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

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JoVE

Dear Dr. Singh,

Thank you very much for your review of our manuscript entitled **"Evaluation of Biomarkers in Glioma by Immunohistochemistry on Paraffin Embedded 3D-Glioma Neurosphere Cultures".** We are now enclosing the revised manuscript for your consideration and a document that addresses each editorial comment individually.

We hope that our revised manuscript will now be acceptable for publication in JoVE. Please let me know if you have any further questions or issues.

Yours sincerely,

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1 **TITLE:**
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3 Neurosphere Cultures
4
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25 **KEYWORDS:**
26 Genetically engineered, glioma, neurospheres, 3D-cultures, immunostaining, biomarkers,
27 paraffin embedding
28
29 **SUMMARY:**
30 Neurospheres grown as 3D cultures constitute a powerful tool to study glioma biology. Here we
31 present a protocol to perform immunohistochemistry while maintaining the 3D structure of
32 glioma neurospheres through paraffin embedding. This method enables the characterization of
33 glioma neurosphere properties such as stemness and neural differentiation.
34
35 **ABSTRACT:**
36 Analysis of protein expression in glioma is relevant for several aspects in the study of its
37 pathology. Numerous proteins have been described as biomarkers with applications in diagnosis,
38 prognosis, classification, state of tumor progression, and cell differentiation state. These analyses
39 of biomarkers are also useful to characterize tumor neurospheres (NS) generated from glioma
40 patients and glioma models. Tumor NS provide a valuable *in vitro* model to assess different
41 features of the tumor from which they are derived and can more accurately mirror glioma
42 biology. Here we describe a detailed method to analyze biomarkers in tumor NS using
43 immunohistochemistry (IHC) on paraffin-embedded tumor NS.
44

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[16-18]. 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

89　fluorescent spectra. The overall goal of this method is to preserve the 3D structure of glioma cells
90　through a paraffin embedding process and enable characterization of glioma neurospheres using
91　immunohistochemistry.
92
93　**PROTOCOL:**
94　All methods described here have been approved by the Institutional Animal Care and Use
95　Committee (IACUC) of the University of Michigan.
96
97　**1. Generation of Brain Tumor Neurospheres Derived from a Mouse Model**
98
99　1.1 Before the dissection procedure, prepare neural stem cell media (NSC Media: DMEM/F12
100　with 1x B27 supplement, 1x N2 supplement, 1x normocin, and 1x antibiotic/antimycotic
101　supplemented with human recombinant EGF and basic-FGF at concentrations of 20 ng/mL each).
102　Fill a 1.5 mL tube with 300 μL of NSC media and reserve for later.
103
104　1.2 Select a mouse with a large tumor.
105
106　NOTE: The size of a tumor can be monitored by bioluminescence if the plasmid or virus injected
107　encodes luciferin. Usually, a large tumor has a bioluminescence of > 10^6 photons/s/cm^2/sr.
108
109　1.3 Euthanize the mouse with an overdose of isoflurane: infuse a tissue paper with isoflurane and
110　introduce the tissue in a euthanization chamber, avoiding direct contact with the mice to prevent
111　irritation. Wait until the mice do not show a pedal reflex (usually 5-10 min). Decapitate the mouse
112　and dissect the brain as described in Calinescu *et al.*[13].
113
114　1.4 If the tumors are fluorescent, use a dissecting microscope equipped with a fluorescent lamp
115　to identify the tumor mass within the brain. With fine forceps, dissect the tumor (usually 5-7 mm
116　in diameter) from the normal brain tissue.
117
118　1.5 Place the dissected tumor in the 1.5 mL tube with NSC media (step 1.1). Homogenize the
119　tumor with a disposable plastic pestle that fits into the walls of the microcentrifuge tube by
120　applying gentle pressure.
121
122　1.6 To dissociate the cell clumps, add 1 mL of enzyme-free dissociation media and incubate for 5
123　min at 37 °C.
124
125　1.7 Filter the cell suspension through a 70 μm cell strainer and wash the strainer with 20 mL of
126　NSC media.
127
128　1.8 Centrifuge at 300 x g for 5 min, decant the supernatant, and re-suspend the pellet into 6 mL
129　or 15 mL of NSC media, depending on the size of the pellet.
130
131　1.9 Plate the tumor cell suspension into a T-75 culture flask and culture at 37 °C in a tissue culture
132　incubator with an atmosphere of 95% air and 5% CO_2.

