Chapter 15

A Microplate-Based Bioluminescence Assay of Mitochondrial Calcium Uptake

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Abstract

Mitochondrial Ca^{2+} homeostasis is crucial for regulating vital functions such as respiration or apoptosis. Targeted aequorins are excellent probes to measure subcellular Ca^{2+}. Ca^{2+} concentration in mitochondria ($[Ca^{2+}]_M$) is low at rest (about 10^{-7} M) and can increase to the micromolar or even approach the millimolar range, upon cell activation. Here we describe a new quantitative luminescent protocol to directly measure mitochondrial Ca^{2+} uptake, optimized for high throughput. The sensitivity of the method allows detection of changes in either the capacity or the affinity of mitochondrial Ca^{2+} transport.

Key words Aequorin, Screening, Calcium uniporter, Plate-reader

1 Introduction

Dynamic changes in mitochondrial Ca^{2+} levels are essential for regulating critical cell functions such as respiration or apoptosis. Ca^{2+} accumulates into the mitochondrial matrix through the mitochondrial Ca^{2+} uniporter (MCU), a calcium channel located at the inner mitochondrial membrane. This low-affinity/high capacity transporter is driven by the mitochondrial membrane potential and is inhibited by ruthenium red. MCU activity is counteracted by mitochondrial Ca^{2+} exit through Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers, both sensitive to CGP-37157. $[Ca^{2+}]_M$ is low at rest (about 10^{-7} M) and can increase up to micromolar, or even millimolar levels, following the rise in cytosolic Ca^{2+} concentration upon cell activation.

Quantification of mitochondrial Ca^{2+} levels depends on the selective localization of the Ca^{2+} probe. In contrast to fluorescent synthetic dyes, genetically encoded Ca^{2+} indicators can be targeted to specific subcellular locations, including the mitochondrial matrix. Aequorin is a calcium-sensitive photoprotein isolated from the jellyfish *Aequorea victoria*, which has been used as a calcium indicator for decades. The apoprotein is reconstituted to the active

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protein in the presence of molecular oxygen and coelenterazine. Binding of Ca^{2+} ions to the three highly conserved EF-hand motifs of aequorin induces a conformational change that leads to the oxidation of the cofactor coelenterazine and the emission of blue light.

Although several fluorescent assays to monitor Ca^{2+} dynamics have already been developed for high-throughput screening, the use of aequorin bioluminescence offers several advantages: (1) Unlike fluorescent probes, aequorin does not require excitation by light, thus avoiding cytotoxic effects caused by radiation. (2) Fluorescent compounds that could potentially interfere in a fluorescence-based assay, can be used in a luminescence screening. (3) Aequorin displays a high signal-to-noise ratio, typically in the range of 100–1000, in comparison with 5–10 for fluorescent sensors. This is due to the combination of a very low background—mammalian cells do not contain bioluminescent proteins—and a high dynamic range. (4) The affinity of aequorin for Ca^{2+} can be engineered to conform to the expected concentration by combining aequorin mutants of reduced affinities with different synthetic coelenterazines. These combinations permit to cover a Ca^{2+} concentration range from 10^{-8} to 5×10^{-3} M. (5) Aequorin can be specifically targeted to a given subcellular location by fusing its gene to a minimal targeting sequence. In this protocol, GFP–aequorin is targeted to the mitochondrial matrix by fusing it to the signal peptide of the *cytochrome c oxidase*.

Here we describe a luminescent-based protocol developed for high-throughput screening of mitochondrial Ca^{2+} uptake. The novel assay offers a number of advantages: First, it is based on mitochondrial-targeted apo-aequorin reconstituted with coelenterazine *n*, a combination that allows measuring Ca^{2+} levels in the range expected in the mitochondrial matrix (1–50 μM) during moderate increases of $[Ca^{2+}]_C$. Second, selective permeabilization of the plasma membrane allows direct experimental access to the mitochondrial membrane and avoids potential false positive hits caused by chemical compounds whose targets are upstream MCU. Finally, the method provides quantitative measurements, allowing the luminescence signal to be calibrated in $[Ca^{2+}]_M$.