Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and *in vivo*

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Normal cells can permanently lose the ability to proliferate when challenged by potentially oncogenic stress, a process termed cellular senescence. Senescence-associated beta-galactosidase (SA-βgal) activity, detectable at pH 6.0, permits the identification of senescent cells in culture and mammalian tissues. Here we describe first a cytochemical protocol suitable for the histochemical detection of individual senescent cells both in culture and tissue biopsies. The second method is based on the alkalinization of lysosomes, followed by the use of 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), a fluorogenic substrate for βgal activity. The cytochemical method takes about 30 min to execute, and several hours to a day to develop and score. The fluorescence methods take between 4 and 8 h to execute and can be scored in a single day. The cytochemical method is applicable to tissue sections and requires simple reagents and equipment. The fluorescence-based methods have the advantages of being more quantitative and sensitive.

INTRODUCTION

Nearly half a century ago, normal somatic human cells were shown to undergo only a limited number of doublings in culture before irreversibly arresting proliferation by a process termed replicative or cellular senescence[1]. Subsequent studies showed that, in addition to the permanent arrest of cell division, senescent cells acquired striking phenotypic changes, including resistance to apoptosis and an altered pattern of gene expression[2]. Further studies also showed that a senescent phenotype can be induced by exposure to a variety of stresses, such as oxidizing or DNA damaging agents (also termed stress-induced senescence or SIPS)[3], or the expression of activated oncogenes (also termed oncogene-induced senescence or OIS)[2,4]. It is now clear that this permanent growth arrest, termed here cellular senescence, is a potent tumor suppressive mechanism that occurs *in vivo* in several vertebrate species, including rodents and humans. Moreover, there is evidence that senescent cells can accumulate in renewable tissues with age and at sites of age-related pathologies, such as osteoarthritis[5] and atherosclerosis[6–8].

In 1995, we showed that senescent cells expressed a β-galactosidase activity that was detectable at pH 6.0: the now so-called 'senescence-associated β-galactosidase' activity (SA-βgal)[9]. This enzymatic activity was distinct from the acidic β-galactosidase activity, present in all cells and detectable at pH 4.0. SA-βgal is very easily detected. The first method of detection was a cytochemical assay using the chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal), which yields an insoluble blue compound when cleaved by β-galactosidase. The SA-βgal could be distinguished from the acidic (lysosomal) β-galactosidase activity by using a citric acid/sodium phosphate buffer at pH 6.0. SA-βgal activity was strongly associated with senescent cells, since it was not detectable in quiescent cells or terminally differentiated cells, although there are exceptions[9]. SA-βgal activity was also detected in human skin biopsies, and was therefore used as a biomarker of cellular senescence *in vivo*. SA-βgal activity is now a widely used biomarker in studies of cellular senescence in culture and *in vivo*[5,8,10–12]. In 1995, it was unclear whether SA-βgal was a distinct enzyme that was induced at senescence and active only at pH 6.0 or whether it was a consequence of increased expression and/or altered activity of the lysosomal β-galactosidase.

In 2000, we demonstrated that SA-βgal activity was because of an increase in the abundance of the lysosomal enzyme, probably linked to the increased lysosomal biogenesis observed in senescent cells[13]. Indeed, an increased number of lysosomes and elevated lysosomal activity had been associated with replicative senescence for many years[14–16]. To ascertain the lysosomal origin of SA-βgal activity, we increased the pH of lysosomes using lysosomal inhibitory drugs such as chloroquine or bafilomycin A1. Chloroquine is a weak base that concentrates in lysosomes, raising the pH to approximately 6. Bafilomycin A1 is a specific inhibitor of vacuolar-type H^+-ATPase and inhibits lysosomal acidification[17]. We then incubated the cells with 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), a fluorogenic substrate for β-galactosidase. This compound is membrane-permeable and nonfluorescent before hydrolysis; after hydrolysis of the galactosyl residues by β-galactosidase, the compound emits green fluorescence upon excitation and remains confined within the cell. Using this method of detection, we found an increase in β-galactosidase activity as a function of replicative age both in the absence and presence of lysosomal inhibitors[13]. Furthermore, although in the presence of inhibitors the absolute level of β-galactosidase activity was reduced by more than 50-fold, the relative residual increase in fluorescence between young and senescent cells was equivalent to that seen without lysosomal alkalinization.

In 2006, Lee *et al.*[18] confirmed the lysosomal origin of SA-βgal activity, showing that it resulted from increased expression of *GLB1*, the gene encoding the classic lysosomal enzyme. Lysosomal β-D-galactosidase is a hydrolase that cleaves the terminal β-galactose from ganglioside substrates and other glycoconjugates[19]. Defects in the *GLB1* gene are the cause of GM1-gangliosidosis and Morquio

B syndrome[20]. Multiple transcripts encoding different isoforms have been identified for *GLB1*. The SA increase in activity is because of an increase in the levels of *GLB1* mRNA and protein[13,18,21], probably resulting from the need to compensate for the accumulation of damaged non-degradable macromolecules and organelles in secondary lysosomes. Thus, although lysosomal β-D-galactosidase has an optimal pH of 4.0, the magnitude of the increase in senescence is sufficient for detection at the suboptimal pH of 6.0. In several models of stress-induced senescence, transforming growth factor-β1 positively regulated the appearance of SA-βgal positive cells[21].

