Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58040R2
Full Title:	Nondestructive monitoring of degradable scaffold-based tissue-engineered blood vessel development using optical coherence tomography
Keywords:	vascular tissue engineering; bioengineering; optical coherence tomography; polyglycolic acid; biodegradation; mechanical conditions
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TITLE:

Nondestructive Monitoring of Degradable Scaffold-Based Tissue-Engineered Blood Vessel Development Using Optical Coherence Tomography

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KEYWORDS:

Bioengineering, optical coherence tomography, vascular tissue engineering, polyglycolic acid, biodegradation, mechanical conditions

SUMMARY:

A stepwise protocol for nondestructive and long-period monitoring of vascular remodeling and scaffold degradation in a real-time culture of biodegradable polymeric scaffold-based tissue-engineered blood vessels with pulsatile stimulation is presented, using optical coherence tomography.

ABSTRACT:

Engineered vascular grafts with structural and mechanical properties similar to natural blood vessels are expected to meet the growing demand for arterial bypass. The characterization of the growth dynamics and remodeling process of degradable polymer scaffold-based tissue-engineered blood vessels (TEBVs) with pulsatile stimulation is crucial for vascular tissue engineering. Optical imaging techniques stand out as powerful tools for monitoring the vascularization of engineered tissue, enabling high-resolution imaging in real-time culture. This paper demonstrates a nondestructive and fast real-time imaging strategy to monitor the growth and remodeling of TEBVs in long-term culture by using optical coherence tomography (OCT). The geometric morphology is evaluated, including the vascular remodeling process, the wall thickness, and a comparison of TEBV thickness at different culture time points and in the presence of pulsatile stimulation. Finally, OCT provides practical possibilities for the real-time observation of the degradation of polymer in reconstructing tissues under pulsatile stimulation or not and in each vessel segment, compared with the assessment of polymer degradation using

a scanning electron microscope (SEM) and a polarized microscope.

INTRODUCTION:

TEBVs are of the most promising material as an ideal vascular graft[1]. In order to develop grafts that are clinically useful, with structural and functional properties that are similar to those of native vessels, multiple techniques have been designed to maintain vascular function[2,3]. Although there have been engineered vessels with acceptable patency rates during implantation and in phase III clinical study[4], long-term culture and high costs also show the necessity of monitoring the development of TEBVs. Trying the understand extracellular matrix (ECM) growth, remodeling, and adaptation processes in TEBVs in the biomimetic chemo-mechanical environment can provide crucial information for the development of vascular tissue engineering.

The ideal strategy to track the development of small-diameter engineered vessels[5] should be nondestructive, sterile, longitudinal, three-dimensional, and quantitative. TEBVs under different culture conditions could be assessed by this imaging modality, even including changes before and after vascular transplantation. Strategies to describe features of living engineered vessels are needed. Optical imaging techniques allow the visualization and quantification of tissue deposition and biomaterials. Other advantages are the possibility to enable deep-tissue and label-free imaging with high resolution[6,7]. However, image-specific molecules and less easily accessible optical equipment for real-time monitoring are significant practical obstacles, which have limited the extensive application of nonlinear optical microscopy. OCT is an optical approach with intravascular imaging modality as a widely-used clinical tool to guide cardiac interventional therapy[8]. In the literature, the method of OCT has been reported as a way to assess the wall thickness of TEBVs[9,10], coupled with affirmative imaging modalities for vascular tissue-engineering research, whereas the dynamics of engineered vascular growth and remodeling has not been observed.

In this manuscript, we detail the preparation of biodegradable polymeric scaffold-based TEBVs for a four-weeks culture. Human umbilical arteries vascular smooth muscle cells (HUASMCs) are expanded and seeded into porous degradable polyglycolic acid (PGA) scaffolds in the bioreactor. Biodegradable polymers play the role of a temporary substrate for the tissue engineering and have a certain degradation rate[11]. In order to ensure an appropriate match between scaffold degradation and neo-tissue formation, ECM and PGA scaffolds are crucial factors for effective vascular remodeling. The perfusion system simulates the biomechanical microenvironment of native vessels and maintains a consistent deformation under pressure stimulation.

The aim of the presented protocol is to describe a relatively simple and nondestructive strategy for TEBVs imaging and the long-term monitoring of culture. This protocol can be utilized for the visualization of morphological changes and thickness measurements of engineered vessels under different culture conditions. Additionally, the analyses of polymer-based materials degradation in the tissue-engineering scaffolds can be performed for the identification. By combining the use of an SEM and a polarized microscope in this protocol, a correlation and quantification of ECM distribution and PGA degradation can be made, which can facilitate assessing scaffold degradation combined with OCT imaging.

PROTOCOL:

1. Degradable PGA Scaffold-based Tissue-engineered Vessels Culture

1.1. PGA scaffold fabrication

1.1.1. Sew PGA mesh (19 mm in diameter and 1 mm thick) around silicone tubing sterilized by ethylene oxide (17 cm in length, 5.0 mm in diameter, and 0.3 mm thick) using a 5-0 suture.

