Protocol refinement for quenching autofluorescence of red blood cells in FFPE sections of organ samples from cattle, pigs and chickens

Maksim V. Bytov, *Irina M.* Petrova, *Sergey L.* Khatsko, *Olga V.* Sokolova*, and *Irina* A. Shkuratova

FSBSI "Ural Federal Agrarian Scientific Research Centre, Ural Branch of Russian Academy of Sciences", Sverdlovsk region, Ekaterinburg, Russia

Abstract. One of the most common problem that researchers encounter when using fluorescence to visualize immunohistochemistry is the autofluorescence of the studied organ tissue sections and cell cultures. Autofluorescence quenching is necessary for a wide variety of organs and tissues, as well as for different methods of fixation and histochemical processing of sections. In addition to autofluorescence quenching, it is necessary to take into account the need for histological readability of tissue sections when using counterstains afterwards. Such protocol refinement for fluorescent immunohistochemistry for chicken, porcine and cattle tissues was carried out for the first time, as well as the use of a dimethyl sulfoxide (DMSO) solution with ethanol as Sudan Black B (SBB) solvent. Incubation of sections in SBB was chosen as the simplest and most nonspecific one. The most effective dissolution of the dye is achieved at a concentration of 0.3% SBB in a solution of 70% ethanol and absolutized DMSO in a 4:1 v/v ratio. The most thorough removal of SBB solution excess is achieved by rinsing the sections 5 times with 70% ethanol and then rinsing the sections with TBST (tris-buffered saline and Tween-20) buffer 5 times.

1 Introduction

Fluorescence microscopy is one of the most popular methods for visualizing immunohistochemical staining of organ tissue sections and cell cultures. The use of fluorescently labeled secondary antibodies avoids the blocking step of endogenous peroxidases which is required when using, for example, chromogenic substrates for bright-field microscopy imaging. On the other hand, the use of secondary fluorescently labeled antibodies or antibodies conjugated with horseradish peroxidase to react with fluorescently labeled tyramide (tyramide signal amplification) has its advantages. That includes avoiding the presence of unwanted background that appears when staining with chromogens such as 3,3'-diaminobenzidine (DAB) and interfering with microscopy of tissues with counterstain.

* Corresponding author: nauka_sokolova@mail.ru

However, one of the most common problem that researchers encounter when using fluorescence to visualize immunohistochemical staining is the autofluorescence of the studied organ tissue sections and cell cultures. Autofluorescence quenching is required when studying cell cultures [1], fresh frozen sections [2]; as well as fresh formaline fixed sections [3] and more than half-century old FFPE-cassettes [4]. Besides red blood cells autofluorescence (RBC) because of oxy- and desoxyhemoglobin [5, 6], autofluorescence of matrix and cells of connective tissues due to collagen, elastin, ceratin and NADH, as well as different forms of hemoglobin has also been reported [7, 8]. In general, the range of autofluorescent compounds is wide, as are the methods of their quenching [9], including ready-to-use solutions such as TrueBlack (Biotium, USA). However, improvement of existing methods using a combination of techniques continues [5, 10].

The number of papers on organ tissues of chickens, pigs and cattle using IHC research techniques is scarce. The study of many biological processes and mechanisms, which have already been demonstrated in humans and model organisms such as rats and mice, remains to be conducted on agricultural animal species. This aspect is especially interesting from the comparative physiology point of view [11-13].

The effectiveness of incubating sections in Sudan Black B has been previously shown [14-17], including the use of mere DMSO as a solvent [18]; TrueBlack® in DMSO, Biotium) however, not using DMSO as an additional solvent to ethanol. In addition, such protocol aspects have not been thoroughly studied in tissue sections from chickens, cattle and pigs. Moreover, an effective and delicate protocol for removing Sudan Black B solution from sections after incubation is required to achieve maximum clearing of excess dye while maintaining quenched autofluorescence.

