Video Article

Time-lapse Imaging of Primary Preneoplastic Mammary Epithelial Cells Derived from Genetically Engineered Mouse Models of Breast Cancer

Rebecca E. Nakles[1], Sarah L. Millman[1], M. Carla Cabrera[1], Peter Johnson[1,2], Susette Mueller[1,2], Philipp S. Hoppe[3], Timm Schroeder[3], Priscilla A. Furth[1,2,4,5]

[1]Department of Oncology, Georgetown University
[2]Lombardi Comprehensive Cancer Center, Georgetown University
[3]Stem Cell Dynamics, Helmholtz Zentrum München - German Research Center for Environmental Health
[4]Department of Medicine, Georgetown University
[5]Department of Nanobiomedical Science and WCU Research Center of Nanobiomedical Science, Dankook University

Correspondence to: Priscilla A. Furth at paf3@georgetown.edu

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Abstract

Time-lapse imaging can be used to compare behavior of cultured primary preneoplastic mammary epithelial cells derived from different genetically engineered mouse models of breast cancer. For example, time between cell divisions (cell lifetimes), apoptotic cell numbers, evolution of morphological changes, and mechanism of colony formation can be quantified and compared in cells carrying specific genetic lesions. Primary mammary epithelial cell cultures are generated from mammary glands without palpable tumor. Glands are carefully resected with clear separation from adjacent muscle, lymph nodes are removed, and single-cell suspensions of enriched mammary epithelial cells are generated by mincing mammary tissue followed by enzymatic dissociation and filtration. Single-cell suspensions are plated and placed directly under a microscope within an incubator chamber for live-cell imaging. Sixteen 650 µm x 700 µm fields in a 4x4 configuration from each well of a 6-well plate are imaged every 15 min for 5 days. Time-lapse images are examined directly to measure cellular behaviors that can include mechanism and frequency of cell colony formation within the first 24 hr of plating the cells (aggregation versus cell proliferation), incidence of apoptosis, and phasing of morphological changes. Single-cell tracking is used to generate cell fate maps for measurement of individual cell lifetimes and investigation of cell division patterns. Quantitative data are statistically analyzed to assess for significant differences in behavior correlated with specific genetic lesions.

Video Link

The video component of this article can be found at http://www.jove.com/video/50198/

Introduction

Genetically engineered mouse models are tools to study and understand how different genetic lesions contribute to the risk of developing breast cancer. For example, genetically engineered mice have shown that the combination of three factors: loss of the full-length breast cancer 1, early onset (*Brca1*) gene in mammary epithelial cells, tumor protein p53 (*Tp53*) germ-line haploinsufficiency, and mammary epithelial cell targeted up-regulated Estrogen receptor alpha (ERa) expression results in the development of mammary cancer in 100% of *Brca1*$^{\textit{floxed (f)11/f11/Mouse Mammary Tumor Virus (MMTV)-Cre/p53+/-/tetracycline-operator (tet-op)-ER/MMTV-reverse tetracycline transactivator (rtTA}}$ mice by 12 months of age in comparison to the lower percentages reported in *Brca1*$^{\textit{f11/f11/MMTV-Cre/p53+/}}$- mice without ERa over-expression (~50-60%) and *Brca1*$^{\textit{f11/f11/MMTV-Cre}}$ mice without *Tp53* haploinsufficiency (<5%).[1]

Dynamic time-lapse imaging of the behavior of preneoplastic primary mammary epithelial cells reveals differences in cell behavior that are less easily appreciated in static tissue sections. Alterations in proliferation and differentiation are observed in primary mammary cells from human BRCA1 mutation carriers.[2] Creation of single-cell suspensions of primary mammary epithelial cells from normal and genetically engineered mice are generated through enzymatic disassociation of resected mammary gland tissue.[3] Time-lapse images are viewed to assess mechanism and timing of cell colony appearance and incidence of morphological changes in cells including epithelial-mesenchymal transition (EMT) and apoptosis. Generation of cell fate maps, quantification of the length of time between cell divisions (cell lifetimes), and determination of patterns of cell division are facilitated by use of single-cell tracking. Timm's Tracking Tool (TTT) is publically available software used to generate single-cell fate maps. Its utility in elucidating mechanisms of cell fate has been established[4,5] examining normal hematopoietic stem cell development[6-9] and the generation of neurons.[10]

Protocol

1. Overall Scheme

1. Generate primary cultures of preneoplastic mammary epithelial cells from mammary glands of genetically engineered and control wild-type mice.
2. Capture live-cell images every 15 min using Volocity image acquisition software (version 5.3.1, PerkinElmer, Waltham, MA) for up to 5 days.
3. View time-lapse images directly to assess timing and mechanism of epithelial cell colony formation, incidence of apoptosis, and phasing of morphological changes.
4. Convert Volocity generated .TIFF stacks to .JPG files using MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices, LLC Sunnyvale, CA) and rename digital image files (THE Rename 2.1.6, http://www.softpedia.com/get/System/File-Management/THE-Rename.shtml) to enable compatibility with Timm's Tracking Tool software (TTT, http://www.helmholtz-muenchen.de/en/isf/hematopoiesis/software-download/index.html).
5. Using TTT, generate cell fate maps for determination of the time between cell divisions (cell lifetimes) and patterns of cell division (symmetric versus asymmetric).
6. Analyze quantitative data from steps 3 and 5 above to determine if there are statistically significant differences between different genetically engineered mouse models and/or wild-type controls.

