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Quantification of tumor cell adhesion in lymph node cryosections

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TITLE:

Quantification of Tumor Cell Adhesion in Lymph Node Cryosections

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KEYWORDS:

metastasis, lymph node, cancer progression, cell adhesion, integrins, extracellular matrix

SUMMARY:

Here, we describe a simple and inexpensive method that allows the quantification of adhesive tumor cells to lymph node (LN) cryosections. LN-adherent tumor cells are readily identified by light microscopy and confirmed by a fluorescence-based method, giving an adhesion index that reveals the tumor cell-binding affinity to LN parenchyma.

ABSTRACT:

Tumor-draining lymph nodes (LNs) are not merely filters of tumor-produced waste. They are one of the most common regional sites of provisional residence of disseminated tumor cells in patients with different types of cancer. The detection of these LN-residing tumor cells is an important biomarker associated with poor prognosis and adjuvant therapy decisions. Recent mouse models have indicated that LN-residing tumor cells could be a substantial source of malignant cells for distant metastases. The ability to quantify the adhesivity of tumor cells to LN parenchyma is a critical gauge in experimental research that focuses on the identification of genes or signaling pathways relevant for lymphatic/metastatic dissemination. Because LNs are complex 3D structures with a variety of appearances and compositions in tissue sections depending on the plane of section, their matrices are difficult to replicate experimentally *in vitro* in a fully controlled way. Here, we describe a simple and inexpensive method that allows the quantification of adhesive tumor cells to LN cryosections. Using serial sections of the same LN, we adapt the classic method developed by Brodt to use nonradioactive labels and directly count the number of adhering tumor cells per LN surface area. LN-adherent tumor cells are readily identified by light microscopy and confirmed by a fluorescence-based method, giving an adhesion index that reveals the cell-binding affinity to LN parenchyma, which is suggestive evidence of molecular alterations in the affinity binding of integrins to their correlate LN-ligands.

INTRODUCTION:

Cancer metastasis is the main reason for treatment failure and the dominant life-threatening aspect of cancer. As postulated 130 years ago, the metastatic spread results when an elite of disseminated tumor cells (DTCs, the "seeds") acquire specific biological abilities that allow them to evade primary sites and establish malignant growth at distant sites (the "soil")[1]. Recently, several novel concepts regarding the "seed and soil" relations have emerged, such as the induction of premetastatic niches (conceptualized as a "fertile soil" needed for "seeds" to thrive), self-seeding of primary tumors by DTCs, "seed" dormancy at secondary organs and the parallel progression model of metastasis[2].

For most solid malignancies, DTCs can reside and be detected in many mesenchymal organs, such as bone marrow and lymph nodes (LNs) in patients with or without evidence of clinical metastasis. Because tumor-draining LNs are the first location of the regional spread of DTCs, LN status is an important prognostic indicator and is often associated with adjuvant therapy decisions[3]. For some tumor types, the correlation between LN status and worse outcomes is strong, including head and neck[4,5], breast[6], prostate[7], lung[8], gastric[9], colorectal[10,11] and thyroid cancers[12].

LNs are small ovoid organs of the lymphatic system, that are covered with reticular cells and enclosed with lymphatic vessels. These organs are absolutely necessary for the functioning of the immune system[13]. LNs act as attractant platforms for immune circulating cells, bringing the lymphocytes and antigen-presenting cells together[14]. However, LNs also attract circulating tumor cells. Over decades, LNs were pictured as passive routes of transportation for metastatic tumor cells. However, recent studies have indicated that tumor cells may also be guided towards LNs by chemotactic (chemokines) and/or haptotactic (extracellular matrix elements) cues secreted by the lymphatic endothelium[15]. As examples, overexpression of the CCR7 receptor in tumor cells facilitates the guidance of metastatic melanoma cells towards tumor-draining LNs[16]. In addition, extracellular LN proteins provide an adhesive scaffold for the recruitment and survival of circulating tumor cells[17]. In fact, tumor-draining LNs provide fertile soil for the seeding of DTCs, which can be maintained in proliferative or dormant states by specific LN microenvironmental signals[18]. The final fate of these LN-residing DTCs is controversial; some works suggest that these cells are passive indicators of metastatic progression[19], while others propose that they are more likely founders of resistance (by self-seeding primary sites) and/or act as cellular reservoirs for metastases (spreading "seeds" for tertiary cancer growth)[20,21]. Recently, using preclinical models, it has been demonstrated that a fraction of these LN-residing DTCs actively invaded blood vessels, entered into the blood circulation and colonized the lungs[21].

Considering that the presence of cancer cells in LNs is a marker for cancer aggressiveness and invasiveness, in this study, we optimized a classic method developed by Brodt[22] to quantitatively measure tumor cell adhesion to LNs in vitro. The use of a fluorescence-based assay allowed us to develop a low-cost, rapid, sensitive and environmentally friendly (nonradioactive) protocol for the detection of adhesive alterations between tumor cells and LN

cryosections. Using the MCF-7 breast cancer cells expressing different levels of NDRG4 gene expression and rat LN frozen sections to exemplify the method, we showed that this protocol allowed a good correlation between tumor cell adhesion to LNs in vitro and LN metastasis observed in breast cancer patients[24].

PROTOCOL:

LNs were recovered from fresh carcasses of healthy adult Wistar rats sacrificed by cervical dislocation. We followed the NIH Guidelines for Pain and Distress in Laboratory Animals and all procedures were approved by the Ethics Committee and Animal Research of the Research and Education Institute of the Sírio-Libanês hospital (CEUA P 2016-04).

NOTE: All fresh frozen tissues are considered biohazardous and should be handled using appropriate biosafety precautions.

