Chapter 10

Sudan Black B, The Specific Histochemical Stain for Lipofuscin: A Novel Method to Detect Senescent Cells

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Abstract

The Sudan-Black-B (SBB) histochemical stain is well known to specifically react against lipofuscin, an aggregate of oxidized proteins, lipids, and metals. Lipofuscin is related to many ageing processes. It is also known to accumulate in senescent cells. We recently proved that lipofuscin detection, when applying the SBB staining, is highly specific for the visualization of senescent cells. Here, we present in detail this SBB method that can detect senescent cells in any material, irrespective of its preparation. This provides unique advantages not only in understanding physiological processes and the pathophysiology of various diseases but also in estimating the response to therapeutic interventions.

Key words Sudan-Black-B, Lipofuscin, Senescence, Histochemical stain, Archival material, Paraffin, Fresh material

1 Introduction

Cellular senescence is a fundamental feature of normal development and homeostasis, but it occurs also in many pathological conditions such as ageing, cancer, and other diseases [1–9]. This cellular state is either imposed by age-dependent telomere attrition (Replication Stress) or by various stress signals [4–12]. Oncogene Induced Senescence (OIS) is a type of Stress Induced Premature Senescence (SIPS) that is well established to act as an antitumor barrier [5–12]. Senescence exhibits also a "dark" site as senescent cells can promote tumor progression via the senescence-associated secretory phenotype (SASP) [9, 13].

The accurate recognition of senescent cells is essential not only in understanding their precise role in the aforementioned normal and pathophysiological processes but also in monitoring and assessing the outcome of applied therapies. Therefore, the employment of a reliable, convenient, and easy-in-use senescence biomarker that can be used in the frame of clinico-pathological studies is crucial [2, 9]. These investigations mainly deal with archival

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(formalin fixed-paraffin embedded, FFPE) samples. The current, most widely used biomarker for detecting cellular senescence is senescence-associated β-galactosidase activity (SA-β-gal) in sub-optimal pH [2, 14]. Its routine application however exhibits various disadvantages. The most important is the requirement of fresh frozen tissue that must be rapidly processed to preserve its enzymatic activity [14]. Thus, it is not applicable on archival (fixed) material and its use is rather laborious, driving the need for the establishment of more convenient senescence biomarkers [10, 15, 16].

Lipofuscin is considered a “hallmark” of aging [17]. Aged tissues are known to excessively contain senescent cells that commonly exhibit lipofuscin aggregation [17, 18]. However, it cannot be considered an absolute senescent marker. It concentrates within cells also under degenerative circumstances [18]. The material consists of oxidized and cross-linked proteins, lipids, and metals that accumulate in the cytosolic compartment of non-dividing cells, mainly in the lysosomes due to its nonsoluble and nondegradable nature [18–20]. Lipofuscin can be detected under a fluorescencent microscope due to its natural autofluorescent features as well as by histochemical techniques [21–23]. No specific antibody exists, so far.

The SBB technique is a well-known histochemical stain that has been used for many years for the identification of lipofuscin [24, 25]. It is an easy and rapid assay that provides reliable and reproducible results when used in a wide range of applications. It works also in frozen material, and therefore can be complementarily used with SA-β-gal when investigating senescence [26]. SBB is a highly lipophilic agent that exhibits high affinity to the lipid compartment of lipofuscin [19, 27, 28]. The positive SBB-lipofuscin reaction reveals blue-black intracellular granules in cells and frozen tissues and brown to black granules in FFPE tissues under the light microscope [29]. The SBB stain has also the unique property of “masking” the autofluorescence of lipofuscin. The latter feature can be used as a control of the method accuracy [29].

In our previous study, we merged the techniques of Gatenby et al. and *Rasmussen* for Sudan-Black-B (SBB) staining and developed the current SBB methodology that was proven to allow optimal lipofuscin visualization and further specific senescent cell detection in cellular and tissue material [27–29]. When comparing the findings with SA-β-gal staining, both techniques matched [29]. Most importantly, our SBB staining procedure was verified to identify senescent cells even in paraffin-embedded tissues, a unique feature that can be exploited in senescence studies with putative applications in basic and clinical research, diagnosis, and therapy [27].

