Journal of Visualized Experiments

Rapid generation of primary murine melanocyte and fibroblast cultures

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59468R1
Full Title:	Rapid generation of primary murine melanocyte and fibroblast cultures
Keywords:	melanocyte; Mouse; skin; isolation; Cell Culture; fibroblast
Corresponding Author:	Christin Elizabeth Burd Ohio State University Columbus, Ohio UNITED STATES
Corresponding Author's Institution:	Ohio State University
Corresponding Author E-Mail:	burd.25@osu.edu
Order of Authors:	Brandon Michael Murphy
	Tirzah J. Weiss
	Christin Elizabeth Burd
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Columbus, OH, USA

Cover Letter

THE OHIO STATE UNIVERSITY
WEXNER MEDICAL CENTER

Christin E. Burd, PhD
Assistant Professor
Molecular Genetics
Cancer Biology and Genetics
The Ohio State University
James Comprehensive Cancer Center

918 Biological Research Tower
460 West 12th Avenue
Columbus, OH 43210-1240
Phone: (614) 688-7569
E-mail: burd.25@osu.edu

Dr. Bing Wu
Review Editor, *Journal of Visualized Experiments*

Re: JoVE559468
Originally submitted as: "*Rapid generation of murine melanocyte cultures"*

March 21st, 2019

Dr. Wu,

We appreciate the opportunity to submit our revised manuscript, now titled "***Rapid generation of primary murine melanocyte and fibroblast cultures****"*, for publication as an article in *JoVE*. This work is original and not under consideration elsewhere. The authors have no financial conflicts to disclose.

The reviewers expressed enthusiasm for the clear and "streamlined" nature of our original protocol, stating that it had potential to be of "great benefit to the pigment cell research community". They also made several astute suggestions, which were used to enhance the quality and potential impact of our work. Noteworthy changes to our protocol include:

- Improved isolation and validation methods to ensure the purity of resulting melanocyte cultures.
- Time course experiments to document melanocyte purity throughout the first ten days of culture.
- Confirmation that older pups (p.n. days 0-4) can be used for melanocyte isolation.
- Addition of Table 1, which indicates how reagents should be scaled for high-throughput experiments.
- Updates to the protocol notes to address general questions posed by the reviewers.

During the revision of this protocol we discovered that it was possible to simultaneously derive primary fibroblast cultures from our melanocyte preparations. Recognizing the potential utility of a method allowing for the concurrent isolation of two different skin cell types, we revised our manuscript accordingly. The result is a truly unique protocol that enables the consistent and simultaneous generation of primary melanocyte and fibroblast cultures from a single mouse. Moreover, by decreasing the technical savvy and time required for primary cell isolation, this protocol can be easily scaled for high-throughput experiments. We are confident that these advantages will be of interest to a wide variety of researchers studying skin biology and appreciate your consideration of this article for publication in *JoVE*.

Sincerely,

Christin E. Burd, Ph.D.

TITLE:

Rapid Generation of Primary Murine Melanocyte and Fibroblast Cultures

AUTHORS AND AFFILIATIONS:

Brandon M. Murphy[1], Tirzah J. Weiss[1] and Christin E. Burd[1,2]

[1]Department of Cancer Biology and Genetics, The Ohio State University, Columbus, OH, USA

[2]Department of Molecular Genetics, The Ohio State University, Columbus, OH, USA

Corresponding Author:

Christin E. Burd

Email: burd.25@osu.edu

Email Addresses of Co-authors:

Brandon M. Murphy (Brandon.Murphy@osumc.edu)

Tirzah J. Weiss (Tirzah.Weiss@osumc.edu)

KEYWORDS:

melanocyte, fibroblast, mouse, skin, isolation, cell culture

SUMMARY:

This protocol outlines a rapid method to simultaneously generate melanocyte and fibroblast cultures from the skin of 0-4 day old mice. These primary cultures can be maintained and manipulated in vitro to study a variety of physiologically relevant processes, including skin cell biology, pigmentation, wound healing and melanoma.

ABSTRACT:

Defects in fibroblast or melanocyte function are associated with skin diseases, including poor barrier function, defective wound healing, pigmentation defects and cancer. Vital to the understanding and amelioration of these diseases are experiments in primary fibroblast and melanocyte cultures. Nevertheless, current protocols for melanocyte isolation require that the epidermal and dermal layers of the skin are trypsinized and manually disassociated. This process is time consuming, technically challenging and contributes to inconsistent yields. Furthermore, methods to simultaneously generate pure fibroblast cultures from the same tissue sample are not readily available. Here, we describe an improved protocol for isolating melanocytes and fibroblasts from the skin of mice on postnatal days 0-4. In this protocol, whole skin is mechanically homogenized using a tissue chopper and then briefly digested with collagenase and trypsin. Cell populations are then isolated through selective plating followed by G418 treatment. This procedure results in consistent melanocyte and fibroblast yields from a single mouse in less than 90 min. This protocol is also easily scalable, allowing researchers to process large cohorts of animals without a significant increase in hands-on time. We show through flow cytometric assessments that cultures established using this protocol are highly enriched for melanocytes or fibroblasts.

INTRODUCTION:

Mammalian skin is a multilayered organ that protects the body from foreign pathogens and ultra violet irradiation (UVR). The skin also plays a critical role in homeostatic processes such as wound healing, temperature regulation and vitamin D production[1-3]. Mammalian skin consists of three major cell types: melanocytes, fibroblasts and keratinocytes. These cell types populate different layers of the skin, with keratinocytes making up the epidermis, fibroblasts residing in the dermis and melanocytes localizing to the epidermal-dermal junction and hair follicles[4]. Here, we detail a simple procedure that enables the concurrent generation of primary melanocyte and fibroblast cultures from murine skin.

Melanocytes are pigment-producing cells found in many locations throughout the human body, including the basal epidermis, iris, cochlea, brain and hair follicles[5]. The primary function of melanocytes is to generate and secrete melanin-containing vesicles called melanosomes[5,6]. Melanosomes contain two major classes of melanin: brown/black eumelanin and yellow/red pheomelanin[6,7]. Biochemical processes within the melanocyte regulate the relative abundance of each melanin species and help to determine hair, skin and eye color[8,9]. Melanin also serves to absorb UVR and protect sun-exposed tissues from mutagenesis[10].

Melanocyte dysfunction can cause pigmentary defects and increase skin cancer susceptibility. For example, the hyper-pigmented skin patches characteristic of melasma are the result of focal melanin overproduction, whereas germline genetic mutations which compromise genes involved in melanin synthesis lead to albinism[11,12]. Intimate knowledge of melanocyte biology is required to develop strategies that will correct such pigmentary defects and ultimately improve the psychosocial well-being of individuals afflicted with these diseases. Deficits in melanin production and/or the preferential synthesis of pheomelanin are also associated with increased skin cancer risk[10]. This risk is believed to result from reduced UVR protection[6,13]. Thus, methods to enhance or restore eumelanin production in melanocytes may reduce the incidence of skin cancer in these populations.

Mesenchymal fibroblasts establish the connective tissue and structural support for all organs of the body, including the dermal layer of the skin[14]. Excretion of proteins such as collagen, elastin, laminin and fibronectin enable fibroblasts to form the extracellular matrix (ECM) that is essential for tissue integrity[1,14]. Fibroblasts also play essential roles in processes such as wound healing, inflammation, angiogenesis and cancer formation/progression[1,15,16].

Similar to melanocytes, defects in fibroblast activation and function can promote tumorigenesis and disease. For example, inappropriate fibroblast activation commonly leads to the formation of fibrosis, resulting from the enhanced deposition of excess ECM components into the surrounding tissue. As fibroblasts maintain much of the body's structural integrity, fibrosis promotes diseases that affect numerous tissues and organs, including idiopathic pulmonary fibrosis, systemic sclerosis, liver cirrhosis and cardiac fibrosis[15]. Fibroblasts also play a critical role in cancer[16]. Cancer associated fibroblasts (CAFs) are the most abundant non-malignant cells in the microenvironment of many tumors. CAFs have been shown to promote tumor proliferation, progression and therapeutic resistance by modulating tissue stiffness, local cytokine production and immune function[16].

