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Laboratory studies

Sulforhodamine 101 selectively labels human astrocytoma cells in an animal model of glioblastoma

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ABSTRACT

Sulforhodamine 101 (SR101) is a useful tool for immediate staining of astrocytes. We hypothesized that if the selectivity of SR101was maintained in astrocytoma cells, it could prove useful for glioma research. Cultured astrocytoma cells and acute slices from orthotopic human glioma (n = 9) and lymphoma (n = 6) xenografts were incubated with SR101 and imaged with confocal microscopy. A subset of slices (n = 18) were counter-immunostained with glial fibrillary acidic protein and CD20 for stereological assessment of SR101 co-localization. SR101 differentiated astrocytic tumor cells from lymphoma cells. In acute slices, SR101 labeled 86.50% (±1.86; $p < 0.0001$) of astrocytoma cells and 2.19% (±0.47; $p < 0.0001$) of lymphoma cells. SR101-labeled astrocytoma cells had a distinct morphology when compared with *in vivo* astrocytes. Immediate imaging of human astrocytoma cells *in vitro* and in *ex vivo* rodent xenograft tissue labeled with SR101 can identify astrocytic tumor cells and help visualize the tumor margin. These features are useful in studying astrocytoma in the laboratory and may have clinical applications.

1. Introduction

Sulforhodamine 101 (SR101) is a red fluorescent dye that has been used in neuroscience research for the rapid and specific labeling of astrocytes [1,2]. Its underlying mechanism is not completely understood, but much like glial fibrillary acidic protein (GFAP), SR101 labels astrocytic cells and has been used to rapidly label rodent astrocytoma cells in culture [3]. If SR101 also labels glioblastoma (GBM) and other tumor cells of astrocytoma lineage, it could provide a more timely alternative to GFAP for identifying astrocytoma cells in glioma models. Furthermore, it may allow the rapid and definitive differentiation of glioma from other tumors, such as lymphoma, during intraoperative diagnosis. We aimed to establish whether the selective staining of astrocytic tumors by SR101 is a reliable and reproducible method for rapidly identifying human astrocytoma cells in cell culture and animal models, and to test this against a negative control central nervous system (CNS) lymphoma animal model. We hypothesized that the combined use of live-cell imaging with targeted fluorophores could provide a rapid method for confirming the astrocytic lineage of tumors. If so, SR101 may prove to be a rapid alternative to GFAP immunohistochemistry in such models.

2. Materials and methods

2.1. Cell culture

We acquired human glioma cell line U251 and human CNS lymphoma cell line MC116 from American Type Culture Collection. The cell lines were maintained in culture with Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% fetal bovine serum (FBS), and Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 20% FBS (all from Invitrogen, Grand Island, NY, USA). Cells were grown at 37°C in a humidified incubator under 5% CO_2.

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2.2. In vitro SR101 labeling

U251 glioma cells were labeled by incubating 100,000 cells on a collagen-coated glass-bottom dish (MatTek, Ashland, MA, USA). After 24 hours, the medium was replaced with artificial cerebrospinal fluid (aCSF) containing 2 μM SR101 (Sigma-Aldrich, St Louis, MO, USA) for 20 minutes, followed by two 5 minute washes with standard aCSF.

2.3. Animals

Fifteen male Crl:NIH-Foxn1rnu rats (5 weeks of age) were obtained from The Charles River Laboratories International (Wilmington, MA, USA). Experiments were performed in accordance with the guidelines and regulations set forth by the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Barrow Neurological Institute of St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA.

2.4. Intracranial implantation

Rats were anesthetized by intramuscular injection of a mixture of 10 mg/kg xylazine and 80 mg/kg ketamine (Wyeth, Madison, NJ, USA) and placed in a small animal stereotactic headframe (Model 900, David Kopf Instruments, Tujunga, CA, USA). A 10 mm incision was made starting between the animal's eyes to expose bregma. A burr hole was made 3.5 mm lateral to bregma. U251 (nine rats) or MC116 cells (six rats) were infused at a depth of 4.5 mm below the surface of the brain after the syringe (Hamilton, Reno, NV, USA) was advanced 5.0 mm to create a 0.5 mm pocket. The cell suspension was infused using a UMP3-1 UltraMicroPump microinjector (WPI, Sarasota, FL, USA) set to a volume of 10 μL with an infusion rate of 3 μL/minute. The needle was withdrawn 2 minutes after the injection to minimize backflow of the cell suspension. The burr hole was covered with bone wax, the skin incision was sutured, and the rats were allowed to recover.

