学校编码：10384　　密级

学号：20051403065

厦门大学

博　士　学　位　论　文

超高灵敏流式分析技术在单线粒体检测中的应用

Applications of high-sensitivity flow cytometry for single mitochondria analysis

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论文提交日期：　2012 年 07 月

论文答辩日期：　2012 年 08 月

2012 年 7 月

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摘 要

线粒体是细胞生命活动的控制中心，它不仅是细胞的能量中心，而且在多种诱因引发的细胞凋亡信号转导中起着重要的调节作用。以线粒体为切入点的信号转导研究，无论对于揭示细胞凋亡发生过程的分子机制，还是研究小分子化合物诱导肿瘤细胞凋亡的作用机理，均具有重要价值。与全细胞分析相比，单个线粒体水平上的分析能够排除其他细胞器的干扰，揭示线粒体功能之间的异质性，并有助于线粒体亚类的发现。然而，由于线粒体尺寸微小，与其功能相关的生化物质含量低以及缺乏灵敏的检测手段等原因，单个线粒体水平上的检测一直以来都是一个富有挑战性的课题。

本课题组自行研制的超高灵敏流式检测仪（High-sensitivity flow cytometer, HSFCM），其灵敏度可达商品化流式细胞仪的数百倍以上，已在单个纳米颗粒水平的检测以及多个化学生物学体系中突显独特的应用价值。本论文主要基于 HSFCM 的灵敏度, 建立一个能在单细胞器水平对线粒体进行多参数分析的平台。文中首先利用荧光探针对线粒体内膜、核酸进行标记，在 HSFCM 上建立了单个线粒体的检测模式；进而采用免疫荧光的方法对线粒体蛋白进行标记，利用 HSFCM 尝试了单线粒体水平的蛋白检测；并在此基础上，利用三通道的 HSFCM 开展了单个线粒体的多参数分析，在线粒体水平实现了对其纯度、结构和功能完整性的全面考察。最后在全面建立的多参数分析平台上，将体系进一步推广至凋亡相关蛋白的检测，并初步开展了抗肿瘤药物作用机理的研究，主要内容如下：

1. 建立以 HeLa 肿瘤细胞为模型的线粒体提纯方法，采用绿色荧光探针 NAO 对线粒体内膜上的心磷脂进行特异性标记，利用 HSFCM 实现了对单个线粒体的检测；采用红色荧光探针 SYTO 62 对线粒体的核酸进行标记，尝试用 NAO 和 SYTO 62 两种荧光探针对线粒体进行同时标记，在 HSFCM 上初步建立了单个线粒体的检测模式（第二章）。

2. 采用免疫荧光的方法对线粒体的外膜穿孔蛋白（porin）进行标记，优化线粒体膜蛋白检测的染色方案；通过优化免疫荧光的样品制备如细胞器的固定、打孔条件等，以位于线粒体内膜及膜间隙的细胞色素 c 蛋白（cyt c）为模型，建

立了适用于线粒体内部蛋白的免疫荧光标记方法，从而为 HSFCM 应用于单个线粒体水平的线粒体蛋白检测奠定了基础（第三章）。

3. 在已经建立的线粒体特异性生化物质荧光标记、线粒体蛋白免疫荧光标记和相应检测模式基础之上，将三通道 HSFCM 应用于单个线粒体的多参数分析。利用 HSFCM 检测 NAO 和 SYTO 62 荧光探针同时标记的线粒体，实现了对单个线粒体纯度和结构完整性的考察；对线粒体的 porin 和 cyt c 同时进行免疫荧光标记，利用 HSFCM 实现了对单个线粒体功能完整性的考察；并在线粒体水平考察了 Ca^{2+}刺激所引起的细胞色素 c 的释放和线粒体的外膜损伤（第四章）。

4. 鉴于 Bcl-2 家族蛋白在线粒体介导的细胞凋亡中发挥的重要作用，我们利用 HSFCM，在线粒体水平对凋亡相关蛋白 Bax 及 Bcl-2 进行了半定量检测，并以此为基础，初步开展了线粒体凋亡通路相关抗肿瘤药物如：星形孢菌素、桦木酸等作用机理的研究（第五章）。

