Video Article

Evaluation of Biomarkers in Glioma by Immunohistochemistry on Paraffin-Embedded 3D Glioma Neurosphere Cultures

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Abstract

Analysis of protein expression in glioma is relevant for several aspects in the study of its pathology. Numerous proteins have been described as biomarkers with applications in diagnosis, prognosis, classification, state of tumor progression, and cell differentiation state. These analyses of biomarkers are also useful to characterize tumor neurospheres (NS) generated from glioma patients and glioma models. Tumor NS provide a valuable *in vitro* model to assess different features of the tumor from which they are derived and can more accurately mirror glioma biology. Here we describe a detailed method to analyze biomarkers in tumor NS using immunohistochemistry (IHC) on paraffin-embedded tumor NS.

Video Link

The video component of this article can be found at https://www.jove.com/video/58931/

Introduction

Gliomas are primary solid tumors of the central nervous system classified by their phenotypic and genotypic characteristics according to the World Health Organization[1]. This classification incorporates the presence or absence of driver mutations, which represent an attractive source of biomarkers[2]. Biomarkers are biological characteristics that can be measured and evaluated to indicate normal and pathological processes, as well as pharmacological responses to a therapeutic intervention[3]. Biomarkers can be detected in tumor tissue and cells derived from glioma, enabling its biological characterization in different aspects. Some examples of glioma biomarkers include mutated isocitrate dehydrogenase 1 (IDH1), a mutant enzyme characteristic of lower-grade gliomas associated with better prognosis[4]. Mutated IDH1 is frequently expressed in combination with TP53 and alpha-thalassemia/mental retardation syndrome X-linked (ATRX)-inactivating mutations, defining a specific glioma subtype[4,5]. ATRX-inactivating mutations also occur in 44% of pediatric high-grade gliomas[2,6] and have been associated with aggressive tumors and genomic instability[6,7]. In addition, pediatric diffuse intrinsic pontine gliomas (DIPG) are differentiated in subgroups according to the presence or absence of a K27M mutation in histone H3 gene H3F3A, or in the HIST1H3B/C gene[1,6]. Therefore, the introduction of molecular characterization permits the subdivision, study, and treatment of gliomas as separate entities, which will more allow the development of more targeted therapies for each subtype. In addition, the analysis of biomarkers can be used to evaluate different biological processes such as apoptosis[8], autophagy[9], cell cycle progression[10], cell proliferation[11], and cell differentiation[12].

Genetically engineered animal models that harbor the genetic lesions present in human cancers are critical for the study of signaling pathways that mediate disease progression. Our laboratory has implemented the use of the Sleeping Beauty (SB) transposase system to develop genetically engineered mouse models of glioma harboring specific mutations that recapitulate human glioma subtypes[13,14]. These genetically engineered mouse models are used for generating tumor-derived NS, which enable *in vitro* studies in a 3D system, mirroring salient features present in the tumors *in situ*[15]. Also, the orthotopical transplantation of NS into mice can generate secondary tumors that retain features of the primary tumor and mimic the histopathological, genomic, and phenotypic properties of the corresponding primary tumor[15].

In serum-free culture, brain tumor cells with stem cell properties can be enriched, and in the presence of epidermal growth factors (EGF) and fibroblast growth factors (FGF), they can be grown as single cell-derived colonies such as NS cultures. In this selective culture system, most differentiating or differentiated cells rapidly die, whereas stem cells divide and form the cellular clusters. This allows the generation of a NS culture that maintains glioma tumor features[16,17,18]. Glioma NS can be used to evaluate several aspects of tumor biology, including the analysis of biomarkers that have applications in diagnosis, prognosis, classification, state of tumor progression, and cell differentiation state. Here, we detail a protocol to generate glioma NS and embed the 3D cultures in paraffin to be used for IHC staining. One advantage of fixing and embedding glioma NS is that the morphology of NS is maintained better compared to the conventional cytospin method[19], in which glioma NS are subjected to stressful manipulation for cell dissociation and become flattened. In addition, embedding quenches any endogenous fluorophore expression from genetically engineered tumor NS, enabling staining across the fluorescent spectra. The overall goal of this method

is to preserve the 3D structure of glioma cells through a paraffin embedding process and enable characterization of glioma neurospheres using immunohistochemistry.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan.

