Electronic Supporting Information (ESI)

Live imaging of cellular dynamics using a multi-imaging vector in single cells†

Kyoungsook Park,‡ [a] Jinyoung Jeong‡[a,b] and Bong Hyun Chung*[a,b]

[a] BioNano Health Guard Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB)

[b] Nanobiotechnology Major, School of Engineering, University of Science and Technology (UST) , 125 Gwahangno, Yuseong, Daejeon 305-806, Republic of Korea.

Experimental Details

Plasmid construction. DNA polymerase (Takara Bio Inc., Japan) was used to perform the PCRs, and all PCR-amplified sequences were verified using DNA sequencing. The Escherichia coli strain DH5α was used as the host for subcloning, and cultures were grown in LB medium at 37 °C, with 50 mg/ml ampicillin added to the plasmid-bearing strains. Restriction enzymes and modifying enzymes were obtained from Roche Applied Science (Germany) and used in accordance with the supplier's instructions. DNA was prepared using a QIAEX II gel extraction kit (Qiagen, Germany). Primers were purchased from GenoTech Corp. (Korea) (Table S2). The Bax clone (Clone ID: hMU011946) was provided by the Korea Human Gene Bank, Medical Genomics Research Center, KRIBB, Korea. The AIF clone (MHS6278-211690533) was purchased from Open Biosystems (Thermo Scientific, USA). The piRFP670-N1 plasmid (Addgene ID: 45457) was provided by Addgene, a non-profit plasmid repository (USA). All plasmids were verified using DNA sequencing (GenoTech Corp., Korea).

Multi-imaging vector (MIV) construction. Oligonucleotides encoding 3 distinct 2A peptides and general restriction enzyme sites were synthesized by Bioneer (Korea) (Fig. S3). The products were digested using HindIII/XbaI and cloned into the pcDNA4 His/Max A vector (Invitrogen, USA).

Multi-labeling vector (MLV) construction. We constructed the multi-labeling vector in the following manner to insert 4 individual fluorescent proteins fused with localized signals into the multi-imaging vector. First, ECFP-NLS/MIV was constructed as follows: ECFP was amplified from pECFP-C1 (Clontech, USA) with primers 1 and 2; the resultant fragment was then digested with EcoRV/PstI and inserted into MIV. Primer 2 included three copies of the nuclear localization signal (NLS, DPKKKRKV) from simian virus 40 large T-antigen for nuclear localization. Second, Mito-DsRed2: ECFP-NLS/MIV was constructed as follows: DsRed2 was amplified from pDsRed2-N1 (Clontech, USA) with primers 3 and 4 and then digested with BamHI/BglII. A synthesized oligomer of the mitochondrial targeting sequence (MTS) derived from subunit VIII of human cytochrome C oxidase was digested with HindIII/BamHI, and the resultant DNA fragments were inserted into ECFP-NLS/MIV. Third, Mito-DsRed2: ECFP-NLS: Memb-iRFP670/MIV was constructed as follows: membrane targeting iRFP670 was amplified from piRFP670-N1 with primers 5 and 6, and the resultant fragment was then digested with EcoRI/XbaI and inserted into Mito-DsRed2: ECFP-NLS/MIV. Primer 5 included 20 amino acids from neuromodulin (KLNPPDESGPGCMSCKCVLS) for membrane targeting. Finally, MLV (Mito-DsRed2: EGFP-Endo: ECFP-NLS: Memb-iRFP670/MIV) was constructed as follows: EGFP was amplified from pEGFP-N1 (Clontech, USA) with primers 9 and 10 and then digested with KpnI/EcoRI. Human RhoB GTPase (endosomal specific marker, 576 bp, synthesized by Bioneer, Korea) was amplified with primers 7 and 8 and then digested with EcoRI/SalI. The resultant DNA fragments were cloned into the Mito-DsRed2: ECFP-NLS: Memb-iRFP670/MIV vector to generate MLV.

