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Determining the macropinocytic index of cancer cells through a quantitative image-based assay

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Abstract

Macropinocytosis serves as an internalization pathway for extracellular fluid and its contents and is upregulated in oncogene-expressing cells. Recently, we have revealed a functional role for macropinocytosis in fueling cancer cell growth through the internalization of extracellular albumin, which is degraded into a usable source of intracellular amino acids. Assessing macropinocytosis has been challenging in the past due to the lack of reliable assays capable of quantitatively measuring this uptake mechanism. Here, we describe a protocol for visualizing and quantifying the extent of macropinocytosis in cancer cells both in culture and *in vivo*. Using this approach, the 'Macropinocytic Index' of a particular cancer cell line or subcutaneous tumor can be ascertained within 1–2 days. The protocol can be carried out with multiple samples in parallel and can be easily adapted for a variety of cell types and xenograft/allograft mouse models.

INTRODUCTION

Macropinocytosis is an endocytic process that mediates the uptake of extracellular fluid into large intracellular vesicles known as macropinosomes. Macropinosomes are heterogeneous in size and shape and serve to internalize large volumes of extracellular fluid along with the associated membrane. This endocytic process is thought to be initiated by the formation of actin-dependent protrusions of the plasma membrane, known as membrane ruffles. Plasma membrane contacts between individual ruffles or between a ruffle and the plasma membrane, followed by membrane fission, results in the formation of a macropinosome. The newly formed macropinosomes can be targeted to lysosomes or can be recycled back to the plasma membrane[1]. Functionally, macropinocytosis plays a role in immunity as antigen-presenting cells utilize this endocytic process to sample their external environment[2]. Additionally, macropinocytosis also represents a cellular route of entry for numerous bacteria and viruses[3,4].

In cancer, macropinocytosis is stimulated by oncogenes, such as Ras[5] and Src[6–8], and we have recently linked this form of fluid-phase uptake to cancer cell metabolism and nutrient

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AUTHOR CONTRIBUTIONS

C.C., R.F. and D.B.-S. contributed to the experimental design. C.C. performed the experiments and data analysis. C.C., R.F. and D.B.-S. wrote the manuscript.

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internalization[9]. The majority of the nutrients required to sustain tumor cell growth are present in extracellular fluids and approximately 70% of the solubilized substances are proteins, with serum albumin being the most abundant. We determined that oncogene-stimulated macropinocytosis is an entry route for extracellular albumin. In this context, the internalized albumin is subsequently targeted for lysosomal degradation, releasing the constituent amino acids, which then have the capacity to support the metabolic and biosynthetic needs of the tumor cells. Therefore, macropinocytosis is an important nutrient delivery pathway that cancer cells use to drive their proliferation and growth.

Methods to detect and quantify macropinocytosis

We have developed and optimized techniques for detecting and quantifying macropinocytosis both *in vitro* and *in vivo*. Our protocol is based on the incorporation of fluorescently-labeled, lysine-fixable, 70 kDa high molecular weight dextran from the extracellular medium into large, discrete macropinosomes, which can be visualized microscopically as fluorescent intracellular puncta. Using these techniques, the extent of macropinocytosis, or what we have termed the 'Macropinocytic Index', for a particular cancer cell line or subcutaneous tumor can be determined. Specifically, the macropinocytic index is a two-dimensional parameter that quantifies the percentage of cell area occupied by dextran-positive macropinocytic compartments within a given timeframe of dextran uptake. At the cellular level, the macropinocytic index is a read-out of not only macropinocytic rate, but also of macropinosome size. Ascertaining the macropinocytic index of a particular cell line or tumor has the potential to shed light on the metabolic properties of the sample. For example, if a tumor exhibits a high macropinocytic index, then this may be an indication that the tumor relies on macropinocytosis for its growth and survival. Indeed, we found that the inhibition of macropinocytosis in tumors with a high macropinocytic index selectively leads to a decrease in tumor growth in a xenograft mouse model of pancreatic cancer[9]. Macropinocytosis has also been studied in the context of drug delivery and has been implicated as a delivery mechanism for a number of therapeutics including cell penetrating peptides, nanoparticles, and microbe-based biotherapies[10–12]. Therefore, the macropinocytic index may represent a predictive biomarker in the effective usage of these forms of cancer therapy.

The quantification of macropinocytosis can be achieved through either microscopic or non-microscopic methods. For microscopic methods, macropinocytosis is fluorescently visualized and quantified in either fixed or live cells. The protocol described herein illustrates the microscopic visualization and quantification of macropinosomes in fixed cells via image-based analysis in ImageJ. Previous reports have described methods for quantifying macropinocytosis in fixed sample. The quantification method utilized by Haga, *et al.*[13] depends on subjective categorization (i.e. qualitatively assigning cells into low to high macropinocytosis categories). The method developed by Wang, *et al.*[14] is similar to our protocol in that it can be applied to any cell monolayer; however, their procedure involves an additional step to consider only transfected cells. Our protocol has wide applicability to both cultured cells and xenograft tumor models. A particular consideration of our protocol is that the method requires fixed samples. Hence, the analysis captures a snapshot of the process rather than the dynamic nature of macropinocytosis in real time. A quantification

method entailing the imaging of macropinocytosis in live cells through phase contrast microscopy and observing the phase-bright macropinosomes that emerge downstream of membrane ruffles has been recently reported[15]. However, this method lacks the selectivity provided by a fluorescent macropinocytosis marker and is limited to the analysis of cultured cells, precluding the analysis of macropinocytosis in xenograft tumors.

