

A novel 3D culture model recapitulates primary FL B cell features and promotes their survival

By Lamaison C et al.

SUPPLEMENTAL MATERIALS AND METHODS

Reagents and antibodies

Alginate sodium salt from brown algae, D-Sorbitol, CaCl₂ hexahydrate, EGTA, and paraformaldehyde were supplied by Merck (Fontenay-sous-Bois, France). Matrigel was provided by Corning (UGAP, France). Propidium iodide, Phalloidin-Alexa 594, Fixable Viability Dye eFluor 506, calcein red-AM and NucRed Dead were from Thermo Fisher Scientific (Courtaboeuf, France). Hoechst 33258 and ABT-199 were purchased from Tocris Bioscience (Lille, France). The comprehensive cancer center pharmacy department of the Institut Bergonié kindly provided doxorubicin. Hypoxyprobe-1 Times Kit was supplied by Euromedex (Souffelweyersheim, France). Celltiter-Glo 2.0 luminescent cell viability assay was from Promega (Charbonnières-les-bains, France). APC Annexin V, mouse anti-human CD20-PE and mouse anti-human CD19-BV421 were supplied by BD Biosciences (Le Pont de Claix, France). Rabbit polyclonal anti-laminin, rabbit monoclonal anti-human collagen I and chicken polyclonal anti-GFP antibodies were supplied by Abcam (Paris, France). Mouse anti-human CD20 APC-H7 and mouse anti-human CD73 PE were from eBiosciences (Thermo Fisher Scientific). Monoclonal mouse anti-human Ki67 was from Dako Agilent Technologies (Les Ulis, France) and rabbit polyclonal anti-cleaved caspase 3 antibody was provided by Cell Signaling Technology (Ozyme, Saint Cyr l'école, France). All Alexa-conjugated secondary antibodies were purchased from Thermo Fisher Scientific.

Cells

Stromal cells (TSC) were isolated as previously described^{1,2} from human tonsils collected from children undergoing routine tonsillectomy, considered as surgical wastes. GFP-expressing TSC cells were established after transduction with lentivirus carrying the pUltra (gift from Malcolm Moore (Addgene plasmid # 24129; <http://n2t.net/addgene:24129>; RRID: Addgene_24129), which contained the enhanced

GFP gene under the control of Ubc promoter and produced as previously described³. TSC were transduced in RPMI (Thermo Fisher Scientific) supplemented with 2% FBS (Dutscher, Brumath, France) containing pUltra concentrated lentiviral supernatant at a MOI of 4. After 24 hours, the medium was replaced with standard culture medium. After one week, eGFP^{hi} TSC were isolated using a cell sorter (FACS Aria, BD Biosciences). For all experiments, TSC and GFP-TSC were used from passage 9 (P9) to P13. SUDHL4 and DOHH2 cell lines were obtained from the DSMZ cell collection (Braunschweig, Germany). HLY1 cell line was kindly provided by Dr F. Meggetto (CRCT, Toulouse, France). Cells were routinely cultured in RPMI supplemented with 10% FBS under humid atmosphere containing 5% CO₂, with medium changed 3 times a week. One day prior to encapsulation, cells were cultured in DMEM supplemented with 10% FBS. Once cellular capsules were formed following the protocol described below, they were cultured in 75 cm² flasks in DMEM supplemented with 10% FBS, with medium changed 2 times a week. Depending on the type of experiments, capsules were sampled and transferred to dedicated culture supports for imaging, or treated in bulk conditions and subsequently analyzed.

Limiting Dilution Assay

B cell lines (HLY1, DOHH2, SUDHL4) were seeded as 10, 3, and 1 cell per well in 96 wells (n=3), and at 0.1 cell per well in 192 wells (n=3) and were cultured for 21 days before counting of positive wells. Results were statistically evaluated using the Extreme Limiting Dilution Analysis (ELDA) method⁴.

Encapsulation procedure

Cell encapsulation was performed by adapting already published protocols⁵⁻⁷. Briefly, the microfluidic device was printed with EnvisionTEC Micro Hi-Res Plus 3D printer (Arkety3D, Le Raincy, France) using the resin HTM140V2 (EnvisionTEC), with the following printing parameters: burn-in range thickness 400µm, base plate of 300µm, and exposure time 3000ms. The printed device was washed using propanol and air-dried. In parallel, a glass capillary was pulled using a micropipette puller (P30, Sutter Instrument, Micro Mecanique SAS, Saint Germain en Laye, France), then cut and polished with micro-abrasive films (1µm grain, 3M) to reach the desired tip diameter (typically between 130 and 180 µm). This glass tip, which serves as a nozzle for the encapsulation device, was hydrophobized (1H,1H,2H,2H-

Perfluorooctyltrimethoxysilane, ABCR) following standard protocols⁸ and glued to the exit of the microfluidic plastic device with epoxyglue EA M-31CL (Loctite).

To run an encapsulation, the microfluidic device was connected to three syringes (10MDR-LL-GT SGE, Analytical Science, Milton Keynes, UK) mounted on pumps (Nemesys, Cetoni GmbH, Korbussen, Germany) for flow rate control. Three different solutions were injected into the three coaxial cones of the device. The outermost cone contained the alginate solution, which was prepared by dissolving 2% wt/vol sodium alginate (Protanal LF200S) in water and by adding 0.5mM SDS surfactant (VWR International, Fontenay-sous-Bois, France). The solution was filtered at 1 μ m (glass filters, Pall Life Science) and stored at 4 °C. The intermediate cone contained a 300 mM sorbitol (Merck) solution. The innermost cone contained cells suspended in a sorbitol solution or cells/Matrigel/sorbitol solution (when ECM is needed) in a ratio of 2:1:2 (v/v). For encapsulation of lymphoma cell lines or stromal cells only (mono-culture), a suspension of 2x10⁶ cells were resuspended in culture DMEM (Thermo Fisher Scientific). 200 μ L of the cell suspension was injected into a cooling part to maintain Matrigel liquid. The flow rates were set to 45mL/h, 45mL/h and 30mL/h for the alginate, sorbitol, and cell suspension, respectively. The device was positioned 40-50 cm above a petri dish containing 100mM CaCl₂ solution for capsule gelling and collection. To improve capsule shape and monodispersity, an alginate charging part and copper ring (21mm OD, RadioSpares), both connected to a high voltage (2 kV) generator (Stanford Research PS350, Acal BFI, France) were introduced⁷. The charged formed droplets passing through the copper ring under electrical tension get deflected as they crossed the ring, creating a splay-like jet that prevented capsule coalescence. Once formed inside the calcium bath, capsules were washed with DMEM and transferred to cell culture medium.

