in vitro or in vivo models related to WMT invasion. Here, a simplified model has been proposed in which cultured rodent neurons were patterned on laminin-coated surfaces, and fluorescent GBM stem-like cells were seeded on top of the neurons. A grid-shape pattern was used in this study to improve the analysis of tumor cell attachment, invasion, and proliferation. GBM cells migrated more efficiently on top of the neurons than directly on the matrix, which was laminin in these experiments. The cell shape changed throughout the recording process, and the cell surface area increased at the same time. GBM stem-like cells are likely to be attracted by neuron tracts via the activation of specific signaling pathways (i.e., NOTCH2/SOX2)[4] or secreted factors and signals from the neurons themselves. This system is well-suited to analyze the molecular exchanges between GBM cells and neurons, which may include metabolites, neurotransmitters, or cytokines.

Recently, Venkatesh and collaborators described the formation of a neuroglioma synapse in which glutamate activates its receptor, AMPAR[6]. Are similar processes involved during WMT invasion of GBM cells? This can be investigated with this experimental system using pharmacological inhibition or genetic approaches. The following critical points should be noted. First, during the PEGylation steps, the substrate should not be allowed to dry out to avoid affecting the integrity of the anti-adhesive coating. Of note, it is possible to extensively wash the PEG-SVA solution with ultrapure water and to dry it out under a stream of nitrogen before storing it at 4 °C in the dark. Second, the macro developed for analyzing GBM cell migration on neurons is in an open-access mode and is compatible with FiJi software. Although this macro and its updates are available on GitHub, it requires an appropriate calibration for detecting cells. Hence, it may be useful to check the samples manually as a quality control while starting the analysis. The flexibility of the system used here allows different shapes of the pattern, with parallel lines separating the neurons to different extents. With this approach, the shape of several cerebral structures can be mimicked, as observed in the corpus callosum—the largest white matter structure in human brain—where WMT invasion is mainly observed. Alternatively, the same UV-light projection apparatus has been shown to structure UV-sensitive non-adhesive hydrogels in a z-controlled manner[14], allowing the standardization of spheroid formation.

In this context, 3D neurospheres could be generated to test GBM invasion. This technique can be also applied for patterning other cerebral cells, such as brain endothelial cells, to reproduce vessel-like shape or mimic microglial or other immune cells. Thus, synergistic or inhibitory effects of cerebral cells can be observed when co-cultured with GBM cells. One limitation of this study is the use of embryonic rat neurons in co-culture with human GBM cells, which may not mimic true physiological conditions. One way to overcome this drawback would be to use human induced pluripotent stem cell-derived neurons to avoid species cross-reactivity[15]. However, GBM cells quickly adhere and efficiently migrate on rat neurons, as shown in these experiments. It has also been demonstrated than rat cerebral cells (Schwann cells) could be efficiently co-cultured with human neurons[16]. Other methods include the use of 3D nanofibers, which offer a good model to study glioma cell migration[11], but limit cell-cell contact as nanofibers are considered non-living structures.

Furthermore, 2D cultures are reductionistic, simplify the observation of cellular processes, and may limit their validity for the in vivo context[10]. Thus, 3D co-cultures of GBM cells and neurons are better representatives of the in vivo situation. Complex brain organoids, such as mini-brains[17], have been used in confrontation-culture invasion assays[18]. The main advantage of the strategy described herein is the reproducibility of the co-culture approach, i.e., the primary neurons are geometrically constrained on size-controlled micropatterns, and the interaction with the injected GBM cells cannot occur elsewhere. Furthermore, the spatial organization of neurons can be tuned because of the versatility of the UV-projection system, allowing for further optimization. Ultimately, the development and validation of such biomimetic approaches could also help in reducing the number of animal models used in biomedical research.

ACKNOWLEDGMENTS:
This work was supported by Fondation ARC 2020, Ligue Contre le Cancer (Comite de la Gironde), ARTC, Plan Cancer 2021, INCA PLBIO. Alveole is supported by Agence Nationale de

la Recherche (Grant Labex BRAIN ANR-10-LABX-43). Joris Guyon is a recipient of fellowship from the Toulouse University Hospital (CHU Toulouse).

DISCLOSURES:

The authors declare that they have no conflicts of interest.

