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.

Please track the changes in your word processor (e.g., Microsoft Word) or change the text color to identify all of the manuscript edits. When you have revised your submission, please also upload a separate document listing all of changes that address each of the editorial and peer review comments individually with the revised manuscript. Please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.

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](http://www.editorialmanager.com/jove) and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.

Sincerely,

Mala Mani,  
Science Editor

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**Editorial comments:**

• Your manuscript has been modified by your editor, please maintain the current formatting throughout the manuscript. **Please use the updated manuscript located in your Editorial Manager account (under “File Inventory”) for all subsequent revisions**.  
  
• Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.  
  
• JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.  
  
• Please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."  
  
• 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.]