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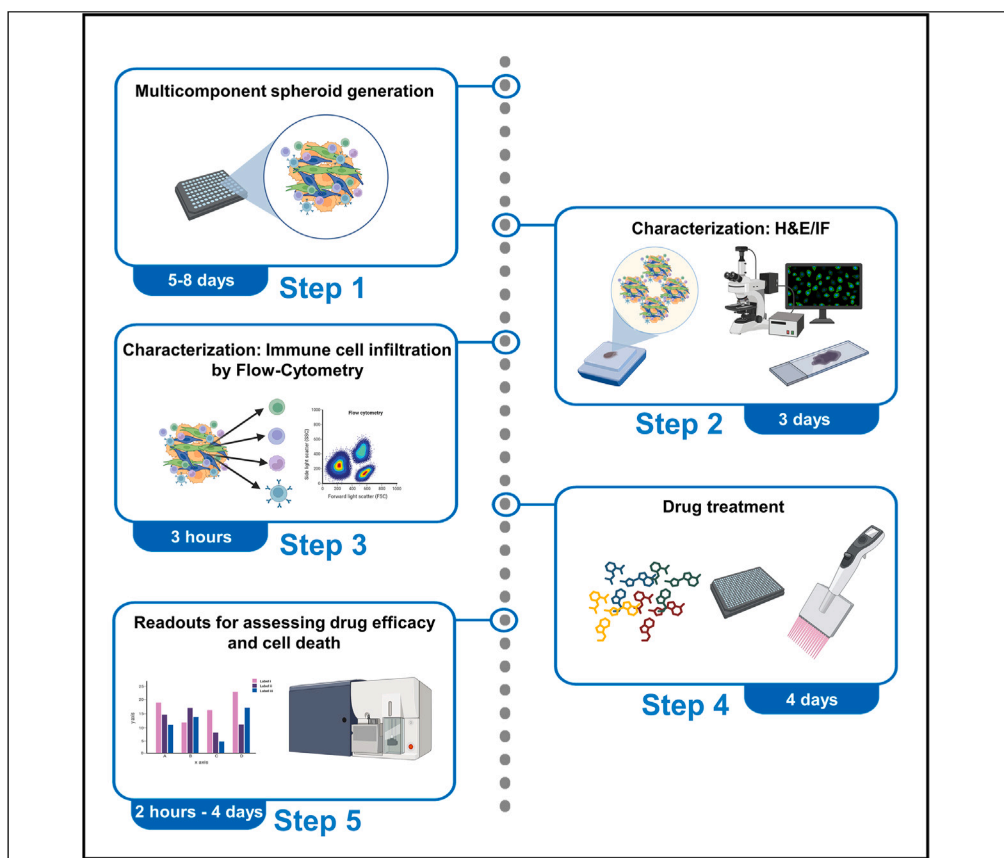
Protocol to generate scaffold-free, multicomponent 3D melanoma spheroid models for preclinical drug testing



Three-dimensional (3D) models play an increasingly important role in preclinical drug testing as they faithfully mimic interactions between cancer cells and the tumor microenvironment (TME). Here, we present a protocol for generating scaffold-free 3D multicomponent human melanoma spheroids. We describe steps for characterizing models using live-cell imaging and histology, followed by drug testing and assessment of cell death through various techniques such as imaging, luminescence-based assays, and flow cytometry. Finally, we demonstrate the models' adaptability for co-cultures with immune cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Generation of a multicomponent 3D melanoma co-culture scaffold-free spheroid model

Functional co-culture of 3D melanoma spheroids with PBMCs

Characterization of multicomponent 3D model using immunofluorescence and flow cytometry

Assessment of drug efficacy in multicomponent 3D models using kinetic and endpoint assays

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Protocol to generate scaffold-free, multicomponent 3D melanoma spheroid models for preclinical drug testing

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SUMMARY

Three-dimensional (3D) models play an increasingly important role in preclinical drug testing as they faithfully mimic interactions between cancer cells and the tumor microenvironment (TME). Here, we present a protocol for generating scaffold-free 3D multicomponent human melanoma spheroids. We describe steps for characterizing models using live-cell imaging and histology, followed by drug testing and assessment of cell death through various techniques such as imaging, luminescence-based assays, and flow cytometry. Finally, we demonstrate the models' adaptability for co-cultures with immune cells.

BEFORE YOU BEGIN

In the past, two-dimensional (2D) cultures of cancer cells have been the main pre-clinical model for the evaluation of drug efficacy. However, the absence of a correct three-dimensional (3D) tumor architecture, which influences the complex interplay of different cell types and their exchange of nutrients and metabolites has resulted in gross misinterpretations of *in vivo* drug effects. 3D cultures better mimic physiological properties of tumor cells allowing for more reliable *in vitro* drug testing,[1,2] especially when different cell types of the tumor microenvironment (TME) are incorporated in multicomponent 3D spheroid models.[3,4] Their high reproducibility allows upscaling to high-throughput drug screening for drug discovery as well as studies investigating the mechanisms of drug responses.[5–11]

In this protocol, we describe the generation of scaffold-free 3D spheroid models in which melanoma cells are co-cultured with endothelial cells, and different types of fibroblasts derived from skin, lung, and liver, representing the sites of common melanoma metastasis. Furthermore, peripheral blood mononuclear cells (PBMCs) were introduced to mimic involvement of the immune system. Detailed sequential seeding protocols were adapted to melanoma cells (Figure 1) allowing for versatile combination of different cell types of choice as has previously been reported for other cancer types.[12] Our advanced Melanoma Multicomponent Spheroid (MMS) models support a more rational assessment of drug effectiveness than standard 2D tissue cultures on plastic surfaces resulting in strikingly different responses to certain drugs. Moreover, compatibility with histological staining methods (immunofluorescence, IF) and cellular assays such as flow cytometry makes these 3D models useful surrogates for the examination of cancer cells specific traits and drug pharmacodynamics. The use of either fluorescently labeled or unlabeled cell lines (Table 1) depends on the planned read-out assay and should be decided beforehand. We recommend using unlabeled cell lines for immunocytochemical staining and flow cytometry-based assays, while labeled cell lines are perfectly suited for microscopy-based imaging and fluorescence-based drug response assays.

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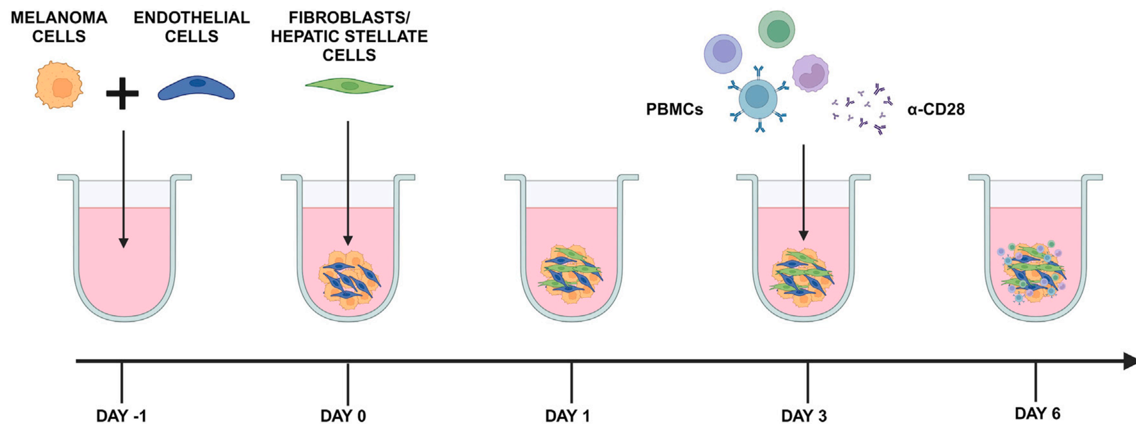

Figure 1. Sequential seeding protocol for Melanoma Multicomponent Spheroid (MMS) generation

Day −1, melanoma and endothelial cells are seeded at a 1:3 ratio in a U-bottom ULA microplate (384- or 96-wells). One day later (Day 0), 3 parts of fibroblasts or hepatic stellate cells are added atop (final ratio: 1:3:3). After 3 days of culture, MMSs can be co-cultured with PBMCs at a 1:10 ratio. Anti-CD28 antibody added for co-stimulatory signals for activation of immune cells. MMSs seeding can be performed in 384- or 96-well plates according to the chosen assays. Created with BioRender.com.

Institutional permissions

Appropriate precautions must be taken when handling human isolated PBMCs and cell lines transduced with lentiviral vectors (Table 1). All experiments involving the use of such material should be performed in accordance with biosafety level 2 (BSL2) guidelines, with dedicated class 2 biosafety cabinets and wearing appropriate protective equipment. Leukopaks for PBMC isolation were obtained from the Croix Rouge Luxembourg under the ethical agreement UNILUX-2023-001.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified anti-human CD28 (mouse IgG1) 5 μg/mL	BioLegend	302902
Anti-human CD45 (mouse IgG1) 4 μL/100 μL of FACS buffer	BioLegend	368507
Anti-human CD8a (mouse IgG1) 1.7 μL/100 μL of FACS buffer	BioLegend	301014
Anti-human CD4 (mouse IgG1) 5 μL/100 μL of FACS buffer	BioLegend	300555
Anti-human CD56 (mouse IgG1) 5 μL/100 μL of FACS buffer	BioLegend	392409
Anti-human CD3 (mouse IgG1) 4 μL/100 μL of FACS buffer	BioLegend	300429
Anti-human CD11b (mouse IgG1) 5 μL/100 μL of FACS buffer	BioLegend	301345
Anti-human CD16 (mouse IgG1) 0.6 μL/100 μL of FACS buffer	BioLegend	302043
Anti-human CD14 (mouse IgG1) 5 μL/100 μL of FACS buffer	BioLegend	367111
Anti-human CD206 (mouse IgG1) 5 μL/100 μL of FACS buffer	BioLegend	321125
Anti-human CD163 (mouse IgG1) 5 μL/100 μL of FACS buffer	BioLegend	333605
Anti-Ki67 (rabbit IgG) 1:200	Abcam	ab16667
Anti-CD31 (mouse IgG1) 1:200	Cell Signaling Technology	3528S
Anti-αSMA (rabbit IgG) 1:200	Cell Signaling Technology	19245
Anti-S100A4 (rabbit IgG) 1:250	Cell Signaling Technology	13018S
Alexa Fluor 488, goat anti-mouse IgG, IgM, IgA (H + L) 1:1,000	Life Technologies	A-10667
Alexa Fluor 647, donkey anti-rabbit IgG, IgM, IgA (H + L) 1:1,000	Life Technologies	A-31573
Chemicals, peptides, and recombinant proteins		
RPMI 1640 + GlutaMAX	Gibco (Thermo Fisher Scientific)	61870036
FBS	Gibco (Thermo Fisher Scientific)	10270106
100× Penicillin/Streptomycin	Gibco (Thermo Fisher Scientific)	15140122

