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Quantitative assessment of macropinocytosis in mTORC1-hyperactive cells using flow cytometry

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TITLE:

Quantitative Assessment of Macropinocytosis in mTORC1-hyperactive Cells Using Flow Cytometry

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SUMMARY:

This protocol provides experimental tools to evaluate macropinocytic uptake of nutrients (carbohydrate and protein) by mTORC1-hyperactive cells. Detailed steps to quantify the uptake of fluorescently labeled dextran and bovine serum albumin (BSA) are described.

ABSTRACT:

Macropinocytosis is a highly conserved, actin-dependent endocytic process that allows the uptake of extracellular material, including proteins and lipids. In proliferating cells, macropinocytosis can deliver extracellular nutrients to the lysosome, processed into critical macromolecule building blocks. Recent studies have highlighted the dependence of multiple cancers on macropinocytosis, including breast, colorectal and pancreatic cancer. Ras mutations are thought to be the driver events behind macropinocytosis initiation, leading to the activation of cellular anabolic processes via the mTORC1 signaling pathway. Significantly, mTORC1 can also be activated by macropinocytosis independently of Ras. Therefore, macropinocytosis represents a metabolic vulnerability that can be leveraged to target macropinocytic tumors by limiting their access to nutrients therapeutically.

In Tuberous Sclerosis Complex (TSC) and Lymphangioleiomyomatosis (LAM), mTORC1-hyperactivation leads to enhanced macropinocytosis and metabolic reprogramming. Here, we describe a flow cytometry-based protocol to assess macropinocytosis in mammalian cells quantitatively. *TSC2*-deficient MEFs are employed, which exhibit aberrant activation of mTORC1 and have been shown to have increased macropinocytosis compared to *TSC2*-expressing cells. Cells treated with pharmacologic inhibitors of macropinocytosis are incubated with fluorescently labeled, lysine-fixable, 70 kDa dextran, or fluorescently labeled bovine serum albumin (BSA) assayed by flow cytometry. To date, robust image-based techniques have been developed to quantitatively assess macropinocytosis in tumor cells *in vitro* and *in vivo*. This analysis provides a

quantitative assessment of macropinocytosis in multiple experimental conditions and complements existing image-based techniques.

INTRODUCTION:

Macropinocytosis is an endocytic process dedicated to the bulk uptake of extracellular material followed by the formation of macropinosomes, either recycled to the plasma membrane or fusing with lysosomes to degrade the internalized cargo[1,2]. Although cargo uptake is non-selective, macropinocytosis is a multi-step process, tightly regulated by Rab GTPases and membrane phospholipids[3,4]. Notably, cancer cells employ macropinocytosis to internalize extracellular nutrients, including proteins, polysaccharides and lipids. Macropinocytosis in cancer cells is activated by oncogenes downstream of Ras or v-Src as a mechanism to support their proliferation, especially under nutrient stress conditions[5,6]. Therefore, macropinocytosis represents a new therapeutic approach for targeting cancer cells by disrupting nutrient uptake pathways[7,8].

In Tuberous Sclerosis Complex (TSC) and Lymphangioleiomyomatosis (LAM), loss of function mutations in *TSC1* or *TSC2* leads to hyperactivation of the mammalian/mechanistic target of rapamycin complex 1 (mTORC1)[9]. Aberrant mTORC1 activation is known to drive extensive metabolic reprogramming, including glucose and glutamine uptake and utilization, enhanced nucleic acid synthesis, lipid synthesis and autophagy[10,11]. To compensate for these increased anabolic demands, mTORC1-hyperactive cells increase the uptake of exogenous nutrients via macropinocytosis and enhance lysosomal degradation of internalized cargo[12]. In recent work, we identified ritanserin, an inhibitor of diacylglycerol kinase alpha (DGKA) as an agent that selectively inhibits the proliferation of *TSC2*-deficient cells[13]. DGKA is a lipid kinase that metabolizes diacylglycerol to phosphatidic acid (PA)[14]. PA is a crucial second messenger molecule that also plays a vital role in maintaining cell membrane homeostasis. Surprisingly, ritanserin strongly inhibits macropinocytosis by reprogramming phospholipid metabolism in *TSC2*-deficient cells. Therefore, targeting the nutrient uptake pathway of macropinocytosis in *TSC2*-deficient cells may provide novel therapeutic approaches in TSC and LAM.

