Response to Editor and reviewer’s comments:

Editor:

1.Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Please watch for missing articles (a, an, the, etc.) as well as subject verb agreement. The manuscript would benefit from copyediting for grammar, as there are minor grammatical errors throughout.

**Response:** We had a native English speaker double checked the grammar and corrected the grammar mistakes in the manuscript.

2. Additional detail is required in a number of places:

-2.5: About how long are the segments incubated?

**Response:** We added “for 4 to 6 hours”.

-3.3: What concentration of neutral proteinase?

**Response:** The concentration of neutral proteinase is 50 units/ml.

-4.6: Are the cells split between the two flasks from 4.1, or are they all plated in a single flask as indicated?

**Response:** Yes. The cells are split between the two flasks from 4.1.

3. References: Some DOI missing, journal titles are not abbreviated. 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.

**Response:** We added those missing DOIs into the references.

Reviewer #1:

*Minor Concerns:*

On Figure 1, it appears that angiogenesis is present. Capillary like structures are visible. In older literature this approach was used to study angiogenesis in vitro. The authors should comment on this phenomenon. Is it important to harvest cells before formation of capillary-like structures?

**Response:** We agree that this approach is used to study angiogenesis in vitro and the formation of capillary like structure indicates angiogenesis capacity of the aorta segment. Based on our experience, if we remove the segments after the capillary like structure fully develops, the incidences of smooth muscle cells contamination increase dramatically. So we feel that the best time point to remove the segment is when the network starts to be visible but not developed. In this way, we are able to harvest as many as endothelial cells while keeping the contamination by smooth muscle cells to minimum. We rephrased the protocol and put this comment into the discussion.

It would be also good to provide additional endothelial marker like eNOS.

**Response:** Thank you very much for your suggestion. We added the staining for additional endothelial marker eNOS.

Reviewer #2:

1.The authors used Dil-ac-LDL uptake, Lectin binding, and CD31 staining to characterize the endothelial cells, which is fine. However, it would further strengthen the manuscript if the authors can expend their discussion to include other phenotypical characteristics of endothelial cells, such as other surface antigen expression (i.e. vWF factor, CD144), functional genes (i.e. eNOS, VEGFR) and assessments (tube formation, etc).

**Response:** Thank you very much for the advices. We added discussion about endothelial cell characteristics in the manuscript.

2. Long abstract, line 5: The sentence "the thoracic aorta is quickly removed the mouse body" is confusion. Please rephrase.

Response: We rephrase the sentence to be “the thoracic aorta is quickly removed from the mouse body”.

3. Page 10, line 32: Please remove "that".

**Response:** We removed “that”.

4. Page 11, line 341: Please remove "a".

**Response:** We removed “a”.

Reviewer #3:

1. To characterize the cultured cells, the authors are recommending to use fluorescence. They are currently suggest a specific wavelength (i.e. 519 nm for green fluorescence), but they may include a mentioning that an appropriate wavelength can be used (i.e 518-535 nm according to the instrument).

**Response:** Thank you very much for the suggestion. We replaced “519 nm” for “518-535nm” in the revised manuscript.

*Reviewer #4:*

1. Manuscript has not been written well since there are numerous grammar problems inside. Please have the native English speaker check the grammar.

**Response:** Thank you for the suggestion. We had a native English speaker double checked the grammar and corrected the grammar mistakes in the manuscript.

1. This method is actually a explant method for isolating vascular cells. People normally apply this method to isolate vascular smooth muscles (VSMCs). As the author claimed in the manuscript, the contamination of other cell types cannot be excluded. Although the time to remove the aortic ring is the key, VSMCs start to expand from the ring at day3. Thus, the chance of the VSMC contamination is probably very high. It is necessary to provide the staining for VSMC maker such as smooth muscle actin or calponin to ascertain if there are VSMCs.

**Response:** Thank you for your comments. Yes, people use this method to isolate vascular smooth muscle cells, except that there are two important differences: 1) In the culture of VSMCs, the endothelium layer is mechanically removed before the segments are seeded onto the matrix. In this protocol, every effort is made to ensure that endothelium is intact, viable, and the endothelium has maximum chance to attach to the matrix, e.g. using PBS containing heparin to stable the endothelial cells, avoiding stretching when isolating the aorta, opening up the aortic ring and having the endothelial side facing down. 2) In the culture of VSMCs, the segments are kept in VSMC favoring culture medium, while in the culture of endothelial cells, the segments are kept in endothelial cell-favoring culture medium. However, we also agree that the above mentioned efforts cannot completely exclude the contamination of smooth muscle cells.

We added the staining for calponin as suggested. In our experiment we observed that most of the cells were negative for calponin.

1. The authors only provided CD31 staining to characterize EC, please examine other EC specific gene expression such as VEGFR2, eNOS and VE-Cadherin for further confirmation.

**Response:** Thank you very much for the suggestion. We added the staining for VEGFR2, eNOS and VA-Cadherin in the revised manuscript.