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Preparation of extracellular matrices produced by cultured and primary fibroblasts

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Abstract

Fibroblasts secrete and organize extracellular matrix (ECM), which provides structural support for their adhesion, migration, and tissue organization, besides regulating cellular functions such as growth and survival. Cell-to-matrix interactions are vital for vertebrate development. Disorders in these processes have been associated with fibrosis, developmental malformations, cancer, and other diseases. This unit describes a method for preparing a three-dimensional matrix derived from fibroblastic cells; the matrix is three-dimensional, cell and debris free, and attached to a two-dimensional culture surface. Cell adhesion and spreading are normal on these matrices. This matrix can also be compressed into a two-dimensional matrix and solubilized to study the matrix biochemically.

Culturing fibroblasts on traditional two-dimensional (2-D) substrates induces an artificial polarity between lower and upper surfaces of these normally nonpolar cells. Not surprisingly, fibroblast morphology and migration differ once suspended in three-dimensional (3-D) collagen gels (Friedl and Bocker, 2000). However, the molecular composition of collagen gels does not mimic the natural fibroblast (i.e., mesenchymal) microenvironment. Fibroblasts secrete and organize ECM, which provides structural support for their adhesion, migration, and tissue organization, in addition to regulating cellular functions such as growth and survival (Buck and Horwitz, 1987; Hay, 1991; Hynes, 1999; Geiger et al., 2001). Cell-to-matrix interactions are vital for vertebrate development. Disorders in these processes have been associated with fibrosis, developmental malformations, cancer (i.e., desmoplastic tumor microenvironment), and other diseases (Rybinski et al., 2014).

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Key References

Cukierman et al. (2001). See above.

Vlodavsky I, Lui GM, Gospodarowicz D. 1980. Morphological appearance, growth behavior and migratory activity of human tumor cells maintained on extracellular matrix versus plastic. *Cell*. 19(3):607–16. See *UNIT 10.4*

The source for procedures and materials described in this unit.

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NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All cell-culture incubations should be performed in a 37°C, 10% CO₂ humidified incubator.

NOTE: When primary human cells are harvested and during their experimental usage, institutional biosafety procedures needed for working with unfixed human materials should be followed.

This unit describes methods for generating tissue culture surfaces coated with a fibroblast-derived 3-D ECM produced and deposited by both established and primary fibroblasts. The matrices closely resemble *in vivo* mesenchymal matrices and are composed mainly of fibronectin fibrillar lattices. Utilizing *in vivo*-like 3-D matrices as substrates allows the acquisition of information that is physiologically relevant to cell-matrix interactions, structure, function, and signaling, which differ from data obtained by culturing cells on conventional 2-D substrates *in vitro* (Cukierman et al., 2001).

These protocols were initially derived from methods described in *UNIT 10.4*, which were modified to obtain fibroblast-derived 3-D matrices and to characterize cellular responses to them. The basic approach is to allow fibroblasts to produce their own 3-D matrix (see Basic Protocol). For this purpose, fibroblasts are plated and maintained in culture in a confluent state. After 5 to 9 days, unextracted 3-D matrix cultures can be sorted into normal or activated (i.e., myofibroblastic, fibrotic or desmoplastic) phenotypes (see Support Protocol 1) or matrices are denuded of cells, and cellular remnants are removed. Such extraction results in an intact fibroblast-derived 3-D matrix that is free of cellular debris and remains attached to the culture surface (see Figure 10.9.1). The fibroblast-derived 3-D matrices are then washed with Dulbecco's phosphate-buffer solution (DPBS⁺) and can be stored 2 to 6 weeks at 4°C or up to 3 weeks frozen at -80°C. Moreover, to analyze the effect of matrix pliability on cellular behavior, prepared 3-D matrices can be rigidified by chemical cross-linking (see Support Protocol 2 and *UNIT 17.10*).

Additionally, to evaluate the quality and functionality of the fibroblast-derived 3-D matrices, support protocols present a variety of procedures for measuring matrix production phenotypes such as matrix thickness and fiber alignment/orientation (see Support Protocol 1), as well as cell responsiveness to the 3-D matrix microenvironment (see Support Protocols 3 to 6). The rapid cell attachment of fibroblasts plated within the matrix can be quantified. By plating isolated fibroblasts in the 3-D matrix (Support Protocol 3), the acquisition of an *in vivo*-like spindle-shaped morphology can also be measured (Support Protocol 4). To ascertain whether fibroblasts respond to the 3-D microenvironment when plated within specific (NIH-3T3) matrices, the phosphorylation level of non-receptor focal adhesion kinase (FAK) pY³⁹⁷ can be quantified by immunoblotting (see Support Protocol 6). In addition, the ability of tumor- or cancer-associated fibroblast-derived matrices to induce and maintain an active (i.e., desmoplastic) phenotype in quiescent naïve fibroblasts can be queried by calculating levels (via western blot and/or semi-quantitative immunofluorescence) and localization (using immunofluorescence) of myofibroblastic markers such as alpha-smooth muscle actin (see Support Protocols 5 and 6).

This unit will also describe how to mechanically compress the fibroblast-derived 3-D matrices to obtain 2-D substrate controls (see Support Protocol 7). Moreover, a support protocol will illustrate how to solubilize the fibroblast-derived 3-D matrices to produce a matrix-derived protein mixture for additional 2-D coating controls and for subsequent biochemical analysis of the matrices (see Support Protocol 8 and Commentary). Lastly, there are two support protocols designed for the isolation of primary fibroblasts from fresh tissue samples to produce additional types of fibroblast-derived 3-D matrices (see Support Protocols 9 and 10).

BASIC PROTOCOL: PREPARATION OF EXTRACELLULAR MATRICES PRODUCED BY CULTURED OR PRIMARY FIBROBLASTS

Any fibroblastic cell that has overcome growth inhibition by contact can be used. Nevertheless, preconditioned NIH-3T3 cells probably constitute the best example (for the harvesting of primary fibroblasts, see Support Protocols 9 and 10). NIH-3T3 cells must be routinely cultured in high-glucose Dulbecco's modified Eagle medium supplemented with 10% calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin unless otherwise specified. Never allow cultured NIH-3T3 cells to become completely confluent while maintaining stock cultures. Once cells reach 80% confluence (about once per week), subculture at a 1:20 dilution. However, prior to plating for matrix deposition, NIH-3T3 cells should be adapted (i.e., preconditioned) to grow in 10% fetal bovine serum rather than calf serum for the cells to adopt an optimal phenotype needed for matrix production (see Critical Parameters).

Depending on the laboratory equipment available and on the anticipated uses of the fibroblast-derived 3-D matrices, a suitable surface on which the matrices will be produced (e.g., glass-bottom dishes, coverslips, or tissue culture dishes) must be selected as follows:

1. Disposable glass bottom dishes (MatTek) can be utilized for real-time fluorescent experiments or for quality assessment assays (e.g., cell attachment and cell shape) using an inverted fluorescent or confocal microscope (see Support Protocols 3 and 4).
2. Coverslips (e.g., 12-mm no. 1.0) can be used for immunofluorescence experiments in which samples are fixed and mounted on microscope slides (see Support Protocols 1, 3, 4 and 5), or for mechanical (e.g., 18-mm no. 1.0 or 1.5) compression of the fibroblast-derived 3-D matrices to be used as control 2-D surfaces (see Support Protocol 7).
3. Regular tissue culture dishes (e.g., 35-mm diameter) can be used for *in vivo* observations using an inverted microscope, for matrix solubilization (Support Protocol 8) and further characterization, and/or for biochemical analyses (Support Protocol 6). Tissue culture dishes are also used for real-time cell motility analyses (Cukierman, 2005).

Materials

NIH-3T3 cells (ATCC) or primary fibroblasts (see Support Protocols 8 and 9)

Confluent medium with fetal bovine serum (FBS; see recipe)

Trypsin/EDTA; 0.25% (w/v) trypsin/0.03% (w/v) EDTA solution (see recipe)

0.2% (w/v) gelatin solution (see recipe)

Ethanol (absolute)

Dulbecco's phosphate-buffered saline with Ca⁺⁺ and Mg⁺⁺ (DPBS⁺;
APPENDIX 2A)

DPBS, Ca^{++} and Mg^{++} free (DPBS⁻, *APPENDIX 2A*)

1% (v/v) glutaraldehyde in DPBS⁺ (see recipe)

1 M ethanolamine (see recipe)

Matrix medium with ascorbic acid (see recipe)

Extraction buffer (see recipe), 37°C

10U/ml DNase I (Roche) in DPBS⁺, optional

Penicillin/streptomycin (Invitrogen)

Fungizone (amphotericin B; Invitrogen)

37°C, 10% CO₂ humidified incubator

15-cm dishes (or 75 cm² culture flasks) plus the specific culture vessels for matrix production

Inverted phase-contrast microscope

6-well tissue culture plates or 35-mm dishes (optional)

22-, 18-, 12-, 7- or 5-mm circular high-quality coverslips (Carolina; optional)

Bacterial 6-, 12-, 24- or 48- multi-well petri plates for preparing matrices on coverslips, optional

Parafilm strips

Small, sterile fine-pointed tweezers (e.g., Dumont no. 4), optional

Prepare cell cultures

- 1** Start with a semi-confluent (80% confluent) culture of NIH-3T3 cells cultured in 10 to 12 ml confluent medium containing fetal bovine serum (see Critical Parameters) on a 15-cm culture plate, or primary fibroblastic cells in 75 cm² culture flasks (see Support Protocols 9 and 10); discard the culture medium by rinsing with 10 ml DPBS⁻ pre-heated at 37°C and aspirate.

- 2** Rinse the cell layer briefly with 1.5 ml of 0.25% trypsin/0.03% EDTA (trypsin/EDTA), pre-heated at 37°C, per 15-cm dish. Then gently aspirate off the solution.

This rinse will remove traces of serum that contains trypsin inhibitors.

- 3** Add enough 37°C pre-heated trypsin/EDTA solution to cover the cell layer, quickly aspirate excess liquid, and observe under an inverted microscope at room temperature until the cells have started to detach from the culture dish (1 to 3 min).

Gently tapping the culture dish can facilitate this process.

- 4** Collect the cells in 10 ml of confluent medium with serum.

The presence of serum in the medium inactivates trypsin remnants.

- 5** Add 2 ml of the suspended cells and 10 to 12 ml of confluent medium to a 15-cm plate and culture for 2 to 3 days (until semi-confluent, up to ~80% confluence).

As many as five 15-cm culture dishes (or 75 cm² culture flasks) may be used.

Prepare surfaces for matrix deposition

Although not strictly required, both gelatin coating (steps 6 and 7) and cross-linking of gelatin (steps 8 through 11) with glutaraldehyde stabilize anchoring of the matrices to the culture surface, and could critically improve final yield. However, the resulting matrices may be thinner than those obtained without gelatin cross-linking. Therefore, matrix thickness should be determined for each fibroblastic cell type, and a decision to follow the optional steps should be made each time.

- 6a** *For tissue culture dishes:* Add 2 ml of 0.2% gelatin solution to a 35-mm tissue culture dish surface to be used for fibroblast-derived 3-D matrix deposition and incubate for 1 hr. at 37°C.

