Video Article

Fully Human Tumor-based Matrix in Three-dimensional Spheroid Invasion Assay

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Abstract

Two-dimensional cell culture-based assays are commonly used in in vitro cancer research. However, they lack several basic elements that form the tumor microenvironment. To obtain more reliable in vitro results, several three-dimensional (3D) cell culture assays have been introduced. These assays allow cancer cells to interact with the extracellular matrix. This interaction affects cell behavior, such as proliferation and invasion, as well as cell morphology. Additionally, this interaction could induce or suppress the expression of several pro- and anti-tumorigenic molecules. Spheroid invasion assay was developed to provide a suitable 3D in vitro method to study cancer cell invasion. Currently, animal-derived matrices, such as mouse sarcoma-derived matrix (MSDM) and rat tail type I collagen, are mainly used in the spheroid invasion assays. Taking into consideration the differences between the human tumor microenvironment and animal-derived matrices, a human myoma-derived matrix (HMDM) was developed from benign uterus leiomyoma tissue. It has been shown that HMDM induces migration and invasion of carcinoma cells better than MSDM. This protocol provided a simple, reproducible, and reliable 3D human tumor-based spheroid invasion assay using the HMDM/fibrin matrix. It also includes detailed instructions on imaging and analysis. The spheroids grow in a U-shaped ultra-low attachment plate within the HMDM/fibrin matrix and invade through it. The invasion is daily imaged, measured, and analyzed using ilastik and Fiji ImageJ software. The assay platform was demonstrated using human laryngeal primary and metastatic squamous cell carcinoma cell lines. However, the protocol is suitable also for other solid cancer cell lines.

Video Link

The video component of this article can be found at https://www.jove.com/video/59567/

Introduction

Conventional two-dimensional (2D) cell culture studies have considerably contributed to cancer research. Currently, researchers are shifting more towards three-dimensional (3D) cell culture assays to better mimic the in vivo conditions[1]. The 3D cancer cell culture more accurately reflects the complex tumor microenvironment in terms of cell-cell and cell-matrix interactions, gene expression profiles, drug sensitivity, and signaling pathway activity[2,3].

Several 3D cell culture models are used in cancer research such as tumor tissue explant, tumor on a chip, and multicellular tumor spheroids[3,4]. Multicellular tumor spheroids are now widely used, as they mimic several features of the in vivo conditions in human tumors[1,5]. When the spheroid diameter is greater than 500 µm, it even has hypoxic regions and a necrotic center, representing thus the in vivo tumor situation[2].

Many synthetic (e.g., polydimethylsiloxane) and animal-derived (e.g., rat tail type I collagen and mouse sarcoma-derived matrix, Matrigel, referred to as MSDM) matrices have been developed for 3D cell culture assays[3,6,7,8]. Thus far, none of the commercially available matrices have originated from human tumor tissue. Therefore, they lack the features of the human tumor microenvironment, which has significant effects on cancer cell invasion processes[8].

Myogel (human myoma-derived matrix, referred to as HMDM) is extracted from human uterus leiomyoma tumor tissue[9]. It has been shown that the protein content of HMDM differs significantly from that of MSDM. In fact, 66% of HMDM proteins are different from MSDM proteins. On the other hand, some proteins, such as laminin, type IV collagen, heparan sulfate proteoglycans, nidogen, and epidermal growth factor, are present in both matrices[10]. Additionally, the mouse differs from the human in enzyme contents, with humans having 78 fewer proteases than mice[11].

Fibrin has been widely used alone or in combination with other materials as a scaffold material[12]. In 3D cell culture assays, commercially available human fibrinogen and thrombin are combined to form a fibrin hydrogel[12].

This protocol describes an improvement of the previously introduced 3D tumor spheroid invasion assay[7].This new protocol applies human tumor-derived matrix instead of mouse-derived tumor matrix. It also involves imaging and analysis techniques using ilastik and Fiji ImageJ software. This protocol could be used for spheroid assay of several different solid cancer cell lines. It offers a biologically relevant tool to develop novel anti-cancer therapies and to study the effects of specific molecules on cancer cell invasion.

Protocol

1. Generation of Multicellular Tumor Spheroids

NOTE: The protocol is demonstrated here with UT-SCC-42A and -42B cell lines but it could also be applied using other cell lines.

