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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※ 運用螢光方法研究 Rab3A 的訊息傳遞機制 ※

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計畫類別：□個別型計畫 □整合型計畫

計畫編號：NSC89－2311－B－040－005－

執行期間：88 年 8 月 1 日至 89 年 7 月 31 日

計畫主持人：林崇智

共同主持人：高閬仙

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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

運用螢光方法研究 Rab3A 的訊息傳遞機制

Studies in Signal Transduction Mechanism of Rab3A by Fluorescence Techniques

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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 以綠螢光蛋白（GFP）結合，在 SK-N-SH 神經瘤細胞中表現，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) GFP-Rab3A 的真核表達載體的構築，(2) 建立轉染神經細胞株的條件，(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 SK-N-SH 細胞中的分布。與過去免疫螢光染色結果相似，GFP-Rab3A 在細胞呈點狀分布，且集中在核周圍與神經纖維末端，證明 GFP-Rab3A 不因為 GFP 而使其分布改變。隨後分析 GFP-Rab3A 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，大多分布在神經纖維呈靜止狀態，此顯示小光點可能為準備分泌的囊泡，顯示 Rab3A 可能作用於囊泡的導引與膜融合反應。大光點分布在細胞本體，呈不規則的結構，少部份大光點會移動（0.5 微米/秒）與互相融合，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。

關鍵詞：Rab3A，GFP，調控式胞吐

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.

Rab3A is fused with green fluorescence protein (GFP) and the fusion protein is expressed in neuroblastoma cell line, SK-N-SH. Transfected cells are grown in a chambered coverlass and observed by inverted fluorescence microscopy. Fluorescence indicates subcellular localization of Rab3A and is recorded by microphotography. Photograph is scanned and NIH Image and Photoshop analyze translocalization of Rab3A. Part of Rab3A is cytosolic and over the whole cell except nucleus, and this is quite different from GFP distribution. Some Rab3A is associated with small organelles, i.e. vesicles, to form small dots, and these dots are concentrated in perinuclear area and neurites. Sizes of dots in perinuclear area, several microns, are larger than those in neurites, 0.3-1 microns. Structures of dots in perinuclear area are various, but those in neurites are very unique. Small dots in neurite terminus are very steady and this seems to indicate that these dots are in ready-to-release pools. Although these results are similar to distribution of synaptic vesicle associated proteins in PC12 cells, these dots need more evidence to know their exact location. Several larger dots are very mobile among neurites and cell body, and some of them are fused each other or break down into small vesicles, and this seems to indicate that Rab3A may also play a role in vesicles recycle.

In this study, we have setup a system to track Rab3A translocalization in living cells, this system will be applied to know the exact locations of Rab3A during exocytosis and find out the role of Rab3A in regulation of exocytosis.

Keywords: Rab3A, GFP, regulated exocytosis

二、計畫緣由與目的

Rab3 proteins are members of small G proteins. Four Rab3 proteins have been identified so far, Rab3A, Rab3B, Rab3C and Rab3D (Baldini et al., 1992). Rab3 proteins are preferentially localized in neuronal or secretory cells. Several lines of evidence suggest that Rab3 proteins are involved in docking and fusion steps of regulated secretory pathway (Holz et al., 1994; Johannes et al., 1994; Geppert et al., 1994). However, there is very little direct evidence about the exact molecular roles of Rab3 proteins in regulated exocytosis, especially the place where they locate and when they interact with their target proteins during secretion. If Rab3A involves in docking and fusion, it must locate near vesicles and plasma membrane. Tracking Rab3A in living cells during secretion provides important direct evidence to explain the exact place where it acts. Thus, a working system to tack Rab3A in vivo is setup first in the first year project. First, Rab3A needs to be visualized to track in vivo. There are several methods to make particular proteins visible, including fluorescent dyes and immunohistochemistry. But these methods have limitations, ex. immunohistochemistry is used in permable or fixed cells and fluorescent dyes are not very specific. Recently, people found that GFP (green fluorescent protein) is a good tool to label proteins for studying protein translocalization. GFP is isolated from jelly

fishes, Aequorea victoria, and its 65-67 amino acid residues are cyclized and oxidized to form the fluorophore, which adsorb 475nm light to emit 507 fluorescence. There are three GFP mutants with different colors, blue, yellow and cyan. Fluorescence quantum yield is very high (0.72-0.85); fluorescence intensity is very strong and lasts over 24hr. (Cubitt et al., 1995; Tsien et al., 1993). Because of stability and strong intensity of GFP, GFP is a good tool to label proteins to study protein translocalization. Besides, proteins fused to different GFP variants are also very useful for protein-protein interaction studies, because of FRET (fluorescence resonance energy transfer). When two fluorescent groups is close enough (below 10nm), fluorescence emitted from one fluorescent group will be transferred to the other to emit fluorescence of the other fluorescent group. Adam and their colleagues label PKA (protein kinase A) regulatory and catalytic subunits with fluorescein and rhodamine. In absence of cAMP, regulatory and catalytic subunits are associated, thus excited fluoresceins in regulatory subunits of PKA will tranfer energy to rhodamine to emit fluorescnece. Once cAMP increases, regulatory and catalytic subunits are dissociated, and rhodamine fluorescence decreases (Adams et al., 1991). Miyawaki and their colleagues fuse BFP and GFP to calmodulin and M13 (one of calmodulin-binding proteins), and these fusion proteins are tagged with organelle-specific localization signal peptides to express these proteins in specific organelles. Because binding calmodulin with M13 is calcium-dependent, fluorescence by FRET will reflect calcium concentration in particular organelles (Miyawaki et al., 1997). Rab3A is fused to GFP and GFP fluorescence indicates where Rab3A is.

