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The use of mouse mammary tumor cells in an in vitro invasion assay as a measure of oncogenic cell behavior

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To JoVE Editorial Staff,

Thank you very much for inviting me to submit an article for consideration to JoVE. Over the years I have found the videos and articles in your journal very useful for my own research as well as for instructional use in the classroom. I believe my article entitled '**The use of mouse mammary tumor cells in the transwell invasion assay as a measure of aggressive cell behavior and metastasis potential'** will be a good fit for your journal and format. In this article I present the procedure for doing a transwell invasion assay in the context of using this assay to study cancer cell behavior and early stages of the metastasis pathway. I also provide ideas for other applications that may be useful for readers since this method is very versatile and adaptable to areas of cell and molecular biology.

I have some names of potential reviewers who are familiar with this field and for whom I have great respect if you are interested in asking them to review: Stephen P. Ethier, Ph.D. MUSC, Gilbert Howlett Smith, Ph.D. NIH, Lewis Chodosh M.D. Ph.D. UPenn, and Joseph R. Testa, PhD Fox Chase Cancer Center.

Thank you very much for your consideration. Feel free to contact me with any questions.

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TITLE:

The Use of Mouse Mammary Tumor Cells in an In Vitro Invasion Assay as a Measure of Oncogenic Cell Behavior

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KEYWORDS:

Cell Migration, Cell Invasion, Cell Behavior, Metastasis, Boyden Chamber, Breast Cancer, Oncogene

SHORT ABSTRACT:

The in vitro cell invasion assay is used to measure the potential of cancer metastasis by quantifying the cellular potential for invasion and migration using cell culture inserts containing protein matrix. Cells are challenged to migrate through the protein matrix and a porous membrane, towards a chemoattractant, and then quantified by light microscopy.

LONG ABSTRACT:

The in vitro invasion assay uses a protein-rich matrix in a Boyden chamber to measure the ability of cultured cells to pass through the matrix and a porous membrane in a process analogous to the initial steps of cancer cell metastasis. The tested cells can be altered for the gene expression or treated with inhibitors to test for changes in the invasion potential. This experiment tests the aggressive phenotype of the mouse mammary tumor cells to discover and characterize the potential oncogenes that promote cell invasion. This technique, however, can be versatile and adapted to many different applications. The experiment itself can be done in one day and the results are acquired by light microscopy in less than a day. The results include counts of the number of invading cells for comparison and analysis. The in vitro invasion assay is a rapid, inexpensive, and clear-cut method for determining cell behavior in a culture that can be used as an initial assessment before more involved in vivo assays.

INTRODUCTION:

The in vitro invasion assay can be a useful tool when measuring a cell's ability to migrate through a protein-coated membrane, analogous to the first steps in metastasis. A key feature of malignant cancer cells is their ability to migrate through and invade nearby tissues. Cancer that has spread or metastasized poses more treatment challenges and has lower rates of long-term survival, while localized tumors are easier to treat and have higher rates of long-term survival. In order to

metastasize, cancer cells must leave the primary tumor and migrate into the circulatory or lymphatic system, a process which requires passing through the extracellular matrix and basement membrane[1]. In the process called the epithelial mesenchymal transition (EMT), the tumor cells must break cell-cell contacts, migrate directionally, and invade nearby blood or lymph vessels. The initial steps of this metastasis cascade are of great interest since these steps are what can make cancer deadlier. The genetic and epigenetic factors involved in the early steps of metastasis are the focus of a great amount of research, but accurate and reliable experimental tools are needed to test these early steps both in vivo and in vitro.

Tools to measure changes in cell migration such as wound healing (scratch) assays or growth in 3D environments such as soft agar assays can partially address the need for experimental methods of measuring early steps of metastasis, but an assay to measure invasion is more challenging since the process occurs in the body within a complex tumor microenvironment. For the purposes of screening drugs or gene alterations to determine important factors in invasion and metastasis, a system that can be used in vitro with cultured cells and mimic the challenges faced by metastatic cells in vivo is the invasion assay[2,3]. Breast cancer is the most commonly diagnosed type of cancer in women and the second leading cause of cancer death in women, so understanding the genes responsible for breast cancer cell invasion and metastasis is critically important for public health. Moreover, mouse cells are a useful model system for studying breast cancer and its progression.

The in vitro invasion assay is based on the Boyden Chamber assembly where two chambers of growth media are separated by a porous membrane[3]. To mimic the tumor microenvironment, a protein-rich gel is also included to separate cells in one chamber from a chemoattractant in the other and act as a basement membrane barrier. In order to migrate towards the chemoattractant, cells must first pass through the protein-rich barrier then pass through the porous membrane – a process analogous to how metastatic cells migrate through stroma. The protein-rich gel can be altered based on the needs of the experiment, but usually consists of collagen, or basement membrane extract (e.g., Matrigel)[4]. It is a complex mixture of proteins, proteoglycans and growth factors, but mostly consists of laminins and collagen IV [4,5]. Cells must then pass through a porous membrane typically made of polycarbonate, polyester, or polytetrafluoroethylene (PTFE). Membranes may be purchased commercially with or without a protein gel (typically collagens), or the gel may be purchased separately and added. The pore size can be adjusted based on the cell size. While pore sizes are available from 0.4 - 8.0 µm, only pores from 3.0 - 8.0 µm are large enough for cell migration. The invasion assay has been used to determine the effectiveness of inhibitors on the ability of cells to migrate and invade. While lacking the exact tumor microenvironment that is present in vivo, the in vitro invasion assay is beneficial at screening many conditions in a short time while minimizing the need for animal models. The goal of these experiments is to compare gene expression of suspected oncogenes and determine the effects on cancer cell behavior and disease aggressiveness using the in vitro invasion assay and other tests. Overall, the invasion assay provides consistent, quantitative, and rapid results for determining metastatic potential while also being a relatively inexpensive, straightforward, and adaptable method.

