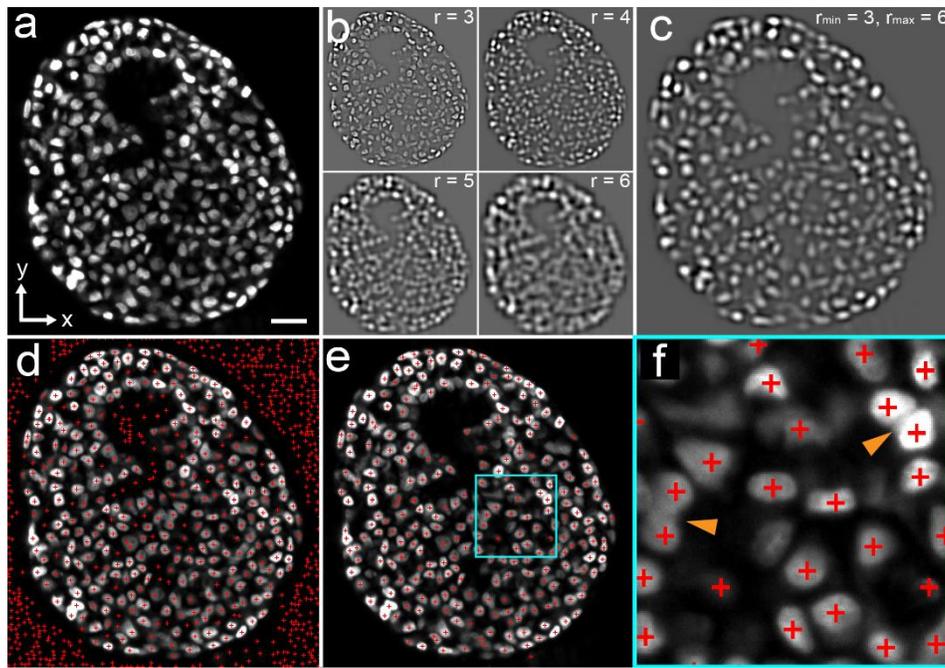


Supplementary Information

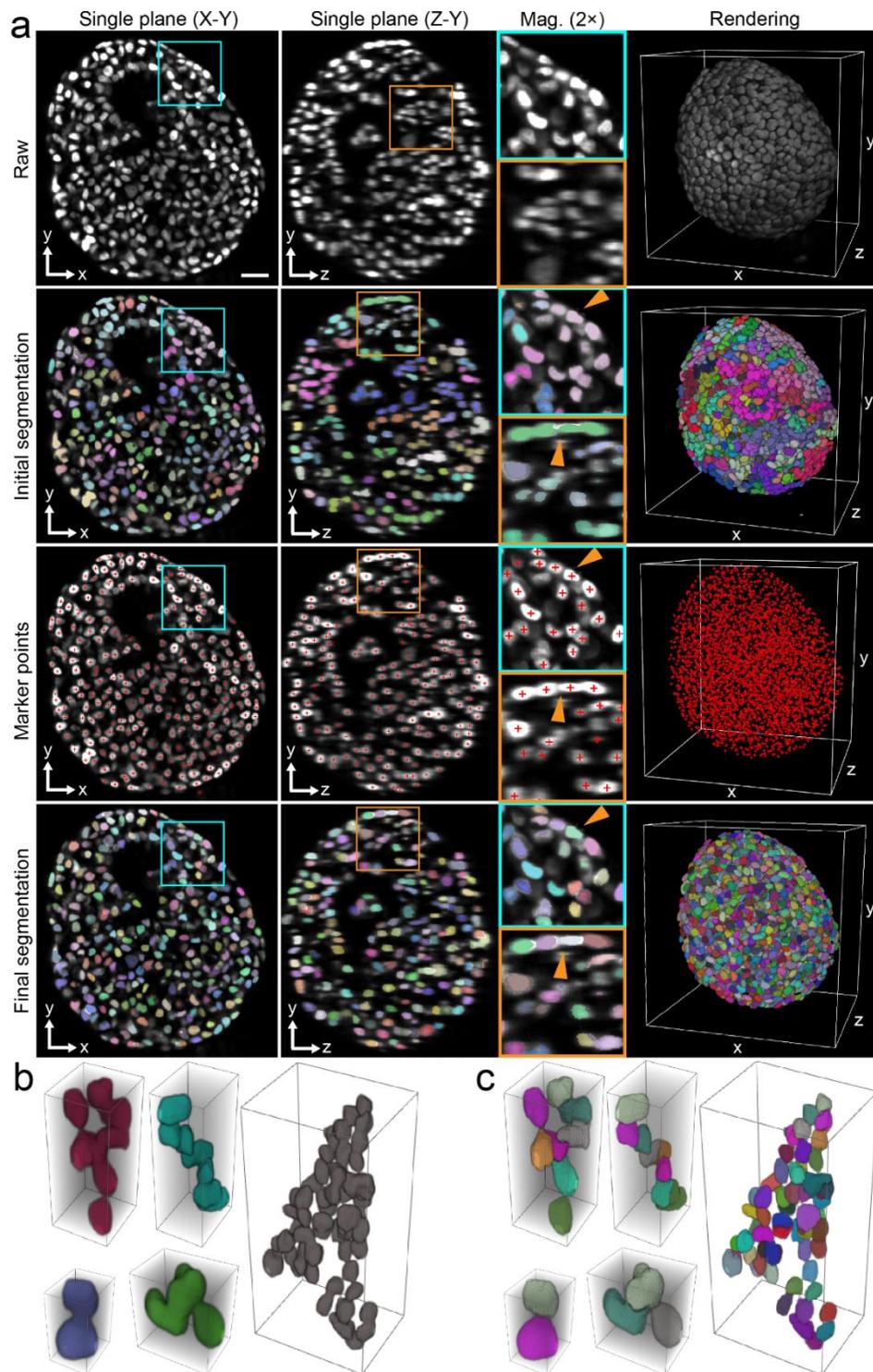
Multiscale image analysis reveals structural heterogeneity of the cell microenvironment in homotypic spheroids

Alexander Schmitz[°], Sabine C. Fischer^{°*}, Christian Mattheyer, Francesco Pampaloni and Ernst H.K. Stelzer

