Title

A practical toolkit to study aspects of the metastatic cascade *in vitro*

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Abstract

While metastasis – the spread of cancer from the primary location to distant sites in the body – remains the principle cause of cancer death, it is incompletely understood. It is a complex process, requiring the metastatically successful cancer cell to negotiate a formidable series of interconnected steps, which are described in this paper. For each step, we review the range of *in vitro* assays that may be used to study them. We also provide a range of detailed, step-by-step protocols that can be undertaken in most modestly-equipped laboratories, including methods for converting qualitative observations into quantitative data for analysis. Assays include: (1) a gelatin degradation assay to study the ability of endothelial cells to degrade extracellular matrix during tumour angiogenesis; (2) the morphological characterisation of cells undergoing epithelial-mesenchymal transition (EMT) as they acquire motility; (3) a 'scratch' or 'wound-healing' assay to study cancer cell migration; (4) a transwell assay to study cancer cell invasion through extracellular matrix; and (5) a static adhesion assay to examine cancer cell interactions with, and adhesion to, endothelial monolayers. This toolkit of protocols will enable researchers who are interested in metastasis to begin to focus on defined aspects of the process. It is only by further understanding this complex, fascinating and clinically relevant series of events that we may ultimately devise ways of better treating, or even preventing, cancer metastasis. The assays may also be of more broad interest to researchers interested in studying aspects of cellular behaviour in relation to other developmental and disease processes.

Keywords

metastasis, *in vitro* assays, cell migration/invasion, adhesion to the endothelium, gelatin degradation assay, image analysis

Author contributions

Paschalia Pantazi: Conceptualization, Investigation, Methodology, Writing - Original draft, **Emanuela Carollo:** Writing - Original draft, **David Raul Francisco Carter:** Writing - Review and Editing, Supervision **Susan Ann Brooks:** Conceptualization, Writing - Review and Editing, Supervision

Introduction

Metastasis is a clinical milestone in cancer progression that dramatically increases the risk of death in cancer patients. More than 90% of cancer-related deaths are due to metastatic disease (Weigelt et al., 2015). It is often undetectable at the point of diagnosis, only becoming clinically apparent when it is well established. By this stage, it is usually multifocal, and is often resistant to therapy. Because it is usually diagnosed at a relatively late stage, the mechanisms of metastasis are technically difficult to study in humans. Animal models of cancer are both complex and costly, requiring specialist facilities, and also carry significant ethical implications. Moreover, they have limitations in terms of accurately reflecting the disease in humans (for example, reviewed by Cekanova and Rathore (2014)). Metastasis remains, therefore, very incompletely understood.

The process of metastasis is accepted to involve a complex cascade of events that can be arbitrarily divided into sequential steps, illustrated in Figure 1 and described in more detail in the following sections (Brooks et al., 2010; Nicolson, 2015). At the primary tumour site, low oxygen levels lead to the formation of new blood vessels (angiogenesis, Figure 1A), the nutrient and oxygen suppliers that also serve as a pathway to other sites in the body (Bergers and Benjamin, 2003). The next step in the metastatic cascade is the dissemination of cells from the primary tumour (Figure 1B). This process requires abrogation of cell-cell and cell-basement membrane adhesion, and occurs through changes in the expression of a range of adhesion molecules, including integrins, cadherins, and catenins (Byers et al., 1995). Epithelial to mesenchymal transition (EMT) allows epithelial cancer cells to acquire a more motile, mesenchymal phenotype, enabling them to invade more effectively. They also become resistant to anoikis, a process by which epithelial cells that lose anchorage dependency are normally subjected to programmed cell death (Frisch et al., 2013). Tumour cells with increased motility can degrade the extracellular matrix by releasing matrix metalloproteinases (MMPs) (Savagner, 2001). Subsequently, the cells degrade and invade the local basement membrane and enter neighbouring tissues. Cell protrusions, such as invadopodia, aid their motility (Friedl and Wolf, 2010). Then, cells break through the blood vessel walls (intravasation) and move into the circulation (Figure 1C). Three mechanisms have been proposed through which tumour cells are able to cross the endothelium: abrogation of cell-cell junctions, passage through/within an endothelial cell, and use of reactive oxygen species to produce a permeant perforation in the endothelium (Tremblay et al., 2008). In the circulation, tumour cells need to survive blood flow and escape immune attack until they encounter the capillary bed of the target organ site (Figure 1D). Here, they may become mechanically trapped in the narrow bore of the vessel, or may interact with the endothelium through specific adhesive interactions. Then, cells exit the blood vessel (using similar mechanisms to those described above) and invade the local tissue (Chambers et al., 2002) (Figure 1E). Here, a reversal of EMT occurs, where cells undergo mesenchymal to epithelial transition (MET), a process resulting in re-establishment of the initial epithelial cell phenotype (Yao et al., 2011). Once established at the new site, tumour cells may remain dormant for months or years until they adapt to local conditions and can flourish to establish new tumour foci.

In theory, metastasis would be halted, and most cancer deaths avoided, if any one of these steps could be blocked (Poste and Fidler, 1980). It is for this reason that *in vitro* studies that allow focussed study of individual components of the process can be powerful and illuminating. In this paper, we provide a detailed overview of each step in the metastatic cascade, as outlined above, suggest a range of assay approaches that can be used to study each step *in vitro*, and give detailed but simple protocols for each that can be readily optimised in most laboratories.

Angiogenesis

Angiogenesis, the formation of new blood vessels and capillaries from the existing vascular system, is a process that is operative throughout life. Newly synthesised vasculature is essential to provide nutrients and metabolites to tissues during growth and repair (Adair and Montani, 2010). In cancer too, blood vessels are the nutrient and oxygen suppliers of an evolving tumour but also serve as a pathway to other sites in the body. Angiogenesis is an intrinsic mechanism for tumour growth and progression, mediated by a range of cancer-derived signals (Bergers and Benjamin, 2003). More specifically, angiogenic factors such as the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and various cytokines can activate the otherwise quiescent adult endothelial cells (Benelli et al., 2006). In cancer, these signals are induced by a hypoxic tumour microenvironment (Muz et al., 2015). In a step-wise process, activation of endothelial cells leads to an altered gene expression landscape, which results in increased proliferation, migration, and degradation and invasion of surrounding tissues (Alghisi and Ruegg, 2006). Endothelial cells secrete matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) to degrade the basement membrane of the existing vessel and the surrounding tissues. These are secreted by filopodia, which are the leading structures in the migration movement of the endothelial cells. Other cells, following the leading ones, proliferate and align to form a tubular structure (the new capillary), which is then perfused with blood and serves as a new nutrient supply to the tumour mass (Bielenberg and Zetter, 2015). All of the steps described above could potentially be a target of anti-cancer therapy or other interventions, and can be assessed *in vitro*. A range of such assays are described below, and a summary critique of their strengths and limitations is given in **Table 1**.

Various **proliferation assays** can be employed to evaluate the proliferation rate of endothelial cells in response to a stimulus. For example, DNA synthesis proliferation assays like the [3H]-thymidine or the 5-bromo-2'-deoxy-uridine (BrdU) incorporation assay allow the detection of newly formed DNA and can be adapted for flow cytometry, microscopy, or high throughput screening. Other proliferation assays include metabolic proliferation assays (e.g. MTT assay, where live cells metabolise the yellow tetrazolium (3-(4,5-di**m**ethyl**t**hiazol-2-yl)-2,5-diphenyl**t**etrazolium bromide) to the insoluble purple formazan, then the cells are lysed and viability is measured as absorbance at 570 nm), luminescent cell viability assays (e.g. luciferase-based ATP assay to measure ATP usage in live cells), fluorescent dye proliferation assays (e.g. using carboxyfluorescein succinimidyl ester (CFSE), a non-fluorescent cell permeable dye, which, when in the cell, is hydrolysed by esterases and fluoresces; with each cell division, the fluorescence intensity is halved allowing quantification of cell proliferation), and the simple method of cell counting using trypan blue to distinguish live from dead cells. These methods to assess proliferation are analysed in detail by Romar et al. (2016).

Alternatively, variations of zymography protocols exist, where the active and the latent form of matrix metalloproteinases (usually MMP2 and MMP9) can be detected by gel staining or using enzyme linked immunosorbent assay (ELISA) in order to assess the cells' capacity to degrade the surrounding matrices (Eccles et al., 2016).

One of the most commonly used tests for angiogenesis is the **tube formation assay**, which examines the cells' ability to form 3D luminal structures to create capillaries. It is understood that sprouting or *de novo* formation of capillaries is a complex process that requires the crosstalk of endothelial cells with surrounding cells and extracellular matrix. Most tube formation protocols include the use of Matrigel®, a commercially available extracellular matrix-rich compound prepared from tumour cells containing high amounts of laminin (Madri et al., 1988).

Finally, the **migration** and **invasion assays** described in the following sections for tumour cells could be adapted to assess the migration rate and the invasive capacity of endothelial cells under an angiogenic stimulus – for example, hypoxia. A technique that could be categorised as an invasion assay is the gelatin degradation assay, illustrated in Figure 2A, and which can be used to assess the capacity

of endothelial cells to degrade the extracellular matrix (here simply represented by gelatin). In this assay, endothelial cells are seeded on top of a gelatin layer, and the secretion of proteolytic enzymes by the cells acts to degrade this layer. Now, when the gelatin is fluorescent, degradation appears as dark spots, and is quantifiable using microscopy. A detailed protocol for this method is provided below, and examples of results illustrated in Figure 2B.

Protocol: Gelatin degradation assay

1. Prepare gelatin coated coverslips.
    a) Sterilise glass coverslips (13 mm in diameter, thickness #1.5) by dipping them in 70% v/v ethanol using forceps. Make sure coverslips are clean, without any dust or scratches.
    b) Incubate the coverslips in 20% v/v nitric acid (HNO_3) for 2 h at room temperature (RT). Then, wash coverslips in water for 4 h, replacing the water every 20 min. Keep washing the coverslips until the pH rises to 6-7.

        You can place the coverslips in a glass receptacle on a shaking rack to speed the wash and ensure that the HNO_3 is washed away completely.
    c) Place the coverslips on a 24 well cell culture plate (one per well) and work in a sterile environment to avoid contamination.
    d) Incubate the coverslips with 250 µl/well of 50 µg/ml poly-L-lysine in phosphate buffered saline (PBS) for 15 min at RT. Then, remove the poly-L-lysine excess and wash twice with PBS.

        You can adjust the volume of poly-L-lysine depending on the coverslip/well size. Make sure to fully cover the coverslip with the solution.
    e) Incubate the coverslips in a 0.15% solution of glutaraldehyde for 10 min at RT. Then, remove the glutaraldehyde and dispose of it according to local regulations. Wash coverslips twice with PBS and sterilise with 70% v/v ethanol.

