REVIEWER #1

We thank all the reviewers for their insightful comments and helpful suggestions. Based on your recommendations, we have significantly expanded and improved the manuscript. We are extremely proud of the revised version of our work. In the revised manuscript, the new or modified sections of the text are in purple. Below, please find our responses to how each of your queries were addressed.

Other labs may not equip with the exact instruments, such as Cytation 5, as described in the protocol, for example. It would be good if authors could make some additional comments on adaptation of using similar instruments.

In light of the reviewer's comments, we have added general guidelines in sections 4 and 5 to be used for the adaptation of our protocol to other multi-mode plate readers.

REVIEWER #2

We thank all the reviewers for their insightful comments and helpful suggestions. Based on your recommendations, we have significantly expanded and improved the manuscript. We are extremely proud of the revised version of our work. In the revised manuscript, the new or modified sections of the text are in purple. Below, please find our responses to how each of your queries were addressed.

It would be good to include a discussion on the possibility of assessing cargo other than dextran. Could this assay be used to quantify macropinocytic uptake of fluorescent proteins (albumin) or lipids?

In light of the reviewer's comments, we have modified the discussion to include the mention of other cargo and how our protocol may be used to assess their internalization.

How do the authors normalize for differences in cell size? What happens when a drug treatment alters the size of the cells being assessed?

For determining the macropinocytic index for a drug treatment where the size of the cells is not affected we do not take cell size into account. However, when comparing different cell lines where cell sizes differ or evaluating a drug treatment that might alter the size of the cells, this parameter would need to be accounted for. We have outlined in the discussion that in these cases, a slight modification to the protocol can be made where the macropinocytic index is computed using total cell area in the field using a cell mask dye, phase contrast or bright field images instead of the DAPI-determined cell number.

REVIEWER #3

We thank all the reviewers for their insightful comments and helpful suggestions. Based on your recommendations, we have significantly expanded and improved the manuscript. We are extremely proud of the revised version of our work. In the revised manuscript, the new or modified sections of the text are in purple. Below, please find our responses to how each of your queries were addressed.

Line 79 : cancer cells does not make sense as cell lines are 'cancerous'. The authors must distinguish this better from primary cells

In light of the reviewer's comment, we have rephrased the sentence to indicate that the protocol can be used for adherent cells in general.

Line 80 : Since this is a protocol largely on assay development aimed at automated high content analysis, the authors must mention 384 well plates. It will be good if they can adapt this protocol to 384 well plates, but they must definitely mention the advantages of 384 wp, and the additional considerations 384 wp handling entail over 96 wp

The reviewer makes an excellent point that referring to this protocol as "high-throughput" is not appropriate. We have completely revamped the protocol to indicate that our method is for 'automated image acquisition and analysis' to distinguish it from a high-content analysis protocol. We now mention in the discussion the possibility to adapt the protocol to high-content analysis, including 384-well and 1536-well analyses, and we have included suggestions of actions that can be substituted by robotics.

Line 97 : This being a detailed protocol, can the authors also mention the possibility of conjugating dextran to fluorophores. This generally gives researchers high flexibility in terms of fluorophores. They can mention here the rationale (or citation) for why 20 mg/mL is a good stock concentration, and also what is the expected final conc (i.e, if this stock will be 100x, 1000x etc)

Although possible, we prefer not to mention the possibility of conjugating dextran since we have no experience with this process and therefore may include instructions that can negatively affect the reproducibility of the assay.

Line 106 : Details of coverslips such as size (that can fit into 24 wp), and coverslip numbers will be important to have here

A more detailed mention of the coverslips characteristics has now been included.

Line 127 : The time will also depend on the cell types, some mention on cells that adhere strongly compared to other cells will be good

Given the variety of cell lines and variability between cell lines, we have generalized the mention of trypsin incubation time and now recommend that this should be empirically determined.

Line 135 : There are guidelines on the number of cells to be seeded to reach near confluency the next day. While this number does change with cell type, the guidelines must be referred to here, and typical seeding density for common cell lines such as HeLa, 3T3, HEK can be mentioned here for both 24 wp and 96 wp.

Given the variety of cell lines and variability between cell lines, we have generalized the mention of cell number as these can vary greatly and now recommend that this should be empirically determined.

