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行政院國家科學委員會補助專題研究計畫成果報告

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※ PC12 細胞於胞吐時其 Rab3A 與 Rab3A/Rabphilin3A 複合 ※
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計畫類別：■個別型計畫 □整合型計畫
計畫編號：NSC90－2311－B－040－006
執行期間：90 年 8 月 1 日至 91 年 7 月 31 日

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執行單位：中山醫學院生命科學系

中 華 民 國 91 年 10 月 15 日

行政院國家科學委員會補助專題研究計畫成果報告

PC12 細胞於胞吐時其 Rab3A 與 Rab3A/Rabphilin3A 複合物分布之動態分析

Dynamic Analysis of Localization of Rab3A and Rab3A/Rabphilin3A During Exocytosis in PC12 Cells

計畫類別：■個別型計畫　□整合型計畫
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一、中文摘要

Rab3A 被認為是一種參與調控式胞吐的小 G 蛋白。根據目前的研究認為 Rab3A 的作用是在囊泡的導引與膜融合，但是沒有直接證據證明其作用的位置與實際參與的生化反應。為了解決這個問題，首先一定要了解 Rab3A 在活體細胞中的位置與其在胞吐作用時的分佈變化。本計劃將 Rab3A 以螢光蛋白（Enhanced Cyan Fluorescent Protein, ECFP）結合，在 PC12 嗜鉻性瘤細胞中表現，以螢光顯微鏡來觀察 Rab3A 在活體細胞中的位置與其在胞吐作用時的分佈變化。

本年度成功地完成計劃所需的基本實驗配備，實驗材料與實驗條件，包括(1) 分泌囊泡標記蛋白之螢光融合蛋白（NPY-GFP、Chg-DsRed2、VAMP2-YFP）與 Rab3A 結合蛋白之螢光融合蛋白（YFP-Rabphilin3A）的真核表達載體的構築），(2) 建立分泌與蛋白質分布同步觀察的螢光顯微鏡系統，(3) 建立觀察螢光蛋白質動態分布的顯微影像分析系統。根據這些基礎工作所得的實驗條件，進行觀察 GFP-Rab3A 在 PC12 細胞中的分布。由免疫螢光顯微影像發現融合蛋白可以被 Rab3A 的抗體辨認。此外，本結果與過去免疫螢光染色結果相似，EGFP-Rab3A 在細胞呈點狀分布，且集中在核周圍與細胞膜附近。當 ECFP-Rab3A 與分泌囊泡標記之螢光融合蛋白（NPY-GFP）共同表現時，發現 ECFP-Rab3A 與標記融合蛋白有類似的分布，顯示 ECFP-Rab3A 位於分泌囊泡上。此兩項實驗結果證明螢光融合蛋白並不會影響 Rab3A 的正確位置。當以高鉀溶液刺激同時表現 NPY-GFP 與 ECFP-Rab3A 的 PC12 細胞，雙光螢光顯微鏡紀錄，發現 ECFP-Rab3A 螢光隨著 NPY-GFP 螢光下降而下降，此一結果顯示 ECFP-Rab3A 分布在胞吐時有轉移的現象。為了更精密地了解此種移動是移出聚焦面還是離開分泌囊泡擴散至細胞質，以內全反射螢光顯微鏡（Total Internal Reflection Fluorescence Microscopy, TIRFM）觀察 ECFP-Rab3A 在 PC12 細胞中的動態分布。在此之前，先觀察分泌囊泡標記蛋白之螢光融合蛋白（NPY-GFP），以了解我們的 TIRFM 系統是否可以觀察單一囊泡的分泌時的各步驟，結果發顯此 TIRFM 系統可以觀察分泌囊泡的移動、定位、膜融合。確定此 TIRFM 的表現之後，以此 TIRFM 分析 EYFP-Rab3A 在 PC12 細胞中的動態分布，並以 PowerPoint 進行初步分析。光點

的大小與形狀，發現其大小約 0.3 微米到 1 微米之間，小光點其形狀近似圓形，大多分布在細胞膜附近呈靜止狀態，此顯示小光點可能為準備分泌的囊泡。有一部份囊泡從細胞內部往細胞膜附近移動，顯示 Rab3A 可能作用於囊泡的導引與膜融合反應。部分光點有互相融合，有部分光點會從細胞膜像細胞中心移動，此種現象類似內胞食體（endosome）的行為，此顯示出 Rab3A 亦有可能參與突觸囊泡的回收與生成。當以高鉀刺激細胞時，發現 EYFP-Rab3A 的螢光會增強而後模糊減弱，似乎證明 EYFP-Rab3A 會導引分泌囊泡而後從囊泡離開。然而此一現象亦有可能是細胞底部因加藥而貼近玻璃所致，需要進一步以反射干涉顯微鏡進一步檢測。
關鍵詞： Rab3A、螢光蛋白、NPY-GFP、胞吐、內全反射螢光顯微鏡、螢光顯微鏡、PC12 細胞

Abstract

Rab3A is a small GTP-binding protein thought to regulate regulated exocytosis. Recent investigations indicate Rab3A plays a role in docking and fusion steps of exocytosis, but there is no direct evidence to prove where Rab3A acts. To solve this problem, tracking Rab3A localization during exocytosis in vivo need to be established. In this study, we have setup a simple system to detect Rab3A translocalization in vivo.

