Video Article

An Orthotopic Glioblastoma Mouse Model Maintaining Brain Parenchymal Physical Constraints and Suitable for Intravital Two-photon Microscopy

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Abstract

Glioblastoma multiforme (GBM) is the most aggressive form of brain tumors with no curative treatments available to date.

Murine models of this pathology rely on the injection of a suspension of glioma cells into the brain parenchyma following incision of the dura-mater. Whereas the cells have to be injected superficially to be accessible to intravital two-photon microscopy, superficial injections fail to recapitulate the physiopathological conditions. Indeed, escaping through the injection tract most tumor cells reach the extra-dural space where they expand abnormally fast in absence of mechanical constraints from the parenchyma.

Our improvements consist not only in focally implanting a glioma spheroid rather than injecting a suspension of glioma cells in the superficial layers of the cerebral cortex but also in clogging the injection site by a cross-linked dextran gel hemi-bead that is glued to the surrounding parenchyma and sealed to dura-mater with cyanoacrylate. Altogether these measures enforce the physiological expansion and infiltration of the tumor cells inside the brain parenchyma. Craniotomy was finally closed with a glass window cemented to the skull to allow chronic imaging over weeks in absence of scar tissue development.

Taking advantage of fluorescent transgenic animals grafted with fluorescent tumor cells we have shown that the dynamics of interactions occurring between glioma cells, neurons (e.g. Thy1-CFP mice) and vasculature (highlighted by an intravenous injection of a fluorescent dye) can be visualized by intravital two-photon microscopy during the progression of the disease.

The possibility to image a tumor at microscopic resolution in a minimally compromised cerebral environment represents an improvement of current GBM animal models which should benefit the field of neuro-oncology and drug testing.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51108/>

Introduction

Glioblastoma multiforme appears as the most aggressive form of brain tumor in adults with a median survival of 12 months and a 5-years survival rate of 5%. Clinical management relies on surgery, radiotherapy and chemotherapy often used in combination. However, the effects of these treatments remain palliative¹⁻³.

Up to now, most of neuro-oncology studies rely on techniques that are only able to provide a static view and performed on large cohorts of tumor bearing animals sacrificed at different time-points (see for example^{4,5}). The recent development of follow-up methods based on intravital imaging allows studying glioma growth and the interactions between tumor cells and their pathophysiological microenvironment on the same animal over time. This opens the way to exclusive piece of information that was so far unachievable⁶. Transgenic animals expressing fluorescent tags in cells of interest may be used to study specific interactions between tumor cells and e.g. neurons in this paper.

Over the past decade, intravital two-photon microscopy⁷ has become a gold standard in fundamental neuro-oncology studies and preclinical trials^{8,9} for its ability to perform deep intravital observation of mouse brain (>500 µm below the dura-mater) with a micrometric spatial resolution¹⁰. Using intravital two-photon microscopy with orthotopical animal models implanted with a chronic cranial window¹¹, it is possible to follow the tumor progression over time on the same mouse^{9,12}.

One of the major drawbacks of these previously published animal models is however that they do not mimic the physical constraints that govern tumor growth as the dura-mater is not sealed after the injection of the cell suspension^{9,13,14}. Glioma cells may leak in the extradural space transforming an orthotopic glioma model into a heterotopic one.

The animal model presented here consists in the injection of a spheroid of fluorescent glioma cells in the cerebral cortex at a depth of 200 μm followed by the sealing of the dura-mater with a cross-linked dextran gel hemi-bead and histo-compatible glue. The tumor growth is then restricted to the brain parenchyma that maintains pathophysiological physical constraints. A chronic glass window implanted above the tumor allows an easy optical access for intravital two-photon microscopy. Using transgenic animals expressing fluorescent tags in cells of interest it is possible to perform a follow-up of the glioma growth over time and to study its interaction with its microenvironment (here with neurons and vasculature highlighted with fluorescent dextrans).

Protocol

All experimental procedures were performed in accordance with the French legislation and in compliance with the European Community Council Directive of November 24, 1986 (86/609/EEC) for the care and use of laboratory animals. The research on animals was authorized by the Direction Départementale des Services Vétérinaires des Bouches-du-Rhône (license D-13-055-21) and approved by the ethical committee of Provence Cote d'Azur n°14 (Project 87-04122012).

