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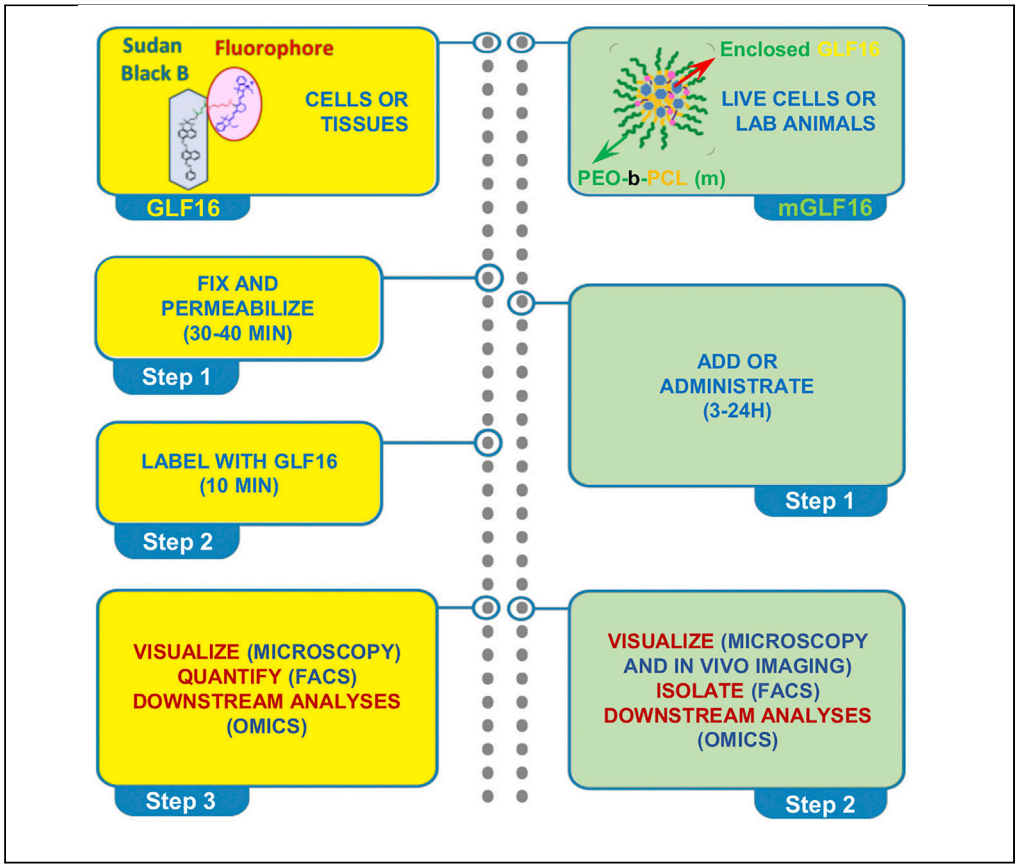
One-step rapid tracking and isolation of senescent cells in cellular systems, tissues, or animal models via GLF16



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Highlights

Steps described to detect senescent cells in any biological material

Compatible with multimarker analyses

Sorting of live senescent cells for cell culture and/or downstream applications

In vivo and *ex vivo* monitoring of senescence in murine models

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One-step rapid tracking and isolation of senescent cells in cellular systems, tissues, or animal models via GLF16

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SUMMARY

Identification and isolation of senescent cells is challenging, rendering their detailed analysis an unmet need. We describe a precise one-step protocol to fluorescently label senescent cells, for flow cytometry and fluorescence microscopy, implementing a fluorophore-conjugated Sudan Black-B analog, GLF16. Also, a micelle-based approach allows identification of senescent cells *in vivo* and *in vitro*, enabling live-cell sorting for downstream analyses and live *in vivo*

tracking. Our protocols are applicable to cellular systems, tissues, or animal models where senescence is present.
For complete details on the use and execution of this protocol, please refer to Magkouta et al.[1]

BEFORE YOU BEGIN

Cellular senescence is associated with cell cycle arrest and deregulated metabolism, and therefore, is inextricably linked to protein macromolecular damage[2] that leads to lipofuscin accumulation.[3] Lipofuscin (meaning "dark fat" deriving from both Greek and Latin) is a complex of undegradable aggregates of proteins, lipids as well as metals formed as a consequence of translational deregulation during stressful insults[3,4] and could be therefore considered the ''dark matter'' of senescent cells. Therefore, lipofuscin accumulation is one of the hallmarks of cellular senescence present in all types of senescent cells.[5,6] Lipofuscin detection by a recently synthesized fluorophore-conjugated Sudan Black-B analog, named GLF16, can therefore, be a reliable, quick and straightforward method of senescent cell identification.

The following protocols describe all the relevant steps for GLF16-dependent senescence detection in the Li-Fraumeni- $p21^{WAF1/Cip1}$ Tet-ON cellular system. For the same purpose, we also made use of the HBEC-CDC6 Tet-ON7 cellular system,[7] splenocytes, and prostate tumor cells.

Before proceeding with any animal model, the experimental design should be approved by the relevant Veterinary Administration Bureau of each country in order to certify that any animal handling is under compliance to national law and international Directives. Mice should be acclimatized for at least one week before initiation of the experiments, with food and water accessible *ad libitum*.

Institutional permissions

Experimental testing in mice described here was performed under compliance with the national law and the EU Directives and approved by the Veterinary Administration Bureau, Prefecture of Athens, Greece (Decision No: 548032, 08/05/2023).

Preparation and characterization of GLF16 stock solution and micelle encapsulation

Timing: 2–8 h

1. To prepare GLF16 stock solution, weigh 4 mg of GLF16 and dilute into 500 μL of DMSO (solution 1). Prepare aliquots and store up to 12 months at 4°C, protected from prolonged exposure to light. GLF16 working solution should be prepared as follows:
   a. Add to 25 μL of solution 1 an equal volume of Tween-20.

