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·基础研究·

基于 VN210/VC210 的双分子荧光互补定量分析 cofilin-actin 相互作用的特异性 *

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摘要 目的：利用双分子荧光互补技术定量分析 cofilin-actin 的特异性相互作用。**方法：**首先构建表达 VN210（编码荧光蛋白 Venus 1-210 氨基酸）和 VC210（编码荧光蛋白 Venus 210-238 氨基酸）探针的质粒，通过梯度剂量转染检测该探针对的自组装能力；其次构建 VN210/VC210 与 bJun、bFos、Fos△ zip、cofilin（WT）、cofilin（S3E）和 actin 的融合表达载体，在 HeLa 细胞中分别过表达不同载体组合，以 VN210-bJun/bFos-VC210 组作为阳性对照，以 VN210-bJun/bFos△ zip-VC210 组作为阴性对照，使用多功能细胞观测显微镜观察并拍摄荧光图像；最后使用多功能微孔板检测仪，对对照组进行波谱扫描，确定最适合检测的激发光波长和发射光波长，再以此选择波长检测实验组中可观察到荧光信号组合的荧光强度，进行统计分析。**结果：**(1) VN210/VC210 在各转染剂量组中均未观察到荧光信号；(2) 阳性对照组可观察到荧光信号，阴性对照组未观察到荧光信号；实验组中，VN210-cofilin(WT)/actin-VC210 组荧光信号较强，VN210-cofilin(S3E)/actin-VC210 和 VN210/actin-VC210 组荧光信号较弱，其余组均观察不到荧光信号；(3) 产生荧光信号的实验组中，VN210-cofilin(S3E)/actin-VC210 和 VN210/actin-VC210 组间的荧光信号无显著差异，但分别与 VN210-cofilin(WT)/actin-VC210 组具有显著差异。**结论：**VN210/VC210 双分子荧光互补技术可定量检测 cofilin-actin 的特异性相互作用。

关键词：双分子荧光互补；定量分析；cofilin-actin 相互作用

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Quantitatively Analyzing the Specific Interaction of Cofilin-actin by VN210/VC210-based Bimolecular Fluorescence Complementation Technique*

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ABSTRACT Objective: To quantitatively analyze the specific interaction of cofilin-actin using bimolecular fluorescence complimentary technique. **Methods:** Firstly, the plasmids expressing VN210 (coding fluorescent protein Venus 1-210 amino acids) and VC210 (coding fluorescent protein Venus 210-238 amino acids) probes were constructed, and their self-assembly ability was detected by gradient dose of transfection experiment. Then, the fusion expression vectors of VN210/VC210 with bJun, bFos, bFos△ zip, cofilin(WT), cofilin (S3E) and actin were constructed. After co-transfections of the different pairs of vector combinations into HeLa cells, their fluorescent signals were measured. Among the combinations, VN210-bJun/bFos-VC210 was used as a positive control and VN210-bJun/bFos△ zip-VC210 as a negative control. The fluorescent signals were observed and recorded by the multifunctional cell observation microscope. Finally, the microplate detector was used to scan the spectrum of the control group to determine the optimal excitation and emission wavelengths, and the differences of the fluorescence signals observed in the experimental groups were statistically analyzed. **Results:** 1 Fluorescent signals were not observed in VN210/VC210 group under various plasmid transfection doses. 2 In the control groups, the fluorescence signals were observed in VN210-bJun/bFos-VC210 group but not in VN210-bJun/bFos△ zip-VC210 group. In the experimental groups, the fluorescence signal of VN210-cofilin (WT)/actin-VC210 group was stronger, while those of VN210-cofilin (S3E)/actin-VC210 and VN210/actin-VC210 groups were weaker, and fluorescence signals were not observed in other groups. 3 There was no difference of fluorescence signals between VN210-cofilin (S3E)/actin-VC210 and VN210/actin-VC210 groups, but a significant difference between VN210-cofilin (WT)/actin-VC210 and VN210-cofilin (S3E)/actin-VC210. **Conclusions:** The specific interaction between cofilin and actin can be quantitatively detected by VN210/VC210 bimolecular fluorescence complementation.