1. Lymphadenectomy and cryosectioning

1.1. Place fresh carcasses of adult Wistar rats (180–220 g) lying in dorsal recumbency on a clean dissection board at room temperature.

NOTE: LNs must be collected up to 30 min post euthanasia.

1.2. Spray the rat carcass with 70% isopropyl alcohol and use sterilized instruments for LN harvesting.

1.3. Lift the abdominal skin with the aid of tweezers and open a cavity with a medial incision without damaging the underlying tissue, exposing the abdominal viscera. Pull out the intestine and the thoracic and abdominal LNs become visible (**Figure 1**).

1.4. Carefully excise the LNs from each rat with the use of blunt tip scissors to avoid injuring the superior mesenteric artery lying behind.

NOTE: Depending on the location of the resected lymph node, it is necessary to clean other tissues adhered to it, such as mesenteric tissue.

1.5. Harvest LNs into 15 mL conical tubes containing 5 mL of sterile phosphate buffered saline (PBS).

1.6. Properly discard the rat carcasses.

1.7. Remove fresh LNs from the PBS, roll and dry the node on a dry filter paper. Place it in a small Petri dish and add embedding solution for frozen tissue specimens (O.C.T.) for 2 min.

1.8. Transfer and orientate the LN face down in a desired position in the base of a cryomold, with just enough O.C.T to cover it. Avoid bubbles near the tissue. The sectioning surface is the

bottom of the cryomold.

1.9. Immediately snap-freeze the cryomold in a Styrofoam cooler with dry ice. When there is still a small part of unfrozen O.C.T. (~20-35 s), transfer the sample to aluminum foil and place it in a cooler with dry ice while continuing to freeze other samples. At the end, store all samples at -80 °C until sectioning.

1.10. Section the LN with a cryostat adjusting section thickness to 5-8 µm. Transfer crysections onto microscope slides.

NOTE: Before sectioning, remove the frozen samples from the -80 °C freezer and allow them to equilibrate to the temperature in the cryostat microtome chamber at -22 °C for approximately 30 min. LN-containing slides can be stored at -80 °C for up to one month.

2. Cellular labeling with fluorescent dyes

NOTE: Fluorescent dyes are widely used in cell biology. We prefer to use the long-chain dialkylcarbocyanines labeling (DiI(C_{18}), excitation 549 nm, Emission 565 nm) because they are bright, stable and can be added directly to culture media, does not affecting cell viability or cell adhesive properties[25,26].

2.1. Dissociate cells growing under ideal conditions (i.e., in complete medium) and resuspend in serum-free medium at a density of 10^6 cells/mL.

2.2. Add 1 mL of cell suspension (10^6 cells) to a 15-mL conical tube and label with DiI(C_{18}) (2 µg/mL) for 10 min at 37 °C.

NOTE: After 5 min, gently agitate the tubes to avoid cell sedimentation and understaining of sedimented cells. Larger densities require longer incubation times for uniform staining. An optimal incubation time for cell staining varies with cell line. It can be better quantified using the conventional FL2 flow cytometry detection channel (**Figure 2A**).

2.3. Centrifuge the labeled suspension tubes at 300 x *g* for 4 min.

2.4. Remove the supernatant and wash twice in 10 mL of serum-free medium. Recover the cells as red pellets. Resuspend the cells at 10^6 cells/mL in serum-free medium with 0.1% bovine serum albumin (BSA).

3. Precoating dishes with poly-L-lysine solution or BSA as a seeding control (optional)

NOTE: We used cell culture dishes precoated with PLL as positive loading-control surfaces to ensure that different experimental groups of tumor cells were seeded at the same number, as well as BSA-coated surfaces as negative controls.

3.1. Under sterile conditions, to prepare PLL- or BSA-coated wells, add 300 µL of PLL (0.1% w/v in H_2O) or BSA (diluted at 2.5% w/v in H_2O) directly to the 24-well plate and incubate overnight at 4 °C.

3.2. Remove solution by aspiration, gently rinse the surface with sterile PBS and air-dry the plate at room temperature in the tissue-culture hood before cell seeding.

NOTE: The final volumes of PLL or BSA must be adjusted according to the area of different well plates.

4. Seeding fluorescent-labeled tumor cells on LN cryosections or PLL/BSA-coated wells

NOTE: As experimental controls, we used (1) cell culture dishes precoated with PLL or BSA and (2) consecutive sections of the same LN per experiment (see this detail in **Figure 2D**), where the latter will minimize regional variations in extracellular matrix (ECM) composition of each LN section, which in turn can dictate the cell adhesion rate. For the following tumor cell adhesion assay, select high quality and sequential LN cryosections.

4.1. Gently wash the cryosections twice with PBS and rehydrate with PBS for 15 min at room temperature.

4.2. Block unspecific adhesion to cryosections with 2.5% BSA for 30 min at 37 °C. Use immunohistochemistry wash chambers and lamina cradles to ensure that the entire O.C.T was removed during washes and incubations.

4.3. Drain the excess BSA on a dry paper towel, dry the outline of LN sections with a cotton swab and encircle the sections using a PAP pen.

4.4. For the tumor cell adhesion assay, add 100 µL of cell suspension (from step 2.4) to each encircled LN section or well in the 24-well PLL-coated plates and incubate for 1-2 h at 37 °C.

NOTE: The final volume of cell suspension needs be adjusted according to the area of different encircled LNs.

4.5. Gently wash off non-adherent cells four times with PBS. Fix the remaining adherent fluorescent cells with 3.7% formaldehyde in PBS for 15 min at room temperature.

5. Manual quantification of the adhesive index

NOTE: The adhesive index (i.e., tumor cells/LN mm^2) was achieved using a 10X objective and manually counting the number of tumor cells, readily identified by light microscopy and confirmed by a fluorescence microscopy (**Figure 2D**), per lymph node areas of several independent fields (obtained using National Institute of Health's ImageJ/FIJI software).

