Cell Culture and Coculture for Oncological Research in Appropriate Microenvironments

Apekshya Chhetri,[1,10] Shirisha Chittiboyina,[1,2,10] Farzaneh Atrian,[1,10] Yunfeng Bai,[1,10] Davide A. Delisi,[1] Rahim Rahimi,[3,4] John Garner,[5] Yuri Efremov,[4,6] Kinam Park,[5,7,8] Rabih Talhouk,[9] and Sophie A. Lelièvre[1,2,8,11]

[1]Department of Basic Medical Sciences, Purdue University College of Veterinary Medicine, West Lafayette, Indiana
[2]3D Cell Culture Core (3D3C) Facility, Birck Nanotechnology Center, Discovery Park, Purdue University, West Lafayette, Indiana
[3]Department of Materials Engineering, Purdue University, West Lafayette, Indiana
[4]Birck Nanotechnology Center, Discovery Park, Purdue University, West Lafayette, Indiana
[5]Akina, Inc., West Lafayette, Indiana
[6]School of Mechanical Engineering, Purdue University, West Lafayette, Indiana
[7]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, Indiana
[8]Center for Cancer Research, Purdue University, West Lafayette, Indiana
[9]Department of Biology, American University of Beirut, Beirut, Lebanon
[10]First coauthors
[11]Corresponding author: *lelievre@purdue.edu*

With the increase in knowledge on the importance of the tumor microenvironment, cell culture models of cancers can be adapted to better recapitulate physiologically relevant situations. Three main microenvironmental factors influence tumor phenotype: the biochemical components that stimulate cells, the fibrous molecules that influence the stiffness of the extracellular matrix, and noncancerous cells like epithelial cells, fibroblasts, endothelial cells, and immune cells. Here we present methods for the culture of carcinomas in the presence of a matrix of specific stiffness, and for the coculture of tumors and fibroblasts as well as epithelial cells in the presence of matrix. Information is provided to help with choice and assessment of the matrix support and in working with serum-free medium. Using the example of a tissue chip recapitulating the environmental geometry of carcinomas, we also highlight the development of engineered platforms that provide exquisite control of cell culture parameters necessary in research and development.

Keywords: cancer • collagen I • epithelium • extracellular matrix • fibroblast • tumor

How to cite this article:
Chhetri, A., Chittiboyina, S., Atrian, F., Bai, Y., Delisi, D. A., Rahimi, R., Garner, J., Efremov, Y., Park, K., Talhouk, R., & Lelièvre, S. A. (2019). Cell culture and coculture for oncological research in appropriate microenvironments. *Current Protocols in Chemical Biology*, e65. doi: 10.1002/cpch.65

INTRODUCTION

Several decades of research have clearly established that the extracellular matrix (ECM), i.e., a water-rich meshwork of proteins and polysaccharides, does not simply support

CURRENT PROTOCOLS A Wiley Brand
Current Protocols in Chemical Biology e65
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpch.65

cells. Rather, it provides a dynamic microenvironment, owing to the presence of various signaling molecules and to the specific strength of its network of fibers that feed biochemical and mechanotransduction pathways within cells. There are two major types of ECM: (i) the interstitial matrix that surrounds cells and tissues, and (ii) the basement membrane, a specialized form of ECM that separates epithelial cells from the surrounding stroma (Bonnans, Chou, & Werb, 2014; Theocharis, Skandalis, Gialeli, & Karamanos, 2016). Among the interstitial fibrous proteins, collagen I is the most abundant, and its density and cross-linking greatly contribute to microenvironmental stiffness (Lin & Gu, 2015).

Even though the basic components of natural matrices are the same, differences exist depending on the type of tissue or organ, and these differences participate in dictating the specific phenotype and function of cells. Importantly, as proposed in the early 1980s (Bissell, Hall, & Parry, 1982), a body of literature has confirmed the existence of a bidirectional influence between the cells (all the way to the cell nucleus) and the ECM that participates in tissue development and the progression of diseases like cancers. Therefore, studying cell and tissue phenotypes *in vitro*, regardless of normal or diseased status, is best served by a physiologically relevant microenvironment in cell cultures. At minimum, ECM molecules should be provided as inducers for cells to secrete and organize a complete matrix, hence leading to tissues with an architecture that resembles the situation *in vivo*. When cell culture promotes *in vivo*–like tissue architecture, it is called three-dimensional (3D) cell culture.

Differences between tumors and normal tissues encompass not only cell phenotypes, but also the microenvironment. The tumor microenvironment constitutes an active field of research considering its important capacity to readily control the behavior of tumor cells (Spill, Reynolds, Kamm, & Zaman, 2016). The remodeling of the ECM that contributes to increased matrix stiffness is a phenomenon that was revealed more than a decade ago (Paszek et al., 2005), and which is now commonly observed in tumors (Fullár et al., 2015; Leight, Drain, & Weaver, 2016). Incorporating a defined ECM stiffness in models of cancer reproduced *in vitro* has become an important goal of 3D cell culture. In addition to an increased density of fibrous ECM molecules produced via secretion by cells, a major mechanism that participates in local remodeling of the tumor microenvironment is degradation of collagen by metalloproteinases (Conlon & Murray, 2018). Collagenase is one such enzyme, essential for the degradation of collagen *in vivo*, but also used *in vitro* to release tumor nodules from cell culture.

The tumor microenvironment also comprises cells contributing to tumor behavior, e.g., stromal cells or fibroblasts, specifically known as cancer-associated fibroblasts (CAFs), which participate in ECM remodeling, as well as immune cells (Cohen et al., 2017). A standard method to study the interaction of cells in the microenvironment with tumors *in vitro* is to conduct co-cultures (i.e., cultures of different cell types in the same culture vessel) enabling paracrine exchange and alterations in matrix stiffness as appropriate. Differences in phenotype for stromal as well as tumor cells have been shown in co-cultures compared to monocultures, illustrating the mutual influence of these cells in promoting cancer progression (Northey, Przybyla, & Weaver, 2017).

In this article, we present two sets of protocols related to the study of epithelial glandular cancers (or carcinomas), which represent the bulk of the global cancer burden. In the first set, Basic Protocol 1 discusses the importance of the choice of the ECM and its stiffness for the culture of tumors cells. Specifically, it presents the culture of cancer cells within a collagen I gel of chosen stiffness. It is followed by Alternate Protocol 1 describing drip culture with an Engelbreth-Holm-Swarm (EHS) gel of preinvasive cells that require contact with a basement membrane, and Alternate Protocol 2 describing how a nonbiological gel can be used for the culture of tumors on top of the gel. The accompanying

Table 1 Stock, Working and Final Concentrations of Cell Culture Additives with Their Storage Conditions

		Initial stock concentration		Aliquot with working stock concentration		Final concentration
Additive	Company		Expiration at −80°C		Expiration at 4°C	In cell culture medium
Prolactin	Sigma L-6520	30.03 I.U./ml (1 mg/ml)	1 year	1 mg/ml	1 month	5 µg/ml
Insulin	SIGMA I-4011	2 mg/ml	6 months	100 µg/ml	1 month	250 ng/ml
β-estradiol	SIGMA E-2758	8 mg/ml (0.03 M stock)	1 year	2.67×10^{-5} µg/ml	1 month	2.67×10^{-8} µg/ml (or 0.1 nM)
Hydrocortisone	SIGMA H-0888	5 mg/ml (1.4×10^{-2} M)	1 year	0.5 mg/ml	1 month	0.5 µg/ml (or 1.4 µM)
Sodium selenite	BD-Biosciences 354201	20 mg/ml	3 months	2.6 µg/ml	1 week	2.6 ng/ml
Transferrin	SIGMA T-2252	20 mg/ml	3 months	20 mg/ml	1 month	10 µg/ml
Epidermal growth factor	Corning Life Sciences 354001	20 µg/ml	3 months	20 µg/ml	1 week	5 ng/ml
Fibroblast growth factor	ThermoFisher PHG0264	10 µg/ml	1 year	10 µg/ml	1 week	2.5 ng/ml
Transforming growth factor- β	ThermoFisher PHG9204	300 ng/ml	1 year	60 ng/ml	1 week	7.5 pg/ml
Soybean trypsin inhibitor	SIGMA, T-6522 type I-S	10 mg/ml	6 months	10 mg/ml	2 weeks	0.18 mg/ml

Support Protocol 1 details preparation steps for the collagen I gel, Support Protocol 2 outlines a method for selecting an appropriate matrix for the type of cells to be placed in culture, and Support Protocol 3 describes one possible way to measure matrix stiffness with cell cultures. The second set of protocols presents examples of cultures pertaining to integrated studies with non-neoplastic cells from the tumor microenvironment known to control cancer behavior. Specifically, Basic Protocol 2 presents (i) the coculture of tumor nodules with fibroblasts within a collagen I matrix and (ii) the coculture of tumors within hemichannels, mimicking portions of mammary ducts, in the presence of non-neoplastic epithelial cells that are located near carcinomas. It is followed by Support Protocol 4, which explains the calculation of cell concentrations for seeding in coculture based on the ratio of cell types observed *in vivo*, and by Support Protocol 5, which presents important information on how to wean cells off serum and assess the impact on cell phenotypes. In the Reagents and Solutions section, we have also included detailed information on the preparation of the additives that we use for the serum-free culture of the cells presented in these protocols.

NOTE: The cell culture protocols presented in this article require specific steps and good organization to prepare the appropriate cell culture medium if one wishes to work with serum-free medium. Please read the Reagents and Solutions section and review Table 1 in order to adequately prepare for serum-free cell culture.

BASIC PROTOCOL 1

CULTURE OF CANCER CELLS IN COLLAGEN I MATRIX OF SPECIFIC STIFFNESS LEVEL

The purpose of this method is to place cancer cells in a microenvironmental context that provides an optimal level of constraints for them to display their phenotype. For instance, cancer cells have different degrees of invasive capabilities, and a matrix too stiff or not stiff enough would influence such capabilities. A similar issue might occur with proliferation capabilities. Most cancer cells make their own ECM components, but carcinomas (the frequent cancers of glandular epithelial origin) grow within the interstitial matrix that normally delineates tissues in an organ; the basis for such matrix is collagen. There are many types of collagens depending on the organ (Kular, Basu, & Sharma, 2014). The protocol detailed below is focused on collagen type I (collagen I), the major constituent of the microenvironment of carcinomas. We will use examples of breast carcinomas that recapitulate ductal carcinoma in situ (DCIS), a noninvasive form of cancer, and invasive ductal carcinomas (IDC) with low and high aggressiveness based on their invasive and metastatic potentials. The steps included in the protocol are the management of cells prior to culture within collagen, the preparation of collagen and embedding of cells (to provide a layer thin enough for direct immunostaining), the observation of cells within collagen, and the release of cells from collagen for further desired analyses.

Materials

HMT-3522 T4-2 cells at 80% confluence in 25-cm^2 (T-25) or 75-cm^2 (T-75) tissue culture flasks in H14 serum-free medium:

IDC T4-2 cells belong to the HMT-3522 series containing non-neoplastic S1 cells, preinvasive S2 cells, and invasive T4-2 cells (Briand, Petersen, & Van Deurs, 1987; Briand, Nielson, Madsen, & Peterson, 1996; Rizki et al., 2008) and were developed in H14 serum-free medium (see Reagents and Solutions and Table 1). They can be obtained from the European Collection of Authenticated Cell Cultures (ECACC; Catalog #98102212), from Sigma-Aldrich (cells are from ECACC), or by contacting Mina J. Bissell (Lawrence Berkeley National Laboratory, Berkeley, California). The T4-2 cells were established when, after 238 passages, the S2 cells in the series became tumorigenic in mice. Re-inoculation of these cells into mice led to

formation of another tumor from which the T4-2 cell line was ultimately derived (Briand et al., 1996).

H14 serum-free medium (also see Reagents and Solutions and step 6 below):

Note that Table 1 contains all cell culture additives used in the different protocols in this article. For the specific list of additives depending on the cell line, refer to the protocol steps.

Dulbecco's Modified Eagle Medium/F12 modification (DMEM/F12; Thermo Fisher Scientific, Catalog #12400-024; also see recipe and Table 1)

Prolactin (see recipe and Table 1)

Insulin (see recipe and Table 1)

Hydrocortisone (see recipe and Table 1)

β-Estradiol (see recipe and Table 1)

Sodium selenite (see recipe and Table 1)

Transferrin (see recipe and Table 1)

Collagen I (PhotoCol® kit for preparing tunable collagen I; Advanced Biomatrix, Catalog #5201-1KIT).

Trypsin-EDTA (0.25% trypsin/1 mM tetrasodium EDTA; Gibco, Catalog #25200-056)

Soybean trypsin inhibitor (SBTI; T-6522 type I-S; BD Biosciences Catalog #354201; SBTI is prepared in Milli-Q water for a 10 mg/ml stock; see Table 1 and Reagents and Solutions for details)

Collagenase (Advanced Biomatrix, Catalog #5030-50 mg bottle; see annotation to step 22, below)

Phosphate-buffered saline [PBS; prepared in the laboratory as a 10× stock (76 g NaCl, 18.76 g Na_2HPO_4, 4.14 g NaH_2PO_4, completed to 1000 ml with deionized water, with pH adjusted to 7.4, and autoclaved)]

Dulbecco's Modified Eagle Medium/F12 modification (DMEM/F12; Thermo Fisher Scientific, Catalog #12400-024; see recipe)

Light microscope

Laminar flow hood

Ice bucket

Cell culture–compatible plastic tubes (15- and/or 50-ml; Falcon; ThermoFisher, Catalog #352095)

4-well chambered slides (used for direct immunostaining of cultures; Falcon; ThermoFisher, Catalog #354104)

Petri dishes to contain the chambered slides (VWR Catalog #734-2321)

Additional reagents and equipment for preparing collagen type I (Support Protocol 1)

Management of cells prior to culture within collagen I

1. Use T4-2 cells at 80% confluence from a T-25 or T-75 flask depending on the number of cells needed for an experiment. The number of cells depends on the type of culture vessel (refer to Table 2 for information on cell numbers). Usually, 4 to 5 million cells are expected from a T-75 flask at 80% confluence.

   It is essential to control the passage number of these cells for experiments, and we recommend keeping all experiments within a window of 10 passages, as phenotypic drifts are typically observed in 3D cell cultures when cells are used beyond a certain number of passages. DO NOT allow the cells to exceed 70% to 80% confluency before using, or for routine passages; otherwise, you will enrich the population with cells that are less aggressive. This phenotypic drift is usually only seen in 3D culture, and by the time the drift in phenotype is observed, it is impossible to recover the original phenotype. To avoid phenotypic drift, it is very important to perform standard cell culture for passages very carefully, with always the same number of cells seeded per flask for each passage, and always the same confluence chosen at which to split the cell population for

Table 2 Example of Cell Seeding Depending on the Culture Mode

Culture container	Surface area	T4-2; S2; MDA-MB-231 cells	H-14 medium	S1 cells
		Standard 2D culture		
4-well plate/well	2.01 cm^2	23,500	300 µl	47,000
6-well plate/well	9.08 cm^2	106,000	1.2 ml	212,000
12-well plate/well	3.8 cm^2	44,500	500 µl	89,000
35 mm dish	9.62 cm^2	112,500	1.3 ml	225,000
60 mm dish	28.27 cm^2	330,000	3.8 ml	660,000
T-25 flask	25 cm^2	291,500	3 ml	583,000
T-75 flask	75 cm^2	875,000	10 ml	1,750,000
		Embedded culture in collagen I		
4-well plate/well	2.01 cm^2	86,700	500 µl	
4-well slide/well	1.44 cm^2	62,100	500 µl	
35-mm dish	9.62 cm^2	415,100	1.5 ml	
60-mm dish	28.27 cm^2	1,220,000	4.5 ml	
		Drip culture with EHS gel		
4-well slide/well	1.44 cm^2	25,000	400 µl (on 60-µl gel coat)	50,000
35-mm dish	9.62 cm^2	200,000	1.2 ml (on 500-µl gel coat)	400,000
60-mm dish	28.27 cm^2	600,000	3.8 ml (on 1.5-ml gel coat)	1,200,000

[a]For 2D culture, cancer cell seeding for the indicated cell lines is usually 11,700 cells/cm^2 and non-neoplastic epithelial cell seeding is usually 23,300 cells/cm^2; for EHS gel-drip culture, cancer cell seeding for the indicated cell lines is usually 17,400 cells/cm^2 and 34,700 cells/cm^2 for non-neoplastic epithelial cells on a gel coat of 42 µl/cm^2 and with 5% final EHS gel concentration in the cell culture medium; for collagen I–embedded culture, cancer cell seeding for the indicated cell lines is usually 43,150 cells/cm^2, in 55 µl of gel/cm^2, on a thin gel coat of 14 µl/cm^2

propagation (Plachot et al., 2009; Vidi, Bissell, & Lelièvre, 2013; see Table 2 for cell seeding concentration in 2D culture). 3D cell culture is usually done no sooner than after one passage of the cells in standard 2D culture, if they were thawed from their liquid nitrogen storage.

2. Observe the cells using a light microscope. At 80% confluence, the T4-2 cells typically form large islands. Cells within the islands are of irregular (but not fusiform) shape and sizes around 20 µm. Cells make contact within these islands, so it looks like a continuous sheet of cells (Vidi et al., 2013).

   If the cells do not look as they do usually, or if there are many floating (detached) cells, do not use that flask.

3. Sterilize the laminar flow hood with UV at least 30 min prior to use.

   We do not use antibiotics for cell culture. The use of antibiotics is not recommended for cell culture, as it might lead to the survival of resistant bacteria that could devastate cultures in the long run. It is better to use sterile cell culture elements (containers,

pipettes, etc.), thoroughly clean every piece of equipment before placing it in the hood, and clean the hood before and after use (making use of UV as appropriate).

4. Place an ice-filled bucket inside the laminar flow hood.

   The bucket must be cleaned by wiping it down with ethanol before taking it inside the laminar flow hood.

5. Prepare collagen I according to Support Protocol 1. The amount to be prepared depends on the culture vessel and experiment (see Table 2 for detailed information).

   This step takes between 10 and 20 min depending on whether different degrees of gel stiffness need to be prepared.

Removal of cells from their 2D culture device in preparation for embedding in collagen

6. Prepare fresh H14 medium (DMEM/F12 medium including additives) as outlined in the Reagents and Solutions section. For T4-2 breast cancer cells, in this protocol we use the following final concentrations in cell culture medium (see Table 1):

   5 μg/ml (or 0.15 IU/ml) prolactin
   250 ng/ml insulin
   1.4 μM hydrocortisone
   0.1 nM β-estradiol
   2.6 ng/ml sodium selenite
   10 μg/ml transferrin.

   Note that this cocktail works well for the T4-2 and MDA-MB-231 cell lines that we will use as examples of breast IDC in this protocol.

7. Detach cells with trypsin. To do so, discard the medium from the flask and add 750 μl (for T-75) or 250 μl (for T-25) of 0.25% trypsin/1 mM EDTA and spread evenly on the cell layer for a quick rinse at room temperature.

