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# Direct monitoring of the expression of the green fluorescent protein–extracellular signal-regulated kinase 2 fusion protein in transfected cells using capillary electrophoresis with laser-induced fluorescence detection

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**Abstract**

Proper subcellular localization of the extracellular signal-regulated kinases (ERKs) is important in regulating physiological functions such as proliferation and differentiation in the pheochromocytoma cell line (PC12 cells). Thus, a direct visualization method is necessary to observe ERK localization within the cell or in crude cellular extracts. In this paper, a determination method was established for the detection of ERK2 localization in PC12 cells using green fluorescent protein (GFP) and capillary electrophoresis with laser-induced fluorescence (LIF). GFP as a reporter or labeling tag for gene expression in biochemistry and cell biology was used for the detection of ERK2 localization in PC12 cells. PC12 cells were transfected with GFP–ERK2 plasmid construct that was inserted into a variant GFP gene (enhanced green fluorescent protein), and successfully expressed GFP–ERK2 fusion proteins. GFP–ERK2 fusion proteins were detected within 5 min by CE analysis using an uncoated fused-silica capillary with LIF. Optimum conditions for GFP–ERK2 fusion proteins detection were 100 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid buffer containing 100 m*M* sodium dodecylsulfate, pH 11, running at 20 °C. This result offers new opportunity in screening for the determination of localization of intracellular components, protein–protein interactions and kinase activity within the cells.


*Keywords:* Pheochromocytoma cell line; Proteins; Green fluorescent protein; Enzymes; Extracellular signal-regulated kinase

## 1. Introduction

Two extracellular signal-regulated kinase (ERK) isoforms, ERK1 (p44) and ERK2 (p42), have been well characterized in mammals, and are important signaling pathway components regulating cellular proliferation and maintenance of a differentiated phenotype. ERKs are activated by many different extracellular stimuli such as peptide growth factors and phorbol esters [1–3]. For the enhancement of ERK activity in response to mitogenic stimulation, ERKs are phosphorylated on threonine-183 and tyrosine-185 residues in a TEY motif. The phosphorylation of both residues on ERKs leads to maximal enzyme activation, and covalently bound phosphates are required to maintain high activity. After ERK activation, a key step in the signaling mechanism of ERK cascades is the translocation of ERKs into the nucleus. The ERKs then catalyze the phosphorylation of a number of cellular protein

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targets, which include transcription factors and other protein kinases [4,5]. The proper subcellular localization of various components including ERKs has been shown to play an important role in regulating the physiological functions of ERK cascades. In the pheochromocytoma cell line (PC12 cells), transient activation of ERKs by epidermal growth factor causes cell proliferation [6], where they differentiate in response to nerve growth factor, which acts partly by inducing a sustained activation of ERKs [7]. Thus it is important to understand the nuclear translocation mechanism of ERKs. In cell biology, there were many attempts to characterize the subcellular localization of ERKs in the cells using the fluorescent microscopy.

A popular use of fluorescent microscopy led to many fluorescent tags being used to check the localization of intracellular components and protein–protein interactions. Recently, numerous applications for a group of proteins with native fluorescence properties known as green fluorescent protein (GFP) have been found. A main advantage of GFP is that their excitation spectra are in the visible range and thus have an excitation line that is compatible with the frequently used argon ion laser. A wild-type GFP, originally isolated from the jellyfish *Aequorea victoria* [8], is an acidic, globular protein with a molecular mass of 30 000, a maximum excitation wavelength at 395 nm and a smaller excitation peak at 475 nm [9]. A wild-type GFP and its variants can easily be detected in the cellular environment without use of chemical tagging and disturbance to the cell. Enhanced-GFP (EGFP), a variant of GFP, that contains the double-amino acid substitution of phenylalanine 64 to leucine and serine 65 to threonine, is 35 times more intensely fluorescent than the wild-type GFP [10], and has also been extensively used as reporters in gene expression. In several reports, the fluorescence of GFP-fusion proteins analyzes protein transport in the secretary pathway [11], functions as a marker of gene expression in bacterial, plants, and mammalian cells, and detects protein–protein interactions by electrophoretic methods. The classical gel electrophoretic approaches, however, have the disadvantages of being time-consuming, difficult to automate, and are hampered by high consumption of materials [12].

