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Real-time monitoring of human glioma cell migration on DRG axon-oligodendrocyte co-cultures

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Dear Editor,

We are submitting our manuscript entitled: "**Real-time monitoring of human glioma cell migration on DRG axon-oligodendrocyte co-cultures**" and would appreciate it if you would consider it for publication in "JoVE". This is an original study that was not submitted before for publication.

Human glioblastoma is one of the most aggressive and lethal human tumors due to the presence of tumor-propagating glioma stem cells and the highly migratory nature of these cells. Despite the wealth of knowledge regarding the genetic background of this disease, studies on glioma cell migration are hindered by the lack of efficient *in vitro* or *in vivo* migration models.

Here we present a novel approach for the study of human glioma cell migration on myelinated and non-myelinated axons. We developed an *ex vivo* system containing Dorsal Root Ganglion axon-oligodendrocyte co-cultures and then seeded these cultures with patient derived glioma cells. We discovered that glioma cells interact with axonal tracks and migrate along the myelinated and non-myelinated axons thus mimicking the common clinical scenario of tumor infiltration in human glioblastoma. In our co-culture model, we observed that glioma cells interact with neighboring axons through extensive formation of pseudopodia.

On behalf of all authors, I would like to note that there is no conflict of interest to disclose.

Thank you for your time and consideration.

Sincerely,

Nikos Tapinos MD, PhD

TITLE:

Real-Time Monitoring of Human Glioma Cell Migration on Dorsal Root Ganglion Axon-Oligodendrocyte Co-Cultures

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KEYWORDS:

Glioma stem cells, migration, ex vivo model, co-culture, DRG axons, myelin

SUMMARY:

Here we present an ex-vivo mixed monolayer culture system for the study of human glioma cell (hGC) migration in real-time. This model provides the ability to observe interactions between hGCs and both myelinated and non-myelinated axons within a compartmentalized chamber.

ABSTRACT:

Glioblastoma is one of the most aggressive human cancers due to extensive cellular heterogeneity and the migration properties of hGCs. In order to better understand the molecular mechanisms underlying glioma cell migration, an ability to study the interaction between hGCs and axons within the tumor microenvironment is essential. In order to model this cellular interaction, we developed a mixed culture system consisting of hGCs and dorsal root ganglia (DRG) axon-oligodendrocyte co-cultures. DRG cultures were selected because they can be isolated efficiently and can form the long, extensive projections which are ideal for migration studies of this nature. Purified rat oligodendrocytes were then added on purified rat DRG axons and induced to myelinate. After confirming the formation of compact myelin, hGCs were finally added to the co-culture and their interactions with DRG axons and oligodendrocytes was monitored in real-time using time-lapse microscopy. Under these conditions, hGCs form tumor-like aggregate structures that express GFAP and Ki67, migrate along both myelinated and non-myelinated axonal tracks and interact with these axons through the formation of pseudopodia. Our ex vivo co-culture system can be used to identify novel cellular and molecular mechanisms of hGC migration and could potentially be used for in vitro drug efficacy testing.

INTRODUCTION:

Glioblastoma is one of the most aggressive and lethal tumors of the human brain. The current standard of care includes surgical resection of the tumor followed by radiation[1] plus concomitant and adjuvant administration of temozolomide[2]. Even with this multi-therapeutic approach, tumor recurrence is inevitable[3]. This is partly due to the extensive migratory nature of the tumor cells, which invade the brain parenchyma creating multiple finger-like projections within the brain[4] that make complete resection unlikely.

In recent years, it has become evident that the aggressiveness of glioblastoma is due, in part, to the presence of a population of cancer stem cells within the tumor mass[5,6], which exhibit high migratory potential[7,8], resistance to chemotherapy and radiation[9,10] and the ability to form secondary tumors[11]. GSCs are capable of recapitulating original polyclonal tumors when xenografted to nude mice[5].

Despite the wealth of knowledge regarding the genetic background of glioblastomas, studies on glioma cell (GC) migration are currently hindered by a lack of efficient in vitro or in vivo migration models. Notably, while glioma cell-axonal interactions modulated by cellular and environmental factors are a core component of glioma invasion, to our knowledge there is currently no experimental system with the ability to model these interactions[12-14]. To address this deficiency, we developed an ex vivo culture system of primary hGCs co-cultured with purified DRG axon-oligodendrocytes that results in elevated expression of differentiated tumor markers as well as extensive migration and interaction of hGCs with myelinated and non-myelinated fibers. This ex vivo platform, due to its compartmentalized layout, is suitable for testing the effects of novel therapeutics on hGC migration patterns.

PROTOCOL:

The protocols for collection, isolation, and propagation of patient-derived human glioma cells were approved by the IRB committee of Rhode Island Hospital. All animals were maintained according to the NIH Guide for the Care and Use of Laboratory Animals. All animal use protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital.

1. Media and buffer preparations

1.1. Prepare 50 mL of Neurosphere Media: 1x Neuronal basal medium w/o vitamin A, 1x serum free supplement w/o vitamin A, 2 mM L-glutamine, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic-fibroblast growth factor (FGF), 2 µg/mL heparin, and 1x antibiotic-antimycotic (anti-anti).

1.2. Prepare 50 mL of supplemented Neuronal Basal Medium: 1x Neuronal basal medium, 1x serum free supplement, 4 g/L D-glucose, 2 mM L-glutamine, and 50 ng/mL nerve growth factor (NGF).

1.3. Prepare 500 mL of N2B2 Medium: 1:1 Dulbecco's Modified Eagle Medium-Ham's F12

Nutrient Mixture (DMEM-F12), 1x Insulin-Transferrin-Selenium (ITS-G), 66 mg/mL bovine serum albumin (BSA), 0.1 mg/mL transferrin, 0.01 mg/mL biotin, 6.29 mg/mL progesterone, 5 µg/mL N-Acetyl-L-cystine (NAC), and 1 mM putriscine. Aliquot and freeze at -20 °C.

1.4. Prepare 500 mL of C-Medium: 1x Minimum Essential Medium (MEM), D-glucose (final 4 g/L), 10% fetal bovine serum (FBS), and 2 mM L-glutamine. Aliquot and freeze at -20 °C. Add nerve growth factor (NGF) fresh before use (50 ng/mL).