REFERENCES:

1. Shergalis, A., Bankhead, A., Luesakul, U., Muangsin, N., Neamati, N. Current challenges and opportunities in treating glioblastoma. *Pharmacology Reviews.* **70**, 412–445 (2018).
2. Scherer, H. J. The forms of growth in gliomas and their practical significance. *Brain.* **63**, 1–35 (1940).
3. Zagzag, D. et al. Hypoxia- and vascular endothelial growth factor-induced stromal cell-derived factor-1α/CXCR4 expression in glioblastomas. *American Journal of Pathology.* **173**, 545–560 (2008).
4. Wang, J. et al. Invasion of white matter tracts by glioma stem cells is regulated by a NOTCH1–SOX2 positive-feedback loop. *Nature Neurosciences.* **22**, 91–105 (2019).
5. Venkataramani, V. et al. Glutamatergic synaptic input to glioma cells drives brain tumour progression. *Nature.* **573**, 532–538 (2019).
6. Venkatesh, H. S. et al. Electrical and synaptic integration of glioma into neural circuits. *Nature.* **573**, 539–545 (2019).
7. Boyé, K. et al. The role of CXCR3/LRP1 cross-talk in the invasion of primary brain tumors. *Nature Communications.* **8**, 1571 (2017).
8. Daubon, T. et al. Deciphering the complex role of thrombospondin-1 in glioblastoma development. *Nature Communications.* **10**, 1146 (2019).
9. Gritsenko, P. G. et al. p120-catenin-dependent collective brain infiltration by glioma cell networks. *Nature Cell Biology.* **22**, 97–107 (2020).
10. Guyon, J. et al. A 3D spheroid model for glioblastoma. *Journal of Visualized Experiments: JoVE.* (158) (2020).
11. Rao, S. S. et al. Mimicking white matter tract topography using core–shell electrospun nanofibers to examine migration of malignant brain tumors. *Biomaterials.* **34**, 5181–5190 (2013).
12. Visweshwaran, S. P., Maritzen, T. A simple 3D cellular chemotaxis assay and analysis workflow suitable for a wide range of migrating cells. *MethodsX.* **6**, 2807–2821 (2019).
13. Qian, H., Sheetz, M. P., Elson, E. L. Single particle tracking. Analysis of diffusion and flow in two-dimensional systems. *Biophysics Journal.* **60**, 910–921 (1991).
14. Pasturel, A., Strale, P.-O., Studer, V. Tailoring common hydrogels into 3D cell culture templates. *Advance Healthcare Materials.* **9**, e2000519 (2020).
15. Dolmetsch, R., Geschwind, D. H. The human brain in a dish: the promise of iPSC-derived neurons. *Cell.* **145**, 831–834 (2011).
16. Clark, A. J. et al. Co-cultures with stem cell-derived human sensory neurons reveal regulators of peripheral myelination. *Brain.* **140**, 898–913 (2017).
17. Linkous, A. et al. Modeling patient-derived glioblastoma with cerebral organoids. *Cell Reports.* **26**, 3203-3211.e5 (2019).
18. Han, M. et al. Interfering with long non-coding RNA MIR22HG processing inhibits glioblastoma progression through suppression of Wnt/β-catenin signalling. *Brain.* **143**, 512–530 (2020).

