Journal of Visualized Experiments

A 3D Spheroid Model as a More Physiological System for Cancer-Associated Fibroblasts Differentiation and Invasion In Vitro Studies

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60122R1
Full Title:	A 3D Spheroid Model as a More Physiological System for Cancer-Associated Fibroblasts Differentiation and Invasion In Vitro Studies
Keywords:	spheroids, CAF, 3D model, differentiation, immunofluorescent staining, qPCR, flow cytometry, invasion, extracellular matrix gel
Corresponding Author:	A Dr. Cavaco Westfalische Wilhelms-Universitat Munster Münster, GERMANY
Corresponding Author's Institution:	Westfalische Wilhelms-Universitat Munster
Corresponding Author E-Mail:	acmcavaco@gmail.com
Order of Authors:	A Dr. Cavaco
	Johannes Eble
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Germany

Westfälische Wilhelms-Universität Münster

medizinische fakultät
Westfälische Wilhelms-Universität Münster

Institut für Physiologische Chemie und
Pathobiochemie ◆ Universitätsklinikum Münster
◆ 48149 Münster

Institute for Physiological Chemistry und Pathobiochemistry
Univ.-Prof. Dr. J. Eble
Director of Physiological Chemistry
Waldeyerstraße 15 ◆ 48149 Münster

Telephone: (02 51) 83 – 5 55 91
Secretary: (02 51) 83 – 5 55 92
Fax: (02 51) 83 – 5 55 96
E-Mail: acmcavaco@gmail.com
URL: physiolchem.klinikum.uni-muenster.de/

Münster, April 11th, 2019

To the Editor of

JoVE, Journal of Visualized experiments

Dear Editor,

As a high visibility journal, it would be for us a great opportunity to publish in your journal. By sharing this protocol for a 3D model for *in vitro* study of the differentiation of cancer associated fibroblasts (CAFs), as well as the interplay between CAFs and cancer cells in tumour invasion, we hope to reach other scientists and help them improve their experimental design. Futhermore, after an extensive comparison of different methods to study both CAF and cancer cells biology, we explain the limitations of the methods and highlight the advantages of the present protocol and how to analyse the obtained data. We believe that we describe for the first time how to dissociate spheroid for downstream assays such as real time qPCR and flow cytometry analysis.
We would be happy if you would consider our manuscript for publication in JoVE.

Cordially,

Ana Cavaco

TITLE:

A 3D Spheroid Model as a More Physiological System for Cancer-Associated Fibroblasts Differentiation and Invasion In Vitro Studies

AUTHORS & AFFILIATIONS:

Ana C. Martins Cavaco[1, †], Johannes A. Eble[1]

[1]Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, Münster, Germany

[†]Present address: Instituto de Medicina Molecular, Av. Professor Egas Moniz, Lisboa, Portugal

Corresponding Author:

Johannes A. Elbe (johannes.eble@uni-muenster.de)

Email Address of Co-Author:

Ana C. Martins Cavaco (acmcavaco@gmail.com)

KEYWORDS:

spheroids, CAF, 3D model, differentiation, immunofluorescent staining, qPCR, flow cytometry, invasion, extracellular matrix gel

SUMMARY:

The goal of this protocol is to establish a 3D in vitro model to study the differentiation of cancer-associated fibroblasts (CAFs) in a tumor bulk-like environment, which can be addressed in different analysis systems, such as immunofluorescence, transcriptional analysis and life cell imaging.

ABSTRACT:

Defining the ideal model for an in vitro study is essential, mainly if studying physiological processes such as differentiation of cells. In the tumor stroma, host fibroblasts are stimulated by cancer cells to differentiate. Thus, they acquire a phenotype that contributes to the tumor microenvironment and supports tumor progression. By using the spheroid model, we have set up such a 3D in vitro model system, in which we analyzed the role of laminin-332 and its receptor integrin α3β1 in this differentiation process. This spheroid model system not only reproduces the tumor microenvironment conditions in a more accurate way, but also is a very versatile model since it allows different downstream studies, such as immunofluorescent staining of both intra- and extracellular markers, as well as deposited extracellular matrix proteins. Moreover, transcriptional analyses by qPCR, flow cytometry and cellular invasion can be studied with this model. Here, we describe a protocol of a spheroid model to assess the role of CAFs' integrin α3β1 and its ectopically deposited ligand, laminin-332, in differentiation and in supporting the invasion of pancreatic cancer cells.

INTRODUCTION:

The tumor microenvironment is a very complex niche and extremely important for the maintenance and progression of the tumor cells[1]. It is formed not only by the cancer cells but also by stromal fibroblasts. The tumor cells are surrounded by a stroma that is specific and different from the stroma of normal tissues[2]. Laminin-332 is an extracellular matrix protein ectopically expressed in the stroma of different tumors, such as of pancreatic adenocarcinoma[3]. Moreover, the biochemical composition of the ECM and also its biophysical properties, such as rigidity and tension, change within the tumor bulk[4]. This tumor stroma, or "reactive stroma", is caused by an adaptation of fibroblasts to the neighboring cancer cells and by the recruitment of other very important players that develop a favorable and supportive environment for tumor progression. The differentiation of stromal fibroblasts results in cancer-associated fibroblasts (CAF). These cells can be identified using different markers such as α-smooth muscle actin (αSMA)[5] or neural/glial antigen 2 (NG2)[6].

The most suitable in vitro model to recapitulate the tumor microenvironment (TME) with CAFs is difficult to select. The method to mimic physiological parameters of the TME in a cost-efficient and reproducible way must be considered for such a model system. Within the TME, different processes, such as proliferation, differentiation, migration and invasion of the different cell types occur. These cellular processes can be performed individually with different methods. However, the experimental conditions must consider the cellular interactions with the tumor stroma ECM, since the stiffness of the substratum influences the CAF differentiation process. R.G. Wells commented on the impact of matrix stiffness on cell behavior and highlighted that cytoskeletal organization and differentiation status observed in in vitro cultured cells might be artefactual[7]. Different stimuli seem to be involved in CAF differentiation, including mechanical tension[5,7]. To avoid this, 2D soft substrates could be possible approaches for differentiation studies, as they circumvent the problem of the stiff culture dish plastic. A soft 2D surface, on which fibroblasts can be grown, can be collagen-I coated polyacrylamide gels, whereby the gel stiffness can be manipulated by the concentration of polyacrylamide and the gel cross-linker. The adhesion and formation of αSMA-rich stress fibers are enhanced in fibroblasts along with the gel stiffness[8]. These results stress the importance of soft substrate scaffolds for more physiological in vitro differentiation models. However, in our hands the experimental reproducibility and imaging of these gels were challenging. To overcome these shortcomings, we changed the 2D soft substrate system for a 3D spheroid model for differentiation and invasion studies. This model is more clinically relevant and, similar to an in vitro organoid, recapitulates in vivo cell-cell interactions, ECM production and deposition, as well as cell behavior[9].

Spheroids are formed when cells lack a substrate to adhere to. When the cells are left without an adhesive surface, they aggregate to form a more or less spherical structure. If the spheroids are composed of one type of cell, they are called homospheroids; if composed of two or more different cell types they form heterospheroids.

Among the different methods for spheroid preparation, we perform the protocol using non-adherent round bottom 96-well plates. It is very effective with respect to the costs. Here, we produce both homospheroids of fibroblasts, CAF or CAFs lacking the integrin α3 subunit to examine the differentiation process and heterospheroids of CAFs or integrin α3 KO CAFs and

pancreatic duct carcinoma cells (AsPC-I and PANC-I) to study the invasion into the surrounding matrix.

The aim for these studies was to use primary CAFs isolated from human pancreatic carcinoma biopsies. However, the biopsies to obtain the cells are scarce and for this reason, the CAFs used in these studies have been immortalized using lentivirus containing HTERT. They are called iCAFs, and their normal counterparts, primary human pancreatic fibroblasts, are termed iNFs. The human pancreatic fibroblasts and the pancreatic duct carcinoma cells, AsPC-I and PANC-I, are commercially available.

This protocol was used to study the effect of the laminin-332-integrin interaction in the CAF differentiation process. To prove specificity of this interaction and its function, inhibitor compounds were used: BM2, a monoclonal antibody that blocks the integrin binding site the laminin-332 α3 chain[10], or lebein 1, a snake venom derived compound that blocks the laminin-binding integrins $\alpha3\beta1$, $\alpha6\beta1$ and $\alpha7\beta1$[11,12].

For the invasion assay, cells had been transduced with lentivirus containing cDNA encoding either mCherry (iCAFs and integrin α3 KO iCAFs) or GFP (AsPC-I and PANC-I) to distinguish the different cell types in the heterospheroids. The transduction of the cells to immortalize them and/or to label them with fluorescent protein (mCherry and GFP) expression is described in a previous study[13], that should be consulted for further information.

PROTOCOL:

1. 3D spheroids as an in vitro model for fibroblast/CAF differentiation using different TGF-β1 inhibiting compounds of cell-matrix interaction

1.1. Mix 1 part of 6 mg/mL methylcellulose solution and 3 parts of cell culture media, MEM supplemented with 1% of heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin to obtain the spheroid formation solution.

1.2. Resuspend freshly thawed immortalized normal fibroblasts (iNFs), immortalized CAF (iCAFs) or integrin $\alpha3\beta1$ KO iCAF (passage up to 25) in the spheroid formation solution. If preparing one 96 well plate, prepare an excess of spheroid formation solution, 10 mL and add 75,000 cells, keeping in mind that each spheroid is composed by 750 cells, and that 100 µL are distributed per each well of the plate.

