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Monitoring cancer cell invasion and T-cell cytotoxicity in 3D culture

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TITLE:
Monitoring Cancer Cell Invasion and T-Cell Cytotoxicity in 3D Culture

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KEYWORDS:
3D cell culture, cancer cells, co-culture, collagen, cytotoxicity, invasion, immunofluorescence, immunohistochemistry, t-cells, tumor spheroid

SUMMARY:
The presented approach simultaneously evaluates cancer cell invasion in 3D spheroid assays and T-cell cytotoxicity. Spheroids are generated in a scaffold-free agarose multi-microwell cast. Co-culture and embedding in type I collagen matrix are performed within the same device which allows to monitor cancer cell invasion and T-cell mediated cytotoxicity.

ABSTRACT:
Significant progress has been made in treating cancer with immunotherapy, although a large number of cancers remain resistant to treatment. A limited number of assays allow for direct monitoring and mechanistic insights into the interactions between tumor and immune cells, amongst which, T-cells play a significant role in executing the cytotoxic response of the adaptive immune system to cancer cells. Most assays are based on two-dimensional (2D) co-culture of cells due to the relative ease of use but with limited representation of the invasive growth phenotype, one of the hallmarks of cancer cells. Current three-dimensional (3D) co-culture systems either require special equipment or separate monitoring for invasion of co-cultured cancer cells and interacting T-cells.

Here we describe an approach to simultaneously monitor the invasive behavior in 3D of cancer cell spheroids and T-cell cytotoxicity in co-culture. Spheroid formation is driven by enhanced cell-

cell interactions in scaffold-free agarose microwell casts with U-shaped bottoms. Both T-cell co-culture and cancer cell invasion into type I collagen matrix are performed within the microwells of the agarose casts without the need to transfer the cells, thus maintaining an intact 3D co-culture system throughout the assay. The collagen matrix can be separated from the agarose cast, allowing for immunofluorescence (IF) staining and for confocal imaging of cells. Also, cells can be isolated for further growth or subjected to analyses such as for gene expression or fluorescence activated cell sorting (FACS). Finally, the 3D co-culture can be analyzed by immunohistochemistry (IHC) after embedding and sectioning. Possible modifications of the assay include altered compositions of the extracellular matrix (ECM) as well as the inclusion of different stromal or immune cells with the cancer cells.

INTRODUCTION:

Despite significant improvements in cancer immunotherapy over the past decade, our mechanistic understanding of sensitivity and resistance to treatments are still fairly poor[1]. It is well-established that tumors display substantial heterogeneity, and that the dynamic interactions of the tumor cells with their microenvironment as well as with the immune cells, impact tumor cell death, invasive behavior and response to treatments that include immunotherapy[1-3]. As one arm of the adaptive immune system, T-cells execute cell-specific cytotoxicity. The analysis of T-cell recognition and response to cancer cells provides mechanistic insights into resistance and sensitivity to immune modulatory treatments.

In vitro modeling and monitoring interactions between cancer and T-cells in an appropriate environment has been challenging and so far, resulted in limited mechanistic insights. Most cell-based assays rely on a two-dimensional (2D) environment, that lacks key features that are critical for recapitulating the three-dimensional (3D) in vivo physiology[4-6], namely spatial cell-cell interactions, contact with the extracellular matrix (ECM)[7], dynamic metabolic demand, increased hypoxia due to mass growth[8], and effects of the tumor microenvironment (TME)[9]. On the other hand, there are still a number of shortcomings with the currently used three-dimensional (3D) co-culture and invasion assay systems: (1) the time consuming nature of spheroid generation and harvest[5,10], (2) the lack of control over spheroid size, shape and cell density[11-12], (3) the low-throughput type assays, (4) the requirement for special equipment[13-14], (5) the need to transfer the co-culture into distinct environments for different assays[15-17]. In particular, transferring of a co-culture assay often leads to disruption of spheroids and loss of the co-culture integrity. This applies especially for “loose” spheroids with reduced cell-cell adhesion. For example, most 3D invasion assays require that spheroids are harvested after their initial formation and then resuspended in ECM[14-16]. This resuspension step results in a loss of control over the distance between spheroids. Since distance between tumor spheroids impacts their invasive behavior, this loss of control introduces high inter-assay variance and reduces the reproducibility. Furthermore, the application of cell fractionation assays by consecutive centrifugation steps for assessment of the peripheral and tumor spheroid infiltrating immune cells is limited to tumor cell populations that generate more stable spheroids[17].

Concept and approach

Our approach addresses the above-mentioned deficiencies using an "All-in-One"—3D spheroid co-culture model, which does not require the transfer of spheroids for subsequent assays. We adapted a spheroid formation device (see **Table of Materials**) to generate an assay for simultaneously monitoring invasive behavior of cancer cells and cytotoxicity of co-cultured T-cells. This method is user-friendly, inexpensive and allows for quick and easy handling in a relatively high-throughput 3D setting. Dependent on the type of device used, up to 81 large uniformly-sized spheroids can be generated in a single pipetting step with control over the individual spheroid size by modifying the number of cells seeded. Spheroid formation is forced by enhanced cell-cell interactions in scaffold-free agarose multi-well casts with U-shaped bottoms. We adapted this 3D system for dynamic cell-based functional studies as well as endpoint molecular and biochemical assays that include fluorescence activated cell sorting (FACS), immunofluorescence (IF) or immunohistochemistry (IHC) staining as well as gene expression analysis of the intact 3D co-culture.

For **functional studies**, embedding spheroids in type I collagen within the agarose casts results in invasion of cancer cells from equidistant spheroids and permits monitoring essential cell line-specific features, such as single cell vs. collective cell migration[18,19]. Furthermore, the collagen matrix is easily separated from the agarose cast, resulting in a 1–2 mm thick patch containing multiple spheroids, which can be further processed for IF-staining and imaging by confocal microscopy. This can reveal distinct cell invasion and cell-matrix interactions in a high-throughput screening. Also, cells in the collagen matrix can be isolated after collagen digestion and single cell dissociation for subsequent cell cultivation or analysis.

For **IHC analysis of spheroids**, after fixation and sectioning of the agarose cast, proteins or other molecules of interest are detectable whilst maintaining the geographic positions of the spheroids. In the approach described here, spheroids are directly embedded in Hydroxyethyl agarose processing gel within the agarose cast and the gel serves as a "lid" to retain the spheroids at the bottom of the microwells. After paraffin embedding of the agarose cast [20], serial horizontal sectioning is performed with the bottom of the cast serving as the starting point.

This approach contrasts with conventional IHC sectioning of spheroids that requires harvesting of cells before embedding in Hydroxyethyl agarose processing gel[21] and risks disruption of spheroids thus losing the spatial arrangement of cells. Also, cell fractionation by centrifugation for assessing whether immune cells infiltrated or remained peripheral to tumor spheroids[17] is avoided by direct embedding.

Furthermore, 3D co-culture can be performed by admixing tumor, stromal or immune cells, and thus studying tumor cell crosstalk or recapitulating different tumor microenvironments for analyzing cell-cell interactions including co-cultures with endothelial cells[16].

This 3D spheroid co-culture setting can be used to perform co-culture of different cell types present in the tumor microenvironment and to assess the effects of altered ECM elements. Besides type I collagen, other ECM components (e.g., matrigel, matrigel/collagen mixtures, fibronectin), can be used since tumor cell invasion is impacted by the abundance of different

substrates[22]. Also, the microwells of the agarose cast are suitable for spheroid formation of primary cell lines and for cells with low cell-cell adhesion.

PROTOCOL:

A list and explanation of some frequently used words throughout the protocol can be found in **Supplementary File 1**.

1. Generation of spheroids

1.1) Prepare and autoclave 2% agarose in 1x PBS (e.g., 1 g agarose in 50 mL of 1x PBS) and autoclave 35- and 81-microwell rubber molds.

