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Title: Measuring Single-Cell Aging with an Imaging-Based Biomarker of Chromatin and Epigenetic Aging

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**

   If **Yes**, we will need you to record using screen recording software.

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: 26
Number of Shots: 56 (30 SC)

Introduction

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

INTRODUCTION:

1.1. **Krystal Ortaleza:** We developed imaging-based chromatin and epigenetic age (ImAge), a novel technique designed to quantify aging and rejuvenation at single-cell resolution.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What are the current experimental challenges?

1.2. **Krystal Ortaleza:** DNA methylation clocks estimate biological age but depend on linear regression, need large cohorts, and destroy samples, making them costly and unsuitable for large-scale or longitudinal studies.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

CONCLUSION:

What research gap are you addressing with your protocol?

1.3. **Josue A. Lopez:** This protocol enables researchers to investigate age-associated changes in chromatin and epigenetic organization at single-cell resolution across various cell types and tissues.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

What advantage does your protocol offer compared to other techniques?

1.4. **Josue A. Lopez:** Our imaging-based method is non-destructive, preserves chromatin structure, allows repeated measurements, and is more cost-effective for large-scale or longitudinal studies.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.1*

What questions will future research focus on?

1.5. **Josue A. Lopez:** Future research will focus on adding more epigenetic markers and studying other aging-related intranuclear structures.

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:

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Josue A. Lopez, Postdoc, Scintillon Institute**: (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.

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Josue A. Lopez, Postdoc, Scintillon Institute**: (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. *Suggested B-roll: 4.2.1*

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute

Protocol

2. Extraction and Preparation of Nuclei

Demonstrator: Krystal Ortaleza

2.1. To begin, transfer the frozen organs and tissues to a pre-chilled mortar placed over dry ice **[1]**. Pour liquid nitrogen over the frozen tissue **[2]** and grind it thoroughly using a pre-chilled pestle until a uniformly fine powder is obtained **[3]**.

2.1.1. WIDE: Talent placing the frozen tissue into a pre-chilled mortar set on dry ice.

2.1.2. Talent carefully pouring liquid nitrogen over the tissue.

2.1.3. Talent using a pre-chilled pestle to grind the frozen tissue.

2.2. Using a pre-chilled metal spatula, aliquot the ground tissue into multiple tubes **[1]**.

2.2.1. Talent scooping the powdered tissue with a pre-chilled metal spatula into labeled tubes.

2.3. After extracting the nuclei, mix them with Trypan Blue in an equal ratio in a separate tube **[1-TXT]**. Transfer ten microliters of the mixture to a hemocytometer or load it into an automated cell counter **[2-TXT]**.

2.3.1. Talent adding Trypan Blue to the nuclei suspension and mixing gently. **TXT: Use commercially available nuclear isolation kits**

2.3.2. Talent loading ten microliters of the nuclei-Trypan Blue mixture into a hemocytometer or automated counter. **TXT: Dilute the sample in PBS + 0.5% BSA to obtain 10,000 nuclei/30 µL**

2.4. Enter and verify all metadata including sample IDs, conditions, and biomarkers in the Platemap and export as CSV file **[1]**.

2.4.1. Talent working at the computer table, entering parameters in the Platemap.

2.5. Now, dispense ten thousand nuclei per well in a twenty to thirty microliter volume into a 384-well plate **[1]**. Centrifuge the plate at 1000 *g* with acceleration setting nine and deceleration setting four for ten minutes at four degrees Celsius **[2]**. Next, fix the nuclei by adding an equal volume of eight percent paraformaldehyde in PBS and incubate for

ten minutes at room temperature **[3]**.

2.5.1. Talent pipetting nuclei suspension into each well of the plate.

2.5.2. Talent placing the well plate into the centrifuge and setting parameters for one thousand g at four degrees Celsius.

2.5.3. Talent adding paraformaldehyde solution to the wells and keeping the plate aside.

2.6. After removing the fixative, add thirty microliters of 0.1 molar glycine in PBS and incubate for five minutes at room temperature **[1]**. Remove the glycine solution and wash the wells twice with 100 microliters of PBS **[2-TXT]**.