2.5. Acute slices

Twenty-eight days after implantation, rats were deeply anesthetized using the xylazine/ketamine mixture as described previously. They were immediately decapitated, and their brains were removed. Immediately, coronal slices (350 μm thick) were cut from the cerebral cortex on a Leica VT1200 vibratome (Leica Biosystems, Nussloch, Germany), in aCSF containing the following (in mM): 126 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$ and 10 glucose, pH 7.4. Slices were then incubated at room temperature in aCSF containing 2 μM SR101 for 20 minutes followed by a 10 minute wash in aCSF. A two-tailed paired *t*-test with alpha set to 0.05 was used to compare mean fluorescence intensity between tumor cells and reactive astrocytes.

2.6. Co-labeling

SR101 is not amenable to fixation; we therefore used a fixable version of SR101 (Texas Red Hydrazide; Sigma-Aldrich) for these experiments. The staining pattern of fixable SR101 fluorophore mimics the staining pattern of the standard nonfixable SR101 [1,2]. For clarity, the use of the fixable version of SR101 is clearly indicated by the term "fixable-SR101" throughout the text. Fixable-SR101 and the nonfixable version had similar staining patterns, but the intensity and cellular staining of the fixable version was weaker than that of the standard SR101.

Acute xenograft slices were incubated with fixable-SR101, washed at room temperature, and fixed with 4% paraformaldehyde for 12 hours at 4°C. The sections were rinsed in phosphate-buffered saline, permeabilized with 0.3% Iriton, and blocked with CAS block (Invitrogen) [4]. The GBM xenograft slices (nine slices from three animals) were incubated in anti-GFAP primary antibody (EMD Millipore, Billerica, MA, USA; 1:500) for 12 hours, and the lymphoma sections (nine slices from three animals) were incubated in anti-CD20 primary antibody (EMD Millipore; 1:250) for 12 hours. Sections were then rinsed and incubated with Alexa-Fluor488 secondary antibody (Invitrogen), followed by 4′,6-diamidino-phenylindole (DAPI; Invitrogen) nuclear counterstain and mounted on slides with vectashield (Vector Laboratories, Burlingame, CA, USA) and number 1.5 coverslips (VWR, Radnor, PA, USA).

2.7. Stereology

We adapted standard stereology approaches to quantify tumor cells labeled with fixable-SR101 and GFAP or CD20 antibodies [5,6]. We selected one rostral, midline, and caudal acute slice from each brain containing tumor incubated with fixable-SR101. Glioma slices were immunofluorescently stained for GFAP, and slices containing lymphoma were stained for CD20. In each slice, 10 randomly selected tumor regions (150 μm^2) were optically sectioned to 50 μm with a Zeiss 710LSM (Carl Zeiss Surgical, Oberkochen, Germany). The first 5 μm of each image stack was discarded to minimize counts from cells damaged during sectioning. A maximum intensity projection image was generated from the remaining 45 μm, and a stereology dissector was overlaid onto the image. Cells within the dissector and those in contact with its left and bottom edges were counted for either GFAP or CD20 positivity and for SR101 positivity.

The percent overlap between immunostaining and SR101 positivity was calculated. Two-tailed *t*-tests with alpha levels of 0.05 were used to determine statistical differences. A paired *t*-test was used to determine if staining localization between antibody and SR101-labeling differed between cell types. An unpaired *t*-test was used to compare fixable-SR101 staining between glioma and lymphoma models.

2.8. Imaging

SR101-labeled samples were placed in uncoated number 1.5 glass-bottom dishes and positioned on the stage of a Zeiss 710 laser scanning confocal microscope equipped with a 40 ×/1.2 NA water immersion objective (Carl Zeiss Surgical). We imaged SR101 by exciting the fluorophore with a 561 nm diode laser and collecting 595 nm to 625 nm emissions. The confocal aperture was set to one Airy unit for imaging. The laser and gain values were set to fill the dynamic range of the photomultiplier tube, and the frame size was set to sample at Nyquist. Images were collected in 8 and 12-bit format absent of nonlinear processing.