The purpose of this study is to refine protocol for autofluorescence quenching in FFPE organ tissue sections of chickens, pigs and cattle, as well as to minimize the consequences of its use. Incubation of sections in Sudan Black B solution prepared without overnight stirring was chosen as the simplest and most nonspecific method.

2 Materials and Methods

To visualize RBC autofluorescence it is preferable to use vascularised organs or tissues with active microcirculation. Therefore, bovine, chicken and porcine liver as well as porcine myocardium and chicken small intestine were studied. To refine protocol for autofluorescence quenching, samples of tissue (≤5 mm thick) were fixed with 10% buffered neutral formaldehyde with a ratio 1:20 of tissue volume to formaldehyde volume (3 samples of each tissue). Tissue samples then were washed in running water, dehydrated and paraffin blocks were prepared. Paraffin sections with a thickness of 3-4 μm were cut. The sections were placed on glass with an adhesive coating SuperFrostPlus (Apexlab LLC), then put on a drying table (60 °C) until the paraffin melted.

To find the optimal protocol for quenching autofluorescence of red blood cells, sections were prepared and stained using the following steps:

1. The sections were placed in a Tris-EDTA buffer solution heated to 96 °C in a water bath for 20 minutes to perform temperature unmasking of antigens using HIER, pH = 9 (PrimeBioMed LLC);
2. After incubation, the sections were left in the buffer to cool to room temperature, then were washed with distilled water;
3. The sections were incubated in H_2O_2 solution for 10 minutes and placed in distilled water for 2 minutes;
4. The sections were dried and outlined with a hydrophobic marker;
5. Sections were incubated with TBST buffer (Servicebio) for 1 minute.

After incubation in TBST control sections (without quenching of autofluorescence of red blood cells) were covered with glass slips using LumiMount mounting medium (Lumiprobe Ltd) and microscoped in the green channel of fluorescent illumination. Assessing of protocols was performed using bright-field microscopy and fluorescence microscopy in the green illumination channel and images were taken with an ADF PRO 03 camera (ADF Optics Co. LTD) on Olympus BX43 microscope (Olympus Co. LLC).

Sections with further quenching of autofluorescence of RBC were incubated in TBST buffer, after which a freshly prepared solution of Sudan Black B was applied. The concentration of 0.3% SBB was dissolved: in a solution of 70% ethanol (group of sections B, C, D); in a 70% ethanol and absolute DMSO solution in a ratio of 4:1 v/v (section group A). Sections coated with SBB solutions were incubated for 25 minutes in the dark and then the solutions were washed off.

Three protocols for removing SBB from sections were tested:

- rinsing 5 times with a 70% alcohol and absolute DMSO solution (9:1 v/v) and subsequently with TBST buffer 5 times (section group C);
- rinsing with absolute DMSO 2 times and subsequently with TBST buffer 5 times (section group B);
- rinsing 5 times with a solution of 70% alcohol and subsequently with TBST buffer 5 times (section group A);
- rinsing 7-10 times with TBST buffer (section group D).

After rinsing with TBST buffer sections were covered with glass slips using LumiMount mounting medium (Lumiprobe Ltd) and microscoped. Assessing of protocols was performed using bright-field microscopy and fluorescence microscopy in the green illumination channel and images were taken with an ADF PRO 03 camera on Olympus BX43 microscope.

3 Results

Incubation of bovine liver samples with SBB in ethanol and DMSO solution leads to quenching of erythrocyte autofluorescence. When examining samples in a bright-field, no accumulation of dye granules was detected, which indicates the effective dissolution of the dye. The protocol of rinsing off the dye with ethanol and then with a TBST buffer turns out to be the most effective, but also gentle, which is indicated by the absence of fluorescence in cells – group of sections A (Fig. 1).

