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Microscopic Methods for Analysis of Macrophage Induced Tunneling Nanotubes

Kiersten P. Carter¹, Jeffrey E. Segall^{1,2}, Dianne Cox^{1,2,3,+}

¹Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

²Gruss-Lipper Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY 10461, USA

³Department of Molecular and Developmental Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave, Gruss MRRC 306, Bronx, NY 10461, USA

Abstract

Macrophages are known to play multiple roles in the breast cancer microenvironment including the promotion of tumor cell invasion that is dependent on soluble factors or through direct contact. Macrophages can also enhance the production of Tunneling Nanotubes (TNTs) in tumor cells which can be mimicked using macrophage conditioned medium. TNTs are long thin F-actin that connect two or more cells together that have been found in many different cell types including macrophages and tumor cells and have been implicated in enhancing tumor cells functions such as invasion. Here we describe basic procedures used to stimulate tumor cell TNT formation through macrophage conditioned medium along with methods for quantifying TNTs.

Keywords

Tunneling Nanotubes; Macrophages; Tumor cell; Microscopy

1. Introduction

Metastasis is the leading cause of death in breast cancer. It has been shown that tumor progression can be enhanced when macrophages interact with tumor cells, resulting in an increase in migration, co-invasion, and intravasation of the tumor cells leading to metastasis (1, 2). Macrophages are immune cells that respond to inflammation through cytokines secreted by the resident tissue. They aid in removal of debris and can secrete cytokines that either recruit other immune cells or dampen the immune response (3). Additionally, tumor associated macrophages have been shown to take part in a paracrine interaction with tumor cells where tumor cell secretion of Colony Stimulating Factor 1 (CSF1) induces macrophages to secrete factors such as Epidermal Growth Factor (EGF) that promote tumor invasion and progression (4, 5).

*address correspondence to: Dianne Cox: dianne.cox@einstein.yu.edu.

It has recently been shown that macrophages and tumor cells also employ a novel means of communication through structures known as Tunneling Nanotubes (TNTs) or membrane nanotubes (6). TNTs are long thin, membranous F- actin containing structures that connect two cells and allow transfer of signals. They can be as long as hundreds of microns but vary in diameter from 50 nm to 800 nm and can either be closed (no cytoplasmic connection between cells) or open (cytoplasmic connection between cells)(diagramed in Figure 1). When TNTs are open they can transport cytoplasmic signals, mRNA, miRNA, vesicles and other organelles between cells. In addition, TNTs can be homotypic (connecting to cells of the same type) or heterotypic (connecting to cells of different types). For example, when in co-culture tumor cells and macrophages can form heterotypic TNTs that are important for co-invasion (7). It has also been shown that macrophage conditioned medium can stimulate homotypic TNTs in breast tumor cells *in vitro* (8, 9). However, quantitation of TNTs can be challenging. Some studies quantify the number of TNT- like protrusions per cell (10), in others every TNT connection per cell is counted (8), while we and others only count the number of TNT connected cell pairs (11). Additionally, some studies utilize fixation and subsequent immunofluorescent microscopy to identify TNTs, which is known to reduce TNT numbers through breakage especially for the thinner TNTs. These differences make it difficult to compare results between studies. Here we describe a method to quantify TNTs by live cell imaging microscopy as an attempt for standardization of TNT scoring.

2. Materials

2.1 Cell Culture and Reagents

- Murine monocyte/macrophage RAW264.7 subline LR5 (12) (Note 1)
- Macrophage medium– RPMI 1640 with L – glutamine, 10% heat – activated newborn Serum, 1% penicillin/streptomycin solution
- Rat adenocarcinoma MTLn3 cells (Note 2)
- MTLn3 medium– Minimal Essential Medium (α -MEM) with L – glutamine, 15% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (Note 3).
- Sterile 10 mM EDTA/PBS (pH 7.4)
- 15 mL Falcon tubes
- 0.22 μ m syringe filter
- Eppendorf centrifuge 58 10 R
- 10 cm tissue culture treated dishes
- 35mm Glass Bottom No. 1.5, uncoated, gamma – irradiated Mat–Tek dishes
- Hemocytometer

¹)Primary murine bone marrow or human monocyte derived macrophages have also been successfully used.

