Real-time Imaging and Quantitative Analysis of Doxorubicin Transport in Cylindrical MDCK Microvessels

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Supplementary Information

Video 1. Perfusion of a MDCKII-w/t microvessel with 100 μM Lucifer yellow for 45 minutes at low shear stress followed by 10 minutes of washout. The video shows overlays of fluorescence and phase contrast images at a 60 s frame rate.

Video 2. Perfusion of a MDCKII-w/t microvessel with 100 μM doxorubicin for 13 minutes at low shear stress followed by 6 minutes of washout. The video shows overlays of fluorescence and phase contrast images at a 60 s frame rate.

1. **Fabrication of Microvessels**
2. **Using epifluorescence imaging for kinetic analysis**
3. **Distributions of Doxorubicin and Lucifer Yellow in the ECM**
4. **Verification of MDCKII-w/t doxorubicin permeability in transwells using HPLC**
5. **Lucifer Yellow Permeability in PDMS Microvessels**

1. Fabrication of Microvessels

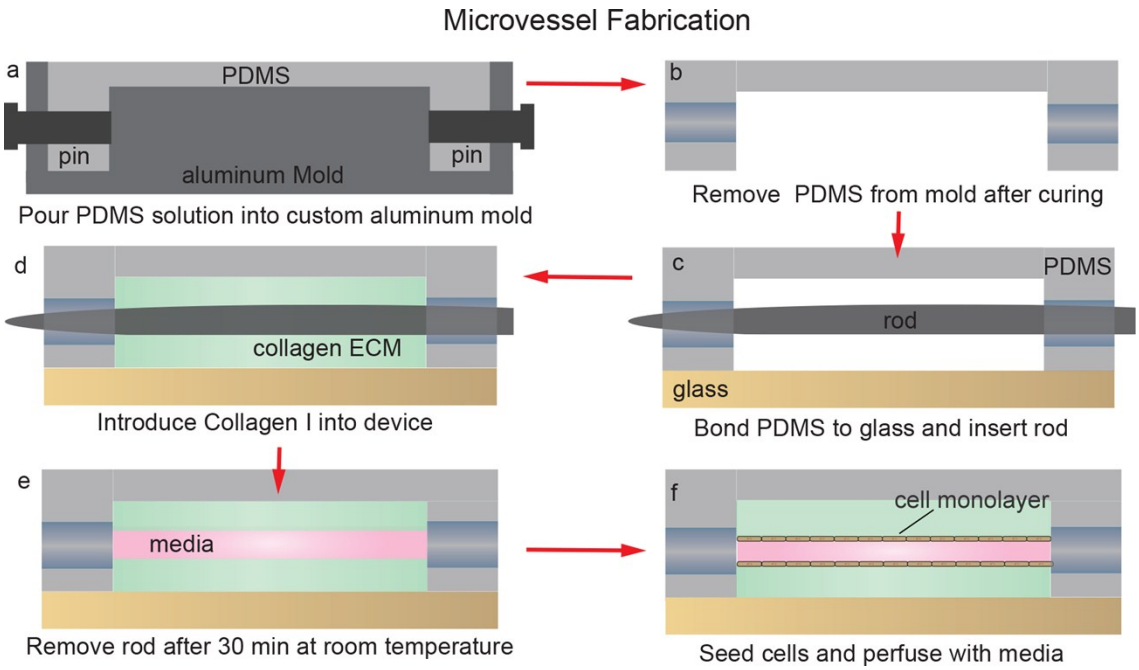

Fig. S1. Steps in the fabrication of microvessels. (a) First, PDMS is poured into a custom made aluminum mold. (b) After baking, the PDMS is removed. (c) The PDMS is plasma bonded to a glass coverslip, creating a hollow rectangular channel between the PDMS and glass. A rod is inserted into channel. (d) The channel is filled with a solution form of rat tail collagen type I that encases the rod. (e) The collagen is then polymerized at room temperature and the rod is removed. (f) The channels are seeded with MDCK.2, MDCKII-w/t, or MDCKII-MDR1 cells and then perfused with growth media at low shear stress until microvessels are formed.

Fabrication. A custom-made aluminum mold was used to fabricate a PDMS housing with a rectangular channel. Inlet and outlet ports for connection to tubing and reservoirs at each end of the rectangular channel were formed using a hole punch (McMaster-Carr, Princeton, NJ). The PDMS housing was then bonded to a glass coverslip. A nitinol rod 150 µm in diameter (McMaster-Carr, Princeton, NJ) was located in the center of the channel. The rod defines the cylindrical channel in which the microvessel is formed. Prior to collagen introduction, devices and flow components were sterilized in an autoclave. The extracellular matrix was formed from a 7 mg mL^{-1} solution of rat tail type I collagen (BD Biosciences, San Jose, CA) using the manufacturer's recommended neutralizing protocol in 1N NaOH, 10X PBS, and distilled water.