For non-microscopic methods, the most commonly reported macropinocytosis assay involves uptake of the soluble enzyme Horseradish peroxidase (HRP). Quantification in this procedure measures HRP enzymatic activity in cell lysates derived from cells that have internalized the enzyme[16]. This approach is suitable for quantifying macropinocytosis across multiple conditions or cell lines; however, this method does not provide any visual confirmation of the process nor does it provide structural information in the manner that image-based procedures do. Moreover, it is unclear how specific an HRP uptake assay is considering the extent that HRP adheres to the cell surface[17]. A second non-microscopy-based quantitative method for the analysis of macropinocytosis utilizes Flow Activated Cell Sorting (FACS)[18]. For this approach, cells are pulsed with fluorescently labeled dextran, cells are washed, trypsinized, and fixed, and dextran uptake is subsequently quantified via FACS. While this method allows for robust quantification of macropinocytosis on a sample by sample basis, it does not provide any structural information on a cellular level. Furthermore, the temperature and time required for the trypsinization step, which is necessary to create the cell suspensions for FACS analysis, are not optimal; hence our protocol takes advantage of a low temperature incubation immediately following the uptake step, which introduces a blockade in trafficking events, allowing for the maximal retention of the internalized dextran[19].

EXPERIMENTAL DESIGN

Choice of cell lines

The protocol described herein was optimized for use in the human pancreatic adenocarcinoma cell lines, MIA PaCa-2 and BxPC-3. However, the protocol has been very easily adapted to other cell lines taking into account some important considerations. Optimization of our procedure has been performed using moderate cell confluence conditions (~75% confluent). Thus, cell lines that require either sparse growth conditions or maintenance as a confluent sheet may require further optimization. The procedure involves several washing steps prior to fixation; therefore, the cell lines used should be readily adherent to cover glass to ensure that they are not displaced during the washes. To circumvent poor adhesion to the cover glass, the cover glass could be coated with an extracellular matrix protein to increase cell adherence; however how this would affect usage of our procedure would have to be determined. Additionally, this protocol has been optimized to assess levels of macropinocytosis that occur under serum-starved conditions. Serum starvation was employed to distinguish the inherent levels of macropinocytosis within the cell lines analyzed from the non-autonomous effects on macropinocytosis exerted by the growth factors present in serum-supplemented media. However, this protocol can be adapted to assay cells in serum- or growth factor-supplemented media by further optimizing the concentration and incubation time of the fluorescent dextran.

Choice of dextran conjugate

We have optimized the described assays for use with fixable 70 kDa dextran that has been conjugated with either fluorescein isothiocyanate (FITC) or tetramethlyrhodamine (TMR). However, our preference for the assay is the TMR conjugate because, unlike FITC, it is generally insensitive to the low pH attained by the macropinosome as it matures[20]. We have utilized 70 kDa molecular weight dextrans to label macropinosomes because the macropinosomes labeled by these dextrans fall within the classically defined size range of 0.2–10 μm in diameter[21]. These high molecular weight dextrans are selectively internalized into macropinosomes whereas lower molecular weight dextrans show less fidelity in their internalization and may label other endocytic pathways[22]. Depending on the timing of trafficking events in the particular cell line or tumor being analyzed, it is possible that the macropinocytosed dextran may label other endocytic compartments, such as endosomes that have fused with macropinosomes downstream of internalization. Therefore, if the macropinocytic contents are rapidly targeted to the endosome system or the lower molecular weight dextrans must be employed, then a shorter dextran incubation time and the diameter of the labeled macropinosomes should be taken into consideration when quantifying the results.

Analysing xenografts

For determining the macropinocytic index of xenograft tumors, the tumors can be generated using established procedures, such as the protocol developed by Kim et al.[23] The *in vivo* methodology in our protocol has been designed to be utilized in the study of subcutaneous heterotopic human pancreatic cancer xenografts in immunodeficient mice. However, it can be easily adapted to other heterotopic organ models and to heterotopic allograft tumors. For computing the macropinocytic index of subcutaneous tumors, immunological staining of the tissue with an antibody that selectively labels the tumor cells is necessary to ensure that the macropinosomes visualized are a feature of the transplanted cells rather than the host cells. In pancreatic tumor xenografts, we have successfully used anti-cytokeratin 8 staining to specifically label the human pancreatic epithelial cells within the tumor[9]. Therefore, to adapt this protocol to other subcutaneous tumors, a tumor cell-specific marker must be identified prior to analysis.

Controls

In addition to their large size and the ability to internalize high molecular weight dextran, macropinosomes are also defined by their unique sensitivity to amiloride and its derivatives, a class of Na+/H+ exchange inhibitors[21]. Amilorides inhibit macropinocytosis by lowering the submembranous pH, which in turn interferes with the actin remodeling that is required for plasma membrane ruffling[24]. Therefore, in order to further validate macropinocytosis-specific uptake, an important experimental control in the analysis of macropinocytosis is to evaluate the effects of amiloride treatment. Treatment with the amiloride derivative 5-(N-ethyl-N-isopropyl) amiloride (EIPA) is the most effective and selective method currently used to pharmacologically inhibit macropinocytosis[25]. For cell culture experiments, the dose of EIPA required to inhibit macropinocytosis must be empirically determined; however, we have observed robust inhibition typically in the range of 25 to 100 μM. It should be noted

that EIPA doses at the higher end of this range tend to be cytotoxic to a subset of cell lines and whether this is the case for a particular cell line of interest must be determined. To validate the specificity of EIPA in a cell line of interest, the effects of EIPA treatment on clathrin-mediated endocytosis can be assessed by performing a transferrin uptake assay[26]. At high doses, EIPA may exhibit autofluorescence when imaging using a filter set for collecting blue or green wavelength fluorescence; therefore we recommend the use of TMR-Dextran with EIPA treatment as opposed to FITC-Dextran, whenever possible. Furthermore, EIPA autoflourescence may appear when DAPI images are collected, thus it is advised to use short exposure times to collect DAPI images in EIPA treated cells to minimize this effect. To inhibit macropinocytosis *in vivo*, we have successfully employed osmotic pumps (Alzet) implanted subcutaneously in mice to systemically deliver EIPA at a steady dose over the course of a 3–7 day experiment[9]. Such a delivery mode is advantageous because of the short half-life of EIPA once exposed to circulating plasma[27]. An additional control, especially in the case of a low macropinocytic index, is to simultaneously assess macropinocytosis in a cell line expressing an oncogenic form of Ras. This can be achieved by transfection of oncogenic Ras into the particular cell line of interest or by using an oncogenic Ras-expressing cell line that has been established to have a high macropinocytic index, such as MIA PaCa-2, T24 or NIH 3T3 $KRas^{V12}$ cells[9].

