HELDA
HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

UNIVERSITY OF HELSINKI

https://helda.helsinki.fi

An Approach to Study Melanoma Invasion and Crosstalk with Lymphatic Endothelial Cell Spheroids in 3D Using Immunofluorescence

Alve, Sanni

Humana press
2021

Alve , S , Gramolelli , S & Ojala , P M 2021 , An Approach to Study Melanoma Invasion and Crosstalk with Lymphatic Endothelial Cell Spheroids in 3D Using Immunofluorescence . in KM Hargadon (ed.) , MELANOMA . Methods in Molecular Biology , vol. 2265 , Humana press , pp. 141-154 . https://doi.org/10.1007/978-1-0716-1205-7_11

http://hdl.handle.net/10138/341582
https://doi.org/10.1007/978-1-0716-1205-7_11

acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

An Approach to Study Melanoma Invasion and Crosstalk with Lymphatic Endothelial Cell Spheroids in 3D using Immunofluorescence

Sanni Alve[1], Silvia Gramolelli[1], Päivi M. Ojala[1,2]*

[1] Translational Cancer Medicine Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland

[2] Section of Virology, Department of Infectious Disease, Faculty of Medicine, Imperial College London, London, United Kingdom

*Correspondence to: paivi.ojala@helsinki.fi

Running Head: Study of melanoma-LEC crosstalk in 3D by immunofluorescence

i. Chapter Title: An Approach to Study Melanoma Invasion and Crosstalk with Lymphatic Endothelial Cell Spheroids in 3D using Immunofluorescence

ii. Summary/Abstract

Three-dimensional (3D) cell culture has allowed a deeper understanding of complex pathological and physiological processes, overcoming some of the limitations of 2D cell culture on plastic and avoiding the costs and ethical issues related to experiments involving animals. Here we describe a protocol to embed single melanoma cells alone or together with primary human lymphatic endothelial cells in a 3D-crossliked matrix, to investigate the invasion and molecular crosstalk between these two cell types, respectively. After fixation and staining with antibodies and fluorescent conjugates, phenotypic changes in both cell types can be specifically analysed by confocal microscopy.

iii. Key Words: Lymphatic endothelial cells, melanoma, 3D cell culture, co-culture, invasion, sprouting, molecular crosstalk, fibrin matrix, immunofluorescence

1. Introduction

In living tissues, cells are organized in a 3-dimensional (3D) environment where complex cell-cell and cell-matrix interactions occur in all directions and have a pivotal role in many physiological and pathological processes. Therefore, standard 2D monolayer cell culture approaches are insufficient to properly understand these processes and instead require the use of different animal models, which are costly and involve ethical issues. Despite the indisputable importance of these *in vivo* approaches, they do not easily allow dissection of molecular interactions between the tumor and its microenvironment. The development of 3D cell culture methodology has, to some extent, filled the gap between 2D cell culture and animal models, providing an experimental platform that resembles more closely the *in vivo* tissue in terms of cellular communication and organization, without the high costs and issues related to experiments involving animals. Several studies have highlighted how the morphology, proliferation, and differentiation, as well as the gene and protein expression, of many cell types is modified by the 3D environment, resembling more closely the *in vivo* situation (reviewed in [1,2]). In 3D matrices the diffusion of cytokines, nutrients, and growth factors also occurs in a more physiological fashion. Another remarkable advantage of this technique is that gene expression can be easily manipulated, and the effects of this manipulation can be readily observed when cells are embedded in 3D matrices [1]. The 3D cultures with relevant extracellular matrix components mimic the content of the actual microenvironment for the cancer and provide more relevant results. In particular, the 3D cell culture approach has provided a low-cost and efficient platform to better understand the progression of cancer and to find new molecules for cancer therapy [3,4].

The 3D cell culture offers many advantages also when studying the effects of the molecular crosstalk between different cell types as it allows a deeper interaction and communication in all spatial dimensions. Cancer cells can be embedded in crosslinked

matrices alone (as single cells or tumor spheroids) or together with other cell types that compose the tumor microenvironment. This has allowed researchers to reveal and dissect the contribution of the different stromal components (*e.g.* endothelial cells, fibroblasts, immune cells, different types of extracellular matrices) to cancer progression [5-7,4,8].

