Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary information

Multi-size spheroid formation using microfluidic funnels

Mohana Marimuthu,[acdf] Nassim Rousset,[a] Amelie St-Georges-Robillard,[abcd] Muhammad A. Lateef,[cd] Mikaël Ferland,[g] Anne-MarieMes-Masson[cde] and Thomas Gervais*[abcd]

[a]Department of Engineering Physics, Polytechnique Montréal, Canada

[b]Institut de géniebiomédical, Polytechnique Montréal, Canada

[c]Centre de recherche du Centre hospitalier de l'Université de Montréal, Canada

[d]Institut du cancer de Montréal, Canada

[e]Department of Medicine, Université de Montréal, Canada

[f]Department of Biotechnology, Alagappa University, India (Current affiliation)

[g]Département de Génie Informatique et Génie Logiciel, Polytechnique Montréal, Canada

* Corresponding author: Dr. Thomas Gervais

Email: thomas.gervais@polymtl.ca

Chip Fabrication

1. Mould micromachining:

The SIMSS chip is comprised of four PDMS layers, namely the HD layer (combination of layer 1: pinhole and layer 2: seeding channel), the cell funnelling layer, and the vent layer (Fig. 1a). Each PDMS layer was fabricated via moulding onto poly(methyl methacrylate) (PMMA) structures manufactured using flat end mills with various diameters and flute configurations in a computerized numerical control (CNC) machine (EMCO PC Mill 55, EMCO GmbH, Hallein, Austria). Inverse designs of each layer were produced with CATIA (DassaultSystemes, Paris, France). The designs were then processed in FeatureCAM (AutoDesk, San Rafael, USA), to generate the codes for the CNC milling process. A 3 mm flat end mill was used for milling the funnel layer and 1 mm flat end mill was used to fabricate the HD layer (pinhole and seeding channel) and for vent layer, combination of 3 mm and 1 mm flat end mills were used. Generated PMMA moulds were cleaned with isopropanol and the sides were polished using a commercial plastic polishing starter kit (Novus^{TM} plastic polish, Minneapolis, USA). Then these moulds were used to cast the different PDMS layers. Liquid PDMS (Sylgard® 184 silicone elastomer kit, Dow Corning, Midland, USA) prepared at 10:1 ratio of base polymer to curing agent was poured into the moulds of each layer, degassed and kept in an oven at 80°C for 1.5 h. Followed by, the polymerized PDMS layers were carefully peeled off from the moulds and both sides were cleaned using adhesive tape.

2. Layers of SIMSS chip:

Figure S3 shows the schematic of a layer-by layer-SIMSS chip design configuration. The HD layer has three parallel seeding channels with 2 mm width and 1 mm height and each channel has a series of 8 consecutive pinholes, with 1.5 mm and 0.75 mm top and bottom end diameters, respectively. It is designed such that the top of the pinholes is assembled within the 2 mm-wide channel. The cell-funnelling layer has a series of 8 funnels with constant 1 mm bottom diameters, but with varying apex angles of 12, 19, 26, 33, 40, 47, 54, and 61 degrees. These 8 funnels in series were replicated thrice in parallel such that the bottom end of each funnel points towards a pinhole. The vent layer design uses a cylindrical and a conical vent assembled on top of each funnel. The geometry of each vent was designed such that a 0.2 mm high cylinder with 1 mm diameter was patterned on top of 0.5 mm high cylinder with 2 mm diameter. The double cylindrical sandwich was assembled on top of a cone with 1.3 mm height and its base and top diameters were of 5 mm and 2 mm,

respectively. Finally, a single inlet and three outlets were punched in the cell-funnelling layer using biopsy punches.

3. Bonding of Layers:

The bottom side (with 1 mm end of funnels) of the cell-funnelling layer and the top side (with seeding channels and pinholes) of the HD layer were exposed to an atmospheric plasma (Enercon Industries Corp., Menomonee Falls, USA), and assembled and bonded carefully to locate the 1 mm end of funnel into the 1.5 mm end of the pinholes. Similarly, the bottom side of the vent layer and the top side of the cell-funnelling layer were plasma exposed and assembled to locate the vents on top of each funnels. Each SIMSS chip consists of 24 funnels to synthesize triplicates of 8 different sizes of spheroids. From a top view, the centre of each pinhole aligns with the centres of funnels and vents approximately. Lateral distance, centre-to-centre, between two pinholes is 10 mm.

4. SIMSS chip holder:

To hold each SIMSS chip for cell culture and imaging applications, a CO_2 laser cutter (Speedy 300 LASER Engraver, Trotec Inc., Langley, Canada) was used to cut pieces of PMMA. The PMMA pieces were then bonded using dichloromethane solvent to fabricate the holder for the SIMSS chip.