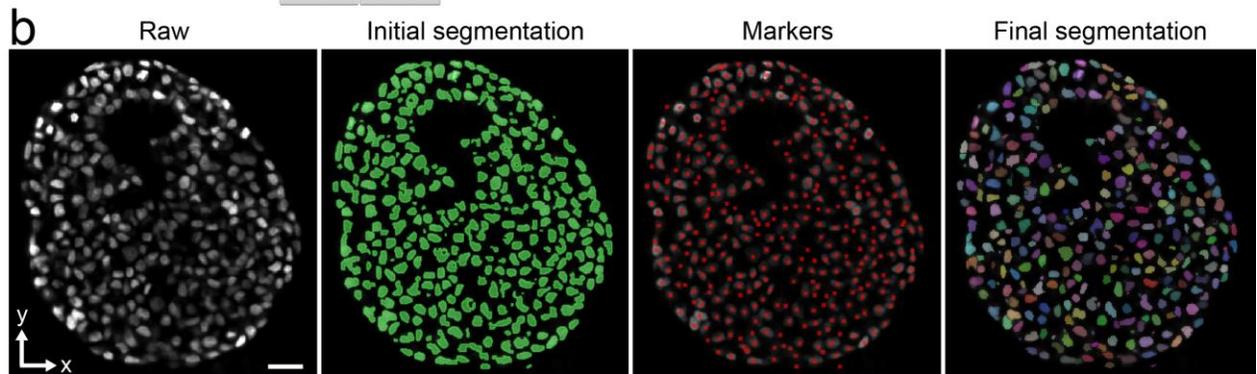
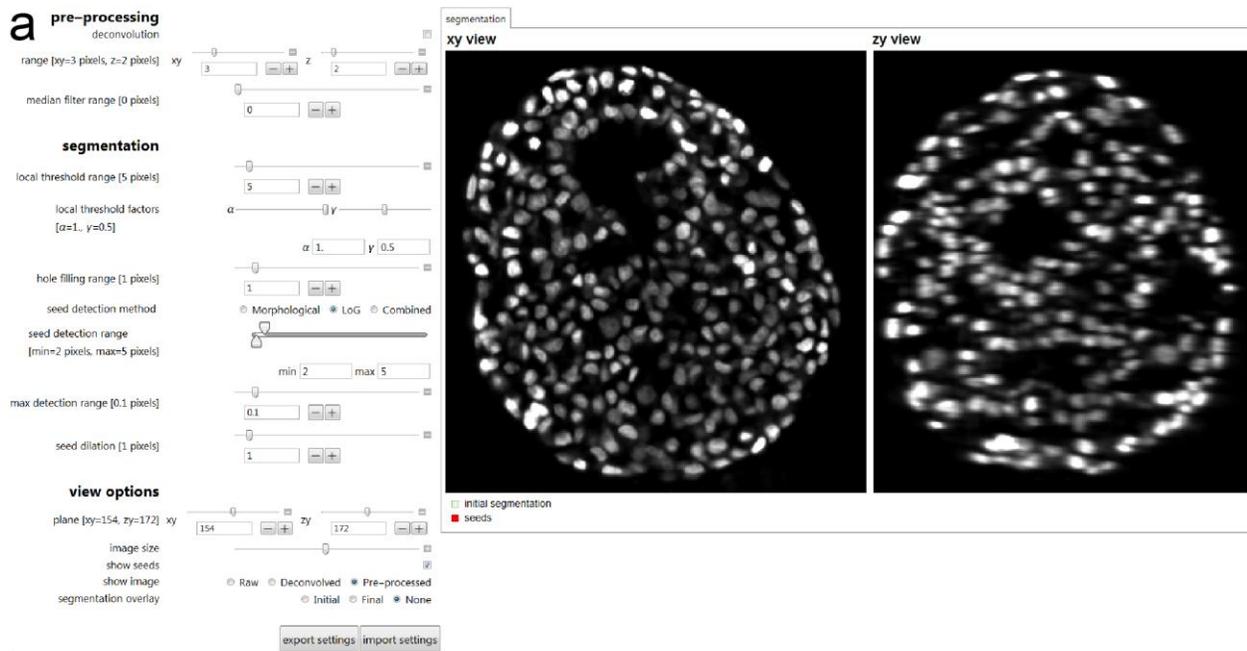
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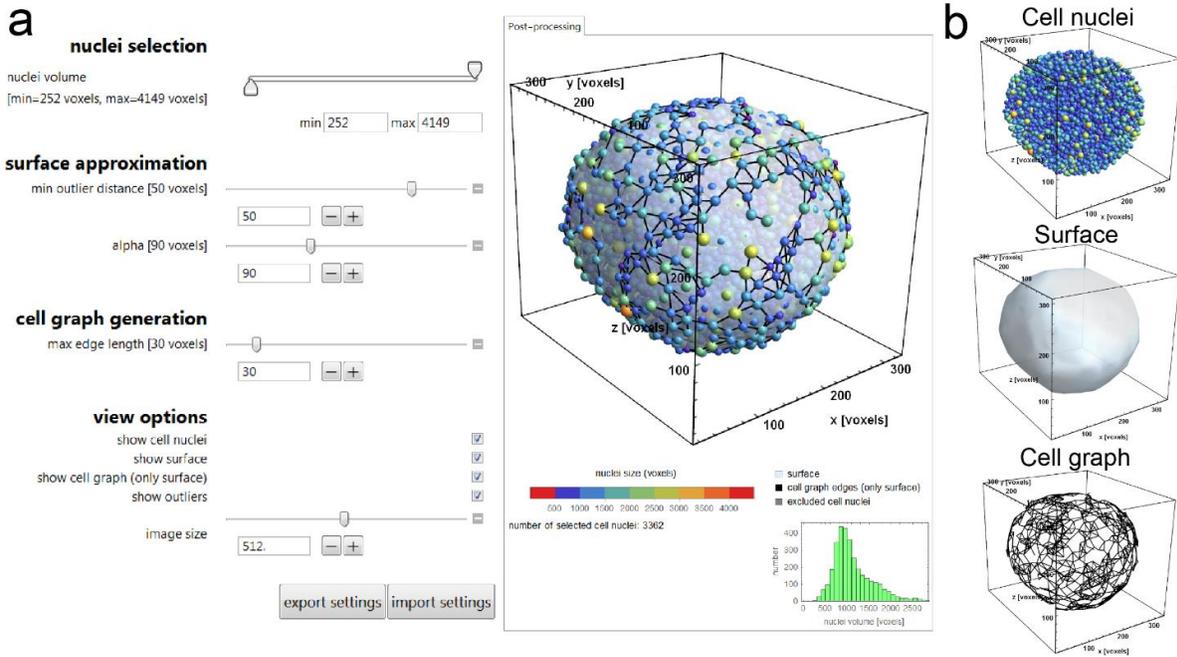
Supplementary Figure 1 Marker point detection with the Laplacian of Gaussian filter. (a) Sub-region of a single plane of the raw volume along X-Y. (b) Resulting images of the Laplacian of Gaussian (LoG) filter applied to the image in (a) for different values of the LoG filter range r in pixels. (c) The response images for different values of r are combined to provide a maximum response image. (d) Marker points detected by an extended local maxima search displayed as red crosses in the raw image. (e) Detections in the background region are discarded by multiplying the detected maxima image with the initial segmentation result. (f) Magnification of the indicated region (cyan box) in panel e. The marker point detection method accurately detects cell nuclei. For apparently touching cell nuclei (orange arrowheads), unique marker points are identified that define starting points for the subsequent watershed algorithm. Please note that for the purpose of this illustration, the marker point detection was applied in two dimensions. In the pipeline it is applied in three dimensions Scale bar: 25 μm .