Multi-monitoring vector (MMV) construction. We generated the multi-monitoring vector in the following manner to replace EGFP-Endo and Memb-iRFP670 of the MLV with AIF-EGFP and Bax-iRFP670, respectively. First, Bax was amplified from Bax-pOTB7 with primers 11 and 12 and then digested with EcoRI/SmaI; iRFP670 was also amplified from piRFP670-N1 with primers 13 and 6 and digested with SmaI/XbaI. Memb-iRFP670 of the MLV was replaced with these products to generate Mito-DsRed2: EGFP-Endo: ECFP-NLS: Bax-iRFP670/MIV. AIF was obtained by performing PCR with primers 14 and 15, followed by digestion with KpnI/SalI. EGFP was amplified from pEGFP-N1 (Clontech, USA) with primers 16 and 17 and then digested with XhoI/SalI. EGFP-Endo of the Mito-DsRed2:

EGFP-Endo: ECFP-NLS: Bax-iRFP670/MIV was substituted with these products to generate the MMV.

EGFP-Endo construction. We constructed EGFP-Endo vector in the following manner EGFP was amplified from pEGFP-N1 (Clontech, USA) with primers 9 and 10 and then digested with KpnI/EcoRI. Human RhoB GTPase (endosomal specific marker, 576 bp, synthesized by Bioneer, Korea) was amplified with primers 7 and 8 and then digested with EcoRI/SalI. The resultant DNA fragments were cloned into the pcDNA 3 vector to generate EGFP-Endo.

Cell culture. Human cervical carcinoma (HeLa) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10 % (v/v) fetal bovine serum and 1 % (w/v) penicillin/streptomycin at 37 °C under 5 % CO_2. For plasmid transfection, HeLa cells were plated at a density of 5000 cells/well in a μ-slide 8-well plate (Ibidi, Germany). Plasmid transfection was performed using Lipofectamine™ 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. After transfection, cells were cultured in the presence of 5 μM staurosporine (STS) or 0.5 mM etoposide. After exposure to the anticancer drugs, we fixed the cells with 10 % formaldehyde for 10 min. After washing with 1x PBS, we mounted the cells on microscope slides using fluorescent mounting medium (Vector Lab., USA) and visualized the cells under confocal fluorescence microscopy.

Immunofluorescence. To conform endosomal labeling with EGFP-Endo, twenty-four hours after transfection with EGFP-Endo, we fixed cells with 4 % paraformaldehyde for 10 min, washed them with 1x PBS, and blocked them with 5 % goat serum (Vector Lab., USA) in PBS for 1 hr at 37 °C. We washed and incubated with anti-EEA1 antibody (Abcam, England) overnight at 4 °C and then with Alexa Fluor 555-conjugated secondary antibody (Cell signaling, USA) for 1h in the dark at RT. After washing with 1x PBS, we mounted the cells on microscope slides using fluorescent mounting medium with DAPI (Vector Lab., USA) and visualized the cells with confocal fluorescence microscopy.

Western blotting analysis. To detect protein cleavage event, HeLa cells were transiently transfected with multi-monitoring vector. Twenty-four hours after transfection, the cells were collected and lysed in 150 mM NaCl, 1 % NP-40, 0.5 % deoxycholic acid, 0.1 % SDS in 50

mM Tris-HCl (pH 8) with 0.5 ml protease inhibitor (Roche Applied Science, Germany). Cell lysates (50 μg) were separated by 12 % SDS-PAGE with a molecular weight standard and transferred onto nitrocellulose membranes. After blocking with 5 % skim milk for 1hr at RT, blots were incubated overnight at 4 °C with anti-AIF antibody (1:1000, Cell signaling, USA) or anti-Bax antibody (1:250, Santa Cruz Biotechnology, USA), which were detected using horseradish peroxidase (HRP)-conjugate secondary antibodies (Thermo Scientific Inc., USA) and ECL reagent (Thermo Scientific Inc., USA).

Confocal imaging. Cells were observed with a laser scanning microscope (LSM) 510 META confocal microscope system (Carl Zeiss, Germany) using a c-Apochromat 40x1.2 w objective. Images were taken using an excitation/emission band pass filter (or long pass filter) set at 405/420-480 nm, 488/505-550 nm, 543/560-615 nm, and 633/650 nm for ECFP, EGFP, DsRed2, and iRFP670, respectively.`

Table S1. The excitation/emission maxima of the fluorescent proteins.