Capillary electrophoresis (CE) has proven to be a powerful separation tool, and is therefore being used as a useful separation method for biomaterial analysis [13,14]. Also when combined with appropriate technique and laser-induced fluorescence (LIF) detection, CE can provide high sensitivity. Therefore, CE–LIF can perform rapid separation with high detection sensitivity. In addition, only a minute amount of sample ranging from units to tens of nanoliters, is applied for single CE analysis. CE–LIF has also been applied to human growth factor, insulin, neurotransmitters, single cell, and proteins analysis. In cell biology studies especially, the investigation of GFP fusion protein is increasingly carried out with the CE–LIF method. In this study, the objective of the work is to develop a method of GFP–ERK2 fusion protein determination in PC12 cell extracts using CE–LIF. First, we studied the condition of running buffer for the analysis of GFP–ERK2 fusion protein using CE. We compared it with the conventional Western blot analysis method in order to validate our developed CE–LIF method for detection of GFP–ERK2 fusion protein in PC12 cells. The GFP–ERK2 fusion protein used is the genetic construct that was fused to the 3′-end of the gene expressing green fluorescent protein (GFP), resulting in GFP-ERK2 fusion protein as shown in Fig. 1.

## 2. Experimental

### *2.1. Apparatus*

All experiments were carried out with an automated A P/ACE 5000 CE system (Beckman Instruments, Fullerton, CA, USA) fitted with an LIF detector. The 488-nm line of a 5 mW argon ion laser detector was used as the excitation source of the LIF detector, and the emitted fluorescence was collected at 520 nm. Data were analyzed using Beckman System Gold software, version 8.1. Confocal image was obtained using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany). For gel electrophoresis and Western blot analysis, samples were run on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at 150 V for 90 min in a Mini Protean 3 unit (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose

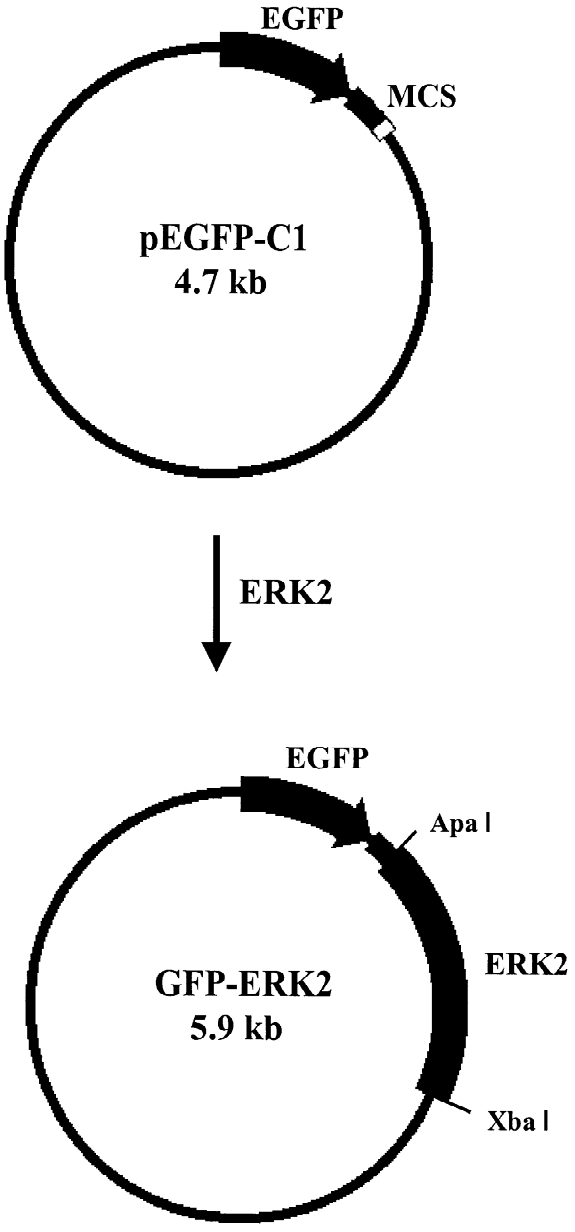


Fig. 1. Scheme of GFP–ERK2 plasmid construct. Wild-type ERK2 (base 22–1096) was ligated into *Apa* I and *Xba* I sites downstream from green fluorescent protein (GFP) gene of pEGFP-C1 that is a mutant GFP.

membrane (Schleidher and Schuell, Keene, NH, USA) with a Bio-Rad Mini-Trans Blot cell in transfer buffer for 90 min at 200 mA.

