Supplemental Methods and Figures

Aggregate size and growth rate analysis from phase contrast images

Aggregate sizes were quantified from phase contrast images. Images were manually segmented in ImageJ and the cross sectional area of each aggregate was measured. The aggregate radius was calculated from the cross sectional area, assuming each cross section to be circular and using the formula: $radius = \sqrt{\frac{area}{\pi}}$.

Growth rate was defined as change in radius between D1 and D4, normalized by the initial radius. This metric was calculated on a population-averaged basis, e.g.

$$Growth\ rate_{device\ 1} = \frac{mean\ D1\ radius_{device\ 1} - mean\ D4\ radius_{device\ 1}}{mean\ D1\ radius_{device\ 1}}$$

We chose to calculate this on a population averaged basis due to the inability to measure the size of a single batch aggregate on both D1 and D4.

Fluorescent image analysis

For each aggregate, a single two-channel *z* slice approximately 15 µm in from the surface was analyzed using custom-written MATLAB code. The two-channel image was separated into individual single-channel images, corresponding to the nuclear stain (Hoechst) and Oct4 antibody staining. As Oct4 is localized to the cell nucleus, we segmented nuclei in order to quantify Oct4 expression for each cell. To segment the nuclei, we used a modified version of a method developed by Lou, et al.[1] Starting from the greyscale nuclear stain image, contrast adjustment was performed as a preprocessing step. Next, we applied a Gaussian filter to the image, calculated the Hessian matrix of the resulting image, and calculated the first eigenvalue of the Hessian matrix. Then, a threshold was applied to the first eigenvalue of the Hessian matrix to eliminate dim pixel values that would otherwise result in poor segmentation. The resulting image was used as a mask to segment the contrast adjusted greyscale nuclei image. This typically yielded slightly under-segmented nuclei, so a watershed transform was applied to the masked greyscale nuclei to yield a final, binary image of segmented nuclei. The image of segmented nuclei was then used to identify and segment nuclei in both the nuclear and Oct4 individual greyscale images.

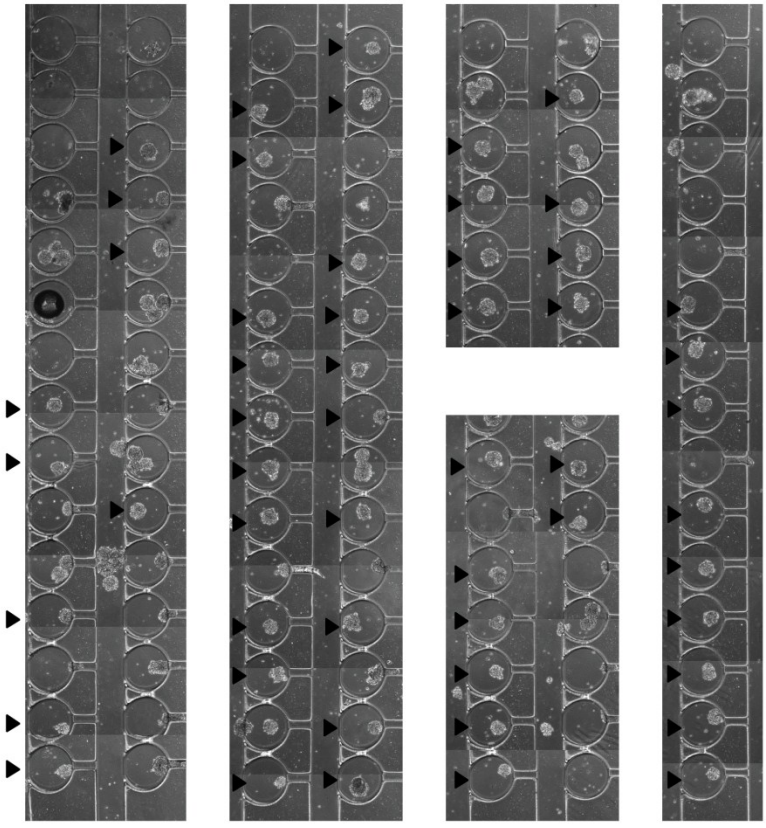
To quantify Oct4 expression for each cell, the mean Oct4 pixel intensity was calculated and normalized by the mean Hoechst pixel intensity for the same cell. This yielded a metric for cellular Oct4 expression (Ratio of Oct4/Hoechst). To obtain a metric for mean Oct4 expression for a single aggregate (Ratio of Oct4/Hoechst), the cellular metric was averaged for all cells in the aggregate.