In this study, we have accomplished basic reagents, facility and experimental conditions; including construct of expressing vectors of fluorescent protein-tagged organelle markers （NPY-EGFP、Chg-DsRed2、VAMP2-EYFP）and Rab3A-binding protein (EYFP-Rabphilin3A), simultaneous detection system for secretion and protein translocalization, and detection and analysis system for dynamic protein localization. Based on these accomplishments, we detect protein localization of Rab3A in PC12 cells during exocytosis. Cells expressing EGFP-Rab3A are analyzed by immuohistochemistry, EGFP-Rab3A can be recognized by anti-Rab3A antibody. Besides, subcellular localization Rab3A is similar to previous investigations that Rab3A-associated compartments are punctuated and concentrate in cell membrane and perinuclear region. When ECFP-Rab3A and NPY-EGFP, one of secretory vesicle markers, are co-expressed in PC12 cells, ECFP-Rab3A and NPY-EGFP are co-localized. These two results indicate that fluorescent protein doesn't interfere normal subcellular localization of Rab3A. When cells are stimulated with high potassium, fluorescent intensity of NPY-EGFP decrease due to release of NPY-EGFP in to cytosol. Interestingly, ECFP-Rab3A decreases, too. This indicates that Rab3A translocalize during exocytosis. To verify whether translocalization of ECFP-Rab3A is moving out of the focal plane or dissociating from secretory vesicles and diffusing into cytosol, TIRFM (Total Internal Reflection Fluorescence Microscopy) is used for detection of dynamic localization of ECFP-Rab3A during secretion. Before this study, we use NPY-EGFP, soluble content of secretory vesicles, to confirm our TIRFM capable of detecting all steps of single exocytosis, and find that our system can detect movement, docking and fusion of vesicles during secretion. After this, we use TIRFM to detect behavior of EYFP-Rab3A in living PC12 cells and analyze dynamic protein localization by PowerPoint. Sizes of fluorescent dots are ranging from 0.3micron to 1micron. Fluorescent dots are static near plasma membrane, and these may be docked vesicles. Some fluorescent dots move from center of the cell to plasma membrane, and this indicates that Rab3A involves in docking of vesicles. Some of vesicles move oppositely and fuse each other, and these phenomena are similar to those of endosomes. Therefore, Rab3A seems to also play a role in recycle of vesicles. When high potassium stimulates cells expressing EYFP-Rab3A, fluorescence of EYFP-Rab3A increases and becomes blurring. This may be due to Rab3A moving toward plasma membrane and dissociating from secretory vesicles. But this observation may be also due to increasing membrane associated with coverslip when high potassium is applied. And we need to use reflection interference contrast microscopy to rule out this possibility.
Keywords: Rab3A, GFP, regulated exocytosis, TIRFM, fluorescence microscopy

二、緣由與目的

Rab3A is one member of small G protein family expressed in neuronal cells and secretory cells. The Rab3A protein is thought to be involved in regulated exocytosis. There are several lines of evidence that prove that Rab3A proteins involve in vesicle-plasma membrane fusion. [5, 6,7, 8] However other reports suggested that Rab3A might involve in docking of vesicles to plasma membrane and recycle of secretory vesicles rather than vesicle-plasma membrane fusion. [2] The reason why investigations cannot precisely point at which Rab3 proteins act in these sequential processes is problems of methods. Overexpression of Rab3A and its mutants might cause unexpected artifacts, ex. Inhibitory effect of secretion might be due to non-specific depletion of other small G protein effectors. Adding GTP and its analogues might also activates or inactivates small G proteins and trimeric G proteins, and makes results hard to be interpreted. Combining electrophysiological and transgenic techniques can provide results with very high temporal resolution but no further detail molecular roles of roles of Rab3A in regulated exocytosis, especially location of Rab3A and how Rab3A interacts with its effectors to affect exocytosis. Reduction of LTP in Rab3A knockout mice may prove that Rab3A involves in the

late step of exocytosis, but the mechanism of Rab3A in the late step of exocytosis remains unknown. [6] Biochemical analysis of synaptosomes or cells stimulated by secretory agents may resolve more detail molecular mechanism, but exocytosis events happen within second and it is hard to detect molecular details at each steps of exocytosis. [4, 13] Besides, part of molecules dissociates from membranous compartments during preparation of samples, and some of molecular information will be lost. Therefore, the exact role and molecular mechanism of Rab3A in exocytosis needs more tools to resolve.