1.5. Prepare 200 mL of Papain Buffer: 1x Earle's Balanced Salt Solution (EBSS), 100 mM Mg_2SO_4, 30% Glucose, 0.25 M EGTA, and 1 M $NaHCO_3$.

1.6. Prepare 10 mL of DMEM-ITS-G Medium: 1x DMEM, 0.5% BSA, and 1x ITS-G.

1.7. Prepare 200 mL of PB Buffer: 1x Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+} and 0.5% BSA. Degas the buffer before use and keep on ice.

2. Isolation and culture of Glioma Stem Cell Neurospheres

2.1. Isolation of Neurospheres

2.1.1. Collect IRB approved and patient consented fresh human glioblastoma (GBM) tissue from the operating room. Transfer GBM samples on ice in DPBS containing 2x anti-anti to a BL2 certified biological safety cabinet.

2.1.2. Transfer one 1 cm^3 GBM sample with minimal necrosis or red blood cell contamination to a 60 mm plate and cut into 1 mm^3 fragments; remove any excess DPBS.

2.1.3. Digest the tissue with 5 mL of collagenase/dispase (1 mg/mL) for 30 min at 37 °C and gently swirl the dish every 5-10 min.

2.1.4. Transfer digested fragments to a 50 mL tube and triturate by pipetting with a 10 mL pipette several times to dissociate the tissue.

2.1.5. Add an equal volume of Neurosphere Media and triturate the tissue again; allowing the large fragments to settle.

2.1.6. Remove media without tissue fragments and pass slowly through a 70 µM cell strainer placed over a fresh 50 mL tube.

2.1.7. Repeat steps 2.1.5-2.1.6 until all the tissue has been dissociated, changing the cell strainer as needed.

2.1.8. Spin the cell suspension at 110 *x g* for 10 min.

2.1.9. Remove the supernatant and resuspend the pellet in 10 mL of ACK lysing buffer to remove red blood cell contamination. Incubate for 10 min at room temperature.

2.1.10. Spin the cell suspension at 110 *x g* for 5 min.

2.1.11. Remove the supernatant and resuspend cells in 10 mL of Neurosphere Media. Take 10 µL of the cell suspension and dilute with 10 µL of trypan blue. Count 10 µL of the cell suspension using an automated cell counter or hemocytometer and plate in 10 mL of Neurosphere Media at a density of 3 x 10^6 cells in 100 mm suspension culture plates.

2.1.12. Add 2 mL of fresh Neurosphere Media to the culture 2-3 times a week.

NOTE: Neurospheres will form in suspension within 3-4 weeks. After subculturing for 2-4 times, confirm the stemness via the limiting dilution assay and tumor recapitulation via xenographic transplantation in immunocompromised mice as previously described [22].

2.2. Sub-culturing Neurospheres

2.2.1. When spheres form and reach a diameter between 200-500 µm, transfer neurospheres to a 15 mL tube and spin at 110 *x g* for 5 min.

2.2.2. Resuspend neurospheres in 1 mL of pre-warmed cell detachment solution.

2.2.3. Transfer to a 1.5 mL tube and incubate at 37 °C for 5 min.

2.2.4. Set a P200 pipette to 150 µL and triturate by pipetting up and down to dissociate neurospheres.

2.2.5. Transfer dissociated cells to a 15 mL tube. Add 4 mL of Neurosphere Media and spin at 300 *x g* for 5 min.

2.2.6. Remove the supernatant and resuspend cells in 5 mL of Neurosphere Media. Take 10 µL of the cell suspension and dilute with 10 µL of trypan blue. Count 10 µL of the cell suspension using an automated cell counter or hemocytometer and plate at a density of 1 x 10^6 cells/60 mm dish in a final volume of 4 mL.

2.2.7. Refresh media 2 times a week and subculture cells as needed. Freeze whole neurospheres when desired in Neuronal basal media w/o vitamin A supplemented with 10% DMSO. Neurospheres may be used for experiments after P4.

3. Compartmented culture of Rat Dorsal Root Ganglia (DRGs), Oligodendrocytes (OPCs) and hGCs

3.1. Preparation of compartmented culture dishes

NOTE: Perform the following steps the days before the planned harvest of the DRGs.

3.1.1. Assemble compartmented culture dishes.

3.1.2. Dilute collagen stock solution to 500 µg/mL in sterile distilled H_2O; mix thoroughly.

3.1.3. With a sterile transfer pipette, fill a 35 mm culture dish with 2 mL of collagen solution; remove the solution, leaving a thin film of collagen behind and place it into the next 35 mm dish. Repeat this process, adding more collagen solution as needed, until all dishes have been coated.

3.1.4. Once all plates are coated, place the plates in a 245 mm x 245 mm culture tray and lay three 1 mm x 1 mm gauze pads in the center of the tray.

3.1.5. To polymerize the collagen, wet gauze pads with 1 mL of concentrated ammonium hydroxide and cover the trays for 15 min.

3.1.6. Remove gauze pads and allow the 35 mm dishes to dry in the laminar flow hood.

3.1.7. While dishes are drying, load the barrel of the syringe grease applicator with high vacuum grease. Place the compartmented chambers in a large mouth media bottle filled with distilled water. Sterilize both by autoclaving and allow to cool.

3.1.8. File off the point of an 18-G needled to make a blunt tip. Sterilize in 70% ethanol. Attach the needle to the grease syringe.

3.1.9. Sterilize the pin rake by soaking in 70% ethanol; allow it to air-dry in the laminar flow hood.

3.1.10. Remove the lid from a dry, collagen coated 35 mm dish. Hold the dish between the thumb and pointer finger. Hold the pin rake with the other hand. Apply a firm pressure to create even 200 µM wide scratches across the center of the dish.

3.1.11. Using a pasture pipette, place two drops of Supplemented Neuronal basal Medium in the center of the scratches.

3.1.12. Repeat steps 3.1.10-3.1.11 until all dishes have been scratched.

3.1.13. Dry the compartmented chambers in a laminar flow hood.

3.1.14. With sterile hemostatic forceps, grasp one compartmented chamber by the center divider. Flip the hemostatic forceps so the bottom of the chamber is facing up.

3.1.15. Apply silicone grease to the compartmented chamber, starting at the top. Ensure that grease is placed neatly and overlaps at all corners.

3.1.16. Remove the lid from a 35 mm dish. Invert the dish and place the scratches over the chamber. Tap down on the bottom of the plate gently with a pair of forceps.