Primary cell cultures provide researchers with genetically tractable models to identify and mitigate melanocyte and fibroblast defects that lead to disease. However, current methods to establish melanocyte cultures are time consuming and technically challenging. The need for trypsinization and delicate separation of the epidermis and dermis contributes to variability in experimental yield and makes it difficult to perform large-scale experiments. Furthermore, protocols to simultaneously isolate melanocytes and fibroblasts from whole skin are currently lacking in the field.

We have developed a method to reduce the processing steps, variability and time required to establish melanocyte and fibroblast cultures from the same whole skin sample. Using a mechanical homogenization method followed by a brief digestion, our strategy significantly decreases the amount of hands-on time required to isolate primary melanocytes while enabling concurrent fibroblast isolation. The increased speed, efficiency and consistency of this protocol, in combination with the ability to isolate melanocytes from 0-4 day old mice, provides researchers the flexibility to perform a wider array of experiments than previously possible.

PROTOCOL:

Obtain approval from your institutional animal ethics committee before commencing this or any other study involving animals. Experiments performed in this protocol were approved by the Ohio State University's Institutional Animal Care and Use Committee (IACUC, protocol #2012A00000134).

1. Protocol preparation

NOTE: The following reagent preparation instructions are appropriate for the generation of 9 cm^2 melanocyte and fibroblast cultures *from a single mouse*. Refer to the reagent preparation guide in **Table 1** for larger scale isolations.

1.1 Prepare 1.5 mL of Collagen Solution containing 50 µg/mL collagen in 0.1 M glacial acetic acid.

1.2 In a laminar flow cabinet, coat one well of a 6-well cell culture dish with 8 µg/cm^2 (1.44 mL) Collagen Solution. Ensure that the well is completely covered by the Collagen Solution. Incubate the dish at 37 °C for 3 h or at 4 °C overnight. Prior to use, wash the collagen-coated well twice with 1.35 mL of sterile phosphate buffered saline (PBS) (150 µL of PBS per 1 cm^2).

1.3 Assemble the tissue chopper in a laminar flow cabinet by carefully placing a chopping disk on the tissue chopper platform and securing a sterile blade to the movable arm.

1.4 Prepare 3 mL of 1x Antibiotic/Antimycotic Solution by diluting 100x antibiotic/antimycotic stock solution in sterile PBS.

1.5 Prepare 3 mL of fresh Skin Digestion Buffer containing 10% fetal bovine serum, 1%

penicillin/streptomycin solution, 1% L-glutamine, 10 mg/mL collagenase type I, 0.25% porcine trypsin and 0.02 mg/mL deoxyribonuclease I in RPMI 1640.

1.6 Prepare 6 mL of fresh Melanocyte Media containing 10% fetal bovine serum, 7% horse serum, 1% penicillin/streptomycin solution, 1% L-glutamine, 0.5 mM di-butyryl cyclic AMP (dbcAMP), 20 nM tetradecanoylphorbol 13-acetate (TPA) and 200 pM cholera toxin (CT) in Nutrient Mix F-12 Ham's media.

NOTE: Concentrated dbcAMP, TPA and CT stock solutions can be made, aliquoted and stored for > 1 year at -80 °C. Base media lacking these components can be stored for up to 1 month at 4 °C.

1.7 Prepare 4 mL of Fibroblast Media containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine in Dulbecco's Modified Eagle Medium. This media can be stored at 4 °C for up to 1 month.

1.8 Prepare 40 mL of 4% Paraformaldehyde Solution by diluting 16% paraformaldehyde in PBS. Store any excess at 4 °C for 1 month or -20 °C for up to 1 year.

1.9 Prepare a 1% saponin stock solution by mixing 0.5 g of saponin in 50 mL of PBS at 37 °C until the saponin has completely dissolved. Sterilize the solution for long-term storage using a 50 mL syringe fitted with a 0.2 µm PES filter. The resulting saponin stock solution can be kept at 4 °C for 1 month.

1.10 Prepare 200 µL of 0.1% Saponin Solution per mouse by diluting 1% saponin stock solution in PBS containing 3% bovine serum albumin (BSA).

1.11 Prepare 1 mL of Viability Solution by diluting 1 µL of fixable viability dye in 1 mL of PBS.

2. Melanocyte and fibroblast isolation

2.1 Euthanize 0 to 4 day-old male and/or female C57Bl/6J pups by decapitation and remove extremities from the euthanized mice using surgical scissors.

2.2 In a laminar flow cabinet, briefly roll the trunk of each mouse in a sterile Petri dish containing 70% ethanol. Remove the trunk from the ethanol and place it into an empty, sterile Petri dish.

2.3 Using surgical scissors, sterilized in 70% ethanol, make an incision in the skin on the ventral side of the trunk starting from the neck to the tail. Peel the skin from the trunk of the mouse using sterile forceps.

2.4 Place the skin dermis side down in a 6-well dish containing 3 mL of 1 x Antibiotic/ Antimycotic Solution and incubate at room temperature for 2-3 min.

2.5 Turn on the tissue chopper with the following settings in place: Slice thickness: 1 µm; Blade force: ~60% of the maximum; Speed: ~50% of the maximum.

2.6 Transfer the skin, dermis side down, to a sterile tissue chopper disk and pass the skin completely through the activated tissue chopper 3 times.

2.7 Transfer the homogenized skin to a sterile, 15 mL conical tube containing 3 mL of Skin Digestion Buffer. Mix the resulting suspension by pipetting up and down 10-15 times with a P1000 micropipette.

2.8 Cap the conical tube and incubate the sample in a 37 °C water bath for 15 min, inverting the tube every 3-5 min.

2.9 Pellet the cells in the skin homogenate by centrifuging the conical tube in a swinging bucket rotor at 750 *x g* for 5 min at room temperature.

2.10 Using a P1000 micropipette, slowly and completely remove the Skin Digestion Buffer being careful not to disturb the pellet.

NOTE: A portion of whole skin can be saved at this stage and used as a control for Step 3: Confirmation of Cellular Purity. Strain these cells through a 70 µm cell strainer and then begin at step 3.5 for further processing.

2.11 Thoroughly resuspend the cell pellet in 1 mL of Melanocyte Media by pipetting up and down 15-20 times with a P1000 micropipette. Add the resulting cell solution dropwise to an uncoated well of a 6-well dish containing 1 mL of Melanocyte Media.

2.12 Place the plated skin homogenate in a tissue culture incubator set at 37 °C and 5% CO_2. Allow the cultures to incubate for 40 min.

NOTE: During this time, some fibroblasts in the skin homogenate will adhere to the uncoated dish while the melanocytes and keratinocytes remain in suspension.

2.13 Transfer the culture supernatant from the uncoated dish to one well of a pre-washed, collagen-coated 6-well dish. Add G418 to the media such that the final concentration is 100 ng/mL.

2.14 Add 2 mL of Fibroblast Media to one well of the uncoated dish, now containing adherent fibroblasts.

2.15 Incubate both cultures overnight in a tissue culture incubator set at 37 °C and 5% CO_2.

2.16 Separately aspirate the media and any debris from each culture, 16-24 h after plating. Wash each dish twice with 1 mL of sterile PBS, and then add 2 mL of fresh Melanocyte Media

plus 100 ng/mL G418 to the melanocyte culture and 2 mL of fresh Fibroblast Media to the fibroblast culture.

2.17 After the melanocyte cultures have been treated with G418 for 48 h, wash the cells twice with 1 mL of sterile PBS and add 2 mL of fresh Melanocyte Media without G418 to the culture.

NOTE: As fibroblasts in the melanocyte culture continue to die off post-G418 treatment, wash the dish with sterile PBS to remove dead cells and add fresh Melanocyte Media. Melanocyte and fibroblast cultures should be passaged when they reach 70-80% confluency.

3. Confirmation of cellular purity

3.1 Aspirate the media from each culture and carefully wash each dish with 1 mL of sterile PBS.

3.2 Add 0.7 mL of 0.25% trypsin to each culture and incubate the cultures in trypsin at 37 °C and 5% CO_2 for 1 min.

3.3 Dislodge the cells by pipetting the trypsin against the bottom of the dish using a P1000 micropipette.

3.4 Add 0.7 mL of the appropriate media to each trypsinized culture and transfer the cell solution into a 1.5 mL microcentrifuge tube.

