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TITLE:

Automated Imaging and Analysis for the Quantification of Fluorescently Labeled Macropinosomes

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SUMMARY:

Automated assays using multi-well microplates are advantageous approaches for identifying pathway regulators by allowing the assessment of a multitude of conditions in a single experiment. Here, we have adapted the well-established macropinosome imaging and quantification protocol to a 96-well microplate format and provide a comprehensive outline for automation using a multi-mode plate reader.

ABSTRACT:

Macropinocytosis is a non-specific fluid-phase uptake pathway that allows cells to internalize large extracellular cargo, such as proteins, pathogens, and cell debris, through bulk endocytosis. This pathway plays an essential role in a variety of cellular processes, including the regulation of immune responses and cancer cell metabolism. Given this importance in biological function, examining cell culture conditions can provide valuable information by identifying regulators of this pathway and optimizing conditions to be employed in the discovery of novel therapeutic approaches. The study describes an automated imaging and analysis technique using standard laboratory equipment and a cell imaging multi-mode plate reader for the rapid quantification of the macropinocytic index in adherent cells. The automated method is based on the uptake of high molecular weight fluorescent dextran and can be applied to 96-well microplates to facilitate assessments of multiple conditions in one experiment or fixed samples mounted onto glass coverslips. This approach is aimed at maximizing reproducibility and reducing experimental variation while being both time-saving and cost-effective.

INTRODUCTION:

The non-specific endocytic pathway of macropinocytosis allows cells to internalize a variety of

extracellular components, including nutrients, proteins, antigens, and pathogens, through bulk uptake of extracellular fluid and its constituents[1]. Though important for the biology of numerous cell types, increasingly, the macropinocytosis pathway is described to play an essential role in tumor biology, where, through macropinocytic uptake, tumor cells are able to survive and proliferate in the presence of a nutrient-depleted microenvironment[2,3]. The uptake of extracellular macromolecules, including albumin and extracellular matrix, and necrotic cell debris, provides an alternative nutrient source for biomass production by creating amino acids, sugars, lipids and nucleotides through macropinosome and lysosome fusion-mediated cargo catabolism[4–8].

The induction and regulation of macropinocytosis are complex and can vary depending on cellular context. Thus far, several inducers of macropinocytosis have been identified and include ligands, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), galectin-3, and Wnt3A[9–13]. In addition, culturing conditions that mimic the tumor microenvironment can trigger activation of the pathway. Pancreatic ductal adenocarcinoma (PDAC) tumors are nutrient-deprived, especially for the amino acid glutamine, which causes both cancer cells and cancer-associated fibroblasts (CAFs) to rely on macropinocytosis for survival[7,13–15]. Moreover, tumor stresses, such as hypoxia and oxidative stress, can activate this scavenging pathway[16]. In addition to the numerous extrinsic influencers that can induce macropinocytosis, a variety of intracellular pathways control macropinosome formation. Oncogenic Ras-mediated transformation is sufficient to initiate the macropinocytic machinery, and multiple cancer types exhibit oncogenic Ras-driven constitutive macropinocytosis[4,5,9,17]. Alternatively, wild-type Ras activation and Ras-independent pathways have been identified to activate macropinocytosis in cancer cells and CAFs[10,11,15,18]. The use of various *in vitro* models in combination with inhibitor treatments has resulted in the identification of several macropinocytosis modulators, which include sodium-hydrogen exchangers, the small GTPase Rac1, phosphoinositide 3-kinase (PI3K), p21-activated kinase (Pak), and AMP-activated protein kinase (AMPK)[4,13,15]. However, given the multitude of described factors and conditions that regulate macropinocytosis, it is conceivable that many more modulators and stimuli remain undiscovered. The identification of novel modulators and stimuli can be facilitated by automated assessment of a multitude of conditions in a single experiment. This methodology can shed light on the factors involved in macropinosome formation and may allow for the discovery of novel small molecules or biologics that target this pathway.

Here, we have adapted our previously established protocol for determining the extent of macropinocytosis in cancer cells *in vitro* to a 96-well microplate format and automated imaging and quantification[19,20]. This protocol is based on fluorescent microscopy, which has become a standard in the field to determine macropinocytosis *in vitro* and *in vivo*[4–7,9–13,15–22]. Macropinosomes can be distinguished from other endocytic pathways through their ability to internalize large macromolecules, such as high-molecular-weight dextran (i.e., 70 kDa)[2–4,20–23]. Thus, macropinosomes can be defined through uptake of extracellularly administered fluorophore-labeled 70 kDa dextran. As a result, macropinocytic vesicles manifest as intracellular clusters of fluorescent puncta with sizes ranging from 0.2–5 µm. These puncta can be microscopically imaged and subsequently quantified to determine the extent of

macropinocytosis in the cell - 'the macropinocytic index'.

In this protocol, the essential steps to visualize macropinosomes in adherent cells *in vitro* on a 96-well microplate and coverslips using standard laboratory equipment are described **(Figure 1)**. In addition, the directions to automate the image acquisition and quantification of the macropinocytic index using a cell imaging multi-mode plate reader are provided. This automation reduces time, cost, and effort compared to our previously described protocols[19,20]. In addition, it avoids unintentionally biased imaging acquisition and analysis and thereby enhances reproducibility and reliability. This method can easily be adapted to different cell types or plate readers or be utilized to determine alternative macropinosome features, such as size, number, and location. The herein described method is especially suitable for the screening of cell culture conditions that induce macropinocytosis, the identification of novel modulators, or optimization of drug concentrations of known inhibitors.

[Place **Figure 1** here]

PROTOCOL:

1. Preparation of materials

1.1. Dissolve 70 kDa dextran labeled with FITC or tetramethylrhodamine (TMR) in PBS to obtain a 20 mg/mL solution. Store the aliquots at -20 °C.

1.2. Dissolve Hoechst 33342 in DMSO to obtain a 10 mg/mL solution. Store the aliquots at -20 °C.

1.3. Dissolve DAPI in ddH_2O to obtain a 1 mg/mL solution. Store the aliquots at -20 °C.

CAUTION: DAPI is a potential carcinogen and should be handled with care.

1.4. On the day of fixation, prepare fresh 3.7% ACS grade formaldehyde in PBS.

CAUTION: Formaldehyde is a fixative, known carcinogen and is toxic if inhaled. Make the solution in a chemical fume hood to avoid inhalation and handle with care.

1.5. Prepare acid-washed coverslips.

1.5.1. Use a 500 mL beaker and water bath to heat 28 g of borosilicate glass coverslips with a 12 mm diameter and #1.5 thickness for 24 h at 56 °C in 100 mL of 1 M HCl. Seal the beaker with plastic wrap to avoid extensive evaporation.

CAUTION: HCl is a strong acid that is highly corrosive and should thus be handled with care and used in a chemical fume hood to avoid inhalation.

1.5.2. Wash the coverslips with distilled water. Repeat the wash 4 times. Then wash the coverslips with 95% ethanol. Repeat the wash 4 times.

1.5.3. Store the acid-washed coverslips in a cell culture dish at room temperature for future use by maintaining sterility through submersion in 95% ethanol. Seal the dish with parafilm to avoid extensive evaporation.

2. Preparation of cells

2.1. Using a confluent 10 cm tissue culture plate with the adherent cells of interest, aspirate the media and rinse the cells with 5 mL of DPBS, prewarmed at 37 °C.

2.2. Detach the cells from the plate by adding 1.5 mL of prewarmed 0.25% trypsin and incubating at 37 °C.

NOTE: The trypsin incubation time required to detach the cells of interest should be empirically determined and can be confirmed by observing detachment under a conventional light microscope.

2.3. Collect the cells in a 15 mL centrifuge tube and add 4.5 mL of complete media to quench the trypsin.

2.4. Pellet the cells by centrifugation for 3 min at 200 x *g* and aspirate the supernatant.

2.5. Resuspend the cell pellet in an adequate volume of prewarmed complete media to obtain a single cell suspension for seeding.

2.6. Proceed to seed the cells on a 24-well plate with coverslips or a 96-well microplate format (**Figure 1**).

NOTE: The number of cells to be seeded should be empirically determined for each cell line as proliferation rates and size vary between cell lines. This protocol has been optimized for adherent cancer cells with 80% cell confluency on the day of macropinosome labeling. Cell confluency may affect macropinocytic capacity, and this should also be determined empirically.

2.6.1. 24-well plate with coverslip format

2.6.1.1. Add coverslips to a 24-well tissue culture plate, use forceps to grip a single coverslip from the ethanol bath. Tap the coverslip to the inside wall of the plate to remove excess ethanol and place the coverslip flat on the bottom of a well.

2.6.1.2. Let the ethanol evaporate and wash the coverslip 2 times with DPBS.

2.6.1.3. Seed the cells on top of the coverslip by adding 500 µL of the cell suspension to

each well. Place the cells in a 37 °C cell incubator with 5% CO_2 until cell confluency reaches 60%–80% on the day before macropinosome labeling.

2.6.1.4. The day before macropinosome labeling, aspirate the media from the wells and add 500 µL of prewarmed serum-free media to each well and place the cells in a 37 °C cell incubator with 5% CO_2 for 16–24 h.

NOTE: As residual serum can affect the macropinocytic capacity of cells, as well as inhibitor activity, removal of serum can be improved by gently rinsing the cells 1 or 2 times with 500 µL of prewarmed DPBS.

2.6.2. 96-well microplate format

2.6.2.1. Transfer the cell suspension to a 25 mL reagent reservoir. Using a multichannel pipette (8 or 12 channels), seed 100 µL of the cell suspension to each well of a black 96-well high-content screening microplate with optically-clear cyclic olefin or glass-bottom.

