Title

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Journal

Current Protocols, 3(2)

ISSN

2691-1299

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Publication Date

2023-02-01

DOI

10.1002/cpz1.665

Peer reviewed

Published in final edited form as:

Curr Protoc. 2023 February ; 3(2): e665. doi:10.1002/cpz1.665.

Murine-Derived Glioma Organoids and Cell Line Culture Systems

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Abstract

Research models in cancer have greatly evolved in the last decade, with the arising of several new methods both *in vitro* and *in vivo*. While *in vivo* models remain the gold standard for preclinical studies, these methods present a series of disadvantages, such as a high cost and long periods of time to produce results compared with *in vitro* models. We have previously developed a method named **M**osaic **A**nalysis by **D**ual **R**ecombinase-mediated cassette exchange (MADR) that generates autochthonous gliomas in immunocompetent mice through the transgenesis of personalized driver mutations, which highly mimic the spatial and temporal tumor development of their human counterparts. Due to the control of single-copy expression of transgenes, it allows for comparing the visualization of tumor cells and non-tumor cells. Here we describe a method to generate murine-derived glioma organoids (MGOs) and cell line cultures from these murine models by physical and enzymatic methods for *in vitro* downstream applications. Tumor cells can be readily distinguished from non-tumor cell populations in both organoids and monolayer cell cultures and isolated due to the use of personalized fluorescent reporter transgenes.

Keywords

MADR; Glioma; Organoids; Biobank; Personalized driver mutations

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CONFLICT OF INTEREST STATEMENT:
Cedars-Sinai has filed for patent protection for in vivo dual recombinase-mediated cassette exchange and disease models thereof.

INTRODUCTION:

Brain cancer research has increasingly made great progress due to the generation of novel platforms both *in vitro* (Azzarelli, 2020; Hubert et al., 2016; Jacob et al., 2020; Linkous et al., 2019) and *in vivo* (Hambardzumyan, Parada, Holland, & Charest, 2011; Kim et al., 2019; Liu et al., 2011). Genetically engineered mouse models (GEMMs) are outstanding systems to faithfully mimic tumor generation and development in gliomas (Hambardzumyan et al., 2011). Nevertheless, GEMM generation is expensive and laborious. Moreover, the intrinsic heterogeneity of brain tumors and the increasing list of tumor-driver genes can hardly be modeled in acceptable time frames by GEMMs. Alternatively, platforms for direct delivery of tumor-driver genes relying on genetically engineered viruses or by electroporation (EP) have quickly adapted and brought efficient and faster methods to create highly personalized brain tumor models (e.g., EP with transposons (Breunig et al., 2015; Xu et al., 2017)). Unfortunately, viral and transposon-derived methods are exposed to processes of random genomic integration, transgene silencing, and transgene copy number variability (Garrick, Fiering, Martin, & Whitelaw, 1998; Gibson, Seiler, & Veitia, 2013; Woods et al., 2003). Additionally, CRISPR/Cas9 allows multiple gene knockouts in mice (Chen, Rosiene, Che, Becker, & LoTurco, 2015), but with the risk of unintended off-target genomic alterations (Kosicki, Tomberg, & Bradley, 2018). To overcome these limitations, we developed mosaic analysis with dual recombinase-mediated cassette exchange (MADR) technology. The MADR platform allows accurate control of single-copy transgenesis with genetic labeling of recombined cells (Kim et al., 2019; Rincon Fernandez Pacheco, Sabet, & Breunig, 2020). Moreover, any cell line (human or murine) can be readily adapted to work as a MADR acceptor cell line allowing more personalized and fine-tunned genetic engineering (Ayala-Sarmiento, Kobritz, & Breunig, 2020). MADR technology permits the combination of gain-of-function (GOF) and loss-of-function (LOF) genetic alternations in immunocompetent rodents, allowing for the precise generation of different types of murine gliomas that resemble and behave like their human counterparts *in vivo* (Kim et al., 2019). MADR tumor models can become a high-throughput preclinical platform for functionalizing many tumor driver mutations that provides a quick pipeline for preclinical drug testing.

Despite the fact that *in vivo* studies remain the gold standard for preclinical studies, *in vitro* two-dimensional (2D) and three-dimensional (3D) cell cultures present advantages, such as higher control over the variants affecting the results. Therefore, *in vitro* methods are relevant systems for high-throughput drug testing and molecular mechanism research platforms. Successful protocols of human 3D glioma cultures have been described (Azzarelli, 2020; Hubert et al., 2016; Jacob et al., 2020; Lee et al., 2006; Linkous et al., 2019), with glioblastoma organoids (GBOs) being one of the easiest to establish (Jacob et al., 2020). However, the high interpatient heterogeneity of brain tumors, the increasing number of reported tumor-driver genes, and the inherent difficulties to obtain viable human samples, compared with mouse-based models, highlight the necessity of murine-based organoids as a primary system to study these pathologies.

Therefore, this protocol focuses on describing the generation of 3D murine-derived glioma organoids (MGOs) (Basic Protocol 1), and 2D glioma cell line cultures (Basic Protocol

2) obtained from MADR mouse fluorescent-gliomas as alternatives for *in vitro* glioma research. This protocol can be potentially extended to different types of murine-derived gliomas.

CAUTION: Maintain mice and euthanize them according to an institutionally approved animal study protocol.

BASIC PROTOCOL 1: Generation of 3D Murine-derived Glioma Organoids

In this protocol, we describe in detail the steps to generate 3D Murine-derived Glioma Organoids (MGOs) from autochthonous mouse brain tumors developed by MADR. Figure 1 provides an overview of the generation of MGOs, and the subsequent cell lines developed from the organoids. Different fluorescent signals can be observed depending on the reporter transgenes expressed in the parental tumor.

