

# Fabrication of Three-Dimensionally Addressable Microfluidic Plumbing by Stereolithographic 3D Printing

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## Abstract

Thick cell cultures could be fed, sampled and corrected from the inside if every point in their volume had its own microfluidic port that a computer could open independently, an ability we call addressable. Our group previously built such addressable plumbing in two dimensions. Extending it to three dimensions requires channels and valves that reach every culture chamber along all three axes, whereas layer-bonded soft lithography places channels and valves in one or two planes. Here we report how to fabricate that plumbing in one piece by stereolithographic 3D printing. In the plumbing, every chamber has an inlet and an outlet channel on each axis, a valve at its mouth on either side, and a bypass channel that carries the flow around the chamber when those valves close. Fluid carrying cells or chemicals can therefore reach any chamber in a row without entering the chambers before it, and valves on the bypasses let neighboring chambers exchange molecules by diffusion while the rest of the lattice stays isolated. Printing that plumbing posed three problems. First, a monolithic print cannot tell a valve from any thin wall between two channels, so the layout keeps unrelated channels apart. Second, a chamber reached from six directions needs valves in two orientations that, to our knowledge, no one had printed before: a membrane printed standing vertically, and a control pressure arriving perpendicular to the membrane. The vertical membranes split along the seams between print layers, and the perpendicular-control valves leaked. Flow simulation traced the leak to pressure concentrated at the center of the membrane. Moving the control channel's entry to the side of the valve chamber, and backing the vertical membrane with a reinforcement whose layer seams are offset from its own, made both valves seal and open under pneumatic control. Third, most printed membrane valves rely on resins formulated in-house. We printed one complete unit module of the lattice as a single cube in a custom poly(ethylene glycol) diacrylate resin and again in an off-the-shelf commercial resin. Both cubes resolved their channels, bypass loops and ports as drawn, and dyes loaded into the control and flow sides of the commercial-resin cube stayed in separate networks. The plumbing is built for patterned bone-cartilage co-cultures monitored through their own ports, which a later version of this preprint will report.

**Keywords:** addressable microfluidics; 3D printing; stereolithography; digital light processing; membrane valves; monolithic fabrication; commercial photopolymer resins; tissue engineering scaffold.

## 1. Introduction

Cell experiments underpin biology, drug development and biomanufacturing, and the field is moving from flat dishes to three-dimensional (3D) cultures because flat environments misrepresent how cells behave in tissue.<sup>1-3</sup> That move, however, runs into three bottlenecks once a culture grows thicker than a few hundred micrometers. First, nothing circulates inside an artificial construct, so nutrients reach, and waste leaves, only its outer shell. Engineered vessels about a millimeter in diameter extend that reach, and yet cells survive only about 200 to 300  $\mu\text{m}$  from each vessel, which caps viable constructs near one centimeter in thickness.<sup>4-8</sup> Second, analyzing a thick culture means destroying it. Light for confocal microscopy penetrates roughly 100  $\mu\text{m}$  into tissue, so the standard readouts remain histology sections and plate-reader assays on crushed samples, each of which yields one time point averaged over the entire construct.<sup>9-12</sup> Third, cells are hard to reach once they are inside. Perfusion, the usual way in, distributes a drug or a new cell type to every space the fluid can reach, and clogged regions receive less of the payload than open ones. Targeting one location therefore means an injection, which damages the culture around it.<sup>13-15</sup>

Our group has answered all three bottlenecks in two dimensions. We built an automated microfluidic device whose 4 x 4 grid of individually actuated ports opens into one shared culture chamber. A computer can therefore deliver cells or chemicals to a chosen location, withdraw culture fluid or a micro-biopsy from another, and remove cells from a third, all while the culture keeps growing.<sup>16,17</sup> With that device we seeded mesenchymal stem cells and fibroblasts in a prescribed pattern, trapped single cells, cleared cells from chosen locations by local trypsinization, and biopsied differentiated osteoblasts for staining outside the culture. An implantable version has since reported on biomaterial integration inside a living animal without sacrificing the host.<sup>18</sup> Each of those devices, however, addresses one plane, and a thick culture needs the same ports throughout its volume. That need raises two questions: what plumbing can address any point in three dimensions without an impractical number of external connections, and how to fabricate such plumbing. This paper answers the second question, for the plumbing that answers the first.

The plumbing question is one of scaling. The simplest layout gives every port its own supply channel, so the channel count grows with the product of the grid dimensions and the channels crowd the space between neighboring ports. Large-scale microfluidic integration solved that problem with rows of flow channels crossed by columns of valve-actuating channels, which brings the count down to the sum of the dimensions.<sup>19,20</sup> Two addressable layouts descend from that idea, and each pays a price. In the first, every chamber sits on a row channel with a thin bypass around it, and a ring-shaped valve decides whether flow enters the chamber or passes by.<sup>21-23</sup> Payloads in that layout move only along a row, and reaching another row means traversing the entire device. In the second layout, every other column of chambers is left empty to serve as a bypass, which lets payloads turn corners.<sup>24</sup> That freedom costs half of the chambers. Moving one payload in that layout also means shifting its row neighbors out of the way and routing it around through the empty column, and during that detour the payload disperses and can cross-contaminate.

