Researchers need *in vitro* models that mirror the biology of organisms. Primary fibroblasts play essential roles in wound healing and are present in many tissues. They are widely used in studies of cell cycle control, reprogramming, and aging. Though extraction protocols exist, alternatives that maximize use of available resources are useful. Here, we present our protocol for extracting primary fibroblasts from adult mouse ear pinnae, an often-discarded source of primary cells, which consistently yields large, pure numbers of primary fibroblasts.

Juan I. Bravo, Minhoo Kim, Bérénice A. Benayoun
juanb@usc.edu (J.I.B.)
berenice.benayoun@usc.edu (B.A.B.)

**Highlights**

- Primary fibroblasts are isolated from adult mouse ear pinnae, an often-unused tissue
- Fibroblasts can be isolated from mice of varying sex, age, and genotype
- Fibroblast cultures are highly pure, with >90% THY1.2 or PDGFRα staining

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**Protocol**

**Protocol for isolation of adult mouse ear pinnae-derived primary fibroblasts**

1. **Ear Harvest**
   - ~30 min Chopping

2. **Tissue Adherence**
   - ~2 weeks

3. **Fibroblast Migration**
   - ~1.5 weeks Expansion

4. **Primary Fibroblast Culture**
Protocol for isolation of adult mouse ear pinnae-derived primary fibroblasts

Juan I. Bravo,1,2,7,* Minhoo Kim,1 and Bérénice A. Benayoun1,3,4,5,6,8,*

1Leonard Davis School of Gerontology, University of Southern California, Los Angeles, CA 90089, USA
2Graduate Program in the Biology of Aging, University of Southern California, Los Angeles, CA 90089, USA
3Neuroscience Graduate Program, University of Southern California, Los Angeles, CA 90089, USA
4USC Norris Comprehensive Cancer Center, Epigenetics and Gene Regulation, Los Angeles, CA 90089, USA
5Molecular and Computational Biology Department, USC Dornsife College of Letters, Arts and Sciences, Los Angeles, CA 90089, USA
6USC Stem Cell Initiative, Los Angeles, CA 90089, USA
7Technical contact
8Lead contact
*Correspondence: juanb@usc.edu (J.I.B.), berenice.benayoun@usc.edu (B.A.B.)
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SUMMARY
Researchers need in vitro models that mirror the biology of organisms. Primary fibroblasts play essential roles in wound healing and are present in many tissues. They are widely used in studies of cell cycle control, reprogramming, and aging. Though extraction protocols exist, alternatives that maximize use of available resources are useful. Here, we present our protocol for extracting primary fibroblasts from adult mouse ear pinnae, an often-discarded source of primary cells, which consistently yield large, pure numbers of primary fibroblasts.

BEFORE YOU BEGIN
The protocol below describes the specific steps for extracting primary cells from a cohort of 20 mice, most often C57BL/6Nia mice derived from the NIA (National Institute on Aging) aging mouse colony, from which we normally harvest multiple tissues (including ear pinnae). Times listed will increase or decrease if mouse cohorts are larger or smaller, or if additional tissues are or are not harvested. We have successfully extracted primary ear pinnae fibroblasts from mice as young as 3 months and as old as 29 months. This protocol has also been successfully applied with both male and female mice, as well as with transgenic mice. Thus far, we have not found a condition where this protocol does not work.

Prior to starting, prepare the necessary solutions for the “Tissue Collection” step and autoclave tweezers, razor blades, scissors, and any other tools that will be used to handle samples. Refer to the key resources table and materials and equipment section for the necessary recipes.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Pacific Blue Rat Monoclonal Anti-mouse CD90.2 [Thy1.2] Antibody (Clone 30-H12) | BioLegend | Cat#105323; RRID: AB_1877204 |
| PE Rat Monoclonal Anti-mouse CD140a [PDGFRα] Antibody (Clone APAS) | BioLegend | Cat#135905; RRID: AB_1953268 |
| APC Rat Monoclonal Anti-mouse CD45 Antibody (Clone 30-F11) | BioLegend | Cat#103112; RRID: AB_312977 |