1. Spheroids Preparation

1. Preparation of the agarose-coated Petri dishes

- Mix 1 g of agarose with 100 ml of cell culture medium not supplemented with fetal bovine serum. Use a microwave oven (850 Watts for 40 sec, then 3 x 15 sec; stir the solution in between each microwave session) to boil the solution and to completely dissolve the agarose. Autoclave this 1% agarose solution for 20 min at 120 °C to insure sterility. **Note:** Take care to completely dissolve the agarose before the autoclave protocol. Inhomogeneity may compromise the formation of spheroids.
- Once retrieved from the autoclave, pour 10 ml of the 1% agarose solution in 100 x 20 mm Petri dishes. Leave the Petri-dishes 20 min at room temperature to allow the 1% agarose solution to solidify.
- Add 10 ml of PBS above the agarose to maintain humidity. Seal the lid of the agarose-coated Petri dishes by wrapping them with parafilm to avoid evaporation. Petri dishes can be stored for up to 2 months at 4 °C if properly humidified by the PBS layer.

2. Spheroid culture

- In a 75 cm² flask, culture the glioma cells in their recommended medium as a monolayer.
- Replace the PBS from one of the 1% agarose-coated Petri dish by 1.5 ml of "preconditioned medium" collected from the flask containing the 2D monolayer of glioma cells.
- Remove the medium from the flask containing the 2D monolayer of glioma cells.
- Gently rinse the monolayer with 10 ml of PBS.
- Add 2 ml of 0.05% of a Trypsin/EDTA solution and incubate the flask 4 min at 37 °C. **Note:** The incubation time may vary according to the cell line used.
- Add 8 ml of culture medium to stop the action of Trypsin, gently flush the cell suspension. **Note:** Take care not to flush air in the cell suspension as it increases cell death.
- Put 6 ml of the cell suspension in a sterile vial.
- Centrifuge the vial 4 min at 800 rpm.
- Remove the supernatant and gently suspend the cell sediment in 3 ml of culture medium.
- Seed the cell suspension in the prepared agarose-coated Petri dish.
- Put the Petri dish at 37 °C in a 5% CO₂ atmosphere for 2 to 3 days until spheroids of desired diameter are formed.

2. Spheroid and Window Implantation

1. Preparation of the injection system

- Clean glass capillaries (1 mm in diameter) by sonicating in 70% ethanol for 10 min. Rinse two times with water prior placing inside an incubator until dry.
- Pull several cleaned glass capillaries with a pipette puller in order to prepare a small stock.
- Break the tip of the pulled capillary by scratching the extremity on a piece of tissue paper to obtain a beveled extremity of the desired size: typically an external diameter of 300-350 μm and an internal diameter of 250-300 μm . During this shaping process, control the diameter of the capillary using a macroscope whose ocular is equipped with a graduated mira.
- Connect the non-beveled extremity of the capillary to a 3 way manifold using a piece of plastic tubing whose inner diameter fits the outer diameter of the capillary.
- Using plastic tubing, connect the two other ways of the manifold to a 25 μl microliter syringe and to a 1 ml syringe loaded with histo-compatible mineral oil.
- Adjust the manifold selector to establish a pathway between the microliter syringe and the 1ml syringe. Remove the piston of the microliter syringe and inject mineral oil backward into the microliter syringe until it leaks out by pushing the piston of the 1ml syringe. Replace the piston of the microliter syringe while taking care not to leave air bubbles in the tube.
- Adjust the manifold selector to establish a pathway between the capillary and the 1 ml syringe. Fill the capillary with mineral oil until it leaks out from the tip while taking care not to leave air bubbles in the tube.
- Adjust the manifold selector to establish a connection between the microliter syringe and the capillary. Expel about 10 μl of mineral oil.
- Dip the capillary tip into PBS solution and using the gauge of the microliter syringe, suck in 5 μl of PBS in the capillary. Note that the meniscus between oil and PBS is clearly visible.