5.1. Use a fluorescent microscope with a 10X objective to take separate TIFF images in two channels corresponding to the bright and red-fluorescent fields (**Figure 2D**). Name and save these images systematically.

5.2. Start ImageJ/FIJI, open the images and set the scale. It is necessary to use a calibration scale (e.g., a micrometric ruler 1 mm) (**Figure 2C**).

5.3. Open the photo of the micrometric ruler (or a stage micrometer), select the **Straight line tool** and draw a straight line that defines a known distance.

5.4. In the **Analyze** menu, select **Set Scale**. The **Distance in pixels** will be filled based on the length (in pixels) of the line drawn in step 5.3. The **Known distance** will be filled with the real distance (in this case, in millimeters) and the unit of length in the Unit of length field (in this case, in millimeters).

5.5. Click on **Global** (this calibration applies to all images opened in this ImageJ/FIJI session) and press **OK**.

5.6. Lymph node area quantification: Select the **Wand tool** and by double clicking, open the **Wand tool** settings. Set mode to **8-connected**. Click in the photo and set the tolerance until select all lymph nodes in the photo and press **OK**. To measure the area, open **Analyze | Measure** (**CTRL + M**). The area is expressed in the units set earlier.

5.7. Tumor cell quantification: Open the light microscopy/fluorescence images in FIJI software. Select **Plugins | Analyze | Cell Counter | Cell Counter**. Click on the photo to be quantified and press **Initialize** button in the cell counter window. Select counter type (1-8) and click on the cells in the photo. To initialize the next photo, presses the **Reset** button in the cell counter window, open the new photo and repeat all steps.

NOTE: LN adhesion index is expressed as the number of adherent tumor cells per LN covered area (cells/mm^2).

REPRESENTATIVE RESULTS:

We illustrate the assay by evaluating the LN adhesive potential of red fluorescent MCF-7 breast cancer cells expressing different levels of the NDRG4 gene (referred to as NDRG4-positive and NDRG4-negative cells), a negative modulator of beta1-integrin clustering at the cell surface[24], by examining the fractions of rat LN-adherent tumor cells. Examples of the raw images of this protocol are shown in **Figure 2**. As observed in **Figure 2B**, the morphology of adherent cells is rounded in shaped, and they are heterogeneously dispersed throughout the LN. The LN adhesive index is 2-fold higher in NDRG4-negative MCF-7 cells (877 ± 124 cells/mm^2 of LN) compared to that in corresponding NDRG4-positive MCF-7 cells (412 ± 76 cells/mm^2 of LN, $p = 0.03$) (**Figure 2D**).

FIGURE AND TABLE LEGENDS:

Figure 1. Stepwise procedure for the isolation of the rat mesenteric LNs. (**a**) Ventral midline skin incision: euthanized rats were placed in dorsal recumbency position and a 30-50 mm midline incision was made in the skin overlying the mid abdomen, exposing the abdominal viscera (liver, small intestine, cecum and bladder). (**b**) The small intestine was gently pulled out from abdominal cavity exposing rat mesenteric LNs embedded in visceral adipose tissue. (c) Gross anatomy of the dissected gastrointestinal tract after removal. (d) Dissected mesenteric lymph nodes from the connecting adipose tissue.

Figure 2. Representative results of tumor cell adhesion to rat lymph node sections. (**A**) Illustrative flow cytometry analysis showing the intensity of DiI(C_{18}) labeling (upper quadrant) compared with nonlabelled cells (lower quadrant). (**B**) Light (left) and fluorescent (right) microscopy images of red-labeled MCF-7 cells adherent to LN sections after the washing step. (**C**) After adhesion assay, attached cells on coverslips are manually quantified by using a calibration scale to estimate the lymph node area and fluorescent microscopy to direct cell counting. (**D**) NDRG4 knockdown in MCF-7 breast tumor cells increases lymph node adhesion. Representative images of red fluorescent MCF-7 cells (NDRG4-positive or NDRG4-negative DiIC18-labelled cells) 30 min after seeding on 5 µm rat lymph node sections. The LN adhesive index is expressed as the number of adherent tumor cells per LN covered area (cells/mm^2). Scale bar: 200 µm. *$p < 0.05$

DISCUSSION:

Lymphatic system dissemination of cancer cells requires a variety of complex cell-driven events. They initiate with cell detachment from primary tumor and the remodeling of the extracellular matrix (ECM) architecture, and are supported by persistent chemotaxis and active migration through the afferent lymphatics en route to the sentinel LNs. If cancer cells adhere and survive in LNs, they can easily spread to other secondary organs. Here we describe an easy method for rapid and low-cost functional analysis of specific adhesive interactions between tumor cells and frozen LNs.

Structurally, LNs are discrete sponge-like masses of dense and extensive networks of ECM fibers, frequently referred to as “reticular fibers”, which act as paths for cell migration and as conduits for rapid delivery of soluble factors (antigens and/or chemokines) within the LN parenchyma[27]. The preserved reticular fibers of the frozen LNs of the assay support haptotactic signals and provide scaffolds for tumor cell adhesion in vitro. These fibers are made up primarily of structural proteins, such as collagens I and III, and by secondary ECM elements, such as fibronectin, tenascin, laminin, vitronectin and heparan sulfate proteoglycans[28,29]. Following cell adhesion, most of these LN-derived ECM factors provide molecular cues that determine cell survival (proliferative or dormancy states) or cell death (anoikis) through integrin-mediated signals.

