Journal of Visualized Experiments

Isolation and Characterization of Adult Cardiac Fibroblasts and Myofibroblasts

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60909R2
Full Title:	Isolation and Characterization of Adult Cardiac Fibroblasts and Myofibroblasts
Section/Category:	JoVE Biology
Keywords:	Fibroblast, myofibroblast, magnetic beads, alpha SMA, cardiac injury, fibrosis, flow cytometry, GFP
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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.	Nashville, TN, USA

Title: Isolation and Characterization of Adult Cardiac Fibroblasts and Myofibroblasts

Senior Review Editor, JoVE
November 21st, 2019

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And

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TITLE:

Isolation and Characterization of Adult Cardiac Fibroblasts and Myofibroblasts

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KEYWORDS:

isolation, αSMA, fluorescence activated cell sorting, fibroblasts, myofibroblast, collagen gel, immunofluorescence, MEFSK4

SUMMARY:

Obtaining a pure population of fibroblasts is crucial to studying their role in wound repair and fibrosis. Described here is a detailed method to isolate fibroblasts and myofibroblasts from uninjured and injured mouse hearts followed by characterization of their purity and functionality by immunofluorescence, RTPCR, fluorescence-assisted cell sorting, and collagen gel contraction.

ABSTRACT:

Cardiac fibrosis in response to injury is a physiological response to wound healing. Efforts have been made to study and target fibroblast subtypes that mitigate fibrosis. However, fibroblast research has been hindered due to the lack of universally acceptable fibroblast markers to identify quiescent as well as activated fibroblasts. Fibroblasts are a heterogenous cell population, making them difficult to isolate and characterize. The presented protocol describes three different methods to enrich fibroblasts and myofibroblasts from uninjured and injured mouse hearts. Using a standard and reliable protocol to isolate fibroblasts will enable the study of their roles in homeostasis as well as fibrosis modulation.

INTRODUCTION:

Cardiac fibroblasts, cells of mesenchymal origin, play a significant role in maintaining the electrical conduction and mechanical forces in the heart in addition to the maintenance of cardiac architecture during homeostasis[1]. Following injury, these cells are activated, expand, and produce extracellular matrix (ECM) proteins[2]. Many preclinical studies have revealed fibroblasts as critical cellular regulators that maintain the structural integrity of an injured heart[3] as well as main effector cells responsible for unchecked production and deposition of ECM proteins, resulting in stiff scar formation and heart failure[24]. Fibroblasts are a

heterogenous group of cells, making it challenging to dissect their reparative function from pro-fibrotic maladaptive properties. Recently, the functional heterogeneity of two distinct fibroblast subtypes following myocardial injury have been defined, indicating the possibility of isolating different fibroblast subtypes and studying their role in wound healing[5].

Obtaining a pure fibroblast population is crucial in delineating their functional role in repair and fibrosis. However, the presence of multiple fibroblast markers that recognize other cell types make it challenging to isolate a substantially pure fibroblast population[6]. Several elegant studies have devised clever ways to isolate cardiac fibroblasts from uninjured and injured myocardium. The most popular and well-established method of enriching fibroblasts is through selective adhesion following enzymatic tissue digestion[7].

Additionally, fluorescence-activated cell sorting (FACS) of fibroblasts based on cell surface antigens has been successfully described[8]. In the study, following enzymatic digestion, the mesenchymal cells were sorted as lineage-negative (Lin: $Ter119^{-}CD45^{-}CD31^{-}$) and gp38-positive ($gp38^{+}$) from mouse hearts. $Gp38^{+ve}$ cells were confirmed to be fibroblasts based on their co-expression of col1α1 and other mesenchymal markers. Although most tissue digestion is completed after dissecting out the ventricle in a Petri dish, a recent study has investigated the use of a direct needle enzyme perfusion of the left ventricle to isolate myocytes and non-myocytes which include fibroblasts[9]. Fibroblasts were then isolated by selective adhesion in this case.

This protocol describes the isolation and enrichment of fibroblasts using three methods. The first is an already established method involving selective adhesion of fibroblasts following enzymatic digestion. The second method is used to primarily isolate injury-induced alpha smooth muscle expressing myofibroblasts. The third method involves sequential, magnetic depletion of an enzyme-digested cardiac cell suspension of hematopoietic and endothelial cells. Following depletion, fibroblasts/myofibroblasts are isolated based on the presence of the antigen MEFSK4 using magnetic beads. Recently, MEFSK4 has been described as an antigen present on quiescent as well as activated fibroblasts, making it a suitable marker for fibroblast identification and isolation. Naturally, all the methods described here have unique limitations. It is therefore highly recommended to check the purity of the isolated cell population by flow analysis, immunostaining, and semi-quantitative real-time PCR. However, these methodologies can be expanded upon, and additional markers can be added in order to exclude other contaminating populations prior to utilizing the fibroblast and myofibroblast populations for crucial experiments.

PROTOCOL:

This study strictly upholds the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Vanderbilt University Institutional Animal Care and Use Committee approved the protocol (protocol number: M1600076-01).

1. Heart dissection

1.1. Solution preparation

1.1.1. KHB buffer

1.1.1.1. Using a stir bar, slowly dissolve 9.4 g of Krebs-Henseleit buffer (KHB) powder in 900 mL of DDI water.

NOTE: Buffer will precipitate if stirred too quickly or for too long. KHB buffer must be cold during fibroblast isolation.

1.1.1.2. Add 2.9 mM $CaCl_2$ and 24 mM $NaHCO_3$. Adjust the pH to 7.2–7.3 and dilute to a volume of 1 L with dH_2O.

1.1.1.3. Using a sterile filter (0.22 µm), store at 4 °C for up to 4 weeks. Keep on ice or at 4 °C for duration of isolation.

1.1.2. Collagenase digestion cocktail

1.1.2.1. Prepare digestion cocktail the day of fibroblast isolation. Determine the appropriate volume of digestion cocktail according to the number of hearts; 5 mL of cocktail per 1 heart.

1.1.2.2. Prepare collagenase blend (see **Table of Materials**) and DNase I according to the manufacturer's instructions.

1.1.2.3. For 20 mL of total digestion cocktail, add 17.5 µL of DNase I, 180 µL of 1 M HEPES, and 500 µL of collagenase to an empty 50 mL conical tube.

1.1.2.4. Add a sufficient volume of Hank's Balanced Salt solution (HBSS) with Ca^{2+} and Mg^{2+} to obtain a 20 mL total volume.

1.1.3. Red blood cell (RBC) lysis buffer

1.1.3.1. Determine the total volume needed based on number of hearts (5 mL/heart). Dilute the 10x RBC lysis stock buffer to 1x using dH_2O.

1.1.4. Fibroblast media: 10% FBS in DMEM F-12

1.1.4.1. Add 10% FBS to DMEM-F12 with L-glutamine and HEPES. Add 10 U/mL penicillin/streptomycin, 2.5 µg/mL anti-fungal, and 2.5 µg/mL mycoplasma prophylactic (see **Table of Materials**). Store at 4 °C.

1.2. Heart dissection

1.2.1. Prepare a 6 well plate on ice with 2 mL of cold KHB per well to store hearts in during dissection. Utilize autoclaved surgical scissors and forceps.

1.2.2. Euthanize mice at 12 weeks of age or older by isoflurane overdose, and follow with cervical dislocation.

1.2.3. Alternatively, for activated fibroblast isolation, induce myocardial infarction in 12 week-old mice by coronary artery ligation[10]. Euthanize the mice 8–10 days following injury.

1.2.4. Spray body with 70% ethanol and orient so the ventral side is facing the experimenter. Pin or restrain appendages to prevent interference.

1.2.5. Cut the abdominal skin and muscle open but avoid piercing the liver. Cut vertically towards the sternum, and carefully open the thorax while avoiding piercing of the heart. Continue to cut through the ribcage to expose the heart.