Choose 35-, 60-, or 100-mm dishes to be used in this protocol. For 60- or 100-mm dishes, scale up the volumes of all reagents added from 2 ml to 4 and 8 ml, respectively.

- 6b** *For coverslips placed in multi-well plates:* Pre-sterilize by flaming the coverslips after dipping in anhydrous ethanol (absolute). Then place coverslip in a tissue culture dish and rinse with DPBS⁺. Incubate coverslips in a 0.2% gelatin solution for 1 hr. at 37°C.

- 7** Aspirate gelatin and add 2 ml DPBS⁺.

- 8** Aspirate DPBS⁺ and add 2 ml of 1% glutaraldehyde (pre-diluted in DPBS⁺) to each dish or well and incubate 30 min at room temperature.

- 9** Wash coverslips (or culture dishes) three times for 5 min each using 2 ml of DPBS⁺.

- 10** Add 2 ml of 1 M ethanolamine to each coverslip (or dish) and incubate 30 min at room temperature.

- 11** Repeat Step 9.

This step is a good point in which to stop if time does not permit cell seeding. Dishes can be stored for 1 to 3 days at 4°C under sterile conditions.

- 12** Aspirate DPBS⁺ from dishes and replace with 2 ml matrix medium. If the medium appears purple (signifying basic pH), repeat steps 11 and 12 to remove all traces of ethanolamine.

At this point, the surfaces are ready to be seeded with matrix-producing fibroblasts.

When using coverslips, it is recommended to transfer coverslips to individual wells of multi-well bacterial plates. Coverslip sizes will vary from 22- to 5-mm according to the area available in the selected multi-well plates.

When using coverslips for 3-D matrix deposition, multi-well bacterial petri dishes are preferred over tissue culture plastic dishes because the fibroblasts do not adhere well to bacterial petri plastic. Consequently, there is preferential fibroblastic growth on pretreated glass coverslips instead of on the surface of the petri plastic, conditions conducive to enhancing matrix production on the coverslip. Placing coverslips on bacterial petri plastic also facilitates lifting the coverslip off the dish surface with tweezers during matrix extraction (see step 20) because any cells growing underneath the glass coverslips are easily dislodged.

Allow cells to deposit matrix

- 13** Repeat steps 1 to 3 to harvest cells from a semi-confluent dish (or flask).

This protocol was developed for NIH-3T3 cells. Nevertheless, other fibroblastic cell lines can be used. For example, the same protocol can be followed using human or other primary fibroblastic cells, such as naïve or cancer-associated fibroblasts (see Support Protocols 9 and 10).
- 14** Collect cells from each dish (or flask) in 10 ml of matrix medium, count cells (*UNIT 1.1*), and dilute to a final concentration of 2.5×10^5 cells/ml.
- 15** Aspirate medium (from step 12) and seed 5×10^5 cells in 2 ml of matrix medium per 35-mm dish and culture for 24 hr.

Use as many dishes as needed; there should be enough cells for ~100 35-mm plates from each 15-cm semi confluent dish. Remember to scale up or down volumes (without altering cell concentrations) according to the selected plates; from 2 ml up to 4 or 8 ml or down to 1, 0.5, 0.25 ml for 60- or 100-mm single well or 12, 24, or 48 multi-well plates, respectively.
- 16** After 12-18 hrs, carefully aspirate the medium from cells and replace with fresh matrix medium containing 50 µg/ml of ascorbic acid.

For some primary cell lines, using 500 µg/ml of ascorbic acid on this first step can increase matrix thickness.

NOTE: On this first day suggested higher ascorbic acid concentration can be detrimental to NIH-3T3 cells. The decision of whether or not to use this dose should be independently determined for each fibroblastic cell type.

- 17** Ascorbic acid rapidly degrades in culture, hence changing medium with freshly made matrix medium every 48 (for NIH-3T3s) or 24 hrs. (for primary fibroblasts), by removing half of the media volume and replenishing it with fresh media, containing 100 µg/ml ascorbic acid (to achieve the needed 50 µg/ml of ascorbic acid in the total volume) is needed. Continue this process for a total of 5 to 9 days (times depend on cell type used; 5 days should suffice for NIH-3T3s) counting from step 16.

At this time, the matrix should be sufficiently thick to achieve three-dimensionality (10 µm, see below). This 3-D matrix is ready to be characterized for phenotypic evaluation, or extracted (see below) for testing of assorted matrix induced cell responses (see Figure 10.9.1 A).

Matrices should be extracted after they reach a thickness of at least 10 µm. The time required for each fibroblast cell type to produce a matrix of this thickness may vary. Therefore, this time period must be determined empirically for each cell type. Time course experiments are recommended to establish this time.

- 18** Optional; confirm the phenotypic quality of the “unextracted” matrix by conducting an immunofluorescent assay to visualize cells and matrix characteristics (see Support Protocol 1). Alternatively, proceed to step 19 below.

The unextracted matrices should look similar to the one shown “before extraction” in Figure 10.9.1A

Remove cells from matrix

- 19** Carefully aspirate the medium and rinse gently two times with 2 ml DPBS⁻, by placing the pipet against the dish wall rather than at the bottom of the dish where the matrices and cells are located.
- 20** Gently add 1 ml of pre-warmed (37°C) extraction buffer.
- If coverslips are being used, gently lift the coverslips with the fine-pointed tweezers (or a syringe needle) so that extraction buffer reaches under the coverslip. This step will ensure that the matrix deposited on the coverslip will be separated successfully from the matrix deposited on the bottom of the culture dish. This will facilitate subsequent handling of the coverslips without tearing the delicate matrix. In fact, placing the coverslip into a dry well prior to extraction is recommended, but this step needs to be conducted with great care to avoid sample damage.
- 21** Observe the process of cell lysis using an inverted microscope. Incubate at 37°C until no intact cells are visualized (~3 to 5 min; see Figure 10.9.1B).

Remove cellular debris

- 22** Without aspirating, slowly add 2 to 3 ml DPBS⁻ to dilute the cellular debris. Gently pipet the DPBS⁻ on the side of the dish to avoid disturbing the newly formed matrix. Store dishes in DPBS⁻ overnight at 4°C to avoid disturbing the matrix.
- The above dilution process should be carried out gently to prevent turbulence that may cause the freshly denuded matrix-layer to detach from the surface.
- 23** As cautiously as possible (using a pipet), aspirate the diluted cellular debris, but without completely aspirating the liquid layer so that the matrix surface remains hydrated at all times.
- Do not attempt to aspirate the whole volume. This will prevent removing or damaging the matrix layer.
- 24** Gently add another 2 ml of DPBS⁻ and gently aspirate as described in steps 22 and 23. Repeat two additional times only using DPBS⁺, always avoiding liquid turbulence or disturbing the matrix.
- 25** (*Optional*) If necessary, minimize DNA debris by treating the freshly made matrices with DNase I. Add 2 ml DNase I and incubate 30 min at 37°C.
- 26** At the end of the incubation, aspirate the enzyme and wash two times with 2 ml DPBS⁺.
- 27** Cover the matrix-coated plates (or coverslips) with at least 3 ml DPBS⁺ supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone. Seal with Parafilm® strips and store for 2 to 6 weeks at 4°C.
- For signal transduction assays, store the matrix-coated plates in serum-free medium (see Commentary).
- Matrices can also be stored for about 3 weeks at -80°C (longer times have not yet been tested) without compromising matrix integrity, when compared with matrices from the same batch stored at 4°C. To store matrices at -80°C, rinse matrix dishes two times with sterile, nanopure H₂O and carefully aspirate all the liquid. Then label dishes to indicate the date of freezing for future reference, seal with Parafilm®, and place at -80°C. When needed, thaw matrix dishes at room temperature and rehydrate with DPBS⁺ prior to use.
- NOTE: Treating matrices with nanopure H₂O may alter the native conformation of ECM proteins. Hence, matrix functions may be altered following freezing/thawing procedures, and control experiments are recommended prior to introducing this step to routine procedures.
- 28** Evaluate the integrity of the matrices directly before use. Examine for matrix integrity using an inverted phase-contrast microscope.

Matrices should remain attached to the culture surface and appear similar to the example in Figure 10.9.1B.

SUPPORT PROTOCOL 1: PHENOTYPIC EVALUATION OF UNEXTRACTED ECM CULTURES

Prior to using a newly deposited and extracted matrix batch, it is recommended to evaluate the quality and type of matrix production (i.e., normal vs. desmoplastic cancer-associated or fibrotic) by measuring unextracted culture phenotypical characteristics such as matrix thickness, alignment and/or levels of assorted myofibroblastic marker expressions. For this, a basic indirect immunofluorescent assay designed for querying alpha-smooth muscle actin (α -SMA) expression and patterning (Gupta et al., 2011), as well as ECM parallel fiber orientation (Amatangelo et al., 2005), is recommended.

Materials

Dulbecco's phosphate-buffered saline (DPBS⁺; *APPENDIX 2A*)

DPBS⁺ with Tween-20 (DPBS-T; see recipe)

Fixing/Permeabilization solution (see recipe)

Fixing solution (see recipe)

Fibroblast-derived 3-D matrix produced onto 12-mm no. 1.0 coverslips (see Basic Protocol)

100% Donkey serum stock (Jackson ImmunoResearch Laboratories)

Odyssey® Blocking Buffer (LI-COR Biosciences, P/N 927-40000) with 1% donkey serum (see recipe)

Primary antibody cocktail: anti- α -Smooth Muscle Actin (α -SMA) mouse antibody [1:300] (Sigma-Aldrich, catalog number: A2547), anti-fibronectin rabbit antibody [1:200] (for murine samples use Abcam, catalog number: ab23750; for human samples use Sigma, catalog number: F3648.). Antibodies are diluted in Odyssey® Blocking Buffer.

Secondary antibody cocktail: anti-rabbit Cy5-conjugated and anti-mouse Rhodaminered-conjugated affinity purified F(ab')₂ donkey fragments [both 1:100] (Jackson ImmunoResearch Laboratories, catalog number: 54557 and 54831, respectively). Antibodies are diluted in Odyssey® Blocking Buffer.

SYBR green reagent for nuclei labelling (Invitrogen, Catalog number: S7567) 1:10,000 dilution.

Small fine-pointed tweezers (e.g., Dumont no. 4)

Light-protected humid chamber (i.e., dark plastic box)

Parafilm® squares (a minimum of 2)

Prolong Gold anti-fade reagent (Invitrogen)

Glass microscope slides

Confocal microscope (objectives 40× or 60×), equipped with a Krypton/Argon laser that includes three lines, 488, 568, and 647 nm, for fluorescence excitation of dye-labeled samples (e.g. Confocal spinning disk Ultraview, Perkin-Elmer Life Sciences), complemented with CCD camera and image acquisition software (e.g., Velocity 6.3.0, Perkin-Elmer Life Sciences) for 8-16bit images acquisition. *Alternatively*, any microscope equipped with epi-fluorescent capabilities, excitation and emission filters to match the above-mentioned fluorophores, and a digital camera capable of acquiring monochromatic 8-16 bit images can be used.

