

Submission ID #: 70588

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Title: Senescence Detection Using Reflected Light in Adipose Stromal Vascular Fraction

Authors and Affiliations:

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

Current Protocol Length

Number of Steps: 18

Number of Shots: 52

Interviews

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

INTRODUCTION:

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

- 1.1. **Leo Westerberg:** We study adipocyte senescence across different fat depots and metabolic conditions like obesity and hyperinsulinemia.

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. **Leo Westerberg:** Traditional brightfield-based SABG assays are subjective and limit multiplexing; our new protocol ensures objectivity and allows multiplexing.

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. **Leo Westerberg:** This protocol enables sensitive, quantitative detection of SA-beta-gal activity while allowing simultaneous imaging of other senescence or cellular markers.

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. **Leo Westerberg:** The main challenge is the initial microscope setup, however once optimized, the same acquisition settings can be reused across experiments.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

1.5. **Leo Westerberg:** Future studies can combine this method with subcellular markers to investigate how SA-beta-gal activity relates to different cellular processes.

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.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What is the key takeaway you hope viewers remember after watching your video article?

1.6. Leo Westerberg, PhD Student: (authors will present their testimonial statements live)

What value do you expect this video article to have for other researchers working in your field?

1.7. Leo Westerberg, PhD Student: (authors will present their testimonial statements live)

Ethics Title Card

This research has been approved by the Regional Ethics Committee in Stockholm

Protocol

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

2. X-gal Staining of Human Adipose-Derived Stromal Vascular Cells

Demonstrator: Leo Westerberg

2.1. To begin, retrieve the tube containing the human adipose tissue [1]. Homogenize ten grams of adipose tissue [2] and add 0.05% (*zero-point zero five percent*) type one collagenase enzyme to isolate 1.0×10^6 (*one million*) stromal vascular fraction or SVF (*S-V-F*) cells [3].

2.1.1. WIDE: Talent taking out a tube containing human adipose tissue.

Videographer's Note: 2.1.1.5 is an extra shot (close up)

2.1.2. Talent homogenizing the tissue to disrupt it.

2.1.3. Talent adding collagenase solution to the tube containing the tissue.

2.2. Aliquot five hundred thousand cells into each of three individual 1.5-milliliter microcentrifuge tubes [1]. Add one milliliter of 2 percent paraformaldehyde to each tube for cell fixation [2] and incubate on a rocker for 20 minutes at room temperature [3]. Centrifuge at 600 *g* for ten minutes [4] and remove the supernatant [5].

2.2.1. Talent adding 0.5×10^6 cells into each 1.5 mL tubes.

2.2.2. Talent adding 2% PFA in 1.5 ml tube containing cell suspension.

2.2.3. Talent placing the tube on a rocker.

2.2.4. Talent placing the tube in a centrifuge. *Videographer: Please capture the digital meter on the centrifuge showing the centrifuge speed and time.*

2.2.5. Talent discarding the supernatant from the tube.

2.3. Prepare 6 milliliters of X-gal (*X-gal*) staining solution using the senescence β (*beta*) - galactosidase staining kit according to manufacturer's instructions [1]. Add one milliliter of the staining solution each into three fresh tubes [2] and adjust the pH (*P-H*) to five, six and eight using 1 molar hydrochloric acid and 1 molar sodium hydroxide solutions [3]. Use a calibrated pH meter to verify the pH [4].

