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In Vitro 3D Cell-cultured Arterial Models for Studying Vascular Drug Targeting Under Flow

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Corresponding Author:	Netanel Korin Technion Israel Institute of Technology Faculty of Biomedical Engineering Haifa, Technion ISRAEL
Corresponding Author's Institution:	Technion Israel Institute of Technology Faculty of Biomedical Engineering
Corresponding Author E-Mail:	korin@bm.technion.ac.il
Order of Authors:	Maria Khoury
	Mark Epshtein
	Netanel Korin
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TITLE:

In Vitro 3D Cell-cultured Arterial Models for Studying Vascular Drug Targeting Under Flow

AUTHORS AND AFFILIATIONS:

Maria Khoury, Mark Epshtein, Netanel Korin

Department of Biomedical Engineering, Technion - IIT, Haifa 32000, Israel

Email addresses of co-authors:

Maria Khoury (mariakh@campus.technion.ac.il)
Mark Epshtein (epshmark@campus.technion.ac.il)

Corresponding author:

Netanel Korin (korin@bm.technion.ac.il)

KEYWORDS:

Biomedical engineering, 3D printing, Arterial models, Nanomedicine, Cardiovascular diseases, Atherosclerosis, Vascular targeting

SUMMARY:

Here, we present a new protocol to study and map the targeted deposition of drug carriers to endothelial cells in fabricated, real-sized, three-dimensional human artery models under physiological flow. The presented method may serve as a new platform for targeting drug carriers within the vascular system.

ABSTRACT:

The use of three-dimensional (3D) models of human arteries, which are designed with the correct dimensions and anatomy, enables the proper modeling of various important processes in the cardiovascular system. Recently, although several biological studies have been performed using such 3D models of human arteries, they have not been applied to study vascular targeting. This paper presents a new method to fabricate real-sized, reconstructed human arterial models using a 3D printing technique, line them with human endothelial cells (ECs), and study particle targeting under physiological flow. These models have the advantage of replicating the physiological size and conditions of blood vessels in the human body using low-cost components. This technique may serve as a new platform for studying and understanding drug targeting in the cardiovascular system and may improve the design of new injectable nanomedicines. Moreover, the presented approach may provide significant tools for the study of targeted delivery of different agents for cardiovascular diseases under patient-specific flow and physiological conditions.

INTRODUCTION:

Several approaches have recently been applied utilizing 3D models of human arteries[1–5]. These models replicate the physiological anatomy and environment of different arteries in the human body *in vitro*. However, they have been mainly used in cell biology studies. Current studies on vascular targeting of particles to the endothelium include *in silico* computational simulations[6–8],

in vitro microfluidic models[9–11], and *in vivo* animal models[12]. Despite the insights they have provided, these experimental models have failed to accurately simulate the targeting process that occurs in human arteries, wherein blood flow and hemodynamics constitute dominant factors. For example, the study of particle targeting to atherosclerotic regions in the carotid artery bifurcation, which are known for their complex recirculation flow pattern and wall shear stress gradient, may impact the journey taken by the particles before they reach the endothelium[13–16]. Therefore, these studies must be performed under conditions that replicate the physiological environment, *i.e.*, size, dimension, anatomy, and flow profile.

Recently, this research group fabricated 3D-reconstructed human arterial models to study the deposition and targeting of particles to the vasculature[17]. The models were based on geometrical 3D replicas of human blood vessels, which were then cultured with human ECs that subsequently lined their inner walls. In addition, when subjected to a perfusion system that produces physiological flow, the models accurately replicated physiological conditions. The perfusion system was designed to perfuse fluids at a constant flow rate, using a peristaltic pump in both closed and open-circuit configurations (**Figure 1**). The system can be used as a closed-circuit to map particle deposition and targeting to the cells seeded inside the carotid model. In addition, it can be used as an open circuit to wash out non-adherent particles at the end of the experiments and to clean and maintain the system. This paper presents protocols for the fabrication of 3D models of the human carotid bifurcation, design of the perfusion system, and mapping of the deposition of targeted particles inside the models.

PROTOCOL:

NOTE: This protocol describes the fabrication of a 3D model of the carotid artery and can be applied to generate any other artery of interest by simply modifying the geometric parameters.

1. Design and fabrication of a 3D bifurcation of the human carotid artery model

1.1. Choose images from patients or previously studied geometries of the human carotid artery bifurcation, and create a computer-aided design model of the mold that needs to be printed.

NOTE: The carotid artery bifurcation has one inlet and two outlets. It is important to design a 3D mold frame around the artery and temporary printing supports between the frame and the artery mold (**Figure 2A–C**).

1.2. Print the geometries using a 3D printer. Cut the temporary printing supports, and use sandpaper to polish and smooth the molds, especially in the areas where the supports were cut. Rinse the model with acetone and the sanded model with isopropyl alcohol to remove the plastic dust, and allow to completely dry in a chemical hood for 2–3 h.

NOTE: Here, the printed molds were made of clear resin v4 (**Figure 2D,E**).

1.3. To easily dissolve the plastic, spray the molds with transparent lacquer inside a chemical hood, and allow to air-dry for 1 h. Repeat this 3x.

NOTE: Here, **2X Ultra Cover Clear Spray** was used, but any other kind should be suitable as long as it is not wood lacquer. Confirm that there is no exposed plastic left because the plastic may react with the silicone and prevent it from properly solidifying. The quality of the sprayed surface will determine the quality of the surface of the final silicone model.

1.4. Cut transparent rectangular slides/strips of smooth plastic of the same dimensions as the mold frame, and glue them using the lacquer to the frame on all sides and from one side of the frame, such that it will be sealed at the bottom and open at the top. Apply the lacquer using a paint brush inside a chemical hood, and allow the slides to completely dry for at least 24 h (**Figure 2F**).

1.5. To prepare the silicone rubber mixture, add liquid silicone with its curing agent (mass ratio 1:10) in a plastic plate, and stir thoroughly with a wooden stick to ensure complete mixing. For the carotid model, add 54 g of the silicone and 6 g of its curing agent.

1.6. Cool the mixture for 15 min at 4 °C, then degas it in a vacuum desiccator until all the air bubbles have been eliminated. Place the mold in the desiccator (with the open side facing up), slowly pour the silicone mixture into the mold, and again remove the air bubbles until the mixture is clear.

1.7. Let the mold stand with the silicone overnight at room temperature (if possible, leave it in the desiccator without vacuum). If the mixture has not fully dried, incubate it at 60 °C for another few hours.