1.2.6. Using forceps, gently lift the heart out of the chest, cutting away any lung or excess tissue attached to the outside of the heart. Remove the ventricle and place in one well of 6 well plate with cold KHB. Continue to dissect hearts in this manner until all samples have been isolated.

1.3. Enzymatic dissociation of heart

1.3.1. Using forceps, repeatedly squeeze and agitate the heart in KHB to remove excess blood. Transfer the heart to a clean sterile 10 cm^2 plate. Using a single edge blade, quickly mince the heart into small pieces.

1.3.2. Add 1 mL of collagenase digestion cocktail and continue mincing until pieces are small enough to transfer with a 1 mL micropipette. Transfer pieces to a 50 mL conical tube with a 1 mL micropipette. Wash plate 2x with 2 mL of collagenase digestion cocktail.

NOTE**:** Cutting off a portion of the pipette tip can help collect larger pieces of heart that may otherwise become stuck in the pipette tip.

1.3.3. Incubate conical tube at 37 °C for 30 min with rocking or agitation. Secure tube as needed. Resuspend 10x with a 5 mL pipet until the contents are homogenous, and incubate the conical tube at 37 °C for 15 min with rotation or agitation.

1.3.4. Resuspend 10x with a 10 mL pipet until homogenous. Prime a 40 µm cell strainer by wetting the filter with 1–2 mL of KHB buffer on top of a new 50 mL conical tube. Add 25 mL of KHB buffer to digestion suspension, resuspend, and filter through a primed 40 µm cell strainer. Change filter as needed.

NOTE: As filtration slows, lightly tapping tube or using a 1 mL micropipette to draw suspension from underside of filter can help the cell suspension pass through a partially blocked 40 µm cell

strainer.

1.3.5. Centrifuge at 400 x *g* and 4 °C for 10 min. Remove the supernatant and resuspend the pellet in 1x RBC lysis buffer (5 mL/heart). Incubate for 2 min at room temperature (RT).

1.3.6. Centrifuge at 400 x *g* and RT for 10 min. Remove supernatant then wash by resuspending pellet in 1 mL of KHB buffer.

1.3.7. Add 9 mL of KHB buffer and filter through primed 40 µm cell strainer into new 50 mL conical tube. Centrifuge at 400 x *g* and RT for 10 min.

1.3.8. Remove the supernatant, resuspend in 1 mL of fibroblast media or PBS, and determine the cell number. Fibroblasts can be isolated by the three different methods described below.

2. Isolation of fibroblasts from single cell suspension

2.1. Fibroblast isolation: differential plating (Figure 1)

2.1.1. Prepare a 6-well plate by adding 2 mL of fibroblast media per well and swirling the plate to cover the well bottom.

2.1.2. Resuspend cells in 1 mL of media per heart. Plate 1 mL of cell suspension per well such that one heart (theoretically) is being plated per well. For example, six hearts would be resuspended in 6 mL of media, then plated into six wells with 1 mL of cell suspension per well.

2.1.3. Swirl plate to evenly distribute cells. Add an additional 1–2 mL media per well for a total volume of up to 5 mL per well, and incubate at 37 °C for 4 h.

2.1.4. Fibroblasts will be separated by selective adhesion after 4 h. Remove unattached and dead cells by removing media. Wash attached cells with 2 mL of PBS and add 2–4 mL sterile fibroblast media per well. Incubate at 37 °C until confluent. Change media every 2–4 days.

2.2. Fibroblast Isolation: FACS using a GFP reporter mouse model (Figure 1)

2.2.1. FACS buffer

2.2.1.1. Prepare 15 mL of 5% fetal bovine serum (FBS) in dPBS without Ca^{2+} and Mg^{2+}. Store on ice or at 4°C.

2.2.2. FC blocker solution

2.2.2.1. Prepare 0.5 µL FC blocker (purified anti-mouse CD16/CD32) in 25 µL of FACS (1:50 dilution). 25 µL of FC blocker solution is required per sample. Store on ice or at 4 °C.

2.2.3. Sample preparation and antibody staining

NOTE: Antibody dilutions can be prepared in advance, but this may risk light or temperature exposure.

2.2.3.1. Prepare 7AAD or Ghost dye violet 510 that is 2x the required dilution in FACS buffer. See **Table 1** for antibodies and dilutions.

2.2.3.2. Resuspend 500,000 freshly isolated cells in 25 µL of FC blocker solution to prevent nonspecific binding of the FC antibody region to an FC receptor. Incubate for 5 min at RT.

2.2.3.3. Add 7AAD or Ghost dye violet 510 antibody to the final volume of 25 µL of cell suspension. Incubate for 30–60 min on ice in the dark.

2.2.3.4. Wash by resuspending in 1 mL of FACS buffer. Centrifuge at 500 x *g* for 5 min at 4°C.

2.2.3.5. Resuspend pellet in recommended flow cytometry sample volume of FACS buffer (300 µL) and transfer to flow cytometry tube.

2.2.4. Fluorescence-activated cell sorting (FACS)

NOTE: GFP-αSMA reporter mice were a contribution from Dr. Ivo Kalajzic[11]. This mouse expresses GFP under the alpha smooth muscle cell (αSMA) promoter. αSMA has been used as a marker of activated fibroblasts (myofibroblasts) [12].

2.2.4.1. For activated fibroblast (αSMA expressing myofibroblast) isolation, induce myocardial infarction in 12 week-old GFP-αSMA mice by coronary artery ligation. Sacrifice the mice 8–10 days following injury.

2.2.4.2. Following single cell suspension from the injured mice hearts, sort αSMA^{+ve} fibroblasts for green fluorescent protein (GFP). Use unstained uninjured fibroblasts to set the background signal in the GFP channel post-compensation.

2.2.4.3. Gate for live GFP^{+ve} cells by gating for $7AAD^{-ve}/GFP^{+ve}$ cells or Ghost dye violet $510^{-ve}/GFP^{+ve}$ cells and sort GFP expressing αSMA^{+ve} myofibroblasts. Collect the cells in fibroblast media.

2.3. Fibroblast isolation: magnetic bead-based isolation of fibroblasts (Figure 1)

2.3.1. Equilibration buffer

NOTE: Always prepare fresh buffer for isolations.

2.3.1.1. Prepare 0.5% BSA and 2 mM EDTA in PBS. Degas the buffer by stirring the solution

while attaching a vacuum to the lid of the container.

NOTE: Stirring removes excess gas from the solution that is then removed through the vacuum such that bubbles will not clog the separation column upon usage.

2.3.2. Magnetic labeling: $CD45^+$ hematopoietic cells

2.3.2.1. Centrifuge isolated cells from mice hearts at 500 x *g* for 5 min, then remove the supernatant.

2.3.2.2. Resuspend the cell pellet in 1 mL of equilibration buffer. Count the cells using a hemocytometer.

2.3.2.3. Centrifuge the cell suspension as above and resuspend cell pellet in 90 µL equilibration buffer per 1 x 10^7 total cells. Add 10 µL of $CD45^+$ magnetic beads per 1 x 10^7 total cells. Mix well and incubate for at least 15 min at 4°C.

2.3.2.4. Wash cells by adding 2 mL equilibration buffer per 1 x 10^7 total cells, then centrifuge at 500 × *g* for 10 min at 4°C.

2.3.2.5. Remove supernatant, count the cells using a hemocytometer and resuspend up to 1 x 10^7 total cells in 2 mL of equilibration buffer. If more than 10^7 cells are present, scale the buffer linearly. Pass cells through a 40 µm filter to prevent cell aggregations from clogging the separation column matrix.

2.3.3. Magnetic separation: $CD45^+$ hematopoietic cells

2.3.3.1. Place separation column in the magnetic field of a suitable separator and equilibrate column with at least 3 mL of PBS.

2.3.3.2. Collect unlabeled cells in the flowthrough (FT), and wash column 3x with 3 mL of equilibration buffer. Collect washes with FT. Remove the column from the separator. Place the column on a 15 mL conical tube.

