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Title: Lysosomal Profiling with LysoTracker for Quantitative Assessment of Cellular Senescence in Human Fibroblasts

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**

Videographer: Please record the computer screen for the shots labeled as SCREEN. The screen should be in focus and the fonts should be clearly visible.

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? Same building, 2 different floors with elevator access.

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: 14

Number of Shots: 31 (4 SC and 1 Lab Media)

Interviews

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

Authors: 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. **Javier Estrada Toro:** We study cellular senescence and its characterization, focusing on how features like lysosomal changes can serve as live-cell senescence markers.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

NOTE: Display the full name “Javier Estrada Toro” in the entire protocol wherever that name appears

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

- 1.2. **Javier Estrada Toro:** Conventional senescence markers are often endpoint-based and binary, whereas live-cell imaging reads senescence as a continuous, quantitative signal across conditions.
 - 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. **Javier Estrada Toro:** This protocol enables researchers to measure lysosomal remodeling in living cells, providing a quantifiable, single-cell readout of senescence burden and progression.
 - 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. **Javier Estrada Toro:** It is important to keep the staining and imaging settings identical across wells, since intensity comparisons are valid only within one session.

- 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. **Javier Estrada Toro:** Future studies can extend this procedure to three-dimensional models, additional cell types, and validation across different microscope systems and workflows.
- 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 problem in your field of research does your JoVE article address?

- 1.6. **Javier Estrada Toro, Research Project Assistant**: (authors will present their testimonial statements live)

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

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

- 1.7. **Javier Estrada Toro, Research Project Assistant**: (authors will present their testimonial statements live)

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

Protocol

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

2. Live-Cell Lysosomal and Nuclear Staining

Demonstrator: Javier Estrada Toro

Author's NOTE: Show 2.4 at the beginning of the protocol here, before 2.1

- 2.1. Warm the complete culture medium to 37 degrees Celsius [1]. Prepare a 75-nanomolar LysoTracker Deep Red working solution in the medium [2-TXT], and protect it from light [3].
 - 2.1.1. WIDE: Talent placing the complete culture medium in a 37 degrees Celsius incubator/waterbath. *Videographer: Please obtain a WIDE and a CU shot for this*
 - 2.1.2. Talent adding the LysoTracker Deep Red stock and warm media to a tube. **TXT: Stock LysoTracker: 1 mM**
 - 2.1.3. Talent wrapping the tube with aluminum foil.
- 2.2. Mix the working solution by gentle inversion [1].
 - 2.2.1. Talent gently inverting the foil-wrapped tube.
- 2.3. Select matched pre-senescent and senescent IMR-90 fibroblast cohorts prepared using a defined senescence induction method [1-TXT]. Confirm the senescent phenotype by monitoring growth characteristics and morphology [2].
 - 2.3.1. Talent removing 2 labeled dishes (Sen-low and Sen-High) from the incubator and placing them on the work surface. **TXT: Senescence induction: Serial passaging, stress or treatment exposure** *Video editor: Please show this text overlay when the VO says "and senescence induction method"*
 - 2.3.2. **LAB MEDIA:** Side-by-side views of pre-senescent and senescence-high IMR-90 cells highlighting enlarged, flattened morphology in

senescent cells. **Authors:** Please provide some microscopic images for non-senescent and senescent cells to show morphological differences **NOTE:** Add missing media title card

NOTE: Move 2.4 at the beginning of the protocol before 2.1

- 2.4. To begin, seed IMR-90 fibroblasts in a 24-well plate [1] and incubate them at 37 degrees Celsius in a 5% carbon dioxide incubator until they form a subconfluent monolayer [2]. Record the seeding density and the time between plating and lysosomal staining for each experiment [3].
 - 2.4.1. Talent adding the cells to a plate for staining.
 - 2.4.2. Talent placing the plate in the incubator.
 - 2.4.3. Talent noting the seeding density and the interval between plating and staining on the dish or a notebook.
- 2.5. Aspirate the spent medium from each well [1]. Gently add the LysoTracker working solution along the wall of each well, avoiding disturbance to the monolayer [2].
 - 2.5.1. Talent aspirating the spent medium from each well.
 - 2.5.2. Talent adding the LysoTracker working solution along the wall of each well.
- 2.6. Incubate the cells with LysoTracker for 30 minutes at 37 degrees Celsius in a 5 percent carbon dioxide incubator [1].
 - 2.6.1. Talent placing the cells in the incubator.
- 2.7. During the final 10 minutes of LysoTracker incubation, add Hoechst 33342 directly to the wells at the manufacturer-recommended working concentration [1] and mix by gently rocking the plate [2].
 - 2.7.1. Talent adding a Hoechst 33342 to the wells.
 - 2.7.2. Talent gently rocking the plate.
- 2.8. Remove the staining medium at the end of the incubation [1] and rinse the cells once with pre-warmed PBS to reduce background fluorescence [2].
 - 2.8.1. Talent removing the staining medium.