1.8. Once the mixture is fully dry, remove the transparent slides, and immerse the model in absolute acetone for 48 h in a chemical hood until the plastic is fully dissolved. Remove any plastic leftovers with a wooden stick. To evaporate the acetone trapped inside the model, incubate it at 60 °C for at least 4 days before cell seeding.

2. Cell culture and seeding in models

2.1. Prepare three connectors for the carotid model: one for the inlet and two for the outlets (see **Table of Materials**). Sterilize the model and the connectors by ultraviolet irradiation in a biological hood for 20 min.

2.2. Coat the models with 4 mL of 100 µg/mL fibronectin (in 1x phosphate-buffered saline, (PBS)) for 2 h at 37 °C or overnight at 4 °C. Inject the fibronectin solution into the model through the inlet using a 5 or 10 mL plastic syringe. Remove the fibronectin through the outlet, and wash the model with EC medium.

2.3. Suspend 2.5 × 10^6 cells/mL human umbilical vein endothelial cells (HUVECs, passage < 6), and fill the model with 4 mL of the cell suspension using a 5 or 10 mL plastic syringe (**Figure 3A**). Place the model on a rotator inside an incubator (37 °C) at a speed of 1 rpm for 48 h to ensure homogeneous seeding. Make sure the model is well-attached to the rotator (**Figure 3B**). Change the medium after 24 h inside a biological hood, and return to the rotator inside the incubator for another 24 h.

NOTE: After 24 h, the cells are seeded and can be imaged using a microscope.

2.4. Remove the model from the rotator, and wash with 1x PBS using a 10 mL plastic syringe. To fix the cells, incubate the cells with 4 mL of 4% paraformaldehyde (PFA) to the model for 15 min inside a chemical hood, and then rinse 3x with PBS. Add 4 mL of PBS, and store at 4 °C until the experiment (**Figure 3C**). Stain the cells inside the model using standard staining protocols (*e.g.,* nuclear staining with 4',6-diamidino-2-phenylindole (DAPI), **Figure 3D**).

3. Design of the perfusion system

3.1. Merge the two inlets into a single 4 mm ID tube and again into two 6 mm ID tubes, which connect to the peristaltic pump. Merge the two 6 mm ID tubes coming out of the peristaltic pump into a single 4 mm tube, and connect it to an oscillation damper to eliminate any oscillations from the pump. Use a 250 mL narrow-mouthed bottle with a three-orifice lid as the damper.

3.2. Connect one orifice to the inlet from the pump, close the second with a cork that is used for pressure venting in emergencies, and extend the third orifice (which is the outlet) to the bottom of the bottle.

3.3. Connect the damper to the inlet of the cultured carotid model using the outlet tubing. Merge the two outlets of the model to one tubing, which will be the outlet of the system.

3.4. Split the outlet tubing to two outlet tubes (one for the closed circuit and the other for the waste container in the open circuit). Attach a plastic clamp to each tube.

NOTE: The combination of open/closed clamps will determine whether the system is in a closed or open circuit configuration. As shown in **Figure 1**, if clamps a and d are close while b and c are open, the system is a closed circuit; the reverse brings the system to open circuit configuration.

3.5. Prepare three containers: one that can hold 300 mL fluid (for closed-circuit) and two others of 1 L each: one for washing and the other for waste (for open circuit).

4. Closed-circuit configuration: perfusion experiment and imaging

4.1. Add 300 mL of PBS to the closed-circuit container, which is sufficient to fill the entire system, including the tubing and the model. Place one inlet tube and one outlet tube (open clamps b and c) inside the container.

4.2. Fill the 1 L washing container with distilled water (for washing at the end of the experiment), and leave the other 1 L waste container empty. Place the other inlet and outlet tubing (close clamps a and d) in the washing and waste containers, respectively.

4.3. Take the fixed cell cultured carotid model out of 4 °C storage, and empty the PBS. Connect the inlet and outlets of the carotid as described in steps 3.3–3.4. Do not leave the model dry for a long time. Once the model is connected, activate the pump to perfuse fluid.

4.4. Place the carotid model under the microscope. Open the tubing before the inlet and after the outlet of the carotid model. Set the peristaltic pump at 10 rpm, and turn it on. Increase the speed in increments of 5 rpm, every 4–5 min. Make sure there are no leakages.

4.5. At 100 rpm, which equals the maximum flow rate of the physiological waveform of the human carotid artery (~400 mL/min), add fluorescent carboxylated polystyrene (PS) particles (2 µm, at a concentration of 1.6 µg/mL) to the 300 mL of PBS in the closed-circuit container. Image the region of interest every 10 s for 1.5 h (still single images or video as needed).

5. Open-circuit configuration: the washing step

5.1. Open the clamps of the washing and waste tubes in the 1 L containers (clamps a and d), and immediately close the clamps of both the inlet and outlet tubes in the 300 mL container (clamps b and c) to change the system from a closed to open circuit configuration.

5.2. Let most of the water flow from the washing container to the waste container at 100 rpm. Before it is completely transferred, press stop on the peristaltic pump, and close the tube clamps before the inlet and after the outlet of the carotid model.

5.3. Using the appropriate filters, capture images of the model at the region of interest to show the deposition and adhesion of particles to the cells. Disconnect the carotid model. Carefully and slowly add 4 mL of PBS with a 10 mL syringe through the model's inlet.

5.4. Connect a "dummy model", (which is also a silicone rubber 3D carotid model used only for washing, without cultured cells) instead of the carotid model, and rinse the system. Add another 1 L of water, and wash the system again until all the water is transferred from the washing container to the waste. Turn off the peristaltic pump.

6. Data acquisition and analysis

6.1. Acquire a digital movie of particle deposition at the region of interest with the images taken during the experiment, using a customized software code (see the **Table of Materials**).

6.2. For mapping of the deposition of the particles along the model, tile multiple images to cover the examined region of interest (**Figure 4A,B**).

NOTE: A customized software code can be written to quantify the number of adhered particles at a site of interest (a sample file has been provided as **Supplemental Information**)[17].

