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Article

A Modular, Cost-Effective and Pumpless Perfusion Assembly for Long-Term Culture of Engineered Microvessels

Shashwat S. Agarwal ¹, Jacob C. Holter ^{2,4}, Travis H. Jones ¹, Brendan T. Fuller ^{2,4}, Joseph W. Tinapple ², Joseph M. Barlage ³ and Jonathan W. Song ^{1,4,*}

¹ Department of Mechanical and Aerospace Engineering, The Ohio State University, Columbus OH 43210

² Department of Biomedical Engineering, The Ohio State University, Columbus OH 43210

³ Department of Biomedical Education and Anatomy, The Ohio State University, Columbus OH 43210

⁴ The Comprehensive Cancer Center, The Ohio State University, Columbus OH 43210

* Correspondence: to: song.1069@osu.edu

Abstract: Continuous perfusion is necessary to sustain microphysiological systems and other microfluidic cell cultures. However, most of the established microfluidic perfusion systems, such as syringe pumps, peristaltic pumps, and rocker plates, have several operational challenges and may be cost-prohibitive, especially for laboratories with no microsystems engineering expertise. Here, we address the need for a cost-efficient, easy-to-implement, and reliable microfluidic perfusion system. Our solution is a modular pumpless perfusion assembly (PPA), which is constructed from commercially available, interchangeable, and aseptically packaged syringes and syringe filters. The total cost for the components of each assembled PPA is \$1-2 USD. The PPA retains the simplicity of gravity-based pumpless flow systems but incorporates high resistance filters that enable slow and sustained flow for extended periods of time (hours to days). The perfusion characteristics of the PPA were determined by theoretical calculations of the total hydraulic resistance of the assembly and experimental characterization of specific filter resistances. We demonstrated that the PPA enabled reliable long-term culture of engineered endothelialized 3-D microvessels for several weeks. Taken together, our novel PPA solution is simply constructed from extremely low cost and commercially available laboratory supplies and facilitates robust cell culture and compatibility with current microfluidic setups.

Keywords: modular microfluidics; microphysiological systems; xurography; hydraulic resistance

1. Introduction

Perfusion is integral to several microfluidic-based applications, such as microphysiological systems (MPS) [1,2], single cell capture [3], mimicking perspiration [4,5], and on chip sorting, positioning, and stimulation of *c. elegans* [6]. In the context of MPS and 3-D microtissues, perfusion ensures sufficient nutrient delivery, waste removal, and mechanical stimuli for sustaining long-term cell culture conditions for biological mechanistic and therapeutic screening studies. To meet these needs, researchers have commonly turned to externally controlled machines such as syringe pumps [7], peristaltic pumps [8,9], and rocker plates [10,11]. While these external controls have been widely implemented by microfluidic researchers, they are also subject to several operational challenges. For instance, syringe pumps and peristaltic pumps interface with microfluidic devices through tubing, which—depending on the material—can be prone to non-specific adsorption of analytes from the fluid being perfused and are subject to dead volume [12,13]. Moreover, insertion of tubing in microfluidic devices can introduce air bubbles, which often cause device failure by blocking channels and/or creating flow instabilities. Rocker systems provide a pumpless and tubeless approach for

perfusion where flow occurs in microdevices resting on moving plates. However, the nature of the flow controlled by rocker plates is typically oscillatory and bidirectional, which may not be appropriate based on the physiology of interest: e.g., blood microcirculation where intravascular flow under normal conditions is unidirectional and steady [14].

The challenges associated with equipment-based perfusion apparatuses have prompted researchers to develop easy-to-use perfusion setups that do not require an external power source and are tubeless [15-20]. For instance, a very simple and commonly used approach is to insert a fluid filled pipette tip [21] directly into the inlet port to create a hydrostatic pressure differential. However, in this setup, changes in pressure are transient and rapidly deplete, resulting in temporally non-uniform flow that lasts for no longer than several seconds or minutes. Beebe and colleagues [22-24] proposed a passive pumping mechanism to create surface tension driven flow through the introduction of different sized droplets on the inlet and outlet ports of a microfluidic device. While this pumping platform is easy to fill, readily multiplexed, and compatible with conventional fluid handling, the pressure differential quickly equilibrates; it is also prone to flow-control problems [25]. Komeya et al. [16] reported an interesting and bespoke pumpless design that uses a resistance microchannel between the fluid reservoir and the cell culture region of interest to sustain hydrostatic pressure head driven flow for up to several days. The resistance channel can be modified to control flow rates, and this design was used for long-term cultivation of endothelialized lumens [26]. However, this method requires fabrication of custom resistance microchannels using specialized facilities, such as a cleanroom, which may limit accessibility and translation to laboratories with no prior microfluidic and/or lithographic expertise.

The purpose of the present study is to develop a perfusion system that can benefit all biomedical researchers, including ones from non-microfluidic labs, interested in long-term cultivation of MPS. To this end, our solution should be easy to fabricate and implement, low-cost, accessible, and reliable in sustaining 3-D microtissues. Here we present a pumpless perfusion assembly (PPA) that meets all these requirements. The PPA is assembled from components (or modules) consisting of sterile, pre-packaged (or “off-the-shelf”) syringes and syringe filters. These components are readily available from commercial suppliers and can be obtained at a total cost of \$1-2 USD per PPA. The PPA retains the simplicity of gravity-based pumpless flow systems but incorporates high resistance filters that enable slow and sustained flow for long periods (hours to days). The PPA is highly modular and can readily incorporate syringe filters of different properties reported by the commercial vendor (e.g., diameters, porosity, and pore sizes). We developed and validated our PPA approach by using several flow characterization schemes, where we observed that the flow rate of each PPA was a property of the syringe filter diameter and the pore size. We demonstrated that perfusion with the PPA can culture and sustain endothelialized 3-D microvessels for up to 21 days. Notably, we engineered 3-D microvessels using an inexpensive and versatile rapid prototyping technique recently developed by our group using desktop craft cutting, or xurography [27]. Thus, the PPA provides a very simple, extremely low cost, and robust perfusion solution using commercial off-the-shelf components for reliably culturing vascularized MPS using laboratory methods that should be accessible to most labs worldwide.

