High-throughput analysis of single hematopoietic stem cell proliferation in microfluidic cell culture arrays


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Note: Supplementary Software 1 and Supplementary Videos 1 and 2 are available on the Nature Methods website.
Supplementary Figure 1. Schematic of the iso-osmotic microfluidic cell culture array. The iso-osmotic bath is filled with medium and pressurized by gravity to avoid formation of air bubbles. If necessary, the bath content can be replaced by removing the plug and introducing fresh medium from the syringe. The array inlet and outlet are pressurized by air and the control lines are connected to solenoid actuators.
Supplementary Figure 2. Flow and shear stress profiles during cell loading. (a) The velocity magnitude (m s\(^{-1}\)) during cell loading is modeled for a total flow rate of 1 \(\mu\)l min\(^{-1}\). The maximum velocity in the flow channels does not exceed 1.2 \(\times\) 10\(^{-3}\) m s\(^{-1}\). (b) For the same conditions, the shear stress (Pa) on the flow channel walls is modeled. The maximum shear stress exerted on the cells is 0.26 Pa.
Supplementary Figure 3. Cell immobilization during medium exchange. (a) Manual cell counts (ND13 cells) in individual chambers before and after medium exchange (2 µl min\(^{-1}\) for 10 min) remained similar (slope=1.02, \(R^2=0.998\)). (b) Variations in clone size after medium exchange. The time between both image sets was approximately 15 minutes (5 min acquisition + 10 min perfusion). In this timeframe, it is frequent to see cells dividing or die. Small differences are due to cell death or division and not to the addition or loss of cells. (c) The shear exerted on the cells while at the bottom of the chamber during medium exchange is calculated for a total flow rate of 2 µl min\(^{-1}\). The maximum shear stress does not exceed 3.1 \(x\) 10\(^{-4}\) Pa.
Supplementary Figure 4. Recovery of selected clones. (a) Individual clones can be recovered from individual chambers by delaminating the cover layer of the device and transpiercing the membrane with a micropipette. (b) The efficiency of recovery was measured by counting the number of cells in individual chambers and recounting the number of cells that were successfully transferred in a well. On average, 91% of the cells from individual colonies of different sizes could be recovered (slope= 0.983, R2=0.998). (c) Cell losses from recovery ranges from 0 to 5 cells per clone.
Supplementary Figure 5. Cell death in the absence of medium exchange is inversely correlated to the number of cells per chamber. Nanovolume chambers at different seeding density were isolated with no medium feeding and no iso-osmotic bath in batch mode. As expected, due to nutrient limitation and metabolite accumulations, the maximum cell concentration was inversely correlated with the initial number of cells in each chamber.
Supplementary Figure 6. Mature myeloid population derived from lineage negative ND13 cells. ND13 cells were stained for Gr-1, Mac-1 and B220 and sorted by flow cytometry. The lin⁻ fraction was cultured for 9 days and gave rise to a new lin⁺ population.
Supplementary Figure 7. Microfluidic cell culture array for temporal stimulation and parallelization of experiments. This microfluidic cell culture array contains 6,144 chambers and can support up to 8 different conditions simultaneously. Two populations of cells can be loaded from the left side of the array using the pump located downstream of the array. The multiplexer can be used to direct cells in specific rows and obtain a more even distribution of cells across the different conditions. Up to 6 different medium conditions can be loaded from the right side of the array. Only the top half of the array was used to study murine HSCs due to the relatively small cell numbers. The 6 different conditions were distributed across the array as shown. Medium was automatically exchanged every 2 hours for each condition.
Supplementary Figure 8. Individual growth curves of primary murine HSCs under different Steel factor exposure conditions. Growth curves were generated using the enhanced bifocal image analysis algorithm. The analysis was started after 21 hours to allow small quiescent cells to reach a suitable size for detection by image analysis.
**Supplementary Figure 9. Medium exchange control.** The microfluidic cell culture array was loaded with medium supplemented with PE-TexasRed-streptavidin and the inlet was replaced by medium only. Pictures of the last 3 columns of the array were taken during perfusion and fluorescence intensity was quantified with Image J. This experiment shows that perfusing 4-fold the volume of the array (26 µl) is sufficient for complete medium exchange. Each data point represents the average of 9 wells and error bars represent the standard deviation.
Supplementary Figure 10. Automated image analysis algorithm for cell quantification. (a) Cells segmentation scripts were written in MATLAB (MathWorks). Segmentation was accomplished through three main steps: chamber segmentation (A-E), cell-containing region segmentation (F-J), and then single cell isolation (K-O). First, the individual chambers are segmented from the image background. This step of the segmentation is accomplished by applying a bandpass filter (B) and then creating a binary image through an automatically determined threshold (C). The resulting binary image is enhanced by removing objects touching the image borders and suppressing noise by removing small objects (D). Finally, the chambers are segmented from the rest of the background by filling in the holes created by the edges of the chambers. Next, the regions containing cells are separated from the rest of the chamber. This is achieved by first applying a local standard deviation filter to enhance the highly variable regions (G). The noise in the filter response is then suppressed by removing small regions, and this result is converted into a binary image through an empirically determined threshold (H). Any holes in this result are then filled in to create the final region mask (I). To segment the individual cells from the rest of the group, a bandpass filter is applied to the output of a local standard deviation filter applied to the image (K). A top hat filter is then used to enhance the edges (L), and the bounded regions are subsequently filled (M). This result is then converted to a binary image using an automatically determined threshold, and further enhanced by removing small objects (N). (b) Comparison between automated and manual cell counts. The straight line represents the 1:1 slope. Deviations at higher cell numbers are caused by the shadow around the edges some chambers. The enhanced bifocal algorithm can correct this error.
Supplementary Figure 11. Bifocal image analysis algorithm. (a) Comparison between automated and manual cell counts. The straight line corresponds to a linear least square regression. (b) Absolute differences between the algorithm and manual counts.
Supplementary Note 1