Fluorescent proteins	Excitation maximum (nm)	Emission maximum (nm)
ECFP	433(major), 453(minor)	475, 501
EYFP	513	527
DsRed2	563	582
iRFP670	643	670

Table S2. Primer sequences.

Primer	Sequence
1	CGGGATATCATGGTGAGCAAGGGCGAGGAGC
2	AACTGCTGCAGTACCTTTCTCTTCTTTTTTGGATCTACCTTTCTCTTCTTTTTTG GATCTACCTTTCTCTTCTTTTTTGGATCGAACTCCTTGTACAGCTCGTCCATGC
3	CGGGATCCACCGGTCGCCACCATGGCCTCCTCCGAGAACG
4	GAAGATCTCAGGAACAGGTGGTGGC
5	CGGAATTCATGCTGTGCTGTATGAGAAGAACCAAACAGGTTGAAAAG AATGATGAGGACCAAAAGATCGGAAGCATGGCGCGTAAGGTCGATC
6	GCTCTAGATTAGCGTTGGTGGTGGGCGGC
7	GGGGTACCATGGTGAGCAAGGGCGAGG
8	CGGAATTCCTTGTACAGCTCGTCCATGC
9	CGGAATTCCGGAAGAAACTGGTGGTTG
10	ACGCGTCGACAGCACCTTGCAGCAGTTGATG
11	CGGAATTCGCGGTGATGGACGGGTCC
12	TCCCCGGGGCCCATCTTCTTCCAG
13	TCCCCGGGATGGCGCGTAAGGTCGATC
14	GGGGTACCATGTTCCGGTGTGGAGG
15	ACGCGTCGACGTCTTCATGAATGTTGAATAG
16	CCGCTCGAGATGGTGAGCAAGGGCGAGG
17	ACGCGTCGACCTTGTACAGCTCGTCCATGC

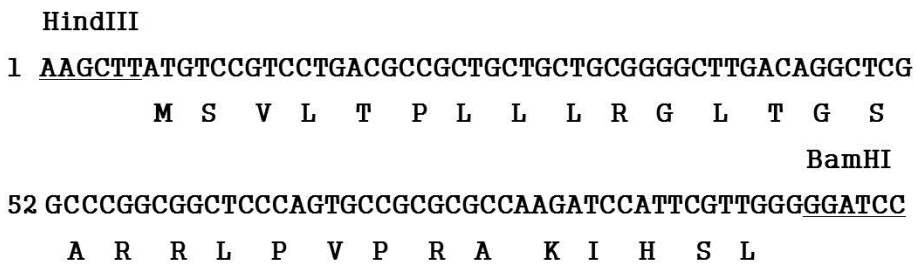

Fig. S1 The mitochondrial targeting sequence derived from the subunit VIII of human cytochrome C oxidase.

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  1 CGGAAGAAAC TGGTGGTTGT TGGTGATGGA GCCTGTGGAA AGACATGCTT
 51 GCTCATAGTC TTCAGCAAGG ACGAGTTCCC AGAGGTGTAT GTGCCCACAG
101 TGTTTGAGAA CTATGTGGCA GATATCGAGG TGGATGGAAA GCAGGTAGAG
151 TTGGCTTTGT GGGACACAGC TGGCCAGGAG GACTACGACC GCCTGCGGCC
201 GCTCTCCTAC CCGGACACCG ACGTCATTCT CATGTGCTTC TCGGTGGACA
251 GCCCGGACTC GCTGGAGAAC ATCCCCGAGA AGTGGGTCCC CGAGGTGAAG
301 CACTTCTGTC CCAATGTGCC CATCATCCTG GTGGCCAACA AAAAAGACCT
351 GCGCAGCGAC GAGCATGTCC GCACAGAGCT GGCCCGCATG AAGCAGGAAC
401 CCGTTCGCAC GGATGACGGC CGCGCCATGG CCGTGCGCAT CCAAGCCTAC
451 GACTACCTCG AGTGCTCTGC CAAGACCAAG GAAGGCGTGC GCGAGGTCTT
501 CGAGACGGCC ACGCGCGCCG CGCTGCAGAA GCGCTACGGC TCCCAGAACG
551 GCTGCATCAA CTGCTGCAAG GTGCTA
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Fig. S2 DNA sequence of the human RhoB GTPase sequence.