2.3.3.3. Flush out magnetically labeled $CD45^+$ cells by pipetting 5 mL of equilibration buffer onto the column and firmly plunging the cells with a plunger supplied with the column.

2.3.3.4. Centrifuge eluent as well as FT/wash fractions at 500 × *g*. Count the cells using a hemocytometer.

2.3.4. Magnetic labeling and separation: $CD31^+$ endothelial cells

2.3.4.1. Repeat protocol for $CD45^+$ magnetic labeling and separation (sections 2.3.2–2.3.3), except use $CD31^+$ magnetic beads to incubate with the FT and wash portions from the $CD45^+$

isolation.

2.3.5. Magnetic labeling and separation: MEFSK4^{+} fibroblasts

2.3.5.1. Repeat protocol for CD45^{+} magnetic labeling using the MEFSK4 anti-feeder-APC antibody instead of magnetic beads. Centrifuge the FT and Wash portions from the CD31^{+} isolation at 500 × *g* for 5 min, then remove supernatant. Resuspend the cell pellet in 1 mL of equilibration buffer, and count the cells using a hemocytometer.

2.3.5.2. Add 10 µL of MEFSK4 anti-feeder-APC antibody per 1 x 10^7 cells. Incubate for at least 15 min at 4 °C.

2.3.5.3. Wash MEFSK4 antibody bound cells by adding 5 mL of equilibration buffer per 1 x 10^7 total cells, then centrifuge at 500 × *g* for 5 min.

2.3.5.4. Remove supernatant and resuspend in anti-APC beads, using the same volume of MEFSK4 anti-feeder-APC antibody used. Incubate on ice for 15 min at 4 ˚C.

2.3.5.5. Centrifuge at 500 × *g* for 10 min. Remove supernatant, resuspend in 2 mL of equilibration buffer per 1 x 10^7 total cells, and proceed with magnetic separation as previously described (section 2.3.3). Magnetically separated MEFSK4^{+ve}/ CD45^{-ve}/CD31^{-ve} fibroblasts can be used for purity analyses and other downstream applications.

3. Purity and functionality analysis of isolated fibroblast population

3.1. FACS population purity analysis: αSMA-GFP cell analysis (Figure 2)

3.1.1. Resuspend freshly isolated cells in 25 µL of Fc blocker solution to prevent the nonspecific binding of antibodies. Incubate for 5 min at RT.

3.1.2. Optional: add 25 µL of 7AAD or Ghost dye violet 510 to cell suspension. Incubate for 30–60 min on ice in the dark.

3.1.3. Wash by resuspending in 1 mL of FACS buffer. Centrifuge at 500 x *g* for 5 min.

3.1.4. Resuspend pellet in 50 µL of FACS buffer. Add CD31-PE, CD45-APC, and AN2/NG2 antibodies directly to cell suspension. Incubate for 15 min on ice **(Table 1)**.

3.1.5. Wash by resuspending in 1 mL FACS buffer. Centrifuge at 400 × *g* for 5 min.

3.1.6. Add donkey anti-rat AlexaFluor 405 secondary antibody to the cells labeled with unconjugated primary antibody. Incubate for 30 min on ice.

3.1.7. Wash by resuspending in 1 mL of FACS buffer. Centrifuge at 400 x *g* for 5 min.

3.1.8. Resuspend pellet in recommended flow cytometry sample volume of FACS buffer (300 µL) and transfer to a labeled flow cytometry tube for flow analysis.

NOTE: FACS analysis to characterize the expression of MEFSK4 antigen on isolated fibroblasts is described in section 3.3.

3.2. Fibroblast purity analysis: immunofluorescence (Figure 3A)

3.2.1. Seed 30,000 primary fibroblasts (P0-P1) per well on coverslips placed in a 24 well plate and culture until 80% confluent. Or concentrate sorted CD45+ and CD31+ cells (30,000 cells per well) on a coverslip by cytospin at 400 × *g* (a method used to deposit cells directly and evenly onto a coverslip in a 24 well plate).

3.2.2. Fix cells with cold acetone for 15 min. Wash 3x times with PBS.

3.2.3. Block slides in 10% goat serum. Incubate slides with primary antibodies overnight (**Table 2**).

3.2.4. Wash slides 3x in PBS. Incubate secondary antibodies for 2 h (**Table 2**).

3.2.5. Counterstain slides, and mount with one drop of DAPI in slow-fade mounting media.

3.3. FACS population purity analysis: MEFSK4 probing (Figure 3B)

3.3.1. Resuspend freshly isolated cells in 25 µL of FC blocker solution to prevent the nonspecific binding of antibodies. Incubate for 5 min at RT.

3.3.2. Optional: add 25 µL of 7AAD or Ghost dye violet 510 to cell suspension. Incubate for 30–60 min on ice in the dark.

3.3.3. Wash by resuspending in 1 mL of FACS buffer. Centrifuge at 500 x *g* for 5 min.

3.3.4. Resuspend pellet in 50 µL of FACS buffer. Add MEFSK4 antibody directly to cell suspension. Incubate for 15 min on ice **(Table 1)**.

3.3.5. Wash by resuspending in 1 mL of FACS buffer. Centrifuge at 400 x *g* for 5 min.

3.3.6. Add rat IgG-APC to the cells (**Table 1**). Incubate for 30 min on ice.

3.3.7. Wash by resuspending in 1 mL of FACS buffer. Centrifuge at 400 x *g* for 5 min.

3.3.8. Resuspend pellet in recommended flow cytometry sample volume of FACS buffer (300 µL) and transfer to flow cytometry tube for flow analysis.

3.4. Fibroblast purity analysis: semi-quantitative real-time rtPCR (Figure 3C)

3.4.1. RNA isolation and semiquantitative real-time PCR

3.4.2. Following the enrichment of fibroblasts, isolate RNA using an RNA isolation kit (see **Table of Materials**). Follow the manufacturer's instructions.

3.4.3. Complete first strand DNA synthesis using a cDNA synthesis kit (see **Table of Materials**), following the manufacturer's instructions.

3.4.4. Perform semiquantitative real-time PCR[5].

3.5. Fibroblast functionality analysis: collagen gel contractility assay (Figure 4)

3.5.1. Collagen solution

3.5.1.1. Prepare 20 mM HEPES and 44 mM $NaHCO_3$ in DMEM. Add 1.67 mg of type 1 rat collagen per 1 mL of DMEM with HEPES and $NaHCO_3$.

3.5.2. TGFβ-supplemented DMEM

3.5.2.1. Prepare 10% FBS in DMEM supplemented with antibiotics and anti-fungal. Add TGFβ to a final concentration of 1 ng/mL.

3.5.3. Cell/collagen mixture and plating

3.5.3.1. Prepare cell suspension (P3-P5) and determine required volume to obtain 3.3 x 10^5 cells.

3.5.3.2. Add suspension volume with 3.3 x 10^5 cells to enough collagen solution to obtain 1 mL total volume.

NOTE: Rat collagen type 1 concentration should now be 1.5 mg/mL.

3.5.3.3. In a 48 well plate, seed 300 µL of cell-collagen mix per well (~1 x 10^5 cells/well). Incubate at 37 °C for 15–20 min until gelled.

3.5.3.4. Use a 30 G needle to help separate gel from the well walls. Add 600 µL of TGFβ and FBS supplemented DMEM to each well. Image plates on a reflective scanner at 24 h and 48 h.

