

Chapter 15

In Vivo Assay for Tumor Cell Invasion

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Summary

We describe an in vivo invasion assay that enables the collection of invasive cells from the primary tumor. In addition to determination of the endogenous, unstimulated invasive properties of cells in vivo, the assay can take advantage of the chemotactic properties of cancer cells. Microneedles are filled with a mixture of extracellular matrix components such as Matrigel with or without a chemoattractant such as EGF, and then introduced into the primary tumor of a rat or mouse that is generated either by orthotopic injection of carcinoma cell lines or by a transgene such as polyoma Middle T. Over the course of 4 h the invasive cell population enters the needles while the animal is kept under anesthesia. At the end of the collection time, the invasive cells are extruded from the microneedles and can be analyzed in terms of the number and type of cells that invade in response to defined stimuli. By including pharmacological inhibitors in the needle, signaling pathways contributing to in vivo invasion can also be identified. This assay leads to a better understanding of the cell types and signaling involved in the tumor microenvironment, and has the potential to be applied to a variety of in vivo models.

Key words: Invasion, Chemotaxis, Tumor microenvironment, In vivo, Chemoattractant

1. Introduction

An important characteristic of cancer cells is their ability to detect chemoattractant gradients and move in response to them (1, 2). It has been shown that chemotaxis plays a critical role in metastasis, where cancer cells detect chemoattractant gradients found within the tumor microenvironment (3, 4) and distant tissues (5), and respond to these signals by leaving the primary tumor, entering the circulation and colonizing other organs (6, 7). In vitro studies of chemotaxis using devices such as the Boyden

microchemotaxis chamber allow for accurate measurements under controlled conditions, but often are restricted to studying cell motility on flat, 2-D surfaces. Increasingly sophisticated 3-D invasion assays help to provide more realistic, accessible models to study cell motility and invasion (8, 9), but still remain limited approximations that require comparison to cell behavior in a true *in vivo* microenvironment. To most accurately study the *in vivo* process of invasion at the cellular level, the direct collection of tumor cells *in vivo* was central to designing what we term the *in vivo* invasion assay (10). This assay enables the direct stimulation and collection of cells that have been conditioned and grown in their natural microenvironments. With this assay, such cells are directly collected from living animals. The assay can be used for a variety of purposes, including (1) measuring invasion under various conditions or stimuli, (2) evaluation of invasive cell types, (3) pharmacological dissection of invasion mechanisms *in vivo*, and (4) further characterization of the specific properties of the invasive cells. In the remainder of this section we provide some examples of these uses.

Measurements from the *in vivo* invasion assay have been shown to correlate with the metastatic properties of cancer cells in a xenograft model of breast cancer, with more metastatic cells invading 15-fold more efficiently than less metastatic cancer cells in response to an EGF gradient, correlating with their *in vitro* chemotactic responses to EGF (10). Transgenic models of breast cancer have also been examined, and the invasiveness of breast cancer cells to chemoattractants such as TGF- α and heregulin among others was evaluated in the polyoma Middle T model (11).

Identification of the invasive cells by immunofluorescence led to a remarkable and unexpected result. Both cancer cells and macrophages invaded into the needles, leading to the discovery of a paracrine communication between these cell types during invasion. Chemoattractant-stimulated secretion of CSF-1 by cancer cells can activate macrophages to secrete EGF, resulting in further stimulation of the cancer cells. This positive feedback loop can result in the propagation of the chemotactic signal (11). Inhibitors included in the needles with the chemoattractants that blocked either EGF or CSF-1 signaling lent further support to this model of paracrine signaling between tumor cells and macrophages (11). In addition to analyzing the invasive properties, further characterization of cell-cell and cell-stroma interactions in those tumors was achieved by intravital multiphoton imaging of the tumors during the invasion assay (11).

The collected invasive cells can be extruded from the needles and then characterized using other assays. Carcinoma cells from such *in vivo* invasion assays were subjected to cDNA microarray analysis and compared to the average primary tumor population (12,

13). Gene expression characterization of the invasive cancer cells collected using this assay has shown that invasive cancer cells activate motility pathways that are important for cells to leave the primary tumor, and are nonapoptotic and nonproliferative (12, 14). This method provides a way of overcoming the problem of identifying the gene expression signatures or other properties of a relatively small number of invasive cells within a primary tumor.