2.8.2. Talent adding pre-warmed PBS to the cells and removing it.

2.9. Immediately after the rinse, add pre-warmed phenol red-free culture medium [1]. Proceed directly to live cell imaging, keeping the time from buffer exchange to data acquisition consistent across all wells [2 and 3].

2.9.1. Talent adding pre-warmed phenol red-free culture medium or an imaging buffer.

2.9.2. Talent placing the plate on a live imaging microscope.

2.9.3. SCREEN: Acquiring live cell images. **Videographer's NOTE:** The screen + microscope shots are all at the end since they were in a different location

Videographer: Please record the computer screen for the shots labeled as SCREEN. The screen should be in focus and the fonts should be clearly visible.

3. Senescence Associated- β -Gal Staining

3.1. Label the parallel wells in the culture plate for beta-galactosidase staining to compare highly senescent cells with the pre-senescent controls [1-TXT].

3.1.1. Talent labeling the wells for senescence-associated beta-galactosidase staining validation. **TXT: SA- β -Gal staining is an optional procedure**

3.2. Complete live cell imaging for the validation wells before fixation [1]. Record the stage coordinates during live imaging to relocate the same fields after fixation [2].

3.2.1. SCREEN: live images of the sen-low and sen-high validation wells.

3.2.2. SCREEN: Hover the cursor over the stage coordinates on the screen during acquisition. **Videographer's NOTE:** The info listed on 3.2.1 + 3.2.3 is shown on screen during 2.9.3 so did not shoot separately per Javier

3.3. Add the fixation solution from the senescence beta-galactosidase staining kit, containing 2% formaldehyde and 0.2% glutaraldehyde, to the cells [1]. Cover the plate with the lid and incubate for 10 to 15 minutes at room temperature [2].

- 3.3.1. Talent adding the fixation solution to the cells.
- 3.3.2. Talent closing the plate lid and keeping the plate aside.
- 3.4. Remove the fixative, and rinse the wells twice with PBS [1]. Then, add the beta-galactosidase staining solution containing the X-gal substrate at pH 6 to each well [2].
 - 3.4.1. Talent removing the fixative and adding PBS.
 - 3.4.2. Talent adding the senescence-associated beta-galactosidase staining solution to each well.
- 3.5. Seal the plate with parafilm to limit evaporation during incubation [1] and place the plate in a 37 degrees Celsius dry incubator without carbon dioxide for 12 to 16 hours [2].
 - 3.5.1. Talent sealing the plate with parafilm.
 - 3.5.2. Talent placing the plate in the dry incubator.
- 3.6. After fixation and staining, place the plate on the microscope stage and relocate the same imaging fields using the recorded stage coordinates [1].
 - 3.6.1. Talent placing the plate of the microscope stage and relocating the wells marked/imaged earlier by adjusting coordinates.
- 3.7. Inspect the wells under a brightfield microscope and document the staining pattern [1]. Acquire representative images for each condition and determine the fraction of senescence-associated beta-galactosidase-positive cells per well [3].
 - 3.7.1. Talent placing the sample under a brightfield microscope.
 - 3.7.2. SCREEN: Acquiring representative images.

Results

4. Results

4.1. Senescent cells showed bright, spatially expanded perinuclear lysosome-enriched regions [1], whereas pre-senescent controls showed lower overall signal with discrete punctate structures [2].

4.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the bright red signal in the senescent cells in B.*

4.1.2. LAB MEDIA: Figure 2. *Video editor: Highlight the small red puncta below the left blue signal in the pre-senescent cells in B.*

4.2. Senescent conditions showed a higher fraction of β -Gal-positive cells [1] than pre-senescent controls [2].

4.2.1. LAB MEDIA: Figure 3. *Video editor: Show last column only “Beta gal stain” and highlight the dark β -Gal-stained cells in the senescent row.*

4.2.2. LAB MEDIA: Figure 3. *Video editor: Show last column only “Beta gal stain” and highlight the pre-senescent image*

4.3. A strong correlation was observed between LysoTracker intensity and senescence-associated β -Gal-positive area across the replicate wells [1].