(Continued on next page)
### MATERIALS AND EQUIPMENT

**Note:** Reagents from alternative suppliers may alter the efficiency of fibroblast extractions and should be validated prior to long-term use.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Dulbecco’s PBS (DPBS), 1x without calcium and magnesium | Corning | Cat#21-031-CV |
| PBS with 10% bovine albumin | Sigma-Aldrich | Cat#SRE0036-500ML |
| Hanks’ Balanced Salt Solution (HBSS) | Gibco | Cat#14175095 |
| 100X Antibiotic-antimycotic | Gibco | Cat#15240-062 |
| Gentamicin sulfate (10 mg/mL) | MP Biomedicals | Cat#C10503050 |
| Normocin (50 mg/mL) | InvivoGen | Cat#ant-nr-2 |
| Penicillin-streptomycin-L-glutamine 100X | Corning | Cat#30-009-CI |
| DMEM | Corning | Cat#15-013-CV |
| L-Glutamine (200 mM) | Corning | Cat#25-009-CI |
| Fetal bovine serum (FBS) | Sigma-Aldrich | Cat#F0926-500ML |
| 0.25% Trypsin | Cytiva | Cat#SV30031.01 |
| 0.05% Trypsin | Corning | Cat#25-052-CI |
| DMSO | Sigma-Aldrich | Cat#D2730-100ML |
| 2-Propanol | Sigma-Aldrich | Cat#19076-2.5L |
| Propidium Iodide | Alfa Aesar | Cat#P14055-A6 |
| Mouse Fc-blocking reagent | Miltenyi Biotec | Cat#130-092-575 |
| autoMACS® Rinsing Solution | Miltenyi Biotec | Cat#130-091-222 |
| MACS® BSA Stock Solution | Miltenyi Biotec | Cat#130-091-376 |

**Deposited data**
- Raw and analyzed flow cytometry data: This paper [https://doi.org/10.6084/m9.figshare.13661051.v1](https://doi.org/10.6084/m9.figshare.13661051.v1)

**Experimental models: organisms/strains**
- Mouse: C57BL/6Nia: wild type (4 and 20 months) | National Institute on Aging | N/A

**Software and algorithms**
- Flowlogic v8 | Miltenyi Biotec | Cat#160-002-087

**Other**
- 6-Well Suspension Culture Plates | Genesee Scientific | Cat#25-100
- 100 mm Tissue Culture Dishes | VWR | Cat#10062-880
- 100 mm Petri Dishes | Genesee Scientific | Cat#32-107G
- 15 mL Centrifuge Tubes | VWR | Cat#89039-664
- 50 mL Centrifuge Tubes | VWR | Cat#89039-656
- 5 mL Polystyrene Round-Bottom Tubes | Falcon | Cat#352054
- Cryovials | Bioland Scientific | Cat#TUBEC020-32
- Razor Blades | Genesee Scientific | Cat#38-101
- 70 μm MACS SmartStrainers | Miltenyi Biotec | Cat#130-110-916 or Cat#130-098-462
- Mr. Frosty Freezing Container | Thermo Scientific | Cat#5100-0001
- Countess™ Cell Counting Chamber Slides (includes 0.4% Trypan blue solution) | Invitrogen | Cat#C10228
- Countess II FL Automated Cell Counter | Invitrogen/Applied Biosystems | Cat#AMOAF1000
- MACSQuant Analyzer 10 | Miltenyi Biotec | Cat#130-096-343
Note: The solution can be prepared in advance and stored at 4°C. We recommend storing tissue wash buffer for no longer than 6 months.

**Tissue Wash Buffer**

| Reagent                     | Amount   |
|-----------------------------|----------|
| DPBS                        | 500 mL   |
| Antibiotic-Antimycotic 100× | 5 mL     |
| Gentamicin Sulfate          | 2.5 mL   |

**Initial Growth Media**

| Reagent                     | Amount   |
|-----------------------------|----------|
| DMEM                        | 400 mL   |
| FBS                         | 100 mL   |
| L-Glutamine                 | 5 mL     |
| Antibiotic-Antimycotic 100× | 5 mL     |
| Gentamicin Sulfate          | 2.5 mL   |
| Normocin                    | 1 mL     |

⚠ CRITICAL: Normocin is only used during Passage 0 in order to further prevent microbial growth.

Note: The solution can be prepared in advance and stored at 4°C. We recommend storing initial growth media for no longer than 6 months. Warm initial growth media to 37°C before use.

**Expansion Growth Media**

| Reagent                     | Amount   |
|-----------------------------|----------|
| DMEM                        | 400 mL   |
| FBS                         | 100 mL   |
| L-Glutamine                 | 5 mL     |
| Antibiotic-Antimycotic 100× | 5 mL     |
| Gentamicin Sulfate          | 2.5 mL   |

Note: Since antibiotics are known to alter mitochondrial function (Kalghatgi et al., 2013), we reduce the amount of antibiotics present in the expansion growth media by omitting Normocin, and we use this media for Passages 1–3.

Note: The solution can be prepared in advance and stored at 4°C. We recommend storing expansion growth media for no longer than 6 months. Warm expansion growth media to 37°C before use.