MATERIALS

REAGENTS

- Cell line or xenograft to be analysed plus appropriate controls. CRITICAL See Experimental Design section for further guidance. CAUTION Experiments using live rodents must conform to Institutional and National regulations.
- DMEM (Gibco, cat. no. 11965-092)
- RPMI (Gibco, cat. no. 11875-093)
- Sodium Pyruvate (Mediatech Cellgro, cat. no. 25-000-CI)
- HEPES (Corning Cellgro, cat. no. 25-060-CI)
- 1% Penicillin/Streptomycin (Gibco, cat. no. 15140-122)
- Fetal Bovine Serum (FBS, Gibco, cat. no. 10438-026)
- Trypsin-EDTA (Gibco, cat. no. 25200-056)
- 1 M HCl (Sigma, cat no. 7647-01-0)
- 70 kDa TMR-Dextran, lysine fixable (Invitrogen Molecular Probes, cat. no. D1818)
- 70 kDa FITC-Dextran, lysine fixable (Invitrogen Molecular Probes, cat. no. D1822)
- 5-(N-ethyl-N-isopropyl) amiloride (EIPA) (Invitrogen Molecular Probes, cat. no. e-3111)
- PBS (Gibco, cat. no. 14190)

- 4’,6-Diamidine-2’-phenylindole dihydrochloride (DAPI) (Sigma, cat. no. D9542)
- DAKO fluorescent Mounting Media (DAKO, cat. no. S3023)
- 37% ACS reagent grade Formaldehyde Solution (Ricca Chemical Company, cat. no. 50-00-0)
- O.C.T. Compound (Tissue-Tek, cat. no. 4583)
- Normal Chicken Serum (Jackson ImmunoResearch, cat no. 003-000-001)
- Albumin from bovine serum (BSA, Sigma-Aldrich, cat no. A7906)
- Troma I Antibody (recognizes Cytokeratin-8, Developmental Studies Hybridoma Bank)
- Alexa Fluor 594 Chicken Anti-Rat IgG (Molecular Probes, cat no. A-2147)
- Dry ice
- Athymic mice (Taconic NCRNU, homozygous)

EQUIPMENT

- 37°C/5% CO_2 cell culture incubator
- Vacuum aspirator
- 12 mm (0.13–0.17 mm thick) circular cover glass (Fisherbrand, cat no. 12-545-80)
- Zeiss Axiovert inverted 200M wide-field microscope equipped with a 63X 1.4 NA Phase objective, filter sets for DAPI, FITC, and Rhodamine, and a CCD camera
- 1 cc 28G ½ inch insulin syringe (Becton Dickinson, cat no. 329410)
- Standard dissection tools
- Microtome cryostat
- 24 well plates (Becton Dickinson Falcon, cat no. 353047)
- Cryomold (Tissue-Tek Cryomold, cat no. 4557)
- 3x1 inch (0.93–1.05 mm thick) glass slides (Becton Dickinson Gold Seal, cat no. 3010)
- Forceps
- Digital calipers (Fisher World Precision, cat no. 111–100)
- Liquid blocker pen (Daido Sangyo Co, Super PAP Pen)
- 24x50 mm rectangular cover glass (VWR, cat no. 48393081)
- High-Density Polyethylene Staining Jar (Sigma, S5641)
- Image J software (http://imagej.nih.gov/ij/)

REAGENT SETUP

CRITICAL All solutions and buffers must be free of detergent and therefore should be prepared in glassware thoroughly cleaned with ethanol or new plasticware. All solutions containing cell culture media should be pre-warmed to 37°C prior to usage. Prepared media containing FBS can be stored at 4°C for 1–2 weeks.

MIA PaCa-2 complete media—This media consists of DMEM supplemented with 10% (vol/vol) FBS, 25 mM HEPES, and 100 U/ml Penicillin/Streptomycin.

MIA PaCa-2 serum-free media—This media consists of DMEM supplemented with 25 mM HEPES and 100 U/ml Penicillin/Streptomycin.

BxPC-3 complete media—This media consists of RPMI supplemented with 10% (vol/vol) FBS, 25 mM HEPES, 1 mM Sodium Pyruvate, and 100 U/ml Penicillin/Streptomycin.

BxPC-3 serum-free media—This media consists of RPMI supplemented with 25 mM HEPES, 1 mM Sodium Pyruvate, and 100 U/ml Penicillin/Streptomycin.

Dextran stock solution Dissolve—25 mg of fluorescently labeled 70 kDa fixable dextran in 1.25 mL of PBS to give a 20 mg/ml Dextran final concentration. Store 100 μL aliquots of this stock in the dark at −20°C.

CRITICAL Ensure that the dextran is completely dissolved by pipetting up and down. Incomplete solubilization will negatively affect downstream microscopic visualization.

Dextran cell culture incubation media—Dilute the Dextran stock solution to 1 mg/ml final concentration in the appropriate serum-free medium.

CRITICAL The dextran cell culture incubation media should be prepared on the day of the experiment.

Fixation buffer Dilute—37% ACS reagent grade Formaldehyde Solution in PBS to a final concentration of 3.7% (v/v). CRITICAL Freshly prepare the solution on the day of the experiment.

DAPI stock solution Dissolve—5 mg of DAPI in 0.5 mL of ddH_20 to a final concentration of 10 mg/ml. Store 5 μL aliquots at −20°C.

DAPI solution Dilute—one 5 μL aliquot of DAPI stock solution with 495 μL of ddH_20 and stored at -20°C.