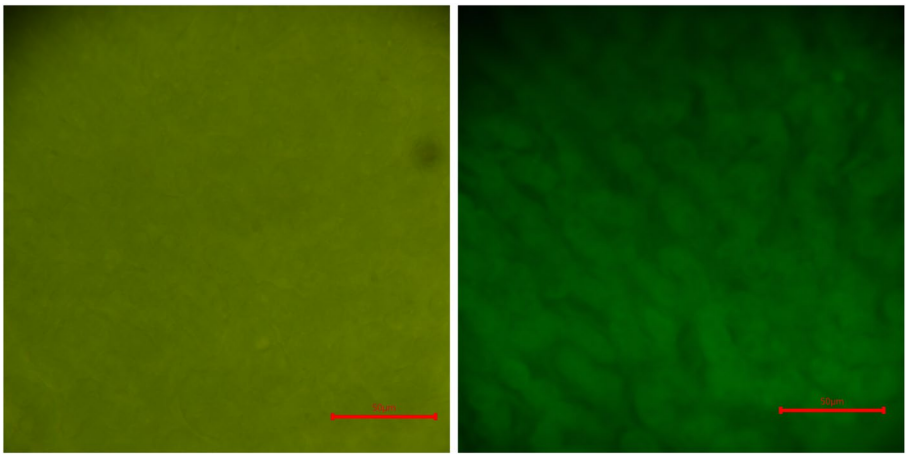

Fig. 1. Bovine liver: absence of dye granules (bright-field microscopy, left) and absence of autofluorescence of red blood cells (green illumination channel, right).

Incubation of chicken liver samples with SBB in ethanol solution does not lead to quenching of erythrocyte autofluorescence. However, this is probably due to the technique of rinsing off the dye: when sections are treated with DMSO solution and TBST buffer, SBB is overly washed off, and, as a consequence, the appearance of autofluorescence of erythrocytes is present – section group B (Fig. 2). Even though dye granules are not detected with bright-field microscopy, this method of SBB rinsing is not efficient for autofluorescence quenching, although the absence of DMSO in the dye solution reduces the efficiency of Sudan Black B dissolution.

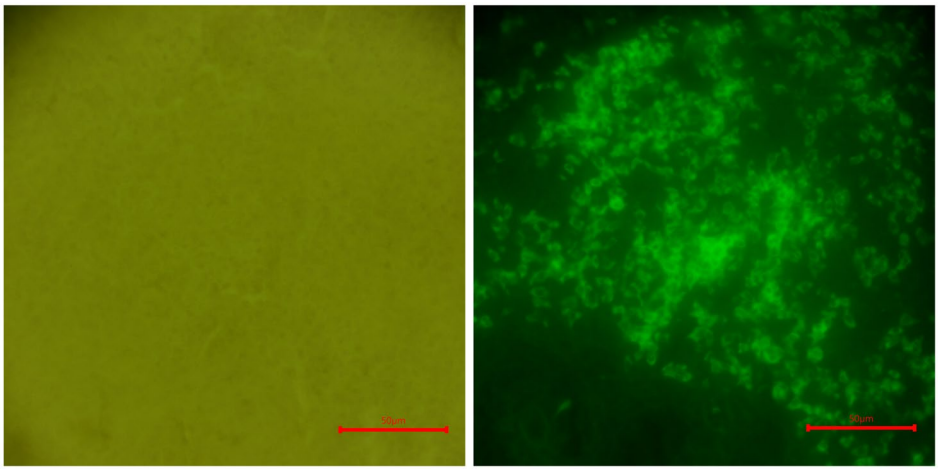

Fig. 2. Chicken liver: absence of dye granules (bright-field microscopy, left), but presence of autofluorescence of red blood cells (green illumination channel, right).

A similar autofluorescence pattern is observed for sections of porcine liver incubated with Sudan Black B dissolved in ethanol. However, dye granules are detected by bright-field microscopy, which indicates that the rinsing of SBB with ethanol and DMSO was not sufficient enough to remove Sudan granules, but too intense for the dye to quench the autofluorescence of erythrocytes – section group C (Fig. 3).