   Note: i. GLF16 can be weighted non-aseptically. ii. Given that Tween-20 is viscous, use wide bore tips (otherwise cut the narrow end of a regular 100 μL tip), keep the tip inside solution for longer while holding the pipette in an upright position.

   b. Dissolve with PBS up to 1 mL. This is the working stock solution of 200 μg/mL (solution 2), store up to 10–12 days at 4°C in dark condition.
      i. Prepare your GLF16 diluent (2.5% DMSO/2.5% Tween-20/PBS). You can use this to prepare the working solutions (see next step) and perform the washing steps.
      ii. Prepare the working solution using the GLF16 diluent. In case of immunofluorescence staining we suggest a working solution of 50–70 μg/mL, while for flow cytometry analyses concentration may vary from 10 μg/mL (in case of large cells e.g., fibroblasts, tumor cells) to 2 μg/mL (for small cells with limited cytoplasm e.g., lymphocytes).

2. To prepare micelle enclosed GLF16 (**mGLF16**) for isolation and tracking of live senescent cells follow these steps:
   a. Dissolve 5 mg PEO-b-PCL in 1 mL chloroform.
   b. Add 1 mL of GLF16 (stock solution 0.01 mg/mL), which is prepared by directly weighing GLF16 and dissolving it in 1 mL of pure acetone.
   c. Transfer the mixture into a round flask (such as #201–1355 by VWR) connected to a rotary evaporator (Hei-VAP series CORE-Heidolph). Apply vacuum until the formation of the polymeric film and for 20 more minutes in order to remove the traces of the solvent (1 mbar). A polymeric thin film will be formed by slow removal of the solvent at 42°C. In this step, the polymeric film could be stored at 4°C, and the hydration process could be done in the next few days.
   d. Hydrate the polymeric film stored in round flask (#201–1355 by VWR) in 1 mL HPLC-grade water, by slowly stirring for 1 h, in a water bath at 42°C.
   e. The concentration of PEO-b-PCL in the final micellar dispersion was 5 mg/mL and the concentration of GLF16 was 0.01 mg/mL.
   f. Use a probe sonicator (such as (Bandelin sonopuls, homogenizer, HD3200) to equilibrate micelles by applying twice 3-min-long sonication cycles interrupted by a 3-min resting period, allow micelles to anneal for 30–60 min at room temperature.

Characterization of PEO-b-PCL:GLF-16 (micellar GLF16: m-GLF16)

Timing: 3–4 h

3. Upon micelle preparation Dynamic Light Scattering (DLS) is applied to study crucial physicochemical parameters such as size -R_h (Rh: Hydrodynamic radius) and size distribution –PDI (Polydispersity Index).
   a. A 100 μL aliquot of the micellar dispersion is diluted into 3 mL HPLC-grade water and DLS measurements are performed at a fixed temperature (25°C) and at a fixed scattering angle of 90°.
   b. The morphological characterization is achieved through Transmission Electron Microscopy (TEM) (TEM, CM20, Philips, Amsterdam, Netherlands). A droplet (3 μL) of the final micellar dispersion (control sample) or the m-GLF16 dispersion is directly applied to the grid without any further dilution.

Preparation of the senescence system of study (cellular or animal model)

Timing: 8–10 days for cellular models, 14–18 days for animal models

4. Preparation of Li-Fraumeni-p21$^{WAF1/Cip1}$ Tet-ON cellular system for acquiring senescent cells. The below protocol describes the steps to be followed for inducing senescence through p21$^{WAF1/Cip1}$ induction in the Li-Fraumeni-p21$^{WAF1/Cip1}$ Tet-ON cellular system that is cultured based on a previously described protocol.[8] (Timing: 8–10 days) In detail.
   a. Thaw a vial of cryopreserved Li-Fraumeni-p21$^{WAF1/Cip1}$ Tet-ON cells containing 2–3 million cells by diluting the 1 mL of cell suspension (in 10% DMSO) in 5 mL of DMEM (10% tetracycline free FBS, 2 mM L-Glutamine, 1% Penicillin/Streptomycin solution).
   b. Collect cells by spinning down at 280 × *g* for 5 min at 20°–22°C (using a centrifuge such as Eppendorf 5804R), and resuspend them in 10 mL of DMEM (10% tetracycline free FBS).
   c. Seed cell suspension onto 10-cm cell culture dish. Freezing/thawing cycles do not significantly affect cell viability. However, let cells recover for 48 h before inducing senescence. In the case that cells are analyzed by immunofluorescence/confocal microscopy, cells may be seeded onto uncoated coverslips (see key resources table) at a density of 2 mL/well.

Note: FBS used in the culture should be Tetracycline-free, otherwise residual tetracycline (present in conventional FBS) may cause p21 $^{WAF1/Cip1}$ upregulation.

   d. Monitor cells daily and replace medium every other day until they reach 60–70% confluency.

Note: Cells can be split up using 3 mL of Trypsin (0.02%) for 2–4 min at 37°C) to 1:2 to propagate and acquire the desired cell number for the experiment.

e. Senescence induction: Remove medium and replace with fresh one (2 mL or 10 mL if seeded in 6-well or 10-cm dishes, respectively) containing 25 μg/mL Doxycycline (AppliChem, Darmstadt, Germany). Cells undergo senescence upon 6 days of doxycycline treatment. Cells should appear relatively enlarged in size. Media containing doxycycline should be replenished every two days.

Note: Make sure positive (70–90% senescent cells) and negative (exponentially proliferating cells) control groups are included. This way the selectivity and specificity of the staining will be determined. Positive control may include cells treated with 50–800 μM H_2O_2 for 72 h (treatment should be daily repeated) or exposed to a single dose of 10 Gray (Gy) using a gamma irradiator or any other condition that consistently induces high levels of senescent cells.

5. Bleomycin induced lung fibrosis murine model for *in vivo* monitoring of senescence.
   a. Anesthetize 8–10 week-old C57BL/6 mice by intraperitoneal injection of 150 μL of anesthesia/analgesia cocktail (solution of 5 mg/mL xylazine, 10 mg/mL ketamine in water for injection, or other alternatives such as isoflurane).
   b. Wait for 5–10 min until mice do not react to toe pinch

⚠ CRITICAL: Steps i–iii below are critical.

   i. Mice should be carefully monitored for excessive depression of cardiac rhythm or respiration or being insufficiently anesthetized, this would subject them to severe stress.
   ii. Thermal support is crucial during anesthesia. Put mice onto a heating blanket (45°C).
   iii. Protect the animals' eyes from corneal drying and/or trauma by applying an ophthalmic ointment.

   c. Administer 30 μL of 0.5 mg/kg body weight of bleomycin solution (3 units/μL) intratracheally using a 1 mL syringe bearing a plastic gavage tube of 2 mm diameter. In detail, place the mouse on an intubation platform (such as platform MIP, Penn Century Inc.) so that it is hanging from its top front teeth on the platform. Open the animal's mouth and gently pull out the tongue using a sterile swab. Locate the trachea, peer into the mouth and carefully slide the gavage tube into the trachea.

