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ADVANCED MATERIALS

Supporting Information

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Assembly of Multi-Spheroid Cellular Architectures by Programmable Droplet Merging

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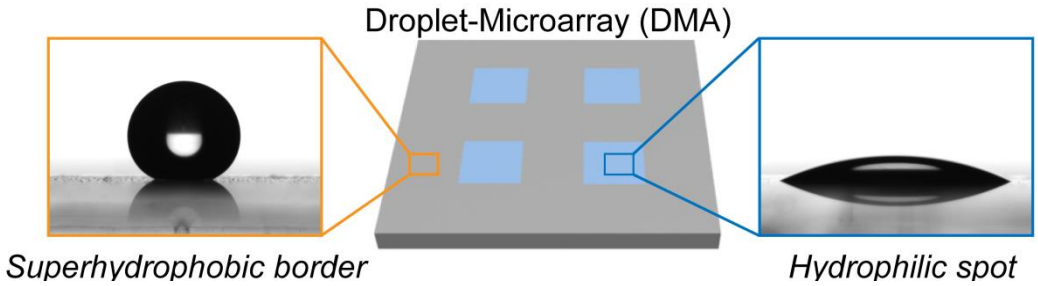


Figure S1. The DMA slide had superhydrophobic borders and hydrophilic spots. The inserted pictures were contact angle images for water droplet. The contact angles (CA) were 151.7° and 24.1° respectively.