1.3. If cytokines or integrin inhibitors are included in the study, add these compounds to the cell suspension, in order to embed them into the spheroids during their formation. Note that iNFs are stimulated with 10 ng/mL of TGF-β1 in order to trigger differentiation. Where appropriate, add inhibitor compounds together with TGF-β1, such as 20 µg/mL BM2 or 10 µg/mL of lebein 1.

1.4. Prepare CAF homospheroids in the same way but without the TGF-β1 stimulus, since they are already differentiated. Prepare the integrin α3 KO CAF homospheroids without the addition of any compound.

1.5. Distribute the spheroid formation solution onto a round bottom 96 multi-well plate by adding 100 µL of the solution in each well. Every time the spheroid solution is distributed in the wells, mix well by pipetting up and down, several times.

1.6. Place the plate in the cell culture incubator for 24 h to allow one spheroid to be formed in each well.

1.7. Collect the spheroids after about 24 h and use them for different downstream purposes: immunofluorescent staining, real time (RT) q-PCR and flow cytometry. To detect expression of intra- and extracellular antigens, including deposition of ECM proteins within the spheroid, use immunofluorescent staining. After disintegration of spheroids into single cells, quantify membrane-anchored receptor by flow cytometry. To detect gene activation and transcriptional changes, use RT q-PCR of mRNA isolated from the spheroid cells.

2. Immunofluorescent staining of spheroids

2.1. Collect the spheroids after about 24 h into one 1.5 mL reaction tube per experimental condition or per protein to be analyzed. For example, place the spheroids of iNFs stimulated with TGF-β1 in a tube different from the control iNFs, which were not treated. To avoid rupture of spheroids due to too strong shear forces, cut the end of the pipette tip to enlarge the orifice. Include at least 5-10 spheroids in one reaction tube, to allow replicates and take into account the possible losses during the washing steps.

2.2. Centrifuge the spheroids with a bench top centrifuge for about 30-60 s at 1,000 x *g*. Carefully remove the methylcellulose-containing supernatant by pipetting, without disturbing the pelleted spheroids.

2.3. Wash with 50 µL of 1x PBS. Repeat the centrifugation step and carefully remove the PBS by pipetting, as described in 2.2.

2.4. Depending on the protein to be stained, fix with 50 µL of 4% paraformaldehyde in PBS for 20 minutes at room temperature (for αSMA, NG2 and integrin α3β1) or with 50 µL of methanol for 10 min at -20 °C (for laminin-332 subunits).

2.5. Repeat the washing step as explained in steps 2.2-2.3.

2.6. Permeabilize the cells with 0.1% Triton-X in PBS for 4 min at room temperature.

2.7. Repeat the washing step as explained in steps 2.2-2.3 three times.

2.8. Incubate the spheroids in PBS containing 5% FBS and 2% BSA, for 1 h at RT to block non-specific protein interaction sites.

2.9. Incubate the spheroids with 30 µL of primary antibodies in PBS, 2.5% FBS and 1% BSA at 4 °C overnight. The following primary antibodies were used: anti-αSMA Cy-3 conjugated and anti-NG2 at 5 µg/mL; BM2 anti-laminin α3, 6F12 anti-laminin β3 and anti-laminin γ2 20 µg/mL.

2.10. Repeat the washing step as explained in steps 2.2-2.3 three times.

2.11. Incubate the spheroids with 30 µL of secondary antibodies in PBS, 2.5% FBS and 1% BSA at RT for 90 min. The following secondary antibodies used were: Alexa 488 anti-rabbit and anti-mouse (5 µg/mL).

2.12. Repeat the washing step as explained in steps 2.2-2.3 three times.

2.13. Incubate the cells with 30 µL of DAPI staining solution for 4 min at room temperature.

2.14. Repeat the washing step as explained in steps 2.2-2.3.

2.15. Place the spheroids on a glass slide, in a drop of PBS, using a glass Pasteur pipet.

2.16. Image the spheroids by fluorescence microscopy in a laser scanning microscope using a magnification of 10x. Take different optical cross-section of the spheroid at different optical planes of a z-stack (15-20 stack planes). To establish the laser settings in the microscope, use a spheroid composed of cells negative for the antigen of interest or a spheroid stained only with the secondary antibody. Reuse the same settings for all the samples that will be compared.

2.17. Analyze the microscopic images with ImageJ software as published previously[14]. The steps are summarized in **Supplemental Figure 1**. As background, determine the integrated signal density of a cell-free region of interest (ROI). Calculate the total corrected cell fluorescence (TCCF) as:

$$TCCF = integrated\ signal\ density - (area\ of\ selected\ spheroid \times mean\ fluorescence\ of\ the\ background) \quad \text{(Equation 1)}$$

2.18. Determine the total corrected fluorescence (TCF) of the spheroids as the TCCF normalized to the area of spheroid and the number of stacks.

$$TCF = \frac{TCCF\ of\ spheroid}{area\ of\ spheroids \times number\ of\ stacks} \quad \text{(Equation 2)}$$

3. RT q-PCR of homospheroids

3.1. To perform RT-qPCR, collect at least 288 spheroids (corresponding to three 96-well plates) into a 50 mL reaction tube. Centrifuge for 5 min at 1,000 x *g* and carefully remove the methylcellulose-containing supernatant by pipetting without disturbing the pelleted spheroids.

3.2. Wash with 1 mL of 1x PBS and transfer the spheroids to a 1.5 mL reaction tube. Centrifuge the spheroids with a bench top centrifuge for about 30-60 s at 1,000 x *g*. Carefully remove the PBS by pipetting, without disturbing the pelleted spheroids.

3.3. Dissociate the spheroids with 250 µL of 4 mg/mL collagenase B, 50 µg/mL DNase I, 2% BSA, 1 mM $CaCl_2$ in PBS at 37 °C for about 30-45 min by gently vortexing and pulsing intermittently for a few seconds every 5 min.

3.4. Repeat the washing step as explained in step 3.2.

3.5. Isolate the total RNA from the cells of disintegrated spheroids using a commercial RNA isolation kit, according to the manufacturers' instructions.

3.6. Reverse transcribe the isolated mRNA into cDNA with a commercial kit, according to manufacturers' instructions.

3.7. Quantify the transcription levels in replicates by real-time PCR. The primers used were: α-SMA: Fw 5'-CCGACCGAATGCAGAAG GA-3', Rev 5' ACAGAGTATTTGCGCTCCGAA-3'[15]; Laminin α3 chain: Fw 5'-GCTCAGCTGTTTGTGGTTGA-3', Rev 5'-TGTCTGCATCTGCCAATAGC-3'[16]; Laminin β3 chain: Fw 5'-GGCAGATGATTAGGGCAGCCGAGGAA-3' Rev 5'-CGGACCTGCTGGATTAGGAGCCGTGT-3'[17]; Laminin γ2 chain: Fw 5'-GATGGCATTCACTGCGAGAAG-3' Rev 5'-TCGAGCACTAAGAGAACCTTTGG-3' [16]; NG2: Fw 5'-CTGCAGGTCTATGTCGGTCA-3' Rev 5'-TTGGCTTTGACCCTGACTATG-3'[18]; integrin α3 subunit: Fw 5'-AGGGGACCTTCAGGTGCA-3' Rev 5'-TGTAGCCGGTGATTTACCAT-3'[19]; TOP-1: Fw 5'-CCAGACGGAAGCTCGGAAAC-3' Rev 5'-GTCCAGGAGGCTCTATCTTGAA-3'[20].

3.8. Correct the Ct values for the Ct-value of an endogenous reference gene such as TOP-1 using the equation: $2^{-\Delta\Delta Ct}$. ΔΔCt = ΔCt (target sample) – ΔCt (reference sample) and ΔCt= Ct of target gene or reference gene – TOP-I.

4. Flow cytometry analysis of integrin expression

4.1. For flow cytometric analysis, collect at least 288 spheroids (corresponding to three 96-well plates) into a 50 mL reaction tube. Centrifuge for 5 min at 1,000 x *g* and carefully remove the methylcellulose-containing supernatant by pipetting.

4.2. Wash with 1 mL of 1x PBS and transfer the spheroids to a 1.5 mL reaction tube. Repeat the centrifugation step and carefully remove the PBS by pipetting.

4.3. Dissociate the spheroids as described in step 3.3.

4.4. Repeat the washing step as explained in step 4.2.

4.5. To block non-specific binding sites and to prevent binding of detection-relevant antibodies to Fcγ-receptors (CD16, CD32, and CD64), incubate the cells in 100 µL of PBS containing 2% horse serum (or serum of another animal species, the antibodies of which will not be used in the experiment) and 0.1% BSA for 20 min at 4 °C with rotation.

4.6. In case of staining intracellular markers, fix/permeabilize the cells as described under step 2.2-2.5.

4.7. Incubate the cells with the primary antibodies in 100 µL of PBS containing 2% horse serum and 0.1% BSA for 30 min at 4 °C with rotation. Dilute the primary antibody to 10 µg/mL.

4.8. Wash with 100 µL of PBS + 0.1% BSA three times, with centrifugation steps in the top bench centrifuge at 1,000 x *g* in between.

4.9. Incubate the cells with the secondary antibodies in 100 µL of PBS containing 2% horse serum and 0.1% BSA for 30 min at 4 °C with rotation. Dilute secondary antibody to 10 µg/mL.

4.10. Repeat the washing step as explained in step 4.7.

4.11. Analyze 2.0 x 10^4 stained cells in a flow cytometer. Gate for single live cells via the FSC-SSC dot plot and analyze the fluorescence of the gated cells on the channel appropriate for the selected fluorophore.