Neutralized collagen solutions were injected into the rectangular channels. During collagen neutralization and injection, all solutions and devices were kept on ice. The collagen was then left at room temperature to form a gel around the template rod. The template rod was slowly removed, from the gelled matrix leaving behind a cylindrical channel connecting the inlet and outlet ports. Plastic adapters (removed from Precision Glide Needles, BD Biosciences) were inserted into the inlet and outlet ports, allowing for easy attachment and detachment of tubing. Next, syringe casings (cut from 20 mL syringes, BD Biosciences) that served as solution reservoirs were attached to the plastic adaptors at the inlet and outlet ports. A height difference of about 10 mm between inlet and outlet reservoirs resulted in a flow rate of about 5 $\mu L\ min^{-1}$, corresponding to a shear stress of 2 dyne cm^{-2} for a 150 μm diameter microvessel. For experiments at 9 dyne cm^{-2}, the syringe casings were replaced by petri dishes that could be raised and lowered to vary the flow rate. Cells were seeded into the channel at a density of approximately 10^7 cells mL^{-1}, and were perfused with culture medium at 37 ˚C and 5% CO_2 until fully confluent. The time from cell seeding to confluence was dependent on cell type, and ranged from 1 to 4 weeks.

Analysis of fluorescence intensity in the lumen and ECM. Fluorescence images for Lucifer yellow experiments were obtained at 10X magnification over a region of about 800 μm x 650 μm with the microvessel oriented at the center of the imaging window and the focal plane oriented at the vertical center of the microvessels. Fluorescence images for doxorubicin were obtained at higher magnification (20X) over a region of about 400 μm x 325 μm with the microvessel centered at the top of the imaging window to capture half the lumen and ECM. For analysis of the total intensity in the lumen and ECM, the background intensity was measured over a period of 5 - 10 minutes prior to introducing the solute. The background intensity (typically about 25% of the total intensity after injection of the solute) was subtracted from the measured intensity following introduction of the solute.

Analysis of fluorescence intensity in ECM. To obtain the fluorescence intensity in the ECM as a function of time, we defined a region of interest that excludes the lumen and cells (Figure 2h). The fluorescence intensity was plotted beginning with the first measurement after injection, corresponding to 20 s after injection of Lucifer yellow or doxorubicin. The ECM intensity from

the first measurement after solute injection was subtracted from the measured intensities, and plots were normalized to the lumen intensity after injection. Plots of the fluorescence intensity in the ECM as a function of distance from the cells are shown in Figure S2.