3.5 Pellet the cells by centrifugation at 750 *x g* for 2 min at room temperature. Carefully remove and discard the supernatant using a P1000 micropipette.

NOTE: Steps 3.1 – 3.5 can be used to passage melanocyte and fibroblast cultures. The resulting pellet should be resuspended in the appropriate media and placed in a new, uncoated (fibroblasts) or pre-washed, collagen-coated (melanocytes) dish.

3.6 Resuspend the cell pellet in 0.5 mL of ice-cold PBS.

3.7 Enumerate and transfer 0.5 million cells to a pre-chilled 1.5 mL microcentrifuge tube.

3.8 Repeat step 3.5 and then resuspend the cells in 100 µL of Viability Solution. Incubate the cell suspension for 30 min at 4 °C in the dark.

3.9 Repeat steps 3.5 – 3.6 twice.

3.10 Repeat step 3.5, then fix the cells by resuspending the pellet in 100 µL of ice cold 4% Paraformaldehyde Solution. Incubate the suspension for 15 min at room temperature in the dark.

3.11 Repeat step 3.5 twice, each time resuspending the pellet in 0.5 mL of 3% BSA in 1x PBS.

3.12 Repeat step 3.5, then resuspend the cell pellet in 100 µL of 0.1% Saponin Solution. Incubate the suspension for 15 min at room temperature in the dark.

3.13 Repeat step 3.5, then resuspend the cell pellet in 100 µL of 0.1% Saponin Solution containing 0.5 µg of rabbit anti-gp100 antibody, 0.5 µg of rabbit anti-Fibroblast-specific protein 1 (FSP1) antibody and 0.025 µg of mouse anti-Cytokeratin 14 (K14) antibody. Incubate the suspension for 1 h at room temperature in the dark.

NOTE: While the K14 antibody was purchased pre-conjugated to Alexa Fluor 647, the gp100 and FSP1 antibodies were conjugated to CF 555 and CF 488, respectively. The staining of control populations should also commence at this step. Control cell populations should be isolated from culture and processed as described in steps 3.5 – 3.12.

3.14 Repeat step 3.11 twice, to remove any unbound antibody.

3.15 Repeat step 3.5, then resuspend the cell pellet in 200-400 µL of 3% BSA in 1x PBS.

3.16 Pass the cell solution through a 40 µm cell strainer into a 5 mL polystyrene round-bottom tube and analyze the strained cells by flow cytometry.

REPRESENTATIVE RESULTS:

Male and female C57Bl/6J mice were euthanized on postnatal days 0-4 and the truncal skin was subjected to mechanical dissociation as described above. After chopping, the skin formed a viscous slurry lacking any sign of structural tissue. Centrifugation of this slurry resulted in the formation of a large cell pellet at the bottom of the conical tube and a layer of adipose floating on top of the supernatant. This adipose layer was discarded with the supernatant while the remaining cell pellet was resuspended and transferred to an uncoated well of a 6-well dish. The high density of cells caused the media to appear cloudy. However, after 40 min of incubation, larger cell and tissue conglomerates were observed in the media and adherent fibroblasts could be seen with an inverted light microscope (**Figure 1**).

The non-adherent cells from the fibroblast culture were transferred to one well of a collagen-coated 6-well dish in order to isolate melanocytes. Addition of G418 killed any remaining fibroblasts in the homogenate, leaving numerous dead cells floating in the media for the next 5 days (**Figure 2A-C**). After 4-5 days of growth, the plated melanocytes began to take on a stereotypical dendritic phenotype with melanocytic granules (**Figure 2C**). This phenotype persisted after passaging of the culture (**Figure 2D**). While clusters of contaminating keratinocytes were initially observed in the melanocyte cultures (**Figure 2A**), these populations were lost upon subsequent passaging.

Flow cytometric analyses were used to confirm the purity of the resulting primary melanocyte and fibroblast cultures. C5N mouse keratinocytes[17], day 10 primary melanocyte cultures and day 6 primary fibroblast cultures were stained with: anti-gp100 (differentiated melanocytes), anti-

Cytokeratin 14 (K14; keratinocytes) and anti-Fibroblast-specific protein 1 (FSP1; fibroblasts) (**Figure 3**). In these cells, gp100 staining was specific to the primary melanocytes, while FSP1 positivity was only observed in fibroblasts (**Figure 3A,B**). Expression of K14 was limited to C5N keratinocytes (**Figure 3C**). Additional analyses were performed to determine the purity of our melanocyte cultures 1, 2, 3, 4 and 10 days after isolation (**Figure 4**). Combined fibroblast and keratinocyte contamination never exceeded 25% of the total cells in culture (**Figure 4B,C**). Nevertheless, dramatic increases in gp100 were not observed until the fourth day in culture (**Figure 4A**). We attribute this observation to the reduced expression of gp100 in melanocyte precursors (i.e., melanoblasts), many of which appear fully differentiate by day 10 in culture (**Figure 4A**). In summary, these data demonstrate that our rapid isolation protocol simultaneously produces cultures enriched for melanocytes and fibroblasts.

FIGURE AND TABLE LEGENDS:

Table 1: Reagent preparation guide for different cohort sizes.

Figure 1: Representative images of primary murine fibroblast cultures. Shown are 20X images of fibroblasts immediately following isolation (**A**), as well as 24 (**B**) and 48 (**C**) h post-isolation.

Figure 2: Representative images of primary melanocyte cultures. Shown are 20X images of melanocyte cultures 1 (**A**), 2 (**B**) and 4 days (**C**) post-isolation. Contaminating keratinocytes are indicated by the arrow in 'A'. (**D**) Representative image of primary melanocyte cultures after passaging.

Figure 3: Flow cytometric assessments of culture purity. Representative histograms showing gp100 (A; differentiated melanocytes), FSP1 (B; fibroblasts) and K14 (C; keratinocytes) positivity in primary melanocytes (day 10), primary fibroblasts (day 6) and C5N keratinocytes. After gating on live cells, 10,000 events were analyzed from each population. Single color controls were used for compensation and the resulting data was visualized with FlowJo software.

Figure 4: Time course analysis of melanocyte purity. Representative histograms showing melanocyte purity 1-10 days after initial isolation. Samples were stained, analyzed and graphed as described in **Figure 3**. Positive and negative control populations were as follows: gp100—human melanocytes (+), primary murine fibroblasts (-); FSP1—primary murine fibroblasts (+), human melanocytes (-); K14—C5N cells (+), human melanocytes (-). The average positivity and standard deviation for at least three distinct melanocyte cultures is shown in the upper right-hand corner of each graph.

DISCUSSION:

The in vitro culture of primary melanocytes and fibroblasts has led to significant advancements in our understanding of skin biology and disease. This protocol improves upon prior melanocyte isolation methods by reducing the time and technical savvy needed to generate consistent melanocyte cultures while allowing for the simultaneous isolation of skin fibroblasts. A novel, time-saving element of this procedure is that the dermis and epidermis do not need to be

separated. Instead, skin cells are isolated using the consistent chopping force and granularity of a mechanical tissue chopper along with selective media and plating techniques. By reducing processing complexity and hands-on time, this procedure also enables researchers to conduct large-scale experiments efficiently.

We recommend several steps to enhance the yield and consistency using this protocol. First, prior to step 2.4, we advise using curved forceps to scrape away any adipose tissue from the dermal side of the skin. This process will reduce the fat cake formed after centrifugation. Next, proper mechanical dissociation of the skin is critical to the success of this protocol and can be enhanced by rotating the homogenizer disk ~60° after each pass of the blade. Another key step is to ensure that all of the Skin Digest Buffer is removed in step 2.10. Failure to do so will hinder cell adhesion to the dish and significantly decrease yield. Because the pellet is not firmly affixed to the bottom of the 15 mL conical tube in step 2.10, the cells can be easily aspirated if the supernatant is not slowly removed. If thorough removal of the Skin Digestion Buffer is a challenge, we suggest washing the pellet in 2-3 mL of Melanocyte Media and then repeating steps 2.9-2.10 prior to plating. Finally, when resuspending the cell pellet in step 2.11, it is important to pipet vigorously in order to break up any clumps and ensure that the cells are evenly distributed on the surface of the cell culture dish. Debris and dead cells will be observed in the culture during the first few days after isolation. These dead cells may hinder the growth of the culture and should be removed by washing the dish with PBS and then adding fresh media.

