Video Article

Quantification of Breast Cancer Cell Invasiveness Using a Three-dimensional (3D) Model

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Abstract

It is now well known that the cellular and tissue microenvironment are critical regulators influencing tumor initiation and progression. Moreover, the extracellular matrix (ECM) has been demonstrated to be a critical regulator of cell behavior in culture and homeostasis *in vivo*. The current approach of culturing cells on two-dimensional (2D), plastic surfaces results in the disturbance and loss of complex interactions between cells and their microenvironment. Through the use of three-dimensional (3D) culture assays, the conditions for cell-microenvironment interaction are established resembling the *in vivo* microenvironment. This article provides a detailed methodology to grow breast cancer cells in a 3D basement membrane protein matrix, exemplifying the potential of 3D culture in the assessment of cell invasion into the surrounding environment. In addition, we discuss how these 3D assays have the potential to examine the loss of signaling molecules that regulate epithelial morphology by immunostaining procedures. These studies aid to identify important mechanistic details into the processes regulating invasion, required for the spread of breast cancer.

Video Link

The video component of this article can be found at https://www.jove.com/video/51341/

Introduction

Migration and invasion of individual or collective cells are two hallmarks of cancer, and required for the metastatic spread of cancer cells[1-4]. The ability of cancers cells to initiate metastasis depends on their capability to migrate and invade into the neighboring tissue using invadopodia to degrade the basement membrane of the cells. Invadopodia are dynamic actin-rich matrix degradation protrusions that enable degradation of the extracellular matrix through the release of matrix-degrading proteases[5]. Cancer cell invasion involves the degradation of the matrix followed by the migration of the cancer cells and this is accompanied by a reorganization of the three-dimensional (3D) matrix environment[2]. Thus, to penetrate through the matrix, a cell must transform its shape and interact with the extracellular matrix (ECM)[2].

The maintenance of breast tissue integrity depends on tightly controlled tissue architecture since cell–ECM and cell–cell adhesion junctions influence gene expression and disruption of epithelial polarity can lead to the onset of cancer[6-10]. However, most *in vitro* migration and invasion assays such as transwell chamber assays or wound-scratch assays are two-dimensional (2D) and hence these neglect the intricate interactions between cells and their adjacent environment[3,6,8,11-14]. Considerable morphological and functional diversities including variations in cellular morphology, cellular differentiation, cell-matrix adhesions and gene expression patterns have been detected by culturing cells in 3D cultures that are commonly lacking in 2D assays[2,6,8,11]. Thus, uses of 3D assays are significantly beneficial in recapitulating a more physiological *in vivo* condition, leading to a better translation of ground-breaking findings in basic research to the clinic[6-10]. However, it should be noted, despite the many advantages gained with the use of 3D cultures, this model cannot capture all of the complexities of the *in vivo* tumor microenvironment that includes various cell types. However, it is possible to incorporate stromal cells into the 3D models (for example, fibroblasts, leukocytes, and macrophages) to study the effect of tumor-stromal interactions on cancer cell adhesion and invasion[15-17].

Breast epithelial cells in culture grow most effectively when ECM proteins such as laminin and collagen are present. With this known, a commercially available matrix mixture has been derived from Engelbreth-Holm-Swarm (EHS) murine tumor and is known as Matrigel basement membrane matrix[2,8]. A number of techniques have been established to grow epithelial cells as 3D colonies in basement membrane matrix[2,8]. The 3D basement membrane matrix model is effective for establishing both malignant and non-malignant breast cell growth, resembling what is occurring in the *in vivo* environment[18,19]. MCF10A cells are non-malignant mammary epithelial cells. When grown in basement membrane matrix, these cells exhibit *in vivo* traits of normal breast cells and undergo controlled cell proliferation, cell polarization, and apoptosis to establish the lumen space[8,12,20]. Furthermore, the appearance of cell nuclei of MCF10A cells forming acini in 3D cultures more closely resemble those of

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mammary epithelial cells in tissue than those cultured in monolayer[21]. Studies by Bissell and colleagues were the first to reveal that malignant breast cells can be differentiated from non-malignant breast cells when grown in a laminin-rich surroundings, since the malignant cells display a highly disorganized phenotype, increased proliferation, decreased cell-to-cell adhesion, increased expression of mesenchymal markers and an increase in the number of invasive structure formed[3,6,22].

