Video Article

# Induction and Validation of Cellular Senescence in Primary Human Cells

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## Abstract

Cellular senescence is a state of permanent cell cycle arrest activated in response to different damaging stimuli. Activation of cellular senescence is a hallmark of various pathophysiological conditions including tumor suppression, tissue remodeling and aging. The inducers of cellular senescence *in vivo* are still poorly characterized. However, a number of stimuli can be used to promote cellular senescence *ex vivo*. Among them, most common senescence-inducers are replicative exhaustion, ionizing and non-ionizing radiation, genotoxic drugs, oxidative stress, and demethylating and acetylating agents. Here, we will provide detailed instructions on how to use these stimuli to induce fibroblasts into senescence. This protocol can easily be adapted for different types of primary cells and cell lines, including cancer cells. We also describe different methods for the validation of senescence induction. In particular, we focus on measuring the activity of the lysosomal enzyme Senescence-Associated β-galactosidase (SA-β-gal), the rate of DNA synthesis using 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, the levels of expression of the cell cycle inhibitors p16 and p21, and the expression and secretion of members of the Senescence-Associated Secretory Phenotype (SASP). Finally, we provide example results and discuss further applications of these protocols.

## Video Link

The video component of this article can be found at https://www.jove.com/video/57782/

## Introduction

In 1961, Hayflick and Moorhead reported that primary fibroblasts in culture lose their proliferative potential after successive passages[1]. This process is caused by the sequential shortening of telomeres after each cell division. When telomeres reach a critically short length, they are recognized by the DNA-damage response (DDR) that activates an irreversible arrest of proliferation — also defined as replicative senescence. Replicative senescence is currently one of the many stimuli that are known to induce a state of permanent cell cycle arrest that renders cells insensitive both to mitogens and to apoptotic signals[2,3]. The senescence program is normally characterized by additional features including high lysosomal activity, mitochondrial dysfunction, nuclear changes, chromatin rearrangements, endoplasmic reticulum stress, DNA damage and a senescence-associated secretory phenotype (SASP)[3,4]. Senescent cells have multiple functions in the body: development, wound healing and tumor suppression[2]. Equally, they are known to play an important role in aging and, paradoxically, in tumor progression[5]. The negative, and partially contradictory, effects of senescence are often attributed to the SASP[6].

Recently, it was shown that elimination of senescent cells from mice leads to lifespan extension and to elimination of many of the aging features[7,8,9,10,11,12]. In the same way, multiple drugs have been developed to either eliminate senescent cells (senolytics) or to target the SASP[13,14]. The anti-aging therapeutic potential has recently attracted more attention to the field.

The study of mechanisms associated to cellular senescence and the screenings for pharmacological interventions heavily rely on *ex vivo* models, particularly on human primary fibroblasts. While there are some common features activated by diverse senescence inducers, a large variability in the senescence phenotype is observed and dependent on various factors including cell type, stimulus and time point[3,15,16,17]. It is imperative to consider the heterogeneity for studying and targeting senescent cells. Therefore, this protocol aims to provide a series of methods used to induce senescence in primary fibroblasts by using different treatments. As it will be explained, the methods can easily be adapted to other cell types.

Apart from replicative senescence, we describe five other senescence-inducing treatments: ionizing radiation, ultraviolet (UV) radiation, doxorubicin, oxidative stress and epigenetic changes (namely promotion of histone acetylation or DNA demethylation). Both, ionizing radiation and UV-radiation cause direct DNA damage and, at the appropriate dose, trigger senescence[18,19]. Doxorubicin also causes senescence mainly through DNA damage by intercalating into the DNA and disrupting topoisomerase II function and thus halting DNA repair mechanisms[20]. The expression of genes essential for senescence is normally controlled by histone acetylation and DNA methylation. As a consequence, histone deacetylase inhibitors (*e.g.*, sodium butyrate and SAHA) and DNA demethylating (*e.g.,* 5-aza) agents trigger senescence in otherwise normal cells[21,22].

Finally, four of the most common markers associated to senescent cells will be explained: activity of the senescence associated-β-galactosidase (SA-β-gal), rate of DNA synthesis by EdU incorporation assay, overexpression of the cell cycle regulators and cyclin-dependent kinase inhibitors p16 and p21, and overexpression and secretion of members of the SASP.

## Protocol

# 1. General Preparation

1. Prepare D10 medium. Supplement DMEM medium-Glutamax with 10% FBS and 1% penicillin/streptomycin (Final concentration: 100 U/mL).
2. Prepare sterile PBS. Dissolve the tablets in water according to manufacturer's instructions. Sterilize by autoclave.
3. Prepare 1x trypsin. Dilute 5 mL of Trypsin-Versene EDTA/10x 1:10 in 45 mL of sterile PBS.
   Note: Throughout the protocol, we use cell culture conditions that are closer to the physiological conditions for primary fibroblasts. This means that we incubate cells at 37 °C and 5% $CO_2$ as is normally done but using 5% $O_2$ instead of the "standard" 20% $O_2$.
4. Handle all samples in sterile conditions by using a laminar flow hood, lab coat and gloves. Keep cells at 20% $O_2$ (room conditions) only while being handled.