Potential applications

The SA-βgal assay is very useful for testing whether different conditions or compounds can induce or inhibit the appearance of senescent cells. Potential applications include determining the replicative potentials of normal cell populations in culture and determining the abilities of drugs or genetic manipulations to induce a senescence response in culture or *in vivo* (tissue samples). In addition, SA-βgal can be used to study the appearance of senescent cells after different types of stresses[22–24] and to identify the potential antiaging protective effects of compounds[17].

Limitations

The limitation of the assay is that SA-βgal activity is associated with cellular senescence, but is not completely specific. For example, cells held at confluence can express the activity, as can some differentiated cells, such as adult melanocytes[9]. There are other markers of cellular senescence, although all of them are also not completely specific. These other markers include expression of the $p16^{INK4a}$ tumor suppressor protein, development of distinct domains of heterochromatin (senescence-associated heterochromatin foci) and stable nuclear foci containing DNA damage response proteins[2]. Moreover, certain biomarkers of senescence can depend upon one another. For instance, in human keratinocytes, loss of functional $p16^{INK4a}$ precludes the appearance of SA-βgal[25,26]. It is therefore advisable to identify senescent cells by more than one method, where possible.

Here, we describe two detailed protocols for SA-βgal detection. Both these assays (cytochemical and fluorescence-based) are in widespread use and there are, to our knowledge, no other widely used assays for SA-βgal. The cytochemical assay is particularly simple and quick, and allows histochemical detection in tissue samples[9]. The fluorescence-based assay is more sensitive, and, coupled to flow cytometry, provides a quantitative measure of βgal activity for each cell in the population, enabling a more accurate evaluation of differences in activity within cell populations and also between cultures of different replicative ages[13,21]. Both assays can be used in combination with immunological methods to identify SA changes in gene expression or to evaluate the senescence status of different subpopulation within a heterogeneous sample.

Experimental design

Cell culture. Senescence-associated beta-galactosidase activity and cellular senescence have been correlated in several cell types. SA-βgal activity is undetectable in immortal cells, except when they are genetically or chemically manipulated to undergo senescence. Culture and senescence induction conditions have been abundantly described elsewhere[9,13,18,21]. It is advisable to prepare positive and negative controls for each experiment. A typical positive control might be a culture of normal human fibroblasts induced to senesce by oxidative stress, ionizing radiation, oncogenic RAS or any convenient means, as described in the literature. A negative control might be a culture of early passage human fibroblasts or cancer cells.

The cytochemical assay must be carried out on subconfluent cell populations in culture, as it was reported that confluence can sometimes induce SA-βgal activity. Tissue sections can also be stained for SA-βgal activity[9]. In this case, it is crucial that the tissue be frozen in liquid nitrogen immediately after surgical resection and then quickly imbedded in optimal cutting temperature embedding medium (OCT) and stained. Indeed, storage of tissue samples at −80 °C for even a few hours can completely destroy the enzyme activity. It is also important to avoid incubating the samples in a CO_2 incubator. The 5–10% CO_2 in these incubators will lower the pH of the buffer.

Cytochemical or histochemical detection of SA-βgal activity. Cytochemical or histochemical detection of SA-βgal activity requires incubation of fixed cells or tissue (see **Box 1** for details) with the chromogenic βgal substrate X-gal in a buffer at pH 6.0. A blue color develops in some cells within 2 h, but staining is maximal after 12–16 h. After the staining, the cells are washed with phosphate-buffered saline (PBS) and viewed by bright field or phase contrast microscopy. The fixed and stained cells can also be rinsed with methanol and air-dried before microscopic viewing. The proportion of cells positive for SA-βgal activity can be easily determined by counting the number of blue cells in the total population. The blue staining is fairly stable and remains detectable in fixed and dried specimens for several months, if protected from light.

Concerning the histochemical detection of SA-βgal in fixed tissue, 4-micron sections are recommended. It is important to note that the fixation condition may vary depending on the tissue. The fixation condition presented in the protocol is suitable for skin sections. Fixation conditions must be kept as mild as possible, as long incubation times or harsh conditions may destroy SA-βgal activity.

Fluorescence detection of SA-βgal activity. In this procedure, the internal pH of lysosomes is increased to ~pH 6 using lysosomal inhibitory drugs such as chloroquine or bafilomycin A1. Cells are incubated with C_{12}FDG, a β-galactosidase substrate that becomes fluorescent after cleavage by the enzyme. This procedure can be done using living cells, and SA-βgal positive cells can be detected and quantified by flow cytometry. This procedure can also be carried out without lysosomal alkalinization, generally producing equivalent results. However, the choice to exclude the alkalinization step may depend on the particular experimental design and/or cell type, and therefore should be assessed by carrying out preliminary staining experiments in the absence and presence of bafilomycin A1.

