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Optimized protocol for identifying senescence-associated heterochromatin foci (SAHF) as markers of cellular senescence in blood samples



Tsupykov O.^{1,2}, Ustymenko A.^{2,3}, Kyryk V.^{2,3}, Skarzhevskiy O.², Parkhomenko O.²

¹Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine, Kyiv, Ukraine

²M. D. Strazhesko National Scientific Center of Cardiology, Clinical and Regenerative Medicine, National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine

³D. F. Chebotarev State Institute of Gerontology, National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine

*Corresponding author's e-mail: tsupykov@gmail.com

ABSTRACT

Senescence-associated heterochromatin foci (SAHF) are distinct nuclear structures formed in senescent cells and involved in the permanent suppression of genes promoting cell proliferation. SAHF are composed of repressive chromatin marks and specific proteins. Their detection serves as a reliable hallmark of cellular senescence and holds potential clinical applications in aging research, cancer biology, regenerative medicine, and disease monitoring – including use as a marker of high cardiovascular risk in patients who have experienced acute cardiac conditions. This article reviews the current methods for SAHF detection in cultured cells and introduces an optimized protocol for identifying SAHF-positive cells in peripheral blood samples, which may facilitate non-invasive monitoring of senescence in vivo.

KEY WORDS: SAHF; cellular senescence; heterochromatin; DAPI; fluorescence microscopy

Cellular senescence is a state of permanent cell cycle arrest induced by various cellular stresses, such as DNA damage, oxidative stress, telomere shortening, and oncogene activation [1]. First described by Hayflick and Moorhead in the 1960s, senescence was initially thought to be a simple proliferative endpoint [2]. However, it is now considered as a complex biological program with both beneficial and detrimental effects. On the one hand, senescence prevents the propagation of damaged cells, contributing to tumor suppression; on the other hand, it promotes tissue dysfunction and inflammation during aging via the senescence-associated secretory phenotype (SASP) [3].

Among the most distinct nuclear changes in senescent cells is the formation of senescence-associated heterochromatin foci (SAHF) [4]. SAHF were first identified in 2003 by Narita et al. as numerous compact DNA-dense structures in the nuclei of senescent cells, distinct from the chromatin organization of quiescent or proliferating cells [5]. They discovered that senescent cell nuclei display 30–50 distinct, brightly DAPI-stained DNA foci that are easily distinguishable from the chromatin pattern seen in young cells (**Figure 1A**) [5].



Fig. 1. SAHF organization: A – DAPI-stained nucleus of a senescent cell; B – schematic illustration depicting SAHF localization within the nucleus, with the inset providing an enlarged view. SAHFs correspond to chromosome territories (CT, white) encircled by the interchromosome domain compartment (ICD, grey). C – chromatin architecture scheme in young cells versus SAHF-containing senescent cells, where SAHF chromatin is structured into an H3K9me3-enriched core (red) surrounded by an H3K27me3-enriched ring (green). Modified from [4, 6].

SAHFs are encircled by a DAPI-negative interchromosome domain (ICD) region, which serves as a site for RNA transcription. Each SAHF represents a compacted chromosome territory (CT) enclosed within this ICD compartment (**Fig. 1B**) [4].

SAHFs exhibit a layered chromatin structure, with a central histone H3 trimethylated on lysine 9 (H3K9me3)-rich core surrounded by a facultative heterochromatin mark H3K27me3 ring that insulates it from active transcription zones (**Fig. 1C**) [6]. This organization implies that SAHF assembly involves both chromatin type clustering and spatial segregation into distinct concentric domains.

Disruption of SAHF can lead to abnormal cell growth. Emerging studies suggest SAHF also limit DNA damage responses, possibly protecting senescent cells from apoptosis, and may have functional relevance in aging tissues *in vivo* [7, 8].

Several chromatin-associated proteins participate in SAHF architecture. Notably, macroH2A, a histone variant linked to X chromosome inactivation, is enriched in SAHF [9]. However, its incorporation follows SAHF formation and does not initiate it, suggesting a role in structural maintenance rather than assembly. Overexpression of macroH2A in proliferating cells fails to trigger SAHF formation, although macroH2A accumulates to senescent levels [10].

In contrast, histone H1 – typically involved in chromatin compaction – is significantly reduced in various types of senescence, including those triggered by telomere attrition, oxidative stress, or oncogenic signaling [11]. This loss, likely driven by protein degradation rather than reduced synthesis, correlates with SAHF frequency. Interestingly, as H1 declines, non-histone proteins HMGA1 and HMGA2 become more abundant [12]. These proteins bind to AT-rich DNA via AT-hooks, and their increased presence suggests they displace histone H1 on linker DNA, contributing to the unique chromatin configuration of senescent cells. This structural rearrangement may enhance chromatin resistance to enzymatic digestion and reinforce transcriptional repression [13].