Recent improvement of reliable fluorescence probes and cell imaging, optical methods can detect protein localization and exocytosis with very high temporal and spatial resolutions, even distinguish each step of exocytosis, including moving, docking and fusion. [1, 12] Almers and his colleagues fuse NPY (neuropeptide Y, a soluble peptide in secretory vesicles) GFP (Green fluorescent protein of *A. Victoria*) to detect secretion activity by epifluorescence and TIRFM (Total Internal Reflection Fluorescence Microscopy). Docked vesicles containing GFP-tagged NPY at the particular focal plan will be released when cells are evoked with secretory stimulators, and fluorescent intensity will drop when GFP-tagged NPY diffuse out of the focal plane. After secretion, fluorescent intensity will increase when new GFP-tagged NPY containing vesicles move to the focal plane. [9, 15] Co-expressing with other fluorescent proteins and fluorescent probes, ex. Rab3A, makes simultaneous detection of protein translocalization, cytosolic calcium and secretion possible. Similar approach has bee developed by Rothman and his colleagues. [11, 14] They construct pH-sensitive GFP (pHluorin) and this new version of GFP has very low fluorescence excited at 470nm in acidic condition (pH 5.5), and fluorescence increase in alkaline condition. Because of sensitivity to pH, pHluorin is fused with organelle marker proteins to detect pH of organelles. In secretory vesicles, there are lots of proton pumps to make vesicles acidic. During secretion, vesicles fused to membrane and internal content will be neutralized by extracellular medium. Using this particular property, pHluorin is fused with VAMP2 (vesicle associated membrane protein 2; a v-SNARE of synaptic vesicles) to let pHluorin locate in the lumen of synaptic vesicles, named as superecliptic synaptopHluorin, to detect exocytosis. Fluorescent intensity is low before exocytosis, because lumen of vesicles is acidic. During exocytosis, fused synaptic membrane exposes this protein to neutral pH condition, fluorescent intensity of this fusion protein increases during secretion. After secretion, fluorescent intensity decreases due to recycle of synaptic vesicles. Therefore, this method can detect both exocytosis and recycle of synaptic vesicles. Increased fluorescent intensity also indicates location of fused vesicles.

Locations of proteins reveal functions of proteins. For examples, if Rab3A plays a role in docking and inhibits fusion, Rab3A needs to locate at synaptic vesicles during docking and dissociate form vesicles during membrane fusion. Accoding to Jahn's investigations, Rab3A dissociates from synaptic vesicles during secretion, and free Rab3A are in GDP-form. Besides, Rab3A that binds to synaptic vesicles is GTP-bound form, and binding activity will be decreased by high calcium concentration and enhanced by GDI (GDP dissociation inhibitor). [3, 4, 13] But these findings have been done in synaptosomes and analyzed by biochemical methods; these results cannot fully the real behaviors of Rab3A in living cells. Here, we co-express ECFP (enhanced cyan fluorescent protein)-tagged Rab3A with NPY-EGFP in PC12 cells, and localization of Rab3A during secretion is visualized by epi-fluorescence microscopy, confocal microscopy and TIRFM to test whether Rab3A dissociates from secretory vesicles in living cells.

三、研究報告應含的內容

Construct mammalian vector to express fluorescent protein-tagged fusion proteins. Restriction sites are added to genes of interest, including Rabphilin3A, Chromograinin A, VAMP-2 and NPY, by PCR, and then PCR products are cloned into pGEMT to amplify PCR products. Because C-terminus of Rab3A determines its subcellular distribution, Rab3A is fused to C-terminus of fluorescent protein to avoid that fluorescent protein interferes its location. In contrast, the marker proteins of secretory vesicles are fused to the N-terminus, because their target sequences are in their N-terminus. Inserts are cut and cloned into fluorescent protein-tagged fusion expressing vector. The correct clone is picked by restriction enzyme digestion. Fig.1 indicates that correct vectors to express fluorescent protein-tagged fusions have been constructed.

Characterization of subcellular localization of fluorescent protein-tagged Rab3A

In some cases, GFP interferes structure of proteins and makes fusion proteins lose normal functions and subcellular localizations. (Personal communications with Dr. Piston at Vanderbilt University) Therefore, characterization of EGFP-Rab3A is the first step for this study.