The fabrication method shaped both layouts. Soft lithography bonds separate flat layers, so channels, bypasses and valves share one or two planes, and flow can approach a chamber only from within those planes. Stereolithographic 3D printing removes that constraint by building the entire device, valves included, as one monolithic part.<sup>25,26</sup> Several groups have printed membrane valves this way, with the membrane lying flat on the build plate and the control pressure arriving in the plane of the membrane.<sup>27-30</sup> A chamber approached from six directions, however, needs valves on vertical channels as well, which

means either a membrane standing on edge or a control pressure arriving perpendicular to the membrane. To our knowledge, no one had printed and operated either orientation before this work.

The resin is a second constraint, separate from the layout. A printed valve needs a membrane thin and flexible enough to deflect under pneumatic pressure inside a body rigid enough to carry the channel network, and resin vendors publish little that predicts whether a given resin will do both. Most printed membrane valves to date, including those that have lasted a million actuations, were therefore made in resins formulated in-house, typically poly(ethylene glycol) diacrylate with a tailored photoinitiator and ultraviolet absorber.<sup>27,31</sup> Those groups formulated their own resins to resolve small features, and for a laboratory outside them a custom resin can prove to be difficult, expensive and slow to reproduce. For that reason, we have worked toward valves in off-the-shelf commercial resins. In a separate study, we showed that one measurement of how far a printed membrane flexes predicts the valve geometry that seals.<sup>32</sup> With that framework, we made valves in FunToDo NanoClear, a clear resin sold for rigid parts, and in Asiga Dental IBT, a dental tray material. The NanoClear valves blocked flow completely and lasted one million actuation cycles at all but the widest channel width tested.

Here we report how to fabricate three-dimensionally addressable plumbing by stereolithographic 3D printing. Section 2 describes the plumbing that the prints must realize, which we term row-column-stack addressable plumbing with distinct bypasses, and the design rules that make it printable. Section 4 then reports the two steps of fabrication. The first is valves in the two new orientations, each with its failure mode and the change of geometry that removed it. The second is one complete unit module of the plumbing, printed as a single cube in a custom poly(ethylene glycol) diacrylate resin and in NanoClear. The architecture is the subject of a United States patent application.<sup>33</sup> Its use for patterned bone-cartilage co-cultures monitored through their own ports is under way and will be reported in a later version of this preprint.

## **2. The Plumbing to Be Printed**

### **2.1. Reaching Any Neighbor without Sacrificing a Chamber**

The new layout is easiest to read in one plane, beside the two layouts it replaces (see Figure 1). In the row-wise layout (see Figure 1-LEFT), each chamber sits on a row channel, a thin bypass loops around it, and a ring-shaped valve around the chamber either admits the flow or diverts it through the bypass. That layout confines a payload to its row. In the sacrificial-column layout (see Figure 1-CENTER), payloads turn corners because every other column of chambers is left empty to carry the detour, at the cost of half the chambers. The new layout (see Figure 1-RIGHT) keeps the distinct bypass of the first and the two-direction reach of the second. Every chamber sits at the crossing of a row channel and a column channel. Each channel enters the chamber through one side valve and leaves through another, and a bypass loops around the chamber from the inlet side to the outlet side. When the two side valves on an axis close, flow along that axis takes the bypass and the chamber stays untouched. When they open, the chamber receives or releases its payload along that axis. A payload can therefore travel from any chamber to any neighbor, row-wise or column-wise, through the bypasses of the chambers in between. Those chambers keep their contents undisturbed, and every chamber in the grid holds a payload at the same time.

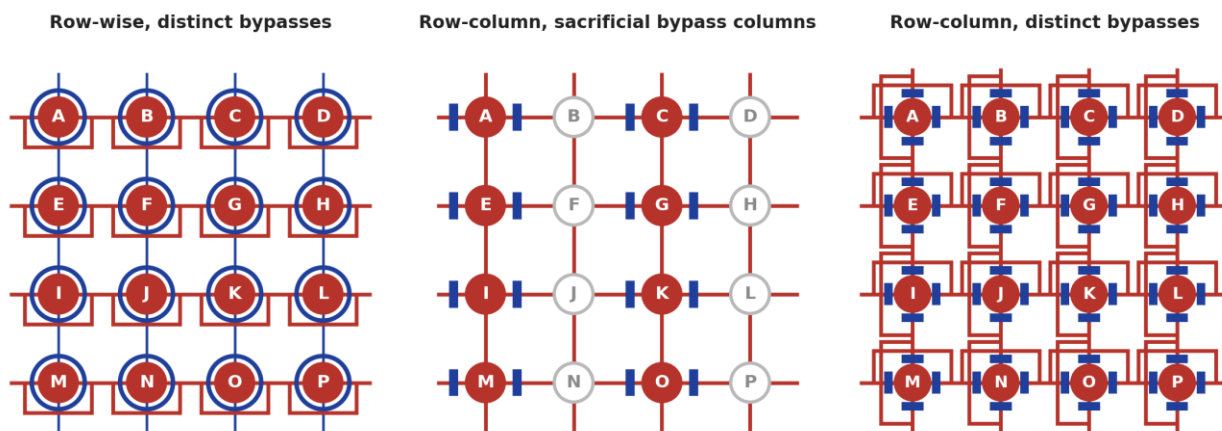


**Figure 1** Three addressable layouts, drawn for the same 4 x 4 grid, with its 16 positions lettered A to P in every panel. LEFT: row-wise addressable plumbing with distinct bypasses, with a ring valve around each chamber. CENTER: row-column addressable plumbing with sacrificial bypass columns, in which chambers sit only in alternate columns and the gray positions carry the detours instead of a payload. RIGHT: row-column addressable plumbing with distinct bypasses, with a valve on each of the four sides of every chamber. Red = flow channels, bypasses and chambers; blue = valves (vertical blue lines in LEFT = control lines of the ring valves); gray = positions given up as detours. Control lines of the other panels, flow directions and the third dimension are omitted for clarity.