2 Materials

2.1 Biological Samples and Their Accompanying Materials

1. Cells (From Aspiration or Cell Culture).
2. Cover Slips.
3. Fixative Solutions (*see* **Note 1**).
4. 1–5% (wt/vol) Paraformaldehyde/PBS Solution: Dissolve 1–5 g of Paraformaldehyde (PFH) in 100 mL of PBS in a glass beaker (*see* **Note 2**). Heat and stir the mixture until it becomes transparent. Let the solution cool and adjust pH to the value of 7.4 (*see* **Notes 3–5**).
5. Phosphate Buffered Saline (PBS; 10×): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4 (*see* **Note 6**).
6. Incubation Chambers for Cover Slips.
7. Tissue samples (OCT-Frozen or Fixed in 10% Buffered Formalin Solution and Paraffin Embedded/FFPE).
8. OCT-Tissue Freezing Media.
9. Superfrost Slides.
10. 10% Buffered Formalin Solution (ready to use).
11. 1% (vol/vol) formaldehyde/PBS: Add in a volumetric cylinder, of appropriate size, 10 mL of Buffered Formalin Solution 10%, adjust then volume to 100 mL with PBS, and stir (*see* **Notes 3–5**).
12. Positively Charged Glass Slides.
13. Coplin Jars.
14. Glass Beaker.
15. Volumetric Cylinder.
16. Thin edged Forceps.

2.2 SBB Solution Components

1. Sudan Black B reagent.
2. 70% Ethanol solution: add in a volumetric cylinder, of appropriate size, 70 mL of 100% ethanol and adjust volume with distilled water to 100 mL. Cover the cylinder with parafilm and stir (*see* **Note 3**).
3. Parafilm.
4. Filter paper.
5. Frittered glass filter of medium porosity.
6. Airtight Dye Container.
7. Dissolve 0.7 g of Sudan Black B in 100 mL of 70% ethanol in a glass beaker and cover it with parafilm. Stir the solution thoroughly overnight at room temperature (*see* **Note 7**). Filter next through filter paper and then filter again through frittered

glass filter of medium porosity with suction. The dye is then ready to use and can be stored for a short period in the airtight container (*see* **Note 8**).

2.3 SBB Staining Components and Accompanying Instruments

1. Xylene.
2. Gradually Decreased (96, 80, 70, and 50%) Ethanol Solutions (*see* **Note 9**).
3. Syringe.
4. 25 mm filter of medium porosity.
5. Soft paper (dry or dipped in ethanol).
6. Nuclear Fast Red reagent.
7. Aluminum Sulfate reagent (*see* **Notes 5** and **10**).
8. Thymol reagent (*see* **Note 11**).
9. Glycerol.
10. Tris Buffered Saline (TBS; 10×): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4 (*see* **Note 6**).
11. Light Microscope (*see* **Note 12**).
12. Fluorescent Microscope (*see* **Note 13**).

2.4 Preparation of Accompanying Counterstain and Mounting Components

2.4.1 Preparation of 0.1% Nuclear Fast Red Counterstain

1. Dissolve 5 g of Aluminum Sulfate in 100 mL distilled water in a glass beaker. Use heat and stir to dissolve until the mixture becomes transparent. Add then 0.1 g Nuclear Fast Red and continue stirring until the solution boils at 100 °C (*see* **Note 14**). Cool and filter through filter paper. Add a few grains of Thymol (*see* **Note 11**).

2.4.2 Preparation of 40% (vol/vol) Glycerol/TBS Mounting Medium

Add in a volumetric cylinder, of appropriate size, 40 mL of Glycerol, adjust then volume to 100 mL with TBS, and thoroughly stir (*see* **Notes 3** and **15**).

3 Methods

Prepare all solutions using deionized water and store all reagents at room temperature (unless otherwise indicated). All the following incubations are performed at room temperature (unless otherwise specified) in chambers (cover slips) and coplin jars (glass slides). Follow accurately all safety regulations during manipulations and waste disposal instructions when disposing waste materials.

3.1 Preparation of Material Under Investigation

3.1.1 Cells

Mount cells on cover slips and fix them in 1–5% (wt/vol) Paraformaldehyde/PBS solution for 5 min in room temperature. Then wash three times (approximately 1 min) with PBS (*see* **Note 16**).

3.1.2 Tissue Samples

1. FFPE sections: Cut thin paraffin sections and mount them on positively charged glass slides. Store them at 40 °C until staining.
2. Frozen sections: Cut thin OCT-frozen-sections, mount on superfrost slides (*see* **Notes 16** and **17**).