# 2. Induction of Senescence

1. **Replicative Senescence**
   1. While handling cells or any material that will be in contact with them (pipets, flasks, media, *etc.*) work under sterile conditions, by using a laminar flow hood, lab coat and gloves. Keep cells at 20% $O_2$ (room conditions) only while being handled.
   2. Seed 7 x $10^5$ viable primary fibroblasts in a low population doubling (PD) in a T75 flask (~9.3 x $10^3$ cells/$cm^2$) containing 10 mL of D10.
   3. Grow the cells in a cell incubator at 37 °C with 5% $CO_2$ and 5% $O_2$ until they reach 70–80% confluence (3–4 days for proliferating cultures in a low PD).
   4. Detach the cells using 3 mL of 1x trypsin and incubating for ~5–7 min in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$. Monitor the cells regularly with a cell culture microscope to check the detaching process.
   5. When cells are spherical, stop the trypsin reaction by adding 9 mL of D10. Do not incubate longer than 10 min.
   6. Spin the cells at 300 x *g* for 5 min. Cells will form a pellet at the bottom of the tube, while debris and smaller particles will remain in the supernatant.
   7. Remove the supernatant and dissolve the cell pellet in 1 mL of D10 and perform a cell count using an automated cell counter according to manufacturer's instructions or a Neubauer chamber for manual counting. While counting, include an assay to check the viability of the cells (*e.g.*, Trypan Blue exclusion[23]).
   8. Calculate the cumulative PD using this formula:
      $PD_{new}$ = 3.32*(LOG(cell number total)-(LOG(cell number seeded)) + PDold
      Cell number total = all cells counted: dead and alive.
      Cell number seeded = number of viable cells seeded (8x$10^5$ cells).
      $PD_{old}$ = population doubling at the moment of seeding.
      $PD_{new}$ = population doubling at the moment of counting (after incubation).
      NOTE: If 7 x $10^5$ primary fibroblasts (PD 35.2) were seeded on a T-75 flask and, after 4 days they reach 80% confluence and are split again, counting now 1.3 x $10^6$ total cells (dead+alive).
      $PD_{new}$ = 3.32*(LOG(1,300,000 cells)-LOG(700,000))+35.2
      $PD_{new}$ = 36.1
   9. Reseed 8 x $10^5$ cells in a new T75, repeating steps 2.1.2–2.1.8.
      NOTE: After multiple consecutive passages, the culture will take longer to get confluent until cells stop dividing at all. Once cells stop dividing, test for senescence markers and/or harvest for downstream applications.
   10. Consider that the bigger size of senescent cells may cause the culture to appear full and yet have a low cell count. Thus, senescence can be assumed when the PD is stable and other senescence markers appear in the culture (see protocols 3.1–3.5).
       Note: Use proliferating primary fibroblasts as control.

2. **Ionizing Radiation-induced Senescence**
   1. While handling cells or any material that will be in contact with them (pipets, flasks, media, *etc.*), work under sterile conditions by using a laminar flow hood, lab coat and gloves. Keep cells at 20% $O_2$ (room conditions) only while being handled.
   2. Seed 7 x $10^5$ viable primary fibroblasts in a low PD in a T75 flask (~9.3 x $10^3$ cells/$cm^2$) containing 10 mL of D10.
   3. Incubate the cells overnight in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$.
   4. Expose the cells to 10 gray of gamma irradiation according to the instructions of the machine in use.
   5. Aspirate the medium from the cells and replace with 10 mL of D10.
   6. Incubate the cells in 10 mL of D10 in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$ for another 10 days replacing the medium regularly, approximately every 3 days.
   7. After 10 days, test for senescence markers and/or use the cells for downstream applications.
      NOTE: Use proliferating primary fibroblasts of the same PD (before irradiation) as control.

3. **Ultraviolet (UV) radiation-Induced Senescence**
   1. While handling cells or any material that will be in contact with them (pipets, flasks, media, *etc.*), work under sterile conditions by using a laminar flow hood, lab coat and gloves. Keep cells at 20% $O_2$ (room conditions) only while being handled.
   2. Seed 1.5–2 x $10^5$ viable primary fibroblasts in a low PD in one well of a 6-well plate (1.5–2.0 x $10^4$ cells/$cm^2$), add 2 mL of D10 medium.
   3. Place the cells in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$ and allow them to adhere to the plastic for at least 5 h.
   4. Take off the medium from the cells. Place the 6-well plate in the middle of the UV-radiation chamber and take the plastic lid off. Irradiate with UVB, 20–30 mJ/$cm^2$.

5. Add 2 mL of medium per well.
6. Incubate the cells in 2 mL of D10 in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$ for another 7 days replacing the medium regularly, approximately every 3 days.
   NOTE: After 7 days, cells can be tested for senescence markers and used for downstream applications. Use proliferating primary fibroblasts of the same PD (before irradiation) as control.

4. **Doxorubicin-induced Senescence**
   1. Prepare 1,000x Doxorubicin stock solution: Make a 250 µM stock of Doxorubicin in 1x PBS, filter-sterilize the solution and aliquot in 500 µL per sterile tube. Store the doxorubicin stock at -80 °C.
   2. While handling cells or any material that will be in contact with them (pipets, flasks, media, *etc.*), work under sterile conditions by using a laminar flow hood, lab coat and gloves. Keep cells at 20% $O_2$ (room conditions) only while being handled.
   3. Seed 7 x $10^5$ viable primary fibroblasts in a low PD in a T75 flask (~9.3 x $10^3$ cells/$cm^2$) containing 10 mL of D10.
   4. Incubate the cells overnight in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$.
   5. Dilute 11 µL of the 1,000x doxorubicin stock solution in 11 mL of D10 to a final concentration of 250 nM**.**
   6. Aspirate the medium from the cells and replace with 10 mL of D10 + doxorubicin.
   7. Incubate the cells for exactly 24 h in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$.
   8. Aspirate the medium from the cells and carefully wash once with 10 mL of D10.
   9. Incubate the cells in 10 mL of D10 for another 6 days replacing the medium regularly, approximately every 3 days.
   10. At day 7, test for senescence markers and/or use for downstream applications.
       NOTE: As control, use proliferating primary fibroblasts of the same PD treated for 24 h with vehicle (PBS) 1:1,000 in D10.