Two additional adaptations of this protocol are also presented—one allows the measurement of SA-βgal activity by fluorescence microscopy, and the other allows the use of a microfluidic chip such as that employed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). In the microfluidic chip protocol, living cells are detected by the cell permeable pH indicator carboxynaphtofluorescein diacetate (CBNF), which emits red fluorescence when de-esterified in living cells. Although expressing the results as

BOX 1 | CYTOCHEMICAL STAINING FOR TISSUE SECTIONS

1. Flash freeze the tissue in liquid nitrogen after resection or isolation.
! CAUTION Liquid nitrogen is a dangerous material that can cause severe burns. Hand protection and goggles are to be worn when dispersing and handling liquid nitrogen.
! CAUTION Experiments using human or animal tissue should comply with institutional and national guidelines.
▲ CRITICAL STEP It is crucial that the tissues are directly flashed frozen and stained. Even overnight storage of tissue samples at −80 °C can destroy the enzyme activity.
2. Immediately embed the tissue in OCT compound (embedding medium for frozen tissue sections).
3. Immediately section (4 µm) the frozen tissues and mount onto superfrost slides.
4. Fix the tissue. For human skin, best fixation condition is 1% (wt/vol) formaldehyde in PBS for 1 min at room temperature (20–25 °C).
! CAUTION Hazardous. Formaldehyde is a toxic and corrosive solution. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Use a fume hood.
5. Remove the fixative and wash the cells three times, for approximately 1 min at room temperature, with enough PBS to completely immerse the slides.
6. Incubate overnight at 37 °C with enough staining solution to cover the slides.
! CAUTION Staining solution contains $K_4[Fe(CN)_6]$ $3H_2O$ and $K_3[Fe(CN)_6]$, which are irritants (higly toxic after hydrolysis) for humans and dangerous for the environment. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution and discard in an appropriate manner.
7. View blue-stained senescent cells by light microscopy and count manually.
▲ CRITICAL STEP Do not incubate in a CO_2 incubator.

median fluorescence intensities (MFIs) is suitable in most instances, in cases where suboptimal doses of the stressor induce senescence only in a small number of cells in the population, determination of the percentage of positive cells rather than the MFI should be used.

In the fluorescence microscope protocol, we describe the use of the TOPRO-3 dye to stain the nucleus. Other nuclei-specific dyes (e.g., DAPI) can also be used.

Relative advantages and disadvantages. Cytochemical detection of SA-β gal (Step 1A) requires less time and equipment than the fluorescence-based assays (Step 1B–D). Only a phase-contrast microscope is necessary. In addition, the cytochemcial assay can be used to detect senescent cells in tissues. The disadvantage is that cells must be counted 'manually' by the investigator at the microscope, which could require hours if several conditions are tested, and is subjective. Automated scoring and counting of cytochemically stained cells should be possible, although to our knowledge has not yet been reported.

Fluorescence-based detection is probably the best solution for the quantitation of SA-βgal positive cells at both the population and single cell levels. Further, these methods permit staining of living cells. The disadvantage of these methods is the need for a flow cytometer (option B), microfluidics analyzer (option C) or fluorescence microscope (option D) which are not available in all laboratories. The main advantage of the microfluidics analyzer is that it is much cheaper than a fluorescence-activated cell sorter (FACS). Among the three fluorescence-base detection methods, however, most laboratories have access to at least one method.

MATERIALS

REAGENTS

(**A**)

- WI-38 fetal lung human fibroblasts (Coriell Cell Repositories, cat. no. AG06814)
- Basal medium Eagle (BME, Gibco, cat. no. 41010-026)
- Fetal calf serum (FCS, Gibco, cat. no. 10270-106)
- Trypsin 25% (Gibco, cat. no. 25050-14)
- Human umbilical vein endothelial cells (HUVEC) (Biowhittaker, cat. no. C2517)
- Endothelial cell growth medium 2 (EGM-2) (Biowhittaker, cat. no. CC3162)
- Formaldehyde 37% (wt/vol) (Merck, cat. no. 1.04003) **! CAUTION** Hazardous. Formaldehyde is a toxic and corrosive solution. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Use a fume hood.
- Glutaraldehyde 50% (wt/vol) (Fluka, cat. no. 49629) **! CAUTION** Hazardous. Glutaraldehyde is toxic, corrosive and dangerous for the environment solution. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Use a fume hood. Discard in an appropriate manner.
- Sodium chloride (NaCl; Merck, cat. no. 567440)
- Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$; Merck, cat. no. 106346)
- Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$; Merck, cat. no. 106580) **! CAUTION** Eye and skin irritant. Handle with care. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution.
- Di-potassium hydrogen phosphate trihydrate($K_2HPO_4.3H_2O$; Merck, cat. no. 105099) **! CAUTION** Eye and skin irritant. Handle with care. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution.
- Potassium dihydrogen phosphate (KH_2PO_4; Merck, cat. no. 104873)
- Potassium hexacyano-ferrate (II) trihydrate ($K_4[Fe(CN)_6].3H_2O$; Merck, cat. no. 104984) **! CAUTION** Irritant (higly toxic after hydrolysis) for humans and dangerous for the environment. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Discard in an appropriate manner.
- Potassium hexacyano-ferrate (III) ($K_3[Fe(CN)_6]$; Merck, cat. no. 104973) **! CAUTION** Harmful for humans and dangerous for the environment. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Discard in an appropriate manner.
- Citric acid monohydrate ($C_6H_8O_7.H_2O$; Merck, cat. no. 100244) **! CAUTION** Irritant. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Use a fume hood.
- Magnesium chloride hexahydrate ($MgCl_2.6H_2O$; Merck, cat. no. 105833)
- 5-Bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal; AppliChem GmbH, cat. no. A1007)
- N,N-dimethylformamide (Sigma Aldrich, cat. no. 33120) **! CAUTION** Dimethylformamide has toxic and harmful vapors. Wear personal

protective clothing (e.g., nitrile or latex gloves, lab coat, goggles) when handling solution. Use a fume hood.
- Liquid nitrogen (Air Liquide) ! CAUTION Liquid nitrogen is a dangerous material that can cause severe burns. Hand protection and goggles are to be worn when dispersing and handling liquid nitrogen.
- OCT compound (Sakura Finetek, cat. no. 4583)