Figure 1

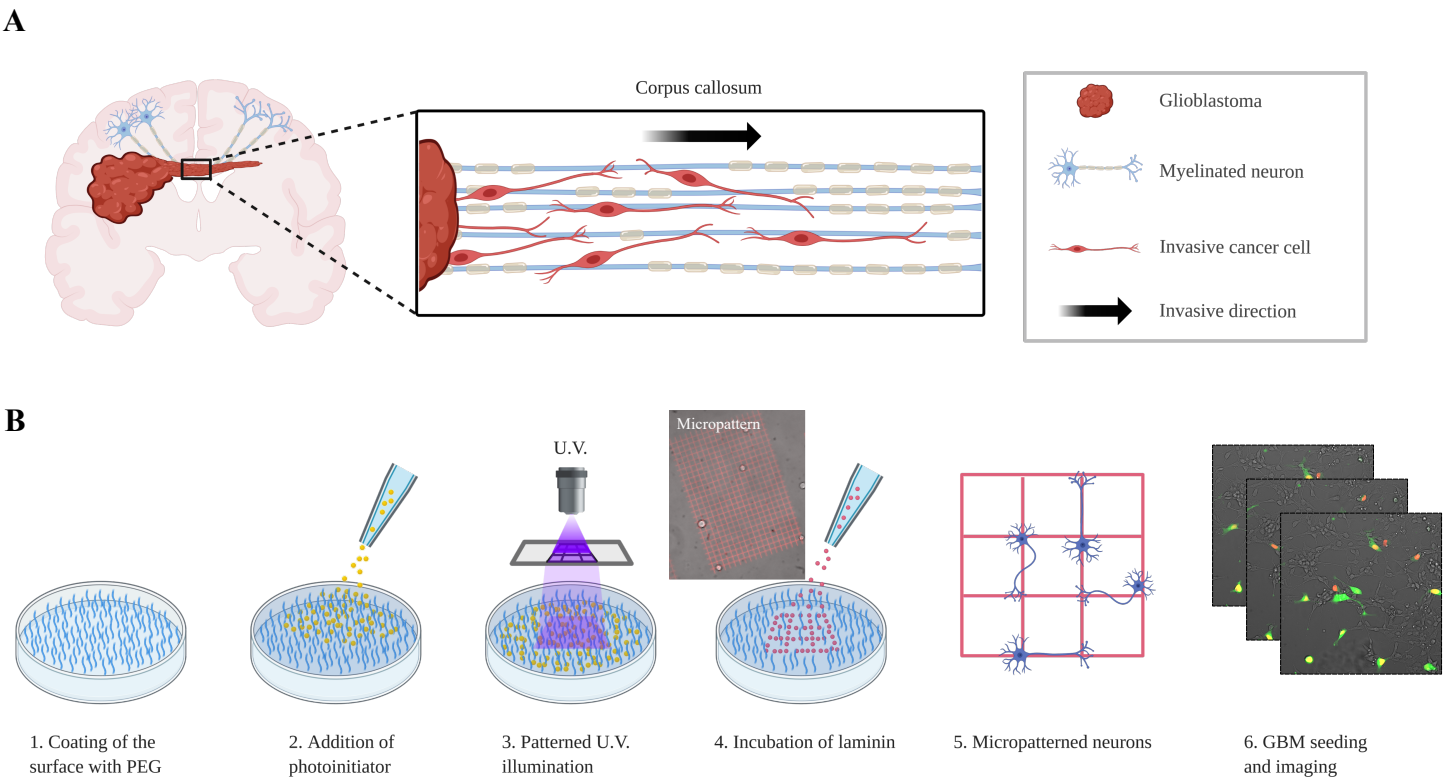


Figure 2

Click here to access/download;Figure;