Because of fast exocytosis and small secretory vesicles, imaging system needs both high spatial resolution and temporal resolutions. For high spatial-resolution and time-resolution, evanescent wave fluorescence microscopy will be a better choice than conventional confocal microscopy, because confocal microscopy needs time for high spatial-resolution and it is not a good idea to apply to measure kinetics of exocytosis. Evanescent wave only illuminates a thin layer of interface between the cell and medium, about 300nm, thus this system avoid interference from out-of-focus fluorescence to give confocal microscopic grade resolution. According to previous investigations, evanescent wave fluorescence microscopy can detect single exocytosis of chromaffin granule (Fig. 2 and 3, Steyer and Almers, 1999). Because of limited budget, we modify Steyer's setup method, only put an annular mask and high NA objective to regulate incident angle of the light beam to generate evanescent wave, and replace laser and CCD by mercury lamp and conventional camera. Although our imaging system is not as perfect as Steyer's, it is enough to detect Rab3A translocalization and provide some basic results.

In this study, we have setup a system to track Rab3A translocalization in living cells, this system will be applied to know the exact locations of Rab3A during exocytosis and find out the role of Rab3A in regulation of exocytosis.

三、結果與討論

Construct mammalian vector to express GFP-Rab3A. BamHI sites are added to Rab3A 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 GFP to avoid that GFP interferes its location. The cloning sites of EGFP-C1 is in C-terminus of GFP, therefore, pEGFP-C1 is used to fuse Rab3A. Rab3A containing BamHI ends is cut and cloned into pEGFP-C1, and Rab3A is fused to C-terminus of GFP. The correct clone is picked by restriction enzyme digestion. Fig. 4 indicates that correct vector to express GFP-Rab3A has been constructed.

Expression of GFP-Rab3A in neuronal cell line. Because SK-N-SH cells have good adherence to poly-L-lysine coated coverslips, they provide longer time and stability for experiments. Besides, adherent cells have thinner thickness to avoid out-of-focus blur to reduce spatial resolution of microscopy, SK-N-SH cells are used as study material for our project. Before transfection with pEGFP-Rab3A into SK-N-SH cells, cells are transfected with pEGFP-Rab3A by Tfx-50, one of liposomes, to get the optimal transfection condition. The optimal charge/DNA ratio is 2, DNA amount is 1μg per 35mm dish, and cell density is 50-80% confluent. Either low cell density, SK-N-SH cells grow ill at low cell density, or too much Tfx reagents, their deregent effect lyse cells, reduce transfection efficiency. At optimal condition, transfection efficiency is 20-50% (Fig. 5).

Spatial resolution test of imagine system. Acridine orange is a fluorescent dye specific to nucleus and acidic compartments, ex lysosomes. Because lysosomes are ranging 1μm to several microns and intensity of acridine orange is strong, acridine orange-stained cells may be good to test our imaging system. Both SK-N-SH cells and CHO cells have red dots ranging several microns after acridine orange staining. From this test, though there is blur due to out-of-focus fluorescence, resolution of our microscopy system reaches sub-micron level and this is enough to observe protein translocalization of Rab3A proteins (Fig. 6).

Subcellular localization of GFP and GFP-Rab3A in SK-N-SH cells.

Cells are transfected with pEGFP-C1 (A and B) or pEGFP-Rab3A (C-E), and observed by fluorescence microscopy. Fluorescence indicates subcellular localization of Rab3A and is recorded by microphotography. Photograph is scanned and NIH Image and Photoshop analyze translocalization of Rab3A. GFP locates over the cell and the brightest area is nuclear area due to thickness of nucleus. The processes are very sharp, and this indicates that our imaging system can be used to study cell motility (Fig. 7A-B). Part of Rab3A is cytosolic and over the whole cell except nucleus, and this is quite different from GFP distribution. Some Rab3A is associated with small organelles, i.e. vesicles, to form small dots, and these dots are concentrated in perinuclear area and neurites. Sizes of dots in perinuclear area, several microns, are larger than those in neurites, 0.3-1 microns. Structures of dots in perinuclear area are various, but those in neurites are very unique. Small dots in neurite terminus are very steady and this seems to indicate that these dots are in ready-to-release pools. Comparing to previous results of endogenous Rab3A visualized by immunohistochemistry

(Lin et al., 1997), GFP-Rab3A has similar subcellular distribution to that of endogenous Rab3A and this indicates that GFP doesn't interfere localization Rab3A (Fig. 8C-E). These results are similar to distribution of synaptic vesicle proteins, ex VAMP and chromograinin B, in PC12 cells, this indicates that GFP-Rab3A associated with synaptic or secretory vesicles. Comparing with EM investigations, synaptic vesicles are ranging 30nm, 1/10 of small fluorescent dots found in SK-N-SH cells. It may be that lots of synaptic vesicles are clustered at active zones or fused synaptic vesicles are retrieved back to endosomes. Although our results indicate that Rab3A is possibly associated to synaptic or secretory vesicles, fluorescent dots need more evidence to know their exact location.