        As glutaraldehyde is highly toxic, make sure to use an appropriate fume hood and dispose of it carefully.
    f) Sterilize a piece of parafilm with 100% ethanol. Make a solution consisting of 1 part of 0.1% fluorescein isothiocyanate (FITC)-porcine skin gelatin and 9 parts of 0.2% gelatin in PBS. Make 20 µl droplets on the parafilm and invert the coverslip onto the droplet, so that the gelatin can uniformly spread over the entire surface of the coverslip. Incubate the coverslips for 10 minutes at RT. Place coverslips back into the cell culture plate and wash twice with PBS.

        From this time on, work in darkness or minimise exposure of the coverslip to bright light
    g) Incubate coverslips in 5 mg/ml sodium borohydride ($NaBH_4$) for 15 min at RT and then wash twice with PBS.

        You will notice that $NaBH_4$ creates hydrogen bubbles. Agitate the plate to prevent the bubbles from lifting the coverslips. Dispose of $NaBH_4$ according to local regulations. Coverslips can be used the same day (see next passage) or be temporarily stored in PBS containing 200 units/ml penicillin at 4°C in the dark.
2. Culture cells on gelatin-coated coverslips.

a) Incubate the coverslips with complete media in a humidified tissue culture incubator at 37°C with a 5% carbon dioxide (CO_2) atmosphere, to equilibrate, for 2 h.

b) Culture the appropriate number of cells on the coverslips.

The number of cells may vary depending on the characteristics of each cell line and optimisation may be required prior to the experiment.

c) Incubate the coverslips in a cell culture incubator for the appropriate amount of time. Afterwards, remove the media and wash the coverslips twice with PBS.

The incubation time depends on the characteristics of each cell line and the conditions used in the experiment. Usually, the time required for gelatin degradation ranges between 8 and 72 h.

3. Fix and image the samples.

a) Fix with a 4% solution of paraformaldehyde (PFA), pH 6.9, for 20 min at RT and wash cells twice with PBS.

Dispose of PFA according to the local regulations.

b) Incubate the coverslips with PBS containing 3% w/v bovine serum albumin (BSA) + 0.1% v/v Triton X-100 for 1 h at RT in the dark, or protected from light.

c) Remove BSA/Triton/PBS solution and incubate with phalloidin (in PBS containing 0.3% w/v BSA and 0.1% v/v Triton X-100). Then, remove phalloidin and wash twice with PBS.

Phalloidin concentration and incubation time may vary depending on the cell lines used. Phalloidin is used to ease the selection of the cells during image analysis.

d) Add a drop of mounting medium to a glass microscope slide for each coverslip. Pick up each coverslip using forceps and place it onto the drop of mounting medium. You can image the slides straight away using a fluorescence microscope or store them at 4°C in the dark for several weeks, or even longer.

Image using excitation/emission 491/516 nm for FITC and the appropriate excitation/emission for phalloidin (this depends on the fluorophore that is conjugated with phalloidin, here red or far red would be suitable). If possible, image the whole coverslip or take representative images for each one (sufficient images should be acquired for the statistical analysis).

4. Analyse images using FIJI

a) Import images into FIJI. FIJI is an open-source platform for biological-image analysis (Schindelin et al., 2012).

You can drag the images on the FIJI window, or from the FIJI menu bar select File > Open…

b) Convert images to 8-bit (if they are not already in that format).

From the FIJI menu: Image > Type > 8-bit

c) Using the free hand tool from the FIJI toolbar, draw around each cell. Save ROIs (regions of interest) into the ROI manager (from the FIJI menu: Edit > Selection > Add to Manager).

To draw around the cell, use the phalloidin channel (or the transmitted light). Save the ROIs.

d) Keep the FITC channel only.

From the FIJI menu: Image > Duplicate. Keep only channel 1 (FITC channel) and if you wish, rename the new image.

e) Convert to binary.

From the menu: Image > Adjust > Threshold > Apply. Set the parameters so that you create a mask for the dark spots on the images.

f) Measure dark spots per cell.

Apply the ROIs you have saved before in/from the ROI manager. From the FIJI menu: Analyze > Analyze Particles > OK

g) For each cell, you have the number of dark spots, and also the area that the dark spots cover. You can normalise to the size of the cells *(to calculate cell area, apply ROI from the ROI manager and from the FIJI menu select Analyze > Mea*sure). Then, compare the area of dark spots per cell in each treatment and perform the appropriate statistical tests for your experiment. Sample results are illustrated in Figure 2B.

Epithelial-mesenchymal transition, EMT

EMT is the transformation of epithelial cells into mesenchymal cells (Hay, 1995). It is observed in healthy tissues during the embryonic development and after tissue injury, and also in cancer (Kalluri and Weinberg, 2009). During EMT, epithelial cells lose their polarity by eliminating cell adhesion structures such as gap junctions. Cell adhesion molecules including CDH1 (E-cadherin) are downregulated and are replaced by other molecules, such as N-cadherin, which grant more transient cell-cell and cell-matrix adhesions (Nakajima et al., 2004). The morphology of the cells gradually changes from cuboidal to spindle-shaped. This results from alterations in cytoskeleton dynamics due to actin rearrangement and the exchange of cytokeratin intermediate filaments with vimentin (Micalizzi et al., 2010). In general, the molecular composition of the cells dramatically shifts during EMT to facilitate these morphological and functional changes. Some of the pathways that are activated during EMT are the Ras (Janda et al., 2002), PI3K (phosphoinositol–3 kinase) (Bakin et al., 2000), Jagged1/Notch (Zavadil et al., 2004), and mitogen-activated protein kinase (MAPK) (Bakin et al., 2002) pathways. Cells that have undergone EMT can revert to their original epithelial phenotype through the reverse process mesenchymal-epithelial transition (MET), highlighting the plasticity of the cells during cancer metastasis (Kalluri and Weinberg, 2009).

Cells bearing the mesenchymal phenotype have a distinctive gene expression landscape compared with cells with the epithelial phenotype. Several genes are upregulated in cells that have acquired the mesenchymal phenotype, including cell-surface proteins (N-cadherin, α5β1 integrin, αVβ6 integrin, syndecan-1), cytoskeletal markers (FSP1, α-SMA, vimentin, β-catenin), extracellular matrix (ECM) proteins (a1(I) collagen, α1(III) collagen, fibronectin, laminin 5), transcription factors (snail1, snail2, ZEB1, CBF-A/KAP-1, Twist, LEF-1, Ets-1, FOXC2, Goosecoid), and miRNAs (miR10b, miR-21); while epithelial markers (E-cadherin, ZO-1, cytokeratin, α1(IV) collagen, laminin 1, miR-200) are downregulated (Zeisberg and Neilson, 2009).

A summary critique of different approaches for assessing EMT is given in **Table 2**. **Real-time qPCR, western blot, ELISA, flow cytometry** and **immunohistochemistry for EMT markers** are common methods to examine whether tumour cells have lost their epithelial phenotype. Indeed, commercial companies offer arrays with pre-set or customisable targets for high throughput detection of EMT. Good protocols for flow cytometry and immunohistochemistry for EMT are given by Strauss et al. (2013).

Alternatively, EMT can be assessed through the **morphological characterisation of cells**, and a protocol is given here which is both simple and inexpensive. During EMT, cancer cells acquire an irregular non-polarised shape, which is significantly different from that of epithelial cells. This is illustrated diagrammatically in Figure 3A, while Figure 3B shows phase contrast images of T47D breast cancer cells (left) with a cuboidal (epithelial) morphology, and non-malignant human breast hTERT-HME1 cells (right) with elongated (mesenchymal) morphology. There are several approaches that can

be used to convert a visual observation into a measurable trait, with the aspect ratio (length/width) and circularity (4π*area/perimeter^2) being the most commonly used. In the protocol given here, we use the FIJI image processing package to achieve this.

Protocol: Morphological analysis of cells

1. Sterilise glass coverslips (13 mm in diameter, thickness #1.5) by dipping them in 70% v/v ethanol using forceps. To allow the coverslips to air dry, place them vertically in a 24-well cell culture plate. Then, lie them flat, one per well.

   This protocol is for fixing cells. However, images of live cells can be acquired and analysed in the same way.

2. Culture the cells of interest on the coverslips.
3. Perform experiment (i.e. treatment with agents/drugs of interest).
4. Fix with a 4% solution of PFA, pH 6.9, for 20 min at RT, then wash the cells 3 times with PBS. Add a drop of mounting medium to glass microscope slide for each coverslip. Pick up each coverslip using forceps and place it onto the drop of mounting medium. You can image the slides straight away using a standard light microscope or store them for analysis later.

   Dispose of PFA according to the local regulations. Optionally, you can stain the cells with a fluorescent dye (e.g. 2µM Cell Mask, 2µM PKH26, or 10µM CFSE for 5 min, then wash 3 times with PBS, then fix). This will ease the selection of the cells during image analysis; however, a fluorescence microscope needs to be available. Note: the fixative should not alter the cell morphology, therefore, avoid alcohols, which shrink cells due to dehydration.

5. Analyse cell morphology using FIJI.
   a) Import images into FIJI.

      You can drag the images on the FIJI window or from the FIJI menu bar: File > Open…

   b) Using the free hand tool from the FIJI toolbar, draw around each cell. Save ROIs into the ROI manager (from the FIJI menu: Edit > Selection > Add to Manager).

      It is good practice to save the ROIs and the images for future reference. If a fluorescent dye like CFSE has been used, then ROIs may be selected from the FIJI menu bar as following: Process > Binary > Make Binary, Process > Binary > Fill Holes, Analyze > Analyze Particles (Settings: 0-Infinity; Circularity 0-1; Show: Overlay Outlines; Add to Manager; In Situ Show).

   c) From the FIJI menu: Analyze > Set Measurements (click on Area, Shape descriptors, and Area fraction). This needs to be done only at the start of the analysis. Then, for each measurement, select Analyze > Measure.

   d) In the Results window use the AR (aspect ratio) and/or the Circ. (circularity) values to draw comparisons between treatments. Sample results are illustrated in Figure 3B.

      Make sure all images are the same size, or calibrated so that comparison is possible. The aspect ratio (length/width) dictates elongation of a cell, while circularity is defined as 4π(Area)/ (Perimeter)^2, and can take any value between 0 and 1, with 1 representing a perfect circle.*

Cell migration

Cell migration is the movement of cells upon a substrate. Migration is a common trait of cells during embryogenesis (Keller, 2005), vascularisation (Rossant and Howard, 2002), wound healing and

immune surveillance (Friedl and Weigelin, 2008), as well as cancer metastasis (Paul et al., 2017). Cancer cells can migrate individually or collectively, as determined by transforming growth factor beta (TGF-β) signalling (Matise et al., 2012). Single cell migration can be either amoeboid (resembling the movement of an amoeba) or mesenchymal (the cell is polarised and interactions with the substrate are transient in order to facilitate an actin contraction and trail retraction mode of movement) (Lauffenburger and Horwitz, 1996).