8. Immediately remove the trypsin rinse from the flasks (only dead cells will have time to go into suspension).

9. Add 1 ml or 330 μl of 0.25% trypsin/1 mM EDTA to the T-75 or T-25 flask, respectively, and gently spread the solution. Incubate the flask at 37°C for no more than 5 to 10 min.

   We recommend that you use a timer and remove the flask from the incubator at the 5-min mark to check for floating cells with a microscope after gently tapping on the sides of the flask. Continue the incubation for up to five more minutes if the cells are not detached; within 10 min the trypsin should have detached most of the cells.

10. After the incubation period is over, add 3 ml (T-25) or 9 ml (T-75) of DMEM/F12 containing SBTI at 0.18 mg/ml final concentration. Mix the solution with the pipettor two or three times, rinsing the entire surface where the cells were cultured, and ensure that all the cells are detached and within the solution.

    When the cells are removed from their culture vessels, trypsin is used as for other cells, but since there are no serum proteins in the culture to inactivate the trypsin, SBTI needs to be added to cancel the effect of trypsin after the few minutes incubation necessary to detach the cells. Once an aliquot of stock SBTI (10 mg/ml) is thawed, it is stored in the fridge for no more than two weeks.

11. Add 100 μl of the above cell suspension to a prelabeled microcentrifuge tube for cell counting. Count cells using a hemacytometer (see Current Protocols article: Phelan & May, 2015).

Based on the number of cells counted, the necessary volume of suspension can be calculated for cell seeding purposes. For detailed information on cell seeding concentration and numbers see Table 2.

12. Centrifuge the cells from step 10 for 5 min at 3000 × *g*, at room temperature, and resuspend the cell pellet in 5 µl of H-14 medium.

    There should be no significant dilution effect in the collagen I gel. The final volume here usually corresponds to no more than 10% of the total volume in which the cells are embedded in the next step.

Embedding of cells in collagen I (thin layer)

13. Fill an ice bucket dedicated to the "cell culture" room with ice and transfer the collagen-containing tube (see Support Protocol 1) from the 4°C refrigerator to the ice bucket inside the laminar flow hood, if you have stored the collagen I there after preparing it (see step 5).

14. Label the tissue culture vessels inside the laminar flow hood (e.g., 4-well chambered slides are used for immunostaining purposes) with indication of the cell type, the collagen stiffness, and any specific treatment for each well as appropriate; also indicate the date.

15. Coat the surface of each well of the culture vessel with an ultrathin layer of collagen I (14 µl/cm^2, i.e., 20 µl per well of 4-well chambered slide) and incubate at 37°C for 5 min until the gel becomes opaque.

    For the coating step, first add a few drops of the calculated volume of the prepared collagen solution so that all the surface of the well is covered. Then, use the tip of the pipettor to mix the drops together by gently moving the tip back and forth between the drops. If you keep the tip attached to the micropipettor, ensure that the plunger is pushed down to the first notch all the time and avoid drawing back the solution into the tip. Alternatively, some people prefer to keep a small volume of solution in the tip (then the plunger is not pushed all the way down to the first notch) so that it can finally be added all around the surface of the well. When doing that, the remaining collagen solution is slowly released to prevent bubble formation (as usual, stop pushing on the plunger when you reach the second notch, to avoid making bubbles).

16. Take 10 µl of collagen and add this volume into the ~5 µl of cell suspension (see step 12), mixing up-and-down with the plunger only once (see below regarding how to take and release the collagen I solution). This step will allow the cells to get accustomed to the collagen environment. Then, add the necessary volume of collagen (55 µl/cm^2, thus 80 µl per well of a 4 well chambered slide, see Table 2; you may subtract the initial 10-µl volume that was just added). As the collagen solution is viscous, you will have to push the plunger of the micropipettor down and stop at the second notch, then release the plunger just a little and wait a few seconds for the solution to go up the tip, release the plunger a little more, etc., until the whole volume is taken. Carefully release the collagen solution into the cell suspension, using slow release by maintaining pressure on the plunger of the micropipettor. Do not add the last drop of the solution, as this would create bubbles. Now, mix the solution up and down with the plunger only once. After the two-step addition of collagen I to the cells, take the entire volume of collagen solution containing the cells and slowly release the solution on top of the well, which is precoated with collagen. Add two drops next to each other in the well (and mix the drops together by moving the tip of the micropipettor across the drops if necessary, with no more than one or two back-and-forth tip movement to avoid disturbing the cells). You should do this very gently, also dragging the gel solution to the sides and edges of the well.

17. Incubate the chambered slides at 37°C in the cell culture incubator for 25 min.
18. After incubation, carefully add 500 µl of H-14 culture medium by bringing the pipette tip close to the side of the culture vessel, without disturbing the gel.
19. Place the 3D cultures in the cell culture incubator and replace the cell culture medium every 2 to 3 days unless the experiment necessitates changing the medium every 24 hr (depending on the type of reagent used for certain treatments).
20. Keep the cells in culture for the necessary length of time.

    To obtain tumors, we usually wait at least 3 days before stopping an experiment, since they are formed by cell division and not aggregation of cells; however, routinely we leave our cells in culture for 8 days so that we can compare with other cells that require longer culture times. T4-2 cells can be kept in culture for weeks, since the tumors they form do not grow beyond day 15 or so (there is an equilibrium between the percentages of cells that divide and cells that die at some point).

Release of cells from collagen I

After the number of days of culture necessary for the experiment, if the cells are not to be used for immunostaining but are going to be used instead for additional cell culture (see Basic Protocol 2) or for the preparation of cell extracts (e.g., protein, RNA, DNA), the tumors need to be released from the collagen I gel.

21. Aspirate the medium gently without touching the surface of the gel. It is desirable to ensure that all the medium has been removed from the gel by tilting the chambered slide at an angle to facilitate medium aspiration.
22. Calculate the desired volume (78 µl/cm^2) of collagenase needed based on the surface area of the culture vessel.

    Collagen requires the use of a dislodging enzyme, collagenase, to break the gel. It is used at a final concentration 1 mg/ml diluted from a stock concentration of 5 mg/ml prepared in phosphate-buffered saline (PBS). Before adding collagenase, aspirate the cell culture medium, then briefly wash with prewarmed (37°C) PBS to remove excess salt, minerals, etc., as these components might interfere with collagenase activity

23. Add the collagenase solution directly on top of the cell culture, covering the entire surface of the culture vessel.
24. Incubate the culture vessel at 37°C for 45 min.
25. After the incubation period, observe the solution with a microscope to ensure that the cells or the multicellular nodules are floating. The cell culture vessel can be further incubated with collagenase for 15 min if necessary.
26. Remove the floating nodules from the culture vessel with a 1-ml pipette tip (avoid taking the gel; it is recommended to not aspirate the solution into the pipet tip from the bottom of the dish, as most cells or nodules are floating on the surface).
27. Place the collected cells or nodules into a microcentrifuge tube, then centrifuge the tube 5 min at 3000 × *g*, room temperature.
28. Remove the supernatant carefully using a small pipette tip connected to a vacuum source, ensuring that the pellet remains at the bottom of the tube.
29. Wash the pellet three times with DMEM/F12 culture medium, centrifuging 5 min at 3000 × *g*, room temperature, between washes.

    Importantly, after each centrifugation, wash the pellet without resuspending or dislodging it (do not move the solution up and down in the pipet tip; this will prevent the pellet from

being stuck to the wall of the pipet tip). The washing step is important to remove as much of the collagenase solution as possible.

30. After the 3rd wash, the 4th wash can be performed with PBS if the cells are to be lysed for analysis, with medium again before proceeding with reseeding, or by trypsinization and individual cell reseeding based on the planned experiment.

ALTERNATE PROTOCOL 1

ENGELBRETH-HOLM-SWARM (EHS) GEL DRIP CULTURE

A characteristic of the DCIS type of carcinomas that grow within the breast ducts without invading the interstitium is an intact basement membrane, as shown by immunostaining. Culturing of these cells usually works well with Engelbreth-Holm-Swarm (EHS)–based gels like Matrigel™ that provide the initial signaling for the cells to display basal polarity. This type of polarity is measured by the presence of a continuous staining for β4- or α6-integrin and basement membrane components collagen IV and laminin 5 at the outer side of the tumor nodule (Plachot et al., 2009; Fig. 1). It is expected that in the presence of EHS gel, DCIS cells will form tumors with no signs of invasiveness (e.g., no extensions away from the tumor nodule) and display basal polarity (as described above). There is an abundant literature regarding cell culture with EHS-derived gels. We have published protocols (Vidi et al., 2013) and we have made aspects of the technique available online (see Internet Resources). One of the characteristics of EHS gels is that they can be used as a drip (see protocol below) and not solely as an embedded culture (Plachot et al., 2009), hence saving a considerable amount of gel and reducing background signal from the matrix with immunofluorescence staining.

Additional Materials *(also see Basic Protocol 1)*

HMT-3522 S2 cells:

In the example chosen for this protocol we will use DCIS S2 cells from the HMT-3522 progression series (see Basic Protocol 1 materials list). These cells can be obtained from

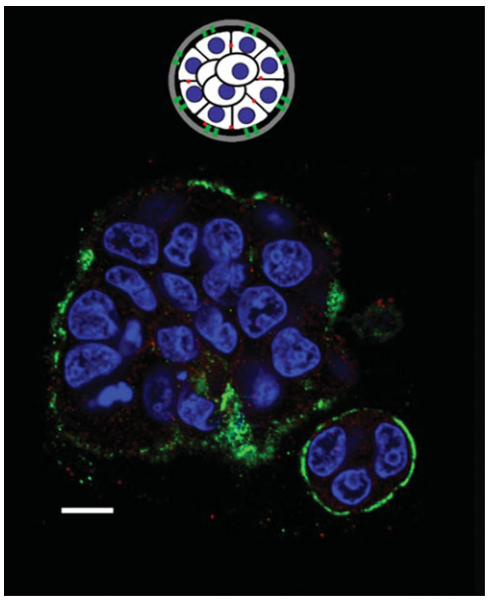


Figure 1 Basal polarity in a DCIS model. HMT-3522 S2 cells were cultured with the EHS-gel drip method for 10 days and immunostained for basal polarity marker α6-integrin (green). Two nodules are shown, revealing the heterogeneity in nodule appearance (size) in this population of cells. Nuclei are stained with DAPI (blue). The drawing illustrates the organization of cells within these nodules. Size bar, 10 μm.

the European Collection of Authenticated Cell Cultures (ECACC; Catalog #98102211), from Sigma-Aldrich (cells are from ECACC), or by contacting Mina J. Bissell (Lawrence Berkeley National Laboratory, Berkeley, California). The S2 cells were selected after omitting EGF from the culture medium at passage 118 of non-neoplastic S1 cells spontaneously immortalized in cell culture (Briand et al., 1987).

EHS-matrix Matrigel (Corning Biologics, Matrigel Basement Membrane; Discovery Labware, Catalog #354234)

1. Thaw a 1-ml aliquot of EHS-derived gel by placing it on ice overnight at 4°C (in the fridge). The thawing date should be indicated, and the tube should be kept continuously on ice in the refrigerator until expiration after 1 month.

   While holding the EHS-gel bottle, care should be taken to not touch the bottom surface, as the solution can solidify quickly at higher than ice-cold temperature. The expiration date on the EHS gel lot is important to consider, since cells might not properly differentiate anymore if the product starts to degrade.

2. Prepare the cell culture hood, observe the cells, prepare the H14 medium, and detach and count the cells prior to performing the drip culture, as described in Basic Protocol 1, steps 2 to 4 and 6 to 12.

   As for collagen I (see Basic Protocol 1, step 5), place an ice bucket filled with ice inside the laminar flow hood when working with EHS-derived gel. For S2 breast cancer cells, we use the same culture medium as for T4-2 cells [5 μg/ml (or 0.15 IU/ml) prolactin, 250 ng/ml insulin, 1.4 μM hydrocortisone, 0.1 nM β-estradiol, 2.6 ng/ml sodium selenite, and 10 μg/ml transferrin in DMEM/F12; see Table 1). Importantly, we do not add EGF (although we do with their precursor S1 cells); otherwise, S2 cells would not thrive (regardless of the culture conditions).

   It is important to keep in mind that the HMT-3522 cells are sensitive to the type of culture vessel. They are fine when cultured on Falcon vessels, but they do not thrive on Corning cell culture vessels (we have made this observation with cell culture flasks, for instance, but we do not know the reason for the peculiar sensitivity of these cells).

3. Calculate the number of cells needed for the "number of vessels of the same surface area + 1 vessel" ($N + 1$) so that you do not run short of volume needed for the last vessel—indeed, the slight miscalibration of micropipettors and loss of microdrops on the surface of the tips lead to loss of liquid in the main solution.

   When using an EHS-gel drip method, the cells can be pooled for several wells or culture vessels (since there is no embedding) using the N+1 approach.

4. Coat the surface of each culture vessel with a thin layer of EHS-derived gel (42 μl/cm^2) using a pipette tip (see Internet Resources for movies illustrating this and other procedures) and incubate at 37°C for 15 to 30 min until the gel is formed.

   The volume of EHS-derived gel for different culture vessels is indicated in Table 2.

5. Centrifuge the calculated volume of cells (N+1 for the same vessel types) and resuspend cells in half of the total volume of medium that will be required for the culture.

6. Mix the solution (up-and-down in the pipette tip) several times to resuspend the cell pellet completely in the culture medium.

7. Add the volume of cell suspension necessary for each vessel, drop-by-drop, all over the surface of the coated gel using a micropipettor. Mix the stock cell suspension a couple of times up-and-down in the tip or pipette in-between the seedings of the culture vessels.

8. Allow the cells to settle to the bottom of the well or dish where they have been seeded by leaving the culture vessel undisturbed in the hood for 5 to 10 min.
9. In the meantime, prepare a solution containing 10% EHS-derived gel in cell culture medium utilizing the remaining half of the medium. The volume of EHS gel and the medium to be added can be calculated based on Table 2. Mix the solution gently (only once), to avoid bubbles as much as possible.
10. Add the 10% EHS-derived solution drop-by-drop over the entire surface of the culture vessel, on top of the seeded cells such that it covers both the center and the periphery of the culture area (see Internet Resources). Change the cell culture medium regularly (every 2 to 3 days), but do not add the EHS-derived gel drip anymore.

To add volumes of solution to small culture vessels, it is important that the pipettor be held correctly and placed properly without disturbing the gel or spilling the medium out of the culture dish. The other hand could be used to support the pipettor and keep the hand holding the pipettor steady. If there are any bubbles formed when adding the medium to the gel, especially when adding the last drop, the bubbles can usually be removed using the pipette tip. However, if a bubble is difficult to remove, it is better to leave it so that the distribution of cells is not disturbed.

ALTERNATE PROTOCOL 2

CULTURE OF TUMOR NODULES ON TOP OF A NONBIOLOGICAL GEL

With certain gels, it is possible to obtain a rapid formation (in ~3 or 4 days) of tumors of acceptable size for drug screening on top of the substratum. This allows high-throughput culture and easy imaging and analysis. It is simple to seed the cells by adding them directly on top of the gel (no preformation of tumors in a biological gel is necessary). This method is preferred for nonbiological gels that provide necessary stiffness for the microenvironment, without additional reagents, as long as tumors are capable of making their own matrix. Upon culture of tumor cells with this method, nodules with shape and organization characteristic of the chosen tumor type should be obtained. Nonbiological gels that are preferred are those for which the stiffness can be chosen to adapt to the type of tumor cells. Indeed, although cells are not embedded in the gel, we have observed that they can still sense that there is a different stiffness underneath them. Here we give an example of the A3DH gel that we tested with T4-2 IDC cells for possible use in high-throughput drug screening.

Additional Materials *(also see Basic Protocol 1)*

A3DH gel (Akina Inc.)

1. Prepare the solution of A3DH gel by solubilizing 0.25 g of A3DH gel in 5 ml of DMEM/F12 cell culture medium for a final concentration of 5% (w/v). Then, refrigerate the hydrogel at approximately 2° to 8°C for ~48 hr to reach a state of dissolution.

The A3DH material is a stearate-modified methylcellulose. This polymer dissolves readily at cold temperatures and forms a gel at biological temperatures (37°C), providing for a thermogelling material that has appropriate biocompatibility and physicomechanical properties for the formation of spheroids with tumor cells.

2. Observe the cells and prepare the cell culture hood as in steps 2 to 4 of Basic Protocol 1.
3. Coat 26.3 µl per cm^2 of the gel solution in a manner similar to that described for EHS gel culture (see Alternate Protocol 1, step 4).
4. Place the culture vessels in the cell culture incubator at 37°C for 90 min.

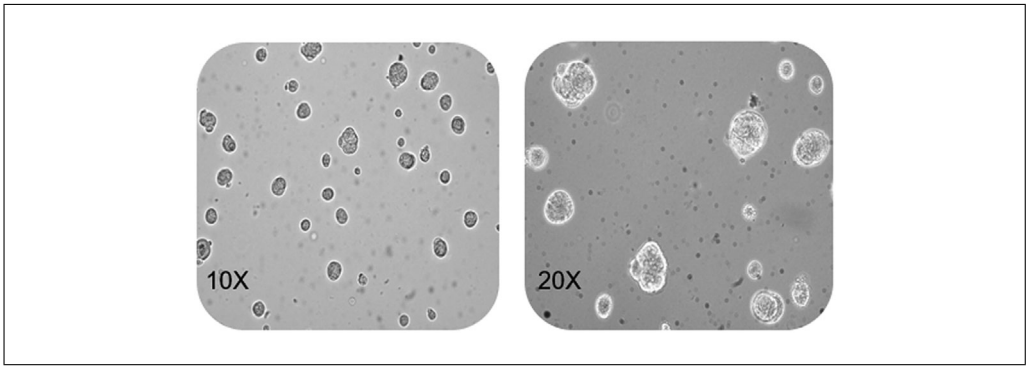


Figure 2 T4-2 cells cultured on the A3DH gel form tumor nodules. Each well of a 12-well plate was coated with 100 μl of the A3DH solution and incubated at 37°C for 90 min. Cancer cells were seeded on the gel (79,000 cells per well) and cultured for 48 hr. Shown are bright-field microscope images taken with 10× and 20× objectives.

5. Add cells on the surface of the gel-coated culture vessel, drop-by-drop, in the volume of liquid necessary for cell culture.

   Here the cancer cells are cultured on top of the gel without the need for a drip or embedding (Fig. 2). This technique might not be an option to study invasiveness, since tumor nodules are formed on top of the nonbiological gel and the necessary attractants for invasion are not present in the gel.

COLLAGEN TYPE I PREPARATION

SUPPORT PROTOCOL 1

Here we report a method used to prepare tunable collagen I from Advanced Biomatrix. Other types of collagen I matrix are available, like the Collymers (Geniphys Inc.) that we use for stiffness measurement in Support Protocol 3. The gels vary depending on the origin of the collagen I and the level of purification. Collagen obtained from both companies is polymerized at acidic pH and room temperature, leading to the formation of a gel. Polymerization via the kits purchased from these suppliers provides an appropriate ECM of a determined stiffness. The preparation follows the manufacturer's instructions based on the stiffness needed. The resulting stiffness can be confirmed by measuring it as described in Support Protocol 3.