Image analysis/editing software:

ImageJ (<http://imagej.nih.gov/ij/>)

MetaMorph® (Molecular Devices). *Optional*

Photoshop® (Adobe). *Optional*

ImageJ' OrientationJ plugin (<http://bigwww.epfl.ch/demo/orientation/>)

Excel software (Microsoft)

Immunofluorescence

The following protocol requires careful manipulation of samples to avoid damaging or detaching of the unextracted ECM cultures.

- 1 Aspirate media from unextracted ECM cultures, which were produced onto 12 mm coverslips (i.e., placed in 24 well-plates from Basic Protocol step 18) and rinse each sample twice using 0.5 ml of DPBS⁺.
- 2 Add 0.35 ml (per well) of fixing/permeabilization solution and incubate for 5 min at room temperature (RT). Gently remove this solution and add 0.3 ml of fixing solution for 20 min at RT.
- 3 Remove solution and rinse samples twice with 0.6 ml of DPBS-T.
- 4 Remove liquid and add 0.35–0.50 ml of Odyssey® Blocking Buffer containing 1% (v/v) donkey serum and incubate for 60 min at RT, while rocking gently.
- 5 During the blocking incubation above, set a Parafilm® square inside of a light-protected humid chamber, and carefully place a 15–25 µl drop of primary antibody cocktail for each experimental sample.

Use a container with a lid to set the humid chamber: Place a square of Parafilm® centered inside the container and attach it to the surface of the chamber by pressing firmly. Leave enough space between the edge of the square and the wall of the container for placing pieces of wet paper towel in order to maintain a humid environment throughout antibody incubations.

- 6 Using the tweezers, gently transfer the recently blocked coverslip to the humid chamber; removing half the volume from the well aids in identifying the edge of coverslips. Carefully touch the edge of the coverslip onto a dry paper towel to gently remove the excess of liquid from the coverslip and proceed to place it, matrix side down, onto the pre-deposited 25 μ l drop (from step 5). Close the chamber lid and incubate for 60 min at RT.
- 7 Carefully remove each coverslip and transfer back to a 24-well plate, matrix side up, for washing. It is important to avoid sample drying during this process.
- 8 Using care to avoid sample damage, wash samples with 1 ml DPBS-T by gently rocking for 5 min at RT. Repeat three times.
- 9 Repeat steps 5 and 6, but this time the 25 μ l drops will consist of the secondary antibody cocktail and the incubation time will last for no longer than 45 min at RT.
- 10 Repeat steps 7 and 8.
- 11 Remove DPBS-T and add 0.35 ml of SYBR green reagent per well. Incubate for 15 min at RT protecting samples from light.
- 12 Repeat step 8.
- 13 Remove DPBS-T and rinse twice using double distilled water.
- 14 Deposit a 7 μ l drop of Prolong Gold anti-fade reagent (avoid the presence of air bubbles) onto a glass slide. Proceed to mounting each coverslip by collecting from the multi-well plate and gently touching the edge of the coverslip on an absorbent paper towel (removing liquid excess) prior to placing, matrix face down, onto the Prolong Gold drop. Make sure to touch the drop with the coverslip edge and slowly lower the coverslip onto the drop avoiding capturing air bubbles. Allow samples to cure overnight by placing in a dry location which is protected from light.

If image acquisition is not to be conducted immediately after overnight curing or for sample storage purposes, samples can be placed in a container that is protected from light and from humidity at -80 degrees. If samples were frozen, make sure that these are dry and equalized to RT prior to image acquisition.

NOTE: Do not place samples in storage if Prolong Gold was not completely dry/cured.

Image acquisition

- 15 Utilize a confocal (or an epifluorescence) microscope equipped with precise z-control capabilities. Use appropriate excitation and emission filters (*APPENDIX 1E*).
- 16 Utilizing a 40 $\times$ or 60 $\times$ objective, acquire a z-plane stack of images for the corresponding wavelength of each marker (488nm for nuclei; 568nm for α -

SMA; and 647nm for fibronectin). Acquisition of z-stacks should be done using a μm -calibrated and motorized stage. Set the top and bottom positions of the stage in a way that it encompasses locations that are slightly above and slightly underneath the sample. Set up z-stepping distances as suggested by the objective manufacturer. Repeat this step at a minimum of five different locations per coverslip.

It is important to maintain constant exposure times, lasers powers and other tunable acquisition settings during scanning for each color/ marker. This point is particularly important if images will be used in semi-quantitative analyses. If the acquisition software is calibrated properly, the count of fibronectin z-positive slices, together with the preset z-slice distance calculations, will render estimated 3-D matrix thickness values per image stack. These should be averaged for each experimental condition (i.e. coverslip).

- 17 Export monochromatic 3-D maximum reconstructed (8-16 bit TIFF file) images representative of 0 or 180 degree renderings of the original z-stacks, for further analysis.

For a fast visual assessment, 8bit format reconstituted images should suffice. Nonetheless, 16 bit monochromatic z-stacks constitute the original intact data while their corresponding maximum 3-D reconstitution renderings will serve for accurate analyses (i.e., using Image J).

NOTE: If Velocity 6.3.0 software is being used for image acquisition, under Export Options choose: “multiple files with one image per file, with channels separate and planes merged”.

ECM fiber orientation analysis

This section will convey means for measuring ECM fiber alignment. Also, it will instruct on means of orientation normalization of both visual and quantitative data outputs to allow common sample averaging and comparisons between assorted experimental conditions.

- 18 Download the OrientationJ plugin from <http://bigwww.epfl.ch/demo/orientation/> and follow instructions for its ImageJ installation.

This website contains all the needed information to conduct fiber alignment measurements.

- 19 Open a 16 bit monochromatic image, corresponding to the fibronectin channel, using ImageJ.

Analyses could also be conducted using 8 bit monochromatic images.

Nonetheless, the provided settings are intended for 16 bit usage. All images are required to encompass identical dimensions as these were acquired under identical conditions.

- 20** To obtain the mode ECM fiber angle, select the OrientationJ Dominant Direction option available at the OrientationJ plugin.
- Keep this number in reference as it will be needed for data normalization purposes.
- 21** Click again the OrientationJ plugin, but this time select OrientationJ Distribution. In the OrientationJ Analysis window, set the Gaussian window σ number of pixels at 3 and select Gaussian Gradient from the provided options. Both Min. Coherency and Energy should be set at 0%. In the Color survey options, select Orientation for Hue, Coherency for Saturation and Original-image for Brightness.
- Settings could be changed to match experimental needs as long as these are kept identical throughout the entire experiment. If experiments will be conducted at different times and these will be compared to one another, identical settings for both acquisition and analysis should be maintained.
- 22** Run the analysis to obtain image (hue/saturation-color coded image overlaid onto the original image), graphical (Gaussian distribution curve representation of the orientation distributions) and numeral outputs (List) (See Figure. 10.9.2 G, H and K).
- 23** To obtain the numeral outputs, click the List option in the graph window. This will render a 2 column chart of the data, where X corresponds to degree angle orientations and Y to the number of objects (i.e., fibers) distributed for each given angle/orientation. Proceed to transfer the 2 column data to an Excel spread sheet (a single sheet per image and one file per condition are recommended) and plot as shown in example presented in Figure 10.9.2 K.

Normalization of ECM fiber orientation

- 24** To normalize the data obtain in Step 23, the mode angle annotated in step 20 should be used for correcting the mode value to equal 0° while the rest of the angles should be adjusted accordingly.
- For example, if the mode angle obtained from step 20 equals x° , all angles should be corrected by subtracting x° thus normalizing all image mode angles to 0° .
- 25** To maintain the angle distributions between -90° and 90° , angles need to be corrected by adding 180° to angles smaller than -90° , or subtracting 180° to angles greater than 90° from step 24. The resulting list could be plotted using Excel to obtain a new corrected distribution curve (See Figure 10.9.2 L).
- In order to compare experimental conditions, it is recommended to plot the averaged data from all the images, with their calculated standard deviations. The data can also be used to calculate percentage

of angle distributions at a given degree distance from the mode angle (see step 26).

- 26** Optional: To calculate the total percentage of fibers within a certain range (e.g., -10° and 10° range); divide the distribution of each angle within that range, by the total distribution of all the angles to obtain their percentage. Calculate the corresponding percentage for each image and proceed to compare these values among the assorted experimental condition using median values and standard deviations.

As an example for this, desmoplastic (i.e., myofibroblastic) unextracted 3-D cultures tend to present more than 50% of ECM fibers distributed within 10° from their mode (Amatangelo, 2005).

- 27** Similarly to normalization of number outputs, image Hue values can be adjusted to display a common color (i.e., cyan) representative of the image ECM fibers' mode angle (compare images in Figure 10.9.2 G and H with images in Figure 10.9.2 I and J, respectively). To achieve this, multiply the value obtained from step 20 by 2 (i.e., $2x^\circ$) and use Photoshop's Hue function to correct the image by a value that equals $-2x$.

If this step was conducted correctly the resulting image should display a cyan color representative of the normalized mode angle as shown in Figure 10.9.2 I and J.

NOTE: For publication purposes the intensity and saturation levels of resulting (Hue adjusted) images can be fine-tuned as long as these are conducted in an identical manner throughout all images selected as representative of assorted experimental conditions.

Nuclei shape and α -SMA assessments

Procedures for nuclei shape measurements are depicted in Morphometric Analyses described in Support Protocol 4 (steps 22 to 24), only nuclei -as opposed to cell shapes- are to be assessed. For α -SMA evaluations, follow instructions for intensity assessment in Support Protocol 5 step 12.

SUPPORT PROTOCOL 2: FIXING EXTRACTED MATRICES FOR LACK OF PLIABILITY ANALYSES

For certain experiments designed to analyze the effect of rigidity or pliability of the decellularized matrix on cell behavior, it is necessary to chemically rigidify the prepared matrices. To this end, matrices are fixed with 1% glutaraldehyde prior to cell plating and analysis.

Materials

Tissue culture dishes or coverslips with matrix

Dulbecco's phosphate-buffered saline (DPBS⁺; APPENDIX 2A)

1% (v/v) glutaraldehyde in DPBS⁺ (see recipe)

1 M ethanolamine (see recipe)

Penicillin/streptomycin

Fungizone

Parafilm

1. Aspirate DPBS⁺, add 2 ml of 1% glutaraldehyde (pre-diluted in DPBS⁺) to each tissue culture dish or well, and incubate 30 min at room temperature.
2. Wash coverslips or culture dishes three times, for 5 min each, with 2 ml DPBS⁺ at room temperature.
3. Add 2 ml of 1 M ethanolamine to each dish and incubate 30 min at room temperature.
4. Repeat the DPBS⁺ washes (step 2).
5. Cover the matrix-coated plates (or coverslips) with at least 3 ml PBS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone. Seal with Parafilm. Store up to 2 or 6 weeks at 4°C or at –80°C as described for NIH-3T3 matrices (see Basic Protocol, step 27).