DAPI staining buffer—Dilute DAPI solution 1:100 in PBS.

CRITICAL The DAPI staining buffer should be prepared on the day of the experiment.

CAUTION DAPI is a carcinogen; avoid contact with skin.

Normal Chicken Serum—Resuspend the freeze-dried chicken serum in 2 mL PBS. Can be aliquoted and stored at -20°C.

Blocking solution—10% Normal Chicken Serum (v/v) and 2% BSA (w/v) prepared in PBS. CRITICAL Freshly prepare the solution on the day of the experiment.

Primary antibody solution—Dilute Troma-I antibody 1:250 in Blocking solution. CRITICAL Freshly prepare the solution on the day of the experiment.

Secondary antibody solution—Dilute Alexa Fluor 594 Chicken Anti-Rat antibody 1:500 in Blocking solution. CRITICAL Freshly prepare the solution on the day of the experiment.

EQUIPMENT SETUP

- **Circular Cover Glass** Heat cover slips in a covered glass beaker in 1M HCl at 50°C for 24–48h. Cool them to room temperature (22.5°C) and rinse four times with ddH_2O. Rinse four times with 95% EtOH and store in 95% EtOH at room temperature.
- **Epifluorescence microscopy setup** Our setup consists of a Zeiss Axiovert inverted 200M fluorescence microscope (Carl Zeiss AG) equipped with a 63X 1.4 NA Phase oil objective and a CCD camera (1388 X 1040 Axiocam MR Rev3, Zeiss). DAPI (absorption/emission maxima ~358/461) is detected with the excitation filter 365/50, the 395 beamsplitter and the emission filter 445/25. The FITC-dextran fluorescence (absorption/emission maxima ~ 494/521) is detected with the excitation filter 485/10, the 510 beamsplitter and the emission filter 540/25. TMR-Dextran (absorption/emission maxima ~ 555/580) or secondary antibody conjugated Alexa fluor 594 (absorption/emission maxima ~590/617) is detected with the excitation filter 545/15, the 570 long pass beamsplitter and the emission filter 620/30.

PROCEDURE

Labeling of macropinosomes

1 The visualization of macropinosomes in tumor cells, either in cell culture conditions (option A) or *in vivo* (option B), is based on the ability of cells to internalize extracellular fluid containing fluorescently-tagged 70 kDa high molecular weight dextran, an established marker of macropinocytosis. Option A requires the addition of either FITC-dextran or TMR-dextran to the culture medium and is applicable to any adherent cell line. Option B involves the intratumoral injection of FITC-dextran into subcutaneous xenograft tumors in immunodeficient mice and can be employed to detect macropinosomes in tumors derived from any cancer cell line. For option B, the protocol is described for FITC-dextran; however, TMR-dextran can be just as effectively utilized, but it must be ensured that the fluorescent secondary antibodies that are

subsequently used are compatible for microscopic analysis (i.e. are not imaged on the same channel).

(A) Labeling of macropinosomes in cultured cells—Timing 2–4 days

i. Working in a sterile cell culture hood, place a circular cover glass into each well of a 24-well tissue culture plate, as required, and rinse 3 times in PBS.

ii. Split cell line(s) of interest in complete media into wells containing the circular cover glass. The optimal cell density should be such that the cells will be 60–70% confluent within 2–3 days post-seeding.

iii. When cells reach ~65% confluency, remove complete growth media and incubate the cells in the appropriate serum-free media for 12–16 hours.

iv. Replace serum-free media on cells with Dextran cell culture incubation media. Incubate in a 37°C/5% CO_2 cell culture incubator for 30 min.

v. Place 24-well plate containing cells on ice and carefully aspirate media from each well. Gently wash each well with 2 mL of ice cold PBS for a total of 5 washes.

CRITICAL STEP Ensure complete rinsing of the samples, as insufficient washing will yield high levels of fluorescent background and negatively impact downstream microscopic visualization.

B. Labeling of macropinosomes in xenograft tumors—Timing 3–5 weeks.

i. Establish xenograft tumors in the flanks of 4 week old female nude athymic mice (NCr-*Foxn1nu*) using the appropriate protocol that is in accordance with relevant guidelines and regulations specific to your institution. Refer to an existing protocol for details, such as that by Morton and Houghton[28]. Allow subcutaneous tumors to attain an average volume of 300–500 mm^3.

ii. Place the tumor-bearing athymic mouse into the anesthesia chamber following the manufacturer's recommended settings. After the mouse is sufficiently anesthetized, remove it from the chamber and place it (ventral side down) in an appropriately-sized nose cone, configured according to the manufacturer's recommended settings.

iii. Use a 1 cc 28G ½ inch insulin syringe to inject 100 µL of the Dextran stock solution (FITC-dextran) directly into the tumor.

CRITICAL STEP Ensure that the needle has entered the tumor mass prior to expulsion of the dextran stock solution. For stiff tumors, it is imperative to inject the dextran solution as slowly as possible. If properly injected, the tumor should have a slight yellow coloring due to the fluorescent moiety of the dextran. If the dextran is inadvertently injected subcutaneously, then coloration is visualized in the flank of the mouse instead, indicating a failed dextran injection and the tumor should be re-injected.

iv. Place the animal in a clean cage and observe for 10–15 min to ensure recovery from the anesthetic.

v. 30–60 minutes post-dextran injection, euthanize the mouse in accordance with relevant guidelines and regulations. We use a CO_2 euthanasia chamber with a stainless steel cover with the tube attached to the CO_2 regulator. CO_2 at a low flow rate (20% of the chamber volume per minute) is recommended. Low flow rate of CO_2 should be maintained until the animals become unconscious, then the flow rate can be increased to minimize the time to death (maximum of 5 minutes). The flow should be maintained for at least 1 minute after apparent clinical death. They should remain in the chamber or cage until absence of respiration and heartbeat is observed. Animals are removed from the chamber and cervical dislocation is performed to confirm death

vi. Immediately dissect the tumor and place in a cryomold containing O.C.T. Compound. Ensure that the tumor is completely covered by the O.C.T. Compound and place the cryomold atop dry ice. Once completely frozen (10–15 min), store the sample at −80°C for at least 24 hours.

