Organs-on-a-Chip

InVADE: Integrated Vasculature for Assessing Dynamic Events

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Drug screening with simplified 2D cell culture and relevant animal testing fail to predict clinical outcomes. With the rising cost of drug development, predictive 3D tissue models with human cells are in urgent demand. Establishing vascular perfusion of 3D tissues has always been a challenge, but it is necessary to mimic drug transport and to capture complex interorgan crosstalk. Here, a versatile multiwell plate is presented empowered by built-in microfabricated vascular scaffolds that define the vascular space and support self-assembly of various parenchymal tissues. In this configuration, assembly and organ-specific function of a metabolically active liver, a free-contracting cardiac muscle, and a metastatic solid tumor are demonstrated, tracking organ function using noninvasive analysis techniques. By linking the 3D tumor and the liver tissue in series, it is demonstrated that the presence of liver tissue is crucial to correctly reveal the efficacy of a chemotherapeutic drug, Tegafur. Furthermore, the complete cancer metastasis cascade is demonstrated across multiple organs, where cancer cells escaping from the solid tumor can invade a distant liver tissue connected through a continuous vascular interface. This combinatory use of microfabricated scaffold onto a standard cell culturing platform can offer important insights into the mechanics of complex interorgan biological events.

1. Introduction

In vitro 2D monolayer cell-culture has long played a major role in the screening of new drugs in the pharmaceutical industry. However, conventional 2D models fall short on replicating the cellular responses and variable nature of a 3D multicellular microenvironment.^[1–3] Thus, preclinical

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screening often fails to predict clinical outcome, resulting in high cost and high risk late stage drug failure. In fact, only 1 in 5000 promising drug candidates lead to a successful clinical approval.^[4] To curb the high cost of drug development, long lists of drug candidates need to be narrowed early in the development process with cheap, safe, and predictive methods. Researchers have attempted to address the limitations of over-simplified 2D culture with 3D cultures in customized microenvironments to capture features of living organs by incorporating physiologically relevant factors such as biomolecule permeation, [5,6] biomechanical stimulation,^[7,8] and cell migration.^[9] Numerous studies have reported that the consideration of relevant physiological environmental factors can lead to improved cell morphology, maturation, and functionality, improving model predictability.^[10-12]

Although these organ-mimetic devices excel in creating controlled microenvironments, they are often limited to modeling one subsection or aspect of the human organ, such as (i) the vascular/epithelial interface,^[13–17] (ii) the parenchymal tissue,^[18–22] or (iii) the dynamics of multiorgan interaction.^[23–25] Integration of multiple aspects of human physiology into a single platform is met with several engineering challenges. For instance, modeling the tissue interface barrier requires a stable physical interface to support cell growth, whereas generating functional high density parenchymal tissues requires an amenable matrix for tissue remodeling. Moreover, linking multiple 3D organ models requires a continuous 3D vascular interface with minimal dead volume to capture interorgan crosstalk and even interorgan cell trafficking.

We recently reported a bioscaffold (referred to as AngioChip) shown to support a stable vascular barrier within a functional parenchymal tissue self-assembled through an extensive matrix remodeling process.^[26] Here, we significantly expanded the capability and usability of these microfabricated scaffolds by adapting them in a ready-to-use 96-well plate platform and demonstrated the assembly of mini tissues representing a metabolically active liver, a free-contracting cardiac muscle, or a metastatic solid tumor. Moreover, by linking two organ models in series with a single vascular scaffold, for the first time we captured the complete cancer invasion-metastasis



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Figure 1. InVADE platform and tissue assembly. a) A schematic diagram that summarizes the key aspects of the scaffold multiwall plate technology. b) Illustration of components of the scaffold 96-well plate. c) SEM of the tissue chamber for the liver model with a scaffold suspended across. Scale bar, 1 mm. d) SEM of the tissue chamber for the heart or tumor models with a scaffold, attached with four cantilevers, suspended across the tube. Scale bar, 1 mm. SEM of the scaffold showing e) the main channel and f) the microholes on the side channel walls. Scale bar, 200 μ m. g) Illustration of the scaffold seeded with endothelial cells and parenchymal cells showing the spatial configuration of the coculture environment. h) Image of the scaffold multiwall plate including both heart and liver chambers. A pair of carbon electrodes was embedded into the base plate adjacent to the heart chamber for electrical stimulation. i) Schematics of the cell seeding process.

cascade^[27] including cancer cells escaping from the engineered 3D tumor, circulating through the common vasculature, and infiltrating the 3D liver tissue downstream. Modeling this interorgan biological event was realized by the presence of all three aspects of human physiology: a dense parenchymal

tissue, a stable vascular barrier, and an interorgan connection through a common vasculature on a single platform, each of which was enabled by advances in scaffold fabrication and played important roles at different stages of the cancer metastasis cascade (**Figure 1**a).



2. Results

The core capability of our integrated vasculature for assessing dynamic events (InVADE) platform originates from the convergence of microfabricated scaffolds with standard cell-culture plate. The base material for the scaffolds was constructed with a synthetic highly elastic polymer, poly(octamethylene maleate (anhydride) 1,2,4-butanetricarboxylate) (here referred to as 1,2,4 polymer).^[28] 1,2,4 polymer is amendable to both ultraviolet (UV) and heat polymerization. Complex suspended and hollow microstructures can be fabricated with this material using the 3D stamping method introduced previously.^[26] To create a versatile platform to culture tissues with a vascular interface, we fabricated a luminal structure with 50 µm wall thickness and an inner luminal dimension of 100 µm. Fifteen micrometer microholes were patterned onto the channel walls to improve channel permeability and allow for cell migration and endothelial cell sprouting. Moreover, microcantilevers were strategically incorporated onto the tube through the same fabrication process. These microstructures allowed us to noninvasively probe the passive and active contraction of the parenchymal tissues.