Quantification of macropinocytic uptake *in vitro* and *in vivo* can provide crucial insights into macropinosome formation regulation and accelerate discovery of molecular mechanisms while identifying novel therapeutic approaches[2,6]. To date, multiple methodologies have been developed that adequately quantify macropinocytic uptake of fluorescent dextran both *in vitro* and *in vivo*[2,15]. Here we describe a flow cytometry-based approach to directly assess the amount of internalized dextran and albumin in mTORC1-hyperactive cells (**Figure 1**). This method can be utilized to analyze multiple experimental conditions in parallel and complements existing image-based approaches.

[**Insert Figure 1 here**]

PROTOCOL:

1. Cell treatment

Day 1

1.1. Seed *TSC2*-deficient and *TSC2*-expressing mouse embryonic fibroblasts (MEFs) in triplicate, in each well of a six-well tissue culture plate using DMEM, supplemented with 10% FBS. Cells should be 60-70% confluent by day 3.

1.1.1. Seed additional control wells for each drug condition that will not be stained with FITC-Dextran or TMR-BSA.

Day 2

1.2. Carefully aspirate media and rinse cells twice with PBS at room temperature.

1.2.1. Treat cells with vehicle (DMSO), 100 µM of phosphatidic acid (PA), 25 µM of EIPA, 10 µM of ritanserin, or a combination of PA and ritanserin. Use DMEM supplemented with 1% FBS.

NOTE: DMSO volume should equal the maximal volume of solvent used in treating conditions. For example, if 10 µL of EIPA is used, the volume of DMSO in the vehicle conditions should also be 10 µL.

Day 3

1.3. Replace media with serum-free DMEM containing the abovementioned drugs and 0.5 mg/mL of FITC-Dextran or 0.5 mg/mL of TMR-BSA 16 h post-treatment. Incubate the cells in a 37 °C/5% CO_2 cell culture incubator for 60 min.

NOTE: To minimize photobleaching of the fluorophores, FITC-Dextran and TMR-BSA tubes should be wrapped in aluminum foil and the experiment should be performed in a cell culture cabinet with the lights turned off.

1.4. Aspirate media and wash twice with ice-cold PBS.

1.4.1. Detach cells using 500 µL of trypsin. Place cells in 37 °C/5% CO_2 cell culture incubator for 2-3 min.

NOTE: Ensure all cells are detached by observing them under a brightfield microscope.

1.4.2. Using a clean pipette tip every time, collect cells in 1.5 mL tubes using 1% FBS supplemented DMEM on ice.

1.4.3. Pellet cells by centrifugation at 425 x *g* for 2 min at 4 °C.

1.4.4. Aspirate the supernatant.

1.5. Resuspend the cell pellet using 50 µL of 2% paraformaldehyde.

CAUTION: Paraformaldehyde is highly toxic and should be handled under an appropriate fume hood. Formaldehyde-containing waste should be disposed of according to institutional guidelines.

1.5.1. Incubate cells at room temperature for 10 min.

1.5.2. Add 1 mL of ice-cold PBS to each tube and resuspend the cell pellet gently. Place the tubes on ice.

1.6. Centrifuge cells at 425 x *g* for 2 min at 4 °C.

1.7. Aspirate the supernatant.

1.7.1. Resuspend cell pellet in 300 µL of ice-cold PBS.

1.7.2. Transfer cells to the appropriate FACS tubes on ice.

1.7.3. Proceed with flow cytometry.

2. Flow cytometry

2.1. Briefly vortex the cells before inserting them into the FACS sample holder.

2.2. Using low-speed flow, gate live cells from each unstained sample (negative control) using appropriate laser power as seen in **Figure 2A.** This step differs between instruments and will need to be optimized for each experiment. Adjust the power for both forward scatter (FSC) and side scatter (SSC) so that live cells are distinct from debris or cell clusters.

2.3. Using the autogate feature, select the live cell populations for each sample avoiding cell debris and cell clusters.

NOTE: All cell events (regardless of gating) are ultimately recorded by the cytometer. Therefore selecting a gate at this step is not critical.

2.4. Record the fluorescence intensity from each sample using the appropriate green or red lasers.

3. **Flow cytometry analysis**

3.1. Gate cells using forward and side scatter parameters. Apply the same gate to all samples.

3.2. Create histograms for fluorescence in each sample as in **Figure 2B**.

3.3. Within each cell gate calculate the mean/median fluorescence intensity for all samples.

3.4. Export data for appropriate statistical analysis.

NOTE: During data analysis, the mean/median fluorescence intensity of each sample should be normalized by subtracting the unstained sample values.