In light of the clinical observation that in solid cancers, such as melanoma, the presence of peri- and intratumoral lymphatic vessels predicts poor outcome due to metastatic dissemination (reviewed in [9]), we have established 2D and 3D co-culture models to investigate the crosstalk between melanoma and lymphatic endothelial cells (LECs) (**Fig.1**) [7]. Besides representing a possible route for dissemination of the cancer cells from the primary tumor, LECs actively communicate with cancer cells inducing pro-metastatic changes and enhancing their ability to invade within the surrounding tissues [10,7]. This co-culture system has made it possible to score not only for cancer cell growth but also invasion into lymphatics and changes in cell fate that could be directly connected to melanoma progression.

Here we describe two different experimental protocols that allow the dissection of the molecular basis of the crosstalk between LECs and melanoma cells: 3D co-culture of melanoma cells with preformed LEC spheroids in crosslinked fibrin matrix (workflow described in **Fig.2A**) and embedding of the melanoma cells after co-culturing with LECs in 2D or from monotypic cultures into 3D cross-linked fibrin matrix (workflow described in **Fig.2B**). For these studies we chose fibrin matrix because it is commonly found in the melanoma perivascular tumor spaces. Cells are cultured in the fibrin droplets for four days, after which the droplets are fixed, stained with antibodies and/or fluorescent markers, and imaged by confocal microscopy (workflow described in **Fig. 2**). While the 3D co-culture allows to directly observe the interplay between the preformed LEC spheroids and the melanoma, the embedding of melanoma cells into 3D matrix, after the LEC co-culture in 2D

(also called LEC priming), permits observation of the LEC-interaction induced phenotypic changes in melanoma. In fact, we observed that in the 3D co-cultures the melanoma cells are attracted towards the LEC spheroids and adhere to them, likely mimicking the intravasation of the tumour cells into the peritumoral lymphatics (**Fig. 1B**). Furthermore, the pro-metastatic changes induced by the LEC priming in 2D induced the melanoma cells to change their growth phenotype in 3D from a sphere-like (typical for the monotypic, not LEC-primed melanoma cells) to elongated and sprouting, suggestive of an increased invasive potential (**Fig. 3**).

2. Materials

Prepare all the solutions (thrombin and aprotinin aliquots, agarose stock solution, blocking buffer, and washing solutions) in sterilized, deionized water. Prepare all the stock solutions and reagents and perform all the procedures involving living cells in a cell culture hood (with laminar flow) using sterile equipment and reagents suitable for cell culture to avoid contamination. After fixation, staining of the fibrin droplets can be carried out under non-sterile conditions.

2.1 Separation of the two cell types from 2D melanoma-LEC co-cultures

In Figure 3 we show as an example of the phenotypic comparison of monotypic melanoma vs. LEC co-cultured, separated melanoma cultured in fibrin droplets (*see* **Note 4**). The melanoma cells can come from co-cultures consisting of different cell types, but the materials needed as well as the separation procedure works for most cell types.

1. Fluorescently labeled melanoma cells (e.g. melanoma cells expressing a fluorescent reporter protein such as EGFP or labeled with a fluorescent cell tracer prior to the start of co-culture).

2. Cell growth medium suitable for co-culture. For the melanoma-LEC 2D co-cultures we have used Endothelial cell growth medium (EGM-2 Basal Medium supplemented with EGM-2 MV Microvascular Endothelial Cell Growth Medium SinleQuots supplements but excluding VEGF; Lonza).
3. PBS.
4. FACS tubes complemented with a strainer cap.
5. Falcon tubes.

2.2 Preparation of fibrin droplets

Fibrin droplets are prepared for embedding of melanoma cells or LEC spheroids in fibrin and for establishing 3D melanoma-LEC co-cultures (**Fig. 3, 4**).

1. Thrombin from human plasma: Prepare a 800 U/mL stock solution of thrombin in sterile, deionized water. Store in 10 μL aliquots (= 8U/aliquot) at -20 °C.
2. Aprotinin: Dissolve aprotinin in sterile, deionized water to a final concentration of 20 μg/μL. Store in 40 μL aliquots (= 800 μg/aliquot) at -20 °C.
3. Human lyophilized fibrinogen, plasminogen depleted.
4. Hank's balanced salt solution (HBSS): 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgSO_4$-$7H_2O$, 0.5 mM $MgCl_2$-$6H_2O$, 0.3 mM Na_2HPO_4-$2H_2O$, 0.4 mM KH_2PO_4, 6 mM D-glucose, 4 mM $NaHCO_3$.
5. 6-well cell culture dishes.
6. Growth medium appropriate for the cells under study, supplemented as necessary (e.g. RPMI-1640 or DMEM supplemented with 10% fetal bovine serum [FBS]).
7. Trypsin.
8. Microcentrifuge tubes.
9. Falcon tubes.

