Supplementary Information for

Microfluidic scaffolds for tissue engineering

NAK WON CHOI, MARIO CABODI, BRITTANY HELD, JASON P. GLEGHORN,
LAWRENCE J. BONASSAR and ABRAHAM D. STROOCK

1School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, New York 14853, USA
2Present address: Center for Nanoscience and Nanobiotechnology, Boston University, Boston, Massachusetts 02215, USA
3Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853, USA
4Department of Biomedical Engineering, Cornell University, Ithaca, New York 14853, USA
5Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, New York 14853, USA

*e-mail: ads10@cornell.edu; lb244@cornell.edu

Supplementary Methods

Jig for fabrication and operation of microfluidic scaffolds:

Supplemental Fig. 1 shows a photo with all of the parts used in the jigs for molding and operating microfluidic scaffolds:
**O-rings (Supplementary Fig. 1i):** an O-ring (McMaster-Carr®, Robbinsville, New Jersey) was inserted in a 2-mm recess on the bottom of an aluminum part (Supplementary Fig. 1ii). O-rings served as gaskets to prevent CaCl$_2$ solution from leaking during the cross-linking step.

**Aluminum parts (Supplementary Fig. 1ii, v, vi, vii, and ix):** machined aluminum parts served as rigid frames to provide thermal stability in autoclave, and to guide assembly of sealed microfluidic scaffold.

**Perforated aluminum support (Supplementary Fig. 1iii):** perforated aluminum sheets (812 µm thick, 838 µm in diameter round perforations; Small Parts, Inc., Miami Lakes, Florida) were cut, and screw holes and injection ports were drilled. Tygon tubing (1 mm in inner diameter; Small Parts, Inc., Miami Lakes, Florida) was glued on one of the ports using cyanoacrylate adhesive (Super Glue Corp., Rancho Cucamonga, California).

**Plastic sheets:** Plastic shim (AccuTrex Products, Inc., Canonsburg, Pennsylvania) were cut, and screw holes and windows were created with a scalpel.

**Poly(dimethylsiloxane) (PDMS) stamp (Supplementary Fig. 1viii):** a mold was pre-assembled with a lithographically defined SU-8 master (see Methods in main text) and jig parts for top layer of the microfluidic scaffold (Supplementary Fig. 1ii, vi, and vii). PDMS (Sylgard 184, Dow Corning, Midland, Michigan) was poured as a pre-polymer into the mold and cured in two steps: 24 h at room temperature and followed by 1 h at 65 °C. This two-step process was necessary to avoid deformation of the stamp due to thermal expansion during the initial cross-linking of the pre-polymer.

CAD drawings of jig parts are available upon request.
Assembly of molds for calcium alginate:

Coating with poly(ethylenimine): to promote adhesion of calcium alginate, a solution of poly(ethylenimine) (PEI) (MW 750,000; branched.; Sigma-Aldrich, St. Louis, Missouri) was made at 0.1 % [v/v] in water. Components v and xi were oxidized for 1 min in a plasma cleaner/sterilizer (Model PDC-001, Harrick Plasma, Ithaca, New York) at the highest RF power. Immediately following oxidation, ~ 28 µL/cm² (corresponding to a total of 250 µL) of the solution of PEI was applied onto the surfaces of each part. These parts were allowed to dry in a sterile hood overnight.

Mold for top layer of calcium alginate: the PDMS stamp was oxidized for 1 min in the plasma cleaner/sterilizer. This oxygen plasma treatment was performed immediately before each fabrication run in order to increase wettability of the PDMS stamp by uncross-linked alginate during the injection step. A mold for the top layer of the scaffold was formed by stacking the following parts (Supplementary Fig. 1): i, ii, iii, iv, v, vi, viii, and vii.

Mold for bottom layer of calcium alginate: a mold for the bottom layer of the scaffold was formed by stacking the following parts (Supplementary Fig. 1): i, ii, iii, iv, v, vi, xi, and ix.

