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In Vivo Study of Human Endothelial-Pericyte Interaction Using the Matrix Gel Plug Assay

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Corresponding Author:	Vinicio De Jesus Perez Stanford Stanford, CA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	vdejesus@stanford.edu
Corresponding Author's Institution:	Stanford
Corresponding Author's Secondary Institution:	
First Author:	Ke Yuan
First Author Secondary Information:	
Other Authors:	Ke Yuan
	Mark E Orcholski
	Ngan F. Huang
Order of Authors Secondary Information:	
Abstract:	Angiogenesis is the process by which new blood vessels are formed from existing vessels. New vessel growth requires coordinated endothelial cell proliferation, migration, and alignment to form tubular structures followed by recruitment of pericytes to provide mural support and facilitate vessel maturation. Current in vitro cell culture approaches cannot fully reproduce the complex biological environment where endothelial cells and pericytes interact to produce functional vessels. We present a novel application of the in vivo matrix gel plug assay to study endothelial-pericyte interactions and formation of functional blood vessels using severe combined immune deficiency mutation (SCID) mice. Briefly, matrix gel is mixed with a solution containing endothelial cells with or without pericytes followed by injection into the back of anesthetized SCID mice. After 14 days, the matrix gel plugs are removed, fixed and sectioned for histological analysis. The length, number, size and extent of pericyte coverage of mature vessels (defined by the presence of red blood cells in the lumen) can be quantified and compared between experimental groups using commercial statistical platforms. Beyond its use as an angiogenesis assay, this matrix gel plug assay can be used to conduct genetic studies and as a platform for drug discovery. In conclusion, this protocol will allow researchers to complement available in vitro assays for the study of endothelial-pericyte interactions and their relevance to either systemic or pulmonary angiogenesis.
Author Comments:	June 28th, 2016 Avital Braiman, Ph.D. Director of Editorial Journal of Visualized Experiments 1 Alewife Center, Suite 200

	Cambridge, MA 02140 Dear Dr. Braiman, Please find enclosed our revised manuscript entitled "In vivo study of human endothelial-pericyte interaction using the matrix gel plug assay" by Yuan et al that we would like it to be re-considered for publication in Journal of Visualized Experiments. Your comments and those of the reviewers were highly insightful and enabled us to greatly improve the quality of our manuscript. In the following pages are our point-by-point responses to each of comments, I believe we have fully addressed all the questions and concerns raised by reviewers. We hope that revisions in the manuscript and our accompanying responses will be sufficient to make it suitable for publication in the JOVE. We look forward to hearing from you a positive response.
Additional Information:	
Question	**Response**
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STANFORD UNIVERSITY SCHOOL OF MEDICINE
269 CAMPUS DRIVE, Grant S-140B, STANFORD, CA 94305-5162
(650) 723-0318 · Fax (650) 1-650-723-6272· EMAIL: vdejesus@stanford.edu

Vinicio A. de Jesus Perez, M.D.

Assistant Professor of Medicine and Staff Physician
Stanford University Pulmonary Hypertension Clinic
Stanford University Medical Center

March 17th, 2016
Avital Braiman, Ph.D.
Director of Editorial
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Dr. Braiman,

Please find enclosed our revised manuscript entitled " *In vivo* study of human endothelial–pericyte interactions by implantation of matrix gel" by Yuan et al that we would like it to be re-considered for publication in Journal of Visualized Experiments.

Your comments and those of the reviewers were highly insightful and enabled us to greatly improve the quality of our manuscript. In the following pages are our point-by-point responses to each of comments, I believe we have fully addressed all the questions and concerns raised by reviewers.

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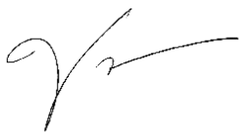
We look forward to hearing from you a positive response.

Yours sincerely,



Vinicio A. de Jesus Perez, MD

TITLE:
In Vivo Study of Human Endothelial–Pericyte Interaction Using the Matrix Gel Plug Assay in Mouse

AUTHORS:
Yuan, Ke
Divisions of Pulmonary and Critical Care Medicine and Stanford Cardiovascular Institute
School of Medicine
Stanford University
Stanford CA, USA
keyuan@stanford.edu

Orcholski, Mark E.
Divisions of Pulmonary and Critical Care Medicine and Stanford Cardiovascular Institute
School of Medicine
Stanford University
Stanford CA, USA
marko1@stanford.edu

Huang, Ngan F.
Stanford Cardiovascular Institute,
VA Palo Alto Health Care System,
Department of Cardiothoracic Surgery,
School of Medicine,
Stanford University,
Stanford CA, ngantina@stanford.edu

de Jesus Perez, Vinicio A.
Divisions of Pulmonary and Critical Care Medicine and Stanford Cardiovascular Institute
School of Medicine, Stanford University,
Stanford CA USA
Email: vdejesus@stanford.edu

CORRESPONDING AUTHOR:
de Jesus Perez, Vinicio A.

KEYWORDS:
Angiogenesis, Endothelial cells, Pericytes, matrix gel plug, *in vivo* assay, mice

SHORT ABSTRACT:
We present a protocol to study human endothelial-pericyte interactions in mouse using a variation of the matrix gel plug angiogenesis assay.

