Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61775R1
Full Title:	Isolation and functional assessment of human breast cancer stem cells from cell and tissue samples
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Additional Information:	
Question	**Response**
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TITLE:

Isolation and functional assessment of human breast cancer stem cells from cell and tissue samples

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KEYWORDS:

Breast cancer stem cell (BCSC), fluorescence-activated cell sorting (FACS), colony forming assay, mammosphere assay, 3D culture model, in vivo tumor model

SUMMARY:

This experimental protocol describes the isolation of BCSCs from breast cancer cell and tissue samples as well as the in vitro and in vivo assays that can be used to assess BCSC phenotype and function.

ABSTRACT:

Breast cancer stem cells (BCSCs) are cancer cells with inherited or acquired stem cell-like characteristics. Despite their low frequency, they are major contributors to breast cancer initiation, relapse, metastasis and therapy resistance. It is imperative to understand the biology of breast cancer stem cells in order to identify novel therapeutic targets to treat breast cancer. Breast cancer stem cells are isolated and characterized based on expression of unique cell surface markers such as CD44, CD24 and enzymatic activity of aldehyde dehydrogenase (ALDH). These $ALDH^{high}CD44^{+}CD24^{-}$ cells constitute the BCSC population and can be isolated by fluorescence-activated cell sorting (FACS) for downstream functional studies. Depending on the scientific question, different in vitro and in vivo methods can be used to assess the functional characteristics of BCSCs. Here, we provide a detailed experimental protocol for isolation of human BCSCs from both heterogenous populations of breast cancer cells as well as primary tumor tissue obtained from breast cancer patients. In addition, we highlight downstream in vitro

and in vivo functional assays including colony forming assays, mammosphere assays, 3D culture models and tumor xenograft assays that can be used to assess BCSC function.

INTRODUCTION:

Understanding the cellular and molecular mechanisms of human breast cancer stem cells (BCSCs) is crucial for addressing the challenges encountered in breast cancer treatment. The emergence of the BCSC concept dates back to the early 21st century, where a small population of $CD44^{+}CD24^{-/low}$ breast cancer cells were found to be capable of generating heterogenous tumors in mice[1,2]. Subsequently, it was observed that human breast cancer cells with high enzymatic activity of aldehyde dehydrogenase ($ALDH^{high}$) also displayed similar stem cell-like properties[3]. These BCSCs represent a small population of cells capable of self-renewal and differentiation, contributing to the heterogenous nature of bulk tumors[1-3]. Accumulating evidence suggest that alterations in evolutionarily conserved signaling pathways drive BCSC survival and maintenance[4-14]. In addition, the cell extrinsic microenvironment has been shown to play a pivotal role in dictating different BCSC functions[15-17]. These molecular pathways and the external factors regulating BCSC function contribute to breast cancer relapse, metastasis[18] and development of resistance to therapies[19-21], with the residual existence of BCSCs post-treatment posing a major challenge to the overall survival of breast cancer patients[22,23]. Pre-clinical evaluation of these factors is therefore very important for identifying BCSC-targeting therapies that could be beneficial for achieving better treatment outcomes and improved overall survival in breast cancer patients.

Several in vitro human breast cancer cell line models and in vivo human xenograft models have been used to characterize BCSCs[24-29]. The ability of cell lines to continuously repopulate after every successive passage makes these an ideal model system to perform omics-based and pharmacogenomic studies. However, cell lines often fail to recapitulate the heterogeneity observed in patient samples. Hence, it is important to complement cell line data with patient-derived samples. Isolation of BCSCs in their purest form is important for enabling detailed characterization of BCSCs. Achieving this purity depends on the selection of phenotypic markers that are specific to BCSCs. Currently, the $ALDH^{high}CD44^{+}CD24^{-}$ cell phenotype is most commonly used to distinguish and isolate human BCSCs from bulk breast cancer cell populations using fluorescence activated cell sorting (FACS) for maximum purity[1,3,26]. Furthermore, the properties of isolated BCSCs such as self-renewal, proliferation, and differentiation can be evaluated using in vitro and in vivo techniques.

For example, in vitro colony forming assays can be used to assess the ability of a single cell to self-renew to form a colony of 50 cells or more in presence of different treatment conditions[30]. Mammosphere assays can also be used to assess the self-renewal potential of breast cancer cells under anchorage-independent conditions. This assay measures the ability of single cells to generate and grow as spheres (mixture of BCSCs and non-BCSCs) at each successive passage in serum-free non-adherent culture conditions[31]. Additionally, 3-Dimensional (3D) culture models can be used to assess BCSC function, including cell-cell and cell-matrix interactions that closely recapitulate the in vivo microenvironment and allow investigation of the activity of potential BCSC-targeted therapies[32]. Despite the diverse applications of in vitro models, it is difficult to model the complexity of in vivo conditions using only in vitro assays. This challenge can be

overcome by use of mouse xenograft models to evaluate BCSC behavior in vivo. In particular, such models serve as an ideal system for assessing breast cancer metastasis[33], investigating interactions with the microenvironment during disease progression[34], in vivo imaging[35], and for predicting patient-specific toxicity and efficacy of antitumor agents[34].

This protocol provides a detailed description for the isolation of human $ALDH^{high}CD44^{+}CD24^{-}$ BCSCs at maximum purity from bulk populations of heterogenous breast cancer cells. We also provide a detailed description of three in vitro techniques (colony forming assay, mammosphere assay, and 3D culture model) and an in vivo tumor xenograft assay that can be used to assess different functions of BCSCs. These methods would be appropriate for use by investigators interested in isolating and characterizing BCSCs from human breast cancer cell lines or primary-patient derived breast cancer cells and tumor tissue for the purposes of understanding BCSC biology and/or investigating novel BCSC-targeting therapies.

PROTOCOL:

Collection of patient-derived surgical or biopsy samples directly from consenting breast cancer patients were carried out under approved human ethics protocol approved by the institutional ethic board. All mice used to generate patient-derived xenograft models were maintained and housed in an institution approved animal facility. The tumor tissue from patient-derived xenograft models using mice were generated as per approved ethics protocol approved by the institutional animal care committee.

1. Preparation of cell lines

1.1 Perform all cell culture and staining procedures under sterile conditions in a biosafety cabinet. Use sterile cell culture dishes/flasks and reagents.

1.2 Maintain human breast cancer cells at 37 °C with 5% CO_2 in defined media supplemented with fetal bovine serum (FBS) and necessary growth factors specific to each cell line.

1.3 Maintain mouse NIH3T3 fibroblast cell cultures (for use in colony forming assays) at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS.

1.4 For all cultures, replenish the old media every 2-3 days with fresh media. Once the cultures reach 75-80% confluency, subculture into multiple sterile cell culture flasks.

2. Preparation of breast cancer tumor tissue

2.1 Collect the patient-derived surgical or biopsy samples directly from consenting breast cancer patients under a human ethics protocol approved by the institutional ethics board.

2.2 Subsequently, collect and generate tumor tissue from patient-derived xenograft models using mice under an animal ethics protocol approved by the institutional animal care committee.

2.3 Collect all tumor tissues under sterile conditions into a 50 mL sterile conical tube containing 30 mL DMEM:F12 media, keep on ice, and process the samples as described below within 2 h of collection.

3. Generation of single cell suspensions of breast cancer cells

3.1 Aspirate media from the flask containing a monolayer of breast cancer cells that is 60-80% confluent (cell lines of choice). Wash the cells with 1x phosphate buffered saline (PBS). Aspirate PBS and add appropriate cell dissociation solution (e.g., Trypsin:EDTA; just enough to cover the monolayer of cells) and incubate for 5 min at room temperature (recommended) or at 37 °C.

3.2 Add 5 mL of culture media to neutralize the activity of cell dissociation solution.

3.3 Transfer the resulting dissociated cell solution to a 50 mL conical tube and centrifuge at 1000 x *g* for 5 min.

3.4 Discard supernatant and resuspended the cell pellet in 5 mL of 1x PBS. Count the cells using a hemocytometer and a microscope.