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前言

研究蛋白质 - 蛋白质相互作用（Protein-Protein Interactions，PPIs）有助于分析蛋白质的功能[1-5]，其方法包括双分子荧光互补（Bimolecular Fluorescence Complementary，BiFC）技术[6,7]。该技术能够在普通荧光显微镜下直接观察多数活细胞中的 PPIs 及定位，操作简易，是可视化活细胞中 PPIs 便捷有效的方法[8-10]。其原理是两个非荧光片段分别与一对相互作用的蛋白质融合表达后，可通过后者的相互作用促进前者靠近并组装成完整荧光蛋白，从而能够被激发产生荧光，而非相互作用蛋白不能通过该方式产生荧光[11]。文献报导，cofilin 在第三位丝氨酸残基的磷酸化修饰抑制其 actin 结合能力[12,13]，基于荧光蛋白 Venus 构建的 VN210/VC210 探针应用于 BiFC 可定性判断 cofilin 与 actin 的相互作用[14]。由于我们在前期实验中发现，相互作用组和非相互作用组均可产生荧光信号，前者较强，后者较弱且与 VN210/actin-VC210 组的荧光信号基本一致。因此，为了定量分析 cofilin-actin 相互作用的特异性，我们使用多功能微孔板检测仪先通过对照组确定最适激发光和发射光波长（495 nm 和 530 nm），再检测产生荧光信号的三个实验组的荧光强度，通过定量数据的统计分析检测 cofilin 与 actin 的特异性相互作用。我们的结果表明，基于 VN210/VC210 探针的 BiFC 技术可定量检测 cofilin-actin 的特异性相互作用。

1 材料与方法

1.1 材料

HeLa 细胞购自中国医学科学院基础医学研究所（Institute of Basic Medical Sciences & Chinese Academy of Medical Sciences，IBMS & CAMS）细胞资源中心。pcDNA3.1(+)为本实验室保存。质粒大量提取试剂盒（PL14）和 DNA 凝胶回收试剂盒（DR01）购自北京艾德莱生物技术有限公司。限制性内切酶、DNA 连接试剂盒（6022）和 DNA 聚合酶（R044A）购自大连宝生物工程有限公司。琼脂糖（75510-019）购自 Invitrogen 公司。琼脂（PB10028）购自北京普博欣生物科技有限公司。Trans5α 感受态细胞（CD201）和 Trans2K Plus DNA Marker（BM-111）购自北京全式金生物技术有限公司。

1.2 方法

1.2.1 细胞培养 配制含 10%胎牛血清（Fetal Bovine Serum，FBS）的达尔伯科改良的伊格尔（Dulbecco's Modified Eagle Medium，DMEM）培养基。从液氮罐中取出冻存的 HeLa 细胞，37 ℃水浴迅速解冻，离心机离心（室温，800 r/min，5 min），弃上清，加入配制好的培养基混匀后接种于培养皿中，于 37 ℃、5% CO_2 培养箱中培养。当细胞密度达到 80%~90%时，用 0.25%胰酶消化传代。

