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Protocol

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Sequential orthogonal assays for longitudinal and endpoint characterization of three-dimensional spheroids

Eva Blondeel[1,2], Sam Ernst[1,2,3], Felix De Vuyst[1,2], Ákos Diósdi[4], Cláudio Pinheiro[1,2], Diogo Estêvão[5,6], Pekka Rappu[7], Robin Boiy[1,2], Sándor Dedeyne[1,2], Ligia Craciun[8], Vera Goossens[9], Jonas Dehairs[10], Tânia Cruz[5,6], Dominique Audenaert[9], Wim Ceelen[2,3], Michael Linnebacher[11], Tom Boterberg[2,12], Jo Vandesompele[2,13], Pieter Mestdagh[2,13], Johan Swinnen[10], Jyrki Heino[7], Peter Horvath[4,14,15,16], Maria José Oliveira[5,6], An Hendrix[1,2], Pieter De Metter[8] & Olivier De Wever[1,2] ✉

Abstract

Spheroids are reaggregated multicellular three-dimensional structures generated from cells or cell cultures of healthy as well as pathological tissue. Basic and translational spheroid application across academia and industry have led to the development of multiple setups and analysis methods, which mostly lack the modularity to maximally phenotype spheroids. Here we present the self-assembly of single-cell suspensions into spheroids by the liquid overlay method, followed by a modular framework for a multifaceted phenotyping of spheroids. Cell seeding, supernatant handling and compound administration are elaborated by both manual and automated procedures. The phenotyping modules contain a suite of orthogonal assays to analyze spheroids longitudinally and/or at an endpoint. Longitudinal analyses include morphometry with or without spheroid or cell state specific information and supernatant evaluation (nutrient consumption and metabolite/cytokine production). Spheroids can also be used as a starting point to monitor single and collective cell migration and invasion. At an endpoint, spheroids are lysed, fixed or dissociated into single cells. Endpoint analyses allow the investigation of molecular content, single-cell composition and state and architecture with spatial cell and subcellular specific information. Each module addresses time requirements and quality control indicators to support reproducibility. The presented complementary techniques can be readily adopted by researchers experienced in cell culture and basic molecular biology. We anticipate that this modular protocol will advance the application of three-dimensional biology by providing scalable and complementary methods.

Key points

- This protocol describes the self-assembly of single-cell suspensions into spheroids by the liquid overlay method, alongside a modular framework of orthogonal assays to phenotype spheroids both longitudinally and at an endpoint.
- This method ensures production and long-term culture of spheroids with defined size, morphology and composition. In addition, the workflow addresses a comprehensive number of spheroid metrics including supernatant analysis.

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A full list of affiliations appears at the end of the paper. ✉e-mail: Olivier.Dewever@UGent.be

Introduction

Many insights of biology are based on experimentation with three-dimensional (3D) models that mimic the cellular context of a tissue. Spheroids are reaggregated multicellular 3D structures commonly used in both fundamental and translational research across academia and industry. Cells in a single-cell suspension self-assemble above a nonadhesive surface into a spheroid showing physiological gradients of nutrients and oxygen[1]. Spheroids can be not only homocellular but also heterocellular, containing elements from the (tumor) microenvironment, including fibroblasts, endothelial cells and immune cells, enabling architectural and spatial mimicking of (cancer) tissue. Spheroids secrete products (proteins, metabolites, etc.) and consume nutrients that may act as indicators of spheroid state. Hence, the 3D spheroid model has emerged as a powerful tool to overcome the correlation mismatch between the preclinical and clinical situation by providing more relevant and predictive data. This is an essential feature in drug testing; compounds that retain their cytotoxic effect in spheroids can contribute to a reduction/replacement in animal testing and a higher success rate in clinical trials[2]. 3D spheroids and other 3D cell cultures possess several in vivo features of tumors, such as cell–cell interaction, hypoxia, drug penetration, response and resistance and production/deposition of extracellular matrix (ECM)[3]. Despite promising recent insights[4,5], solid proof that cells growing in 3D spheroids behave similarly to tissues or organs is still very much lacking and further research is needed in the field.

Development of the protocol

We previously developed a knowledgebase and transparency tool for Minimum Information in Spheroid IDentity (MISpheroID). With this initiative, we identified pronounced heterogeneity and lack of reporting in the methodological setup of spheroids. Empirical evaluation and interlaboratory validation of selected variations in spheroid methodology (cell line, culture media, formation method and size) revealed a striking impact on spheroid phenotype[1] (Box 1). This emphasizes the need for minimum reporting standards and transparent protocols for spheroid setup in combination with complementary quantitative assays to phenotype spheroids[6]. Despite the widespread use of spheroids, modular workflows for multifaceted spheroid characterization are currently limitedly available.

The liquid overlay method is the most implemented technique according to the MISpheroID knowledgebase and is based on the principle of seeding cells on a nonadherent surface[1]. The use of multiwell ultra-low attachment (ULA) plates overcomes the difficulties of the other methods by ensuring production and long-term culturing of spheroids with defined size, morphology and composition. Moreover, the technique is compatible with automatization and allows single-well/single-spheroid monitoring and analysis. Uniform spherical geometry gives the opportunity to couple structure to function and to perform quantitative analyses (e.g., growth,

BOX 1

Transparent reporting of spheroid experiments using MISpheroID

Transparent reporting is a prerequisite for the successful interpretation and reproducibility of experiments. We strongly encourage spheroid researchers to make use of the crowdsourcing MISpheroID (Minimum Information in Spheroid IDentity) knowledgebase. This is a free online tool (https://mispheroid.org/) where researchers can upload the experimental parameters of their published or unpublished spheroid experiments, after which they will receive a MISpheroID string that can be linked to the publication. This MISpheroID string comprises four experimental parameters; cell line, culture medium, formation method and size, which have been empirically proven to be minimum information in spheroid experiment interpretation and repeatability[1]. In addition, researchers can freely consult this database using a range of search parameters to compare general and specific information. The query result list is accompanied by a MISpheroID string and Pubmed ID.

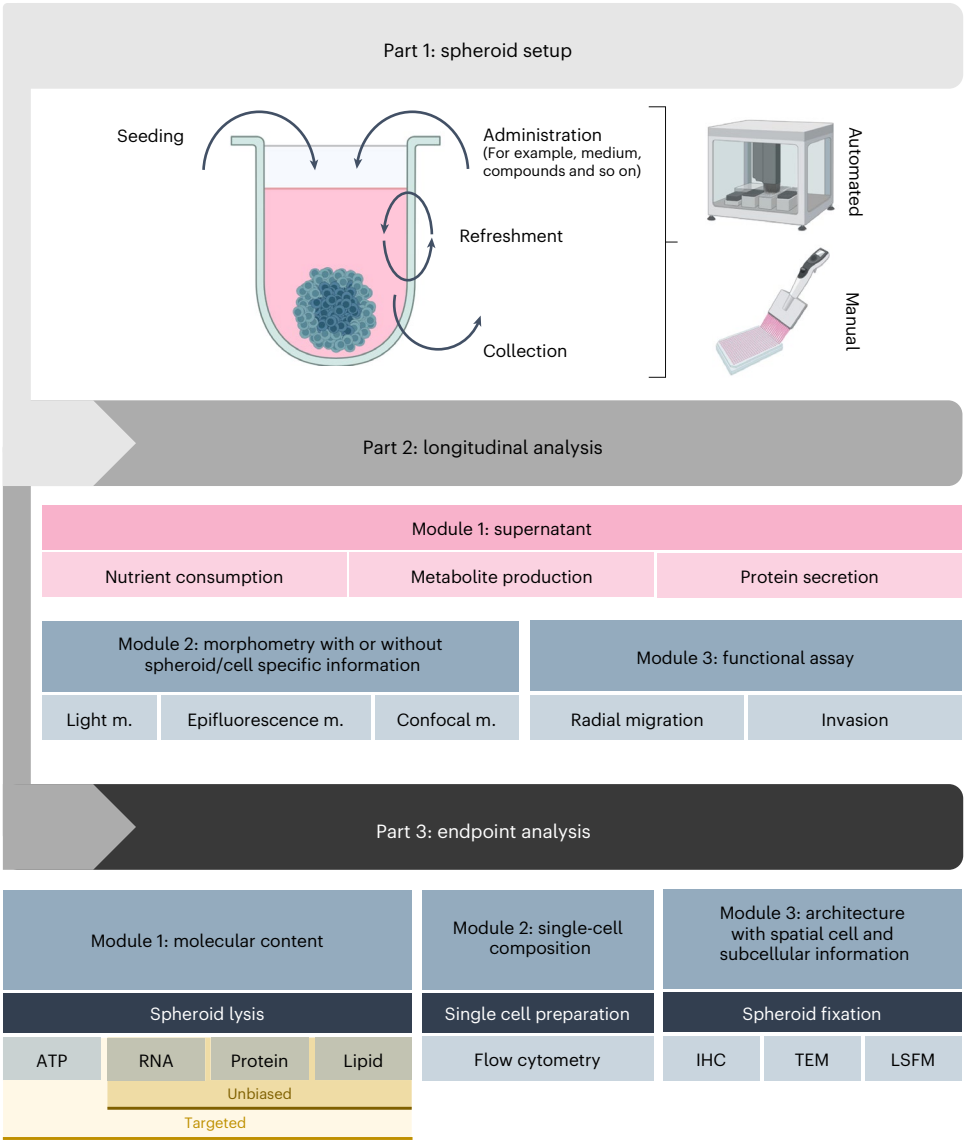

Fig. 1 | Overview of the protocol. The protocol starts with spheroid setup followed by a modular framework for multifaceted longitudinal and endpoint phenotypic analyses. M, microscopy.

morphology and invasiveness). The reproducibility of this technique was demonstrated in an interlaboratory study[1] and makes it an ideal model for robust (therapy) readouts.

In this protocol, we present a step-by-step procedure for the bulk production and reproducible handling of spheroids combined with a suite of orthogonal assays organized into different phenotyping modules. This unique modular workflow comprises a collection of protocols for the longitudinal and endpoint analysis of spheroids and their supernatant (Fig. 1). Via this modular framework approach, one can get an extensive data output through time and resource-efficient efforts. The proposed protocol workflow distinguishes itself from previously published protocols that address a limited number of spheroid metrics or disregard the supernatant.

Applications of the method

The workflow can be used for established as well as early-passage patient-derived cell cultures of different tissue types. The spheroid formation protocol can be extended to cells of different

genetic origin (species and tissue type) from both healthy and pathological tissues. In addition, spheroids are a suitable platform to study the cross-talk and interactions between cell types (e.g., cancer cells, cancer-associated fibroblasts, immune cells and endothelial cells) with direct cell–cell contact[7]. The procedures can be performed in 96- and 384-well ULA plates, and reproducibility is ensured through the implementation of automated liquid handlers, allowing high-throughput compound screening to be performed. Furthermore, the presented pipeline can be readily adopted by researchers working with other 3D models (e.g., patient-derived tissue fragments and organoids) by making small adaptations to fit the model. Taken together, we anticipate that the protocol will advance 3D biology research by providing scalable, straightforward methods. Spheroids can be used for fundamental mechanistic studies in cell biology and can also be used in biomarker discovery, disease modeling, drug discovery and functional precision medicine. The target audience of spheroid analysis is broad, including the fields of oncology, stem cell biology, drug discovery, toxicity testing, regenerative medicine and tissue engineering.

Comparison with other methods

Spheroids were first introduced in the early 1970s by Sutherland et al.[8]. Since then, a plethora of spheroid formation methods has emerged, which all take advantage of the natural tendency of various cell types to aggregate. Spinner flasks and rotating wall vessels are agitation-based culture methods in which the cells are continuously stirred or rotated respectively, thereby preventing adherence to the culture vessel and promoting cell-to-cell collision. These techniques are useful when large numbers of spheroids are required. However, these spheroids are heterogeneous in size and morphology, and the constant agitation causes shear stress[9,10]. The clinostat method avoids these problems through slow rotation around a horizontal axis and, hereby, reducing gravitational forces[10,11]. Nonetheless, the continuous rotation and bulk production of spheroids makes real-time monitoring of single spheroids by microscopy difficult. The hanging drop method is a highly reproducible method that is capable of generating individual and uniformly sized spheroids. The hanging drops are formed by surface tension of the fluid and the hydrophobic plate surface. Owing to gravity, the cells gather at the liquid–air interface and aggregate. However, despite efforts to make this method compatible with scaling up and automatization[12], the handling (e.g., media exchange and drug treatment) of these spheroids is still difficult and laborious. In addition, these cultures mostly have a short follow-up time or are transferred to ULA plates after 72 h owing to the small volumes (20–30 µL) of the hanging drops[9].

Limitations of the protocol

A limitation of the protocol is that the spheroid culture model cannot be applied to all cell types. There is the obvious lack of relevance for suspension cell lines (e.g., leukemia), but not all early-passage cell cultures or established cell lines form spheroids with spontaneous tight cell–cell interactions (Extended Data Fig. 1a). However, to achieve spheroids of even these cells, medium supplementation with additives (e.g., methylcellulose and type I collagen) is suggested. Methylcellulose is an inert, viscoelastic polymer and, at a concentration of 1–3%, increases the viscosity of the culture medium and stimulates compaction of aggregated cells. Some cell cultures require ECM components such as type I collagen to stimulate cell-matrix-dependent spheroid compaction. Type I collagen in a nongel-forming concentration of 1–10 µg/mL enables compact spheroid formation of MDA-MB-231 triple-negative breast cancer cells[13]. In all, cell number, medium viscosity and/or matrix components should be empirically tested to obtain compact spheroids (Extended Data Fig. 1a). The addition of viscoelastic polymer or ECM to stimulate spheroid compaction also affects spheroid mechanical properties. Measuring the stiffness of spheroid cultures is crucial for understanding their mechanotransduction, which influences differentiation, migration, proliferation and drug/nutrient penetration. Techniques available to assess the mechanical properties and stiffness of spheroids vary in terms of resolution, scale and complexity and include atomic force microscopy[14], compression testing[15], magnetic resonance elastography[16] and micropipette aspiration[17]. Their detailed description is beyond this protocol.

A second limitation of the protocol is the relatively high cost of commercially produced ULA plates. However, these plates ensure consistent reproducible spheroid formation and analysis[1], which is in contrast to the cheaper alternative where plates with a cell repellent surface (e.g., agarose and poly(2-hydroxyethyl methacrylate) (poly-HEMA)) are prepared in-house. ULA plates from different vendors other than described in this protocol can be used; however, prior testing is required.

Furthermore, ULA plates require manual or robot-assisted partial refreshments for long-term spheroid cultivation. More recent emerging methods are the microfluidic systems (spheroid on a chip), which are, contrary to the ULA plates, nonstatic and offer continuous automatic perfusion of fresh medium. Despite the fact that this is a promising technique, it has not yet reached full maturity. The technique is more complex, has a higher need of material and cost and is more difficult to scale up, and the generated spheroids are size-limited and harder to retrieve after formation.

Lastly, not all equipment is necessarily available within one laboratory such as an automated liquid handler or live cell imager. Although we recommend their use to reduce hands-on costs and promote repeatability and scalability, we additionally provide a manual dispensing and imaging protocol.

Overview of the procedure

This protocol includes three parts: (1) spheroid setup, (2) longitudinal analysis and (3) endpoint analysis (Fig. 1).

The first part of the protocol discusses the preparation of spheroids starting from established, as well as early-passage patient-derived cell cultures using the process of spontaneous reaggregation in ULA plates. The protocol describes both manual as well as robotic single-cell seeding (Steps 1–13), supernatant collection and partial refreshments (Steps 14–17), compound administration (Steps 18–22) and spheroid collection (Steps 23–24) (Fig. 2 and Supplementary Video 1 and 2). Note that the order of the optional partial supernatant refreshments and compound administration can be changed.

The second part of the protocol describes the orthogonal assays to comprehensively analyze spheroids longitudinally for their supernatant composition (Part 2, Module 1), morphometry with or without spheroid or cell state specific information (Part 2, Module 2) and functional activity (Part 2, Module 3). Longitudinal follow-up circumvents the limitations of single-time point readouts, which only provide a snapshot and make it likely to miss key phenotypes and misinterpret treatment effects. Supernatant collection and preparation before analysis (including metabolite consumption and production and protein secretion) is reported (Fig. 3). Morphometric spheroid analysis can be performed with the use of light microscopy,

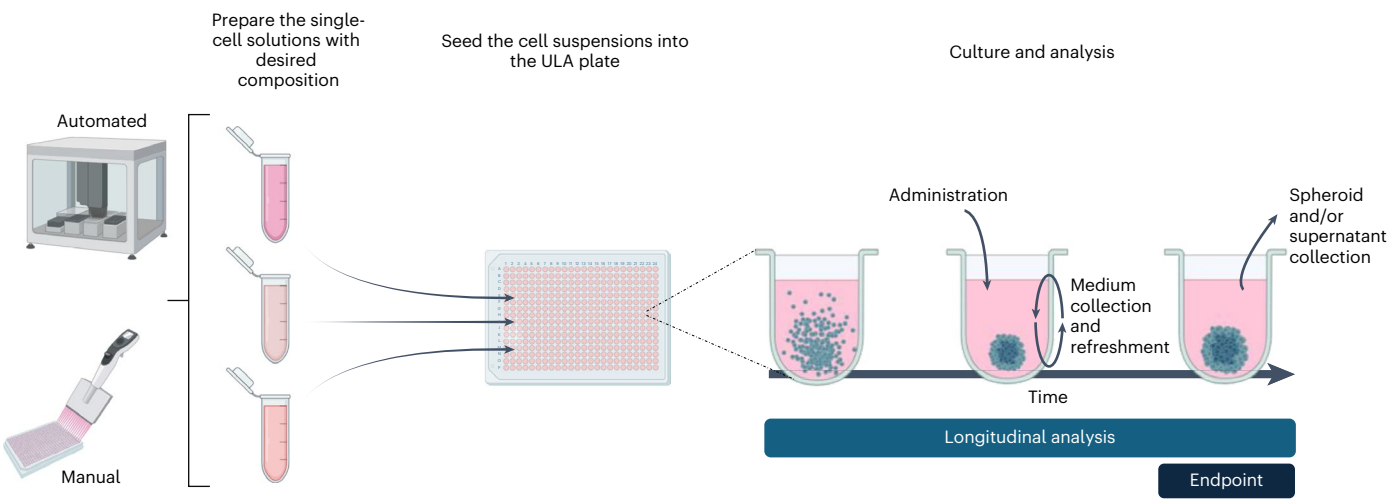

Fig. 2 | Spheroid setup. The protocol describes both manual as well as robotic single-cell seeding and spheroid harvesting, supernatant collection and refreshments and compound administration in and from ULA plates.