Dynamic subcellular localization of GFP-Rab3A. Cells prepared as described in Fig.7., and serial photos are taken at speed 8s/frame. Most of GFP-Rab3A in SK-N-SH cells are in dots and still (Fig. 8A). Several larger fluorescent spots move between cell body and neurites (Fig. 8B, arrow heads), and sometimes these spots fuse each other (Fig. 8B, yellow arrowheads). Several larger dots are very mobile among neurites and cell body, and some of them are fused each other or break down into small vesicles, and this seems to indicate that Rab3A may also play a role in vesicles recycle.

Table 1 Properties of GFP Variants

GFP Variant	Fluor. Intensity	Excit./Emis. Maxima (nm)	Half-life
EGFP	35x	488/509	>24 h
EYFP	35x	513 /527	>24 hr
EBFP	1x	380/440	>24 hr
ECFP		433 (453)/ 475(501)	>24 hr

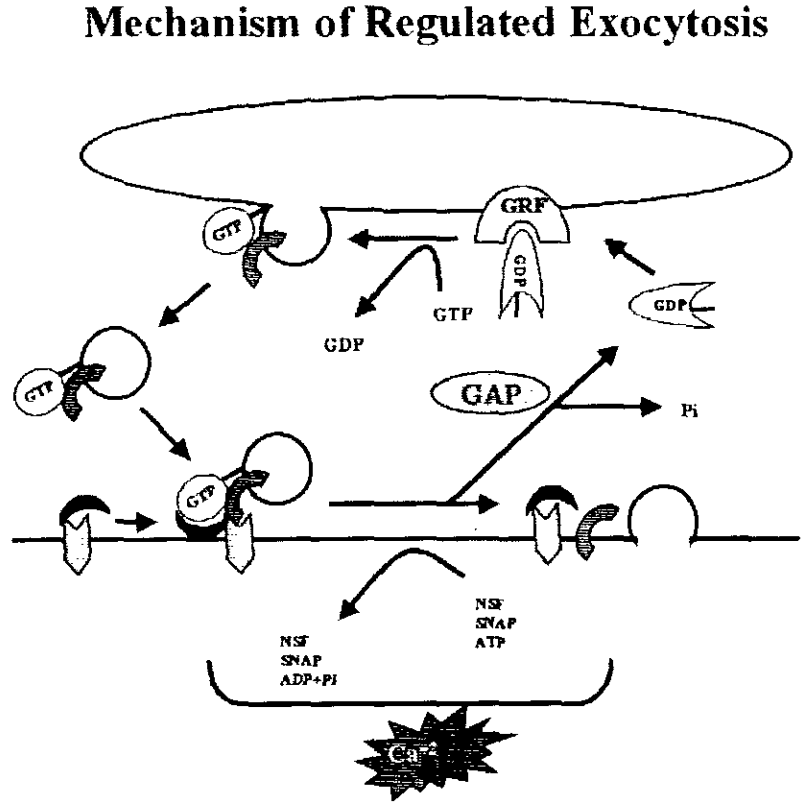


Fig. 1. The current hypothetical model of regulated exocytosis

Methods for Measuring Exocytosis- Evanescent Wave Fluorescence

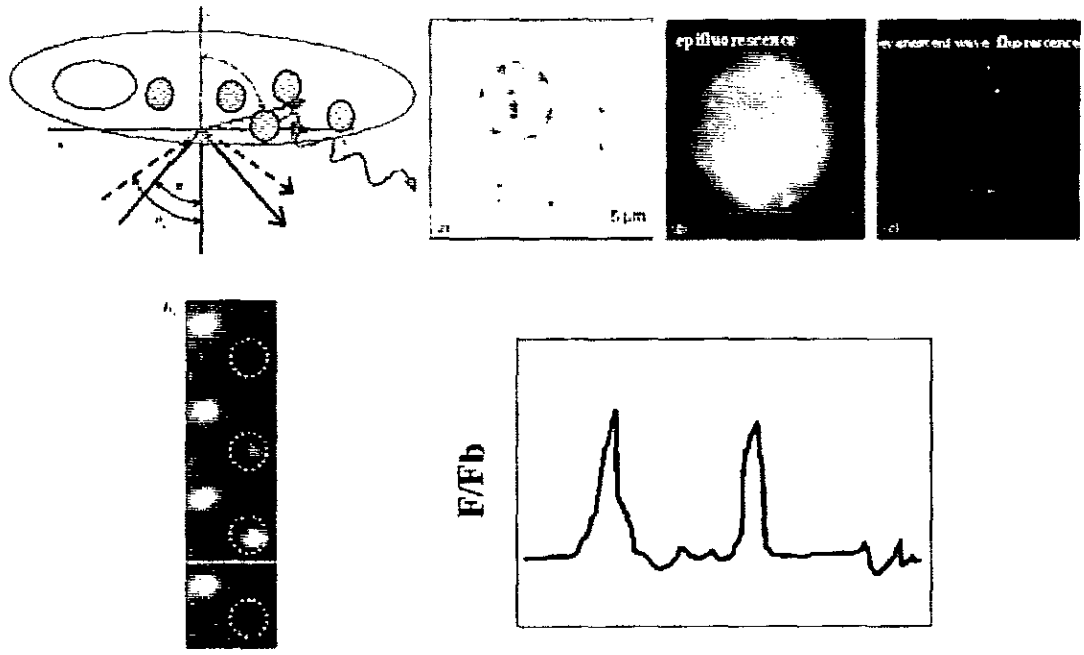


Fig.2 Basic illustration and application of evanescent fluorescence microscopy

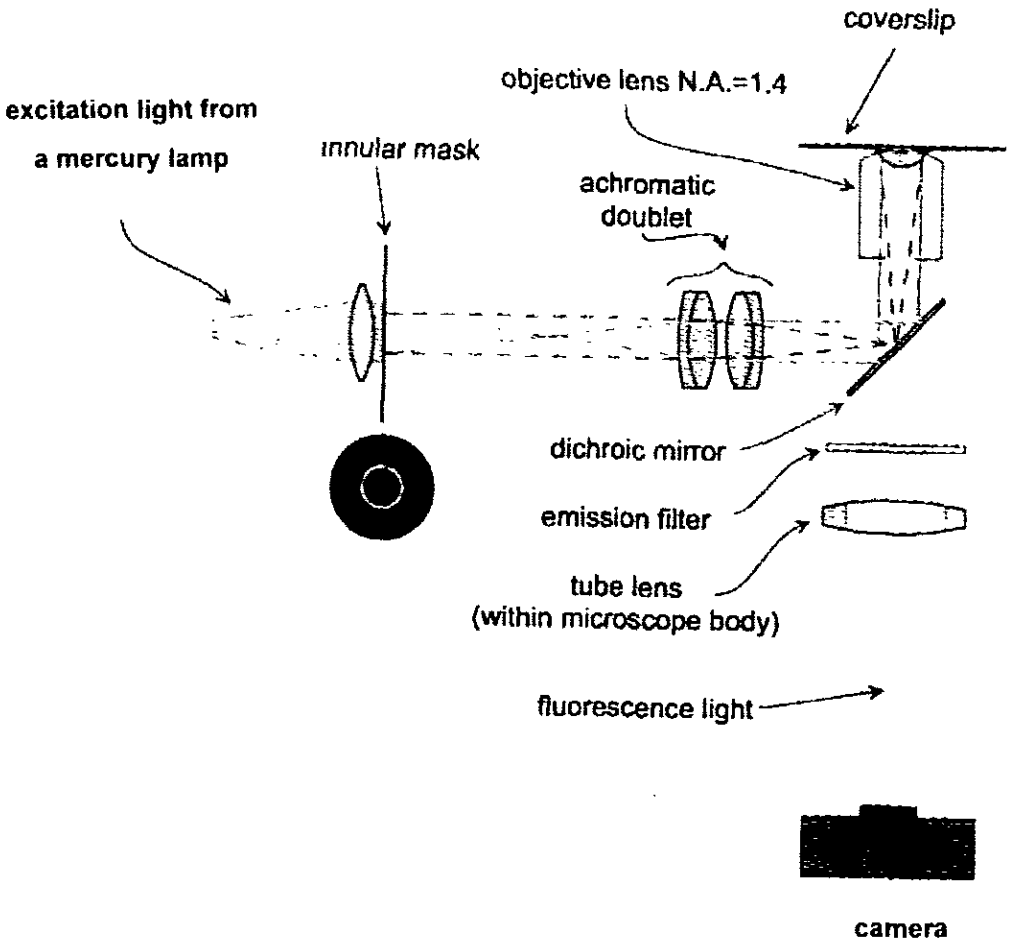
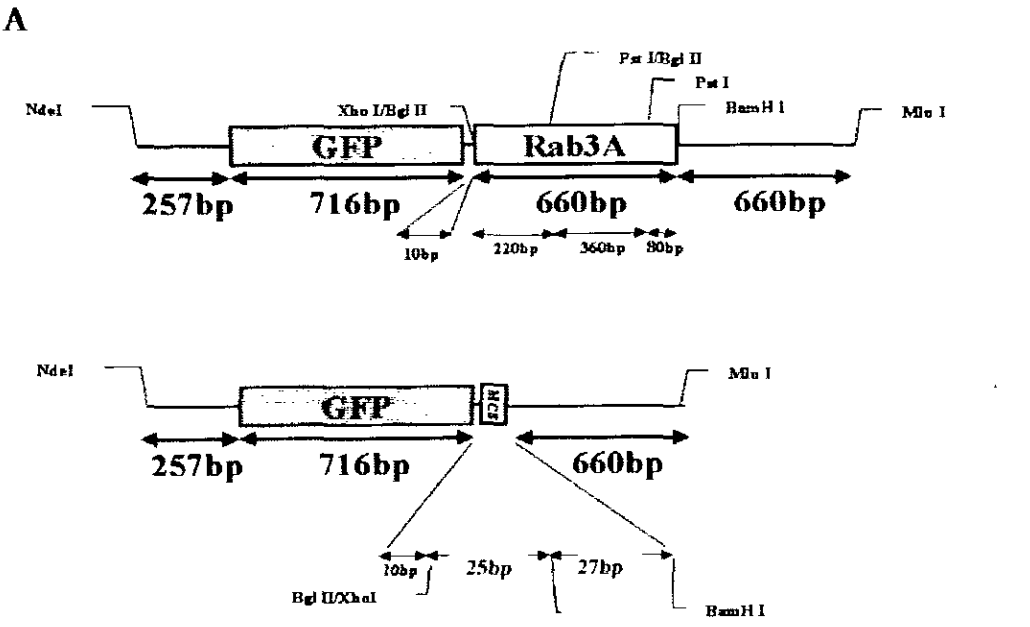