10. Fix the capillary on a 3 axis micromanipulator fitting under the surgery microscope. This system will be used to target the capillary to the injection site under visual control.

2. Spheroid implantation

1. Lightly sedate the mouse by inhalation of Isoflurane in an induction chamber (1.5% in air during 1 to 1.5 min).
2. Anesthetize the animal with an intra-peritoneal injection of a mixture of Ketamine/Xylazine (120 mg/kg, 12 mg/kg). Note that anesthesia often reduces the body temperature of the animal. It is recommended to proceed with surgery in a room at 26 °C and to keep the animal warm with an underlying thermocontrolled heating pad.
3. Apply eye ointment to avoid desiccation of the eyes.
4. Shave the scalp of the animal.
5. Place the animal in a stereotactic frame using ear bars and a mouthpiece.
6. Clean the skin with povidone iodine (3% soap, then 10% solution).
7. Make a 1 cm long incision longitudinally in the middle of the scalp with a scalpel. Using scissors cut and remove the skin above the parietal bones.
8. With a scalpel blade gently remove the periosteum above the skull. Generously apply cyanoacrylate on top of the bone to generate a rough surface for later cement adherence.
9. Drill the parietal bone under a surgical microscope to generate a craniotomy of 4 mm diameter. Generously add ice-cold PBS containing Penicillin (1,000 U/ml) and Streptomycin (1 mg/ml) during the whole procedure to prevent heating. Remove the small bone fragments with forceps and a wet sterile gauze. Take care to drill at least 1mm away from parietal skull sutures to avoid hemorrhages.
10. Thin the bone at the border of the craniotomy where the glass window will be sealed. Aim is to ensure later planar positioning of the glass window with maximal contact surface between the brain and the glass. Slowly remove the bone using a forceps and clean the exposed dura-mater with the PBS solution.
11. Take a 5 mm diameter round glass coverslip, clean it with alcohol and dry it with tissue paper. Try to use the coverslip as a lid for the craniotomy and confirm that a large flat surface of the brain gets into contact with the glass. If necessary remove the coverslip to further thin the side bones. Proceed until the brain can get squeezed flat if gently applying pressure on the coverslip once in place.
12. Clean again the coverslip with alcohol, dry it with tissue paper, and save it apart.
13. With a 26 gauge needle make a hole in the dura-mater in the center of the craniotomy yet avoiding main blood vessels. Make sure not to damage the brain parenchyma as the purpose of this step is just to open the dura-mater.
14. Gently clean the dura-mater with PBS solution to remove hemorrhagic blood. Cover with a piece of tissue paper while preparing the spheroid injection.
15. With the spheroid injection system prepared in step 2.1, suck a round spheroid fitting the capillary inner diameter (approximately 200-250 µm) from the Petri dish prepared in step 1.2. Spheroid should come along with roughly 5 µl culture medium. It should fall down to the tip of the capillarity under its weight.
16. Remove the wet tissue paper used to cover the craniotomy and position the animal under the injection system.
17. Lower the injection pipette until it touches the hole made in the dura-mater. Then, lower it again by 250 µm. Wait 30 sec.
18. Slowly inject the spheroid using the piston of the microliter syringe (2-3 µl) while pumping out the excess liquid with a thin piece of tissue paper. Wait 30 sec and then gently lift the injection pipette by 50 µm toward the surface. Wait again 30 sec and repeat the lifting procedure by steps of 50 µm until the surface. Waiting times avoids extraction of the spheroid that would stick to the pipette.
19. Confirm the presence of the spheroid in the brain using a fluorescence microscope.
20. Mix 100-300 µm diameter cross-linked dextran gel beads with PBS solution in a Petri dish. Wait 1min and fish some hydrated beads. Place them on the bone adjacent to the craniotomy.
21. Under macroscopic control, choose one bead with a diameter similar to the size of the dura-mater opening. Cut the cross-linked dextran gel bead in two halves using forceps.
22. Gently put a cross-linked dextran gel hemi-bead into the injection hole with the convex face toward the spheroid. Take care not to remove the spheroid and press gently downward until the frontier of the concave get into contact with the surrounding dura-mater.
23. Put a drop of cyanoacrylate glue on a glass slide; dip the tip of a toothpick into the drop to take a small amount of glue. Quickly seal the edges of the cross-linked dextran gel hemi-bead to the adjacent dura-mater by stamping glue around. Take care to perform fast and accurate movements in order to avoid gluing the cross-linked dextran gel hemi-bead to the toothpick. Avoid excess of glue that can spread and impede if not prevent imaging once dried. Wait 2 min for the glue to dry and then clean the craniotomy and the surrounding bone with PBS solution.