First, we use anti-Rab3A and rhodamine-conjugated secondary antibody to visualize Rab3A, including endogenous and EGFP-Rab3A in PC12 cells. Fluorescence of EGFP-Rab3A has same protein subcellular localization as that of Rab3A visualized by immunohistochemistry (Fig. 2A). Then, we co-express NPY-EGFP, a water-soluble marker protein in secretory vesicles, with either ECFP-Rab3A or ECFP-RhoB (an endosome marker).

There are two possible explanations for this dissociating from secretory vesicles. But this

ECFP-Rab3A co-localizes with NPY-EGFP, but ECFP-RhoB doesn't (Fig.2B and C). To avoid fluorescence bleeding, we carefully examine our dual color epi-fluorescence microscopy. Cells are transfected with either pECFP-Rab3C or pEYFP-Rab3A, and observe their fluorescence excited at different wavelengths (430nm for ECFP and 513nm for EYFP). There is no fluorescence excited at wrong excitation wavelength (Fig. 3). These two results indicate that fluorescent protein has no effect on subcellular localization of Rab3A.

Detect exocytosis in PC12 cells by epi-fluorescence microscopy. To detect subcellular localization of Rab3A and secretion simultaneously needs to setup an optical method to detect exocytosis. According to Almer's experimental design (Fig. 3A), fluorescent content in vesicles will be released and fluorescent intensity of the focal plane decreases when cells are stimulated for secretion. After secretion, new vesicles arrive at the focal plane, and fluorescent intensity increases. [9, 15] In our hands, PC12 cells expressing NPY-EGFP are stimulated with high K+; the fluorescent intensity of NPY-EGFP decreases immediately and increases slowly, similar to Almer et al's results.

Translocalization of Fluorescence-tagged Rab3A during exocytosis. Because NPY is fused with enhanced green fluorescent protein, Rab3A fuses to another GFP variant, ECFP, for dual colors epi-fluorescence microscopy. To fit CFP/YFP dual filter set, EGFP is excited by 500nm, and this causes fluorescent intensity is lower than that excited at 470nm. But, this is still enough to detect release of NPY-EGFP enhanced by 3×3 binning. Similar secretion results can be obtained by this system, and surprisingly fluorescent intensity of ECFP-Rab3A decreases during release of NPY-EGFP and increases after secretion. (Fig. 4C) This kind of phenomena is not due to fluorescence bleeding, because out filter set is capable of distinguishing EYFP and ECFP (Fig. 3). Besides, in PC12 cells co-expressing EGFP and ECFP-Rab3A, fluorescent intensity EGFP has no change as that of ECFP-Rab3A during secretion. (Fig. 4D) Therefore, fluorescent intensity change of ECFP-Rab3A is stimulus-dependent and this kind of change cannot be found in EGFP (data not shown).

There are two possible explanations for this phenomenon. First, ECFP-Rab3A-associated vesicles move out of the focal plane. Second, ECFP-Rab3A dissociates from fused vesicles and diffuses out of the focal plane. If first explanation is correct, ECFP-Rab3A-associated vesicles move away from the plasma membrane. If the second is correct, ECFP-Rab3A won't co-localize with fused secretory vesicles. The following experiments, we use TIRFM to verify this phenomenon.

Test of capability of TIRFM in detection of exocytosis. TIRFM was used to detect fusion events near cell surface. The advantages of TIRFM are its high spatial and temporal resolution of images, and low photo-damages to cells by incident light beam [1, 12]. Since the high NA objectives were developed few years ago, the objective-based TIRFM high NA objectives have been used to monitor the events occur at the sample surface. This system has been used to detect all steps in single exocytosis successfully [15], and TIRFM at NTU is objective-based model (Fig. 5A and B).

First, we use NPY-EGFP to test the capability of TIRFM at NTU. Comparing with ep-fluorescence microscopy, TIRFM provides more crispy images shown in Fig. 5C and D. When cells are stimulated with high potassium, fluorescent intensity of some vesicles increases gradually (moving toward plasma membrane), reaches highest intensity (docking) and then disappears (fusion) abruptly. These results indicate that TIRFM system is capable of detecting steps of each exocytosis. (Fig. 5E)