LONG ABSTRACT:
Angiogenesis is the process by which new blood vessels are formed from existing vessels. New vessel growth requires coordinated endothelial cell proliferation, migration,

and alignment to form tubular structures followed by recruitment of pericytes to provide mural support and facilitate vessel maturation. Current *in vitro* cell culture approaches cannot fully reproduce the complex biological environment where endothelial cells and pericytes interact to produce functional vessels. We present a novel application of the *in vivo* matrix gel plug assay to study endothelial-pericyte interactions and formation of functional blood vessels using severe combined immune deficiency mutation (SCID) mice. Briefly, matrix gel is mixed with a solution containing endothelial cells with or without pericytes followed by injection into the back of anesthetized SCID mice. After 14 days, the matrix gel plugs are removed, fixed and sectioned for histological analysis. The length, number, size and extent of pericyte coverage of mature vessels (defined by the presence of red blood cells in the lumen) can be quantified and compared between experimental groups using commercial statistical platforms. Beyond its use as an angiogenesis assay, this matrix gel plug assay can be used to conduct genetic studies and as a platform for drug discovery. In conclusion, this protocol will allow researchers to complement available *in vitro* assays for the study of endothelial-pericyte interactions and their relevance to either systemic or pulmonary angiogenesis.

INTRODUCTION

Angiogenesis is the process by which new blood vessels are formed from a pre-existing vascular network[1] and is the focus of ongoing research across many areas ranging from normal development to disease. This dynamic process involves the proliferation and migration of endothelial cells (ECs) and recruitment of pericytes to construct a vascular tube that is directed toward the site that needs oxygen and nutrient delivery[2]. To study this process requires an equally dynamic assay, most importantly one that can recapitulate the three-dimensional nature of tube formation. *In vitro* 3D matrix assays have been developed to address this need and have worked well to allow researchers to define the discrete steps in space and time in which angiogenesis takes place[3-6]. However, these *in vitro* 3D matrix models are limited to studying non-perfused vessels and therefore lack critical components pertinent to the angiogenesis process (e.g. circulating growth and inhibitory factors, unnatural tension/forces across the vascular bed) and fail to simulate the complex environment present in live tissue. To address this limitation, several *in vivo* angiogenesis assays have been developed[7], including the matrix gel plug assay which will be the focus of our report[8,9].

The matrix gel plug assay is a well-established *in vivo* angiogenesis assay that appeals to researchers as it provides a robust platform to test the roles of different cells and substances in angiogenesis. Matrix gel is a commercially available basement membrane solution that is secreted by the Engelbreth-Holm-Swarm (EHS) mouse sarcoma cell line that solidifies into a gel-like material at 37 °C. The matrix gel can be mixed with cells and/or substances, such as growth factors, and injected subcutaneously into the mouse. The host ECs will invade the plug over 14 days, form a vascular network, and become perfused with the host's blood. To date, matrix gel plug assays have focused exclusively on the study of endothelial cell behavior during angiogenesis, however, to the best of our knowledge no effort has yet been made to determine whether this assay can be used to co-culture endothelial cells and pericytes to study how these two cell types interact during angiogenesis. Specifically,

understanding the relationship between ECs and pericytes is valuable for studying diseases where blood vessel loss is pathologic, including microvascular ischemia and peripheral vascular disease[10 11,12].

Here, we describe a protocol that introduces human-derived pericytes to the matrix gel mixture along with human ECs and fibroblast growth factor (bFGF). This mixture can then be injected subcutaneously in the dorsum of SCID mice to allow formation of fully functional, pericyte coated, hybrid vessels. Our protocol describes how to prepare matrix gel plugs containing human ECs either with or without human pericytes, placement into SCID mice and how to analyze the histological sections for critical angiogenesis endpoints.

PROTOCOL:

Ethics Statement: Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee at Stanford University School of Medicine.

Note: Animals are under anesthetization with 3% vaporizer isoflurane and 3% supply of O_2 gas. Use of vet ointment on eyes may help to prevent dryness while under anesthesia.

1. Cell preparation

1.1. Grow human endothelial and pericyte cell cultures in 100 mm plates with 10 mL of the appropriate media. Replace 10 mL of fresh medium every 2-3 days until cells reach 80% confluency.

1.1.1. Culture endothelial cells (ECs) in complete endothelial cell media (ECM) supplemented with company supplied 5% fetal bovine serum (FBS), 10% penicillin/streptomycin and 10% endothelial cell growth supplement.

1.1.2. Culture pericytes in complete pericyte media (PM) supplemented with company supplied 2% FBS, 10% penicillin/streptomycin and 10% pericyte growth supplement.

1.2. Prepare cell mixtures

1.2.1. Calculate the required number of cell for the experimental and control groups.

Note: Each plug in the experimental group contains one million ($1X10^6$) human ECs and two hundred thousand ($2X10^5$) pericytes. For the control group, each plug contains 1.2 million human ECs only. The negative control group has matrix gel only. Each group should include at least four plugs, which would require two mice as plugs can be injected into each side of a mouse's lower back. Three plugs would serve as triplicate for the experiment and the fourth could be used in case one fails. Prepare 25% extra cells to match the extra volume of gel.

1.2.2. Collect and count cells from culture plates.

1.2.2.1. To detach cells, remove media and wash once with room temperature 1X phosphate buffered saline (PBS). Remove PBS, add 1 ml 0.25%Trypsin/2.21 mM EDTA, and incubate for 1 min. Wash cells off the plate by adding 2 mL medium and collect in a 15 mL tube.

1.2.2.2. Count cell using a hemocytometer according to the manufacturer's instructions.

1.2.2.3. Centrifuge the appropriate number of cells into a pellet at 400 x g for 5 min. For the experimental group, centrifuge both ECs and pericytes down together into one pellet.