2.3 Preparation of LEC spheroids

1. Human dermal microvascular endothelial cells (primarily representing lymphatic endothelial cells) (Lonza)
2. 96-well U-bottom dishes.
3. Low melting point agarose stock solution (0.5 % low melting point agarose): Mix 0.5 g of low melting point agarose in 100 mL of sterile deionized water. Mix by using a magnetic stirrer and heat to around 50 – 70 °C until fully dissolved. Store the stock at room temperature (*see* **Note 1**).
4. 200 µL wide bore pipette tips.
5. Multichannel pipette.
6. Sterile multichannel pipette reagent reservoir (e.g. a plastic tray).
7. Endothelial cell growth medium (EGM-2 Basal Medium supplemented with EGM-2 MV Microvascular Endothelial Cell Growth Medium SinleQuots supplements but excluding VEGF; Lonza).

2.4 Indirect immunofluorescence (IF) staining and imaging of fibrin droplets

1. Phosphate-buffered saline (PBS)
2. 4% paraformaldehyde (PFA) in PBS.
3. 48-well dish.
4. Spatulas.
5. 1:2 ice-cold acetone-methanol: Prepare in advance and store at -20 °C.
6. Hoechst solution: 1 µg/mL of Hoechst 33342 in PBS. Store at 4 °C, protected from light.
7. Texas-Red-X Phalloidin (Invitrogen), at a working dilution of 1:200 in blocking buffer.
8. Primary antibodies suitable for IF (*see* **Note 2**). For the detection of PECAM as shown in **Fig. 2**, we use mouse anti-human CD31, clone JC70A (Dako) at a working dilution of 1:500 in blocking buffer.

9. Secondary antibodies conjugated with fluorophores, for example Alexa Fluor (*see* **Note 3**). For detection of the primary anti-PECAM antibody, we use Alexa Fluor 594 Goat anti-Mouse Cross-absorbed Secondary Antibody (Thermo Fisher) a working dilution of 1:800 in blocking buffer.
10. Parafilm.
11. Glass slides.
12. 50x20 mm coverslips.
13. Mowiol mounting medium. Dissolve 5 g of Mowiol 4-88 in 20 mL of 100 mM Tris-HCl, pH 8.0, in a glass beaker, cover the beaker, and mix by using a magnetic stirrer for 16 h. Add 10 mL of glycerol and continue mixing for an additional 16 h. Remove undissolved Mowiol by centrifugation at 11 000 g for 5 min. Store aliquots (1 or 2 mL) at - 20 °C.
14. Blocking buffer: 15% FBS, 0.3% Triton-X in PBS. Measure 7.5 mL of heat-inactivated FBS and 150 µL of Triton-X 100. Add PBS to a volume of 50 mL, mix by using a magnetic stirrer until the solution is homogeneous. The same solution can be used for both primary and secondary antibody incubations, and it can be stored at 4 °C o/n for 4 days.
15. Washing solution: 0.3% Triton-X in PBS. Add 150 µL of Triton-X 100 to 50 mL of PBS. Store at 4 °C.
16. Fluorescence microscope.

3. Methods

3.1 Separation of the two cell types in melanoma-LEC 2D co-cultures

1. Detach the cells in the monotypic and melanoma-LEC 2D co-cultures by trypsin treatment (170,000 U/mL for 5-8 min at 37 °C) and collect them into EGM.
2. Centrifuge the cells at 450 g for 4 min. Remove supernatant.

3. Resuspend the cells into 200 µL of PBS. If necessary, to obtain a single cell suspension, filter the cells through a FACS tube complemented with a strainer cap.
4. Sort the cells with FACS using the filter sets compatible with the melanoma cell fluorescent label. Collect the cells into Falcon tubes containing 2 mL of EGM.