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
DMEM + GlutaMAX	Gibco (Thermo Fisher Scientific)	31966021
MCDB131	Gibco (Thermo Fisher Scientific)	10372019
Hydrocortisone	Sigma-Aldrich (Merck)	H0888-1G
L-glutamine (200 mM)	Gibco (Thermo Fisher Scientific)	25030024
Animal-free recombinant human EGF	PeproTech	AF-100-15-100UG
PBS tablets	Life Technologies	18912014
Trypsin-EDTA (0.05%)	Gibco (Thermo Fisher Scientific)	25300054
Benzonase Nuclease	Merck	E1014-25KU
DAPI	Invitrogen (Thermo Fisher Scientific)	D1306
Mayer's hematoxylin	Merck	MHS32-1L
Eosin Y-solution 0.5% aqueous	Merck	1098441000
Acetic acid	Carl Roth	7332.1
NeoClear	Merck	109843
Eukitt Quick-hardening mounting medium	Merck	03989-100mL
Fluoromount-G mounting medium	Life Technologies	00-4958-02
ROTI Histofix	Carl Roth	P.087.3
Agarose	Carl Roth	2267.3
Trametinib	Selleckchem	S2673
Staurosporine	Cayman Chemical	81590-10
Propidium iodide	Sigma-Aldrich	81845-25MG
Triton X-100	Carl Roth	3051.3
Tris	Carl Roth	4855.3
EDTA	Carl Roth	8040.3
Tween 20	Carl Roth	9127.2
Citric acid monohydrate	Carl Roth	5110.5
Sodium hydroxide	Carl Roth	6771.1
TrypLE Express enzyme 1×, phenol red	Life Technologies	12605010
Accutase solution	Merck	A6964-500mL
Critical commercial assays		
CellEvent Caspase-3/7 detection reagent	Thermo Fisher Scientific	C10433
CellTiter-Glo 3D cell viability assay	Promega	G9682
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	Invitrogen	L34975
Experimental models: Cell lines		
SKmel147 (human)	Prof. Dr. Jochen Utikal	N/A
NHDF (human)	PromoCell	C-12300
MRC-5 (human)	ATCC	CCL-171
LX-2 (human)	Merck	SCC064
HMEC-1 (human)	ATCC	CRL-3243
Buffy Coat of healthy human donors	Croix Rouge Luxembourg	N/A
Software and algorithms		
CellPathfinder software	Yokogawa	CellPathFinder
GraphPad Prism version 10.0.0	GraphPad Software	GraphPad Prism
Gen5	Agilent BioTek	N/A
BioRender	BioRender.com	BioRender
FlowJo version 10.10.0	BD Biosciences	FlowJo
Other		
CellVoyager CV8000	Yokogawa	N/A
Cytation 10	Agilent BioTek	N/A
BioSpa8 robotic arm and automated incubator	Agilent BioTek	N/A
Cytation 5	Agilent BioTek	N/A
96-well plate U bottom ULA clear faCellitate	faCellitate GmbH	F202003
384-well black/clear round bottom ultra-low attachment spheroid microplate	Corning	4516

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PrimeSurface Ultra-low attachment plates: 384 well, U bottom, clear plates	S-BIO	MS-9384UZ
Coverslips 24 × 60 mm	Carl Roth	H878.2
Epredia slides Superfrost PLUS	Fisher Scientific	11877802
Nunc Lab-Tek II Chamber Slide System	Thermo Fisher Scientific	154534PK
CellTrics 50 μm cell strainer	Sysmex	04-004-2327

MATERIALS AND EQUIPMENT

CellVoyager CV8000 (Yokogawa) configuration

The CellVoyager CV8000 high content imaging system with spinning disk technology was used to acquire confocal pictures of live MMSs over time. The instrument is equipped with solid lasers with the following excitation wavelengths: 405/488/561 nm and emission filters: 445/45 nm, 525/50 nm, 600/37 nm. Set the incubation temperature to 37°C.

Cytation 10 (Agilent BioTek) configuration

To robustly assess the efficacy of drugs, assays measurements are executed using automated microplate readers. The Cytation 10 confocal and wide-field microscopy with multimode plate reading with spinning disk technology for confocal images acquisition was used to acquire pictures of IF. The instrument is equipped with a 590 nm LED and Texas Red filter cube (Excitation 586/15 nm, Emission 647/57 nm) and was employed for tracking melanoma fluorescence changes over time. Moreover, the instrument is equipped with a laser combiner (spectral range 398–643 nm) and a DAPI filter cube (Excitation 390/40 nm, Emission 442/42 nm), a GFP filter cube (Excitation 472/30 nm, Emission 520/35 nm), and a TRITC filter cube (Excitation 556/20 nm, Emission 600/37 nm). The instrument is also equipped with a BioSpa8 robotic arm and automated incubator (Agilent BioTek).

Cytation 5 (Agilent BioTek) configuration

The Cytation 5 automated microplate reader is equipped with a luminescence detector and was used to acquire the luminescence signals emitted by 3D Cell Titer Glo.

BD LSRFortessa cell analyzer configuration

All flow cytometry-based assays were acquired on the 4-laser BD LSRFortessa cell analyzer with the BD FACSDiva software and analyzed using the FlowJo v10.10 software. The specific laser/detector setup is presented in the table below.

Laser	Filter	Dye
Violet (405 nm)	780/60	BV785
	710/50	BV711
	610/20	BV605
	450/50	BV421
Green (488 nm)	530/30	FITC
	670LP	PerCP Cy5.5
Yellow-Green (561 nm)	582/15	PE
	780/60	PE Cy7
	610/20	PE Dazzle 594
	582/15	PE
Red (640 nm)	670/20	APC
	780/60	APC Cy7

Table 1. Cell lines

Cell line	Source	Reporter
SKmel147[a]	Prof. Dr. Jochen Utikal[f]	unlabeled/mCherry[g]
NHDF[b]	PromoCell	unlabeled/GFP[g]
MRC-5[c]	ATCC (CCL-171)	unlabeled/GFP[g]
LX-2[d]	Merck	unlabeled/GFP[g]
HMEC-1[e]	ATCC (CRL-3243)	unlabeled/BFP[g]

[a]Metastatic Melanoma cell line.
[b]Normal Human Dermal Fibroblasts.
[c]Human Fetal Lung Fibroblasts.
[d]Hepatic Stellate Cells.
[e]Human Microvascular Endothelial Cells-1.
[f]University Medical Center Mannheim, Germany.
[g]Depending on the readout method for assay, either unlabeled or mCherry labeled cells were used. Please refer to description of cells lines used in particular steps of the protocol.

1% agarose solution

- Weigh 1 g of agarose and transfer to a bottle.
- Add 100 mL of PBS 1×.
- Boil in microwave to dissolve, the agarose should appear transparent.
- Store at 21°C–25°C up to 6 months.

CellEvent Caspase-3/7 detection reagent

- Reconstitute each vial of lyophilized reagent with 100 μL cell culture grade DMSO to obtain a 500 μM stock solution. Aliquoted solution can be stored in −20°C for several months.

Propidium iodide

- Reconstitute lyophilized powder with 25 mL of cell culture grade H_2O to obtain a 1 mg/mL stock solution. Aliquoted solution can be stored in 4°C for several months.

Melanoma cell medium

Reagent	Final concentration	Amount
RPMI 1640 + GlutaMax	N/A	500 mL
Fetal Bovine Serum	10%	50 mL
Penicillin/Streptomycin 100×	1%	5 mL

Note: Melanoma cell medium can be prepared in advance and can be stored for up to 1 month at 4°C.

Fibroblast/Hepatic Stellate Cell medium

Reagent	Final concentration	Amount
DMEM+ GlutaMax	N/A	500 mL
Fetal Bovine Serum	10%	50 mL
Penicillin/Streptomycin 100×	1%	5 mL

Note: Fibroblast/Hepatic Stellate Cell medium can be prepared in advance and can be stored for up to 1 month at 4°C.

Endothelial cell medium		
Reagent	Final concentration	Amount
MCDB131	N/A	500 mL
Fetal Bovine Serum	10%	50 mL
Penicillin/Streptomycin 100×	1%	5 mL
Hydrocortisone	1 μg/mL	10 mL
L-Glutamine (200 mM)	10 mM	25 mL
EGF	10 ng/mL	N/A

Note: Add EGF freshly and only for the required amount of medium used to culture endothelial cells in the flask of choice.

Note: Endothelial cell medium can be prepared in advance and can be stored for up to 1 month at 4°C.

PBMC thawing medium		
Reagent	Final concentration	Amount
RPMI 1640 + GlutaMax	N/A	500 mL
FBS	10%	50 mL
Penicillin/Streptomycin 100×	1%	5 mL
Benzonase Nuclease 25KU, >250 UI/μL	0.1 μL/mL	50 μL

Note: Add Benzonase Nuclease freshly and only for the required amount of medium.

Note: PBMC thawing medium can be prepared in advance and can be stored for up to 1 month at 4°C.

Note: PBMCs have been isolated from blood of healthy donors using Ficoll Paque Plus and a density gradient centrifugation protocol and stored in liquid nitrogen until used.

FACS Buffer		
Reagent	Final concentration	Amount
PBS 1×	1×	Up to 100 mL
Bovine Serum Albumin	1%	1 g

Antigen Retrieval solution		
Reagent	Final concentration	Amount
Sodium Citrate	10 mM	1.92 g
Tween-20	0.05%	0.5 mL
H_2O, Type II		1 L

TBST 0.1%		
Reagent	Final concentration	Amount
TBS 10×	1×	100 mL
Triton X-100	0.1%	1 mL
H_2O, Type II		900 mL

Blocking solution		
Reagent	Final concentration	Amount
TBST 0.1%	N/A	N/A[a]
FBS	10%	N/A[a]

[a]Total amount is prepared accordingly for the total number of slides. Use 200 μL to cover one full slide.