3.1.17. Gently flip the plate over by using the hemostatic forceps. Release the forceps.

3.1.18. Place a mound of grease at the base of the center compartment. Fill each chamber with Supplemented Neuronal Basal Medium (NB) and check for leaks. Seal leaks with silicone grease as needed.

3.1.19. Continue assembling all culture dishes and store overnight at 37°, 5% CO_2.

3.2. Isolation of Rat DRG Neurons and Culture in the Compartmented Chamber

3.2.1. Sacrifice a timed pregnant E16 Sprague-Dawley rat by CO_2 asphyxiation or by chemical overdose.

3.2.2. Place the animal in the supine position on a clean surface; disinfect the abdomen with 70% ethanol.

3.2.3. Grasp the skin of the lower abdomen with forceps and lift gently.

3.2.4. Using scissors, make an "I" incision along the midline of the animal, taking care not to puncture the muscles of the abdominal wall.

3.2.5. With a clean pair of forceps, grasp the muscle wall and make a transverse incision with a clean pair of scissors using caution not to puncture the uterus or intestines.

3.2.6. With a pair of blunt forceps, grasp the uterus and gently lift straight up out of the peritoneal cavity.

3.2.7. Using fresh, sterile scissors, clip the connective tissue at the base of each uterine horn.

3.2.8. Place the entire uterus in a sterile 100 mm tissue culture dish.

3.2.9. Carry the uterus to a laminar flow hood for dissection.

3.2.10. Remove embryos from the uterus, one at a time, by clipping through the amniotic sac and gently teasing the embryo out and into a 60 mm dish containing 5 mL of L-15 with 1x pen-strep.

3.2.11. With fine forceps, place 3-4 embryos into a 60 mm dish containing 5 mL of L-15 with 1x pen-strep.

3.2.12. Working under a dissecting microscope and with one embryo at a time, euthanize by

decapitation.

3.2.13. Lie the embryo ventral side up and remove the limbs and the tail.

3.2.14. With fine forceps, make a midline incision in the animal.

3.2.15. Remove internal organs and tissue to expose the dorsal structures, particularly the spinal cord which should be visible upon completion.

3.2.16. Place one blade of micro-dissecting scissors between the vertebral column and the spinal canal and carefully cut through the vertebral column to expose the spinal cord. Take care not to clip through the spinal cord.

3.2.17. With fine forceps, lightly grasp the rostral end of the spinal cord and slowly lift out of the embryo. The DRGs will be attached to the spinal cord.

3.2.18. Transfer spinal cords and attached DRGs to a 35 mm dish containing 2 ml of L-15 with 1x pen-strep. Place on the ice.

3.2.19. Once all spinal cords have been isolated, use fine forceps to individually pluck DRGs from the spinal cord. Place DRGs into a fresh 35 mm dish.

3.2.20. If nerve roots are present on DRGs, clip them away.

3.2.21. Remove prepared 35 mm dishes from the 37 °C, 5% CO_2 incubator. Remove the media from the previous day. Place 80 µL of Supplemented Neuronal Basal Media (NBF) containing 10 µM 5-Fluoro-2'-deoxyuridine (FUDR) in the center compartment and 250 µL of media in each outer compartment.

3.2.22. Place 2 ganglia in each center compartment. Return dishes to the 37 °C, 5% CO_2 incubator.

3.2.23. The following day add 2.5 mL of NBF.

3.2.24. Feed the cultures following the schedule in **Table 1**, altering the schedule based on the day chosen to begin the DRG prep.

3.2.25. On day 21 (or when axons reach the end of the distal compartments), either seed cultures with a GBM neurosphere (Proceed to step 3.2.26) and live image or myelinate the cultures with oligodendrocytes cultures (Proceed to step 3.3) and then seed with a GBM neurosphere after myelination.

3.2.26. Replace Supplemented NB Medium in each distal compartmented chamber that will be seeded with a GBM neurosphere with Supplemented NB Medium containing 10% FBS.

3.2.27. With a P20 pipette set to 10 µL, remove one GBM neurosphere from the culture dish. The GBM neurosphere should measure approximately 200 microns in size.

3.2.28. Place the tip of the pipette in the distal chamber and expel the GBM neurosphere slowly so that it gently falls onto the axons in the portion of the distal chamber that is closest to the center chamber, over the axons near the center chamber. Be careful not to let the pipette tip disrupt the axons.

3.2.29. Leave the culture in the biosafety cabinet for 1 h at room temperature to allow the GBM neurosphere to attach.

3.2.30. Once the neurosphere has attached, very carefully replace the media in the distal compartment with Supplemented NB Medium.

3.2.31. Live image cultures for 3-7 days, adding media as needed. In this case, a controlled CO_2 live cell enclosure attached to the microscope (e.g., Zeiss Axiovert) was used to continuously monitor cell migration for 7 days using brightfield. Images were acquired every 10 min using the associated software. Repeat the same protocol using hGCs that have been stably transfected to express GFP and images were captured using a combination of brightfield and 488 nm laser.

NOTE: Neurospheres may be transduced with lentivirus or transfected with plasmids or siRNAs. Compartments may also be treated with desired small molecule inhibitors to study migration.

3.3. Myelination of DRG Axons with Oligodendrocytes

3.3.1. The day before oligodendrocyte isolation, replace the NB Medium in the DRGs with C-Medium.

3.3.2. Before dissection, place 10 mL of Papain Buffer into a 60 mm dish in the incubator to equilibrate.

3.3.3. In a laminar flow hood, fill one 100 mm dish and one 60 mm dish with ice cold HBSS. Place on ice.

3.3.4. Sacrifice a P2 rat pup by decapitation. Remove the skin using a pair of scissors. After the skin is removed, cut the skull along the midline with a pair of fine scissors.

3.3.5. Gently remove the skull with fine forceps. Using a spatula, gently scoop the brain from the bottom of the skull and transfer to an inverted tissue culture plate lid.

3.3.6. Remove the cerebellum and divide the cerebrum into two cerebral hemispheres. Remove the olfactory bulbs, hippocampus, and basal ganglia below the cerebral cortex of each hemisphere. Place the cerebral cortex in the 100 mm dish containing HBSS. Repeat steps 3.3.4-3.3.6 for the remaining animals.

3.3.7. Working with one cortex at a time, remove the meninges with fine Dumont forceps. Place all meninges-free cortices into fresh 60 mm dishes with HBSS.

