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# Sudan Black B treatment reduces autofluorescence and improves resolution of *in situ* hybridization specific fluorescent signals of brain sections

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**Summary.** Interference by autofluorescence is one of the major concerns of immunofluorescence analysis of *in situ* hybridization-based diagnostic assays. We present a useful technique that reduces autofluorescent background without affecting the tissue integrity or direct immunofluorescence signals in brain sections. Using six different protocols, such as ammonia/ethanol, Sudan Black B (SBB) in 70% ethanol, photobleaching with UV light and different combinations of them in both formalin-fixed paraffin-embedded and frozen human brain tissue sections, we have found that tissue treatment of SBB in a concentration of 0.1% in 70% ethanol is the best approach to reduce/eliminate tissue autofluorescence and background, while preserving the specific fluorescence hybridization signals. This strategy is a feasible, non-time consuming method that provides a reasonable compromise between total reduction of the tissue autofluorescence and maintenance of specific fluorescent labels.

**Key words:** Autofluorescence, Sudan Black B, Paraffin sections, Human brain, Fluorescence microscopy

## Introduction

Fluorescence *in situ* hybridization (FISH) is a powerful tool for the study of chromosome structure and function, being a worldwide method for pathological diagnosis and cancer research. This technique is widely employed in experimental and clinical studies due to its high resolution and good immunostaining of the tissue

constituents. The major advantage of this technique resides in its unique ability to provide an intermediate degree of resolution between DNA analysis and chromosomal investigations while retaining information at the single-cell level.

One of the major restrictions in fluorescence microscopy is tissue autofluorescence. Interference by autofluorescence (AF) is troublesome during immunofluorescence analysis, especially when photographic documentation of the fluorescent microscopic findings and even quantification of immunofluorescence are attempted. Tissue AF can arise from cell endogenous fluorophores, as chlorophyll in plants and flavins, porphyrins and lipofuscin which are usually present in mitochondria and lysosomes in several cell types, as well as components of extra-cellular matrix (collagen, elastin) and from some exogenous dyes.

The diversification of the original FISH protocol into the impressive number of procedures available has promoted throughout the years FISH accuracy and versatility. FISH can be routinely used in formalin-fixed paraffin-embedded (FFPE) tissues for retrospective analysis, thus avoiding the need for cell culture. However, this method is hampered by the interference of background signals with specific hybridization fluorescence. Background signals can be caused by natural material (lipofuscin persists even in paraffin sections), fixative-induced tissue autofluorescence (aldehydes), or introduced by nonspecific hybridization of probe sequences.

In the past years, there have been several reports describing different protocols to reduce or eliminate background signals using different reagents and treatments. In fact, different treatments and dyes such as Trypan blue, methylene blue, Pontamine Sky Blue, CuSO<sub>4</sub> and Sudan Black B have been used to quench background autofluorescence (Cowen and Haven, 1985; Mosiman et al., 1997; Clancy and Cauller, 1998). The

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efficacy of the processes depended on the tissue type and processing technique, but no general recipe to control tissue autofluorescence was available thus far, mainly for FFPE tissue used as routine material in many laboratories. For example, Baschong and colleagues (2001) showed that treatment with Sudan Black B (SBB) was the most efficient method to reduce AF in myocardium in FFPE sections, and the combination of ammonia/ethanol, sodium borohydride ( $\text{NaBH}_4$ ) and SBB was the most efficient on paraffin sections of cartilage and on frozen sections fixed in formaldehyde or glutaraldehyde. These authors also described that ammonia/ethanol was useful to remove part of the formaldehyde derived artifacts, as well as to reduce the tissue background in archival bone marrow sections. The use of  $\text{NaBH}_4$  to control the autofluorescence of glutaraldehyde-related artifacts was also described by others (Johnson, 1987; Baschong et al., 1997, 1999; Tagliaferro et al., 1997; Clancy and Cauller, 1998; Southern et al., 2000).

