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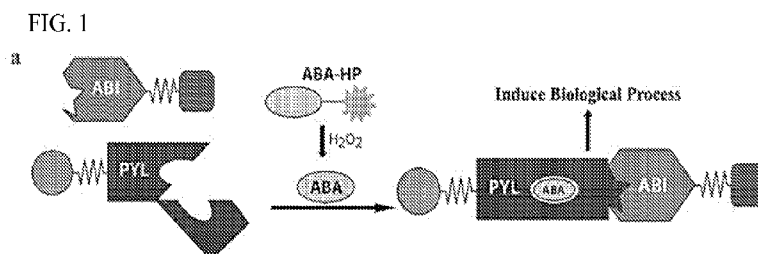
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(54) Title: COMPOSITIONS AND METHODS FOR CONTROLLING CELLULAR FUNCTION



(57) Abstract: This disclosure describes compositions and methods that involve a first modular component and a second modular component. The first modular component includes a first target molecule coupled to a first dimerizing moiety. The second modular component includes a second target molecule coupled to a second dimerizing moiety. The first dimerizing moiety dimerizes with the second dimerizing moiety when the first dimerizing moiety binds a chemical induced proximity (CIP) inducer.

COMPOSITIONS AND METHODS FOR CONTROLLING CELLULAR FUNCTION

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CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application Serial No. 61/941,091, filed February 18, 2014, and U.S. Provisional Patent Application Serial No. 62/091,181, filed December 12, 2014, each of which is incorporated herein by reference.

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SUMMARY

This disclosure describes, in one aspect, a composition that includes a first modular component and a second modular component. The first modular component includes a first target molecule coupled to a first dimerizing moiety. The second modular component includes a second target molecule coupled to a second dimerizing moiety. The first dimerizing moiety dimerizes with the second dimerizing moiety when the first dimerizing moiety binds a chemical induced proximity (CIP) inducer.

In some embodiments, the composition further includes an activatable inactive chemical-induced proximity inducer. In some of these embodiments, the composition can further include a compound that converts the activatable inactive chemical-induced proximity inducer to an active chemical-induced proximity inducer. In some of these embodiments, the first dimerizing moiety is bound to the active chemical-induced proximity inducer and dimerized with the second dimerizing moiety. In some of these embodiments, the compound that converts the activatable inactive chemical-induced proximity inducer to an active chemical-induced proximity inducer comprises H₂O₂.

In some embodiments, the chemical-induced proximity inducer can include ABA or GA.

In another aspect, this disclosure describes a cell that includes any of the compositions summarized above and a polynucleotide whose expression is modulated by a complex that includes the first modular component dimerized to the second modular component. In some embodiments, the polynucleotide can include a polynucleotide endogenous to the cell. In some embodiments, the polynucleotide can include a polynucleotide exogenous to the cell.

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In another aspect, this disclosure describes a complex that includes a first modular component and a second modular component. The first modular component generally includes a first target molecule coupled to a first dimerizing moiety and an active chemical-induced proximity inducer bound to the first dimerizing moiety. The second modular component
5 generally includes a second dimerizing moiety dimerized to the first dimerizing moiety of the first modular component and a second target molecule coupled to the second dimerizing moiety.

In yet another aspect, this disclosure describes a method that generally involves introducing any composition summarized above into a cell, introducing a activatable inactive chemical-induced proximity inducer into the cell, and contacting the cell with a compound that
10 activates the activatable inactive chemical-induced proximity inducer. In some of these embodiments, the cell further includes a polynucleotide whose expression is modulated by a complex of the first modular component dimerized to the second modular component. In some of these embodiments, the polynucleotide can include a polynucleotide endogenous to the cell. In other embodiments, the polynucleotide can include a polynucleotide exogenous to the cell.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should
15 20 not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. (a) General strategy for H₂O₂-inducible protein proximity to control biological processes. (b) Synthesis of ABA-HP and its conversion to ABA in the presence of H₂O₂. (c) 1
25 mM ABA-HP was treated with or without 5 mM H₂O₂ in 50% HEPES/DMSO for different time at 37°C, and detected by HPLC. (d) 100 μM ABA-HP was treated with 100 μM reductive H₂S, reactive oxygen species (ROS) including H₂O₂, •OH, tert-butyl hydroperoxide (TBHP), tBuO• (•OH and •OtBu were generated by reaction of Fe²⁺ with H₂O₂ or TBHP, respectively), and ClO⁻, as well as 100 μM of common cellular metal ions for four hours at 37°C. The results are the
30 averages of three independent experiments.

FIG. 2. (a) DNA constructs for H₂O₂-induced ABA-mediated biological controls: (i) transcriptional control, (ii) nuclear exporting, (iii) ruffle formation. (b) EGFP expression in CHO cells (10 h): (i) no drug; (ii) ABA 10 μ M; (iii) ABA-HP 10 μ M; (iv) ABA-HP 10 μ M + H₂O₂ 10 μ M; (v) ABA-HP 10 μ M + H₂O₂ 50 μ M; (vi) ABA-HP 10 μ M + H₂O₂ 100 μ M. (c) Nuclear exporting of eGFP in CHO cells (30 min): (i) no drug; (ii) ABA 10 μ M; (iii) ABA-HP 10 μ M; (iv) ABA-HP 10 μ M + H₂O₂ 100 μ M. (d) Ruffle formation of CHO cells (30 min): (i) no drug; (ii) ABA 10 μ M; (iii) ABA-HP 10 μ M; (iv) ABA-HP 10 μ M + H₂O₂ 100 μ M. Scale bar is 100 μ m for (b) and 20 μ m for (c) & (d). Images shown are representative from three independent experiments.

FIG. 3. (a) Synthesis of GA-HP and its conversion to GA in the presence of H₂O₂. (b) 5 mM GA-HP was treated with or without 10 eq of H₂O₂ in 50% HEPES/DMSO for different time at 37°C and analyzed by HPLC. (c) DNA constructs used for H₂O₂-induced GA-mediated nuclear exporting of eGFP. (d) Nuclear exporting of eGFP in CHO cells (30 min): (i) no drug; (ii) GA-AM 10 μ M; (iii) GA-H₂O₂ 10 μ M; (iv) GA-H₂O₂ 10 μ M + H₂O₂ 100 μ M. Scale bar is 20 μ m for (d). Images shown are representative from three independent experiments.

FIG. 4. Time dependent cleavage of ABA-HP by H₂O₂ detected by HPLC. 1 mM ABA-HP was treated with 5 mM H₂O₂ in 50% HEPES/DMSO (10 mM HEPES, pH 7.4) from 0 to 240 min at 37°C. ABA-HP dimer (characterized by mass spectrometry, FIG. 13) was generated by incubation at 37°C in 50% HEPES/DMSO for two hours and kept stable 24 hours later. (a) Representative HPLC chromatograms from three independent experiments. (b) Quantitation of generated ABA from ABA-HP in the presence of H₂O₂. The results were quantified by integrating the peak area corresponding to the ABA peak over the total areas of all peaks to give the generated ABA %. The shown result was the average from three independent experiments.

FIG. 5. Reaction selectivity of ABA-HP (100 μ M) against H₂S and ROS (100 μ M) in 50% HEPES/DMSO. The results were analyzed by HPLC after four-hour incubation at 37°C. H₂S was generated by Na₂S in HEPES buffer (pH 7.4). •OH and •OtBu were generated by reaction of Fe²⁺ with H₂O₂ or tert-butyl hydroperoxide (TBHP), respectively. Shown HPLC chromatograms are representative from three independent experiments. The results were quantified by integrating the peak area corresponding to the ABA peak over the total areas of all peaks to give the generated ABA % as shown in Fig. 1d.

FIG. 6. Reaction selectivity of ABA-HP (100 μ M) against common cellular metal ions (100 μ M) in 50% HEPES/DMSO. The results were analyzed by HPLC after four-hour incubation in 37°C. Shown HPLC chromatograms are representative from three independent experiments. The results were quantified by integrating the peak area corresponding to the ABA peak over the total areas of all peaks to give the generated ABA % as shown in Fig. 1d.

FIG. 7. Reaction selectivity of ABA-HP (100 μ M) against Cu^+ and Cu^{2+} (10 μ M) in 50% HEPES/DMSO. The results were analyzed by HPLC after four-hour incubation in 37°C. (a) Shown HPLC chromatograms are representative from three independent experiments. (b) The results were quantified by integrating the peak area corresponding to the ABA peak over the total areas of all peaks to give the generated ABA % as shown.

FIG. 8. Dose-dependent and time-dependent GFP expression controlled by H_2O_2 in HEK 293T eGFP reporter cells. Cells were treated with indicated molecules and eGFP expression was observed under fluorescence microscope after indicated times. The scale bar is 100 μ m. Images shown are representative from three independent experiments.

FIG. 9. Time dependent cleavage of GA-HP by H_2O_2 detected by HPLC. 5 mM GA- H_2O_2 was treated with 50 mM H_2O_2 in 50% HEPES/DMSO (10 mM HEPES, pH 7.4) from 0 to 120 minutes at 37°C. Shown HPLC chromatograms are representative from three independent experiments.

FIG. 10. ^1H -NMR of compound 2.

FIG. 11. ^1H -NMR of compound 3 (ABA-HP).

FIG. 12. ^{13}C -NMR of compound 3 (ABA-HP).

FIG. 13. MS of ABA-HP and ABA-HP dimer.

FIG. 14. ^1H -NMR of compound 4 (GA-HP).

FIG. 15. ^{13}C -NMR of compound 4 (GA-HP).

FIG. 16. MS of GA-HP.

FIG. 17. MS of partial hydrolyzed GA-HP.

FIG. 18. Characterization data of new compounds. **Compound 2**: ^1H -NMR (300.13 MHz, CDCl_3), δ (ppm): 1.340 (12H, s), 4.488 (2H, s), 7.378-7.404 (2H, d, $J = 7.8\text{Hz}$), 7.772-7.798 (2H, d, $J = 7.8\text{Hz}$); **Compound 3** (ABA-HP): ^1H -NMR (300.13 MHz, CDCl_3), δ (ppm): 0.997 (3H, s), 1.097 (3H, s), 1.334 (12H, s), 1.896 (3H, s), 2.004 (3H, s), 2.165 (1H, s), 2.254-2.311 (1H, d, $J = 17.1\text{Hz}$), 2.443-2.500 (1H, d, $J = 17.1\text{Hz}$), 5.155 (2H, s), 5.802 (1H, s), 5.917

(1H, s), 6.130-6.183 (1H, d, J = 15.9Hz), 7.345-7.372 (2H, d, J = 8.1Hz), 7.785-7.812 (2H, d, J = 8.1Hz), 7.855-7.910 (1H, d, J = 16.5Hz). ¹³C-NMR (75.48 MHz, CDCl₃), δ (ppm): 18.9, 21.3, 23.1, 24.3, 24.8, 41.5, 49.8, 65.7, 79.6, 83.8, 118.3, 127.0, 127.2, 128.1, 135.0, 136.5, 139.1, 149.7, 162.4, 165.6, 197.7; **Compound 4** (GA-HP): ¹H-NMR (300.13 MHz, CDCl₃), δ (ppm):
 5 1.198 (3H,s), 1.338 (12H,s), 1.632-2.172 (9H, m), 2.795-2.831 (1H, d, J = 10.8Hz), 3.200-3.236 (1H, d, J = 10.8Hz), 4.127 (1H, s), 4.876 (1H, s), 5.117-5.231 (3H, m), 5.867-5.910 (1H, dd, J = 3.6, 9.3Hz), 6.280-6.313 (1H, d, J = 9.9Hz), 7.318-7.345 (2H, d, J = 8.1Hz), 7.778-7.805 (2H, d, J = 8.1Hz). ¹³C-NMR (75.48 MHz, CDCl₃), δ (ppm): 14.8, 17.3, 25.1, 38.4, 43.1, 45.1, 50.8, 51.0, 51.4, 53.1, 53.7, 67.1, 69.9, 75.4, 78.3, 84.2, 90.8, 107.8, 127.7, 132.7, 133.0, 135.3, 138.5,
 10 157.0, 172.3, 179.0. ESI-MS, m/z (M+Na)⁺ 585.

FIG. 19. Light activation of caged ABA can dimerize two proteins of interest (POIs) and induce downstream cellular processes. The induced proximity of any two POIs can lead to a wide variety of biological effects.

FIG. 20. Synthesis and photo-cleavage of Caged ABA. A) ABA was conjugated to the
 15 DMNB protecting group in a one-step synthesis. Irradiation with 365 nm light removes the DMNB group and results in the release of free ABA. B) HPLC analysis of the photo-cleavage reaction showed that irradiation of 100 μM ABA-DMNB can regenerate free ABA within 2 min.

FIG. 21. Photo-triggered uncaging of ABA-DMNB can induce gene expression. A) Constructs and mechanism of ABA-dependent transcription initiation of a reporter gene. B)
 20 Induction of EGFP expression by 10 μM ABA, ABA-DMNB or UV-irradiated ABA-DMNB in EGFP reporter 293T cells. Scale bar 100 μm. C) Testing the stability of ABA-DMNB. CHO cells transfected with inducible luciferase expression constructs were incubated with ABA or ABADMNB for 12 and 24 h in the dark. D) Uncaging in cell culture and dosage dependence of light-controlled luciferase expression. CHO cells transfected with inducible luciferase constructs
 25 were treated with increasing dosages of ABA, or ABA-DMNB with or without UV irradiation. For C) and D), the relative luciferase expression fold changes were calculated based on transfected cells with no drug treatment. Error bars are SD (N=3).

FIG. 22. Photo-uncaging of ABA-DMNB can induce protein translocation. A) Constructs expressing EGFP-PYL and nuclear export sequence (NES)-tagged ABI. B) CHO cells were
 30 transfected with these constructs to test the light induced EGFP translocation. Under conditions with no drug or with ABADMNB without irradiation, the EGFP-PYL was distributed throughout

the cell. With the addition of ABA or the irradiation of added ABA-DMNB, the EGFP-PYL was exported out of the nucleus. After repeated washing with fresh media, the dimerization was reversed and EGFP-PYL diffused back into the nucleus. Scale bar 10 μ m. C) Quantitative analysis and statistics of EGFP-PYL translocation in EGFP expressing cells. Transfected and treated CHO cells were fixed and analyzed under a fluorescence microscope for nuclear export. Cells were categorized as showing nuclear export of EGFP when the intensity in the nucleus was less than 60% of that in the cytoplasm. Cells were counted from three separate experiments with N > 50 for each experiment.

FIG. 23. Caged ABA can be released to induce signal transduction to produce morphological changes. A) Constructs and mechanism of ABA-inducible membrane localization of Tiam1 and the initiation of Rac1 signaling. B) Examples of cell morphology classified as non-ruffled (top) or ruffled (bottom). Scale bar 10 μ m. C) Quantitative analysis and statistics of induced ruffling. Cells were counted and classified based upon the appearance of ruffled or non-ruffled morphology in EGFP expressing cells. Percentages of cells showing the ruffled morphology were calculated from three experiments with N > 50 for each experiment. Cultures that were given ABA or ABA-DMNB with irradiation showed a higher percentage of ruffled cell compared to cultures that were given ABA-DMNB without irradiation or no drug.

FIG. 24. ABA-DEACM for live cell experiments. A) Synthesis and photocleavage of ABA-DEACM by irradiation with 405 nm light. B) HPLC analysis of irradiated ABA-DEACM showed complete cleavage within 4 min. C) Quantitative analysis of EPFG-PYL nuclear export in live cell imaging under a confocal microscope. EGFP-PYL was monitored for 20 min and the ratio of the fluorescent intensity of the nucleus to the cytoplasm was calculated as a function of time. Grey bar indicates the period of irradiation with 405 nm laser equipped on the confocal microscope. D) Images of CHO cells incubated with ABA-DEACM before (0 sec) and after (620 sec) irradiation at 4 sec. Scale bar 10 μ m. The data in C) and D) are representative data from three independent experiments.

FIG. 25. Photo-uncaged ABA-DEACM induced cytoskeletal remodeling. A) CHO cells were transfected with myr-ABI and EGFP-PYL-Tiam1 constructs and irradiated with 405 nm light with or without the presence of ABA-DEACM. No cytoskeletal remodeling was observed for cells that were irradiated without the presence of ABA-DEACM. On the contrary, the formation of filopodia and lamellipodia (arrows) was observed within 15 min of irradiation

with 405 nm light in the presence of ABA-DEACM. Scale bar 10 μ m. Images shown are representative results repeated in three wells for three independent experiments. B) The enlarged images of the outlined region in A) at different time points during the time course of imaging after uncaging. Membrane ruffling can be observed starting at 5 min.

5 FIG. 26. Chemical stability of ABA-DMNB. ABA-DMNB was incubated in 70% HEPES buffer/30% DMSO at 37°C for 24 h in the dark. The samples were analyzed by HPLC. The peak at 24 h is identical to the one at 0 h indicating no chemical decomposition of ABA-DMNB in the aqueous solution.

10 FIG. 27. Uncaging of ABA-DMNB. ABA-DMNB was dissolved in DMSO at 10 μ M and irradiated with 365 nm light for indicated time (x-axis) from the fluorescence microscope and analyzed by HPLC. The areas under the curves were integrated to determine the relative amount of absorbance of each molecule present at 250 nm. The molar absorptivity of both free and caged ABA at 250 nm was measured, which was used to calculate the concentration of each species from the intensity of absorbance at 250 nm. The relative concentration of each compound was
15 used to calculate the percent concentration of free ABA relative to the concentration of total ABA species (both caged and uncaged). This result showed that ABA can be rapidly regenerated from the photo-uncaging of ABA-DMNB within a min.

20 FIG. 28. Biological activity of regenerated ABA isomers. CHO cells were transfected with the ABA-inducible luciferase reporter constructs. Cultures were incubated for 24 h with ABA isomers that were produced through photo-cleavage and purified from HPLC. Cells were incubated with 10 μ M stock cis ABA, regenerated 2-cis ABA, regenerated 2-trans ABA, and 10 μ M each of regenerated 2-cis and 2-trans ABA. The relative luciferase expression fold-changes were calculated based on cells with no drug treatment. Error bars are SD (N=3).

25 FIG. 29. Light-induced EGFP expression in cell culture. HEK 293T EGFP reporter cells were plated for 24 h and then incubated with ABA, ABA-DMNB, or ABA-DMNB with UV irradiation at indicated concentrations. Images were taken with the fluorescence microscope 12 h after molecules were added.

30 FIG. 30. Photo-cleavage of ABA-DEACM and ABA-DMNB by 405 nm light. 60 μ L of ABA-DEACM in DMSO at 10 μ M was irradiated with 405 nm light for the indicated amount of time (x-axis) in a 96-well plate. 50 μ L of each sample was run through HPLC for analysis. The areas under the curves were integrated to determine the relative amount of absorbance of each

molecule present at 250 nm. The molar absorptivity of both free and caged ABA at 250 nm was measured, which was used to calculate the concentration of each species from the intensity of absorbance at 250 nm. The relative concentrations of each compound were used to calculate the percent concentration of free ABA relative to the concentration of total ABA species.

FIG. 31. Photo-cleavage of ABA-DMNB and ABA-DEACM by 365 nm light. 100 μ L of ABA-DMNB and ABA-DEACM in DMSO at 10 μ M were irradiated by the fluorescence microscope with 365 nm light for the indicated amount of time (x-axis) in a 24-well plate. 50 μ L of each sample was run through the HPLC for analysis. The areas under the curves were integrated to determine the relative amount of absorbance of each molecule present at 250 nm. The molar absorptivity of both free and caged ABA at 250 nm was measured, which was used to calculate the concentration of each species from the intensity of absorbance at 250 nm. The relative concentrations of each compound were used to calculate the percent concentration of free ABA relative to the concentration of total ABA species. The results showed that ABA-DEACM cannot be cleaved effectively under 365 nm light and selective uncaging of ABA-DMNB is possible in the presence of ABA-DEACM.

FIG. 32. Chemical stability of ABA-DEACM. ABA-DEACM was incubated in 70% HEPES buffer/30% DMSO at 37°C for 24 h in the dark. The samples were analyzed by HPLC and absorbance was monitored at 250 nm. The peak at 24 h is identical to the one at 0 h indicating no chemical decomposition of ABA-DEACM in the aqueous solution.

FIG. 33. Cellular stability of ABA-DEACM. CHO cells were transfected with ABA-inducible luciferase reporter constructs. Cells were then incubated with 10 μ M ABA or ABA-DEACM in the dark for 12 or 24 h. The cell lysates were used for luciferase assay. The relative luciferase expression fold changes were calculated based on cells with no drug treatment. Error bars are SD (N=3).

FIG. 34. Photo-uncaging of ABA-DEACM to induce EGFP nuclear export. CHO cells were transfected with the ABA-inducible EGFP nuclear export constructs and then treated with 10 μ M ABA or ABA-DEACM with or without 405 nm light irradiation. Cells were then fixed on slides and analyzed under a fluorescence microscope. Cell were categorized as displaying nuclear export of EGFP when the fluorescent intensity of the nucleus was less than 60% of the intensity of the cytoplasm. Percentages of cells showing nuclear export of EGFP were calculated from three experiments where N > 50 for each experiment.

FIG. 35. Photo-uncaging of ABA-DEACM to induce ruffling. CHO cells were transfected with the ABA-inducible Tiam1 membrane localization constructs and then treated with 10 μ M ABA or ABA-DEACM with or without 405 nm light irradiation. Cells were then fixed on slides and analyzed under a fluorescence microscope. Cells were counted and classified based upon the appearance of ruffled or non-ruffled morphology. Percentages of cells showing a ruffled morphology were calculated from three experiments where $N > 50$ for each experiment.

FIG. 36. Exemplary Fe^{2+} -sensing ABA-based inducer compounds.

FIG. 37. Data characterizing the ABA-based Fe^{2+} sensing compounds shown in FIG. 36. (A) stability data; (B) reactivity data; (C) reactivity of Compound 1 and Compound 4 (FIG. 36) toward varying concentrations of Fe^{2+} ; (D) selectivity of Compound 1 (FIG. 36) for various metals.

FIG. 38. Cell stability data for Compound 13 (FIG. 36).

FIG. 39. Cell stability data for Compound 14 (FIG. 36).

FIG. 40. Exemplary sensing probes for chemical stimuli H_2S , HOCl , HG^{2+} , O_3 , Cu^{2+} , and Co^{2+} .

FIG. 41. A schematic diagram of a dual stimulus SIP system. Cellular activity output is generated only when both cellular stimuli, H_2O_2 and Fe^{2+} are sensed by their respective SIP inducers, ABA- H_2O_2 and GA- Fe^{2+} , respectively.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Cells constantly sense their microenvironments and integrate perceived signals to generate proper cellular responses. Synthetic biology aims to engineer novel cellular functions by assembling molecular parts that enable a cell to detect and process specific signals to produce predictable and desired biological outputs. These efforts not only contribute to the understanding of how sophisticated cellular behaviors are built, but also can provide therapeutic applications.

This disclosure describes a novel chemical strategy, referred to herein as Stimulus-Induced Proximity (SIP), that enables a cellular signal (i.e., H_2O_2) to control chosen cellular events in mammalian cells. This strategy combines reactivity-based small molecule sensing with the chemical-induced proximity (CIP) technology and can control transcription, protein translocation, and/or membrane ruffle formation by H_2O_2 . This new strategy can be a useful tool

for synthetic biologists and ultimately can be applicable to the development of gene and cell therapies.

CIP technology has been developed to regulate various biological processes using exogenous chemical inducers. A chemical-induced proximity inducer can dimerize two unique inducer-binding adaptor proteins that are fused individually to two other proteins of interest. Depending on the choice of proteins of interest, different downstream biological events can be controlled by the exogenous chemical-induced proximity inducers. The modular nature of the chemical-induced proximity technology provides generality in design and diversity in application by being compatible for use with various target proteins of interest. These characteristics of the chemical-induced proximity technology allow one to control the association of target proteins of interest and, therefore, place downstream processes under the control of a chemical-induced proximity system inducer. Several orthogonal chemical-induced proximity systems using different chemical inducers, including rapamycin, FK506, abscisic acid (ABA), gibberellic acid (GA) and other synthetic ligands, have been developed for broad biological and biomedical applications.

The ability to engineer novel signaling pathways that enable cells to perceive and respond to endogenous cellular signals and generate desired outcomes provides advantages over controlling biological events by artificial ligands. For example, cells can be equipped with the ability to differentiate diseased and healthy microenvironments in patients, and respond by producing therapeutic effects accordingly. Existing approaches to connecting upstream cellular signals to downstream biological outcomes mainly rely on natural or laboratory evolved nucleic acids or proteins, and consequently a great deal of effort is usually expended in the creation of new functions. In contrast, this disclosure describes a unique chemical strategy to achieve this goal by modifying the chemical-induced proximity (CIP) technology to be under the control of selected endogenous cellular signals.

We modified CIP technology because the CIP technology provides diversity and generality in controlling cellular processes. Other common small molecule-inducible systems (e.g., tetracycline-inducible or hormone-inducible systems) are typically limited to control of transcription. In contrast, the stimulus-induced proximity (SIP) technology described herein provides the diversity and generality typically associated with methods that involve chemical-induced proximity.

A SIP inducer is generated by chemically modifying a chemical-induced proximity inducer with a residue that abolishes the protein dimerizing activity of the inducer. The modified inducer remains inactive until being exposed to the chosen signal molecule, which promotes the chemical cleavage of the masking group. Having been thus regenerated, the original inducer is free to trigger the desired biological effects (FIG. 1a). This strategy takes advantage of the accumulated knowledge in the development of various chemical reactivity-based small molecule-sensing fluorescent probes aimed at detecting and reporting endogenous cellular signaling molecules such as, for example, H_2O_2 , H_2S , NO , O_3 , and certain metal ions.

One exemplary model embodiment involves modifying the plant abscisic acid (ABA) chemical-induced proximity system. As shown in FIG. 1a, ABA binds selectively to the PYR_1 -like (PYL) protein to create a surface that can subsequently bind the ABI protein. Based on its crystal structure, ABA is imbedded within, and makes extensive contacts with, the PYL pocket. Thus, any chemical modification of ABA may disrupt its binding to PYL and therefore abolish the induced association between PYL and ABI. By modifying ABA with a group that can be removed by a particular SIP stimulus, the ABA-based chemical-induced proximity activity can be controlled by the SIP stimulus. The carboxylic acid group on ABA, which is involved in hydrogen bonding to PYL, provides a target domain for attaching a masking group.

One exemplary embodiment involves an H_2O_2 -inducible ABA-mediated chemical-induced proximity system. H_2O_2 is a reactive oxygen species, serves as an important secondary messenger, and is involved in several biological processes and diseases. To transform the ABA-inducible chemical-induced proximity system into an H_2O_2 -inducible stimulus-induced proximity system, we synthesized an H_2O_2 -responsive boronated ABA, designated as ABA-HP in FIG. 1b. ABA-HP can convert back to ABA upon exposure to H_2O_2 . Monitored by HPLC, we observed that ABA-HP was stable in biological buffer at 37°C throughout the 24-hour observation period (FIG. 1c). Upon the addition of H_2O_2 (5 eq), ABA-HP started to convert back to ABA within minutes and the transformation was completed in four hours (FIG. 1c, 4a, and 4b). To test the selectivity of ABA-HP towards H_2O_2 relative to other reactive oxygen species, cellular metals, and/or signaling molecules, ABA-HP was incubated with different molecules, including H_2O_2 , $\text{HO}\cdot$, tert-butylhydroperoxide (TBHP), $\text{tBuO}\cdot$, ClO^- , H_2S , or various metal ions for four hours and the products were analyzed by HPLC. ABA-HP was stable against all molecules tested at $100\text{ }\mu\text{M}$ (FIG. 1d, FIG. 5, and FIG. 6), except Cu^+ and Cu^{2+} , which produced

a minimal amount of cleavage but were later shown to have no effects at the more physiologically relevant concentration of 10 μ M (FIG. 7).

To determine if the ABA generated from the H₂O₂-induced cleavage of ABA-HP is biologically active, we tested whether it could induce downstream biological processes. We first investigated H₂O₂-induced transcriptional activation using an HEK 293T stable cell line, in which enhanced GFP (eGFP) expression occurs only in the presence of functional ABA. The cell line was created with both the ABA-responsive split transcriptional activator DNA fragment (VP-PYL and GAL4DBD-ABI linked by IRES; Liang et al., 2011, *Sci. Signal.* 4, rs2) and an inducible eGFP DNA fragment (with 5 $\times$ UAS) inserted into the genome (FIG. 2a). The *in vivo* H₂O₂ concentrations of normal or pathologic tissue samples varies from less than 10 μ M to greater than 300 μ M, with pathologic tissues typically exhibiting higher levels of H₂O₂.