1. Wash the UT-SCC-42A and -42B cells with 6 mL of phosphate-buffered saline (PBS), add 0.05% trypsin-EDTA (3 mL for 75 cm^2 flask), and place the flask in a cell culture incubator (37 °C, 5% CO_2, 95% humidity) for 2-5 min.
2. Check that the cells have detached under a microscope. Then add complete Dulbecco's modified Eagle's medium (DMEM) media (DMEM + 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 250 ng/mL amphotericin B, 0.4 µg/mL hydrocortisone, and 50 µg/mL ascorbic acid) to neutralize the enzyme (6 mL for 75 cm^2 flask) and transfer the cell suspension into a 15 mL conical tube.
   NOTE: Select the cell culture medium that suits the cells being studied.
3. Centrifuge the cell suspension at 200 x *g* for 5 min.
4. Remove the supernatant and suspend the cell pellet in 2-5 mL of complete DMEM.
5. Count the cells and dilute the cell suspension with complete DMEM to a final concentration of 20,000 cells/mL.
   NOTE: Optimal cell count must be determined for each cell line.
6. Dispense 50 µL of the cell suspension into each ultra-low attachment 96-well round bottom plate well for a final concentration of 1,000 cells per well.
7. Transfer the plate to the cell culture incubator (37 °C, 5% CO_2, 95% humidity). Four days later, visually confirm tumor spheroid formation with an inverted microscope and proceed with the assay. Ensure that there is only one spheroid per well.
   NOTE: The time taken to form a spheroid varies between different cell lines.

2. Three-dimensional Spheroid Invasion Assay

NOTE: Prepare 2x solution because the gel will be diluted 1:1 when added into the wells.

1. Thaw HMDM on ice and fibrinogen stock solution in a water bath maintained at 37 °C. Do not disturb the fibrinogen until it is completely solubilized and do not put the solution on ice; precipitation will occur.
2. Mix together the appropriate volume of each reagent: 1 mg/mL HMDM (final concentration: 0.5 mg/mL), 0.6 U/mL thrombin (final concentration: 0.3 U/mL), 66.6 mg/mL aprotinin (final concentration: 33.3 ug/mL), and 1 mg/mL fibrinogen (final concentration: 0.5 mg/mL).
   NOTE: Add fibrinogen just before dispensing the mixture into the wells and work quickly; it will form a gel in a few minutes. Treat just a few wells at a time.
3. Add 50 µL of the gel into each well. Direct the tip towards the inside wall of the well and pipet slowly. Avoid air bubbles (using the reverse pipetting technique) and try not to move the spheroid from the center of the well.
4. Return the plate back to the cell culture incubator and allow the HMDM/fibrin matrix to solidify for 30 min, and gently add 100 µL of complete DMEM into each well on top of the gel.

3. Imaging

1. Image the spheroids daily using an inverted light microscope. Alternatively, use automatic imaging systems.

4. Image Segmentation with Ilastik

1. Open ilastik and select **Pixel Classification** workflow (**Figure 1A**). It classifies the pixels based on annotations made by the user. Save ilastik project (.ilp) to computer.
2. Add images for analyses. Click **Input Data** and **Add New** and then choose images (**Figure 1B**).
3. **For feature selection, click Feature Selection and Select Features (Figure 1C, red rectangle).**
   NOTE: The selected features should roughly correspond to the visual properties that separate the objects from the background, and they will be used for training the classifier.
   1. Select features by clicking the boxes. The selected boxes will turn green (**Figure 1C**, blue rectangle).
      NOTE: Here the user can select from several different feature types and scales. Color/Intensity should be selected to separate objects based on color or brightness. Edge should be selected to separate objects based on brightness or color gradients. Texture is an important feature if the objects in the image have a special textural appearance. For this assay, Color/Intensity (sigma 0) and Edge (sigma 6) are used.
4. **For training, click Training and in the Training section there are two labels: Label 1 and Label 2 (Figure 1D, red rectangle). If there is only one label, add a new label by pressing Add label (Figure 1D, blue rectangle).**
   1. Mark the background with one of the labels (**Figure 1D**, yellow) and the cells with the other label (**Figure 1D**, blue).
   2. Train the software for the first 10% of the images. Choose next image from **Current View**.

3. After the cells and the background are marked (of the first image), press **Live Update** (**Figure 1D**, purple rectangle).
   NOTE: With the next image, ilastik will automatically perform the analysis according to the previous images.

5. After training is done and ilastik has analyzed all images, click **Prediction Export**. Choose **Simple Segmentation** from **Source**; Do not choose **Probabilities** or anything else (**Figure 1E**).
6. Choose the desired output file format from **Choose Export Image Settings…** in the Prediction Export section (**Figure 1E**). Export the results of the labeling by clicking **Export All** (**Figure 1F**). The files will be exported to the same folder with the original images.
   NOTE: In this experiment, .h5 format is used.