Assembly of cartridge for operation of microfluidic scaffold:

Once cross-linked with CaCl₂ solution, the top and bottom layers of calcium alginate were assembled into the functional microfluidic scaffold. The complete cartridge was formed as a stack of the following parts (Supplementary Fig. 1): ii, x, xii, v with the top layer of calcium alginate inside the window, xiii, v with the bottom layer of alginate inside the window, xi, vi, and ix.
Supplementary Figure 1  Assembling jig parts used to fabricate microfluidic scaffolds.
Supplementary Note

Process viability of microfluidic scaffolds seeded with L2 and HepG2 cell line

Cell culture: HepG2/C3A (human hepatocytes) cells were purchased from the American Type Culture Collection (ATCC). HepG2/C3A cell lines were cultured in Eagle’s Modified Essential Medium (MEM) (Invitrogen Co., Carlsbad, California) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Co., Carlsbad, California) and 1 mmol/L sodium pyruvate (Sigma-Aldrich, St. Louis, Missouri), at 37°C and 5% CO₂. Cells were harvested using 0.25% trypsin / ethylenediaminetetraacetic acid (EDTA) (Invitrogen Co., Carlsbad, California) and quenched with MEM to re-suspend the cells. Cells were fed every 3-4 days and passed every 6-7 days.

L2 (rat lung epithelial) cells were purchased from the ATCC. L2 cell lines were cultured in 50% [v/v] Dulbecco’s Modified Eagle’s Medium and 50% [v/v] Ham’s F-12 medium (DMEM/F-12) (Invitrogen Co., Carlsbad, California) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Co., Carlsbad, California), at 37°C and 5% CO₂. Cells were harvested using 0.25% trypsin / ethylenediaminetetraacetic acid (EDTA) (Invitrogen Co., Carlsbad, California) and quenched with MEM to resuspend the cells. Cells were fed every 3-4 days and passed every 6-7 days.

Seeding HepG2/C3A or L2 cells into alginate solution: MEM with a 2 mmol/L GlutaMAX-I supplement (Sigma-Aldrich, St. Louis, Missouri) or DMEM/F-12 was added to charcoal-filtered Protanal LF 10/60, a low viscosity alginate with a mean G/M ratio of 70% (FMC Biopolymer, Drammen, Norway), to yield a 4% [w/v] solution. The alginate solution was allowed to incubate at 37°C for 12-24 h. The alginate solution was then added to a HepG2/C3A
or L2 cell pellet to achieve a desired seeding density. Cells were re-suspended in the alginate by slow stirring with a sterile spatula.

Supplementary Figure 2  Process viability of microfluidic scaffolds. Fluorescence micrographs of cells seeded in 4 % [w/v] calcium alginate and stained by 5 µmol/L calcein-AM (green – live) and 0.5 µmol/L ethidium homodimer-1 (red – dead).  

(a) HepG2 cells ($2 \times 10^6$ cell/mL) The image was taken using a black/white CCD camera (Model Retiga 1300, QImaging, Surrey, Canada).  

(b) L2 cells ($1 \times 10^6$ cell/mL) The image was taken using a RGB CCD camera (Model DP70, Olympus America Inc., Center Valley, Pennsylvania).

Effect of shear imposed during the seeding and injection step on cell viability

After isolation, cells were pelleted in a centrifuge tube (50 mL). A 4 % [w/v] alginate solution was poured onto the pellet and the cells were mildly re-suspended using a spatula (30 rpm for ~ 1 min). The cell-seeded 4 % [w/v] alginate solution was taken into a syringe (5 mL) and injected through 3 cm of tygon tubing into the molding jigs at ~ 150 µL/min. It was observed that the standard method employed for injection molding chondrocytes in lower concentration alginate (Lee, C. S. D. et al. Integration of layered chondrocyte-seeded alginate hydrogel scaffolds. Biomaterials 28, 2978-2993 (2007)) led to significant loss in viability when used in 4 % [w/v] alginate solution. We believe that this difference is due to increased viscosity of 4 % [w/v] alginate solution.
**Theoretical background of diffusivity measurement in a microfluidic scaffold**