2.3.1. Talent preparing the X-gal staining solution using the senescence β -gal kit.

2.3.2. Talent adding 2mL staining solution to each of the 3 tubes.

2.3.3. Talent adjusting the pH using 1M HCl and 1M NaOH.

2.3.4. Talent verifying the pH using pH meter.

- 2.4. Add the pH-adjusted X-gal staining solution to each of the labelled tubes containing five hundred thousand cells [1]. Incubate at 37 degrees Celsius for 24 hours without carbon dioxide [2].
 - 2.4.1. Talent adding the pH-adjusted X-gal solutions to each of the labelled tubes with 0.5×10^6 cells.
 - 2.4.2. Talent placing the tubes in a 37 °C incubator.
- 2.5. Centrifuge the cells at 600 *g* for ten minutes [1]. After removing the supernatant, wash the cells with PBS [2] and centrifuge again at 600 *g* for 10 minutes [3].
 - 2.5.1. Talent placing the tubes with staining solutions in the centrifuge.
 - 2.5.2. Talent adding PBS to the cells
 - 2.5.3. Talent placing the tubes in the centrifuge and closing the lid
- 2.6. Once supernatant is removed, incubate the cells with CD31 (*C-D-thirty-one*) or IgG (*I-G-G*) control primary antibody at four degrees Celsius overnight [1-TXT]. Wash the cells thrice using 0.1 percent PBST (*P-B-S-T*) for ten minutes at room temperature on a rocker to remove unbound primary antibody [2] and centrifuge at 600 *g* for ten minutes [3].
 - 2.6.1. Talent incubating the three tubes with primary antibody overnight at 4 °C. **TXT: Primary Ab: CD31 (1:200) or IgG control (1:333) in 5% NDS**
 - 2.6.2. Talent placing the cell containing tube on a rocker after adding PBST.
Videographer: Obtain multiple reusable takes of 2.6.2 as these will be used later in the protocol
 - 2.6.3. Talent placing the tubes in a centrifuge.
- 2.7. After discarding the supernatant, incubate the cells with a secondary antibody for one hour at room temperature on a rocker [1-TXT]. Centrifuge at 600 *g* for ten minutes to remove secondary antibody staining solution [2]. Wash the cells thrice using PBST and then centrifuge as demonstrated earlier [3].
 - 2.7.1. Talent adding secondary antibody to the tubes. **TXT: Secondary Ab: Anti-mouse Alexa Fluor 555 (1:500) and Wheat Germ Agglutinin (1:200) in 5% NDS**
 - 2.7.2. Talent placing the tubes in a centrifuge.
 - 2.7.3. *Reuse 2.6.2 and 2.6.3*
- 2.8. Resuspend cells in the antifade mountant [1]. Pipette the cell suspension onto microscope slides and cure overnight at room temperature to immobilize the cells [2].
 - 2.8.1. Talent resuspending the cells in mountant.

- 2.8.2. Talent pipetting the cell suspension onto glass slides and setting them aside.
- 2.9. The next day, add 100 microliters of 100 percent glycerol [1], place a coverslip on top [2], and seal it with nail polish [3].
 - 2.9.1. Talent adding 100 µL of 100% glycerol.
 - 2.9.2. Talent carefully placing a coverslip on top of the glycerol drop. **Note: Shots 2.9.1 and 2.9.2 are combined.**
 - 2.9.3. Talent sealing the cover slip on slide using nail polish.

3. Microscopy and SA-β-gal scoring

Demonstrator: Leo Westerberg and Laurent Barbe

- 3.1. Place the prepared slide on the stage of confocal microscope [1].
 - 3.1.1. Talent placing the prepared slide on the confocal microscope stage.
- 3.2. In the microscope software interface, select the **Lightpath** option and adjust the beam splitter setting to T90/R10 (*T-ninety-R-ten*) [1]. Next, verify the cut-off wavelength values for the secondary dichroic mirrors, or SDMs and the mirror setting [2].
 - 3.2.1. SCREEN: 70588_screenshot_02-(1). 00:00 – 00:05
 - 3.2.2. SCREEN: 70588_screenshot_02-(1). 00:06 – 00:21
- 3.3. In the **Detector** menu, click on the **Dye & Detector Select** option to assign channels [1] and excitation wavelengths [2]. Then, set the laser power intensity percentage values for each specific laser in the range of 10 to 15 percent [3].
 - 3.3.1. SCREEN: 70588_screenshot_01-(1). 00:00 – 00:06
 - 3.3.2. TEXT ON A PLAIN BACKGROUND

Use 633 nm laser illumination and collect reflected light between 635–645 nm

Excitation Wavelengths:

 - Hoechst: 405 nm
 - Wheat germ Agglutinin: 488 nm
 - CD31: 555 nm
 - 3.3.3. SCREEN: 70588_screenshot_01-(1). 00:23 – 00:33
- 3.4. In the **Series** menu, under the **LSM** section, toggle the **Z** (*zee*) option to **ON** [1]. Set the Z step size to the system optimum of 0.74 micrometer [2]. Next, under the **Z section** menu, enter the value for **Start** input field and define a Z stack based on the membrane signal to capture the full cell volume [3]. Under the **Acquire** panel, enter

the file name and file save location in the LSM input field and click on **LSM Start** to initiate image acquisition and save the files in. oir (*dot-O-I-R*) native microscope format [4]. After acquisition, use the Z-navigation arrows to verify the signal and focal distribution of different dyes across the entire Z-stack [5].

