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PROTOCOL FOR: An optimized procedure for quantitative analysis of mitophagy with the mtKeima system using flow cytometry

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1 Works for me dx.doi.org/10.17504/protocols.io.bimhkc36

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ABSTRACT

Abstract: Mitophagy is the process by which mitochondria are selectively targeted and removed via autophagic machinery to maintain mitochondrial homeostasis in the cell. Recently, the development of flow cytometry-based assays that utilize the fluorescent mtKeima reporter system have allowed for quantitative assessment of mitophagy at a single cell level. However, clear guidelines for appropriate flow cytometry workflow and downstream analysis are lacking, and studies using flow cytometry in mtKeima-expressing cells often display incorrect and arbitrary binary mitophagic or non-mitophagic cutoffs that prevent proper quantitative analyses. In this paper, we propose a novel method of mtKeima data analysis which preserves subtle differences present within flow cytometry data, in a manner that ensures reproducibility.

Methods Summary: When used in conjunction flow cytometry, the fluorescent mtKeima reporter system facilitates the assessment of mitophagy at a single cell level. However, current experimental design and analysis methods inadequately capture biological shifts in measured cell populations. In this report, we propose an outline for the transfection of mtKeima into a cell monolayer, cell preparation and downstream analysis using flow cytometry and associated software, with emphasis on a novel data processing technique.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Winsor NJ, Killackey SA, DJ Philpott, SE Girardin. An optimized procedure for quantitative analysis of mitophagy with the mtKeima system using flow cytometry. BioTechniques, accepted (2020).

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KEYWORDS

mtKeima, mitophagy, ratio gating, gating strategies, flow cytometry assays

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GUIDELINES

- Utilize cell culture media appropriate to particular cell lines.
- Standard cell culture equipment, a flow cytometer equipped with a Violet 405nm and Yellow/Green 561nm laser with 605 or 610/20 filters and appropriate flow cytometry analysis software, such as FlowJo are all required.

MATERIALS

NAME	CATALOG #	VENDOR
Tissue Culture Plate, 6 Well	TCP20-6.SIZE.1	Bio Basic Inc.
pCHAC-mt-mKeima	72342	addgene
TrypLE™ Express Enzyme	12604013	Thermo Fisher Scientific
PBS		
Rainbow calibration beads	URCP-38-2K	SpheroTech
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation	L10119	Thermo Fisher
HEK293T	CRL-3216	ATCC
Cell Culture Media (as required by cell line)		

DISCLAIMER:

Nathaniel J Winsor and Samuel A Killackey contributed equally to this work.

Cell transfection and staining:

1

Plate 1 x 10^6 wild-type (WT) HEK293T cells per 6 well plate overnight.

HINT: As mitophagic stimuli can decreases cell viability, we recommend pooling a minimum of two wells (2 x 10^6cells) per condition to ensure adequate cell numbers.

2

Transfect 0.4 µg/well of pCHAC-mt-mKeima Red(AddgenePlasmid #72342)with a 5:1 ratio of Fugene HD: plasmid (in µg) overnight

3

Treat cells with desired stimulus, dosage and timing.

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4

At conclusion of time point, aspirate media and add 500µL of TrypLE Express cell dissociation reagent (Gibco 12604-013) to each well, and incubate for 10 minutes at 37˚C, 5% CO2.

5

Collect cells and dilute in ten times total TrypLE volume with cold PBS (Wisen 311-425-CL) to inactivate cell dissociation reagent.

6

Pellet cells at 500xg for 5 minutes, and repeat PBS wash as above.

7

Aspirate PBS and incubate cells with 100µl of a viability stain (1:1000, Thermo Fisher, near-IR LIVE/DEAD L10119) for 10 minutes at room temperature, according to manufacturer's instructions.

8

Pellet stained cells at 500g for 5 minutes and resuspend in 200µL of cold PBS for flow cytometry analysis. Run flow analysis immediately.

ATTENTION: To ensure high cell viability, we recommend timing cell collection with cytometry analysis to ensure that dissociated cells are not left in PBS for more than one hour.

Cytometer settings:

9 Run cells on cytometer equipped with a Violet 405nm and Yellow/Green 561nm laser with 605 or 610/20 filters. If using a viability stain, ensure that the chosen cytometer is equipped with appropriate laser line.

HINT: When designing experiments be sure to always include relevant non-transfected (mtKeima-) and relevant unstained controls (fluorescence-minus-one, FMO) to facilitate flow cytometry gating. At minimum, a non-transfected control is required to facilitate accurate gating of mtKeima+ populations. Likewise, if using a viability stain, a relevant mtKeima+ viability FMO control population should be included to ensure accurate gating of viable cells. If the chosen treatments do not induce high levels of cell death, it may be useful to create a dead cell population by briefly fixing the cells in ethanol and to mix this population with an unfixed population at a 50:50 ratio prior to viability staining.