PROTOCOL:

All experiments and methods were performed as authorized by Villanova University Institutional Animal Care and Use Committee (IACUC).

1. Gene expression in cultured mouse mammary tumor cells

1.1 First, prepare the cell lines to be tested.

1.2 Use a breeding colony of BALB/cV mice. These mice carry the BALB/cV strain of mouse mammary tumor virus, transmitted to pups in milk[6,7]. Fifty percent of breeding females develop mammary tumors by 10 months of age.

1.2.1 To establish a tumor cell line, sacrifice a tumor-bearing dam using an IACUC-approved protocol. Soak the abdominal skin in 70% EtOH and remove the tumor under sterile conditions in a laminar flow hood. Tumors are subcutaneous, so take care to avoid puncturing the peritoneum.

1.2.2 Place tumor fragments from non-necrotic areas in a petri dish with a small amount of sterile Hanks Balanced Saline Solution (HBSS) and mince very finely with a sterile razor blade.

1.2.3 Transfer the dispersed cell clumps to a T25 cell culture flask containing 5 mL Dulbecco's Modified Eagle Medium or DMEM (4.5 g/L glucose, phenol red, and L-glutamine) supplemented with 50% Fetal Bovine Serum or FBS and 1% antibiotic/antimycotic solution. Grow cells in treated cell culture flasks in an incubator with 5% CO_2 and 100% humidity at 37 °C.

1.2.4 Wash off non-adherent cells after 24-48 h and replace the DMEM with 50% FBS and 1% antibiotic/antimycotic solution. Return the cells to the incubator.

1.2.5 Replace liquid culture media every 3 days.

1.2.6 Split or passage the cells when they are 90% confluent. Remove culture media and wash adherent cells with HBSS (without calcium or magnesium). Incubate cells with 0.25% Trypsin-EDTA solution for 1-5 min at 37 °C until cells are rounded and detached. Dilute cells 1:10 in fresh culture media and add to a new flask. A T-25 cell culture flask requires 5-8 mL culture media, 5 mL of HBSS to wash, and 1 mL of trypsin-EDTA to passage. Return the flask to the incubator.

1.2.7 Reduce the FBS concentration to 40% after the first passage, then 30% after the second passage, then 20% after the third passage, and finally 10% for all subsequent passages. The process of establishing growth in standard DMEM medium with 10% FBS requires four passages and 2 - 8 months.

1.3 Once the cell line(s) are established, alter the expression of suspected oncogenes by transfection of shRNA targeting mRNA or CRISPR for non-essential genes.

1.3.1 Establish antibiotic-resistant stable cell lines with individual clones that are verified by western blot and/or RT-qPCR for consistent results, however, transient transfections can work as well since the assay only requires 22 h. Control cell lines with non-specific target sequences are also required.

2. In vitro invasion assay

2.1 Grow adherent mouse mammary tumor cells in a T25 flask until 70-90% confluent for Day 1 of the experiment.

NOTE: Other cell lines or experimental conditions may require different cell culture media. For example, if hormone-responsive breast cancer cells are being tested, charcoal-filtered serum may be needed to remove compounds that activate estrogen or other hormone receptors.

2.2 Prepare the Boyden chamber inserts by warming them to room temperature (from -20 °C storage) for about 20 min in a cell culture hood. Avoid freeze-thaw cycles for unused inserts. Use three inserts (replicates) to generate statistically valid data for each cell line for each experiment.

2.2.1 Add pre-warmed 37 °C serum-free DMEM media to the well and then the insert. For inserts designed for 24-well dishes, add 500 µL of serum-free DMEM to the well and then 500 µL of serum-free DMEM to the insert so the porous membrane and gel are hydrated on both sides.

NOTE: For larger inserts designed for a 6-well dish, use 2 mL of serum-free DMEM on both sides.

2.3 Place the dish with inserts into a 37 °C cell culture incubator with 5% CO_2 and 100% humidity for at least 2 h to thoroughly hydrate and acclimate.

2.4 Prepare cells by removing the growth media and rinsing the cells with 5 mL HBSS.

2.4.1 Remove the HBSS and add 1 mL 0.25% trypsin-EDTA solution for 1 - 5 min at 37 °C or until cells appear rounded or show signs of detachment from the flask.

2.4.2 Gently tap the flask to detach all the cells. Resuspend the cells in 5 mL of DMEM + 10% FBS and transfer to a sterile 15 mL centrifuge tube.

2.4.3 Centrifuge the cells at 1,000 *x g* for 5 min to gently pellet the cells. Remove the media and replace with 5 mL serum-free DMEM to resuspend the cells.

3.4.4 Repeat the centrifugation and suspension in serum-free media twice more for a total of 3 washes.

2.4.5 Thoroughly resuspend the cells in the final 5 mL of serum-free DMEM and ensure there are no clumps of cells.

2.5 Determine the cell concentration using a hemocytometer. Count only viable cells. Dilute the cell suspension to 5 x 10^4 cells/mL in 5 mL of serum-free DMEM.