2. Generation of Primary Mammary Epithelial Cell Cultures

1. Prepare Complete Media for isolation of single mammary epithelial cells following a variation of manufacturer's instructions (EpiCult-B Mouse Medium Kit, Stemcell Technologies, Vancouver, BC). Combine 450 ml Epi-Cult-B medium, 50 ml Epi-Cult B proliferation supplement, 5% Fetal Bovine Serum (FBS), 50 µg/ml Penicillin/Streptomycin (PenStrep) and 10 ng/ml Epidermal Growth Factor (EGF).
2. Prepare Dissociation Media by combining 1 part of a 10x Collagenase/Hyaluronidase mixture with 9 parts Complete Media from 2.1.
3. Euthanize mouse and immediately proceed to necropsy. Place mouse on its back on a styrofoam necropsy platform and secure all four limbs so ventral skin is taut. Saturate ventral skin and hair, including that overlying the limbs, with 70% ethanol. Expose #2/3 (thoracic) and #4/5 (inguinal) mammary glands by making a midline incision through the skin (do not enter the peritoneum) that is extended into a Y-incision through the medial skin of each front limb and an upside down Y incision through the medial skin of each rear limb.
4. Using blunt dissection, separate the skin from underlyingperitoneum, pull back on both sides of the skin until it is taut, and secure it to the styrofoam necropsy platform using pins. Starting from the outer side, use blunt dissection with judicious use of dissection scissors to isolate the intact inguinal and/or thoracic mammary glands from underlying connective tissue and muscle. Place no more than two mammary glands in a 10 cm sterile Petri dish and move to tissue culture hood.
5. In tissue culture hood, examine glands for mammary lymph nodes that are identified as small, well-circumscribed nodules with a yellowish color. Remove lymph nodes from the surrounding gland using sterile scalpel and forceps. Mince mammary tissue into ~1 mm^3 cubes using two sterile scalpels, one in each hand.
6. Place minced mammary tissue in 5 ml Dissociation Media, mix by gently pipetting up and down 2-3 times using a 10 ml pipette, and incubate overnight (up to ~16 hr) in a 37 °C incubator with 5% CO_2 in a 50 ml conical tube with loosened cap.
7. The next morning, prepare a cold mixture of 1 part Hanks' Balanced Salt Solution (HBSS) containing 2% FBS and 4 parts buffered Ammonium Chloride Solution to promote red blood cell lysis (HFAmCl) as well as cold HBSS supplemented with 2% FBS (HF). Pre-warm Trypsin-EDTA (0.25%), 5 mg/ml Dispase in Hank's Balanced Salt Solution Modified, 1 mg/ml DNase I, and Complete Media.
8. Remove the conical centrifuge tube with the mammary tissue from the incubator and gently pulse vortex 2-3 sec two times. Centrifuge the tube at 450 × g for 5 min (room temperature or 4 °C), and discard the supernatant.
9. Resuspend the pellet in a minimum of 10 ml HFAmCl and centrifuge at 450 × g for 5 min (room temperature or 4 °C) and discard supernatant.
10. Add 3 ml Trypsin-EDTA to the pellet and mix first with a 5 ml pipette and then with a P1000 micropipettor until stringy (1-3 min). Add 10 ml HF, centrifuge at 450 × g for 5 min (room temperature or 4 °C) and discard supernatant.
11. Add 2 ml of 5 mg/ml Dispase and 200 µl of 1 mg/ml DNase I to the pellet and mix with a P1000 micropipettor for 1 min. Sample should be cloudy but not stringy. If stringy, add 100 µl of DNase I and mix again.
12. Add 10 ml cold HF, strain through a 40 µm cell strainer and centrifuge at 450 × g for 5 min (room temperature or 4 °C) and discard supernatant.
13. Resuspend the final pellet in Complete Media. Quantify the total number of cells (using a hemocytometer or Coulter Counter). Two mammary glands yield approximately 1×10^6 to 1×10^7 cells. Plate primary epithelial cells in a flat-bottomed 6-well plate at a density of 1.5×10^5 cells per well.

3. Live-cell Imaging

1. Immediately after plating the cells, place the 6-well plate securely on a microscope stage within a 5% CO_2/saturated humidity/37 °C temperature incubation chamber. Adjust the condenser for Kohler illumination and center the phase rings. For the experiments presented here, an inverted Nikon Eclipse TE300/PerkinElmer Spinning Disc Confocal microscope system in widefield phase contrast mode with a 10x objective lens was used.
2. Open image acquisition software, create and name a library for the time-lapse images, and save to a location with sufficient space for large files. The experiments presented here utilized Volocity image acquisition software (version 5.3.1, PerkinElmer, Waltham, MA).
3. Allow the plate to equilibrate in the microscope incubator for a minimum of 15 min. Lower the lenses to avoid stage-lens interference. Under the "Stage" heading, select "Calibrate Stage." Reacquire the focal plane, set Z=zero under the x, y, z tab and mark imaging points by selecting "Add point" under the "Stage" heading. Place points in the middle of each well of the 6-well plate for imaging as phase contrast imaging may be distorted near the periphery of plastic wells. Arrange points in a square in 4 x 4 fields (650 µm x 700 µm fields), each with a small overlap (about 5%). Save the selected points under "Stage."