2.9. Rapid uptake

To establish the time course of SR101 uptake, U251 astrocytoma cells were visualized using the confocal microscope as previously described. Astrocytoma cells were cultured on number 1.5 glass-bottom dishes and placed on the stage of an inverted confocal microscope. Time lapse images were collected 30 seconds prior to addition of SR101 to 7 minutes post-incubation. Change in fluorescence intensity of astrocytoma cells and background was calculated with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

3. Results

3.1. SR101 labels human astrocytoma cells and reactive astrocytes

To investigate the potential of SR101 for identifying human astrocytoma cells, we used differential interference contrast with fluorescence overlay to image human astrocytoma cell line U251 after incubation in SR101 (six cultures). The fluorophore filled the cytoplasm of the cultured cells and clearly delineated cell nuclei (Fig. 1A, B).

Next, human astrocytoma cells (U251 cell line) were implanted into the caudate-putamen of six nude rats and allowed to grow for 4 weeks before the rats were sacrificed. This orthotopic xenograft model consistently produced astrocytic tumors as previously characterized [7,8]. Live cell confocal images of acute slices from the cerebral cortex of the implanted animals (Fig. 1C) treated with SR101 showed cells in the tumor core markedly labeled with the fluorophore. The cells were easily distinguished from low level background staining (Fig. 1D–F).

Confocal microscopy imaging of the acute slices treated with SR101 also showed distinct tumor margins that contained SR101-positive astrocytoma cells and reactive astrocytes (Fig. 1G, H). The fluorescence intensity from the astrocytoma cells and reactive astrocytes was quantified. The mean fluorescence intensity did not differ between the two cell types (Fig. 1I). However, reactive astrocytes were easily distinguished based on their distinct morphology (Fig. 1H). Therefore, SR101 can rapidly identify astrocytes and astrocytoma cells in cell culture and animal models, and it can effectively define tumor margins in an animal model.

3.2. Fixable-SR101 labels GFAP-positive human astrocytoma cells and reactive astrocytes

We used confocal microscopy to compare the efficacy and localization of SR101 and GFAP staining. Confocal images from the tumor core regions showed numerous cells simultaneously filled with fixable-SR101 and labeled by GFAP (Fig. 2A–D). In general, GFAP and SR101 labeled the same cells, although