5. **Oxidative Stress-induced Senescence**
   1. While handling cells or any material that will be in contact with them (pipets, flasks, media, *etc.*) work under sterile conditions by using a laminar flow hood, lab coat and gloves. Keep cells at 20% $O_2$ (room conditions) only while being handled.
   2. Seed 7 x $10^5$ viable primary fibroblasts in a low PD in a T75 flask (~9.3 x $10^3$ cells/$cm^2$) containing 10 mL of D10.
   3. Incubate the cells overnight in a cell incubator at 37 °C with 5% $CO_2$ and 5% $O_2$.
   4. Prepare a solution of ~200 µM hydrogen peroxide in D10 medium by adding 22.6 µL of 30% hydrogen peroxide in 11 mL of D10.
      NOTE: Optimize the treatment for the cell type of interest by making a curve dose-response to evaluate toxicity.
   5. Aspirate the medium from the cells and add 10 mL of the freshly prepared D10 medium+hydrogen peroxide. Incubate for 2 h at 37 °C with 5% $CO_2$ and 5% $O_2$.
   6. Aspirate the medium from the cells and wash once with fresh D10 without hydrogen peroxide.
   7. Add 10 mL of D10 without hydrogen peroxide.
   8. Incubate for 48 h in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$.
   9. Repeat steps 2.5.4–2.5.8 two times more for a total of three treatments.
   10. Check for senescence markers or harvest for downstream applications.
       Note: As control, use proliferating cells of the same PD treated with 22.6 µL of sterile water in D10 for 2 h.

6. **Epigenetically-induced Senescence**
   1. Prepare stock and working solutions for the small molecule(s) to use according to **Table 1**. Filter-sterilize the solutions. Aliquot in sterile tubes. Store at -20 °C.
   2. While handling cells or any material that will be in contact with them (pipets, flasks, media, *etc.*) work under sterile conditions, for instance, by using a laminar flow hood, lab coat and gloves. Keep cells at 20% $O_2$ (room conditions) only while being handled.
   3. Seed 7 x $10^5$ viable primary fibroblasts in a low PD in a T75 flask (~9.3 x $10^3$ cells/$cm^2$) containing 10 mL of D10.
   4. Incubate the cells overnight in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$.
   5. Prepare 11 mL of D10 medium + working solution for the desired treatment. The exact dilution per treatment can be seen in **Table 1**.
      1. Prepare 11 µL of 1 mM SAHA working solution in 11 mL of D10.
      2. Prepare 44 µL of 1 M sodium butyrate working solution in 11 mL of D10.
      3. Prepare 11 µL of 10 mM 5-aza working solution in 11 mL of D10.
         Note: Optimize the treatments for the cell type of interest by, for instance, making a curve dose-response to evaluate toxicity.
   6. Add D10 medium + working solution for the desired treatment to the culture.
   7. Incubate for 24 h in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% $O_2$.
   8. Repeat steps 2.6.4–2.6.6 two times more, for a total of three treatments.
   9. Change medium for simple D10 without drug.
   10. After 3 days more, cells become senescent and ready for testing of senescence markers and downstream applications.
       NOTE: As control, use proliferating primary fibroblasts of the same PD treated for three days with D10 medium+vehicle. The D10 medium+vehicle needs to be refreshed every 24 h during those three days. The vehicle depends on the treatment used: 1:1,000 DMSO for SAHA and 5-aza and 1:250 sterile water for Sodium Butyrate.

## 3. Markers of Senescence

1. **Preparation**
   1. Prepare 20 mg/mL X-gal: Dissolve 20 mg of X-gal in 1 mL of dimethylformamide or DMSO. Store at -20 °C protected from light.
   2. Prepare 0.1 M citric acid solution: Dissolve 2.1 g of citric acid monohydrate in 100 mL of water. Store at room temperature.
   3. Prepare 0.2 M sodium phosphate solution: Dissolve 2.84 g of sodium dibasic phosphate or 3.56 g of sodium dibasic phosphate dehydrate in 100 mL of water. Store at room temperature.
   4. Prepare 0.2 M citric acid/sodium phosphate pH 6.0: Dissolve 36.85 mL of 0.1 M citric acid solution and 63.15 mL of 0.2 M sodium phosphate. Adjust exactly to pH 6.0. Store at room temperature.

5. Prepare 100 mM potassium ferrocyanide: Dissolve 2.1 g of potassium ferrocyanide in 50 mL of water. Store at 4 °C protected from light.
6. Prepare 100 mM potassium ferricyanide: Dissolve 1.7 g of potassium ferricyanide in 50 mL of water. Store at 4 °C protected from light.
7. Prepare 5 M sodium chloride: Dissolve 14.6 g of sodium chloride in 50 mL of water. Store at room temperature.
8. Prepare 1 M magnesium chloride: Dissolve 4.8 g of magnesium chloride in 50 mL of water. Store at room temperature.
9. Prepare 2% formaldehyde + 0.2% glutaraldehyde in PBS: Dissolve 800 µL of 25% glutaraldehyde and 12.5 µL of 16% formaldehyde in 100 µL of PBS. Store at room temperature protected from light.
10. Prepare staining solution fresh according to **Table 2.**

