

Submission ID #: 70595

Scriptwriter Name: Sri Lakshmi Priya

Project Page Link: <https://review.jove.com/account/file-uploader?src=21531523>

Title: A Standardized Protocol for Inducing Stress-Induced Premature Senescence in Primary Human Melanocytes Using Tert-butyl Hydroperoxide

Authors and Affiliations:

Amina Hassan^{1,2}, Lena Guerrero-Navarro^{1,2}, Ines Martic^{1,2}, Lieve Declercq³, Maria Cavinato^{1,2},

¹Institute for Biomedical Aging Research, Universität Innsbruck

²Center for Molecular Biosciences Innsbruck (CMBI)

³European Innovation Center

Corresponding Authors:

Maria Cavinato maria.cavinato-nascimento@uibk.ac.at

Email Addresses for All Authors:

Amina Hassan	amina-gharib.hassan@uibk.ac.at
Lena Guerrero-Navarro	lena.guerrero-navarro@uibk.ac.at
Ines Martic	ines.martic@uibk.ac.at
Lieve Declercq	lievedeclercq@proya.com
Maria Cavinato	maria.cavinato-nascimento@uibk.ac.at

Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

If **Yes**, can you record movies/images using your own microscope camera?

Enter Yes or No.

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye.**

Enter make and model of microscope.

If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

Scope shots:

2.1.2.

2.4.2.

4.8.1

4.8.2.

NOTE: These were filmed by the videographer

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations? **No**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 27

Number of Shots: 41

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Answers are slightly edited in accordance with journal guidelines. Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Maria Cavinato:** Our research focuses on investigating the senescent phenotype of melanocytes and its contribution to extrinsic skin aging.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Maria Cavinato:** Established senescence models use fibroblasts and fail to capture melanocyte-specific biology. This protocol induces tBHP-mediated oxidative stress in primary human melanocytes.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Maria Cavinato:** This model allows researchers to study how senescent melanocytes affect pigmentation and communicate with surrounding cells via SASP inflammatory factors.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Maria Cavinato:** The challenge in this protocol is precise timing. tBHP must be applied twice daily with at least a four-hour interval.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Maria Cavinato**: Following this protocol, senescence can be characterized using SA- β -galactosidase staining, immunofluorescence, RT-qPCR, and Western blotting of established senescence markers.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This study utilized commercially obtained de-identified primary human neonatal melanocytes collected with informed donor consent in compliance with established ethical guidelines

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Seeding Melanocytes

Demonstrator: Amina Hassan

- 2.1. To begin, gather the required reagents [1]. After thawing the melanocytes for 3 days, ensure that the cells reach 80 to 90 percent confluency on the seeding day [2].
 - 2.1.1. WIDE: Talent waking into the room with a tray of reagents. **Videographer:** Please obtain CU shot as well for this shot.
 - 2.1.2. SCOPE: Show adherent melanocytes at 80 to 90 percent confluency
- 2.2. Aspirate the medium from the flask [1]. Wash the cells twice with DPBS (D-P-B-S) [2].
 - 2.2.1. Talent aspirating the medium from the culture flask.
 - 2.2.2. Talent adding DPBS to the flask.
- 2.3. Add 2 milliliters of Trypsin-EDTA (E-D-T-A) solution to detach the cells [1]. Place the flask in the incubator for 5 minutes at 37 degrees Celsius with 5 percent carbon dioxide [2].
 - 2.3.1. Talent adding Trypsin-EDTA solution into the culture flask.
 - 2.3.2. Talent placing the flask into the incubator.
- 2.4. Gently tap the flask [1] and under the microscope observe the cells to confirm complete detachment [2].
 - 2.4.1. Talent gently tapping the culture flask.
 - 2.4.2. SCOPE: Show detached cells lifting from the flask surface under the microscope.
- 2.5. Add 1.5 times the volume of trypsin inhibitor to stop the reaction [1].
 - 2.5.1. Talent adding trypsin inhibitor into the flask containing detached cells.
- 2.6. Transfer the cell suspension to a 15 milliliter conical tube [1]. Dilute the suspension with DPBS to a final volume of 10 milliliters [2].
 - 2.6.1. Talent transferring the cell suspension into a 15 milliliter conical tube.
 - 2.6.2. Talent adding DPBS into the conical tube containing cell suspension.
- 2.7. Centrifuge the cell suspension at a relative centrifugal force of 100 for 5 minutes [1].
 - 2.7.1. Talent placing the conical tube into the centrifuge.

- 2.8. Aspirate the supernatant [1] and resuspend the cell pellet in medium to the desired volume [2].
 - 2.8.1. Talent aspirating the supernatant from the conical tube.
 - 2.8.2. Talent resuspending the cell pellet in fresh medium.
- 2.9. Seed 6 lakh cells per 10 centimeter dish for tert-butyl hydroperoxide or tBHP (T-B-H-P) treated plates [1]. Seed 4 lakh cells per plate for the control plates [2].
 - 2.9.1. Talent seeding cells into a 10 centimeter dish labeled tBHP.
 - 2.9.2. Talent seeding cells into a plate labeled control.

