UC Office of the President
Recent Work

Title
Automated Imaging and Analysis for the Quantification of Fluorescently Labeled Macropinosomes.

Permalink
https://escholarship.org/uc/item/63s955tj

Authors
Galenkamp, Koen MO
Galapate, Cheska Marie
Zhang, Yijuan
et al.

Publication Date
2021-08-01

DOI
10.3791/62828

Peer reviewed

Automated imaging and analysis for the quantification of fluorescently-labeled macropinosomes

Koen M.O. Galenkamp[*,1], **Cheska Marie Galapate**[1], **Yijuan Zhang**[1], **Cosimo Commisso**[*,1]

[1]Sanford Burnham Prebys Medical Discovery Institute, NCI-designated Cancer Center

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. Here, we describe 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.

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.

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 is 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 to 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, we describe the essential steps to visualize macropinosomes in adherent cells *in vitro* on a 96-well microplate and coverslips using standard laboratory equipment

(Figure 1). In addition, we provide the directions to automate the image acquisition and quantification of the macropinocytic index using a cell imaging multi-mode plate reader. 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.

PROTOCOL:

1. Preparation of materials

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

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

Dissolve DAPI in ddH$_2$O to obtain a 1 mg/mL solution. Store aliquots at −20 °C.

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

On the day of fixation, freshly prepare 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.

Prepare 24-well tissue culture plate with acid-washed coverslips.

Using a 500 ml beaker and water bath, heat 28 g of borosilicate glass coverslips with a 12 mm diameter and #1.5 thickness for 24 hours 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.

Wash the coverslips with distilled water. Repeat 4 times.

Wash the coverslips with 95% ethanol. Repeat 4 times.

The acid-washed coverslips can now be stored 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

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

Detach the cells from the plate by adding 1.5 mL prewarmed 0.25% trypsin and incubate 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.

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

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

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

From this point, the cells can be seeded 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.

24-well plate with coverslip format

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.

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

Seed the cells on top of the coverslip by adding 500 μL of 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.

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 hours.

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 prewarmed DPBS.

96-well microplate format

Transfer the cell suspension to a 25 mL reagent reservoir and, using a multi-channel pipette (8 or 12 channels), seed the cells into a black 96-well high-content screening microplate with optically-clear cyclic olefin or glass bottom by adding 100 μL of 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.

The day before macropinosome labeling, remove and discard the media from each well using a multi-channel pipette (8 or 12 channels) or a multi-channel aspiration adapter for standard tips.

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

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.

NOTE: 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

24-well plate with coverslip format

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 minutes.

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.

Aspirate the media and gently but quickly wash the cells five times with ice-cold PBS using a precooled wash bottle.

NOTE: Firmly shaking the plate by hand during washes may aid in dislodging dextran aggregates that become stuck to the coverslips.

Fix the cells by adding 350 μL of 3.7% formaldehyde for 20 minutes.

Aspirate the fixation solution and wash the cells with PBS twice.

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

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

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

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

NOTE: When gripping the coverslips, excess PBS can be removed by gently tapping the side of the coverslips to a Kimwipe.

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

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.

96-well microplate format

Aspirate the wells using a multi-channel 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 minutes.

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.

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

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 2X.

NOTE: Cells that weakly attach to the imaging microplate may detach during this process. If required, wells can also be aspirated with a multi-channel aspiration adapter or more gently washed with PBS using a multi-channel pipette.

NOTE: 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.

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

Remove the fixation solution and wash the cells with PBS twice using the submerging and flicking technique. Stain the nuclei with 40 μL of 2 μg/mL DAPI in PBS per well.

After 20 minutes, rinse the cells thrice with ice-cold PBS using the submerging and flicking technique described above.

Following the disposal of the PBS from the last rinse, remove any residual PBS by tapping the microplate upside-down onto a Kimwipe and add 100 μL of fresh PBS to each well using a 25 ml reagent reservoir and multi-channel pipette. Cells can now be imaged or stored 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).

Before imaging, let the plate equilibrate to room temperature and wipe the plate dry with a Kimwipe.

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 we will describe how to acquire images using a Cytation 5. However, the protocol below can be tailored to each individual instrument by adhering to the following guidelines:

Create an automation protocol in which images will be acquired 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 autofluoresence. 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.

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.

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

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

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

24-well plate with coverslip format

Start the Cytation 5 plate reader and insert the microscope slides upside down using the slide holder.

Open the Gen5 microplate reader and imaging software and create a new protocol by clicking 'Protocols', and 'Create New…'. Double click 'Procedure' and select the plate type.

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

Now access the imaging settings by selecting 'Actions', 'Image', 'Inverted imager' and clicking 'OK'.