Using a 3D stamping technique, scaffolds were constructed in parallel allowing for fabrication of up to ten tubes in a single bonding step. These tubes were then assembled onto a hot embossed polystyrene-based plate patterned with tissue chambers. Each assembled scaffold was suspended across one tissue



chamber and connected to an inlet and outlet well. A bottomless 96-well plate was then attached onto the base plate sealed with polyurethane glue.^[29] The complete assembly method and dimensions of the microfabricated scaffold are shown with scanning electron microscopy (SEM, Figure 1b–f). This platform allows us to perfuse up to ten hepatic and ten cardiac/ tumor tissues in a single platform. Carbon electrodes were also embedded into the base plate to provide electrical stimulation to cardiac tissues simultaneously (Figure 1h).

Tissue assembly in each chamber is a multistep process performed with a standard pipetting technique (Figure 1i). Endothelial cells were first seeded into the tube and allowed to attach and proliferate overnight. Parenchymal cells were then cast with fibrin gel around tubes suspended across the tissue chamber, yielding a defined 3D coculture environment (Figure 1g). Tissues were perfused with hydrostatic gravity driven flow, removing the need for external pumps and bubble traps. This flow dynamic of scaffold is demonstrated by perfusing green fluorescent polystyrene beads (Movie S1, Supporting Information). This approach also allowed us to use as little as 300 μ L of culture media to continuously perfuse a single tissue. Furthermore, contrary to the closed microfluidic channel-based systems, the open 96-well plates allowed easy tissue extraction and staining.

To demonstrate the scaffold (Figure 2a,b) is permeable to large molecules, we perfused 70 kDa Tetramethylrhodamineisothiocyanate (TRITC)-dextran (red) through the inlet of the



Figure 2. Blood vessel model. a) Brightfield image of the scaffold built into the multiwell plate. Scale bar, 200 μ m. b) Brightfield image of scaffold seeded with cardiac cells on day 1. Scale bar, 200 μ m. Fluorescent images showing the perfusion of c) TRITC-dextran (70 kDa) and d) CFDA through the scaffold and diffusing into the parenchymal space seeded with cardiac cells. Scale bar, 200 μ m. Confocal images of an endothelialized scaffold e) without or f) with microholes on the side walls immunostained for CD31 (red). Human umbilical cord endothelial cells (HUVECs) were used. Scale bar 100 μ m. g) Illustration of the perfusion scheme of the endothelialized scaffolds in permeability tests. h) Quantification of scaffold permeabilities with or without endothelial cells or side holes (average ± s.d., n = 3). – denotes significant difference between groups with p < 0.05. Media distribution under passive gravity driven perfusion in scaffold i) with or j) without microholes on scaffold side-walls in 96-well plate format over 18 h (average ± s.d., n = 3).





scaffold and recorded molecular diffusion into the cardiac tissue space with time-lapse images (Figure 2c). The TRITC-dextran dye diffused into the parenchymal space within minutes. Next, we perfused a cell tracker dye, carboxyfluorescein diacetate (CFDA), to label live cells with green fluorescence. We observed clear convection-diffusion transport followed by metabolic conversion as cells in the parenchymal space light up within 20 min (Figure 2d).

The endothelialized lumen of the scaffold was highlighted with CD31 immunostaining that showed a confluent layer of endothelial cells covering the luminal surface (Figure 2e). Endothelial cell sprouting through the microholes on the side channel wall of the scaffold was also observed (Figure 2f). Media perfusion through the tube demonstrated the transfer of culture media from the inlet well to the outlet well over time in both cell-free and endothelialized scaffolds with minimal volume change in the main well (Figure 2i,j). We examined the permeability of the cell-free and endothelialized scaffold to large fluorescent molecules (70 kDa TRITC-dextran). Endothelial cells significantly reduced the permeability of the scaffold ($3.0 \times 10^{-6} \pm 0.85 \times 10^{-6}$ cm s⁻¹), compared to the cell-free lumens. Endothelialized scaffold had permeability comparable to that of capillaries to proteins (4.3×10^{-6} cm s⁻¹)^[30] (Figure 2h).

Around the endothelialized scaffold, we assembled a vascularized liver tissue by casting human hepatocytes mixed with human endothelial cells at a 9:1 ratio in fibrin gel (**Figure 3**a). Over 7 d of culture, endothelial cells self-assembled into a rudimentary network in the parenchymal space. The cross-section of liver tissue reveals endothelial sprouting through the microholes of the scaffold, an early stage of angiogenesis (Figure 3b,c). Hepatic aggregates were seen throughout the tissue constructs and cells expressed albumin and E-cadherin at intercellular junctions (Figure 3f). Media dilution of secreted factors is an issue prevalent in many organ-on-a-chip devices, which could have profound effect on the microenvironment and modeling of biochemical interaction in multiorgan models.^[31] To allow the secretion and accumulation of biochemical factors and minimize factor dilution, we used small amounts of culture media under perfusion culture. Using a programmable tilt stage, 300 µL of culture media were perfused repeatedly through the liver tissue to allow metabolite accumulation in the tissue vascular space (Figure 3d).

Using a urea quantification assay, we measured significant amount of urea metabolite secretion and accumulation in both the parenchymal space and the vascular space starting on day 7 for 2 d (Figure 3e). Similarly, when perfusing terfenadine from the vascular space, we showed the delivery of the drug (terfenadine) as well as the secretion of its metabolite (fexofenadine) (Figure 3i). This suggests the metabolic activity and active biomolecular secretion of the hepatic tissues were maintained for at least 1 week. To demonstrate cell viability and distribution within the parenchymal space, we transversely sectioned the liver tissue after 8 d of culturing and performed live (CFDA) and dead (propidium iodide, PI) staining (Figure 3g,h). The intensity and stained area (4'6-diamidino-2-phenylindole (DAPI) staining for all cells, Figure 3c) was quantified throughout the entire depth of the parenchymal space. The result shows minimal differences in cell viability at different depths of the tissue compared to the tissue surface, as expected. This indicates it is possible to construct a tissue as thick as 600 µm with culture media feeding from the top, bottom, and inner surface of a tissue (Figure S2, Supporting Information).



