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Supporting Information

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Supporting Information

for

Stable Long-Term Intracellular Labelling of Biological Cells by Fluorescently Tagged Cationic Magnetoliposomes

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Transmission electron microscopy: the average diameter of the iron oxide cores and MLs were measured by transmission electron microscopy (TEM). An aqueous dispersion of pure, lauric acid-stabilized iron oxide cores or of MLs was drop-cast on to a carbon copper grid, which was air-dried at room temperature before loading into the microscope (Zeiss, 10C A). For MLs, uranylacetate was used as a negative contrast agent to aid visualization of the phospholipids.

For cell internalisation experiments, cells were plated in 6-well plates (density: $1x10^4$ cells $well^{-1}$) containing coverslips (3 mm diameter). After 24 h incubation at 37°C in a 5% CO_2 atmosphere, the medium was replaced by complete medium containing 3.33% DSTAP-containing MLs (100 µg Fe mL^{-1}) and incubated as above for 24 h. The cells were then washed twice with phosphate-buffered saline (PBS), rinsed with sodium cacodylate and fixed for 1 h at room temperature with glutaraldehyde (2.5%) in sodium cacodylate (0.1 M, pH 7.4). The cells were rinsed with sodium cacodylate for several hours at room temperature and kept in this solution overnight at 4°C, then were brought to room temperature, rinsed once with sodium cacodylate and dehydrated by incubation in stepwise-increasing concentrations of alcohol (30-100%). The coverslip on which the cells were attached was mounted on a polypropylene epoxy resin which was polymerised inside a gel-like capsule; after polymerization (72 h at 60°C), the capsule was removed using liquid nitrogen and a thin film of resin containing the cells cut out and uranyl acetate was added.

Cell cultures: 3T3 fibroblasts were cultured in complete medium consisting of Dulbecco's Modified Eagle's medium (DMEM 31966-021) containing Glutamax™ (4 mM), pyruvate and D-glucose (4.5 g L^{-1}; Gibco) supplemented with foetal calf serum (10%) and penicillin/streptomycin (1%, all from Invitrogen). Cells were cultured in 75 cm^2 Falcon culture flasks (Greiner Bio-One) at 37°C in a humidified atmosphere with 5% CO_2. On reaching 80–90% confluency, they were trypsinized and split 1/3. Geneswitch™-Chinese Hamster Ovary (CHO) cells were cultured in F-12 Nutrient Mixture (Ham 31765-021, Gibco) containing foetal calf serum (10%), Glutamax™ (2mM), penicillin/streptomycin (1%) and hygromycin B (100 µg mL^{-1}, Invitrogen, Belgium). Neuroblastoma (N2a) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM 31966-021) containing Glutamax™ (4mM) pyruvate and D-glucose (4.5 g L^{-1}, Gibco) supplemented with foetal calf serum (5%) and penicillin/streptomycin (2%, Invitrogen). Human embryonic kidney (HEK293) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM 41965-039) containing L-glutamine and D-glucose (4.5 g L^{-1}, Gibco) supplemented with foetal calf serum (10%), Glutamax™ (2 mM), penicillin/streptomycin (2%) and non-essential amino acids (1%, 11140-035; Gibco). Human umbilical vein endothelial cells (HUVEC) were cultured in EBM-2/EGM-2 culture medium (Clonetics, San Diego, CA). All cells were cultured in 75 cm^2 Falcon culture flasks (Greiner Bio-One) and incubated at 37°C in a humidified atmosphere with 5% CO_2, and were trypsinized and split 1/3 at 80–90% confluency.

Cell viability and cellular iron content: Cellular viability was determined using the MTT assay, while cellular iron content was measured by protein determination using the bicinchoninic acid (BCA) assay and spectrophotometric determination of the total iron concentration using Tiron, as described by Soenen et al. [1]. The absorbance was measured on an ELISA plate reader (FLUOstar Optima plate reader, BMG LABTECH, Offenburg, Germany) at the appropriate wavelength (MTT and BCA 570 nm; Fe 490 nm).