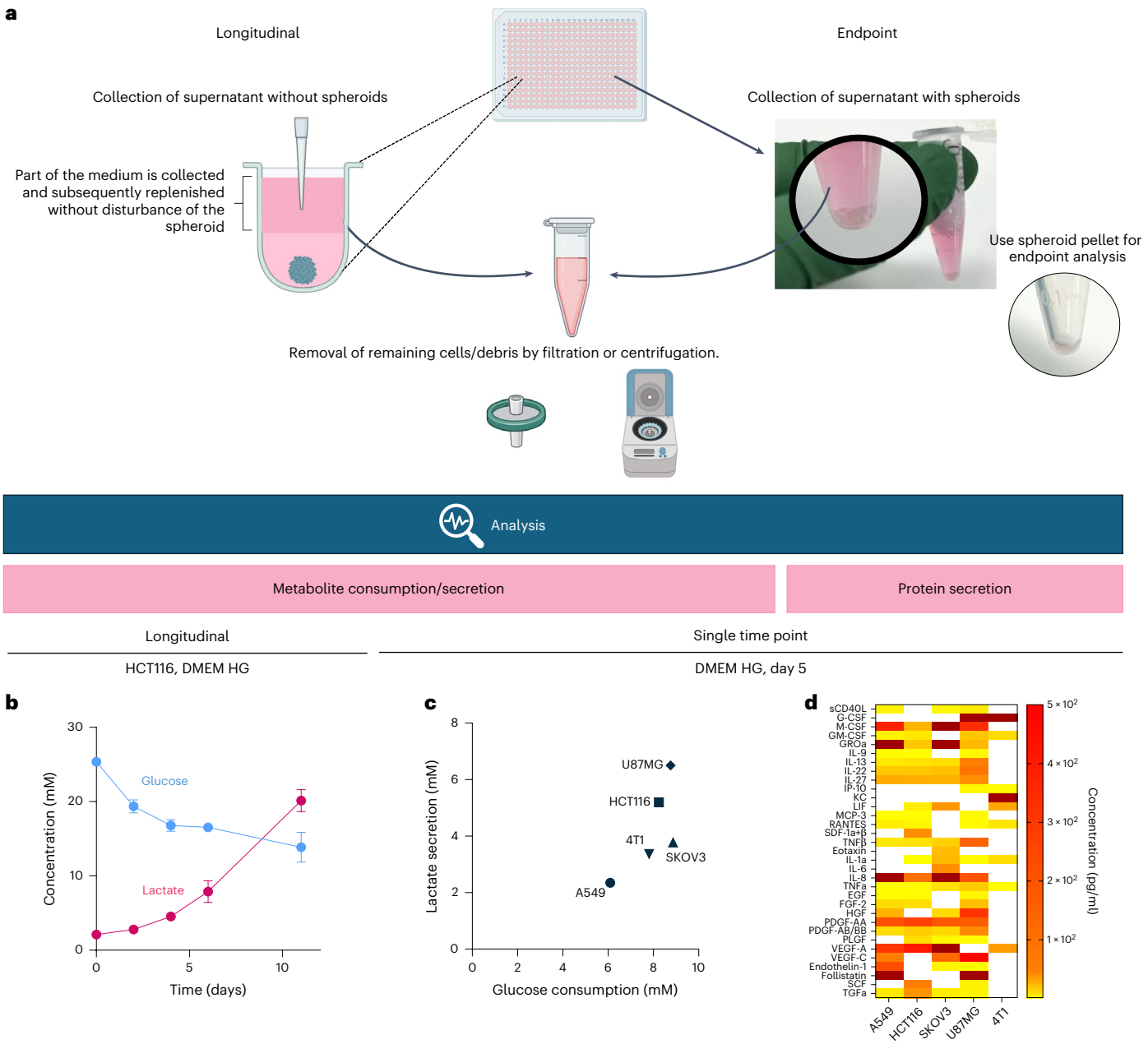

Fig. 3 | Supernatant analysis. a, The spheroid supernatant can either be collected longitudinally during partial medium refreshments or at endpoint and is subsequently cleared for remaining cells and/or debris by filtration or centrifugation. Next, the collected supernatant can be analyzed for metabolite consumption and secretion and protein secretion. **b**, Longitudinal follow-up of glucose and lactate levels in HCT116 (seeded in DMEM HG) spheroid supernatant when no medium refreshments are performed. Every dot indicates the mean, and the error bars indicate the standard deviation ($N = 4$, $n = 8$). **c**, Glucose consumption and lactate secretion of A549, HCT116, SKOV3, U87MG and 4T1 spheroids after 5 d of culture in DMEM HG. Every dot indicates the mean ($N \geq 2$, $n = 4$)[1]. **d**, Protein secretion signatures (luminex analysis) of A549, HCT116, SKOV3, U87MG and 4T1 spheroids after 5 d of culture in DMEM HG ($N \geq 3$, $n = 8$). Secreted proteins that are below the detection range are indicated in white[1]. **d** adapted from ref. 1, Springer Nature Limited.

while spheroid or cell state specific information can be visualized with epifluorescence or confocal microscopy by using fluorescent tracers (Fig. 4). Functional activity analysis includes the radial migration assay and the invasion assay (Fig. 5).

The third part of the protocol describes endpoint analyses, which allow investigation of molecular content (Part 3, Module 1), single-cell composition and state (Part 3, Module 2) and

architecture with spatial cell and subcellular information (Part 3, Module 3). Spheroid lysis and dissociation procedures before targeted and omics analysis of adenosine triphosphate (ATP) (CellTiter-glo 3D), RNA (quantitative PCR with reverse transcription (RT–qPCR) and RNA sequencing), protein (western blot and proteomics) and lipid (dedicated kits and lipidomics) content, are listed (Fig. 6). Moreover, we describe single-cell generation of spheroids to allow a more detailed molecular characterization (e.g., flow cytometry) (Fig. 7). Visualizing the molecular content in the native spheroid architecture can be performed by fixating the spheroids followed by immunohistochemical (Fig. 8), transmission electron microscopic (Fig. 9) or light-sheet fluorescence microscopic (Fig. 9) processing.

Experimental design

Spheroid setup

The protocol starts with the enzymatic dissociation of two-dimensional (2D) adherent cell cultures to single cells. The single cells are subsequently seeded in 96- or 384-well ULA plates in the desired cell density and culture medium. As previously reported by our group, there are critical aspects in spheroid setup (cell line, culture medium and spheroid size) that influence the outcome of a spheroid experiment that should first be considered and optimized[1]. In addition, when working with cocultures to dissect features of tissue microenvironment cross-talk, the optimal ratio of seeding cell populations should be determined beforehand owing to differences in cell growth rates, intercellular adhesion and survival. Culture medium will need to be adapted to support all included cell types. The use of fluorescent protein-expressing cell cultures easily allows the differential identification of the different cell types in spheroids.

For longer follow-up times, especially with lower culture volumes (e.g., 384-well plates), partial refreshment of the medium is advised to prevent nutrient depletion and/or metabolite accumulation. The time point of the required partial refreshments is dependent on the experimental setup (e.g., cell line, culture medium, seeding cell density, etc.) and can be determined based on nutrient and/or metabolite measurements of the supernatant (Part 2, Module 1). Medium evaporation can be avoided by covering the ULA plate with a semipermeable membrane, which still allows gas exchange.

Longitudinal analysis

Spheroids consume and secrete products (proteins, nutrients, metabolites, etc.) in the culture medium making it a primary sample source for spheroid metabolism investigation and biomarker discovery[1]. Supernatant analysis is not limited to the assays described (targeted metabolite and luminex analysis) but can extend to metabolomics, lipidomics, proteomics, transcriptomics, extracellular vesicle analysis, etc. Supernatant collection and analysis can coincide with all phenotypical assays that require collection of the spheroids as described throughout the protocol (Fig. 3).

The most used spheroid characterization methods, according to the MISpheroID knowledgebase, are microscopy-based techniques[1]. Several live-imaging instruments equipped with environmental controls are on the market (e.g., Cytation, IncuCyte, Opera, etc.) to perform individual spheroid monitoring and coupled integrated image analysis. Spheroid morphometric curves can be generated based on kinetic microscopic monitoring and generally comprise an initial compaction phase followed by a growth phase (Fig. 4a). During the compaction phase the single cells aggregate and develop tighter cell–cell contacts to form a compact spheroid (individual cells are difficult to discriminate, and there is an absence of spaces or voids) (Extended Data Fig. 1b) after which the spheroid size increases. The two phases can be distinguished by the point of inflection of the growth curve, which is for most cell lines ~day 2 following single cell seeding (Extended Data Fig. 1c). Moreover, similar to how imaging is used in patients to determine tumor size and, thus, therapy response, monitoring spheroid size can be used as an informative readout for drug efficacy.

Additional morphometric parameters (e.g., perimeter and circularity) can give extra information on spheroid phenotype and compound effect (e.g., disintegration), all evaluated through light microscopy imaging combined with image segmentation software (Fig. 4b). Spatiotemporal dynamics of biological processes and cell interactions can be assessed with

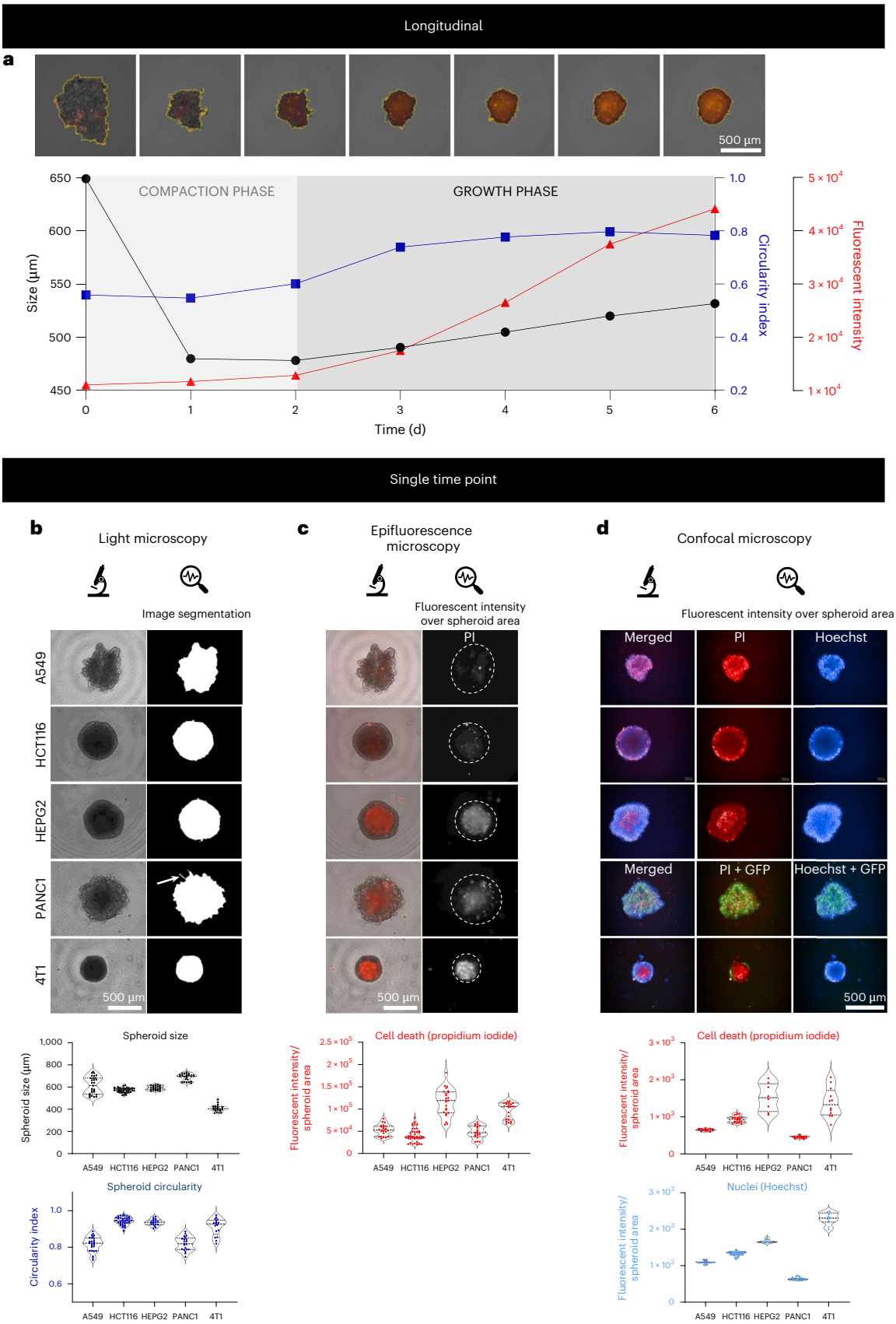
Longitudinal
a
500 µm
COMPACTION PHASE
GROWTH PHASE
Size (µm)
Circularity index
Fluorescent intensity
Time (d)
Single time point
b
Light microscopy
Image segmentation
c
Epifluorescence microscopy
Fluorescent intensity over spheroid area
PI
d
Confocal microscopy
Fluorescent intensity over spheroid area
Merged
PI
Hoechst
PI + GFP
Hoechst + GFP
A549
HCT116
HEPG2
PANC1
4T1
500 µm
Spheroid size
Spheroid size (µm)
Spheroid circularity
Circularity index
Cell death (propidium iodide)
Fluorescent intensity/ spheroid area
Nuclei (Hoechst)

Fig. 4 | Morphometric analysis with or without spheroid/cell specific information. a, Longitudinal follow-up of HEPG2 spheroid morphometry (spheroid size and circularity) with indication of the presence of cell death by propidium iodide (PI) staining. Size (black line), circularity (blue line) and RFP fluorescent intensity by PI (red line) over spheroid area (yellow border in the spheroid images) are visualized over 6 d of spheroid culture. A compact spheroid is formed at time point of the inflection point (smallest spheroid size) in the size curve, which is followed by growth of the spheroid. **b**–**d**, Single time point analysis on light (**b**), epifluorescence (**c**) ($N \geq 3$, $n = 8$) and confocal images (**d**) ($n \geq 8$) of A549, HCT116, HEPG2, PANC1 enhanced green fluorescent protein (eGFP) and 4T1 eGFP spheroids. Light microscopic images suffice to perform morphometric (e.g., spheroid size and circularity) analysis based on image segmentation[1]. Automated segmentation is not always performed perfectly as indicated by the white arrow in the PANC1 image segmentation. Epifluorescent and confocal images with fluorescent markers (propidium iodide (red) indicating cell death, Hoechst (blue) indicating nuclei, eGFP (green) indicating eGFP-expressing cells) allow the investigation of fluorescent intensity over the spheroid area (indicated by the white dotted line in the epifluorescent images)[1]. Horizontal bars in violin plots indicate the median. **b** and **c** adapted from ref. 1, Springer Nature Limited.

fluorescence microscopy with the use of fluorescence-based probes or fluorescent tracer protein-expressing cells (Fig. 4c,d). For example, the addition of 2 µg/mL of propidium iodide to the spheroid culture medium allows the quantification of dead cells (e.g., cell death in the center of the spheroid or cytotoxicity due to therapy) over time. However, dye-induced cytotoxicity and dye fluorescent lifetime should be taken into consideration for long-term follow-up (e.g., propidium iodide can be added during single-cell suspension seeding and is suited for longitudinal imaging; calcein AM or Hoechst have to be added just before spheroid imaging and are not suited for longitudinal follow-up due to cytotoxicity). Note that light microscopy and epifluorescent microscopy images are 2D projected images of the 3D object, which may fail to capture the full complexity of spheroids. Confocal microscopy can acquire higher resolution images with optical sectioning. However, penetration depth is limited to less than 100 µm (Supplementary Video 3). For higher penetration depth, other imaging approaches such as light-sheet fluorescence microscopy (LSFM) should be used ('Endpoint analysis' section).

Light microscopy can also monitor the migratory and invasive capacity of cells escaping from spheroids. In brief, during the radial migration and invasion assays, compacted spheroids are placed on a 2D tissue culture substrate or in a type I collagen matrix respectively, after which the single or collective migration or invasion of cells from the spheroids is recorded[18] (Fig. 5). To mimic the laminin-rich basement membrane together with the fibrillar type I collagen tumor microenvironment, a combination of Matrigel and type I collagen can be used[19]. Invasion occurs in all directions as can be imaged through optical sectioning (Extended Data Fig. 2). Be aware that not all cell types have invasive properties.

Endpoint analysis

Next to spheroid supernatant and morphometric analysis, ATP analysis can inform on the metabolic activity of the spheroid and is widely used to evaluate a therapeutic intervention[1]. Targeted or unbiased nucleic acid, protein and lipid-based investigations can further elucidate the underlying molecular pathways and phenotypically characterize the spheroids[1,20–22] (Fig. 6). These lysis-based approaches are powerful but have a disadvantage: they average cellular heterogeneity observed within a spheroid.

Spheroid dissociation into single cells through enzymatic digestion followed by flow cytometry or single-cell omics allows investigation of tissue biology at the single-cell level. Through flow cytometry, populations of cells can be distinguished on the basis of their physical and targeted molecular characteristics[23] (Fig. 7). This technique can be used for simple detection of the dead and living cell populations by live/dead stainings or can detect different cell populations (e.g., heterocellular spheroids) or cell states (e.g., proliferating, quiescent and apoptotic). Single-cell and single-nucleus RNA sequencing can perform unbiased single-cell gene expression measurements at high resolution of the whole spheroid.

Visualizing these expression profiles in their spatial context makes it possible to understand the cellular makeup of every cell within its original tissue architecture. Fixation of the spheroid and actual sectioning followed by histology (hematoxylin and eosin), immunohistochemistry (IHC) or transmission electron microscopy (TEM) allows investigatation of spheroid architecture with spatial cell (hematoxylin and eosin and IHC) or subcellular (TEM) resolution[24] (Figs. 8 and 9b).

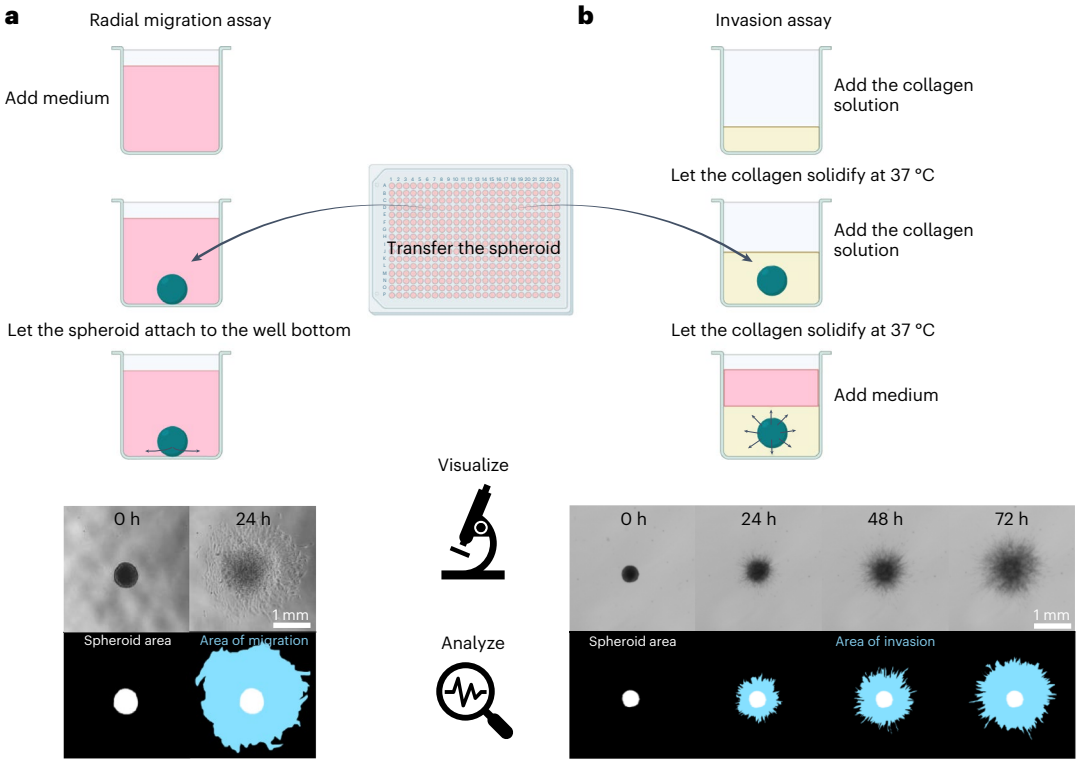

Fig. 5 | Functional assays. a,**b**, The migratory and invasive capacity of SW1353 spheroids is monitored in the radial migration and invasion assays respectively. During the radial migration assay, a compacted spheroid is placed on an adherent substrate and the single and collective migration of cells from the spheroid onto the substrate is recorded with a light microscope (**a**). During the invasion assay, a compacted spheroid is placed into a type I collagen matrix, and the single and collective invasion of cells from the spheroid into the matrix is recorded with a light microscope (**b**). Areas of migration or invasion (total area minus the original spheroid area, indicated in blue) are determined based on image segmentation.

The tissue processing and subsequent embedding before sectioning requires the spheroids to be placed in a matrix (e.g., agarose), which prevents spheroid loss. A spheroid pellet can be placed in a small volume of agarose ensuring the presence of different spheroids in one section and at different spheroid depths. The contrast agent used in the TEM procedure allows easier spheroid spotting during sectioning. A spheroid microarray places all spheroids at the same plane, which benefits the simultaneous sectioning of different (treatment) conditions. Conventional IHC typically labels only one marker per section. Multiplex immunofluorescence techniques have emerged to circumvent these constraints, allowing simultaneous detection of multiple markers on a single section and the comprehensive study of cell composition, cellular function and cell–cell interactions. Although the combination of the slices can be used to reconstruct the 3D conformation, technical caveats remain (loss of one or more sections).

To circumvent the challenges of photobleaching, light scattering and consequent limitations on penetration depth, LSFM combines tissue clearing with optical sectioning to allow high-resolution 3D imaging to characterize neighborhood associations in a true 3D context[25,26] (Fig. 9c and Supplementary Video 4 and 5).