⚠ CRITICAL: Intratracheal administration requires a skilled operator. Short tachypnea (2–3 s) indicates successful intratracheal delivery of bleomycin.

   d. Animals should be monitored until complete recovery.
   e. Two weeks later mice develop lung fibrosis and their lungs are abundant in senescent cells.[9]
   f. Animals can be then prepared for *in vivo* imaging as described below. In case there is no access to specialized equipment for small animal imaging, mice should be euthanized and lungs can be perfused with 10% (v/v) formalin solution, excised, embedded in paraffin or OCT and processed for immunofluorescence analysis of the tissue.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-p16^{INK4A}	Abcam	Cat# ab54210; RRID: AB_881819
Rabbit monoclonal anti-Ki67	Abcam	Cat# ab16667, RRID: AB_302459
Alexa Fluor 488 goat anti-rabbit	Abcam	Cat# ab150077, RRID: AB_2630356
Alexa Fluor 568 goat anti-mouse	Abcam	Cat# ab175473, RRID: AB_2895153

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Doxycycline	AppliChem	A2951; CAS: 24390-14-5
Dulbecco's modified Eagle's medium (DMEM)	Thermo Fisher Scientific	11965092
Phosphate-buffered saline (PBS) 1×	Biowest	L0615-500
Fetal bovine serum (FBS)	Thermo Fisher Scientific	10270–106
Tetracycline-free fetal bovine serum	Biosera	FB-1001T/500
Paraformaldehyde (PFA)	Merck	104005; CAS: 30525-89-4
Bleomycin (sulfate)	Cayman	13877.5MG
Tween 20	Sigma-Aldrich	P1379; CAS: 9005-64-5
Triton X-100	Acros Organics	327372500
Sheep serum	Merck	S22-100ML
Br-PEG3-Ms (PEO-b-PCL)	BOC Sciences	CAS: 2702323-73-5
RNasin ribonuclease inhibitor	Promega	N2615
Critical commercial assays		
TrueVIEW Autofluorescence Quenching Kit	Vector Laboratories	SP-8400-15
RNA lysis buffer	Promega	Z3051
Experimental models: Cell lines		
Li-Fraumeni-p21WAF1/Cip1 Tet-ON	Galanos P. et al. 2016[8]	Generated by our group
Experimental models: Organisms/strains		
Mouse: C57BL/6 (8- to 10-week-old)	BSRC Al. Fleming (Vari, Greece)	N/A
Bleomycin induced lung fibrosis	This paper	Generated by our group
Software and algorithms		
Kuant	N/A	N/A
Other		
Coverslips	Knittel Glass	302974
6-well plates	Greiner	657185
Slide moisture chamber, Black	VWR	76278-848
10 cm cell culture plates	SPL Life Sciences	20101

MATERIALS AND EQUIPMENT

- Solution 1: 4 mg of GLF16, 500 μL of DMSO, store at 4°C up to 6 months, protected from prolonged exposure to light.
- Solution 2: 25 μL of solution 1, 25 μL Tween-20, 950 μL PBS, store at 4°C, protected from prolonged exposure to light.
- GLF16 diluent: 2.5 mL DMSO, 2.5 mL Tween-20, 95 mL PBS, store at 4°C, protected from prolonged exposure to light.
- Anesthesia/Analgesia cocktail: 1 mL of xylazine, 2 mL ketamine, 17 mL water for injection, store at 20°–22°C up to one week.
- Bleomycin solution: 600 units Bleomycin, 200 μL water for injection, store at −20°C up to 6 months.
- Formalin: 10 mL 40% formaldehyde in 90 mL distilled water, store at 20°C–22°C. This solution is irritating, corrosive and toxic, and should be prepared in a fume hood.
- 4% paraformaldehyde (PFA): 4 g paraformaldehyde in 86 mL distilled water and 1 drop of NaOH 10 N. Dissolve at 60°C. Let the solution cool down at 20°C–22°C, add 10 mL 10× PBS and adjust pH to 7.4. Bring volume up to 100 mL. Filter with a (#4) Whatman paper. Store at 4°C for a few days otherwise freeze at −20°C for future use. This solution is irritating, corrosive and toxic and should be prepared under a fume hood.

STEP-BY-STEP METHOD DETAILS

Detection of senescent cells in formalin-fixed paraffin embedded samples or cultured cells

Timing: 2 days for FFPE samples, 1 day for cells cultured onto coverslips

The protocol below describes the steps that will lead to visualizing senescent cells under a fluorescent or confocal microscope together with protein markers of interest. In our case, the senescence-related $p16^{INK4A}$ and Ki67 markers were used. However, the protocol can be readily adjusted for any other marker of interest.

1. In the case of formalin-fixed paraffin embedded (FFPE) samples:
   a. Deparaffinize sections by:
      i. Incubation at 60°C for 30 min.
      ii. Washing in Xylene for 15 min at 20°–22°C.
   b. Gradual rehydrate in:
      i. EtOH 100% for 15 min at 20°–22°C.
      ii. EtOH 96% for 10 min at 20°–22°C.
      iii. EtOH 80% for 5 min at 20°–22°C.
      iv. EtOH 70% for 3 min at 20°–22°C.
      v. EtOH 50% for 3 min at 20°–22°C.
   c. Wash three times with 1× PBS.
   d. Pre-heat citrate acid solution (10 mM, pH = 6) at 100°C for 5 min in a steamer or microwave depending on the datasheet of the applied primary antibodies.
   e. Submerge slides into pre-heated citrate acid solution at 100°C for 18 min for retrieval of antigenic epitopes even in the case of GLF16 single staining.
      i. Let it cool for 20 min on ice.
      ii. Wash three times with PBS.