136 : Why is 3-4 days after seeding preferable?

We prefer to allow cells to equilibrate to their new surroundings after seeding. However, the times required for each cell line may vary and thus we have removed the mention of time.

162 : The last sentence is not clear and must be removed

The sentence has been removed.

164 : The rationale for doing this wash 24 hours before macropinosome labeling is not clear

Washing with PBS before changing to serum-free media can help to remove residual serum which may affect the macropinocytic capacity of the cells or the effects of administered compounds (**Figure 1**). We now make mention of these effects of serum.

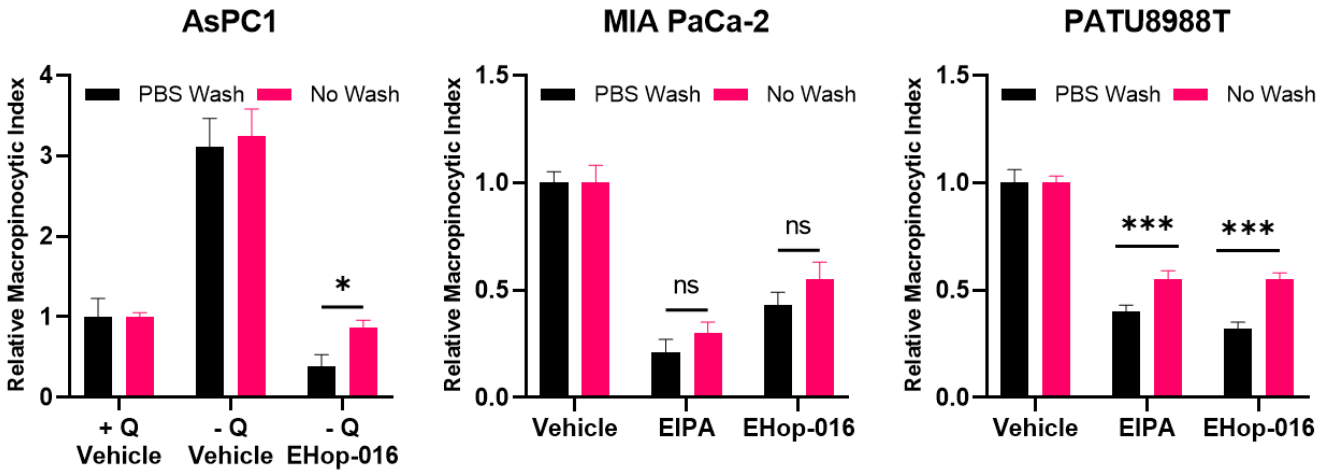


Figure 1: Omitting the wash step before addition of the serum-free media may affect the macropinocytic index. AsPC1, MIA PaCa-2, and PATU8988T cells were cultured on a 96-well microplate and the day before the dextran uptake assay subjected or not to 2 PBS washes, before the addition of serum free media. Cells were treated for 24 hours with 10 µM EHop-016 or for 30 minutes with 75 µM EIPA. AsPC1 cells were deprived of glutamine (-Q) or not (+Q). Bar graphs show the average and S.D. of a single experiment with 4 replicates. Statistical significance was determined by two-way ANOVA. ns, not significant, *, $P < 0.05$, ***, $P < 0.001$.

171 : Use of serum-free media for 24 hours is not good for the cells, and will influence other processes such as autophagy. These caveats must be mentioned

A note has been added to mention these caveats.

178 : Why collect and save 190 ul conditioned media, this will be cumbersome to do for the entire 24 well plate. Why not just aspirate 310 ul media to leave 190 ul behind. Alternatively, why not add the labelled dextran to fresh media to reach the desired final concentration and add this to the cells after aspirating all the media ?

What advantage, if any, is provided by the conditioned media?

We thank the reviewer for pointing out that this step may be cumbersome and based on the recommendation have simplified the protocol, which for the tested cell lines did not change the results (**Figure 2**). We generally recommend reusing conditioned media since cells may secrete factors that can affect their macropinocytic capacity (See Figure 5A of the protocol). Moreover, using fresh media may wash out added treatments. To point this out we have added a note to the protocol.