3. Tert-Butyl Hydroperoxide (tBHP) Treatment

- 3.1. Prepare a 10 millimolar working solution from the tBHP stock using DPBS or media [1].
 - 3.1.1. Talent adding tBHP solution in a tube containing DPBS or media.
- 3.2. Add 100 micromolar tBHP to the cells for 1 hour per treatment [1].
 - 3.2.1. Talent adding tBHP solution into the culture plate.
- 3.3. Move the plate back and forth to distribute the tBHP evenly throughout the medium [1].
 - 3.3.1. Talent gently moving the plate back and forth.
- 3.4. Place the cells in the incubator for precisely 1 hour at 37 degrees Celsius with 5 percent carbon dioxide [1].
 - 3.4.1. Talent placing the culture plate into the incubator.
- 3.5. Aspirate the medium to terminate the tBHP treatment [1].
 - 3.5.1. Talent aspirating the treatment medium from the culture plate.
- 3.6. Wash the plates twice with pre-heated DPBS at 37 degrees Celsius [1].
 - 3.6.1. Talent adding pre-heated DPBS to the plates.
- 3.7. Add the appropriate volume of fresh medium to the plates [1].
 - 3.7.1. Talent adding fresh medium into the culture plates.
- 3.8. Place the plates in the incubator and allow the cells to recover for at least 4 hours before the next treatment [1].
 - 3.8.1. Talent placing the culture plates back into the incubator.
- 3.9. Repeat the treatment procedure later on the same day for the second treatment [1]. Continue the treatment regimen and maintain the cells in culture until Day 9 to assess senescence markers [2].

- 3.9.1. Talent adding t-BHP to the previously treated culture plates.
- 3.9.2. Shot of labeled culture plates in the incubator.

4. Growth Curve Analysis and Senescence Assessment

Demonstrator: Lena Guerrero-Navarro

- ~~4.1. Count the tBHP treated cells and control cells [1].~~
 - ~~4.1.1. SCREEN: Show the cells on the Casy counter computer—~~

Authors: Please create screen capture videos of the shots labeled as SCREEN, create a screenshot summary, and upload the files to your project page as soon as possible: <https://review.jove.com/account/file-uploader?src=21531523>
- 4.2. After counting the treated cells, reseed them at the same initial density or if fewer than 6 lakh cells are obtained from the tBHP-treated plates, reseed all collected cells [1].
 - 4.2.1. Talent reseeding cells into fresh culture plates.
- 4.3. Count the cells on Days 4, 9, and 15 of culture [1].
 - 4.3.1. SCOPE: Show melanocytes in the counting chamber.
- 4.4. On Day 9, wash the cells twice with PBS [1]. Fix the cells using 2 percent formaldehyde and 0.4 percent glutaraldehyde [2].
 - 4.4.1. Talent adding PBS to the culture plate.
 - 4.4.2. Talent adding the fixation solution onto the cells.
- 4.5. Wash the fixed cells twice with PBS [1].
 - 4.5.1. Talent adding PBS to the cells.
- 4.6. Incubate the fixed cells in a staining solution at pH 6 [1] for 18 hours at 37 degrees Celsius in the absence of carbon dioxide [2].
 - 4.6.1. Talent adding the staining solution onto the fixed cells.
 - 4.6.2. Talent placing the stained culture plate into the incubator.
- 4.7. Wash the cells twice with PBS [1].
 - 4.7.1. Talent washing the stained cells with PBS.
- 4.8. Using light microscopy, image and count the number of blue-stained cells [1]. Divide it by the total number of cells within the same field [2]. Express the results as the percentage of positive cells [3].

- 4.8.1. SCOPE: Show blue stained cells under light microscopy.
- 4.8.2. SCOPE: Show the total cell population within the microscopic field.
- 4.8.3. Show a printed data sheet showing the final percentage of positive cells.

- 4.9. Perform immunofluorescence staining [1], RT-qPCR [2], and Western blotting to further assess senescence markers in tBHP-treated melanocytes [3].
 - 4.9.1. Shot sequence showing immunofluorescence imaging being performed in the laboratory.
 - 4.9.2. Shot of RT-qPCR running.
 - 4.9.3. Shot of Western blot membrane imaging.