   Note: In the case of multi marker staining, adjust antigen retrieval conditions according to antibodies' datasheet. However, retrieval should not last less than 18 min.

   f. Place your sections into a humidified black box for immunofluorescence staining (slides moisture black chamber). Incubate tissue sections with sheep serum (1/40 diluted in PBS) for 1 h in 20°–22°C. Wash thrice with PBS. If using GLF16 only, omit this step.
   g. Apply 100 μL of 70 μg/mL GLF16 on each section and incubate for 10 min in the dark.
   h. Wash three times (10 min each), with GLF16 diluent buffer.
   i. Apply 0.3% Triton-X 100-PBS onto sections for 4 min and wash thrice with PBS. If you are using GLF16 only, proceed to step 11.
   j. Apply primary antibody/ies diluted in PBS for 1 h at 20°–22°C and incubate in a humidified box.
   k. Next wash three times with 1× PBS, 10 min each.
   l. Apply secondary antibody/ies diluted in PBS for 1 h at 20°–22°C and incubate in a humidified box.
   m. Next wash three times with 1× PBS, 10 min each.
   n. Decrease the tissue autofluorescence using an autofluorescence quenching kit (such as the vector TrueVIEW Autofluorescence Quenching Kit, cat. no.: SP-8400-15.) Wash three times with 1× PBS
   o. Counterstain using 50–100 μL of DAPI (Biotium, Fremont, CA), depending the size of tissue section, for 3–4 min. Wash with water for 1 min and mount samples using Mowiol solution (cat. no 81381, Sigma-Aldrich).
2. In the case of cells cultured onto coverslips:
   Remove culture media (from 6 wells plates) and wash twice with 1× PBS. Transfer coverslips onto 24-well plates.
   a. Fix cells in 4% paraformaldehyde (PFA) for 15 min at 4°C.

   ⚠ CRITICAL: Use fresh PFA solution (store aliquots at −20°C) as it tends to degrade and lose its properties.

b. Remove fixative (discard in an appropriate container for chemical waste) and wash cells with 1× PBS.
c. Permeabilize your cells using 0.25%–0.3% Triton-X-100 diluted in 1× PBS for 18 min.
d. Remove permeabilization solution and wash three times with 1× PBS.
e. Incubate coverslips with sheep serum (1/40, diluted in PBS) for 1 h at 20°–22°C and wash three times with 1× PBS.

Note: If using GLF16 only, omit this step.

f. Apply 100 μL of 20–70 μg/mL GLF16 working solution (diluting GLF16 stock solution using GFL16 diluent) and incubate for 10 min in the dark.
g. Wash three times using the GLF16 diluent (10 min each time).
h. Incubate cells with 0.3% Triton-X 100 diluted in 1× PBS for 3 min; Wash twice with PBS. If you are using GLF16 only, proceed to step 13.
i. Apply primary antibody/ies for 1 h at 20°–22°C. (We have used the Rabbit polyclonal anti-$p16^{INK4A}$, ab54210 and the Rabbit monoclonal anti-Ki67, ab16667, both purchased from Abcam Inc. Cambridge, UK).
j. Wash three times with 1× PBS, 10 min each.
k. Apply secondary antibody/ies for 1 h at 20°C–22°C. (We have used the Alexa Fluor 488 goat anti-rabbit ab150077 or the Alexa Fluor 568 goat anti-mouse ab175473, both purchased from Abcam Inc. Cambridge, UK).
l. Wash three times with 1× PBS, 10 min each.
m. Counterstain using DAPI (40043, Biotium) for 3–4 min.
n. Wash with PBS for 10 min and mount samples using Mowiol solution. Allow 18 h for stabilization and polymerization of the mountant.
o. Analysis: GLF16 has an absorbance/emission max ratio of 629 nm/743 nm.
i. Set up your instrument accordingly and make sure you do not use any overlapping dyes such as Alexa Fluor 700.

Note: Representative pictures of GLF16 staining alone or combined with senescence associated markers are presented in Figures 1 and 3, respectively.

Flow cytometry and cell sorting

Timing: 3 h

The following protocols provide guidance toward (a) evaluating/quantifying senescent cell populations in a sample using flow cytometry or (b) isolation/sorting of live senescent cells using a cell sorter for subsequent downstream applications (cell culture, cell-based functional assays, transcriptomics etc.).

3. Protocol for multimarker staining and flow cytometry analysis of senescent cells.
   a. Harvest cells from culture. Use 10-cm dishes in order to obtain at least 3 × 10^6 cells.
   b. Centrifuge (Eppendorf 5804R) at 280 × *g* for 5 min at 20°–22°C.
   c. Wash once in 5 mL 1× PBS at 20°–22°C.
   d. Resuspend cell pellet in 2 mL of 4% PFA and incubate cells for 15 min at 20°–22°C (fixation).

⚠ CRITICAL: Make sure the cell pellet is well-dissociated otherwise the fixative will result in cross-linking among cells.

   e. Centrifuge at 280 × *g* for 5 min at 20°–22°C. Resuspend cell pellet in 3 mL PBS. Repeat centrifugation.