Fluorescence and confocal microscopy: Samples to be examined by fluorescence or confocal microscopy were treated similarly. Cells were plated (density: $5x10^4$ cells/well in 500 µL) in Lab-Tek™ Chamber Slides™ (4 wells per plate) and incubated at 37°C in a 5% CO_2 atmosphere for 24 h, after which the medium was removed, the cells washed with PBS (2 x 500 µL) and fresh complete medium added containing the appropriate MLs at the desired concentration (100 µg of Fe mL^{-1}). The mixture was incubated for the desired time at 37°C in 5% CO_2, after which the medium con-

taining the non-internalized MLs was removed and the cells washed with PBS (2 x 500 µL) and fixed for 30 minutes at room temperature with paraformaldehyde (2%, 500 µL). The fixation mixture was removed and the cells washed with PBS (2 x 500 µL) prior to detaching the wells from the Permanox™ slide and mounting the plate by addition of 1 drop of Prolong® Gold anti-fade reagent with DAPI (Molecular Probes) per well and overnight incubation at room temperature. The slide was then mounted in either a fluorescence microscope (Eclipse TE2000, Nikon, Japan) or a confocal microscope (LSM510 confocal laser-scanning microscope, Zeiss, Germany). The specific conditions for each experiment are described in the appropriate paragraphs below.

Intracellular ML localisation: Co-localisation of the inner lipid layer of the MLs and endosomal structures was examined by incubating 3.33% DSTAP-containing MLs containing 1% β-BODIPY® FL C_5-HPC in their inner lipid layer (see above) with 3T3 fibroblasts for 4 h at 37°C in 5% CO_2. The medium containing the non-internalized MLs was removed and the cells washed with PBS (2 x 500 µL), incubated with Lyso-tracker Red (60 nM, 500 µL $well^{-1}$) for 1 h at room temperature, washed twice with PBS (500 µL) and fixed prior to examination by confocal microscopy. Retention of an intact phospholipid bilayer was examined by incubation of 3.33% DSTAP-containing MLs containing 1% β-BODIPY® FL C_5-HPC in the inner lipid layer and 0.1% DPPE-Lissamine™ Rhodamine B in the outer lipid layer (see experimental section) with 3T3 fibroblasts for 4 h at 37°C in 5% CO_2, after which the medium was removed, cells were washed twice with PBS (500 µL) and fresh medium, not containing any MLs, was added to allow continued incubation during 48 hours in order to allow any cell-bound particles to internalize. Subsequently, the medium was removed and cells were washed twice with PBS (500 µL) prior to fixation and confocal microscopy. In both cases, co-localisation was examined using the MBF-plugin, produced by Tony Collins, of the ImageJ software (NIH, USA).

Examination of ML exocytosis: To study possible exocytosis of the MLs, two 4-well plates of 3T3 fibroblasts were incubated with 3.33% DSTAP-containing MLs labelled with 0.25% DPPE-Lissamine™ Rhodamine B for 4 h at 37°C in 5% CO_2, then the medium containing the non-internalised MLs was removed and the cells washed with PBS (2 x 500 µL) and incubated at 37°C and 5% CO_2 in fresh medium in the absence of MLs. After 0, 5, 10, 15, 20, 25, 30, 45, 60 or 120 minutes of incubation, 1 plate of cells was washed and fixed as described above, while the other was incu-

bated with 0.4% (w/v) Trypan Blue (Gibco) for 5 minutes at room temperature, then washed and fixed, prior to fluorescence microscopy analysis. The relative fluorescence was measured using ImageJ. In each condition tested, at least 5 separate pictures per well of 4 different wells were examined to obtain statistically significant information.

Long-term stability of cell labelling: 3T3 fibroblasts were incubated for 24 h at 37°C in 5% CO_2 with 3.33% DSTAP-containing MLs (100 µg of Fe mL^{-1}) labelled with 0.25% DPPE-Texas Red® and were then cultured in fresh medium in the absence of MLs and were split 1/3 whenever confluency reached 80-90%. At 0, 2, 5, 10, 20, 25 and 30 days post ML-incubation, samples of the cells were plated in Lab-Tek™ Chamber Slides™, incubated for 24 h at 37°C and 5% CO_2 and fixed as described above. The presence of fluorescently-tagged MLs in the cell was then investigated by fluorescence microscopy.