We tested the response of our system to H₂O₂ at physiologically relevant concentrations under cell culture conditions. The eGFP reporter cells were treated with no drug, or 10 μ M of ABA, ABA-HP, or ABA-HP exposed to H₂O₂ (1, 5 or 10 eq) for five to 24 hours. The expression of eGFP was monitored at specific time points using a fluorescence microscope. EGFP expression was not observed within a 10-hour period following the addition of ABA-HP alone (FIG. 2b). This result suggested that ABA-HP is stable within cells for at least 10 hours and thus cleavage at the ester linkage by cellular esterases is minimal. With extended incubation (24 hours), eGFP expression was indeed observed (FIG. 8), which indicates further structural modification of the boronic probe may be desirable for applications that involve extended incubation. Alternatively, for cellular experiments, cell culture media can be exchanged regularly with media containing fresh ABA-HP and thus restrict the build-up of esterase-cleaved ABA.

When either ABA or the combination of ABA-HP and H₂O₂ were added, eGFP expression could be observed after five hours (FIG. 2b and FIG. 8), which is evidence for the H₂O₂-induced cleavage of ABA-HP to generate functional ABA *in situ*. Varying levels of eGFP production were evident when different amounts of H₂O₂ were added (FIG. 2b). Different levels of H₂O₂ reproduced differential levels of ABA and resulted in a dose-dependent eGFP expression. This property can be useful for converting different levels of cellular signals (e.g. H₂O₂) into different levels of transcriptional activity or for developing a ratiometric protein-based cellular H₂O₂-reporting system.

Various cellular events are regulated by the dynamic sub-cellular translocation of proteins. To test if protein translocation can be controlled by H₂O₂, we used a known ABA-inducible eGFP nuclear exporting system consisting of NES-ABI and PYL-eGFP DNA constructs (FIG. 2a; Liang et al., 2011, *Sci. Signal.* 4, rs2). CHO cells were transfected with both plasmids for 24 hours and then incubated without additive, or with 10 μM of ABA, ABA-HP, or ABA-HP plus 10 eq of H₂O₂ for one hour. The sub-cellular location of PYL-eGFP fusion protein was observed with a fluorescence microscope. PYL-eGFP showed pan-cellular distribution in the absence of ABA and was localized out of the nucleus when ABA was added (FIG. 2c). ABA-HP alone did not change the sub-cellular location of eGFP-PYL. However, the addition of ABA-HP plus H₂O₂ caused rapid nuclear export (FIG. 2c). This result expanded the scope of H₂O₂-controlled processes beyond transcription, thus beyond that achieved by other inducible systems.

To further investigate whether a complex biological process can be triggered by H₂O₂-induced protein dimerization, we examined the membrane ruffle formation resulting from localized Rho GTPase activation. Tiam1 is a guanine exchange factor for Rac, a member of Rho GTPase. The membrane translocation of Tiam1 activated Rac signaling pathway and induced ruffle formation, which is involved in cell migration and morphogenesis. We constructed an ABA-inducible Tiam1 membrane localization system, which consists of DNA plasmids encoding the membrane-localized ABI (myr-ABI; Liang et al., 2011, *Sci. Signal.* 4, rs2) and the cytoplasmic eGFP/PYL-fused Tiam1 (PYL-eGFP-Tiam1) (FIG. 2a). CHO cells were transfected with these plasmids for 24 hours and then incubated for one hour without additive, or with 10 μM of ABA, ABA-HP or ABA-HP plus 10 eq of H₂O₂. The ruffle formation in cells was analyzed by the confocal microscopy. Only cells treated with either ABA or the combination of ABA-HP and H₂O₂ showed obvious ruffle formation (FIG. 2d). This demonstrated that the feasibility and utility of stimulus-induced proximity to engineer a novel cell signaling pathway to translate a cellular stimulus or signal (e.g. H₂O₂) into a complex cellular event (e.g., ruffle formation) that is not normally associated with this signal.

The same strategy can be applied to other chemical-induced proximity inducers such as, for example, gibberellin (gibberellic acid, GA). GA induces binding between GAI and GID1 proteins similar to the way in which ABA induced binding between PYL and ABA. GA is not cell permeable and has therefore been modified as the acetoxymethoxy ester (GA-AM) to cross the cell membrane and subsequently be cleaved by the esterase to regenerate GA. A carboxylate

group on GA is involved in its native biological function, but a minor modification that converts the carboxylate into a nonhydrolyzable hydroxamate abolishes the native chemical-induced proximity activity of GA. Thus, modifying the carboxylate with the H₂O₂-sensitive boronic probe can render the resulting compound, GA-HP, inactive for chemical-induced proximity until
5 GA is regenerated by exposure to H₂O₂. We synthesized GA-HP by coupling the boronic group to GA (FIG. 3a). We tested the chemical stability of GA-HP, and its conversion back to GA upon H₂O₂ addition, using HPLC (FIG. 3b and FIG. 9). GA-HP did not release GA when incubated in biological buffer for 24 hours even though the boronic group itself was partially hydrolyzed to give an inert product. GA was rapidly regenerated upon adding H₂O₂ (10 eq), and the conversion
10 was completed in two hours.

To examine whether the regenerated GA is functional within the cell, we made DNA constructs to encode a nuclear exported GID1 (NES-GID1) and a pan-cellular eGFP-tagged GAI (eGFP-GAI) (FIG. 3c). CHO cells were transfected with these plasmids for 24 hours and then incubated for one hour without additive or with 10 μM of GA-AM, GA-HP or GA-HP plus 10 eq
15 of H₂O₂ for one hour. The sub-cellular location of the PYL-eGFP protein was observed with a fluorescence microscope (FIG. 3d). Only cells treated with either GA-AM or the combination of GA-HP and H₂O₂, showed nuclear exportation, which indicated that the functional GA was regenerated upon H₂O₂ addition. Given that GA cannot cross the cell membrane, the observed intracellular effects suggested that the H₂O₂-induced cleavage of GA-HP occurred inside the
20 cells. The successful implementation of this chemical-induced proximity modifying strategy with GA demonstrates the potential generality of this method.

The while occasionally described herein in the context of an exemplary embodiment in which inducer molecule is ABA, the methods described herein can be practiced using any suitable inducer molecule. Exemplary alternative inducer compounds include, for example, any
25 compound that can dimerize proteins. Such compounds include, for example, rapamycin, auxins, FK506, or a protein dimerizing analog or derivative thereof.

The ABA-based dimerization system has many features, including rapid response kinetics, fast rate of reversibility, wide dose responsive range, no known toxicity issues and excellent bioavailability. Moreover, ABA and rapamycin systems are orthogonal and can be used
30 simultaneously to control two independent cellular events. In an alternative exemplary

application, one can use a light-inducible ABA-based CID system using a photocaged ABA that enables dose-dependent photo-control of cellular processes (FIG. 19).

As discussed above, ABA binds with the PYL protein to form a complex that subsequently recognizes ABI. Structural modifications of ABA, especially those that involve
5 incorporating bulky groups, may disrupt its binding to PYL and, therefore, inhibit its ability to promote subsequent PYL-ABI dimerization. Consequently, incorporating a photo-removable group can result in an ABA derivative that would be incapable of binding to PYL. Furthermore, the carboxylic acid moiety in ABA, which forms hydrogen bonds with PYL, can be a site to which a chosen photo-removable group can be attached.

10 4,5-Dimethoxy-2-nitrobenzyl (DMNB) and other nitrobenzene groups have been used to cage small molecules because they can be removed by 365 nm light. In one exemplary embodiment, a light-inducible system can involve an ABA derivative, ABA-DMNB, that includes a DMNB ester group (FIG. 20A). HPLC analysis shows that ABA-DMNB is stable at 37°C in biological buffer in the dark for 24 hours (FIG. 26). To examine the efficiency of the
15 photo-uncaging process, solutions of ABA-DMNB (10 µM and 100 µM) were irradiated with 365 nm UV light emitted from a fluorescent microscope. HPLC analysis of the photolysate showed that the ABA-DMNB was cleaved to regenerate free ABA following irradiation for 120 seconds (FIG. 20B and FIG. 27). In addition to the ABA product, an additional product was generated at the same time in near equal amount during the photochemical process (FIG. 20B).
20 We purified the by-product by HPLC and confirmed by mass spectrometry analysis that the by-product was indeed an ABA isomer, and the NMR data revealed the by-product to be the 2-*trans* isomer of ABA. Surprisingly, when uncaged ABA was subjected to the same photo-cleavage condition very little 2-*trans* isomer was produced, which suggests that the DMNB group may serve as a photosensitizing group to facilitate the isomerization of the ABA backbone under the
25 applied irradiation condition (FIG. 20B).

The dimethoxynitrosobenzaldehyde released from the uncaging process of ABA-DMNB was not detected under the wavelength of 250 nm, which was used in the HPLC analysis. This is consistent with nitrosobenzene derivatives having low absorptivity at 250 nm. These results show that photo-uncaging of ABA-DMNB occurs rapidly to produce ABA in biologically
30 relevant concentrations.

To confirm that the photo-regenerated ABA is biologically functional, we determined whether it can be employed to induce PYL-ABI dimerization in an ABA-inducible transcriptional activation system using an established HEK 293T inducible EGFP reporter cell line. The cell line was created with both the ABA-responsive split transcriptional activator DNA fragment (VP-PYL and GAL4DBD-ABI linked by IRES; Liang et al., 2011, *Sci. Signal.* 4, rs2) and an inducible EGFP DNA fragment (with 5 × UAS and the IL-2 minimal promoter) inserted into the genome (FIG. 21A). EGFP expression was activated through the recruitment of VP16AD to the EGFP gene in the presence of ABA. 24 hours after plating the cells, 10 μM of ABA, pre-irradiated ABA-DMNB products, or non-irradiated ABA-DMNB were separately added to the cells and incubated for an additional eight hours. EGFP expression was then determined using a fluorescence microscopy. Both ABA and pre-photocleaved ABA-DMNB induced EGFP production (FIG. 21B), which indicate that the uncaging of ABA-DMNB gives biologically functional ABA. In contrast, 10 μM of non-irradiated ABA-DMNB did not induce EGFP expression (FIG. 21B). Thus, ABA-DMNB would be stable in cells under the experimental condition and unable to induce PYL-ABI dimerization in the absence of light.

The cellular stability of ABA-DMNB was explored further using a more sensitive and quantitative luciferase assay system, which employs an inducible luciferase construct (FIG. 21A). CHO cells were transfected with the ABA-inducible luciferase constructs for 24 hours and then 10 μM of ABA-DMNB was added and incubated in dark for 12 or 24 hours. Cells were then harvested and analyzed by using the luciferase assay. The results showed that very little luciferase expression took place after a 12-hour incubation period (FIG. 21C), an observation that is consistent ABA-DMNB not activating dimerization. Furthermore, only a minimal level of luciferase was induced following a 24-hour incubation, which demonstrates that ABA-DMNB is relatively stable under the cell culture condition despite possessing an ester that could potentially be hydrolyzed.

From the HPLC analysis of the ABA-DMNB photocleavage (FIG. 20B), a near equal amount of 2-*trans* ABA isomer was formed during the uncaging process. The 2-*trans* isomer exhibits measurably lower biological activity than the *cis* isomer. Based on the crystal structures of the ABA-PYL complex, it may be that the 2-*trans* isomer may not fit the PYL pocket as well as the *cis* isomer, which may result in a lower dimerization efficiency and, therefore, decreased downstream activity. To confirm that the generated 2-*trans* ABA isomer would not interfere with

the dimerization induced by the regenerated *cis*-ABA, we tested the induced expression of luciferase by the *cis*-ABA isomer, the 2-*trans* ABA isomer, or a mixture of the ABA isomers. In this experiment, CHO cells were transfected with the ABA-inducible luciferase constructs for 24 hours and then 10 μ M of the stock *cis*-ABA, photo-regenerated *cis*-ABA, photo-regenerated *trans* ABA isomer, or a mixture of regenerated *cis*-ABA and regenerated *trans*-ABA (10 μ M each) was added and incubated for an additional 24 hours. Cells were then harvested and subjected to the luciferase assay. The regenerated *cis*-ABA gave a similar activity as the stock ABA and the 2-*trans* isomer exhibited reduced activity (FIG. 28). The mixture of the *cis* and *trans* isomers induced luciferase expression comparable to the *cis*-ABA alone, suggesting that the *trans* isomer does not attenuate the effects of the *cis*-ABA.

The ABA-inducible luciferase assay also was used to determine if ABA-DMNB is effectively photo-cleaved in cell culture. Different quantities of ABA and ABA-DMNB (FIG. 21D) were independently added to transfected CHO cells. The cells that received ABA-DMNB were either kept in the dark or irradiated using 365 nm UV light for 120 seconds. Twelve hours after either ABA or ABA-DMNB was added and the cells were irradiated, cells were harvested and subjected to the luciferase assay. Cells that were treated with ABA or those with ABA-DMNB followed by irradiation induced luciferase expression (FIG. 21D). Similarly, the HEK 293T-inducible EGFP reporter cell line showed EGFP expression when cultures containing ABA-DMNB were irradiated (FIG. 29). Thus, ABA-DMNB can be uncaged in cell culture to allow light-induced controls of transcriptional activation in live cells.

ABA gives dose-dependent induction of gene expression and ABA generated by irradiation of ABA-DMNB also displays this property. Irradiation of transfected cells with the addition of increasing concentrations of ABA-DMNB leads to corresponding increases in the level of luciferase expression (FIG. 21D). A concern of using UV-triggered reactions in cells is phototoxicity. However, based on cell morphology and the growth rate, no obvious cytotoxicity was observed under the irradiation conditions used to cleave ABA-DMNB. Finally, the DMNB-derived photoproduct does not cause observable toxicity at the concentrations used in the experiments described above.

The ability to use light to control protein translocation through ABA-DMNB uncaging was evaluated next. Plasmids expressing EGFP-tagged PYL and nuclear export sequence (NES) peptide-linked ABI (FIG. 22A) were transfected into CHO cells. The transfected cells were then

either treated or not treated with ABA, ABA-DMNB, or ABA-DMNB and irradiated for 60 seconds. Fluorescence microscopy analysis was used to determine subcellular locations of EGFP fusion proteins. Cells treated with ABA or with ABA-DMNB followed by UV irradiation showed markedly decreased intensities of EGFP in the nuclei within 15 minutes (FIG. 22B and 22C). On the other hand, cells that were either not treated with ABA-DMNB or with ABA-DMNB but not subjected to UV irradiation showed EGFP distribution throughout the whole cells. Furthermore, the observed nuclear export of EGFP fusion protein can be readily reversed by washing away the uncaged ABA using fresh media. Thus, protein translocation can be induced by light through photo-uncaged ABA and can be readily reversed.

To examine the reversibility of the photo-induced EGFP nuclear exportation process, cells that were treated with ABA or ABA-DMNB followed by UV irradiation were washed with fresh culture media not containing drugs and subjected to fluorescence analysis. Induced nuclear exportation of EGFP was reversed following three washes (within 30 minutes) (FIG. 22B, 22C).

Next, we examined the induction of ruffle formation through the activation of the Rac1 signaling pathway in order to determine if the light-activated ABA system can be used to regulate a complex biological process. The GTP exchange factor, Tiam1, when presents at the cell membrane, activates Rac1 to initiate a signaling pathway that leads to cytoskeletal remodeling that forms filopodia and/or lamellipodia. To activate Rac1 signaling and induce membrane ruffling, light and ABA-DMNB were used to control membrane localization of Tiam1 (FIG. 23A). A construct encoding a membrane localized ABI (myr-ABI) and one expressing a constitutively active Tiam1 fused to EGFP and PYL were employed for this purpose (FIG. 23A). Following the transfection of CHO cells with both plasmids for 24 hours, 10 μ M of ABA-DMNB was added and the treated cell culture was irradiated with 365 nm light from a fluorescent microscope. Cells were then fixed on slides and analyzed under a fluorescence microscope. Cells were categorized as non-ruffled if they did not have observable cytoskeletal remodeling or as having ruffles if they displayed distinct lamellipodia and filopodia (FIG. 23B). The cultures that received ABA or ABA-DMNB with irradiation showed a greater percentage of cells displaying ruffling compared to the cultures that were incubated with ABA-DMNB without irradiation or were given no drug (FIG. 23C). Some background ruffling when overexpressing constitutively active Tiam1 was observed even when no drug was added. These observations

demonstrate that the strategy of combining light and a caged ABA can be employed to control cytoskeletal remodeling through the initiation of Rac1 signaling.

A confocal microscope is often used to achieve greater precision in light-induced processes and could be used to cleave caged ABA more precisely. However, a confocal
5 microscope is commonly equipped with a 405 nm laser, which cannot efficiently uncage ABA-DMNB (FIG. 30). To prepare another caged ABA that can be cleaved by 405 nm light, we conjugated ABA to the [7-(diethylamino)coumarin-4-yl]methyl (DEACM) group to give ABA-DEACM (FIG. 24A). The design of ABA-DEACM demonstrates the flexibility of the small molecule caging system, in which different caging groups can be easily installed as needed by
10 using simple chemical processes rather than lengthy protein engineering steps that are otherwise needed in existing protein-based methods. Studies of photo-cleavage of ABA-DEACM using a 405 nm LED revealed that ABA-DEACM can be cleaved rapidly to give ABA within a few minutes (FIG. 24B, FIG. 30). As observed in the case of ABA-DMNB uncaging, the isomerized ABA was also produced, which suggested that the addition of a caging group may in general
15 sensitized and promoted the isomerization of ABA, although the mechanism is unclear. The absorptivity of the coumarin photo-cleavage product at the detection wavelength (250 nm) was measured and was shown to be low ($\epsilon=0.23$), and the coumarin by-product was therefore not seen in the HPLC analysis. Moreover, ABA-DEACM cannot be cleaved efficiently by 365 nm light (FIG. 31), which is employed to activate the ABA-DMNB caging system. This observation
20 suggests that DMNB and DEACM can potentially be utilized as orthogonal caging groups on orthogonal CID inducers to independently control two cellular events.

An evaluation of the stability of ABA-DEACM using HPLC and the luciferase assay showed that it is stable chemically and in cell culture (FIG. 32 and FIG. 33). Photo-uncaged ABA-DEACM also showed the ability to induce EGFP-PYL nuclear export (FIG. 34) and Rac1
25 signaling activation/ruffle formation in cells (FIG. 35). To test the uncaging process in live cell experiments, EGFP nuclear export experiments were carried out by transfecting CHO cells with EGFP-PYL and NES-ABI constructs. The cells were then either treated with 10 μ M ABA-DEACM and with or without 405 nm light irradiation under a confocal microscope, or with no drug but irradiated. The subcellular location of EGFP was followed for 20 minutes after
30 irradiation. The nuclear export of EGFP fusion proteins was observed within a few minutes when treated with ABA-DEACM followed by irradiation, but not in the cases of no drug or no

irradiation (FIG. 24C, 24D). We also tested the use of ABA-DEACM in live cell imaging to induce cell morphology changes. We transfected CHO cells in a culture chamber with myr-ABI and EGFP-PYL^{Tiam1} constructs. Cells that were irradiated in the absence of ABA-DEACM did not show any cytoskeletal remodeling (FIG. 25A). On the other hand, cells that were incubated with ABA-DEACM and irradiated showed formation of filopodia and lamellipodia within 15 minutes (FIG. 25A, 25B). These results demonstrate that the local uncaging of ABA-DEACM produced a sufficient level of ABA, which rapidly dimerized PYL-fusion and ABI-fusion proteins before diffused away, to illicit desired biological responses.

Another exemplary embodiment involves an iron-responsive inducible system. To establish an Fe²⁺-responsive gene inducible system, we developed a fluorescent Fe²⁺-sensing unit that can be used to cage an inducer. The Fe²⁺-sensor links an N-aryl-hydroxylamine moiety to a naphthalimide fluorophore. This Fe²⁺-sensing unit is selectively responsive to Fe²⁺ but is not responsive to other cell signals. This sensor can be used to report the endogenous labile Fe²⁺ in, for example, astrocytes and in ischemic rat brain tissue samples.

Various N-phenyl-hydroxylamine moieties with different substitutions on the benzene ring were synthesized and are shown in FIG. 36. Exemplary benzene ring substitutions that can tune the reactivity towards Fe²⁺ include, for example, p-NO₂, m-NO₂, o-NO₂, p-CN, p-Ac, o-Cl, o-I, or o-Me. HPLC analysis following iron-mediated cleavage *in vitro* revealed that, generally, substitutions that include an electron-withdrawing group facilitate cleavage while substitutions that include an electron-donating group increase the stability of the caged ABA against Fe²⁺ (FIG. 37B). The differential electron-withdrawing/donating character and the position of each group can affect cleavage efficiency and/or the chemical stability of the caged compounds (FIG. 37A, FIG. 37C). Representative caged ABAs were selective for iron among other common abundant metals in cells (FIG. 37D). The caged ABAs also showed selectivity towards Fe²⁺ vs Fe³⁺ (FIG. 37B). One can include both an electron-withdrawing substitution and an electron-donating substitution on the same molecule at different positions to fine-tune the reactivity and selectivity.

Stability of the Fe²⁺-sensing unit can be increased by increasing the steric hindrance near the ester in the Fe²⁺-sensing unit to block esterase activity that can prematurely degrade the sensing unit. The o-Me substitution (FIG. 36, Compound 8) is stable in cells. Additional bulky groups can be installed to further increase the stability of the compound in cells. Moreover, one or more electron-withdrawing and/or electron-donating groups can be installed on the benzene ring to

modulate the reactivity towards Fe^{2+} . For example, Compound 8 in FIG. 36 is chemically stable, exhibits Fe^{2+} reactivity, and exhibits selectivity (FIG. 37A, FIG. 37B). Thus, this compound can be a suitable molecular scaffold to further develop alternative iron-sensing inducers.

Thus, this disclosure describes various exemplary embodiments of a stimulus-induced proximity strategy that can be customized to control different downstream cellular events. Stimulus-induced proximity inducers can be easily generated and respond to various stimuli (e.g., H_2O_2 , light, and Fe^{2+}) rapidly and specifically at physiologically relevant concentrations. In various applications, a sensing unit for an endogenous cellular signaling molecule can be linked to ABA, GA, or another suitable chemical-induced proximity inducer to tailor their responding specificity toward a selected signal.

As discussed above, while certain exemplary embodiments have been described in detail in the context of a stimulus-induced proximity system in which the inducer includes abscisic acid (ABA) or gibberellic acid (GA), a stimulus-induced proximity system can be designed having an inducer that includes any polypeptide capable of heterodimerizing or homodimerizing a protein or proteins of interest. Accordingly, a stimulus-induced proximity system can include, as an inducer, an auxin, rapamycin, FK506, FK1012, cyclosporin A, coumermycin A1, methotrexate-SLF conjugate, trimethoprim-SLF conjugate, fusicoccin, or a protein dimerizing analog or derivative of any of the foregoing. As used herein, "SLF" refers to synthetic ligand for FKBP (FK506 binding protein).

Also, while certain exemplary embodiments have been described in detail in the context of a stimulus-induced proximity system in which the stimulus includes H_2O_2 , Fe^{2+} , or light, the stimulus may be any chemical or physical stimulus for which a suitable sensing probe exists. Thus, alternative exemplary stimuli include, for example, H_2S , NO (or other cellular secondary messengers) HOCl, HOBr, O_3 (or other reactive oxygen species (ROSs)), Hg^{2+} , Cu^+ , Co^{2+} , Ca^{2+} , Zn^{2+} (or other cellular or environmental metal ions), or various proteases. The presence of the stimulus, whether chemical or physical, can induce bond cleavage that unmask the inducer, producing the designed cellular activity. Exemplary sensing probes for H_2S (Lippert et al., 2001, *J. Am. Chem. Soc.* 133:10078-10080; Peng et al., 2011, *Angew. Chem. Int. Eng.* 50:9672-9675; Xuan et al., 2012, *Angew. Chem. Int. Eng.* 51:2282-2284; Liu et al., 2011, *Angew. Chem. Int. Eng.* 50:10327-10329), HOCl (Setsukinai et al., 2003, *J. Biol. Chem.* 278:3170-3175), O_3 (Garner et al., 2009, *Nat. Chem.* 1:316-321), Hg^{2+} (Song et al. 2008, *J. Am. Chem. Soc.*

130:16460-16461; Ando et al., 2011, *J. Am. Chem. Soc.* 133:2556-2566), Cu⁺ (Taki et al., 2010, *J. Am. Chem. Soc.* 132:5938-5939), and Co²⁺ (Au-Yeng et al., 2012, *Chem. Commun.* 48:5268-5270) are illustrated in FIG. 40. Exemplary sensing probes for various proteases are set forth in Table 1. Sensing activity can be tuned by chemically modifying the probing units to meet the targeted cellular signal concentrations.

Table 1. Caged ABA-based inducer compounds for proteases

Protease	Constructs (SEQ ID NO:)	Drug	Disease
Peptide-based prodrugs			
Caspase-3	Asp-Glu-Val-Asp-Pro-PABC-X (SEQ ID NO:11) CAR-Lys-Gly-Ser-Gly-Asp-Val-Glu-Gly-X (SEQ ID NO:12)	DOX, PH-A	Cancer
Cathepsin B	N-L-Leu-X (SEQ ID NO:13)	DNR, DOX	Cancer, RA
CP	Arg-X, Ala-X, Asp-X (SEQ ID NO:14)	MTX	Cancer
FAP	BHQ3-Lys-Gln-Glu-Gln-Asn-Pro-Gly-Ser-Thr-X (SEQ ID NO:15)	PH-A	Cancer
Kallikrein 2	Gly-Lys-Ala-Phe-Arg-Arg-X (SEQ ID NO:16)	TPG	Cancer
MMP-2/-9/-14	Glu-Pro-Cit-Gly-Hof-Tyr-Leu-X (SEQ ID NO:17)	DOX	Cancer
MMP-7	BHQ3-Lys-Arg-Ala-Leu-Gly-Leu-Pro-Gly-X (SEQ ID NO:18) BHQ3-(D-Glu) ₈ -Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser-(D-Arg) ₈ -Lys-X (SEQ ID NO:19)	PH-A	Cancer
Plasmin	D-Ala-Phe-Lys-X (SEQ ID NO:20) D-Val-Leu-Lys-X (SEQ ID NO:21) D-Ala-Phe-Lys-(PABC)-X (SEQ ID NO:22)	ara-C, AT-125, DOX, PM	Cancer, RA
PSA	Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-X (SEQ ID NO:23) Mu-His-Ser-Ser-Lys-Leu-Gln-EDA-X (SEQ ID NO:24) 4-O-(Ac-Hyp-Ser-Ser-Chg-Gln-Ser-Ser-Pro)-X (SEQ ID NO:25) HO ₂ C(CH ₂) ₃ CO-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-X (SEQ ID NO:26) N-gluaryl-(4-hydroxypropyl)-Ala-Ser-chGly-Gln-Ser-Leu-X (SEQ ID NO:27)	DOX, 5-FudR, VNB, TPG, L12ADT	Cancer
TOP	B-Ala-L-Leu-L-Ala-L-Leu-X (SEQ ID NO:28)	DOX	Cancer
uPA	D-Ala-Phe-Lys-PABC-X (SEQ ID NO:29)	DOX	Cancer
Macromolecular prodrugs			
Cathepsin B	PEG-L-Lys-X (SEQ ID NO:30) Poly-L-glutamic acid-X (SEQ ID NO:31) HPMAcp-Gly-Phe-Leu-Gly-X (SEQ ID NO:32) ALB-Lys-Lys-Phe-D-Ala-EMC-X (SEQ ID NO:33) ALB-EMC-D-Ala-Phe-Lys-Lys-X (SEQ ID NO:34)	Ce6, DNR, DOX, 5-FU, MTX, PtD, PTX, SN-392, TNP-470	Cancer
Cathepsin K	HPMAcp-Gly-Gly-Pro-Nle-4AB-X (SEQ ID NO:35)	ALN, PGE1	Bone disease
MMP-2/-9	DEX-Gly-Ile-Leu-Gly-Val-Pro-X (SEQ ID NO:36) ALB-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln-X (SEQ ID NO:37)	DOX, MTX	Cancer
Plasmin	ALB-EMC-D-Ala-Phe-Lys-Lys-X (SEQ ID NO:38)	DOX	Cancer, RA
PSA	ALB-EMC-Arg-Arg-Ser-Ser-Tyr-Tyr-Ser-Gly-X (SEQ ID NO:39) HMPAcp-morpholinocarbonyl-Ser-Ser-Lys-Tyr-Gln-Leu-X (SEQ ID NO:40)	DOX, TPG	Cancer
Thrombin	PEG-poly-L-Lys-X (SEQ ID NO:41) Poly-L-Lys-Gly-D-Phe-Pip-Arg-Ser-Gly-Gly-Gly-Gly-X (SEQ ID NO:42)	Ce6, PH-A	Cancer, RA
Trypsin	Poly-L-Lys-X (SEQ ID NO:43) Poly-L-Lys-Gly-Ala-Ser-D-Arg-Phe-Thr-Gly-X (SEQ ID NO:44)	PH-A	Cancer

uPA	ALB-EMC-Gly-Gly-Gly-Arg-Arg-X (SEQ ID NO:45)	DOX	Cancer
Targeted prodrugs			
Cathepsin B	c1F6-Val-Cit-X (SEQ ID NO:46) cAC10-Val-Cit-X (SEQ ID NO:47) Pep42-Val-Cit-X (SEQ ID NO:48) GAL-HPMAcp-Gly-Phe-Leu-Gly-X (SEQ ID NO:49)	DOX, MMAE, PTX	Cancer
Plasmin	RGD-4C-D-Ala-Phe-Lys-(PABC)-X (SEQ ID NO:50)	MTX	Cancer, RA

The sensing probe and inducer may be linked through a cleavable ester or amide linkage. The cellular stability of the linkages can be enhanced by chemically introducing a bulky substituent on the probes that can sterically interfere with access to the linkage.

