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An In Vitro One-Dimensional Assay to Study Growth Factor-Regulated Tumor Cell–Macrophage Interaction

Ved P. Sharma, Brian T. Beaty, Dianne Cox, John S. Condeelis, and Robert J. Eddy

Abstract

Growth factor-dependent pairing and motility between tumor cells and tumor-associated macrophages on extracellular matrix (ECM) fibers of the tumor microenvironment have been shown to enhance intravasation and metastatic spread of breast carcinomas. We describe an in vitro motility assay that combines time-lapse wide-field microscopy and micro-patterned linear adhesive substrates to reconstitute the in vivo behavior between macrophages, tumor cells, and ECM fibers in orthotopic rodent tumor models observed by intravital imaging. Commercially available linear stripes of 650 nm dye-labeled fibronectin microlithographed onto glass cover slips are sequentially plated with fluorescently labeled MTLn3 tumor cells and bone marrow-derived macrophages and time-lapse imaged for up to 8 h. Incubation with pharmacological inhibitors during the assay can identify important paracrine or autocrine signaling pathways involved in the macrophage–tumor cell interaction. This high-resolution motility assay will lead to a more detailed description of immune cell–tumor cell behavior as well as interrogating additional cell types within the tumor microenvironment which use cytokine/growth factor paracrine signaling interactions to facilitate intravasation and metastasis.

Keywords

EGF; CSF-1; Breast carcinoma cells; Macrophage; Live-cell imaging; Time-lapse microscopy

1 Introduction

The establishment of distant secondary tumors or metastases from the primary tumor is correlated with poor prognosis in breast cancer patients. During metastasis, cells escape the primary tumor, undergo invasion by migrating through the surrounding tissue stroma, and eventually intravasate through the vascular endothelium. Once in the circulation, the tumor cells can extravasate and seed the formation of secondary tumors in various target organs [1]. Although tumor cells can undergo random and directional motility, the process of invasion and dissemination are more efficient when the tumor cells undergo directed cell migration [2]. The phenomenon of chemotaxis, defined as directional migration towards an increasing gradient of a chemokine or a growth factor, is a common form of directed cell

migration occurring in tumors. Chemotaxis not only influences the migratory behavior of tumor cells but also serves to define the tumor microenvironment [1].

The migratory behavior of individual cells and cell–cell interactions within the tumor microenvironment can be observed in vivo with high spatial and temporal resolution by intravital multiphoton microscopy. Intravital imaging of mouse mammary tumors has revealed that tumor cell migration is coordinated by the surrounding tumor microenvironment composed of a variety of cell types including fibroblasts, adipocytes, vascular endothelial cells, and infiltrating immune cells [1]. Recently, a role has been uncovered for macrophages during both in vitro and in vivo mammary tumor cell invasion and metastasis that is dependent on a paracrine interaction between epidermal growth factor (EGF) secreted by macrophages and colony-stimulating factor (CSF-1) secreted by tumor cells. Inhibitors of EGF or CSF-1 signaling disrupt this interaction and decrease tumor cell velocity and protrusion, validating the requirement for an intact paracrine loop [3 – 5].

In addition to the cellular component, the tumor microenvironment comprises the extracellular matrix secreted by various tumor-associated cell types including fibroblasts and endothelial cells. Intravital imaging using second harmonic generation has revealed that extracellular matrix molecules, such as type I collagen, are arranged into a network of linear fibers approximately 2–5 μm in width. MTLn3 tumor cells expressing GFP not only increase their velocity on collagen fibers, but in addition they exhibit a coordinated streaming motility in close association with these fibers [6, 7]. Intriguingly, these streams were found to contain co-migrating macrophages organized into arrays of alternating tumor cells and macrophages [1] while the migration of tumor cells within streams is more efficient than random motility with accompanying increases in various cell motility parameters including velocity, net path length, and directionality [2, 4, 7]. The EGF/CSF-1 paracrine loop is critical for the co-migration of tumor cells and macrophages in streams and is thought to play a significant role in organizing and promoting directed migration to the vasculature and subsequent intravasation during metastasis [2, 8].

In order to further investigate the ability of the tumor microenvironment to organize macrophage–tumor cell streaming behavior observed by intravital imaging [8], we developed an in vitro motility assay that employs a linear micro-patterned adhesive stripe to mimic the fibrillar nature of the tumor extracellular matrix. We found that MTLn3 cells plated onto 2.5 or 5 μm wide stripes of fibronectin or type I collagen micro-patterned onto a glass cover slip supported high-velocity cell motility at rates comparable to in vivo values measured on collagen fibers of similar width. We observed that MTLn3 cells plated on fibronectin or type I collagen stripes migrated with higher velocity than on planar matrix-coated substrates and displayed enhanced lamellipodial protrusion and increased motility upon local interaction and pairing with bone marrow-derived macrophages (BMMs) [9].