Dynamic analysis of subcellular localization of Rab3A in living PC12 cells by TIRFM. After confirming imaging capability of TIRFM, we use this system to detect dynamic protein localization of EGFP-Rab3A in living PC12 cells. Acquired time-lapse images will be analyzed by PowerPoint (Fig. 6A). Sizes of Rab3A-associated compartments are ranging 0.3micron to 1micron. Fluorescent dots are static near plasma membrane, and these may be docked vesicles. (Fig 6B-3) Some fluorescent dots move from center of the cell to plasma membrane, and this indicates that Rab3A involves in docking of vesicles. (Fig. 6B-2) Some of vesicles move oppositely and fuse each other, and these phenomena are similar to those of endosomes. (Fig. 6B-1, 7Ac-d) Therefore, Rab3A seems to also play a role in recycle of vesicles. Besides of detection of lateral movement, fluorescent intensity of vesicles also indicates vertical distance to coverslip. When high potassium stimulates cells expressing EYFP-Rab3A, fluorescent intensity of some vesicles increases (Fig. 7Ab-c), but also fluorescent intensity of some vesicles decreases (Fig. 7Aa-b). This indicates that Rab3A-associated compartments move forward and backward plasma membrane. Overall fluorescence of EYFP-Rab3A increases and becomes blurring (Fig. 8), this may be due to Rab3A moving toward plasma membrane and dissociating from secretory vesicles. But this observation may be also due to increasing membrane associated with coverslip when high potassium is applied. And we need to use reflection interference contrast microscopy to rule out this possibility.

四、計畫成果自評

In this study, we successfully set up an epi-fluorescence microscopy system to detect protein localization and secretion simultaneously in living PC12 cells. Combining with TIRFM, we can detect detail dynamic protein localization of Rab3A during secretion. This system provides more direct and simple way to study this phenomenon in living cells

without further complicated interpretations and experiments.

In early investigations, Jahn's group analyzes localization of Rab3A in stimulated and resting synaptosomes by subcellular fractionation and westernblot; Rab3A dissociates from synaptic vesicle membrane and keep GTP-form when it associates with membrane. [4, 13] But, it is still possible that this kind of change may be due to artifacts during proceeding experiments. Therefore, they setup a in vitro system to prove that only GTP-bound Rab3A associates with synaptic vesicles and Rab3A dissociates from synaptic vesicles due to increase of calcium. [3] But this system is very complicated and these results still cannot fully prove that dynamic localization of Rab3A during secretion in living cells. Several groups use Rab3A mutants [7, 8], application of different GTP analogues [10], and knockout mice to study the roles in regulated exocytosis [5, 6], and find that GTP-bound Rab3A associates with synaptic vesicles to inhibit fusion step of secretion and Rab3A. Therefore, Rab3A should hydrolyze GTP and dissociate from the synaptic vesicles to precede fusion step of secretion. These kinds of studies have been done in living cells and animals, but these results only provide indirect evidence for dynamic localization of Rab3A during secretion. Moreover, these kinds of approaches are very complicated and needs more efforts to interpret results.

Our system not just provide the easy way to detect and quantitate translocalization of Rab3A but also can be applied to detect translocalization of other proteins during secretion. Combining Fluor-3 and ECFP-Rab3A, our system can be expanded to detect cytosolic calcium effect on translocalization. Using tetanic toxin, an inhibitor for membrane fusion, this system also can investigate whether dissociation of Rab3A from synaptic vesicles is fusion-dependent.

Limitation of our system is the choice between temporal and spatial resolution. Because fluorescence of cells co-expressing two different fusion proteins is low, this will need more time to capture enough signal for good spatial resolution. But exocytosis events are very fast; this system cannot capture translocalization Rab3A with very high resolution. Confocal microscopy can provide good spatial resolution, but it needs time for scanning images and our problem still remains. Two-photon excitation microscopy can provide images in very good temporal and spatial resolution, but it is very expensive for us. The possible solution is TIRFM (total internal reflection fluorescence microscopy), because this system only illuminates 30-300nm thick at the interface between the cell and coverslip to avoid out-of-focus fluorescence and detect good images at very high speed. In our lab, we have used TIRFM to detect dynamic localization of Rab3A in living PC12 cells. [16] Right now, we are trying to setup dual colors TIRFM and increase detection efficiency by image intensifier and high-speed CCD camera. We hope to know exact localization of Rab3A during exocytosis in the following projects.

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Fig. 1 Restriction Map of DsRed2-fused secretory marker expressing vectors. A. Chromogranin A is fused with N-terminus of DsRed2. Construct is digested by HindIII to generate 1.3Kbp insert. BamHI digestion profile shows that chromogranin A correctly fused to N-terminus of DsRed2 according to restriction map near multiple cloning sites. B. Full-length VAMP2 is fused with N-terminus of DsRed2. Construct is digested by HindIII to generate 0.4Kbp insert. BamHI digestion profile shows that VAMP2 correctly fused to N-terminus of DsRed2 according to restriction map near multiple cloning sites. C. Rabphilin3A are correctly fused to C-terminus of EYFP.