REPRESENTATIVE RESULTS:

Ritanserin inhibits macropinocytosis in *TSC2*-deficient cells

We have previously shown that macropinocytic uptake of nutrients is increased by three-fold in *TSC2*-deficient cells compared to *TSC2*-expressing cells[16]. In TSC and LAM, macropinocytosis is mediated via diacylglycerol kinase alpha (DGKA). The metabolic product of DGKA is phosphatidic acid (PA), a crucial component of cellular membranes. Therefore, we hypothesized that inhibition of DGKA by ritanserin might impact macropinocytic vesicular trafficking in *TSC2*-deficient cells. To determine the role of ritanserin on macropinocytic uptake of exogenous nutrients, uptake assays using FITC-Dextran (0.5 mg/mL, 70 kDa) or TMR-BSA (0.5 mg/mL) were performed. 70 kDa dextran was chosen because this size carbohydrate molecule is exclusively taken up by macropinocytosis, and not by other forms of endocytosis[17]. Ritanserin treatment (10 µM, 16 h) inhibited uptake of dextran by 90% ($p<0.0001$) in *TSC2*-deficient cells and had no impact on *TSC2*-expressing cells (**Figure 2B**). PA supplementation (100 µM, 16 h) restored macropinocytosis, indicating that macropinocytosis in *TSC2*-deficient cells is mediated via DGKA. The established macropinocytosis inhibitor EIPA (25 µM, 16 h) decreased macropinocytosis by ~60% ($p<0.0001$) in *TSC2*-deficient cells. To further characterize the role of ritanserin on macropinocytosis, the macropinocytic uptake of exogenous BSA was quantified. Uptake of fluorescently labeled BSA (TMR-BSA, 0.5 mg/mL) was decreased by ~70% ($p<0.0001$) upon ritanserin treatment (10 µM, 16 h, **Figure 2C**). Similarly, macropinocytic uptake of BSA was rescued by adding back PA (100 µM, 16 h), indicating that ritanserin inhibits macropinocytosis by depleting PA in the cellular membranes of *TSC2*-deficient cells. Although BSA can also be internalized by endocytosis (hydrodynamic radius: BSA=3.3–4.3 nm vs. dextran=6.49 nm[18,19]), EIPA and ritanserin inhibited its macropinocytic uptake. Therefore, BSA can be used as a complementary cargo to assess protein uptake via macropinocytosis in mammalian cells. These results demonstrate the potential of using flow cytometry to identify novel therapies that target macropinocytosis in mammalian cells using exogenous dextran and BSA as cargo.

[**insert Figure 2 here**]

FIGURE AND TABLE LEGENDS:

Figure 1. Workflow for the assessment of macropinocytosis in mammalian cells. Cells are seeded in six-well plates and subsequently treated with compounds of interest. Fluorescent dextran or BSA are added for 60 min, and the uptake is inhibited by washing with ice-cold PBS.

Cells are fixed using paraformaldehyde, and fluorescence intensity is quantified by flow cytometry. Cells are gated, and data are analyzed with the appropriate software.

Figure 2. Ritanserin inhibits macropinocytosis in *TSC2*-deficient cells. (A) Cell gating of *TSC2*-deficient cells stained with FITC-Dextran (left panel). Representative fluorescence of FITC-Dextran from *TSC2*-deficient cells treated with DMSO, ritanserin (10 µM; 1 h), or EIPA (25 µM; 16 h, right panel). **(B)** Macropinocytosis is enhanced (3-fold) in $TSC2^{-/-}$ MEFs compared to $TSC2^{+/+}$ MEFs. Ritanserin (10 µM; 16 h) inhibited the macropinocytic uptake of dextran (0.5 mg/mL, FITC-Dextran) selectively in $TSC2^{-/-}$ MEFs. Phosphatidic acid (PA, 100 µM) restored macropinocytosis of ritanserin-treated $TSC2^{-/-}$ MEFs to levels compared to untreated cells. **(C)** Exogenous protein uptake (0.5 mg/mL, TMR-BSA) was increased in ritanserin (10 µM; 16 h) treated $TSC2^{-/-}$ MEFs compared to $TSC2^{+/+}$ MEFs. PA (100 µM) partially rescued macropinocytosis in $TSC2^{-/-}$ MEFs. As expected, EIPA (25 µM;16 h) inhibited macropinocytic dextran and BSA uptake. Data represented as mean ±SD from three biological replicates. Statistical significance was assessed using two-way ANOVA with Bonferroni correction with ***, $p < 0.001$; ****, $p < 0.0001$. This figure has been adapted from: Cancer Research. 2021 Feb 16; canres.2218.2020. doi: 10.1158/0008-5472.CAN-20-2218.