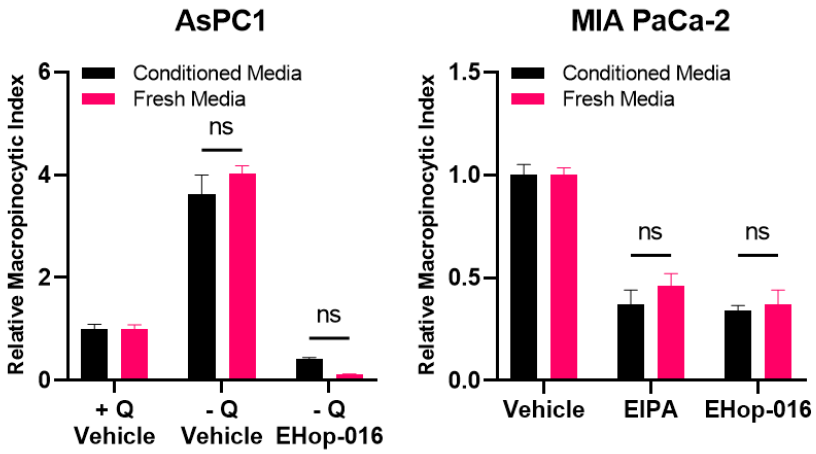


Figure 2: Using fresh media for the addition of dextran does not affect the results compared to reusing conditioned media. AsPC1 and MIA PaCa-2 were cultured on a 96-well microplate and cells were treated for 24 hours with 10 µM EHop-016 or for 30 minutes with 75 µM EIPA. AsPC1 cells were deprived of glutamine (-Q) or not (+Q). Bar graphs show the average and S.D. of a single experiment with 4 replicates. Statistical significance was determined by two-way ANOVA. ns, not significant.

193 : These are fairly standard protocols that can also be performed without the silicone isolators or DAOK fluorescence mounting media. These alternatives must be mentioned, and any specific advantage these specified reagents have can be included.

To prepare the coverslips for automated imaging, their localization should be reproducible. We have determined that silicone isolators are a great way to achieve this since it avoids drifting of coverslips during drying of the mounting media. The mounting media that we recommend in the protocol is a hardening mounting media that fixes the coverslips in their position. Nevertheless, we agree that there is other mounting media available that can be used for this purpose and have therefore removed the DAKO name.

208 : Once again, saving conditioned media to put it back without a sound rationale is cumbersome, and needs an additional transition plate. This is not necessary. Instead, either certain volume can be aspirated out leaving behind the required volume in the well, or fresh media containing dextran can be added to the wells after aspirating the entire volume

We thank the reviewer for pointing out that this step may be cumbersome and based on the recommendation have simplified the protocol, which for the tested cell lines did not change the results (**Figure 2**). We generally recommend reusing conditioned media since cells may secrete factors that can affect their macropinocytic capacity (See Figure 5A of the protocol). Moreover, using fresh media may wash out added treatments. To point this out we have added a note to the protocol.

219 : This is unclear...in general adding smaller volumes into 96 well plates could introduce errors and is a source of well to well variability. This point should be mentioned.

Based on the reviewer's recommendation, we have adjusted the protocol to add the dextran together with fresh media.

225 : This step could lead to errors...manually flicking plate to remove media is not advisable on live cells. The method of rinsing as mentioned here is also not standard practice for high content screen, and will render the assay not scalable.

The possibility of using automated plate washer must be mentioned here. At the level of 1 or 2 numbers of 96 well plates, efficient washing can be achieved using a multi-channel pipette, particularly an automated one. This must be mentioned.

The method for washing, as mentioned here, is not acceptable and could result in uncontrollable variations both within and across plates.

Rinsing plates by submersion is not an uncommon procedure and has already been recommended for macropinocytosis quantification assays (Williams and Kay, *JoVE* 2018). To address the concerns of the reviewer we have extensively tested whether the proposed rinsing method affects the attachment of the cells. Similar to using a multi-channel pipette for washing, the proposed rinsing method by submersion causes minimal detachment of cells (**Figure 3A,B**). However, to minimize the number of steps, we have reduced the number of washes after dextran addition to 2 washes and have made mention of the possibility of using a multi-channel pipette for washing when required.