Determination of cell confluence in capsule

Capsules containing cells were imaged on brightfield at different timepoints after encapsulation. The diameter of the capsule and the thickness of the alginate walls were then measured manually by image analysis using ImageJ software on more than 100 capsules per time point. The diameter and wall thickness were plotted over time and allowed the determination of the confluence and post confluence periods. Confluence was reached when the alginate wall thickness decreased and the capsule diameter increased, but that roundness of cells was still visible. Post-confluence was

reached when the alginate wall thickness was to its minimum and the capsule diameter to its maximum with cell roundness no more visible.

Spheroid growth measurement

At various post-encapsulation culture times, we selected 200 capsules in each 3D culture conditions. Capsules were dissolved by incubation in PBS containing 1 mM EGTA and the spheroids were mechanically dissociated by pipetting. When spheroids were too cohesive, trypsin-EDTA was added to improve cell dissociation. The cell suspension was centrifuged and re-suspended in 200 μ L of PBS and 50 μ L of Precision Count Beads (BioLegend, Ozyme). The number of cells was then determined in a given cell suspension volume of 100 μ L using flow cytometry (FACS Calibur, BD biosciences) according to the manufacturer instructions after GFP-stromal cell backgating when necessary. To evaluate the cell death in spheroids, propidium iodide (PI) (10 μ g/mL) was added to the cell suspension and PI fluorescence was analyzed by flow cytometry using Cell Quest or FlowJo (BD biosciences).

Immunostaining of spheroids

In capsulo staining

Capsules were fixed in DMEM w/o phenol red (Pan Biotech, Dutscher), containing 4% paraformaldehyde for 20 min at room temperature (RT). After rinsing, capsules were incubated for 30 min in DMEM w/o phenol red/ 0.1% triton X-100. Following a step of saturation with a solution containing DMEM w/o phenol red/ BSA 1% /FBS 2% for 1h at RT, capsules were incubated with primary antibody overnight at 4°C under shaking. Primary antibody was revealed by incubation of capsules with secondary Alexa-coupled antibody for 2h at RT. Nuclei were stained by incubation of capsules with Hoechst 33258 for 30 min at RT. To determine the role of Matrigel in the stromal network establishment, immunofluorescence using a rabbit anti-human pan laminin antibody revealed by goat anti rabbit Alexa 633 secondary antibody, was performed to visualize Matrigel, stromal cells were GFP positive and nuclei was stained with Hoechst 33258. Z-stacks were acquired using a Zeiss LSM 510 meta confocal microscope with a 25x objective (N.A 0.7). 3D rendering was obtained using UCSF Chimera software (RBVI, San Francisco, USA).

FFPE staining

To analyze protein expression inside spheroids, immunofluorescence on Formalin-Fixed Paraffin-Embedded (FFPE) spheroids sections (3-5 µm thick) were performed. Alginate shells were dissolved and spheroids were fixed as described above. Spheroids were then pre-embedded in 2% agarose and further embedded in a paraffin block. Immunofluorescence staining was then performed as previously described⁹. Images were acquired using a Zeiss LSM 510 meta confocal microscope (Zeiss, Göttingen, Germany) with a 25x objective (N.A 0.7). Then images obtained were processed using Fiji.

Hypoxia detection in spheroids

To determine hypoxia in spheroids, hypoxyprobe-1 100 times kit (HPI, Euromedex) was used. Briefly, spheroids at confluence or post-confluence were incubated for 2h with 200µM pimonidazole. Spheroids were then fixed and embedded in paraffin. Pimonidazole immunostaining was performed on spheroid sections according to the supplier recommendations. Images were acquired using a Nikon ECLIPSE Ci-L equipped with a Nikon DS-Ri2 camera controlled by NIS-Element software (Nikon-Ci, version BR 4.50.00) (Nikon, Champigny sur Marne, France).

3D cell morphology quantification

Capsules were fixed at various post-encapsulation culture times and each capsule was placed in an agarose well of the Universlide microscopy chamber⁹, which allowed immobilization of the capsules as required for stable z-stack acquisition and medium throughput 3D imaging. Cell nuclei were stained with Hoechst 33258 and stromal cells were visualized by GFP expression. Images were acquired using a Leica SP8 confocal microscope with a 20X dry objective (NA 0.7). To measure the evolution of TSC complexity over time two parameters were measured: the sphericity defined as

$\sqrt[3]{\frac{36 \times \pi \times \text{Volume of particle}^2}{\text{Surface of particle}^3}}$ and the branchness defined as $\frac{\text{Volume of particle convex hull}}{\text{Volume of particle}}$. To

obtain these values, we applied a locally adjusted threshold to the green signal, performed 3D segmentation (3D suite plugin, Fiji¹⁰). For the two parameters, the values of all “particles” contained in one capsule were averaged. Thus, each capsule was characterized by one mean value for sphericity and branchness and 10-18 capsules were analyzed per time point.

Cell co-culture and cell sorting:

2D co-cultures were performed over 3 days. 3×10^5 DOHH2 ($1.5 \times 10^5/\text{mL}$) were plated on 5×10^4 TSC / cm^2 in 6 well plates. For cell sorting, cells were trypsinized (Thermo Fisher Scientific) for 12 min at 37°C and washed with DMEM 10% FBS. 3D co-cultures were performed over 10 days. For cell sorting, after dissolution of the alginate shells, cells were rinsed with DMEM 10% FBS and centrifuged for 7 min at 1700 rpm, incubated with trypsin at 37°C for 15 min, and rinsed with medium DMEM 10% FBS. Cells were stained for 20 min on ice with mouse anti-human CD20 APC-H7 and mouse anti-human CD73 PE. After DAPI staining, cell sorting was processed by a FACS Aria II (BD Biosciences).