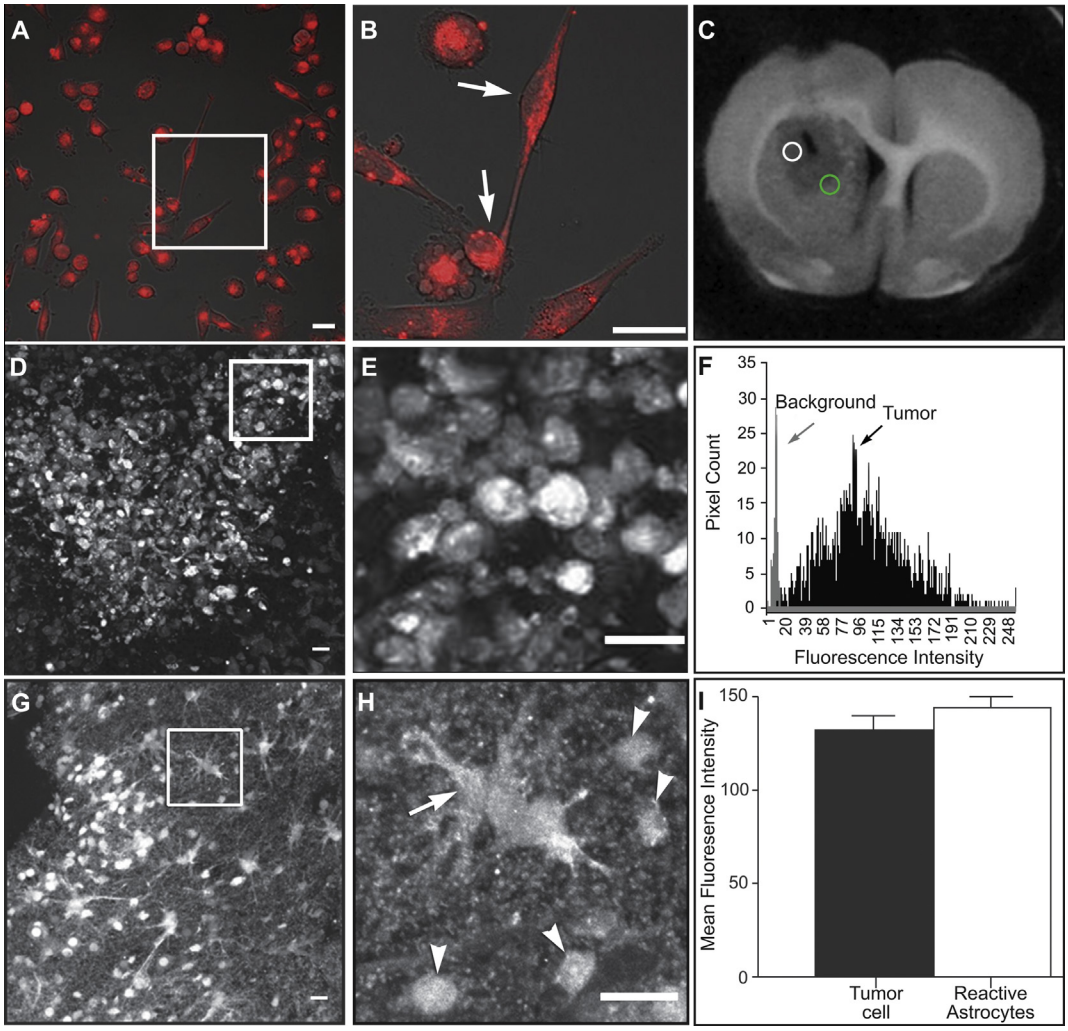

Fig. 1. Non-fixable sulforhodamine 101 (SR101) labels human astrocytoma cells in culture and identifies tumor core and margin in rodent xenografts. (A–B) U251 astrocytoma cell culture. (A) Differential interference contrast image with fluorescent overlay of human U251 astrocytoma cells incubated with SR101. (B) High magnification of inset in (A) demonstrates cytoplasmic filling of cells and delineation of cell nuclei (arrows). (C–H) Acute slices from rodents intracranially implanted with U251 cells. (C) Acute slice containing U251 derived tumor. Representative core and margin regions identified by white circle and green circle, respectively. (D) Confocal fluorescence image of SR101-labeled tumor core. (E) High magnification of inset in (D) shows typical morphology of U251cells. (F) Histogram of SR101 fluorescence distribution in (E) between tumor core and background. Note the clear distinction in mean fluorescence intensity (MFI) between tumor (102.84) and background (8.74). (G) Image of acute slice tumor margin with SR101-labeled cells. (H) Inset of morphologically identified reactive astrocyte (arrow) surrounded by glioma cells (arrowheads) near tumor margin from (G). (I) MFI of U251 cells and reactive astrocytes normalized to background (three acute slices from three rats) showing no significant difference in MFI between the two cell types. Scale bars = 20 μm. *Used with permission from Barrow Neurological Institute.*