In this chapter, we describe technical details on the use and application of commercially available and customizable cover slips micro-patterned with stripes of extracellular matrix (ECM) of varying widths as an in vitro motility assay. This simplified approach uses conventional time-lapsed, wide-field fluorescence microscopy and is designed to analyze multiple adjacent fields simultaneously to maximize data collection from a single micro-

patterned cover slip. Furthermore, this assay can be used to quantify individual cell motility parameters such as speed, persistence, lamellipodial protrusion, and retraction at high resolution as well as explore the effect of various ECM molecules on these parameters. Our findings validate the use of linear micro-patterned substrates to reconstitute in vivo tumor cell–macrophage pairing and stream formation and can be applied to autocrine and paracrine chemokine/growth factor signaling in a variety of tumor-associated cells.

2 Materials

2.1 Preparation of CYTOO Motility Chips

1. CYTOOchip Motility FN650 (CYTOO Inc., cat# 10-031-10-06).
2. CYTOOchip Motility A (CYTOO Inc., cat# 10-031-00-06).
3. CYTOOchamber 4 wells (CYTOO Inc., cat# 30-011).
4. Silicone glue (DAP Aquarium Sealant, 100 % silicone).
5. Glass-bottom 35 mm petri dish (MatTek, cat# P35G-1.5-14-C).
6. Razor blade.
7. Phosphate buffer saline (PBS).
8. FITC-conjugated bovine collagen I (Sigma) solution: Dilute FITC-conjugated bovine collagen in PBS to 40 µg/ml.

2.2 MTLn3 Preparation

1. MTLn3 [10]: TagRFP-cortactin-expressing MTLn3 [11].
2. Culture medium: MEM-alpha (Life Technologies) containing 5 % fetal bovine serum (FBS) and penicillin/streptomycin solution.
3. 0.05 % Trypsin–EDTA.
4. Imaging medium: L-15 (Life Technologies, cat# 21083) containing 5 % FBS.

2.3 BMM Preparation

1. Murine BMMs [12].
2. BMM culture medium: MEM-alpha (Life Technologies) containing 15 % FBS, 1 % penicillin/streptomycin, and 36 ng/ml (2.5 nM) recombinant human CSF-1.
3. Bacterial petri dishes (Falcon).
4. CellTracker™ Green CMFDA (Life Technologies, cat# C7025): Each vial of CellTracker™ Green contains 50 µg dye. Make 5 mM stock solution by adding 21.5 µl of DMSO and mixing by pipette 3–4 times. The stock solution can be kept at –20 °C for up to 3 months.

2.4 Live Imaging of Tumor Cell– Macrophage Interaction

1. Imaging medium: L-15 medium (Life Technologies) containing 5 % FBS.

2. EGFR inhibitor, Iressa (AstraZeneca).
3. EGFR inhibitor, Tyrphostin AG 1478 (Cell Signaling Tech., cat#9842).
4. MCSF receptor antibody, AFS98 (Novus Biologicals, cat# NBP1-43363).
5. Epi-fluorescence microscope (DeltaVision), equipped with a 20x objective, a heat enclosure to maintain temperature at 37 °C, a CoolSNAP HQ² CCD camera and a high precision X and Y scanning stage for capturing time-lapsed images from multipoint locations.

3 Methods

3.1 CYTOOchip- MatTek Dish Assembly

A CYTOO cover slip, with dimensions of 20 × 20 mm², attached to the bottom of a 35 mm glass-bottom petri dish having a 14 mm well in the center (*see* Notes 1 – 3 ; Fig. 1), as follows:

1. Carefully remove the glass cover slip from a 35 mm glass-bottom MatTek dish using a razor blade.
2. With the bottom side of the MatTek dish facing up, apply small amount of silicone glue all around the hole.
3. Remove the CYTOOchip Motility out of the blister pack and place, ECM side down, centered on the hole with a pair of sterile forceps. Gently press with the forceps to ensure a good seal with the applied silicone glue. Leave the dish at room temperature for 6 h to let the glue dry. Store dishes at 4 °C, and use them within 1–2 days.