Use the 40X PL FL Phase objective with 'Wide FOV' and 'Autofocus binning'.

For the first channel, select the LED cube that corresponds to the dextran fluorophore-label (GFP or RFP). Unclick 'Auto Exposure' and optimize exposure settings by clicking the microscope icon button. When the appropriate exposure settings have been determined click 'Save settings'.

NOTE: It is recommended to 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.

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

Set the autofocus settings for each fluorescence channel by selecting '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 '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.

NOTE: 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.

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 '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.

To complete the adjustment of imaging settings, click 'OK'.

Image the coverslips by selecting 'Create a new experiment and read now' from the 'Protocol Tools'. Save the protocol and experiment when prompted.

96-well microplate format

Start the Cytation 5 plate reader and insert the microplate.

Open the Gen5 microplate reader and imaging software and create a new protocol by clicking 'Protocols', and 'Create New…'. Double click 'Procedure' and select the plate type.

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

Now access the imaging settings by selecting 'Actions', 'Image', 'Inverted imager' and clicking 'OK'.

Use the 40X PL FL Phase objective with 'Wide FOV' and 'Autofocus binning'.

For the first channel, select the LED cube that corresponds to the dextran fluorophore-label (GFP or RFP). Unclick 'Auto Exposure' and optimize exposure settings by clicking the microscope icon button. When the appropriate exposure settings have been determined click '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, it is recommended to 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 optimization of the exposure setting in the macropinosome quantification, as the fluorescence has decreased as a result of bleaching during the optimization.

NOTE: It is recommended to 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.

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

Set the autofocus settings for each fluorescence channel by selecting '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 '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.

NOTE: For the laser autofocus option to work properly the bottom of the plate should be clean and it is thus recommended to dry and wipe the plate with a Kimwipe before imaging.

NOTE: 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.

Set the horizontal and vertical offset to zero and under 'Single image' select 'Montage' with 'No overlap' and use 3×3 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, we do not

observe differences in data interpretation between a 2×2 or 4×4 photo frame, although variation between replicate samples may increase when taking less pictures (Figure 5A and 5B). 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 hours to scan completely using a 3×3 frame. A 2×2 and 4×4 frame will halve or double that time, respectively.

To complete the adjustment of imaging settings, click 'OK'.

Now, image the plate by selecting 'Create a new experiment and read now' from the 'Protocol Tools'. Save the protocol and experiment when prompted.

5.   Determining the macropinocytic index

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:

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

Using an image with high dextran signal, determine the minimum intensity signal setting that is required to select only the macropinosomes, frequently called the threshold function. Do the same for the DAPI image, here selecting the nuclei.

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.

For the DAPI image, use the selection to determine the number of nuclei in the image, which reflects the number of cells present.

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

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

Below, we will describe how to analyze the images acquired with a Cytation 5 using the Gen5 built-in software. We have validated this built-in analysis pipeline and have not detected differences in computation relative to Fiji/ImageJ (Figure 6A). Follow these instructions for determining the macropinocytic index when using Gen5 software:

After the imaging is completed, select an image with a high level of macropinocytosis and remove background signal by clicking 'Process…' (Supplementary Figure 1A) and selecting the 'Image Preprocessing' option.

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.

For the DAPI channel use 'Auto' preprocessing and 1 smooth cycle. Click 'OK' and add the image preprocessing step to the protocol by clicking 'ADD STEP'.

Now select the processed image, under the 'Image' roll-out (Supplementary Figure 1B) and click the 'Analyze…' button (Supplementary Figure 1C).

Under 'ANALYSIS SETTINGS', set the 'Type' to 'Cellular Analysis', select the DAPI channel and click 'Options' (Supplementary Figure 2A).

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 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 size may need to be adjusted depending on the cell line and are most commonly set to be 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.

Next optimize the settings for selecting the macropinosome fluorescent puncta by applying a secondary mask. Use the "Measure within a Secondary mask" function and expand the primary mask by 40 μm depending on the size of the cells.

Use the 'Threshold' function and 'Threshold in mask' method to select the dextran positive areas. Click '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).

After creating appropriate masks to select nuclei and macropinosomes, click the 'Calculated Metrics' tab and select 'Select or create object-level metrics of interest'.

Remove all metrics present and add the 'Integral' and 'Area' metrics for analysis of the secondary mask. Click 'OK' and select 'Calculate' and 'Show' for the new metrics.

When finished click 'OK' and select 'ADD STEP' to add the analysis and calculations to the protocol.

Save the finalized protocol for future use by clicking 'File' and 'Save Protocol As…'.