2. Materials and Methods

2.1. Modular Pumpless Perfusion Assembly Using Ready-Made and Widely Available Laboratory Components

We constructed the two-component and modular pumpless perfusion assembly using standardized and aseptically packaged (i.e., sterile) laboratory consumables (**Figure 1**). The first component of the PPA is a trimmed syringe with the plunger removed, henceforth referred to as the liquid hopper. We used syringes of varying volume capacity (e.g., 20 mL and 60 mL) and trimmed to a height suitable for benchtop experimentation, incubation, or microscopy. The open top nature of the liquid hopper enables efficient media exchange via pipetting. The second component is a

hydrophilic syringe filter, which provides the main resistive element of the PPA. Syringes were obtained from Henke Sass Wolf (Germany) and BD (New Jersey, USA), and syringe filters containing semi-porous membranes of cellulose acetate (CA) were sourced from Tisch Scientific (Ohio, USA). These CA syringe filters were selected because of their anti-fouling (i.e., low protein binding affinity) and hydrophilic properties consistent with typical cell culture media filtration [28].

The syringe filter of the PPA was pre-wetted by pushing 5 mL of an aqueous solution through the filter using an attached syringe and plunger (**Figure 1A i**). Sufficient pre-wetting is critical to eliminate any residual air trapped within the filter membrane—which would cause additional resistance—thus ensuring a continuous fluid circuit. For cell culture applications, pre-wetting can be done by cell-specific culture media or a buffered solution (e.g., PBS). Once the filter is properly wetted, the syringe is disconnected, and the filter is visually inspected for any air bubbles in the inlet or outlet of the housing (**Figure 1A ii**). If present, air bubbles are pipetted out. The PPA is fully assembled by connecting the liquid hopper to the pre-wetted syringe filter (**Figure 1A iii**). As standardized laboratory consumables, the syringe and syringe filter have Luer lock fittings, which ensures a leak-proof connection. The Luer slip outlet of the filter allows for insertion and press-fitting into a 4-mm-diameter inlet port of a PDMS microfluidic device. Finally, liquid is added in the liquid hopper up to the desired pressure head height.

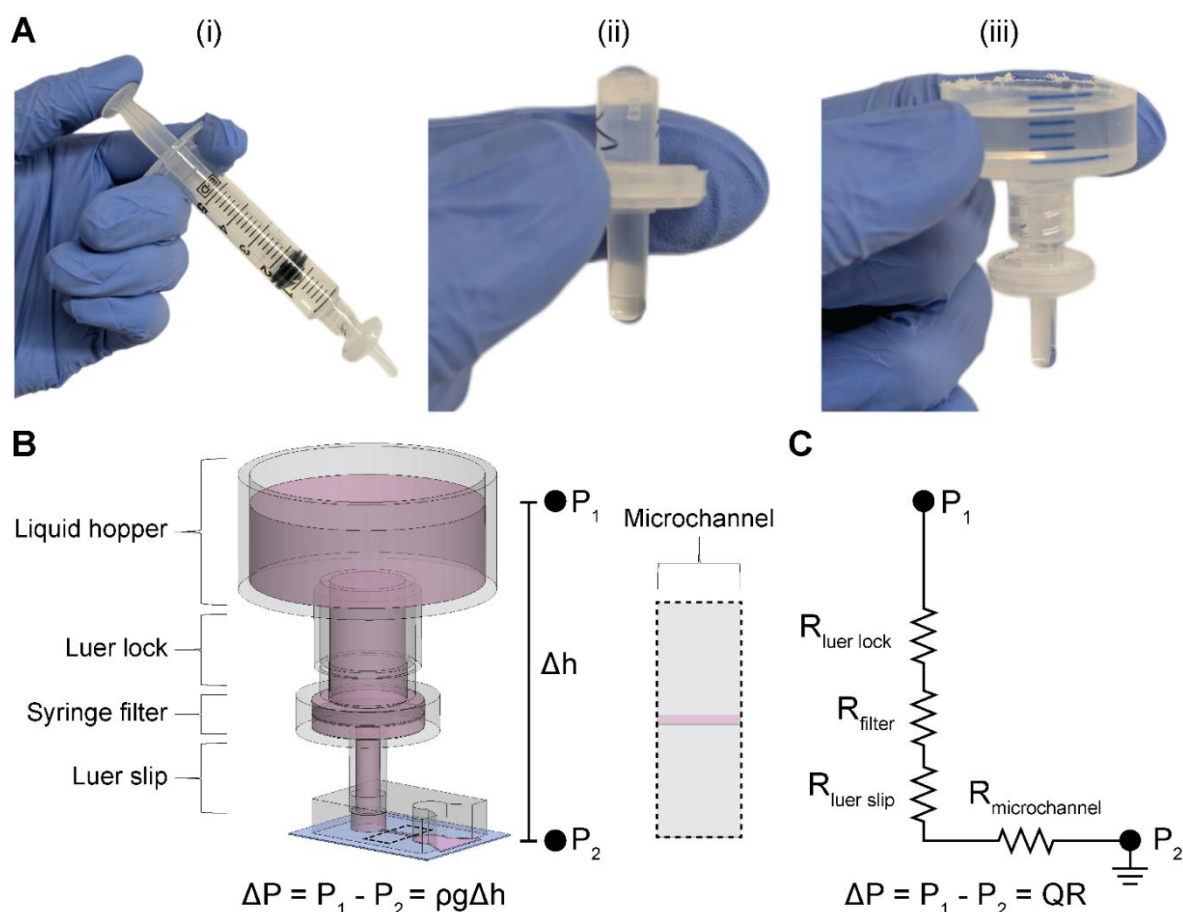


Figure 1. Pumpless perfusion assembly (PPA). **A**) Detailed stepwise instructions for modular PPA: **(i)** the syringe filter is pre-wetted, **(ii)** the syringe filter is inspected for air bubbles, **(iii)** the liquid hopper is attached to wetted syringe filter to form the PPA. **B**) Schematic of the PPA press-fitted into the inlet port of a straight-channel PDMS microdevice illustrating the gravity-based pressure head ($P_1 - P_2$), which drives fluid flow. A slit was cut at the outlet to allow pooling of perfused liquid. Dashed region represents the top view of the straight microchannel. **C**) Equivalent electrical circuit analog for the schematic shown in **B** demonstrating the hydraulic resistances in the system.