Materials:

GBO medium (see recipe in Reagents and Solutions)

Monolayer cell culture medium (see recipe in Reagents and Solutions)

Neurobasal medium (Gibco, ThermoFisher Scientific, cat. no. 21103049)

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, ThermoFisher Scientific, cat. no. 11320033)

Glutamax (100x) (Gibco, ThermoFisher Scientific, cat. no. 35050061)

MEM Non-Essential Amino Acids Solution (100x) (Gibco, ThermoFisher Scientific, cat. no. 11140050)

B27 Supplement without Vitamin A (50x) (Gibco, ThermoFisher Scientific, cat. no. 12587010)

N-2 Supplement (100×) (Gibco, ThermoFisher Scientific, cat. no. 17502048)

Penicillin-Streptomycin-Amphotericin (100x) (Gibco, ThermoFisher Scientific, cat. no. 15240062)

2-Mercaptoethanol (1000x, Gibco, cat. no. 21985023)

Human insulin solution (2.5mg/ml) (Sigma-Aldrich, MilliporeSigma, Produce no. I9278)

Red blood cell (RBC) lysis buffer (Sigma-Aldrich, MilliporeSigma, Produce no. R7757)

Sterile PBS pH 7.4 (Gibco, ThermoFisher Scientific, cat. no. 10010023)

DMSO (Sigma-Aldrich, MilliporeSigma, Produce no. D2650)

CELLstart-CTS substrate (Invitrogen, ThermoFisher Scientific, cat. no. A1014201)

Adult Mouse Brain Slicer Matrix (Zivic Instruments. cat. no. BSMAS001-1)

Razor blades (Avantor VWR, cat. no. 89237-604)

Disposable scalpel (QuickMedical, cat. no. 2975#11)

Spring Scissors (Fine Sciences Tools. cat. no. 15000-03)

1000 µl wide-bore pipet tips (Thomas Scientific. cat. no. 1141L56)

Ultra-low attachment 6-well culture plates (Corning, cat. no. 3471)

S3 Live Imaging Incucyte Instrument (Sartorius. cat. no. 4647)

Stereo microscope (Zeiss Stemi 508, or equivalent)

Stereo microscope fluorescence module (Kramer Fluorescence module, or equivalent)

Tissue chopper (McIlwain, or equivalent)

Orbital Shaker (VWR Standard 1000, or equivalent)

Fluorescence microscope (ECHO Revolve, or equivalent)

Protocol steps with *step annotations*

Brain harvesting

1. Euthanize mice that underwent glioma induction and decapitate.
2. Peel the skin and carefully open the skull, exposing the brain. Using small scissors, maintain superficial and horizontal cuts to avoid brain tissue damage.
3. Detach the brain from the skull and meninges and place it in a 5ml conical tube of cold sterile PBS.

Note: The age, sex, and/or strain of the mice are not important to get Murine-Derived Glioma Organoids.

Brain slicing

4. Pre-wet the mouse brain coronal slicer with 1 ml of cold sterile PBS to avoid tissue adherence.
5. Mount the brain dorsal side up in the mouse brain slicer (Figure 2A).
6. Generate 0.5-1 mm thick coronal brain sections by inserting the first razor blade in the slot right after the olfactory bulbs and the second razor blade before the cerebellum. Then, insert new razor blades one by one into the remaining slots. (Figure 2B).
7. Carefully lift all the razors in unison and separate the slices in a petri dish with ice-cold PBS (Figure 2C).

Glioma dissection

8. Place the coronal sections under a stereoscope with a fluorescence module to visualize the tumor (Figure 2D).

9. Use a scalpel or surgical scissors to dissect the tumor tissue from the brain parenchyma guided by the differential fluorescence.

Note that the different models of brain tumors generated with the MADR technology and background mice strains will lead to differential fluorescent markers which can be used as a guide to dissect and analyze the tumor cell growth in comparation with non-tumor tissue (Figure 4A, C and E).

10. Collect all the tumor tissue fragments and place them in a plate compatible with a tissue chopper, eliminating as much PBS as possible from the plate to promote the adherence of the tissue to the plate.

11. Cut the tumor into approximately 300 μm diameter pieces using a tissue chopper.

Note that while a tissue chopper is strongly recommended to improve the viability of tissue due to the decrease in time, it is not mandatory to use this equipment. Tumor tissue can be fragmented with microdissection scissors or with 2 scalpels doing a scissor motion. However, it is more time consuming with the latter.

12. Resuspend the tumor fragments in 4 ml of ice-cold PBS. Use a 1000 ml pipet with wide-bore tips to avoid extra mechanical damage.

13. Collect the tumor pieces in a 15ml conical tube with ice-cold PBS.

14. Let the tumor pieces settle by leaving the 15 ml conical tube on ice for 3-5 minutes.

All of the following steps should be performed under sterile conditions and under a biological safety hood.

15. Carefully aspirate the PBS without disturbing the pellet and add 12ml of RBC lysis buffer.

16. Incubate for 10 minutes at room temperature on a shaker.

17. Let the tumor pieces pellet on ice.

18. Aspirate the RBC buffer and wash tumor pieces with 10 ml of ice-cold PBS by inverting the tube 5 times.

Generation of glioma organoids

19. After letting the tumor pieces pellet, discard the PBS by carefully aspirating and resuspend the fragments in 4 ml of GBO medium.

20. Transfer the tumor fragment suspension into one or two wells of an ultra-low attachment 6-well culture plate using 1000 μl wide-bore pipet tips or a 5 ml pipet to avoid mechanical damage (Figure 3A).

21. Incubate the tumor fragments on an orbital shaker rotating at ~120 rpm at 37°C, 5% CO_2 and 90% humidity.

22. Change the media every 48 hours by:

    a. Transferring the tumor fragments from each well to a 5ml canonical tube and let the tumor pieces settle by gravity for 3-5 minutes

    b. Carefully aspirate the media and add 4 ml of fresh GBO medium.

    c. Transfer back the tumor fragment suspension to the original well using a 1000 µl wide bore pipet tips or a 5ml pipet.

