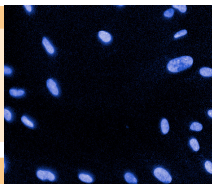
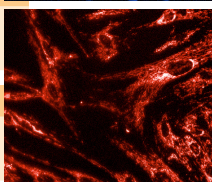
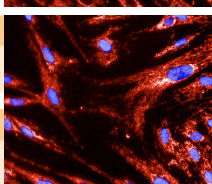
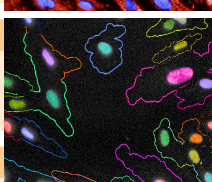
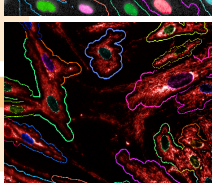
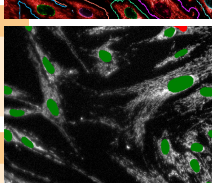
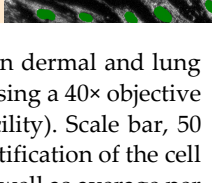
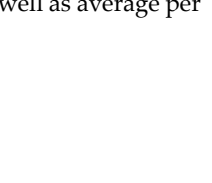


Supplementary Figure S1

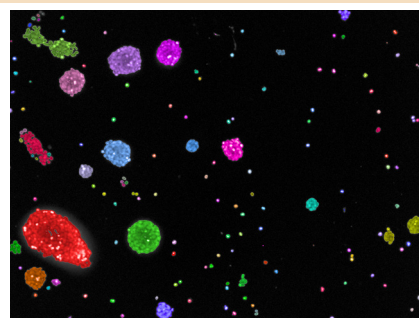
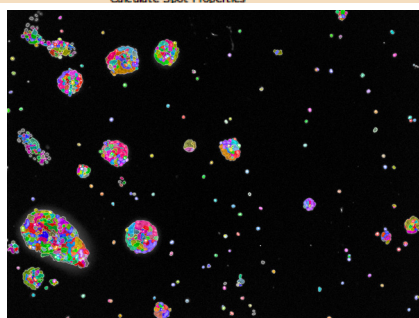
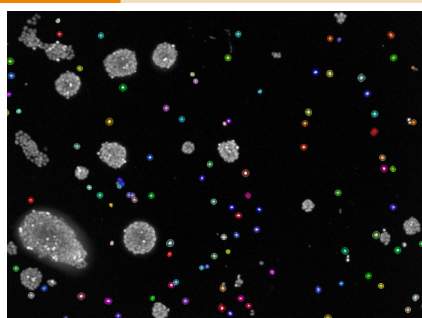
Find Nuclei	Input Channel : HOECHST 33342 ROI : None	Method Method : C Common Threshold : <u>0.2</u> Area : > 30 μm^2 Splitting Coefficient : <u>1</u> Individual Threshold : <u>0.32</u> Contrast : > <u>0.32</u>	Output Output Population : Nuclei	
Calculate Morphology Properties	Input Population : Nuclei Region : Nucleus	Method Method : Standard Area Roundness	Output Property Prefix : Nucleus	
Calculate Intensity Properties	Input Channel : HOECHST 33342 Population : Nuclei Region : Nucleus	Method Method : Standard Mean	Output Property Prefix : Intensity Nucleus HOECHST 33342	
Select Population	Input Population : Nuclei	Method Method : Filter by Property Nucleus Roundness : > <u>0.7</u>	Output Output Population : Nuclei Selected	
Find Cytoplasm	Input Channel : TMRM Nuclei : Nuclei Selected	Method Method : A Individual Threshold : <u>0.08</u>	Output	
Select Cell Region	Input Population : Nuclei Selected	Method Method : Resize Region [%] Region Type : Ring Region Outer Border : <u>-45</u> % Inner Border : <u>-35</u> %	Output Output Region : BG	
Calculate Intensity Properties (2)	Input Channel : TMRM Population : Nuclei Selected Region : Cytoplasm	Method Method : Standard Mean	Output Property Prefix : Intensity TMRM	
Calculate Intensity Properties (3)	Input Channel : TMRM Population : Nuclei Selected Region : BG	Method Method : Standard Mean	Output Property Prefix : Intensity BG TMRM	
Calculate Properties	Input Population : Nuclei Selected	Method Method : By Formula Formula : A-B Variable A : Intensity TMRM Mean Variable B : Intensity BG TMRM Mean	Output Output Property : Delta TMRM	
Select Population (2)	Input Population : Nuclei Selected	Method Method : Filter by Property Delta TMRM : > 0	Output Output Population : TMRM positive	