2.2. Resistance Characterization of the PPA Using Microfluidic Perfusion Experiments

We performed microfluidic experiments and analysis to determine the flow rates and resistances of the modular PPA. These experiments point to the objective of comparing our measured hydraulic resistances to the resistances which were calculated from the manufacturer’s specifications, henceforth referred to as manufacturer’s hydraulic resistances. We incorporated four different syringe filters in the PPA to characterize the modularity of this component: 0.22 μm and 0.45 μm pore sizes for both 4 mm and 13 mm diameter filters, henceforth denoted as 4_{0.22}, 4_{0.45}, 13_{0.22}, and 13_{0.45}. These specific filters were selected in part because we were able to obtain the corresponding manufacturer-specified pressure head (Δ*P*) and flow rates (*Q*), which were used to calculate the manufacturer’s hydraulic resistances (*R*) (**Table 1**).

Table 1. Manufacturer’s hydraulic resistances. Values for filter diameter, pore size, flow rate, and pressure head were obtained from the manufacturer’s specifications.

Filter Diameter (mm)	Pore Size (μm)	Flow Rate (<i>Q</i>) (@Δ <i>P</i> = 10 psi) (mL/min)	Resistance (<i>R</i>)* (Pa·s/m ³)
4	0.22	1.0 – 1.4	3.54 ± 0.59 × 10 ¹²
4	0.45	7 – 9	5.25 ± 0.66 × 10 ¹¹
13	0.22	11 – 17	3.09 ± 0.66 × 10 ¹¹
13	0.45	19 – 56	1.45 ± 0.72 × 10 ¹¹
*Calculated using <i>R</i> = Δ <i>P</i> / <i>Q</i> , Equation 1			

To experimentally obtain hydraulic resistances, henceforth referred to as measured hydraulic resistances, we measured the total pass-through volume through the microchannel of a PDMS microdevice delivered by the PPA over a discrete timeframe (**Figure 1B**). The single, straight-channel microfluidic device used in these experiments has been previously described by our group and was fabricated with PDMS soft lithography [29]. The height, width, and length of the microchannel in the silicon master mold is 150 μm, 500 μm, and 5 mm, respectively. The microchannel was replica molded by pouring PDMS pre-polymer onto the silicon wafers at a 10:1 base to curing agent ratio and cured overnight in a 65°C oven. 4-mm-diameter biopsy punches were used to core the inlet and outlet ports of the microchannel. The PPA was attached to the inlet port of the straight channel microdevice pre-filled with double distilled water (ddH₂O). A 20 mL syringe (BD, 302830) was chosen as the aspect ratio of the liquid hopper allowed for a significant hydrostatic pressure head (~100 mmH₂O) to drive perfusion into the microchannel, in combination with perceptible change of liquid meniscus within the hopper. Moreover, a slit was made at the outlet port, and the device was submerged in a pool of ddH₂O to establish a continuous fluid circuit and to minimize back pressure that would cause inaccurate measurements. The pass-through volume through the microchannel was determined from the drop in height of the ddH₂O within the liquid hopper, photographs of which were taken at the beginning and end of experiments. The experimental time for each filter was informed by the results from our numerical calculations performed using the experimentally applied pressure head (100 mm) and radius of the liquid hopper (9.125 mm, constructed from a 20 mL syringe) (**Section 2.3**) to allow for at least 300 μL of pass-through volume in each filter. Finally, the measured hydraulic resistance of each filter was determined based on the measured pass-through volume and time by using a closed-form solution of filter resistance as a function of volume drop and experimental time described in **Section 2.3**.

2.3. Computational Prediction of Hydraulic Resistance and Volumetric Flow Rate

Volumetric flow rate can be written as proportional to the applied pressure and inversely proportional to the resistance of the system. In our setup, the applied pressure is due to the pressure head in the liquid hopper. The flow rate can then be written as:

$$Q = \frac{\Delta P}{R_{system}} = \frac{\rho g \Delta h}{R_{system}} \tag{1}$$

where Q is the volumetric flow rate and ΔP is the pressure differential between the inlet and outlet defined by ρ , the fluid density, g , the acceleration due to gravity, and Δh , the height difference of the inlet and the outlet. The total hydraulic resistance of the system, R_{system} , is calculated by summing all resistances in series using a corresponding electrical circuit analogy as shown in **Figure 1C**:

$$R_{system} = R_{luer\ lock} + R_{filter} + R_{luer\ slip} + R_{microchannel} \quad (2)$$

$R_{luer\ lock}$ and $R_{luer\ slip}$ are calculated assuming Hagen-Poiseuille flow in a circular cross section (e.g., a pipe) [30], which is given by:

$$R_{pipe} = \frac{128\mu L}{\pi d^4} \quad (3)$$

where μ is the dynamic viscosity of the fluid, L is the length of the pipe, and d is the inner diameter of the pipe. Based on **Equation 3**, $R_{luer\ lock}$ of our setup was $2.21 \times 10^7 \text{ Pa}\cdot\text{s}\cdot\text{m}^{-3}$. Moreover, $R_{luer\ slip}$ for 4 mm and 13 mm filters were $3.94 \times 10^7 \text{ Pa}\cdot\text{s}\cdot\text{m}^{-3}$ and $6.85 \times 10^6 \text{ Pa}\cdot\text{s}\cdot\text{m}^{-3}$, respectively. Furthermore, we determined the resistance of our cylindrical microvessels to be $5.57 \times 10^{10} \text{ Pa}\cdot\text{s}\cdot\text{m}^{-3}$ from **Equation 3**.