3. Window implantation

1. Put the 5 mm diameter round coverslip above the craniotomy (see 2.2.12). The edges of the coverslip must be on the thinned skull at the exterior of the craniotomy. The coverslip must be in contact with the dura-mater and the thinned bone on the edges. It is very important to have a direct contact between the dura-mater and the glass coverslip otherwise scar tissue may develop and impede optical clarity.
2. With a tissue, absorb the PBS on the coverslip edges so that solution does not fully cover the craniotomy when applying a small pressure in the center of the coverslip. This will ensure that bone, glass and brain are glued together on the side of the region of interest.
3. Maintain a small pressure at the center of the coverslip with a forceps while gently applying cyanoacrylate at the border between the bone and the coverslip. The glue will spontaneously spread until the interface with PBS solution. Solution will act as a barrier given the hydrophobicity of the glue. Sealing should be effective in less than a minute but take an extreme care not to move the coverslip until it is fixed. This would otherwise result in cyanoacrylate leakage on the dura-mater hence lead to a failure of the surgery.
4. In case glue would have spread on top of the glass, remove it using a micro-scalpel blade by doing spiral movements as it may otherwise reduce optical clarity. Watch out not to break the glass coverslip during the procedure due to excessive pressure.
5. Consolidate the glass fixation by applying dental cement on the edges of the coverslip toward the adjacent skull. Make sure to cover the whole exposed skull until the scalp. Using extra cement, build up side walls around the coverslip to create the pool required for immersion of the objective. The dental cement will cure in less than 10 min.

3. Post-operative Care and Preparation for Imaging

1. Post-operative care

1. Remove the mouse from the stereotactic frame, inject Dexamethazon (0.2 mg/kg) and Rimadyl (5 mg/kg) s.c. over the pelvic regions and lay her in a cage with a warm tissue-nest.
2. Monitor the animal until the effects of anesthesia have vanished. In general 2 to 3 hr after surgery, mice are fully mobile. Make sure that the animal has an easy access to food and water. Provide the animal with agarose containing glucose (agarose 3%, glucose 3%).
3. Monitor the animal weight every day and inject Dexamethazon (0.2 mg/kg) and Rimadyl (5 mg/kg) s.c. for the first 10d after surgery. For ethical considerations, euthanize the mice when loss exceeds 15% of its original weight. Intracranial pressure increases with tumor size leading to motor impairments for the animal. This is tumor cell line dependent and occurs at various delays post-surgery. Special care should be taken to characterize the endpoint of the experiment with the cell line used.

2. Preparation for imaging

1. Lightly sedate the mouse by inhalation of Isoflurane in an induction chamber (1.5% in air for 1 to 1.5 min).
2. Anesthetize the animal with an intra-peritoneal injection of a mixture of Ketamine / Xylazine (100 mg/kg, 10 mg/kg). Such an anesthesia typically allows 45 min of observation. To perform longer imaging sessions, mouse is placed under continuous inhalation of Isoflurane in air (0.25-0.75%).
3. Apply eye ointment to avoid desiccation of the eyes.
4. Put the animal on a stereotactic frame and block the skull with the earbars.
5. To visualize the blood vessels, a fluorescent dye can be injected intravenously.
6. If the window appears dirty, it can be cleaned by gently removing the debris with a thin blade and the corner of a tissue. Do not use alcohol to clean the window as it is not always compatible with dental cement and it can cool the brain below the window.