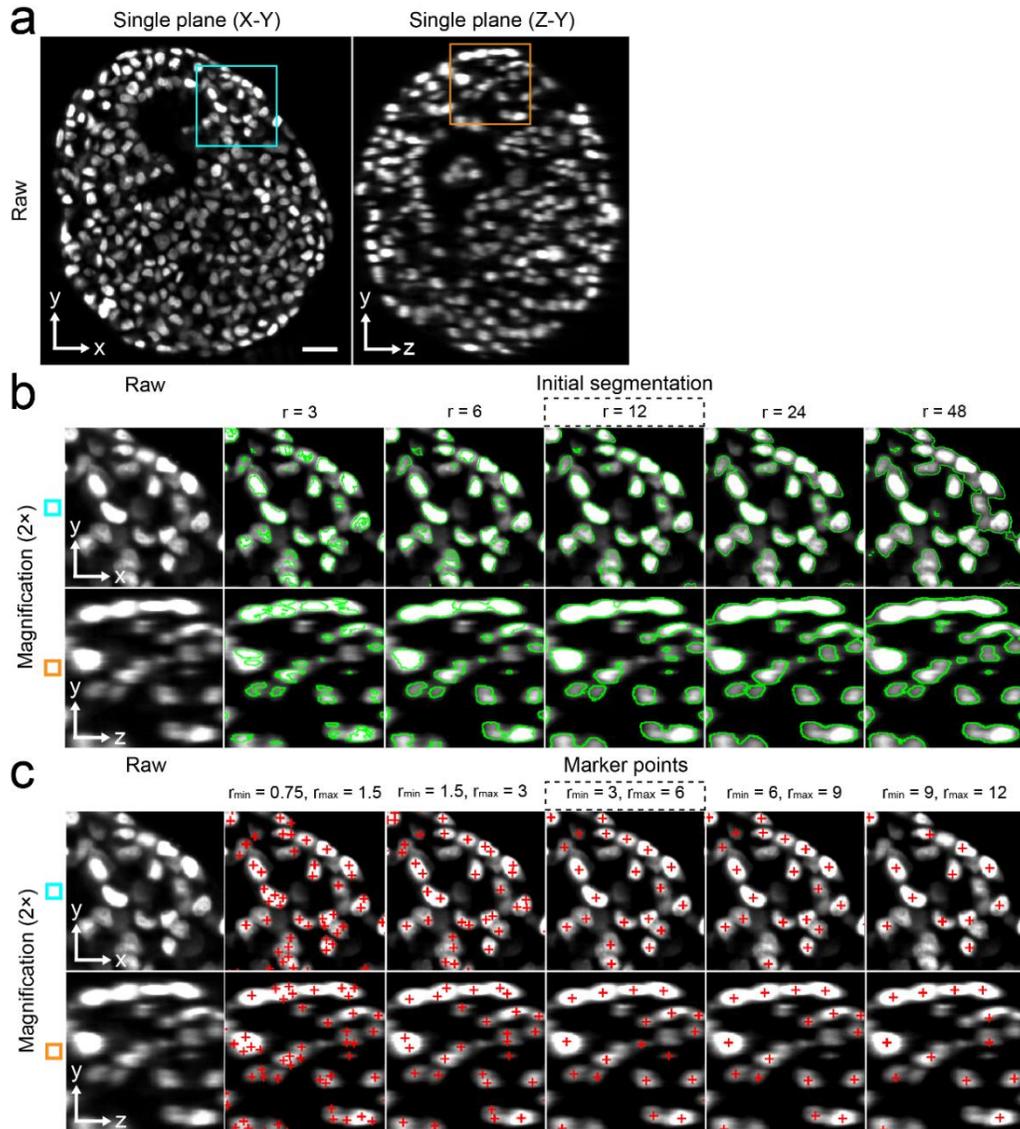
Supplementary Figure 2 Overview and evaluation of the steps of the cell nuclei segmentation. The individual steps of the cell nuclei segmentation are shown for dataset S9. Shown are single planes along X-Y (first column), Z-Y (second column) at about the center of the spheroid, two-fold magnifications (Mag. 2 \times) of regions indicated by cyan and orange boxes (third column) and three-dimensional renderings (fourth column). The obtained components after the initial segmentation are colored and overlaid on the raw image (second row). The range parameter r for the initial segmentation was set to 12 pixels. The initial segmentation identifies the foreground region in the image, but fails to separate apparently touching cell nuclei (orange arrowheads). Unique marker points (indicated by red crosses) are identified by the LoG filter (third row). Parameter values for r_{min} and r_{max} were set to three and six, respectively. In the final segmentation (fourth column), apparently touching cell nuclei are separated (orange arrowheads). Three-dimensional renderings of clusters of connected cell nuclei after connected component labelling of the initial segmentation (b) and after the final segmentation (c). Different colors represent individual components. Please note that to generate the images in the third row, the marker point detection was performed in two dimensions. In the image analysis pipeline, the marker detection is three-dimensional. Scale bar: 25 μ m.



