Supporting Information for Albrecht, Underhill, et al.

Supplemental Materials and Methods

Cell Culture. HeLa and PtK2 cell lines harboring fluorescent reporters were generated as previously described. HeLa and PtK2 cells were maintained at 37°C in a modified CO2-independent medium containing Leibovitz’s L-15 Medium with L-glutamine (Invitrogen, 21083027) supplemented with 4.5 mg mL\(^{-1}\) glucose, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 100 U mL\(^{-1}\) penicillin, 100 µg mL\(^{-1}\) streptomycin, 10 mM HEPES buffer, and 10% fetal bovine serum (FBS). For cell culture maintenance outside of the microfluidic array, 10 µg mL\(^{-1}\) phenol red was added to monitor pH.

Mouse ES cells were maintained in 5% CO\(_2\) at 37°C. The Oct4/EGFP reporter mouse ES cell line and the H2B-EGFP fusion mouse ES cell line were provided by Dr. Douglas Melton’s laboratory (Harvard University) and cultured on mitomycin-C growth arrested mouse embryonic fibroblast (MEF) feeder layers in Knockout-DMEM (GIBCO) media supplemented with 15% ES-grade fetal bovine serum (Millipore), 2 mM L-glutamine (GIBCO), 1 mM nonessential amino acids (GIBCO), 1.1 mM β-mercaptoethanol (Sigma), 1 × penicillin/streptomycin (GIBCO), and 1000 units mL\(^{-1}\) LIF (ESGRO, Millipore) and passaged every 2-3 days. For feeder-free adaptation, ES cells were passaged into 0.1% gelatin coated plates with sequential 2-fold reductions in MEF density, in ES media containing LIF.

Fabrication of Microfluidic Devices. The microwell array was fabricated in poly(dimethylsiloxane) (PDMS), cast from mold masters prepared by photolithography. The photoresist SU8-50 (Microchem) was spin-coated on cleaned 4” silicon wafers for 30 s at 2000 rpm for a 50 µm thick layer. The wafer was softbaked (65°C for 6 min and 95°C for 20 min), and then placed into soft contact with a high-resolution transparency photomask (5080 dpi, Pageworks) and exposed to UV light (365nm, 300 mJ cm\(^{-2}\)). Following a hardbake to complete crosslinking (65°C for 1 min and 95°C for 5 min), the wafer was allowed to cool and developed in SU8 developer (Microchem). For 100 µm thick features, spin speed decreased to 1000 rpm, UV exposure increased to 450 mJ cm\(^{-2}\), and 95°C softbake and hardbake times extended to 30 min and 10 min, respectively. The silicon/SU8 mold masters were then replicated to form several monolithic plastic masters using a casting method described elsewhere.

Once mold masters were fabricated, PDMS (Sylgard 184; Dow Corning) was prepared by mixing the PDMS prepolymer and crosslinker in a 10:1 ratio, and degassing for 1 h to remove air bubbles. PDMS was poured into the mold masters to a depth of 5 mm and cured at 65°C for 3 h. Holes for medium reservoirs and tubing connections were cored using a 2.5 mm dermal punch (AccuDerm). Next, the patterned PDMS block was irreversibly bonded to a 35 x 50 mm #1 glass coverslip (Electron Microscopy Sciences, #63771-01, pre-cleaned) using an oxygen plasma system (PlasmaPreen; Terra Universal). Before bonding, glass surfaces were cleaned and activated with 1 min plasma exposure (150 watts, 750 mTorr at 2 L min\(^{-1}\) 21% oxygen flow),
whereas PDMS surfaces were activated for 7 s at 5 watts. Bonded devices were baked at 65°C overnight to improve bonding strength and stabilize material properties.

Devices utilized in the ES cell studies were fabricated in a similar manner with the following modifications. The SU8-50 photoresist thickness was increased to 100 µm resulting in a doubling of the height of the device channels, and the PDMS casting depth was reduced to 1 mm. A second layer of bulk, unpatterned PDMS (9 mm thickness), containing a centered 1 cm diameter cut-out section corresponding to the microwell imaging region, was oxygen plasma bonded on top of the previously cast 1 mm device layer prior to bonding to the glass coverslip. This layer enabled cell loading of inlets and provided support for tubing connections, while maintaining a minimal thickness of PDMS above the microwell region to promote adequate gas transfer (ESI Fig. 6).

**Cell Seeding in Imaging Devices.** Microfluidic devices were sterilized by wiping external surfaces with 70% ethanol and exposing them to UV-C radiation for 1 h in a tissue culture hood. Devices were placed in a vacuum desiccator for 5 min before filling to eliminate air bubbles after fluid loading and to remove residual byproducts of sterilization (e.g., ethanol and UV-generated ozone). Next, 20 µL warmed culture medium was added to each inlet reservoir and briefly aspirated through the fluidic network. Medium-loaded devices were incubated at 37°C for ≥8 h to provide a cell-adhesive surface via adsorption of serum proteins to the glass substrate. Devices for ES cell experiments were incubated at 37°C for ≥8 h with a 0.1% gelatin solution prior to medium incubation.