2.6.1. Talent pipetting thirty microliters of glycine solution into each well.

2.6.2. Talent removing glycine and adding PBS to the well. **TXT: Cells can be stored at 4 °C if required**

3. Immunolabeling the Nuclei

3.1. Remove the PBS from each well **[1]** and add thirty microliters of blocking solution containing two percent BSA and 0.5 percent Triton X-100 in PBS **[2]**. Incubate the plate for one hour at room temperature **[3]**.

3.1.1. Talent aspirating the PBS from all wells.

3.1.2. Talent pipetting thirty microliters of blocking solution into each well.

3.1.3. Close-up of the plate being kept aside on the work bench.

3.2. After removing the blocking solution **[1]**, incubate the sample with twenty-five microliters of primary antibody solution at four degrees Celsius **[2-TXT]**.

3.2.1. Talent removing the blocking solution.

3.2.2. Talent placing the sealed plate into a four-degree Celsius refrigerator. **TXT: Primary Ab: Mouse anti-H3K27ac (1:250); Rabbit anti-H3K27me3 (1:250); Diluted in blocking buffer (0.4 - 2 µg/mL)**

3.3. Next, wash the wells three times with one hundred microliters of PBS, each wash lasting three minutes at room temperature **[2]**.

3.3.1. Talent adding PBS to the wells using a multichannel pipette.

3.4. Add twenty-five microliters of secondary antibody solution **[1-TXT]** and incubate for two hours at four degrees Celsius **[2]**.

3.4.1. Talent pipetting twenty-five microliters of the secondary antibody solution into each well. **TXT: Secondary Abs: Alexa 488 Donkey Anti-Mouse (1:250); Alexa 555 Donkey Anti-Rabbit (1:250); Diluted in blocking buffer (1 - 10 µg/mL)**

3.4.2. Close-up of the plate being placed at four degrees Celsius.

3.5. Then, wash the wells three times with one hundred microliters of PBS, each wash lasting three minutes at room temperature **[1]**. Stain the nuclei with DAPI or Hoechst by adding thirty microliters of the dye at a concentration of 0.01 milligram per milliliter **[2]** and wash the plate once with one hundred microliters of PBS to remove excess dye **[3-TXT]**.

3.5.1. Talent aspirating antibody and adding PBS to the wells.

3.5.2. Talent placing the plate in the incubator.

3.5.3. Talent adding PBS to the wells. **TXT: Image the cells on a high-content imager**

4. System Software Configuration

Demonstrator: Kenta Ninomiya

NOTE: For the screen captures, please speed up the footage as required.

4.1. Download the workflow scripts from the GitHub repository **[1-TXT]**. Once the download is complete, unzip the file to extract its contents **[2]**.

4.1.1. SCREEN: Show the GitHub repository page with the **Code > Download ZIP** option being selected. 4.1.mp4, 00:00-00:29. **TXT: https://github.com/terskikh-lab/ImAge_workflow**

4.1.2. SCREEN: Show the downloaded ZIP file being unzipped to a local folder, revealing the ImAge_workflow directory. 4.1.mp4, 00:29-00:46.

4.2. Organize the cloned ImAge *(image)* repository so that the folder structure matches the format assumed by the workflow for correct script execution **[1]**. Then, open the **Terminal** application if operating on Linux or macOS, or the **WSL** application if using Windows **[2]**.

4.2.1. SCREEN: Show the folder structure being arranged to match the required layout, with subfolders such as “data,” “scripts,” and “results” properly positioned. 4.2.1._part1.mp4, 0:08-0:35.

4.2.2. SCREEN: Show a user opening the **Terminal** on macOS or Linux, or **WSL** on Windows. 4.2.2.mp4. 0:08-0:27.

4.3. Change the working directory to the cloned repository folder by typing the required command in the terminal and pressing **Enter [1]**. Create a new computational environment named *ImAge_workflow (image workflow)* using Conda with Python version 3.10 **[2]** and press **Enter [3]**.