3.2 Staining Procedure

1. Incubate cover-slips with fixed cells in 70% ethanol for 2 min.
2. Dehydrate gradually OCT-frozen-sections (nonfixed or fixed) until 70% ethanol.
3. Dewax FFPE tissue sections with xylene and hydrate them gradually until 70% ethanol.
4. Put a drop from freshly prepared SBB on a clean slide with the use of a syringe that carries a 25 mm filter of medium porosity. (*see* Fig. 1a).
5. Turn down the cover-slip with the cells or tissue (using thin edged forceps) on the slide and attach them on the SBB drop in a way that the material faces down the drop of the dye on the slide (*see* **Note 18**), (*see* Fig. 1b).
6. Then observe the reaction under the light microscope (*see* **Notes 12** and **19**). Optimal staining with no precipitation routinely appears within 2–8 min (*see* **Notes 19–22**) (*see* Fig. 2).
7. Detach and lift with caution (with a tip or thin-edged forceps) the cover slip or the slide and wipe with the help of a soft paper

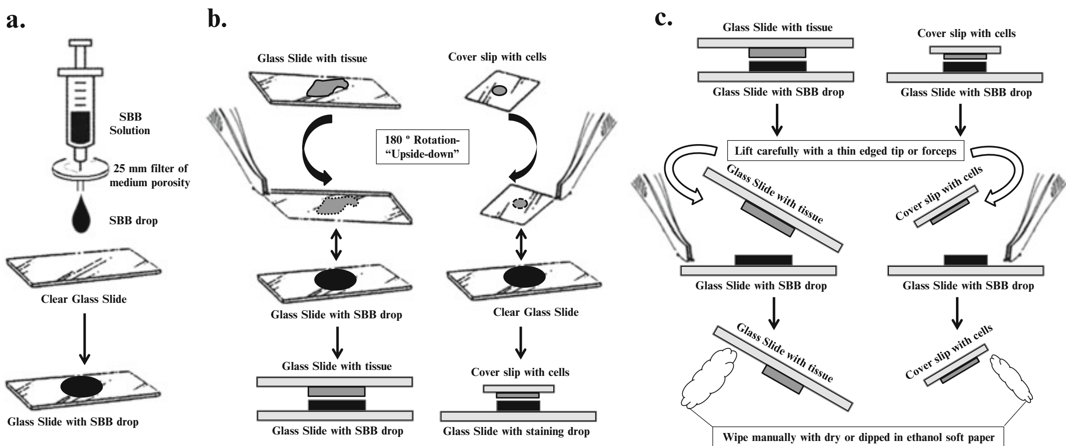
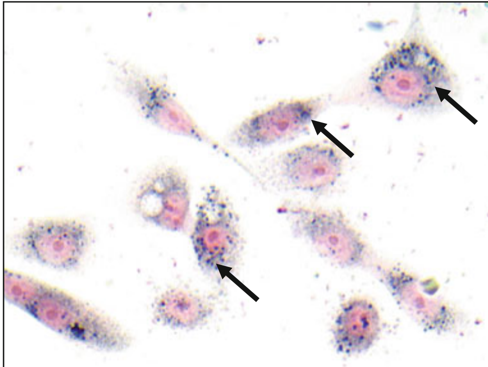
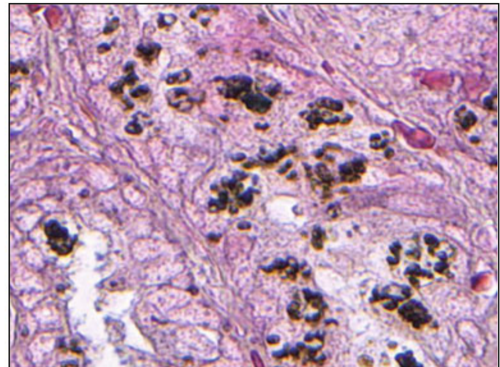
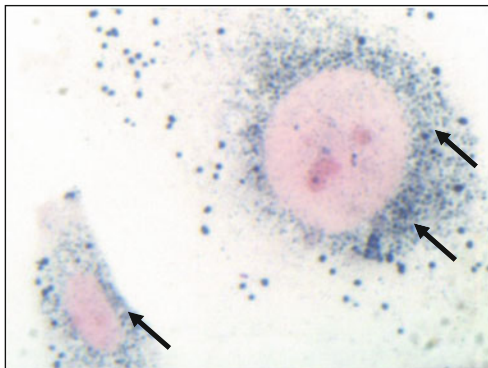