1. Generation of Brain Tumor Neurospheres Derived from a Mouse Model

1. Before the dissection procedure, prepare neural stem cell media (NSC Media: DMEM/F12 with 1x B27 supplement, 1x N2 supplement, 1x normocin, and 1x antibiotic/antimycotic supplemented with human recombinant EGF and basic-FGF at concentrations of 20 ng/mL each). Fill a 1.5 mL tube with 300 µL of NSC media and reserve for later.
2. Select a mouse with a large tumor.
    NOTE: The size of a tumor can be monitored by bioluminescence if the plasmid or virus injected encodes luciferin. Usually, a large tumor has a bioluminescence of > 10^6 photons/s/cm^2/sr.
3. Euthanize the mouse with an overdose of isoflurane: infuse a tissue paper with isoflurane and introduce the tissue in a euthanization chamber, avoiding direct contact with the mice to prevent irritation. Wait until the mice do not show a pedal reflex (usually 5-10 min). Decapitate the mouse and dissect the brain as described in Calinescu *et al.*[13].
4. If the tumors are fluorescent, use a dissecting microscope equipped with a fluorescent lamp to identify the tumor mass within the brain. With fine forceps, dissect the tumor (usually 5-7 mm in diameter) from the normal brain tissue.
5. Place the dissected tumor in the 1.5 mL tube with NSC media (step 1.1). Homogenize the tumor with a disposable plastic pestle that fits into the walls of the microcentrifuge tube by applying gentle pressure.
6. To dissociate the cell clumps, add 1 mL of enzyme-free dissociation media and incubate for 5 min at 37 °C.
7. Filter the cell suspension through a 70 µm cell strainer and wash the strainer with 20 mL of NSC media.
8. Centrifuge at 300 x g for 5 min, decant the supernatant, and re-suspend the pellet into 6 mL or 15 mL of NSC media, depending on the size of the pellet.
9. Plate the tumor cell suspension into a T-25 or T-75 culture flask and culture at 37 °C in a tissue culture incubator with an atmosphere of 95% air and 5% CO_2.
10. After 3 days, transfer the healthy suspended NS and re-plate them into a T-25 or T-75 tissue culture flask. If necessary, add more NSC media to the culture.
    NOTE: There will usually be a mixed population of cells, some will be dead, some will have adhered, and some will be forming NS that will be floating in the media

2. Maintaining and Passaging the NS

NOTE: Prevent overgrowth and maintain cells at relatively high density. Confluence is determined by spheres size (black centers of large spheres mean overgrowth). The growth rate of the NS will depend on the oncogenes used to generate tumors.

1. Once the NS culture has reached confluency, collect the culture in a sterile centrifuge tube and pellet the NS at 300 x g for 5 min.
2. Discard the supernatant, add 1 mL of the cell detachment solution, and pipette to re-suspend the pellet.
3. Incubate at 37 °C for 5 min, flicking the tube every 2 min to help cell dissociation.
    NOTE: When NS reach confluency, they can be usually seen macroscopically as small white spheres. To assure cell dissociation, at least all the visible NS must have disappeared.
4. Add 5 mL of HBSS 1x and pipette several times. Pellet cells at 300 x g for 5 min. Discard the supernatant. Re-suspend the pellet in 5 mL of NSC media supplemented with recombinant EGF and basic-FGF.
5. Add 1 mL of cells to 15 mL of NSC media and culture in a T-75 flask at 37 °C in a tissue culture incubator with an atmosphere of 95% air and 5% CO_2.
6. Add growth factors (EGF and basic-FGF) every 3 days. If the media turns light orange and the cells are not yet confluent, change the media. To do this, centrifuge the NS at 300 x g for 4 min and re-suspend in NSC media supplemented with growth factors.
    NOTE: Depending on the size of the pellet formed, cells may need to be split into more flasks.