Staining Solution 1		
Reagent	Final concentration	Amount
CellEvent Caspase-3/7 Detection Reagent 500 μM	500 nM	2.5 μL
PBS 1×	1×	2.5 mL

Final volume depends on the number of analyzed samples. This example describes preparation of Staining Solution for 10 samples (250 μL per sample).

Staining Solution 2		
Reagent	Final concentration	Amount
Propidium Iodide 1 mg/mL	1 μg/mL	2.5 μL
PBS 1×	1×	2.5 mL

Final volume depends on the number of analyzed samples. This example describes preparation of Staining Solution for 10 samples (250 μL per sample).

STEP-BY-STEP METHOD DETAILS

Generation of melanoma multicomponent spheroids (MMSs)

Timing: 5–8 days

The first part of the protocol describes the generation of melanoma multicomponent spheroids (MMSs) by a sequential seeding method to ensure proper distribution of fibroblasts/hepatic stellate cells within the spheroids. In the first step, only melanoma and endothelial cells are seeded together, followed by addition of fibroblasts or hepatic stellate cells (HSCs) after 24 h (Figure 1). This protocol utilizes an optimized 1:3:3 ratio of melanoma cells: endothelial cells: fibroblasts or HSCs. Live cell imaging (if using fluorescently labeled cells) visualizes the cell type organization within the MMSs (Figures 2A–2D). Furthermore, this protocol allows the co-culture with PBMCs to recapitulate the immune microenvironment of a tumor.

Note: Before starting this procedure, all cell lines should be about 80% confluent in 2D culture flasks.

Alternatives: Seeding of melanoma, endothelial cells, fibroblasts or HSCs can be adapted to 96-well U bottom Ultra-Low Attachment (ULA) plates. Cellular ratios are fixed to 1:3:3 (melanoma cells: endothelial cells: fibroblasts or HSCs) with 2500 melanoma cells per well. Dispensed volume/well of cell suspension per step is 100 μL/well for a final total volume of 200 μL/well.

Day −1: Melanoma and endothelial cell seeding

Note: Pre-warm to 37°C Melanoma cell medium, Endothelial cell medium, PBS 1×, Trypsin-EDTA.

1. Harvest cells and create a single cell suspension for each cell line.

   Note: Reported volumes correspond to a T75 flask.

   a. Remove the culture medium.

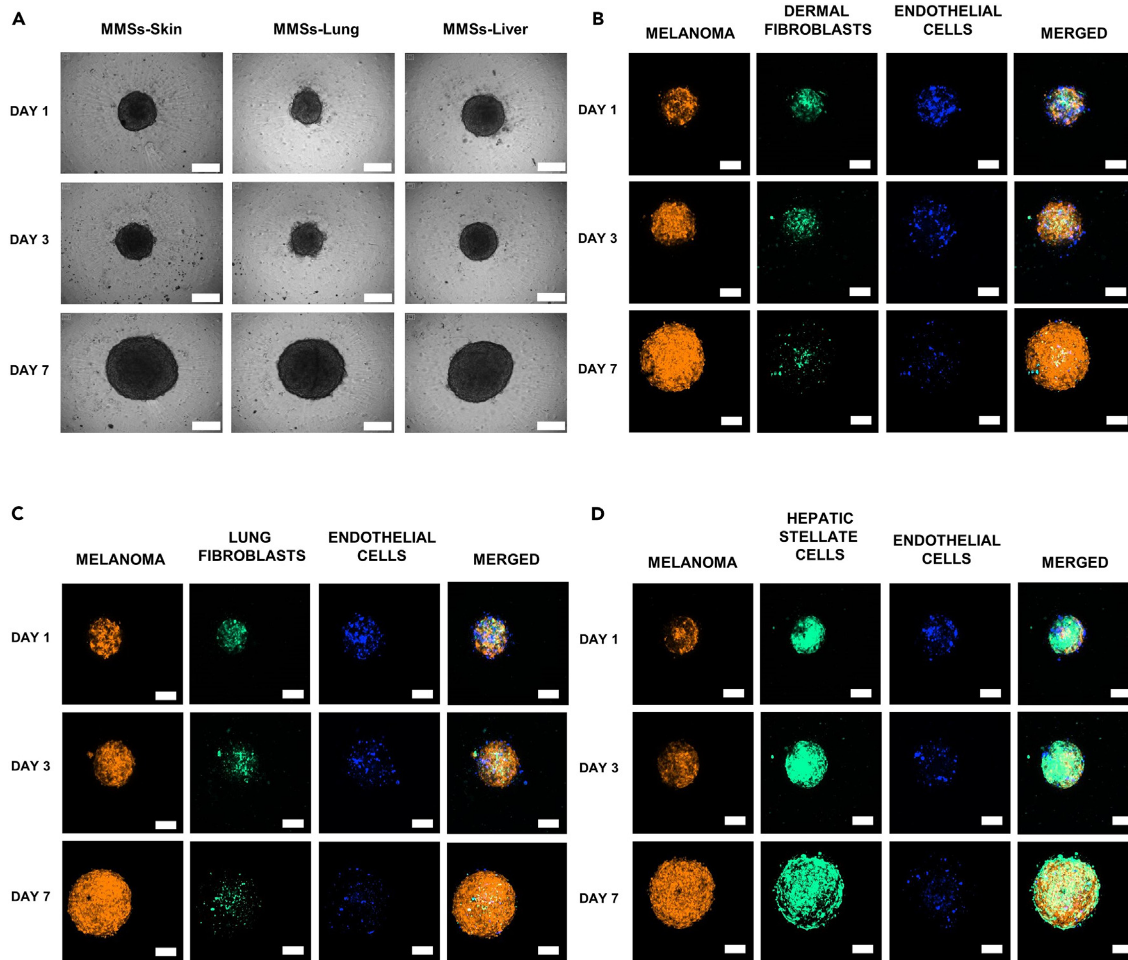

Figure 2. Live-cell images of MMSs

(A) Bright-field pictures of whole MMSs at day 1, 3 and 7 of culture.

(B–D) Cell lines constitutively expressing fluorescent proteins (see Table 1 "reporter") were used. Fluorescently labeled (orange-mCherry) melanoma cells, (blue-BFP) endothelial cells, and either (green-GFP) dermal fibroblasts (B), or lung fibroblasts (C) or hepatic stellate cells (D). In each condition, melanoma cells proliferate over time. Confocal images were taken at maximum intensity projections of multiple z-stacks. Objective 10×. Scale bar = 200 μm.

b. Wash with 5 mL of pre-warmed PBS 1×.
c. Incubate with 3 mL of Trypsin-EDTA for up to 5 min to detach the cells.
d. Visually monitor cell detachment by using a bench-top microscope.
e. Add 12 mL of medium to neutralize Trypsin-EDTA (medium to trypsin-EDTA ratio 4:1) and transfer the solution to a 50 mL conical tube.

Note: For Trypsin-EDTA neutralization, use the dedicated medium for each cell type.

f. Centrifuge cells for 3 min at 300 × *g*.
g. Remove supernatant, add 10 mL of pre-warmed culture medium and resuspend cells.
h. Count viable cells with trypan blue staining or an equivalent method.

2. Preparing the cell culture plates.

Note: To reduce evaporation of medium from wells, avoid using external rows and columns of plates for spheroid culture. Instead, fill them with PBS 1× to an equal or greater volume than the medium. Apply the same to all unused wells on the plate.

   a. 384 well plates: Add 80–90 µL of PBS 1× in all external wells of the plate (columns 1, 24, and rows A, P).
   b. 96 well plate: Add 200–250 µL of PBS 1× in all external wells of the plate (columns 1, 12, and rows A, H).
3. Seeding of melanoma and endothelial cells in 384-well U-bottom ULA plates.
   a. Prepare a cell suspension containing 500 melanoma cells/40 µL and 1500 endothelial cells/40 µL in a 50 mL conical tube in Melanoma cell medium.
   b. Centrifuge cells at 300 × *g* for 3 min and remove the supernatant.
   c. Resuspend the cell pellet in Melanoma cell medium and use appropriate volume for seeding in the desired number of wells.
   d. Pipet gently up and down 3–5 times until the cell pellet is fully resuspended.
   e. Seed 40 µL/well of cell suspension.
   f. Centrifuge the plate at 500 × *g* for 5 min.
   g. Incubate at 37°C and 5% CO_2 for 24 h.

Note: To facilitate the seeding and to avoid variability, a multichannel pipette can be used during the seeding steps.

Day 0: Fibroblast/HSC seeding

Note: Pre-warm to 37°C Melanoma cell medium, Fibroblast/Hepatic Stellate Cells (HSC) medium, PBS 1×, Trypsin-EDTA.

4. Perform enzymatic dissociation of Fibroblasts/HSCs as described for dissociation of melanoma and endothelial cells (step 1a–h).
5. Seeding of fibroblasts or HSCs in a 384-well U-bottom ULA plate on top of melanoma-endothelial cell spheres.
   a. Prepare a cell suspension in Fibroblast/HSC medium containing 1500 dermal fibroblasts or lung fibroblasts or HSCs/well in a 50 mL conical tube.
   b. Centrifuge cells at 300 × *g* for 3 min and remove the supernatant.
   c. Resuspend the cell pellet in Melanoma medium by gently pipetting up and down. The volume of medium is calculated by multiplying the number of well seeded by 40 µL, plus 20% as death volume.
   d. Add 40 µL/well of cell suspension to the of melanoma-endothelial cell spheres.

Note: To facilitate the seeding and to avoid variability a multichannel pipette can be used during the seeding steps.

   e. Centrifuge the plate at 500 × *g* for 5 min.
   f. Incubate at 37°C and 5% CO_2 for 3 days.

Note: Days of culture depend on many factors such as the type of cells, assay and readout. We decided to culture our MMS for 3 further days after fibroblast/HSC addition to have a final spheroid size of >250–300 µm diameter (Figure 2A), which mimics the proliferative (outer layer) and apoptotic/quiescent (inner layer) of the tumor *in vivo*,[13] as well as the perfusion of molecules and drugs.[5] If the spheroids are out of suggested range, we suggest testing different number of melanoma cells while maintaining the same cellular ratio.

6. Live cell imaging of MMSs over time with a high-resolution confocal microscope (CellVoyager CV8000).
   a. Select the 10× objective.
   b. Apply z-stack acquisition (ascending/descending distance and slicing intervals are set according to the spheroid sizes).