5. Spheroid capture chip:

To capture the spheroids made in SIMSS chip by contact transferas shown in Supplementary video 2for further analysis, spheroid capture chip was designed (Fig. S2). The chip has series of cylindrical wells, with 6 mm diameter and 5 mm height, compatible with either 8- or 24-well SIMSS chip. Bottom of each cylindrical well has a conical trap which is an inverse design of the vent layer. Cylindrical well layer and reversed vent layer were plasma exposed and bonded to obtain spheroid capture chip. Advantage of spheroid capture chip instead of conventional well plate is that the spheroids descend down into the conical trap instead of sticking to the corners or walls of the well which facilitates the collection of transferredspheroids. In order to contact transfer the spheroids, spheroid capture chip should be filled up to the surface and upon contact with the bottom of the SIMSS chip, spheroids are pulled down in the conical trap as shown in the supplementary video 2. The same phenomenon was described earlier by Cavnar et al., for contact transfer of spheroids to a customized trap chip.

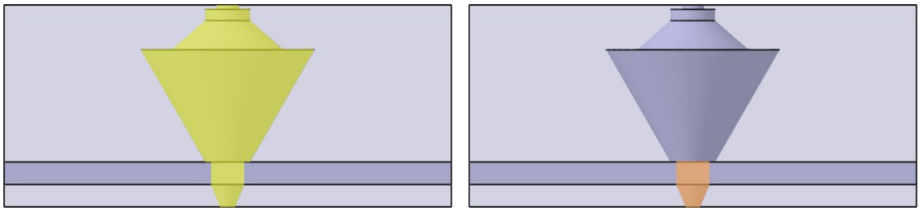

Figure S1:*Schematics showing how the enrichment number is calculated.The enrichment number corresponds to the volume occupied by the pinhole, the volume of the channel over the pinhole, the funnel, and the vent (in yellow) divided by the volume of the pinhole and the volume of the channel over the pinhole (in orange).*

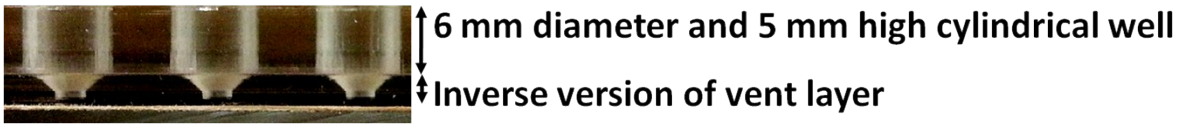

Figure S2: *Picture of 3 wells of spheroid capture chip with the dimensions.*

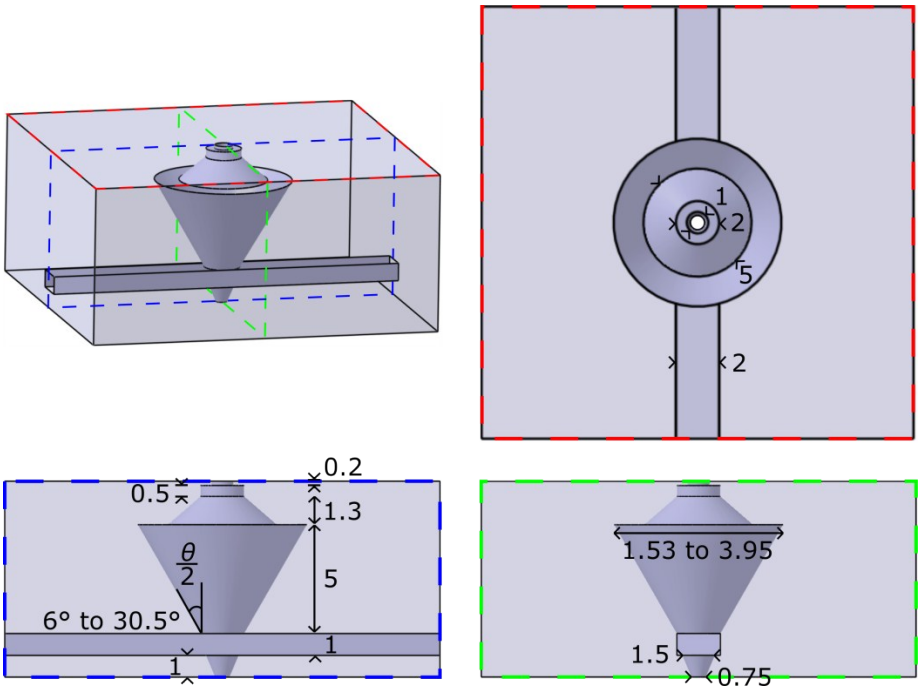

Figure S3: *Schematics showing the microfluidic chip dimensions. A) Sub-unit consisted of a pinhole, its cone concentrator and a top vent. B) Top view. C) Side view showing the different heights of the device. D) Side view showing the width and diameters of the different parts of the device. All distances in mm.*