133

134   1.10 After 3 days, to remove dead cells, transfer the cell suspension into a 15 mL conical tube
135   and collect only the cells that sink to the bottom using a micropipette.

136

137   NOTE: There will usually be a mixed population of cells, some will be dead, some will have
138   adhered, and some will be forming NS that will be floating in the media (the cells that sink are
139   the healthy NS). Transfer the healthy NS and re-plate them into a T-25 or T-75 tissue culture flask.
140   If necessary, add more NSC media to the culture.

141

142   **2. Maintaining and Passaging the NS**

143

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

147

148   2.1 Once the NS culture has reached confluency, collect the culture in a sterile centrifuge tube
149   and pellet the NS at 300 x g for 5 min.

150

151   2.2 Discard the supernatant, add 1 mL of the cell detachment solution, and pipette to re-suspend
152   the pellet.

153

154   2.3 Incubate at 37 °C for 5 min, flicking the tube every 2 min to help cell dissociation.

155

156   NOTE: When NS reach confluency, they can be usually seen macroscopically as small white
157   spheres. To assure cell dissociation, at least all the visible NS must have disappeared.

158

159   2.4 Add 5 mL of HBSS 1x and pipette several times. Pellet cells at 300 x g for 5 min. Discard the
160   supernatant. Re-suspend the pellet in 5 mL of NSC media supplemented with recombinant EGF
161   and basic-FGF.

162

163   2.5 Add 1 mL of cells to 15 mL of NSC media and culture in T-75 flasks at 37 °C in a tissue culture
164   incubator with an atmosphere of 95% air and 5% CO_2.

165

166   2.6 Add growth factors (EGF and basic-FGF) every 3 days. If the media turns light orange and the
167   cells are not yet confluent, change the media. To do this, centrifuge the NS at 300 x g for 4 min
168   and re-suspend in NSC media supplemented with growth factors.

169

170   NOTE: Depending on the size of the pellet formed, cells may need to be split into more flasks.

171

172   **3. Freezing NS for Long-term Storage**

173

174   3.1 When the NS culture becomes confluent, dissociate the cells as described in steps 2.1-2.4.
175   Once the pellet has been re-suspended in HBSS, remove an aliquot and count the cells.

176

177   3.2 Centrifuge cells at 300 x g for 4 min and remove as much as possible of the supernatant.

178

179   3.3 Re-suspend the pellet in freezing media (heat-inactivated FBS, 10% DMSO). It is
180   recommended to freeze between 1×10^6 and 3×10^6 cells per vial per mL.

181

182   3.4 Divide the re-suspended cells in as many cryovials as needed and slowly cool down the vials
183   by first transferring them to ice, then to -20 °C, then to -80 °C, and finally the next day to a liquid
184   nitrogen storage container.

185

186   **4. Fixation of Tumor NS**

187

188   4.1 Culture tumor NS in a T-25 flask for 2-3 days until cells are confluent (~ 3×10^6 cells). Collect
189   tumor NS in a 15 mL conical tube from a least one T-25 confluent flask. Pellet at 300 x g for 5 min.

190

191   4.2. Re-suspend the pellet in 1 mL of 10% formalin and keep at room temperature (RT) for 10
192   min. Add 5 mL of 1x HBSS and pellet the cells as done in step 3.2. Repeat this step one more time.

193

194   4.3. Place the 15 mL conical tube containing the pellet on ice.

195

196   **5. Paraffin Embedding of Tumor NS**

197

198   5.1 Re-suspend the washed NS pellet in 500 µL of 2% warm liquid agarose and mix gently by
199   pipetting.

200

201   5.2 Immediately place the agarose-embedded NS on ice until the agarose polymerizes. Remove
202   the agarose piece harboring the NS. Place the polymerized agarose piece with the NS into the
203   cassette for tissue processing (**Figure 2**).

204

205   5.3 Continue with tissue processing (using an automatic paraffin processor) and paraffin
206   embedding (using an embedding station).