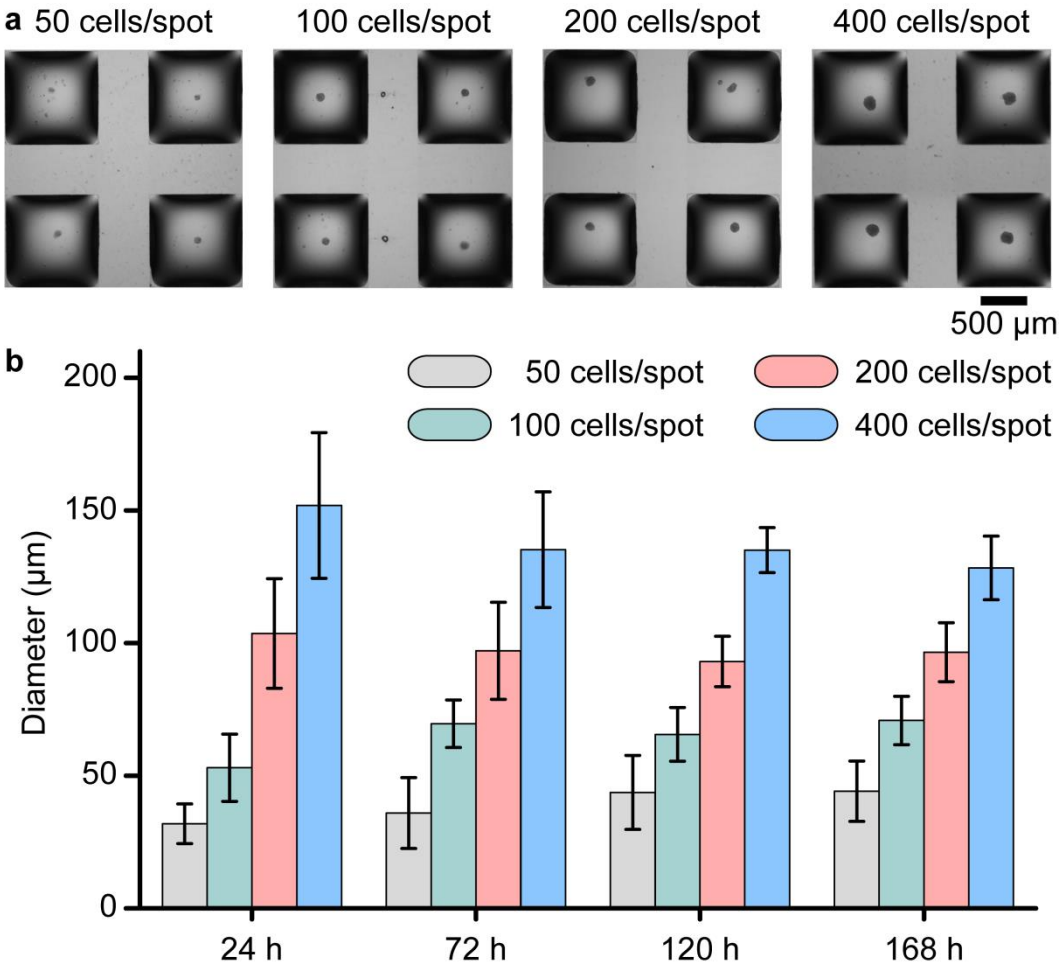


Figure S2. Size distribution of HepG2 spheroids after 24 h, 72 h, 120 h and 168 h. The bigger spheroids could obtain with high cell concentration seeding with I-DOT. a) Typical images of spheroids formed on the DMA with different cell numbers in hanging droplets (50, 100, 200, and 400 cells/spot). b) Measured diameter of spheroids formed on the DMA with different cell numbers (n=40).

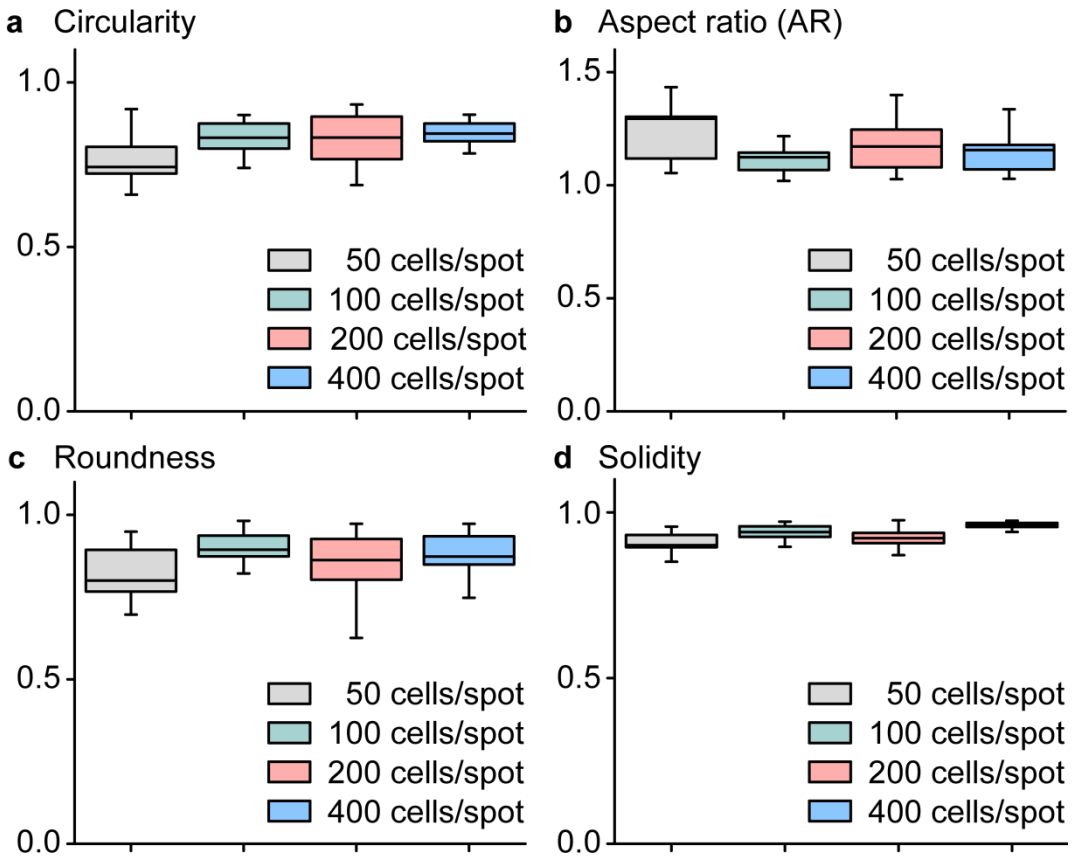


Figure S3. The circularity (a), aspect ratio (b), roundness (c), solidity (d) of prepared spheroids on the DMA with different cell numbers. (50, 100, 200, and 400 cells/spot, the cultivation time was 5 days, n = 30).