2. **Senescence-associated β-galactosidase Staining**
   1. For each sample, seed 1 x $10^4$ cells in at least one well of a 24-well plate (5.2 x $10^3$ cells/$cm^2$) containing 500 µL of D10 so that the cells are sparse. Treatments (if applicable) can be performed directly on this plate or, alternatively, cells treated already can be re-seeded into a 24-well plate.
   2. Incubate the cells overnight at 37 °C with 5% $CO_2$ and 5% $O_2$.
   3. Wash cells two times with 500 µL of PBS.
   4. Fix 3–5 min at room temperature using 500 µL/well of 2% formaldehyde + 0.2% glutaraldehyde in PBS.
   5. Wash cells two times with 500 µL of PBS.
   6. Prepare fresh staining solution, according to the number of samples to stain.
   7. Add staining solution (500 µL/well) and seal the plate with parafilm to avoid evaporation.
      NOTE: Evaporation may cause crystals to form and hinder the observation under the microscope.
   8. Incubate cells in the dark (*e.g.* covered in aluminum foil) at 37 °C in a dry incubator (without $CO_2$) for 12–16 h.
      Note: $CO_2$ may affect the pH and therefore modify the results. Some cell types may require shorter incubation times.
   9. Wash two times with 500 µL of PBS.
   10. Assess the results. Positive cells present a blue (mostly) perinuclear staining under a normal light microscope (**Figure 1A**).
   11. For quantification, observe at least 100 cells per sample and count the number of positive cells. Since senescent cells change shape, it is often difficult to define the cell boundaries and to count the cells. Counterstain with DAPI to facilitate visualization and quantification of individual cells (**Figure 1B**). Quantify the samples (percentage of positive cells versus total amount of cells) using a fluorescent microscope (**Figure 1C**). Take multiple pictures of the same sample so that at the end you can count at least 100 single-cells and evaluate the percentage of SA-β-gal positive cells in them.
   12. Compare results of senescent cells versus their appropriate control for the treatment used.
       NOTE: A co-staining with EdU on the same sample is possible. Consider that cells should then be seeded on coverslips.

3. **EdU Incorporation Assay**
   1. Put a coverslip/well in a 24-well plate according to the number of samples to assess.
   2. Seed 1 x $10^4$ cells in at least one well of a 24-well plate (5.2 x $10^3$ cells/$cm^2$) per condition containing 500 µL of D10 so that the cells are sparse. Treatments (if applicable) can be performed directly on this plate or, alternatively, already treated cells can be re-seeded into a 24-well plate.
   3. Incubate the cells overnight at 37 °C with 5% $CO_2$ and 5% $O_2$.
   4. Make a 20 µM solution of EdU in D10 (1:250) according to the number of samples to treat (250 µL per sample).
   5. Remove half of the medium (250 µL) from each well to treat and replace it with the D10 + EdU solution that was just prepared. Final EdU concentration is 10 µM.
   6. Incubate 18–24 h in a cell culture incubator at 37 °C with 5% $CO_2$ and 5% O2. Use the same incubation time in control and senescent cells.
   7. Wash 2x with 500 µL of PBS.
   8. Fix the cells for 10 min with 500 µL of 4% formaldehyde in PBS.
   9. Incubate 5 min in 500 µL of 100 mM Tris (pH 7.6).
   10. Permeabilize the cells for 10 min in 500 µL of 0.1% Triton X-100 in PBS.
   11. Wash 3x with PBS.
   12. Prepare 50 µL of label mix for each coverslip, adding the components in the following order: 44.45 µL of PBS, 0.5 µL of Cu(II)$SO_4$, 0.05 µL of sulfo-Cy3-azide, 5 µL of sodium-ascorbate.
   13. Put 50 µL of the label mix on a piece of parafilm. Lift the coverslip with the aid of a pair of tweezers and a needle and let it rest on top of the label mix, with the surface containing the cells facing downwards. Ensure that there are no bubbles and that the whole surface of the coverslip is touching the label mix. Incubate for 30 min in the dark.
   14. Put the cells back in the wells of the 24-well plate and wash them three times with PBS.
   15. Mount with mounting media (including DAPI to visualize nuclei) onto glass slides and let them dry overnight.
   16. Visualize the EdU incorporation using a fluorescent microscope. Use a filter appropriate for Cy3 (excitation/emission: 552/570 nm).
   17. Take multiple pictures of the cells for later quantification of at least 100 cells per condition (Cy3 and DAPI).
   18. Quantify the percentage of cells that incorporated EdU by using the following formula:
       EdU positive cells (%) = (EdU positive cell count (Cy3)/total cell count (DAPI)) * 100
   19. Compare results of senescent cells versus their appropriate control for the treatment used.
       Note: A co-staining with SA-β-gal on the same sample is possible. Consider that cells should still be seeded on coverslips.

4. **Gene Expression of p16, p21 and SASP**
   1. Prepare primer-sets of Genes of Interest (50 µM each primer).
      NOTE: A qPCR of p16, p21 and some relevant SASP factors is informative of the senescence status. The protocol described here makes use of the Universal Probe Library (UPL) system for relative quantification using a real time-PCR. **Table 3** shows an overview of the primers used for detection of the CDK inhibitors p16 and p21 and of the most relevant SASP members, as well as for Tubulin and Actin, which serve as reference genes for the assay. The last column of **Table 3** enlists the particular UPL probe to be used for each assay.
   2. Prepare separate qPCR reaction mix for the desired assays. Always include the reference gene as well according to **Table 4**.

3. Run all samples in duplicate or triplicate.
4. Load 7.5 µL/well of the qPCR reaction mix on a 384-well plate.
5. Add ~5 ng of cDNA dissolved in 2.5 µL of RNase-free water.
   NOTE: It is preferable to have similar amounts of cDNA for all the samples to be compared. This can be achieved by using equal amounts of RNA for the reverse transcription.
6. Cover the plate with a seal and make sure that it sticks correctly covering evenly all the wells on the plate.
7. Spin the plate at 2,000 x *g* for 1 min.
8. Run the plate in the Lightcycler for 40 cycles using the following protocol:
   95 °C for 7 min
   40 cycles of 95 °C for 5 s and 60 °C for 30 s
   37 °C for 1 min
9. For the analysis of the results, use the method proposed for Livak and colleagues to analyze qPCR data[24]. Use either Tubulin or Actin as reference genes to calculate the ΔCt value and use the appropriate control to calculate the for the ΔΔCt value for the specific senescence-inducing treatment.