Fig. 3 Illustration of evanescent wave fluorescence microscopy system



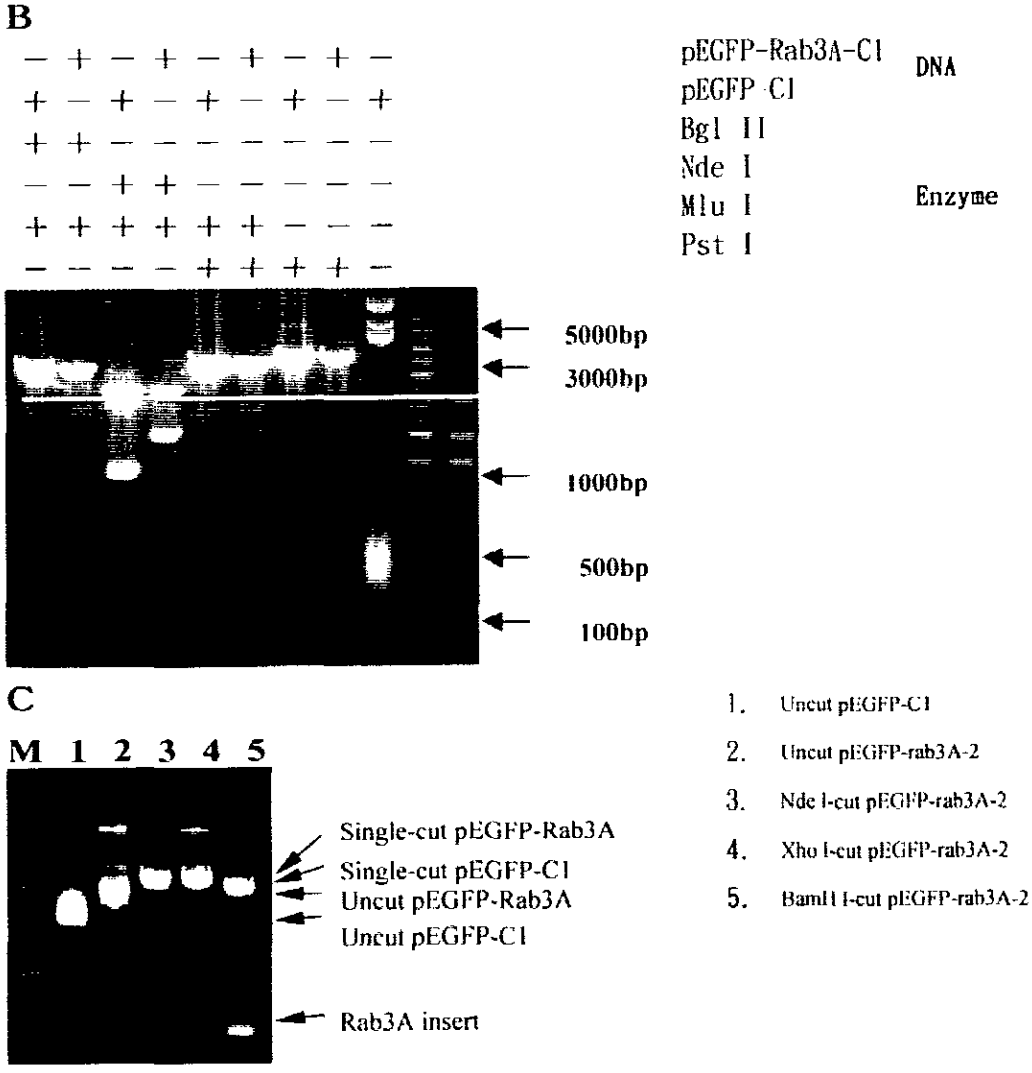


Fig. 4. Restriction Map of EGFP and EGFP-fused Rab3 expressing vectors.
A. Restriction map of EGFP and EGFP-fused Rab3 expressing vectors that are near multiple cloning sites. B and C. Restriction enzyme digestion profiles of EGFP and EGFP-fused Rab3 expressing vectors. Length of all restriction fragments are in right sizes as shown in A.