Supplementary Figure S1. Workflow of the analysis protocol of high-content microscopy images of human dermal and lung fibroblasts. TMRM and Hoechst fluorescence images of human fibroblasts (HDFa, IMR-90) were acquired using a 40× objective with the automated high-content fluorescence imaging Operetta® system (PerkinElmer; HiTS@UniPD facility). Scale bar, 50 μm . Image analysis was performed in a stepwise manner: nuclei detection was followed by automated identification of the cell area; a ring region surrounding the cell boundaries was used as background for each object. Single object as well as average per well fluorescence intensity was calculated for each timepoint.

Supplementary Figure S2

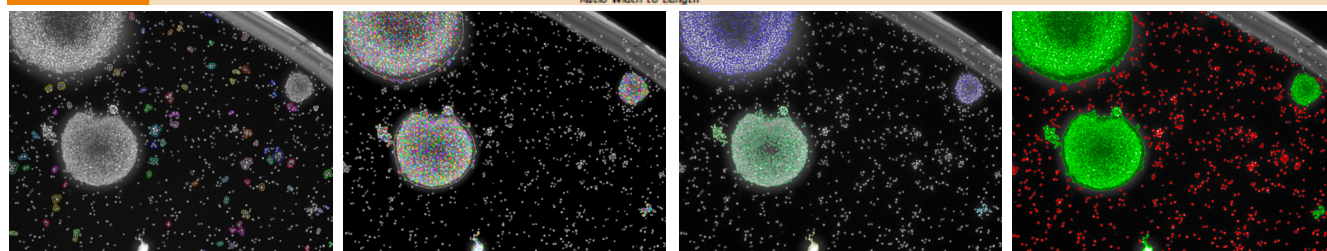
A

Find Image Region	Channel : HOECHST 33342 ROI : None	Method : Common Threshold Threshold : <u>0.3</u> Split into Objects Area : <u>>2</u> px ²	Output Population : Cell Clusters Output Region : Cluster ROI
Calculate Morphology Properties	Population : Cell Clusters Region : Cluster ROI	Method : Standard Area Roundness Width Length Ratio Width to Length	Output Properties : Cluster ROI
Find Nuclei	Channel : HOECHST 33342 ROI : Cell Clusters ROI Region : Cluster ROI	Method : M Diameter : <u>12</u> µm Splitting Coefficient : <u>0.35</u> Common Threshold : <u>0.09</u>	Output Population : Nuclei
Find Cytoplasm (2)	Channel : TMRM Nuclei : Nuclei	Method : A Individual Threshold : <u>0.05</u> Restrictive Region : Cluster ROI	
Select Region	Population : Nuclei Region : Cytoplasm	Method : Resize Region [%] Outer Border : <u>±60</u> % Inner Border : <u>±20</u> %	Output Region : BG
Calculate Morphology Properties (4)	Population : Nuclei Region : Nucleus	Method : Standard Area Roundness Width Length Ratio Width to Length	Output Properties : Nucleus
Select Population (2)	Population : Cell Clusters	Method : Filter by Property Cluster ROI Area [µm ²] : <u>≤ 150</u>	Output Population : Single Cell
Calculate Intensity Properties (3)	Channel : HOECHST 33342 Population : Single Cell Region : Nucleus	Method : Standard Mean Contrast	Output Properties : Intensity single cells HOECHST 33342
Calculate Intensity Properties (7)	Channel : TMRM Population : Single Cell Region : Cytoplasm	Method : Standard Mean	Output Properties : Single cell TMRM intensity
Select Region (2)	Population : Single Cell Region : Cell	Method : Resize Region [%] Outer Border : <u>±80</u> % Inner Border : <u>±40</u> %	Output Region : Bg single cell
Calculate Intensity Properties	Channel : TMRM Population : Single Cell Region : Bg single cell	Method : Standard Mean	Output Properties : Intensity Bg single cell TMRM
Calculate Properties	Population : Single Cell	Method : By Formula Formula : A-B Variable A : Single cell TMRM intensity Mean Variable B : Intensity Bg single cell TMRM Mean	Output Property : Delta TMRM Single Cell
Select Population (3)	Population : Cell Clusters	Method : Filter by Property Cluster ROI Area [µm ²] : <u>> 150</u> Cluster ROI Area [µm ²] : <u>< 1100</u> Boolean Operations : F1 and F2	Output Population : Grouped Cells
Calculate Intensity Properties (5)	Channel : HOECHST 33342 Population : Grouped Cells Region : Nucleus	Method : Standard Mean	Output Properties : Intensity Nucleus Grouped HOECHST 33342
Find Spots	Channel : HOECHST 33342 ROI : Grouped Cells	Method : D Detection Sensitivity : <u>1</u> Splitting Coefficient : <u>0.9</u> Background Correction : <u>0.7</u> Calculate Spot Properties	Output Population : Nuclei Spot Grouped Cells