23. Rounded structures will begin to form depending on the age and type of tumor; thus, it is possible to observe spheroids from 5 to 21 days post induction (Figure 3B).

Note that the generation and size of MGOs will vary at different time points depending on the type and/or maturity of the parental tumor, see critical parameters section.

24. When the organoids reach a diameter of ~500 µm they must be cut in pieces to avoid ischemic necrosis in the center of the organoid:

    a. Transfer the tumor organoids from each well to a tissue-chopper compatible plate and absorb the media to let the organoids slightly adhere to the plate.

    b. Cut the spheroids into approximately 300 µm diameter pieces using a tissue chopper or fine scissors.

    c. Recover the fragmented organoids with 4ml of GBO medium and place the suspension in a new ultra-low attachment 6-well culture plate

    d. Incubate on an orbital shaker rotating at ~120 rpm at 37°C, 5% CO_2 and 90% humidity.

25. Organoid fragments will grow back as spheres in 5-7 days after cutting them (Figure 4B, D, and F).

Biobank

1. Cut the organoids into ~300 µm diameter pieces and transfer them into a 5 ml canonical tube.

2. Wash the organoid pieces with 4 ml of sterile prewarmed PBS by inverting the tube 5 times and letting the pieces settle down in the bottom of the tube.

3. Carefully aspirate the supernatant.

4. Incubate the fragments with GBO medium supplemented with 10 µM Y-27632 (ROCK inhibitor) for 30 minutes at room temperature on a shaker.

5. Let the pieces settle down by gravity for ~ 5 min and carefully collect the supernatant.

6. Add GBO medium supplemented with 10 µM Y-27632 and 10% DMSO.

7. Incubate for 15 minutes at room temperature on a shaker.
8. Transfer 30 to 40 MGOs into each cryovial in 1 ml of media with Y-27632 and DMSO.
9. Place the cryovials in a CoolCell freezing container at −80°C for 24 hours.
10. Store the cryovials in liquid nitrogen for long term storage.

Glioma organoid recovery

1. Thaw cryovials in a 37°C water bath and transfer the organoids to a 15 ml conical tube.
2. Add 14 ml of prewarmed GBO medium supplemented with 10 μM Y-27632.
3. Incubate at room temperature for 10 minutes on a shaker.
4. Let the organoids settle down for 5 minutes and aspirate the medium carefully.
5. Add 4 ml of fresh GBO medium supplemented with 10 μM Y-27632.
6. Transfer the suspension into a well of an ultra-low attachment 6-well culture plate.
7. Incubate the organoids on an orbital shaker at ~120 rpm, 37°C, 5% CO_2 and 90% humidity for 24 hrs.
8. Change the media and resuspend the organoids in GBO medium without Y-27632.
9. Incubate the organoids on an orbital shaker at ~120 rpm, 37°C, 5% CO_2 and 90% humidity.

BASIC PROTOCOL 2: Generation of 2D glioma monolayer cell lines

In this protocol, we describe in detail the steps to generate 2D glioma monolayer cell lines from 3D MGOs. Enzymatic and physical dissociation of the MGOs is a fast methodology to generate 2D cell lines. Different fluorescent signals can be observed depending on the reporter transgenes expressed in the parental MGOs.

Materials:

GBO medium (see recipe in Reagents and Solutions)

Monolayer cell culture medium (see recipe in Reagents and Solutions)

Human EGF (Shenandoah, Part no.100-26)

Recombinant Human FGF-basic (Peprotech, ThermoFisher Scientific, cat. no. 100-18B)

Human PDGF-AA (Shenandoah, Part no. 100-16)

Sterile PBS pH 7.4 (Gibco, ThermoFisher Scientific, cat. no. 10010023)

CELLstart-CTS substrate (Invitrogen, ThermoFisher Scientific, cat. no. A1014201)

Trypsin-EDTA (Gibco, ThermoFisher Scientific, cat. no. 25200056)

StemPro Accutase Cell Dissociation Reagent (Gibco, ThermoFisher Scientific, cat. no. A1110501)

1000 µl pipet tips (TipOne, USA Scientific Inc, cat. no 1122-1830)

25cm^2 Cell Culture Treated Flasks (Corning, ThermoFisher Scientific, 10-126-28)

S3 Live Imaging Incucyte Instrument (Sartorius. Cat. No. 4647)

Tissue chopper (McIlwain, or equivalent)

Protocol steps with *step annotations*

Glioma organoid dissociation for cell culture monolayer

1. Before starting with dissociation, coat a T25 flask with fresh CELLstart-CTS diluted in sterile PBS (1:50) and incubate at 37°C, 5% CO_2 and 90% humidity for 1-2 hours. Aspirate CELLstart-CTS and add monolayer cell culture medium.
2. Transfer the organoids to a 5 ml conical tube and let them settle for 3-5 minutes.
3. Carefully aspirate the media without disrupting the pellet.
4. Wash with 10 ml of prewarmed PBS, letting the organoids settle back, and carefully remove the supernatant.
5. Add 1 ml of Accutase or Trypsin-EDTA (0.25%) to the pellet and incubate 5 minutes at 37°C.
6. Resuspend the suspension using a 1000 µl pipet with normal tips (no wide-bore tips) to speed up the dissociation.
7. Incubate for 3 minutes at 37°C and resuspend the suspension again.
8. Add 4ml of monolayer cell culture medium to the suspension and centrifuge at 500 rcf for 4 minutes.
9. Carefully aspirate the supernatant.
10. Resuspend the pellet in 2 ml of sterile PBS.
11. Filter the suspension through a 40 µm cell strainer.
12. Add 2 ml of monolayer cell culture medium to the filtered suspension.
13. Centrifuge at 500 rcf for 4 minutes and discard the supernatant.
14. Resuspend the pellet in the appropriate volume of monolayer cell culture medium and transfer the cells to the flask previously coated with CELLstart-CTS.

