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博士学位论文

流式细胞术对细菌双杂交系统中蛋白-蛋白相互作用的定量分析

Quantitative analysis of protein-protein interaction in BACTH system using flow cytometry

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摘　要

蛋白-蛋白相互作用是所有生命活动的基础，其研究有助于人们更好地理解蛋白质功能、构建相互作用网络、发现药物靶点、发展疾病诊断和治疗的方法。现有的蛋白-蛋白相互作用研究方法主要分为体外检测和体内检测两种。等温滴定量热法、表面等离子体共振法、荧光偏振法等是常用的体外检测方法，这类方法往往需要使用昂贵的仪器检测以及繁琐的蛋白纯化与标记过程；而酵母双杂交、蛋白质片段重组、合成致死等体内检测方法则只需在细胞和基因水平操作，不仅省去了蛋白质纯化步骤，而且能够真实地反映蛋白质在细胞中的相互作用情况。其中，酵母双杂交系统作为最常用的蛋白-蛋白相互作用研究方法，具有灵敏度高，适用范围广，操作相对简便等优点。其原理是利用待研究蛋白的相互作用使原本独立表达的酵母转录因子的两个结构域在空间上接近，从而激活下游报告基因（如lacZ，HIS等）的转录表达，并通过分析报告基因的表达情况来判断蛋白质之间是否存在相互作用。然而该方法依旧存在假阳性、假阴性率高，研究的蛋白质必须定位于细胞核内等缺点。基于腺苷酸环化酶重构的细菌双杂交（Bacterial adenylate cyclase-based two hybrid， BACTH）系统是一种新发展的体内蛋白质相互作用检测技术，它利用蛋白质相互作用介导的腺苷酸环化酶催化结构域功能重建催化合成cAMP，进而激活报告基因表达来检测蛋白-蛋白相互作用。相比于酵母双杂交系统，该技术不仅大大降低了假阳性和假阴性率，缩短了实验周期，并且不需要所研究的蛋白定位于细胞核，因此适用于膜蛋白的相互作用研究。但是，无论酵母双杂交还是细菌双杂交，常规的报告基因检测方法，如-半乳糖苷酶活性测定，营养缺陷筛选等，都只能对相互作用进行定性分析，无法定量地检测相互作用强度。而且这类检测结果均基于大量细胞的集权平均，掩盖了细胞之间的异质性。利用流式细胞术在单颗粒水平对细胞进行快速、多参数定量分析的优势，结合免疫荧光染色技术，本论文发展了一种基于BACTH系统的蛋白-蛋白相互作用定量检测方法，主要包括以下内容：

第一章为文献综述。主要介绍了蛋白-蛋白相互作用的研究意义及研究方法，并对BACTH系统作了着重介绍。接着，从单细胞分析的重要性出发，详细介绍了流式细

胞术及其在蛋白质相互作用研究领域的应用。本章的最后部分简要介绍了本论文的选题思路以及研究内容。

第二章为双标签BACTH系统实验体系的构建。在BACTH体系中，报告基因的表达水平除了与相互作用蛋白之间的亲和力大小相关，还受细胞内相互作用蛋白浓度的直接影响。为获得相互作用强度的相关信息，必须同时对报告基因和相互作用蛋白的表达量进行定量分析。为此我们在两个相互作用蛋白与腺苷酸环化酶催化域片段（T18和T25）之间分别插入Flag-tag和His-tag标签，以便利用流式细胞仪在单细菌水平考察相互作用蛋白的表达量。选择相互作用已知、与细菌外膜稳定性相关的蛋白对Pal和TolB作为模型，构建实验体系。通过共转化将含有目的蛋白的双杂交质粒转入报告细菌E.coli BTH101中，使其同时表达融合蛋白T18-Flag-Pal与T25-His-TolB。采用Western blot对融合表达的标签蛋白进行检测，以确定双标签BACTH系统的成功构建。利用传统的平板显色以及比色定量法检测-半乳糖苷酶活性，确保BACTH实验体系的成功构建。

第三章为细菌胞质蛋白流式检测方法的建立。作为一种革兰氏阴性菌，大肠杆菌有较为复杂的膜结构，除部分小分子外，大多数物质无法自由跨过细菌膜结构进入胞质内，所以针对细菌胞质蛋白的荧光标记通常需要对细菌进行破膜处理。鉴于传统流式细胞仪的散射光通道难以实现细菌与背景信号的有效分辨，为保证检测结果的准确性，实验条件的优化均在本实验室自行搭建的超高灵敏流式检测装置（High sensitivity flow cytometry， HSFCM）上进行。我们首先采用-半乳糖苷酶的荧光底物C12FDG对经破膜处理的双杂交细菌进行染色，初步建立超高灵敏流式检测装置在单细菌水平上检测蛋白质相互作用的方法。接着为了实现相互作用蛋白表达量的定量检测，我们利用免疫荧光染色法，通过对细菌进行固定、破膜处理，并用Flag-tag和His-tag的单克隆抗体结合荧光标记的二抗对与之融合表达的相互作用蛋白对分别进行特异性标记。由于荧光底物虽能检测-半乳糖苷酶，但是水解产物部分泄漏后重新吸附导致的荧光信号强度平均化，以及固定处理造成的酶活性下降，使其无法与免疫荧光染色兼容，所以我们选择对报告基因-半乳糖苷酶也进行免疫荧光标记，并在此基础上对其和相互作用蛋白分别用两种不同荧光染料标记的二抗进行免疫荧光染色，实现了两者的双参数同时检