Abnormalities of the cell environment can influence tumor formation[20]. The 3D culture method can be used to effectively study the communication that occurs between the tumor cells and their surrounding environment and determine how protein expression influences such communication[14,20,21,23]. This article provides a detailed methodology to grow MDA-MB-231 breast cancer cells in 3D cultures to analyze invasiveness, and to study the loss of epithelial morphology using an epithelial marker laminin, a component of the cell basement membrane[18,19,24,25]. The detailed procedures provide the ability to accurately and reproducibly quantify stellate (invasive) structure formation by any invasive cancer cell and is not limiting to the common breast cancer cell lines (such as MDA-MB-231, Hs578T, MCF-7, or T47D). Thus, this assay can serve as a platform for evaluating how protein expression in cells or treatment with pro- or anti-invasive compounds regulate extracellular matrix degradation, by single or multiple cells.

Protocol

1. Three-dimensional Culture of Breast Cancer Cells in Basement Membrane Matrix (The Embedment Technique)

1. Handling Matrigel basement membrane matrix: Thaw on ice overnight at 4 °C. Basement membrane matrix is liquid at low temperature but solidifies at room temperature. Keep basement membrane matrix on ice (**Figures 1A-B**).
2. Cover the Confocal No.1 glass-bottom dish with 50 µl of basement membrane matrix by means of spreading the matrix using the tip of a P-200 Pipetman in a spiral pattern (**Figure 1C**). Use caution when spreading the basement membrane matrix to avoid the formation of air bubbles. Likewise, avoid spreading the matrix to close to the borders of the glass-bottom dish to prevent meniscus formation. If inexperienced handling basement membrane matrix, then precooled the dish, P-200 Pipetman and pipettes can be on ice (alternatively left overnight in a fridge at 4 °C). This step offers additional time to spread the basement membrane matrix before solidification. Place the dish(es) in a cell culture incubator (at 37 °C with 5% CO_2) for at least 30 min to enable the basement membrane matrix to solidify (**Figure 1D**).
3. While the matrix is undergoing solidification, trypsinize a 70-80% confluent 100-mm plate of cells. Once the cells have begun lifting off of the plate, resuspend them in 10 ml of RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS), to inactivate the trypsin (**Figure 1E**). Then transfer the resuspended cells into a 15 ml conical tube (**Figure 1F**).
4. Spin the cells (present in conical tube) at 100 x g for 3 min in a dedicated cell culture centrifuge (**Figures 1G-H**).
5. While the cells are being spun down, aliquot 50 µl of matrix into an 1 ml microcentrifuge tube (note: each glass-bottomed dish is allocated one microcentrifuge tube; hence, if the experiment requires 3 dishes, then it follows that 3 microcentrifuge tubes are necessary as well), and then place the tube on ice (**Figure 1B**).
6. Aspirate the medium from the conical tube from step 1.4, whilst leaving the pellet undisturbed (**Figure 1I**). Resuspend cells (that have been spun down) in 1 ml of FBS-supplemented RPMI medium (**Figure 1J**).
7. Once the cells are counted using a hemocytometer (**Figure 1K**), or a cell particle counter aliquot 2.5 x 10^4 cells into the microcentrifuge tube and top it off using appropriate media so as to obtain total volume of 50 µl (**Figure 1L**).
8. Mix the cells from step 1.7 (25,000 cells in 50 µl) with the matrix-containing microcentrifuge tube from step 1.5 in a 1:1 ratio; final volume will be 100 µl (**Figure 1M**).
9. Gently plate 100 µl of the matrix:cell mixture from step 1.8 onto the solidified basement membrane matrix-coated dish from step 1.2 (**Figure 1N**). This allows for cells to be embedded in basement membrane matrix.
10. Transfer the dish(es) into the cell culture incubator (at 37 °C with 5% CO_2) and allow the matrix:cell mixture to solidify for at least 30 min.
11. Once the matrix:cell mixture is solidified, add 2 ml of FBS-supplemented RPMI media to the dish (**Figure 1O**) and take the dish back the incubator where it will be stored for the remainder of the experiment (**Figure 1P**).
12. Change media every day for a period of 5 days (or as per required for an assay; the duration of the assay for MDA-MB-231 cells is 5 days in length).
13. Using a light microscope, take differential interference contrast (DIC) images of the MDA-MB-231 colonies suspended in basement membrane matrix (**Figure 3A**). Image 20 representative areas at 10X objective once a day for five days to determine colony morphogenesis (**Figure 3C**).
14. Analyze images *blindly* to determine cell colony stellate formation (**Figure 3B**). A colony is deemed to be stellate if one or more projections from the spheroid of cells are perceived. To determine the percentage of invasive stellate colonies, divide the number of stellate colonies by the total number of cell colonies per acquired image, and then average the stellate colonies percentages of the 20 images for each day.