PAUSE POINT The sample can be frozen for up to 6 months at −80°C.

Processing the samples

2 This step of the protocol is composed of processing the cells or tissue samples in preparation for microscopic analysis. Option A describes the procedure utilized to process the cell culture samples, while Option B outlines the methodology used to process the frozen tumor specimens.

A. Processing cell culture samples—Timing 1 day

i. Add Fixation buffer to the cells for 30 min at room temperature.

ii. Rinse cells 3 times with PBS.

iii. Incubate cells in DAPI staining solution for 15 minutes at room temperature.

CAUTION DAPI is a carcinogen; avoid skin contact.

iv. Rinse cells 3 times with PBS.

v. Apply one drop of DAKO mounting media to glass slide. Lift cover glass from wells using forceps and gently place cover glass on top of DAKO cell-side down.

vi. Store freshly mounted slides on a flat surface in the dark for 12–18 hours, allowing them to dry.

CRITICAL STEP Fluorescent dextrans are light sensitive; therefore, it is imperative to protect the slides from light as much as possible.

PAUSE POINT Once dry, slides can be stored for up to 2 weeks at −20°C.

B. Processing of frozen tumors samples—Timing 1 day

i. Slice the still frozen tissue using a microtome, and mount the frozen sections on a glass slide. Store these slides at −80°C.

PAUSE POINT The frozen slides can be stored for up to 3 months at −80°C.

ii. Place the slides flat (tissue section side up) in an opaque container. Air-dry the slides at room temperature for 15 min, preferably in an air circulation hood with the top of the container slightly ajar.

CRITICAL STEP Fluorescent dextrans are light sensitive; therefore, it is imperative to protect the slides from light as much as possible.

iii. Add 50 mL of fixation buffer to an opaque polypropylene staining jar. To a separate jar, add 50 mL PBS. Place the slides into the jar of PBS and incubate for 30 seconds. Lift and dip each slide five times rapidly into the PBS and immediately transfer to the jar containing the fixation buffer. Incubate at room temperature for 15 min.

CRITICAL STEP Rinsing the slides in PBS immediately prior to fixation helps reduce nonspecific background caused by the fluorescent dextran.

CRITICAL STEP After fixation, do not allow the slides to dry out.

iv. Move the slides to a clean staining jar containing PBS. Incubate for 3 min.

v. For each slide, use a liquid blocker hydrophobic pen to draw a circle around the tissue section allowing several millimeters of space between the liquid blocker and the tissue. Place the slide into a clean staining jar containing PBS. Incubate for 3 min.

vi. Perform one additional PBS wash as above.

vii. Place each slide flat (tissue section side up) in an opaque container. Carefully add 100 μL of Blocking solution directly to the tissue section. The liquid blocker encircling the sample will prevent spreading of the blocking solution. Place the cover on the container. Incubate at room temperature for 1 hr.

CRITICAL STEP Conventional staining techniques often include a detergent-based permeabilization step. Do not use any detergents when analyzing dextran-containing macropinosomes, as tissue permeabilization substantially decreases macropinosome labeling.

viii. Remove the blocking solution from the slide by gently tapping the slide onto a paper towel. Carefully add 100 μL of primary antibody solution to the tissue section. Incubate for 1 hr at room temperature.

CRITICAL STEP This protocol has been optimized for the Troma I antibody and the dilution factor for your tumor cell-specific antibody may vary and must be empirically determined.

ix. Remove the primary antibody solution from the slides by gently tapping onto a paper towel and move the slides to a staining jar containing 50 mL of PBS. Perform 3 PBS washes (5 min each) in clean staining jars.

x. Place each slide flat (tissue section side up) in an opaque container. Carefully add 100 μL of secondary antibody solution to the tissue sample. Incubate at room temperature for one hour.

xi. Remove the secondary antibody solution from the slides by gently tapping onto a paper towel and move the slides to a staining jar containing 50 mL of PBS. Perform 3 PBS washes (5 min each) in clean staining jars.

xii. Place each slide flat in an opaque container. For nuclear staining, add 100 μL of DAPI staining buffer to the tissue section. Incubate at room temperature for 15 min.

CAUTION DAPI is a carcinogen; avoid contact with skin.

xiii. Remove the DAPI staining buffer from the slides by gently tapping onto a paper towel and move the slides to a staining jar containing 50 mL of PBS. Perform 3 PBS washes (5 min each) in clean staining jars.

xiv. Remove excess PBS from the slide using a Kimwipe. Place the slide on a flat surface and add one or two drops of DAKO mounting medium directly to the tissue section. Immediately place a rectangular micro cover glass gently on top of the sample. The mounting medium should spread to the edges of the cover glass. If it does not, gently tap the cover glass.

xv. Dry slides in the dark at room temperature for 18 hours.

PAUSE POINT Stained slides can be can be stored for up to 2 weeks at −20°C.

Capturing images and data analysis

Timing 30 min to 1 hour per individual replicate sample (10 fields)

3 Perform image acquisition. We utilize an Axiovert 200 inverted fluorescent microscope (Zeiss) equipped for phase contrast and with a standard optical filter set including DAPI, FITC and Rhodamine. Capture images using a 63X phase objective and the AxioVision software platform. Prior to capturing images, use the automated exposure time feature to determine the approximate exposure time to be applied to all the samples for each individual channel being analyzed. Typically, the FITC and Rhodamine exposure times range from 100–1000 ms and manually setting the exposure time lower than the automated exposure time can ensure that the images are not overexposed. To ensure reliability of downstream quantification, obtain at least 10–20 random fields for every sample or tissue section.

CRITICAL STEP To fairly evaluate the extent of macropinocytosis across various cell culture samples, it is necessary to utilize the same exposure time for every sample and to capture random images.