Table S1. List of measured circularity, aspect ratio, roundness, and solidity of prepared spheroids on the DMA with different cell numbers.

Cell numbers/spot	Circularity		Aspect ratio		Roundness		Solidity	
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
50	0.74273	0.11803	1.29393	0.29554	0.80063	0.12953	0.90117	0.04865
100	0.83183	0.04628	1.12377	0.08465	0.8943	0.06211	0.9405	0.02166
200	0.83243	0.06817	1.17073	0.12703	0.86283	0.08315	0.9219	0.02882
400	0.8449	0.03333	1.15603	0.12662	0.87343	0.08114	0.96203	0.0087

Supplementary Note 1: Determine conformations of different spheres

```
Initialize empty listOfMatrices

Run n times:

Initialize one sphere
//Build random sphere conformation
For i in range(NumberOfSpheres -1):
      Add sphere at random position on the surface of any existing
sphere.
      Check for colision with other sphere
            If no colision:
                  keep this sphere
            Else:
                  remove sphere and add new with different random
             numbers

//Check if new conformation
For all permutations of Sphere indices:
      Calculate neighborhood matrix
      Check if neighborhood matrix is in listOfMatrices
If not found any match:
      Found new conformation
      Add matrix to listOfMatrices
```

In order to specify the possible conformations that an ensemble of N connected spheroids can have, an algorithm was developed to sample this phase space. Pseudocode of the algorithm to determine the number of confirmations for aa given number of spheres. The length of the listOfMatrixes corresponds to the number of confirmations found. n is the number of iterations. The algorithm starts with one sphere at the surface of that sphere another sphere is added. The position is chosen by randomly sampling the three dimensional angle space *via* spherical coordinates. To add further spheres a random existing sphere is selected and at its surface a new sphere is placed at randomly chosen angles. The new sphere may overlap with existing spheres, which is to be avoided, so a check is performed if the sphere collides with one of the existing spheres. If a collision is found this sphere is removed and another attempt with different random numbers is made. This is performed until no collisions occur and the

new sphere is added. This cycle is performed until the desired number of spheres NumSpheres is reached. The resulting ensemble of spheres is now a candidate of a new conformation.

To identify conformations a NumSphere × NumSpheres neighborhood matrix is created, if a pair of cells is touching the corresponding matrix elements are set to one. Spheres have a radius of 1, of the spheres are within the distance of 2.2 it is assumed they touch. This neighborhood matrix is compared to all already found matrices, if there are no matches the conformation is new and also saved. Spheroids are assumed to be indistinguishable; therefore the identification of a state has to be independent of the numbering. This is achieved by comparing the neighborhood matrices of all possible permutations of the numbering of the current conformation the already found conformations. Only if none of the possibilities match the preexistent possibilities, the conformation is recognized as new. This loop is performed until no new conformations are found for a large number of iterations. The random process does not ensure to find all possible conformations, since the phase space grows exponentially with the number of spheres. Up until five spheres the sampling has certainly converged, for higher numbers of spheres the number found serves as a lower bound.