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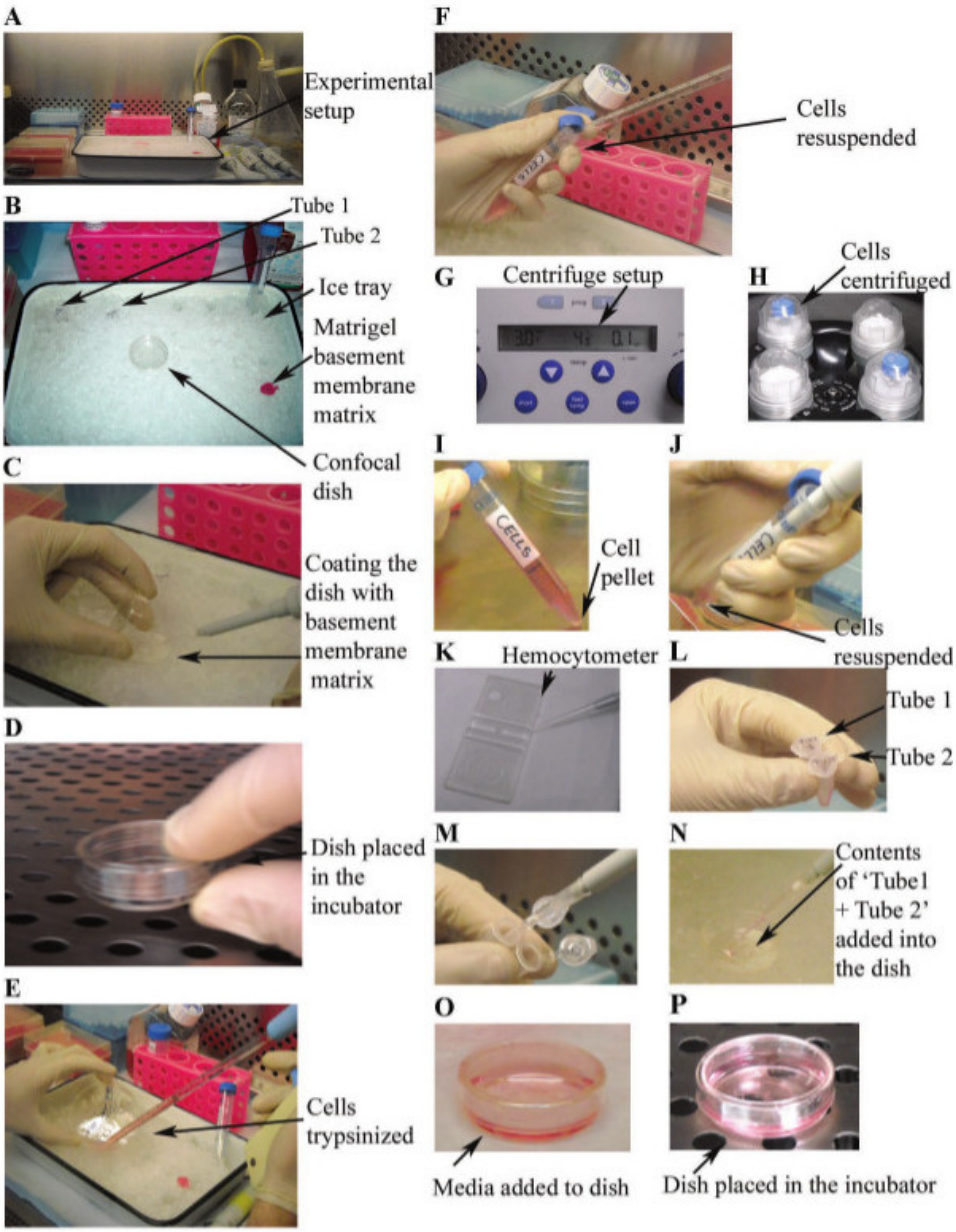