3.4.1. SCREEN: 70588_screenshot_04-(1). 00:00 – 00:04

3.4.2. SCREEN: 70588_screenshot_04-(1). 00:05 – 00:13

3.4.3. SCREEN: 70588_screenshot_03-(1). 00:04 – 00:11

3.4.4. SCREEN: 70588_screenshot_05-(1). 00:00 – 00:12

3.4.5. SCREEN: 70588_screenshot_06-(1). 00:00 – 00:13 *Video Editor: This is the highest resolution the authors could provide for this screen capture*

- 3.5. Upload the saved .oir file to **ImageJ** (*Image-J*) [1]. In the **Bio-Formats Import options** pop-up window, click **OK** [2]. Click on **Image** menu to open the file [3] and from the drop-down options, select **Color**, then **Split Channels** option to open the different channels as separate windows [4].

3.5.1. SCREEN: 70588_screenshot_07-(1). 00:01– 00:03

3.5.2. SCREEN: 70588_screenshot_07-(1). 00:04– 00:06

3.5.3. SCREEN: 70588_screenshot_07-(1). 00:07– 00:13

3.5.4. SCREEN: 70588_screenshot_07-(1). 00:14– 00:22

- 3.6. Next, adjust the brightness-contrast and optimize the LUT (*lut*) values using the **Adjust** option under the **Image** menu for enhanced visualization of the image [1]. Use the **Z-axis scroll bar** at the bottom of the frame to traverse the optical sections and verify cellular features throughout the stack [2].

3.6.1. SCREEN: 70588_screenshot_07-(1). 00:23– 00:35

3.6.2. SCREEN: 70588_screenshot_07-(1). 00:36– 00:42

- 3.7. To generate summed intensity projections of the three-dimensional confocal image, select the **Z Project** function under **Image** menu and **Stack** option [1]. In the Z-Projection dialogue box, define the start and stop slice ranges and select the projection type and click **OK** to obtain the final 2D (*2-D*) composite image [2]. Next, navigate to the **Image** menu, select '**Type**' option to adjust the image type to 16-bit (*sixteen-bit*) [3], then save and export the images as 16-bit TIFF (*tiff*) files [4].

3.7.1. SCREEN: 70588_screenshot_08-(1). 00:00– 00:05

3.7.2. SCREEN: 70588_screenshot_08-(1). 00:06– 00:12

3.7.3. SCREEN: 70588_screenshot_09-(1). 00:00– 00:05

3.7.4. SCREEN: 70588_screenshot_09-(1). 00:05– 00:19

- 3.8. Import the saved images into CellProfiler version 4.2.8 (*four-point two point eight*) for automated cell segmentation, quantification of the reflected light intensity for each cell and exporting the intensity measurement values to a spreadsheet [1].

3.8.1. SCREEN: 70588_screenshot_10-(1). 00:00– 00:09

- 3.9. In the spreadsheet, define the threshold for senescence-associated beta-galactosidase positivity as the sum of average reflected light intensity of the pH eight condition plus three standard deviations [1]. Classify the cells in the pH six condition as senescence-associated beta-galactosidase positive, if their intensity values exceed the established threshold [2].