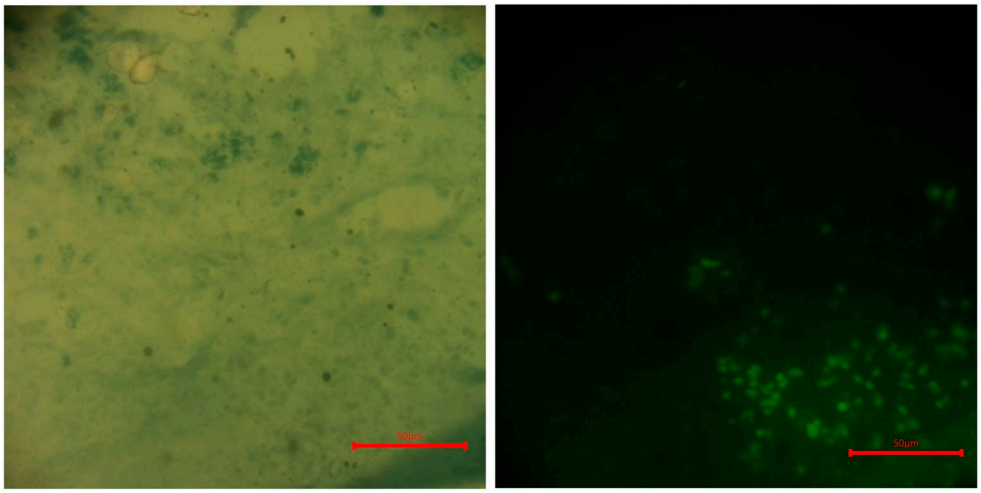

Fig. 3. Porcine liver: the presence of SBB granules (bright-field microscopy, left) and the presence of autofluorescence of erythrocytes (green illumination channel, right).

When sections of porcine heart muscle were incubated with SBB ethanol solution, quenching of erythrocyte autofluorescence was achieved. However, when examining the samples with bright-field microscopy, a large number of dye granules were detected. This is due to the fact that the section samples were rinsed off only with TBST buffer after incubation with the dye. With such treatment the dye is not sufficiently removed from the sections, which leads to suppression of fluorescence, but complicates the histological readability of such samples – group of section D (Fig. 4).

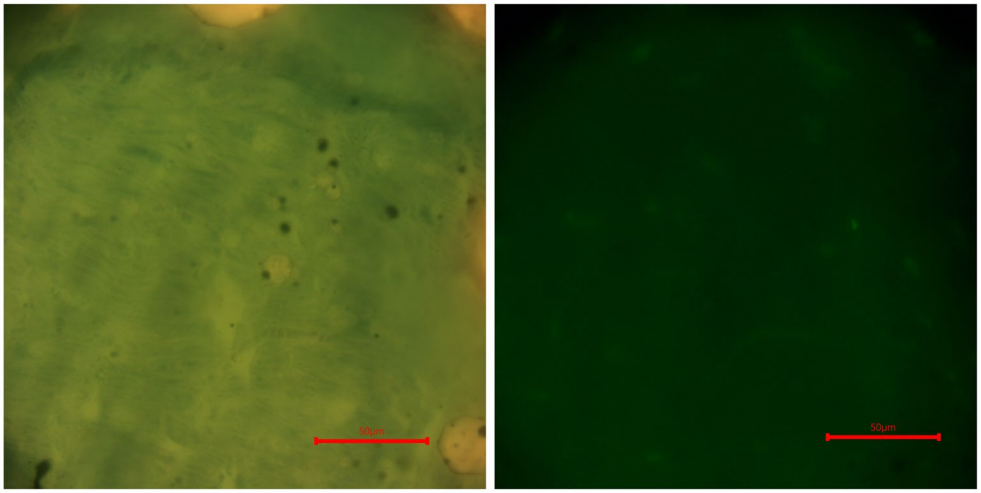

Fig. 4. Porcine myocardium: insufficient removal of SBB dye and undissolved granules (bright-field microscopy, left), but sufficient quenching of autofluorescence of red blood cells (green illumination channel, right)