NOTE: This concentration yields 2,500 cells per insert in a 24-well dish. This number of cells is enough for aggressive cancer cells with high rates of migration and invasion. Less aggressive cell lines may require more cells, up to 10,000 cells per insert for observable invading cells. If reduced cell invasion is expected, consider maximizing invading cells by increasing cell number. If increased cell invasion is expected, begin with fewer cells.

2.6 Remove the cell culture dish from the incubator and gently aspirate the media from the insert. Lift the insert and aspirate the media from the well. Working quickly, add the chemoattractant to the lower chamber. For a 24-well dish, add 750 µL of DMEM with 5% FBS.

2.6.1 Place the insert in the well and add the cells to the insert. For a 24-well dish, use 500 µL of cell suspension. For a 6-well dish insert, use 2.5 mL of chemoattract and 2 mL of cells. Ensure that no bubbles of air are present on either side of the membrane.

2.6.2 Return the dish to the cell culture incubator for 22 h.

2.7 After 22 h, fix and stain the cells. Prepare a solution of 1% paraformaldehyde in 1x phosphate buffered saline (PBS) for fixation.

2.7.1 In a clean 24-well cell culture dish, add 1 mL of fixative to individual wells so there is one well for each insert.

2.7.2 Prepare the staining solution (freshly made) of 0.1% crystal violet (w/v) in a solution of PBS with 10% ethanol (v/v). Similarly, add 1 mL of staining solution to a clean well in a 24-well culture dish for each insert.

2.7.3 Remove each insert one at a time with forceps and place a sterile cotton swab inside the insert and swab the upper side of the membrane to remove unmigrated cells.

2.7.4 Repeat with a second cotton swab. The membrane is rather strong, so gentle pressure while swabbing does not compromise the integrity of the membrane.

2.7.5 Remove any remaining media from the inside of the insert and add 750 µL of PBS to wash away detached cells.

2.7.6 Remove the PBS and repeat the wash. Place the insert into a well containing fixative to fix the migrated cells on the underside of the membrane. Repeat for all inserts.

2.7.7 Fix the inserts for 15 min at room temperature.

2.7.8 Following fixation remove the insert. Wash the insert again with 750 µL PBS.

2.7.9 Place the insert into the well with the staining solution to stain all migrated and fixed cells. Stain the cells for 15 min at room temperature.

NOTE: For 6-well dish inserts, use 2 mL of fixative solution, 2 mL staining solution, and 2 mL of PBS wash.

2.8 Use a beaker with distilled water to destain the inserts. Remove the inserts and dip into the distilled water until the water running off the insert is clear.

2.8.1 Remove any excess water droplets and place the inserts onto a filter paper sidewise to air dry (usually overnight).

2.9 Prepare the membrane for imaging by labeling a clean glass microscope slide for each insert and place a small drop of microscope immersion oil in the center of the slide.

2.9.1 Detach the membrane from the insert using a scalpel to cut around the perimeter of the membrane on the inside of the plastic insert.

2.9.2 Remove the membrane using forceps and place on the top of the oil drop on the slide to hold it in place.

NOTE: The membranes are very thin and very light, so they can easily be lost by gentle air flow.

3. Imaging and analysis

3.1 Since the porous membranes are clear and staining cells with crystal violet give them contrast, use a compound light microscope to view the cells. Use a camera and/or software to quantify for many samples but is not necessary. View cells at 5x, 10x, or 20x magnification. For quantification, use multiple, non-overlapping images at 10x magnification. Alternatively, count all migrated cells on the membrane at 10x magnification.

3.2 Determine the total number of invading cells or cells per area for all samples. For each experiment, perform each condition in the assay with three replicate inserts and repeat multiple times for statistically useful results.

3.3 When comparing different cell lines with different growth rates and migration rates, do a parallel experiment to compare cells that invade through a protein matrix and compare to a Boyden Chamber assembly without protein matrix (migrated cells only). Calculate percent invasion for each cell line (Number of invaded cells/number of migrated cells x 100%).

NOTE: This approach can help compare invasion rate to migration rates. If comparing different conditions applied to the same cell line, calculating invasion alone is more relevant calculations.

The outer edge of the membrane sometimes has a higher number of cells than the central areas and is, therefore, less accurate. If this occurs, exclude those cells from the quantification and ensure that all cells are removed by a cotton swab in a repeat experiment.

REPRESENTATIVE RESULTS:

This method of in vitro invasion through a protein matrix was used to assess the aggressive phenotypes and oncogenic cell behaviors of mouse mammary tumor cells with altered expression of the zinc finger protein ZC3H8[8]. In conjunction with other approaches that also examine cell migration and growth in 3D environments, it was found that higher levels of expression of Zc3h8 in tumor cell lines, or by promoter-mediated expression from a plasmid, resulted in rapid rates of cell proliferation, fast migration, growth in 3D environments, and increased invasion in the in vitro invasion assay[8]. Conversely, decreased expression by shRNA constructs resulted in less aggressive proliferation, migration, and invasion[8]. These results were confirmed in vivo where higher expression of Zc3h8 produced larger tumors that appeared rapidly, while decreased expression produced fewer tumors that were smaller and less frequent[8].

To expand that this work, expression plasmids that can rescue shRNA-mediated knockdown of Zc3h8 expression were stably transfected in mouse mammary cells to evaluate if aggressive cell growth and behavior could be reestablished in these cells. All knockdown and rescue of expressions were verified by western blot or RT-qPCR[8]. An invasion assay was used with 5,000 cells per chamber in a 24-well dish and documented with photographs as shown in **Figures 1** and **Figure 2**. **Figure 3** shows the results of the invasion assay that demonstrate how cell invasion decreased upon shRNA knockdown of Zc3h8 expression, but that invasion is rescued when the expression is rescued. These data show that the invasion assay can provide a rapid method for testing cell lines in vitro before embarking on more expensive and lengthy approaches.

