Published in final edited form as:

Curr Protoc Cell Biol. ; 70: 10.19.1–10.19.11. doi:10.1002/0471143030.cb1019s70.

Preparation of High-Density Fibrillar Collagen Matrices that Mimic Desmoplastic Tumor Stroma

Vira V. Artym^{1,*}

¹Laboratory of Cell and Developmental Biology, NIDCR, NIH, Bethesda, MD 20892

Abstract

The stroma of invasive tumors becomes enriched in dense fibrillar collagen as a result of the desmoplastic reaction. This desmoplastic collagen exerts profound effects on tumor and normal cells. In view of these findings, it is important to develop novel *in vitro* cell systems that mimic this desmoplastic extracellular matrix in order to permit cell studies under *in vivo*-like conditions. This Unit provides a protocol and troubleshooting guide for the preparation of high density fibrillar collagen (HDFC) matrices that closely model the desmoplastic collagenous matrix of malignant tumors. It then describes the use of this HDFC matrix for *in vitro* cell studies of invadopodia formation and function in extracellular matrix invasion. In addition, this Unit also provides a detailed protocol for immuno-labeling of invadopodial proteins and detection of HDFC matrix degradation associated with invadopodia to permit invadopodia visualization using fluorescence microscopy.

Keywords

High-density fibrillar collagen; HDFC matrix; desmoplastic stroma; fibrosis; collagen type I; invadopodia; invasion

INTRODUCTION

Desmoplasia is a common hallmark of multiple types of malignant tumors. It is characterized by extensive depositions of fibrillar collagen type I, causing dense fibrosis around tumors. The desmoplastic collagenous environment promotes tumor progression and metastasis, and it correlates with poor prognosis in patients (Provenzano et al., 2008; Conklin and Keely, 2012). Therefore, *in vitro* modeling of the desmoplastic collagen environment has provided a valuable tool for investigating the regulatory roles of tumor ECM in multiple cellular activities of cancer-associated cells (Artym et al., 2015).

This unit provides a complete protocol for assembly of high density fibrillar collagen (HDFC) matrices, a novel *in vitro* model of desmoplastic collagen (see Basic Protocol 1). HDFC matrix is a layer of densely packed fibrillar collagen type I compressed by centrifugation (Figure 1, A). The average thickness of the HDFC layer is about 5 μ m (Figure 1, B). This minimal thickness makes HDFC matrix an excellent cell culture substrate for

*Corresponding author: Tel: (301) 451-6498; FAX: (301) 402-0897; vartym@mail.nih.gov.

application of different microscopy techniques. It provides sufficient depth and volume for invasion by cellular protrusions, and yet it is thin enough with uniform depth to facilitate fluorescence microscopy of live and fixed cell samples. HDFC closely mimics desmoplastic collagen found in invasive tumors (Figure 1, C and D) and has been found to induce high numbers of invadopodia in both tumor cells and normal fibroblasts (Artym et al., 2015). This Unit also provides guidance for cell culture to obtain invadopodia induction, as well as an immuno-staining protocol for visualization of invadopodia and its function in HDFC degradation by means of fluorescence microscopy (see Basic Protocol 2).

BASIC PROTOCOL 1

PREPARATION OF HIGH-DENSITY FIBRILLAR COLLAGEN MATRICES

This protocol describes the assembly of a layer of HDFC matrix on the glass bottom of a MatTek dish. There are two major steps in the production of HDFC matrix: (1) polymerization of a thin 3D layer of high-concentration fibrillar collagen on the glass bottom of the MatTek dish; (2) physical compaction by centrifugation of the 3D collagen into a densely packed fibrillar collagen layer of HDFC matrix. Upon completion of this compaction, the HDFC matrix can be left untreated and be simply equilibrated against PBS or tissue culture medium prior to addition of cells. Otherwise, the HDFC matrix can be treated with a paraformaldehyde solution to produce a stiffer cross-linked HDFC matrix.

Materials—Solution of rat tail collagen type I (Corning; formerly BD Biosciences), neutralized to pH 7.4 using ice-cold 10X DMEM and 10X reconstitution buffer, and reconstituted to a final concentration of 8.16 mg/ml as described in the protocols from Artym and Matsumoto in Chapter 10, Unit 10.18. Prepare this fresh collagen solution immediately before use, and keep on ice.

