

An improved and cost-effective methodology for the reduction of autofluorescence in direct immunofluorescence studies on formalin-fixed paraffin-embedded tissues

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Interference by autofluorescence is one of the major shortcomings of immunofluorescence analysis by confocal laser scanning microscopy (CLSM). CLSM requires minimal tissue autofluorescence and reduced unspecific fluorescence background, requisites that become more critical when direct immunofluorescence studies are concerned. To control autofluorescence, different reagents and treatments can be used. Until now, the efficacy of the processes described depended on the tissue type and on the processing technique, no general recipe for the control of autofluorescence being available. Using paraffin sections of archival formalin-fixed murine liver, kidney and pancreas, we have found that previously described techniques were not able to reduce autofluorescence to levels that allowed direct immunofluorescence labelling. In this work, we aimed at improving currently described methodologies so that they would allow reduction of the autofluorescent background without affecting tissue integrity or direct immunofluorescence labelling. We have found that the combination of short-duration, high-intensity UV irradiation and Sudan Black B was the best approach to reduce autofluorescence in highly vascularised, high lipofuscins' content tissues, such as murine liver and kidney, and poorly vascularised, low lipofuscins' content tissues such as the pancreas. In addition, we herein show that this methodology is highly effective in reducing autofluorescent background to levels that allow detection of specific signals by direct immunofluorescence.

Key words: direct immunofluorescence, paraffin sections, autofluorescence, liver, kidney, confocal laser scanning microscopy.

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Natural and fixative-induced tissue autofluorescence has long posed a problem in fluorescence-labelling studies, as specific signals can be masked. Fluorescence is the property of some molecules to absorb light at a particular wavelength and to subsequently emit light of a longer one. Natural fluorescence is mainly due to the presence of endogenous flavins, reduced NAD(P)H, lipofuscins, reticulin fibres, collagen and elastin. Flavins and NAD(P)H are coenzymatic redox carriers that play an important role in most metabolic pathways. NAD(P)H fluorescence results from hydrogen uptake at position 4 of its planar pyridine ring (Andersson *et al.* 1998). Lipofuscins, which are localized in lysosomes, consist of malonaldehyde which is a major product of lipid peroxidation. Malonaldehyde is known to react with amino acids and proteins to yield conjugated Schiff bases and hence fluorescent products (Baschong *et al.* 2001) (Table 1). Collagen/elastins, which are typical constituents of blood vessels, are also a source of autofluorescence (Billinton *et al.* 2001) (Table 1). Collagen is also an important component of stroma and connective tissue. Reticular fibres are also an important source of green autofluorescence. The degree of natural fluorescence relates to the tissue type. Tissues such as kidneys and liver can be very challenging, as these organs have extremely high metabolic rates with constant NADP-NADPH turnover, high accumulation of flavins and lipofuscins, and are highly vascularised, thus containing high amounts of collagen and elastin, and their parenchyma, especially that of liver, are rich in reticular fibres. These characteristics are not shared with tissues, such as the pancreas, that have low accumulation of both flavins and lipofuscins, and reduced vascularisation.

Tissue fixatives, which are used for preservation of morphological details, may further augment background fluorescence. Neutral buffered formalin (the fixative used in this work) is the most widely used

Table 1. Emission wavelength of the fluorophores responsible for intrinsic and fixative-induced autofluorescence.

Autofluorescence source	Emission wavelength (nm)	Excitation wavelength (nm)
Flavins	500-560	360-520
NAD(P)H	440-470	340-460
Lipofuscins	450-650	345-360
Collagen/elastins	470-520	330-400
Formaldehyde induced fluorescence	420-470	355-435

fixative in a routine setting. Formaldehyde forms covalent bonds between adjacent amine-containing groups through Schiff acid-base reactions. As a consequence, fluorescent products are formed, resulting in an intense fluorescent background (Beisker *et al.* 1987) (Table 1). These products may also unspecifically react with antibodies.

The molecules that contribute to intrinsic and induced autofluorescence are responsible for an emission of fluorescence between 450 and 650 nm that overlaps the emission wavelength of fluorophores widely used in immunofluorescence studies, such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE).