2. Matrix gel preparation

2.1. Thaw out matrix gel completely at 4 °C for 4 h or overnight to obtain a homogenous solution. No mixing is required.

2.2. Aliquot matrix gel in 1.5 ml tubes and store at 4 °C.

2.3. Pre-chill sterile 1.5 ml tubes and 28G 1cc insulin syringes at 4 °C. Caution: Keep matrix gel on ice at all times.

2.4. Mix cold matrix gel with bFGF at a volume ratio of 1:100 (final concentration of bFGF= 0.5 μg/ml; bFGF stock solution= 50 μg/ml) After mixing matrix gel solution by pipetting up and down several times, incubate on ice for 30-60 min before mixing with the cells in step 3.1.

Note: Thaw the stock bFGF before mixing. Calculate the number of plugs needed for injection. For e.g., each plug requires 200 μL matrix gel. Therefore, prepare 25% extra gel.

3. Mix matrix gel with cells

3.1. For each group, resuspend the cell pellet (containing appropriate cell number) with the calculated volume of matrix gel containing bFGF. Mix gently to avoid foaming and leave in the 15 mL tube on ice until mice are prepared for injection.

4. Mouse preparation

4.1. Anesthetize 2 SCID mice for 5 min in 3% Isoflurane plus 3% O_2 in an induction chamber. Focus on one experimental group, or 2 mice, at a time.

4.2. Remove one mouse from the induction chamber, place on a heating pad ventral side down and quickly attach a snout nozzle to the mouse's face via a non-rebreathing system to supply anaesthesia throughout the injection procedure. Switch the gas flow from the induction chamber to the non-rebreathing system and lower the gas pressure to 1.5% Isoflurane and 1.5% O_2.

4.3. Remove hair from both lateral hind regions (around 1 cm diameter) with a shaver or hair removal cream.

4.4. Wipe the skin area with a 70% alcohol pad.

4.5. Return the mouse to the induction chamber and repeat steps 4.2-4.4 with the second mouse.

5. Matrix gel injection

5.1. Prepare one mouse for injection by attaching to the non-rebreathing system and placing on the heating pad ventral side down.

5.2. Load the matrix gel into a pre-chilled 28G 1cc insulin syringe one group at a time.

5.2.1. Load the complete 1 mL volume of matrix gel for all four plugs (200 μL per plug plus 25% extra) into the syringe.

5.2.2. Make sure to inject the matrix gel into the mice within 3 min of loading the cells into the syringe to prevent the cells from settling to the side of the syringe.

5.2.3. Lay full syringe on a bed of ice whenever not in use to keep matrix gel cold to prevent gelation.

5.3. Lift the back skin to locate subcutaneous space, then inject 200 μL of the matrix gel slowly and evenly into the subcutaneous space of the back posterior of the rib cage.

5.4. Remove the injection needle slowly, being careful to prevent matrix gel from leaking out of the injection site. A bump will form at the site of injection. Wipe injection site with an alcohol pad.

5.5. Outline the bump using a permanent marker to identify the bump after 14 days. After some hair grows back, it will be easier to find the bump.

5.6. Repeat injection steps 5.2-5.5 on the other side of mouse's back, then leave the mouse on its back for 1 min to allow gelation of the matrix gel on the heat pad.

6. Matrix gel plug isolation: 14 days after injection

6.1. Euthanize the mice humanely by exposing the animals to >5 min of carbon dioxide inhalation followed by cervical dislocation to assure death.

6.2. Remove the matrix gel plug from the mouse's back.

6.2.1. Gently remove regrown hair around injection site where the plug is located by repeating step 4.3. The marker line indicates the size of original bump under the skin.

6.2.2. Excise the plug along with the surrounding skin and muscle layers attached on the top and bottom sides of the plug, respectively.

6.2.2.1. Using fine surgical scissors, make an incision on the marked border of the plug that is perpendicular to the skin and cut through to the muscle underneath the plug. Then, carefully cut around the periphery of the plug along the marked border.

6.2.2.2. Remove the plug, keeping it sandwiched between the skin and muscle layers. Immediately rinse in a small beaker with 1X PBS to wash away excess blood. The plug is now ready to be fixed (next step, 6.3).

6.3. Fix the plug in 4% paraformaldehyde.

6.3.1. Place the entire plug, including the skin and muscle layers, in a 50 mL tube with 25 mL of 4% paraformaldehyde for 24 h at room temperature without rocking. The next day, transfer the plug into 25 mL of 70% ethanol for another 24 h before paraffin embedding.

Note: Handle paraformaldehyde with caution. Wear gloves.

7. Paraffin process the matrix gel plug

7.1. Prepare the plug for paraffin embedding.

7.1.1. Orient the plug as shown in Figure 1a; place the plug in a tissue cassette so that the side of the plug is facing up and both the skin and muscle layers are visible across the surface of the tissue block that will be cut first during sectioning.

7.2. Paraffin embed the plug according to standard paraffin embedding protocols[13].

7.3. Cut 8-10 μm thick sections off of the tissue block and mount onto microscope slides according to standard paraffin sectioning protocols[13].

7.4. Store the slides in a cool dry place at room temperature until ready for staining.

8. Stain sections with Hematoxylin and Eosin stain (H&E)[13]

8.1. De-paraffinize the tissue sections by passing the slides through 100% xylene twice, 100% ethanol, 95% ethanol, 70% ethanol and lastly 1X PBS, each for 5 min at room temperature. Leave slides in 1XPBS until the next step.

8.2. Pipette 100 μL H&E solution onto the section and incubate for 1-5 min, or until there is clear contrast between blue nuclei and pink cytoplasm in a cell as viewed under an upright light microscope at 10x magnification. Be careful not to let any H&E solution touch the objective.