(**B**), (**C**) and (**D**)
- Bafilomycin A1 (Sigma, cat. no. B1793) ! CAUTION Irritant. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat, goggles) when handling solution. Use in a fume hood.
- C_{12}FDG (Molecular Probes, Invitrogen, cat. no. SKU#D-2893)
- CBNF (Molecular Probes, Invitrogen, cat. no. C-13196)
- Dimethyl sulfoxide (DMSO) (Acros Organics, cat. no. AC-29 552) ! CAUTION Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat, goggles) when handling solution. Use in a fume hood.
- Paraformaldehyde (Merck, cat. no. 104005) ! CAUTION Harmful. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat, goggles) when handling solution. Use a fume hood.
- Triton X-100 (Merck, cat. no. 112298) ! CAUTION Harmful. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat, goggles) when handling solution.
- Bovine serum albumin (BSA; PAA Laboratories GmbH, cat. no. K41-017)
- Topro-3 (Molecular Probes, Invitrogen, cat. no. SKU#T3605) ! CAUTION Possible mutagen. Wear personal protective clothing (e.g., latex gloves, lab coat and goggles) when handling solution.
- RNase (ICN, cat. no. 9973)
- Mowiol (Aldrich, cat. no.32,459)

EQUIPMENT

(**A**)
- Phase-contrast or bright field microscope (Leica) or similar microscope
- Superfrost microscope slides (VWR, cat. no. 631-0102)
- Microscope cover glasses (coverslips) (Assistant, cat. no. 1001/13)

(**B**)
- FACSCalibur (Becton Dickinson) or FC500 MPL (Beckman Coulter) or similar flow cytometer
- Cellquest (Becton Dickinson) or MXP/CXP (Beckman Coulter) or equivalent software

(**C**)
- Bioanalyzer (Agilent Technolgies)
- Cell Fluorescence LabChip kit (Agilent Technologies)
- Microfluidic chip (Agilent Technologies)

(**D**)
- Fluorescence microscope (Leica) or confocal microscope

REAGENT SETUP

Phosphate buffer (500 mM) Prepare a phosphate buffer (500 mM) containing 500 mM $K_2HPO_4.3H_2O$ + 500 mM KH_2PO_4. Adjust the pH to 7.4. This solution is stable several weeks at 4 °C.

PBS buffer Prepare a PBS buffer by dissolving 140 mM NaCl in 10 mM phosphate buffer. This solution is stable several for months at room temperature (20–25 °C).

(**A**)

Fixation solution Dilute 2% formaldehyde (vol/vol) and 0.2% glutaraldehyde (vol/vol) in PBS buffer. This solution is stable for 1 month at room temperature. ! CAUTION Hazardous. Formaldehyde and glutaraldehyde are toxic and corrosive solutions. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution and use in a fume hood.

Citric acid solution Make a 100 mM $C_6H_8O_7.H_2O$ solution in distilled water. This solution is stable for several weeks at room temperature. ! CAUTION Irritant. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Use a fume hood.

Sodium phosphate (dibasic) solution Dissolve 200 mM $NaH_2PO_4.H_2O$ or $Na_2HPO_4.2H_2O$ in distilled water. This solution is stable for several weeks at room temperature. ! CAUTION Eye and skin irritant. Handle with care. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution.

0.2 M citric acid/Na phosphate buffer Mix 36.85 ml of 100 mM citric acid solution with 63.15 ml of 200 mM sodium phosphate (dibasic) solution. Verify that pH is 6.0. This solution is stable at room temperature for 1 month. ▲ CRITICAL The pH of citric acid/Na phosphate buffer must be at 6.0.

X-gal solution Dissolve 20 mg ml^{-1} X-gal in N,N-dimethylformamide. This solution must be freshly prepared or can be stored for 2 weeks at −20 °C. ! CAUTION Dimethylformamide is toxic and harmful. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Use a fume hood.

Potassium hexacyano-ferrate (II) trihydrate solution Make a 100 mM $K_4[Fe(CN)_6]$ $3H_2O$ solution in distilled water. This solution is stable for several months at 4 °C. ! CAUTION Dangerous for the environment. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Discard in an appropriate manner.

Potassium hexacyano-ferrate (III) solution Make a 100 mM $K_3[Fe(CN)_6]$ solution in distilled water, stable. This solution is stable for several months at 4 °C. ! CAUTION Dangerous for the environment. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Discard in an appropriate manner.

Sodium chloride solution Dissolve 5 M NaCl in distilled water. This solution is stable for 1 month at room temperature.

Magnesium chloride hexahydrate solution Dissolve 1M $MgCl_2.6H_2O$ in distilled water. This solution is stable for 1 month at room temperature.

