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A Migration/Invasion Workflow Using an Automated Live-Cell Imager

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TITLE:

A Migration/Invasion Workflow Using an Automated Live-Cell Imager

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KEYWORDS:

migration, invasion, live-cell imaging, breast cancer, metastasis, extracellular matrix (ECM) gel

SUMMARY:

The current protocol describes an integrated method investigating cancer cell migration and invasion on a single platform in real-time, providing an easily reproducible and time-efficient option to study cell mobility and morphology.

ABSTRACT:

Cancer cell mobility is crucial for the initiation of metastasis. Therefore, investigation of the cell movement and invasive capacity is of great significance. Migration assays provide basic insight of cell movement at a 2D level, whereas invasion assays are more physiologically relevant, mimicking *in vivo* cancer cell dislodgment from the original site and invading through the extracellular matrix. The current protocol provides a single workflow for migration and invasion assays. Together with the integrated automated microscopic camera for real-time HD images and built-in analysis module, it gives researchers a time-efficient, simple and reproducible experimental option. This protocol also includes substitutions for the consumables and alternative analysis methods for users to choose from.

INTRODUCTION:

Cell migration and invasion are important biological processes that enable normal functions in the human body, such as wound closure, invasion of placenta into the uterus and mammary gland morphogenesis¹⁻³. The human body has precise and strict control of these biological events;

however, there are some exceptions. Malignant tumors, for example, are able to escape this safeguard, exhibit abnormal proliferation and invade into neighboring tissue, which is called metastasis. Metastasis is the major cause of cancer-related mortality⁴.

Breast cancer is the most commonly diagnosed cancer in women, and is the second-highest cause of cancer-related death among women in developed countries worldwide⁵. Breast cancer originates from ducts or lobules that consist of one or more layers of epithelial cells. In the normal breast, epithelial cells adhere to one another and to the basement membrane through membrane proteins such as E-cadherin and integrins⁶. However, invasive breast cancer cells have lost their polarity and cell-cell adhesion, and classically undergo epithelial mesenchymal transition (EMT) and gain the ability to move. After extravasation, these cells move across the extracellular matrix (ECM) and enter the blood vessel or the lymphatic system, followed then by intravasation and metastatic growth⁷. Understanding the mechanisms by which this occurs is of great significance, since metastasis is the most common cause of cancer-related mortalities and is closely related to cancer cell migration/invasion. To visualize the movement of cancer cells, migration and invasion assays are ideal models to study 2D and 3D cell movement, respectively. Migration directly assesses the movement of the cells whereas invasion involves interaction with the microenvironment and the ability to degrade biological barriers. The two processes are not fully independent of one another, as migration is a requirement of invasion.

Several methods have been developed to study migration and invasion. As reviewed by Kramer et al., migration assays such as wound healing, fence and micro-carrier assays generate a cell-free area to allow cells to move into, assessing the change of area; whereas, transwell and capillary assays are based on the number of cells that move toward an attractant⁸. For invasion assays, an ECM environment has to be set up with ECM gel or collagen for instance, and 3D movement can be assessed by monitoring the invasion area, distance and cell counts (e.g. transwell assay, platypus assay)⁸. Another type of invasion assay is to combine the invasive cells with non-invasive cells and assess the behavior of the invasive cells (e.g. spheroid assays). The above methods have their pros and cons, and a way that is easy to approach, easy to repeat, and to combine the migration assay and invasion assay in a similar workflow is preferential in experimental design.

This protocol describes the measurement of cell migration and invasion using a live-cell imager. It is a real-time cell monitoring system installed in a standard cell culture incubator. It takes high definition images according to the set scanning intervals and measurements by applying appropriate masks to the cells or fluorescent targets. The module of migration/invasion assay includes using a 96-pin scratch tool, which is suitable for making homogeneous scratch wounds on a cell monolayer in a 96-well plate. The mechanism is based on in vitro wound healing assays, monitoring 2D cell movement on a plastic or coated surface. Invasion or 3D movement across an additional ECM within the scratch wound can also be assessed. A brief workflow is illustrated in **Figure 1**.

PROTOCOL:

NOTE: Two cell lines should be handled separately. The following procedures should be applied to one single cell line if not specified.

1. Optimize cell density prior to wounding

1.1. Culture adherent cells in T75 cm² tissue culture flasks to about 80% confluence in phenol-red free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200 mM L-glutamine and 2 µg/mL insulin at 37 °C with 5% CO₂ (standard incubation conditions for most cancer cell lines, culture formulations are cell line-dependent).

1.2. Remove the culture media by pipetting off into a waste container. Pipet 2 mL of pre-warmed 0.1% trypsin-EDTA to briefly rinse the cell monolayer and pipet off. Add another 2 mL of 0.1% trypsin-EDTA and place the flask under standard incubation conditions at 37 °C for 5 min.

1.3. Gently tap the flask to ensure detachment of the cells and then add 10 mL of pre-warmed culture media to stop the proteolytic reaction.

1.4. Transfer the cell suspension into a 15 mL centrifuge tube and spin down at 200 x g for 5 min at room temperature (RT). Carefully remove the supernatant without agitating the cell pellet and resuspend with another 10 mL pre-warmed culture media.

2. Counting cell number using an automated cell counter (or any counting methods)

2.1. Dilute 200 µL of resuspended cell suspension with 800 µL of 1x Dulbecco's Phosphate buffered Saline (DPBS) in a 1.5 mL centrifuge tube.

2.2. Attach a 60 µm cell count sensor to the cell counter, hold down the toggle and merge the tip into the cell suspension. Slowly release the toggle until the cell suspension is successfully drawn into the sensor. Cell concentration is displayed in cells/mL.

2.3. Calculate the cell number in the 10 mL cell suspension.

3. Cell plating

3.1. Plate cells at a range of cell densities (40,000–90,000 cells/well) in triplicate in a 96-well plate.

3.2. Place the plate into the live-cell imager, and schedule scanning every 2 h for 24 h.

3.2.1. In the software, click **Schedule scans** from the task list. In the drawer setup pane, determine the position of the plate, click **Add vessel** and choose the plate type. In the **Scan Setup** pane, choose or edit the scan pattern according to the experimental plate setup, and set **Scan Type** as **Standard**.

3.2.2. Right click on the **Timeline** and select **Set Intervals**. Set **Add Scans Every** to 2 h at a 24-h schedule. Click **Apply**.

4. Determine the optimal cell seeding density for the migration assay

4.1. Stop scanning after 24 h, apply the confluence processing analysis tool to the HD-phase contrast images automatically collected, and generate a cell proliferation curve against time.

4.1.1. Determine 3–6 representative images and place them in a new **Image Collection**.

4.1.2. Determine a proper mask as **Processing Definition**.

4.1.3. Launch an analysis job.

4.1.4. Determine optimized cell density according to confluence against time (approximately 100% confluence within 6 -18 h depending on when the migration assay commences).

NOTE: The amount of time it takes to grow the cells to confluence varies depending on the seeding dilution.