Here, we demonstrate the assay using xenogeneic rat LNs and a human breast tumor cell line. Alternatively, other sources of LNs could be used. The composition of rat, mouse or human LNs includes the same structural and functional proteins that are part of native mammalian ECM, all preserving similar binding sites that are necessary for cell adhesion[23]. Importantly, the only

critical step is to use consecutive slices of the same LN per experiment to minimize regional variations in ECM composition of each LN section, which in turn could dictate the cell adhesion rate.

A drawback of the assay is that it does not recapitulate the first steps of lymphatic dissemination, only reflecting the adhesive strength of tumor cells to LNs. For example, seeding less aggressive breast tumor cells on LN sections, like the MCF-7 (**Figure 2**) or the T47D tumor cell lines[24], lead to a strong adhesion to LN sections in vitro, at similar levels than the observed for the high aggressive MDA-MB-231 tumor cells (data not shown). However, it is well known that orthotopic MCF-7 xenograft tumors cannot reach sentinel LNs, while MDA-MB-231 tumors spontaneously metastasize to them[30]. Clearly, the main bottleneck for MCF-7 cells LN-metastasis formation occur in steps before they reach and adhere to LNs, like the inability of MCF-7 cells efficiently escape from the primary tumors. So, the strength of the assay described here is not establish direct correlations with LN-metastatic potential, but is a simple method to quantify the adhesive properties of a tumor cell in a more realistic ECM in vitro. By using frozen tissues, the cryosections represent the natural complexity of LNs in terms of structure and composition, which would be impossible to recreate using synthetic techniques, particularly those using purified ECM proteins.

Additional limitations of the method are (1) it does not allow the evaluation of the chemotactic potential of factors secreted by LNs and that (2) it does not inform on whether the cell-specific adhesion to LN sections is a result of preferential binding to ECM proteins, cells or any other structures present in LN sections. However, we felt that this approach could be relevant and must be seriously considered for particular applications, but were beyond the scope of this particular manuscript. For example, in a recent study, we identified the N-Myc downstream-regulated gene 4 (NDGR4) as a mechanistic biomarker of LN metastasis in breast tumors[24]. Mechanistically, tumor cells lacking NDRG4 expression increase adhesion to cryosections of LNs by favoring the assembly of β1-integrin receptors at the leading edge of breast tumor cells. Furthermore, using additional controls, like dishes coated with purified ECM proteins, we uncovered that differential adhesion to LN sections is a result of selective association with vitronectin[24].

Finally, it is worth noting that this method is not restricted to LNs sections and could be set-up to assess cell adhesion to different living organs, like cryosections of spleen or lungs. Metastatic cells exhibit organotropism and measurements of adhesive strength in frozen sections of different organs in vitro, could be a useful mean for predict organ-specific cancer dissemination.

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DISCLOSURES:

The authors have nothing to disclose.