ASSESSING THE QUALITY AND FUNCTIONALITY OF EXTRACTED FIBROBLAST-DERIVED THREE-DIMENSIONAL MATRICES

The quality and functionality of decellularized fibroblast-derived 3-D matrices can be tested by studying the responses triggered in newly re-plated cells by the assorted matrices. The assays presented in this section were designed to evaluate fibroblastic cell responses to a given 3-D matrix. The first two assays: induction of rapid cell attachment (see Support Protocol 3), and acquisition of spindle-body shape morphology (Support Protocol 4), are based on examination of fluorescently labeled cells plated on 3-D matrices. The pre-labeling with a fluorescent dye is required to enhance the observation of cells within fibroblast-derived 3-D matrices. Matrix quality and functionality can be assessed by indirect immunofluorescent semi-quantitative evaluation of markers such as α -SMA (Support Protocol 5). Also to analyze levels of activated [FAKpY³⁹⁷/total FAK] in normal fibroblasts re-plated within NIH-3T3 derived matrices, the ratio of FAKpY³⁹⁷/total FAK is compared to the level of FAKpY³⁹⁷/total FAK of the same cells plated on a traditional fibronectin-coated tissue culture dish (2-D surface; see Support Protocol 3, cell attachment assay). Typically, a 1.5- to 4-fold reduction in FAK pY³⁹⁷/total FAK in normal fibroblasts re-plated into NIH-3T3 matrices is observed (Cukierman 2001; see Support Protocol 6).

SUPPORT PROTOCOL 3: Cell Attachment Assay

Human or mouse fibroblasts can be used to evaluate the cell adhesion-promoting activity of the fibroblast-derived 3-D matrices. It has been reported that these *in vivo*-like 3-D matrices

(NIH-3T3) are about six-fold more effective than 2-D substrates in mediating cell adhesion as quantified by a 10-min cell attachment assay (Cukierman et al., 2001). The following protocol utilizes NIH-3T3 cells; nevertheless other fibroblastic cells can be used similarly. Briefly, cell nuclei are pre-labeled to avoid any background staining from DNA debris on the 3-D matrix. The live pre-labeled cells are rinsed free of excess dye, trypsinized, and plated on the fibroblast-derived 3-D matrix or onto control fibronectin-coated surfaces to be assessed. After 10 min, non-attached cells are washed away, and attached cells are quantified by counting nuclei.

Materials

Semi-confluent fibroblasts (human or mouse) in a 15-cm dish

Confluent medium with fetal bovine serum (see recipe)

Hoechst 33342 stock solution (see recipe)

Dulbecco's phosphate-buffered saline⁺ (DPBS⁺; *APPENDIX 2A*), at both 4°C and room temperature

Trypsin/EDTA solution (see recipe)

Glass-bottom no. 1.5 dishes (MatTek Corporation): three containing fibroblast-derived 3-D matrix (see Basic Protocol) and three with pre-coated 2-D fibronectin (see recipe)

Fixing solution (see recipe)

15-ml polypropylene conical tubes

Tissue culture centrifuge equipped with rotor suitable for conical 15-ml tubes

Fluorescence inverted microscope equipped with an appropriate CCD camera and set of filters to visualize Hoechst 33342 (see *APPENDIX 1E*)

Image analysis software capable of counting objects (optional)

Label cells

- 1** Start with a semi-confluent 15-cm culture dish containing fibroblasts (mouse or human); aspirate and discard the culture medium.
- 2** Add 20 ml of confluent medium containing 40 µl of Hoechst 33342 stock solution (1:500) to the cells. Incubate 15 min at 37°C.
- 3** Wash four times with 10 ml DPBS⁺ at room temperature, about 1 min each time.

Harvest cells

- 4** Rinse 2 times with 10 ml DPBS⁻ at room temperature.
- 5** Add enough 37°C pre-heated trypsin/EDTA solution to cover the cell layer, aspirate excess liquid, and observe under an inverted microscope until cells are detached from the culture dish (1 to 3 min).

Gently tapping the culture dish can facilitate this process.

Prepare cell suspension

- 5 Collect the cells in 10 ml of confluent medium into a 15-ml polypropylene conical tube and take a sample for counting (*UNIT 1.1*).
- 6 Pellet the cells by centrifuging 5 min at $100 \times g$, room temperature.
- 7 Discard the supernatant and gently re-suspend the cells with confluent medium to a final concentration of 3.5×10^5 cells/ml.
- 8 Rotate cells in suspension for 20 min at 37°C .

Allow cells to attach

- 9 Carefully place a 150- μl drop of cell suspension onto the glass-bottom part of the dishes coated with 3-D matrix or 2-D matrix controls. Incubate 10 min at 37°C .
- 10 Remove from the incubator and tilt the dishes slightly to dislodge the medium droplet containing unattached cells from the glass portion onto the plastic portion of the dish and then aspirate.
- 11 Rinse the dishes by slowly adding (to the plastic portion of the dishes) 3 ml of 4°C DPBS⁺.

Fix cells

- 12 Aspirate DPBS⁺ carefully and add 2 ml of fixing solution. Incubate 20 min at room temperature.
- 13 Aspirate and add 2 ml DPBS⁺ at room temperature.

Visualize and analyze attached cells

- 14 Using an inverted fluorescence microscope with appropriate excitation wavelength and excitation and emission filters (*APPENDIX 1E*), acquire five random images of the nuclei from each one of the six dishes utilizing a 10 $\times$ or 20 $\times$ objective and count the nuclei.

Counting of the nuclei can be done automatically utilizing commercially available image analysis software capable of counting objects (e.g., MetaMorph from Universal Imaging Corporation). If the counting is done automatically, then images should be acquired with a 10 $\times$ objective. However, if the nuclei are to be counted manually, then a 20 $\times$ objective is recommended.

The mean number of cells attached to the fibroblast-derived 3-D matrix should be up to six-fold higher than the number attached to the 2-D matrix control. This result will confirm the quality of the NIH-3T3-derived 3-D matrix (Cukierman et al., 2001).

SUPPORT PROTOCOL 4: Determination of Cell Shape

Human or mouse fibroblasts can be used to evaluate induction of spindle-shaped cell morphology promoted by a good-quality *in vivo*-like 3-D matrix, while additional increases in spindle-shaped morphology are achieved in matrices produced by active (i.e., myofibroblastic) cells. It is well established that fibroblasts will acquire an *in vivo*-like spindle-shaped morphology in cell-derived 3-D matrices about 5 hrs. after plating (Cukierman et al., 2001). The protocol consists of pre-labeling live fibroblast membranes with a fluorescent dye and incubating the cells on fibroblast-derived 3-D matrices or controls for a period of 5 hrs. After this period of time, the fibroblast-derived 3-D matrix promotes a spindle-shaped morphology resembling *in vivo* fibroblast morphology, thereby confirming the quality of the 3-D matrices.

Materials

Fibroblast-derived 3-D matrix-covered coverslips (see Basic Protocol)

Fibronectin 2-D matrix-coated coverslips (see recipe)

2% (w/v) heat-denatured BSA (see recipe)

Dulbecco's Phosphate-buffered saline (DPBS⁺; APPENDIX 2A) Semi-confluent 15-cm dish of fibroblasts

Trypsin/EDTA solution (see recipe)

Confluent medium with fetal bovine serum (see recipe)

1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) stock solution (see recipe)

Fixing solution (see recipe)

Prolong Gold mounting medium (Invitrogen)

35-mm tissue culture dishes or 6-well plates

Inverted microscope

15-ml polypropylene conical tubes

Tissue culture centrifuge equipped with rotor suitable for 15-ml conical tubes

Fine-point forceps (e.g., Dumont 4)

Glass microscope slides

Fluorescent microscope equipped with digital camera

Image analysis software capable of measuring elliptical Fourier parameters

Block nonspecific cell binding with BSA

- 1 Cautiously place fibroblast-derived 3-D matrix and control-coated coverslips (matrix face up) into 35-mm tissue culture dishes (or 6-well plates).

- 2 Block nonspecific cell binding by adding 2 ml of 2% heat-denatured BSA and incubate for 1 hr. at 37°C.
- 3 Rinse all blocked coverslips with 2 ml DPBS⁺.

At this point, coverslips are ready to be seeded with the pre-labeled cells.

Label cell membrane with DiI

- 4 Start with a semi-confluent 15-cm dish of fibroblasts; aspirate and discard the culture medium.
- 5 Quickly rinse the cell layer briefly with 1.5 ml trypsin/EDTA pre-heated at 37°C.

Optional: use 10 ml DPBS⁻ pre-heated at 37°C prior to conducting this rinse. This will remove traces of serum that contain trypsin inhibitors.
- 6 Add enough 37°C pre-heated trypsin/EDTA solution to cover the cell layer, aspirate excess liquid, and observe under an inverted microscope until cells are detached from the culture dish (1 to 3 min).
- 7 Collect the cells in 10 ml of confluent medium containing 4 µg/ml DiI into a 15-ml polypropylene conical tube.
- 8 Incubate the cells with the dye in suspension by rotating gently for 30 min at 37°C.
- 9 Pellet the cells by centrifugation 5 min at 100 × *g*, room temperature.
- 10 Discard the supernatant by aspiration, and gently re-suspend the cells in confluent medium to a final volume of 10 ml.
- 11 Repeat steps 9 and 10 four additional times to remove any remaining free dye.
- 12 Count cells (*UNIT 1.1*) and dilute (with confluent medium) to a final concentration of 1 × 10⁴ cells/ml.

Plate labeled cells

- 13 Carefully aspirate DPBS⁺ from the coverslips in step 3.
- 14 Add 2 ml of the diluted cell suspension (from step 12) to the dishes containing coverslips and incubate 5 hr. at 37°C.

For fast qualitative analysis, cells can be observed and photographed at the end of 5 hrs. with an inverted fluorescence microscope (see APPENDIX 1E for wavelength information).
- 15 Aspirate medium and rinse with 2 ml DPBS⁺.

Fix cells

- 16 Aspirate DPBS⁺ and fix with 1 ml of fixing solution for 20 min at room temperature.
- 17 Aspirate fixing solution and rinse with 2 ml DPBS⁺.
- 18 Rinse with 2 ml water to eliminate residual salt.
- 19 Carefully lift coverslip with fine-point forceps and gently discard excess liquid by touching the edge of the coverslip onto a paper towel.
- 20 Mount coverslips (cells face down) on a droplet (~20 µl) of Prolong Gold mounting medium placed on a glass microscope slide.
- 21 Allow mounted samples to dry in the dark for ~1 hr. at room temperature.

At this point, samples are ready for morphometry analysis, or they can be stored overnight in the dark at room temperature before transferring to 4°C.

Perform morphometric analysis

- 22 Acquire fluorescent digital images, slightly over-exposing to visualize the contour of the cells (for wavelength, see *APPENDIX 1E*).

Use a magnification that will allow visualization of an entire cell in each image. Randomly capture images of at least 12 cells per sample and a minimum of 36 cells per substrate.
- 23 Perform the measurements for both the length (span of the longest cord) and the breadth (caliper width) of each cell.
- 24 Calculate the inverse axial ratio by dividing length by breadth.

The inverse axial ratio corresponds to the elliptical form factor (EFF) morphometric parameter found in the integrated morphometry analysis (IMA) function of MetaMorph software (Universal Imaging Corporation).