Figure 1. Three-dimensional culture of MDA-MB-231 breast cancer cells in basement membrane matrix (the embedment technique). A-B) A schematic of the experimental setup (performed in the fume hood). **C)** The well of the glass-bottomed dish coated with 50 µl of basement membrane matrix. **D)** Dish(es) placed in a cell culture incubator (at 37 °C with 5% CO_2) to permit the matrix to solidify for at least 30 min. **E)** Cells were trypsinized. **F)** Cells were resuspended into a 15 ml conical tube. **G-H)** Cells (present in conical tube) were spun down at 100 x g for 3 min in a tissue culture centrifuge. **I)** Cell pellet. **J)** Cells (that have been spun down) were resuspended in 1 ml of FBS-supplemented RPMI medium. **K)** Cells were counted using a hemocytometer. **L)** 2.5 x 10^4 cells aliquoted into the microcentrifuge tube and topped off using appropriate media so as to obtain total volume of 50 µl. **M)** Mix cells from step 1.7 (25,000 cells in 50 µl) with the matrix-containing microcentrifuge tube from step 1.5 in a 1:1 ratio; final volume will be 100 µl. **N)** Gently plate 100 µl of the matrix: cell mixture from step 8 onto the solidified matrix-coated dish from step 1.2. **O)** Once the matrix:cell mixture is solidified, add 2 ml of FBS-supplemented RPMI media to the dish. **P)** Place the dish in the incubator where it will be stored for the remainder of the experiment.

2. Examination of Morphogenic Features of 3D Cultures with Immunofluorescence

1. Set up an ice tray, cold phosphate buffered solution (PBS), 20% acetone: 80% methanol (fixative solution), and 3% bovine serum albumin (BSA; blocking solution) (**Figure 2A**) before taking out the dish(es) from the incubator.
2. Place the dish(es) on the ice tray and aspirate media, then wash 3x with 2 ml of cold PBS (**Figure 2B**).
3. Once the last PBS wash is aspirated, add 2 ml of 20% acetone: 80% methanol solution into the dish(es) (**Figure 2C**) to fix the cells for 20 min at 4 °C (either on ice, or in a refrigerator) (**Figure 2D**).
4. Once the 20 min of fixation is terminated, bring the dishes back at room temperature, aspirate the fixative, and subsequently wash 3x with PBS. Once the final PBS wash is aspirated, add 2 ml of 3% BSA to the dish(es) to block for at least 30 min at room temperature (**Figure 2E**).