RNA-seq analysis

RNA was extracted from $\text{CD20}^{\text{pos}}\text{CD73}^{\text{neg}}$ sorted B cells after 3 days (2D) or 10 days (3D) of *in vitro* culture with or without TSC. HLY1 in 2D (H2D, n=3), HLY1 in 3D (H3D, n=3), HLY1 with TSC in 2D (HT2D, n=3), HLY1 with TSC in 3D (HT3D, n=3), DOHH2 in 2D (D2D, n=4), DOHH2 with TSCs in 2D (DT2D, n=3), and DOHH2 with TSC in 3D (DT3D, n=5) were used for RNA-seq analysis. RNA was extracted using the Nucleospin RNA XS Micro kit from Macherey Nagel (Hoerdt, France). Libraries were prepared using the SMARTer® Low Input RNA kit (Takara). After quality control, all libraries were sequenced on an Illumina HiSeq2500 platform at 100-bp paired-end generating more than 66 million reads per sample with 100% of sequences achieving > Q20 Phred quality scores. The sequence quality was checked by FastQC v0.11.5. The RNA-seq reads were aligned to the reference genome GRCh38 release 90 using STAR v2.5.2a¹¹. Read summarization and gene counts were performed using the featureCounts function from the Subread v1.4.6 R package¹². Data normalization and differential gene expression were performed with the DESeq2 v1.26.0 R package. The DESeq2 HTSFilter v1.26.0 was applied on the normalized counts. Significant genes were defined as $p < 0.05$ after adjustment for multiple testing by False Discovery Rate (FDR) correction and used in downstream analysis. Data analysis was performed using R version 3.6.1 (www.R-project.org; R Foundation for Statistical Computing). Principal Component Analysis (PCA) was visualized using the Factoextra v1.0.7 R package on HTS filtered data normalized by variance stabilizing transformation from the DESeq2 package.

Enrichment for transcription factor targets in differentially expressed genes between FL B cells and tonsil centrocytes was performed with Enrichr (<https://maayanlab.cloud/Enrichr/>) by interrogating the TRRUST Transfection Factor 2019 database and results were represented as barplots. Gene Set Enrichment Analysis^{13,14} (GSEA version 4.1.0) was used for the analysis of DOHH2 and HLY1 GEP, to identify the gene sets that were enriched between two conditions. GSEA preRanked analysis was conducted on the ranked gene lists generated for each pairwise comparison between two conditions, the ranking being applied on the statistics of the Wald test (DESeq2 R package). The gene sets corresponded to the hallmark gene sets from MSigDB version 7.4 and the gene sets from TRRUST v2 human database (version 16/04/2018). In addition, the results were compared to those obtained by comparing using Affymetrix microarrays the gene expression profile of purified FL B cells and purified centrocyte (purified centrocytes, GSE136248, n=5; purified FL B cells, GSE85233, n=10) after Limma analysis with a FDR of 5% and an absolute fold change >1.5^{15,16}.

RT-qPCR arrays

For RT-qPCR, first-strand cDNA was synthesized using 1 µg of total RNA (DNase-treated) in a 20 µl reverse transcriptase reaction with PrimeScript RT reagent kit gDNA eraser (Takara, Ozyme) according to the instructions of the manufacturer. All real-time PCR reactions were performed in a 20 µl mixture containing 1/10 volume of cDNA preparation using Taq'Ozyme HS (Ozyme) according to manufacturer protocol with 5X FG Power Sybr Green PCRF (ABI Applied Biosystems, Fisher Scientific). The gene-specific primers were as follows: hGAPDH-for-5'CCTGGTATGACAACGAATTT-3' and hGAPDH-rev-5'-GTGAGGGTCTCTCTCTTCCT-3'; Collagen 1-for-5'-TCT-GCG-ACA-ACG-GCA-AGG-3' and Collagen 1-rev-5'-GAC-GCC-GGT-GGT-TTC-TTG-3'; Laminin-for-5'-TCG-TCA-ACG-CCG-CCT-TCA-A-3' and Laminin-rev-5'-GTG-TCG-GCC-TGG-TTG-TTG-TA-3'. All qPCR experiments were performed using the Applied Biosystems StepOne Plus Real-Time system (Perkin-Elmer, Applied Biosystems). All the amplifications were done using SYBR Green PCR Master Mix (Applied Biosystems). The thermal cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 30s. Melting curve analysis of every qPCR was conducted after each cycle. Samples were amplified at least in triplicate and non-template samples were used as controls. The

relative expression of mRNA was calculated using $2^{-\Delta\text{CT}}$ method ($\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}}$) and the fold change of mRNA expression level was calculated using $2^{-\Delta\Delta\text{CT}}$ method ($\Delta\Delta\text{Ct} = \Delta\text{Ct}_{3\text{D}} - \Delta\text{Ct}_{2\text{D}}$) in 3D compared with 2D condition. All data were normalized to internal control GAPDH.

Encapsulation and culture of primary FL cells

After thawing of FL LN, FL B cells were isolated using the B-cell isolation kit II (Miltenyi, Paris, France). Cell purity was checked using with mouse anti-human CD20-PE and mouse anti-human CD19-BV421 in the presence of a viability dye (VD-506) and analyzed on a Canto II flow cytometer (BD Biosciences). Cell viability was always >90% with >99% CD20^{pos}CD19^{pos} B cells.

For encapsulation, 12×10^6 FL cells were resuspended in MEM- α culture medium (Thermo Fisher Scientific) with 2×10^6 GFP-TSC in the presence of Matrigel. The encapsulation procedure was the same as that described above. Capsules were then cultured in 25cm² flasks in MEM- α culture medium supplemented with 10% FBS under a humid atmosphere containing 5% CO₂. For 2D cultures, 150×10^3 primary FL cells were plated alone or on 20×10^3 GFP-TSC in 96-well plates over 11 days in MEM- α culture medium supplemented with 10% FBS under a humid atmosphere containing 5% CO₂. At various times, FL B cells cultured in capsules or in 2D were counted by flow cytometry using precision count beads (Biolegend) as described above.

Live imaging of FL B cells co-encapsulated with TSC

Staining: Capsules were stained for 25 min at 37°C with calcein-red-AM (1 μ M), NucRed Dead (2 drops) and Hoechst 33342 (2 μ g/mL) as viability, dead cell and nuclei markers, respectively. Capsules were washed in DMEM without Phenol Red and deposited in glass-bottom dishes (Dutscher) precoated with Poly-D-Lysin to favor alginate adhesion to the substrate.

Microscopy: Acquisitions were conducted on two different setups. Capsules at Day 3 were imaged on a Leica SP8 WLL2 (Leica Microsystems, Mannheim, Germany), with a DMI6000 stand. Illumination provided by a tunable supercontinuum laser was focused to the sample through a Leica 40X dry 0.85 NA (HCX Plan Apo CS). Images were reconstructed from signals collected with Hybrids and PMT detectors. Capsules

at Day 14 were imaged with a Leica DMI8 stand (Leica Microsystems, Wetzlar, Germany) coupled to a Yokogawa spinning-disk module (CSU-W1 T2, Yokogawa Electric Corporation, Tokyo, Japan). Illumination diodes (405 nm, 488 nm, 561 nm, and 642 nm) were focused to the sample with a Leica 40X dry 0.85 NA (HCX Plan Apo CS). Images were acquired with a sCMOS Prime 95B camera (Photometrics, Tucson, USA). All the devices are piloted by MetaMorph (Molecular Devices, Sunnyvale, USA). Both experiments were conducted at 37°C under a controlled environment. Acquisition time being less than half an hour for each capsule.