Various methods and technologies have been developed to assess the migratory capacity of cells *in vitro*, and a summary critique is given in **Table 3**. The **transwell chamber migration assay** (Boyden, 1962) is very commonly used. The assay system comprises two compartments separated by a cell permeable porous membrane (Brooks, 2001). The cells are seeded in transwells, usually in serum free medium, and allowed to migrate for a period of time. The lower chamber contains medium supplemented with serum or an appropriate attractant. Cells migrate and pass through the pores of the membrane. The diameter of the pores is smaller than the cell size to avoid non-specific cell passage. The cells that have migrated to the underside of the membrane are then stained and counted.

Spheroids or cells cultured on microcarrier beads retain some of their *in vivo* structural characteristics. To test their migration capacity, the spheroids or the microcarrier beads are placed in conventional cell culture dishes and the cells start attaching to the surface, moving concentrically outwards (Knupfer et al., 2001; Rosen et al., 1990). Alternatively, single cell movement can be monitored by time lapse microscopy (Zantl and Horn, 2011). The cell culture dish can also be coated with **colloidal gold particles** and, as the cell moves, it will leave a clear path across the gold, which can be measured (Niinaka et al., 2001).

A range of technically quite simple assays assess the migration of cells into a gap or cleared zone in a cancer cell monolayer. To ensure that the gap closure is due to cell migration and not multiplication of cells, a proliferation assay is usually performed in parallel and under the same conditions. A **cell exclusion zone assay,** uses an insert to create a gap in the monolayer (Ashby and Zijlstra, 2012). Once the insert is removed, cell migration into the gap can be measured over time (Liang et al., 2007). Similar to the cell exclusion zone assay, the **fence or ring assay** allows the culture of a confluent monolayer in a confined space. Once the barrier (fence or ring) are removed, the cells migrate radially to the surrounding space. The space occupied by cells at a given time (or over time) can be measured to calculate migration area (Ashby and Zijlstra, 2012). The most commonly used assay of this type, however, is the **'scratch' or 'wound healing' assay** (Todaro et al., 1965), and a detailed protocol is given below. According to this method, cells are cultured in a cell culture dish/plate to confluence and an artificial 'wound' is created by scratching the monolayer with a sharp object such as a pipette tip. The 'healing' of the 'scratch' or 'wound' is then assessed as cells migrate to close this area, illustrated in Figure 4. This assay is inexpensive and requires no special equipment. However, its reproducibility is highly dependent on the accuracy of the operator (Kam et al., 2008). In the protocol given below, we give a detailed explanation on how to convert the microscopy images acquired into quantitative data for analysis using the MRI wound healing tool in FIJI.

New technological advancements that allow the continuous monitoring of cultures can significantly improve the reproducibility and time efficiency/management of migration and chemotaxis experiments. Imaging systems that can be inserted in a tissue culture incubator range from the simple Cytosmart (Lonza) to the more advanced Incucyte (Sartorius) and xCelligence RTCA analyzers (Agilent), which have the capacity to image several positions in a single or multiple plates over long periods of time, in brightfield or fluorescence modes. The Celigo imaging cytometer (Nexcelom Bioscience) is similar to the last two but comes as a bench top instrument with the capacity to be connected to an incubator. The BioStation CT Live Cell Screening System (Nikon) is a 'full package' instrument consisting of the incubator and a built-in LED illumination based inverted microscope. Another popular live cell imaging system is the Livecyte (phasefocus), which uses quantitative phase imaging to capture

high quality images of label-free samples. These new technologies can be used not only for migration but for a plethora of other assays, providing automation and advanced software applications.

Protocol: 'scratch' assay

1. Culture cells in 24-well plates until they reach 100% confluency.

   24-well plates are suggested here, due to ease of handling (well diameter: 15.6 mm). Replicates can be tested on the same plate. Also, relatively small volumes are required, which can be important when testing compounds that are expensive or limited. However, other plate formats can be used depending on the experimental needs.

2. Using a p200 pipette tip, press the bottom of the well firmly and drag the tip from one side to the other, interrupting the cell monolayer and producing a straight 'scratch' (illustrated in Figure 4). Optionally, perform another 'scratch' at right angles to the first, to result in a 'cross'. This will make it easier to image exactly the same field of view when images are acquired at different time points using a standard cell culture microscope.

   Other sizes of pipette tips could be used depending on the cell line. For example, some cells are very large or migrate very quickly, making the p1000 tip a better option. Also, using a ruler to guide and stabilise the pipette tip when performing the 'scratch' can reduce uneven 'scratches'. If the 'cross' approach is not to be used, then mark the bottom of the plate using a permanent marker before starting the experiment; draw a straight line at right angles to the future 'scratch'; then, use that mark to always image the same area of the well.

3. Wash 2-3 times with the desired buffer (PBS or medium) to remove any floating cells. Add fresh complete medium to the wells. Acquire the initial image, which will be used as time 0. Place the plate in the incubator and image at regular time points thereafter.

   Before taking the first image, test substances can be added to the medium (e.g. drugs, growth factors, etc). For the images, the use of a low power objective is recommended (5x or 10x) to include as wide a field of view as possible. Capture the images as quickly as possible when using a standard benchtop optical microscope to minimize the time the plate is outside of the incubator. The time of gap closure depends on the cell lines under investigation, and some initial optimization might be necessary to establish the optimal imaging intervals for each cell line.

4. Analyse images using FIJI.

   a) Create a stack of images for each sample/replicate/well over time.

      Place the images you acquired of one sample over time in a single folder. From the FIJI menu bar select File > Import > Image Sequence. Select the first image and click 'open'. In the pop-up window, specify the number of images. Do not scale the images. Convert to 8-bit Grayscale, sort names numerically, and press 'ok'.

   b) Use the MRI_Wound_Healing_Tool in FIJI.

      For how to install the plugin, visit http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool. Right click on the 'm' button and select your settings empirically, so that the software recognises only the space not covered by cells, as shown in Figure 4B. Left click on the 'm' button. The results window will give the surface area of the 'scratch' in every image. This will be in pixels if the images are not calibrated.

5. Calculate migration. Use the area of each image to plot 'scratch' area over time. To calculate the rate of 'scratch' closure, the ratio of area at time point 0 by area at time point x should be used. The ratio will be affected by unequal 'scratch' widths between replicates. Alternatively, the migration area can be calculated by subtracting the 'scratch' area at time point X from the 'scratch' area at time-point 0. In this case, the 'scratch' should not be completely closed at the final time point.

Cell invasion

Invasion is the penetration of cells into surrounding tissues. In cancer biology, tumour cells degrade the ECM and bypass physical barriers such as the basement membrane or the endothelium to reach the circulatory system to finally find their way to distant organs. Cell movement and remodelling of ECM are key features of cell invasion. Various techniques have been developed to study cell invasion through 3D matrices *in vitro* and a summary critique is given in **Table 4**.

A migration assay modified to study cell invasion is the **ORIS™ 3D invasion assay** (Bertier et al., 2018). Here, similar to the exclusion zone migration assay described in the previous section, the cells are seeded around an insert leaving a gap or exclusion zone. The cells and the gap are then covered/coated with a matrix of basement membrane extract. To close the gap, the cells need to invade the matrix, and the rate of invasion can then be quantified.

Collagen gel invasion assays have also been used to assess invasive capability of single cells, or spheroids. One example is the vertical gel 3D invasion assay, where a monolayer of cells is seeded on top of the gel and allowed to invade into the gel over time (Nystrom et al., 2005). This assay has been successfully used to mimic skin cancer invasion into the subcutaneous tissues (Fusenig et al., 1983). Single cell movement through the ECM can be monitored via time-lapse microscopy and special software or plugins for FIJI such as TrackMate (Tinevez et al., 2017) can be used to analyse cell behaviour. Spheroids, which better represent *in vivo* cell clusters have also been used in gel invasion assays to measure the astral outward invasion of the cluster (Del Duca et al., 2004). Other, more elaborate, spheroid invasion assays, such as the confrontation assay, have been used to assess the invasion capacity of one cell type (spheroid A) into another (spheroid B) (Hattermann et al., 2011).

Matrix metalloproteinases and other molecules are upregulated in invasive carcinomas to help degrade ECM components (Nabeshima et al., 2002). The **gelatin degradation assay** examines this trait of invasive cells. Here, coverslips are coated uniformly with a fluorophore-conjugated gelatin and the cells are seeded on top. Where the cells degrade the fluorescent gelatin, dark spots are visible and can be quantified (Diaz, 2013).

One of the most widely used assays is the **transwell invasion assay**. The basic principle is the same as that described for the transwell chamber migration assay previously (Marshall, 2011). However, here, a thin layer of extracellular matrix component(s) (Matrigel®, laminin, or collagen) is added to the transwells and cells need to degrade it in order to pass through the pores to the other side of the membrane. Here, they are stained and counted, illustrated in Figure 5A. Here, we propose a detailed protocol to perform and analyse transwell invasion assays. This protocol uses commercially available pre-coated inserts, which significantly helps decrease the variation caused by pipetting errors within the experiment, and cells are counted manually using the cell counter plug-in in FIJI, Figure 5B. The protocol can be adapted for inserts coated in-house.

Protocol: Transwell invasion assay

1. Prepare the Matrigel® plates. Allow the plates to equilibrate to RT for 30 min. Add 500 µl pre-warmed serum-free medium in both the inserts and the wells. Incubate plates in a humidified tissue culture incubator at 37°C with 5% CO_2 atmosphere for 2 h.

   This protocol is for commercially available pre-coated inserts. If you wish to prepare the inserts yourself, a protocol is given by Hall and Brooks (2014).

2. Discard medium from the wells and transwells. Add 750 µl medium supplemented with 10-20 % v/v foetal bovine serum or other chemoattractant into the wells. Plate 20,000 – 100,000 cells in each transwell in 500 µl serum free medium. Place the cells back in the incubator and allow to invade for 12 – 72h.

Different cells exhibit different invasion capabilities. The seeding cell number, the time of invasion, and the chemoattractant concentration should be optimised in advance. Use a range of concentrations and time-points to define the optimal conditions for your experiment.

3. Wash the cells by adding PBS in the insert and in the well underneath. Stain the cells with 1 µg/ml Hoechst 33342 fluorescent dye in PBS for 2 min.
4. Wash the cells by adding PBS in the insert and in the well underneath. Fix with a solution of 4% PFA, pH 6.9, for 20 min at RT. Wash as described previously.
5. Remove the non-invaded cells and the Matrigel® layer using a moistened cotton swab, wiping the inner surface of the transwell.