This protocol outlines a method to generate primary melanocyte and fibroblast cultures from a single mouse. However, the procedure can be effectively adjusted to accommodate larger cohorts of mice (see **Table 1**). For larger cohorts, combining skin homogenate from 4 – 5 pups results in a 30-40% confluent 10 cm dish of melanocytes within 4 – 5 days of isolation. Several techniques can be used to improve the efficiency of this protocol when working with multiple animals. First, we typically euthanize the animals in sets of four, processing two animals at a time. We have found that homogenized skin from two pups can be combined at steps 2.7 – 2.8 by increasing the volume of Skin Digest Buffer to 6 mL. To save time, we begin isolating skin from the next pair of pups while the first set is undergoing homogenization and digestion. This approach ensures that the cell populations are isolated soon after euthanasia and reduces the processing time required for multiple animals. When working with older mice (i.e., postnatal days 2-4), we have found that passing the skin a fourth time through the active tissue chopper (step 2.6) enhances skin homogenization and cellular yield. Using this technique, we see no age-dependent difference in the melanocyte or fibroblast yield.

This protocol details how to simultaneously generate fibroblast and melanocyte cultures from the same skin sample. We have observed that keratinocytes in the initial melanocyte culture remain adhered to the dish when short-term trypsinization is performed along with the forceful dislodgement of melanocytes (see steps 3.2 – 3.3). While we have not optimized propagation methods for these remaining cells, we hypothesize that continued culture of such adherent populations in keratinocyte media supplemented with 100 ng/mL G418 could result in an enriched cell population.

ACKNOWLEDGMENTS:

The authors thank the Damon Runyon Foundation (Innovation Award #38-16 to C.E.B.) and Pelotonia (B.M.M.) for financial support. We are grateful to C. Haines and C. Wormsbaecher who provided comments to improve the manuscript text. This work benefitted from the Ohio State Comprehensive Cancer Center's Analytical Cytometry Shared Resource which is supported by NIH P30 CA016058.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

REFERENCES:

1 Rittie, L. Cellular mechanisms of skin repair in humans and other mammals. *Journal of Cell Communucation and Signal.* **10** (2), 103-120, doi:10.1007/s12079-016-0330-1 (2016).

2 Romanovsky, A.A. Skin temperature: its role in thermoregulation. *Acta Physiologica (Oxf).* **210** (3), 498-507 (2014).

3 Holick, M.F., Smith, E., & Pincus, S. Skin as the site of vitamin D synthesis and target tissue for 1,25-dihydroxyvitamin D3. Use of calcitriol (1,25-dihydroxyvitamin D3) for treatment of psoriasis. *Archives of Dermatology.* **123** (12), 1677-1683a (1987).

4 Yamaguchi, Y. & Hearing, V.J. Physiological factors that regulate skin pigmentation. *Biofactors.* **35** (2), 193-199, doi:10.1002/biof.29 (2009).

5 Yamaguchi, Y. & Hearing, V.J. Melanocytes and their diseases. *Cold Spring Harbor Perspectives in Medicine.* **4** (5), doi:10.1101/cshperspect.a017046 (2014).

6 Brenner, M. & Hearing, V.J. The protective role of melanin against UV damage in human skin. *Photochemistry and Photobiology.* **84** (3), 539-549, doi:10.1111/j.1751-1097.2007.00226.x (2008).

7 Thody, A.J. *et al.* Pheomelanin as well as eumelanin is present in human epidermis. *The Journal of Investigative Dermatology.* **97** (2), 340-344 (1991).

8 Swope, V.B. & Abdel-Malek, Z.A. MC1R: Front and Center in the Bright Side of Dark Eumelanin and DNA Repair. *International Journal of Molecular Science.* **19** (9), doi:10.3390/ijms19092667 (2018).

9 Barsh, G.S. What controls variation in human skin color? *PLoS biology.* **1** (1), E27, doi:10.1371/journal.pbio.0000027 (2003).

10 Abdel-Malek, Z. *et al.* The melanocortin-1 receptor is a key regulator of human cutaneous pigmentation. *Pigment Cell Research.* **13 Suppl 8**, 156-162 (2000).

11 Gronskov, K., Ek, J., & Brondum-Nielsen, K. Oculocutaneous albinism. *Orphanet Journal of Rare Diseases.* **2**, 43, doi:10.1186/1750-1172-2-43 (2007).

12 Kwon, S.H., Hwang, Y.J., Lee, S.K., & Park, K.C. Heterogeneous Pathology of Melasma and Its Clinical Implications. *International Journal of Molecular Sciences.* **17** (6), doi:10.3390/ijms17060824 (2016).

13 D'Orazio, J., Jarrett, S., Amaro-Ortiz, A., & Scott, T. UV radiation and the skin. *International Journal of Molecular Science.* **14** (6), 12222-12248, doi:10.3390/ijms140612222 (2013).

14 Bonnans, C., Chou, J., & Werb, Z. Remodelling the extracellular matrix in development and disease. *Nature Reviews Molecular Cell Biology.* **15** (12), 786-801, doi:10.1038/nrm3904

(2014).
15 Kendall, R.T. & Feghali-Bostwick, C.A. Fibroblasts in fibrosis: novel roles and mediators. *Frontiers in Pharmacology.* **5**, 123, doi:10.3389/fphar.2014.00123 (2014).
16 Kalluri, R. The biology and function of fibroblasts in cancer. *Nature Reviews Cancer.* **16** (9), 582-598, doi:10.1038/nrc.2016.73 (2016).
17 Dickson, M.A. *et al.* Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Molecular and Cellular Biology.* **20** (4), 1436-1447 (2000).