Expertise needed to implement the protocol

The presented complementary techniques can be readily adopted by researchers experienced in cell culture and basic molecular biology. Further downstream analysis (metabolomics, luminex, lipidomics, (single-cell) transcriptomics, proteomics, confocal microscopy, IHC, TEM, LSFM, etc.) may require support or outsourcing.

Materials

Biological materials

Early-passage patient-derived cell cultures. For example, we used:

- HROC383 (colorectal cancer cells)[27] (CLS, cat. no. 300873), https://scicrunch.org/resolver/RRID:CVCL_VQ99
- HROC40 (colorectal cancer cells)[28] (CLS, cat. no. 300822), https://scicrunch.org/resolver/RRID:CVCL_1G01
- M28/2 (ovarian cancer cells) (CLS, cat. no. 300305)[29], https://scicrunch.org/resolver/RRID:CVCL_XX32

Established cell lines; for example, we have used:

- AT-3 (mouse mammary cancer cells) (Merck, cat. no. SCC178), https://scicrunch.org/resolver/RRID:CVCL_VR89
- A549 (lung cancer cells) (ATCC, cat. no. CCL-185), https://scicrunch.org/resolver/RRID:CVCL_0023
- CAOV-3 (ovarian cancer cells) (ATCC, cat. no. HTB-75), https://scicrunch.org/resolver/RRID:CVCL_0201
- HCT116 (colorectal cancer cells) (ATCC, cat. no. CCL-247), https://scicrunch.org/resolver/RRID:CVCL_0291
- HEK293T (human embryonic kidney cells) (ATCC, cat. no. CRL-11268), https://scicrunch.org/resolver/RRID:CVCL_0063
- HeLa (cervical cancer cells) (ATCC, cat. no. CCL-2), https://scicrunch.org/resolver/RRID:CVCL_0030
- HEPG2 (liver cancer cells) (ATCC, cat. no. HB-8065), https://scicrunch.org/resolver/RRID:CVCL_0027
- MCF-7 eGFP (breast cancer cells) (ATCC, cat. no. HTB-22)[30], https://scicrunch.org/resolver/RRID:CVCL_W971
- MDA-MB-231 (breast cancer cells) (ATCC, cat. no. HTB-26), https://scicrunch.org/resolver/RRID:CVCL_0062
- PANC1 (pancreatic cancer cells) (ATCC, cat. no. CRL-1469), https://scicrunch.org/resolver/RRID:CVCL_0480
- SKOV3 (ovarian cancer cells) (ATCC, cat. no. HTB-77), https://scicrunch.org/resolver/RRID:CVCL_0532
- SW1353 (sarcoma cells) (ATCC, cat. no. HTB-94), https://scicrunch.org/resolver/RRID:CVCL_0543
- SW837 (rectal cancer cells) (ATCC, cat. no. CCL-225), https://scicrunch.org/resolver/RRID:CVCL_1729
- T47D (breast cancer cells) (ATCC, cat. no. HTB-133), https://scicrunch.org/resolver/RRID:CVCL_0553
- U87MG (glioblastoma cells) (ATCC, cat. no. HTB-14), https://scicrunch.org/resolver/RRID:CVCL_0022
- 4T1 (mouse mammary cancer cells) (ATCC, cat. no. CRL-2539), https://scicrunch.org/resolver/RRID:CVCL_0125

  ▲ **CAUTION** All precautions must be made to avoid mycoplasma, yeast, fungus or bacterial contamination during cell manipulations. In addition, cultures should be regularly checked to ensure their authenticity.

  The T cells and monocytes used in the heterocellular spheroids (Fig. 7) were isolated from buffy coats of healthy donors.

  ▲ **CAUTION** Experiments involving human samples must be in compliance with ethics review board regulations and must obtain informed consent, as locally applicable.

Reagents

Common reagents

- (Ca^{2+}- and Mg^{2+}-free) phosphate-buffered saline, pH 7.2 (PBS; Thermo Scientific, cat. no. 11540546)

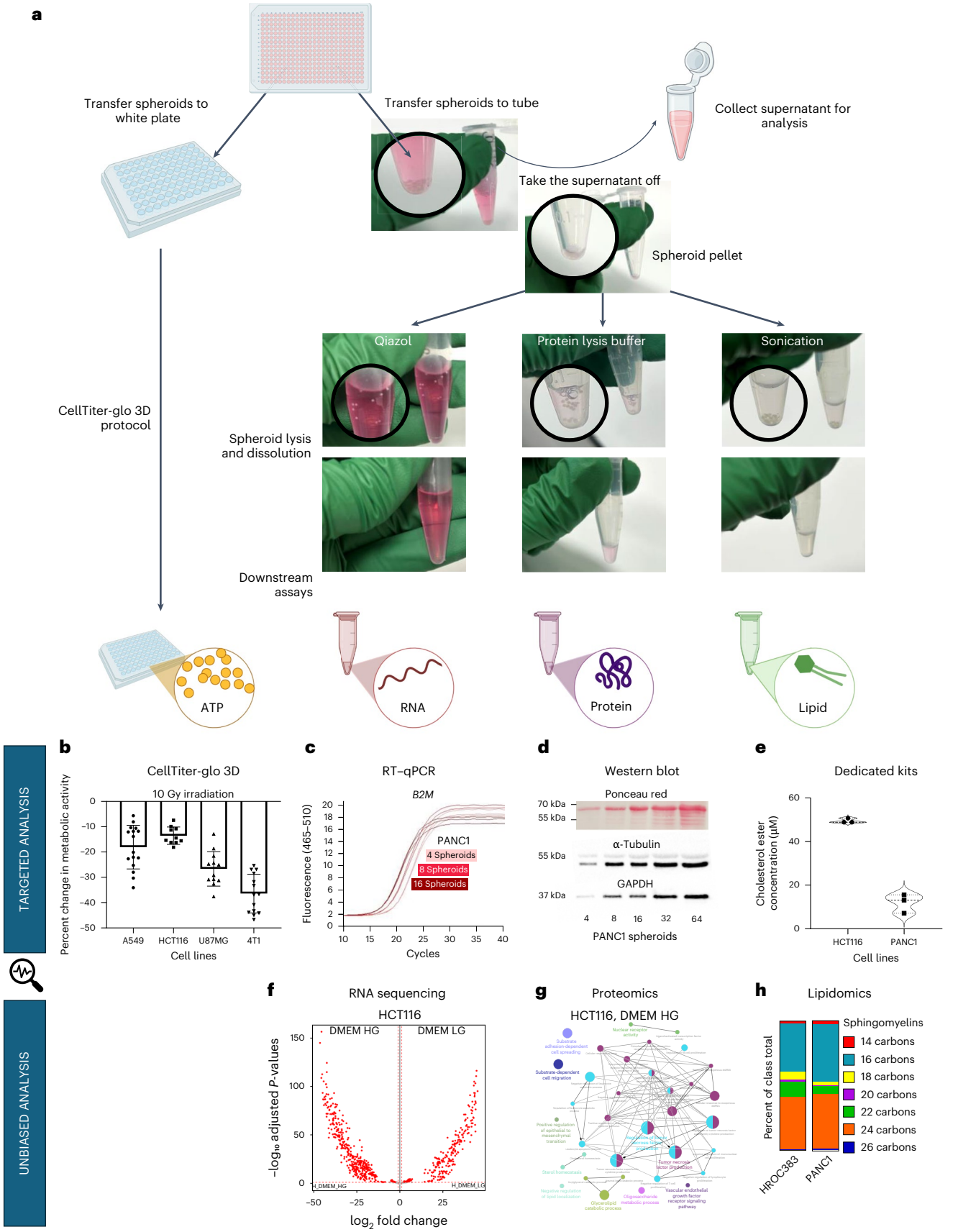
a
Transfer spheroids to white plate
Transfer spheroids to tube
Collect supernatant for analysis
Take the supernatant off
Spheroid pellet
CellTiter-glo 3D protocol
Spheroid lysis and dissolution
Qiazol
Protein lysis buffer
Sonication
Downstream assays
ATP
RNA
Protein
Lipid
TARGETED ANALYSIS
UNBIASED ANALYSIS
b
CellTiter-glo 3D
10 Gy irradiation
Percent change in metabolic activity
A549
HCT116
U87MG
4T1
Cell lines
c
RT–qPCR
B2M
PANC1
4 Spheroids
8 Spheroids
16 Spheroids
Fluorescence (465–510)
Cycles
d
Western blot
Ponceau red
70 kDa
55 kDa
α-Tubulin
55 kDa
GAPDH
37 kDa
4
8
16
32
64
PANC1 spheroids
e
Dedicated kits
Cholesterol ester concentration (μM)
HCT116
PANC1
Cell lines
f
RNA sequencing
HCT116
DMEM HG
DMEM LG
−log10 adjusted P-values
log2 fold change
g
Proteomics
HCT116, DMEM HG
h
Lipidomics
Percent of class total
HROC383
PANC1
Sphingomyelins
14 carbons
16 carbons
18 carbons
20 carbons
22 carbons
24 carbons
26 carbons

Fig. 6 | Analysis of molecular content. a, Spheroids are transferred to a white plate (ATP analysis) or tube (RNA, protein and lipid analysis) and subsequently lysed and dissolved in the appropriate lysis buffer or through sonication. Through preexisting protocols targeted and/or unbiased ATP, RNA, protein and lipid analysis can be performed. **b**, Irradiation effect in A549, HCT116, U87MG and 4T1 spheroids measured by the change in metabolic activity (ATP) compared to non-irradiated spheroids. Horizontal bar indicates the median, error bars indicate standard deviation ($N = 2$, $n = 8$)[1]. **c**,**d**, RT–qPCR cycles of reference gene *B2M* (**c**) and ponceau red protein staining and western blot bands of housekeeping proteins alpha-tubulin and GAPDH of increasing numbers of PANC1 spheroids (**d**). **e**, Cholesterol ester concentrations (measured with Promega's cholesterol/cholesterol-ester glo kit) of HCT116 and PANC1 spheroids. The horizontal bar indicates the median ($N = 3$, $n = 1$). **f**, A volcano plot of differently expressed genes of HCT116 spheroids ($N = 4$, $n = 8$) cultured in DMEM HG versus DMEM LG[1]. **g**, Gene ontology biological process interaction networks (ClueGo) based on differently expressed proteins of HCT116 spheroids ($N = 4$, $n = 16$) cultured in DMEM HG (compared with HCT116 spheroids cultured in DMEM LG). **h**, Chain length of sphingomyelins in HROC383 and PANC1 spheroids ($N = 4$, $n = 25$). **b** and **f** adapted from ref. 1, Springer Nature Limited.

- Dulbecco's modified Eagle medium, high glucose (DMEM HG; Thermo Scientific, cat. no. 41965039)
- Dulbecco's modified Eagle medium, low glucose (DMEM LG; Thermo Scientific, cat. no. 31885023)

Part 1: spheroid setup

- Trypsin–EDTA (0.05%) (Thermo Scientific, cat. no. 11580626)
- Trypan blue 0.4% (Thermo Scientific, cat. no. T10282)

Part 2: longitudinal analysis

Module 2: morphometry with or without spheroid/cell specific information

- Stainings for epifluorescence/confocal microscopy; for example, we used:
  - Propidium iodide (Merck Life Science, cat. no. P4864, 2 µg/mL)
  - Hoechst 33342 (Thermo Scientific, cat. no. 62249, 4 µM)

Module 3: functional assays using spheroids

- Type I collagen, acid extracted rat tail (BD Biosciences, cat. no. 354236)
- Potassium chloride (KCl, Merck Life Science, cat. no. 60128)
- Potassium dihydrogen phosphate (KH_2PO_4, Merck Life Science, cat. no. P5655)
- Sodium chloride (NaCl, Chem-Lab, cat. no. CL00.1429.1000)
- Sodium bicarbonate ($NaHCO_3$, Merck Life Science, cat. no. 106329)
- Disodium hydrogen phosphate heptahydrate ($Na_2HP0_4.7H_2O$, Merck Life Science, cat. no. 7782-85-5)
- MEM 10× (Thermo Scientific, cat. no. 11430030)
- Sodium hydroxide (NaOH, Merck Life Science, cat. no. 221465)

Part 3: endpoint analysis

Module 1: molecular content

- CellTiter-glo 3D (Promega, cat. no. G9681)
- ATP (Promega, cat. no. P1132)
- miRNeasy Micro Kit (Qiagen, cat. no. 217084)
  ▲ **CAUTION** Qiazol is toxic, causes skin and eye irritation and can cause genetic defects. Please handle with appropriate safety gear.
- Triethylammonium bicarbonate (Thermo Scientific, cat. no. 90114)
- Sodium dodecyl sulphate (SDS; Merck Life Science, cat. no. L3771)
  ▲ **CAUTION** SDS causes skin and eye irritation. Please handle with appropriate safety gear.
- Pierce water, liquid chromatography–mass spectrometry (LC-MS) grade (Thermo Scientific, cat. no. 51140)
- Ultrapure water obtained from a Milli-Q system
- Primers for RT–qPCR; for example, we used:
  - B2M forward primer: TGCTGTCTCCATGTTTGATGTATCT (IDT, custom preparation (100 nmol duplex, standard desalting))
  - B2M reverse primer: TCTCTGCTCCCCACCTCTAAGT (IDT, custom preparation (100 nmol duplex, standard desalting))

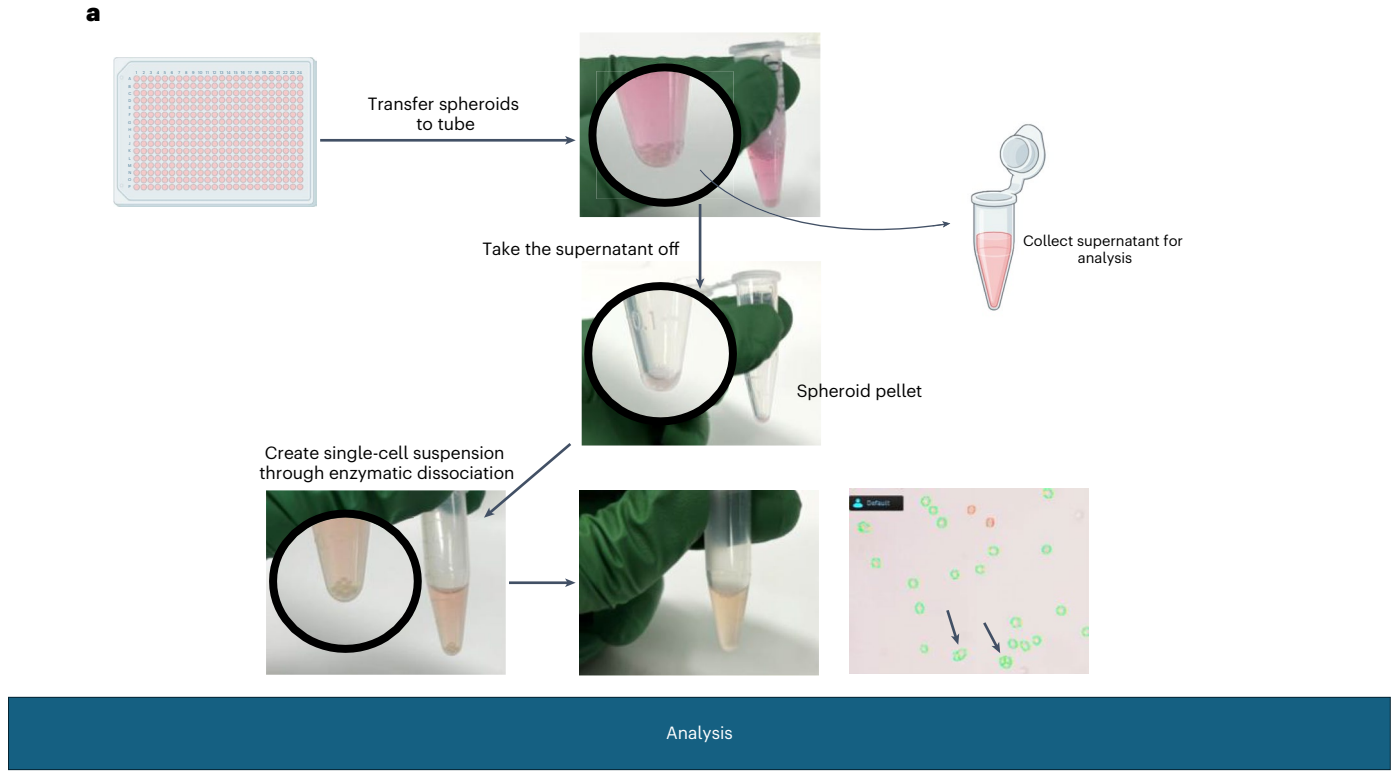

Fig. 7 | Analysis of single-cell composition and state. a, Spheroids are collected in a tube, the supernatant is carefully aspirated and a single-cell suspension is created through enzymatic dissociation. Complete dissolution of the spheroids can be visually confirmed (no visible structures remain and the solution becomes more opaque) and the presence of living (green) and dead (red) single cells and a minimal amount of doublets and triplets (indicated by arrows) can be confirmed by the use of an automated cell counter. **b**–**e**, Flow cytometry can further distinguish different populations: cells versus debris (**b**), single cells versus cell clusters (**c**), living cells versus dead cells (**d**) and T cells ($CD3^+$ staining), monocytes ($CD14^+$ staining) and SW837 cancer cells (double negative)) (**e**). Q, quadrant.

- Antibodies for western blot; for example, we used:
  - Anti-GAPDH (Merck Life Science, cat. no. G8795), https://scicrunch.org/resolver/RRID:AB_1078991
  - Anti-alpha-tubulin (Merck Life Science, cat. no. T5168), https://scicrunch.org/resolver/RRID:AB_477579
- SingleShot lysis kit (BioRad, cat. no. 1725080)
- Pierce 660 nm Protein Assay Kit (Life Technologies, cat. no. 22662)

Module 2: single-cell composition and state

- TrypLE select enzyme 1× (Gibco, cat. no. 12563011)
- Bovine Serum Albumin (NZYtech, cat. no. MB04602)

- Sodium azide (Thermo Scientific, cat. no. 5/2380/48)
  ▲ **CAUTION** Sodium azide is fatal if swallowed, in contact with skin or inhaled. Please handle with appropriate safety gear.
- Pharmingen transcription factor buffer set (Thermo Scientific, cat. no. BDB562574)

Module 3: architecture with spatial cell and subcellular information

- 4% Paraformaldehyde in PBS (Santa Cruz, cat. no. sc-281692)
  ▲ **CAUTION** Formaldehyde can cause cancer and causes skin and eye irritation. Please handle with appropriate safety gear.
- Agarose (Merck Life Science, cat. no. A9539)
- 70% Ethanol (Chemlab, cat. no. CL02.0539)
- 100% Ethanol (Chemlab, cat. no. CL00.1807)
- Xylene (Chemlab, cat. no. CL00.2401)
  ▲ **CAUTION** Xylene is harmful when in contact with skin or inhaled. Please handle with appropriate safety gear.
- Paraffin (Leica, cat. no. 39602012)
- Triton X-100 (Merck Life Science, cat. no. T8787)
  ▲ **CAUTION** Triton X-100 can be corrosive and cause acute toxicity. Please handle with appropriate safety gear.
- Sucrose (Merck Life Science, cat. no. S0389)
- 50% Glutaraldehyde (Electron Microscopy Sciences, cat. no. 16316)
  ▲ **CAUTION** Glutaraldehyde can cause acute toxicity when swallowed or inhaled and causes skin and eye irritation. Please handle with appropriate safety gear.
- 20% Paraformaldehyde aqueous solution electron microscopy grade (Electron Microscopy Sciences, cat. no. 15713)
  ▲ **CAUTION** Formaldehyde can cause cancer and causes skin and eye irritation. Please handle with appropriate safety gear.
- Sodium cacodylate trihydrate (Electron Microscopy Sciences, cat. no. 12300)
  ▲ **CAUTION** Sodium cacodylate trihydrate can cause cancer and is toxic when swallowed or inhaled. Please handle with appropriate safety gear.
- Osmium tetroxide 4% aqueous solution (Electron Microscopy Sciences, cat. no. 19190)
  ▲ **CAUTION** Osmium tetroxide can cause acute toxicity when swallowed or inhaled, can cause genetic defects and causes skin and eye irritation. Please handle with appropriate safety gear.
- Potassium ferrocyanide (Electron Microscopy Sciences, cat. no. 20150)
- Uranyl acetate (Electron Microscopy Sciences, cat. no. 22400)
  ▲ **CAUTION** Uranyl acetate is fatal when swallowed or inhaled. Please handle with appropriate safety gear.
- Ultrapure water obtained from a Milli-Q system
- HistoGel (Thermo Scientific, cat. no. HG-4000-012)
- 100% Propylene oxide (Electron Microscopy Sciences, cat. no. 20401)
  ▲ **CAUTION** Propylene oxide can cause acute toxicity when in contact with skin or inhaled, can cause genetic defects, may cause cancer and causes eye irritation. Please handle with appropriate safety gear.
- EMbed-812 (Electron Microscopy Sciences, cat. no. 14120)
- Stainings and antibodies for histology/IHC; for example, we used:
  - Hematoxylin (Merck Life Science, cat. no. 105174)
  - Erythrosin B, also known as eosin B (Thermo Scientific, cat. no. 409450250, 0.5% (wt/vol))
  - Anti-Ki-67 (Roche, cat. no. 790-4286), https://scicrunch.org/resolver/RRID:AB_2631262
  - Anti-cleaved caspase-3 (Cell Signaling Technology, cat. no. 9661), https://scicrunch.org/resolver/RRID:AB_2341188
- Stainings for LSFM; for example, we used:
  - DRAQ5, 1:10,000 (BioLegend, cat. no. 424101)
  - Flash Phalloidin Green 488, 1:200 (BioLegend, 424201)