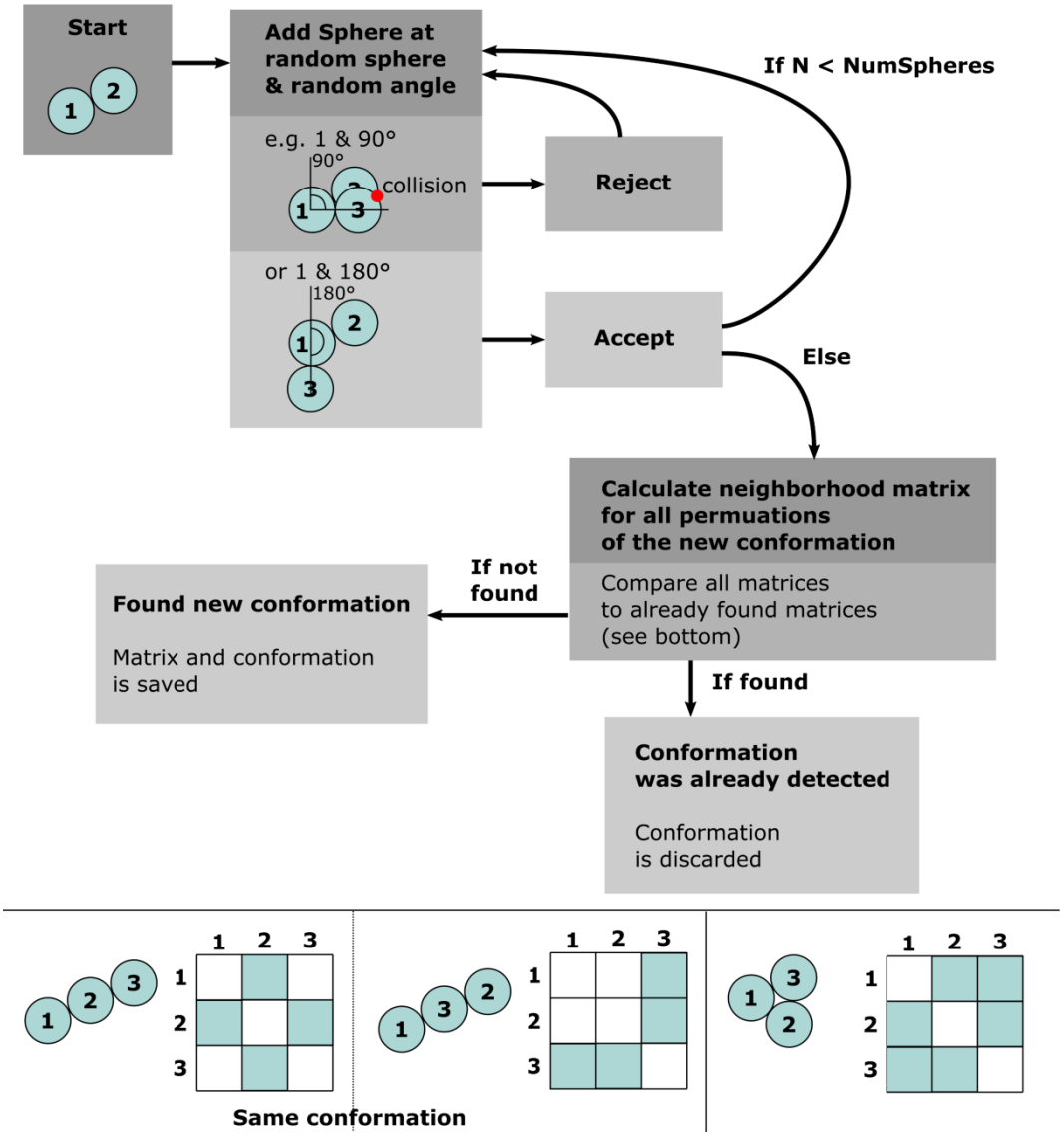
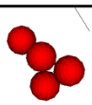


Figure S4. Simulation method to determine conformation space. Depicted for two dimensions for illustration, algorithm is implemented in three-dimensional space.

Table S2. Number of conformations for the number of spheres. (Output of the scanning algorithm. n indicates the number of iterations.)

Number of spheres	Number of conformations
1	1
2	1
3	2 (n=10000)
4	6 (n=10000)
5	20 (n=10000)
6	>98 (n=8*10^6)
7	>250 (n=300000)

3 spheres		4 spheres					
							
85 %	15 %	48 %	19 %	28 %	4 %	1 %	0.3 %

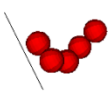
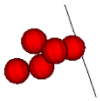
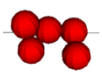
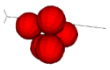
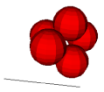
5 spheres							
							
20 %	14 %	27 %	13 %	7 %	2 %	2 %	5 %
							
0.6%	4 %	2 %	0.1 %	1 %	0.9 %	0.2 %	0.3 %
							
0.1 %	0.01 %	0.04 %	0.01 %				

Figure S5. Simulation of the combination and probability of 3, 4, or 5 spheres.

