

# *In vivo* staining of neocortical astrocytes via the cerebral microcirculation using sulforhodamine B

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## 1 Introduction

Intravital imaging of the neuronal-glial-vascular network, or part of it, would undoubtedly provide useful information on brain physiology and pathology.<sup>1-5</sup> The neuronal-glial-vascular interactions are fundamental to sustain brain activity,<sup>4,6</sup> and changes herein have an important impact on brain metabolism and homeostasis. Two-photon excitation microscopy has the potential to investigate and image *in vivo* brain physiology and cellular processes, thanks to its high-resolution imaging in combination with deep tissue penetration.<sup>7,8</sup> Two-photon microscopy has already been used *in vivo* for vascular imaging<sup>7,9,10</sup> and for different applications in neuroscience,<sup>11</sup> namely imaging of astrocytic and neuronal Ca<sup>2+</sup> dynamics.<sup>12-16</sup>

**Abstract.** Staining and imaging glial cells *in vivo* while observing the microvasculature could help understand brain physiology, namely neuronal-glial-vascular communication. Two-photon excitation microscopy provides a means to monitor these interactions at the cellular level in living animals, but the cells of interest must be fluorescent. Injecting dyes intravenously is a rapid and quasi noninvasive method to stain cells in the brain. It necessitates that the dye is soluble in the blood plasma and crosses the blood brain barrier (BBB). We demonstrate here, using two-photon imaging, that sulforhodamine B (SRB) crosses the BBB and stains *in vivo*, specifically mouse astrocytes. This is confirmed by experiments on primary neurons and astrocytes cultures showing the preferential SRB staining of the latter. SRB is rapidly eliminated from the blood, which allows repeated injections in longitudinal studies. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3041163]

**Keywords:** neocortical astrocytes; sulforhodamine B; *in vivo* staining; two-photon microscopy.

Paper 08006RR received Jan. 8, 2008; revised manuscript received Oct. 10, 2008; accepted for publication Oct. 13, 2008; published online Dec. 15, 2008.

Different methods have already been used to stain brain cells: the multicell bolus loading method,<sup>12,15,16</sup> use of transgenic animals with cells expressing fluorescent proteins,<sup>17</sup> and intravenous injection of the dye.<sup>18</sup> For instance, Nimmerjahn et al.<sup>19</sup> demonstrated that sulforhodamine 101 (SR101) specifically stains mouse cortical astrocytes via local dye loading, and Bacskai et al.<sup>18</sup> presented a new fluorescent amyloid-beta ligand injected intravenously (IV method) and enters the brain parenchyma.

The IV method is more problematic in the brain than in systemic organs<sup>20</sup> due to the presence of the blood brain barrier (BBB), which drastically restricts exchanges between the blood and brain tissue. The dye must be soluble in the blood plasma as well as be able to cross the BBB. Its capacity to cross BBB depends on physicochemical (molecular weight, charge, lipophilicity) and physiological parameters (affinity for carrier mechanisms).<sup>21</sup>

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In this paper, it is reported that sulforhodamine B (SRB), when injected intravenously, crosses the mouse BBB and stains mouse cortical astrocytes. The SRB (MW=559 Da) is an amphiphilic fluorescent dye. It belongs to the xanthenes family, which contains different fluorescent markers used *in vivo* for biological staining: rhodamine 123 and rhodamine 6G for mitochondrial stain,<sup>22,23</sup> SR101 for astrocytes staining,<sup>19</sup> and sodium fluorescein for human ocular angiography. SRB has never been used *in vivo* but only for colorimetric cytotoxic assays, because of its binding capability to basic amino acid residues under mildly acidic conditions.<sup>24</sup> Finally, it has a very low toxicity (LD50: 10300 mg/kg).<sup>25</sup> For all previous reasons, we expected SRB to be a good candidate for the staining of cells in cerebral tissue via blood circulation.

This study presents the first complementary physiological characterizations of SRB. Next, the specificity of SRB staining is analyzed on primary cell cultures of neurons and astrocytes. Finally, the method for staining mouse cortical astrocytes using *in vivo* two-photon microscopy is described.

## 2 Materials and Methods

### 2.1 Animal Care Guidelines

All experimental procedures were performed in accordance with the French Government guidelines for the care and use of laboratory animals (licenses 380702, A 3851610004, and B 3851610003).