**Freezing Media**

| Reagent                     | Amount   |
|-----------------------------|----------|
| Expansion Growth Media      | 9 mL     |
| DMSO                        | 1 mL     |
**Note:** Prepare fresh freezing media immediately prior to freezing cells.

| Long-term culture media | Amount | Amount |
|-------------------------|--------|--------|
| DMEM                    | 450 mL | 400 mL |
| FBS                     | 50 mL (10%) | 100 mL (20%) |
| Penicillin-Streptomycin-L-Glutamine 100× | 5 mL | 5 mL |

**Note:** After Passage 3, fibroblasts can be grown on 10% FBS media to limit their growth rate. Alternatively, they can be maintained on 20% FBS media if faster growth is desired. Warm long-term culture media to 37°C before use. We recommend storing long-term culture media for no longer than 6 months at 4°C.

**Note:** Long-term culture media with 10% FBS can be used to neutralize trypsin at any step in this protocol.

| Resuspension buffer (for flow cytometry analysis) | Amount |
|---------------------------------------------------|--------|
| autoMACS® Rinsing Solution                        | 225 mL |
| MACS® BSA Stock Solution                          | 25 mL  |

**Note:** The final buffer composition corresponds to phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA. We recommend storing resuspension buffer for no longer than 6 months at 4°C.

| Flow cytometry staining buffer | Amount |
|--------------------------------|--------|
| Resuspension buffer            | 950 µL |
| Fc blocking reagent            | 50 µL  |

**Note:** Prepare fresh staining buffer immediately prior to running cells through the flow cytometer.

**STEP-BY-STEP METHOD DETAILS**

**Tissue collection**

© Timing: 3 h (to process 20 animals)

This step details how to collect and store mouse ear pinnae tissue that harbors primary fibroblasts.

1. Aliquot 5–10 mL of tissue wash buffer into one sterile 15 mL centrifuge tube for each sample. Alternatively, aliquot 15 mL if tissue samples will be shipped. Maintain centrifuge tubes on ice.
2. Euthanize each C57BL/6Nia mouse according to the procedures approved by your Institutional Animal Care and Use Committee (IACUC). In our lab, mice are euthanized by CO₂ asphyxiation followed by cervical dislocation.
Note: Our cohorts of 20 animals consist of young (4 months) and old (20 months) male and female C57BL/6Nia mice.

3. After ensuring euthanasia, liberally spray the mouse with 70% ethanol, making sure that the fur on and around the ear pinnae is wet.

△ CRITICAL: Mouse fur is a rich source of mycobacteria and other microbes. Aside from acting as a disinfectant, the ethanol makes it easier to shave fur off any part of the corpse.

4. Using a razor blade, shave as much of the fur on and around each ear pinna as possible. Once complete, cut off each ear pinna with sterilized scissors and transfer tissues to the corresponding centrifuge tube on ice. Clean your tweezers and scissors with ethanol between each animal.

Note: Though the ear pinnae are not fur rich, shaving the fur that is present reduces the chances of contamination.

Note: If other tissues are being harvested, the ear pinnae can be cut and shaved elsewhere in order to increase tissue harvesting efficiency. However, shaving the ear pinnae in this manner may be slightly more difficult.

5. Continue collecting ear pinnae tissue from the remaining mice, ensuring that tissue remains submerged in tissue wash buffer and that centrifuge tubes remain on ice.

Note: To simplify handling of the tissue, we recommend collecting each ear pinna as a single piece. However, cutting each ear pinna into smaller pieces should not alter the extraction efficiency.

Note: For each independent animal, we collect both ear pinnae in a single tube. However, we have successfully extracted primary fibroblasts using a single ear pinna. Hereafter, we consider the contents of any given tube as an independent sample.

Pause point: If tissues will be shipped, seal the 15 mL centrifuge tubes with parafilm and place in an appropriate box filled with ice or cold packs. Alternatively, tubes can be maintained at 4°C for a few days prior to the next step. We have extracted primary fibroblasts successfully and in high yields after storing samples up to 3 days. The next set of steps will involve extracting, expanding, and freezing primary fibroblasts over the next ~3.5 weeks (Figure 1).

Ethanol washes, tissue dicing, initial plating, and cell extraction

© Timing: 3 h (to complete plating tissues)

This step details how to disinfect, dice, and maintain ear pinnae tissue in order to promote primary fibroblast isolation and growth while limiting microbial contamination.

6. Prepare 6-well suspension plates in order to conduct an ethanol-ethanol-buffer sequential wash of the tissues. For each independent animal, fill two wells each with 5 mL of 70% ethanol and one well with 5 mL of tissue wash buffer.

7. To ease handling of tissues, remove tubes from the fridge or from ice and transfer tissues, along with some buffer, to a separate, empty set of 6-well suspension plates (Figure 2A). Clean tweezers with 70% ethanol prior to the transfer of each independent sample.
8. Begin the sequential wash by transferring tissues to the first ethanol-filled well and wash for 5 min at 18°C–25°C using a rocker (Figure 2B).