The mean inverse axial ratio induced by a high-quality NIH-3T3-derived 3-D matrix should be about three-fold greater than that induced by the 2-D fibronectin control (Cukierman et al., 2001). Spindle shape morphology EFF ratios should significantly increase when comparing NIH-3T3 to matrices produced by activated (i.e., myofibroblastic desmoplastic/fibrous) fibroblasts (Amatangelo et al., 2005).

SUPPORT PROTOCOL 5: Evaluation of α-SMA phenotype

When fibroblast-derived matrices are produced by activated (i.e., myofibroblastic) cells, the matrices will prompt increased levels of α-SMA content (Amatangelo et al., 2005) and stress fiber-like localization (Gupta et al. 2011) compared to levels induced by normal or

control (i.e., NIH-3T3) produced matrices. This protocol depicts how to conduct indirect immunofluorescent semi-quantitative assessments of markers such as α -SMA.

Materials

Fibroblast-derived 3-D matrix-covered 12-mm no. 1.0 coverslips (see Basic Protocol)

2% (w/v) heat-denatured BSA (see recipe)

Three - 24 well culturing plates

Dulbecco's Phosphate-buffered saline (DPBS⁺; *APPENDIX 2A*)

DPBS, Ca⁺⁺ and Mg⁺⁺ free (DPBS⁻; *APPENDIX 2A*)

DPBS⁺ with Tween (0.05%) (DPBS⁺-T; see recipe)

Double-distilled water

Semi-confluent 15-cm dish of fibroblasts or 75-cm² flask

Trypsin/EDTA solution (see recipe)

Complete medium with fetal bovine serum (see recipe)

Fixing solution (see recipe)

Fixing/Permeabilization solution (see recipe)

100% Donkey serum stock (Jackson ImmunoResearch Laboratories)

Odyssey® Blocking Buffer (LI-COR Biosciences, P/N 927-40000) with 1% donkey serum (see recipe)

Primary antibody solution: anti- α -Smooth Muscle Actin (α -SMA) mouse antibody [1:300] (Sigma-Aldrich, catalog number: A2547) diluted in Odyssey® Blocking Buffer.

Secondary antibody solution: anti-mouse Rhodamine-red-conjugated affinity purified F(ab')₂ donkey fragment [1:100] (Jackson ImmunoResearch Laboratories, catalog number: 54831), diluted in Odyssey® Blocking Buffer.

Fluorescent labeled Phalloidin solution: 5 μ l of Oregon Green® Phalloidin (Life Technologies) diluted in 200 μ l DPBS⁺ -Tween-20 0.05% (see recipe).

Prolong Gold mounting medium (Invitrogen)

Inverted microscope

Fine-point forceps (e.g., Dumont 4)

Glass microscope slides

Fluorescent microscope (objectives 20 $\times$ or 40 $\times$), equipped with digital camera and image acquisition software

Optional: Confocal microscope (objectives 40× or 60×), equipped with a Krypton/Argon laser with three lines, 488, 568, and 647 nm, for fluorescence excitation of dye-labeled samples (e.g. Confocal spinning disk Ultraview, Perkin-Elmer Life Sciences), complemented with CCD camera and image acquisition software (e.g., Velocity 6.3.0, Perkin-Elmer Life Sciences) for 8-16bit images acquisition. Image analysis software, capable of measuring elliptical Fourier parameters, pixel intensity (integrated intensity) and pixel colocalization between two images:

ImageJ (<http://imagej.nih.gov/ij/>)

MetaMorph® (Molecular Devices). *Optional*

Excel software (Microsoft)

Indirect Immunofluorescent Assay

The steps for this protocol are described using cell-derived 3-D matrix deposited on 12-mm no. 1.0 coverslips in a 24-wells plate (See Basic Protocol).

Block nonspecific cell binding with BSA

- 1 Cautiously place fibroblast-derived 3-D matrix that were produced onto 12 mm coverslips and control-coated coverslips, matrix face up, into individual wells of 24-well plates.
- 2 Block nonspecific cell binding by adding 0.5 ml of 2% heat-denatured BSA and incubate for 1 hr. at 37°C.
- 3 Rinse all blocked samples using 1 ml DPBS⁺.

At this point, coverslips coated with cell-derived 3-D matrices are ready to be seeded (re-plated) with the cells that are intended to be tested for their responses to assorted matrices.

Harvest cells for re-plating

- 4 Start with a semi-confluent 15-cm dish of fibroblasts; aspirate and discard the culture medium.
- 5 Quickly rinse the cell layer briefly with 1.5 ml 37°C pre-heated trypsin/EDTA.
Optional: use 10 ml DPBS[−] pre-heated at 37°C prior to conducting this rinse. This will remove traces of serum that contain trypsin inhibitors.
- 6 Add enough trypsin/EDTA solution to cover the cell layer, aspirate excess liquid, and observe under an inverted microscope until cells are detached from the culture dish (1 to 3 min).
- 7 Collect the cells in 10 ml of confluent medium, count cells (UNIT 1.1) and dilute (with confluent medium) to a final concentration of 1.0×10^4 cells/ml.

- 8 Use samples from step 3 above and replace DPBS⁺ with 0.5 ml, corresponding to 5,000 cells per well, from step 7 onto 3-D matrices.
- 9 Incubate cells within matrices for 16-18 hr. at 37°C.
- 10 Remove media and rinse samples with 0.5 ml of DPBS⁺.
- 11 Follow Support Protocol 1, steps 2 to 17, but this time using the primary and secondary solutions described in Support Protocol 5, while Phalloidin substitutes the use of SYBR Green. At the end of this process, monochromatic images should be available.

Analysis of indirect immunofluorescent images

- 12 Determine the intensity value (mean gray value or mean integrated intensity) of α -SMA for each image, and calculate the organization of α -SMA by questioning the percentage of colocalization over the Phalloidin positive pixels in the corresponding accompanying monochromatic image.

Highly functional desmoplastic (i.e., activated) 3-D matrices should induce above 65% increased expression of α -SMA levels compared to levels induced by control matrices (i.e., NIH-3T3). Similarly, a high quality desmoplastic ECM will prompt increased percentages of α -SMA positive areas that are localized at phalloidin positive areas when compared to normal (i.e., NIH-3T3) controls.

SUPPORT PROTOCOL 6: Lysis of Re-Plated Fibroblasts for Western Blot Analyses

To assure the quality of a batch of assorted fibroblasts-derived 3-D matrices (i.e., NIH-3T3), the levels of FAK activity (FAKpY³⁹⁷) must be down-regulated at least 1.5 fold (Cukierman et al., 2001) when compared to classic 2-D cultures. Likewise, matrices produced by normal versus desmoplastic fibroblasts (i.e., tumor-associated/myofibroblastic) induce quantitatively distinct responses, such as changes in α SMA expression levels. This protocol describes how to lyse normal human or murine primary fibroblasts after re-plating within 3-D matrices for biochemical analysis by immunoblotting (see *UNIT 6.2*). In brief, re-plated normal fibroblasts are lysed and subjected to immunoblot analysis. Cell lysate extracts can also be stored for later analyses (see step 11).

Materials

Matrix-coated 35-mm dishes (see Support Protocol 2)
 Fibronectin-coated 35-mm dishes
 Cell suspension from confluent cultures of fibroblasts
 Confluent medium with fetal bovine serum (see recipe)
 Lysis buffer (modified RIPA) reagent (see recipe) supplemented with protease and phosphatase inhibitors (see recipe), ice cold

Normal human or murine fibroblasts re-plated in 3-D matrix dishes (see Basic Protocol, step 27)

DPBS, Ca^{++} and Mg^{++} free (DPBS⁻; *APPENDIX 2A*), ice cold

Dry ice/isopropanol bath

5× sample buffer supplemented with β -mercaptoethanol

Anti-FAKpY³⁹⁷ and anti-total FAK (see recipes)

Anti α -SMA

Glutaraldehyde-3-phosphate dehydrogenase (GAPDH)

37°C, 10% CO_2 humidified incubator

Cell scraper (Costar, Fisher Scientific)

1.5-ml microcentrifuge tubes (Eppendorf)

Sonicator (e.g., Branson Sonifier 150)

Scion image software beta version 4.03

Additional reagents and equipment for calculating the amount of 5× sample buffer supplemented with β -mercaptoethanol (*UNIT 6.1*) and detecting proteins by immunoblotting (*UNIT 6.2*)

Re-plate fibroblasts within 3-D matrices

- 1 Block nonspecific binding in 3-D matrices deposited in a 35-mm dish (or 6-well multi-well dish) with BSA following steps 1–3 from Support Protocol 4.

When using 2-D substrates, pre-coat 35-mm dishes with 1 ml of 5 $\mu\text{g}/\text{ml}$ fibronectin or other matrix protein (see recipe for pre-coated 2-D fibronectin dishes) for 1 hr. at 37°C. Alternatively, use uncoated dishes in DPBS⁺ for 2-D control substrates.
- 2 Plate 2 ml of cell suspension at a final concentration of 1×10^5 cells/ml in confluent medium for each dish and incubate overnight (~16hr) in a 37°C, 10% CO_2 humidified incubator (see Basic Protocol, steps 1 to 4).

Lyse cells within matrices

- 3 Supplement 10 ml of ice-cold lysis buffer with protease and phosphatase inhibitors.
- 4 Carefully aspirate confluent medium from fibroblasts re-plated in 3-D matrix dishes (optional samples from Basic Protocol, step 18 can also be used in this protocol).
- 5 Gently add ice-cold DPBS⁺ and repeat steps 4 and 5 for a total of two washes.
- 6 Carefully aspirate again and tip the dishes for 1 min to accumulate the excess DPBS⁺ on one side of the dish (~30° to bench top).

It is important to remove all traces of DPBS⁺ to prevent diluting lysates with DPBS⁺ for the purpose of maintaining a uniform volume of lysate for different samples. This will yield a relatively consistent protein loaded onto SDS-PAGE gels for immunoblotting (see UNIT 6.2).

- 7 Carefully aspirate the excess DPBS⁺ to avoid detaching of the matrix layer.
- 8 Place the dishes (usually a 35-mm dish) on ice and add 200 to 300 μ l of ice-cold lysis buffer.

For larger dishes, add a proportionally larger volume of lysis buffer.

- 9 Incubate on ice for 5 min with gentle rocking.

Collect the lysate

- 10 Scrape the cells and matrix from the dish with the cell scraper. Then, tilt the dish toward one side and collect the lysate mixture into a 1.5-ml microcentrifuge tube.
- 11 Sonicate each tube of cell lysate using the remote setting of 3 (medium power) for 30 sec for each tube.
- 12 Centrifuge the lysates 15 min at $16,100 \times g$, 4°C.
- 13 Carefully remove the supernatant and transfer to a fresh, labeled tube. If not used immediately, quick freeze the lysates in a dry ice/isopropanol bath and store for 1 to 2 weeks at –80°C.

To quick-freeze cell lysates, prepare a dry ice/isopropanol bath by adding ~100 ml of isopropanol to a 400-ml beaker and placing in an ice bucket containing dry ice pellets in the fume hood. Allow the isopropanol to cool for 30 min. Add 1.5-ml microcentrifuge tubes containing freshly prepared lysates to a tube-rack and lower the rack into the isopropanol so that the lysate volume is completely immersed. The lysates should be frozen almost immediately (smaller aliquots are better). Finally, quickly transfer the tubes to a –80°C freezer. Samples are stable for 1 to 2 weeks. Each individual protein should be tested since there is some variability.