3.2 Embedding of single melanoma cells in fibrin (see Note 4)

1. Prepare fibrinogen solution just before the use. Dissolve fibrinogen in HBSS to the concentration of 6 mg/mL. For most experiments, a final volume of 3 mL is usually enough. Dissolve solution in a 37 °C water bath for 10-15 min. (*see* **Note 5**).
2. Prepare thrombin working solution just before the use. Mix one thrombin aliquot and one aprotinin aliquot in 2mL of HBSS. Keep on ice until used.
3. Count the cells from section **3.1** and resuspend them in growth medium to a concentration of $1e^6$ cells/mL. This will yield about 5,000 cells per fibrin droplet, though the cell number can be adjusted, depending on the growth rate of the cells. To make four droplets with 5000 cells in each at once, transfer 20 µL of the prepared cell dilution into a microcentrifuge tube (*see* **Note 6**).
4. Mix 100 µL of the prepared fibrinogen solution with the cells by pipetting up and down.
5. Add 100 µL of the thrombin working solution. Mix by pipetting up and down 5-6 times (*see* **Note 7**).
6. Using another pipette adjusted to 50 µL in advance, pipet the mixture on the well of a 6-well cell culture dish as a droplet. From the mixture made above it is possible to prepare four droplets at once, and these should fit on the same well (*see* **Note 8**).
7. Once you have pipetted all the droplets on the dish, incubate for 30 min in the cell incubator at 37 °C with 5% CO_2 without adding cell culture medium to allow proper fibrin formation.

8. Add cell culture medium supplemented with 50 μg/mL of aprotinin (= 1 aliquot/16 mL of medium). 3mL of medium/well is usually enough (*see* **Note 9**).
9. Culture the cells in the incubator at 37 °C with 5% CO_2 until the desired level of cell growth is achieved. In most cases four days is optimal (*see* **Note 10**).

3.3 LEC spheroid preparation and embedding into fibrin

1. Heat 0.5% agarose in a microwave oven. Heat at 400 W in few second pulses and mix by gently shaking the bottle in between. Avoid boiling (*see* **Note 11**).
2. Pipet the melted agarose on a 96-well U-shaped dish using a multichannel pipette and a sterile reagent reservoir. Dispense 100 μL/well.
3. Wait a few seconds and remove the agarose using the multichannel pipette. Be careful not to touch the bottom of the well. Instead, tilt the plate and remove the agarose from the walls of the plate.
4. Remove the remaining agarose by inverting the dish and tapping it a few times against a clean tissue paper.
5. Let the dish cool down without the lid for 15 min under the cell culture hood. Afterwards, insert the lid and keep the plate at RT o/n to let the agarose solidify (*see* **Note 12**).
6. Detach the LECs by trypsin treatment (170,000 U/mL for 5-8 min at 37 °C) and collect them into EGM. After counting the cells, prepare a cell suspension of $5e^4$ cells/mL.
7. Dispense 100 μL/well (=5000 cells/well) of the cell suspension on the agarose-coated 96-well U-bottom dish using a multichannel pipette and a sterile reagent reservoir. Be careful not to scratch the bottom of the well.
8. Incubate the cells o/n in the cell incubator (at 37 °C with 5% CO_2) to allow spheroid formation (*see* **Note 13)**.
9. Next day, prepare fibrinogen and thrombin working solutions as described in **steps 1** and **2** of **Section 3.2**.

10. Collect the spheroids in microcentrifuge tubes, using a multichannel pipette and wide bore tips to avoid breaking them. You can collect around 4-6 spheroids into the same tube, and these can go into one fibrin droplet (*see* **Note 14**).
11. Centrifuge at 200 x g for 2 min. Carefully remove the supernatant with suction (*see* **Note 15**).
12. Add 25 μL of fibrinogen solution and 25 μL of thrombin working solution to the LEC spheroids, and mix by pipetting up and down once using a wide bore tip (*see* **Note 16**).
13. Pipet one droplet to a 6-well dish using wide bore tip. Although droplets are prepared and pipetted one at a time, four droplets can be pipetted per well.
14. Follow **steps 7-9** from **Section 3.2**.

3.4. Preparation of 3D Melanoma-LEC co-cultures

A schematic diagram of this workflow is shown in **Fig. 4**.