### 2.2. Reagents

Sodium dodecylsulfate (SDS), 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS), sodium hydroxide, and sodium hydrogen carbonate were obtained from Sigma (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), lipofectAMINE 2000, Optimum minimum essential medium (Opti-MEM), fetal bovine serum (FBS), horse serum, trypsin, penicillin-G and streptomycin solution, and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA), rabbit anti-GFP polyclonal antibody and goat anti-rabbit immunoglobulin G (IgG) conjugated horseradish peroxidase (HRP) antibody from Santa Cruz Biotechnology (Delaware, CA, USA). Pheochromocytoma cell line, PC12 cells were obtained from American Type Culture Collection (Rockville, MD, USA).

### 2.3. GFP–ERK2 plasmid construct

GFP empty vector was purchased from Clontech (Palo Alto, CA, USA), and the cDNA of rat ERK2 (bases 22–1096) that was ligated into *Apa* I and *Xba* I sites downstream from the GFP gene of pEGFP-C1 (Clontech) was kindly provided by Dr Rony Seger from the Department of Biological Regulation at the Weizmann Institute of Science. Fig. 1 shows GFP–ERK2 plasmid construct, and GFP–ERK2 plasmid was transfected into the PC12 cells using lipofectAMINE 2000 reagent, and expression of GFP–ERK2 was visually confirmed with a confocal microscope (Fig. 2).

### 2.4. Transfection and cell preparation

PC12 cells were cultured in complete DMEM, which contained 10% horse serum, 10% FBS, 100 IU/ml penicillin-G and 50 μg/ml streptomycin solution. The cells were maintained in T-75 $cm^2$ culture flasks at 37 °C in a humidified incubator with $CO_2$–air (5:95) until harvesting for the experiment [16,17]. Prior to plating, culture dishes were treated with 1 mg/ml collagen and then rinsed with the medium. The cells were plated and grown on a 100-mm dish until 70–80% confluent. In each 100-mm dish, various concentrations of GFP–ERK2

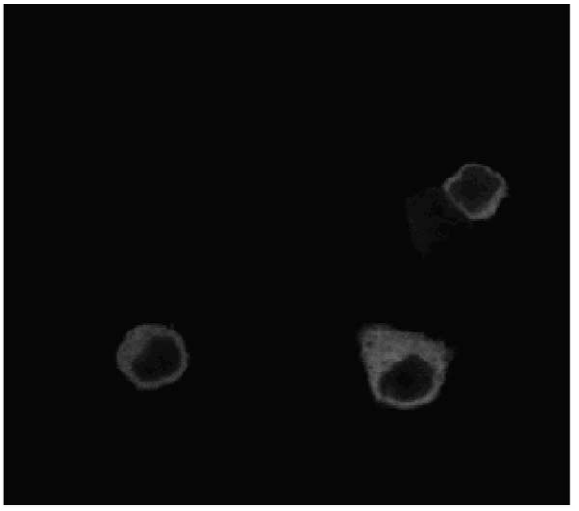

Fig. 2. Confocal laser scanning microscope image of GFP–ERK2 fusion protein expression in PC12 cells. GFP–ERK2 plasmid transfection using LipofectAMINE 2000 into PC12 cells. After transfection, cells were fixed with 3% paraformaldehyde, and then wash several times using PBS. The expression of GFP–ERK2 was visually confirmed with a confocal microscope.

plasmid were transfected into the PC12 cells for 32 h using lipofectAMINE 2000, and the cells were serum starved (0.1% serum) for 16 h. After serum starvation, the cells were washed twice with ice-cold PBS and harvested with cell lysis buffer [50 m*M* β-glycerophosphate, pH 7.3, 1.5 m*M* ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA), 1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1 m*M* sodium vanadate, protein inhibitor cocktail] followed by homogenization and centrifugation (15 000 *g*, 15 min, 4 °C). The supernatants were collected and kept frozen at −20 °C until use.