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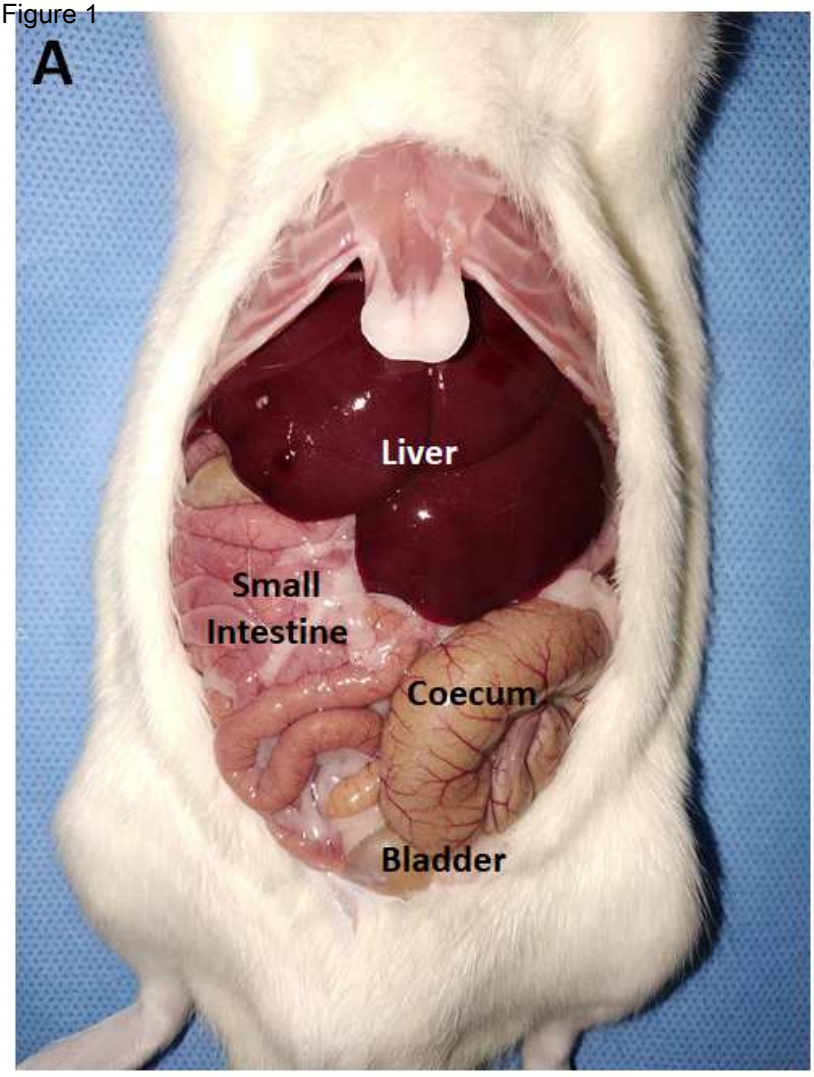
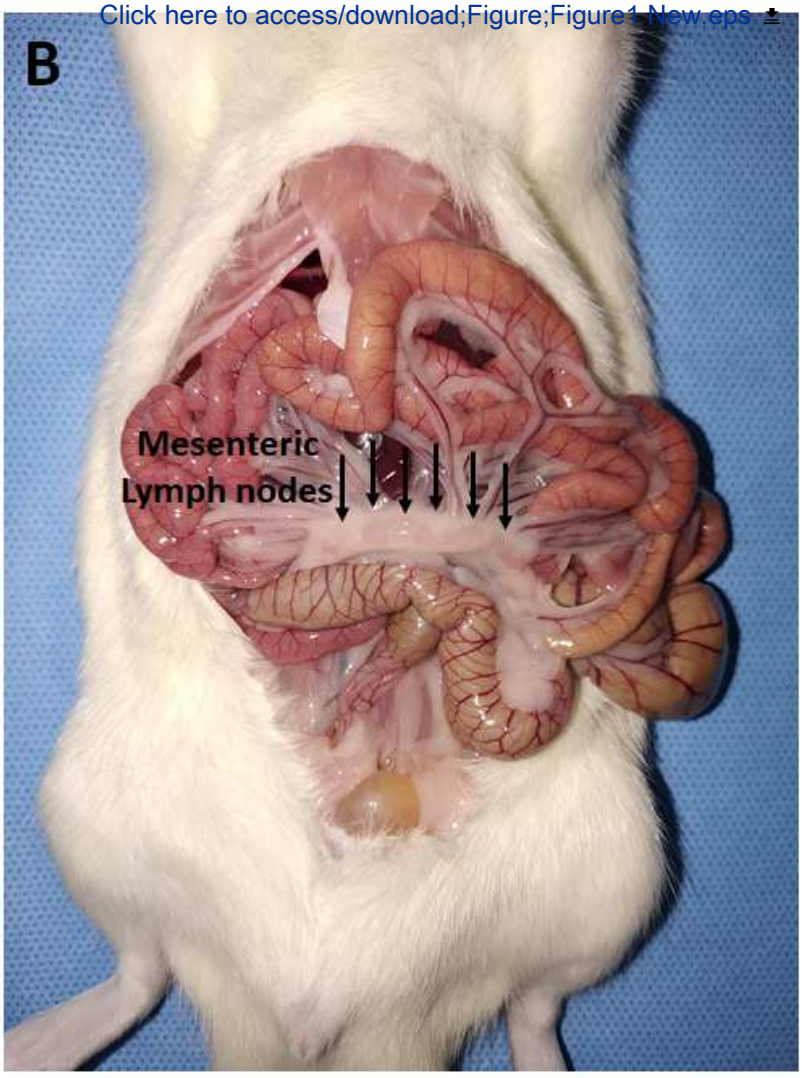
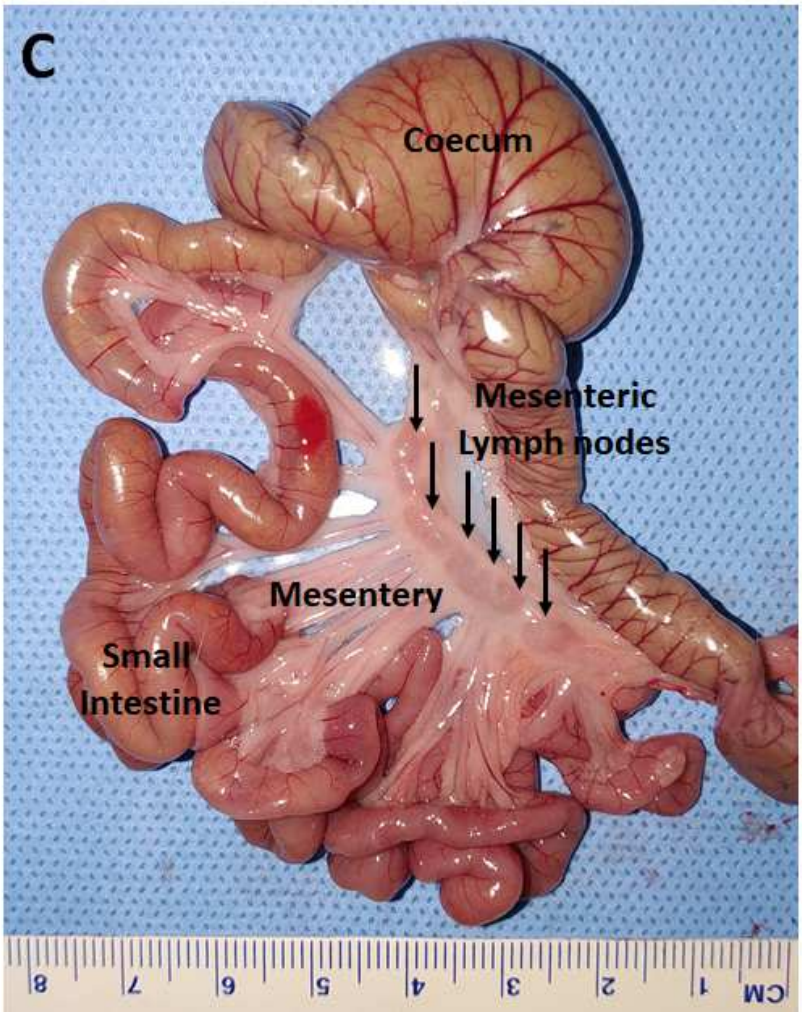
Figure 1







