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(54) Title: LASERTAG: A TOOLKIT ALLOWING THE SPACE-SPECIFIC RECOVERY, CONTROL AND MODIFICATION OF SINGLE CELLS AND BIOLOGICAL MOLECULES IN VIVO

(57) Abstract: In some aspects, the disclosure relates to self-ligating protein tags conjugated to a photocaging molecule and methods of their use.

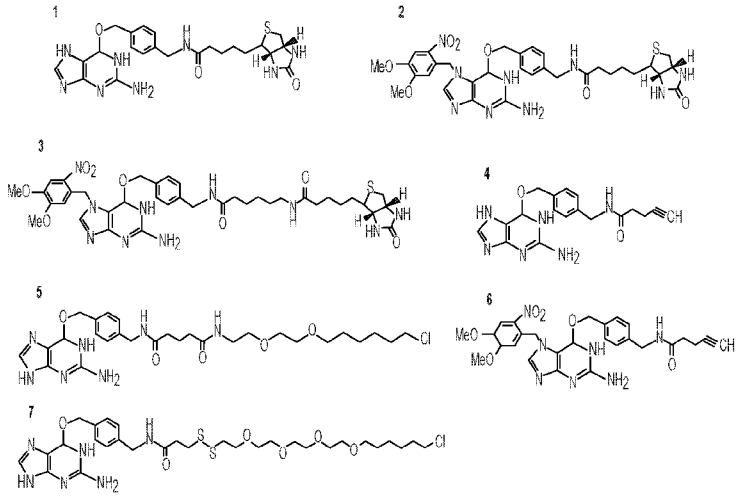


FIG. 4

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LASERTAG: A TOOLKIT ALLOWING THE SPACE-SPECIFIC RECOVERY, CONTROL AND MODIFICATION OF SINGLE CELLS AND BIOLOGICAL MOLECULES IN VIVO

RELATED APPLICATIONS

This Application claims the benefit under 35 U.S.C. 119(e) of U.S. provisional patent application serial number USSN 62/082,122, filed November 19, 2014, and entitled "LASERTAG: A TOOLKIT ALLOWING THE SPACE-SPECIFIC RECOVERY, CONTROL AND MODIFICATION OF SINGLE CELLS AND BIOLOGICAL MOLECULES IN VIVO", the entire teachings of which are incorporated herein by reference.

FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant number 1R21 DA035612-01 awarded by the National Institutes of Health. The government has certain rights in the invention.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

This invention was made pursuant to a joint research agreement between the parties, Cold Spring Harbor Laboratory and Mt. Sinai School of Medicine.

BACKGROUND OF INVENTION

A large number of biological processes are highly local in nature. Whether they occur in cells or whole organisms, many phenomena are influenced by the spatial context in which they take place, and they can often be understood only by taking into account the relative position of their components, and their interactions in space and time.

The study of local events calls for tools able to operate in specific spatial domains, yet many techniques in molecular biology and biochemistry can be applied only to samples that are obtained from the lysis of heterogeneous cell mixtures. Conventional microscopy allows the *in situ* monitoring and manipulation of processes with exquisite spatial detail, but it is not suited for high-throughput applications. Accordingly, the capability to perform molecular biology and biochemical techniques within specific spatial domains requires development of specific tools for those purposes.

SUMMARY

Described herein are compositions and methods useful for space-specific identification, recovery, control or modification of single cells and biological molecules *in vivo*.

In some aspects, the disclosure relates to a photochemically caged dimerizer of Formula I:

P — BG — L — CA

(Formula I),

wherein:

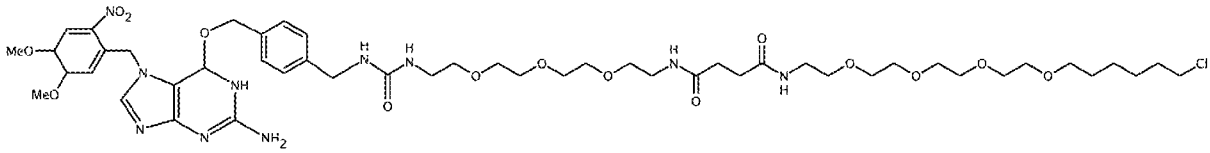
(a) P is a photocaging moiety selected from the group consisting of alpha-carboxy-2-nitrobenzyl; 4,5 dimethoxy 2-nitrobenzyl; 4,5, dimethoxy 2-nitrophenyl; and 6-nitroveratryloxycarbonyl; and bound to BG at position N_7, optionally by an ester bond;

(b) BG is O6-Benzylguanine;

(c) L is a linker that comprises at least one amide bond; at least one polyethylene glycol (PEG) moiety; or at least one amide bond and at least one PEG moiety; or at least one disulfide bond or a single tetrafluorophenylene group, and,

(d) CA is a chloroalkane.

