Supporting Information

Mitochondria penetrating peptide conjugated TAMRA for live-cell long-term tracking

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S1. Materials and Methods

Material and peptide synthesis

All peptides and 6-(tetramethylrhodamine-5-(and-6)-carboxamido)hexanoic acid conjugates D-Argine-Phenylalanine-D-Argine- Phenylalanine- D-Argine-Phenylalanine-NH_2 (rFrFrF) (TAMRA-rFrFrF) were synthesized and purified by AnaSpec, EGT Corporation (Fremont, CA, USA). All other chemical reagents were purchased from Fisher Scientific, and used as received unless otherwise noted. Carbon dioxide (99.9% purity) was purchased from Airgas. All reagents and solvents were purchased from commercial suppliers and used without further purification.

Photophysical properties measurements

Linear absorption, fluorescence, and excitation spectra of TAMRA and TAMRA-rFrFrF were investigated in PBS at room temperature. The absorption spectra were measured with a Tecan Infinite M200 PRO plate reader spectrometer in 1 cm path length quartz cuvettes. The fluorescence and excitation spectra were obtained using an Edinburgh Instruments FLS980 fluorescence spectrometer.

Cell culture

HeLa and pig kidney LLC-PK1 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator.

Cell viability

To assess the cytotoxicity of unconjugated-rFrFrF and TAMRA-rFrFrF, HeLa and LLC-PK1 cells were cultured in DMEM cell medium supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin at 37 °C and 5% CO_2. Cells were placed in 96 well plates and incubated until there were no fewer than 5×10^3 cells per well for the experiments. Next, cells

were incubated with different concentrations of rFrFrF (1, 2.5, 5, 10, 20, 40, and 80 μM) and TAMRA-rFrFrF (1, 2.5, 5, 10, 20, 40, and 80 μM) for an additional 22 h, where the values within parentheses refer to the nominal concentrations of the peptides. After that, 20 μL of the CellTiter 96 Aqueous One solution reagent (for MTS assay) was added into each well, followed by further incubation for 2 h at 37 °C.[1]

Cytotoxicity of Mitotracker Green and Red was performed same procedure as unconjugated-rFrFrF and TAMRA-rFrFrF in HeLa cells with a various concentration of (0.1, 0.25, 0.5, 1, 2, 4, 8, and 16 μM).

The respective absorbance values were recorded with a Tecan Infinite M200 PRO plate reader spectrometer at 490 nm. Cell viabilities were calculated on the basis of the following equation,

$$Cell\ Viability(\%) = \frac{Abs^{s}{}_{490nm} - Abs^{D}{}_{490nm}}{Abs^{c}{}_{490nm} - Abs^{D2}{}_{490nm}} \times 100\%$$

where Abs^{s}_{490nm} is the absorbance of the cells incubated with different concentrations of experimental probe solutions, Abs^{D}_{490nm} is the absorbance of cell-free well containing only dye at the concentration that was studied, Abs^{c}_{490nm} is the absorbance of cells alone incubated in the medium, and Abs^{D2}_{490nm} is the absorbance of the cell-free well.

Oxygen consumption rate (OCR) measurement

Oxygen consumption rates were measured for HeLa cells by using Seahorse XFe96 Analyzer (Agilent, Santa Clara, CA, US). HeLa cells were placed in 96 well Seahorse XF96 cell culture plates at a density of 2×10^4 cells/well, and incubated DMEM supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin at 37 °C and 5% CO_2 for 16 h. Next, to the cells were added 5μM TAMRA-rFrFrF (in 1mM DMSO stock solution) for 6 h. A negative control was treated with DMSO only. After washing out TAMRA-rFrFrF, cells were incubated with XF Assay Medium for 1 h. Measurement of OCR was performed at baseline and following sequential injections of a) 1 μM oligomycin, (final conc. 0.8 μg/mL) (20 μL of 10 drops), an ATP synthase inhibitor, b) final conc. 2 μM FCCP (22 μL of 10 drops), a mitochondrial uncoupler, and c) 0.5 μM Rotenon + 0.5 μM antimycin A, (25 μL of 10 drops), a complex III and complex I inhibitor, respectively. This enabled the measurement of the OCR coupled to ATP production, as well as the maximal and the mitochondrial OCR, respectively.[2]

Colocalization study

To investigate the efficiency and specificity of TAMRA-rFrFrF, HeLa and LLC-PK1 cells were employed. All cells were seeded on confocal dish (MatTek) at the density of 4×10^4 cells per dish and incubated for 24 h at 37 °C. Stock solutions of TAMRA, rFrFrF, and TAMRA-rFrFrF dissolved in DMSO were prepared at a nominal concentration of 10^4 μM. The stock solution was diluted to 5 μM with the DMEM respectively and freshly placed over cells for a 1.5 h incubation period. Cells were washed three times with PBS and then post-incubated with fresh medium at 37 °C for various time periods (0 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 18 h, 1 d, 2 d, and 3 d). After post-incubation, cells were further incubated with