B

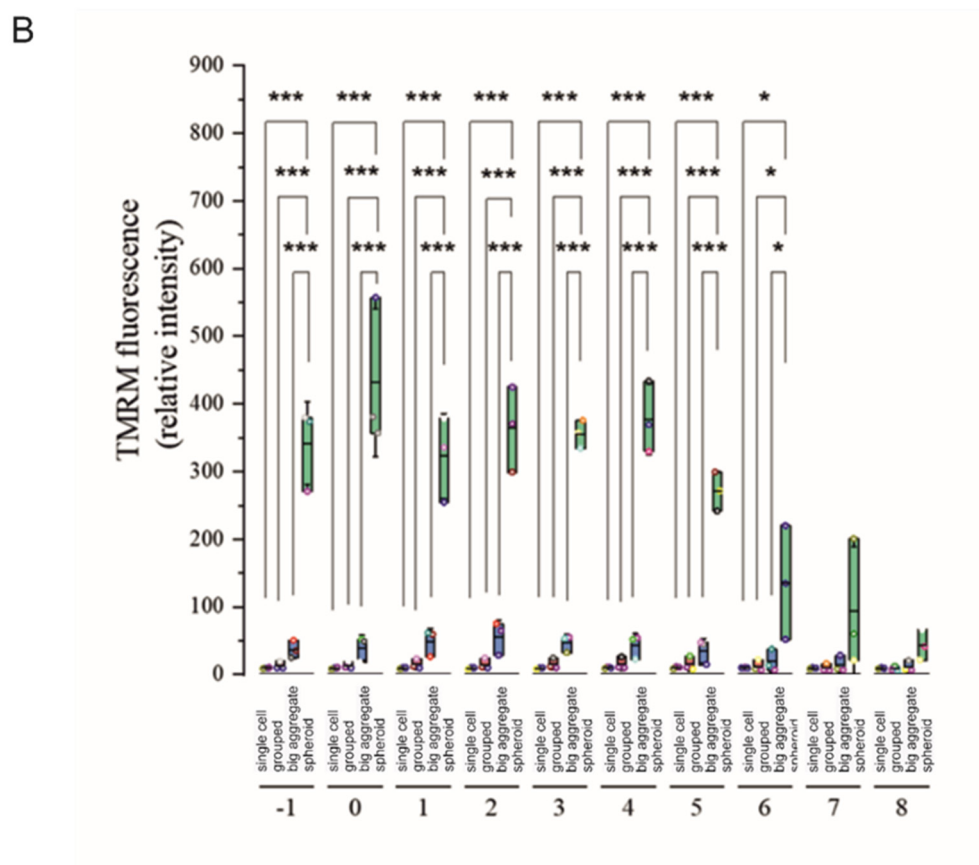
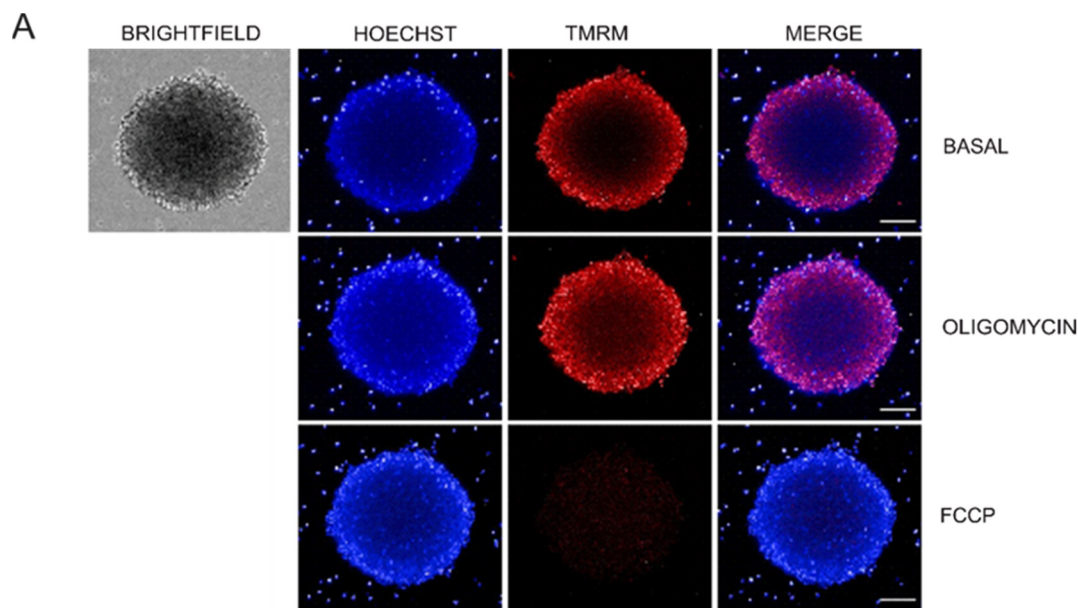
Calculate Intensity Properties (13)	Channel : HOECHST 33342 Population : Grouped Cells Region : Spots	Method : Standard Mean	Output Properties : HOECHST Nuclei spot Grouped
Calculate Intensity Properties (9)	Channel : TMRM Population : Grouped Cells Region : Cytoplasm	Method : Standard Mean	Output Properties : Intensity Cytoplasm Grouped TMRM
Select Region (3)	Population : Grouped Cells Region : Cell	Method : Resize Region [%] Outer Border : ± 80 % Inner Border : ± 40 %	Output Region : Grouped Cells Bg
Calculate Intensity Properties (2)	Channel : TMRM Population : Grouped Cells Region : Grouped Cells Bg	Method : Standard Mean	Output Properties : Intensity Grouped Cells Bg TMRM
Calculate Properties (2)	Population : Grouped Cells	Method : By Formula Formula : A-B Variable A : Intensity Cytoplasm Grouped TMRM Mean Variable B : Intensity Grouped Cells Bg TMRM Mean	Output Property : Delta TMRM grouped cell
Select Population (4)	Population : Cell Clusters	Method : Filter by Property Cluster ROI Area [μm^2] : ≥ 1100 Cluster ROI Roundness : > 0.8 Boolean Operations : F1 and F2	Output Population : spheroids
Select Population	Population : Cell Clusters	Method : Filter by Property Cluster ROI Area [μm^2] : > 1100 Cluster ROI Roundness : ≤ 0.8 Boolean Operations : F1 and F2	Output Population : Big Spheroids
Calculate Intensity Properties (10)	Channel : HOECHST 33342 Population : Big Spheroids Region : Nucleus	Method : Standard Mean	Output Properties : Intensity Big spheroids HOECHST 33342
Calculate Intensity Properties (11)	Channel : TMRM Population : Big Spheroids Region : Cytoplasm	Method : Standard Mean	Output Properties : Intensity Cytoplasm spheroidsTMRM
Calculate Morphology Properties (3)	Population : Big Spheroids Region : Cluster ROI	Method : Standard Area Roundness Width Length Ratio Width to Length	Output Properties : Big Spheroids
Select Region (5)	Population : Big Spheroids Region : Cell	Method : Resize Region [%] Outer Border : ± 80 % Inner Border : ± 40 %	Output Region : Big Spheroids Bg