5. Days 1 and 2: Preparation for migration and invasion assays

5.1. On Day 1, coat the plate for invasion assay.

NOTE: ECM gel should always be handled on ice and with tips that have been placed in the fridge overnight.

5.1.1. Dilute ECM gel with ice-cold culture media to 100 µg/mL and add 50 µL of diluted ECM gel/media into designated wells.

5.1.2. Place the plate at standard incubation conditions overnight.

5.2. On day 2, gently aspirate the excess media. Plate cells with optimized cell densities into 2 96-well plates designated for the migration assay (uncoated) and the invasion assay (coated) in triplicates following sections 1–3 in the late afternoon (in the current protocol, optimized seeding densities for ZR75-1 and MDA-MB-231 were 90,000/well and 50,000/well, respectively).

5.3. Place plates at standard incubation conditions overnight.

6. Day 3: Wound scratch

6.1. Spray and wipe the scratch tool and 2 washing boats with 70% ethanol before placing them in the biosafety cabinet. Fill the washing boat 1 and 2 with exactly 45 mL of sterile (autoclaved) distilled water and 70% ethanol respectively.

6.2. For sterilization, place the scratch tool pin block (top) on washing boat 1 and 2 for 5 min each.

6.3. Start with the migration assay plate.

6.3.1. Move the plate containing cells from the incubator and make sure no well is dry to avoid damage of the scratch tool. Remove the plate cover and insert into the base plate holder of the scratch tool, and carefully place the top part onto the base part by guiding dowels. Press and hold the black lever, and meanwhile carefully lift the pin block. Gaps in each well are usually visible with the naked eye and under the microscope.

6.3.2. Quickly soak the pins in water; this is sufficient to clean the scratch tool prior to scratching the invasion assay plate if plate setup is identical. Otherwise, repeat the sterilization steps with sterile distilled water and then 70% ethanol for 5 min each.

6.4. Wash the plate 1 or 2 times with pre-warmed culture media to avoid detached cells or cell sheets reattaching to the well.

6.5. Add 100 μ L of fresh warm media into designated wells with or without treatments.

6.6. Additional steps for invasion assay.

6.6.1. Place the invasion assay plate at 4 °C for 5 min to equilibrate and carefully aspirate the cold media.

6.6.2. Dilute ECM gel with ice-cold culture media to 5 mg/mL, add 50 μ L of diluted ECM gel into designated wells, and place under standard incubation for 30 min.

6.6.3. Add 100 μ L of warmed-up media with or without testing compounds.

6.7. Place the plate into live-cell imager and let it equilibrate for 5 min. Choose **Vessel Type** as imagelock plate. Set **Scan Type** as **Scratch Wound**, choose or edit **Scan Pattern** according to the experimental plate setup (1 image/well and wide mode) and schedule 24-h repeat scanning every 1–2 h for 72 h until the wounds are healed.

6.8. To clean the scratch tool, put the top pin block in each of the following solutions (45 mL in washing boats) for 5 min: 0.5% detergent 1 (see **Table of Materials**), 1% detergent 2 (see **Table of Materials**), sterile distilled water and 70% ethanol. Place the scratch tool back onto its base plate and store in a dust-free environment.

7. Data analysis

7.1. Stop scanning the designated plate after all the wounds have healed by choosing the

experimental plate on the **Drawer Setup** and clicking **Remove Vessel**.

7.2. Collect 3–6 representative images spanning a range of sound percentages, including images right after the wound has been made, wound closure by 10% and 50%.

7.3. To determine a proper processing definition, use **Segmentation Adjustment, Cleanup and Filters** to apply appropriate **Scratch Wound Mask** and **Confluence Mask**. Use **Preview current/all** to view the accuracy of the masks.

7.4. Launch the analysis job.

7.5. Data can be analyzed within the software or exported for further analysis. Three metrics provided by the software can be used to evaluate the HD-phase images: wound width (μm), wound confluence (%) and relative wound density (%). Comparisons will be discussed in the following section.

REPRESENTATIVE RESULTS:

This migration/invasion assay is based on the wound healing assay, which evaluates the rate of the cells moving into a cell-free area created by the 96-pin scratch tool. The difference between the migration and invasion assays are that migration assays measure cells moving on the tissue-culture treated plastic surface and invasion measures cells moving across ECM gel.

The scratch tool is designed to make consistent scratch wounds in the designated plate and the live-cell imager is designed to take real-time high-resolution images with the scratch wounds in the middle. The two breast cancer cell lines used in the current protocol are ZR75-1 and MDA-MB-231, categorized as luminal B and triple negative subtypes respectively⁹. The scratch wounds generated by the 96-pin scratch tool are commonly between 700–900 μm but may vary among different cell lines (**Figure 2**).

For the migration assay, three metrics are provided: (1) Wound width (μm) is the average distance between cell sheets beside the wound (**Figure 3**). (2) Wound confluence (%) is the confluence within the wounding area (**Figure 3**). The ideal initial wound confluence should be close to 0%. (3) Relative wound density (%) is a background-subtracted algorithm [$\% \text{Relative wound density} = 100 * (w(t) - w(0)) / (c(t) - w(0))$], t = at time t , w = density of wound region, c = density of cell region], measuring the density of the wound region relative to the density of the cell region.

Three eligible metrics can be achieved based on the needs. The relative wound density and wound confluence imply the speed of cells occupying the scratch wound area, and they almost overlap in both cell lines. But the two cell lines showed very different wound healing abilities, where at the completion (circa 50 h) of the wound healing process of MDA-MB-231 cells, ZR75-1 cells had about 50% wound coverage (the two metrics above) and over 400 μm remaining wound width, indicating a lower migration ability of the ZR75-1 cells (**Figure 4**).

The migrating behaviors of the two cell types were different and can be compared based on the recorded images. Lamellipodia (blebbing protrusions) were observed in MDA-MB-231 cells at the migration front at the beginning of migration, which was a typical sign of cytoskeleton rearrangement, directing cell movement toward the cell-free region¹⁰ (**Figure 5**). ZR75-1 cells showed no sign of cell migration at the same time-point (**Figure 5**).

The metric Relative Wound Density is recommended by the manufacturer for cell invasion, as it is a 3D assay, and confluence-based analysis is not applicable. Within 50 h, the relative wound density of MDA-MB-231 cells was saturated (100%), whereas the relative wound density of ZR75-1 cells did not change over time, indicating the highly invasive phenotype of MDA-MB-231 cells and the non-invasiveness of ZR75-1 cells (**Figure 6**).

MDA-MB-231 cells also behaved differently while invading through ECM gel (**Figure 7**). Compared to cells with bleb at the migration front, invading cells showed an elongated morphology with leading protrusions.

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow of migration and invasion assay in the current protocol.

Figure 2: Performance of the scratch tool on breast cancer cell lines ZR75-1 and MDA-MB-231 (upper panel) with the measured initial wound width and initial wound confluence (lower panel).

Figure 3: Migration assay illustrated by HD phase contrast images and blended mode with masks in MDA-MB-231. Blue, scratch wound; yellow, cells surrounding the scratch wound; pink, cells that moved into the scratch wound at 24 h after scratch. Scale bars are 300 μ m.