207

208   **6. Sectioning of Paraffin-embedded NS**

209

210   6.1 Set the water bath to 42 °C and insert the blade on the microtome carefully using two paint
211   brushes to make sure it is leveled. Use this blade only for trimming.

212

213   6.2 Place the paraffin block on the microtome. With the non-dominant hand, press down on the
214   lever located on left-hand side that controls the thickness of each section. Press to the second
215   stop that indicates a 30 µm thickness.

216

217   6.3 Begin trimming the block (*i.e.*, removing excess paraffin) by turning the coarse hand wheel
218   with the right hand until the surface of the sample is reached. At this point, release the lever that
219   controls the trim thickness to either 10 µm or, to completely trim, to 5 µm. Stop trimming when
220   the surface of the sample is reached.

221

222 6.4 Fill an ice box with ice and add just enough water so that when the paraffin block is placed
223 on ice, the water acts as a film around the paraffin block, protecting it from directly touching the
224 ice. Incubate the paraffin block on ice for 20 min.

225

226 6.5 Discard the old blade and insert a new one for sectioning. Dry block using gauze, then place
227 it on the microtome.

228

229 6.6 With the non-dominant hand, obtain a pair of curved forceps that will be used to pull the
230 paraffin ribbon. Keep a damp paint brush near the right hand, which will be used to transfer the
231 paraffin ribbon to the water bath. Begin to section by turning the coarse hand wheel with the
232 right hand at a fast pace. Use the forceps in the left hand to hold the paraffin ribbon.

233

234 6.8 Use the damp paint brush to transfer the paraffin ribbon to the water bath.

235

236 6.9 Use the curve of the forceps to break apart the sections by superficially placing the forceps in
237 between two paraffin sections, then pushing the two sections away gently.

238

239 NOTE: This motion should be entirely on the surface of the paraffin section. Do not press down
240 on the section.

241

242 6.10 Use the damp paint brush to push the separated paraffin sections onto positively charged
243 adhesion microscope slides.

244

245 6.11 Allow the slides to dry overnight inside a chemical hood before storing them in slide boxes.
246 The storage of slides depends on the type of stain performed.

247

248 NOTE: If performing 3,3'-diaminobenzidine (DAB) staining, the slides can be stored at RT.
249 However, if immunofluorescence staining is performed, slides should be stored in the dark at   -
250 20 °C to reduce photo-bleaching.

251

252 **7. Immunohistochemistry (IHC) of Paraffin-embedded NS**

253

254 7.1. Melt paraffin by placing the slides in a metal rack and incubating them at 60 °C for 20 min.

255

256 7.2. Deparaffinize the slides by incubation in a rehydration train at RT: 3x xylene for 5 min, 100%
257 2x ethanol for 2 min, 95% 2x ethanol for 2 min, 70% 1x ethanol for 2 min, and 1x deionized water
258 for 2 min. Hereon, keep the slides in tap water until antigen retrieval, as drying out will cause
259 non-specific antibody binding.

260

261 7.3. Incubate the slides in citrate buffer (10 mM citric acid, 0.05% non-ionic detergent, pH 6) at
262 125 °C for 30 s and at 90 °C for 10 s. Let them cool down, then rinse the slides 5 times with distilled
263 water.

264

265   7.4. Incubate slides at RT in 0.3% H_2O_2 for 30 min.
266

267   7.5. Wash the slides 3 times in washing buffer (0.025% detergent in TBS) for 5 min with gentle
268   agitation.
269

270   7.6. Incubate the slides at RT with blocking solution (10% horse serum, 0.1% BSA in TBS) for 30
271   min.
272

273   7.7. Incubate the slides overnight at 4 °C in a humidified chamber with primary antibody prepared
274   in dilution solution (0.1% BSA in TBS). Wash the slides 3 times in washing buffer for 5 min with
275   gentle agitation.
276

277   7.9. Incubate the slides at RT for 1 h with biotinylated secondary antibody prepared in dilution
278   solution. Wash the slides 3 times in washing buffer for 5 min with gentle agitation.
279

280   7.11. Incubate the slides at RT in the dark for 30 min with avidin-biotinylated horseradish
281   peroxidase complex. Wash the slides 3 times in washing buffer for 5 min with gentle agitation.
282