3.9.1. SCREEN: 70588_screenshot_11. 00:00– 00:16.

3.9.2. SCREEN: 70588_screenshot_12. 00:00– 00:15.

Results

4. Results

- 4.1. Reflected light senescence-associated beta galactosidase staining of human SVF cells produced clear and reproducible patterns across three pH levels [1]. At pH 5, most cells showed a strong signal due to high endogenous enzyme activity [2].
 - 4.1.1. LAB MEDIA: Figure 1: *Video Editor: Please emphasize the columns labelled Ac, Bc, Cc corresponding to pH 5, pH 6 and pH 8.*
 - 4.1.2. LAB MEDIA: Figure 1: *Video Editor: Please emphasize the panels in the row labelled pH 5.*
- 4.2. At the assay standard value of pH 6, the signal intensity and frequency decreased, allowing for the specific identification of senescence-associated beta-galactosidase cells [1]. At pH 8, the signal was minimal [2] and this served as a baseline to define the threshold for positivity [3].
 - 4.2.1. LAB MEDIA: Figure 1: *Video Editor: Please emphasize the panels in the row labelled pH 6.*
 - 4.2.2. LAB MEDIA: Figure 1: *Video Editor: Please emphasize the panels in the row labelled pH 8.*
 - 4.2.3. LAB MEDIA: Figure 1: *Video Editor: Please emphasize the red dotted line in the panel labelled Cc.*
- 4.3. Detection sensitivity testing showed that the reflected light imaging outperformed brightfield across all pH conditions [1]. At pH 5, reflected light showed strong X-gal precipitates [2], whereas brightfield only caught the largest deposits [3].
 - 4.3.1. LAB MEDIA: Figure 2: *Video Editor: Please emphasize the columns labelled Reflected and Bright field corresponding to pH 5, pH 6 and pH 8 sequentially.*
 - 4.3.2. LAB MEDIA: Figure 2: *Video Editor: Please emphasize the column labelled "Reflected" in the pH 5 row.*
 - 4.3.3. LAB MEDIA: Figure 2: *Video Editor: Please emphasize the column labelled "Bright field" in the pH 5 row.*
- 4.4. At pH 6, reflected light [1] identified positive cells that were too weak or diffuse to see in brightfield [2]. At pH 8, reflected light imaging detected minimal signal consistent with low enzymatic activity [3], outperforming brightfield in identifying X-gal precipitates at intermediate and lower activity levels [4].
 - 4.4.1. LAB MEDIA: Figure 2: *Video Editor: Please emphasize the column labelled "Reflected" in the pH 6 row.*
 - 4.4.2. LAB MEDIA: Figure 2: *Video Editor: Please emphasize the column labelled "Bright field" in the pH 6 row.*
 - 4.4.3. LAB MEDIA: Figure 2: *Video Editor: Please emphasize the column labelled "Reflected" in the pH 8 row.*
 - 4.4.4. LAB MEDIA: Figure 2: *Video Editor: Please emphasize the column labelled "Bright field" in the pH 8 row.*

Pronunciation Guide:

1. Adipose
Pronunciation link: <https://www.merriam-webster.com/dictionary/adipose>
IPA: /'ædəˌpoʊs/
Phonetic Spelling: a·duh·pohs
2. Homogenize
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenize>
IPA: /hə'mɑːdʒəˌnaɪz/
Phonetic Spelling: huh·maa·juh·nyz
3. Collagenase
Pronunciation link: <https://www.howtopronounce.com/collagenase>
IPA: /kə'lædʒəˌneɪs/
Phonetic Spelling: kuh·laa·juh·nays
4. Stromal
Pronunciation link: <https://www.merriam-webster.com/dictionary/stromal>
IPA: /'stroʊməl/
Phonetic Spelling: stroh·muhl
5. Vascular
Pronunciation link: <https://www.merriam-webster.com/dictionary/vascular>
IPA: /'væskjələr/
Phonetic Spelling: vas·kyuh·luhr
6. Paraformaldehyde
Pronunciation link: <https://www.howtopronounce.com/paraformaldehyde>
IPA: /ˌpærəfɔːr'mældəˌhaɪd/
Phonetic Spelling: pa·ruh·for·mal·duh·hyde
7. Microcentrifuge
Pronunciation link: <https://www.howtopronounce.com/microcentrifuge>
IPA: /ˌmaɪkroʊ'sentrɪˌfjuːdʒ/
Phonetic Spelling: my·kroh·sen·truh·fyooj
8. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
9. Supernatant
Pronunciation link: <https://www.howtopronounce.com/supernatant>