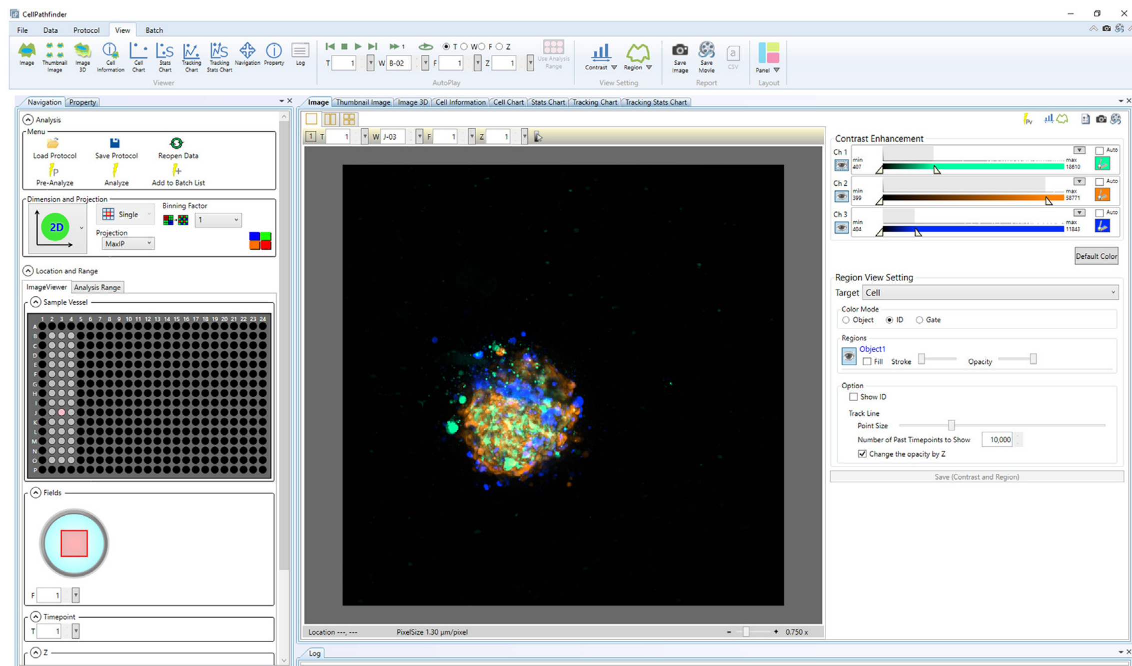

Figure 3. CellPathfinder Software

Overview of the main control board of the CellPathfinder software for visualization and analysis of MMSs pictures acquired using the CellVoyager CV8000 (Yokogawa) confocal microscope.

Note: We suggest applying slicing intervals ranging between 5 and 10 μm to have higher image resolution after Maximum Intensity Projection.

Note: Images are sequentially acquired using bright-field and fluorescence channels at predefined time points (see Figure 2).

c. Apply Maximum Intensity Projection (MIP) using the CellPathfinder Software (Figure 3).

Note: The generated MMSs can be used directly for histological characterization and drug testing or alternatively, co-culturing with PBMCs followed by flow-cytometry based characterization can be added.

Day 3: PBMC seeding

Note: PBMCs are added in a 1:10 ratio (1:10 ratio of MMSs to PBMCs) to pre-formed MMSs in 384-well or 96-well plates by adjusting the cell quantity and volume of the culture medium. For the subsequent steps, MMSs were generated in 96-well U-bottom ULA plates to facilitate subsequent flow cytometric analyses.

Note: Pre-warm to 37°C Melanoma cell medium and PBMC thawing medium.

7. Quickly thaw a cryotube containing PBMCs in a water bath at 37°C by gently swaying.
8. Add 500 μL of pre-warmed PBMC thawing medium dropwise into the cryovial.
9. Transfer the cell suspension dropwise to a 15 mL conical tube containing 5 mL of pre-warmed PBMC thawing medium.

10. Fill up the conical tube to 10 mL with PBMC thawing medium.
11. Centrifuge cells 300 × *g* for 8 min at 21°C–25°C.
12. Resuspend the cell pellet in 5 mL of PBMC thawing medium and incubate for 5–10 min at 37°C.

⚠ CRITICAL: Addition of Benzonase Nuclease to PBMC thawing medium will prevent clumping caused by DNA released from dead cells and increases PBMC recovery.

13. Add further 10 mL of PBMC thawing medium and centrifuge at 300 × *g* for 8 min at 21°C–25°C.
14. Remove the supernatant and resuspend the cell pellet in 10 mL Melanoma cell medium.
15. Adding PBMCs to a 96-well plate with pre-established melanoma-endothelial-fibroblast/HSC MMSs:
    a. Count the viable PBMCs using trypan blue staining or an equivalent method.
    b. Add the PBMC suspension to a 50 mL conical tube with a volume containing ten times the total cell number than that was used to generate MMSs (175000 PBMCs/well).
    c. Centrifuge cells 300 × *g* for 8 min at 21°C–25°C.
    d. Remove the supernatant and resuspend the cell pellet in an appropriate volume of Melanoma cell medium containing anti-CD28 antibody at final concentration of 5 μg/mL. The volume of medium is calculated by multiplying the number of well seeded by 100 μL, plus 20% as death volume.

    Note: Add anti-CD28 antibody to ensure necessary co-stimulatory signals for activation of immune cells.

    e. Remove 100 μL/well of medium from the 96-well plate.
    f. Add 100 μL/well of PBMC cell solution to reach a volume of 200 μL/well.
    g. Incubate at 37°C and 5% CO_2 for 3 days in an incubator.

Histological characterization of MMS

⏲ Timing: 3 days

Below, we describe the procedure for formalin-fixation and paraffin embedding (FFPE) of MMSs for histological analysis. We apply hematoxylin/eosin staining for structural evaluation, and immunofluorescence (IF) to visualize different cell populations by using antibodies listed in Table 2.

Note: MMSs were generated in a 96-well plate to augment the quantity of spheroids for downstream readouts.

16. MMSs collection.
    a. Begin from step 5f.

    ⚠ CRITICAL: To transfer spheroids without damaging them, use wide orifice tips.

    b. Using a multichannel pipet, collect the spheroids from the plates and transfer them to a reagent reservoir.

    ⚠ CRITICAL: Double-check the plate to ensure that all spheroids have been collected.

    c. Transfer the spheroids to a 15 mL conical tube and wait 5 min until spheroids sink to the bottom of the tube.
    d. Remove the supernatant and wash with 5 mL PBS 1×.

Table 2. Antibodies for immunofluorescence

Antibody	Source	Isotype	Dilution
Ki-67	Rabbit	IgG	1:200
PECAM-1/CD31	Mouse	IgG1	1:200
S100A4	Rabbit	IgG	1:200
a-SMA	Rabbit	IgG	1:250
Alexa 647	Rabbit	IgG	1:1000
Alexa 488	Mouse	IgG	1:1000

Note: Either aspirator with glass Pasteur pipet or plastic serological pipet can be used to remove the supernatant.

e. Add 5 mL of HISTOFIX and incubate for 30 min at 21°C–25°C.
f. Remove HISTOFIX and wash with 5 mL PBS 1×.
g. Repeat the wash once more.

Pause point: Fixed spheroids can be stored in PBS 1× at 4°C for several days. However, it is advisable to proceed with the next steps as soon as possible to minimize the loss of antigen signal.

17. Embedding and cutting.
    a. Heat the agarose 1% solution in a microwave until the melting point is reached and let it cool down for 5 min at 21°C–25°C.
    b. Remove PBS 1× from the conical tube completely by making sure not to touch the spheroids.
    c. Add 300 μL of agarose 1% solution to the spheroids.
    d. Transfer the spheroids in a chamber of a Lab-Tek II Chamber Slide.
    e. Place the μ-Slide 8 well on ice and let the agarose solidify for 5 min.
    f. Carefully transfer the agarose block into a 50 mL conical tube.
    g. Fix the agarose block by adding 15 mL HISTOFIX and incubate for 3–4 h at 21°C–25°C or ON at 4°C.

Note: In one agarose block, up to 96 MMSs can be pooled together.

h. Remove HISTOFIX solution and wash with 5 mL PBS 1×.
i. Remove PBS 1× and add 20 mL of 70% Ethanol to the conical tube.

Pause point: Agarose blocks can be stored at 4°C in 70% Ethanol for several weeks. However, it is recommended to proceed as soon as possible with paraffin embedding.

j. Remove the 70% Ethanol.
k. Transfer the block into an embedding cassette and transfer the cassette to a container with a magnetic stirrer.
l. Add 500 mL 100% Ethanol and incubate for 2 h with continuous stirring.
m. Repeat previous step once by removing and replacing with fresh 100% Ethanol.
n. Add 500 mL NeoClear (Xylene substitute) solution and incubate for 1–2 h with continuous stirring.
o. Remove NeoClear solution.
p. Transfer the agarose block into an embedding mold, cover with liquid paraffin and incubate for 2 h at 60°C.
q. Exchange the paraffin inside the embedding mold.
r. Let the paraffin solidify on a cooling plate or on ice.

Note: Solidified FFPE blocks are stored at 21°C–25°C long term until needed for sectioning.

s. Cut 4–5 μm thick sections with a microtome slicer and place them on a microscope slide.

⚠ CRITICAL: If paraffin sections were cut more than a month before staining is performed, incubate the slides at 40°C–42°C from 16 to 20 h.

18. Rehydration of paraffin sections.

Note: Rehydration of paraffin sections steps must be performed inside r a fume hood or a ventilated staining table.

Note: Use Coplin jars for rehydration and dehydration steps. Prepare jars with appropriate volumes of descending percentages of Ethanol, TBST, hematoxylin and eosin solutions.

   a. Place the slides in a slide holder and immerse the slides in NeoClear solution for 10 min, repeat once.
   b. Transfer slide and immerse in 100% Ethanol for 5 min.
   c. Transfer slide and immerse in 95% Ethanol for 5 min.
   d. Transfer slide and immerse in 70% Ethanol for 5 min.
   e. Transfer slide and immerse in 50% Ethanol for 5 min.
   f. Transfer slide and rinse with TBST for 5 min
19. Hematoxylin and eosin staining.
   a. Rinse with TBS.
   b. Incubate in Mayer's hematoxylin for 30–40 s.
   c. Rinse with TBS.
   d. Incubate in Eosin solution for 30 s.
   e. Rinse with TBS.
   f. Immerse in 95% Ethanol for 2 min.
   g. Immerse in 100% Ethanol for 2 min.
   h. Immerse in NeoClear solution for 2 min.
   i. Leave the slides to dry at 21°C–25°C .
   j. Mount the slices by adding 1–2 drops of mounting medium (like Eukitt) per slide and cover with coverslips.
20. Immunofluorescence (IF): Begin from step 18f.
   a. Wash with TBST in a coplin jar.
   b. Transfer slides in a coplin jar filled with the appropriate antigen retrieval solution (ARS).