Figure2_pdf.pdf

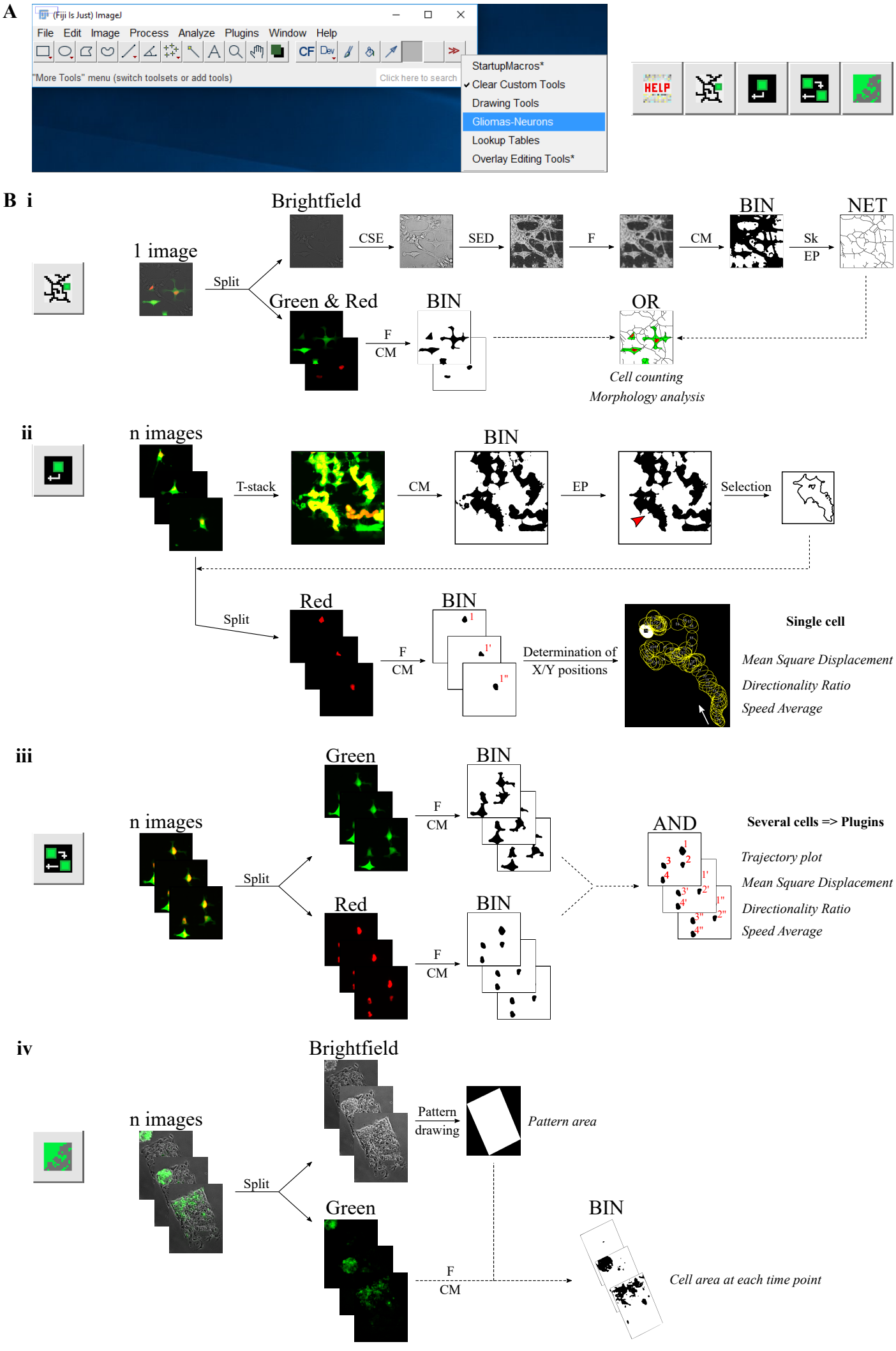
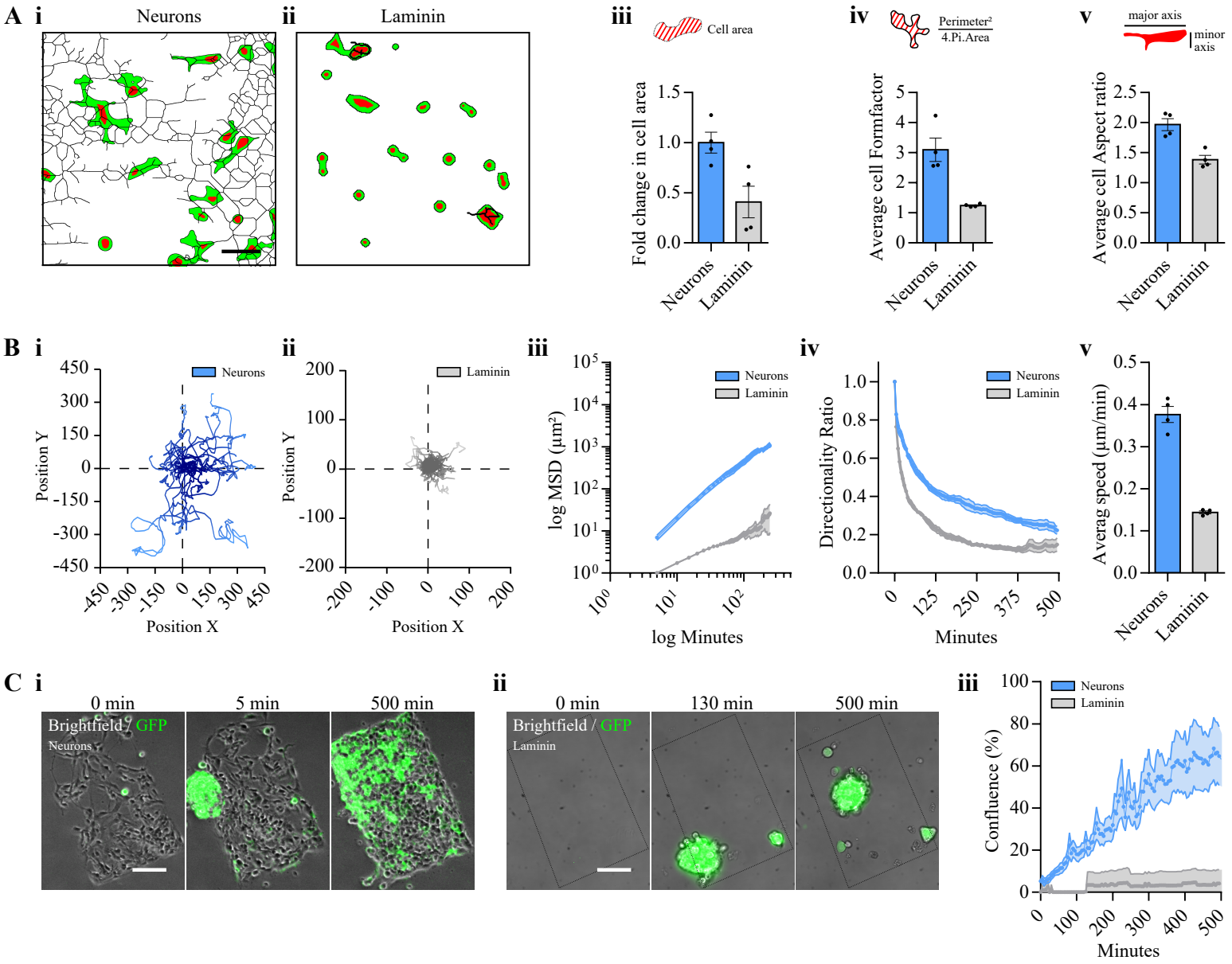