5. Area Analysis with Fiji ImageJ

1. **Scale setting**
   1. Open the original image with a scale bar in Fiji ImageJ (**Figure 2A**). Ensure that the image has the same size and dimension as the analyzed images. Use the Line Selection tool to draw a line of a known length (**Figure 2B**).
      NOTE: This protocol is executed by using Fiji ImageJ 1.51 (64-bit) and ilastik -1.3.2rc.
   2. Click **Analyze** and **Set Scale** (**Figure 2C**). Set the known distance in the **Known Distance** field and set the proper unit and click **Global** (**Figure 2D**).

2. **Installing the macro**
   1. If a plugin **ilastik** is not installed, follow these steps: click **Help**, **Update…**. On the open window **ImageJ Updater**, click **Manage Update Sites**. On the open window **Manage Update Sites**, click **next to ilastik** and then click **Close**. Then click **Apply Changes** in the window **ImageJ Updater**. This process can take some time. The next window will be **Information** with the text **Updated Successfully**. Click **OK** and restart ImageJ.
   2. Click **Plugins**, **Macros**, and **Install** (**Figure 3A**). Then choose the counter.ijm file (see **Supplemental Information**) and click **Open**.
      NOTE: The macro was written especially for measuring the area. The macro should be installed every time the software is opened.

3. **Area analysis**
   1. Analyze images when all plugins are installed. Click **Plugins** and scroll down to **ilastik** (**Figure 3C**). Choose **Import HDF5**, choose the file with .h5 format, click **Open** and **Load and apply LUT** (**Figure 3C,D**).
   2. Press the **a** button from the keyboard (macro) and the area will appear in the **Summary** window as **Total Area** (**Figure 3E**).

Representative Results

Four days after UT-SCC-42B (metastatic laryngeal SCC cell line) cell seeding, the matrix (HMDM/fibrin, MSDM, or collagen) was added on the formed spheroids (day 0) and the invasion was monitored for three days. Images here were obtained daily using an inverted microscope (**Figure 4**).

Once embedded, cells in the HMDM/fibrin matrix started to invade rapidly after one day and extended into the matrix (**Figure 4A**). Cells in the MSDM did not invade into the surrounding matrix, instead forming an asymmetrical structure (**Figure 4B**). On the other hand, cells in the collagen invaded slightly, but due to the matrix shrinkage the analysis was difficult (**Figure 4C**). In some collagen wells, the spheroids even disappeared after three days (**Figure 4C**).

Figure 5 illustrates the quantification of the cell invasion in HMDM/fibrin of the primary laryngeal SCC cell line UT-SCC-42A and the corresponding metastatic cell line UT-SCC-42B. The video (**Movie 1**) taken using a live-cell analysis system of the HMDM/fibrin spheroid shows the movement of the SCC cells within the matrix where they form strands followed by the other cells.

Figure 1: Image segmentation. Selected steps of image segmentation using ilastik. Please click here to view a larger version of this figure.