Phosphate-buffered saline (PBS) pH 7.4, at room temperature 4% paraformaldehyde/5% sucrose in PBS at room temperature (see recipe below) High glucose-DMEM (HyClone) cell culture medium, sterile and at room temperature Ice bucket with ice

35 mm MatTek dish with 20 mm diameter glass bottom (MatTek Part No. P35G-0-20-C), pre-chilled on ice

10 µl plastic pipette tips

10 µl pipetter

Incubator with temperature set to 37°C

Phase contrast inverted microscope with 10X objective and a stage insert to permit examination of 35 mm tissue culture dishes

MatTek dish holder, a custom-designed assembly that is assembled from two covers of Corning Costar six-well cell culture plates and two strips of Time tape (see Figure 2) Centrifuge with swinging bucket rotors, such as the Sorvall Legend RT or Eppendorf 5810R, equipped with microplate centrifuge adapter and temperature pre-set to 20°C

1. Place a MatTek dish covered with lid on ice and chill it for 15 min.
2. Neutralize the collagen solution to pH 7.4 and reconstitute to a final concentration of 8.16 mg/ml solution of rat tail collagen. Keep the tube with collagen solution on ice.

Measure the pH of collagen solution using pH paper. 10 μ l of collagen solution is required to coat one 35 mm MatTek dish with a 20 mm diameter glass bottom. However, in order to avoid bubble formation, reconstitute the collagen in much larger volume, such as 350 μ l. Then, centrifuge the collagen solution at 9,100 g and 4°C for 3 min to eliminate any air bubbles introduced during pipetting.

3. Deposit a 10 μ l drop of ice-cold collagen solution on the glass bottom of the MatTek dish and spread it with a 10 μ l plastic pipette tip to completely coat the glass surface.

Keep the MatTek dish on ice while gently spreading the collagen solution on the glass surface in all directions to achieve even coating of the whole glass surface, taking care to avoid spreading the collagen onto the adjacent plastic dish. Avoid introducing air bubbles while spreading the collagen.

4. Leave the MatTek dish on ice for 10 minutes. This incubation period facilitates flattening of the upper surface of the collagen layer.
5. Transfer coated MatTek dish into an incubator pre-set to 37°C and incubate for 30 min to allow the collagen to polymerize into a fibrillar meshwork.

Different types of incubators that can maintain a temperature of 37°C can be used for collagen incubation to achieve polymerization. However, the incubation time will need to be adjusted depending on the humidity and CO₂ level of the individual incubator. Both humidity and CO₂ concentration can influence the rate of collagen polymerization as well as the thickness of the polymerized collagen fibrils. Extensive discussion about choosing the appropriate incubation time is provided in the Troubleshooting section. The goal of selecting this incubation time is to prepare a 3D collagen layer that will remain uniform during the centrifugation step.

6. Examine the collagen layer using phase contrast microscopy to confirm the formation of a uniform fibrillar collagen network covering the glass portion of the dish.

The neutralized and reconstituted solution of collagen is a mixture of globular collagen subunits that appears as a translucent substance when viewed under a phase contrast microscope. Polymerized fibrillar collagen can be visualized as a rough reticular meshwork under a phase contrast microscope (Figure 3, A). When inspecting the integrity of the layer of fibrillar collagen, scan multiple fields of the MatTek dish with the phase

contrast microscope. If transparent areas devoid of fibrils are detected (Fig. 3C), refer to the Troubleshooting section to resolve problems.

7. Secure the MatTek dishes in a custom holder by sandwiching them between two Corning six-well plate covers (Figure 2, A – C). Insert each MatTek dish within the circular grooves outlining the wells etched on the plate cover. Use strips of Time tape to fasten the two plate covers to one another with the MatTek dishes sandwiched inside (Figure 2, D).

Prepare a parallel custom-designed holder that holds the appropriate number of empty MatTek dishes to be used as a balance for centrifugation. The simple MatTek holder described here can be used as a prototype to manufacture custom MatTek dish holders made of plastic or rubber material. The concept of this custom holder is to provide a solid object that fits into the swinging centrifuge adapters, has circular openings to hold each MatTek dish firmly in place, and has a lid that secures the covers of the MatTek dishes.

8. Place the MatTek dish holders containing MatTek dishes into the microplate centrifuge adapters of the centrifuge and centrifuge at 3,500g for 20 min to flatten the collagen meshwork into a relatively 2D layer.

Set the temperature control of the centrifuge to 20°C one day in advance. It is crucial to have the centrifuge and its microplate adaptors fully equilibrated at 20°C in order to prevent detachment of the fibrillar collagen layer from the dish glass surface during centrifugation.

9. Upon completion of centrifugation, examine the MatTek dish with phase contrast microscopy to identify any defects and imperfections in the flattened fibrillar collagen matrix.

Note, if the incubation time in step 5 is properly adjusted, then the HDfC matrix generated by centrifugation will uniformly cover the entire glass surface of the MatTek dish. Phase contrast microscopy visualizes HDfC as a tightly compacted fibrillar collagen meshwork (Figure 3, B).