1.2.2 载体构建 根据荧光蛋白 Venus 的开放阅读框序列合成编码 Flag-VN210（编码 Venus 1-210 氨基酸）和 VC210（编码 Venus 210-238 氨基酸）-HA 的 DNA 序列，克隆入 pcDNA3.1(+)载体，分别命名为 pcDNA3.1-VN210 和 pcDNA3.1-VC210。将合成的 bJun 序列（GeneBank 序列号：X17163；编码 257-334 氨基酸）克隆入 pcDNA3.1-VN210 载体构建 pcDNA3.1-VN210-bJun。将合成的 bFos 序列（GeneBank 序列号：NM_022197；编码 118-211 氨基酸）和 bFos△zip（编码 118-211△179-193 氨基酸）克隆入 pcDNA3.1-VC210 载体分别构建 pcDNA3.1-bFos-VC210 和 pcDNA3.1-bFos△zip-VC210。将合成的 cofilin 序列（GeneBank 序列号：XM_034651872；编码 1-166 氨基酸）克隆入 pcDNA3.1-VN210 载体构建 pcDNA3.1-VN210-cofilin（WT），再通过定点突变技术得到 pcDNA3.1-VN210-cofilin（S3E），该突变体的第三位丝氨酸残基突变为谷氨酸残基。将合成的 actin 序列（GeneBank 序列号：NM_007393；编码 1-375 氨基酸）克隆入 pcDNA3.1-VC210 载体构建 pcDNA3.1-actin-VC210。

1.2.3 细胞转染与检测 HeLa 细胞接种于孔板中并在培养箱中培养过夜。次日按照质粒（μg）和聚乙烯亚胺（Polyethylenimine，PEI）（μL）1:2 的比例转染细胞，除特别标注外，每个质粒的转染浓度为 0.25 μg/mL。细胞在培养箱中培养约 24 h 后，弃上清，加入适量 PBS，使用多功能细胞观测显微镜 EVOS（美国 ThermoFisher Scientific，Auto 2）拍照，使用多功能微孔板检测仪（美国 Biotek，Synergy H1）测量荧光强度。

1.3 统计学分析

采用 GraphPad Prism 8 统计软件进行分析处理。两组间比较采用独立样本 t 检验，$*P<0.05$ 为具有统计学意义。

2 结果

2.1 VN210/VC210 的自组装能力与质粒转染剂量无关

利用不同剂量的增强型绿色荧光蛋白（Enhanced Green Fluorescent Protein，EGFP）表达质粒和 VN210/VC210 质粒对分别转染 HeLa 细胞。如图 1 所示，EGFP 产生荧光的细胞比例随着质粒转染剂量的增加而升高，但 VN210/VC210 的不同转染剂量组均观察不到明显的荧光信号。这一结果提示，VN210/VC210 探针基本不发生自组装，也与转染剂量无关。

2.2 基于 VN210/VC210 探针检测 cofilin-actin 的相互作用

根据上述实验结果，进一步利用 VN210/VC210 探针检测 cofilin-actin 的相互作用及其特异性。按照图 2 所示的不同质粒组合转染 HeLa 细胞，结果显示，阳性对照组（VN210-bJun/bFos-VC210）可观察到明显的荧光信号，而阴性对照组（VN210-bJun+bFos△zip-VC210）基本观察不到荧光信号。对于实验组而言，除 VN210-cofilin(WT)/actin-VC210 相互作用组荧光信号较强外，VN210-cofilin (S3E)/actin-VC210 和 VN210/actin-VC210 组均观察到较弱荧光信号，其余各组观察不到荧光信号。这些结果表明，VN210/VC210 探针可以检测 cofilin-actin 之间特异的相互作用。

2.3 定量分析 VN210-cofilin(WT)/actin-VC210 相互作用的特异性

为了定量分析上述三个实验组（VN210-cofilin (WT)

/actin-VC210、VN210-cofilin(S3E)/actin-VC210 和 VN210/actin-VC210)产生荧光信号的强度,首先利用多功能微孔板检测仪对阳性对照组和阴性对照组的细胞进行波谱扫描,得到能够区分二者的最适激发和发射光波长,它们分别是 495nm 和 530nm(图 3 A、B)。再利该仪器检测上述三组和无转染组细胞中的荧光信号强度,进行统计分析。如图 3 C 所示,VN210-cofilin(S3E)/actin-VC210 和 VN210/actin-VC210 两组间相对荧光强度无统计学差异,但均与 VN210-cofilin(WT)/actin-VC210 间具有显著统计学差异,即 VN210-cofilin(WT)/actin-VC210 组产生更强的荧光信号。该结果进一步说明,VN210/VC210 探针可以定量检测 cofilin-actin 的特异性相互作用。