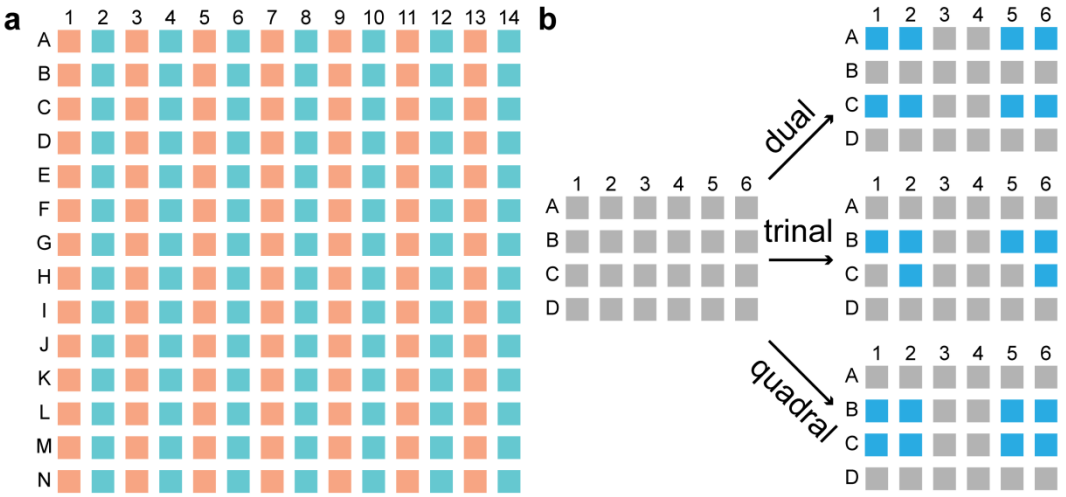


Figure S6. Printing program for two different cell types. a) The odd columns and even columns represent two different cell lines seeded on the DMA. For example, the HEK 293T (TOP-GFP) cells are printed on odd columns and the Wnt3a transfected HEK 293T cells are seeded on even columns. b) The printing program used for merging different number of droplets. The gray squares represent 200 nL medium initially printed. The blue spots represent the second printing step: 900 nL for each in fusing 2 or 4 droplets, 850 nL for each in fusing 3 droplets.