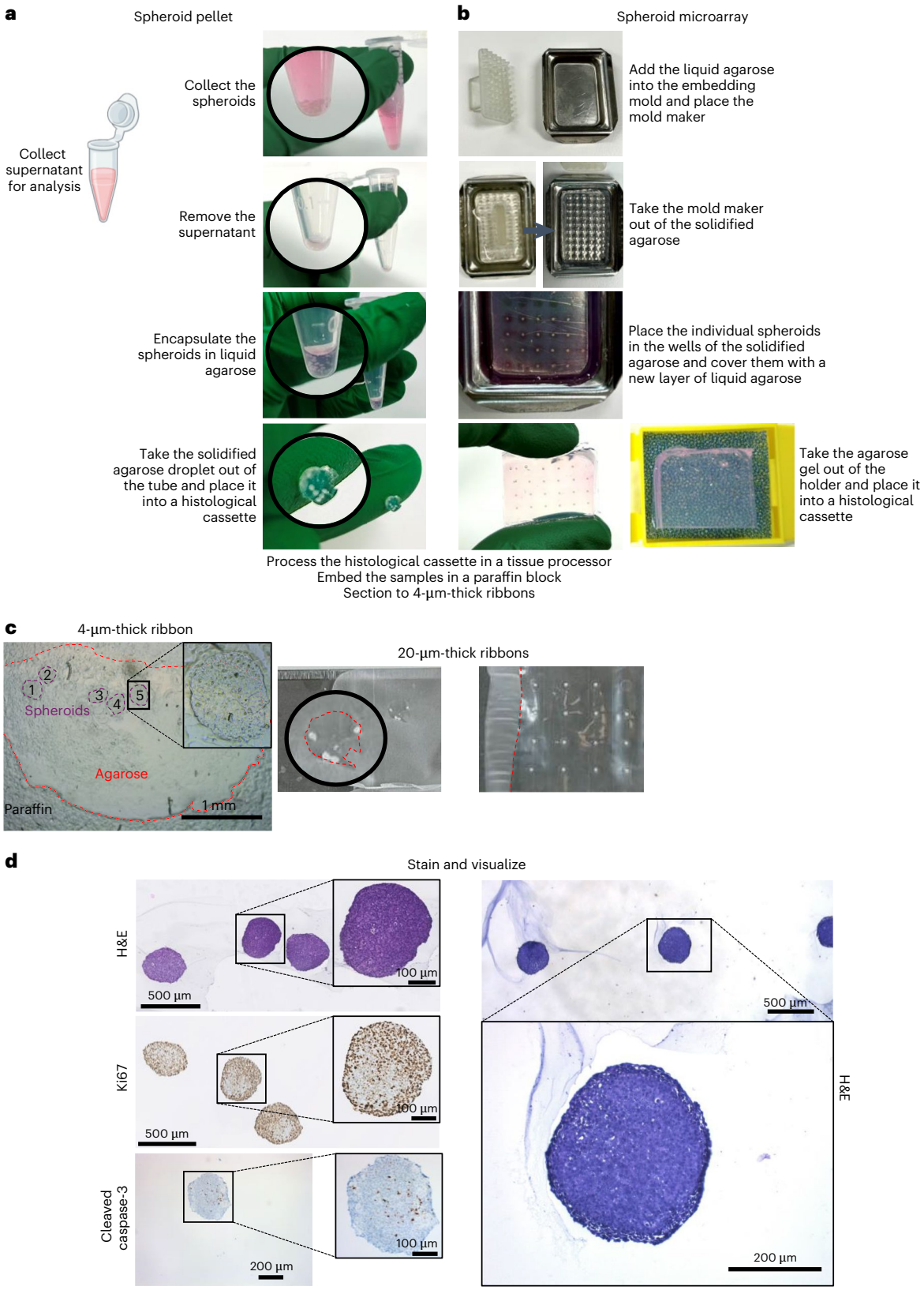
a
Spheroid pellet
Collect the spheroids
Collect supernatant for analysis
Remove the supernatant
Encapsulate the spheroids in liquid agarose
Take the solidified agarose droplet out of the tube and place it into a histological cassette
b
Spheroid microarray
Add the liquid agarose into the embedding mold and place the mold maker
Take the mold maker out of the solidified agarose
Place the individual spheroids in the wells of the solidified agarose and cover them with a new layer of liquid agarose
Take the agarose gel out of the holder and place it into a histological cassette
Process the histological cassette in a tissue processor
Embed the samples in a paraffin block
Section to 4-µm-thick ribbons
c
4-µm-thick ribbon
20-µm-thick ribbons
1
2
3
4
5
Spheroids
Agarose
Paraffin
1 mm
d
Stain and visualize
H&E
Ki67
Cleaved caspase-3
500 µm
100 µm
200 µm
H&E

Fig. 8 | Architecture with spatial cell information: histology and IHC. **a**,**b**, The spheroids have to be placed in agarose to prevent loss during the tissue processing and embedding and to be able to section at the height of the spheroids; the spheroids can either be collected in a tube and subsequently encapsulated in an agarose droplet (**a**) or placed in an agarose mold, which allows the sectioning of multiple spheroid conditions at the same time (**b**). **c**, The encapsulated spheroids are dehydrated, embedded in paraffin and sectioned to 4-µm-thick ribbons. Sectioning at the height of the spheroids can be checked on 20-µm-thick ribbons with the naked eye (the white structures in the agarose (encircled by the red dotted line)) or on the 4-µm-thick ribbons with a light microscope. **d**, The spheroids (HCT116) are histologically stained (hematoxylin and eosin (H&E)) or stained through IHC (Ki67 indicating proliferating cells, cleaved caspase-3 indicating apoptotic cells) and visualized.

Equipment

Common equipment

- 1.7 mL Eppendorf tube (Eppendorf, cat. no. A14934)
- 5 mL Eppendorf tube (Eppendorf, cat. no. A26755)
- 15 mL centrifuge tube (Nerbe Plus, cat. no. 02-502-8001)
- 5 mL serological pipette (Nerbe Plus, cat. no. 12-441-9105)
- 10 mL serological pipette (Nerbe Plus, cat. no. 12-461-9108)
- 10 µL pipette tips (Nerbe Plus, cat. no. A32506)
- 100 µL pipette tips (Vacutest Kima, cat. no. A12505)
- 1 mL pipette tips (Vacutest Kima, cat. no. A17313)
- Multichannel pipette tips (Sartorius, cat. no. LH-B790204)
- Motorized pipette controller
- Single-channel, adjustable volume pipettes
- Multichannel, adjustable volume pipette
- CO_2 incubator
- Laminar air flow (class II)
- Fume hood
- Benchtop centrifuge
- Hot plate
- Ice machine
- Balance
- −20 °C freezer
- −80 °C freezer

Part 1: spheroid setup

- Water bath
- 25 cm^2 tissue flasks (Avantor, cat. no. 734-2312)
- P250 pipette tips (liquid handler) (Axygen, cat. no. FX-250-R-S)
- P1000 pipette tips (liquid handler) (Beckman Coulter, cat. no. B01123)
- Cell counting chamber slides (Thermo Scientific, cat. no. C10228)
- Automated cell counter
- 96-well PrimeSurface ULA plate (S-bio, cat. no. MS-9096UZ)
- 384-well PrimeSurface ULA plate (S-bio, cat. no. MS-938UZ)
- Liquid handler (Biomek 4000, Beckman Coulter, cat. no. 369651)
  ▲ **CRITICAL** To ensure sterility during the seeding of the single-cell suspension, the liquid handler should be equipped with a positive-pressure high efficiency particulate air (HEPA) enclosure.
  ▲ **CRITICAL** The scripts to seed the spheroids, collect and refresh supernatant, administer compounds and collect spheroids (Part 1) are a list of commands, which have to be designed according to the user's needs and depend on the liquid handler used.
- Breath-easy sealing membrane (Merck Life Science, cat. no. Z380059)

Part 2: longitudinal analysis

Module 1: supernatant

- 0.22 µm filter (Novolab, cat. no. A37111)
- 2 mL syringe (BD, cat. no. 307727)

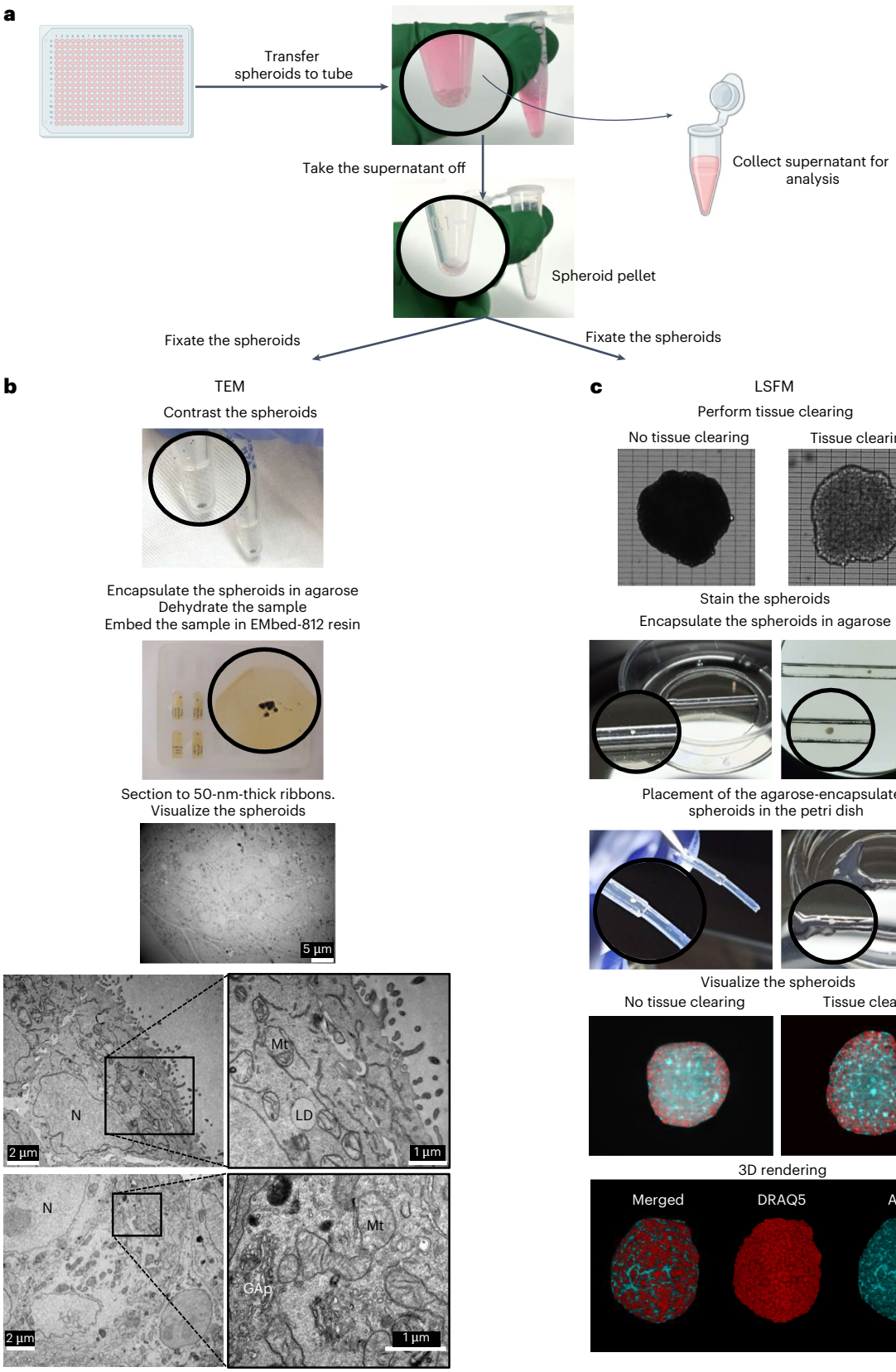
a
Transfer spheroids to tube
Take the supernatant off
Collect supernatant for analysis
Spheroid pellet
Fixate the spheroids
Fixate the spheroids
b
TEM
Contrast the spheroids
Encapsulate the spheroids in agarose
Dehydrate the sample
Embed the sample in EMbed-812 resin
Section to 50-nm-thick ribbons.
Visualize the spheroids
5 µm
N
Mt
LD
2 µm
1 µm
N
Mt
GAp
2 µm
1 µm
c
LSFM
Perform tissue clearing
No tissue clearing
Tissue clearing
Stain the spheroids
Encapsulate the spheroids in agarose
Placement of the agarose-encapsulated spheroids in the petri dish
Visualize the spheroids
No tissue clearing
Tissue clearing
200 µm
3D rendering
Merged
DRAQ5
Actin
100 µm

Fig. 9 | Architecture with spatial cell and subcellular information: LSFM and TEM. a, Spheroids are collected in a tube, supernatant is carefully aspirated and the spheroids are fixed. **b**, The spheroids are contrasted and encapsulated in agarose, dehydrated, embedded in EMbed-812 resin, sectioned to ultrathin 50 nm sections and subsequently visualized by a transmission electron microscope (N, nucleus; Mt, mitochondria; GAp, Golgi apparatus; LD, lipid droplet). **c**, Tissue clearing before LSFM renders the spheroids transparent and enables the visualization of the whole spheroid. Stained (DRAQ5 (red) indicating nuclei, Flash Phalloidin Green 488 (blue) indicating actin) T47D spheroids are encapsulated in agarose and subsequently visualized by a light-sheet fluorescence microscope.

Module 2: morphometry with or without spheroid/cell specific information

- 384-well spheroid microplates (for confocal imaging) (Corning, cat. no. 3830)
- Live cell imager (Cytation 5, BioTek Agilent, cat. no. CYT5MFAV)
- Automated CO_2 incubator (BioSpa, BioTek Agilent)
- Light microscope
- Epifluorescence microscope
- Confocal microscope (Opera Phenix, Revvity, cat. no HH14001000)

Module 3: functional assays using spheroids

- 24-well flat bottom plate (Thermo Scientific, cat. no. 142475)
- Live cell imager (Cytation 5, BioTek Agilent, cat. no. CYT5MFAV)
- Light microscope

Part 3: endpoint analysis

Module 1: molecular content

- White 96-well flat bottom plate (Fischer Scientific, cat. no. 236108)
- Orbital shaker
- Plate reader (Synergy HTX, BioTek Agilent)
- Handheld sonicator

Module 3: Architecture with spatial cell and subcellular information

- Microwave
- Forceps (vwr, cat. no. 232-0096)
- Histological cassette (vwr, cat. no. 10022-344)
- Tissue processor (HistoCore Pearl, Leica Biosystems)
- Embedding machine
- Cold plate
- Microtome
- Embedding mold (vwr, cat. no. LEIH3803081)
- Mold maker (inhouse 3D printed, adapted based on a described design[31]) (Supplementary Fig. 1)
- Foam pad (Simport, cat. no. M476)
- Superfrost Plus Gold Adhesion Microscopic slides (Epredia, cat. no. K5800AMNZ72)
- U-shaped glass capillary (Leica, cat. no. 158007061)
- Cellview cell culture petri dish (Greiner, cat. no. 627861)
- Light-sheet fluorescence microscope (Leica SP8)
- TEM-suitable mold (Science Services, cat. no. E70900)
- Ultramicrotome
- 200 mesh copper grids (Electron Microscopy Sciences, cat. no. G200-Cu)
- Transmission electron microscope (JEM-1400, Jeol)

Reagent setup

Type I collagen solution

To prepare a 1 mg/mL solution, mix (1) 4 volumes of 3.25 mg/mL rat tail type I collagen, (2) 5 volumes of calcium and magnesium-free Hanks buffered saline solution (CMF-HBSS), (3) 1 volume of minimum essential medium (MEM) 10×, 4) 1 volume of $NaHCO_3$ 0.25 M, (5) 0.3 volumes of NaOH 1 M and (6) 2.7 volumes of culture medium (vol/vol) on ice in a laminar air flow (adjust volumes to own requirements). Add the different components in

this described order. The concentration of the stock type I collagen is variable, adapt the volume of type I collagen with CMF-HBSS if necessary. Volumes of MEM, NaHCO3, NaOH and culture medium remain constant. Store prepared type I collagen solution at 4 °C for ≤8 weeks while keeping sterile.

CMF-HBSS
Dissolve 0.8 g KCl, 0.12 g KH_2PO_4, 17.2 g NaCl, 0.2 g $NaHCO_3$, 0.18 g $Na_2HP0_4{\cdot}7H_2O$ in 2 L of distilled water (wt/vol) and filter–sterilize (0.22 µm). Store at 4 °C for ≤1 year while keeping sterile.

0.25 M $NaHCO_3$ solution
Dissolve 0.25 M $NaHCO_3$ in CMF-HBSS (wt/vol) and filter–sterilize (0.22 µm). Store at 4 °C for ≤1 year while keeping sterile.

1 M NaOH solution
Dissolve 1 M NaOH in CMF-HBSS (wt/vol) and filter-sterilize (0.22 µm). Store at 4 °C for ≤1 year while keeping sterile.

Protein lysis buffer
Mix 50 mM triethylammonium bicarbonate (vol/vol) and 5% SDS (wt/vol) in Pierce water. Store at 4 °C for ≤1 year.

Flow cytometry staining protocol (fluorescent-activated cell sorting, FACS) buffer
Dissolve 2% bovine serum albumin in PBS (wt/vol). Add 0.1% sodium azide to prevent contamination. Store at 4 °C for ≤1 month.

Agarose solution
Dissolve 2% agarose in distilled water (wt/vol) and cook for 2 min in a microwave. Store at room temperature (20–22 °C). The solution (solidified at room temperature) can be reused several times by boiling again in a microwave.

Triton X-100 solution
Make 0.1–2% Triton X-100 in PBS (vol/vol). Store at room temperature for ≤1 year.

Sucrose solution
Make 10–50% sucrose in distilled water (wt/vol). Store at 4 °C for ≤1 month.

Sodium cacodylate trihydrate buffer
Dissolve 0.1 M sodium cacodylate trihydrate in distilled water (wt/vol), pH should be set at 7.3. Store at 4 °C for ≤3 months.

Fixation solution
Mix 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate trihydrate buffer (vol/vol). Prepare fresh before every use.

Contrast solution 1
Mix 1% osmium tetroxide (vol/vol) and 1.5% potassium ferrocyanide (wt/vol) in 0.1 M sodium cacodylate buffer. Store at 4 °C for ≤3 months.

Contrast solution 2
Dissolve 1% uranyl acetate in MilliQ water (wt/vol). Store at room temperature for ≤1 month.

EMbedding 3:1 solution
Dilute (3:1 dilution) EMbed-812 resin in propylene oxide (vol/vol). Prepare fresh before every use.

EMbedding 1:1 solution
Dilute (1:1 dilution) EMbed-812 resin in propylene oxide (vol/vol). Prepare fresh before every use.

EMbedding 1:3 solution
Dilute (1:3 dilution) EMbed-812 resin in propylene oxide (vol/vol). Prepare fresh before every use.