Actually, the use of Sudan Black B (SBB) in tissue from central nervous system (CNS) was previously described by Schnell and colleagues (1999) who used one human bone marrow and brains of rhesus monkeys and rats in an immunocytochemistry and retrograde axonal tract tracing study. At the same time, Romijn and colleagues (1999) showed that the staining of human brain sections with SBB after immunolabeling for neuropeptides blocked tissue AF. Previously, Belichenko and colleagues (1996) had described the advantages of using SBB to reduce fluorescence in human brains using indirect immunofluorescence in a confocal microscopy study. More recently, Viegas and colleagues (2007) reported that the combination of short-duration, high-intensity UV irradiation and SBB tissue treatment was the best approach to reduce autofluorescence in sections of murine liver, kidney, and pancreas. However, none of these studies addressed the use of SBB in FISH assays or in FFPE brain sections

Hence, we were interested to improve and standardize the current methodologies that would allow the reduction of autofluorescent background without affecting tissue integrity or direct FISH labeling, and a systematic study comparing SBB with other tissue treatments has yet to be performed. The aim of our study was to identify the best methodology for reducing tissue AF among six different tissue treatments, such as ammonia/ethanol, SBB, photobleaching with ultra-violet light and various combinations of these in archival formaldehyde-fixed and frozen brain sections. Our results show that the use of 0.1% SBB in 70% ethanol is the best approach to reduce/eliminate tissue autofluorescence and background in human brains.

## **Materials and methods**

### *Tissue sections*

Neutral formalin-fixed paraffin-embedded tissues stored for 6 months to 9 years were retrieved from the

archives of Department of Pathology at Ribeirão Preto School of Medicine, University of São Paulo from temporal lobe of patients operated for clinically intractable temporal lobe epilepsy (TLE). Four- $\mu\text{m}$  thick sections were obtained on a rotatory microtome, mounted on slides coated with 3-aminopropyl-triethoxysilane (Sigma, St Louis, MO, USA) and air-dried overnight. Sections were deparaffinized in an oven at 60°C overnight, immersed in three 10 min changes of fresh xylene (Merck, Darmstadt, Germany) at 60°C, dehydrated in ethanol (Merck) series, and pretreated in heat pretreatment solution (Invitrogen Corp., Carlsbad, CA, USA) at 96°C for 10 min.

For comparison purposes two temporal lobes obtained at autopsy from adults without known neurological disease were also used. Cause of death was from acute cardiac failure and sepsis, and frontal neocortices were collected between 4 and 11 h after death. None of the autopsy cases showed macroscopic signs of cerebral pathology. Sections of the neocortex were quickly removed at the beginning of the necropsy and immediately frozen in liquid nitrogen, and stored at -80°C until use. Sections (4  $\mu\text{m}$ ) were obtained using a rotatory cryostat at -15°C (Microm<sup>®</sup>, HM 505 E), mounted on slides coated with 3-aminopropyl-triethoxysilane (Sigma) and fixed for 25 minutes in a phosphate buffered saline (PBS) solution with 2% formaldehyde (Sigma).

Finally, the sections were washed and subjected to digestion with digestion enzyme solution (Invitrogen) for 15 min at room temperature, rinsed in distilled water, and air-dried. Slides were then treated in different test solutions for reducing the autofluorescent background, as described below.

### *Ammonia/ethanol*

The slides were incubated with 0.25% ammonia (Merck) in 70% ethanol for 1 hour at room temperature, followed by three washes of 5 minutes each in PBS solution with 0.02% Tween 20 (BioRAD Lab., Hercules, CA, USA).

### *Sudan Black B*

Tissue sections were immersed 0.1% SBB (Sigma) and 70% ethanol (Merck) for 20 minutes at room temperature. In order to remove the excess SBB, the slides were washed three times, for 5 minutes each in a PBS solution with 0.02% Tween 20 (BioRAD).

### *Photobleaching*

Tissue sections were irradiated with ultra-violet light (UV, T30W/G30T8, Philips), for 2 hours at room temperature. The UV irradiation was performed after the removal of the paraffin and/or fixation of the frozen sections. The exposition was direct at tissue section, at a distance of 20 cm from the light source and the wavelength of emission was 254 nm.