5. During the blocking period prepare dilutions of primary (laminin V 1:100) and secondary antibodies (at appropriate dilution) dissolved in 3% bovine serum albumin (BSA) blocking buffer. Please note that 400-500 µl the 'BSA + primary antibody' solution should be added directly onto the matrix: cell mixture.
6. Once the 30 min of blocking have expired, add the primary antibodies and incubate for at least 1 hr at room temperature (**Figure 2F**). Please note that no PBS washes should be performed following the 30 min blocking period.
7. Upon completion of step 2.6, remove the 'BSA + primary antibody' solution, and wash 3x with PBS.
8. Add the secondary antibody dissolved in 3% BSA (at appropriate dilution) directly onto the matrix: cell mixture, cover the dishes and incubate for 1 hr at room temperature (**Figure 2G**).
9. Remove the 'BSA + secondary antibody' solution, and wash 3x with PBS.
10. Add 2 ml of Hoechst 33258 (1:10,000) dissolved in PBS for nuclei staining, and incubate for 5 min under aluminum foil (or cover with an opaque container to limit photobleaching) (**Figure 2H**).
11. Once the 5 min of incubation with Hoechst 33258t have expired, wash the dish(es) 5x with PBS.
12. Add mounting medium directly onto the matrix: cell mixture in the confocal dish and cover with glass coverslip carefully to prevent inducing any disruptions to the integrity of the colonies in the basement membrane matrix:cell mixture (**Figure 2I**).
13. Allow the dish(es) to dry overnight (or 24 hr) at room temperature (**Figure 2I**). Once dry, the dish(es) can be stored at -20 °C, but it is highly recommended that they should be imaged as quickly as possible.
14. Acquire images using a fluorescent microscope with appropriate laser wavelengths of antibodies used (**Figures 3D-E**).

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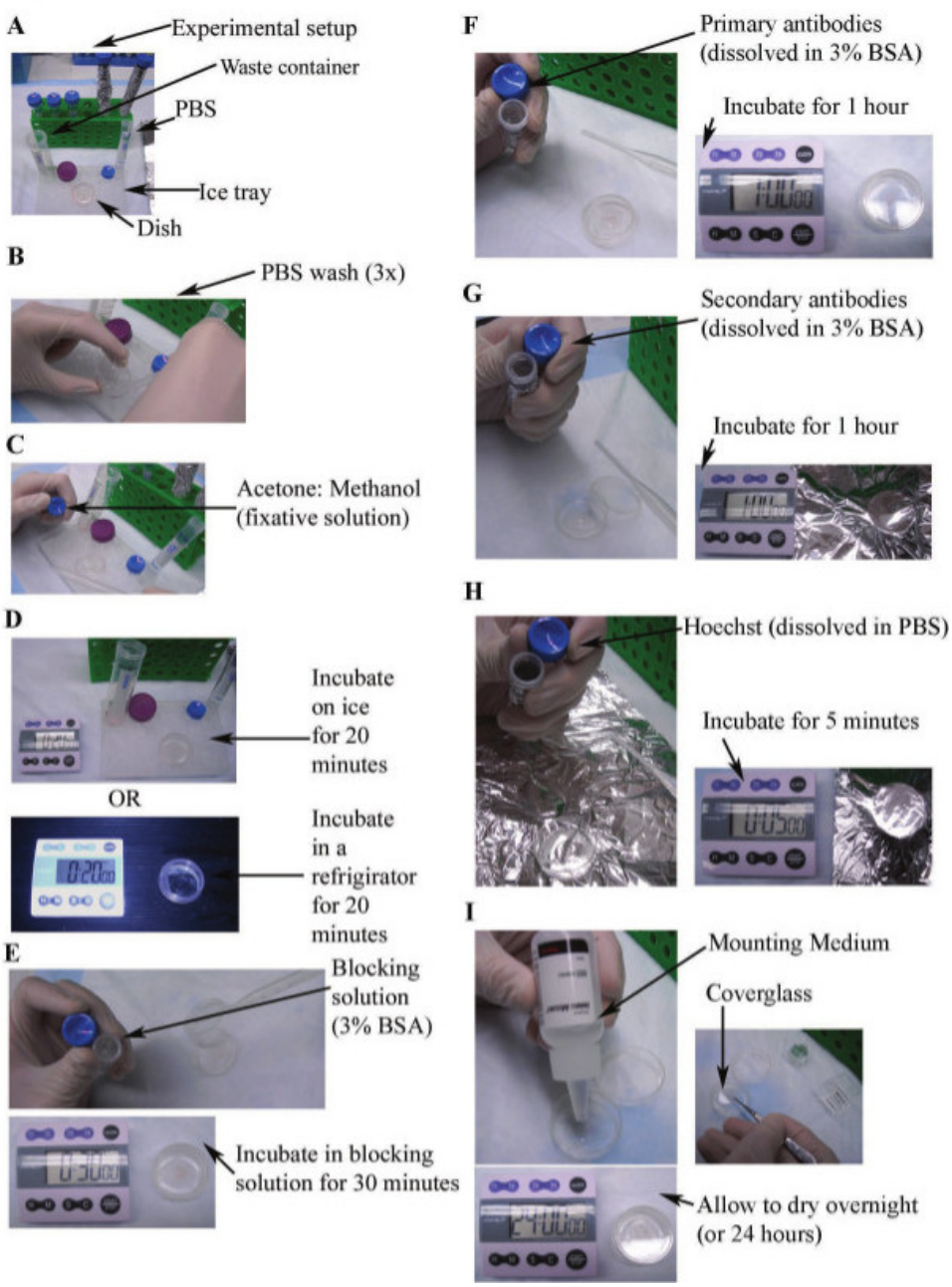