15. Visualize the organoid-derived monolayer cell culture under a fluorescent microscope or Incucyte for imaging. Different morphology and fluorescent signal can be observed depending on the tumor model and fluorescent reporter transgene (Figure 5).

Note: Optionally, a non-enzymatic culture monolayer generation approach can be used in an experimental design where enzymatic digestions could undermine the pursuit results. To this end, culture MGOs in CELLstart-*CTS coated plates with monolayer cell culture media, this will promote the attachment of the organoids to the plate. Immediately, the cells will start to migrate to the coated surface of the plate, generating a monolayer culture without the necessity of enzymatic digestions*, video 1. *This process can be sped up by cutting the organoids with a tissue chopper* obtaining around 100-200 μm pieces *before seeding them in the coated cell culture plate.*

REAGENTS AND SOLUTIONS:

GBO medium

To prepare 500ml of GBO medium mix as previously described for human GBOs (Jacob et al., 2020):

• Neurobasal media	235 ml
• Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12	235 ml
• Glutamax (100x)	5 ml
• MEM Non-Essential Amino Acids Solution (100x)	5 ml
• B27 Supplement without Vitamin A (50x)	10 ml
• N-2 Supplement (100X)	5 ml
• Penicillin-Streptomycin-Amphotericin (100x)	5 ml
• Human insulin solution (2.5mg/ml)	125 μl
• Filter the media through a 0.2 mm vacuum filter and add 500 μM of sterile 2-Mercaptoethanol.	
• Store up to 1 month at 4°C.	

Monolayer cell culture medium

Prepare working media by adding to 50 ml of GBO medium:

- Human EGF (to a final concentration of 20 ng/ml)
- Human FGF (20 ng/ml) and heparin (2 μg/ml)
- Human PDGF-AA (10 ng/ml) must be added to the media when generating cell lines from tumor models that include the transgene of the constitutively active mutant variant $Pdgfra^{D842V}$
- Filter the media through a 0.2 mm vacuum filter.
- Store up to 1 month at 4°C.

COMMENTARY:

Background Information:

In a recent study, we devised a transgenic method that allows us to manage single-copy integration of genetic elements into targeted loci, allowing us to construct autochthonous mouse brain tumors with great precision in completely immunocompetent animals (Kim et al., 2019). Despite our success in creating several forms of brain tumors *in vivo*, the derivation of cell line cultures from parental tumors has shown inconsistent results, which is greatly reliant on the type of tumor modelled. For example, diffuse pediatric gliomas carrying K27M mutation in the histone 3.3 with oncogenic mutations in Pdgfra and Trp53, rapidly adapt to 2D culture conditions. However, a similar genetic paradigm model but carrying a G34R mutation in place of the K27M or even tumors generated with a wild type histone 3.3, do not develop well in 2D culture and the media necessary to maintain these cultures become exceedingly complicated and costly. There have been successful approaches for human glioma cultures, generally in 3D rather than 2D monolayer cell cultures, with the glioblastoma organoids (GBOs) technique being the simplest and least expensive. (Azzarelli, 2020; Hubert et al., 2016; Jacob et al., 2020; Lee et al., 2006; Linkous et al., 2019). Furthermore, GBOs preserve not only intra-tumor heterogeneity but also tumor microenvironment cells, which are critical in tumor progression and to faithfully model this disease *in vitro* (Jacob et al., 2020; Yao et al., 2020). We sought to adapt the GBOs methodology to autochthonous mouse gliomas to generate murine-derived glioma organoids, or MGOs, which offer several benefits over GBOs or other human cell line systems. MGOs have the majority characteristics of the parental tumors generated by MADR technology, they can be personalized based on the different patient driver mutations, specific fluorescent reporters allow distinguishing tumor cells from non-tumor cells, collection and resection of tumor samples can be done with ease and expedience, and they can be created at different stages of tumor development. MGOs cell composition evolves over time as the tumor cells outcompete the tumor-microenvironment cells. Therefore, while tumor cell heterogeneity is well maintained in MGOs, non-tumor cells slowly disappear once the organoids have been established. In general, it is recommended to use the organoids for *in vitro* research within low passages (<5), considering a passage every time MGOs must be cut in pieces to avoid core necrosis. This recommendation is especially relevant for studies that are targeting non-tumor populations or the interactions of these cells with the tumor mass. Because MGOs are more diverse and prolific, they may be used in a broader range of downstream applications, such as biobanks, drug testing, fundamental research, and so on. MGOs can also be used to construct 2D monolayer cell cultures. Although this approach shows how to make MGOs from autochthonous mouse brain tumors, it may be relevant to other types of murine-derived gliomas.

Critical Parameters:

An important factor that influences the successful generation of MGOs, is the maturity of the parental tumor. MGOs generated from mature tumors from moribund mice, tend to grow faster and generate spheroids in less than a week. Organoids generated from immature tumors can take longer to generate spheroids (from 2 weeks to a month) and show a slower growth ratio.

For 2D monolayer cell cultures, it is very important to add growth factors, e.g.: FGF, and EGF, to keep a viable cell line. We have found that monolayer cell cultures without factors do not establish, and cells tend to die in a couple of weeks.

It is very important to work and manipulate all the tissues, MGO's, and cell lines in a biosafety cabinet to avoid contamination.

Troubleshooting:

Understanding Results:

Rounded structures typically appear one to two weeks after the initial tumor has been processed. It may take longer depending on the tumor's maturity. MGOs are fully established when they are completely spherical. Because MGOs are derived from MADR-induced autochthonous mouse brain tumors, they share many traits with their parents. Therefore, depending on the fluorescent reporter that each cell carries, it is straightforward to identify tumor cells from non-tumor cells (see figures 3, 4, and video 1). As shown in Figure 5, this "simple" property is valuable for tumor cell purification as well as other downstream studies. We determined that cultivating the chopped tissue is impractical if no MGOs appear after 1 month of induction.