Control of ML dilution in non-proliferative cells: To establish a non-dividing cell population, cells were plated in 96-well plates ($5x10^4$ cells $well^{-1}$ in 200 µL) and incubated for 24 h at 37°C in 5% CO_2, then in medium containing tubulin polymerization inhibitor II (TPI, 1, 5 or 20 µM) for up to 4 days, after which cell viability and cell numbers were checked as described above to determine the optimal concentration of TPI. ML uptake was examined by incubating cells plated in 96-well plates ($5x10^4$ cells per well in 200 µL) with TPI (1 µM) for 24 h at 37°C in 5% CO_2, after which fresh medium containing 1 µM TPI and various types of MLs (0, 1.66, 3.33, 6.66 or 16.66% DSTAP) at a concentration of 100 µg Fe mL^{-1} was added. After 2, 4, 8 or 24 h incubation at 37°C in 5% CO_2, cell viability and cellular iron content were checked as described above.

Mechanism of endocytotic uptake: The effect of various endocytosis inhibitors on cell viability was checked by plating the cells in 96-well plates ($5x10^4$ cells $well^{-1}$ in 200 µL), incubating them for 24 h at 37°C in 5% CO_2 and replacing the medium with medium containing 3 concentrations of the different inhibitors for 4 h at 37°C in 5% CO_2. The concentrations tested were 20, 50 and 100 µM for nocodazole (Noco), 10, 25 and 75 µg mL^{-1} for cytochalasin D (Cyt D), 10, 25 and 75 µM for monodansyl cadaverine (MDC), 2, 5 and 20 µg mL^{-1} for filipin III (Fil) and 100, 200 and 500 µg mL^{-1} for genistein (Gen). For ML uptake experiments, 3T3 fibroblasts were incubated with medium containing the inhibitors at the above concentrations for 30 minutes at 37°C

in 5% CO_2, then the medium was replaced by fresh medium containing 3.33% DSTAP-containing MLs and the mixture incubated for 2 or 4 h at 37°C in 5% CO_2, then cell viability and cellular iron content were checked. The conditions leading to optimal results (the lowest concentration giving maximal inhibition) were selected and mixtures of various inhibitors made at these concentrations (20 µM Noco + 25 µg mL^{-1} of Cyt D; 25 µM MDC + 5 µg mL^{-1} of Fil; 25 µM MDC + 200 µg mL^{-1} of Gen; 5 µg mL^{-1} of Fil + 200 µg mL^{-1} of Gen). Using these mixtures, cellular iron content and cell viability were examined as described above. Cellular iron content and cell viability were also checked on cells incubated with 3.33% DSTAP-containing MLs for 2 and 4 h at 4°C.