As many parameters need to be taken into account, reduction of tissue autofluorescence has proven very difficult. Depending on the cause of autofluorescence, different methodologies have been used (Cowen *et al.* 1985; Mera *et al.* 1980; Neumann *et al.* 2002; Schnell *et al.* 1999). It was suggested that the use of chemical procedures, such as treatment with ammonia/ethanol, could be a good approach to reduce autofluorescence (Baschong *et al.* 2001). Autofluorescence quenching using dyes, such as Sudan Black B, Pontamine Sky Blue and Trypan Blue, among others, may also constitute a good alternative (Baschong *et al.* 2001, Cowen T *et al.*, 1985, Mosiman VL *et al.*, 1997, Schnell *et al.* 1999). A third approach is the use of photochemical methods, such as photobleaching (Billinton *et al.* 2001), through which, the molecular structure of a fluorophore is changed so that it loses its ability to fluoresce. The use of instruments, such as optimised filter sets, confocal laser scanning microscopy (CLSM) and post-measurement image correction also allow discrimination of autofluorescence through the use of mathematical models (Steinkamp *et al.* 1986; Van de Lest *et al.* 1995). The use of optimised filter sets is particularly suited for the reduction of NAD(P)H-associated autofluorescence

background. So far, tissue type and processing techniques have dictated the effectiveness of the methodologies used for quenching of autofluorescence. Until now, no methodology has been shown to sufficiently reduce the autofluorescent background in organs with different characteristics, for the detection of direct immunofluorescence signals. In fact, the application of the previously described methods to kidney, liver and pancreas tissue sections did not allow the detection of specific immunofluorescent signals due to the autofluorescent background still observed. In this work, we aimed at the improvement of these methodologies so that they would permit drastic reduction of autofluorescence without compromising direct immunofluorescence labelling in formalin-fixed, paraffin-embedded murine kidney, liver and pancreas. A total reduction of the autofluorescent background was attained when kidney, liver and pancreas tissue sections were pre-treated with short-duration (2 hours), high-intensity (30 W) photobleaching and Sudan Black B.

Materials and Methods

Tissue sections

Murine kidneys, livers, and pancreata were collected from six-month old C57Bl.6/*lpr/lpr* mice, fixed in 10% paraformaldehyde and embedded in paraffin. Tissue sections of 5 μ m were cut and mounted onto glass slides that were previously coated with poly-L-lysine (5 mg/mL) (Sigma). For removal of paraffin, the slides were immersed in xylene (twice for 10 min), rehydrated with graded ethanol (5 min in 100% and 5 min in 95% ethanol) and transferred to deionised water.

All mice used in this work were bred and kept in the animal housing facilities of the Institute for Molecular and Cell Biology (Porto, Portugal), under specific pathogen free conditions. This work was approved by the Animal Welfare Division of the Portuguese Veterinary Council.

Ammonia/ethanol

The slides were incubated with 0.25% ammonia in 70% ethanol for 1 hour at room temperature, in a wet chamber. For rehydration, the slides were immersed in 50% ethanol for 10 min, at room temperature, in a wet chamber and transferred to MHB buffer (calcium free Hank's buffer containing 2 mM EGTA, 5 mM 2-morpholino-ethanesulfonic acid), pH 6.2-6.4, for 20 min, at room temperature.

Sudan Black B

Tissue sections were immersed in 0.1% Sudan Black B (Sigma) in 70% ethanol for 20 min, at room temperature, in a wet chamber. For the removal of Sudan Black B excess, the slides were washed 3 times, for 5 min, in phosphate buffered saline (PBS) 0.02% Tween 20. A final jet wash with PBS 0.02% Tween 20 was required for the complete removal of Sudan Black B excess.

Photobleaching

Before removal of paraffin, tissue sections were irradiated with UV light (30 W, 253 nm to 400 nm discrete emission, Philips), at room temperature, and hourly monitored for reduction of the autofluorescent background, after which they were transferred to PBS.

Proteolytic digestion

Tissue sections were incubated with proteinase K (20 µg/mL) (Quiagen) in PBS, for 10 min, in a wet chamber, at room temperature. After antigen retrieval, the slides were washed in PBS 0.02% Tween 20.