Control sections of chicken small intestine tissue that were not incubated with SBB demonstrate intense autofluorescence of red blood cells (Fig. 5)

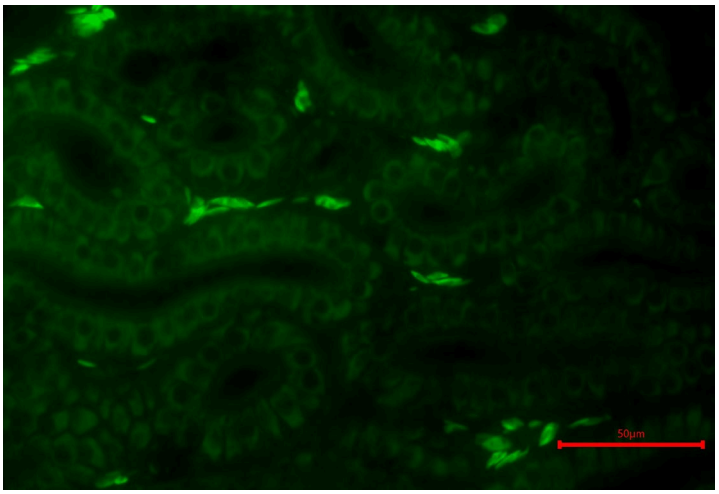

Fig. 5. Chicken small intestine: control sections were not incubated with Sudan Black B. Red blood cells show bright autofluorescence.

4 Conclusion

Many papers on IHC exploit different and non-standardized techniques to reduce tissue autofluorescence. It is necessary to have a unified treatment protocol while performing fluorescent IHC on different animals especially since SBB treatment efficiency does not seem to have a dependency on tissue type. Out of several tested protocols for staining sections with Sudan Black B the most optimal one was determined. The protocol, during which the minimum content of dye granules and the maximum quenching of red blood cell autofluorescence is observed, consists of two parts. The most effective dissolution of the SBB is achieved at a concentration of 0.3% in a 70% alcohol with absolute DMSO solution in a 4:1 v/v ratio. The most thorough removal of excess SBB solution while maintaining autofluorescence quenching is achieved by rinsing the sections 5 times with 70% alcohol and subsequently with TBST buffer 5 times.

Acknowledgements

The work was carried out within the framework of the RSF project No. 23-16-00117 “Spatial characteristics of transcriptomic and metabolomic features of premature structural and functional degeneration of organ tissues of farm animals and poultry.”
The research was approved by the Institutional Ethics Committee of the FSBSI “Ural Federal Agrarian Scientific Research Centre, Ural Branch of RAS” (protocol dated April 18, 2024).