Wafer Fabrication Protocol

Each new microfluidic design is created with a drawing software such as AutoCAD. A micro-pump is located downstream of the array to avoid crushing the cells and control the speed during the loading process. Depending on the application, microfluidic cell culture arrays can contain from 1,600 to 6,144 chambers in the order of ~4 nl each. Multiplexers, isolation valves and hydration lines can be added when necessary to offer a better control of the microenvironment. Designs are printed at 20,000 dpi on transparent masks. The fabrication of molds on a silicone substrate is performed using common photolithography techniques as described below.

Flow wafer

Flow channels
1. Dehydrate a wafer for 10-15 minutes at 150 °C.
2. Treat the wafer with vapor phase HMDS for at least 2 minutes.
3. Pour SPR220-7.0 resist on half the diameter of the wafer.
4. Ramp at 500 rpm for 10 seconds, then spin at 1,500 rpm for 90 seconds.
5. Pre-bake the wafer at 115 °C for 120 seconds.
6. Expose for 30 s.
7. Wait 30 minutes to rehydrate the resist.
8. Develop in MF319 primary bath for around 5-10 minutes, then rinse in an MF319 secondary bath.
9. Rinse with DI water and dry the wafer with compressed nitrogen.
10. Ramp from room temperature to 190 °C and leave overnight for hard bake.

Aim: 11-13 µm after reflow

Inlet channels

1. Pour SU8-50 resist on half the diameter of the wafer.
2. Ramp at 500 rpm for 30 seconds, then spin at 2,500 rpm for 30 seconds.
3. Soft bake the wafer for 2 minutes at 65 °C, 10 minutes at 95 °C, and 2 minutes at 65 °C.
4. Expose for 7s.
5. Perform a post-exposure bake for 2 minutes at 65 °C, 10 minutes at 95 °C, and 2 minutes at 65 °C.
6. Develop in an SU8 developer primary bath for around 4 minutes, then rinse in a SU8 developer secondary bath.
7. Rinse with IPA and dry the wafer with compressed nitrogen.