The microchannel resistance, $R_{microchannel}$, was derived using analytical solutions corresponding to the microchannel geometry. For the simple rectangular microchannel we used [30]:

$$R_{microchannel} = \frac{12\mu L}{1 - 0.63(h/w)} \cdot \frac{1}{h^3 w} \quad (4)$$

where h is the length of the short wall and w is the length of the long wall. For our straight-channel device, the microchannel resistance was estimated to be $3.90 \times 10^{10} \text{ Pa}\cdot\text{s}\cdot\text{m}^{-3}$. Moreover, since $R_{microchannel} \gg R_{luer\ lock}$ and $R_{microchannel} \gg R_{luer\ slip}$, **Equation 2** can be reduced to:

$$R_{system} = R_{filter} + R_{microchannel} \quad (5)$$

Equation 5 can be substituted into Equation 1 to express flow rate, Q , in terms of the pressure head and the dominant hydraulic resistances of the system:

$$Q = \frac{\rho g \Delta h}{R_{filter} + R_{microchannel}} \quad (6)$$

Since the pressure head in the liquid hopper decreases with time, any flow rate measurement would be a time-averaged value. Hence, **Equation 6** cannot be directly employed to compute measured hydraulic resistance for all filters tested. Instead, we sought to define the measured hydraulic resistance as a function of discrete pressure head measurements. This required calculating the instantaneous pressure head over time, where cumulative volume drop can then be quantified from the difference in heights given the cross-sectional area of the liquid hopper.

The rate of change in pressure head can be written as an ordinary differential equation:

$$\frac{dh}{dt} = -\frac{Q(t)}{A} \quad (7)$$

where h is the pressure head, t is time, $Q(t)$ is the instantaneous flow rate, and A is the cross-sectional area of the liquid hopper. Substituting Q from **Equation 6** into **Equation 7** and recognizing that **Equation 7** is of the form of an exponential decay equation, one can write the closed form solution as:

$$h(t) = h_0 e^{-\frac{\rho g t}{A(R_{filter} + R_{microchannel})}} \quad (8)$$

where h_0 is the initial pressure head at time $t = 0$. A custom MATLAB script was written to compute the time dependence of fluid height within the liquid hopper and by extension the time-dependent flow rates for all syringe filters, using the manufacturer's hydraulic resistances. These calculations were conducted to inform our choice of syringe filter to use for long-term culturing of 3-D engineered microvessels.

To extract measured filter hydraulic resistances from the experimentally observed pass-through liquid volumes, R_{filter} was solved for, given **Equation 8**, leading to the following equation:

$$R_{filter}(\Delta V, \Delta t) = - \left[\frac{\rho g \Delta t}{A} \log^{-1} \left(1 - \frac{\Delta V}{A h_0} \right) \right] - R_{microchannel} \quad (9)$$

where ΔV is the experimentally observed pass-through liquid volume and Δt is the experimental time duration.

Similarly, the time taken to reach a specific pressure head can be solved for from **Equation 8**. We chose to solve for the time required for the liquid hopper to be depleted, which results in the following equation:

$$t_{empty}(A, R_{filter}) = - \frac{A(R_{filter} + R_{microchannel})}{\rho g} \log \left(\frac{h_b}{h_0} \right) \quad (10)$$

where h_b is the height of the base of the liquid hopper. To generate a contour plot of half-time of liquid hopper heights, an array of varying liquid hopper radii ranging from 5 – 25 mm were used along with an array of theoretical filter resistances ranging from 1×10^9 – 1×10^{13} Pa·s·m⁻³. The bottom of the liquid hopper, h_b , was measured to be 30 mm above the microdevice outlet.

2.4. Human Umbilical Vein Endothelial Cell Culture and Preparation

Commercially available human umbilical vein endothelial cells (HUVECs, Lonza) were purchased and maintained using endothelial cell growth medium-2 (EGM-2, Lonza). Cell passage numbers of 5–10 were used in this study. Cells were cultured in a humidified incubator at 37°C and 5% CO₂ with media exchange every two days. Cells were harvested by washing with 1x Dulbecco's phosphate-buffered saline (PBS) without Mg/Ca (1x DPBS, Gibco) followed by detachment using 0.05% Trypsin-EDTA 1x (Gibco) for 3–4 minutes. Subsequently, trypsin was neutralized with 10% FBS in DMEM and cells were centrifuged at 200 RCF for 5 minutes. HUVECs were resuspended in media at a concentration of 50×10^3 cells per μ L in preparation to seed the lumens of the microfluidic devices.

2.5. Preparation of 3-D Engineered Microvessels and Integration of PPA for Long-Term Culture

We demonstrated the applicability of the PPA by enabling long-term culture of engineered microvessels constituted with HUVECs. To achieve this, we employed a xurography-based microfluidic device made from PDMS to engineer 3-D, cylindrical microvessels, as previously described by our group [27]. Briefly, the device was constructed from 5 layers of PDMS bonded together by plasma oxidation and then bonded to a glass slide. A central channel was cut via xurography, and ports were formed by boring circular holes with biopsy punches. To ensure an adequate connection with the PPA, the top-most layer of the PDMS device was made to be 6 mm in thickness with a 4-mm-diameter inlet port; this facilitated a press-fit connection between the PPA and the device. After fabrication, microdevices were pre-coated with poly(dopamine) (PDA) to enhance collagen gel attachment to PDMS [31]. Type I collagen was added to the central ECM chamber at a concentration of 3 mg mL⁻¹ (Corning, 354249) with a 250- μ m-diameter nitinol wire running through the gel chamber from inlet to outlet ports. Following collagen polymerization the wire was removed, thereby leaving a hollow cylindrical lumen encased in hydrogel. 24 hours after seeding the lumen with HUVECs, the PPA was filled with 5 mL of cell media and it was press-fitted into the inlet port of the microfluidic device to enable perfusion, as shown in **Figure 3A**. The syringe filter in the PPA was replaced daily to ensure precise flow rates. However, we suspect this was not necessary, and further testing would be needed to determine the maximum number of days a particular filter can maintain its flow rate profile. Phase contrast images of the microvessel were taken daily (Invitrogen, EVOS XL Core). It is important to press fit the PPA in the microdevice with minimal force to prevent mechanical perturbation of the gel or microvessel.