10. Add 2 ml of a solution of 4% paraformaldehyde/5% sucrose in PBS at room temperature to the MatTek dish to completely cover the HDfC matrix. Using a pipette gently add PBS solution to the plastic edge of the dish and avoid releasing the solution directly to the matrix surface. Incubate at room temperature for 20 min. This will chemically cross-link the flattened fibrillar collagen network with formaldehyde in order to produce a covalently cross-linked HDfC matrix.

Alternatively, skip this step if an untreated HDfC matrix is needed for the experiment. Instead, add 2 ml of PBS to the HDfC matrix in the MatTek dish and proceed to step 12.

11. Wash the chemically cross-linked HDfC matrix with 3 ml of sterile PBS three times. Use a pipette held at the side of the dish to insure gentle addition of the PBS to the plastic side of the dish avoiding pouring the PBS on to the matrix surface.

Gently aspirate used for wash PBS from the dish with an aspirating pipette tip carefully placed at the edge of the dish and avoiding matrix.

Perform this step in a tissue culture hood in order to avoid bacterial or fungal contamination of the HDFC matrix. Always gently add PBS to the edge of the MatTek dish and avoid adding it directly to the HDFC matrix. Doing so will avoid detaching or damaging the HDFC layer.

12. Incubate the matrices with sterile HyQ-DMEM cell culture medium at room temperature for 20 min to block non-specific reactive sites and to equilibrate with culture medium.

Perform this step in a tissue culture hood to avoid bacterial or fungal contamination of HDFC matrix. If desired, sterile 1% heat-denatured bovine serum albumin in PBS can be used instead of HyQ-DMEM to block residual reactive sites in cross-linked HDFC matrix.

13. Wash the matrices with 3 ml of sterile PBS two times. After the last wash, add 2ml of sterile PBS to the MatTek dish. For each addition of the PBS to the dish, exercise caution to add the PBS to the edge of the dish avoiding pouring PBS on to the matrix. For each aspiration of the PBS, position pipette tip at the edge of the dish avoiding damaging of the matrix.

Perform this step in a tissue culture hood to avoid contamination of the HDFC matrix.

14. Use a piece of Parafilm to seal the lid onto MatTek dish containing HDFC matrix.

Dishes with matrices can be stored at 4°C for several weeks. Aseptic techniques must be observed to prevent bacterial contamination.

BASIC PROTOCOL 2

USING HDFC AS AN *IN VITRO* MODEL TO STUDY INVADOPODIA

HDFC matrix, which mimics the tumor desmoplastic environment found *in vivo*, offers a convenient *in vitro* model for studies of invadopodia. It has been demonstrated that both untreated and chemically cross-linked versions of HDFC matrix can induce abundant invadopodia in a wide range of tumor cells (Artym et al., 2015). HDFC can be used to study invadopodia formation under a variety of different cell culture conditions, such as in the absence or presence of serum in cell culture media or the addition of a variety of growth factors or extracellular matrix proteins to serum-free tissue culture medium. It has been established that fibronectin from the serum used in cell culture media binds to the HDFC surface and can be degraded at the invadopodia. Therefore, focal loss of fibronectin labeling can serve as an indicator of the extent and type of matrix degradation at invadopodia (Artym et al., 2015). In addition, invadopodia-forming cells degrade the fibrillar collagen that constitutes HDFC. Immuno-labeling of fibrillar collagen cleavage sites by antibody against the resultant 1/4 collagen type I fragment permits detection of cleaved collagen, though it is practical only using HDFC matrices that are not treated with chemical cross-linker. Moreover, the presence of serum in HDFC cell culture medium decreases the detection

signal from the anti- $\frac{1}{4}$ collagen type I fragment antibody, possibly due to blocking of an epitope required for immuno-labeling by serum protein(s).

This protocol provides guidelines for tumor cell incubation on HDFC matrix and detection of an invadopodial response by means of visualization of invadopodia and their function in fibrillar collagen degradation using fluorescence confocal microscopy. The classical invadopodial marker used in this protocol is the presence of co-localized actin and cortactin immuno-staining (Figure 4). Invadopodia degradative function in extracellular matrix remodeling is visualized by fibrillar collagen cleavage detected by immuno-labeling for the $\frac{1}{4}$ collagen type I fragment (Figure 4).