In some embodiments, the photochemically caged dimerizer is represented by Formula II:



(Formula II),

wherein:

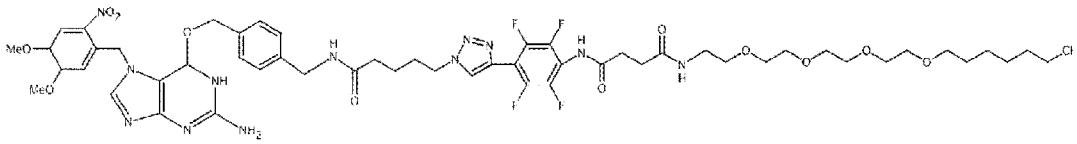
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by a carbonyl bond to L;

(c) L consists essentially of, in the following order: three PEG moieties; two amide bonds, and four PEG moieties; and,

(d) CA is a chloroalkane comprising at least six carbon molecules.

In some embodiments, the photochemically caged dimerizer is represented by Formula III:



(Formula III)

wherein:

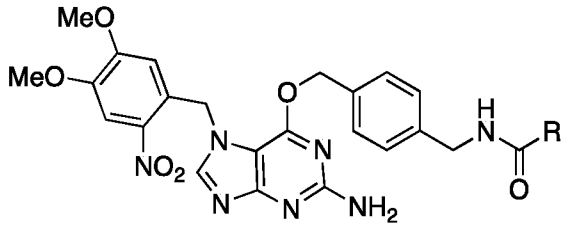
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by an amide bond to L;

(c) L consists essentially, of in the following order: 1,2,3-triazole group; tetrafluorophenylene group, two amide bonds, four PEG moieties: and,

(d) CA is a chloroalkane comprising at least six carbon molecules.

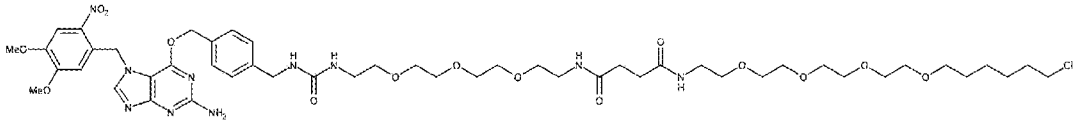
In some embodiments, the disclosure relates to a photochemically caged dimerizer represented by Formula IV:



(Formula IV),

wherein R is selected from the group consisting of an alkyne moiety, an azide moiety, biotin, and a fluorescent moiety.

In some embodiments, the photochemically caged dimerizer is represented by Formula VII:



(Formula VII),

wherein:

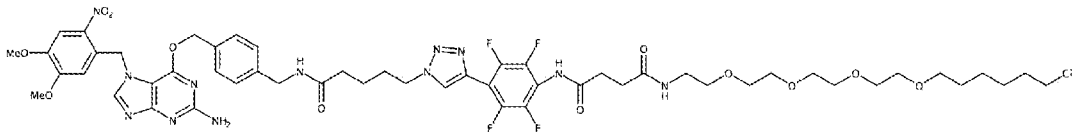
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by a carbonyl bond to L;

(c) L consists essentially of, in the following order: three PEG moieties; two amide bonds, and four PEG moieties; and,

(d) CA is a chloroalkane comprising at least six carbon molecules.

In some embodiments, the photochemically caged dimerizer is represented by Formula VIII:



(Formula VIII)

wherein:

(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by an amide bond to L;

(c) L consists essentially, of in the following order: 1,2,3-triazole group; tetrafluorophenylene group, two amide bonds, four PEG moieties; and,

(d) CA is a chloroalkane comprising at least six carbon molecules.

The disclosure also relates to a method of removing a cell component, such as a target protein or a target nucleic acid, from a single cell or from individual cells in a pool of cells. In the method of removing a protein, the method comprises (a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is not the protein to be removed and binds a protein to be removed, referred to as a target protein (on or within a single cell or individual cells in the pool); and (2) a photochemically caged SNAP-HALO dimerizer of any one of formulas I, II, or III, thereby producing a cell comprising SNAP-tagged protein bound to the target protein and (2); (b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon

density sufficient to uncage (that uncages) the photochemically caged SNAP-HALO dimerizer, thereby producing an uncaged SNAP-HALO dimerizer (BG-L-CA, designated below as DIMERIZER), under conditions under which the uncaged dimerizer binds to SNAP and forms a complex of Formula V:

SNAPTAG Protein —— **DIMERIZER**

(Formula V);

(c) contacting the complex of Formula V with a separation agent comprising HALO protein, under conditions under which the complex of Formula V binds to HALO protein of the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula Va:

SNAPTAG Protein —— **DIMERIZER** —— **HALOTAG**

(Formula Va); and,

(d) separating SNAPTAG Protein of Formula Va from other cellular components based on binding of the complex to the HALO protein. In Formula Va, SNAPTAG Protein is a SNAP-tagged protein bound to target protein on or within a cell, DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b) and HALOTAG is the separation agent comprising HALO protein.