8.3. Wash H&E solution off of the slide by dunking the slide in a large beaker of tap water, then place the slide in a rack in the beaker and flush with running water for 2 min.

8.4. Move to step 9.8 to mount the slides.

9. Stain other slides for human capillaries and pericytes

9.1. De-paraffinize the tissue sections by passing the slides through 100% xylene twice, 100% ethanol, 95% ethanol, 70% ethanol and lastly 1X PBS, each for 5 min at room temperature. Leave slides in 1XPBS until the next step.

9.2. Boil the sections in antigen retrieval solution.

9.2.1. In a 2 L beaker, prepare boiling antigen retrieval solution using 1x citrate buffer (pH 7.0) at 95 °C on a heating block.

9.2.2. Once boiling, place the slides in a metal rack and submerge in the boiling antigen retrieval solution for 20 min.

9.2.3. Carefully remove the beaker from the heating block with the slides still submerged and allow the solution to slowly return to room temperature for about 30 min.

9.2.4. Place the slides in 1XPBS until next step.

9.3. Block the sections in blocking buffer (1X PBS, 5% Goat Serum, 0.3% Triton X-100).

9.3.1. Draw a hydrophobic circle around the edges of each section with a PAP pen.

9.3.2. Place the slides in a humidity tray with distilled water at the bottom of the tray.

9.3.3. Pipette 100 μL blocking buffer onto the sections making sure all of the tissue is covered by fluid (may require more than 100 μL).

9.3.4. Incubate sections with blocking buffer for 1 h.

9.4. Incubate sections with primary antibody.

9.4.1. Prepare one solution with two primary antibodies to probe each section for CD31 (ECs) and smooth muscle actin (pericytes) diluted in dilution buffer (1X PBS, 1%BSA, 0.3% Triton X-100).

Note: Here, anti-CD31 concentration is 1:50 and anti-smooth muscle actin concentration is 1:300.

9.4.2. Incubate the sections overnight at 4 °C with 100 μL primary antibodies in a humidity tray containing distilled water.

9.5. Wash the slides three times in 1X PBST (1X PBS +0.1% Tween20) for 5 min each wash.

9.6. Incubate sections with secondary antibody.

9.6.1. Prepare a solution with a fluorophore conjugated goat anti-rabbit secondary antibody to recognize the anti-CD31 rabbit antibody diluted in dilution buffer.

9.6.1.1. Note: The smooth muscle actin primary antibody is already conjugated with a CY3 fluorophore, so no secondary antibody staining is needed. Goat-anti rabbit secondary antibody concentration is 1:250.

9.6.2. Incubate the sections for 1 h with 100 μL secondary antibody in the humidity tray containing distilled water.

9.7. Wash the slides three times in 1X PBST for 5 min each wash.

9.8. Mount the slides.

9.8.1. Pipette 2-3 drops (~40-60 μL) of antifade mounting solution with DAPI nuclear staining onto each section.

9.8.2. Gently place a no. 1.5 cover slip over the sections and mounting solution, allowing the fluid to spread out over the sections and to the edges of the cover slip. Gently use a finger to press out air bubbles that may be located over the section.

9.8.3. Let the slides dry for at least 1 h before handling.

10. Quantify capillary density and structure[14]

10.1. Count the number of capillaries in H&E stained sections, expressed as #/mm^2.

10.1.1. Acquire photos of four different fields on an upright light microscope under 40x magnification.

Note: Capillaries are identified as tubes filled with red blood cells.

10.1.2. Alternatively, instead of manually counting capillaries, use image analysis software (e.g. Wimasis Image Analysis) to quantify various angiogenesis parameters such as vessel number, average vessel length and diameter, and branch points.

10.2. Analyze capillary structure in immunofluorescent stained sections.

10.2.1. Acquire photos of four different fields on an inverted fluorescent microscope with FITC and TxRed filter cube.

10.2.1.1. Use a FITC cube to image ECs by excitation at 488 nm wavelength and use a TxRed cube to image pericytes by excitation at 594 nm wavelength.

REPRESENTATIVE RESULTS

Representative H&E and immunofluorescent staining of matrix gel plug sections are shown in Figure 2. Sections from EC only plugs display some vessels that are mostly not perfused with blood (Figure 2 top left, black arrows) whereas plugs containing both

ECs and pericytes display several perfused vessels with larger diameters and complete pericyte coverage, as evidenced by positive SMA staining immediately adjacent to CD31-positive ECs. These results suggest that pericytes play a substantial role in nascent blood vessel formation and that this dynamic process can be recapitulated and easily analyzed in an *in vivo* model.

FIGURE LEGENDS

Figure 1. Tissue orientation when embedded in a tissue cassette. After the matrix gel plug (yellow) is isolated, it is in between the mouse's muscle (purple) and skin (green) layers. The plug is placed in the tissue cassette as shown so that all three layers can be seen across the surface that will be cut first during sectioning. A) View from the top of the cassette, B) View from the side of the cassette.

Figure 2. Representative H & E (top row) and immunofluorescence (bottom row) of matrix gel. Images show the appearance of ECs alone (left images) and ECs plus healthy pericytes (Pc) (right images). Human and murine ECs are labeled in green (bottom images) and pericytes (α smooth muscle actin (αSMA)) are labeled in red (bottom right), and nuclei are stained blue with DAPI stain. Scale is 25 μm. This figure has been modified from a previous publication[8].

DISCUSSION

The matrix gel plug assay has proven to be a convenient and powerful method to evaluate gene regulation in angiogenesis, angiogenic and antiangiogenic compounds *in vivo*, and to supplement *in vitro* tests. Here, we describe in detail a novel matrix gel plug assay of human angiogenesis that investigates the interaction between endothelial cells and pericytes during vessel formation.