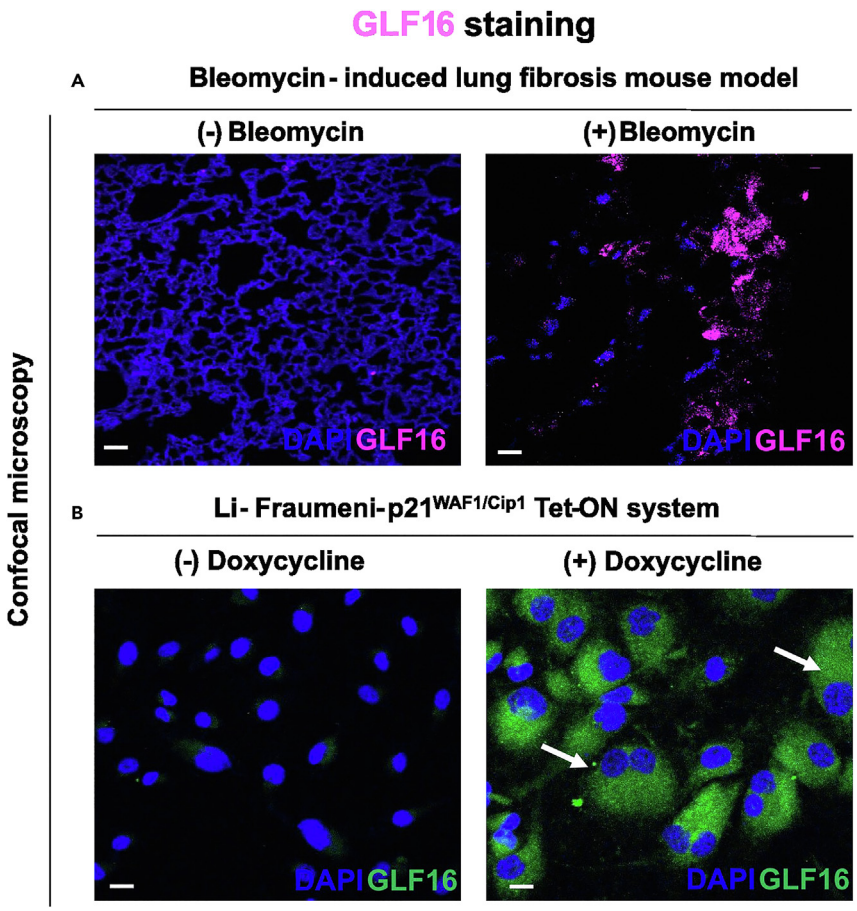

Figure 1. GLF16 staining of lung tissue sections and cellular models of induced senescence

(A) Representative pictures of GLF16 staining of lungs of bleomycin (right) or vehicle (left) treated mice. Objectives 10× and 40×, Scale bars: 50 μm (-Bleomycin), 10 μm (+Bleomycin). Pictures were originally published in Magkouta, Veroutis, Pousias, Papaspyropoulos et al., 2023).

(B) Representative pictures of Li-Fraumeni$^{p21WAF1/Cip1}$ Tet-ON cells treated with doxycycline (right) or not (left). Lipofuscin aggregates are clearly visualized by GLF16, as depicted by white arrows. Objectives 40×, Scale bar: 10 μm.

f. Permeabilize cells by suspending the cell pellet in 0.3% Triton-X/PBS (1 mL) and incubating it for 15 min at 20°–22°C.

Optional: Steps i–iv below are optional.

i. You can alternatively permeabilize cells by adding 900 μL of ice cold methanol (100%).
ii. Addition of methanol should be performed slowly in pre-chilled cells with agitation using a vortex (at low speed).
iii. Adjust methanol concentration at 90% by adding 100 μL of water (pre-chilled).
iv. Permeabilize for at least 10 min.

Note: Cells in 90% methanol can be stored at −20°C until analysis (up to one month).

g. Wash with 2 mL PBS at 20°–22°C.
h. Centrifuge at 280 × *g* for 5 min at 20°–22°C.
i. Incubate samples with 100 μL of GLF16 (2–10 μg/mL) for 8 min, at 20°–22°C, while shaking gently. Avoid direct exposure to light.