Immunofluorescence labelling

Kidneys from autoimmune glomerulonephritis-prone mice were collected, fixed in 10% paraformaldehyde and embedded in paraffin. Tissue sections were photobleached (30 W) for 2 hours at room temperature. After removal of paraffin and proteolytic digestion, tissue sections were blocked in 10% foetal bovine serum (FBS) in PBS, for 1 hour, at room temperature, and incubated with 0.1% Sudan Black B in 70% ethanol, for 20 min, in a wet chamber, at room temperature. Afterwards, the slides were incubated with FITC-labelled goat anti-mouse IgG₁ (cat. n° 1070-02), goat anti-mouse IgG₂ (cat. n° 1090-02) or goat anti-mouse IgG₃ (cat. n° 1100-02) monoclonal antibodies (all from Southern Biotechnologies) overnight, at 4 °C, in a wet chamber. These antibodies react with the heavy chain of mouse IgG₁, IgG₂ and IgG₃, respectively. As a control, slides were incubated in PBS alone. The slides were then washed 3 times in PBS 0.02% Tween 20 for 5 min and mounted with VectaShield immunofluorescence mounting medium (Vector Laboratories, Inc).

Evaluation of autofluorescence and of immunolabelling

For image acquisition, a confocal laser scanning

microscope (BioRad, MRC60C) was used. The settings for contrast, brightness, pinhole, acquisition mode and scanning time were maintained throughout the work. The illumination system consisted of a Kr-Ar laser, a 20x plan-neofluor objective (Olympus) and two filter sets. One of the filter sets included an excitation filter of 488 nm and an emission filter at 520 nm. The second filter set included an excitation filter of 568 nm and a barrier filter of 585 nm. Tissue sections were evaluated under transmission light to check for any structural abnormality. Epifluorescence was used to evaluate autofluorescence and immunolabelling. All the tissue sections were evaluated with each of the two filters sets.

Results

Control of autofluorescence background

Natural autofluorescence of different tissues

We have found that formalin-fixed murine kidney, liver and pancreas tissue sections presented an intense autofluorescence, irrespective of the filter set used (Figure 1A-C). Of notice, the autofluorescent background of pancreas tissue sections was not as bright as that observed for kidney and liver tissue sections.

Ammonia/ethanol

Ammonia/ethanol has been used to decrease fixative-induced fluorescence in decalcified bone marrow preparations (Baschong *et al.* 2001). When applied to kidney, liver and pancreas tissue sections the autofluorescence was not reduced regardless of the filter set used for excitation (Figure 1D-F).

Sudan Black B

Sudan Black B is widely used to quench natural autofluorescence, namely that of lipofuscin (Baschong *et al.* 2001). When excitation was performed at 488 nm, we have observed a drastic reduction of the autofluorescent background in kidney, liver and pancreas tissue sections. However, when tissue sections were exposed to light of 568 nm excitation wavelength the autofluorescent background was not decreased in any of the tissue sections under study (Figure 1G-I).

Combined use of ammonia/ethanol and Sudan Black B

When ammonia/ethanol and Sudan Black B were combinedly applied to kidney tissue sections, auto-