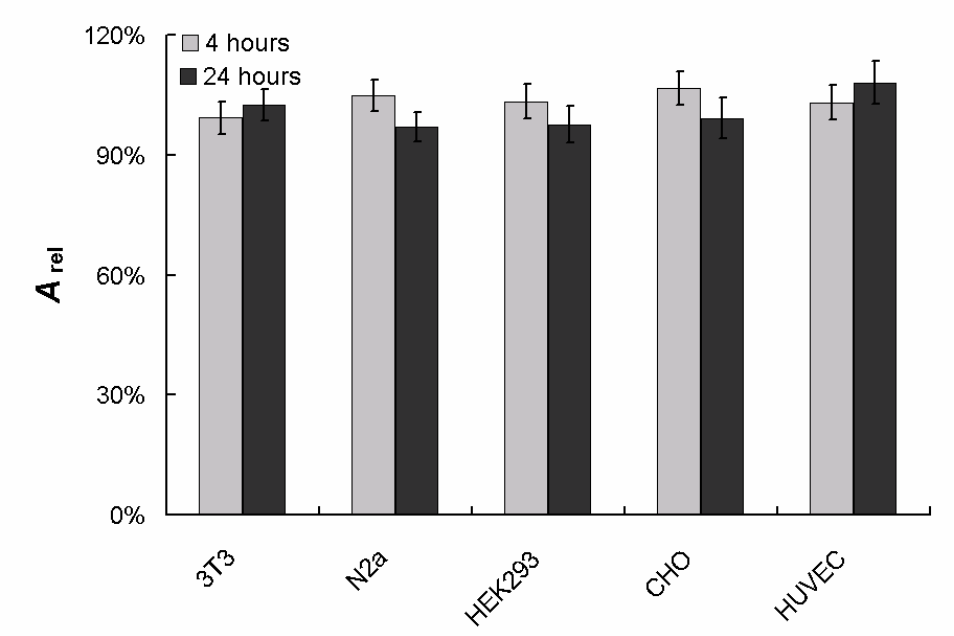

Figure S1. Relative viability at 4 or 24 h of different cell types (3T3 fibroblasts, N2a neuroblastoma cells, HEK-293 cells, Geneswitch™-CHO cells and primary HUVEC cells) incubated with 3.33% DSTAP-containing MLs. The viabilities are given relative to that of control cells not incubated with MLs, but otherwise treated identically. The error bars indicate the mean ± SEM (n = 10); A_{rel}: absorbance relative to that of untreated control cells.

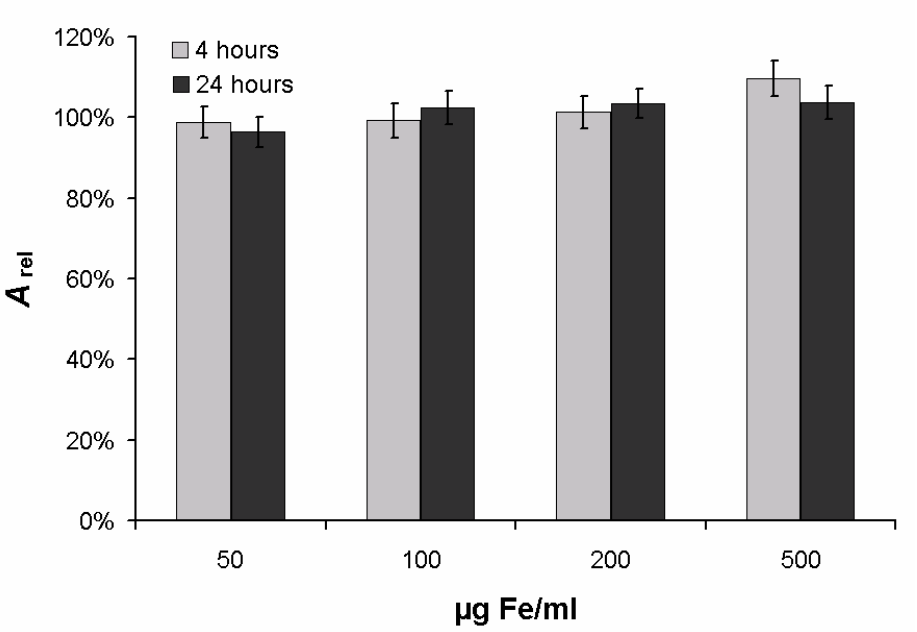

Figure S2. Relative viability at 4 or 24 h of 3T3 fibroblasts incubated with different concentrations of 3.33% DSTAP-containing MLs 50, 100, 200 and 500 µg Fe mL^{-1}). The viabilities are given relative to that of control cells not incubated with MLs, but otherwise treated identically. The error bars indicate the mean ± SEM (n = 10); A_{rel}: absorbance relative to that of untreated control cells.