CAUTION: Avoid using low-melting agarose for generating the agarose casts for IHC processing.

1.2) Prepare agarose casts

NOTE: 35-microwell casts are used for invasion, immunofluorescence (IF) and cell isolation assays. 81-microwell casts are used for cytotoxicity, immunohistochemistry (IHC), cell isolation and RNA extraction assays.

1.2.1) After the agarose has been autoclaved, let molten agarose cool to about 60–70 °C. In a cell culture hood, use aseptic technique and pipette 500 µL of molten agarose into an 81-microwell rubber mold per well or 330 µL into a 35-microwell rubber mold per well.

CAUTION: Avoid creating bubbles while mixing or pipetting agarose. Remove any bubbles by gently pipetting.

1.2.2) After the agarose is solidified, carefully flex the rubber mold to remove the agarose cast from it. Hereby, place hands above the appropriate well-plate and let the agarose cast drop right into one well of the well-plate.

NOTE: Flex the rubber mold at different positions in order to avoid over flexing. It might be helpful to flex the rubber mold and push gently from the bottom at the same time.

1.2.3) To equilibrate the agarose casts, add cell culture medium, consisting of DMEM supplemented with 10% fetal bovine serum (2.5 mL/well for 12-well plate and 1 mL/well for 24-well plate). Put the well-plate into a cell culture incubator (37 °C, 5% CO_2) and incubate for 1 h.

1.3) Meanwhile, prepare cell culture for seeding.

NOTE: The total cell seeding number per agarose cast is to be decided by the investigator. For murine primary pancreatic cancer cell lines, 35,000 cells/35-microwell cast and 81,000 cells/81-microwell cast (in average 1000 cells/spheroids) are seeded for all following assays. The average diameter of a spheroid is about 150–200 µm after 48 h.

1.4) Remove cell culture medium surrounding the agarose cast with a P1000 pipette first by tilting the well-plate. Then carefully remove the medium in the seeding chamber.

1.5) Carefully seed the prepared tumor cell suspension drop-wise into the cell seeding chamber. Carefully put the well-plate back into the cell culture incubator (37 °C, 5% CO_2) for 15 min.

NOTE: During this step the cells will settle into the microwells of the agarose cast.

1.6) Add additional medium to the outside of the agarose cast (2.5 mL/well for 12-well plate and 1 mL/well for 24-well plate).

1.7) Put the well-plate back into the cell culture incubator (37 °C, 5% CO_2) for 48 h.

NOTE: Usually it takes up to a few hours for the cells to form spheroids in the microwells. In general, solid tumor cell spheroids are formed after 24 h and are stable after 48 h. However, this can vary between different cell lines. If necessary, change cell culture medium by carefully tilting the well-plate with the agarose casts and removing the surrounding cell culture medium with a P1000 pipette. Then carefully add fresh medium by pipetting it along the wall of the well. Usually it is not necessary to change the media within the casts due to its small volume and the risk of removing the spheroids.

2. Co-culture with T-cells

2.1) Resuspend the required number of T-cells in 75 µL (35-microwell cast) or in 190 µL (81-microwell cast) of the appropriate T-cell culture medium (RPMI supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin).

2.2) Remove the cell culture medium surrounding the agarose cast with a P1000 pipette first by tilting the well-plate. Then slowly and carefully remove the medium within the cast by gently targeting one corner of the seeding chamber with a P200 pipette while keeping the well-plate tilted with the other hand.

2.3) (Critical step 1) As there is a risk of removing spheroids, control for loss of spheroids by comparing the number of spheroids within the microwells before and after removing the medium by viewing under the microscope at 10x magnification.

2.4) Carefully seed the T-cell suspension drop-wise into the seeding chamber of the agarose cast by holding the P200 pipette about 0.5 cm above the cast.

2.5) (Critical step 2) There is a risk of flushing out the spheroids while adding the T-cells. Therefore, it is critical to add the T-cells very slowly and about 0.5 cm above the seeding chamber.

NOTE: One possibility to decrease the number of flushed out spheroids is by pointing the pipette to one corner of the seeding chamber while adding the T-cells, thus only risking the spheroids in this corner to be flushed out.

2.6) Carefully place the well-plate back into the cell culture incubator (37 °C, 5% CO_2) for 15 min.

2.7) Take the well-plate out from the incubator and add fresh cell culture medium (RPMI supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin) around the agarose cast by slowly pipetting it along the wall of the well.

2.8) Put the well-plate back into the cell culture incubator **for 48 h**.

3. Embedding of 3D co-culture into type I collagen matrix

3.1) Prepare neutralized type I collagen

3.1.1) Dilute the stock collagen with serum free base medium (RPMI) to a final working concentration of 3 mg/mL. For every 100 µL of diluted collagen, add 11 µL of 10x PBS and 1.2 µL 1 M sodium hydroxide (NaOH).

3.1.2) Keep on ice and incubate (neutralize) for 1 h.

3.2) Remove the cell culture medium surrounding the agarose cast with a P1000 pipette first by tilting the well-plate. Then, slowly and carefully remove the medium within the cast by gently targeting one corner of the seeding chamber with a P200 pipette while keeping the well-plate tilted with the other hand.

NOTE: Here, it is critical to completely remove the cell culture medium surrounding the agarose cast.

3.3) Carefully pipette the neutralized collagen I mix drop-wise into the seeding chamber of the agarose cast by holding the P200 pipette about 0.5 cm above the cast.

3.4) Put the well-plate immediately into the cell culture incubator for 4 min (35-microwell cast) or 5 min (81-microwell cast).

CAUTION: (Critical step 3) It is critical to strictly keep to the given incubation time. Otherwise, the invasive behavior might not be reproducible.

3.5) Invert the well-plate and leave it flipped in the incubator for 1 h.

NOTE: The casts will stay attached to the bottom of the well-plate due to surface tension. In case special cell culture medium with high serum concentration (e.g., RPMI supplemented with 20%

fetal bovine serum) is used, an additional washing step with 1x PBS after removing the medium in the well might be necessary to increase surface tension.

3.6) Take the well-plate out from the incubator and invert it back. Add fresh cell culture medium (RPMI supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin) around the agarose cast by slowly pipetting it along the wall of the well.

3.7) Put the well-plate back into the cell culture incubator for 48 h.

4. Cytotoxicity assay

4.1) Prepare 3 mL of 1x PBS with 2% fetal bovine serum per agarose cast.

4.2) After co-culture for 4 d, remove the cell culture medium surrounding the agarose cast with a P1000 pipette by slightly tilting the well-plate with the other hand.

NOTE: The total time period of co-culture is to be decided by the investigator.

4.3) Distribute 1 mL of 1x PBS + 2% FBS with a P1000 pipette into the seeding chamber to dislodge the spheroids from the microwells.

4.4) Repeat step 4.3 twice with the volume in the well and transfer it into a 15 mL tube.

4.5) Centrifuge the tube at 300 x *g* for 10 s at room temperature (RT).

4.6) Carefully remove the supernatant with a P1000 pipette.

4.7) Wash the cells by adding 1 mL of 1x PBS with 2% FBS and centrifuge at 300 x *g* for 1 min at RT.

4.8) Repeat the washing step (step 4.7).

4.9) Remove the supernatant and add 1 mL of cell dissociation enzymes solution (see **Table of Materials**).

4.10) Use a P200 pipette and pipette up and down to break the cell clusters.

4.11) Incubate the cells for 20 min in the cell culture incubator (37 °C, 5% CO_2).

4.12) Repeat step 4.10.

NOTE: Take ~10 µL out and seed on a cell culture dish to observe (under the microscope at 10x magnification) how well the spheroids have dissociated in single cells. If necessary, add 5 min of further incubation.