4. Under "Stage" select "Make focus map" and follow the prompts to set the focus of each point. Set image acquisition timing to capture 4 pictures per hour (every 15 min). This can be adjusted depending on the requirements of the specific experiment. Save the focus map under "Stage".
5. Right click on the right side tool bar "Image Acquisition" and save the imaging settings. Press the record button to begin the imaging. After 1 hr, check the focus of the images to see if any adjustment is needed.
6. Monitor and continue live imaging for 5 days, ending when cells become confluent.

4. Direct Viewing of Time-lapse Images to Assess Timing and Mechanism of Initial Epithelial Cell Colony Formation, Incidence of Apoptosis, and Phasing of Morphological Changes

1. Direct viewing of time-lapse images can be performing using any compatible video viewing software (*e.g.* http://www.real.com/realplayer or other freeware or commercially available software). Images can be viewed in .TIFF format at this step or after conversion to .JPG files (see Step 5).
2. To determine mechanism of initial colony formation, start with a single-image stack representing one imaging site on the plate. In initial frames, cells will be floating. Follow serial images to determine when the first epithelial cell adheres to the plate. At this step, epithelial cells can be identified and differentiated from fibroblasts by their more cuboidal morphology. Follow this single epithelial cell through serial images and follow its fate over the subsequent 24 hr. Record if it becomes surrounded by additional epithelial cells or undergoes cell division or apoptosis. If surrounded by epithelial cells, the mechanism of colony formation can be determined directly by viewing if the surrounding cells are derived from floating cells that then aggregate with the initial adherent cell(s) or from division of the initial adherent cell. Record the number of colonies formed by cell aggregation versus cell division during the first 24 hr.
3. To determine the number of initially adherent cells that undergo apoptosis over a specific time period, follow serial images for the appearance of classic features of apoptosis developing in a cell that has been adherent (http://www.alsa.org/research/about-als-research/cell-death-and-apoptosis.html), and the number of apoptotic cells identified during the entire duration of the imaging or within specific time frames recorded.
4. Over time, epithelial cells in culture alter their morphology from an initial cuboidal shape to a more elongated appearance consistent with EMT. Follow serial images to determine the day/hour after plating when this occurs.

5. Image File Modification

1. Through the Volocity software, export the image files to TIFF stacks. Here MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices, LLC Sunnyvale, CA) was used to open the TIFF stack files and they were then saved in MetaMorph as single .JPG images.
2. Rename the image folders and files according to the requirements for Timm's Tracking Tool software (http://www.helmholtz-muenchen.de/fileadmin/ISF/PDF/Haematopoese/publications/TTT_Operating_Instructions_July_2009_.pdf). Use a freeware program like THE Rename 2.1.6 (http://www.softpedia.com/get/System/File-Management/THE-Rename.shtml) to rename files in groups. Create one folder containing all folders and files of the experiment and name the folder (*e.g.* EXPERIMENTNAME).
3. Create a subfolder for each point/position where the culture was imaged. Name the subfolders: EXPERIMENTNAME_pPOSITIONNUMBER. The position number should have 3 digits. Example: 072511RN_p001. Put all the image files of that individual position in the subfolder.
4. Add the timepoint (using 5 digits) and channel number (since there is only one bright field channel, use "0") at the end of each file name in this format:

   EXPERIMENTNAME_pPOSITIONNUMBER_tTIMEPOINT_wCHANNELNUMBER.jpgExample: 072511RN_p001_t00001_w0.jpg
5. Generate a log file for each position by first downloading the free program TTTlogfileconverter from the TTT website. Start the TTTlogfileconverter program and select the experiment folder. Input an image interval (in seconds). For a picture taken every 15 min, enter 900 sec. Then press the green covert log files button.

6. Generation of Cell Fate Maps of Single Cells Using TTT

1. Create a folder called TTTexport and a subfolder called TTTfiles where all cell tracking data will be stored following TTT software instructions (http://www.helmholtz-muenchen.de/fileadmin/ISF/PDF/Haematopoese/publications/TTT_Operating_Instructions_July_2009_.pdf).
2. Start the TTT program, select user initials, and click "Continue."
3. Press the "Set NAS," select the user created "TTTexport" folder, and click Ok. In the browser, select an experiment folder, select a position folder, and then press "Load position" button.
4. Set ocular factor to "10x." Load the number of images required (loading all images is recommended for first time).
5. In cell editor window, select "New colony" under the File menu. In the Movie window, press the "Track cell" or F2 to begin tracking a cell.
6. Identify a cell in the Movie window and use mouse to place cursor over the cell. A circle with the number of the cell should appear after F2 has been clicked. Use the "0" key on the number pad of the keyboard to track the placement of the cell and advance to the next picture. Move the cursor to follow the placement of each cell through each frame. To delete a track and return to the previous image press "Del" on the number pad. To move forward frames without tracking the cell press "3," and to move backward without tracking press "1" on the number pad.
7. To mark a cell division click the "Division" button in the Movie window. To mark cell death click the "Cell death" button in the Movie window. Once a division has occurred, the daughter cells can be tracked in the same cell fate map by right clicking on the circle symbol of the designated daughter cell in the Cell editor window to start tracking mode automatically.
8. Press F10 to save the tree. Each tree will save in the designated output folder (TTTExport). To start a new cell fate map, select "New colony" under the "File" then "Open" menu in the Cell editor window.
9. Tabulate cell lifetime data after gathering from "Cell data" tab in the Cell editor window.