NOTE: Observe for cell clumping in the hemocytometer. Repeat cell dissociation step if single cell suspension has not formed.

3.5 After cell counting, re-centrifuge the cell suspension at 1000 x *g* for 5 min, discard supernatant, and resuspend the cell pellet in ALDH substrate buffer at a concentration of 1 x 10^6 cells/mL.

4. Generation of single cell suspension from tissue samples

4.1 Mince the tumor tissue with surgical blades using a crisscross technique to obtain smaller pieces of approximately 1 mm in size. Transfer the tissue pieces into a fresh 50 mL conical tube containing 10 mL dissociation buffer. Seal the conical tube with parafilm and incubate at 37 °C in a shaker incubator for 40 min.

NOTE: If there is not a shaker incubator, place the tube in a 37 °C water bath and mix the tube by vortexing every 5-10 min.

4.2 Pellet the digested tissue by centrifuging sample at 530 x *g* for 5 min. Discard the supernatant and add 5 mL of trypsin. Pipette up and down using 1 mL pipette (set to 750 µL mark) to disrupt the pellet and incubate in a 37 °C water bath for 5 min. After incubation, pipette up and down vigorously to release single cells.

4.3 Top up the total volume in the tube to 25 mL with DMEM:F12 media and centrifuge at

1000 x *g* for 5 min. Discard the supernatant and resuspend the pellet in 1 mL of dispase/DNase. Incubate in a 37 °C water bath for 5 min.

4.4 Top up the total volume in the tube to 10 mL with PBS. Mix by pipetting up and down, pass the resulting cell suspension through a 40 µm cell strainer attached to a fresh 50 mL conical tube. Centrifuge at 1000 x *g* for 5 min.

4.5 Discard supernatant and resuspend the cell pellet in 5 mL of 1x PBS. Count the cells and complete preparation of the cell suspension as described in steps 3.4 and 3.5.

5. Isolation of breast cancer stem cells (BCSCs)

5.1 Label flow tubes for the unstained control, single cell staining controls (DEAB control, ALDH, CD44-PE, CD24-PE-Cy7, 7AAD), the negative control tube (stained with DEAB, CD44-PE, CD24-PE-Cy7 and 7AAD), fluorescent minus one (FMO) control and the 'sort' tube (stained with ALDH, CD44, CD24 and 7AAD).

5.2 Transfer 500 µL (0.5 x 10^6 cells) of the cell suspensions from step 3.5 or step 4.5 to each tube that is labelled cells only, CD44, CD24 and 7AAD. Place the tubes on ice until use.

5.3 Transfer 2 mL of sample (2 x 10^6 cells) to respective 'ALDH' tube. Add 5 µL of DEAB to the 'DEAB control' and 'negative control' tubes and cap it tightly. Add 10 µL of ALDH substrate to the 'ALDH' tube, mix well by vortexing, and immediately transfer 500 µL to corresponding 'DEAB control' and 'negative control' tube. Recap the 'DEAB control', 'negative control' and 'ALDH tubes' and incubate at 37 °C for 30-60 min (do not exceed 60 min).

NOTE: The optimal incubation time may require optimization depending on the cell line. Always protect the ALDH substrate and the tubes containing stained cells from light.

5.4 Following incubation, centrifuge all samples for 5 min at 250 x *g*. Resuspend the cells in 500 µL of ALDH substrate buffer. Add manufacturer-recommended or user-optimized concentration of anti-CD44-PE and anti-CD24-PE-Cy7 antibody cocktail and incubate at 4 °C for 30 min. Add anti-CD44-PE and anti-CD24-PE-Cy7 antibodies to respective 'CD44' and 'CD24' labelled tubes.

5.5 Following incubation, centrifuge all samples at 250 x *g* for 5 min. Resuspend the cells in 500 µL of ALDH substrate buffer. Incubate the 'negative control' tube, 'Sort tube' and the '7ADD' tube with 7AAD (suggested concentration: 0.25 µg/1 x 10^6 cells) for 10 min on ice.

NOTE: The ALDH activity is detected in the green fluorescent channel, therefore a fluorochrome with a different compatible emission spectrum should be used. Where spectral overlap is observed during multi-parameter flow cytometry, single color controls and FMO control should be used as a guide to allow compensation between fluorochromes to minimize the spill over of

fluorescent signal into other channels.

5.6 Set up the analysis protocol on the FACS instrument in preparation for sample analysis. Create scatter plots (forward vs side scatter, forward scatter vs fluorescent channels).

5.7 Using the unstained control, adjust the photomultiplier to separate debris from whole cell population and adjust the fluorescent voltage to move the whole cell population around the first log scale (10^1). Using the DEAB control, move the whole cell population within the second log scale (10^2) by adjusting the green fluorescent voltage channel.

5.8 Analyze all the single staining controls first (ALDH, CD44-PE, CD24-PE-Cy7) and 7AAD and FMO control, adjusting the voltage to separate stained from unstained cells and to minimize the spillage of fluorescent signals into other channels.

5.9 Gate on the positive population for each single stained cell sample. Using the negative control tube, gate for viability (7AAD negative), $ALDH^{low}$ and $ALDH^{high}$ cell populations (representative gating strategy shown in **Figure 1B**).

5.10 Analyze multiparameter stained samples of interest to isolate BCSCs. Using the viable $ALDH^{low}$ and $ALDH^{high}$ gates, select for $CD44^{+}CD24^{-}$ (BCSC) and $CD44^{-}CD24^{-}$ (non-BCSC) cell population respectively (**Figure 1B**).

5.11 Collect viable BCSCs and non-BCSCs in collection media in sterile collection tubes (populations from two representative cell lines shown in **Figure 2A&B**). Use sorted cells for downstream in vitro and in vivo assays as described below.

NOTE: In addition to in vitro and in vivo assays described below, BCSCs can be validated by measuring the expression of pluripotent markers such as SOX2, OCT4 and NANOG via standard immunoblotting techniques.

(*Insert Figures 1, 2 here*)

6. Colony forming assay

6.1 Resuspend the cells of interest (sorted cells from step 5.11 or unsorted cells from steps 3.5 or 4.5) in complete media.

6.2 Label three flow tubes for 1 x 10^2, 2 x 10^2 and 5 x 10^2 cells. Add 2 mL of complete media and transfer the appropriate cell number (sorted from step 5.11 or unsorted cells from steps 3.5 or 4.5) in respective tubes. Mix the cell solutions thoroughly by pipetting it up and down 5 times.

6.3 Plate the cells in a 6-well plate and distribute the cell suspension by gently swirling the plates to obtain uniform distribution of cells.

6.4 Incubate the plates in a 37 °C, 5% CO_2 incubator until colonies appear (where colonies = ≥50 cells per colony). Carefully replenish media twice a week without disturbing colony formation.

6.5 Aspirate media and wash once with 1 mL PBS. Add 0.5 mL of 0.05% crystal violet solution into each well and incubate the plate for 30 minutes. Remove excess crystal violet stain by washing with 2 mL of water. Repeat the washing step until background staining has been removed.

6.6 Using a microscope at 4x and 10x magnification, count and record the total number of colonies generated (representative images shown in **Figure 3A**).

6.7 Calculate the frequency of colony formation as follows: Frequency (%) = (# of colonies formed/number of cells seeded) x 100. For example, if 25 colonies are generated from 1 x 10^2 cells, then the Frequency of colony formation is, Frequency = (25/100) x100 = 25%.

6.8 Alternatively, replace steps 6.1 to 6.4 with an alternate method involving co-culture with fibroblasts, which provide a microenvironmental support for BCSCs through production of necessary growth and survival factors.

6.9 Pre-coat cell 60 mm culture dishes with type I bovine collagen (1 in 30 dilution of 3 mg/mL collagen). Allow collagen to polymerize for 30 min in a 37 °C incubator. Aspirate the unpolymerized collagen and wash the plate twice with 1x PBS. Cover the collagen-coated plate with 1 mL of PBS and set it aside at room temperature until use.