Table S1 Repeated analysis to evaluate the required number of scenarios. Example using the diameters of OV90 spheroids on day 5

Number of randomized scenarios	% of significant *p*-values ($p < 0.05$)			Relative standard deviation (%)
	Run 1	Run 2	Run 3	
10	80.00	90.00	100.00	9.07
1 000	85.60	85.60	87.60	1.09
5 000	85.90	86.54	85.78	0.39
10 000	87.07	86.45	86.78	0.29

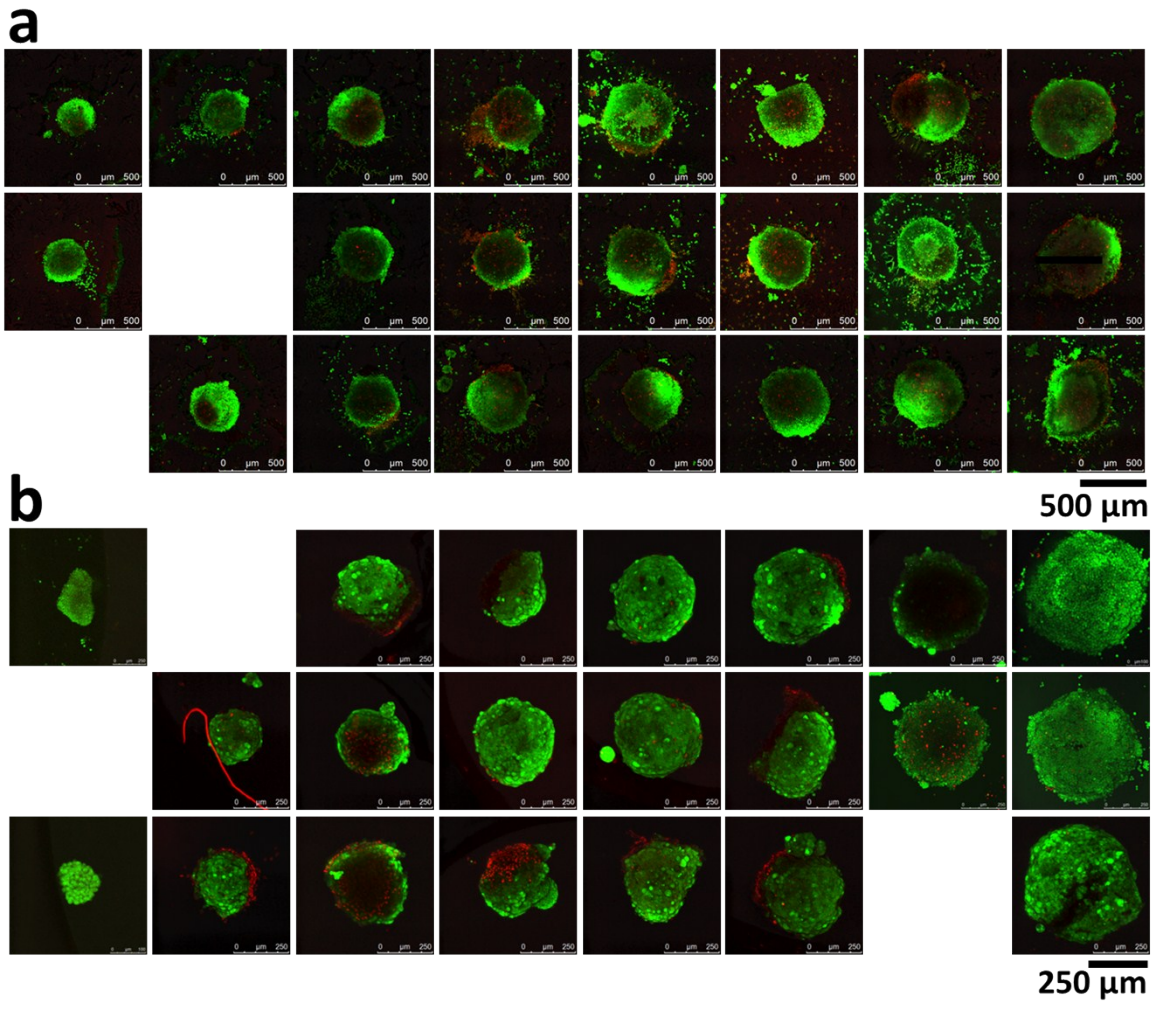

Figure S4: *3D projectionconfocal microscopic images of spheroids after 7 days of culture. (a) OV90 spheroids and (b) TOV112D spheroids (green: live cells (CTG); red: dead cells (PI)).*