1.1.2. Sew polytetrafluoroethylene (ePTFE, 1 cm in length) with a 4-0 suture onto each end of the PGA mesh, overlapped by 2 mm.

1.1.3. Dip PGA scaffolds in 1 mol/L NaOH for 1 min to adjust the spatial structure of the mesh and soak it with tissue culture grade water 3x for 2 min each. Each time, gently pat dry the scaffold with a tissue paper. Then, dry up the scaffolds in a hood with a blower for 1 h.

1.2. Assembly of the bioreactor and the Y-junction for OCT imaging

1.2.1. Soak the self-developed glass cylindrical bioreactor (10 cm in diameter and 11.7 cm in height, with four lips inside and four side-arms outside the reactor as shown in **Figure 1**), PGA scaffolds, silicone tube (with an external diameter of 5 mm and a thickness of 0.3 mm), biocompatible tubes, and connectors in a 95% ethanol tank for 2 h.

1.2.2. Pull the PGA scaffold through the side-arms of the bioreactor connected to one side with a connector, as well as to another side with the Y-junction used to deliver OCT guidewire. Assemble another PGA scaffold in the bioreactor in the same way. Please refer to **Figure 1**.

1.2.3. Fit ePTFE to the bioreactor lips by tightening it with 5-0 sutures.

1.2.4. Put the bioreactor in the ethanol tank again for 1 h and dry up overnight in a hood with the blower on.

1.3. Seeding of HUASMCs and static bioreactor conditioning

1.3.1. Isolate HUASMCs from human umbilical arteries by standard explant techniques.

1.3.2. Expand and maintain the cells in smooth muscle cell growth medium composed of DMEM medium, 20% fetal bovine serum, 2.36 mg/mL HEPES, 100 U/mL penicillin G, 50 µg/mL proline, 20 µg/mL alanine, 50 µg/mL glycine, 1.5 µg/mL CuSO4, 50 µg/mL ascorbic acid, 10 ng/mL basic fibroblast growth factor, and 10 ng/mL platelet-derived growth factor.

1.3.3. Seed HUASMCs at a concentration of 5 x 10^6 cells/mL in the above culture medium onto the PGA scaffolds.

1.3.4. Insert one feeding tube (5 mm in diameter, 15 cm in length) and three short tubing segments (5 mm in diameter, 7 cm in length) for gas exchange through the silicone stopper lid. Attach PTFE 0.22-μm filters to each air change tube and one heparin cap to the feeding tube.

1.3.5. Put a stir bar (1.5 cm in length) in the bioreactor with a stirring speed of 13 rounds/min. Assemble the glass bioreactor, silicone stopper lid, and PGA scaffold into the culture system.

1.3.6. Allow HUASMCs to adhere for 45 min by leaning the bioreactor every 15 min with stand, to the left and right. The reactor ports and joints are all sealed with paraffin film.

1.3.7. Fill the culture chamber with 450 mL of the HUASMCs culture medium. Place the overall bioreactor in a humidified incubator with 5% CO_2 at 37 °C.

1.3.8. Connect the Luo-Ye pump, phosphate buffered saline (PBS) bag, and driver with the biocompatible tubes to create the perfusion system. Open the driver to fill the tubes with PBS.

1.3.9. Press the stop button and turn off the power of the drive device. Grow the seeded scaffolds under static culture for one week.

1.3.10. Change the culture medium every 3 - 4 d by aspirating half of the old medium through the feeding tube and refilling the reactor with an equivalent amount of fresh culture medium.

1.4. Preparation of the perfusion system for OCT imaging

1.4.1. Pump fluids in the PBS bag to circulate them through the biocompatible tubes and back to the bag.

1.4.2. Open the power of the driver and regulate the pump setting with a frequency of 60 beats/min and an output systolic pressure of 120 mmHg. Adjust the mechanical parameters according to the needs of the tissue-engineering vascular culture.

1.4.3. Click the run button to make the perfusion system work. Provide the above fixed pulsatile stimulation to the vessels for 3 weeks by iteratively pressurizing the biocompatible tubes[10,12] after 1 week of static culture.

2. Performing Optical Imaging with OCT

2.1. Use a light source to ensure the axial resolution of 15 - 20 μm and the image depth of 1 - 2 mm to identify the structure of the TEBVs based on the frequency-domain OCT intravascular imaging system[9].

2.2. Turn on the power switch and open the image capture software.

2.3. Connect the fiber optic imaging catheter to the drive-motor and optical controller (DOC) with the catheter automatic retreat function.

2.4. Set the parameters of the image acquisition rate to 50 frames/s with an automatic pullback speed of 10 mm/s.

2.5. Attach the imaging catheter to the Y-junction *via* the heparin cap with an 18-G needle.

2.6. Place the catheter into the silicone tube and identify the suture tightness of the PGA mesh before loading the PGA scaffold onto the bioreactor.