Materials

Advanced Biomatrix kit (PhotoCol®, Catalog #5201-1KIT (formerly #5201-1EA):
  Acetic acid, 20 mM (Advanced Biomatrix, Catalog #5079-50ML)
  Neutralizing solution (Advanced Biomatrix, Catalog #5205-10ML)
  Pure collagen I (PhotoCol^{R}, Advanced Biomatrix, Catalog #5198-100MG)
Ice bucket containing ice

1. Fill an ice bucket with ice to keep the collagen solution cold.

   While holding the collagen bottle, care should be taken not to touch the bottom part, as the solution can solidify quickly at higher than ice-cold temperature.

2. Calculate the volume of pure collagen I to be placed into a prelabeled microcentrifuge tube based on the desired matrix stiffness and the entire culture surface needed for the experiment.

   Refer to the manufacturer's guidelines to determine the volume of collagen, acetic acid, and neutralizing solution to be added, which vary depending on the matrix stiffness

desired for a specific experiment. For instance, to embed T4-2 cells in collagen I of stiffness 900 Pa in one well of a 4-well chambered slide, 200 µl of the collagen solution is desirable.

A photoinitiator component is used when photo cross-linking is desired to reach certain degrees of stiffness, as per the manufacturer's instructions. Here we present a protocol that does not make use of the photoinitiator.

3. Remove the needed volume of solution carefully and gently, since collagen is a viscous polymer, to avoid the formation of bubbles during the process. Place the microcentrifuge tube on ice when not handled.

   During handling, make sure that the bottom of the tube is not touched, to avoid raising the temperature of the solution. To take the volume of collagen I solution needed, bring the pipette tip inside the collagen stock bottle, making sure not to touch the neck of the bottle with the end of the tip. The collagen is viscous; thus, insert the pipette tip sufficiently inside the bottle (although not too deep). When you 'aspirate' the needed volume, wait a few seconds with the pipette tip plunged inside the solution for the needed volume to come up into the tip, then carefully remove it from the bottle. Take a microcentrifuge tube and slowly release the contents of the pipette tip at the very bottom of the tube without touching that part of the tube with your hand, in order not to raise the temperature too quickly. Make sure not to release all the collagen from the pipette tip. As you are nearing the last drop of polymer in the pipette tip, stop its release (in other words, do no push through the second notch with the micropipette plunger). Acting in this way will prevent the formation of bubbles. Place the microcentrifuge tube immediately on ice.

4. Immediately replace the collagen I solution in the refrigerator at 4°C after the desired volume has been taken, take the 20 mM acetic acid stock solution out of 4°C storage into the laminar flow hood, and place on ice.

5. Add the required volume of acetic acid, depending on the stiffness chosen, to the microcentrifuge tube containing the collagen I. Place the tube on ice.

   Take the necessary volume of acetic acid from its storage bottle. Carefully and slowly release the acetic acid into the collagen solution in the microcentrifuge tube. Make sure that you are releasing the content onto the side wall toward the bottom of the tube (not directly within the gel at the very bottom of the tube; otherwise a bubble will form). Do not release the last drop (stop at the second notch when pushing on the plunger), to avoid bubble formation.

6. Place the acetic acid back in the refrigerator after use.

7. Bring the neutralizing solution into the cell culture hood.

   The neutralizing solution is stored at room temperature.

8. Add the required volume of neutralizing solution carefully to the microcentrifuge tube containing the mixture of collagen I and acetic acid, in a similar manner to the acetic acid.

   The neutralizing solution is used to reach a final pH of 7.0 to 7.4. In the mixing steps with acetic acid and neutralizing solution, mixing up-and-down with the pipettor should only be done once, to ovoid bubble formation.

9. Place this final solution on ice or in the 4°C refrigerator until used for the experiment.

   If gel coating will be delayed by more than 30 min after to the preparation of the solution, we usually wait to add the neutralizing solution so that there is no gel formation with time in the tube. We only add the neutralizing solution when we know that we will coat the gel within 30 min of preparation.

CHOOSING THE APPROPRIATE MATRIX DEPENDING ON THE TYPE OF CANCER

For each type of cancer, it is important to identify the matrix stiffness that should be used. In the protocol below, we give a step-by-step plan to obtain matrix stiffness information and help choose an appropriate matrix. A literature search will be necessary to gather some of the necessary information. If information on stiffness is not available in the literature, measurement from real tumors is feasible with the appropriate knowledge and equipment (see Support Protocol 3). If the stiffness and composition of the matrix have been correctly chosen, the tumors should display phenotypic (notably architectural) traits that are similar to those observed *in vivo*.

Materials

ECM molecules and culture medium depending on the type of tumors
Cancer cells of interest
Phosphate-buffered saline [PBS; prepared in the laboratory as a 10× stock (76 g NaCl, 18.76 g Na_2HPO_4, 4.14 g NaH_2PO_4, completed to 1000 ml with deionized water, with pH adjusted to 7.4, and autoclaved)]
Fixative, e.g., 4% paraformaldehyde solution in PBS (Santa Cruz Biotechnology Catalog #SC281692)
Stains of interest, e.g., hematoxylin & eosin (H&E)

Computer with internet access
Histology resources (book, online information, a pathologist)
4-well chambered slides (used for direct immunostaining of cultures; Falcon, ThermoFisher Catalog #354104 354104)
4-well plate (Nunc ™, ThermoFisher, Catalog #176740)

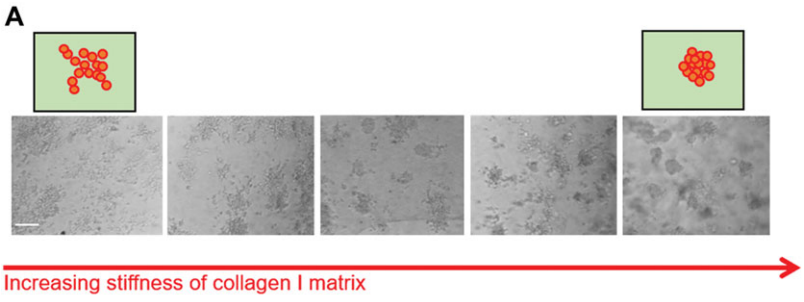
1. Determine the origin of the cells to be used (e.g., preinvasive or invasive cancer, subtype of a specific cancer).

   If the cancer type is preinvasive, a basement membrane–type molecule (e.g., laminin) will be usually necessary to provide initial signaling for basal polarity to be established. It might also be necessary to have a stiffness higher than that found in normal tissue using collagen I, as is the case, for instance, for breast DCIS, which may require a matrix twice as stiff as normal (Lopez, Kang, You, McDonald, & Weaver, 2011). EHS-based gels typically have a stiffness close to the normal breast stroma (i.e., Young's modulus of 700 to 800 Pa measured by indentation of unconstrained matrix; see Support Protocol 3). To reach a stiffness around 1400 Pa and to maintain basal polarity, we mix basement membrane component laminin 111 (76 μg/ml) with the collagen of appropriate stiffness prior to embedding the cells (Chittiboyina et al., 2018).

2. Proceed with a literature search to identify articles that report measurements of matrix stiffness

   If this information is available, make sure it is the matrix that was measured and not the whole tumor, as stiffness would be different between the two types of samples. It is also important to know how the measurement was performed, since results might vary greatly depending on how the measurements were performed (see Support Protocol 3). Very often the information might be in term of fold increase rather than absolute values, which is useful if we know the matrix stiffness of the normal tissue for the location site.

3. Perform an additional literature search to determine any specific content of the ECM in the case of an invasive type of cancer (e.g., molecules other than collagen I that are abundant in the microenvironment of this type of cancer). If necessary, talk with a pathologist, since there are tumors for which histochemical staining has been performed to identify the matrix content.



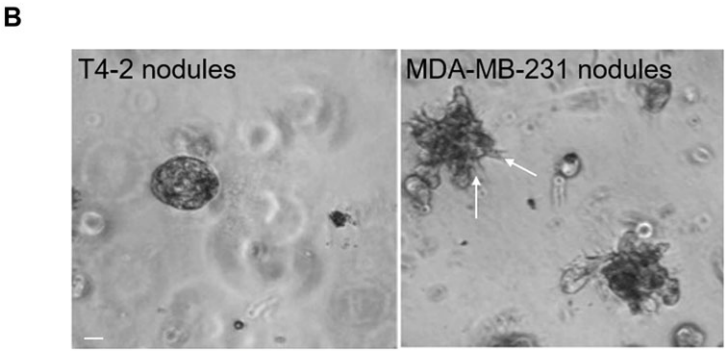


Figure 3 Influence of matrix stiffness on cancer cells. (**A**) T4-2 cells were seeded in collagen I with a range of stiffness degrees (100 to 1500 Pa). Bright-field images at day 6 of culture show increasing cohesion of cancer cells with increasing matrix stiffness. Drawings display the cellular organization at low and high stiffness degrees. Size bar, 100 μm. (**B**) T4-2 cells (poorly invasive) and MDA MB-231 (highly invasive) cells were seeded within the collagen I matrix with stiffness adjusted to 2000 Pa to mimic the *in vivo* tumor environment. Bright-field images are shown for day 6 of culture. Arrows indicate invasive arms formed by the nodules from MDA MB-231 cells. Scale bar, 25 μm.

The type of molecules to include beyond collagen I vary depending on the type of cancer; these molecules might be fibronectin, tenascin, hyaluronic acid, etc. It might not be necessary to add these molecules at first, since the cancer cells may be able to synthetize and secrete them. Adding these molecules should only be done if the use of standard collagen I at the expected stiffness is not giving an in vivo–like phenotype upon immunostaining and/or histological analysis.

4. Test the selected matrix stiffness, possibly performing a range of stiffness degrees (if the protocol for these cells was not established in the literature) with cancer cells of interest. Starting from individual cells as in Basic Protocol 1, follow the growth of tumors over the first 6 days of culture and take pictures if possible. If tumors grow to reach at least 100 μm in size, you may stop the cultures for analysis.

   The characteristics of IDC types of breast carcinomas are invasiveness and a stiffer matrix (at least 2000 Pa if measured on unconstrained samples; Lopez et al., 2011). For instance, the T4-2 cells are mildly invasive and are a triple-negative subtype of breast cancer [estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) negative]. The formation of tumors and the invasive phenotype of these cells greatly depend on the collagen I matrix stiffness (Fig. 3A). In contrast to T4-2 cells, triple-negative MDA-MB-231 cells (available from American Type Culture Collection, ATCC, HTB-26™) are highly aggressive (which is also characterized by resistance to treatment; Amaro et al., 2016). This aggressiveness is easily visible when comparing T4-2 and MDA-MB-231 cells for the same matrix stiffness of 2000 Pa (Fig. 3B). Note that these tumors grow relatively fast (over a few days), but other types of tumors might take a couple of weeks to reach an acceptable size for experiments.

5. Remove the cell culture medium, wash once quickly with PBS, and add a fixing solution to the cultures for immunofluorescence staining and for future embedding in paraffin, sectioning and hematoxylin & eosin (H&E) staining. Then, proceed with (immuno)staining (protocols depend on the laboratories and/or the antibodies) or give the samples to the histology laboratory.

   For direct fluorescence immunostaining, tumors are usually grown in a thin layer of collagen I in 4-well chambered slides (see Basic Protocol 1 and Table 2). The fixing solution used depends on the molecules to be stained. Typically, measurement of proliferation (e.g., Ki67) and apoptosis (e.g., caspase 3) might be done along with any characteristic marker of the cancer of interest. We often use 4% paraformaldehyde solution as a fixative. For embedding of the cell cultures in paraffin, tumors are usually cultured in 4-well plates. Then, we give the fixed samples to the tissue core or histology facility for further processing.

6. Proceed with the analysis of the fluorescence staining (around 150 to 300 cells per sample over several tumors for stainings that can be assessed on a per cell basis; Chittiboyina et al., 2018).

7. Send the H&E-stained slide or a scan of the slide (e.g., using Aperio Digital Pathology) prepared in the histology laboratory to a pathologist for review if the histological analysis of the tumor cannot be done at the tissue or histology core.

MEASUREMENT OF MATRIX STIFFNESS *IN VITRO*

SUPPORT PROTOCOL 3

In this protocol, we present one of the possible methods for measuring matrix stiffness. In this process, the sample (in the form of a disc) is compressed at a constant rate between two flat plates while allowed to freely expand in the radial direction. The deformation speed can range from 0.1 to 10 mm/min and the applied force is constantly recorded. The stiffness is determined by the extrapolated slope from the normalized stress versus strain curve.

Materials

Type I Oligomer solution and Collymer polymerization kit (collagen formulation from Geniphys, Oligomer-PD, Catalog #CM1001):
- 100 mg Oligomer-PD in 0.01 M hydrochloric acid
- 10× Polymerization Buffer PLUS
- 0.1 M sodium hydroxide
- Polymerization Supplement

Dulbecco's Modified Eagle Medium/F12 modification (DMEM/F12; Thermo Fisher Scientific, Catalog #12400-024; see recipe)

4-well plates (Nunc ™ Thermo Fisher Scientific, Catalog #176740)

Universal Testing System (eXpert 4000 micro test system, ADMET, Inc): has a custom-designed sample compression stage made of acrylic, which was designed for characterizing mechanical properties of soft materials (e.g., tissue, hydrogel)

Scooping device for the gel (e.g., a weighing spatula or spoon)

Additional reagents and equipment for preparation of collagen I gels (Support Protocol 1)

1. Prepare 800 µl of collagen I–based gel at the desired stiffness for each well following the instructions provided by the manufacturer of the gel system. We prepare three replicates using three different wells in the same 4-well plate. (refer to Support Protocol 1 for gel preparation)

   The stiffness of collagen I gel is linked to the density of fibers. This is the reason why increased stiffness is achieved in part by increasing the concentration of collagen I in

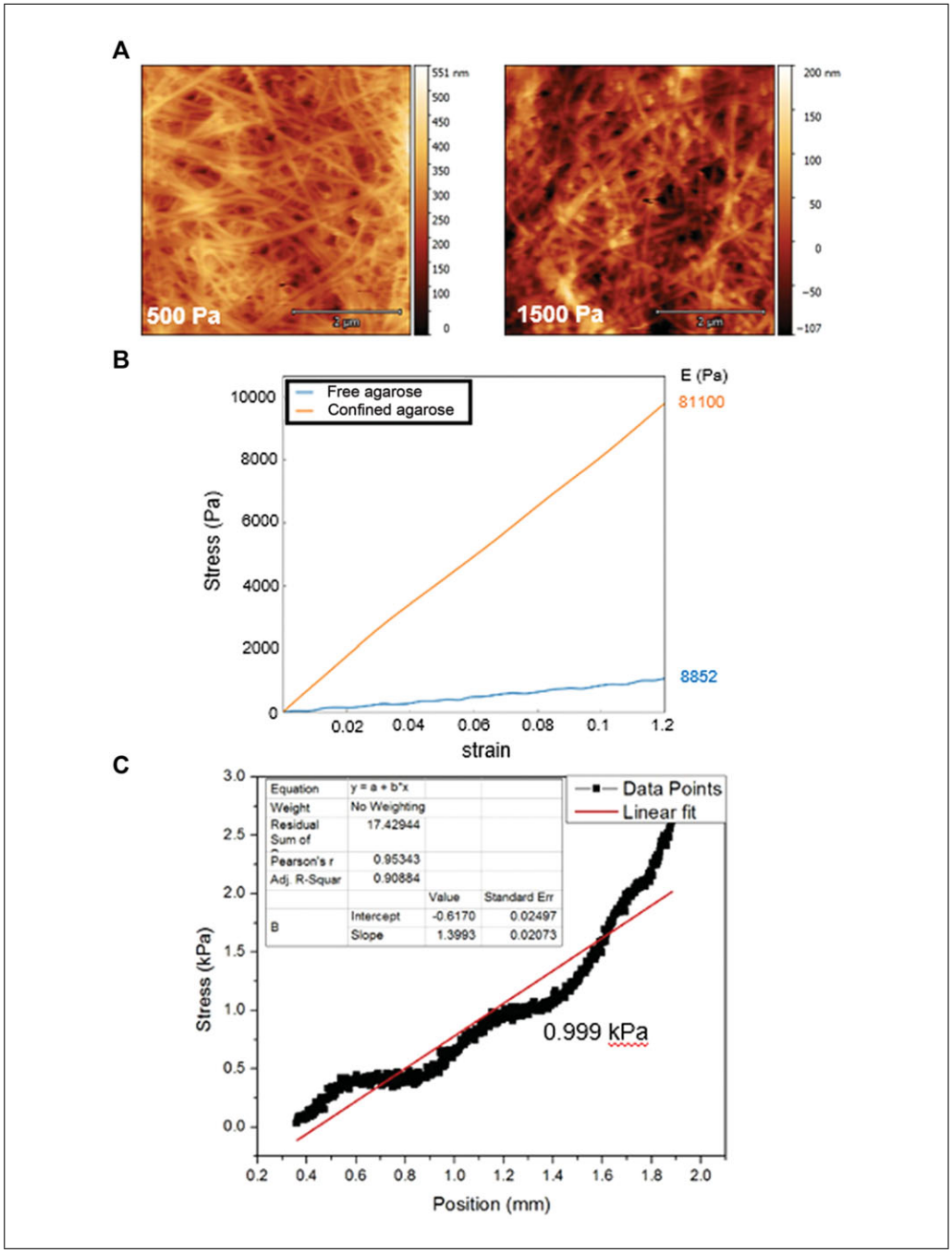


Figure 4 Assessment of collagen I organization. (**A**) Collymers gel blocks were prepared in a 4-well chambered slide at 500 and 1500 Pa according to the manufacturer's instructions. Gel blocks were incubated with H14 medium for 3 days before removal from the wells. The blocks were then transferred into a clean container, washed three times with PBS, and dried at 37°C before being subjected to imaging via scanning of the gel surface with an MFP-3D-BIO atomic force microscope (Asylum Research, Oxford Instruments). Topography images are presented with vertical scale shown as the color bar. (**B**) Constrained agarose gel sample (sample is not released from the container, orange line) compared to free agarose gel sample (released from the container, blue line) shows one order of magnitude difference for the recorded stress or stiffness (Pa) under increasing strain with the universal testing system. (**C**) Example of stiffness measurement on unconstrained collagen I gel prepared to achieve a stiffness of 1000 Pa, measured at 0.999 kPa or 999 Pa (= average slope of stress vs. strain); R^2 (*R-Squar* in the inset table) shows how linear the data points are (close to 1 is ideally linear), indicating that the Young's modulus measured is within the elastic region of the material (i.e., there is no destruction).

the solution. This increased density can be observed by atomic force microscopy (AFM) (Fig. 4A).

2. Incubate the collagen I solution at 37°C for 20 min to form a gel.
3. Add cell culture medium on top of the gel until the day of measurement (here we use DMEM/F12 since it is the medium of the cells that will be used with that type of gel in a future experiment).

   In the example shown here, we did measurements on day 8, to mimic the length of time that the cell culture experiment of interest will run.

4. Remove the cell culture medium and place the gel (e.g., by scooping it out with a spatula) on the disc-shaped base plate of the Universal Testing System.