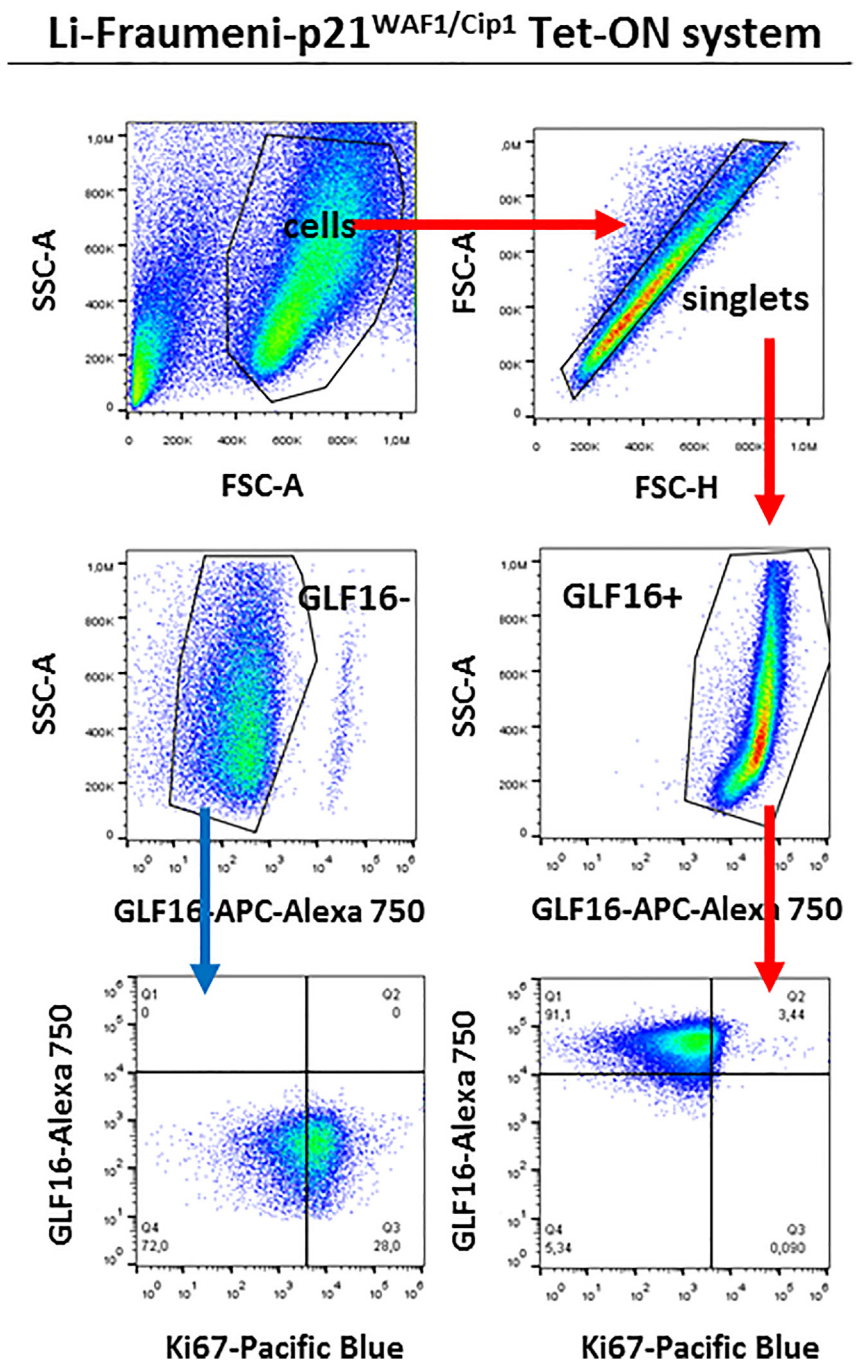

Figure 2. Flow cytometry application of GLF16

Representative gating strategy to identify senescent cells based on GLF16 staining profile. Total cell populations may be first gated on a forward scatter (FSC)/side scatter (SSC) plot so that debris is excluded from the analysis. Singlets should be subsequently identified using an FSC-A/FSC-H scatter plot. GLF16-positive and -negative populations can be subsequently selected either by GLF16 alone or combined with other markers, such as the proliferation marker Ki67.

j. Add 2 mL of GLF16 diluent and centrifuge at 280 × *g* for 5 min at 20°–22°C. Repeat washing step once, using the GLF16 diluent, to ensure proper removal of the compound.

k. Incubate with the primary conjugated antibody/ies for 30 min at 20°–22°C. Avoid direct light exposure.

l. Collect cells by centrifugation at 280 × *g* for 5 min at 20°–22°C.
m. Resuspend cell pellet in flow cytometry buffer (PBS / 5% FBS).
n. Analyze in a flow cytometer, such as Attune Nxt Flow Cytometer by Thermo Scientific (Figure 2).

4. Protocol for isolation/sorting of live senescent cells in culture for downstream analyses
   a. Remove media from cell cultures and wash with warm 1× PBS.
   b. Incubate cells with 0.0166–0.166 μg/mL of m-GLF16 for 3 h (37°C, 5% CO_2).
   c. Remove culture medium and wash with warm 1× PBS to remove excessive m-GLF16.
   d. Collect cells by centrifugation at 280 × *g* for 5 min at 20°–22°C.
   e. Wash once in 5 mL 1× PBS at 20°–22°C.
   f. Resuspend cell pellet in flow cytometry buffer (PBS / 5% FBS) or BD FACS Pre-Sort Buffer (Cat. # 563503) in case the cells are more fragile for handling.
   g. Use a sterile 40 μm cell strainer (e.g., 35 μm cell strainer cap BD Falcon Cat #2235) to ensure that a single cell suspension is prepared immediately before the sample is passed through a cell sorter.

   Note: Filtration of the sample through the filter mesh of the cell strainer will most probably decrease the number of cells in the suspension.

   h. Prepare sterile 5 mL Falcon tubes (12 × 75 polystyrene tubes e.g., BD Labware Cat # 352054) containing 1 mL cold DMEM-10% FBS (or the appropriate culture medium) in case cells will be subsequently used for cell cultures or 5 mL sterile and RNase free tubes containing cold lysis buffer (such as #Z3051, Promega Corp. Madison, WI) and RNase inhibitor (1 unit/μL of RNasin Ribonuclease Inhibitor, Promega Corp. Madison, WI) for subsequent bulk RNA-seq analysis.
   i. Collect the senescent cell population of interest using a cell sorter (BD FACS Aria III, Becton Dickinson) (0.8–1 × 10^6 cells would provide sufficient RNA for bulk RNA-seq analysis).

⚠ CRITICAL: Unstained cells as well as stained non-senescent (proliferating) cells (negative) should be included in the analysis to help determine the positive signal for GLF16. Include a positive control using a cell population that is known to be abundant in senescent cells, for example cells treated with fresh hydrogen peroxide or a DNA-damaging agent (such as cisplatin or cytarabine) to ensure proper performance of the experimental set up and help to determine flow cytometry gates.

In vivo monitoring of senescence

Timing: 2 days

The following steps are required for monitoring senescence *in vivo* in lab animals.

5. Anesthetize mice using a 150 μL of anesthesia/analgesia cocktail (solution of 5 mg/mL xylazine, 10 mg/mL ketamine in water for injection). Wait for 5–10 min until mice do not react to toe pinch.
6. Administer 1 mg of m-GLF16 micellar dispersion (200 μL of the 5 mg/mL m-GLF16 solution) intravenously (e.g., the tail vein or through the retro orbital plexus) using an insulin syringe of a 30G needle.

⚠ CRITICAL: injection rate should be very low otherwise embolism may occur.

7. Monitor mice until complete recovery.
8. 24 h later anesthetize mice as described previously, unless the imaging device is supplied with a gas anesthesia system.