In the method of removing a protein, the method comprises (a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is the protein to be removed; and (2) a photochemically caged SNAP-HALO dimerizer of any of formulas I, II, or III, thereby producing at least one cell comprising SNAP-tagged protein and (2); (b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged SNAP-HALO dimerizer, thereby producing an uncaged SNAP-HALO dimerizer (BG-L-CA, designated below as DIMERIZER), under conditions under which the uncaged dimerizer binds to SNAP and forms a complex of Formula V:

SNAPTAG Protein —— **DIMERIZER**

(Formula V); and,

(c) contacting the complex of Formula V with a separation agent comprising HALO protein, under conditions under which the complex of Formula V binds to HALO protein of the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula Va:

SNAPTAG Protein —— **DIMERIZER** —— **HALOTAG**

(Formula Va); and,

(d) separating SNAPTAG Protein of Formula Va from other cellular components based on binding of the complex to the HALO protein. In Formula Va, SNAPTAG Protein is a SNAP-tagged protein to be removed, DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b) and HALOTAG is the separation agent comprising HALO protein.

In some embodiments, the disclosure also relates to a method of removing a cell component, such as a target nucleic acid, from a single cell or from individual cells in a pool of cells. In the method of removing a nucleic acid, the method comprises (a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that binds a nucleic acid to be removed, referred to as a target nucleic acid (within a single cell or individual cells in the pool); and (2) a photochemically caged SNAP-HALO dimerizer of any one of formulas I, II, or III, thereby producing a cell comprising SNAP-tagged protein bound to the target nucleic acid (2); (b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged SNAP-HALO dimerizer, thereby producing an uncaged SNAP-HALO dimerizer (BG-L-CA, designated DIMERIZER), under conditions under which the uncaged dimerizer binds to SNAP and forms a complex of Formula V:

SNAPTAG Protein —— **DIMERIZER**

(Formula V); and,

(c) contacting the complex of Formula V with a separation agent comprising HALO protein, under conditions under which the complex of Formula V binds to HALO protein of the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula Va:



(Formula Va); and,

(d) separating SNAPTAG Protein of Formula Va from other cellular components based on binding of the complex to the HALO protein. In Formula Va, SNAPTAG Protein is a SNAP-tagged protein bound to target nucleic acid on or within a cell, DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b) and HALOTAG is the separation agent comprising HALO protein.

In some embodiments, individual cells in a pool of cells comprising (1) and (2) are identified and light is delivered to those cells, but not to other cells in the pool.

In some embodiments of the method, the region of interest is a whole cell, all or a portion of the cell membrane or a subcellular compartment. In some embodiments, light is delivered for from about 1 second to about 1 minute. In some embodiments, the wavelength, power, and length of exposure of the delivered light results in two-photon excitation and uncaging of the dimerizer. In some embodiments, the wavelength, power and length of exposure of the delivered light results in one-photon excitation and uncaging of the dimerizer.

In some embodiments, the wavelength of the light delivered is from about 300 nm to about 800 nm. In some embodiments, the wavelength is about 365 nm. In some embodiments, the wavelength is about 405 nm. In some embodiments, the wavelength is about 488 nm. In some embodiments, the wavelength of the light delivered is from about 700 nm to about 800 nm. In some embodiments, the light is delivered by laser and the energy of the laser ranges from about 0.1 $\mu J/\mu m^2$ to 5 $\mu J/\mu m^2$. In some embodiments, the power of the laser ranges from about 0.1 $mW/\mu m^2$ to about 5 $mW/\mu m^2$.

Separation of the target protein or nucleic acid is carried out by methods that rely on binding of the complex of Formula V (which includes the target protein or nucleic acid to be separated) to the separation agent comprising HALO protein in order to form the complex of

Formula Va. In some embodiments, the cell or pool of cells is lysed before the separation step (c). In some embodiments, the separation agent comprising HALO protein is a bead. In some embodiments, the separation agent comprising HALO protein is a resin. In some embodiments, the separation step is performed by affinity chromatography, immunoprecipitation, or flow cytometry.

In some embodiments, the cells are incubated with SNAP-tagged protein, HALO-tagged protein and photochemically caged SNAP-HALO dimerizer for between about 30 minutes and about 12 hours before the delivery of light. In some embodiments, the region of interest to which light is delivered is a whole cell, all or a portion of the cellular membrane or the membrane of a subcellular compartment.

In some embodiments, light is delivered for between about 1 second and about 1 minute. In some embodiments, the delivery of light results in two-photon excitation and uncaging of the dimerizer. In some embodiments, the delivery of light results in one-photon excitation and uncaging of the dimerizer. In some embodiments, the wavelength of the light delivered is from about 300 nm to about 800 nm. In some embodiments, the wavelength of the light delivered is about 365 nm. In some embodiments, the wavelength of the light delivered is about 405 nm. In some embodiments, the wavelength of the light delivered is about 488 nm. In some embodiments, wherein the wavelength of the light delivered is from about 700 nm to about 800 nm.