ATTENTION: Ensure that voltages are set accurately by using necessary unstained and stained controls. We have found that CCCP can induce high levels of fluorescence, particularly in the lysosomal channel between control and stimulated populations. Therefore, it is crucial to set voltages using both stimulated and unstimulated controls, as well as relevant single-stained samples, in order to ensure sufficient voltage to acquire data, but not set so high as to force the majority of mtKeima+ events off-scale. We recommend the

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use of linear axes when determining voltages for the violet 405nm and yellow/green 561nm lasers used with the mtKeima plasmid. Unlike logarithmic or biexponential axes,linear axes facilitate the visualization of more subtle differences in fluorescence, at the expense being able to capture disparate populations. However, unlike antibody staining of distinct cell populations, fluorescent differences are comparatively minor in the mtKeima assay, and as such non-linear axes can collapse and obscure relevant population differences. Once voltages have been determined in an initial experiment, we recommend using calibration particles (Spherotech, URCP-38-2K) at the beginning of each flow-cytometry session to ensure voltages are consistent (+/- 10%) across previous replicates.

ATTENTION: Traditionally, flow cytometers rely on algorithms to clearly separate populations when there is an overlap between distinct signals from different fluorochromes, in a process known as compensation. This requires the use of compensation controls, which are usually single-stained cells or beads, that facilitate the calculation of compensation values. However, in the case of mtKeima, as both YG610 and V605 emissions are encoded on the same plasmid, it is impossible to create the necessary single-positive compensation controls. Thus, if mtKeima is to be combined with other stains, compensation must be calculated manually. Alternatively, if mtKeima is used in conjunction with only one additional stain, such as a viability marker, it is simpler to use uncompensated data with the viability stain in a distinct laser line. We recommend using a far-red stain to decrease autofluorescence. However, it is still necessary to verify that there is minimal overlap between the viability stain and mtKeima+ channels, prior to running samples.

Gating and data export:

10 Separate cells from debris by optical gating based off forward and side scatter area

11 Select single cells based off the ratio of forward scatter area to forward scatter height.

12 Within the single cell population, select live cells as defined by low LIVE/DEAD staining, when graphing the total singe cell population by the viability dye laser line by forward or side scatter area.

13 Within the live population, graph appropriate mtKeima laser lines (V605 by YG610), and define the mtKeima+ population through the use of a non-transfected control.

14

Collect approximately 25,000 live, mtKeima+ events per condition and export to a flow analysis software

Determining frequency of lysosomal mitochondria:

15

After re-gating in the chosen cytometry analysis software, export 20,000 raw values of both the lysosomal and cytosolic events to a spreadsheet/graphing program, such as Microsoft Excel. Due to variation between in-cytometer gating and gating of exported flow cytometry standard (FCS) files, we have found it better to collect slightly more than 20,000 events to ensure that the number of analyzed events are consistent across experiments. While investigators can opt to capture a different number of events, we have found that measuring 20,000 cells allows for consistent readouts

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between biological replicates while diminishing run time on the flow cytometer, and initial numbers of cultured cells.

HINT: If using Flowjo, the raw coordinates values can be extracted as follows. To begin, open the context menu for the sample group of interest by right clicking on the name of the group. Select 'Export/ Concatenate Group' from the menu options, a secondary window will appear titled 'Group: Export or Concatenate'. In Flowjo 10.6 there are currently three export options; FCS, and two comma-separated values (CSV) options, CSV-channel and CSV-scale. As CSV is a spreadsheet format, select either of the CSV options from the drop-down menu. We opt to use CSV-scale values as it represents the uncompressed form of the data, however differences between channel and scale values are negligible for this analysis pipeline. It is still best to avoid alternating between formats across replicates. Next, select 20000 events to be exported by setting 'include no more than: 20000' in the include events option box. Then, under parameters select 'custom set of parameters'.A secondary window will appear titled 'Custom Parameter Set'. Within this dialogue box, select the lysosomal and cytosolic channels. After selecting 'OK' the dialogue box will close. Finally, click 'export'. Exported CSV files can then be opened in a spreadsheet/graphing software such as Microsoft Excel.

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Calculate the lysosomal/cytosolic ratio for all events. This value can then be averaged, or individual replicates from distinct experiments can be pooled (example: n=3, 60K events) such that both the mean and spread of data can be analyzed.

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