²)We have successfully employed other cancer cell lines in this method.

³)If there is no CO₂ control for microscope, 10 mM HEPES can be added to the medium or Leibovitz's L-15 medium can be used to maintain appropriate pH control.

- 0.05% Trypsin, 0.53 mM EDTA without sodium bicarbonate
- Buffer with Divalent (BWD) –20 mM HEPES, 125 mM NaCl, 5 mM KCl, 5 mM dextrose, 10 mM NaHCO₃, 1 mM KH₂PO₄, 1 mM CaCl₂, and 1mM MgCl₂, pH 7.4.

2.2 Microscopy

Any inverted fluorescent microscope may be used. We use an Olympus IX71 inverted microscope with planapo phase contrast 60X NA objective with a Cooke Sensicam and Photometrics Coolsnap, HQ.

3. Methods

3.1 Preparation of macrophage conditioned medium

1. Thaw frozen vial of RAW/LR5 macrophages by placing in a 37°C water bath for 2 to 3 minutes.
2. Dilute DMSO by adding mostly thawed cell suspension to 15 mL Falcon tube with 9 mL of appropriate medium.
3. Centrifuge at 400g, 25°C, for three minutes.
4. Aspirate supernatant and resuspend pellet in 10 mL of macrophage medium, place cell solution into a 10 cm tissue culture treated dish in incubator at 37°C with 5% CO₂.
5. Wait until cells reach 70 to 80% confluence (Note 4).
6. Harvest cells by quickly rinsing cells once with PBS to remove floating cells and medium components followed by incubation in 2 mL of sterile 10 mM EDTA/PBS at 37°C/5% CO₂ for approximately 5 to 10 minutes until cells are detached.
7. Add 2 mL of macrophage medium to plate and transfer the 4 mL of cell suspension to a 15 mL Falcon tube and centrifuge at 400g, 25°C, for 3 minutes.
8. Aspirate supernatant and resuspend pellet in 1 mL of macrophage medium.
9. Determine the cell concentration using the hemocytometer.
10. Plate RAW/LR5 cells in MTLn3 medium so that the cells are at approximately 90% confluency. For RAW/LR5 cells we normally plate 9×10^5 cells per 10 cm dish but this number would vary depending on the type of macrophages used and the size of dish (Note 5).
11. Incubate at 37°C in a 5% CO₂ incubator overnight.

⁴)To improve consistency between experiments we have found that it is best to start with healthy cells, so it is essential to not overgrow the cells.

⁵)The number of macrophages to be plated is variable and depends on the macrophage type and should be adjusted for 90% confluency. Additionally, when using other tumor cell lines in this assay it is essential to generate the macrophage conditioned medium in the appropriate medium for each line to be used in the assay.

12. Next day, collect medium without lifting cells using a 10 mL syringe and filter with a 0.22 μ m syringe filter. After filtering, the macrophage conditioned medium can be used immediately, place in -20°C freezer for use later in the week, or kept at -80°C for long-term storage.

3.2 Tumor Cell TNT induction with Macrophage Conditioned Medium

1. Thaw rat adenocarcinoma MTLn3 cells as described in 3.1 steps 1-3 but using MTLn3 medium.
2. Grow cells in a 10 cm plate in 10 mL of MTLn3 medium in a 37°C incubator with 5% CO_2 until cells reach 70 to 80% confluence. Be careful not to allow cells to become too confluent (Note 4)
3. Aspirate medium and wash with 2 mL of 1X PBS. Lift cells using 2 mL of 0.05% Trypsin, 0.53 mM EDTA. Wait 10-15 minutes or until cells are in suspension.
4. Add 2 mL of MTLn3 medium to plate to inactivate the trypsin and transfer the 4 mL of cell suspension to a 15 mL Falcon tube.
5. Centrifuge at 400 g, 25°C for 3 mins and aspirate supernatant. Resuspend pellet in 1 mL of MTLn3 medium.
6. Plate 1×10^5 tumor cells in a 35mm Glass Bottom No. 1.5, uncoated, gamma-irradiated Mat-Tek dish per condition.
7. To determine the basal number of tumor cell TNTs, MTLn3 cells are plated in 2 mLs of MTLn3 medium and placed in a 37°C incubator with 5% CO_2 overnight.
8. To determine the effect of macrophage conditioned medium on tumor cell TNTs- MTLn3 cells are plated in a 50:50 ratio of MTLn3 medium: macrophage conditioned medium. Place dish in a 37°C incubator with 5% CO_2 overnight.