Calculate Intensity Properties (12)	Channel : TMRM Population : Big Spheroids Region : Big Spheroids Bg	Method : Standard Mean	Output Properties : Intensity Big Spheroids Bg TMRM
Calculate Properties (4)	Population : Big Spheroids	Method : By Formula Formula : A-B Variable A : Intensity Cytoplasm spheroidsTMRM Mean Variable B : Intensity Big Spheroids Bg TMRM Mean	Output Property : Delta TMRM Big Spheroids
Find Spots (3)	Channel : HOECHST 33342 ROI : Big Spheroids	Method : D Detection Sensitivity : 1 Splitting Coefficient : 0.3 Background Correction : 0.089 Calculate Spot Properties	Output Population : Nuclei spot Big spheroids
Calculate Intensity Properties (14)	Channel : HOECHST 33342 Population : Big Spheroids Region : Spots	Method : Standard Mean	Output Properties : HOECHST Nuclei spot Big Spheroids
Calculate Intensity Properties (6)	Channel : HOECHST 33342 Population : spheroids Region : Nucleus	Method : Standard Mean Contrast	Output Properties : Intensity spheroids HOECHST 33342
Calculate Intensity Properties (9)	Channel : TMRM Population : spheroids Region : Cytoplasm	Method : Standard Mean	Output Properties : Intensity TMRM spheroids
Find Spots (2)	Channel : HOECHST 33342 ROI : spheroids	Method : D Detection Sensitivity : 1 Splitting Coefficient : 0.3 Background Correction : 0.7 Calculate Spot Properties	Output Population : Nuclei spot Spheroids
Calculate Intensity Properties (15)	Channel : HOECHST 33342 Population : spheroids Region : Spots	Method : Standard Mean	Output Properties : HOECHST Nuclei spots Spheroids
Calculate Intensity Properties (4)	Channel : TMRM Population : spheroids Region : Spheroids Bg	Method : Standard Mean	Output Properties : Intensity Spheroids Bg TMRM
Calculate Properties (3)	Population : spheroids	Method : By Formula Formula : A-B Variable A : Intensity TMRM spheroids Mean Variable B : Intensity Spheroids Bg TMRM Mean	Output Property : Delta TMRM Spheroids
Calculate Morphology Properties (2)	Population : Big Spheroids Region : Cluster ROI	Method : Standard Area Roundness Width Length Ratio Width to Length	Output Properties : Big Spheroids