In some embodiments, the light is delivered by laser and the energy of the laser ranges from about 0.1 $\mu J/\mu m^2$ to 5 $\mu J/\mu m^2$. In some embodiments, the power of the laser ranges from about 0.1 $mW/\mu m^2$ to about 5 $mW/\mu m^2$.

The length of time for which cells are incubated with the components described herein can vary. In some embodiments, the cells are incubated with the photochemically caged SNAP-HALO dimerizer for between about 30 minutes and about 12 hours. Photoactivation occurs by the delivery of light. In some embodiments, light is delivered for between about 1 second and about 1 minute. In some embodiments, the delivery of light results in two-photon excitation and uncaging of the dimerizer. In some embodiments, the delivery of light results in one-photon excitation and uncaging of the dimerizer. In some embodiments, the wavelength of the light delivered is from about 300 nm to about 800 nm. In some embodiments, the wavelength of the light that is delivered is about 365 nm. In some embodiments, the wavelength of the light that is delivered is about 405 nm. In some

embodiments, the wavelength of the light that is delivered is about 488 nm. In some embodiments, the wavelength of the light that is delivered is between about 700 nm to about 800 nm.

In some aspects, the disclosure relates to a method of delivering a viral vector to the surface of a cell or cells, the method comprising: (a) expressing a SNAP-tagged surface protein in a cell or cells, wherein SNAP is on the extracellular side of the plasma membrane of the cell or cells; (b) contacting the cells with a docking-defective viral vector expressing the HALO protein on the envelope; (c) incubating the cell or cells with at least one photochemically caged SNAP-HALO dimerizer described herein; and, (d) photoactivating a region of interest, under conditions under which the photochemically caged SNAP-HALO dimerizer is uncaged and binds to both SNAP and HALO, thereby producing a SNAP-HALO heterodimer. The formation of the SNAP-HALO dimer brings the viral vector into contact the extracellular side of the cell on which the surface protein bearing a SNAP-tag is expressed.

In some embodiments, the surface protein expressed on the extracellular side of the cell or cells is the CD4-2 transmembrane domain. In some embodiments, the viral vector comprises a docking-defective fusagenic protein, optionally a mutant version of the sindbis virus fusion protein. In some embodiments, the docking-defective viral vector further comprises a transgene to be delivered. In some embodiments, the viral vector is a lentiviral vector.

In some embodiments, the cells are incubated with the photochemically caged SNAP-HALO dimerizer for between about 30 minutes and about 12 hours. In some embodiments, the photoactivation occurs by the delivery of light. In some embodiments, light is delivered for between about 1 second and about 1 minute. In some embodiments, the delivery of light results in two-photon excitation and uncaging of the dimerizer. In some embodiments, the delivery of light results in one-photon excitation and uncaging of the dimerizer. In some embodiments, the wavelength of the light delivered is from about 300 nm to about 800 nm. In some embodiments, the wavelength of the light delivered is about 365 nm. In some embodiments, the wavelength of the light delivered is about 405 nm. In some embodiments, the wavelength of the light delivered is about 488 nm. In some embodiments, wherein the wavelength of the light delivered is between about 700 nm to about 800 nm.

In some embodiments, the light is delivered by laser and the energy of the laser ranges from about 0.1 $\mu J/\mu m^2$ to 5 $\mu J/\mu m^2$. In some embodiments, the power of the laser ranges from about 0.1 $mW/\mu m^2$ to about 5 $mW/\mu m^2$.

In some embodiments, the disclosure provides a method for removing a protein, the method comprising:

(a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is not the protein to be removed and binds a protein to be removed, referred to as a target protein (on or within a single cell or individual cells in the pool); and (2) a photochemically caged dimerizer of any one of formulas I-III and VII-VIII, or ligand of formula IV, thereby producing a cell comprising SNAP-tagged protein bound to the target protein and (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged dimerizer or ligand, thereby producing an uncaged "tagging molecule" (designated below as "TAG"), under conditions under which the uncaged tagging molecule binds to SNAP and forms a complex of Formula IX:

SNAPTAG Protein ——— **TAG**

(Formula IX);

(c) contacting the complex of Formula IX with a separation agent comprising (1) a HALO protein (*e.g.*, if the tag has a structure corresponding to formulas I-III or VII-VIII), or if the tag has a structure corresponding to formula IV, a streptavidin,), or an alkyne, under conditions under which TAG binds to the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula Va:

SNAPTAG Protein ——— **TAG** ——— **SEPARATION AGENT**

(Formula IXa); and,

(d) separating the complex of Formula IXa, which comprises SNAPTAG Protein, and therefore the target protein, from other cellular components based on binding of the complex to the separation agent. In Formula IXa, SNAPTAG Protein is a SNAP-tagged protein bound to target protein on or within a cell, TAG is an uncaged SNAP-HALO dimerizer or a SNAP

ligand produced in (b) and SEPARATION AGENT is the separation agent comprising a HALO protein, streptavidin, azide, or alkyne.