4 Save the images in ZVI format. TIFF format can also be used.

5 Select an image from your collection that visually contains bright, discrete macropinocytic puncta. Load the ZVI file into Image J. There should be separate images for each acquired channel of the image.

6 Duplicate the image containing the labeled macropinosomes using the Image>Duplicate function. Tile the images side-by-side. One of the images will be used as a reference image and the other will be processed.

7 Perform a background subtraction on the image to be processed by selecting Process>Subtract Background. Set the rolling ball radius to 5 pixels. If any other options are selected with a check mark, unselect them.

8 To adjust the thresholds of the image, select Image>Adjust>Threshold. To optimize the threshold settings such that the macropinosomes are specifically selected, select "Dark background" and click the "Auto" button. If, by comparing to the reference image, all of the macropinosomes are covered with a red threshold signal, then the reference image may be closed and the threshold value recorded. This recorded threshold value is the threshold to be set for all the images. Select "Apply."

CRITICAL STEP For images with low fluorescent signal or high background fluorescence, it may be necessary to manually set the threshold values to detect the macropinosomes. To do this, set the top threshold sliding bar just to the right of the peak count value. Use the reference image to ensure the accuracy of the threshold adjustment.

9 **Compute** the 'Macropinocytic Index' of the sample by determining the total macropinosome area in relation to the total cell area for each field ('Field Index') and then determining the average across all the fields. Calculate the 'Total Cell Area' parameter for the *in vitro* setting with a phase contrast image as described in Option A or in the xenograft tumor setting from images obtained by counterstaining sections with a tumor-cell specific marker as described in Option B.

A. Determining the 'Total Cell Area' for a cell monolayer

i. To determine the 'Total Cell Area' in ImageJ, click on the phase contrast image. Select the "Polygon selections" tool. Use the polygon tool to outline the regions of the image that are covered by cells and then select Analyze>Measure to determine the area of each region. Record the value for the "Area" parameter. If there is more than one region per image, then sum the regions to obtain 'Total Cell Area.'

B. Determining the 'Total Cell Area' for a xenograft tumor

i. To determine the 'Total Cell Area' in ImageJ, click on the image displaying the immunostaining for the tumor-cell specific marker. Select the "Polygon selections" tool and outline the areas of the image containing tumor-cell specific fluorescence. Determine the area of the field covered by tumor cells by selecting Analyze>Measure. Record the "Area" parameter. This is the 'Total Cell Area' for the field.

10 Next, overlay the polygon-shaped selection onto the thresholded binary image containing the macropinosomes. Do this by clicking on the binary image and

selecting Edit>Selection>Restore Selection. Compute the area of the macropinosomes within the selection by selecting the Analyze>Analyze Particles feature. Keep the default settings for Size (0-Infinity) and Circularity (0.00–1.00). The boxes for “Clear results” and “Summarize” should be selected. Click “OK.” Several parameters are displayed. Record the “Area” for this image. If there are multiple cell area selections, then repeat this for each unique polygonal selection. This is the ‘Total Particle Area’ for the field.

11 To compute the ‘Field Index’ for each field, divide the ‘Total Particle Area’ by the ‘Total Cell Area’ and multiply by 100. Repeat this procedure for all the images originating from the sample and compute the ‘Macropinocytic Index’ of the sample by averaging the ‘Field Index’ column.

TIMING

Steps 1A and 2A, labeling macropinosomes in cultured cells and processing samples: 2–4 days for cell culture + 3 hours labeling macropinosomes and processing samples + 12–18 hours coverglass bonding to slides prior to imaging.

Steps 1B and 2B, labeling macropinosomes in xenograft tumors and processing samples: 3–5 weeks establishing xenograft tumors + 3 hours labeling macropinosomes and tumor dissection + 3 days processing samples

Steps 3–10, capturing images and data analysis: 4–12 hours

TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

The image-based assays described herein allow for the visualization and quantification of macropinosomes *in vitro*, as well as in tumor xenografts *in vivo*. Because our experimental setup relies on signal thresholding, it is imperative to capture random images from one set of experiments at the same exposure times, which can vary from experiment to experiment, in the same imaging session. In our experience, the best method of determining the optimal exposure time is to utilize the automated exposure feature available with all imaging platforms on an experimental sample that visually exhibits a high level of macropinocytosis. This will allow for an accurate assessment of macropinocytosis when setting the threshold value in the ImageJ software.

As an example of the *in vitro* implementation of this protocol, we have assessed the extent of macropinocytosis in two pancreatic cancer cell lines. Sample images from the various steps of quantification are shown in Figure 1. The DAPI channel is utilized to visualize the number of cells and using a 63X objective lens, a typical field may contain 20–40 cells, depending on the confluence of the cells. The phase contrast image allows for the determination of cell area, which is outlined in yellow. For demonstrative purposes, we compared the extent of macropinocytosis in MIA PaCa-2 and BxPC-3 cells, cell lines that we have previously established to have a high and low macropinocytic index, respectively.

Images depicting TMR-dextran fluorescence from representative fields for each cell line are shown. The threshold value for this particular experiment was determined by utilizing the automated threshold feature of ImageJ for one of the MIA PaCa-2 images and this threshold value was then applied to all the images in the experiment. In the threshold image, TMR-positive macropinosomes are identified by the software and colored red. Once the threshold is applied, the result is a binary image, which is then analyzed using the analyze particles feature. The process is repeated for each image, and the total particle area and total cell area is then recorded and tabulated as shown in Table 2. For simplicity, we multiply the “Field Index” values by 100. To successfully employ this protocol for cell monolayers, we recommend analyzing between 200 and 300 cells, which typically consists of 10–20 fields. The macropinocytic index values for cancer cell lines typically range from 0 to 0.5 for low macropinocytic cells and are >1 for highly macropinocytic cells. These values can vary by up to 20% from experiment to experiment, which can be improved by increasing the sample size, and we typically observe an average variation between 5–10% from experiment to experiment. When analyzing several cell lines or different conditions, the macropinocytic index can be displayed graphically using the raw or relative values.

