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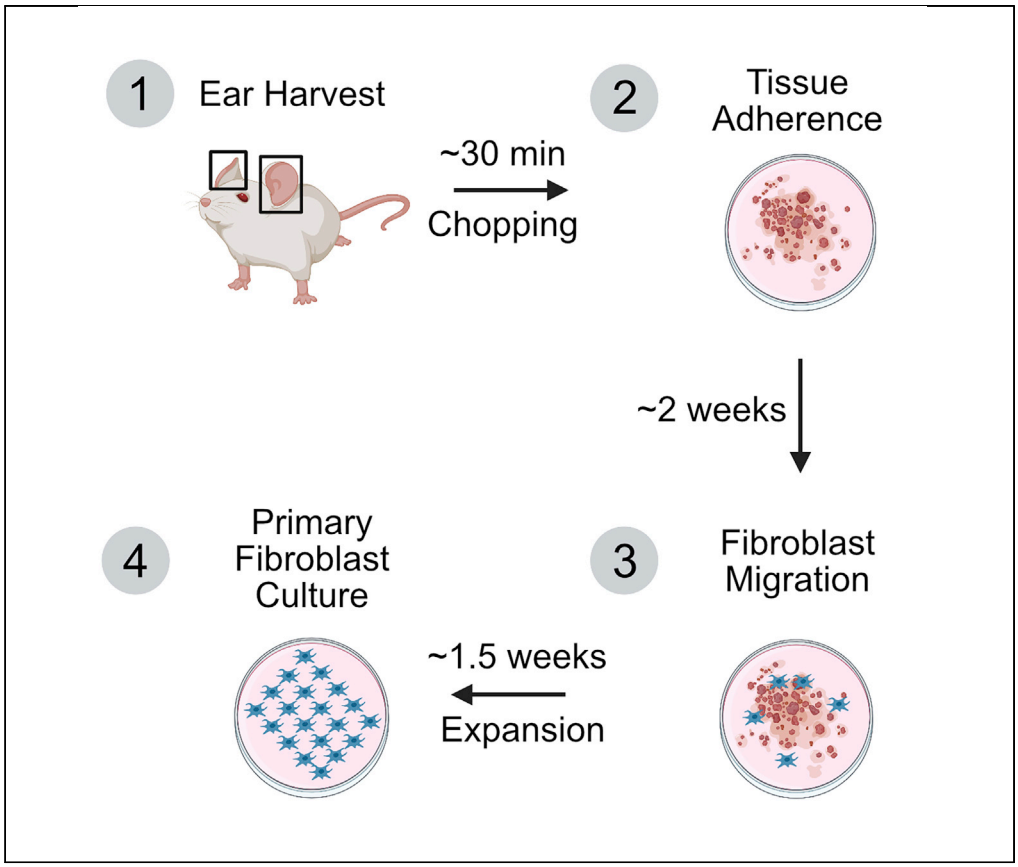
Protocol for isolation of adult mouse ear pinnae-derived primary fibroblasts



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.

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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 for isolation of adult mouse ear pinnae-derived primary fibroblasts

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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 APA5)	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*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's PBS (DPBS), 1× 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
100× Antibiotic-antimycotic	Gibco	Cat#15240-062
Gentamicin sulfate (10 mg/mL)	MP Biomedicals	Cat#IC10503050
Normocin (50 mg/mL)	InvivoGen	Cat#ant-nr-2
Penicillin-streptomycin-L-glutamine 100×	Corning	Cat#30-009-CI
DMEM	Corning	Cat#15-013-CV
L-Glutamine (200 mM)	Corning	Cat#25-005-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#472301-100ML
2-Propanol	Sigma-Aldrich	Cat#190764-2.5L
Propidium Iodide	Alfa Aesar	Cat#J66584-AB
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
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#AMQAF1000
MACSQuant Analyzer 10	Miltenyi Biotec	Cat#130-096-343

MATERIALS AND EQUIPMENT

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

Tissue Wash Buffer	
Reagent	Amount
DPBS	500 mL
Antibiotic-Antimycotic 100×	5 mL
Gentamicin Sulfate	2.5 mL

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.

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		
Reagent	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)	
Reagent	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	
Reagent	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 CO_2 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.