   Use gentle but firm movements, making sure you wipe the whole surface of the transwell, including the periphery.
6. Cut around the membrane with a scalpel, holding the membrane with forceps. For each membrane, add a drop of mounting medium onto a coverslip. Place the membrane with the cells facing down onto the coverslip. Add another drop of mounting medium on top of the membrane, then place a glass microscope slide on top.

   The weight of the slide will help remove bubbles. Alternatively, you can place a drop of mounting medium onto a glass microscope slide, then add the membrane with the cells facing up. Finally, adding another drop of mounting medium and a coverslip on top.
7. Use a 405 nm laser (Hoechst 33342 has excitation/emission 361/497 nm) for microscopy. If possible, image the whole membrane. Otherwise, acquire an adequate number of random fields of view per membrane.
8. Analyse images using FIJI. Calculate invasion. Sample results are illustrated in Figure 5B.
   a) Import images into FIJI.

      You can drag the images on the FIJI window or from the FIJI menu bar: File > Open…

   b) Convert images to 8-bit (if they are not already in this format).

      From the FIJI menu: Image > Type > 8-bit

   c) Count the nuclei manually.

      From the FIJI menu: Plugins > Analyze > Cell Counter

      Click on each nucleus. This will add to the nuclei counted, and the total number will appear on a separate window.

      Alternatively, count nuclei automatically.

      From the FIJI menu: Image > Adjust > Threshold > Apply

      From the FIJI menu: Analyze > Analyze Particles > OK

      Modify default parameters empirically to detect only, and all, nuclei as single objects. Click the Summary box (this will give you the number of total nuclei counted) and optionally the 'add to Manager' box to see whether the plugin has counted all the nuclei.

   d) Compare numbers of invaded cells between treatments or calculate percentage invasion *((mean number of cells invading though Matrigel® insert membrane / mean number of cells invading though insert membrane without Matrigel®) x100)* and invasion index *(% invasion of treatment group / % invasion of control group).*

Adhesion to the endothelium

Leukocytes are directed towards the site of inflammation during infection or tissue damage during the innate immune response. This is a well-orchestrated process involving chemoattraction, rolling adhesion (loose tethering adhesion to the activated endothelium), firm adhesion, and transendothelial migration (Granger and Kubes, 1994). Similarly, in cancer, circulating tumour cells make transient interactions with the endothelium, which slow down their rolling speed, and then stable adhesions are developed that lead to the cell's exit from the vessel (Brooks et al., 2010). It has been estimated that only 0.01% of the circulating tumour cells are able to form secondary foci at a new site (Tremblay et al., 2008), making the interaction with the endothelium a strong prerequisite for successful metastasis.

Assays that examine cell adhesion to endothelial monolayers *in vitro* are performed under either static or flow conditions and a summary critique of the different approaches is given in **Table 5**. **Adhesion under flow** can be studied in a parallel-plate fluid flow chamber which simulates physiological flow rates and shear stresses (Lane et al., 2012; Orr et al., 2000). Briefly, endothelial cells are cultured on glass coverslips to model the inside surface of a blood vessel, and these are then mounted in a flow chamber as described by Orr et al. (2000). In a more contemporary approach, the cells are cultured in specialised slides with a single inlet and a single outlet, before being mounted into the flow chamber. The endothelial cell monolayer may be stimulated using pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNFα), to better facilitate the attachment of tumour cells. A syringe pump system is utilised to draw tumour cells, previously labelled and in suspension, over the endothelial monolayer at a constant flow rate. Cells adhering to endothelial cells are captured by video microscopy. These assays require specialist flow adhesion facilities and are technically quite complex to run.

A simpler adaptation of this was described by Bapu et al. (2014), who used a basic rocking platform to mimic flow over endothelial monolayers in a setting similar to the static adhesion assays described below. **The rocking adhesion assay** provides a simple experimental setup to study adhesion under sweeping flow, but without the need for expensive and technically complex equipment.

Static adhesion assays better represent cells that have been trapped or arrested in small capillaries in the circulation. As previously, a monolayer of endothelial cells is cultured on a coverslip or in a culture dish and activated using pro-inflammatory cytokines. Tumour cells are then labelled using a fluorescent dye (for example, carboxyfluorescein succinimidyl ester, CFSE), dissociated from the culture flask, and simply introduced onto the endothelial monolayer where they are left to interact for a set time period. The non-bound cells are then removed, and a coverslip is mounted onto a slide (Lowe and Raj, 2015; Wilhelmsen et al., 2013), illustrated in Figure 6A. Representative or whole coverslip images can be acquired using a fluorescent microscope. Cells may attach as single entities or as clusters, which influences the method of analysis. This also models the physiological situation where circulating tumour cells can arrest and transverse capillaries as thin as 7um in clusters (Au et al., 2016). Here, we provide a detailed protocol for the static adhesion assay along with instructions on image analysis using the Analyze Particles or cell counter plugins from FIJI, Figure 6B.

Protocol: Static adhesion assay

1. Preparation of endothelial cells.
    a. Sterilise glass coverslips (13 mm in diameter, thickness #1.5) by dipping them in 70% v/v ethanol using forceps. To allow the coverslips to air dry, place them vertically in a 24-well cell culture plate. Then, lay them down, one per well.

b. Coat the coverslips with 0.2 % w/v bovine gelatin in PBS, pH 7.4. Add about 400 µl gelatin per well and incubate for 2 h in a humidified tissue culture incubator at 37°C with 5% CO_2 atmosphere. Then, remove excess gelatin.

c. Seed endothelial cells (for example, human umbilical endothelial cells, HUVEC) in complete medium directly onto the coated coverslips and culture until completely confluent. Cells need to have formed a confluent monolayer on the day of the experiment.

d. On the day of the experiment, activate endothelial cells by incubating them with 10 ng/ml TNF-α in medium for 2 h.

2. Stain the cells of interest with 10 µM CFSE in medium for 10 min. Wash the cells with PBS to remove excess dye. Then add serum free medium, and detach cells from the culture flask using a cell scraper. Count cells using a standard protocol.

   Additionally, or alternatively, use a nuclear dye (i.e. Hoechst 33342) to visualise cells. Any cell dye that allows cell counting could be used (e.g. membrane dyes). Pipette cells up and down to break clumps after cell scraping, or use a cell strainer.

3. Place a coverslip bearing a confluent endothelial cell monolayer into each well. Add 60,000 cancer cells of interest in 500 µl serum free medium to each well. Allow the cancer cells to adhere for 30 min in a humidified tissue culture incubator at 37°C with 5% CO_2 atmosphere.

   The number of cells added on the endothelial cell monolayer and the incubation time should be tested in advance for each cell line of interest.

4. Wash away unbound cancer cells using PBS. Fix with 400 µl of a solution of 4% PFA for 20 min at RT. Then, wash cells 3 times with PBS. For each coverslip, add a drop of mounting medium onto a glass microscope slide. Pick up each coverslip using forceps and place it onto the drop of mounting medium. You can image the slides straight away or store them at 4°C.

   Dispose of PFA according to the local regulations. Image using (excitation/emission 492/517 nm) for CFSE labelled cells. If possible, image the whole coverslip or take representative images for each one (ensure you have sufficient images for the statistical analysis).

5. Analyse images – calculate adhesion. Adhesion can be calculated as the number of cells attached or the area occupied by cells of interest per field of view. Sample results are given in Figure 6B.

   a) If single cells are obvious and easy to count, or a nuclear dye has been used, then use either the 'Analyze Particles' plugin *(from the FIJI menu bar select Image > Adjust > Threshold > Apply and then Analyze > Analyze Particles, in this case set the parameters so that the software measures single cells or nuclei),* or use the 'Cell counter' plugin *(From the FIJI menu bar select Analyze > Cell Counter, and click on each cell or nucleus).*

   b) If the cells have attached in clusters, and the counting of single cells is difficult, measure the area occupied by cells per field of view *(from the FIJI menu bar select Image > Adjust > Threshold > Apply and then Analyze > Analyze Particles, in this case set the parameters so that the software measures the clusters and make sure the field of view remains constant among the different treatment groups).*

Conclusion

Cancer metastasis remains the most important cause of the burden of cancer-related mortality, and there is a pressing need to understand its complexities such that, ultimately, we can better treat or even prevent it. It is extraordinarily difficult to study metastasis in the clinical situation because, at the point of diagnosis, metastasis has either already taken place – even though frank metastatic disease may not become apparent until months or even years later – or it will be effectively prevented by

timely surgical removal of the primary tumour. While metastasis is undoubtedly highly complex, it is helpful to envisage it as a series of interrelated steps, including: angiogenesis to supply the primary tumour with oxygen and nutrients and to remove waste products, as well as supplying a means of egress; EMT, migration and invasion, such that the cancer cells can enter the blood circulatory system; and then interaction between cancer cell and endothelium prior to extravasation and establishment of a tumour at a new, distant site. It is possible to study each individual step in some detail using simple *in vitro* assay systems. In this paper, we have provided an overview of this complex and fascinating – although deadly – process, and also provided a toolkit of protocols by which researchers who are new to the field can begin to unravel its secrets.

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Figure legends

Figure 1. **The steps of the metastatic cascade:** (A) Angiogenesis, development of a blood supply to the primary tumour. (B) Disaggregation of cancer cells from the primary tumour mass, followed by invasion of the surrounding tissue. (C) Intravasation of cancer cells into the vasculature and haematogenous dissemination. (D) Cancer cells adhere to the endothelium of the vasculature. (E) Cancer cells extravasate and invade into the local tissue to form secondary tumour foci.

Figure 2. **Degradation of the extracellular matrix assessed using a gelatin degradation assay.** (A) Schematic overview of the gelatin degradation assay. Coverslips are coated uniformly with a fluorophore-conjugated gelatin. Endothelial cells are seeded on top of the gelatin-coated coverslips and then labelled with phalloidin. Where the cells have degraded the fluorescent gelatin, there are easily quantifiable dark spots. (B) An example image of a gelatin degradation assay (left). The lines define the borders of two cells. The image is converted to binary and the dark spots are measured for one of the cells using the Analyze Particles plugin from FIJI (right). The results are given in the table below the image.

Figure 3. **Overview of the changes in cell shape during EMT/MET.** (A) Schematic representation of the morphological changes during EMT/MET highlighting the major (x) and minor (y) axes in a fitted ellipse in each case. (B) Example phase contrast images of T47D cells (left) with cuboidal morphology, and hTERT-HME1 cells (right) with elongated morphology. Random cells have been selected using the free hand tool in FIJI and their characteristics (area, circularity, aspect ratio) are reported in the tables below.

Figure 4. **Cell migration assessed using a 'scratch' or 'wound healing' assay**. (A) Schematic overview. A 'scratch; or 'wound' is made in a dense cell monolayer using a vertically held pipette tip. Over time, the cells migrate to close this area. (B) Example phase contrast images of hTERT-HME1 cells 'wounded' in a two directional (cross) pattern. The 'wound' has been marked and the cell-free area has been calculated at each time-point using the MRI Wound Healing tool in FUJI. The area of the 'wound' is given in the top left hand of each image.