MitoTracker Green (MT Green)， Lysotracker Green (LT Green), or ER-tracker Green (ER Green) separately before cell imaging.[3] Cells were then washed with PBS three times, and then the live cell imaging solution (Molecular Probes) was added to confocal dishes. Fluorescence images were obtained using an inverted Olympus IX70 microscope coupled with a FITC filter cube (Ex: 482/40; DM: 506; Em: 534/40) for MT green, LT green, or ER Green, and a Texas Red filter cube (Ex: 562/40; DM: 593; Em: 624/40) for TAMRA or TAMRA-rFrFrF, respectively. Pearson's correlation coefficients for TAMRA and TAMRA-rFrFrF were calculated using Fiji, a freely available image processing software.

To further compare long-term tracking advantage of TAMRA-rFrFrF, incubation time-dependent cell imaging of MT green was performed in HeLa cells. Cells were incubate with 100 nM of MT green for 15 min at 37 °C. Cells were washed three times with PBS and then post-incubated with fresh medium at 37 °C for various time periods (0 h, 1 h, 2 h, 4 h, 6 h and 12 h). After post-incubation, cells were further incubated with 100 nM of Hoechst 33258 for 10 min and washed out with PBS before cell imaging.

Mitochondria uptake measurement

To evaluate the uptake of TAMRA-rFrFrF by mitochondria, 1×10^6 cells were incubated with 5 μM TAMRA-rFrFrF for 1.5 h at 37 °C. After washing twice with PBS, post-incubation was performed at 37 °C for 2 h, 4 h, 6 h, 12 h, 1 d, 2 d, and 3 d. Subsequently, mitochondria were extracted according with a Mitochondria Isolation Kit for cultured cells (Thermo Fisher Scientific, Waltham, MA, US), and diluted to 2 mL using PBS. The fluorescence intensity was measured using an Edinburgh Instruments FLS980 fluorescence spectrometer. Isolated mitochondria with no TAMRA-rFrFrF treatment served as the blank.[4]

S2. Supporting Figures

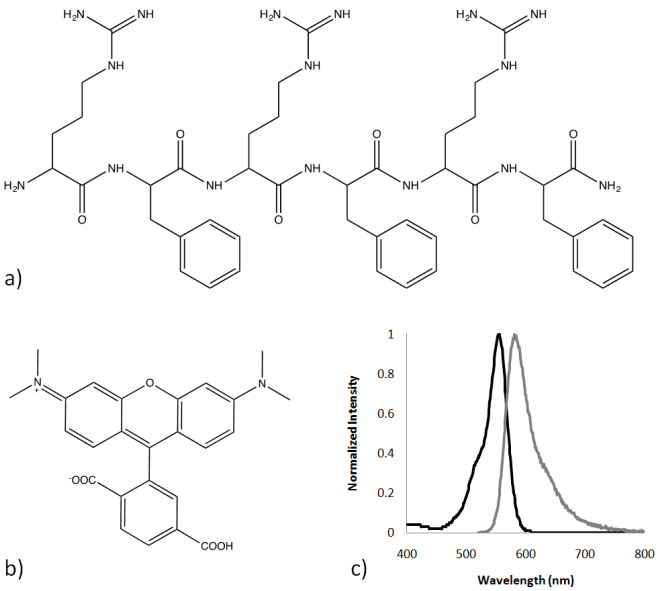


Figure S1. a) Molecular structure of unconjugated rFrFrF, b) molecular structure of TAMRA, c) normalized absorption and emission spectra of TAMRA-rFrFrF in PBS.

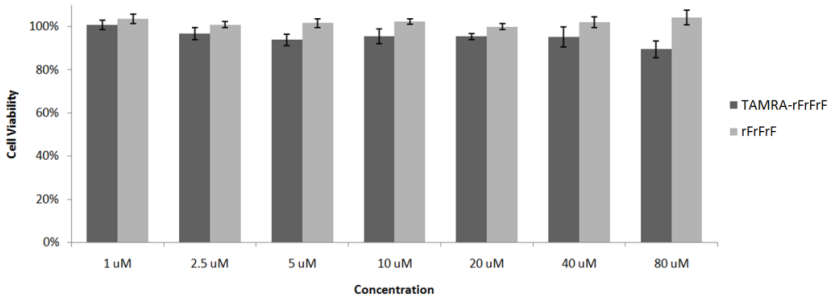


Figure S2. Cell viability of HeLa cells after treatment with TAMRA-rFrFrF and unconjugated rFrFrF at different concentrations. HeLa cells with no treatment was classified as 100%.