Figure 3. Liver tissue model. a) Brightfield image of a scaffold seeded with human hepatocytes (HepG2). Scale bar, 400 μ m. Confocal fluorescent images of a liver tissue immunostained for CD31 (red) and DAPI from b) a top view and c) a cross-sectional view. Scale bar, b,c) 200 μ m. d) Illustration of the perfusion scheme for long-term perfusion culture of 3D tissue models. e) Quantification of urea secretion in culture media from the liver tissues while feeding the tissue with ammonium biocarbonate (10 μ m) under perfusion over 2 d (average ± s.d., *n* = 3). f) Confocal fluorescent images of a liver tissue immunostained for albumin (green) and E-cadherin (red). Scale bar, 100 μ m, (inset) 50 μ m. A cross-sectional view of a g) perfused and h) static liver tissue with live and dead fluorescent staining on day 7. Scale bar 200 μ m. i) Quantification of drug distribution (drug: Terfenadine) and metabolism (metabolite: Fexofenadine) in the liver tissue model via mass spectrometry (average ± s.d., *n* = 3).





Cardiac tissues were formed from human induced pluripotent stem cell derived cardiomyocytes. Over 4 d, cardiomyocytes gradually remodeled the fibrin matrix and compacted around the scaffold, grabbing and bending the cantilever in the process (Figure 4a). Immunostaining for the contractile protein, sarcomeric- α -actinin, and the structural protein, F-actin shows cardiomyocytes elongate along the tube and between the cantilevers (Figure 4b,c) as a result of the induced tension in the matrix. The 3D of the tissue is demonstrated in a circular tissue cross-section centered around the scaffold (Figure 4d,e). The cantilevers attached onto the scaffold and later embedded into the cardiac tissue allowed us to determine the frequency and force of tissue contraction in a noninvasive manner using beam bending theory. Spontaneous beating of the cardiac tissue can be tracked 2 d after seeding the human iPSC-derived cardiomyocytes on to the platform (Movie S2, Supporting Information).

Using an external force probe, we first experimentally correlated the displacement of the cantilever tip to measured force as a relation to material elasticity (Figure 4f). Since both the beam bending theory and the experimental data suggest a linear correlation, we fitted the force-displacement curve with a linear equation (Figure 4g). This equation was then used to correlate cantilever tip displacement to tissue contraction force. We derived this correlation curve by measuring 16 cantilevers situated on multiple scaffolds (Figure 4h). The sensitivity of the current system allows us to measure force range from 0.02 to 80 µN. However, our tissues showed an average contraction force of $3.3 \pm 1.7 \mu N$ under spontaneous contraction. We also looked at how the cantilever displacement and force correlation may change over 2 week period when immersed in culture media. We found no significant changes in the mechanical properties of the cantilever over this experimental period



Figure 4. Cardiac muscle model. a) Brightfield images of the cardiac tissue remodeling process around the scaffold over time. Scale bar, 200 μ m. b,c) Confocal fluorescent images of a cardiac tissue immunostained for sarcomeric- α -actinin (green) and a zoom-in tissue section immunostained for c) sarcomeric- α -actinin (green) and F-actin (red). Scale bar, b) 200 μ m, c) 30 μ m. Confocal fluorescent images of a cardiac tissue cross-section immunostained for d) F-actin (red) and e) sarcomeric- α -actinin (green). Scale bar, d,e) 200 μ m. f) Illustration of the cantilever mechanical test. g) Representative mechanical testing curve showing a linear correlation between cantilever displacement and measured force. h) A fitted linear curve showing the average correlation between cantilever displacement and measured force of 16 cantilevers from four different scaffolds (average ± s.d., n = 4). Inset, Equation shows the definition of a (coefficient of linear regression) correlating measured forces with cantilever displacements i) Changes in the mechanical property of the cantilever or a 2 week period (average ± s.d., n = 4). j) Representative traces of cantilever displacements (for μ stimulation under perfusion. k) Quantification of the changes in beating frequency and force of contraction under epinephrine stimulation. I) Brightfield image of embedded carbon electrodes adjacent to a cardiac tissue. m) A representative trace of cantilever displacement on a cardiac tissue being paced with increasing stimulation frequency by the built-in carbon electrodes.



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Figure 5. Solid tumor model and multiorgan integration. a) Fluorescent images of cancer cells remodeling around a scaffold over a 6 d period. Scale bar, 200 μ m. b) Quantification of extravasated GFP+ cancer cells near the scaffold outlet over 6 d. c) Fluorescent images of extravasated GFP+ cancer cells near the scaffold outlet over 6 d. c) Brightfield image of a joint scaffold with an assembled 3D tumor and a liver tissue connected through a built-in continuous vascular interface. d) Quantification of extravasated GFP+ cancer cells or control cells at the scaffold outlet (average ± s.d., n = 5). HUVECs were used as control cells due to their lack of metastasizing capability. * denotes significant difference between groups with p < 0.05. e) Quantification of lactate dehydrogenase (LDH) concentration in culture media under Tegafur (100 μ m) perfusion in the duo-organ model (average ± s.d., n = 6). – denotes significant difference between groups with p < 0.05. f) Quantification of GFP gene expression in liver tissues after 6 d perfusion culture with either a GFP+ control tissue or a GFP+ tumor in the duo-organ model (average ± s.d., n = 8). g) Confocal z-stack of a liver tissue infiltrated with GFP+ cancer cells. White arrows indicate GFP+ cells. Insets show GFP+ cells in both the vascular space and the parenchymal space.

(Figure 4i), suggesting the stability of the scaffold can support repeated force measurement over time.