1. Prepare LEC spheroids as described in **steps 1-11** of **Section 3.3**.
2. Prepare fibrinogen and thrombin working solutions as described in **steps 1** and **2** of **Section 3.2**.
3. Detach the melanoma cells by trypsin treatment (170,000 U/mL) for 2-5 min at 37 °C and collect into growth medium, count the cells, and prepare a cell suspension of $1e^6$ cells/mL.
4. Next, mix in a new microcentrifuge tube or Falcon tube the melanoma cell suspension with fibrinogen working solution for as many droplets as needed. For each droplet, 5 μL of the cell suspension (= 5000 cells/droplet) and 25 μL of the fibrinogen working solution are needed.
5. Pipet 25 μL of the thrombin working solution in the tube containing the LEC-spheroids. Carefully pipet up and down to mix the solution without breaking the spheroids.

6. Add 25 μL of the melanoma cell/fibrinogen solution prepared in **step 4** of this section. Using a wide bore pipette tip, mix by pipetting up and down once.
7. Pipet the droplet onto a 6-well dish using a wide bore tip.
8. Follow **steps 7-9** of **Section 3.2**.

3.5 Staining of fibrin droplets

1. Remove growth medium and fix the cells with freshly thawed 4% PFA in PBS. Incubate 20-30 min at RT. Make sure to add enough PFA-PBS to fully cover the droplets (5 mL/well are usually sufficient).
2. Remove PFA and wash once with 5 mL of PBS per well. Add at least 5 mL of PBS into the wells so that the fixed droplets do not dry. The fixed droplets can be stored for a few weeks at 4 °C in PBS.
3. Carefully remove the droplets from the 6-well dish using a spatula and move them into a 48-well dish. Add 500 μL of PBS. Two or three droplets of the same sample type can be put on the same well and stained with the same antibodies.
4. Remove PBS and add 500 μL of ice-cold acetone-methanol mix. Incubate for 1 min, remove acetone-methanol, and wash twice with 500 μL of PBS (*see* **Note 17**).
5. Remove PBS and add 500 μL of blocking buffer. Incubate 1h at RT.
6. Meanwhile, prepare optimized reagents and primary antibody dilutions for proteins of interest (for example fluorescently labelled phalloidin to visualize the cell morphology or endothelial markers such as PECAM to label the LECs) in blocking buffer and keep on ice until use. For one well, a 150 μL volume of diluted primary antibodies is needed.
7. Remove the blocking solution and add the diluted primary antibody to the wells containing the droplets. Carefully check that droplets are fully covered. Wrap the plate with Parafilm to avoid evaporation of the buffer and incubate at 4 °C o/n.

8. Remove primary antibody solution. Wash the droplets with washing solution, at least 500 µL per wash. Do at least 3 washes for 15 min (*see* **Note 18**).
9. Prepare optimized secondary antibody dilutions in blocking buffer. Keep on ice until use.
10. Remove the washing buffer and add the diluted secondary antibodies in a volume of 150 µL per well. Wrap the plate with Parafilm to avoid evaporation of the buffer and incubate at 4°C o/n. From this point on, keep the droplets protected from light as much as possible (use aluminum foil to wrap the plate).
11. Remove the secondary antibody solution and wash at least 3X for 15 min with washing solution.
12. Incubate for 15-30 min in 500 µL of Hoechst 33342 solution.
13. Wash at least 2X for 15 min with washing solution.
14. Wash 15 min in PBS.
15. Rinse the droplets with deionized water.
16. Using spatulas, transfer the droplets to a microscope glass slide (two or three droplets can be put onto the same slide).
17. Pipet thawed Mowiol mounting medium on top of the droplets, taking care to avoid making bubbles. Insert a glass objective slide on top (*see* **Note 19**).
18. Keep the slides at least o/n at RT to allow Mowiol to solidify. Keep the slides protected from light.
19. Image the droplets with a fluorescence microscope. The best choice is to use a confocal laser scanning microscope with a z-stack imaging option.

4. Notes

1. 0.5 % agarose solidifies when stored at room temperature (RT). Before use, the agarose solution can be heated in a 400 W microwave oven for a few times in few second pulses

to have a liquid solution. Check the solution often and mix it by gentle shaking, being careful not to boil the liquid. If the liquid boils, wait for a couple of minutes for the agarose to cool down before proceeding.