3. Freezing NS for Long-term Storage

1. When the NS culture becomes confluent, dissociate the cells as described in steps 2.1-2.4. Once the pellet has been re-suspended in HBSS, remove an aliquot and count the cells.
2. Centrifuge cells at 300 x g for 4 min and remove as much as possible of the supernatant.
3. Re-suspend the pellet in freezing media (heat-inactivated FBS, 10% DMSO). It is recommended to freeze between 1 x 10^6 and 3 x 10^6 cells per vial per mL.
4. Divide the re-suspended cells in as many cryovials as needed and slowly cool down the vials by first transferring them to ice, then to -20 °C, then to -80 °C, and finally the next day to a liquid nitrogen storage container.

4. Fixation of Tumor NS

1. Culture tumor NS in a T-25 flask for 2-3 days until cells are confluent (~ 3 x 10^6 cells). Collect tumor NS in a 15 mL conical tube from a least one T-25 confluent flask. Pellet at 300 x g for 5 min.
2. Re-suspend the pellet in 1 mL of 10% formalin and keep at room temperature (RT) for 10 min. Add 5 mL of 1x HBSS and pellet the cells as done in step 3.2. Repeat this step one more time.

3. Place the 15 mL conical tube containing the pellet on ice.

5. Paraffin Embedding of Tumor NS

1. Re-suspend the washed NS pellet in 500 µL of 2% warm liquid agarose and mix gently by pipetting.
2. Immediately place the agarose-embedded NS on ice until the agarose polymerizes. Remove the agarose piece harboring the NS. Place the polymerized agarose piece with the NS into the cassette for tissue processing (**Figure 2**).
3. Continue with tissue processing (using an automatic paraffin processor) and paraffin embedding (using an embedding station).

6. Sectioning of Paraffin-embedded NS

1. Set the water bath to 42 °C and insert the blade on the microtome carefully using two paint brushes to make sure it is leveled. Use this blade only for trimming.
2. Place the paraffin block on the microtome. With the non-dominant hand, press down on the lever located on left-hand side that controls the thickness of each section. Press to the second stop that indicates a 30 µm thickness.
3. Begin trimming the block (*i.e.*, removing excess paraffin) by turning the coarse hand wheel with the right hand until the surface of the sample is reached. At this point, release the lever that controls the trim thickness to either 10 µm or 5 µm. Stop trimming when the surface of the sample is reached.
4. Fill an ice box with ice and add just enough water so that when the paraffin block is placed on ice, the water acts as a film around the paraffin block, protecting it from directly touching the ice. Incubate the paraffin block on ice for 20 min.
5. Discard the old blade and insert a new one for sectioning. Dry block using gauze, then place it on the microtome.
6. With the non-dominant hand, obtain a pair of curved forceps that will be used to pull the paraffin ribbon. Keep a damp paint brush near the right hand, which will be used to transfer the paraffin ribbon to the water bath. Begin to section by turning the coarse hand wheel with the right hand at a fast pace. Use the forceps in the left hand to hold the paraffin ribbon.
7. Use the damp paint brush to transfer the paraffin ribbon to the water bath.
8. Use the curve of the forceps to break apart the sections by superficially placing the forceps in between two paraffin sections, then pushing the two sections away gently.
   NOTE: This motion should be entirely on the surface of the paraffin section. Do not press down on the section.
9. Use the damp paint brush to push the separated paraffin sections onto positively charged adhesion microscope slides.
10. Allow the slides to dry overnight inside a chemical hood before storing them in slide boxes. The storage of slides depends on the type of stain performed.