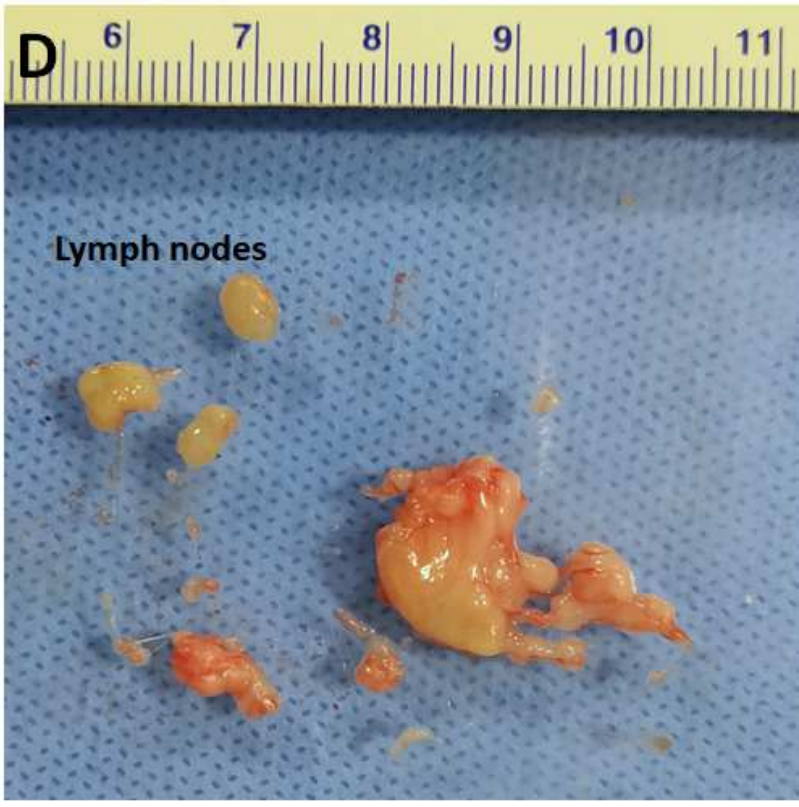


Figura 2

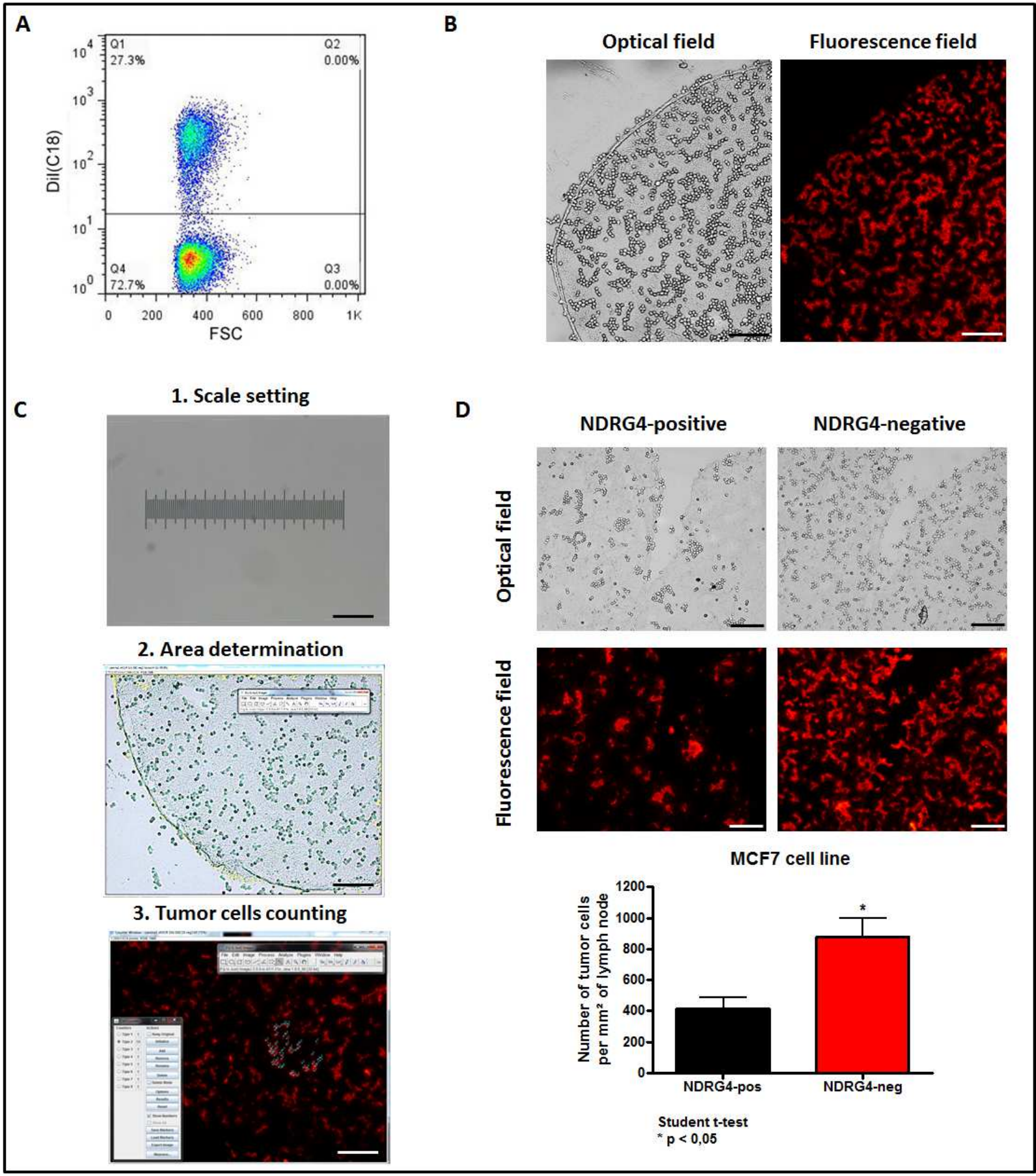


Table of Materials

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Name of Material/ Equipment	Company	Catalog
15 mL Conical Tubes	Corning	352096
2-propanol	Merck	109634
Benchtop Laminar Flow	Esco Cell Culture	
Bin for Disc	Leica	14020139126
Bovine Serum Albumin	Sigma-Aldrich	A9647-100
Cell culture flask T-25 cm^2	Corning	430372
Cryostat	Leica	CM1860 UV
Cryostat-Brush with magnet	Leica	14018340426
DiIC18 Cell Traker Dye	Molecular Probes	V-22885
Fetal Bovine Serum (FBS)	Life Technologies	12657-029
Fluorescence microscope	Nikon Eclipse 80	
Forma Series II CO2 incubator	Thermo Scientific	
Formaldehyde	Sigma-Aldrich	252549
High Profile Disposable Razor	Leica	14035838926
Incubation Cube (IHC)	KASVI	K560030
Inverted microscope	Olympus	CKX31
Isofluran 100 mL	Cristália	
Liquid Bloquer Super Pap Pen	Abcam, Life Science Reagents	ab2601
Optimal Cutting Temperature "OCT" compound	Sakura	4583
Phosphate-buffered Saline (PBS)	Life Technologies	70011-044
Poly-L-lysine	Sigma-Aldrich	P8920
RPMI	Gibco	31800-022
Serological Pipettes 1 mL	Jet Biofil	GSP010001
Serological Pipettes 10 mL	Jet Biofil	GSP010010
Serological Pipettes 2 mL	Jet Biofil	GSP010002
Serological Pipettes 5 mL	Jet Biofil	GSP010005
Serological Pipettes 50 mL	Jet Biofil	GSP010050
Serological Pipettor Easypet 3	Eppendorf	
Tissue-Tek cryomold	Sakura	4557
Trypan Blue 0.4%	Invitrogen	T10282
Trypsin	Instituto Adolfo Lutz	ATV

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Author(s): Elisa HF Jandrey, Mayra A Kuroki, Anamaria A Camargo, Erico T Costa

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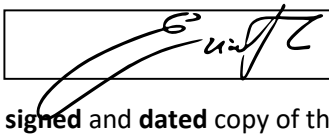
Signature:	
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INSTITUTO SÍRIO-LIBANÊS DE ENSINO E PESQUISA

São Paulo, 22th Oct 2019.

Rebuttal document JoVE60531
Dr. Bing Wu
Review Editor *JoVE*

Re: Revisions required for your JoVE submission JoVE60531 - [EMID:4fc1e164d71dfd01]

Dear Dr. Wu,

Thank you for your message informing us that our manuscript entitled "Quantification of tumor cell adhesion to lymph node cryosections" (JoVE60531), has been editorially and peer-reviewed for JoVE. We have followed your recommendations for video production and formatting, and we are also very grateful for the reviewers' comments and thoughtful suggestions.

Based on these, we have carried out additional experiments and revised the manuscript accordingly. Please, find attached a point-by-point response to reviewers' concerns. We revised the manuscript using the tracked changes function of Word to help the reviewers identify our changes.

We believe we have produced an improved version of our manuscript, and we hope the reviewers now deem it acceptable for publication in JoVE.

Sincerely,

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Reply to the Editor's comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

R: Thank you, Dr. Wu, for your comments on the language. We now have proofread the manuscript by a professional native speaker.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e., "This figure has been modified from [citation]."