4.3.1. SCREEN: Display the command being typed in the terminal and being entered. 4.3.mp4, 00:00-00:12.

4.3.2. SCREEN: Show the command being entered in the terminal and the Conda environment creation progress. 4.3.mp4, 00:13-00:44.

4.3.3. SCREEN: Command being executed. 4.3.mp4, 00:45-00:49.

4.4. Activate the newly created Conda environment with the command conda activate ImAge_workflow **[1]**. Install Poetry, a Python package manager, within the activated environment using the pip installer **[2]**. Then, install all prerequisite Python packages required for the workflow using Poetry **[3]**.

4.4.1. SCREEN: Show the command being typed conda activate ImAge_workflow. 4.4.1and2.mp4, 00:00-00:09.

4.4.2. SCREEN: Show Poetry installation with "pip install poetry". 4.4.1and2.mp4, 00:09-00:24.

4.4.3. SCREEN: Display terminal output showing Poetry resolving dependencies and installing necessary packages. 4.4.3.mp4, 0:01-0:30.

4.5. Now, configure CUDA *(C-U-D-A)* within the Conda environment to enable graphics processing unit functionality during the segmentation step **[1-TXT]**.

4.5.1. SCREEN: Show user entering the three CUDA configuration commands sequentially in the terminal. 4.5.mp4, 00:00-00:20. **TXT: Applicable for Linux or Windows systems equipped with an NVIDIA GPU**

5. Computational Analysis of Imaging Data

5.1. Open the **Terminal** application **[1]** and change the folder to the cloned **[2]**. Activate the Conda environment by typing the command below and pressing **Enter [1]**. 5.1-5.9.mp4: 00:00-00:05

5.1.1. SCREEN: Show the **Terminal** window being launched.

5.1.2. SCREEN: Show the cd /PATH_TO_CLONED_REPOSITORY/ImAge-main command

being entered.

5.1.3. SCREEN: Show executing conda activate ImAge_workflow

5.2. Open the workflow/main.py *(workflow-main)* file in a code or text editor such as VS code or **Notepad** to configure the analysis parameters **[1]**. Set the project name by assigning the variable p to a short, file system–safe identifier used to organize outputs **[2]**.

5.2.1. SCREEN: Show workflow/main.py opened in the editor with the parameters section visible. 5.1-5.9.mp4: 00:05-00:16.

5.2.2. SCREEN: Show the editor where p = brain_3ages_k27me3 is typed and saved. 5.1-5.9.mp4: 00:16-00:35.

5.3. Set the list of channels for feature extraction in chs, including the segmentation channel if features from that channel are required **[1]**.

5.3.1. SCREEN: Show the editor where chs = ['DAPI', 'H3K27me3', 'H3K27ac'] is entered. 5.1-5.9.mp4: 00:35-01:03.

5.4. Using imageIndex and the channel label as the key, map the image channel identifiers from filenames to the exact, case-sensitive platemap column header saved earlier **[1]**.

5.4.1. SCREEN: Show the editor where imageIndex = {'1': 'Channel1', '2': 'Channel2', '3': 'Channel3'} is entered, with a side panel previewing the platemap column headers. 5.1-5.9.mp4: 01:03-01:22.

5.5. Then, set data locations by assigning orgDataLoadPath *(org-data-load-path)* to the dataset root **[1]**, orgDataSubFolder *(org-data-sub-folder)* to the image subfolder if needed **[2]**, and resultsSavePath *(results save path)* to the output root **[3]**.

5.5.1. SCREEN: Show the editor where orgDataLoadPath is filled in. 5.1-5.9.mp4: 01:03-01:35.

5.5.2. SCREEN: Show the editor where orgDataSubFolder path is filled in. 5.1-5.9.mp4: 01:35-01:45.

5.5.3. SCREEN: Show the editor where resultsSavePath is filled in. 5.1-5.9.mp4: 01:45-01:56.

5.6. Confirm the filename extension in imageFileFormat *(image file format)* **[1]**, then edit imageFileRegEx *(image file reg-ex)* to use Python named capturing groups for the

required parts including row, column, field, z *(zee)*-position, and channel **[2-TXT]**.