Figure 4: Comparison of three algorithms (wound width, relative wound density and wound confluence of breast cancer cell lines ZR75-1 (left) and MDA-MB-231 (right)). All experiments represent the mean of three independent experiments, \pm the S.D. Scale bars are 300 μ m.

Figure 5: Migration front of ZR75-1 and MDA-MB-231 cells 4 h after scratch. Arrows, lamellipodia at the migration front.

Figure 6: Quantification of invasion using metric Relative Wound Density (%) of breast cancer cell line ZR75-1 (left) and MDA-MB-231 (right). All experiments represent the mean of three independent experiments, \pm the S.D.

Figure 7: Different cellular characteristics of MDA-MB-231 cells in migration (left) and invasion (right).

Figure 8: An example of a scratch wound that cannot be analyzed by the integrated migration module from a 96-well plate.

DISCUSSION:

Migration and invasion are important parameters to assess the mobility of cancer cells. By using the 96-pin scratch tool, it is possible to conduct wound healing assays in 2D and 3D simultaneously. Apart from facilitating automatic scanning, providing a stable cell culture environment with minimum disruption, the scratch assay conducted using the 96-pin scratch tool provides consistent scratch wounds, enabling experiments that are more robust and reproducible. The 96-well plate format gives additional options of either increasing the number of cell lines or different drug treatments. In addition, cell morphology changes and dynamic movement can be recorded for further analysis.

The migration/invasion assay is designed to be easily applicable with a few prerequisites. A confluent cell monolayer is critical prior to wounding to ensure a directed cell movement toward the gap. It is therefore highly recommended to optimize cell seeding density, and a timeframe between 6–18 h for complete wound closure is typically ideal. The ability of the experimental cells attaching to the surface and the proliferation rate will affect this timeframe. Prolonged incubation time may lead to strong cell-cell adhesion and generate crooked bilateral cell fronts due to removal of cell sheets. A biomatrix coating will be helpful with less adhesive cell lines, to decrease the waiting time. Cells can be starved before wounding. Starvation timing is similar to scratch timing, which is when the confluence reaches nearly 100%, and the starvation media and duration are cell type dependent. Cells can also be transfected, but seeding density has to be determined for a prolonged time before wounding, considering incubation time of the transfection process. Wounding can be achieved within a few seconds using the 96-pin scratch tool. Defined-sized pins guarantee a precise migration/invasion starting line and distance. Importantly, the top and bottom sections must be well-aligned to complete a full scratch across the wells, so that the live-cell images don't include either terminal of the scratch wounds. The scratch wounds can be easily observed through naked eyes on the confluent cell monolayer and can be quickly checked under a bright field microscope. Washing of the wells should be performed immediately and gently to avoid reattachment of dislodged cells and removal of bilateral cells. If one washing is sufficient to clean up the desired cell-free zone, there is no need for an additional wash.

Migration assays can be finalized by adding culture media with or without treatments and are ready to scan, whereas a few additional steps are needed to be taken into account for an invasion assay. Unlike cell migration, invading cells penetrate through ECM, which more closely reflects *in vivo* cell movement better. The principal of the current protocol is to generate an ECM-surrounded environment by using ECM gel. Cells are seeded on ECM gel-coated wells and after wounding, ECM gel is layered on the cells and the scratch wound. When handling ECM gel, it is better to keep everything cold to avoid gelation. Unevenly distributed ECM gel can cause problems when focusing the microscope. Due to the viscous nature of ECM gel, it is possible to generate bubbles, but the bubbles can be easily removed by aspirating 70% ethanol through a spray bottle. To generate fine but not excessive spray, simply unscrew the cap of the spray bottle and spray off the excess 70% ethanol within the straw pointing elsewhere. By spraying at a

distance above and horizontal to the plate, the fine 70% ethanol spray is sufficient to eliminate the bubbles.

The first scan should commence as soon as possible following wounding, to capture the initial cell-free wound area, and this is important for establishing the criteria for the analysis. The scan intervals depend on the cell type, as more invasive cell lines have a more rapid wound closure rate.

This migration/invasion application is relatively straightforward, and by using the built-in analysis module, cell movement can be graphed kinetically with 3 metrics: the wound width, wound confluence and the relative wound density (**Figure 3**). All three can be used to assess migration. Wound width describes the average distance in μm when the bilateral cell sheets move relatively parallel, and is independent of the initial wound boundary. Wound confluence and relative wound density, on the contrary, require the initial wound boundary in the calculation, and describe the confluence within the wound area and the cell density relative to the unwounded area, respectively. For the invasion assay, relative wound density is recommended, since it computes the wound area in reference to the bilateral cell sheets. Readers can choose the one that best represents their needs.

This scratch wound migration/invasion assay can be convenient, but it requires the 96-pin scratch tool, designated plates, and the live-cell imager with the integrated migration/invasion module to reproduce the typical results presented in this protocol, and these can be expensive. Cost-effective substitutions such as a flat bottom 96-well multiwell plate can be used and can generate decent scratch wounds (data not shown). However, the performance of the scratch tool was not as good as when the designated plate was used, and some wounds could not be picked up by the scanning mode (**Figure 8**), thus unable to correctly define the initial wound area. Additionally, the scratch tool can be used to create scratch wounds, with analysis either manually, by image processing programs, or using other end-point visualization and analysis tools. They provide more flexibility to locate the initial wounds, and therefore are not limited to the designated plates. On the other hand, the migration/invasion assay combined with live-cell imaging in the current protocol is less time-consuming and less error-prone. This could be particularly useful for experimental consistency and increased efficiency.

In conclusion, this migration/invasion assay is quick, easy and robust. Users can choose from our protocol and others based on their need and budget.