The coefficient of variation (CV) was used to describe the heterogeneity in Oct4 expression among cells within a single aggregate or among aggregates within a culture

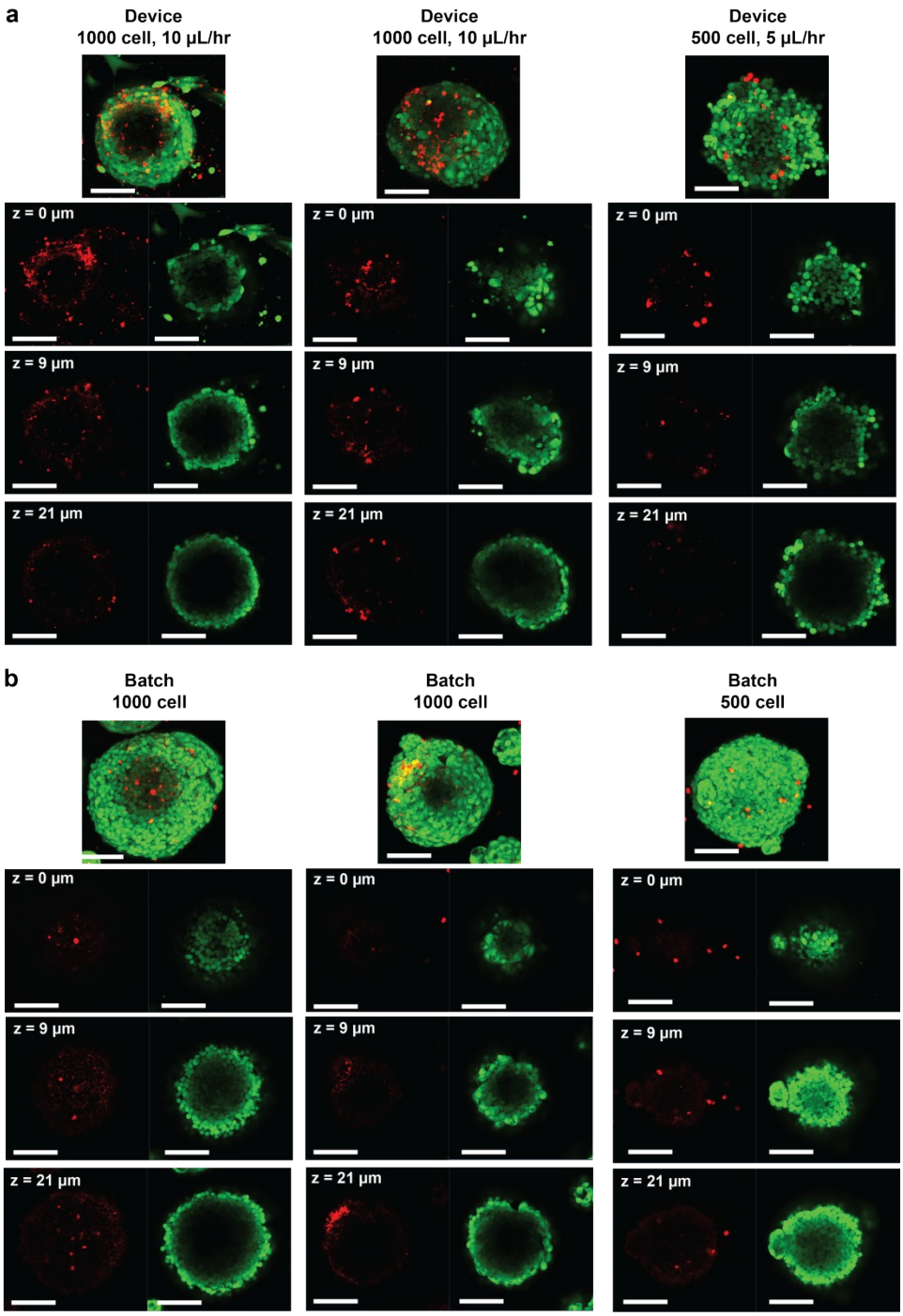
platform. In the former case, the CV was defined as the standard deviation in cellular Oct4 expression for a given aggregate, divided by the mean value for all cells in that aggregate. In the latter case, the CV was defined as the standard deviation in aggregate Oct4 expression for all aggregates in a given culture platform, divided by the mean value for all aggregates.