R: We have modified Figure 1 accordingly, and now all figures from the revised manuscript are original.

3. Please only highlight the protocol steps for filming. Please do not highlight any text in Introduction, Representative Results or Discussion.

R: These instructions were followed as recommended.

4. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations.

R: Thank you for the suggestions. We updated the discussion session accordingly.

5. Please ensure that the references appear as the following. 6. Please do not abbreviate journal titles for references. 7. Please sort the items in alphabetical order according to the name of material/equipment. 8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). Any text that cannot be written in the imperative tense may be added as a “Note.” 9. Please add a one-line space between each of your protocol steps. 10. Please use h, min, s for time units.

R: Thank you for all these points. In the revised manuscript, we have adjusted these specific issues that you highlighted. Thank you very much for the time and energy you spent on our behalf.

Reply to Reviewer #1

Manuscript Summary: This manuscript describes a method to examine tumor derived factors that promote adhesion of tumor cells to molecules, cells and structures present in lymph nodes. This method has advantages relative to in vitro tumor adhesion assays and also limitations as described by the authors. The authors were transparent when discussing the advantages and disadvantages of the research tool that they developed. The methods were easy to follow and a very relevant example of how to apply their assay was provided.
R: Thank you very much for your kind words about our paper.

Minor Concerns:
The manuscript does require a good amount of copy-editing.
R: We have made careful modifications to the original manuscript. The manuscript was proofread by a professional to minimize spelling and grammar errors.

Reply to Reviewer #2

Manuscript Summary: This technical paper is based on a recent publication by the same authors in which NDRG4 promoter hypermethylation was identified as a biomarker of metastatic breast cancer (Jandrey et al NPJ Breast Cancer 2019). In that paper, the authors have shown that MCF-7 cells in which NDRG4 was silenced using shRNA had an increased adhesion to LN cryostat sections. Increased β1 integrin clustering at the leading edge of tumor cells resulting in increased cell adhesion and migration towards vitronectin was identified as the underlying mechanism.

Here, the authors provide a step-by-step detailed description of the adhesion assay used, supplementing previously published data. The assay described is a further optimization of a previously published technique (i.e. Brodt et al). They propose that it can be used as a low-cost, rapid, sensitive and environmentally friendly protocol with clinical relevance to lymph node metastasis in breast cancer patients.

Critique:

The description of the assay is very clear. This adhesion assay could be useful to other investigators who wish to establish a functional in vitro correlate of lymphatic metastasis as part of a broader profiling of metastatic cancers.