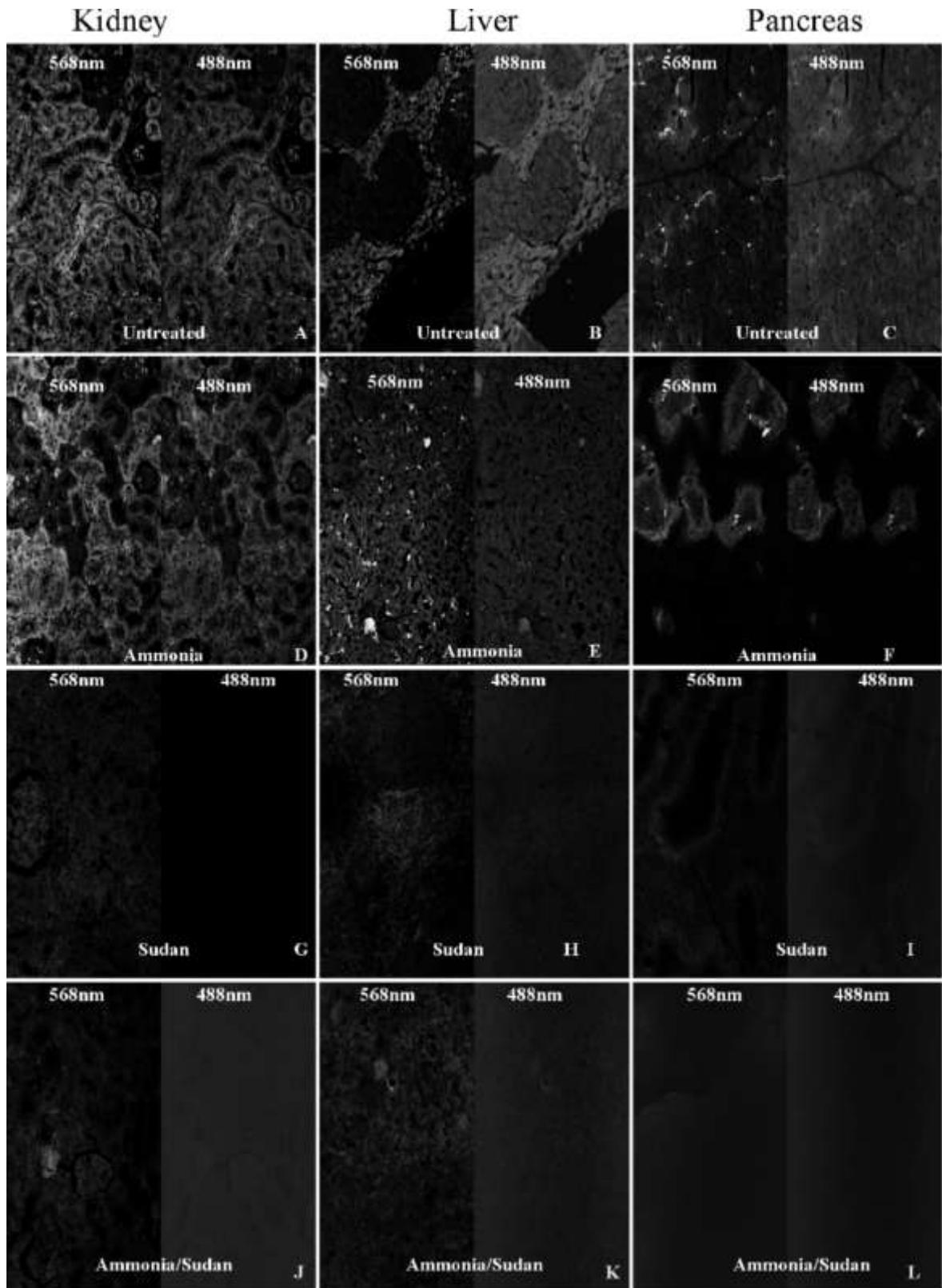


Figure 1. Influence of tissue type and processing in histochemical control of autofluorescence on formalin-fixed, paraffin-embedded tissues. Untreated controls: kidney (A), liver (B) and pancreas (C). Treatment with ammonia/ethanol did not reduce autofluorescence regardless of the wavelength of excitation (D, E, F). After treatment with Sudan Black B, reduction of autofluorescent background was attained when excitation was performed at 488 nm, but not at 568 nm (G, H, I). Treatment with ammonia/ethanol and Sudan Black B was inefficient in kidney (J), but reduced autofluorescence in pancreas (L). No effect was observed when this treatment was applied to liver tissue sections (K). (x200, CLSM).

fluorescence was increased regardless of the filter set used. In contrast, in pancreas tissue sections treatment with ammonia/ethanol and Sudan Black B reduced the autofluorescent background to some extent. As for the liver, treatment with ammonia/ethanol and Sudan Black B had no effect in reduction of the autofluorescent background (Figure 1J-L).