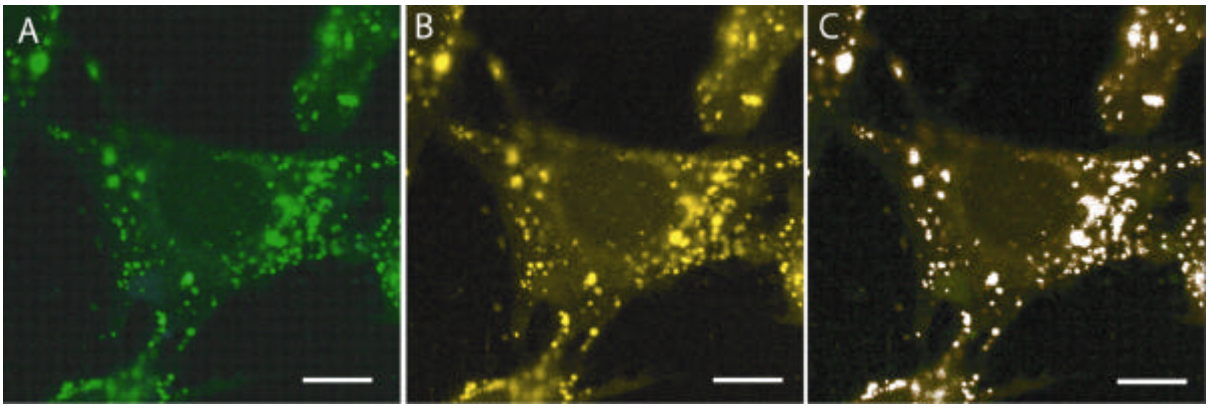

Figure S3. Confocal microscopic images of 3T3 fibroblasts labelled by incubation for 4 h with bifluorescent [inner lipid layer, 1% β-BODIPY® FL C_5-HPC (green, A); outer lipid layer, 0.1% (DPPE)-Lissamine™ Rhodamine B (yellow, B)] 3.33% DSTAP-containing MLs, followed by 48 h of continued incubation in ML-free medium. Colocalisation (white, C) was examined by using ImageJ software. Scale bars: 10 µm.

Note that, due to the green autofluorescence of the 3T3 fibroblasts, colocalisation was analysed using the green fluorescent signal of unlabelled cells as a threshold

level. Also, the signal intensity of the BODIPY FL-signal could not be increased much due to the quenching of the proximate iron oxide cores. These phenomena might contribute to weakening of the BODIPY FL-signal, resulting in a more dominant red colouring of the MLs due to the strong Texas Red® signal.

References

[1] S.J.H. Soenen, J. Baert, M. De Cuyper, *ChemBioChem* **2007**, *8*, 2067-2077.

Colour versions of the figures from the main text.