REPRESENTATIVE RESULTS:

This paper presents a new protocol to map the deposition of particles inside real-sized 3D human artery models, which may provide a new platform for drug delivery research. Using a 3D printing technique, a model of the human carotid bifurcation artery was fabricated (**Figure 2**). The model was made of silicone rubber and seeded with human ECs (**Figure 3**). Importantly, this protocol enabled the replication of physiological conditions, especially with respect to fluid dynamics. A perfusion system was designed to infuse particles to the carotid bifurcation under constant flow at the magnitude of the physiological waveform characteristic of the carotid. **Figure 1** presents the perfusion system, which consists of the peristaltic pump, an oscillation damper, the cultured bifurcation model, tubing, and fluid containers.

To map the deposition and adhesion of the perfused particles, the arterial model was imaged under a stereomicroscope, both at the end of the experiment and after washing (step 5.3). The images were captured using 2x objective and tiled together to form a whole image of the model. Then, the number of adhered particles was calculated using a customized software code. To examine the formation of the recirculation pattern at the bifurcation, 10 µm fluorescent glass beads were infused into the model. **Figure 4A** shows the recirculation, which suggests that the conditions inside the model mimic physiological conditions.

To map the deposition of particles inside the model, 2 µm fluorescent carboxylated PS particles were infused, and their adhesion to the ECs was imaged (**Figure 4B,C**). These particles adhered differently at various regions along the model—more adhesion was observed out of the recirculation area, where wall shear stress is high. These results have been previously discussed to show that the adhesion of particles is a function of the model's geometry, particle surface characteristics, and shear stress[17]. These deposition maps are relatively simple and may be quickly obtained for screening drug carriers' affinity and targeting under physiological conditions in patient-specific models.

FIGURE AND TABLE LEGENDS:

Figure 1: The perfusion system. A perfusion system was designed to perfuse fluids under constant flow. It is comprised of (1) a peristaltic pump, (2) an oscillation damper, (3) the cultured 3D arterial model, and three glass containers: two with a 1 L capacity (4 and 6) and a third that can hold 300 mL fluid (5). The system can operate in two configurations: (i) an open circuit, in which clamps a+d are open and b+c are closed, or (ii) a closed circuit, in which clamps a+d are closed and b+c are open.

Figure 2: Fabrication process of a 3D carotid artery bifurcation model. (**A–C**) Human carotid bifurcation, the mold frame around the artery, and temporary printing supports were designed. (**D, E**) The geometries were printed using a 3D printer. (**F**) The temporary printing supports were cut, and the model was sanded and sprayed with lacquer. Then, transparent rectangular slides

were glued to the frame from all sides. Silicone rubber was cast when the glue was dry. Abbreviation: 3D = three-dimensional.

Figure 3: Seeding of ECs inside 3D models of the carotid artery. (**A**) Real-sized 3D model of the human carotid bifurcation made of silicone rubber. The model was cultured with human ECs and filled with cell medium. (**B**) The cultured model was placed on a rotator at 37 °C for 48 h. (**C**) Images of the cultured ECs inside the 3D model in brightfield and (**D**) with DAPI for nuclear staining in blue. Scale bars = 10 µm. Abbreviations: ECs = endothelial cells; DAPI = 4',6-diamidino-2-phenylindole; 3D = three-dimensional.

Figure 4: Perfusing and mapping the adhesion of particles. (**A**) Streak-line image of streamlines and recirculation (dashed rectangle) generated upon perfusion of 10 µm fluorescent glass particles at a constant flow of 400 mL/min through the model. (**B**) Deposition map of the 2 µm fluorescent carboxylated PS particles (in red) inside the 3D-cultured model. Scale bar = 2 mm. (**C**) Adhesion of the particles (in red) to the cultured ECs (in blue–DAPI) inside the model at a 10x magnification. Scale bar = 10 µm. Abbreviations: PS = polystyrene; 3D = three-dimensional; ECs = endothelial cells; DAPI = 4',6-diamidino-2-phenylindole.

DISCUSSION:

Current approaches to study vascular targeting of particles fall short in replicating the physiological conditions present in the human body. Presented here is a protocol to fabricate 3D-reconstructed models of human arteries to study particle targeting to the ECs lining the artery under physiological flow applied using a customized perfusion system. When choosing the material for 3D printing, it is best to use a clear plastic to avoid pigment transfer to the silicone model, which should be as transparent as possible. In addition, it is important to choose a material that does not dissolve in acetone, but instead becomes soft and brittle and can subsequently easily be removed from the model.

The presented 3D models are made of silicone rubber, a transparent silicone, mixed with its curing agent. It is important to ensure that the mixture is always at room temperature or below, otherwise the crosslinking between the silicone and the curing agent will begin before the degassing and casting onto the molds. Although polydimethylsiloxane can also be used to fabricate such models (1:10 ratio with its crosslinker), silicone rubber is more durable. After immersing the model in acetone to dissolve the plastic, it is crucial to incubate it at 60 °C for at least 4 days to ensure full evaporation of any acetone residue. If any acetone remains trapped inside the model, the cells will not grow properly. Changing the medium after 24 h and fixation of the cells after 48 h are the two steps that involve manual injection of fluid using a 10 mL syringe. It is therefore important to perfuse slowly, otherwise the cells might be washed out.

The perfusion system has two inlets and two outlet tubes. Each tube has a plastic tube clamp for flow control. Most of the tubing system is comprised of 4 mm inner dimeter (ID) tubes, except for the tubes that are clamped in the pump, which are 6 mm ID tubes. The ID of the tubes clamped in the pump will determine the maximum flow rate that can be achieved in the system. This perfusion system can also generate a pulsatile waveform by superposing oscillations on the

constant mean flow[17] by connecting the outlet of the damper to an oscillator assembly, which superposes the oscillatory part of a desired waveform on the constant flow rate produced by the peristaltic pump. This configuration enables operation under oscillatory flow or under constant flow conditions when the oscillator is turned off.

In this paper, the perfusion system was customized based on experiments with the 3D human carotid artery bifurcation. Therefore, if other arterial models or other tubing are used, the amounts of fluids and flow rate may require adjustments. In such cases, the system and flow rate will have to be calibrated, while ensuring no cell detachment from the model walls. It is very important to gradually increase the flow rate in the peristaltic pump to guarantee that the cells are not washed away with the flow. Moreover, it is crucial to ensure that the entire system, including the tubing, the model, as well as the container are filled with fluids (*e.g*., in this case, it was filled with a total volume of 300 mL of fluid).In addition, before and after each experiment, the system should be washed with distilled water in an open-circuit configuration.