On this platform, we also demonstrated the control of cardiac tissue contraction through both biochemical and electrical stimulation. When a beta-adrenergic agonist, epinephrine, was perfused through the internal vasculature, we observed an immediate increase in tissue contraction frequency. The spontaneous beating frequency of the cardiac tissues increased by $19\% \pm 6\%$ under drug perfusion but showed no significant increase in contraction force (Figure 4j). To pace the tissue under electrical stimulation, we embedded a pair of carbon fiber electrodes next to the tissue chamber and within the polystyrene base plate (Figure 4l). By connecting the electrodes to an external stimulator, we were able to control the contraction of the tissue in the multiwell plate setup, showing the gradual increase in tissue contraction under pacing (Figure 4m). We previously showed electrical stimulation under increasing frequency can improve cardiac tissue maturation;^[18] this platform will allow us to implement this protocol in a 96-well plate format under vascular perfusion in the future.

In a similar approach, we assembled a 3D solid tumor around the vascularized constructs. Metastatic cancerous cells remodeled the matrix when seeded in fibrin gel around the scaffold (**Figure 5**a). Using a Green Fluorescent Protein (GFP)-expressing breast cancer cell-line (MDA-MB-231), we tracked cell migration over time and observed the invasion of GFP-cancer cells into the vascular space and escaping through the scaffold outlet. Over a course of 6 d, the presence of cancer cells at the outlet rose after day 2 and increased continuously (Figure 5b). To demonstrate the significance of multiorgan interaction in drug screening, we connected the 3D tumor model with the liver model in series (Figure 5c). There were no differences in the number of cells between the two tumor models with or without liver tissues on day 6 prior to the application of drugs (Figure S3, Supporting Information). When perfused with a common chemotherapeutic drug, Tegafur (administrated with uracil in combination^[32]), a stronger tumor toxicity was seen in the presence of liver tissue (Figure 5e). These differences highlight the significant role of liver metabolism in modulating drug effects and underline the ability of our platform to recapitulate interorgan crosstalk in vitro.

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With the duo-organ model, we demonstrated the direct coupling of tumor with another organ model in series to capture the entire cancer invasion-metastasis cascade. First, we confirmed that the cancer cells can invade the vascular space and escape from the outlets in the duo-organ model (Figure 5d). This is quantified and compared against a nonmetastatic tissue, constructed of endothelial cells alone, which showed minimal cells escaping into the outlets (Figure 5d). To quantify the presence of cancer cells in the liver tissue, we performed qPCR analysis on the GFP gene specific to the cancer cells used. An elevated GFP-gene expression was observed in the tumor + liver model only, while both the control tissue + liver and liver alone yield zero expression (Figure 5f). To visualize the infiltrated cancer cells in the 3D liver tissue, we cleared the liver tissue with a passive CLARITY technique^[33] and scanned the tissue construct with a confocal microscope, revealing the localized presence of cancer cells within and around the vascular interface (Figure 5g and Figure S1, Supporting Information). This visual evidence confirms that cancer cell infiltration of the liver organ took place in our platform in agreement with the qPCR analysis.

3. Discussion

Our previous work with AngioChip required significantly more cells per tissue (2 million vs 200 000) and had a significantly lower throughput compared to the AngioChip platform, with only three tissues grown in a bioreactor with a footprint of a 96-well plate. In this work, we transformed the AngioChip platform into a user-friendly InVADE multiwell plate. The InVADE plate does not require bulky external setups and allows for multiplexed perfusion and screening of up to 20 3D tissues in parallel. In this format, the platform along with built-in scaffolds can be prefabricated, assembled, and stored as the off-the-shelf product ready for use. Furthermore, the platform is completely polydimethylsiloxane (PDMS)-free, avoiding the prevalent drug absorption issue in many microfluidic devices.^[29,34,35] Culture media and reagent usage is also minimized to reduce dilution of secreted growth factors. The flexibility to incorporate microfeatures into the scaffold provides a range of additional possible functionalities tailored to the biological function of each organ model, such as incorporation of cantilevers to measure the force of contraction as demonstrated here. These modifications of the scaffold do not alter the device setup and operation procedure, opening the possibility to construct a variety of tissue models in a similar manner.

The combination of dense parenchymal tissue, stable vascular barrier, and interorgan connection through a continuous vascular interface on a single platform is necessary to capture this complex cancer invasion-metastasis cascade, which is an interorgan biological event previously modeled only in parts. As shown, all three aspects of the human physiology played an important role at different stages of the invasion-metastasis cascade. The continuous vascular interface linking multiple organs contains minimal dead volume, which allowed cells to effectively escape from one organ and travel to another organ under physiological flow. This advance is rooted in both the fabrication and integration of a multicentimeter long vascular scaffold that span across multiple compartments of a standard well plate. This type of interorgan cell-trafficking has not been demonstrated previously and can potentially be expanded to model interorgan migration of circulating immune cells and others. Previous work has demonstrated complex biochemical signaling between different organs.^[23-25,36] However, the technical criteria to allow cell trafficking between organs are different. For instance, devices with built-in pneumatic pumping system to drive flow could damage cells traveling in the flow circuit. In addition, the fluid circuit design needs to minimize turns, incorporate bubble traps and mixing chambers all of which introduce significant dead volume prone to trapping cells.

Compartmentalized vascular and parenchymal space also allows for use of tissue-specific media formulations in each organ-compartment to facilitate robust multiorgan coculture. As we scale this platform to connect more organ models, tissue volume scaling will be an important consideration. Fortunately, our platform allows many scaling parameters to be changed independently. The size of the parenchymal tissue is controlled by seeding density and tissue chamber size. The surface area of the tissue interface and the blood-residence time of each tissue are controlled by the design of the vascular scaffold and can be tuned independently. Nonetheless, allometric scaling^[31] based on organ size might be impractical. The field is moving toward functional scaling^[23,24,31,37,38] where the basal metabolic rate of the individual organ is used to gauge tissue size. Our platform allows the recirculating vascular media to be analyzed for a specific secreted factor, such as urea or albumin that is present in the human blood. We expect to see an initial transition period to homeostasis as shown previously in other platforms.^[39] Tissue size can therefore be optimized to match the concentration of these secreted factors in the vascular space to the physiological level.