Staining solution Prepare the staining solution containing 40 mM citric acid/Na phosphate buffer, 5 mM $K_4[Fe(CN)_6]$ $3H_2O$, 5 mM $K_3[Fe(CN)_6]$, 150 mM sodium chloride, 2 mM magnesium chloride and 1 mg ml^{-1} X-gal in distilled water. This solution must be prepared freshly. ▲ CRITICAL This must be freshly prepared just before the staining.

(**B**), (**C**) and (**D**)

0.1 mM bafilomycin A1 Dissolve bafilomycin A1 in DMSO to a final concentration of 0.1 mM. This solution is stable for several months at −20 °C.

20 mM C_{12}FDG Dissolve C_{12}FDG in DMSO to a final concentration of 20 mM. This stock solution is stable and protected from light for several months at −20 °C.

2 mM C_{12}FDG Dilute the 20 mM stock solution of C_{12}FDG 1:10 with fresh cell culture medium to make a 2 mM working solution just before its addition into the cell culture.

4% (wt/vol) paraformaldehyde Dissolve paraformaldehyde in PBS to a final concentration of 4% (wt/vol). This solution is stable for 1 month at −20 °C. ! CAUTION Harmful. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution. Use a fume hood.

1% (vol/vol) triton X-100 Mix triton X-100 (vol/vol) in PBS to a final concentration of 1% (vol/vol). This solution must be prepared freshly. ! CAUTION Harmful. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution.

3% (wt/vol) BSA Dissolve BSA in PBS to a final concentration of 3% (wt/vol). This solution must be freshly prepared.

Topro-3 solution Dissolve 80× Topro-3 in 2 mg ml^{-1} RNase, prepare the solution fresh. ! CAUTION Possible mutagen. Wear personal protective clothing (e.g., latex gloves, lab coat and goggles) when handling solution.

EQUIPMENT SETUP

(**B**)

The procedures described in this section are intended for workers with some experience in flow cytometry. For further details, refer to the operating instructions of the individual instrument.
- Set up the instrument for acquisition. Adjust photomultiplier settings and gains to record forward scatter (FSC), side scatter (SSC) and fluorescein fluorescence (FL1).
- Run a nonlabeled cell suspension in setup mode. Using a dot plot display of FSC versus SSC, adjust the instrument threshold on the FSC channel to exclude small debris.
- Continue to run the nonlabeled cell suspension. Use a dot plot display of FSC versus FL1 and/or a histogram display of green fluorescence to adjust the FL1 voltage so that all events fall within the first decade of the logarithmic scale.
- Switch the instrument to acquisition mode and run the remainder of the tube collecting 10,000 events. The acquired data from this tube will be used at the analysis stage to determine the value of the autofluorescence signal.
- Run the labeled tubes in acquisition mode one after the other without altering the instrument settings.

PROCEDURE

1| Senescence-associated beta-galactosidase can be measured using option A, a chromogenic assay; option B, a fluorescence-based assay using flow cytometry; option C, a fluorescence-based assay using microfluidic detection; or option D, a fluorescence-based assay using microscopy.

(A) Chromogenic assay ● TIMING 15 min/2 d

(i) Wash subconfluent cells twice with enough PBS to comfortably cover the cells (~2 ml per 35 mm dish) for ~30 s per wash.

(ii) Add enough fixation solution to submerge the cells (1–2 ml per 35 mm dish); incubate for 5 min at room temperature.
! CAUTION The fixation solution contains formaldehyde and glutaraldehyde, which are toxic and corrosive solutions. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solutions and use in a fume hood.
▲ CRITICAL STEP Longer incubation times may destroy the SA-βgal activity.

(iii) Remove the fixation solution and wash the fixed cells twice with PBS as described in (i).
? TROUBLESHOOTING

(iv) Add the staining solution (1–2 ml per 35 mm dish).
! CAUTION Staining solution contains $K_4[Fe(CN)_6]$ $3H_2O$ and $K_3[Fe(CN)_6]$, which are irritants (higly toxic after hydrolysis) for humans and dangerous for the environment. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution and discard in an appropriate manner.

(v) Incubate overnight (12–16 h) at 37 °C.
▲ CRITICAL STEP Do not incubate the cells in a CO_2 incubator.
▲ CRITICAL STEP Blue color is detectable in some cells within 2 h, but staining is maximal after 12–16 h.

(vi) After the incubation, wash the cells for ~30 s twice with PBS (2 ml per 35 mm dish), and once with methanol (1 ml per 35 mm dish) and allow the dish to air dry.
■ PAUSE POINT The dried dishes can be kept for several months at room temperature, if protected from light.

(vii) View by bright field or phase contrast microscopy.
? TROUBLESHOOTING

(B) Fluorescence-based assay using flow cytometry ● TIMING 4–5 h

(i) To induce lysosomal alkalinization, pretreat subconfluent cells with 100 nM bafilomycin A1 for 1 h in fresh cell culture medium (2 ml per 35 mm dish) at 37 °C, 5% CO_2.
! CAUTION Bafiolmycin is an irritant. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution and use in a fume hood.

(ii) Add 33 µl of 2 mM C_{12}FDG working solution to the cell culture medium to give a final concentration of ~33 µM and continue the incubation for 1–2 h.

(iii) Remove the solution and wash the cell monolayers twice for ~30 s per wash with 2 ml of PBS at room temperature.