7. Immunohistochemistry (IHC) of Paraffin-embedded NS

1. Melt paraffin by placing the slides in a metal rack and incubating them at 60 °C for 20 min.
2. Deparaffinize the slides by incubation in a rehydration train at RT: 3x xylene for 5 min, 100% 2x ethanol for 2 min, 95% 2x ethanol for 2 min, 70% 1x ethanol for 2 min, and 1x deionized water for 2 min. Hereon, keep the slides in tap water until antigen retrieval, as drying out will cause non-specific antibody binding.
3. Incubate the slides in citrate buffer (10 mM citric acid, 0.05% non-ionic detergent, pH 6) at 125 °C for 30 s and at 90 °C for 10 s. Let them cool down, then rinse the slides 5 times with distilled water.
4. Incubate slides at RT in 0.3% H_2O_2 for 30 min.
5. Wash the slides 3 times in washing buffer (0.025% detergent in TBS) for 5 min with gentle agitation.
6. Incubate the slides at RT with blocking solution (10% horse serum, 0.1% BSA in TBS) for 30 min.
7. Incubate the slides overnight at 4 °C in a humidified chamber with primary antibody prepared in dilution solution (0.1% BSA in TBS). Wash the slides 3 times in washing buffer for 5 min with gentle agitation.
8. Incubate the slides at RT for 1 h with biotinylated secondary antibody prepared in dilution solution. Wash the slides 3 times in washing buffer for 5 min with gentle agitation.
9. Incubate the slides at RT in the dark for 30 min with avidin-biotinylated horseradish peroxidase complex. Wash the slides 3 times in washing buffer for 5 min with gentle agitation.
10. Incubate the slides with DAB-H_2O_2 substrate brands here for 2-5 min at RT.
11. Rinse 3 times with tap water. Dip (1-2 s) the slides in hematoxylin solution to counterstain, and rinse them thoroughly in tap water until clearing.
12. Dehydrate the slides as follows: incubate in distilled water for 2 min, dip 3 times in 80% ethanol, incubate in 95% ethanol 2 times for 2 min each, incubate in 100% ethanol 2 times for 2 min each, and finally in xylene 3 times for 3 min each.
13. Coverslip the slides with a xylene-based mounting medium and let them dry on a flat surface at RT until they are ready for imaging.
    NOTE: If performing 3,3'-diaminobenzidine (DAB) staining, the slides can be stored at RT. However, if immunofluorescence staining is performed, slides should be stored in the dark at -20 °C to reduce photo-bleaching.

Representative Results

To monitor tumor development and evaluate if the tumor has reached the size necessary to generate NS, we analyzed the luminescence emitted by tumors using bioluminescence. This allows the study of transduction efficiency in the pups and tumor progression by the luminescence signal of luciferase (**Figure 1A** and **1B**). When the tumor size reaches a signal of 1 x 10^7 photons/s/cm2/sr (**Figure 1B**), the brain can be processed for NS generation. Another advantage of this system is that the coding sequence of different fluorescent proteins can be added to the plasmids used for gene delivery and tumor generation. We can then confirm the expression of the inserted genetic alterations, using a dissecting microscope equipped with a fluorescent lamp (**Figure 1C**). The NS generated from wt-IDH1 (**Figure 1D**) and mIDH1 (**Figure 1E**) tumors are identified by the characteristic spherical morphology, and the expression of fluorophores associated with the specific genetic lesion (*i.e.*, GFP associated with shATRX and shp53, and Katushka associated with mIDH1; **Figure 1D** and **1E**). To proceed with the IHC on NS, cells are fixed, then embedded in agarose and paraffin (**Figure 2**). Embedding the NS in paraffin blocks has several advantages, including the preservation of 3D structures of the NS and allowing for long-term storage. The embedded NS can be stained for specific glioma biomarkers. Using this technique, we analyzed the expression of ATRX, Ki67 and OLIG2 (oligodendrocyte differentiation marker) (**Figure 3A-3C**). We confirmed the low expression levels of ATRX protein in our NS cultures (**Figure 3A**), which is a feature of the LGG glioma subtype currently being studied[4]. Also, Ki-67, a well-described biomarker of cell proliferation, was positive in the mIDH1 NS (**Figure 3B**), demonstrating active proliferation, which is a key feature of tumor cells. Interestingly, cells localized in the center of the larger glioma NS clusters were negative for Ki-67 (**Figure 3B**), indicating that they were not proliferating, unlike the Ki-67 positive cells localized to the periphery. This phenomenon may be due to the fact that peripheral cells do not have access to nutrients from the growth media. When they reach this larger size, the glioma NS should be dissociated and passaged. Finally, wt-IDH1 NS were positive for Olig2, a transcription factor participating in oligodendrocyte differentiation (**Figure 3C**).
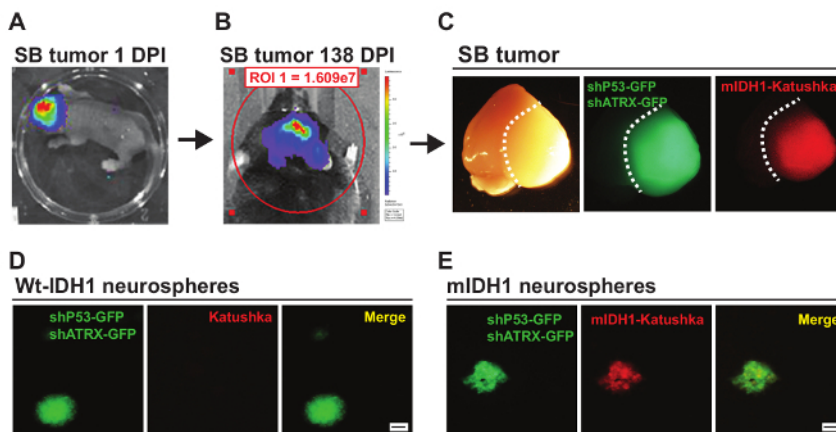