- 5 The stimulus-induced proximity system described herein allows one to construct a novel cell signaling pathway by coupling a selected signal input to a chosen biological output. By networking these signaling pathways and applying desired computation algorithms (e.g., through Boolean logic gates), new cellular functions can be engineered to carry out sophisticated decision-making and to give proper biological outputs based on cellular microenvironments.
- 10 FIG. 41 illustrates a more complex system in which a biological output occurs only after dual inputs—H₂O₂ and Fe²⁺—are detected by the individual SIP sensor-inducer for each, ABA-H₂O₂ and GA-Fe²⁺, respectively.

- The stimulus-controlled systems described herein, whether controlled by a chemical stimulus based or a physical stimulus (e.g., light) can be used to regulate cellular processes such
- 15 as, for example, transcription, protein translocation, signal transduction, and/or cytoskeletal remodeling. In particular, the light-controlled systems allow for dosage-dependent regulation and/or independently regulating multiple cellular events using light of different wavelengths. As a result, the strategy can be used to expand the repertoire of light-controlled methods that can be exploited to control and/or manipulate biological systems.

- 20 The term “and/or” means one or all of the listed elements or a combination of any two or more of the listed elements; the terms “comprises” and variations thereof do not have a limiting meaning where these terms appear in the description and claims; unless otherwise specified, “a,” “an,” “the,” and “at least one” are used interchangeably and mean one or more than one; and the recitations of numerical ranges by endpoints include all numbers subsumed within that range
- 25 (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

In the preceding description, particular embodiments may be described in isolation for clarity. Unless otherwise expressly specified that the features of a particular embodiment are incompatible with the features of another embodiment, certain embodiments can include a combination of compatible features described herein in connection with one or more
5 embodiments.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The present invention is illustrated by the following examples. It is to be understood that
10 the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

15 Chemical synthesis.

Synthesis of compound 2: To 4-(bromomethyl)phenylboronic acid (200 mg, 0.93 mM, Sigma-Aldrich, St. Louis, MO) in 1 mL toluene was added pinacol (165 mg, 1.40 mM, Sigma-Aldrich, St. Louis, MO), then heated to reflux with Dean-Stark trap and stirred for three hours. The solvent was removed by rotavapor, and the product was purified through column
20 chromatography and obtained as white solid (250 mg, 90.6%).

Synthesis of ABA-HP: To ABA (44 mg, 0.17 mM, Gold Biotechnology, Inc., St. Louis, MO) in 10 mL CH₃CN was added compound 2 (55 mg, 0.19 mM) and DBU (55 µL, 0.37 mM, Sigma-Aldrich, St. Louis, MO), then stirred at room temperature for four hours. The solvent was removed by rotavapor, then the product was purified by column chromatography and obtained as
25 white solid (50 mg, 62.5%).

Synthesis of GA-HP: To GA (100 mg, 0.29 mM, Alfa Aesar, Ward Hill, MA) in 20 mL CH₃CN was added compound 2 (102.9 mg, 0.35 mM) and DBU (52 µL, 0.35 mM, Sigma-Aldrich, St. Louis, MO), then stirred at room temperature for four hours. The solvent was removed by rotavapor, and the product was purified by column chromatography, obtaining as
30 white solid (97 mg, 60%).

Characterization data is shown in FIGS. 10-17.

Reverse-phase HPLC analysis.

Chemical stability and reactivity of ABA-HP towards H_2O_2 : ABA-HP chemical stability: 1 mM ABA-HP (in DMSO) was incubated in 50% HEPES/DMSO (10 mM HEPES, pH 7.4) for 24 hours at 37°C. HPLC results were detected at 0 minutes, 20 minutes, 40 minutes, 60 minutes, 80 minutes, 100 minutes, 120 minutes, 240 minutes, and 24 hours. ABA-HP reactivity towards H_2O_2 : 1 mM ABA-HP was incubated with 5 mM (5 eq) H_2O_2 in 50% HEPES/DMSO (10 mM HEPES, pH 7.4) at 37°C. HPLC results were detected at 0 minutes, 20 minutes, 40 minutes, 80 minutes, 100 minutes, 120 minutes, and 240 minutes (FIGS 1c, 4a and 4b). 100 mM H_2O_2 was diluted with 11 μ L of 30% (10 M) stock H_2O_2 (VWR) and 989 μ L of dd H_2O . All showed concentrations were the final concentration. HPLC chromatograms were acquired using an ULTIMATE 3000 LC System (Thermo Fisher Scientific, Waltham, MA) with ACCLAIM 120 Å, C18, 3 μ m analytical (4.6 $\times$ 100 mm) column (Thermo Fisher Scientific, Waltham, MA). Chromatographic conditions: eluent A: 0.1% v/v TFA in water; eluent B: 0.1% v/v TFA in acetonitrile. ABA in ethanol or ABA-HP in DMSO was eluted at a flow rate of 0.750 ml/min monitored at a wavelength of 260 nm. 0-12 min (linear): 95% A, 5% B to 5% A, 95% B; 12-15 min: 5% A, 95% B; 15-17 min (linear): 5% A, 95% B to 95% A, 5% B. Generation of ABA was quantified by the peak area of ABA out of the total peak area.

Reaction selectivity versus different molecules: 100 μ M ABA-HP was incubated with 100 μ M (1 eq) of different molecules in 50% HEPES/DMSO (pH 7.4) at 37°C for 4 hours and detected by HPLC. H_2S was generated by 100 μ M Na_2S solution in HEPES buffer, which can slowly release H_2S . $\bullet OH$ and $\bullet OtBu$ were generated by reaction of Fe^{2+} with H_2O_2 or tert-butyl hydroperoxide (TBHP), respectively. HPLC condition was the same as the stability and reactivity test of ABA-HP.

Chemical stability and reactivity of GA-HP towards H_2O_2 : GA-HP chemical stability: 5 mM GA-HP (in DMSO) was incubated in 50% HEPES/DMSO (10 mM HEPES, pH 7.4) for 24 hours at 37°C and detected by HPLC. GA-HP reactivity towards H_2O_2 : 5 mM GA-HP was incubated with 50 mM (10eq) H_2O_2 in 50% HEPES/DMSO (10 mM HEPES, pH 7.4) at 37°C. HPLC results were detected at 5 minutes, 30 minutes, 60 minutes, 90 minutes, and 120 minutes. HPLC chromatograms were acquired using an ULTIMATE 3000 LC System (Thermo Fisher Scientific, Waltham, MA) with ACCLAIM 120 Å, C18, 3 μ m analytical (4.6 $\times$ 100 mm) column

(Thermo Fisher Scientific, Waltham, MA). Chromatographic conditions: eluent A: 0.1% v/v TFA in water; eluent B: 0.1% v/v TFA in acetonitrile. Solutions in DMSO were eluted at a flow rate of 1.000 ml/min monitored at a wavelength of 206 nm. 0-12 min (linear): 95% A, 5% B to 5% A, 95% B; 12-16 min: 5% A, 95% B; 16-18 min (linear): 5% A, 95% B to 95% A, 5% B.

5

Cloning and plasmid construction.

All DNA fragments were amplified by PCR (Polymerase chain reaction) from other intermediate constructs with the enzyme of Phusion DNA Polymerase (New England Biolabs, Ipswich, MA), or PfuUltra II Fusion HotStart DNA Polymerase (Agilent Technologies, Santa Clara, CA) under S1000 thermal cycler with Dual 48/48 Fast Reaction Module (Bio-Rad Laboratories, Inc., Hercules, CA). DNA fragments were inserted into the vector using T4 DNA Ligase (New England Biolabs, Ipswich, MA) or In-Fusion HD Enzyme Premix (Clontech Laboratories, Inc., Mountain View, CA). All the restriction enzymes used below are purchased from New England Biolabs.

15

PYL-eGFP-Tiam1 construct: Derived from pSV40-VP16-PYL-IRES-Gal4DBD-ABI (Liang et al., 2011, *Sic. Signal.* 4, rs2) by inserting a PYL fragment using EcoRI and AscI sites, a Tiam1-SG linker fragment using AscI and NotI sites, and an eGFP-SG-linker fragment using AscI site.

20

PYL Fragment: PCR amplified by primers

CCGACAGAATTCGCCACCATGACCCAGGACGAGTTTACCCAG (SEQ ID NO:1) and
CCGACAGGCGCGCCGCTGCCGCCGTTTCATAGCCTCAGTAATGCT (SEQ ID NO:2)

25 Tiam1-SG linker fragment: PCR amplified by primers

GCTATGAACGGCGCGCCAAGTGCTGGTGGTAGTGCTGGT (SEQ ID NO:3) and
CTAGAGTCGCGGCCGCTCAGATCTCAGTGTTTCAGTTTC (SEQ ID NO:4)

eGFP-SG-linker fragment: PCR amplified by primers

30 CCGACAGGCGCGCCAGGTGGATCTGGAGGTTTCAGGTGGATCTGGAGGTGTGAGCAAGGGCGAGG
AGCTG (SEQ ID NO:5) and

CCGACAGGCGCGCCCTTGTACAGCTCGTCCATGCC (SEQ ID NO:6)

NES-GID1 construct: generated from NES-ABI (Liang et al., 2011, *Sic. Signal.* 4, rs2) by inserting GID1 fragment using MluI and NotI sites.

5

GID1 fragment: PCR amplified by primers

CCGACAACGCGTGGATCTGGTGGAGCTGCGAGCGATGAAGTTAAT (SEQ ID NO:7) and

CCGACAGCGGCCGCTCAACATTCCGCGTTTACAAACGC (SEQ ID NO:8)

10 EGFP-GAI construct: generated from eGFP-PYL (Liang et al., 2011, *Sic. Signal.* 4, rs2). GAI fragment was inserted by AscI and blunt end ligation into AscI and blunted NotI site on the vector.

GAI fragment: PCR amplified by primers

15 CCGACAGGCGCGCCAGGATCTGGTGGAAAGAGAGATCATCATCATCAT (SEQ ID NO:9) and
CCGACAGGATCCTCAAGGATTAAGGTCGGTGAGCAT (SEQ ID NO:10)

Mammalian cell culture and transfection.

All cells were cultured in DMEM medium (GIBCO, Life Technologies, Carlsbad, CA) supplemented with 10% FBS, 2 mM GlutaMAX (Life Technologies, Carlsbad, CA), 100 U/ml
20 penicillin (Life Technologies, Carlsbad, CA) and 100 µg/ml streptomycin (Life Technologies, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO₂.

EGFP expression experiments: HEK 293T (GFP) cells were seeded 24 hours prior to treatment in 24-well plates at 100 K/well. 10 µM ABA-HP was added to the cells 10 minutes
25 prior to the addition of 10 µM ABA and 10, 50, or 100 µM of H₂O₂. Images were taken for living cells at 5 hours, 7 hours, 10 hours and 24 hours.

NES localization experiments: CHO cells were seeded in 24-well plates at 50 K/well with coverslip for 24 hours. 0.4 µg NES-ABI and 0.2 µg eGFP-PYL (Fig 2a, ii) of DNA plasmid were used for each well, with 30 µL of Opti-MEM (Life Technologies, Carlsbad, CA) and 1.8 µL of
30 PEI. After incubation at room temperature for 15 min, the mixture was added to the cells and

cultured for 24 hours. Then, 10 μ M ABA-HP was added to the cells 10 minutes prior to the addition of 10 μ M ABA and 100 μ M of H₂O₂, or 10 μ M GA-HP was added to the cells 10 minutes prior to the addition of 10 μ M GA-AM and 100 μ M of H₂O₂. Slides were made 30 minutes after addition of compounds and images were taken the next day.

5 Ruffle generation experiment: CHO were seeded in 24-well plates at 50 K/well with coverslip for 24 hours. 0.1 μ g PYL-eGFP-Tiam1 and 0.1 μ g myr-ABI (Fig 2a, iii) of DNA plasmid were used for each well, with 30 μ L of Opti-MEM (Life Technologies, Carlsbad, CA) and 1.8 μ L PEI. After incubation at room temperature for 15 min, the mixture was added to the cells and cultured for 24 hours. Then, 10 μ M ABA-HP was added to the cells 10 minutes prior to
10 the addition of 10 μ M ABA and 100 μ M of H₂O₂. Slides were made 30 minutes after addition of compounds and images were taken the next day.

Fluorescence microscopy.

15 An Axio Observer D1 fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany) outfitted with HBO 100 microscopy illumination system (Carl Zeiss AG, Oberkochen, Germany; GFP: excitation 470/40 and emission 525/50) was used to analyze GFP expression and NES localization. A 20 $\times$ objective was used to analyze GFP expression. A 63 $\times$ objective was used for NES localization.

20 A LSM 510 META Confocal Microscope (Carl Zeiss AG, Oberkochen, Germany) with 40 $\times$ and 63 $\times$ oil-immersion objectives to analyze ruffle formation. Fluorophore channels in all experiments were adjusted to the same intensity ranges. Acquisition times ranged from 100 to 1000 ms.

Example 2

25 HPLC analysis: Reversed-phase HPLC was performed on an ACCLAIM 120 (4.6 $\times$ 100 mm) C18 column (Thermo Fisher Scientific, Waltham, MA) with an ULTIMATE 3000 pump system, including a Variable Wavelength Detector 3100, Degasser 1210, and Autosampler SPS 3000 (Thermo Fisher Scientific, Waltham, MA). A mixture of water and acetonitrile containing 0.1 % TFA was used as the eluent. Absorbance at 250 nm was used to monitor the elution of the
30 molecules. The method used an increase in acetonitrile from 5% to 95 % over 15 minutes to elute the molecules at a flow rate of 0.7 mL/min. The peaks for the molecules were integrated by using

Chromeleon software (Thermo Fisher Scientific, Waltham, MA). The molar absorptivity of both free and caged ABA at 250 nm were measured, which was used to calculate the concentration of each species from the intensity of absorbance at 250 nm. The relative concentration of each compound was used to calculate the percent concentration of free ABA relative to the concentration of total ABA species (both caged and uncaged).

Cell culture and transfection: CHO cells and HEK 293T EGFP reporter cells (provided by Dr. Gerald R. Crabtree) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Life Technologies, Carlsbad, CA) with 10 % FBS (Atlanta Biologicals, Inc., Norcross, GA), 1× GlutaMAX (GIBCO, Life Technologies, Carlsbad, CA), and 1× penicillin/streptomycin (Pen/Strep; GIBCO, Life Technologies, Carlsbad, CA). 15,000-50,000 cells were plated in a 24-well or 8-well plate for 24 hours before transfection. DNA constructs (0.1 µg-0.5 µg) were added to 50× (v/w) Opti-MEM (GIBCO, Life Technologies, Carlsbad, CA), and then 3× (v/w) PEI (Polysciences, Inc., Warrington, PA) was mixed with the DNA. The mixture was incubated for 20 minutes at room temperature before adding it to cell cultures. The cells were grown for one day after transfection before experiments were performed.

DNA plasmid construction: Construction of the 5FL, 5IG, SV-VPiGA, NES-ABI, GFP-PYL, and myr-ABI plasmids has been described previously. (Liang et al., 2011, *Sic. Signal.* 4, rs2) The PYL-eGFP-Tiam1 construct was constructed as described in Example 1.

Photo-irradiation: Irradiation at 365 nm was performed with an Axio Observer (Carl Zeiss AG, Oberkochen, Germany) microscope with an HBO103 W/2 mercury arc lamp (Carl Zeiss AG, Oberkochen, Germany). Irradiation was performed by using a DAPI filter (Carl Zeiss AG, Oberkochen, Germany), set with peak excitation at 365 nm (power density: 23 mW cm²) and a spectral width of 50 nm. No objective lens was used for whole-well irradiation, which created an area of illumination that nearly completely covered one well of a 24-well plate. Light was transmitted through the bottom of the well of polystyrene plate. Irradiation at 405 nm was performed by using an adjustable focus violet purple laser pointer (LazerPoint SKU 0733579), with an excitation wavelength of 405 nm and 1000 mW intensity positioned 8 cm above the bottom of either a 96-well or 24-well plate and irradiated through the polystyrene lid. All samples for HPLC analysis were irradiated in DMSO to prevent evaporation of solvent and

changes in sample concentration. Irradiation of cell cultures was performed in 24-well plates containing culture medium (500 μ L).

Luciferase assay: Cells from 24-well plates were washed with PBS and lysed with Reporter lysis buffer (100 μ L; Promega Corp., Madison, WI) by incubation and gentle shaking at room temperature for 10 minutes after a freeze/thaw cycle. Cell lysates were centrifuged at 15,000 rpm in an Eppendorf 5424 Centrifuge with lysate used for the luciferase assay (25 μ L). Luciferase assay reagent (100 μ L, 5 mg luciferin (Gold Biotechnology, Inc., St. Louis, MO) and coenzyme A (7 mg; Sigma-Aldrich, St. Louis, MO) in Luciferase Assay Buffer (33 mL, 20 mM tricine, 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5 \text{H}_2\text{O}$, 2.67 mM MgSO_4 , 0.1 mM EDTA, 33.3 mM dithiothreitol, and 0.53 mM ATP in water) was added to lysates. Luciferase assay reagent was added through the auto-injector of a GLOMAX-Multi Detection System (Promega Corp., Madison, WI), and the signal was detected by the instrument with a 1.5 s delay and 0.5 s integration time. All experiments were conducted in triplicate.

Slide preparation: Cells were grown on glass coverslips in 24-well plates. The coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde (PFA, 300 μ L, prepared in PBS) at room temperature for 20 min. The cells were then washed twice with PBS and incubated with $1 \times$ DAPI in the dark at the room temperature for 5 min. After a final wash with PBS, the coverslips were mounted on a glass slide with VECTASHIELD (Vector Laboratories, Inc., Burlingame, CA) mounting media and allowed to stand for two hours in the dark before imaging.

Fluorescence microscopy imaging: Slides were imaged with an Axio Observer (Carl Zeiss AG, Oberkochen, Germany) microscope or with an LSM 510 Meta confocal microscope (Carl Zeiss AG, Oberkochen, Germany) mounted on an AxioObserver inverted microscope with a $63\times$ oil objective. Images were taken with DAPI and GFP channels.

Live cell confocal microscopy irradiation and imaging: EGFP fluorescence of CHO cells was detected with an LSM 510 Meta confocal mounted on an AxioObserver inverted microscope (Carl Zeiss AG, Oberkochen, Germany). ABA-DEACM was uncaged by using a 405 nm UV laser (25 mW) set to 25 % power for approximately three seconds. To image, fluorescence was excited with the 488 nm line of an argon laser (30 mW) with laser power attenuated to 50 %. EGFP emission was collected with a FITC filter. Live cells were plated in 8-well coverslip-bottom culture chambers in medium (200 μ L) and maintained at 37°C with an objective lens

heater (Bioprotechs Inc., Butler, PA). Culture medium was exchanged with OptiMEM (GIBCO, Life Technologies, Carlsbad, CA) with caged ABA or no drug prior to imaging. Images were acquired every 10 to 20 seconds in different experiments with a 63×5/1.2 NA water objective.

Statistical analysis of cell population: Cell were categorized as displaying nuclear export of EGFP when the fluorescent intensity of the nucleus was less than 60 % of the intensity of the cytoplasm. Cells were categorized as ruffled when they displayed broad extensions identifiable as lamellipodia or filopodia from the GFP fluorescence from membrane-localized EGFP-PYL-Tiam1. Cells were counted from three separate experiments, with $N > 50$ for each experiment.

Image analysis of fluorescence intensity in nuclear export experiments: Generated images were analyzed for fluorescence intensity by using Slide Book v.6 software (Intelligent Imaging Innovations, Inc., Denver, CO). Equal sized regions of interest were analyzed from the cytoplasm and the nucleus to compare fluorescent intensity of EGFP in three cells for each condition from images taken every 20 seconds for a duration of 20 minutes.

Example 3

Mammalian Cell Culture: HEK 293 EGFP reporter cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Life Technologies, Carlsbad, CA) with 10% Fetal Bovine Serum (FBS, GIBCO, Life Technologies, Carlsbad, CA), 1× Glutamate (100× from GIBCO, Life Technologies, Carlsbad, CA), and 1× Penicillin/Streptomycin (Pen/Strep, 100× from GIBCO, Life Technologies, Carlsbad, CA). Cells were plated with the starting concentration of 200,000 cells per well in a 24-well plate (Greiner Bio-One GmbH; Frickenhausen, Germany) for a day, and then were treated with either 10 μM of ABA-Fe and 20 μM of DFO, 10 μM of ABA-Fe only, or 10 μM of ABA-Fe and 100 μM of Fe^{2+} aqueous solution.

Imagines for green fluorescence were taken for living cells every two hours after adding in those molecules by microscopy illumination system (Axio Observer; Carl Zeiss AG, Oberkochen, Germany). Each type of the experiment was carried out as duplex.

Reverse-phase HPLC Analysis

Chemical stability of ABA-Fe molecules: 100 μ M ABA-Fe (in 50% HEPES/DMSO buffer, pH=7.4) was incubated at 37°C for one hour before HPLC detection. The percentages for molecule cleavage were quantified by the peak area of ABA out of the total peak area.

ABA-Fe reactivity and selectivity towards Fe^{2+} : 100 μ M ABA-Fe with 1 mM Fe^{2+} (in 50% HEPES/DMSO buffer, pH=7.4), and 100 μ M ABA-Fe with 1 mM Fe^{3+} (in 50% HEPES/DMSO buffer, pH=7.4) were incubated at 37°C for one hour before HPLC detection. The percentages for molecule cleavage were quantified by the peak area of ABA out of the total peak area.

HPLC chromatograms were acquired using an ULTIMATE 3000 LC System (Thermo Fisher Scientific, Waltham, MA) with ACCLAIM 120 Å, C18, 3 μ m analytical (4.6 $\times$ 100 mm) column (Thermo Fisher Scientific, Waltham, MA). Chromatographic conditions: eluent A: 0.1% v/v TFA in water; eluent B: 0.1% v/v TFA in acetonitrile. ABA in ethanol or ABA-HP in DMSO was eluted at a flow rate of 0.750 ml/min monitored at a wavelength of 260 nm. 0-12 min (linear): 95% A, 5% B to 5% A, 95% B; 12-15 min: 5% A, 95% B; 15-17 min (linear): 5% A, 95% B to 95% A, 5% B. Generation of ABA was quantified by the peak area of ABA out of the total peak area.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are

approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A composition comprising:

a first modular component comprising a first target molecule coupled to a first dimerizing moiety; and

5 a second modular component comprising a second target molecule coupled to a second dimerizing moiety;

wherein the first dimerizing moiety dimerizes with the second dimerizing moiety when the first dimerizing moiety binds a chemical induced proximity (CIP) inducer.

10 2. The composition of claim 1 further comprising an activatable inactive chemical-induced proximity inducer.

3. The composition of claim 2 further comprising a compound that converts the activatable inactive chemical-induced proximity inducer to an active chemical-induced proximity inducer.

15 4. The composition of claim 3 wherein the first dimerizing moiety is bound to the active chemical-induced proximity inducer and dimerized with the second dimerizing moiety.

5. The composition of any one of claims 1-4 wherein the chemical-induced proximity inducer comprises abscisic acid (ABA) or gibberellic acid (GA).

20 6. The composition of any one of claims 1-5 wherein the compound that converts the activatable inactive chemical-induced proximity inducer to an active chemical-induced proximity inducer comprises H_2O_2 or Fe^{2+} .

25 7. A cell comprising:

the composition of any one of claims 1-6; and

a polynucleotide whose expression is modulated by a complex comprising the first modular component dimerized to the second modular component.

8. The cell of claim 7 wherein the polynucleotide comprises a polynucleotide endogenous to the cell.

9. The cell of claim 7 wherein the polynucleotide comprises a polynucleotide exogenous to the cell.

10. A complex comprising:

a first modular component comprising:

a first target molecule coupled to a first dimerizing moiety; and

an active chemical-induced proximity inducer bound to the first dimerizing moiety; and

a second modular component comprising:

a second dimerizing moiety dimerized to the first dimerizing moiety of the first modular component; and

a second target molecule coupled to the second dimerizing moiety.

11. A method comprising:

introducing the composition of claim 1 into a cell;

introducing a activatable inactive chemical-induced proximity inducer into the cell; and

contacting the cell with a compound that activates the activatable inactive chemical-induced proximity inducer.

12. The method of claim 11 wherein the cell further comprises a polynucleotide whose expression is modulated by a complex comprising the first modular component dimerized to the second modular component.