## 2.2. Independent Side Valves and Bypass Valves: Exchange by Diffusion

Two refinements turn the array from a delivery network into a co-culture tool. First, the two side valves on an axis actuate independently rather than as a pair, so that a chamber can open toward one neighbor while staying closed toward the other. Second, each bypass carries its own pair of valves. With a side valve open toward a neighbor and the bypass closed, the two chambers communicate by diffusion in the absence of flow, and nothing escapes into the bypass and the rest of the array. Figure 2 shows the two refinements in the row-wise layout, where they are easiest to follow (see Figure 2). In the standard version (see Figure 2-LEFT), one ring valve per chamber admits or diverts the flow. In the refined version (see Figure 2-RIGHT), the linear valves in columns 1 to 8 open or close each side of each chamber, and the valves in rows i to iv block the bypasses of a row. Opening columns 4 and 5 while closing row i, for example, lets chambers B and C exchange signals by diffusion while the bypasses stay sealed. The three-dimensional unit module of Section 2.3 applies the same two refinements along all three axes, so the exchange can be switched on along any combination of directions and switched off again later in the experiment.

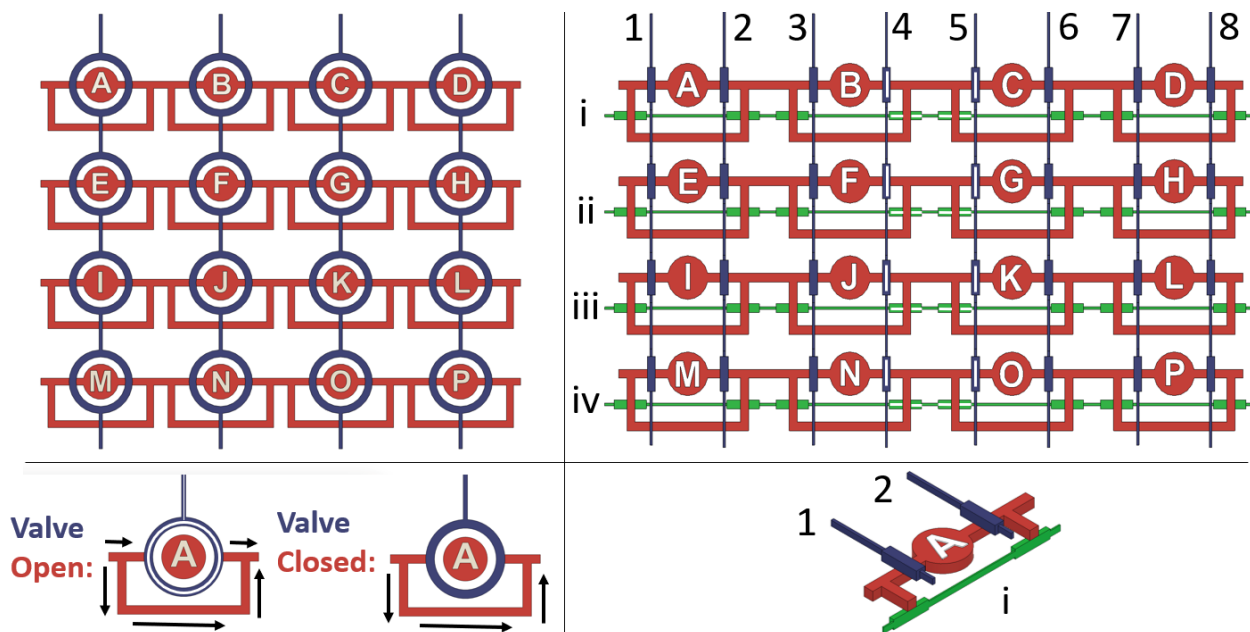


**Figure 2** Independent side valves and bypass valves, shown for the row-wise layout of a 4 x 4 grid. LEFT: standard row-wise layout with one ring valve per chamber; the insets show the open state (flow enters the chamber) and the closed state (flow takes the bypass), with arrows marking the flow direction. RIGHT: the same grid with a linear side valve on each side of every chamber (columns 1 to 8) and a bypass-blocking valve on every bypass (rows i to iv); the inset shows one chamber in three dimensions with its two side valves (1, 2) and its bypass valve (i). Red = flow channels, bypasses and chambers; blue = side valves and their control lines; green = bypass valves and their control lines.

### 2.3. The Unit Module in Three Dimensions

The third axis repeats the same pattern a third time (see Figure 3). The unit module is a spherical chamber with one pair of flow channels per axis and one bypass per pair (see Figure 3A). A sphere gives the cells the same curvature in every direction, which is why we prefer it to a cylinder, the easier shape to make. The three bypasses are spread as far apart as the geometry allows, for a fabrication reason. A monolithic print carries no label that distinguishes a valve from ordinary plumbing, so wherever only a thin wall separates two channels, that wall becomes an unintended squeeze valve once the system is pressurized. A side valve on each of the six flow channels (see Figure 3B) makes the module the three-dimensional counterpart of the layout in Figure 1-RIGHT. A valve on each bypass loop (see Figure 3C) adds the exchange mode that Section 2.2 describes. Modules tile in all three directions (see Figure 3D). The row channel of one module continues into its row neighbor, the column channel into its column neighbor, and the stack channel into the module above, and the bypasses of neighboring modules overlap in space to keep the array compact.