4.13) Add 4 mL of full cell culture medium (RPMI supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin) and mix by inverting the tube 3–4 times.

4.14) Centrifuge at 400 x *g* for 4 min at RT.

4.15) Remove the supernatant and resuspend the cells in FACS buffer for Annexin V staining of apoptotic cells.

NOTE: From here on any FACS staining and cell analysis can be performed.

5. Hydroxyethyl agarose processing gel embedding for IHC sectioning

NOTE: Here it is critical to avoid using low-melting agarose for generating the agarose casts.

5.1) At the end of the co-culture, remove the cell culture medium surrounding the agarose cast with a P1000 pipette first by tilting the well-plate. Then slowly and carefully remove the medium within the cast by gently targeting one corner of the seeding chamber with a P200 pipette while keeping the well-plate tilted with the other hand.

5.2) Slowly pipette 10% formalin first in the seeding chamber by gently targeting one corner of the seeding chamber with a P200 pipette. Then add 10% formalin to the outside of the agarose cast until it is completely covered. Fix the agarose cast in 10% formalin for 1 d.

5.3) Remove the formalin on the next day.

5.4) Carefully pipette 210 µL (81-microwell cast) or 100 µL (35-microwell cast) of pre-warmed and liquefied hydroxyethyl agarose processing gel (see **Table of Materials**) drop-wise into the seeding chamber of the agarose cast by holding the P200 pipette about 0.5 cm above the cast.

5.5) Let the hydroxyethyl agarose processing gel solidify for 10 min at RT.

5.6) Transfer the agarose cast to 1x PBS.

NOTE: For longer storage, embed the cast in 70% ethanol to prevent contamination.

5.7) Dehydrate the gel sections through an ethanol series (1 h each: 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol [3x changes each]). Then clear in a clearing solution 3x for 1 h each (e.g., aliphatic hydrocarbons [e.g., Clearite], or xylenes), and infiltrate with molten paraffin (3x for 1 h each), either manually or in a tissue processer. Embed the cast in paraffin wax for subsequent sectioning at 5 µm per section.

5.8) Remove wax by putting slides in xylenes, 3x for 3 min each, then rehydrate tissue sections through a graded alcohol series: 100% ethanol for 2 min, 95% ethanol for 1 min, 80% ethanol for 30 s and 70% ethanol for 30 s, then place in water.

5.9) Perform heat induced epitope retrieval in a vegetable steamer at 100 °C for 20 min, followed by 20 min of cooling in a 10 mM sodium citrate pH 6.0 solution and block endogenous peroxidases with H_2O_2.

5.10) Block the sections with normal goal serum and expose them to anti-CD8 antibody (dilution 1/25) overnight.

5.11) Apply anti-rabbit-HRP conjugated secondary antibodies (see **Table of Materials**) and develop the staining using 3,3'-Diaminobenzidine (DAB) chromagen. Counterstain nuclei with an 11% Harris hematoxylin solution (see **Table of Materials**).

6. Monitoring and analyzing spheroid invasion in co-culture

NOTE: The time-point of imaging spheroid invasion into the collagen I matrix is to be decided by the investigator. Acquire cell culture images using an inverted microscope with 10x magnification. The ideal time-point is dependent on the cell line being tested, as well as the ECM component. More invasive cell lines will begin to spread into the collagen within a few hours after adding the collagen. Since the T-cells in the co-culture might prevent a full view on the egress from the spheroids at very early time-points, generally images are taken at 0 h (as reference), 24 h and 48 h after adding the collagen.

6.1) Quantitate invasiveness by manually counting the number of “spikes” coming out from the spheroid and/or using image analysis software, e.g., Image J.

6.1.1) Analyze invasion as the number of “spikes” from the spheroid by manually counting the number of “spikes” of a spheroid.

NOTE: It is to be decided by the investigator which protrusions from the spheroids are considered as “spikes”. A decision criterion could be the length of the “spike” measured from the edge of the spheroid.

6.1.2) Analyze invasion as the invasion area relative to the size of the spheroid.

6.1.2.1) Use the **Freehand Draw Tool** (Image J) to trace the border of the total area (invasion + spheroid area).

6.1.2.2) Click **Analyze** on the top menu, then click **Measure** to display the area measurement.

6.1.2.3) Trace the border of the total spheroid area.

6.1.2.4) Click **Analyze** on the top menu, then click **Measure** to display the area measurement.

6.1.2.5) Copy the measured results list into a spreadsheet and calculate the total invasion/spheroid by using the formula: total invasion = total area/spheroid area.

7. Immunofluorescence staining

7.1) Prepare the following.

7.1.1) Prepare 5.4% formalin (5 mL) using 2.3 mL of 1x PBS and 2.7 mL of 10% formalin.

7.1.2) Prepare 0.5% Octoxynol (50 mL) using 50 mL of 1x PBS and 2.5 mL of 10% Octoxynol (store at RT).

7.1.3) Prepare IF Buffer (200 mL) using 200 mL of 1x PBS, 200 mg bovine serum albumin (BSA), 4 mL of 10% Octoxynol, 1 mL of 10% polysorbate 20 (warm up to RT before use; filter and store at 4 °C).

7.1.4) Prepare blocking solution (5 mL) using 5 mL IF Buffer and 500 µL of goat serum (warm up to RT before use).

7.1.5) Keep 8-well chamber slides, glass coverslips, and mounting media ready.

7.2) Day 0

7.2.1) Fix the whole agarose cast, including the spheroids, overnight in 5.4% formalin at RT in a humidified chamber.

7.3) Day 1

7.3.1) Remove the cell culture medium surrounding the agarose cast with a P1000 pipette by tilting the well-plate.

7.3.2) Transfer the collagen patch into an 8-well chamber slide by grasping a corner of the collagen matrix within the seeding chamber of the agarose cast with sleek pointed tip tweezers and peel it off the agarose cast in a single, confident movement.

NOTE: The collagen patch should be easily separated from the agarose cast. Otherwise, use a P1000 pipette to carefully add culture medium in the area of the seeding chamber or invert the agarose cast and gently shake the well-plate to dislodge the collagen matrix.

7.3.3) Add 250 µL of 0.5% Octoxynol (see **Table of Materials**) per well. Incubate for 1 h at RT.

7.3.4) Aspirate the Octoxynol and add 250 µL of blocking solution per well. Block for 1 h at RT.

7.3.5) Meanwhile, dilute the primary antibody (dilution for anti-keratin 8: 1/500; Phalloidin 546: 1/200; Hoechst: 1/1000) in the blocking solution, calculating for 250 µL per well.

7.3.6) Add the primary antibody in blocking solution and place the chamber slide in a humidified chamber for overnight incubation at RT.

7.4) Day 2

7.4.1) Remove the primary antibody in blocking solution by aspirating.

7.4.2) Wash 3 times by adding 300 µL of IF Buffer and let it sit for 5 min at RT.

NOTE: Just let it sit, there is no need to put it on a shaker.

7.4.3) Dilute the secondary antibody in blocking solution (for anti-keratin 8: anti-rat, dilution 1/500).

7.4.4) Add 250 µL of secondary antibody in blocking solution to each well and incubate for 1 h at RT. From here on, protect the samples from light.

7.4.5) Remove the secondary antibody in blocking solution by aspirating.

7.4.6) Wash 3 times with 300 µL of IF Buffer by adding and let it sit for 5 min at RT.

7.4.7) Rinse the samples with 1x PBS and aspirate it.

7.4.8) Carefully remove the walls of the chamber slide so that only the glass slide at the bottom remains.

7.4.9) Add at least 200 µL of mounting media to a glass coverslip and slowly drop the coverslip over the sample.

NOTE: Avoid creating bubbles as this will impact the image quality. Bubbles can be prevented by gently place the cover slip on the long edge of the slide at a 45° angle or slowly lowering the cover slip on the slide.