6.10 Label three flow tubes for 1 x 10^3, 5 x 10^3 and 1 x 10^4 cells. Add 4 mL of colony forming assay media and transfer the appropriate number of cells (sorted from step 5.11 or unsorted cells from steps 3.5 or 4.5) into the respective tubes. Add irradiated mouse NIH3T3 fibroblasts (4 x 10^4 cells/mL of media). Mix cell solutions thoroughly by pipetting it up and down 5 times.

6.11 Aspirate the PBS from the collagen-coated culture dish from step 6.1 and plate the cell mixture onto each of the cell culture plates as described in step 6.3.

6.12 Incubate the plates in a 37 °C, 5% CO_2 incubator and leave them undisturbed for 7-10 days or until colonies form, without replenishing the media. Count and record the total number of colonies generated as described in steps 6.6 and 6.7.

7. Mammosphere assay

7.1 Resuspend the cells of interest (sorted cells from step 5.11 or unsorted cells from steps 3.5 or 4.5) in complete mammosphere media and plate cells at a seeding density of 5 x 10^2 cells/cm^2 area in a 96 well ultra-low attachment cell culture plate.

NOTE: Cell seeding density should be optimized for different cell lines.

7.2 Incubate the culture plates for 5-10 days in a 37 °C incubator with 5% CO_2. Carefully replenish media twice a week without disturbing mammosphere formation.

7.3 After incubation, count the number of mammospheres generated in each well using a microscope; where mammospheres are defined as breast cancer cell clusters greater than 100 μm in diameter (representative images shown in **Figure 3B**).

7.4 Calculate the mammosphere formation efficiency (MFE) as follows: MFE (%) = (number of mammospheres per well)/ (number of cells seeded per well) x 100 (i.e., if 5 mammospheres are generated by 1 x 10^2 cells in a well, then MFE = (5/100) x 100 = 5%).

7.5 To subculture mammospheres, carefully transfer the media containing mammospheres content into a fresh 50 mL conical tube and centrifuge media at 1000 x *g* for 5 min. Carefully remove the supernatant, resuspend the cell pellet in 500 μL of trypsin, and incubate for 5 min at room temperature.

7.6 Discard the supernatant and resuspend the pellet in 1 mL of complete mammosphere media. Count the cells using a hemocytometer and re-plate the cells in an ultra-low attachment cell culture plate as described in step 7.1.

NOTE: In addition to sub-culturing, the mammosphere-derived cells can be also analyzed further by FACS to assess BCSC phenotype and/or obtain pure populations of BCSCs for other downstream assays.

7.7 To determine the number of mammosphere-initiating cells contained within your cell populations, use an alternate method involving sphere limiting dilution analysis (SLDA). Plate cells in serial dilutions of high to low cell numbers in a 96 well ultra-low attachment cell culture plate, with the highest dilution resulting in less than one cell per well.

7.8 Incubate the culture plate for 10-14 days in a 37 °C incubator with 5% CO_2 and leave them undisturbed to avoid cell aggregation.

7.9 After incubation, count the number of mammospheres generated in each well using a microscope; where mammospheres are defined as breast cancer cell clusters greater than 100 μm in diameter. Calculate the sphere-initiating frequency and significance using Extreme Limiting Dilution Analysis (ELDA) online software (http://bioinf.wehi.edu.au/software/elda/).

8. 3D culture model

8.1 Depending on the experimental question, use basement membrane extract (BME) with or without growth factors (reduced). In order to evaluate the effect of individual growth factor on cancer cells, use growth factor reduced BME. It also helps in minimizing the non-specific effects of endogenous growth factors present in BME.

NOTE: BME solidifies above 10 °C. Always keep BME on ice even during the thawing step.

8.2 Carefully add 50 µL of BME per well in a 96-well plate without creating air bubbles and allow it to polymerize at 37 °C for 1 h. After 10 min of incubation, add PBS to avoid drying of the gel layer.

8.3 Resuspend the sorted cells from step 5.11 or unsorted cells from steps 3.5 or 4.5 at a concentration of 5 x 10^3 to 5 x 10^4/200 µL in 3D culture media.

8.4 Once the BME has polymerized, remove PBS, add 200 µL of cell suspension to each well and incubate in 37 °C incubator with 5% CO_2. Add PBS to the surrounding wells to avoid evaporation of the media.

NOTE: The optimal number of cells for plating should be determined prior to setting of the experiment. Depending on the experimental question, BCSCs can be cultured alone or with other cells types (fibroblasts/endothelial/immune cells etc.).

8.6 Add fresh media to the culture plates twice weekly. Maintain cultures for 10-14 days prior to analyzing the formation of organoids (representative images shown in **Figure 3C**).

8.7 For sub-culturing, carefully aspirate the media and add 200 µL of dispase to each well containing cells. Incubate the plate in a 37 °C incubator for 1 h. Halfway through the incubation period (30 min), take out the plate, gently pipette the dispase solution up and down 5 times, and place back in the incubator for a further 30 min.

8.8 After 1 h, transfer the dissociated cell solution to a flow tube. Wash the well with 1x PBS containing 2% FBS (fPBS) and transfer it to the flow tube. Centrifuge the tube at 1000 x *g* for 5 min. Carefully aspirate the supernatant and add 500 µL of trypsin, incubate at 37 °C for 5 min. Inactivate trypsin by adding equal amount of fPBS and centrifuge at 1000 x *g* for 5 min.

8.9 Discard the supernatant and resuspend the pellet in 1 mL of 3D culture media. Count the cells and re-plate required number of cells in the BME as in steps 8.2 to 8.4.

NOTE: Multiple wells can be pooled to further analyze or sort the cell population of interest.

(Insert Figure 3 here)

NOTE: Perform animal experiments under an animal ethics protocol approved by the institutional animal care committee.

9. In vivo xenograft model

9.1 In order to determine the tumor initiation capacity of breast cancer stem cells, prepare cells (sorted population from step 5.7 or unsorted populations from steps 3.5 or 4.5) using a limiting dilution approach. Serially dilute cells in PBS using between 1 and 5 different dilution groups, with doses as low as 0.01-0.2 x 10^2 cells/100 µL and as high as 1 x 10^6 cells/100 µL.

NOTE: Unsorted/whole population cells can be used as a control. The number of dilution groups used will depend on the desired scientific outcome (e.g. if only testing tumorgenicity then 1 group at a higher cell number may be used, whereas when calculating tumor-initiating capacity, it is optimal to test 5 limiting dilution doses).

9.2 To generate xenograft models from human breast cancer cells, use immunocompromised female mice (athymic nude [*nu/nu*], nonobese diabetic/severe combined immunodeficient [NOD/SCID] or NOD/SCID IL2γ [NGS] strains).

NOTE: Although a minimum of 4 animals per group can be used, 8-12 animals per group is recommended to obtain robust results particularly for limiting dilution analysis.

9.3 Perform standard mammary fat pad (MFP) injections using 100 µL/mouse of each cell preparation, under sterile conditions in a biosafety cabinet.

NOTE: For optimal breast tumor growth and spontaneous metastasis to distant organs, the thoracic MFP is recommended. Alternatively, the inguinal MFP can also be used.

9.5 Post-injection, monitor the mice on a daily basis for general health and tumor growth at the site of injection. Upon detection of a palpable tumor, begin measuring the tumor size by calipers in two perpendicular dimensions and record weekly until endpoint.

NOTE: The experimental end point is determined based on the regulations laid out the institutional animal ethics protocol; typically, endpoint by euthanasia is usually required once tumor volumes reach 1500 mm^3. For BCSC populations and/or higher cell doses (e.g. >1 x 104 cells), this endpoint will likely be reached within 4-8 weeks of MFP injection. For very low cell doses and/or non-BCSC cell populations, tumor growth should be allowed to progress for up to 8 months post-injection.

9.6 From these measurements, calculate the tumor volume using the following formula: Volume in mm^3 = 0.52 x $(width)^2$ x length. If using a limiting dilution approach, calculate tumor-initiating capacity and significance using ELDA online software (http://bioinf.wehi.edu.au/software/elda/).

