**Mito-Keima assay to assess mitophagy**

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**Buffers and reagents:**

* + Growth media: DMEM with 10% FBS, 4.5 g/l Glucose (Sigma, G8769), 1x GlutaMAXTM (ThermoFisher, 35050061), 1x MEM NEAA (ThermoFisher, 11140-050), 25 mM HEPES (1688449)
  + Antimycin-A (Sigma, A8674; made up in 100% Ethanol to 20 mg/ml), Oligomycin (Calbiochem, 495455; made up in DMSO to 10 mg/ml) and qVD (MedChemExpress, HY-12305; made up in DMSO to 10 mM)
  + 1x PBS
  + FACS buffer: 10% FBS, 0.5 mM EDTA in 1xPBS

**Procedures:**

1. HeLa cells were seeded the day before the treatment day in 24 well plates (each well contained 0.5 ml of growth media; 120,000 cells were seeded for penta KO expressing BFP-Parkin, mito-Keima (mtKeima) and GFP-OPTN or -NDP52; the number of cells of other cell lines were adjusted so that the next day they are all in similar confluency with penta KO expressing BFP-Parkin, mtKeima and GFP-OPTN or -NDP52).

Control cells include unstained cells (without any fluorescence proteins), cells that only express GFP-OPTN or GFP-NDP52 (to set up compensation so that GFP signal doesn’t bleach into mtKeima signal.

1. The next day, make sure the seeded cells are spreading out (not concentrated in the middle of the well because this can affect the results).
2. Aspirate off the old media and treat each well with 0.5 ml of growth media containing 4 μM Antimycin A, 10 μM Oligomycin and 10 μM QVD for indicated times (make sure all drugs are vortexed well, mix the media well after adding each drug). Treat the longest time points first.
3. 2 hours prior to harvesting, feed the untreated wells with 1 ml of warm growth media.
4. Aftrer treatment, harvest the cells by trypsinisation.

* Pre-chill eppies on ice.
* Aspirate the media thoroughly from the wells, wash the wells with 0.5 ml of 1x PBS (make sure swirl around after adding the PBS to wash the cells properly).
* Aspirate 1x PBS and add 150 μl of trypsin and incubate at 37 oC for 2-5 min (check under microscope to make sure all the cells were trypsinised properly).
* Add 300 μl of growth media to each well. Mix well with a P1000 and transfer the cells to cold eppies.
* Centrifuge the eppies at 1000x g for 2 min at 4 oC. Aspirate off the liquid.

1. Resuspend the cell pellets in 150 μl of FACS buffer, transfer to pre-chilled FACS tubes and keep the samples on ice in the dark for analysis.