7.4.10) Put the mounted samples in a dark and dry place and let it sit overnight. Imaging can be performed on the following day.

NOTE: The coverslip might initially shift a bit once placed over the collagen patch. Let the glass slide sit on an even area for a few minutes. The coverslip will flatten out the sample so that there will be no gap left between the glass slide and coverslip over time.

8. Isolation of cells from the collagen matrix

8.1) Prepare the following.

8.1.1) Prepare 1 mg/mL collagenase 4 in serum-containing cell culture medium (store aliquoted collagenase 4 dilutions at -20 °C)

8.1.2) Prepare 2.5% BSA in 1x PBS and coat all tubes and pipette tips with it (filter BSA solution before use).

8.1.3) Prewarm the BSA solution and cell culture medium before use.

8.2) Ready 2 mL of collagenase 4 solution (1 mg/mL) to digest a maximum of three collagen matrices from 35-microwell agarose casts.

8.3) Transfer up to three 75 µL collagen matrices from 35-microwell agarose casts into a 2.5% BSA pre-coated 15 mL tube. Grasp a corner of the collagen matrix within the seeding chamber of the agarose cast with sleek pointed tip tweezers and peel it off the agarose cast in a single, confident movement.

8.4) Bevel-cut the tip of a P1000 tip with scissors. Pre-coat the remaining tip with 2.5% BSA and break down the collagen matrix as much as possible by pipetting up and down. Incubate the tube for 15 min at 37 °C.

8.5) (Critical step 4) At every 5 min, check whether the collagen matrix has dissolved. Pipette up and down again before taking 10 µL of the sample out and seed it on a cell culture dish for observing under the microscope at 10x magnification. Add another 5 min if necessary.

8.6) Fill up the tube with 10 mL of prewarmed cell culture medium (DMEM supplemented with 10% fetal bovine serum). Gently mix by inverting the tube 3–4 times.

8.7) Centrifuge the tube at 400 x *g* for 4 min at RT.

8.8) Carefully remove the supernatant and leave 2 mL of volume.

8.9) (Critical step 5) Leave 2 mL after the first centrifugation step as there might still be undissolved collagen at the bottom of the tube carrying cells along them.

8.10) Perform two washing steps by adding 10 mL of serum-containing cell culture medium each time. Centrifuge each time at 400 x *g* for 4 min at RT.

8.11) After the last washing step, remove as much medium as possible and leave just the cell pellet.

8.12) Resuspend the cell pellet in 1 mL of cell dissociation enzymes solution (see **Table of Materials**).

8.13) Use a P200 pipette and pipette up and down to break the cell clusters.

8.14) Incubate the cells for 20 min in the cell culture incubator (37 °C, 5% CO_2).

8.15) Repeat step 8.13).

NOTE: Take ~10 µL out and seed on a cell culture dish to observe under the microscope at 10x magnification to view how well the spheroids have dissociated into single cells. If necessary, add 5 min of further incubation.

8.16) Add 4 mL of cell culture medium (DMEM supplemented with 10% fetal bovine serum) and mix by inverting the tube 3–4 times.

8.17) Centrifuge at 400 x *g* for 4 min at RT.

8.18) Remove the supernatant. From here onwards, FACS staining or cell culture can be performed.

9. RNA extraction from the collagen matrix

9.1) Prepare guanidinium thiocyanate with phenol (see **Table of Materials**), 100% chloroform, 70% RNase-free ethanol, RNA extraction kit (see **Table of Materials**), RNase-free 1.5 mL tubes, RNase-free 15 mL tubes and RNase-free ddH_2O.

9.2) Transfer up to twelve 190 µL collagen matrices from 81-microwell agarose casts into a 15 mL tube. Grasp a corner of the collagen matrix within the seeding chamber of the agarose cast with sleek pointed tip tweezers and peel it off the agarose cast in a single, confident movement.

NOTE: The matrices can also be frozen at -80 °C until ready for RNA extraction.

9.3) Add 1 mL guanidinium thiocyanate with phenol (see **Table of Materials**) to the collagen matrices in the 15 mL tube.

NOTE: The guanidinium thiocyanate with phenol must completely cover the collagen matrices.

9.4) Vortex the tubes for 10–20 s.

9.5) Homogenize the matrices with 20 G needles and 5 mL syringes until they are completely dissolved.

9.6) Let the matrices sit for 5 min at RT.

9.7) Add 200 µL of pure chloroform to the matrices and shake the tube vigorously for 15 s.

9.8) Immediately transfer the mix to 1.5 mL tubes.

9.9) Let the mix sit for at least 5 min at RT until the phases are separated.

9.10) Centrifuge the 1.5 mL tubes at 12,000 x *g* for 15 min at 4 °C.

9.11) Fill a new 1.5 mL tube with 500 µL of 70% ethanol.

NOTE: Dependent on the volume of the aqueous phase after centrifugation (see step 9.12) this might not be 500 µL. The amount of 70% ethanol should be equal to the volume of the aqueous phase.

9.12) Carefully transfer the upper aqueous phase of the centrifuged samples to the 70% ethanol-filled tube and mix thoroughly by pipetting up and down.

9.13) (Critical step 6) Be careful not to disturb the layers at the bottom of the guanidinium thiocyanate with phenol separation while removing the upper phase, as this will contaminate the RNA.

9.14) Add the sample to an RNA extraction column (included in the RNA extraction kit, see **Table of Materials**). From here on, follow the manufacturer's protocol for RNA purification from cells.

REPRESENTATIVE RESULTS:

The 3D co-culture model allows for different assays shown in **Figure 1A**, which can be combined or modified as needed. In our established experimental setup, tumor and T-cells are co-cultured for 2 days followed by initiation of the invasion assay for selection of invasive and/or resistant tumor cells (**Figure 1B**). On day 4 the quantitation of invasion is performed and "survivor" cells are isolated from the collagen matrix or directly processed for RNA[23] or DNA extraction from matrix (**Figure 1B**). Embedding the 3D culture in type I collagen within the microwells of the agarose cast allows monitoring and analysis of invasion by using Image J to first demarcate the total area using the software's freehand draw tool and then calculate the ratio over the demarcated spheroid area (**Figure 2A**), and/or by counting the number of "spikes" leaving the spheroid. Using two primary murine pancreatic cancer cell lines (cell line 1 and cell line 2), different spheroid shapes and invasive behavior were observed and quantified accordingly (**Figure 2B**). Cell line 1 shows a more compact spheroid formation and "spiky" invasion, comparable to single cell invasion, whereas cell line 2 forms more loose spheroids and shows a collective invasion pattern (**Figure 2C**). Co-culture was performed with two different tumor clonal cell lines seeded at the same time (**Figure 3A–E**) to follow their interactions during subsequent assays, and with tumor cells and T-cells upon tumor spheroid formation (**Figure 3F–J**). For detailed assessment of the invasive behavior, immunofluorescent staining was performed (**Figure 4**). After separation of the collagen matrix from the agarose cast and transfer to a glass

slide (**Figure 4A–B**), confocal imaging was performed in a high-throughput manner (**Figure 4C–J**). The size and consistence of the agarose cast allow embedding of the whole 3D culture system in paraffin for serial sectioning and immunohistochemistry (IHC) staining for quantifying the spatial relation between tumor and T-cells (**Figure 5**). T-cells present in the section can be identified and further characterized by cell surface marker staining exemplified here for CD8 (**Figure 5D,E**). T-cells that have infiltrated the tumor spheroid can be counted relative to those that remained in the periphery of the spheroid. **Figure 5D,E** shows examples of distinct infiltration of T-cells into tumor spheroids grown from tumor cell line 1 (**D**) and cell line 2 (**E**). **Table 1** shows typical experimental setups for the assays, and the yield of representative and analyzable samples at the end of the experiment for each protocol.