9.7 Alternatively, to humanely extend the endpoint, surgically remove primary tumors and continue to monitor mice for health and/or development of spontaneous metastasis in distant organs. Use resected tumor tissue for the generation of serial xenotransplants.

9.8 At endpoint, harvest tissue from primary tumors and distant organs (lymph nodes, lung,

liver, brain, bone) and carry out histopathological and/or immunohistochemical analysis or dissociated the tumor tissue and use in the in vitro assays described in sections 6-8.

REPRESENTATIVE RESULTS:

The described protocol allows isolation of human BCSCs from a heterogenous population of breast cancer cells, either from cell lines or from dissociated tumor tissue. For any given cell line or tissue sample, it is crucial to generate a uniform single cell suspension to isolate BCSCs at maximum purity as contaminating non-BCSC populations could result in variable cellular responses, especially if the study aim is to evaluate the efficacy of therapeutic agents targeting BCSCs. Application of a stringent sorting strategy will minimize the presence of contaminating non-BCSCs and result in the ability to collect the proportion of breast cancer cells with stem cell-like characteristics that display a cellular phenotype that distinguishes them from bulk population of cancer cells. Human breast cancer cells that exhibit enhanced ALDH enzymatic activity, express high levels of the cell surface marker CD44, and low/negative expression of CD24 have an $ALDH^{high}CD44^{+}CD24^{-}$ phenotype and can be classified as BCSCs. The proportion of BCSCs within the bulk population can vary between cell lines or patients (**Figure 2**), and often depends on disease stage, with more aggressive breast cancer usually displaying a higher proportion of BCSCs[26,36,37].

Isolated BCSCs can be used to perform different in vitro and in vivo assays where their behavior and function can be compared to that of the bulk and/or non-BCSC populations. For example, the ability of a single breast cancer cell to self-renew and generate colonies of 50 cells can be assessed by colony-forming assays (**Figure 3A**). The ability of BCSCs to self-renew under anchorage-independent experimental conditions can be assessed by mammosphere assays, where variable sphere number, size, and sphere-initiating capacity can be analyzed and correlated with the presence and function of BCSCs (**Figure 3B**). It is important to determine the seeding cell densities for different breast cancer cell lines or breast tumor samples to obtain optimal results. This is particularly important when performing SLDA, as higher cell densities could lead to cell aggregation resulting in misinterpretation of cellular activity.

Culturing breast cancer cells in BME allows BCSCs to form 3D structures that recapitulate in vivo conditions (**Figure 3C**). 3D culture of breast cancer cells in the presence of other microenvironmental cell types such as fibroblasts, endothelial cells, and/or immune cells has the added capacity for investigating the role of microenvironment in 3D growth of BCSCs[38,39]. The specific cell numbers required to generate 3D organoids may vary depending on the cell line or patient tumor source, and thus it is important to optimize the culture conditions and cell numbers prior to any large-scale experiments.

Finally, in vivo mouse xenograft models can be used to understand the differences in growth (**Figure 4**) self-renewal, differentiation and/or tumor-initiating ability of BCSCs in vivo compared to non-BCSCs or bulk cell populations. Often, the in vitro cellular responses observed in the presence of exogenous factors or therapeutic agents is not representative of in vivo setting, suggesting that in vitro observation should be complimented with in vivo studies whenever feasible. Using in vivo xenograft models, the cellular heterogeneity and tumor architecture is

preserved and thus these models can serve as a system that closely mimics the microenvironment in human patients. In vivo LDA can be performed to determine the proportion of tumor-initiating cells in a given mixed population of cancer cells (BCSCs or non-BCSCs)[40,41]. The range of cell dilutions used should be optimized and will depend on the frequency of initiating cells in the cell population of interest. Ideally these dilutions should include doses that result in 100% tumor formation, down to cell doses with no tumor formation and a reasonable range in between. The frequency of tumor-initiating cells in primary samples can be variable, and in instances where breast tumors have very low numbers or heterogenous populations of tumor-initiating cells, performing LDA can be particularly challenging[42]. In these cases, injecting larger number of cells would be more appropriate for understanding breast cancer biology.

(Insert Figure 4 here)

FIGURE AND TABLE LEGENDS:

Figure 1: **FACS gating strategy for isolation of BCSCs from breast cancer cell lines and tissue samples**. (**A**) Flowchart describing the procedure of BCSC isolation. (**B**) Representative FACS plots showing the sort strategy used to isolate viable BCSCs and non-BCSCs from a heterogenous pool of cells. MDA-MB-231 human breast cancer cells are concurrently labeled with 7-AAD, CD44-APC, CD24-PE and the ALDH substrate. Cell subsets were isolated using a four-color protocol on a FACS machine. Cells are selected based on expected light scatter, then for singlets, and viability based on 7-AAD exclusion. Cells are then analyzed for ALDH activity and the top 20% most positive are selected as the $ALDH^{high}$ population, while the bottom 20% of cells with the lowest ALDH activity were deemed to be $ALDH^{low}$. Finally, 50% of the $ALDH^{low}$ cells are further selected based on a $CD44^{low/-}CD24^{+}$ phenotype, and 50% of the $ALDH^{high}$ cells are selected based on $CD44^{+}CD24^{-}$ phenotype. This figure has been adapted from Chu et al.[17].

Figure 2: **BCSCs proportions are variable in different breast cancer cell lines**. Representative image showing the differential proportion of BCSCs and non-BCSCs in (**A**) SUM159 and (**B**) MDA-MD-468 triple negative breast cancer cell lines following labelling and sorting as described in **Figure 1**.

Figure 3: **In vitro assays to assess BCSC cell function**. In vitro assays were performed as described in protocol sections 6.1 to 6.5 (**A**), 7.1 to 7.4 (**B**), or 81. to 8.4 + 8.6 (**C**). (**A**) Representative image showing the colonies generated by MDA-MB-231 human breast cancer cells; (**B**) Representative images showing mammosphere formation by MCF7, SUM159, or MDA-MB-468 human cell lines as well as patient-derived LRCP17 breast cancer cells. (**C**) Representative images showing the 3D structures formed by MCF7 and MDA-MB-231 breast cancer cells in 3D cultures models.

Figure 4: **In vivo xenograft assays to assess BCSC function.** MDA-MB-231 breast cancer cells were isolated by FACS as described in Figure 1 and injected into the right thoracic mammary fat pad of female NSG mice as described in protocol sections 9.1 to 9.8 ($5x10^5$ cells/mouse; 4 mice/cell population). Primary breast tumor growth kinetics are shown for $ALDH^{hi}CD44^{+}CD24^{-}$ (■) versus $ALDH^{low}CD44^{low/-}CD24^{+}$ (□) populations. Data represented as the mean ± S.E.M. * = significantly different tumor size than respective $ALDH^{low}CD44^{low/-}$ subsets at the same time-point ($P < 0.05$).

This figure has been adapted from Croker et al.[26].

DISCUSSION:

Breast cancer metastasis and resistance to therapy have become major cause of mortality in women worldwide. The existence of a sub-population of breast cancer stem cells (BCSCs) contributes to enhanced metastasis[26,43-46] and therapy resistance[21,47,48]. Therefore, the focus of future treatments should aim at eradicating BCSCs to achieve better treatment outcomes, and this requires accurate methods for isolating and characterizing the functional characteristics of BCSCs using both in vitro and in vivo methods.

Immortalized cell lines derived from different subtypes of breast cancer have proven to be feasible models to study breast cancer biology including the isolation and characterization of BCSCs[26,49,50]. The high proliferative capacity and unlimited expansion ability of cell lines provides an ideal model system for performing studies that are highly reproducible and technically straightforward. However, due to the clonal origin of cell lines, they may fail to recapitulate the heterogeneity exhibited by different patients and/or by cancer cells within tumor tissue. In addition, genetic alterations can be acquired during serial passaging of cell lines and may induce genotypic or phenotypic changes that can confound experimental results[51]. In contrast, primary patient-derived cells, despite their limited proliferative and expansion ability, may provide a more accurate model to that observed in vivo. However, such samples may be more difficult to acquire and be more technically challenging to work with. All of these factors should be considered when choosing a starting model system with which to isolate and characterize BCSCs.