For cell line studies, cell suspensions were prepared by trypsinization of PtK2 or HeLa cells from culture flasks and passage through a 40 µm cell strainer to remove cell clumps. Each inlet well was emptied of medium and loaded with 5 µL cell suspension (15 x 10⁶ mL⁻¹), periodically mixing the reservoir to maintain a uniform suspension density. After cells have passed though the fluidic network (< 1 min), both inlet and outlet wells were quickly emptied and refilled with 12 µL medium. By balancing medium volume in the inlets and outlet, fluid flow in the microfluidic network ceased, allowing cells to settle onto the glass microwell surface and attach. Seeded devices were placed in a 37°C incubator for several hrs to overnight for complete cell attachment.

For the establishment of MEF-ES co-cultures within the device, mitomycin-C growth-arrested MEF cells were passed through a cell strainer following trypsinization, and loaded into 0.1% gelatin coated devices at a density of 2 x 10⁶ mL⁻¹ using the technique described above. Following an attachment period of 2.5 h, fresh medium was added to the device, and the seeded device was incubated overnight at 37°C. The following day, mouse ES cells were loaded at 2 x 10⁶ mL⁻¹ into devices containing MEF feeder layers, and similarly allowed to adhere for 2.5 h prior to the addition of fresh ES medium. A subsequent 2 h incubation at 37°C was performed prior to the attachment of tubing connections for medium perfusion. For feeder-free ES cell experiments, ES cells adapted to feeder-independent culture were loaded at 5 x 10⁶ mL⁻¹ into 0.1% gelatin coated devices and allowed to adhere for 2.5 h prior to medium change.
Shear Forces during Medium Exchange. The miniaturization of cell culture from large plates to microwells may incur a penalty of diminished nutrient transfer and physical perturbation via shear flow. Cells in microwells are surrounded by a medium volume about 1-2 orders of magnitude lower than in standard tissue culture. Thus, microwells require more frequent medium replenishment or continuous flow. The small channel dimensions also increase shear forces during flow, affecting cell fate and function at levels around 1 – 10 dyne cm$^{-2}$ depending on cell type. For a given flowrate, shear stress on adherent cells decreases by chamber height squared, such that doubling microwell height allows ~4-fold greater medium flow with the same applied shear stress.

To understand how shear forces and nutrient exchange vary with microwell height and medium perfusion rate, we analyzed a finite element model of a single microwell (Fig. 1C) using FEMLAB 3 (COMSOL). Supplemental Table 1 lists model calculations for a 1 Pa pressure drop across a 50 or 100 µm tall microwell. While shear stress at the cell attachment surface is nearly equivalent for both microwell heights given the same pressure drop, we find that shear stress at a given flowrate is 3.5 times lower for the taller microwells. During a manual medium change (Fig. 1E), flow increases rapidly when medium is added into the inlet reservoir and decreases exponentially as inlet and outlet reservoir volumes equilibrate. By measuring outlet volume over time, we calculated the initial flowrates and exponential time constants for both microwell heights (ESI Table 1). Using these parameters, we estimated the maximum shear stress and medium exchange rates for several flow protocols (ESI Table 2).

We cultured PtK2 and HeLa cells in 50 µm tall devices with manual medium changes every 12 h. This perfusion rate corresponds to 0.25 nL cell$^{-1}$ day$^{-1}$ for a microwell containing 30 cells, similar to standard bulk tissue culture of these cells (~0.3 nL cell$^{-1}$ day$^{-1}$). We estimate a maximum 0.33 dyn cm$^{-2}$ shear stress during manual feeding, below typical limits for cell perturbation, and no adverse effects on viability or cell division were noted in either cell type.

Embryonic stem (ES) cells are more metabolically active and require more frequent medium changes than PtK2 or HeLa cells, and they showed diminished survival under these feeding conditions. Therefore, we made a series of modifications to the device and feeding protocol to balance nutrient exchange and shear stresses, outlined in Supplemental Table 2. First, the microwell height was doubled to 100 µm to increase the local medium volume per microwell, and the thickness of PDMS was reduced to 1 mm to increase oxygen diffusion to the cells (ESI Fig. 6). Next, we increased the rate of medium exchange to ES cultures by connecting a syringe pump to automatically perfuse fresh medium at faster intervals. Quick medium exchanges (8 µL min$^{-1}$ for 1 min) at 4 h intervals also resulted in declining ES cell viability and proliferation, either due to elevated shear stresses during the rapid but brief flow, or by still insufficient nutrient exchange. Similar results were seen with longer, slower medium flow (24 µL h$^{-1}$ for 1 h every 4 h), despite a 20-fold decrease in shear stress. However, a continuous flow of 1 µL h$^{-1}$ provided a large volume of medium per cell at a very low shear (~0.001 dyn cm$^{-2}$)
and maintained long-term ES cell viability and proliferation. These conditions were used for all subsequent ES cell experiments.