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow, analyses and timeline of experiments. (**A**) An 81-microwell and 35-microwell rubber mold are filled with 2% agarose in 1x PBS to generate an agarose cast with multiple microwells. Spheroids are formed upon cell seeding into the chambers of the agarose cast in a single pipetting step. Co-culture with T-cells is performed within the same cast. Functional monitoring and potential assays are shown. (**B**) Timeline of experiments. Tumor cells are co-cultured with autologous T-cells for 2 days allowing for a maximum interaction between both cell types. The invasion assay is initiated after two days. Endpoint analyses are performed after further two days to monitor the invasive and survival phenotype of tumor cells as well as the proliferation and survival of T-cells.

Figure 2: Quantification of invasion. Invasion can be quantified by image analysis, e.g., using Image J software and counting the number of "spikes" per spheroid. (**A**) Calculation of the total invasion area as a ratio of total area to the spheroid area. Scale bar: 300 µm. (**B**) Examples of two different primary murine pancreatic cancer cell lines (cell line 1 and 2) in spheroid formation at different magnifications and invasion into type I collagen. (**C**) Analysis of invasion is performed by counting the number of spikes per spheroid (left diagram; error bars: 2.63 for cell line 1, 1.47 for cell line 2) and calculate the invasion area as described (right diagram; error bars: 0.36 for cell line 1, 1.28 for cell line 2).

Figure 3: Co-culture with dye-labeled tumor and T-cells. (**A–E**) Mix of two differently dye-labeled primary murine pancreatic cancer clonal cell lines (green and red) and magnified view of one representative microwell (**B–E**). (**F–J**) Co-culture of pre-labeled tumor (green) and T-cells (red). (**G–J**) Magnified view of one representative microwell shows one tumor–T-cell co-culture upon tumor spheroid formation.

Figure 4: Immunofluorescence staining. Immunofluorescence (IF) staining was performed after separating the collagen matrix from the agarose cast. (**A–B**) After IF staining, the collagen patches are transferred to a glass slide and covered with glass coverslips. (**C–F**) Example of an IF-stained collagen patch including tumor spheroids with (**C**) Hoechst, (**D**) keratin 8, (**E**) phalloidin and (**F**) overlay. Panels (**G–J**) Show the respective magnified view of a single spheroid. Scale bar = 300 µm.

Figure 5: Immunohistochemistry sectioning. The agarose cast with the 3D culture immersed in hydroxyethyl agarose processing gel, was embedded in paraffin, sectioned and processed for immunohistochemistry (IHC) staining. (**A**) Paraffin block used for horizontal sectioning that starts from the bottom of the agarose cast to obtain serial sections of multiple tumor cell/T-cell co-cultures within a single cast; scale bar = 5 mm. (**B**) H&E-stained section of an agarose cast containing 3D co-culture of tumor and T-cells. Scale bar: 1 mm. (**C**) Magnified view of an H&E-stained co-culture within the agarose cast. CD8 staining of T-cells co-cultured with sensitive (**D**) and resistant (**E**) tumor cells. Scale bars in C–E = 200 µm.

Table 1: Typical experimental setup and yield for the protocols. The table shows the typical experimental setup for each protocol and the typical yield of analyzable samples (number of cells, spheroids or RNA concentration) at the end of the experiment, respectively. IHC= immunohistochemistry; IF= immunofluorescence.

DISCUSSION:

The method presented here describes 3D tumor spheroid generation, which allows co-culture with T-cells, cell-based functional and molecular assays, as well as a variety of monitoring and analysis possibilities using a single device. The major advantage of our approach is that it does not necessitate transfer of the 3D culture to a separate assay and maintains the integrity of the 3D culture throughout the assays.

The workflow presented here can be modified as needed. The incubation times for spheroid formation, T-cell co-culture or cytotoxicity assay, may need to be altered for different experimental conditions or cell lines.

There are a few steps throughout the assays, which require close adherence to the protocol. These are in general: removing the cell culture medium before adding a second cell line for co-culture, as well as embedding the 3D culture in ECM or hydroxyethyl agarose processing gel within the agarose casts. It is absolutely critical to slowly and carefully remove the medium of the seeding chamber by tilting the well-plate and targeting one corner of the chamber with a micropipette. As long as the agarose casts contain cells, we recommend to always remove any medium in the well with a micropipette, instead of using a pipettor. Adding a second cell line and embedding in ECM or hydroxyethyl agarose processing gel needs to be performed slowly and drop-wise in order to prevent flushing out the cells in the microwells. The most critical step of the collagen invasion assay is the incubation time before inverting the casts in the well-plate with the wells. Reducing the time might cause the collagen to drop out from the cast, and exceeding the time might cause the culture to be pressed down to the bottom of the microwells, resulting in unevenly distributed spheroid invasion. Inverting the cast enables cells in the microwells to completely submerge in the liquid collagen before it polymerizes and solidifies. The surface tension between the agarose cast and the plastic bottom of the well-plate, generates a “hanging-drop”[15,24] during the 3D spheroid invasion.

It is important to note that the incubation time before inverting the casts has been established for embedding in type I collagen. With other ECM components, this step must be adjusted accordingly. Of note, complete removal of residual media should not be attempted to avoid an inadvertent loss of cells present in the microwells. This residual media results in a slight dilution of the added ECM. This needs to be taken into consideration for the analysis and adaptation from other assay systems.

The co-culture described here allows for a maximum tumor/T-cell interaction before the invasion of tumor cells is assayed: Tumor and T-cells are co-cultured for 2 days before embedding in type I collagen and then identifying surviving and invasive tumor cells over the next two days (**Figure 1B**). Of note, when the co-culture was initiated while T-cells were resuspended in collagen I, T-cells failed to show an impact on tumor cell invasion and cytotoxicity. This effect might be due to the T-cells being more distributed in collagen and thus less concentrated around the spheroids. This suggests that the direct interaction between tumor and T-cells during the two days of co-culture prior to the invasion assay are critical for assessment of T-cell mediated effects on the tumor cells.

Nevertheless, some of the advantages of this 3D model come with disadvantages. This high-throughput setting allows easy and quick seeding of cells into the agarose cast in one pipetting step, resulting in the generation of a multitude of uniformly sized spheroids within one cast. However, since the spheroids are all located within the same cast, they can also be easily removed, e.g., during removal of the cell culture medium within the seeding chamber and while adding T-cells for co-culture. Therefore, the amount of yield for each protocol is strongly dependent on the technical skills and experience of the investigator, but also on the type of assay being performed. As **Table 1** suggests, a possible loss of spheroids of about 50% at the end of an experiment must be taken into consideration. Moreover, during the seeding step, cells might not equally distribute over the area of the seeding chamber, resulting in representative spheroids within the agarose cast. From our experience, this effect increases with the size of the agarose cast. Accordingly, replicates must be considered while planning out the experiment.

The spatiotemporal interaction of cells within the 3D system can be assessed by time lapse imaging, which presents another monitoring option in this model. Furthermore, the small diameter of the microwells and the single pipetting step to seed cells, are suitable for performing single cell cloning. Lastly, patient's samples (e.g., from biopsies) can be analyzed in the assay due to the small number of cells required for the microwells and the high-throughput feature of the 3D system. The inclusion of stimulating or blocking drugs (e.g., anti-PD-1 or anti-PD-L1) to probe the tumor cell/T-cell interaction is a logical extension of the assay.