5.6.1. SCREEN: Show the editor where imageFileFormat is verified (cursor hovering over the correct extension). 5.1-5.9.mp4: 01:56-02:13.

5.6.2. SCREEN: Show imageFileRegEx being edited to include any one named groups. 5.1-5.9.mp4: 02:13-02:54. **TXT: (?P<row>\d+); (?P<col>\d+); (?P<field>\d+); (?P<zposition>\d+); (?P<channel>\d+)**

5.7. Now, set the segmentation channel in segmentation_ch *(segmentation C-H)* to the nuclear channel used for object detection **[1]**.

5.7.1. SCREEN: Show the editor where segmentation_ch = 'DAPI' is entered.

5.1-5.9.mp4: 02:54-03:07.

5.8. Decide whether to use illumination correction by setting illumiCorrection *(illumi correction)* **[1]** and set to True to compute and apply BaSiC *(basic)* models **[2-TXT]**.

5.8.1. SCREEN: Show the editor where illumiCorrection is clicked. 5.1-5.9.mp4: 03:07-03:11.

5.8.2. SCREEN: True is selected and commented to indicate the choice. 5.1-5.9.mp4: 03:11-03:35. **TXT: Set to False to process raw images**

5.9. Then, set the physical voxel size in micrometers in voxel_dim *(voxel dim)* as [z, y, x] *(zee-Y-X)* using microscope metadata **[1]**. Save the file and close the editor after verifying each parameter **[2]**.

5.9.1. SCREEN: Show the editor where voxel_dim = [z, y, x] is entered. 5.1-5.9.mp4: 03:35-03:50.

5.9.2. SCREEN: Show the user saving main.py and closing the editor. 5.1-5.9.mp4: 03:50-03:58.

5.10. Finaly, run the workflow script to obtain ImAge readouts and perform validation, keeping the terminal open while the script is running **[1]**. Visualize results and export raw ImAge readouts for additional analysis in external software **[2]**.

5.10.1. SCREEN: Show the terminal executing Python workflow/main.py with progress messages appearing. 5.10.1.mp4, 00:00-00:35.

5.10.2. SCREEN: Show the terminal running Python workflow/visualization.py and rendering plots or summaries. 5.10.2.mp4, 00:00-00:40.

Results

6. Results

6.1. Representative images of induced pluripotent stem cells showed clearly separated nuclei stained with DAPI **[1]**, trimethylated histone H3 at lysine 27 **[2]**, and acetylated histone H3 at lysine 27 **[3]**.

6.1.1. LAB MEDIA: Figure 3. *Video editor: Highlight the DAPI panel.*

6.1.2. LAB MEDIA: Figure 3. *Video editor: Highlight the panel H3K27me3*

6.1.3. LAB MEDIA: Figure 3. *Video editor: Highlight the panel H3K27ac*

6.2. ImAge analysis of liver and skeletal muscles revealed a significant difference between young **[1]** and old samples **[2]**, with old-OSKM mice showing reduced ImAge **[3]** compared to old, indicating partial reprogramming **[4]**.

6.2.1. LAB MEDIA: Figure 4B,C. *Video editor: Highlight the 'Young' data plots*

6.2.2. LAB MEDIA: Figure 4B,C. *Video editor: Highlight the 'Old' data plots*

6.2.3. LAB MEDIA: Figure 4B,C. *Video editor: Highlight the 'Old-OSKM' data plots*.

6.2.4. LAB MEDIA: Figure 4B,C. *Video editor: Highlight the 'Old' data plots*

6.3. Analysis of individual animals indicated that some old-OSKM samples displayed significantly reduced ImAge values compared to old mice **[1]**.

6.3.1. LAB MEDIA: Figure 4D, E. *Video editor: Highlight the data plots for "4" under Old-OSKM in D and "3" and "5" under Old-OSKM group in E*.