NOTE: All Immunofluorescence experiments were performed on a flow cytometry machine equipped with three lasers (405 nm, 488 nm, and 640 nm). Data were acquired using a flow data acquiring software (**Table of Materials**). Further data analysis was performed using flow

data analysis software. AlexaFluor 405 and Ghost Dye Violet 510 were excited with the 405 nm laser and collected using a 450/50 BP and 525/50 BP filter, respectively. GFP and PE were excited by the 488 nm laser and collected using the 530/30 BP and 575/26 BP filters, respectively. Either APC or AlexaFluor 647 was excited by the 640 nm laser and collected using a 670/14 BP filter. All cell sorting experiments were performed on a flow cytometry machine (**Table of Materials**) equipped with four lasers (405 nm, 488 nm, 561 nm and 640 nm). 7-AAD was excited using the 561 nm laser and collected with a 670/14 BP filter. GFP and Ghost Dye Violet 510 were collected using the same laser/filter combinations as described above. All sorting experiments utilized a 100 um nozzle with a 17 psi pressure configuration for increased downstream viability of the target cells.

REPRESENTATIVE RESULTS:

Flow gating scheme demonstrating myofibroblast isolation using αSMA-GFP reporter mice

Uninjured hearts showed no detectable GFP^+ cells in αSMA-GFP reporter mouse model; hence, they were used to establish a gate for the background signal of the GFP channel post-compensation (**Figure 2**). αSMA^+ cells were sorted based on the presence of GFP expression from the injured left ventricle 10 days following MI. A small percentage of endothelial (GFP+/CD31+ cells; SD = 3.8% ± 0.0164; n = 5) and hematopoietic (GFP+/CD45+ cells; SD = 3.18% ± 0.0112; n = 5) cells also expressed GFP in the injured αSMA-GFP mouse hearts (**Figure 2A**). However, GFP+/CD31-/CD45- cells did not express AN2, a pericyte marker.

Uninjured (quiescent) and injured (activated, αSMA^+GFP+) cells expressed fibroblast markers

GFP+ cells isolated from αSMA-GFP mice expressed αSMA, collagen type 1 alpha-1 chain (COL1α1), vimentin, and periostin when analyzed by IF analysis. Uninjured fibroblasts isolated by selective adhesion expressed vimentin but did not demonstrate expression of the activated fibroblast markers: αSMA, periostin, and COL1α1 (**Figure 3A**). Both uninjured and activated fibroblasts expressed the MEFSK4 antigen when analyzed by flow analysis (**Figure 3B**). Magnetically isolated MEFSK4+ve cells from uninjured mice hearts expressed markers of fibroblasts: Col1a1, pdgfrα, and periostin. In contrast, magnetically isolated CD45 and CD31 positive cells had negligible expression of fibroblast markers.

Fibroblasts and myofibroblasts demonstrated the ability to contract collagen

In cell culture on stiff plastic, fibroblasts have been shown to contract collagen gels in the presence of TGFβ, demonstrating their functional capability of contraction[13,14]. This in vitro characteristic of fibroblasts is very similar to the connective tissue contraction that happens during tissue repair as well as other biological processes. Both uninjured fibroblasts, isolated by selective adhesion, and myofibroblasts, isolated and sorted from αSMA-GFP mice, demonstrated an ability to contract collagen (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of fibroblast isolation using three different approaches. A. differential plating, **B.** GFP+ cell sorting of αSMA positive cells, and **C.** magnetic bead based isolation of

fibroblasts. Representative bright field of the cells in culture following differential plating. Scale bar = 50 µM.

Figure 2: FACS analysis of single cells isolated from αSMA-GFP mice hearts following MI. (A) Representative FACS gating scheme demonstrating GFP+ cells co-expressing CD31, CD45, or AN2 from αSMA-GFP mice injured hearts 10 days after myocardial infarction (MI). **(B)** Graphical quantification of the presented FACS data for post-MI hearts; n = 5 experiments were performed independently (***p < 0.0001 as calculated using one-way ANOVA with Tukey's multiple comparisons test). This figure is adapted from Saraswati et al.[10].

Figure 3: Purity analyses of fibroblasts isolated from uninjured and injured mice hearts. (A) Immunofluorescence staining of cell populations (P0) from heart of uninjured, or injured αSMA-GFP mice sorted by FACS. Both uninjured and activated cells express fibroblast (FB) markers, such as COL1α1, and vimentin, but not hematopoietic marker CD45 or the endothelial marker CD31. Cells isolated from injured αSMA-GFP mice heart expressed activated fibroblast markers, αSMA, and periostin, which were not present in the cells isolated from uninjured mice hearts. Nuclei were stained with DAPI, n = 3 experiments were performed independently. Scale bar = 100 µm. (**B)** Representative FACS overlay histogram of uninjured and activated fibroblasts (P3–P5) showing the expression of the fibroblast marker MEF-SK4. For a negative control, rat IgG was used, n = 2 experiments were performed independently. **(C**) Relative fold change of *Col1α1*, *Pdgfrα*, and *Postn* transcripts in uninjured MEKSK4+ve fibroblasts, n = 3 experiments were performed independently (*p < 0.05 as calculated using two-way ANOVA with Tukey's multiple comparisons test). (A) and (B) are adapted from Saraswati et al.[10].

Figure 4: Functional characterization of fibroblasts isolated from uninjured and injured mouse hearts. Representative figure of collagen gel contraction in the presence of uninjured and injured activated αSMA^{+} fibroblasts (P3–P5). The graph represents the percentage change in the initial gel area after 24 h and 48 h of contraction when incubated with uninjured and injured activated αSMA^{+} fibroblasts, n = 2 experiments were performed independently. This figure is adapted from Saraswati et al.[10].

Table 1: FACS dyes and antibodies.

Table 2: Immunofluorescence primary and secondary antibodies.

DISCUSSION:

Fibroblasts are a heterogenous group of cells, identified by diverse set of markers. The protein markers that have been used to identify fibroblasts are discoidin domain receptor 2 (DDR2), fibronectin, vimentin, collagen I and III, and Thy1[15-20]. Whereas vimentin has been used to identify uninjured quiescent cardiac fibroblasts, fibroblast specific protein 1, αSMA, and periostin have been shown to identify injury-induced activated fibroblasts, with αSMA being the most common marker to detect activated fibroblasts[6,12,21]. Additionally, Tcf21 and MEFSK4 proteins have gained recent recognition in recognizing both quiescent fibroblasts found in

uninjured cardiac tissue as well as activated fibroblasts including myofibroblasts found in injured mouse hearts[21,22].

This protocol utilizes three different approaches to isolate and enrich fibroblasts and activated fibroblasts including myofibroblasts. The fibroblast's ability to preferentially adhere to plastic is used in the first approach for isolation. Following enzymatic digestion with liberase, the single cell suspension of cells is seeded on a plastic dish to preferentially adhere. The inability of many non-fibroblast cells to adhere to the polystyrene surface of Petri dishes allows us to remove all media from the dish and remain with a relatively pure population of fibroblasts. A caveat of using this technique is although fibroblasts will preferentially adhere to the polystyrene dish, some contaminating non-fibroblast cells may also attach, leaving a non-homogenous population of cells.

The second isolation technique utilizes FACS to separate αSMA expressing myofibroblasts from other cells. In the transgenic mouse model employed here, GFP is exclusively expressed along with αSMA, so myofibroblasts containing αSMA can be detected by a FACS machine through the fluorescent capabilities of GFP. This isolation procedure enables us to obtain a population of cells that is approximately 99% myofibroblast. The purity analyses of these cells have been extensively described by Saraswati et al.[10].

The third isolation technique is an efficient way to isolate both uninjured and activated fibroblasts by magnetic bead-based separation of MEFSK4 expressing cell. By allowing a single cell suspension to bind to anti-CD45 and anti-CD31 magnetic beads and become immobilized in a matrix due to magnetic field effects, this allowed the separation of any hematopoietic as well as endothelial cells that may have contaminated the fibroblast isolation. As MEFSK4 has been recently used as a reliable marker to identify fibroblasts, an antibody that will bind to MEFSK4 expressing cells is able to be applied. After binding a magnetic bead to the antibody, creating a complex that allows isolation of fibroblasts, the magnetic bead-cell complex is passed through a matrix in a magnetic field, and a highly enriched fibroblast population is obtained. The purity of the isolated fibroblast population should be assessed by immunostaining, RTPCR, and flow cytometry analyses.