Figure 2. Examination of 3D cultures by immunofluorescence. A) A schematic of the experimental setup. **B)** Dish(es) placed on the ice tray and media aspirated; subsequently cells were washed three times with 2 ml of cold PBS. **C)** 2 ml of 20% acetone: 80% methanol solution added into the dish(es). **D)** Fix the cells for 20 min at 4 °C (either on ice, or in a refrigerator). **E)** 2 ml of 3% BSA added to the dish(es) to block for at least 30 min at room temperature. **F)** Once the 30 min of blocking have expired, add the primary antibodies and incubate for at least 1 hr at room temperature. **G)** Secondary antibodies dissolved in 3% BSA (at appropriate dilution) were added directly onto the basement membrane matrix:cell mixture; subsequently dishes covered and incubated for 1 hr at room temperature. **H)** 2 ml of Hoechst (1:10,000; dissolved in PBS) added to the dishe(es) and cells incubated for 5 min under aluminum foil. **I)** Mounting medium added directly onto the matrix: cell mixture in the confocal dish and the dish is covered with glass coverslip. Dish(es) left to dry overnight (or 24 hr) at room temperature.

Representative Results

An example of the MDA-MB-231 cells invading in 3D matrix is illustrated in **Figure 3C**. The cells are embedded in matrix (Day 1), and start forming invasive (stellate) structures by Day 3, and completely invade into the matrix by Day 5 (**Figure 3C**). The number of stellate colonies formed are counted, and expressed as a percentage of total number of colonies per dish (invasive and non-invasive). Additionally, since measurements are done daily for the five days, the rate of invasion can also be evaluated.

Establishing a timeline of morphogenetic events provides a basis to test numerous parameters in these assays. For example, breast cancer cells can be treated with anti-cancer drugs or drugs that may promote cytoskeletal re-arrangement, and the effects of these drugs on invasion

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can be ascertained, compared to the unstimulated cells and vehicle controls[24]. Alternately, the capacity of breast cancer cells to form invasive protrusions can be quantified upon genetically modifying the cells to express a potential oncogene or reduced gene expression using RNA interference (such as shRNA)[18,24,25].

A major benefit of using 3D cell cultures as an experimental tool is the capability to examine the spatial and temporal features of important signaling molecules during cell morphological changes. By utilizing immunofluorescence staining, the expression of these molecules can be visually detected within the 3D culture. In **Figure 3E,** we show representative invasive (stellate) colonies of the MDA-MB-231 cells displaying a loss of membrane integrity and diffuse localization of the basement membrane protein laminin V[24]. In stark contrast to what is observed in the breast cancer cells, laminin V was localized to an intact basement membrane layer enclosing the mammary acini of untreated non-malignant MCF10A cells (**Figure 3E**)[24].