Procedure: Part 1: spheroid setup

● **TIMING Steps 1–13: 30 min to 3 h**

1. Culture cells under 2D conditions at 37 °C in an atmosphere of 5–10% (dependent on the culture medium used) CO_2 in air.
2. Prewarm trypsin/EDTA solution, Ca^{2+}- and Mg^{2+}-free PBS and culture medium in a 37 °C water bath.
   ▲ **CRITICAL STEP** To ensure sterility, the next steps should be performed in a laminar air flow with a positive-pressure HEPA enclosure.
3. Prepare a single-cell suspension from an exponentially growing culture (usually 80% confluence is used) by mild enzymatic dissociation, using a Ca^{2+}- and Mg^{2+}-free PBS wash followed by a short incubation (<2 min) with trypsin/EDTA solution at 37 °C.
   ▲ **CRITICAL STEP** Optimize the trypsinization procedure for every cell type to obtain a single-cell suspension with minimal presence of cell doublets or clusters.
4. Detach the cells by tapping the cell culture flask and neutralize the trypsin with three times the volume of prewarmed culture medium with serum.
5. Transfer the single-cell suspension from the culture flask to a 15 mL Eppendorf tube with a serological pipette.
6. (Optional) When a switch in culture conditions is required (e.g., different medium, nutrient concentrations, serum concentration, etc.); wash the cell culture twice by centrifugating the single cells at 200*g*, aspirating the supernatant without disrupting the single-cell pellet and resuspending the single-cell pellet in the desired culture conditions.
7. Determine the number of viable cells per mL via a cell counter. Viability is assessed by trypan blue.
8. Dilute the cell suspension volume (n) in a volume of medium (m) for every cell line involved in the culture, using the following calculations

$$\frac{a \times b}{c} = n$$

and

$$(d \times b) - n = m,$$

where a is the desired number of cells per well, and b is the number of wells (technical replicates, one well per spheroid).
▲ **CRITICAL STEP** Include additional wells to account for volume loss.
The variable c is the number of viable cells per milliliter (determined with the cell counter), and d is the volume per well (working volume of 80 µL per well in a 384-well plate and 200 µL per well in a 96-well plate).
▲ **CRITICAL STEP** Between different cell cultures, the seeding cell number does not correlate to size after spheroid formation (Fig. 4b). Thus, to obtain spheroids of a specific diameter for use in a particular downstream assay, the seeding cell density for the respective cell culture needs to be empirically determined.
▲ **CRITICAL STEP** If compound treatments are planned during spheroid culture (Step 20), count working volumes of 60 µL per well in a 384-well plate and 180 µL per well in a 96-well plate. A total of 20 µL of compound dissolved in culture medium will be administered at those later time points.

▲ **CRITICAL STEP** For cocultures; divide the working volume by the number of cell types involved in the culture and combine these cell culture volumes next.

9. (Optional) Add 2 µg/mL of propidium iodide to the culture medium if cell death should be visualized or quantified.
10. Seed the wells of the ULA plate with the single-cell suspension, using the volume per well from Step 8. Follow option A for robot-assisted seeding, or follow option B for manual seeding.
    (A) **Robot-assisted seeding of the ULA plates**
    ▲ **CRITICAL** To ensure sterility during the seeding of the single-cell suspension, the liquid handler should be equipped with a positive-pressure HEPA enclosure.
    (i) Start the robot (e.g., Biomek 4000 automated workstation) and software.
    (ii) Open the single-cell suspension seeding script.
    (iii) Place the tip boxes, pipette and tube rack in one of the 12 deck positions of the workstation.
    (iv) Place the single-cell suspensions in the tube rack and the ULA plate on the thermal exchange unit (37 °C).
    (v) Start the single-cell suspension seeding script (Supplementary Video 1 shows a part of the robot-assisted seeding).
    ▲ **CRITICAL STEP** Add in the script up- and down-pipetting steps in the tube of the single-cell suspension to ensure a homogenous cell suspension.
    ▲ **CRITICAL STEP** It is recommended to fill any empty wells with sterile PBS to avoid excessive evaporation.
    (B) **Manual seeding of the ULA plates**
    ▲ **CRITICAL** To ensure sterility, the seeding of the single-cell suspension should be performed in a laminar air flow with a positive-pressure HEPA enclosure.
    (i) Place the ULA plate on a hot plate (37 °C).
    (ii) Seed every well with the cell suspension.
    ▲ **CRITICAL STEP** Ensure that the suspension is homogeneous by gently pipetting up and down before seeding.
    ▲ **CRITICAL STEP** It is recommended to fill any empty wells with sterile PBS to avoid excessive evaporation.
11. Place a semipermeable sealing membrane on the plate to prevent evaporation.
12. Place the seeded ULA plate in an incubator at 37 °C and 5–10% (dependent on the culture medium used) CO_2 in air.
13. Ensure compact and uniform spheroid formation and growth during culture by microscopic evaluation (Part 2, Module 2).
    ▲ **CRITICAL STEP** Exclude spheroids which are formed around foreign particles (e.g., dust) from further analysis (Extended Data Fig. 3).
    ◆ **TROUBLESHOOTING**

(Optional) Perform partial medium refreshment

● **TIMING Steps 14–17: 20–80 min**

14. Take the ULA plate out of the incubator and remove the semipermeable membrane from the ULA plate in a laminar airflow.
15. Refresh the medium. Follow option A for robot-assisted or option B for manual.
    ▲ **CRITICAL STEP** To avoid aspirating and consequently losing the spheroid during these steps or critically reducing spheroid-secreted growth factors, we recommend leaving 40 µL of medium in a 384-well and 100 µL of medium in a 96-well plate.
    (A) **Robot-assisted medium refreshments**
    ▲ **CRITICAL** To ensure sterility during medium refreshments, the liquid handler should be equipped with a positive-pressure HEPA enclosure.
    (i) Start the robot (e.g., Biomek 4000 automated workstation) and software.
    (ii) Open the supernatant collection script (with liquid-level sensing) (Supplementary Video 2 shows a part of the robot-assisted supernatant collection).

(iii) Place the tip boxes, pipette and tube rack in one of the 12 deck positions of the workstation.
(iv) Place the empty Eppendorf tubes in the tube rack and the ULA plate on the thermal exchange unit (37 °C).
(v) Start the supernatant collection script.
- Collected supernatant can be analyzed (Part 2, Module 1).

(vi) Open the spheroid medium addition script.
(vii) Place the fresh medium in the tube rack and keep the ULA plate on the thermal exchange unit (37 °C).
▲ **CRITICAL STEP** Make sure the fresh medium has the same composition as the seeding medium (e.g., with addition of propidium iodide).
(viii) Start the spheroid medium addition script.

(B) **Manual medium refreshments**
▲ **CRITICAL** To ensure sterility, medium refreshments should be performed in a laminar air flow with a positive-pressure HEPA enclosure.
(i) Place the ULA plate on a hot plate (37 °C).
(ii) Collect the supernatant at the liquid–air surface without disturbing the spheroid.
- Collected supernatant can be analyzed (Part 2, Module 1).

(iii) Replace the same volume of medium that you collected with fresh medium.
▲ **CRITICAL STEP** Make sure the fresh medium has the same composition as the seeding medium (e.g., with addition of propidium iodide).

16. Place a new semipermeable membrane on the plate to prevent evaporation.
17. Place the ULA plate back in an incubator at 37 °C and 5–10% (dependent on the culture medium used) CO_2 in air.

(Optional) Administering compounds

● **TIMING Steps 18–22: 15–40 min**

▲ **CRITICAL** Follow this step to administer compounds (e.g., drugs, fluorescent staining, etc.) during spheroid culturing.

18. Prepare compound solutions at the desired concentrations, taking into account the dilution (e.g., when 20 µL of a compound solution is added to 60 µL of cell suspension already present in the well, the concentration of the compound solution should be four times as high as the desired end concentration).
▲ **CRITICAL STEP** In case of drug screenings, include a control condition.
19. Take the ULA plate out of the incubator and remove the semipermeable membrane from the ULA plate in a laminar airflow.
20. Administer the compound. Follow option A for robot-assisted or option B for manual.

(A) **Robot-assisted compound administration**
▲ **CRITICAL** To ensure sterility during compound administration, the liquid handler should be equipped with a positive-pressure HEPA enclosure.
(i) Start the robot (e.g., Biomek 4000 automated workstation) and software.
(ii) Open the spheroid drug administration script.
(iii) Place the tip boxes, pipette and tube rack in one of the 12 deck positions of the workstation.
(iv) Place the Eppendorf tubes containing the compound or control solutions in the tube rack and the ULA plate on the thermal exchange unit (37 °C).
(v) Start the spheroid drug administration script.

(B) **Manual compound administration**
▲ **CRITICAL** To ensure sterility, compound administration should be performed in a laminar air flow with a positive-pressure HEPA enclosure.
(i) Place the ULA plate on a hot plate (37 °C).
(ii) Add the compound and control solutions to the appropriate wells.

21. Place a new semipermeable membrane on the plate to prevent evaporation.
22. Place the ULA plate back in an incubator at 37 °C and 5–10% (dependent on the culture medium used) CO_2 in air.

(Optional) Spheroid collection

● **TIMING Steps 23–24: 15 min to 1 h**

23. Take the ULA plate out of the incubator and remove the semipermeable membrane from the ULA plate in a laminar airflow.
24. Collect the spheroids. Follow option A for robot-assisted or option B for manual.

▲ **CRITICAL STEP** The 100 µL pipette tips are wide enough to collect most spheroids without damage. The 1,000 µL pipette tips can be an alternative for bigger spheroids.

(A) **Robot-assisted spheroid collection**

▲ **CRITICAL** If the spheroid collection is an endpoint handling, sterility might not be required. In case sterility is required, the liquid handler should be equipped with a positive-pressure HEPA enclosure.

(i) Start the robot (e.g., Biomek 4000 automated workstation) and software.
(ii) Open the spheroid collection script (with liquid-level sensing).
(iii) Place the tip boxes, pipette and tube rack in one of the 12 deck positions of the workstation.
(iv) Place the empty Eppendorf tubes in the tube rack and the ULA plate on the thermal exchange unit (37 °C).
(v) Start the spheroid collection script.

(B) **Manual spheroid collection**

▲ **CRITICAL** If the spheroid collection is an endpoint handling, sterility might not be required. In case sterility is required, spheroid collection should be performed in a laminar air flow with a positive-pressure HEPA enclosure.

(i) Place the ULA plate on a hot plate (37 °C).
(ii) Collect the supernatant together with the spheroid by pipetting up the whole well volume.

▲ **CRITICAL STEP** The collection of the spheroid can be followed visually (Extended Data Fig. 4).

◆ **TROUBLESHOOTING**

Procedure: Part 2: longitudinal analysis

Module 1: Supernatant

▲ **CRITICAL** To calculate consumption, production and secretion of metabolites or proteins in the medium, it is essential to include a control. This control is the same culture medium, without suspended cells, undergoing all steps (incubation, filtration, etc.) similar to the test conditions.

● **TIMING Steps 1–2: 30–90 min**

1. Collect the supernatant. The spheroid should not be aspirated. Supernatant should be collected at the liquid-air surface without disrupting the spheroid and replaced by culture medium (Part 1, medium refreshment).
   - In case of an endpoint analysis (Part 3) the entire well volume together with the spheroid can be aspirated (Part 1, spheroid collection).
2. Separate remaining cells and cellular debris from the supernatant. Follow option A to filter the supernatant and option B to use a centrifuge.

▲ **CRITICAL STEP** Removal of remaining cells or cellular debris should be performed before freezing the supernatant owing to intracellular content (e.g., cytokines and metabolites) release by bursting of the cells during the freeze–thaw procedures.

(A) **Filter the supernatant**

(i) Attach a 0.22 µm filter to a syringe.
(ii) Transfer the supernatant in the syringe.
(iii) Gently push the supernatant through the filter into a new Eppendorf tube.

BOX 2

Metabolite and secretome evaluation using dedicated protocols or kits

Metabolite evaluation: metabolite evaluation can be performed by metabolomics or dedicated kits (e.g., Promega's Metabolite Detecting Assays) following manufacturer's instructions.

Secretome evaluation: secretome evaluation can be performed by Luminex, individual or multiplex ELISA or dedicated kits (e.g., Promega's Lumit Immunoassays) following manufacturer's instructions.

Longitudinal (Fig. 3b) or single time point (Fig. 3c,d) consumption, production or secretion should be calculated based on the 'starting' levels of the control.

(B) **Centrifuge the supernatant**

(i) Centrifuge the supernatant at 200*g* for 5 min.

(ii) Transfer the supernatant into a new Eppendorf tube.

▲ **CRITICAL STEP** Gently aspirate the supernatant at liquid–air surface while steering away from the bottom of the Eppendorf tube to avoid transfer of pelleted cells/cell debris.

■ **PAUSE POINT** Supernatant can be stored at −80 °C for <4 weeks in case the intended aim is to perform secreted protein analysis (growth factors, cytokines and chemokines have a short half-life, avoid freeze–thaw cycles) or for <1 year in case the intended aim is to perform metabolite analysis.

3. Perform metabolite and secreted protein analysis with dedicated protocols or kits following manufactures instructions (Box 2).

◆ **TROUBLESHOOTING**

Procedure: Part 2: longitudinal analysis

Module 2: morphometry with or without spheroid/cell specific information

1. Image spheroids. Follow option A for robot-assisted imaging and option B for manual imaging.

▲ **CRITICAL STEP** Corning 384-well spheroid microplates are compatible with (longitudinal) confocal imaging. The spheroids can be formed, cultured and imaged in these plates without the need for a transfer to other (flat bottom) plates, compatible with confocal microscopy.

◆ **TROUBLESHOOTING**

(A) **Robot-assisted imaging**

● **TIMING 10 min to 1 h**

▲ **CRITICAL** The live cell imager/incubator should be equipped with environmental controls (temperature, CO_2) that are essential for kinetic live cell imaging.

(i) Start the live cell imager (e.g., Cytation 5 in combination with the BioSpa) and software.

(ii) Place the ULA plate in the holder of the imager (one time point imaging) or the attached automated incubator with robotic arm (discontinuous kinetic imaging).

(iii) Schedule one time point or discontinuous kinetic imaging with the necessary channels (brightfield or phase contrast with or without combinatory fluorescent channels, e.g., red fluorescent protein (RFP) channel for propidium iodide).

BOX 3

Image analysis

Morphometrics: morphometrics such as spheroid size and circularity can be calculated based on image segmentation, where the availability of brightfield or phase contrast images suffices. In image segmentation, every pixel in an image is assigned a label based on certain characteristics (e.g., intensity), and in this way, the spheroid can be distinguished from the background. Image segmentation can be performed inherently by the microscope software by changing a few parameters such as intensity to ensure the best possible segmentation (Fig. 4a, yellow border) or by uploading the images in open-source image processing programs such as AnaSP[34] or ImageJ (Fig. 4b, background (black) and spheroid (white) distinguishments). Image segmentation depends on image quality, background and intensity difference. Note that automated segmentation is not always performed perfectly (Fig. 4b, arrow), in this case manual segmentation (drawing the border manually) can be a valuable but more time-consuming alternative. Based on the image segmentation, data such as area, diameter, perimeter, circularity, etc. can be extracted with the same software (Fig. 4b).

To calculate the area of migration and invasion (blue in Fig. 5), the initial spheroid area at the start of the assay (white) should be subtracted from the area after migration or invasion has occurred.

Spheroid/cell specific information: spheroid or cell specific information can be captured by epifluorescence or confocal microscopy through fluorescent protein expressing cell cultures (e.g., enhanced green fluorescent protein (eGFP) (Fig. 4d) or staining (e.g., propidium iodide to stain damaged or dead cells (Fig. 4a,c,d), nuclei staining by Hoechst (Fig. 4d)). Data extraction out of these images can be performed inherently by the microscope software (e.g., fluorescent intensity over spheroid area (Fig. 4a,d)) or can be performed by uploading the fluorescent intensity images (in gray scale) in open-source image processing programs such as ImageJ (e.g., measure fluorescent intensity over spheroid area (white oval (Fig. 4c)).

(B) **Manual imaging**

● **TIMING 20 min to 2 h**

(i) Turn on the microscope and software.

(ii) Place the ULA plate in the holder of the microscope and start taking pictures with the necessary channels (brightfield or phase contrast with or without combinatory fluorescent channels, e.g., RFP channel for propidium iodide).

(iii) Place the ULA plate back in the incubator in case continuous follow-up is required.

2. Perform image analysis either with the software of the microscope or through the use of open-source image processing programs (Box 3).

◆ **TROUBLESHOOTING**

Procedure: Part 2: longitudinal analysis

Module 3: functional assays using spheroids; radial migration assay

● **TIMING Steps 1–4: 3.5 h**

▲ **CRITICAL** Assays to evaluate migration should be performed at time points when the spheroids are compact (Extended Data Fig. 1).

▲ **CRITICAL** To ensure sterility, next steps should be performed in a laminar air flow with a positive-pressure HEPA enclosure.

1. Prewarm the culture medium in a warm water bath at 37 °C.

   ▲ **CRITICAL STEP** To ensure sterility, the next steps should be performed in a laminar air flow with a positive-pressure HEPA enclosure.

2. Add 700 µL of the culture medium in every well of a flat bottom 24-well plate.

3. Pipet 50 µL supernatant together with the spheroid in one smooth movement from the ULA well (Part 1, manual spheroid collection) and transfer it carefully (push out slowly) in the center of the well of the 24-well plate. The pipetting and the placement of the spheroid should be visible with the naked eye but can also be checked under a microscope.

   ▲ **CRITICAL STEP** To ensure migration in all directions and to avoid a shadow of the well walls during imaging, the spheroid should be placed as close as possible to the center of

the well. For the same reason, 24-well plates (bigger well area) are preferably used over 96-well plates.

4. Carefully place the 24-well plate in an incubator at 37 °C and 5–10% CO_2 in air.
   ▲ **CRITICAL STEP** Avoid swirling of the plate and vibration of the incubator (e.g., harshly closing the door) to ensure that the spheroids stay in the center of the well.
5. Image the migration of the cells out of the spheroid onto the plastic after spheroid attachment to the plastic (normally after ~3 h). This can either be performed automatically with a live cell imager or manually with a brightfield or phase contrast microscope (Part 2, Module 2).
   ▲ **CRITICAL STEP** A picture should be taking immediately after spheroid attachment to the plastic and, thus, before any migration occurred. The size of the spheroid in this reference picture needs to be calculated and used for the analysis of the migration area.
   ▲ **CRITICAL STEP** To ensure the observation of migration and not proliferation, the imaging duration should maximally be one cell cycle (~24 h).
6. Perform image analysis with the software of the microscope or manually through the use of open-source image processing programs (Box 3).
   ◆ **TROUBLESHOOTING**

Procedure: Part 2: longitudinal analysis

Module 3: functional assays using spheroids; invasion assay

● **TIMING Steps 1–8: 85 min**

▲ **CRITICAL** Assays to evaluate invasion should be performed at time points when the spheroids are compact (Extended Data Fig. 1).

▲ **CRITICAL** To ensure sterility, next steps should be performed in a laminar air flow with a positive-pressure HEPA enclosure.

1. Prepare a 1 mg/mL type I collagen solution (total volume of 500 µL/well × total amount of wells).
   ▲ **CRITICAL STEP** Keep the liquid cold until addition to the well plate to avoid premature gelification.
2. Add 250 µL of the type I collagen solution to every well of the flat bottom 24-well plate.
3. Place the 24-well plate at 37 °C until the solution is gelified (~20 min).
4. Carefully add 250 µL of the prepared collagen type I solution to the wells of the flat bottom 24-well plate.
   ▲ **CRITICAL STEP** Work fast. To avoid gelification of this second layer of collagen before the spheroid is placed, perform Step 4 and 5 per two wells and repeat until the whole plate is filled.
5. Pipet 30 µL supernatant together with the spheroid in one smooth movement out of the ULA well (Part 1, manual spheroid collection) and place it carefully (push out slowly) in the center of the well, where the spheroid will sink into the liquid collagen type I solution. The pipetting and the positioning of the spheroid should be visible with the naked eye but can also be checked under a microscope.
   ▲ **CRITICAL STEP** To ensure invasion in all directions and to avoid a shadow of the well walls during imaging, the spheroid should be placed as close as possible to the center of the well.
6. Place the 24-well plate in an incubator at 37 °C and 5–10% CO_2 in air until the solution is gelified (~20 min).
   ▲ **CRITICAL STEP** Avoid swirling of the plate and vibration of the incubator (e.g. harshly closing the door) to ensure that the spheroids stay in the center of the well.
7. Prewarm the culture medium in a warm water bath at 37 °C.
8. Carefully add 500 µL of medium on top of the gel.
   ▲ **CRITICAL STEP** Pipet the medium against the wall of the wells to avoid damage to the gel.