Analysis: Image analysis and quantifications were performed with Fiji (an ImageJ with tool boxes, <https://fiji.sc/>) and Imaris 9.3 (Bitplane, Oxford Instruments). Briefly, cell centres were spotted on the Hoechst channel, and nuclei were tagged as TSC or Dead if colocalized with respectively GFP or NucRed Dead channels, remaining nuclei were therefore identified as B cells.

Chemotherapeutic toxicity measurement and doxorubicin diffusion in spheroids

To compare the drug toxicities on lymphoma cells cultured in suspension and in spheroids, 0.5×10^6 cells/well co-cultured or not with 5×10^4 GFP-TSC /cm² or 100 confluent spheroids were distributed in 6-well plates and treated or not with doxorubicin for 24h. Cell death was then assessed by flow cytometry after Annexin V-APC labelling according to the manufacturer instructions (BD Biosciences). To determine the half-maximal inhibitory concentration (IC₅₀) of doxorubicin, 20 000 cells per well or 100 spheroids at confluence per well were plated in 96-well plates, with various concentrations of doxorubicin for 24h. Doxorubicin cytotoxicity was determined using the luminescent cell viability assay CellTiter-Glo® 3D Cell viability assay (Promega, Charbonnières-les-bains, France). Luminescence levels were quantified using the FlexStation® 3 (Molecular Devices, USA).

The uptake of doxorubicin by cells cultured in suspension or in capsules was determined using the fluorescent properties of doxorubicin ($\lambda_{ex}=470\text{nm}$; $\lambda_{em}=560\text{nm}$)¹⁷. Cells cultured in suspension or in capsule before or at confluence were incubated for 4h with doxorubicin (1 $\mu\text{mol/L}$) at 37°C and 5% CO₂. Then, capsules were dissolved as previously described and cells were harvested, washed in PBS and analysis of cell fluorescence reflecting doxorubicin uptake was done by flow cytometry (BD biosciences Canto II).

Statistical analyses

All data were expressed as means \pm SEM. The significance of differences was calculated using the parametric ANOVA or the non-parametric U-Mann and Whitney or Wilcoxon tests, as appropriate. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Statistical analyses were performed using GraphPad Prism 8 software.

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MOVIE LEGENDS

Movie 1: Dissolution of alginate shell of capsules containing SUDHL4 spheroid.

Alginate shell of capsule containing SUDHL4 spheroid obtained after 10 days in culture was dissolved in a bath of PBS/EGTA. The images were acquired every 15 seconds until complete dissolution of capsule using an epifluorescence microscope (Leica DMI8).

Movie 2: Dissolution of alginate shell of capsules containing HLY1 spheroid.

Alginate shell of capsule containing HLY1 spheroid obtained after 10 days in culture was dissolved in a bath of PBS/EGTA. Movie was recorded using a CKX53 Olympus microscope.

Movie 3: Establishment of the stromal cell network. TSC expressing GFP and DOHH2 expressing dTomato were co-encapsulated in presence of matrigel and placed in an Universlide in DMEM containing 10% FCS. The images were acquired immediately after encapsulation every 30 minutes for 12 hours using an epifluorescence microscope (Leica DMI8) equipped with a thermostatic and CO₂ chamber (Live on Stage, Leica). Scale bar: 100µm.

Movie 4: Spatial organization of lymphoma spheroid. 3D reconstruction from optical sections using confocal microscopy of capsule containing DOHH2 with Mg and GFP-TSC cultured for 6 days. Nuclei were stained in blue by Hoechst 33258.

Movie 5: Spatial organization of follicular lymphoma (FL1) spheroid. 3D reconstruction of multicolor live/dead (calcein red in yellow and NucRed Dead in red) staining from optical sections using confocal microscopy of capsule containing FL1 B cells with Mg and GFP-TSC cultured for 3 days. Nuclei were stained in blue by Hoechst 33258.

Movie 6: Spatial organization of follicular lymphoma (FL2) spheroid. 3D reconstruction of multicolor live/dead (calcein red in yellow and NucRed Dead in red) staining from optical sections using confocal microscopy of capsule containing FL1 B cells with matrigel and GFP-TSC cultured for 3 days. Nuclei were stained in blue by Hoechst 33258.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Distribution of (A) the alginate microcapsule size and (B) the number of SUDHL4 cells in capsules immediately after encapsulation.

Figure S2: Limit dilution assays of DOHH2, HLY1 and SUDHL4 cells: A log-fraction plot of the limiting dilution results of DOHH2 (black), SUDHL4 (green), HLY1 cell lines (red). The slope of the line is the log-active cell fraction. The dotted lines give the 95% confidence interval.

Figure S3: Determination of the cell confluence and post-confluence in capsules. Capsules containing SUDHL4 (A) or HLY1 (B) were imaged on brightfield at different times after encapsulation. The diameter of the capsule and the thickness of the alginate walls were then measured manually by image analysis on more than 100 capsules per time point. The diameter (blue line) and wall thickness (red line) are plotted over time and allow the determination of the confluence (light grey) and post confluence (dark grey) periods (data plotted are Mean \pm SEM). Representative images of capsules at D8 (confluence) and D12 (post confluence) at low (scale bar=100 μ m) and high (scale bar=25 μ m) magnification are show below the graphs for both SUDHL4 (A) and HLY1 capsules (B).

Figure S4: Evaluation of cell proliferation and cell death in confluent and post-confluent capsules. Proliferative cells were stained using Click-it EdU cell proliferation kit (ThermoFisher Scientific) as manufacturer's recommendations. Dead cells were detected by propidium iodide labelling. Then, the percentages of proliferative (EdU+) and dead (PI+) cells were measured by flow cytometry.

Figure S5: Detection of hypoxia in SUDHL4 and HLY1 spheroids. Pimonidazole immunocytochemistry of spheroids. Spheroids at confluence or post-confluence were treated for 2h with pimonidazole 200 μ M. Hypoxia was revealed by hypoxyprobe-1 detection kit (brown color) and cells were counterstained with hematoxylin. Scale bar: 50 μ m.

Figure S6: Dose-effect of doxorubicin in SUDHL4 and HLY1 cultured in suspension (2D) or 3D. Cells or spheroids (w/o Mg and TSC) at confluence were seeded in 96-well plates with various concentrations of doxorubicin for 24h or 48h and the IC50 have been determined using the luminescent cell viability assay CellTiter-Glo® 3D cell viability assay (Promega, USA).