5. **Protein expression and secretion of IL6**
   1. Seed 5–10 x $10^4$ cells in at least one well of a 6-well plate (5.2–10.5 x $10^3$ cells/$cm^2$) per condition containing 2 mL of D10. Treatments (if applicable) can be performed directly on this plate or, alternatively, cells treated already can be re-seeded into a 24-well plate. Let it stand overnight after seeding.
   2. Remove the medium and replace for 2 mL of DMEM medium without FBS. Incubate in normal conditions for 24 h.
   3. Collect the medium in a 15 mL tube.
   4. Centrifuge the sample at 300 x g for 5 min. Medium can be stored at -80 °C until processed.
   5. Follow manufacturer's instructions to perform the ELISA.
   6. Compare results of senescent cells versus the appropriate control for the specific senescence-inducing treatment

## Representative Results

### Enrichment of SA-β-gal staining in senescent fibroblasts

B-galactosidase (β-gal) is a lysosomal enzyme that is expressed in all cells and that has an optimum pH of 4.0[25,26]. However, during senescence, lysosomes increase in size and, consequently, senescent cells accumulate β-gal. The increased amounts of this enzyme make it possible to detect its activity even at a suboptimal pH 6.0[25,27]. **Figure 1A** shows representative images of the SA-β-gal staining in proliferating versus senescent primary fibroblasts. Cells also look enlarged and with an irregular cell body. As mentioned, it can be hard to distinguish individual cells, so that a co-staining with DAPI facilitates visualization and cell counting (**Figure 1B**). It is necessary to take pictures in a fluorescence microscope to be able to observe the DAPI staining. This means that pictures on the bright field channel will be taken in black/white, so that the "blue" staining of the SA-β-gal will appear black on the pictures. Of note, not all the cells within a sample are positive for β-gal. The efficiency of senescence induction is highly dependent on the stimulus- and cell type/strain used. The protocols described here yielded >50% β-gal positive cells in primary fibroblasts (BJ and WI-38) in our hands.

### Fewer cells incorporate EdU after induction of senescence

EdU is an analog of the nucleoside thymidine that, during active DNA synthesis, will be incorporated into the DNA[28]. The incorporation of EdU into DNA can be visualized after performing the Copper-Catalyzed Azide-Alkyne cycloaddition (CuAAC) to the EdU, reaction that cannot be performed in regular thymidine because it lacks the alkyne group[28]. In this particular protocol, a Sulfo-Cy3-azide is being used. If the coupling of the azide to the alkyne has taken place, cells will display fluorescence under a Cy3 filter (**Figure 2A**). It is important to take into account that by performing the EdU incorporation assay, cells that are proliferating can be distinguished from non-proliferating cells. The non-proliferating cells can be either quiescent or senescent, meaning that the EdU incorporation assay cannot discriminate between these two types of cell cycle arrest.

### Senescent Fibroblasts upregulate the CDK inhibitors p16 and p21

Senescent cells make use of inhibitors of the CDKs to stop the cell cycle[29]. Particularly p16 and p21 are often measured as markers of senescent cells[3]. Either one or both markers are normally upregulated in senescent cells, and the upregulation is often measured at the transcriptional level. It is encouraged to use both markers simultaneously since some cells do not upregulate p16 at the transcriptional level and p21 is a universal but not specific marker for senescence[15,17,30]. **Figure 3** shows representative relative quantifications of p16 and p21 mRNA in fibroblasts induced to senescence. Other techniques such as immunostaining and/or western blotting to detect protein levels are also possible.

### Senescent Fibroblasts display a SASP

Most senescent cells transcriptionally upregulate several genes encoding for secreted proteins, a phenomenon called SASP[6]. The SASP includes factors involved in inflammation, *e.g.*, interleukins and chemokines, or in extracellular matrix (ECM) degradation, *e.g.*, MMPs, but it is highly heterogeneous. Induction of SASP factors can be evaluated by measuring either mRNA expression levels via qPCR or levels of secreted protein via Enzyme-Linked Immuno Sorbent Assay (ELISA). **Figure 4** shows a representative image showing the upregulation of IL6 both at the transcriptional and secreted levels. We used IL6 only as a representation; however, it is encouraged to measure multiple members of the SASP from the suggested list on protocol 3.4.