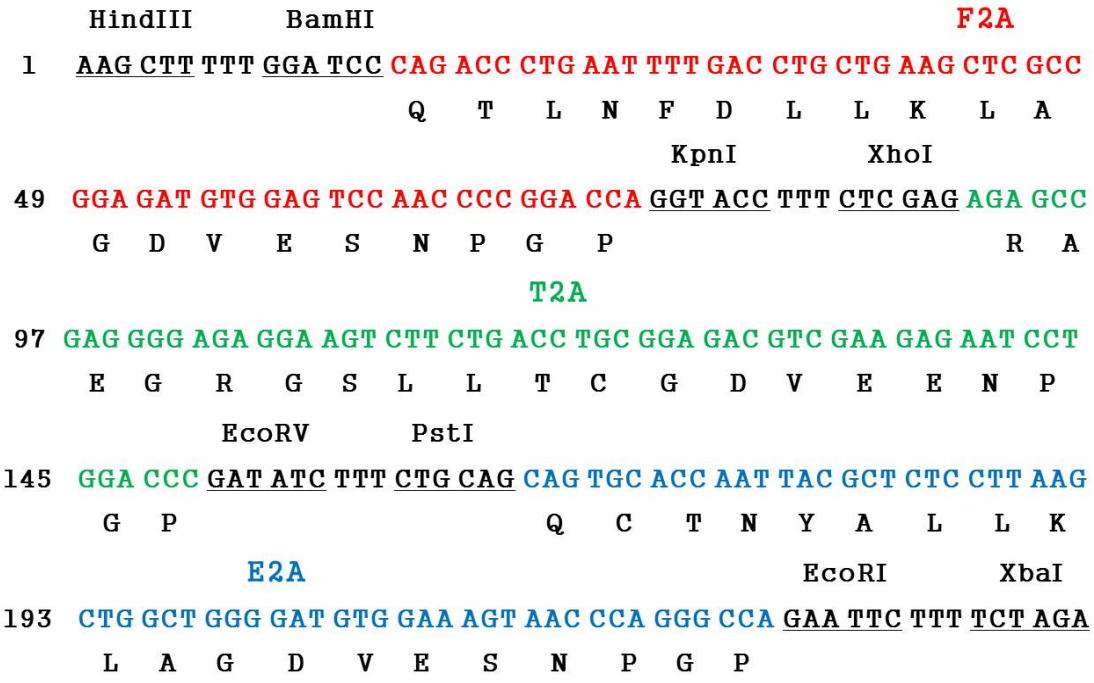

Fig. S3 DNA sequence of multi-imaging vector.

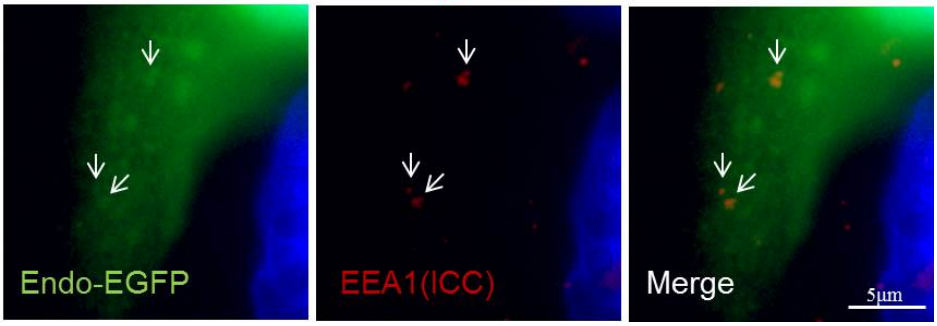

Fig. S4 Subcellular localization of endosomal targeting marker-fused EGFP in HeLa cells. Immunofluorescence images showed the subcellular localization of EGFP-Endo. EGFP and early endosomal marker (EEA1, red) indicates endosomes and DAPI (blue) indicates nuclei. Immunofluorescence images were obtained with fluorescence microscopy. Scale bar = 5 μm.