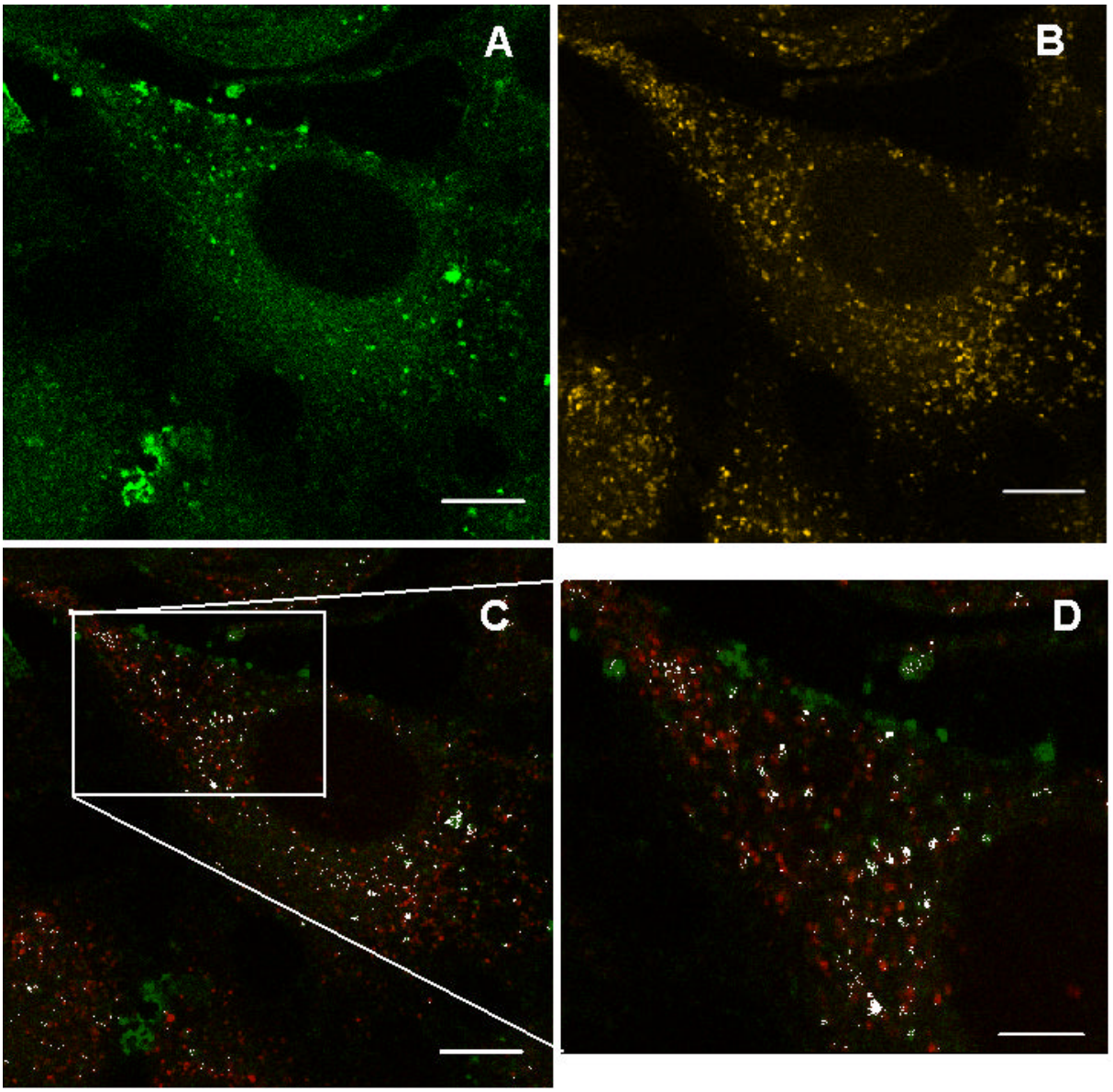

Figure 5.

