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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,

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