For simplicity, we present analysis for early times after the injection of the dye into the scaffold ($t < H^2 / D_{s,g}$) for which the diffusion occurs in a domain that is effectively infinite in extent along the $x$-axis; the experiments presented in Fig. 3 are in this regime. Using methods of image sources, this approach (in particular, the integration over $x$) can also be shown to be appropriate for all times in a domain bounded by parallel, impermeable boundaries. At early times, the time-dependent concentration distribution due to an instantaneous source, $M(x, y)$ at $t = 0$ can be expressed in integral form as:

$$c(x, y, t) = \frac{1}{4\pi D_{s,g} t} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} M(x', y') \exp \left[ -\frac{(x-x')^2 + (y-y')^2}{4D_{s,g} t} \right] dx' dy'. \quad (1)$$

In equation 1, the axes are as defined as in Fig. 3a in the text, with the $x$-axis into the page, through the thickness of the scaffold. For fast injection (high $Pe$ and high $Bi$), we neglect variations along $z$. We have verified that the fluorescence intensity, $I(y, t)$, in micrographs taken from above at low magnification (~10x), as in Fig. 3a and 3b, is linearly proportional to the concentration of dye and the thickness of the scaffold. Therefore, we expect the following relationship between intensity and concentration:

$$I(y, t) = \alpha \int_{0}^{H} c(x, y, t) dx \approx \alpha \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} M(x', y') \exp \left[ -\frac{(x-x')^2 + (y-y')^2}{4D_{s,g} t} \right] dx' dy' dx$$

$$= \alpha \int_{-\infty}^{\infty} \frac{M_x(y')}{\sqrt{4\pi D_{s,g} t}} \exp \left[ -\frac{(y-y')^2}{4D_{s,g} t} \right] dy'. \quad (2)$$

In equation 2, $\alpha$ is a constant that depends on the intensity of the illumination, quantum yield of the fluorophor, the length of the exposure, and the sensitivity of the camera, and $M_x(y')$ is the $x$-integrated distribution of the initial source. In the second step, we have used the assumption of
early times to extend the bounds of the integral along $x$ to $\pm \infty$. In the absence of variations along $z$, $I(y,t) = \tilde{I}_z(y,t)$, the $z$-averaged intensity in Fig. 3b. Performing a continuous Fourier transform on this $x$-integrated intensity in equation (2) gives the following expression for the amplitude of the fundamental Fourier mode: $\hat{I}(k_0, t) = \hat{M}_x(k_0) e^{-t/\tau}$ where the time constant for the decay, $\tau = 1/k_0^2D_{s,p}$ and $\hat{M}_x(k)$ is the Fourier transform of $M_x(y')$, defined as

$$\hat{M}_x(k) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} M_x(y') e^{-iky'} dy'.$$

**Effective diffusion in a porous material**

To clarify our interpretation of the dynamics of fluorescein and FITC-BSA as “unhindered” pore diffusion (Fig. 3), we introduce the basic concepts of pore diffusion: for any solute that can move freely within and between the pores of a porous material, the effective macroscopic diffusivity will be related to its free diffusivity by a single coefficient, $C_{pore} < 1$, that is related to the geometry of the pores:

$$D_g = C_{pore}D_w = \left( \frac{l}{l_{eff}} \right) \left( \frac{A_{eff}}{A} \right) D_w,$$  \hspace{1cm} (1)

where $l$ [m] is a macroscopic length over which diffusion occurs within the porous material, $l_{eff}$ [m] is an effective length of the path of the solute through pores, $A$ [m$^2$] is a macroscopic cross-sectional area of the porous material through which the solute motion, $A_{eff}$ [m$^2$] is a reduced area available for flow of the solute, and $D_w$ [cm$^2$/s] is the diffusivity of the solute in water (Scheidegger, A. E. *The physics of flow through porous media* (University of Toronto,
Toronto, 1960). Our experiments with fluorescein and FITC-BSA indicate that \( C_{\text{pore}} \sim 0.5 \) for 4% calcium alginate.

We also highlight the observations that indicate conclusively that mass transfer within the gel is diffusive (not convective): 1) the quantitative agreement between purely diffusive model and transient profiles (Fig. 3); note that Darcy convection of the solute would lead to a sharp solute front and non-diffusive temporal scaling (Neeves, K. B., Lo, C. T., Foley, C. P., Saltzman, W. M. & Olbricht, W. L. Fabrication and characterization of microfluidic probes for convection enhanced drug delivery *J. Control. Release* **111**, 252-262 (2006)) and 2) the measured coefficients of pore diffusion scale in the expected way with molecular weight and free diffusivity; if a convective mechanism were at play, there should be no dependence of the rate of broadening on molecular weight.