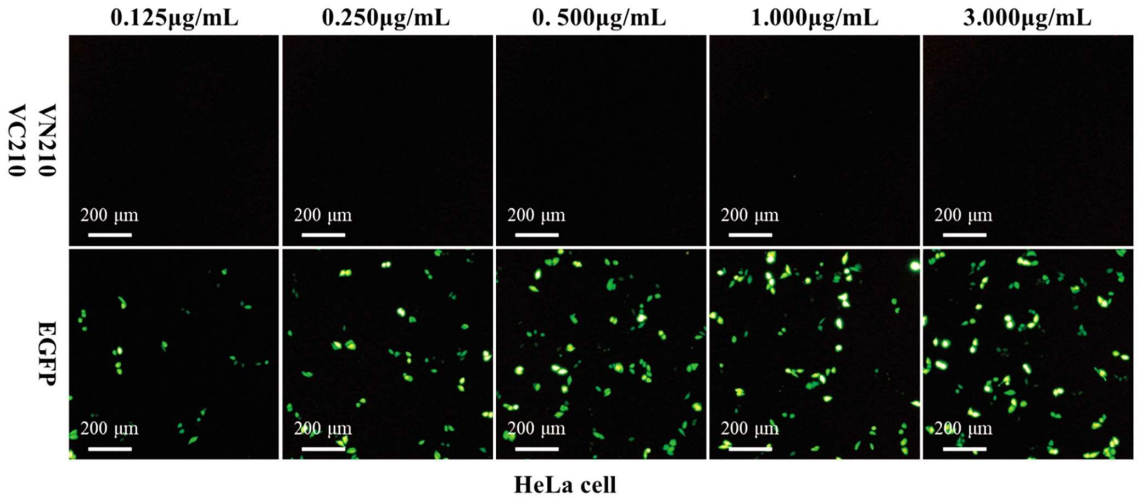

图 1 VN210/VC210 探针对自组装能力的检测

Fig.1 Detection of self-assembly ability of VN210/VC210 probe pair

Note: The fluorescent images of EGFP and VN210/VC210 probe pair at different plasmid dose were taken.