Time Considerations:

It will take around 1-2 hours from tumor harvest to incubation of the chopped tissue. MGOs form after one to four weeks of incubation, depending on the maturity of the parental tumor. Finally, the generation of monolayer cell lines takes around 30 minutes, and an established monolayer cell line takes about one to two weeks depending on the purity of the tumor and the necessity of cell sorting.

ACKNOWLEDGMENTS:

We acknowledge support from TJ's Dream Team HEADing for a Cure, a Samuel Oschin Comprehensive Cancer Institute (SOCCI) Cancer Research Forum Award, the Board of Governors RMI of Cedars-Sinai (to J.J.B.). J.J.B. was supported by NIH grants R33CA202900, R33CA236687, P50CA211015-05S1, R01NS121617, and R03NS101529; DoD CDMRP grant KC200178, American Cancer Society grant RSG-16-217-01-TBG; the Saban Research Institute Team Science Grant, a Hyundai Hope on Wheels Grant, and SOCCI Jack Mishkin Discovery, P&G, and Cancer Biology Awards.

DATA AVAILABILITY STATEMENT:

The data that support the protocol are available from the corresponding author upon reasonable request.

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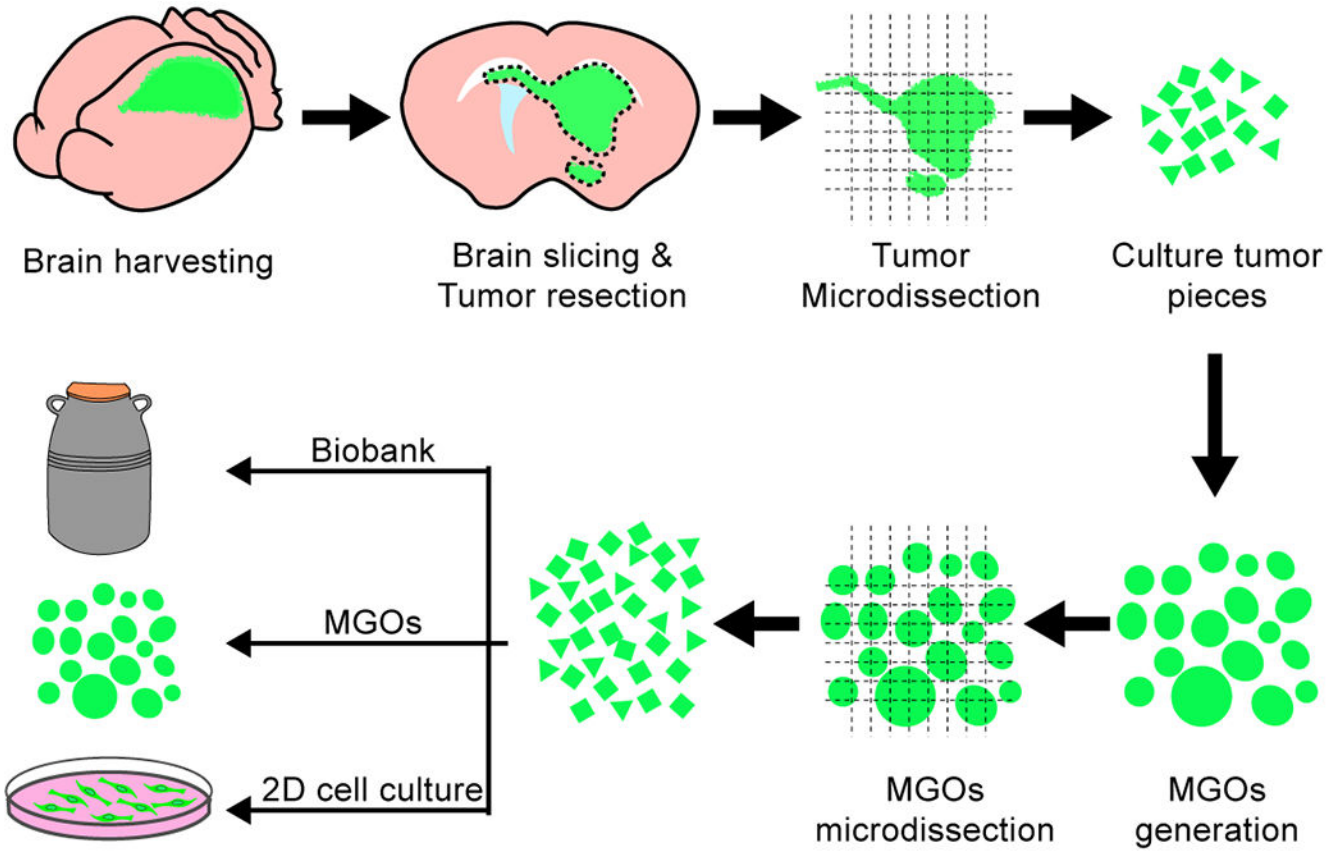

Figure 1.

General diagram of the protocol: Murine brains with fully developed MADR fluorescent gliomas are dissected. Tumor tissue is recovered and fragmented to generate MGOs that can be further expanded by fragmentation and used in research, stored in a biobank, or further enzymatically digested to generate a 2D cell culture.