9. Image the invasion of the cells out of the spheroid into the collagen gel. This can either be performed automatically with a live cell imager or manually with a brightfield or phase contrast microscope (Part 2, Module 2).
   ▲ **CRITICAL STEP** A picture should be taking immediately after gelification, thus, before any invasion occurred. The size of the spheroid in this reference picture needs to be calculated and used for the analysis of the invasion area.
   ▲ **CRITICAL STEP** To be able to follow invasion in three dimensions, *z*-stacking is preferred when possible.
10. Perform image analysis with the software of the microscope or manually through the use of open-source image-processing programs (Box 3).
    ◆ **TROUBLESHOOTING**

Procedure: Part 3: endpoint analysis

Module 1: molecular content; ATP content

● **TIMING Steps 2–10: 50–95 min**

1. Thaw the CellTiter-glo 3D reagent at 4 °C overnight and equilibrate to room temperature by placing the reagent in a 22 °C water bath before use for ~30 min.
2. Take the ULA plate out of the incubator and remove the semipermeable membrane from the ULA plate.
3. (Optional) Remove supernatant from the wells of the ULA plate without disrupting the spheroid, after which the collected supernatant can be analyzed (Part 2, Module 1). This optional step will further reduce the required volume of cellTiter-glo 3D reagent (Step 6). Make sure that the spheroid together with at least 40 µL of supernatant is still present in every well of the ULA plate.
4. Transfer the remaining supernatant with the spheroids from the ULA plate (Part 1, spheroid collection) to a white 96-well flat bottom plate.
   ▲ **CRITICAL STEP** Opaque walls are required to avoid luminescent signal crossover.
   ▲ **CRITICAL STEP** Include a negative control to determine assay background.
5. (Optional) Include ATP standard curve (the instructions are covered in the manufacturer's manual) when absolute ATP values need to be determined.
6. Add an equal volume of cellTiter-glo 3D reagent in every well with a multichannel pipette.
   ▲ **CRITICAL STEP** Work in a dark environment.
7. Shield the plate from light.
8. Mix the contents by placing the plate on an orbital shaker for 5 min.
9. Place the shielded plate at room temperature for 25 min.
10. Read out the luminescent signal with a plate reader.
    ▲ **CRITICAL STEP** The final ATP concentration depends on cell line, culture conditions, seeding cell number and culture time (see Table 1).
    ◆ **TROUBLESHOOTING**
11. Analyze the results (Box 4).

Table 1 | ATP concentrations with varying cell types, seeding cell numbers, culture media and spheroid ages

ATP concentration (µM/spheroid)		1,000 cells per well		4,000 cells per well	
		DMEM HG	DMEM LG	DMEM HG	DMEM LG
PANC1	Day 2	0.36	0.38	1.36	1.42
	Day 6	1.02	1.10	1.96	1.76
HCT116	Day 2	0.28	0.19	1.02	0.77
	Day 6	1.60	1.34	2.33	1.56

BOX 4

Molecular content analysis

ATP: The cellular adenosine triphosphate content is a measure for metabolic activity and can be an indicator of treatment efficacy (e.g., change in metabolic activity after radiotherapy, Fig. 6b). Change in metabolic activity should be calculated based on the ATP levels of control spheroids.

RNA/protein: RNA and protein concentrations should be quantified and made equal over all samples before targeted RNA/protein (RT–qPCR/RT–dPCR/western blot) (Fig. 6c,d) or unbiased transcriptome/proteome (RNA sequencing/proteomics) (Fig. 6f,g) analysis. RNA concentrations and integrity can be determined by several spectrophotometers or electrophoresis devices available on the market (e.g., BioDrop and FragmentAnalyzer) respectively. Protein concentrations can be quantified with SDS-compatible assays (e.g., Pierce 660nm Protein Assay Kit).

Several open-source software are available to perform and visualize gene set enrichments (Fig. 6f) and biomolecular interaction networks (e.g., g:Profiler, Cytoscape, etc.) (Fig. 6g).

Lipid: For targeted lipid analysis (e.g., Promega's cholesterol/cholesterol Ester-Glo kit) follow manufacturer's instructions (Fig. 6e). DNA concentrations should be quantified and made equal over all samples before targeted lipid or unbiased lipidome analysis. DNA concentrations can be quantified by several spectrophotometers available on the market (e.g., BioDrop). Based on the lipidomics results, the different lipid species with their features (saturation and chain length) can be evaluated and plotted (e.g., chain length of sphingomyelins as an example in Fig. 6h).

Procedure: Part 3: endpoint analysis

Module 1: molecular content; RNA/protein/lipid content

● **TIMING Steps 1–4: 35–80 min**

▲ **CRITICAL** To avoid cellular changes, the next steps should be performed on ice.

1. Collect *x* spheroids per condition in a cone bottom Eppendorf tube (Part 1, spheroid collection).
   ▲ **CRITICAL STEP** The final RNA/protein/lipid concentration depends on cell line, culture conditions, seeding cell number and culture time. The number of spheroids (*x*) required to have a sufficient concentration should be determined beforehand (Tables 2–7).
2. Centrifuge at 200*g* and 4 °C for 5 min to spin the spheroids down.
3. Take as much supernatant off as possible without disrupting the spheroid pellet.
   • Collected supernatant can be analyzed (Part 2, Module 1).
4. Wash the spheroids three times by adding 1 mL of PBS, centrifuging at 200*g* for 5 min and subsequently removing the PBS without disturbing the spheroid pellet.

Table 2 | RNA concentrations with varying number of spheroids

PANC1, DMEM HG, 4,000 cells per well, day 10					
No. of spheroids	4	8	16	32	64
RNA (ng)	762	1792	3,586.8	6,934.2	10,838.8

Table 3 | RNA concentrations with varying cell types, seeding cell numbers, culture media and spheroid ages

RNA concentration (ng/spheroid)		1,000 cells per well		4,000 cells per well	
		DMEM HG	DMEM LG	DMEM HG	DMEM LG
PANC1	Day 2	84.91	82.04	182.18	174.79
	Day 6	190.93	195.83	302.05	255.68
HCT116	Day 2	60.21	92.23	256.73	215.95
	Day 6	342.48	304.5	587.13	436.28

Table 4 | Protein concentrations with varying number of spheroids

PANC1, DMEM HG, 4,000 cells per well, day 10					
No. of spheroids	4	8	16	32	64
Protein (µg)	14.4	27.8	49.9	72.3	129.3

Table 5 | Protein concentrations with varying cell types, seeding cell numbers, culture media and spheroid ages

Protein concentration (µg/spheroid)		1,000 cells per well		4,000 cells per well	
		DMEM HG	DMEM LG	DMEM HG	DMEM LG
PANC1	Day 2	0.71	0.52	1.81	2.03
	Day 6	1.5	2.38	2.06	2.03
HCT116	Day 2	1.01	0.59	2.76	1.78
	Day 6	2.23	2.07	3.15	3.49

Table 6 | DNA concentrations with varying number of spheroids

HCT116, DMEM HG, 4,000 cells per well, day 6					
No. of spheroids	4	8	16	32	64
dsDNA (ng)	2,416	5,270	29,850	61,240	99,420

Table 7 | DNA concentrations with varying cell types, seeding cell numbers, culture media and spheroid ages

dsDNA concentration (ng/spheroid)		1,000 cells per well		4,000 cells per well	
		DMEM HG	DMEM LG	DMEM HG	DMEM LG
PANC1	Day 2	507.5	546.25	1,522.5	1,505
	Day 6	938.75	993.375	1,841.25	1,651.25
HCT116	Day 2	190.5	304	863.875	856.375
	Day 6	1,121.375	1,248.5	1,973.75	1,045.75

5. Perform spheroid lysis. Follow option A (Box 5) for RNA analysis, option B for protein analysis and option C for lipid analysis.
   (A) **RNA**
   ● **TIMING Step 5A(i–iii): 2 h**
   (i) Add 700 µL of Qiazol Lysis Reagent (part of the miRNeasy Micro Kit) to the spheroid pellet.
   (ii) Vortex 20 times or more for complete spheroid dissolution. Dissolution of the spheroids can be followed by the naked eye.
   ▲ **CRITICAL STEP** Incomplete spheroid lysis may lead to reduced RNA yields and misrepresented RNA signatures.
   ■ **PAUSE POINT** Samples can be stored in Qiazol Lysis Reagent at −80 °C for <1 year until further RNA purification.
   (iii) Follow the miRNeasy Micro Kit protocol for purification of total RNA.
   ■ **PAUSE POINT** Store the RNA eluates at −80 °C for <1 year until RNA analysis.
   (iv) Perform RT–qPCR or digital PCR with reverse transcription (RT–dPCR) (targeted RNA analysis) or RNA sequencing (unbiased transcriptome analysis) (Box 4).
   (v) Analyze the results.

BOX 5

SingleShot Cell Lysis Kit for generation of cell lysates that are suitable for RNA analysis without RNA purification

The SingleShot Cell Lysis kit is a direct cell lysis method that does not require RNA purification. In this way, gene expression quantification can be performed in a less time-consuming and labor-intensive manner. Crude lysates may be less sensitive compared with standard RNA extraction but result in accurate gene expression measurements[35].

Procedure

Optimized protocol for spheroids:

● **TIMING Steps 1–5: 20 min**

1. Add 20 µL of SingleShot reagent to the spheroid pellet.
2. Pipet up and down thoroughly for complete spheroid disruption.
   ▲ **CRITICAL STEP** Incomplete spheroid lysis may lead to reduced RNA yields and misrepresented RNA signatures.
3. Incubate for 10 min at room temperature.
4. Incubate for 5 min at 37 °C.
5. Incubate for 5 min at 75 °C.
   ■ **PAUSE POINT** Samples can be stored at −80 °C for <1 year until RNA analysis.
6. Perform RT–qPCR or RT–dPCR (targeted RNA analysis) or RNA sequencing (unbiased transcriptome analysis) (Box 5).
   ▲ **CRITICAL STEP** RNA concentration cannot be determined on crude lysates. RT–qPCR with reference genes can be performed before RNA sequencing to ensure equal input.
7. Analyze the results.

(B) **Protein**

● **TIMING Step 5B(i–iii): 10 min**

(i) Add a minimum of 50 µL of protein lysis buffer to the spheroid pellet.
▲ **CRITICAL STEP** In case of a large spheroid pellet, a higher volume might be required to dissolve it completely.

(ii) Vortex 20 times or more for complete spheroid dissolution. Dissolution of the spheroids can be followed by the naked eye.
▲ **CRITICAL STEP** Incomplete spheroid lysis may lead to reduced protein yields and misrepresented protein signatures.
■ **PAUSE POINT** Samples can be stored at −80 °C for <1 year.

(iii) Clear the sample by centrifuging at 13,000*g* at 4 °C for 5 min and collect the supernatant in a new Eppendorf tube. The remaining pellet can be discarded.
■ **PAUSE POINT** Samples can be stored at −80 °C for <1 year until further protein analysis.

(iv) Perform western blot (targeted protein analysis) or proteomics through mass spectrometry (unbiased proteome analysis) (Box 4).

(v) Analyze the results.

(C) **Lipid**

● **TIMING Step 5C(ii–iii): 10 min**

(i) Freeze the spheroid pellet at −80 °C.
▲ **CRITICAL STEP** Freezing and thawing of the spheroid pellet causes better lysis.
■ **PAUSE POINT** Samples can be stored at −80 °C for <3 months.

(ii) Add a minimum of 100 µL of ice-cold MilliQ water to the spheroid pellet.
▲ **CRITICAL STEP** In case of a large spheroid pellet, a higher volume might be required to sonicate and dissolve it completely.

(iii) Lyse the spheroid pellet with a handheld sonicator. Dissolvement of the spheroids can be followed by the naked eye.
▲ **CRITICAL STEP** Incomplete spheroid lysis may lead to reduced DNA yields (normalization to DNA concentration) and misrepresented lipid signatures.
■ **PAUSE POINT** Samples can be stored at −80 °C for <3 months until further lipid analysis.

(iv) Perform lipidomics (unbiased lipidome analysis) (Box 4).

(v) Analyze the results.

Procedure: Part 3: endpoint analysis

Module 2: single-cell composition and state

● **TIMING Steps 1–7: 60–105 min**

1. Collect *x* spheroids per condition in a cone bottom Eppendorf tube (Part 1, spheroid collection).
   ▲ **CRITICAL STEP** The final single-cell number depends on cell line, culture conditions, seeding cell number and culture time. The number of spheroids (*x*) required to have a sufficient number of single cells should be determined beforehand (Tables 8–9).
2. Centrifuge at 200*g* for 5 min to spin the spheroids down.
3. Take as much supernatant off as possible without disrupting the spheroid pellet.
   • Collected supernatant can be analyzed (Part 2, Module 1).
4. Wash the spheroids three times by adding 1 mL of PBS, centrifuging at 200*g* for 5 min and subsequently removing the PBS without disturbing the spheroid pellet.
5. Add 300 µL of TrypLE select enzyme and incubate for 10 min at 37 °C.
   ▲ **CRITICAL STEP** In case of incomplete dissolution of the spheroids, mechanically dissociate the spheroids by pipetting up and down with a 200 µL pipette. Extensive mechanical dissociation should be avoided since it induces cell death.
   ▲ **CRITICAL STEP** Spheroid dissolution may require optimization according to the cell type used.
   ▲ **CRITICAL STEP** All following steps should be performed on ice.
   ◆ **TROUBLESHOOTING**
6. Wash twice by adding 1 mL of cold FACS buffer, centrifuging for 5 min at 200*g* and 4 °C and carefully removing the supernatant without disturbing the single-cell pellet.
7. Resuspend the single-cell pellet in cold FACS buffer.
8. Perform single-cell (e.g., flow cytometry, single-cell RNA sequencing) analysis according to existing protocols (Box 6).
   ◆ **TROUBLESHOOTING**
9. Analyze the results

Table 8 | Single cells with varying number of spheroids

	PANC1, DMEM HG, 4,000 cells per well, day 6				
No. of spheroids	4	8	16	32	64
Viable cells	17,792	42,004	81,008	183,018	468,047

Table 9 | Single cells with varying cell types, seeding cell numbers, culture media and spheroid ages

Live and dead cells (cells/spheroid)			1,000 cells per well		4,000 cells per well	
			DMEM HG	DMEM LG	DMEM HG	DMEM LG
PANC1	Day 2	Viable	1,320	1,504	4,500	6,600
		Dead	44	51	90	110
	Day 6	Viable	3,956	2,531	6,375	7,481
		Dead	1,429	1,650	2,531	1,099
HCT116	Day 2	Viable	3,075	3,075	10,331	13,313
		Dead	219	469	771	219
	Day 6	Viable	14,963	14,738	29,063	21,675
		Dead	3,300	2,963	2,644	4,613

BOX 6

Single-cell composition and state

Targeted single-cell analysis (flow cytometry): First, after spheroid dissociation, the single cells should be incubated with stainings that can enter the cell (e.g., live/dead stainings) or with fluorescent-conjugated antibodies that can detect antigens at the cell surface. Unstained and fluorescence minus one controls should be included to set appropriate gates and single-stain controls should be added to correct for fluorescent spillover. Flow cytometry samples can be fixed (and permeabilized) using dedicated kits (e.g., Pharmingen Transcription Factor Buffer Set) or 4% paraformaldehyde.

Based on the gating strategies (size, intracellular complexity and fluorescence intensities of stainings) different cell populations can be determined (Fig. 7b–e).

Unbiased single-cell omics (e.g., single-cell RNA sequencing): before single-cell omics, FACS can either be performed for quality control purposes to select for intact live cells or can be used to sort populations of interest within the spheroid (e.g., proliferating cells). Samples from different time points can be combined in one run by using dedicated fixation procedures for (single-cell) RNA sequencing purposes.

Based on the differently expressed omics profile, different cell populations can be determined.

Procedure: Part 3: endpoint analysis

Module 3: architecture with spatial cell and subcellular information; histology and IHC

● **TIMING Steps 2–6: 35 h**

▲ **CRITICAL** To avoid cellular changes, all steps until fixation should be performed on ice.

1. To use spheroid pellets, follow option A. To use spheroid microarrays, follow option B.

(A) **Spheroid pellet**

● **TIMING Step 1A(i–x): 25–49 h**

(i) Collect 10–30 spheroids per condition in a cone bottom 1.7 mL Eppendorf tube (Part 1, spheroid collection).

(ii) Leave the spheroids to sediment for 2 min.

▲ **CRITICAL STEP** Sedimentation of the spheroids due to gravity can be followed visually. Avoid centrifugation as it might change spheroid morphology and induce clumping.

(iii) Take as much supernatant off as possible without disrupting the spheroid pellet.

- Collected supernatant can be analyzed (Part 2, Module 1).

(iv) Dissolve 2% agarose in MilliQ water by boiling for 2 min in a microwave.

▲ **CRITICAL STEP** Prepare a higher volume (e.g., 20 mL) than required. Small volumes cool down fast and will solidify too early.

▲ **CRITICAL STEP** Place the warm agarose solution on a hot plate to avoid fast cooling and solidification.

(v) Add a minimum of 20 µL of the liquid agarose solution to the spheroids, ensuring that the agarose solution completely incapsulates the spheroids.

▲ **CRITICAL STEP** Work fast to avoid solidification of the agarose solution in the pipette tip.

▲ **CRITICAL STEP** Avoid the formation of air bubbles.

▲ **CRITICAL STEP** Higher spheroid numbers may require a higher volume of agarose to completely encapsulate them.

◆ **TROUBLESHOOTING**

(vi) Let the agarose droplet incapsulating the spheroids solidify.

▲ **CRITICAL STEP** Place 5 min at 4 °C for faster solidification.

◆ **TROUBLESHOOTING**

(vii) Fix the spheroids by adding 500 µL of 4% paraformaldehyde for 24–48 h at 4 °C.
▲ **CRITICAL STEP** Fixation of the spheroids (Step 1A(vii and viii)) can also be performed before placing the spheroids in agarose (Step 1A(iv–vi)).

(viii) Take the paraformaldehyde off.

(ix) Take the solidified agarose droplet with spheroids out of the Eppendorf tube with forceps.
▲ **CRITICAL STEP** To facilitate this process, the Eppendorf Tube can be cut off close to the agarose droplet.
◆ **TROUBLESHOOTING**

(x) Place the solidified agarose droplet with spheroids in a histological cassette.
■ **PAUSE POINT** The histological cassettes can be kept fully submerged in 70% ethanol until further tissue processing.

(B) **Spheroid microarray**

● **TIMING Step 1B(i–xiii): 25–49 h**

(i) Dissolve 2% agarose in demi water by boiling for 2 min in a microwave.
▲ **CRITICAL STEP** Prepare a higher volume (e.g., 20 mL) than required. Small volumes cool down fast and will solidify too early.
▲ **CRITICAL STEP** Place the warm agarose solution on a hot plate to avoid fast cooling and solidification.

(ii) Add the liquid agarose in the embedding mold and place the mold maker.

(iii) Let the agarose solidify.
▲ **CRITICAL STEP** Place 5 min at 4 °C for faster solidification.
◆ **TROUBLESHOOTING**

(iv) Carefully remove the mold maker out of the agarose gel. If required, remove excess agarose with a scalpel.

(v) Transfer one spheroid from the ULA plate into each well of the solidified agarose gel.
▲ **CRITICAL STEP** This placement of the spheroid into the agarose wells can be followed visually.
▲ **CRITICAL STEP** It is crucial to transfer an excess of culture medium into the agarose wells, as the spheroids will sink to the bottom of the wells. In case the spheroids are placed in the wells without transfer of culture medium, the spheroids will not sink out to bottom, they will stick to the wall of the wells and will consequently be at different heights during sectioning.

(vi) Once the spheroids have sunk to the bottom of the wells, remove the excess culture medium by pipetting it off the agarose gel.

(vii) Reheat the premade agarose solution until boiling.
▲ **CRITICAL STEP** Place the warm agarose solution on a hot plate to avoid fast cooling and solidification.

(viii) Cover the wells of the agarose gel with a layer of liquid agarose.

(ix) Let the agarose solidify.
▲ **CRITICAL STEP** Place 5 min at 4 °C for faster solidification.

(x) Take the solidified agarose gel with spheroids out of the embedding mold and cut to the desired size.
▲ **CRITICAL STEP** To know which spheroids are placed where, indicate the position of the gel by for example cutting off a corner.