(iv) Harvest the cells by trypsinization or by any other appropriate means[27] followed by centrifugation at 100–250*g* (depending on the cell type) for 5 min at 4 °C.

(v) Resuspend the cells in ice-cold PBS at a concentration of ~1×10^6 cells ml^{-1}. A minimum of 0.4 ml cell suspension is required for analysis.

(vi) Run the cell suspension immediately in a FC500 MPL or similar flow cytometer. Acquire and analyze the data using MXP/CXP or an equivalent software.

(vii) Data processing to estimate the relative βgal activity: on a two-parameter display of FSC versus SSC set up an analysis region that excludes dead cells and subcellular debris. Depict the events within this region in a green fluorescence histogram where the *y* axis indicates cell number and the *x* axis indicates C_{12}-fluorescein fluorescence intensity in log scale. On this histogram, estimate the relative β-galactosidase activity (compared with positive or negative control cells) using the MFI of the population. Use a nonlabeled sample to determine the autofluorescence and if this value is significant subtract it from the MFI of the labeled samples.
? TROUBLESHOOTING

(viii) Data processing to estimate the percentage of positive cells: using an appropriate negative control as a reference (e.g., early passage nonstressed cells) divide the fluorescence histogram into two compartments by setting up a boundary between the negative (dim fluorescence) and positive cells (bright fluorescence). Estimate the percentage of positive cells by dividing the number of events within the bright fluorescence compartment by the total number of cells in the histogram. This determination can be also carried out on a two-parameter display of FSC versus C_{12}-FL1 in the same manner.
? TROUBLESHOOTING

(C) Fluorescence-based assay using microfluidic detection ● TIMING 6 h 30 min

(i) To induce lysosomal alkalinization, pretreat subconfluent cells with 100 nM bafilomycin A1 for 1 h in fresh cell culture medium (2 ml per 35 mm dish) at 37 °C, 5% CO_2.
(ii) Add 33 µl of C_{12}FDG to the pretreatment medium and continue the incubation for 2 h.
(iii) Remove the solution and wash the cell monolayers twice for ~30 s per wash with 2 ml of PBS at room temperature.
(iv) Add 2 ml of 10 µM CBNF diluted in PBS and incubate for 30 min at room temperature.
(v) Remove the solution and wash the cell monolayers twice for ~30 s per wash with 2 ml of PBS at room temperature.
(vi) Harvest the cells by adding 200 µl of trypsin. Incubate for 5 min at 37 °C.
(vii) Resuspend the cells in 1 ml of fresh cell culture medium containing 10% FCS.
(viii) Centrifuge for 4 min at 2,000*g*, room temperature.
(ix) Remove the supernatant and resuspend the cell pellet in 40 µl of cell buffer provided in the Cell Fluorescence LabChip kit.
(x) Run the microfluidic chip according to the manufacturer's instructions.
(xi) Use a dot plot tab to analyze the results. In the dot plot tab, single events (cells) are shown as they are detected, displayed as dots. Their red fluorescence (CBNF staining = living cells) intensity is mapped on the *y* axis and their blue fluorescence (C_{12}FDG staining = SA-βgal positive cells) is mapped on the *x* axis.
(xii) Add a region (rectangle) to the upper right part of the dot plot (limits: 10^0 in the *y* and *x* axis) to quantify the number of living cells that are SA-βgal positive. The same region must be added to the control and test plots.

(D) Fluorescence-based assay, fluorescence microscopy ● TIMING 6 h 30 min

(i) Twenty-four hours before staining, seed the cells at 50% confluence (for human fibroblasts: 10,000 cells per coverslip) in 24-well culture plates containing sterile coverslips. Culture for 24 h at 37 °C, 5% CO_2.
(ii) To induce lysosomal alkalinization, remove the culture medium and pretreat the subconfluent cells with 100 nM bafilomycin A1 for 1 h in fresh cell culture medium (2 ml per 35 mm dish) at 37 °C, 5% CO_2.
(iii) Add 33 µl of C_{12}FDG to the pretreatment medium and continue the incubation for 2 h.
(iv) Remove the solution and wash the cell monolayers twice for ~30 s per wash with 2 ml of PBS at room temperature.
(v) Fix the cells for 10 min with 4% (wt/vol) formaldehyde at room temperature.
! CAUTION Formaldehyde is a toxic and corrosive solution. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat or goggles) when handling solution and use in a fume hood.
(vi) Remove the fixative and wash the cell monolayers three times for ~30 s per wash, with 2 ml of PBS at room temperature.
(vii) Permeabilize the cells in 1 ml of 1% (vol/vol) triton X-100 for 5 min at room temperature.
! CAUTION Triton X-100 is harmful. Wear personal protective clothing (e.g., nitrile or latex gloves, lab coat and goggles) when handling solution.
(viii) Remove the triton and wash the cells twice with 2 ml of 3% (wt/vol) BSA at room temperature.
(ix) Stain the nuclei by reversing the coverslip onto a drop of ~30 µl of Topro-3 solution and incubate the cells for 30 min at room temperature.
! CAUTION Possible mutagen. Wear personal protective clothing (e.g., latex gloves, lab coat and goggles) when handling solution.
(x) Mount the coverslips in Mowiol and observe with a TCS confocal microscope.