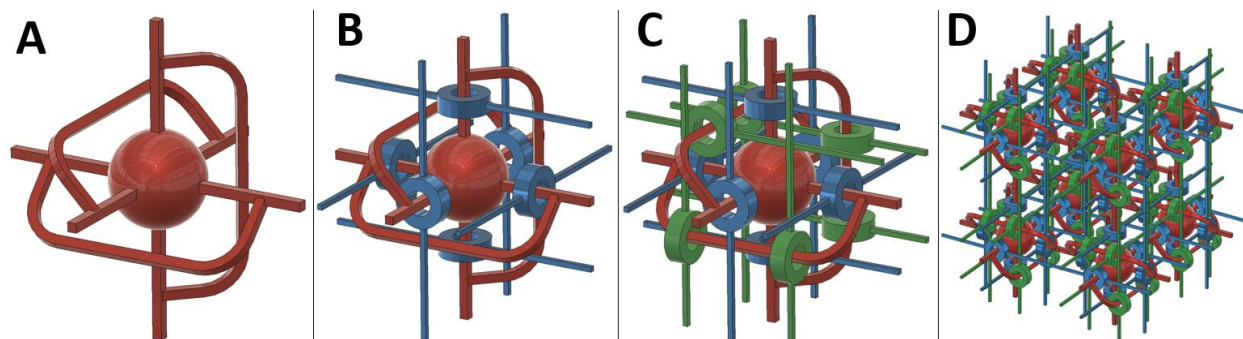


**Figure 3** Unit module of the row-column-stack addressable plumbing and its array. (A) Spherical chamber with three pairs of flow channels and three bypass loops; all valves omitted. (B) The same module with a side valve on each flow channel. (C) The same module with bypass-blocking valves added on each loop. (D) A 2 x 2 x 2 array of the module in (C). Red = chamber, flow channels and bypasses; blue = side valves and their control lines; green = bypass valves and their control lines. The array size is arbitrary.

## 2.4. Operation and External Control

Because every side valve and bypass valve opens on its own, one array can run as a single interconnected thick culture, as isolated chambers for screening, or as several isolated sub-volumes, and the controller can switch between those modes during an experiment.<sup>33</sup> Each of those valves, however, needs its own pressurized line, and at the roughly 1 mm<sup>2</sup> footprint of a port in our two-dimensional device one cubic centimeter of scaffold would hold about a thousand addresses. A dedicated solenoid per line would not scale to that number. The design therefore feeds the lines through a combinatorial multiplexer, a valve matrix in which each line carries valves on a distinct half of a small set of control lines, so that venting one particular half opens exactly one line.<sup>20,34</sup> Sixteen control lines select any one of 12,870 lines in that scheme. Connecting printed scaffolds to the multiplexer belongs to the application of Section 5 and will be reported with it.

## 2.5. Design Rules for Layer-by-Layer Printing

The architecture does not depend on one valve type. The drawings above use ring-shaped squeeze valves, and the patent application lists square squeeze valves, Quake-style membrane valves and their dome-shaped variant, ribbon valves, membrane valves and a miniaturized squeeze valve as interchangeable alternatives, to be chosen by whatever is most convenient to print and to operate.<sup>33</sup> Four modifications of the plumbing near the valves answer specific constraints of layer-by-layer printing, and the application draws each of them. (i) **Reinforced vertical membranes:** a membrane standing on edge is built from stacked layers whose seams act as planes of weakness, so a reinforcement whose seams are offset from the membrane's, or a change of layer thickness or of exposure next to the membrane, makes delamination less likely. (ii) **U-turns for vertical channels:** a vertical flow channel detours through a U-shaped turn so that a horizontal squeeze valve, which is easier to print, can block one arm of the U, or both arms symmetrically when the valve sits at the center of the U. (iii) **Doormats:** a narrowing of the channel profile under the valve shortens the distance the membrane has to travel to seal. (iv) **Right-angle bypasses:** bypass loops made only of straight horizontal and vertical segments avoid the diagonal and curved features that layer-by-layer printers render least well. Section 4.1 reports which of these the printed valves needed.

# 3. Materials and Methods

## 3.1. Parametric Design

The plumbing was drawn as parametric scripts in OpenSCAD version 2021.01, a script-based CAD environment. One file builds the one-, two- or three-dimensional module from a flag and tiles it into an array of any size, with the chamber shape, channel width, channel height, bypass position and valve position as parameters. The same approach generated arrays of valves in which flow-channel and control-channel dimensions, valve chamber sizes and membrane thicknesses were swept within a single print, so that many geometries could be tested from one build (see Figure 4). SolidWorks Student Edition 2023 was used for preliminary drawings and for flow animations. Needle ports were drawn as 0.63 mm holes to

receive 23-gauge blunt stainless-steel dispensing needles of 0.642 mm outer diameter. The 0.012 mm interference seals the port.