2.7. Place the catheter tip over the region of interest. Adjust the pullback device and check for the image quality[8].

2.8. Acquire images at 1, 4, 7, 10, 14, 17, 21, and 28 days in culture for each individual TEBV and save them sequentially with a real-time observation of the TEBV microstructure, including surface morphology, internal structure, and composition.

2.9. Repeat the measurement 3x to get a reliable measurement of the engineered vessels each time. Capture a series of images throughout testing using the image capture software.

3. Imaging Analysis

3.1. Use image analysis software to measure the TEBV wall thickness. Select the image to be analyzed. Click the tracking tool to automatically identify the inner side of the TEBV with the software and manually sketch the outer side. A diagram of thickness will appear on the screen.

3.2. Repeat the measurement for 5x to get a reliable measurement of the constructs. The OCT analysis was performed by two independent investigators blinded to the obtained information.

4. Harvest of the TEBVs and Tissue Processing

4.1. Open the silicone stopper lid placed over the bioreactor when the culture is finished and discard the culture medium. Lose the ePTFE from the bioreactor lips and cut the silicone tubes from the outer side of the ePTFE with scissors. Harvest TEBVs from the bioreactor and cut them into sections for a scanning electron microscopy examination.

4.2. Take out the rest of the TEBVs and cut them into 4-µm thick sections. Pull out the supporting silicone tube and fix the sections with 1% paraformaldehyde. Perform routine histological staining with Masson's trichrome and Sirius red to examine the morphology of collagen and PGA[10,13,14].

4.3. To assess the PGA content and collagen component, observe histologic samples with Sirius red staining through a polarized microscope. PGA remnants are clearly demarcated through

birefringence and the remnant area can be quantified based on the cross-sectional area[10].

REPRESENTATIVE RESULTS:

The three-dimensional culture system consists of a culture chamber in the bioreactor and a perfusion system with a closed fluid cycle[10,13] (**Figure 1**). The OCT imaging catheter was inserted into the distal end of the Y-junction and pulled back into the silicone tube for imaging. OCT imaging was first used to delineate the structural characterization of biodegradable polymeric scaffold-based TEBVs during bioreactor cultivation.

Figure 2 shows the process of engineered vascular remodeling through this cross-sectional imaging of tissue microstructure in real-time. Geometric morphology was evaluated, including wall thickness, degradable PGA content, and a comparison of the TEBV thickness at different culture time points, as well as in the presence of pulsatile stimulation. A trend of decreasing thickness of, and dramatic changes in, the engineered tissue within the first two weeks of culture was seen, suggesting signal-rich PGA gradual degradation and a transition of the structure of new tissue from loose to tight. At 21 days in culture, the vasculature had formed a smooth structure, with an extracellular matrix evenly distributed and high signal components mostly dissipated. The wall thickness of TEBVs with an even signal increased gradually after three weeks of culture. This remodeling occurred earlier and the morphological changes manifested more obviously in the dynamic group (**Figure 3**). Thereby, OCT enables the imaging of engineered vascular morphology to be visualized *in situ* and in real-time in the course of long-running culture.

Figure 4 compares OCT images with histopathological finds of TEBVs after 4 weeks of culture. Masson's trichrome staining demonstrates collagen fibers distributed in a certain direction along with PGA remnants in the media layer of the engineered vessels (**Figure 4B**). Sirius red staining revealed PGA remnants and a collagen component by using a polarized microscope (**Figure 4C**). Scanning electron micrographs of engineered vessels with a compact microstructure were compared with the histological assessment (**Figure 4D**). Taken together, the OCT images showed PGA with different sizes and a porous network structure. The structure of the PGA scaffold had no obvious change and was swollen because it came into direct contact with the culture medium in an early stage of the culture. However, the signal intensity of the PGA was reduced. PGA components were disintegrated and replaced with cells and extracellular matrix. Fewer fragments were seen over a four-week period. SEM images of cross-sectional engineered vessels demonstrated fiber rupture to the extension of the incubation time. The material and extracellular matrix composites had a honeycomb-like structure with less transparency and were more compact.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the tissue-engineering vascular culture system, consisting of a culture chamber in the bioreactor and a perfusion system for OCT imaging. The pulsatile pump provided a stable fluid flow simulating the biomechanical microenvironment. The OCT imaging catheter was pulled back into the silicone tube in the culture chamber.

Figure 2: The microstructure of tissue-engineered blood vessels during culture. Over culture-time, signal-rich PGA gradually degraded, and the structure of new tissue went from loose to tight. The TEBVs had a smooth surface and an abundant extracellular matrix evenly distributed after four weeks of culture. It showed the process of engineered vascular remodeling through cross-sectional images in real-time. This figure has been modified from Chen *et al*.[10].

Figure 3: Comparison of TEBV wall thickness change during vascular remodeling in dynamic and static groups obtained from OCT measurements. The error bars indicate standard error. This figure has been modified from Chen *et al*.[10].