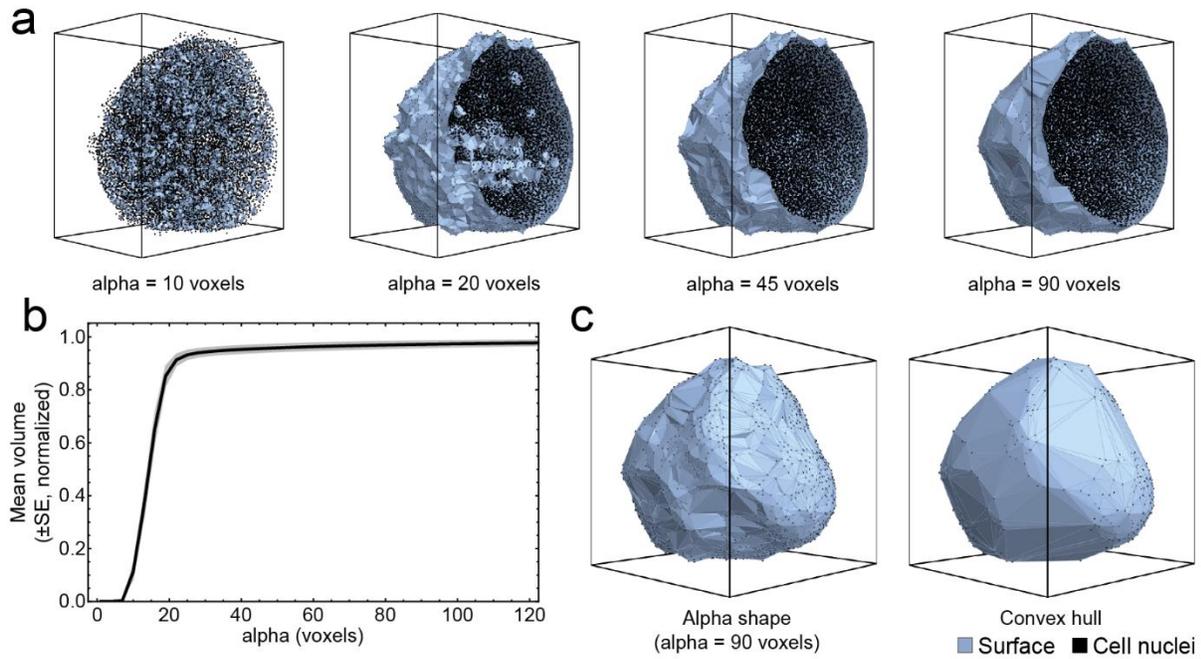
Supplementary Figure 3 User interface for cell nuclei segmentation. (a) Main user interface with control elements to adjust values of the segmentation parameters and two panels of the current image plane in X-Y and Z-Y. (b) Different modes of visualization overlaid on the raw image. The initial segmentation, the detected marker points and the final segmentation result after watershed. Note that the program will display the segmentation result for the current image plane and continuously update the displayed image. The chosen settings are then used for three-dimensional segmentation. The dataset shown is S9. Scale bar: 25 μ m.



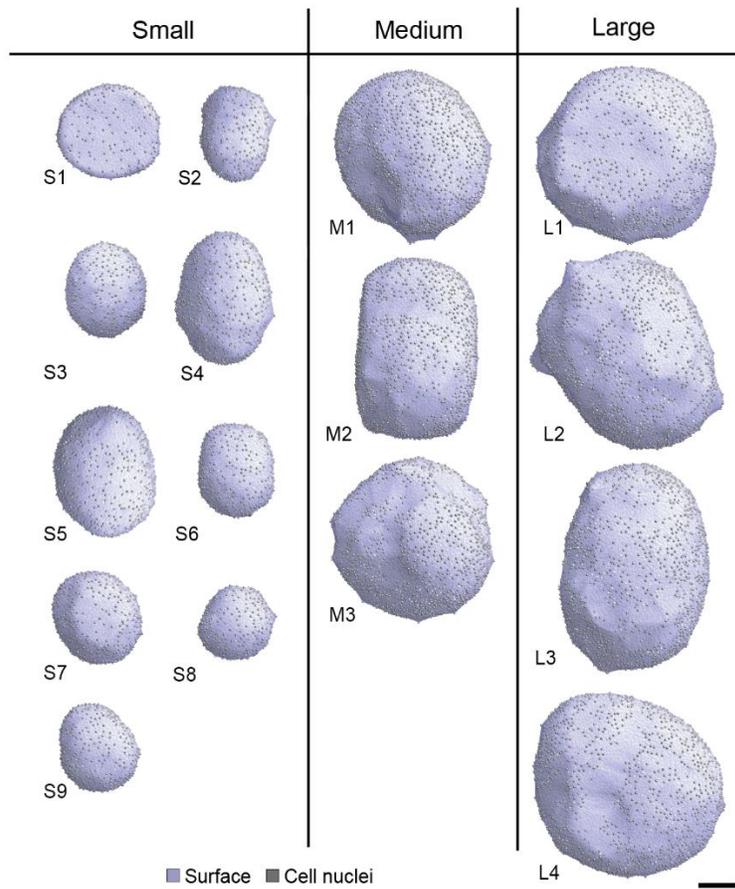
Supplementary Figure 4 User interface for post-processing. (a) Main user interface with control elements to adjust the values of the post-processing parameters. The resulting selection of cell nuclei as well as the surface of the alpha shape and the cell graph are continuously updated and shown on the right. (b) Different modes of visualization. The images depict cell nuclei rendered as spheres and colored according to the volume, the alpha shape surface and the cell graph for cell nuclei on the surface. The dataset shown is S9.