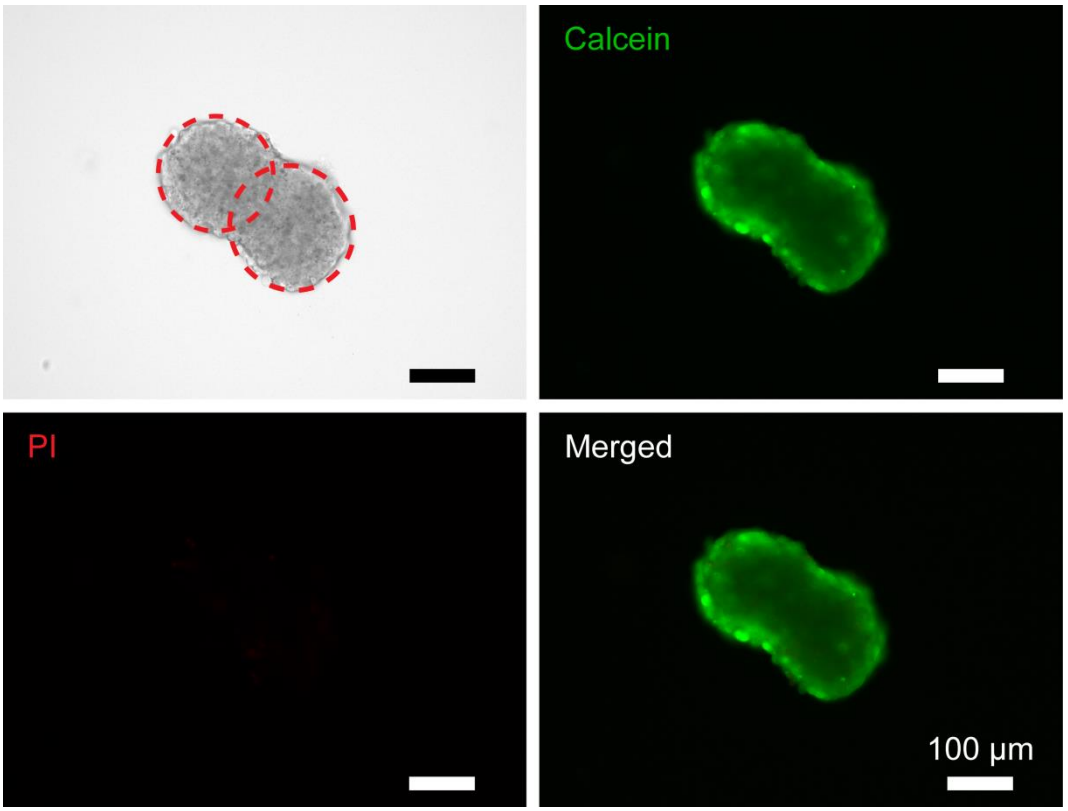


Figure S7. The cell viability of merged spheroids was showed by live/dead staining after 24 h post-fusion. The HepG2 cells were stained by Calcein (green fluorescence) and PI (red fluorescence).

Supplementary Note 2: Simulate merging of two spheroids

The fusion of two spheroids consisting of 400 cells each was simulated using the simulation package Nastja-CellsInSilico. More details on simulation method and the underlying energy function can be found in previous work.[1]

Two spheres of cells with 150 µm diameter were placed in a simulation box with surrounding liquid. The fusion was driven by the cell-cell adhesion between the individual cell and a repulsive interaction between cells and liquid.

The simulations are based on the cellular Potts model, which is a lattice-based model for tissue dynamics simulations. Each biological cell occupies a connected area on a three-dimensional lattice. The dynamics are determined a Hamiltonian energy function:

$$H = \sum_{Cells} \lambda_{Vcell}(V_0 - V)^2 + \sum_{Cells} \lambda_{S\,cell}\ (S_0 - S)^2 + \sum_{Cells} \sum_{neighbors} J_{cell-neighor} * \Delta S * \left(1 - \delta(cell, neighbor)\right) + \lambda_{motility} * RandomVector$$

Consisting of the energy terms for:

Adhesion:

Cell – cell and cell – liquid interactions. Energy terms are proportional to the shared surface of two cells or a cell and the liquid.

Surface:

Surface constraining term for each cell with a square potential. $\lambda_{S\,cell}$ defines the cell surface compressibility. The surface is calculated via the marching cubes algorithm.

Volume:

Volume constraining term for each cell, defining the number of voxels a single cell occupies on the lattice. $\lambda_{V\,cell}$ defines the cells volume compressibility.

Motility:

To model thermal fluctuations as well as undirected cell migration each cell has an independent vector potential that it is coupled to. The direction of this vector is randomly reassigned 200 time steps. The cell is coupled to that potential via the coupling constant $\lambda_{motility} = 60$. This results in a random walk around the position of the cell.

Monte Carlo Step:

The system propagation is performed by incremental changes on cell surfaces. In a Monte Carlo Step the value of a voxel can be changed to be the value of one the nearest neighbor values. The energy difference of that change is evaluated and the step is accepted or rejected using the metropolis algorithm.

Timestep:

The timestep is defined by so called Monte Carlo Sweeps (MCS). In each MCS a randomly distributed subset consisting of $\frac{1}{25}$ of all voxels in the grid is iterated through and a Monte Carlo Step is attempted on each of these voxels.

A Monte Carlo Sweep (=MCS) is a sweep over the field that performs a Monte Carlo Step on $\frac{1}{25}$ of all voxel in the simulated field. So, the Monte Carlo Step is local time propagation at one time and a Sweep is the propagation of the entire field.