After the data analysis has finished, the data can now be used for determining the macropinocytic index by selecting the metrics of interest and exporting the data. The macropinocytic index can be determined 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 to calculate 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.

The protocol for imaging and including the subsequent automated analysis can now be saved and reused 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.

NOTE: 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.

Prepare the cells as in section 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.

Without removing the media from the well, add one well-volume of treatment solution to each well.

Shake the plate to assure proper mixing.

Incubate the cells for the desired amount of time.

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. It may therefore be preferred to add dextran directly to the wells without aspirating or alternatively re-add the treatments or reuse the conditioned media for preparation of the dextran solution.

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 minutes 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 hours (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, 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, we used the

96-well microplate format protocol to determine constitutive macropinocytosis in a cell line previously not assessed in our lab, the PATU8998T PDAC cells. To determine whether these cells exhibit macropinocytosis, we studied the effect of two known inhibitors of dextran uptake: EHop-016 and EIPA. As illustrated, the dose response experiment gradually decreased the macropinocytic index at higher drug concentrations (Figure 6A and 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.

DISCUSSION:

The quality of the experiments and data acquisition highly depend 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 and 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 by 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 work-around 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 with an evaluation of 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 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 artefacts 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, adaption of this protocol to high-content screening would greatly facilitate the identification of 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS:

REFERENCES:

1. Lin XP, Mintern JD & Gleeson PA Macropinocytosis in Different Cell Types: Similarities and Differences. Membranes. 10 (8), 21, (2020).

2. Recouvreux MV & Commisso C Macropinocytosis: A Metabolic Adaptation to nutrient Stress in Cancer. Frontiers in Endocrinology. 8, (2017).

3. Zhang YJ & Commisso C Macropinocytosis in Cancer: A Complex Signaling Network. Trends in Cancer. 5 (6), 332–334, (2019). [PubMed: 31208695]

4. Commisso C et al. Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. Nature. 497 (7451), 633–+, (2013). [PubMed: 23665962]

5. Kim SM et al. PTEN Deficiency and AMPK Activation Promote Nutrient Scavenging and Anabolism in Prostate Cancer Cells. Cancer discovery. 8 (7), 866–883, (2018). [PubMed: 29572236]

6. Jayashankar V & Edinger AL Macropinocytosis confers resistance to therapies targeting cancer anabolism. Nature Communications. 11 (1), (2020).

7. Kamphorst JJ et al. Human Pancreatic Cancer Tumors Are Nutrient Poor and Tumor Cells Actively Scavenge Extracellular Protein. Cancer Research. 75 (3), 544–553, (2015). [PubMed: 25644265]

8. Olivares O et al. Collagen-derived proline promotes pancreatic ductal adenocarcinoma cell survival under nutrient limited conditions. Nature Communications. 8, (2017).

9. Seguin L et al. Galectin-3, a Druggable Vulnerability for KRAS-Addicted Cancers. Cancer discovery. 7 (12), 1464–1479, (2017). [PubMed: 28893801]

10. Redelman-Sidi G et al. The Canonical Wnt Pathway Drives Macropinocytosis in Cancer. Cancer Research. 78 (16), 4658–4670, (2018). [PubMed: 29871936]

11. Tejeda-Munoz N, Albrecht LV, Bui MH & De Robertis EM Wnt canonical pathway activates macropinocytosis and lysosomal degradation of extracellular proteins. Proceedings of the National Academy of Sciences of the United States of America. 116 (21), 10402–10411, (2019). [PubMed: 31061124]

12. Schmees C et al. Macropinocytosis of the PDGF beta-receptor promotes fibroblast transformation by H-RasG12V. Molecular Biology of the Cell. 23 (13), 2571–2582, (2012). [PubMed: 22573884]

13. Lee S-W et al. EGFR-Pak Signaling Selectively Regulates Glutamine Deprivation-Induced Macropinocytosis. Developmental Cell. 50 (3), 381–+, (2019). [PubMed: 31257175]

14. Recouvreux MV et al. Glutamine depletion regulates Slug to promote EMT and metastasis in pancreatic cancer. Journal of Experimental Medicine. 217 (9), (2020).

15. Zhang Y et al. Macropinocytosis in Cancer-Associated Fibroblasts is Dependent on CaMKK2/ ARHGEF2 Signaling and Functions to Support Tumor and Stromal Cell Fitness. Cancer discovery. 10.1158/2159-8290.Cd-20-0119, (2021).

16. Su H et al. Cancer cells escape autophagy inhibition via NRF2-induced macropinocytosis. Cancer cell. 10.1016/j.ccell.2021.02.016, (2021).

17. Bar-Sagi D & Feramisco JR Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science (New York, N.Y.). 233 (4768), 1061–1068, (1986).