Analyze lysates

- 14 To analyze the matrix by immunoblotting, calculate the amount of 5 $\times$ sample buffer supplemented with β -mercaptoethanol (UNIT 6.1) that is needed to make a 1 $\times$ final concentration after addition to the sample; and add that amount to appropriate lysate samples.
- 15 Analyze signaling proteins by immunoblotting and immunodetection (UNIT 6.2) using antibodies to FAKpY³⁹⁷ and total FAK or, for α -SMA, to α -SMA and GAPDH.

For analysis of phosphoproteins, incubate primary antibodies with TBST with a final concentration of 5% (w/v) BSA.

For total FAK, α -SMA, GAPDH and other non-phosphorylated protein epitopes, primary antibodies, secondary antibodies, and blocking buffer, use 5% (w/v) nonfat dried milk in TBST as the diluent.

16. Scan individual protein bands corresponding to FAKpY³⁹⁷ or total FAK (or α -SMA and GAPDH) and quantify their optical densities using the version 1.48v or later of publicly available ImageJ by means of the Analyze>Gel macro. To adjust for sample loading, quantify glutaraldehyde-3-phosphate dehydrogenase (GAPDH, 40 kDa) as a total cellular protein control.

The software can be downloaded from the ImageJ Web site at: <http://imagej.nih.gov/ij/>

The average protein yield of the matrix and fibroblastic proteins (lysate) is ~0.5 to 2 mg per 35-mm dish.

PREPARATION OF TWO-DIMENSIONAL CONTROLS

Any given cell response induced by *in vivo*-like fibroblast-derived 3-D matrices could be due to the three-dimensionality of the matrix, its molecular composition, or a combination of both. The following two support protocols provide methods for obtaining suitable 2-D control matrices with the same molecular composition as the 3-D matrices.

SUPPORT PROTOCOL 7: Mechanical Compression of the Fibroblast-Derived 3-D Matrix

This protocol describes how to apply pressure to the fibroblast-derived 3-D matrix to collapse the matrix to a flat substrate. Mechanical compression of the 3-D matrix ensures that all natural components of the 3-D matrix are present, lacking only the element of three-dimensionality. Briefly, the 3-D sample is compressed using a known weight applied to a given area. The surface that comes into contact with the matrix is covered with a Teflon film to prevent sticking and to avoid tearing the flattened matrix as the weight is retracted.

NOTE: Any other materials fulfilling the same purpose can be substituted.

Materials

Superglue

Absolute ethanol, optional

Fibroblast-derived 3-D matrix on 22-mm circular coverslips

Dulbecco's phosphate-buffered saline (DPBS⁺; see *APPENDIX 2A*)

Flat platform large enough to rest on the ring (see Figure 10.9.3)

Suitable spacer smaller in width than the diameter of the ring but longer in height than the depth of the ring (see Figure 10.9.3)

12-mm round coverslips (Carolina)

Teflon film: protective overlay composed of: 0.001-in. FEP film, on 0.008-in. vinyl film, with adhesive back (use to cover laboratory bench-tops, Cole-Parmer Instrument Company)

Cork borer (12-mm diameter)

Biological hood equipped with UV light

Stand equipped with a horizontal ring

Lifting laboratory jack

Parafilm

Weight (~158 g)

35-mm dishes

Inverted phase-contrast microscope

Construct weight holder for matrix compression

- 1** Glue the flat platform to the spacer in such a way that the spacer will protrude slightly beyond the bottom of the ring when the platform is placed on the ring (Figure 10.9.3).
- 2** Glue four 12-mm round coverslips to the end of the spacer (one on top of the other) as an extension of the spacer using superglue. Allow enough time for the superglue to completely dry.

This will facilitate penetration of the coverslip portion into the matrix while avoiding contact between the matrix and the rest of the spacer, and it defines the area of compression.
- 3** Cut a Teflon circle (12-mm diameter) with the cork borer.
- 4** Cover the last coverslip with the Teflon film.
- 5** Sterilize materials by exposing them to a UV light in a biological hood for several hours with the Teflon film facing the light.

If the compressed matrices are to be in contact with cells for only short periods of time (e.g., for the 10-min cell attachment assay), rinsing the Teflon film with ethanol and air-drying should be sufficient to prevent contamination.
- 6** Place the glued platform with spacer on the ring portion of the stand with the Teflon facing down.
- 7** Cover the flat upper surface of the laboratory jack with Parafilm and position the jack under the ring.

- 8 Set the weight on the platform and level the ring so that the Teflon film is situated parallel to the surface of the jack (see Figure. 10.9.3).

Mechanically compress the 3-D matrix

- 9 Position the fibroblast-derived 3-D matrix-coated coverslip (matrix face up) onto the jack directly underneath the Teflon film.
- 10 Slowly raise the laboratory jack until the matrix contacts the Teflon film and the platform rises above the ring. Wait for 2 min.

At this point, the entire weight should be resting on the matrix, compressing it at a specific weight per unit area.
- 11 Slowly lower the jack until the platform rests once again on the ring, and the compressed matrix is separated from the Teflon film.
- 12 Place the coverslip with the compressed matrix into a 35-mm dish. Carefully add 2 ml DPBS⁺ and examine by phase-contrast microscopy to confirm continued integrity of the compressed matrix.

SUPPORT PROTOCOL 8: Solubilization of Fibroblast-Derived 3-D Matrix

This protocol describes how to solubilize fibroblast-derived 3-D matrix to generate a protein mixture that can be used for subsequent coating of surfaces or biochemical analysis. Briefly, the matrices are treated with a guanidine solution to denature and solubilize the matrix components, thereby producing a liquid mixture that can be stored and used for coating surfaces.

Materials

Fibroblast-derived 3-D matrices on 35-mm dishes (see Basic Protocol)

Solubilization reagent (see recipe)

Cell scraper (e.g., rubber policeman, Costar brand, Fisher Scientific)

1.5-ml microcentrifuge tubes

Rotator at 4°C

Microcentrifuge

Prepare dishes

- 1 Aspirate DPBS⁺ from fibroblast-derived 3-D matrix-covered dishes.
- 2 Tip the dishes for 1 min to accumulate the excess DPBS⁺ on one side of the dish (~30° to bench top).
- 3 Aspirate the excess DPBS⁺ carefully to avoid detaching the matrix layer.

Solubilize matrix

- 4 Place the dishes on ice and add 300 µl of solubilization reagent. Incubate 5 min on ice.
- 5 Scrape the dish with a cell scraper toward one side of the dish and pipet the mixture into a 1.5-ml microcentrifuge tube.
- 6 Add an additional 200 µl solubilization reagent. Rotate 1 hr. at 4°C.

Collect solubilized matrix

- 7 Microcentrifuge 15 min at $12,000 \times g$, 4°C.
- 8 Transfer the supernatant into a fresh 1.5-ml microcentrifuge tube. Store the solubilized matrix in solubilization reagent indefinitely at 4°C.

The average protein concentration is 1 to 3 mg/ml.

ISOLATION OF PRIMARY FIBROBLASTS FROM FRESH TISSUE SAMPLES

NIH-3T3 cells are particularly well-suited for mesenchymal cell-derived matrix production because they are homogeneous and provide batch-to-batch consistency. When grown in FBS, their ability to grow at high densities and lack of contact inhibition allows the NIH-3T3 cells to produce a thicker matrix, usually 10 µm, which is optimal for cell studies of 3-D cultures (see Critical Parameters). However, primary fibroblasts and other fibroblastic cell lines are also suitable for the production of cell-derived matrices. This protocol describes harvesting of primary fibroblasts from fresh tissue samples (i.e., fresh unfixed cancer surgical samples), either from normal or neoplastic regions, by using one of two possible approaches. The first is based on fibroblasts' motile capabilities allowing cells to migrate or "crawl" out of the processed tissue (Support Protocol 9). This approach renders homogeneous fibroblastic populations. In the second approach, depicted in Support Protocol 10, an enzymatic tissue digestion facilitates the fast isolation of heterogeneous fibroblastic cells. Both approaches are useful and should be used according to homogenous or heterogeneous as well as time restrictions prompted by the intended specific cell's needs.

NOTE: At the end of either of these protocols, cells should be tested (i.e., via western blot) to assure they express fibroblastic markers such as vimentin but lack expression of epithelial markers such as pan-cytokeratin.

SUPPORT PROTOCOL 9: Isolation of primary fibroblasts by non-enzymatic procedure**Materials**

Fresh tissue samples (murine or human surgical)

Dulbecco's Phosphate-buffered saline (DPBS⁺; *APPENDIX 2A*) supplemented with antibiotics (see recipe), 4°C

Complete fibroblastic medium with 10–15% fetal bovine serum (FBS; see recipe)

Ciprofloxacin (Invitrogen), optional
Fungizone (amphotericin B; Invitrogen), optional
Trypsin/EDTA solution (see recipe)
BSA (Fraction V, Sigma-Aldrich)
Sterile DMEM-3% BSA-pen/strep (see recipe)
100-mm tissue culture dishes
Dissecting scissors, tweezers, and scalpels (Fisher Scientific)
12-well or 6-well tissue culture plates
Sterile laminar flow hood
75-cm² or 25-cm² tissue culture flasks
Tissue Culture Incubator: 37°C, 5–10% (v/v) humidified CO₂ incubator
Inverted phase-contrast microscope

Prepare tissue samples

From this point forward, all solutions and equipment coming into contact with tissue samples or living cells must observe sterile and aseptic conditions all the times. Cell culturing must be maintained at 37°C, in a 5% CO₂ humidified incubator.

Collect the tissue samples in 50 ml polypropylene tubes with 25 ml ice cold DPBS⁺ supplemented with antibiotics. Process the tissues as quickly as possible after surgery.

1. Rinse tissue samples obtained immediately after surgery (human or murine) three times in a 100-mm tissue culture dish with pre-cooled (to 4°C) DPBS⁺ supplemented with antibiotics.
2. Aspirate supplemented DPBS⁺, finely chop the tissue sample into 1-mm² pieces using a sterile scalpel, assisting with sterile tweezers (see Amatangelo et al., 2005).

Optional: These samples can now be used to follow Support Protocol 10.
3. Using sterile dissecting scissors and/or scalpel, make multiple scratches into the plastic surface of a 12-well or 6-well tissue culture dish in a star-like configuration.
4. Wash the dish two times with 1 ml (12-well plate) or 2 ml (6-well plate) DPBS and gently press tissue pieces into the indentations created by the scratches.
5. Allow plate to dry for 5 min under the sterile laminar hood.

6. Gently add 1 ml (12-well plate) or 2 ml (6-well plate) confluent medium with FBS to each of the wells, ensuring that the tissue samples remain attached to the scratched surfaces. Incubate 2 to 7 weeks. Replace the confluent medium every other day until primary fibroblasts emerge from the tissue pieces.

This process normally takes 2 to 7 weeks depending on the tissue source.