Photobleaching

Photobleaching has been used to decrease fixa-

tive-induced fluorescence. It was reported that the fluorescent background decreased as irradiation time increased (Neumann *et al.* 2002). Kidney, liver and pancreas tissue sections were submitted to UV irradiation and reduction of fluorescence was hourly monitored. We have found that in kidney, liver and pancreas tissue sections reduction of the background fluorescence reached its peak after two hours of UV irradiation (Figure 2A-B). Nevertheless, some fluorescence was still observed. Interestingly, with regards to pancreas tissue sec-

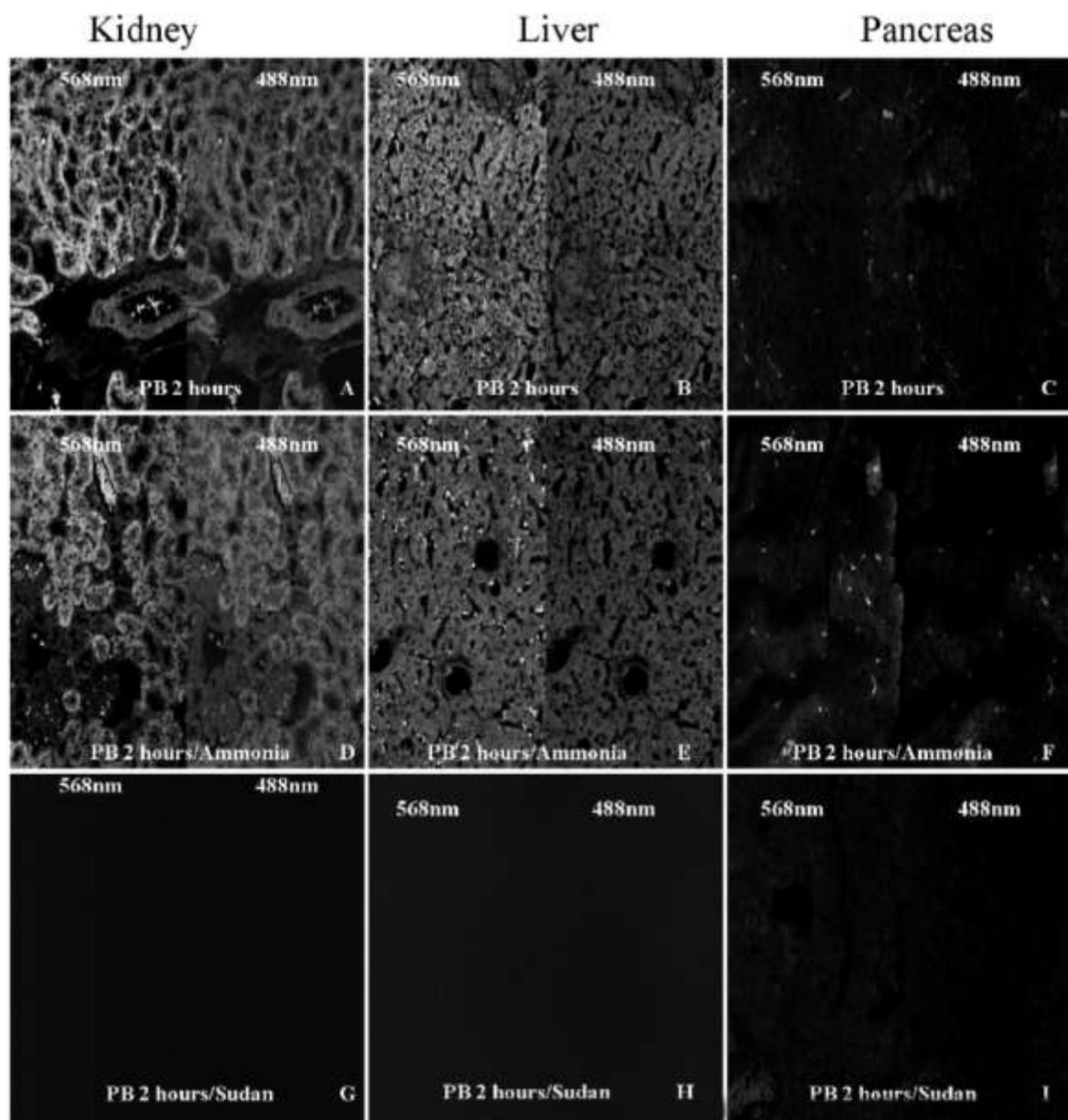


Figure 2. Histochemical control of autofluorescence on formalin-fixed, paraffin-embedded kidney, liver and pancreas tissue sections. Reduction of fixative-induced autofluorescence in kidney, liver and pancreas tissue sections reached its maximum after 2 hours of high-intensity (30 W) photobleaching (A, B, C). Combined use of photobleaching and ammonia/ethanol was inefficient in all tissue sections under study (D, E, F). Photobleaching of kidney, liver and pancreas tissue sections and treatment with Sudan Black B quenched the autofluorescent background (G, H, I). (x200, CLSM).

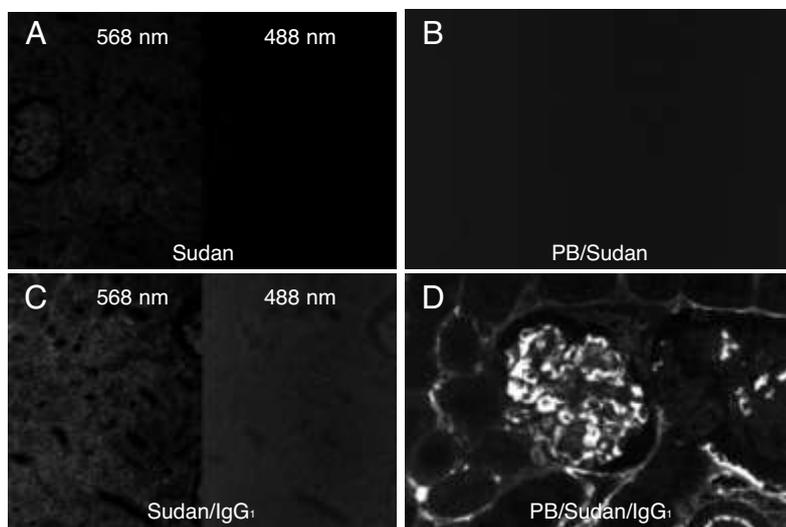


Figure 3. Immunofluorescence labelling of formalin-fixed, paraffin-embedded kidneys of autoimmune glomerulonephritis-prone mice. Kidney tissue sections were pretreated with Sudan Black B either alone (A) or in combination with photobleaching (B). For immunolabelling, a FITC-labelled goat anti-mouse IgG₁ monoclonal antibody was used (C,D). Specific green fluorescence (488nm) indicates the presence of IgG₁ mediated immune complexes. Immune complex deposits could only be detected after treatment of tissue sections with a combination of photobleaching and Sudan Black B (D). (x200, CLSM).