1. Nitrogen
   Pronunciation link: https://www.merriam-webster.com/dictionary/nitrogen
   IPA: /ˈnaɪtrədʒən/
   Phonetic Spelling: nye·truh·jun
2. Aliquot
   Pronunciation link: https://www.merriam-webster.com/dictionary/aliquot
   IPA: /ˈælɪˌkwɑːt/
   Phonetic Spelling: al·uh·kwaht
3. Nuclei
   Pronunciation link: https://www.merriam-webster.com/dictionary/nucleus
   IPA: /ˈnuːkliaɪ/
   Phonetic Spelling: noo·klee·eye

4. Trypan
   Pronunciation link: https://www.merriam-webster.com/medical/trypan%20blue
   IPA: /ˈtraɪˌpæn/
   Phonetic Spelling: try·pan
5. Hemocytometer
   Pronunciation link: https://www.merriam-webster.com/dictionary/hemocytometer
   IPA: /ˌhiːməsaɪˈtɑːmɪtər/
   Phonetic Spelling: hee·muh·sy·tah·muh·ter
6. Metadata
   Pronunciation link: https://www.merriam-webster.com/dictionary/metadata
   IPA: /ˈmɛtəˌdeɪtə/, /ˈmɛtəˌdætə/
   Phonetic Spelling: meh·tuh·day·tuh / meh·tuh·da·tuh
7. Paraformaldehyde
   Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde
   IPA: /ˌpærəfɔːrˈmældəˌhaɪd/
   Phonetic Spelling: pair·uh·for·mal·duh·hyde
8. Glycine
   Pronunciation link: https://www.merriam-webster.com/dictionary/glycine
   IPA: /ˈglaɪsiːn/
   Phonetic Spelling: gly·seen
9. Immunolabeling
   Pronunciation link: https://www.merriam-webster.com/medical/immunolabeling
   IPA: /ˌɪmjənoʊˈleɪbəlɪŋ/
   Phonetic Spelling: im·yoo·noh·lay·buh·ling
10. Triton
    Pronunciation link: https://www.merriam-webster.com/dictionary/triton
    IPA: /ˈtraɪtən/
    Phonetic Spelling: try·tun
11. Antibody
    Pronunciation link: https://www.merriam-webster.com/dictionary/antibody
    IPA: /ˈæntɪˌbɑːdi/
    Phonetic Spelling: an·tuh·bah·dee
12. Histone
    Pronunciation link: https://www.merriam-webster.com/dictionary/histone
    IPA: /ˈhɪstoʊn/
    Phonetic Spelling: his·tohn
13. Acetylated
    Pronunciation link: https://www.merriam-webster.com/dictionary/acetylated
    IPA: /əˈsiːtəˌleɪtɪd/
    Phonetic Spelling: uh·see·tuh·lay·tid
14. Pluripotent
    Pronunciation link: https://www.merriam-webster.com/dictionary/pluripotent
    IPA: /ˌplʊrɪˈpoʊtənt/
    Phonetic Spelling: plur·ih·poh·tunt

15. Centrifuge
    Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
    IPA: /ˈsɛntrəˌfjuːdʒ/
    Phonetic Spelling: sen·truh·fyooj
16. Paraffin (as in paraformaldehyde fixation context)
    Pronunciation link: https://www.merriam-webster.com/dictionary/paraffin
    IPA: /ˈpærəfɪn/
    Phonetic Spelling: pair·uh·fin
17. Computational
    Pronunciation link: https://www.merriam-webster.com/dictionary/computational
    IPA: /ˌkɑːmpjəˈteɪʃənəl/
    Phonetic Spelling: kom·pyuh·tay·shuh·nul
18. Reprogramming
    Pronunciation link: https://www.merriam-webster.com/dictionary/reprogramming
    IPA: /ˌriːˈproʊgræmɪŋ/
    Phonetic Spelling: ree·proh·gram·ing
19. Acetylation
    Pronunciation link: https://www.merriam-webster.com/dictionary/acetylation
    IPA: /əˌsiːtəˈleɪʃən/
    Phonetic Spelling: uh·see·tuh·lay·shun
20. Trimethylated
    Pronunciation link: https://www.merriam-webster.com/medical/trimethylated
    IPA: /ˌtraɪˈmɛθɪleɪtɪd/
    Phonetic Spelling: try·meth·uh·lay·tid