   With 800 μl of gel, the collagen sample disc of 16 mm diameter is ~5 mm in height after transfer onto the base plate.

5. Set up the conditions on the Universal Testing System so that the top plate is moved at a constant rate of 6 mm/min.

   In this setup, the base plate is stationary while the other plate (top plate) is attached to the load. Compression measurements that are done directly on the gel inside a constraining container (e.g., a well) might result in incorrect measurements (i.e., with higher Young's modulus). This effect can be explained by the mechanical constraints that are induced by the wall of the culture well while applying force onto the gel. Indeed, when measuring stiffness of the Collymers (collagen I) gel, we found that results from the unconstrained samples were comparable to the reference (e.g., the proposed stiffness achieved based on the gel preparation according to the manufacturer's instructions).

6. Extract the elastic modulus of each sample from the recorded stress/strain data (Fig. 4B,C).

   The measurement of the Young's modulus of the gel is based on data recorded throughout the whole process that begins with the contact of the probe with the surface of the gel and stops at the contact of the probe with the surface of the container at the bottom of the gel. In our experience, the depth of 0.5 cm for the gel provides more reliable measurements compared to thinner gels.

COCULTURE OF CANCER CELLS WITH NONCANCER CELLS TO CREATE A MORE COMPLEX MICROENVIRONMENT

BASIC PROTOCOL 2

The purpose of the coculture section is to familiarize scientists with the questions and tasks related to culture of cancer cells with other cells from their surroundings. As described in the introduction, different cell types have been shown to influence carcinomas, such as non-neoplastic epithelial cells, resident stromal cells, or immune cells. The mode of coculture depends on the scientific question under investigation, since coculture can be done within the same matrix or in separate chambers. Coculture in separate chambers is reported in many publications either using an insert in a cell culture well or using microfluidics to connect distinct cell culture chambers with fluid that can be exchanged from one chamber to another (Goers, Freemont, & Polizzi, 2014; Mi et al., 2016; Lee et al., 2018). However, with matrix stiffness being important for the behavior of all cell types, many projects will also require coculturing cells in the same physical microenvironment (which of course could be replicated in separate chambers as well), enabling cells to be near each other and even make contact as they would *in vivo*. The first set of steps detailed below ("a" steps) provide information on coculture of tumors with fibroblasts within a collagen I matrix. The second set of steps ("b" steps) present the coculture, on a tissue chip, of tumors with differentiated non-neoplastic cells lining the ducts where carcinomas usually grow.

In the examples chosen for this protocol, we will use non-neoplastic epithelial S1 cells from the HMT-3522 progression series, HMT-3522 IDC T4-2 cells, and human fibroblasts (HMS-32).

Materials and cells

HMS-32 human fibroblasts (human-TERT immortalized human mammary stromal cell line from a reduction of mammoplasty patient #32; a kind gift from Brittney-Shay Herbert, IU Simon Cancer Center; Shay, Van Der Haegen, Ying, & Wright, 1993; Shay, Tomlinson, Piatyszek, & Gollahon, 1995; Subbaramaiah et al., 2016)

Culture medium for fibroblasts (also see Reagents and Solutions and step 1, below):

Note that Table 1 contains all cell culture additives used in the different protocols in this article. For the specific list of additives depending on the cell line, refer to the protocol steps.

Dulbecco's Modified Eagle Medium/F12 modification (DMEM/F12; Thermo Fisher Scientific, Catalog #12400-024; also see recipe and Table 1)

Insulin (see recipe and Table 1)

Hydrocortisone (see recipe and Table 1)

Sodium selenite (see recipe and Table 1)

Transferrin (see recipe and Table 1)

Epidermal growth factor (Corning Life Sciences (previously sold through BD Biosciences Catalog #354001)

Epidermal growth factor (EGF; see recipe and Table 1)

Fibroblast growth factor (FGF; see recipe and Table 1

Transforming growth factor β (TGF-β; see recipe and Table 1)

HMT-3522 S1 cells and T4-2 cells (Briand et al., 1987; Briand et al., 1996) cultured in H14 serum-free medium (see Reagents and Solutions and Table 1). They can be obtained from the European Collection of Authenticated Cell Cultures (ECACC; Catalog #98102212 for T4-2 cells and #98102210 for S1 cells) or from Sigma-Aldrich (cells are from ECACC) or by contacting Mina J. Bissell (Lawrence Berkeley National Laboratory, Berkeley, California).

H14 serum-free medium (also see Reagents and Solutions and step 6 of Basic Protocol 1):

Note that Table 1 contains all cell culture additives used in the different protocols in this article. For the specific list of additives depending on the cell line, refer to the protocol steps.

Prolactin (see recipe and Table 1)

Insulin (see recipe and Table 1)

Hydrocortisone (see recipe and Table 1)

β-Estradiol (see recipe and Table 1)

Sodium selenite (see recipe and Table 1)

Transferrin (see recipe and Table 1)

Epidermal growth factor (Corning Life Sciences (previously sold through BD Biosciences Catalog #354001)

Trypsin-EDTA (0.25% 1 mM tetrasodium EDTA; Gibco, Catalog #25200-056)

Soybean trypsin inhibitor (SBTI, T-6522 type I-S; BD Biosciences, Catalog #354201)

1 mg/ml laminin 111 (Corning Life Sciences, Catalog: #354239)

T-25/T-75 flasks (Falcon, ThermoFisher, Catalog #353133 for T-75 and 353108 for T-25)

Microscope

Ice bucket containing ice

Centrifuge

Hemacytometer
Hemichannel-embedded chips (Vidi et al., 2014)
37°C mini oven
6-well plates (Nunc™ ThermoScientific, Catalog #147065)
15-ml conical centrifuge tubes (Falcon, ThermoFisher)

Additional reagents and equipment for preparing tumors in collagen I matrix (Basic Protocol 1), preparation of collagen I (Support Protocol 1), and basic cell culture techniques including counting cells with a hemacytometer (Phelan & May, 2015)

Coculture of tumors in the presence of fibroblasts

In this protocol, we are mixing tumor cells and fibroblasts for embedding in collagen I matrix. Once fibroblasts are detached from their cell culture flask and counted, and tumors are removed from collagen I and counted, the two cell types are mixed to mimic approximately the cell-to-cell ratio that is observed *in vivo*. For triple-negative breast IDC, the ratio has been estimated as one fibroblast for five cancer cells. Therefore, to use this ratio with preformed tumors, it is essential to first estimate the number of tumor cells per tumor nodule. The calculation of this number is described in Support Protocol 4. Upon completion of this protocol, there should be distinguishable tumors and fibroblasts in the culture vessel.

1a. Prepare T-25 or T-75 flasks with fibroblasts in culture. HMS-32 cells are seeded in T-25 flasks at 5000 cells/cm^2, and the DMEM/F12 medium with additives is changed every 2 to 3 days. For these cells, we use the following (final concentrations in medium):

250 ng/ml insulin
1.4 μM hydrocortisone
2.6 ng/ml sodium selenite
10 μg/ml transferrin
5 ng/ml EGF
2.5 ng/ml FGF
7.5 pg/ml TGF-β.

Cell propagation for a new passage or an experiment is done when confluence has reached ~80% (usually after 6 days of culture). The HMS-32 cell line is also weaned off serum (see Support Protocol 5). Cells are always used in the same window of 10 passages, so we can compare among experiments and maintain the phenotype.

2a. Prepare tumors with T4-2 cells in collagen I in H14 medium as described in Basic Protocol 1, culturing for 5 days (which leads to tumors of 150 μm in diameter on average).

The culture vessel used should be commensurate in size with the number of nodules needed for the experiment.

The density of tumors obtained from 3D culture should be measured for each cell line (or primary cells) of interest to prepare for the coculture, which will require the proper stromal cell-tumor cell ratio. Typically, with T4-2 cells, we obtain 700 nodules per cm^2 when seeded at the usual concentration for collagen I–embedded culture.

3a. On the day of the coculture experiment, observe the separate cultures with fibroblasts and tumor nodules under the microscope to ensure that both are in good shape. There is no point in pursuing the experiment if one of the cell types is not behaving as expected normally.

4a. Prepare the culture medium for the co-culture by adding the additives necessary for the HMS-32 and T4-2 cells from the working stocks to reach the necessary final

concentrations for each additive (see Table 1). Keep the medium in the water bath at 37°C until use.

Some of the additives are common to both cell types and certain additives are specific, but all additives for both cell types can be mixed (i.e., all necessary additives for both cell types are included in the DMEM/F12 medium).

5a. Release the tumor nodules from collagen I as described in Basic Protocol 1, steps 21 to 30, and immediately count them using a hemacytometer (Phelan & May, 2015).

This takes up to 45 to 60 min of incubation at 37°C.

Collagen requires the use of a dislodging enzyme, collagenase, to break the gel. It is used at final concentration 1 mg/ml from a stock concentration of 5 mg/ml prepared in PBS. Before adding collagenase, aspirate the cell culture medium, then briefly wash with prewarmed (37°C) PBS to remove excess salt, minerals, etc., as these components might interfere with collagenase activity.

6a. Half-way through the incubation time of the tumor nodules with collagenase, place a clean ice bucket containing ice inside the hood for the preparation and handling of the collagen solution; follow Support Protocol 1 for the preparation of the collagen at the needed Young's modulus (or stiffness degree).

7a. After incubating the tumor nodules with collagenase at 37°C for 45 min to 1 hr, wash the nodules three times, each time with 0.6 to 1 ml of culture medium to get rid of the solution (collagen I + collagenase), centrifuging 5 min at 1000 × *g*, room temperature, after each wash.

The volume for the washes depends on the size of the pellet; e.g., for a pellet of cells from a 35-mm dish we use 0.6 ml; for a pellet of cells from a 60-mm dish, we use 1 ml. Once floating nodules are observed (using a microscope), they can be collected with a 1-ml pipette; however, care should be taken to not include any gel that might be left (although this is not frequent) along with the nodules (avoid taking the nodules from the bottom of the culture vessel in that case).

8a. Resuspend the tumor nodules in one ml of co-culture medium (see step 4) and take an aliquot of 50 µl to count the number of nodules with a hemacytometer (see Current Protocols article: Phelan & May, 2015).

This count should be done during the incubation time of the fibroblasts with trypsin (see next step).

9a. Just before counting the tumor nodules, add 300 µl trypsin-EDTA to fibroblasts in a 25-cm^2 cell culture flask as per Basic Protocol 1, steps 7 to 9.

There should be enough time during the 10 min of trypsin treatment to not only count the tumor nodules but also coat the surface of the culture vessel to be used for the coculture with the thin coat of collagen I (14 µl/cm^2; see Basic Protocol 1, step 15).

10a. Place the culture vessels thin-coated with collagen I in the cell culture incubator until use (at least 5 to 10 min).

11a. After 10 min of incubation with trypsin, observe the fibroblasts under the microscope to see if they are detached completely from the surface of the flask.

12a. If fibroblasts are completely detached, immediately add 3 ml of the freshly prepared medium for co-culture (containing additives) and 60 µl SBT1 from 10 mg/ml working stock solution.

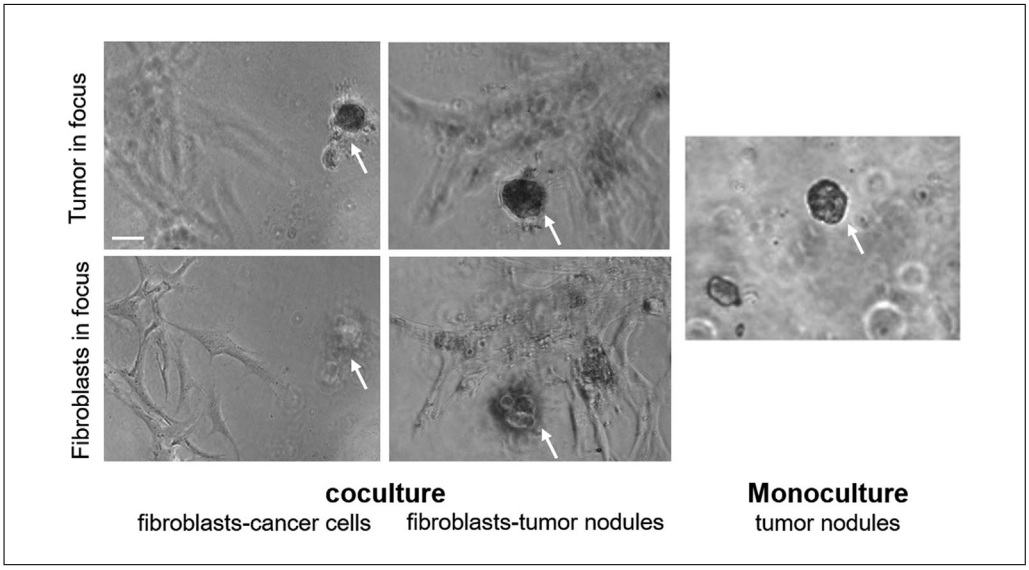


Figure 5 Coculture of IDC cells and fibroblasts. Coculture: Bright-field images of HMS-32 cells with T4-2 nodules with either cells mixed from day 1 of culture and kept for 6 days in collagen I (2000 Pa; fibroblasts-cancer cells) or fibroblasts mixed with tumor nodules (collected on day 3 of monoculture in a collagen I matrix) and cocultured for 3 days in collagen I (2000 Pa; fibroblasts-tumor nodules). The arrows point to tumor nodules. Images are shown with either the tumor in focus or the fibroblasts in focus. Monoculture: Bright-field image of T4-2 cells cultured alone in collagen I (2000) for 6 days to match the cell culture length of the T4-2 cells used for the cocultures. Scale bar, 25 μm.

13a. Spin down the fibroblasts for 5 min at 3000 × *g*, room temperature. Remove the supernatant carefully with vacuum aspiration and resuspend the pellets in 1 ml of freshly prepared medium for co-culture.

This step should be done carefully, by mixing up-and-down only a few times to avoid the risk of losing cells via their sticking to the tip of the pipette.

14a. Take an aliquot of 50 μl to count the number of fibroblasts with the hemacytometer (Phelan & May, 2015), and determine their concentration.

15a. Place the necessary volumes of fibroblasts and tumor nodules separately into two prelabeled microcentrifuge tubes and centrifuge for 5 min at 3000 × *g*, room temperature.

16a. Resuspend each pellet in 20 μl of cell co-culture medium containing the additives.

17a. Mix the fibroblast and the tumor solutions in a labelled microcentrifuge tube and proceed with embedding in collagen I as described in Basic Protocol 1, steps 16 to 20.

Note that we use a higher volume than usual (5 to 10 μl) to dissociate the pellets because we need 50,000 fibroblasts per well of a chambered slide (close to seven-fold the amount used for monoculture) in order to have a fibroblast/tumor cell ratio of 1/5 (see Support Protocol 4), and we are starting from tumor nodules (instead of single cells). This increase in volume for cell/tumor suspension is necessary due to the size of the pellets. The cells may be distinguished with a vital dye by staining one of the two cell types prior to embedding (each dye needs to be tested in 3D culture for a specific cell type to make sure that it is not cytotoxic), or cells expressing fluorescent molecules (e.g., GFP) may be used.

In Figure 5, we show cocultures with fibroblasts seeded with IDC cells from day 1 and with IDC tumors preformed in collagen I for 3 days prior to coculture. The fibroblasts are from a noncancerous ECM environment and seem to repress the development of

the tumor nodules compared to the control monoculture (see fibroblasts-cancer cell coculture compared to monoculture of cancer cells for representative images).

Coculture of tumors with non-neoplastic epithelial cells

Here we present a coculture protocol used for drug screening in our laboratory in which non-neoplastic HMT-3522 S1 cells (Briand et al., 1987) are seeded on hemichannels made of acrylic—called disease-on-a-chip (DOC)—and coated with the ECM molecule laminin 111. Preformed tumors are added onto the DOC as described in a previous publication (Vidi et al., 2014). This system is useful for rapid seeding of cells in a tissue geometry that matters for drug sensitivity. It is not used for measuring invasive potential, as tumor cells cannot go through the acrylic support. Other options to maintain the possibility of observing an invasive phenotype are being developed in our laboratory.

1b. Obtain or prepare acrylic-based hemichannels of 100 μm in width to mimic the diameter of the terminal ducts in the breast (where tumors arise normally). The in-house preparation of these chips has been described previously (Vidi et al., 2014).

2b. Sterilize the chips.

This could be done under UV in the cell culture hood, but we currently use ethylene oxide exposure in an Anprolene Sterilizer model AN74i (Andersen Products Inc.). Sterilization is performed with that instrument in accordance with the manufacturer's instructions for a 24-hr sterilization cycle, and ETO exposure is confirmed by observing color change on the enclosed Anpro Dosimeter (Andersen Products Inc.). The sterilized products are aseptically handled and packaged in sterilized materials in a UV laminar flow hood (Labconco Purifier Class II biosafety cabinet, Model 36204-00) wiped down with 70% ethanol (Decon Laboratories).

3b. The day before cell seeding, dilute 8.3 μl of 1 mg/ml laminin 111 stock in 91.7 μl of DMEM/F12 to obtain a final solution of 133 μg/ml. Add 100 μl of this laminin solution per chip (2.3 × 2 cm). Spread the solution drop-by-drop over the DOC gently but rapidly, ensuring that the entire surface is covered.

To spread the laminin, move the tip back and forth in different directions.

4b. Let the chips dry in a mini oven overnight at 37°C.

5b. Sterilize the chips again the following day for 30 min under UV irradiation in the laminar flow hood.

6b. Place each chip in a well of a 6-well plate (surface per well is 9.08 cm^2).

7b. Prepare the H14plus culture medium by adding the additives from the working stocks to reach the necessary final concentrations for each additive (see Table 1). The medium is kept in the water bath at 37°C until use.

Both cell culture media can be mixed (i.e., all necessary additives for T4-2 cells and S1 cells are combined in DMEM/F12 medium). Additives are the same for these two cells lines [5 μg/ml (or 0.15 IU/ml) prolactin, 250 ng/ml insulin, 1.4 μM hydrocortisone, 0.1 nM β-estradiol, 2.6 ng/ml sodium selenite, and 10 μg/ml transferrin], except that S1 cells also need 5 ng/ml EGF (therefore we call this medium H14plus).

8b. Detach S1 cells from their cell culture flask using trypsin/EDTA and add DMEM/F12 medium treated with SBTI as described in Basic Protocol 1, steps 9 to 10.

S1 cells are typically in culture in the seeding flask for 8 to 11 days before being used for propagation and/or an experiment (refer to Table 2 for information on cell numbers for seeding). Detaching non-neoplastic cells at earlier than 8 days would enrich the population for cells that rapidly spread on the flask surface; these cells usually have less

potential for phenotypically normal differentiation, and a drift in phenotype would occur after a few weeks. A good 2D culture is characterized by several islands of cells in which the cells that are delineating the islands have a cobblestone appearance with nuclei against the inside of the island (Vidi et al., 2013). This typical organization of cells in the island might not occur until day 8. These cells never reach 100% confluency; by day 8 of 2D culture they have filled between 60% and 70% of the culture surface. Usually, 5 to 7 million S1 cells are expected from a T-75 flask with that type of confluence.