**Enzymatic consumption of calcein-AM**

Experiments with cells in suspension were performed to study the enzymatic consumption of calcein-AM by primary chondrocytes in suspension. Isolated chondrocytes and solutions of calcein-AM with various concentrations (i.e., 1.25, 2.5, 5, 10, 20 \( \mu \text{mol/L} \)) were dispensed into wells of a 96-well plate. In addition, cells without calcein-AM, calcein-AM without the cells, and completely pre-cleaved calcein (Carlo, D. D., Aghdam, N. & Lee, L. P. Single-cell enzyme concentrations, kinetics, and inhibition analysis using high-density hydrodynamic cell isolation arrays. *Anal. Chem.* **78** (14), 4925-4930 (2006)) without the cells were dispensed for estimating background, autohydrolysis, and intracellular concentrations of calcein respectively. Fluorescence was measured in a microplate reader (Model Synergy™ HT, BioTek, Winooski, Vermont) every minute for 1 h. The intensity of both the background and
autohydrolyzation were subtracted from the raw fluorescence intensity of calcein. After converting the corrected intensity to concentration of calcein (product concentration, $P$ [mol/L]) production rate at early times (i.e., 5 ~ 15 min when $P$ linearly increased over time) was obtained. It was found that the enzymatic kinetics was first order with respect to the concentration of calcein-AM (substrate concentration, $S$ [mol/L]) with a rate constant, $k_{eff} = \rho_{cell}k_{cell}^{sup}$, where the cellular rate constant, $k_{cell}^{sup} = 5.9 \times 10^{-11}$ mL/cell·s.

Supplementary Figure 3  Schematic illustration of diffusion-reaction process for calcein-AM

Transient enzymatic transformation of calcein-AM in 3-D

The visualization of the Krogh length is directly associated with enzymatic production of calcein. Given that $\frac{dP}{dt} = \rho_{cell}k_{cell}S$, a distribution of calcein within microfluidic scaffolds can be expressed as $P(x,y,z,t) = \int_0^t \rho_{cell}k_{cell}S(x,y,z,t) dt$, where $P$ [mol/L] and $S$ [mol/L] are concentration of calcein and calcein-AM respectively. If the distribution of calcein-AM reaches a steady state in a short time relative to the length of the experiment, the distribution of calcein...
reduces to $P_{ss}(x,y,z,t) \approx \rho_{cell}k_{cell}tS_{ss}(x,y,z)$. The corresponding fluorescence intensity of calcein can be written as $I(x,y,z,t) = \alpha P_{ss}(x,y,z,t) = \alpha\rho_{cell}k_{cell}tS_{ss}(x,y,z)$ where $\alpha$ is again a constant relating concentration and intensity. In addition, the observed intensity is the integrated intensity along the $x$-axis: $I(y,z,t) \equiv \int_0^H I(x,y,z,t)\,dx$. Supplementary Fig. 4 presents the transient distributions of calcein (a) and their collapse at long times, when normalized by the time of injection (b). This behavior verifies that the distribution of calcein-AM reaches a steady state at long times and that measuring the concentration of $P$ via fluorescence provides a measure of the steady state distribution of reactant.

**Supplementary Figure 4** Transient distributions of calcein and their collapse at long times. 

- a, Plot of $z$-averaged intensity, $\bar{I}_z(y,t)$ measured in Fig. 4a at times (20 min (dark grey), 40 min (red), 60 min (green), 80 min (blue), 100 min (dark red), 120 min (dark green), 140 (dark blue), 160 min (pink)).
- b, Plot of $z$-averaged intensity divided by time [h], $\bar{I}_z(y,t)/t$. The plot was obtained from a single sample. The experimental data points were mean values of fluorescence intensity (321 pixels in $z$-direction) in images. Standard deviations were smaller than symbols.