## Sudan black B and in situ hybridization

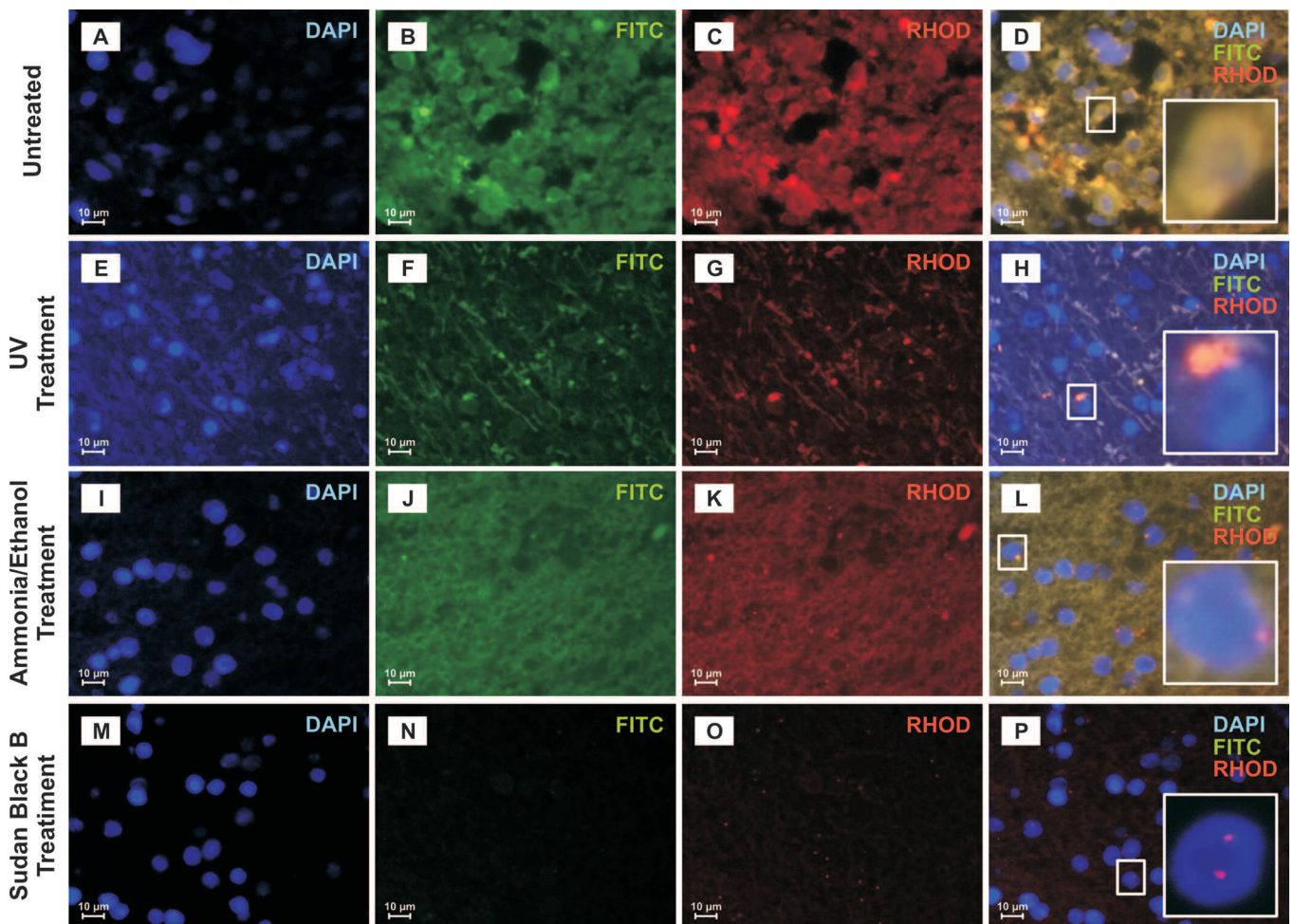
### Immunofluorescence labeling

The centromeric probes Chromosome X alpha satellite, directly labeled with Red Fluorophore (Cytocell, Cambridge, UK) were used to evaluate the applicability of our method to the reduction of autofluorescent background without affecting directly the specific immunofluorescence labeling. The probe mixture was applied to slides, which were then cover-slipped and sealed with rubber cement. Probes and target DNA were denatured simultaneously at 95°C for 6 minutes using a SpotLight® CISH™ Hybridizer (Invitrogen, 120 VAC, 50-60 Hz), followed by overnight incubation at 37°C in a humid atmosphere and subsequent washes in a stringent solution (0.1% NP-40/2x SSC). Nuclei were counterstained with 10 µL of DAPI-II anti-fade solution (Vysis, Illinois, USA).

### Evaluation of autofluorescence and of immunolabelling

Images were acquired through an AxioCam digital camera and analyzed with the AxioVision 4.1 software (Carl Zeiss, Oberkochen, Germany) using the Axioskop2 epifluorescence microscope (Carl Zeiss) with the DAPI, FITC and Rhodamine filters. All tissue sections were evaluated using these three different filter sets.

Semiquantitative scoring of the reduction/elimination of the tissue autofluorescence was independently performed by two observers (V.C.O. and D.L.C.S.) on a 4-tiered scale (0, no reduction; 1, moderate reduction; 2, very apparent; 3, near to complete reduction). This study was approved by our Institutional Ethical Committee (Proc. No HCRP 3168/07).