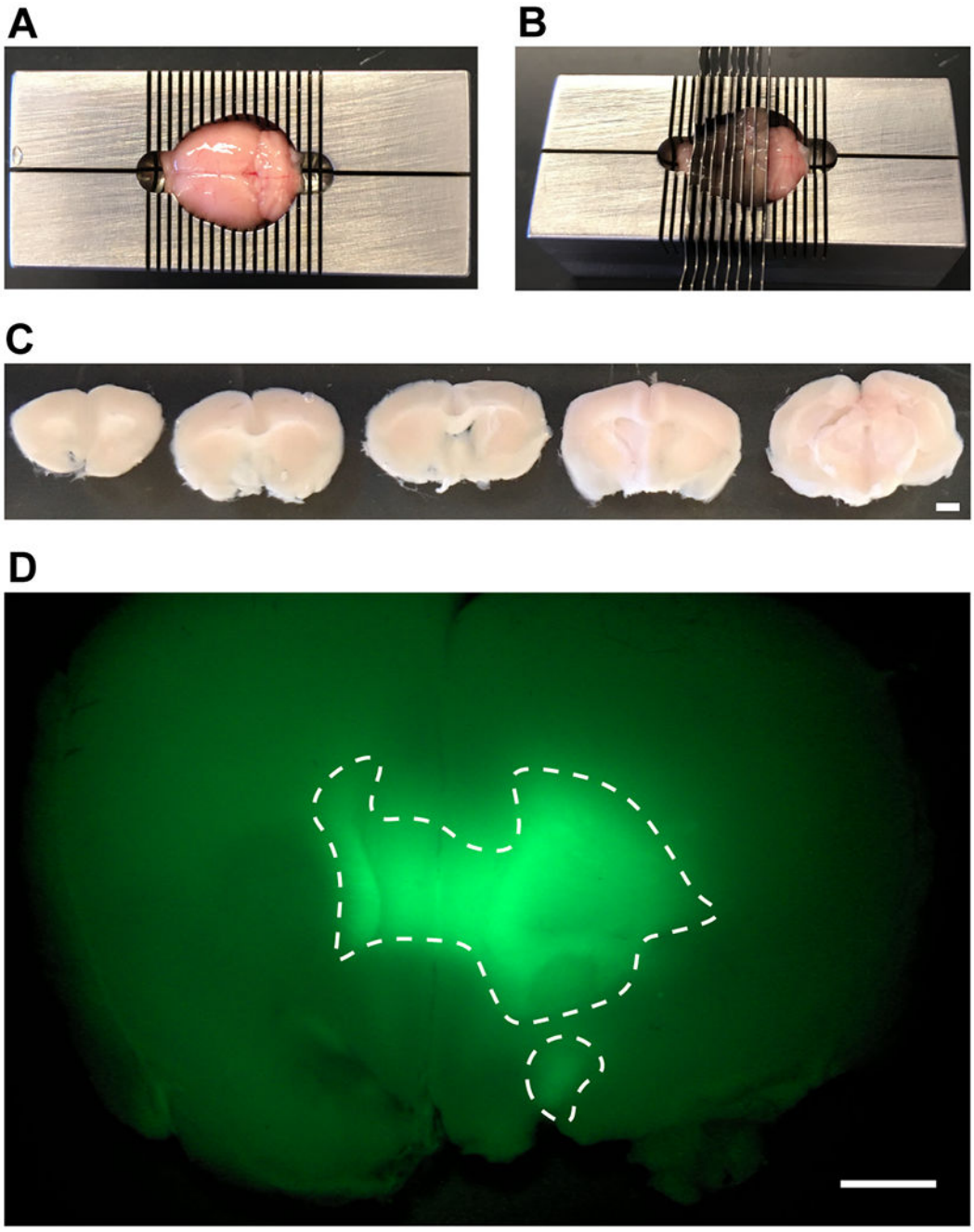

Figure 2.

A) Adult mouse brain mounted in coronal mouse brain slicer. B) Slicing of mouse brain using razor blades. C) Mouse coronal sections ordered from anterior to posterior. D) Fluorescent stereoscope image of green-fluorescent mouse glioma highlighted in dashed lines in a coronal section. Scale bars represents 1mm in both 2C and D.

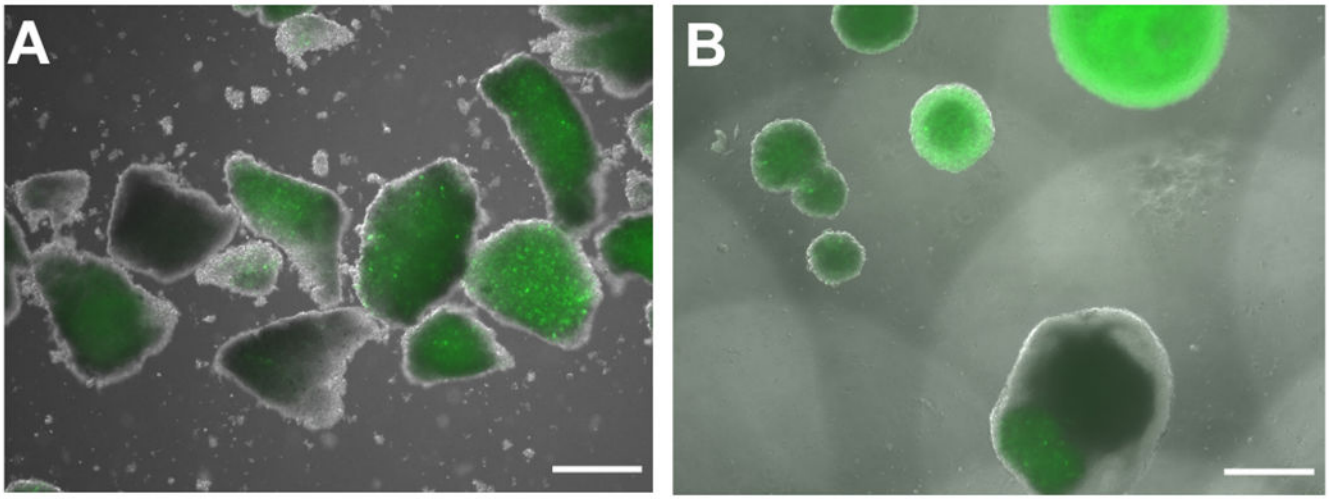

Figure 3.

Generation of MGOs. A) Tumor tissue fragments generated by the tissue chopper after tumor microdissection present sharp edges. B) Rounded MGOs after 2-3 weeks of culture. Tumor cells can be observed by their expression of the fluorescent reporter Mtfp1. Scale bars represent 500μm.