Peclet number calculation

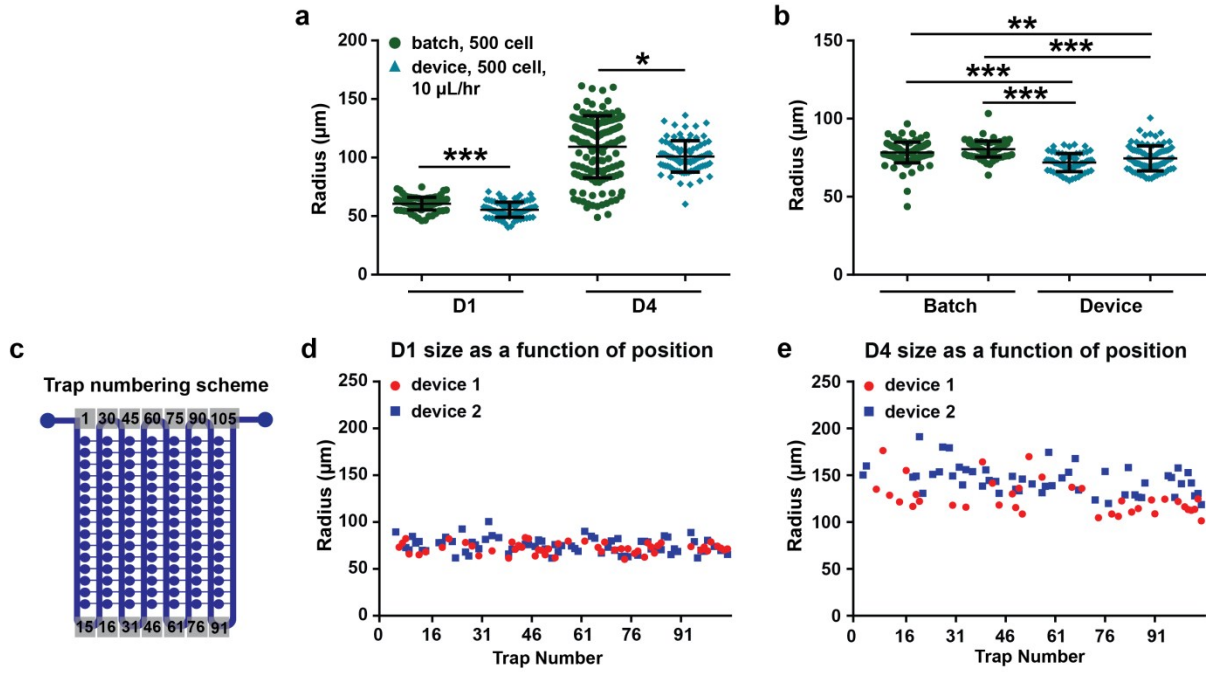
We calculated the Peclet number (Pe) using the equation $Pe = \frac{Lv}{D}$ where L is the characteristic length, v is the fluid velocity, and D is the diffusion coefficient. We assumed L to be the width of the main channel (300 µm). We calculated v ~ 30 µm/s using the relationship $v = \frac{Q}{A}$ where Q is the volumetric flow rate through the device (10 µL/hr) and A is the cross-sectional area of the main channel ((300 µm)^2). We assumed D ~ 10^{-6} cm^2/s for a small molecule.[2] Using these values yielded Pe ~ 90.