In summary, the in vivo invasion assay has provided us with vital information about how in vitro chemotactic properties of breast cancer cells correlate with their in vivo invasive and metastatic characteristics. The assay provides the conditions to generate a gene signature specific to the invasive population, as opposed to the average primary tumor. It was critical for the discovery of a paracrine interaction between breast carcinoma cells and macrophages which is important for their invasive properties. It is therefore evident that this in vivo invasion assay has many potential applications, such as testing effects of different chemoattractants, inhibitors, or ECM composition on invasion. This assay is not limited to studying breast cancer, and can be applied to a wide range of tumor models, whether created by cell lines injected orthotopically into different sites or tumors generated by different transgenes. This assay can potentially be applied to any tissue that has a migratory cell population.

2. Materials

2.1. Instruments for Needle Cell Collection Setup

1. *Micromanipulator.* We use the Narishige model MN-151 as a relatively inexpensive but stable micromanipulator.
2. *Needle holder.* We use a customized holder for the needles (Fig. 1). A 4 cm × 5 mm stainless steel bar is turned on a lathe so that the first disc is shaped to be 1 mm in width × 4 mm in diameter. The next part of the bar is shaped to be a 3 mm × 3 mm spacer, followed by another 1 mm × 4 mm disc, followed by a 5 mm × 3 mm spacer. Three equidistant 0.5 mm holes are drilled into each of the discs to hold the 25-gauge guiding needles. The bar is attached to a 12.5 mm × 1 mm disc with three equidistant 1-mm holes. It is silver soldered to a 12 cm × 4 mm stainless steel bar that is threaded at the far end. A Plexiglas block of 1 cm × 1.5 cm × 0.5 cm is tapped to take the threaded end of the bar. The block allows the holder to be placed in a Narishige model MN-151 micromanipulator.
3. Hamilton needles, 33 gauge (Fisher Scientific, Pittsburgh, PA).

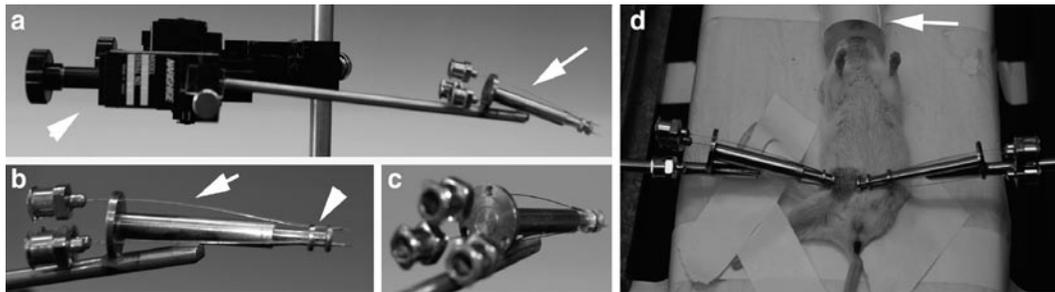


Fig. 1. In vivo invasion instruments and setup. (a) Apparatus used for the in vivo invasion assay. *Arrow* points to microneedle holder. *Arrowhead* points to Narishige model MN-151 micromanipulator. (b) Closeup of microneedle holder. *Arrow* points to 33-gauge microneedles. *Arrowhead* points to 25-gauge guide needles. (c) Rear view shows how microneedles are placed within the holder. (d) Experimental setup of microneedles inserted into an orthotopically grown mammary tumor in an SCID mouse. *Arrow points* to nose piece for delivery of Isoflurane anesthesia.

4. *Guide needles.* The guide needles are made by cutting standard 25-gauge needles at the plastic base. Prior to cutting, a wire is inserted into the needle to avoid closing the hollow part of the needle.
5. Vaporizer for Isoflurane, model 100 (Surgivet, Waukesha, Wisconsin).
6. Mapleson E. breathing circuit (VetEquip, Pleasanton, CA).
7. Experimental platform for laying down the rodent during the procedure. Use the styrofoam holders of 15-mL or 50-mL conical tubes, wrapped in a bench disposable diaper (Kendall).