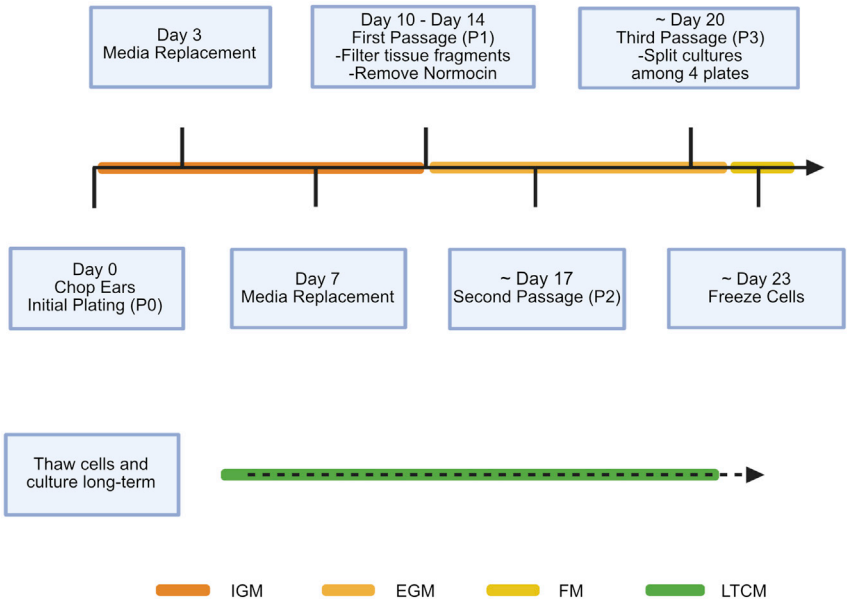

Figure 1. Timeline of primary fibroblast culture protocol

Primary fibroblasts migrate out of the diced ear pinnae tissue over the course of 10 – 14 days. After filtering out the tissue, these cells are expanded and passaged three times. Cultures are frozen at the end of passage 3 and thawed when needed for experiments. P0 – P3 correspond to Passages 0 – 3. IGM: initial growth media. EGM: expansion growth media. FM: freezing media. LTCM: long-term culture media.

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% CO_2 (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,

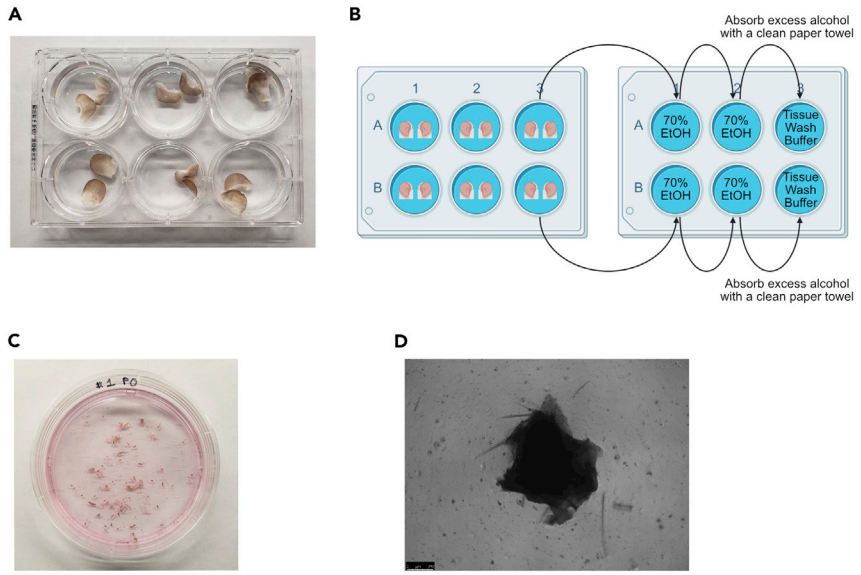

Figure 2. Ear pinnae tissue before and after dicing

(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.

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% CO_2.
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.