7. Statistical Analyses

1. Use either Student's t-test (if comparing two genotypes) or ANOVA (if comparing > two genotypes) to determine if differences in parametric data such as numbers of initial cell colonies, cell lifetimes or hours until the appearance of EMT between different genotypes are statistically significant. Apoptotic frequency can be compared as the number/total number plated cells using Student's t-test or ANOVA or with Mann-Whitney or Kruskal-Wallis when derived as a percentage of adherent cells.
2. Perform power tests using data from analyses of initial experiments to determine how many experimental replicates need to performed and cell fate maps generated from each replicate for adequate statistical power using available freeware (*e.g.* http://statpages.org/). In the experiments presented here, cell cultures derived from three mice per genotype were sufficient to determine if there were statistically significant differences in cell colony formation and cell lifetimes when specific genetic modifications were made.

Representative Results

Epithelial and fibroblast cells can be distinguished by cell morphology. Epithelial cells have a cuboidal shape (**Figure 1A-B**) and form cell colonies (**Figure 1A**). Fibroblasts, a type of stromal cell, have an elongated morphology (**Figure 1C**).

Cells were rounded and floating at the onset of imaging (**Figure 2A-D**). After attachment to the plate they became flat and demonstrated a cuboidal-type appearance (**Figure 2E, H**). By 4 days of culture the majority of epithelial cells elongated into an EMT-like morphology (**Figure 2I-L**). This change occurred with the same chronology in all genotypes studied. Some digital images were blurry because Kohler illumination and phase ring alignment was not set correctly (**Figure 2D, H, L**).

The floating cells developed into defined individual epithelial cell colonies by 24 hr after plating (**Figure 3A, B**). Serial image analysis revealed that these colonies were generated by cell aggregation rather than cell division. While disruption of *Brca1* by itself did not alter the number of colonies formed, addition of *Tp53* haploinsufficiency significantly reduced the number of colonies formed and addition of ERα over-expression significantly increased the number of colonies formed (**Figure 3C**).

It is critical to monitor the digital images acquired and adjust the focus map frequently during the first 24 hr as cells attach to the plate and then at least twice daily to ensure the cells remain in focus and cultures are not contaminated. Accuracy of analyses is compromised if imaged cells are not in focus (**Figure 4**).

In the representative experiment presented here, a critical question was to determine if changes in expression levels of genes known to impact mammary cancer risk (*Brca1, Tp53*,and estrogen receptor 1 (*Esr1*)[1]) altered the number of hours between cell divisions (cell lifetime) or impacted the patterns of division (symmetric versus asymmetric) in non-cancerous primary mammary epithelial cells. To accomplish this, individual cell fate maps (trees) were generated using TTT. Examples of representative cell fate maps for each of the four genotypes tested are illustrated (**Figure 5A-D**). The four genotypes of the cells tested were wild-type (no genetic manipulation) (**Figure 5A**), loss of full-length *Brca1* (*Brca1*$^{f11/f11/MMTV\text{-}Cre}$) (**Figure 5B**), loss of full-length *Brca1* in combination with *Tp53* haploinsufficiency (*Brca1*$^{f11/f11/MMTV\text{-}Cre/p53+/-}$) (**Figure 5C**), and loss of full-length *Brca1* in combination with *Tp53* haploinsufficiency and gain of *Esr1* (*Brca1*$^{f11/f11/MMTV\text{-}Cre/p53+/-/MMTV\text{-}rtTA/tet\text{-}op\text{-}ER}$) (**Figure 5D**). Generation of cell fate maps was stopped when cells became confluent (~3-4 generations) since unequivocal tracking of single cells was no longer possible after that. Generation 0 (time to first cell division) was not included in the analyses of cell lifetimes because duration of generation 0 was longer and more variable than subsequent generations. Generations 1, 2, 3, and 4 were grouped together for the final analyses because there were no significant differences in mean cell lifetimes or Standard Error of the Mean (SEM) between these generations. Comparisons of mean cell lifetimes between the different genotypes (**Figure 5E**) revealed that loss of full-length *Brca1* reduced the number of hours between cell divisions from 21.3 ± 1.6 hr (wild-type) to 16.5 ± 1.0 hr (*Brca1*$^{f11/f11/MMTV\text{-}Cre}$). Notably, although loss of one *Tp53* allele in addition to loss of full-length *Brca1* is associated with a significant increase in mammary cancer development1, mean cell lifetime was unchanged (16.3 ± 1.2 hr) compared to mice lacking *Brca1* only. Interestingly gain of *Esr1* in *Brca1*$^{f11/f11/MMTV\text{-}Cre/p53+/-/MMTV\text{-}rtTA/tet\text{-}op\text{-}ER}$ mice restored cell lifetime durations to near wild-type levels (19.3 ± 2.1 hr), albeit these mice have the highest incidence of mammary cancer development of all the models studied here.1 These findings indicated that loss of full-length *Brca1* was not invariably associated with a shortened cell lifetime, instead this was modified by over-expressed ERα. Possible next-step experiments utilizing this technology could be dose-response experiments using different estrogens, other growth factors, or candidate therapeutics to measure their impact on cell lifetime in the different genetic backgrounds. In contrast to the impact on cell lifetime duration, none of the genetic alterations studied here altered cell division patterns.