3.2 Collagen I Coating of CYTOOchip Motility A

1. Remove the activated CYTOOchip out of the blister pack, and glue it to the bottom of a 35 mm glass-bottom dish, as described in Subheading 3.1.
2. Put 200 µl of the FITC-conjugated collagen solution in the center well to cover the activated CYTOOchip. Incubate for 2 h at room temperature.
3. Wash three times with PBS, store at 4 °C, and use within 1–2 days.

3.3 Preparation of Carcinoma Cells

1. Put 2 ml of culture media on ECM (FN650 or FITC-collagen I)-coated CYTOOchip-MatTek dish, and incubate in the cell culture incubator for 30 min.

¹As an alternate to attaching the CYTOO cover slip to the bottom of a MatTek dish, 1- or 4-well CYTOO chambers can be used, where CYTOO cover slip is sandwiched between the bottom frame and the main body of the CYTOO chamber and held firmly by the magnetic force. Make sure that the silicone gasket is attached to the main body of the chamber before attaching it to the bottom frame; this ensures a leakproof assembly.

³A 4-well CYTOO chamber combined with multiple-position imaging is very useful for testing up to four different conditions on a single CYTOOchip in one imaging session. The only drawback is that all the 2D areas on the chip are covered by the chamber walls and therefore not available for imaging.

2. Trypsinize a 10 cm culture dish containing either parental or TagRFP-cortactin-expressing MTLn3 cells with 1 ml of 0.05 % trypsin–EDTA solution for 5 min in a cell culture incubator at 37 °C containing 5 % CO₂. Add 9 ml of culture media to stop the reaction.
3. Aspirate media from the CYTOOchip-MatTek dish, put 2 ml of MTLn3 cells (5×10^4 cells/ml) into the dish, and transfer it to a cell culture incubator at 37 °C containing 5 % CO₂.
4. After 3–4 h, check the cells using a tissue culture microscope. Cells should nicely line up in the 1D areas with enough space in between for the cells to freely move in either direction.
5. Wash floating cells once with imaging media, aspirate, then add 2 ml of imaging media, and transfer to an epi-fluorescence microscope.

3.4 Macrophage Preparation

1. Culture BMMs in bacterial petri dishes at 37 °C in a 5 % CO₂ incubator [13] (*see* Note 4).
2. To render BMMs quiescent [12], deprive cells of CSF-1 by changing to CSF-1-free BMM medium and incubating cells for 16 h prior to experiment.
3. Harvest BMMs by incubating in 10 mM EDTA/PBS for 10 min at 37 °C in a 5 % CO₂ incubator to lift the cells. Then pellet cells by centrifugation at $400 \times g$ for 5 min at room temperature.
4. Resuspend BMMs in CSF-1-free culture medium, and transfer cells to a 15 ml centrifuge tube. BMMs can be kept suspended in the tube inside cell culture incubator for up to 6 h.
5. Label BMMs with CellTracker™ green dye 1 h before they are ready to be added to carcinoma cells on the CYTOOchip. Add the dye at 1:1,000 (final concentration, 5 μ M) to 5×10^6 BMMs in 1 ml of media, and incubate for 30 min in a cell culture incubator.
6. Wash two times, and resuspend BMMs in 2 ml of imaging media (5×10^4 cells/ml).

3.5 Live Imaging of Tumor Cell– Macrophage Interaction

1. To maintain good focus during live imaging, turn the microscope heat ON at least 2–3 h before starting to image.
2. Place the CYTOO chamber with attached TagRFP-cortactin MTLn3 cells on the microscope. Adjust the focus on the cells, and set up for multiple-position imaging (*see* Note 5). For longer duration time lapses, imaging of multiple adjacent fields is recommended because cells frequently move in and out of the field (*see* Note 6).

⁴Do not use regular tissue culture-treated dish for BMMs or the cells will adhere too tightly and will be difficult to replate. BMMs should be cultured in bacterial petri dishes.

3. After 2–3 h of time-lapse imaging of TagRFP-cortactin MTLn3 cells alone (Figs. 1d, e and 2) to establish baseline carcinoma cell motility, add 1×10^5 BMMs and continue imaging.
4. BMMs start attaching to the dish (mostly on the ECM but also on nonadhesive areas) within 5 min of addition. Continue imaging for 6 h. Instances of tumor cell–macrophage pairing (Fig. 3a, b) and streaming, consisting of an alternating pattern of tumor cell and macrophages (Fig. 3c), can be seen starting from 1 h after the addition of BMMs.
5. To evaluate the role of EGF–CSF-1 paracrine signaling pathway, EGFR inhibitors (1 μ M Iressa or 5 μ M AG-1478) or CSF-1 receptor function blocking antibody, AFS98 (50 μ g/ml), is added at the indicated concentrations and imaging is continued for 6 h.