13. The method of claim 12 wherein the polynucleotide comprises a polynucleotide endogenous to the cell.

14. The method of claim 12 wherein the polynucleotide comprises a polynucleotide exogenous to the cell.

FIG. 1

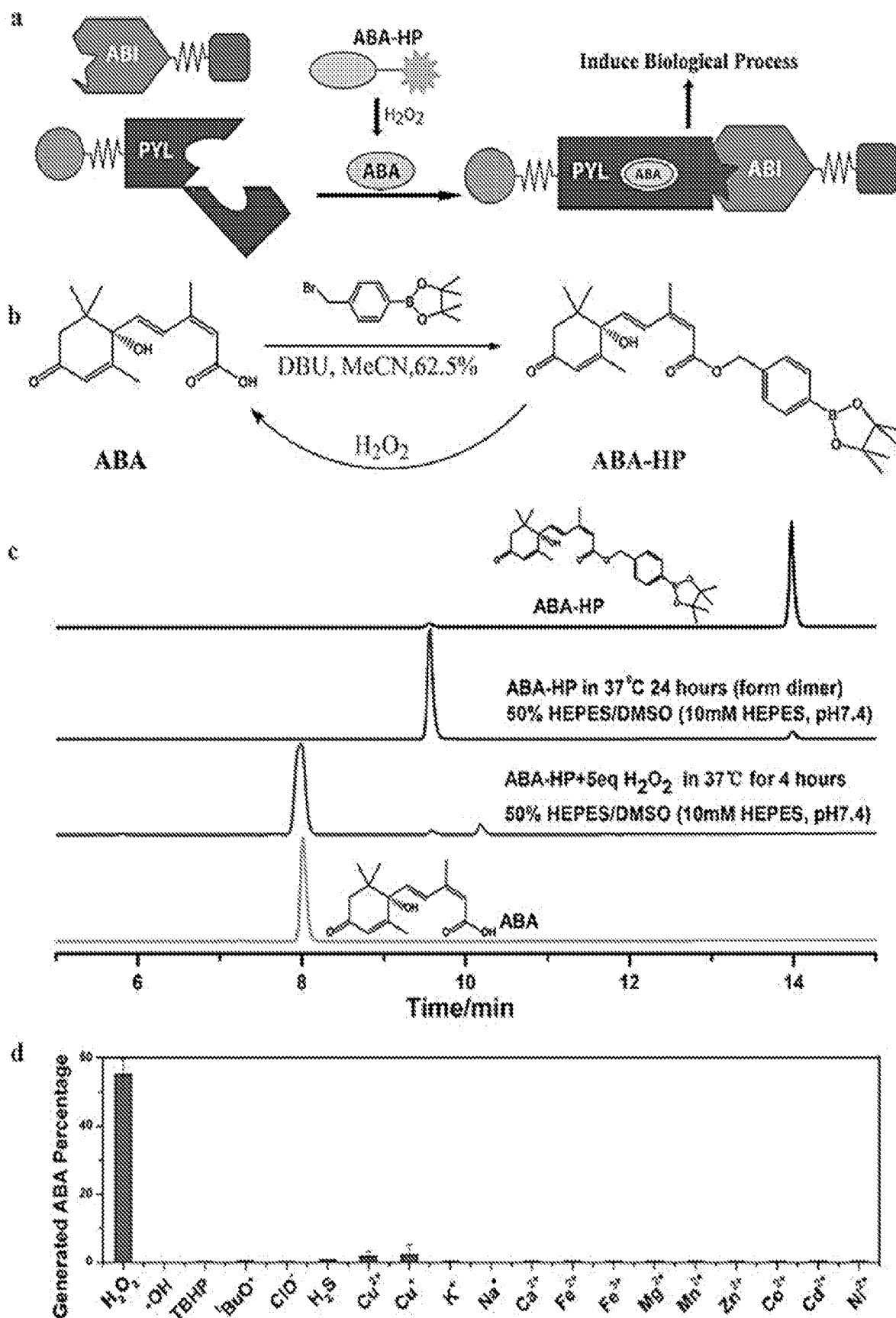


FIG. 2

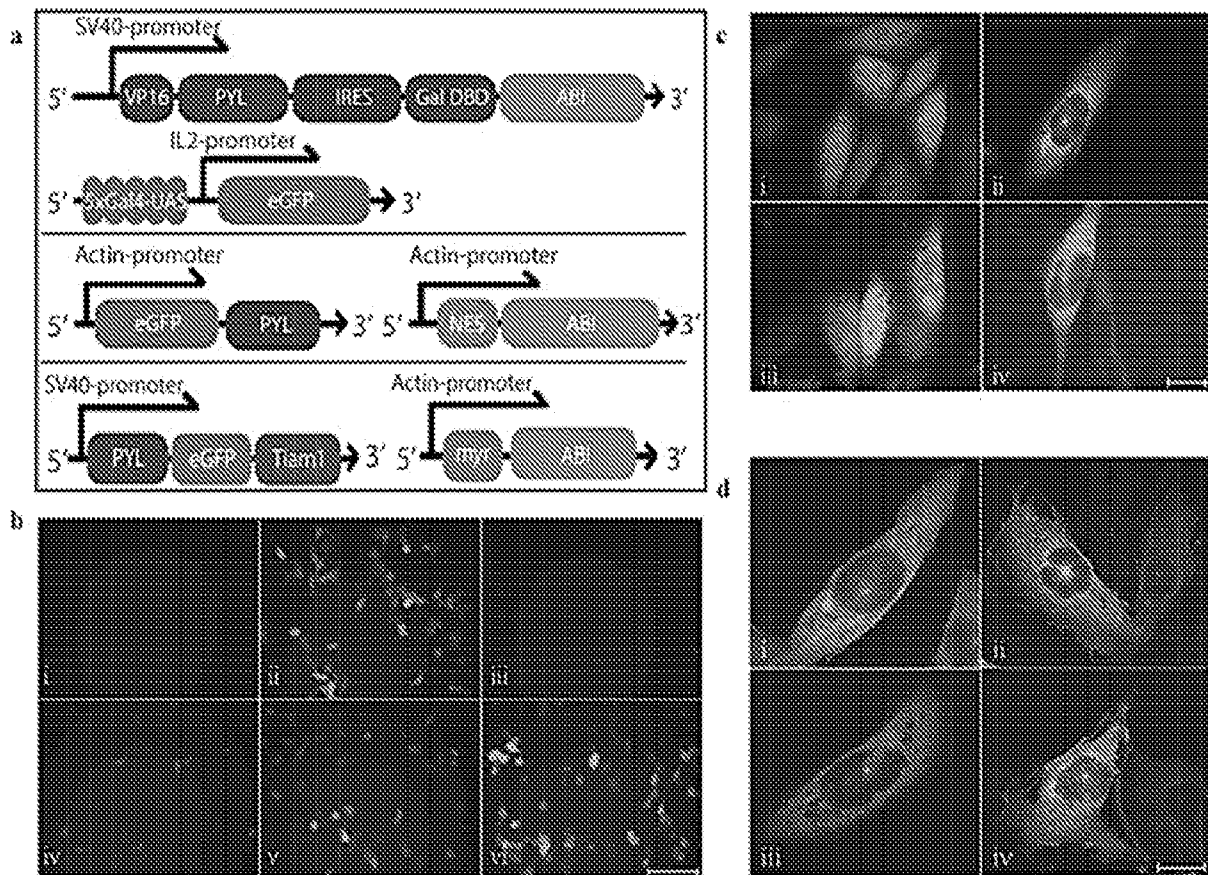


FIG. 3

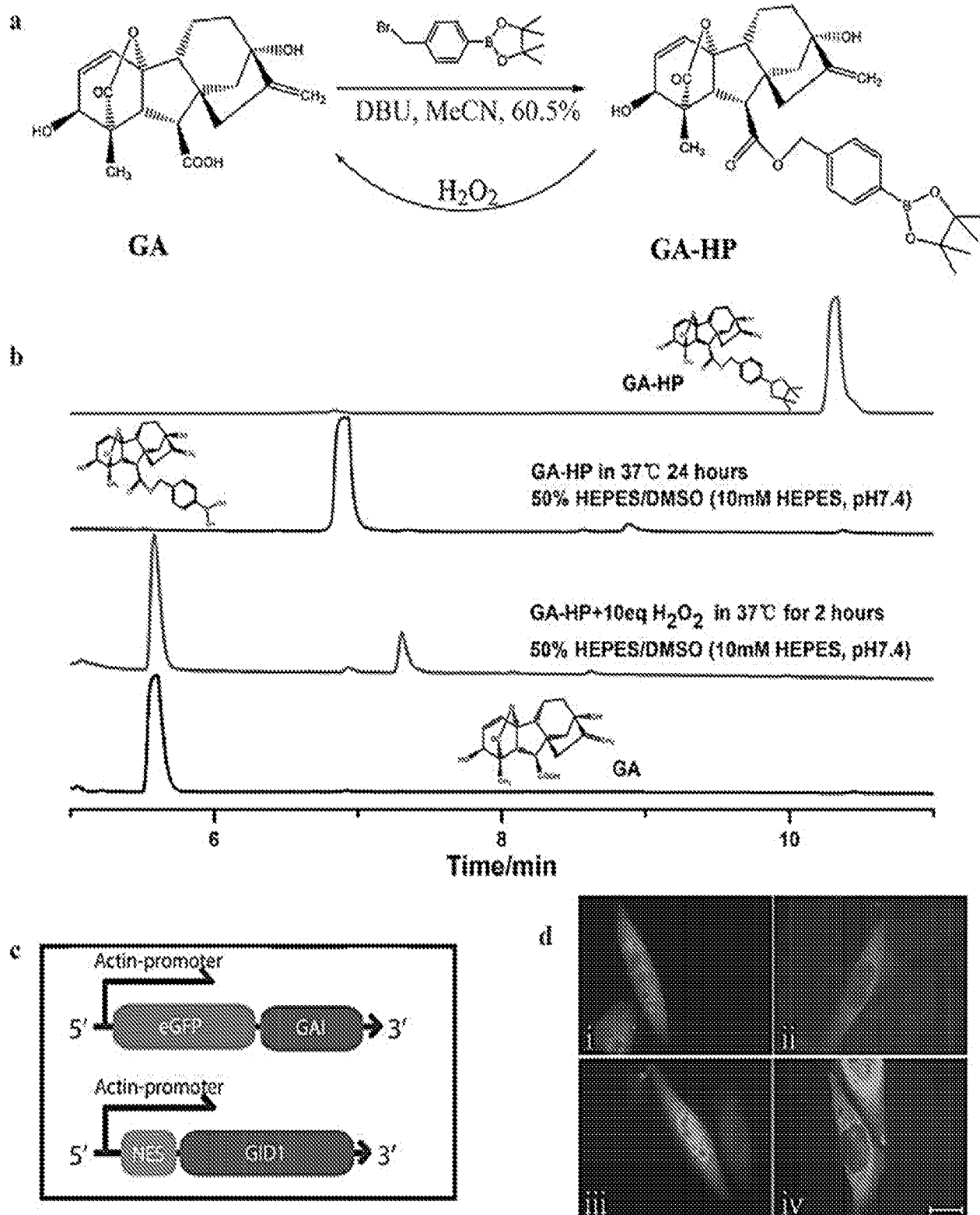


FIG. 4

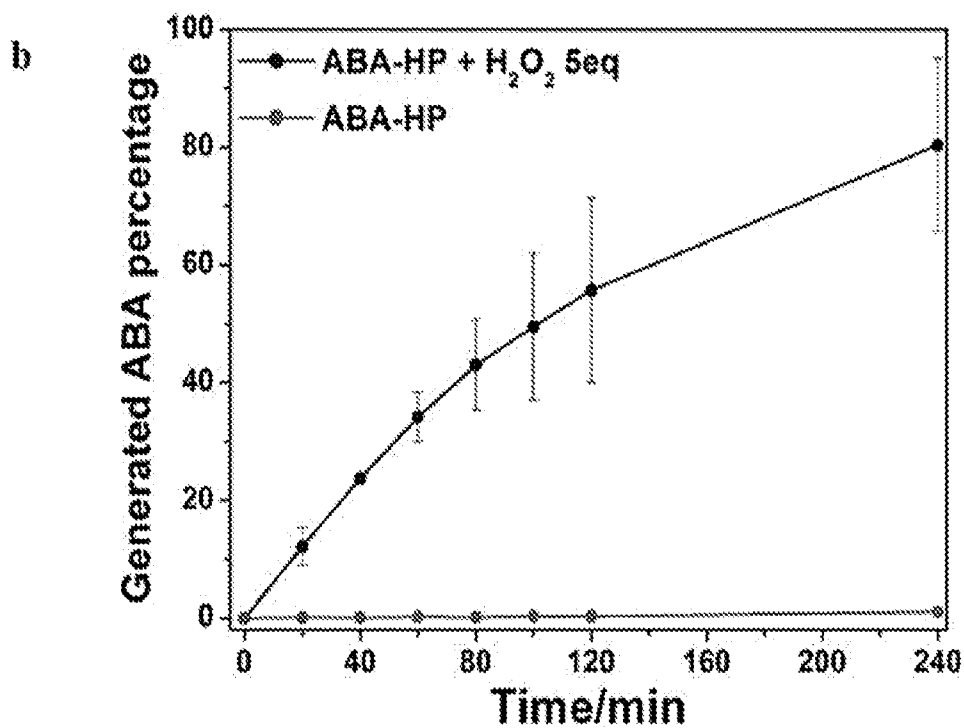
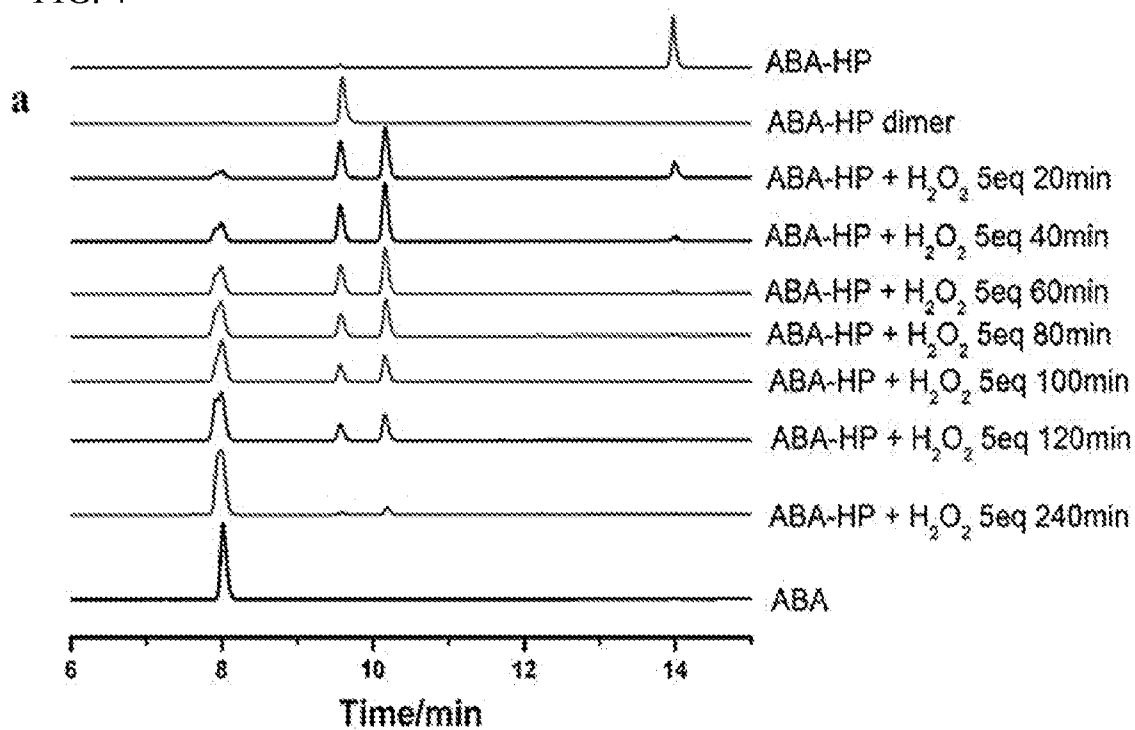


FIG. 5

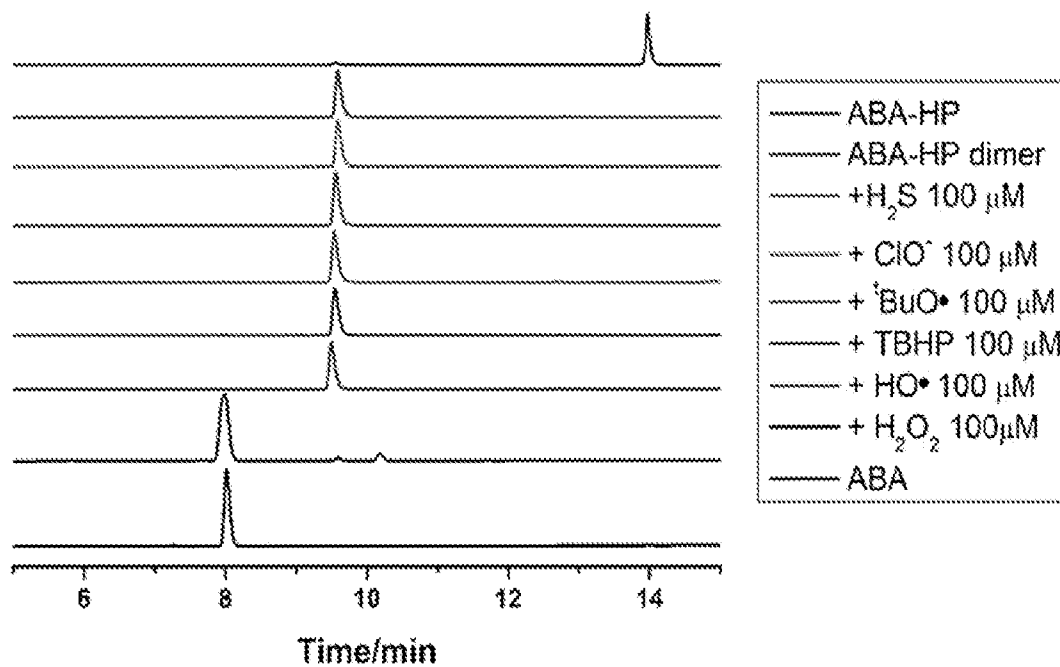


FIG. 6

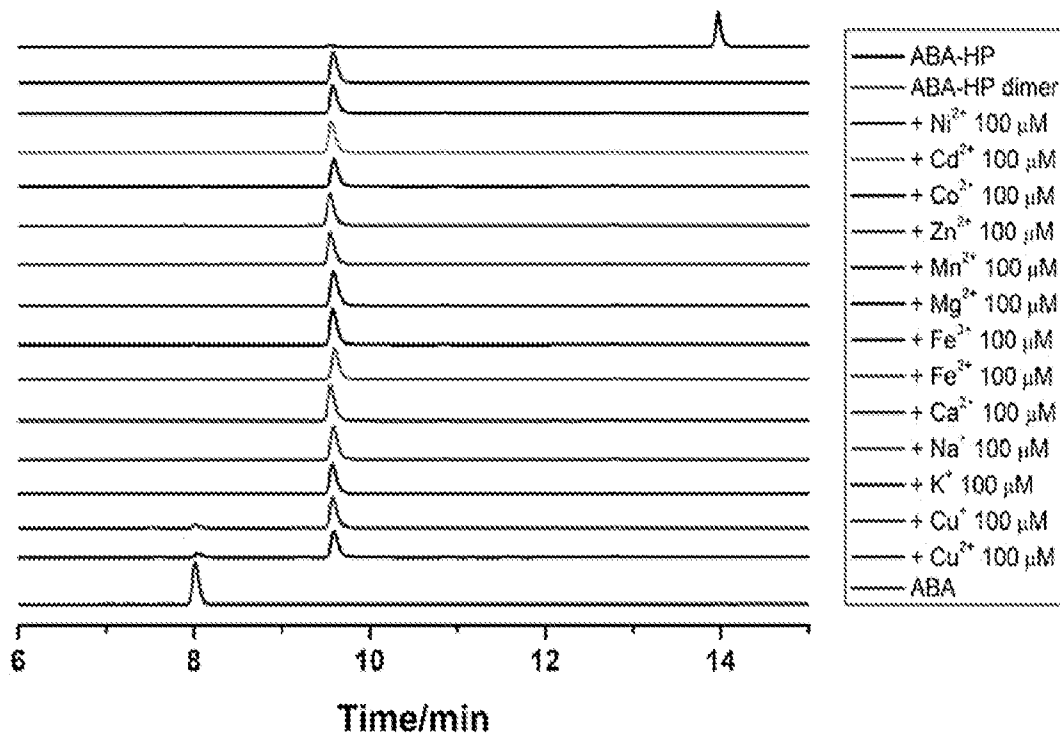
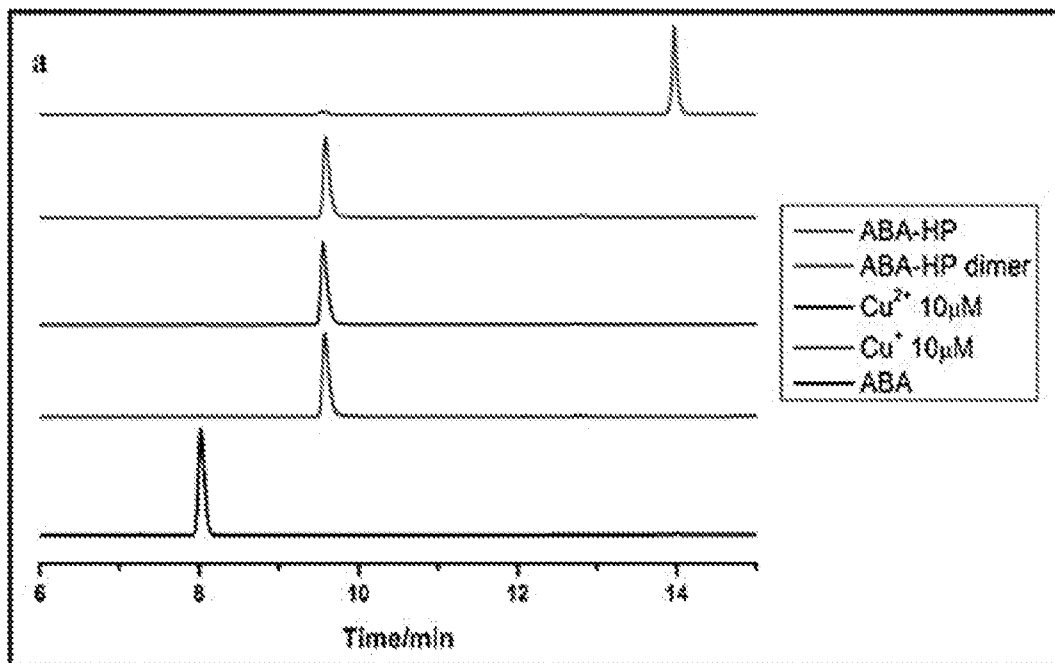