Materials—MatTek dish coated with HDFC matrix not treated with chemical cross-linker

Complete cell culture medium consisting of HyQ-DMEM and 10% fetal bovine serum, sterile and at 37°C

Trypsin-EDTA, sterile and at 37°C

HyQ-DMEM, sterile and at 37°C

4% paraformaldehyde/5% sucrose in PBS at 37°C

PBS, sterile and at room temperature

0.5% Triton X-100 in PBS at room temperature

2% bovine serum albumin in PBS at room temperature (see recipe)

Primary antibody solution (see recipe)

Secondary antibody solution (see recipe)

75 cm² cell culture flask of MDA-MB-231 breast carcinoma cells at ~60-70% confluence

50 ml polypropylene centrifuge tube

Tissue culture incubator with 10% CO₂, humidification, 37°C temperature

Tissue culture phase contrast microscope

Cell culture table top centrifuge

1. Warm HyQ-DMEM, complete cell culture medium, and trypsin-EDTA to 37°C in a water bath.
2. Gently aspirate PBS from a MatTek dish coated with non-crosslinked HDFC matrix. Add 2 ml of HyQ-DMEM to the side of the MatTek dish and place the dish into a tissue culture incubator with 10% CO₂ at 37°C.
3. Retrieve a flask of MDA-MB-231 cells from a tissue culture incubator.

The flask with MDA-MB-231 cells must be prepared in advance. Any other cell line could be used for plating on HDFC matrices. However, the cells require to be growing in the flasks at the density of about 70-80% final confluence at the time of being re-plated on the HDFC matrix to ensure that they are in the growing phase. If one desires to defrost a new aliquot of the cells for the plating on HDFC matrix, then the cells must be growing in the culture for at least one week with required passaging of the cells that maintain at maximum of 70-80% final confluence of the cells in the flasks. Overgrowing of the cell lines to the state of 100% confluence in the flask decreases the potential of the cells to produce invadopodia in general.

4. Now proceed with detaching cells from the flask surface to yield cell suspension for plating cells on HDFC matrix. For this, in a tissue culture hood, gently aspirate the cell culture medium from the flask of MDA-MB-231 cells.
5. Add 8 ml of trypsin-EDTA to the flask to completely cover the cells with a thin layer of trypsin-EDTA solution. Incubate the cells at room temperature. Periodically, gently tilt the flask from side-to-side to swirl and mix the trypsin-EDTA covering the whole cell layer.
6. Observe cells using a tissue culture phase contrast microscope during this incubation. As soon as cells start to shrink away from the substrate, return the cell culture flask to the tissue culture hood, and stop further trypsinization by adding 20 ml of complete cell culture medium supplemented with 10% fetal bovine serum to the flask.
7. Gently pipet the cell culture medium up and down inside the flask, directing the flow of liquid against the bottom of the flask in order to detach the cells.
8. Collect the cell suspension in a centrifuge tube and centrifuge at 1,000 g for 5 minutes to form a pellet of dissociated, trypsinized cells.

After applying fresh trypsin-EDTA to the flask, it requires only 1 min incubation at room temperature on average for the cells to start retracting and shrinking enough for detachment.

9. Gently aspirate the cell culture medium supernatant from the centrifuge tube avoiding dislodging the pellet of cells.
10. Gently add 20 ml of HyQ-DMEM to the cells and mix by pipetting to re-suspend the cells.
11. Place the tube with cell suspension into a centrifuge and centrifuge the cells to form a pellet again.
12. Aspirate the cell culture medium from the centrifuge tube and re-suspend the cells in 10 ml of HyQ-DMEM.
13. Count cells and prepare an aliquot of 1×10^5 cells in 2ml of HyQ-DMEM.

14. Retrieve the MatTek dish from the incubator and gently aspirate HyQ-DMEM. Add 2 ml of cell suspension to the MatTek dish containing HDFC matrix.
15. Place the MatTek dish into an incubator set to 10% CO₂ and 37°C. Incubate cells for 3 hours, allowing the cells to attach and interact with HDFC matrix.
16. Retrieve the MatTek dish from the incubator and place it on a piece of Kimberly-Clark paper tissue (Kimwipe) in the hood.

Place the MatTek dish on a piece of paper to prevent contact of the coverglass bottom of the dish with a cold surface, since a rapid decrease in temperature can destabilize cellular protrusions, including invadopodia. Do not keep cell samples sitting at room temperature. Start fixing cell samples as described in the next step as soon as possible.

17. Gently aspirate the cell culture medium from the MatTek dish and add 1 ml of 4% paraformaldehyde/5% sucrose in PBS down the side of the dish to cover the cells cultured on the HDFC matrix.
18. Incubate 20 min at room temperature.
19. Gently aspirate fixative from the MatTek dish and add 2 ml of PBS to the dish.
20. Repeat PBS wash two more times by aspirating old PBS from the dish and adding 2 ml of fresh PBS to the dish each time.
21. Gently aspirate PBS and add 1 ml of 0.5% Triton X-100 in PBS to permeabilize the cells for immuno-staining of intracellular proteins.
22. Incubate 10 min at room temperature.
23. Gently aspirate the Triton X-100 solution from the MatTek dish.
24. Wash sample two times with 2 ml of PBS by aspirating the liquid from the dish and adding fresh PBS. No soaking time is necessary.
25. Perform a final wash by adding fresh PBS and incubating the sample for 30 min at room temperature.
26. Gently aspirate the PBS and add 1 ml of 2% bovine serum albumin in PBS to the dish to block the sample and prevent non-specific binding of the antibodies. Incubate 30 min at room temperature.
27. Gently aspirate blocking solution and add 1 ml of primary antibody solution. Incubate for 60 min at room temperature.