3.6 Analysis of Time-Lapse Movies for Cell Motility Parameter Calculations

1. Open fluorescent time-lapse movies in ImageJ. Multiple timelapse movies of adjacent areas can be stitched together in ImageJ by combining two fields at a time with the command Image > Stacks > Tools > Combine. A montage of 6–7 adjacent fields totaling approximately 2 mm of continuous stripe was regularly analyzed (Fig. 2).
2. For measuring carcinoma cell motility parameters, threshold the RFP-cortactin channel using the command Image > Adjust > Threshold.
3. A custom ImageJ macro (available upon request), based on outlining a cell with the Wand tool and determining the cell centroid in each frame, was used to calculate the average and maximum tumor cell speed.
4. Based on the formula described earlier [14], average migration persistence was calculated as $\text{speed}/[1 + (100/360) \times \text{angle}]$, where angle is the directional change in degrees. On 1D stripes, the angle value is either 0 or 180, depending on whether the cell persists in the same direction or changes direction.
5. For displaying the images of tumor cell–macrophage interaction, fluorescent and phase channels were merged using the command Image > Color > Merge Channels.

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⁵On a DeltaVision epi-fluorescence microscope, equipped with a 20 $\times$ objective and Photometrics CoolSnap HQ² CCD camera, the width of a field of view is about 329 μ m. Up to 6–8 fields were imaged by successively moving each field by this distance in $x - y$ direction.

⁶One of the challenges in analyzing thresholded time-lapse movies is when adjacent cells touch each other. To overcome this, cells were manually separated by drawing a black line (value 0, same as the background) between the cell boundaries with a pencil tool in every frame where cells were touching each other.

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Fig. 1.

An overview of the in vitro motility assay utilizing linear adhesive micro-patterned substrates. **(a)** A cartoon showing a single $20 \times 20 \text{ mm}^2$, $175 \text{ }\mu\text{m}$ (#1.5) thick CYTOOchip™ Motility FN650 (CYTOO, Inc.) affixed to a 35 mm diameter, 14 mm microwell dish (MatTek Corporation). Each chip is micro-patterned with four quadrants of parallel linear stripes of fibronectin labeled with 650 nm dye in widths ranging from 2.5, 5, and 10 to 20 μm with 192 stripes for each width. **(b)** A zoomed area of one quadrant of the CYTOOchip showing fibronectin-650 stripes of different widths. The chip includes an area of planar 2D area of fibronectin-650 that is continuous with the 2.5 μm linear stripes so that a direct comparison of 1D and 2D motility can be made. **(c)** A zoomed area of CYTOOchip with 2.5 μm fibronectin-650 stripes continuous with the 2D area. A cartoon of an elongated cell attached to the fibronectin-650 stripe is also shown. **(d, e)** TagRFP-cortactin- expressing MTLn3 cells showing elongated morphology on **(d)** 2.5 μm and **(e)** 5 μm fibronectin-650 linear stripes. Merge images of phase, RFP, and far-red channels are shown. Scale bars, 25 μm

Fig. 2.

Demonstration of combining multiple adjacent fields of view in ImageJ. TagRFP-cortactin-expressing MTLn3 cells were plated on CYTOOchip Motility FN650 for 3–4 h. Adjacent fields of view with MTLn3 cells on linear stripes were imaged in phase, RFP, and far-red channels on a DeltaVision microscope with a 20 $\times$ objective (field-of-view width = 329 μ m). Merged images (phase, RFP, and far-red channels) of four adjacent fields, totaling a length of 1,316 μ m, were combined as described in Subheading 3.6. Scale bar, 50 μ m

Fig. 3.

Examples of tumor cell–macrophage pairing and streaming on 1D adhesive substrates.

TagRFP-cortactin- expressing MTLn3 cells were plated for 3–4 h on CYTOOchip Motility FN650. After time-lapse imaging for 1.5–2 h, CellTracker green-labeled BMMs were added and imaged for an additional 6 h. **(a)** Single-tumor cell (*red*) and macrophage (*green*) pairing on 2.5 μm fibronectin- 650 (*blue*) stripe. **(b)** Single-tumor cell (*red*) and macrophage (*green*) pairing on 5 μm fibronectin- 650 (*blue*) stripe. **(c)** Multiple-tumor cells (*red*) and macrophages (*green*) were capable of forming streams of alternating tumor cells and macrophages on fibronectin- 650 stripe (*blue*) like those observed in vivo [8, 9]. Scale bars, 25 μm