There are a few novel and critical steps in this protocol. Cells in low passage (passage 1-4) are preferable because young cells will be more viable during the 14 day experimental setting. The number of endothelial cells used is minimum one million and the ratio of EC to pericyte is 5:1; however, increased pericyte cell number will enhance its coverage on capillaries. For the experimental group of endothelial cells alone, the total number of cells injected should be the sum of endothelial cells and pericytes, which is 1.2 million, therefore, the total cell number per plug is consistent. It is important to calculate the appropriate amounts of cell numbers and matrix gel volume. The basic formula is 1.2 million cells in 200 μL of matrix gel. In addition, before injection, keep pipet tips, syringes, matrix gel, and cell pellets on ice at all times. Since matrix gel is viscous and in order to avoid air bubbles forming during cell suspension, cut about 1 cm off of the tip of 1 mL pipet tips to allow for better flow. Do not mix matrix gel with cells until mice are ready to be injected. Each plug injection should take less than one minute. One mouse can carry two plugs, one on each side of its back. When extracting the plugs make sure to keep the plug sandwiched between the muscle and skin layers, then the matrix gel plug will be well collected after isolation.

The drawbacks of the matrix gel plug assay are that it is time consuming, costly, and involves tedious and delicate steps such as injection and plug isolation. If these steps

were to fail, the results would be ruined and the process would have to be repeated again with new mice and materials.

However, the data is reproducible and provides flexibility in terms of experimental design. For example, growth factors or inhibitors could be administered to the plug at different stages of vascular development, which can be used for drug validation. Furthermore, incorporation of other cell types, such as growth-arrested cells, transfected cell lines or tumor cell lines, into the matrix gel plug is another possibility for manipulating the endothelial cell phenotype during angiogenesis. Our protocol utilizes this flexibility and introduces human-derived pericytes along with ECs to allow formation of fully functional, pericyte covered, hybrid vessels *in vivo*. Our protocol also describes how to analyze the histological sections from these plugs for critical angiogenesis endpoints, including tube number and tube length, with these two cell types present.

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DISCLOSURES
No competing financial interests enclosed.

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Figure 1. Tissue orientation when embedded in tissue cassette. After matrix gel (yellow) is isolated, it is in between the muscle (purple) and skin (green) layers. A)