FIGURE AND TABLE LEGENDS:

Figure 1: Invasion assay components. (**A**) Boyden chamber insert for a 24-well tissue culture dish. (**B**) A 24-well tissue culture dish used for fixation, staining, and washing after 22 h incubation with cells. Numbers 1, 2, and 3 are replicates of a single cell line.

Figure 2: Invasion assay flowchart with the time scale.

Figure 3: Sample invasion assay results of a mouse mammary tumor cells altered for expression of Zc3h8, which changes the oncogenic phenotype. (**A, B**) Cells isolated from mouse mammary tumors were stably transfected with shRNA targeting a control sequence of mRNA or targeting Zc3h8 mRNA. (**C**) The later cell line was then rescued by expressing recombinant Zc3h8 designed to be unaffected by shRNA. Cells are stained with crystal violet and captured at 10x magnification using a light microscope. Scale bar represents 500 µm. (**D**) Quantification showing that reduced expression of Zc3h8 decreased the number of invading cells and the metastasis potential. Rescue of gene expression rescues higher rates of cell invasion. Values represent the total number of invading cells from a 24-well invasion assay insert. For each replicate in the experiment, the average number of invading cells was calculated. This was repeated for three experiments. Error bars indicate standard error of the mean.

DISCUSSION:

The in vitro invasion assay is an inexpensive, rapid, quantitative, and straightforward method for studying the factors promoting cancer cell invasion. Breast cancer is the most commonly diagnosed cancer among women. Of the three major subtypes of breast cancer, triple negative, (or ER-, PR-, HER2/*neu*-), is the most aggressive, most likely to metastasize, and most deadly [9]. Therefore, understanding the genes and expression that result in metastasis can help find new therapeutic targets and genetic markers for the disease. While many of the genes important for cancer cell invasion and metastasis have been identified and characterized, expression levels and activity of metastasis drivers versus metastasis suppressors may be a critical aspect of disease progression[9,10].

Beyond gene expression, the in vitro invasion assay has also been used to study the role of microRNA and other regulators in either promoting or preventing cancer cell invasion[11,12]. The in vitro invasion setup method can be used for the study of inhibitors, multi-cell type tumor environments, CRISPR-edited cells, or short-term changes to cellular growth environments. The versatility and adaptability make this assay very advantageous.

The invasion assay may be used in a first step in the analysis of genes and factors that contribute to or prevent tumor progression. For instance, Yan et al. (2010) used the in vitro invasion assays to define the role of GATA-3 in suppressing the EMT by the highly aggressive breast cancer cell line MDA-MB 231[13]. They were then able to show that this suppression correlated with a decreased ability to form metastases in an in vivo assay[13]. Potential therapeutic strategies can be initially characterized by the ability of pathway inhibitors to limit invasion through Matrigel, also correlating with the effect of these inhibitors on tumor formation in animal models. The in vitro invasion assay can be used for more in-depth analysis of known and potential oncogenes and interacting partners. For example, molecular dissection of known functional motifs of an oncoprotein or rapid analysis of mutations can be done with the in vitro invasion assay as an initial screen or assessment of significance. This can provide valuable insight into critical domains as well as a functional understanding of cell phenotypes at the molecular level.

While the Boyden Chamber invasion assay has many advantages, there are limitations. For instance, the invasion assay only looks at intravasation, one of the initial steps of metastasis, but not the later steps when cancer cells colonize secondary locations. Therefore, only a partial view of metastasis potential can be concluded. The 22 h length of the assay, while flexible, cannot exclude some cell division that could skew subtle changes in measuring invasion of asynchronous cell populations. Inhibitors such as Mitomycin C can be used to prevent cell division in the case of rapidly diving cells. Lastly, the use of 5% FBS solution for chemotaxis will slowly diffuse over time and equilibrate between both the upper and lower chambers. The density of the protein gel slows this diffusion and presents the cells with the option of migrating laterally across the gel and membrane (haplotaxis) or through the protein matrix and through the pores towards the higher concentrations of FBS (chemotaxis). Alternative chemotaxis agents can be substituted or shorter time allowances for invasion can be used to measure only the cells that invaded prior to equilibration. It is the flexibility, not the rigidity, of the in vitro invasion assay that allows for

customization that makes this assay so useful. Future adaptations of this assay include a large-scale screening of compounds, gene expression changes, and assessment of allele-specific drug effectiveness. Furthermore, a dual chamber system with circulating media could challenge cells to invade through a protein matrix, traverse a liquid environment, and reestablish on a second protein matrix at a secondary location. Lastly, a more challenging membrane can be used with the in vitro invasion assay system such as a monolayer of non-invasive cells that would be more difficult for cancer cells to cross.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