Blood can also be perfused into the models using the perfusion system[17]. In this case, extra caution must be exercised to prevent any leakage, especially if human blood is used. Moreover, the washing step is crucial as bleach must be perfused at the end of the experiment to ensure full wash out of the blood. After the bleach, water should be perfused as mentioned in the protocol. It is important to note that in this paper, carboxylated PS particles were used, which have a uniform composition and a narrow size distribution. Moreover, these particles adhere to the cells primarily through electrostatic interactions. However, other drug nanocarriers may be used, and specific targeting should be examined as well with ligand-labeled particles, *e.g*., to anti-intercellular adhesion molecule 1 and anti-platelet endothelial cell adhesion molecule 1, which will increase particle accumulation to ECs at the site of interest.

In addition, in this protocol, ECs were fixed prior to connecting the models to the perfusion system and injection of the particles. The adhesion of particles to fixed cells represents the first stage in the binding process and therefore, experiments with live cells need to be performed, where internalization of particles may occur during later stages of the adhesion process. This protocol can be used to fabricate 3D arterial models for the study of drug carriers under physiological conditions. The outlined approach may assist in the study of delivery of agents under patient-specific conditions.

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DISCLOSURES:
The authors declare no conflicts of interest.

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Figure 1

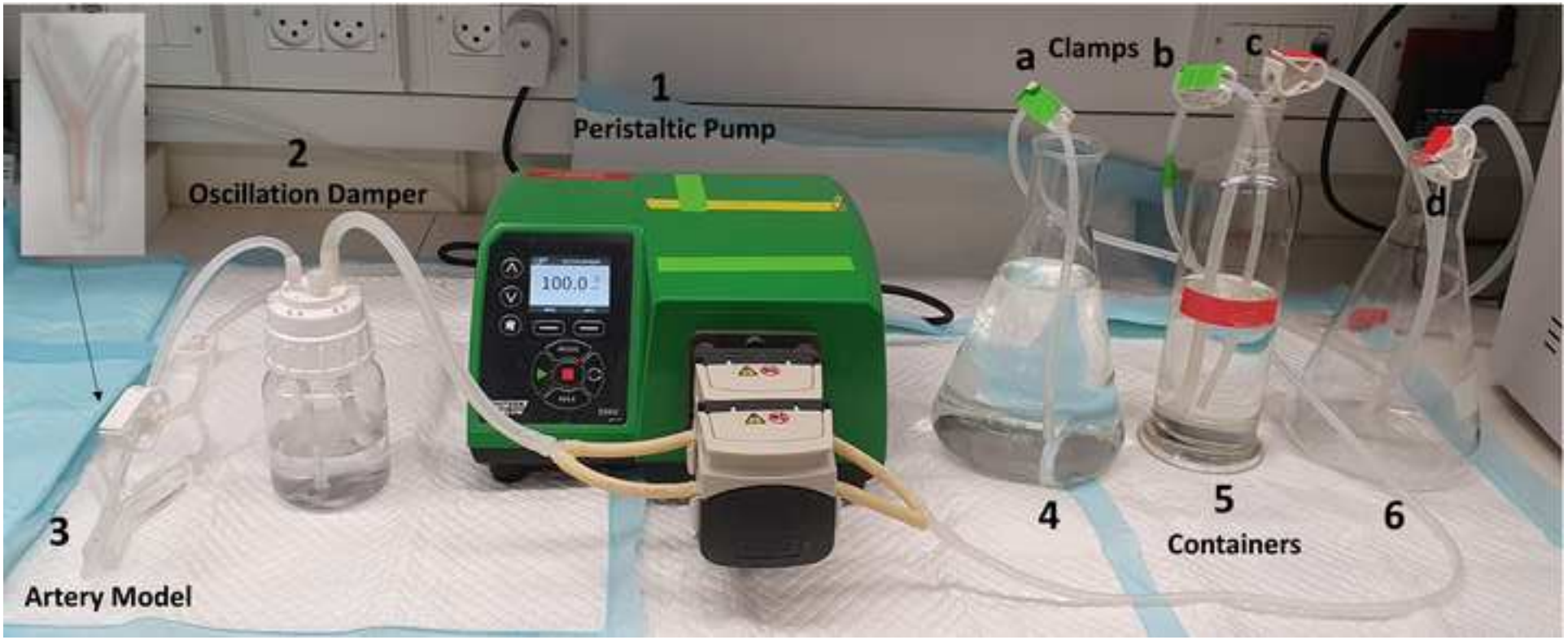

Figure 2

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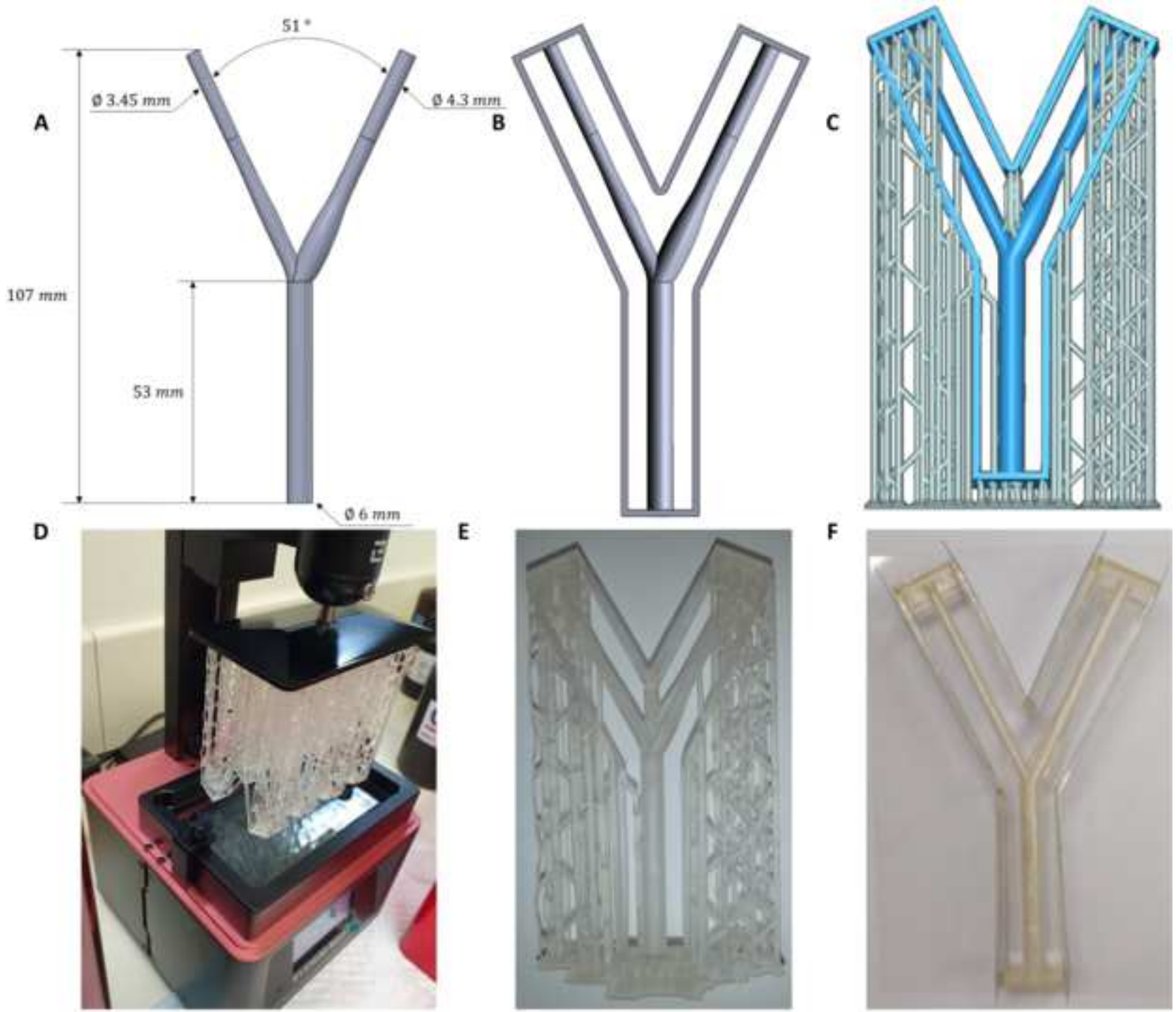