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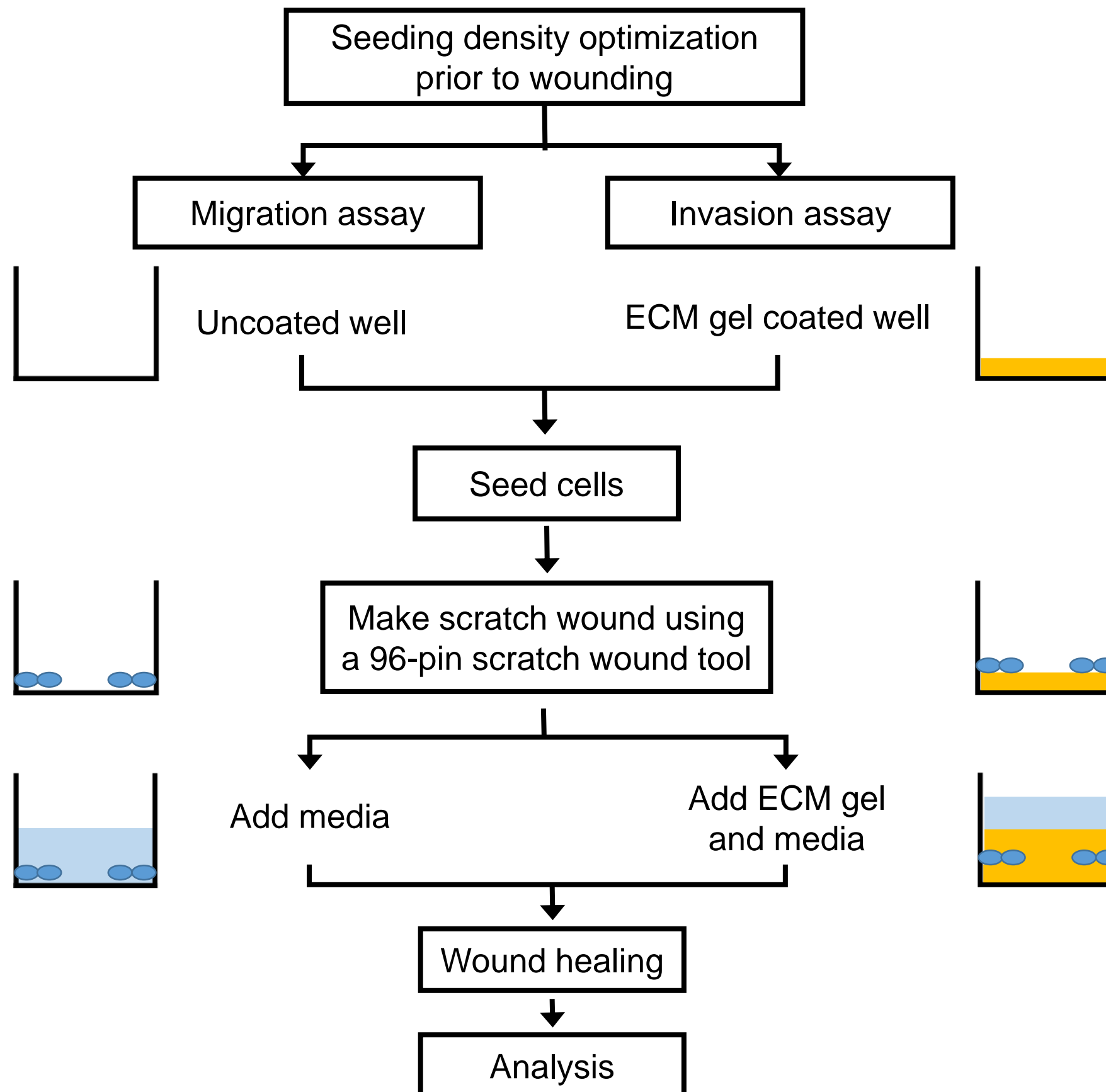
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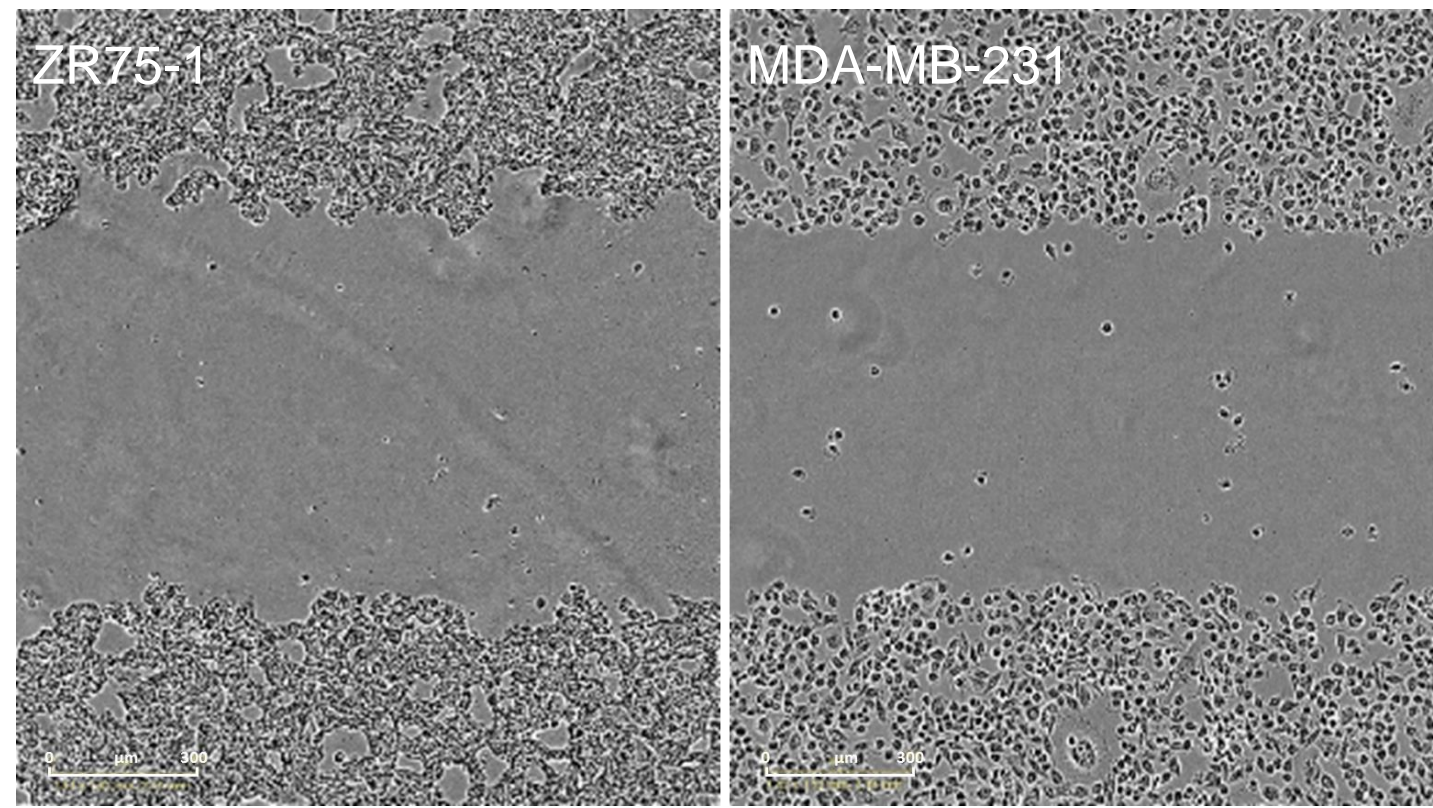
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The authors declare that they have no competing financial interests.