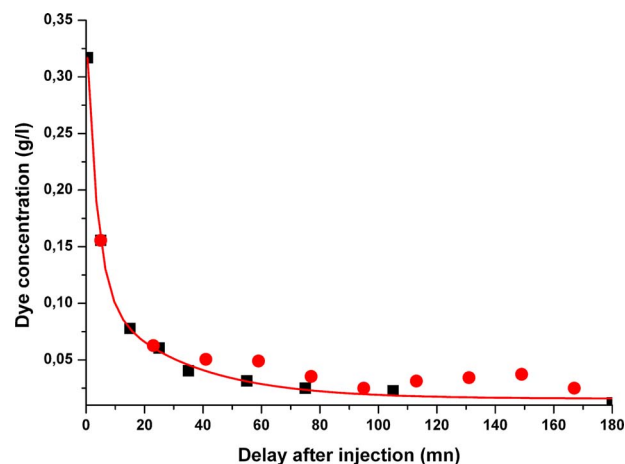
### 2.2 Physiological Characterizations

#### 2.2.1 Sulforhodamine B binding to albumin

Dye binding to plasma proteins (principally albumin) prevents BBB crossing. To measure the unbound dye fraction in the presence of albumin, we prepared an aqueous solution of albumin (30 g/l, Molekula, Germany) in 0.1-M phosphate buffer (pH 7.4). SRB (Radiant Dye, Germany) was added to the albumin solution (final concentration: 0.2 g/l). The solution was dialyzed for 24 h at 37 °C using cellulose dialysis tubing (cutoff: 1000 Da, Fisher Scientific AG, Germany). Finally, the SRB concentration was measured in the dialysate using a spectrofluorophotometer (lambda 9, Perkin-Elmer, Waltham, Massachusetts) and the unbound plasma dye concentration was deduced. Control measurements were performed on a SRB solution without albumin.

#### 2.2.2 Sulforhodamine B clearance from the mouse vascular compartment

To study the SRB clearance from the mouse vascular compartment, mice (Swiss/IOPS CD1, 38 to 43 g, Charles River Laboratories, France) were anesthetized using a gas mixture of isoflurane/air (5% for induction, 2% for maintenance). The catheter was inserted into the jugular vein and 100  $\mu$ l of a SRB solution (5 mg·ml<sup>-1</sup> in saline buffer) was injected. At different times for 3 h after injection (see Fig 1), a blood sample of 40  $\mu$ l was collected. The blood sample was diluted 40 times and homogenized in heparinized physiological saline. One milliliter of every sample was centrifuged and absorbance of the supernatant was measured at 560 nm



**Fig. 1** SRB clearance from the mouse vascular compartment. Evolution of SRB concentration in the mouse vascular compartment after injection of 100  $\mu$ l of a SRB solution (5 g/L) at time 0. Square and round dots are associated with two different mice whose SRB clearances are very similar.

### 2.3 Cell Cultures

#### 2.3.1 Primary astrocytic cultures

Astrocytic cultures were performed as described by Raponi et al.<sup>26</sup> Hemispheres from whole forebrains of P2 newborn mice (Charles River Laboratories, France) were separated and dissociated by trypsinization and mechanical trituration in Hanks balanced saline solution. Microglial cells were removed by preplating the cell suspension on noncoated petri dishes for 15 min. The resulting cell suspension was then replated on noncoated Falcon petri dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After 4 days, oligodendrocyte progenitor cells and neuroblasts growing on top of the developing astroglial layer were selectively removed by flushing.<sup>27</sup> The percentage of nonastrocytic cells never exceeded 2.5% of the cells.

#### 2.3.2 Primary neuron cultures

Primary cerebellar granular neuron cultures were prepared from 6-day-old S/IOPS NMRI mice (Charles River Laboratories, France) as described by Trioulier et al.<sup>28</sup> Cerebella were removed and cut before a 10-min incubation at 37 °C in 0.25% trypsin EDTA in DMEM. The cerebella were dissociated by trituration, and dissociated cells were centrifuged for 5 min at 500 g. Cells were plated on poly-D-lysine precoated dishes and incubated at 37 °C in K25+S medium. One day later, cytosine- $\beta$ -D-arabinooside (10  $\mu$ M, Sigma, France) was added in the medium to prevent the growth of non-neuronal cells.