18. Mishra R et al. Stromal epigenetic alterations drive metabolic and neuroendocrine prostate cancer reprogramming. Journal of Clinical Investigation. 128 (10), 4472–4484, (2018).

19. Commisso C, Flinn RJ & Bar-Sagi D Determining the macropinocytic index of cells through a quantitative image-based assay. Nature Protocols. 9 (1), 182–192, (2014). [PubMed: 24385148]

20. Galenkamp KMO, Alas B & Commisso C Quantitation of Macropinocytosis in Cancer Cells. Methods in molecular biology (Clifton, N.J.). 1928 113–123, (2019).

21. Wang JTH, Teasdale RD & Liebl D Macropinosome quantitation assay. MethodsX 1 36–41, (2014). [PubMed: 26150932]

22. Lee S-W, Alas B & Commisso C Detection and Quantification of Macropinosomes in Pancreatic Tumors. Methods in molecular biology (Clifton, N.J.). 1882 171–181, (2019).

23. Williams T & Kay RR High-throughput Measurement of Dictyostelium discoideum Macropinocytosis by Flow Cytometry. Jove-Journal of Visualized Experiments. 10.3791/58434 (139), (2018).
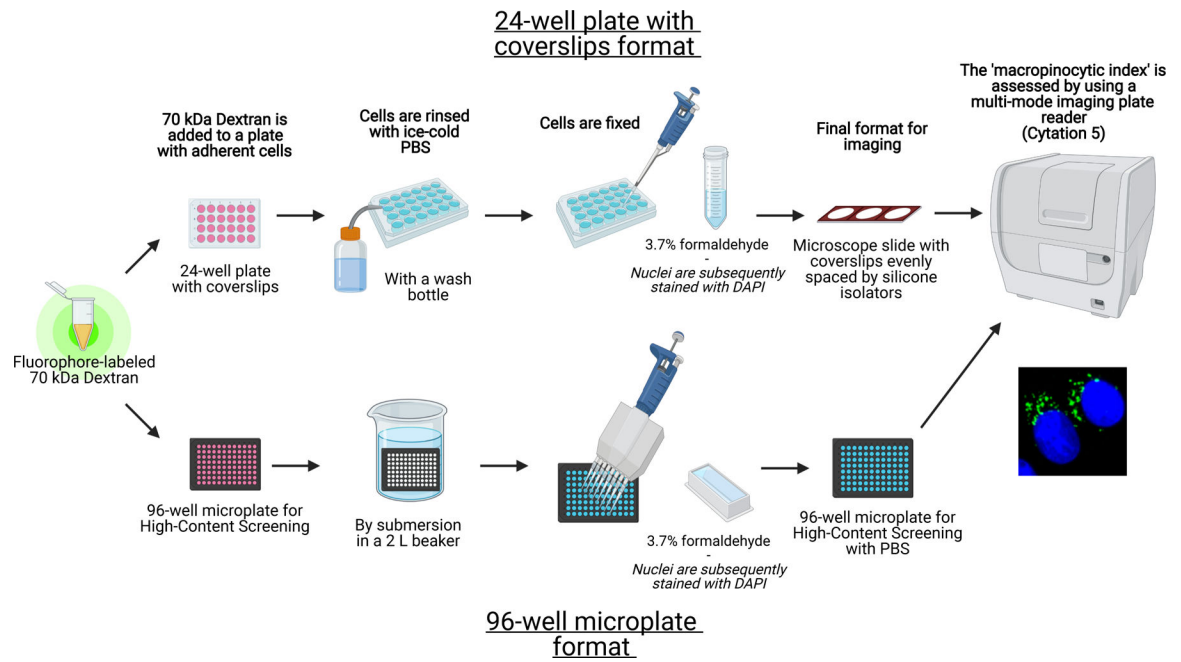
24-well plate with coverslips format

70 kDa Dextran is added to a plate with adherent cells

24-well plate with coverslips

Cells are rinsed with ice-cold PBS

With a wash bottle

Cells are fixed

3.7% formaldehyde - *Nuclei are subsequently stained with DAPI*

Final format for imaging

Microscope slide with coverslips evenly spaced by silicone isolators

The 'macropinocytic index' is assessed by using a multi-mode imaging plate reader (Cytation 5)