9. Transfer tissues to the second ethanol-filled well and wash for 5 min at 18°C–25°C using a rocker (Figure 2B).

10. Once the second ethanol wash is complete, use tweezers to temporarily remove tissues from their wells and press them against a clean paper towel to absorb excess ethanol. Once excess ethanol has been removed, immediately transfer tissues to the third well with tissue wash buffer (Figure 2B). Temporarily store plates at 4°C until ear pinnae are ready to be diced.

11. Fill a new beaker with fresh 70% EtOH to maintain autoclaved tweezers. Place all needed materials in the biosafety cabinet. From the fridge, remove and process the samples one 6-well plate at a time.

12. For each independent sample, transfer only the tissue from both ear pinnae to a sterile 10 cm petri dish. Add a few drops of tissue wash buffer to keep the tissue moist. If needed, use an autoclaved razor blade to trim any remaining fur.

13. With a second pair of autoclaved razor blades, and away from any fur, dice the tissue into many smaller fragments. Aim for most fragments to be 1–3 mm x 1–3 mm in size.

14. Once the tissue is fragmented, resuspend the diced tissue with 3 mL of initial growth media and transfer to a 10 cm tissue culture dish. Gently agitate the plate to distribute tissue fragments and incubate the plate in a humidified incubator at 37°C and 5% CO₂ (Figures 1, 2C, and 2D).

15. Continue trimming, dicing, transferring, and incubating the remaining samples. Incubate the plates without disturbance for three days.

**CRITICAL:** During the first three days, primary fibroblasts will migrate out of some tissue fragments and adhere to the tissue culture plate. As more primary fibroblasts crawl out,
The tissue fragments will themselves become increasingly attached to the plate. Any disturbances to the plate will inhibit attachment and reduce extraction efficiencies.

**Note:** We extract primary fibroblasts from tissue fragments of both ear pinnae of an experimental animal. However, we have successfully extracted cells using only one pinna.

16. Check the plates after three days. At this time, a few primary fibroblasts may be observed under the microscope (Figures 3A and 3B). Additionally, tissue fragments that are not floating in solution are indicative of a likely successful cell extraction. If there are signs of microbial contamination, such as the media turning yellow or turning opaque or visible fungal outgrowths, discard immediately.

**Note:** We have not experienced contamination issues with this protocol thus far. Omitting the ethanol steps or the inclusion of Normocin, however, has increased the likelihood of contamination in our hands.

17. Remove the remaining media and gently add 10 mL of fresh initial growth media (Figure 1). Minimize agitation of tissue fragments that have attached to the plate. Continue incubating the plates in a humidified incubator at 37°C and 5% CO2.

18. Remove old media and add fresh initial growth media again at seven days (Figure 1). Larger, more noticeable clusters of primary fibroblasts should be noticeable around many tissue fragments (Figures 3C and 3D). Continue culturing cells for, at most, another week, making sure to replace initial growth media every 2–3 days. Once many confluent clusters are observed on a plate (Figures 3E and 3F), continue to the next step. If no or few dense clusters are observed after two weeks, the extraction was likely unsuccessful for those samples, and they can be discarded.

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**Figure 2. Ear pinnae tissue before and after dicing**

(A) Ear pinnae are initially shaved to remove excess fur and are transferred to 6-well plates prior to washing with 70% ethanol.

(B) Ear pinnae are sequentially washed twice in 70% ethanol, cleaned of excess ethanol, and washed in tissue wash buffer.

(C) After washing with ethanol, ear pinnae tissue is diced into finer pieces, which are then cultured with 3 mL of initial growth media to promote fibroblast adherence.

(D) Initially, no cells are visible when plates are visualized under a microscope. Scale bar, 250 μm.
Critical: Large, elongated, “myocyte-like” cells sometimes co-extract with primary fibroblasts. Since these cells do not grow efficiently in the outlined conditions, passaging the cultures several times before storage or usage will deplete cultures of these alternative cell types and enrich for primary fibroblasts (Figures 4A and 4B).

Figure 3. Primary fibroblast extraction over 10 days
The number of fibroblasts that migrate out of each tissue fragment is a function of time. A few cells may be seen after 3 days of culture (A and B), though cells become easily visible after 7 days (C and D). After ~10 days (E and F), the clusters of cells surrounding each tissue fragment begin becoming confluent, and cells should be passaged soon after. Cells are visualized at (A, C, and E) 5x and (B, D, and F) 10x magnification on the left and right images, respectively. Scale bar, 100 μm or 250 μm respectively.