The simulation is run on 45 cores on a single node on the JUWELS supercomputer for 12 hours to achieve 500 kMCS. The dynamics of the enclosing angle between the two spheres as well as the neck diameter were analyzed and plotted against time using Python.

Table S3. Parameter overview of the 3D simulations using CellsInSilico.

Volume V_0	4400 μm^3	l_V	5
Surface S_0	1800 μm^2	l_S	5
Adhesion	Cell Cell 60 Cell Medium -60	Temperature	60
Motility vector recalculation time	200MCS	Simulation box	180x180x450 μm^3
Motility coupling	60		

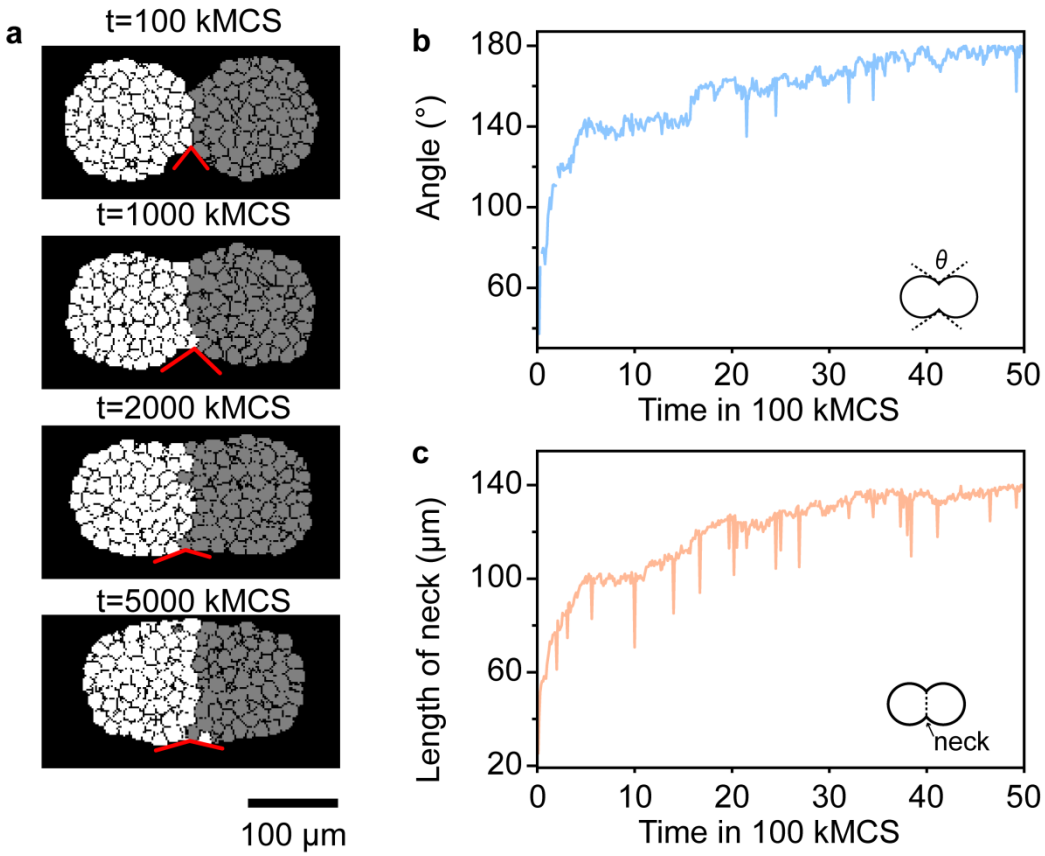


Figure S8. Simulation results of spheroids fusion process. a) Two dimensional slices through the center of the spheroids. Red lines indicate the measured angle. b) Plot of the enclosing angle of the two spheroids and the neck width over time.

