

Colocalization Studies of Cytochrome-c with Mitochondria

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Key Words

- Mitochondria
- Fluorescent
- Markers
- FCCP
- Cytochrome-c
- Colocalization

Abstract

Mitochondria play an important role in regulating cellular apoptosis, as well as being the center of energy metabolism of cells. Several proteins such as cytochrome-c (cyt-c), Bcl, Bax, Smac, and AIF that are vital in regulating apoptosis are resident in the mitochondria of normal, healthy cells. In cells undergoing apoptosis, mitochondrial transmembrane potential ($\Delta\Psi_m$) is affected and several of the above mentioned proteins are also released into the cytoplasm, changing their localization with respect to the mitochondria. Using a fluorescent imaging approach, we have studied the colocalization of cyt-c with mitochondria in HeLa cells treated with compounds that affect the $\Delta\Psi_m$. Untreated and compound treated HeLa cells were stained with a fluorescent dye that stains mitochondria. Localization of cyt-c in these cells was visualized using a cyt-c antibody in combination with a fluorescent dye conjugated secondary antibody. Using a fully automated imaging application capable of measuring fluorescent intensity and morphological features at the single cell level, we computed Pearson's correlation coefficient as a measure of colocalization of cyt-c with

mitochondria in addition to several other measurements correlating cyt-c colocalization with mitochondria. Our results show that when cells are treated with compounds affecting $\Delta\Psi_m$, there is a dose dependent decrease in the colocalization of cyt-c with the mitochondria. These studies indicate that although $\Delta\Psi_m$ is affected by these compounds, some of the cyt-c is still retained in the mitochondria, suggesting that these compounds at the doses tested do not induce complete translocation of cyt-c from mitochondria to the cytoplasm.

Materials and Methods

HeLa (ATCC #) at 6,000 cpw were plated in 96 well microtiter plates. Cells were allowed to attach for four hours at 37°C and 5% CO₂. Cells were then treated with varying doses of compounds (Sigma Aldrich, St. Louis, MO, USA) that affect $\Delta\Psi_m$ or compounds that induce apoptosis for an additional 17-18 hours at 37°C and 5% CO₂.

Cells were stained with 0.5 mM MitoTracker Orange and 0.5 mg/mL of Hoechst-33342 for 30 minutes. Cells were then fixed with warmed 4% formaldehyde (37° C) for 15 minutes at room temperature.

Cells were then permeabilized followed by a blocking buffer and incubated with anti-cytochrome-c (polyclonal anti-sheep) for one hour at room temperature.

After washing, the cells were incubated with Alexa 488 conjugated to donkey-anti-sheep IgG and incubated for one hour at room temperature. The cells were then washed with HBSS, and then imaged and analyzed on a Thermo Scientific Cellomics® ArrayScan® V^{TI} HCS Reader running the Thermo Scientific Cellomics Colocalization BioApplication.

Description of the Colocalization BioApplication

The Colocalization BioApplication is an automated image analysis algorithm for the ArrayScan V^{TI} platform. The BioApplication allows automatic quantification of fluorescently stained cells, to determine colocalization of targets with markers defining various cellular regions. The BioApplication computes and reports several colocalization relevant measurements including Pearson's correlation coefficient, Mander's coefficient and other coefficients based on intensity and area colocalization of the target with marker proteins or dyes.

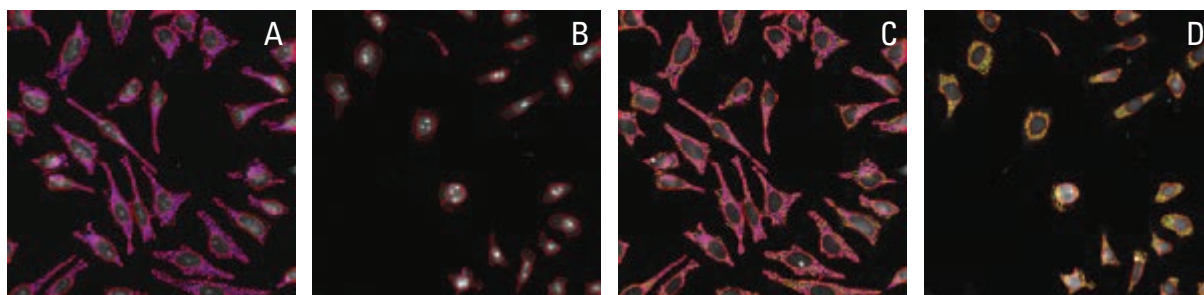


Figure 1. Effect of FCCP on the Colocalization of Cytochrome-C with Mitochondria in HeLa Cells

HeLa cells stained for mitochondria (A, B; pink overlays) and cytochrome-c (C, D; yellow overlays). Cells are untreated (A, C) or treated with 100 mM FCCP (B, D) for 17 h. Untreated cells show higher levels of colocalization of cytochrome-c with mitochondria compared to cells treated with FCCP. Images were obtained on an ArrayScan V^{TI} using a 20X objective.