283   7.13. Incubate the slides with DAB-H_2O_2 substrate brands here for 2-5 min at RT.
284

285   7.14. Rinse 3 times with tap water. Dip (1-2 s) the slides in hematoxylin solution to counterstain,
286   and rinse them thoroughly in tap water until clearing.
287

288   7.16. Dehydrate the slides as follows: incubate in distilled water for 2 min, dip 3 times in 80%
289   ethanol, incubate in 95% ethanol 2 times for 2 min each, incubate in 100% ethanol 2 times for 2
290   min each, and finally in xylene 3 times for 3 min each.
291

292   7.17. Coverslip the slides with a xylene-based mounting medium and let them dry on a flat surface
293   at RT until they are ready for imaging.
294

295   **REPRESENTATVE RESULTS:**
296   To monitor tumor development and evaluate if the tumor has reached the size necessary to
297   generate NS, we analyzed the luminescence emitted by tumors using bioluminescence. This
298   allows the study of transduction efficiency in the pups and tumor progression by the
299   luminescence signal of luciferase (**Figure 1A** and **1B**). When the tumor size reaches a signal of 1 x
300   10^7 photons/s/cm2/sr (**Figure 1B**), the brain can be processed for NS generation. Another
301   advantage of this system is that the coding sequence of different fluorescent proteins can be
302   added to the plasmids used for gene delivery and tumor generation. We can then confirm the
303   expression of the inserted genetic alterations, using a dissecting microscope equipped with a
304   fluorescent lamp (**Figure 1C**). The NS generated from wt-IDH1 (**Figure 1D**) and mIDH1 (**Figure 1E**)
305   tumors are identified by the characteristic spherical morphology, and the expression of
306   fluorophores associated with the specific genetic lesion (*i.e.*, GFP associated with shATRX and
307   shp53, and Katushka associated with mIDH1; **Figure 1D** and **1E**). To proceed with the IHC on NS,
308   cells are fixed, then embedded in agarose and paraffin (**Figure 2**). Embedding the NS in paraffin

309 blocks has several advantages, including the preservation of 3D structures of the NS and allowing
310 for long-term storage. The embedded NS can be stained for specific glioma biomarkers. Using
311 this technique, we analyzed the expression of ATRX, Ki67 and OLIG2 (oligodendrocyte
312 differentiation marker) (**Figure 3A-3C**). We confirmed the low expression levels of ATRX protein
313 in our NS cultures (**Figure 3A**), which is a feature of the LGG glioma subtype currently being
314 studied[4]. Also, Ki-67, a well-described biomarker of cell proliferation, was positive in the mIDH1
315 NS (**Figure 3B**), demonstrating active proliferation, which is a key feature of tumor cells.
316 Interestingly, cells localized in the center of the larger glioma NS clusters were negative for Ki-67
317 (**Figure 3B**), indicating that they were not proliferating, unlike the Ki-67 positive cells localized to
318 the periphery. This phenomenon may be due to the fact that peripheral cells do not have access
319 to nutrients from the growth media. When they reach this larger size, the glioma NS should be
320 dissociated and passaged. Finally, wt-IDH1 NS were positive for Olig2, a transcription factor
321 participating in oligodendrocyte differentiation (**Figure 3C**).
322
323 **FIGURE AND TABLE LEGENDS:**
324 **Figure 1**: **Glioma neurospheres generated from GFP- and Katushka-positive sleeping beauty**
325 **(SB) brain tumor**. (A) Luminescent signal of a neonate mouse injected with SB-transposase
326 plasmid in combination with plasmids harboring genetic lesions to induces glioma
327 (NRAS/shP53/shATRX/IDH1-R132H). (B) Luminescence indicating tumor development at 132
328 days post-injection (DPI). (C) A bright-field and fluorescent image of a brain harvested from a
329 mouse that reached the end-point stage. The tumor area is delineated by the dotted line and
330 positive for the fluorescent reporters, GFP and Katushka. (D and E) Tumor neurospheres
331 generated from SB brain tumors expressing GFP associated to shP53 and shATRX; and Katushka
332 associated to IDH1-R132H. Scale bar = 100 μm.
333
334 **Figure 2**: **Representation of the workflow for paraffin-embedded glioma NS.** NS are collected
335 from a t-25 flank into a 15 mL conical tube and fixed in 10% formalin. Then, NS are washed with
336 HBSS and embedded in 2% agarose. Polymerized agarose containing glioma NS are placed in a
337 cassette for tissue processing and paraffin embedding.
338
339 **Figure 3**: **Representative results of immunohistochemistry with HRP-DAB detection of**
340 **biomarkers**. (A-C) IHC performed on paraffin-embedded mIDH1 glioma NS stained for ATRX (A)
341 and Ki67 (B), and wt-IDH1 glioma NS stained for OLIG2 (C). Red arrows indicate positive cells for
342 the biomarker staining. Scale bar = 50 μm.
343
344 **DISCUSSION:**
345 In this article, we detail a versatile and reproducible method to perform immunohistochemistry
346 on paraffin-embedded glioma NS, which maintain the characteristic 3D structure of tumor cells
347 growing in the brain *in situ*. This technique possesses the following advantages over using cells
348 plated onto glass or plastic surfaces: i) preservation of the 3D structure of NS; ii) avoiding of stress
349 of cell dissociation before analysis of protein expression; iii) quenching of any endogenous
350 fluorophore expression from genetically engineered tumor NS, enabling staining across the
351 fluorescent spectra; and iv) long-term storage.
352