Figure 1

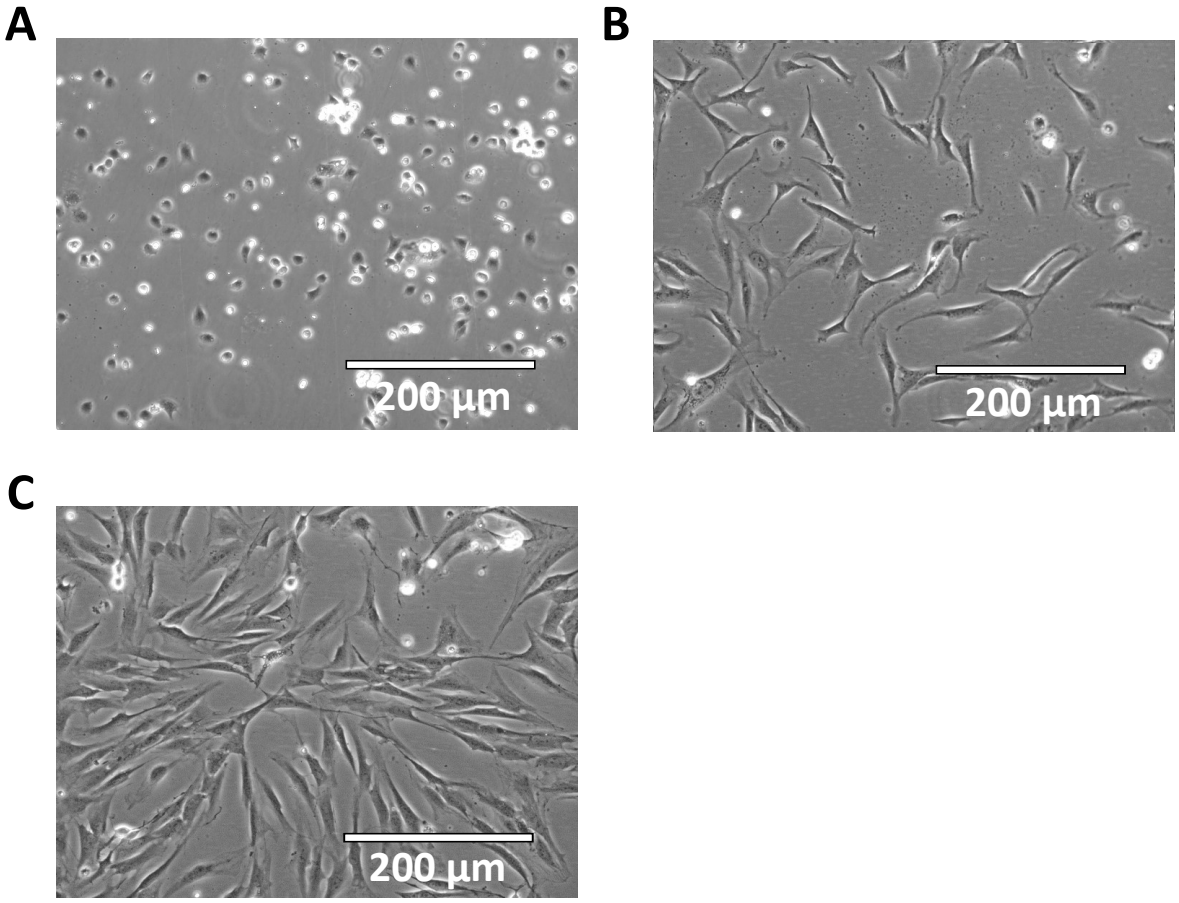


Figure 2

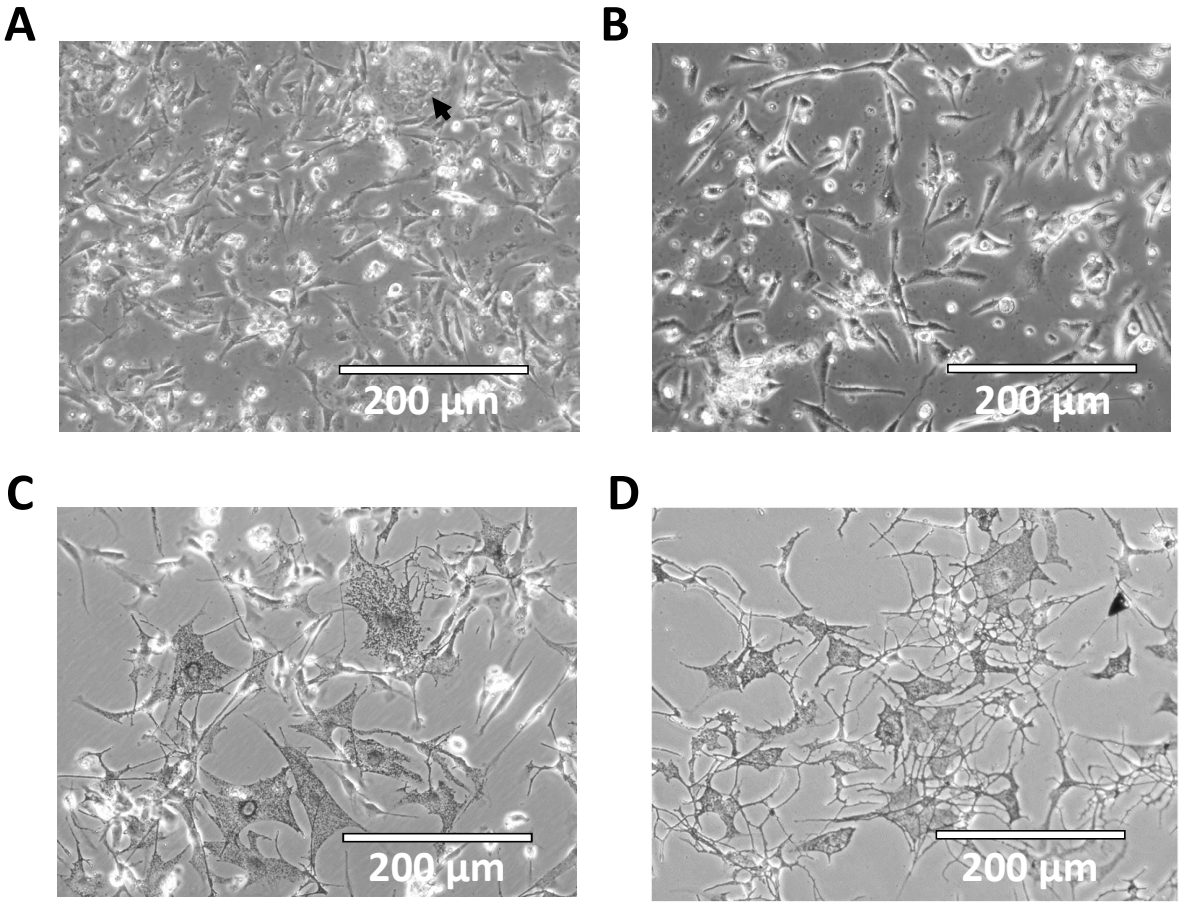


Figure 3

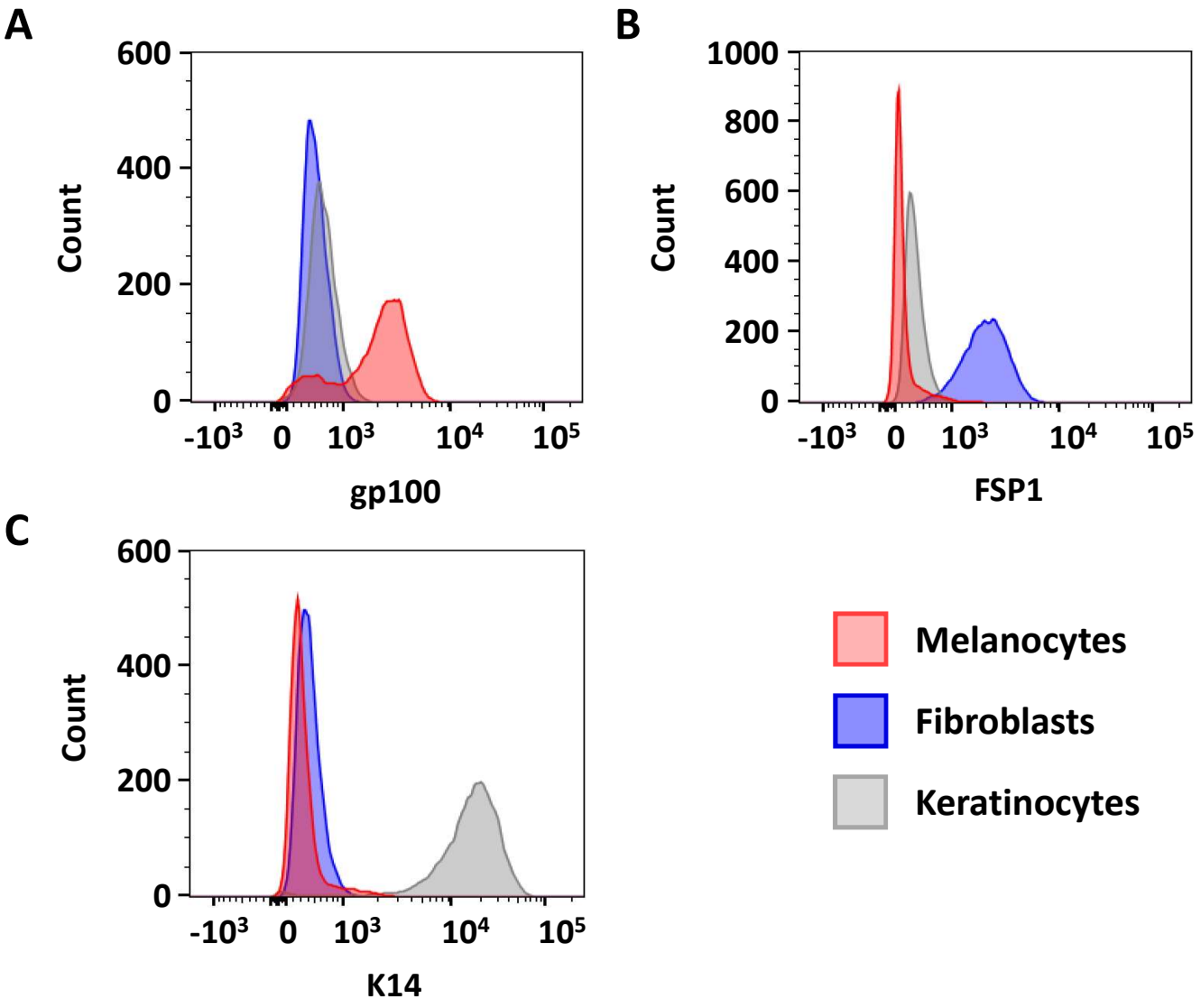


Figure 4

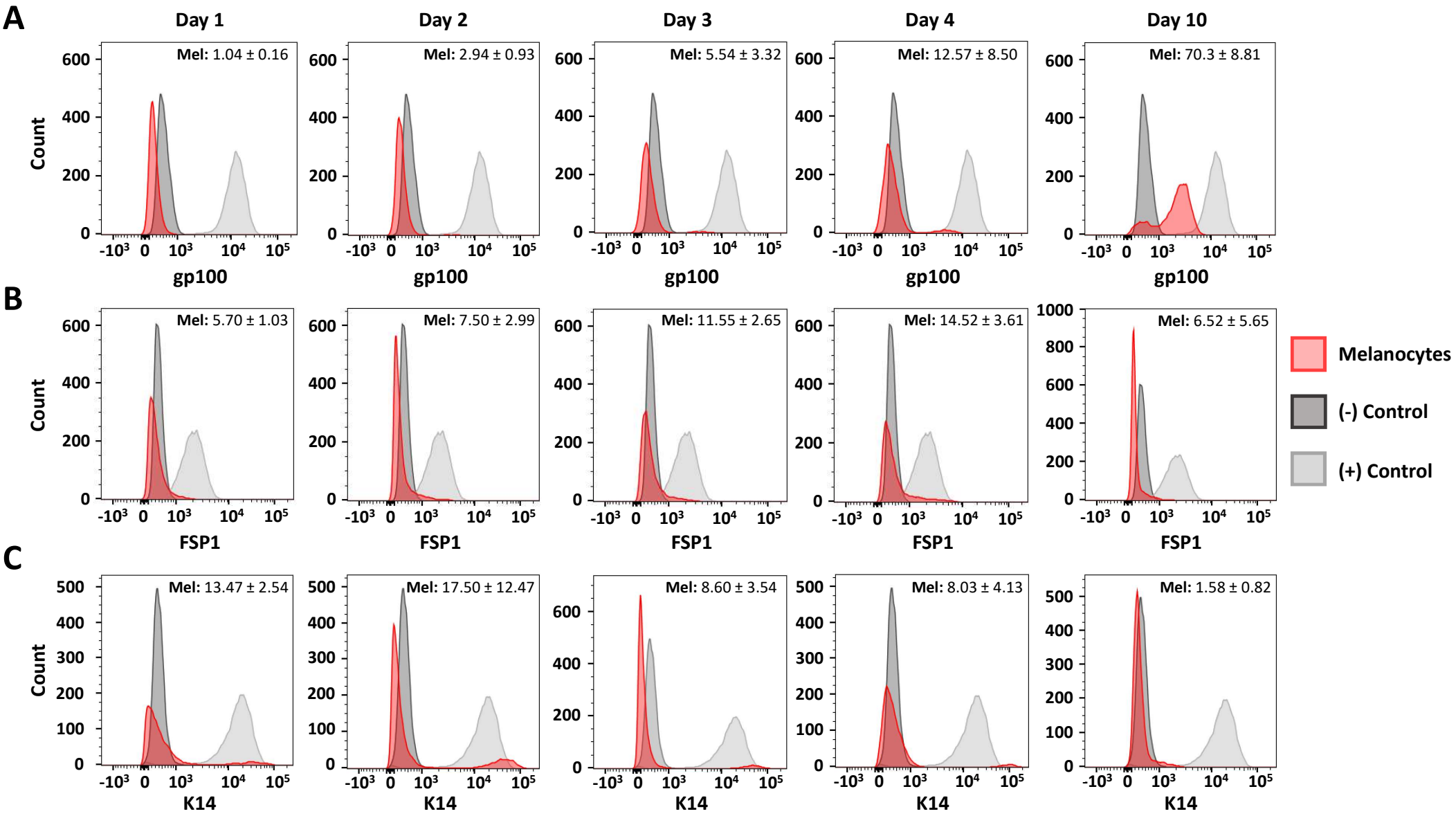


Table of Materials

Click here to access/download;Table of Materials;Murphy et al_JoVE Materials 03.21.2019.xls

Name of Material/ Equipment	Company	Catalog Number
0.2 µm PES sterile syringe filter	VWR	28145-501
10 cm cell culture dish	Corning	430167
40 µm cell strainer	Fisher Scientific	22363547
5 mL polystyrene round-bottom tubes	Fisher Scientific	352008
6-well cell culture dish	Sigma-Aldrich	SIAL0516
70 µm cell strainer	Fisher Scientific	22363548
Antibiotic Antimycotic Solution (100x)	Sigma-Aldrich	A5955
Bovine Serum Albumin	Fisher Scientific	BP9706-100
CF 488A Mix-n-Stain Antibody Labeling Kit	Biotium	92273
CF 555 Mix-n-Stain Antibody Labeling Kit	Biotium	92274
Cholera Toxin	Sigma-Aldrich	C8052
Collagen from rat tail	Sigma-Aldrich	C7661
Collagenase Type I	Worthington Biochemicals	LS004156
Corning Penicillin/Streptomycin Solution	Fisher Scientific	30-002-CL
Cytokeratin 14 Antibody Alexa Fluor 647	Novus Biologicals	NBP2-34403AF647
Deoxyribonuclease I	Worthington Biochemicals	LS002058
Di-butyryl cyclic AMP	Sigma-Aldrich	D0627
Dulbecco's Modified Eagle Medium	Gibco	12800-082
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8537
eBioscience Fixable Viability Dye eFluor 780	Thermo Fisher	65-0865-14
Ethanol, 200 proof	Fisher Scientific	22032601
Fetal Bovine Serum	Sigma-Aldrich	12306C
FSP1/S100A4 antibody	Millipore Sigma	07-2274
G418 Disulfide	P212121	LGB-418-1
Glacial Acetic Acid	VWR	VWRV0714