2. Using epifluorescence imaging for kinetic analysis

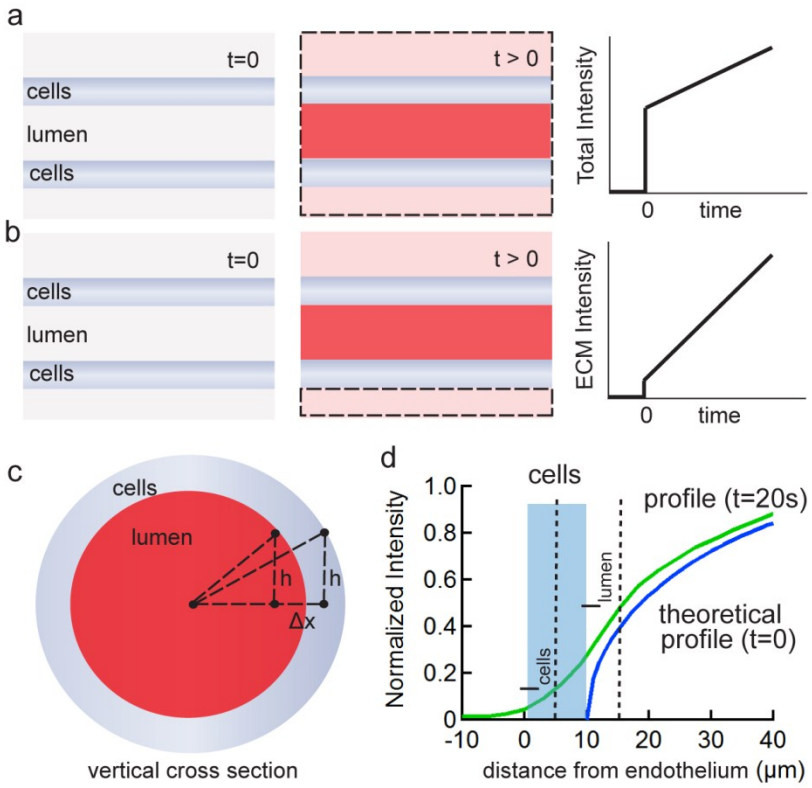

Figure S2: Consequences of epifluorescence imaging in cylindrical vessels. (a) Calculating permeability from total fluorescence. At t = 0, an increase in intensity occurs when a fluorescent solute is introduced into the lumen, followed by a linear increase as the solute permeates into the ECM at a constant rate. (b) When separately measuring the solute concentration in the ECM, a small increase in intensity occurs at t = 0 due to the small amount of scattering from the solute in the lumen, followed by a linear increase. (c) Schematic illustration showing the location for measuring the lumen intensity. Measuring the lumen intensity at a distance Δx from the center of the cells in the vessel wall ensures that the intensity is collected from the same volume in the lumen and cells. (d) Theoretical profile based on the projected intensity of Lucifer yellow in a 150 μm MDCKII-w/t vessel at t = 0 (blue line), and the actual profile at the first time point after injection (t = 20 s). Dotted lines show the position where the cell intensity is measured and the optimal position for measuring the lumen intensity (Δx = 10 μm).

Traditionally, permeability is calculated from epifluorescence measurements of total fluorescence intensity. At t = 0, a rapid increase in intensity occurs when a fluorescent solute is introduced into the lumen, followed by a linear increase as the solute permeates into the ECM at a constant rate while the concentration in the lumen remains constant (**Figure S2a**). When separately measuring the solute intensity in the ECM (**Figure S2b**), a small increase in intensity occurs at t = 0 due to a small amount of scattering of the fluorescence from the solute in the lumen, followed by a linear increase as the solute permeates into the ECM.

For detailed mechanistic analysis of transport processes, the solute concentration in the cells in the vessel wall must be normalized to the concentration in the lumen. However, due to the cylindrical geometry, the fluorescence intensity is dependent on the projected height of the lumen and hence is not constant throughout the lumen (**Figure S2c,d**). Therefore, in order to relate the concentration in the cells to the concentration in the lumen, it is important to measure the lumen intensity at the point along the lumen that contains the same volume as the cells. In Figure S2c, this point is located at a distance of Δx from the center of the cells, and is a function of the cell thickness (d), and vessel radius (r):

$$\Delta x = r - \frac{1}{2}\left(\sqrt{(\Delta r - 2r)(5\Delta r - 2r)} + \Delta r\right)$$

Note that when $\Delta r << r$, $\Delta x \approx \Delta r$.

For a 150 μm diameter vessel with a cell thickness of 10 μm, the relative cell intensity should be calculated using the lumen intensity at a distance of $\Delta x \approx 10$ μm from the center of the cells (**Figure S2d**). Figure S2d shows the theoretical epifluorescence intensity profile along the lumen of a 150 μm vessel with a cell thickness 10 μm (blue curve). In the absence of scattering the intensity should be equal to zero at the lumen-cell interface at t = 0. The actual intensity

profile of Lucifer yellow in an MDCKII-w/t vessel at the first time point after injection (t = 20 s) is also shown (green curve). Dotted lines show the position where the cell intensity is measured and the optimal position for measuring the lumen intensity (Δx = 10 μm). Assuming that all of the intensity in the cells at t = 20 s results from scattering, we can estimate an upper limit of the scattering error as ~20% of the measured lumen intensity.