Supplementary Figure S2. Workflow of analysis protocol in high-content microscopy of LUHMES cells and 3D spheroids. Imaging of mitochondrial membrane potential and nuclei in LUHMES cells was performed with the automated platform (Op-eretta® High-Content Imaging System, PerkinElmer; HiTS@UniPD facility). A) Hoechst staining was used for image segmentation and detection of ROIs. B) The Hoechst-positive ROIs of the LUHMES cells were categorized into 4 groups: single cells,

grouped, big aggregates and spheroids, as shown in the right column. The classes were distinguished based on the size of the nuclei clusters, and TMRM intensity was calculated per each class.

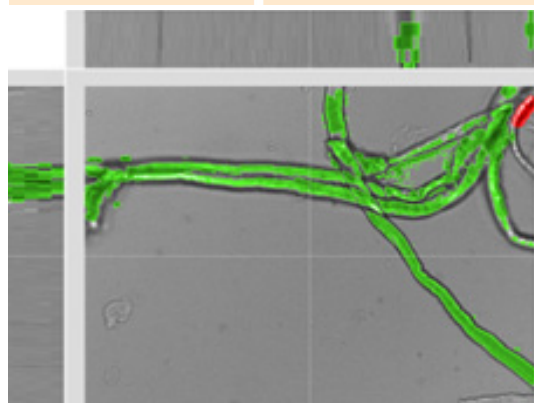
Supplementary Figure S3



Supplementary Figure S3. Representative images of 3D Spheroids in all the channels and statistical analysis of the 4 different groups of cells. (A) Representative images of mitochondria and nuclei staining in LUHMES 3D Spheroids at three different timepoints, corresponding to basal conditions, exposure to oligomycin and FCCP. (B) Box-plot graph of TMRM fluorescence intensity values (arbitrary units) representing of $\Delta\Psi_m$ changes occurring in the four populations at each time-point. Data represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$, *** $p < 0.001$. Standard ANOVA procedures followed by multiple pairwise comparison adjusted with Bonferroni corrections were performed using GraphPad software (GraphPad Software, San Diego, CA, USA).