Horse Serum	Fisher Scientific	26050088
HyClone L-Glutamine	Fisher Scientific	SH3003402
McIlwain Tissue Chopper	Ted Pella	10180
Melanoma gp100 antibody	Abcam	ab137078
Nutrient Mix F-12 Ham's Media	Sigma-Aldrich	N6760
Phorbol 12-Myristate 13-acetate	Sigma-Aldrich	P8139
Pierce 16% Formaldehyde	Thermo Fisher	28908

Porcine Trypsin	Sigma-Aldrich	85450C
RPMI 1640 media	Sigma-Aldrich	R8758
Saponin	Sigma-Aldrich	S-7900
Tissue Chopper Blade	Ted Pella	121-6
Tissue Chopper Plastic Disk	Ted Pella	10180-01
Trypsin	VWR	VWRL0154-0100

Comments/Description

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Rapid generation of murine melanocyte cultures
Author(s):	Brandon M. Murphy, Tirzah J. Weiss and Christin E. Burd

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Christin E. Burd, PhD
Department:	Molecular Genetics, Cancer Biology and Genetics
Institution:	The Ohio State University
Title:	Assistant Professor
Signature:	
Date:	11/30/18

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

We are grateful to the Editor and the Reviewers for taking the time to provide insightful comments that led to improvements in both the quality and potential impact of our manuscript. A point-by-point response to each critique appears below.

Editorial comments:

E Q1. ***Please expand the Summary to briefly describe the applications of this protocol.***
The summary has been revised as requested. See lines 31-33.

E Q2. ***Please remove commercial language: McIlwain, BD LSRFortessa, etc.***
Commercial language has been removed.

E Q3. ***Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.***
An ethics statement is now included on lines 119-122.