Consideration of microvascular architecture is also important to regulate the microenvironment of tumors and many other solid organs. Previous work established vascularized tumor models by taking advantage of the intrinsic ability of endothelial cells to self-assemble into a perfusable microvascular network in vitro.^[40,41] These studies helped to determine the culture conditions for microvascular assembly and tumor coculture. As we illustrated in our platform, the scaffold also supports vascular sprouting. This is an important feature that may allow us to establish a hierarchical network with a dynamic microvasculature to interact and regulate tumor microenvironment in the future. Establishing microvascular perfusion through the parenchymal tissue could also lead to improved biomolecular exchange between the vascular and parenchymal space, potentially improving the metabolic efficiency of model livers.

4. Conclusion

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The field of organ-on-a-chip has made tremendous progress since the publication of lung-on-a-chip by Ingber and coworkers,^[15] which demonstrated high-level organ-like functionality can be achieved through an engineering approach. As the field advances further, it is also clear that device usability and assay compatibility are as important as the sophistication of the tissue model in leading to user adoptability.^[42] We believe that our InVADE platform strikes a balance between these two factors and offers sophisticated versatile tissue models that is also product-ready.

5. Experimental Section

Scaffold Fabrication and InVADE Multiwell Plate Assembly: Both layers of the scaffolds, top and bottom, were first designed using the AutoCad software and micropatterned onto individual SU8-silicon wafers using standard soft lithography techniques. The top layer of scaffold was 50 μm in height and the bottom layer was 100 μm in height. The micro side holes of the scaffold were patterned on the bottom layer through AutoCad drawing. Four cantilevers on the cardiac/tumor scaffold were also designed onto the bottom layer through AutoCad software and the thickness of the cantilever was 0.1 mm. The pattern of each layer of the scaffold was then micromolded onto PDMS at 1:10 cure agent to monomer ratio. After curing for 2 d at room temperature, the patterned PDMS molds were capped to a flat PDMS sheet for the top mold and to a glass slide for the bottom mold. Poly(octamethylene maleate (anhydride) 1,2,4-butanetricaboxylate) prepolymer (1,2,4 polymer), a highly elastic polyester material, mixed with poly(ethylene glycol) dimethyl ether (PEGDM, Sigma) at 80:20 (w/w) along with 5% (w/w) ultraviolet initiator (Irgacure 2959, Sigma) was injected and perfused through both PDMS molds. After overnight perfusion, the channels of the patterned PDMS molds were filled with 1,2,4 prepolymer mixture and the prepolymer mixture was crosslinked under ultraviolet light at an intensity of 14.5 mJ s⁻¹ for 8 min. The PDMS sheets were carefully removed and the crosslinked polymer for the top layer of scaffold remained on the patterned PDMS mold and the crosslinked polymer for the bottom layer of scaffold transferred onto the glass slide. Afterward, the crosslinked polymers of top and bottom layers were aligned and stamped together under a customized ultraviolet mask aligner and were bonded to form a closed circuit channel under ultraviolet exposure for 10 min. The bonded scaffolds remained on the glass slide after the uncapping of the PDMS mold. Incubation at 80 °C for overnight or another ultraviolet exposure for 15 min was performed to ensure 1,2,4 prepolymer was fully crosslinked. The scaffolds were released from the glass slide by immersing in phosphate buffered saline (PBS) and lifting off by razor blade. The scaffolds were washed overnight with 70% ethanol to leach out PEGDM and were kept in PBS at 4 °C until assembly onto the microfluidic plate device.

The base to serve as the multiwell microfluidic plate device was also designed using the AutoCad software. The design of the base was intended to resemble a 96-well plate format. The microstructured surface of the base was designed to hold ten of each hepatic and cardiac/tumor scaffolds within one plate system. Microposts of 200 μ m in height were incorporated into the hepatic and cardiac/tumor chambers in order to lift the scaffolds from the bottom of the base plate and to allow for the entire encapsulation of the scaffolds during the seeding

of hepatic, cardia, or tumor tissues within a fibrin hydrogel. Trenches of specific dimensions (liver chamber: $4 \times 3 \times 2$ mm³, cardiac/tumor chamber: $2.5 \times 2 \times 2$ mm³) were also patterned to include the inlet and outlet chamber where the inlet and outlet of the scaffolds were aligned. The base plate design was patterned onto an SU8-silicon wafer using standard soft lithography and was transferred onto a PDMS mold (1:5 cure agent to monomer ratio). The PDMS mold was then plasmatreated and bonded onto a new silicon wafer to serve as the master for hot embossing fabrication. The patterned microstructure of the base was translated onto a polystyrene sheet (0.05" thick, Plaskolite) through EVG 520 hot embosser. For plates with electrodes incorporated onto the cardiac chamber, a pair of carbon electrodes were first laid parallel on the PDMS-Silicon wafer master on top of the cardiac chamber and were sandwiched by two polystyrene sheets. The electrodes were then hot-embossed into the polystyrene base sheets under the same hot embossing process. Similarly, duo organ scaffold and its corresponding base plate were designed through AutoCad and prepared by soft lithography microfabrication, with the liver scaffold jointed in series to the cardiac/tumor scaffold.

After positioning the scaffolds into their respective chambers on the polystyrene base, a 96-well bottomless plate (square shaped well, Greiner) was bonded onto the top of the base and assembled into the InVADE microfluidic plate device. Polyurethane (GSP 1552-2, GS Polymers) was used to bind the polystyrene base and bottomless plate component together. The microfluidic plate device was then incubated at 80 °C for 2 h to ensure quick crosslinking of the polyurethane glue. After assembling the scaffold onto the plate device, the plate device was treated with 70% ethanol for 1 h and exposed to UV for 30 min as a sterilization process. All InVADE microfluidic plate devices were kept in dry condition and stored at room temperature before use.