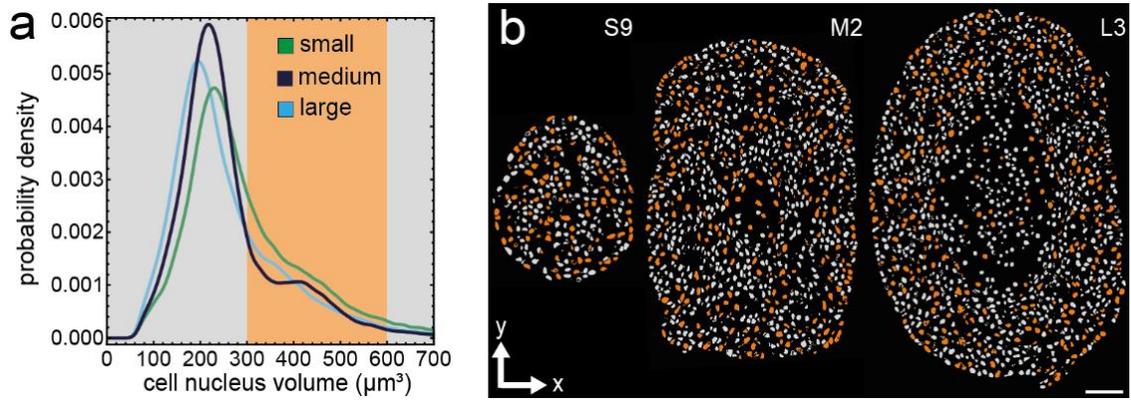
Supplementary Figure 5 Evaluation of parameter values for initial segmentation and marker point detection. (a) Representative single planes along X-Y and Z-Y of dataset S9 at the center of the spheroid. Scale bar: 25 μm . The results of initial segmentation (outlined in green) for different values of the range parameter r (b) and the results of marker point detection (depicted by red crosses) for different values of the parameters r_{min} and r_{max} (c) are shown in two-fold magnification for the regions indicated by cyan and orange boxes in panel a. The parameter values that were used in our study are indicated by a dashed box. Please note that the initial segmentation was applied as described in the Methods section, whereas for illustration purposes the marker point detection was applied in two dimensions. In the image analysis pipeline, the marker point detection is performed in three dimensions. A reasonable starting value for the range parameter of the initial segmentation is the average diameter of cell nuclei (about 12 pixels in the underlying datasets). However, the initial segmentation proved to be robust towards small deviations. Over-segmentation and under-segmentation occur for large deviations. For the marker point detection, reasonable values for the parameters for r_{min} and r_{max} are the minimal and maximal radius of cell nuclei in the dataset (three pixels for r_{min} and six pixels for r_{max}). The marker point detection is more sensitive to the choice of the parameter values. For example, lower values (e.g. $r_{min}=1.5, r_{max} = 3$) lead to the detection of parts of cell nuclei, whereas for higher values (e.g. $r_{min}=9, r_{max} = 12$) clusters of cell nuclei are detected instead of individual cell nuclei.



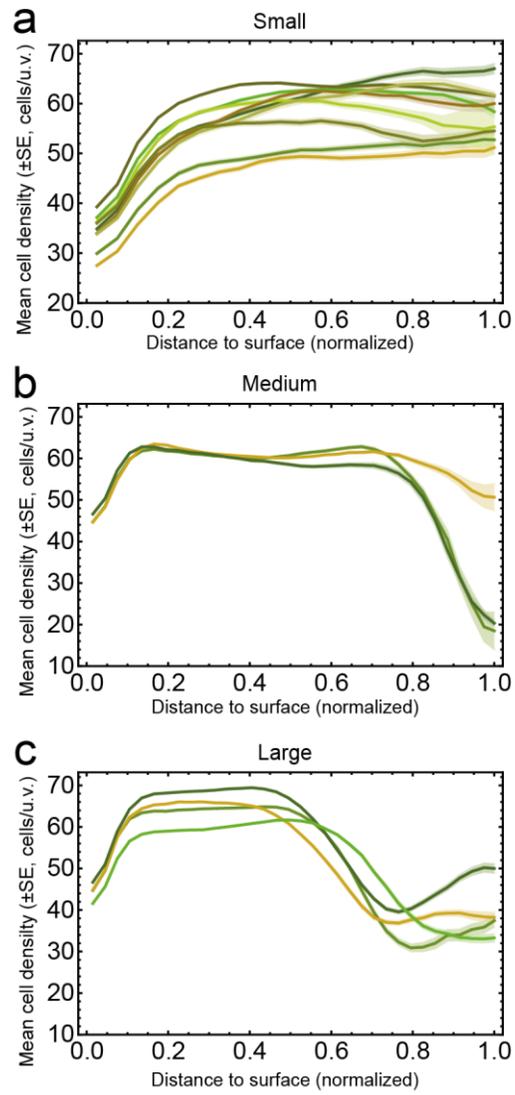
Supplementary Figure 6 Surface approximation using alpha shapes. (a) Alpha shape surface of dataset M3 for different values of the parameter alpha. Cell nuclei centroids are plotted as black points. (b) Mean volume of all datasets normalized to the volume of the convex hull as a function of alpha. The shaded region indicates the standard error of the mean (SE). (c) Alpha shape surface for alpha set to 90 voxels (left) and convex hull (right) for dataset M3.