E Q4. ***In steps 1.1, 1.8: Please list an approximate volume to prepare.***
Appropriate preparation volumes are now included for all protocol solutions (See Protocol Section 1. Protocol Preparation). In addition, we added a table which indicates how protocol reagents should be scaled for high-throughput experiments (Table 1).

E Q5. ***In step 1.2: How large is the culture dish?***
The size of the culture dish is now included on line 133.

E Q6. ***In step 2.1: Please specify the gender and strain of the pups.***
Mouse gender and strain are now included on line 176.

E Q7. ***Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.***
Protocol steps were combined where appropriate.

E Q8. ***Please include single-line spaces between all paragraphs, headings, steps, etc.***
Single-line spaces now separate all paragraphs, headings and steps.

E Q9. ***After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.***
Appropriate steps for filming are highlighted in the revised protocol.

E Q10. ***Lines 221-229: Please remove these lines from the manuscript. They are not necessary.***
These lines, referencing an attached Table of Materials, have been removed.

E Q11. ***References: Please do not abbreviate journal titles.***
All journal titles are now provided in full.

E Q12. ***Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.***
Materials in the Table are now listed in alphabetical order.

Reviewer #1:

R1 Q1. ***(It) might be good to provide guidance in the protocol about how much volume of the collagen solution to make (per animal equivalent).***

This was a great suggestion. We have included a new table (**Table 1**) to clearly indicate the reagent volumes required for large-scale preparations.

R1 Q2. ***Is there any need to swirl plates occasionally during the incubation?***

We have found that swirling is unnecessary as long as the collagen solution completely covers the dish during the entire incubation period. We now emphasize that the dish must remain completely covered throughout the coating procedure on lines 133-137.

R1 Q3. ***Are there any alternatives to the "McIlwain Tissue Chopper"? Seems like manually chopping the skin tissue with a straight-edged razor blade would be an acceptable substitute. This might be nice to comment on so that investigators would not necessarily have to purchase a MTC.***

We have looked into the availability of other tissue choppers/homogenizers and unfortunately there are few commercial alternatives to the "McIlwain Tissue Chopper". In our experience, the strong chopping force and 1 µm sectioning size provided by the tissue chopper are critical for obtaining consistent melanocyte yields. We have found that manual chopping is unable to provide the same consistency, force and granularity of homogenization as the mechanical chopper. Nevertheless, we have removed all commercial language from the protocol as similar choppers have been available in the past.

R1 Q4. How long in advance can the digestion buffer be made? Can it be frozen into aliquots or need it be made fresh each time?

We have only prepared the digest buffer immediately before melanocytes isolation and have not attempted to freeze aliquots of digest buffer.

R1 Q5. How long is the melanocyte growth media good for at 4 degrees? Can it be frozen into aliquots?

A comparable melanocyte medium is used by the Bennett lab (Godwin et al, *Current Protocols in Cell Biology*, 2014; PMID: 24894835). We now include protocol notes based upon their storage suggestions (lines 153-155). Specifically, complete growth medium should be made fresh. However, base medium containing serum, antibiotics and L-glutamine can be stored at 4 °C for up to one month. If supplemental mitogen stocks are frozen at -20 °C these can be thawed and added to make fresh, complete growth medium.

R1 Q6. ***Do pups need to be harvested on their day of birth? Other isolation protocols call for pups 0-3 days old.***

Initial attempts to isolate melanocytes from mice older than postnatal day 0 were performed during the early stages of protocol development. Therefore, we revisited this question using our new dual (fibroblast & melanocyte) isolation method. We found that melanocyte cultures can be effectively generated from mice up to 4 days of age. Fibroblast cultures can also be isolated up until postnatal day 4. Our revised manuscript has been updated to include these findings (See Protocol Section 2 and lines 402-405 of the Discussion).

R1 Q7. ***Can the pup carcasses be sterilized before extremity amputation (as an alternative to after amputation)?***

We do not have any reason to believe that sterilization of the carcass before extremity amputation would affect the protocol. We typically use the same scissors for euthanasia and extremity amputation, therefore we like to sterilize the mice after these procedures in order to avoid contamination of our cell culture hood.

R1 Q8. ***(The protocol details) very specific instructions to this one kind of tissue chopper. Wonder if the protocol can incorporate more "generalizable" instructions (such as "chop the tissue at least ___ times until skin pieces are roughly ____ sized").***

We have made the protocol instructions more generalized, but we are not aware of any current alternatives that provide the same precision, force and granularity. Please refer to response R1 Q3 for more details.

R1 Q9. ***(The) degree of melanocyte purity (and fibroblast/keratinocyte percentages) should be indicated at various time points after collection; their method by flow is fine, but the percentages should be included in the manuscript. Certainly in our experience, even though epidermal suspensions are grown in melanocyte-selective media similar to the one listed in this protocol, there seems to always be some degree of co-culture with other types of skin cells (mainly dermal fibroblasts, even long-term after several weeks in culture).***

This point is now addressed in Figure 4 and on lines 328-329 of the results. Specifically, we performed flow cytometry to quantify the melanocyte, keratinocyte and fibroblast content of our cultures 1, 2, 3, 4 and 10 days post-isolation. We found a low amount of fibroblast and keratinocyte contamination in our early melanocyte cultures (<25% combined). However, levels of these contaminating cells decreased over time and were lost completely upon passaging.

R1 Q10. ***It is not clear why this method is not successful in generating melancoytes in animals >d0. I would have thought that since this method does not require separation between epidermis and dermis (which limits applicability to pups <3-4 days old because of the challenges of separating epidermis from dermis once hair sprouts through the epidermis), then could it be used in older animals (for example using the skin of ears/tail that maintains interfollicular epidermal melanocytes)?***

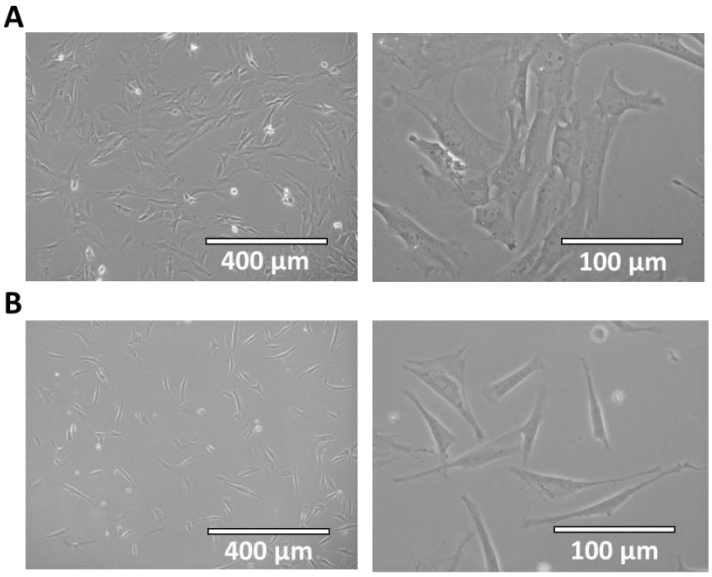
Please refer to comment R1 Q6. Since we were unable to isolate melanocytes from mice older than postnatal day 4 we do not believe our protocol would enable melanocyte isolation from adult mice.

Reviewer #2:

R2 Q1. It is unclear why this method works to purify primary melanocytes away from keratinocytes and fibroblasts. As the authors write in their introduction, current practices require both physical separation of epidermis and dermis in addition to selective culturing practices such as selective media, treatment with G418, or selective disassociation. This protocol uses homogenized full skin and media that would be expected to be permissive to at least fibroblast, if not also keratinocyte, growth. In the discussion, can the authors discuss the theory behind this isolation or speculate as to why it works? Would it be expected to work with other species?

We thank the reviewer for raising these astute concerns which led to important protocol modifications.