Optional: As an additional measure to prevent contamination of freshly isolated surgical samples, culture half of the tissue pieces in confluent medium supplemented with 250 ng/ml to 2.5 µg/ml Fungizone and 10 µg/ml ciprofloxacin.

7. After fibroblasts are grown to ~70% confluence in multi-well dishes, remove the tissue pieces.
8. Trypsinized fibroblasts with trypsin/EDTA and passage into a 75-cm² tissue culture flask (see Basic Protocol, steps 1 to 5).
9. Once fibroblasts reach confluence in a 75-cm² flask, harvest and freeze them for future experimental analysis, and/or use them to produce fibroblast-derived matrices, preferentially between passages 2 and 6 (see Basic Protocol).

The authors start counting passages once the fibroblasts are initially transferred into a 15-cm dish. The fibroblasts are stable by morphological and biochemical criteria to at least passage 6. Morphological criteria include an elongated cell shape and the shape of the nuclei by Hoechst staining. For tumor-associated fibroblasts, elliptically shaped nuclei typical of myofibroblastic cells have been observed. They have not yet been genetically characterized.

To assure that the primary isolated cells correspond to a fibroblastic cell population, it is highly recommended to evaluate via immunofluorescence (See Protocol 1) and/or immunoblotting and immuno-detection (Support protocol 6 and UNIT 6.2) a minimum two fibroblastic markers, for example: vimentin (positive in fibroblastic cells), and cytokeratins (negative in fibroblastic cells).

SUPPORT PROTOCOL 10: Isolation of primary fibroblasts by enzymatic tissue digestion

Materials

Fresh tissue samples (murine or human surgical)

Dulbecco's Phosphate-buffered saline (DPBS⁺; *APPENDIX 2A*) supplemented with antibiotics (see recipe), 4°C

Complete fibroblastic medium with 10% fetal bovine serum (FBS; see recipe)

Trypsin/EDTA solution (see recipe)

Fungizone (amphotericin B; Invitrogen), optional

BSA (Fraction V, Sigma-Aldrich)

Sterile DMEM-3% BSA-pen/strep (see recipe)

Sterile 10X Collagenase-3 (Worthington)

100-mm tissue culture dishes

Dissecting scissors, tweezers, and scalpels (Fisher Scientific)

12-well or 6-well tissue culture plates

Sterile laminar flow hood

75-cm² or 25-cm² tissue culture flasks

Tissue Culture Incubator: 37°C, 5–10% (v/v) humidified CO₂ incubator

Inverted phase-contrast microscope

Harvesting cells

From here on, all solutions and equipment coming into contact with tissue samples or living cells must observe sterile and aseptic conditions. Cell culturing must be maintained at 37°C, in a 5% CO₂ humidified incubator.

Collect the tissue samples in 50 ml polypropylene tubes with 25 ml ice cold DPBS⁺ supplemented with antibiotics. Process the tissues as quickly as possible after surgery following steps 1 and 2 from Support Protocol 9.

1. Begin enzymatic digestion by incubating minced tissue samples in 8 mL Sterile DMEM-3% BSA-pen/strep (see recipe) complemented with 2 mL of Collagenase-3 (10×). Incubate overnight at 37°C in a 5% CO₂ humidified incubator.
2. Mechanically disrupt the remaining tissue by carefully pipetting several times the suspension against the dish bottom.
3. Collect the digested tissue into a 50 mL polypropylene tube and centrifuge at 200 × g for 10 min.
4. Remove the supernatant and re-suspend in 10 mL complete fibroblast medium.
5. Filter the cell-suspension through sterile 500 µm nylon mesh to remove big tissue pieces. Proceed to filtrate two additional times: first through a 100 µm and then through a 40 µm cell strainer.

6. Transfer the final filtrate into a 75-cm² tissue culture flask or into a smaller culture dish, if the size of original tissue was small. Place in the incubator for 2–3 hr.
7. Carefully aspirate the medium to remove non-adherent cells and debris. Add 7 ml of complete fibroblastic medium with FBS.
8. Culture the cells until they reach 80% confluence. Change media every third or fifth day by removing half of the volume and replenishing with a similar volume using fresh medium with FBS.
9. Once fibroblasts reach confluence in a 75-cm² flask, harvest and freeze them for future experimental analysis, and/or use them to produce fibroblast-derived matrices, preferentially between passages 2 and 6 (see Basic Protocol).

The authors start counting passages once the fibroblasts are initially transferred into a 15-cm dish. The fibroblasts are stable by morphological and biochemical criteria to at least passage 6. Morphological criteria include an elongated cell shape and the shape of the nuclei by Hoechst staining. For tumor-associated fibroblasts, elliptically shaped nuclei typical of myofibroblastic cells have been observed. They have not yet been genetically characterized.

To assure that the primary isolated cells correspond to a fibroblastic cell population, it is highly recommended to evaluate via immunofluorescence (See Protocol 1) and/or immunoblotting and immunodetection (Support protocol 6 and UNIT 6.2) a minimum two fibroblastic markers, for example: vimentin (positive in fibroblastic cells), and cytokeratins (negative in fibroblastic cells).

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Anti- α SMA

Use a 1:5000 dilution of anti- α SMA (Sigma-Aldrich) in 5% (w/v) nonfat dried milk (Carnation, Fisher Scientific)/TBST (see UNIT 6.2; see recipe for TBST). Store up to 12 months at –20°C.

Anti-FAKpY³⁹⁷

Make a 1:1000 to 1:2500 dilution of anti-FAKpY³⁹⁷ (Biosource International or Covance) in 5% (w/v) BSA (Sigma)/TBST (see UNIT 6.2; see recipe for TBST). Store up to 12 months at –20°C.

Anti-total FAK

Use a 1:2500 dilution of anti-total FAK (Upstate Cell Signaling Solutions) in 5% (w/v) nonfat dried milk (Carnation, Fisher Scientific)/TBST (see *UNIT 6.2*; see recipe for TBST). Store up to 12 months at -20°C .

Confluent medium with fetal bovine serum

High-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with:

10% (v/v) fetal bovine serum (*APPENDIX 2A*)

100 U/ml penicillin 100 $\mu\text{g/ml}$ streptomycin

Sterilize by 0.2- μm filtration in a stericup filter. Store for 1 month at 4°C .

For surgical or fine needle aspirates tissue samples, 250 ng/ml to 2.5 $\mu\text{g/ml}$ Fungizone can be added to the cultures.

In some cases primary fibroblasts will benefit from using 15% (v/v) fetal bovine serum as opposed to 10%.

Culture medium with calf serum

High-glucose Dulbecco's modified Eagle medium supplemented with:

10% (v/v) calf serum

100 U/ml penicillin

100 $\mu\text{g/ml}$ streptomycin

Sterilize by 0.2- μm filtration in a stericup filter. Store for 1 month at 4°C

Dil solution

Dilute 2.5 mg/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) stock solution in ethanol to 4 $\mu\text{g/ml}$ with confluent medium (see recipe) and sterilize by filtration using a 0.22- μm filter. Store up to 12 to 24 months at -20°C .

DMEM-3%BSA-pen/strep

High-glucose Dulbecco's modified Eagle medium supplemented with:

3% BSA (add 3 g of BSA to 100 ml of DMEM, warm up the solution at 37°C for fast dissolving)

100 U/ml penicillin

100 $\mu\text{g/ml}$ streptomycin

Sterilize by 0.2- μm filtration in a stericup filter. Store for 1 month at 4°C

DPBS⁺

Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*) containing:

1 mM CaCl_2

1 mM MgSO_4

Store 6 to 12 months at room temperature

DPBS⁺ supplemented with antibiotics

DPBS⁺ (see recipe) containing:

100 U/ml penicillin

100 $\mu\text{g/ml}$ streptomycin

2.5 $\mu\text{g/ml}$ Fungizone (amphotericin B; Invitrogen), optional

10 $\mu\text{g/ml}$ ciprofloxacin

Store 1 to 2 weeks at 4°C

DPBS⁻

Dulbecco's phosphate-buffered saline (DPBS; APPENDIX 2A) lacking:

CaCl_2 and MgSO_4

Store 6 to 12 months at room temperature

DPBS⁺ -Tween-20 0.05%

100 ml DPBS⁺ containing:

50 μl Tween-20 (Sigma-Aldrich)

Ethanolamine, 1 M

Prepare a 1 M solution of ethanolamine (Sigma-Aldrich) sterile water by adding 0.062 ml of ethanolamine per milliliter of water. Filter sterilize through a 0.2- μm filter unit. Prepare fresh.

Extraction buffer

Dulbecco's phosphate-buffered saline, Ca^{++} and Mg^{++} free (DPBS; APPENDIX 2A, lacking: CaCl_2 and MgSO_4); containing:

0.5% (v/v) Triton X-100

20 mM NH_4OH

Store up to 1 month at 4°C

Fixing solution

Into a 50-ml polypropylene conical tube, add:

2 g sucrose

10 ml 16% (w/v) solution paraformaldehyde (EM-grade from Electron Microscopy Sciences)

Dulbecco's phosphate-buffered saline (DPBS⁺; *APPENDIX 2A*) to a final volume of 40 ml

Store in the dark at room temperature for up to 1 week.

Fixing and permeabilization solution

Into a 50-ml polypropylene conical tube, transfer:

20 ml from the Fixing solution and add

20 μ L of Triton X-100.

Store in the dark at room temperature for up to 1 week.

Gelatin solution, 0.2% (w/v)

Prepare a 0.2% (w/v) gelatin solution in DPBS⁺ (*APPENDIX 2A*). Autoclave the solution, cool, and filter through a 0.2- μ m filter. Prepare fresh.

Glutaraldehyde solution in DPBS⁺, 1% (v/v)

Dilute a 25% stock of glutaraldehyde (Sigma) to 1% glutaraldehyde in DPBS⁺ (*APPENDIX 2A*). Thaw a 10-ml aliquot of the stock and dilute to a final volume of 250 ml in DPBS⁺. Filter sterilize through a 0.2- μ m filter unit and store in 50-ml aliquots at -20°C .

Heat-denatured BSA, 2% (w/v)

Dissolve 2 g BSA fraction V (Sigma) in 100 ml water and filter sterilize using a low-protein-binding 0.22- μ m filter. Store indefinitely at 4°C .

Just prior to use, heat the amount needed 5 min at 65°C or until the solution starts to appear slightly opaque (not milky). Cool to room temperature before using for blocking procedures. Do not store the heat-denatured BSA.

Hoechst 33342 stock solution

Prepare a 2 mM (MW 615.9 g) Hoechst 33342 (bisbenzimidazole H 33342 fluorochrome, trihydrochloride; Calbiochem) solution in water. Store at 4°C , protected from light.