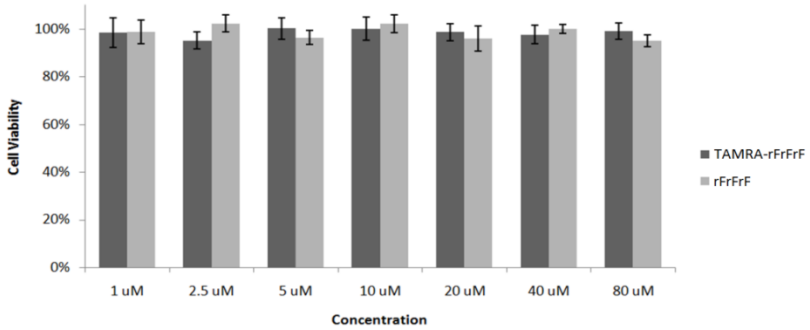


Figure S3. Cell viability of LLC-PK1 cells after treatment with TAMRA-rFrFrF and unconjugated rFrFrF at different concentrations. LLC-PK1 cells with no treatment was classified as 100%.

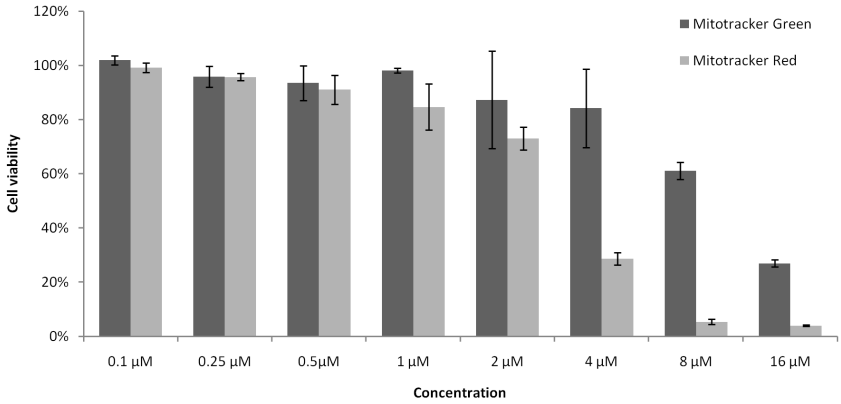


Figure S4. Cell viability of HeLa cells after treatment with Mitotracker Green and Red at different concentrations. HeLa cells with no treatment were classified as 100%.

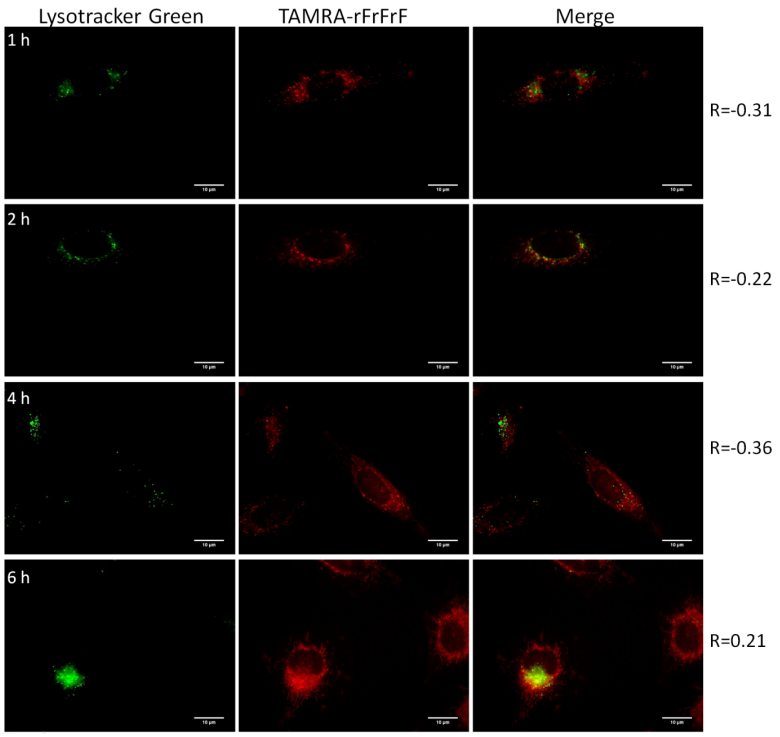


Figure S5. HeLa cells were incubated with 5 μM TAMRA-rFrFrF for 1.5 h, and, after washing out the TAMRA-rFrFrF, an extra incubation of the cells at 37 °C for various times (post-incubation time). Then, cells were incubated with 200 nM of Lysotracker Green for 1 h and washed out before cell imaging. Pearson's Value is labeled as R