2.6.2.2. Place the cells in a 37 °C cell incubator with 5% CO_2 until cell confluency reaches 60%–80% on the day before macropinosome labeling.

2.6.2.3. The day before macropinosome labeling, remove and discard the media from each well using a multichannel pipette (8 or 12 channels) or a multichannel aspiration adapter for standard tips attached to a vacuum pump.

2.6.2.4. Using a reagent reservoir and multichannel pipette (8 or 12 channels), gently add 100 µL of prewarmed serum-free media to each well. Place the cells in a 37 °C cell incubator with 5% CO_2 for 16–24 h.

NOTE: As residual serum can affect the macropinocytic capacity of cells, as well as inhibitor activity, removal of serum can be improved by gently rinsing the cells 1 or 2 times with 100 µL prewarmed DPBS. Depending on the conditions to be studied, serum-free media is recommended to reduce the effects of growth factors that can induce macropinocytosis and which are normally present in serum. However, it should be considered that serum starvation may affect other cellular processes, such as proliferation and autophagy.

3. Macropinosome labeling

3.1. 24-well plate with coverslip format

3.1.1. Aspirate the wells and add back 200 µL of serum-free media with 1 mg/mL fluorophore-labeled high-molecular-weight (70 kDa) dextran. Place the cells in a 37 °C cell incubator for 30 min.

NOTE: Depending on the conditions to be studied, instead of using fresh media, reusing the

conditioned media for dextran loading may be preferred as it would contain secreted or supplemented factors, such as EGF or inhibitor compounds, respectively, that can affect the macropinocytic capacity of the cells.

3.1.2. Aspirate the media and gently but quickly wash the cells 5 times with ice-cold PBS using a precooled wash bottle. Firmly shake the plate by hand during washes to aid in dislodging dextran aggregates that become stuck to the coverslips.

3.1.3. Fix the cells by adding 350 µL of 3.7% formaldehyde and incubating for 20 min. Then, aspirate the fixation solution and wash the cells with PBS twice.

3.1.4. Stain the nuclei with 350 µL of 2 µg/mL DAPI in PBS. After 20 min, aspirate the DAPI solution and wash the cells with PBS thrice.

3.1.5. Adhere silicone isolators side-by-side on a microscope slide to obtain even spacing and reproducible localization of the coverslips, required for imaging automation (**Figure 2A,B**).

NOTE: The entire microscope slide can be populated with a total of 3 isolators.

3.1.6. For each coverslip, add a drop of hardening fluorescence mounting media on the microscope slide within the open space of the isolator (**Figure 2C**). Pick up a coverslip using forceps and remove excess PBS by gently tapping the side of the coverslips on a lint-free wipe.

3.1.7. Place the coverslip upside down on the drop of mounting media (**Figure 2D**). Gently tap the coverslip using closed forceps to remove bubbles from the mounting media (**Figure 2E**).

3.1.8. Store the slides in a dark environment and allow the mounting media to dry at room temperature, typically taking 16–24 h. Slides may now be imaged or stored at -20 °C for up to 2 weeks.

3.1.9. Before imaging, remove the isolators from the microscope slide. Let the slides equilibrate to room temperature and clean the coverslips using a cotton-tipped applicator wetted with ammonia-free glass cleaner. Subsequently, use a clean cotton-tipped applicator wetted with 70% ethanol to clean and leave the coverslip dry.

[Place **Figure 2** here]

3.2. 96-well microplate format

3.2.1. Aspirate the wells using a multichannel aspiration adapter attached to a vacuum and add 40 µL of serum-free media with 1 mg/mL fluorophore-labeled high-molecular-weight (70 kDa) dextran back to the wells. Incubate the cells in a 37 °C cell incubator for 30 min.

NOTE: Depending on the conditions to be studied, instead of using fresh media, reusing the

conditioned media for dextran loading may be preferred as it would contain secreted or supplemented factors, such as EGF or inhibitor compounds, respectively that can affect the macropinocytic capacity of the cells.

3.2.2. Dispose of the media in the microplate by manually flicking the plate upside down into an empty 5 L beaker (**Figure 3A**).

3.2.3. Rinse the cells in the microplate by slowly submerging the plate vertically, at a slight angle, into a 2 L beaker filled with ice-cold PBS (**Figure 3B**) and subsequently dispose of the PBS in the microplate by flicking the plate upside down into the 5 L beaker (**Figure 3C**). Repeat 2 times.

NOTE: Cells that weakly attach to the imaging microplate may detach during this process. If required, wells can also be aspirated with a multichannel aspiration adapter or more gently washed with PBS using a multichannel pipette. Processing one 96-well microplate will require approximately 2 L of ice-cold PBS. If more plates are to be analyzed, use a larger beaker, and add 1 L of ice-cold PBS for each additional plate or refresh the ice-cold PBS as necessary.

3.2.4. Following the disposal of the PBS from the last rinse, fix the cells for 20 min at room temperature by adding 100 µL of 3.7% formaldehyde in PBS to each well using a 25 mL reagent reservoir and a multichannel pipette (**Figure 3D**).

3.2.5. Remove the fixation solution and wash the cells with PBS twice using the submerging and flicking technique. Stain the nuclei with 100 µL of 2 µg/mL DAPI in PBS per well.

3.2.6. After 20 min, rinse the cells thrice with ice-cold PBS using the submerging and flicking technique described above (step 3.2.3). Remove any residual PBS by tapping the microplate upside-down onto a lint-free wipe and add 100 µL of fresh PBS to each well using a 25 mL reagent reservoir and multichannel pipette. Image the cells now or store them covered from light at 4 °C for up to one week.

NOTE: Alternatively, a solution of glycerol in PBS (instead of PBS) can be used for imaging and storage to better stabilize the fluorescence (**Figure 4A**).

3.2.7. Before imaging, let the plate equilibrate to room temperature. Wipe the cell culture plate dry with a lint-free wipe.

[Place **Figure 3** here]

4. Automated macropinosome imaging

Images of macropinosomes can be captured using a standard fluorescent microscope, as previously described[19,20]. However, such a procedure can be improved upon in terms of efficiency through automation, especially when assessing numerous different cell culture conditions. Automation of image acquisition can be accomplished via a cell imaging multi-mode plate reader,

which decreases effort by reducing handling procedures and, importantly, increases data reproducibility and reliability by acquiring images in an unbiased fashion. Multiple imaging systems are commercially available, and directions will differ between instruments. Here, acquiring images using a Cytation 5 is described. However, the protocol below can be tailored to each individual instrument by adhering to the following guidelines:

4.1. Create an automation protocol to acquire the images with a 40x air objective in the wavelength channel of the dextran fluorophore (FITC/TMR) and DAPI.

NOTE: The commonly used macropinocytosis inhibitor EIPA exhibits autofluorescence in the FITC channel, especially when previously excited in the DAPI channel. Other compounds being tested might also display autofluorescence. To circumvent this issue, setting the image acquisition to occur first in the channel with the highest excitation wavelength (FITC/TMR) and second in the DAPI channel helps to avoid this occurrence.

4.2. Optimize exposure settings using a sample predicted to have the highest level of macropinocytosis to avoid overexposure, which may result in saturation of the signal and loss of intensity data. Use focus settings that readily and consistently locate the sample to produce high-quality images.

4.3. Acquire multiple images across each well or coverslip to account for sample variability and obtain an accurate representation of the sample.

4.4. Once the imaging settings have been determined, use the same settings for each sample within the experiment.

4.5. Follow these instructions for macropinosome image acquisition when using a Cytation 5 and Gen5 software:

4.5.1. 24-well plate with coverslip format

4.5.1.1. Start the plate reader and insert the microscope slides upside down using the slide holder.

4.5.1.2. Open the microplate reader and imaging software, create a new protocol by clicking on **Protocols**, and **Create New**. Double click on **Procedure** and select the plate type.

NOTE: If the plate type is not available, add the plate type to the software by clicking on **System** > **Plate Types** > **Add Plate**, and using the plate dimensions as provided by the manufacturer. For ease of use, the template for two microscope slides with three coverslips spaced using the silicone isolators is provided in **Supplementary File 1**.

4.5.1.3. To access the imaging settings, select **Actions** > **Image** > **Inverted imager** and click on **OK**. Use the 40x, PL FL Phase objective with **Wide FOV** and **Autofocus Binning**.

4.5.1.4. For the first channel, select the LED cube corresponding to the dextran fluorophore-label (GFP or RFP). Unclick **Auto Exposure** and click the microscope icon button to optimize exposure settings. When the appropriate exposure settings have been determined, click on **Save Settings**.

NOTE: Adjust the exposure settings using a sample predicted to have the highest level of macropinocytosis to avoid overexposed images, which can result in saturation of the signal and loss of intensity data.

4.5.1.5. Repeat the previous step for the second channel using the DAPI LED cube.

4.5.1.6. Set the autofocus settings for each fluorescence channel, select **Focus Options**. Unselect the default focus method and use **Autofocus with Optional Scan** and **Autofocus without Optional Scan** for the dextran-fluorophore and DAPI channel, respectively. Click on **OK** to save the settings.

NOTE: The scan distance can be reduced to 200 µm and the increment to 20 µm to increase autofocus efficiency. The optional scan is required for adequate autofocus on samples that have low fluorescence. This normally occurs when analyzing conditions with low macropinocytosis, such as when macropinocytosis is inhibited or not innately present.

4.5.1.7. Use the **Define Beacons** option to automate the acquisition of images at different regions of the coverslip. Click the microscope icon and add beacons by clicking in the image window and moving the stage to the next region. When the appropriate number of regions have been selected, move to the next coverslip and repeat the process. To finalize, click on **Save Settings**.