Lysis buffer (modified RIPA) reagent

50 mM Tris-Cl, pH 8.0 (*APPENDIX 2A*)

50 mM NaCl

1% (w/v) deoxycholic acid, sodium salt (Fisher)

48 mM NaF

1% (w/v) glycerol (Fluka)

1% (w/v) Triton X-100 (Sigma)

Adjust to 100 ml with MilliQ H₂O

Store 3 to 6 months at 4°C

Matrix medium with ascorbic acid

Confluent medium (see recipe) containing:

L-ascorbic acid sodium salt (Sigma) at a final concentration of 50 µg/ml

Filter sterilize with a 0.2-µm filter

Prepare fresh

Ascorbic acid should be freshly prepared just prior to use as a 1000× stock concentration of 50 mg/ml in DPBS⁺ to yield a final concentration of 50 µg/ml. Remove a 5- to 10-ml aliquot of medium, add ascorbic acid, and after filtering, add the ascorbic acid-containing medium back to the total volume of medium. In cases where a 500 µg/ml final concentration of ascorbic acid is added on the first day after cell plating, the stock is diluted only 100-fold instead of 1000-fold (see Basic Protocol, steps 16 and 17).

SYBR green solution

Dilute SYBR green (Invitrogen, Catalog number: S7567) 1:10,000 in DPBS⁺ -Tween-20 0.05% (see recipe above)

Phalloidin solution

Dilute 5 µl of Oregon Green® Phalloidin (Life Technologies) in 200 µl DPBS⁺ -Tween-20 0.05% (see recipe above).

Pre-coated 2-D fibronectin dishes

In phosphate-buffered saline (*APPENDIX 2A*), prepare a 5 µg/ml solution of human plasma fibronectin (see UNIT 10.5 or Sigma). Immediately add 1 ml per 35-mm tissue culture dish and incubate for 1 hr. at 37°C. Remove remaining fibronectin solution and rinse once with DPBS⁺. Prepare diluted fibronectin solution fresh for each experiment.

The above procedure can be used with any desired protein for coating dishes or coverslips. If solubilized matrix mixture is to be used (see Support Protocol 6), coat with a 30 µg/ml protein concentration.

Protease and phosphatase inhibitors

1 mM sodium pyrophosphate

1 mM nitrophenol phosphate

5 mM benzamidine

1 mM PMSF

1 mM sodium orthovanadate

Serine/threonine phosphatase cocktail inhibitor 1 (100 μ l/10 ml lysis buffer; Sigma)

Tyrosine phosphatase inhibitor cocktail 2 (100 μ l/10 ml lysis buffer; Sigma)

Prepare fresh

Solubilization reagent

5 M guanidine containing:

10 mM dithiothreitol

Store indefinitely at 4°C

Tris-buffered saline with Tween (TBST)

10 mM Tris-Cl, pH 8.0 (*APPENDIX 2A*)

150 mM NaCl

0.5% (v/v) Tween-20 detergent (Sigma)

Adjust pH to 8.0 with 12 M HCl

Store up to 2 weeks at 4°C

Trypsin/EDTA solution

2.5 g trypsin

0.2 g EDTA

8 g NaCl

0.4 g KCl

1 g glucose

0.35 g NaHCO₃

0.01 g phenol red

Bring up to 1 liter with H₂O

Sterilize by filtration with a 0.2- μ m filter and store up to 3 months at -20°C

COMMENTARY

Background Information

Extracellular matrix (ECM) was historically regarded as a passive scaffold that stabilizes the physical structure of tissues. With time, it became evident that the ECM is much more than a simple physical scaffold. The ECM is a dynamic structure capable of inducing (and responding to) a large variety of physiological cell responses regulating the growth, migration, differentiation, survival, and tissue organization of cells (Buck and Horwitz, 1987; Hay, 1991; Hynes, 1999). Integrins are receptors for matrix molecules and can mediate these cell responses by inducing the formation of membrane-associated multi-

molecular complexes. These integrin-based structures (cell-matrix adhesions) mediate strong cell-substrate adhesion and transmit information in a bi-directional manner between ECM and the cytoplasm. There are three main cell-to-matrix adhesions. The “focal adhesion” mediates firm linkage to relatively rigid substrates (Burridge and Chrzanowska-Wodnicka, 1996). Focal adhesions cooperate with “fibrillar adhesions” that generate fibrils from pliable fibronectin (Katz et al., 2000; Pankov et al., 2000). Fibroblasts require culture for several days at high cell density to generate 3-D matrices and evolve “three-dimensional-matrix adhesions.” The requirements for producing 3-D matrix adhesions include three-dimensionality of the ECM, integrin $\alpha_5\beta_1$, fibronectin, other matrix component(s), and pliability of the matrix (Cukierman et al., 2001). The fibroblast-derived matrix provides an *in vivo*-like 3-D environment for cultured fibroblasts, thereby restoring their normally nonpolar surroundings. The fibroblast-derived 3-D matrix can be used as a suitable *in vitro* system to investigate *in vivo*-like fibroblast-to-matrix interactions, such as 3-D matrix adhesion signaling.

Critical Parameters

The phenotype of cultured NIH-3T3 fibroblasts as monitored by cell morphology is extremely important for the successful preparation of 3-D matrix-coated dishes. The fibroblasts should be well-spread and flat under sparse culture conditions. If elongated cells are commonly observed in the cell population, re-cloning of the cell line may be necessary to achieve greater phenotypic homogeneity. The NIH-3T3 line obtained from ATCC (ATCC# CRL-1658) has this morphology and produces an excellent matrix. The NIH-3T3 cells must be maintained routinely as sub-confluent cultures in a medium containing calf serum to retain the correct phenotype. However, if the matrix deposition at confluence is performed in the presence of calf serum, the resultant matrices are thicker but less stable and more likely to detach from the surface than matrices obtained after culture in fetal bovine serum. Therefore, NIH-3T3 cells should be changed to medium containing fetal bovine serum prior to matrix deposition. While the NIH-3T3 cells are being adapted for matrix production in FBS, they take on a more uniform polygonal morphology and are not as contact inhibited as those grown in calf serum. NIH-3T3 cells do not normally take on a very elongated morphology unless they are cultured within 3-D matrices. To pre-adapt the NIH-3T3 cells to fetal bovine serum-containing medium, it is recommended to culture the cells for 15 to 20 passages before plating for matrix deposition.

The Basic Protocol can be modified for other fibroblastic cell lines capable of secreting and assembling fibronectin-based matrices. As described in Support Protocol 7, the authors have adapted this protocol for the isolation of primary fibroblasts obtained from human and/or murine surgical tissue samples. Fibroblasts can be isolated from tissue samples after ~2 to 7 weeks in culture. In some cases, the resulting matrix may be too thick or dense to obtain efficient extraction. In such cases, more prolonged cell extraction may be needed with extensive DNase treatment until no cell debris is detected. The lack of contaminating cellular debris (in the case of NIH-3T3 cells) in the matrices has been confirmed by immunoblotting and immunofluorescence staining for cellular proteins like actin or GAPDH.

Pre-coating surfaces with gelatin promotes fibronectin binding and results in smooth layers of relatively homogenous matrices that will not detach from the surface.

The thickness of NIH-3T3-derived 3-D matrices is measured using a confocal microscope without dehydration of the matrix (no mounting or fixing). The resultant thickness observed varies between 8 and 20 μm . Basic molecular characterization of the matrices revealed the presence (among other molecules) of fibronectin organized in a fibrillar mesh, collagen I and III but not IV, and small traces of non-organized laminin and perlecan.

The integrity of these 3-D matrices must be confirmed prior to every use. This can be accomplished by using phase-contrast microscopy and discarding any matrices that are torn or detached (see Figure 10.9.1 B). Moreover, if matrices are to be used for short-term signal transduction assays under serum-depleted conditions, then freshly made matrices must be utilized. Matrices that have been stored at 4°C or –80°C (up to 2 to 6 weeks) should be used only after assessment of their integrity by phase-contrast microscopy. Freshly prepared or stored matrices can be used to test the induction of cellular responses in the presence of serum such as attachment, morphology, motility, proliferation, and for immunofluorescence staining. Additionally, biochemical analysis of the 3-D matrix can be assessed by immunoblotting to test for cell responsiveness to three-dimensionality by phospho-FAK down regulation (see Support Protocol 6).

Anticipated Results

The Basic Protocol is based on the ability of densely cultured fibroblasts (start up at $\sim 2.5 \times 10^5$ cells/ml) to coat any available tissue culture surface by deposition of their natural matrix, which gradually forms a 3-D matrix. This intact, naturally produced ECM is similar in its molecular organization to mesenchymal fibronectin-based extracellular matrices *in vivo* (Cukierman et al., 2001). The basic approach is to allow cells to deposit their own ECM followed by removal of cells, while avoiding procedures that may alter or denature the native ECM constituents and supra-molecular organization.

One NIH-3T3 semi-confluent (80%) cultured 15-cm dish can yield enough cells to coat 100 35-mm tissue culture dishes.

Time Considerations

The adaptation step after switching NIH-3T3 cell medium to fetal bovine serum for future matrix deposition requires culturing the cells for 15 to 20 passages. This adaptation process could take between 5 and 22 weeks depending on the rate of NIH-3T3 cell proliferation and the dilution factor per passage. The rate of NIH-3T3 proliferation is dependent upon many factors, including the growth-promoting abilities of the fetal bovine serum, which unfortunately is largely batch-dependent. Therefore, the time required for adapting NIH-3T3 cells to grow in FBS should be determined empirically. At this point, the NIH-3T3 cells are between 20 and 25 passages, and can be cultured in FBS for at least 20 to 25 additional passages, resulting in a total of 50 passages. After that, their morphology starts to become more spindle-shaped, and, therefore, they can no longer form optimal matrices. When the NIH-3T3 cells become too spindle-shaped, they fail to form uniform monolayers that upon extraction can ultimately produce uneven matrix coverage. Matrix production will require

between 5 and 9 days. About 2 to 7 weeks are required from the time of tissue isolation to harvesting primary fibroblasts.

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Figure 10.9.1. Fibroblast-derived 3-D matrices before and after extraction process. **(A)** Culture at day 5 prior to matrix extraction. **(B)** The resulting fibroblast-derived 3-D matrix. Panels **C** and **D** are magnified insets from **A** and **B**, respectively. Bars represent 50 μm .

Figure 10.9.2.

Unextracted fibroblast-derived 3-D matrix phenotypes. (A–F) Representative confocal indirect immunofluorescent reconstructed images, corresponding to assorted unextracted human pancreatic primary fibroblasts-derived 3-D matrix cultures. Normal vs. desmoplastic (i.e., myofibroblastic activated) monochromatic matrix (i.e., fibronectin; A–B) and α -SMA (C–D) images are shown while corresponding nuclei images are in the inserts. The same overlaid images are shown in panels E and F; green fibronectin, red α -SMA and blue nuclei. G and H panels depict the OrientationJ software’s visual output obtained from A and B. Colored bars to the right represent the angles revealed by the software, while the corresponding mode angle color is shown for each image. I and J are the resultant Hue-corrected images displaying mode angle in Cyan representing normalized image/color (i.e., 0°). Note how color variations are diminished in the desmoplastic example where the majority of fibers are parallel oriented. Bars represent 50 μ m. Graphs in K constitute numeral outputs from G and H while L corresponds to the normalized distributions (analogous to I and J).

Figure 10.9.3. Diagram showing the components of the mechanical compression device. (a) Weight. (b) Flat platform. (c) Spacer. (d) 12-mm coverslips. (e) Teflon film. (f) Ring stand. (g) Fibroblast-derived 3-D matrix to be mechanically compressed. (h) Lifting laboratory jack.