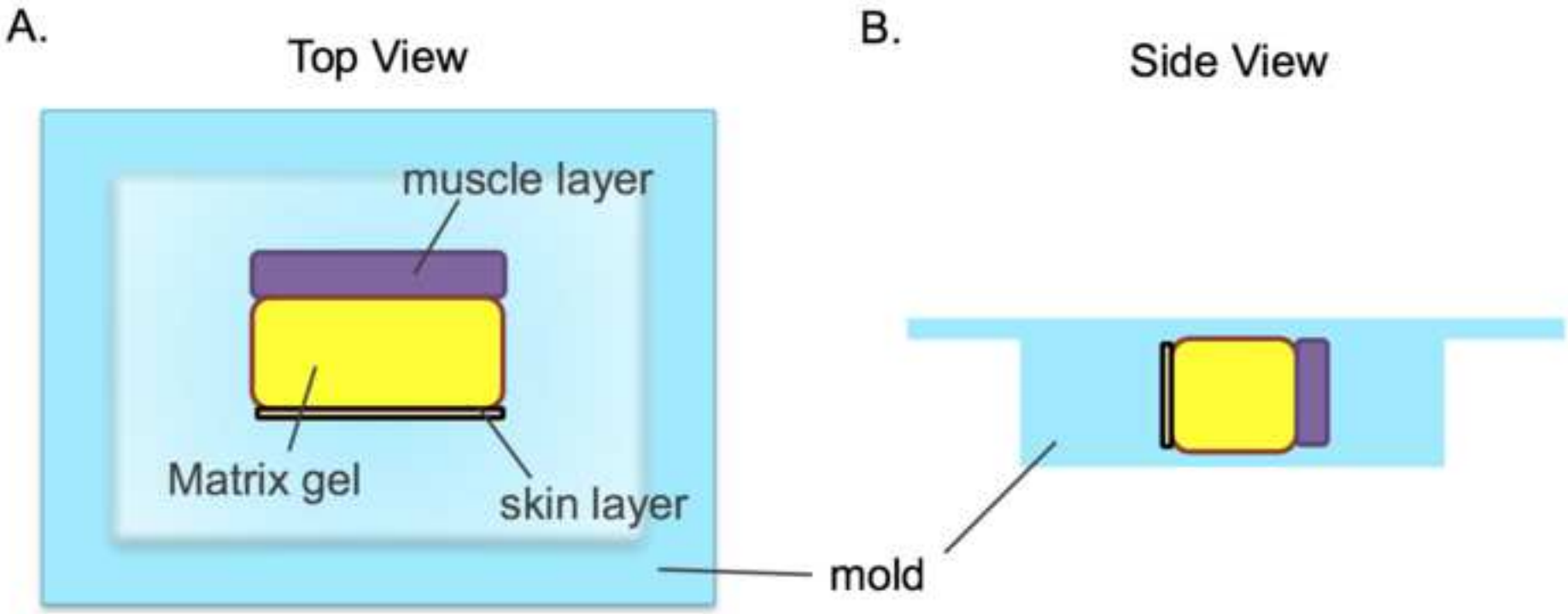
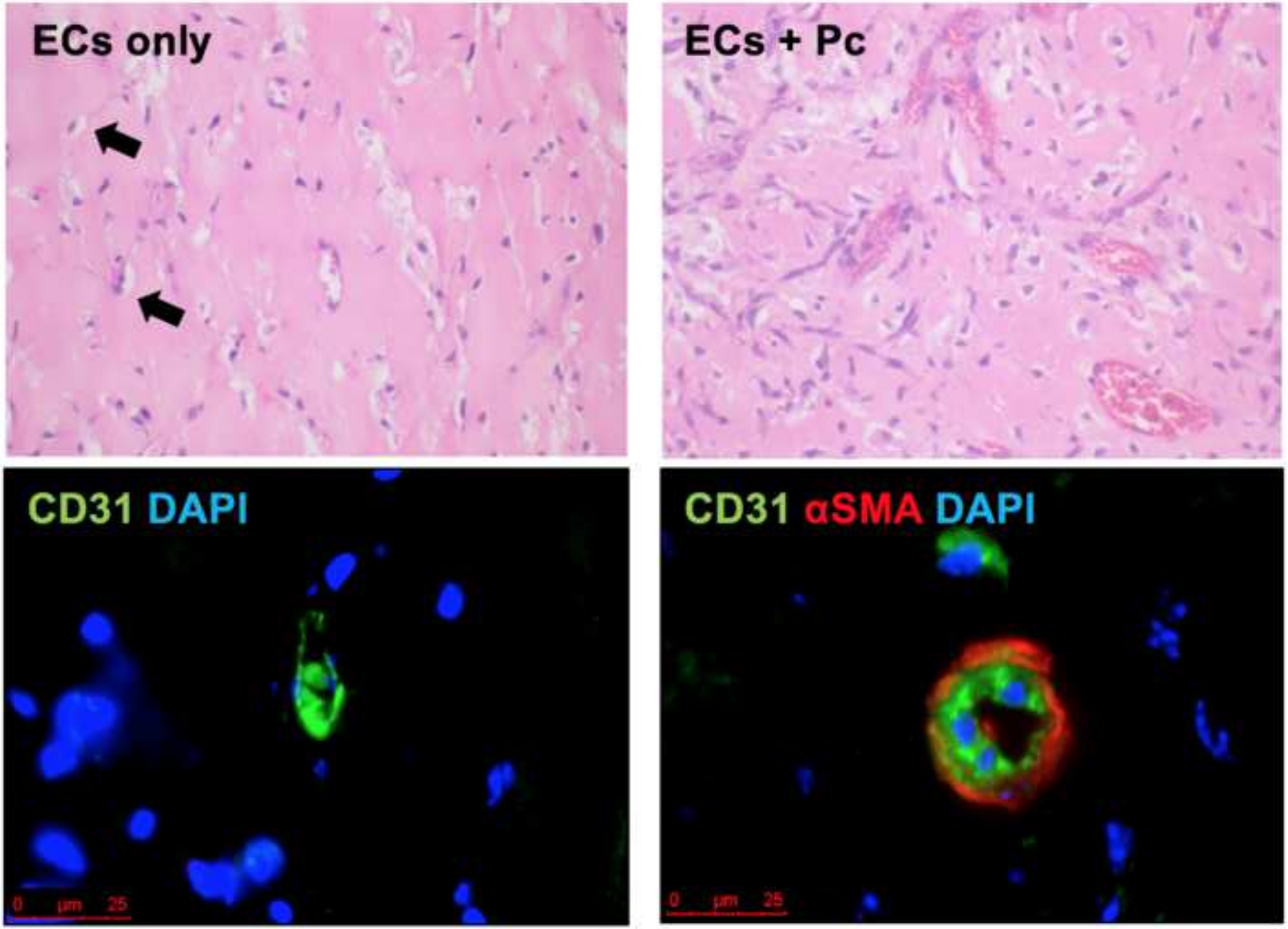


Figure 2. Representative H & E (top row) and immunofluorescence (bottom row) images of matrigel plug. Images show the appearance of ECs alone (left images) and

Click here to download Figure Figure 2.png



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
PBS (Phosphate buffered saline)	Corning	21-031-CV	
0.25% Trypsin/0.53mM EDTA	Corning	25-053-CI	
Endothelial Cell Media (ECM) kit	Sciencell	1001	includes media, EC growth s
Pericyte Media (PM) kit	Sciencell	1201	includes media, Pericyte gr
primary human pulmonary microvascular endothelial cells	Pulmonary Hypertension Breakthrough Initiative		this cell type is also availabl
primary human pulmonary pericytes	Pulmonary Hypertension Breakthrough Initiative		this cell type is not available
bFGF (basic Fibroblast Growth Factor)	Peprotech	100-18B	stock solution is 50ug/ml in
Matrigel Basement Membrane Matrix	BD	356237	
28G 1cc Insluin Syringe	BD	329410	
SCID (Severe Combined Immune Deficiency) mice	The Jackson Laboratory	5557	NOD.SCID IL2R gamma kno
1.5mL microcentrifuge tubes, sterile	Thermo Fisher Scientific	05-408-129	
15 mL screw top tubes, sterile	BD Biosciences	352096	
PAP pen	Life Technologies	8899	
hemocytometer	ThermoFisher Scientific	02-671-6	
Nair hair removal cream	Walmart		
anti-human CD31 primary antibody	LifeSpan Biosciences	LS-B4737	working solution is 1:50
anti-human Smooth Muscle actin CY3 primary fluorescent antibod	Sigma	C6198	working solution is 1:300