5. Invasion assay using heterospheroids

5.1. Prepare the spheroid formation solution for heterospheroids, containing the different cell types and corresponding mediums, as summarized in **Table 1**. The cells had previously been transduced with lentivirus containing vectors for either mCherry or GFP expression.

5.2. Fill 100 µL of the spheroid formation solution in each well of the plate and incubate the plate in the cell culture incubator for 24 h to allow one spheroid to be formed in each well.

5.3. Prepare 60 µL of gelatinous matrix mixture containing 2 mg/mL of rat tail collagen I, 30% of commercial gelifying matrix and 2.5 µg/mL of laminin-332 in a 1.5 mL reaction tube. Rapidly distribute 15 µL in 3 wells of the µ-slide angiogenesis chamber. Embed one spheroid in each well already containing the gel.

5.4. If necessary, rapidly adjust the location of the spheroid to the center of the well with a pipette under the microscope.

5.5. Repeat steps 5.2-5.3 for the next heterospheroids and repeat again for the homospheroids.

5.6. Incubate the gels in the incubator for 1 h to solidify and, then, gently overlay the gels with cell culture medium.

5.7. Image the spheroids by fluorescence microscopy in a laser scanning microscope. Take different optical cross-section of the spheroid at different optical planes of a z-stack and at different time points of invasion (e.g., 0 h, 24 h, 48 h and 72 h).

5.8. Analyze the images by counting the number of invading cancer cells. Invading cells are those ones that have left the spheroid and entered the invasive area. The invasive area is defined as the ring area between the outer and inner perimeters given by the furthest invaded cell and by the spheroid border of the spheroid at the beginning of the invasion experiments, respectively, as outlined in **Supplemental Figure 2**.

REPRESENTATIVE RESULTS:

The results of this experimental design are published in Martins Cavaco et al.[13], which is recommended for further reading on the conclusions that were drawn from these experiments.

Figure 1, a representative image of an immunofluorescent spheroid, shows the immunostaining of the integrin $\alpha 3$ subunit of both immortalized normal fibroblasts and immortalized CAFs (**Figure 1A**), as well as the immunofluorescence quantification (**Figure 1B**) and the transcriptional levels of the integrin $\alpha 3$ subunit gene by qPCR (**Figure 1C**). This panel of results demonstrates that integrin $\alpha 3$ is up-regulated in iCAFs, as compared to the normal counterpart. This proved that integrin $\alpha 3\beta 1$ can be considered a marker for pancreatic fibroblasts differentiation. This was also demonstrated by flow cytometry studies[13].

FIGURE AND TABLE LEGENDS:

Figure 1. Expression of integrin α3 subunit by iNFs and iCAFs. The integrin α3 subunit is upregulated by iCAFs, reflecting its potential as a differentiation marker for pancreatic CAFs. (**A**) Immunofluorescent staining of homospheroids of normal pancreatic fibroblasts (iNFS) compared to the homospheroids of iCAF shows an increased signal of the integrin α3 subunit in the differentiated cells. (**B**) The fluorescence signal of the cells in the spheroid was quantified with the Z-stack images of the 3D spheroid, using the ImageJ software. Means ± SEM of three independent experiments are shown and compared by t-test (*, $p < 0.1$). (**C**) The cells obtained from the dissociated spheroids were analyzed for their transcriptional levels of integrin α3 subunit. Means ± SEM of ΔΔCt-values as fold changes from two independent experiments are shown and compared by t-test. Although not reaching significance level of 0.1, the transcriptional levels of integrin α3-encoding mRNA are higher in iCAFs than in iNFs.

Table 1. Cell composition of hetero- and homospheroids. The number of cells necessary to assemble the hetero- and homospheroids as well as the corresponding medium which should be used for the spheroid formation solution are summarized in the following table.

Supplemental Figure 1. Sequential steps for quantification of the immunofluorescent staining of a protein of interest in spheroids, using ImageJ.

Supplemental Figure 2. Sequential steps for quantification of the number of invading cancer cells in the invasion assay, using ImageJ.

DISCUSSION:

To develop an appropriate in vitro model to study CAF differentiation is a challenging task. After employing different approaches, we concluded that a 3D spheroid model is the more practical, physiological and clinically relevant model, in which the interplay between pancreatic carcinoma cells with immortalized CAFs can be studied. This model prevented spontaneous differentiation of fibroblasts, due to artefactual stressors such as stiffness of the cell culture plastic, at least in short-term culture conditions (up to 48 h). Although the exact stiffness of the spheroid used in these studies was not measured, Ito et al. evaluated the stiffness of human umbilical vein endothelial cells and mesenchymal stem cell heterospheroids using a robot integrated microfluidic chip[22]. The average stiffness-index of the heterospheroids was measured 1 and 3 days after assembly. The size of the spheroids was different, the MSC seemed to increase their aggregation capacity, which resulted in a decrease in size over time (from 135.5 µm to 99.8 µm)[21,22]. Consequently, the stiffness of the spheroid also increased from 5.0 x 10^{-3} to 7.5 x 10^{-3} Pa[22]. However, the stiffness of the spheroids composed of a heterotypic population of CAFs and AsPC-I might possess mechanical properties and stiffness values different from the ones of homospheroids made of a single population of fibroblasts/CAFs. This is still unknown and might be different from what was described by Ito et al[22]. The stiffness also depends on the amount and cross-linkage of cell-produced and deposited ECM, on the different cell-cell interactions within the spheroid, on the number of cells within the spheroid or on the duration of culture.

Like any other model, the experimental design involves some variables that need optimization such as the number of cells necessary to form the spheroids, the time points of treatment and microscopic imaging. The described experimental design was optimized with the aim of having a spheroid size, which would have minimal influence in the diffusion of nutrients and oxygen, as there is a diffusion limit due to mass transport limitations[9]. For example, the transport of oxygen in spheroids with a size greater than 150-200 µm is significantly affected, as well as the diffusion of glucose and lactate, in aggregates bigger than 300 µm[9,18]. Spheroids with diameters larger than 400–500 µm usually present a concentrically layered structure consisting of a necrotic core surrounded by a viable layer of quiescent cells and an outer rim of proliferating cells[9,24]. To avoid the limited diffusion of compounds, and to eliminate the problem of the inaccessible spheroid core, cells were treated with TGF-β1, BM2 and lebein-1 prior to spheroid formation, when the cells are individually suspended in the spheroid formation solution. Moreover, to allow the availability of oxygen and nutrients to most of the cells in the spheroid and to prevent the formation of a necrotic core, the number of cells per spheroid was reduced to 750-1000 cells, which form spheroids with a diameter of 140-200 µm.

It is important to thoroughly mix the different cell types in the spheroid formation solution, so the spheroids are composed by approximately the same number of cells, avoiding significant size

differences between the spheroids. Nevertheless, sometimes some spheroids can be slightly bigger than the other and that will result in more Z-stacks acquired in the microscope. Some spheroids might also deviate from the perfectly spherical form, but this is also taken in account when the ROI of the spheroid is delineated. These differences of size and shape are accounted in the normalized quantification values as explained in the next paragraph. Another important factor regarding the shape of the spheroid is the cell type. For example, fibroblasts and CAFs form spheroids with a nearly spherical form, while AsPC-I and PANC-I cells form a more disperse aggregate of cells.

For imaging the spheroids, cryosections of fixed spheroids can also be used, however the integrity of the spheroid may be compromised upon sectioning, depending on the cell type and the immunostaining protocol. Alternatively, the staining of the whole spheroid in its 3D structure, proved to be a simpler option. Acquiring microscopic Z-stacked pictures of the spheroid as a 3D structure takes on average 5-15 min per spheroid.

The immunofluorescent signals in images of spheroids are difficult to quantify as they represent planes of 3D structures. The method used was based on a previously described one, where the authors extrapolated and considered the spheroid to be a cell[14]. In this way, the fluorescence signal is corrected using **Equation 1**.

As background, the integrated signal density of a cell-free region of interest (ROI) was determined. The measured integrated signal density is the sum of the intensity values of the pixels within the selected ROI. Since spheroids are 3D structures, confocal images from different stacks are acquired. To process such images one can measure the fluorescent signal in each stack individually or use the sum of all stacks into a Z-projection. All the quantifications can be performed using ImageJ. The total corrected fluorescence (TCF) of the spheroids is determined as the normalized TCCF, considering the area of spheroid and the number of stacks, using **Equation 2**. This accounts for the varying sizes of spheroids and the discrepancy in the spherical shape. Analyzing the immunofluorescent staining of the spheroids using this method can take up to 5 min per spheroid.

Similarly, invasion of cells from spheroids into the surrounding stromal matrix can be analyzed by different algorithms. A straightforward method was previously described by Nowicki et al, in which the invasive cancer cells were counted[23]. In order to discriminate the invasive cells from the ones that do not invade, it is important to establish a spatial limit beyond which the cells are considered to have left the spheroid structure. Therefore, the starting point is the limit that corresponds to the perimeter of the spheroid images acquired at the initial time point (time 0), when the spheroids were embedded into the gel. The cell that invades the gel the furthest is considered the maximal distance an invasive cell can reach, and thus, it defines the outer rim of the invasion region. This method counts the invasive cancer cells that are present in the invading area, using the counting tool from ImageJ, and the ROI corresponding to the spheroid at time 0 h is added to the ROI manager and then transposed to the image of the same exact spheroid acquired 48 h or 72 h later. For this reason, it is important that the spheroid remains as close to

the center of the planes as possible, during the different times of acquisition. Counting the number of cells can take up to 5 min per spheroid.

Another important consideration is to distinguish the different cell types in the heterospheroid. This can be performed using live cell fluorescent dyes, which can be problematic when the cell type has a high cell division rate or if the experiment is to be performed for long periods of time. The more robust and reliable way to distinguish the two different populations is to develop cell lines expressing fluorescent proteins such as mCherry and GFP. The delivery of the expression vectors can be performed using lentivirus, which results in stable expression of these proteins.