Scanning Electron Microscope and Mechanical Analysis of Scaffold: The microstructures of the scaffolds were assessed by imaging the scaffolds with SEM. The scaffolds were not washed with 70% ethanol or kept in PBS prior to imaging with SEM. The scaffolds were first cross-sectioned transversely to expose the main channels of the scaffolds and were then sputter coated with gold for 120 s to achieve a 20 nm thickness using a standard sputter coater. All images were captured with the Hitachi S-3400 scanning electron microscope (Hitachi High Technologies).

The mechanical properties of the cantilevers of the cardiac scaffolds were tested using the CellScale MicroSquisher under the SquisherJoy software program. The force by the probe (0.1524 μm in diameter) was calculated by correlating cantilever deflection through the probe displacement as suggested by the manufacturer. The cantilevers of scaffolds tested were aligned on the microfluidic plate device and submerged in endothelial growth medium. All four cantilevers of the same scaffolds were assayed and a minimum of five different scaffolds were mechanically tested. All experiments were carried out at room temperature.

Cells Maintenance, Scaffold Endothelization, and Tissues Formation: The human umbilical vein endothelial cell (HUVEC) and human hepatocellular carcinoma (HepG2) were obtained from American Type Culture Collection and cultured with endothelial growth medium (EGM2, Lonza) and Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 1% (v/v) GlutaMAX, and 1% (v/v) penillicin-streptomyocin, respectively. GFP expressing human breast carcinoma cell line (MDA-MB-231/ GFP) was purchased from Cell Biolabs, Inc. and cultured with DMEM supplemented with 10% FBS, 0.1×10^{-3} M MEM nonessential amino acids, 2×10^{-3} $_{\rm M}$ 1-glutamine, and 1% penillicin–streptomyocyin. The human cardiomyocytes were obtained from differentiation of human pluripotent stem cell (hPSC) line BJ1D. The cellular differentiation of the hPSC to cardiomyocytes and maintenance of the cardiomyocytes were reported previously.^[26] The hPSC-derived cardiomyocytes were cultured with StemPro34 media supplemented with the required supplement nutrients and 1% glutamine.

To enhance endothelial cell attachment to the internal lumen of the scaffold, the scaffolds were first coated by perfusing 0.2% (w/v) sterile gelatin (from porcine skin, Type A, Sigma) for 2 h at 37 °C and followed

by overnight perfusion of endothelial medium at 37 °C. After overnight priming of the internal network, HUVECs were first seeded into the lumen of scaffold by perfusing 6–10 μ L of concentrated endothelial cell suspension (25 \times 10⁶ cells mL⁻¹) on either end of the scaffold. Endothelial cells were drawn and packed the lumen of scaffold by means of negative pressure and attachment of cells was performed at static conditions for 1-1.5 h at 37 °C. The InVADE microfluidic device should be in a flat position during cell attachment. Unattached endothelial cells were then flushed by adding endothelial growth media into the wells to initiate perfusion. Perfusion in the InVADE microfluidic device was achieved by pressure-head difference between inlet, tissue, and outlet wells. The attached endothelial cells were allowed to proliferate overnight and form confluent network under the continuous flow. The continuous flow in the scaffold is maintained by placing the InVADE microfluidic device on a rocker (with a 20° tilt angle) that is automated to reverse the flow every 3 h.

After endothelialization of the internal scaffold lumens, parenchymal cells were seeded and allowed to assemble into tissue that is representative of their respective organs. Endothelial medium was removed completely from the wells prior to seeding of parenchymal cells to the tissue well. When forming hepatic tissue, human HepG2 cells along with HUVEC (150 \times 10⁶ cells mL⁻¹, HUVEC population density is 10% of total cell population) were encapsulated within fibrinogen. The incorporation of HUVEC into hepatic tissue was to study vascular network within the hepatic tissue and endothelial sprouting from cells within internal network of scaffold to the parenchymal space. Seven microliter of the encapsulated cells was premixed with 2 μ L of thrombin. Immediately thereafter and prior to crosslinking of the fibrinogen, 7 μ L of the cell fibrinogen-thrombin mixture was dropped on top of the scaffold. As a control, fibrinogen encapsulated with HUVEC (15×10^6 cells mL⁻¹) was seeded into the parenchymal space to study self-assembling vessel networks in the parenchymal space. Similarly, human cardiac tissue and human malignant breast tumor was prepared by encapsulating hiPSCderived cardiomyocytes (80×10^6 cells mL⁻¹) or MDA-MB-231/GFP $(80 \times 10^6 \text{ cells mL}^{-1})$ within fibrinogen. Due to smaller scale of cardiac/ tumor scaffold, only 2 µL of the fibrinogen-thrombin premix was seeded on top of the scaffolds. After 15 min of gelation at 37 °C, endothelial medium was added to the inlet and outlet wells and respective tissue medium was added in the tissue well, initiating perfusion within the scaffold. Tissue characterization and drug studies were performed 7 d after initial seeding of the parenchymal cells. Media change was performed daily and similar perfusion rate was maintained throughout the cultivation process.