As SAHFs are becoming increasingly relevant to diagnostics and therapeutic monitoring, there is a need for simple, reproducible, and accessible detection techniques. While cellular senescence has been extensively characterized in cultured cells, its investigation in living human tissues remains limited due to technical challenges in detecting senescent cells *in vivo* [14]. Most clinical assessments rely on peripheral blood samples, which are the most accessible material in routine diagnostics. This review introduces fluorescent-based methods for detecting SAHF-positive nuclei in human peripheral blood samples – a minimally invasive and cost-effective approach that could be implemented in clinical laboratories.

Established laboratory methods for SAHF detection

SAHF are typically detected using fluorescence microscopy in cultured fibroblasts following induction of senescence [15-18].

When working with cultured cells, it is important to track their shift from an elongated, spindle-shaped form to a broad, flattened appearance, as this change indicates the onset of senescence [17]. SAHF formation varies by cell type and is most often linked to oncogene-driven senescence, meaning not all senescence models will display these structures [19].

Fibroblast cultures are maintained on coverslips until they reach the target confluence for SAHF assessment. Cells are gently rinsed with PBS to remove culture medium, with special care to avoid detaching loosely adherent senescent cells [17]. Fixation is carried out in 4% formaldehyde for approximately 10 minutes at room temperature, followed by PBS washes. After fixation with formaldehyde, nuclei are stained by incubating the cells with 4',6-diamidino-2-phenylindole (DAPI) solution (typically 0.1–0.3 µg/mL) for several minutes, then rinsed again in PBS to remove unbound dye. Coverslips are mounted with anti-fade mounting medium, avoiding air bubbles. Prepared slides can be stored at –20 °C for short periods, although fluorescence signal may gradually decline. Under a fluorescence microscope, senescent

fibroblasts display characteristic punctate DAPI-positive foci (SAHF), in contrast to the diffuse nuclear staining of non-senescent cells [18].

In addition to DAPI, SAHF detection also involves immunofluorescent labeling of histone modifications and chromatin-associated proteins characteristic of SAHF, such as trimethylated histone H3 at lysine 9 (H3K9me3), heterochromatin protein 1 isoforms (HP1α, HP1β, HP1γ), and the histone variant macroH2A [5]. These markers co-localize with DAPI-bright foci, confirming the presence of transcriptionally inactive heterochromatin typical for senescent cells.

Proposed cytological method for SAHF detection in venous blood

To enable clinical application, we developed a method to detect SAHF by DAPI staining in human peripheral blood cells using conventional cytology and fluorescence microscopy (**Table 1**).

Table 1. Protocol for identifying SAHF-positive cells in peripheral blood samples

	Steps	Notes
1	Place 10 µL of venous blood onto one end of a clean microscope slide. Use a cover glass at ~30–45° angle to create a thin smear. Allow to air-dry at room temperature.	Too large a drop → smear will be too thick. Too small a drop → smear will be too short. Lower angles → longer, thinner smears. Steeper angles → shorter, thicker smears.
2	Fix dried smears in vapors of freshly prepared 4% (w/v) formaldehyde solution at room temperature for 30 minutes.	Use a closed chamber.
3	Stain with DAPI (final concentration 0.105 µg/mL (300 nM) in PBS) for 3 minutes at room temperature.	Because DAPI dissolves poorly in PBS, use sonication if needed to help it go into solution. Using excessive DAPI or extending the staining time can reduce the clarity needed to differentiate SAHF from the surrounding chromatin.
4	Wash slides in PBS for 5 minutes.	Because senescent cells attach poorly to coverslips, PBS wash must be performed with extreme care. If such rinsing risks dislodging the cells, it is acceptable to omit the wash of the slides in PBS.
5	Dry the slides and apply a coverslip with an anti-fade mounting medium.	Ensure that no air bubbles are trapped beneath the coverslips during mounting.
6	Examine under a fluorescence microscope: – capture images of DAPI-stained nuclei; – count at least 100 nuclei per sample.	In senescent cells, DAPI typically produces a punctate nuclear pattern, whereas in non-senescent cells DAPI staining appears evenly distributed throughout the nucleus (Fig. 2).
7	Calculate the percentage of SAHF-positive nuclei: $\% \text{ SAHF-positive} = (\text{Number of SAHF-positive cells} / \text{Total counted cells}) \times 10^0$.	Slides may be kept at –4 °C for as long as one month, but the fluorescence intensity can gradually diminish during storage.