3.3.8. Dice the cortical tissue into 1 mm^3 pieces. Place on ice.

3.3.9. Place equilibrated Papain Buffer into a 15 mL tube. Add 200 units of papain and 2 mg L-cysteine. Filter sterilize and add 200 µL of sterile DNase I.

3.3.10. Remove HBSS from the diced brain tissue and replace with Papain Buffer from 3.3.2. Place dish in 37 °C, 5% CO_2 incubator for 80 min, gently shaking every 15 min.

3.3.11. Transfer the digested tissue to a 50 mL tube and add 2 mL of C-medium.

3.3.12. Triturate with a 5 mL serological pipette to dissociate the tissue; allow larger pieces of tissue to settle. Remove supernatant and place into a sterile 15 mL tube.

3.3.13. Add 2 mL of C-Medium and repeat trituration with a 5 mL serological pipette once more. Switch to a 1 mL pipette tip and triturate until the tissue is completely dissociated. Transfer to the 15 mL tube.

3.3.14. Spin the triturated tissue at 300 *x g* for 15 min. Carefully remove supernatant and resuspend pellet in 8 mL of DMEM-ITS-G Medium.

3.3.15. Pre-wet a sterile 30 µM cell strainer with 2 mL of PB Buffer. Place cell strainer over a 50 mL tube and filter the cell suspension 1 mL at a time. Rinse the filter with 5 mL of PB Buffer.

3.3.16. Transfer the cell suspension to a 100 mm bacteriological plate; incubate for 15 min in a 37 °C, 5% CO_2 incubator to allow microglia to attach.

3.3.17. Remove media and place into a 15 mL tube. Rinse the plate gently with 2 mL of DMEM-ITS-G medium and transfer to the 15 mL tube.

3.3.18. Resuspend the cell suspension gently. Take 10 µL of the cell suspension and dilute with 10 µl of trypan blue. Count 10 µl of the cell suspension using an automated cell counter or hemocytometer.

3.3.19. Spin the cell suspension at 300 *x g* for 10 min.

3.3.20. Aspirate supernatant completely and resuspend cells in 70 µL of PB Buffer for every 1 x 10^7 cells. Mix well and incubate at 4 °C for 10 min.

3.3.21. Add 20 µl of anti-A2B5 microbeads for every 1 x 10^7 cells. Mix well and incubate at 4 °C.

3.3.22. Add 1-2 mL of PB Buffer for every 1 x 10^7 cells and centrifuge at 300 *x g* for 10 min in a 4 °C centrifuge.

3.3.23. Aspirate supernatant completely. Resuspend up to a total of 10^8 cells in 500 µL of PB Buffer. Keep on ice.

3.3.24. Place a magnetic bead column in the magnetic field of a separator.

3.3.25. Prepare the column by rinsing with 500 µL of PB Buffer. Apply the cell suspension to the column. Discard flow-through in a waste container (this contains un-labeled cells).

3.3.26. Wash the column with 500 µL of PB Buffer 3 times. Discard flow-through.

3.3.27. Remove the column from magnetic separator and place in a collection tube.

3.3.28. Pipette 1 mL of PB Buffer onto the column. Flush the magnetically labeled cells by firmly pushing the plunger into the column.

3.3.29. Add 4 mL of C-Medium to the cell fraction and spin at 300 *x g* for 10 min.

3.3.30. Remove supernatant and resuspend in 5 mL of N2B2 Medium. Take 10 µL of the cell suspension and dilute with 10 µL of trypan blue. Count 10 µL of the cell suspension using an automated cell counter or hemocytometer.

3.3.31. Plate oligodendrocytes at a concentration of 150,000 cells per compartmented chamber containing a DRG with fully extended axons.

3.3.32. The following day change the media in the compartmented chamber to N2B2 to allow for myelination. Replace media every 2-3 days. Myelination will be complete in 14 days.

3.3.33. On day 14, seed culture with a GBM neurosphere as in 3.2.26-3.2.32. Maintain myelinated cultures in N2B2 Medium for the duration of any experiments.

NOTE: The protocol is presented as a flow chart schematic in **Figure 1.**

REPRESENTATIVE RESULTS:

In order to study the interaction of hGCs with axons, we generated purified DRG axons as previously described[15-18]. These purified DRG axons were then seeded with hGCs, which formed GFAP+/Ki67+ tumor-like structures integrated within the axonal network, while individual hGCs migrated either in association or between the axons (**Figure 2**). To determine how hGCs interact with myelinated axons, we seeded DRG axon cultures with purified rat oligodendrocytes and induced myelination as previously described[19-21]. Addition of hGCs on the myelinated DRG-oligodendrocyte co-cultures showed that hGCs migrate in association with the myelinated axons

and away from the tumor mass through the formation of pseudopodia as shown in our recent paper (**Figure 3**)[22]. Oligodendrocyte myelination can be determined using Myelin Basic Protein (MBP) staining of the cultures. To quantify the migration of hGCs in these cultures we measured the total area of the culture occupied by the migrating hGCs using Image J software[22].

FIGURE LEGENDS:

Table 1: Feeding schedule of DRG cultures.

Figure 1: Schematic summary of the protocol.

Figure 2: hGCs form tumor-like structures in co-culture with DRG axons. The culture was fixed and stained for the tumor markers GFAP (red) and Ki67 (green), while the axons were stained with Neurofilament (blue). Scale bar: 200 µm. This figure has been modified from our recently published paper[22].

Figure 3: hGCs migrate along myelinated axonal tracks. hGCs expressing the green fluorescent protein (GFP) migrate along myelinated axonal tracks stained red for MBP in the hGC-DRG-oligodendrocyte culture system. Scale bar: 200 µm.

DISCUSSION:

Migration studies for hGCs may be performed by using Boyden chamber systems or scratch assays. However, while these experiments fail to give any information regarding the interactions of tumor cells with other surrounding tissues, the present system can recapitulate GC interactions with myelinated and non-myelinated fibers. Furthermore, to study tumor formation and end-point migration, organotypic slice cultures of the rodent brain or in vivo implantation of glioma cells into the rodent brain or flank have previously been utilized[23-25]. More recent efforts on three-dimensional modeling of glioma cells have utilized systems like collagen layers[26-28], astrocyte-based scaffolds[29,30], extracellular matrix layers[31], electrospun nanofibers[32], and hydrogels[33]. While these experiments produce satisfactory end-point results regarding migration, they lack the ability to be studied in real-time by high-resolution microscopy.

