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厦门大学

硕 士 学 位 论 文

超高灵敏流式检测技术
在线粒体融合定量检测中的应用

Quantitative Measurement of Mitochondrial Fusion *in vitro* by High-Sensitivity Flow Cytometry

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摘 要

线粒体是高度动态的细胞器，在真核细胞能量代谢和信号转导中发挥重要的调控作用。线粒体融合分裂与细胞代谢、线粒体自噬、细胞凋亡等多种生命活动息息相关，融合分裂失衡会导致多种人类疾病，包括神经退行性疾病、心血管疾病、癌症等。因此，建立快速的线粒体融合检测方法并对该过程进行定量检测显得尤为重要。目前线粒体融合的检测手段主要是采用荧光显微镜和透射电子显微镜对线粒体融合现象进行观察，但这两种方法都在检测速度和定量方面存在很大的局限性。线粒体融合的相关研究迫切需要发展高灵敏、高通量的单线粒体水平检测技术。

结合瑞利散射和鞘流单分子荧光检测技术，我们课题组研发了超高灵敏流式检测技术（high sensitivity flow cytometry, HSFCM），实现了单个线粒体的散射和多色荧光信号的同时检测，检测速率高达每分钟上万个颗粒。本论文旨在利用实验室自行研制的超高灵敏流式检测装置，结合基于质粒转染的线粒体荧光标记手段，建立线粒体融合的高通量定量检测方法，并将其应用于线粒体融合与膜电位和氧化还原平衡的研究。第一章对线粒体融合分裂的分子机制、意义及其与人类疾病关系进行介绍，并简介线粒体融合的研究手段以及超高灵敏流式检测技术。实验部分的主要内容如下：

1. 建立线粒体荧光标记的高通量评估方法。分别将 pAcGFP1-Mito 质粒和 pDsRed2-Mito 质粒瞬时转染至人宫颈癌 HeLa 细胞中，再用抗性筛选试剂 G418 分别筛选出两种稳定转染细胞。单线粒体水平的荧光定量分析表明，相比瞬时转染和染料标记，稳定转染具有荧光亮度高且标记稳定性好的独特优势，能更好地应用于需要线粒体稳定标记的线粒体融合研究中（第二章）。
2. 建立线粒体融合的定量检测方法。分别从两种稳定转染细胞中提取表达绿色荧光蛋白或红色荧光蛋白的线粒体，等量混合后诱导线粒体融合，采用 HSFCM 在单颗粒水平对样品中线粒体的散射、绿色和红色荧光信号进行同时检测。如果能同时检测到颗粒的绿色和红色荧光信号，就可以判定这是一个融合线粒体。 在优化的融合条件下，测得的线粒体融合效率约为 22~23%，

与文献报道相吻合。此外，采用透射电子显微镜观察到线粒体融合中间体。相比于 TEM 检测，线粒体融合的 HSFCM 测定在速度和定量表征方面具有独特的优势（第三章）。

3. 线粒体融合定量检测方法的应用。分别考察了线粒体融合与膜电位、氧化还原平衡的相互关系。实验结果表明，融合过程需要线粒体维持膜电位；在一定工作浓度范围内，氧化剂能促进线粒融合，而抗氧化剂会抑制线粒体融合（第四章）。
4. 由于线粒体融合过程与多种细胞活动息息相关，我们希望能将所建立的线粒体融合定量检测方法用于探究参与调控线粒体融合的其他影响因素。论文的结尾总结了现有的研究工作，并对后续的研究方向提出了初步展望（第五章）。

关键词：超高灵敏流式检测技术；单线粒体分析；稳定转染；线粒体融合

Abstract

Mitochondria play a central role in the regulation of energy metabolism and signal transduction in eukaryotic cells. Besides, mitochondria are highly dynamic organelles that continually undergo fusion and fission. Mitochondrial dynamics actively contributes to a lot of cellular activities, such as cell metabolism, mitophagy and apoptosis, and dysfunction of which will lead to several human diseases, including neurodegenerative diseases, cardiovascular disease and cancers. As a result, developing a quantitative method for the rapid measurement of mitochondrial fusion is urgently needed. The techniques commonly used for mitochondrial fusion study are confocal microscopy and transmission electron microscopy (TEM), both of which are of low-throughput and limited quantification capability. A sensitive, high-throughput, and quantitative method for mitochondrial fusion measurement at the single-mitochondrion level is in high demand.

By combining Rayleigh scattering with strategies for single-molecule fluorescence detection in a sheathed flow, our laboratory have developed a high sensitivity flow cytometer (HSFCM) which can achieves real-time light-scattering and multicolor fluorescence detection of single mitochondrion at a throughput of up to 10000 particles per minute. Employing the unique advantage of HSFCM in the sensitive, rapid, and quantitative multiparameter analysis of individual mitochondria, here, we aim to develop a high-throughput method for the quantitative measurement of mitochondrial fusion via the integration of mitochondria labeling with transfected plasmid. The applications of the as-developed method in the relationship study between mitochondrial fusion and the mitochondrial membrane potential and redox homeostasis have been conducted. The first chapter introduces the mechanisms and significance of mitochondrial fusion and fission, along with their relationship with human diseases. The conventional methods for mitochondrial fusion measurement are

described and technology of high sensitivity flow cytometry is introduced. The experimental parts contents of this dissertation are summarized as follows:

1. High-throughput assessment of mitochondrial fluorescence labeling at the single-particle level. Plasmid encoding mitochondrial-targeted fluorescent proteins, pAcGFP1-Mito and pDsRed2-Mito, were transiently transfected to HeLa cells, respectively. Stable transfection was achieved by G418 selection. The strong fluorescence intensity and good labeling stability make stable transfection an excellent method to label mitochondria stably (Chapter 2).
2. Development of high-throughput quantitative method for mitochondrial fusion measurement *in vitro*. Mitochondria isolated from HeLa cells with stable transfection of pAcGFP1-Mito or pDsRed2-Mito plasmid were mixed at equal concentration. The HSFCM was used to analyze the sample, and the side scatter, green fluorescence, and red fluorescence of individual mitochondria were measured simultaneously. The event with concomitant detection of both the green and red fluorescence signals was identified to be a fused mitochondrion. The fusion condition and the HSFCM detection condition were optimized. Mitochondrial fusion buffer was used to promote mitochondrial fusion and the fusion efficiency was measured to around 22~23%. These results agreed well with the literature reports. On the other hand, we observed the structure of intermediate state by using TEM measurement. Compared with TEM measurement, the HSFCM exhibits unique advantages in the rapid and quantitative measurement of mitochondrial fusion (Chapter 3).
3. Applications of the as-developed HSFCM method for the quantitative measurement of mitochondrial fusion *in vitro*. The relationships of the fusion efficiency and mitochondrial membrane potential or redox homeostasis were studied. Results show that mitochondrial membrane potential is essential for mitochondrial fusion, and oxidants effectively induce mitochondrial fusion whereas antioxidants inhibit mitochondrial fusion (Chapter 4).
4. Due to the essential roles of mitochondrial dynamics in cellular activities, in the future we hope to apply the as-developed HSFCM method for the quantitative

measurement of mitochondrial fusion to study other factors involved in the regulation of mitochondrial fusion. Prospects on the future development are discussed in the end of this dissertation (Chapter 5).

Keywords: high sensitivity flow cytometry; single mitochondrial analysis; stable transfection; mitochondrial fusion

第一章 绪论

1.1 线粒体结构与功能简介

在真核细胞中，线粒体不仅是细胞的能量工厂，还是多种信号转导的调控中枢。单个细胞内含有的线粒体的数目可以从几十个到数千个不等[1]，能量需求越高的细胞含有的线粒体数目越多[2]。线粒体尺寸微小，直径一般为 0.5-1.0 μm，长度为 1.5-3.0 μm，且形态和功能存在高度的异质性（heterogenity）[3-7]。

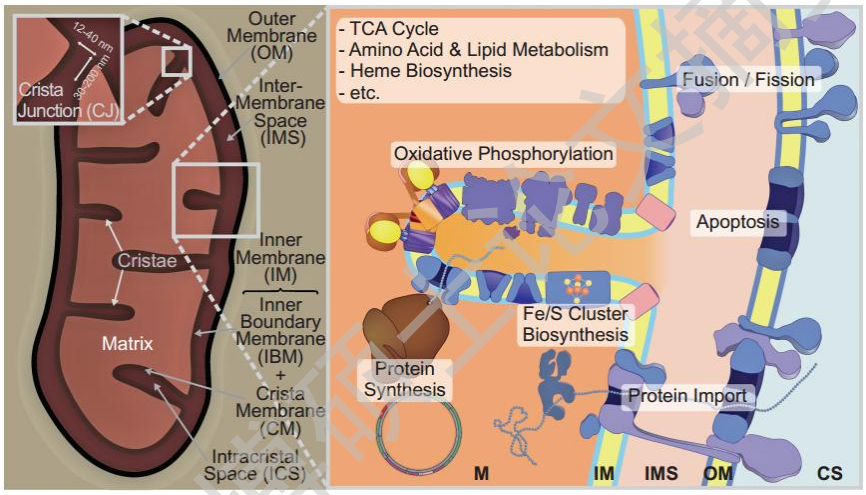

图 1.1 线粒体结构示意图[8]。M，基质；IM，内膜；IMS，膜间隙；OM，外膜；CS，胞浆。

Fig. 1.1 Scheme of a mitochondrion[8]. M, matrix; IM, inner membrane; IMS, intermembrane space; OM, outer membrane; CS, cytosol.

线粒体的结构如图 1.1 所示[8]，其由双层膜包被，划分为四个部分：外膜（outer membrane, OM）、膜间隙（intermembrane space, IMS）、内膜（inner membrane, IM）和基质（matrix, M）。外膜将线粒体与胞浆隔离，可以通过电压依赖性阴离子通道（voltagedependent anion channel, VDAC）[9]与胞浆进行代谢物传递，借助外膜转运酶（translocase of the outer membrane, TOM）[10]转运核编码蛋白质。内膜可以分为内边界膜（inner boundary membrane, IBM）和嵴（cristae）[11]。内边界膜上含有将蛋白运转至基质的内膜转运酶（translocase of the inner membrane, TIM）