Despite their shear sensitivity, ES cells were initially introduced into the devices by manual pipetting, just as for seeding HeLa and PtK2 cells. We used this cell loading method for convenience, as shear stresses are minimal on flowing cells prior to attachment.

**Medium Changes.** For manual medium changes, all reservoirs were emptied and prewarmed culture medium was added to one reservoir per fluidic circuit. Fresh medium flowed though the fluidic network by gravity until >3 network volumes (1 – 3 µL each) passed. All reservoirs were then emptied and refilled with fresh medium.

To remotely perfuse medium without disturbing the microfluidic device during long time-lapse experiments, media-filled containers (cut-off syringes) and tubing were attached to the reservoirs (Fig. 1e, right panel). Sterile wide-bore (1/16” ID) tubing interfaced with medium reservoirs on the microfluidic device via metal tubing (12 gauge heavy wall, 12.5mm; New England Small Tube). Bubble-free connections were made by overfilling each reservoir with medium and ensuring a small drop protruded from the metal fitting before insertion into the reservoir. In some experiments, sterile vacuum grease was applied to the metal fitting to ensure a leakproof seal.

For continuous perfusion, a syringe pump (Chemyx Fusion 200) pulled culture medium through the microwell array network. To minimize any pulsatile flow from the syringe pump, we used small 1 mL syringes (to increase the pump motor step rate) and wide-bore tubing (to increase compliance and damping). Smooth flow was observed even at low flowrates (1 µL h⁻¹).

**Automated image analysis.** The high-throughput quantification of metaphase nuclei and total nuclei area was performed utilizing an image analysis pipeline developed with CellProfiler™ open-source software⁵. The pipeline consisted of the following features. First, individual image frames from the time-lapse acquisition were loaded, rescaled, and masked based on a thresholding algorithm. The total area of the masked region was then quantified as a measure of total nuclei area. For identification of metaphase nuclei, fluorescent objects within a typical diameter range and above an intensity threshold were identified, and then further filtered based on 7 object intensity and shape measurements (maximum intensity, mean intensity, area, perimeter, form factor, solidity, and eccentricity). To track individual cell division events, we quantified the number of metaphase nuclei per frame, or per series of frames. Since a single metaphase nucleus can span several sequential time-lapse frames, it was important to count such events only once. To do so, the x-y positional coordinates of metaphase nuclei were identified, and those spatially registered with contiguous prior frames were not counted.
References

Supplemental Movie Captions

Supplemental Movie 1. Near-simultaneous parallel imaging of multiple cell types and soluble conditions in a single multiplexed time-lapse experiment. Montaged fluorescent image sequence demonstrating the proliferation of H2B- and tubulin-labeled PtK2 cells exposed to NZ (red labels) or DMSO control (green) conditions in 24 distinct microwells. Images were acquired at 40X magnification, at 5 min intervals, for 54 h. Scale bar, 100 μm. All movies are encoded with the XviD codec, available at [http://www.xvidmovies.com/codec](http://www.xvidmovies.com/codec).

Supplemental Movie 2. Near-simultaneous parallel imaging of embryonic stem cell proliferation within multiple microwells. Montaged fluorescent image sequence demonstrating the time lapse analysis of H2B-EGFP mouse ES cell proliferation under self-renewing conditions (+LIF, MEF feeder layer) for 24 distinct microwells in parallel. Images were acquired at 20X magnification, at 3 min intervals, for 60 h.

Supplemental Movie 3. Proliferation of embryonic stem cells with MEF feeder cells within microfluidic culture. Overlaid fluorescent and phase contrast image sequences for a single example microwell selected from ESI Movie 2. Images were acquired at 20X magnification, at 3 min intervals, for 60 h.
**Supplemental Table 1. Fluid flow and shear stress calculations**