Fluorophore-labeled 70 kDa Dextran

96-well microplate for High-Content Screening

By submersion in a 2 L beaker

3.7% formaldehyde - *Nuclei are subsequently stained with DAPI*

96-well microplate for High-Content Screening with PBS

96-well microplate format

Figure 1: Schematic of the automated assay to determine the 'macropinocytic index' in adherent cells.
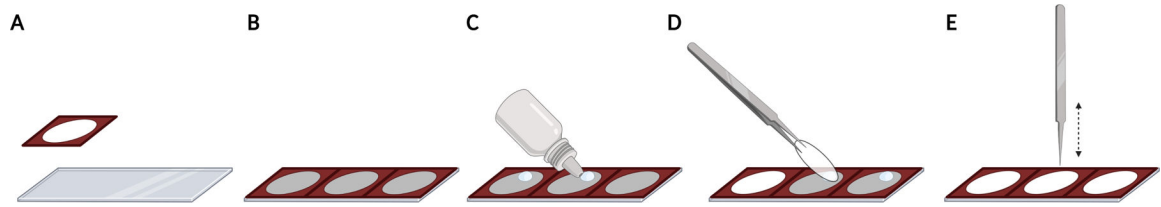
Figure 2: Placing coverslips on a microscope slide with silicone isolators.
(A) Silicone isolators are pressed and adhered to a microscope slide. **(B)** The entire microscope slide can be populated with a total of 3 isolators, resulting in even spacing and reproducible localization of the coverslips. **(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 a closed forceps to remove bubbles.
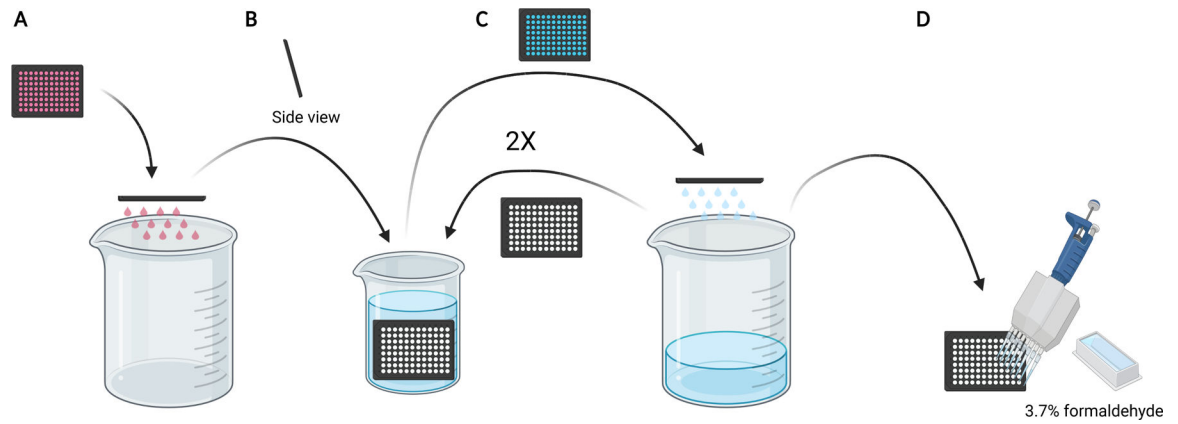
Figure 3: Rinsing the 96-well microplate to prepare for fixation.
(A) Empty the microplate of media into a 5 L beaker by manually flicking. **(B)** Vertically and at a slight angle, slowly submerge the microplate in a 2 L beaker filled with ice-cold PBS. **(C)** Empty the microplate of PBS into the 5 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 multi-channel pipette.