ACKNOWLEDGMENTS:

We acknowledge Barbara Schedding's help in preparing the BM2 and lebein-1. We acknowledge Àgnes Noel for sharing her expertise in spheroid assays. We thank Sonja Schelhaas and Michael Schäfers for their help in handling lentiviral transfection under S2 conditions. We acknowledge Sabine von Rüden's assistence in preparing CAFs from pancreatic cancer tissue.

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under the REA grant agreement n◦ (316610) to J.A.E. Moreover, J.A.E. and A.C.M.C. was financially supported by the Deutsche Forschungsgemeinschaft (DFG) within the Cells-in-Motion Cluster of Excellence (EXC 1003-CiM). This project was also supported by Wilhelm Sander Stiftung (grant: 2016.113.1 to J.A.E.).

DISCLOSURES:

The authors declare no conflict of interest. This material reflects only the author's views and the European Union is not liable for any use that may be made of the information contained therein.

REFERENCES:

1. Pietilä, M., Ivaska, J., Mani, S.A. Whom to blame for metastasis, the epithelial-mesenchymal transition or the tumor microenvironment? *Cancer Letters*. **380** (1), 359–368, doi: 10.1016/j.canlet.2015.12.033 (2016).
2. Heldin, C.-H., Rubin, K., Pietras, K., Ostman, A. High interstitial fluid pressure - an obstacle in cancer therapy. *Nature Reviews. Cancer*. **4** (10), 806–813, doi: 10.1038/nrc1456 (2004).
3. Tani, T. et al. Pancreatic carcinomas deposit laminin-5, preferably adhere to laminin-5, and migrate on the newly deposited basement membrane. *The American Journal of Pathology*. **151** (5), 1289–1302 (1997).
4. Eble, J.A., Niland, S. The extracellular matrix in tumor progression and metastasis. *Clinical & Experimental Metastasis*. doi: 10.1007/s10585-019-09966-1 (2019).
5. Tomasek, J.J., Gabbiani, G., Hinz, B., Chaponnier, C., Brown, R.A. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nature Reviews. Molecular Cell Biology*. **3** (5), 349–363, doi: 10.1038/nrm809 (2002).
6. Sugimoto, H., Mundel, T.M., Kieran, M.W., Kalluri, R. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biology & Therapy*. **5** (12), 1640–1646, doi: 10.4161/cbt.5.12.3354 (2006).

7. Wells, R.G. The role of matrix stiffness in regulating cell behavior. *Hepatology (Baltimore, Md.)*. **47** (4), 1394–1400, doi: 10.1002/hep.22193 (2008).
8. Cavaco, A., Rezaei, M., Niland, S., Eble, J.A. Collateral Damage Intended—Cancer-Associated Fibroblasts and Vasculature Are Potential Targets in Cancer Therapy. *International Journal of Molecular Sciences*. **18** (11), 2355, doi: 10.3390/ijms18112355 (2017).
9. Mehta, G., Hsiao, A.Y., Ingram, M., Luker, G.D., Takayama, S. Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. *Journal of Controlled Release: Official Journal of the Controlled Release Society*. **164** (2), 192–204, doi: 10.1016/j.jconrel.2012.04.045 (2012).
10. Rousselle, P., Lunstrum, G.P., Keene, D.R., Burgeson, R.E. Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *The Journal of Cell Biology*. **114** (3), 567–576 (1991).
11. Eble, J.A., Bruckner, P., Mayer, U. Vipera lebetina venom contains two disintegrins inhibiting laminin-binding beta1 integrins. *The Journal of Biological Chemistry*. **278** (29), 26488–26496, doi: 10.1074/jbc.M301860200 (2003).
12. Kusuma, N. et al. Integrin-dependent response to laminin-511 regulates breast tumor cell invasion and metastasis. *International Journal of Cancer*. **130** (3), 555–566, doi: 10.1002/ijc.26018 (2012).
13. Martins Cavaco, A.C. et al. The Interaction between Laminin-332 and α3β1 Integrin Determines Differentiation and Maintenance of CAFs, and Supports Invasion of Pancreatic Duct Adenocarcinoma Cells. *Cancers*. **11** (1), 14, doi: 10.3390/cancers11010014 (2019).
14. Ansari, N., Müller, S., Stelzer, E.H.K., Pampaloni, F. Quantitative 3D cell-based assay performed with cellular spheroids and fluorescence microscopy. *Methods in Cell Biology*. **113**, 295–309, doi: 10.1016/B978-0-12-407239-8.00013-6 (2013).
15. Goldberg, M.T., Han, Y.-P., Yan, C., Shaw, M.C., Garner, W.L. TNF-alpha suppresses alpha-smooth muscle actin expression in human dermal fibroblasts: an implication for abnormal wound healing. *The Journal of Investigative Dermatology*. **127** (11), 2645–2655, doi: 10.1038/sj.jid.5700890 (2007).
16. Kim, B.G. et al. Laminin-332-Rich Tumor Microenvironment for Tumor Invasion in the Interface Zone of Breast Cancer. *The American Journal of Pathology*. **178** (1), 373–381, doi: 10.1016/j.ajpath.2010.11.028 (2011).
17. Chen, J. et al. Overexpression of β3 Chains of Laminin-332 is Associated With Clinicopathologic Features and Decreased Survival in Patients With Pancreatic Adenocarcinoma. *Applied Immunohistochemistry & Molecular Morphology*. **23** (7), 516–521, doi: 10.1097/PAI.0000000000000115 (2015).
18. Huang, C.-C. et al. Hypoxia-induced therapeutic neovascularization in a mouse model of an ischemic limb using cell aggregates composed of HUVECs and cbMSCs. *Biomaterials*. **34** (37), 9441–9450, doi: 10.1016/j.biomaterials.2013.09.010 (2013).
19. Wang, L., Wang, L., Gu, Y., Shu, Y., Shen, Y., Xu, Q. Integrin α6high Cell Population Functions as an Initiator in Tumorigenesis and Relapse of Human Liposarcoma. *Molecular Cancer Therapeutics*. **10** (12), 2276–2286, doi: 10.1158/1535-7163.MCT-11-0487 (2011).
20. Makizumi, R., Yang, W.-L., Owen, R.P., Sharma, R.R., Ravikumar, T.S. Alteration of Drug Sensitivity in Human Colon Cancer Cells after Exposure to Heat: Implications for Liver

Metastasis Therapy using RFA and Chemotherapy. *International Journal of Clinical and Experimental Medicine*. **1** (2), 117–129 (2008).
21. Saleh, F.A., Whyte, M., Genever, P.G. Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional in vitro model. *European Cells & Materials*. **22**, 242–257; discussion 257 (2011).
22. Ito, K., Sakuma, S., Kimura, M., Takebe, T., Kaneko, M., Arai, F. Mechanical characterization system using on-chip probe with wide range actuation. *2016 International Symposium on Micro-NanoMechatronics and Human Science (MHS)*. 1–2, doi: 10.1109/MHS.2016.7824204 (2016).
23. Curcio, E., Salerno, S., Barbieri, G., De Bartolo, L., Drioli, E., Bader, A. Mass transfer and metabolic reactions in hepatocyte spheroids cultured in rotating wall gas-permeable membrane system. *Biomaterials*. **28** (36), 5487–5497, doi: 10.1016/j.biomaterials.2007.08.033 (2007).
24. Lin, R.-Z., Lin, R.-Z., Chang, H.-Y. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology Journal*. **3** (9–10), 1172–1184, doi: 10.1002/biot.200700228 (2008).
25. Nowicki, M.O. et al. Lithium inhibits invasion of glioma cells; possible involvement of glycogen synthase kinase-3. *Neuro-Oncology*. **10** (5), 690–699, doi: 10.1215/15228517-2008-041 (2008).

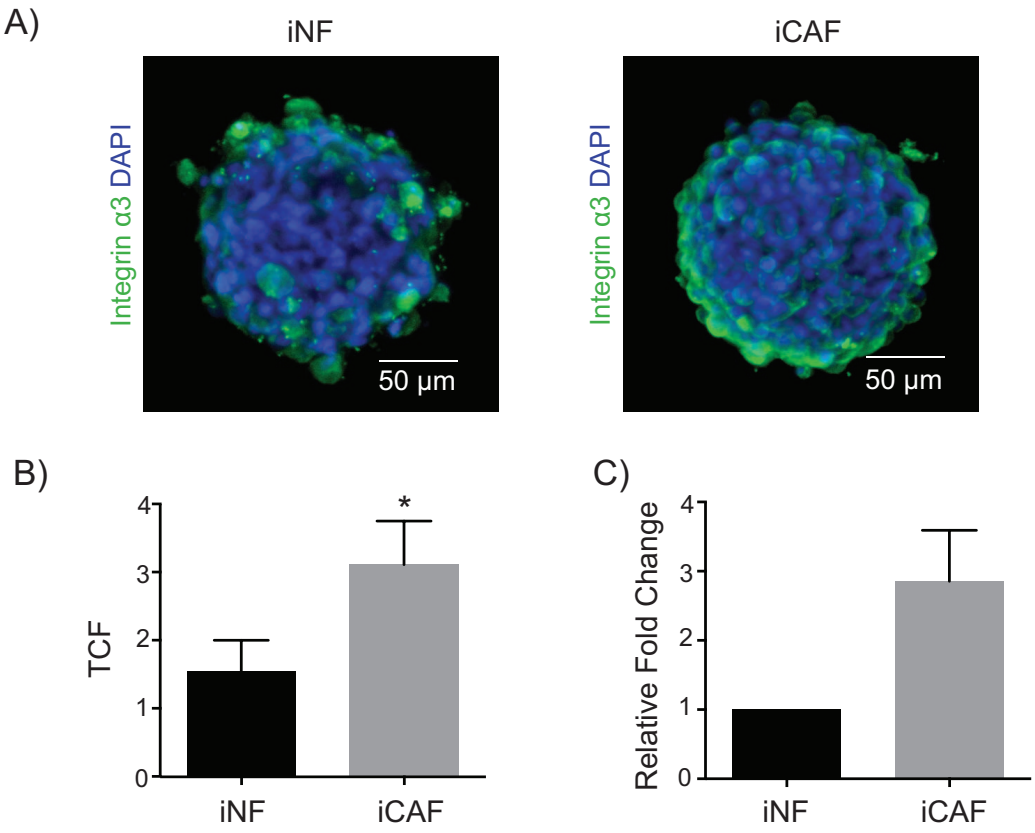


Figure 1.