Figure S7: Comparative gene expression profile of B-NHL cell lines maintained in 2D or 3D culture with or without TSC. HLY1 and DOHH2 were maintained either in 2D with TSC (HT2D, n=3; DT2D, n=4), in 2D without TSC (H2D, n=3; D2D, n=3), in 3D with Mg+TSC (HT3D, n=3; DT3D, n=5) and in 3D without TSC (H3D, n=3) before sorting of CD20^{pos}CD73^{neg} cells and analysis of gene expression profile by RNAseq. Unsupervised principal component analysis of RNAseq data for HLY1 (A) and DOHH2 (B).

Figure S8: Stromal cells trigger a decrease in DOHH2 proliferation. DOHH2 cells maintained without (D2D) or with (DT2D) TSC in 2D culture were studied by RNAseq and genes upregulated in the absence of TSC were analyzed by GSEA using HallMark MSigDB database.

Figure S1

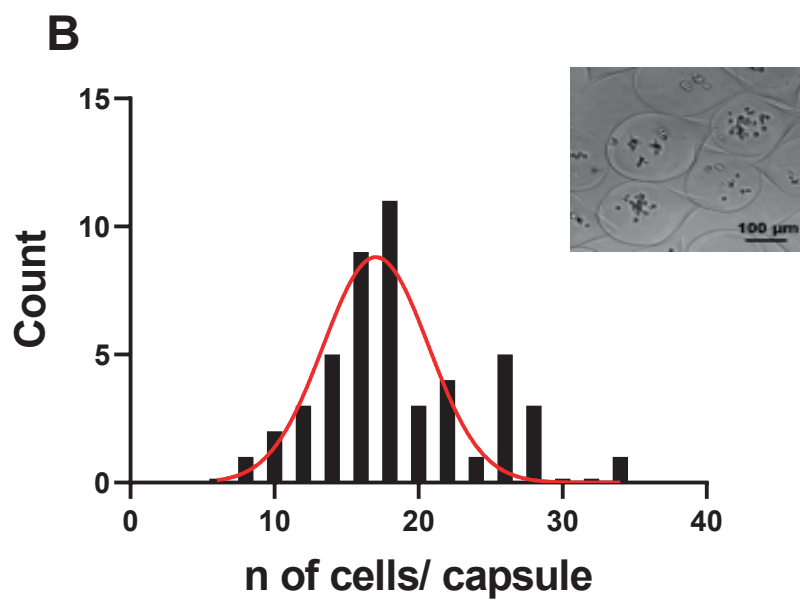
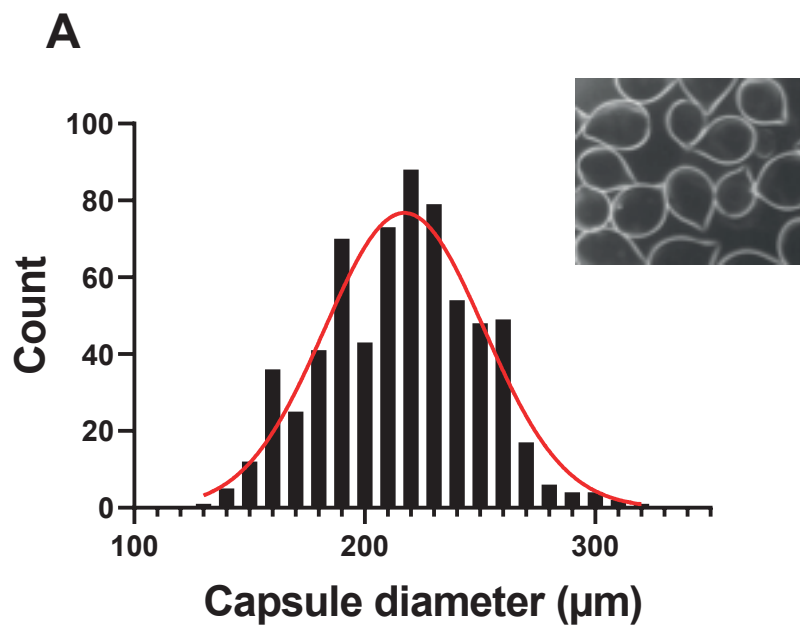


Figure S2

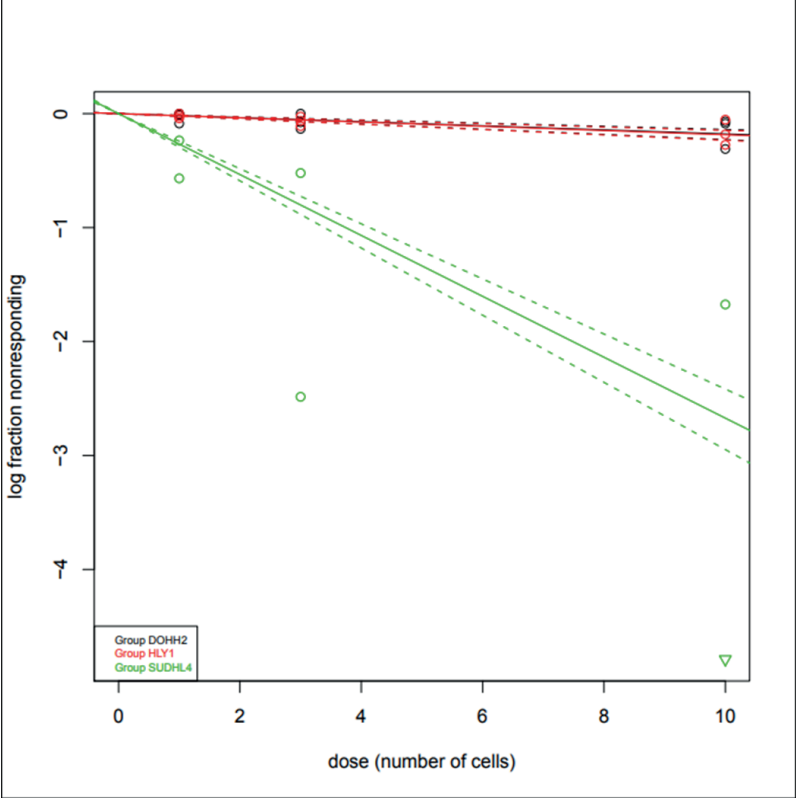
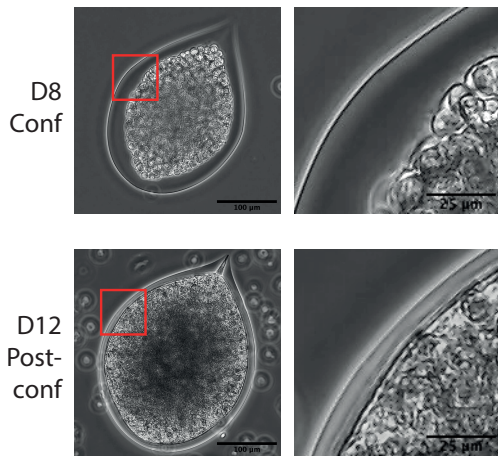
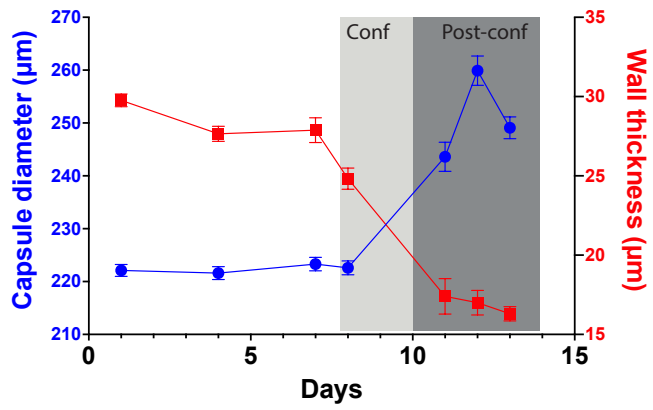