**Fig. 1.** Histochemical treatments for controlling tissue autofluorescence on FFPE brain sections. Untreated control sections (A, B, C, D). Treatment with UV light (E, F, G, H) and treatment with ammonia/ethanol (I, J, K, L) provided a slight reduction of the autofluorescence regardless the wavelength of excitation. After treatment with Sudan Black B (M, N, O, P) a total reduction of autofluorescent background was observed. Wavelength of excitation: DAPI, 360 nm; FITC, 488 nm and Rhodamine, 568 nm. Scale bar: 10 µm.

## Results

### Control of autofluorescence background

#### Natural autofluorescence of brain tissues

FFPE sections and frozen untreated brain tissue revealed intense autofluorescence when visualized with each filter set. This autofluorescence masked the fluorescence of the in situ hybridization labeling (Figs. 1A-D, 3A-D).

#### Photobleaching

Exposition to UV light provided a slight autofluorescence reduction in paraffin-embedded formalin-fixed tissue sections regardless of the wavelength of excitation (Fig. 1E-H). Treatment had no effect in the reduction of the autofluorescent background in frozen brain tissues, similarly to untreated control slides (Fig. 3E-H). Regarding the direct fluorescence of in situ hybridization, a decrease in the ability to visualize the signal was observed in both frozen and deparaffinized sections.

#### Ammonia/ethanol

There was a slight reduction in the autofluorescence

for all tissues examined regardless of filter type used (Figs. 1I-L, 3I-L).

#### Sudan Black B

A significant reduction of the tissue autofluorescence was observed in both FFPE and frozen tissue brain sections when SBB treatment was used (Figs. 1, 3M-P).

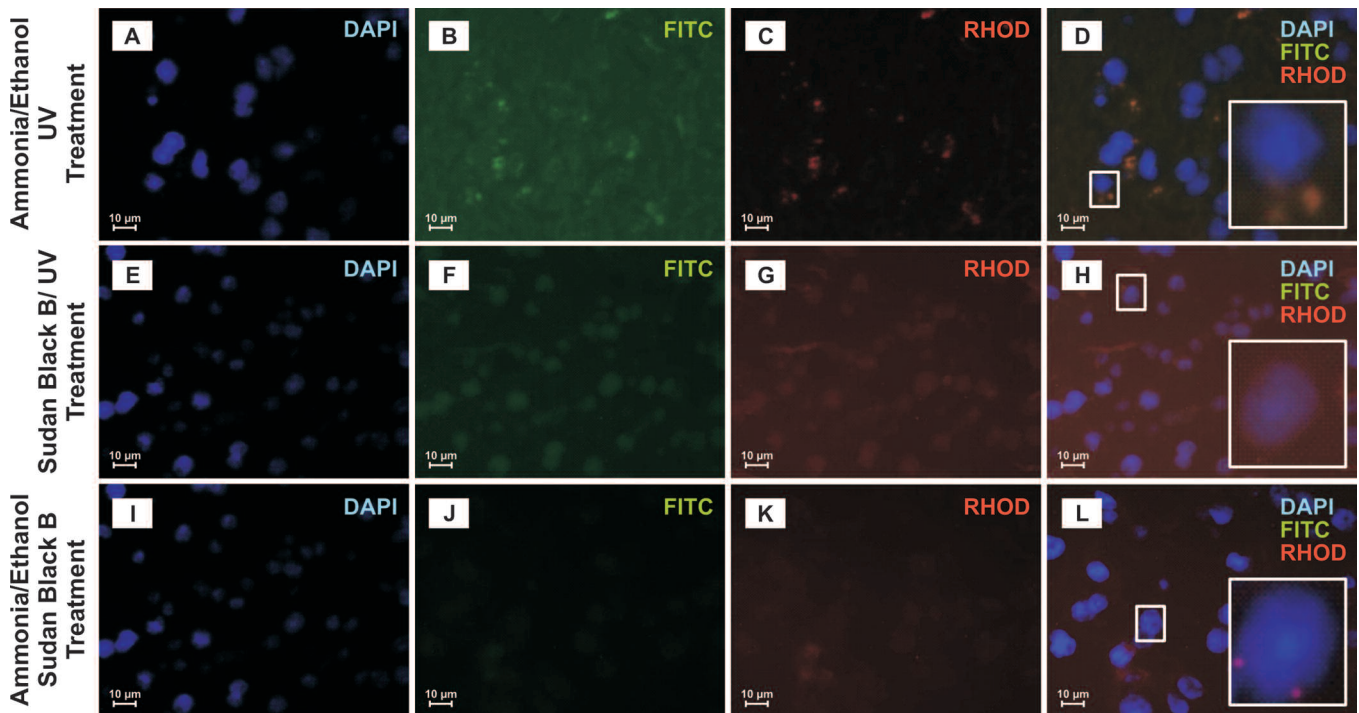
#### Combined use of Photobleaching and Ammonia/Ethanol

This combination reduced autofluorescence more efficiently than when we used ammonia/ethanol or UV light alone. However, like the UV treatment, a decrease in the ability to visualize the specific FISH signals was observed (Figs. 2A-D, 4A-D).