NOTE: To obtain a good representation of macropinocytosis across the sample, select approximately 20 beacons that are evenly distributed across the coverslip (**Figure 4B**). Less beacons can be used, but some images may have to be excluded from image analysis after acquisition due to quality discrepancies, such as when the image is out-of-focus or contains bubbles or fluorescent blotches and smudges.

4.5.1.8. To complete the adjustment of imaging settings, click on **OK**. To image the coverslips, select **Create a New Experiment and Read Now** from the **Protocol Tools**. Save the protocol and the experiment when prompted.

[Place **Figure 4** here]

4.5.2. 96-well microplate format

4.5.2.1. Start the plate reader and insert the microplate.

4.5.2.2. Open the microplate reader and imaging software, create a new protocol by clicking on **Protocols**, and **Create New**. Double click on **Procedure** and select the plate type.

NOTE: If the plate type is not available, add the plate type to the software by clicking on **System** > **Plate Types** > **Add Plate**, and using the plate dimensions as provided by the manufacturer. For ease of use, the template for the CellCarrier-96 Ultra Microplates from PerkinElmer is provided in **Supplementary File 2**.

4.5.2.3. To access the imaging settings, select **Actions** > **Image** > **Inverted Imager** and click on **OK**. Use the 40x PL FL Phase objective with **Wide FOV** and **Autofocus Binning**.

4.5.2.4. For the first channel, select the LED cube corresponding to the dextran fluorophore-label (GFP or RFP). Unclick **Auto Exposure** and click the microscope icon button to optimize exposure settings. When the appropriate exposure settings have been determined, click on **Save Settings**.

NOTE: During the optimization of the exposure settings, significant fluorescence bleaching can occur. This can result in settings that cause overexposure when a new field is imaged. Therefore, validate the exposure settings by checking a yet unexposed field and assuring that no saturation of the signal occurs at the selected settings. Do not include the wells used for the optimization of the exposure setting in the macropinosome quantification, as the fluorescence has decreased as a result of bleaching during the optimization. Adjust the exposure settings using a sample predicted to have the highest level of macropinocytosis to avoid overexposed images, which can result in saturation of the signal and loss of intensity data.

4.5.2.5. Repeat the previous step for the second channel using the DAPI LED cube.

4.5.2.6. Set the autofocus settings for each fluorescence channel, select **Focus Options**. Unselect the default focus method and use **Laser Autofocus**. Capture a reference scan after determining the focal plane for optimal visualization of macropinosomes and nuclei. Click on **OK** to save the settings.

NOTE: The scan distance can be reduced to 400 µm and the increment to 3 µm to increase autofocus efficiency. For the laser autofocus option to work properly, clean the bottom of the plate, dry and wipe the plate with a lint-free wipe before imaging. The laser autofocus is a superior method for focus as it requires minimal time to find the focal plane. Other focus methods may be used, but, since no anti-fade has been added to the wells, these methods may cause significant bleaching of the samples which will negatively impact the collection of data.

4.5.2.7. Set the horizontal and vertical offset to zero and under **Single Image** select **Montage** with **No Overlap** and use 3 x 3 images, depending on how many cells are desired to be included in the analysis.

NOTE: Depending on the size and density of the cells, more or less images can be taken to obtain

a representative assessment of macropinocytosis throughout the sample. Assessing macropinocytosis in AsPC-1 or MIA PaCa-2 cells under varying conditions, no differences in data interpretation between a 2 x 2 or 4 x 4 photo frame are observed, although variation between replicate samples may increase when taking less pictures (**Figure 5A,B**). Increasing or decreasing the size of the frame will affect the time it takes to scan the plate. Depending on the exposure time, a full 96-well microplate will take around 1–1.5 h to scan completely using a 3 x 3 frame. A 2 x 2 and 4 x 4 frame will halve or double that time, respectively.

4.5.2.8. To complete the adjustment of imaging settings, click on **OK**.

4.5.2.9. To image the plate, select **Create a New Experiment and Read Now** from the **Protocol Tools**. Save the protocol and the experiment when prompted.

[Place **Figure 5** here]

5. Determining the macropinocytic index

NOTE: The 'macropinocytic index' is the extent of cellular macropinocytosis that is determined by quantifying fluorescent dextran uptake per cell using microscopic imaging[19]. To this end, the acquired images are used to determine the amount of internalized dextran by measuring the total fluorescence intensity or fluorescence-positive area and the total number of cells as determined by DAPI staining. This analysis can be performed with open-source image processing and analysis software, such as Cell Profiler or FIJI/ImageJ, as previously described[19,20]. However, when working with a multi-mode plate reader the software provided with the instrument may include built-in analysis applications that can be used for the purposes of computing the macropinocytic index. In some cases, the built-in software analysis pipeline may not be completely apparent to the user. It is therefore recommended to validate the software at an early stage by comparison with a non-automated procedure, such as Cell Profiler or FIJI/ImageJ. This protocol can be adapted to other image processing and analysis software tools by adhering to the following general instructions:

5.1. For the DAPI and corresponding dextran image, subtract the background by applying the appropriate function, frequently called the rolling ball function. Adjust the settings so that the background noise is minimized and there is minimal to no subtraction effect on the DAPI and dextran signal.

5.2. Using a field with high dextran signal, determine the intensity signal settings, frequently called the threshold function, to select the nuclei and determine the minimum intensity signal setting required to select only the macropinosomes.

5.3. For the dextran image, calculate the total fluorescence within the created macropinosome selection or use the selection to determine the total area positive for dextran.

5.4. For the DAPI image, use the selection to determine the number of nuclei in the image to

reflect the number of cells present.

5.5. To determine the macropinocytic index, divide the total dextran fluorescence or area by the number of cells determined by DAPI.

5.6. Repeat these analysis steps for all the acquired images applying the same numerical settings throughout.

5.7. Analysis of the images acquired.

NOTE: The built-in analysis pipeline was validated and detected no differences in computation relative to Fiji/ImageJ (**Figure 6A**). Follow these instructions for determining the macropinocytic index when using Gen5 software:

5.7.1. After the imaging is completed, select an image with a high level of micropinocytosis. Remove the background signal, click **Process** (**Supplementary Figure 1A**) and select the **Image Preprocessing** option.

5.7.2. For the dextran channel, deselect **Auto** and use a rolling ball diameter of 5 µm, prioritize fine results and smooth the image with 1 cycle.

5.7.3. For the DAPI channel, use **Auto** preprocessing and 1 smooth cycle. Click on OK and add the image preprocessing step to the protocol; click **ADD STEP**. Next, select the processed image under the **Image** roll-out (**Supplementary Figure 1B**) and click the **Analyze** button (**Supplementary Figure 1C**).

5.7.4. Under **ANALYSIS SETTINGS**, set the **Type** to **Cellular Analysis**. Select the DAPI channel and click on **Options** (**Supplementary Figure 2A**).

5.7.5. For the primary mask, using the processed DAPI image, create a mask to select single nuclei. Use the dark background and **Auto** option. Additionally, determine which settings allow for mask selection of single nuclei and when finished, click on the **Apply** button to determine if the mask is applied appropriately.

NOTE: Activating the **Split Touching Objects** and **Fill Holes in Masks** options may work best for selecting single nuclei. Minimum and maximum object sizes may need to be adjusted depending on the cell line and are most commonly set in the 5–40 µm range. **Primary Edge Objects** can be included, and the entire image should be analyzed. The slider can be applied to adjust the mask selection to the signal intensity.

5.7.6. Next, apply a secondary mask to optimize the settings for selecting the macropinosome fluorescent puncta. Use the **Measure within a Secondary Mask** function and expand the primary mask by 40 µm depending on the size of the cells.

5.7.7. Use the **Threshold** function and **Threshold in Mask** method to select the positive dextran areas. Click on **Apply** to determine whether the settings are applied correctly.

NOTE: To determine the threshold value, use the **View Line Profile** tool (**Supplementary Figure 2B**) and draw a line over a dextran-positive area (**Supplementary Figure 2C**). Use the measured intensity to determine the best setting to create a mask that selects macropinosomes and excludes background signal (**Supplementary Figure 2D**).

5.7.8. After creating appropriate masks to select nuclei and macropinosomes, click on the **Calculated Metrics** tab and select **Select or Create Object-Level Metrics of Interest**.

5.7.9. Remove all metrics present and add the **Integral** and **Area** metrics for analysis of the secondary mask. Click on **OK** and select **Calculate** and **Show** for the new metrics. When finished, click on **OK** and select **ADD STEP** to add the analysis and calculations to the protocol.

5.7.10. Save the finalized protocol for future use, click **File** and **Save Protocol As**.

5.7.11. After the data analysis has finished, use the data, select the metrics of interest and export the data to determine the macropinocytic index. Determine the macropinocytic index as follows:
Dextran fluorescence per cell = Object Int_2[Dextran fluorophore]
Dextran area per cell = Object Area_2[Dextran fluorophore]

NOTE: For the 24-well plate with coverslips format, the metrics reflect the mean of the average macropinocytic index per image. Alternatively, the macropinocytic index can be calculated manually for the entire sample by dividing the sum of the 'Area' or 'Integral' for all images by the total 'Cell Count'. The difference between these approaches in calculating the macropinocytic index is minimal in most settings. For the 96-well microplate format, the macropinocytic index is calculated as the average for the entire sample.

5.7.12. Save the protocol for imaging and the subsequent automated analysis. Reuse the protocol for future experiments with the same fluorophores.

NOTE: When using the laser autofocus function, a new reference scan must be taken when a different cell line is to be analyzed since nuclei and macropinosomes are possibly localized to a different plane. Each time a new experiment is performed using a previously determined protocol, the exposure settings for that experiment must be optimized.