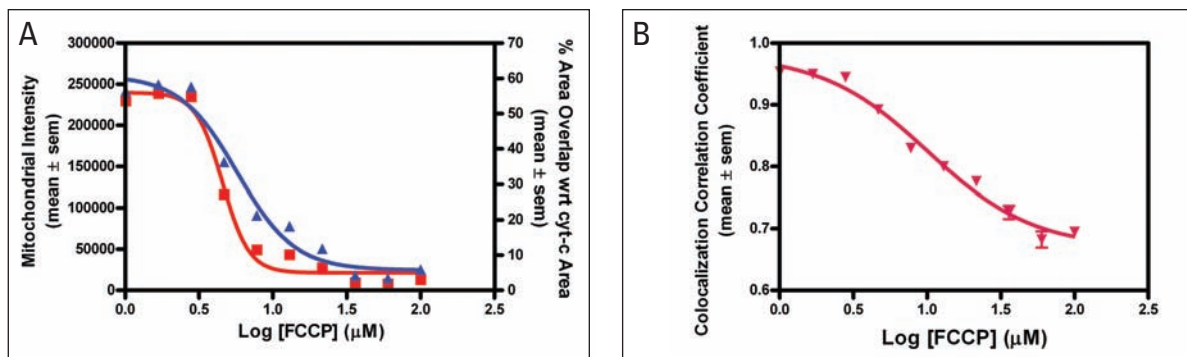


Figure 2. Dose Response of FCCP on the Colocalization of Cytochrome-c with Mitochondria in HeLa Cells Total mitochondrial intensity (indicator of $\Delta\Psi_m$), % colocalizing area of cytochrome-c with mitochondria (panel A) and Pearson's Colocalization Coefficient of cytochrome-c with mitochondria (panel B) in HeLa cells treated with varying concentrations of FCCP were plotted to determine EC50 concentrations. EC50 concentrations were calculated to be 4.56 mM, 5.75 mM and 10.11 mM respectively for decrease in mitochondrial intensity, % colocalizing area of cytochrome-c with mitochondria and Pearson's Colocalization Coefficient. The plots shows that a decrease in $\Delta\Psi_m$ is associated with a decrease in the other measurements.

Table 1 Z' values (8) were computed from 3 individual 96 well microplates of HeLa cells. In each plate, 48 wells were left untreated and 48 wells were treated with 100 mM FCCP for 17 hours and cells were stained for mitochondria and cytochrome-c as described earlier.

Assay Performance			
Measured Feature	Z' factor	SD	COV
Mitochondria Intensity	0.637	0.025	3.95
% overlap cytochrome c	0.630	0.017	2.75
Pearson's Colocalization Coefficient	0.390	0.020	5.13

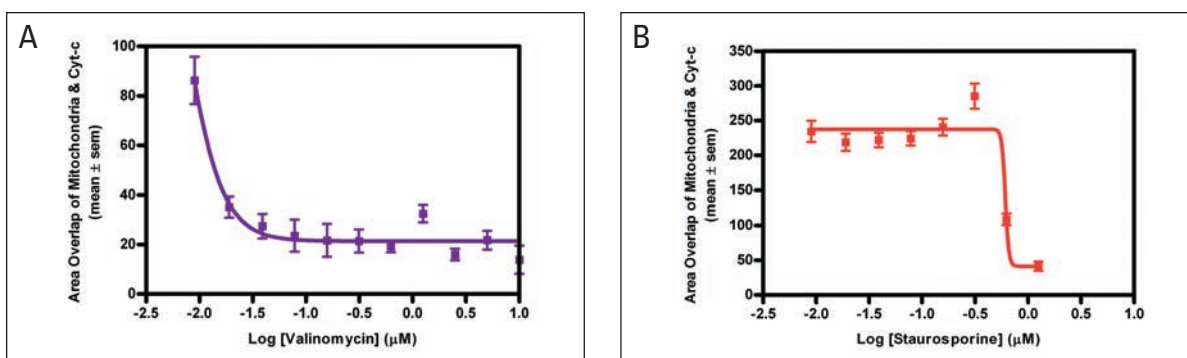


Figure 3. Dose Response of Valinomycin and Staurosporine on the Colocalization of Cytochrome-c with Mitochondria in HeLa Cells HeLa cells were treated with compounds affecting in $\Delta\Psi_m$ (valinomycin) or inducing apoptosis (staurosporine) for 17 hours and stained for mitochondria and cytochrome-c as described earlier. Graphs in Figure 3 show the change in the colocalized area in pixels of cytochrome-c with mitochondria as a function of the concentration of the compound. The plots show that with valinomycin (panel A) low concentrations are sufficient to cause a large decrease in the colocalizing area, while with staurosporine (panel B) large changes in colocalization occur at much higher doses.

Conclusions

- Preliminary data obtained clearly shows that the decrease in colocalization of cytochrome-c with the mitochondria can be quantified using the Colocalization BioApplication.
- A decrease in Pearson's correlation coefficient follows the same trend as the decrease in $\Delta\Psi_m$.

- Images show that at higher concentrations of FCCP, HeLa cells still show cytochrome-c staining, even though mitochondrial staining is largely absent.
- Valinomycin, a K⁺ ionophore that affects $\Delta\Psi_m$, and staurosporine an inducer of apoptosis both affect the colocalization of cytochrome-c with mitochondria.

- The Colocalization BioApplication performs robustly with high Z' values and low COVs, suggesting that it can be used for screening compounds that affect localization of proteins and other macromolecules in cells.

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