The volume of the antibody solutions can be decreased to 700 µl if necessary in order to conserve a precious antibody used for immuno-labeling. Phalloidin conjugated to a fluorescent dye can be added to the primary antibody solution for visualization of the actin cytoskeleton. For the purpose of this protocol, the descriptions of primary antibody solutions are for rabbit anti-cortactin and mouse anti-collagen cleavage site antibodies, as well as fluorescently tagged phalloidin (see Reagents and Solutions list).

28. Wash the sample with 2 ml of PBS three times, each time incubating the sample for 5 min at room temperature.
29. Gently aspirate PBS from the dish and apply 1 ml of secondary antibody solution. Incubate 30 min at room temperature.

For the purpose of this protocol, descriptions of secondary antibody solutions are provided for fluorescently conjugated goat anti-rabbit and anti-mouse antibodies (see Reagents and Solutions list).

30. Wash the sample with 2 ml of PBS three times. For each wash with the PBS, add 2ml of the PBS to the dish and incubate the dish for 5 min at room temperature. After 5 min of incubation, gently aspirate used PBS from the dish, and replace it with 2 ml of fresh PBS.
31. Aspirate PBS from the last washing step and add 1 ml of fresh sterile PBS to prepare the final sample for confocal microscopy in PBS.
32. Examine the sample using confocal fluorescence microscopy.

It is recommended to proceed to fluorescence confocal microscopy immediately after immuno-labeling is complete. The length of time that the sample can be stored in PBS depends on the affinity of the antibody used for immuno-staining, as well as avoiding any potential risk of bacterial contamination. For many antibodies, the fluorescence signal will be lost during storage due to molecular diffusion. For some high affinity antibodies and fluorescently tagged phalloidins, the sample can be stored overnight at 4°C and examined in the morning of the next day.

REAGENTS AND SOLUTIONS

10X DMEM—To make a 10X solution of DMEM with phenol red, dissolve a packet of DMEM powder (GIBCO) in one-tenth of the standard volume of distilled water. Centrifuge out any precipitate that forms due to incomplete solubility of this high-concentration DMEM, e.g., at 15,300 g for 10 min. Filter the supernatant through a 0.22 µm vacuum sterilizing filter to sterilize the DMEM solution. Aliquot the filtered solution and store at –20°C. This solution can be stored frozen for one year. Always defrost a fresh aliquot on ice before using it to neutralize and reconstitute collagen solutions.

10X Reconstitution buffer—Prepare 10X reconstitution buffer by dissolving 2.2 g of sodium bicarbonate and 4.8 g of HEPES in 100 ml of distilled water. Filter this buffer through a 0.22 µm vacuum filter to sterilize. Aliquot and store aliquots at –20°C for one year. Always defrost a fresh aliquot on ice before using it to neutralize and reconstitute collagen solutions.

4% (v/v) paraformaldehyde/5% (w/v) sucrose in PBS—Prepare a fresh solution of 4% paraformaldehyde (electron microscopy grade) and 5% sucrose in PBS. Filter the solution through a 0.22 µm vacuum filter. Preheat this solution to 37°C in a water bath before fixing cells.

0.5% (v/v) Triton X-100 in PBS—Prepare a 0.5% solution of Triton X-100 in PBS by diluting a 10% stock solution of Triton X-100 in PBS with stirring. Filter the solution through a 0.22 µm vacuum filter. Store up to 2 weeks at room temperature.

2% (w/v) bovine serum albumin in PBS—Prepare a 2% solution of bovine serum albumin in PBS. Filter the solution through a 0.22 µm vacuum filter. Always prepare fresh before use. Do not store.

Primary antibody solution—Prepare a fresh solution of primary antibody at 10 µg/ml final concentration in 2% bovine serum albumin in PBS. Always make it fresh and do not store. For this protocol, a fresh solution of anti-cortactin rabbit monoclonal antibody (Epitomics, catalog number 2067-1), mouse polyclonal antibody against collagen type I ¼ cleavage site (raised in-house and described in Artym et al., 2015), and rhodamine-phalloidin (Invitrogen) was prepared and used. Follow manufacturer suggestions for final concentrations for fluorescently tagged phalloidins.

Secondary antibody solution—Prepare a fresh solution of secondary fluorescently conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 2% bovine serum albumin in PBS. Always make it fresh and do not store. For this protocol, a fresh solution of goat anti-rabbit Cy2-conjugated and goat anti-mouse Cy5 conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc.) was prepared and used. Follow the manufacturer's suggestions for the final concentrations for fluorescently labeled secondary antibodies.