#### Combined use of Photobleaching and Sudan Black B

For brain tissue sections, we observed a reduction in autofluorescence regardless of the excitation wavelength used. In frozen sections, this treatment was efficient in reducing the autofluorescence only with the Rhodamine filter.

The reduction of the tissue background using this protocol did not provide better results compared to the



**Fig. 2.** Combined treatments for controlling tissue autofluorescence on FFPE brain sections. Note that combined treatment with ammonia/ethanol/UV (A, B, C, D) provided a reduction of autofluorescent background and was more efficient than ammonia/ethanol or UV light alone. Combined treatment with SBB / UV (E, F, G, H) and ammonia/ethanol / SBB (I, J, K, L) were efficient at reducing autofluorescence, but they did not provide better results than treatment with SBB alone. Wavelength of excitation: DAPI, 360 nm; FITC, 488 nm and Rhodamine, 568 nm. Scale bar: 10 µm.

## Sudan black B and in situ hybridization

tissue treatment with SBB alone. Moreover, a decrease in the ability to visualize the fluorescence *in situ* hybridization signals was observed, similarly to seen in the UV treatment (Figs. 2E-H, 4E-H).

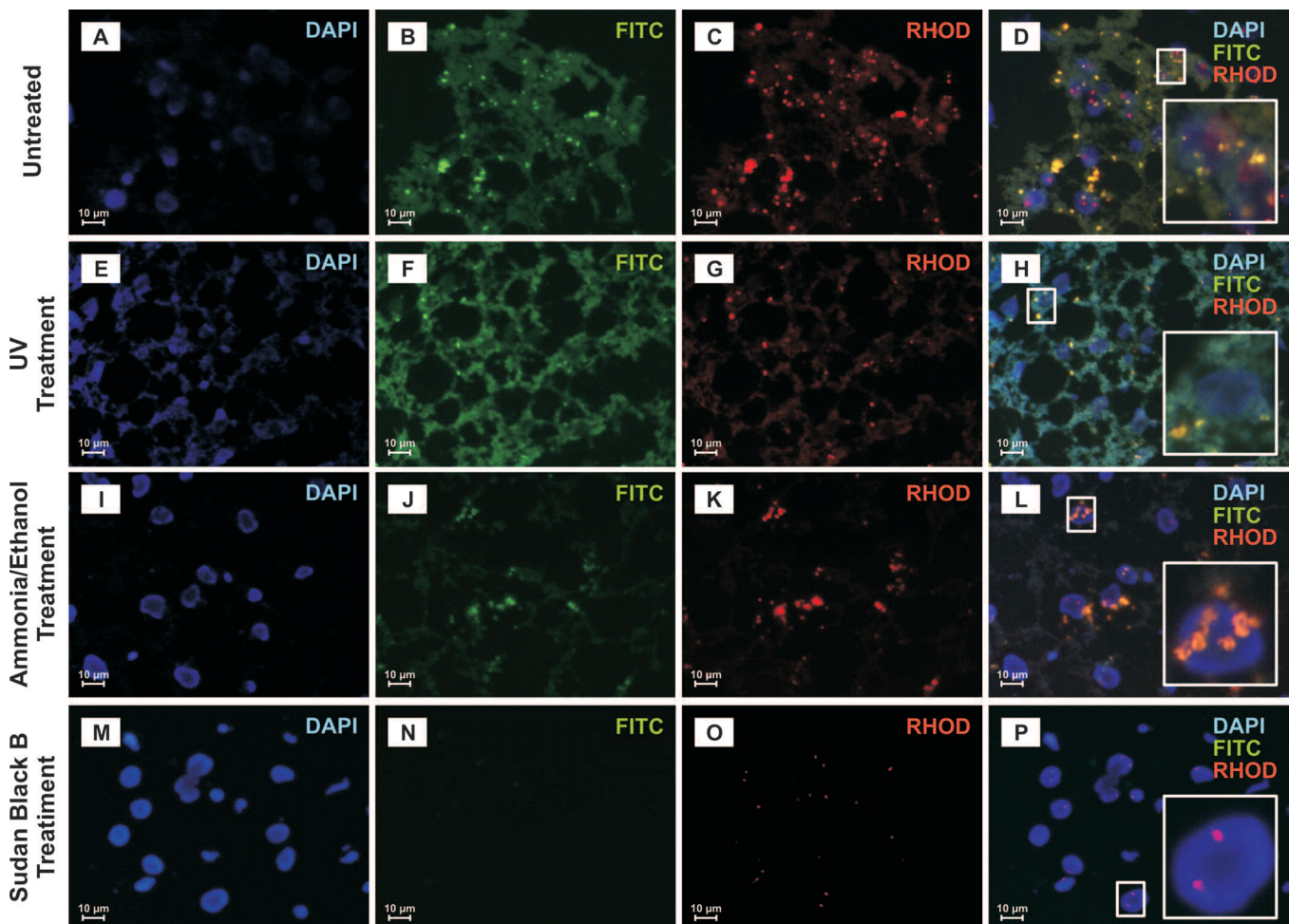
Combined use of ammonia/ethanol and Sudan Black B