Here, we have demonstrated a new approach to studying the migration of hGCs by preparing a DRG co-culture in a compartmented chamber. If access to fresh GBM tissue is available, hGCs can be isolated and cultured reliably. Although hGCs take a long time to form neurospheres initially, the cultures are easy to maintain and subculture once the spheres form. To ensure the success of the hGC culture, the tissue should be processed as quickly as possible. The time between resection and processing should be minimized as much as possible, and samples should always be transported on ice.

The DRG is also easy to isolate, extends its axons longer than cortical neurons and can be easily myelinated in culture with oligodendrocytes. hGC neurospheres or dissociated hGCs are co-cultured in the distal compartments of the chambers, allowing for live cell imaging and real-time quantification of cellular interactions with axons and myelinated fibers. Additionally, this

protocol is not limited to only DRG cultures, as cortical neurons may also be isolated and myelinated with few modifications to this protocol. This model may also be used to study other forms of brain cancer.

While the DRG is relatively simple to isolate and maintain, the addition of the compartmented chamber is time consuming and creates a multitude of technical limitations that require training and practice to succeed. Critical to the success of this protocol is uniform collagen coating and scratching of the culture dishes because uneven or excessively thick collagen tends to peel. Extreme care should also be taken while placing silicone grease on the compartmented chambers. If even pressure is not used while dispensing the silicone grease, there will be gaps along the bottom of the compartmented chamber that will require sealing with excess grease to prevent media leakage. Additionally, minimum pressure should be used when adhering the compartmented chambers to the culture dish floor. If axons fail to cross out of the middle compartment after week one and the DRG looks otherwise healthy, it is likely that too much pressure was applied when placing the compartmented chamber or an excess amount of grease was used.

hGC migration along myelinated and non-myelinated axonal tracts in the brain has not been well described due to the lack of efficient and reproducible ex vivo models. We describe here the development of an innovative ex vivo culture system to study how glioma cells migrate along axons and myelin, a crucial component in developing new treatments that specifically target tumor cell migration. Our culture system is versatile since there is fluidic isolation between compartments, facilitating the ability to study various novel treatments or differing concentrations of substances that affect glioma cell migration. An additional advantage of our co-culture system is the ability to monitor hGC migration and the effects of the various treatments in real-time. The protocol described here is currently being used as the basis for the development of more sophisticated biomimetic 3-dimensional ex vivo systems using exclusively human cellular components that could be used to assess toxicology and efficacy of novel drugs.

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DISCLOSURES:
The authors have nothing to disclose.

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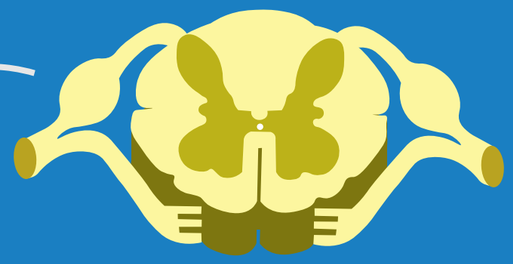
1 DRG isolation

Spinal cord dissection from embryo



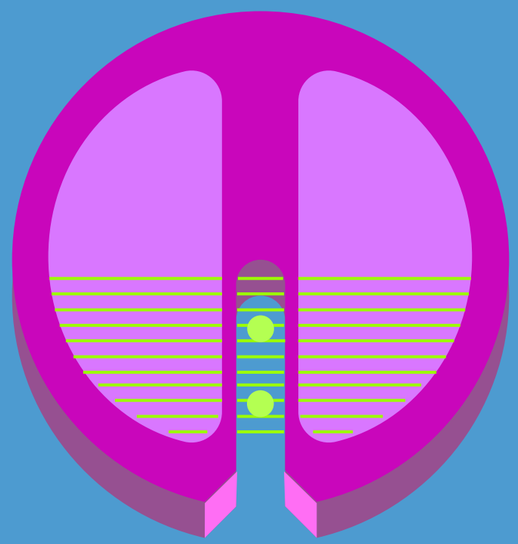
Isolate DRGs from spinal cord

Coat scratched plate with collagen and polymerize

Seed 2 DRGs in central chamber

2 DRG seeding and myelination

Allow axonal growth along scratches
(20 days)



Axon myelination of seeded DRGs

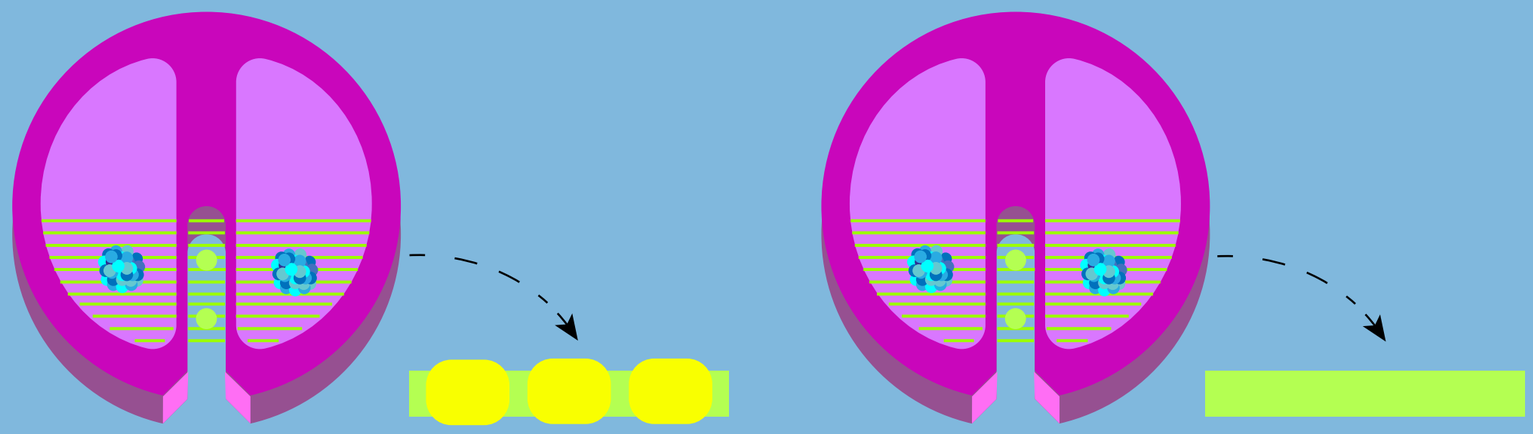
Seed 150,000 oligodendrocytes per chamber to allow for DRG axon myelination
(14 days)