6. Addition of treatments

NOTE: Cell treatments (small molecules, biologics, growth factors, metabolites etc.) can be incorporated at any stage of the protocol, and the precise timing will depend on the goals and aims of the study.

6.1. Prepare the cells as in section 2.

6.2. Just before adding the treatments of interest, prepare the treatments and the appropriate controls at twice their final concentrations in serum-free media. Prepare the treatments in a volume equal to the volume of the number of replicate wells being assessed.

NOTE: Given the role that secreted factors can play in controlling cellular functions, it may be preferred to dilute the treatments of interest in conditioned media. For these purposes, it may be helpful to seed additional plates, such as 6-cm or 10-cm cell culture dishes, when preparing cells as described in section 2 to generate the conditioned media for the preparation of the treatment solutions.

6.3. Without removing the media from the well, add one well-volume of treatment solution to each well. Shake the plate to ensure proper mixing. Incubate the cells for the desired amount of time.

6.4. Continue with section 3.

NOTE: When adding the dextran, the use of fresh media causes the removal of the added treatments, which may affect the level of macropinocytosis. Therefore, it may be preferred to add dextran directly to the wells without aspirating or alternatively re-adding the treatments or reusing the conditioned media to prepare the dextran solution.

[Place **Figure 6** here]

REPRESENTATIVE RESULTS:

When the steps and adjustment of the above-described protocol are followed accordingly, the final experimental results should provide information about whether the studied cell culture conditions or inhibitors induce or reduce macropinocytosis in the cell line of interest. To strengthen the validity of these findings, the inclusion of control conditions will allow for the scrutinization of the results to determine whether the experiment has been completed successfully. Macropinocytosis induction controls will provide information about the relative level of macropinocytosis. For this purpose, the ligand EGF is most commonly used[13]. In AsPC-1 PDAC cells, adding EGF at 100 ng/mL for 5 min before adding the dextran activates macropinocytosis (**Figure 5A**). Moreover, autocrine EGF activation of macropinocytosis can be induced by depriving the cells of glutamine for 16–24 h (**Figure 5A**). Alternatively, other inducers may be incorporated depending on the cell type and available literature; these may include PDGF or Wnt3A[10–12]. Not all cells behave similarly, and *KRAS* mutant cells may exhibit constitutive macropinocytosis[13]. One example is the MIA PaCa-2 PDAC cell line, which does not respond to EGF treatment. Here, the addition of known macropinocytosis inhibitors will validate that the observed and quantified fluorescent puncta are indeed macropinosomes. The inhibitory controls most commonly used include the sodium-hydrogen exchanger inhibitor EIPA, which is a specific macropinocytosis inhibitor (**Figure 5B**)[4]. Other controls may also be included, such as the Rac1 inhibitor EHop-016 (**Figure 5B**), the Pak inhibitor FRAX597, or the PI3K inhibitor LY294002; however, their effect on macropinocytosis may be model specific[13].

For cell lines that have previously not been assessed for macropinocytosis and inhibitors, dose-response curves are an excellent approach to confirm macropinocytosis and to determine optimal drug concentration for future use. For these purposes, the 96-well microplate format protocol was used to determine constitutive macropinocytosis in a cell line previously not assessed in the lab, the PATU8998T PDAC cells. To determine whether these cells exhibit macropinocytosis, the effect of two known inhibitors of dextran uptake (EHop-016 and EIPA) were studied. As illustrated, the dose-response experiment gradually decreased the macropinocytic index at higher drug concentrations (**Figure 6A,B**), thereby confirming the existence of constitutive Rac1-dependent macropinocytosis in these cells. In addition, the results provide information for optimal drug concentrations to be used for future experiments when studying macropinocytosis in this cell line.

[Place **Figure 7** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the automated assay to determine the 'macropinocytic index' in adherent cells. Created using BioRender.

Figure 2: Placing coverslips on a microscope slide with silicone isolators. (**A**) Silicone isolators are pressed and adhered to a microscope slide. (**B**) The entire microscope slide can be populated with a total of 3 isolators, resulting in even spacing and reproducible localization of the coverslips. (**C**) For each coverslip, add a drop of fluorescence mounting media on the microscope slide within the open space of the isolator. (**D**) Using forceps, pick up a coverslip from the 24-well plate and place it upside down on the drop of mounting media. (**E**) When bubbles are present between the coverslip and microscope slide, gently tap the coverslip using closed forceps to remove bubbles. Created using BioRender.

Figure 3: Rinsing the 96-well microplate to prepare for fixation. (**A**) Empty the microplate of media into a 5 L beaker by manually flicking. (**B**) Vertically and at a slight angle, slowly submerge the microplate in a 2 L beaker filled with ice-cold PBS. (**C**) Empty the microplate of PBS into the 5 L beaker by manually flicking. Repeat the wash steps as described in **B** two times. (**C**) After emptying the PBS in the microplate for the last time, add 100 µL 3.7% formaldehyde to the wells, using a multichannel pipette. Created using BioRender.

Figure 4: Optimization of conditions for image acquisition. (**A**) Increasing the glycerol concentration increases TMR-dextran fluorescence, as determined in AsPC-1 cells treated with EGF. (**B**) Example coordinates of imaging beacons for automatic image acquisition when using the 24-well plate with coverslips format. The bar graph shows the average relative fluorescence with SEM of 5 experiments. Statistical significance was determined by two-way ANOVA, relative to PBS. ** $p < 0.01$; *** $p < 0.001$.

Figure 5: Control conditions for assessing macropinocytosis in PDAC cells. (**A**) AsPC-1 cells display macropinocytosis in response to 100 ng/mL EGF stimulation for 5 min or glutamine

deprivation for 24 h. For image acquisition, picture frames of 4 x 4, 3 x 3, 2 x 3, or 2 x 2 were taken to determine the influence of the number of photos on data quality. (**B**) MIA PaCa-2 cells show constitutive macropinocytosis that is inhibited by 30-min treatment with 75 µM EIPA or 2-h treatment with 10 µM EHop-016. Picture frames were taken as in **A**. Scale bar = 25 µm. Bar graphs show the average relative macropinocytic index with SD of 1 experiment with 4 replicates. Statistical significance was determined by two-way ANOVA relative to the +Q or vehicle condition. *** $p < 0.001$

Figure 6: Performing a dose-response curve for macropinocytosis inhibitors. Example data obtained when testing known macropinocytosis inhibitors in a new cell line. PATU8998T cells were used for the 96-well microplate format and treated for 2 h and 30 min with the indicated concentrations of (**A**) EHop-16 and (**B**) EIPA, respectively. Comparison of results obtained through image analysis by the Gen5 software or ImageJ shows no significant differences between the two approaches as indicated by ns in (**A**). Scale bar = 25 µm. Bar graphs show the average and SD of a single experiment with 4 replicates. Statistical significance was determined by one- or two-way ANOVA, compared to untreated conditions. * $p < 0.05$; *** $p < 0.001$.

Figure 7: Trouble-shooting conditions that may be encountered. (**A**) The image is overexposed, as indicated in pink. (**B**) The image is out-of-focus. (**C**) The image contains dextran blotches or smudges. (**D**) The image contains residue. (**E**) The image contains a bubble. (**F**) The cells show drug-induced autofluorescence. Scale bar = 25 µm.

Supplementary Figure 1: Screenshot of image preprocessing. (**A**) **Process** button. (**B**) **Image** roll-out. (**C**) **Analyze** button.

Supplementary Figure 2: Screenshot of image quantification settings. (**A**) **ANALYSIS SETTINGS**. (**B**) View Line Profile tool. (**C**) Line over dextran positive area. (**D**) View Line Profile results

DISCUSSION:

The quality of the experiments and data acquisition highly depends on the quality of the reagents, the optimization of the settings, and the cleanliness of the coverslips and microplate. The final results should give minimal variation between replicates; however, biological variations do naturally occur or may otherwise be caused by a number of factors. Cell density may cause cells to respond better or worse to macropinocytosis inducers or inhibitors. It is, therefore, crucial to adhere to the 80% confluency as proposed here in the protocol. Alternatively, it is well documented by microplate manufacturers that media evaporation occurs on a 96-well microplate. Here, the outer wells are subject to more evaporation relative to the inner wells and thereby may affect macropinocytosis. Thus, the choice can be made to not include the outer wells in the analysis and instead fill these wells with PBS to build a 'buffer wall' to protect the inner wells from extensive evaporation.

It is highly recommended to visually inspect images for each condition to determine if the acquisition was completed successfully. This may be done during the imaging of the first set of conditions to ascertain that the applied settings are indeed correct and allow for intervention if

required. Specific issues that can occur are as follows; the images are overexposed (**Figure 7A**), the images are out-of-focus (**Figure 7B**), the images contain blotches of fluorescent dextran (**Figure 7C**) or residue (**Figure 7D**), the images contain bubbles (**Figure 7E**), or autofluorescence is visible in the dextran or DAPI channel (**Figure 7F**). For the first two issues, overexposure and out-of-focus (**Figure 7A,B**), the image acquisition settings have to be adjusted, and the acquisition has to be repeated. In addition, the plate and coverslips should be cleaned for the autofocus to work properly. If only a few images are out-of-focus, the specific images or the entire well may also be excluded from the analysis using the 'Mask' image function. This same function can be applied to exclude images that contain blotches in the dextran channel (**Figure 7C**), impurities (**Figure 7D**), or bubbles (**Figure 7E**). In addition, make sure that the samples have been washed well, the dextran has completely been dissolved, which can be improved by heating the dextran solution at 37 °C and vortexing, and that the experiment is performed with freshly prepared and clean reagents. Finally, drug-induced autofluorescence can occur and is quite common for the macropinocytosis inhibitor EIPA which fluoresces in the FITC and DAPI channel, especially when previously excited in the DAPI channel. A workaround is the acquisition of the FITC channel before DAPI. Alternatively, TMR-dextran can be utilized instead of FITC-dextran, or for the exposure settings, the light intensity and exposure time can be lowered while increasing the gain.