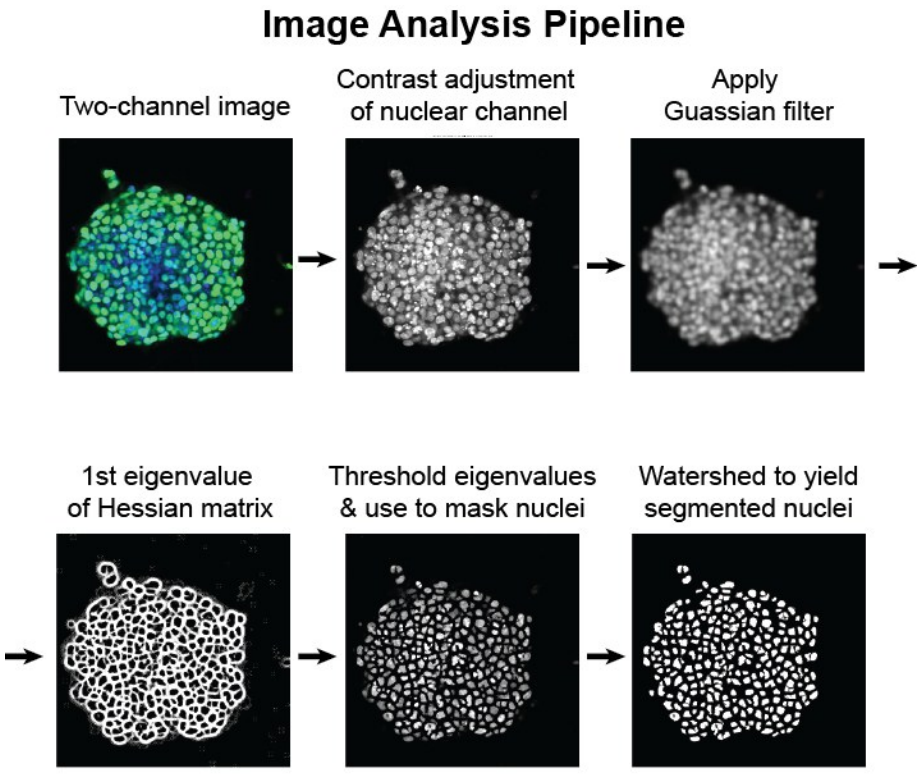
Supplemental Figure 1. *Stitched representative image of a device loaded with 1000 cell aggregates. Black arrows indicate singly loaded traps.*