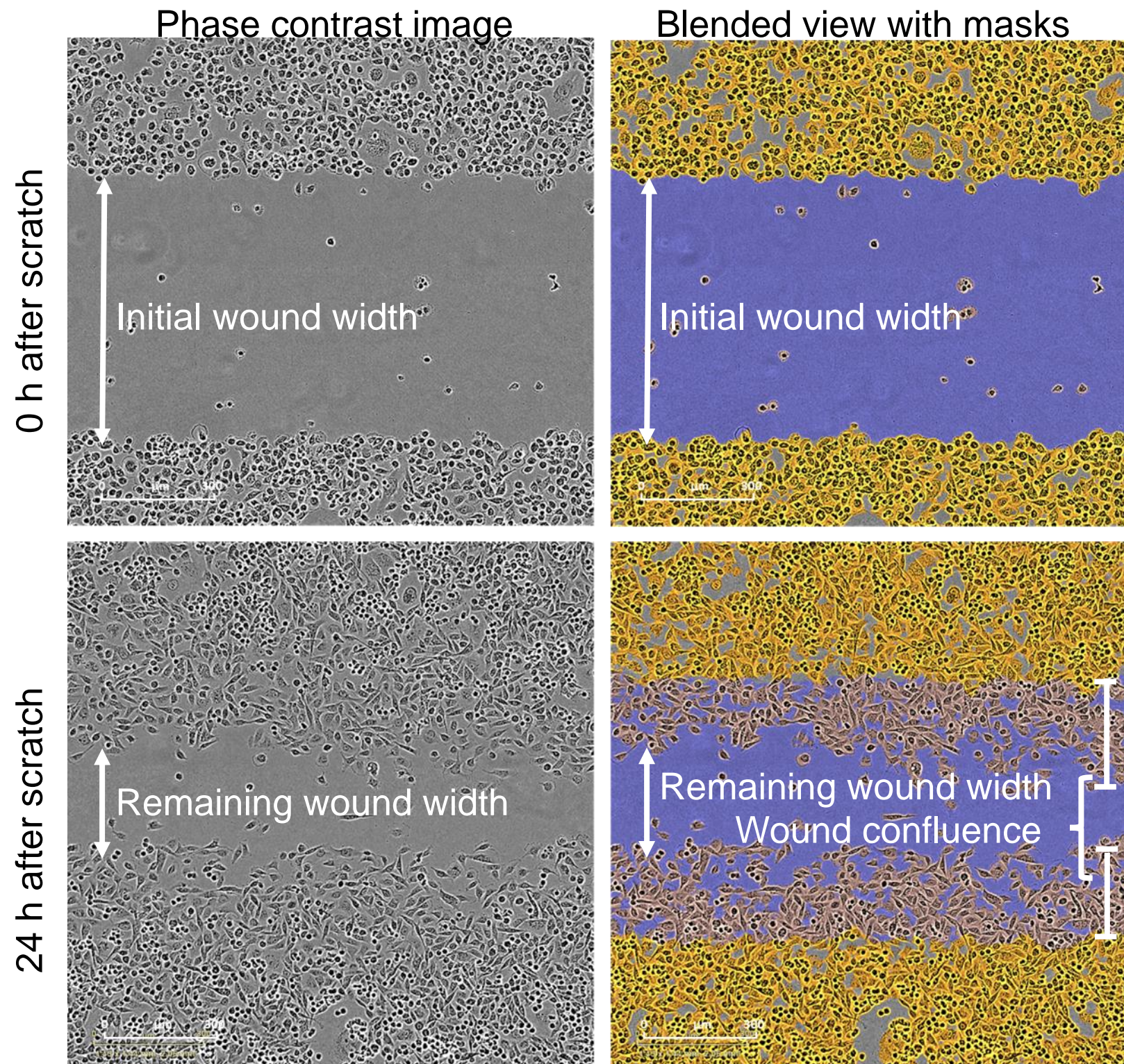
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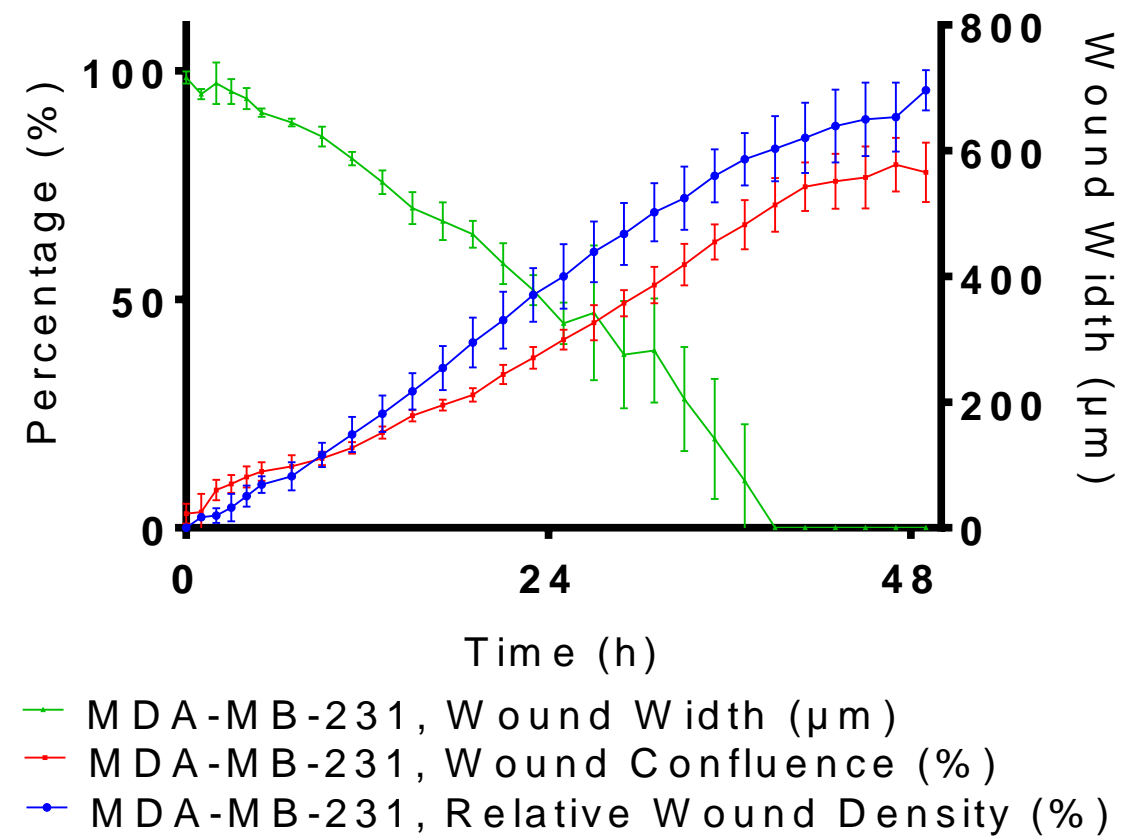
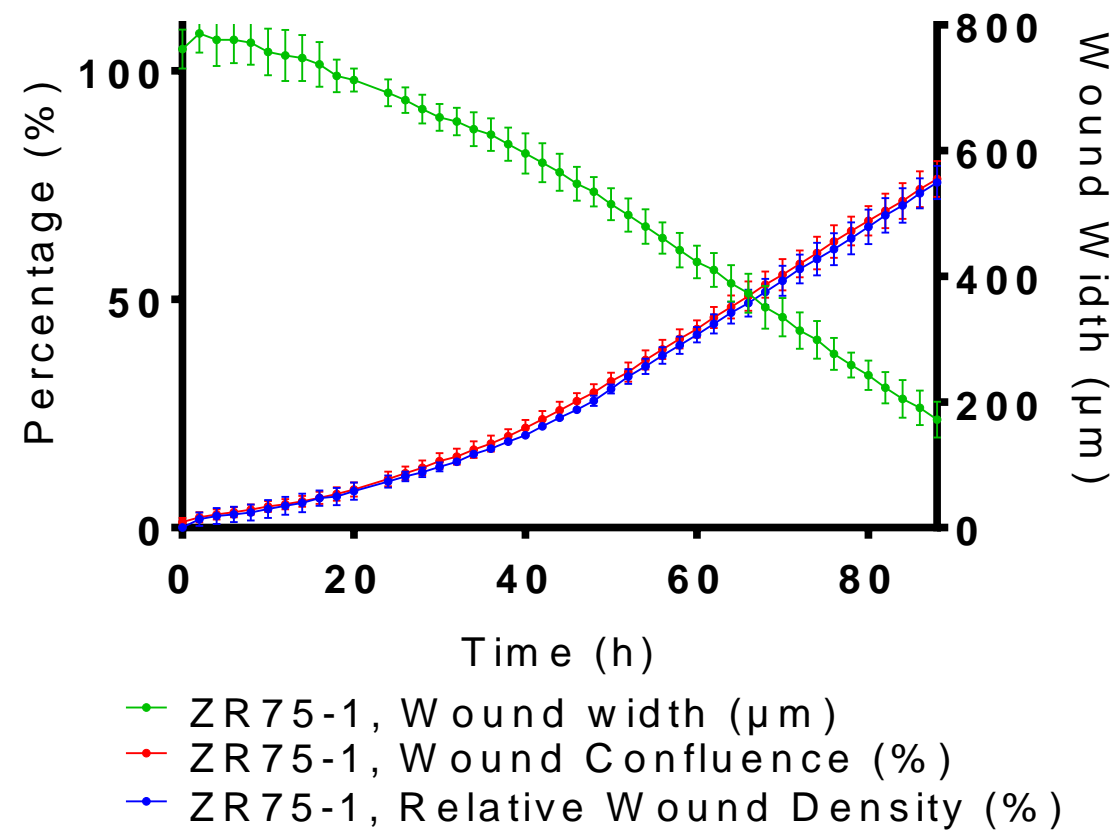