353 A critical step of this technique is to ensure that the cells are re-suspended well in the agarose to
354 ensure that they are homogenously distributed and not clumped together. One limitation of this
355 technique is the time-consuming steps related to cell culturing and NS processing before the IHC
356 staining procedure. A second limitation is the high number of cells needed to execute this
357 method. If the number of cells is less than 1×10^6 cells, there will be too few cells per field of view
358 NS clusters in each section. Therefore, it could be difficult to reach the ideal number of cells if
359 the glioma cell culture is slow-growing. We used a confluent T-25 flask containing at least 3.0 x
360 10^6 cells. The number of cells used can be modified as desired; however, it is necessary to obtain
361 a NS pellet of appropriate size for the embedding procedure. After this, the embedded NS can be
362 manipulated as a regular paraffin-embedded block, and it can then proceed to sectioning and
363 IHC staining to analyze protein expression by immunofluorescence or IHC using DAB staining.
364
365 After sectioning of the blocks and following the protocol for IHC, sections should be
366 counterstained with hematoxylin (blue). If tissue expresses the target protein, a brown signal
367 should be visible (**Figure 3**). If after IHC the sections are too brown (high background), this could
368 be due to 1) non-specific biding of the secondary antibody, 2) an incomplete quenching of the
369 endogenous peroxidase, or 3) insufficient blocking. These issues can be solved by 1) using a
370 negative control slide (incubated only with a secondary antibody) to check non-specific binding
371 of the secondary antibody, 2) increasing the peroxidase quenching time, or 3) increasing the
372 blocking incubation period. On the other hand, if no signal is detected, it is possible that the
373 epitopes are still masked, which may be related to the type of buffer used for antigen retrieval.
374 In our experience, we have had satisfactory staining using citrate buffer, but this can be tissue-
375 and antibody-dependent, and different formulations for antigen retrieval buffers can be used
376 such as 0.1 M Tris-HCl (pH 8.0) buffer[21]. These are some of the most common issues related to
377 IHC staining, but if other difficulty arises, troubleshooting should be carried out as with any other
378 IHC (*i.e.*, trying different dilutions of the primary and secondary antibodies, different blocking
379 reagents, different blocking times, *etc*).
380
381 An alternative method to evaluate protein expression using paraffin-embedded cells was
382 previously described by Hasselbach *et al.*[20]. This method does not include the agarose steps,
383 which in this protocol enable adequate special distribution of the 3D cell clusters. We also
384 describe an accurate method for NS culturing, passaging, and freezing for long-term storage. This
385 method also illustrates how to collect, fix, and embed NS without altering 3D structure (**Figure
386 2**).
387
388 Since the NS can be generated from glioma tissue obtained from patients and glioma animal
389 models, this technique constitutes a powerful tool that can be used to analyze biomarker
390 expression in different glioma suptypes and validate the original models. Here we describe the
391 technique with NS originated from genetically engineered mice using the SB transposase system
392 (**Figure 1**). However, this technique can also be used to analyze protein in paraffin-embedded
393 human glioma cells that grow as 3D cultures without modifications to the protocol, other than
394 the cell culture conditions. So, this method may also be applied to 3D cultures obtained from
395 transplantable animal models, human PDX models, and other genetically engineered models.
396 Lastly, this method may be used with other types of cancer, both mouse models and human

397 cancers, in addition to gliomas.
398

DISCLOSURES:

The authors have nothing to disclose.