4.3.1. LAB MEDIA: Figure 2D.

4.4. The mean LysoTracker intensity within the positive mask was higher in senescent cultures [1] than in pre-senescent cultures, making it the most robust field-level discriminator between the two conditions [2].

4.4.1. LAB MEDIA: Table 1. *Video editor: Highlight the values for “Mean*

Lyso intensity” column for high passage senescent row.

4.4.2. LAB MEDIA: Table 1. *Video editor: Highlight the values for “Mean Lyso intensity” column for low passage pre-senescent row.*

4.5. The integrated LysoTracker intensity per nucleus was also higher in senescent cultures in the representative field shown [1].

4.5.1. LAB MEDIA: Table 1. *Video editor: Highlight the values for “Integrated LysoTracker intensity per nucleus (a.u./nucleus)” for the high-passage senescent row.*

1. LysoTracker
Pronunciation link: <https://www.merriam-webster.com/dictionary/LysoTracker>
IPA: /'laɪsoʊˌtrækər/
Phonetic Spelling: ly·soh·trak·er
2. Lysosomal
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysosomal>
IPA: /ˌlaɪsəˈsoʊməl/
Phonetic Spelling: ly·suh·soh·muhl
3. Senescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/senescent>
IPA: /səˈnesənt/
Phonetic Spelling: suh·nes·uhnt
4. Fibroblast
Pronunciation link: <https://www.merriam-webster.com/dictionary/fibroblast>
IPA: /ˈfaɪbrəˌblæst/
Phonetic Spelling: fy·broh·blast
5. Phenotype
Pronunciation link: <https://www.merriam-webster.com/dictionary/phenotype>
IPA: /ˈfiːnəˌtaɪp/
Phonetic Spelling: fee·nuh·type
6. Morphology
Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>
IPA: /mɔːrˈfɑːlədʒi/
Phonetic Spelling: mor·fol·uh·jee
7. Subconfluent
Pronunciation link: <https://www.merriam-webster.com/dictionary/subconfluent>

- IPA: /ˌsʌbkənˈfluːənt/
Phonetic Spelling: sub·kuhn·floo·uhnt
8. Monolayer
Pronunciation link: <https://www.merriam-webster.com/dictionary/monolayer>
IPA: /ˈmɑːnoʊˌleɪər/
Phonetic Spelling: mon·oh·lay·er
9. Hoechst
Pronunciation link: <https://www.howtopronounce.com/hoechst>
IPA: /hɛkst/
Phonetic Spelling: hekst
10. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊˈresəns/
Phonetic Spelling: floo·res·uhns
11. Galactosidase
Pronunciation link: <https://www.merriam-webster.com/dictionary/galactosidase>
IPA: /gəˌlæktˈɑːsɪˌdeɪs/
Phonetic Spelling: guh·lak·tos·ih·days
12. Formaldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/formaldehyde>
IPA: /fərˈmældəˌhaɪd/
Phonetic Spelling: fer·mal·duh·hyde
13. Glutaraldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/glutaraldehyde>
IPA: /gluːˌtærˈældəˌhaɪd/
Phonetic Spelling: gloo·tar·al·duh·hyde
14. Parafilm
Pronunciation link: <https://www.howtopronounce.com/parafilm>
IPA: /ˈpærəˌfɪlm/
Phonetic Spelling: par·uh·film
15. Perinuclear
Pronunciation link: <https://www.merriam-webster.com/medical/perinuclear>
IPA: /ˌperiˈnuːkliər/
Phonetic Spelling: peh·ree·noo·klee·er
16. Punctate
Pronunciation link: <https://www.merriam-webster.com/dictionary/punctate>
IPA: /ˈpʌŋktet/
Phonetic Spelling: punk·tayt
17. Replicate
Pronunciation link: <https://www.merriam-webster.com/dictionary/replicate>
IPA: /ˈrepləˌkeɪt/
Phonetic Spelling: rep·luh·kayt
18. Integrated
Pronunciation link: <https://www.merriam-webster.com/dictionary/integrated>

IPA: /'ɪntəˌɡreɪtəd/

Phonetic Spelling: in·tuh·gray·tuhd

19. Nucleus

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

IPA: /'nuːkliəs/

Phonetic Spelling: noo·klee·uhs

20. Aspirate

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

IPA: /'æspəˌreɪt/

Phonetic Spelling: as·puh·rayt