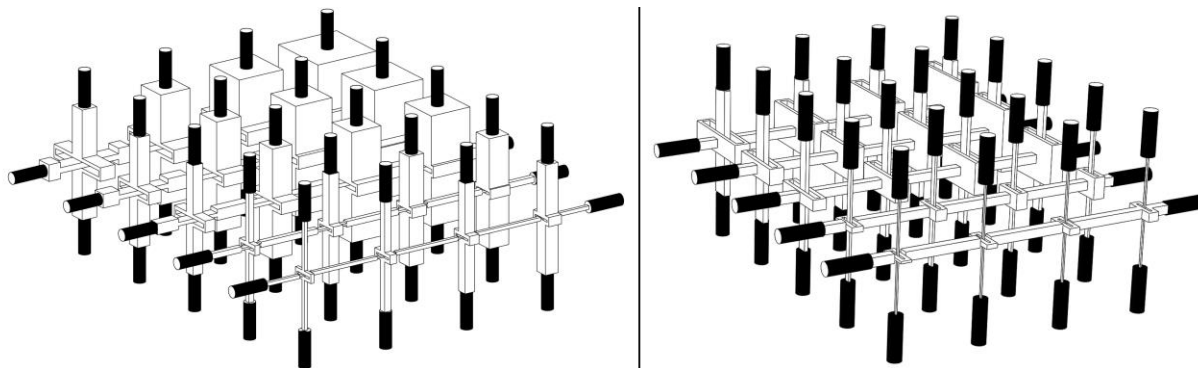


**Figure 4** Parametric valve arrays generated for one print. LEFT: array of valves with horizontal membranes actuated by vertical control channels (Type II in Table 1). RIGHT: array of valves with vertical membranes actuated by horizontal control channels (Type III). Within each array the channel dimensions, membrane thicknesses and control chamber sizes vary from valve to valve; black cylinders = needle ports.

### 3.2. Resins

The valve arrays and the first of the two printed unit modules were printed in a custom photopolymer resin of poly(ethylene glycol) diacrylate of molecular weight 258 (PEGDA-258, Sigma-Aldrich) with 1% (w/w) phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (Irgacure 819, Sigma-Aldrich) as photoinitiator and an ultraviolet-absorbing dye to confine the cure to the exposed layer. Among the printers, resins and photoinitiators we iterated through, that formulation printed the most reliable and longest-lasting integrated valves. Three further formulations were screened for the scaffold body and the cell-contact surfaces: a PEGDA-PEGMEMA blend for flexible elements, silicone methacrylate-based blends for durable body sections, and a methacrylated gelatin (GelMA) blend for the chamber surfaces that cells attach to.

The second printed unit module was printed in NanoClear (FunToDo), an off-the-shelf commercial photopolymer resin sold for clear rigid parts, which our framework study had already made into working valves.<sup>32</sup>

### 3.3. 3D Printing

Parts were printed on Asiga digital light processing (DLP) printers (Asiga, Sydney, Australia). The valve arrays were printed on an Asiga Pro4K65 DLP printer with a 385 nm light-emitting diode at 15.1 mW cm<sup>-2</sup>, about 80% of the machine's maximum power, after that printer was acquired in the second year of the project to speed up prototyping. Devices were printed on silanized glass slides rather than directly on the build plate, which gave a flat optical window for microscopy, protected the device during removal, and let the slide, slightly larger than the plate, be lifted off with a razor blade. Slides were silanized by immersion for 2 h in 10% 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich) in toluene, rinsed, and stored in fresh toluene until use. Before printing, resin was applied to the slide, the slide was fixed to the build plate, and a 30 s exposure anchored it.

### 3.4. Post-Processing

For the PEGDA devices, unpolymerized resin was drawn out of the valves and channels by applying vacuum to the pneumatic and fluidic ports, the interiors were flushed with isopropyl alcohol at 1 to 5 mL

min<sup>-1</sup> so that the flow would not tear the membranes, and a second vacuum step removed the residual alcohol. Devices were then post-cured for 15 to 20 min under a 430 nm light-emitting diode (Thorlabs, Newton, NJ, USA) delivering 21.3 mW cm<sup>-2</sup> at the curing plane. That wavelength lies outside most of the absorption band of the resin's ultraviolet absorber, so the curing light penetrates deep into the device, and inside the photoinitiator's band, so polymerization continues.

### 3.5. Flow Simulation

Pressure distributions on the valve membranes were computed with COMSOL Multiphysics version 6.2 as steady laminar flow of air through the control channel and valve chamber of the printed geometry, with a 172 kPa (25 psi) inlet, matching the bench control pressure, an atmospheric outlet and no-slip walls, on a tetrahedral mesh refined around the membrane.

### 3.6. Pneumatic Control

Valves were actuated by computer-controlled solenoid valves built on the open-source programmable pneumatic setup of Brower et al.,<sup>35</sup> driven from a MATLAB interface that also runs the microscope,<sup>36</sup> and later by a custom compact controller for solenoids, pressure and temperature developed for this scaffold (patent pending).

### 3.7. Flow Visualization

Flow and valve action were imaged on a Microqubic 3D MRCL700 microscope (Microqubic AG, Zug, Switzerland) in transmitted light with the manufacturer's medium-zoom lens, and the images and videos were background-subtracted and contrast-enhanced in ImageJ Fiji.<sup>37</sup> Food coloring perfused through the channels showed which ports delivered and which stayed sealed.