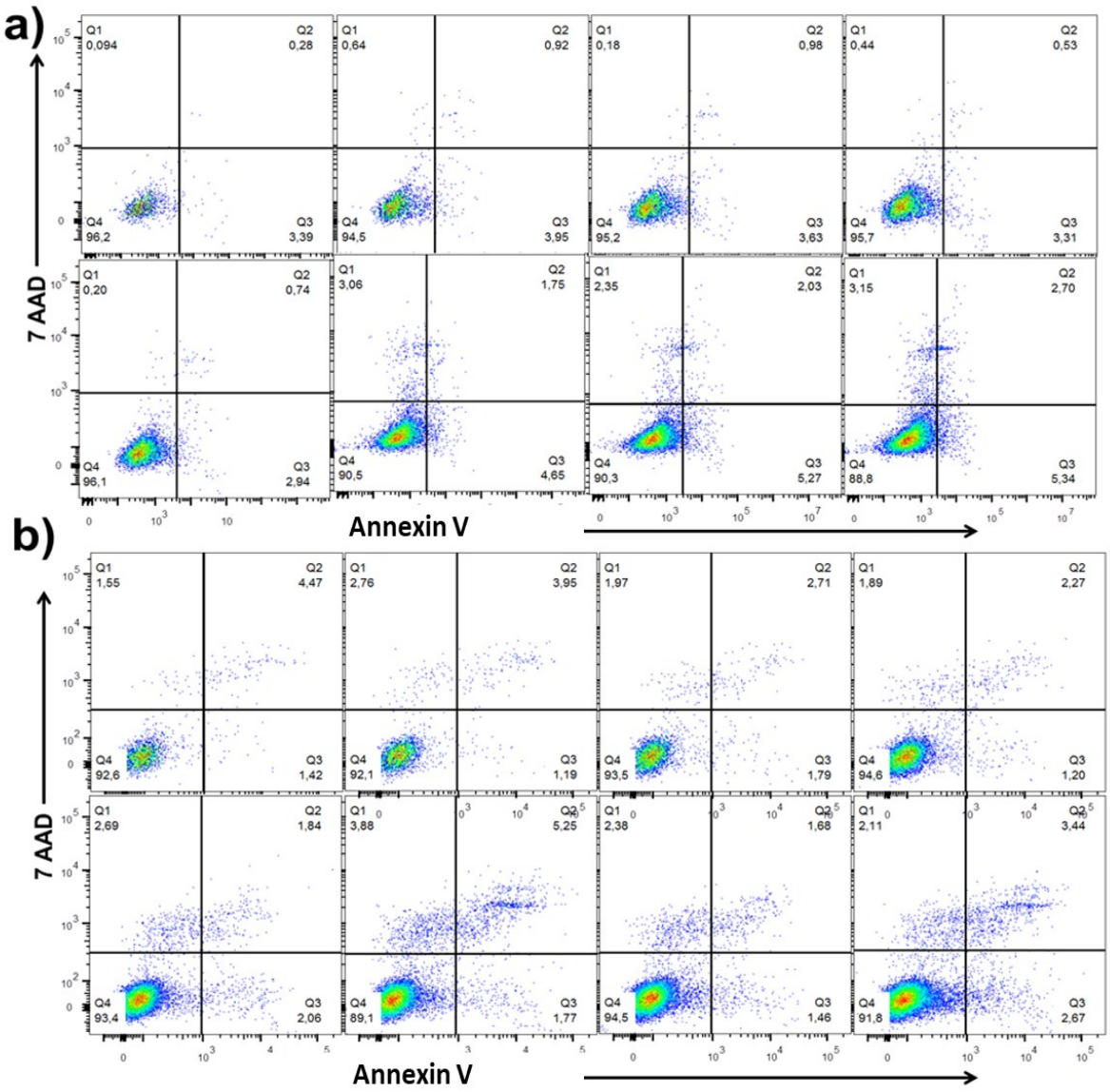

Figure S5: *Spheroid viability assay using flow cytometry. Single spheroids from each enrichment number were analysed. (a) OV90 spheroids and (b) TOV112D spheroids.*