### 2.5. Western blot analysis

Transfected PC12 cells were serum-starved (16 h), and harvested as described above. Same volumes of protein samples were loaded with several concentrations of GFP–ERK2 plasmid such as 1 and 3 μg in order to obtain clear bands. The nitrocellulose membrane was probed with a 1:2000 dilution of rabbit anti-GFP polyclonal antibody and then probed with a 1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase antibody.

### 2.6. Capillary electrophoresis

An untreated fused-silica capillary column of 27 cm (effective length of 20 cm)×50 μm I.D. was purchased from Beckman Instruments. Sample injections were performed under pressure for 5.0 s at 3447.38 Pa. The separation conditions involved an applied voltage of 12 kV and the current was 50 μA using a running buffer consisting of 100 m*M* CAPS with 100 m*M* SDS at pH 11. Preparation of a new capillary prior to use, involved a rinse with 0.5 *M* sodium hydroxide (15 min), water (30 min), and running buffer (15 min). To obtain reproducible results, the capillary was washed between runs with sodium hydroxide (7 min) and running buffer (15 min).

## 3. Results and discussion

In this study, we investigated the optimum capillary electrophoretic condition for the detection of GFP–ERK2 fusion protein in cells. The GFP–ERK2 fusion protein samples driven from PC12 cells were extracted and the samples were injected after 10 times dilution with PBS, without clean-up of the sample prior to the CE run. The application of CE to biological samples such as the extracted cell sample and biofluid is more difficult due to the adsorption of sample components to the capillary wall, which affects the reproducibility of the assay. In order to overcome such a problem, our studies were conducted with SDS, a micellar electrokinetic chromatography (MEKC) method, which prevents the adsorption of proteins to the capillary walls [18]. CE was carried out with running buffer containing 100 m*M* SDS in an uncoated capillary (27 cm×50 μm I.D.) at a constant field strength of 12 kV. In the CE system, since the pH of the running buffer has a key effect on the precise detection of the analytes, the selection of running buffer pH is very important in obtaining accurate data. We therefore studied the effects of the running buffer pH on sensitivity using a phosphate buffer of various pH values ranging from 7.0 to 12.0. Because GFP plasmid construct was a mutant of GFP, enhanced-GFP (EGFP), it was shown to be pH-sensitive both in vivo and in vitro. The mutant GFP plasmid retains fluorescence in the

pH range of 7.0–11.5 [19]. When the pH was lower than 8.0, GFP−ERK2 was not detected in the CE system. At a pH level higher than 8.0, an increase in sensitivity was observed upon increasing pH but a decrease in sensitivity was observed with the use of a pH buffer higher than 11.0. Therefore, we obtained fairly good efficiency and a highly sensitive peak at pH 11.0. The results are shown in Fig. 3. In order to study the effect of the running buffer type, we tested using 100 m*M* CAPS buffer containing 100 m*M* SDS at pH 11.0 instead of phosphate buffer containing 100 m*M* SDS at pH 11.0 as a running buffer. The result showed that CAPS buffer has a higher resolution compared to that of phosphate buffer (data not shown). Therefore, based on the results of our experiment, we decided that a 100 m*M* CAPS buffer containing 100 m*M* SDS (pH 11.0) was the optimal running buffer pH condition. The use of CAPS buffer also presented some distinct advantages. First, the use of zwitterionic compounds such as CAPS, as the running buffer enables it to act as a shield against the capillary walls. In addition, the 100 m*M* CAPS buffer has a low current at high ionic strength compared to phosphate buffer. Fig. 4 shows electropherograms of the GFP–ERK2 fusion protein and GFP protein in 100 m*M* CAPS buffer containing 100 m*M* SDS, pH 11. The peak of GFP protein appeared within 3 min in Fig. 4A. The electropherogram