tions a near to total reduction of the fluorescent background was achieved after 2 hours of UV irradiation (Figure 2C). We have observed that tissue integrity was not affected by photobleaching (data not shown).

Combined use of Photobleaching and Ammonia/Ethanol

When photobleaching and ammonia/ethanol were applied to the tissue sections under study, we have found that they did not reduce the autofluorescent background regardless of the filter set used. In fact, in kidney, liver and pancreas tissue sections the autofluorescent background resembled that of the untreated controls (Figure 2D-F).

Combined use of Photobleaching and Sudan Black B

We have found that the combined use of irradiation with UV light (30 W) for 2 hours and incubation with Sudan Black B resulted in a total reduction of the autofluorescent background in kidney, liver and pancreas tissue sections, regardless of the excitation wavelength used (Figure 2G-I).

Immunofluorescence labelling

To evaluate whether the pretreatments to which the tissue sections were subjected compromised direct immunofluorescence labelling, we have selected kidneys from autoimmune glomerulonephritis-prone mice and detected immune complexes on kidney tissue sections using FITC-labelled monoclonal antibodies. Treatment of kidney tissue sections with ammonia/ethanol, combined with either photo-

bleaching or Sudan Black B, did not reduce the autofluorescent background to levels that would allow detection of immunofluorescence labelling (data not shown). Similar observations were made when kidney tissue sections were pre-treated with Sudan Black B alone (Figure 3A, 3C). In contrast, we have found that the combined use of photobleaching and Sudan Black B reduced the autofluorescent background to levels that allowed detection of specific fluorescence labelling signals (Figure 3B, 3D). In addition, we have found that the combined use of photobleaching and Sudan Black B did not compromise the immune staining as the staining pattern observed was similar to that widely illustrated in the literature.