Figure 5. **Cell invasion through extracellular matrix assessed using a transwell invasion assay.** (A) Schematic overview. The transwells are coated with a thin layer of Matrigel ® and the cells are seeded on top in serum free medium (SFM). The lower chamber contains medium supplemented with serum or an appropriate chemoattractant. Cells migrate and pass through the pores of the membrane. The cells that are on the underside of the membrane are then stained and counted, while the non-invaded cells on the upper surface are wiped away using a cotton swab. (B) An example fluorescence image of a whole membrane. Here, nuclei of invaded cells were labelled with Hoechst 33342 fluorescent dye and counted manually using the Cell Counter plugin in FIJI.

Figure 6. **Adhesion to an endothelial monolayer assessed using a static adhesion assay**. (A) Schematic overview. A monolayer of HUVEC endothelial cells is cultured and activated using pro-inflammatory cytokines (e.g. TNFα) to better facilitate the attachment of tumour cells. Tumour cells are then labelled, using a fluorescent dye (e.g. CFSE) and applied on to the endothelial monolayer and allowed to interact. The non-bound cells are then removed, and the cells that have adhered are counted. (B) An example composite image of a static adhesion assay (left). Labelled tumour cells adherent to the endothelial layer (phase contrast image). The fluorescence image is converted to binary (right) and the clusters of tumour cells (highlighted) are measured using the Analyze Particles plugin from FIJI.

Table headings

Table 1 **Summary critique of assays for angiogenesis**

Assay type	Summary description	Advantages	Limitations	References
DNA synthesis Proliferation assays	Labelling of the newly formed DNA	Population or individual cell level quantitation	Toxicity, may affect cell physiology	(Duque and Rakic, 2011)
Metabolic proliferation assays	Cells metabolise a specific substrate to a coloured product	High throughput	Toxicity, dependence on mitochondrial enzymes, low sensitivity	(Prabst et al., 2017)
Luminescent cell viability assays	Measurement of ATP content in live cells	High throughput, high sensitivity	Reproducibility may be low	(Posimo et al., 2014)
Fluorescent dye proliferation assays	The fluorescence of each cell is reduced by half after each division	High throughput, not an end-point assay	Possible fluorescence interference	(Lyons et al., 2013)
Cell counting using dye exclusion test	Dead cells incorporate dye, and are distinguished from live cells using a light microscope	Accurate quantitation, inexpensive	Time-consuming, low throughput, unable to distinguish between healthy cells and cells that are alive but lost function	(Strober, 2001)
Tube formation assay	Endothelial cells form capillary structures under angiogenic stimuli	Short culture time, potential to be high throughput	Reproducibility may be low and dependent on cell types and matrices	(DeCicco-Skinner et al., 2014)
Endothelial cell migration assays	see Table 3 Summary critique of assays for cell migration			

Endothelial cell invasion assays	See Table 4 Summary critique of assays for cell invasion

Table 2 **Summary critique of assays for epithelial mesenchymal transition, EMT**

Assay type	Summary description	Advantages	Limitations	References
Assessing mRNA expression of EMT markers using PCR and real-time qPCR	The mRNA levels of target EMT marker genes is measured against housekeeping genes	Quick test or validation experiment, potential to be high throughput	mRNA presence does not guarantee functional phenotype	(Minafra et al., 2014)
Assessing protein expression of EMT markers using western blot / ELISA / flow cytometry / immunohistochemistry	The protein levels of target EMT marker genes is detected by specific antibodies	More conclusive than mRNA expression, potential to be high throughput	Reproducibility may be low and dependent on cell type, especially when only a selection of markers is tested	(Mikesh et al., 2010; Strauss et al., 2013)
Morphological characterization of cells	Assessment of cell morphology using microscopy	Inexpensive, potential to be high throughput, single cell level analysis	Reproducibility may be low and dependent on cell types and matrices	(Abdulla et al., 2013; Nelson et al., 2008)

Table 3 **Summary critique of assays for cell migration**

Assay type	Summary description	Advantages	Limitations	References
Wound healing / scratch assay / exclusion zone assay / fence/ring assay	Space filling collective migration	Standardised techniques, no special equipment needed, inexpensive	Affecting the neighbouring cells, reproducibility may be low	(Ashby and Zijlstra, 2012; Chen and Nalbantoglu, 2014; Todaro et al., 1965)
Transwell chamber migration assay	Vertical single cell migration	Commercially available, variations of the assay exist to fit the researcher's needs, relatively high throughput	Relatively high cost, end-point assay, assessment of single cell movement only	(Boyden, 1962; Harisi et al., 2009)
Microcarrier bead/ spheroid migration assay	Vertical cell movement away from the bead/spheroid	Recapitulates *in vivo* characteristics	Non-standardised, reproducibility may be low depending on cell type	(Knupfer et al., 2001; Rosen et al., 1990)
Individual cell tracking/colloidal particle assay	Migrating cells leave a measurable trail on a colloidal gold coated surface	Can be real-time, measures random motility including direction and speed	Non-standardised, reproducibility may be low, assessment of single cell movement only	(Niinaka et al., 2001)

Table 4 **Summary critique of assays for cell invasion**

Assay type	Summary description	Advantages	Limitations	References
Gelatin degradation assay	Proteolytic gelatin degradation for vertical movement	Assessment of the cell's capacity to produce enzymes capable of degrading ECM, inexpensive	Non-standardised, reproducibility may be low	(Diaz, 2013)
Transwell invasion assay	Vertical chemotaxis assessing 3D invasion	Commercially available, variations of the assay exist to fit the researcher's needs, relatively high throughput	Relatively high cost, assessment of single cell movement only, distance of invasion is not considered	(Marshall, 2011)
ORIS™ 3D invasion assay	Space filling invasion	Standardised method	Specialised equipment required, high cost	(Bertier et al., 2018)
Collagen gel invasion assays (spheroid, vertical, cell tracking)	3D invasion of spheroid, cell layer or single cells into ECM	Real-time monitoring of 3D cell movement	Non-standardised, reproducibility may be low	(Hattermann et al., 2011; Nystrom et al., 2005)

Table 5 **Summary critique of assays for cell adhesion to endothelial monolayers**

Assay type	Summary description	Advantages	Limitations	References
Flow adhesion assay	Adhesion of cells on an endothelial cell layer under shear flow conditions	Recapitulates *in vivo* shear forces	Specialised equipment required, technically complex, time consuming, relatively high cost,	(Lane et al., 2012; Orr et al., 2000)
Rocking adhesion assay	Adhesion of cells on an endothelial cell layer under sweeping flow conditions	Allows for some flow to mimic physiological characteristics, technically simple, multiple replicates possible	Does not recapitulate *in vivo* shear forces	(Bapu et al., 2014)
Static adhesion assay	Adhesion of cells on an endothelial cell layer under no flow conditions	Easy set-up, no special equipment needed, recapitulates static flow as in capillary beds, multiple replicates possible	Does not recapitulate *in vivo* shear forces	(Lowe and Raj, 2015)