To compute the macropinocytic index, nuclear DAPI staining is utilized to determine the cell number in each image. This staining procedure is easy to apply and is a reliable readout for the number of cells and thus for the calculation of the relative dextran uptake per cell. One caveat to this approach is that it does not consider differences in cell size, which may occur when comparing different cell lines or in response to pharmacological treatments. In these cases, where cell size is suspected to affect the macropinocytic index, the cell area can be used for normalization of dextran uptake, as previously described[4]. This can be achieved by slightly modifying the protocol to include a cell mask stain after fixation or incorporating phase contrast or bright field imaging. It is also highly recommended to follow up any findings using the described assay to evaluate whether macropinocytosis is a nutrient supply route that contributes to cellular fitness in the particular cell-based system being employed. This may be achieved by assessing proliferative capacity, survival, or viability in nutrient-depleted conditions with and without the addition of extracellular albumin[4,7]. Additionally, a DQ-BSA pulse-chase assay can provide evidence for whether the changes in macropinocytosis translate to changes in albumin degradation in lysosomes. Like high molecular weight dextran, DQ-BSA is internalized through macropinocytosis and is delivered to the lysosomes, where it fluoresces after proteolytic digestion[4]. Given the similarities to fluorescent dextran uptake, the described method can be adapted to assess DQ-BSA uptake. Likewise, this protocol may be used to evaluate the uptake of other fluorescently tagged cargo, such as albumin, lipids, or necrotic cell debris known to enter cells through macropinocytosis. On all occasions, these adaptations should be in line with the manufacturer's protocols, and experimental controls should be used to validate the assay.

Microscopic image analysis and quantification is a favored approach to assess macropinocytosis and has become the standard in the field[4–7,9–13,15–22]. It allows for the visual inspection of the samples and can provide additional information about macropinosome number, size, and location. Moreover, the possibility to visually inspect images permits the assessment of cell

fitness and viability and identification of artifacts and/or autofluorescence caused by drug administration. Compared to other proposed quantification methods, such as flow cytometry, these data could otherwise be lost or overlooked and may cause false positives or negatives[23]. However, imaging is more labor-intensive and more prone to biased data acquisition as flow cytometry. Here, we have overcome these obstacles through automation of the protocol, thereby reducing bias, labor, cost, and time.

Many modulators of macropinocytosis most likely remain undiscovered and, given the importance of the macropinocytic pathway in certain pathologies, such as cancer, their discovery could potentially be of great importance for the development of novel therapeutic approaches[2,3]. The identification of these modulators can be achieved through high-content screening of compounds, for which the herein proposed method may function as a cornerstone. The outlined protocol is aimed at performing the experiments with standard laboratory equipment. However, the 96-well plate format may be optimized and adapted for high-content screening. Increasing the well format to 384 or 1536-well microplates would allow the user to increase the number of compounds that can be tested on one plate. Moreover, robotization of cell seeding, plate washing, compound and dextran administration would reduce manual handling and well-to-well variability, and thereby improve scalability. Ultimately, an adaption of this protocol to high-content screening would greatly facilitate identifying new factors that regulate macropinocytosis.

Altogether, the herein proposed method is an excellent approach to determine the level of macropinocytosis in cells of interest, the identification of regulators of the process, and the optimization of drug concentrations for known inhibitors. Also, the protocol can serve as the starting point for assessing macropinocytic uptake of other cargo besides dextran, and high content screening aimed at identifying lead compounds that could potentially result in the development of novel therapeutic strategies.

ACKNOWLEDGMENTS:

This work was supported by NIH/NCI grants (R01CA207189, R21CA243701) to C.C. KMO.G. is a recipient of a TRDRP Postdoctoral Fellowship Award (T30FT0952). The BioTek Cytation 5 is a part of the Sanford Burnham Prebys Cell Imaging Core, which receives financial support from the NCI Cancer Center Support Grant (P30 CA030199). Figures 1–3 were created using BioRender.

DISCLOSURES:

C.C. is an inventor on an issued patent titled ‘‘Cancer diagnostics, therapeutics, and drug discovery associated with macropinocytosis,’’ Patent No.: 9,983,194.

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Figure 1

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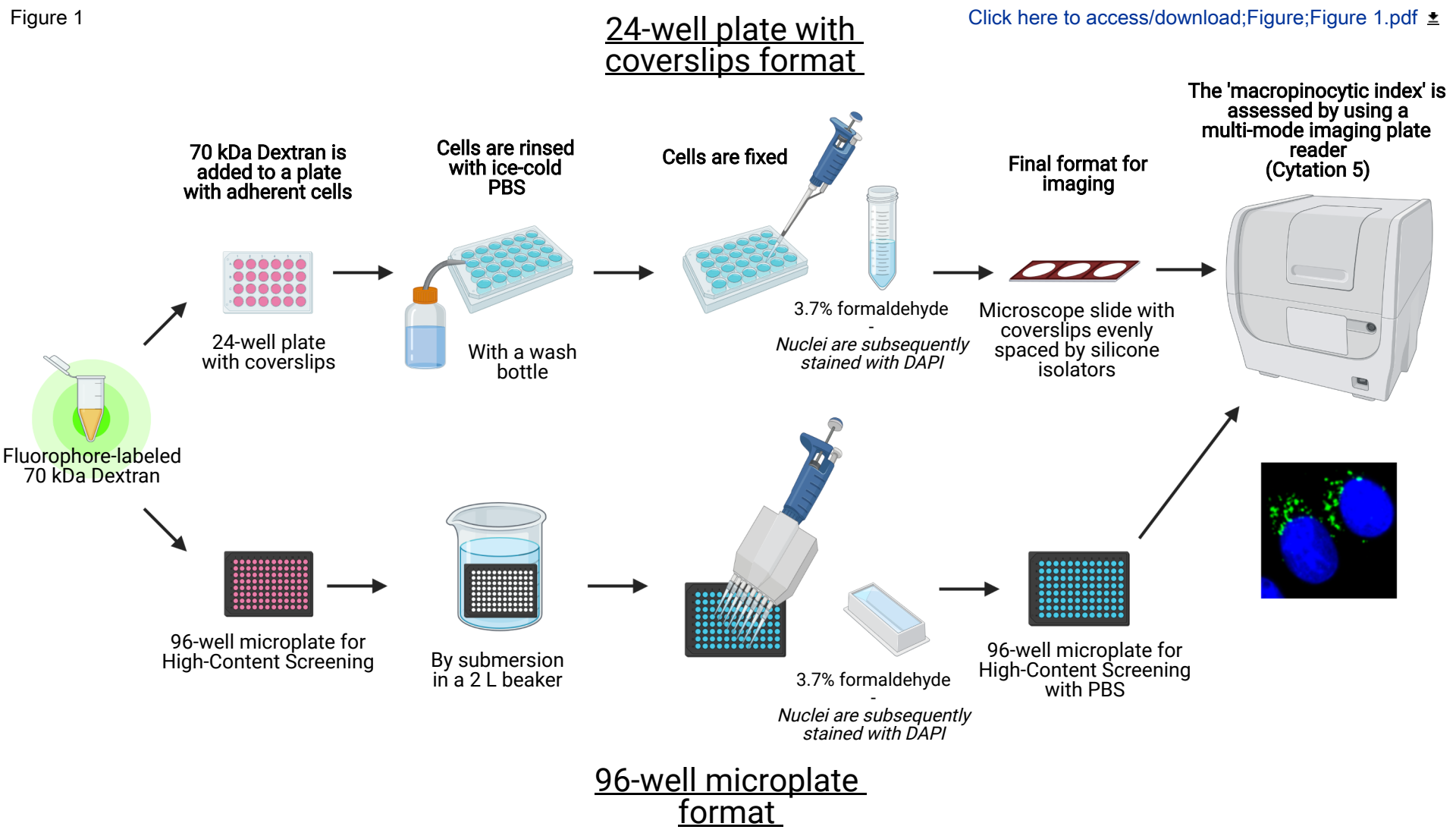

Figure 2

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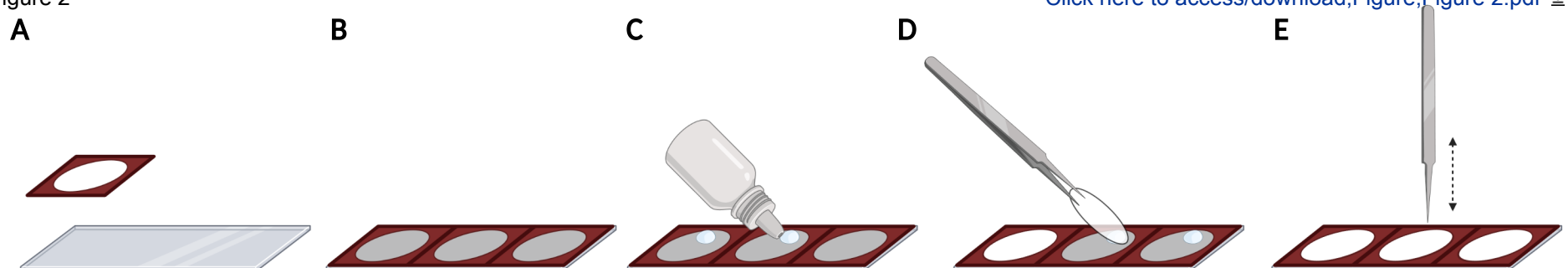