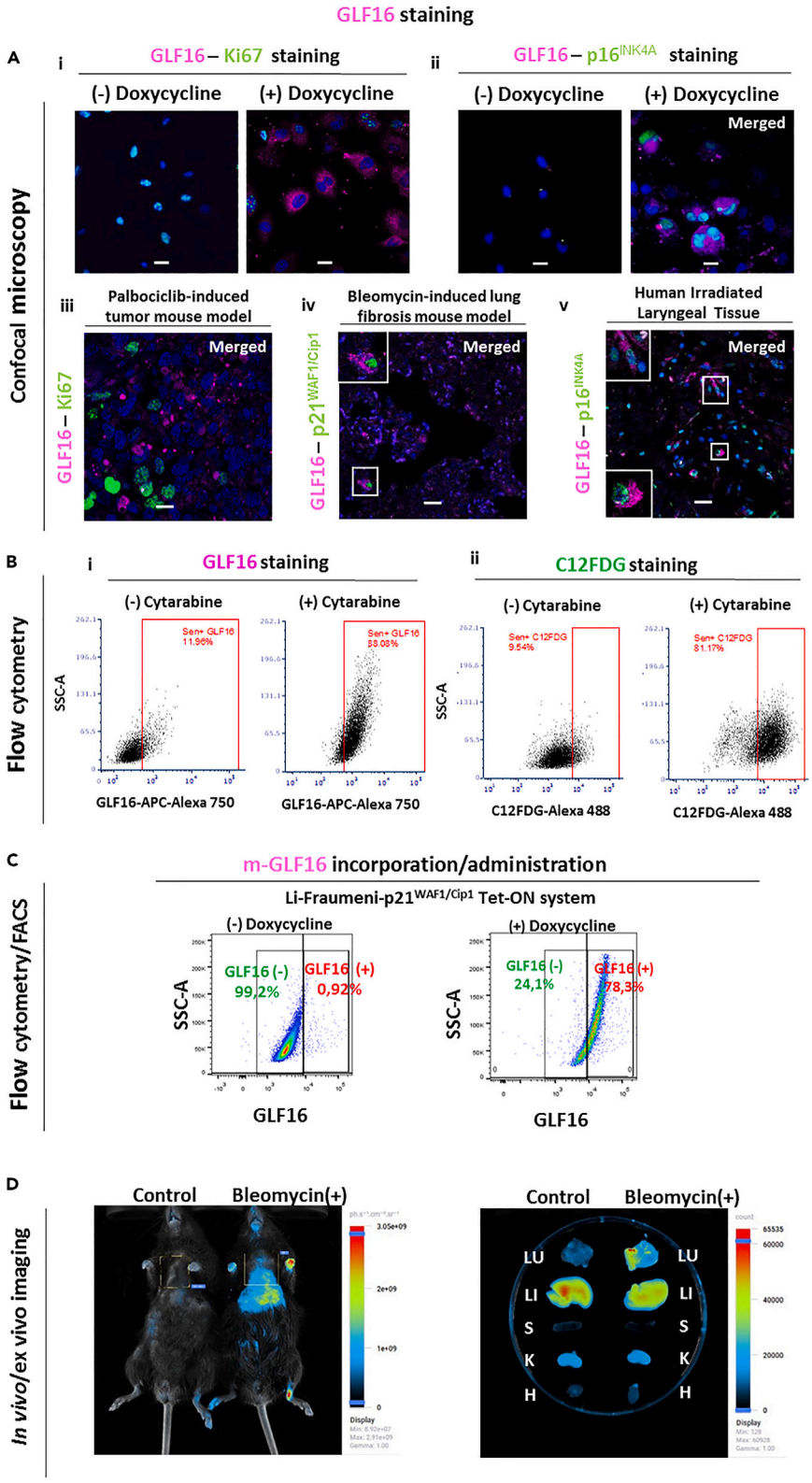
GLF16 staining
A
i
GLF16 – Ki67 staining
(-) Doxycycline
(+) Doxycycline
ii
GLF16 – p16INK4A staining
(-) Doxycycline
(+) Doxycycline
Merged
Confocal microscopy
iii
Palbociclib-induced tumor mouse model
Merged
GLF16 – Ki67
iv
Bleomycin-induced lung fibrosis mouse model
Merged
GLF16 – p21WAF1/Cip1
v
Human Irradiated Laryngeal Tissue
Merged
GLF16 – p16INK4A
B
i
GLF16 staining
(-) Cytarabine
(+) Cytarabine
Sen+ GLF16 11.96%
Sen+ GLF16 88.08%
SSC-A
GLF16-APC-Alexa 750
ii
C12FDG staining
(-) Cytarabine
(+) Cytarabine
Sen+ C12FDG 9.54%
Sen+ C12FDG 81.17%
SSC-A
C12FDG-Alexa 488
Flow cytometry
C
m-GLF16 incorporation/administration
Li-Fraumeni-p21WAF1/Cip1 Tet-ON system
(-) Doxycycline
(+) Doxycycline
GLF16 (-) 99,2%
GLF16 (+) 0,92%
GLF16 (-) 24,1%
GLF16 (+) 78,3%
SSC-A
GLF16
Flow cytometry/FACS
D
Control
Bleomycin(+)
Control
Bleomycin(+)
LU
LI
S
K
H
In vivo/ex vivo imaging

Figure 3. Representative pictures of expected outcomes upon

(A) GLF16 and $p16^{INK4A}$ or Ki67 fluorescent staining of fixed cells (upper panel) or tissues known to be abundant in senescent cells [lower panel (pictures were originally published in Magkouta, Veroutis, Pousias, Papaspyropoulos et al., 2023)] Objectives (i, ii) 20×, 40×, (iii) 63×, (iv, v) 40×. Scale bars (i-iii) 10 μm, (iv, v) 25 μm).

(B) Comparative flow cytometry analysis of senescent THP-1 cells (treated with 200 nM Cytarabine every other day for 7 days) and respective controls (untreated cells) using (i) GLF16 or (ii) C12FDG compounds.

(C) Representative flow cytometry analysis of cells incubated with m-GLF16.

(D) *In vivo* (left) and *ex vivo* (right) monitoring of senescence in the bleomycin-induced lung fibrosis model, Lu: Lung, Li: Liver, S: Spleen, H: Heart, K: Kidney.

⚠ CRITICAL: Intensity of fluorescence signal can be greatly compromised by the presence of animal hair, so mice should be either shaved or chemically depilated in the area of interest before conducting measurements. Either manipulations should be performed under caution otherwise skin damage may be caused. Any skin damage or wound shall falsely create positive signal due to the presence of hemoglobin.

9. Place mice onto the imaging platform (Newton 7.0 FT-500, Vilber, France) making sure that the area of study (in our case, the chest area should be analyzed in prone position) faces the detection camera.
10. Set excitation filter at 640 nm and emission at 750 nm. Aperture: 16, Sensitivity: high. Exposure time is calculated automatically.

Note: Exposure time varies (from milliseconds to seconds or minutes) depending on the animal model and the equipment used and should be adjusted based on negative control animals of the study.

11. Select the Region Of Interest (ROI). Fluorescence intensity should better be represented as photons per second per square centimeter per steradian.
12. Mice can be now euthanized and vital organs together with the region of interest (in our case, the lungs) can be excised, put on a transparent plastic culture plate and measured.