2.6. Fluorescence Microscopy

We used a protocol developed by our group to fix and stain 3-D engineered microvessels [27]. Briefly, between day 7 - 14, cells in the microvessels were fixed with 4% paraformaldehyde (PFA) for

30 min at room temperature (RT), permeabilized with 0.2% Triton X-100 for 30 min at RT, blocked with blocking buffer for 30 min at RT, and subsequently stained for nuclei (DAPI, Sigma-Aldrich, D9542), VE-cadherin (monoclonal antibody, BD Biosciences, 561567), and F-actin (phalloidin, Fisher Scientific, A12379). Microvessels were washed three times between each step with washing buffer: 0.1% Tween 20 in 1x PBS. Blocking buffer consisted of 1% BSA in washing buffer. VE-cadherin was stained overnight at 4°C, phalloidin was stained for 60 min at RT, and lastly DAPI was counterstained for 10 min. Images were taken using a Nikon A1R Live Cell Imaging Confocal Microscope controlled with NIS-Elements Software.

3. Results

3.1. Validation of the Hydraulic Resistances of Syringe Filters: Implications for Flow Modulation

First, we determined the average hydraulic resistance of syringe filters integrated in the PPA, with the objective of comparing measured and manufacturer's hydraulic resistances. Experiments were conducted with PPAs connected to microdevices containing a simple, straight microchannel (**Figure 1B**). We assessed filters of different diameters and pore sizes which came with manufacturer-specified flow rates at a constant pressure head of 10 psi (**Table 1**). The flow rates and pressure head were used to calculate the manufacturer's hydraulic resistances of the syringe filters. We chose the following filters: 4_{0.22}, 4_{0.45}, 13_{0.22}, and 13_{0.45} to study the effects of filter area and pore size on volumetric flow. In addition, these filter pore sizes are widely available for purchase as they are commonly used for membrane filtration of particulates. For instance, 0.22 µm pore size membranes are the industry standard for sterile-filtration of cell culture media to eliminate contaminating bacteria [32].

Using the manufacturer's average hydraulic resistance values for each filter, we quantified the time dependence of flow rate (**Figure 2A**) using the closed-form solution for instantaneous pressure head (**Equation 8**). These calculations were performed using the initial experimentally applied pressure head (100 mmH₂O) and radius of the liquid hopper (9.125 mm, constructed from a 20 mL syringe). The results informed the experimental time for measuring at least 300 µL of pass-through volume in each filter. Subsequently, we measured volume drop in the liquid hopper over the predetermined experimental time with the same initial pressure head for all aforementioned filters. The volume drop in the liquid hopper is equivalent to the pass-through volume, neglecting evaporation effects. Subsequently, **Equation 9** was used to calculate the measured hydraulic resistances for all filters tested.

A comparative assessment of the measured and manufacturer's hydraulic resistances is shown in **Figure 2B**. It can be observed that the measured hydraulic resistances mostly lie within the range of manufacturer's hydraulic resistance values. In addition, the ratio of measured resistances for 13 mm to 4 mm filters was found to be $13_{0.22}:4_{0.22} = 0.102$ for 0.22 pore size and $13_{0.45}:4_{0.45} = 0.120$ for 0.45 pore size. These ratio values closely match the inverse ratio of the manufacturer-listed filtration areas, $A_4:A_{13} = 0.115$. Furthermore, the ratios of measured filter resistances for 0.45 µm to 0.22 µm pore size at the same filter diameter were found to be $4_{0.45}:4_{0.22} = 0.221$ and $13_{0.45}:13_{0.22} = 0.262$. These ratio values closely match the inverse ratio of theoretical pore area $A_{0.22}:A_{0.45} = 0.239$. Both observations are consistent with the notion that hydraulic resistance is inversely proportional to cross-sectional area. Notably, we confirmed resistance of filters at much lower pressures (~ 10-fold lower) compared to the 10-psi pressure used by the manufacturer, indicating a linear dependence of flow rate to pressure head as given in **Equation 1**. These observations validated our approach to quantify hydraulic resistance as well as demonstrated that commercial syringe filters show proportional resistances based on filtration area and pore size.

We also observed from the numerical calculations (**Figure 2A**) that for all filters there was a non-linear decay of flow rate with time. This non-steady flow rate is a consequence of a depleting pressure head within the liquid hopper, the rate of which depends on the radius of the hopper and the filter hydraulic resistance. It is important to point out that once the hopper is depleted, the air-liquid interface at the inner radius of the Luer lock (1 mm) causes the fluid to experience capillary arrest

whereby the capillary force counteracts the remaining hydrostatic pressure thereby achieving equilibrium and causing the flow to stop. One advantage of this phenomenon of capillary arrest is that the liquid hopper can directly be refilled with media without having to disconnect the PPA from the microdevice to reestablish a continuous fluid circuit. The time taken for the liquid hopper to deplete, as given in **Equation 10**, was calculated for a range of hydraulic resistances and hopper radii, in the context of the PPA connected to our straight channel microdevice (**Figure 2C**). The contour plot illustrates the modular nature of our PPA, enabling researchers to modulate flow rate and consequently the flow rate decay by swapping the syringe hopper or syringe filter in the PPA. This notion is supported by the estimated time taken for flow arrest in a 60 mL syringe hopper compared to a 20 mL syringe hopper for each syringe filter. This outcome is due to the larger radius of the 60 mL syringe (13 mm) as compared to the 20 mL syringe (9.125 mm) which results in a slower head loss for the same instantaneous volumetric flow rate. Thus, the flow rate can be considered comparatively “steadier” for a liquid hopper with a larger radius. Moreover, increasing filter membrane diameter or pore size decreases overall filter resistance, resulting in more rapid head loss. Syringe filters with larger diameters and pore sizes would be better suited for short-term experiments where a high flow rate is required for only a short duration. In contrast, high resistance syringe filters are well suited for longer-term studies which require quasi-steady flow rate conditions.