Figure 1: Glioma neurospheres generated from GFP- and Katushka-positive sleeping beauty (SB) brain tumor. (A) Luminescent signal of a neonate mouse injected with SB-transposase plasmid in combination with plasmids harboring genetic lesions to induce glioma (NRAS/shP53/shATRX/IDH1-R132H). (B) Luminescence indicating tumor development at 132 days post-injection (DPI). (C) A bright-field and fluorescent image of a brain harvested from a mouse that reached the end-point stage. The tumor area is delineated by the dotted line and positive for the fluorescent reporters, GFP and Katushka. (D and E) Tumor neurospheres generated from SB brain tumors expressing GFP associated to shP53 and shATRX; and Katushka associated to IDH1-R132H. Scale bar = 100 µm. Please click here to view a larger version of this figure.
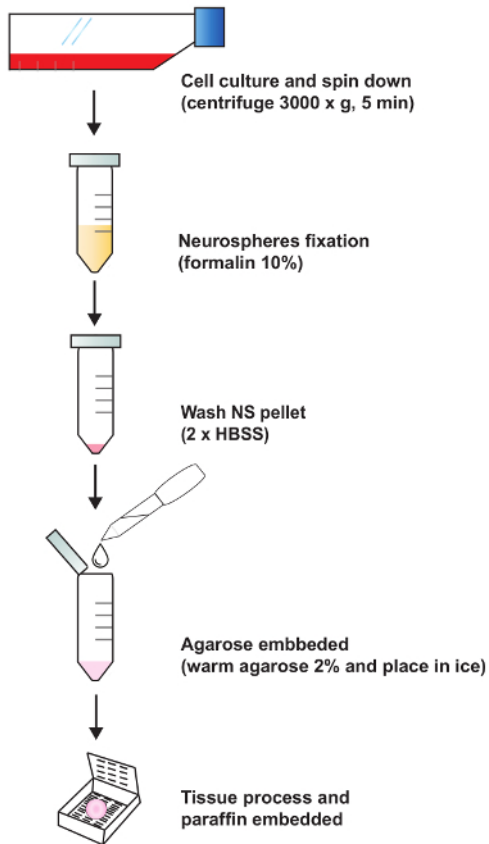
Figure 2: Representation of the workflow for paraffin-embedded glioma NS. NS are collected from a T-25 flask into a 15 mL conical tube and fixed in 10% formalin. Then, NS are washed with HBSS and embedded in 2% agarose. Polymerized agarose containing glioma NS are placed in a cassette for tissue processing and paraffin embedding. Please click here to view a larger version of this figure.
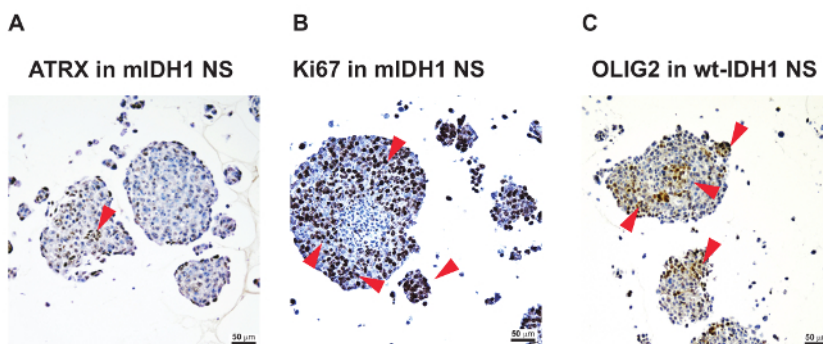

Figure 3: Representative results of immunohistochemistry with HRP-DAB detection of biomarkers. (A-C) IHC performed on paraffin-embedded mIDH1 glioma NS stained for ATRX (A) and Ki67 (B), and wt-IDH1 glioma NS stained for OLIG2 (C). Red arrows indicate positive cells for the biomarker staining. Scale bar = 50 µm. Please click here to view a larger version of this figure.