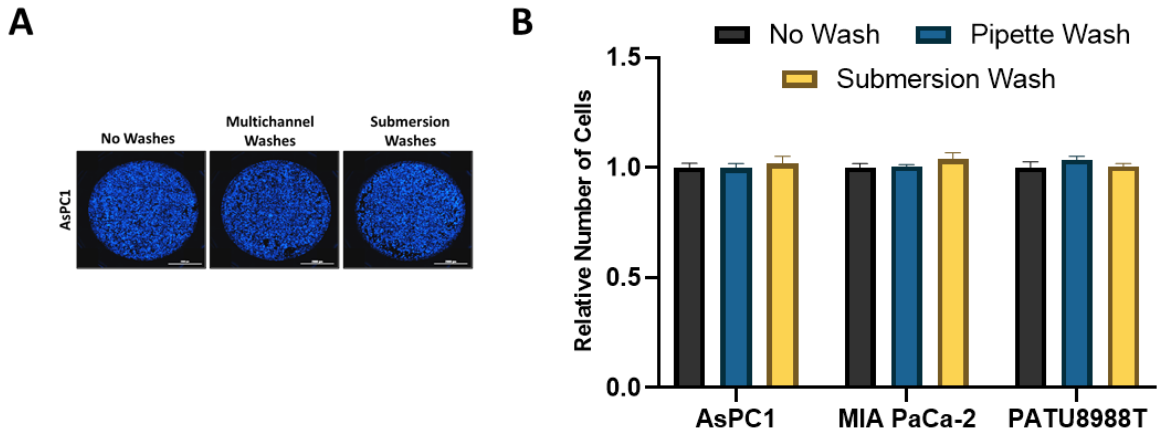


Figure 3: Washing the cells by submersion does not change the number of cells in the area of image acquisition. AsPC1 cells were cultured on a 96-well microplate and subjected or not to washes by using a multi-channel pipette or by submersion. Thereafter, nuclei were stained with DAPI. Shows whole well images of the different wash conditions. (**B**) AsPC1, MIA PaCa-2 and PATU8988T cells on 96-well plates were subjected or not to multichannel pipette or submersion washes after which the relative number of cells in the area of image acquisition for determining the macropinocytic index were determined by DAPI staining. Bar graphs show the average and S.D. of a single experiment with 4 replicates.

242 : What is the advantage of adding Hoechst in 96 well plate and DAPI in 24 well plate?

Why not stain the 96 well plate with dapi after fixation? The use of different dyes for the same assay in different formats can be confusing.

We thank the reviewer for pointing out this inconsistency and have updated the protocol to use DAPI for both formats.

258 : Why should the imaging be done with cytation? Does this offer advantages over other readers? Can the protocol be made general, so that users who do not have cytation but a different imaging reader can also use?

In light of the reviewer's comment and the possible limited availability of this specific instrument to all readers, we have added general guidelines in sections 4 and 5 to be used for the adaptation of our protocol to other multi-mode plate readers.

350 : The imaging conditions are instrument specific. Basic QC criteria to be assessed prior to high content imaging should be mentioned. For example, equal cell density in all fields, histograms of fluorsence intensities in the channels of interest, flat field corrections, shift between channels (particularly if the reader has multiple cameras) etc

In light of the reviewers' comments, we have determined that labeling our protocol as "high-throughput" was not appropriate. We have now modified the scope of the protocol to serve as a guide for performing the assay with standard laboratory equipment.

370 : What does substantial mean? What are the negative controls in the assay? How is the assay window determined?

We thank the reviewer for pointing out the fluidity of this word and have therefore rephrased the sentence to state “select an image with a high level of macropinocytosis”.

425 : The need for doing these assays in 96 well plates must be mentioned. Usually this is for a screen. So it is important to assess well to well variations, identify patterns across rows or columns, or other plate effects.