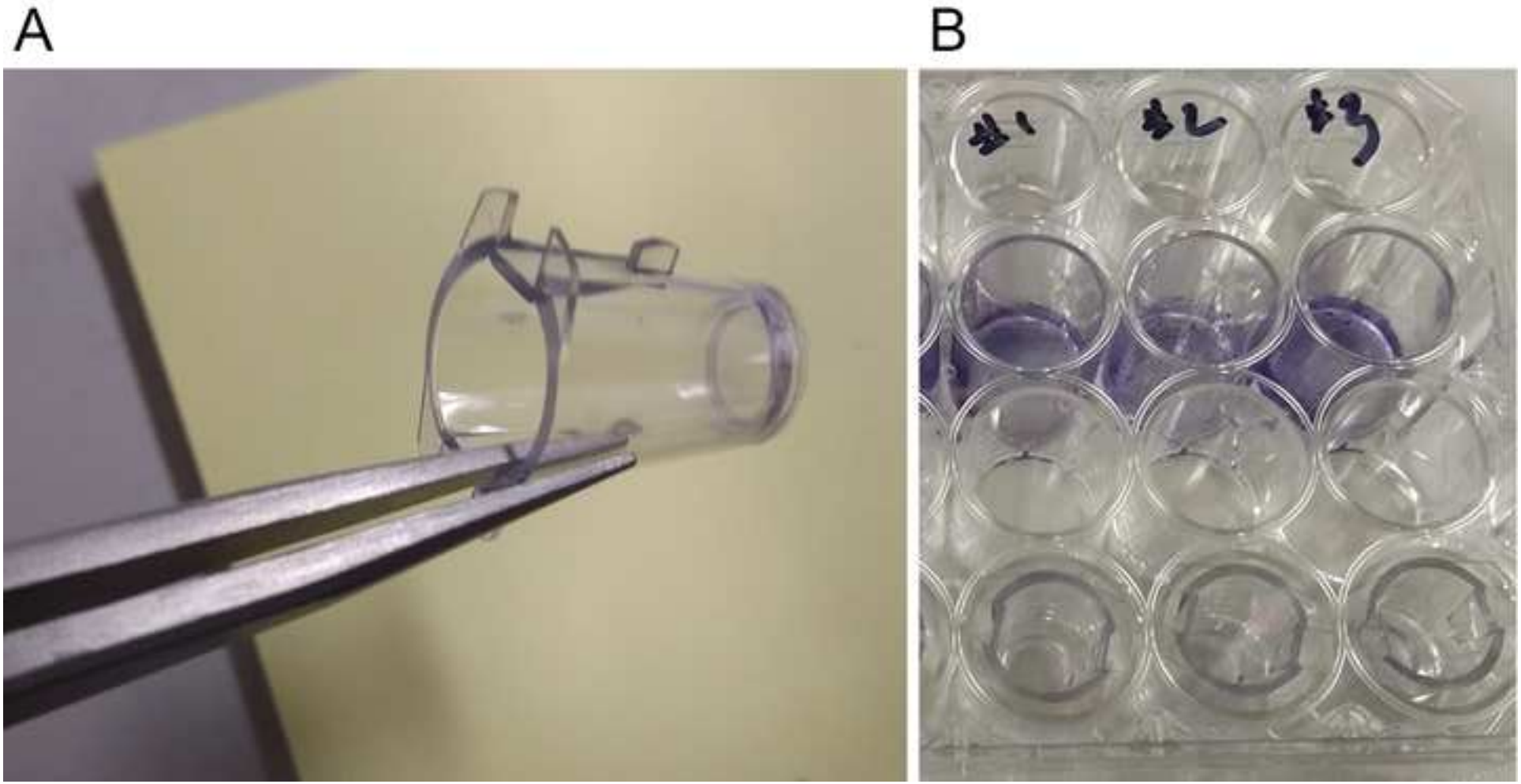


Figure 2