References

1. H. Pandit, A. Valentin, M. Angel, C. Deleage, C. Bergamaschi, J. Bear, R. Sowder, B.K. Felber, G.N. Pavlakis, iScience, **26**, 2 (2023). https://doi.org/10.1016/j.isci.2023.105929
2. L. Luo, H. Uehara, X. Zhang, S.K. Das, T. Olsen, D. Holt, J.M. Simonis, K. Jackman, N. Singh, T.R. Miya, W. Huang, F. Ahmed, A. Bastos-Carvalho, Y.Z. Le, C. Mamalis, V.A. Chiodo, W.W. Hauswirth, J. Baffi, P.M. Lacal, A. Orecchia, N. Ferrara, G. Gao, K. Young-Hee, Y. Fu, L. Owen, R. Albuquerque, W. Baehr, K. Thomas, D.Y. Li, K.V. Chalam, M. Shibuya, S. Grisanti, D.J. Wilson, J .Ambati, B.K. Ambati, Elife, **2**, e00324 (2013). https://doi.org/10.7554/eLife.00324
3. J. Terreros-Roncal, M. Flor-García, E.P. Moreno-Jiménez, C.B. Rodríguez-Moreno, B. Márquez-Valadez, M. Gallardo-Caballero, A. Rábano, M. Llorens-Martín, Hippocampus, **33**, 4, 271-306 (2023). https://doi.org/10.1002/hipo.23474
4. J. Kajimura, R. Ito, N.R. Manley, L.P. Hale, J Histochem Cytochem, **64**, 2, 112-24 (2016). https://doi.org/10.1369/0022155415610792
5. W. Qin, M. Luo, K. Wang, M. Yang, H. Sheng, G. He, Microscopy, **67**, 6, 345-355 (2018). https://doi.org/10.1093/jmicro/dfy038
6. N.C. Whittington, S. Wray, Curr Protoc Neurosci, **81**, 2.28.1-2.28.12 (2017). https://doi.org/10.1002/cpns.35
7. J. Epstein, J. Sciubba, *Strategies for oral cancer detection*, (Elsevier, 2009). https://doi.org/10.1016/B978-1-4160-5202-9.50009-0
8. M.H. Armitage, J. Solliday, Microscopy Today, **28**, 5, 30-38 (2020). https://doi.org/10.1017/S1551929520001340
9. J.W. Hickey, E.K. Neumann, A.J. Radtke, J.M. Camarillo, R.T. Beuschel, A. Albanese, E. McDonough, J. Hatler, A.E. Wiblin, J. Fisher, J. Croteau, E.C. Small, A. Sood, R.M. Caprioli, R.M. Angelo, G.P. Nolan, K. Chung, S.M. Hewitt, R.N. Germain, J.M.

Spraggins, E. Lundberg, M.P. Snyder, N.L. Kelleher, S.K. Saka. Nat Methods, **19**, 3, 284-295 (2022). https://doi.org/10.1038/s41592-021-01316-y

10. S. Nazir, R.P.G. Charlesworth, P. Moens, P.F. Gerber, J Immunol Methods, **496**, 113097 (2021). https://doi.org/10.1016/j.jim.2021.113097
11. E. Seroussi, M. Knytl, F. Pitel, D. Elleder, V. Krylov, S. Leroux, M. Morisson, S. Yosefi, S. Miyara, S. Ganesan, M. Ruzal, L. Andersson, M. Friedman-Einat, Int J Mol Sci. **20**, 18, 4489 (2019). https://doi.org/10.3390/ijms20184489
12. F. Mercati, C. Dall'Aglio, L. Timperi, P. Scocco, E. De Felice, M. Maranesi, Eur J Histochem. **63**, 1, 2993 (2019). https://doi.org/10.4081/ejh.2019.2993
13. T. Brément, C. Cossec, C. Roux, A.C. Knol, B. Dréno, A. Khammari, P. Bourdeau, V. Bruet, J Comp Pathol. **167**, 82-90 (2019). https://doi.org/10.1016/j.jcpa.2018.10.179
14. V.C. Oliveira, R.C. Carrara, D.L. Simoes, F.P. Saggioro, Jr C.G. Carlotti, D.T. Covas, L. Neder, Histol Histopathol, **25**, 8, 1017-24 (2010). https://doi.org/10.14670/HH-25.1017
15. Z. Zhang, H. Fan, W. Richardson, B.Z. Gao, T. Ye, Eur J Histochem, **67**, 4, 3812 (2023). https://doi.org/10.4081/ejh.2023.3812
16. S. Wang, X. Ren, J. Wang, Q. Peng, X. Niu, C. Song, C. Li, C. Jiang, W. Zang, M. Zille, X. Fan, X. Chen, J. Wang, Front Immunol., **14**, 1168292 (2023). https://doi.org/10.3389/fimmu.2023.1168292
17. Y. Sun, H. Yu, D. Zheng, Q. Cao, Y. Wang, D. Harris, Y. Wang, Arch Pathol Lab Med. **135**, 10, 1335-42 (2011). https://doi.org/10.5858/arpa.2010-0549-OA
18. W.M. Frederiks, A. Slob, J.J. Frank, J. Tas, (Acta Histochem Suppl. **24**, 259-265 (1981).