In conclusion, the 3D spheroid co-culture model presented here provides a flexible framework for monitoring cancer cell invasion and cytotoxicity of co-cultured T-cells in a biologically relevant setting. The resulting crosstalk can be visualized while maintaining the integrity of the 3D culture and thus provide mechanistic insights into tumor cell – T-cell interactions.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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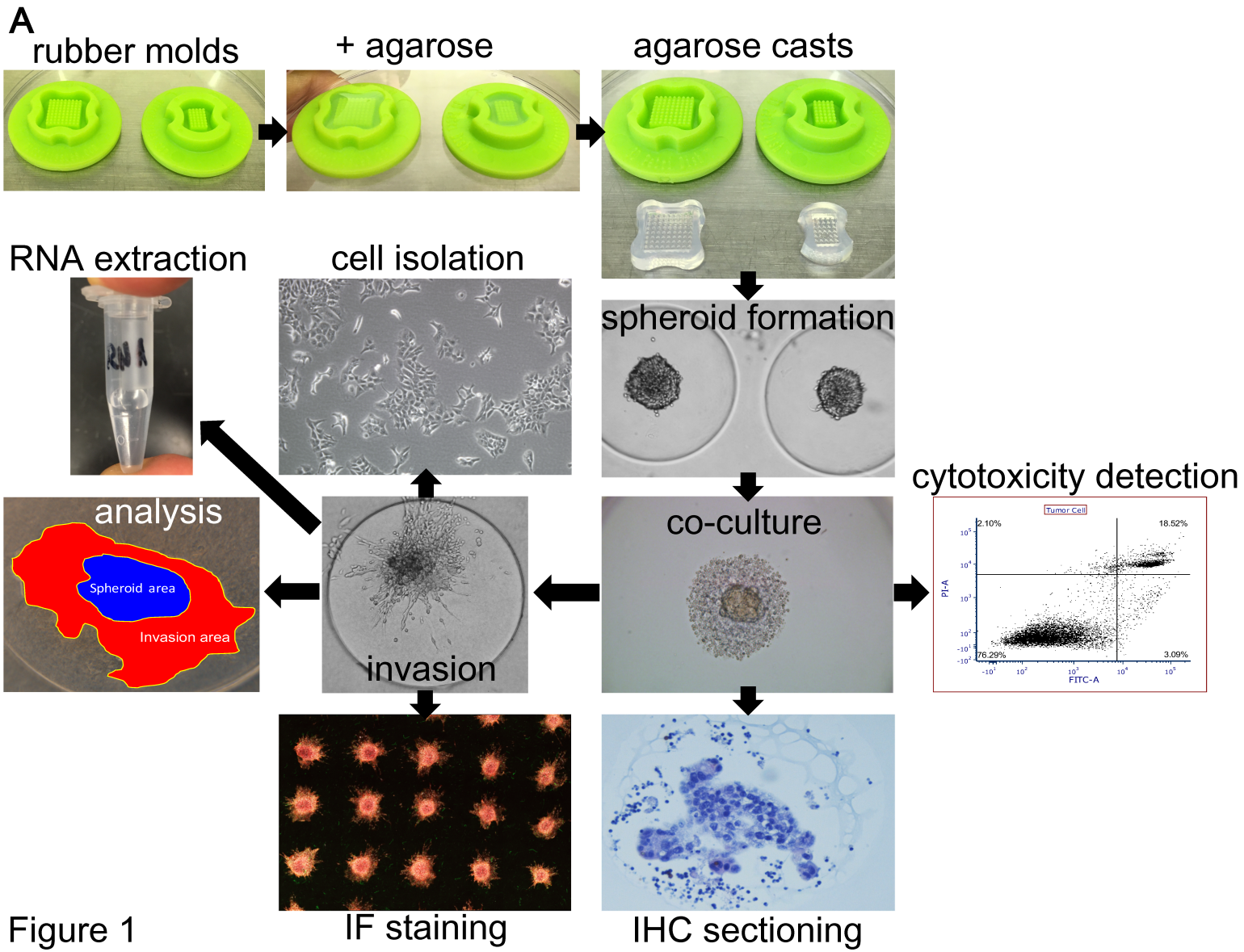