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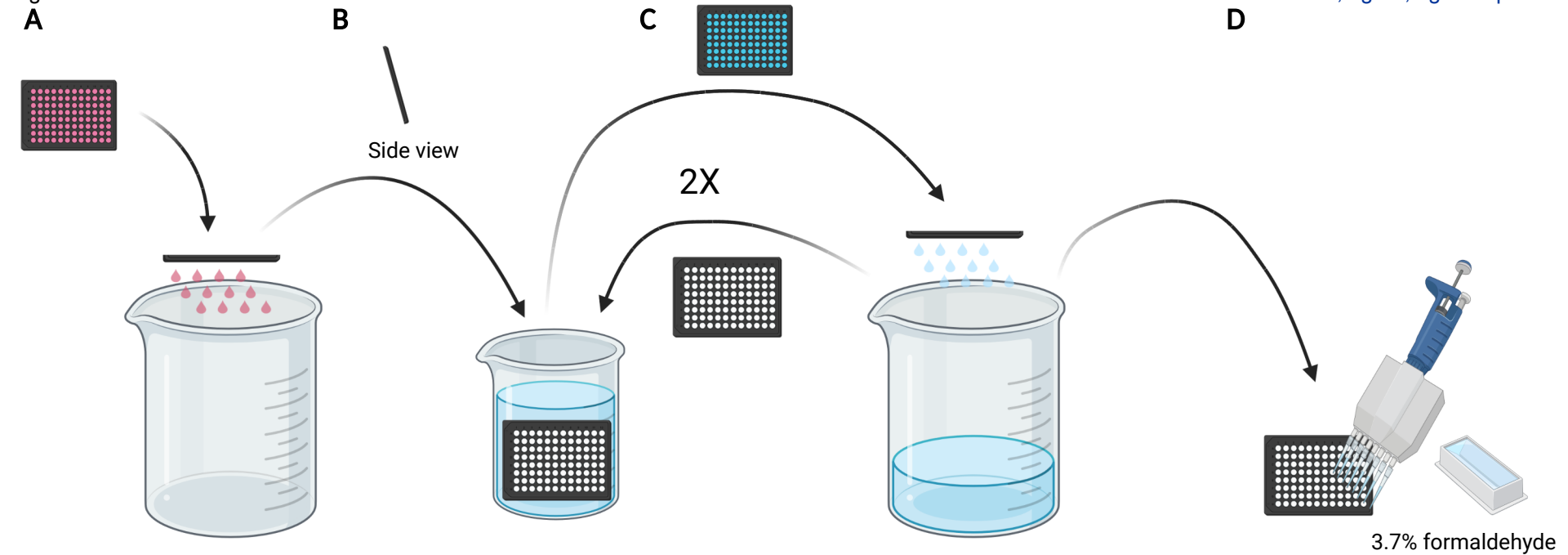

Figure 4

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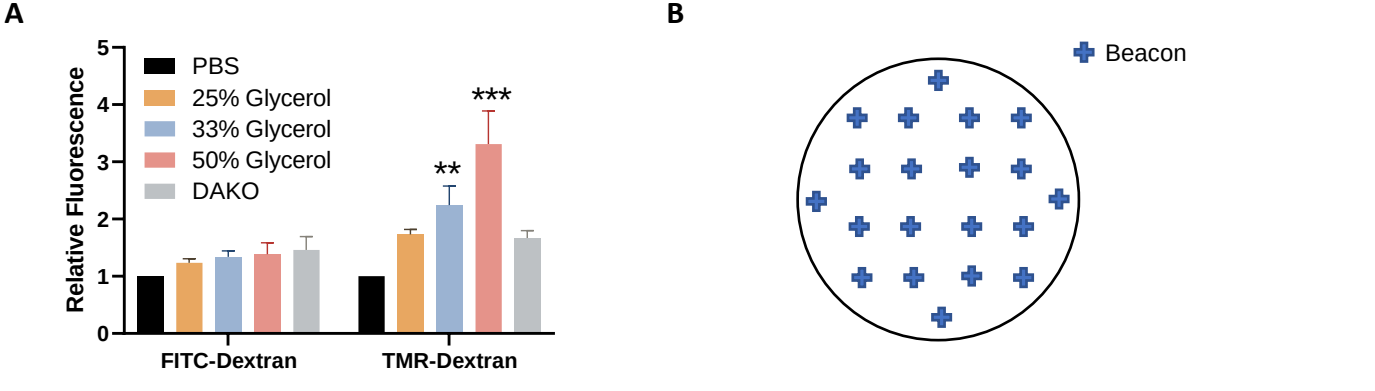

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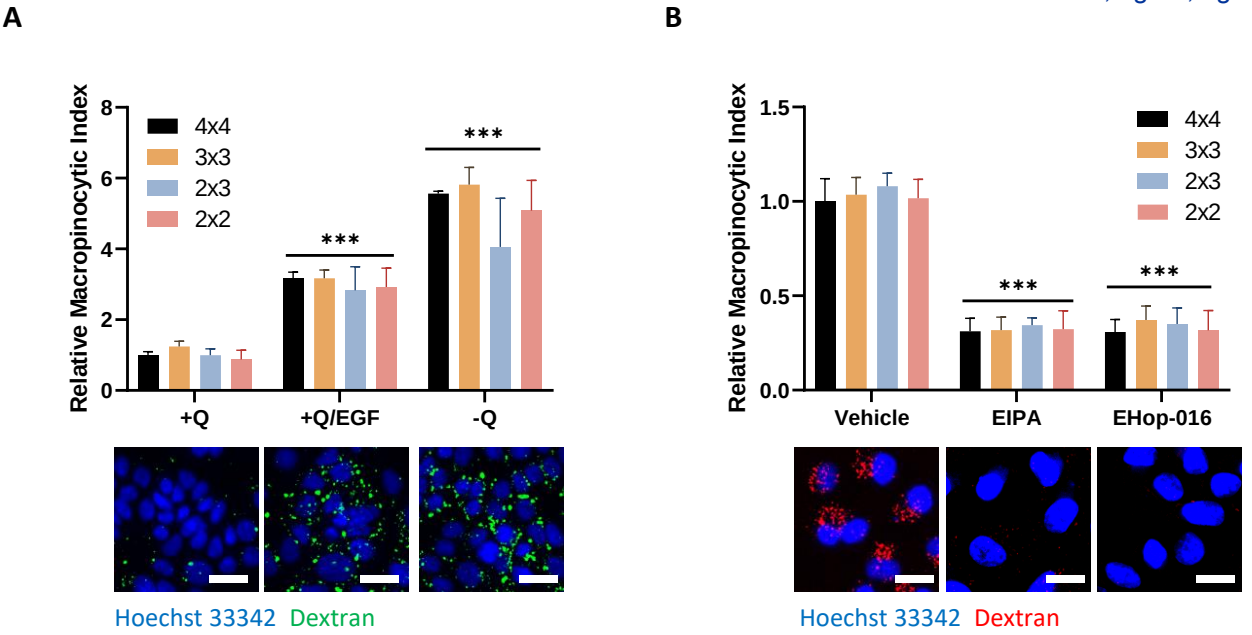