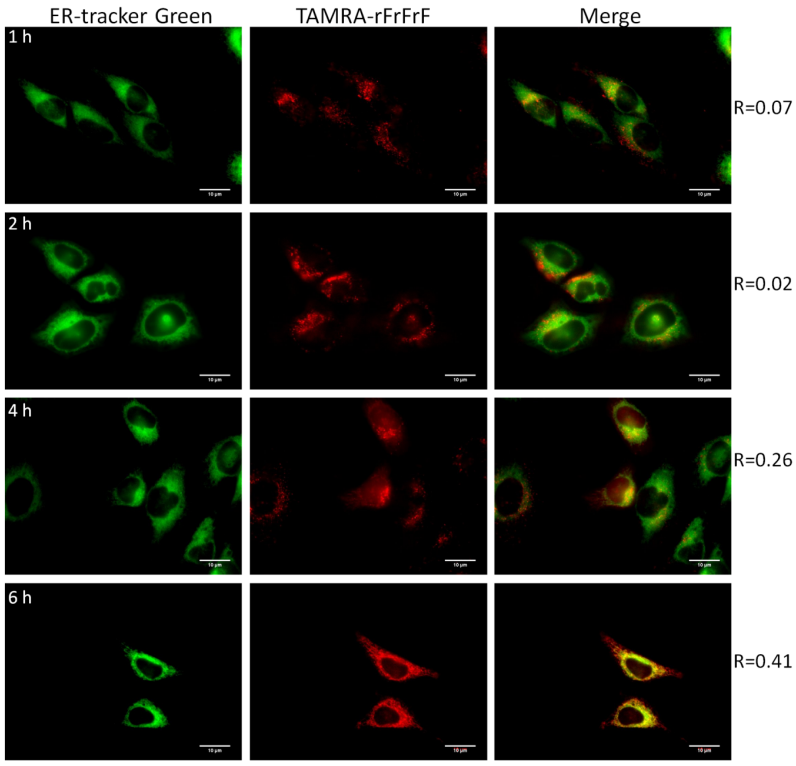


Figure S6. HeLa cells were incubated with 5 μM TAMRA-rFrFrF for 1.5 h, and, after washing out the TAMRA-rFrFrF, an extra incubation of the cells at 37 °C for various times

(post-incubation time). Then, cells were incubated with 1 μM of ER-tracker Green for 30 min and washed out before cell imaging. Pearson's Value is labeled as R