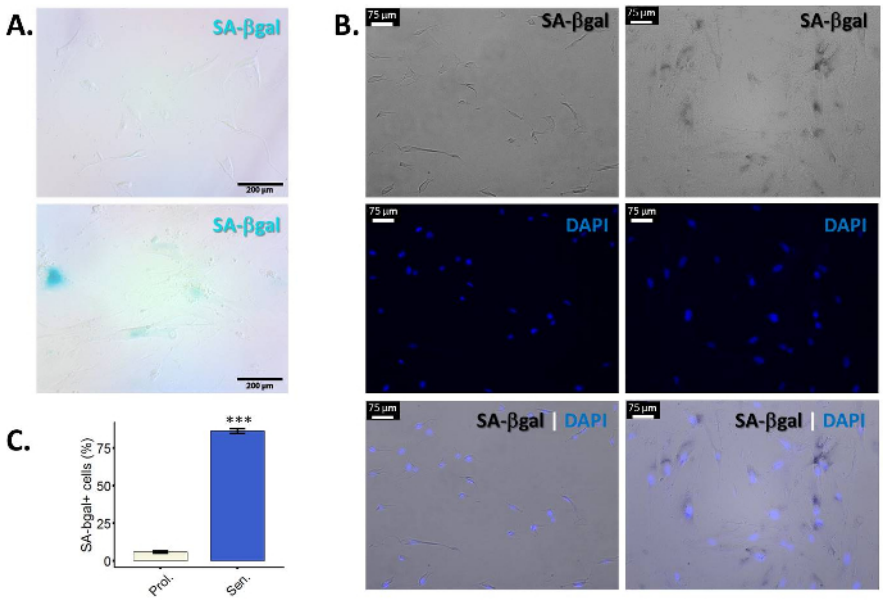


**Figure 1: Enrichment of SA-β-gal staining in senescent primary fibroblasts.** BJ primary foreskin fibroblasts (PD 34.1) were induced to senescence by exposing them to ionizing radiation (10 Gy). Cells were stained for SA-βgal ten days after irradiation. (**A**) Representative results for the SA-βgal staining in BJ primary fibroblasts either untreated (up) or exposed to ionizing radiation (down). Final magnification: 100X. (**B**) Representative figure of SA-β-gal co-stained with DAPI for BJ primary fibroblasts either untreated (three left panels) or exposed to ionizing radiation (three right panels). The DAPI staining (blue) helps to visualize individual cells facilitating the quantification. Pictures taken on bright-field appear in black/white. Therefore, in these particular pictures the SA-β-gal staining will look like black perinuclear spots. Final magnification: 100X. (**C**) Quantification of SA-β-gal positive cells in proliferating (Prol., white) BJ fibroblasts versus ionizing irradiated-treated counterparts (Sen., blue). Quantification was performed by using three biological replicates with error bars showing the standard error of the mean. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values. (n = 3, ± SEM, *** = ***p*** value <0.01). Please click here to view a larger version of this figure.

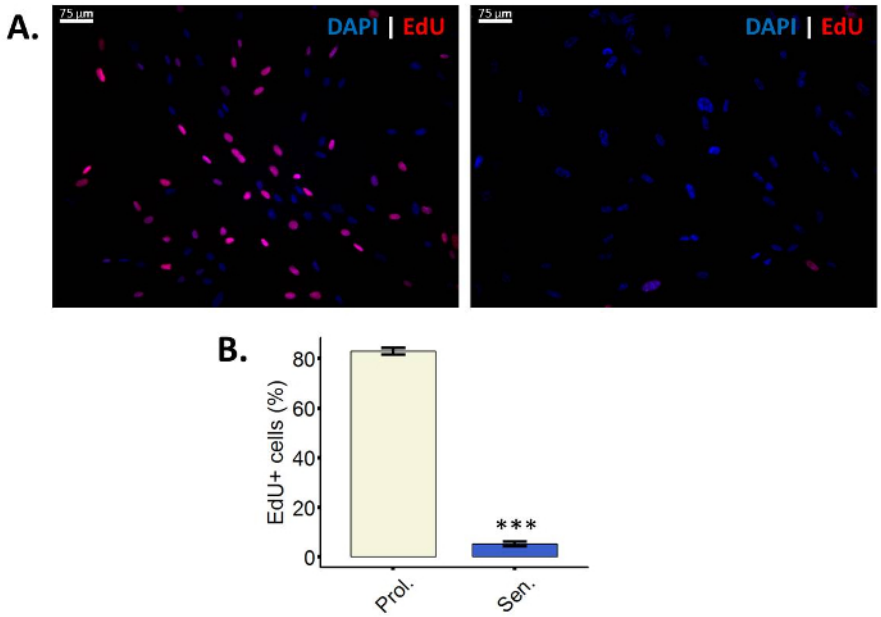


**Figure 2: Fewer cells incorporate EdU after induction of senescence.** (**A**) Representative image of the EdU incorporation assay in proliferating WI-38 fibroblasts PD 43.86 (left) and their ionizing irradiated counterparts (right). Final magnification: 100X. (**B**) Quantification of EdU positive cells in proliferating (Prol., white) BJ fibroblasts (PD 38.7) versus their irradiated counterparts (Sen., blue). Quantification was performed by using three biological replicates with error bars showing the standard error of the mean. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (n = 3, ± SEM, *** = ***p*** value <0.01). Please click here to view a larger version of this figure.

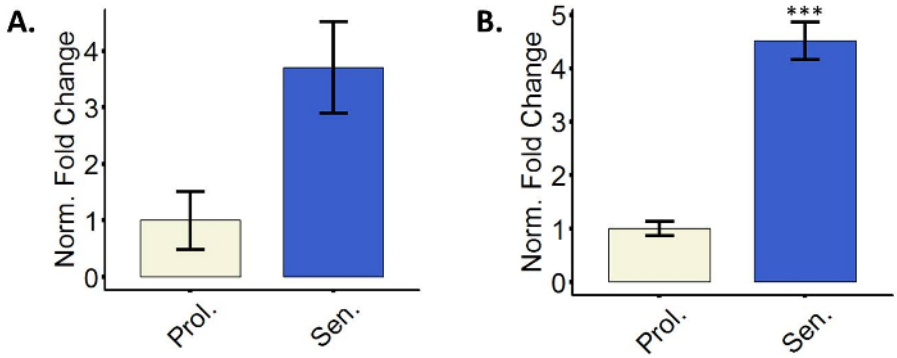


**Figure 3: Senescent fibroblasts upregulate the CDK inhibitors p16 and p21.** (**A**) Quantification of p16 mRNA expression in proliferating (Prol., white, PD 35.3) or 5-aza-treated BJ cells (Sen., blue). (**B**) Quantification of p21 mRNA expression in proliferating (Prol., white, PD 35.3) or 5-aza-treated BJ cells (Sen., blue). Quantification was performed by using three biological replicates (each with two technical replicates) with error bars showing the standard error of the mean. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (n = 3, ± SEM, *** = ***p*** value <0.01). Please click here to view a larger version of this figure.