Table 1

Heterospheroids		
Cells type (passage up to 25)	Number of cells	Medium (1 part methylcellulose +…)
mCherry-iCAFs + GFP-AsPC-I	400 CAF + 400 pancreatic cancer cells	1,5 parts cell MEM with 10% of FBS and 1% pen/strep + 1,5 parts of RPMI with 10% of FBS and 1% pen/strep
mCherry-α3KO-iCAFs + GFP-AsPC-I		
mCherry-iCAFs + GFP-PANC-I		1,5 parts cell MEM with 10% of FBS and 1% pen/strep + 1,5 parts of DMEM with 10% of FBS and 1% pen/strep
mCherry-α3KO-iCAFs + GFP-PANC-I		
Homospheroids		
Cell type (passage up to 25)	Number of cells	Medium (1 part methylcellulose +…)
mCherry-iCAFs	400 cells	3 parts MEM supplemented with 10% of FBS and 1% pen/strep
mCherry-α3KO-iCAFs		
GFP-AsPC-I		3 parts RPMI with 10% of FBS and 1% pen/strep
GFP-PANC-I		3 parts DMEM with 10% of FBS and 1% pen/strep

Name of Material/ Equipment	Company	Catalog Number
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAI	SIGMA-ALDRICH	D9542-10
6F12 anti-Laminin β3 subunit Mouse (monoclonal)		
A3IIF5 Anti-α3 integrin subunit Mouse (monoclonal)		
Acetone	SIGMA-ALDRICH	32201
Albumin Fraction V - BSA	AppliChem	A1391
Alexa fluor 488 Goat (polyclonal) anti-Mouse	Invitrogen	A11029
Alexa fluor 488 Goat (polyclonal) Rabbit	Invitrogen	A11034
Anti-laminin γ2 subunit Mouse (monoclonal)	Santa Cruz	sc-28330
Anti-NG2 Rabbit (polyclonal) Millipore, AB5320	Millipore	AB5320
Anti-α-SMA-Cy3 Mouse (monoclonal)	SIGMA-ALDRICH	C6198
AsPC-1 cell line	ATCC	
Bench centrifuge	Fisher Scientific	50-589-620
BM2 anti-laminin α3 subunit Mouse (monoclonal)		
Calcium Chloride ($CaCl_2$)	Fluka	21074
Centrifuge	Thermo Scientific	
Centrifuge tubes 50 mL	Corning	430290
Collagenase B	Roche	11088831001
Collagen-I, rat tail	Gibco	A10483-01
Confocal microscope	Zeiss	
DMEM (High glucose 4.5 g/L)	Lonza	BE12-604F
Dnase I	Roche	10104159001
Flow Cytometer	BD Biosciences	
Gelifying matrix	ThermoFisher Scien	A1413202
Goat IgG, isotype	DAKO	X 0907

Horse Serum	SIGMA-ALDRICH	12449-C
Human Primary Pancreatic Fibroblasts	PELOBiotech	PB-H-6201
Incubator	Heraeus	B6060
Laminin-332	Biolamina	LN332
MEM	SIGMA-ALDRICH	M4655
Microplate, 96 wells, U-bottom	Greiner Bio-One	650101
Microscope Slides	Thermo Scientific	J1800AMNZ
Mouse IgG, isotype	SIGMA-ALDRICH	I8765
Multi axle rotating mixer	CAT	RM5 80V
PANC-I	ATCC	
Paraformaldehyde	Riedel-de Haën	16005
Penicillin/streptomycin	Gibco	15140-122
QuantiTect Reverse Transcription Kit	Qiagen	205310
Rat IgG, isotype	Invitrogen	10700
Reaction tubes, 1.5 mL	Greiner Bio-One	616201
Real-time PCR cycler	Qiagen	
RNeasy Mini Kit	Qiagen	74104
Rotor Gene SYBR Green PCR Kit	Qiagen	204074
RPMI	Lonza	BE12-702F
TritonX-100	SIGMA-ALDRICH	X100RS
Vórtex	Scientific Industries	
µ-Slide Angiogenesis, uncoated	Ibidi	81501

Comments/Description

Homemade

Kindly provided by Prof. M. Hemler, Dana Faber-Cancer Institute, Boston

Kindly given by prof. Jorg Haier's Lab

Sprout

Kindly provided by Prof. Patricia Rousselle, CNRS, Lyon

Multifuge 1S-R

LSM 700 and 800

FACSCaliburTM

Matrigel, Geltrex

Kindly given by Prof. Jorg Haier's Lab

Rotor-Gene Q

Add glucose to 4.5 g (0.2 um filter) and 1% sodium pyruvate

Vortex-Genie 2

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	3D spheroid model: leaving the 2D approach behind and following a more physiological system for CAF differentiation and invasion in vitro studies.
Author(s):	Ana C. Martins Cavaco, Johannes A. Eble

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

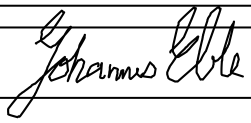
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the **internal** laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Johannes A. Eble
Department:	Institute of Physiological Chemistry and Pathobiochemistry
Institution:	University of Münster
Title:	Prof. Dr.
Signature:	
Date:	April, 11th, 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Westfälische Wilhelms-Universität Münster

medizinische fakultät
Westfälische Wilhelms-Universität Münster

Institut für Physiologische Chemie und Pathobiochemie ◆
Universitätsklinikum Münster ◆ 48149 Münster

To

Dr. Phillip Steindel

Review Editor

THE JOURNAL OF VISUALIZED EXPERIMENTS

Institute for Physiological Chemistry und Pathobiochemistry

Waldeyerstraße 15 ◆ 48149 Münster

Telephone: (02 51) 83 – 5 55 91
Secretary: (02 51) 83 – 5 55 92
Fax: (02 51) 83 – 5 55 96
E-Mail: acmcavaco@gmail.com

Lisbon, May 10th, 2019

Dear Dr. Steindel,

Dear Editor,

please find attached the revised version of our manuscript “3D spheroid model: leaving the 2D approach behind and following a more physiological system for CAF differentiation and invasion *in vitro* studies”.

We acknowledge the accurate and helpful peer review comments. All suggestions and comments of the reviewers have been addressed in the new version of our manuscript, which has improved thereby in its solidity and significance. All alterations in the text are highlighted in bold as well as the part of the protocol that is going to be included in the video.

Our point-to-point answers to all suggestions made by the reviewers are described below. We hope the manuscript is now suitable for publication in JOURNAL OF VISUALIZED EXPERIMENTS.

Yours sincerely,

Ana C. M. Cavaco

Rebuttal letter

Editorial comments:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
The text was proofread and we have hopefully corrected any spelling or grammar mistakes.

2. Please use American spellings (e.g., 'tumor', 'favorable').
The text was changed to American English.

3. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.
The manuscript was formatted according to the required guidelines.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Sigma-Aldrich, Thermo Fisher, Peproech, Falcon, RNeasy, Quantitect, Quantifast, Matrigel, etc.
All the references for commercial trademarks and company names were removed.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
The protocol encompasses about 6 pages and the steps that should be part of the video were highlighted in bold, making up to approximately 2 pages.

2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:

1. 2.2: What centrifuge speed (in x g)?
The relative centrifugal force (RCF) is 1000 × g. It was added to the text.

Figures, Tables, and Figure Legends:

1. Please remove the embedded figure from the manuscript. All figures should be uploaded separately to your Editorial Manager account.
The figure was removed from the manuscript.

2. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.
The table was removed from the manuscript.

3. Figure 1: What do the error bars and asterisk represent?

As explained in the legend of figure 1: * stands for $p < 0.1$. The error probability p fell under the significance level of 0.1 = 10 %, when the means ± SEM of three independent experiments were compared with the respective controls by t-test.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
All the materials were added to the table of materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The submitted article provides a description of a technique to create cancer spheroids with cancer-associated or normal fibroblasts. All the approaches to create 3D microenvironment as a cancer model are highly relevant nowadays, and so is the presented technique. However, there are some fundamental issues with how the manuscript was written and submitted. It is unclear of how the proposed technique is an advancement or improvement of already established cancer-fibroblasts models (including for example Khawar et al 2019 Neoplasia, Tsai et al 2018 BMC Cancer, Malik et al 2018 Matrix Biol). Notwithstanding, the described technique is straightforward and relevant to different cancer subtypes. Authors focus on pancreatic cancer 3D spheroids, and also highlight specific molecular targets (laminin-332, alphaSMA or NG2) that can be studied using this model.
Throughout the text, some issues were identified that should be addressed to increase the quality of the paper and support the technique.