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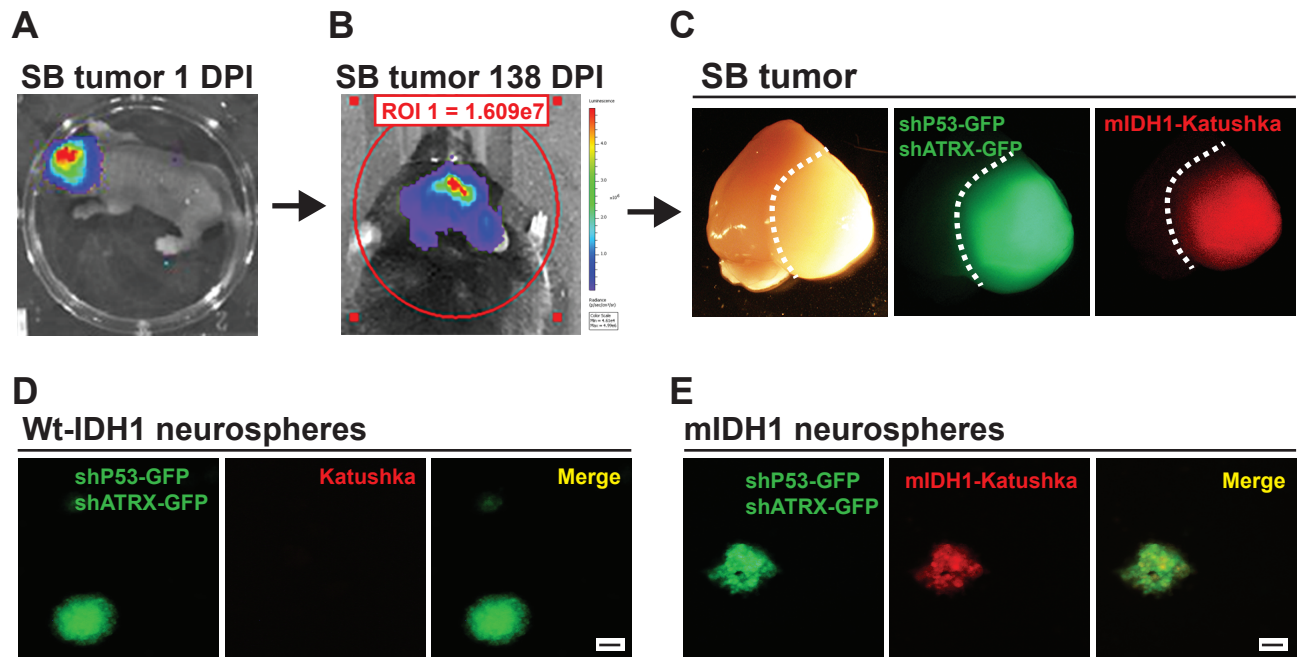
459