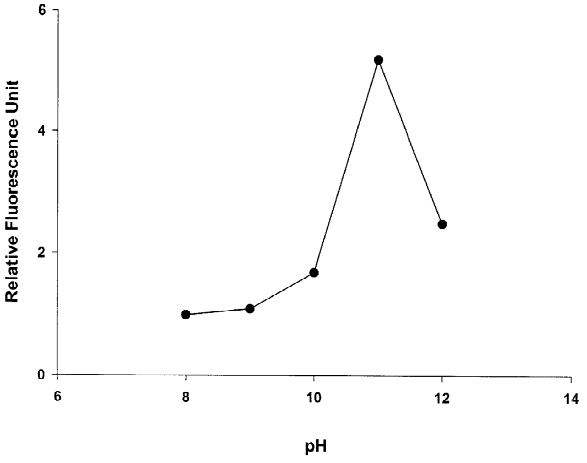


Fig. 3. Influence of fluorescence of GFP–ERK2 upon different pH values of 100 m*M* phosphate buffer containing 100 m*M* SDS. CE conditions: 27 cm×50 μm I.D. untreated fused-silica capillary, 12 kV, LIF detector (excitation, 488 nm; emission, 520 nm).

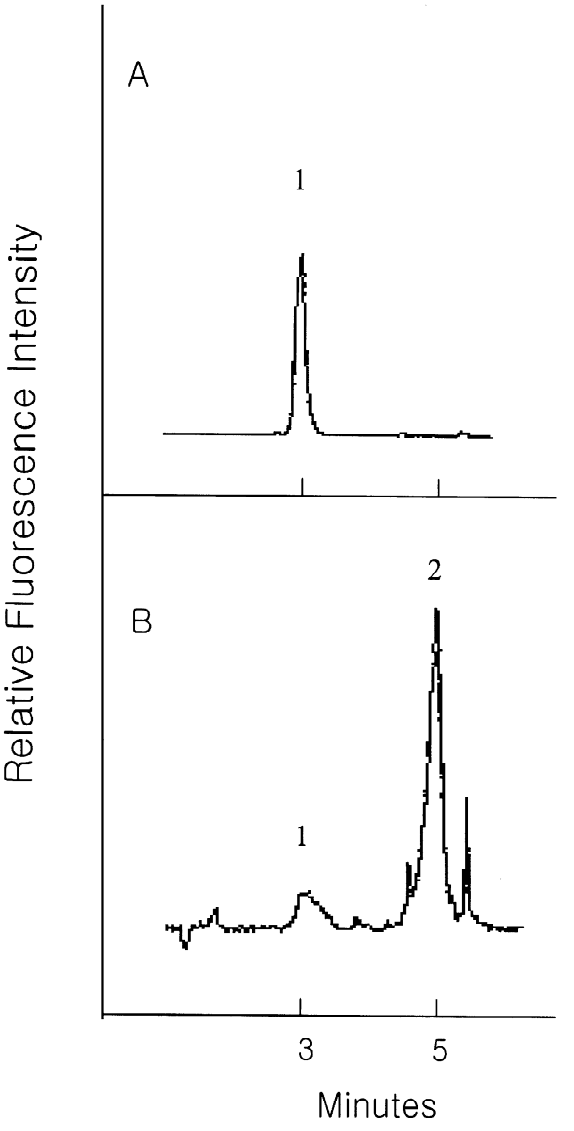


Fig. 4. Electropherograms of the (A) GFP vector and (B) GFP–ERK2 in optimized conditions. CE conditions: 27 cm×50 μm I.D. untreated fused-silica capillary, 100 m*M* CAPS buffer containing 100 m*M* SDS, 12 kV, LIF detector (excitation, 488 nm; emission, 520 nm). Peak 1, GFP vector; peak 2, GFP–ERK2.

resulting from the injection of the GFP–ERK2 showed a major GFP–ERK2 fusion protein peak at 5.0 min, and the minor GFP peak at 3 min, respectively, in Fig. 4B.

Under optimized analysis conditions, the reproducibility of migration time and peak area were

investigated for the GFP–ERK2. The relative standard deviations (RSDs) of migration time and peak area were 5.15 and 7.71%, respectively.