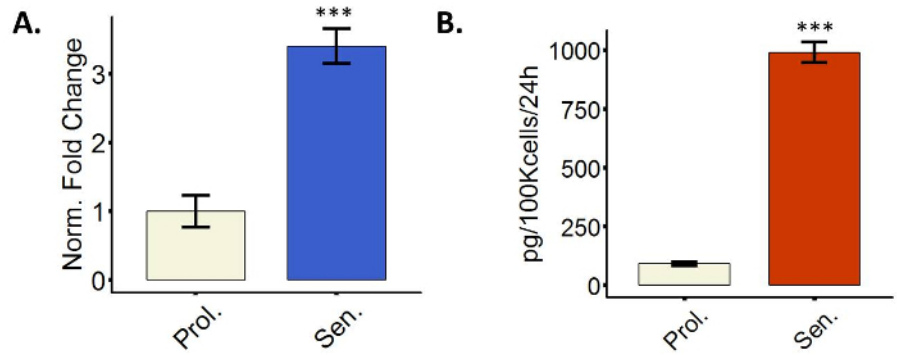


**Figure 4: Senescent fibroblasts display a Secretory Phenotype (SASP).** (**A**) Quantification of IL6 mRNA expression in BJ fibroblasts either proliferating (Prol., white, PD 38.7) or induced to senescence by ionizing radiation (Sen., blue). (**B**) Quantification of IL6 protein expression in proliferating PD 38.6 (Prol., white) or ionizing radiation-treated WI38 fibroblasts (Sen., blue). Quantification was performed by using three biological replicates with error bars showing the standard error of the mean. In the case of the qPCR data, each biological replicate had two technical duplicates. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (n = 3, ± SEM, *** =***p*** value <0.01). Please click here to view a larger version of this figure.

| | Diluent | Stock solution | Working solution | Dilution for Treatment | Final Concentration |
|---|---|---|---|---|---|
| **SAHA** | DMSO | 100 mM | 1 mM | 1:1,000 | 1 µM |
| **Sodium butyrate** | Sterile water | --- | 1 M | 1:250 | 4 mM |
| **5-aza-2'-deoxycytidine (5-aza)** | DMSO | 100 mM | 10 mM | 1:1,000 | 10 µM |

**Table 1: Stock and Working Solutions for the Different Treatments used for Epigenetically-induced Senescence.**

| Component | Volume | Final concentration |
|---|---|---|
| **20 mg/mL X-gal** | 1 mL | 1 mg/mL |
| **0.2 M citric acid/sodium phosphate buffer ph 6.0** | 4 mL | 40 mM |
| **100 mM potassium ferrocyanide** | 1 mL | 5 mM |
| **100 mM potassium ferricyanide** | 1 mL | 5 mM |
| **5 M sodium chloride** | 0.6 mL | 150 mM |
| **1 M magnesium chloride** | 0.04 mL | 2 mM |
| **Water** | 12.4 mL | - |
| ***Total*** | **20 mL** | |

**Table 2: Composition of the Staining Solution used for Senescence Associated (SA)-β-gal staining.**

| Target | Forward Primer (5'--> 3') | Reverse Primer (5'--> 3') | UPL probe |
|---|---|---|---|
| Tubulin | CTTCGTCTCCGCCATCAG | CGTGTTCCAGGCAGTAGAGC | #40 |
| Actin B | ccaaccgcgagaagatga | ccagaggcgtacagggatag | #64 |
| P16 | GAGCAGCATGGAGCCTTC | CGTAACTATTCGGTGCGTTG | #67 |
| P21 | tcactgtcttgtaccttgtgc | ggcgtttggagtggtagaaa | #32 |
| IL6 | CAGGAGCCCAGCTATGAACT | GAAGGCAGCAGGCAACAC | #45 |
| IL8 | GAGCACTCCATAAGGCACAAA | ATGGTTCCTTCCGGTGGT | #72 |
| IL1a | GGTTGAGTTTAAGCCAATCCA | TGCTGACCTAGGCTTGATGA | #6 |
| CXCL1 | CATCGAAAAGATGCTGAACAGT | ATAAGGGCAGGGCCTCCT | #83 |
| CXCL10 | GAAAGCAGTTAGCAAGGAAAGGT | GACATATACTCCATGTAGGGAAGTGA | #34 |
| CCL2 | AGTCTCTGCCGCCCTTCT | GTGACTGGGGCATTGATTG | #40 |
| CCL20 | GCTGCTTTGATGTCAGTGCT | GCAGTCAAAGTTGCTTGCTG | #39 |
| PAI1 | AAGGCACCTCTGAGAACTTCA | CCCAGGACTAGGCAGGTG | #19 |
| MMP1 | GCTAACCTTTGATGCTATAACTACGA | TTTGTGCGCATGTAGAATCTG | #7 |
| MMP3 | CCAGGTGTGGAGTTCCTGAT | CATCTTTTGGCAAATCTGGTG | #72 |
| MMP9 | GAACCAATCTCACCGACAGG | GCCACCCGAGTGTAACCATA | #53 |