## 4. Results and Discussion

### 4.1. Valves in Every Orientation

A module with ports on all six faces needs valves in orientations that layer-by-layer printing had not produced before. Two attributes define four possible configurations: whether the membrane lies flat (horizontal) or stands on edge (vertical), and whether the control pressure arrives parallel or perpendicular to it (see Table 1). The horizontal membrane with parallel control is the configuration of every 3D-printed membrane valve we know of, and it served as our benchmark. We set aside the vertical membrane with parallel control, because its purpose, a vertical control channel, is served without a vertical membrane by the horizontal membrane with perpendicular control. That left two configurations to realize, and we expected each to fail in its own way. A membrane standing on edge is built from stacked layers whose seams run across it, so pressure on one face can open a seam on the other and let the valve leak. A control pressure arriving perpendicular to a membrane concentrates its load at the membrane's center, and a membrane only tens of micrometers thick can tear there.

**Table 1** Membrane-valve configurations in layer-by-layer 3D printing, by membrane orientation and direction of the control pressure.

| Type | Membrane   | Control pressure | Expected failure | Reported before | Studied here |
|------|------------|------------------|------------------|-----------------|--------------|
| I    | Horizontal | Parallel         | None             | Yes             | Benchmark    |

|     |            |               |                                        |    |                                   |
|-----|------------|---------------|----------------------------------------|----|-----------------------------------|
| II  | Horizontal | Perpendicular | Tearing at the center                  | No | Yes                               |
| III | Vertical   | Perpendicular | Delamination at the seams, and tearing | No | Yes                               |
| IV  | Vertical   | Parallel      | Delamination at the seams              | No | No; its purpose is met by Type II |

Both expectations held. Every valve of the naive Type II design leaked when pressurized, whatever its flow-channel and control-channel dimensions, valve chamber size, membrane thickness or exposure setting within the swept arrays. The flow simulation located the cause: the control pressure peaked at the center of the membrane (see Figure 5-LEFT). Degassing the resin, discarding prints with visible bubbles, washing at 1 to 5 mL min<sup>-1</sup> and reducing the exposure over the membrane did not remove the leak, which left the geometry as the cause to change. Moving the control channel's inlet from the top center of the valve to its side, and the opposite control channel to the opposite side for symmetry (see Figure 6), placed most of the pressure on the flow channel's side wall rather than on the membrane, and the simulation then showed a uniform load on both membranes (see Figure 5-RIGHT). Valves of that geometry sealed, and Video S1 shows one of them opening and closing under its 1.1 s actuation cycle (see Video S1).

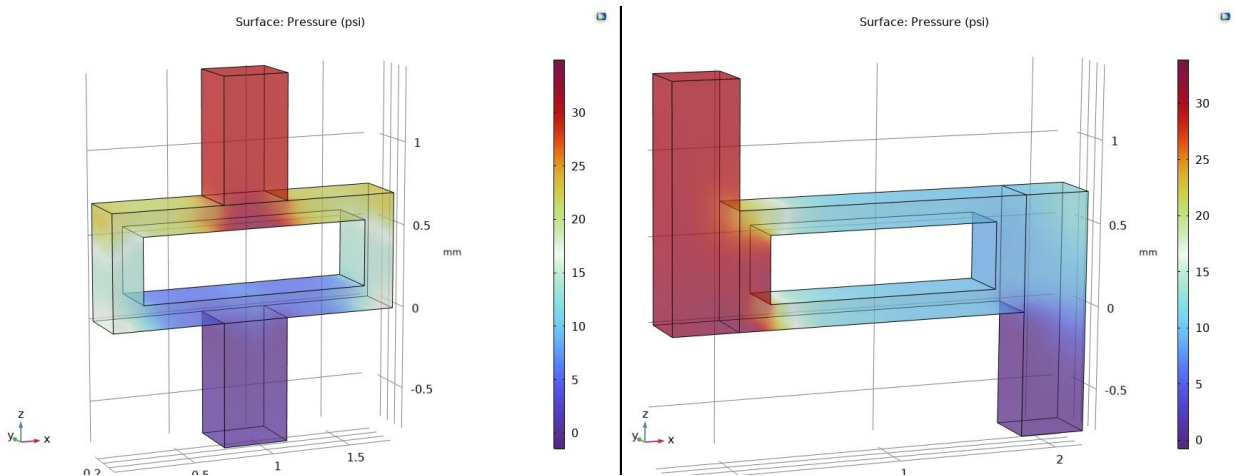


**Figure 5** Simulated pressure on the membranes of a Type II valve at a 25 psi control pressure. LEFT: naive design, with the control channel entering at the top center of the valve. RIGHT: redesign, with the control channels entering at the sides. Color scale = surface pressure in psi; axes in mm. The flow channel passes horizontally through the valve.

The redesign keeps the control channels perpendicular to the membrane, which is the whole point of the Type II valve, and only moves where they meet the valve chamber (see Figure 6). The membrane therefore still sees a control pressure arriving from above, but the jet of that pressure lands on the side wall, and the membrane is loaded by the chamber pressure alone.