FACS is a commonly used technique to isolate cells of interest based on cell surface marker expression[52,53]. Based on cell surface antigens (CD44 and CD24) and ALDH enzymatic activity, human BCSCs can be isolated at high purity from both breast cancer cell lines and tumor tissues[1,2]. The sorting efficiency determines the purity of sorted sample, and it is recommended that users analyze a small portion of sorted sample incubated with viability dye to check the efficiency of sorting[53,54]. The sorting efficiency can be confounded by many factors including the presence of cell clumps, a high number of dead or dying cells, improper compensation of the fluorochromes and/or damage to cell surface antigens due to sensitivity to trypsin or collagenase during pre-sorting dissociation steps[53-55]. Therefore, generation of a proper single cell suspension and use of appropriate cell dissociation techniques will increase the sorting efficiency. While performing multiparameter cell sorting, it is important to choose fluorochromes that minimizes spectral overlap. In some cases, where spectral overlap cannot be avoided, a control that contains all the fluorochromes except one (fluorescence minus one, FMO) should be used to minimize the spillover of fluorescent signals into other channels[54]. Alternatively, the spectral overlap can reduced by immunomagnetically isolating cell populations prior to final FACS isolation of cells of interest[56].

In vitro assays such as the colony-forming and mammosphere assays described in this protocol have been extensively used to study the self-renewal and proliferative ability of BCSCs[57-62]. Additionally, these assays can be used to assess the activity of different therapeutic drugs on BCSC function. Several evolutionarily conserved signaling pathways have been implemented in

BCSC maintenance[63], and both colony-forming[64-66] and mammosphere assays[64,67] have been used to assess the value of therapeutic disruption of these pathways as an intervention to block BCSC intrinsic signaling and reduce BCSC activity and disease progression. Colony forming assay using primary cells can be challenging due to low cell density, variation between samples and lack of its adaptability to isolated in vitro conditions. These challenges can be overcome by culturing BCSCs on a soft agar layer or by coculturing them with fibroblasts on a collagen-coated cell culture dish[68-70]. In addition, supplementing growth factors into the culture media (such as FGF7[71]) could also improve the colony-forming ability of cells isolated from tissue samples. In addition, over-digestion of tissue using collagenase or trypsin during single cell suspension generation step can result in low to zero colony-forming ability and reduce mammosphere-forming efficiency[31]. In both assays, care should be taken to incubate the assay plates undisturbed to avoid disruption of colony or sphere structures as they are forming. It is also recommended that users extend the incubation period for primary cells (relative to cell lines) as it might take longer for these cells to form colonies or spheres.

Multiple lines of evidence have demonstrated the critical role of extracellular matrix (ECM)[15,17,72] and stromal components, such as fibroblasts, immune cells, endothelial cells and adipocytes in influencing BCSC functions[15]. Thus, the 3D culture model we describe in this protocol can provide a useful experimental system for helping to recapitulate the in vivo tumor microenvironment in an in vitro setting. Although the 3D culture system closely resembles the tumor microenvironment in cancer patients, long term maintenance of cells as organoids can be difficult. In addition, optimization of the 3D culture conditions and the ability to accurately investigate self-renewal and differentiation ability of BCSCs is challenging[73]. The efficiency of organoids formed in 3D culture system depends on the growth factors supplemented in the culture media[74]. Absence of key components (for example, ROCK inhibitor) could lead to reduced or no organoid formation[74]. Media should be replenished every 3-4 days to maintain optimal cellular function and the sustainability of the culture. In order to recapitulate in vivo conditions and response, it is always important to allow the cells to form organoids prior to any kind of exogenous treatment[75]. Cells derived from patient samples should be giving sufficient time to form organoids, particularly if the objective is the evaluate drug response[75].

While these in vitro methods are attractive and accessible experimental tools for characterizing BCSC function, tumor heterogeneity and the effect of tumor microenvironment on BCSC behavior cannot be studied with complete effectiveness. These in vitro assays should therefore be complemented with in vivo xenograft models whenever feasible in order to further validate experimental findings related to BSCS biology and/or response to novel therapeutics. Different in vivo models have been used study BCSC tumorigenicity and metastasis. Ectopic (subcutaneous engraftment) and orthotopic (MFP engraftment) mouse models have been used to generate breast tumors and assess longitudinal changes in tumor growth over time[50]. Although both in vivo injection approaches can be used to study BCSC biology, the native stromal and vasculature-related components of the MFP allow more accurate recapitulation of primary breast tumor progression as observed in patients, and thus MFP injection is preferred[76,77,78]. Finally, the use of immunocompromised mice is required for engraftment of human BCSCs and tumor growth, and this prevents incorporating the role of immune cells in tumorigenesis and metastasis studies[79].

More recently, this limitation has been addressed through the use of humanized mice in which a human immune system is reconstituted via bone marrow transplantation prior to the initiation of xenograft studies[80-82]. However, these models are expensive and technically challenging, and thus are still not commonly used[83].

In summary, here we have provided a protocol for the isolation of human BCSCs from both breast cancer cell lines and patient-derived tumor tissue samples. We have also described in vitro and in vivo protocols for downstream assays that can be used to study BCSC function, with the ability to be optimized for different breast cancer cell sources and the flexibility to be performed under different experimental conditions. These protocols will be useful for investigators interested in cancer stem cells, breast cancer biology and therapeutic development, with the ultimate goal of improving patient outcomes in the future.

ACKNOWLEDGMENTS:

We thank members of our laboratory for their helpful discussions and support. Our research on breast cancer stem cells and the tumor microenvironment is funded by grants from the Canadian Cancer Research Society Research Institute and the U.S. Army Department of Defense Breast Cancer Program (Grant # BC160912). V.B. is supported by a Western Postdoctoral Fellowship (Western University), and both A.L.A. and V.B. are supported by the Breast Cancer Society of Canada. C.L. is supported by a Vanier Canada Graduate Scholarship from the Government of Canada.

DISCLOSURES:

The authors have nothing to disclose.

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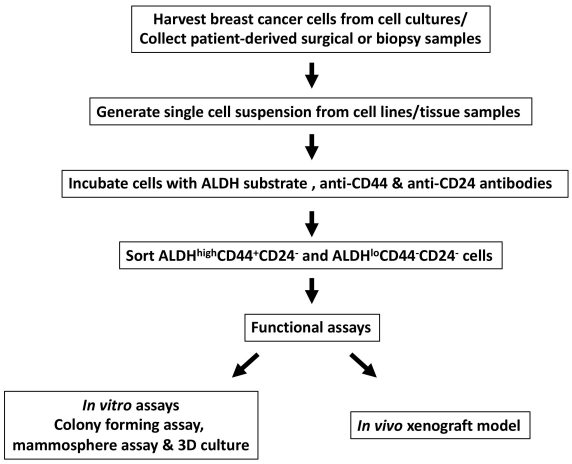
Figure 1