In some embodiments, the disclosure provides a method of removing a protein, the method comprising:

(a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is the protein to be removed; and (2) a photochemically caged dimerizer of any one of formulas I-III and VII-VIII, or ligand of formula IV, thereby producing at least one cell comprising SNAP-tagged protein and (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged dimerizer or ligand, thereby producing an uncaged "tagging molecule" (designated below as "TAG"), under conditions under which the uncaged tagging molecule binds to SNAP and forms a complex of Formula IX:

SNAPTAG Protein —— TAG

(Formula IX);

(c) contacting the complex of Formula IX with a separation agent comprising either 1) a HALO protein (if the tag has a structure corresponding to formulas I-III or VII-VIII), or 2) a streptavidin or an azide (if the tag has a structure corresponding to formula IV), under conditions under which TAG binds to the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula IXa:

SNAPTAG Protein —— TAG —— SEPARATION AGENT

(Formula IXa); and,

(d) separating the complex of Formula IXa, which comprises SNAPTAG Protein, and therefore the target protein, from other cellular components based on binding of the complex to the separation agent. In Formula IXa, SNAPTAG Protein is a SNAP-tagged protein bound to target protein on or within a cell, TAG is an uncaged SNAP-HALO dimerizer or a SNAP ligand produced in (b) and SEPARATION AGENT is the separation agent comprising a HALO protein, streptavidin, azide, or alkyne.

In some embodiments, the disclosure relates to a method of removing a cell component, such as a target nucleic acid, from a single cell or from individual cells in a pool of cells. In the method of removing a nucleic acid, the method comprises:

(a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that binds a nucleic acid to be removed, referred to as a target nucleic acid (within a single cell or individual cells in the pool); and (2) a photochemically caged dimerizer of any one of formulas I-III and VII-VIII, or ligand of formula IV, thereby producing a cell comprising SNAP-tagged protein bound to the target nucleic acid (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged dimerizer or ligand, thereby producing an uncaged "tagging molecule" (designated below as "TAG"), under conditions under which the uncaged tagging molecule binds to SNAP and forms a complex of Formula IX:

SNAPTAG Protein ——— TAG

(Formula IX); and,

(c) contacting the complex of Formula IX with a separation agent comprising either a HALO protein (if the tag has a structure corresponding to formulas I-III or VII-VIII), or if the tag has a structure corresponding to formula IV, a streptavidin, an azide, under conditions under which TAG binds to the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula IXa:

SNAPTAG Protein ——— TAG ——— SEPARATION AGENT

(Formula IXa); and,

(d) separating the complex of Formula IXa, which comprises SNAPTAG Protein, and therefore the target nucleic acid, from other cellular components based on binding of the complex to the separation agent. In Formula IXa, SNAPTAG Protein is a SNAP-tagged protein bound to target nucleic acid on or within a cell, TAG is an uncaged SNAP-HALO dimerizer or a SNAP ligand produced in (b) and SEPARATION AGENT is the separation agent comprising a HALO protein, streptavidin, azide, or alkyne.

In some embodiments, individual cells in a pool of cells comprising (1) and (2) are identified and light is delivered to those cells, but not to other cells in the pool.

In some embodiments of the method, the region of interest is a whole cell, all or a portion of the cell membrane or a subcellular compartment. In some embodiments, light is delivered for from about 1 second to about 1 minute. In some embodiments, the wavelength, power, and length of exposure of the delivered light results in two-photon excitation and uncaging of the dimerizer. In some embodiments, the wavelength, power and length of exposure of the delivered light results in one-photon excitation and uncaging of the dimerizer.

In some embodiments, the wavelength of the light delivered is from about 300 nm to about 800 nm. In some embodiments, the wavelength is about 365 nm. In some embodiments, the wavelength is about 405 nm. In some embodiments, the wavelength is about 488 nm. In some embodiments, the wavelength of the light delivered is from about 700 nm to about 800 nm. In some embodiments, the light is delivered by laser and the power of the laser ranges from about 0.1 $mW/\mu m^2$ to 5 $mW/\mu m^2$.

Separation of the target protein or nucleic acid is carried out by methods that rely on binding of the complex of Formula V or IX (which includes the target protein or nucleic acid to be separated) to the separation agent comprising HALO protein in order to form the complex of Formula Va or IXa. In some embodiments, the cell or pool of cells is lysed before the separation step (c). In some embodiments, the separation agent comprising HALO protein is a bead. In some embodiments, the separation agent comprising HALO protein is a resin. In some embodiments, the separation step is performed by affinity chromatography, immunoprecipitation, or flow cytometry.

In some embodiments, the cells are incubated with SNAP-tagged protein, HALO-tagged protein, photochemically caged SNAP-HALO dimerizer (or photochemically caged SNAP-ligand), or any combination of the foregoing, for between about 30 minutes and about 12 hours before the delivery of light. In some embodiments, the region of interest to which light is delivered is a whole cell, all or a portion of the cellular membrane or the membrane of a subcellular compartment.