Note: Ex vivo measurement of vital organs acts in a complementary manner to verify the emission of fluorescence signal measured *in vivo*. In addition to this, the *ex vivo* measurement provides a more accurate quantitative measurement of the fluorescence signal emitted by the organ of interest.

EXPECTED OUTCOMES

As presented in Figures 1 and 3, since GLF16 binds to lipofuscin, the signal is expected to be detected in the cytoplasmic region. In some cases, lipofuscin granules can be clearly visualized (as depicted by white arrows, Figure 1B). GLF16 confers similar results to compounds already available (e.g., C12FDG, a fluorescent SA-β-gal substrate) (Figure 3B) Micelle m-GLF16 incorporation is expected to be highly efficient, approximately 70–80% (Figure 3C). Fluorescence signal during *in vivo* monitoring varies depending on the animal model and the equipment used. Hairless or wounded areas may present high autofluorescence (see animals' paws in Figure 3D left). In *ex vivo* imaging, high signals are expected in the liver and the kidneys, since these organs participate in the excretion of the micelles (Figure 3D right).

LIMITATIONS

Although the GLF16 compound can be applied in any biological sample (fixed or non-fixed), its use requires special equipment for analysis, such as fluorescent or confocal microscope, small animal imager, flow cytometer etc. Nevertheless, such equipment is now standard in most research facilities. Similarly, m-GLF16 requires access to TEM and a chemical laboratory and should be expended within a short period of time (up to one week).

In lipid–rich tissues that are embedded in OCT, a vague, diffused cytoplasmic reaction commonly occurs, corresponding to the large intracellular lipid droplets. In contrast, when embedded in paraffin, GLF16 will not interfere with lipid droplets since they are removed by the alcohols and xylene-based solutions used during the embedding process. In the latter case, lipofuscin, due to its nature (heavily oxidized lipids and lipoproteins among other constituents), remains intact and can be easily detected.[5,6]

TROUBLESHOOTING

Problem 1

PEO-b-PCL is difficult to dissolve (Step 2a during micelle encapsulation).

Potential solution

- Increase solvent (chloroform) up to 5 mL.

Problem 2

The polymeric film of PEO-b-PCL and GLF16 mixture is unevenly formed inside the round flask (Step 2c during micelle encapsulation).

Potential solution

- Redilute the film in 1 mL of pure acetone and 2 mL of chloroform and repeat the process.

Problem 3

The staining yields a high background and non-specific signal under the microscope.

Potential solution

- Apply lower concentration of GLF16 solution or prepare fresh.
- Add an extra washing step using fresh GLF16 diluent solution to remove excess compound.
- Optimize microscope settings to avoid overexposure.
- In the case of multiple staining the incubation time of blocking buffer can be increased or the concentration of the secondary antibody emitting in the red spectrum area can be decreased, in order to avoid overlapping of this signal with GLF16.
- Make sure that background autofluorescence has been successfully diminished using a quenching kit.

Problem 4

Staining of high-lipid OCT embedded tissues yields a diffused cytoplasmic reaction difficult to distinguish from the lipofuscin signal.

Potential solution

- Estimate your signal based on the morphological features of lipid droplets that allow even a non-experienced observer to discriminate them against lipofuscin. Particularly, lipid cells consist of one or few large central lipid droplets that occupy the entire cytoplasm, displacing the nucleus at the periphery. Lipofuscin staining with GLF16 is reflected by a granular staining pattern, either perinuclear or occupying the entire cytoplasm. Lipofuscin granules are of variable size but in any case are smaller than the aforementioned large lipid droplets.
- Perform serial section analysis with GLF16 and other lipid detecting dyes (for instance Oil Red O Stain) to distinguish the detection of lipids from lipofuscin true positive signal.
- Compare your GLF16 staining pattern in your OCT embedded tissue with GLF16 staining of the same tissue upon fixation and paraffin embedding (so that the lipids are diminished).

- In case you still find it hard to discriminate the lipid staining pattern from lipofuscin you can implement the guideline algorithmic approach to verify the area of senescent cells.[6]

Problem 5

There is no positive staining for GLF16 cells.

Potential solution

- Make sure that antigenic epitopes were successfully retrieved. In the case of a weak signal, increase incubation time of antigen retrieval buffer for another 5 min. For immunocytochemistry experiments, no signal or weak signal may be due to improper permeabilization of cells. This step may be extended for 3–4 min.
- Apply a higher concentration of GLF16 solution.
- Increase incubation time of GLF16 for 3–5 min. GLF16 may be also applied one more time after the washing step of the first application.
- Check the microscope settings, use the correct detector and filter.
- Keep samples at 4°C and observe them within 7 days.
- Include positive controls or verify that samples contain senescent cells using other senescence-associated markers.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Professor Vassilis G Gorgoulis (vgorg@med.uoa.gr).

Technical contact

Technical information and advice can be provided upon request by the lead contact, Professor Vassilis G Gorgoulis (vgorg@med.uoa.gr).

Materials availability

The compounds generated in this study are available upon request from the lead contact, Dr. Vassilis G. Gorgoulis (vgorg@med.uoa.grvgorg@med.uoa.gr).

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

S.M., D. Veroutis, A. Pousias, and A. Papaspyropoulos contributed to writing the *in situ* protocol; N. Pippa, N. Lougiakis, M.G., M.C., S.P., N. Pouli, and P.M. contributed to writing the key resources and troubleshooting, N. Lagopati and A. Polyzou contributed to graphical abstract preparation; S.M., D. Veroutis, K.G., K.K., S.F., E.K., A.K., D. Valakos, and G.V. contributed to figures preparations; S.M., D. Veroutis, A. Papaspyropoulos, K.G., A.K., N. Lagopati, A. Polyzou, and P.V. contributed to writing

the flow cytometry and cell sorting protocol; S.M., D. Veroutis, A. Pousias, A. Papaspyropoulos, N. Pippa, S.P., and P.V. contributed to writing the *in vivo* protocol; and D.T., R.P., M.D., K.E., R.D.M., and V.G.G. contributed to writing and supervising the work.

DECLARATION OF INTERESTS

Pending patents: (1) Greek Patent Application 20230100019 and (2) University of Dundee 20230100019, regarding SBB analogs hydrophilic compounds on chemical synthesis, method(s), and application(s) use.

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