No axon myelination

Seed one neurosphere per myelinated chamber

Seed one neurosphere per unmyelinated chamber

3 Neurosphere seeding on DRG axons



Treat with drug of choice followed by live imaging
(3-7 days)

Figure 2

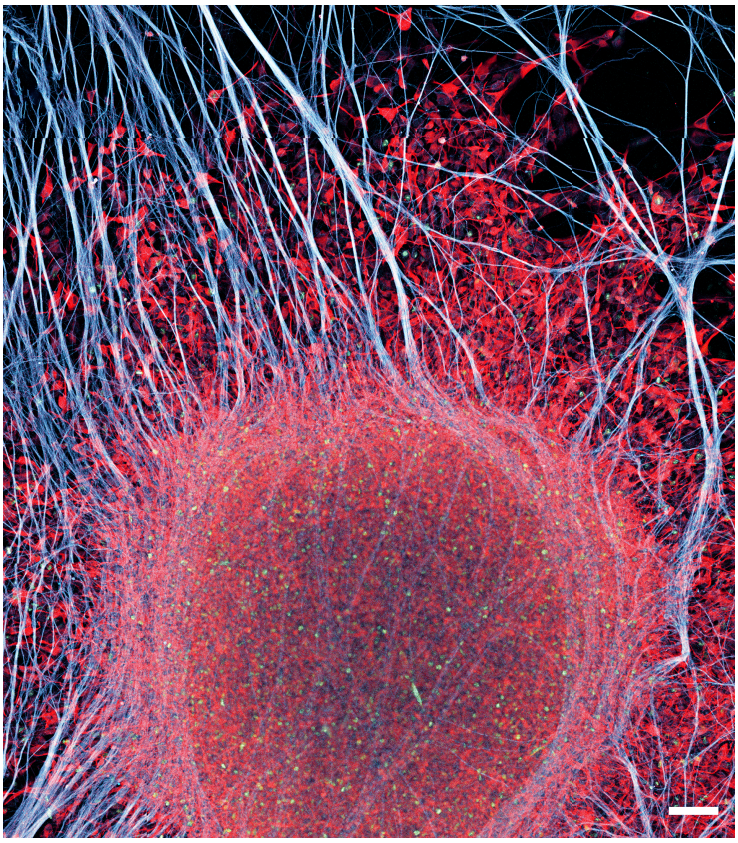


Figure 3

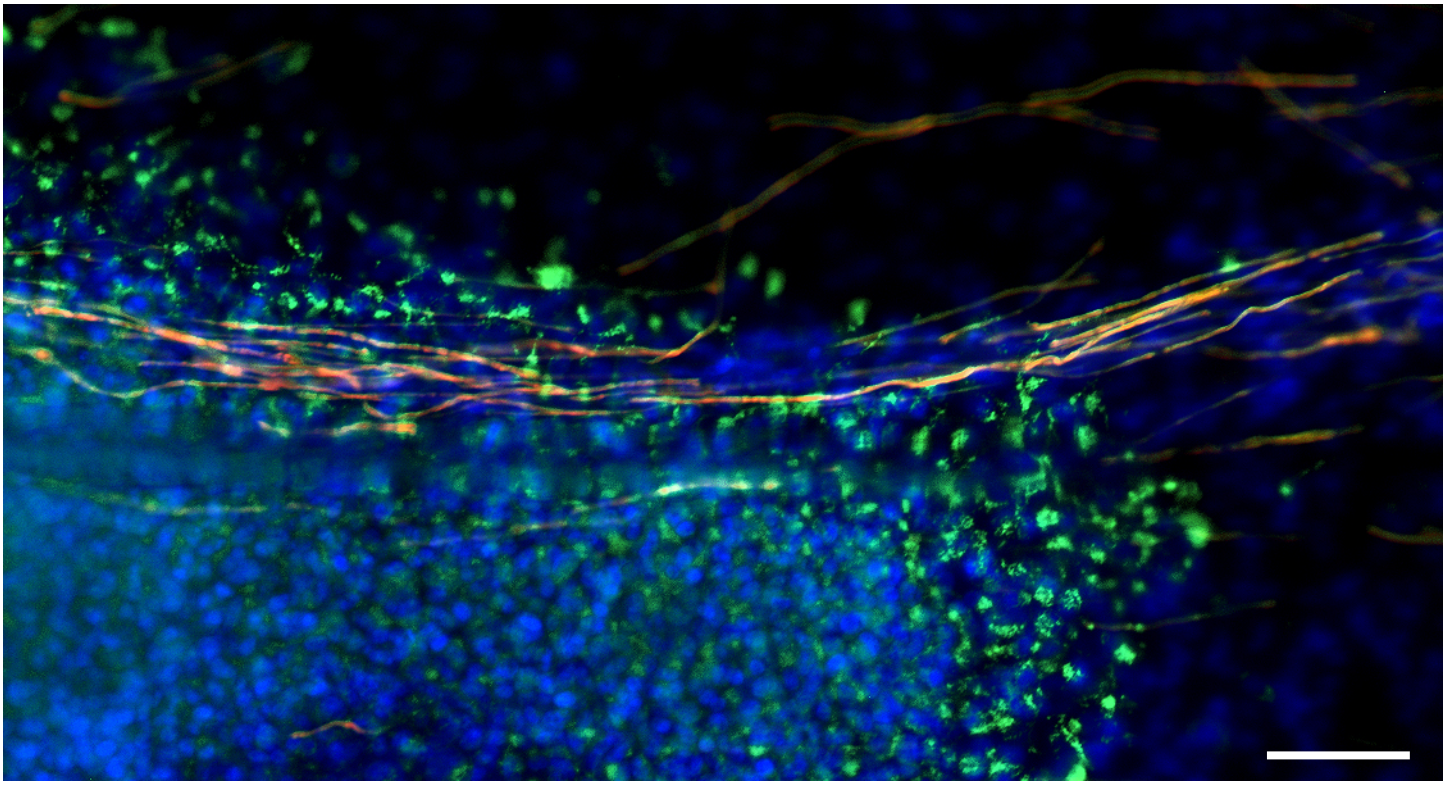


Table 1

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	MONDAY	WEDNESDAY	FRIDAY
DAY 1			NBF
WEEK 1	NB	NBF	NB
WEEK 2	NBF	NB	NB
WEEK 3	NB	NB	