2.2. Animal Models Used for Cell Collection

1. *Xenograft model of breast cancer:* our laboratory uses the rat mammary carcinoma MTLn3 cells (10). This cell line is grown in α -MEM supplemented with 5% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin solution (Life Technologies). The tumor cells are grown to 70–85% confluence before being harvested. Cells are detached using PBS-EDTA and scraped using a rubber policeman. 5×10^5 cells are injected into the right fourth mammary fat pad from the head of 5-to-7-week-old female severe combined immunodeficient (SCID) mice (National Cancer Institute, Bethesda, MD). Tumors are allowed to grow for 4 weeks before cell collection when the tumor is about 2 cm in diameter (see Note 1).
2. *Transgenic model of breast cancer.* FVB mice transgenic for the polyoma virus middle T (PyVT or PyMT) oncogene under the mouse mammary tumor virus (MMTV) long terminal repeat 206 (LTR), generated as previously described (15), are used for in vivo invasion assays at 12–14 weeks of age when the tumors are about 2 cm in diameter (see Note 2).

2.3. Reagents for Cell Collection

1. *L15-0.3% BSA solution*. Leibovitz's L-15 Medium (1×), no phenol red, stored at 4°C protected from light (Invitrogen, Carlsbad, CA). Bovine serum albumin, Fraction V, ethanol precipitated, stored at 4°C (Fisher Scientific). Prepare L15-0.3% BSA solution fresh for every experiment by first preparing a 6.9% BSA solution in PBS, sterile filtered using 0.20- μ m sterile syringe filter. Keep the L15-0.3% BSA solution on ice covered with aluminum foil.
2. Matrigel basement membrane (Becton Dickinson, Franklin Lakes, NJ). Store at -80°C in 200–500 μ L aliquots. Thaw single aliquots overnight at 4°C when you are ready to use. Always keep on ice to prevent gel formation. Store the thawed Matrigel aliquot at 4°C. Stock concentration of 9.7 mg/mL, diluted 1:10 in the needles.
3. Ethylenediamine tetraacetic acid, pH 8.0 (EDTA, Invitrogen). Prepare 1 mM stock solution by diluting with double-distilled deionized water. Store at room temperature.
4. Epidermal growth factor (EGF, Gibco Life Technologies). Dilute in PBS to make 50-mM stock aliquots and store stock at -80°C. Thaw an aliquot on ice the day of the experiment and dilute as needed with L15-0.3% BSA. Once EGF has been diluted with L15-0.3% BSA it should not be refrozen; discard at the end of the day.
5. Isoflurane, USP (Baxter, Deerfield, IL).
6. Oxygen tanks (M-E) with regulator (TechAir, Connecticut).

2.4. Reagents for Cell Counting

1. 4',6-Diamidino-2-phenylindole, dilactate (DAPI, Sigma, St. Louis, MO). Prepare a 10 mg/mL stock solution in ddH₂O and a 0.5 μ g/mL working solution in PBS. Store both at 4°C protected from light.
2. Microscope cover slips, 24 mm \times 50 mm \times 1.0 mm (Thermo Scientific, Waltham, MA).
3. Sterile disposable 1-mL syringes with Luer slip tip (Fisher Scientific).

2.5. Reagents for Cell Typing by Immunofluorescence

1. Formalin neutral-buffered phosphate 10% (BioWhittaker, Walkersville, MD).
2. Poly-L-lysine hydrobromide (Sigma). Prepare a 0.1 mg/mL stock solution in water. Store at -20°C.
3. MatTek dishes (MatTek, Ashland, MA). To prepare the MatTek dish for cell plating, clean the dish in a culture hood by incubating with 1 M HCl for 5 min, rinse extensively with PBS, and then add 95% EtOH solution and incubate for another 5 min. Rinse the dish with PBS.