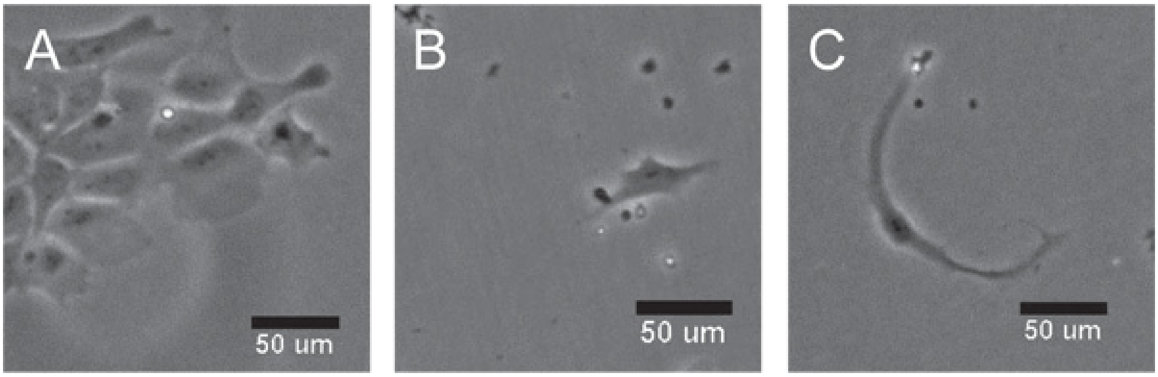

Figure 1. Appearance of an epithelial cell colony (A), epithelial cell (B), and fibroblast (C) from time-lapse imaging. Epithelial cells appear more cuboidal while fibroblasts appear more elongated. Size bars=50 µm.