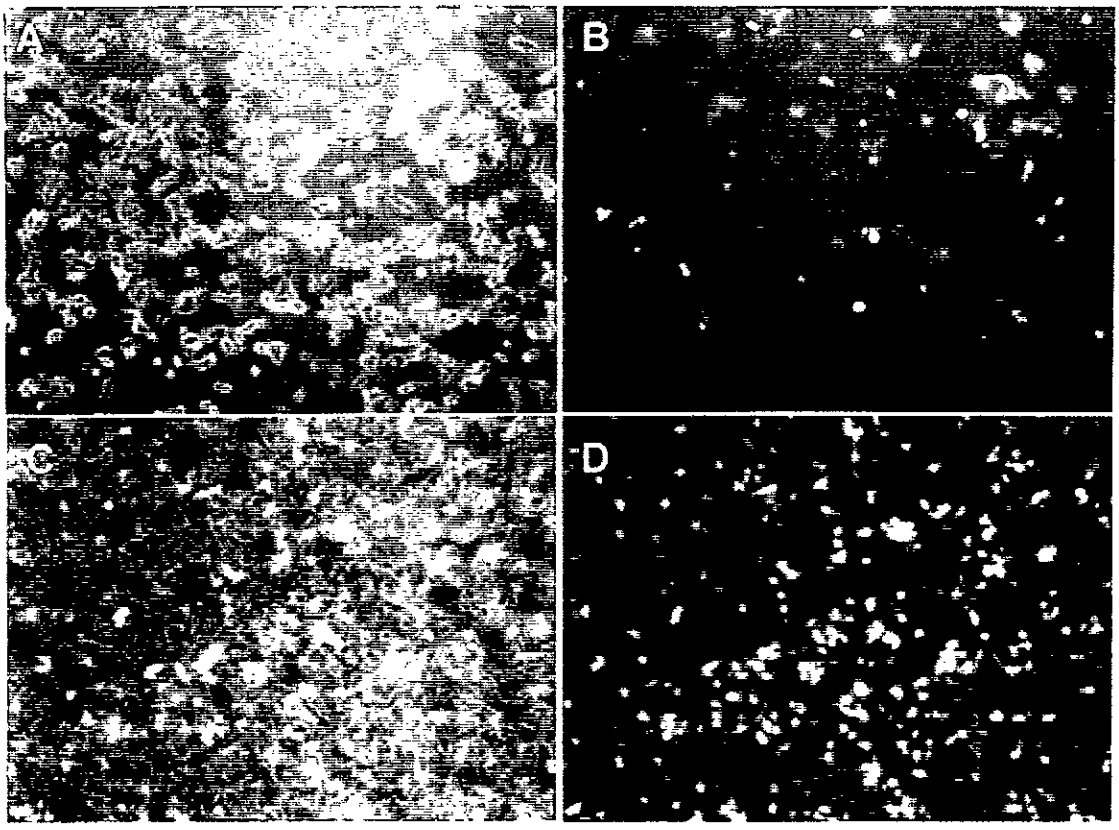


Fig. 5. Transfection of SK-N-SH cells with pEGFP-C1 by Tfx50. SK-N-SH cells are transfected with pEGFP-C1 at different charge/DNA ratio (A and B: 4; C and D: 2) and culture for 48hr. After culture, cells are observed by fluorescence (B, C) and phase-contrast (A, D) microscopy.