DISCUSSION:

Here, we describe a quantitative approach to assess macropinocytosis using flow cytometry. This method provides an accurate and rapid measurement of the fluorescently labeled macropinocytic cargo dextran and albumin. Prior studies have carefully characterized the macropinocytic index of cancer cells using confocal microscopy approaches[15,20]. Although these methods accurately quantify the internalization and size spatial distribution of macropinosomes, they require the analysis of multiple images for each experimental condition. In contrast, the technique described here allows for the simultaneous assessment of multiple experimental conditions and time points. Additionally, the flow cytometry approach provides an absolute quantification of internalized dextran, which is advantageous to current microscopic techniques.

Since macropinocytosis is a dynamic process, the duration of incubation with fluorescently labeled dextran or BSA should be optimized for each cell line of interest. In our experience and agreement with previous studies, maximal uptake of dextran and BSA requires 30-60 min of incubation and remains stable for up to 180 min[15,21]. To allow for an accurate assessment of macropinocytosis, it is recommended that initial experiments should include several time points. Additionally, appropriate positive and negative controls should be included in each experiment. EIPA, the Na^+/H^+ channel inhibitor, has been traditionally used to inhibit macropinocytosis[22]. Here we show that ritanserin, a DGKA inhibitor, blocks macropinocytosis in *TSC2*-deficient cells and can therefore be used as a tool compound in macropinocytosis studies. It should also be noted that FITC-Dextran can only be used on fixed cells to avoid quenching of the fluorophore in acidic organelles[23,24]. Finally, the size of fluorescent dextran (70 kDa) is an essential factor, especially since smaller size dextrans can be internalized via clathrin-mediated endocytosis[17].

Consideration for the cell size is critical when measuring fluorescence emitted by dextran or BSA, as larger cells will most likely internalize increased amounts of extracellular cargo. To this extent,

drug treatments might also affect cell size leading to misinterpretation of results. To counteract this, the fluorescence of unstained samples corresponding to each drug treatment should be measured during each experiment. These values can then be subtracted from the mean/median fluorescence intensity values of stained samples. It should be noted that the method described here is low throughput and will need to be modified if a high throughput format is required. Recently described methods for the high throughput measurement of macropinocytosis could potentially be adapted to be used in mammalian cells and ultimately allow the screening of compound libraries[25].

In summary, this method provides a highly reproducible assessment of macropinocytosis that can be readily applied in multiple research areas, including pancreatic, breast and lung cancer.

ACKNOWLEDGMENTS:

The LAM Foundation Career Development Award. **Figure 1** was created with BioRender.com. Critical reading was performed by Hilaire C. Lam.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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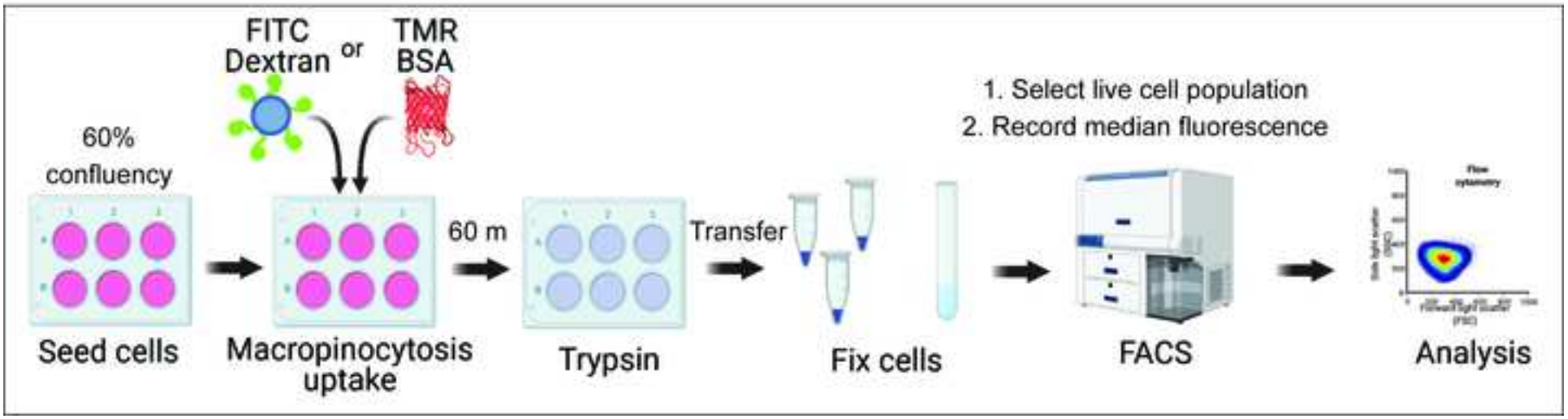