Aim: 40 µm
Chambers

8. Pour SU8-100 resist on half the diameter of the wafer.
9. Ramp at 500 rpm for 10 seconds, then spin at 1,300 rpm for 50 seconds.
10. Soft bake the wafer for 5 minutes at 65 °C, 70 minutes at 95 °C, and 5 minutes at 65 °C.
11. Expose for 25 s.
12. Perform a post-exposure bake for 5 minutes at 65 °C, 18 minutes at 95 °C, and 5 minutes at 65 °C.
13. Develop in an SU8 developer primary bath for around 20 minutes, then rinse in a SU8 developer secondary bath.
14. Rinse with IPA and dry the wafer with compressed nitrogen.
15. Ramp up and down from room temperature to 135 °C for 20 minutes.

Aim: 160 µm

Control wafer

1. Dehydrate a wafer for 10-15 minutes at 150 °C.
2. Pour SU8-50 resist on half the diameter of the wafer.
3. Ramp at 500 rpm for 10 seconds, then spin at 4,200 rpm for 40 seconds.
4. Soft bake the wafer for 2 minutes at 65 °C, 4 minutes at 95 °C, and 2 minutes at 65 °C.
5. Expose for 2 minutes.
6. Perform a post-exposure bake for 2 minutes at 65 °C, 6 minutes at 95 °C, and 2 minutes at 65 °C.
7. Develop in an SU8 developer primary bath for around 2 minutes, then rinse in a SU8 developer second bath.
8. Rinse with IPA and dry the wafer with compressed nitrogen.
9. Ramp up and down from room temperature to 135 °C for 20 minutes.

Aim: 25 µm
Supplementary Note 2

Device Fabrication Protocol

Cleaning
1. Place control wafers in plastic box with TMCS (can clean flow wafers with PDMS, but that requires degassing) for at least 2 minutes.
2. Pour 15.0 g RTV-A and 1.5 g RTV-B (10:1 ratio) per wafer into plastic cup, place cup in mixing machine, and mix together.
3. While machine mixing, wrap 1 Petri dish per wafer with aluminum foil.
4. After mixing is done, remove wafers from TMCS box and place in Petri dishes.
5. Pour PDMS onto each wafer and tilt dish so that wafer is covered with PDMS and that PDMS overflows on the foil.
6. Place in 80 °C oven for at least 20 minutes.

Flow Layer
7. Place flow wafers in plastic box with TMCS for at least 2 minutes.
8. Pour 12.5 g RTV-A and 2.5 g RTV-B per wafer into 5:1 plastic cup, place cup in mixing machine, and mix together.
10. After mixing is done, remove wafers from TMCS box and place in aluminum holders. Press down wafer on the bottom by folding the aluminum foil on top of wafer edges.
11. Pour PDMS onto each wafer, and level the aluminum holder with 2 micropipette tips.
12. Place into degasser machine, pressurize, and degas for until no visible bubbles are left. Prepare control layer during that time.
13. Remove from degasser and level again with 2 micropipette tips. Let sit for at least 15 min.
14. Place in 80 °C oven for 18 minutes.

Control Layer
15. Cut around cleaned wafer with surgical knife and peel off PDMS to release the cleaned wafer.
16. Place cleaned control wafer in plastic box with TMCS for at least 2 minutes.
17. Pour 15.0 g RTV-A and 0.75 g RTV-B into 20:1 plastic cup, place cup in mixing machine, and mix together.
18. Turn on gas and vacuum for spinner.
19. Ensure spinner recipe ramps in 5 seconds to 500 rpm, dwells at 500 rpm for 10 seconds, ramps to 1630 rpm in 10 seconds, dwells at 1630 rpm for 60 seconds, and ramps down to 0 rpm in 5 seconds.
20. Place wafer carefully on centre of spinner chuck, close lid and secure with copper slab, and execute spinner recipe.
21. After spinning is finished, remove wafer from spinner and place in clean, new Petri dish. Let sit for at least 15 minutes.