9b. Count cells with a hemacytometer (Phelan & May, 2015) and calculate the cell concentrations. Spin down the number of each type of cell needed for the experiment for 5 min at 3000 × *g*, room temperature.

For the DOC, we add a higher concentration of cells compared to usual 2D culture (23,300 cells/cm^2), which for the surface of the chip (2.3 × 2 cm) corresponds to 318,000 cells (~3-fold the usual concentration).

10b. Resuspend the cell pellet in 1 ml of H14plus medium (times the number of chips + 1, if more than one chip is used) in a 15-ml Falcon tube.

11b. Add 1 ml of this solution gently (after mixing with the pipettor once or twice) drop-by-drop over the hemichannels.

To add the cells onto the DOC, gently release this solution on top of the laminin-coated hemichannels from one end to another following a 'zig zag' pattern to cover all the channels.

12b. Allow the cells to settle down on the DOC surface for 10 min (leave the chip in the laminar flow hood without moving it).

13b. After 10 min, add 2 ml of H14plus cell culture medium (containing the additives) around the DOC gently, to let the medium progressively rise and submerge the chip.

IMPORTANT NOTE: *Ensure that the medium is added from around the side of the dish so that the cells are not disturbed. Do not add the medium directly on top of the cells.*

14b. Culture the cells on the chips in the 6-well plate in the cell culture incubator for 8 days. Change medium every 2 to 3 days by adding it next to the DOC so that it gently covers the chip.

After 8 days, S1 cells should be basoapically polarized and out of the cell cycle. This complete differentiation can be confirmed with immunostaining for proliferation marker Ki67, basal polarity marker β4-integrin, and apical polarity marker ZO-1 (Vidi et al., 2014). Note that the proper imaging of basoapical polarity requires confocal microscopy and stacking of optical sections to display the orthogonal view (xz).

15b. Seed the T4-2 cells in collagen as described in Basic Protocol 1 for 3 days to initiate tumor formation (thus, start this 3D culture while the S1 cells have been in culture on the DOC for 5 days).

16b. After 3 days of collagen I culture, dislodge the T4-2 nodules from the collagen I gel with collagenase and wash them as described in Basic Protocol 1, steps 21 to 30.

17b. Take 50 µl of the solution with nodules and mix with 50 µl of 0.25% trypsin/EDTA, then incubate at 37°C for a few minutes to separate cells.

18b. Determine the cell concentration with a hemacytometer (Phelan & May, 2015) and calculate the volume to use so that there are 16,600 cells/ml of medium in the chosen culture vessel (i.e., the desired number of cancer cells is 50,000 cells per chip in 3 ml of medium to give enough nodules for one DOC device).

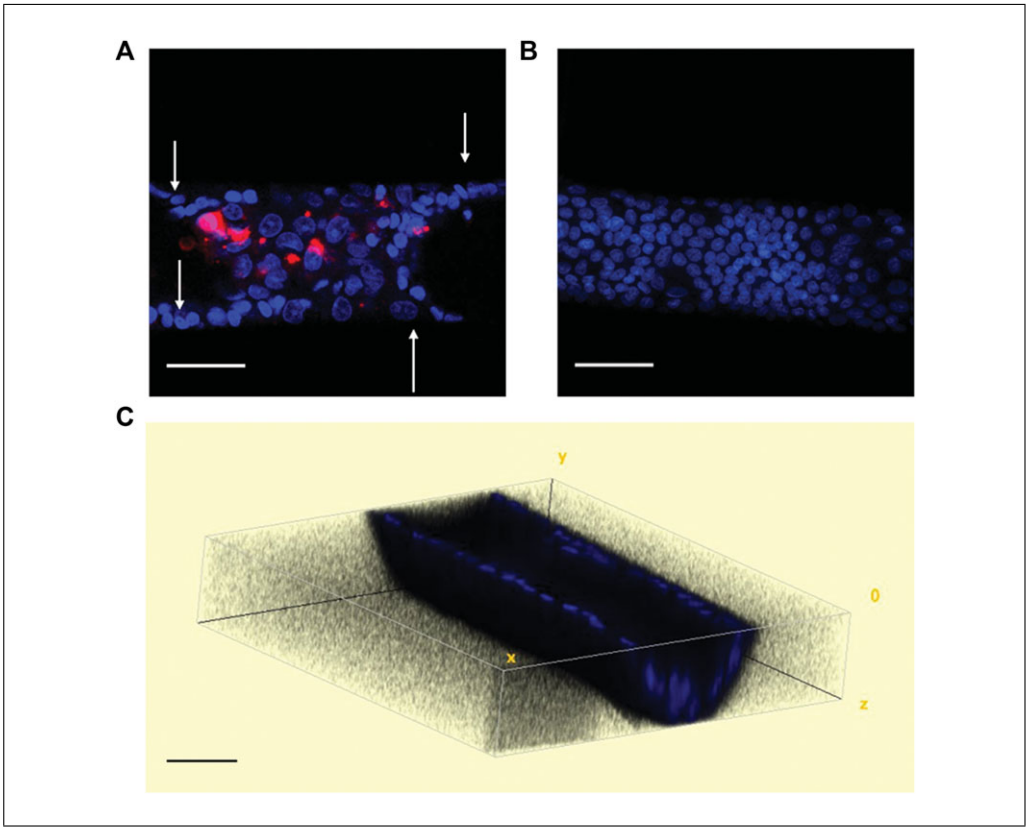


Figure 6 Coculture of non-neoplastic epithelial cells and cancer cells in the DOC. (**A**) Immunofluorescence image resulting from the staining of T4-2 tumors with dil prior to their seeding in the hemichannel. Non-neoplastic S1 cells were cultured on acrylic hemichannels covered with laminin 111 for 10 days to sustain their proliferation and differentiation. Tumor nodules (3 days old, prepared in 3D culture) were stained with dil (red) and seeded in the hemichannels for coculture with S1 cells. Cell nuclei were stained with DAPI (blue). Arrows point to areas with S1 cells only (this image is focused on the top of the hemichannel). (**B**) Image focused on the bottom portion of a hemichannel of the DOC containing only the monolayer of S1 cells. (**C**) Reconstituted hemichannel with 3D view based on the stacking of optical sections of the layer of S1 cells (shown using the 3D viewer of ImageJ; only the cells delineating the limits of the hemichannel in this image are shown). Size bar, 50 μm.

It seems that for consistency between chips for an experiment, it is better to count cells rather than nodules, as this gives a more accurate idea of the number of cancer cells present on the chip, even if they are distributed in many nodules. Importantly, if individual tumor cells were added directly onto the S1 cell layer, no tumor would grow, as it seems that the non-neoplastic cells in that culture condition prevent the proliferation of tumor cells.

19b. After the volume needed to have 16,600 cells/ml (for 3 ml) has been calculated, spin down the nodules for 5 min at 1000 × *g*, room temperature, and gently resuspend the pellet in 500 μl of H14plus cell culture medium.

20b. Add the medium containing the cells onto the entire DOC surface drop-by-drop.

21b. Allow the nodules to settle down on the surface of the DOC for 5 min.

22b. Add the rest of the culture medium (2.5 ml) slowly to the side of the DOC so that it rises to submerge the chip.

If the DOC is floating in the culture well, try to push it down to the bottom using a pipette tip.

To distinguish the two cell types, cells transfected with a fluorescent molecule (e.g., EGFP, dsRED, etc.) might be used. Alternatively, tumor nodules can be stained with diI (ThermoFisher Scientific, Catalog #D-282; stock solution, 1 mg/ml), a vital dye that is not toxic for the HMT3522 cells in 3D culture (Fig. 6). Briefly, incubate the number of nodules necessary for the experiment (following counting) in 1 to 2 ml of DiI solution at a final concentration of 1 µg/ml (from stock concentration 1 mg/ml) in the cell culture medium [the volume depends on the quantity of cells; e.g., 1 ml if cells are from a 35-mm dish, 2 ml if cells are from a 60-mm dish] for each culture vessel, in a 15-ml Falcon tube for 30 min in the cell culture incubator at 37°C. Then, wash the cell pellets three times with DMEM before adding the nodules drop-by-drop and the remaining volume of medium as explained above.

CALCULATION OF FIBROBLAST SEEDING CONCENTRATION FOR COCULTURE WITH TUMORS IN COLLAGEN I

SUPPORT PROTOCOL 4

The outcomes of the coculture of cells logically result from "cell type–to–cell type" interactions. These interactions might have inhibitory or stimulatory impact depending on the ratio of cells of different types. Therefore, we propose a protocol that helps calculate the seeding concentration of cancer cells and cells in the tumor microenvironment for coculture, based on the ratio of these cells in real tissue that was estimated by a pathologist. This approach will provide a means to best mimic a physiologically relevant situation.

Here we give an example for the ratio of triple-negative IDC cells to fibroblasts, which, according to a pathologist, should be 5:1.

Materials

Sample cultures of tumors made by the cells of interest (see Basic Protocol 1)
Microscope with eyepiece reticle
Calculator

1. Measure the diameter of cancer cells of interest in 3D cell culture.

   This measurement can be done with an eyepiece reticle based on cell membrane staining such as E-cadherin or other adhesion complex protein (and DAPI staining to identify cells). The microscope needs to be calibrated with a stage micrometer before using the eyepiece reticle for measurements (see Internet Resources). The corresponding length in µm depends on the lens magnification [4×, 10×, 20×, 40×, etc.], i.e., the distance between the division lines on the reticle changes with the magnification (reticle division/objective lens value = distance between lines on the reticle). The reticle division depends on the length of the reticle and the number of divisions: length of the reticle in mm/number of divisions= reticle division. A minimum of 50 cells should be analyzed to get an average of the cell size. In the case of T4-2 cells, the calculated diameter is 20 µm.

2. Measure tumor size in the 3D culture conditions to be used for the coculture (e.g., collagen I of a given stiffness and a given length of culture).

   You may use the same cultures as in step 1 and proceed similarly with the eyepiece reticle to analyze approximately 50 tumor nodules. In the case of T4-2 cells, after 5 days of culture in collagen I (2000 Pa), the average tumor diameter is 125 µm.

3. Count the number of tumor nodules per cm^2 under the 3D culture conditions of interest.

   You may use the same samples as in steps 1 and 2, but it is better to have at least three different wells from which to count in order to have an average number.

4. Calculate the average number of tumor cells per nodule under the chosen 3D culture conditions.

Volume of tumor = 4/3 × (3.14 × (125/2)^3) = 1,022135 μm^3 (125 is the tumor diameter)

Volume of a cell = 4/3 × (3.14 × (20/2)^3 = 4,182 μm^3 (20 is the cell diameter)

Volume of a tumor nodule/Volume of a cell = 1,022135/4,182 = 244

Thus, the number of cancer cells per nodule is 244.

5. Calculate the seeding density of fibroblasts in the coculture with tumors.

There are on average 1000 nodules per chambered slide well of T4-2 in collagen I culture (2000 Pa) after 5 days. Thus, there are 244 × 1000 = 244,000 cancer cells/well. This number is almost four times the number of T4-2 cells usually seeded per well (62,100). This is calculated as follows:

T4-2: 43,150 cells/cm^2; number of cells per well of a chambered slide = 43,150 × 1.44 cm^2 [surface of a well] = ~62,100 cells/well/100 μl of collagen spread (14 μl/cm^2 thin coat of collagen + 55 μl/cm^2 of collagen for embedding; note that 20%, thus 20 μl, of that volume is a thin coat done prior to adding the cells to 80% of the volume, hence 80 μl).

Since the tumor nodules/fibroblast ratio is 5:1, the number of fibroblasts required for the coculture is ~50,000 per well (244,000/5) of a 4-well chambered slide if we add 1000 nodules per well as compared to the usual 7200 fibroblasts per well (since seeding is usually 5000 cells/cm^2).

SUPPORT PROTOCOL 5

WEANING CELLS OFF SERUM

We present a simple method to wean cells off serum so that coculture can be optimized to readily measure the impact of cells on each other. Weaning off serum requires careful and daily observation of cells in 2D culture as the percentage of serum is progressively reduced and the concentration of additives is progressively raised. In the end, the phenotypic traits of the cells should not be dramatically changed, unless it is for an improvement. Here we illustrate the weaning off procedure and test with a stromal cell line.

Materials

HMS-32 human fibroblasts (human-TERT immortalized human mammary stromal cell line from a reduction of mammoplasty patient #32; a kind gift from Brittney-Shay Herbert, IU Simon Cancer Center; Shay et al., 1993; Shay et al., 1995; Subbaramaiah et al., 2016)
DMEM/F-12 (Thermo Fisher Scientific, Catalog #12400-024; see recipe)
Fetal bovine serum (FBS; ATCC #302020)
Insulin (see recipe and Table 1)
Hydrocortisone (see recipe and Table 1)
Sodium selenite (see recipe and Table 1)
Transferrin (see recipe and Table 1)
Epidermal growth factor (EGF; see recipe and Table 1)
Fibroblast growth factor (FGF, see recipe and Table 1)
Transforming growth factor (TGF, see recipe and Table 1)
Trypsin-EDTA (0.25% 1 mM tetrasodium EDTA; Gibco, Catalog #25200-056)
Collagen, type I (Advanced Biomatrix: PhotoCol®, Catalog #5201-1KIT for preparing tunable collagen I)

Additional reagents and equipment for collagen embedding of cells (Basic Protocol 1) and basic cell culture techniques including counting cells with a hemacytometer (Phelan & May, 2015)

1. Initiate the process of serum wean-off after seeding the cells for a new passage in a flask by culturing them in 0.75× fetal bovine serum (FBS)–supplemented medium

(i.e., 75% of the usual serum concentration) combined with 25% of chemically defined serum-free medium (i.e., 25% of the normal concentration of each necessary additive).

Weaning off serum for cell line(s) previously cultured in 10% FBS in medium can usually be accomplished in two continuous cell passages through a sequential FBS reduction process, although depending on the cell lines it might take longer. We have weaned several breast cell lines (e.g., MDA-MB-231, MCF10A) and fibroblasts (HMS-32) off serum; the only cell line that we could not accustom to a serum-free medium is MCF7 (these cells have been cultured for a long time without a standard protocol, and it seems that there are now many strains, depending on the laboratories, according to experts on these cells).

2. Feed the cells every 2 days after initial cell seeding with medium that contains sequentially reduced FBS and gradually increased defined chemicals at each feeding step, i.e., the medium for first-time feeding should contain 0.5× FBS-supplemented medium (i.e., 50% of the usual serum concentration) and 50% chemically defined serum-free medium (i.e., 50% of the normal concentration of each necessary additive). Likewise, the medium for the second-time feeding should contain 0.25× FBS-supplemented medium (i.e., 25% of the usual serum concentration) and 75% chemically defined serum-free medium (i.e., 75% of the normal concentration of each necessary additive).

3. Detach cells from the flask with 0.25% trypsin/EDTA after they reach the usual confluence for propagation (e.g., it usually takes 6 days of culture for fibroblasts to reach 70% to 80% confluence) and seed a new generation of cells in 0.10× FBS–supplemented medium (i.e., 10% of the usual serum concentration) and 90% chemically defined medium (i.e., 90% of the normal concentration of each necessary additive), to avoid potential crisis in FBS-free medium.

   It might be advisable to start the new flask with a 25/75 ratio of FBS and chemically defined medium and then go to 10/90 at the next feeding stage). It is also important to add SBTI after trypsin/EDTA, as described in Basic Protocol 1, step 10.

4. After the cells are fed twice with the same medium (thus, after seeding in a new flask and one feeding step), feed the cells with 100% of chemically defined medium for the third feeding and thereafter.

   If the cells appear to suffer during the weaning-off procedure, place them back at the last FBS/chemically defined medium ratio that showed a healthy cell culture, until the cells recover. Then, continue reducing FBS content with smaller decreases in percentages (hence, more intermediate steps). If the cells look good but simply do not proliferate as fast as before, the culture might be normal (as long as cells still proliferate), or if they are stalled, a similar process, with "stepping back" as explained in the text above, can be adopted.

5. Embed cells that were weaned off serum and control cells (that were kept in their initial FBS-containing medium throughout the weaning off procedure) in collagen I using the method presented in Basic Protocol 1.

   The density of cells at seeding is an important parameter to consider for each cell line or source of primary fibroblasts. The density of cells is assessed via observation by bright-field microscopy as shown with the example of NIH-3T3 cells, which are fibroblasts of murine origin (Fig. 7A).

6. Keep cells in culture for 6 days, then stain them with phalloidin (for actin microfilaments) and DAPI (for the cell nucleus).

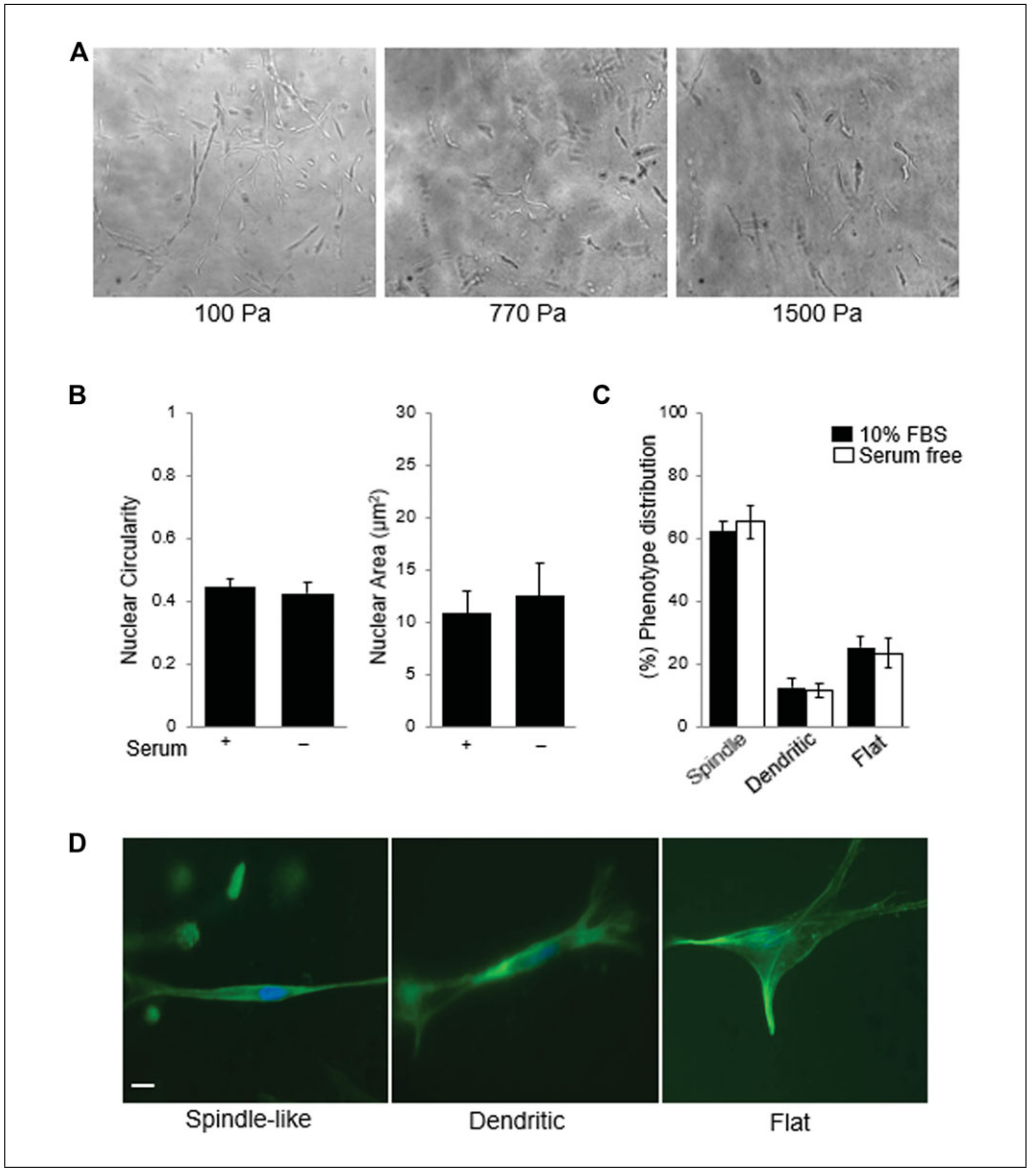


Figure 7 Influence of culture conditions on the phenotype of fibroblasts. (**A**) NIH3T3 cells were embedded in Collymers prepared at different stiffness degrees (100, 770, 1500 Pa) with a seeding density of 1250 cells/cm^2 and cultured in DMEM/F12 medium supplemented with 10% FBS. Note the differences in cell density in the plane of focus and in orientation of the cells in the matrix depending on matrix stiffness. Images were taken with an Olympus IX70 microscope (objective 10×) on day 5 of culture. (**B**-**D**). HMS-32 cells were cultured in collagen I matrix of stiffness 770 Pa for 6 days at a seeding density of 5000 cells/cm^2. Bar graphs show the analysis of nuclear circularity and size with and without serum (**B**) and the phenotypic distribution of mammary stromal fibroblasts (**C**). Fluorescence images representing three phenotypes of HMS-32 cells (spindle-like, dendritic, and flat) are shown with nuclei stained with DAPI (blue) and the actin cytoskeleton stained with phalloidin (green) (**D**). $n = 3$ with 150 cells analyzed per group. Scale bar, 5 μm.