Representative Results

Once the surgical protocol is performed (**Figure 1**), animals can be observed by means of fluorescent microscopy over weeks until sacrifice. An inflammatory reaction may be observed after the surgery that disappears within one or two weeks. Tumor growth can be observed by various microscopy techniques including fluorescent macroscopy and two-photon microscopy (**Figure 2**). Example images depicted here were realized on a fluorescence macroscope and a two-photon microscope coupled to a femtosecond pulsed infrared tunable laser and home-modified to allow animal positioning below the 20X water immersion objective (1.0NA).

The tumor development was estimated over time using a fluorescence macroscope combining oblique illumination for bright field reflectance imaging and epifluorescence emission (**Figures 2A-2C**). Thresholding of the fluorescence easily gives access to the tumor area while superficial vasculature detach as dark structures out of the white background of the visible light image. On the other hand intravital two-photon microscopy allows z-sectioning and orthogonal reconstructions of large fields of view with subcellular resolution (see dendrites in **Figure 2D**). Using transgenic animals expressing fluorescent proteins in cells of interest (for example, Thy1-CFP mice that express the Cyan Fluorescent Protein in neurons, green pseudo-color in **Figure 2D**) it is possible to observe the tumor progression in its neuronal micro-environment. However, it must be noted that tumor cell density can limit the photon penetration depth as well as the emitted photon detection due to increased diffusion. This is clearly visible in **Figure 2D** as the spatial resolution starts to decrease at 200 μm below the dura-matter in the tumor but not in the healthy brain parenchyma. Moreover, the second-harmonic generation signal arising from organized patterns of non-centrosymmetric molecules such as type I and III collagen provides a signature of the dura-mater (cyan pseudo-color in **Figure 2D** and arrows). It must be noted that with our surgical protocol, the tumor grows below the dura-mater in the brain parenchyma where its development is governed by the physical constraints of the brain. The sealing of the dura-mater with a cross-linked dextran gel hemi-bead prevents the escape of glioma cells in the extradural space.

When followed by intravital two-photon microscopy the tumor progression between different time-points can be compared at cellular resolution. Imaging the same area at D16 and D27 post-surgery (**Figure 3A**) we found some blood vessels as the one highlighted by arrows that were stable over a period of 11 days; on the other hand the tumor front clearly infiltrated into the healthy parenchyma over the same period. To evaluate tumor progression, its margin observed at D27 was outlined with a yellow dotted line and overlaid on the picture taken at D16. A 520 μm shift of the front edge was observed consistently with the reported proliferative profile of the GL261 glioma model¹⁵. The patterns of the vascular network inside the tumor were very different on D16 and D27 indicating that significant vascular remodeling occurs in pathological areas (**Figure 3A**). At the tumor margins, we also found cases of glioma cells detaching from the tumor core as expected from an invasive tumor. The microscopic resolution of our images showed not only that these cells emit cytoplasmic protrusions to sense the environment but also that they can use blood vessels as a matrix for their progression (arrowheads and z-reconstruction in **Figure 3B**). Two-photon microscopy thus allows to study cellular interactions in a truly physiological context. Using transgenic animals where each cell population of interest is identified by its own fluorescent reporter it becomes possible to look for the existence of physical interactions between tumor cells and their environment as key regulators of disease progression (**Figure 3B**, right).

Figure 1. Overview of the surgery. The craniotomy is performed on the left parietal bone (A). The limits of the left parietal bone are outlined with a dotted line and the removed part is filled in yellow (B). Once the craniotomy performed, the parietal cortex is exposed (C), the area for the injection of the spheroid is selected taking care to avoid main blood vessels (arrow in D) and a 26 gauge needle is used to open the dura-matter (arrow in E). The spheroid is injected and the hole in the dura-matter is clogged by a cross-linked dextran gel hemi-bead sealed with cyanoacrylate glue (arrow in F). A glass coverslip is glued to the bone while keeping contact with the brain. The coverslip is further cemented to the exposed skull to ensure long term solid attachment of the cranial window for chronic observations (G-I). The whole protocol is briefly schematized in J. [Click here to view larger image.](#)