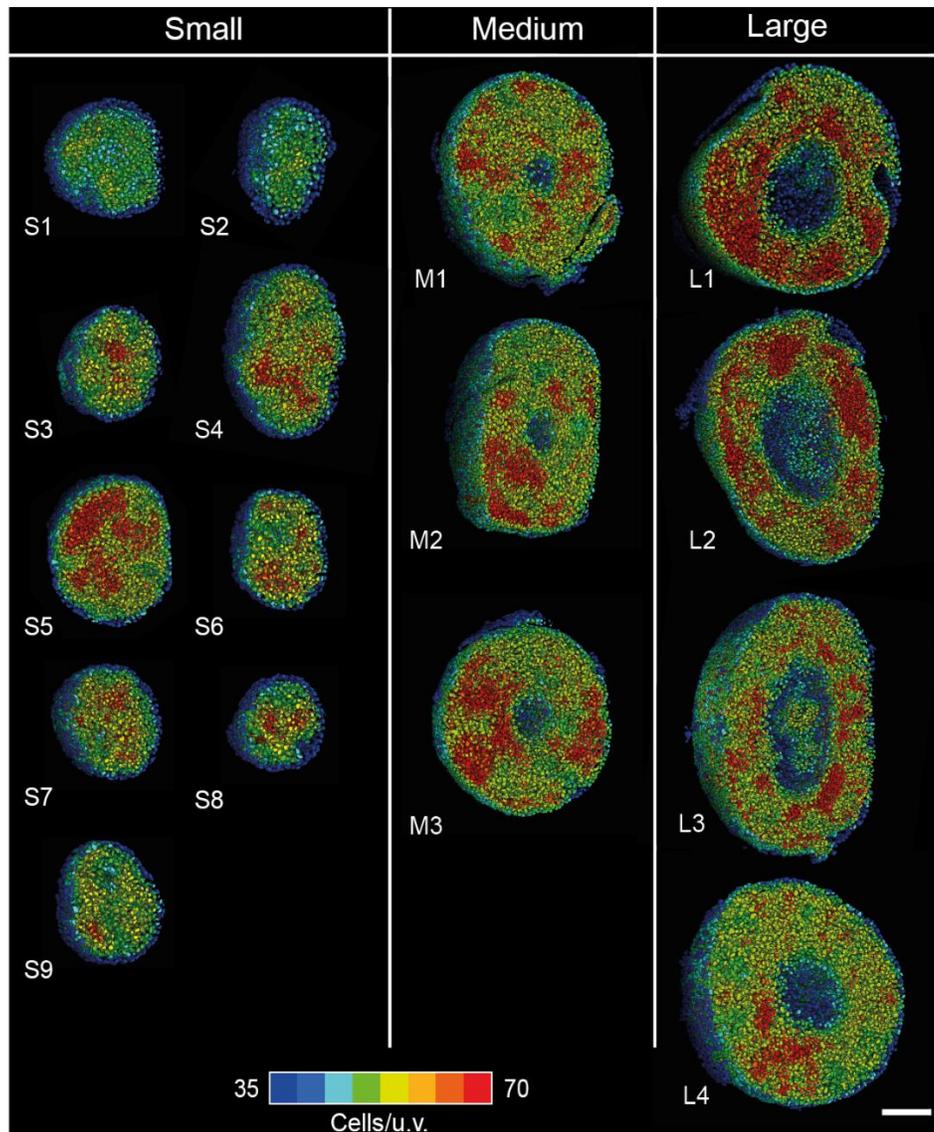
Supplementary Figure 7 Surface approximation for all datasets. For a complete list of datasets see Supplementary Table 4. Scale bar: 50 μm .



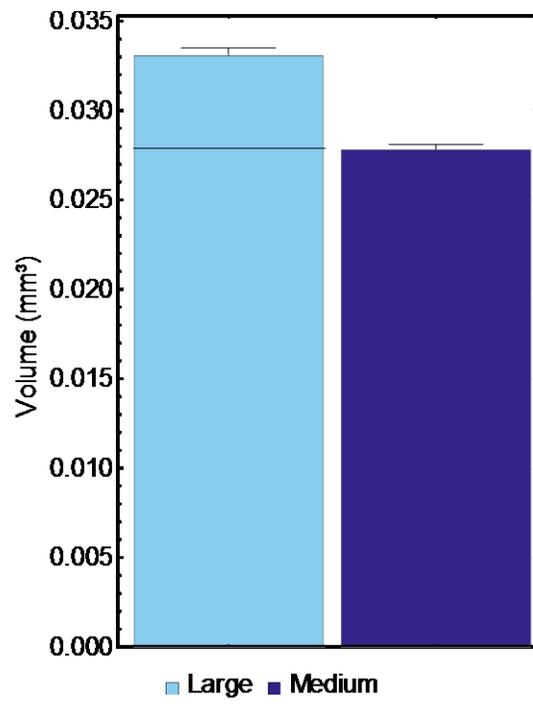
Supplementary Figure 8 Spatial localization of cell nuclei with a volume between 300 and 600 μm^3 . (a) Smoothed histogram of the cell nuclei volume distribution of small, medium and large spheroids. Cell nuclei with volumes in the region highlighted in orange (300 to 600 μm^3) were colored in the segmentation images to analyze their spatial localization. (b) Single planes of datasets S9, M2 and L3 at the center of the spheroid. All cell nuclei with a volume of less than 300 μm^3 or greater than 600 μm^3 are depicted in gray. The cell nuclei with a volume between 300 μm^3 and 600 μm^3 are colored in orange. Scale bar: 50 μm .



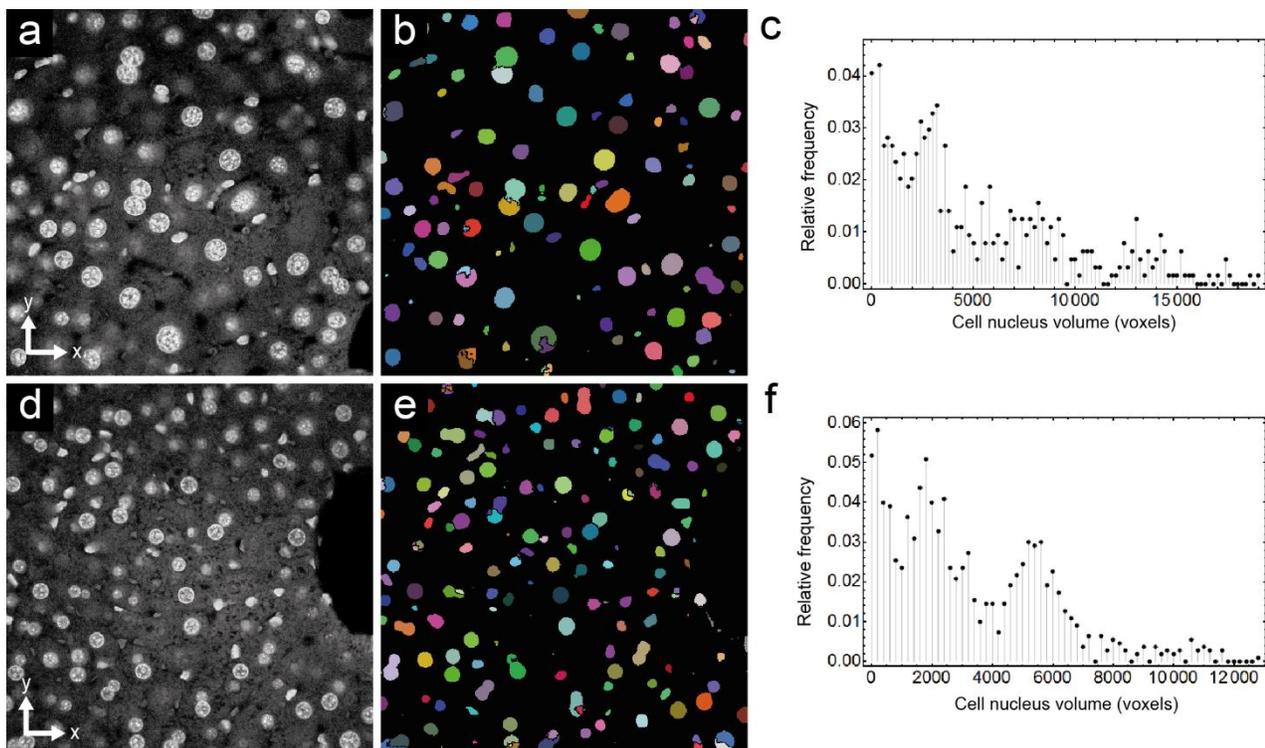
Supplementary Figure 9 Plots of the mean cell density versus the normalized distance to the surface for small (a), medium (b) and large (c) spheroids.