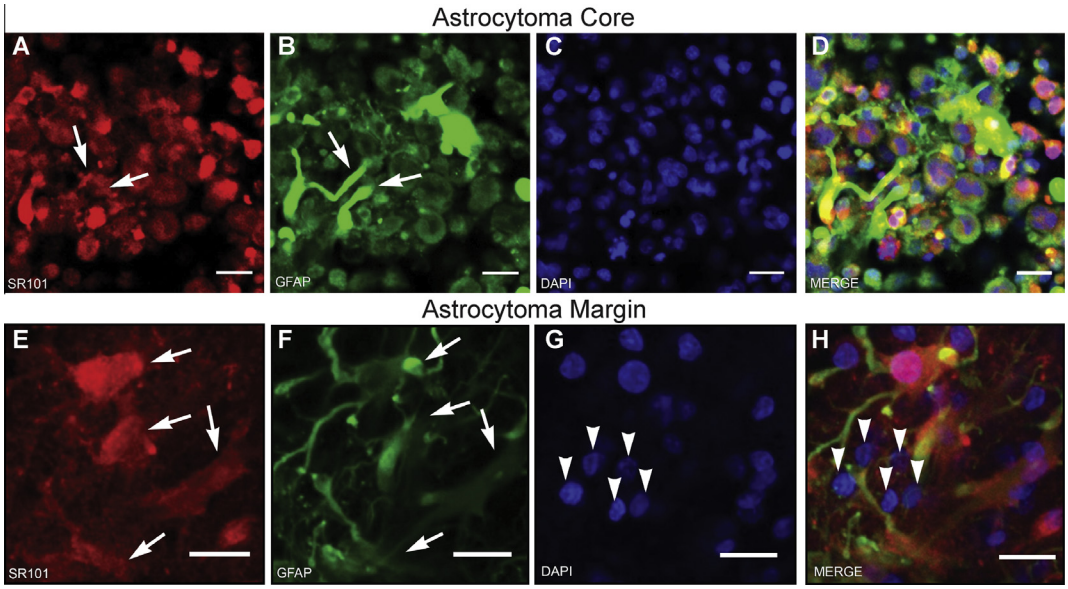

Fig. 2. Fixable-sulforhodamine 101 (SR101) co-localizes with the astrocytic marker glial fibrillary acidic protein (GFAP). (A) Confocal imaging of rodent xenograft acute slices incubated in fixable-SR101. Following incubation, slices were fixed and stained with (B) GFAP and (C) 4′,6-diamidino-phenylindole (DAPI). Images are from the core of the astrocytoma. Fixable-SR101 fills the cell bodies of GFAP-positive cells in the tumor core and weakly fills astrocytic processes (arrows). (D) GFAP, DAPI and SR101 overlap considerably in the merged image. (E–H) Images from the margin of the astrocytoma. Fixable-SR101 fills cell bodies of GFAP-positive cells at the tumor margin. Solid arrows identify (E) SR101-positive and (F) GFAP-positive cells. Note the appearance of (G) DAPI positive cells (arrowheads) unlabeled by SR101 or GFAP that are selectively observed at the astrocytoma margin. (H) Merged image from tumor margin. Scale bars = 20 μm. *Used with permission from Barrow Neurological Institute.*

fixable-SR101 stained some cellular processes less intensely than GFAP (Fig. 2A, B, D). Merged images from the tumor core showed that most cells were positive for both fixable-SR101 and GFAP, indicating that SR101 effectively labels astrocytic tumor cells.

We compared the staining pattern of fixable-SR101 and GFAP to determine if reactive astrocytes could be differentiated from neoplastic astrocytes to help identify tumor borders. Imaging of rat brain regions adjacent to human astrocytoma cells showed that the staining patterns of fixable-SR101 and GFAP were similar (Fig. 2E, F). However, GFAP labeled membrane processes more thoroughly than the fixable-SR101. Cells in the peripheral regions contained extensive membrane projections that could be readily differentiated from cells within the tumor core that lacked this feature. When additional cells in these regions were stained with DAPI (Fig. 2G), groups of cells were negative for both fixable-SR101 and GFAP (Fig. 2G, H). These findings suggest a mixed cell population of tumor cells and nontumor brain cells typical of regions outside the tumor core. Together, the data indicate that SR101-positive cells are the GFAP-positive astrocytoma cell population. Furthermore, fixable-SR101 provides morphological information that appears to differentiate astrocytoma cells from reactive astrocytes.

3.3. SR101 differentiates astrocytoma from lymphoma

In contrast to the findings from astrocytoma cells, confocal imaging indicated minimal SR101 signal from the human CNS lymphoma cell line MC116 (Supp. Fig. 1). In acute slices from astrocytoma and CNS lymphoma animal models incubated with fixable-SR101, we quantified co-localized GFAP for astrocytoma slices and CD20 for lymphoma slices. In astrocytoma tumor regions, fixable-SR101 labeled the majority of cells (Fig. 3A–D, I). The frequency of co-localization of SR101 and GFAP was 86.50% (Fig. 3K, Table 1), with a mean of 22.30 SR101-positive cells and 20.58 GFAP-positive cells per stereology dissector region of interest (ROI) ($p = 0.0004$, Fig. 3I, Table 1). In contrast, fixable-SR101 labeled only a very small number of cells from ROI in CNS lymphoma acute slices (Fig. 3E). SR101 and CD20 co-localized poorly (2.19%), and there were significantly more CD20-positive cells than fixable-SR101-positive cells ($p < 0.0001$) (Fig. 3J, Table 1). However, SR101 labeled a small number of cells in CNS lymphoma tissue that were not CD20-positive and that morphologically resembled reactive astrocytes (Fig. 3E, F). SR101 distinguished astrocytoma from lymphoma tissue and co-localized with GFAP more frequently than CD20 (Fig. 3K). This finding demonstrates the strong relationship of SR101 to GFAP-positive cells in astrocytoma, and the ability of SR101 to differentiate an astrocytic from a non-astrocytic tumor such as CNS lymphoma.