#### 2.3.3 In vitro studies

5  $\mu$ l of a SRB solution (10 mg/ml) was added to the 5-ml culture medium as previously described. Observations were made by two-photon microscopy (see next). A 60 $\times$  water-immersion objective (0.90 NA, LUM Plan FI/ IR Olympus, Japan) was directly immersed in the medium, and acquisition was performed from 3 min to 2 h after addition of the SRB

on each petri dish. The temperature was maintained at 37° C. Cell nuclei were stained by adding 40  $\mu\text{l}$  of a Hoechst 33342 solution (1 mg/ml–Sigma, France). Observations on six different petri dishes with astrocytes and three with neurons are presented. The SRB dye in the extracellular compartment diffuses inside the whole intracellular compartment of the astrocytes, thus a distinction of the cell shapes from the background is impossible. Therefore, on a different petri dish of the same cell culture, the cell membranes were stained with the lipophilic tracer 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4'-chlorobenzenesulfonate (DiD, Invitrogen France).

## 2.4 *In Vivo Two-Photon Tridimensional Imaging of Sulforhodamine B Brain Entry and Staining*

### 2.4.1 *Microscopy setup*

Two-photon laser scanning microscopy was performed with a confocal microscope consisting of a Biorad MRC 1024 scan-head and an Olympus BX50WI microscope. Fluorescence was directly epicollected. An 800-nm excitation beam from a Tsunami femtosecond Ti:sapphire laser (5-W pump, Millennia V, Spectra-Physics, Mountain View, California) was focused in the sample using a 20 $\times$  water-immersion objective (0.95 NA, Xlum Plan Fl Olympus, Japan).

The beam was scanned in the  $x$ - $y$  plane to acquire 512  $\times$  512 pixel images in 0.9 s. The  $z$ -scan for variation of the observation depth was realized by vertical motion of the motorized objective. The incident laser intensity was adjusted by using a half-wave plate and a polarizer placed before the microscope, so that the total average power delivered at the surface ranged from 1 to 150 mW. Two channels were simultaneously observed using two added external photomultiplier tubes (PMT) and an appropriate filter set made up of a HQ620/60 Red filter for SRB and a HQ535/30 Green filter for FITC-dextran (staining vasculature) associated with a 585 DCRX Dichroic filter, all from Chroma Technology (Rockingham, Vermont).

Images were acquired using the Biorad exploitation system and displayed using ImageJ (ImageJ., v.1.33, Public Domain Software, available on <http://rsb.info.nih.gov/ij/>, 2005).

### 2.4.2 *Animal preparation*

Mice were anaesthetized with a gaseous inhalation of a mixture of isoflurane/air (2%). For dye injections, craniotomy realization, and two-photon imaging, mice were placed on a stereotaxic frame modified to allow a longitudinal rotation of the animal to optimize laser penetration. Core temperature was maintained at approximately 36 °C using warm water circulating through a pad.

### 2.4.3 *In vivo two-photon tridimensional imaging*

The images of the SRB distribution were acquired at successive depths in the cortex (down to 500  $\mu\text{m}$  below the dura) from 2 h 30 to 5 h ( $n=3$  Swiss nude mice, Charles River Laboratories, France) after injection of the SRB solution (50  $\mu\text{l}$ , 10 mg. $\text{ml}^{-1}$  in saline buffer) through the tail vein. Fifteen minutes before two-photon imaging, a 3-mm-diam craniotomy was carried out above the left parietal cortex; the bone was removed and the exposed cerebral cortex was filled

with a 0.9% saline water solution. Approximately 10 min after surgery, a 70-kDa FITC-Dextran solution (100  $\mu\text{l}$ , 100 mg. $\text{ml}^{-1}$  in saline buffer, Sigma, France) was injected *via* the tail vein to visualize simultaneously microvessels and SRB staining.

## 3 Results

### 3.1 *Physiological Characterizations*

#### 3.1.1 *Sulforhodamine B clearance from the mouse vascular compartment*

The results are presented in Fig. 1. The SRB-concentration decrease has been fitted with the sum of two exponential functions. The two characteristic times were  $t_1=3.6 \pm 0.4$  min and  $t_2=29.9 \pm 7.1$  min. The initial SRB concentration in the blood circulation measured approximately 30 s after injection was 0.3 g/l.