Discussion

In this article, we detail a versatile and reproducible method to perform immunohistochemistry on paraffin-embedded glioma NS, which maintain the characteristic 3D structure of tumor cells growing in the brain *in situ*. This technique possesses the following advantages over using cells plated onto glass or plastic surfaces: i) preservation of the 3D structure of NS; ii) avoiding of stress of cell dissociation before analysis of protein expression; iii) quenching of any endogenous fluorophore expression from genetically engineered tumor NS, enabling staining across the fluorescent spectra; and iv) long-term storage.

A critical step of this technique is to ensure that the cells are re-suspended well in the agarose to ensure that they are homogenously distributed and not clumped together. One limitation of this technique is the time-consuming steps related to cell culturing and NS processing before the

IHC staining procedure. A second limitation is the high number of cells needed to execute this method. If the number of cells is less than 1×10^6 cells, there will be too few cells per field of view NS clusters in each section. Therefore, it could be difficult to reach the ideal number of cells if the glioma cell culture is slow-growing. We used a confluent T-25 flask containing at least 3.0×10^6 cells. The number of cells used can be modified as desired; however, it is necessary to obtain a NS pellet of appropriate size for the embedding procedure. After this, the embedded NS can be manipulated as a regular paraffin-embedded block, and it can then proceed to sectioning and IHC staining to analyze protein expression by immunofluorescence or IHC using DAB staining.

After sectioning of the blocks and following the protocol for IHC, sections should be counterstained with hematoxylin (blue). If tissue expresses the target protein, a brown signal should be visible (**Figure 3**). If after IHC the sections are too brown (high background), this could be due to 1) non-specific biding of the secondary antibody, 2) an incomplete quenching of the endogenous peroxidase, or 3) insufficient blocking. These issues can be solved by 1) using a negative control slide (incubated only with a secondary antibody) to check non-specific binding of the secondary antibody, 2) increasing the peroxidase quenching time, or 3) increasing the blocking incubation period. On the other hand, if no signal is detected, it is possible that the epitopes are still masked, which may be related to the type of buffer used for antigen retrieval. In our experience, we have had satisfactory staining using citrate buffer, but this can be tissue- and antibody-dependent, and different formulations for antigen retrieval buffers can be used such as 0.1 M Tris-HCl (pH 8.0) buffer[21]. These are some of the most common issues related to IHC staining, but if other difficulty arises, troubleshooting should be carried out as with any other IHC (*i.e.*, trying different dilutions of the primary and secondary antibodies, different blocking reagents, different blocking times, *etc*).

An alternative method to evaluate protein expression using paraffin-embedded cells was previously described by Hasselbach *et al.*[20]. This method does not include the agarose steps, which in this protocol enable adequate special distribution of the 3D cell clusters. We also describe an accurate method for NS culturing, passaging, and freezing for long-term storage. This method also illustrates how to collect, fix, and embed NS without altering 3D structure (**Figure 2**).

Since the NS can be generated from glioma tissue obtained from patients and glioma animal models, this technique constitutes a powerful tool that can be used to analyze biomarker expression in different glioma suptypes and validate the original models. Here we describe the technique with NS originated from genetically engineered mice using the SB transposase system (**Figure 1**). However, this technique can also be used to analyze protein in paraffin-embedded human glioma cells that grow as 3D cultures without modifications to the protocol, other than the cell culture conditions. So, this method may also be applied to 3D cultures obtained from transplantable animal models, human PDX models, and other genetically engineered models. Lastly, this method may be used with other types of cancer, both mouse models and human cancers, in addition to gliomas.

Disclosures

The authors have nothing to disclose.

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