Phosphate-buffered saline (PBS)—PBS is available commercially or can be prepared as described for common stock solutions, see **APPENDIX 2A**. Keep the PBS sterile or filter the solution through a 0.22 µm vacuum filter to sterilize before use.

COMMENTARY

Background Information

The mechanisms of invadopodia-mediated matrix degradation and invasion had been studied extensively *in vitro* in applications using of a variety of artificial 2D and 3D extracellular matrices. Amongst the most widely used *in vitro* matrices are: 2D glass or gelatin coated with different ECM proteins in globular conformation, 2D and 3D fibrillar collagen matrices polymerized at relatively low collagen concentrations, and basement membranes (Artym et al., 2009; Weaver et al., 2013). Although these *in vitro* ECM models are convenient and inexpensive, they do not accurately reproduce the *in vivo* dense collagenous environments found in the majority of invasive metastatic tumors.

High density fibrillar collagen (HDFC) matrix was developed to mimic the dense fibrillar network of desmoplastic collagen. Its two-dimensional upper surface and average thickness of only 5 µm greatly facilitates the use of confocal fluorescence microscopy, and its high density of collagen type I and fibrillar composition were shown to dramatically stimulate invadopodia formation in tumor cells and transformation of normal fibroblasts (Artym et al., 2015). The importance of using this HDFC matrix to recreate the fibrotic component of

tumors became obvious from recent phospho-proteomics studies, which revealed unique HDFC matrix signaling pathways supporting invadopodia formation in tumor cells (Artym et al., 2015).

Critical Parameters and Troubleshooting

Maintaining reproducible thickness of HDFC matrices—There are three major factors that affect the thickness of the polymerized collagen layer in this protocol: (a) the volume of collagen solution used for polymerization of the fibrillar collagen layer, (b) the area of glass being coated with this collagen solution, and (c) the smoothness of the top layer of the spread collagen prior to initiating collagen polymerization. Collagen solutions, especially those at high collagen concentrations such as 8.16 mg/ml, are very viscous and, therefore, create difficulties with pipetting small volumes. It is critical to apply precisely 10 μ l of collagen solution to the glass surface of each MatTek dish with 20 mm glass bottom in order to maintain the same collagen-to-glass surface area ratio. If one finds the use of a regular micropipette too challenging for precise pipetting of 10 μ l of collagen solution, then a positive displacement micropipette designed for small volumes can greatly facilitate pipetting and minimize pipetting error. In order to achieve the same collagen-to-glass surface area ratio under conditions of controlled collagen volume, one needs to invest considerable effort into complete spreading of the small collagen volume over the 20 mm diameter glass surface. First of all, each MatTek dish must be pre-chilled on ice so that the inside of glass dish surface remains dry, and minimal water condensation occurs only on the outer surface of the glass adjacent to the ice. To do so, place MatTek dish covered with the lid on ice for 15 min. Prolonged incubation of the covered dishes on ice will result in the buildup of the large condensation droplets on the outer surface of the glass adjacent to the ice. If this cannot be avoided, use paper tissue to wipe off the glass before spreading the collagen. While spreading collagen on the glass surface, press the pipette tip only gently against the glass surface to avoid breaking the glass. Second, slightly tilt the dish to control the spreading and to confirm that no area of the glass surface is left uncoated with collagen solution. If an air bubble forms in the collagen during spreading, use the pipette tip to drive the bubble to the edge of the glass, where it is released. Each MatTek dish with its carefully spread collagen solution must be covered with the lid and incubated on ice for 10 min to allow for smoothening of the collagen layer top. One must ensure that the MatTek dish is leveled on the ice to avoid redistribution of the liquid collagen into tilted areas. A large plastic tissue culture dish can be used for supporting several MatTek dishes on ice. In this case, one must pre-chill this plastic dish on ice before placing MatTek dishes on it.

Polymerization of a thin fibrillar collagen layer that will withstand

centrifugation—It is extremely important to polymerize a thin, sufficiently uniform layer of fibrillar collagen on the glass surface of the MatTek dish that can be compacted by centrifugation into a solid and uniform layer of HDFC matrix. The success of the collagen polymerization for this protocol depends on establishing the proper timing required for assembly of the fibrillar collagen network during incubation in a 37°C incubator. This optimal incubation time should allow sufficient time for uniform polymerization of fibrillar collagen across the whole glass bottom while avoiding over-drying of the polymerized collagen layer. The optimal polymerization time interval will depend on the CO₂ level and