Among the syringe filters considered, the $4_{0.22}$ filter has the highest hydraulic resistance. Consequently, it takes the longest time for liquid hopper depletion compared to all other chosen filters. Since flow rate is directly proportional to the pressure head, this also implies that the $4_{0.22}$ filter offers the steadiest flow, among the filters tested. For instance, the fluid within the liquid hopper depletes in approximately 12 hours for the PPA constructed from a $4_{0.22}$ filter and a 60 mL syringe (**Figure 2C**). In contrast, the same drop takes approximately 30 minutes for the $13_{0.45}$ filter. This positions $4_{0.22}$ filter as having the “most steady” and long-lasting flow rate among the four filters. This analysis informed our choice of using a 60 mL syringe hopper with a $4_{0.22}$ filter as the modular components of PPA used for the long-term culture of endothelialized microvessels.

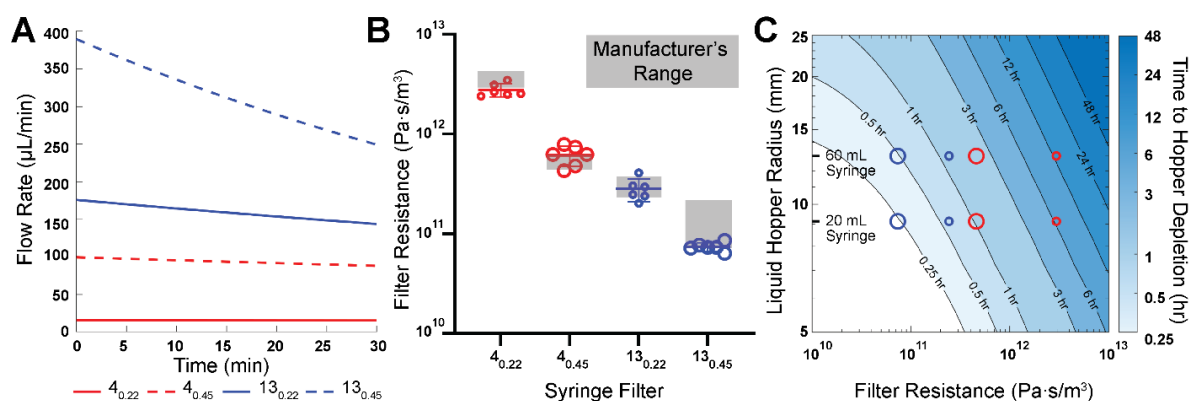


Figure 2. Measured and manufacturer's hydraulic resistances of syringe filters. **A)** Instantaneous flow rate over the first 30 minutes for an initial 100 mmHg pressure head and a 20 mL syringe as the liquid hopper. Values calculated using **Equation 6** with the manufacturer's hydraulic resistances. **B)** Comparative assessment of measured and manufacturer's hydraulic resistances for the four types of syringe filters. **C)** Contour visualizing time taken for depletion of fluid in the liquid hopper for different syringe filters and hopper radius based on **Equation 10** for a 40 mmHg pressure head. Small and large, red and blue circles correspond to syringe filter labels from panel **B**.

3.2. PPA Facilitates Long Term Culture of Engineered Microvessels for Several Days

Next, we employed a PPA with a $4_{0.22}$ filter and 60 mL syringe for long-term culture of HUVEC-lined microvessels. The PPA was press-fitted into the inlet port of the microdevice 24 hours after seeding (**Figure 3A**). **Figure 3B** shows the theoretical instantaneous height of fluid in the liquid

hopper, using the manufacturer's average hydraulic resistance for each tested filter. The height of media in the liquid hopper depletes to the minimum height fastest for 13_{0.45} and slowest for 4_{0.22} due to the relative hydraulic resistances. The minimum pressure head from **Figure 3B** corresponds to the elevation of the base of the liquid hopper.

We also calculated the theoretical instantaneous flow rate and wall shear stress within the microengineered vessel (**Figure 3C**), using the manufacturer's hydraulic resistances for the syringe filters. It was observed that 4_{0.22} has the lowest flow rate and is the only filter with shear stress values below 1 dyn/cm². Although 3 dyn/cm² is typically considered physiological for HUVECs, minimal shear stresses (~0.1 dyn/cm²) are sufficient to maintain HUVEC viability [33]. Ultimately, we selected the 4_{0.22} filter for integration into the PPA for two principal reasons. First, as mentioned above, it takes the longest time to deplete the liquid hopper and needs replenishment only once in ~12 hours (neglecting evaporation effects in a sufficiently hydrated cell culture incubator). Second, since the resistance of the 4_{0.22} filter is an order of magnitude higher than the cylindrical microvessel (comparable to typical resistances for MPS), it shows the "most steady" flow rate of the syringe filters tested, as shown in **Figure 3C**. The instantaneous flow rate results demonstrate only a 4.5% decrease in flow rate over the first 60 minutes for 4_{0.22} filter. These aspects reinforced our decision to use the 4_{0.22} filters for long-term culturing of HUVEC-lined microvessels.

Phase contrast images of a microvessel perfused using this PPA are shown in **Figure 3D** for days 1, 5 and 11. The longest duration for which we cultured HUVEC microvessels was 21 days (data not shown). Following immunofluorescent staining for VE-cadherin, DAPI and actin, we confirmed with confocal microscopy that the perfused engineered microvessel was stable with barrier integrity and with an open circular lumen (**Figures 3E-F**). Interestingly, actin fibers in HUVECs did not appear elongated or aligned in the flow direction. This result can be attributed to the sub-physiological levels of average wall shear stress values ($\tau_w < 1$ dyn/cm²) for 4_{0.22} as shown in **Figure 3C**. It has been observed previously that HUVECs lack any actin stress fibers when exposed to 0.8 dyn/cm² of shear stress [34].