A



B

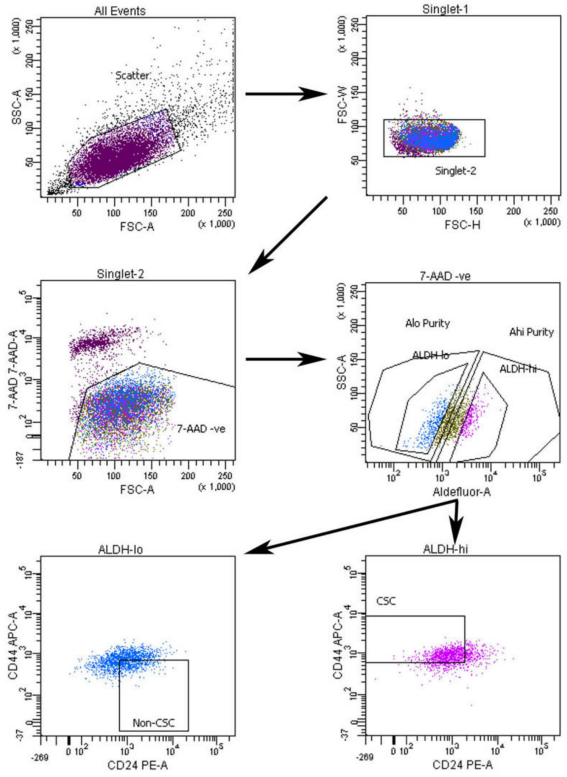


Figure 2

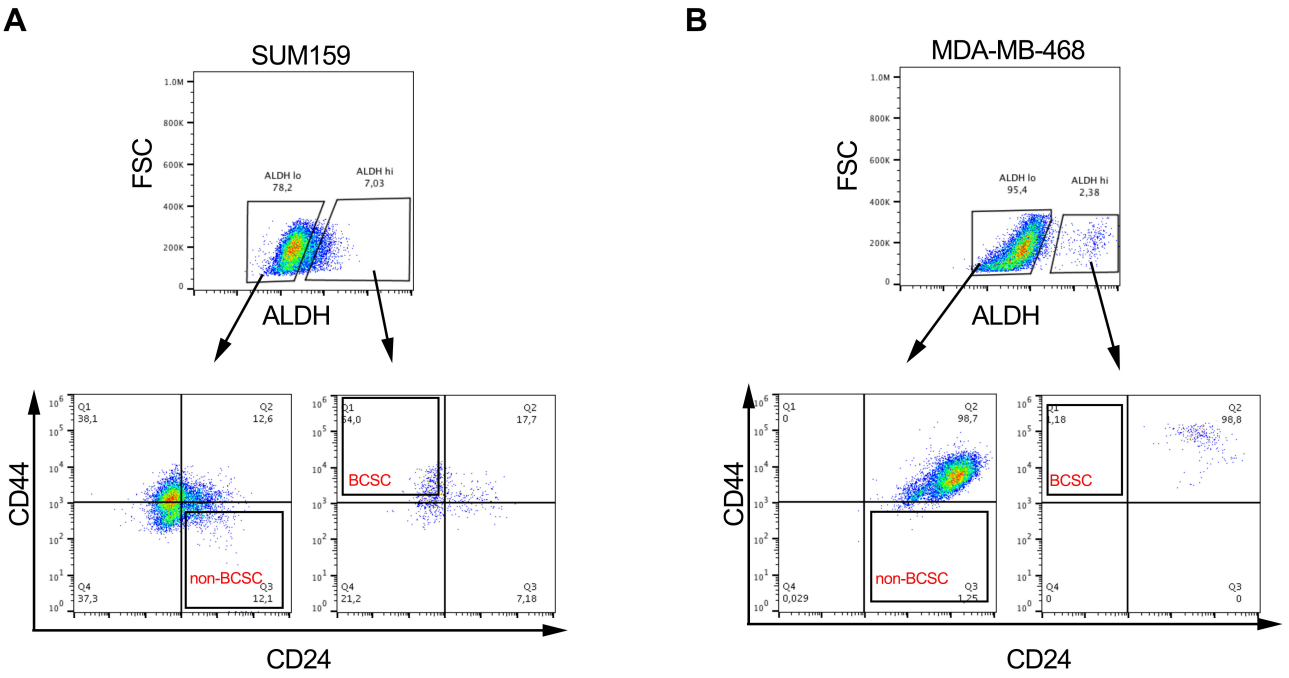


Figure 3

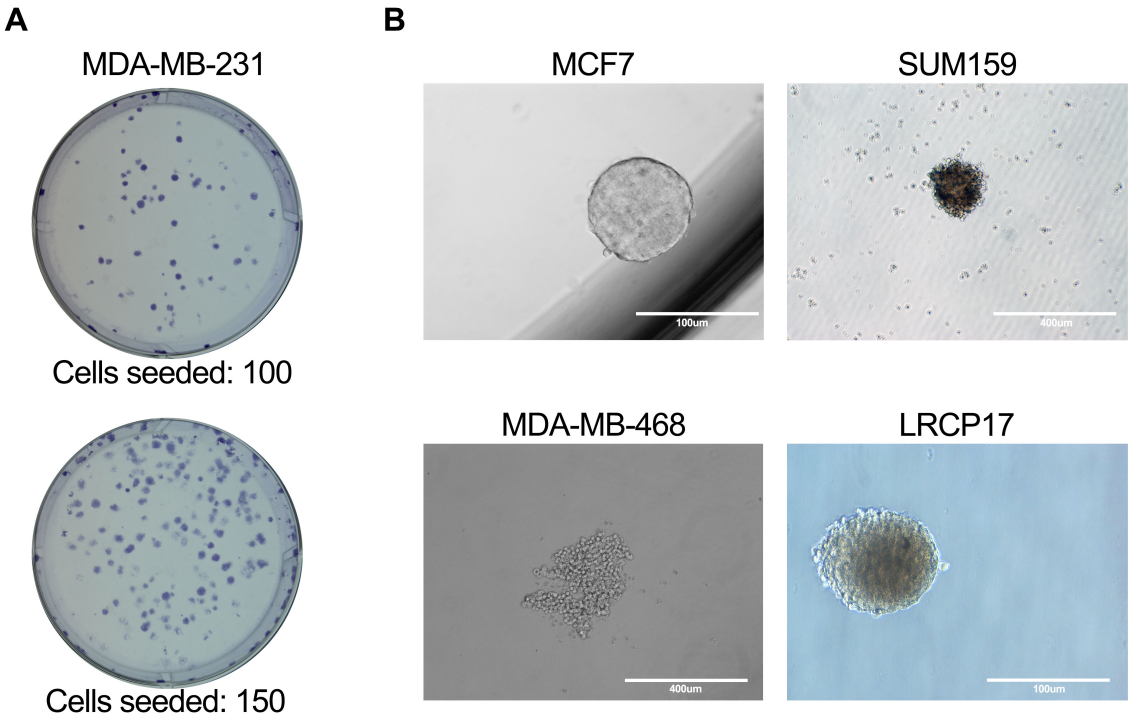
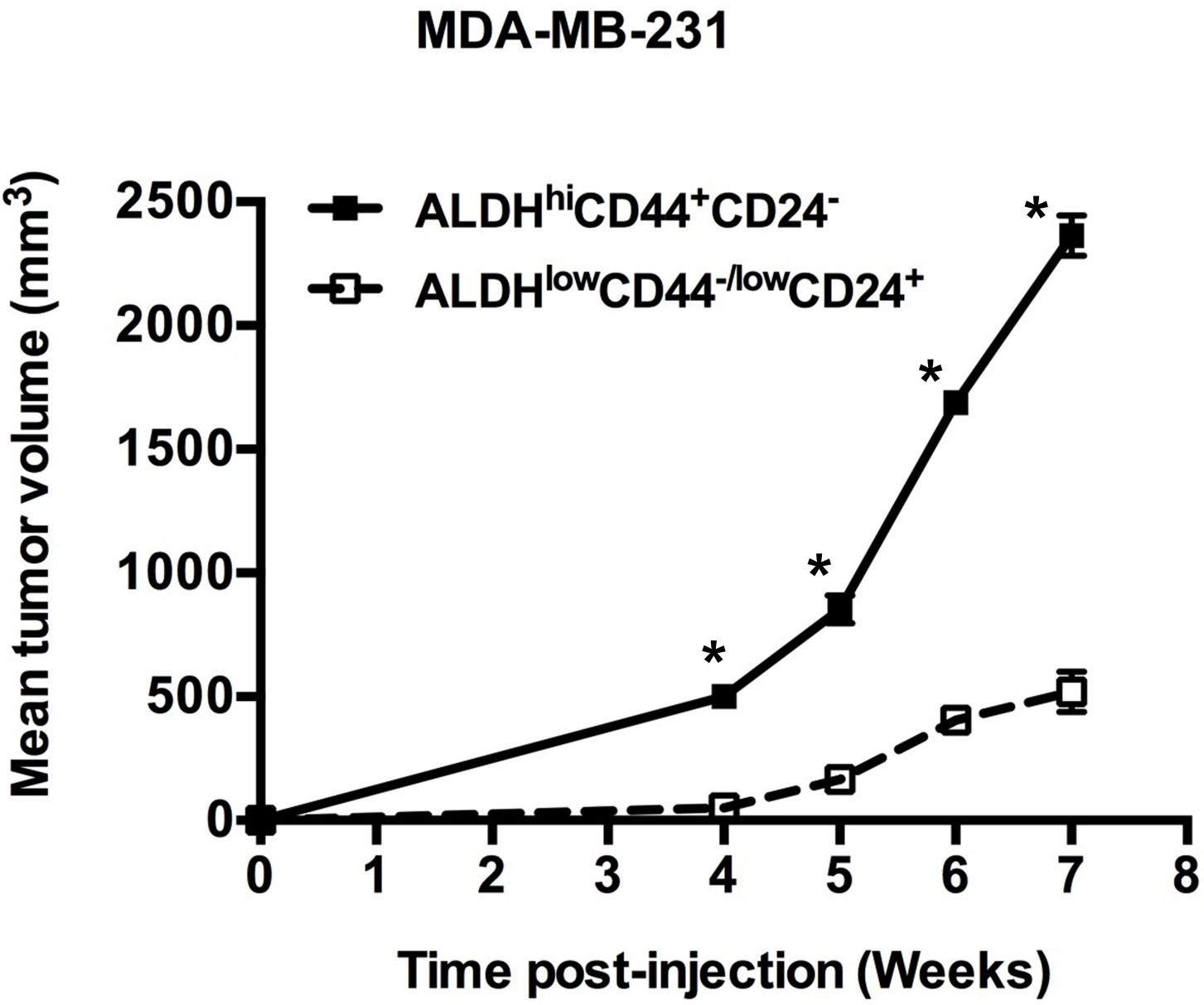