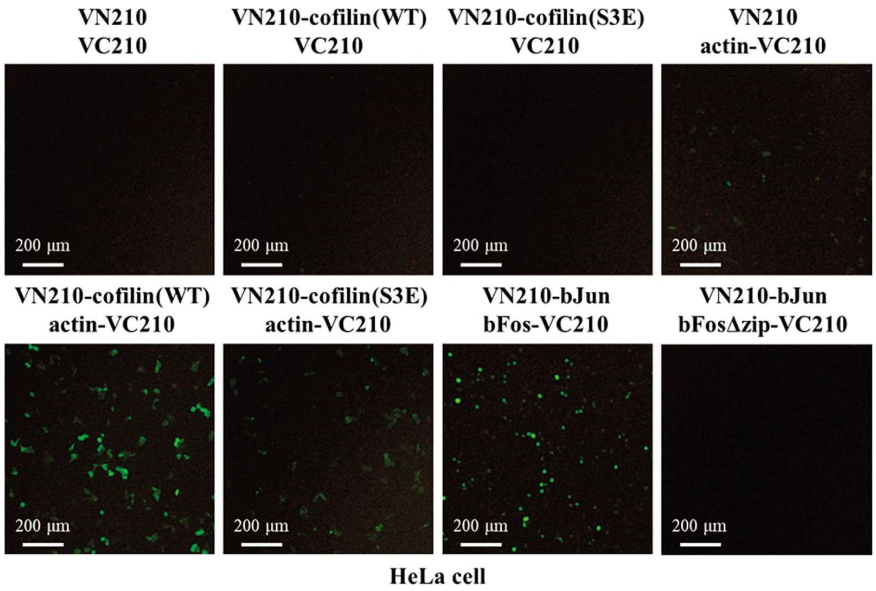

图 2 定性分析 cofilin-actin 的特异性相互作用

Fig.2 The specific interaction of cofilin-actin was qualitatively analyzed

Note: The fluorescent images of VN210/VC210-based fusion protein or non-fusion protein expression cells were captured. The interaction of bJun-bFos and non-interaction of bJun-bFosΔ zip were used as positive and negative controls respectively, and the specific interaction of cofilin-actin was displayed.

3 讨论

PPIs 是产生生物学效应的基础[15-18]。目前已经发展出多种研究 PPIs 的方法[19-22],其中 BiFC 技术由于能够利用荧光显微镜直接地观察活细胞中的 PPIs 及其亚细胞定位而受到越来越多的重视[23,24]。BiFC 检测方法早期使用的黄色荧光蛋白(Yellow Fluorescent Protein,YFP)由于成熟过程比较缓慢、需低温(例如 30℃)拍照以及对酸敏感且被氯离子(Cl^-)淬灭等因素,其应用受到了较多的限制。维多利亚水母绿色荧光蛋白(aequorea Victoria Green Fluorescent Protein,avGFP)为生物学研究提供了广阔的应用前景[25-30],在经过一系列定点突变后得到荧光性能更好的黄色荧光蛋白 Venus,将 Venus 应用于 BiFC 技术不仅提高了荧光效率而且细胞可在生理培养条件下进行荧光拍照[31]。本研究的结果表明,VN210/VC210 探针不仅