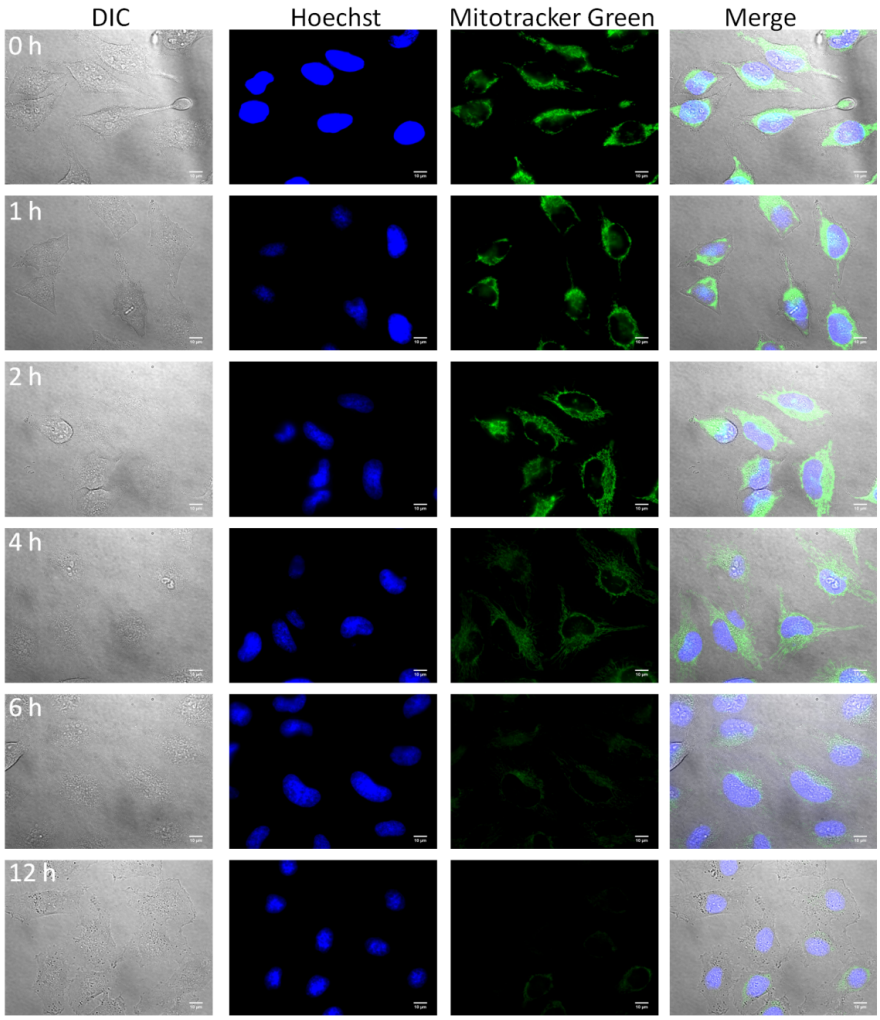
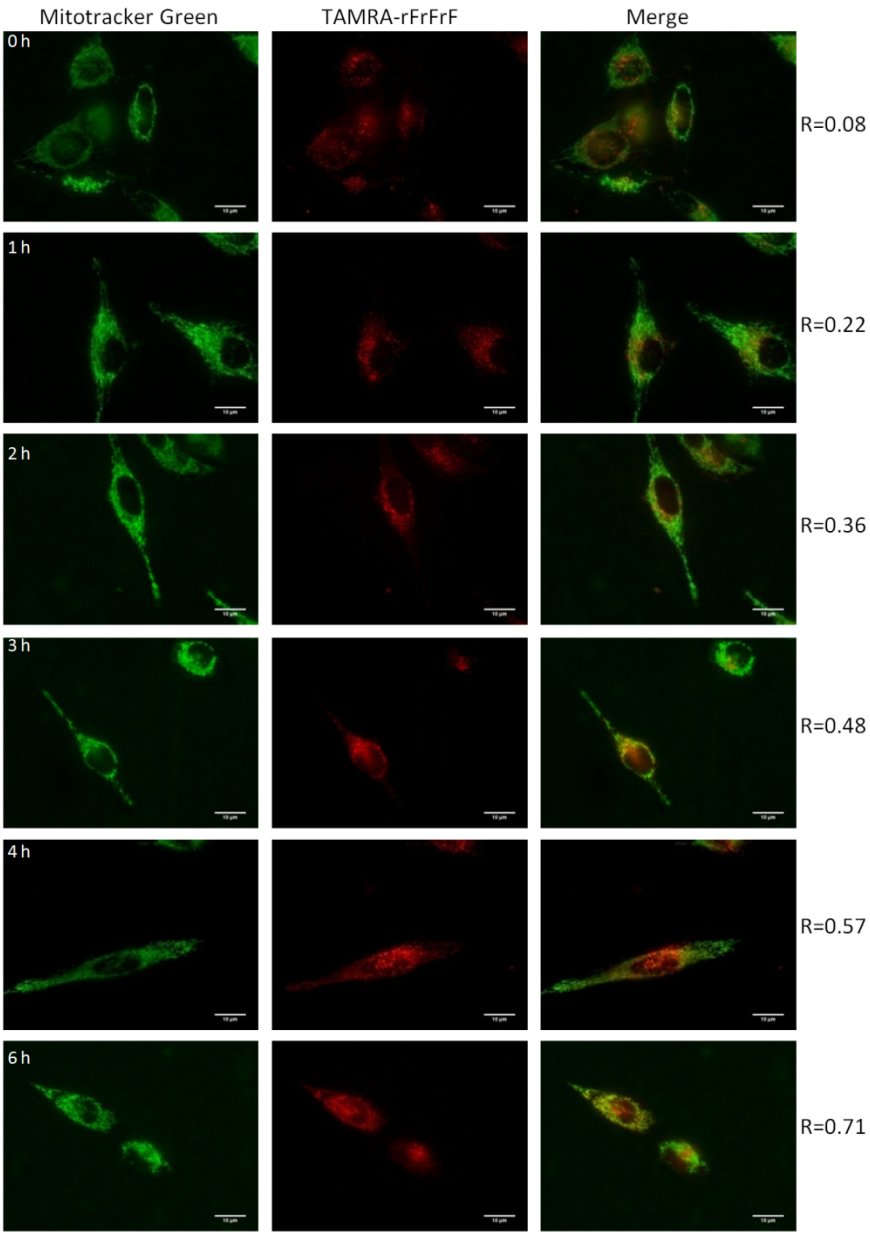