Scaffold Permeability Assessments: Permeability of scaffold was measured by quantifying the diffusion of large fluorescent molecules from inside the vessel to the extracellular matrix of engineered tissue organ. Scaffold with or without 15 µm side holes and scaffold that was either endothelialized or nonendothelialized were subjected to the permeability assessment. Dextran conjugated with TRITC molecule $(M_{\rm W} \approx 70$ kDa, Sigma-Aldrich), initially at 10×10^{-6} м, was perfused from the inlet of the scaffold for 18 h. After 18 h, subsequent media in vessels and parechynmal space were collected and the concentration of TRITC-dextran molecules permeating into the parechynmal space was determined by measuring the fluorescence with SpectraMax i3 spectrophotometer (Molecular Devices) and correlating the measurement to a standard curve. The channel permeability of the scaffold was then calculated from the net rate of diffusion (the amount of fluorescent molecules in parenchymal space in the total time of measurement) in proportion to the luminal surface area of the scaffold. Time-lapse fluorescence images of 10 \times 10⁻⁶ $_{\rm M}$ TRITC-dextran and 100×10^{-6} M CFDA ($M_{\rm W} \approx 555$ Da) perfused through the endothelialized scaffold were also captured to illustrate the qualitative diffusion of molecules and the metabolism of biomolecules by the engineered hepatic tissue. A 4x objective of the Olympus IX81 microscope with an environmental chamber for heat and CO₂ control was used to capture fluorescence images in a 30 min interval.

Metabolic Activity of Hepatic Tissues: Seven days after cultivating the encapsulated HepG2 cells into a 3D engineered hepatic tissue on



the scaffold, the cells were treated with either ammonium bicarbonate (Sigma) or terfenadine (Sigma) to evaluate the metabolic activity of the engineered tissue. To assess the urea metabolized from ammonium bicarbonate, media containing 10×10^{-3} M ammonium bicarbonate were added to the parechynmal tissue space and the scaffold hepatic tissue scaffold was also perfused with the same media from the inlet well. Ammonium bicarbonate was dissolved in a 1:1 ratio of EGM2 and hepatic medium and the perfusion of this biocompound was performed for 48 h, with flow reversal every 3 h on a rocker. After 48 h incubation, the media from the tissue and vascular spaces were collected and frozen at -20 °C. The metabolite urea was quantified using the QuantiChrom Urea assay kit (BioAssay Systems) in accordance to the manufacturer's instructions. In the case of hepatic metabolism of terfenadine, mass spectrometry analysis was used to detect fexofenadine, main metabolite of the terfenadine prodrug, in hepatic tissue scaffolds. The drug compound was dissolved with dimethyl sulfoxide (DMSO) and diluted with EGM2 medium to a final drug concentration of 10×10^{-6} ${\mbox{\scriptsize M}}$ and less than 0.1% DMSO content. Terfenadine was then delivered to the hepatic tissue through the microfluidic scaffolds using the hydrostatic pressure gradient. After 48 h incubation, media from the tissue and vascular spaces were collected and analyzed for the concentration of fexofenadine with liquid chromatography mass spectrometry. The mass spectrometry analysis was performed by BioZone Mass Spectrometry Facility at the University of Toronto.

Functional Characterization of Cardiac Tissues: After day 7 of cultivation, the spontaneous contractions of the engineered cardiac tissues were first recorded under DAPI channel with Olympus IX81 fluorescent microscope under 37 °C. Through autofluorescent signals of the 1,2,4 polymer, the bending and movement of the fluorescent cantilever can be detected. By using Image J (National Institute of Health, NIH) to analyze the displacement of the cantilever from rest position to maximum amplitude, the beating frequency of the engineered cardiac tissue was mapped out with respect to time.

The change in contraction frequency of the cardiac tissues was tested either through cardiac drug stimulation or electrical stimulation. For cardiac drug stimulation, epinephrine $(10 \times 10^{-6} \text{ M}, \text{Sigma})$ was perfused through endothelialized scaffold channels from the inlet chamber. The effect of the drug was assessed by recording contraction of the cardiac tissue under DAPI channel every 5 min for 30 min. A minimum of three engineered cardiac tissues were tested to assay the effect of epinephrine on cardiac cells. For electrical excitability stimulation, electrodes that were incorporated into the scaffold microfluidic plate device were connected to an external electric stimulator (Grass S88x). The spontaneously beating cardiac tissues were first paced at a frequency of 1 pulse per second and the excitation threshold was determined by gradual increase in pacing frequency. The response of the engineered cardiac tissues to electrical stimulation was captured under fluorescent channel as a 5 min video.

Immunofluorescent Staining and Imaging of Engineered Vascular Network, Hepatic Tissues, and Cardiac Tissues: After 7 d of culturing and following respective metabolic and drug evaluation studies, immunofluorescent staining was performed to evaluate the morphology and viability of the engineered vascular network and tissues. For immunostaining the endothelialized scaffold and its sprouting network, the microfluidic device was fixed by flowing 4% paraformaldehyde (PFA) for overnight at 4 °C and followed by 3x PBS wash at room temperature. The vascular network was then blocked with 10% FBS for 1 h and stained with CD31 (Mouse Anti-Platelet endothelial cell adhesion molecule, (Anti-PECAM) 1:200) overnight at 4 °C. The vasculature was then observed by staining with secondary Alexa 649 antimouse IgG (1:200, Rockland). For visualizing the morphological integrity and cell viability of engineered hepatic and cardiac tissues, the scaffolds with their respective hydrogel encapsulated tissues were carefully removed from the microfluidic device. For morphological analysis, both tissues were first fixed overnight at 4 °C with 4% PFA. After washing 3× with PBS, the scaffold cardiac tissues were blocked and permeated simultaneously with 10% FBS and 0.25% Tritonx100 in PBS for 1 h at room temperature. The cardiac tissue was then incubated with antisarcomeric α -actinin



(Mouse, 1:200, Sigma) overnight at 4 °C, followed by secondary incubation of Alexa 488 antimouse IgG (1:200, Sigma) and F-actin staining (Phalloidin 660 coconjugated, Sigma) for 1 h. The hepatic tissues on the scaffold were first blocked with 10% FBS for 1 h at room temperature followed by permeation with ice cold methanol for 2 min. The hepatic tissues were first stained with primary antibodies E-cadherin (Mouse, 1:200, BD BioSciences) and albumin (Goat, 1:200) overnight at 4 °C, and followed by corresponding secondary antibodies of Alexa 649 antimouse IgG (1:200, Sigma) and TRITC antigoat IgG (1:200, Sigma) for 1 h at room temperature. For cell viability analysis, the hepatic tissues were first washed once with PBS. Live and dead staining of the tissues was done by staining the liver tissue with CFDA (1:1000, Invitrogen) and PI (1:75, Invitrogen) in PBS for 30 min at 37 °C. All cross-sectional views of hepatic tissues were visualized by first transversely slicing the hepatic tissue and then performing the respective immunostainings. Quantification of the staining across the depth of each tissue was performed in ImageJ. Confocal images of the vascular network and engineered tissues were captured using Olympus FluoView 1000 laser scanning confocal microscope at the University of Toronto Advanced Optical Microscopy Facility. Typically, z-stacks were acquired with reconstructing scanning steps.