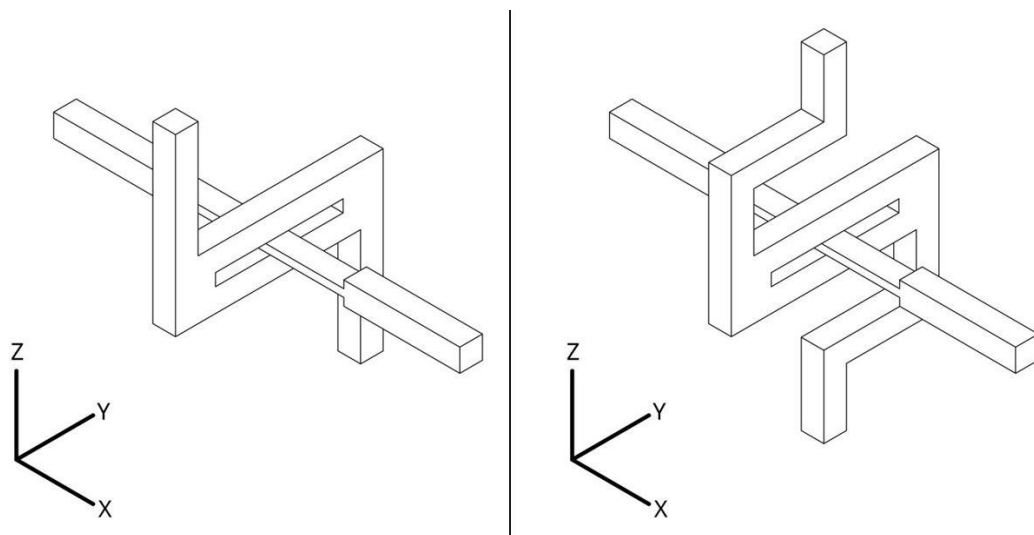


**Figure 6** Type II valve geometry before and after the redesign. LEFT: naive design, with the vertical control channels entering the valve chamber at its top and bottom center. RIGHT: redesign, with the control channels offset to opposite sides of the valve chamber. The flow channel passes through the chamber along the X axis; the control channels run along Z.

For the vertical membranes of Type III, the failure was the one that Table 1 predicts: the membrane delaminated at its layer seams under pressure (see Figure 7-CENTER). A reinforcement whose inter-layer seams are offset from those of the membrane (see Figure 7-RIGHT), together with an adjusted layer thickness and crosslinking time next to the membrane, stopped the delamination, and the vertical valves sealed as well. Video S2 shows one of them under a 0.9 s actuation cycle (see Video S2). With those two configurations in hand, every face of the module can carry a valve, and high-aspect-ratio channels and diagonal bypasses printed in the same builds.

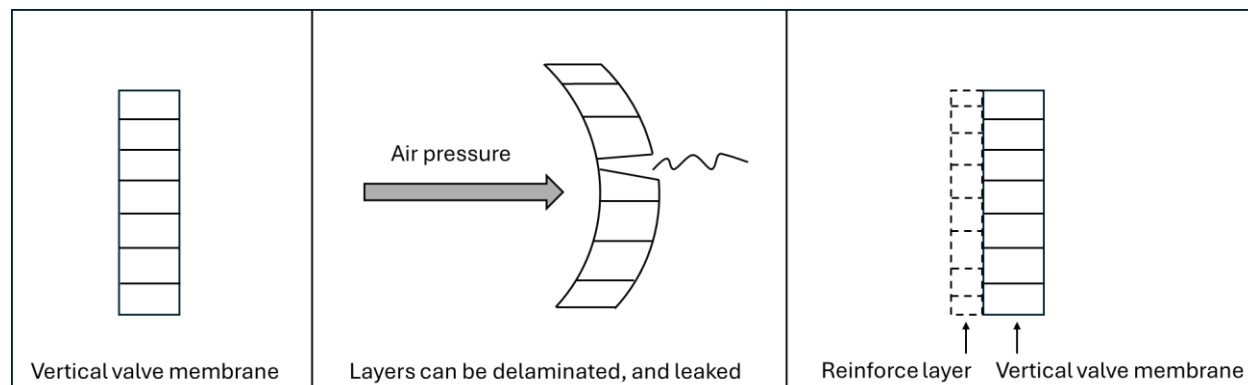


**Figure 7** Vertical valve membrane: failure mode and remedy. LEFT: a membrane standing on edge is built from stacked print layers (horizontal lines = layer seams). CENTER: air pressure on one face opens a seam on the other face and the valve leaks. RIGHT: a reinforcement layer (dashed) whose seams are offset from the membrane's seams backs the membrane.

#### 4.2. One Complete Unit Module, Printed as a Single Cube

We then printed one complete three-dimensional unit module as a single cube, first in the custom PEGDA resin of Section 3.2 and later in NanoClear (see Figure 8). In the PEGDA cube (see Figure 8A), the chamber sits at the center, the flow channels cross it along the two in-plane axes, the bypass loops run around it, and the needle ports open on the faces, with a needle seated in one of them. The close-up (see

Figure 8B) shows the chamber region with its side valves and the layer texture of the printed loop walls. In the NanoClear cube, we filled the control side and the flow side of the plumbing with dyes of two colors, and the two dyes stayed in separate networks in both the perspective view (see Figure 8C) and the top-down view (see Figure 8D). Both modules printed as one part, with their channels, bypass loops and ports resolved as drawn, which is the step the three-dimensional layout required before any array could be tiled. The NanoClear cube also shows that the module does not need a resin formulated in-house to print.