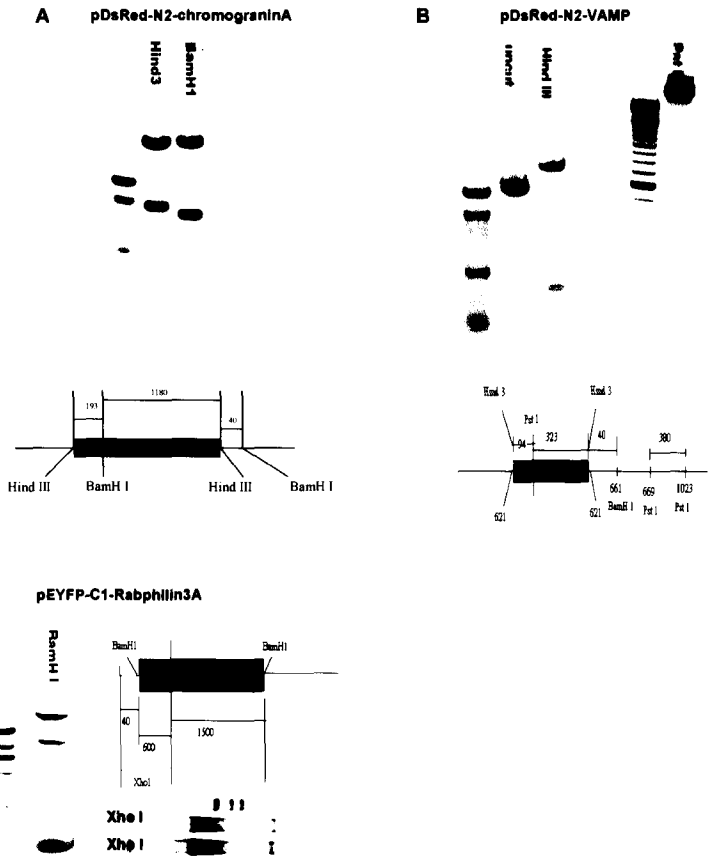
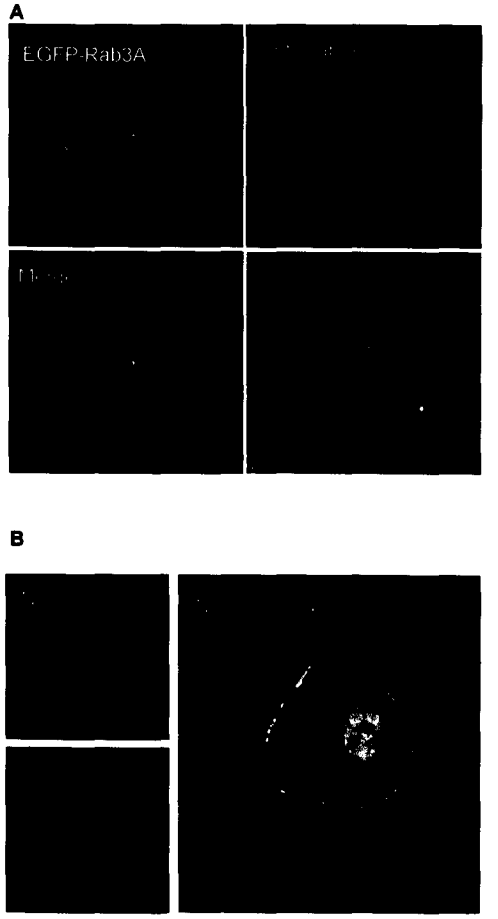


Fig. 2 Characterization of subcellular localization of Rab3A fused with GFP and its variant. **A.** Comparison of subcellular localization of EGFP-Rab3A and immunocytochemistry of Rab3A. PC12 cells tranfected with pEGFP-Rab3A are fixed and immunostained with anti-Rab3A. Then, cells observed by confocal microscopy; green channel for EGFP and red channel for Rhodamin-conjugated secondary antibody. EGFP-Rab3A has the same subcellular localization as that of endogenous Rab3A. **B.** ECFP-Rab3A co-localizes with NPY-EGFP. Cells co-expresses ECFP-Rab3A and NPY-EGFP and are observed by dual colors epi-fluorescence microscopy. Most of ECFP-Rab3A are in punctuated compartments, and co-localized with NPY, a soluble marker in secretory granules. **C.** ECFP-RhoB doesn't co-localize with NPY-EGFP. Cells co-expresses ECFP-RhoB, a small G protein that locates at endosomes, and NPY-EGFP and are observed by dual colors epi-fluorescence microscopy. As expected, ECFP-RhoB is not co-localized with NPY.