Figure 4. “Myocyte-like” cells co-extract with primary fibroblasts
During Passage 0, “myocyte-like” cells may appear. The first three passages are important since primary fibroblasts replicate relatively quickly and become enriched in culture, while other cells have a growth disadvantage and become increasingly depleted. The same cells are visualized at (A) 5x and (B) 10x magnification. Arrows indicate myocyte-like cells. Scale bar, 100 μm or 250 μm respectively.
First passage (P0 -> P1) and fibroblast expansion (P2 and P3)

* Timing: 2 h (per passage of 20 samples)

This step details how to filter purify and maintain cells in order to enrich primary fibroblasts and deplete other cell types.

19. After ~10–14 days, passage the cells for the first time (Figure 1).
   a. Begin by aspirating the culture media.
   b. Wash cells twice with 2.5 mL of HBSS.
   c. Add 3 mL of 0.25% trypsin to each plate and incubate for ~ 6 min at 37°C.

△ CRITICAL: Cells are grown in media with a high concentration of FBS, which, if not diluted sufficiently, will slow or inhibit the trypsinization. At least two washes are recommended.

20. Check the plates. If a few clusters of cells remain attached, these can be liberated by gently tapping the plates. Once most cells are unattached, neutralize the trypsin with 6 mL of 10% FBS-containing media.

21. Transfer solutions to sterile 15 mL centrifuge tubes and spin down at 500 g and at 18°C–25°C for 5 min. Aspirate the supernatant and resuspend the cells in 9 mL of expansion growth media.

22. To remove the tissue fragments from solution, begin by attaching 70 μm MACS SmartStrainers to sterile 15 mL centrifuge tubes, one per sample. Pre-wet the strainers with 1 mL of expansion growth media.

23. Afterwards, pass the 9 mL of cell-tissue suspensions through the strainers and allow the tissues and cells to separate by gravity filtration for a few seconds.

24. Finally, transfer the filtered cell suspensions to 10 cm tissue culture dishes, designating these cells as Passage 1 (Figures 5A and 5B).

25. Continue incubating the plates in a humidified incubator at 37°C and 5% CO2.
   a. Replace the media every 2–3 days until cells reach confluency (Figures 5C and 5D).
   b. Carry out the second passage at ~17 days (Figure 1). Passage the cells 1:4, as described here (without the use of a strainer).
   c. Once cells become confluent during Passage 2 at ~20 days, split each sample among four plates (Passage 3) (Figure 1). The cells from these four plates will be frozen and stored long-term once they reach ~90% confluency.

Cell storage

* Timing: 6 h (to process 4 plates × 20 samples)

This step details how to cryopreserve primary fibroblasts so that cells may be used at a later date.

26. Cells are frozen after ~23 days (Figure 1). Begin by filling the Mr. Frosty Boxes with 2-Propanol, if not already filled. Replace the alcohol after the fifth use.

27. Dissociate the cells by trypsinization as before, neutralize trypsin, combine cell suspensions corresponding to the same sample in a 50 mL centrifuge tube, spin the cells down, and aspirate the supernatants.

28. Resuspend cells in freshly prepared freezing media, 1 mL per plate. Aliquot cell suspensions into cryovials, 1 mL per vial. Place the vials in the Mr. Frosty Boxes and store at −80°C.

Note: The Mr. Frosty Boxes will slowly cool the cells at about −1°C per minute, the optimal rate for cell preservation.
29. Once the Mr. Frosty Boxes have chilled 12–16 h, transfer vials from the –80°C freezer to a liquid nitrogen tank for long-term storage.

Cell thawing

**Timing:** 2 h (to process 20 cryovials)

This step details how to resuscitate primary fibroblasts from cryopreservation so that they may be used for experiments.

30. Pre-warm a bottle of long-term culture media in a water or bead bath at 37°C.

31. Rapidly thaw the contents of frozen vials of fibroblasts in a 37°C water bath. Once thawed, quickly spray the vials liberally with 70% ethanol before transferring to the biological hood.

32. Immediately combine the 1 mL of each cell suspension with 10 mL of pre-warmed long-term culture media in a 15 mL centrifuge tube.

△ CRITICAL: This step is necessary for the removal of DMSO from the media in order to maximize cell health and recovery from cryopreservation.

33. Pellet cells at 500 × g in a centrifuge at 18°C–25°C for 5 min. Discard the supernatant, resuspend cells in 10 mL of pre-warmed long-term culture media, and transfer the resuspended cells to a 10 cm tissue culture dish.

34. After 24 h, the cells are ready to be passaged and used for experiments. From this point on, 0.05% trypsin should be used to minimize cell stress.

Optional: Purity of the obtained cell culture can be confirmed using flow cytometry (see below).
Fibroblast identity validation by flow cytometry (optional)

Timing: 2 h (to process 6 samples and a control)

This step details how to stain cells and assess primary fibroblast culture purity by flow cytometry using a MACSQuant Analyzer 10 benchtop flow cytometer.