Figure S3

A SUDHL4



B HLY1

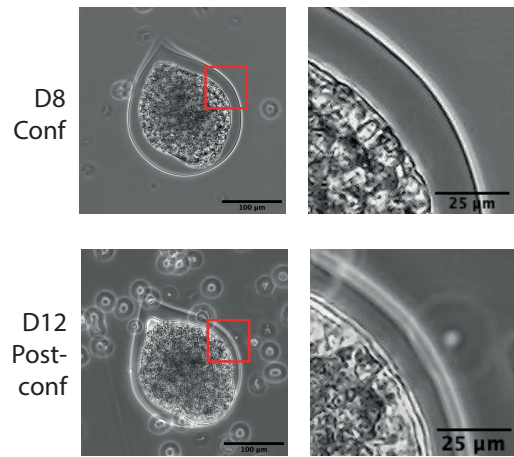
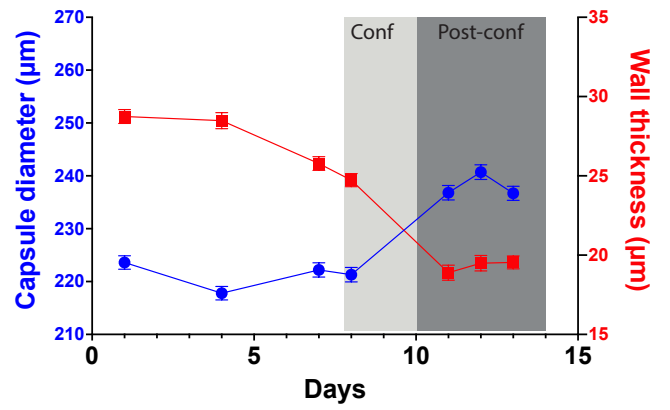


Figure S4

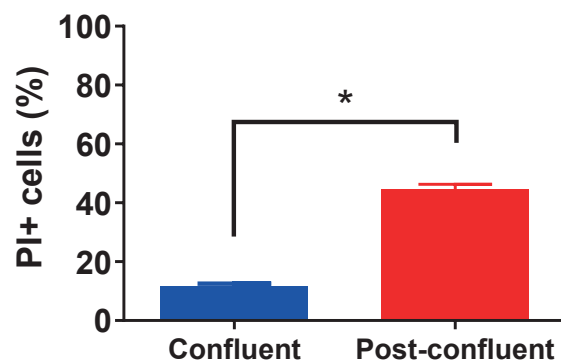
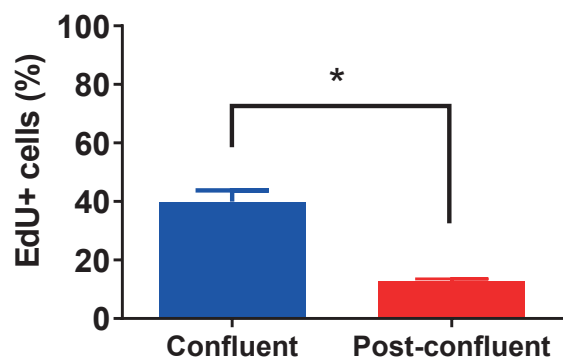