Note: Depending on the antibody used, different antigen retrieval solution (ARS) needs to be employed for heat-mediated antigen retrieval.

   c. Perform heat-mediated antigen retrieval in a microwave. Start on 100% power until boiling point (up to several minutes). Assure that the slides are covered with the ARS.
   d. Incubate in a microwave for a further 10–15 min on 10–20% power at sub-boiling temperature (95°C–98°C).

Alternatives: A water bath at 96°C–100°C can be used, place slides in a plastic jar with ARS and incubate for 30 min.

⚠ CRITICAL: During antigen retrieval the solution may evaporate. Make sure that the level of the solution is always above the level of the slides as the tissue may dry out. Keep a second coplin jar with the same antigen retrieval solution in the microwave or water bath in case the volume in the jar with the slides is dropping.

e. Leave at 21°C–25°C for 30 min to cool down.
f. Rinse 3 times with TBST in a coplin jar for 5 min each.
g. Place the slides in a staining tray and add 200 µL per slide of blocking solution and incubate at 21°C–25°C for 30 min.

⚠ CRITICAL: Place the slides in a humidified chamber to avoid evaporation during blocking, primary and secondary antibodies incubation steps.

h. Remove blocking solution by tilting the slides on paper towels and add 200 µL per slide of primary antibody, diluted in blocking solution.
i. Incubate at 4°C from 16 to 20 h in a humidified chamber.
j. Rinse 3 times with TBST in a coplin jar for 5 min each.
k. Add 200 µL per slide of conjugated secondary antibody solution and DAPI diluted in blocking solution and incubate at 21°C–25°C for 1 h.
l. Mount the slides by adding 2–3 drops of Fluoromount per slide and cover with coverslips.

Note: Use clear nail polish to seal the edges of the coverslip, to prevent dehydration. Mounted slides can be stored in the dark at 4°C for up to 1 month. It is recommended to image slides immediately due to the decay of fluorescent signals.

21. Image acquisition (Cytation 10).
    a. For imaging H&E staining, use a wide field 20× objective and colored bright field.
    b. For IF, use a confocal (spinning disk) microscope and a 20× objective.

MMS characterization: FACS analysis for PBMC interaction with MMSs

⏲ Timing: 3 h

This part of the protocol describes the detection of infiltrating and non-infiltrating immune cells co-cultured with MMSs by flow cytometry. To identify the subpopulations of immune cells (lymphocytes, monocytes, macrophages and Natural killer cells) we composed a panel of specific antibodies as listed in Table 3. MMSs are co-cultured with healthy donor PBMCs for 3 days with a ratio of 1:10 (1:10 ratio of MMSs to PBMCs). One 96-well plate of MMSs co-cultured with PBMCs is used for each specific composition of MMS (MMS-Skin, MMS-lung, MMS-Liver).

Note: MMSs were generated in a 96-well plate to augment the quantity of spheroids for the downstream readouts.

Table 3. Antibody panel for PBMCs

Antibody target	Conjugate	Volume (µL)[a]
CD45	FITC	4
CD8a	APC	1.7
CD4	BV605	5
CD56	PE-594	5
CD3	PerCP 5.5	4
CD11b	BV785	5
CD16	BV711	0.6
CD14	PE-Cy7	5
CD206	BV421	5
CD163	PE	5

[a]The specific volume of antibodies was validated per 1 million of PBMCs in 100 µL FACS buffer.

Note: It is necessary to generate a compensation matrix for all fluorophores used in the assay, to prevent fluorescent signals to spill over between channels. Compensation can be done using mono-stained compensation beads or cells.

22. Begin from step 15g.
23. Dissociation of MMSs co-cultured with PBMCs.
    a. Using a multichannel pipet, collect the spheroids and medium from 96-well plates and transfer them into a reagent reservoir.

    ⚠ CRITICAL: Collect all the medium in the wells to ensure the collection of all non-infiltrating PBMCs.

    b. Transfer all the reagent reservoir's content (spheroid and supernatant) in a 50 mL conical tube and wait approximately 5 min until spheroids sink to the bottom of the tube.
    c. Remove the supernatant, which contains non-infiltrating immune cells into another 50 mL conical tube and leave it at 21°C–25°C until further use.
    d. Wash MMSs with 10 mL PBS 1×.
    e. Centrifuge 300 × *g* for 3 min at 21°C–25°C.
    f. Transfer supernatant into the conical tube containing the non-infiltrating immune cells.
    g. Add 5 mL of a pre-warmed TrypLE Express and Accutase mix (1:1) to the MMSs.
    h. Mix well and incubate for 10 min at 37°C.

    ⚠ CRITICAL: To minimize dissociation-induced cell death, avoid prolonged incubation with the enzyme mix and vortexing MMSs. Proper dissociation of spheroids is ensured by the pipetting described below (Figure 4).

    i. To dissociate the MMSs, use a small tip (10 μL) on top of a bigger tip (1 mL) (Figure 4) and pipet up and down until a single cell solution is obtained.
    j. Add 10 mL PBS 1× to the dissociated MMSs.
    k. Centrifuge both infiltrated PBMCs (dissociated spheroids) and non-infiltrated cells at 300 × *g* for 8 min at 21°C–25°C.
    l. Remove supernatant and resuspend cells in 4 mL PBS 1×.
    m. Using a pipet, pass the whole volume of cell solution through a 50 μm strainer.
    n. Divide each sample into two and transfer to two FACS tubes: 1 mL (or: $^1/_4$ of the sample) for the unstained control and 3 mL (or: $^3/_4$ of the sample) for staining with antibodies.
    o. Centrifuge at 300 × *g* for 8 min at 4°C.
24. Flow cytometry staining and analysis.
    a. Prepare antibody mix in FACS buffer, keep on ice and in the dark until use.
    b. Remove supernatant from both tubes and add 100 μL of antibody panel for PBMCs (volumes per antibody reported in Table 2) to the cells. Add 100 μL FACS buffer for the unstained control tube.
    c. Incubate for 30 min at 4°C in the dark.
    d. Add 1 mL of FACS buffer to each tube and centrifuge at 300 × *g* for 8 min at 4°C.
    e. Remove supernatant and add 300 μL of FACS buffer.
    f. Keep on ice in the dark until acquisition.
    g. Run samples on BD LSRFortessa Cell Analyzer (or equivalent).
    h. Use a gating strategy as represented in Figure 5A.

Drug dispensation in MMSs

⏲ Timing: 4 days

Figure 4. Double plastic tip to enhance MMS dissociation
Using a 100–1000 μL micropipette, a 10 μL plastic tip is inserted on top of a 1000 μL plastic tip.

The fourth part of the protocol describes the drug testing procedure. Depending on the nature of the selected compound and targeted cell types, the drug concentration varies. Here, we use a MEK 1/2 inhibitor (Trametinib) at a concentration of 500 nM. For evaluating the drug effect, vehicle (DMSO 0.1%) and positive controls are included in each experiment. The selected positive control compound and its concentration may vary depending on the experimental criteria. In this protocol, Staurosporine is used as positive control.

Note: The following procedure reports volumes adapted for a 384-well U-bottom plate. However, the same protocol can be implemented in 96-well U-bottom plates by multiplying all volumes by a factor of 2.5.

Note: Pre-warm to 37°C Melanoma cell medium.

25. Begin from step 5f.
26. Check under the microscope that MMSs have an appropriate size (>250–300 μm) and a compact morphology (Figure 2A).
27. Prepare a 2× concentration of the compound to be tested and DMSO solution in Melanoma cell medium. The volume of medium is calculated by multiplying the number of well seeded by 40 μL, plus 20% as death volume.
28. Remove 40 μL/well of medium from the wells assigned to contain the vehicle control and tested compound.

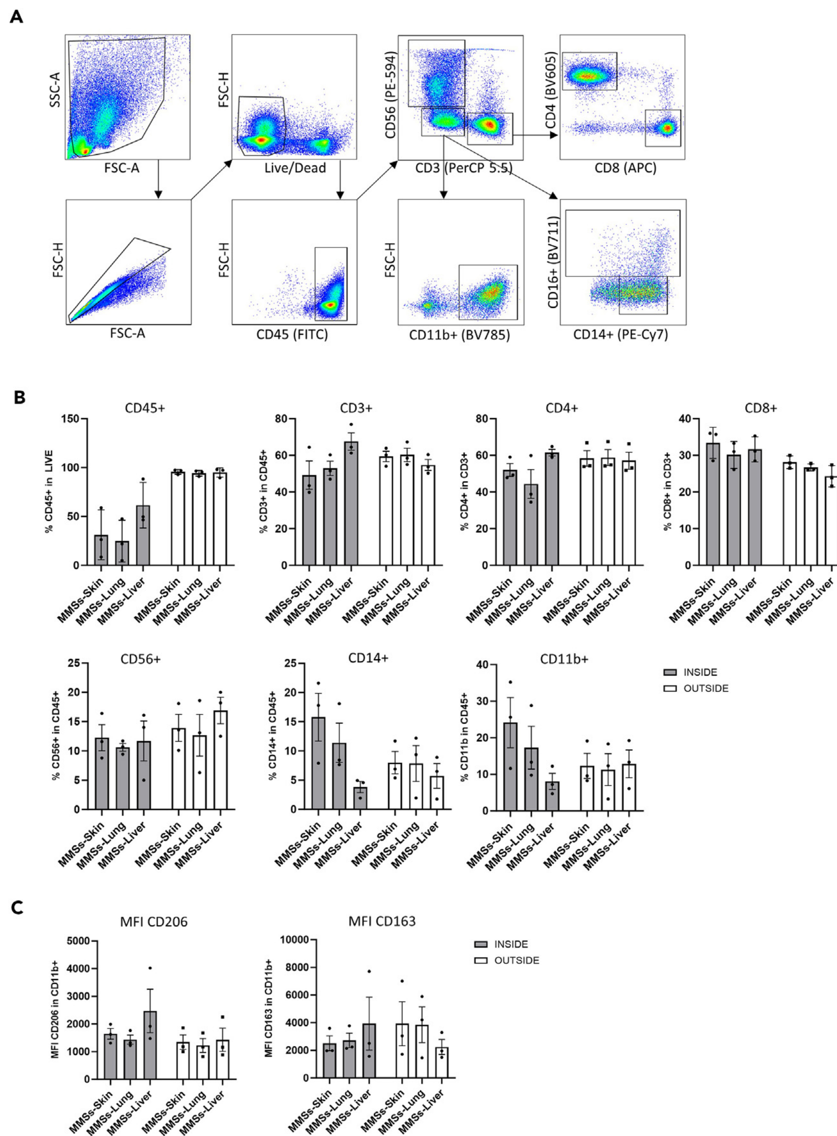

Figure 5. Flow-cytometry for identification of PBMC components

(A) Flow-cytometry gating strategy for identification of different immune cell populations using a specific panel of antibodies (Table 2). Arrows indicated in which order the gating was performed.