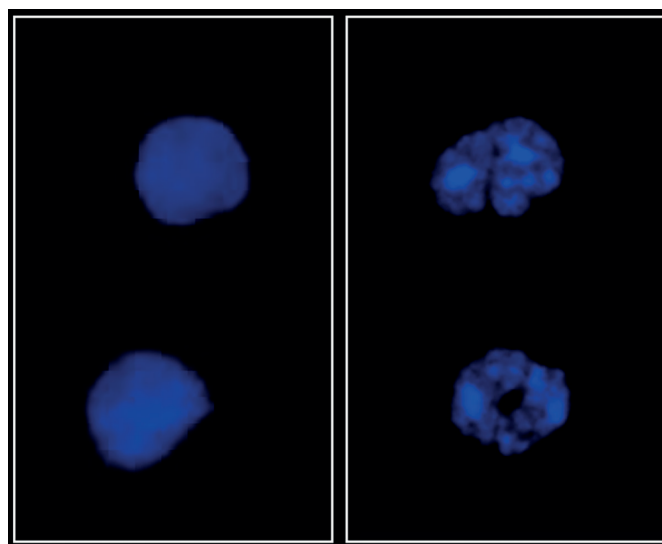


Fig. 2. Fluorescence images of DAPI-stained nuclei from human venous blood cells. A – nuclei without senescence-associated heterochromatin foci (SAHF); B – nuclei containing SAHF, ×400.

Advantages of formaldehyde vapor fixation:

- Gentle process with no liquid movement across the smear, ensuring cells remain undisturbed.
- Reduces the risk of washing away or damaging fragile elements such as platelets or compromised leukocytes.
- Maintains three-dimensional nuclear morphology and preserves delicate chromatin architecture, which is critical for SAHF detection.
- Prevents artefacts that can arise from liquid drying on the slide.

This method has been optimized to preserve nuclear morphology and chromatin integrity, allowing for reliable identification of SAHF based on their bright punctate DAPI fluorescence and characteristic shape.

Our cytological method applied to peripheral blood samples successfully visualized SAHF structures in a subset of mononuclear cells from patients who have experienced acute cardiac conditions (**Fig. 2**). These appeared as bright, condensed DAPI-stained foci resembling canonical SAHF in fibroblast cultures.

The reproducibility of results across different donors suggests that the method may be suitable for monitoring systemic cellular senescence.

Interestingly, the frequency of SAHF-positive nuclei varied across samples, with a higher percentage observed in aged individuals and patients with chronic inflammatory conditions, suggesting that systemic stress may influence circulating senescent cell burden. The quantification protocol enables semi-quantitative analysis, which could be correlated in future studies with markers such as p16, γ H2AX, and SASP components.

Notably, the presence of SAHF is not a universal marker of senescence. Murine fibroblasts and certain human cancer cells may exhibit senescent features without forming visible SAHF [20]. This underscores the importance of multi-parametric analysis when diagnosing or evaluating senescence *in vivo*.

The ability to visualize SAHF directly in blood cells introduces the possibility of using peripheral samples to track senescence dynamics during aging, chemotherapy, radiation exposure, or regenerative therapy. Unlike flow cytometry-based detection of p16 or β -galactosidase, this technique preserves nuclear context and permits morphological assessment alongside fluorescence data.

CONCLUSION

SAHF serve as a robust marker of chromatin remodeling and stable cell cycle exit in senescent human cells. While classical detection relies on complex immunofluorescence protocols in cultured cells, the method described here enables the identification of SAHF in peripheral blood - offering a new direction for clinical senescence diagnostics. This minimally invasive, rapid, and affordable method may facilitate broader applications in translational aging research, oncology, and regenerative medicine.

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Оптимізований протокол виявлення асоційованих із клітинним старінням гетерохроматинових осередків (SAHF) як маркерів клітинного старіння у зразках крові



Цупиков О. М.^{1,2}, Устименко А. М.^{2,3}, Кирик В. М.^{2,3}, Скаржевський О. А.², Пархоменко О. М.²

¹Інститут фізіології ім. О. О. Богомольця Національної академії наук України, Київ, Україна

²ДУ "Національний науковий центр «Інститут кардіології, клінічної та регенеративної медицини імені академіка М. Д. Стражеска Національної академії медичних наук України", Київ, Україна

³ДУ "Інститут геронтології ім. Д. Ф. Чеботарьова Національної академії медичних наук України", Київ, Україна

РЕЗЮМЕ

Асоційовані з клітинним старінням гетерохроматинові осередки (SAHF) – це специфічні ядерні структури, що формуються в клітинах, що старіють, і беруть участь у стабільному пригніченні генів, відповідальних за клітинну проліферацію. SAHF складаються з репресивних епігенетичних міток та специфічних білків. Їх виявлення є надійною ознакою клітинного старіння та має потенційне клінічне застосування в дослідженнях старіння, біології раку, регенеративній медицині та моніторингу перебігу захворювань — зокрема як маркер високого кардіоваскулярного ризику у пацієнтів, що перенесли невідкладні кардіологічні стани. У статті проаналізовано сучасні методи виявлення SAHF у культурі клітин та представлено оптимізований протокол для ідентифікації SAHF-позитивних клітин у зразках периферичної крові, що може сприяти моніторингу клітинного старіння *in vivo*.

Ключові слова: SAHF; клітинне старіння; гетерохроматин; венозна кров; флуоресцентна мікроскопія