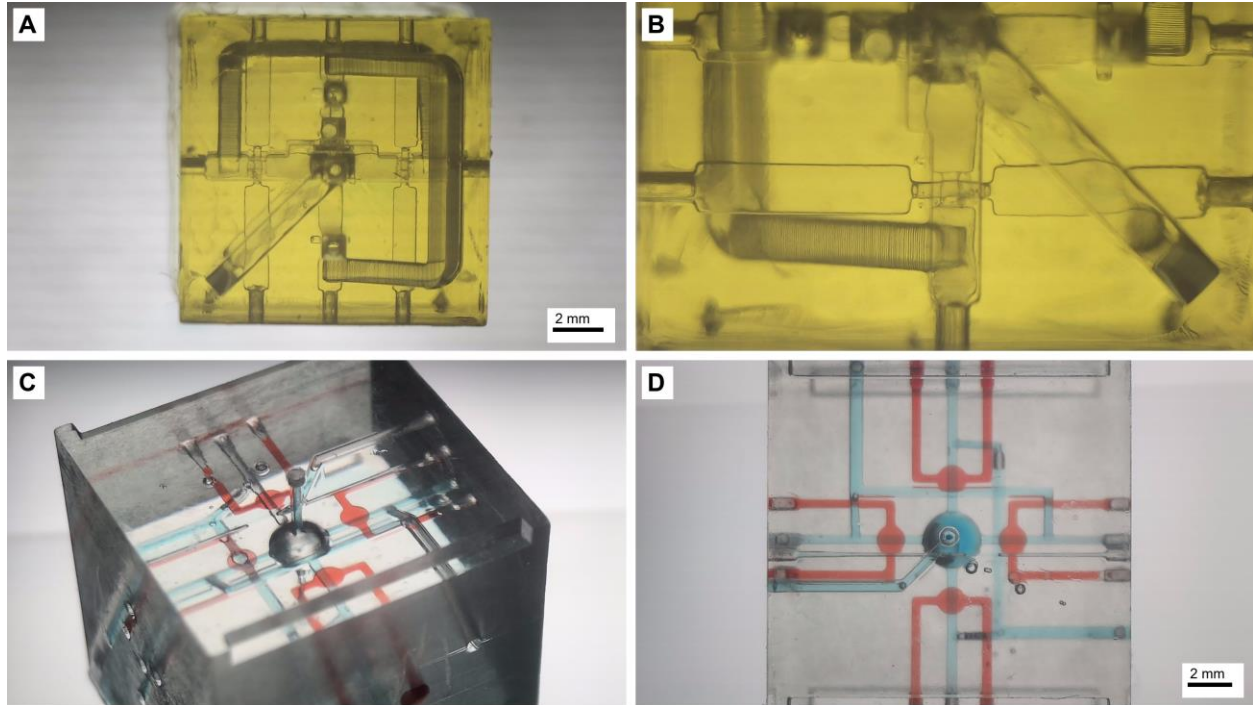


**Figure 8** Printed unit modules in two resins. (A) Transmitted-light view of a unit module printed as a single cube in the PEGDA resin of Section 3.2, showing the chamber at the center, the flow channels crossing it, the bypass loops around it, and the needle ports on the faces; a needle is seated in one port. (B) Higher-magnification view of the chamber region of the module in (A). (C) Perspective view of a unit module printed in NanoClear, with red dye in the control side and blue dye in the flow side and the culture chamber. (D) Top-down view of the module in (C). The dye colors in (C) and (D) are the reverse of the convention of the drawings, where red marks the flow side and blue the control side. Scale bars in (A) and (D) = 2 mm.

## 5. Limitations and Next Steps

The printed plumbing does carry limitations of its own at this stage. Addresses in the printed module sit farther apart than in our two-dimensional device, whose ports were 150  $\mu\text{m}$  in diameter on 100  $\mu\text{m}$  channels, because the printed membranes set the smallest valve. The vertical valves have sealed, but we have not yet measured how long they last under repeated actuation. The resins were chosen for their valves, so the printed scaffold does not yet degrade to make room for tissue. The architecture does not depend on that material, and the patent application anticipates a degradable version.<sup>38</sup>

The next steps follow from those limitations and from the application the plumbing is built for. (i) **Miniaturization and tiling:** the unit module is being shrunk until it tiles into a dense scaffold, with the parametric scripts of Section 3.1 sweeping the geometry within one print, and the scaffold's valve lines are being connected to the combinatorial multiplexer of Section 2.4. (ii) **Patterned co-culture:** primary human osteoblasts and chondrocytes are being delivered through separate addresses into predetermined

patterns, and medium withdrawn through individual ports is assayed outside the culture, so that assays which are destructive in the dish leave the culture intact. A later version of this preprint will report both steps.

## 6. Conclusions

Three-dimensionally addressable microfluidic plumbing can be printed in one piece. The plumbing gives every chamber its own bypass and its own valves on every axis, so a payload reaches any neighbor without passing through other chambers, and valves on the bypasses let chosen neighbors exchange molecules by diffusion. Two changes of geometry made valves print and seal in the two orientations that a six-sided chamber needs. The first moved the control channel's entry off the center of the membrane, and the second backed a vertical membrane with a reinforcement whose layer seams are offset from its own. A complete unit module then printed as a single cube in a custom poly(ethylene glycol) diacrylate resin and in a commercial resin, so the module can be printed without a resin formulated in-house. Its parametric scripts build the one-, two- or three-dimensional version of the module from a flag.

## Supplementary Videos

**Video S1.** Type II valve (horizontal membrane, perpendicular control) in a printed array, opening and closing under a 1.1 s actuation cycle, viewed in transmitted light with green dye in the flow channel.

**Video S2.** Type III valve (vertical membrane) in a printed array, opening and closing under a 0.9 s actuation cycle, viewed in transmitted light with green dye in the flow channels.

## Acknowledgments

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## Conflicts of Interest

R.V. is the founder and president of AltVIVO, Inc., which is commercializing the technology described here, and several of the authors are named inventors on the patent application that covers it.

## Data Availability

The parametric design scripts and the valve videos are available from the corresponding author on reasonable request.

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