Figure 3

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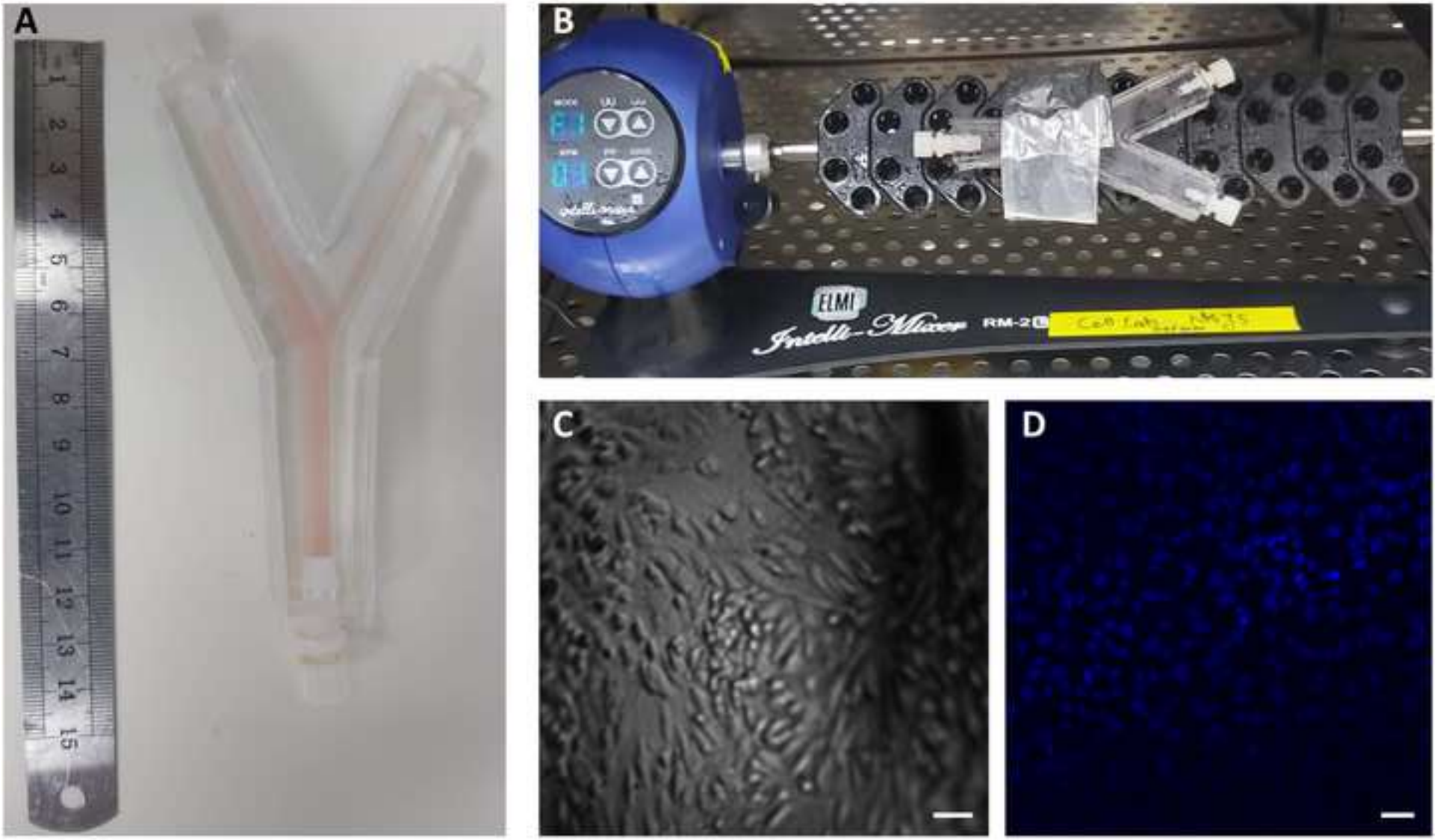