Discussion

Autofluorescence has long posed a problem when immunofluorescent labelling studies are required, especially in the case of direct staining. The fluorescent background can result from natural factors or be induced by fixation. A third source of background fluorescence can result from unspecific binding of antibodies to Fc receptors. As the latter is well documented and routinely dealt with in all immunofluorescence methodologies (Lu *et al.* 1998), it will not be discussed here. The majority of the immunofluorescence protocols described so far used indirect labelling of antigens, which allows amplification of the fluorescent signal and requires a lesser degree of reduction of autofluorescence. Furthermore, they are limited to tissue type and often require a combination of various procedures, as well as constant adaptations. Moreover, the reduction of the autoflu-

orescent background is not always satisfactory. Indeed, when applied to detection of antigens in tissue sections of murine kidney, liver and pancreas, by direct immunofluorescence, these methodologies (Baschong *et al.* 2001, Neumann *et al.* 2002) were far from being effective and autofluorescence still masked immunolabelling. As quenching of autofluorescence in kidney and liver can be very challenging due to their high metabolic rates, high content of flavins, lipofuscins, reticulin fibres and high vascularisation, we have decided to optimise a methodology that would allow detection of direct fluorescent labelling signals and through which the autofluorescent background would always be effectively reduced without the need for constant adjustments.

As mentioned before, tissue autofluorescence may be due to intrinsic factors. In kidney and liver, natural fluorescence is mainly due to NAD(P)H, flavins, lipofuscins and reticulin fibres. NAD(P)H autofluorescence results from hydrogen uptake, whereas that of lipofuscins is due to the conjugated Schiff bases formed between groups of amino acids and proteins (Baschong *et al.* 2001). These natural fluorophores are responsible for the emission of light ranging from 450 nm to 650 nm, wavelengths that overlap those of the fluorophores used in immunofluorescence studies (from 488 nm to 568 nm). One of the approaches used to quench intrinsic autofluorescence is the use of dyes whose absorbance spectrum overlaps the autofluorescence emission. A wide range of dyes can be used to quench background autofluorescence. The choice for the most appropriate dye should be done according to tissue characteristics and assays to be performed. For instance, trypan blue is mostly used to quench autofluorescence when performing flow cytometry assays (Mosiman VL *et al.* 1997). Likewise, Pontamine Sky Blue is used when selective quenching of mesenteric vessels and carotid arteries autofluorescence is required (Cowen T *et al.* 1985). Sudan Black B, among other Sudan dyes, is the most appropriate dye to quench autofluorescence of lipofuscins, fats, triglycerides and lipoproteins (Baschong *et al.* 2001, Schnell *et al.* 1999). A drastic reduction of the autofluorescent background was observed when Sudan Black B was applied to kidney, liver and pancreas tissue sections. However, this reduction was still not sufficient to allow detection of signals by direct immunofluorescence.

Another source of background fluorescence is the use of fixatives, namely neutral buffered formalin,