Figure 3

Click here to access/download;Figure;Figure3_pdf.pdf



Movie 1

Click here to access/download
Video or Animated Figure
Dissoc_cells.mp4

Movie 2

Click here to access/download
Video or Animated Figure
Spheroid.mp4

Table of Materials

Click here to access/download;Table of Materials;Table of Materials-revised (2).xlsx

Name	Company	Catalog number
(3-aminopropyl) triethoxysilane	Sigma	440140-100ML
96-well round-bottom plate	Sarstedt	2582624
Accutase	Gibco	A11105-01
B27	Gibco	12587
Basic Fibroblast Growth Factor	Peprotech	100-18B
Countess Cell Counting ChamberSlides	Invitrogen	C10283
Coverslips	Marienfeld	111580
Dessicator cartridges	Sigma	Z363456-6EA
DPBS 10x	Pan Biotech	P04-53-500
Fiji software, MTrack2 macro	ImageJ	
Flask 75 cm^2	Falcon	10497302
HBSS	Sigma	H8264-500ML
Heparin sodium	Sigma	H3149-100KU
Laminin		114956-81-9
Leonardo software		
MetaMorph Software	Molecular Devices LLC	NA
Methylcellulose	Sigma	M0512
Neurobasal medium	Gibco	21103-049
Nikon TiE (S Fluor, 20x/0.75 NA)		
Penicillin - Streptomycin	Gibco	15140-122
PLPP	Alveole	PLPPclassic_1ml
Poly(ethylene glycol)-Succinimidyl Valerate (mPEG-SVA)	Laysan Bio	VA-PEG-VA-5000-5g
PRIMO	Alveole	PRIMO1
Trypan blue 0.4%	ThermoFisher	T10282
Trypsin-EDTA	Sigma	T4049-100ML

Comments
The amino group is useful for the bioconjugation of mPEG-SVA
Used to prepare spheroids
Stored at -20 °C (long-term) or 4 °C (short-term), sphere dissociation enzyme
Stored at -20 °C, defrost before use
Stored at -20 °C, defrost before use
Used to cell counting
Cell culture substrate
Used to reduce mosture during (3-aminopropyl) triethoxysilane treatment
Stored at 4 °C
Used to analyze pictures
Stored at 4 °C
Promotes neuronal adhesion
loading of envisioned micropatterns
Microscopy automation software
Diluted in NBM for a 2% final concentration
Stored at 4 °C
inverted microscope equipped with a motorized stage
Stored at 4 °C
Photoinitiator used to degrade the PEG brush
Used as an anti-fouling coating
Digital micromirror device (DMD)-based UV projection apparatus
Used for cell counting
Used to detach adherent cells

Dear Editor and Reviewers,

Please find our reply to reviewer comments by a point-by-point in the text below.