4. *Tris-buffered saline, pH 7.4 (TBS)*. Mix 8 g NaCl, 0.2 g KCl, 10 mL 1 M Tris base in 1 L ddH₂O and adjust pH to 7.4 with HCl; store at room temp.
5. TBS-2% FBS solution: dilute fetal bovine serum (FBS) (Gemini, West Sacramento, CA) to 2% with TBS.
6. TBS-1% BSA solution, sterile filter solution using 0.20- μ m sterile syringe filter. Store at 4°C.
7. 0.05% Triton X-100 (Sigma-Aldrich) in TBS. Store at room temp.
8. Primary antibody mixture of rabbit pan-Cytokeratin antibody (Santa Cruz) (1:50 dilution) for carcinoma cells and rat anti-F4/80 (16) (1:25 dilution) for macrophages. Store the undiluted pan-Cytokeratin antibody at 4°C; never freeze. The undiluted F4/80 antibody should be stored at -20°C in several aliquots; prevent frequent freeze-thaws. Use appropriate (antirabbit for the cytokeratin, antirat for the anti-F4/80) secondary fluorescent antibodies, stored as indicated by manufacturer, protected from light.

3. Methods

3.1. Preparing Solutions for Needle Collection

1. 100 μ L total volume is prepared to fill each 33-gauge experimental needle. Thaw all reagents on ice and prepare mixtures on ice using sterile 1.5-mL Eppendorf tubes. Each needle should contain:
 - 0.01 mM final concentration of EDTA in 100 μ L total volume
 - Dilute EGF stock with L15-0.3% BSA to a concentration of 1 mM, add 2.5 μ L of this dilution for a final concentration per needle of 25 nM (the normal concentration we use)
 - L15-0.3% BSA to bring total volume to 100 μ L, including the Matrigel volume
 - 10 μ L Matrigel (added last)
2. Vortex reagents at low speed to ensure proper mixing, getting rid of any bubbles in the mixture by tapping on the tube, place on ice.

3.2. Preparing the Needle Collection Setup

1. Clean setup with 70% ethanol, allow to dry.
2. Insert 25-gauge guide needles into the needle holder.
3. Place 33-gauge blocking needles through the 25-gauge guide needles, letting the tips of the blocking needles surpass the ends of the guide needles by several millimeters.

4. Place the needle holder into the micromanipulator.
5. Clean the 33-gauge experimental needles using a 1-mL syringe containing 70% ethanol. Pressing the plunger push about 0.5 mL of 70% ethanol through each needle. Get rid of any traces of ethanol by repeating the procedure with dH₂O.
6. Using a 1-mL syringe collect the contents of the Eppendorf tube with the appropriate mixture of buffer, Matrigel, and chemoattractant (such as EGF) prepared in **step 3.1** and load the 33-gauge experimental needles slowly until 2–3 drops of liquid are expelled from the needle tip (*see Note 3*).

3.3. Performing the Assay

1. Anesthetize rodent by using 5% Isoflurane, and place it on its back on the experimental platform, with the nose piece attached to the Isoflurane vaporizer placed around the rodent's head to allow comfortable breathing and delivery of Isoflurane (*see Note 4*).
2. Prepare the area of the tumor where the needles will be placed by trimming the hair using scissors, so there is a clear view of the tumor.
3. Using the micromanipulator, slowly advance the guide needles onto the area where the cells will be collected until the guide needles pierce the skin of the tumor (*see Note 5*). Make sure the guide needles truly pierce the skin of the tumor and that the setup is being held very stably (*see Note 6*). Gently push the blocking needles into the tumor until they are about 2 mm in (*see Note 7*). Carefully remove the blocking needles one by one, while holding the tumor in place. Do this paying attention that the guide needles do not move out of place.
4. Insert the experimental needles through the guide needles one by one, gently pushing them into the tumor until you feel resistance. Do not push any further as to avoid getting a tumor biopsy (*see Note 8*). Make sure that you hold the tumor in place each time you insert an experimental needle by supporting the tumor between your thumb and index fingers, so that the whole setup does not shift.
5. After all the experimental needles are placed in the tumor, watch the animal's breathing frequently to ensure proper anesthesia. Lower the Isoflurane level gradually, especially during the first half hour. There is variation from animal to animal, but after the first half hour Isoflurane should be for the most part no higher than 2%, the usual being around 1% and below (*see Note 9*).
6. Monitor the animal for the duration of the needle collection (we typically collect for 4 h), adjusting the Isoflurane level as necessary (*see Note 9*). At the end of the collection, carefully remove each experimental needle holding the tumor in place

by supporting the tumor between your thumb and index fingers. Remove the entire setup from the tumor and euthanize the animal by cervical dislocation.