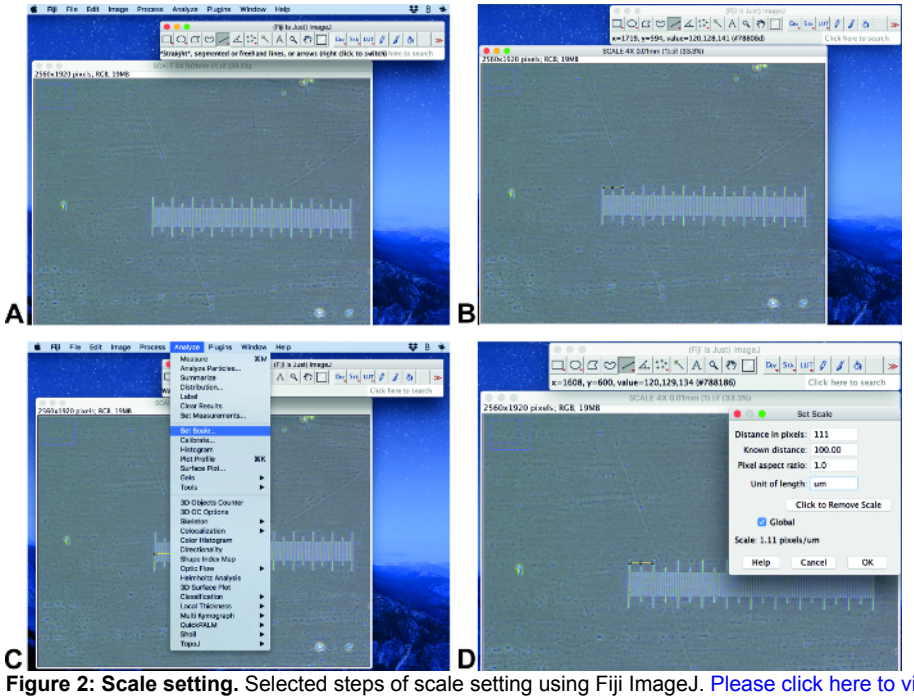


Figure 2: Scale setting. Selected steps of scale setting using Fiji ImageJ. Please click here to view a larger version of this figure.

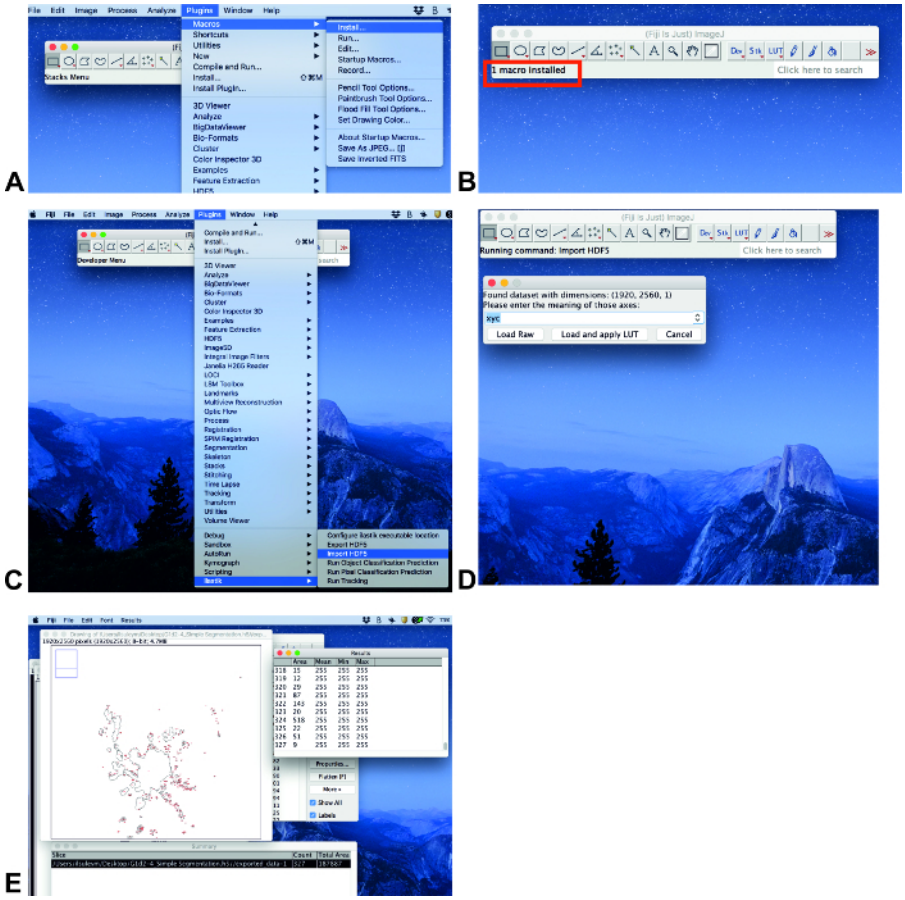


Figure 3: Invasion analysis using ImageJ. Selected steps of image analysis using Fiji ImageJ. Please click here to view a larger version of this figure.