Treating FFPE and frozen brain tissue sections with ammonia/ethanol and SBB decreased autofluorescence

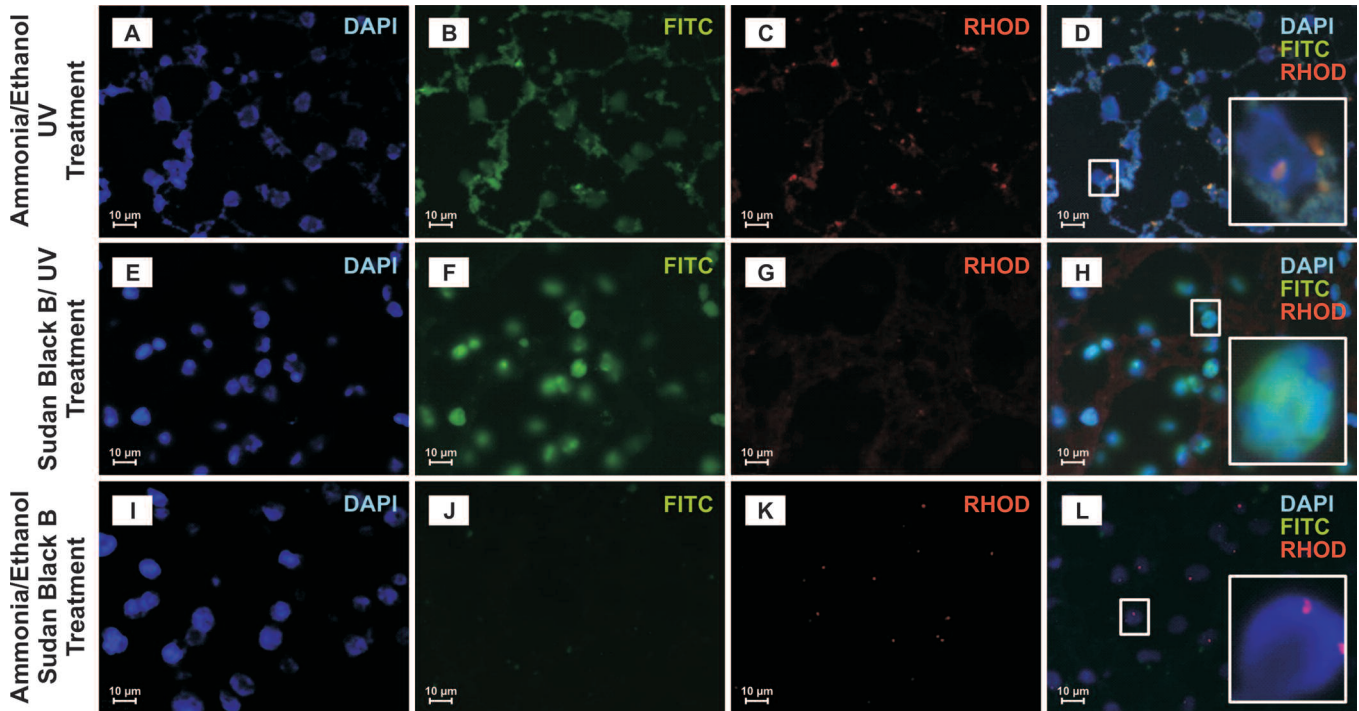
**Table 1.** Effects in the reduction of autofluorescence according to the tissue treatment.