22. Place in 80 °C oven for 18 minutes

_The control and flow layers should go into the oven at the same time._

**Membrane**

23. Cut around cleaned wafer with surgical knife and peel off PDMS.
24. Pour 15.0 g RTV-A and 0.75 g RTV-B into 20:1 plastic cup, place cup in mixing machine, and mix together.
25. Turn on gas and vacuum for spinner.
26. Ensure spinner recipe ramps in 5 seconds to 500 rpm, dwells at 500 rpm for 10 seconds, ramps to 500 rpm in 10 seconds, dwells at 500 rpm for 60 seconds, and ramps down to 0 rpm in 5 seconds*.
27. Place wafer carefully on centre of spinner chuck, close lid and secure with copper slab, and execute spinner recipe.
28. After spinning is finished, remove wafer from spinner and place in clean, new Petri dish.
29. Let sit for at least 15 minutes and align flow/control during that time.
30. Place in 80 °C oven for 12 minutes (13 min after flow/control duo has been placed in the oven)

* A thinner membrane will result in leaky valves while a too thick membrane does not spread evenly on the wafer.

**Flow/Control Alignment**

31. Remove both flow and control wafers from the oven.
32. Cut inside the edge of the flow wafer with a surgical knife, then peel off PDMS layer from silicon wafer.
33. Place control wafer under the microscope.
34. Align flow layer to control layer, trying not to peel off and on too much.
35. Push down any bubbles that remain between the two layers, and place in 80 °C oven for 25 min.

_The blank should come out of the oven at the same time as the flow/control combo. Time out accordingly._

**Membrane/Duo Alignment**

36. Remove both flow/control duo and blank wafers from the oven.
37. Cut around the edge of the control/flow wafer with a surgical knife, then peel off PDMS layer from silicon wafer.
38. Place flow/control duo onto blank layer.
39. Push down any bubbles that remain between the two layers, and place in 80 °C oven for at least one hour.

_Can be left in the oven overnight after this step._
**Bath layer**

40. Pour 40.0 g RTV-A and 4.0 g RTV-B in 10:1 plastic cup, place cup in mixing machine, and mix together.*
41. While machine mixing, prepare aluminum wrap using metal dish.
42. Press down wafer on the bottom by folding the aluminum foil on top of wafer edges.
43. Pour PDMS onto blank wafer, and level the aluminum holder with 2 micropipette tips.
44. Place into degasser machine, pressurize, and degas until no visible bubbles are left.
45. Remove from degasser and level again with 2 micropipette tips.
46. Place in 80 °C oven for 20 minutes.
* This amount of PDMS gives a sufficient height to provide good support structure for inlet and outlet ports.

**Cover layer**

47. Pour 14.0 g RTV-A and 1.4 g RTV-B in 10:1 plastic cup, place cup in mixing machine, and mix together.
48. While machine mixing, prepare aluminum wrap using metal dish.
49. Press down wafer on the bottom by folding the aluminum foil on top of wafer edges.
50. Pour PDMS onto each wafer, and level the aluminum holder with 2 micropipette tips.
51. Place into degasser machine, pressurize, and degas until no visible bubbles are left.
52. Remove from degasser and level again with 2 micropipette tips.
53. Place in 80 °C oven for 20 minutes.

**Chip Assembly**

54. Remove flow/control/membrane wafer, and blank wafers from the oven and let cool for about 5 minutes.
55. Dice layers into individual chips and place the chips on a ball bearing bed, flow layer down.
56. Dice the bath layer, and cut inside to create a bath having the area of the array. Leave enough space for the ports and the edges.
57. Punch holes that go in the corner of each side of the bath.
58. Dice the cover layers into pieces bigger that each chip.
59. Clean all surfaces with scotch-tape.
60. Mix together about 10.0 g RTV-A and 1.0 g RTV-B in 10:1 plastic cup, place in mixing machine, and mix together.
61. Set spinner to spin at 6,000 rpm for 6 minutes.
62. Remove blank wafer and place on spinner, pour on PDMS, and spin.
63. Remove from spinner and place in Petri dish.
64. “Stamp” the bath portion onto the liquid blank wafer and leave for 30 seconds. Make sure to stamp the right side of the bath.
65. Remove from wafer, and stick together with the flow/control portion.
66. Remove bubbles between layers.