Figure 4



Name of Material/ Equipment	Company	Catalog Number
7-Aminoactinomycin D (7AAD)	BD	51-68981E
Acetone	Fisher	A18-1
Aldehyde dehydrogenase (ALDH) substrate	Stemcell Technologies	1700
Basement membrane extract (BME)	Corning	354234
Cell culture plates: 6 well	Corning	877218
Cell culture plates: 60mm	Corning	353002
Cell culture plates: 96-well ultra low attachment	Corning	3474
Cell strainer: 40 micron	BD	352340
Collagen	Stemcell Technologies	7001
Collagenase	Sigma	11088807001
Conical tubes: 50 mL	Fisher scientific	05-539-7
Crystal violet	Sigma	C6158
Dispase	Stemcell Technologies	7913
DMEM:F12	Gibco	11330-032
DNAse	Sigma	D5052
FBS	Avantor Seradigm Lifescience	97068-085
Flow tubes: 5ml	BD	352063
Methanol	Fisher	84124
mouse anti-Human CD24 antibody	BD	561646
mouse anti-Human CD44 antibody	BD	555479
N,N-diethylaminobenzaldehyde (DEAB)	Stemcell Technologies	1700
PBS	Wisent Inc	311-425-CL
Trypsin-EDTA	Gibco	25200-056
Mammosphere Media Composition		
B27	Gibco	17504-44
bFGF	Sigma	F2006
BSA	Bioshop	ALB003

DMEM:F12	Gibco	11330-032
EGF	Sigma	E9644
Insulin	Sigma	16634

3D Organoid Media Composition

A8301	Tocris	2939
B27	Gibco	17504-44
DMEM:F12	Gibco	11330-032
EGF	Sigma	E9644
FGF10	Peprotech	100-26
FGF7	Peprotech	100-19
GlutaMax	Invitrogen	35050-061
HEPES	Gibco	15630-080
N-acetylcysteine	Sigma	A9165
Neuregulin β1	Peprotech	100-03
Nicotinamide	Sigma	N0636
Noggin	Peprotech	120-10C
R-spondin3	R&D	3500
SB202190	Sigma	S7067
Y-27632	Tocris	1254

Comments/Description/Final Concentration

suggested: 0.25 µg/1x10^6 cells

Sold commerically as part of the ALDEFLOUR Assay kit; follow manufacturer's instructions for ALDH substrate preparation
Sold under the commercial name Matrigel

Prepare 1:30 dilution of 3 mg/mL collagen in PBS
1x

Use 0.05% crystal violet solution in water for staining
5U/mL
1x, With L-glutamine and 15 mM HEPES
0.1 mg/mL final concentration

Polypropylene round-bottom tubes

R-phycoerythrin and Cyanine dye conjugated Clone: ML5
R-phycoerythrin conjugated, Clone: G44-26
Sold commerically as part of the ALDEFLOUR Assay kit; follow manufacturer's instructions DEAB preparation
1x, Without calcium and magnesium

1x
10 ng/mL
0.4%

1x, With L-glutamine and 15 mM HEPES
20 ng/mL
5 µg/mL

500 nM
1x
1x, With L-glutamine and 15 mM HEPES
5 ng/mL
20 ng/mL
5 ng/mL
1x
10 mM
1.25 mM
5 nM
5 mM
100 ng/mL
250 ng/mL
500 nM
5 µM

23 August 2020

Dear Dr. Bajaj:

We thank the editor and reviewers for taking time to evaluate our manuscript and provide constructive feedbacks to improve this protocol paper. We have carefully considered all the editorial and reviewers' comments and these have been addressed in the revised manuscript as described in detail below.

Sincerely,
Dr. Alison Allan
Professor, Dept. of Anatomy and Cell Biology
University of Western Ontario

A. Editorial Comments

1. ***"Protocol Language:*** *Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Some examples NOT in the imperative: Lines 104–125, step 7.4, etc*"

Response: The text in the protocol section has been carefully reviewed and revised to ensure that it is written in the imperative voice/tense.

2. ***"Protocol Detail****: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.*
   1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the guidelines of your institutions human research ethics committee.
   2) 5.7–5.11: Provide detailed software steps such as button clicks and menu selections.
   3) 6.6: Mention magnification".

Response:
1) The ethical statements have been added to the protocol to indicate that the protocol follows the guidelines of our institutional human and animal research ethics committee (Line 221-226, and Lines 571-572 in the revised manuscript.
2) During cell sorting experiment, the software and commands used for setting up the sort controls, gates and display plots varies from instrument to instrument depending on the model and manufacturer. therefore, we have not mentioned these details in the current protocol as they would vary from user to user.
3) The magnification has now been included in Step 6.6 in the revised manuscript.

3. ***"Figures**: Fig 4: define the error bars*".

Response: The error bars have now been defined in the Figure 4 legend.

4. ***"References:** Please spell out journal names"*

Response: The full journal names are now included in the Reference section.

5. "***Commercial Language**: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are ALDEFLOUR, Matrigel, etc. Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names".*

Response: The commercial terms have been replaced with generic names and specific commercial products have been referenced in the Table of Materials.

6. ***"Table of Materials:** Please sort in alphabetical order".*

Response: The Table of Materials has now been sorted in alphabetical order.

B. Reviewers' Comments

Reviewer #1:

1. *"Trypsin should be replaced with a different reagent such as TriPLE or Accutase that preserve cells surface protein digestion".*

Response: We agree with this suggestion. However, as per the journal's formatting guidelines, commercial reagent names cannot be used in the protocol and thus we have refrained from mentioning commercial reagent names.

2. *"The protocol for the generation of single suspension from tissue samples should be performed in just one step using the collagenase and DNase combined possibly".*

Response: We thank the reviewer for this suggestion. Although DNase could be used with collagenase during tissue digestion process, in our experience the DNase is much more effectively used just prior to staining for cell sorting in order to ensure the greatest prevention of cell clumping and non-specific uptake of 7AAD dye that can reduce the efficiency of sorting process.

3. *"Centrifuge speed should be reduced from 1000g to 500-750g".*

Response: We thank the reviewer for this suggestion. In optimizing our protocol, we have observed that centrifugation at 1000 x *g* (compared to lower speeds of 500-750 x g) improves overall cell recovery without affecting cell viability. Therefore, we perform cell pelleting at 1000 x *g*.