3. Distributions of Doxorubicin and Lucifer Yellow in the ECM

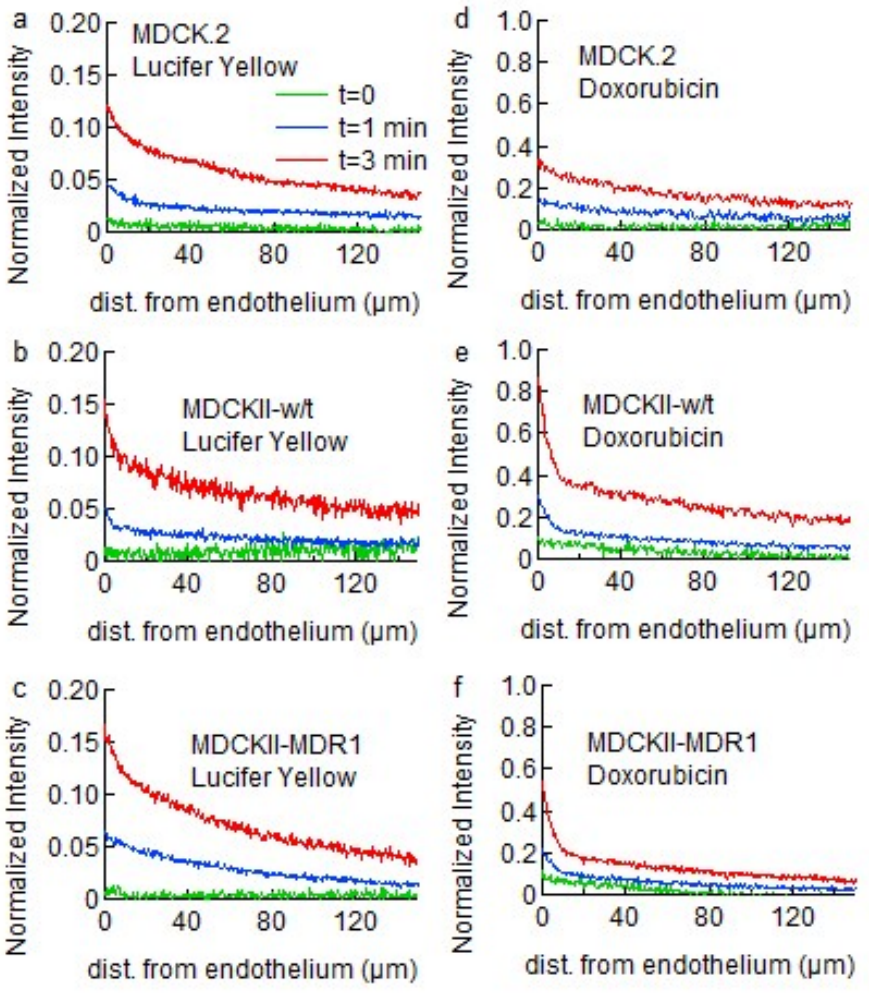

Fig. S3. Normalized intensity of doxorubicin and Lucifer Yellow in the ECM as a function of distance from the microvessel wall. (a-c) Normalized fluorescence intensity of Lucifer yellow as a function of distance from the cells at t = 0 (green), t = 1 min (blue), and t = 3 min (red) for MDCK.2, MDCKII-w/t, and MDCKII-MDR1 cells, respectively. (d-f) Normalized fluorescence

intensity for doxorubicin as a function of distance from the cells at t = 0 (green), t = 1 min (blue), and t = 3 min (red) for MDCK.2, MDCKII-w/t, and MDCKII-MDR1 cells, respectively. Experiments were performed using 100 μM Lucifer yellow and 100 μM doxorubicin.

4. Preparation of transwell samples for analysis with HPLC

200 μL of acetonitrile and 1 μL of daunorubicin (standard) was added to the doxorubicin samples and vials were vigorously rocked for 10 min. Samples were then centrifuged for 10 min at 8000 rpm and the supernatant was extracted. This supernatant was subsequently dried under flow of argon in a water bath at 37 °C. When the samples appeared dry, they were placed in a vacuum chamber for 3 h – overnight to confirm removal of liquid. The samples were then re-suspended in 30% methanol, 70% water. These samples are run at 1 mL min^{-1} using 70% methanol and 30% water with 0.1% formic acid through a C18 BDS Hypersil column.

6. Lucifer Yellow Permeability in PDMS Microvessels

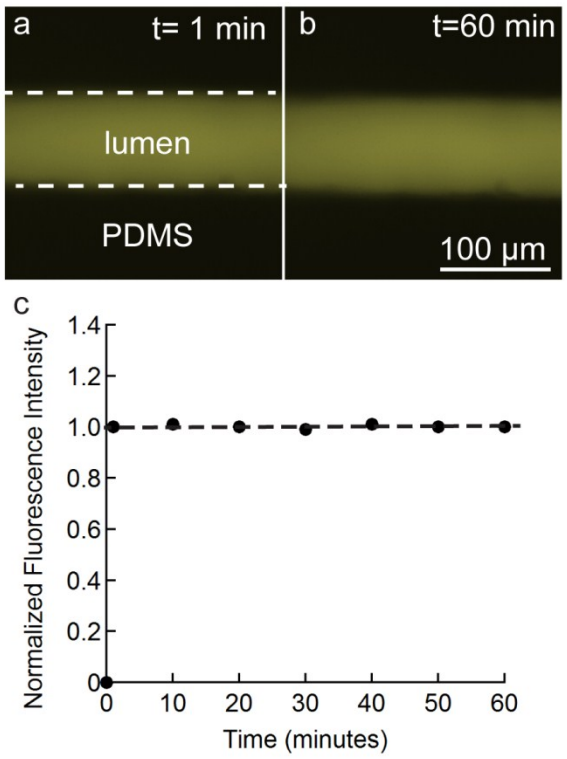

Figure S4. Lucifer Yellow permeability in PDMS microvessels (a,b) Fluorescence images of Lucifer Yellow in cylindrical PDMS tubes at 1 and 60 minutes respectively. (c) The normalized fluorescence intensity in the microvessels after injection of Lucifer yellow. The fluorescence does not increase, confirming that the permeability is zero.