7. Count the number of cells (between 150 and 300 cells per biological replicate) in different cell-shape categories and compare the 'serum-free' condition with the 'serum' condition.

   Here we use the shape parameter, since the phenotype of fibroblasts is partly identified via their shape (spindle-like, dendritic-like and flat). We are also using nuclear morphometry (size and shape), which is a sensitive annunciator of upcoming phenotypic changes (Chittiboyina et al., 2018; Lelièvre & Chittiboyina, 2018). We had first established the necessary density of cells in 3D culture with normal collagen I stiffness, so that cells are resting (i.e., they are negative for Ki67 staining) and they predominantly

adopt an elongated or spindle-like shape under normal matrix stiffness (770 Pa). An increase in the percentage of cells with a flattened phenotype is often an indication of stress-activated fibroblasts (Sampson et al., 2011). Thus, the culture of fibroblasts requires the proper density, shape, and orientation as in vivo. These cells were not drastically affected by the switch to a serum-free medium, as shown by the same ratio of cells with different shapes and nuclear morphometric analysis (Fig. 7B-D).

REAGENTS AND SOLUTIONS

DMEM/F12 medium

For most of our cell culture needs, we use Dulbecco's Modified Eagle's Medium (DMEM/F12, Thermo Fisher Scientific, Catalog #12400-024). Note that this medium can also be purchased as a ready-made solution.

We typically prepare 10 liters of DMEM/F12 medium, starting from powder according to the manufacturer's guidelines. Medium quantity is adjusted depending on the cell culture activity and can be anticipated for a 6-month period knowing that each open bottle should not be kept for more than 1 month (it is stored at 4°C) to avoid changes in pH. The medium is filtered under sterile conditions into 20 screw-capped bottles of 500 ml each. One of the bottles is kept at 37°C in a water bath for 1 day to make sure that there is no contamination of the new batch of medium that was just prepared. It is preferable NOT to share a bottle of medium among different users, in order to avoid cross-contamination.

Additives for the DMEM/F12 medium:

The medium should not be stored with additives for more than 1 day. Therefore, we prepare the medium with additives fresh for each set of experiments on the day of the experiment and/or cell propagation. Below is the list of additives used in the protocols described above and the final concentrations achieved in the cell culture medium. The core additives, transferrin, hydrocortisone, insulin, and sodium selenite [THIS], are usually the basis for all cells; then, we add hormones, growth factors, or other components according to the cell types. Additives have very strict shelf lives, depending on their initial stock concentration and diluted (working) stock concentration, for each appropriate storage temperature. For each additive, a preparation process has been established so that we can have a reasonable dilution to use with the cell culture medium even in small quantities, which sometime necessitates intermediate stock concentrations. Aliquots of the storage concentrations are made with the idea that each person performing cell culture will have his/her own set of additives (not shared with others to reduce risks of cross contamination):

1. Prolactin (Sigma-Aldrich®, Catalog #L-6520); final concentration, 5 µg/ml (see recipe below)
2. Insulin (Sigma-Aldrich® Catalog #I-4011): final concentration, 250 ng/ml (see recipe below)
3. Hydrocortisone (Sigma-Aldrich® Catalog #H-0888); final concentration, 1.4 µM (see recipe below)
4. β-Estradiol (Sigma-Aldrich® Catalog #E-2758); final concentration, 0.1 nM (see recipe below)
5. Sodium selenite (BD Biosciences Catalog #354201); final concentration, 2.6 ng/ml (see recipe below)
6. Transferrin (Sigma-Aldrich® Catalog #T-2252); final concentration, 10 µg/ml (see recipe below)
7. Epidermal growth factor [Corning Life Sciences (previously sold through BD Biosciences Catalog #354001]; final concentration, 5 ng/ml (see recipe below)

8. Fibroblast growth factor (FGF, ThermoFisher); final concentration, 2.5 ng/ml (see recipe below)
9. Transforming growth factor (TGF, ThermoFisher); final concentration, 7.5 pg/ml (see recipe below)

We have also included details of preparation and storage for SBTI (see recipe below), which is an additive necessary to counteract the effect of trypsin/EDTA since there is no serum in the cell culture medium. Once aliquots of additives are in use, they are stored in the refrigerator at 4°C. At that storage temperature, SBTI should not be kept for more than 2 weeks and sodium selenite and EGF should not be kept for more than 1 week; the other additives should not be kept longer than 1 month (Table 1). The length of storage at lower temperatures is indicated in each detailed additive preparation below and in Table 1.

β-Estradiol

Sigma, Catalog #E-2758

Preparation procedure:

- Weigh 10 mg of β-estradiol in a 20 ml sterile beaker. Cover the beaker immediately with aluminum foil (the one used to sterilize the beaker). This step can be performed under non-sterile conditions.
- From here onwards, work must be performed under the laminar flow hood and with the light OFF[1]. Disinfect the beaker and any other material (e.g. ice bucket) appropriately by wiping with a tissue paper sprayed with 70% ethanol, before introducing it into the hood. Also, carefully follow all other rules for working inside the cell culture room.
- It is better to prepare the dark glass vials needed for aliquotting before beginning. We usually UV-sterilize the autoclaved glass vials used for this purpose overnight by opening the lids/caps of the bottles and placing the lid facing upward such that all the surfaces of the bottle including its neck and lids are properly sterilized. You can prepare the labels on the vials before they are UV sterilized; label as many vials as you will need, from a stock of sterile vials only used for preparing additives. The proper labeling should include the additive's code, concentration, and expiration date, and the initials of the person who prepared the additive.
- Add 1.25 ml of cold 95% (190 proof) ethanol[2] to make an 8 mg/ml solution. When completely dissolved, place the beaker on ice, and transfer the solution to a labeled sterile dark glass vial, placed on ice. Do not filter.
- Serially dilute the 8 mg/ml stock in the following manner, using a sterile labeled dark glass vial each time:
    - Take 50 µl of the 8 mg/ml stock and add 1.45 ml of 95% (190 proof) ethanol to make 1.5 ml of 10^{-3} M or 2.67×10^{-1} mg/ml stock.
    - Take 20 µl of the 10^{-3} M and add 1.98 ml of 95% (190 proof) ethanol to make 2 ml of 10^{-5} M or 2.67×10^{-3} mg/ml stock.
    - Take 20 µl of the 10^{-5} M and add 1.98 ml of 95% (190 proof) ethanol to make 2 ml of 10^{-7} M or 2.67×10^{-5} mg/ml working stock.
    - Do not filter any of the stocks; place all vials on ice right after use.
- Make 500-µl aliquots of the 10^{-7} M or 2.67×10^{-5} mg/ml working stock using sterile dark glass vials. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the glass vials[3]. Place each aliquot on ice, immediately after preparation.
- Transfer to the appropriate freezer. Since β-estradiol does not freeze, due to the presence of ethanol, there is no need to place the aliquots on dry ice.

- Note the number of aliquots made, concentration, expiration date. etc., in the usage log for β-estradiol.

Storage and usage:

- The 8 mg/ml (stable for 1 year), 10^{-3} M (stable for 1 year), and 10^{-5} M (stable for 6 months) stocks are always kept at −80°C. Some aliquots of the 10^{-7} M working stock (stable for 6 months) are kept at −20°C for ready availability. The rest of the working stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- To avoid confusion, in the −80°C freezer, it is useful to keep the working stock separated from the concentrated stock.
- Aliquots are removed from −20°C when needed for cell culture and kept at 4°C for a period of up to 1 month.
- Write the date when an aliquot is thawed on the glass vial, for reference for determining the expiration date at 4°C.

The 8 mg/ml, 10^{-3} M, and 10^{-5} M stocks can be used later to prepare more diluted stocks (follow the expiration dates). Remove vials from the freezer and place on ice immediately. All tubes are kept on ice; transfer the tubes to the corresponding freezer once the preparation is done.

[1] β-estradiol is light sensitive. For this reason, only dark glass vials must be used for its preparation.

[2] Ethanol, 95% (190 proof) should be from a commercially available source and ready for use.

[3] Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!

Epidermal growth factor

Previously sold by BD Biosciences; currently sold by Corning Life Sciences (Catalog #354001).

Preparation procedure:

- Work must be performed under a laminar flow hood. Disinfect EGF (100 µg) bottle and any other material (e.g. ice bucket) appropriately by wiping with a tissue paper sprayed with 70% ethanol, before introducing in the hood. Also, carefully follow all other rules for working inside the cell culture room. Place the 100-µg EGF bottle on ice as soon as it is introduced in the hood.
- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV-sterilize the autoclaved 1.5-ml microcentrifuge tubes used for this purpose overnight by opening their caps facing upward such that the all the content of the tube is properly sterilized. You can prepare the labels on the microcentrifuge tubes before they are UV sterilized and label as many tubes as you will need, from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labeling should include the additive's code, concentration, and expiration date, and the initials of the person who prepared the additive.
- Filter 7 ml of sterile deionized Milli-Q water[1] using a 10-ml syringe and a 0.22-µm pore size filter; transfer to an ice-cold 15-ml Falcon tube. Place the tube on ice.
- Disinfect the bottle of EGF thoroughly, especially the neck and top of the bottle. Do this by wiping it with a 70% ethanol–sprayed tissue paper, before and after removing the metal cap (there is a rubber stopper underneath). Avoid spraying the bottle directly. Leave rubber stopper loose and keep the bottle on ice.

- Dissolve the 100 µg of EGF, contained in the bottle, in 5 ml of prefiltered sterile water. When completely dissolved (swirl the bottle), place on ice. If it is easier to handle, transfer the solution to an ice-cold 15-ml Falcon tube and keep on ice. Do not filter.
- Keeping the bottle/tube on ice, make 50-µl and 100-µl aliquots. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the microcentrifuge tube[2]. Place each aliquot on ice, immediately after preparation.
- Fast-freeze all aliquots by placing them on dry ice[3] for about 5 to 10 min. When completely frozen (solution is completely white), transfer to the appropriate freezer.

Storage and usage:

- Some aliquots of the working stock are kept at −20°C for ready availability. The rest of the stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out. Storage at both temperatures is fine for up to 3 months.
- Aliquots are thawed from −20°C when needed and kept at 4°C for a period of up to 1 week.

[1]The stock bottle of sterile deionized Milli-Q water used for the preparation of additives must NOT be used for any other purpose.

[2]Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!

[3]Spray dry ice with 70% ethanol (to decrease the temperature of the ice) before placing the aliquots.

Fibroblast growth factor

ThermoFisher, Catalog #PHG0264

- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV-sterilize the autoclaved 1.5-ml microcentrifuge tubes used for this purpose overnight by opening their caps facing upward such that the surface of the tube is properly sterilized. You can prepare the labels on the microcentrifuge tubes before they are UV sterilized, and label as many tubes as you will need from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labeling should include the additive's code, concentration, and expiration date, and the initials of the person preparing the additive.
- The bottle of FGF should be briefly centrifuged prior to opening. DO NOT VORTEX!
- Weigh 2 mg of BSA in a sterile 20-ml beaker used only for cell culture. Immediately cover the top of the beaker with aluminum foil as soon as you have added the BSA.
- Work must be performed in the laminar flow hood. Disinfect the FGF (10 µg) bottle and any other material including the beaker and ice bucket appropriately by wiping with a tissue paper sprayed with 70% ethanol, before introducing in the hood. Also, carefully follow all other rules for working inside the cell culture room. Place the 10 µg TGF-β bottle on ice as soon as it is introduced in the hood.
- Dissolve weighed BSA in 2 ml of 1× PBS[1] to prepare a 0.1% w/v solution and transfer it to a 10-ml Falcon tube. Filter the solution using a 0.2 µm pore size filter.
- Add 100 µl of sterile Milli-Q[2] water to the FGF bottle to make a concentration of 100 µg/ml (intermediate solution).
- Add all of the intermediate solution (100 µl) to 900 µl 0.1% BSA in PBS to make a concentration of 10 µg/ml (working stock solution).

- Aliquot 50 µl from the working stock solution to microcentrifuge tubes and store them at −20°C. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the microcentrifuge tube[3]. Place each aliquot on ice, immediately after preparation.
- Fast freeze all aliquots by placing them on dry ice[4] for about 5 to 10 min. When completely frozen (solution is completely white), transfer to the appropriate freezer.

Storage and usage:

- Some aliquots of the working stock concentration are kept at −20°C for ready availability. The rest of the stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- Aliquots are thawed once from −20°C, when needed and kept at 4°C for a period of up to 1 week.

[1]The bottle of sterile PBS used for the preparation of additives must NOT be used for any other purpose.

[2]The stock bottle of sterile deionized Milli-Q water used for the preparation of additives must NOT be used for any other purpose.

[3]Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!

[4]Spray dry ice with 70% ethanol (to decrease the temperature of the ice) before placing the aliquots in dry ice.

Hydrocortisone

Sigma, Catalog #H-0888.

- Weigh 50 mg of hydrocortisone in a 20-ml sterile beaker. Cover the beaker immediately with the aluminum foil (the one used to sterilize the beaker). This step can be performed under nonsterile conditions.
- From here onward, work must be performed in the laminar flow hood. Disinfect the beaker and any other material (e.g., ice bucket) appropriately by wiping with a tissue paper sprayed with 70% ethanol before introducing it in the hood.
- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV-sterilize the autoclaved 1.5-ml microcentrifuge tubes used for this purpose overnight by opening their caps facing upward such that the surface of the tube is properly sterilized. You can prepare the labels on the microcentrifuge tubes before they are UV sterilized and label as many tubes as you will need, from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labeling should include the additive's code, concentration, expiration date, and the initials of the person who prepared the additive.
- Dissolve hydrocortisone in 10 ml of cold 95% (190 proof) ethanol[1], to make 1.4×10^{-2} M (5 mg/ml) concentrated stock. When completely dissolved, place the beaker on ice. The solution can be transferred to an ice-cold, properly labeled 15-ml Falcon tube if it is easier to handle. Do not filter.
- Keep the beaker (covered when not in use) or Falcon tube on ice, and prepare 500-µl aliquots separately. Use a new tip for each aliquot and avoid touching the walls of the beaker or microcentrifuge tube[2]. Place each aliquot on ice, immediately after preparation.
- Dilute 0.5 ml of 1.4×10^{-2} M (5 mg/ml) solution in 4.5 ml of cold 95% (190 proof) ethanol in a 15-ml Falcon tube, to make 1.4×10^{-3} M (0.5 mg/ml) working stock.

The Falcon tube containing 95% (190 proof) ethanol can be placed on ice before adding 0.5 ml of 1.4×10^{-2} M (5 mg/ml) solution. Do not filter.

- Keep the Falcon tube on ice to make 500-µl aliquots in the manner described two steps above.
- Transfer to the appropriate freezer. Since hydrocortisone does not freeze, due to the presence of ethanol, there is no need to place the aliquots on dry ice. Note the number of aliquots made, concentration, expiration date, etc., in the usage log for hydrocortisone.

Storage and usage:

- The concentrated stock (5 mg/ml) is always kept at −80°C. Some aliquots of the 10^{-3} M working stock are kept at −20°C for ready availability. The rest of the working stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- To avoid confusion, in the −80°C freezer, it is useful to keep the working stock separated from the concentrated stock.
- Aliquots are removed from −20°C when needed and kept at 4°C for a period of up to 1 month.

The 5 mg/ml stock can be used later to prepare more diluted stocks, but the initial expiration date should be considered. Remove from the freezer and place on ice immediately. Keep on ice and transfer tubes to the corresponding freezer once the dilution is done.

[1]Ethanol 95% (190 proof) should be ready for use, from a commercially available source.

[2]Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!

Insulin

Sigma, Catalog #I-4011

- Weigh 25 mg of insulin in a 40-ml sterile beaker. Cover the beaker immediately with aluminum foil. This step can be performed under nonsterile conditions.
- From here onwards work must be performed in the laminar flow hood. Disinfect the beaker and any other material (e.g, ice bucket) appropriately by wiping with a tissue paper sprayed with 70% ethanol, before introducing in the hood. Place the beaker on ice as soon as it is introduced in the hood.
- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV-sterilize the autoclaved 1.5-ml microcentrifuge tubes used for this purpose overnight, by opening their caps facing upward such that the surface of the tube is properly sterilized. You can prepare the labels on the microcentrifuge tubes before they are UV sterilized, and label as many tubes as you will need from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labelling should include the additive's code, concentration, and expiration date, and the initials of the person who prepared the additive.
- Add 12.5 ml of 5 mM HCl[1] to the insulin, to make a 2 mg/ml concentrated stock. When completely dissolved, transfer to an ice-cold, labeled 15-ml Falcon tube. Filter and transfer to another ice-cold 15-ml Falcon tube, using a 20-ml syringe and a 0.22-µm syringe filter.
- Keep the Falcon tube on ice and transfer 1.5 ml of the 2 mg/ml concentrated stock to an ice-cold, labeled 50-ml Falcon tube for later dilution (see two steps below). Place the tube on ice.