Monitoring Migration of GFP-Expressing Metastatic Breast Cancer Cells: The migration profile of MDA-MB-231/GFP tumor was tracked using the Olympus IX81 fluorescent microscope over a course of 6 d perfusion culture. The outlet wells of scaffolds were monitored at 10x magnification under the fluorescein isothiocyanate (FITC) channel. The dissemination of cancer cells from a minimum of six different MDA-MB-231/GFP tumors was studied. Using the Image J software, the change in percent cell coverage of the outlet wells was quantified.

Joint scaffold was utilized to track the subsequent cancer cells migration from its cancer tissue model and extravasation into an adjacent liver tissue model or the outlet wells of the scaffold. The coverage of the outlet wells of the scaffolds was monitored for 6 d and with HUVEC/GFP as a control tissue on tracking GFP expressing cells detaching from a 3D solid tissue. Nine sets of each model, liver with GFP tumor tissue, and liver with GFP control tissue were monitored. After 6 d perfusion culture, the adjacent liver tissue of each joint scaffold was sacrificed and real time polymerase chain reaction (qPCR) was performed to detect eGFP expression in the liver model.

Two Step gPCR Assay was Performed: The liver tissue samples were first removed from the microfluidic device carefully and placed under a quick freeze with liquid N2. The RNA within tissue was extracted with the Arcturus PicoPure RNA isolation kit (Applied Biosystems) in accordance with manufacturer's procedure. Using the Nanodrop, the concentrations of RNA extracted from the liver tissue were determined. The eluted RNA sample then undergoes reverse transcriptase process using the high capacity cDNA reverse transcription kit (Applied Biosystems). All RNA samples were diluted to a concentration of 35 ng μ L⁻¹ with nuclease free water. The resulting cDNA of each sample was kept at -20 °C. The qPCR assay was performed with cDNA in triplicate in a 20 µL reaction sample using the TaqMan Fast Advanced Master Mix and the Applied Biosystems 7000 Real-Time system. The primers and probe for eGFP were manufactured by Applied Biosystems: forward primer 5'-GAGCGCACCATCTTCTAAG-3', reverse primer 5'-TGTCGCCCTCGAACTTCAC-3', and probe 5'-FAM-ACGACGGCAACTACA-NFQ-3'. Glyceraldehyde 3-phospahte dehydrogenase (GAPDH) was used as the housekeeping gene for the qPCR study, with probe tagged with JOE fluorescent dye in 5' and Tetramethylrhodamine (TAMRA) as the quencher (Applied Biosystems). MDA MB 231/GFP tumor tissue and HUVEC/GFP control tissue were used as positive controls for the qPCR assay and liver tissue with no adjacent GFP tissues served as the negative control in the qPCR assay. A total of 60 cycles were performed using the standard qPCR setup as applied for the TaqMan Fast Advanced Master Mix.

Visualization of GFP-cancer cells dissemination to the liver tissue model was enabled by means of performing tissue optical clearing and capturing with the Nikon A1R confocal microscope in the Advanced Optical Microscopy Facility at University of Toronto. After removing the liver tissues from the joint scaffold, the tissues were soaked in acrylamide hydrogel solution for 5 d at 4 °C, with new hydrogel solution exchanged on a daily basis. The hydrogel solution is made up of 4% acrylamide, 0.25% Azo initiator (VA-44), and 4% paraformaldehyde dissolved in PBS. After 5 d of incubation, the hydrogel was polymerized by incubating at 37 °C for 3 h with a short purge of N₂ gas beforehand. The embedded liver tissue sample was then subjected to an sodium dodecyl sulphate (SDS) clearing solution overnight at 37 °C with gentle shaking. The clearing solution contained 8% SDS with 200 \times 10⁻³ M boric acid dissolved in deionized water and adjusted to pH 8.5 before use. The cleared tissue sample was then further optically cleared with 60% 2,2-thiodiethanol (dissolved in deionized water) for 3 h at room temperature under gentle shaking before confocal imaging.

Breast Cancer Cells Viability under Drug Treatment in a Duo-Organ Model: One hundred micromolar of Tegafur (Sigma) was perfused from the inlet well of a joint scaffold, connecting liver tissue with adjacent breast tumor tissue. The drug perfusion culture was maintained for 2 d before the media from respective chambers are collected for cytotoxicity analysis. The amount of lactate dehydrogenase (LDH) released by the tissue cells was quantified with Cayman LDH cytotoxicity assay kit, as per manufacture protocol. Breast tumor tissue cultured without liver tissue was used as a control for the study. A minimum of six breast tumor tissues cultured with or without liver tissue were seeded on the joint scaffold.

Statistical Analysis: Significant differences between experimental groups were determined using one way Analysis of Variance (ANOVA) followed by pairwise multiple comparison procedures (Tukey test) unless specified otherwise. Normality tests (Shapiro-Wilk) and equal variance tests were performed on all data sets. If data normality or equal variance was not satisfied, Kruskal–Wallis one way ANOVA on Ranks was then performed. p < 0.05 was considered significant for all tests.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

M.R. and B.Z. are amongst cofounders of TARA Biosystems and they hold equity in this company.

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