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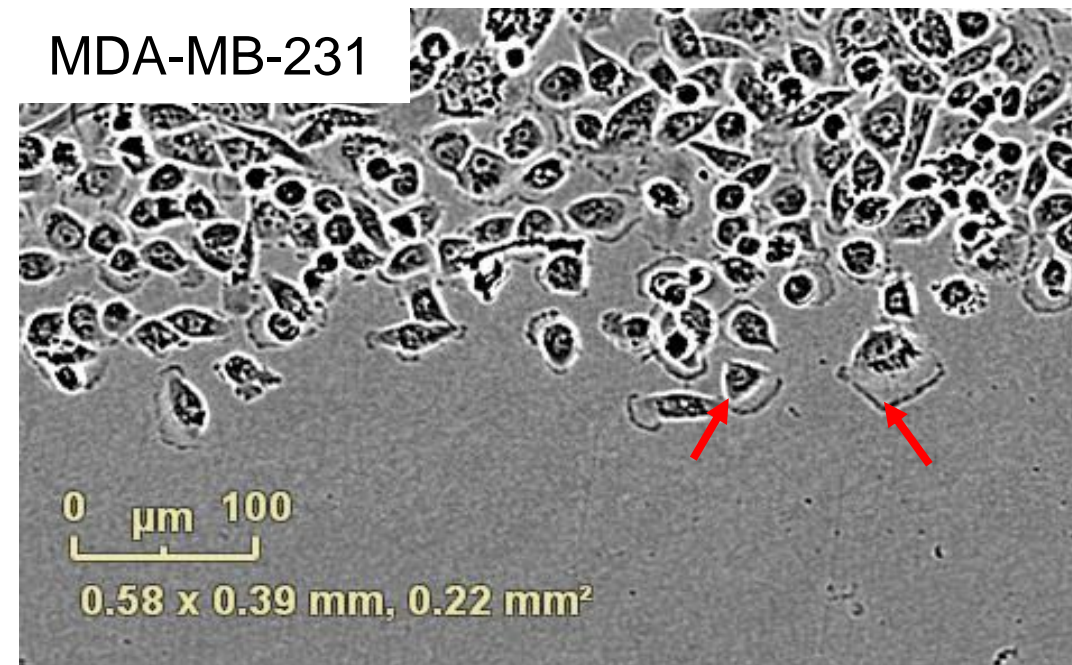
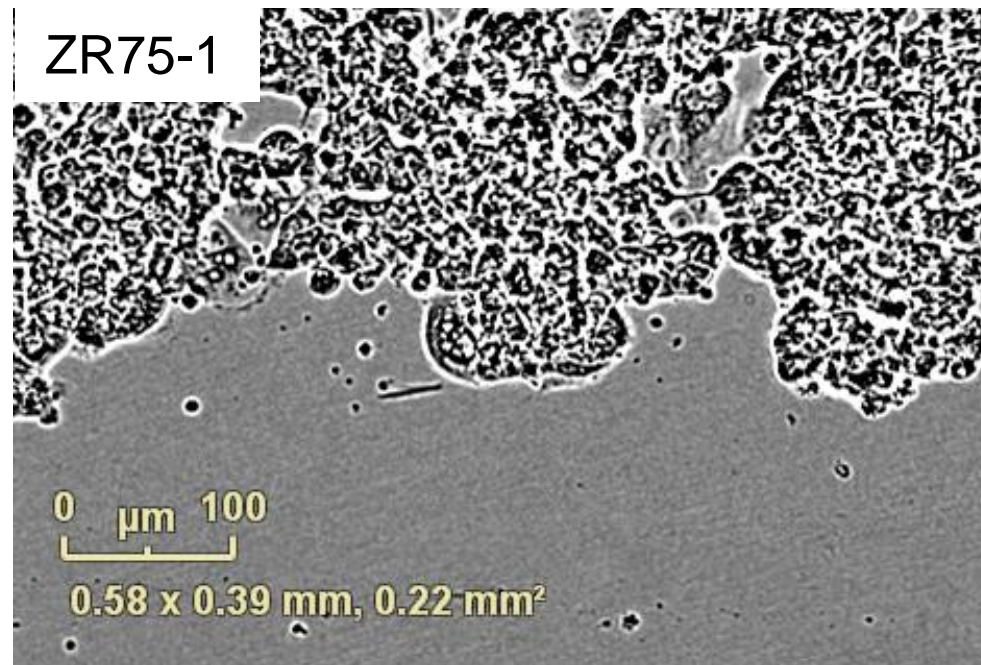