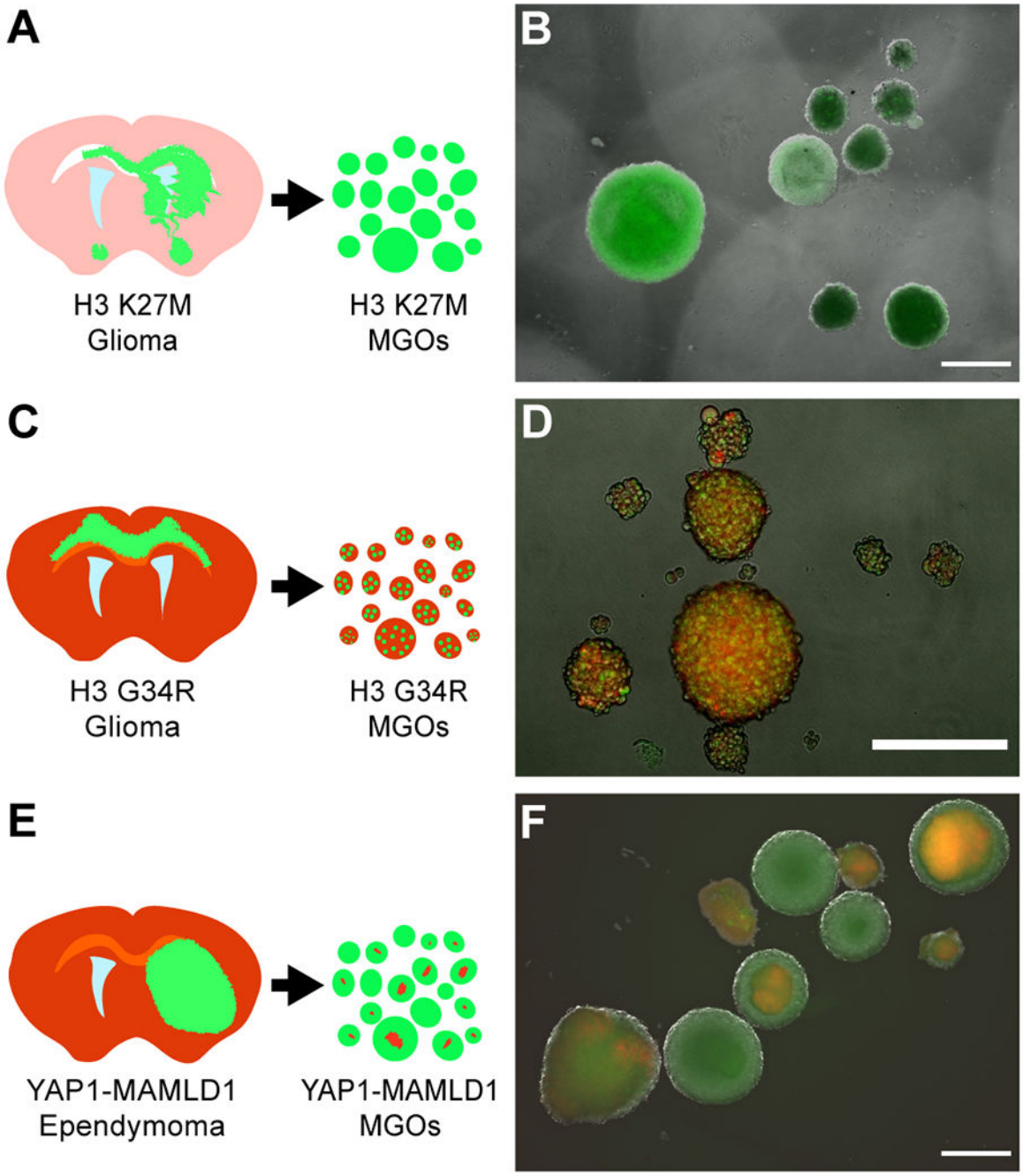

Figure 4.

Diversity of MGOs obtained from different MADR engineered gliomas with distinct fluorescent markers generated in different strains of acceptor mice. A) Scheme showing a Histone 3.3K27M glioma, generated in a non-constitutively fluorescent MADR acceptor mouse, and the subsequent generation of fluorescent MGOs containing cytoplasmic Mtfp1 tumor cells and non-fluorescent tumor-microenvironment cells. B) Epifluorescence image of Histone 3.3K27M MGOs expressing Mtfp1. C) Scheme showing a Histone 3.3G34R glioma, generated in a mTmG mouse constitutively expressing membrane tdTomato

(Muzumdar, Tasic, Miyamichi, Li, & Luo, 2007), and the subsequent generation of fluorescent MGOs containing nuclear EGFP and membrane mScarlet tumor cells and membrane tdTomato tumor-microenvironment cells. D) Epifluorescence image of Histone 3.3G34R MGOs expressing nuclear EGFP and membrane mScarlet. E) Scheme showing a YAP1-MAMLD1 fusion ependymoma, generated in a mTmG mouse, and the subsequent generation of fluorescent MGOs containing cytoplasmic “spaghetti monster” reporter fluorescent protein bright-myc (smFP bright-myc) tumor cells and membrane tdTomato tumor-microenvironment cells. F) Epifluorescence image of YAP1-MAMLD1 MGOs expressing smFP bright-myc (tumor cells) and tdTomato (tumor-microenvironment cells). Scale bars in B, D and F represent 500μm.