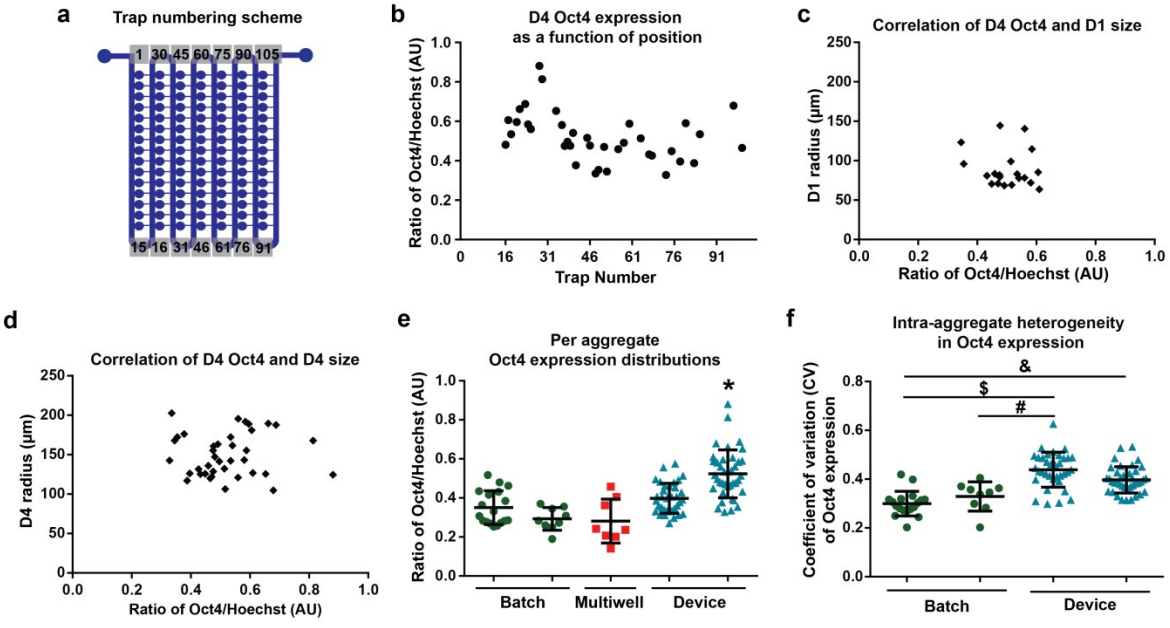
Supplementary Figure 2. Additional viability data. Representative confocal images of day 4 aggregates treated with a LIVE/DEAD stain (live cells: green; dead cells: red). For each condition, the top image is a maximum intensity projection of all z slices imaged for a given aggregate. Underneath, three z slices are shown. a) Images for both 1000 cell starting size aggregates cultured at 10 µL/hr and for 500 cell starting size aggregates cultured at 5 µL/hr. b) Images for 100 cell starting size and 500 cell starting size aggregates cultured in batch. All scale bars: 100 µm.



*Supplementary Figure 3. a) Device culture reduces variability in size for 500 cell starting size aggregates. Aggregate radii were quantified from phase contrast images at day 1 and day 4 of differentiation for 500 cell aggregates cultured under 10 µL/hr media perfusion). Two independent samples are shown for each condition, with n ≥ 100 for batch samples and n ≥ 85 for device samples. * P < 0.01; *** P < 0.0001. b) Size distributions of aggregates captured in devices are similar to batch aggregates. Two replicates per condition are shown for 1000 cell aggregates (device culture: 10 µL/hr media perfusion). ** P < 0.001; *** P < 0.0001 c) Schematic of trap numbering convention used in c and d. c,d) Aggregate radii is plotted as a function of position within the device, for 1000 cell aggregates cultured under 10 µL/hr perfusion. Two devices are shown for days 1 and 4 of differentiation.*



Supplementary Figure 4. Image processing pipeline used to analyze fluorescent images modified from Lou, et al.[1]



Supplementary Figure 5. a) Schematic of trap numbering convention used in b. b) Mean aggregate Oct4 expression is plotted as a function of position within the device. One device is shown. Performing linear regression on this data produced a line of slope -0.002 and an R^2 value of 0.141, indicating no trend between Oct4 expression and position.

*c,d) Mean aggregate Oct4 expression is plotted as a function of aggregate radius at day 1 (c) and day 4 (d). e) Scatter plot shows mean Oct4 expression for individual aggregates cultured within batch, multiwell, or device platforms (raw data corresponding to Figure 5b). Two independent experiments are shown for batch and device conditions (n ≥ 25 each) and one experiment is shown for multiwell condition (n = 8). * P < 0.00001 vs. both batch and multiwell samples, P < 0.0001 vs. other device sample. f) Intra-aggregate heterogeneity in Oct4 expression for device and batch platforms. Two independent experiments are shown for batch and device conditions (n ≥ 25 each). # P < 0.001, $ P < 0.00001, & P < 0.0001.*

References

1. X. Lou, M. Kang, P. Xenopoulos, S. Munoz-Descalzo and A. K. Hadjantonakis, *Stem Cell Reports*, 2014, **2**, 382-397.
2. F. Wang, H. Wang, J. Wang, H.-Y. Wang, P. L. Rummel, S. V. Garimella and C. Lu, *Biotechnology and Bioengineering*, 2008, **100**, 150-158.