(xi) Place the solidified agarose gel with spheroids in a histological cassette.
▲ **CRITICAL STEP** Place a foam pad between the histological cassette and agarose gel on the side of the spheroids to avoid indentation of the cassette into the gel.

(xii) Fix the spheroids by submerging the histological cassette with 4% paraformaldehyde for 24–48 h at 4 °C.

(xiii) Take the paraformaldehyde off.
■ **PAUSE POINT** The histological cassettes can be kept fully submerged in 70% ethanol until further tissue processing.

2. Process the histological cassette in a tissue processor through dehydration in ascending alcohol series followed by infiltration with molten paraffin (40 min incubation steps in 70% ethanol, 80% ethanol and 95% ethanol, two time 1 h incubation steps in 100% ethanol, three times 1 h incubations steps in xylene and three times 1 h incubation steps in paraffin).
3. Embed the paraffin infiltrated agarose gel with spheroids in a paraffin block.
   ■ **PAUSE POINT** The paraffin block can be stored at 4 °C.
4. Section the paraffin block (at the depth of the spheroids) by a microtome to 4-µm-thick ribbons.
   ▲ **CRITICAL STEP** Place the paraffin blocks at 4 °C or on a cold plate (−20 °C) for easier sectioning.
   ▲ **CRITICAL STEP** Sectioning at the height of spheroids can normally be observed by the naked eye when working with a spheroid pellet, especially when the thickness is increased to 20 µm.
   ◆ **TROUBLESHOOTING**
5. Place the paraffin ribbons onto a microscopic slide.
   (C) Wet the microscopic slides before placing the ribbons, ensure the ribbons stretch open on the water and remove the excess of water after the placement.
   ▲ **CRITICAL STEP** Check the presence of spheroid structures under a light microscope.
6. Place the microscopic slides at 37 °C for 24 h to dry.
7. Stain the microscopic slides according to existing protocols.
   ◆ **TROUBLESHOOTING**
8. Image with the appropriate microscope (histology and IHC by light microscopy, immunofluorescence by epifluorescent microscopy).

Procedure: Part 3: endpoint analysis

Module 3: architecture with spatial cell and subcellular information; TEM

● **TIMING Steps 1–27: 110 h**

▲ **CRITICAL** To avoid cellular changes, all steps until fixation should be performed on ice.

1. Collect 5–20 spheroids per condition in a cone bottom Eppendorf tube (Part 1, spheroid collection).
2. Leave the spheroids to sediment for 2 min.
   ▲ **CRITICAL STEP** Sedimentation of the spheroids due to gravity can be followed visually.
3. Take as much supernatant off as possible without disrupting the spheroid pellet.
   • Collected supernatant can be analyzed (Part 2, Module 1).
4. Wash the spheroids twice by adding 1 mL of PBS, let the spheroids sediment for 2 min and subsequently remove the PBS without disturbing the spheroid pellet.
5. Fix the spheroids by adding 500 µL of fixation solution for 48 h at 4 °C.
   ■ **PAUSE POINT** The spheroids may be incubated in the fixation solution up to 7 d at 4 °C.
6. Take off the fixation solution without disturbing the spheroid pellet.
7. Wash the spheroids three times by adding 1 mL of sodium cacodylate trihydrate buffer 0.1 M pH 7.2–7.4, letting the spheroids sediment for 2 min and subsequently removing the buffer without disturbing the spheroid pellet.
8. Add 1 mL of contrast solution 1 and incubate for 1 h at room temperature.
   ■ **PAUSE POINT** The spheroids may be incubated in the contrast solution overnight at 4 °C.
9. Remove as much of the contrast solution 1 as possible without disturbing the spheroid pellet.
10. Wash the spheroids three times by adding 1 mL of MilliQ water, letting the spheroids sediment for 2 min and subsequently removing the water without disturbing the spheroid pellet.
11. Add 1 mL of contrast solution 2 and incubate for 30 min at room temperature.
    ■ **PAUSE POINT** The spheroids may be incubated in the contrast solution overnight at 4 °C.

12. Wash the spheroids two times by adding 1 mL of PBS, letting the spheroids sediment for 2 min and subsequently removing the water without disturbing the spheroid pellet.
13. Dissolve 2% agarose in demi water by boiling for 2 min.
    ▲ **CRITICAL STEP** Prepare a higher volume (e.g., 20 mL) than required. Small volumes cool down fast and will solidify too early.
    ▲ **CRITICAL STEP** Place the warm agarose solution on a hot plate to avoid fast cooling and solidification.
14. Add 200 µL of the liquid agarose solution to the spheroids, ensuring that the agarose solution completely incapsulates the spheroids.
    ▲ **CRITICAL STEP** Work fast to avoid solidification of the agarose solution in the pipette tip.
    ▲ **CRITICAL STEP** Avoid the formation of air bubbles.
    ◆ **TROUBLESHOOTING**
15. Let the agarose solution incapsulating the spheroids solidify.
    ▲ **CRITICAL STEP** Place 5 min at 4 °C for faster solidification.
    ◆ **TROUBLESHOOTING**
16. Take the solidified agarose droplet with spheroids out of the Eppendorf tube with forceps.
    ▲ **CRITICAL STEP** To facilitate this process, the Eppendorf Tube can be cut off close to the agarose droplet.
    ◆ **TROUBLESHOOTING**
17. Dehydrate the agarose droplet with the spheroids by incubation in ascending alcohol series (10 min in a series of 50%, 70%, 80%, 90% ethanol followed by 3 times 10 min in 100% ethanol).
18. Remove the ethanol solution.
19. Add 1 mL of 100% propylene oxide and incubate for 30 min at room temperature.
20. Remove the solution.
21. Transfer the agarose droplet with spheroids into the EMbedding 3:1 solution and leave it agitating for 2 h.
22. Transfer the agarose droplet with spheroids into the EMbedding 1:1 solution and leave it agitating for 2 h.
    ■ **PAUSE POINT** The agarose droplet with spheroids may be left overnight in this solution.
23. Transfer the sample into the EMbedding 1:3 solution and leave it agitating for 2 h.
24. Transfer the sample into EMbed-812 resin and leave it agitating for 2 h.
25. Transfer the resin embedded sample carefully into a TEM-suitable mold and incubate for 48 h at 55 °C.
26. Section the EMbed-812 resin block (at the depth of the spheroids) by an ultramicrotome to 50 nm ribbons.
    ▲ **CRITICAL STEP** The spheroids can be detected by the naked eye due to contrast.
    ▲ **CRITICAL STEP** Place the block at 4 °C or on a cold plate (−20 °C) for easier sectioning.
27. Mount the ultrathin ribbons onto 200 mesh copper grids.
28. Image with a transmission electron microscope.

Procedure: Part 3: Endpoint analysis; Module 3: Architecture with spatial cell and subcellular information; LSFM

● **TIMING Steps 1–6: 120–165 min**

● **TIMING Steps 10–18: 25 min**

▲ **CRITICAL** Steps 11–18 describe the sample preparation suited for the Leica TCS SP8 DLS upright microscope. Different sample holders and sample preparation may be required when operating other light-sheet fluorescence microscopes.

▲ **CRITICAL** To avoid cellular changes, the next steps until fixation should be performed on ice.

1. Collect *x* spheroids per condition in a cone bottom Eppendorf tube (see part 1 spheroid collection).

2. Leave the spheroids to sediment for 2 min.
   ▲ **CRITICAL STEP** Sedimentation of the spheroids due to gravity can be followed visually.
3. Take as much supernatant off as possible without disrupting the spheroid pellet.
   • Collected supernatant can be analyzed (Part 2, Module 1)
4. Fix the spheroids by incubating them with 500 µL of 4% paraformaldehyde for 1.5 h at 4 °C.
5. Take off the paraformaldehyde without disturbing the spheroid pellet.
6. Wash the spheroids twice by adding 1 mL of PBS, letting the spheroids sediment for 2 min and subsequently removing the PBS without disturbing the spheroid pellet.
   ■ **PAUSE POINT** The spheroids can be stored at 4 °C in PBS.
7. Follow option A if tissue clearing is not required. Follow option B for tissue clearing.
   (A) **No tissue clearing**
   ● **TIMING Overnight incubation**
   ▲ **CRITICAL** If only performing nuclear labelling, no additional steps are required; and you can proceed to staining the spheroids (Step 9). Phalloidin staining (actin labelling) requires overnight incubation with Triton X-100 to increase penetration depth.
   (i) Incubate the spheroids in 500 µL of 0.1% Triton X-100 overnight.
   (B) **Tissue clearing**
   ● **TIMING 15 min of handling and 53 h of incubation**
   (i) Incubate the spheroids in 500 µL of 2% Triton X-100 for 48 h.
   (ii) Remove as much of the Triton X-100 as possible without disturbing the spheroid pellet.
   (iii) Add 500 µL of 10% sucrose solution and incubate the spheroids for 1 h.
   (iv) Replace the sucrose solution every hour, increasing by 10% until a final concentration of 50% is reached. Then incubate for a further hour.
8. Wash the spheroids twice by adding 1 mL of PBS, letting the spheroids sediment for 2 min and subsequently removing the PBS without disturbing the spheroid pellet.
9. Stain the spheroids according to existing protocols.
10. Wash the spheroids twice by adding 1 mL of PBS, letting the spheroids sediment for 2 min and subsequently removing the PBS without disturbing the spheroid pellet.
11. Dissolve 2% agarose in demi water by boiling for 2 min.
    ▲ **CRITICAL STEP** Prepare a higher volume (e.g., 20 mL) than required. Small volumes cool down fast and will solidify too early.
    ▲ **CRITICAL STEP** Place the warm agarose solution on a hot plate to avoid fast cooling and solidification.
12. Create a sample holder for the spheroids by placing a U-shaped glass capillary in the center of a Cellview cell culture petri dish.
13. Fill the capillary halfway with agarose and let it solidify.
    ◆ **TROUBLESHOOTING**
14. Position the spheroids on top of the agarose gel.
15. Fill the capillary completely with liquid agarose and let it solidify.
16. Remove the capillary by carefully pushing out the solidified agarose with spheroid from the side.
    ▲ **CRITICAL STEP** The removal of the glass capillary decreases light scattering and increases the signal-to-noise ratio.
17. Place the solidified agarose with spheroids in the center of the petri dish and secure it with a drop of liquid agarose at both ends.
    ▲ **CRITICAL STEP** Avoid adding agarose at the position of the spheroid.
18. Fill the petri dish with immersion fluid.
    ▲ **CRITICAL STEP** Use distilled water for noncleared spheroids and 20% sucrose dissolved in distilled water for cleared spheroids as immersion fluid.
19. Image the complete spheroids through *z*-stacking with a light-sheet fluorescence microscope.

Troubleshooting

Troubleshooting advice can be found in Tables 10–12.

Table 10 | Troubleshooting table; Part 1: Spheroid setup

Step	Problem	Possible reason	Solution
Step 13	There is no compact spheroid formation (Extended Data Fig. 1a)	The used cell line has the inherent problem of not forming tight cell–cell contacts	Supplement the culture medium with additives to stimulate compaction such as methylcellulose (1–3%), which increases the medium viscosity or type I collagen (1–10 µg/mL), which increases cell-matrix dependent aggregation
	There is no spheroid uniformity across replicates	Cells sink to the bottom of the tube during seeding	Resuspend the single-cell suspension regularly during seeding
	The spheroid is formed around a foreign particle (e.g., dust) (Extended Data Fig. 3)	The presence of dust particles in the PBS, trypsin/EDTA, culture medium or on/in the pipette tips	Filter the solutions before use with a 0.22 µm filter and use presterilized individually packed tip boxes
			Exclude the spheroid from analysis when foreign particle is present
Step 24B(ii)	The spheroid is not collected	The spheroid is lost due to up and down pipetting	Do not pipet up and down as you tend to lose the spheroid faster. Collect the spheroid in one smooth movement. Collection of the spheroid can be confirmed visually (Extended Data Fig. 4)
		The total volume is not collected	Increase the settings of the pipette to a volume higher than the well volume

Table 11 | Troubleshooting table; Part 2: Longitudinal analysis

Module: Step	Problem	Possible reason	Solution
Module 1: Step 3	Measured cytokine and chemokine concentrations are low	Secreted cytokines and chemokines are too diluted	Work with smaller well volumes (e.g., opt to work with 384-well instead of 96-well plates)
			Pool spheroid supernatant of same conditions and concentrate using concentration filters (e.g., 3 kDa cutoff)
Module 2: Step 1	Dye is insufficiently penetrated in the spheroid	Incubation time is not long enough	Let the dye penetrate over longer time periods while taking the possible cytotoxicity into account (e.g., Hoechst should be incubated overnight for full penetration into the spheroid)
Module 2: Step 2 Module 3, radial migration: Step 6 Module 3, invasion: Step 10	Automated image segmentation is imperfect (as indicated with a white arrow in Fig. 4b)	Image quality is low or low differences in intensities of the spheroid and the background	Perform manual segmentation

Table 12 | Troubleshooting table; Part 3: Endpoint analysis

Module: step	Problem	Possible reason	Solution
Module 1, ATP: Step 10	Low to no luminescent signal in some wells	Spheroid is not transferred (completely)	Collect the spheroid in one smooth movement and do not pipet up and down as you tend to lose the spheroid faster. Collection of the spheroid can be confirmed visually (Extended Data Fig. 4)
Module 2: Step 5	Incomplete dissociation of the spheroid into single cells (e.g., cancer-associated fibroblasts form tight spheroids that are harder to dissociate)	Dissociation protocol needs to be optimized to the used cell types	Invert the tube every few minutes and mechanically dissociate the spheroids by pipetting up and down with a 200 µL pipette but avoid extensive mechanical dissociation since this induces cell death. In case the spheroids are not completely dissociated, the dissociation procedure can be adjusted by increasing the incubation time and/or by increasing concentration of enzymes and/or change type of enzymes (e.g., collagenase, DNase and trypsin). If trypsin is used, pay attention as this can cause loss of cell surface markers
Module 2: Step 8	No detection of specific cell types (e.g., T cells)	Incomplete pelleting due to small cell sizes	Increase the centrifugation (Step 6) to 500*g* to pellet even small cell types
	High amounts of cellular debris	Dissociation protocol is too harsh for the cells and needs to be optimized to the used cell types	Avoid pipetting or inverting the tube too vigorously. Either increase the incubation time, the enzyme concentration or change the type of enzymes
	Unclear live/dead results	Interference of the proteins and/or sodium azide present in the FACS buffer with the live/dead staining	Perform the live/dead staining in PBS

Table 12 (continued) | Troubleshooting table; Part 3: Endpoint analysis

Module: step	Problem	Possible reason	Solution
Module 3, IHC: Step 1A(v) Module 3, TEM: step 14	Spheroids are not completely incapsulated in the agarose droplet	The presence of a high amount of air bubbles	Place a small volume of liquid agarose onto the droplet, which will fill the holes of the air bubbles and incapsulate the loose spheroids
Module 3, IHC: Step 1A(vi) Module 3, IHC: Step 1B(iii) Module 3, TEM: Step 15 Module 3, LSFM: Step 13	The agarose insufficiently solidifies	The agarose solution did not boil long enough	Boil the agarose solution for two minutes to ensure proper solidification once cooled off
			HistoGel can be used as an alternative to agarose
Module 3, IHC: Step 1A(ix) Module 3, TEM: step 16	Breakage of the agarose droplet	Pinching too hard with the forceps	Place a small volume of liquid agarose onto the pieces to form one whole droplet again, which encapsulates the spheroids
			Cut the Eppendorf tube close to the droplet, which facilitates taking the droplet out
		The agarose solution did not boil long enough	Boil the agarose solution for 2 min to ensure proper solidification once cooled off
			HistoGel can be used as an alternative to agarose
Module 3, IHC: Step 4	The spheroids are not sectioned at the same level	The spheroids did not sink to the bottom of the agarose wells (Module 3, step 1B(v))	Ensure the transfer of culture medium together with spheroid when placing the spheroid into the agarose well, in this way it cannot stick to the well walls. If required, centrifuge the spheroids down at 200*g*
		The paraffin infiltrated agarose gel is curled and dried up (turns white) (Module 3, Step 3)	Make sure that the agarose gel is immediately embedded in a paraffin block after infiltration with paraffin and that it does not cool off
		The paraffin block is not sectioned at the correct angle (Module 3, Step 4)	Adjust the angle of the cutting block
Module 3, IHC: Step 7	Loss of the spheroids while staining	Use of microscope slides which do not ensure sufficient adhesion for spheroid structures	Use the Superfrost Plus Gold Adhesion Microscopic slides (Epredia, cat. no. K5800AMNZ72)

Timing

The timing estimates given here assume that the researcher has basic knowledge of cell culture and molecular biology. The total hands-on time of a spheroid experiment varies considerably based on the plate format (96-well versus 384-well plate), complexity of the experiment (number of variables, e.g., cell lines, seeding cell numbers, medium types, compounds, fluorescent channels, etc.), use of single- or multichannel pipettes and of which modules are being executed (e.g., single time point analysis or combinations of longitudinal and/or endpoint analyses). Timing includes spheroid handling and not further downstream analysis (e.g., metabolomics, Luminex, western blot, proteomics, RT–qPCR, RNA sequencing, lipidomics, flow cytometry, single-cell RNA sequencing, immune or fluorescence staining procedures, data analysis, etc.).

Part 1: spheroid setup

Steps 1–13, spheroid seeding: 30 min to 3 h
(optional) Steps 14–17, partial medium refreshments: 20–80 min
(optional) Steps 18–22, compound administration: 15–40 min
(optional) Steps 23–24, spheroid collection: 15 min to 1 h

Part 2: longitudinal analysis

Module 1: supernatant

Step 1, supernatant collection: 20–80 min
Step 2, cell/debris removal: 10 min

Module 2: morphometry with or without spheroid/cell specific information

Step 1, imaging; (A) robot-assisted imaging: 10 min to 1 h
Step 1, imaging; (B) manual imaging: 20 min to 2 h

Module 3: functional assays using spheroids; (1) radial migration assay
Steps 1–3, spheroid placement: 30 min
Step 4, incubation: 3 h

Module 3: functional assays using spheroids; (2) invasion assay
Steps 1–2, collagen solution preparation and placement: 20 min
Step 3, incubation: 20 min
Steps 4–5, collagen solution and spheroid placement: 20 min
Step 6, incubation: 20 min
Steps 7–8, medium addition: 5 min

Part 3: endpoint analysis

Module 1: molecular content; (1) ATP content
Steps 2–4, spheroid/control transfer: 15 min to 1 h
Steps 6–7, reagent addition: 3 min
Steps 8–9, incubation: 30 min
Step 10, read out: 2 min

Module 1: molecular content; (2) RNA/protein/lipid content
Step 1, spheroid collection: 15 min to 1 h
Steps 2–4, supernatant removal: 20 min
Step 5, spheroid lysis; (A) RNA
Step 5A(i–ii), spheroid lysis: 10 min
Step 5A(iii), RNA purification: 110 min
Box 5: SingleShot cell lysis: 20 min
Step 5, spheroid lysis; (B) protein
Step 5B(i–ii), spheroid lysis: 5 min
Step 5B(iii), sample clearance 5 min
Step 5, spheroid lysis; (C) lipid
Step 5C(ii–iii), spheroid lysis: 10 min

Module 2: single-cell composition and state
Step 1, spheroid collection: 15 min to 1 h
Steps 2–4, supernatant removal: 20 min
Step 5, spheroid dissolution: 10 min
Steps 6–7, enzyme removal: 15 min

Module 3: architecture with spatial cell and subcellular information; (1) histology and IHC
Step 1A, spheroid pellet
Step 1A(i), spheroid collection: 15 min to 1 h
Step 1A(ii–iii), supernatant removal: 3 min
Step 1A(iv–vi), spheroid incapsulation with agarose: 7 min
Step 1A(vii), fixation: 24–48 h
Step 1A(viii–x), placement in a histological cassette: 5 min
Step 1B, spheroid microarray
Step 1B(i–iv), agarose gel preparation: 10 min
Step 1B(v), spheroid transfer: 15 min to 1 h
Step 1B(vi–x), microarray completion: 15 min
Step 1B(xi), placement in a histological cassette: 5 min
Step 1B(xii), fixation: 24–48 h
Step 2, tissue processing: 10 h
Step 3, embedding: 5 min
Steps 4–5, sectioning: 30 min
Step 6, drying: 24 h

Module 3: architecture with spatial cell and subcellular information; (2) TEM
Step 1, spheroid collection: 15 min to 1 h
Steps 2–4, supernatant removal: 10 min
Step 5, fixation: 48 h
Steps 6–7, washing: 10 min
Steps 8–12, contrast agent addition: 110 min
Steps 13–15, spheroid incapsulation with agarose: 5 min
Steps 16–18, dehydration: 70 min
Steps 19–20, incubation with propylene oxide: 30 min
Steps 21–24, embedding: 8 h
Step 25, incubation: 48 h
Steps 26–27, sectioning: 1 h

Module 3: architecture with spatial cell and subcellular information; (3) LSFM
Step 1, spheroid collection: 15 min to 1 h
Steps 2–3, supernatant removal: 5 min
Step 4, fixation: 90 min
Steps 5–6, washing: 10 min
Step 7, tissue clearing; (A) no tissue clearing: optional overnight incubation
Step 7, tissue clearing; (B) tissue clearing: 15 min of handling and 53 h of incubation
Step 8, washing: 5min
Step 10, washing: 5 min
Steps 11–18, positioning the spheroids in agarose: 20 min

Anticipated results

In the first part of the protocol we discussed the preparation and culturing of spheroids. As demonstrated by our previous publication, spheroid phenotype and, thus, consequently, the result of your spheroid analysis, is affected by the chosen spheroid setup[1]. First, cellular characteristics (genetic background) impact spheroid formation and morphology. Different cell lines cultured in the same conditions (seeding cell numbers, medium type, culture time, etc.) will form spheroids with different compaction and sizes (Fig. 4b and Extended Data Fig. 1c). Furthermore, presence of cell death (Fig. 4c), consumption and secretion of metabolites and proteins (Figs. 3c and 3d), migration and invasion capacity and molecular signatures (Extended Data Fig. 5a–c) are cell-type-specific. For example, although the cell seeding number is equal, 4T1 initiates small spheroids with a high level of cell death while A549 and HCT116 form larger spheroids with less cell death (Fig. 4c). Second, the cell seeding number influences spheroid size and consequently phenotype[1]. Note that there is no linear trend between the cell seeding number and spheroid size (Extended Data Fig. 5d). Third, the type of culture medium substantially impacts spheroid phenotype[1]. The metabolic environment strongly influences spheroid morphology, cell death (Extended Data Fig. 5e), functional activity (e.g., spheroid growth, Extended Data Fig. 5f) and molecular content (Figs. 6f and 6g and Extended Data Fig. 5a). During spheroid analysis it must be considered that spheroid morphology and size (Fig. 4a) and metabolic environment (Fig. 3b) are not static but continuously evolve during culture, meaning that spheroid age at time point of evaluation will also influence the experiment outcome. To summarize, selected variations in spheroid methodology have an impact on spheroid phenotype and yield and have to be considered before analysis (Tables 1–9).