Regards,

Dr Thomas Daubon

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Reply: This has been done.

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences (not points) between 10-50 words: "Here, we present a protocol to …"

Reply: This has been changed.

3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Reply: This has been changed

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Harrick Plasma, Ithaca, NY, USA; Sigma-Aldrich, Saint-Quentin Fallavier, France; Peg-SVA, Laysan Bio, Arab, AL, USA; MilliQ; Alveole, France; Nikon Eclsipse Ti-E, France; Metamorph Multidimensional acquisitions etc

Reply: Commercial language has been removed from the main text and we generated a table listing all the products used in our study ("Table of Materials and Reagents").

5. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please specify the method of euthanasia to the text without highlighting these euthanasia steps.

Reply: This part of the protocol will not be filmed by your collaborator. This has been added into the text: Euthanasia of an E18 timed pregnant rat was performed by CO2.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Reply: This has been corrected through all text.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. 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. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Reply: This has been checked.

8. Lines 182-185: Even if this will not be part of your video, please provide enough details to allow readers to replicate this procedure or cite a reference in which this has been described.
Reply: Details have been added into the text ("Do live cell imaging on an inverted microscope equipped with a motorized stage (Nikon TiE) for recording multiple positions, by using multidimensional acquisitions toolbox (MetaMorph). Brightfield and epifluorescence GFP images are acquired every minute during 12 hours with a 20X objective (S Fluor, 20X/0.75 NA) under temperature (37°C) and gas-controlled (5% CO_2) environment").

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
Reply: This has been checked and the text has been highlighted up for video.

10. Please include a section "Figure and Table Legends" to follow the representative results section (before the discussion section) and include the figure legends in this legends section.
Reply: This has been added into the text.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
Reply: This has been done.

12. Please include legends (title plus description) for both movies in the Figure and Table Legends section.
Reply: This has been added into the text.

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Reply: These details have been added into the Discussion.

14. Wherever possible, provide volume and issue numbers for all articles, and do not abbreviate the journal names in the reference list.
Reply: This has been corrected.

Reviewers' comments:

Reviewer #1:
In this manuscript, Guyon et al presents a methodology dedicated to explore migration of glioblastoma cells on patterned neurons. This is an interesting topic.
Reply: We thank the reviewer for this positive comment.

Please find below my comments on the manuscript to be addressed.
The manuscript is focused on migration on patterned neurons, while the manuscript uses the general term of white matter track. It might appear overstated to use this term in the context of this presented approach and might be somehow confusing. The authors are invited to clarify.
Reply: We have changed the text accordingly and toned down our description concerning white matter tract invasion.

In general, figure legends are not detailed enough to fully understand the data presented. The authors should improve this section (especially in regards to figure 3).
Reply: Figure legends have been extended and improved.

We have additional remarks and comments to be addressed:
- introduction. please mention that the manuscript concerns mainly primary glioma (and not metastatic brain tumors).
Reply: This has been added into the text.

- lines 83-87. Again, it appears overstated to compare single-cell and spheroid culture to single-cell and collective migratory behavior. This has to be rephrased.
Reply: This has been rephrased by "The two experimental settings are aimed at recapitulating invasion on neurons which is observed in glioblastoma".

- Ethical parts are succinct and might require more details for experimentation related to both human cells and rat embryonic cells.
Reply: Details have been added into the text.

- line 108. "N2 flow". Could the authors clarify this step?
Reply: This has been changed for "chemical hood".

- line 132. Could the authors precise the specie of the recombinant laminin used here?
Reply: The laminin is from Engelbreth-Holm-Swarm murine sarcoma basement membrane.