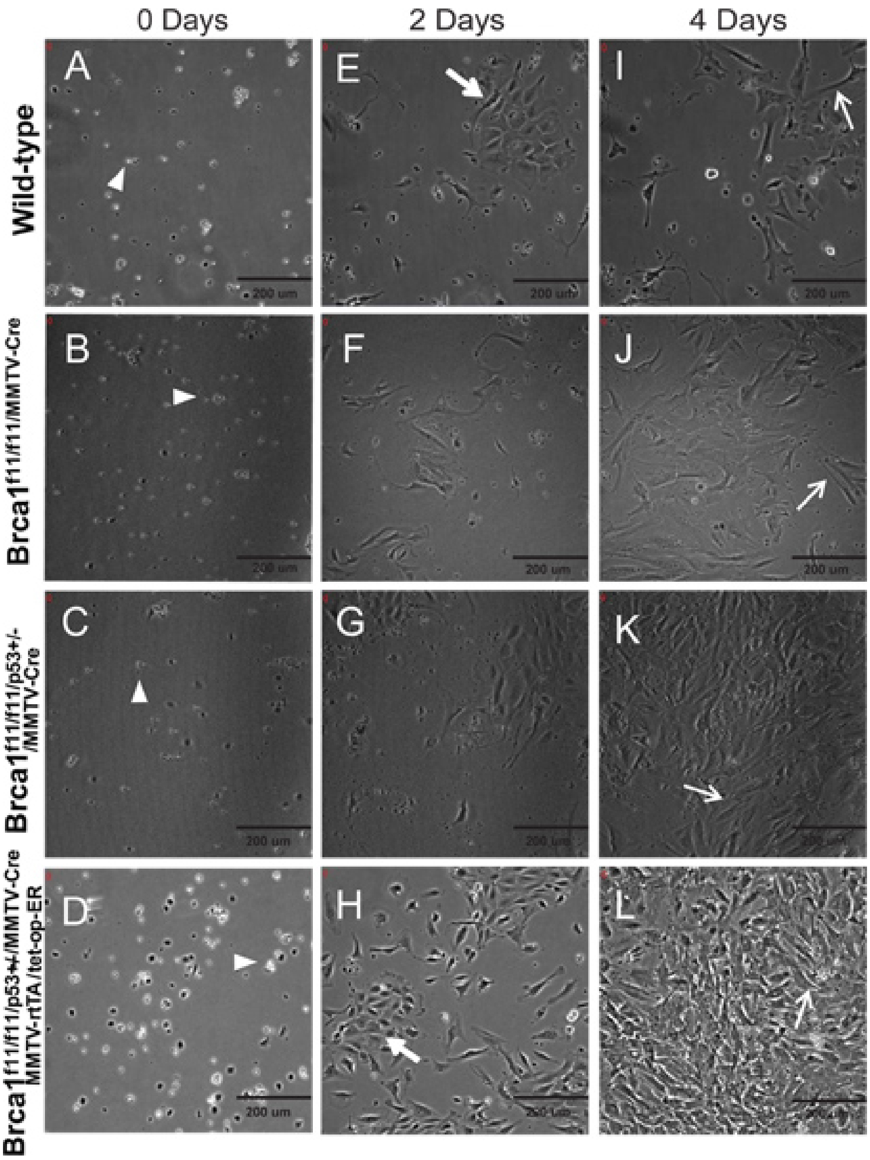

Figure 2. Representative serial time-lapse images from time 0, 2 and 4 days demonstrate changes in cellular morphology over time and differences in appearance with and without Kohler illumination. At time 0 the plated cells are floating (arrowheads A-D), by two days in culture they are adherent and may exhibit a cuboidal-type morphology (thick arrows E, H) and by four days in culture they elongate and demonstrate an EMT-like morphology (thin arrows I-L). Kohler illumination is present in panels A-C, E-G, and I-K. Panels D, H and L illustrate images without Kohler illumination. Images shown are from the same imaging point over time. Size bars=200 µm. Click here to view larger figure.

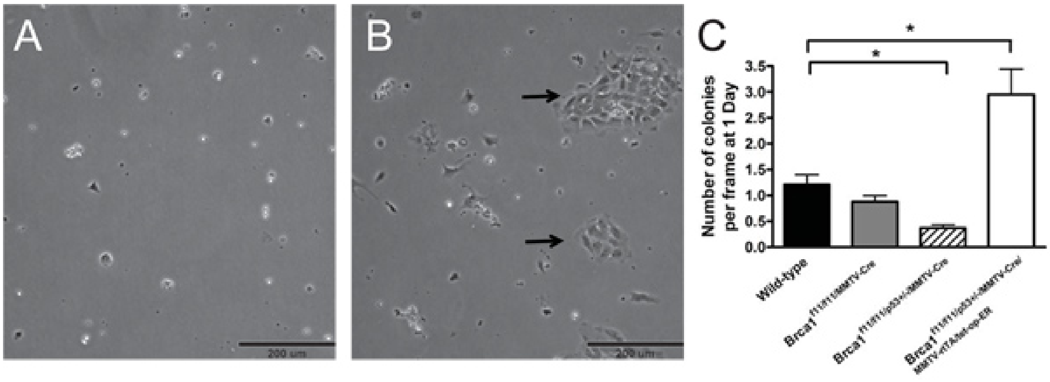

Figure 3. Numbers of epithelial cell colonies at 1 day varied by genotype. (A) Rounded floating cells at the beginning of time-lapse imaging. (B) Two representative epithelial cell colonies (arrows). (C) Numbers of epithelial cell colonies/frame formed at 24 hr varied by genotype. Bar graphs illustrate the mean and standard error of the mean. **p*<0.05, ANOVA. Size bars=200 µm. Cells isolated from mammary glands without palpable tumor from 10- to 12-month-old *Brca1*$^{f11/f11/MMTV\text{-}Cre}$ (n=4), *Brca1*$^{f11/f11/\ MMTV\text{-}Cre/\ p53+/-}$ (n=3), *Brca1*$^{f11/f11/p53+/-/MMTV\text{-}Cre/Tet\text{-}op/\text{-}ER/MMTV\text{-}rtTA}$ (n=3), and wild-type (n=3) mice were imaged for the studies. Five independent imaging experiments composed of different combinations of wild-type and genetically engineered mice were performed. Media contained phenol red. No estrogen was added. Click here to view larger figure.