As with any other technique, there are limitations with the techniques described in this manuscript. The limitation of the selective adhesion protocol and magnetic bead-based isolation is that these methods do not differentiate between quiescent and activated fibroblasts. In order to enrich activated fibroblasts, the isolation should be performed 8–10 days following myocardial infarction. Additionally, it is important to check the purity of the isolation with other fibroblast markers. MEFSK4-positive fibroblast purity has been demonstrated only by RTPCR, it is recommended to test (by immunostaining and flow cytometry analysis) with other fibroblast markers and markers that recognize contaminating cell types including hematopoietic (CD45), endothelial (CD31), and pericytes (AN2). If possible, other fibroblast specific markers could be used to further sort or magnetically isolate the fibroblast population.

Using αSMA-GFP mice to isolate and sort myofibroblasts is a reliable technique to obtain an activated fibroblast population. However, a negligible percentage of hematopoietic and endothelial cells has been observed in the flow analysis. To improve upon this technique, CD45+ve/CD31+ve and AN2+ve cells should be excluded from the $GFP^{+ve}/\alpha SMA^{+ve}$ cell sorting. Since αSMA is a widely accepted marker of myofibroblasts, the αSMA-GFP reporter mouse model is a valuable tool that should be exploited to study myofibroblasts in the context of myocardial injuries.

There are several crucial troubleshooting steps that must be taken into account. Digestion time can be decreased if cell viability and yield is affected. A stir bar should not be used to stir the digestion mixture, as this affects cell viability. The tube should be secured on a rocker or in a shaking incubator to agitate the digestion mixture gently. Resuspending the digested tissue 10x with a 5 mL or 10 mL pipette is crucial for proper dissociation of cells.

Proper red blood cell lysis of the single cell suspension must be utilized if cells are going to be sorted or analyzed by flow cytometry. For magnetic bead isolation, degassing of the buffer is essential to prevent the introduction of any air bubbles in the column. The column used for magnetic bead cell isolation should not be reused between different magnetic bead-conjugated cells. For example, a new column should be used to separate $CD45^+$ cells, which should be discarded after elution of the $CD45^+$ cells, then another new one for the $CD31^+$ cell isolation. In our hands, we have not seen contamination of pericytes in isolated/sorted fibroblasts. However, MEFSK4 has been shown to recognize pericytes[22]. It is therefore recommended to use an additional step to sort out/magnetically deplete pericytes (AN2) from the single cells. Although this protocol is validated in 12 week-old mice, the technique may be used for younger or older mice.

ACKNOWLEDGMENTS:

The authors want to thank Dr. Ivo Kalajzic for the αSMA-GFP mice. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under Award Number R01GM118300 (S.S.), National Institute of Biomedical Imaging and Bioengineering of the NIH under Award Number R21EB019509 (P.P.Y.), and Scientist Development Grant of the American Heart Association under Award Number 17SDG33630187 (S.S.). Flow cytometry analyses were performed at the VUMC Flow Cytometry Shared Resource which is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

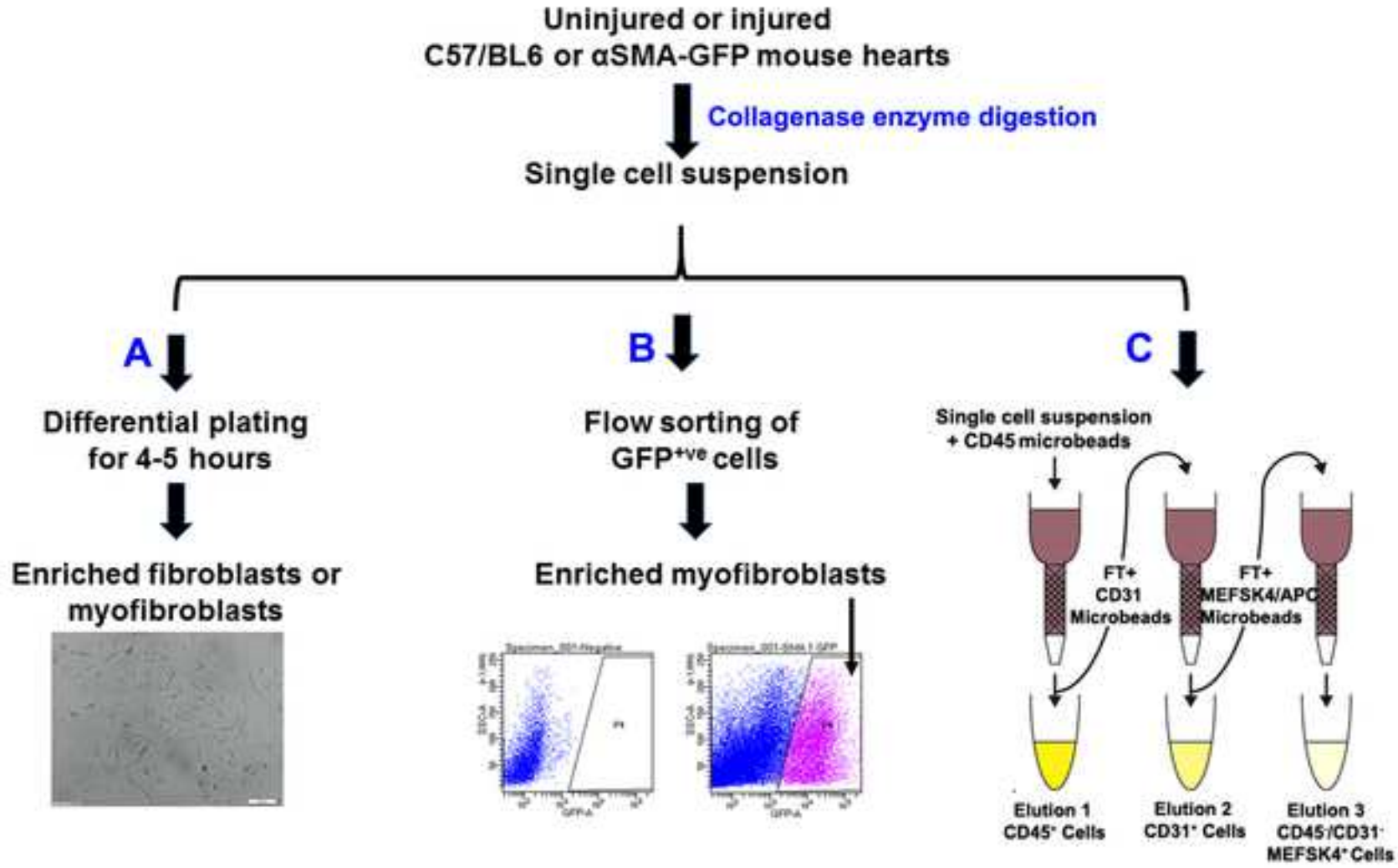
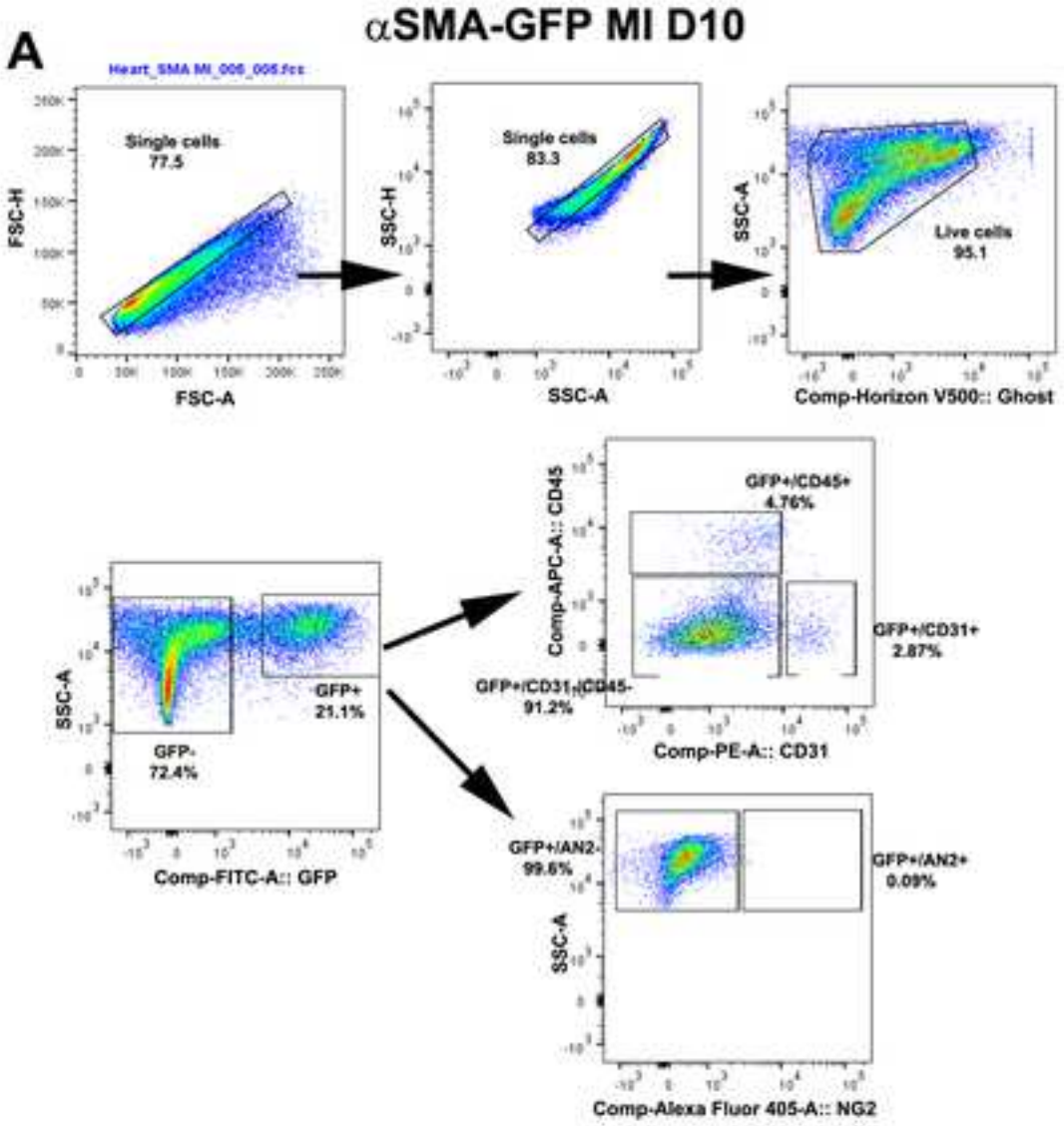