To demonstrate the *in vivo* quantification of macropinocytosis we have evaluated the extent of FITC-dextran uptake in tumor xenografts derived from either MIA PaCa-2 or BxPC-3 cells. In these tumors, the pancreatic epithelial cells are labeled by cytokeratin-8 staining in order to differentiate them from the mouse host cells, such as fibroblasts and immune cells, which may infiltrate the tumor (Figure 2). This cytokeratin-8 staining is utilized to compute the total area occupied by the tumor cells in each field. The thresholding procedure used to identify and quantify the FITC-positive macropinosomes is similar to that performed for the *in vitro* staining. Because each individual intratumoral dextran injection leads to varying degrees of labeling and background fluorescence, the threshold value to be applied to each tumor is unique and applied to all the images that originate from that single tumor. For the xenograft tumors, the total particle area and total tumor area are recorded from the ImageJ analysis and tabulated as shown in Table 3. For simplicity, we multiply the “Field Index” values by 100. To successfully employ this protocol for xenograft tumors, we recommend analyzing at least 300 cells, which typically consists of 5–10 fields/section from 3–5 frozen sections. The macropinocytic index values for xenograft tumors typically range from 0 to 0.5 for low macropinocytic tumors and are >1 for highly macropinocytic tumors. These values can vary by up to 30% from experiment to experiment, which can be improved by increasing the sample size, and we typically observe an average variation between 10–20% from experiment to experiment. When analyzing xenograft tumors derived from several different cancer cell lines or when employing different conditions, the macropinocytic index can be displayed graphically using the raw or relative values.

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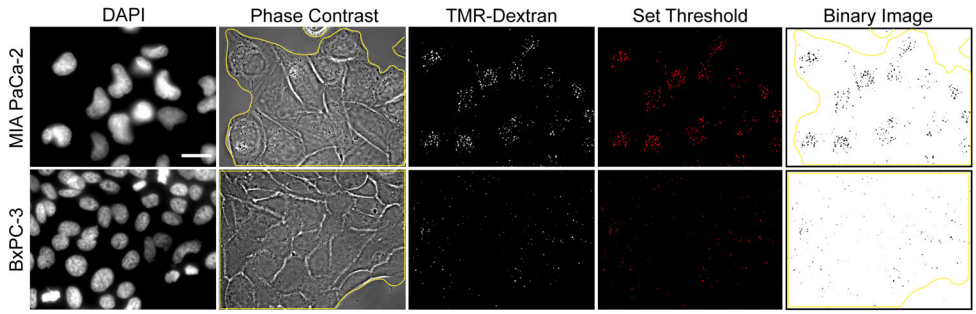

Figure 1. Representative images typically obtained from an *in vitro* macropinocytosis assay

The cells utilized in this particular assay were MIA PaCa-2 (top panel) and BxPC-3 (bottom panel). Nuclei are labeled with DAPI. A phase contrast image is used to determine the area of the field covered by cells. Fluorescent images captured in the rhodamine channel show the TMR-dextran-positive macropinocytic puncta for each field. For display purposes only, the TMR-dextran images were adjusted for brightness and contrast via ImageJ by selecting the B&C auto function. This adjustment does not affect the subsequent thresholding of the images. To determine the threshold value to be applied to all the images, the MIA PaCa-2 image was utilized. Based on this image, a threshold of 133 (on a dark background) was applied to all the images. Prior to accepting the threshold value, ImageJ depicts the detected macropinosomes in red (Set Threshold). Once accepted, the image is converted to a binary image in which the macropinosomes are shown in black on a white background (Binary Image). In the binary image, only particles that fall within the cell-covered area (yellow outline) contribute to the calculation of the ‘Field Index’. Scale bar represents 20 μm.

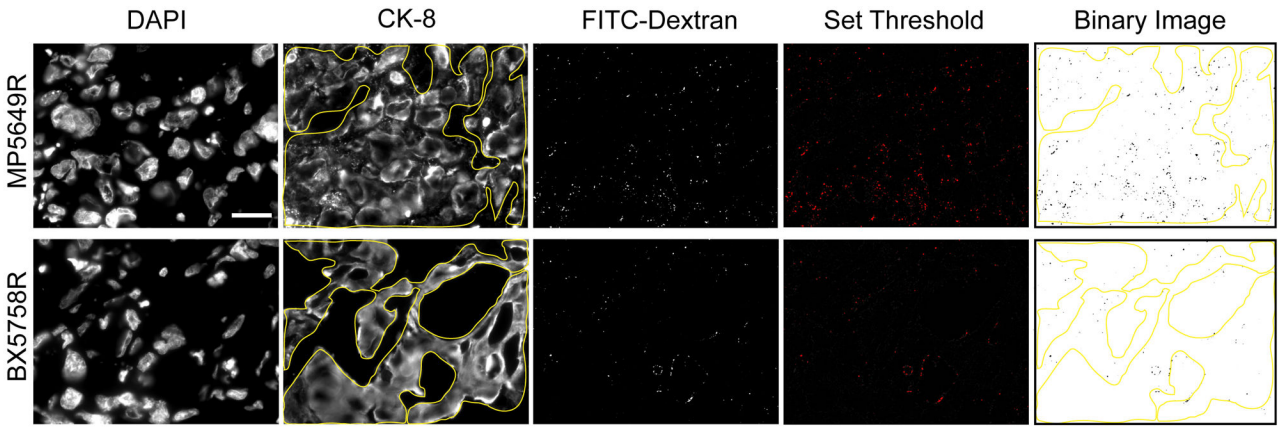

Figure 2. Representative images typically obtained from an *in vivo* macropinocytosis assay