goat anti-rabbit secondary antibody; 488/green	ThermoFisher Scientific	A-11008	working solution is 1:250
Prolong Gold DAPI solution	Cell Signaling	8961S	
microscope slides	VWR	48300-047	
no. 1.5 cover slips	Thermo Fisher Scientific	12-544-D	
citrate buffer 10x	Millipore	21545	
extra fine surgical scissors	Fine Science Tools	14084-08	
Formalin (paraformaldehyde)	Thermo Fisher Scientific	245-685	
Tissue cassettes	Simport	M492-12	
goat serum	Dako	X0907	

supplement, and penicillin/streptomycin, each supplied at the appropriate volume for easy mixing
owth supplement, and penicillin/streptomycin, each supplied at the appropriate volume for easy mixing

le commercially. Cells used at passage 1-4

e commercially, but brain paricytes are. Cells used at passage 1-4
0.1% BSA in PBS, aliquots at 50 uL and stored at -20 degrees

ckout strain is the best strain; 4-6 weeks of age

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Title of Article: In Vivo Study Of Human Endothelial–Pericyte Interactions by Implantation Of Matrigel Plugs In SCID Mice

Author(s): Ke Yuan, Mark Orcholski, Ngan F. Huang and Vinicio de Jesus Perez

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CORRESPONDING AUTHOR:

Name:	Vinicio de Jesus Perez
Department:	School of Medicine
Institution:	Stanford University
Article Title:	In Vivo Study Of Human Endothelial–Pericyte Interactions by Implantation Of Matrigel Plugs In SCID Mice
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Dear Dr. De Jesus Perez,

Your manuscript JoVE54617R2 "In Vivo Study of Human Endothelial-Pericyte Interaction Using the Matrix Gel Plug Assay" has been peer-reviewed and the following comments need to be addressed. Please keep JoVE's formatting requirements and the editorial comments from previous revisions in mind as you revise the manuscript to address peer review comments. Please maintain these overall manuscript changes, e.g., if formatting or other changes were made, commercial language was removed, etc.

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Your revision is due by **Jun 20, 2016.** Please note that due to the high volume of JoVE submissions, failure to meet this deadline will result in publication delays. To submit a revision, go to the JoVESubmission Site and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.

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Mala Mani,
Science Editor
JoVE
1 Alewife Center, Suite 200, Cambridge, MA 02140
tel: 6174019173

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• Please provide references for section 10 (quantification).
Provide reference #14 on section 10.

•Formatting:
-1.5 – Please reference step by entire step number (ie 1.3).
We do not have 1.5??but found 1.4
-Please format centrifuge speeds as “x g” rather than “g” to avoid confusion with units of mass.
Change it at 2.2.2.3
-9.3, 9.4.1 – Should be “Triton X-100”.
Changed

•Grammar:
-2.2.1 – “of in complete”
Not found
-10.1.2 – “images analysis”

Not found
-Line 387 – “staining of matrix gel plug are shown”
Change to “were shown”

-Please end all sentences/steps in a period. Section headings can be left without periods.
All sentences have been punctuated.

•Additional detail is required: 10.2.1.1 – Please specify the wavelengths used.
Added.

•Discussion: Please discuss the future applications of the protocol.
Need to add more.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this manuscript the authors provide in detail a well established technique for understanding the interaction between Endothelial Cells and Pericytes during angiogenesis. This manuscript will be of interest to investigators who wish to understand these interactions during angiogenesis and want to explore the effects of drugs on these interactions. Therefore this methods paper should be published. However, before publication several things need to be addressed as outlined below.

Major Concerns:
1) Line 103-104: The authors talk about "critical angiogenesis endpoints" however they do not specify what these end points are. The authors should specify these endpoints and also state if the proposed technique will be useful in understanding all of the endpoints or only a few?

We added in brief two examples of typical angiogenesis endpoints that can be assessed on histological sections.

2) The authors begin to describe the protocol with 1. Matrix Gel Preparation, but do not use the matrix gel till after step 2 (which involves growing the cells and only then can they use the matrix gel prepared in step 1). I believe it would be more logical to start the protocol with step #1 being Cell Preparation, followed by Step #2, Matrix Gel Preparation.

Agree. We changed the order as suggested.

3) Line 117-118: The matrix gel is thawed to obtain a homogenous solution. Does this solution need to be mixed by vortexing, flicking or pipetting up and down, to obtain this homogenous solution? If so, this should be stated. If not then it should be stated that no mixing is required.

We added “no mixing required.”

4) Line 120: Matrix Gel aliquots are to be stored at -20°C, will this result in refreezing? If so this should be stated and if not then it should be stated that this lower temperature will not result in refreezing.

We changed the temperature to 4 degrees.

5) Line 125-127: It is stated that the stock solution aliquot be stored at -80°C. Will this result in refreezing of the stock solution? If so, then there should be a step inserted here to talk about the need to re thaw, otherwise it should be mentioned that the stock solution will not freeze.

This has been revised.

6) Line 159-160: It is suggested to have "at least four plugs" why at least four plugs are recommended? This should be clarified.

This has been revised.

7) Line 168: Trypsin/EDTA mixture is mentioned but the concentration of EDTA is not specified. This should be specified.

We added 0.25%Trypsin/2.21mM EDTA.

8) Line 228-229: Do you need to mark the bump here as well? I guess you would need to, if so please mention the need to repeat step 5.5.

We added as suggested.

9) Line 239: This step talks about the need for removal of regrown hair. How should this be accomplished? Should the user of the protocol use step

4.3 here? if so this should be specified. Also, for step 4.3, is there a recommended hair removal cream?

Repeat 4.3 was added.
The name of Nair hair removal cream was added and included in the materials spreadsheet.