Figure 6

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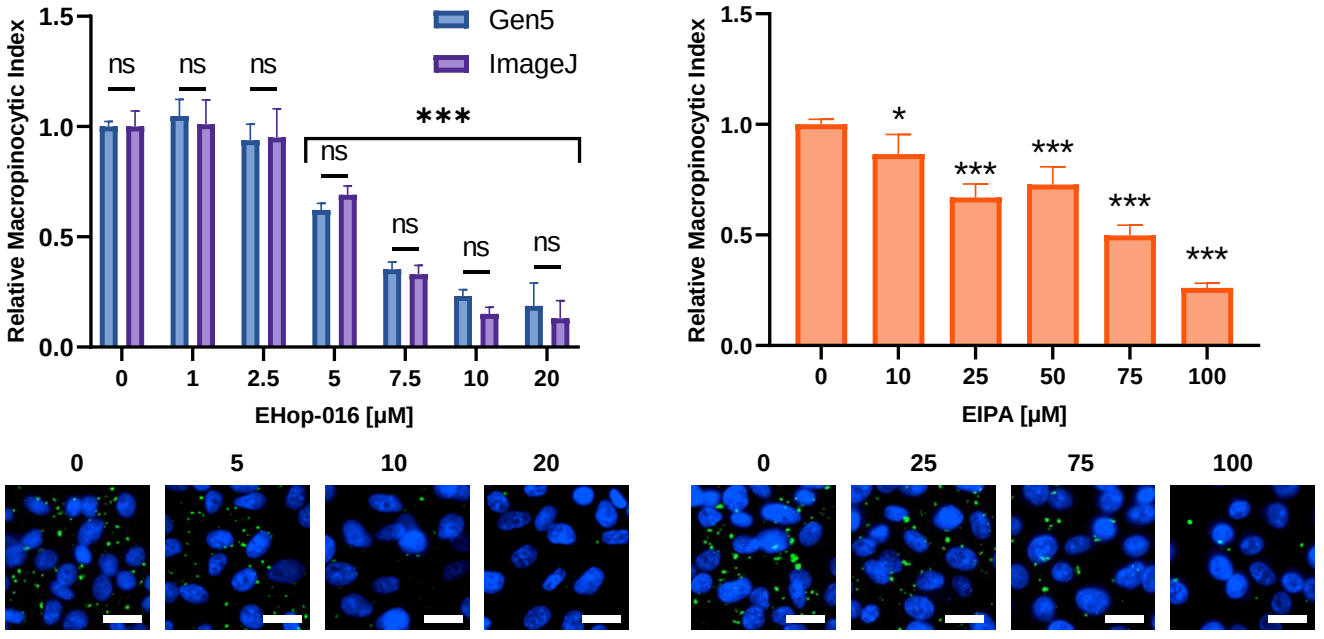
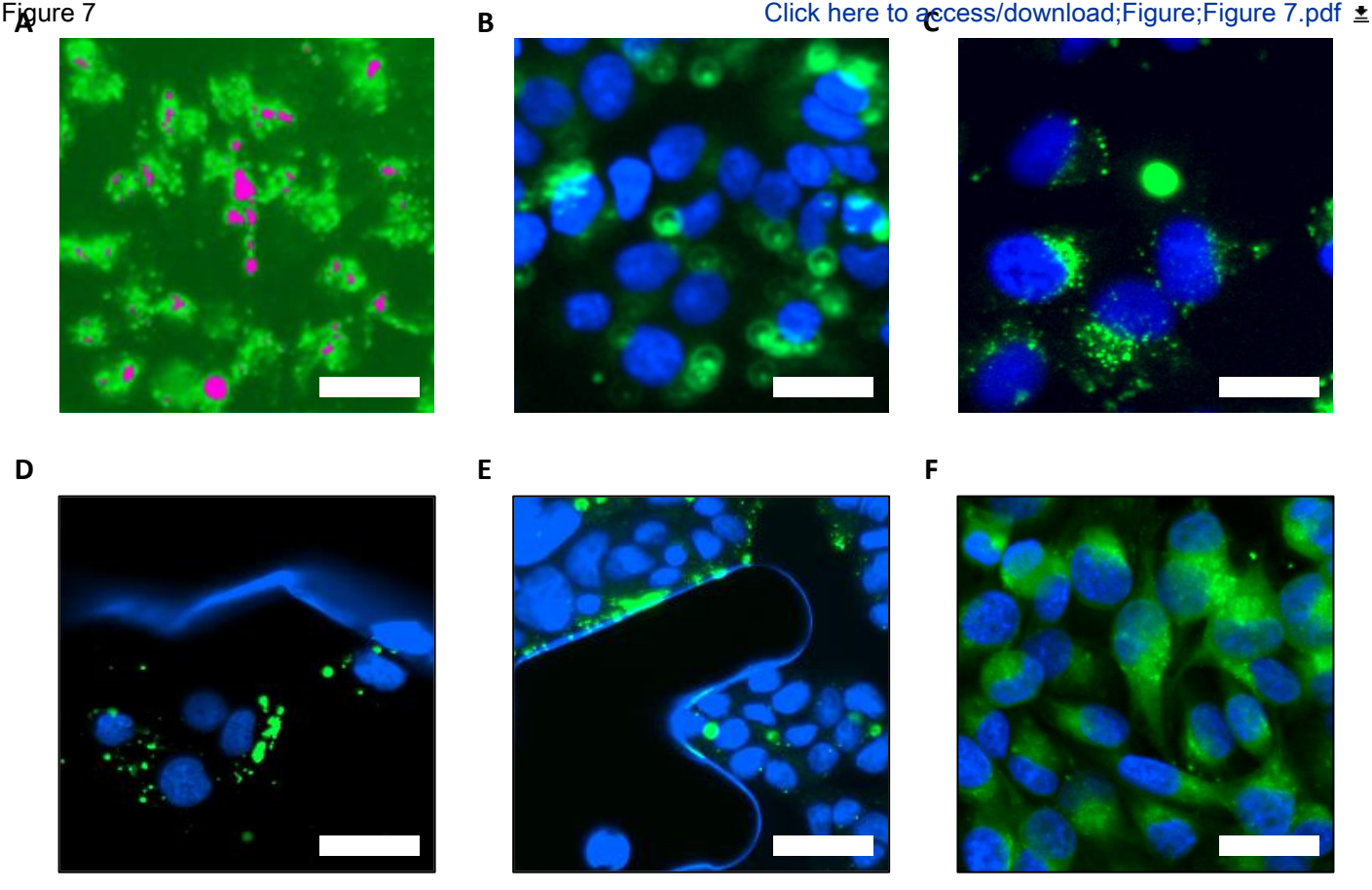


Figure 7



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.

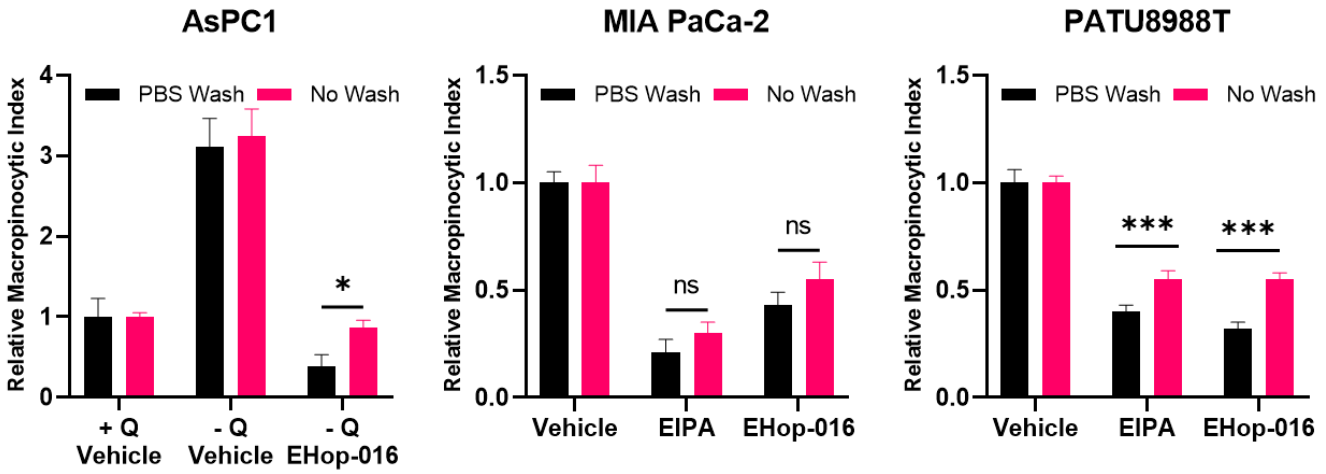


Figure 1: Omitting the wash step before addition of the serum-free media may affect the macropinocytic index. AsPC1, MIA PaCa-2, and PATU8988T cells were cultured on a 96-well microplate and the day before the dextran uptake assay subjected or not to 2 PBS washes, before the addition of serum free media. 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.

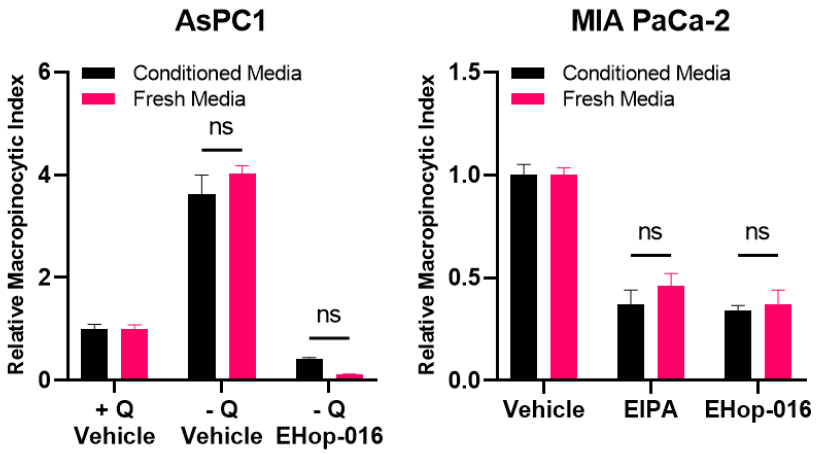


Figure 2: Using fresh media for the addition of dextran does not affect the results compared to reusing conditioned media. AsPC1 and MIA PaCa-2 were cultured on a 96-well microplate and cells were treated for 24 hours with 10 µM EHop-016 or for 30 minutes with 75 µM EIPA. AsPC1 cells were deprived of 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.

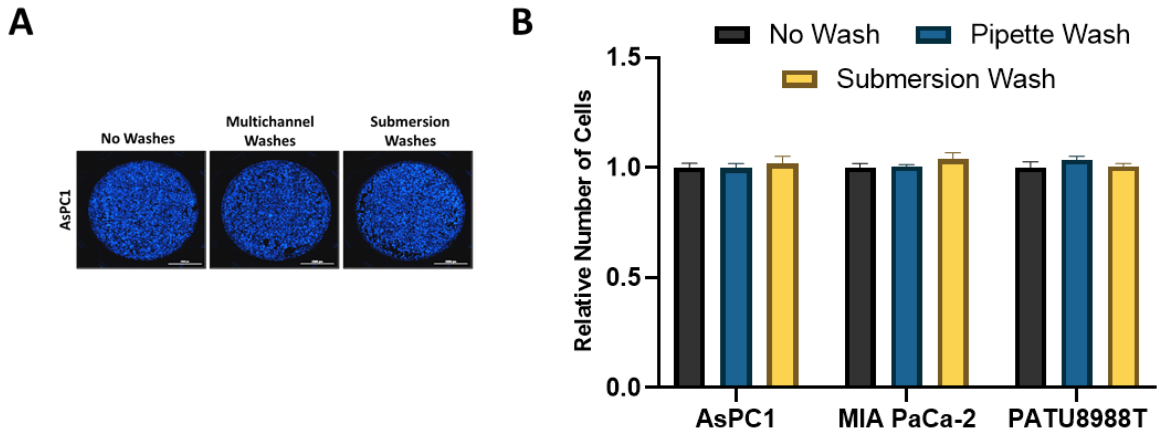


Figure 3: Washing the cells by submersion does not change the number of cells in the area of image acquisition. AsPC1 cells were cultured on a 96-well microplate and subjected or not to washes by using a multi-channel pipette or by submersion. Thereafter, nuclei were stained with DAPI. Shows whole well 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

