

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

张舒越

指导教师姓名: 颜 晓 梅 教授

专 业 名 称: 化学生物学

论文提交日期: 2012 年 07 月

论文答辩日期: 2012 年 08 月

2012 年 7 月

厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其他个人或集体已经发表的研究成果,均在文中以适当方式明确标明,并符合法律规范和《厦门大学研究生学术活动规范(试行)》。

另外,该学位论文为()课题(组)的研究成果,获得()课题(组)经费或实验室的资助,在()实验室完成。(请在以上括号内填写课题或课题组负责人或实验室名称,未有此项声明内容的,可以不作特别声明。)

声明人(签名):

年 月 日

厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文（包括纸质版和电子版），允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

1. 经厦门大学保密委员会审查核定的保密学位论文，
于 年 月 日解密，解密后适用上述授权。

2. 不保密，适用上述授权。

（请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。）

声明人（签名）：

年 月

目 录.....	I
Contents.....	V
摘 要.....	IX
Abstract.....	XI
第一章 绪论	15
1.1 线粒体与细胞凋亡	15
1.1.1 细胞凋亡与疾病	15
1.1.2 线粒体是细胞凋亡的调控中心	17
1.1.3 单线粒体检测的重要性.....	18
1.2 单线粒体分析的主要方法及特点.....	18
1.2.1 原位分析技术	18
1.2.2 毛细管电泳分析技术.....	19
1.2.3 流式细胞术.....	20
1.2.4 膜片钳技术.....	21
1.3 超高灵敏流式检测技术（HSFCM）	21
1.3.1 超高灵敏流式细胞仪简介.....	21
1.3.2 超高灵敏流式检测仪在生物体系检测中的应用	23
1.4 本论文的选题思路及主要研究内容	25
参考文献	27
第二章 HSFCM 应用于单个线粒体检测的模式建立	33
2.1 引言	33
2.2 材料与方法.....	35
2.2.1 仪器与设备	35
2.2.2 实验材料与试剂	37
2.2.3 实验方法.....	38

2.3 结果与讨论	42
2.3.1 实验体系的建立	42
2.3.2 HSFCM 应用于单个线粒体分析的可行性考察	45
2.3.3 NAO 荧光探针标记浓度的优化	49
2.3.4 SYTO 62 核酸荧光探针对线粒体的标记	51
2.3.5 线粒体的双色荧光标记初探	53
2.4 本章小结	54
参考文献	55
第三章 HSFCM 应用于单个线粒体水平的蛋白检测	57
3.1 引言	57
3.2 材料与方法	58
3.2.1 实验材料	58
3.2.2 实验方法	60
3.3 结果与讨论	63
3.3.1 HSFCM 对线粒体穿孔蛋白 (porin) 的检测	63
3.3.2 线粒体免疫荧光标记的优化	66
3.3.3 单线粒体水平 cyt c 和 porin 蛋白的免疫荧光检测.....	69
3.4 本章小结	70
参考文献	71
第四章 HSFCM 应用于单个线粒体的多参数分析	73
4.1 引言	73
4.2 材料与方法	74
4.2.1 实验体系	74
4.2.2 实验材料与试剂	75
4.2.3 实验方法	75
4.3 结果与讨论	77
4.3.1 双荧光探针标记考察线粒体的纯度和结构的完整性.....	77
4.3.2 线粒体蛋白的多参数分析	80
4.4 本章小结	83
参考文献	84

第五章 单个线粒体水平细胞凋亡相关蛋白的检测.....	87
及抗肿瘤药物作用机理的初步探讨	87
5.1 引言	87
5.2 材料与方法.....	90
5.2.1 实验体系.....	90
5.2.2 实验材料.....	91
5.2.3 实验方法.....	92
5.3 结果与讨论.....	95
5.3.1 线粒体水平上调亡蛋白的半定量检测.....	95
5.3.2 Staurosporine 的作用机理研究	101
5.3.3 桦木酸的作用机理探讨	106
5.4 本章小结.....	108
参考文献	109
第六章 总结与展望	113
6.1 HSFCM 应用于单个线粒体检测的总结	113
6.2 后续研究方向展望	114
参考文献	115
作者攻读博士学位期间发表论文及成果	117
致谢.....	118