Figure 2. Tumor growth is restricted to the brain parenchyma and can be monitored over time. **A-C.** Tumor growth (GL261 cell line expressing DsRed2) is observed by macroscopy on the day of surgery (D0, **A**) and at days 21 (**B**) and 27 (**C**). The arrowhead in **C** highlights a drop of dental cement that was not removed during step 2.3.5. **D.** Orthogonal reconstructions obtained from a 3 x 3 field acquired from 0 to 300 μm below the dura-mater in a Thy1-CFP mouse bearing a GL261 tumor by two-photon microscopy at D20 post-surgery. Tumor (red), dura-mater observed by second-harmonic generation (arrows, cyan) and neurons (green). Note that the tumor grows below the dura-mater in the brain parenchyma. [Click here to view larger image.](#)

Figure 3. Vascular remodeling and tumor cells invasion can be monitored over time with cellular spatial resolution by intravital two-photon microscopy. **A.** GL261 tumor (red) observed at D16 and D27 post-surgery after an intravenous injection of Cascade-blue dextran 70 kDa to highlight blood vessels. Dotted line: tumor margin at D27; arrows: example of blood vessels that can be used as landmarks to localize the same area at D16 and D27. **B.** Individual tumor cells (red) invade the healthy brain parenchyma (neurons; green) using blood vessels as a matrix for invasion (arrowheads). Images were taken at depth ranging from 100 to 150 μm below the dura-mater. Dotted line: region where the orthogonal reconstruction depicted in the bottom center panel was realized. [Click here to view larger image.](#)

Discussion

This approach allows the use of optical imaging methods to monitor over days and weeks the growth of an orthotopically implanted glioma. The same animal can subsequently be subjected to virtually any brain imaging modality during the course of the pathology; yet the two-photon microscopy specific preparation offers the unique opportunity to achieve subcellular resolution inside the brain of the living animal. Our protocol presents the advantage to enforce the tumor growth into the cerebral parenchyma rather than extra-durally as it happens if glioma cells can leave the central nervous system through the damaged dura. In absence of physical constraints exerted by the brain environment, tumor cell can indeed proliferate faster as they do not need to infiltrate the parenchyma¹⁶. Previously published methods did not attempt to seal the dura mater after grafting which was all the more problematic than they injected suspension of cells rather than spheroids^{9,13,14}. Suspensions of cells are indeed impossible to apply focally as individual cells are systematically seeded along the injection tract when withdrawing the pipette from the depth to the dura-mater.

The current protocol can be applied on various mouse strains including transgenic and immunosuppressed animals. Various glioma cell lines can be grafted and visualized, including cells directly derived from human biopsies as long as they are transfected to stably express a fluorescent reporter prior grafting. Surgical protocol can be typically performed in 1 hr and cohorts of animals can quickly be obtained. This approach can thus be used to study vascular remodeling, the effects of pharmacological treatments on angiogenesis and tumor growth, as well as the recruitment of immune cells into the tumor. When needed glioma cell migration and dynamic interactions between glioma cells and the microenvironment can be observed in real time for several hours.

As imaging can be done over depths of 500 μm it is possible to cover most of the tumor volume initially implanted 200 μm below the dura-mater. To ensure maximal imaging depth special care must be taken during the surgery to create physical contact between the dura-mater and the glass coverslip. This indeed reduces the formation of scar tissue that would otherwise compromise optical clarity. Absolute sterility of the surgical environment should also be observed to limit the post-surgery inflammation that transiently blurs the window. We anyway advise to wait 15 days

between the surgery and the first observation session to obtain optimal imaging conditions. Thereafter, window was found to remain clear up to one year after the surgery on sham operated animals that underwent the whole protocol except the injection of the tumor spheroid.

In conclusion, the protocol described here represents an improvement of existing orthotopic glioma mouse model dedicated to chronic intravital two-photon microscopy. The superficial injection of a tumor spheroid, the clogging of the injection track with a cross-linked dextran gel hemi-bead and the sealing of the dura-mater enforce tumor development in the brain environment that truly recapitulates the physical constraints normally governing disease progression. Not only this method will serve fundamental studies on glioma pathophysiology but it will also improve the relevance of pharmacological results during preclinical trials.

Disclosures

The authors declare that they have no competing financial interests.

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