Figure 2



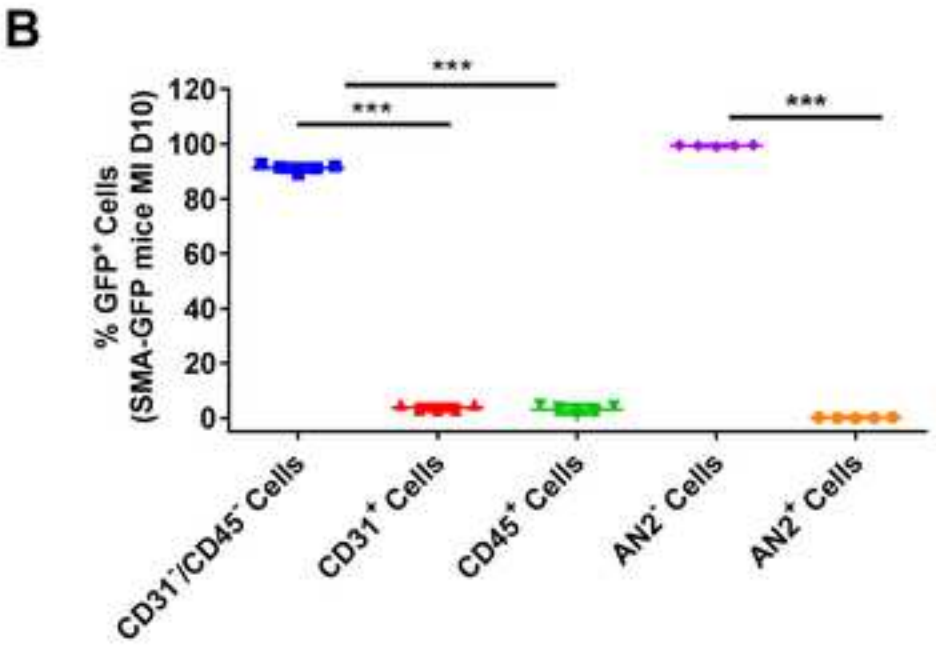


Figure 3

Click here to access/download;Figure;Fig 3-flatten.tif

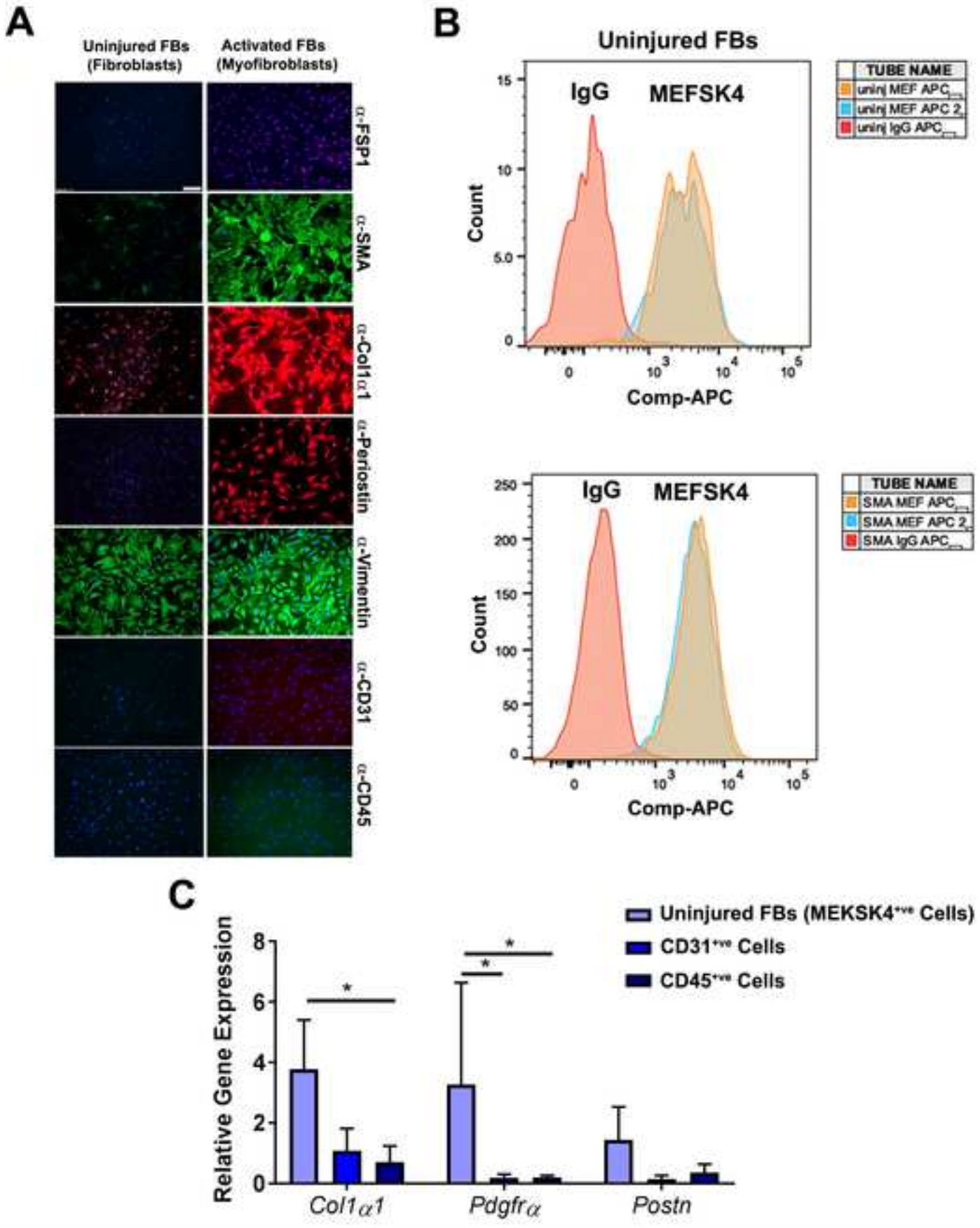
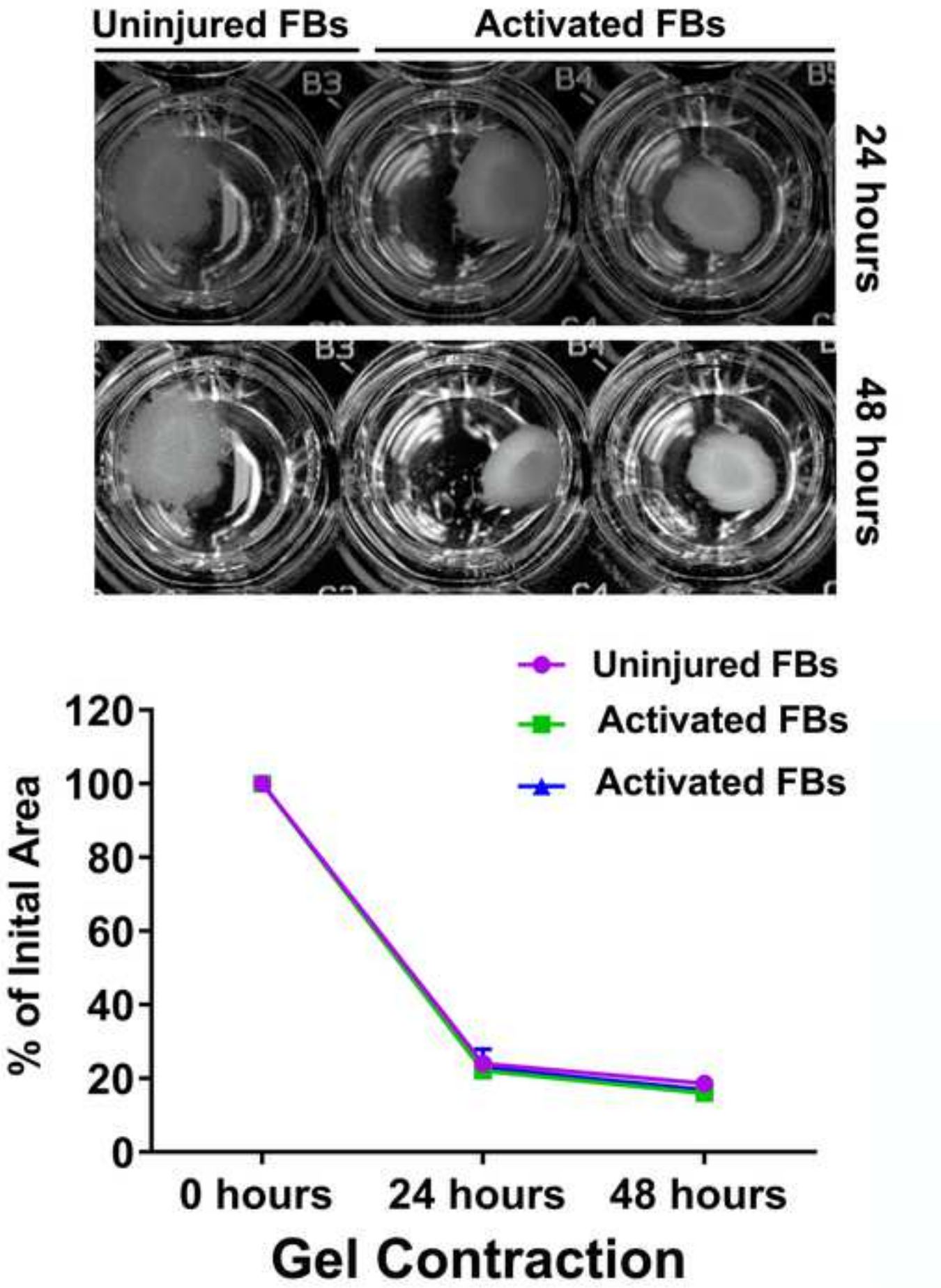


Figure 4



Antibody
7AAD
Ghost dye violet 510
APC-CD45
PE-CD31
anti-AN2/NG2
Donkey anti-rat alexa fluor 405
Anti-feeder cells-APC (MEFSK4)
Rat IgG-APC

Cell Target	Dilution
Dead cells	1:1000
Dead cells	1:1000
Hematopoietic cells	1:200
Endothelial cells	1:200
Pericytes	1:11
Secondary Antibody	1:100
Fibroblasts	1:100
Isotype Control	1:100

Primary Antibody	**Dilution**
α-smooth muscle actin (αSMA)	1:1000
Fibroblast specific protein 1 (FSP1)	1:100
COL 1α1	1:1000
Periostin	1:100
Vimentin	1:200
CD31	1:250
CD45	1:250

Secondary Antibody	**Dilution**
Goat anti-mouse Alexa Fluor 488	1:200
Goat anti-rabbit-FITC	1:200
Goat anti-rabbit-Cy3	1:200
Goat anti-rat Alexa Fluor 488	1:200
Goat anti-rat Alexa Fluor 647	1:200

Name	Company
Reagents	
Acetone	
Anti-fungal (Amphotericin B-solubilized; Fungizone)	Sigma Aldrich
Bovine Serium Albumin (BSA)	Sigma
Calcium chloride	
Citrate Buffer	
Collagenase blend (Liberase Blendzyme 3 TH)	Roche Applied Science
DAPI	
DDI water	