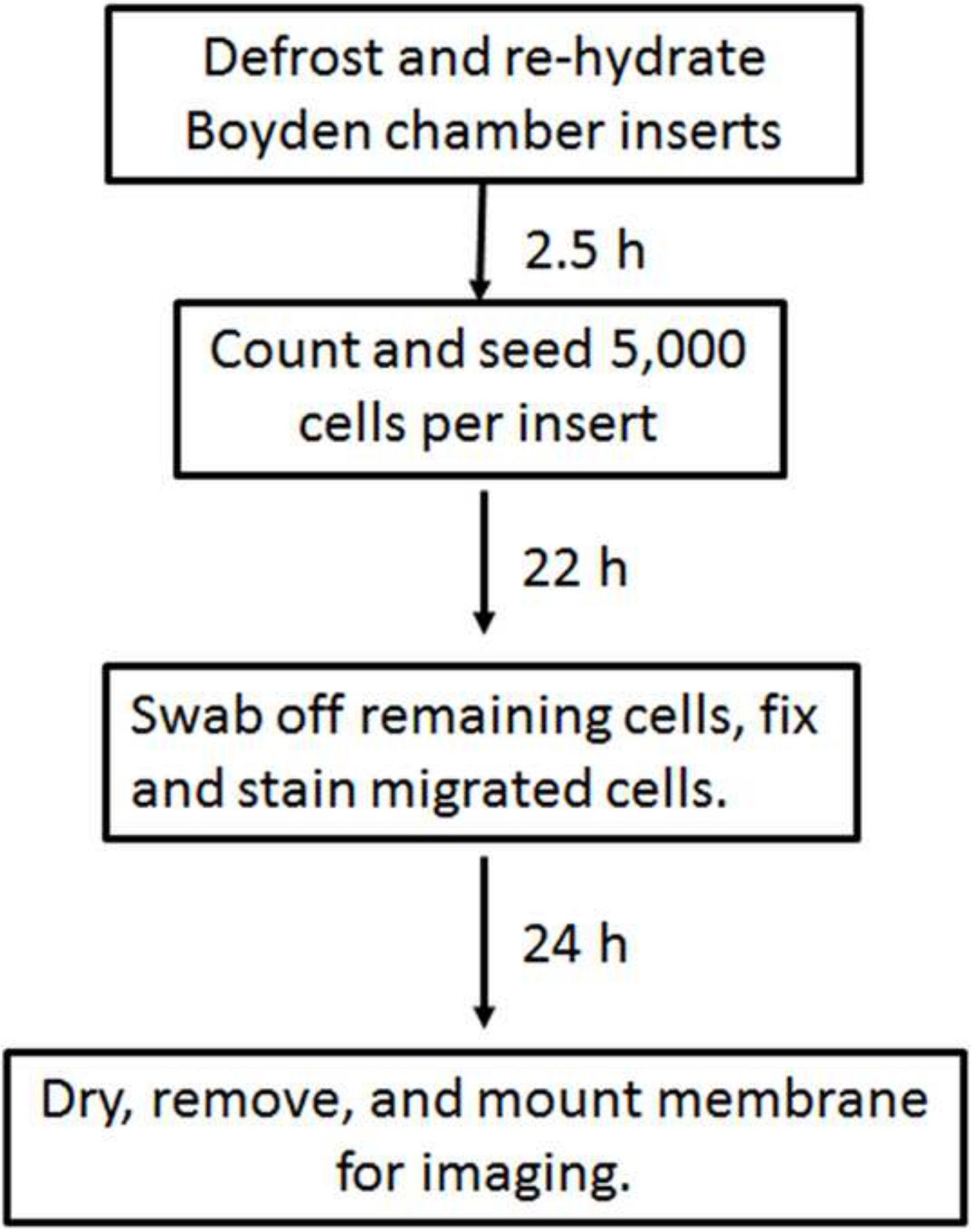
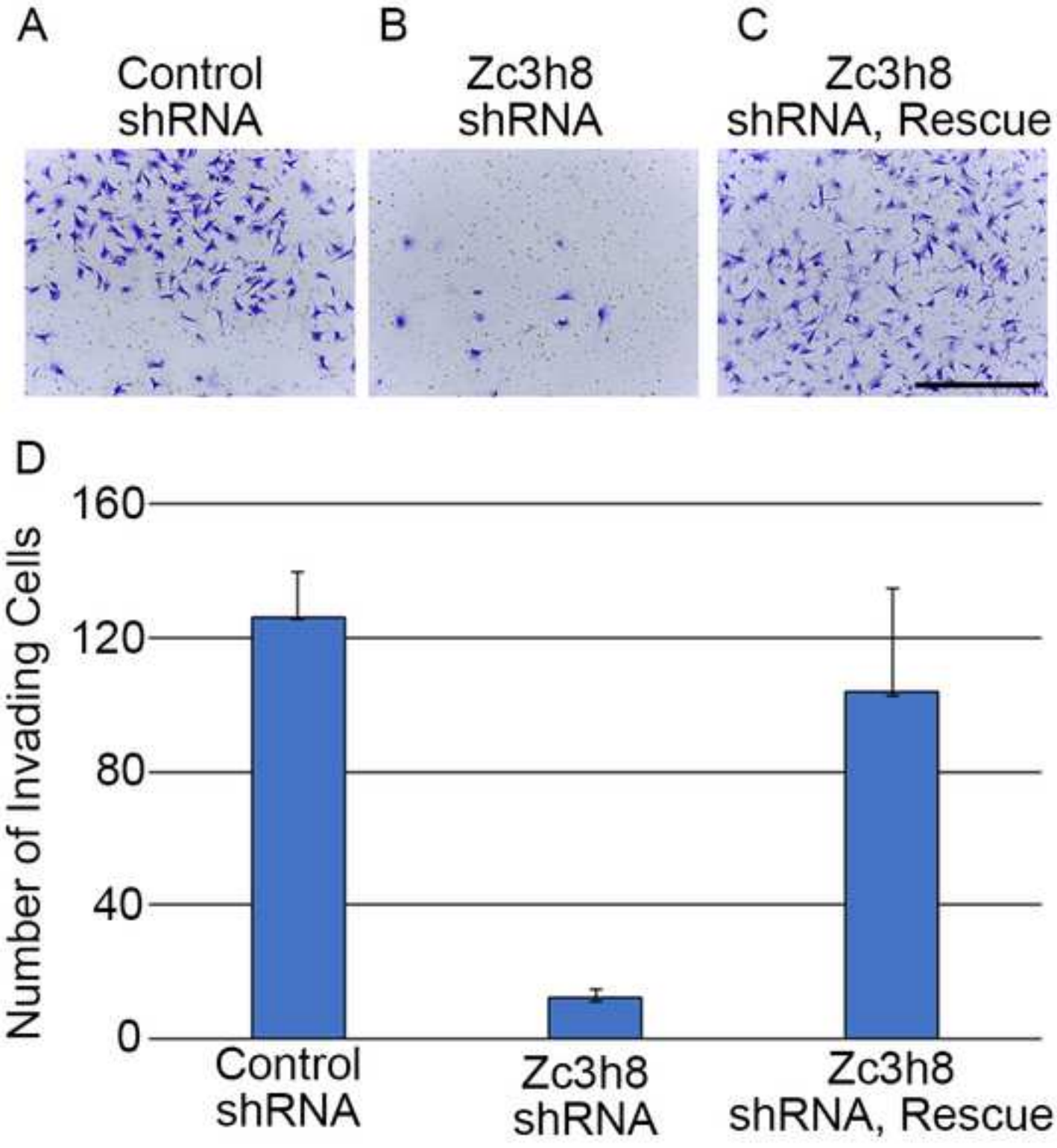