67. Mix together about 10.0 g RTV-A and 1.0 g RTV-B in 10:1 plastic cup, place in mixing machine, and mix together.
68. Set spinner to spin at 6,000 rpm for 6 minutes.
69. Remove blank wafer and place on spinner, pour on PDMS, and spin.
70. Remove from spinner and place in Petri dish.
71. “Stamp” the cover layer portion onto the liquid blank wafer and leave for 30 seconds.
72. Remove from wafer, and stick on top of the bath portion.
73. Remove bubbles between layers

74. Leave chips to cure at room temperature overnight on ball bearings and place them in the oven.

After this step, the chips can be left in the oven.

Hole Punching/Bonding to glass
75. Remove chips from the oven and punch appropriate holes with the hole puncher.
76. Clean glass slides with IPA and PDMS chips with Scotch tape.
77. Use plasma bonder to bond together chips and glass slides (25s).
78. Cure at 80 °C in oven overnight.

The total curing time at 80 °C should equal at least 5 days before testing of chips, and chips should be 12 days old and autoclaved before use for cell culture.
Supplementary Note 3

Mathematical Model Description

Transport Equations:

The simulation of the system was performed with a three-dimensional, steady state, single phase, laminar flow model. The CFD (computational fluid dynamics) simulation has been done using FLUENT 6.3.26 (Fluent Inc). In laminar flow the Navier-Stokes equations describe the momentum transport. Therefore, the conservation of momentum in the micro-bioreactor is described by Eq. (1)

\[
\frac{\partial}{\partial t} (\rho \vec{V}) + \nabla . (\rho \vec{V} \vec{V}) = -\nabla P + \nabla \tau
\]  

(1)

The conservation of mass is described by the continuity equation as follows,

\[
\frac{\partial \rho}{\partial t} + \nabla . (\rho \vec{V}) = 0
\]  

(2)

where \( \rho \) (Kg m\(^{-3}\)) is the fluid density, \( \vec{V} \) (m s\(^{-1}\)) is the velocity vector of the fluid, \( P \) (Pa) is the pressure, and \( \tau \) is the stress tensor. Water has been used as a model to estimate the physical properties of fluid at 37 °C.

Boundary Conditions:

The uniform velocity profile has been defined as the inlet boundary condition. At the outflow boundary, the diffusion fluxes for all flow variables in the direction normal to the exit plane are assumed to be zero. The fluid temperature is assumed to be constant at 37 °C, and a no-slip boundary condition has been specified for the velocity at the walls.
Supplementary Note 4

Rate of Water Loss from the Iso-osmotic Bath

Water vapor loss from the osmotic bath may be modeled as a near-Fickian diffusion and has a flux given by,

\[ J = -D \nabla C \]  \hspace{1cm} (3)

Where D is the diffusion constant of water vapor in PDMS (~8.5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1})\(^1\) and C is the concentration of water vapor in the bulk PDMS.

The iso-osmotic bath covers the area of the array (20 mm \times 11 mm) and has a height of ~5 mm. The majority of vapor loss occurs through the top surface of the chamber that is sealed with a 1 mm thick layer of PDMS and through the long and short sides of the bath that are sealed with 5 mm and 3 mm thick edges of PDMS respectively. This is well approximated as a one-dimensional diffusion for problem given by,

\[ J = -D \Delta C / L \]  \hspace{1cm} (4)

Where L is the thickness of the PDMS sealing the top and 4 sides of the osmotic bath.

We assume a saturated water vapor concentration at 37 °C on the inside surface of the membrane (~ 39.3 mol m\(^{-3}\)). Assuming a 90% relative humidity in the incubator we approximate the water vapor concentration at the outside surface of the chip to be 0.9 \times 10^{-10} \text{ mol m}^{-3} = 35.4 \text{ mol m}^{-3}, giving a total vapor flux of 2.1 \times 10^{-8} \text{ g s}^{-1}. This corresponds to a loss of 13 µl over a 5-days experiment. Given a total osmotic bath volume of 1.1 ml this results in approximately 1.2% change in osmotic strength during an experiment.