(B and C) Quantification of PBMC populations able to interact/infiltrate the MMSs (INSIDE, gray bars) or remaining in the supernatant (OUTSIDE, white bars). Data are mean ± SD of $n = 3$ independent biological replicates. The PBMC populations in the supernatant remain stable across the three MMS types (skin, lung and liver), while the interacting/infiltrating fractions show heterogeneity across the MMS types. (B) MMSs-liver present higher immunogenicity compared to -skin and -lung depicted by CD45+, with slightly higher lymphocyte infiltration (CD3+, CD8+, CD4+, CD56+) but lower monocyte infiltration (CD14+ and CD16+). (C) Median Fluorescence Intensity or MFI is depicted to assess tumor-associated macrophages (CD163 and CD206).

29. Add 40 μL/well of 2× concentrated compound and DMSO solution to the dedicated wells.

Note: Solutions are prepared 2× concentrated to be in a final volume of 80 μL/well.

30. Incubate at 37°C and 5% CO_2 for 3 days.

31. Prepare a 2× concentrated solution for the positive control. Calculate extra solution as dead volume.

Note: Given the strong apoptotic effect of Staurosporine, for endpoint assays (such as 3D Cell Titer Glo) 1 μM Staurosporine can be added 24 h before the end of the assay, while for kinetic assay (such as tracking fluorescence signal emitted by melanoma cells) 200 nM Staurosporine is dispensed at the same time with compound of interest and DMSO solution (step 29).

32. Remove 40 μL/well of medium only from wells assigned to positive controls.
33. Add 40 μL/well of positive control solution to these wells.
34. Incubate at 37°C and 5% CO_2 for 1 day.

Readout assays to evaluate drug efficacy

Timing: 2 h to 4 days

In the last section of the protocol, different assays are described for the evaluation of cell viability and cell death. Viability is assessed through two methods: kinetically, by following the fluorescence intensity of melanoma cells (Table 1) over time and by endpoint measurements using an assay (3D CellTiter-Glo) based on luciferin-ATP-luciferase. Alternatively, a combined staining with propidium iodide (PI) and a DEVD-conjugated dye (CellEvent Caspase-3/7) is applied to detect necrotic and apoptotic cells by flow cytometry.

35. Tracking melanoma fluorescence changes over time

Note: Melanoma cell proliferation in response to drugs is followed by kinetic measurements of the fluorescent signals emitted by fluorescently labeled melanoma cells (Table 1).

a. Begin from step 29.
b. Place the microplate in an automated microplate reader (Cytation 10) with a fluorescence signal acquisition option.

Note: Set the live-cell incubation (37°C) during the plate reading phase to maintain the plates' temperature and prevent water condensation on the lid.

c. Monitor intensity of the fluorescent signal every 4 h (Figures 6A–6C) assisted by an automated robotic station equipped with an integrated incubator (37°C and 5% CO_2).

Note: The detailed Cytation 10 configuration is reported in materials and equipment.

Note: Acquire bright-field pictures (wide field, 4× objective) as a quality control.

36. Cell viability assay: 3D Cell Titer Glo

Note: Reagent preparation is performed according to the manufacturer's guidelines (3D Cell Titer Glo_TechicalManual)

Note: The specified working volumes are intended for a 384-well plate, adjustments can be made to accommodate various types of multi-well plates while maintaining the same volume proportions. According to the manufacturer, a 1:1 ratio between medium and reagent is recommended.

a. Remove 40 μL/well of medium from all wells of a 384-well plate containing MMSs.

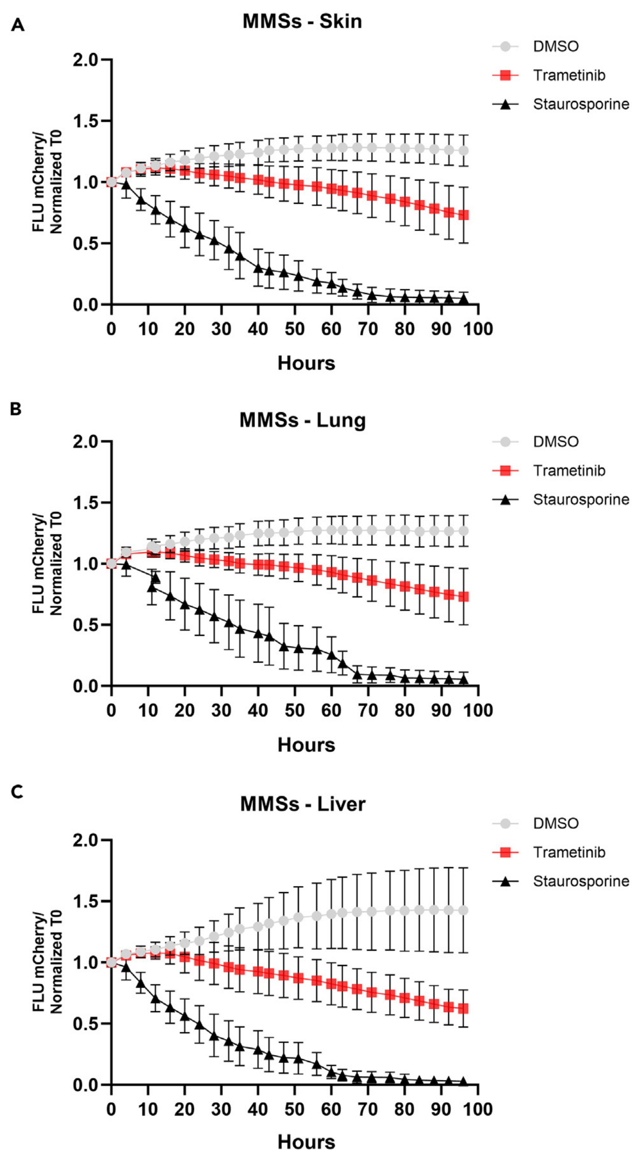

Figure 6. Kinetic assay

The fluorescent signal emitted by labeled melanoma cells is captured every 4 h for the kinetic evaluation of drug efficacy. Plotted is the kinetic profile of MMSs-skin (A), -lung (B) and -liver (C) treated for 96 h with Trametinib 500 nM, DMSO 0.1% and Staurosporine 200 nM. Data are mean ± SD of *n* = 3 independent biological replicates.

b. Remove the whole content of 3-6 wells containing PBS 1× (edge wells) and add 40 μL/well of Melanoma cell medium (these wells will be dedicated to the Blank).

⚠ CRITICAL: Depending on the compound to be tested, cellular connections may be impaired, leading to the formation of fragmented MMSs that can be accidentally aspirated during pipetting. To avoid it remove the medium very slowly without disturbing the cells on the bottom of the wells.

c. Add 40 μL/well of 3D Cell Titer Glo.
d. Incubate at 37°C while shaking (orbital) at 400 rpm for 30 min.
e. Read luminescence signal.

37. Cell death assay: apoptosis and necrosis
    This flow cytometry-based assay allows to determine the ratio of apoptotic and necrotic cells by double staining with a green fluorogenic substrate for activated Caspase 3/7 for apoptosis detection and propidium iodide for necrotic cell death.

Note: MMSs were generated in a 96-well plate to augment the quantity of spheroids for the downstream readouts.

Note: Reagent preparation is performed according to the manufacturer's guidelines. Staining Solutions 1 and 2 are prepared as described in materials and equipment.

⚠ CRITICAL: To minimize dissociation-induced cell death, avoid prolonged incubation with the enzyme mix and vortexing MMSs. Proper dissociation of MMSs is ensured by the pipetting described below.

Note: Pre-warm to 37°C PBS 1× and TrypLE Express and Accutase mix

a. Dissociation of MMSs.
   i. Using a multichannel pipet, collect the spheroids from 96-well plates and transfer them into a reagent reservoir.
   ii. Transfer the MMSs to a 50 mL conical tube and fill up to 50 mL with pre-warmed PBS 1×.
   iii. Centrifuge at 300 × *g* for 5 min at 21°C–25°C.
   iv. Remove supernatant carefully, avoid touching the pellet.
   v. Wash MMSs again with 10 mL PBS 1× and remove the supernatant.
   vi. Add 5 mL of a pre-warmed TrypLE Express and Accutase mix (1:1) to the pelleted MMSs.
   vii. Mix well by pipetting up and down and incubate for 10 min at 37°C.
   viii. To dissociate the MMS, use a small tip (10 μL) on top of a bigger tip (1 mL) (Figure 4) and pipet up and down until no cell clumps are visible anymore.
   ix. Fill up the tube with PBS 1× and centrifuge at 300 × *g* for 5 min at 21°C–25°C.
   x. Remove supernatant and resuspend cells in 2 mL PBS 1×.
   xi. Using a pipet pass the 2 mL cells suspension through a 50 μm strainer to obtain a single cell solution.
   xii. Divide each sample into two FACS tubes: 0.5 mL for unstained control and 1.5 mL for stained sample.
   xiii. Centrifuge at 300 × *g* for 5 min at 4°C.
b. Flow-cytometry staining and analysis.
   i. Prepare Staining Solution 1 (see Table Staining Solution 1) in PBS 1×, keep in the dark until use.
   ii. Remove supernatant and add 250 μL of Staining Solution 1 to the samples. Add 500 μL of PBS 1× to the unstained control tube.
   iii. Incubate for 30 min at 37°C in the dark.
   iv. Add 250 μL of Staining Solution 2 to the sample. Do not add anything to the unstained control tube.
   v. Immediately after adding Staining Solution 2 analyze samples on a BD LSRFortessa Cell Analyzer (or equivalent).
   vi. Use a gating strategy as represented in Figure 7A.