Note: We assess purity by staining for the following: 1) the pan-fibroblast marker CD140a, (also known as PDGFRα) (Lynch and Watt, 2018, Guerrero-Juarez et al., 2019, Salzer et al., 2018), 2) the alternative fibroblast surface marker CD90.2, (also known as THY1.2), which correlate with an activated fibroblast signature (Croft et al., 2019), and 3) the pan-immune marker CD45. We define fibroblasts as CD45⁻ CD140a⁺ cells. See Figure 6A for an overview of the gating strategy and the results for a representative sample.

Figure 6. Flow cytometry analysis of the identity of post-Passage 3 cultures

Passage 3 cryopreserved primary fibroblasts were thawed and analyzed 24 h later by flow cytometry. Data was analyzed with Flowlogic v8.

(A) Gating strategy applied to a representative culture of primary fibroblasts (derived from a young 4 months-old male). Debris is first gated out from the FSC-A vs. SSC-A plot of the entire population. Single cells are then gated based on the FSC-A vs. FSC-H plot. From this singlet population, CD45⁻ cells are obtained by gating on the CD45 vs FSC-A plot. Finally, we use this CD45⁻ population to gate for CD90.2⁺ and CD140a⁺ cells.

(B) Same gating strategy as in (A) applied to a population of peritoneal lavage cells, which should contain a large fraction of immune (CD45⁺) cells.

(C) Fibroblast cultures from three 4 months-old males and females were analyzed by flow cytometry, and a beeswarm plot was generated for the percentage of cells staining for combinations of immune (CD45) and fibroblast (CD90.2 and CD140a) markers.

(D) The same plot as in (C) but for a population of peritoneal lavage cells as a CD45⁺ control population.

Fibroblast identity validation by flow cytometry (optional)

© Timing: 2 h (to process 6 samples and a control)
35. Wash the fibroblast layer twice using 10 mL DPBS.
36. Then, detach cells by applying 2 mL of 0.05% trypsin, and incubate for 7 min in a humidified incubator at 37°C and 5% CO2.
37. Once most cells have detached, neutralize the trypsin with 8 mL of long-term culture media (10% FBS-containing media).
38. Collect detached cells in a 15 mL centrifuge tube, pellet cells at 500 \( g \) and at 18°C–25°C for 10 min in a centrifuge. Wash cells with 10 mL of resuspension buffer.
39. Again, pellet cells at 500 \( g \) and at 18°C–25°C for 10 min in a centrifuge. Resuspend cells in 1 mL of resuspension buffer. Count cells and cell viability using Trypan Blue exclusion and a Countess cell counting apparatus.
40. Aliquot 300,000 cells per culture into 5 mL polystyrene round-bottom tubes for staining. Also, similarly reserve cells for the unstained and single-stained controls to draw the gates. For ease, the unstained and single-stained controls can be combined into an equi-cellular mix of all populations to be tested, and this mix can be used to define the flow cytometry gates.

**Optional:** To have a CD45+ (pan-immune) control for gating, peritoneal lavage cells can be obtained by injecting and then recovering 10 mL of PBS (with 3% BSA) in the peritoneal cavity of a euthanized mouse. Alternatively, a cell line with known expression of CD45 (e.g., RAW267.4) can also be used. See Figure 6B for an application of our gating strategy to a population of peritoneal lavage cells.

41. Pellet cells at 500 \( g \) and at 18°C–25°C for 10 min in a centrifuge. Resuspend cells in 50 \( \mu L \) of flow cytometry staining buffer. Block cells by incubating at 4°C for 10 min.
42. For each sample that will be stained, prepare 50 \( \mu L \) of a 2× antibody solution in flow cytometry staining buffer (Table 1).
43. Add the 50 \( \mu L \) of 2× antibody solution to the 50 \( \mu L \) of blocked cells. Stain cells by incubating at 4°C for 20 min in the dark.
44. Wash away excess antibody by adding 2 mL of resuspension buffer and then pellet cells by centrifuging at 500 \( g \) and at 18°C–25°C for 10 min.
45. Resuspend the pellet in 2 mL of resuspension buffer for a second wash, and again pellet cells by centrifuging at 500 \( g \) and at 18°C–25°C for 10 min.
46. Resuspend cells in 100 \( \mu L \) of resuspension buffer per 100,000 cells.

**Optional:** Add 1 \( \mu L \) of propidium iodide (PI) per 100 \( \mu L \) of sample to quantify and exclude dead cells.