DI water	
DMEM-F12 with L-Glutamine and HEPES	Life technologies
Dnase I(20U/mL)	BioRad
Dulbecco's Phosphate-Buffered Saline (dPBS) without Ca2+ and Mg2+	Gibco
70% Ethanol	
FC Blocker (Purified anti-mouse CD16/CD32)	Tonbo Biosciences
Fetal Bovine Serum (FBS)	Life technologies
10% goat serum	
Hank's Balanced Salt Solution (HBSS) with Ca2+ and Mg2+	Corning
1M HEPES	Corning
Krebs-Henseleit Buffer powder	Sigma
Mycoplasma prophylactic (Plasmocin)	Invivogen
Penicillin/Streptomycin	Thermo Fisher Scientific
1x Phosphate-Buffered Saline (PBS)	
10x Red Blood Cell Lysis Buffer	Miltenyi
Slow-fade Mounting Media	
Sodium azide	
Sodium bicarbonate	
TGFβ	
Trypan Blue Stain (0.4%)	Gibco
Type 1 Rat Collagen	
Antibodies	
7AAD (stock: 1 mg/mL solution in DMSO)	Molecular Probes
CD45-APC	BD Bioscience
CD31-PE	BD Bioscience
CD31	BD Biosciences
CD45	BD Biosciences
COL 1α1	MD Bioproducts
Ghost dye violet 510 (Formulation: 1 uL/test in DMSO)	Tonbo Biosciences
Goat anti-mouse Alexa Fluor 488	Molecular Probes
Goat anti-rabbit-Cy3	Southern Biotech
Goat anti-rabbit-FITC	Jackson Immunoresearch
Goat anti-rat Alexa Fluor 488	Molecular Probes
Goat anti-rat Alexa Fluor 647	Thermo-Fisher