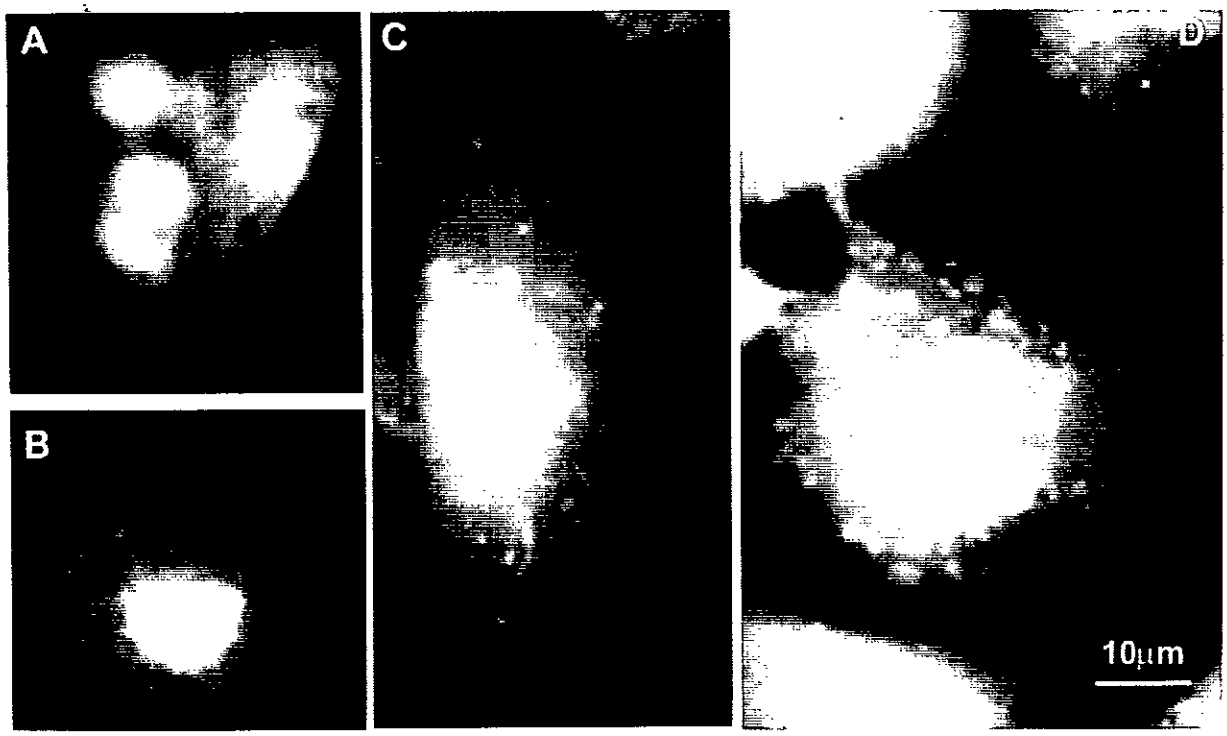


Fig. 6. Spatial resolution of imaging system for the project. SK-N-SH cells (A and B) and CHO cells (C and D) are grown on chambered coverslips for two days, and incubated with 10μM acridine orange for 10min. Stained cells are observed by fluorescence imaging system. Spatial resolution of imaging system is ranging from 0.3μm to several microns (microscope: Axiovert-25CF; objective: 1,000X oiled NA1.4; exposure time: 8s for ASA800 film).

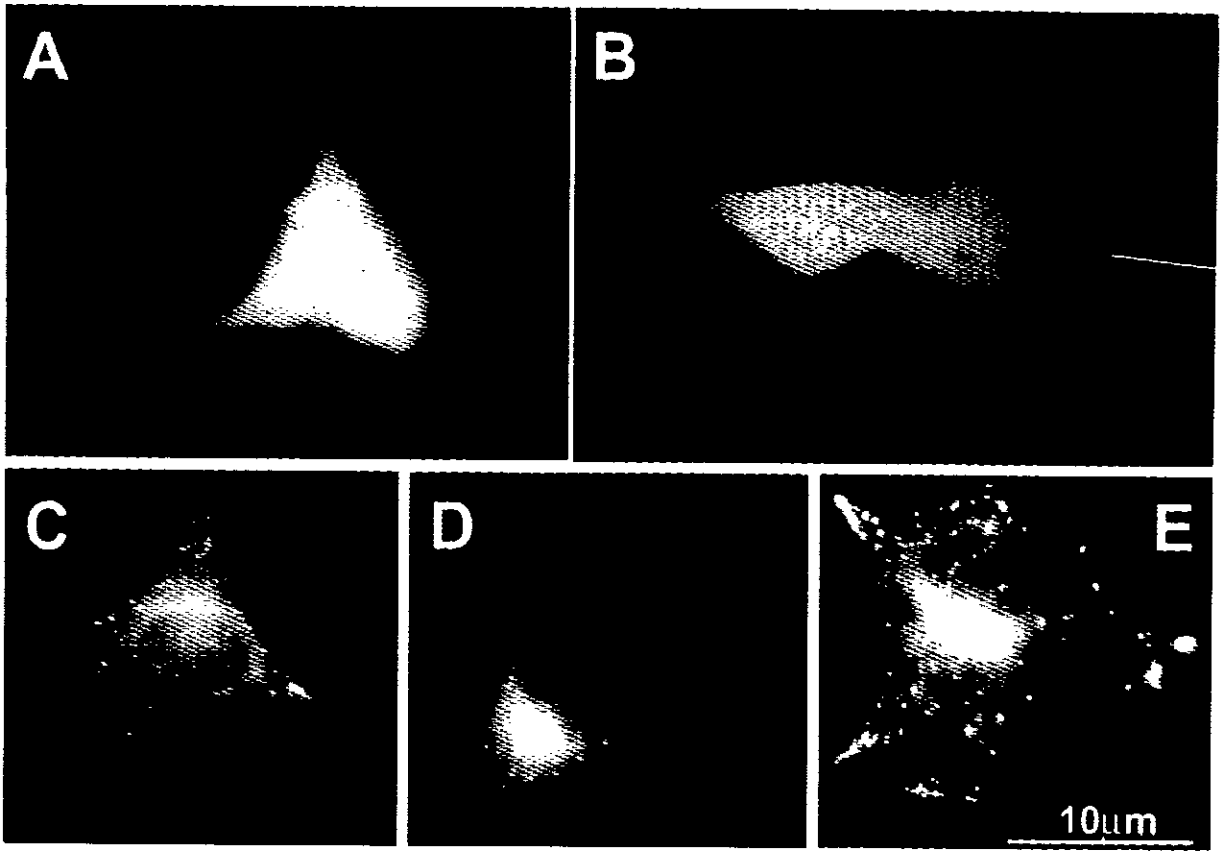


Fig. 7. Distribution of GFP and GFP-Rab3A in SK-N-SH cells. Cells are transfected with pEGFP-C1 (A and B) or pEGFP-Rab3A (C-E), and cultured for O/N. Transfected cells are trypsinized and grown on the poly-L-lysine coated chambered coverslips for two days. Cells are observed by fluorescence microscopy (microscope: Axiovert-25CF; objective: 1,000X oiled NA1.4; exposure time: 8s for ASA800 film).