2. Many primary antibodies suitable for immunofluorescence usually work well also in 3D but this always needs to be tested. Slightly higher concentrations are often needed in 3D when compared to 2D staining. If one wishes to observe the shapes and structures of the cell clusters, fluorescently conjugated phalloidin is a good choice. We have stained LEC spheroids with PECAM antibody to specifically visualize the LECs, however, any other (lymphatic) endothelial marker could be used.
3. Secondary antibodies can be used at a bit higher concentration than recommended for 2D immunofluorescence. In our experiments, 1:800 secondary antibody dilution (AlexaFluor) has worked well.
4. These experiments are easily adjustable and different treatments can be applied to the melanoma cells prior to embedding into 3D (e.g. transfection, transduction, inhibitor and small molecule treatments, co-culture with different cell types followed by sorting). **Fig. 3** shows a comparison of the phenotype of parental melanoma cells grown as a monotypic culture (left panel) to cells after co-culture with LECs in 2D (right panel) prior to embedding into 3D matrix.
5. If it is not possible to weigh fibrinogen under sterile conditions, the fibrinogen-HBSS can be sterilized after dissolving by using a 0.45 μm filter head attached to a syringe.
6. The number of cells to be embedded into fibrin droplets depends largely on their growth rate. Droplets with 5000 cells are good for medium growth rate cells but the cell number can be reduced to 3000 cells or even less for fast growing cells. Mix the cell suspension carefully after trypsinization to ensure that you have a single cell suspension.

7. This step needs to be done fast since fibrin starts to solidify immediately. The colder the enzyme mix is the slower the fibrinogen will solidify, this is why it is important to keep the mix on ice. Be careful not to contaminate with the pipette tips the thrombin solution with fibrinogen solution and vice versa.
8. Because fibrin hardens and becomes insoluble very fast, one cannot use the same pipette that was used in the previous steps for preparing the droplets. Be quick while pipetting the droplets. It is also very important that the droplets do not touch each other or the walls of the well.
9. Although the droplets should be fully solidified by now, they are still fragile; be gentle while pipetting the cell growth medium. The droplets tend to first repel the added medium; to make sure that they will be fully covered, pipet an excess amount of medium in the well and then remove it.
10. It is possible to add soluble drugs, inhibitors, etc. in the growth medium as desired for specific studies.
11. If the agarose solution has boiled during heating, let it cool down for a couple of minutes at RT.
12. If too little agarose is left on the plate, cells may attach on the bottom and will not form a proper spheroid. If 0.5% agarose boils during heating, it is very important to cool it down a bit, since very hot agarose forms a too thin layer on the well. On the other hand, if too much agarose is left on the well, it may be incorporated into the spheroid itself, resulting in peculiar, irregular spheroid shapes.
13. It is highly recommended to prepare a larger number of spheroids for each experiment than actually needed, since usually some are lost or broken during the processing. Some cells may require 48 h to form a proper, compact spheroid.

14. The spheroids are usually visible in the tip, as small white dots, when collected. The easiest way to collect spheroids is to take up some medium from the well into the tip, carefully pipet it back against the wall of the well and then pipet the excess medium back into the pipet tip.
15. Spheroids are very fragile and can be damaged by centrifugation. It is recommended to first test with only a couple of microcentrifuge tubes to ensure that the centrifugation force does not damage them.
16. This step might need a bit more mixing by pipetting up and down to make sure that the spheroids have not clumped together. However, too much mixing will result in fragmentation of spheroids. Check the pipetting result often at the microscope while pipetting the droplets on the dish.
17. There is the risk to pipet the droplet together with the washing liquid since 1 mL pipette tips might have too wide ends to conveniently remove liquids from the well. We find it useful to attach a 10 μL pipette tip (without filter) on top of a 1 mL pipette tip for easier removal of washing liquids. Placing the dish on a dark surface also helps to visualize where the droplets are located in the well.
18. Some antibodies may need more washing. If too much background disturbs the imaging, it is also possible to incubate the droplets in washing buffer overnight at 4°C between the primary and secondary antibody treatments.
19. Add enough mounting medium to fully cover the droplets. Sometimes droplets tend to move under the coverslip before mounting medium has solidified. Adding more mounting medium may help.