In some embodiments, light is delivered for between about 1 second and about 1 minute. In some embodiments, the delivery of light results in two-photon excitation and uncaging of the dimerizer. In some embodiments, the delivery of light results in one-photon excitation and uncaging of the dimerizer. In some embodiments, the wavelength of the light delivered is from about 300 nm to about 800 nm. In some embodiments, the wavelength of

the light delivered is about 365 nm. In some embodiments, the wavelength of the light delivered is about 405 nm. In some embodiments, the wavelength of the light delivered is about 488 nm. In some embodiments, wherein the wavelength of the light delivered is from about 700 nm to about 800 nm.

In some embodiments, the light is delivered by laser and the power of the laser ranges from about 0.1 $mW/\mu m^2$ to 5 $mW/\mu m^2$.

In some aspects, the disclosure relates to a method of delivering a viral vector to the surface of a cell or cells, the method comprising: (a) expressing a SNAP-tagged surface protein in a cell or cells, wherein SNAP is on the extracellular side of the plasma membrane of the cell or cells; (b) contacting the cells with a docking-defective viral vector expressing the HALO protein on the envelope; (c) incubating the cell or cells with at least one photochemically caged SNAP-HALO dimerizer described herein; and, (d) photoactivating a region of interest, under conditions under which the photochemically caged SNAP-HALO dimerizer is uncaged and binds to both SNAP and HALO, thereby producing a SNAP-HALO heterodimer. The formation of the SNAP-HALO dimer brings the viral vector into contact the extracellular side of the cell on which the surface protein bearing a SNAP-tag is expressed.

In some embodiments, the surface protein expressed on the extracellular side of the cell or cells is the CD4-2 transmembrane domain. In some embodiments, the viral vector comprises a docking-defective fusagenic protein, optionally a mutant version of the sindbis virus fusion protein. In some embodiments, the docking-defective viral vector further comprises a transgene to be delivered. In some embodiments, the viral vector is a lentiviral vector.

In some embodiments, the cells are incubated with the photochemically caged SNAP-HALO dimerizer for between about 30 minutes and about 12 hours. In some embodiments, the photoactivation occurs by the delivery of light. In some embodiments, light is delivered for between about 1 second and about 1 minute. In some embodiments, the delivery of light results in two-photon excitation and uncaging of the dimerizer. In some embodiments, the delivery of light results in one-photon excitation and uncaging of the dimerizer. In some embodiments, the wavelength of the light delivered is from about 300 nm to about 800 nm. In some embodiments, the wavelength of the light delivered is about 365 nm. In some embodiments, the wavelength of the light delivered is about 405 nm. In some embodiments,

the wavelength of the light delivered is about 488 nm. In some embodiments, wherein the wavelength of the light delivered is between about 700 nm to about 800 nm.

In some embodiments, the light is delivered by laser and the power of the laser ranges from about 0.1 $mW/\mu m^2$ to 5 $mW/\mu m^2$.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a graphical depiction of photocaging groups and their properties. (QY) Quantum Yield, chemical or fluorescent; (2PCS) two-photon cross section at 720-740 nm; (n.d.) no data; (NB) nitrobenzyl; (DMNB) dimethylnitrobenzyl; (7-MCM) 7-methoxycoumarinylmethyl; (DEACM) 7-dimethylaminocoumarinylmethyl; (DMCM) 6,7-dimethoxycoumarinylmethyl; (pHP) para-hydroxyphenacetyl; (Bbc) 6-bromo-7-hydroxycoumarinylmethyl; (BHQ) 8-bromo-7-hydroxyquinolino; (MNI) 4-methoxy-7-nitroindolino.

Figure 2 depicts one synthesis scheme for photochemically caged benzyl-guanine (BG) derivatives.

Figure 3 depicts synthesis schemes for BG-HALO. Figure 3A depicts one embodiment of a synthesis scheme for BG-HALO. Figure 3B depicts one embodiment of a synthesis scheme for BG-TFP-HALO (TFP denotes a tetrafluorophenylene group).

Figure 4 depicts the structures of (1) BG-biotin; (2) DMNB-BG-Biotin; (3) DMNB-BG-Biotin with an extended linker; (4) BG-alkyne; (5) BG-PEGx2-HALO; (6) DMNB-BG-alkyne; (7) BG-SS-PEGx4-HALO; (8) BG-fluorescein diacetate; (9) DMNB-BG-azide; (10) DMNB-BG-PEGx3-PEGx4-HALO; (11) DMNB-BG-PEGx2-HALO.

Figure 5 demonstrates in vivo validation of BG-HALO dimerizer. A hybrid protein (native MW = 50kDa) including both SNAP-tag and HALO-tag domains separated by a specific protease digestion site was first cleaved by TEV protease, thus releasing the two subunits (SNAP = 20 kDa and HALO = 30 kDa). This mixture was then incubated in the presence of BG-HALO or BG-SS-HALO dimerizer. Interaction of the reagent with both SNAP and HALO reformed the full size protein (lanes 3 and 5).