Fig 1. Nunez et al JoVE 2018

A   SB tumor 1 DPI
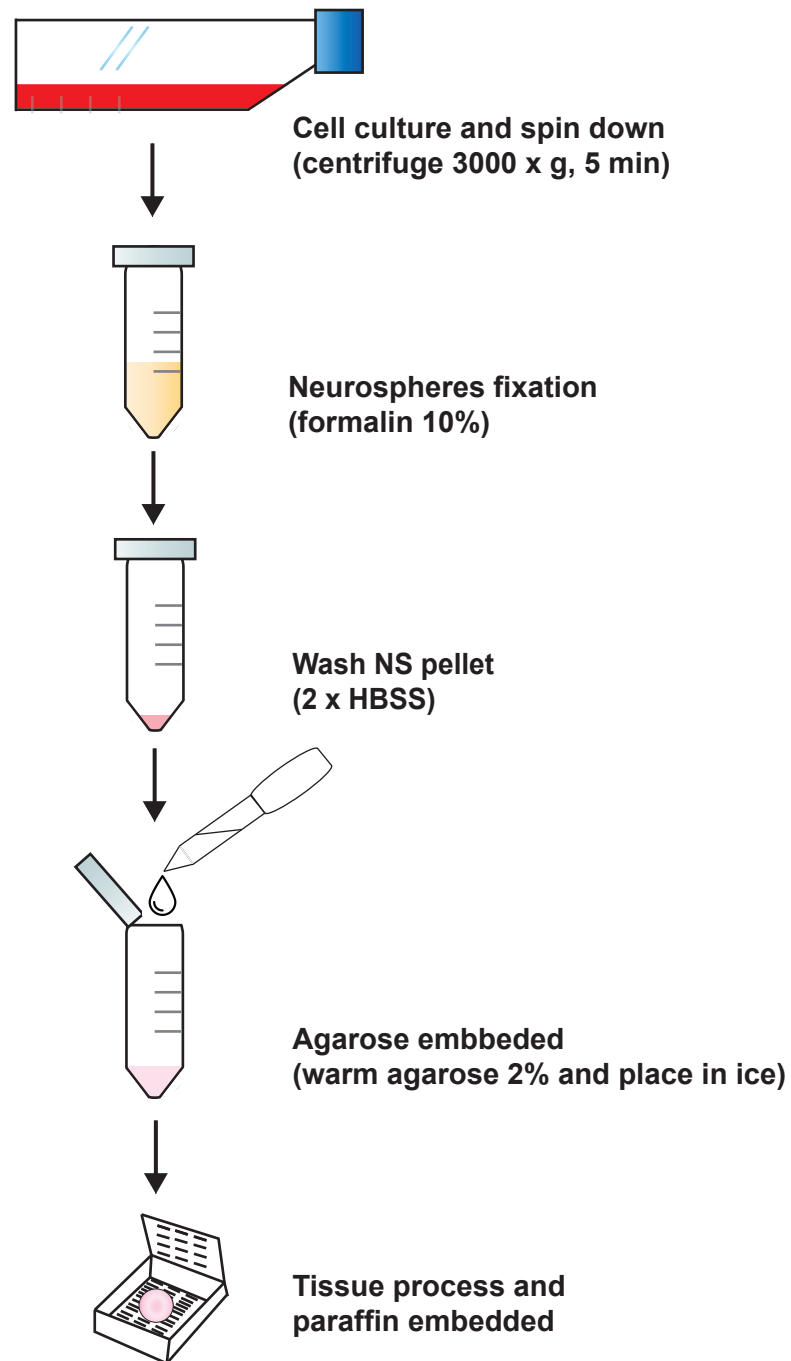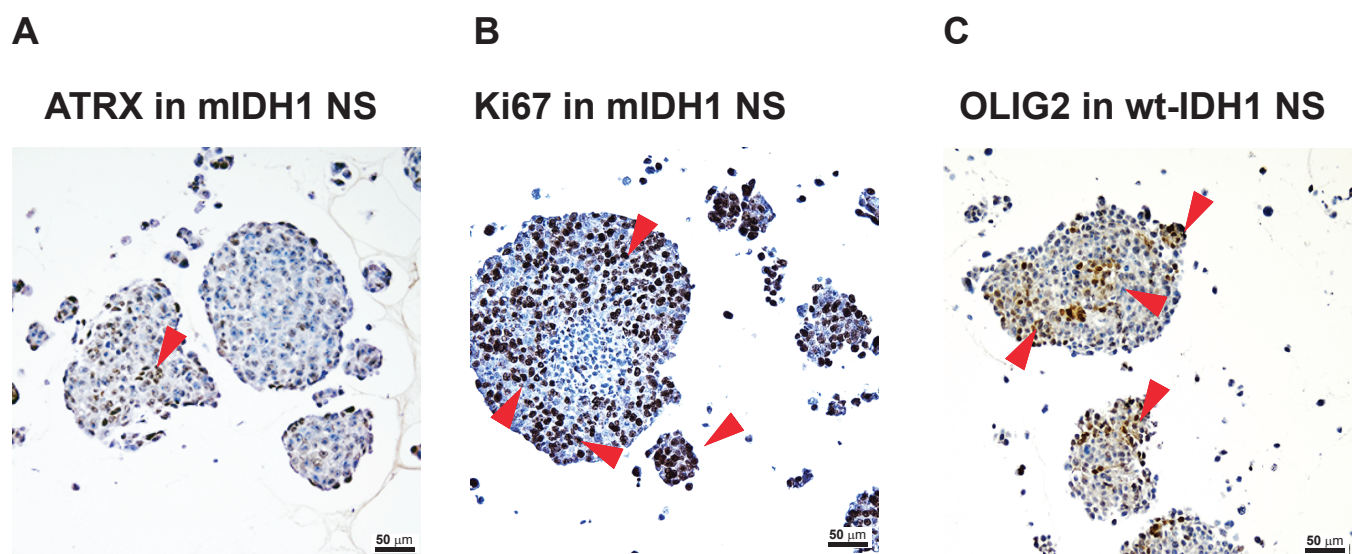
B   SB tumor 138 DPI