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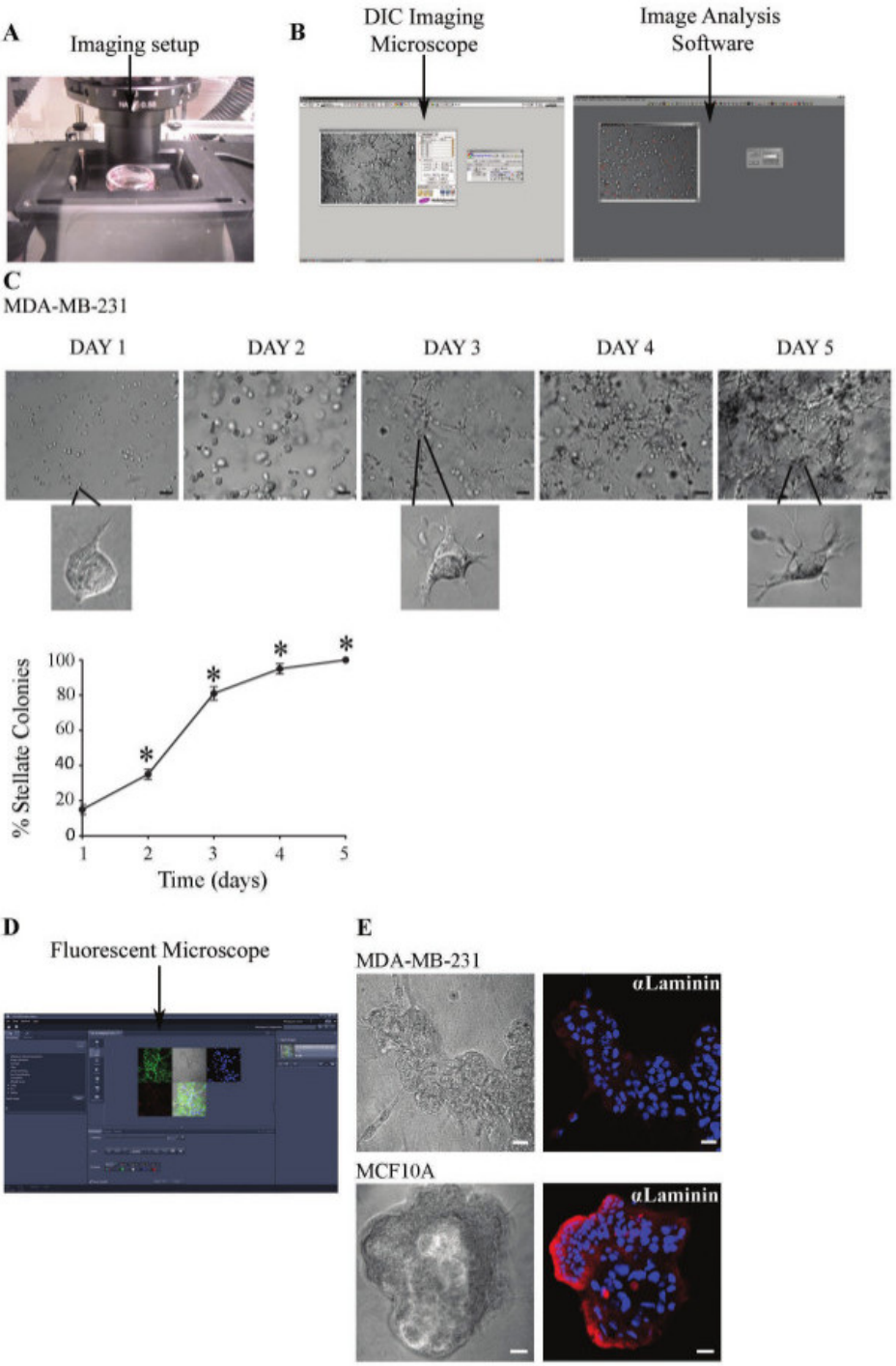

Figure 3. Image acquisition and illustration of representative images. A) Images taken with a microscope. **B)** Differential interference contrast (DIC) imaging microscope used to acquire images and subsequently images analyzed using image analysis software. **C)** Sample representative DIC images of MDA-MB-231 cells (taken once a day for five days) are shown. One-way ANOVA followed by Dunnett's multiple comparison test: *, $P < 0.05$. Scale bar, 100 µm. **D)** Image acquisition using fluorescent microscope. **E)** Sample immunofluorescent images depicting laminin V localization in MDA-MB-231 (grown for 5 days) and MCF10A cells (grown for 12 days) are shown. Scale bar, 20 µm.