Figure S5

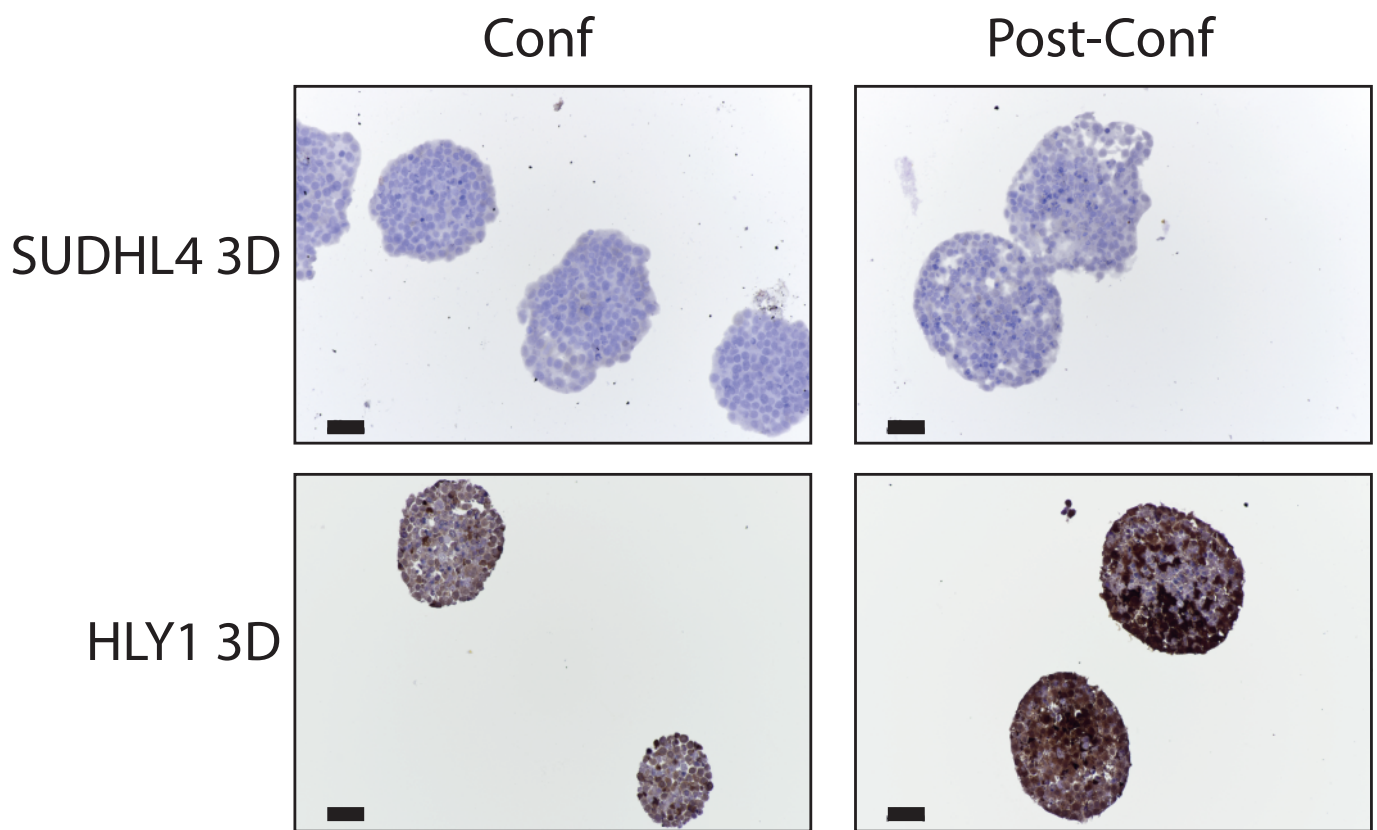


Figure S6

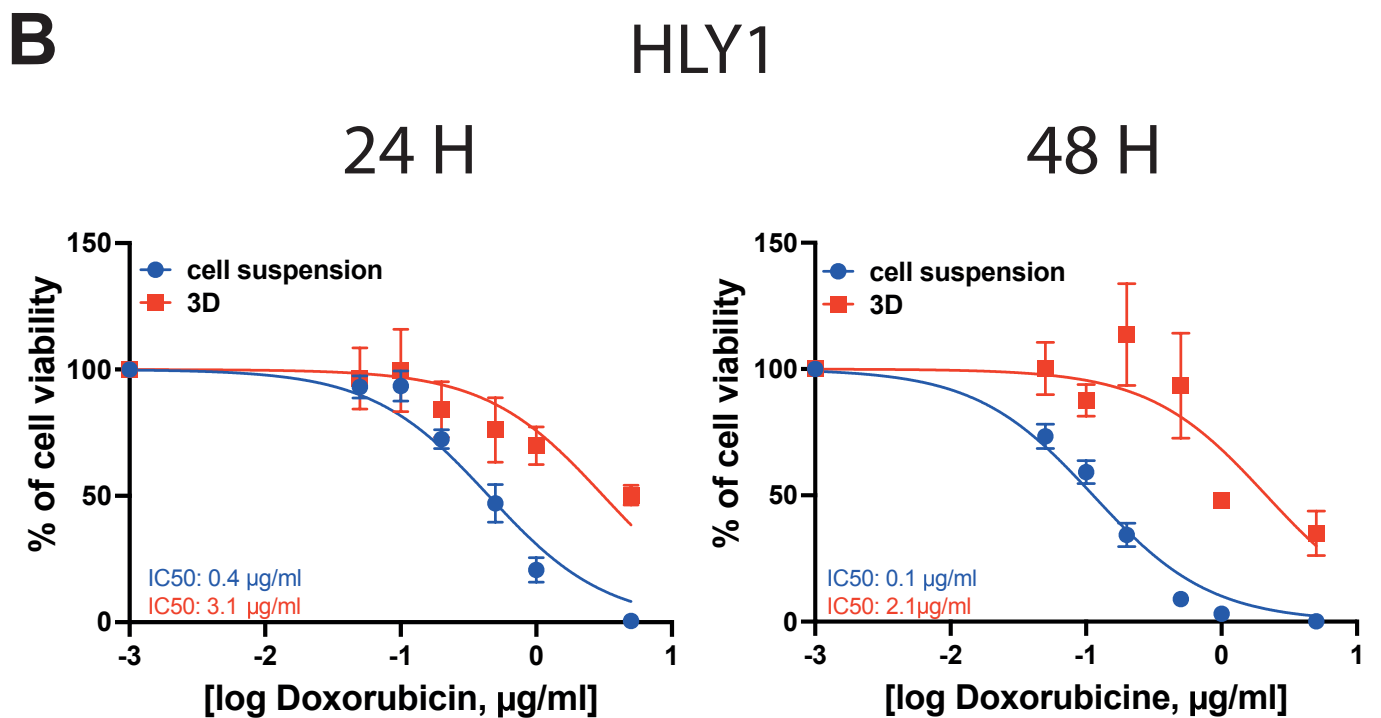
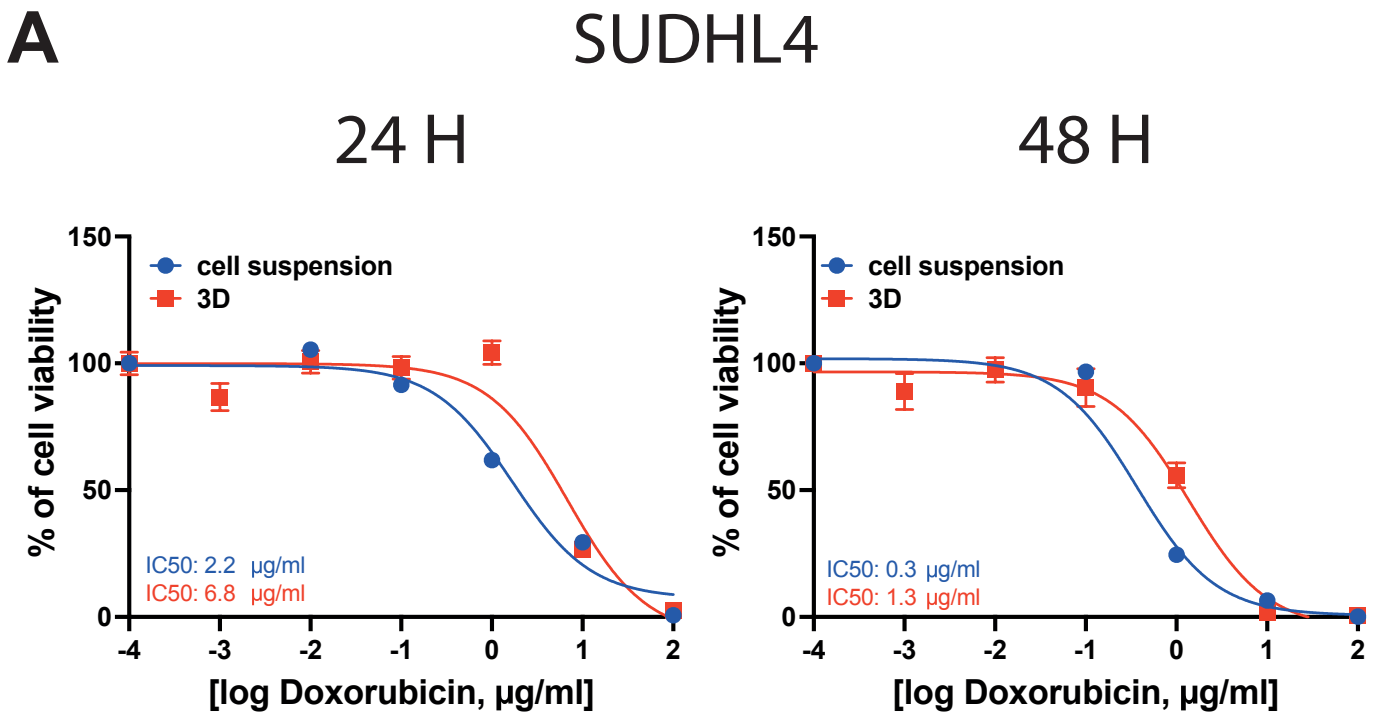
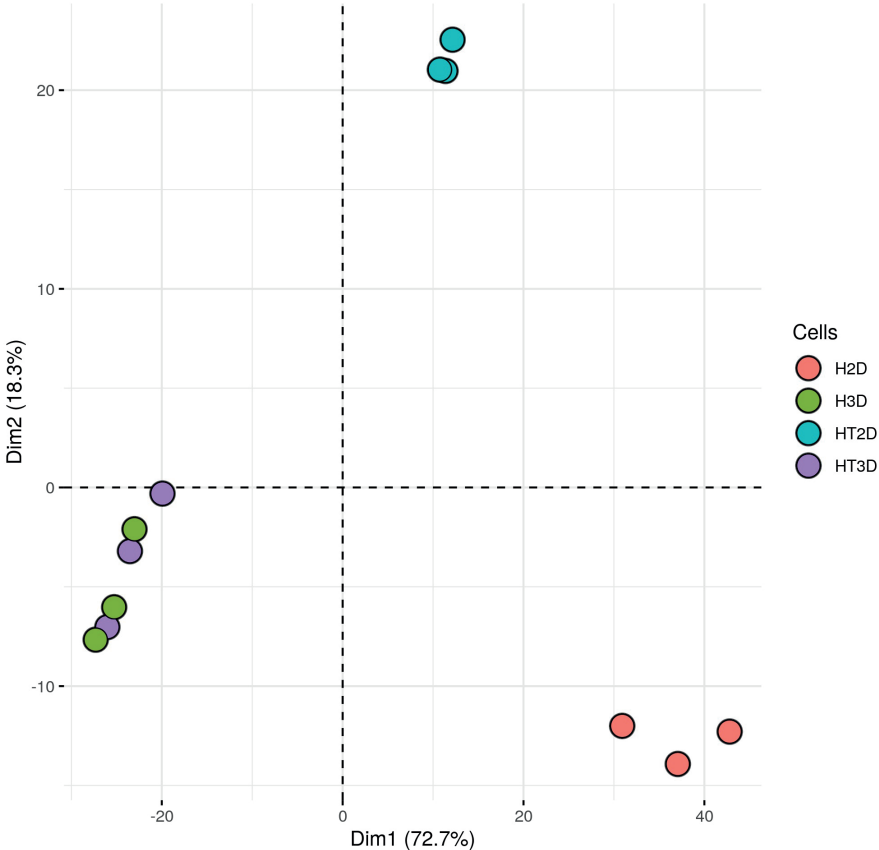