Figure S7. HeLa cells were incubated with 100 nM Mitotracker Green for 15 min, and, after washing out Mitotracker Green, an extra incubation of the cells at 37 °C for various times (post-incubation time). Then, cells were incubated with 100 nM of Hoechst 33258 for 10 min and washed out before cell imaging. Pearson's Value is labeled as R


Mitotracker Green
TAMRA-rFrFrF
Merge
0 h
1 h
2 h
3 h
4 h
6 h
R=0.08
R=0.22
R=0.36
R=0.48
R=0.57
R=0.71

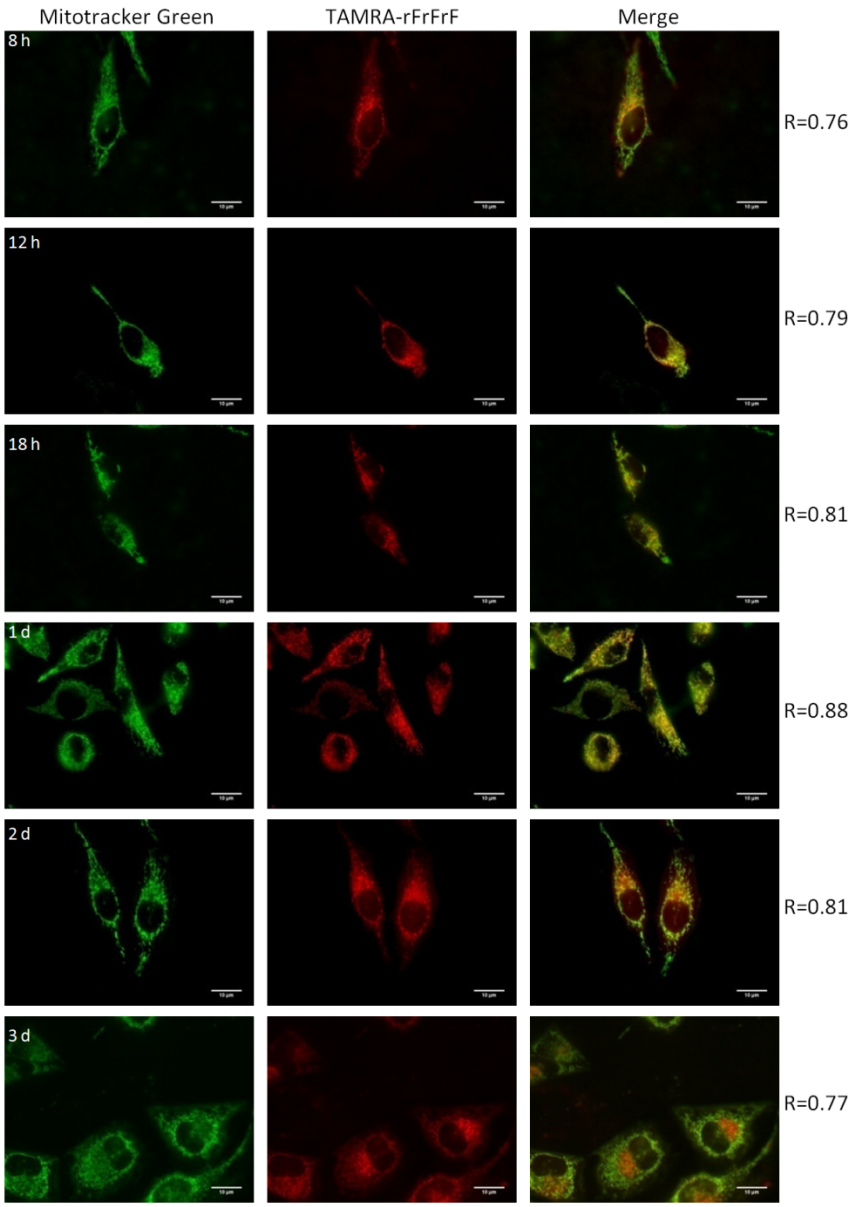
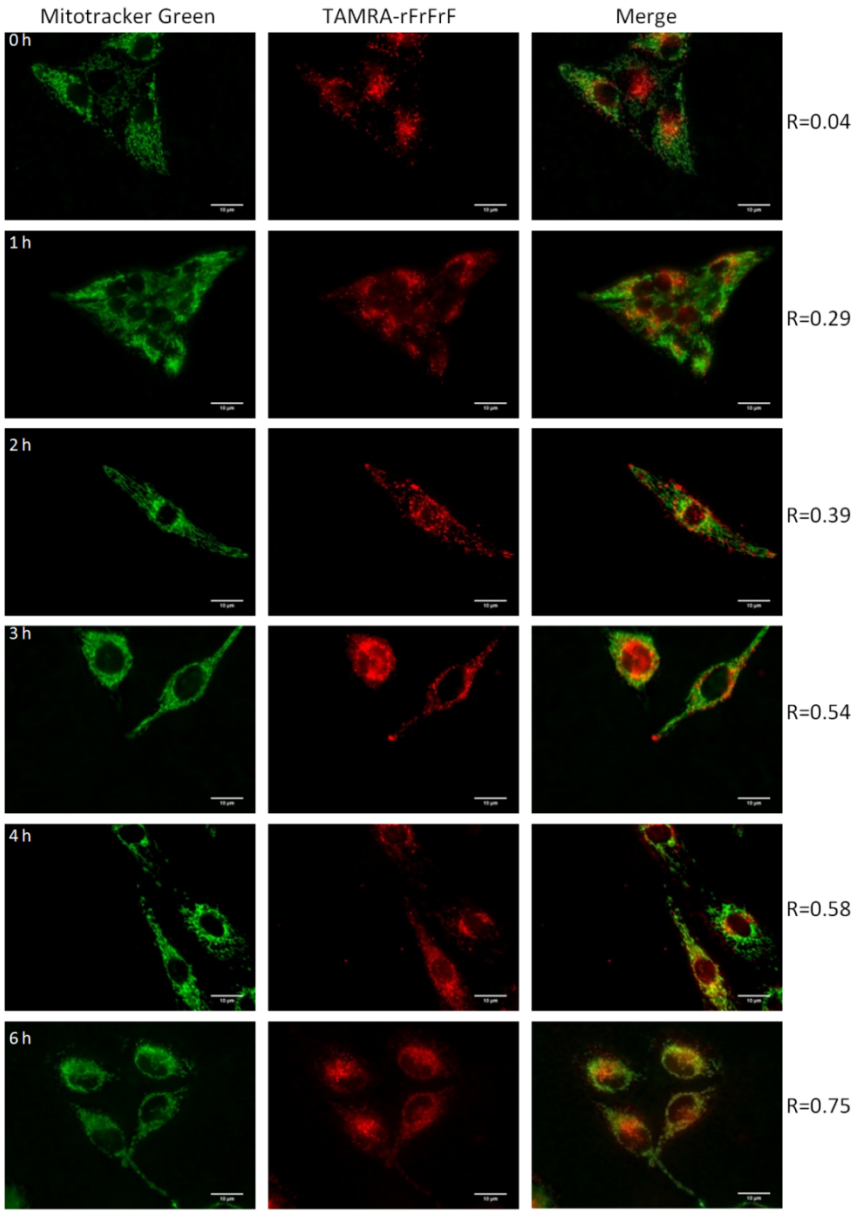