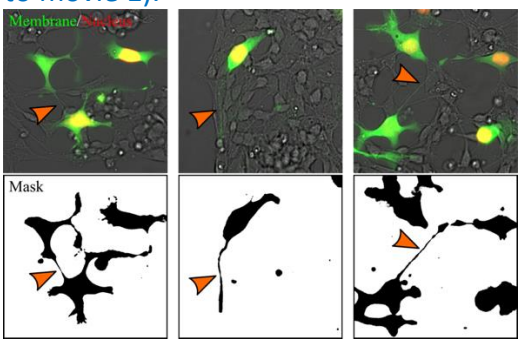
- line 149. Could the authors give an estimated of max death allowed in this assay?
Reply: Details have been added into the text

- line 185. Could the authors clarify 'temperature and gas-controlled environment"?
Reply: Details have been added into the text ("Do live cell imaging on an inverted microscope equipped with a motorized stage (Nikon TiE) for recording multiple positions, by using multidimensional acquisitions toolbox (MetaMorph). Brightfield and epifluorescence GFP images are acquired every minute during 12 hours with a 20X objective (S Fluor, 20X/0.75 NA) under temperature (37°C) and gas-controlled (5% CO_2) environment").

- line 260. This statement is too strong when referring to this figure only. This has to be rephrased and toned down.
Reply: This has been rephrased ("Cells migrated along neuronal extensions, in a random motion").

- line 272. Please clarify how the 93% was calculated (outside the grid or outside neurons).
Reply: The grid corresponds to the neural network obtained by image processing previously described in the Figure 2. When GBM cell overlayed the grid, it was considered on neurons otherwise it was outside neurons. We removed this parameter from the revised manuscript.

- line 273. There is no evidence for microtubes based on the reported assay. Again, this statement is too strong. This has to be rephrased and toned down.
Reply: Microtubes are very fine structures linking two cells, as shown in the images below. We renamed these structures in the text, in accordance to reviewer comment (related now to movie 1).



- line 275-7. Please check figure labeling and clarify "cell 1 versus cell 2".
Reply: This has been removed from our text.

- discussion. The authors are invited to comment on limitations of their model, such as cross-species interaction between human GBM cells and rat embryonic neurons.
Reply: This has been discussed in our manuscript.

- figure legends. In general, the authors are asked to carefully detailed figure legends, acronyms and the way the data are obtained. For instance, what is "formfactor"?
Reply: Figure legends have been improved.
This sentence has been added into the Legends: "Formfactor is the ratio to the circumference to the area normalized to a circle, providing parameters on cell elongation and cell branching". Formfactor action can be easily found in FiJi software.

- Figure 1. It could be nice to see laminin micropattern (without cells).
Reply: This has been added into Figure 1 panel B.

- Figure 3. Define better 'early' versus 'late'.
Reply: These terms have been deleted from the revised manuscript.

As a control, the authors should also provide images of the behavior GBM spheroids on laminin patterns (without neurons).
Reply: This has been added into Figure 3 (replacing cells which were not on neurons) and movie 2

Minor typos
- line 77: cap letter on Invasion
- line 80: matricellular (no dash)
- line 80: check referencing
- line 165: check referencing
- line 183: check brackets
- line 251: these without s

Reply: Thank you for carefully reading our manuscript, we apologized for these typos. This has been corrected.

Reviewer #2:

Manuscript Summary:

This paper describes a protocol to study the migration of glioblastoma stem cells on patterned neuronal cultures. The protocol is well described and would be a useful tool to study the interaction of glioblastoma cells with other cells in the tumor microenvironment. However, there are few concerns that need to be addressed prior to publication.

Reply: We thank the reviewer for these comments.

Major Concerns:

- The authors have not discussed other relevant in vitro models previously employed to mimic white matter (e.g., electrospun nanofibers). This should be included in the introduction.

Reply: This has been discussed into the introduction.

- It looks like the area, speed are in 'px' unit. I suggest they be converted into microns.

Reply: The changes have been made.

- Please include a video of GBM cells on laminin to compare to those seeded on neurons.

Reply: A new video has been added into the manuscript (movies 1 and 2).

Minor Concerns:

- Introduction, Line 55, 56. This should be "only extending the survival rate by few months".
- Introduction, Line 79. This should be "Several factors……..".
- Co-culture of human glioblastoma stem-like cells on neurons, Line 164, 165. Please include references here.

Reply: The changes have been made.