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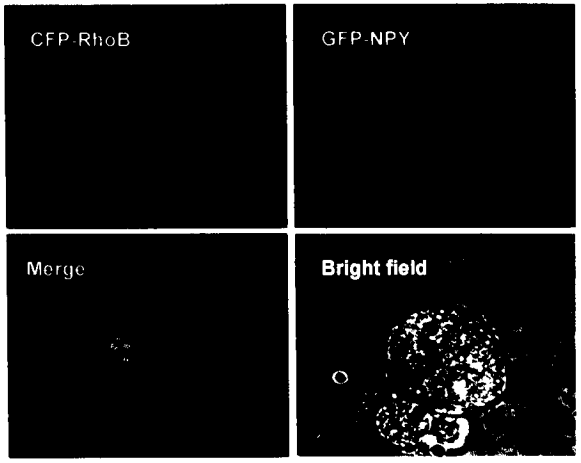

Fig. 3 There is no cross-interference between ECFP and EYFP. Cells are transfected with either pECFP-Rab3C or pEYFP-Rab3A, and observe their fluorescence excited at different wavelengths (430nm for ECFP and 513nm for EYFP). There is no fluorescence excited at wrong excitation wavelength. Even cells transfected with pEYFP-Rab3A are overexposed (**C**), there is no fluorescence excited at 430nm (**D**).

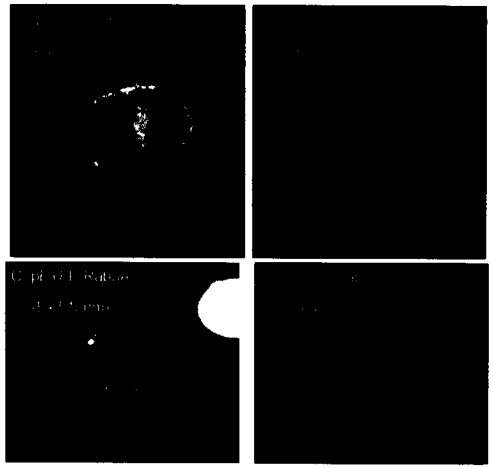

Fig. 4 Detection of subcellular localization of Rab3A during exocytosis in living PC12 cells. **A.** At resting state, vesicles at the focal plane (gray area) are fluorescent (black). When cells are stimulated for secretion, fluorescent content in vesicles will be released and fluorescent intensity of the focal plane decreases. After secretion, new vesicles arrive at the focal plane, and fluorescent intensity increases. **B.** Cells expressing NPY-EGFP are stimulated by either high K^+ or loading buffer. Fluorescent intensity reduces only when cells are stimulated with high K+, and this kind of decrease is due to release of NPY-EGFP to diffuse out of the focal plane (re-do the graph in sigmaplot) **C.** Cells co-expressing NPY-EGFP and EYFP-Rab3A are imaged by dual colors epi-fluorescence microscopy. (please add response of loading buffer and re-do the graph in sigmaplot) When stimulus is applied, decreases of fluorescence of NPY-GFP are as expected but surprisingly the fluorescent intensity of ECFP-Rab3A also drops, too. And this kind of decrease cannot find in EGFP (D, please put data).

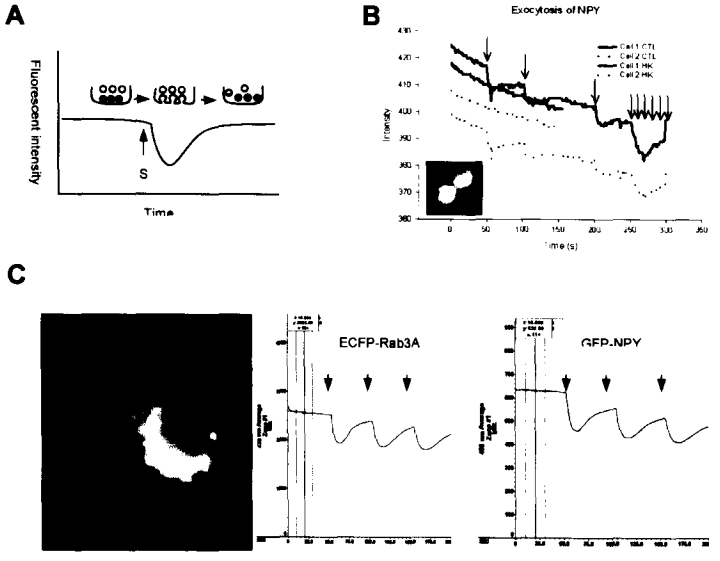

Fig. 5 NPY-EGFP is use to confirm that TIRFM at NTU can detect all steps in exocytosis. A. Illustration of Objective-based TIRFM. B. Fluorescence property changes during exocytosis. When vesicles dock toward plasma membrane, fluorescence intensity increases. Fully-docked vesicles have highest fluorescent intensity. When vesicles fuse to plasma membrane, the area of fluorecence diffuses and blurrs, and fluorescence intensity decreases. C. Localization of NPY-EGFP is clearly puctuated in PC12 cells in TIRFM detection system. D. Comparing with epi-fluorescence microscopy, TIRFM provides more clear images. E. TIRFM at NTU can detect steps of single release of NPY-EGFP, including moving (blue arrow) and fusion (yellow arrow).