Figure S7

A



B

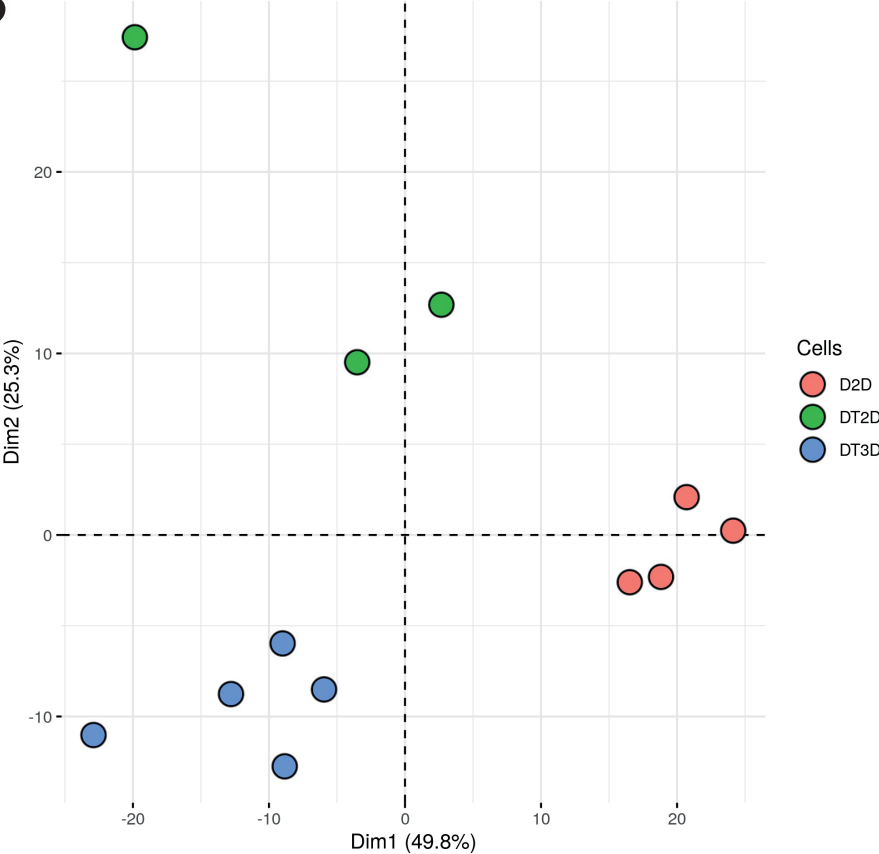


Figure S8