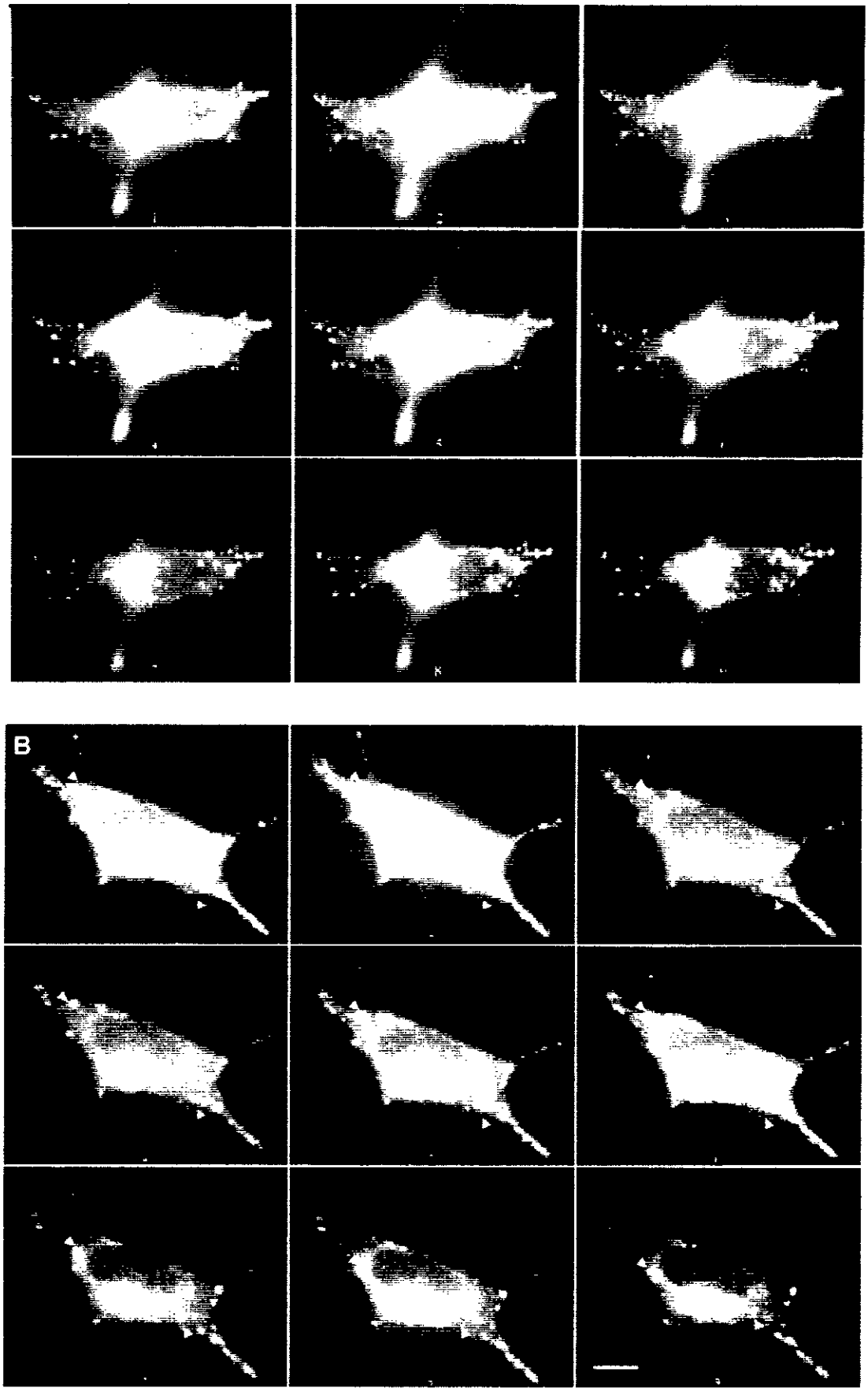


Fig. 8. Translocalization of GFP-Rab3A in SK-N-SH cells. Cells prepared as described in Fig.7., and serial photos are taken at speed 8s/frame. Most of GFP-Rab3A in SK-N-SH cells are in dots and still (A). Several larger fluorescent spots move between cell boy and neurites (B, arrow heads), and sometimes these spots fuse each other (B, yellow arrowheads).

四、計畫成果自評

Because of 921 earthquakes, instruments and lots of prepared reagents, including cells and DNA, were totally destroyed and our project re-started at November. Besides, there is no microscopy to detect protein translocalization, we need to build one. Because of limited budget (most of it is wasted in 921), we build it in alternative way. Because of no CCD camera to record protein localization, we need to capture with camera and scan photos to built up animation of Rab3A translocalization. Our system is working but costs lots of time, and we are afraid that we cannot catch up with other colleagues who have perfect system. If NSC gives more support for us to get better CCD and microscope, we can get more results.

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