In short, the robustness of assay must be thoroughly evaluated. The metrics for such an assessment must be provided, and appropriate references cited

It is true that 96-well assay format is usually employed as a pre-step to developing a HT-compatible assay for 384-well or higher format for screening. However, in our experience, 96-well format can be extremely useful outside of screening. We use this 96-well assay to assess a wide-range of concentrations for inhibitors, growth factors, or metabolites with many replicates per condition being tested. Outside of screening, this assay has vastly improved our data output. In light of the reviewer’s comments, we have now modified the scope of the protocol to serve as a guide for performing the assay with standard laboratory equipment.

443 : Representative images of the inhibitor treatments mentioned in Fig 6 B,C should be shown, this will help readers appreciate the phenotypes produced by inhibiting the pathway. Also useful will be to show a result from analysis, for example a distribution of the number of macropinosomes per cell from a large number of cells, that will be typical for such analyses.

We thank the reviewer for this recommendation, and, in response, we have modified the figure to include representative images of the inhibitor treatments.

REVIEWER #4

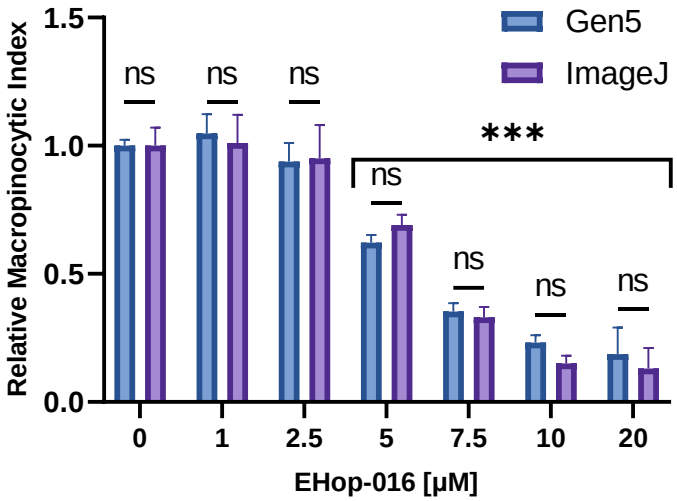
We thank all the reviewers for their insightful comments and helpful suggestions. Based on your recommendations, we have significantly expanded and improved the manuscript. We are extremely proud of the revised version of our work. In the revised manuscript, the new or modified sections of the text are in purple. Below, please find our responses to how each of your queries were addressed.

First, the entire manuscript is based on using the "Cytation 5 Cell ImagingMulti-Mode Reader". There are many different machines that enable researchers to perform quantitative high-throughput analysis of micropinocytosis, and the presented machine is only one (and perhaps not even the best) solution. The protocol is entirely focused on this particular machine (which is to some extent understandable), however it does not state at all how the experimental and analytical settings can be put in place or adapted for other machines. This is not really helpful for scientists as there is most likely only a minority of researchers performing quantitative micropinocytosis analysis who use the machine of the manuscript. I felt that the document is a good reference that could be used by Biotek to promote their machine. A possible rescue of the manuscript would be to state clearly which of the parameters need to be taken into account for the analysis on different systems, and then the used plate reader is presented as one option.

In light of the reviewer's comments, we have added general guidelines in sections 4 and 5 to be used for the adaptation of our protocol to other multi-mode plate readers.

To address this major issue, the authors should do two things: they should mention the possibility that in-built software solutions (depending on the application- sometimes they work perfectly fine and I appreciate the efforts of the companies to offer solutions for complete experimental-analytical pipelines) may not be very reliable, and secondly, they should compare their data with the data obtained through a fully transparent analytical pipeline.

We have improved section 5 to make mention of the possible drawbacks of using built-in analysis applications, make mention of open-source alternatives for image analysis, and provide the recommendations to validate the built-in analysis application. In addition, we provide evidence in Figure 6A (and see below) to show that the Gen5 software produces similar results as ImageJ.



Gen5 software and ImageJ image analysis produces nearly identical results. The effects of EHop-016 administration on dextran uptake in PATU8988T cells was assessed by analyzing images with Gen5 and ImageJ software, as described in the current protocol and in Galenkamp et al. *Methods in Molecular Biology* 2019, respectively. Comparison of both methods does not show significant differences as indicated by ns. Bar graphs show the average and S.D. of a single experiment with 4 replicates. Statistical significance was determined by two-way ANOVA, compared to untreated condition. ***, $P < 0.001$.