Figure 1

B

start

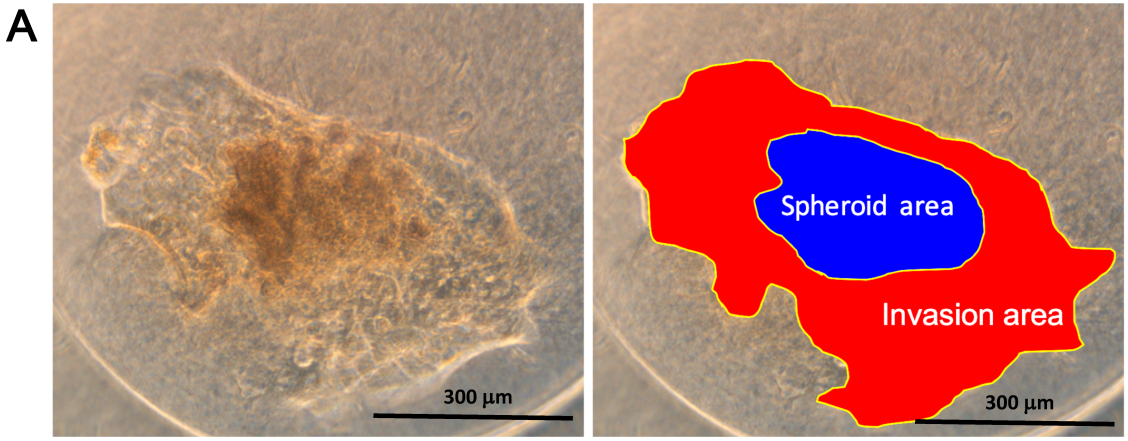
tumor - T-cell co-culture

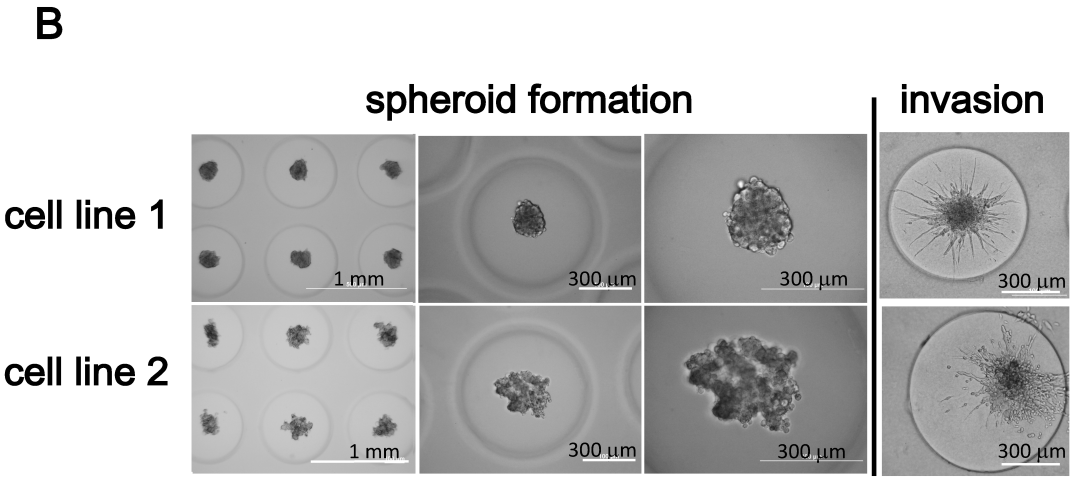
Day 2
invasion assay

selection for invasive/resistant tumor cells

Day 4
analysis,
cell isolation

Figure 2





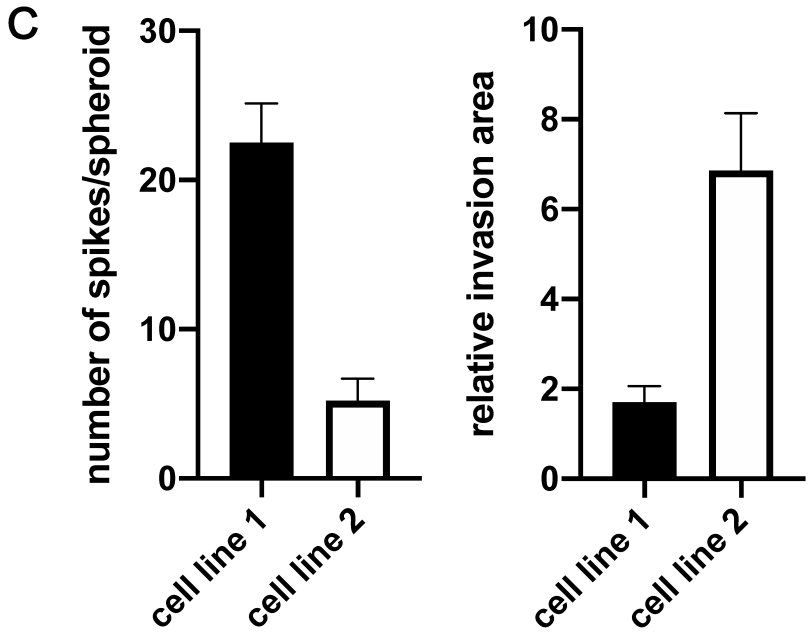


Figure 3

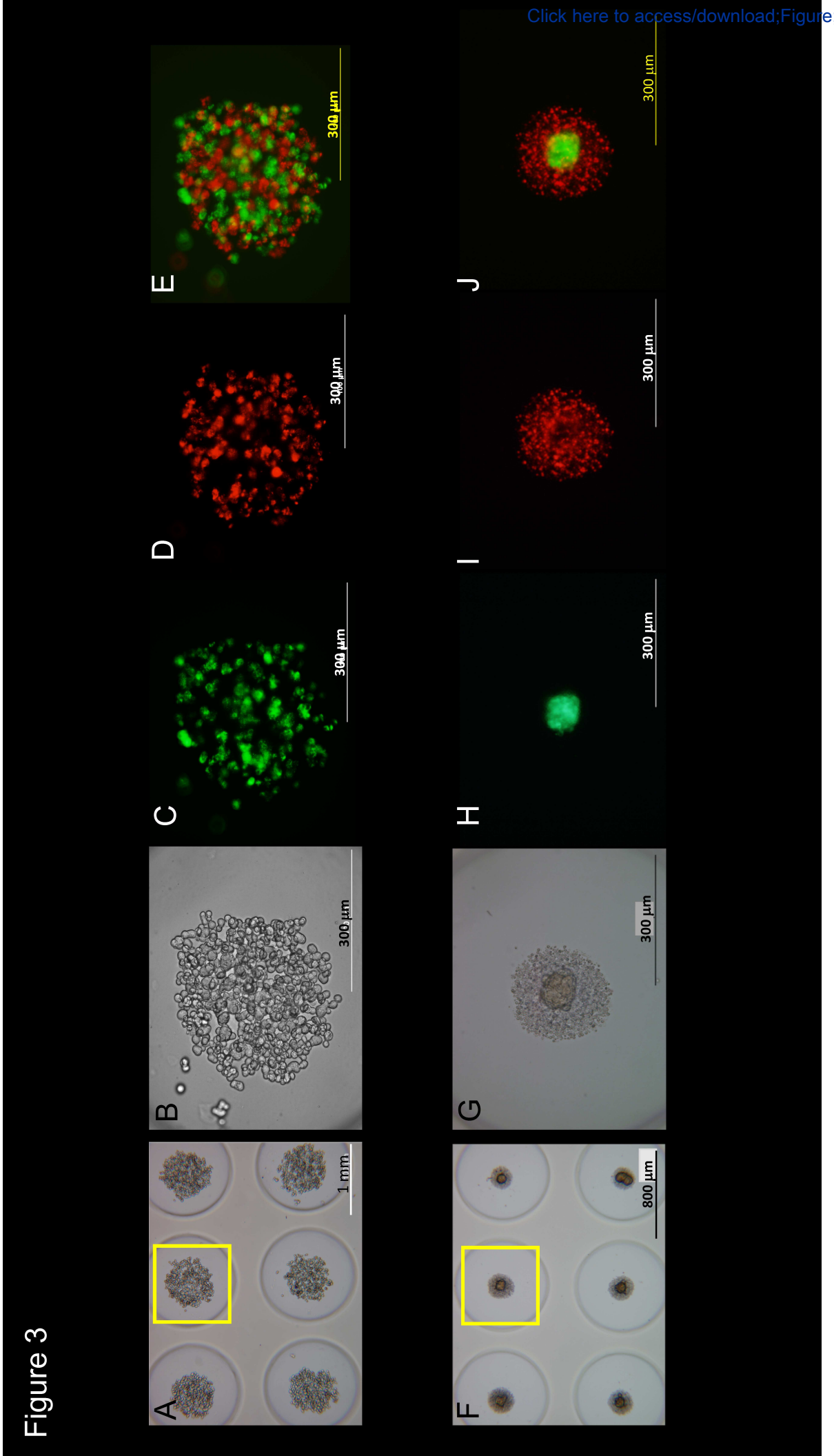
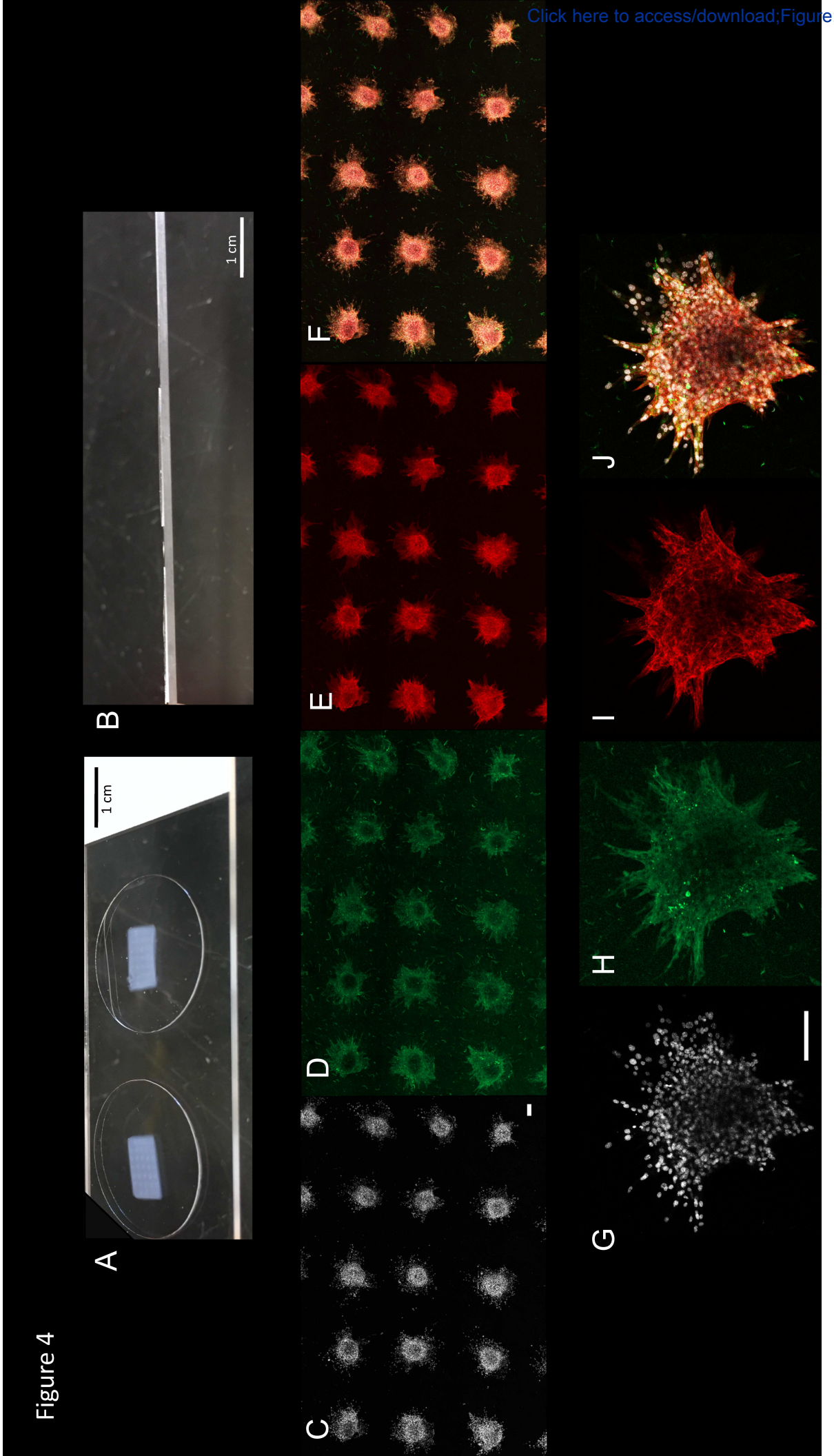