- Make 1.5-ml aliquots with the remaining 2 mg/ml stock. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the microcentrifuge tube[2]. Place each aliquot on ice, immediately after preparation.
- Add 28.5 ml of cold, sterile deionized Milli-Q water[3] to the 2 mg/ml concentrated stock solution contained in the 50-ml Falcon tube (see two steps above), to make a 100 µg/ml working stock. Filter and transfer to another ice-cold 50-ml Falcon tube, using a 60-ml syringe and a 0.22-µm pore-size filter.
- Make 1-ml aliquots of the 100 µg/ml working stock, as described two steps above.
- Fast-freeze all aliquots by placing them on dry ice[4] for about 5 to 10 min. When completely frozen (solution is completely white), transfer to the appropriate freezer.

Storage and usage:

- The 2 mg/ml concentrated stock is always kept at −80°C. Some aliquots of the 100 µg/ml working stock are kept at −20°C for ready availability. The rest of the working stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- To avoid confusion in the −80°C freezer, it is useful to keep the working stock solution separated from the concentrated stock solution.
- Aliquots are thawed once from −20°C, when needed, and kept at 4°C for a period of up to 1 month.

The 2 mg/ml stock can be used to prepare the 100 µg/ml working stock, respecting the expiration date. Remove from the freezer and place on ice immediately.

[1]The 5 mM HCl is prepared using the commercially available HCl (Mallinckrodt, Catalog #2062). The 5 mM solution is prepared in a sterile container in the fume hood and it is only used for preparing insulin. The 5 mM HCl solution is prepared in a sterile deionized water, using a sterile container, in the laminar flow hood, and it is only used for preparing the insulin.

[2]Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!

[3]The stock bottle of sterile deionized Milli-Q water used for the preparation of additives must NOT be used for any other purpose.

[4]Spray dry ice with 70% ethanol (to decrease the temperature of the ice) before placing the aliquots

Prolactin

Sigma, Catalog #L-6520, with name "Luteotropic hormone."

- Freshly prepare 40 ml of 2.22 mg/ml solution of sodium bicarbonate ($NaCHO_3$)[1]. Weigh 88.67 mg of sodium bicarbonate in a 20-ml sterile beaker. Cover the beaker immediately with the aluminum foil (the one used to sterilize the beaker). This step can be performed under nonsterile conditions.
- From here onwards, work must be performed in the laminar flow hood. Disinfect the beaker and any other material (e.g., ice bucket) appropriately by wiping with a tissue paper sprayed with 70% ethanol, before introducing in the hood. Also, carefully follow all other rules for working inside the cell culture room. Place the 1000 IU prolactin bottle on ice as soon as it is brought in the hood.
- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV-sterilize the autoclaved 1.5-ml microcentrifuge tubes used for this purpose overnight by opening their caps facing upward such that the surface of the tube is properly sterilized. You can prepare the labels on the

microcentrifuge tubes before they are UV sterilized and label as many tubes as you will need from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labeling should include the additive's code, concentration, and expiration date, and the initials of the person preparing the additive.

- Add 10 ml of cold sterile deionized Milli-Q water[2] to the sodium bicarbonate. When completely dissolved, transfer to an ice-cold, labeled 50-ml Falcon tube. Add 30 ml of sterile deionized water to make a 2.22 mg/ml solution. Filter and transfer to another ice-cold 50-ml Falcon tube using a 60-ml syringe and a 0.22-µm pore-size filter.
- Disinfect the bottle of prolactin thoroughly, especially the neck and top of the bottle. Do this by wiping it with a 70% ethanol-sprayed tissue paper, before and after removing the metal cap (there is a rubber stopper underneath). Avoid spraying the bottle directly. Leave rubber stopper loose and keep the bottle on ice.
- Add 10 ml of sterile 2.2 mg/ml sodium bicarbonate solution to the 1000 IU of prolactin. Once fully dissolved (swirl the bottle), place the solution on ice. Repeat this twice to make sure to remove all prolactin from the bottle. Transfer to the same 50-ml Falcon tube, kept on ice. Add another 3.3 ml of sodium bicarbonate to the tube to make a 30.3 IU/ml working solution (the total volume for solution must be 33.3 ml). Filter and transfer to another ice-cold 50-ml Falcon tube using a 60-ml syringe and a 0.22 µm pore size filter.
- Keeping the Falcon tube on ice, make 1-ml aliquots. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the microcentrifuge tube[3]. Place each aliquot on ice, immediately after preparation.
- Fast freeze all aliquots by placing them on dry ice[4] for about 5 to 10 min. When completely frozen (solution is completely white) transfer to the appropriate freezer.

Storage and usage:

- Some aliquots of the working stock are kept at −20°C for ready availability. The rest of the stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- Aliquots are thawed once from −20°C, when needed and kept at 4°C for a period of up to 1 month.

An alternative source is biological prolactin from ovine (10 mg), available from the National Hormone and Peptide Program (Harbor-UCLA Medical Center).

[1]The sodium bicarbonate used is cell culture tested from Sigma, Catalog #S-5761.

[2]The stock bottle of sterile deionized Milli-Q water used for the preparation of additives must NOT be used for any other purpose.

[3]Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!

[4]Spray dry ice with 70% ethanol (to decrease the temperature of the ice) before placing the aliquots.

Sodium selenite

Selenous acid, sodium salt, BD Biosciences, Catalog #354201.

Preparation procedure:

- Work must be performed under a laminar flow hood. Disinfect the sodium selenite (100 mg) bottle and any other material (e.g. ice bucket) appropriately by wiping with a tissue paper sprayed with 70% ethanol before introducing it in the hood.

Also, carefully follow all other rules for working inside the cell culture room. Place the 100 mg sodium selenite bottle on ice as soon as it is brought into the hood.

- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV sterilize the autoclaved 1.5-ml microcentrifuge tubes used for this purpose overnight, by opening their caps facing upward such that the tube is properly sterilized. You can prepare the labels on the microcentrifuge tubes before they are UV sterilized and label as many tubes as you will need from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labeling should include the additive's code, concentration, and expiration date, and the initials of the person who prepared the additive.
- Filter 15 ml of sterile deionized Milli-Q water[1] using a 10-ml syringe and a 0.22-µm pore-size filter, and transfer to an ice-cold 15-ml Falcon tube. Place the tube on ice.
- Disinfect the 100 mg bottle of sodium selenite thoroughly, especially the neck and top of the bottle. Do this by wiping it with a 70% ethanol–sprayed tissue paper, before and after removing the metal cap (there is a rubber stopper underneath). Avoid spraying the bottle directly. Leave the rubber stopper lose and keep the bottle on ice.
- Dissolve the 100 mg of sodium selenite contained in the bottle in 5 ml of prefiltered cold, sterile Milli-Q water to make a 20 mg/ml stock. When completely dissolved (swirl the bottle), place on ice. If easier to handle, transfer the solution to an ice-cold 15-ml Falcon tube and keep it on ice. Do not filter.
- Serially dilute the 20 mg/ml stock in the following manner:
  - Dilute 25 µl of the 20 mg/ml stock in 1 ml of cold prefiltered sterile Milli-Q water to make a 500 µg/ml stock.
  - Dilute 26 µl of the 500 µg/ml stock in 5 ml of cold prefiltered sterile Milli-Q water to make a 2.6 µg/ml working stock.
  - Do not filter. Place tubes on ice right after use.
- Keeping the bottle/tube on ice, make 1-ml aliquots of the 20 mg/ml stock[2], 50-µl aliquots of the 500 µg/ml stock[2], and 250-µl aliquots of the 2.6 µg/ml stock. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the microcentrifuge tube[3]. Place each aliquot on ice, immediately after preparation.
- Fast-freeze all aliquots by placing them on dry ice[4] for about 5 to 10 min. When completely frozen (solution is completely white), transfer to the appropriate freezer. Note the number of aliquots made, concentration, expiration date, etc., on the usage log for sodium selenite.

Storage and usage:

- The 20 mg/ml and 500 µg/ml concentrated stocks are always kept at −80°C. Some aliquots of the 2.6 µg/ml working stock are kept at −20°C for ready availability. The rest of the working stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- To avoid confusions in the −80°C freezer, it is useful to keep the working stock separated from the concentrated stocks.
- Aliquots are thawed once from −20°C, when needed and kept at 4°C for a period of up to 1 week.
- Write down on the microcentrifuge tube the date when an aliquot is thawed, for reference to determine the expiration date.

IMPORTANT NOTE: *Sodium selenite is oxygen-sensitive. For this reason, the tubes should not be left opened for long.*

[1] *The stock bottle of sterile deionized Milli-Q water used for the preparation of additives must NOT be used for any other purpose.*

[2]*The 20 mg/ml and 500 µg/ml stocks can be used later to prepare a 2.6 µg/ml working stock. For this reason, it is useful to aliquot them, to avoid repeated freezing and thawing. When preparing a working stock, remove a tube from the freezer and place it on ice immediately. All tubes are kept on ice; once the preparation is done, fast freeze, and transfer the tubes to the corresponding freezer.*

[3]*Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!*

[4]*Spray dry ice with 70% ethanol (to decrease the temperature of the ice) before placing the aliquots on dry ice.*

Soybean trypsin inhibitor

Sigma, Catalog #T-6522, type I-S

- Weigh 100 mg of SBTI into a 20-ml sterile beaker. Cover the beaker immediately with the aluminum foil that was used to sterilize the beaker. This step can be performed under nonsterile conditions.
- From here onwards, work must be performed in the laminar flow hood. Disinfect the beaker and any other material (e.g. ice bucket) appropriately by wiping with a tissue paper sprayed with 70% ethanol before introducing it in the hood. Also, carefully follow all other rules for working inside the cell culture room.
- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV-sterilize the autoclaved 1.5-ml microcentrifuge tubes used for this purpose overnight by opening their caps facing upward such that the entire surface of the tube is properly sterilized. You can prepare the labels on the microcentrifuge tubes before they are UV sterilized, and label as many tubes as you will need, from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labelling should include the additive's code, concentration, and expiration date, and the initials of the person who prepared the additive.
- Add 10 ml of cold sterile deionized Milli-Q water[1] to make a 10 mg/ml stock. When completely dissolved (swirl beaker), place on ice. Filter and transfer to an ice-cold 15-ml Falcon tube using a 10 ml syringe and a 0.22-µm pore size filter.
- Keeping the tube on ice, make 500-µl aliquots of the 10 mg/ml stock. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the microcentrifuge tube[2]. Place each aliquot on ice, immediately after preparation.
- Fast-freeze all aliquots by placing them on dry ice[3] for about 5 to 10 min. When completely frozen (solution is completely white), transfer to the appropriate freezer.

Storage and usage:

- Some aliquots of the working stock are kept at −20°C for ready availability. The rest of the stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- Aliquots are thawed once from −20°C, when needed and kept at 4°C for a period of up to 2 weeks.

[1]*The stock bottle of sterile deionized Milli-Q water used for the preparation of additives must NOT be used for any other purpose.*

[2]*Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!*

[3] *Spray dry ice with 70% ethanol (to decrease the temperature of the ice) before placing the aliquots.*

Transferrin

Sigma, Catalog #T-2252, with name Apo-transferrin.

- Weigh 50 mg of transferrin in a 20-ml sterile beaker. Cover the beaker immediately with the aluminum foil (the one used to sterilize the beaker). This step can be performed under nonsterile conditions.
- From here onwards, work must be performed under the laminar flow hood. Disinfect the beaker and any other material (e.g. ice bucket) appropriately by wiping with a tissue paper sprayed with 70% ethanol, before introducing it in the hood.
- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV sterilize the autoclaved 1.5-ml microcentrifuge tubes used for this purpose overnight by opening their caps facing upward such that the surface of the tube is properly sterilized. You can prepare the labels on the microcentrifuge tubes before they are UV sterilized and label as many tubes as you will need from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labelling should include the additive's code, concentration, and expiration date, and the initials of the person who prepared the additive.
- Add 2.5 ml of cold sterile deionized Milli-Q water[1] to make a 20 mg/ml stock. When completely dissolved (swirl beaker), place on ice. Filter and transfer to an ice-cold 15-ml Falcon tube using a 3-ml syringe and a 0.22 µm pore size filter.
- Keeping the tube on ice, make 250-µl aliquots of the 20 mg/ml stock. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the microcentrifuge tube[2]. Place each aliquot on ice, immediately after preparation.
- Fast-freeze all aliquots by placing them on dry ice[3] for about 5 to 10 min. When completely frozen (solution is completely white), transfer to the appropriate freezer.

Storage and usage:

- Some aliquots of the working stock are kept at −20°C for ready availability. The rest of the stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- Aliquots are thawed once from −20°C when needed and kept at 4°C for a period of up to 1 month.

[1] *The stock bottle of sterile deionized Milli-Q water used for the preparation of additives must NOT be used for any other purpose.*

[2] *Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!*

[3] *Spray dry ice with 70% ethanol (to decrease the temperature of the ice) before placing the aliquots.*

Transforming growth factor β

Thermo-Fisher, Catalog #PHG9204

Preparation procedure:

- It is better to prepare the microcentrifuge tubes needed for aliquotting before beginning. We usually UV-sterilize the autoclaved 1.5 ml microcentrifuge tubes used for this purpose overnight by opening their caps facing upward such that the

entire surface of the tube is properly sterilized. You can prepare the labels on the microcentrifuge tubes before they are UV sterilized and label as many tubes as you will need from a stock of sterile microcentrifuge tubes only used for preparing additives. The proper labeling should include the additive's code, concentration, and expiration date, and the initials of the person preparing the additives.

- Weigh 20 mg of BSA in a sterile 20-ml beaker used only for cell culture. Immediately cover the top of the beaker with aluminum foil after adding the BSA.
- The bottle of TGF-β should be briefly centrifuged prior to opening. DO NOT VORTEX!
- Work must be performed under the laminar flow hood. Disinfect TGF-β (5 μg) bottle and any other material including the beaker and ice bucket appropriately by wiping with a tissue paper sprayed with 70% ethanol, before introducing it in the hood. Place the 5 μg TGF-β bottle on ice as soon as it is brought into the hood.
- Prepare 10 mM citric acid, pH 3.0, in Milli-Q water[1] and filter it using a 0.22 μm pore size filter. (Citric acid can be stored in the dark at room temperature for 2 years).
- Dissolve the weighed BSA in 20 ml of 1× PBS[2] to prepare 0.1% (w/v) solution and transfer it to a 50-ml Falcon tube.
- Add 33 μl of 10 mM citric acid solution to the TGF-β bottle to make a 151.5 μg/ml intermediate solution. Swirl the bottle to dissolve. Do not vortex. Add this solution to a tube containing 16.7 ml of 0.1% BSA in PBS, to obtain a concentration of 300 ng/ml of primary stock solution. This solution can be stored at 2° to 8°C for up to 1 week.
- Aliquot the primary stock solution into 1-ml microcentrifuge tubes with 500 μl in each. Ensure that the tip is changed for each microcentrifuge tube[3].
- To prepare the working solution, take 500 μl of one primary stock solution and add this primary stock in 2 ml of 0.1% BSA in PBS to make 2.5 ml of working stock solution at a concentration of 60 ng/ml.
- Aliquot 50 μl from the working stock solution into each microcentrifuge tube and store the tubes at −20°C. Prepare each aliquot separately. Use a new tip for each aliquot and avoid touching the walls of the microcentrifuge tubes[3]. Place each aliquot on ice, immediately after its preparation.
- Fast-freeze all aliquots by placing them on dry ice[4] for about 5 to 10 min. When completely frozen (solution is completely white), transfer to the appropriate freezer.

Storage and usage:

- Some aliquots of the working stock are kept at −20°C for ready availability. The rest of the stock is kept at −80°C and transferred to −20°C when the −20°C stock runs out.
- Aliquots are thawed once from −20°C, when needed and kept at 4°C for a period of up to 1 week.

[1]The stock bottle of sterile deionized Milli-Q water used for the preparation of additives must NOT be used for any other purpose.

[2]The bottle of sterile PBS used for the preparation of additives must NOT be used for any other purpose.

[3]Should you touch the wall or neck of a container, the tip should be discarded. If a vial containing an aliquot might have become accidentally contaminated (e.g., if there is any doubt that the tip used was not properly handled), it should be discarded. The bottom line is, under suspicion of risk of contamination, better discard than regret!

[4]Spray dry ice with 70% ethanol (to decrease the temperature of the ice) before placing the aliquots.

COMMENTARY

Background Information

Historically, 3D cell culture started with floating type I collagen (a nontunable system usually purified from rat tail; Emerman & Pitelka, 1977). With the discovery of the possibility of extracting the EHS matrix enriched with basement membrane components from rhabdomyosarcomas, 3D cell culture methods switched to the production of spheroids in EHS-derived gel, such as Matrigel™, for the past three decades. However, physical considerations have reoriented 3D cell culture since it was reported that not only the chemical signals, but also the mechanical constraints from the ECM, were paramount for best recapitulating cell behavior. For this reason, we have devoted most of the protocols described in this article to using the possibilities offered by tunable collagen I that are essential, notably for the study of cancer (Fang, Yuan, Peng, & Li, 2014). There have also been several nonbiological gels designed as scaffolds for the culture of tumors (Rijal & Li, 2017). There is a wide disparity among the gels regarding their appropriateness for cell culture; once again, the stiffness degree will be paramount for best mimicking tumor phenotypes. The use of nonbiological gels is possible, without additional reagents, with tumors capable of making their own matrix.

Stiffness measurement is an important part of the protocols using cells for which changes in physical attributes of the microenvironment occur during differentiation and/or disease. There are different methods to measure stiffness, and more methods are becoming available. Atomic force microscopy (AFM) can be used to measure stiffness (Young's modulus); however, measurement by microindentation requires many sampling points to get an average, and it is not convenient for determining the global stiffness of a gel. One of the most common characterization methods for determining the overall mechanical properties of a material is by studying its behavior under stretch until the material ruptures, while continuously recording both the applied stress and strain. Depending on the tested specimen, the material type, and the targeted application, the mechanical stimulus can be applied through tension or through compression. Although tensile mechanical tests are widely used for characterization of different metal- and polymer-based biomedical materials, the samples for this type of test need to be prepared in a certain dumbbell- or ring-shape geometry with some level of integrity, to be clamped between the two holders of the tensile machine. This sample preparation is very challenging for low-strength collagen-based hydrogels having a liquid content above 99%. In this article, we have presented one possibility to apply for the measurement of soft materials, utilizing the compressing plate of the Universal Testing System, but biosensors that can be directly integrated in the matrix are under development, which would enable real-time measurement of stiffness changes.

Another exciting development in 3D culture is the engineering of tissue chips with which microenvironmental parameters can be exquisitely controlled. We have given an example of such a chip—the "disease-on-a-chip" (DOC)—with which we can achieve a reproducible curved geometry within the diameter of a terminal breast duct. There are also chips that make use of microfluidics to modify the tumor microenvironment (Chittiboyina et al., 2018) or to study the interaction between cell types (Rothbauer, Zirath, & Ertl, 2018). The ECM is still an essential part of these tissue chips if the cells cannot make all the components themselves, as we showed with the use of laminin 111, to provide the necessary differentiation stimulus for the non-neoplastic cells.