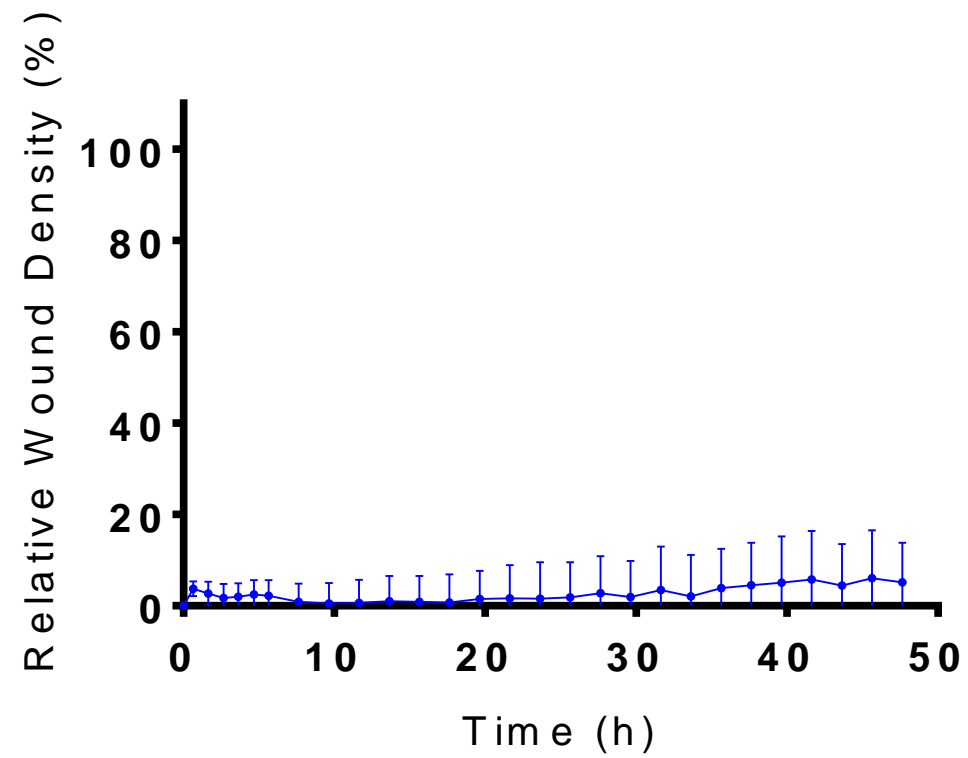


	Average Initial Wound Width (μm)	Average Initial Wound confluence (%)
ZR75-1	877.6	1.7
MDA-MB-231	750.1	2.1