7. Eject the contents of each experimental needle using a 1-mL syringe filled with DAPI onto a microscope slide. The contents of each needle should be emptied with no more than about 100 μL of DAPI. Count the cells from each needle using a fluorescence microscope, counting all cells present within the drop by DAPI stain and/or other stains if cells are fluorescently labeled.
8. Clean experimental needles using a 1-mL syringe filled with 70% ethanol. Flush about 500 μL ethanol through to clean each needle.

3.4. Identification of the Invasive Population

To characterize the invasive population, all the experimental needles should be used in this procedure (usually 3–6, depending on size of tumor) using the same experimental conditions (e.g., needles with chemoattractant) so as to maximize the number of cells available for imaging at the end of the procedure, for each experiment. There is a great loss of cells during this procedure as there are several incubation steps with washes in between.

1. Prepare a MatTek dish for extrusion of the needle collected cells by first rinsing the glass center with 1 M HCL for 5–8 min. Following this, rinse the dish with PBS, aspirate it, and add 95% ethanol solution for 1–2 min. After aspirating the ethanol and rinsing well with PBS, coat the glass part of the dish by adding poly-L-lysine solution for 2 h. At the end, aspirate the poly-L-lysine and allow the dish to dry before using it.
2. After the experimental needles are removed from the tumors carefully at the end of the needle collection, empty the contents of the needles into the precoated MatTek dish using a 1-mL syringe filled with 10% formalin solution. Total volume should not exceed 200 μL . This means that only 2 or 3 drops should be extruded from each needle. Allow the cells to fix for 1 h.
3. To block nonspecific binding, add an equal volume of TBS-2% FBS solution (such that the final concentration is 1% FBS) and leave the dish overnight at 4°C.
4. The next day, remove the blocking solution and rinse 3 times with TBS-1% BSA solution. Keep in mind that all the washes and incubation solutions must be applied and aspirated extremely gently, by adding solution to one side of the glass part and aspirating from the other, never actually making contact with the glass surface where the cells are attached.
5. Permeabilize the cells with 100–200 μL of TBS-0.1% Triton X-100 for 5 min at room temperature.
6. Wash 3 times with TBS-1% BSA.

7. Incubate the collected cells in a mixture of primary antibodies. For our purposes, we usually incubate with an anti-F4-80 antibody (1:25) and anti-pan-Cytokeratin antibody (1:50) in TBS-1% BSA to stain for macrophages and carcinoma cells, respectively. The incubation is done for 1.5 h at room temperature.
8. Wash 3 times with TBS-1% BSA.
9. Incubate in a mixture of appropriate secondary antibodies coupled to different fluorophores at a ratio of 1:300 (*see Note 10*) for 1 h.
10. Wash twice with TBS-1% BSA, and leave in the third wash. When ready, add DAPI and count the cells using a fluorescence microscope.

4. Notes

1. In our experience, the MTLn3 xenograft model is ready to be used for needle collection 4 weeks post injection in the mammary fat pad. At this point, the tumors are 1.5–2 cm in diameter and are not significantly ulcerated thus maximizing the surface area where the needles can be inserted. When using other xenograft models for this assay, one has to take into account the size of the tumor, which should be no smaller than 1 cm in diameter and not too ulcerated to allow the insertion of at least three needles.
2. When using transgenic models of cancer one has to first stage the tumor. For this assay we use tumors that are already invasive to maximize the number of cells that invade the needles. One can also compare the invasive potential of the cancer cells across different stages, but always making sure to use tumors at least 1-cm diameter to allow the insertion of at least three needles. Staging of the tumor can be confirmed after the experiment using H&E sections.
3. To determine the optimal concentration for collecting the invasive population, it is often necessary to perform dose response experiments and try a range of chemoattractant amounts. We have found that for EGF, the optimal concentration in the in vivo invasion assay is 25 nM while for the in vitro Boyden chamber chemotaxis it is 5 nM. The difference can be explained by different diffusion properties of the gradient emanating from a tip of a needle into live tissue vs. EGF in a buffer being directly accessible to migrating cells across pores in a filter. By the same token, the optimal