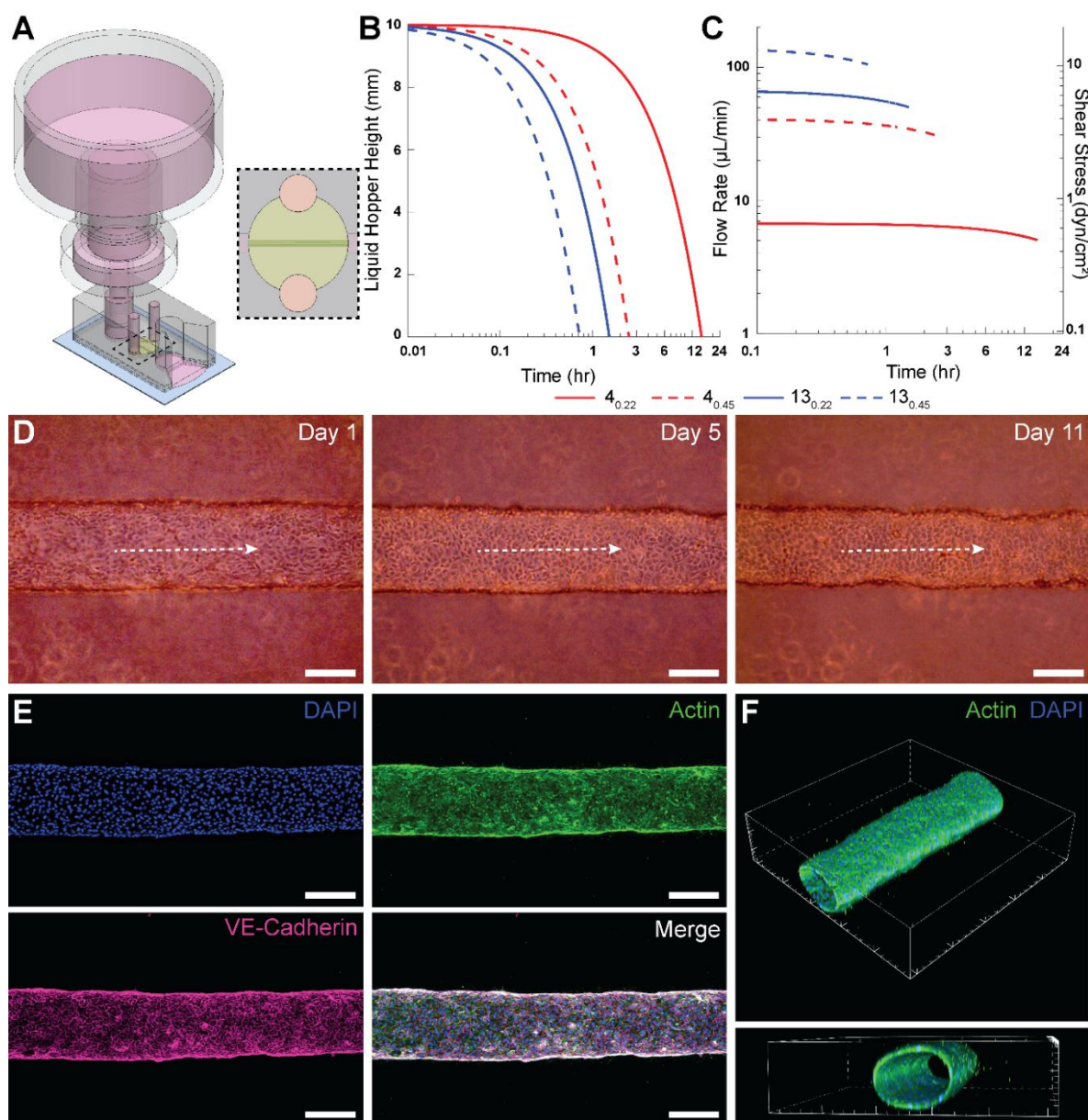


Figure 3. Implementation of the PPA for long-term culture of 3-D engineered microvessels. **A)** Isometric view of PPA constructed from a trimmed 60 mL syringe and a 4_{0.22} filter press-fitted into a PDMS-based device with a cylindrical HUVEC-lined microvessel. A slit was cut at the outlet to allow pooling of perfused cell culture media. Dashed region represents the top view of the perfusable microvessel encapsulated in collagen. **B)** Theoretical instantaneous media height within the liquid hopper for the PPA consisting of modular syringe filters given in Table 1 connected to the microvessel device as shown in A. **C)** Theoretical instantaneous flow rates for the PPA and the directly proportional intravascular wall shear stress within a 250- μ m-diameter cylindrical microvessel. **D)** Phase contrast images of a representative microvessel at Day 1, 5 and 11. Dashed white arrows indicate intravascular flow direction. **E)** Confocal z-projection of an intact cylindrical microvessel fully lined with HUVECs. The vessel was fixed at day 7, and stained for F-actin (phalloidin, green), VE-cadherin (monoclonal antibody, pink), and nuclei (DAPI, blue). **F)** 3-D renders of the microvessel from confocal z-stack to show cellular monolayer and patency. Scale bars in D and E are 200 μ m. The interval of major ticks in F is 200 μ m.

4. Discussion

The limitations of equipment-based perfusion apparatuses prompted us to develop a novel perfusion solution for microfluidic culture applications that meet the following design considerations. First, the flow rates produced by the perfusion setup can be sufficiently well characterized, are predictable, and fall within desirable ranges over a prescribed timeframe for the

intended applications. Second, the setup is low cost without the requirement for the procurement of specialized equipment. Third, the perfusion apparatus is portable, does not require external power or control, and has a minimal footprint such that the entire system (both the microdevice and perfusion apparatus) can be placed within the same controlled environment (e.g., cell culture incubator). Fourth, the setup is associated with lower operational challenges, enabling researchers to pursue perfusion studies more readily. Fifth, the setup is amenable to conventional fluid handling techniques (e.g., pipetting), which may foster scaling up for high-throughput applications and foster adoption by scientists with no prior microfluidic and microfabrication training. Our study puts forth the described PPA that meets all these design considerations.