Figure 6 are micrographs depicting the cell-permeability of BG-HALO dimerizer derivatives.

Figure 7 shows one embodiment of a method for affinity purification using one embodiment of the photochemically caged dimerizers disclosed herein.

Figure 8 shows two embodiments of a method for affinity purification using one embodiment of the BG-HALO dimerizers disclosed herein.

Figure 9 shows data related to chromatin recovery from H2B-SNAP cells by BG-HALO and BG-SS-HALO derivatives. Figure 9A shows capillary electrophoresis of the DNA recovered after reaction of H2B-SNAP cells (SNAP) or JIMT-1 cells (Control) with BG-HALO dimerizer. Recovery is specific for the tagged cells in presence of dimerizer. Figure 9B shows capillary electrophoresis after reaction with BG-SS-HALO. Figure 9C shows qPCR quantitation of the pull-down efficiency in the presence of either BG-HALO or BG-SS-HALO dimerizer.

Figure 10 depicts one embodiment of the pull-down of ribosomes using the BG-HALO dimerizers described herein.

Figure 11 shows the results of a ribosome pull-down assay. Rp110a-SNAP cells were incubated with either the BG-HALO or BG-SS-HALO dimerizer or no dimerizer (control). RNA pull-down was detected through photometry after intercalation of a fluorescent dye.

Figure 12 describes the use of a photochemically caged SNAP-HALO dimerizer for light-dependent viral delivery of genetic material. Figure 12A depicts the structure of a pseudotyped lentiviral vector for use with the SNAP-HALO dimerizer. A fusagen protein lacking the cell-binding function is co-expressed on the virus envelope with the HALO-tag protein. Figure 12B is a graphic depiction of the first step in the light-dependent delivery of viral material. A SNAP-tag protein is expressed on the surface of the cells to which delivery of genetic material is desired; the viral vector carrying the genetic material and expressing a HALO-tag on its surface is then introduced to the cells. Figure 12C depicts the delivery of light that uncages the photochemically caged SNAP-HALO dimerizer, thereby tethering the viral vector to the cell. Figure 12D shows the delivery of the genetic material into the cell after the viral vector has been tethered to the cell by the SNAP-HALO dimerizer.

Figure 13 shows one embodiment of a purification (*e.g.*, DNA pull down) protocol.

Figure 14 shows a micrograph of HEK293 cells stained with BG-Biotin and BG-alkyne. Figure 14A shows that, unlike fluorescent BG derivatives (BG-TMR), BG-biotin does not penetrate cells unless they have been permeabilized. Figure 14B shows that BG-Alkyne can penetrate living cells even if they are not permeabilized.

Figure 15 shows the uncaging of DMNB caged BG derivatives. Figure 15A shows a thin layer chromatography differentiating non-caged BG-biotin and DMNB-caged BG-biotin

in function of their hydrophobicity. Figure 15B shows that caged DMNB-BG-Biotin can be converted into non-caged DMNB-BG-Biotin following light irradiation. Figure 15C shows a micrograph of HEK293 cells incubated with caged DMNB-BG-Biotin in which uncaging was produced in a small area (yellow signs). Biotin was then stained with streptavidin-tetramethylrhodamine. The image indicates that DMNB-BG-biotin can be uncaged through light irradiation in a spatially restricted way with high resolution. Figure 15D shows a micrograph analog to Figure 15C, in which uncaging was instead produced by two-photon excitation.

Figure 16 depicts further embodiments of synthesis schemes for photochemically caged benzyl-guanine (BG) derivatives. R can be an alkyne, azide, biotin or a combination of PEG, trifluorobenzene and chloroalkane.

Figure 17 depicts synthesis schemes for BG-HALO. Figure 17A depicts one embodiment of a synthesis scheme for BG-HALO. Figure 17B depicts one embodiment of a synthesis scheme for BG-SS-HALO (SS indicates a disulfide bond). PBS is phosphate-buffered saline, DMF is dimethylformamide.

DETAILED DESCRIPTION OF INVENTION

As described herein, at least two different types of self-ligating protein tags conjugated to a photocaging molecule may be used as tools to perform various molecular biology and biochemical techniques that may benefit from the formation of heterodimers within the bounds of specific spatial domains.

SNAP-Tag and HALO-tag are engineered proteins that bind to specific small molecules through a covalent bond. The binding is extremely specific for the correct target (*benzyl-guanine* for SNAP and *chloroalkane* for HALO) and is readily formed at physiological conditions in living cells and tissues.

One embodiment relates to a series of modified SNAP ligands bearing a photochemically caged version of benzyl-guanine. Non-limiting examples of photocaging moieties are described in FIG. 1. These molecules do not bind to SNAP unless they are activated by a focused light pulse, such as that produced by a laser beam. Light irradiation can be controlled with great specificity both spatially and temporally and, as a result, it is possible to activate the ligands in very small volumes inside cells or tissues. The resolution exceeds that of single cells, enabling specific activation within sub-cellular compartments.