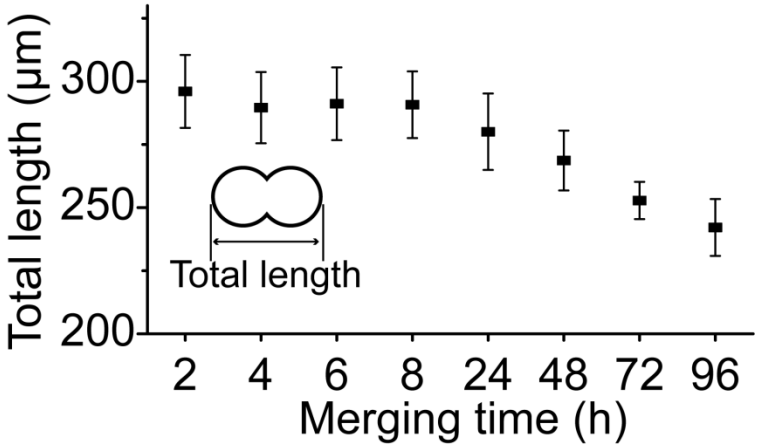


Figure S9. Plot of the total length of merging spheroid (in Figure 3) over the time. n=10 (10 different merged spheroids). Error bars represent SD.

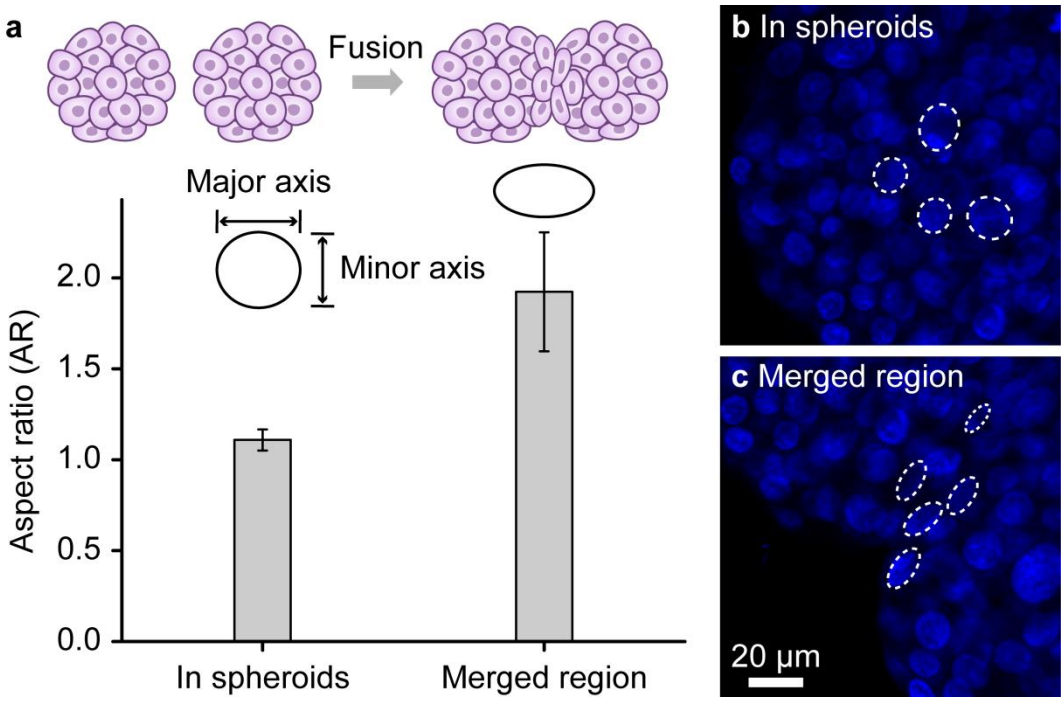


Figure S10. Deformation of nuclei within the merged region of fused spheroids. a) The schematic representation of the process of merging of two spheroids (upper panel), and the graph showing the aspect ratio of cell nuclei in spheroids and in merged region of fused spheroids 24 hours post-fusion. The aspect ratio of nuclei was defined as the ratio of major axis to minor axis. (Error bars represent SD, n = 30). b,c) Fluorescent microscope images of center of individual spheroid (b) and merged region of fused spheroids (c) stained with DAPI (blue fluorescence; cell nucleus).

Reference:

[1] M. Berghoff, J. Rosenbauer, F. Hoffmann, A. Schug, *BMC Bioinformatics* **2020**, *21*, 436.