Name of Reagent/Equipment	Company	Catalog Number	Comments/Description
100 mm Suspension Culture Dish	Corning	430591	
2.5S NGF	ENVIGO	B.5025	
60 mm Suspension Culture Dish	Corning	430589	
ACK Lysing Buffer	Thermo Fisher	A1049201	
Ammonium Hydroxide Solution	Fisher Scientific	A669-500	Concentrated
Animal-Free Recombinant Human EGF	Peprotech	AF-100-15	
Animal-Free Recombinant Human FGF-basic (154 a.a.)	Peprotech	AF-100-18B	
Anti-A2B5 MicroBeads, human, mouse, rat	Miltenyi Biotec	130-093-392	
Antibiotic-Antimycotic (100X)	Thermo Fisher	15240062	
AutoMACS Rinsing Solution (PBS, pH 7.2)	Miltenyi Biotec	130-091-222	
B27 Supplement	Thermo Fisher	17504044	
B27 Supplement, minus vitamin A	Thermo Fisher	12587001	
Bacteriological Plate	BD Falcon	351029	
Biotin	Sigma	B4639	
BSA	Sigma	A9418	
Campenot Chamber	Tyler Research	CAMP-10	
Cell Culture Dish	Corning	430165	35mm X 10mm
Cell Strainer	BD Falcon	352350	70 uM, Nylon
Cell Strainer	BD Falcon	352340	30 uM, Nylon
Collagenase/Dispase	Roche	11097113001	
Cultrex Rat Collagen I	Trevigen	3440-100-01	
D-Glucose	Sigma	G5146	
DMEM	Thermo Fisher	10313021	
DNase I	Sigma	D7291	
Dow Corning High-Vacuum Grease	Fisher Scientific	14-635-5D	
Dumont #5 Forceps	Roboz	RS-5045	
E16 Timed Pregnant Sprague Dawley Rat			
EBSS	Sigma	E7510	
EGTA	Sigma	E3889	
FBS	Hyclone	SH30070.02	
FUDR	Sigma	F0503	
GlutaMAX Supplement	Thermo Fisher	35050061	

Ham's F-12 Nutrient Mix	Thermo Fisher	11765054	
HBSS	Thermo Fisher	14175095	
Hemostatic Forceps	Roboz	RS-7035	
Heparin Sodium Salt, 0.2% in PBS	Stem Cell Technologies	07980	
Hypodermic Needle, 18G	BD	511097	
Insulin-Transferrin-Selenium G	Thermo Fisher	41400045	
L-Cysteine	Sigma	C7477	
L-Glutamine	Thermo Fisher	25030081	
Leibovitz's L-15 Medium	Thermo Fisher	11415064	
MACS BSA Stock Solution	Miltenyi Biotec	130-091-376	
MACS MultiStand	Miltenyi Biotec	130-042-303	
MEM	Thermo Fisher	1190081	
Mg_2SO_4	Sigma	M2643	
MiniMACS Separator	Miltenyi Biotec	130-042-102	
MS Columns plus tubes	Miltenyi Biotec	130-041-301	
NAC	Sigma	A8199	
$NaHCO_3$	Sigma	S5761	
Neurobasal Medium	Thermo Fisher	21103049	
Neurobasal-A Medium	Thermo Fisher	10888022	
Ordinary forceps			
P2 Sprague Dawley Rat Pups			
Papain	Worthington	LS003126	
Penicillin-Streptomycin	Thermo Fisher	15140148	
Pin Rake	Tyler Research	CAMP-PR	
Progesterone	Sigma	P8783	
StemPro Accutase Cell Dissociation Reagent	Thermo Fisher	A1110501	
Syrine Grease Applicator	Tyler Research	CAMP-GLSS	
Transferrin	Sigma	T2036	
Uridine	Sigma	U3003	

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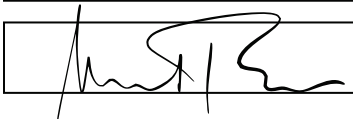
Signature:	
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Dear Editor,

We would like to thank you and the reviewers for the constructive criticism of our paper. We have modified the manuscript in response to the insightful reviewer comments. Furthermore, we have added a flow chart describing our protocol which also includes a schematic representation of the construction of the compartmented chamber.

Here is our point by point response to the reviewer comments:

Reviewer 1

1. English language issues, especially in the abstract

   The manuscript has been copy edited extensively and all English language issues have been resolved.

2. Some typing errors are present in the text (e.g. latin words should be italicized, as "in vitro", and µl should be used instead of ul). Furthermore, acronyms should be defined at first use (e.g. human glioma cells (hGCs) and this acronym should be used throughout the whole text, in the manuscript it appears as hGCs and human GCs…). Also, in page 9, point 3.3.3, it should read "one 100 mm dish and one 60 mm dish" instead of "1 100 mm dish and 1 60 mm dish".

   All abbreviations and acronyms have been defined. Typing errors have been corrected, and numbers have been converted to type.

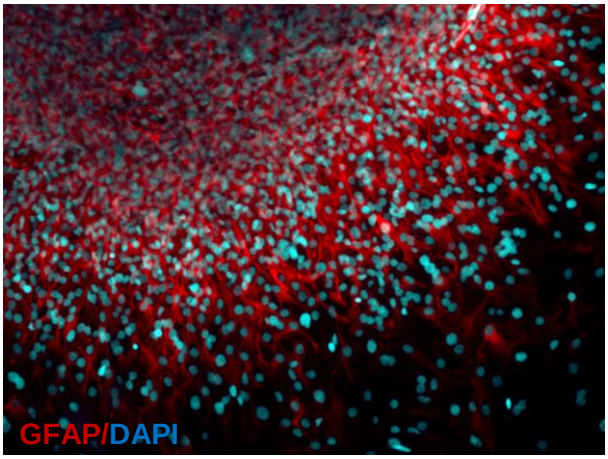
3. Suggest authors to better explain some steps as how they triturate the tissue (page 4, point 2.1.4) and also to provide an abbreviation list

   The process for tissue trituration was expanded upon to include instruction to triturate with a 10 mL pipette. All abbreviations and acronyms are defined in the manuscript as is customary in journal articles, and the authors therefore do not feel as though an abbreviation list will be a beneficial addition to the reader.