The reviewer correctly noted that different authors have similar 3D approaches for different kinds of studies. Here, we described not only the invasion assay using cancer cells and CAFs, but also the possibility to use the spheroids for immunofluorescence detection of extracellular matrix proteins, and for dissociation of spheroids, allowing further studies of flow cytometry and quantitative PCR.
Furthermore, the three papers mentioned by the reviewer do not give detailed protocols for generating spheroids and their subsequent analyses, e.g. by immunofluorescence microscopy. Nor do they discuss the particularities and cautions that must be taken if spheroids are handled. This is of extreme importance, if one wants to use such model.

Major Concerns:

1) Section, lines 68-75 is speculative and without references. How imaging of PAA is challenging? They are transparent and used highly specifically for imaging purposes
We agree with the reviewer, the PAA gels are transparent and allow imaging. However, when using them we faced a couple of difficulties: 1) we could not prepare the functionalized PAA gels in our lab, because activation of PAA gels with the cross-linker, Sulfo-NHS failed, likely because of the fact that a higher power UV light must be used with potential formation of radicals that affect cell behavior. For this reason, we used commercial PAA gels of defined strength. 2) The number of cells that adhere to the gels of lower stiffness is strongly reduced. The low number of adherent cells made it impossible for us to obtain sufficient cell numbers to perform studies such as quantitative PCR or flow cytometry. 3) The PAA gels poured into the 6 well-plates have a long optical path and are almost impossible to be analyzed in an inverted confocal microscope.
For these reasons, we found 3D spheroids a better method because they can easily be generated in the own lab, are less expensive, provide enough cells for downstream applications and are accessible for analysis by both inverted and non-inverted microscopes.

2) The introductory section should be focus more on the development of spheroid techniques and how they have been improved, and how the proposed technique is different to the published one, not on speculation whether CAFs grow differently on stiff or soft substrates, especially as the methodology of the article does not study the stiffness of the grown spheroids.
The reviewer is right. Therefore, we changed the introduction and put less emphasis on the stiffness. We thank the reviewer for the suggestion.

3) Subsection in lines 88-93. It is not clear how the CAFs were validated to make sure the isolated cells were CAFS, have their phenotype changed over time, where they validated after immortalisation and how?

The CAFs were isolated from biopsies using dissociation with collagenase and cycles of trypsin detachment and attachment as well as cultivation in MEM medium. CAFs exhibit a very different cell morphology, compared to the pancreatic cancer cells, they are spindle shape, and a pure culture was obtained at the end of the isolation steps. The phenotype was also confirmed as activated fibroblasts with expression of αSMA and NG2, that are not expressed by normal fibroblasts. Stainings for these 2 markers were performed frequently, including before and after immortalization. Also the immortalized cells were compared to their non-immortalized counterparts for their expression of αSMA and NG2, and routinely verified to be CAFs during the course of the study

4) Protocol: the submitted manuscript would benefit greatly from a detailed description of the derivation of CAFs from biopsies and their validation and comparison against the normal fibroblasts. This is essential part of this paper and should be included here
We agree with the reviewer that immortalization of CAFs and normal fibroblast had been a critical part of the original study. However, within this JoVE article, the main focus of our protocol is the generation of homo- and heterospheroids of these cells. Therefore, we prefer to concentrate on the spheroid technology and to make a reference to the original manuscript of ours, where we, together with our collaborators from the department of surgery, isolated and immortalized the CAFs from pancreatic cancer biopsies.

5) Creating fibroblast homospheroids. It is unclear what is the relevance of creating fibroblast homospheroids. Is the proposed protocol supposed to replace the 2D culture of fibroblast to promote and support their phenotype? If so why the fibroblasts from the spheroids are not then used in the heterospheroids by mixing directly the 3D fibroblasts spheroids with cancer cells? are the fibroblast from 2D frozen cultures used to create 3D cancer-fibroblasts spheroids?
We apologize, if in the introduction was not clear enough, that the 3D spheroid model is meant to replace the 2D culture of normal fibroblasts in this experimental setting. We changed this in the revised manuscript to make this point even clearer. The goal was to keep fibroblasts in their non-differentiated state, so we could then stimulate the differentiation and study the effect of the laminin-332-integrin binding. We agree with the reviewer that this stimulation could have been performed using the co-culture system. However, we wanted to start this study with clearly defined populations of fibroblasts and cancer cells. These populations were verified with markers by PCR or flow cytometry. To this end, higher cell numbers were required and resourcing these primary cells from biopsies by FACS would not warrant the necessary provision of sufficient cell number, because of the low FACS yields. As a control, we stimulated fibroblast to differentiate into CAFs with TGF-β1, the main cytokine present in the tumor microenvironment and involved in CAF differentiation.
To meet the demand of well characterized cells in this study, we expanded both normal fibroblasts and CAFs first in 2D cell culture conditions and froze several vials. For each experiment, a new vial was used to directly prepare the spheroids.

6) Discussion

a. Authors claim that the proposed method prevents spontaneous differentiation of fibroblasts. The spheroids were cultured only for 24hr therefore there is no evidence of the fibroblast differentiation or loss of phenotype in long term culture. Additionally, no evidence was presented from spontaneous differentiation in 2D in their cell lines
In fact, here, we used the spheroids for short term studies. In our previously performed stiffness studies we used mouse fibroblasts, but it was clear that stiffness increased the expression of αSMA expression, the marker for differentiation. In contrast, in the 3D spheroid culture conditions, even the more differentiation-prone mouse fibroblasts showed a decrease in the expression of αSMA, whereas stimulation with TGF-β1 increased expression of αSMA and promoted fibroblast differentiation. This was clarified in the manuscript.

b. Authors compare their spheroids to spheroids based on human endothelial cells and mesenchymal cells. this direct comparison is not appropriate
We agree with the referee, that the stiffness of spheroids cannot be compared unless by physical measurements of the elasticity modulus. However, when we wrote “However, the stiffness of the spheroids composed of either a single population of fibroblasts/CAFs or a heterotypic population of CAFs and AsPC-I might possess different mechanical properties”, we did not intend to make a direct comparison. It is clear that different cell types might cause different stiffness values, depending on the intercellular junction, their force-exerting potential, their capacity to produce extracellular matrix, etc. Nevertheless, it is also accepted that a spheroid irrespective of the cell types will unlikely reach stiffness values as high as the one of the plastic of the cell culture flasks. We rephrased the respective sentences in the manuscript to avoid any inadvertent misunderstanding on this.

c. discussion is largely based on speculations of role of stiffness in spheroids development. As in the protocol the stiffness measurements were not included and are unknown between normal fibroblast spheroids or

cancer associated spheroids, this discussion does not have a link. The maintenance of fibroblasts in spheroids and their differentiation should be discussed, as that's the aim of this paper.
We followed the reviewer's suggestions and changed the discussion in order to less emphasize the stiffness of the spheroids. According to the guidelines of JoVE, we put more focus on the method itself. The results were already published and discussed, and we referred the reader to the paper for complementary information.

d. If authors have access to equipment to measure stiffness of their spheroids that would add important information of the differences between normal and CAFs spheroids and would then contribute to the discussed knowledge.
We fully agree with the reviewer's opinion. We discussed with biophysicists about ways to determine, or at least compare spheroids with respect to their stiffness in a way that can be performed in a biochemical lab without any highly sophisticated physical equipment for routine use. Together, we further pursue this point. Unfortunately, although it is indeed an important and interesting information, we have not yet established an easily applicable method nor set up a routine access to an equipment that measures stiffness. This would be far beyond the scope of this JoVE article.

e. No results were shown for the invasion assay while it has been discussed and speculated
We think that, the important point of the discussion section is that the reader understands how the analysis of this study is performed. The actual results were already published, and the reader should consult the paper. Since it can be confusing to the reader, paragraph 426-435 have been withdrawn.

Minor Concerns:

1) Line 40/41 and 47/48, when mentioning specific markers of pancreatic adenocarcinoma stroma (laminin-332) or CAFs markers (alphaSMA and NG2), the cited literature includes reviews papers (included self-cited review). Authors should also include the original research articles showing the role of those markers in pancreatic cancer or identifying CAFS.
The original research articles were included. We thank the reviewer for pointing it out.

2) In statement in lines 57-59 reference is missing
Following the reviewer's suggestion, we focused less in the stiffness of the substrate and rephrased the text. Now the statement is in lines 61-63 corresponding to the reference 8, which is the review paper in which we included this result.

3) There is no data supporting 3D fibroblast spheroids for the integrin expression
In the present protocol we gave an example of an α3 integrin staining of fibroblasts homospheroids and CAF homospheroids. Complementary data of the flow cytometry analysis of α3 integrin was published in the original article.

4) Why are the fibroblast spheroids grown only for 24 hours. This is not enough culture time for the cells to produce and deposit the relevant matrix proteins (point 1.5 in protocol)
Our studies showed that after 24h it is possible to detect deposition of laminin-332 in the spheroids, by immunofluorescence staining. The gene expression of the 3 chains of laminin-332 in CAFs present in the spheroids was also up-regulated, as compared to the normal fibroblasts. The main reason why we used 24h formation was because we treated the cells with both TGF-β1 and BM2 or lebein1. We could see significant differences already at this time of treatment. To perform the experiment for longer time would require repetitive addition of these compounds. Due to the uneven access of these compounds to all cells in the spheroid (cf. rim and center zones of the spheroids), the treatment would not be the same for all cells. This would allow different populations to arise.
To make the protocol more succinct, we don't include the staining of intracellular antigen or for detecting cell-bound ligands in the flow cytometry protocol.