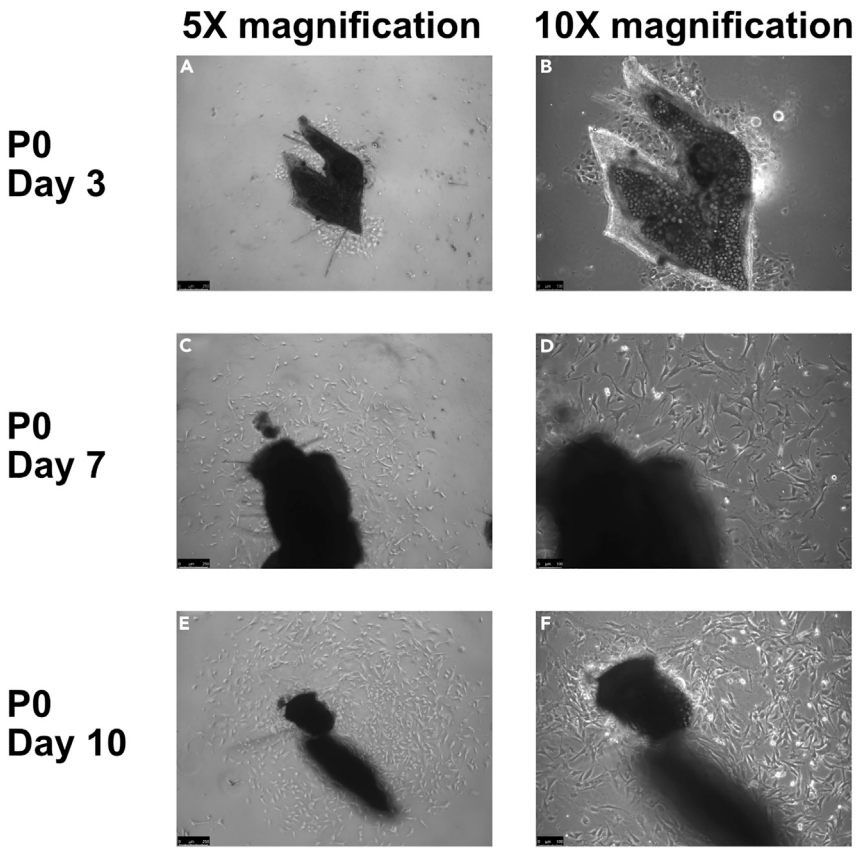

Figure 3. Primary fibroblast extraction over 10 days

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) 5× and (B, D, and F) 10× magnification on the left and right images, respectively. Scale bar, 100 μm or 250 μm respectively.

⚠ 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).

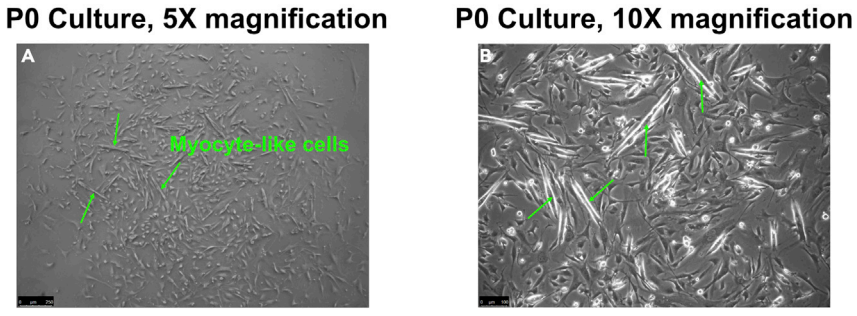

Figure 4. "Myocyte-like" cells co-extract with primary fibroblasts

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) 5× and (B) 10× 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% CO_2.
    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.

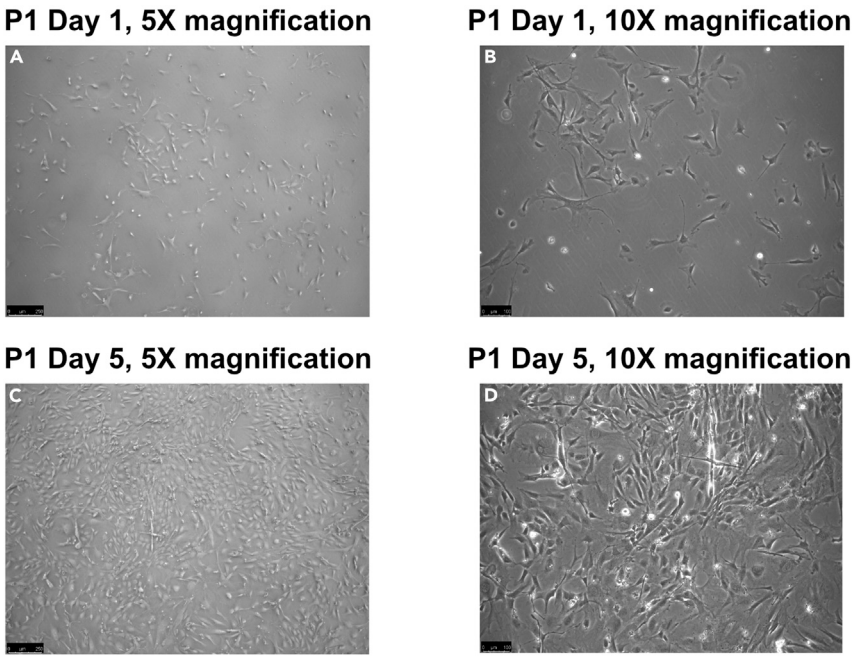

Figure 5. Primary fibroblasts after Passage 1

Primary fibroblasts at one day after Passage 1, visualized at (A) 5× and (B) 10× magnification, and at five days after Passage 1, visualized at (C) 5× and (D) 10× magnification. Note that the cell strainer has removed all tissue fragments previously present in the media. Also note that cells are ready to be passaged a second time within a few days after the first passage. Scale bar, 100 μm or 250 μm respectively.

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).