#### 3.1.2 *Sulforhodamine B binding to plasma proteins*

After 24 h of dialysis of the solution with SRB and albumin, a dialyzable fraction of SRB of 0.6 was measured. This indicates that the majority of the dye in the blood was free. Surprisingly, the control solution (SRB in water+PBS) showed a smaller dialyzable fraction. This could be explained by the tendency of SRB molecules to aggregate and form dimers in an aqueous solution.<sup>29</sup> So it appears that the albumin may efficiently prevent the dimer formation.

### 3.2 *Cell Culture*

In cells culture, the SRB stains the astrocytes 3 min after dye addition. The whole cell (nucleus and cytoplasm) is labeled [Fig. 2(a)]. On the contrary, observations made up to 2 h after the addition of the dye showed that the SRB remains extracellular in primary neurons cultures [Fig. 2(b)].

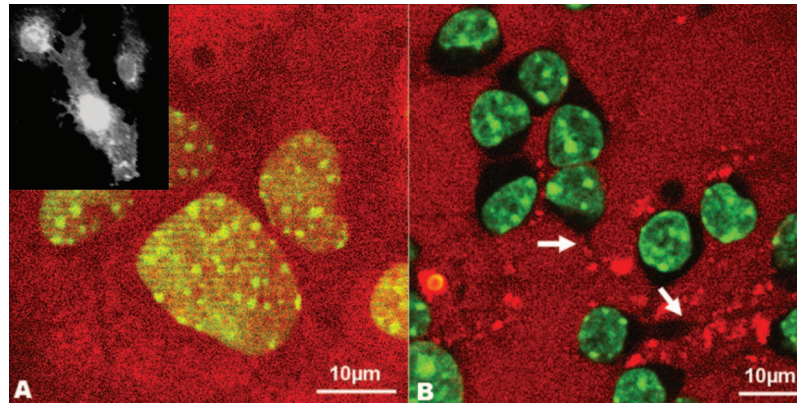
### 3.3 *In Vivo Tridimensional Imaging of Sulforhodamine B Brain Entry and Staining*

#### 3.3.1 *Sulforhodamine B crosses the blood brain barrier*

From 2 h 30 after injection of the SRB solution, a specific staining of cortical cells was observed (see Fig. 3). In these *in vivo* studies, any uptake of SRB due to a traumatic increase of BBB permeability after craniotomy cannot be excluded. The SRB, however, was injected more than two hours before the surgery, when the SRB blood concentration is inferior to 0.02 g/l (see Fig. 1) and would contribute little or nothing to the astrocytes staining. This demonstrates that the SRB crosses most probably the healthy BBB. SRB labeled cells are detected until 5 h after injection.

#### 3.3.2 *Sulforhodamine B stains specific mouse cortical cells*

All cells stained by SRB had common and specific morphological features, such as starshapes with many extensions and end-feet encircling capillaries (see Fig. 3). These features are very similar to those described for neocortex astrocytes in Refs 19 and 30. The volume density of SRB stained cells calculated on a volume of  $299 \times 299 \times 40 \mu\text{m}^3$  between 80 and 120  $\mu\text{m}$  under the dura is  $11.10^3$  cells/ $\text{mm}^3$ .



**Fig. 2** Observation of (a) astrocytes and (b) neurons cell cultures after the addition of a solution of SRB (red) and Hoechst 33342 (green) in the culture medium. The astrocytes are homogenously stained in the nucleus and cytoplasm. For comparison, a similar astrocyte culture (see insert) was stained with a membrane fluorescent dye DiD to better show the cell shapes and cytoplasmatic extensions. The neurons and their neurites (see white arrows) were not stained.

#### 4 Discussions

In this study, we showed that SRB crosses the healthy BBB and stains astrocytes according to morphology and cell studies. The fast SRB elimination from the blood plasma (Fig. 1) and the two-hour delay between the craniotomy and the dye injection allowed us to affirm that the presence of SRB in cortical cells was not due to dye leakage induced by the surgery.