humidity, which influence the speed of collagen fibril assembly and the thickness of individual fibers. Therefore, because CO₂ and humidity settings can vary from incubator to incubator, the optimal polymerization time will need to be established for the specific incubator being used. Test several incubation intervals to determine the optimal incubation time. For this, start with 20 min collagen polymerization and increase the incubation time by 2 min intervals if needed. Examine the polymerized fibrillar collagen layer with phase contrast microscopy to ensure that a uniform fibrillar network has formed across the entire glass bottom of the dish (Figure 3, A). If clear areas of unpolymerized collagen are observed, return the MatTek dish to the incubator for an additional 2 min incubation, then reexamine the fibrillar collagen matrix by phase microscopy. If a uniform fibrillar collagen layer is observed, proceed with the centrifugation step. After the centrifugation is completed, examine the MatTek dish under the phase contrast microscope for uniformity of the HDFC matrix across the entire glass portion of the MatTek dish. Figure 3, B provides a representative image of the HDFC matrix obtained with phase microscopy. If areas of empty glass depleted of matrix or containing damaged HDFC matrix (Figure 3, C) are detected, one needs to go back to the incubation step and increase the incubation time an additional 2 min. However, if the incubation time is too long, the fibrillar collagen layer will dry. This problem can be observed by the naked eye: the collagen layer is not translucent, but instead becomes opaque and whitish. In addition, centrifugation of an overly dry 3D collagen layer yields HDFC matrix with “feather-like” tears (Figure 3, D), and salt crystal formation can be observed. In this case, one needs to decrease the incubation time.

Controls for immuno-fluorescence labeling—The immuno-staining with many different antibodies and anti-sera should yield appropriate immuno-staining patterns without artifacts or nonspecific background staining when using the simple cell fixation and permeabilization protocol provided in *Basic Protocol 2*. Nonetheless, it is recommended to check the binding specificity of the primary and secondary antibodies by performing the following controls: (1) immunostaining of HDFC samples with the secondary antibody alone to confirm the specificity of the secondary antibodies with low background fluorescence; (2) the use of GFP chimeras of the protein being studied to compare and confirm the pattern of endogenous protein localization in the cells on HDFC matrix.

Anticipated Results

Basic Protocol 1—Polymerization of a thin layer of high-concentration fibrillar collagen type I with its subsequent physical compaction by centrifugation will result in the formation of HDFC matrix.

Basic Protocol 2—Incubation of tumor cells on HDFC matrix will lead to induction of invadopodia and invadopodia-mediated HDFC degradation. Chemical crosslinking and fixation of the cells will produce a cell sample appropriate for immuno-fluorescence labeling of HDFC matrix degradation sites as well as invadopodial intracellular proteins required for their visualization by fluorescence confocal microscopy. The HDFC matrices can also be used for live cell imaging of invadopodia formation and function in HDFC matrix degradation with applications of time-lapse fluorescence confocal microscopy. For these

purposes, antibody to collagen cleavage sites needs to be added to the dish with live cells expressing fluorescently-tagged chimeras of invadopodial proteins.

Time Considerations

Basic Protocol 1—It takes about 30 min to prepare a stock of neutralized and reconstituted collagen solution required for the HDCE matrix polymerization, and 2 hours to coat MatTek dishes with HDCE matrix. The great convenience of the HDCE matrix is its prolonged shelf life when stored at 4°C under conditions that prevent drying (each MatTek dish sealed with Parafilm) and bacterial contamination (provided that aseptic techniques and sterile solutions are used).

Basic Protocol 2—It takes about 40 min to detach the cells from tissue culture dishes, to prepare the cell suspension, and to plate the cells on HDCE matrix. Three hours are needed for cell incubation on HDCE matrix in a 37°C tissue culture incubator, and an additional 30 min is required for cell fixation. Immuno-staining of cells and matrix requires about 3 hours. Thus, the whole sequence of steps, starting with cell plating and completing immuno-staining, takes 7 hours. It is highly recommended to start confocal imaging immediately after the immuno-labeling is complete, and it will require at least two hours for collecting imaging data. The imaging time can easily exceed 2 hours if confocal z-stacks are being imaged. Therefore, it is advised to split the protocol into two portions to be completed in two days. That is, one can schedule the cell incubation on HDCE matrix for the first day, and then stop the protocol after fixing the cells and washing them with PBS. The fixed samples can be stored at 4°C overnight. The next day, one can proceed with the steps of cell permeabilization, immuno-labeling, and confocal imaging.

ACKNOWLEDGEMENT

The project described was supported by Pathway to Independence Award K99CA129205 from NCI with ARRA award (V. Artym) and NIH/ NIDCR Intramural project DE 000719.