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
24-well plates	Corning	353504	
Antibiotic-Antimycotic (100X)	ThermoFisher	15240062	
BALB/c mice			
Cell Culture Incubator			
Cell Culture Treated Flasks			
Clinical cenrifuge			
Cotton swab	Puritan	25-806	
Crystal Violet	Sigma Aldrich	C0775	
Distilled water			
DMEM	ThermoFisher	10566-016	high glucose, GlutaMAX
Ethanol			
FBS	Sigma Aldrich	F2442-500ML	
Forcepts			
Glass Slide	VWR	16004-422	
HBSS	ThermoFisher	14025076	no calcium, no magnesium
Hemocytometer			
Imersion oil			
Invasion Chambers (24-well)	Corning	354480	Cat. #354481 for 6-well
Light Microscope			
Lipofectamine Transfection Reagent			
paraformaldehyde	Sigma Aldrich	P6148	
PBS			
Scalpel, disposable			#11
shRNA			
Sterile Transfer pipet			
Trypsin-EDTA	ThermoFisher	25200056	

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Date:	2/19/2019

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To Peer Review at JoVE,

Thank you very much for your editorial and peer reviews. I have made all the suggested changes and believe the manuscript is better as a result of these changes. Below, I will address each major and minor concern with the steps taken to resolve the concern. If you have any more changes or suggestions, please let me know.

John Schmidt

_____

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Authors submitted an open access ALA but indicated standard access in the submission questionnaire. Please be consistent.

Complete. After communicating with the editorial staff and billing department, I have adjusted the submission to standard and resubmitted corrected paperwork.
3. Title: Please remove commercial language (transwell) and shorten the title if possible.

Complete. I shortened the title and removed the term 'transwell.'
4. Keywords: Please remove commercial language (transwell, matrigel). Please ensure that there are 6-12 keywords.

Complete. There are seven key words and commercial language has been replaced.
5. Short Abstract: Please expand it to briefly describe the method.

Complete. The short abstract is more descriptive of the method.
6. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of " transwell" and "Matrigel" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

Complete. I removed commercial language including 'transwell' and 'Matrigel' wherever possible and replaced with 'in vitro invasion' as a more generic name.
7. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Complete. I rephrased each sentence in the Protocol section to be directive and impetrative tense.

8. Please add more details 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. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Complete. I added more detail to many steps of the protocol especially those mentioned by the reviewers. In particular Section 1 of the protocol was re-written and multiple points of sections 2 and 3 were expanded.

9. For culture media and buffer, please spell out at first use and provide composition. If they are purchased, please cite the Table of Materials.

Completed.

10. 1.2: Please describe how to remove tumor cells from mouse mammary tumor virus (MMTV) positive BALB/c mice.

Completed. A large portion of section 1 of the Protocol was re-written to include more details about deriving cells from MMTV positive mice.

11. 1.2.3, 2.4.1: At what temperature are the cells incubated with 0.25% Trypsin-EDTA solution?

Complete.

.

12. 1.3.1: Please describe the actions being performed here in the imperative tense. Please specify the cell lines used.

Complete with all other tense changes.

13. 2.1: Please specify the cell type and growth conditions.

Complete.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

17. Figure 1: Please add panel labels in the figure. Please describe what “#1”, “#2”, and “#3” represent in the figure legend.

Complete.

18. Figure 2: Please remove commercial language (transwell).

Complete.

19. Figure 3A-C: Please include a scale bar at the lower right corner for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

Complete. Scale bars added and verified by objective micrometer.

20. Figure 3: Please define the error bars in the figure legend.

Complete.

21. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Complete. An additional paragraph was added to the discussion that includes limitations, alterations, and potential problems with the protocol.

22. References: Please do not abbreviate journal titles.

23. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

Completed. Some additions made to the list.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors describe a simple and efficient protocol, that I understand to be well-established, for the transwell invasion assay and demonstrate how it can be used to interrogate potential genes involved in cancer cell invasion. The results shown seem credible based on the protocol. I am not entirely convinced that the assay measures invasiveness specifically, but this may be examined with additional controls.

Minor Concerns:
In the original paper of Albini et al. (1987) the performance of cancer cells in the transwell invasion assay was actually reduced compared to normal cells, which is contradictory to their expected invasive potential. Perhaps this is because Matrigel may not be an optimal invasion matrix for cancer cells (Nguyen-Ngoc et al. 2012). I am therefore wondering if the assay measures more chemotaxis/haptotaxis than invasiveness per se. This is not to say that the former does not contribute to the latter, but perhaps the assay may not be specific for invasion-related cancer-specific cellular processes. The authors do mention that the protein-rich gel can be altered based on the needs of the experiment.

Complete. A new paragraph was added to the discussion that addresses these concerns including the distinctions between chemotaxis, haplotaxis, and invasion.

For the experiment, they authors have isolated primary breast tumor cells from "MMTV positive BALB/c mice" (I would prefer some more details on the model and the cells in terms of invasiveness ect.), which they transduced retrovirally to knock down Zc3h8, a putative oncogene they have identified in a previous study, using shRNA. They have included the derivation & modification of cells into the protocol, but not in such detail that it could be replicated without going to their original paper. Therefore, I am wondering if it is justified to include these parts into the step-by-step protocol, which I assume is expected to serve as a stand-alone guide for whatever is described.

Complete. An expanded and more detailed section of the protocol regarding cell derivation was added. Since other reviewer comments and editorial comments requested more information, it was decided to expand rather than exclude this section.