Figure 1

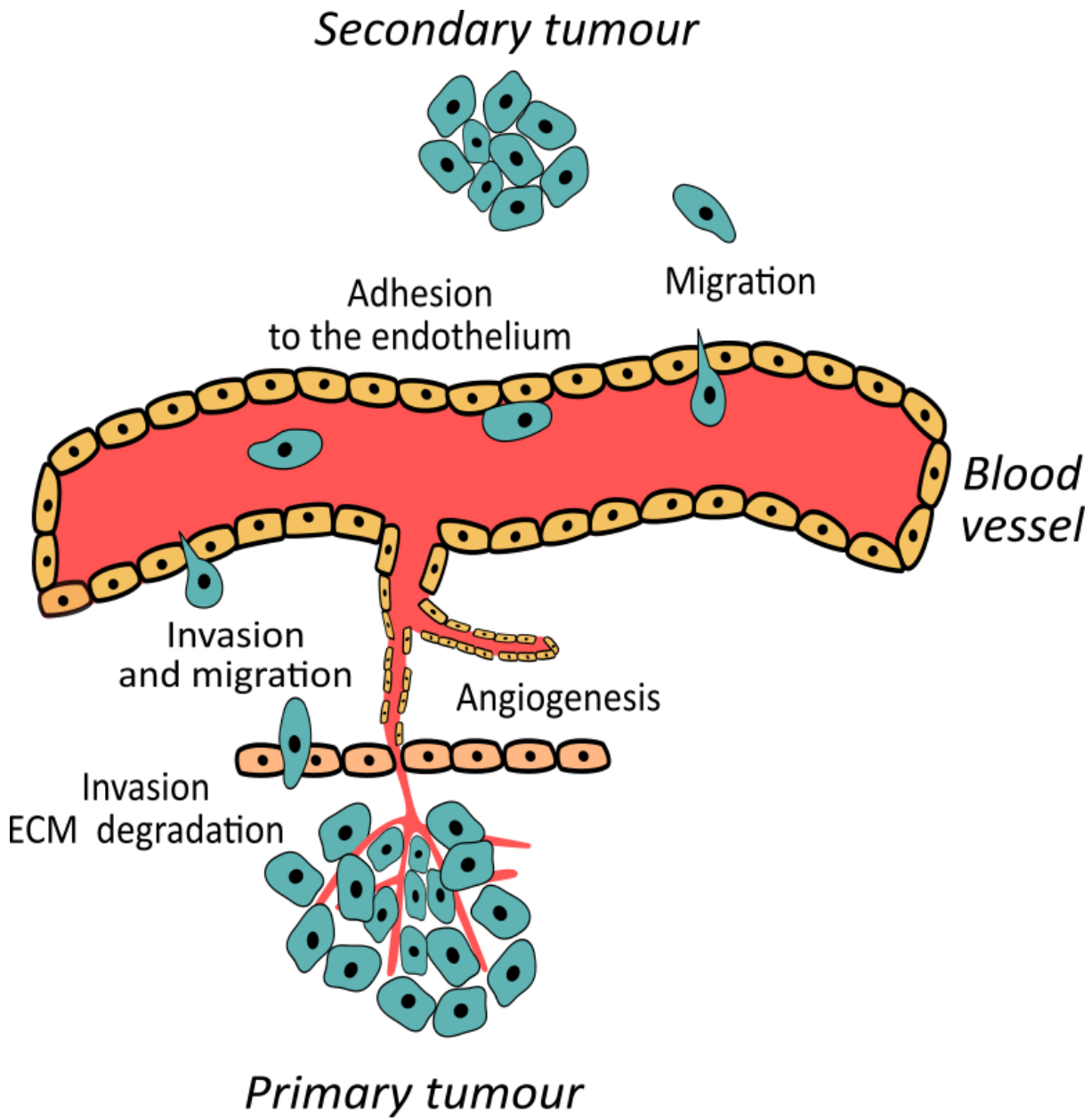
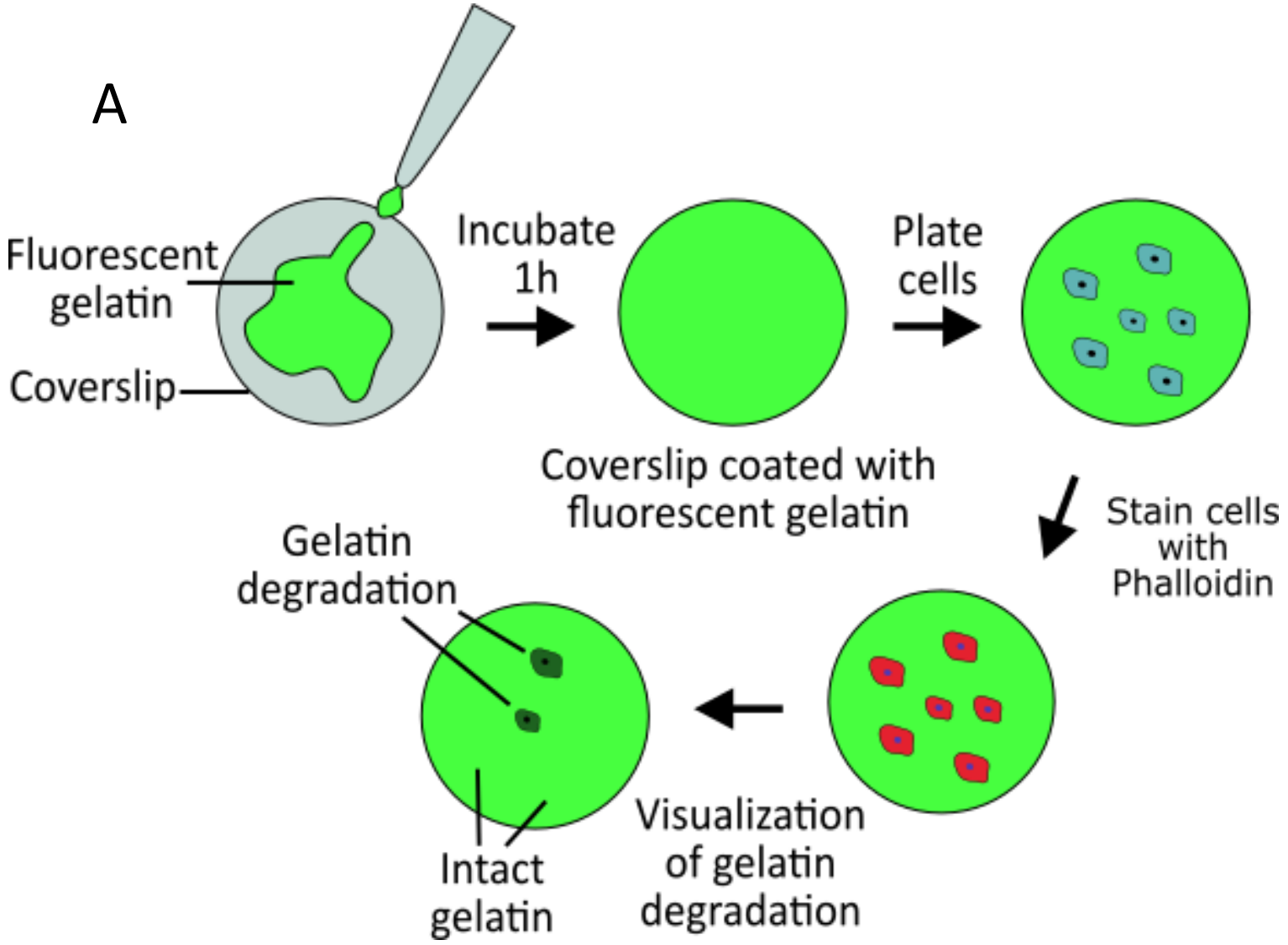
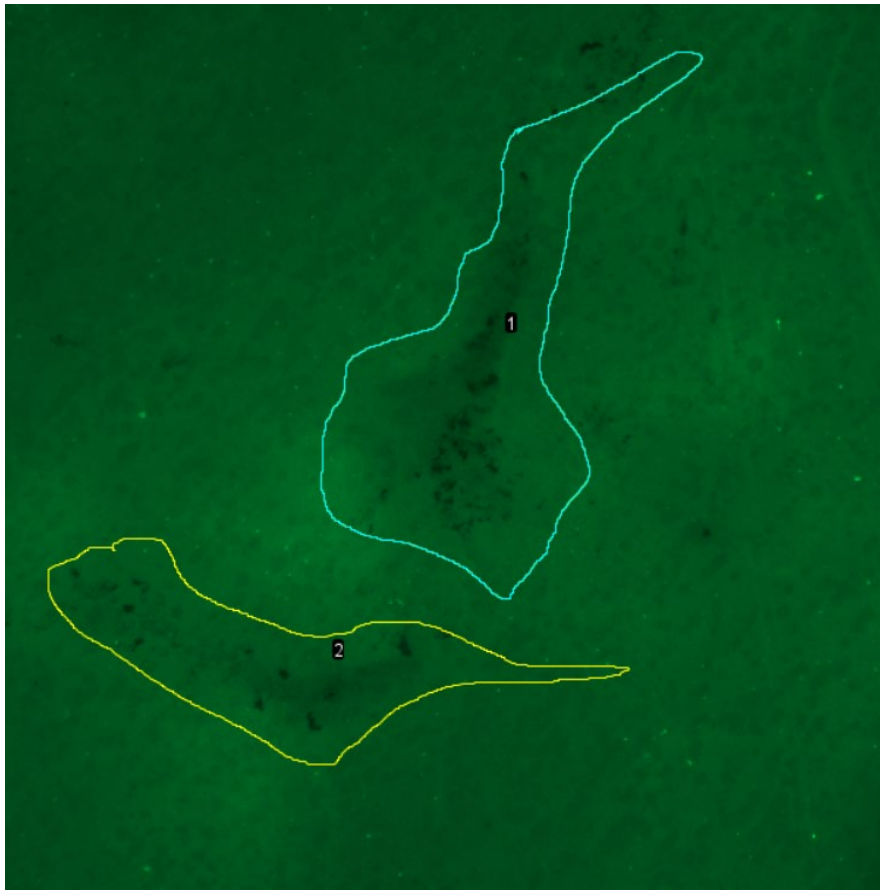
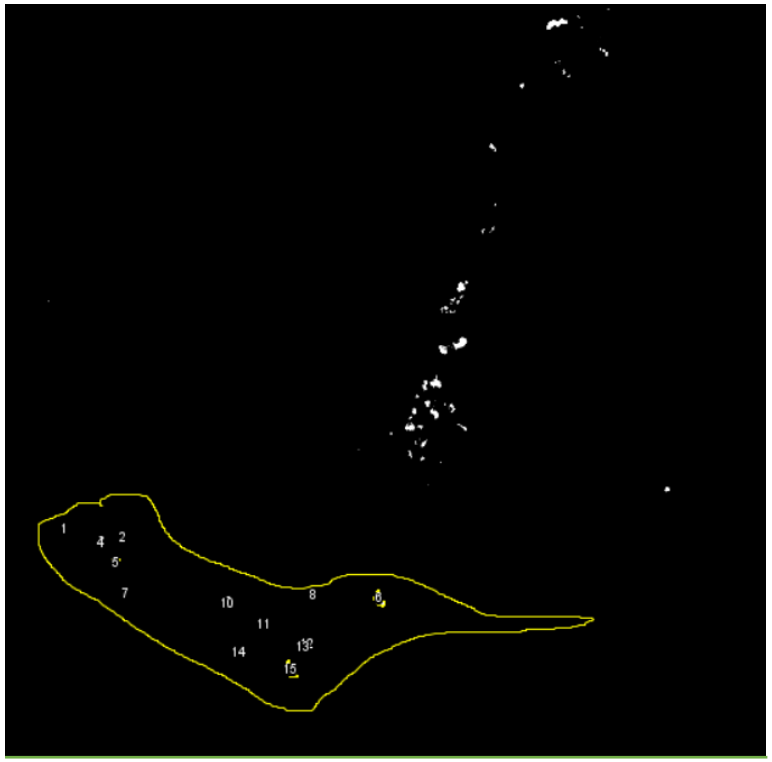

Secondary tumour
Migration
Adhesion
to the endothelium
Blood
vessel
Invasion
and migration
Angiogenesis
Invasion
ECM degradation
Primary tumour

Figure 2A


A
Fluorescent gelatin
Coverslip
Incubate 1h
Plate cells
Coverslip coated with fluorescent gelatin
Stain cells with Phalloidin
Visualization of gelatin degradation
Gelatin degradation
Intact gelatin

B





Slice	Count	Total Area (µm²)	Average size (µm²)	% Area
Gelatine	15	15.7	1.05	0.76