FIG. 7



b

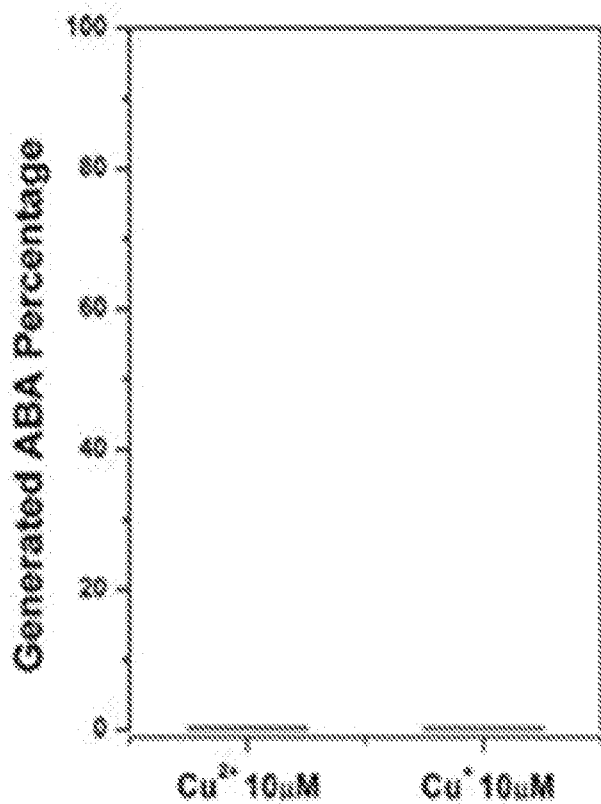


FIG. 8

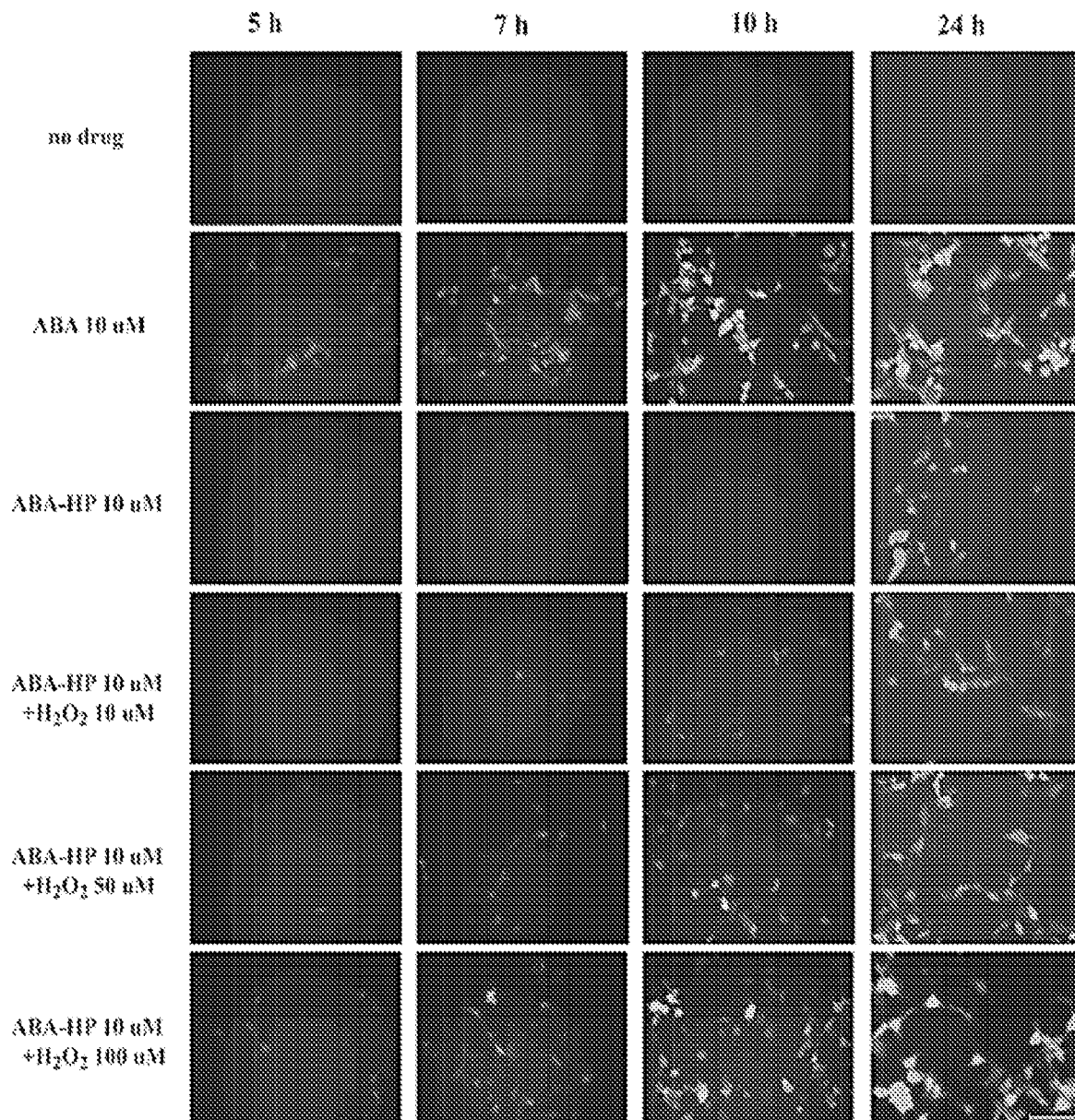


FIG. 9

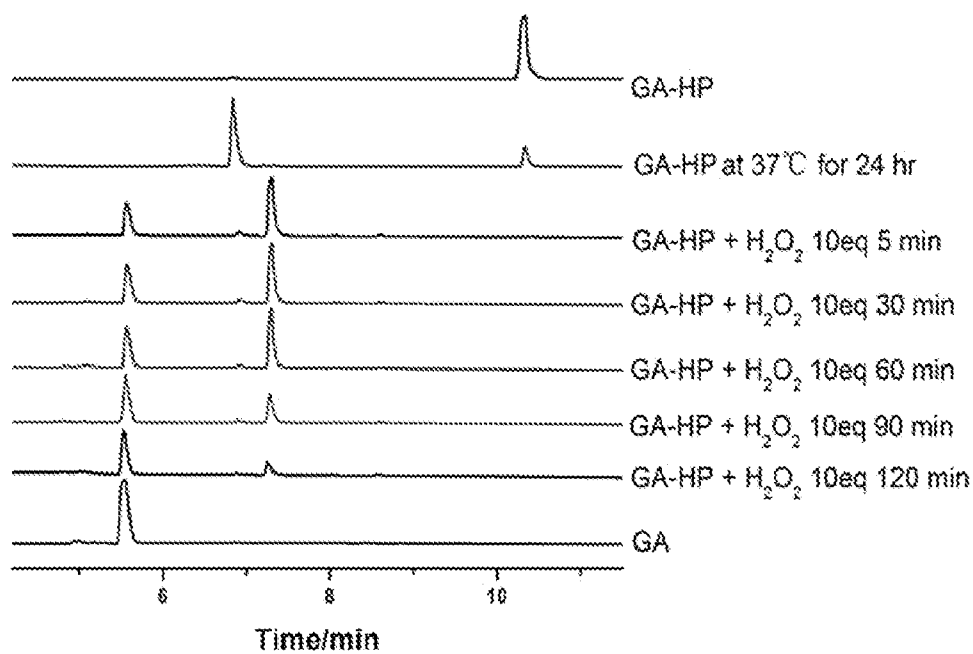


FIG. 10

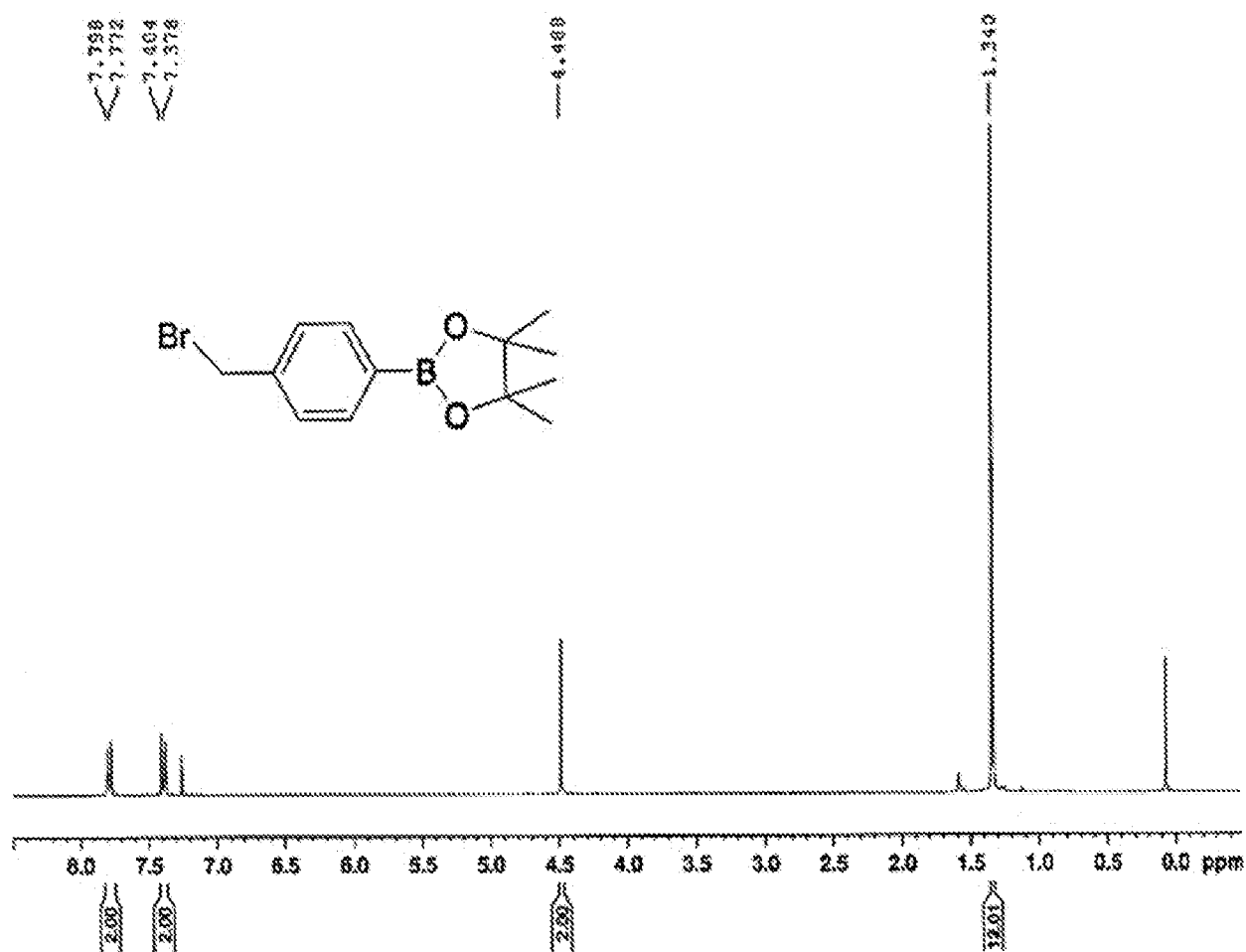


FIG. 11

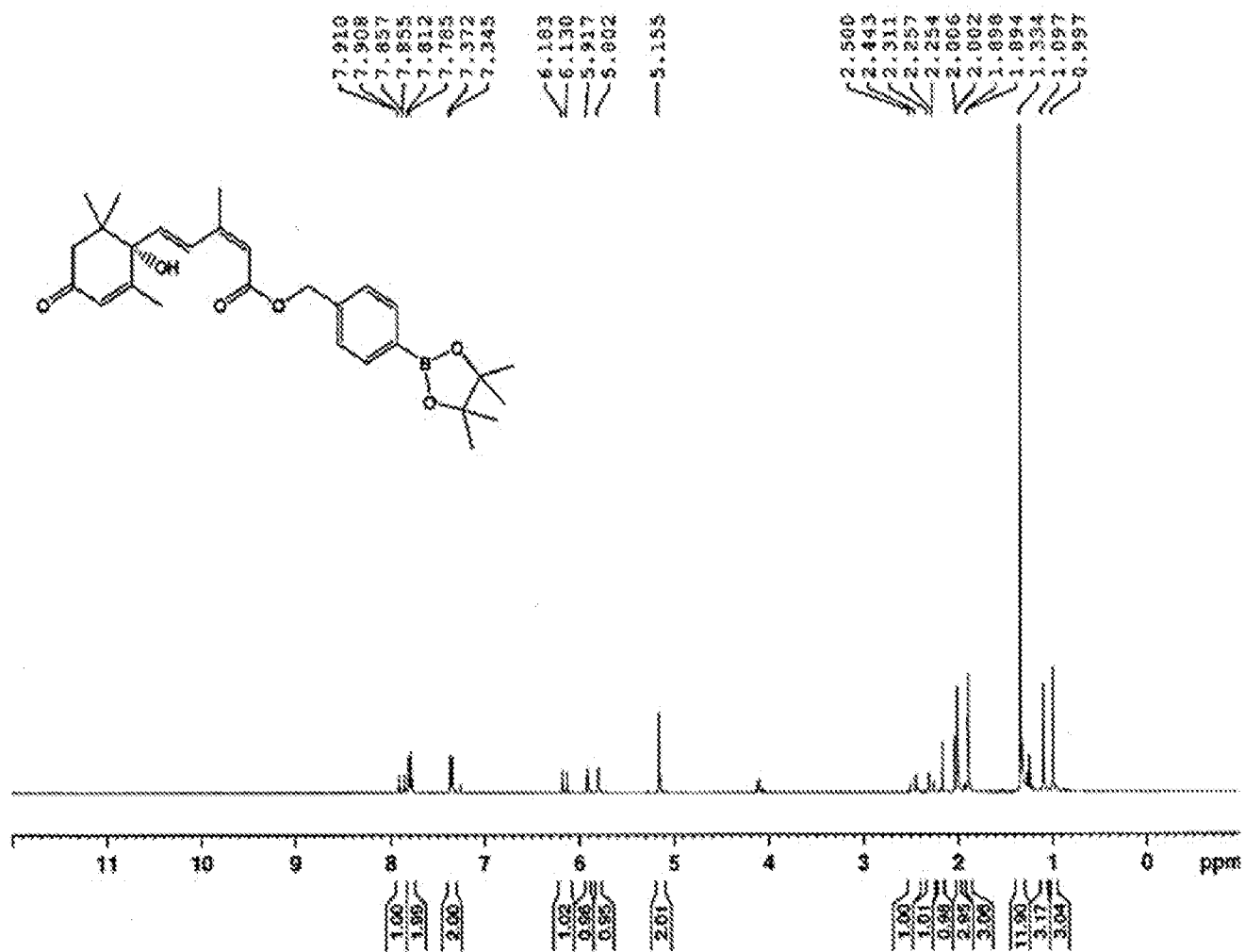


FIG. 12

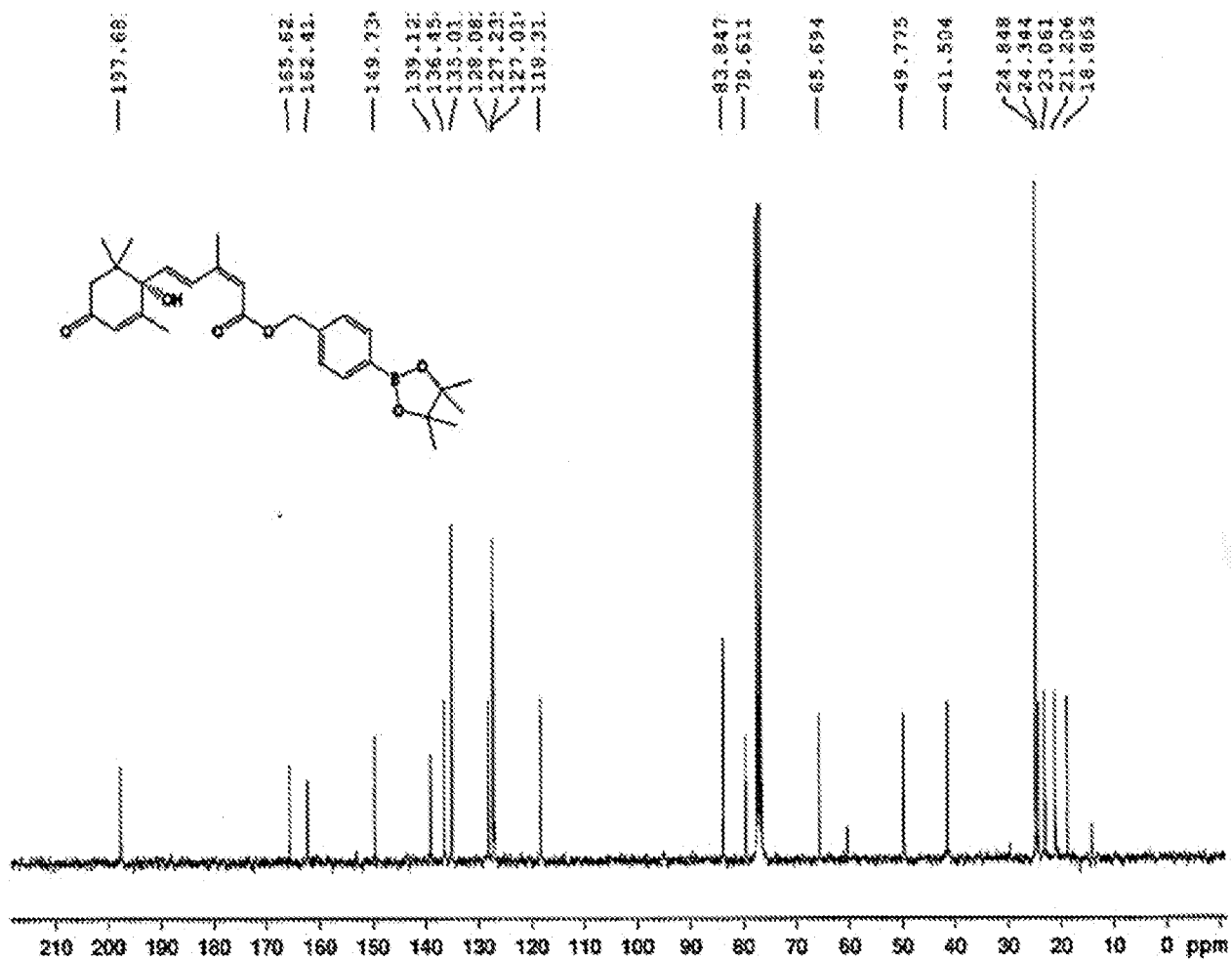


FIG. 13

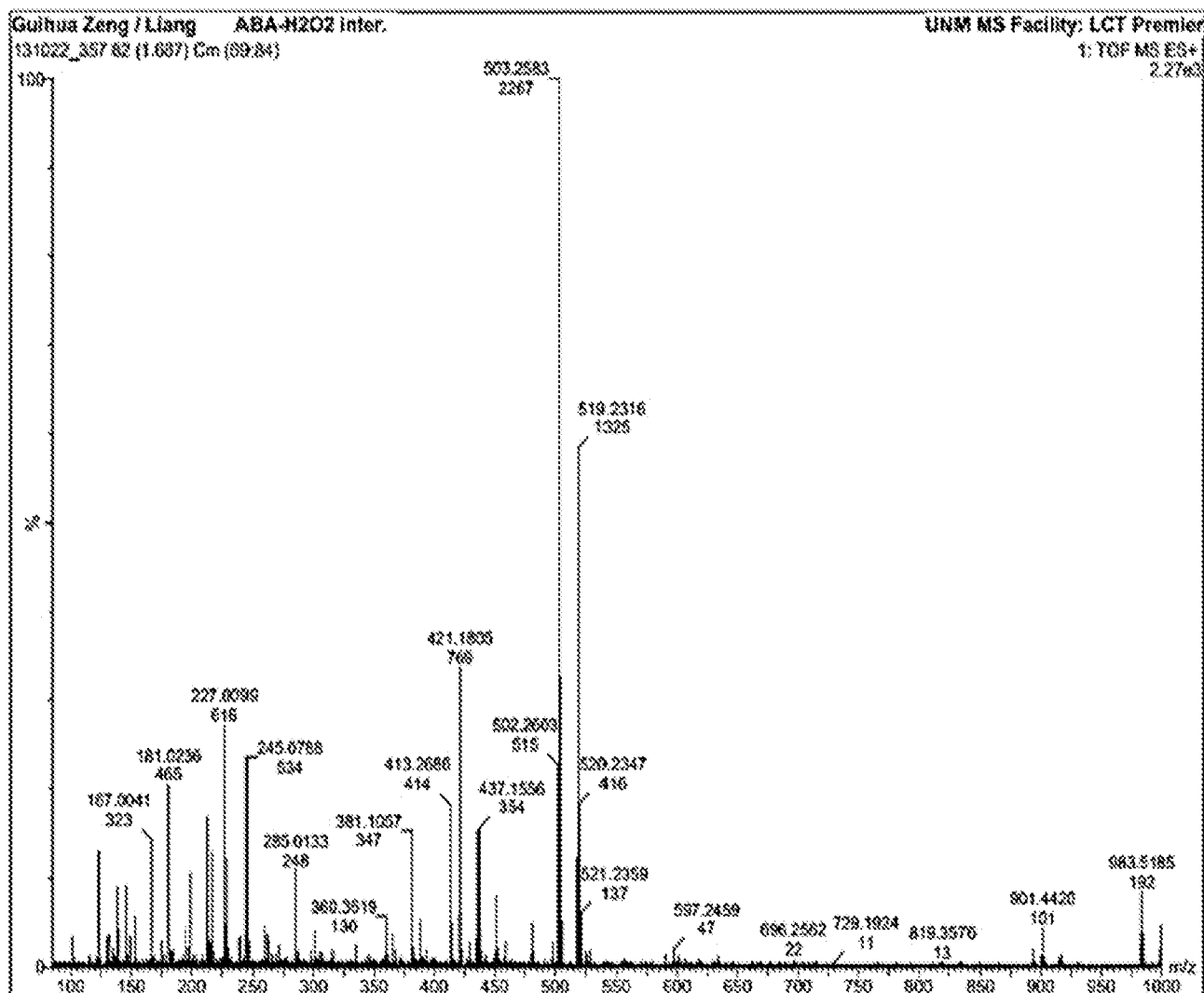


FIG. 14

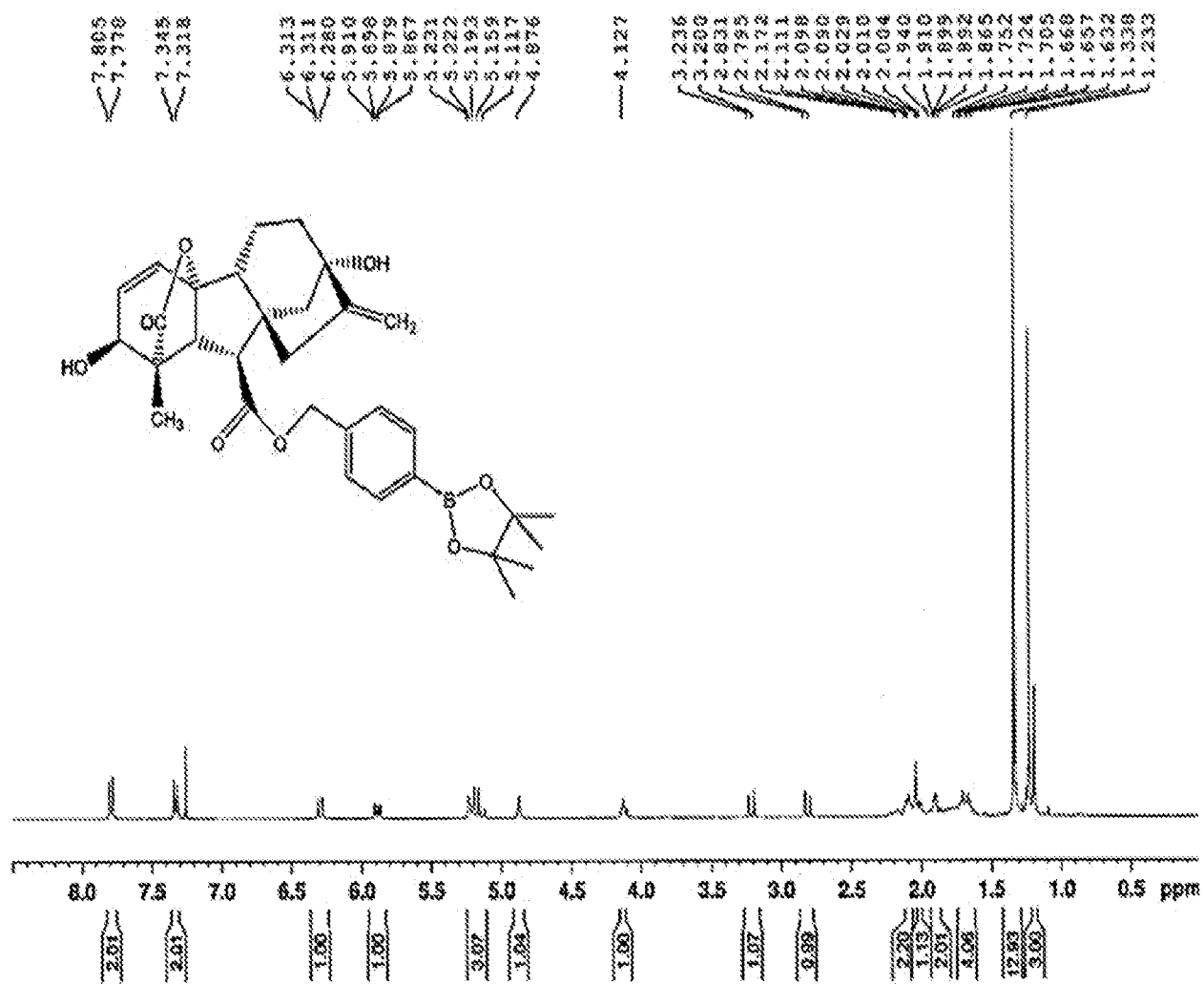


FIG. 15