**Uniform metabolic environment within bulk of microfluidic scaffold**

Supplementary Fig. 5 shows a uniform network with spacing such that relatively homogeneous delivery of calcein-AM is achieved.
Supplementary Figure 5  Uniform metabolic environment within the bulk of a microfluidic scaffold. Fluorescence micrograph of uniformly stained primary chondrocytes (1×10^7 cell/mL) by 5 µmol/L calcein-AM delivered via the embedded microchannels within a microfluidic scaffold (Fig. 1ci).

Analysis of distribution of fluorescent product in cross-sectional images

Cross-sectional images were cropped, and a region of interest was copied into a canvas with black-background; the height of the canvas was twice that of the cropped image (Supplementary Fig. 6). A Radial distribution of calcein about a concentric point in a microchannel was then obtained using a plugin of Radial Profiles provided in ImageJ. Each experimental data point presented in Fig. 4c was acquired by binning three raw data points. We note that the binning process does not affect the global trends and is only for better readability.
**Supplementary Figure 6** Radial distribution of calcein about a microchannel. Fluorescence micrograph of cross-section of top (micropatterned) layer ($7.3 \times 10^7$ cell/mL) of scaffold.

**Computational analysis for diffusion-reaction of calcein-AM within bulk of microfluidic scaffold**

Supplementary Fig. 7 presents numerical calculations of the distribution of a reactive solute in a microfluidic scaffold. This type of calculation was compared to experimentally measured distributions in Fig. 4 of main text.

**Supplementary Figure 7** Distribution of a reactive solute delivered via the embedded microchannels within a microfluidic scaffold with variably spaced microchannels.

Concentration of calcein-AM calculated by finite element method with an assumption of first order kinetics with $D_{calc,g,g} = 2.4 \times 10^{-6}$ cm$^2$/s, $k_{cell} = 1 \times 10^{-11}$ mL/cell·s, and $\rho_{cell} = 2.5 \times 10^7$ cell/mL. Concentration boundary conditions were maintained at the channel walls ($c_{wall} = 1$), and no-flux conditions were maintained at the top and bottom boundaries. The distributions are shown for $t = 120$ min (a) and steady state (b).
Quantitative comparison between experimental and computational results presented in Fig. 4c

To quantitatively compare how well the computational curves fit with the experimental results, square correlation coefficient ($R^2$) and root mean square error (RMSE) are calculated at each cell seeding density:

$$R^2 = \frac{\left[N \sum_{i=1}^{N} (X_{i,\text{experiment}} - X_{i,\text{computation}})^2 \right]}{\left[N \sum_{i=1}^{N} X_{i,\text{experiment}}^2 \right] - \left[\left( \sum_{i=1}^{N} X_{i,\text{experiment}} \right)^2 \right]} = \frac{1}{N \sum_{i=1}^{N} X_{i,\text{computation}}^2}$$

$$\text{RMSE} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (X_{i,\text{experiment}} - X_{i,\text{computation}})^2}$$

where $N$ is a number of data points at a cell seeding density. We note that computational data points, corresponding to experimental ones, were selected at each cell seeding density so that $N_{\text{experiment}} = N_{\text{computation}}$.

Computational analysis for diffusion of non-reactive solute within microfluidic scaffold with two independent networks

Supplementary Fig. 8 presents numerical calculations of the distributions of non-reactive solutes in scaffolds of two different thicknesses. These calculations illustrate that the average sharpness of the gradient achieved varies with the thickness in these cases for which the thickness is large compared to the interchannel spacing.
**Supplementary Figure 8** Steady-state distribution of a non-reactive solute delivered via the embedded microchannels in network 1 and extracted via those in network 2. Computationally calculated concentration profiles within a microfluidic scaffold with the thickness of 2 mm (a) and 1 mm (b). Concentration boundary conditions were maintained at the channel wall ($c_{wall} = 1$ in network 1; $c_{wall} = 0$ in network 2), and no-flux conditions were maintained at the top and bottom boundaries.
Supplementary Figure 9  Spatially resolved delivery of non-reactive solutes via embedded microfluidic networks. Fluorescence micrographs of two-network microfluidic scaffold (acellular) after a, 2 h-delivery of the pH-adjusted buffer through network 1 and 5 μmol/L rhodamine B, dissolved in the pH-adjusted buffer, through network 2, b, 2 h-delivery of 5 μmol/L fluorescein, dissolved in the pH adjusted buffer, through network 1 and rhodamine B through network 2, and c, 2 h-delivery of fluorescein through network 1 and the pH-adjusted buffer through network 2.
Nomenclature