10) Line 245-247: What type of fine scissors should be used (make and model)? Please make a statement that this information is provide in the table. Also, the step 6.2.2.1 should be rewritten as it does not convey this step in a clear fashion.

The surgical scissors we use are listed in the materials spreadsheet. We are not sure how to make step 6.2.2.1 more clear, but I added the word ‘down’ to indicate how to cut in relation to the skin and muscle layers.

11) Line 255-257: Does the fixation step require rocking of the fixative with the plug or this is not needed? Whatever the case this should be clearly stated.

‘No need to rock’ was added.

12) Line 265: Where can the tissue cassette mold be purchased?

They were purchased from Simport, catalog number M492-12. This information has been added to the materials spreadsheet.

13) Line 387: The staining is of Matrix Gel Plug "Sections" and not the entire plug..this should be corrected to state that ....staining of matrix gel plug SECTIONS are shown. Similarly, in Line 388 it should state that SECTIONS FROM EC only plugs displayed some vessels.....

This has been revised.

14) Line 388 it is stated that "EC only plugs displayed some vessels that were mostly perfused with blood" Looking at the figure it is not clear what is being referred to. The above fact should be indicated with a box, arrow or a closeup to show vessels with perfused blood.

We added black arrows pointing to the reference vessels on Figure 2A.

15) Line 391-392: States that " these results demonstrate that pericytes play a substantial role in nascent blood vessel formation". How can the author come to this conclusion based on just the association of pericytes with the ECs? At best, this is suggestive of a role for pericytes in nascent blood vessel formation. This should be corrected.

This sentence was revised as suggested.

16) Line 410-412: Please provide citations for the fact stated here.

Not found

17) Line 416-430: This paragraph should be rewritten for better flow and clarity. For example, why is passage 1-4 preferable? Also, this paragraph lists a set of precautions instead of being a discussion of the representative results. Wouldn't this be better placed in a subheading of precautions?

We added a brief explanation as to why passage 1-4 is preferable.
In the second revision comments, reviewers suggested to put a set of precautions in the discussion section.

18) Figure 2: The edges of individual panels are not aligned, the color for labeling the top panels is not appropriate as it blends into the pink background. The authors can place the green colored font on a background that contrasts clearly with the font color, for example put it on a dark background. Also, if possible then use similar sized fonts for top and bottom panels, increase the font size of bottom panels to the font size used for labeling the top panel. In panel A and B what is being seen should be highlighted through an arrow or a box.

All revised as suggested. Image B was not taken from any area of image A.

19) The authors should consider providing another table outlining the concentrations of solutions used in this protocol.

The concentration of each solution is provided in the materials spreadsheet as well as in the text.

20) Also, the vendor names of some of the materials used is not specified. The names of the missing vendors should be provided.

All materials have been detailed in the materials spreadsheet.

Minor Concerns:
1) Some spelling errors and minor changes:
Line 247: Border instead of Boarder
Line 302: Block instead of Black
Line 439: Instead of "used AS drug validation" consider using "used for drug validation"
2) Line 337: Consider using: "In the morning wash the slides....."
3) Line 383: Insert FILTER to the following :FITC FILTER Cube and TxRed FILTER cube
All revised.

Additional Comments to Authors:
This is an important technique that I would love to see published with the changes suggested above.

Reviewer #2:
Manuscript Summary:
This is an appropriate article for JoVE involving a matrix gel plug angiogenesis assay. Although the assay has been previously described in print format (Refs 7), this submission includes refinements and additional details. The visual demonstration with the JoVE format will be helpful to those who wish to perform the experiments. The authors have provided a detailed description of methods and materials for the experiments. They have discussed appropriate controls and highlighted steps that are particularly critical to the success of the procedure.

Major Concerns:
None

Minor Concerns:
The authors could improve the article with a bit more discussion of pros and cons of this procedure. For example, how does the in vivo matrigel environment differ from the endogenous in vitro environment and the in

vitro matrigel environment? Although the authors touch on some of the issues, a clear paragraph in the discussion would be helpful.

The language used in the discussion has been modified to provide more clarity as to what the pros and cons of the assay are.

Several minor points should be addressed:
l. 86: remove the extra space between 37 and ∘
Corrected
l. 86-9: this is a run-on sentence with poor grammar. Please edit.
Corrected
l. 122: remove the extra space between 20 and ∘
Not found
l. 125: Instructions say to prepare a stock solution, but it appears that the authors purchase this as described. If not, please clarify and indicate source of BSA, if appropriate.
Not found
l. 127: remove the extra space between 80 and ∘
Not found
l. 133-4: Please edit for grammar and clarity.
Not found, the passage at line 133 is void of grammatical errors and is clearly written.
l. 256: Please indicate source of paraformaldehyde in table
This information has been added to the materials spreadsheet.
l. 302: remove the extra space between 95 and ∘
Corrected
l. 302: "black" should be "block"
Corrected
l. 313: indicate source of goat serum in table
Corrected
l. 334: state the temperature of the overnight step
Corrected
l. 398: insert space between Figure and 1
Corrected
l. 398/Fig 1: Consider modifying figure such that the skin layer is a bright yellow for red/green colorblind readers.
Colors have been changed.
l. 411-2: "in vivo" should be in italics
Corrected
l. 416: write out "example" instead of "e.g."

Not found

Additional Comments to Authors:
N/A

[**Editorial recommendation**: Please keep JoVE's protocol requirements in mind as you address the above comment - the protocol must contain sufficient details in order to enable users to accurately replicate your technique. We recommend NOT removing any details from the protocol text.]