3.3 Live Cell Imaging of Tumor Cell TNTs

As noted by us and others, TNTs can be very fragile, and many are broken during fixation. Therefore, we prefer to utilize the live cell imaging method described below. An example of a live cell image of a TNT is shown in Figure 2. It is possible to use fixation followed by fluorescent staining (Note 6).

1. Replace medium with BWD to avoid autofluorescence due to components in the medium.
2. TNTs can be identified by phase contrast microscopy, but it is preferable to include a fluorescent dye that labels the plasma membrane by adding 1 $\mu\text{g/mL}$ Alexa Fluor TM conjugated Wheat Germ Agglutinin or 1:1000 dilution of FM1-43FX directly into the BWD in the Mat-Tek dish immediately before

⁶)We have found that fixation using 3.7% formaldehyde in BWD preserves approximately 50% of the TNTs. However, fixation permits the use of other microscopy techniques such as confocal and super resolution imaging by structured illumination microscopy (7, 14). We routinely use fluorescently labeled phalloidin and Wheat Germ Agglutinin to identify F-actin containing membrane covered connections but other methods can be used including labeling vesicles with a Cell tracker dye (7).

imaging. It is also possible to use tumor cells expressing a genetically encoded fluorescent protein targeted to the membrane, such as mCherry, with a fused CAAX motif (7).

3. Place the dish on the stage of an Olympus IX71 fluorescent microscope using a 60X oil NA 1.43 objective.
4. Focus lens using the coarse focus until cells are in view then use the fine focus adjustment until cells can be clearly seen attached to the substrate. Search the field for thin, tubular structures. To verify that a structure is a TNT at least part of the structure must be off of the substrate, move the fine focus adjustment above and below the plane of focus. When going in and out of the plane of focus, you will see part of the structure fade away while the other part of the structure becomes visible indicating that part of the structure is above the substrate (Note 7).

3.4 Quantification of Tunneling Nanotubes

TNTs are identified and quantified as long thin connections between two cells that are at least 8 μm in length where at least part of the TNT is not in contact with the substrate (as described in (7, 13)). In some cases, the kinetics of TNT precursors or protrusions can be analyzed (Note 8).

1. Cells counted as negative for TNTs must be within one cell body length of another cell without touching any other cell.
2. Each cell containing a TNT that is connected to another cell is counted as positive. A minimum of 128 cells are normally counted per sample and at least 3 independent experiments are needed for statistical analysis. The data reported are the average number of cells that are connected or % of cells with TNTs. An example of this quantitation is shown in Figure 3.
3. Alternatively, the number of the number of TNTs present can be identified as described above and quantified but reported as the number or TNTs per 100 cells.

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⁷)Several characteristics are used to distinguish TNTs from similar structures such as filopodia such as length and attachment to the substrate. Another indicator of a TNT is the presence of cargo that can be seen as bulges in the structure or labeled with a vesicle marker.

⁸)Other possible definitions of TNTs have been used. TNT precursors have been identified as long protrusions that are off the substratum and reach a minimum of 8 μm in length during the imaging period and eventually touch another cell.

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Figure 1:
Schematic of TNTs showing both open and closed TNTs.

Figure 2:
Live cell confocal imaging of MTLn3 cells treated with macrophage conditioned medium and stained with Alexa488 WGA. Cells appear to be out of focus since the image is focused in the plane of the TNT which is suspended above the substrate. Arrow indicates a TNT. Scale bar = 10 μm .

Figure 3:

MTLn3 cells untreated or treated with macrophage conditioned medium overnight were stained with Alexa488 WGA for live cell imaging. Cells connected via a TNT to another cells were quantified as described in 3.4 and plotted as the percentage of cells with TNTs. n=3 * p< 0.05.