47. Run samples through a MACSQuant 10 flow cytometer. Unstained samples and single-stained samples are used to set appropriate gates that have clear positive and negative populations. Debris, dead cells, and doublets are excluded based on scatter signals and optionally PI staining (see Figure 6C for example data on fibroblasts from 3 young female and 3 young male animals).

**EXPECTED OUTCOMES**

Researchers are always in need of in vitro models that mirror the molecular and cellular processes of healthy organisms. Primary cells, derived from the tissues of an organism of interest, provide such a model and serve as a source of true biological replicates compared to standard cell lines. One such
cell type, primary fibroblasts, are present in multiple tissues (e.g., skin, tail, lungs, ears, etc.), play essential roles in wound healing (Eming et al., 2014, Lynch and Watt, 2018), and provide several advantages compared to other primary cells. Since they carry intact (unmutated) cell cycle control genes, they serve as an optimal model for the study of DNA repair and cell cycle control mechanisms. In studies of regeneration, they are widely used and reprogrammed into induced pluripotent stem cells (iPSCs) (Ocampo et al., 2016). Moreover, they retain age-related pro-inflammatory changes, making them a useful model for the study of “inflamm-aging” (Mahmoudi et al., 2019, Campisi, 2013, Franceschi et al., 2018). Additional advantages include 1) the ability to store tissues and extract cells at a later date, 2) their rapid and continued proliferation in culture, and 3) their easy maintenance, requiring no special reagents. Though a number of primary fibroblast extraction protocols exist (Au - Khan and Au - Gasser, 2016, Seluanov et al., 2010, Edelman and Redente, 2018), there is always a need for alternative protocols that maximize use of the resources available.

In our protocol, we extract primary fibroblasts reproducibly, in high yield, and in high purity from adult mouse ear pinnae tissue, which is frequently discarded. Our protocol yields four 10 cm dishes per animal, each dish with an average of 1.5–2.5 × 10⁶ cells at the end of passage 3. Moreover, these cultures appear to be enriched with fibroblasts, with > 90% of cells staining CD45 - CD140a⁺ (Figures 6A and 6C). An even greater percentage of cells staining CD45⁻ CD90.2⁺ supports the notion of pure fibroblast cultures. The observation that over 80% of cells stain CD45⁻ CD140a⁺ CD90.2⁺ suggests that a large fraction of these cultures consists of activated fibroblasts, although not exclusively. These results are in sharp contrast to those of the peritoneal lavage cells used as a positive control for the CD45⁺ gate, which exhibit an expected enrichment for CD45 and a depletion of fibroblast markers (Figures 6B and 6D). We anticipate that others will be able to achieve similarly high yields and purities.

Although not necessary for cell viability, we recommend the freezing step at the end of passage 3 to allow all samples to “catch up” on growth before experiments and avoid biases due to technical differences in the initial culture time for fibroblast derivation. After thawing, these primary fibroblasts can be cultured for ~1 week in 10% FBS media with a media change every 2–3 days before further passaging is needed. Unlike established cell lines, however, it is best to use primary fibroblasts before passage 10 to avoid unwanted senescence, transformation, or other non-physiological responses. Because of the relative ease of the presented procedure, experiments that require large numbers of cells may be performed using cultures from additional animals. Ultimately, this method should simplify the process of interrogating researchers’ questions in a physiologically relevant model primary cell system.

LIMITATIONS
A number of protocols for the isolation of primary fibroblasts from a variety of tissue sources exist, each with their own advantages and disadvantages (see Table 2 for a few comparisons). Our protocol produces high yield, high purity cultures at relatively low cost, partly because no expensive kits are needed, and makes use of common supplies generally found across most cell biology labs. However, this protocol is not designed to quickly (~1 day) isolate primary fibroblasts, requiring ~3.5 weeks to complete. In contrast to many other protocols, we also assess the purity of our cultures by flow cytometry, demonstrating that >90% of cells are either CD140a (PDGFRα) or CD90.2 (THY1.2) positive. In contrast to protocols relying on the selection of specific markers and thus specific fibroblast sub-populations, our protocol can isolate both activated (CD90.2⁺) (Croft et al., 2019) and non-activated fibroblasts. This may be an advantage or disadvantage depending on the researcher’s question and whether they are studying fibroblasts at the whole-population level or whether they are interested in specific sub-populations.

There are other technical factors to consider that may limit the efficacy of this protocol or bias results. Tissue dicing is a highly variable process which may lead to fluctuations in the number of primary
fibroblasts extracted from each animal. To a certain degree, this variability can be reduced by ensuring that all ear pinnae samples are diced until they have the same mushy consistency. The freezing step at the end of passage 3 also allows sample with varying degrees of initial extractions to "catch up" with each other. Additionally, it is possible that factors, such as the ethanol wash and the duration of expansion before usage, may erase phenotypes observed in vivo. If this is the ultimate goal, there are alternative protocols that allow for the extraction and purification of primary fibroblasts within a shorter experimental window, although at much lower yields (Table 2). Nevertheless, age-related RNA, chromatin, and metabolic changes were observed in primary fibroblasts extracted with a variant of this protocol (Mahmoudi et al., 2019). Our method is particularly attractive for studying the impact of strong, retained treatments, such as genetic background or aging, in primary cells.