Figure 4

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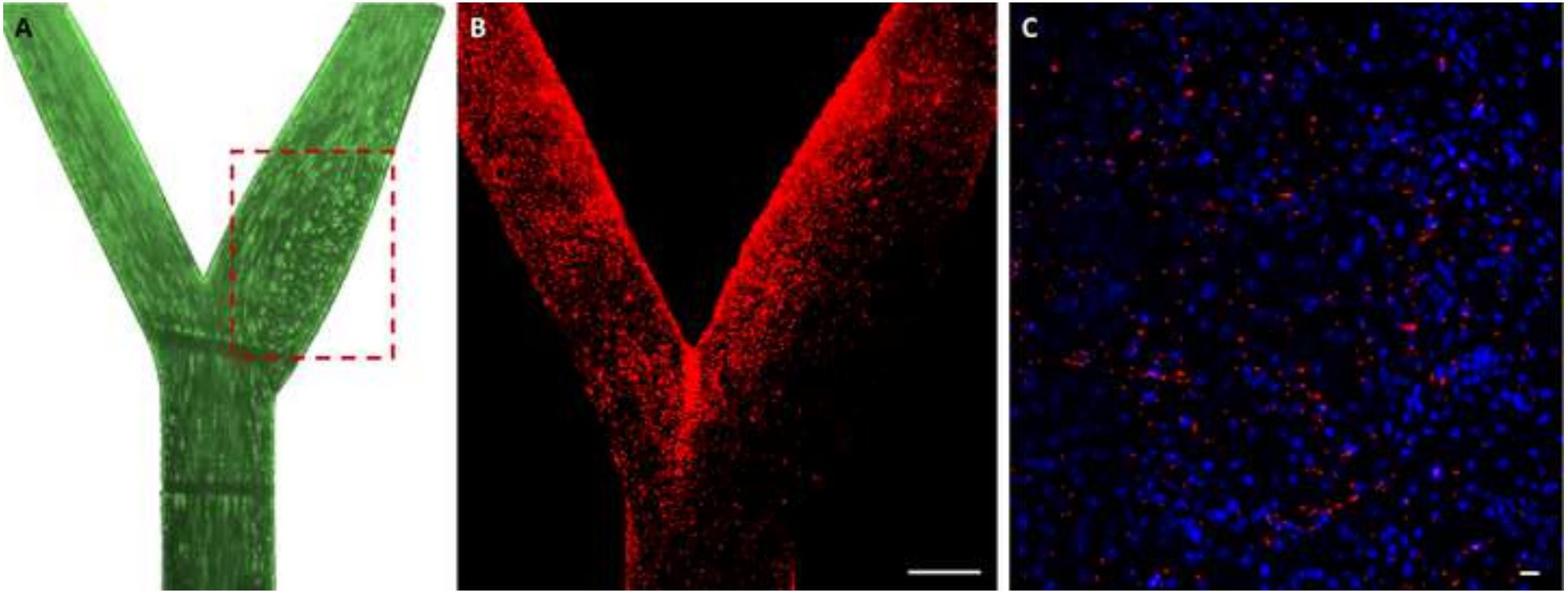

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3D printer	FormLabs	PKG-F2-REFURB	
Acetone, absolute (AR grade)			
Connectors	Nordson Medical	FTLL013-1	Female Luer
		FTLL230-1	Female Luer
		FTLL360-1	Female Luer
		LP4-1	Male Luer Integral Lock
Damper	Thermo-Fisher Scientific	DS2127-0250	Nalgene Polycarbonate, Validation Bottle
Damper Cover	Thermo-Fisher Scientific	2162-0531	Nalgene Filling/Venting Closures
Elastosil Elastosil RT 601 A	Wacker	60003805	
Elastosil RT 601 B	Wacker	60003817	The crosslinker
Endothelial Cell Media	ScienCell	1001	
Fibrontectin	Sigma Aldrich	F0895-5mg	
HUVEC	Lonza	CC-2519	
Isopropyl alcohol, AR grade 99.5%			Remove plastic dust from the sanded model
Lacquer	Rust-Oleum		2X-Ultra cover Gloss Clear
Matlab	Mathworks		https://www.mathworks.com/products/matlab.html
Microscope	Nikon	SMZ25	
Microscope Camera	Nikon	DS-Qi2	
Peristaltic pump	Watson Marlow	530U IP31	With 2 pumpheads: 313D
Plastic tube clamp	Quickun	1-2240-stopvalve-2pcs	
Polystyrene Particles	Thermo-Fisher Scientific	F8827	Diameter = 2 µm
Printer resin	FormLabs	RS-F2-GPCL-04	
Rotator	ELMI Ltd.		Intelli-Mixer RM-2
Solidworks	SolidWorks Corp., Dassault Systèmes		https://www.solidworks.com/
Tubing	Watson Marlow	933.0064.016	Tubing for the pump: 6.4 mm ID
			All the other tubing: Silicon tubing: 4 mm ID

To
Dr. Vidhya Iyer,
Review Editor
JoVE

February 15, 2021

Dear Dr. Vidhya Iyer,

Thank you for considering our manuscript, entitled: "In-vitro 3D Cell-Cultured Arterial Models for Studying Vascular Drug Targeting under Flow", Ref. No.: JoVE62279, for publication in *JoVE - the Journal of Visualized Experiments*.

We thank the editor and reviewers for the careful and thorough reading of our manuscript, the positive evaluation of our study and the constructive comments which helped us improve the scientific value of the manuscript. Correspondingly, we have now revised and modified the text as per the editor and reviewers' suggestions. Additionally, we have added new data to the revised manuscript. Please find below a point-by-point response to the editor and reviewers' comments and details of the revisions in the manuscript (the changes are highlighted in the revised manuscript as well). We believe that in its current form the manuscript is suitable for publication in *JoVE - the Journal of Visualized Experiments*.

Sincerely,
Netanel Korin

Netanel Korin, PhD
Corresponding author
Department of Biomedical Engineering
Technion - Israel Institute of Technology
Haifa, Israel
Tel +972-48294116
email: korin@technion.ac.il

Revision of Manuscript JoVE62279:

"In-vitro 3D Cell-Cultured Arterial Models for Studying Vascular Drug Targeting under Flow"

We are pleased to submit our revised manuscript entitled "In-vitro 3D Cell-Cultured Arterial Models for Studying Vascular Drug Targeting under Flow" to be considered for publication in JoVE - the Journal of Visualized Experiments. We would like to thank the Editor and the Reviewers for their valuable input, which we believe helped improve and strengthen the scope of the paper.

Changes to the text and figure legends are highlighted *in blue font color* in the revised manuscript. A point-to-point response is detailed below:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. E.g. Lines 103, 105, 135: "..at RT/ 60 oC" instead of "..in..", Line 174: "…and imaging" instead of "…and imagine" etc.

Reply: We thank the editor for this comment. The manuscript was re-edited for its English and the grammar issues were corrected.

2. 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 …"

Reply: The summary was rephrased as follows: "Here, we present a new protocol to study and map the targeted deposition of drug carriers to endothelial cells, in fabricated real-sized 3D human artery models, under physiological flow. The presented method may serve as a new platform for targeting drug carriers within the vascular system".