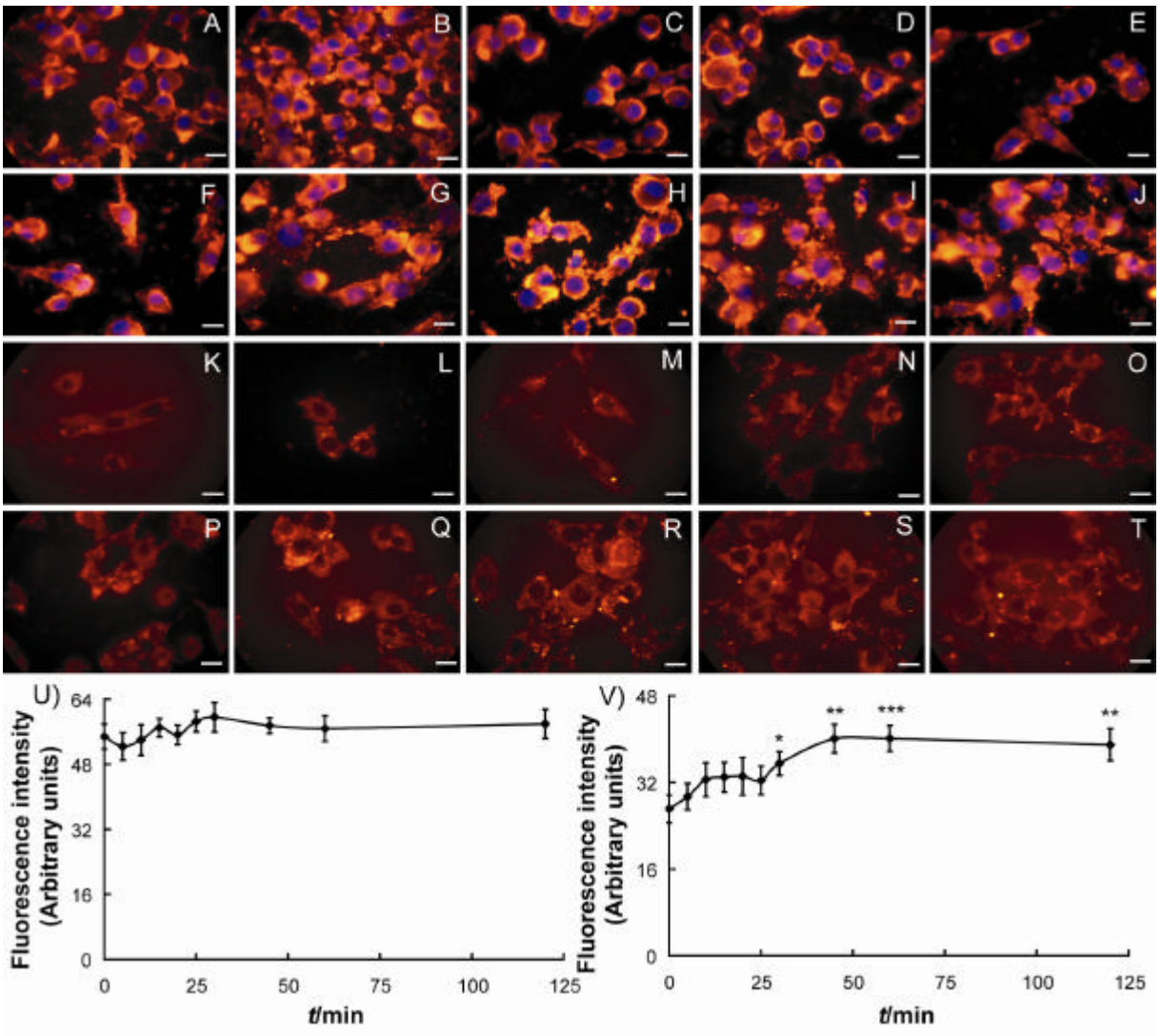

Figure 6.

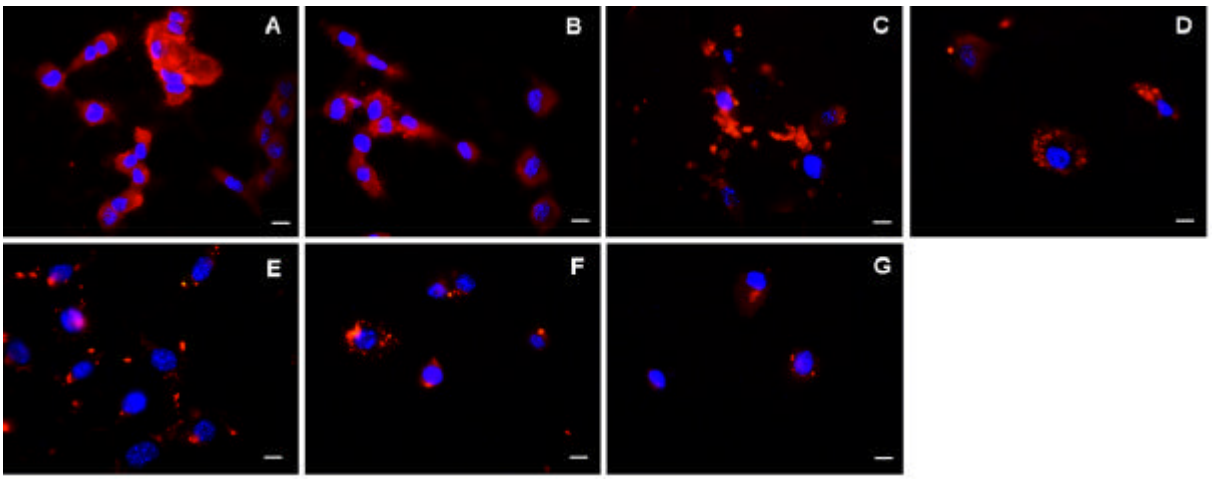

Figure 8.