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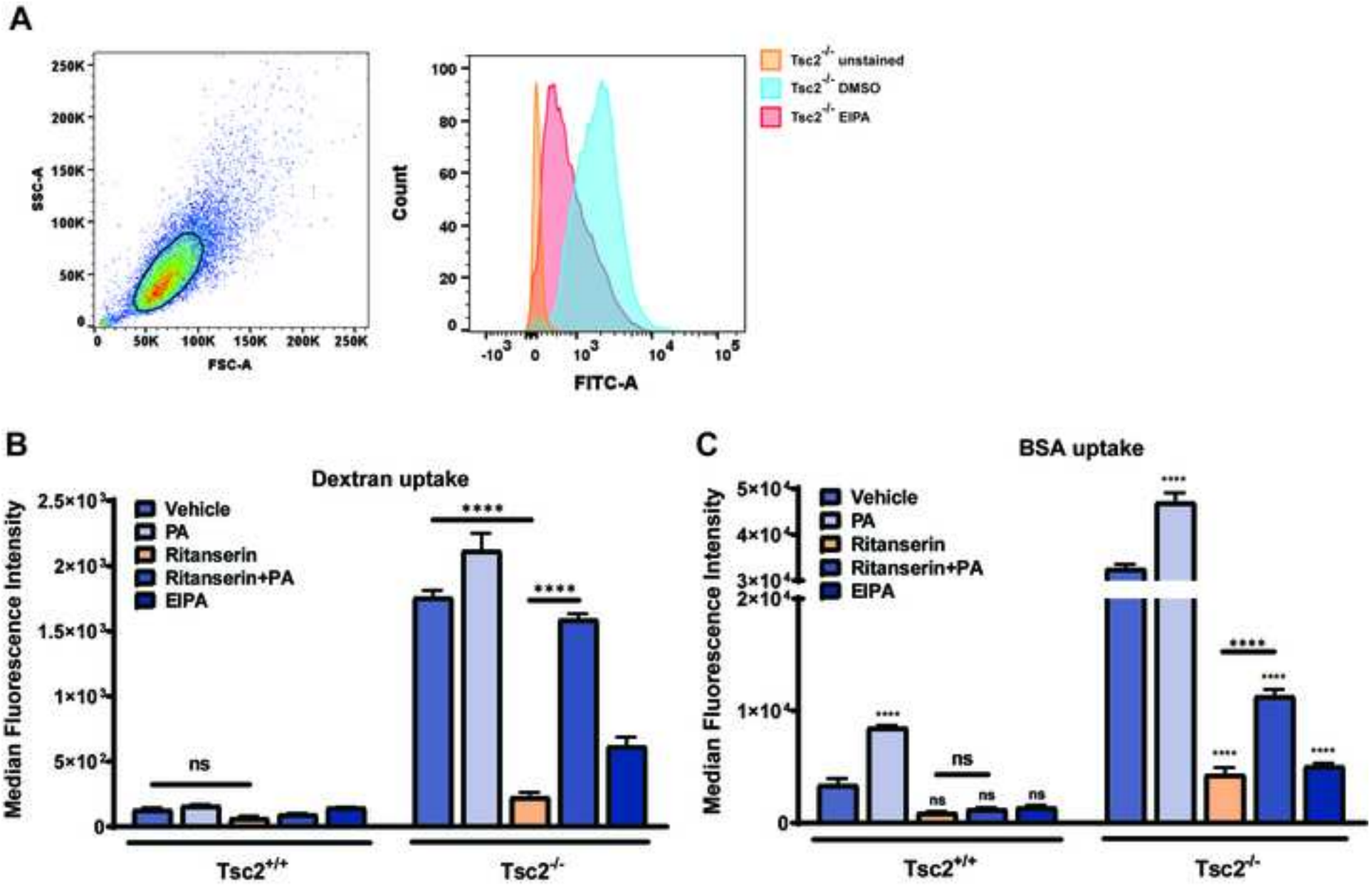