测。

第四章为基于流式细胞仪的BACTH系统蛋白质相互作用定量检测方法的建立。通过流式检测我们发现，BACTH系统中细菌存在两种截然不同的蛋白表达状态，即双稳态现象。表现为一部分细菌同时表达相互作用蛋白和报告基因，而另一部分细菌的两种蛋白表达均无法检测到，两类细菌的比例随细菌培养时间而变化，并且不同单菌落之间存在巨大差异。我们对表达蛋白的这部分细菌进行分析，为了减小细菌个体间由于质粒分配不均匀以及相互作用蛋白表达随机性所造成的相互作用蛋白表达量差异的影响，引入了相对报告基因表达量的概念，即单位相互作用蛋白表达量所产生的报告基因表达量。通过对同一单菌落的不同生长时期以及不同单菌落培养的细菌的相互作用蛋白和报告基因表达量的单细菌水平免疫荧光定量检测，发现对于同一对相互作用蛋白，相对报告基因表达量具有较为恒定的值。为进一步验证相对报告基因表达量与相互作用强度的对应关系，我们设计了与Pal之间亲和力较小的TolB蛋白突变体TolB22-25和TolB22-33¬，分别与Pal蛋白构建双杂交体系，比较三组蛋白相互作用体系的相对报告基因表达量，并最终建立了基于流式细胞仪的BACTH系统蛋白质相互作用定量检测方法。

第五章为基于流式细胞仪的BACTH系统蛋白质相互作用定量检测方法的应用。我们选择了一系列相互作用强度已知的卷曲螺旋（Coiled-coil）蛋白作为研究对象，构建含有这些蛋白质基因的质粒。利用已建立的蛋白质相互作用定量检测方法，比较并证实了文献报道的该系列相互作用蛋白的亲和力与本实验体系测定的相互作用强度存在良好的对应关系。结果表明本方法能方便快速地获得蛋白相互作用强度信息。此外，以Pal和TolB为模型，考察了相互作用抑制剂对BACTH系统报告基因及相互作用蛋白表达量和表达比例的影响。对于常规的筛选方法，某些药物的加入可能会影响细菌蛋白的表达，而常规的报告基因酶活检测难以排除这类干扰。单细菌水平的相互作用蛋白和报告基因同时检测不仅可以规避以上问题，还能从检测结果中获得更多相关信息，所以我们尝试在此基础上建立一种快速、简便、高通量的蛋白质相互作用抑制剂筛选方法。

第六章为总结与展望。总结了本论文的研究内容，并对将来进一步的研究工作进行了展望。

关键词：蛋白质相互作用；BACTH系统；流式细胞术；免疫荧光染色；单细菌检测

Abstract

Protein-protein interactions (PPIs) are fundamental to virtually every aspect of life processes. Characterizing the interacting partners of a protein is important in the understanding of protein function, deciphering biochemical networks, discovering novel drug targets, and developing diagnostic and therapeutic assays. Current approaches for PPI study in vitro include isothermal titration calorimetry, surface plasmon resonance, fluorescence polarization and so on. Compared with these methods, in vivo approaches, such as yeast two-hybrid (Y2H), protein-fragment complementation assay, and synthetic lethality, not only eliminate the cumbersome protein purification and labeling procedures but also measure interactions at their native status. Among these methods, yeast two-hybrid system is the most frequently used method. For the study of the interaction of two proteins of interest, one protein is fused to the DNA-binding domain (DBD) of Gal4 (the bait) and the other protein is fused with the transcription-activating domain (AD) of Gal4 (the prey). The bait fusion binds upstream activating sequences (UAS) of the reporter gene such as lacZ and His. Association of the interacting proteins bring the Gal4 AD to the reporter gene, followed by recruitment of the basal transcriptional machinery, which establishes the gene transcription that can be detected by following analysis. Although numerous protein-protein interactions have been identified by the Y2H system, the false-positive and false-negative rate of the system is relatively high, and the fusion proteins must be targeted to the nucleus. Bacterial two-hybrid based on the reconstitution of adenylate cyclase activity in E. coli (BACTH) is an ideal alternative for Y2H. It is an easy and reliable method with few reported case of false positives or false negatives. Moreover, studying the interactions of membrane proteins was made possible as the BACTH system does not require the hybrid proteins to be located in the nucleus as Y2H. However, both the Y2H

and BATCH methods for PPI detection suffer from some shortcomings. First, lack of quantitation capability renders these methods unsuitable for measuring the affinity between two interacting proteins, nor for the affinity comparison of different PPIs. Second, the widely existed population heterogeneity has been masked by the ensemble measurements using coventional reporter plate or colorimetric assays. Flow cytometry is a powerful technique for the quantitative and multiparameter measurement of single mammalian or plant cells at high speed. Combined with the BACTH system with flow cytometry, a new method for the quantitative analysis of PPIs was developed in this dissertation. The contents are summarized as follows:

In chapter one, the methods for study of PPI are systematically reviewed including the BACTH system. Flow cytometry is introduced in detail followed by a discussion for the significance of PPIs analysis at the single-cell level. The research plan and main contents of this dissertation are proposed.

Chapter two describes the construction of the two-tag BACTH system. Because in the BACTH system, the transcription and expression level of reporter protein is determined not only by the binding affinities between the interacting proteins, but also by the cytoplasmic concentrations of the proteins themselves. It is valuable to assess protein expression level side by side with protein-protein interaction measurement. Therefore, His-tag and Flag-tag were inserted between T25 or T18 domain and the hybrid proteins, respectively. Pal and TolB, two important proteins for bacterial outer membrane integrity were chosen as a model of interacting proteins. The constructed plasmids were cotransformed into reporter bacteria E. coli BTH101. The PPI was detected on a reporter plate or by colorimetric assays. Besides, the expression of target proteins was detected via Western blot using Flag/His-tag specific antibodies. These experiments demonstrate the successful construction of the BACTH system.

Chapter three describes the development of flow cytometric approach for the

detection of bacterial cytoplasmic protein. The membrane of Gram-negative bacteria like E. coli is impermeable for most of the molecules with large molecular weight. It is necessary to permeabilize bacteria before cytoplasmic protein labeling. The optimation of experimental conditions was carried out on a laboratory-built high sensitivity flow cytometer (HSFCM) by taking advantage of its high sensitivity. C12FDG was used as a fluorescent substrate of -gal to stain permeabilized bacteria for the detection of PPI on HSFCM at single-bacterium level. Employing immunofluorescent labeling, the expression level of interacting proteins can be measured by flow cytometry through labeling the epitope tags fused to the hybrid proteins. Due to the leakage of the hydrolysate of C12FDG after permeabilization and enzyme activity decreasing caused by fixation, immunofluorescent staining was chosen to detect -gal. At last, the Flag/His-tag fused with interacting protein pair and the reporter protein -gal of protein-protein interaction were immunofluorescently labeled and analyzed on the flow cytometer simultaneously.

Chapter four describes the quantitative measurement of PPI in the BACTH system using flow cytometry. Bistability phenomenon was observed in the BACTH system via flow cytometric analysis that there existed two distinct populations when bacteria were immunofluorescently stained. Neither the reporter -gal nor the interacting proteins were detected in one population of the bacteria. The ratio of the bacterial population with protein expressed varied with the cultivation time for the same coloby and with different individual colonies. Two parameter detection was conduced for both the interacting protein (His-TolB) and the reporter protein (-gal) at the single-cell level for bacterial samples collected at different cultivation time and from different individual colonies. It was identified that the relative reporter protein expression (RRPE), which is defined as the normalized -gal expression to that of the interacting protein, is constant and can be used to evaluate the interaction strength of protein pairs. To

validate the applicability of RRPE in the measurement of protein interaction affinity, two mutated forms of TolB, which renders lower binding affinity to Pal, were constructed and compared with the wild type TolB using the BACTH systems.

Chapter five describes the applications of the flow cytomteric-based BACTH system for the quantitative measurement of PPIs. To investigate the potential of using RREP measured in the BACTH system for the strength evaluation of protein-protein interactions, five pairs of acid (En) and base (Kn) -helices with various heptad repeats (n) and associate into coiled coils were constructed into the BACTH system. The measured RRPE and the affinity reported in literature yields a good correlation. The as-developed flow cytometric-based BACTH system not only allows rapid in vivo detection of protein interaction in single cells but also provides relative affinity assessment of the protein interaction. In addition, using TolB and Pal as a model, the effect of PPI inhibitor on reporter protein expression was examined. Due to the direct detection of the expression level for both the reporter protein and the interacting protein, the decrease of -gal activity caused by other effect, such as protein expression inhibition, can be excluded. Thus, the as-developed method can be used for the convenient and high-throughput inhibitor screening for PPIs.

In chapter six, the contents of present thesis are summarized and the future prospects of the research are given.

Keywords: protein-protein interaction; BACTH system; flow cytometry; immunofluorescent staining; single-bacteria detection

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第一章

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