● TIMING

Step 1A, Chromogenic assay using light microscopy: 15 min/2 d
Step 1B, Fluorescence-based assay using flow cytometry: 4–5 h/1 d
Step 1C, Fluorescence-based assay using microfluidic detection: 6 h 30 min/1 d
Step 1D, Fluorescence-based assay using fluorescence microscopy: 6 h 30 min/1 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(iii)	Cells wash away during the rinsing steps	Cells are not fixed. The fixation solution was incorrect	Adjust the concentration of formaldehyde and glutaraldehyde depending on the cell type
1A(vii)	No blue cells	Staining solution incorrect	Verify the pH of the solution Use a positive control in case there are no senescent cells in your conditions
1A(vii)	All cells are blue	Staining solution incorrect	Verify the pH of the solution Do not use a CO_2 incubator: CO_2 can decrease the pH of the staining solution under 6.0 through the HCO3−/CO_2 interchange Only senescent cells present in your conditions Use a negative control (e.g., SV-40 WI-38 fibroblasts)
1B(vii)	No green fluorescence signal	Omission of C_{12}-FDG or old stock solution	Confirm lack of fluorescence by examining the cells under a fluorescence microscope. Repeat staining. If cells are still not fluorescent use a fresh C_{12}FDG solution
1B(vii)	No green fluorescence signal	Wrong instrument setup	If microscopic examination shows fluorescent cells, readjust the acquisition parameters on the flow cytometer so that the fluorescence signal becomes detectable
1B(vii) and (viii)	No shift in fluorescence pattern	No senescent cells	Use a positive control in case there are no senescent cells in the conditions

C_{12}FDG, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside.

ANTICIPATED RESULTS

Senescence-associated beta galactosidase activity can be detected whether cells are induced to senesce by replicative exhaustion, genotoxic or other forms of stress, or oncogene activation. For instance, SA-βgal was observed in WI-38 human lung fibroblasts whether replicatively senescent or after stress-induced senescence, caused by repeated sublethal exposure to ethanol or *tert*-butylhydroperoxide[21], and in replicatively senescent HUVECs (**Fig. 1**). WI-38 and HUVECs typically reach replicative senescence after 40–50 population doublings (PDs). The proportion of SA-βgal positive cells increases from 5–15% in the cultures at early PDs and to 65–70% in replicatively senescent cells at late PDs (**Fig. 1a**). After five repeated incubations of fibroblast cultures at early PDs with *tert*-butylhydroperoxide or ethanol, the proportion of SA-βgal positive cells reach around 50% (refs. 21 and 23). Repeated exposure is required to enhance the effect of certain types of stress, like UVB[22] and *tert*-butylhydroperoxide[23] on the increased proportion of SA-βgal positive cells without inducing cell death. Repeated doses (e.g., 10 × 250 mJ cm^{-2} UVB) might be sublethal for fibroblasts, whereas the cumulative dose in a single exposure (in this example: 2,500 mJ cm^{-2} UVB) becomes lethal[22]. Similar conclusions are reached with human keratinocytes treated with different UVB doses[25]. A single sublethal stress with hydrogen peroxide is sufficient[24,28] to reach a similar percentage of SA-βgal positive cells. It was demonstrated that five exposures to *tert*-butylhydroperoxide generate as much telomere shortening as a single exposure to hydrogen peroxide. The telomere shortening observed did not reach the critical telomere shortening described for replicative senescence[29–31]. However, single strand DNA fragments by themselves were shown to induce senescence caused by telomere uncapping[32]. Thus, a possible explanation might be that a certain level of DNA damage is necessary to induce senescence, and that repeated exposure might be needed when the stressor is a weaker oxidant and thus a weaker inducer of DNA damage. Additional means of inducing senescence include ionizing radiation or other means of damaging DNA, high level expression of certain activated oncogenes or tumor suppressor genes or telomere uncapping[33,34].

Examination of cultures at different PDs by flow cytometry (option B) should show a single major fluorescent cell population (**Fig. 1b**). The median C_{12} fluorescein fluorescence of senescent cultures should be expected to increase between three and six-fold relative to that of cultures at early PDs. Cells of intermediate replicative ages should show intermediate levels of fluorescence[13].

Flow cytometry can be employed also to evaluate stress-induced senescence[35]. Although expressing results as MFI is suitable in most instances, in cases where suboptimal doses of the stressor induce senescence in only a small number of