**Table 3: Primer sequences and their corresponding UPL probe for detecting mRNA of Senescence Markers in samples of human origin.**

| Component | Volume/sample |
|---|---|
| Sensifast Probe Lo-Rox mix | 5 µL |
| Primer-set (50 µM) | 0.1 µL |
| UPL probe | 0.1 µL |
| Nuclease-free water | 2.3 µL |
| cDNA (~4 ng) | 2.5 µL |
| **Total** | 7.5 µL |

**Table 4: Composition of the qPCR reaction mix for the UPL system.**

## Discussion

The protocols explained here were optimized for human primary fibroblasts, particularly BJ and WI-38 cells. The protocols for replicative senescence, ionizing radiation and doxorubicin, have been successfully applied to other types of fibroblasts (HCA2 and IMR90) and in other cell types (namely neonatal melanocytes and keratinocytes or iPSC-derived cardiomyocytes) in our laboratory. However, adaptations for additional cell types can be optimized by adjusting some details such as the number of seeded cells, the methods and chemicals to help cells for attaching/detaching to plastic supports and the dosage of the treatment to avoid toxicity.

Even the use of primary fibroblasts poses a number of challenges. Senescent cells are usually more difficult to detach than their proliferating counterparts, and they are often more sensitive to trypsinization or any other type of detaching method, meaning that the viability after detaching is slightly lower than the one of proliferating cells. The choice of the appropriate control for the different senescence-inducing methods is difficult. For instance, for the drug-based treatments such as doxorubicin, we suggest a short treatment with the vehicle: PBS for 24 h in the case of the control samples for doxorubicin-treated cells followed by immediate harvesting/processing. It might be argued that cells induced to senescence go through an extended culture time after the treatment was applied (six extra days of culture for doxorubicin-treated cells) and that control cells should be cultured equal amount of time after removal of PBS. However, such a long culture would allow the cells to divide further, to become over-confluent or to require further passaging and to increase PD. Over-confluence may cause senescence markers, such as SA-β-gal to appear despite cells maintaining their proliferating potential[31]. The increased PD would get them closer to their replication limit (and to replicative senescence) and make them less comparable to their doxorubicin-treated counterparts. A similar situation would apply for the other treatments. We have suggested the controls that we consider more appropriate for each case.

Most of the techniques used to induce cells into senescence seem relatively easy and straight-forward, but many factors can affect the outcome of the experiments. For instance, normal glucose concentration of conventional cell culture media for fibroblasts is 4.5 g/L. However, for some cell types such as stem cells, lower concentrations of glucose extend their proliferative potential[32], while for others higher concentrations may lead to premature senescence[33]. Moreover, as senescent cells are highly metabolic and spend high amounts of energy to produce secreted factors[34], other senescence-associated phenotypes might be affected by oscillations in glucose concentrations.

Another potential variable in the cell culture medium is serum. The composition of the serum is normally not defined and varies according to the animal source and the batch. Particularly, the amount of growth factors and pro-inflammatory proteins can influence senescence[35].

We recommend that the same batch of serum is used for the whole experiment to avoid unnecessary and confounding variability. Yet, some inevitable technical conditions such as the use of serum-free medium used for some ELISA-based protocols can reduce SASP expression.

Oxygen tension is important for the complete senescence induction. Hypoxia can inhibit geroconversion, so that cells do not proliferate but are not irreversibly arrested[36]. However, the most common problem in the experimental setup is not hypoxia but hyperoxia. Indeed, standard culture conditions often use 20% oxygen as "normoxia", but physiological conditions for most cell types are lower. Mouse blastocysts present markers of senescence (SA-βgal and DNA damage) when cultured at 20% oxygen, unlike their *in vivo*-derived counterparts or the same cells cultured at 5% oxygen[37]. Furthermore, mouse fibroblasts cultured at more physiological conditions (3% oxygen) and not at conventional ones (20% oxygen) display a SASP[38]. Here, we used 5% oxygen for all the cultures and experiments and we urge researchers to reconsider the oxygen concentrations used for the particular cell type of interest.

Finally, another factor to consider is the intrinsic heterogeneity of senescent cells. On one hand, different cell types and even cell strains display differences in senescence-associated phenotypes. For instance, some strains of fibroblasts do not upregulate p16 at the transcriptional level upon senescence induction[15,16,17], as it is also shown in **Figure 3A**, where despite seeing an upregulation of p16, this was not statistically significant. P16 is also controlled at the translational and post-translational level, so measuring the protein levels might in some cases demonstrate an increased activity of this CDK inhibitor. However, it may be that some cells simply rely on other CDK inhibitors like p21. We recommend measuring the transcriptional levels of both of them. The exact composition of the SASP also depends on the cell that produces it[3]. Furthermore, some cells constitutively express high levels of β-galactosidase, giving a positive result for SA-β-gal staining that is not necessarily indicative of senescence[3]. In some cases, this problem might be overcome by reducing the incubation time with staining solution during the SA-β-gal staining protocol. As mentioned, over-confluent cells might also stain with the SA-β-gal without them being senescent[31], so we urge researchers to culture cells sparsely for performing this staining. On the other hand, the senescence phenotype itself is not stable[39]. The composition of the SASP and the appearance of other markers of senescence are time-dependent[17,40]. Here, we have suggested the time points after each treatment in which cells are considered fully senescent and that are routinely used in our laboratory. Importantly, measuring markers at a shorter time point might render negative results due to incomplete senescence[40]. Moreover, since in most of the treatments a percentage of cells do not become senescent, using a longer time point might give enough time for the few non-senescent cells to expand and overtake the culture, reducing the expression of senescent markers. In views of the heterogeneity of senescent cells and the multiple caveats of the different markers, we highly encourage researchers to use multiple senescence markers within the same sample.

## Disclosures

N/A

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