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May 18, 2021

Amit Krishnan, Ph.D.
Review Editor
JoVE

RE: JoVE62793

Dear Dr. Krishan,

We are grateful to the Reviewers for their additional comments and suggestions, which we believe have strengthened our manuscript. In the revised manuscript we have addressed all editorial and reviewer comments. Please note that the essential sections for the video are highlighted in gray. Our point-by-point responses are detailed below.

Reviewer #1:

1. *Why did the authors use BSA? Is there evidence that BSA is not taken up by other modes of endocytosis? What is the hydrodynamic radius? Does it add anything that we do not get from 70 kDa dextran. A better justification for the use of BSA is warranted.*

Response: We agree that further clarification is required on the use of BSA as macropinocytic cargo. The hydrodynamic radius of albumin is ~3.3 nm vs 70kDa dextran which is 6.49nm. Therefore, albumin can also be internalized via endocytosis. Since albumin uptake is strongly inhibited upon inhibition of macropinocytosis with EIPA or ritanserin, it can be used as a complementary cargo to assess protein uptake. This is now clearly stated in the discussion (lines 183-186): "*Although BSA can also be internalized by endocytosis (hydrodynamic radius: BSA=3.3–4.3 nm vs dextran=6.49 nm[18,19]) EIPA and ritanserin inhibited its macropinocytic uptake. Therefore, BSA can be used as a complementary cargo to assess protein uptake via macropinocytosis in mammalian cells.*"

2. *It would be useful to have a discussion regarding the ability to measure absolute uptake of dextran by flow. This seems advantageous over microscopy where absolute uptake is more difficult to ascertain. It is worth describing the disadvantages of flow, such as loss of qualitative features, i.e. macropinosome size and number per cell.*

Response: We thank the reviewer for highlighting this important advantage of using flow cytometry compared to traditional microscopy-based techniques. We have included a statement in the discussion to reflect this (lines 222-224): *"Additionally, our flow cytometry approach provides an absolute quantification of internalized dextran, which is advantageous to current microscopic approaches."*

3. *The authors should mention that FITC-dextran can only be used in fixed cells. In live cells FITC will be quenched in acidic macropinosomes.*

Response: We have added the following statement in the discussion (lines 235-236): *"It should also be noted that FITC-Dextran can only be used on fixed cells to avoid quenching of the fluorophore in acidic organelles*[23,24]*."*

Reviewer #2:

1. *If appropriate, it would be good to include short "notes" for some of the steps if there is a particular tip or modification that might be useful.*

Response: We thank the reviewer for this helpful suggestion. We have added useful modifications in the protocol. Please see added notes in the protocol on lines: 98, 104, 111, and 146.

Reviewer #3:

Minor Concerns:

1. *Line 47: please spell "recycled" correctly.*
2. *Line 57: please write also "TSC2" in italics, here and throughout the text.*

Response: These have been corrected.

3. *Lines 113-115: please provide more details.*

Response: This has been corrected. Please see new text in lines 142-150.

4. *Lines 130-132: "In TSC and LAM, macropinocytosis is mediated via diacylglycerol kinase alpha (DGKA) its metabolic product phosphatidic acid (PA), a crucial component of cellular membranes." I cannot read this sentence very well: is there anything missing?*

Response: We thank the reviewer for identifying this omission. The discussion on line 166 now reads: "*In TSC and LAM, macropinocytosis is mediated via diacylglycerol kinase alpha (DGKA). The metabolic product of DGKA is phosphatidic acid (PA), a crucial component of cellular membranes."*

Thank you for your consideration of our work.

Sincerely,

Charilaos Filippakis, Ph.D.
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From: Echikson, Eileen <eileen.echikson@aacr.org>
Sent: Tuesday, May 18, 2021 12:25:53 PM
To: Filippakis, Charilaos,Ph.D. <CFILIPPAKIS@BWH.HARVARD.EDU>
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Thank you,
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