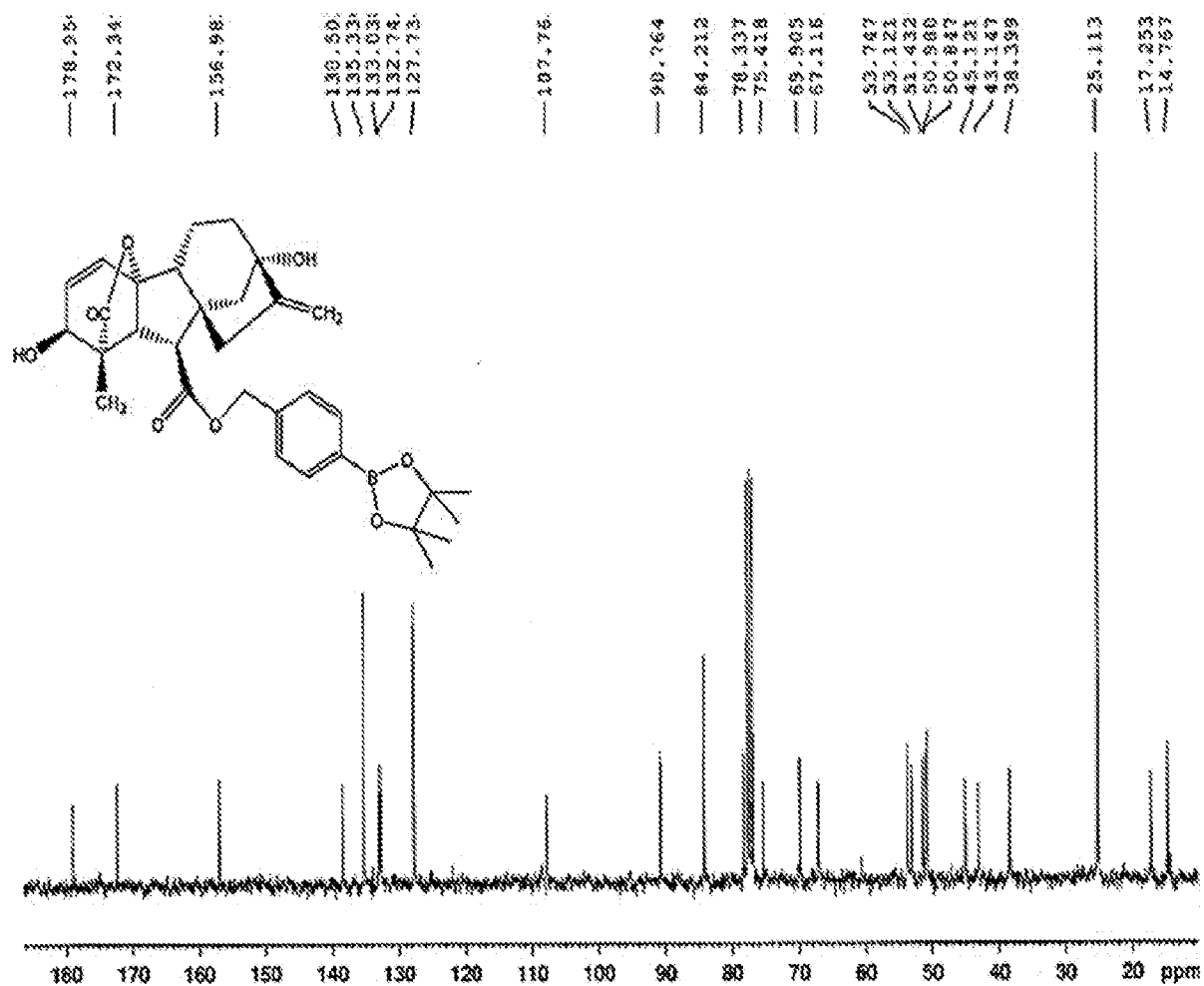


FIG. 16

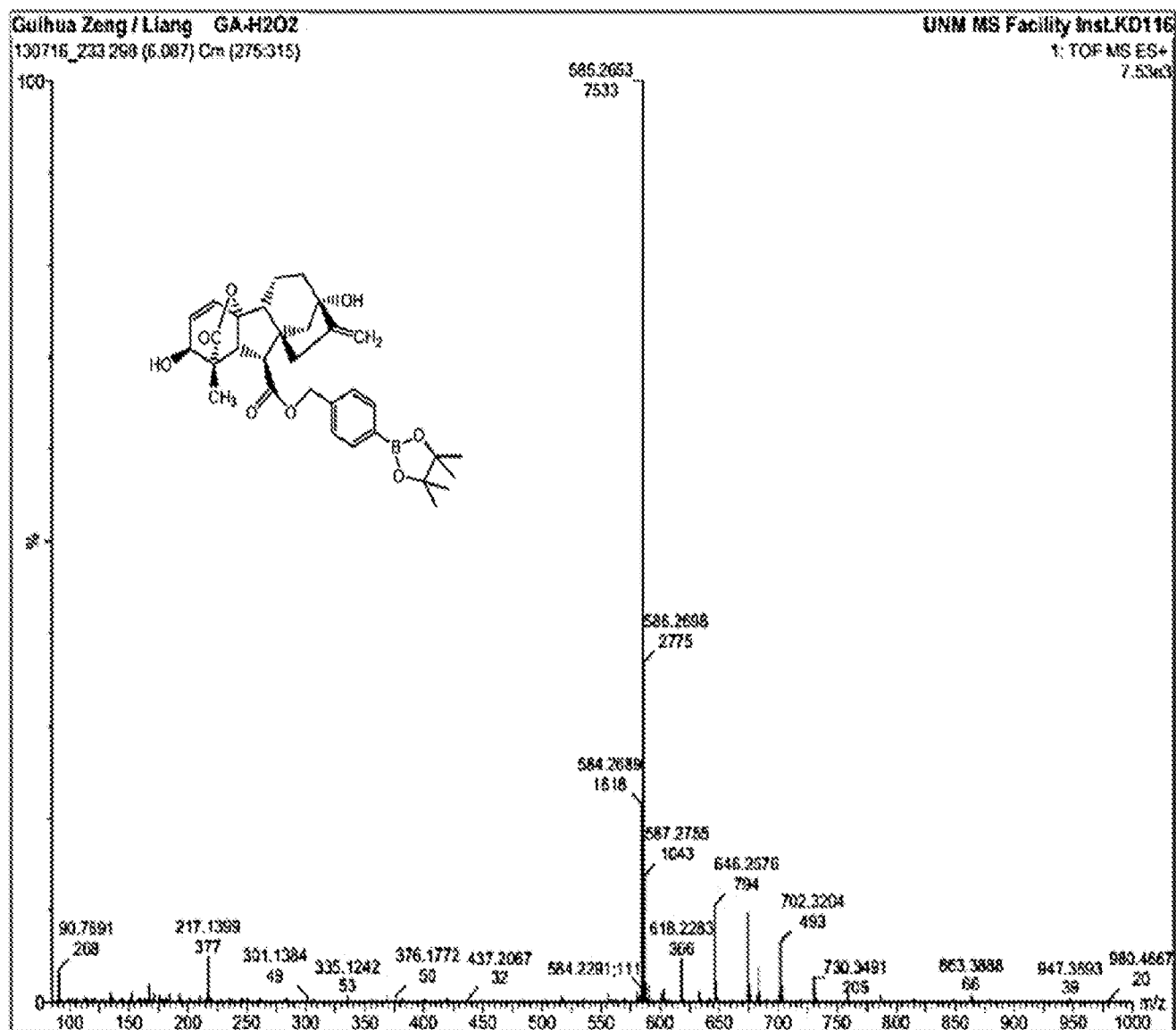


FIG. 17

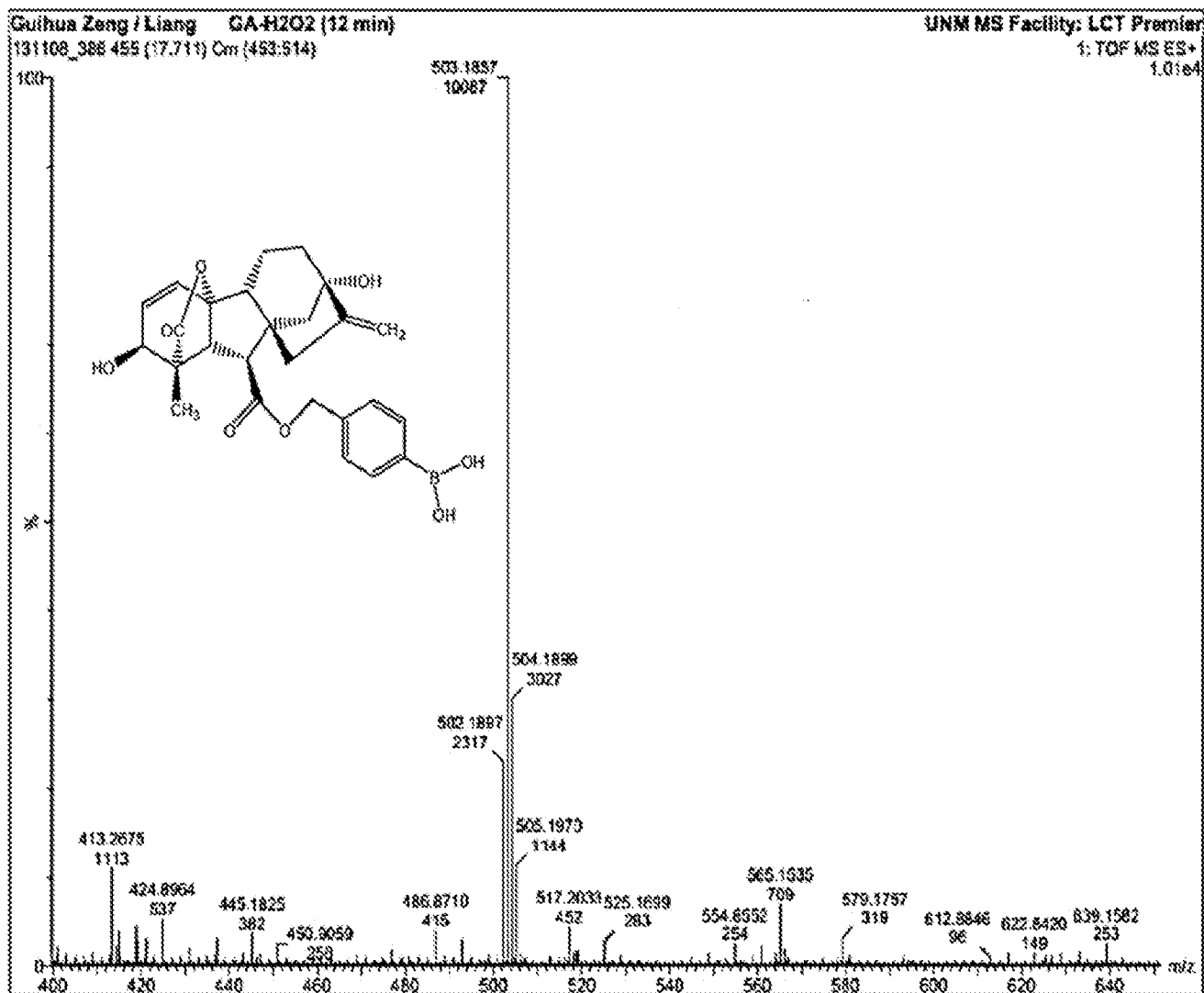


FIG. 18

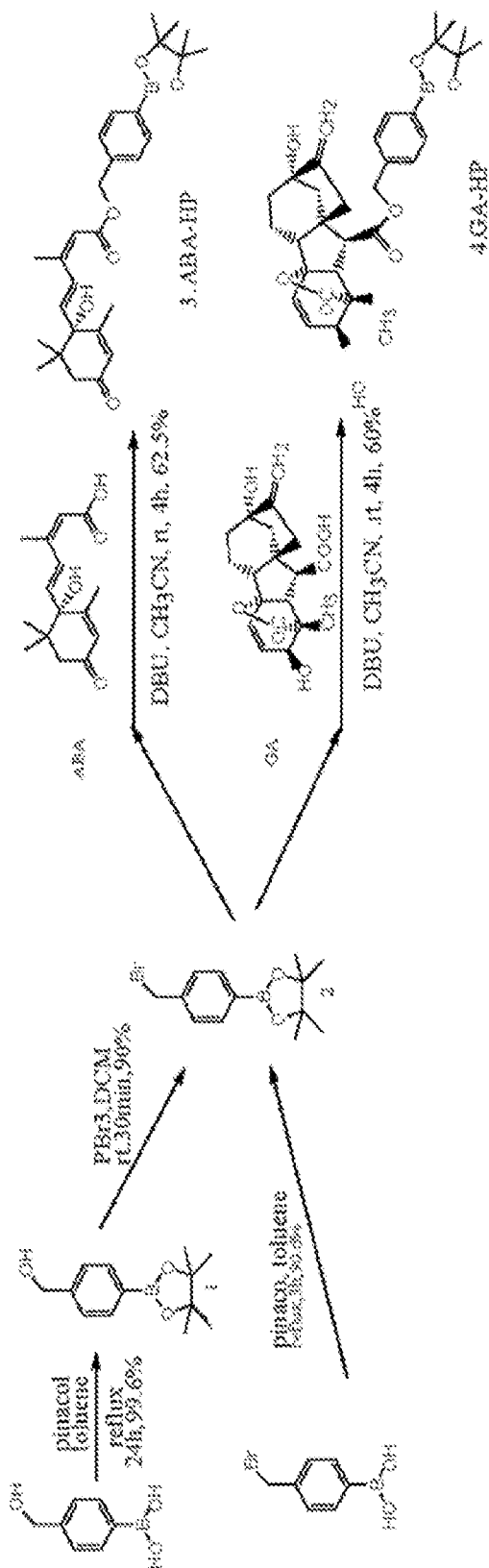


FIG. 19

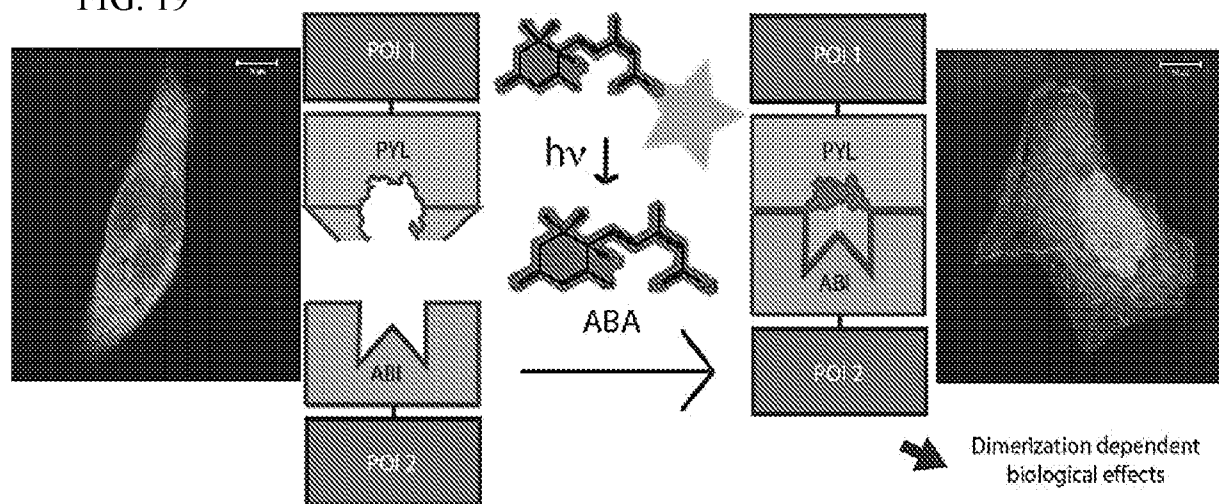


FIG. 20

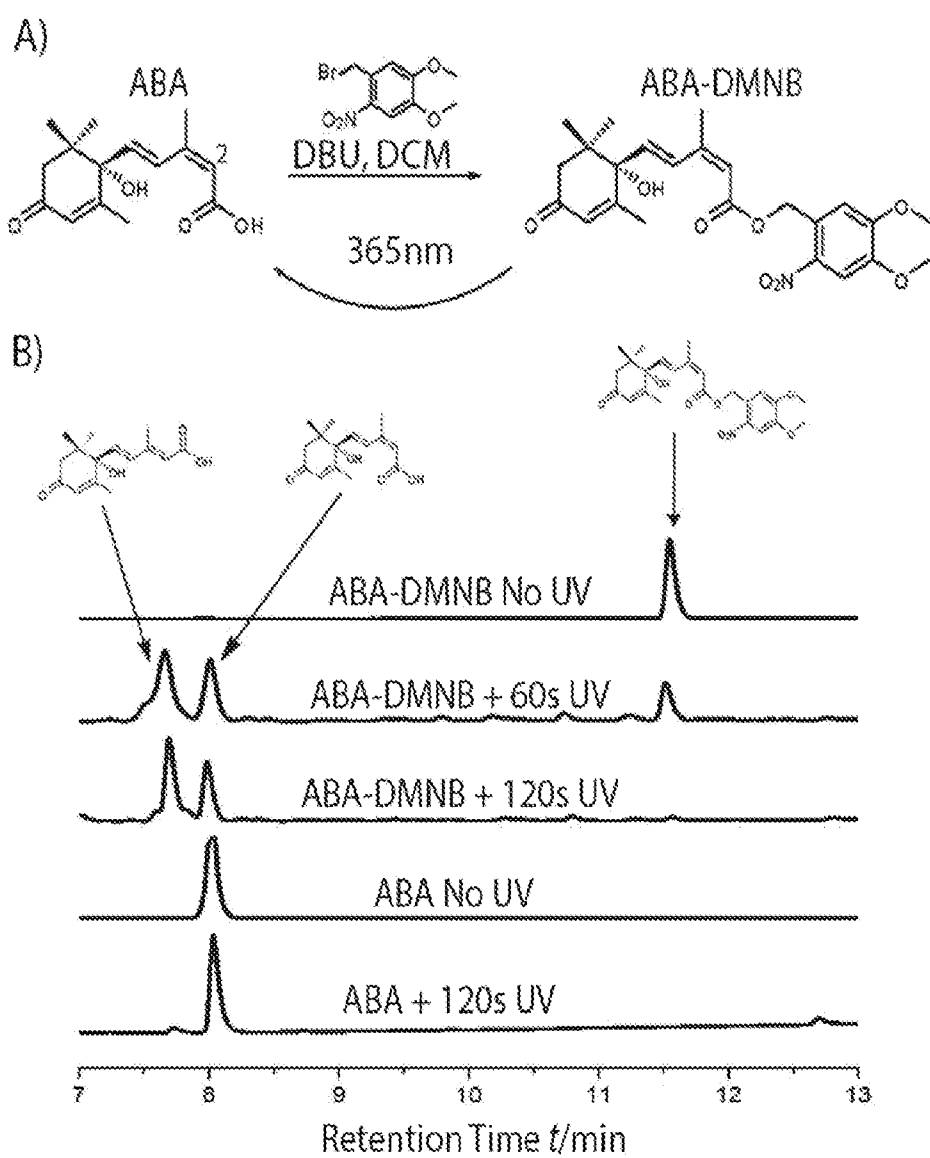


FIG. 21

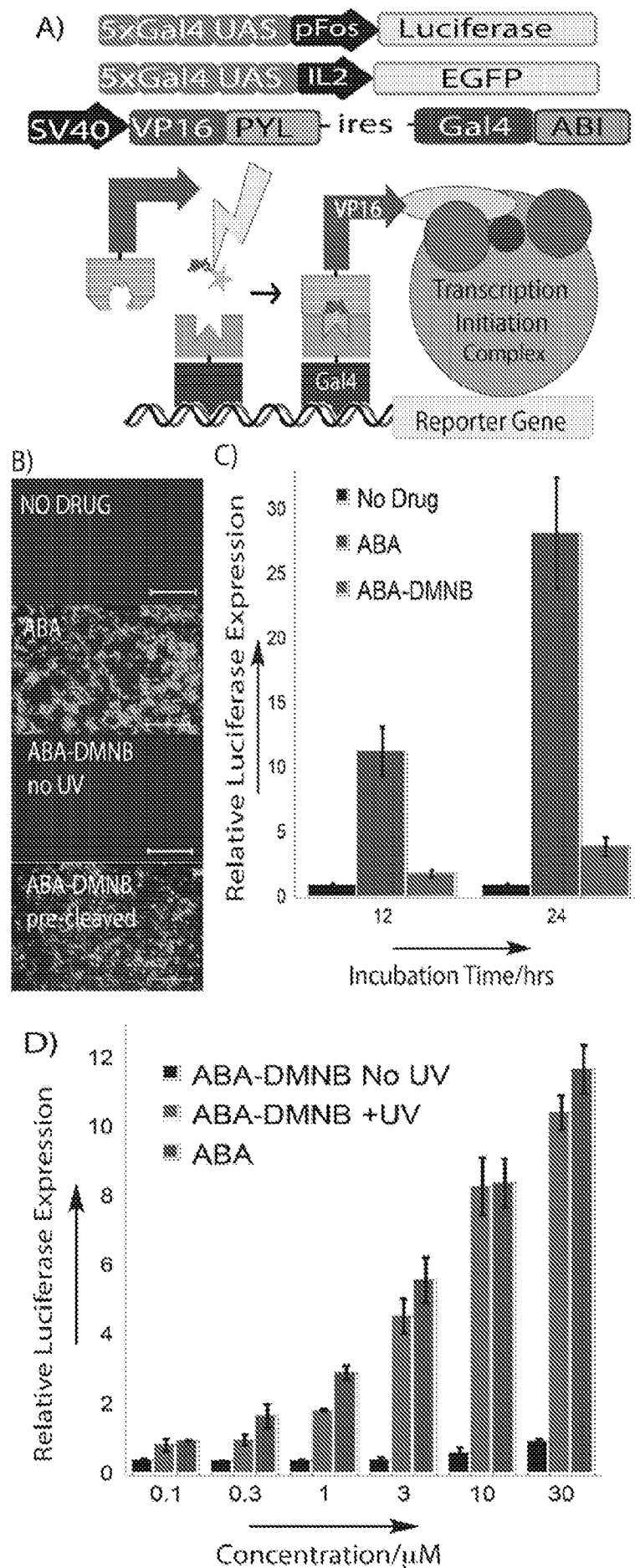


FIG. 22 A)

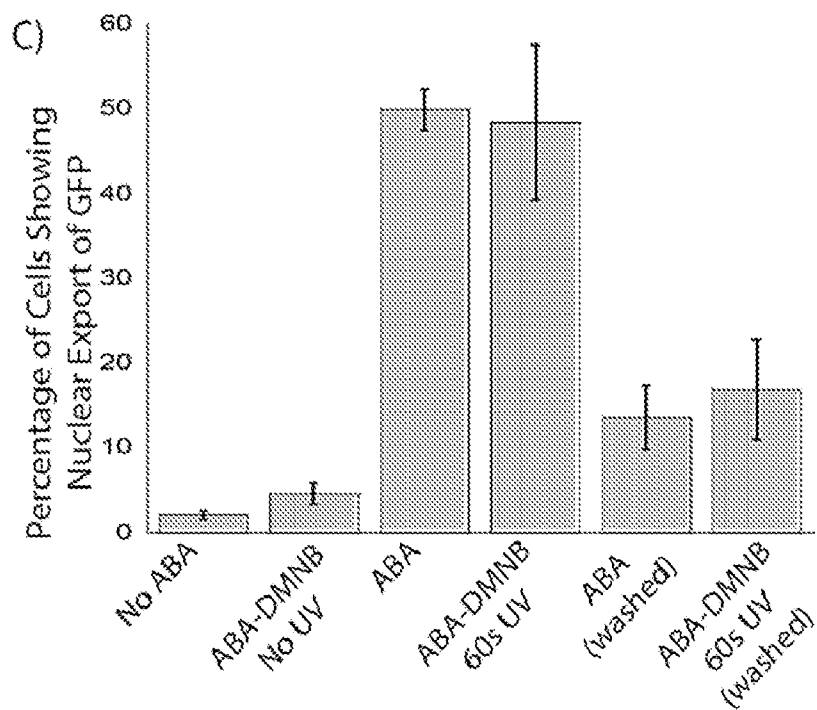
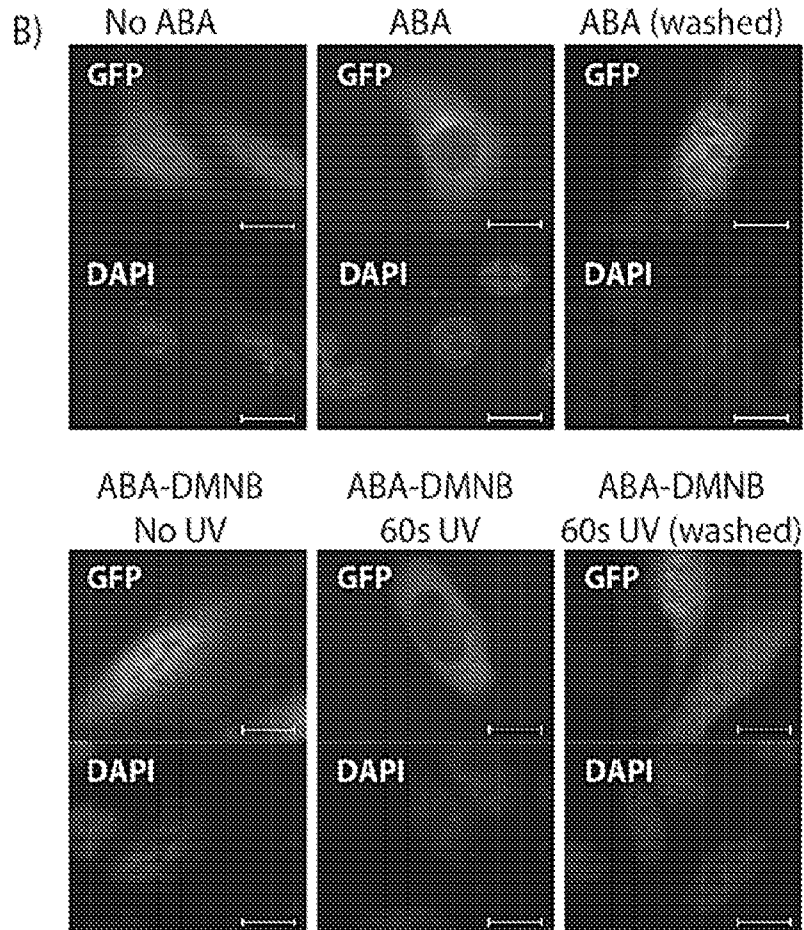