Figure 4



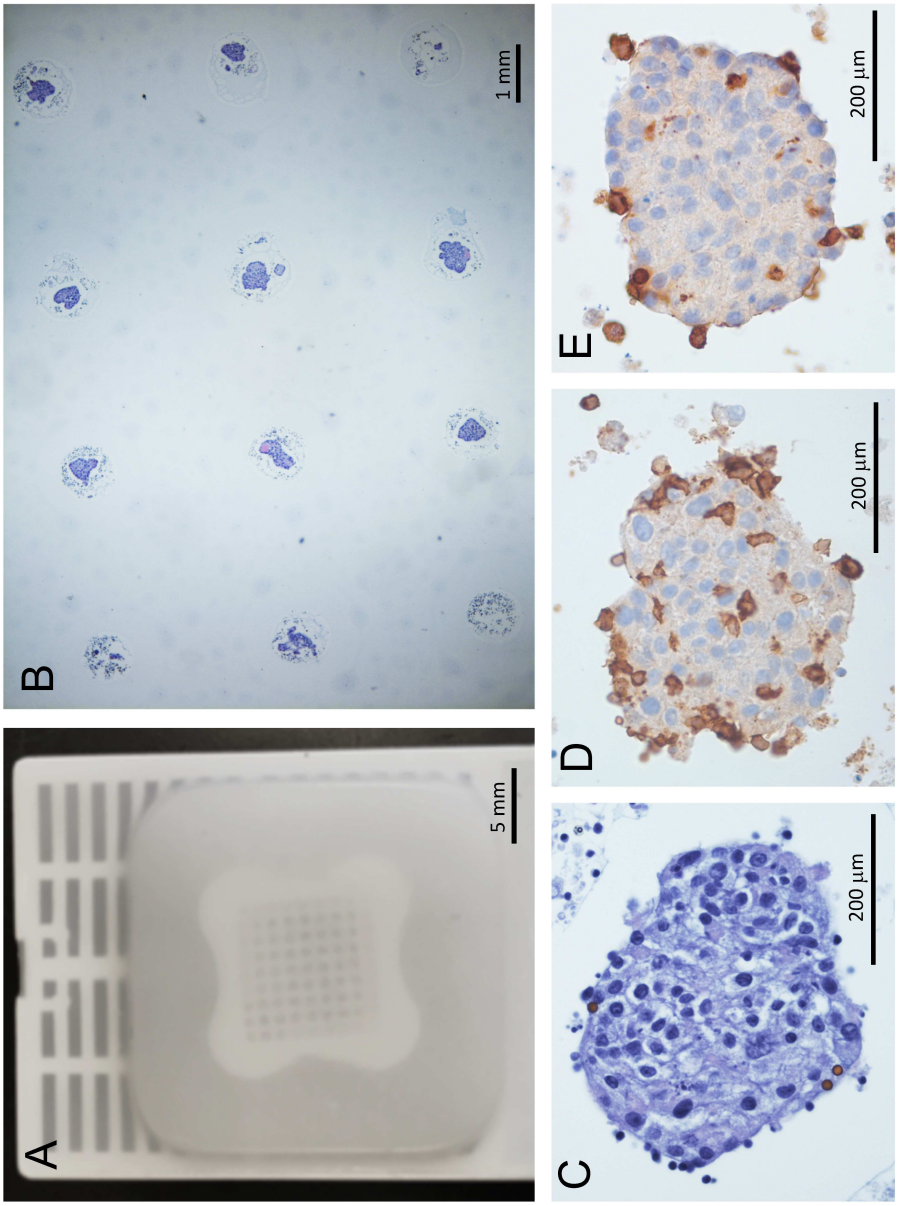


Figure 5

Table 1

protocol	typical setup	cells seeded (per cast)
cytotoxicity assay	2x 81-microwell casts	81,000
IHC assay	1x 81-microwell cast	81,000
Invasion assay	2x 35-microwell casts	35,000
IF assay	2x 35-microwell casts	35,000
Cell isolation from collagen I	2x 35-microwell casts	35,000
RNA extraction from colagen I	12x 81-microwell casts	243,000

typical yield
100,000 cells
40 spheroids
50 spheroids
50 spheroids
50,000 cells
400-600 ng/ul

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
3D Petri Dishes	Microtissues Inc	Z764019 & Z764051
8-well Chamber Slides	Lab-Tek	154534
Agarose Type I, low EEO	Sigma-Aldrich	A6013
anti-rabbit-HRP conjugated secondary antibody	Agilent	K4003
Collagen Type I, Rat Tail, 100 mg	Millipore	08-115
Collagenase Type 4, 1 g	Worthington	LS004188
DMEM, fetal bovine serum	ThermoFisher	11965092, 16000044
Harris hematoxylin	ThermoFisher	SH30-500D
HistoGel	ThermoFisher	HG-4000-012
Hoechst	Life Technologies	H1399
Phalloidin 546	Invitrogen	486624
rabbit anti-CD8 antibody	Cell Signaling	98941
rat anti-keratin 8	DSHB	TROMA-I AB_531826
RNeasy Mini Kit	Qiagen	74104
RPMI	ThermoFisher	11875093
Triton X-100	BioRad	1610407
Trizol	ThermoFisher	15596026
Tween 20	Sigma-Aldrich	P1379
TypLE	ThermoFisher	12604013

Comments/Description
referred to as "rubber molds" in the protocols; 81-microwell & 35-microwell molds
ready to use
referred to as "cell culture medium" in the protocols
referred to as "Hydroxyethyl agarose processing gel" in the protocols
1/1000 dilution
1/200 dilution
1/25 dilution
1/500 dilution
referred to as "RNA extraction kit" in the protocols
for T-cell culture medium
referred to as "Octoxynol" in the protocols
referred to as "guanidinium thiocyanate with phenol" in the protocols
referred to as "polysorbate 20" in the protocols
referred to as "cell dissociation enzymes solution" in the protocols

Rebuttal Letter

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Supplementary Data:

Explanation of some frequently used words throughout the protocol:

well-plate:
A well-plate describes a plate consisting of 12 or 24 wells, in which the agarose casts are placed. 12-well plate: for 81-microwell casts; 24-well plate: for 35-microwell casts

seeding chamber:
The seeding chamber is a squared area within an agarose cast, containing the microwells. Cells are seeded into the seeding chamber and spheroids are formed within the microwells.

microwells/multi-microwells:
Microwells are wells within the seeding chamber of an agarose cast. Spheroids are formed within the microwells after seeding. Also described as 35- or 81-microwell, referring to the number of microwells within the agarose cast.

Chamber Slide:
A Chamber Slide is a glass slide divided into separated wells through plastic walls. The collagen matrix is put in one well of an 8-well Chamber Slide for immunofluorescence staining.