which is the fixative routinely used in the clinical setting. Formaldehyde forms cross-linking methylene bridges and Schiff bases between basic amino acid and protein residues. Although this cross-linking is important for the *in situ* stabilization of proteins, when immunofluorescence studies are concerned, these bridges are a problem due to their fluorescence, which is difficult to eliminate. The emission of light of these fluorescent products occurs near the emission wavelength of FITC. Indeed, we have observed that the fluorescent background of the untreated sections was high. Treatment with ammonia/ethanol (Baschong *et al.* 2001) or photobleaching (Billinton *et al.* 2001) were considered good approaches to quenching fixative-induced fluorescence. Ammonia/ethanol react with free formaldehyde residues consequently reducing the autofluorescent background. Furthermore, these compounds appear to dissolve charged lipid derivatives, phenols or polyphenols, and to hydrolyse weak esters (Baschong *et al.* 2001). Nevertheless, and as shown by Baschong and co-workers (2001), ammonia/ethanol was only efficient in reducing the autofluorescent background in formaldehyde-fixed, decalcified bone marrow preparations but not in non-decalcified bone marrow slides. They propose ammonia/ethanol to be able to thoroughly dissolve deposits formed during the decalcification process by trichloroacetic acid. In addition, degradation of pH-sensitive fluorophores further augments ammonia/ethanol efficacy. So, it was not surprising that ammonia/ethanol was unable to reduce the autofluorescent background in kidney, liver and pancreas tissue sections. In fact, the autofluorescent background resembled that observed in untreated tissue sections. As these approaches did not reduce the autofluorescent background, we have decided to treat tissue sections with ammonia/ ethanol and Sudan Black B, a combination that was described to be effective in reducing autofluorescence in bone marrow tissue sections (Baschong *et al.* 2001). Nonetheless, we have found that when these treatments were applied to the tissues under study, some autofluorescence was still observed. Although the achieved reduction of autofluorescence may be sufficient for indirect immunofluorescence studies, it was still not adequate for direct immunolabelling. Photobleaching reduces fixative-induced fluorescence through continuous and prolonged illumination of tissue sections. Neumann and co-workers (2002) described that quenching of fixative-induced

autofluorescence by UV irradiation was time dependent: longer UV irradiation resulted in greater quenching of autofluorescence. We have found that when kidney, liver and pancreas tissue sections were photobleached, maximal reduction of autofluorescence was achieved after 2 hours of UV irradiation. It should be noted that the intensity of UV irradiation used by Newmann and co-workers (2002) was lower than the one used in our work. This may justify the longer irradiation time used in their work. In addition, they did not directly irradiate tissue sections as we did. Nonetheless, remaining autofluorescence did not allow detection of direct immunofluorescence signals. As such, we have decided to treat tissue sections with a combination of photobleaching and Sudan Black B. Using this approach the autofluorescent background in kidney, liver and pancreas tissue sections was totally eliminated.

To evaluate the applicability of our method in direct immunofluorescence labelling, we have selected kidneys of glomerulonephritis-prone C57Bl.6/*lpr/lpr* female mice. In these mice, glomerulonephritis is the consequence of the deposition of immune complexes in the kidney, which may be detected by immunofluorescence. Thus, kidney tissue sections were labelled with FITC-labelled monoclonal antibodies directed against the heavy chain of mouse IgG₁, IgG₂ and IgG₃. We have found that the reduction of the autofluorescent background, achieved by treating tissue sections with a combination of photobleaching and Sudan Black B, allowed the detection of specific immunofluorescent signals. The staining patterns obtained by these procedures were similar to those described in the literature (Vidal S *et al.* 1994).

In conclusion, we herein describe an improved methodology that besides eliminating autofluorescence in formalin-fixed paraffin-embedded tissue sections, also allows the detection of antigens by direct immunofluorescence using FITC-labelled monoclonal antibodies. Treatment of kidney, liver and pancreas tissue sections with short-duration (2 hours), high-intensity (30 W) photobleaching and Sudan Black B totally eliminated the autofluorescent background, regardless of the filter set used for excitation (488 nm or 568 nm). This quenching of autofluorescence enabled detection of direct fluorescence labelling signals, which are not amplified by the use of a secondary antibody, in contrast to indirect immunofluorescence studies. Another advantage of this methodology is the reduction of the cost

of the experiments as it avoids the use of indirect labelling methods. Additionally, this improved methodology allows performance of direct immunofluorescence studies in formalin-fixed paraffin-embedded archival samples. Finally, we propose that this new methodology (combined use of high-intensity, short-duration photobleaching and Sudan Black B) can be used regardless of tissue type as similar results were obtained for highly vascularised, lipofuscin-rich tissues, such as kidney and liver, and for tissues with reduced vascularisation and low lipofuscin content, such as the pancreas. This approach is even more effective when poorly vascularised and low lipofuscin content tissues, such as the pancreas, are under study.

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