Figure S8. HeLa cells were incubated with 5 μM TAMRA-rFrFrF for 1.5 h, and, after washing out the TAMRA-rFrFrF, an extra incubation of the cells at 37 °C for various times (post-incubation time). Then, cells were incubated with 100 nM of Mitotracker Green for 15 min and washed out before cell imaging. Pearson's Value is labeled as R


Mitotracker Green
TAMRA-rFrFrF
Merge
0 h
1 h
2 h
3 h
4 h
6 h
R=0.04
R=0.29
R=0.39
R=0.54
R=0.58
R=0.75

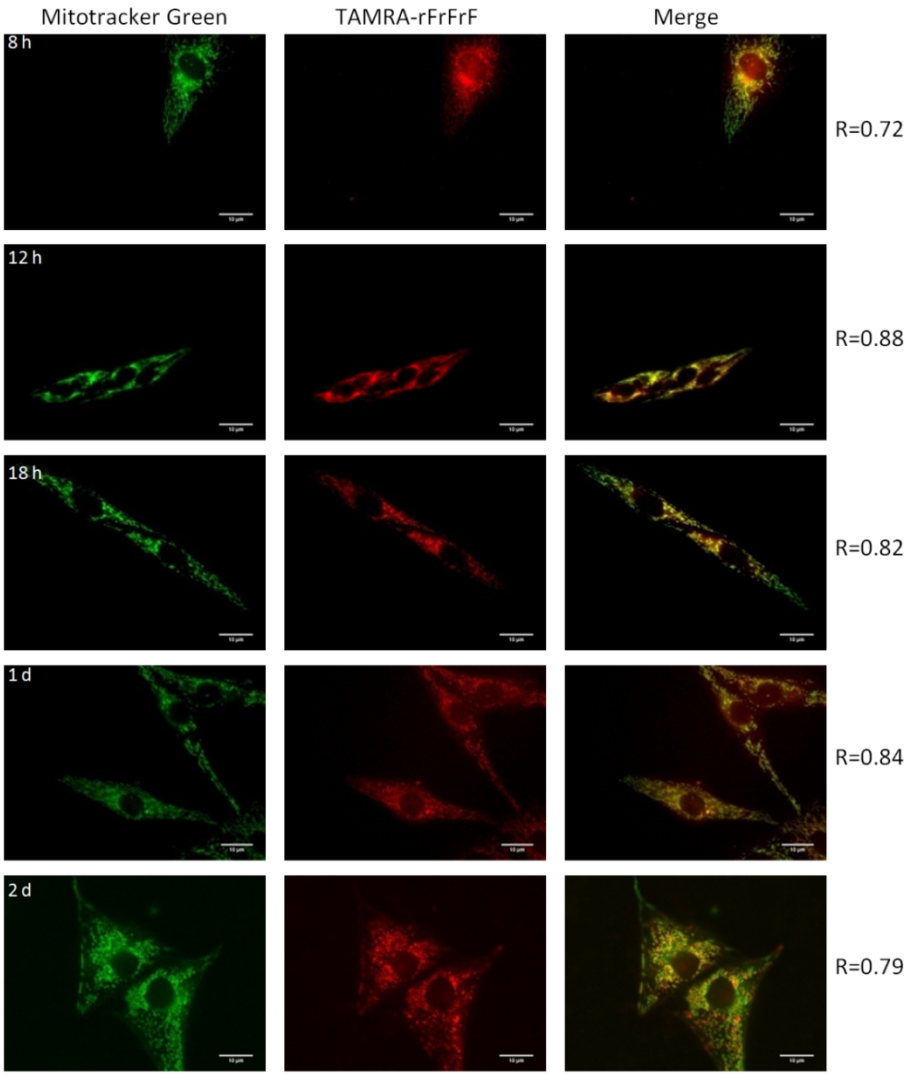


Figure S9. LLC-PK1 cells were incubated with 5 μM TAMRA-rFrFrF for 1.5 h, and, after washing out the TAMRA-rFrFrF, an extra incubation of the cells at 37 °C for various times (post-incubation time). Then, cells were incubated with 100 nM of Mitotracker Green for 15 min and washed out before cell imaging. Pearson's Value is labeled as R.

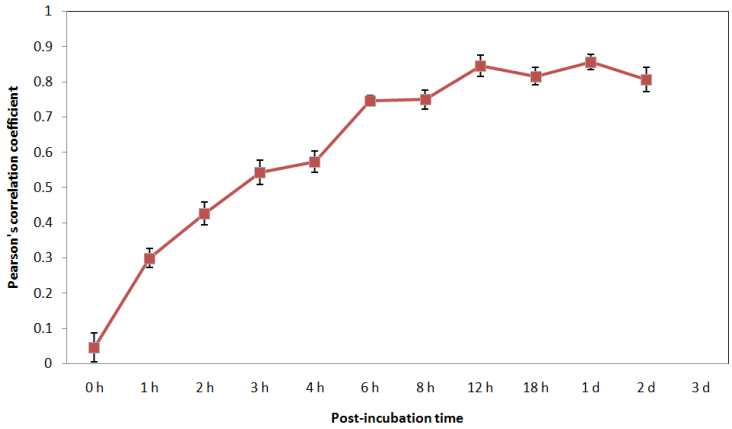


Figure S10. Post-incubation time-dependent Pearson's correlation coefficients of LLC-PK1 cells incubated with 5 μM TAMRA-rFrFrF.

References

(1) Liu, X., Ardizzone, A., Sui, B., Anzola, M., Ventosa, N., Liu, T., Veciana. J., and Belfield, K.D. (2017) Fluorenyl-loaded quatsome nanostructured fluorescent probes. *ACS Omega. 2,* 4112-4122.

(2) Kuntz, E. M., Baquero, P., Michie, A. M., Dunn, K., Tardito, S., Holyoake, T. L., Helgason, G. V., and Gottlieb, E. (2017) Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. *Nature Med. 23,* 1234-1240.

(3) Moriya K., Nagatoshi K., Noriyasu Y., Okamura T., Takamitsu E., Suzuki T., and Utsumi T. (2013) Protein N-myristoylation plays a critical role in the endoplasmic reticulum morphological change induced by overexpression of protein Lunapark, an integral membrane protein of the endoplasmic reticulum. *PLoS One. 8,* e78235.

(4) Zhang, Q., Raoof ,M., Chen, Y., Sumi, Y., Sursal, T., Junger, W., Brohi, K., Itagaki, K., and Hauser, C. J. (2010) Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature. 464,* 104-107.