Fig. 1 Schematic presentation of important steps during the SBB staining procedure (*see* Subheading 3.4, for corresponding steps). (**a**) Step 4. (**b**) Step 5. (**c**) Step 7







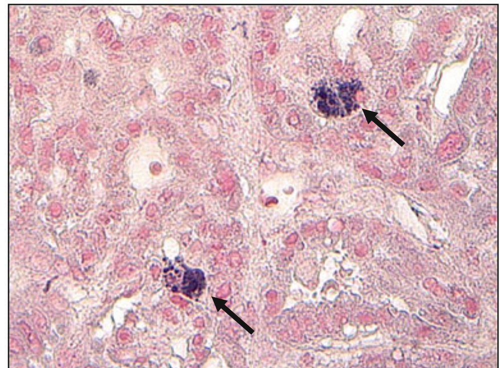


Fig. 2 Representative photos of SBB staining in established senescent models comprising of (**a**) cell lines [30, 31] and (**bii**) formalin fixed paraffin embedded tissue [29]. Arrows indicate positive SBB granules (Lipofuscin) within senescent cells (ai, aii and bii), (*see* **Notes 19** and **21**). Human seminal vesicle tissue was used in positive control staining assays (**bi**). Magnification, (ai and bii): 400×; (aii and bi): 630×. Counterstain: Nuclear Fast Red

(dry or dipped in ethanol) the excessive SBB stain from all sites (*see* **Note 17**) (*see* Fig. 1c).

8. Immerse the slide or cover slip quickly into 50% ethanol solution for a few times and then transfer and wash into distilled water (*see* **Note 23**).
9. Counterstain with 0.1% Nuclear Fast Red for 10 min.
10. Permanently mount in 40% Glycerol/TBS.

4 Notes

1. Various fixative media such as 100% Methanol, 100% Ethanol, and 1–5% (wt/vol) Paraformaldehyde/PBS Solution can be applied.

2. The amount of PFH depends on the desired concentration.
3. If larger volume is needed adjust the values accordingly.
4. The entire procedure must be performed in a flow hood to avoid any contact with fumes.
5. Always wear gloves, mask and glasses.
6. Store at 4 °C.
7. During the entire process the dye container must be air tightly sealed to prevent evaporation of ethanol, which in turn leads to precipitation of the saturated dye solution in cells and tissues.
8. Preferably always prepare a fresh solution before the experiments. Caution: Flammable.
9. Dilute accordingly 100 % Ethanol in distilled water, to reach the desired concentration.
10. Attention: This reagent is photosensitive. Always handle under low light conditions.
11. Thymol is used as a preservative.
12. The light microscope must be equipped with high magnification lenses (×40 and ×63. Magnification lens ×100 is optional).
13. The Fluorescent Microscope can be used in the detection of lipofuscin, as this material exhibits auto fluorescent features. We used the Leica DMRAZ microscope equipped with the Leica DFC350FX camera. The sample is mounted in 40% glycerol/TBS medium, after its appropriate preparation and observed by excitation at 450–490 nm, using a dichromatic mirror at 510 nm and a long-pass filter at 515 nm (*see* ref. [29]). This approach can be used in control experiments as lipofuscin autofluorescence is quenched with the current SBB staining method (*see* ref. [29]).
14. Monitor the temperature with a thermometer.
15. Store at room temperature.
16. Store in PBS until staining, either at room temperature for a short time or at 4 °C for a longer period.
17. If preservation of morphology is not crucial, the slides can be directly placed and stored in PBS. If preservation of morphology is essential, fix them in 1% (wt/vol) formaldehyde/PBS for 1 min. Wash three times (approx. for1 min) with PBS.
18. This step is crucial to avoid evaporation of the dye.
19. Perinuclear and cytoplasmic intracellular aggregates of blue-black granules (in cells and frozen material) or brown to black granules (FFPE material) at high magnification [30] correspond to positive lipofuscin staining (*see* Fig. 2).
20. Positive granules inside the cells are visible usually within only 2 min, but the procedure can be prolonged for 1–2 min for

clearer results. If no staining is evident within 5–6 min, the sample is probably negative.

21. The presence of smaller granules in FFPE tissue might occur due to partial lipid striping of lipofuscin during sample preparation.
22. In order to get familiar and acquire experience with detecting SBB positive cells under the light microscope while performing the staining reaction, without the presence of any counter stain, numerous trials and attempts are needed.
23. This step is crucial to remove "dirt" and clean cover slips and slides.

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