5) Point 1.2. where the cells thawed and places into methylcellulose solution without expanding in 2D? What was the passage of the frozen samples? What was the viability of the cells after thawing? Where the dead cells excluded from the thawed samples?
The cells were thawed, centrifuged and counted with trypan blue, taking into account only the viable cells. Mostly, alive cells actively cluster into spheroid. The formed spheroids were taken from the formation wells, and most of the dead cells were left behind. The cells used were passaged up to 25 times.

6) Point 2.3. how were the spheroids washed? Mixed by pipetting or gently vortex or on a shaker
The spheroids were washed by adding the PBS to the reaction tube, and ressupending the spheroids in the solution. Neither pipetting nor vortexing were used so the spheroid structure was kept intact.

7) Point 2.5 and other points - washing in explained in steps 2.2 and 2.3
Thank you for pointing it out, "… and 2.3." was added.

8) Point 2.9 - dilution of the anti-alpha SMA antibody
The dilution of the antibody was the same as the NG2, 5 µg/mL, "… and…" was added to the phrase. We thank the reviewer for pointing it out.

9) Point 2.16 - what were the parameters of the microscope used, laser power, objective used, steps in the z-stack
The parameters of the microscope varied, depending on the staining and the amount of antigen present in the cell. The laser power will also depend on the laser of the microscope used by the reader. The number of z-stacks might also vary depending on the size of the spheroid, but we added in the text the average range of z-stacks used (15-20). We added the objective to the text, but the other microscopic parameters are subject to the microscopic equipment and will have to be optimized by the reader. Nevertheless, we included in the text that the negative control should be used for optimizing the sets of the microscope.

10) Point 2.17 - It would be beneficial to add more detailed explanation of the analysis, including for a example a diagram of the steps when using ImageJ software or images showing selection of ROIs
We are grateful for this advice and have added this information to the revised manuscript.

11) Section 3. What was the control used for RT qPCR. Were the normal fibroblast spheroids used as the control baseline or 2D cultured cells?
All the cells used for the RT qPCR, both normal fibroblasts and CAFs, were kept as spheroids for 24h, dissociated and directly lysed and the RNA isolated. The RT qPCR values of the normal fibroblasts were taken as the reference value, unless otherwise stated in the figure legend.

12) Point 3.7 - source of the primers, where they designed in-house or pre-made, validation of the primers, primer efficiency?
The primers used were pre-designed and selected from already published data. The references are indicated after each primer.

13) Point 3.8 - is using only TOP-1 as a housekeeping gene enough. Why this house keeping gene was chosen?
This house keeping gene was not specifically chosen because of the spheroid culture condition or the cell type. It is an accepted standard housekeeping gene used in our and numerous other labs. We used only this one housekeeping gene in these experiments due to the limited amount of RNA isolated from the spheroids.

14) Section 4. Was validation done to make sure you don't lose the expression of receptors upon disruption of the spheroids? For extracellular markers, was the analysis done on not-fixed samples? Was live/dead dye included? What was the viability of the fibroblast spheroid?
Cells expressing the integrins of interest in normal cell culture conditions were also assembled in spheroids. Afterwards, for analysis, they were dissociated, stained and characterized by flow cytometry. The cells used for these studies were not fixed, just kept on ice to avoid receptor internalization. Furthermore, we could confirm the up-regulation of integrin $\alpha 3$ in CAFs, compared to the normal fibroblasts. No live/dead dye was included in the spheroid formation step to avoid inadvertent effects on the spheroid behavior. However, for flow cytometric analysis, cells were gated for life cells and cell debris was excluded from analysis.

15) Section 5. Why for the invasion study the homospheroid had 400 cells while for other experiments 750 cells?
Although the homospheroids were not compared to the heterospheroids we decided to use the same total amount of cells used in the heterospheroids, irrespective of cell type. We used an equal number of both cell type to form the 750 cells-spheroids.

16) It is unclear from all the text of what is the obtained size of the spheroids and how reproducible this is. This information should be included.
Thank you for pointing it out. We have added this important information to the discussion section of the revised manuscript.

17) Point 5.7 - imaging parameters should be included
As we have explained in point 9) above, these parameters depend on the microscope.

18) Point 5.8 - more details on the analysis steps

The imageJ steps to follow were added in work-flow chart.

Overall, the manuscript is lacking detailed descriptions of the techniques described, validation of the methodologies, and including representative results and analysis approaches, that are mentioned in the methodology protocols. Authors discuss various variables of the spheroids without having done that specific analysis, like spheroid stiffness or the matrix stiffness, or production of ECM.

We think that, as we have added additional information and details on experimental parameters, the revised protocol is succinct enough that readers can follow it to generate spheroids and to analyze them for the expression of proteins by immunofluorescence microscopy.

I would advice authors to focus on a specific part of the technique, like isolation, culture, and validation of CAFs vs normal fibroblasts, and the validation of fibroblast spheroid assay to show the improvements of that method of culturing and maintenance of fibroblasts phenotype over standard 2D or soft gel methods.

We trust that by revising the manuscript we have focused on one methodological work flow of spheroid formation and immunofluorescent analysis thereof. We have revised the introduction and the discussion sections in line with the reviewer's suggestion and trust that it has improved according to the reviewer's suggestions.

Reviewer #2:

Manuscript Summary:

The aim of this protocol is to develop a 3D in vitro model to study the effect of tumour microenvironment on physiological development of CAFs differentiation. Authors and colleagues have already published their research findings in regards to the role of laminin-332 and its receptor integrin α3β1 in this differentiation process of NF and CAFs. Here they are stating their systematic method of how they use the 3D mono and co-culture (homo or heterospheroids) approach to understand the biology.

Minor Concerns:
This is a relevant protocol to current research obstacle and understating of pancreatic cancer biology and treatment options. Pancreatic cancer is one of the deadliest type of cancer worldwide. Early detection and surgery is still the only suitable path for this cancer and even in a small subgroup of patients despite the surgery, disease comes back and the overall survival remains poor. Therefore understanding the tumors microenvironment of this cancer would lead to better understanding the biology and physiology to move toward better therapy and prolong patient's survival benefits.

As a methodological manuscript, I see a very strong relevance of title and the given methodology. Others have described the 3D spheroids platform in many different types of research oncology and in the most recent years for pancreatic models and CAFs (Mackenzie Lee Goodwin, Sumi Kanthraj Urs, Diane M. Simeone, 2019, Pancreatic Microtumors: A Novel 3D Ex Vivo Testing Platform).

Few points:

1. Authors should statue here, the reason why this method more applicable than already described methods by others.
We trust that the heterospheroid model of tumor cells and CAFs recapitulate the tumor mass much better than the 2D co-culture system. Taken alone the fact that mechanical stiffness of the substrate causes fibroblast differentiation into αSMA-expressing CAF-like fibroblasts, made us explore this heterospheroid model system. Its generation and its analysis by for instance immunofluorescence microscopy allowed us new insights into CAF-differentiation and the role of CAFs' integrin $\alpha 3\beta 1$ in tumor progression. Hence, this manuscript highlights the methodology that brought up these results.

2. There is no description or emphasis on imaging the spheroids before immunofluorescence stating as that is a very important part to make sure that the 3D spheroids keep their integrity. 24hrs of spheroids formation is very short to recapitulate the right 3D condition as cells have only been out of plastic for 24hrs which is not long enough to recapitulate the right physiological as well as environmental condition for the cells.
The period of time is relatively short, but fibroblasts form spheroids very fast and 24h are enough for production and deposition of laminin-332 and for differentiation of fibroblasts upon treatment with TGF-$\beta 1$. Furthermore, to continue the treatment we performed would be necessary to add the compounds to the already formed spheroids. Diffusion of such compounds might not be the same. To keep the same characteristics of the formed spheroid and reproducible results we prepared the heterospheroids in the same

way as the homospheroids. We also trust that the two cell types during the 72h have enough interaction to promote the invasion, as we could see in our results published in the original paper.

3. I would suggest author to attach few images of the spheroids at 24hrs to show that the spheroids formation was completed and repetitive washing and staining procedure does not interrupt the integrity.
The image 1 is a representative image of spheroids that were formed in 24h. They were stained according to the described protocol, so that the integrity of the spheroids was not compromised.

4. The type of instrument for this model are not listed and only mentioned about the analysis on image J. it would be great if the authors would write the timing of how long it takes each images to be acquired and time needed for the analysis too.
Following the referee's suggestions, we have added this information to the revised manuscript.

5. Figures and a list of table presented, however I could not find the figure 2. Figure 1 has not been explaining well specially figure 1c and more information needed.
Figure 2 was originally in the discussion section. We have replaced the former figure 2 by a flow Chart, in which the former figure 2 was included.

6. Overall, I will personally be more interested if there are more images available for the readers to visualise the results in specific invasion as that is a novelty of this methodology paper.
Representative images were added, including of the invasion assay and its quantification.

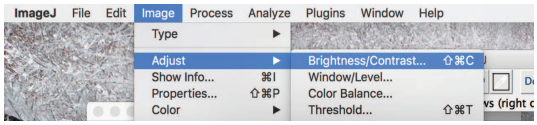
Supplemental Figure 1-1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure_1-1.eps

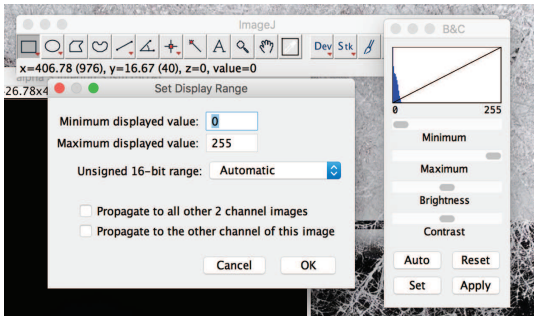
Open the original Z-stack image in the microscope format on ImageJ/Fiji software and follow the steps:

1. Make sure all images you compare display the same values (min. 0 and max. 255)

1.1.