Figure 4: Imaging of biodegradable polymer-based tissue-engineered blood vessels. (**A**) This panel shows an OCT image of a TEBV after four weeks of culture. M = culture medium, S = silicone tube, and the white arrow indicates the TEBV. The red arrow indicates a PGA fragment. (**B**) Masson's trichrome staining demonstrated well-organized collagen fibers along with the residual content of PGA in the media layer of engineered vessels. The scale bar = 100 µm. (**C**) Sirius red staining revealed PGA remnants by using a polarized microscope. The green arrow indicates the PGA fragment. The scale bar = 100 µm. (**D**) Scanning electron micrographs of engineered vessels with a compact microstructure are shown for a comparison with the histological assessment. The scale bar = 50 µm.

DISCUSSION:

To generate engineered vessels with structural and mechanical properties similar to those of native blood vessels can shorten the time for clinical use and is the ultimate goal of vascular engineering. Optical imaging techniques permit the visualization of tissue-engineered vascular-specific components, which cannot monitor individual constructs throughout culture and expose grafts to a culture environment without compromising sterility[7]. In this article, the culture chamber is separated from the perfusion system. The relatively independent perfusion system guarantees a decrease in the risk of pollution during culture and the placement of OCT guidewire. Meanwhile, this intraluminal imaging modality adopted the easy and safety monitoring of TEBVs *in situ* with a high resolution approaching that of histopathology, which makes the assessment of the TEBV growth status more practical and is even expected to be used before or after implant placement.

The current protocol indicates a readily available, fast real-time and nondestructive imaging strategy to evaluate degradable polymer-based engineered vessel development using catheter-based OCT. Through the observation of the dynamic process, some main factors affecting vascular engineering, such as contamination or unmatched cell-material interaction leading to tissue loss, can be detected early on. Critical steps to ensure the efficacy of the protocol include the fabrication of a NaOH-modified PGA scaffold, the successful seeding of HUASMCs in the scaffold, the separation of the sterile culture system from the monitoring system, and a fast catheter operation process.

This technique can be utilized to assess degradation states and the complex structure of PGA scaffolds blended in with new tissue. The polymeric scaffold with a porous network structure

degrades gradually and dominates the process of vascular remodeling in the first three weeks, which are important for cell adhesion and extracellular matrix deposition with a three-dimensioned structure for nutrient exchange and as a signal carrier[15,16]. For the quantification of PGA remnants in engineered vessels clearly identified by Sirius red-stained images, the use of polarized microscopes[17] in degradable scaffold-based vascular engineering has the potential to become the standard evaluation after cultivation. Hence, OCT imaging combined with a polarized microscope might serve as a qualitative and quantitative method for assessing PGA degradation in vascular engineering.

A limitation of this technique is the resolution limit to assess cell proliferation, distribution, and cell-cell and cell-ECM interaction during engineered vascular remodeling. We hope to find suitable methods to investigate the TEBV microstructure at a cellular or subcellular level[18] and quantify growth kinetics. With a quantitative analysis of average optical signals of OCT imaging, we might be more aware of the mechanism of material degradation in vascular engineering. Such experiments are being considered for our future studies.

Overall, the results presented here show that OCT is a readily available, fast real-time, and nondestructive imaging strategy to monitor the growth and remodeling of TEBVs. It is utilized to characterize structural architectural features and the long-term remodeling process of engineered vessels. The application of a polarized microscope, which provided supplementary evidence for the quantification of polymeric remnants in engineered vessels, might be useful for assessing scaffold degradation combined with OCT imaging. Taken together, the current protocol holds promising value for the application of OCT in vascular tissue engineering.

ACKNOWLEDGMENTS:
The authors would like to acknowledge the Science and Technology Planning Project of the Guangdong Province of China (2016B070701007) for supporting this work.

DISCLOSURES:
The authors have nothing to disclose.