Supplementary Figure 10 Local cell density in all datasets. Three-dimensional rendering of segmented cell nuclei colored according to their corresponding cell density value for all small, medium and large spheroids, ranging from blue (35 cells/u.v.) to red (70 cells/u.v.). Renderings were clipped at the center of the spheroids. For a complete list of datasets see Supplementary Table 3. Scale bar: 50 μm .



Supplementary Figure 11 Volume of outer region in medium and large spheroids.



Supplementary Figure 12 Quality of cell nuclei segmentation in two test datasets from mouse liver imaged with a confocal microscope (Friebel *et al.*, *Bioinformatics*, 2015). (a) Single plane 38 along X-Y of test dataset #1 downloaded from (<http://ms.izbi.uni-leipzig.de/index.php/software>, accessed 11/10/2016). (b) The obtained components after cell nuclei segmentation are colored. (c) Plot of the relative frequency of cell nuclei volumes in the whole dataset #1. (d) Single plane 38 along X-Y of test dataset #2 downloaded from (<http://ms.izbi.uni-leipzig.de/index.php/software>, accessed 11/10/2016). (e) The obtained components after cell nuclei segmentation are colored. (f) Plot of the relative frequency of cell nuclei volumes in the whole dataset #2. For both data sets, one voxel corresponds to $0.02 \mu\text{m}^3$.

Supplementary Table 1 Evaluation of segmentation performance for different regions within a spheroid. The performance was measured against a manually determined ground truth for the regions I, II and III. The performance metrics recall, precision and F score are determined from the number of true positives, false negatives and false positives. Values range from 0 (worst performance) to 1 (optimal performance). GT, number of cell nuclei in the ground truth; SC, number of cell nuclei determined by the segmentation; TP, true positives; FN, false negatives; FP, false positives.

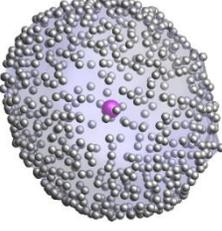
	GT	SC	TP	FN	FP	recall	precision	F score
region I	233	222	198	35	24	0.85	0.89	0.87
region II	269	262	230	39	32	0.86	0.88	0.87
region III	252	225	216	36	9	0.86	0.96	0.91

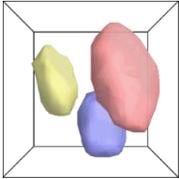
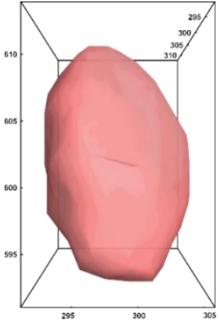
Supplementary Table 2 Parameter names and values used for cell nuclei segmentation, alpha shape and cell graphs. The same parameters values were used for all datasets. Please refer to the Methods section for a detailed description of the parameters.

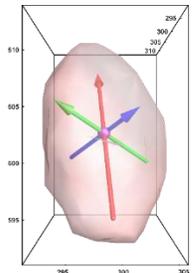
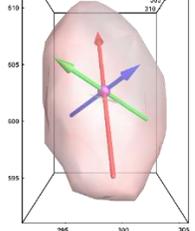
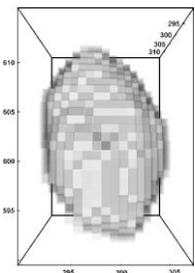
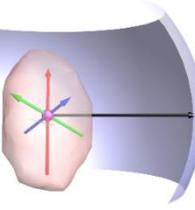
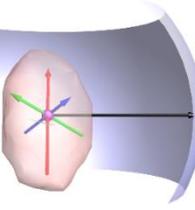
Segmentation	
Parameter	Value
ImageZScalingFactor	3.5
ImageScalingFactor	0.5
NucleiSeedDetectionMinRadius	3
NucleiSeedDetectionMaxRadius	6
NucleiFilterRange	3
NucleiThresholdRange	12
NucleiMeanFactor	1
NucleiSeedDilation	2
NucleiBackgroundFactor	0.25
NucleiMinCount	250
NucleiMaxCount	42,500

Alpha shape / cell graphs	
Parameter	Value
Alpha	90
OutlierDistanceThreshold	20
EdgeDistanceThreshold	40

Supplementary Table 3 Overview of features extracted for the spheroid and individual cells.