Supplementary Figure S4

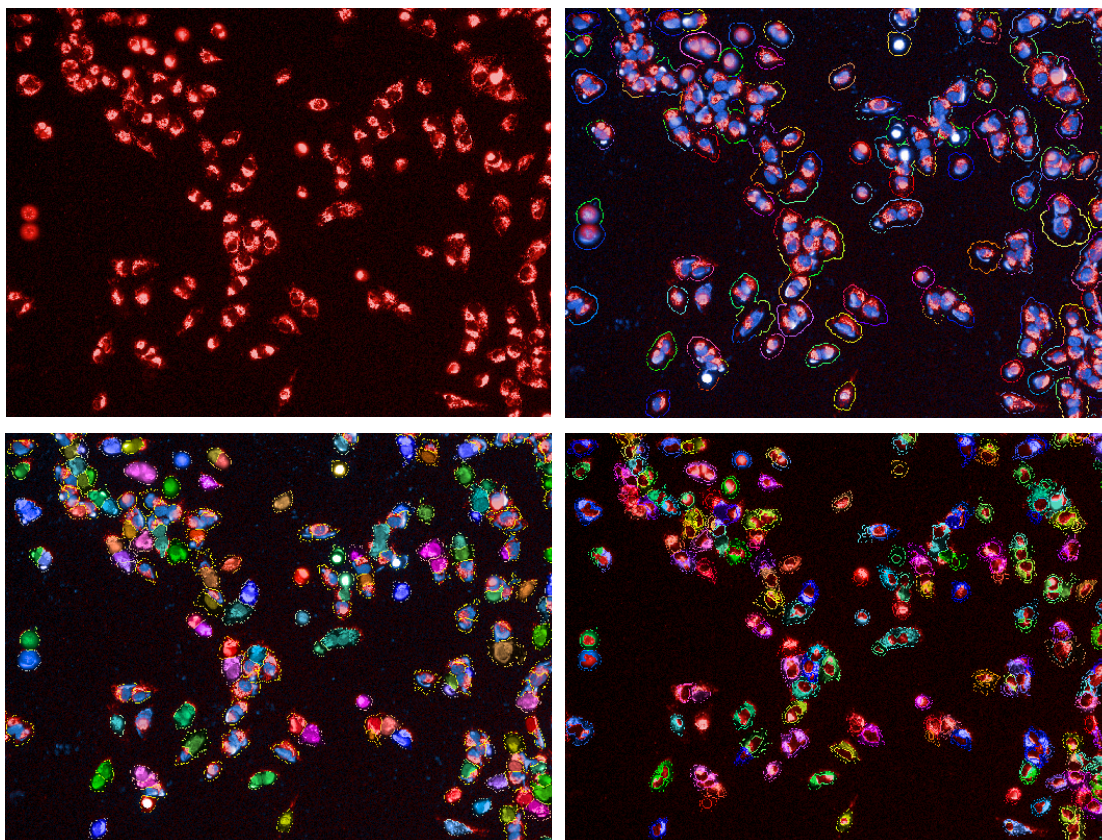
Filter Image	Input Channel : Brightfield	Method Method : Sliding Parabola Curvature : 3	Output Output Image : Sliding Parabola Brightfield Fiber
Find Image Region	Channel : Sliding Parabola Brightfield Fiber ROI : None	Method : Local Threshold Threshold : 0.45 Region Scale : 6 µm Closing : 0.2 µm Filling : Fill Plane-Wise Smoothing : 4 µm Volume : > 4000000 µm ³	Output Output Population : ROIs Output Region : ROIs region
Calculate Morphology Properties (3)	Population : ROIs Region : ROIs region	Method : Standard Volume Surface Area Number of Fragments Equivalent Ellipsoid Axes Object Box Size Sphericity Inner Sphere Radius Object Height Maximum Thickness Footprint Area Maximum Crosssection Area Maximum Inner Disk Radius	Output Property Prefix : ROIs region
Calculate Texture Properties (4)	Channel : Sliding Parabola Brightfield Fiber Population : ROIs Region : ROIs region	Method : PLS Features Filter : Plane Bright Scale XY : 2 px Scale Z : 0 px PSF Aspect Ratio : 3 Kernel Normalization	Output Property Prefix : ROIs region Sliding Parabola Brightfield Fiber
Find Image Region (2)	Channel : TMRM ROI : ROIs ROI Region : ROIs region	Method : Absolute Threshold Lowest Intensity : ≥ 100 Highest Intensity : ≤ INF Closing : 6 µm Filling : Fill Cavities Smoothing : 12 µm Volume : > 300000 µm ³	Output Output Population : Fibers Output Region : Fibers Region