In general, I feel the step-by-step protocol (also other parts of the manuscript) deserves further proof-reading.

1.0 This part of the protocol is not very detailed.
1.2. How were cells "removed" from the mouse tumor. This is not self-explanatory.
1.2.1. How ofter cells were split and what is the purpose of going from 50% to 10%. This is also not self-evident for a non-expert.
1.2.2. This could be grouped with 1.2.1
1.2.3. Should be stated here "for passaging of the cells.."
1.3. When were cells considered to be established? CRISPR was not used here, so it should be removed.
1.3.1 Establishment of clones is not described
2.1. For a non-expert, the purpose of the charcoal-filtered serum is not clear.

Complete. All of these changes were included in the re-write of section 1.

2.2. Are the inserts transferred to a fresh plate? Should there be recommendation about freeze-thaw cycles of matrigel?

Complete. Clarified in text.

2.2.1. Is there a specific order in which solutions should be added into the compartments?

Complete.

2.5. "If your hypothesis includes...." <-I think it would be better to rephrase this

Complete.

2.6. Any discussion about usage of serum as a chemoattractant? Other recommendations?

Complete. This was incorporated into the new discussion paragraph.

2.6.1 Is the insert lifted off from the well when chemoattractant is added?

Complete. Clarified in text.

2.7. "Needed to be" <- rephrase. Used for what? <- for fixation.

Complete.

2.7.2. Freshly made or in advance? Is the solution not filtered?

Complete. Clarified in text.

3.1 I interpret this so that cells can be directly counted by looking into the oculars without taking pictures. Which magnification was used for counting? I recommend that this entire part be rephrased.

Complete. Clarified in text.

3.2. The area is not specified here, but it should be mention in the legend at least.

Complete. Clarified in figure legend and scale bar placed in figure.

.

3.3 This part is actually relevant for what I mentioned above about the specificity of the assay, but the purpose of the control does not come accross clearly based on how it is written here.

Complete. I re-phrased to clarify the distinctions between using a Boyden chamber to measure migrations versus invasion.

Note (after 3.3.). Based on this, what do you then recommend?

Complete.

Figure 3 (legend). Is the the average number of cells per image (shown). What is the unit area? Statistical significance? What does the cV1A01-51 stand for?

Complete. Explained in the figure legend.

These should be primary cells, not a cell line, correct? Scale bars?

Complete. A better description of cell derivation and the inclusion of scale bars.

Reviewer #2:
Manuscript Summary:
The manuscript by Schmidt & Knepper on "The use of mouse mammary tumor cells in the transwell invasion assay as a measure of aggressive cell behavior and metastasis potential" explain in detail the benefits of a transwell migration assay as a surrogate platform to evaluate the metastatic potential of cancer cells in terms of intravasation/extravasation steps.

Major Concerns:
I have no major concerns in the methodology proposed.

Minor Concerns:
I would encourage the authors to include the limitations of the transwell assay as well in their discussion, and delve deeper and cite more relevant literature in their discussion to help the readers understand several other areas where this technology has been used.

Complete. In the discussion, both limitations and other future directions are more thoroughly explored.

Reviewer #3:
Manuscript Summary:
This manuscript discusses the to test the aggressive phenotype of mouse mammary tumor cells to discover and dissect potential oncogenes that promote cell invasion.

Major Concerns:
1. The conclusion is not satisfactory and the title is not provide any message as authors are unable to justify that what is the exact impact. Please elaborate it in terms of a particular Gene knockdown and its impact on migratory effect.

Complete. The title has been revised, the cells have been described more extensively in the protocol, and the impact has been expanded in the discussion.

2. Please also elaborate that how this study can be useful and help for future studies.

Complete. In a new paragraph in the discussion additional future studies have been proposed.

3. Authors established an antibiotic-resistant stable cell lines with individual clones that were verified by western blot and/or RT-qPCR for consistent results but they did not provide any data.

Complete. Since this data has been published previously it cannot be included here, but a citation has been added.

4. There is a missing information about the significance of this gene and hoe the stable knockdown of this gene make a big impact on migration assay.

Complete. See Concern 1 above.

5. Did authors treat the cells with Mitomycin C? As without the treatment is very difficult to say that its proliferative effect also or only Migratory effect.

The use of Mitomycin C is addressed in the discussion.

6. This Boyden Chamber based migration and Invasion method is very familiar in cancer biology field. So authors need to mention that what is the novelty of this protocol.

Complete. More emphasis was added to the importance, adaptability, and ease-of-use of this assay.

7. This manuscript appears OK in principle, but there aren't enough investigation/analysis done, and the overall finding in this manuscript is not novel.

Complete. More elaboration was added to the analysis section of the protocol and the discussion to emphasize potential uses for this assay.

Minor Concerns:
8. The discussion presented here is the elaboration of results. The authors have not sufficiently discussed the results what could be the possible reasons of such results thus obtained supported by previous case studies in literature.

Complete. This is addressed in the additional discussion text.

9. References need to be updated with latest ones (if available).

Complete. New references added.

10. The duration of this experiment is 24 hrs. I hope after 12 hrs the chemotactic phenomena will not be happened as the media in upper and lower chamber will be saturate. So I think author to 10 hrs is sufficient to get better results rather then to wait for one day.

Complete. This is addressed in the new paragraph in the discussion section along with other potential pitfalls and alterations.

Finally this manuscript requires some correction before accepted for publication. The manuscript should be revised for clarity, cohesiveness, and logic flow.

Complete. The manuscript was re-read for clarity and flow.