5. HSFCM 在单个线粒体的检测方面已经获得了一定的应用，在蛋白–蛋白相互作用以及线粒体凋亡通路研究等方面还有着广阔的应用前景。本文的结尾对已有的研究工作进行了总结，并对今后的研究方向进行了初步展望。（第六章）。

关键词：细胞凋亡 超高灵敏流式检测仪 线粒体 多参数分析

Abstract

Mitochondria are one of the most important organelles responsible for cellular activities. They are not only the energy center of a cell, but also play very important roles in apoptosis regulation. Researches of cellular signal transduction pathways mediated by mitochondria are critical in illustrating the molecular process of apoptosis and in studying the mechanism of anti-tumor drugs and small molecules that can induce cell apoptosis. Compared with whole cell analysis, analysis of mitochondria at single organelle level can exclude the interference of other organelles in cells, reveal their functional heterogeneities, and possibly identify a mitochondrial subpopulation. However, due to the small sizes of isolated mitochondrial particles, low content of specific organelle components as well as the lack of sensitive analytical methods, analysis of single mitochondria at the organelle level is still a great challenge.

Very recently, our laboratory built a high sensitivity flow cytometer (HSFCM). Compared with commercial flow cytometers, the HSFCM is several hundred fold more sensitive. Unique values have been demonstrated when applying the HSFCM to the detection of individual nanoparticles and various experimental systems of chemical biology. Based on the high sensitivity of the HSFCM, we attempted to establish a versatile and multiparameter platform for mitochondria analysis at the single organelle level. In this dissertation, we used a series of specific fluorescent probes to label mitochondria inner membrane and nucleic acid, using immuno-staining method to label mitochondria proteins. On the HSFCM, we analyzed various parameters and proteins at single mitochondrial level and were abel to measure the side scatter and two fluorescence signals simultaneously. Based on the established platform for multiparameter analysis of individual mitochondria, we can characterize the purity, structure and functional integrity of isolated mitochondrial samples. In the end, we expanded the application of the HSFCM in the detection of

apoptotic proteins and the investigation of the mechanism of anti-tumor drugs. The contents of this dissertation are summarized as follows:

1. We established an efficient method for mitochondria isolation from cultured cells by using HeLa cells as a model system. The first attempt of single mitochondria analysis by the HSFCM started with mitochondria labeled with NAO, a green fluorescent probe targeting at cardiolipin on the inner membrane of mitochondria. Then we labeled the mitochondria with SYTO 62, a red fluorescent probe binding specifically to nucleic acid. After optimizing the experimental conditions, we developed the approach for the simultaneous staining of a single mitochdrion with both the NAO and SYTO 62 fluorescent probes. Through which, we preliminarily established the platform for multiparameter analysis of single mitochondria on the HSFCM (Chapter 2).

2. By using immune-staining to label porin protein located on the mitochondria outer membrane, we were able to detect mitochondrial proteins at the single organelle level. In order to develop a method suitable for the immuno-staining of all the mitochondrial inner proteins, we optimized the staining procedure through sample fixation and permeabilization by using cytochrome c located on the inner membrane or in the intermembrane space. By then, we lay down the basis of applying the HSFCM for mitochondrial protein detection at single organelle level (Chapter 3).

3. Based on the established fluorescence labeling approaches through specific fluorescent probes or immune-labeling of mitochondrial proteins, we applied the HSFCM for the high-throughput multiparameter analysis of individual mitochondria. By labeling mitochondria with NAO and SYTO 62, we demonstrated the assessment of the purity and structural integrity of individual mitochondria. Then we applied the experimental system for the multiparameter analysis of mitochondrial proteins. By simultaneous labeling of porin and cyt c proteins, we can characterize the functional integrity of individual mitochondria. Cytochrome c release and rupture of outer membrane were observed at the mitochondria level upon Ca^{2+} stimulation (Chapter 4).

4. Because Bcl-2 family proteins play a central role in regulating cell apoptosis,

we successfully applied the HSFCM for the semi-quantitive analysis of apoptosis proteins such as Bax and Bcl-2. Meanwhile, we began the mechanism study of anti-tumor drugs related to mitochondria-mediated apoptosis pathway, such as Staurosporine and Betulinic Acid (Chapter 5).

5. HSFCM holds great potential in many aspects of single mitochondria analysis, especially protein-protein interaction and mitochondria-mediated apoptosis. At the end of this dissertation, future applications of the HSFCM for single mitochondria analysis are discussed, and preliminary designs of several experimental systems are proposed (Chapter 6).

Keywords: Apoptosis; High sensitivity flow cytometer (HSFCM); Mitochondria; Multiparameter analysis