Sections	Treatments											
	AE		SBB		PB		AE /SBB		AE/PB		SBB/PB	
	FITC	RHOD	FITC	RHOD	FITC	RHOD	FITC	RHOD	FITC	RHOD	FITC	RHOD
FFPE sections	1	1	3	3	1	1	3	3	2	2	2	3
Frozen sections	1	1	3	3	0	0	3	3	2	2	1	2

AE, ammonia/ethanol; SBB, Sudan Black B; PB, photobleaching; FITC, wavelength of excitation = 488 nm; RHOD, rhodamine, wavelength of excitation = 568 nm.



**Fig. 3.** Histochemical control of autofluorescence on frozen brain tissue. Observe that treatment with UV light (E, F, G, H) had no effect in the reduction of the autofluorescent background, resembling the untreated controls. Treatment with ammonia/ethanol (I, J, K, L) provided a slight reduction of the autofluorescence regardless of the wavelength of excitation. Note that treatment with SBB (M, N, O, P) led to a total reduction of the autofluorescent background. Controls (untreated sections, A, B, C, D). Wavelength of excitation: DAPI, 360 nm; FITC, 488 nm and Rhodamine, 568 nm. Scale bar: 10 µm.



**Fig. 4.** Combined treatment with ammonia/ethanol/UV (A, B, C, D) on frozen brain tissue provided a reduction of the autofluorescent background and was more efficient than ammonia/ethanol or UV light. Combined treatment with SBB / UV was efficient in reducing the autofluorescent background when Rhodamine filter was used, in contrast to FITC filter (E, F, G, H). Combined treatment with ammonia/ethanol / SBB (I, J, K, L) was efficient in reducing autofluorescence, but it did not provide better results than treatment with Sudan Black B alone. Wavelength of excitation: DAPI 360 nm, FITC 488 nm and Rhodamine 568 nm. Scale bar: 10  $\mu$ m.

regardless of the filter used. However this reduction did not provide better results than when we used Sudan Black B alone (Figs. 2I-L, 4I-L). Table 1 summarizes our results in reducing AF in brain sections using the different approaches.

## Discussion

By comparing six different protocols, this study found that a concentration of 0.1% Sudan Black B in 70% ethanol was the best treatment to reduce or eliminate the tissue AF with preservation of the specific fluorescence hybridization signals in brain sections. Autofluorescence is particularly worrisome in *in situ* hybridization studies on brain sections due to the presence of lipofuscin granules in the cytoplasm of some nerve cell bodies and glial cells which intensely fluoresce according to the microscope filter. Nonspecific autofluorescent staining can mask real FISH signals from chromosome-specific probes in interphase nuclei, particularly in the cerebral cortex, whose large neurons may harbor lipofuscin granules (Correa et al., 1980; Partanen et al., 1980; Santer et al., 1980; Helen, 1983; Kalyuzhny and Wessendorf, 1998). Lipofuscin is an “aging” pigment formed exclusively within lysosomes as a result of the peroxidation of non-degraded materials by reactive oxygen molecules such as the hydroxyl radical,

and it usually appears as small, punctate intracellular structures that are strongly fluorescent under any excitation ranging from 360 nm to 647 nm. In neurons lipofuscin appears to be derived primarily from autophagocytosed mitochondria and its amounts increase with age (Terman and Brunk, 2006), but the composition of lipofuscin granules is quite variable and dependent upon cell type, and even in the same cell type located at different sites in the CNS. For instance, based on their results Schnell and colleagues (1999) pointed out that the chemistry composition of lipofuscin in neurons in the inferior olivary nuclei is different from neurons from other sites in mammalian brains. Nevertheless, the primary constituents of lipofuscin are oxidatively modified protein residues, which are bridged into polymer complexes by acids, and lipid residues such as aldehydes originating from the breakdown of triglycerides, free fatty acids, cholesterol and phospholipids (Terman and Brunk, 2004). The amount of lipofuscin in human cortex is variable among different cells and layers, increasing with age and in some diseases, such as chronic epileptic damage, dementia and neurodegenerative conditions (Braak, 1976; Double et al., 2008).

Sudan Black B ( $C_{26}H_{24}N_4O$ ) is a lysochrome diazo dye used for staining neutral triglycerides and lipids on frozen sections and some lipoproteins on paraffin

## Sudan black B and *in situ* hybridization

sections. Since this dye is the most appropriate to quench autofluorescence of lipofuscins, fats, triglycerides and lipoproteins (Schnell et al., 1999; Baschong et al., 2001), we systematically re-examined the SBB tissue treatments in our routine material of neurosurgical pathology focused on FISH assays, and compared the results with other tissue treatments, such as ammonia/ethanol, SBB in 70% ethanol, photobleaching with UV light and different combinations of them. To this aim, we used FFPE and frozen human brain sections of epileptic cortices that usually exhibit gliosis and lipofuscin accumulation in pyramidal neurons.