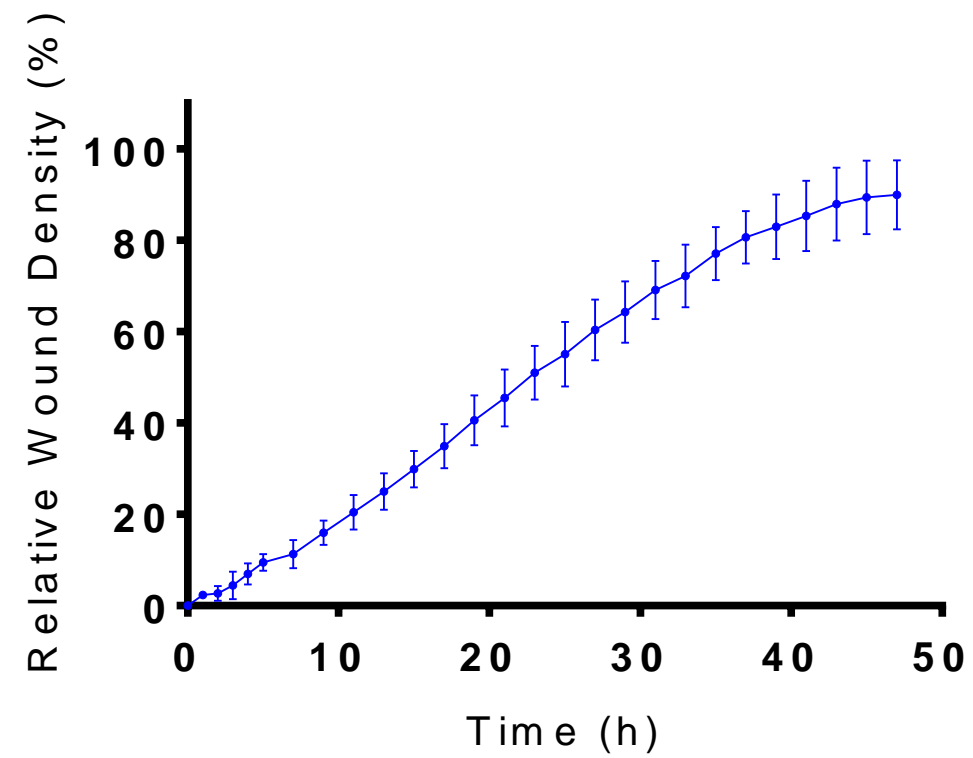




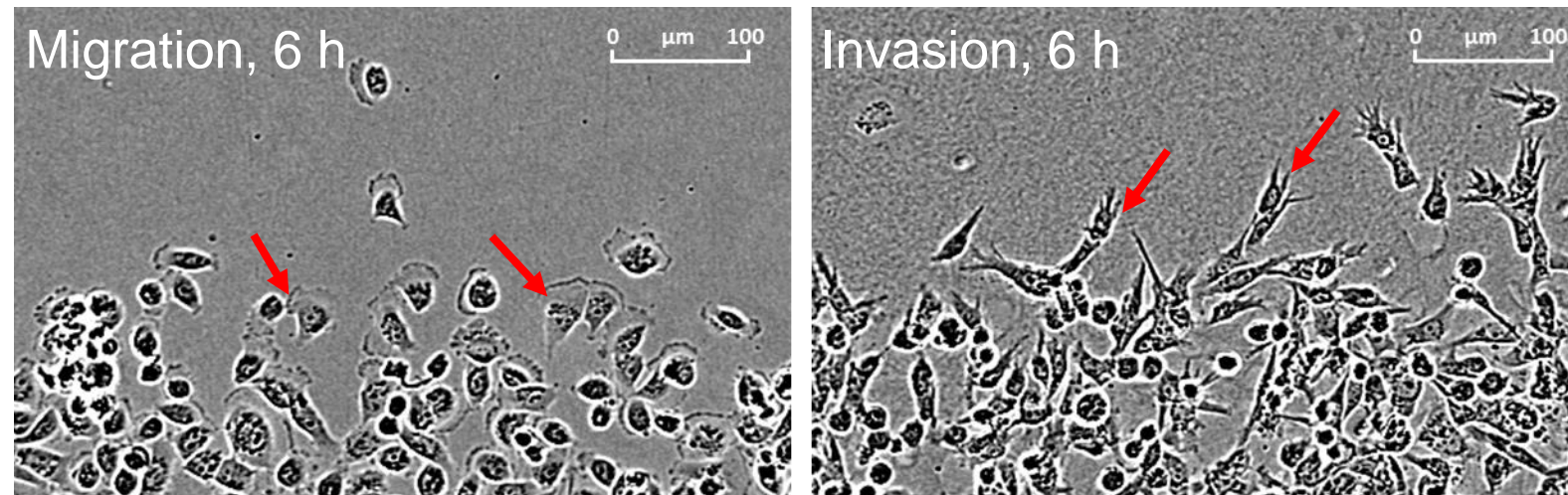


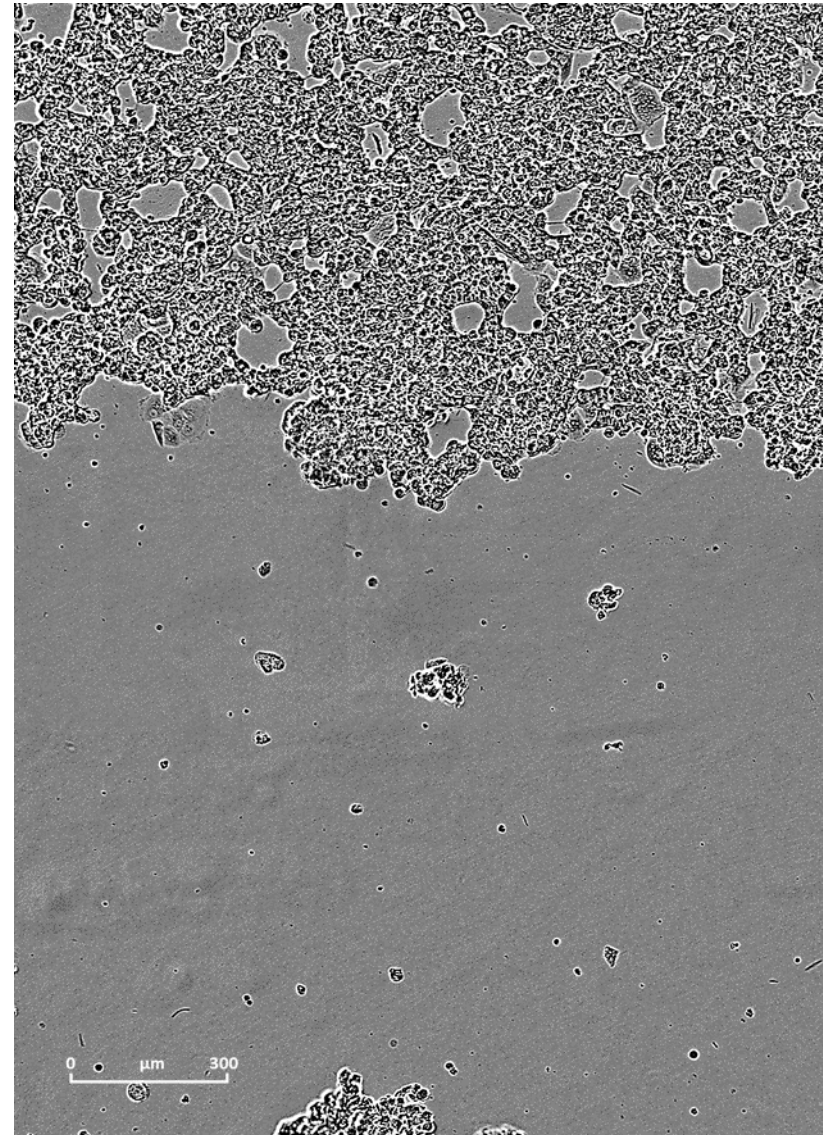


—•— ZR75-1, Relative Wound Density (%)



—•— MDA-MB-231, Relative Wound Density (%)





Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.5% trypsin-EDTA solution (10x)	ThermoFisher Scientific	30028-02	Dilute to 2x in DPBS
Countess II FL Automated Cell Counter	ThermoFisher Scientific	AMQAX1000	Automated cell counter
Detergent 1 (Alconox)	Sigma-Aldrich	242985	0.5% working concentration
Detergent 2 (Virkon S)	VetProduct DIRECT		1% working concentration
Dulbecco's Modified Eagle Medium, no phenol-red	ThermoFisher Scientific	21063-045	Supplimented with 10% FBS, 200 mM L-glutamine, 2 µg/ml insulin
ECM Gel (matrigel)	Sigma-Aldrich	E6909	Growth-factor reduced, phenol red free
Essen ImageLock 96-well plate, flat bottom	Essen	4379	
EVE Counting slides	BioTools	EVS-50	
Fetal bovine serum (FBS)	Bovogen Biologicals	SFBS-F-500ml	
IncuCyte 96-well scratch wound cell invasion accessories	Essen	4444	Including CoolBox, 2x CoolSink
IncuCyte Cell migration kit	Essen	4493	Including the 96-well pin block, 2x wash boats and the software
IncuCyte ZOOM	Essen		Live cell analysis system
Insulin solution human	Sigma-Aldrich	19278-5ML	
L-glutamine solution (100x)	ThermoFisher Scientific	25030-091	
Tissue culture flask, 75 cm ² growth area	Greiner Bio-One	658175	



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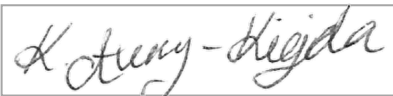
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Article Title:	A simple workflow of migration/invasion using the IncuCyte and WoundMaker	
Signature:		Date: 4/9/18

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FACULTY OF HEALTH



Phillip Steindel, PhD

Review Editor,

c/o JoVE

1 Alewife Center, Suite 200
Cambridge, MA 02140

Friday 31st October 2018

Dear Dr Steindel,

Re: submission JoVE59042R3 “A simple workflow of migration/invasion using an automated live-cell imager”.

Thank you for your email on 1/11/18 regarding the editorial review of our manuscript JoVE59042, and for giving us the opportunity to make the suggested changes to our manuscript. We would like to thank you for your valuable feedback on this manuscript. The revised manuscript has been attached with track changes to demonstrate the changes made to the manuscript. This current document lists all the changes made to address the comments for the revised manuscript, where words marked with a ~~strike through~~ have been removed, and words underlined have been added. Please be advised that all the page and line numbers following refer to the revision document under All Markup view.

- **Editorial comment 1. Short and Long abstract: You mention ‘similar’ platforms and workflows in these sections-similar to what? Please expand.**

This has been corrected as follows:

- Page 2, line 70:invasion on a ~~similar~~ single platform in real-time
- Page 2, line 79:provides a ~~similar~~ single workflow ~~between~~ for.....

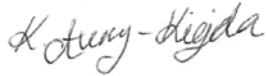
- **Editorial comment 2. Protocol steps 1 and 2: You have highlighted the headers here but none of the details; do you intend to show any of these steps? Note that we can indicate that viewers follow the text protocol if you don’t want to show specific steps in the video.**
 - No, we do not intend to show this in the video, please indicate to viewers to follow the text.

- **Editorial comment 3. Figures 2 and 3: Please include a scale bar.**
 - A scale bar has now included

- **Editorial comment 4. Figures 7 and 8: The scale bars are hard to read; please choose a color that increases contrast.**
 - We have made the changes as required.

We hope that these changes are acceptable to continue the submission process at JoVE.

Yours Sincerely,



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