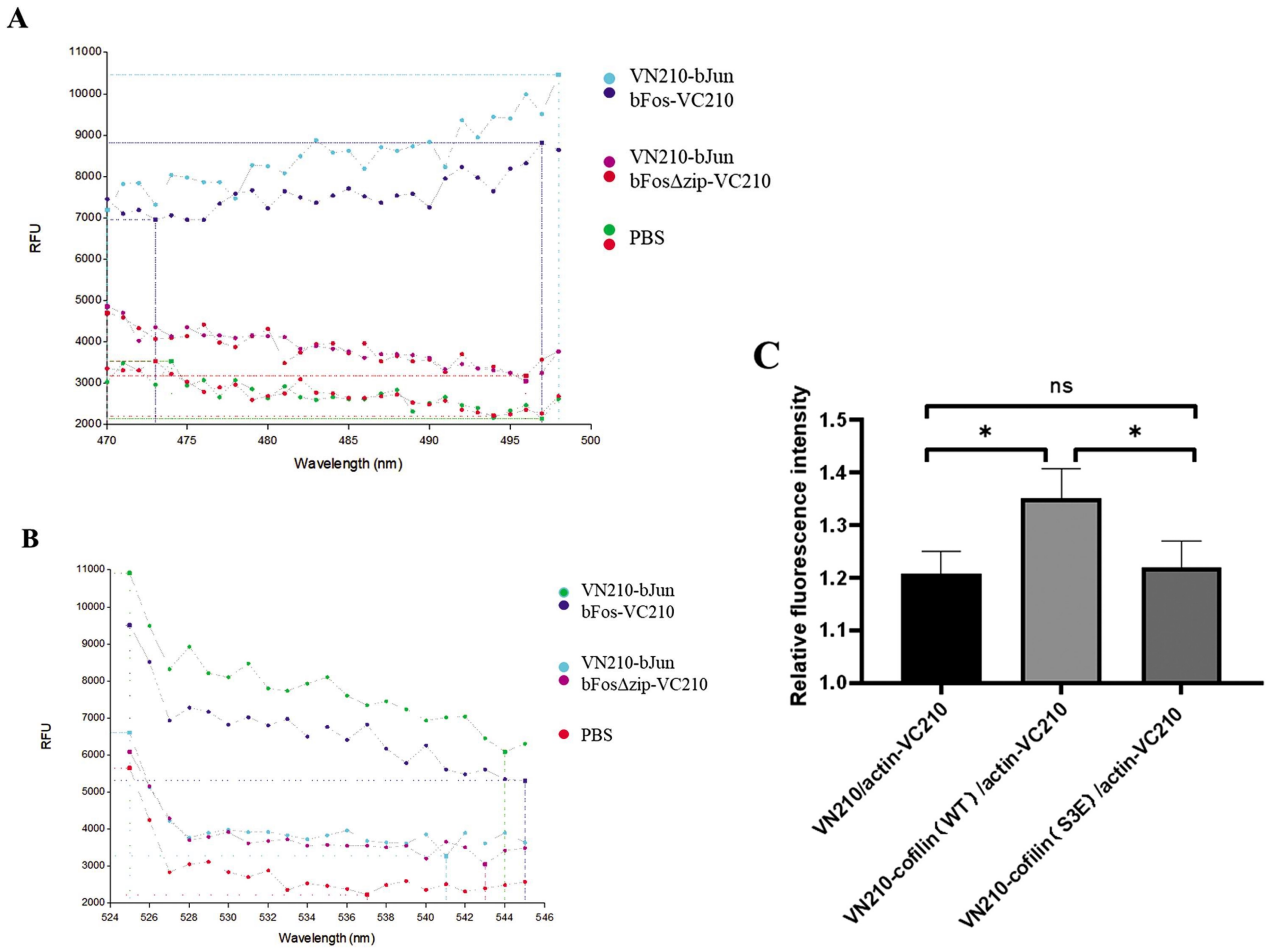

图 3 定量分析 VN210-cofilin(WT)/actin-VC210 相互作用的特异性

Fig.3 Quantitative analysis of the specificity of VN210-cofilin(WT)/actin-VC210 interaction

Note: A. Fixed Emission: 530 nm, Excitation Start: 470 nm, Stop: 500 nm, Step: 1 nm, Optics: Top, Gain: 25. B. Fixed Excitation: 495 nm, Emission Start: 525 nm, Stop: 545 nm, Step: 1 nm, Optics: Top, Gain: 25. C. The ratio of fluorescence intensity measured by each group to that measured by PBS group is expressed as relative fluorescence intensity. ns represents no significant difference,* represents $P < 0.05$.

不通过自组装产生荧光，还能够特异性地检测 cofilin-actin 之间的相互作用。

质粒梯度转染剂量实验表明 VN210/VC210 组无论转染剂量高低均观察不到荧光信号，但 EGFP 组中产生荧光细胞的比例随着质粒转染剂量的增加而升高，提示 VN210/VC210 不发生自组装与目的蛋白表达水平低无关，可能与由自身理化性质决定。

在检测 cofilin-actin 相互作用的实验中，相互作用组无论是 VN210-bJun/bFos-VC210 或 VN210-cofilin(WT)/actin-VC210 均观察到明显的荧光信号，而非相互作用组 VN210-bJun/bFosΔzip-VC210 观察不到荧光信号，VN210-cofilin (S3E)/actin-VC210 组与 VN210/actin-VC210 组观察到较弱但强度接近的荧光信号，提示 actin 可能与 VN210 之间存在特异或非特异的相互作用，这需要设计实验再进一步验证。

鉴于 BiFC 技术具有可视化活细胞中 PPIs 及其亚细胞定位的特点，接下来的研究工作可考虑进一步鉴定 cofilin-actin 相互作用在细胞中的具体位置，将有助于更深入地分析这两种蛋白质相互作用的功能。

总而言之，利用多功能微孔板检测仪通过 495 nm 的激发光和 530 nm 的发射光可定量分析基于 VN210/VC210 的 BiFC 中 cofilin-actin 的特异性相互作用。

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