Periostin	Santa Cruz
Vimentin	Sigma Aldrich
α-smooth muscle actin (αSMA)	Sigma Aldrich
Fibroblast specific protein 1 (FSP1)	Millipore 07-2274
CD45 Magnetic Beads	Miltenyi Biotec
CD31 Magnetic Beads	Miltenyi Biotec
Anti-feeder cells-APC (MEFSK4)	Miltenyi Biotec
anti-APC Beads	Miltenyi Biotec
Rat IgG-APC	Miltenyi Biotec
Donkey anti-rat Alexa Fluor405	Abcam
anti-AN2/NG2	Miltenyi Biotec
Other Materials	
0.22 µm Filter	Thermo Scientific
10 cm2 Cell Culture Dish	Corning
10 mL Pipet	Fisherbrand
40 µm Cell Strainer	Fisherbrand
5 mL Pipet	Fisherbrand
50 mL Conical Tube	Falcon
6-well Plate	Corning
Flow Cytometry Tubes	Falcon
Forceps	
Rocker	
Single Edge Blade	PAL
Surgical Scissors	
GFP-αSMA Reporter Mice	
MACS Separator Magnetic Field	
MACS Separation Column	
Coverslips	
Qaigen Rneasy Mini Kit	Qaigen
Ambion RNAqueous Micro Total Isolation Kit	Ambion
BioRad iScript cDNA Syntehsis Kit	BioRad
48-well Plate	
30G Needle	
3 Laser Flow Cytometry Machine (BD LSRFortessa)	BD Biosciences
4 Laser Flow Cytometry Machine (BD FACSAria III)	BD Biosciences
Flow Data Acquiring Software (BD FACSDiva Software v8.0a)	BD Biosciences
Flow Data Analysis Software (FlowJo Software)	BD Biosciences

Catalog Number	Comments
A9528	
9048-46-8	
11330057	
7326828	
13190-144	
70-0161	
16000044	
21-023-CV	
25-060-Ci	
K3753	
ant-mpp	
15140122	
130-094-183	
15250-061	
A1310	dilution = 1:1000; RRID =
559864	dilution = 1:200; RRID = AB_398672
553373	dilution = 1:200; RRID = AB_394819
553370	dilution = 1:250; RRID = AB_394816
553076	dilution = 1:250; RRID = AB_394606
203002	dilution = 1:1000; RRID =
13-0870	dilution = 1:1000; RRID =
A11029	dilution = 1:200; RRID = AB_138404
4050-02	dilution = 1:200; RRID = AB_2795952
711-165-152	dilution = 1:200; RRID = AB_2307443
A11006	dilution = 1:200; RRID = AB_2534074
A21247	dilution = 1:200; RRID = AB_141778

SC67233	dilution = 1:100; RRID = AB_2166650
V2258	dilution = 1:200; RRID = AB_261856
A2547	dilution = 1:1000; RRID = AB_476701
07-2274	dilution = 1:100; RRID = AB_10807552
130-052-301	
130-087-418	
130-102-900	dilution = 1:100; RRID = AB_2660619
130-090-855	
130-103-034	dilution = 1:100; RRID = AB_2661598
ab175670	dilution = 1:100
130-097-455	dilution = 1:11; RRID = AB_2651235

723-2520
430167
13-678-11E
22363547
13-678-11D
352070
3506
352058

62-0177

74104
AM1931
1708891

Second Rebuttal Document

1. **1.2.2.** I unhighlighted euthanasia as we will not film it.

Agreed.

2. **1.2.3**. Cite a reference.

Cited as "Alternatively, for activated fibroblast isolation, induce myocardial infarction in 12 week old mice by coronary artery ligation {Saraswati, 2019 #298}."

3. **2.2.3.1.** What is the required concentration?

Text changed from "concentration" to "dilution". Dilutions listed in Table 1.

4. **2.2.3.3.** Add both to table of materials along with stock concentrations for each

We added the stock conc of 7AAD (1 mg/mL) and the formulation conc of Violet 510 (1 µL/test) to materials table. Only the formulation conc of the Ghost dye Violet was available from the company.

5. **2.2.3.3**. How much of dye is added?

It is based on the dilution (1:1000) which is mentioned in the Table of Materials and the Table 1.

6. **2.3.2.2.** How and when were the cells counted?

Additional step added to count cells with a hemocytometer.

7. **2.3.3.4.** Mention method.

"Using a hemocytometer" added.

8. **2.3.5.1**. What volume/concentration is added? Reference Table 1 here if you wish for readers to refer to it here.

MEFSK4 anti-feeder-APC antibody is used for both magnetic bead isolation and for FACS analysis, thus Table 1 is not referenced here. When using it for magnetic bead isolation, 10 µL of MEFSK4 antibody is used per 10^7 cells. Additional step added to clarify this.

9. **2.3.5.3**. When are the cells counted?

Counting step added to step 2.3.5.1.

10. **2.3.5.3.** Reference the step numbers

Referenced step 2.3.3.

11. **3.1.2.** Add both to table of materials along with stock concentrations for each

We previously added the stock conc of 7AAD (1 mg/mL) and the formulation conc of Violet 510 (1 µL/test) to materials table, under antibodies. Only the formulation conc of the Ghost dye Violet was available from the company.

12. **3.1.4.** This is not mentioned in table 2. What is the final concentration?

FACs antibodies are in Table 1, not Table 2. Text changed. AN2/NG2 added to reflect Figure 2. FACS MEFSK4 probing is in a separate step 3.3.

13. **3.2.1.** What concentration? Mention culture conditions, media etc

Added 30,000 cells per well in 24-well plate.

14. **3.2.1.** Mention settings

Added speed: 400 xg

15. **3.2.3.** For how long?

This step is not required; hence deleted

16. **3.2.4**. Not present with submission.

IF antibodies included in Table 2. Text changed.

17. **3.2.5.** Not present with submission.

IF antibodies included in Table 2. Text changed.

18. **3.2.6.** Concentration?

Text changed.

19. **3.3.4.** How much? At what concentration?

Table 1 referenced. MEFSK4 probing is a separate FACs analysis than previous FACS analysis in step 3.1. NOTE added to step 3.1 to notify reader of the difference, but antibodies and concentrations for steps 3.1 and 3.3 are included in Table 1.

20. **3.3.6.** Concentration?

Dilutions are mentioned in Table 1.

21. **3.4.4**. Needs a reference

Reference added.

22. **3.5.2.1**. Use a generic name

“fungizone” changed to “anti-fungal”.

23. **3.5.3.4.** Mention generic name for the type of scanner used.

Scanning occurs on a generic scanner. Text changed to clarify.

24. **3.5.2.4.** Remove all commercial names. Add this to the table of materials. I have marked all following instances of commercial names that need replacement in red

Specific names replaced and referenced in the table of materials.

25. **Representative Results.** ?

N removed after 10. Supposed to read “10 days following MI”.

26. **Figure Legends.** Remove the text "figure #" from all figures.

Figure # removed.

27. **Figure 1.** Replace the name Liberase from the figure

Liberase replaced with “Collagenase”.

28. **Figure 2.** This figure is too dense, and text on panels A and B are too small to read. I suggest splitting up this figure into 2 or 3 figures instead. If you choose to have a multipanel figure, the figure legend must have a common title as well.

The figure has been revised. We have removed panel B and rewrote the text.

29. **Figure 2C.** Define *, mention statistical tests used.

Edited.

30. **Figure 3.** Needs a title.

Added a title to all figure legends in bold.

31. **Figure 3C.** Define error bars and *. Mention statistical test used.

Defined.

32. **Troubleshooting.** Please remove the numbered list and use paragraph style instead.

Text changed.

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