length of the invasion assay should be determined. Our group has determined that with EGF in the needles, the maximum number of cells was collected by 4 h (increasing in a linear fashion from time 0 to 4 h), after which the number of cells plateaus.

4. The position of the rodent during the experiment can and should be adjusted depending on the size and the position of the tumor. We commonly use orthotopic tumors in an abdominal mammary fatpad (2nd up from the tail) and place the rodent on its back during the experiment. In a different mammary tumor model such as the MMTV-driven Polyoma Middle T transgenic model, the mouse usually develops large, bulky tumors in the thoracic mammary glands; in such cases the tumor burden may affect proper breathing of the rodent, and we can overcome that by placing the animal on its stomach or on its side (with the largest tumor laying down on the surface), while the tumors remain easily accessible for the insertion of the needles. This trick may also be useful in cases when the animal is breathing heavily under anesthesia – to avoid the tumor shifting back and forth against the needles, one can stabilize it against the surface and additionally tape the tumor down. If the tumor is right in the middle of the rodent's ribcage and is weighing down on it, one can use two needle holders from each side, inserted in such a way as to almost be holding the tumor up.
5. Tumors may have different characteristics, but one should always palpate the tumor and try to select an area that is solid, and avoid the very soft necrotic areas which are unlikely to have viable cells that would be able to invade into the needle, and may also leak necrotic fluid out of the tumor.
6. Any wobbling or movement of the holder with the needles can later result in a failed experiment (for examples biopsy or empty needles).
7. One of the trickiest parts of the procedure is making sure the experimental needles are far enough into the tumor without taking a biopsy of it. How far they are pushed in can depend on how solid or soft the tumor mass is. As a first step, one should always make sure the guide needles are pushed enough against the tumor to have pierced the skin, and following this that the blocking needles are inserted far enough beyond where the tips of the guide needles seem to be. Sometimes forceps should be used to make sure the blocking needles are pushing through the tumor tissue, remembering that this should *not* be done with the experimental

needles. To increase the likelihood of experimental needles being inserted correctly, it is best to use more than one holder with multiple needles in each if the tumor size permits it. Some tumor types are too small to allow more than one holder to be used.

8. Occasionally the needles pierce a blood vessel or a necrotic area inside the tumor; this is unavoidable at the time of setup. If most of the experimental needles are already set up, it is best to not try and reinsert the experimental needle that seems to have been placed unsuccessfully in order to avoid disrupting the entire setup. Depending on the severity of the bleeding, the needle may or may not produce useable data. One should observe whether the bleeding is severe enough to cause premature death of the rodent during the experiment.
9. To avoid the animal dying from an overdose or waking up from insufficient levels of anesthesia, one must constantly observe the animal and adjust the levels of Isoflurane accordingly. As the rodent's breathing slows down (1 breath roughly every 3–5 s, but this is extremely variable from animal to animal), you can start lowering the Isoflurane in 0.5% steps. Constantly monitor the breathing and keep lowering during the first half hour as appropriate. If the rodent starts breathing faster at any point during lowering you should increase the Isoflurane as needed until the animal's breathing slows down again. The way the anesthesia levels are adjusted is rarely identical from one rodent to the next; variations can be due to the rodent's general health condition, its size and age, and also on the setup itself. One must verify that the oxygen tank has sufficient levels of gas and that the vaporizer has an appropriate amount of Isoflurane. It is helpful to watch for too frequent or too infrequent breaths of the animal. It may be helpful to place heating pads under the animal to prolong its survival as well.
10. Adjust ratio as needed for different antibodies.

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