**TROUBLESHOOTING**

**Problem 1**
Low yield of primary fibroblasts.

**Potential solution**
Primary fibroblast yield may be maximized by 1) processing ear pinnae tissue soon after harvesting, when cell viability is likely at its highest, and 2) dicing ear pinnae tissue into really fine fragments, which increases the surface area where fibroblasts may migrate from.

**Problem 2**
Primary cells are not adhering to the plate.

**Potential solution**
It is critical that plates are not moved during the first three days after plating the tissue fragments. Additionally, initial growth media replacements during the first two weeks are carried out with great

| Variables                      | This protocol       | Khan and Gasser (Au - Khan and Au - Gasser, 2016) | Edelman and Redente (Edelman and Redente, 2018) | Miltenyi tumor-associated fibroblast isolation kit | Miltenyi neonatal cardiac fibroblast isolation kit |
|-------------------------------|---------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|--------------------------------------------------|
| Type of Equipment & Reagents  | General cell culture equipment | General cell culture equipment | General cell culture equipment | Miltenyi proprietary kits/equipment | Miltenyi proprietary kits/equipment |
| Relative Cost                 | Low-Intermediate    | Low                                           | Low-Intermediate                              | High                                           | High                                             |
| Source Tissue                 | Ear pinnae          | Ear pinnae or tails                           | Lungs                                        | Tumors                                        | Neonatal heart                                   |
| Hands-on time                 | Intermediate        | Short                                         | Short (digestion) to intermediate (crawl-out method) | Intensive                                     | Intensive                                       |
| Relative Duration             | Long                | Intermediate                                  | Intermediate (digestion) to long (crawl-out method) | Short                                         | Short                                           |
| Relative Cell Yield           | High                | Low                                           | Low (digestion) to Intermediate (crawl-out method) | Low                                           | Low                                             |
| Fibroblast Selection Markers  | None                | None                                          | None                                          | CD90.2 (Negative and positive selection)       | Proprietary (Negative selection)                 |
| Age of animals                | Any                 | Any                                           | Any                                           | Any (if tumors are present)                   | Neonatal only                                    |
| Purity                        | >90% CD140a+ cells  | Unclear; Cultures tested for vimentin staining | Unclear; Cultures tested for phalloidin staining | Unclear; High purity of CD90.2+ and CD45 cells expected | Unclear; High purity of CD90.2+ and CD31+ cells expected |
care to minimize dislodging of attached tissue fragments. Though we check on plates every couple of days during those two weeks, plates are not frequently agitated.

**Problem 3**
Plates become contaminated with bacteria, mold, or fungus a few days after initially plating the tissue fragments.

**Potential solution**
Please ensure that ear pinnae are thoroughly and evenly washed during the ethanol bath. A third ethanol bath may help reduce contamination, though we have not needed more than two. Additionally, consider testing a fresh lot of antibiotic-antimycotic solution, gentamycin, or Normocin.

**Problem 4**
Some samples are growing more slowly compared to other samples.

**Potential solution**
There may be sample-to-sample differences in the initial number of primary cells extracted. Thus, we recommend growing cells to confluency (at passage 1 and 2) before passaging in order to normalize growth rates. This may mean that some samples need to be cultured for additional time prior to passaging. Under some circumstances, you may extract an insufficient number of cells to sustain the culture at a healthy cell density. We discard plates with low cell densities that show no signs of expansion either at passage 0 or at passage 1.

**Problem 5**
Primary cells are growing very slowly.

**Potential solution**
There may be FBS lot-to-lot variability that is creating sub-optimal conditions for cell culture. We recommend testing different FBS lots and using the one that optimizes cell growth.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bérénice A. Benayoun (berenice.benayoun@usc.edu).

**Materials availability**
This study did not generate new or unique reagents.

**Data and code availability**
The raw and processed flow cytometry data generated during this study are available on Figshare under the following DOI: https://doi.org/10.6084/m9.figshare.13661051.v1. This study did not generate any code.

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**AUTHOR CONTRIBUTIONS**
Conceptualization, J.I.B. and B.A.B.; investigation, J.I.B. and M.K.; writing - original draft, J.I.B. and B.A.B.; writing - review and editing, J.I.B., M.K., and B.A.B.; funding acquisition, B.A.B.
DECLARATION OF INTERESTS
The authors declare no competing interests.

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