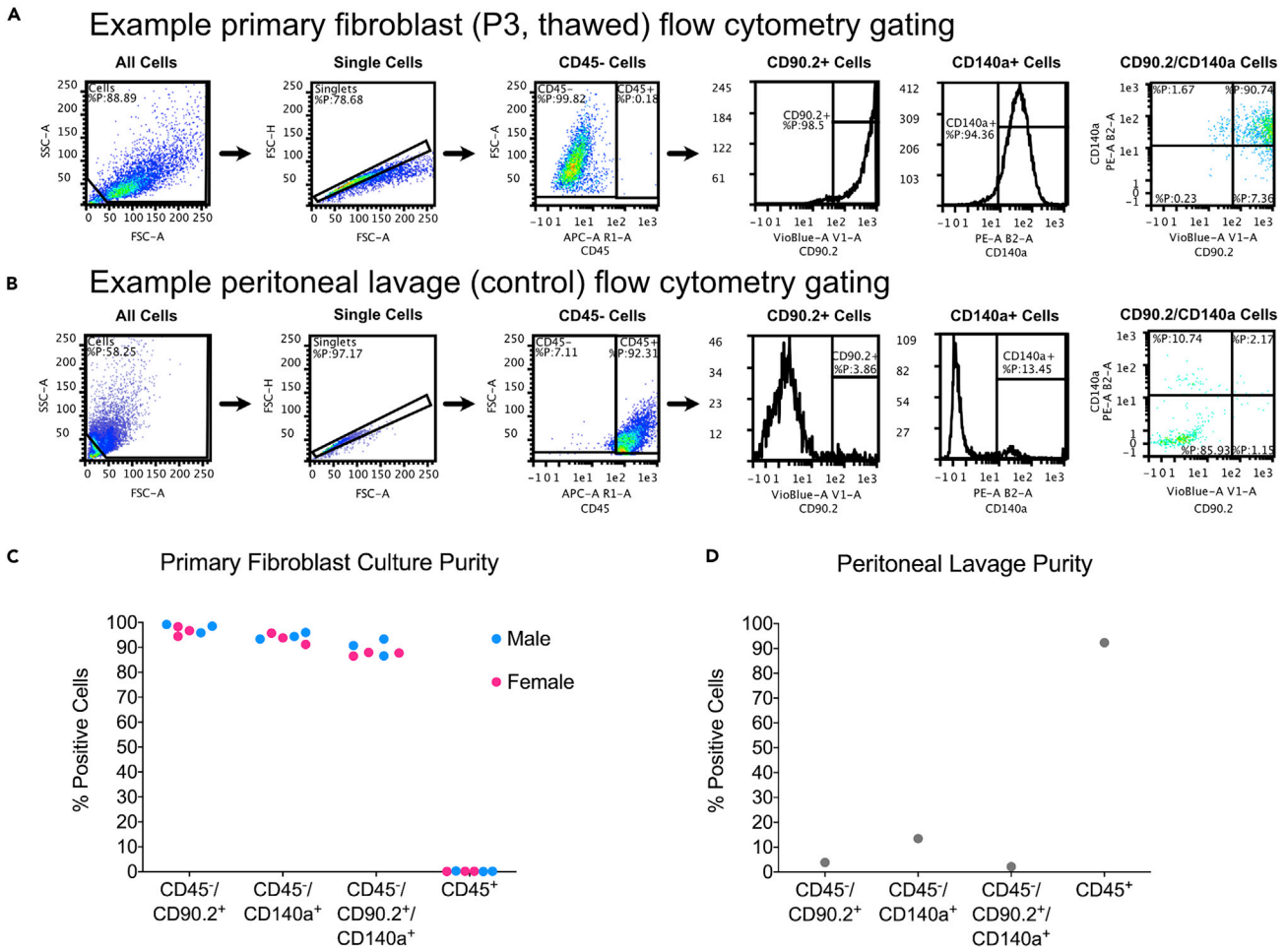

Figure 6. Flow cytometry analysis of the identity of post-Passage 3 cultures

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)

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 correlates 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.

Table 1. Antibody dilutions for fibroblast and immune cell markers

Antibody	Stock conc.	Dilution factor for a 2× solution	Final dilution factor	Final conc.
Pacific Blue anti-mouse CD90.2	0.5 mg/mL	1:25	1:50	10 μg/mL
PE anti-mouse CD140a	0.2 mg/mL	1:40	1:80	2.5 μg/mL
APC anti-mouse CD45	0.2 mg/mL	1:40	1:80	2.5 μg/mL

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% CO_2.
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 μL 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 μL of a 2× antibody solution in flow cytometry staining buffer (Table 1).

43. Add the 50 μL of 2× antibody solution to the 50 μL 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 μL of resuspension buffer per 100,000 cells.

Optional: Add 1 μL of propidium iodide (PI) per 100 μL 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 $1.5–2.5*10^6$ 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 $CD45^-$ $CD90.2^+$ also 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

Table 2. Comparison of select fibroblast isolation protocols

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 >90% $CD90.2^+$ cells <0.5% $CD45^+$ 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

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 samples 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

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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