EXPECTED OUTCOMES

With the present protocol, we have established an *in vitro* 3D scaffold-free melanoma spheroid model that allows efficient evaluation of drugs in a physiologically meaningful context. These 3D spheroid models can be flexibly adapted to various microwell plate formats, ranging from low to medium-throughput (96-well plate) to high-throughput (several 384-well plates) drug screening.

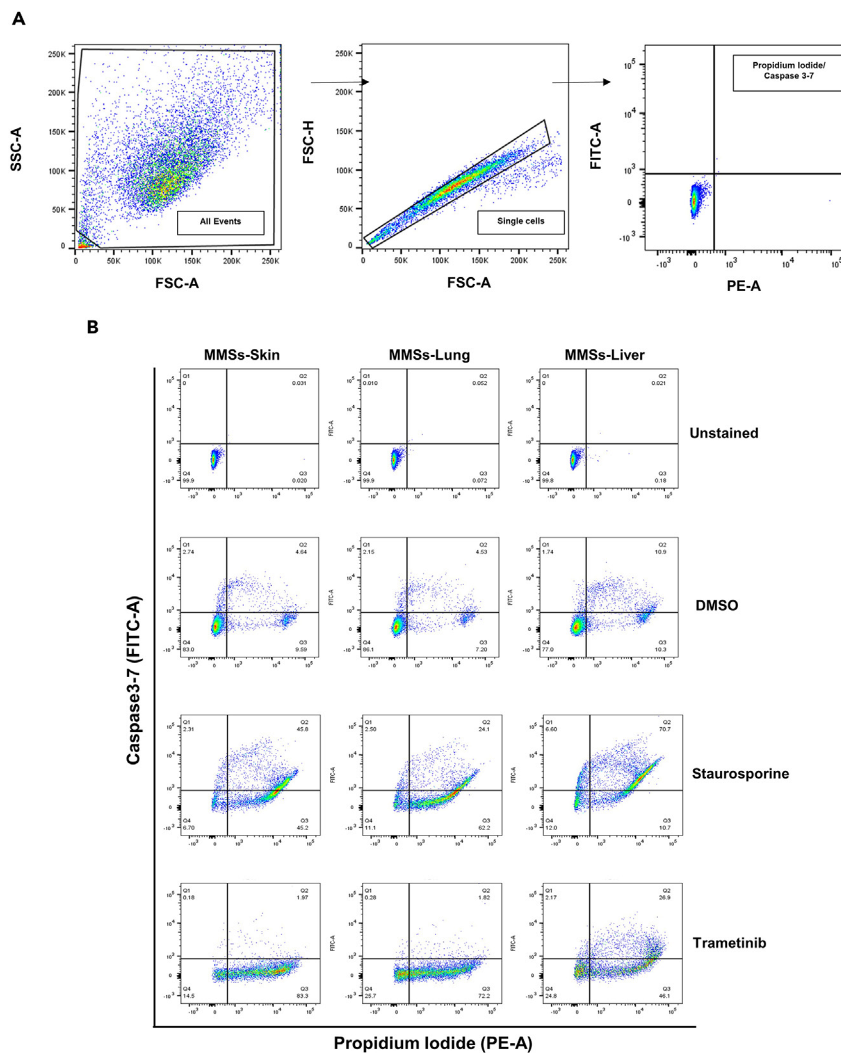

Figure 7. Flow-cytometry apoptosis assay

(A) Gating strategy for flow cytometry apoptosis assays, including a doublet discrimination step. Arrows indicated in which order the gating was performed.

(B) Representative dot plots of an apoptosis assay. Cells were treated with 500 nM Trametinib for 4 days or 1 μM Staurosporine (positive control) for 24 h. 0.1% DMSO was used as a vehicle control. Double staining with Caspase 3–7 and propidium iodide allows to determine the ratio of early apoptotic (positive for Caspase), late apoptotic (positive for both markers) and necrotic cells (positive for propidium iodide).

Sequential seeding (Figure 1) of different co-cultured cells results in 3D structures that far better resemble a tumor in its distinct microenvironment. Dermal and lung fibroblasts display similar growth behavior in MMSs with a tendency to migrate towards the core of the spheres (Figure 2B showing MMS-Skin and 2C showing MMS-lung), while HSCs remain on the outer layer of MMSs (Figure 2D). Histological assays are employed to determine proper cellular morphology and 3D architecture (Figure 8) together with staining of cell-specific markers (Figures 9A–9D). The inclusion of distinct cell types from metastatic target organs can mimic distinct TMEs while the different growth and migration behavior of certain cell types may give important clues to explain drug responses in 3D MMSs, which are more closely reflecting *in vivo* scenarios.

Establishment of co-cultures with PBMCs further increases the level of biological complexity of our model. A panel of antibodies (Table 2) permits the characterization of different PBMC populations

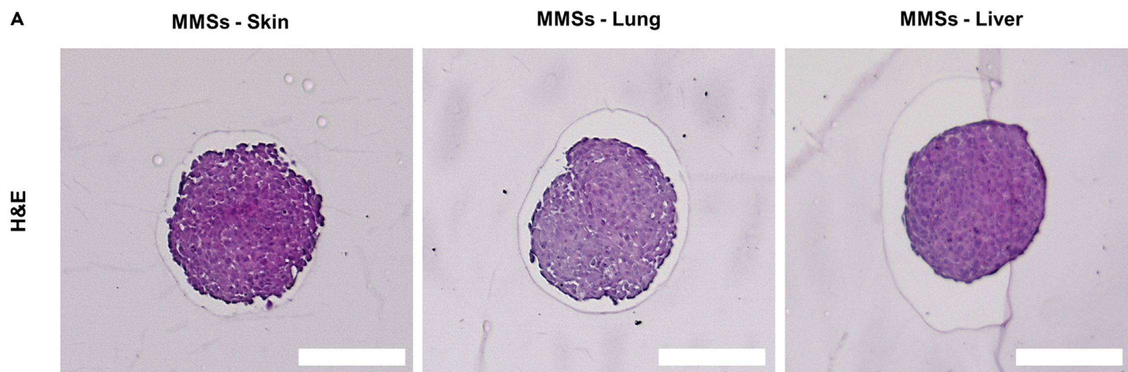

Figure 8. Hematoxylin & Eosin staining of MMSs fixed in formalin and embedded in paraffin (FFPE)
MMSs-skin, -lung, and -liver structure and cellular architecture by Hematoxylin and Eosin (H&E) staining. Objective 20×. Scale bar = 200 μm.

co-cultured with MMSs (Figure 5A). Infiltration of immune cells can be confirmed through flow cytometry of dissociated MMSs (Figures 5B and 5C) providing important information on the involvement of the immune compartment for specific drug responses, including combinations of targeted drugs with immune checkpoint inhibitors or other immune-modulating treatments.

To further increase the flexibility and usability of our models, we have set up several readout assays for kinetic (Figures 6A–6C) and endpoint analysis of cell viability (Figures 10A–10C) and the occurrence of both apoptosis and necrosis (Figure 7B), enabling a detailed evaluation of drug efficacy. Of note, all readout assays can also be applied to mono-component spheroids that only consist of melanoma cells from different genetic backgrounds (such as BRAFmut, NRASmut, and other genetic subtypes) if results need to be obtained faster and only in the cancer cell at hand without the influence of surrounding cell types. Furthermore, if the effect of extracellular matrix needs to be considered, MMSs can also be embedded in the appropriate matrix. We have successfully established protocols to embed multicomponent 3D models in hydrogel.

In conclusion, MMSs are a cost-effective and versatile 3D *in vitro* model for evaluating the efficacy of known or novel drugs or drug combinations. This model incorporates crucial cancer-TME interactions, which influence drug responses, better mirroring the physiological conditions found in early-stage and late-stage metastatic melanoma.

LIMITATIONS

While the application of multicomponent scaffold-free 3D spheroid *in vitro* models offers the opportunity to test novel or newly combined compounds for the treatment of melanoma patients in which cancer cells can interact with cells of the TME, some limitations apply.

For the establishment of this model, commercially available melanoma and stromal cell lines were used. These cell lines exhibit lower heterogeneity compared to patient-derived cells. Hence, the model requires further adaptation when melanoma cells isolated from patient-derived tumors are co-cultured with isolated stromal cells.

For specific readout assays, cells require fluorescent labeling. This might be more difficult to achieve in patient-derived primary melanoma cells compared to established cell lines.

The TME is composed of a vast variety of stromal and immune cells. As a proof of concept, this protocol includes only few stromal cell types such as endothelial cells, fibroblasts/HSCs and PBMCs. Other cell types of interest can be easily substituted paying attention to a potential need for cell type specific media. Immune cell compartments are here portrayed by PBMCs isolated from healthy