Our results provide ample support for the PPA in meeting the first design consideration. We have shown that the experimentally determined hydraulic resistances of the four tested filters fall within the range of the manufacturer's specifications. This allows researchers to use the provided product specifications to reliably predict flow rate and duration of flow for their specific experimental setup. In addition, since flow rate and duration of flow are functions of both components of the PPA, specifically the filter hydraulic resistance and liquid hopper cross-sectional area, users have flexibility in selecting the filter and syringe that meets the requirements for their experimental design. To demonstrate this point, we utilized a 40.22 filter and a 60 mL syringe for long-term perfusion of an engineered microvessel, enabling culture and maintenance of HUVEC vessels for up to 21 days.

Regarding the second design consideration, part of the ambition of this study was to develop a cost-efficient and functional perfusion system that facilitates access to 3-D microfluidic perfusion studies. A recent example that meets these needs is a 3D-printed mini-peristaltic pump, which can be fabricated at a fraction of its commercial counterparts (~\$175 USD) and leverages open-source programmable electronic controllers [9]. Comparatively, we offer a perfusion solution that is incredibly low cost (\$1-2 USD per PPA) and requires no additional capital investment. Indeed, the components used to construct the PPA (syringe and syringe filter) are often presently stocked in most wet labs. Hence, this status may enhance research workflows because an effective PPA can be constructed without waiting for the procurement of materials or equipment installation. Notably, our solution is devoid of any external machines or controllers. The versions of the PPA described in this study used only readily available and prepackaged (or "off-the-shelf") products, thereby employing favorable economies of scale to suppress costs. Nonetheless, our PPA solution also allows for a degree of customization as several manufacturers of syringe filters may be willing to work with researchers to fabricate membranes of different materials, thicknesses, diameter, and pore sizes. All these parameters will have an impact on the effective hydraulic resistance of the syringe filter, which we showed to be the dominant resistive term that determines the flow rates in an integrated PPA-microfluidic setup, for all tested filters. Our study also describes a generalizable microfluidic-based characterization scheme using experiments and numerical calculations. This scheme can be used by others for deriving the perfusion characteristics of other PPAs, including ones that integrate custom syringe filter modules.

A leading cause of detrimental air bubble formation is changes in temperature and pressure between a microfluidic device residing inside an incubator and a perfusion controller placed outside of an incubator and connected via tubing. Perfusion controllers (e.g., a syringe pump) are often much larger than a microfluidic device and cannot be placed inside an incubator for several reasons. One such reason is the heat dissipation from these controllers when operating, which disrupts effective temperature control within an incubator. In contrast, our PPA is only slightly larger than a microfluidic device, thereby enabling us to readily meet the third design requirement. We note that the PPA does add some vertical height to the setup that may be of consideration during microscopy. Nonetheless, as a highly portable and tubeless solution, our PPA intrinsically circumvents the pain points that can cause air bubble formation and experiments to fail. For our long-term culture experiments with 3-D engineered microvessels, we used a PPA constructed with the 40.22 syringe filter and a 60 mL syringe hopper. The perfusion characteristics of this PPA required us to replenish the PPA only once every 24 hours by adding 5 mL of culture media. Thus, our perfusion solution readily

meets the fourth design requirement by posing little to no technical barriers in conducting experiments that culture engineered microvessels. Finally, with respect to the fifth design requirement, the open top of the PPA is amenable to fluid handling—from simple pipetting to scaled-up automation. This is advantageous such that the liquid hopper can be continuously refilled to accommodate steady flow into the microvessel, by maintaining a constant pressure head in the liquid hopper. Moreover, the PPA can be applied to microvessel systems to model intravenous infusion of drugs or inhibitors during therapeutic screening studies.

The described PPA provides a robust approach for enabling long-term cell culture and continuous supply of nutrients and waste removal in MPS. Yet, we recognize that there are several important limitations of the PPA as a microfluidic perfusion solution. First, like most gravity-based perfusion systems, the flow rates of the PPA vary temporally. For the 40.22 syringe filter, we observed only a 4.5% decrease in flow rate over 60 mins. However, certain applications may require narrower fluctuations in flow rates over time. Second, the range of time-averaged flow rates offered by the PPA may be too narrow for certain desired applications. Along these lines, the maximum shear stress levels of 0.65 dyn/cm² that were provided by the PPA in the engineered microvessel studies were much lower than physiological levels (typically > 3 dyn/cm²) [33]. This notion was reinforced by the lack of characteristic endothelial cell alignment and elongation in response to physiological shear stress levels observed in our studies. Our group and others have deep interests in studying the effects of physiological shear stress on endothelial cell morphogenesis, angiogenesis, and vessel remodeling [35,36]. Hence, external pumps and rocker plates may remain the mainstay flow applicators for these types of microfluidic studies. Although we demonstrated the effectiveness of the PPA for long-term culture of HUVECs, the characteristics of the PPA may be applicable for cell culture applications where low shear stress conditions are desirable, such as what was shown previously with CaCo-2 cells [20]. Collectively, unlike most microfluidic perfusion systems, researchers will encounter minimal technical and operational barriers and only nominal monetary cost with the PPA. Hence, we believe these characteristics of the PPA may help enhance the accessibility of MPS, especially to those with no prior microfluidic or microfabrication training.

5. Conclusions

This paper presents a pumpless perfusion assembly obtained by the integration of low-cost, standardized and aseptically packaged laboratory consumables. We demonstrated that the PPA enabled predictable flow rates based on theoretical calculations of the total hydraulic resistance of the assembly and experimental characterization. We also showed that the PPA enabled reliable long-term culture of HUVEC-lined engineered microvessels for several weeks. Hence, we posit that the value proposition of our PPA as a microfluidic perfusion solution is very high due to its ease of construction from extremely low cost and commercially available laboratory supplies, accessibility, robust culture, and compatibility with current MPS setups.

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