IPA: /ˌsuːpərˈnɛtənt/

Phonetic Spelling: soo-pruh-nay-tuhnt

10. Senescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/senescence>

IPA: /sɪˈnɛsəns/

Phonetic Spelling: suh-neh-suhns

11. Galactosidase

Pronunciation link: <https://www.howtopronounce.com/galactosidase>

IPA: /gəˌlæktəˈtoʊsɪˌdeɪs/

Phonetic Spelling: guh-lack-toh-suh-days

12. Hydrochloric

Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrochloric>

IPA: /ˌhaɪdrəˈklɔːrɪk/

Phonetic Spelling: hy-druh-klor-ik

13. Calibrated

Pronunciation link: <https://www.merriam-webster.com/dictionary/calibrated>

IPA: /ˈkælɪˌbreɪtəd/

Phonetic Spelling: kal-uh-bray-tuhd

14. Incubate

Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>

IPA: /ˈɪŋkjəˌbeɪt/

Phonetic Spelling: ing-kyuh-bayt

15. Antibody

Pronunciation link: <https://www.merriam-webster.com/dictionary/antibody>

IPA: /ˈæn.tɪˌbɑːdi/

Phonetic Spelling: an-tee-baa-dee

16. Agglutinin

Pronunciation link: <https://www.howtopronounce.com/agglutinin>

IPA: /əˈɡluːtənɪn/

Phonetic Spelling: uh-gloo-tuh-nin

17. Antifade

Pronunciation link: No confirmed link found

IPA: /ˌæntɪˈfeɪd/

Phonetic Spelling: an-tee-fayd

18. Confocal

Pronunciation link: <https://www.howtopronounce.com/confocal>

IPA: /kən'foʊkəl/

Phonetic Spelling: kuhn·foh·kuhl

19. Dichroic

Pronunciation link: <https://www.merriam-webster.com/dictionary/dichroic>

IPA: /daɪ'krɔɪk/

Phonetic Spelling: dye·kroh·ik

20. Illumination

Pronunciation link: <https://www.merriam-webster.com/dictionary/illumination>

IPA: /ɪˌlu:mə'neɪʃən/

Phonetic Spelling: ih·loo·muh·nay·shuhn

21. Micrometer

Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>

IPA: /maɪ'krɑ:mətər/

Phonetic Spelling: my·kraa·muh·tuhr

22. Navigate

Pronunciation link: <https://www.merriam-webster.com/dictionary/navigate>

IPA: /'nævəˌgeɪt/

Phonetic Spelling: nav·uh·gayt

23. Bio-Formats

Pronunciation link: No confirmed link found

IPA: /'baɪoʊ 'fɔ:rmæts/

Phonetic Spelling: by·oh for·mats

24. Visualization

Pronunciation link: <https://www.merriam-webster.com/dictionary/visualization>

IPA: /ˌvɪʒwələ'zeɪʃən/

Phonetic Spelling: vɪzh·oo·uhl·uh·zey·shuhn

25. Projection

Pronunciation link: <https://www.merriam-webster.com/dictionary/projection>

IPA: /prə'dʒekʃən/

Phonetic Spelling: pruh·jek·shuhn

26. Composite

Pronunciation link: <https://www.merriam-webster.com/dictionary/composite>

IPA: /kəm'pɑːzət/

Phonetic Spelling: kuhm·paa·zuht

27. Segmentation

Pronunciation link: <https://www.merriam-webster.com/dictionary/segmentation>

IPA: /,segmən'teɪʃən/

Phonetic Spelling: seg·muhn·tay·shuhn

28. Quantification

Pronunciation link: <https://www.merriam-webster.com/dictionary/quantification>

IPA: /,kwɑːntəfə'keɪʃən/

Phonetic Spelling: kwon·tuh·fuh·kay·shuhn

29. Senescence-associated

Pronunciation link: No confirmed link found

IPA: /sɪ'nesəns ə'soʊsi,etəd/

Phonetic Spelling: suh·neh·suhns uh·soh·see·ay·tuhd

30. Deviations

Pronunciation link: <https://www.merriam-webster.com/dictionary/deviation>

IPA: /,diːvi'eɪʃənz/

Phonetic Spelling: dee·vee·ay·shuhnz