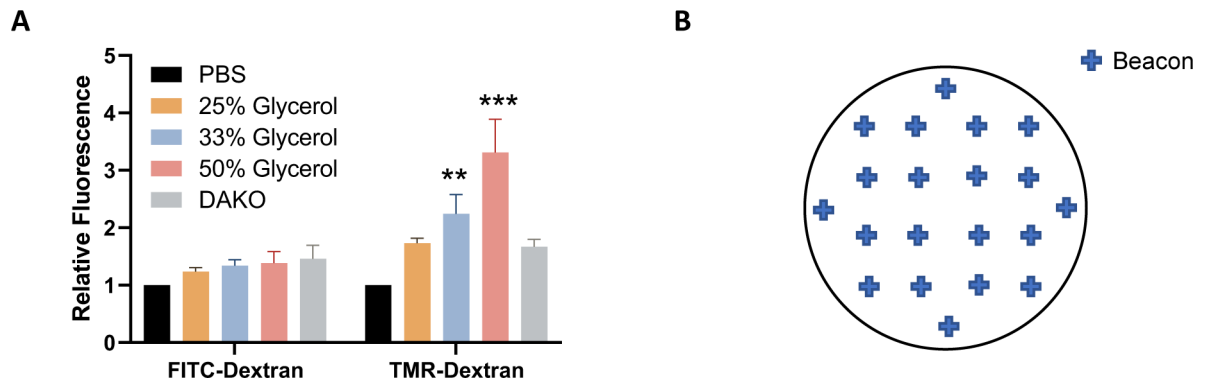
Figure 4: Optimization of conditions for image acquisition.
(A) Increasing the glycerol concentration increases TMR-dextran fluorescence, as determined in AsPC-1 cells treated with EGF. **(B)** Example coordinates of imaging beacons for automatic image acquisition when using the 24-well plate with coverslips format. Bar graph shows the average relative fluorescence with S.E.M. of 5 experiments. Statistical significance was determined by two-way ANOVA, relative to PBS. **, $P < 0.01$; ***, $P < 0.001$.
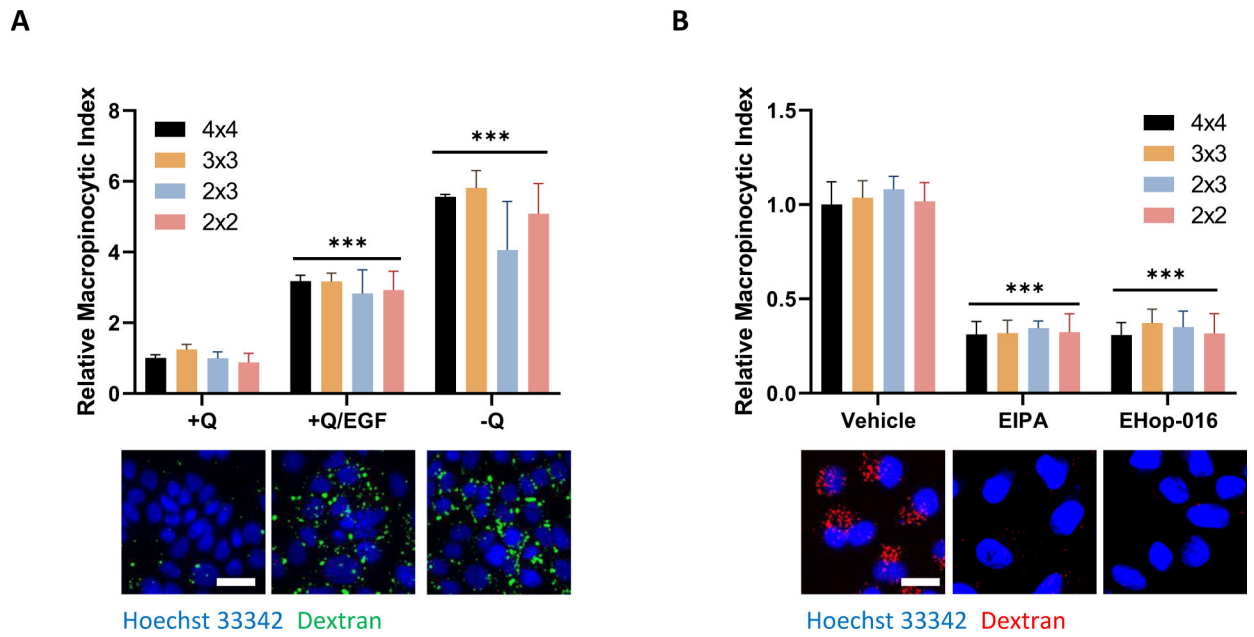
Figure 5: Control conditions for assessing macropinocytosis in PDAC cells.
(A) AsPC-1 cells display macropinocytosis in response to 100 ng/ml EGF stimulation for 5 minutes or glutamine deprivation for 24 hours. For image acquisition, picture frames of 4 by 4, 3 by 3, 2 by 3, or 2 by 2 were taken to determine the influence of the number of photos on data quality. **(B)** MIA PaCa-2 cells show constitutive macropinocytosis that is inhibited by 30-minute treatment with 75 μM EIPA or 2-hour 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 S.D. of 1 experiment with 4 replicates. Statistical significance was determined by two-way ANOVA, relative to the +Q or vehicle condition. ***, $P < 0.001$