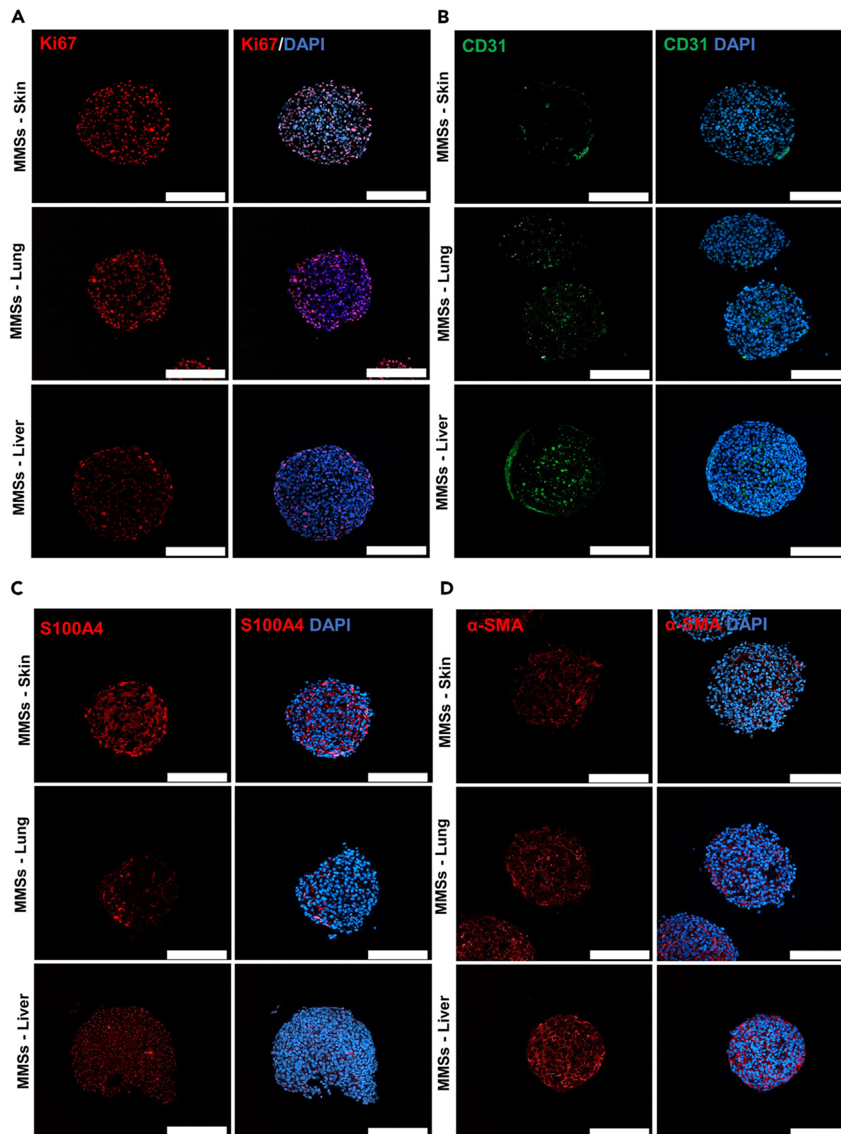

Figure 9. Immunofluorescence staining of MMSs fixed in formalin and embedded in paraffin (FFPE)
Immunofluorescence staining of skin-, lung- and liver-MMSs by (A) Ki67 for cell proliferation, (B) CD31/PECAM-1 for endothelial cell differentiation, (C) S100A4 for melanoma cells identification and (D) α-Smooth Muscle Actin (α-SMA) as a marker for fibroblasts/HSCs activation. Objective 20×. Scale bar = 200 μm.

donors, likely not exhibiting matching HLAs with the melanoma cells used for this protocol. When using patient-derived material, we recommend using PBMCs isolated directly from blood of the same patient, as this approach would provide matching HLAs, which might be relevant for a physiological immune response against the tumor cells when applying immunotherapy.

For this protocol, a single cellular ratio has been chosen for all three distinct melanoma metastasis models (1:3:3). However, depending on the stage of the tumor, the anatomical site, the proliferation rates of TME and cancer cells, these ratios can vary considerably and are expected to dynamically change over time. Therefore, a chosen ratio can only provide a snapshot of a given *in vivo* situation.

The above protocols evaluate the efficacy of tested compounds, primarily focusing on the effect on melanoma cells. Highly proliferative melanoma cells tend to overgrow fibroblasts and endothelial

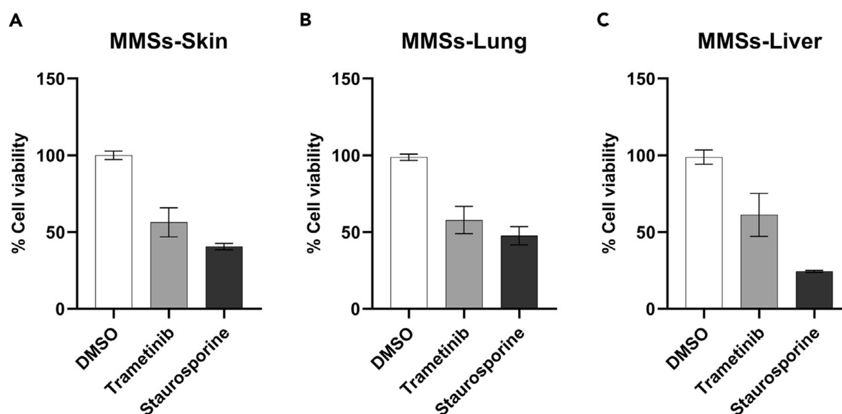

Figure 10. MMSs cell viability
MMS cell viability is assessed using an end-point luminescence-based assay (3D Cell Titer Glo) after 96 h. The tested compound (Trametinib 500 nM), vehicle (DMSO 0.1%) and positive control (Staurosporine 1 μM) are represented for MMSs-skin (A), -lung (B) and -liver (C). Data are mean ± SEM of *n* = 3 independent biological replicates.

cells over time, which reflects the physiological behavior of melanoma tumors. Further adaptations are required to evaluate the effects of test compounds on the TME cells, which would additionally provide an indication of the toxicity of the drug at hand.

TROUBLESHOOTING

Problem 1

Sphere formation issues (During steps 3–5).

Potential solutions

- Use U-bottom ultra-low attachment (ULA) microplates indicated in the Key Research Table. Batches of plates sometimes differ. Try a different batch or provider if problems persist. We recommend the use of U-bottom ULA microplates as outlined in the Key Research Table (KRT).
- Ensure correct and homogeneous mixing of cell solutions before seeding. This reduces the occurrence of incorrect melanoma and endothelial cell ratios per well.
- Ensure the correct centrifugation speed. Incorrect centrifugation speed can either impair cell viability or reduce cell aggregation.
- Melanoma cells (established cell lines and patient-derived cells) have various propensities to grow in 3D displaying lose aggregates to very compact round spheroids. Try different melanoma cells when 3D morphologies are not satisfactory.

Problem 2

PBMCs low viability after thawing (During steps 7–15).

Potential solutions

- Ensure a correct freezing process of PBMCs.
- Precise and quick thawing process reduces exposition time of PBMCs to freezing medium.
- Test PBMCs from a different donor.

Problem 3

PBMC hyperactivation (During step 15).

Potential solutions

- Use PBMCs isolated from a different donor.
- Use HLA-matched PBMCs that can be commercially purchased or use blood from same patient, if patient-derived melanoma cells are used.

Problem 4

A fraction of MMSs can be lost or damaged during the collection for histological and cellular analysis for two main reasons. First, spheroids can stick to the wall of the plastic tips and serological pipets during pipetting. Second, vigorous pipetting might damage the spheroids, which makes them unfit for histological assays (During steps 16, 23, and 37)

Potential solutions

- Pre-wash with PBS 1× the tips used for collecting and transferring the spheroids, which reduces the occurrence of sticky spheroids.
- Pre-coat the tips with pipetting up and down 1 mL of FBS. FBS coating minimizes adhesion of spheroids to plastic surfaces.
- Wash the tips with PBS 1× by pipetting up and down to remove sticky spheroids from the wall of the tips.
- Perform all pipetting steps carefully and with constant movements and treat 3D spheroids gently to avoid damage.

Problem 5

Several problems can be encountered during the embedding and cutting of MMSs. Specifically, breakage of agarose blocks might occur during transferring steps due to their soft consistency. Moreover, incorrect placement of spheroids in agarose and the embedding of agarose blocks into a paraffin cassette may result in unevenly sectioned MMSs (During step 17).

Potential solutions

- Adopt proper and standard procedures for histology staining practices.
- Gently transfer agarose blocks ensuring that all material is prepared and close by.
- Ensure an optimal positioning of spheres in the agarose by gently moving the MMSs with a fine needle.
- Ensure an optimal positioning of the agarose blocks into the paraffin cassettes.

Problem 6

Lack of signal from immunofluorescence staining (During steps 18, 20, and 21).

Potential solutions

- Ensure correct and complete deparaffinization using proper clearing solution. We recommend the use of xylene substitute (see KRT), which has a lower evaporation point than xylene and a less disturbing smell.
- Ensure to use the correct ARS specific for the antibodies used.
- Ensure antibodies have been stored correctly. Possibly, change primary antibodies trying another provider or a different antibody and/or lower dilutions.
- Using different cells and different antibodies, ensure that the antigens/markers of choice are present on the cells.
- Use the appropriate secondary conjugated antibodies ensuring the correct species-specificity and avoid overlapping the conjugate fluorophores emission wavelengths in case of multiplex immunofluorescence.

- Set up proper lasers and filters for confocal microscopy and ensure that settings match the chosen fluorophores.

Problem 7

Low MMS dissociation efficacy for the generation of single cell solutions for flow cytometry analysis (During steps 23 and 37).

Potential solutions

- Efficacy of spheroids dissociation into single cells is affected by cell types and experimental conditions. Under different conditions, cells might produce higher amounts of ECM, such as collagen. In that case, we suggest adding specific ECM-proteases (i.e., collagenase) to increase the efficacy of MMS dissociation or by optimizing the ratio of TrypLE Express and Accutase mix.
- Pay attention to excessive exposition of MMSs to dissociation solution, which might reduce cell viability.

Problem 8

Clogging of the flow cytometer's sample line due to poor dissociation of MMSs or low quantity of single cell events after the doublet exclusion step in data acquisition. (During steps 24 and 37).

Potential solutions

- Ensure proper dissociation of MMSs. In case of using cells producing large amounts of extracellular matrix, collagenase IV at a concentration of 1 mg/mL can be used to improve MMS dissociation.
- Do not skip the straining step, it is crucial to obtain a proper single cell suspension.

Problem 9

No signal detected from conjugated antibodies in FACS (During step 24).

Potential solutions

- Ensure use of proper, flow cytometry validated antibodies. Titration of antibodies is necessary in case of using different antibodies/flow cytometer than suggested in this protocol.
- Re-do the compensation matrix and adjust the lasers' voltage.

Problem 10

Spillover of 3D Cell Titer Glo and medium mix solution from the wells during orbital shaking (During step 36).

Potential solution

- Optimize a proper orbital shaking force for the type of plate, volume dispensed per well and type of MMSs to have optimal sphere lysis and to prevent solution spillover from well to well.

Problem 11

High background staining in confocal microscopy images (During step 21).

Potential solution

- Optimize antibody concentrations and incubation times.
- Use a negative control with only secondary antibody to detect unspecific binding and optimize the concentration of the secondary antibody.

- Decrease the setting of the laser or/and the detector (laser power, gain).
- Extend the washing times or increase stringency of washing buffers by adding detergent (for instance Triton X-100, Tween 20).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Stephanie Kreis (stephanie.kreis@uni.lu).

Technical contact

Further technical information and requests should be directed to and will be fulfilled by the technical contacts, Cristian Angeli (cristian.angeli@uni.lu), Dr. Joanna Patrycja Wroblewska (joanna.wroblewska@uni.lu), and Eliane Klein (eliane.klein@uni.lu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

C.A. conceptualized and designed the MMSs model. C.A., J.P.W., and E.K. designed, performed the experiments, and analyzed the data. C.A., J.P.W., and E.K. wrote the manuscript. S.K. and C.M. edited the manuscript. S.K. acquired the fundings and supervised the whole study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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