4. *"Gating boundaries should be determined using FMO gating controls"*.

Response: We apologize for omitting this detail from the protocol. We have now included this as part of the description of the gating strategy in steps 5.1 and 5.8 of the protocol in the revised manuscript.

5. *"I suggest to improve the quality of the image in Fig.3. The scale is incorrect and MDA-MB-468 cultures do not represent spheres. They seem to be more similar to aggregates of few cells".*

Response: We have improved the quality of the image for the MBA-MB-468 mammosphere image and ensured that the scale bars are accurate. The reason that we included images of mammospheres generated from different breast cancer cell lines and PDXs was to highlight the heterogeneity in size and cellular aggregation patterns across cell lines. In the case of MDA-MB-468 cells, these images are representative of how these cells grow under mammosphere-forming conditions.

Reviewer #2:

1. "*Some protocols are not well described and lack repeatability. In some functional identification assays, the composition of media was not clear enough. For example, what cytokines have been added? Why are these cytokines added*?" .

Response: As per the journal's formatting guidelines; the details of media compositions including cytokines are described in full in the Table of Materials. The media compositions are based on previously published literature which describes the rationale for the media composition in detail. The 3D organoid media composition is based on Sachs et al., 2018 (Reference 74). The mammosphere media composition is based on Ponti et al., 2005 (Reference 62).

2. *"It is not clear whether the cells need to be replenished with culture medium during the long incubation period of the colony forming assay and mammosphere assay".*

Response: We apologize for omission of this detail. The culture medium should be replenished twice per week for the colony forming assay (without fibroblasts) and mammosphere assay. For the colony forming assays with fibroblasts, media is not replenished. This has now been detailed in protocol steps 6.4, 7.2, and 6.12 respectively.

3. "*The presentation of cell concentration and density in some protocols is not standard and inconsistent*".

Response: We have corrected the presentation of cell concentration to be consistent throughout the protocol.

4. *"Some of the reagents used in the experiment are missing key information, such as the manufacturer, model and item number"*.

Response: As per the journal's formatting guidelines, all detailed information for the reagents and materials is presented in the Table of Materials.

Reviewer #3:

1. *"In section 3, subsection 3.1, the authors have mentioned use of trypsin or EDTA. It is usually Trypsin-EDTA that is generally used. Also, why do they incubate at room temperature and not 37˚C"*.

Response: We thank the reviewer for this suggestion and we have now changed the recommended dissociation reagent to Trypsin-EDTA in protocol step 3.1 in the revised manuscript. We prefer performing cell dissociation at room temperature in order to avoid excess damage to the cell membrane that could occur if cells are left for too long at 37˚C in the presence of trypsin.

2. *"Change PE-cy7 to PE-Cy7".*

Response: We have now made this change.

3. *"It would be better if the authors could use a flowchart or some kind of a schematic for the procedure of BCSC isolation"*.

Response: We thank the reviewer for the suggestion. A flowchart depicting the BCSC isolation procedure has now been added as Figure 1A.

4. *"There are no isotype controls used for the CD44-PE and CD24-PE-Cy7. How is it gated in the flow cytometer without these controls"?*

Response: The positive signals generated by stained cells are gated based on unstained controls, single stained controls and fluorescence minus one (FMO) controls rather than isotype controls as we find this approach is more robust and reproducible. The recommended antibodies used for staining are clinical-grade antibodies and their specificity has been tested and confirmed.

5. *"In Section 7 Subsection 7.3, the authors have used 40 µm as a cut off diameter for the assessment of mammospheres. Did the authors optimize this diameter based on the cell line or was it a generalized cut-off diameter? We typically employ 100 µm as the cut off."*

Response: We apologize for this error. We have corrected the cut off diameter to 100 µm in Steps 7.3 and 7.9 of the protocol in the revised manuscript.

6. *"Do the authors have any other validations on BCSCs, such as Western blots or immunofluorescence data for the pluripotency markers SOX2, OCT4, NANOG".*

Response: We thank the reviewer for this suggestion and have now added it at a note to the protocol (Note 5; Lines 206-208) in the revised manuscript.

7. *"In 7.6 Change FACs to FACS".*

Response: This has now been changed.

8. *"9.11- The authors say a serial dilution of 1-20 cells/ml. How long do they wait for tumor initiation? What kind of controls would you use for this experiment, since non-BCSCs are unable to initiate tumor at such low concentrations"?*

Response: Unsorted breast cancer cells can be used as a control to compare the tumor initiation ability of BCSCs in xenograft models, now detailed in Note 11 of the protocol (Lines 326-329). The experimental end point is determined based on the regulations laid out the institutional animal ethics protocol; typically, endpoint by euthanasia is usually required once tumor volumes reach 1500 mm^3. For BCSC populations and/or higher cell doses (e.g. >1 x 10^4 cells), this endpoint will likely be reached within 4-8 weeks of MFP injection. For non-BCSC cell populations and/or very low cell doses (e.g. 0.01-0.2 x 10^2 cells/mouse), tumor initiation/growth should be assessed for up to 8 months post-injection. This is now detailed in Note 14 (Lines 342-347) of the revised manuscript.

9. *"Also, in Figure 4, the authors have shown a figure that has been adapted from a previous publication Croker et al. for in vivo studies. This study has used 5x 105 cells/ml which is very different from what the authors have suggested".*

Response: We have now revised Section 9 of the protocol to provide more details about the cell range that can be used for limiting dilution analysis (as low as 0.01-0.2 x 10^2 cells/mouse, up to 1 x 10^6 cells/mouse); now detailed in Step 9.2 of the protocol. The publication by *Croker et. al* was aimed at assessed simple differences in tumorgenicity and metastasis between BCSC and non-BCSC cell populations (rather than tumor initiating capacity) and therefore used a single cell dose of 5 x 10^5 cells/mouse of each sorted population. This is within the cell dose range described in the protocol.

10. *"Also, the authors need to make sure that they have proper permissions for publishing these results as it could be copyright infringement, even if it is from the same group".*

Response: We have received copyright permission for publishing these results in this JoVE manuscript, and these permissions are now included as part of the supplemental information.

11. *"Do the authors have better images for the mammospheres with a higher magnification?"*

Response: Higher magnification images have been added wherever possible. Unfortunately, generating new figures has become difficult due to the current COVID19 pandemic situation.

12. *"Section 5.5, change 7ADD-7AAD".*

Response: This has now been corrected.

13. *"Do the authors have data on the primary and secondary mammosphere formation abilities of the cells that have been sorted?"*

Response: Unfortunately, the unpublished data on the primary and secondary mammosphere formation generated in our lab cannot be used in this manuscript as it is earmarked for another publication in preparation. Generating a new dataset for this purpose has become difficult due to the current COVID19 pandemic situation.

14. *"Section 6-Would it be possible for the authors to explain the rationale and references for colony formation assay with fibroblasts. What is the purpose of using fibroblasts in this assay?"*

Response: Fibroblast cultures can act as a feeder layer to provide additional microenvironmental support for BCSCs through production of necessary growth and survival factors. Fibroblast feeder layers have been used to study colony forming ability of adult stem/progenitor cells as well as cancer stem cells; e.g. as described in References 68-70.

15. *"The author's section on in vivo xenograft model (section 9) focuses more on tumor cells than breast cancer stem cells. This section is redundant as there are previous JOVE publications that demonstrate mammary fat pad injection. The authors should elaborate on the procedures pertaining specifically to BCSCs".*

Response: We thank the reviewer for this suggestion. Section 9 of the protocol has now been changed to make it more specific to generation of xenograft models using BCSC.

16. *"In the figure legend for figure 3C, include MDA-MB-231"*

Response: The figure legend for Figure 3 has been updated to include this information.

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Expected publication date	Aug 2020
Portions	Figure 8, panel A
Requestor Location	Alison Allan London Regional Cancer Program 800 Commissioners Road East P.O. Box 5010 STN B London, ON N6A 5W9 Canada Attn: Alison Allan
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