	Feature Name	Description	Illustration
Spheroid features	<i>ProximityCellGraph (PCG)</i>	Proximity cell graph $PCG(V, E_{PCG})$ with vertices V and edges E_{PCG} describes the whole spheroid. Vertices V have positions $\{x, y, z\}$ and represent cell nuclei centroids. An edge $(u, w) \in E_{PCG}$ exists if the Euclidean distance between the two vertices u and w is less than a predefined threshold. The edge weight is determined by the Euclidean distance between the two vertices u and w	
	<i>DelaunayCellGraph (DCG)</i>	Delaunay cell graph $DCG(V, E_{DCG})$ with vertices V and edges E_{DCG} describes the whole spheroid. Vertices V have positions $\{x, y, z\}$ and represent cell nuclei centroids. An edge $(u, w) \in E_{DCG}$ exists if it is part of the Delaunay triangulation of V and the Euclidean distance between the two vertices u and w is less than a predefined threshold. The edge weight is determined by the Euclidean distance between the two vertices u and w .	
	<i>Surface</i>	Surface of the spheroid (light blue) constructed from all cell nuclei centroids P using the alpha shapes approach. Points in P located on the surface are shown in gray.	
	<i>Centroid</i>	Geometric center $\{x, y, z\}$ of the spheroid (purple) is the arithmetic mean of all cell nuclei centroids.	
	<i>MinDistanceSurface</i>	Euclidean distance of <i>Centroid</i> to its closest point on <i>Surface</i> .	
	<i>Volume</i>	Total number of voxels of the volume enclosed by and including <i>Surface</i> .	
	<i>SurfaceArea</i>	The number of voxels of <i>Surface</i> .	

	Feature Name	Description	Illustration
Cell nuclei features (segmentation, surface)	<i>Label</i>	Unique integer that identifies a cell nucleus.	
	<i>Mask</i>	A binary three-dimensional array, in which 1s refer to the voxels that are part of a cell nucleus.	
	<i>BoundingBox</i>	Axes-oriented minimal box $\{\{x_{min}, y_{min}, z_{max}\}, \{x_{max}, y_{max}, z_{max}\}\}$ that contains a cell nucleus.	
	<i>Count</i>	Total number of voxels of a cell nucleus.	
	<i>PerimeterCount</i>	Total number of voxels on the surface of a cell nucleus.	
	<i>MinCentroidDistance,</i> <i>MaxCentroidDistance,</i> <i>MeanCentroidDistance</i>	Minimum, maximum and average distance of all voxels to the geometric center of a cell nucleus.	

	<i>Centroid</i>	Geometric center $\{x, y, z\}$ of a cell nucleus (purple sphere).	
	<i>MainAxes</i>	Main axes of orientation $\{\vec{p}_1, \vec{p}_2, \vec{p}_3\}$ of a cell nucleus measured by principal components analysis. In particular, \vec{p}_1 (red) is the vector along the first, \vec{p}_2 (green) along the second and \vec{p}_3 (blue) along the third principal component, respectively.	
	<i>Extension</i>	Extension $\{e_1, e_2, e_3\}$ of a cell nucleus along its three main axes of orientation $\{\vec{p}_1, \vec{p}_2, \vec{p}_3\}$ as the number of voxels, where e_1 is the extension along \vec{p}_1 , e_2 along \vec{p}_2 and e_3 along \vec{p}_3 , respectively.	
	<i>TotalIntensity, MeanIntensity, StandardDeviationIntensity, MinIntensity, MaxIntensity</i>	Total, average, standard deviation, minimum and maximum value of the intensity distribution of a cell nucleus measured in the raw volume. Intensity features are normalized to the interval $[0, 1]$.	
	<i>IntensityCentroid</i>	Intensity weighted geometric center $\{x, y, z\}$ of a cell nucleus.	
	<i>SurfaceDistance, SurfaceNearest</i>	Minimal distance (black arrow) of the cell nucleus centroid (magenta sphere) to the aggregate surface (<i>Surface</i> , light blue) in number of voxels, and the corresponding surface point $\{x, y, z\}$.	
	<i>SurfaceOrientation</i>	Angle in degree between major axis vector \vec{p}_1 (red) of the cell nucleus and the aggregate surface (<i>Surface</i> , light blue). An angle of 0° indicates that \vec{p}_1 is parallel to <i>Surface</i> , whereas for an angle of 90° , \vec{p}_1 is orthogonal <i>Surface</i> .	

	Feature Name	Description	Illustration
Cell neighborhood features (cell graphs)	<i>NeighborCount, MinNeighborDistance, MaxNeighborDistance, MeanNeighborDistance, StandardDeviationNeighborDistance</i>	Number of vertices adjacent to a vertex v (e.g. vertex 10 in the illustration has seven neighbors). Minimum, maximum, mean and standard deviation of the edge weight of all edges incident to a vertex v . These features are derived from both proximity cell graph and Delaunay cell graph.	

Supplementary Table 4 Quantitative measures for all datasets. Based on automated cluster analysis (partitioning around medoids with squared Euclidean distance) of spheroid volume and cell number, the datasets were separated into three groups of small (S1-S9), medium-sized (M1-M3) and large (L1-L4) spheroids.

Dataset	Group	Number of seeded cells	Volume (μm^3)	Number of cells	Median cell nucleus volume (μm^3)	Median absolute deviation of cell nucleus volume (μm^3)
S1	Small	1,000	$5,81 \times 10^6$	4,155	333	77
S2	Small	1,000	$4,15 \times 10^6$	2,883	341	78
S3	Small	1,000	$4,68 \times 10^6$	3,981	263	57
S4	Small	2,000	$1,06 \times 10^7$	8,975	245	57
S5	Small	2,000	$1,18 \times 10^7$	10,311	249	55
S6	Small	500	$4,58 \times 10^6$	3,938	268	71
S7	Small	500	$5,96 \times 10^6$	4,866	260	66
S8	Small	500	$2,71 \times 10^6$	2,326	261	77
S9	Small	500	$3,98 \times 10^6$	3,359	280	66
M1	Medium	5,000	$2,90 \times 10^7$	25,775	234	51
M2	Medium	5,000	$2,88 \times 10^7$	25,908	235	48
M3	Medium	5,000	$2,82 \times 10^7$	25,064	232	53
L1	Large	10,000	$3,80 \times 10^7$	32,898	211	50
L2	Large	10,000	$4,10 \times 10^7$	35,097	211	53
L3	Large	10,000	$4,21 \times 10^7$	38,752	209	54
L4	Large	5,000	$3,87 \times 10^7$	32,222	279	62