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Reply: The reference numbers have been changed to subscripts, according to JoVE references style.

4. Use SI units as much as possible and abbreviate all units: L, mL, μL, cm, kg, h, min, s, etc.

E.g. Line 98: use "g" instead or "gr", Line 123, 132, 135, 157: "mL" instead of "ml" etc.

Reply: The units and abbreviates have been changed as requested.

5. Line 78: Please specify the type/grade of plastic used for mold construction. Also use "..Figure 1 D, E" instead of "Figure 1 D +E".

Reply: We thank the editor for this comment. The plastic used for preparing the molds was added in the protocol as follows: "2. Print the geometries using a 3D printer. Note: in our case, the printed molds were made of clear resin v4". Also, the figures were changed as requested.

6. Line 82: Which alcohol? Concentration?

Reply: Isopropyl Alcohol. This was modified in the manuscript as follows: "5. Rinse the sanded model with isopropyl alcohol AR-grade 99.5 % to remove the plastic dust and allow to completely dry in a chemical hood, for 2-3 h".

7. Line 117: For how long should the sterilization be carried out?

Reply: For 20 min. This was changed in the manuscript as follows: "2. Sterilize the model and the connectors by UV irradiating them in a biological hood for 20 min".

8. Line 121: Remove fibronectin from where? Endothelial cells medium composition?

Reply: Prior seeding the cells, fibronectin (in PBS) was added to the model for 2 h in incubator or overnight in 4 °C. Therefore, before adding the cells, the fibronectin needs to be removed from the model. This was rephrased in the manuscript as follows: "3. Coat the models with 4 ml 100 μg/mL fibronectin (in phosphate-buffered saline, X1 (PBS)) for 2 h, at 37 °C, or overnight, at 4 °C. Inject the fibronectin solution to the model through the inlet, using a 5 or 10 ml plastic syringe. 4. Remove the fibronectin through the outlet and wash the model with endothelial cell medium".

9. Line 122: Please specify the unit for the cell concentration. E.g., /mL

Reply: Cells concentration seeded inside the models was 2.5x106 cells/mL. This was added to the manuscript as follows: "5. Suspend 2.5x106 cells/mL human umbilical vein endothelial cells (HUVECs, passage<6), and fill the model with 4 mL suspended cells, using a 5 or 10 mL plastic syringe".

10. Line 132, 134: 1X PBS?

Reply: Yes. This was added to the manuscript: "8. Remove the model from the rotator and wash with X1 PBS using a 10 mL plastic syringe".

11. Line 182: Please specify the step numbers corresponding to "..as explained above..".

Reply: We thank the editor for this comment. This was modified as follows: "6. Connect the inlet and outlets of the carotid as described in steps 3.4-3.6".

12. Line 184: Which particles? Figure 4 mentions use of fluorescent tags – please provide details about these.

Reply: We thank the editor for this comment. This manuscript presents a new method to fabricate real-sized arterial models and how to map deposition of particles inside the model. Carboxylated Polystyrene (PS) particles (2 µm) were used as representative model particles. This was mentioned in the manuscript as follows: "11. At 100 rpm, which equals the maximum flow rate of the physiological waveform of the human carotid artery (~ 400 mL/min), add fluorescent carboxylated polystyrene (PS) particles (2 µm, at a concentration of 1.6 µg/mL) to the 300 ml PBS in the closed-circuit container".

13. Line 192: Please provide details about the imaging E.g. equipment used, recording format (still image/video) etc.

Reply: For imaging we have used our Nikon stereomicroscope and camera, specified details were added to the Table of Materials. This was added as follows: "12. Image the region of interest every 10 s, for 1.5 h (still single images or video as needed)".

14. Line 208: What is the dummy model?

Reply: We thank the editor for this comment. The dummy model is an elastosil 3D carotid model used only for washing, not cultured with cells. This was clarified in the manuscript as follows: "5. Connect a "dummy model", (which is also an elastosil 3D carotid model used only for washing, without cultured cells), instead of the carotid model and rinse the system".

15. Line 214: Designate this as a "note". Remove the bullet point.

Reply: Done. The bullet point was removed.

16. Please use single line spacing between the protocol steps.

Reply: Done.

17. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." E.g. Line 136: "..stain the cells with.." instead of "cells can be stained…", Lines 147-160 etc.

Reply: We thank the editor for this comment. Sentences were rephrased as requested.

18. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Reply: Three pages have been highlighted.

19. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Reply: We thank the editor for this comment. The representative results were modified as follows: "Here we presented a new protocol to map the deposition of particles inside real-sized 3D human artery models, which may provide a new platform for drug delivery research.

Using a 3D printing technique, a model of the human carotid bifurcation artery was fabricated (Figure 1). The model was made of Elastosil and was seeded with human endothelial cells (Figure 2). Importantly, this protocol enabled replication of the physiological conditions, especially with regards to fluid dynamics. A perfusion system was designed to infuse particles to the carotid bifurcation under constant flow, at the magnitude of the physiological waveform characteristic of the carotid. Figure 3 presents the perfusion system which consists of the peristaltic pump, an oscillation damper, the cultured bifurcation model, tubing and fluids containers.

To map the deposition and adhesion of the perfused particles, the arterial model was imaged under a stereomicroscope, at the end of the experiment and after washing (step 5.3). The images were captured using x2 objective and tiled together to form a whole image of the model. Then, the number of adhered particles was calculated using a customized MATLAB code.

To examine the formation of the recirculation pattern at the bifurcation, 10 μm fluorescent glass beads were infused into the model. In Figure 4A, the recirculation was observed, which suggests that the conditions inside the model are physiological.

To map the deposition of particles inside the model, 2 μm fluorescent carboxylated PS particles were infused and their adhesion to the endothelial cells was imaged (Figure 4B, C). These particles adhered differently at various regions along the model, when more adhesion was observed out of the recirculation area, where wall shear stress is high. We have previously elaborated on these results and showed that the adhesion of particles is a function of the model's geometry, particle surface characteristics and shear stress[17].

These deposition maps are relatively simple and may be quickly obtained for screening drug carriers' affinity and targeting under physiological conditions in patient specific models".

20. In the references, please use a period "." between the initials of the last author and the article title instead of a comma ",". Please ensure that the following style is followed: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).]

Reply: The references format was changed to JoVE formatting style.