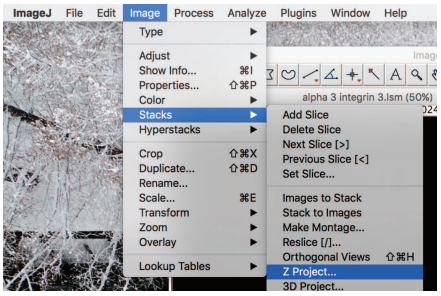


1.2.

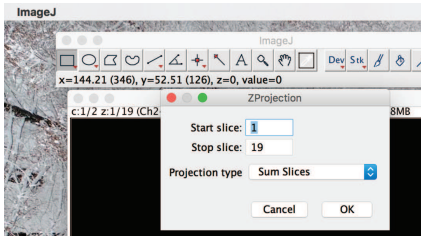


2. Convert the images to stack projection type Sum Slides. At this point enter in the excel datasheet the number of stacks e.g. 19

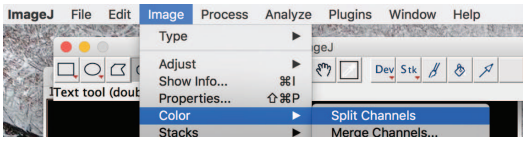
2.1.



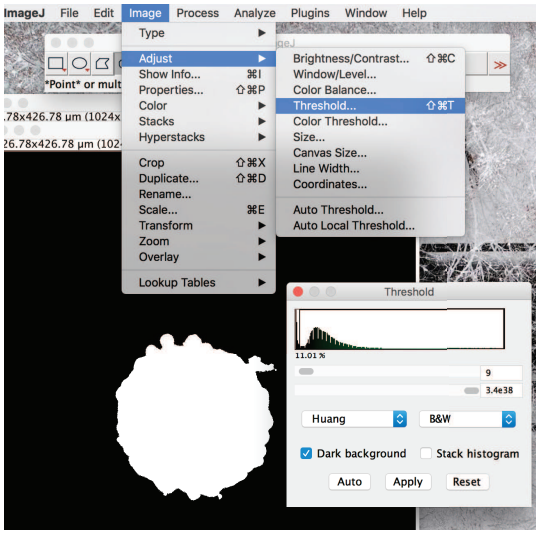
2.2.



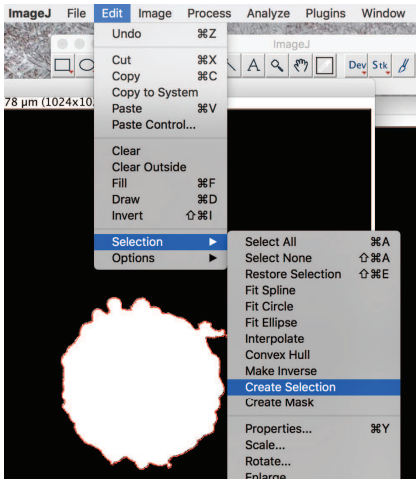
3. Split the channels



4. Select the spheroid area in the image of the channel with the staining of interest. Use e.g. the threshold (Huang*)



5. Create a selection from the threshold image

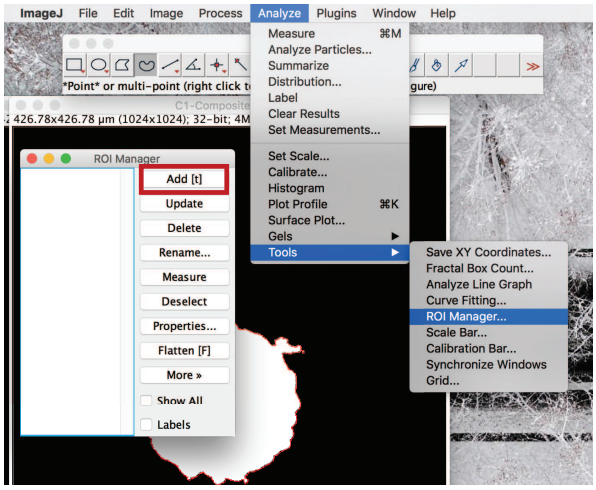


*Huang is an auto-thresholding method. Depending on the staining, one must choose a method that includes the all spheroid, for an accurate measure of the integrated density in the ROI

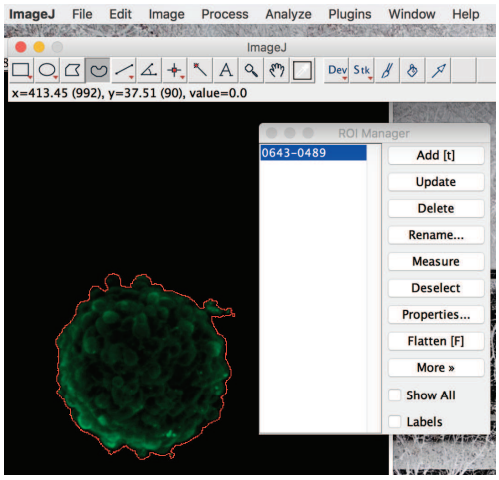
Supplemental Figure 1-2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure_1-2.eps

6. Add the selection to the ROI manager

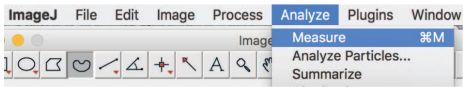


7. Close the threshold image and open the original image again, repeat steps 1-3 and select the staining of interest

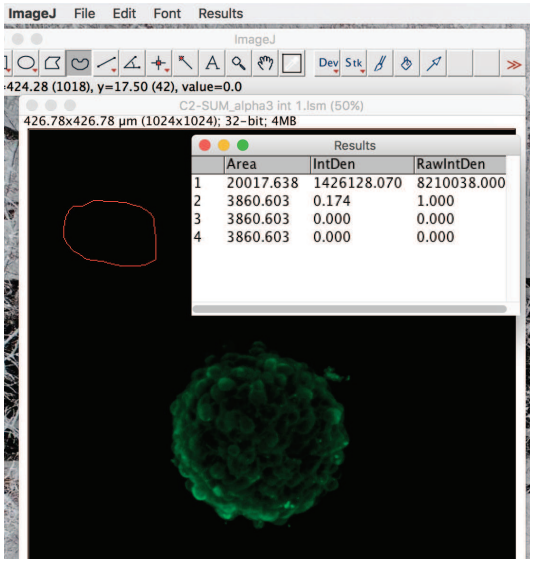


8. Measure the area and the integrated density of the selection (value) as well as the background (values 2-4)

8.1.



8.2.



8.3. Transfer all the values in the excel datasheet and apply equations (1) and (2)

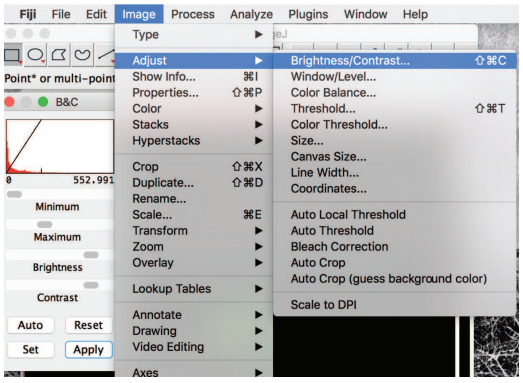
Supplemental Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental_Figure_2.eps

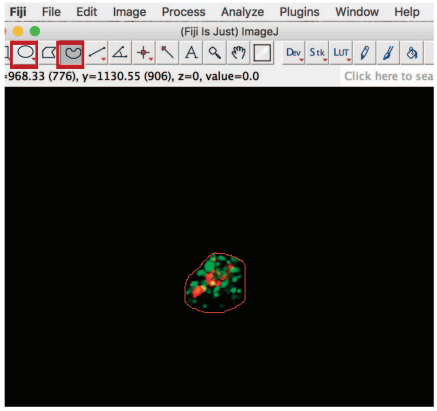
Open the original Z-stack image in the microscope format on ImageJ/Fiji software, the same spheroid at time 0 and time 72h, and follow the steps:

1. Convert the images to stack projection as described in step 2 of flow chart 1

2. If necessary increase the contrast of the images so you can clearly see all the cells



3. Manually delineate the time 0 spheroid rim using the freehands selections or oval tool

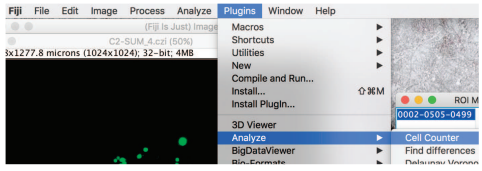


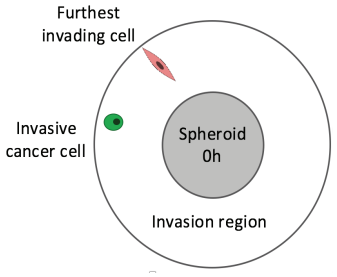
4. Add the selection to the ROI manager as described in step 6 of the flow chart 1

5. Close the image of the spheroid at time 0 and split the channels of the the spheroid at 72h that was also converted to a stack pro-jection as described in step 3 of flow chart 1

6. Use the ROI in the manager in the split image of the channel that depicts the cancer cells or the cells of interest Slightly move the ROI to the center of the spheroid, if thit is not already there. Update the ROI

7. Count the cells in the invasion region using the plugin cell counter





8. Initialize the counter, select the type of counter and count the cells manually by clicking on top of the cell. The number 1 will show on top of the cell