Based on the above experiments, we analyzed the differences in the expression level of GFP–ERK2, depending on the concentration of transfected GFP–ERK2 plasmid into PC12 cells. The experiment showed that PC12 cells transfected with 1 and 3 μg of GFP–ERK2 plasmid were extracted with lysis buffer. As shown in Fig. 5A, the results show increasing peak intensity depending on the concentration of transfected GFP–ERK2 plasmid into PC12 cells. In order to present the feasibility of the CE method for measurement of the GFP–ERK2 fusion protein, we compared this method to such conventional methods as Western blot analysis. The Western blotting method was performed three times in our experiment. In this study, Western blot analysis was carried out using SDS–PAGE and rabbit anti-GFP polyclonal antibody for the analysis of GFP–ERK2 fusion protein. Each well was loaded with protein samples of 30 μl, the loading basis being amount not protein concentration level. The result showed that the bands increased in density with higher concentrations of transfected plasmid as shown in Fig. 5B and this result is in accordance with the results of GFP–ERK2 obtained by CE method. Therefore, we concluded that CE–LIF could be a useful technique to detect the GFP–ERK2 fusion protein in cell extract in this study.

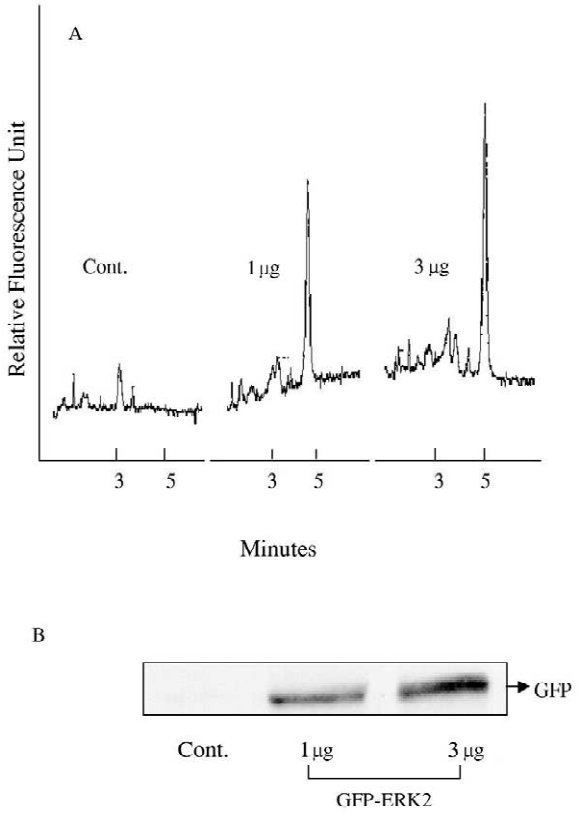


Fig. 5. Expression of the GFP–ERK2 fusion protein in PC12 cells monitored by (A) CE–LIF, (B) Western blot analysis with rabbit anti-GFP polyclonal antibody. CE conditions as in Fig. 4. Cont: non-transfected samples; 1 μg, 3 μg: the concentration of transfected GFP–ERK2 plasmid.

## 4. Conclusion

In the present work, we developed a method for the determination of GFP–ERK2 fusion protein in PC12 cell extracts using CE–LIF. In order to obtain the optimum conditions for detection of GFP–ERK2 fusion protein, the influences of pH and type of running buffer were investigated. From this study, we obtained the optimal conditions as follows: an uncoated fused-silica capillary (27 cm × 50 μm I.D.) with 100 m*M* CAPS buffer of pH 11 containing 100 m*M* SDS at 20 °C, and 12 kV. Under optimized analysis conditions, the RSDs of migration time and peak area were 5.15 and 7.71%, respectively. Compared to the conventional Western blot method, this new assay method is relatively rapid, reproducible, and has many advantages including a few nanoliter sample volume, and did not need a clean-up step for sample preparation.

We reported here a new approach for the detection of GFP-fusion protein in cells. We believe that this assay method could be applied to detect the phosphorylated form and the sublocalization of GFP fusion protein induced by stimulation.

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