5. References

1. Baker BM, Chen CS (2012) Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. J Cell Sci 125(Pt 13):3015-3024. doi:10.1242/jcs.079509

2. Justice BA, Badr NA, Felder RA (2009) 3D cell culture opens new dimensions in cell-based assays. Drug Discov Today 14(1-2):102-107. doi:10.1016/j.drudis.2008.11.006

3. Ravi M, Sah S, Bhammar R (2014) Differences of SiHa (human cancer of cervix) and BMG-1 (brain glioma) cell lines as 2D and 3D cultures. J Cell Physiol 229(2):127-131. doi:10.1002/jcp.24433

4. Thoma CR, Zimmermann M, Agarkova I, Kelm JM, Krek W (2014) 3D cell culture systems modeling tumor growth determinants in cancer target discovery. Adv Drug Deliv Rev 69-70:29-41. doi:10.1016/j.addr.2014.03.001

5. Chen Y, Sun W, Kang L, Wang Y, Zhang M, Zhang H, Hu P (2019) Microfluidic co-culture of liver tumor spheroids with stellate cells for the investigation of drug resistance and intercellular interactions. Analyst 144(14):4233-4240. doi:10.1039/c9an00612e

6. Meier-Hubberten JC, Sanderson MP (2019) Establishment and analysis of a 3D co-culture spheroid model of pancreatic adenocarcinoma for application in drug discovery. In: Moll J, Carotta S (eds) Target identification and validation in drug discovery. Methods Mol Biol, 1953. Humana Press, New York, NY

7. Pekkonen P, Alve S, Balistreri G, Gramolelli S, Tatti-Bugaeva O, Paatero I, Niiranen O, Tuohinto K, Perala N, Taiwo A, Zinovkina N, Repo P, Icay K, Ivaska J, Saharinen P, Hautaniemi S, Lehti K, Ojala PM (2018) Lymphatic endothelium stimulates melanoma metastasis and invasion via MMP14-dependent Notch3 and beta1-integrin activation. Elife 7. doi:10.7554/eLife.32490

8. Alonso-Nocelo M, Raimondo TM, Vining KH, Lopez-Lopez R, de la Fuente M, Mooney DJ (2018) Matrix stiffness and tumor-associated macrophages modulate epithelial to mesenchymal transition of human adenocarcinoma cells. Biofabrication 10(3):035004. doi:10.1088/1758-5090/aaafbc

9. Alitalo A, Detmar M (2012) Interaction of tumor cells and lymphatic vessels in cancer progression. Oncogene 31(42):4499-4508. doi:10.1038/onc.2011.602

10. Ma Q, Dieterich LC, Ikenberg K, Bachmann SB, Mangana J, Proulx ST, Amann VC, Levesque MP, Dummer R, Baluk P, McDonald DM, Detmar M (2018) Unexpected contribution of lymphatic vessels to promotion of distant metastatic tumor spread. Sci Adv 4(8):eaat4758. doi:10.1126/sciadv.aat4758

Figure Captions:

Fig. 1 Representative images of (A) GFP-expressing WM852 melanoma cells (green) co-cultured with LECs in 2D for two days. The dashed line indicates the LEC-melanoma border. Scale bar = 25 µm; (B) preformed LEC spheroids embedded in a 3D crosslinked fibrin matrix for four days alone (right) or together (left) with single, GFP-expressing WM852 melanoma cells (green). Scale bar = 200 µm. Cells are stained as indicated, and nuclei are counterstained with Hoechst 33342.

Fig. 2 Schematic diagram of the assays described. (A) Preformed LEC spheroids (shown in red) alone or together with single melanoma cells (shown in green) are embedded for four days either alone or together in 3D-crosslinked fibrin matrix droplets. (B) Melanoma cells from a monotypic culture (dark green) or co-cultured with LECs in 2D (light green) are, after sorting and separation of the different cell types, embedded for four days in 3D-crosslinked fibrin matrix. After fixation and staining, cells are imaged by confocal microscopy.

Fig. 3 Representative images of melanoma cells embedded in 3D-crosslinked fibrin matrix for four days. Left panel: monotypic WM852 melanoma cells. Right panel: WM852 cells after the LEC co-culture. Cells are stained as indicated, nuclei are counterstained with Hoechst 33342. Scale bar = 50 µm.

Fig. 4 Schematic diagram of the experimental procedure for embedding the preformed LEC spheroids with single melanoma cells in 3D-crosslinked fibrin matrix. LEC spheroids are carefully resuspended in a thrombin + aprotinin solution (indicated as thrombin) while single melanoma cells are suspended in a fibrinogen solution using different pipette tips. The two

solutions are mixed together (indicated as MIX), and the solution is then dispensed on a dish as single 50 μL droplets.