C   SB tumor

shP53-GFP shATRX-GFP   mIDH1-Katushka

D   Wt-IDH1 neurospheres

shP53-GFP shATRX-GFP   Katushka   Merge

E   mIDH1 neurospheres

shP53-GFP shATRX-GFP   mIDH1-Katushka   Merge

Fig 2. Nunez et al JoVE 2018

**Cell culture and spin down
(centrifuge 3000 x g, 5 min)**

**Neurospheres fixation
(formalin 10%)**

**Wash NS pellet
(2 x HBSS)**

**Agarose embbeded
(warm agarose 2% and place in ice)**

**Tissue process and
paraffin embedded**

Figure 3

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Fig 3. Nunez et al JoVE 2018

A

ATRX in mIDH1 NS

B

Ki67 in mIDH1 NS

C

OLIG2 in wt-IDH1 NS

table of materials

Name of Material/ Equipment	Company	Catalog Number
Coated microtome blade HP35	Thermo Scientific	3150734
Microtome RM 2135	Leica	MR2135
Formalin solution, neutral buffered, 10%	Sigma-aldrich	HT501128-4L
Rabbit polyclonal anti-ATRX,	Santa Cruz Biotechnology	sc-15408
Rabbit polyclonal anti-Ki-67	Abcam	Ab15580
Rabbit polyclonal anti-OLIG2	Millipore	AB9610
Goat polyclonal anti-rabbit biotin-conjugated	Dako	E0432
Vectastain Elite ABC HRP kit	Vector Laboratories Inc	PK-6100
BETAZOID DAB CHROMOGEN KIT	Biocare medical	BDB2004 L/price till 12/18
N-2 Supplement	ThermoFisher	17502048
N-27 Supplement	ThermoFisher	A3582801
Accutase® Cell Detachment Solution	Biolegend	423201
AGAROSE LE	GoldBio	A-201-1000
Genesee Sc. Corporation	Olympus 15 ml	21-103
Genesee Sc. Corporation	TC-75 treated Flask	25-209
Genesee Sc. Corporation	TC-25 treated Flask	25-207
DMEM/F12	Gibco	11330-057
HBSS	Gibco^{TM}	14175-103
C57BL/6	Taconic	B6-f

Comments/Description

IHC, 1:250 dilution
IHC, 1:1000 dilution
IHC, 1:500 dilution
IHC, 1:1000 dilution

NS media
balanced salt solution
C57BL/6 mouse

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

Author(s):
Felipe J. Núñez, Flor M. Mendez, M. Belen Garcia-Fabiani, Marta Edwards, Pedro R. Lowenstein, and Maria G. Castro

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Institution: University of Michigan, Medical School

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1- Please do not write the representative result in the legend style. Please explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Thank you very much for this comment. We re-wrote the representative results section in the context of the technique that we described as the editorial reviewer requested.

2- 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
Please ensure that you make different paragraphs for each of the points mentioned above.

We re-organized our discussion according to the editorial reviewer's suggestion

3- Some parts of this can be moved to the representative result section.

We moved parts of the discussion to the representative results section as requested.

4- Please do not abbreviate journal titles.

We added full journal titles as requested.