Abbreviations

2D	two-dimensional
3D	three-dimensional
PBS	phosphate-buffered saline
ECM	extracellular matrix
DMEM	Dulbecco's Modified Eagle Medium
HDCE	High-density fibrillar collagen

LITERATURE CITED

- Common stock solutions, buffers, and media. *Curr. Protoc. Cell Biol.* 2001; 00:2A, A.2A.1–A.2A.10. Appendix 2A.
- Artym VV, Yamada KM, Mueller SC. ECM degradation assays for analyzing local cell invasion. *Methods. Mol. Biol.* 2009; 522:211–9. [PubMed: 19247615]

- Arтым VV, Matsumoto K. Imaging cells in three-dimensional collagen matrix. *Curr. Protoc. Cell. Biol.* Unit 10. 2010; 18:1–20.
- Arтым VV, Swatkoski S, Matsumoto K, Campbell CB, Petrie RJ, Dimitriadis EK, Li X, Mueller SC, Bugge TH, Gucsek M, Yamada KM. Dense fibrillar collagen is a potent inducer of invadopodia via a specific signaling network. *J. Cell. Biol.* 2015; 208(3):331–50. [PubMed: 25646088]
- Campbell CB, Cukierman E, Arтым VV. 3-D Extracellular Matrix from Sectioned Human Tissues. *Curr. Protoc. Cell. Biol.* Unit 19. 2014; 16:1–20.
- Conklin M, Keely P. Why the stroma matters in breast cancer: Insights into breast cancer patient outcomes through the examination of stromal biomarkers. *Cell. Adh. Migr.* 2012; 6(3):249–60. [PubMed: 22568982]
- Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, White JG, Keely PJ. Collagen density promotes mammary tumor initiation and progression. *BMC. Med.* 2008; 6:11. [PubMed: 18442412]
- Weaver AM, Page JM, Guelcher SA, Parekh A. Synthetic and tissue-derived models for studying rigidity effects on invadopodia activity. *Methods. Mol. Biol.* 2013; 1046:171–89. [PubMed: 23868588]

Figure 1. High-density fibrillar collagen (HDFC) matrix mimics tumor desmoplastic collagen. (A) X-Y view of HDFC matrix presented as a maximum-intensity projection of a confocal Z-stack of HDFC matrix. Collagen labeled with Alexa Fluor 568F fluorescent dye was polymerized and centrifuged to generate HDFC matrix and examined with confocal fluorescence microscopy. Images of the Z-stack were filtered with a low pass filter before rendering the maximum-intensity projection using MetaMorph software (Molecular Devices). Scale bar = 10 μ m. (B) X-Z view of HDFC matrix labeled with Alexa Fluor 568F fluorescent dye. Fluorescence confocal z-stack of HDFC matrix was deconvolved using a blind deconvolution algorithm from AutoDeblur software (Media Cybernetics). The deconvolution algorithm was set to 10 iterations. Scale bar = 10 μ m. (C) Desmoplastic collagen deposition visualized with Masson's trichrome stain in blue. Slices of human breast tumor tissue were chemically treated to extract cells and retain the extracellular matrix (for the protocol, see Campbell et al., 2014). Scale bar = 300 μ m. (D) Confocal fluorescence microscopy of extracted human breast tumor matrix immuno-stained for collagen. Scale bar = 10 μ m.

Figure 2.

Custom MatTek dish holder for the centrifugation step in the HDFC matrix preparation protocol. (A) Custom holder in closed configuration: two covers of Corning Costar six-well cell culture plates attached one to another with a strip of Time tape. (B) Custom holder in opened configuration. (C) Opened custom holder loaded with a MatTek dish. (D) Closed custom holder with sandwiched MatTek dish: a second strip of Time tape is used to secure one or more MatTek dishes between the two Corning six-well plate covers.

Figure 3.

Phase contrast microscopy of fibrillar collagen matrices. (A) A thin 3D layer of polymerized fibrillar collagen coated on the glass bottom of a MatTek dish. (B) A layer of HDFC matrix obtained by compacting thin 3D fibrillar collagen with centrifugation. (C) An area of HDFC matrix damaged by centrifugation as a result of incomplete polymerization of thin 3D fibrillar collagen. (D) An area of HDFC matrix damaged during centrifugation as a result of fibrillar collagen drying and crystal formation. Scale bar = 50 μm .

Figure 4.

HDFC matrix is a novel *in vitro* model to study invadopodia. Fluorescence confocal microscopy of MDA-MB-231 breast carcinoma cells incubated on HDFC matrix for 3 hours. Invadopodia are yellow dots of co-localized anti-cortactin (red) and anti-actin (green) fluorescent staining. Individual invadopodia are shown in the insets. Collagen cleaved by invadopodia is visualized with anti-collagen cleavage site antibody (right-hand panel). Fluorescence confocal images were processed with a low pass filter using MetaMorph software. Scale bar = 10 μ m.