Figure 3A

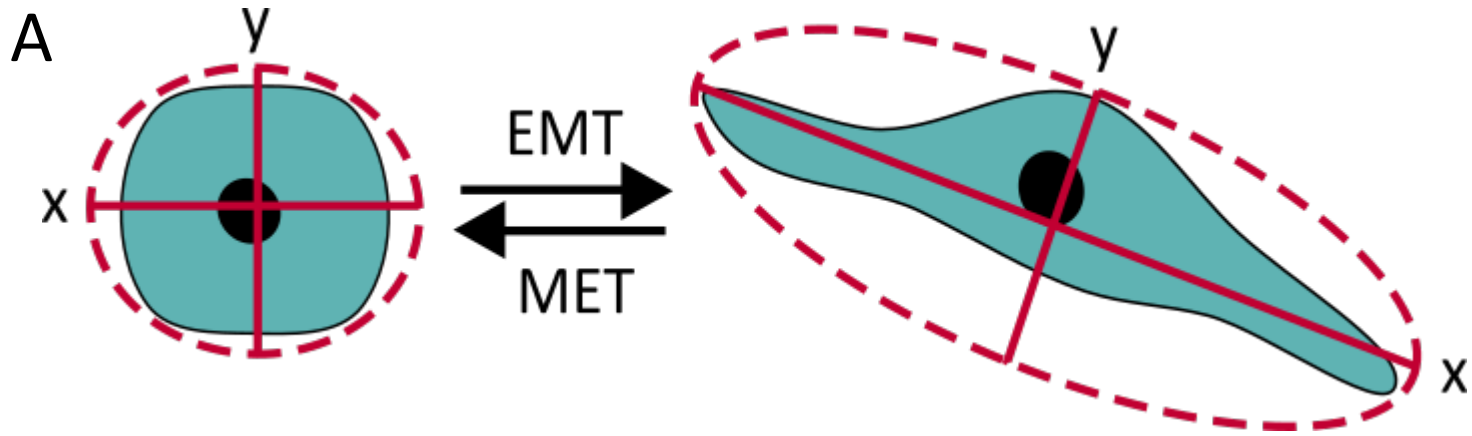
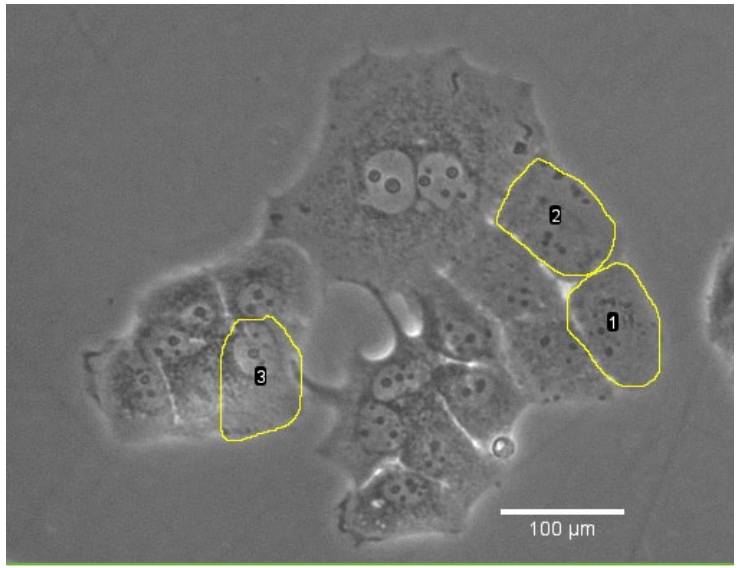
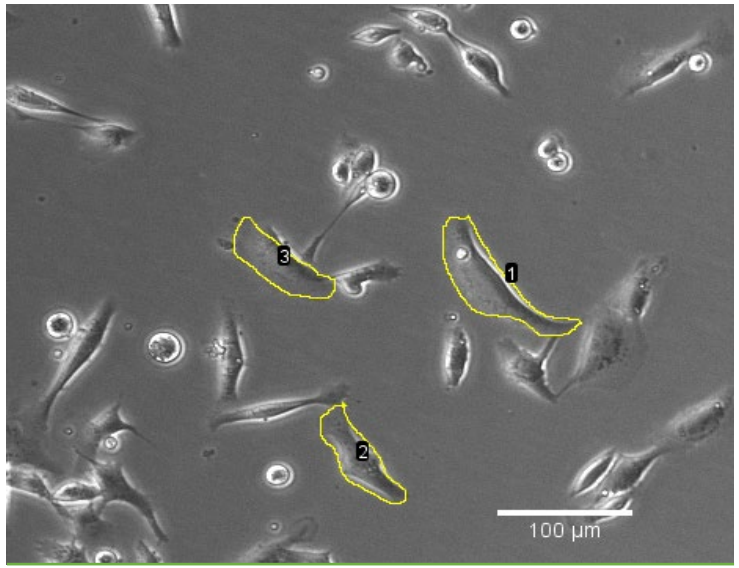


Figure 3B

B



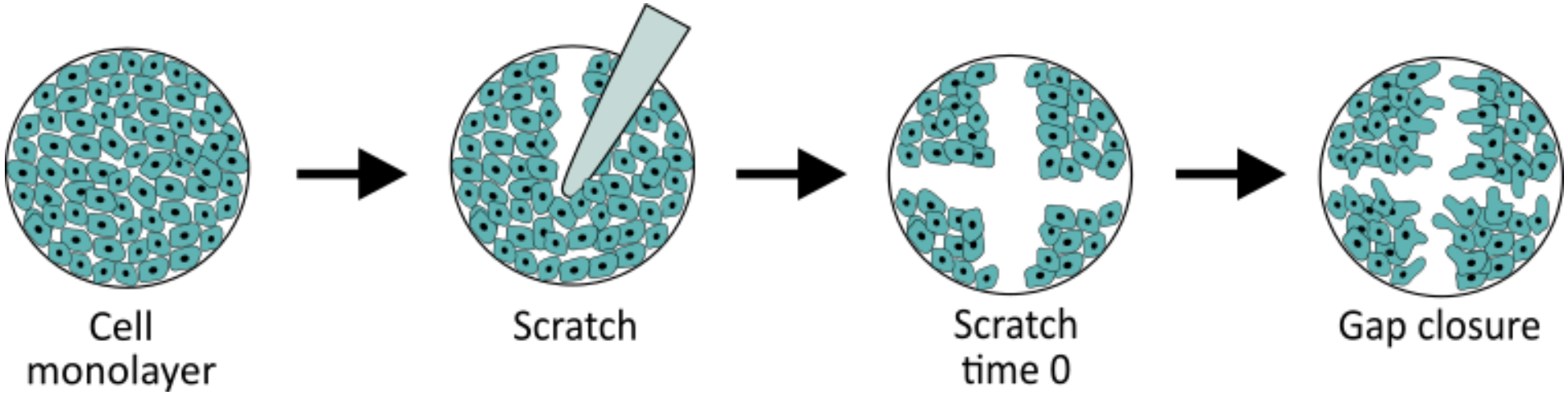
Cell	Area (pixels)	Circ.	AR
1	5122	0.879	1.496
2	5390	0.799	1.539
3	3851	0.768	1.914



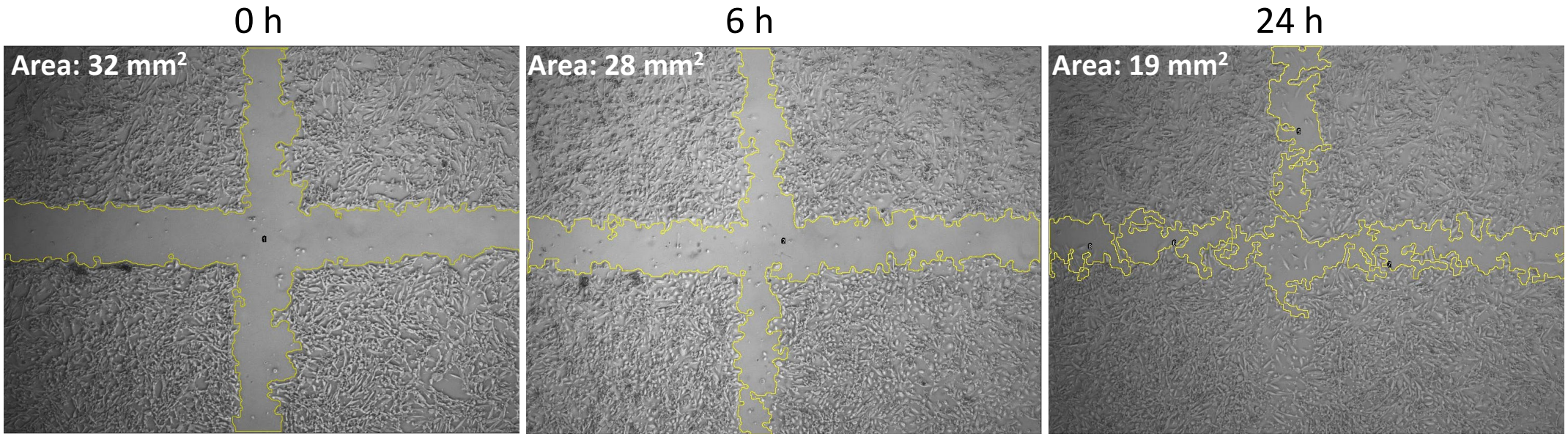
Cell	Area (pixels)	Circ.	AR
1	2080	0.368	3.163
2	1413	0.508	2.370
3	1714	0.575	2.251

Figure 4

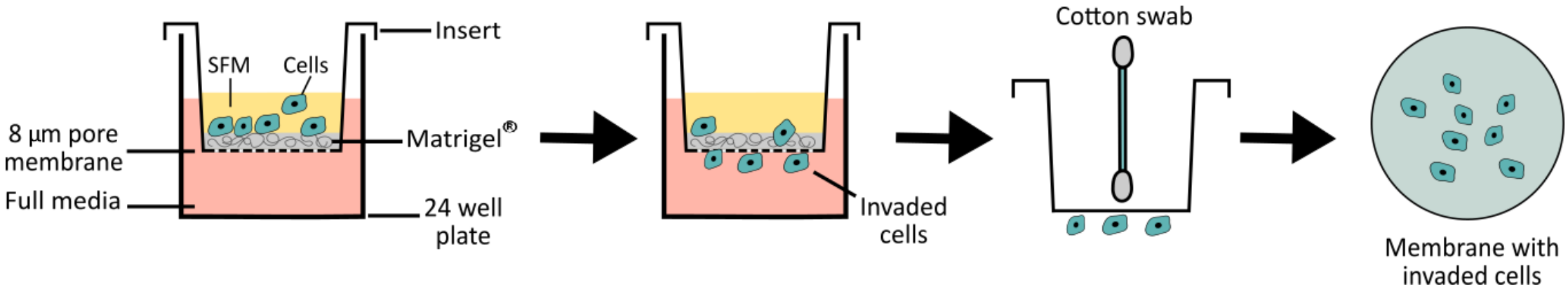
A


Cell monolayer
Scratch
Scratch time 0
Gap closure

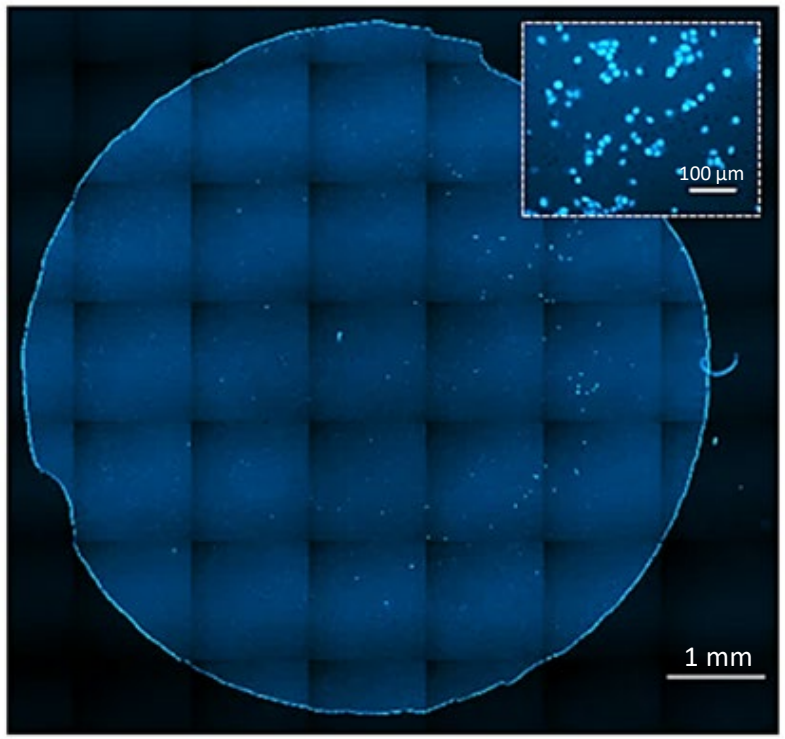
B


0 h
6 h
24 h
Area: 32 mm²
Area: 28 mm²
Area: 19 mm²

A


Insert
SFM
Cells
8 µm pore membrane
Matrigel®
Full media
24 well plate
Invaded cells
Cotton swab
Membrane with invaded cells