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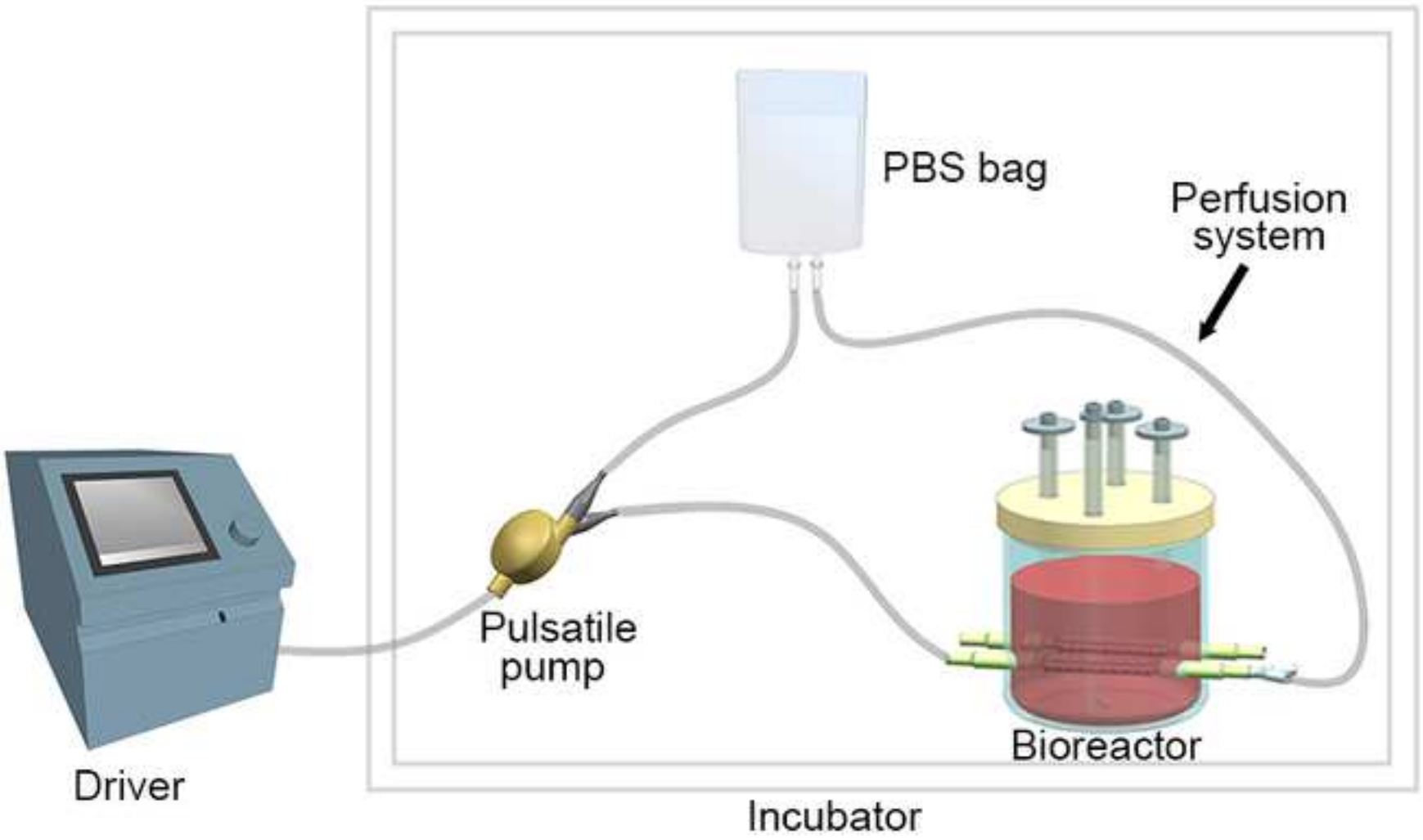

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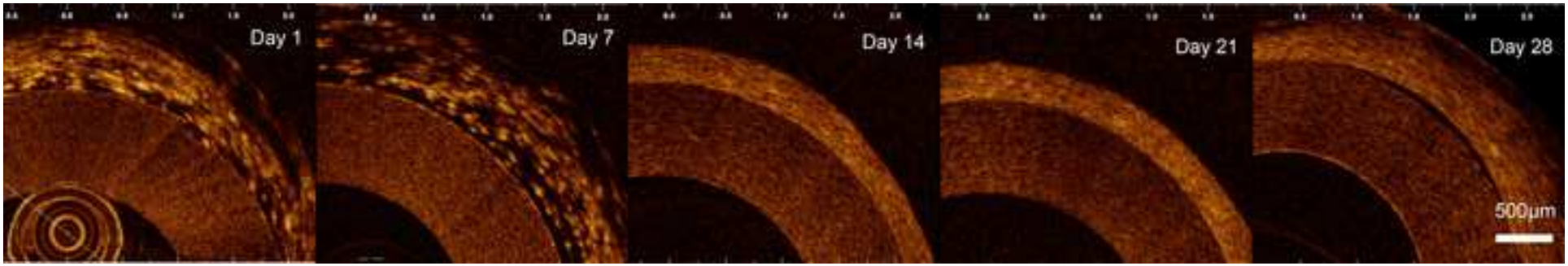