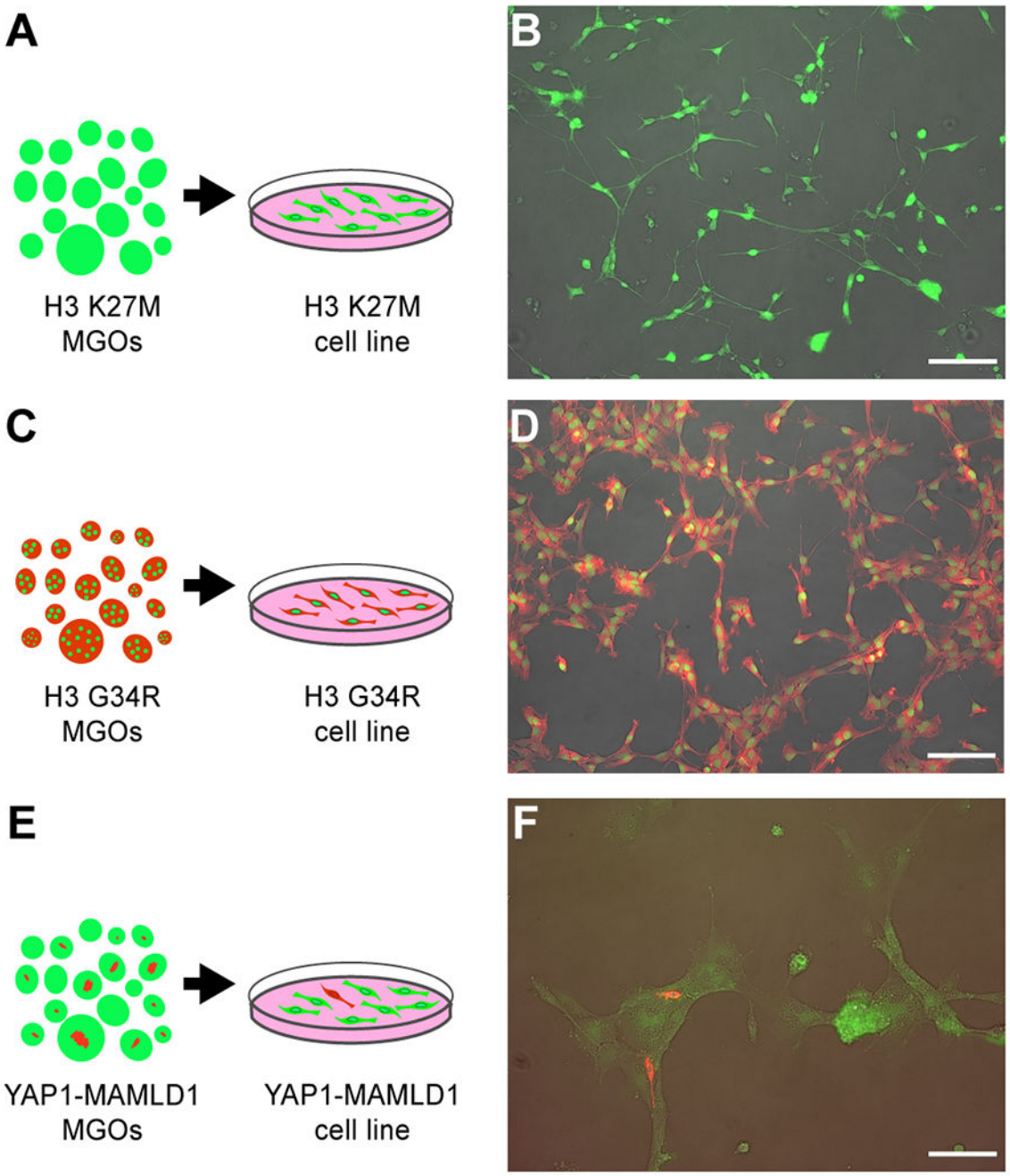

Figure 5.

Generation of 2D cell cultures from different MGOs. A) Scheme showing Histone 3.3K27M MGOs enzymatically dissociated to generate a Mtfp1 fluorescent 2D cell culture. B) Epifluorescence image of a Histone 3.3K27M cell line expressing Mtfp1. C) Scheme showing Histone 3.3G34R MGOs enzymatically dissociated to generate a 2D cell culture where tumor cells can be separated from the tumor microenvironment cells due to the nuclear EGFP fluorescence. D) Epifluorescence image of a Histone 3.3G34R cell line expressing nuclear EGFP and membrane mScarlet. E) Scheme showing YAP1-

MAMLD1 MGOs enzymatically dissociated to generate a 2D cell culture with a mix of tdTomato (tumor microenvironment) and smFP bright-myc (tumor cells) expressing cells. F) Epifluorescence image of a YAP1-MAMLD1 Ependymoma cell line expressing tdTomato (tumor microenvironment cells) and smFP bright-myc (tumor cells). Scale bars in B, D and F represent 100μm.

Video 1.

Live imaging showing non-enzymatic generation of a monolayer cell culture from MGOs by direct culture of the organoids in CELLstart-CTS coated plates. Histone 3.3G34R MGOs attach to the plate and tumor cells migrate from the parental MGOs through the plate surface generating a 2D culture. Tumor cells express EGFP and tumor microenvironment cells express tdTomato.

Table 1.

Troubleshooting Guide for MGO generation, culture, and maintenance.

Problem	Possible Cause	Solution
MGOs become necrotic in the center and disaggregate over time.	When MGOs reach a diameter of ~1mm, their core doesn't receive nutrients to maintain cell viability which necrotizes. The fragments released by these moribund MGOs are usually unable to generate new viable MGOs.	Ensure you are maintaining the MGOs with a diameter smaller than ~1mm and change media frequently to avoid media turning yellow.
Thawed MGOs are unable to grow and die over time.	The state of the MGOs in the moment of freezing is highly important. Unhealthy or excessively big MGOs will have trouble to regrow after freezing and thawing.	We recommend freezing them when the MGOs have become spherical after a maintenance cutting.
MGOs don't become fully spherical, maintaining non-tumor tissue.	Microdissection of the tumor mass was incomplete, fragments of non-tumor tissue remained attached to the tumor.	These non-tumor tissue fragments usually disappear over time, however, if they are more abundant than pure tumor fragments they can delay or block MGO development. Stricter microdissection is advisable to improve MGOs development and viability.