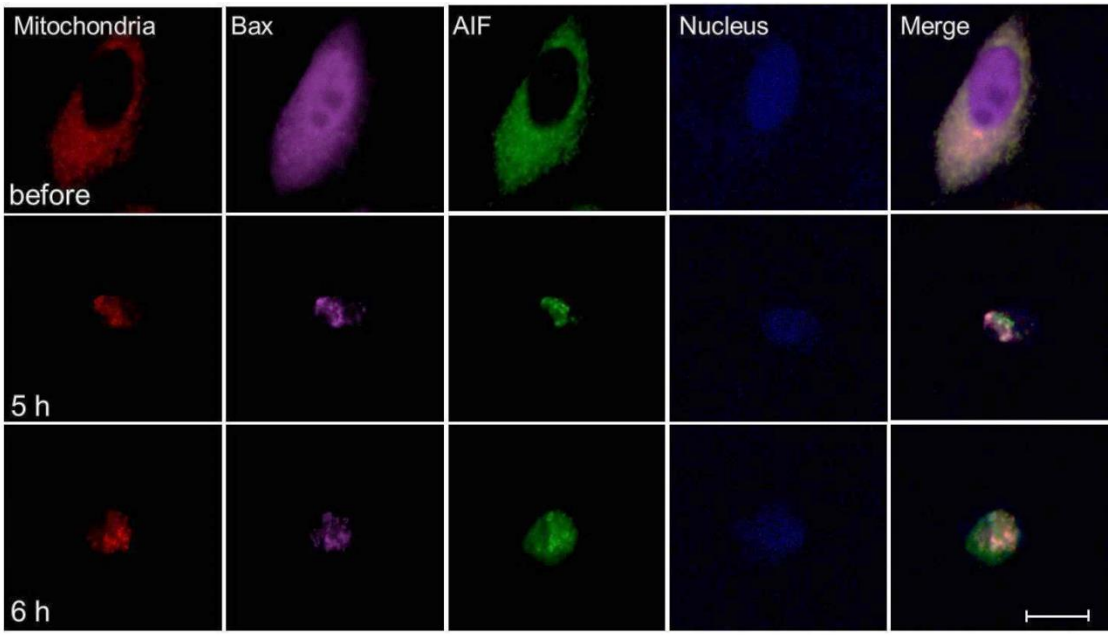

Fig. S5 Multi-monitoring system of AIF-Bax translocation. Fluorescent images of the transiently transfected HeLa cells with multi-monitoring vector. After transfection, HeLa cells were exposed to 0.5 mM etoposide for 6 hrs. Bax was labeled with iRFP670 in purple, AIF-EGFP in green, DsRed2 the mitochondria in red, and ECFP the nucleus in blue, respectively. Scale bar = 20 μm.

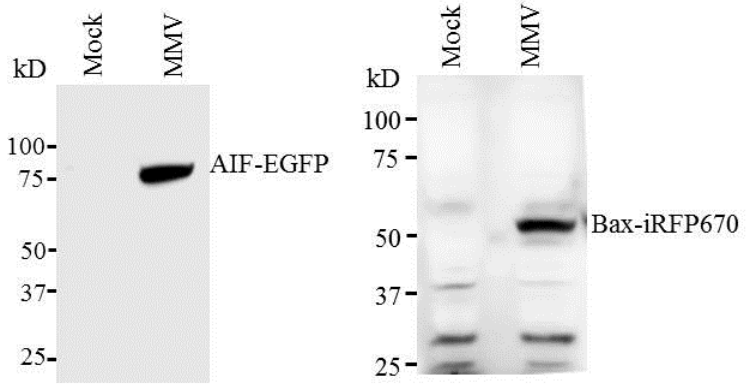

Fig. S6 The cleavage efficiency of the 2A peptides in the multi-monitoring vector.
HeLa cells were transfected with the multi-monitoring vector. Mock transfection served as a control. At 24 hrs after transfection, cells were processed for western blot analysis. The cleavage efficiency was assessed using AIF and Bax antibodies to decorate AIF-EGFP and Bax-iRFP670, respectively.

Movie. S1 Time-lapse image of multi-monitoring vector-transfected cells by taking images every 5 min for 30 min. The movie indicated that this technique made it a live cell imaging without phototoxicity and photobleaching.