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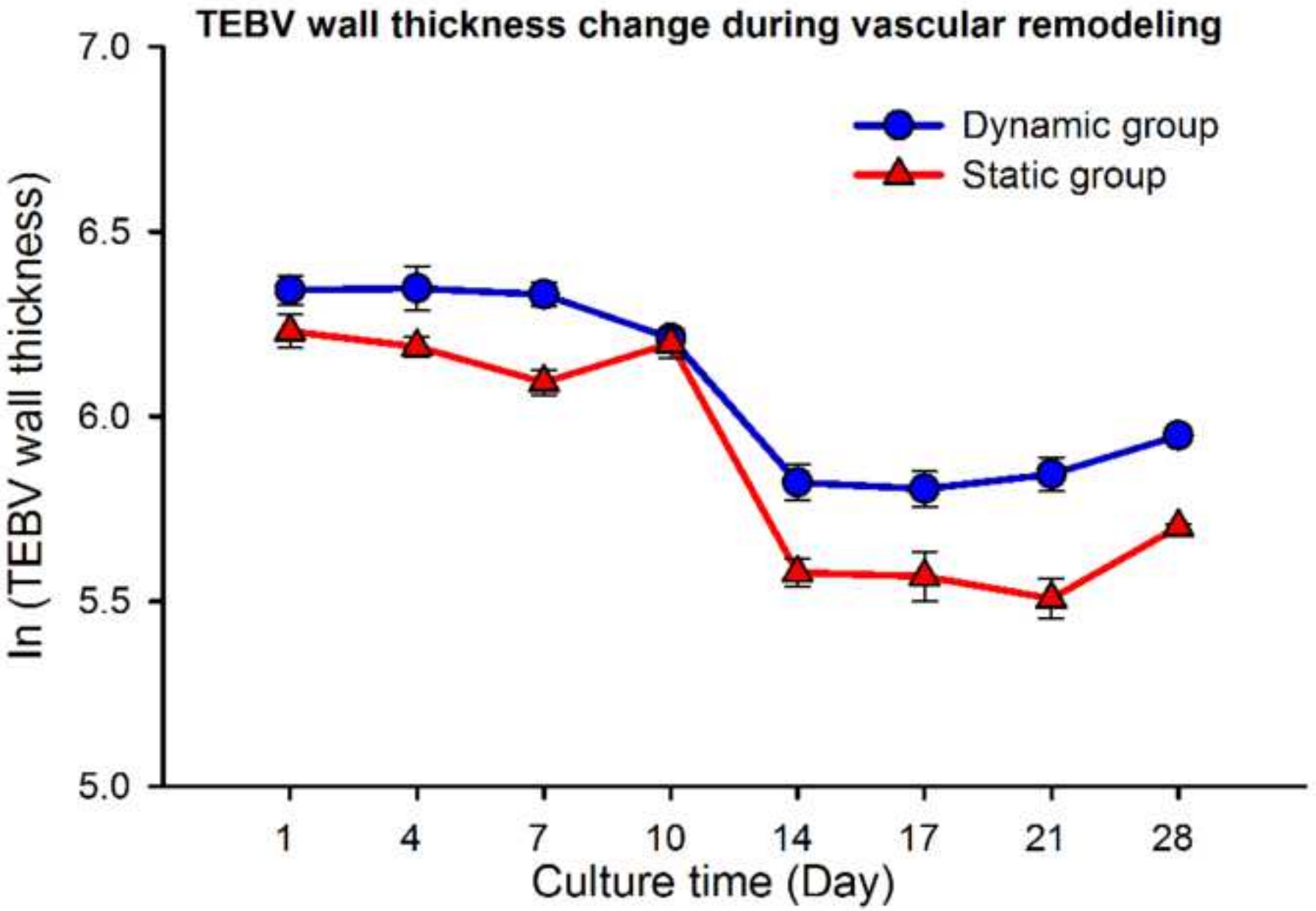

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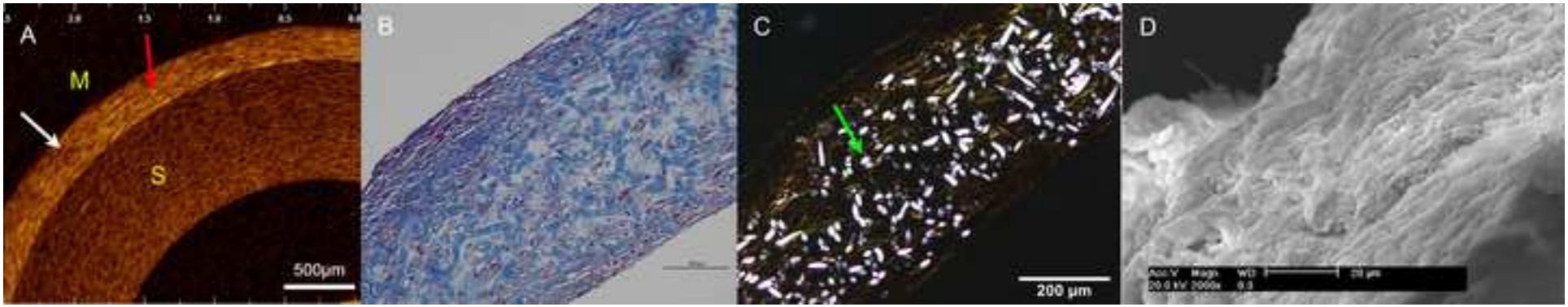

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
PGA mesh	Synthecon		
silicone tube	Cole Parmer		
connector	Cole Parmer		
intravascular OCT system	St. Jude Medical, Inc	Dragonfly model C7	
scanning electron microscopic	Philips	FEI Philips XL-30	
polarized microscope	Olympus	Olympus BX51	
sutures	Johnson & Johnson		
pulsatile pump	Guangdong Cardiovascular Institute		
LightLab Imaging software	St. Jude Medical, Inc		

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Article Title: Nondestructive imaging of tissue-engineered blood vessels using optical coherence tomography

Signature: Zhanyi Lin Date: 2018.02.20

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Responds to Reviewers and Editor:

First of all, we both thank reviewers and editor for the positive and constructive comments and suggestions.