3.4. SR101 uptake is rapid

Astrocytoma cells exposed to SR101 in cell culture took up the fluorophore within 1 minute of incubation. One minute after incubation, astrocytoma cells concentrated SR101 to approximately twice the fluorescence intensity of background, and within 3 minutes contained approximately six times the fluorescence intensity compared to background. These data show the utility of SR101 for rapidly labeling astrocytoma cells in culture (Supp. Fig. 2, 3).

4. Discussion

We have demonstrated a technique for identifying the most common primary brain tumor, astrocytoma, in animal models by *ex vivo* exposure to the fluorescent agent SR101. We tested the specificity of SR101 in human cell culture and orthotopic rodent xenografts. We found SR101-labeling to be preserved in cell culture and animal models of astrocytoma. Compared to GFAP immunocytochemistry, SR101 provided more rapid and equally accurate identification of astrocytic tumors. Our results clearly indicate the potential utility of SR101 for rapidly identifying an astrocytic neoplasm. Compared to *ex vivo* tissue from a negative control animal model, we found SR101 staining could differentiate astrocytoma and CNS lymphoma within 30 minutes of biopsy. Our rapid incubation experiments in cell culture suggest that this time frame could be compressed substantially further.

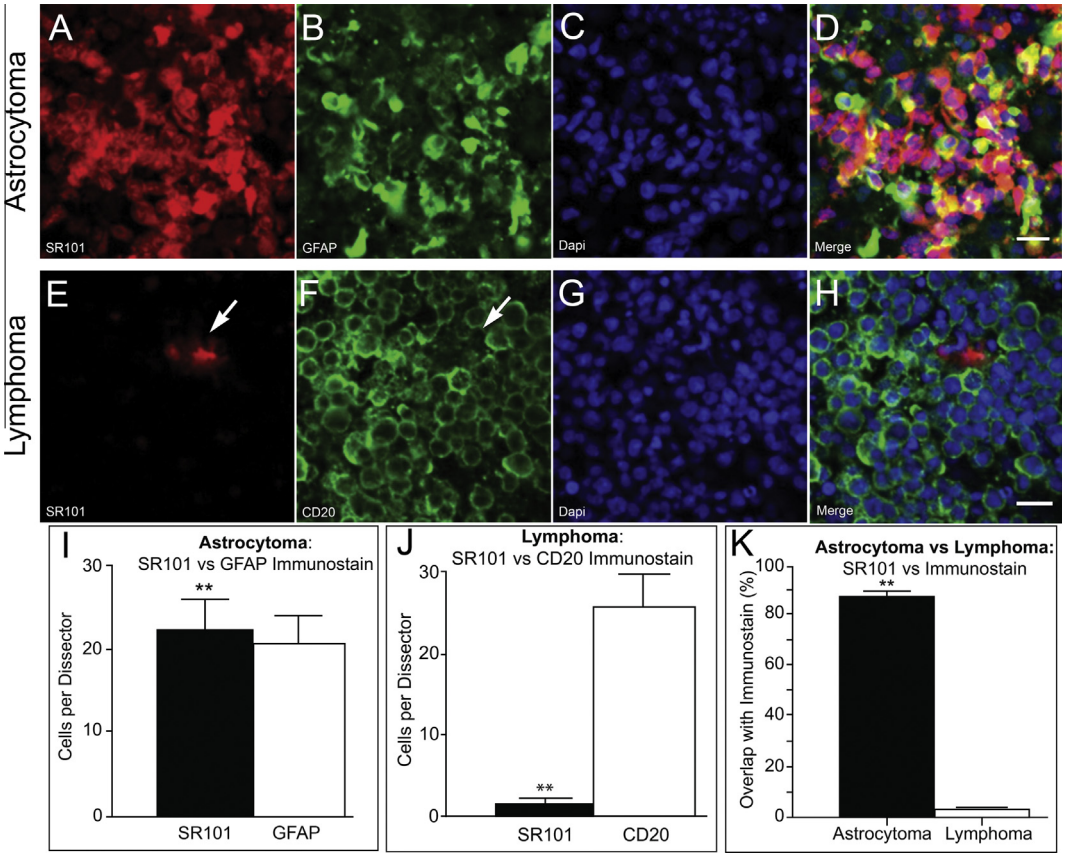

Fig. 3. Sulforhodamine 101 (SR101) rapidly differentiates human astrocytoma from central nervous system (CNS) lymphoma in rodent xenografts. Confocal imaging of acute slices from xenograft animals implanted with astrocytoma cells (U251, top row) or lymphoma cells (MC116, middle row). Slices were stained with fixable-SR101 and specific markers for astrocytoma, glial fibrillary acidic protein (GFAP), or lymphoma (CD20). Nuclei were counterstained with 4′,6-diamidino-phenylindole (DAPI). (A–D) Region from U251 astrocytoma acute slice incubated with fixable-SR101 and counterstained with GFAP and DAPI. (E) SR101 labels a single cell (arrow) in a MC116 xenograft lymphoma region. (F) CD20 immunostaining labels lymphoma cells but does not label region containing SR101-positive cell (arrow). (G) DAPI counterstain of cell nuclei in field of view. (H) Merged lymphoma image indicating poor colocalization of SR101 and CD20. Scale bars = 20 μm. (I–K) Confocal stereology of acute slices. (I) Number of SR101-positive cells slightly outnumber GFAP-positive cells in U251 xenograft astrocytoma regions ($p = 0.0004$). (J) Difference in number of SR101-positive and CD20-positive cells present in MC116 xenograft lymphoma regions is highly significant ($p < 0.0001$). (K) SR101 significantly overlaps with GFAP-positive astrocytoma cells (86.50%) compared with CD20 lymphoma cells (2.19%, $p < 0.0001$). *Used with permission from Barrow Neurological Institute.* vs = *versus.*