In order to develop techniques that are optimal for 3D cell culture, one ought to consider chemical and physical parameters of the cells' microenvironment. Therefore, methods used to create tumor nodules via aggregation (e.g., hanging drop, certain hydrogels, reactors) are less powerful, as they do not reproduce a cellular organization like *in vivo*. In addition, the use of serum-free medium as we present it in these protocols is essential to conducting reproducible experiments, especially those involving treatment with therapeutic drugs and other molecules.

Critical Parameters

The protocols used for 3D cell culture can only work well if the cells are properly handled via standard methods used for propagation of cells in 2D culture (i.e., cell 'passages'). We reported previously the importance of propagating cells from the same confluence and with a set number of cells seeded (Vidi et al., 2013). Indeed, cancer cell populations left in 2D culture for too long will become enriched with cells that are less aggressive. In contrast, non-neoplastic cells that are not given the time to settle in their medium (and are propagated too

soon, prior to reaching the desired confluence) will usually be enriched with cells that have a lesser capability to differentiate. Phenotypic drift that might result from poor cell culture practice may not be seen until cells are in 3D culture, at which time it is usually too late to revert the phenotype to what it used to be.

Other factors that influence the protocols are the cell culture medium conditions. The best results in 3D cell culture are obtained with cells cultured in serum-free medium. A medium with serum does not preclude using 3D culture, but there might be issues with reproducibility due to the change in batch of serum (which contains many unknown components with variable concentrations) and interference of serum components with the reagents used for the treatment of cells, depending on the experiment. For instance, unknown factors in the serum might protect the cells against a particular drug treatment or prevent the recapitulation of the phenotype normally observed *in vivo*. The use of well-chosen additives will provide reproducibility; however, specific attention needs to be given to their concentration, storage, and shelf-life, since deviating from the set conditions is likely to perturb the phenotype or even kill the cells. When using additives, it is essential to indicate the preparation dates for the stocks and replace additives based on these dates (no use beyond expiration should be attempted; otherwise the cells might not proliferate, or their phenotype might drift).

Features of tissue differentiation are the main tools to confirm the validity of the model. These are usually based on immunostaining, and help catch differentiation issues with the cell culture conditions or with the cells themselves. Each set of differentiation features for testing will depend on the type of cells and the expected phenotype (i.e., the phenotype that would represent a physiologically relevant situation). For instance, DCIS tumors are expected to have a measurable level of basal polarity, as we show in Fig. 1.

Troubleshooting

If the desired phenotype is not achieved or not sustained when trying to use cells for which a protocol is not in place and you are developing the protocol, it is important to verify that the cell lines or primary cells are the appropriate choices for the tissue of interest. Then, the conditions for cell culture need to be reviewed both for content and stiffness. If collagen I is the basis for the culture because cells are of mesenchymal type or tumors, it is worth looking into the literature for any additional component that plays a major role and might not be made at all or in enough quantity by the cells (e.g., certain tumors rely also on basement membrane components). The issue might be simpler, such as not achieving the correct stiffness. In that case investigating different stiffness levels might be the solution. For instance, high density of collagen I might induce an orientation of fibroblasts parallel to each other, similar to what is observed in fibrosis, instead of the expected random orientation for certain types of interstitial tissues. Cell density in the matrix of a given stiffness might also be important to consider. As an example, high density of fibroblasts might lead to lack of proliferation arrest (fibroblasts are normally resting cells unless they are stimulated).

If the desired phenotype used to be achieved before, but it is not achieved anymore (e.g., lack of proper cellular organization, increase in cell death, abnormal functional parameters), troubleshooting should start with the preparation of the culture conditions for that experiment (medium, serum or additives) before venturing into cellular issues (e.g., mycoplasma contamination, phenotypic drift) and environmental issues (incubator, etc.). It is essential to keep a log of cell vials thawed and of the batches of medium, additives, serum, and ECM that were bought, in order to trace the issue back in time (Fig. 8). Since culture without serum makes the cells particularly sensitive to any alteration of their environment, when all 'in-house' potential issues have been dismissed as the sources of the problem, it is critical to investigate potential issues with commercially available reagents and any change in their manufacture. The increased sensitivity of cells to their microenvironment is particularly noticeable in 3D culture. As an example, medium prepared with water contaminated with low concentrations of potential toxicants could lead to cell death in 3D culture only, whereas cells could survive in standard 2D culture.

Understanding Results

Results are typically those related to the analysis used with the cells and do not pertain to 3D culture per se, except with differentiation. Anticipated results regarding differentiation are signs of organizational and functional mimicry of physiologically relevant tissues. Therefore, markers of differentiation typical of the tissue of interest should not only be expressed, but should also be localized properly within the tissue (e.g., lateroapical distribution of tight junction markers in the

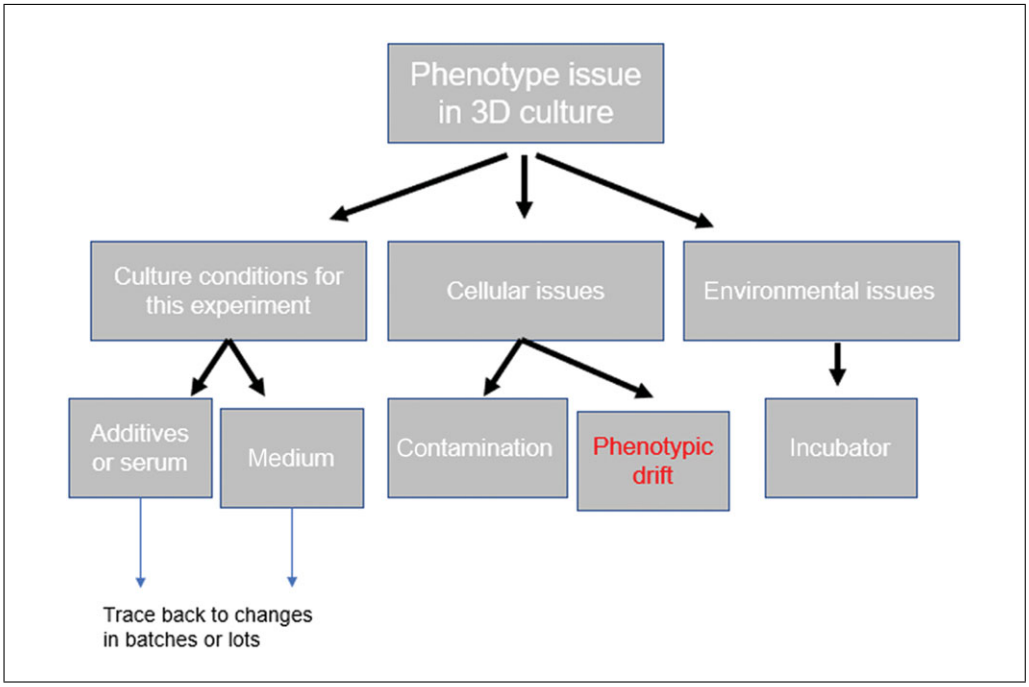


Figure 8 Schematic of troubleshooting steps if the phenotype is not achieved anymore in 3D cell culture. Note that the phenotypic drift is usually an irreversible issue due to mishandling of the cells in standard 2D culture (maintenance and propagation steps).

phenotypically normal epithelium; basal location of β4-integrin in DCIS). To validate the functional aspects of the culture, the activity of major pathways involved in the function or behavior of the cells of interest should correlate with that observed *in vivo* (e.g., activation of a signaling pathway characteristic of a specific subtype of tumors, shutdown of stress-response pathways in aggressive tumors, or activation of certain metalloproteinases in invasive cancers).

There will be unexpected results with 3D cell culture when certain techniques normally used in 2D cell culture and setup with 2D cell cultures are translated for 3D cell culture. For instance, washing steps for immunostaining in 3D cell culture should be longer than in 2D cell culture, to reduce the nonspecific fluorescence background from the matrix. More subtly, there might be altered ability to extract and break materials from cells obtained from 3D culture, especially since the tissue structures are usually kept intact (e.g., no cell separation) before lysis. For instance, DNA breakage for experiments like chromatin immunoprecipitation (ChIP) needs to be optimized in 3D cell culture compared to 2D cell culture for the same cell line in order to get the necessary small size of DNA pieces.

Time Considerations

The minimum amount of time necessary for 3D culture depends on the cell type used. Differentiation requires adequate signaling pathways and gene-expression patterns, and in many cases the presence of a certain architecture of cell assemblies that occurs via the ordered division of cells. Culture for 5 days might be enough for many tumors; however, these tumors will be of small size. We may wait 10 days with the breast cancer cells. Other models in which the cells proliferate very slowly, like certain glioblastomas, may take 2 to 3 weeks to get tumors of acceptable size for experiments. With fibroblasts, the usual amount of time to achieve differentiation and resting (or nonactivated) phenotype is 5 days, but for the phenotypically normal differentiation of epithelial cells, the minimum period of culture is usually 8 days. With co-cultures, the amount of time needed to reach differentiation of all cells involved might take longer, depending on the approach used. Finally, it is important to realize that in contrast to 2D cultures in which cells might keep proliferating and start piling up, 3D cultures can be kept for a long time (over months if necessary), since once the tissues reach their differentiation state, they remain stable unless stimulated. As an example, this type of culture permits chronic treatment to investigate pathways involved in cancer progression.

Acknowledgments

Part of the work presented with the monoculture of fibroblasts was supported by the

Congressionally Directed Medical Research Program/Breast Cancer Research Program (CDMRP/BCRP) (W81XWH-17-1-0250 to S.L.).

Literature Cited

Amaro, A., Angelini, G., Mirisola, V., Isabella, A., Reverberi, D., Matis, S., ... Pfeffer, U. (2016). A highly invasive subpopulation of MDA-MB-231 breast cancer cells shows accelerated growth, differential chemoresistance, features of apocrine tumors and reduced tumorigenicity in vivo. *Oncotarget*, *7*, 68803–68820. doi: 10.18632/oncotarget.11931.

Bissell, M. J., Hall, H. G., & Parry, G. (1982). How does the extracellular matrix direct gene expression? *Journal of Theoretical Biology*, *99*, 31–68. doi: 10.1016/0022-5193(82)90388-5.

Bonnans, C., Chou, J., & Werb, Z. (2014). Remodelling the extracellular matrix in development and disease. *Nature Reviews Molecular Cellular Biology*, *15*, 786–801. doi: 10.1038/nrm3904.

Briand, P., Nielson, K. V., Madsen, M. W., & Peterson, O. W. (1996). Trisomy 7p and malignant transformation of human breast epithelial cells following epidermal growth factor withdrawal. *Cancer Research*, *56*, 2039–2044.

Briand, P., Petersen, O. W., & Van Deurs, B. (1987). A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cellular & Developmental Biology*, *23*, 181–188. doi: 10.1007/BF02623578.

Chittiboyina, S., Rahimi, R., Atrian, F., Ochoa, M., Ziaie, B., & Lelièvre, S. A. (2018). Gradient-on-a-chip with reactive oxygen species reveals thresholds in the nucleus response of cancer cells depending on the matrix environment. *American Chemical Society, Biomaterials Science and Engineering*, *4*, 432–445. doi: 10.1021/acsbiomaterials.7b00087.

Cohen, N., Shani, O., Raz, Y., Sharon, Y., Hoffman, D., Abramovitz, L., & Erez, N. (2017). Fibroblasts drive an immunosuppressive and growth-promoting microenvironment in breast cancer via secretion of Chitinase 3-like 1. *Oncogene*, *36*, 4457–4468. doi: 10.1038/onc.2017.65.

Conlon, G. A., & Murray, G. I. (2018). Recent advances in understanding the roles of matrix metalloproteinases in tumour invasion and metastasis. *The Journal of Pathology*, *247*(5), 629–640. doi: 10.1002/path.5225.

Emerman, J. T., & Pitelka, D. R. (1977). Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *Journal of Society for In Vitro Biology*, *13*, 316–328.

Fang, M., Yuan, J., Peng, C., & Li, Y. (2014). Collagen as a double-edged sword in tumor progression. *Tumor Biology*, *35*, 2871–2882. doi: 10.1007/s13277-013-1511-7.

Fullár, A., Dudás, J., Oláh, L., Hollósi, P., Papp, Z., Sobel, G., ... Kovalszky, I. (2015). Remodeling of extracellular matrix by normal and tumor-associated fibroblasts promotes cervical cancer progression. *BMC Cancer*, *15*, 256. doi: 10.1186/s12885-015-1272-3.

Goers, L., Freemont, P., & Polizzi, K. M. (2014). Co-culture systems and technologies: Taking synthetic biology to the next level. *Journal of the Royal Society, Interface*, *11*. doi: 10.1098/rsif.2014.0065.

Kular, J. K., Basu, S., & Sharma, R. I. (2014). The extracellular matrix: Structure, composition, age-related differences, tools for analysis and applications for tissue engineering. *Journal of Tissue Engineering*, *5*, 1–17. doi: 10.1177/2041731414557112.

Lee, J., Kim, S., Khawar, I. A., Jeong, S., Chung, S., & Kuh, H. (2018). Microfluidic co-culture of pancreatic tumor spheroids with stellate cells as a novel 3D model for investigation of stroma-mediated cell motility and drug resistance. *Journal of Clinical Cancer Research*, *37*, 4. doi: 10.1186/s13046-017-0654-6.

Leight, J. L., Drain, A. P., & Weaver, V. M. (2016). Extracellular matrix remodeling and stiffening modulate tumor phenotype and treatment response. *Annual Review of Cancer Biology*, *1*, 313–334. doi: 10.1146/annurev-cancerbio-050216-034431.

Lelièvre, S. A., & Chittiboyina, S. (2018). Microphysiological systems to study microenvironment cell nucleus interaction: Importance of tissue geometry and heterogeneity. *Microphysiological Systems*, *6*, 23.doi: 10.21037/mps.2018.11.02.

Lin, S., & Gu, L. (2015). Influence of crosslink density and stiffness on mechanical properties of Type I collagen gel. *Materials*, *8*, 551–560. doi: 10.3390/ma8020551.

Lopez, J. I., Kang, I., You, W. K., McDonald, D. M., & Weaver, V. M. (2011). In situ force mapping of mammary gland transformation. *Integrative Biology: Quantitative Biosciences from Nano to Macro*, *3*, 910–921. doi: 10.1039/c1ib00043h.

Mi, S., Du, Z., Xu, Y., Wu, Z., Qian, X., Zhang, M., & Sun, W. (2016). Microfluidic co-culture system for cancer migratory analysis and anti-metastatic drugs screening. *Nature: Scientific Reports*, *6*, 35544. doi: 10.1038/srep35544.

Northey, J. J., Przybyla, L., & Weaver, V. M. (2017). Tissue force programs cell fate and tumor aggression. *Cancer Discovery*, *7*, 1224–1237. doi: 10.1158/2159-8290.CD-16-0733.

Paszek, M. J., Zahir, N., Johnson, G. I., Lakins, J. N., Rozenberg, G. I., Gefen, A., ... Weaver, V. M. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell*, *8*, 241–254. doi: 10.1016/j.ccr.2005.08.010.

Phelan, K., & May, K. M. (2015). Basic techniques in mammalian cell culture. *Current Protocols in Cell Biology*, *66*, 1.1.1–1.1.22. doi: 10.1002/0471143030.cb0101s66.

Plachot, C., Chaboub, L. S., Adissu, H. A., Wang, L., Urazaev, A., Sturgis, J., ... Lelièvre, S. A. (2009). Factors necessary to produce

basoapical polarity in human glandular epithelium formed in conventional and high-throughput three-dimensional culture: Example of the breast epithelium. *BMC Biology*, *7*, 77, doi: 10.1186/1741-7007-7-77.

Rijal, G., & Li, W. (2017). A versatile 3D tissue matrix scaffold system for tumor modeling and drug screening. *Science Advance*, *3*, e1700764. doi: 10.1126/sciadv.1700764.

Rizki, A., Weaver, V. M., Lee, S., Rozenberg, G. I., Chin, K., Myers, C. A., ... Bissell, M. J. (2008). A human breast model of preinvasive to invasive transition. *Cancer Research*, *68*, 1378–1387. doi: 10.1158/0008-5472.CAN-07-2225.

Rothbauer, M., Zirath, H., & Ertl, P. (2018). Recent advances in microfluidic technologies for cell-to-cell interaction studies. *Lab on a Chip*, *18*, 249–270. doi: 10.1039/C7LC00815E.

Sampson, N., Koziel, R., Zenzmaier, C., Bubendorf, L., Plas, E., Jansen-Dürr, P., & Berger, P. (2011). ROS signaling by NOX4 drives fibroblast-to-myofibroblast differentiation in the diseased prostatic stroma. *Molecular Endocrinology*, *25*, 503–515. doi: 10.1210/me.2010-0340.

Shay, J. W., Van Der Haegen, B. A., Ying, Y., & Wright, W. E. (1993). The frequency of immortalization of human fibroblasts and mammary epithelial cells transfected with SV40 large T-antigen. *Experimental Cell Research*, *209*, 45–52. doi: 10.1006/excr.1993.1283.

Shay, J. W., Tomlinson, G., Piatyszek, M. A., & Gollahon, L. S. (1995). Spontaneous in vitro immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome. *Molecular Cellular Biology*, *15*, 425–432. doi: 10.1128/MCB.15.1.425.

Spill, F., Reynolds, D. S., Kamm, R. D., & Zaman, M. H. (2016). Impact of the physical microenvironment on tumor progression and metastasis. *Current Opinion Biotechnology*, *40*, 41–48. doi: 10.1016/j.copbio.2016.02.007.

Subbaramaiah, K., Brown, K. A., Zahid, H., Balmus, G., Weiss, R. S., Herbert, B. S., & Dannenberg, A. J. (2016). Hsp90 and PKM2 drive the expression of aromatase in Li-Fraumeni syndrome breast adipose stromal cells. *The Journal of Biological Chemistry*, *291*, 16011–16023. doi: 10.1074/jbc.M115.698902.

Theocharis, A. D., Skandalis, S. S., Gialeli, C., & Karamanos, N. K. (2016). Extracellular matrix structure. *Advanced Drug Delivery Reviews*, *97*, 4–27. doi: 10.1016/j.addr.2015.11.001.

Vidi, P. A., Bissell, M. J., & Lelièvre, S. A. (2013). Three-dimensional culture of human breast epithelial cells: The how and the why. *Methods in Molecular Biology*, *945*, 193–219. doi: 10.1007/978-1-62703-125-7_13.

Vidi, P. A., Maleki, T., Ochoa, M., Wang, L., Clark, S. M., Leary, J. F., & Lelièvre, S. A. (2014). Disease-on-a-chip: mimicry of tumor growth in mammary ducts. *Lab on a Chip*, *14*, 172–177. doi: 10.1039/C3LC50819F.

Internet Resources

https://nanohub.org/resources/25058/supportingdocs

The 3D Cell Culture (3D3C) facility at Purdue University is compiling short movies under NanoHub on certain aspects of the techniques that we have described in this article. These movies were made with the help of members of the Lelièvre laboratory and the Online Production Managing team of Joe Cychosz.

http://www.microscopeworld.com/t-calibration.aspx

Information on how to use an eyepiece reticle.