Results

5. Results

- 5.1. Following oxidative stress induction, tBHP treatment significantly reduced melanocyte proliferation [1] compared to controls [2].
 - 5.1.1. LAB MEDIA: Figure 2A. *Video editor: Emphasize the entire tBHP curve in the line graph.*
 - 5.1.2. LAB MEDIA: Figure 2A. *Video editor: Emphasize the entire Ctrl curve in the line graph.*
- 5.2. The treated cells reached only 1.4 cumulative population doublings or cPDL (C-P-D-L) by day 9 [1], whereas controls reached 3.1 cPDL [2]. By day 15, treated cells plateaued at 1.9 cPDL [3] while control cells expanded to 5.1 cPDL [4].
 - 5.2.1. LAB MEDIA: Figure 2A. *Video editor: Emphasize the tBHP line at the d9 point around 1.4 cPDL.*
 - 5.2.2. LAB MEDIA: Figure 2A. *Video editor: Emphasize the Ctrl line at the d9 point around 3.1 cPDL.*
 - 5.2.3. LAB MEDIA: Figure 2A. *Video editor: Emphasize the tBHP line at the d15 point around 1.9 cPDL.*
 - 5.2.4. LAB MEDIA: Figure 2A. *Video editor: Emphasize the Ctrl line at the d15 point around 5.1 cPDL.*
- 5.3. Melanocytes treated with tBHP showed increased cell surface area [1] compared to controls at all measured time points [2] with enlargement progressing until day 15 [3] consistent with the enlarged and flattened morphology of senescent cells [4].
 - 5.3.1. LAB MEDIA: Figure 2B. *Video editor: Emphasize all dark teal tBHP bars at day 4, day 9, and day 15.*
 - 5.3.2. LAB MEDIA: Figure 2B. *Video editor: Emphasize all light teal Ctrl bars at day 4, day 9, and day 15.*
 - 5.3.3. LAB MEDIA: Figure 2B. *Video editor: Emphasize the dark teal tBHP bar at day 15.*
 - 5.3.4. LAB MEDIA: Figure 2C. *Video editor: Emphasize the bottom image.*
- 5.4. tBHP-treated melanocytes showed an increase in senescence-associated beta-galactosidase or SA- β -gal (S-A-beta-galactosidase) activity with 32% cells positive cells [1] compared 3% in controls [2]. The rise in SA- β -gal positivity was consistent with a senescent phenotype [3].
 - 5.4.1. LAB MEDIA: Figure 2D. *Video editor: Emphasize the dark teal bar labelled as tBHP.*

- 5.4.2. LAB MEDIA: Figure 2D. *Video editor: Emphasize the light teal bar labelled as Ctrl.*
- 5.4.3. LAB MEDIA: Figure 2E: *Video editor: Emphasize the bottom image.*
- 5.5. Immunofluorescence analysis showed increased gamma-H2AX (*H-two-A-X*) levels in tBHP-treated melanocytes [1] compared with controls [2].
 - 5.5.1. LAB MEDIA: Figure 2G. *Video editor: Emphasize the dark teal bar labelled as tBHP.*
 - 5.5.2. LAB MEDIA: Figure 2G. *Video editor: Emphasize the light teal bar labelled as Ctrl.*
- 5.6. The treated cells showed stronger gamma-H2AX signal [1], than controls [2].
 - 5.6.1. LAB MEDIA: Figure 2F. *Video editor: In the tBHP row, emphasize the bright green spots under γ H2AX and merged.*
 - 5.6.2. LAB MEDIA: Figure 2F. *Video editor: In the Ctrl row, emphasize the bright green spots under γ H2AX and merged.*
- 5.7. Real Time-quantitative polymerase chain reaction analysis demonstrated increased senescence-associated secretory phenotype factor expression [1], with interleukin-8 showing the largest increase [2]. While interleukin-6 and matrix metalloproteinase-1 showed moderate increases [3].
 - 5.7.1. LAB MEDIA: Figure 2H. *Video editor: Emphasize all the dark teal coloured bars labelled as tBHP.*
 - 5.7.2. LAB MEDIA: Figure 2H. *Video editor: Emphasize the dark teal tBHP bar under IL-8.*
 - 5.7.3. LAB MEDIA: Figure 2H. *Video editor: Emphasize the dark teal tBHP bar under IL-6 and MMP-1.*
- 5.8. Western blot analysis confirmed senescence induction [1]. LaminB1 showed a significant reduction in tBHP-treated melanocytes [2] compared to controls [3].
 - 5.8.1. LAB MEDIA: Figure 2I.
 - 5.8.2. LAB MEDIA: Figure 2J. *Video editor: Emphasize the dark teal coloured bar labeled as tBHP.*
 - 5.8.3. LAB MEDIA: Figure 2J. *Video editor: Emphasize the light teal coloured bar labeled as Ctrl.*
- 5.9. Phosphorylated retinoblastoma protein levels were reduced in tBHP-treated melanocytes [1] compared to controls [2].
 - 5.9.1. LAB MEDIA: Figure 2K. *Video editor: Emphasize the dark teal coloured bar labeled as tBHP.*
 - 5.9.2. LAB MEDIA: Figure 2K. *Video editor: Emphasize the light teal coloured bar labeled as Ctrl.*

- 5.10. tBHP treatment increased transcriptional expression of p16 [1] and p21 in melanocytes [2].
 - 5.10.1. LAB MEDIA: Figure 2L. *Video editor: Emphasize the dark teal coloured bar labeled as tBHP under p16.*
 - 5.10.2. LAB MEDIA: Figure 2L. *Video editor: Emphasize the dark teal coloured bar labeled as tBHP under p21.*