Table 1
Co-localization of immunostain-positive and sulforhodamine 101-positive cells from stereology

Tumor	Immunostain	Immunostain positive	SR101 positive	Percent overlap	Total cells counted
Astrocytoma	GFAP	20.58 ± 3.26	22.30 ± 3.55	86.50 ± 1.86	1316
Lymphoma	CD20	25.61 ± 4.05	1.58 ± 0.32	2.20 ± 0.47	1657

GFAP = glial fibrillary acidic protein, SR101 = sulforhodamine 101.
Data are presented as mean ± standard deviation.

We chose a U251 immortalized GBM cell line for this model because, compared to more invasive models, it is easier to distinguish the border zone of the tumor and therefore distinguish tumor cells from native astrocytes. In future studies, additional astrocytoma models will need to be validated for their SR101 avidity.

U251 cells are largely GFAP-positive. GBM contains a heterozygous population of cells that vary in terms of GFAP expression [9]. SR101 labeled slightly more tumor cells in our GBM xenografts than GFAP. This finding resembles data from normal brain showing that SR101 identifies subtypes of astrocytes and precursor cells that are GFAP-negative [2,10]. Future studies on SR101 are needed to identify and characterize GBM precursor cells that uptake SR101. A direct comparison of the sensitivity of SR101 and GFAP in human brain tumors obtained at surgery is being pursued by our group.

These findings have important implications for astrocytoma research, since SR101 can be used to identify astrocytic tumor cells in both *in vitro* and *ex vivo* settings in a more rapid time frame than has previously been possible. In addition, the fact that SR101-labeling appears to occur within minutes could represent an opportunity for clinical neuropathology, in that it could be used intraoperatively to provide a definitive diagnosis that a given tumor is of glioma lineage. While SR101 does not distinguish astrocytoma from astrocytes (also a limitation of GFAP), it is interesting that the selective uptake of SR101 is preserved in malignant astrocytic cells. Further work is required to determine the exact mechanism of uptake. Any variation in the uptake mechanism between astrocytoma and astroctyes could represent an opportunity for a selective therapy.

5. Conclusion

SR101 rapidly and selectively labels human and rodent astrocytic tumor cells. In an orthotopic xenograft model, SR101

staining retained its specificity. This method could provide utility in neuro-oncology research. This technique also provides proof of concept for a method to provide a clinically meaningful immediate *ex vivo* neuropathological diagnosis.

Conflicts of interest/disclosures

The authors declare that they have no financial or other conflicts of interest in relation to this research and its publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jocn.2014.02.007.

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