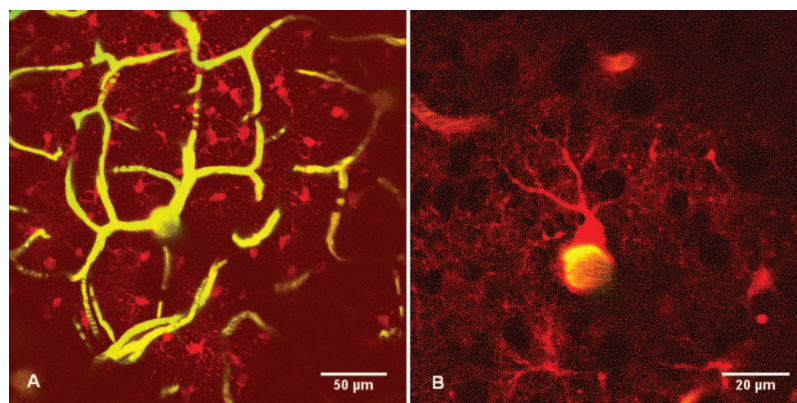
The high water solubility, the rapid SRB clearance from the blood, and its very low toxicity allow injecting of large dye quantities to improve the staining for *in vivo* experiments.

Because the SRB does not bind strongly the albumin protein, its effective molecular weight is mainly 559 Da. The presence of albumin proteins probably limits SRB dimerization. Consequently, a passive diffusion through lipidic membranes could be possible. In the case of passive diffusion, a low water-octanol partition coefficient of the SRB (inferior to 0.3, see <http://club.bhe.free.fr>) would predict a low vascular

permeability to SRB. We measured a partition coefficient of 0.017 with the “shake-flake” method [precognized and described by *OECD, Test Guideline 107, Decision of the Council C(81) 30 final, 1981*]. It is coherent with experimental observations that, despite high initial SRB concentration, it is always necessary to wait at least 2 h to obtain a contrasted cell staining (data not shown).

The experimental observations on cells culture and *in vivo* in the mouse cortex showed that the SRB stains specifically astrocytes. First, the SRB specifically labeled astrocytes in cells culture. Moreover, SRB stained cells showed *in vivo* characteristic features of the astrocytes. Last, the cell density is comparable to the one given by Nimmerjahn et al. in mouse cortex layer 2/3.<sup>19</sup> In our study, the astrocyte density was estimated at the top of layer 2, which is slightly lower than the estimated value of Ref. 19.

The next step for definitively demonstrating the specificity of the SRB staining is to show the colocalization of this stain-



**Fig. 3** Images acquired in the left parietal cortex of a nude mouse at 2 h 30 min after injection of 50  $\mu$ l of a SRB solution (10 mg. $\text{ml}^{-1}$ ). Fifteen minutes before two-photon imaging, a 2-mm-diam craniotomy was performed and a 70-kDa FITC-Dextran solution (100  $\mu$ l, 100 mg. $\text{ml}^{-1}$ , Sigma) was injected intravenously. (a) Z projection of the maximum intensity of a stack of images obtained between 80 and 120  $\mu$ m below the dura. (b) A slice obtained at 85  $\mu$ m below the dura. Cells stained by the SRB (in red) present characteristic features of astrocytes: star-shaped with many extensions and end-feet encircling capillaries. Note thinner red spots coming from astrocyte processes outside the focal plane. The capillaries appeared in yellow (merge of red and green colors).

ing with transgenically labeled astrocytes, *in vivo* in mouse.

This method uses the blood circulation to transport the fluorescent dyes to the cerebral tissue and has the advantage to uniformly stain cerebral tissue due to the dense capillary network. Nevertheless, it is restricted to fluorescent dyes that cross the BBB.

This method of staining astrocytes can be used, for instance, to observe gliosis following an injury, e.g., trauma, focal ischemia, or after radiotherapy. The utilization of a cranial window would enable us to follow gliosis in time, because the low toxicity and the fast clearance of the SRB dye allows repeated injections.

With this method, it is possible to label simultaneously astrocytes, the vascular network, and neurons (expressing, for example, green fluorescent protein) in transgenic animals injected with the SRB and an intravascular dye. This will enable a closer insight in the neuronal-glia-vascular communication directly *in vivo*.

### Acknowledgments

This work was supported by grants from the Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche, the Ligue contre le cancer (comité de l'Isère), the Association pour la recherche sur le cancer, the interdisciplinary CNRS-INSERM-CEA program, Imagerie du petit animal and the Région Rhône-Alpes. The authors warmly thank Regine Farion for biotechnical assistance, and Jean-Christophe Deloulme, Flavie Strappazon, and Yves Goldberg for providing primary cell cultures of astrocytes and neurons, respectively.

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