Tumors used in this assay were derived from MIA PaCa-2 (top panel) or BxPC-3 (bottom panel) cells. Nuclei are labeled with DAPI. The pancreatic epithelial cells are labeled with the Troma I antibody, which labels Cytokeratin-8 (CK-8) and areas of the tumor populated by the CK-8-positive epithelial cells are outlined in yellow. Fluorescent images captured in the FITC channel show the FITC-dextran-positive macropinocytic puncta for each field. The CK-8 and FITC-dextran images were automatically adjusted for brightness and contrast via ImageJ. The automated threshold value was determined for each individual tumor (MIA PaCa-2 = 378, BxPC-3 = 464) and verified visually. When setting the threshold, ImageJ depicts the macropinosomes that fall within the threshold setting as red (Set Threshold) and converts the image to a binary image for quantification (Binary Image). In the binary image, only particles that fall within the CK-8-positive area (yellow outline) contribute to the computation of the ‘Field Index’. Appropriate institutional regulatory board permission was obtained for the animal experiments this data was derived from. Scale bar represents 20 μm.

Table 1

Step	Problem	Possible Reason	Solution
1.A.iii	Lower than anticipated cell confluency after initial seeding or after serum starvation.	Cell line does not adhere well to uncoated cover glass or cells die upon serum starvation.	Coat coverglass with extracellular matrix protein solution or polylysine and evaluate cell adherence on coated coverglass. Reduce serum starvation to 1–3 hours, or place cells in reduced serum media instead of withdrawing serum completely.
3	Automated exposure time feature yields low signal, high background preview with long exposure time (>2000 ms).	Poor Signal to Noise Ratio: Dextran uptake levels are low or dextran signal intensity is low.	Manually reduce exposure time to 100–1000 ms. If macropinosomes can be clearly delineated above background use the manually inputted exposure for subsequent imaging. If little to no macropinosomes are observed, then the cell line might have low macropinocytosis levels and should be compared to cell lines known to possess high macropinocytosis levels as described in the experimental design section. If known high macropinocytosis cell line(s) yield low signal intensity macropinosomes then optimization of macropinosome labeling will be necessary (see TROUBLESHOOTING of Step 6)
6	Very few images contain bright, discrete macropinocytic puncta.	Poor Signal to Noise Ratio: Dextran signal intensity is low or background noise is too high.	If high background is observed, ensure complete washing steps (see steps 1.A.v or 2.B.iii) are performed or add additional PBS washes to reduce non-specific background. An additional low pH buffered wash step may diminish surface bound dextran[29]. Alternatively, reduce the concentration of dextran used. Note that lowering the dextran concentration will also reduce dextran signal intensity in macropinosomes. If low dextran signal intensity in macropinosomes is observed even in known high macropinocytosis cell lines the following may improve results: (i) increase concentration of dextran used for labeling macropinosomes (ii) ensure dextran properly reconstituted or make new dextran stock solution (iii) increase time of dextran uptake. Note that fluorescently labeled fixable dextrans from other sources than those listed in materials section might contain less fluorescent dye molecules per dextran, thus yielding less signal intensity.
6	Dextran aggregates are present in images that are greater in size than macropinosomes (>10 μm).	Dextran Stock Solution not properly reconstituted or insufficient washes post macropinosome labeling.	Ensure that dextran stock solution is properly reconstituted. If problem still occurs see washing steps above for problems with high background.

TABLE 2

Sample quantification of macropinocytic index in two pancreatic cancer cell lines

Cell Line	Field #	Total Particle Area	Total Cell Area	Field Index (10^2)	Macropinocytic Index (Average Field Index)
MIA PaCa-2	1	21978	1410078	1.56	
	2	23375	1410677	1.66	
	3	26431	1410053	1.87	
	4	21510	1199075	1.79	
	5	16487	1254033	1.31	
	6	21283	1289662	1.65	
	7	17001	1075334	1.58	
	8	30134	1318339	2.29	
	9	19847	1180696	1.68	
	10	21385	1292160	1.65	**1.71**
BxPC-3	1	3666	1423160	0.26	
	2	4341	1241958	0.35	
	3	4476	1414136	0.32	
	4	6059	1404568	0.43	
	5	6458	1365115	0.47	
	6	5181	1423610	0.36	
	7	6053	1424160	0.43	
	8	5596	1368867	0.41	
	9	5042	1424160	0.35	
	10	5550	1424160	0.39	**0.38**

TABLE 3

Sample quantification of macropinocytic index in two pancreatic tumor xenografts

Tumor ID	Slide #	Field #	Total Particle Area	Total Cell Area	Field Index (× 10^2)	Macropinocytic Index (Average Field Index)
	1	1	20566	1041428	1.97	
		2	13440	1357569	0.99	
		3	30225	1329736	2.27	
	2	1	13343	759256	1.76	
		2	6477	1249723	0.52	
		3	14755	1353573	1.09	
MP5649R	3	1	10017	1085938	0.92	
		2	10898	1349215	0.81	
		3	20071	1419432	1.41	
	4	1	16077	1153850	1.39	
		2	9409	1393527	0.68	
		3	11755	1195798	0.98	
	5	1	8664	1399512	0.62	
		2	7546	1384801	0.54	
		3	6225	1178729	0.53	**1.10**
	1	1	3898	802440	0.49	
		2	5618	892025	0.63	
		3	1063	749640	0.14	
	2	1	1911	661583	0.29	
		2	2253	635709	0.35	
		3	4336	622023	0.70	
BX5758R	3	1	2221	422281	0.53	
		2	2317	608988	0.38	
		3	4314	722012	0.60	
	4	1	2154	466707	0.46	
		2	1992	807089	0.25	
		3	697	722576	0.10	
	5	1	2560	488012	0.52	

Nat Protoc. Author manuscript; available in PMC 2014 July 18.

Tumor ID	Slide #	Field #	Total Particle Area	Total Cell Area	Field Index (× 10^2)	Macropinocytic Index (Average Field Index)
		2	1184	580171	0.20	
		3	882	602896	0.15	**0.39**