We recommend performing spheroid evaluation after a compact spheroid is formed to replicate 3D tissue architecture. Spheroid initiation and, consequently, evaluation readout is fast (Extended Data Fig. 1c). Furthermore, the ability to form monocellular, as well as heterocellular spheroids (Fig. 7) demonstrates the versatility of this approach. Highly reproducible spheroid phenotyping (both morphological, (Figs. 4a and 4b) and

molecular (Fig. 6)) results should be expected after successfully following the described protocol. However, relatively small changes in spheroid sizes and, consequently, molecular content, over different biological replicates can be caused by cell counting and pipetting errors. We propose to contain this issue by a normalization procedure to a control situation (Extended Data Fig. 5g). Furthermore, we stimulate transparent results reporting and advise researchers to show each analyzed spheroid as an individual data point.

In the second and third part of the protocol, we discuss longitudinal and endpoint spheroid analyses. The described assays provide insights with different levels of detail and the choice of analysis will depend on the application. Image segmentation measurements (Box 3) based on light microscopy images (Fig. 4b and Fig. 5) and metabolic activity (Fig. 6b) are commonly used measures in drug evaluations and can be readily performed in most laboratories. Epifluorescence (Fig. 4c) and confocal (Fig. 4d) images increase spheroid/cell specific information with fluorescent labelling of cells or molecules. With increasing detail, bulk (Fig. 6) and single-cell analysis (Fig. 7) can inform on underlying biology, which can be visualized in the native spheroid architecture by IHC (Fig. 8), TEM (Fig. 9b) and LSFM (Fig. 9c). You can expect a better representation of tissue biology, metabolic profiles and secreted factors when using 3D spheroid models compared to 2D monolayer cultures[1,4,5,32,33]. Longitudinal analysis modules can be combined with each other (e.g., microscopic monitoring while collecting supernatant) and with endpoint analysis modules (e.g., spheroid collecting for proteomic evaluation while collecting supernatant). Moreover, the one spheroid/well allowing single spheroid evaluation together with the plate format (384-well systems) ensure high throughput evaluations.

In sum, following the proposed protocol will lead to fast and robust big data output in a physiologically relevant model.

Data availability

Any additional data required for research purposes are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions

E.B. generated the spheroid cultures. E.B., S.E., F.D.V., A.D., C.P., D.E., P.R., R.B., L.C., V.G. and J.D. performed downstream analyses. E.B. performed the data analysis. E.B. and O.D.W. wrote the manuscript. All authors revised and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Olivier De Wever.

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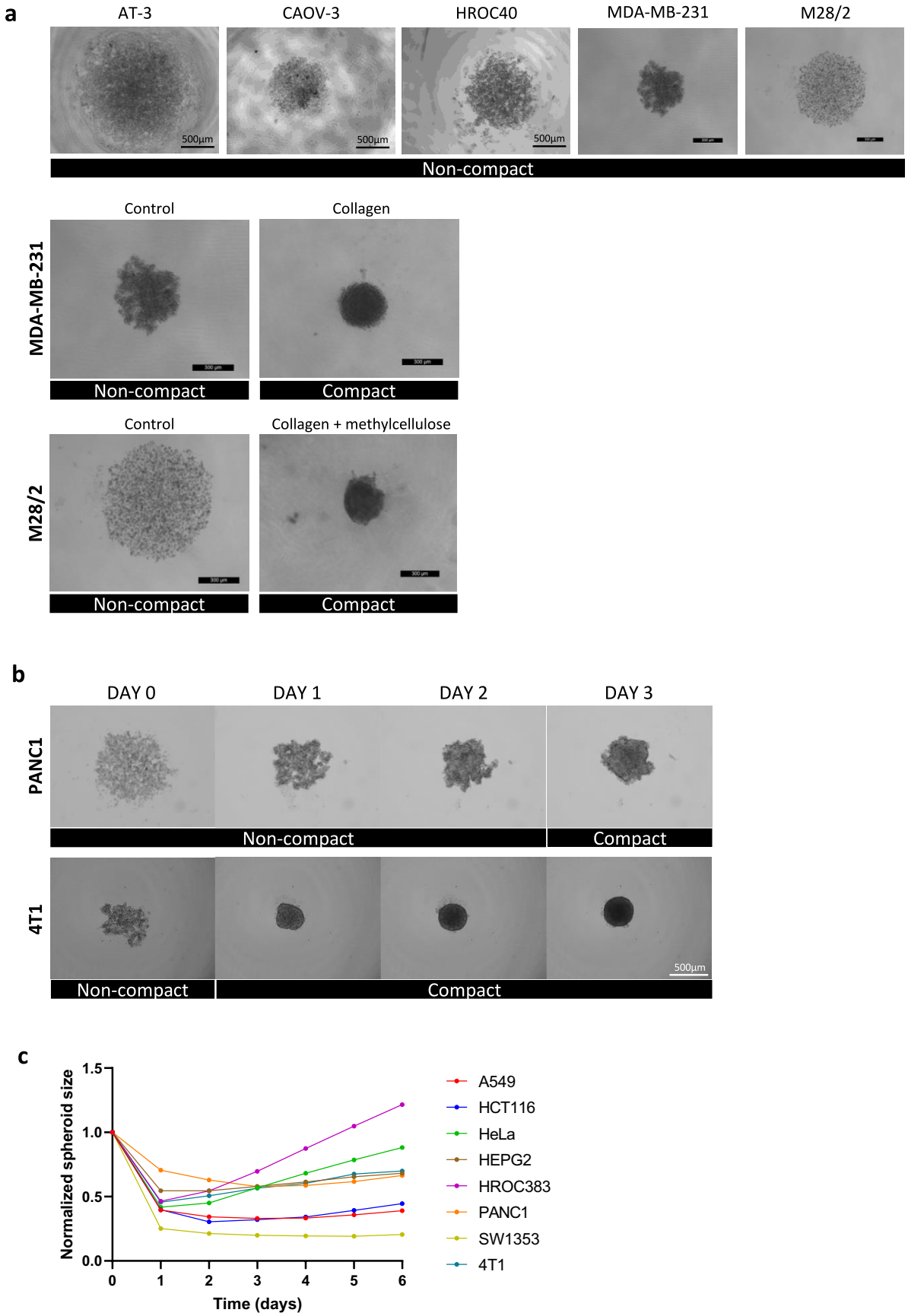
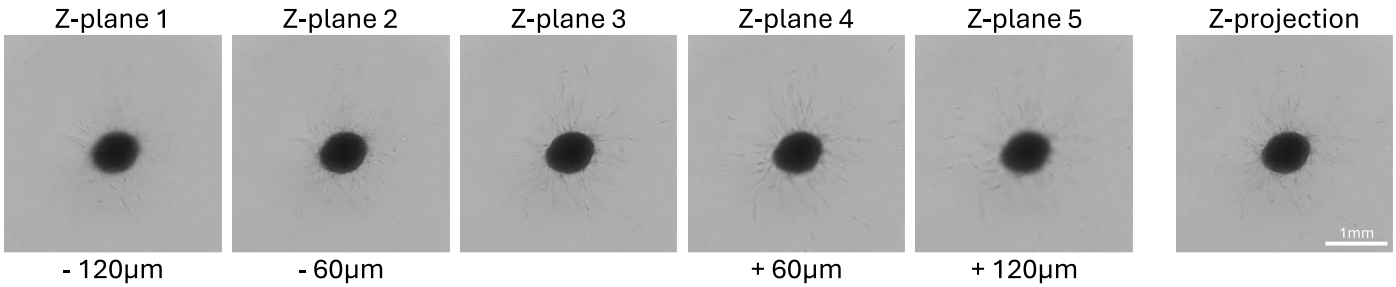
[1]Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Ghent, Belgium. [2]Cancer Research Institute Ghent, Ghent, Belgium. [3]Experimental Surgery Lab, Department of Human Structure and Repair, Ghent University, Ghent, Belgium. [4]Synthetic and Systems Biology Unit, HUN-REN Biological Research Centre, Szeged, Hungary. [5]i3S—Institute for Research and Innovation in Health, University of Porto, Porto, Portugal. [6]Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal. [7]Department of Life Technologies and InFLAMES Flagship, University of Turku, Turku, Finland. [8]CerbaPath, Division CMP, Brussels, Belgium. [9]VIB Screening Core and Ghent University Expertise Centre for Bioassay Development and Screening, Ghent University, Ghent, Belgium. [10]Laboratory of Lipid Metabolism and Cancer, Department of Oncology, KU Leuven, Leuven, Belgium. [11]Clinic of General Surgery, Molecular Oncology and Immunotherapy, University of Rostock, Rostock, Germany. [12]Department of Radiation Oncology, Ghent University Hospital, Ghent, Belgium. [13]OncoRNALab, Department of Biomolecular Medicine, Ghent University, Ghent, Belgium. [14]Single-Cell Technologies Ltd, Szeged, Hungary. [15]Institute of AI for Health, Helmholtz Zentrum München, Neuherberg, Germany. [16]Institute for Molecular Medicine Finland, University of Helsinki, Helsinki, Finland.

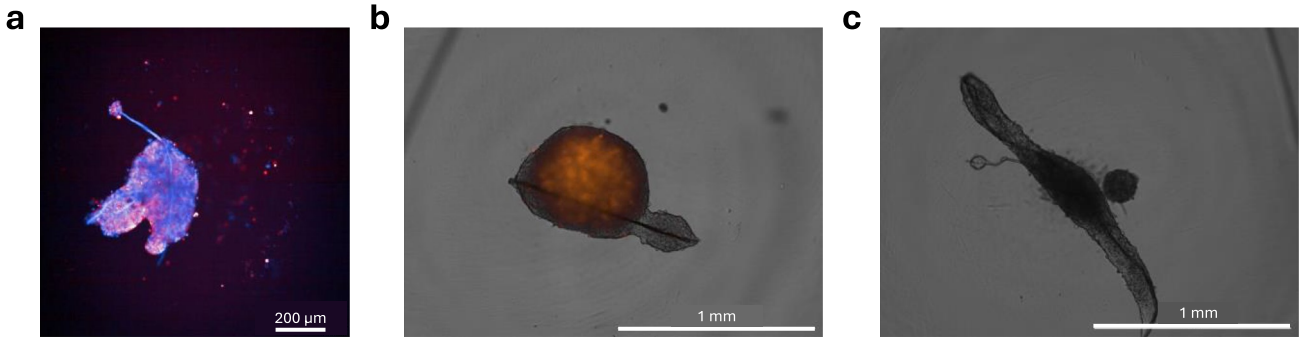


Extended Data Fig. 1 | See next page for caption.

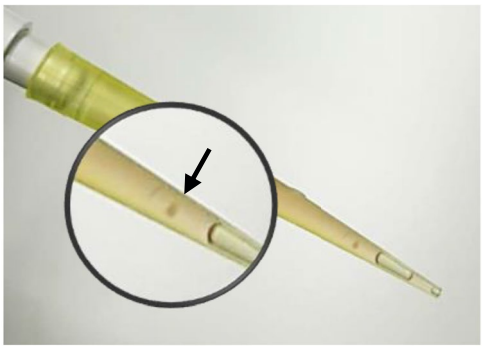
Extended Data Fig. 1 | Spheroid compaction. a, Examples of cell lines that do not form compact spheroids spontaneously. Addition of viscosity enhancers or matrix components (e.g. collagen, methylcellulose) enable compact spheroid (individual cells are difficult to discriminate and there is absence of spaces or voids) formation. **b**, Longitudinal light microscopic monitoring of PANC1 and 4T1 spheroids over 3 d. Compact spheroids are formed 3 d after single-cell seeding for PANC1 while they are formed 1 d after single-cell seeding for 4T1. **c**, Normalized spheroid size curves of various cell lines (2,000 cells per well, DMEM HG) show differences in compaction phases and growth phases.



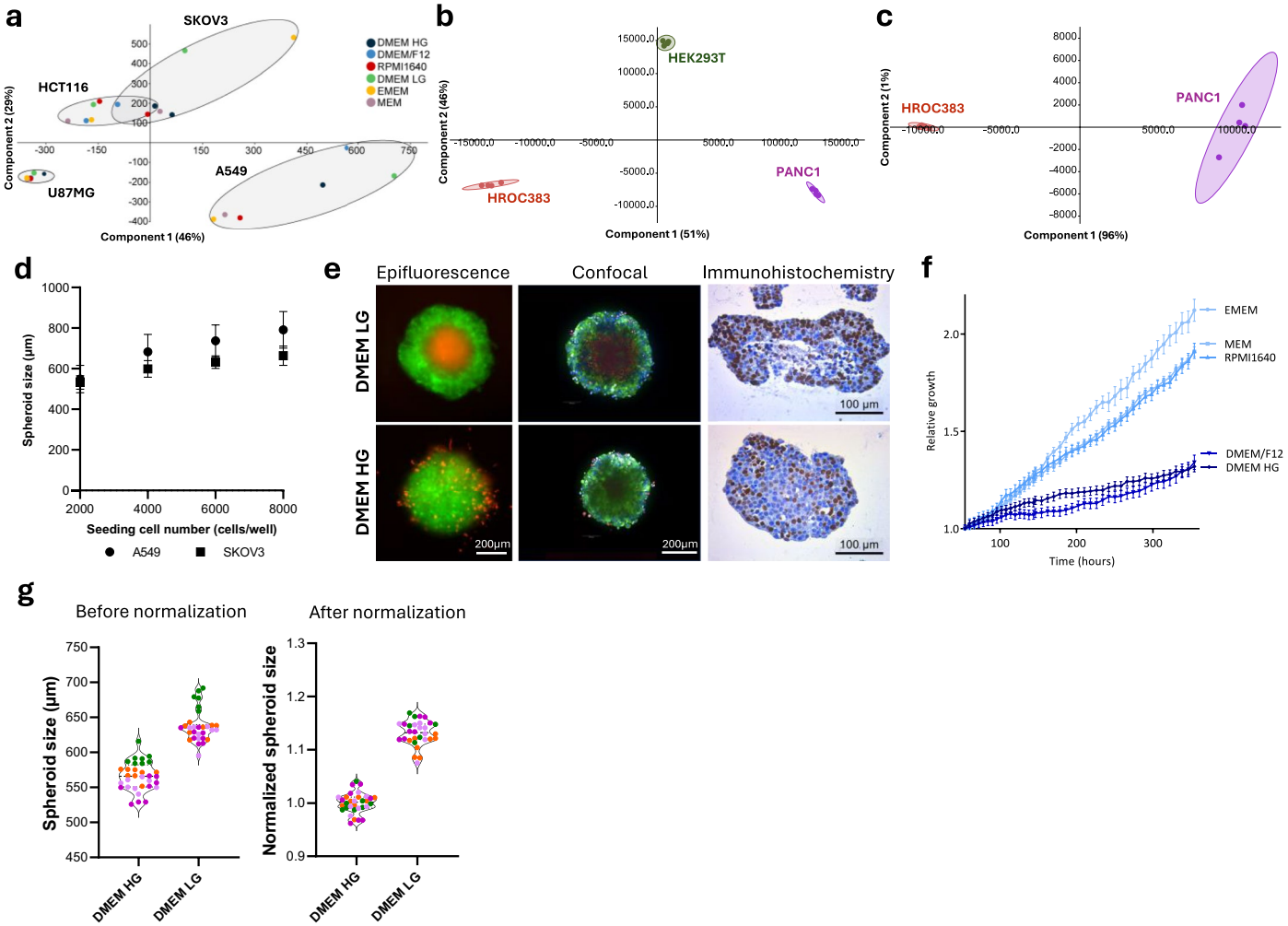
Extended Data Fig. 2 | Imaging of spheroid invasion at different z-planes. Light microscopic images at different z-planes (60 µm steps) illustrate the invasion of single cells and cell clusters into type I collagen in all directions. The z-projection (2D image construction of all z-planes) can be used to calculate area of invasion.



Extended Data Fig. 3 | Examples of spheroid formation around foreign particles. a, Confocal (hoechst (blue), propidium iodide (red)), **b**, epifluorescence (propidium iodide (red)), and **c**) light microscopic images of spheroids formed around a foreign particle (e.g. dust particle).



Extended Data Fig. 4 | Manual spheroid collection. Manual collection of the spheroid out of the well can be visually checked (arrow indicates the spheroid in the pipette tip).



Extended Data Fig. 5 | Anticipated results. Principal component analysis plots of **a**, gene expression profiles of A549, HCT116, SKOV3 and U87MG spheroids cultured in 6 different medium types (every dot is the average of 4 biological replicates)1, **b**, protein expression profiles of HEK293T, HROC383 and PANC1 spheroids cultured in DMEM HG (every dot (biological replicates) represents the protein profile of 16 pooled spheroids), and **c**, lipid content profiles of HROC383 and PANC1 spheroids (every dot (biological replicates) represents the lipid profile of 25 pooled spheroids). **d**, A549 and SKOV3 spheroid sizes correlating with increasing seeding cell numbers. Every dot indicates the mean, error bars indicate standard deviation (N=4, n=8). **e**, Epifluorescent (green (eGFP) indicating viable cells, red (propidium iodide) indicating dead cells), confocal (green (eGFP) indicating viable cells, red (propidium iodide) indicating dead cells, blue (Hoechst) indicating nuclei) and IHC (Ki67 indicating proliferating cells) images of MCF-7/AZ eGFP spheroids. **f**, Relative growth curves of MCF-7/AZ eGFP spheroids cultured in 5 different medium types. Every dot represent the mean, error bars indicate standard deviation (n=32). **g**, Sizes of HCT116 spheroids cultured in DMEM HG and DMEM LG before and after normalisation, biological replicates are indicated by different colours (N=4), every dot is one spheroid ($n \leq 8$). Panel **a** and **d** adapted from ref. 1, Springer Nature Limited.