Calculate Intensity Properties	Channel : TMRM Population : Fibers Region : Fibers Region	Method : Standard Mean Standard Deviation Coefficient of Variance Median	Output Property Prefix : Intensity Fibers Region TMRM
Select Region	Population : Fibers Region : Fibers Region	Method : Resize Region [µm/px] Direction : XYZ Fixed Aspect Ratio : 1 Outer Border XY : -180 µm Outer Border Z : -180 µm Inner Border XY : -150 µm Inner Border Z : -150 µm	Output Output Region : Fibers Resized
Calculate Intensity Properties (2)	Channel : TMRM Population : Fibers Region : Fibers Resized	Method : Standard Mean Standard Deviation	Output Property Prefix : Intensity Bg TMRM
Calculate Properties	Population : Fibers	Method : By Formula Formula : A-B Variable A : Intensity Fibers Region TMRM Mean Variable B : Intensity Bg TMRM Mean	Output Output Property : Delta TMRM
Calculate Texture Properties	Channel : Brightfield Population : Fibers Region : Fibers Region	Method : PLS Features Filter : All Scale XY : 1 px Scale Z : 0 px PSF Aspect Ratio : 2 Kernel Normalization	Output Property Prefix : Fibers Region Brightfield
Select Population (5)	Population : Fibers	Method : Filter by Property Delta TMRM : > 10 Intensity Fibers Region TMRM Mean : > 10 Intensity Fibers Region TMRM CV [%] : > 20 Boolean Operations : F2 and F3 and F4	Output Output Population : True Fibers