FIG. 23

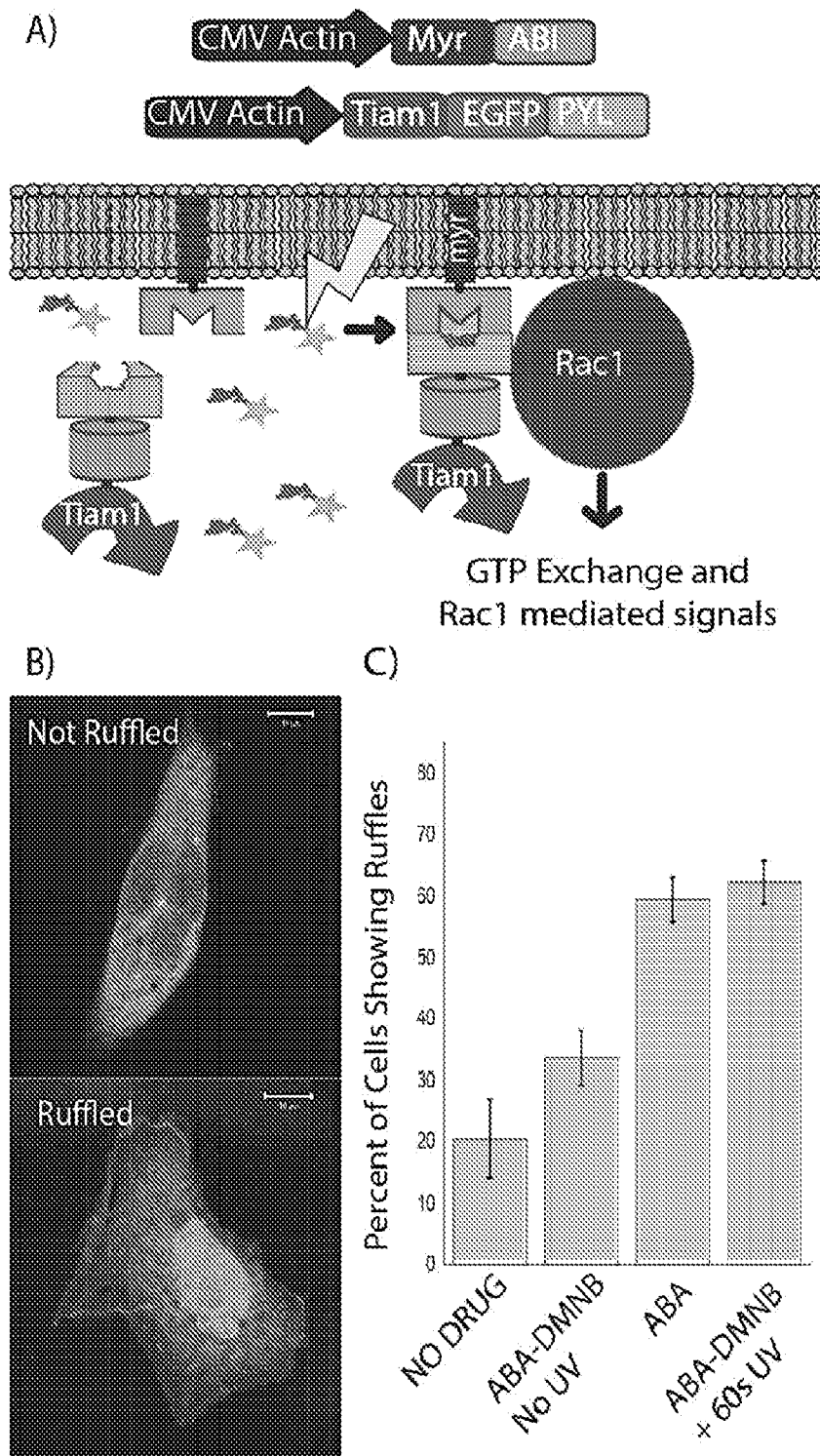


FIG. 24

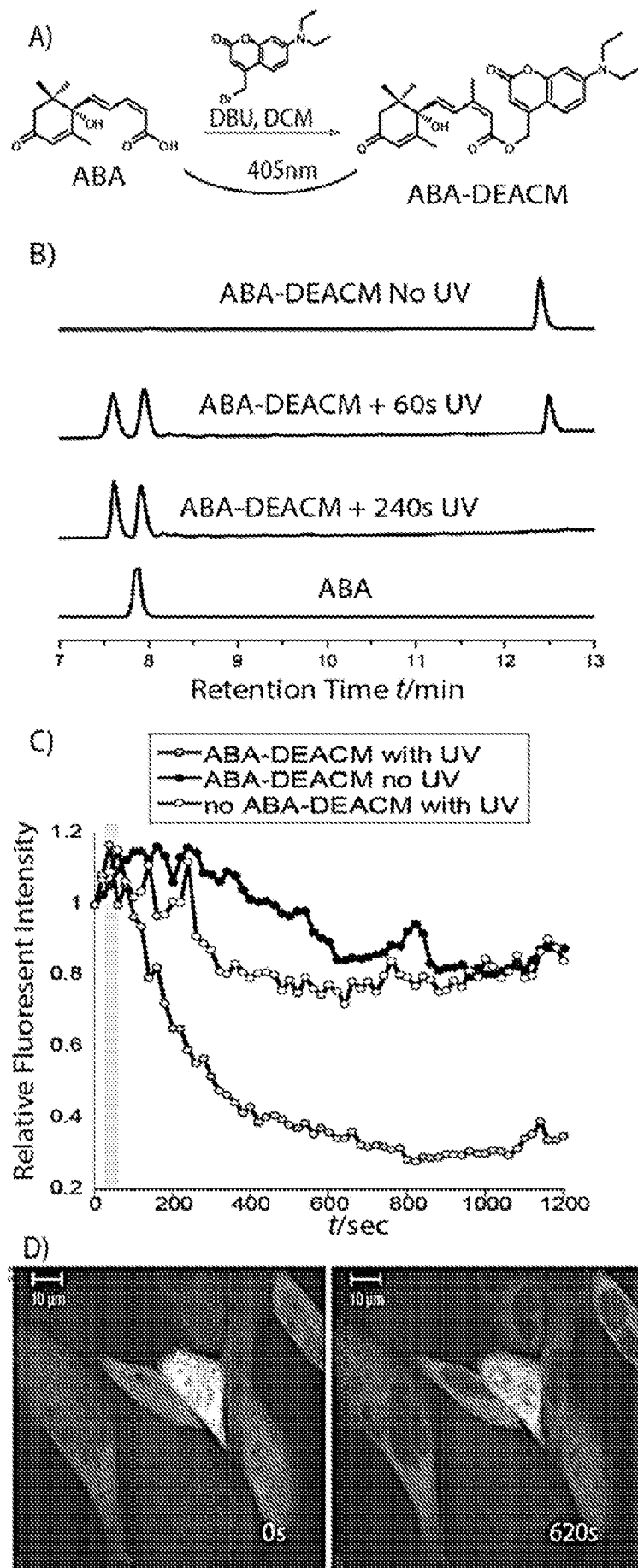


FIG. 25

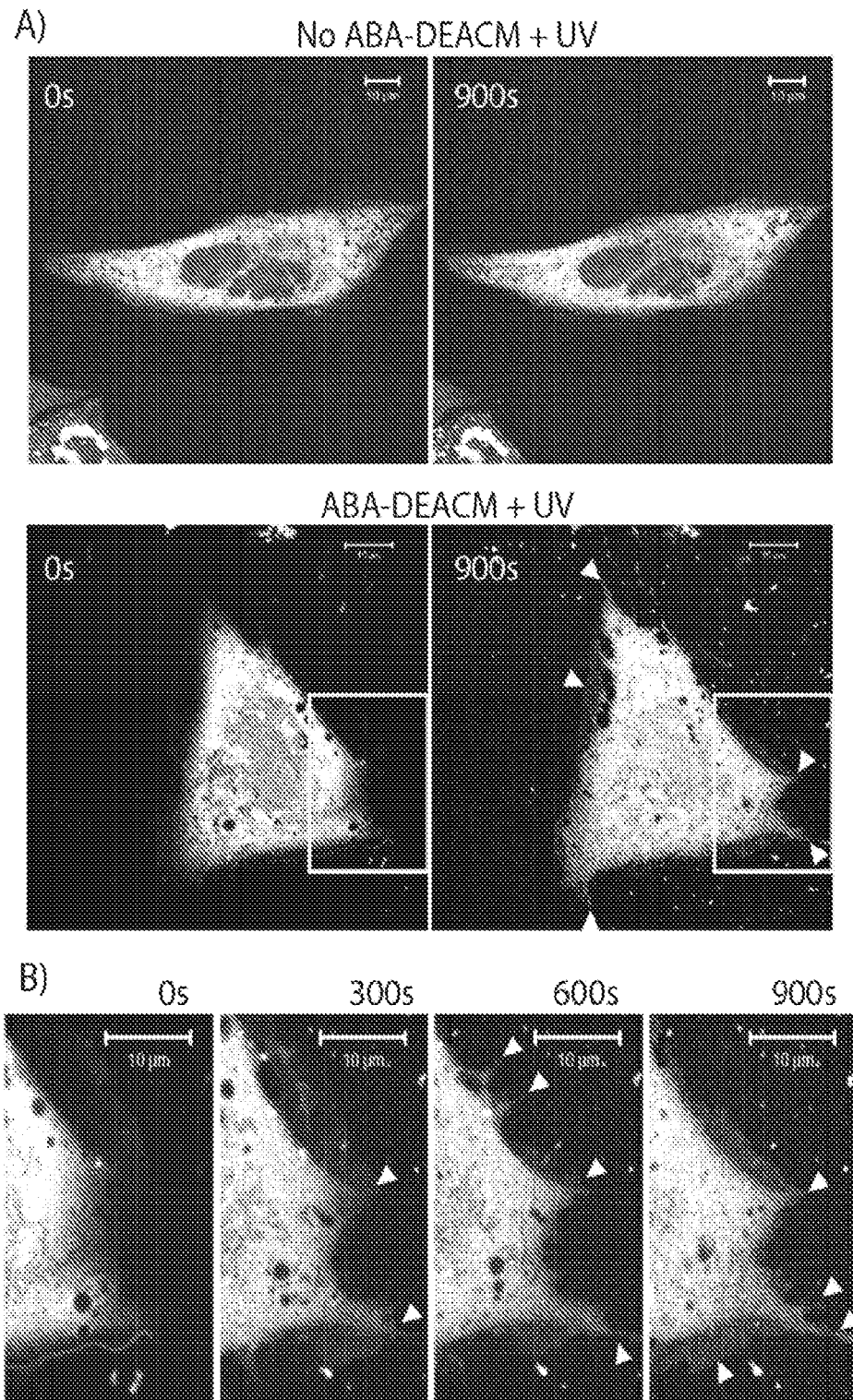


FIG. 26

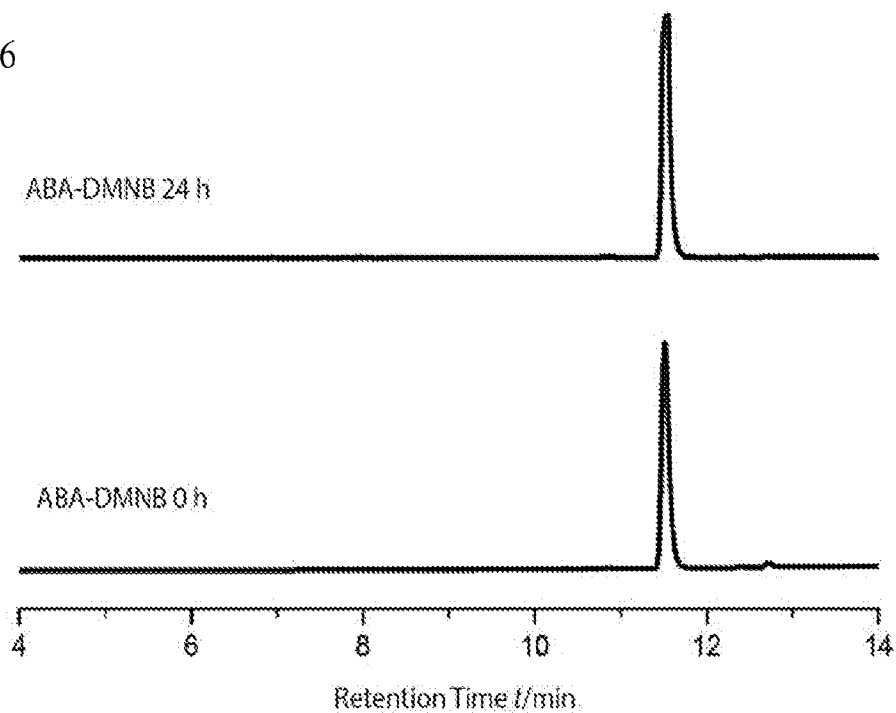


FIG. 27

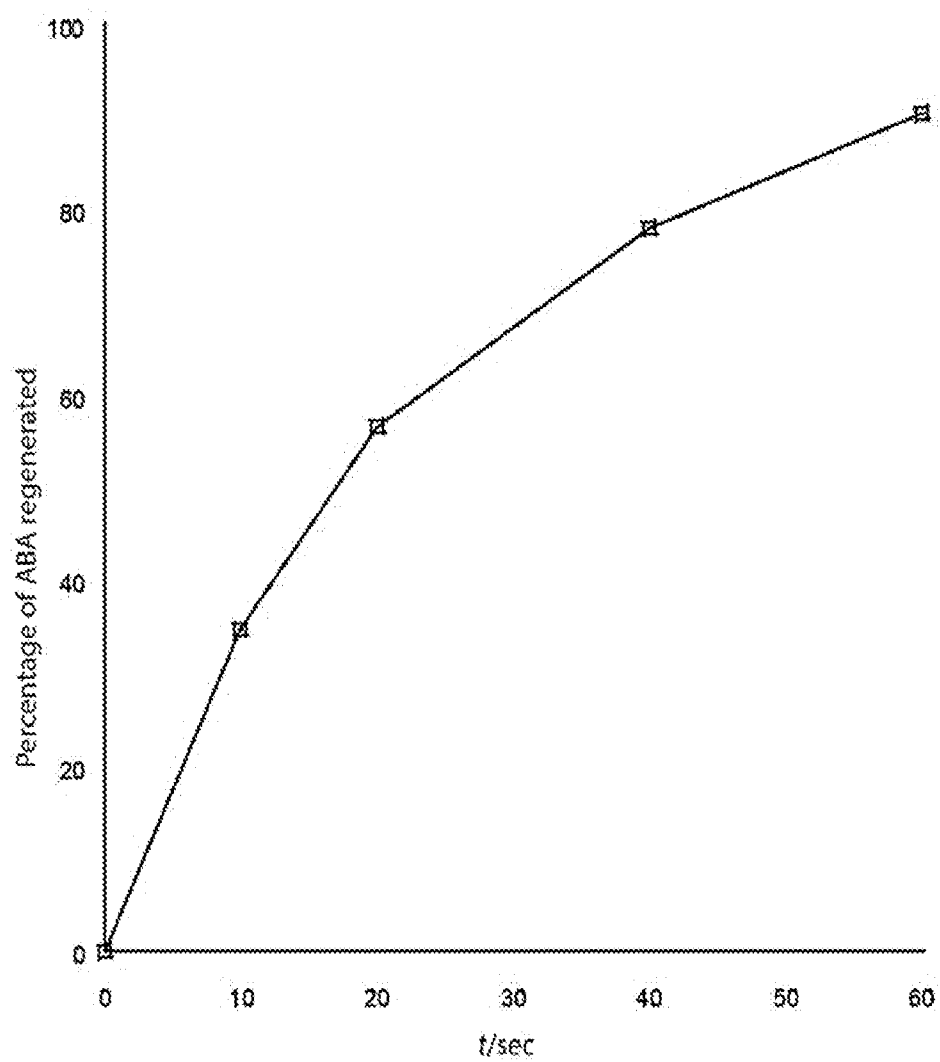


FIG. 28

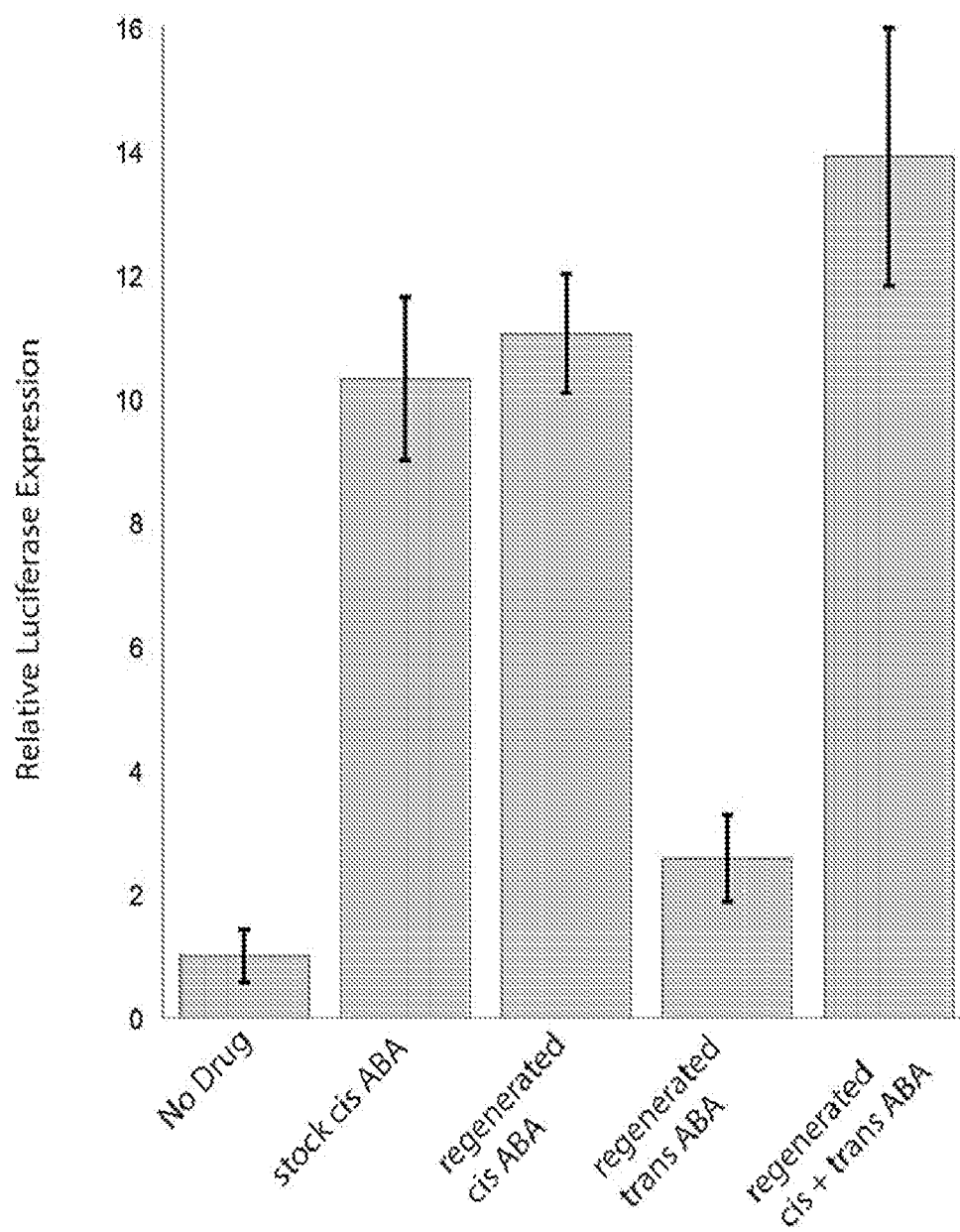


FIG. 29

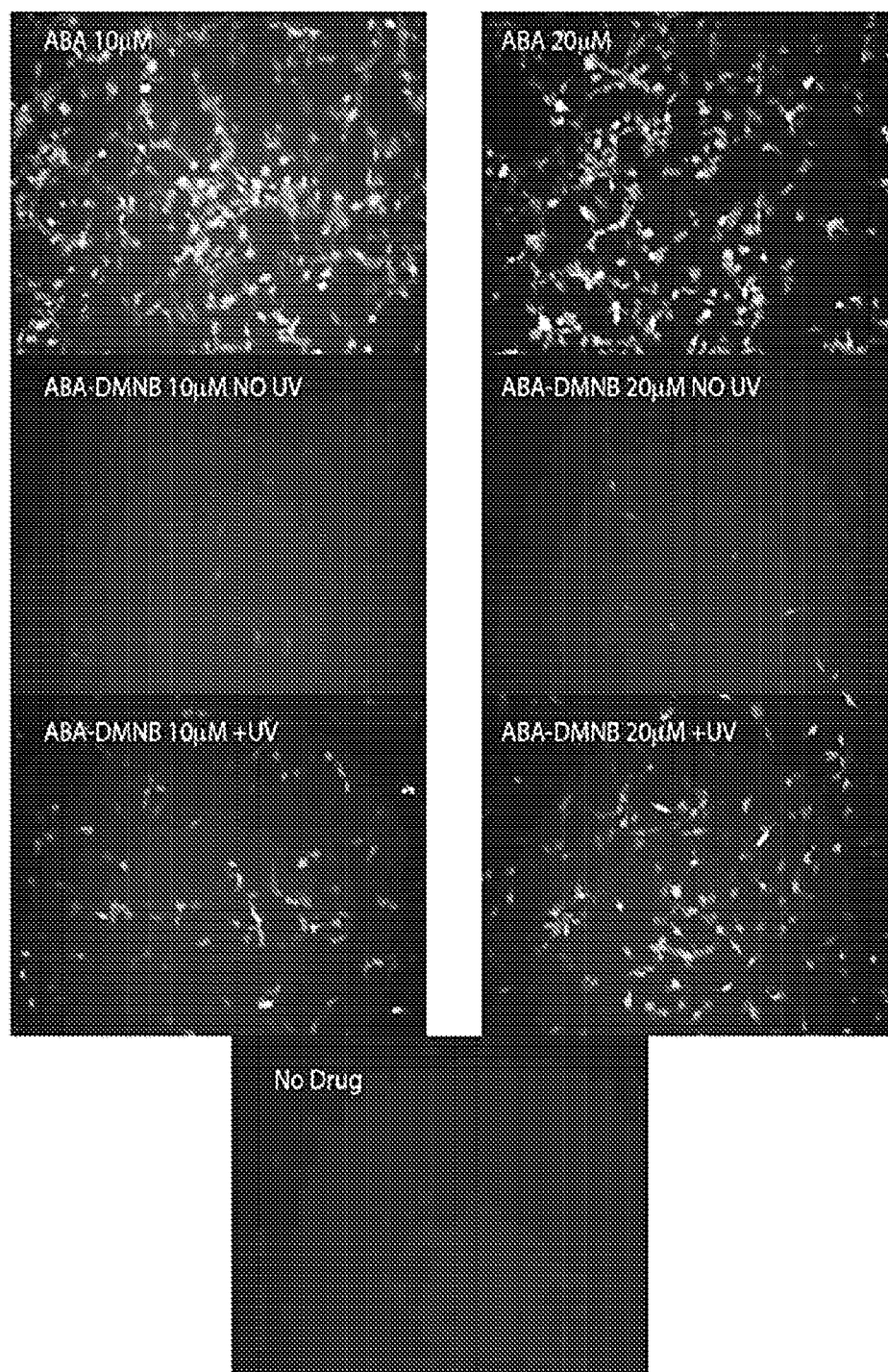


FIG. 30

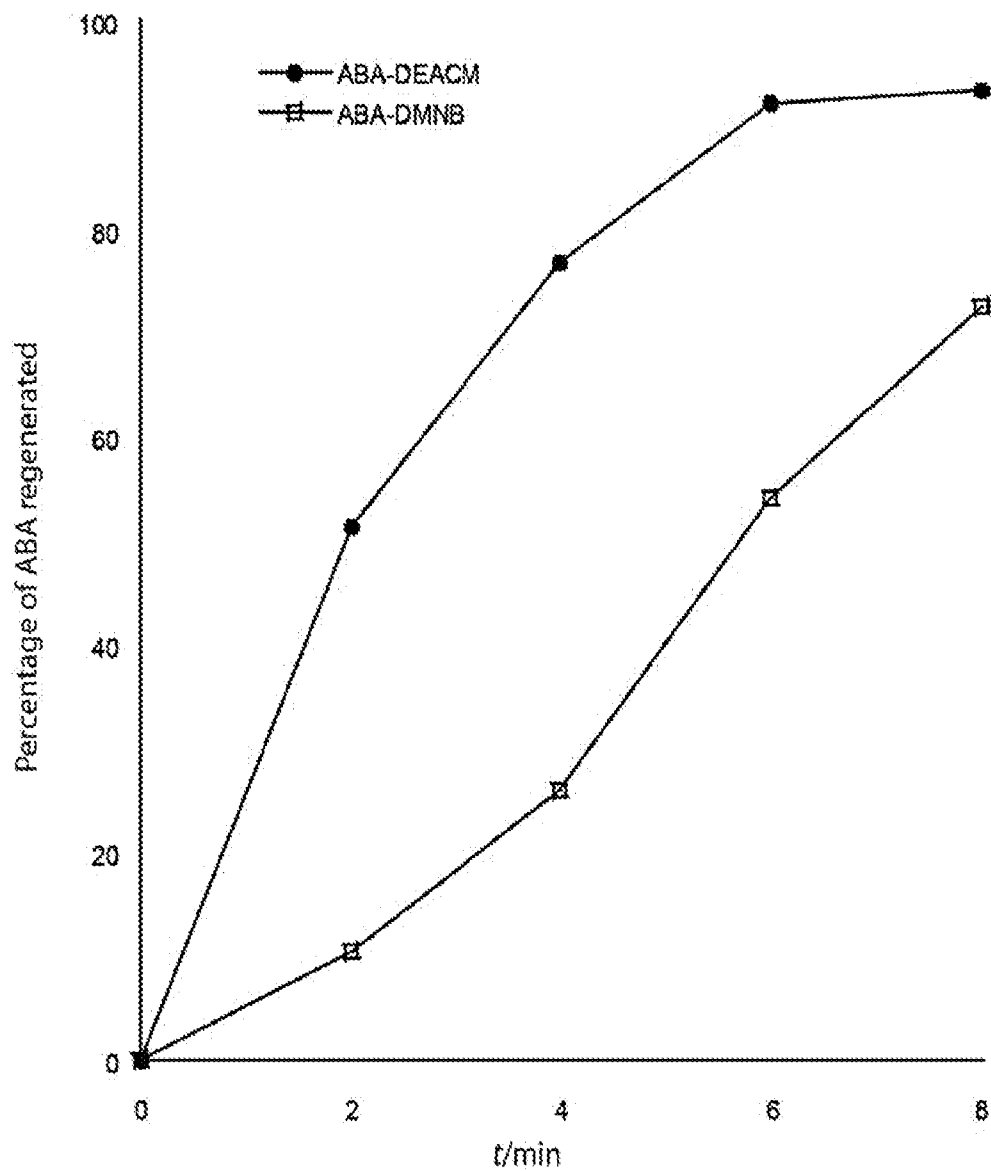


FIG. 31

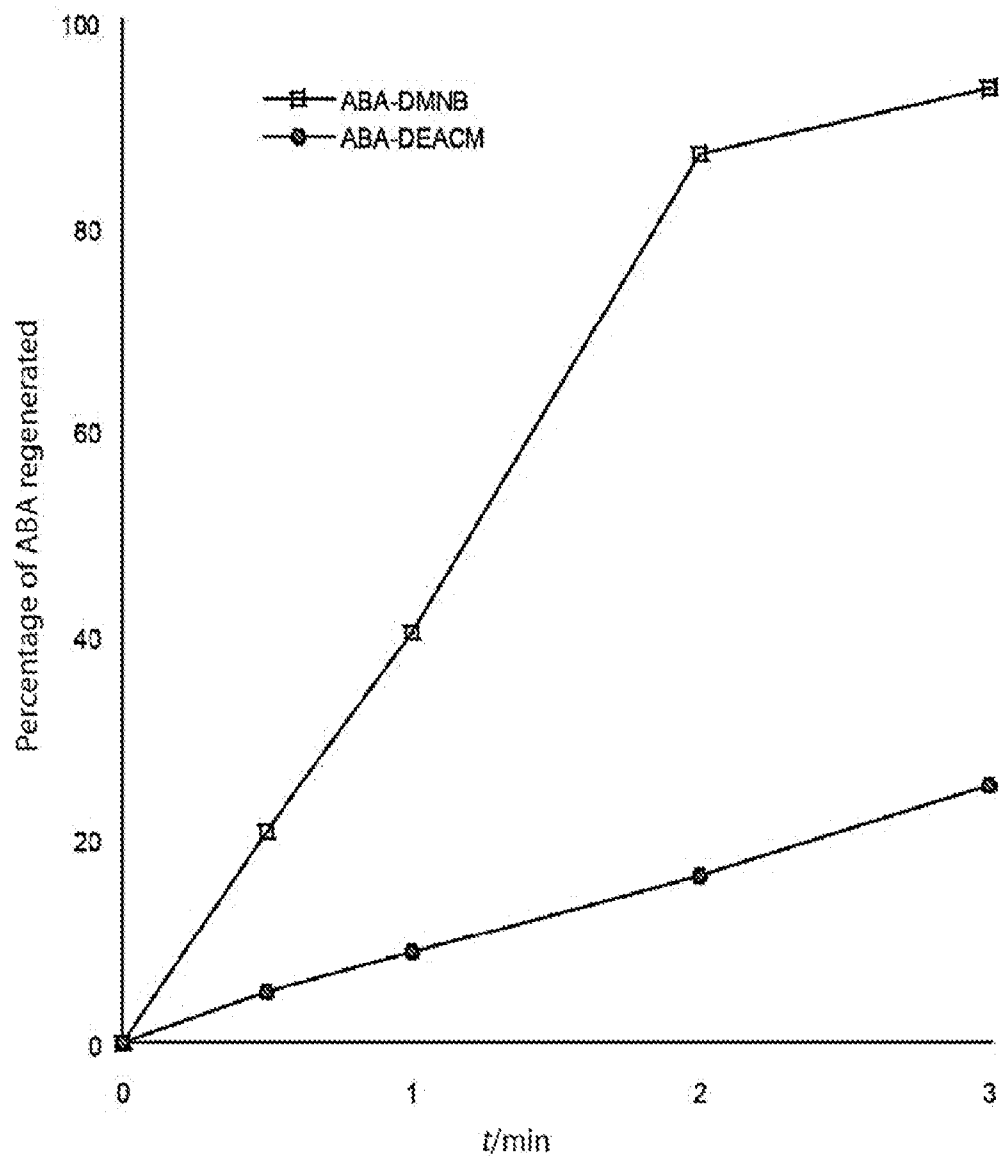


FIG. 32

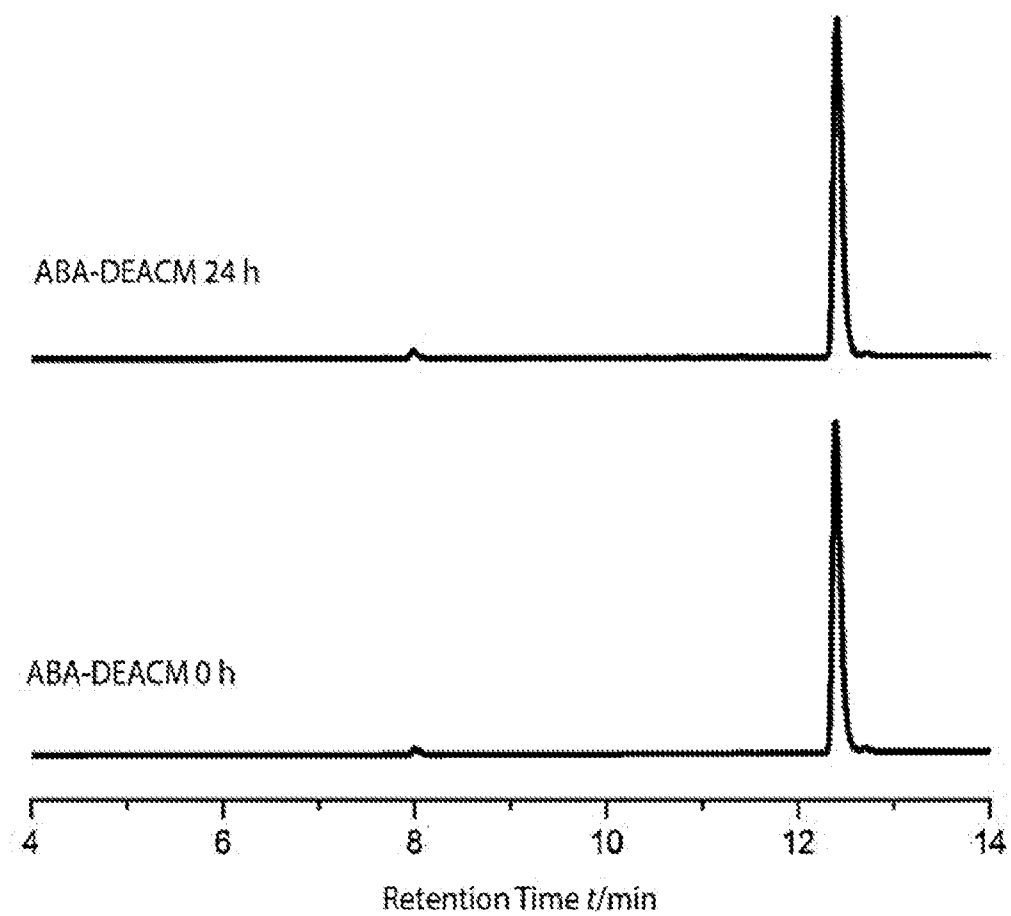


FIG. 33

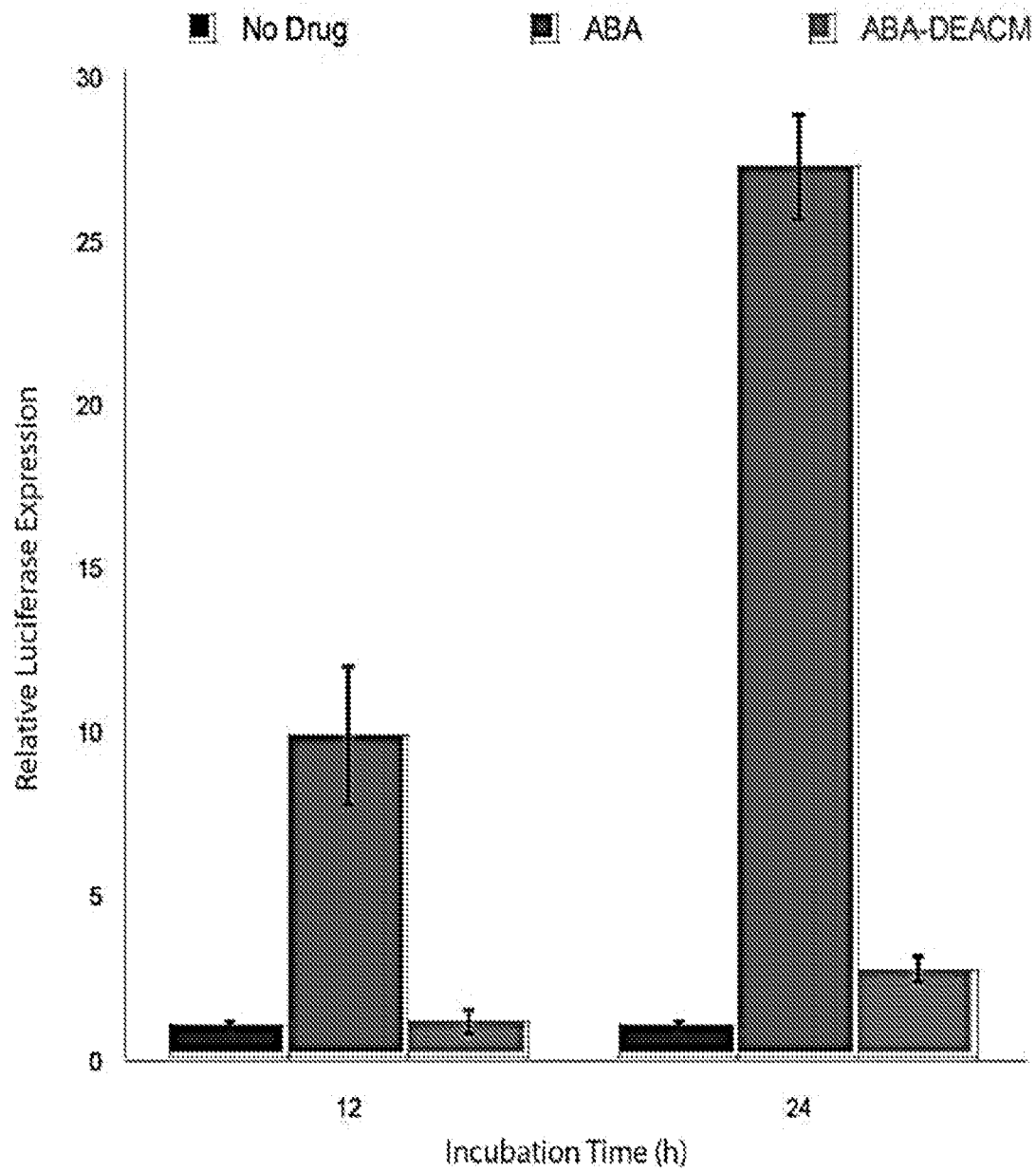


FIG. 34

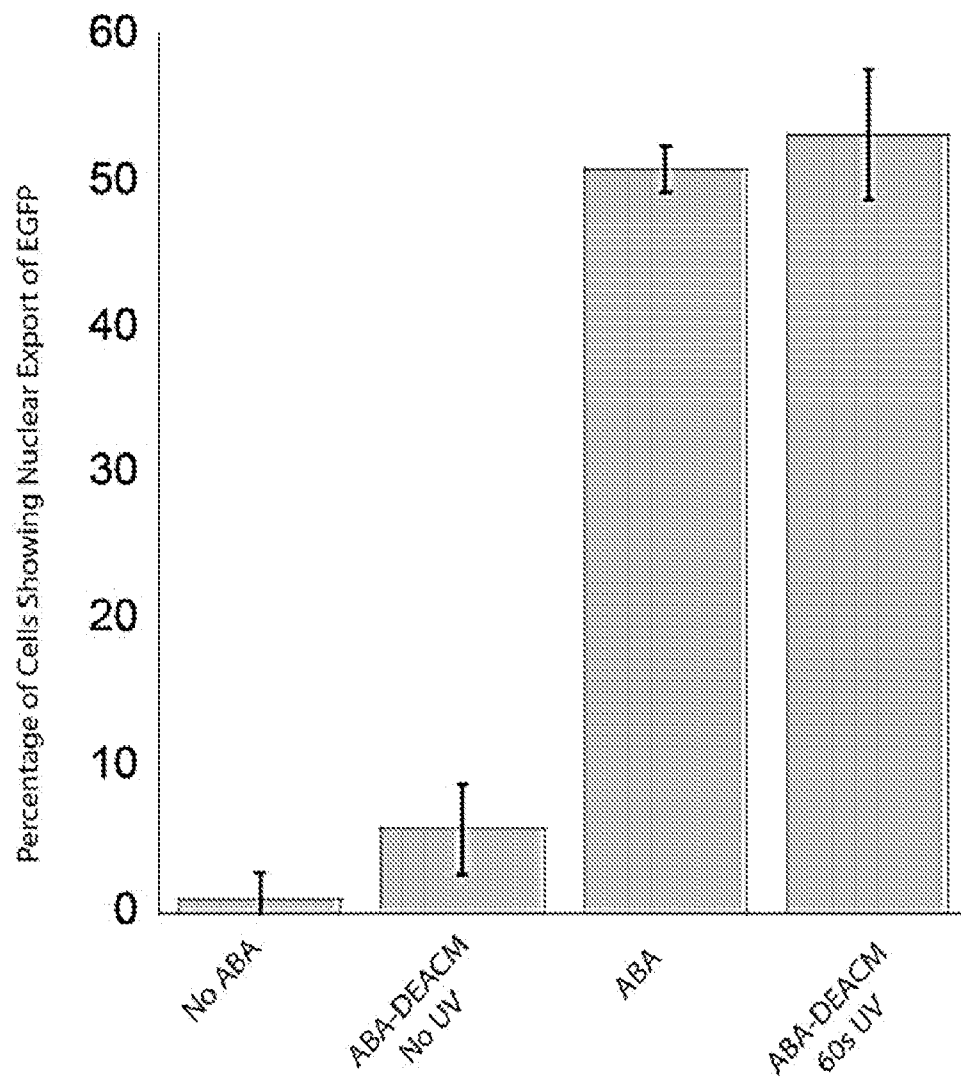


FIG. 35

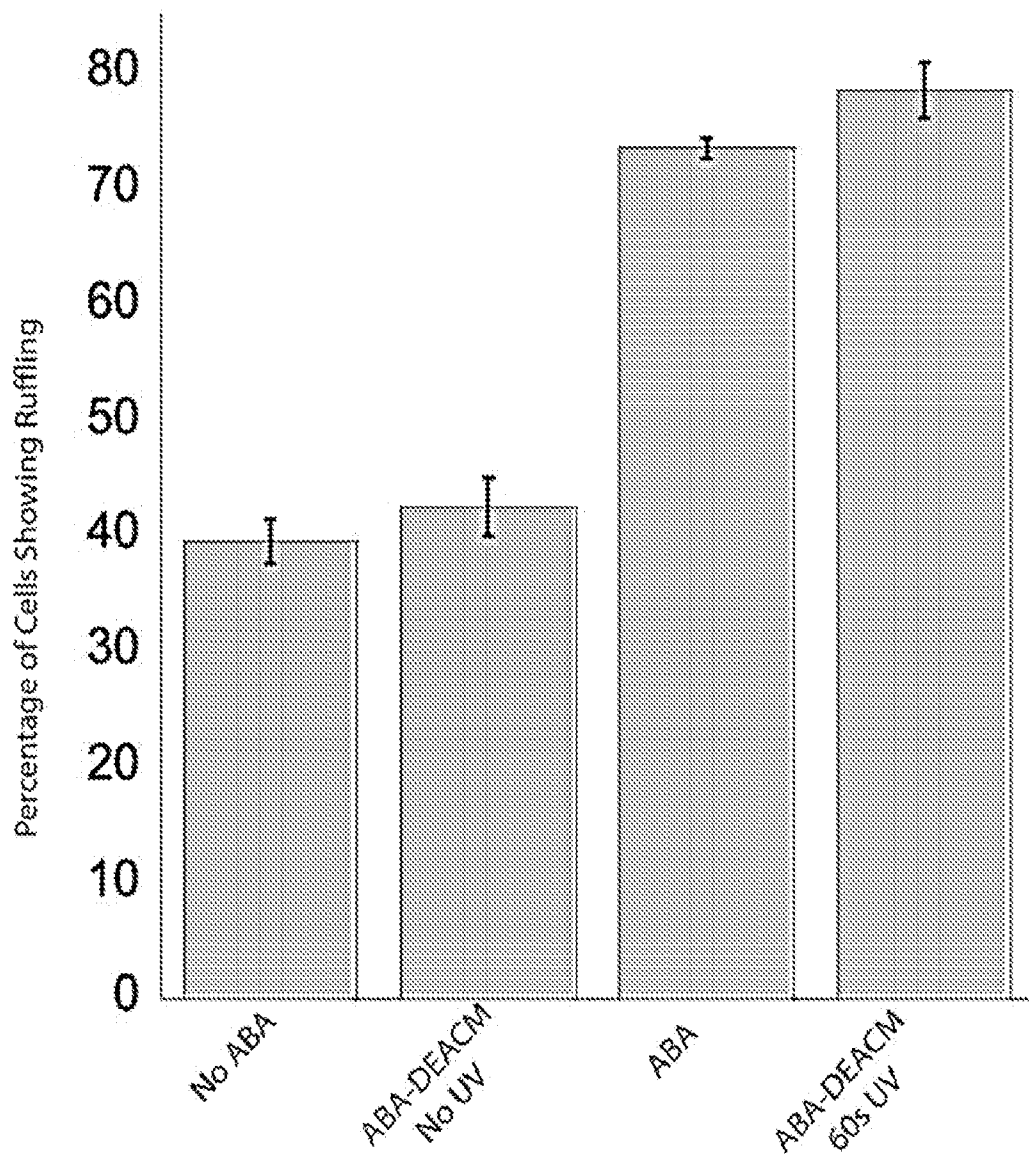
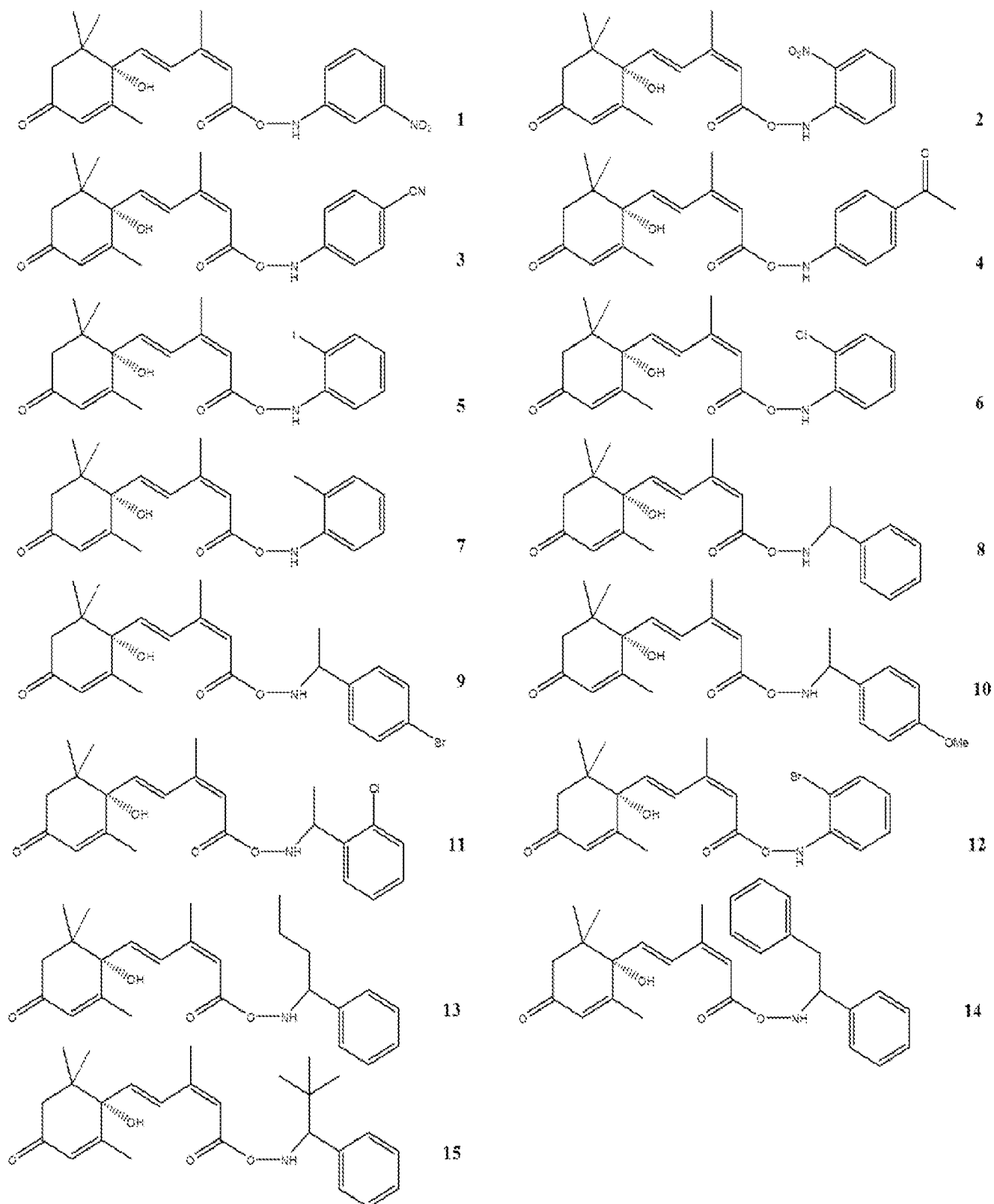