4. Authors claim that co-culture of hGCs with DRG axons induce GFAP+/Ki67+ tumor-like structures formation, however, no negative controls are shown in the manuscript. Is GFAP and Ki67 expression due to co-culture with DRG axons or is an artifact of the culture media/conditions? Furthermore, one of the main characteristics of human GBM is heterogeneity. It would be interesting that authors show some examples with different tumor samples. Is this result reproducible in other hGCs? Did they observed the same with other hGC tumor samples?

   GFAP is a known diagnostic marker of glioblastoma (1). Therefore, expression of GFAP would not be due to culture conditions or co-culture, but is an inherent property of

glioblastoma. Ki-67 is a marker of cellular proliferation (2). Therefore, any cell that is proliferating will stain positive for Ki-67. Expression of GFAP and Ki67 in our co-culture system is not an artifact of culture conditions. There is no negative control per se for these experiments. Glioma cell aggregates express differentiation markers like GFAP in the presence of serum containing media and absence of bFGF, EGF or DRG axons (see image below):



The authors agree that glioblastoma is a very heterogeneous disease and frequently isolate tissue from multiple patients to account for patient to patient variation in our experiments. In our 2018 Oncogene manuscript (3), we report that co-culture of hGCs with DRG axons is reproducible with hGCs of two patients, representing different subtypes of glioblastoma according to TCGA classification.

5. Figure 2 has been already published, but no reference in the text is found (see "Regulation of human glioma cell migration, tumor growth, and stemness gene expression using a Lck targeted inhibitor", Oncogene 2018). Furthermore, in this manuscript already published they described the protocol submitted to JOVE; authors should make reference to this manuscript.

   Manuscript reference for "Regulation of human glioma cell migration, tumor growth, and stemness gene expression using a Lck targeted inhibitor" has been added.

6. Authors should also discuss limitations of this technique.

   The technical limitations of this technique were already discussed at length in the discussion.

Reviewer 2

1. If it is possible, I suggest author provide a flow chart summary including the crucial steps and time points of this methods.

   A flow chart summary has been added.

Reviewer 3

1. The title and abstract are appropriate for this article and the method presented seem to have a great potential in biomaterials science, cell biology and cancer research. Hopefully it can be applied in the broader context (not only for migration studies) - can the authors elaborate on that?

   The protocol described here is not just limited to migration studies. This *ex vivo* co-culture system has the potential to be used for drug discovery. The potential future uses of this model have been expanded upon further in the manuscript.

2. Also, can the authors present the quantities obtained with ImageJ analysis of glioma migration? I assume the quantification might be difficult since cells move in quasi 3D environment? Please, discuss the challenges if possible.

   Migration was quantified by dividing the area of migration by the area of each sphere to account for variations in sphere size. This was done at a single time point post-fixation.

3. All the materials and equipment are appropriately listed in the table and the steps listed in the procedure are clearly explained, with one exemption: Point 2.2.1. "When spheres form and reach a size between 200-500 μm" is this diameter or radius?? Please specify.

   This is diameter. The manuscript has been revised to clarify.

References

1. Reifenberger G, Szymas J, Wechsler W. Differential expression of glial- and neuronal-associated antigens in human tumors of the central and peripheral nervous system. *Acta Neuropathol.* 1987;74(2):105–23
2. Scholzen T, Gerdes J. The Ki-67 protein: From the known and the unknown. *J Cell Physiol.* 2000 Mar;182(3):311-22
3. Zepecki, J.P., Snyder, K.M., Moreno, M.M., Fajardo, E., Fiser, A., Ness, J., Sarkar, A., Toms, S.A., & Tapinos, N. Regulation of human glioma cell migration, tumor growth, and stemness gene expression using a Lck targeted inhibitor. *Oncogene*. 2018 **38**, 1734-1750

Response to the Editor:

Comments A1-A5 were all addressed in the revised manuscript according to the editor's requests.

Comment A6: We added some details on the protocol (3.2.31, 3.2.32) regarding the imaging of migrating cells.

Comment A7 & A9: We don't agree with this comment. To show lack of autofluorescence we must show a culture stained with secondary antibody only. This was done and it is totally black hence we did not include it here or in our published Oncogene paper. In addition, the quality and specificity of staining of axons and glioma cells excludes the possibility of autofluorescence. If the editors want us to include a black image (no primary antibody control) we can certainly send this even though it doesn't make sense to us.

Comment A8: I don't understand this comment. The cells migrate in association with axons and away from the tumor mass. We cannot say anything else than that. There is no particular destination of migration since they don't "follow" any gradient. The editor also asks how they migrated. This is a mechanistic question that has been answered in our published paper and we added language to address this.

Comment 10: Figure legends are updated.

More detailed answers to the original editorial questions:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

   The manuscript has been extensively copy-edited for errors.

2. Please define all abbreviations during the first-time use.

   All abbreviations have been defined.

3. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee as well.

   An updated ethics statement has been included.

4. 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.

All text in the protocol section is written in imperative tense.

5. The Protocol should contain only action items that direct the reader to do something.

   The protocol only contains action item.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

   The protocol is made up of discrete steps.

7. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

   More detail has been added where appropriate.

8. 2: Size of the GBM sample?

   The GBM sample is 1 cm^3; the protocol has been updated to reflect the size of the sample.

9. 2.1.12: How do you ensure that the neurospheres are formed? Do you perform any analysis at this stage? Citations if any?

   We provide citation to our recent paper in the Methods section of the revised manuscript.

10. 3.1.9: Please describe the pin rake.

    The pin rake is a ceramic holder with aligned stainless steel pins and was purchased from Tyler Research Corporation.

11. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please ensure that the highlight is 2.75 pages or less of the Protocol (including headings 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 and is in alignment with the title of the manuscript.

    The highlighted section is within the limit.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

    Oncogene **permits authors to use re-prints. The link to the editorial process can be found here: https://www.nature.com/reprints/permission-requests.html**

13. For the result section, please include some positive and negative controls to ensure that the claims made are correct.
    This was addressed in our response to reviewer 1.

14. 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

    The discuss has been revised to cover this is great detail.

15. Please expand the journal title in the reference section.

    The journal titles have been expanded in the reference section.

16. Please alphabetically sort the materials table.

    The materials list has been alphabetically sorted.