While we have found that a concentration of 0.1% SBB in 70% ethanol in brain sections produced a drastic reduction in tissue AF and preserved the specific fluorescence *in situ* hybridization signals, it must be pointed out that the SBB itself exhibits some autofluorescence, starting at about 630 nm of wavelength (Romijn et al., 1999). However, SBB autofluorescence was not observed because our excitation wavelength was 360-568 nm. Schnell and colleagues (1999) showed that the reduction in lipofuscin-like autofluorescence by using SBB is concentration-dependent. Lower concentrations (<0.01%) of SBB provided insufficient reduction of autofluorescent pigments, whereas higher concentrations (>1%) of SBB gave adequate reduction in lipofuscin-like autofluorescence. We have found that a concentration of 0.1% SBB was strong enough to eliminate autofluorescent pigments. The combination of photobleaching and SBB to the tissue sections also provided a reduction in autofluorescence but it did not provide better results than when we used SBB alone.

Unlike ammonia/ethanol, which may promote the dissolution of negatively charged lipid derivatives, phenols, or degrade weak esters by hydrolysis, and borohydride that interacts with aldehydes and ketones by reducing both reactive groups to the respective alcohols, SBB reduces the background most probably by masking the autofluorescent structures (Romijn et al., 1999). It stains blue-black and given the almost opaque character of its labeling, we agree with Schnell and colleagues (1999) who postulated that SBB may act by obscuring lipofuscin granules without interacting with it at the chemical level.

Neumann and Gabel (2002) showed that longer UV irradiation resulted in a greater quenching of the fixative-induced autofluorescence in brain tissue sections. The time and intensity of UV irradiation used in our study was different from those used by Neumann and Gabel, and this fact could explain our failure to reduce the autofluorescent background using the photobleaching tissue treatment. Viegas et al. (2007) described that the maximal reduction of tissue AF in kidney, liver and pancreas sections by photobleaching was achieved after 2 hours of UV tissue irradiation. We believe that in FFPE and frozen brain tissue sections similar results with UV irradiation are hard to reproduce, and our attempts to address this matter were unsuccessful.

In contrast to previous reports (Schnell et al., 1999; Baschong et al., 2001; Viegas et al., 2007), we have found that treatment with ammonia/ethanol for reducing autofluorescence resulted in only a slight reduction. Therefore, we decided to treat sections with other protocols, such as ammonia/ethanol/UV and ammonia/ethanol/ SBB treatment. The combination ammonia/ethanol/UV was more effective to reduce the AF than when we used ammonia/ethanol or UV light treatment alone. Yet, treatment with the combination ammonia/ethanol/SBB was efficient at reducing the autofluorescence, but did not provide better results than when we used SBB alone. We further observed that when these combined treatments were applied to brain sections, some AF was still present, and the magnitude of the background reduction was not sufficient in several cases to detect specific signals by direct immunofluorescence.

It is important to consider some potential limitations related to methodology, experimental design, and FISH procedures when interpreting our results. First, we used frozen sections of neocortex obtained at autopsy which were frozen directly in liquid nitrogen instead of using isopentane, which is less aggressive for the structure of the tissue. Furthermore, autopsy specimens had a long postmortem delay before the freezing procedure, which certainly added more tissue artifacts. Indeed, the frozen sections seem less conserved than FFPE tissue. However, the intensity and specificity of the signal hybridizations did not differ between frozen and FFPE tissue, and our goal here was to analyze the reduction of tissue autofluorescence with preservation of specific probe labeling. Secondly, as we used a centromeric Chromosome X alpha satellite DNA probe to evaluate the reduction of autofluorescence, and because the amount of X alpha satellites in cells, a stronger hybridization signal is expected in comparison with other probes. Our experimental design assumes that this fact did not interfere in the evaluation of the results since we used the same probe in all assays.

In conclusion, the reduction of autofluorescence intensity using SBB was well suited in both FFPE and frozen brain sections without affecting tissue integrity, while maintaining the specificity of *in situ* hybridization labeling. Nonetheless, all the techniques described above have their advantages and disadvantages, depending on the type of tissue, fixation, processing, and wavelength of excitation light, as well as the design of the study. We conclude that as regards FISH procedures on brain sections, treatment with Sudan Black B is the most effective means to reduce the background and, at the same time, preserve the specificity of fluorescent labels.

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