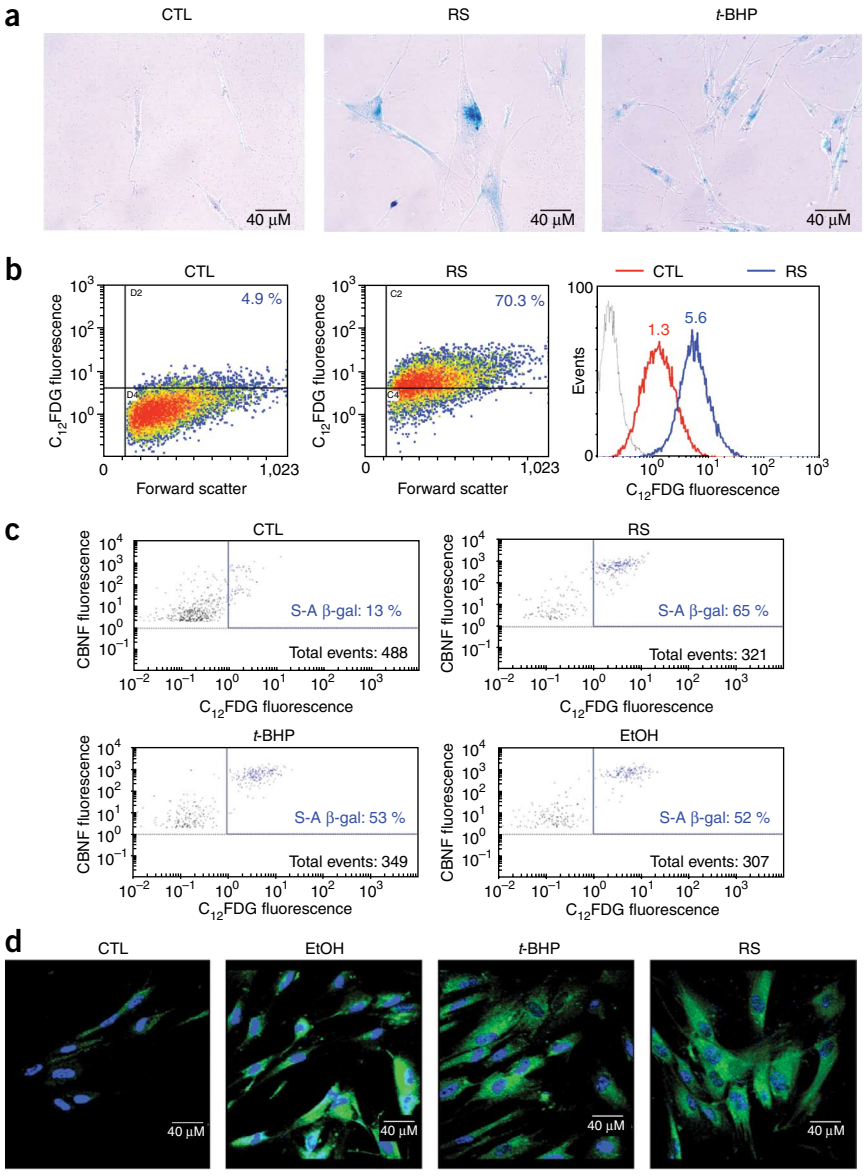

Figure 1 | Senescence-associated beta-galactosidase (SA-βgal) detection in replicative senescence and stress-induced premature senescence. Cytochemical staining of SA-βgal activity in normal WI-38 human fetal lung fibroblasts that were proliferating (control, CTL) either prematurely senescent after five incubations (1 h d^{-1} over 5 d) with 30 μM *tert*-butylhydroperoxide (*t*-BHP), or replicatively senescent (RS)[23] (**a**). Flow cytometric detection of SA-βgal activity in endothelial cells obtained from human umbilical vein[13]. Proliferating second passage (CTL) and RS cultures were pretreated with bafilomycin A1 followed by 1 h incubation with dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG). Density plots of C_{12}-fluorescein fluorescence versus forward scatter depict the percentage of cells with bright fluorescence. C_{12}-fluorescein fluorescence histograms show the relative levels of βgal in CTL and RS cultures; the values above the peaks represent the median fluorescence intensity of the respective populations; the faint (gray) tracing represents the autofluorescence of a culture treated with bafilomycin A1 only (**b**). Dot plots resulting from microfluidic chip detection of SA-βgal in WI-38 cells that were replicatively senescent[21] or senescent because of exposures to *t*-BHP- (dose of 30 μM, 1h exposure d^{-1}, over 5 d) or ethanol- (EtOH) (dose of 5% (vol/vol), 2 h exposure d^{-1}, over 5 d) (**c**). Cells were pretreated with bafilomycin A1 to induce lysosomal alkalinization, incubated with C_{12}FDG, a fluorogenic substrate of β-galactosidase and carboxynaphtofluorescein diacetate (CBNF), a fluorescent cell viability marker. Each dot represents the fluorescence intensities in arbitrary units of CBNF and C_{12}-fluorescein, displayed on a logarithmic scale, of a single cell. Blue region (SA-βgal) marks double-positive cell population. Control cells (CTL) are nonsenescent early passage cells. Fluorescence micrographs of SA-βgal detection in WI-38 cells that are replicatively senescent[21] or induced to senesce by *t*-BHP- (dose of 30 μm, 1 h exposure d^{-1}, over 5 d) or EtOH (dose of 5% (vol/vol), 2 h exposure d^{-1}, over 5 d) (**d**). The cells were pretreated with bafilomycin A1 to induce lysosomal alkalinization, and incubated with C_{12}FDG. Nuclei were stained with TO-PRO-3 (blue). Control cells (CTL) are cells at early population doublings. **c,d** are reprinted with permission from ref. 21, with permission from Elsevier.

cells in the population, determination of the percentage of positive cells rather than the MFI should be used. Expression of the results in this way can be also used when examining cells at different PDs (**Fig. 1a**).

Two modifications of this fluorescence-based protocol are shown in **Figure 1c** (microfluidic chip) and 1D (fluorescence microscope). These two protocols give comparable results with the cytochemical-based protocol[21].

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