Discussion

The development of 3D cell culture techniques has allowed researchers to study the transformation of breast epithelial cells, allowing us to visualize the dramatic morphological changes. Besides analyzing cell invasion, the single or multicellular mammary epithelial spheroids can be used to assess changes in cellular adhesion, proliferation, size, and basal-apical polarity. In contrast to previously reported methodologies where the cells are overlaid with ECM[8], our method embeds the cells in ECM[18,19,24], which allows for multidirectional invasion to be quantified.

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These innovative 3D culture models allow researchers to study phenotypic changes that are not possible when studying cells in monolayer, using traditional transwell chamber invasion assays. Through the incorporation of both biochemical and pharmacological strategies along with confocal immunofluorescence analysis of cell colonies, 3D models have facilitated the ability to carefully analyze morphological alterations with more sophistication and detail. This technique has therefore furthered our understandings of the mechanisms influencing the initiation and advancement of cancer.

We found that the optimal number for plating of MDA-MB-231 cells per confocal dish was determined to be 25,000 cells. Increasing this cell number may potentially result in excessive basement membrane matrix degradation (referred to as 'the fluffing phenomenon'), and thus, would not be recommended. However, higher cell numbers may work if other parameters are adjusted, such as with an accompanying increase in basement membrane matrix volume. Additionally, basement membrane matrix mixtures can vary in concentration from one batch to another. Thus it is important to initially test the mixture for its ability to sustain normal morphogenesis of non-malignant mammary epithelial cells in 3D assays. When imaging 3D cultures, unique problems arise that are not present when imaging cultures grown in monolayer. Given that colonies grown in basement membrane matrix are multi-dimensional, entire structures may be missed when imaging if located outside of the plane of focus. This is overcome by imaging using confocal Z-stacks that enable images to be taken in multiple planes and thus each individual invasive structure of the 3D colony can be imaged.

We have also determined that the 20% acetone : 80% methanol solution is the optimal agent for fixing colonies embedded in basement membrane matrix, compared to using other fixing agents such as paraformaldehyde with Triton-X solution. We have found that the acetone: methanol fixative improves immunolabeling and tends to reduce background autofluorescence. However, optimization of the staining procedure is required in every experiment, in order to determine the ideal antibody dilutions that might differ from those typically employed for monolayer staining. Most antibodies that work on monolayers can be used for 3D immunostaining. However, the length of incubation time with the antibody may vary, and this will have to be determined on an individual basis.

Imaging 3D cultures for immunofluorescence studies can pose some challenge[8]. In order to overcome the problem of 'blurriness/graininess' due to the thickness of the specimen, certain steps can be taken to improve the quality of image such as acquiring Z-stacks of the specimen or increasing exposure time, and increasing frame number average. This is also extremely beneficial to distinguish stellate (invasive) compared to spheroidal morphology rather than acquiring an image in one plane. The Z-stack imaged can also be processed to render a 3D image of the specimen showing the immunostaining. Furthermore, using the MDA-MB-231 or MCF10A cell lines, we successful formed cell aggregates over the time period grown. Thus, we were able to examine the location and changes in expression of cell polarity markers in 3D cultures by immunofluorescence. Alternately, cellular aggregates can be pre-formed before being placed into the basement membrane matrix and the localization of proteins of interest can then be assessed.

To conclude, the variety of possible applications makes the use of 3D cultures a dynamic assay that can be used with a wide spectrum of cells, whether pathological and/or normal in origin allowing for the evaluation of changes in cellular morphology and invasion in fixed samples or in real-time[18,19,24].

Disclosures

We have nothing to disclose.

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