厦门大学博硕士学位论文摘要库

Contents

Abstract (in Chinese)	VI
Abstract (in English)	VIII
Chapter 1. Preface	15
1.1 Mitochondria and apoptosis	15
1.1.1 Apoptosis and diseases	15
1.1.2 The central role of mitochondria in apoptosis	17
1.1.3 Importance of single mitochondria analysis	18
2 Methods and their characteristic for single mitochondria analysis	18
1.2.1 In-situ analysis techniques.....	18
1.2.2 Capillary Electrophoresis	19
1.2.3 Flow Cytometry	20
1.2.4 Patch Clamping.....	21
1.3 High-sensitivity flow cytometry (HSFCM)	21
1.3.1 Introduction of HSFCM	21
1.3.2 Applications of HSFCM in chemical biology	23
1.4 Objective and main contents of this dissertation	25
References	27
Chapter 2. Application HSFCM in single mitochondria analysis	33
2.1 Introduction	33
2.2 Materials and methods	35
2.2.1 Instruments	35
2.2.2 Materials and reagents.....	37
2.2.3 Methods.....	38
2.3 Results and discussion	42
2.3.1 Establishment of experimental system.....	42
2.3.2 Estimation of the capability of HSFCM for single mitochondria analysis	45
2.3.3 HSFCM detection of NAO labeled mitochondria	49

2.3.4 HSFCM detection of NAO labeled mitochondria	51
2.3.5 Attempt of labeling mitochondria with both NAO and SYTO 62.....	53
2.4 Conclusion.....	54
References	55
Chapter 3. Application HSFCM in mitochondrial protein analysis at	
the single orgaelle level.....	57
3.1 Introduction	57
3.2 Materials and methods	58
3.2.1 Materials and reagents.....	58
3.2.2 Methods.....	60
3.3 Results and discussion	63
3.3.1 HSFCM detection of mitochondrial porin protein.....	63
3.3.2 Optimization of immune-staining method of mitochondrial proteins	66
3.3.3 Simultaneous detection of porin and cyt c at single organelle level.....	69
3.4 Conclusion.....	70
References	71
Chapter 4. Applications of HSFCM in Multi-parameter analysis of	
single mitochondria.....	73
4.1 Introduction	73
4.2 Materials and methods	74
4.2.1 Experimental system.....	74
4.2.2 Materials and reagents.....	75
4.2.3 Methods.....	75
4.3 Results and discussion	77
4.3.1 Estimation of purity and structural integrity of single mitochondria upon co-labeling by two fluorescent probes.....	77
4.3.2 Multi-parameter analysis of mitochondrial proteins.....	80
4.4 Conclusion.....	83
References	84

Chapter 5. Apoptic protein detection at single mitochondrial level and study of pharmacological mechanism of anti-tumor drugs	87
5.1 Introduction	87
5.2 Materials and methods	90
5.2.1 Experimental system	90
5.2.2 Materials and reagents.....	91
5.2.3 Methods.....	92
5.3 Results and discussion	95
5.3.1 Semi-quantitive detection of apoptic proteins at single mitochondrial level	95
5.3.2 Study of pharmacological mechanism of Staurosporine.....	101
5.3.3 Study of pharmacological mechanism of Betulinic Acid.....	106
5.4 Conclusion.....	108
References	109
Chapter 6. Summary and prospects.....	113
6.1 Summary	113
6.2 Prospects	114
References	115
Publications	117
Acknowledgements	118

厦门大学博硕士学位论文摘要库

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 able 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 mitochondrion 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-quantitative 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

Degree papers are in the "[Xiamen University Electronic Theses and Dissertations Database](#)". Full texts are available in the following ways:

1. If your library is a CALIS member libraries, please log on <http://etd.calis.edu.cn/> and submit requests online, or consult the interlibrary loan department in your library.
2. For users of non-CALIS member libraries, please mail to etd@xmu.edu.cn for delivery details.

厦门大学博硕士学位论文摘要库