FIG. 36



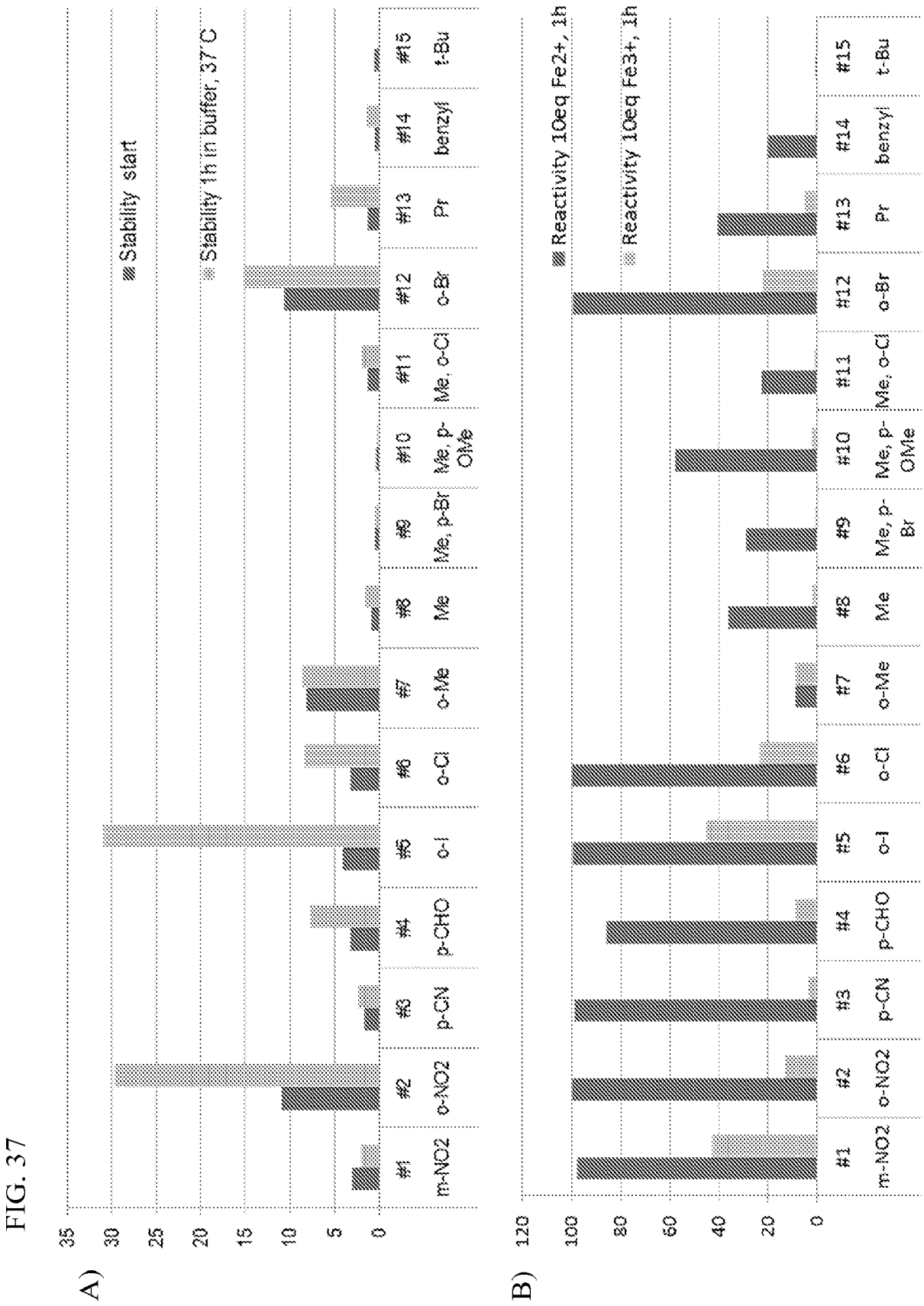


FIG. 37

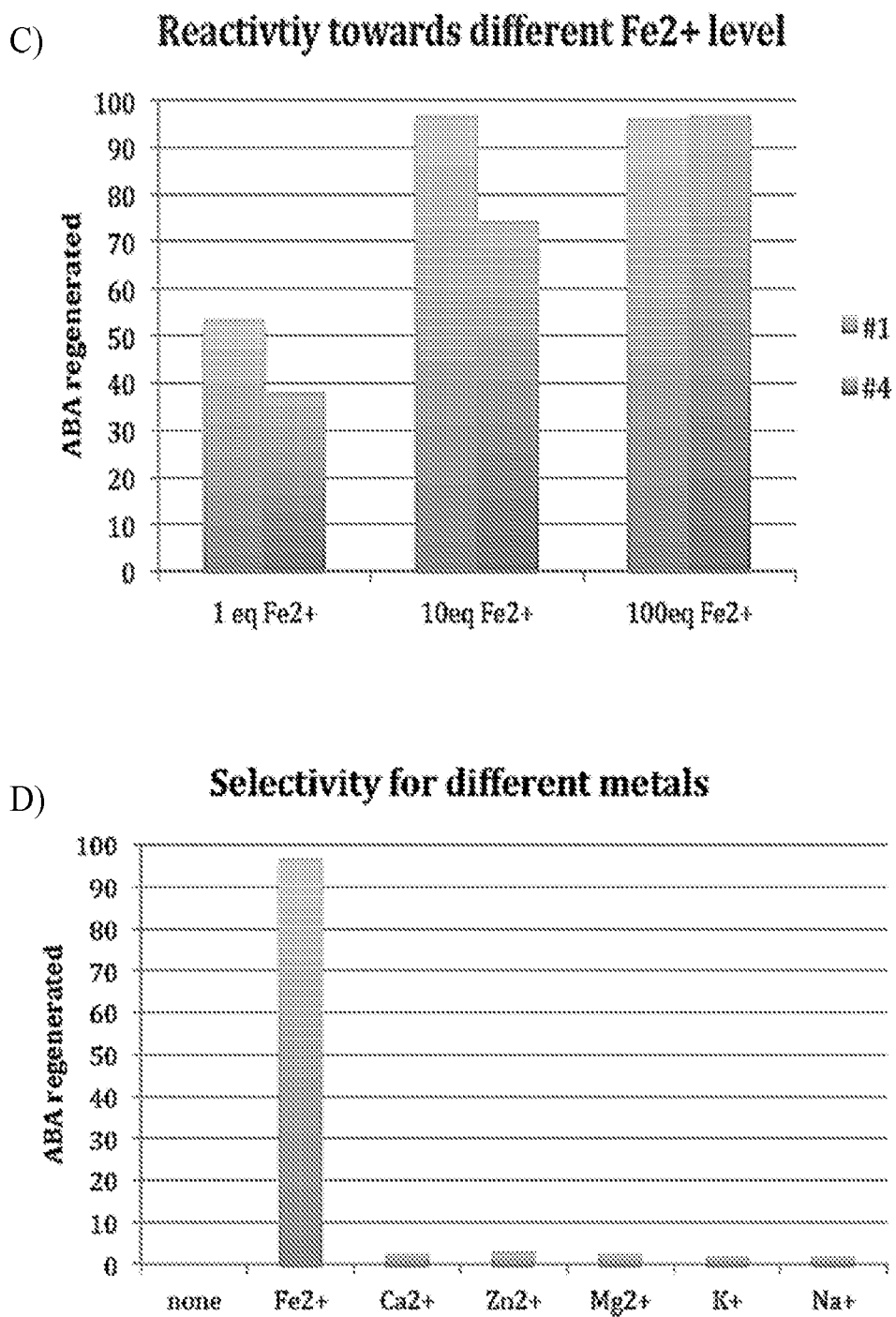


FIG. 38

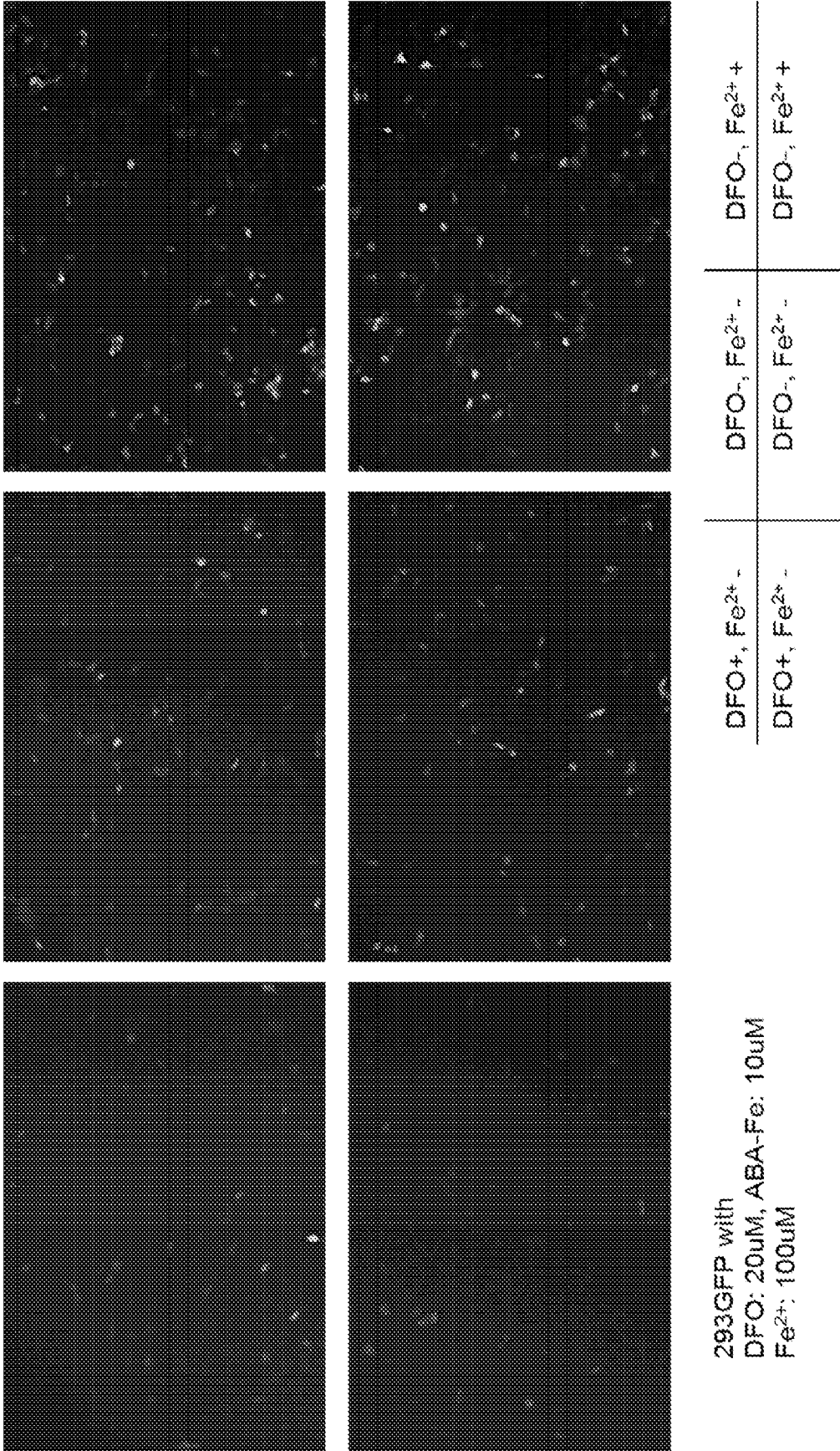


FIG. 39

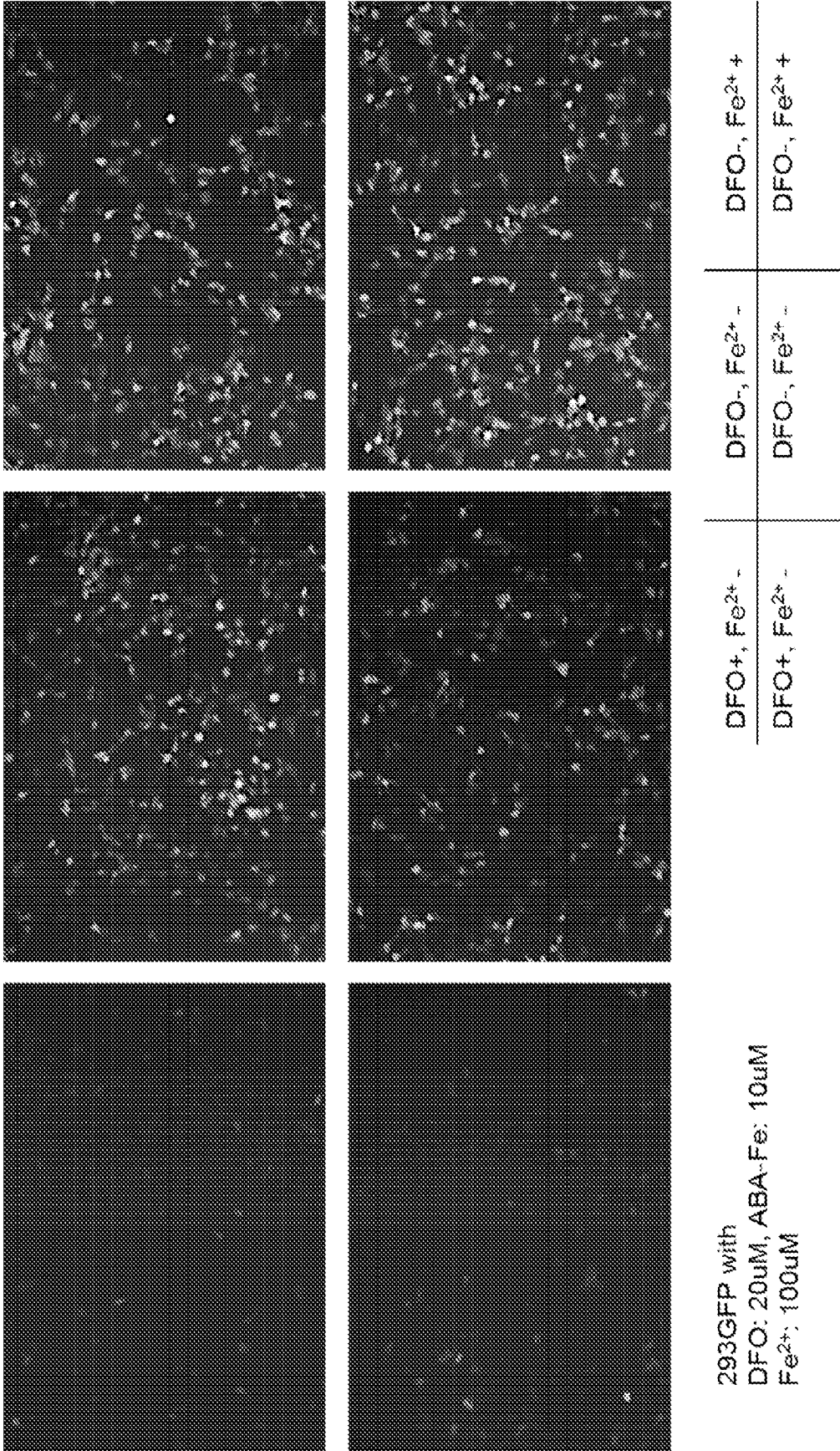


FIG. 40

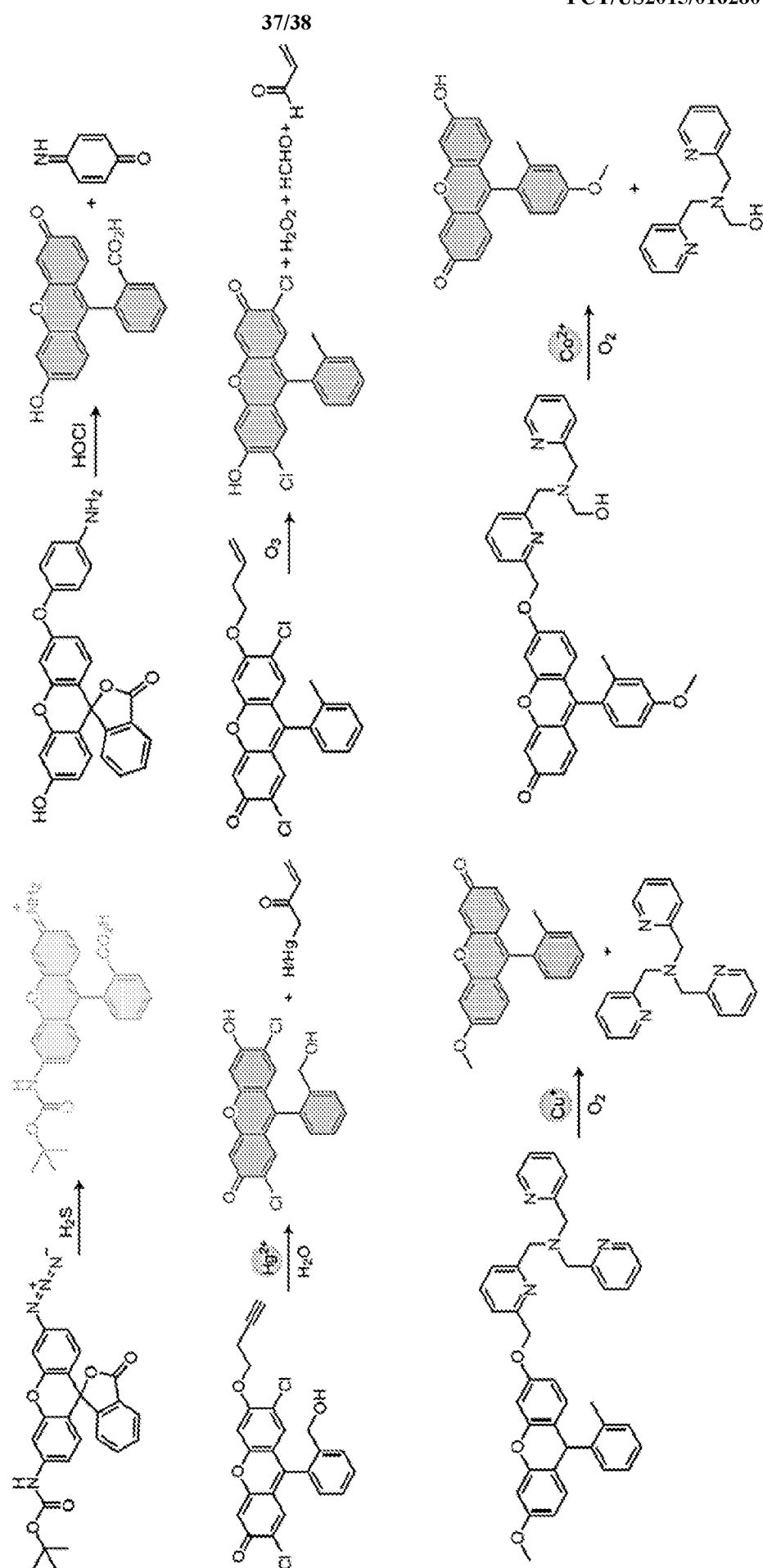
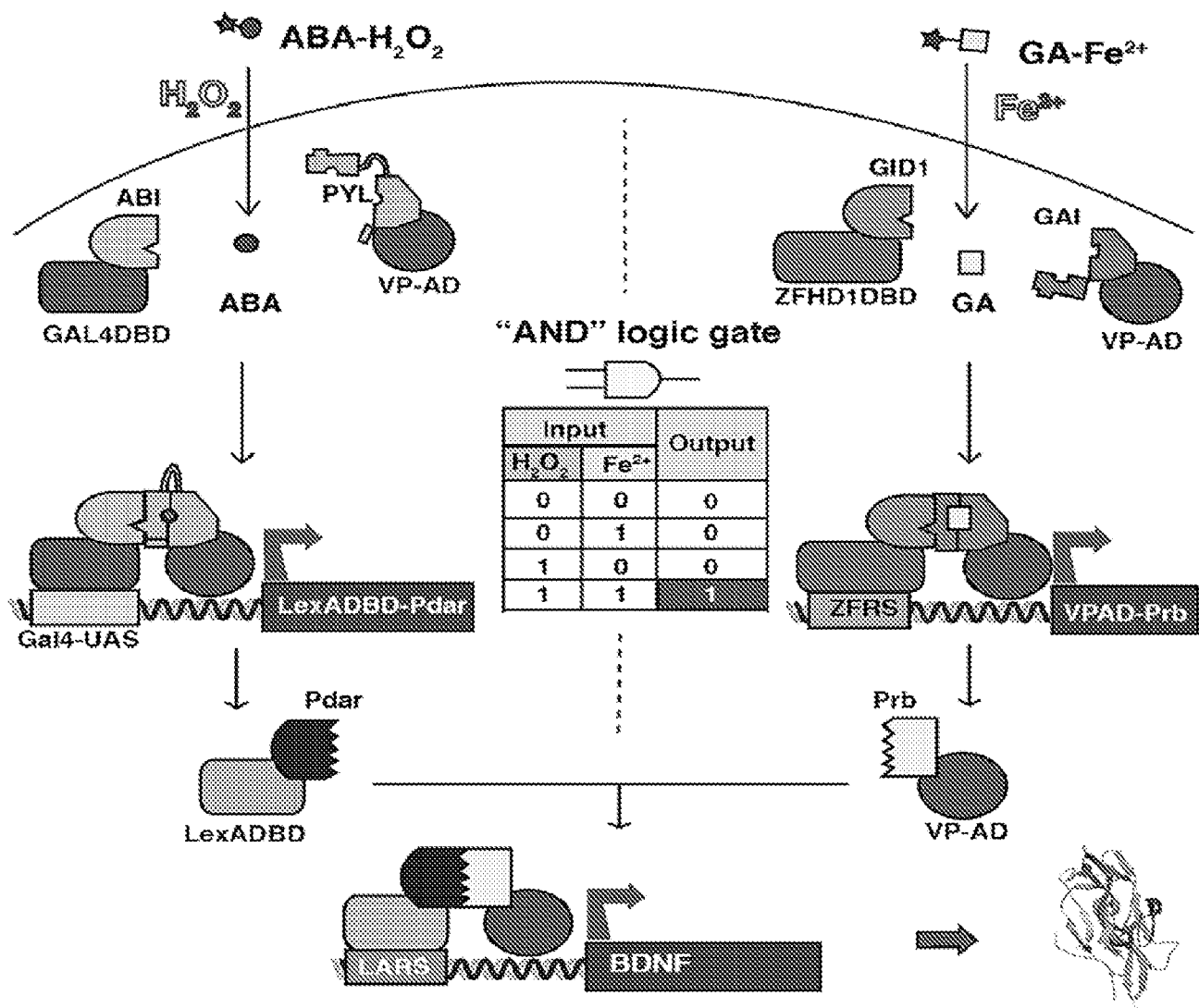


FIG. 41



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/016280**A. CLASSIFICATION OF SUBJECT MATTER****C12N 5/10(2006.01)i, C12N 5/02(2006.01)i, C12N 15/11(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 5/10; C12Q 1/68; G01N 33/68; C12N 5/02; C12N 15/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: chemical induced proximity (CIP), inducer, dimer, active, abscisic acid (ABA), gibberellic acid (GA), H₂O₂, Fe²⁺**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIANG, FU-SEN et al., 'Engineering the ABA plant stress pathway for regulation of induced proximity', Science Signaling, 2011, Vol. 4, Issue 164, Article No. rs2 (internal pp. 1-9) See abstract; pages 1-3, 5-7; and figures 1A-1G.	1,5
A		2-4, 10-14
A	FECHAN, ADRIAN et al., 'Chemically controlled protein assembly: techniques and applications', Chemical Reviews, 2010, Vol. 110, No. 6, pp. 3315-3336 See pages 3316-3321, 3323-3329, 3334-3335; and figures 1-16.	1-5, 10-14
A	MIYAMOTO, TAKAFUMI et al., 'Rapid and orthogonal logic gating with a gibberellin-induced dimerization system', Nature Chemical Biology, 2012, Vol. 8, No. 5, pp. 465-470 See abstract; pages 465-468; and figures 1-4.	1-5, 10-14
A	DEROSE, ROBERT et al., 'Manipulating signaling at will: chemically-inducible dimerization (CID) techniques resolve problems in cell biology', Pflügers Archiv-European Journal of Physiology, 2013, Vol. 465, Issue 3, pp. 409-417 See the whole document, especially figures 1-3; and table 1.	1-5, 10-14

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

29 May 2015 (29.05.2015)

Date of mailing of the international search report

29 May 2015 (29.05.2015)

Name and mailing address of the ISA/KR

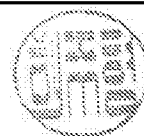
International Application Division
Korean Intellectual Property Office
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Republic of Korea

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Authorized officer

HEO, Joo Hyung

Telephone No. +82-42-481-8150



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/016280

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LYNCH, TIM et al., `Direct interactions of ABA-insensitive (ABI)-clade protein phosphatase (PP) 2Cs with calcium-dependent protein kinases and ABA response element-binding bZIPs may contribute to turning off ABA response`, Plant Molecular Biology, 2012, Vol. 80, Issue 6, pp. 647-658 See the whole document.	1-5,10-14
A	WO 2013-188406 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 19 December 2013 See abstract and claims 1-35.	1-5,10-14
T	ZENG, GUIHUA et al., `Constructing de novo H2O2 signaling via induced protein proximity`, ACS Chemical Biology, Epub. 16 March 2015, DOI: 10.1021/acscchembio.5b00170 (internal pp. A-G) See the whole document.	1-5,10-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/016280

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 8-9
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 8-9 are unclear since they each refer to a multiple dependent claim which does not comply with PCT Rule 6.4(a) (PCT Article 6).

3. ☒ Claims Nos.: 6-7
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2015/016280

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013-188406 A1	19/12/2013	None	