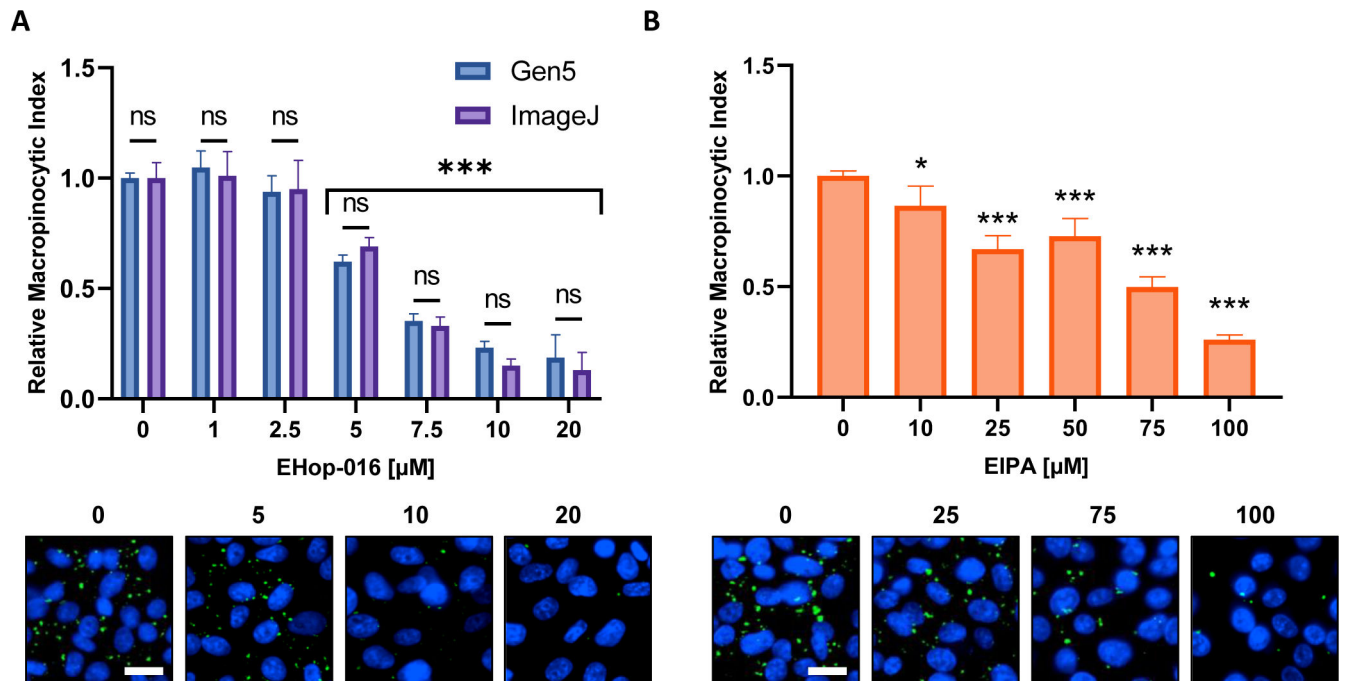
Figure 6: Performing a dose-response curve for macropinocytosis inhibitors.
(**A**) and (**B**) Example data obtained when testing known macropinocytosis inhibitors in a new cell line. PATU8998T cells were used for the 96-well microplate format and treated for 2 hours and 30 minutes with the indicated concentrations of EHop-16 (**A**) and EIPA (**B**), 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 S.D. of a single experiment with 4 replicates. Statistical significance was determined by one- or two-way ANOVA, compared to untreated condition. *, $P < 0.05$; ***, $P < 0.001$.
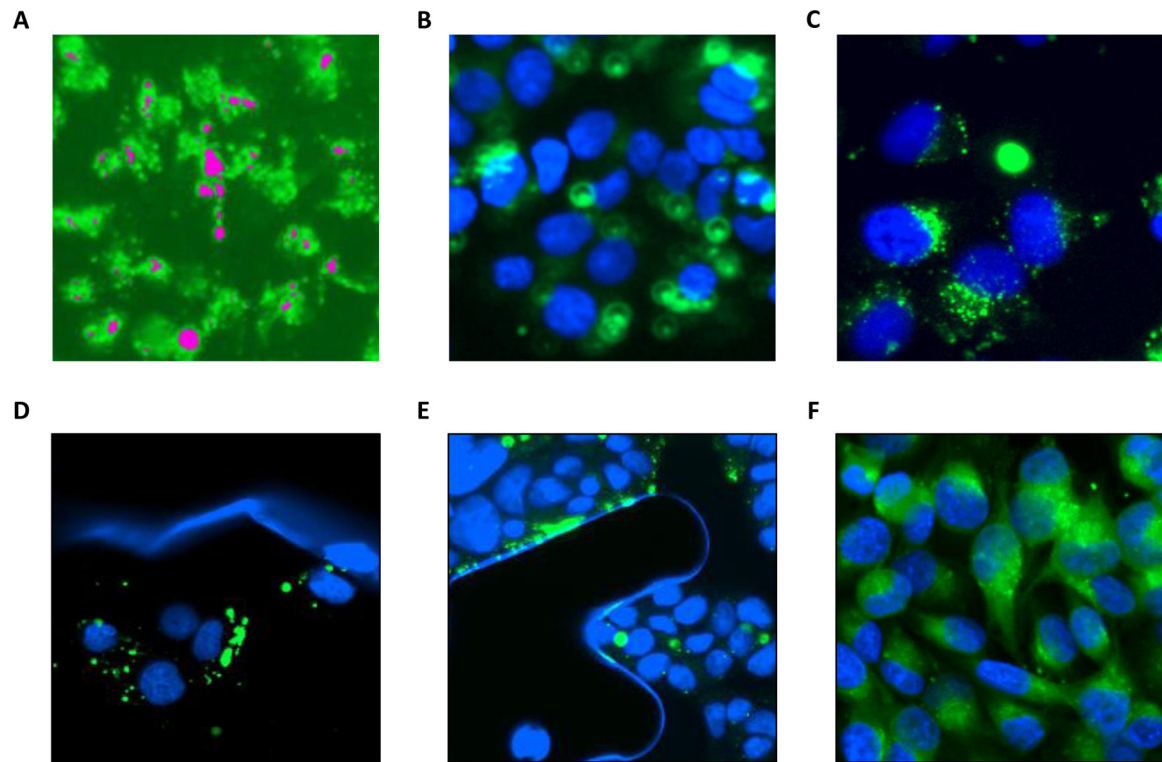
Figure 7: Trouble-shooting conditions that may be encountered.
(A) The image is overexposed, as indicated in pink. **(B)** The image is out-of-focus. **(C)** The image contains dextran blotches or smudges. **(D)** The image contains residue. **(E)** The image contains a bubble. **(F)** The cells show drug-induced autofluorescence.

Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25% Trypsin	Corning	25053CI	0.1% EDTA in HBSS w/o Calcium, Magnesium and Sodium Bicarbonate
1.5 ml Microcentrifuge tube	Fisherbrand	05-408-129	
10-cm Tissue culture dish	Greiner Bio-One	664160	CELLSTAR
15 mL Centrifuge tube	Fisherbrand	07-200-886	
2 L Beaker	Fisherbrand	02-591-33	
24-well Tissue culture plate	Greiner Bio-One	662160	CELLSTAR
25 mL Divided reagent reservoir	Genesee Scientific Corporation	28-123	
25 mL Reagent reservoir	Genesee Scientific Corporation	28-121	
500 ml Beaker	Fisherbrand	02-591-30	
6-cm Tissue culture dish	Greiner Bio-One	628160	CELLSTAR
8-Channel aspiration adapter	Integra Biosciences	155503	
8-Channel aspiration adapter for standard tips	Integra Biosciences	159024	
95% Ethanol	Decon Laboratories Inc	4355226	
Ammonia-free glass cleaner	Sparkle	FUN20500CT	
Black 96-well high-content screening microplate	PerkinElmer	6055300	CellCarrier-96 Ultra
Cotton-tipped applicator	Fisherbrand	23-400-101	
Coverslips	Fisherbrand	12-545-80	12 mm diameter
Cytation 5 Cell Imaging Multi-Mode Reader	Biotek	CYT5FW	
DAPI	Millipore Sigma	5.08741	
Dextran 70 kDa - FITC	Life Technologies	D1822	Lysine-fixable
Dextran 70 kDa - TMR	Life Technologies	D1819	
DMSO	Millipore Sigma	D1435	
DPBS	Corning	21031CV	Without Calcium and Magnesium
Forceps	Fine Science Tools	11251-20	Dumont #5
Formaldehyde, 37%	Ricca Chemical	RSOF0010-250A	ACS Reagent Grade
Glycerol	Fisher BioReagents	BP229-1	
Hardening fluorescence mounting media	Agilent Tech	S302380-2	DAKO
Hoechst 33342	Millipore Sigma	B2261	
Hydrochloric acid (HCl)	Fisher Chemical	A144-212	Certified ACS Plus, 36.5 to 38.0%
Kimwipes	Kimberly-Clark	34155	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Miscroscope slides	Fisherbrand	12-544-1	Premium plain glass
Multichannel pipette	Gilson	FA10013	8 channels, 0.5–10 uL
Multichannel pipette	Gilson	FA10012	12 channels, 20–200 uL
Multichannel pipette	Gilson	FA10011	8 channels, 20–200 uL
Parafilm M	Pechiney	PM996	
Plastic wrap	Kirkland Signature	208733	Stretch-Tite
Silicone isolators	Grace Bio Labs Inc	664107	13mm Diameter × 0.8mm Depth ID, 25mm × 25mm
Slide adapter	Biotek	1220548	
Wash bottle	Fisherbrand	FB0340922C	