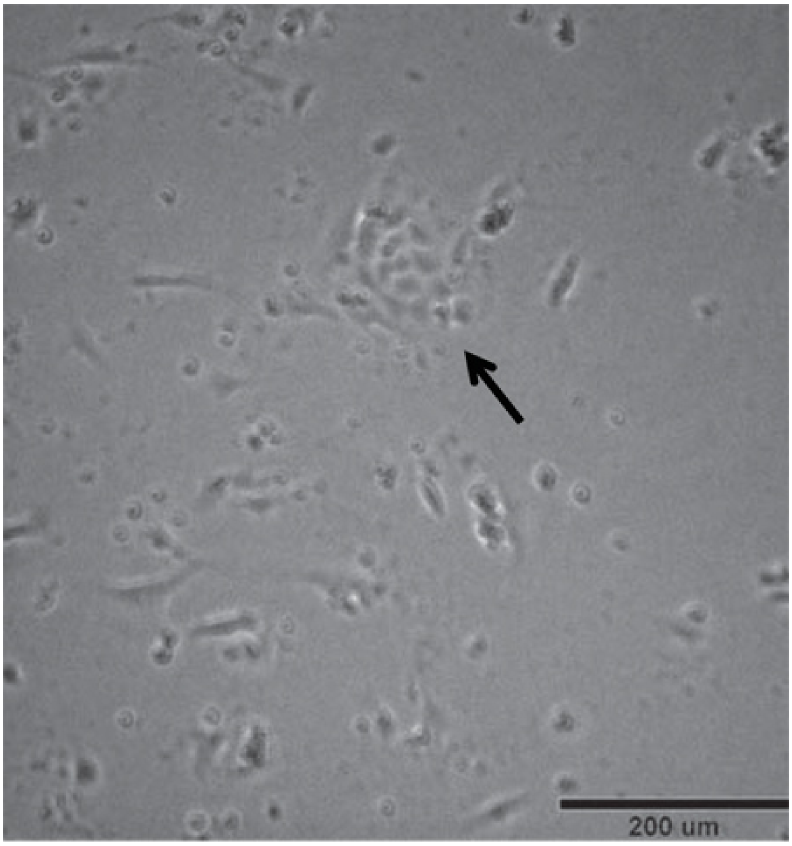

Figure 4. Out-of-focus images cannot be accurately analyzed. Before imaging the plate, the plate should sit in the imaging incubator for 15 min to equilibrate. During the first day of imaging, the focus should be checked and adjusted every few hours. After that it should be checked twice daily but generally remains more stable. Arrow indicates an out-of-focus epithelial cell colony.