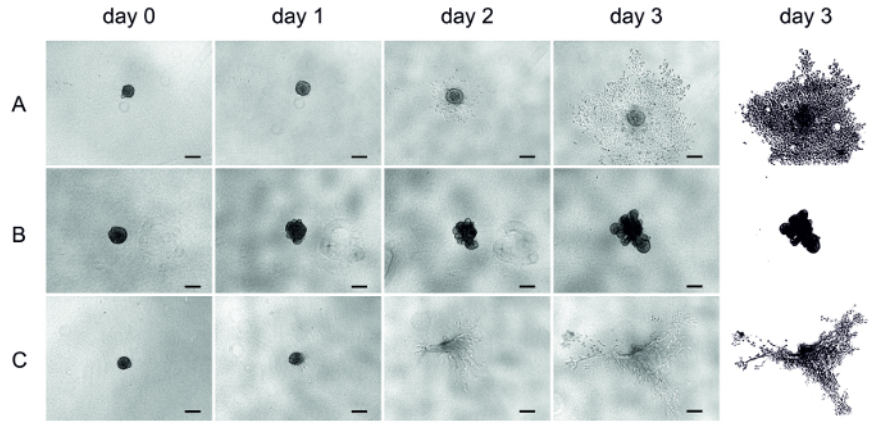


Figure 4: UT-SCC-42B cell invasion in three different matrices. Representative images of SCC cell invasion in the 3D spheroids. Invasion was followed for three days and images were taken daily using an inverted microscope. (**A**) HMDM/fibrin, (**B**) MSDM, (**C**) collagen. The last column represents clarification of day 3 invasion. Scale bar = 200 µm. Please click here to view a larger version of this figure.

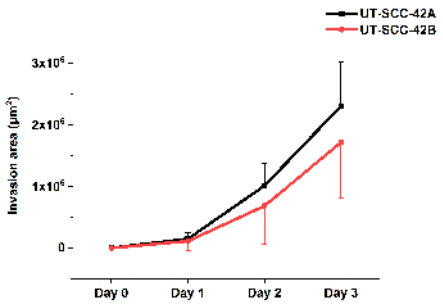
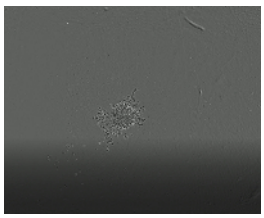


Figure 5: Quantification of SCC cell invasion in HMDM/fibrin. Primary laryngeal UT-SCC-42A and corresponding metastatic laryngeal UT-SCC-42B SCC cell line invasion was analyzed using ilastik and Fiji ImageJ software. Results represent mean ± standard deviation of three independent experiments, each in triplicate. Please click here to view a larger version of this figure.



Video 1: SCC cell movement within the HMDM/fibrin for three days. Please click here to view this video. (Right-click to download.)

Discussion

The presented method provides a 3D human tumor-based assay to evaluate the invasiveness of cancer cells within human tumor microenvironment mimicking matrix. Cancer cell invasion can be easily measured by daily imaging with a standard microscope and by using image analysis software. The method demonstrates cell invasion rather than merely proliferation.

Unlike MSDM, the HMDM/fibrin matrix does not require any temperature control. This method is easy to perform, in particular without the need to transfer the spheroids from one plate to another. It has only one technically sensitive step, the addition of fibrinogen to the matrix. Since fibrinogen starts to gel after a few minutes, adding fibrinogen requires robust pipetting and treatment of only a few wells at a time. There is also a risk of disturbing the spheroid and moving it from its central position in the U-shaped well if pipetting is done in a high-pressure manner. Dislocation of the spheroid can complicate imaging and eventually the analysis.

The assay can be modified by altering the concentration of HMDM or fibrinogen. The cells can also be fixed and stained for subsequent molecular studies. Even though this method can be modified to a fully automated form, it can also be carried out with a normal microscope and two open source software, with no need for costly equipment.

Compared to the traditional MSDM-based 3D assays, this assay could display better the cell invasion properties. Spheroids grown in a basement membrane-containing laminin-rich matrix, such as MSDM, might have more proliferation of cancer cells than actual invasion, rendering it unsuitable for invasion assays in several cancer cell lines due to their low invading capacity. Another frequently used matrix, type I collagen, tends to shrink from the edges during 3D cultivation, which affects spheroid localization and imaging of the wells. Additionally, the differences between the intrinsic invasive properties of the more (HSC-3) and less (SCC-25) aggressive tongue carcinoma cell lines have been demonstrated using human tumor microenvironment mimicking models (myoma discs and its soluble form matrix)[10,13].

The assay is also more ethical than using MSDM since HMDM is extracted from the leftover material of human leiomyoma tumor. Currently, HMDM is the only available human tumor-derived ECM product that appears to be suitable for many cancer-related in vitro assays[8,10]. In the future, animal tissue-derived matrices could be replaced by human tumor-based matrices to reduce the need to sacrifice animals for matrix production.

Replacing 2D cell culture assays with 3D methods provides more accurate information on cancer cell behavior. Currently, there are several 3D models and commercial matrices, but, unfortunately, none are suitable for all cancer cell lines. Researchers should select the most suitable matrix for their particular assay. Optimal matching of assay and matrix can be challenging, but it could significantly increase the reliability of the results.

Disclosures

The authors have nothing to disclose.

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