R: We appreciate your time, comments, and insights. We are delighted to hear that you think our method is relevant, clearly described and contextualized by our previously published data.

Major Concerns:

1. For their studies, the authors selected human breast cancer MCF-7 cells that are known to be non-metastatic in xenotransplanted mice. Moreover, they provide no evidence that NDRG4 silencing in these cells actually alters their lymph-node metastasizing potential (e.g. in an animal model). Thus, while differences in adhesion of the two cell lines were demonstrated, the correlation to LN metastasis is indirect, as it relies on other data showing NDRG4 promoter methylation in clinical specimens. To strengthen the impact of this work, the authors should use cancer cell line pairs for which a difference in lymph node metastasizing potential that correlates with differences in adhesion to the cryostat LN sections can be demonstrated.

R: You are correct that the correlation between our method and LN metastasis is indirect and should be complemented with additional experiments. Lymphatic dissemination of tumor cells requires a series of complex events, like remodeling of cell-cell interactions, local invasion, chemotaxis, cell migration, etc. In my experience, even the use of "pairwise" adhesion assays, using a couple of different cell lineages, will hardly indicate direct correlation with LN-metastatic potential *in vivo*. For example, as you well noticed, even a non-metastatic breast tumor cell line, like MCF-7 (see manuscript) or the T47D tumor cells (data not shown), strongly adhere to LN sections *in vitro*, at similar levels to that observed for the highly metastatic MDA-MB-231 tumor cells (data not shown from our lab). However, it is well known that orthotopic MCF-7 xenograft tumors cannot reach sentinel LNs, while MDA-MB-231 tumors spontaneously metastasize to LNs (Pathak et al., *Cancer Res*, 2006). The main bottlenecks for MCF-7 LN-metastasis formation occur in steps in the metastatic cascade before reaching and adhering to LNs, like the inability of MCF-7 cells to efficiently escape from the primary tumors. This paper aims to carefully describe

a simple method to quantify cell-matrix adhesion properties in a more realistic ECM *in vitro*. The purpose of this assay is not to establish direct correlations with potential LN metastasis but is to allow a rapid and low-cost functional analysis of specific adhesiveness of tumor cells to ECM, cells, and structures present in LN. Nonetheless, to address the review's comment, we included a paragraph in the discussion section highlighting this limitation of the assay.

2. Because the lymph nodes used are derived from rats, the authors should consider using rat breast (or other) carcinoma lines or human lymph nodes, if available. At the very least, potential differences in the stromal components of human and rat lymph nodes should be discussed.

R: We agree with the reviewer that potential differences in the stromal components of human and rat lymph node could impact the performance of the assay. However, we believe that the impact would be minimal as the composition of rat, mouse or human LNs includes the same structural and functional proteins that are part of native mammalian ECM (e.g. collagens, fibronectins, laminins, etc), all preserving similar binding sites that are necessary for cell adhesion (Badylak SF Transpl Immunol 2004). Nevertheless, to address the review's comment, we included a paragraph in the discussion section highlighting potential differences in stromal components and their potential impact in our assay.

Minor Concerns: As a control, the authors used BSA-coated dishes. As an additional and more relevant control, the authors should consider using dishes coated with ECM proteins such as fibronectin, vitronectin and collagen. This will inform on whether the differential adhesion to LN sections is merely a result of differential adhesion to specific ECM proteins, or the LN stroma has additional complexity and constituents (e.g. cells) that could contribute to the differential adhesion.

R: Thanks for raising this critical point about controls. Dishes with purified ECM compounds, or decellularized LNs, certainly could be used, but the results would be cell-type specific, as different cell lines possess different adhesive affinities towards different LN components. The purpose of this paper is to describe a general protocol to evaluate cell-matrix adhesion in frozen LNs. To evaluate whether the differential adhesion to LN sections is a result of binding to

collagen, vitronectin, or other structures present in LN sections, is beyond the scope of this particular article. To address the reviewer's comments these points are now addressed in the revised version of the manuscript.

Reply to Reviewer #3

Major Concerns: Although the method is well described and complete, I suggest to authors to include photographs showing the identification and isolation of lymph nodes, instead of drawings. This is the most challenging step in this methodology and deserves a proper representation.

R: We agree with the reviewer's suggestion. These photographs were incorporated in the revised version of the manuscript (see new Figure 1). Thank you for this relevant suggestion to better illustrates our method.

Minor Concerns:
- Page 1, line 31 - in the second sentence of the abstract, replace carcinomas for cancer.
R: We agree and have changed the sentence accordingly.

Reply to Reviewer #4

Major Concerns: To better represent figure 1, the authors should provide annotations for the location of lymph nodes. (…)
R: Thank you for this valuable comment that Reviewer 3 also pointed out. Instead of drawings, we now included photographs showing the identification and isolation of rat LNs in the revised version of the manuscript (see the new Figure 1).

(…) The discussion should also outline the advantages in utilizing this methods in a clinical setting i.e studies that would benefit in utilizing this method. Authors should also mention if similar methods can be used for different organs and cell types i.e spleen, WBC.
R: The reviewer raised an interesting perspective to use this method in the clinical setting. Metastatic cells exhibit organotropism and measurements of adhesive strength in different organs could be a useful means to predict organ-specific cancer metastasis. We now elaborate on this point in the discussion

section of the revised version of the manuscript. Nevertheless, we are hesitant to take a strong position on this point (like utilizing it in a clinical setting), because metastatic growth requires a series of more complex events, than a simple tissue-specific adhesion.

Minor Concerns: The manuscript requires linguistic revision. i.e line 104-105, line 123, line 158 etc. The protocol section needs revision for imperative statements.

R: Thank you for your recommendations on the language. The manuscript was proofread the manuscript by a native speaker.