<table>
<thead>
<tr>
<th>FEM model calculations(^{(a)})</th>
<th>(h)</th>
<th>50</th>
<th>100</th>
<th>(\mu m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microwell height</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flowrate</td>
<td>(Q)</td>
<td>0.065</td>
<td>0.235</td>
<td>(\mu L \cdot h^{-1})</td>
</tr>
<tr>
<td>Max. velocity(^{(b)})</td>
<td>(V_{\text{max}})</td>
<td>2.59</td>
<td>5.26</td>
<td>(\mu m \cdot s^{-1})</td>
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<tr>
<td>Shear rate at wall(^{(b)})</td>
<td>(\dot{\gamma})</td>
<td>0.207</td>
<td>0.210</td>
<td>(s^{-1})</td>
</tr>
<tr>
<td>Shear stress at wall(^{(b)})</td>
<td>(\tau_w)</td>
<td>0.00143</td>
<td>0.00145</td>
<td>(\text{dyn cm}^{-2})</td>
</tr>
<tr>
<td>Shear stress per flowrate</td>
<td>(\tau_w/Q)</td>
<td>0.022</td>
<td>0.006</td>
<td>(\text{dyn cm}^{-2} \text{ per (\mu L \cdot h^{-1})})</td>
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</table>

<table>
<thead>
<tr>
<th>Flow following manual medium change(^{(c)})</th>
<th>(Q_0)</th>
<th>8.9</th>
<th>33.0</th>
<th>(\mu L \cdot h^{-1})</th>
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</thead>
<tbody>
<tr>
<td>Initial flowrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time constant</td>
<td>(\tau)</td>
<td>203</td>
<td>55</td>
<td>(s)</td>
</tr>
</tbody>
</table>

**Notes:**

(a) Finite element models performed using FEMLAB (Fig. 1c), using the following parameters: viscosity, 0.6915 cP; density, 1 g cm\(^{-3}\); pressure across microwell, 1 Pa; no slip boundary condition on all walls.

(b) In center of microwell.

(c) Medium flowrate (\(Q\)) following manual filling of the inlet reservoir declines exponentially according to: \(Q = Q_0 \exp\left(-t/\tau\right)\). Parameters were estimated from measurements of outlet reservoir volume over time.
### Supplemental Table 2.
Estimated shear stress and medium exchange for various cell culture conditions

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Well height</th>
<th>Medium flow</th>
<th>Max. circuit flowrate</th>
<th>Flow duration</th>
<th>Medium change interval</th>
<th>Max. shear stress (c)</th>
<th>Flow duty cycle</th>
<th>Min. medium exchange rate (d)</th>
<th>Cellular effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtK2, HeLa</td>
<td>50 manual 10 µL (a)</td>
<td>89 (b)</td>
<td>3.4 (b)</td>
<td>12</td>
<td>0.33</td>
<td>~0.5%</td>
<td>0.24</td>
<td>normal division</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 manual 10 µL (a)</td>
<td>330 (b)</td>
<td>0.91 (b)</td>
<td>12</td>
<td>0.34</td>
<td>~0.1%</td>
<td>0.24</td>
<td>declining survival &amp; proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 manual 10 µL (a)</td>
<td>480</td>
<td>1</td>
<td>4</td>
<td>0.50</td>
<td>0.4%</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 syringe pump</td>
<td>24</td>
<td>60</td>
<td>4</td>
<td>0.025</td>
<td>25%</td>
<td>1.0</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>µm</th>
<th>µL h⁻¹</th>
<th>min</th>
<th>h</th>
<th>dyn cm⁻²</th>
<th>nL cell⁻¹ day⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 continuous</td>
<td>0.001</td>
<td>100%</td>
<td>16.7</td>
<td>normal division</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**

Values in bold type indicate parameters used for experimental data in this report.

(a) Medium volume added to inlet reservoir, as in Fig. 1E.

(b) Medium flowrate \( Q \) following manual filling of the inlet reservoir declines exponentially according to: \( Q = Q_0 \exp(-t/\tau) \). For manual medium changes, maximum flowrate is listed as \( Q_0 \), and exchange duration is listed as the exponential time constant, \( \tau \).

(c) For these experiments, each fluidic circuit fed 6 parallel microwell channels. The maximum microwell shear stress is calculated using a microwell flowrate 1/6 of the circuit flowrate.

(d) The rate of medium exchange is elevated during perfusion and lowest during static conditions. For periodic perfusion, the minimum exchange rate is measured as the average medium volume accessible during static conditions per medium change interval per cell:

\[
Q_{\text{min}} = Q_{\text{static}} = \frac{V_{\text{well}}}{I (1 - D_{\text{flow}}) N_{\text{cell}}}
\]

where \( V_{\text{well}} \) is microwell volume, \( I \) is the medium change interval, \( D_{\text{flow}} \) is the duty cycle of the flow period, and \( N_{\text{cell}} \) is the number of cells per microwell. For continuous perfusion, the medium exchange rate is constant:

\[
Q_{\text{flow}} = \frac{Q_{\text{well}}}{N_{\text{cell}}}
\]

where \( Q_{\text{well}} \) is the medium flowrate per microwell.

These calculations assume, as a typical upper limit, \( N_{\text{cell}} = 30 \) cells per well (PtK2, HeLa) or \( N_{\text{cell}} = 60 \) cells per well (ES + MEF).