We first attempted to address these comments by determining if primary fibroblasts would grow in our original melanocyte culture medium. We found that fibroblasts grew efficiently in this medium, but adopted a more dendritic phenotype reminiscent of melanocytes (See **Response Figure 1**). Seeing this phenotype led to concerns about the specificity and sensitivity of our cell type-specific flow cytometry antibodies. Therefore, we performed



Response Figure 1. Primary fibroblast phenotype is media-dependent. Shown are representative 10x (left) and 40x (right) images of primary fibroblasts cultured in **(A)** 10% DMEM or **(B)** Melanocyte Media.

extensive antibody testing and optimization. After this process, we re-ran our analyses and confirmed that a significant number of fibroblasts were contaminating our original melanocyte cultures.

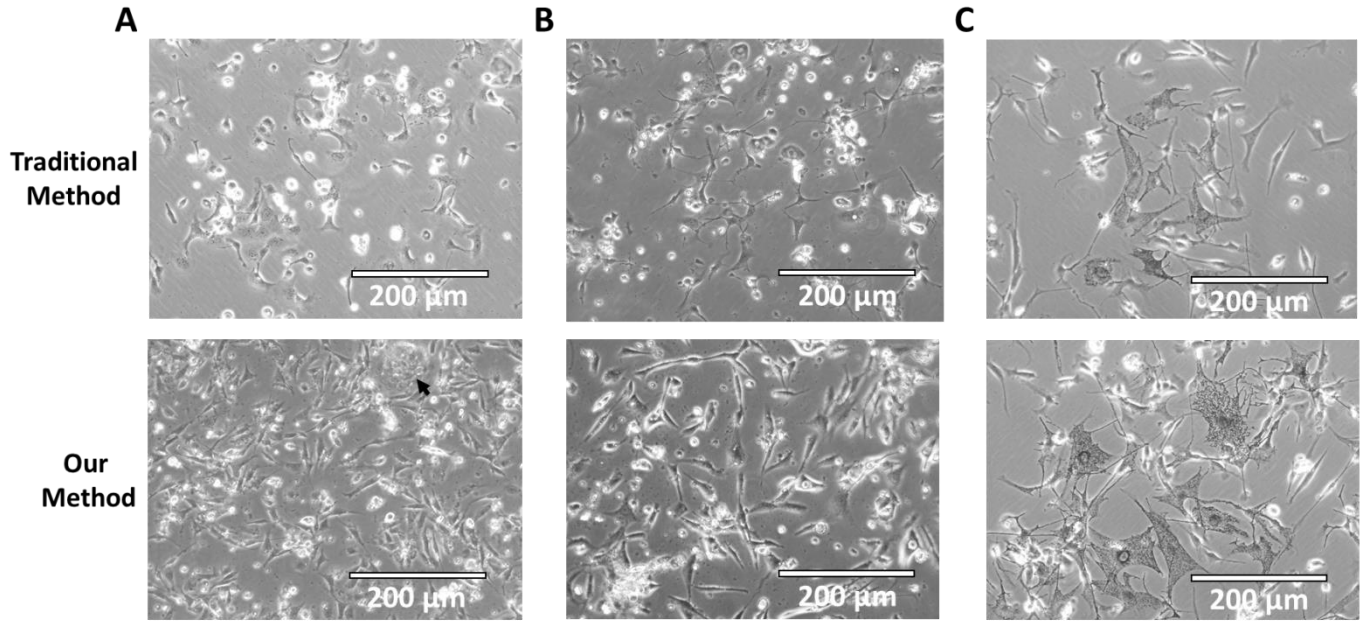
We made several protocol changes to remedy this problem, including selective plating of the skin homogenate and transient G418 treatment (See manuscript **Figures 3 & 4**). At the same time, we discovered that primary fibroblast cultures could be generated as a byproduct of our revised isolation procedure. This ability to produce fibroblast and melanocyte cultures from the same skin sample has become a unique feature of our revised protocol. Although we concede that low-level fibroblast and keratinocyte contamination still remain in our initial melanocyte cultures (<25% combined), we find that these cells are lost during melanocyte propagation (manuscript **Figure 4**).

We have not tried to implement this procedure in other species and do not feel comfortable speculating on its broader utility at this point.

R2 Q2. ***Positive and negative control cell populations are really needed throughout. Assume there will be scientists interested in this protocol with no melanocyte experience. What do pure melanocytes look like when derived with current best practices? What do the potentially contaminating fibroblasts and keratinocytes look like? Showing these images side-by-side with the images in Figure 1 is critical. Using them as controls for the flow cytometry data in Figure 2 would be important as well. Higher magnification images would be appreciated.***

Positive and negative controls are now included in all of our flow cytometry studies (manuscript **Figures 3** and **4**). We have also added new, high magnification images of the resulting fibroblast and melanocyte cultures (manuscript **Figures 1 & 2**). A cluster of contaminating keratinocytes is indicated by an arrow in manuscript **Figure 2A**, but contaminating fibroblasts are more difficult to discern (See **Response Figure 1**).

We find that there is little difference in the phenotype of melanocytes isolated using a traditional protocol versus our new method (See **Response Figure 2**).



Response Figure 2. Comparison of primary melanocyte cultures generated using traditional protocols versus our improved method. Shown are 20x images of primary melanocyte cultures **(A)** 1 day, **(B)** 2 days **(C)** and 4 days post isolation.

R2 Q3. ***Figure 1C, right image shows two patches of keratinocytes (upper right corner and just below the "10x" label). Figure 2B, confirms that the melanocyte population contains keratin expressing cells. It is fine if the melanocyte population is not 100% pure, but the authors should comment on the range and reproducibility of keratinocyte contamination they observe with this protocol.***

Simply highlighting the keratinocytes in the image and using the K10/14 cytometry data to estimate a purity would suffice.

These concerns are now addressed in the Discussion (lines 408-410) as well as manuscript **Figures 2A & 4C**. Quantitative analysis indicates that keratinocytes make up ~15-20% of the initial melanocyte culture and decrease in frequency with passage.

R2 Q4. ***The MITF antibody staining does not appear to have worked. All three cell populations look equally positive. If true, where did the dominant MITF negative population in whole skin go? The authors' K10/14 and FSP1 staining look beautiful and show what these profiles look like when an antibody has been optimized and has no background staining. The authors need to achieve equivalently as convincing profiles with a melanocyte marker. Tyrosinase, DCT, or Sox10 would all be reasonable, among others. As an alternative suggestion, if the authors have access to the mouse lines with Tyr::CreER and an inducible fluorescent reporter, these could be a useful tool for demonstrating the purity of the culture. Either way, the authors need to identify some marker that is specific to the melanocytes in order to provide convincing data on the purity of the cells.***

We were unable to find a flow-compatible MITF antibody that provided sufficient specificity and sensitivity. Therefore, we switched to using a pre-conjugated glycoprotein 100 (gp100) antibody (manuscript **Figures 3A & 4A**). Unfortunately, because gp100 is transmembrane protein involved in melanosome maturation, its expression does not appear to be robust in early melanoblast populations. Nevertheless, strong positivity can be seen in differentiated melanocyte cultures 10 days after plating (manuscript **Figure 4A**).

R2 Q5. ***Can the authors comment on how many cells per unit area are being plated in section 2.14? Assuming that different investigators will experience different efficiencies of cell recovery, it would be useful to know approximately how many cells that authors are plating in this step.***

We quantified skin homogenate following resuspension in step 2.11 and determined that approximately 10.5 million cells were obtained from the skin of a single day 0 mouse. However, as small clusters of cells and tissue were excluded from this quantification we do not feel comfortable stating a specific cell number in the manuscript. We also fear that alternative enumeration methods may be differentially impacted by the lack of sample homogeneity and believe that even the larger pieces of tissue likely contain relevant cells. Thus, we would prefer not to provide enumerated values which could confuse those using the protocol.

R2 Q6. ***The authors mention that this protocol is scalable. A useful addition to the protocol would be a table that lists the steps that need changed and by how much for different desired melanocyte yields*****.**

Please refer to manuscript **Table 1**.

Step #	Reagent	1 pup	5 pups
1.1	Collagen Solution	1.5 mL	4.5 mL
1.2	Plate size	6-well	10 cm
1.4	Antibiotic / Antimycotic Solution	3 mL	15 mL
1.5	Skin Digestion Buffer	3 mL	15 mL
1.6	Melanocyte Media	6 mL	30 mL
1.7	Fibroblast Media	4 mL	20 mL