Editorial comments:

Changes to be made by the Author(s):

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.

Answer: We have paid attention to these issues and fixed them in the revised manuscript.

2. There are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 37-39, 52-57, 59-62, 67-77, 114-118, 160-163, 165-169, 179-181, 184-197, 212-237, 239-242, etc.

Answer: We are so sorry to make this mistake. We fixed this in the revised manuscript.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Answer: We have uploaded the copyright permission file and illustrated those cited figures in the figure legend.

4. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300dpi.

Answer: The figures we uploaded have met the requirement.

5. Please keep the font consistent in all figures, if possible.

Answer: We have made the modification as required.

6. Figure 2/4A: Please provide a scale bar to provide context for the images.

Answer: We have made the modification as required.

7. Figure 3: Please define error bars in the figure legend.

Answer: We have made the modification as required.

8. Please include a space between all numbers and their corresponding units: 45 min, 450 mL, 100µm; etc.

Answer: We are sorry to make these mistakes. We have fixed them in our revised manuscript.

9. Please provide an email address for each author.

Answer: We have changed the email address of some authors.

10. Summary: Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …" Currently it reads a bit awkward.
Answer: Thanks for the suggestion. We have fixed this in our revised manuscript.

11. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Prolene 5-0, Prolene 4-0, LightLab, etc.
Answer: We have fixed this in our revised manuscript and added the supplemented information to the table.

12. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
Answer: We have revised the section of protocol in the manuscript and hope to meet the requirement.

13. Line 82: Please describe how silicone tubing is sterilized.
Answer: We have explained how to sterilize the silicone tubing in the paper.

14. Lines 86-87: What is used to hold the scaffolds in this step? Please use molar concentration for 1 N NaOH.
Answer: We have fixed this in the revised manuscript.

15. Line 89: Please specify the size and type of the glass bioreactor. Also provide more details about the silicone tube and connector (type and size, etc.) used. Should not the PGA scaffolds already include the silicone tube? How large is the tank?
Answer: It has been supplemented the size and type of the glass bioreactor and the size of silicone tube in the manuscript as recommended. Other tubes and connectors have different sizes according to different parts just to assemble the system. The ethanol tank is enough to hold the above supplies without limitation of size.

16. Line 91: Please refer to Figure 1 to guide the readers.
Answer: We have fixed this in the revised manuscript.

17. Line 94: Please specify the location of bioreactor lips and length of ePTFE.
Answer: We have fixed this in the revised manuscript.

18. Line 95: Do you mean soak the bioreactor in the ethanol tank?
Answer: Yes.

19. Line 98: Please describe how and where the vascular smooth muscle cells are obtained. What is the container? What is the composition of the culture medium? How is the seeding process done?

Answer: We have fixed this in the revised manuscript.

20. Line 100: Please specify the length and diameter of the feeding tube and the three short tubing segments for gas exchange.
Answer: The length and diameter of the feeding tube is 15cm and 5mm. The three short tubing segments for gas exchange have the length of 7cm and diameter of 5mm.

21. Line 102: What is the length of the stir bar?
Answer: The length of the stir bar is 1.5cm. We have fixed this in the revised manuscript.

22. Lines 106-107: What is the composition of culture medium? What is the moisture in the incubator? Molecular formulas should include subscripts: e.g., CO2.
Answer: We have fixed this in the revised manuscript.

23. Line 113: What is a Luo-Ye pump? Is a certain flow rate recommended?
Answer: Luo-Ye pump is a pulsatile pump to provide a stable fluid flow simulating the biomechanical microenvironment. In this paper , the flow rate is 154 mL/min and it can be adjusted according to the experimental requirements.

24. Line 116: Please define bpm, beats per minute?
Answer: We have fixed this in the revised manuscript.

25. Line 125: Please add more details to how the pullback device is adjusted to ensure good image quality.
Answer: We have fixed this in the revised manuscript.

26. Lines 143-147: Please add more details to how these are done. For instance, how are the TEBVs collected? How the PGA content is assessed? Alternatively, add references to published material specifying how to perform the protocol action.
Answer: We took the TEBVs out of the bioreactor and performed routine histological staining and scanning electron microscopy examination to figure out the characterization of microstructure and PGA degradation. References are made in the manuscript.

27. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Answer: Thanks for the suggestion. We have revised this section in the manuscript.

28. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than

6 authors, list only the first author then et al.
Answer: We have fixed this section in the revised manuscript.

29. Please do not abbreviate journal titles and include volume and issue numbers for all references.
Answer: We are sorry to make this mistake. We have fixed this in the revised manuscript.

Reviewers' comments:

Reviewer #1:
Chen and colleagues described a protocol to image tissue-engineered blood vessels using optical coherence tomography (OCT). I think this work is useful for people studying tissue-engineered blood vessels and thus merits publication at JOVE. However, I suggest the authors enhance the reported protocol to address the following points.
First, please change the numbering scheme. Right now, it is hard for me to differentiate the different levels of numbering.
Second, it helps the readers to comprehend the protocol if the authors justify and explain the reasons to support each step of the protocol.
Third, the protocol needs more details on the production and use of VSMC. It also needs more details on the OCT system instead of just mentioning the resolution.
Fourth, it seems that only the vessel thickness was measured. If this true, it needs to be clarified throughout the article. As for now, it is unclear how "vascular remodeling" was characterized.
Answer: We are sorry to make it confusing and we fixed this in our revised manuscript. According to the Reviewer's suggestion, we have added more details about VSMCs and the use of OCT imaging. Nondestructive monitoring of degradable scaffold-based tissue-engineered blood vessel development in real-time culture is not simple but important. The geometrical morphology of TEBVs is part of the engineering vascular remodeling and we hope to explore more information to more clearly understand this process.

Reviewer #2:
Manuscript Summary:
the paper describe a technique which should help in following the development of the tissue-engineered blood vessels. The technique looks promising.
Major Concerns: No major concerns
Minor Concerns: No minor concerns
Answer: Thanks for the comment.

Reviewer #3:
Manuscript Summary:
Why is this manuscript so similar to this paper, "In vitro remodeling and structural characterization of degradable polymer scaffold-based tissue-engineered vascular grafts using optical coherence tomography" by Wanwen Chen & Junqing Yang & Wenjun Liao & Jiahui Zhou & Jianyi Zheng & Yueheng Wu & Dongfeng Li & Zhanyi Lin previously published as Cell Tissue Res (2017) 370:417-426?

This is very strange! Is this allowed by the journal?
The two texts have a few differences in their respective author lists, and the text of the manuscript is in worse English, but the figures found in the manuscript to be reviewed are exactly the same as some of the figures in the above-mentioned paper (although with worse resolution). Ironically, this paper was cited in the text.
Other critiques: The manuscript requires significant grammatical edits.
Answer: Thanks for the suggestions. We are so sorry to make this mistake. We have fixed this in our revised manuscript and hope to meet the requirements of the journal.

We have adjusted and uploaded documents according to the editorial office's recommendations. We greatly appreciate both your help and that of the reviewers' concerning improvement to this paper. I hope that the revised manuscript is now suitable for publication. Should you have any questions, please contact us without hesitate.

Responds to Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Answer: We have read the full manuscript and made corresponding modifications.

2. Please use h, min, s, etc for time units.
Answer: We have paid attention to these issues and fixed them in the revised manuscript.

3. Step 1.3.9: What condition is used to grow the scaffolds? Please specify the stimulation.
Answer: In step 1.3.9, the perfusion system is static and does not provide pulsatile stimulation. In order to avoid confusion, we have modified it here in the revised manuscript.

4. 1.4.3: How to pressurize the tubes? What pressure is used?
Answer: The perfusion system was driven by a pulsatile pump, which can be adjusted to the systolic pressure and frequency for different modes of mechanical stimulation. Fluids circulating through biocompatible tubes to the PBS bag act on tubes with a consistent deformation and generate force iteratively. The pressure action mode is determined by the pulsation pump system. Please see the references for details.

5. 3.1-3.2: For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.
Answer: We have paid attention to this issue and fixed it in the revised manuscript.

6. 4.1: How to harvest? Please add more details.
Answer: We are sorry to make it simple and we fixed this in our revised manuscript. Open the silicone stopper lid placed over the bioreactor when the culture is finished and discard the culture medium. Loose ePTFE from bioreactor lips and cut the silicone tubes from the outer side of ePTFE with scissors. Harvest TEBVs from the bioreactor and cut into sections for scanning electron microscopy examination.

7. 4.2: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
Answer: We have fixed this in the revised manuscript. Take out the rest of TEBVs and cut into 4 μm thick sections. Pull out the supporting silicone tube and fix sections with 1% paraformaldehyde. Perform routine histological staining of Masson's trichrome and Sirius red to examine the morphology of collagen and PGA.

8. 4.3: Please add more details to your protocol steps. Please ensure you answer the

“how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
Answer: We have fixed this in the revised manuscript.

We have adjusted and uploaded documents according to the editorial office's recommendations. We hope that the revised manuscript is now suitable for publication. Should you have any questions, please contact us without hesitate.

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