B


100 µm
1 mm

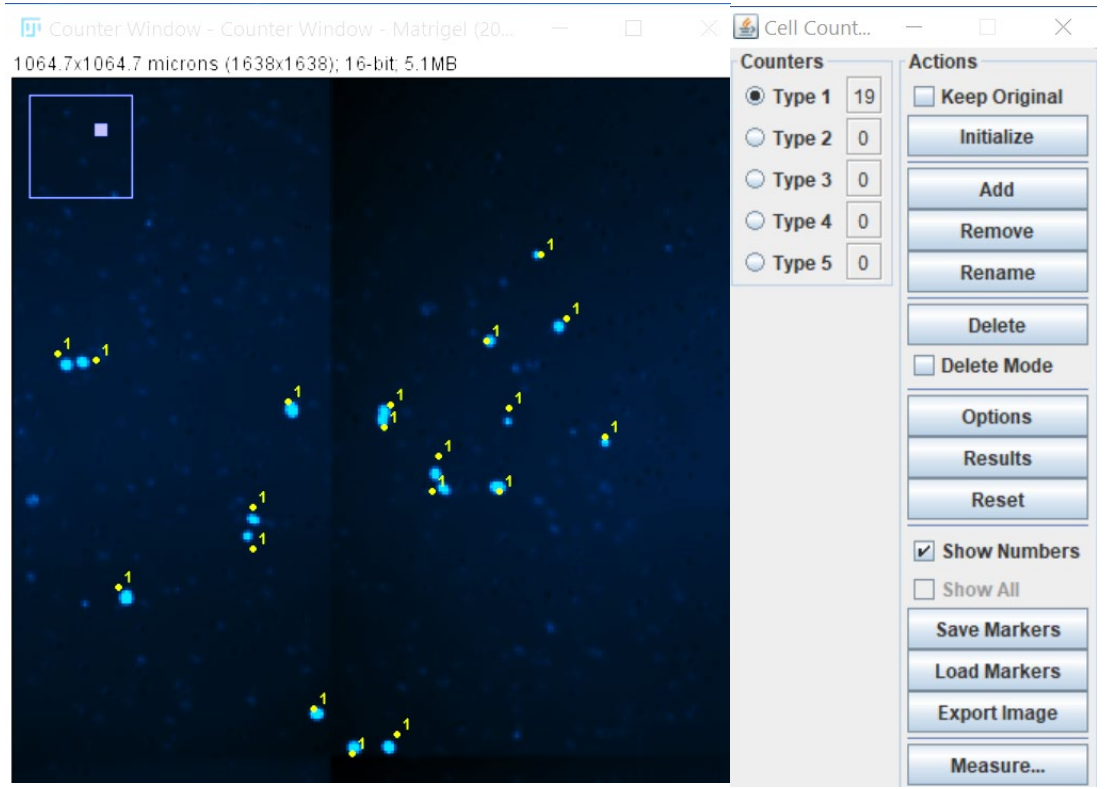
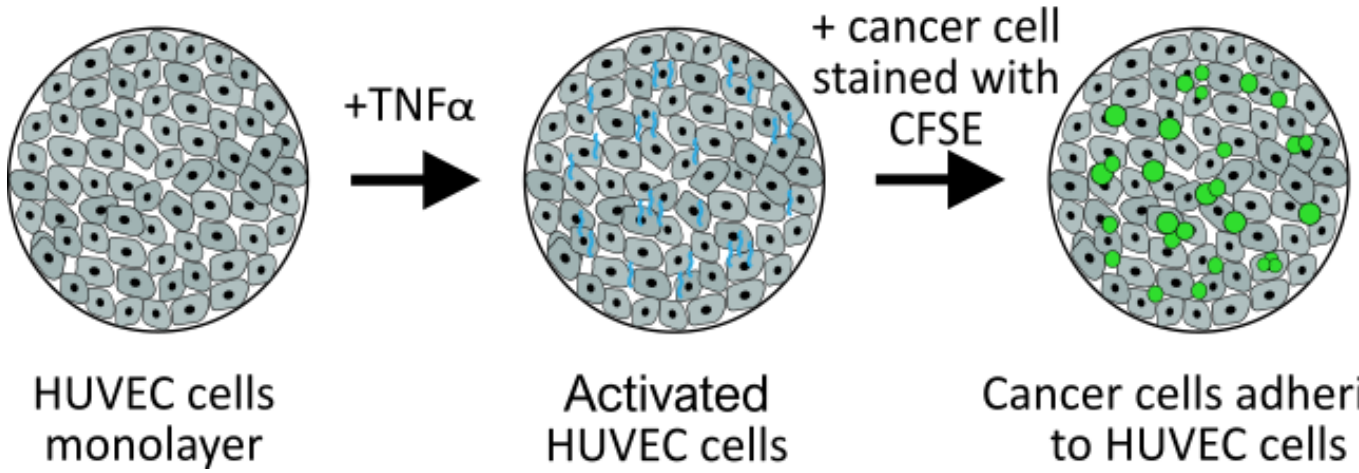
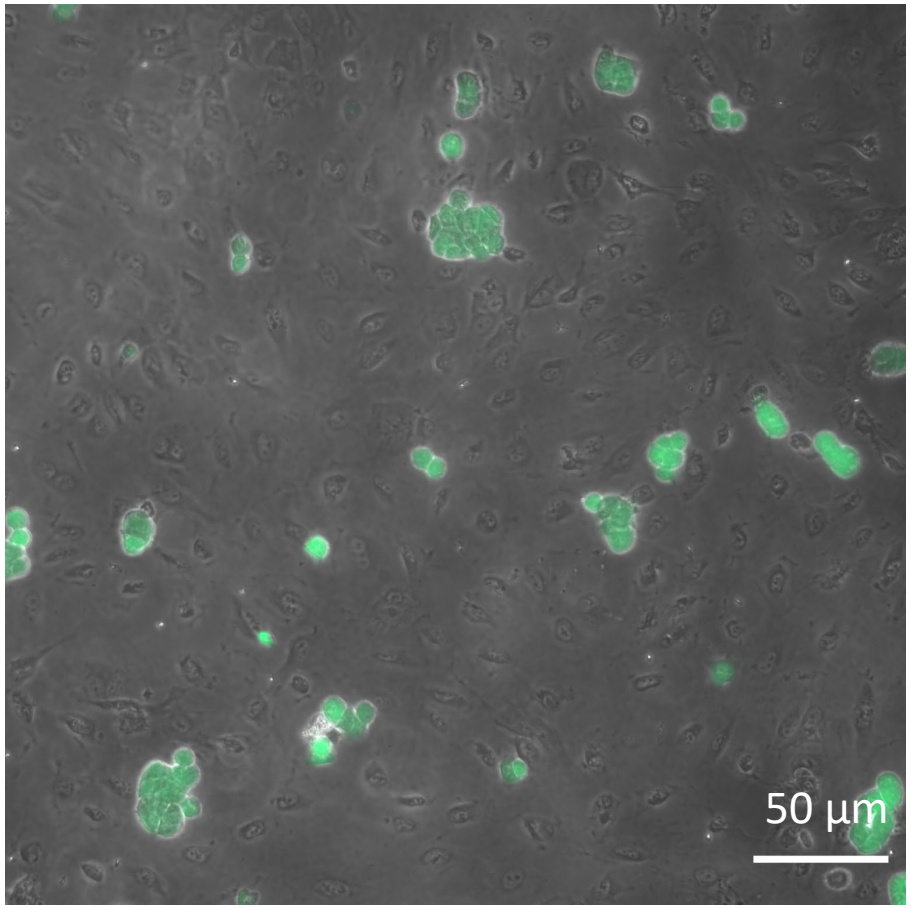

Counter Window - Counter Window - Matrigel (20...
1064.7x1064.7 microns (1638x1638); 16-bit; 5.1MB
Cell Count...
Counters
Type 1 19
Type 2 0
Type 3 0
Type 4 0
Type 5 0
Actions
Keep Original
Initialize
Add
Remove
Rename
Delete
Delete Mode
Options
Results
Reset
Show Numbers
Show All
Save Markers
Load Markers
Export Image
Measure...

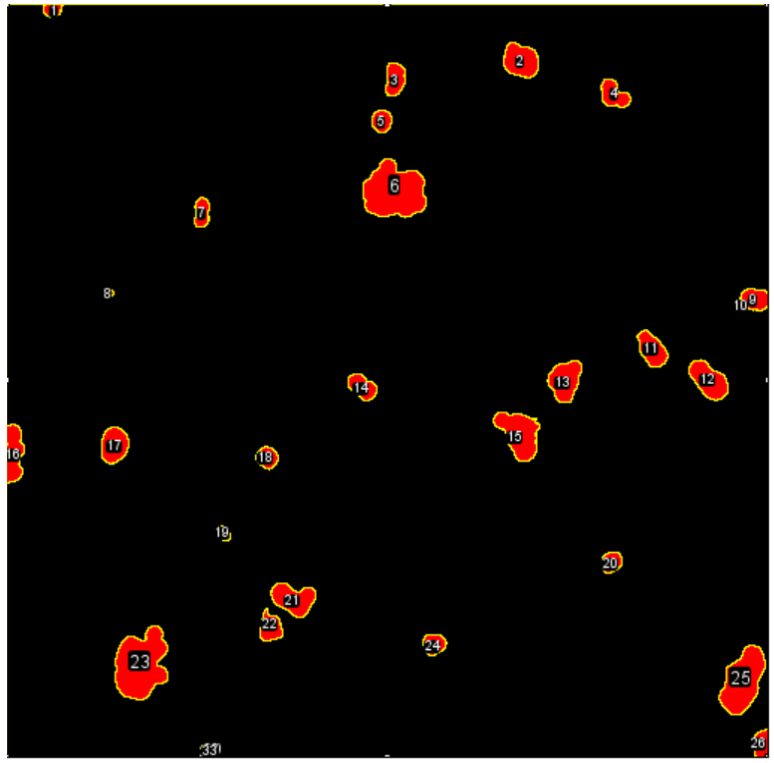
Figure 6

A



B





Slice	Count	Total Area (pixels)	Average size	% Area
Adhesion	33	15622	473	3.5