21. Please sort the Materials Table alphabetically by the name of the material, and do not embed it in the manuscript (Line 251). Also mention the equipment used E.g. microscope, imaging devices etc.

Reply: The table was modified as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The work by M. Khoury et al. introduces a new 3D printing method to create human bifurcated arterial models at anatomical size. They demonstrate the use of such models to study the targeting of vascular cells with various therapeutics. The work is innovative and has the potential to establish a high-throughput robust platform for a wide variety of drug screening assays. There are, however, a number of issues that need to be addressed in order to further improve the submitted manuscript.

Reply: We thank the reviewer for his positive evaluation and comments on the paper.

Major Concerns:

- The authors are generally quite vague about the "particle" used in the study. It is only late in the middle of protocol that they do mention what type of particles they are referring to here, what size/material they are, and what their real significance is. Using polystyrene particles at 2 um size for this study is not making much excitement, as it won't be clinically/biologically relevant. Why not using any of the nanoparticle systems that are actually used for drug delivery purposes (liposome nanocapsules, dendrimers, SPIONs, etc.)? What's the significance of perfusion with PS particles? How does that relate to any specific biological application?

Reply: We thank the reviewer for this important comment. The submitted manuscript presents a new technique to study deposition of particles in real-sized models of human arteries under physiological conditions. In these experiments, carboxylated polystyrene are used as model particles having uniform composition as well as a narrow size distribution, and they adhere to the cells mainly via electrostatic interactions. Indeed, specific targeting and ligand labeled particles, e.g., to anti-ICAM-1, anti-PECAM etc., increase the targeting and delivery of the particles to the site of the disease, i.e., endothelial cells. This also applies to other targeting carriers such as liposome nanocapsules, dendrimers nanoparticles etc.

However, different type of particles and the use of specific ligands have their specific attributes that require a study of their own – which is beyond the scope of our study, that focuses on the general methodology. We believe that our method would serve as a valuable platform to study targeting of other carriers under blood flow, as detailed by the reviewer (e.g., liposome nanocapsules, dendrimers), however these require a comprehensive study in which the specific drug carrier and their targeting approach and characteristics need to be considered and examined (e.g., magnetic targeting). This was added to the manuscript discussion as follows: "It is important to note that in this paper, carboxylated polystyrene particles, which have a uniform composition and a narrow size distribution were used. Moreover, these particles adhere to the cells primarily through electrostatic interactions. However, other drug nanocarriers can be used and, specific targeting, with ligand-labeled particles, e.g., to anti-ICAM-1, anti-PECAM etc., which increase particle accumulation to ECs at the site interest, should be examined as well.".

Moreover, we are currently performing experiments, in collaboration with other groups, where we use the presented protocol and system to study targeting of deformable particles with different moieties (e.g., anti-ICAM1, Sial-Lewis-A) to inflamed EC in our model.

- It is not clear why authors fixed the cell prior to perfusion. The main application for these types of systems would be to do live culture of cells in the models and perfusing them various molecules/particles. If fixed, how could particle targeting be of relevance? A live culture/perfusion and targeting assay would be needed here.

Reply: We thank the reviewer for this comment. In our perfusion experiments we use fixed cells, because we focus on the initial adhesion step of the particles, especially large ones, upon reaching the vessel wall, as previously described by other groups in past studies (see for example: PMID: 19721193). In this work, it was shown that particles, in the first stage of adhesion, bind to live and fixed cells similarly. However, internalization of particles was observed only by live cells in following stages. Therefore, we understand the reviewer's comment and the need to perform such experiments in live-cell models since full replication of the physiological conditions should include targeting to live ECs. 3D models with live cells have been used by other groups, however, they were used for biological studies (see for example: PMID: 24841070) and therefore, we believe our system can be used for targeting experiments with live cells, but this was not the focus of our work. This note was referred in

the discussion by added the following: "In addition, in the presented protocol, ECs were fixed prior to connecting the models to the perfusion system and injection of the particles. Particles' adhesion to fixed cells represents the first stage in the binding process and therefore, experiments with live cells need to be performed, where internalization of particles may occur during later stages of the adhesion process".

- Figure 4 is not clear and properly presented. Did authors use two different particle types with different fluorescence? This needs to be clarified in the caption and with proper labeling in the figure. Further, there is too much background (auto)fluorescence in panel B which makes it really difficult to conclude any rigid observation from this image. A control picture of untreated sample should be presented to compare the background signal. Also, higher magnification images should be provided to show clearly the cells, the particle accumulation, etc..

Reply: We thank the reviewer for this comment, and Figure 4 was updated accordingly. Prior to studying targeting of particles in cell cultured model, we have conducted experiments with 10 µm glass particles to visualize whether the formation of the recirculation occurs at the bifurcation sinus. Figure 4A confirms that using our system, physiological flow structures are obtained as expected. Afterwards, we performed experiments with 2 µm fluorescent carboxylated PS particles (in red) to ECs cultured inside the models (in blue) and tracked their deposition and adhesion (Figure 4B, 4C). Figure 4 legend was changed to: Figure 4: Perfusing and mapping the adhesion of particles. (A) Streak-line image of streamlines and recirculation (dashed rectangle) generated upon perfusion of 10 µm fluorescent glass particles, at a constant flow of 400 mL/min, through the model. (B) Deposition map of the 2 µm fluorescent carboxylated PS particles (in red) inside the 3D cultured model. (C) Adhesion of the particles (in red) to the cultured ECs (in blue – DAPI) inside the model at a 10X magnification."

Regarding the control untreated sample, we include an image of the cultured ECs inside the models without the particles, which is shown in Figure 2D. It is important to notice that Figure 4B shows the deposition of the red fluorescent 2 µm particles with high accumulation at one side of the bifurcation sinus which is characterized with a high shear regime – and it is not an autofluorescence effect.

Minor Concerns:

- Add proper labeling in Figure 3 to introduce all components of the perfusion system in the figure.

Reply: Done.

Reviewer #2:

Manuscript Summary:

The manuscript is well-written and the contents are of interest of a broad readership especially in 3D printing in medical research. The video presentation of the work would be a very interesting one and beneficial.

Reply: We thank the reviewer for his positive evaluation on the paper's contribution to the field.

Major Concerns:

Nil.

Minor Concerns:

The manuscript needs further copyediting for its English.

Reply: We thank the reviewer for this comment. English copyediting was done, and the manuscript was re-edited as requested.