1. $A$ - macroscopic cross-sectional area of a porous material through which solutes flow

2. $A_{eff}$ - reduced area available for flow of solute in a porous material

3. $Bi$ - Biot number

4. $Bi_{fluor}$ - Biot number of fluorescein

5. $Bi_{rhod}$ - Biot number of rhodamine B

6. $c_0$ - concentration of a solute in microchannels [mol/L]

7. $c_{FEM}$ - calculated concentration by finite element method [mol/L]

8. $c_{FEM}^{max}$ - maximum concentration by finite element method [mol/L]

9. $C_{pore}$ - experimentally observed prefactor for effective pore diffusion

10. $D_{BSA,g}$ - effective pore diffusivity of FITC-BSA in scaffold [cm$^2$/s]

11. $D_{BSA,w}$ - diffusivity of FITC-BSA in water [cm$^2$/s]

12. $D_{fluor,g}$ - effective pore diffusivity of fluorescein in scaffold [cm$^2$/s]

13. $D_{fluor,w}$ - diffusivity of fluorescein in water [cm$^2$/s]

14. $D_{s,c}$ - diffusivity of a solute in microchannel [cm$^2$/s]

15. $D_{s,sg}$ - effective pore diffusivity of a solute in scaffold [cm$^2$/s]

16. $D_{s,w}$ - diffusivity of a solute in water [cm$^2$/s]

17. $H$ - scaffold thickness [mm]

18. $h_e$ - microchannel height [$\mu$m]

19. $I_{Exp.}$ - experimentally measured intensity
1. $I_{\text{Exp.}}^{\text{max}}$ maximum experimentally measured intensity

2. $\bar{T}_y(z,t)$ $y$-averaged intensity

3. $\bar{T}_z(y,t)$ $z$-averaged intensity

4. $\hat{\bar{T}}_z$ Fourier transform of $z$-averaged intensity

5. $k_0$ characteristic spatial frequency [$\mu m^{-1}$]

6. $k_c$ mass transfer coefficient in microchannels [cm/s]

7. $k_{\text{cell}}$ cellular rate constant [mL/cell·s]

8. $k_{\text{cell}}^{\text{scaf}}$ cellular rate constant in scaffold [mL/cell·s]

9. $k_{\text{cell}}^{\text{susp}}$ cellular rate constant in suspension [mL/cell·s]

10. $k_{\text{eff}}$ effective rate constant [s$^{-1}$]

11. $l$ macroscopic length of a porous material [m]

12. $l_{\text{eff}}$ effective flow path of solutes through pores [m]

13. $L$ microchannel length [mm]

14. $M$ instantaneous initial source [mol/L]

15. $M_x$ $x$-averaged distribution of instantaneous initial source [mol/L]

16. $\hat{M}$ Fourier transform of $M$

17. $P$ concentration of calcein [mol/L]

18. $P_{\text{ss}}$ concentration of calcein at steady state [mol/L]

19. $Pe$ Peclêt number

20. $Pe_{\text{calc-g}}$ Peclêt number of calcein-AM-green

21. $Pe_{\text{calc-r}}$ Peclêt number of calcein-AM-red
$\textit{Pe}_\text{fluor}$ Peclèt number of fluorescein

$\textit{Pe}_\text{rhod}$ Peclèt number of rhodamine B

$R^2$ square correlation coefficient

$R_G$ radius of gyration [nm]

$S$ concentration of calcein-AM [mol/L]

$S_{ss}$ concentration of calcein-AM at steady state [mol/L]

$t$ time [s] or [min] or [h]

$u_c$ flow speed in microchannels [cm/s]

$V_m$ maximum consumption rate [mol/L·s]

$w_c$ microchannel width [µm]

\textbf{Greek letters}

$\alpha$ constant to convert concentration to fluorescence intensity [L/mol·m]

$\lambda_c$ inter-channel distance [µm]

$\lambda_K$ the Krogh length [mm]

$\rho_{cell}$ cell density [cell/mL]

$\tau$ time constant for decay [s]