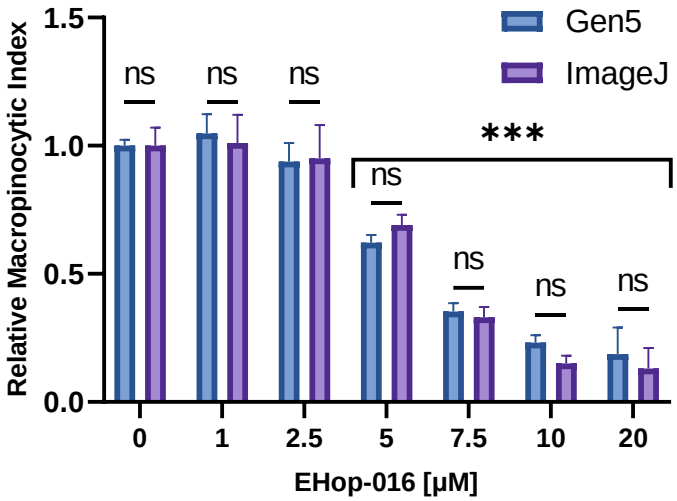
We thank all the reviewers for their insightful comments and helpful suggestions. Based on your recommendations, we have significantly expanded and improved the manuscript. We are extremely proud of the revised version of our work. In the revised manuscript, the new or modified sections of the text are 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. ***, $P < 0.001$.

Supplementary Figure 1

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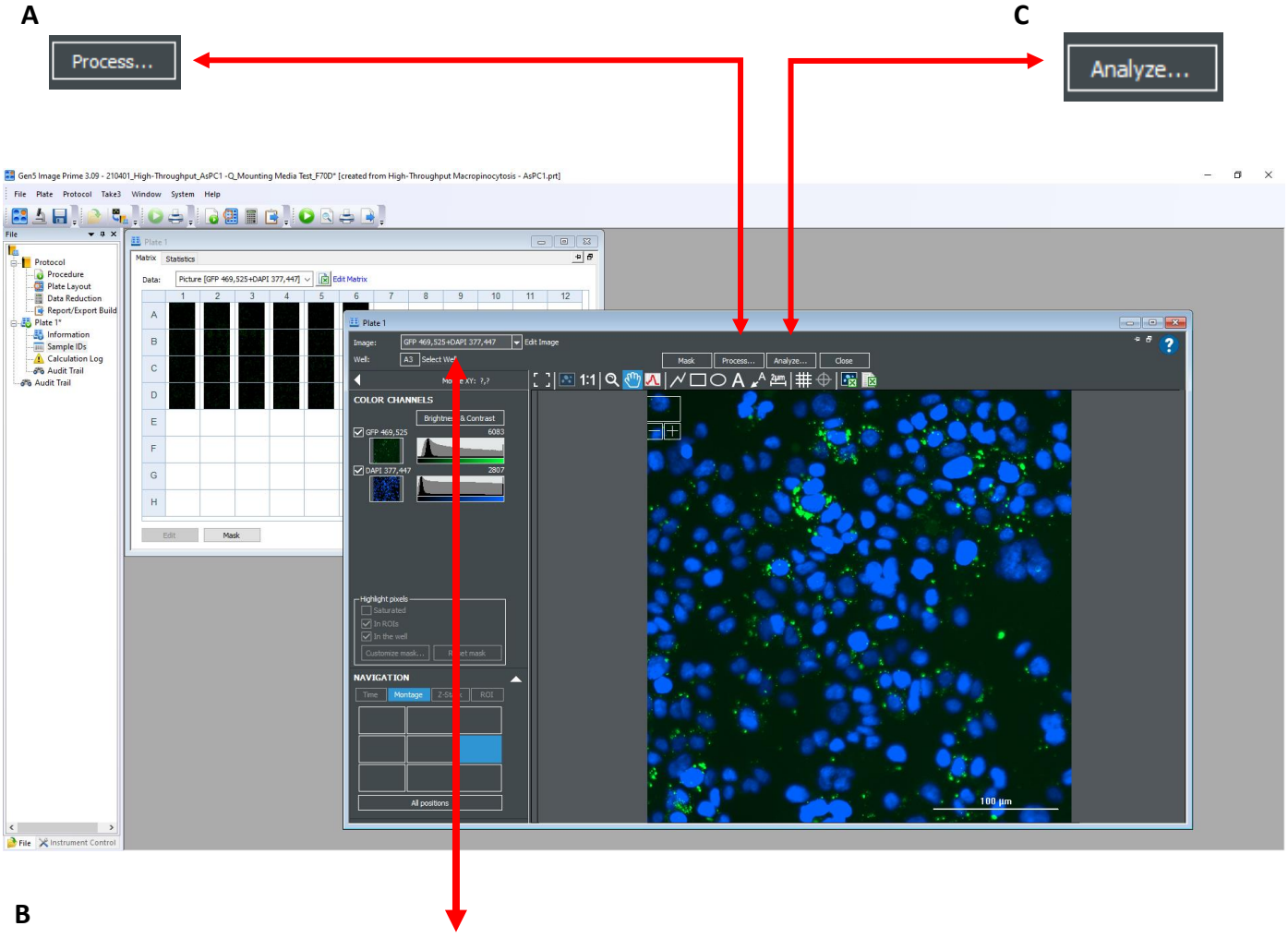

Supplementary Figure 2

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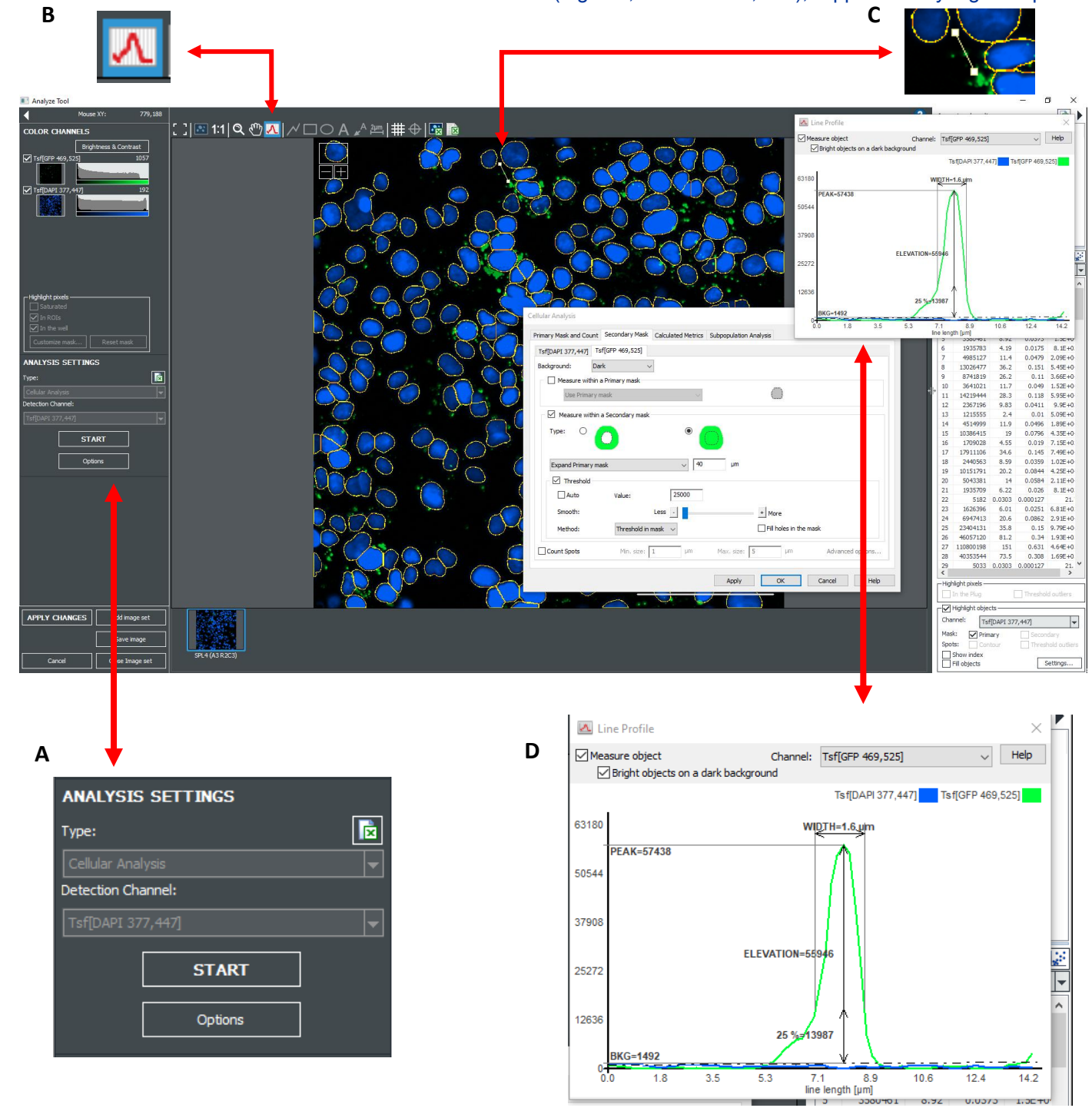

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Supplemental Coding Files

Supplementary Coding File 1_Slide Holder.xml