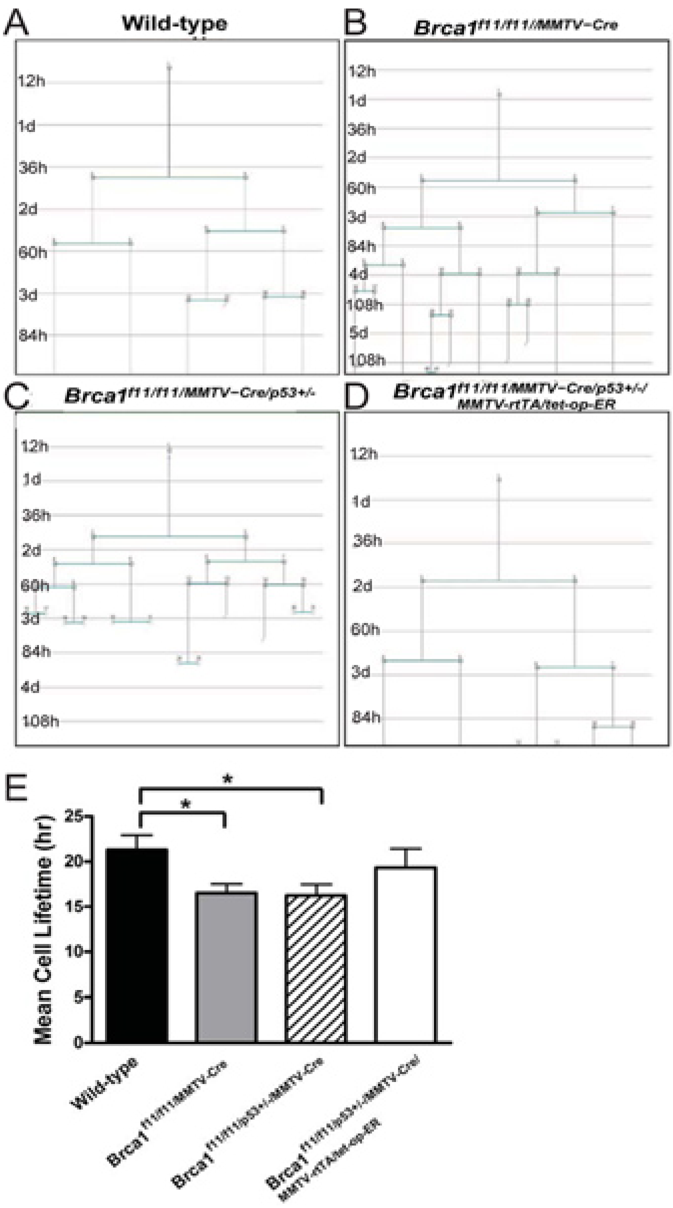

Figure 5. Cell fate maps generated from single-cell tracking using TTT for (A) wild-type, (B) *Brca1*$^{f11/f11/MMTV\text{-}Cre,}$ (C) *Brca1*$^{f11/f11/MMTV\text{-}Cre/p53+/-}$, and (D) *Brca1*$^{f11/f11/MMTV\text{-}Cre/p53+/-/MMTV\text{-}rtTA/tet\text{-}op\text{-}ER}$ mice. Cells that cannot be tracked due to loss out of frame or into a confluent cell layer are indicated by a question mark. When no question mark indicated, tracking was deliberately ended due to cell confluence and inability to unequivocally follow single-cell fate. **(**E) Mean cell lifetimes varied by genotype. Bar graphs illustrate the mean and standard error of the mean cell lifetimes (time between cell divisions) over generations 1-4 for each genotype. Mean cell lifetimes were significantly shorter in mammary epithelial cells derived from *Brca1*$^{f11/f11/MMTV\text{-}Cre}$ and *Brca1*$^{f11/f11/MMTV\text{-}Cre/p53+/-}$ as compared to wild-type mice. *$p<0.05$, ANOVA. Cells isolated from mammary glands without palpable tumor from 10- to 12-month-old *Brca1*$^{f11/f11/MMTV\text{-}Cre}$ (n=4), *Brca1*$^{f11/f11/ MMTV\text{-}Cre/ p53+/-}$ (n=3), *Brca1*$^{f11/f11/p53+/-/MMTV\text{-}Cre/ Tet\text{-}op/\text{-}ER/MMTV\text{-}rtTA}$ (n=3), and wild-type (n=3) mice were imaged for the studies. Five independent imaging experiments composed of different combinations of wild-type and genetically engineered mice were performed. Media contained phenol red. No estrogen was added. Click here to view larger figure.

Discussion

Critical steps

It is important to ensure that mammary glands are harvested from mice of the same age to control for age-related variability in mammary epithelial cell behavior. When plating the cells, the same number of cells should be plated in each well for every experiment. Cells should be relatively sparse when plating so that cultures do not become confluent too quickly making it possible to follow multiple individual cells through serial images. It is essential that the plate be equilibrated in the incubator before imaging because the focus will change after the plate is equilibrated. Once imaging has begun, the focus should be checked frequently and adjusted as needed, especially within the first 24 hr. It is important to have the temperature of the incubator regulated and pre-warmed. The number of people going in and out of the room should be limited as much as possible. We recommend practicing all aspects of setting up the experiment beforehand to ensure reproducibility and quality control. As with all cell culture experiments, sterility is an important issue and standard sterile cell culture practices should be used at all times.

Saving and manipulating the large image files requires a substantial amount of memory space. A shared network drive or portable hard drives can be used. Once files are converted it is important to make sure the images are consistently named correctly and placed in corresponding folders. Frequent saves of digital data should be performed throughout the process.

Limitations

This method uses a 2-D culture system that does not allow for analysis of behavioral differences that might become apparent in a 3-D culture system. Durations of cell lifetimes may only be reproducibly measured after the first observed cell division as the number of hours to first cell division following initial plating of the cells is variable.

Possible modifications

Depending on the requirements of the experiment, time-lapse images can be taken more or less frequently. For example, if transient cellular structures are to be quantified, a 5 sec interval may be more appropriate. Other procedures to generate primary mammary epithelial cell cultures may be used. Any imaging equipment may be used to create the time-lapse imaging, as long as it is able to meet the requirements of the experiment. Alternative systems for single-cell tracking can be employed. For the procedure described here, a standard plastic flat bottom 6-well plate was adequate. Plastic dishes can be used for all long working distance air lenses such as 4x, 10x, and 20x. It is important to choose regions near the center of the plastic dishes when performing phase contrast imaging since the plastic is curved near the edges in most dishes and distorts the light. Coverslip thickness glass bottomed wells would be required for normal working distance immersion lenses (oil, water, glycerol, etc.), which are typically at higher magnification.

Trouble-shooting

When cells are plated, media should be sufficient to maintain cell growth for 5 days to decrease the need for manipulating the plate on the stage; however, if necessary, media can be added. It is recommended that evaporation be kept to a minimum. Evaporation should be managed by placing three to four dishes of sterile deionized water inside the incubator and refilling them when necessary. It is possible to fill the unused wells of the 6-well plate with sterile deionized water.

If images are not loading into the TTT program, first check and make sure the files and folders are arranged and named correctly. Next, check if the log file is in the correct folder (see step 5.5).

Future application or direction after mastering technique

The same general procedure could be used to analyze behavior of other cell types including human primary mammary epithelial cells as well as breast cancer cells. For example, genotypic-specific behavior and/or response to candidate treatments can be analyzed. Alternatively, imaging of fluorescence-activated cell (FAC) sorted cell populations could be performed or fluorescent labels added to monitor specific cell types.

Significance of this technique with respect to existing methods

Addition of time-lapse imaging and analyses of cell behavior over time to traditional cell culture and labeling techniques provides quantitative data from single cells over time rather than only whole population dynamics. This technique allows single cells to be observed continuously, unlike traditional methods that permit observation only at discrete timepoints. Moreover, traditional methods require the cells to be disturbed by taking them in and out of the incubator to view them under a microscope whereas the approach described here allows the cells to be observed without being transferred. Finally, time-lapse imaging provides an enduring record of the cells under observation that can be returned to for further analyses. The use of TTT to track individual cell behavior permits an unequivocal analysis of multiple parameters and does not require the use of a fluorescent label to localize the specific cells under observation. The technique reveals differences in mammary epithelial cell behavior that are difficult to measure in static tissue sections of mammary gland. Cell lifetime measurements quantify the number of hours between mitotic events. This measurement provides specific data on the actual number of hours between cell divisions indicating the length of the cell cycle in hours. A researcher can use this data to determine how specific genetic modifications or culture conditions impact cell cycle length.

Disclosures

The authors declare that they have no competing financial interests.

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