Supplementary Figure S4. Workflow of analysis protocol in high-content microscopy of 3D myofibers. Imaging of mitochondrial membrane potential in isolated muscle fibers from EDL and TA were acquired by automated fluorescence microscopy. White scale bars: 200 µm. Image analysis was performed in a stepwise manner. Brightfield image correction was followed by 3D reconstruction. Texture analysis of the latter allowed the first segmentation, that is detection of image regions containing fibers. These areas have been further segmented based on phenoLOGIC™ analysis (Perkin-Elmer machine learning algorithm), to identify individual fibers. Contracted fibers (likely damaged due to the dissociation process) were discarded based on morphology and texture parameters, yielding the final population named “true fibers”.

Supplementary Figure S5

A



B

Find Nuclei	Input Channel : HOECHST 33342 ROI : None	Method Method : M Diameter : <u>20</u> μ m Splitting Sensitivity : <u>0.46</u> Common Threshold : <u>0.11</u>	Output Output Population : Nuclei
Find Cytoplasm	Input Channel : TMRM Nuclei : Nuclei	Method Method : B Common Threshold : <u>0.38</u> Individual Threshold : <u>0.33</u>	Output
Select Cell Region	Input Population : Nuclei	Method Method : Resize Region [%] Region Type : Cytoplasm Region Outer Border : <u>-25</u> % Inner Border : <u>-5</u> %	Output Output Region : Background
Calculate Intensity Properties	Input Channel : TMRM Population : Nuclei Region : Cytoplasm	Method Method : Standard Mean	Output Property Prefix : TMRM Int Cyt
Calculate Intensity Properties (3)	Input Channel : TMRM Population : Nuclei Region : Background	Method Method : Standard Mean	Output Property Prefix : Intensity Background TMRM
Calculate Properties	Input Population : Nuclei	Method Method : By Formula Formula : A-B Variable A : TMRM Int Cyt Mean Variable B : Intensity Background TMRM Mean	Output Output Property : Delta TMRM
Calculate Morphology Properties	Input Population : Nuclei Region : Cell	Method Method : Standard Area Roundness Width Length Ratio Width to Length	Output Property Prefix : Cell
Calculate Texture Properties (2)	Input Channel : TMRM Population : Nuclei Region : Cytoplasm	Method Method : SER Features Scale : 0 px Normalization by : Kernel SER Spot SER Hole SER Edge SER Ridge SER Valley SER Saddle SER Bright SER Dark	Output Property Prefix : Cytoplasm TMRM
Calculate Texture Properties	Input Channel : HOECHST 33342 Population : Nuclei Region : Nucleus	Method Method : SER Features Scale : 0 px Normalization by : Kernel SER Spot SER Hole SER Edge SER Ridge SER Valley SER Saddle SER Bright SER Dark	Output Property Prefix : Nucleus HOECHST 33342
Calculate Intensity Properties (2)	Input Channel : HOECHST 33342 Population : Nuclei Region : Cell	Method Method : Standard Mean Standard Deviation Coefficient of Variance	Output Property Prefix : HOECHST Intensity
Calculate Morphology Properties (2)	Input Population : Nuclei Region : Nucleus	Method Method : Standard Area Roundness Width Length Ratio Width to Length	Output Property Prefix : Nucleus

Supplementary Figure S5. Workflow of analysis protocol in high-content microscopy of co-cultured melanoma A375 cells and macrophages. A) TMRM and Hoechst fluorescence images of human melanoma A375 cells and macrophages, grown either alone or in co-culture, were acquired by automated fluorescence microscope platform (Operetta® High Content Imaging System, PerkinElmer; HiTS@UniPD facility). Scale bar 100 μ m. B) Hoechst staining was used for image segmentation; several nuclei parameters (texture, intensity, morphology) were measured and considered for the subsequent machine learning analysis.