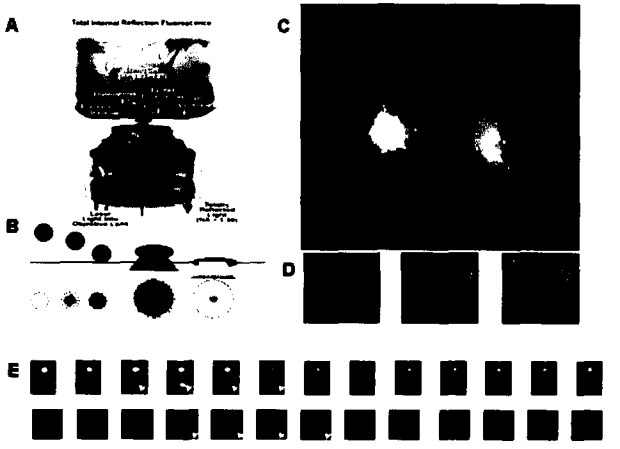

Fig. 6 Manual analysis of dynamic localization of EGFP-Rab3A in living PC12 cells. The process of analysis is shown in A. There are three major kinds of movements. B-1. Vesicles move from membrane to center of the cells. B-2. Vesicles move toward plasma membrane. B-3. Vesicles move alone plasma membrane.

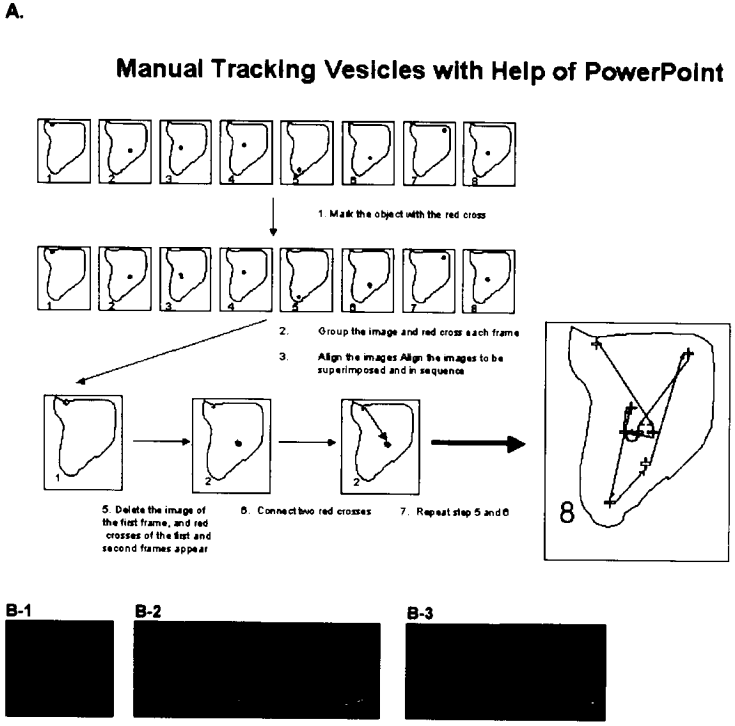

Fig. 7 The fusion event occurred after high K^+ stimulation (depolarization). EGFP-Rab3A fusion proteins were overexpressed in PC12 cells. Under high K^+ stimulation, one secretory vesicle (green arrowhead) was seen to first move away from the focal plan (t = 1.5s), and then back to original focal plane (t = 3s), finally fused with the vesicle (red arrows) on the plasma membrane.

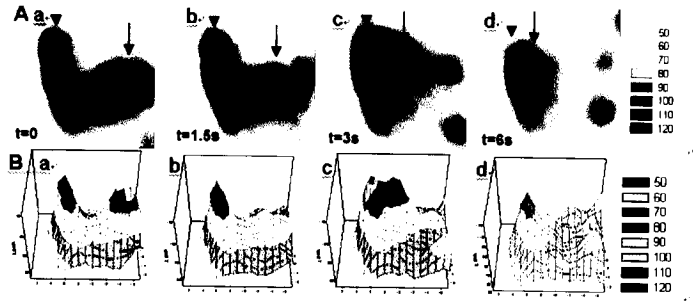

Fig. 8 Rab3A-associated comparments move to plasma membrane when high potassium is applied. The