In some aspects, the disclosure relates to a photochemically caged dimerizer of Formula I:

P — BG — L — CA

(Formula I),

wherein:

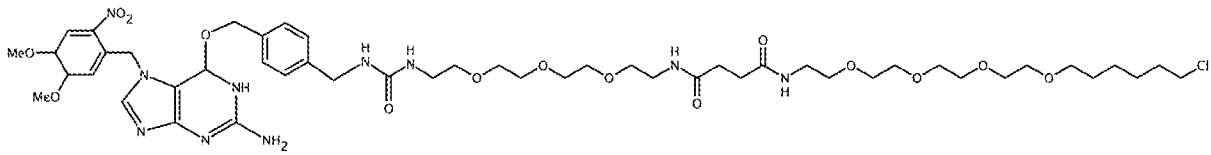
(a) P is a photocaging moiety selected from the group consisting of alpha-carboxy-2-nitrobenzyl; 4,5 dimethoxy 2-nitrobenzyl; 4,5, dimethoxy 2-nitrophenyl; and 6-nitroveratryloxycarbonyl; and bound to BG at position N_7, optionally by an ester bond;

(b) BG is O6-Benzylguanine;

(c) L is a linker that comprises at least one amide bond; at least one polyethylene glycol (PEG) moiety; or at least one amide bond and at least one PEG moiety; or at least one disulfide bond, or a single tetrafluorobenzene group, and,

(d) CA is a chloroalkane.

In some embodiments, the photochemically caged dimerizer is represented by Formula II:



(Formula II),

wherein:

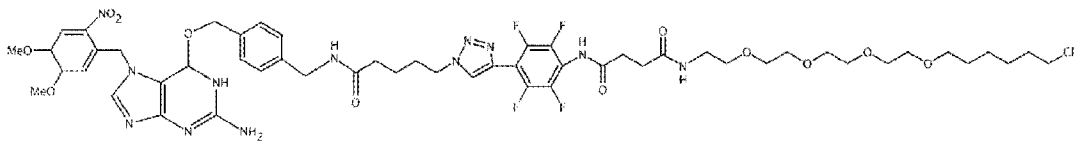
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by a carbonyl bond to L;

(c) L consists essentially of, in the following order: three PEG moieties; two amide bonds, and four PEG moieties; and

(d) CA is a chloroalkane comprising at least six carbon molecules.

In some embodiments, the photochemically caged dimerizer is represented by Formula III:



(Formula III)

wherein:

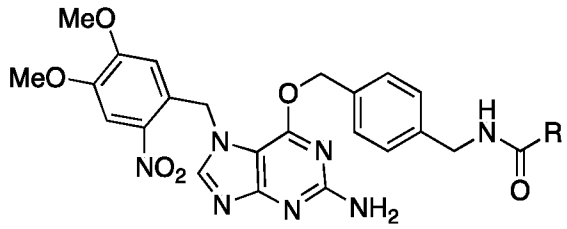
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by an amide bond to L;

(c) L consists essentially of in the following order: 1,2,3-triazole group; tetrafluorophenylene group, two amide bonds, four PEG moieties and

(d) CA is a chloroalkane comprising at least six carbon molecules.

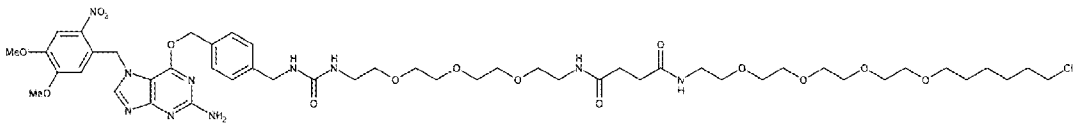
In some embodiments, the disclosure relates to a photochemically caged dimerizer represented by Formula IV:



(Formula IV),

wherein R is selected from the group consisting of an alkyne moiety, an azide moiety, biotin, and a fluorescent moiety.

In some embodiments, the photochemically caged dimerizer is represented by Formula VII:



(Formula VII),

wherein:

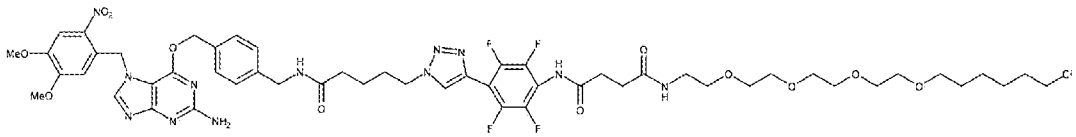
P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

BG is O6-Benzylguanine bound by a carbonyl bond to L;

L consists essentially of, in the following order: three PEG moieties; two amide bonds, and four PEG moieties; and,

CA is a chloroalkane comprising at least six carbon molecules.

In some embodiments, the photochemically caged dimerizer is represented by Formula VIII:



(Formula VIII)

wherein:

(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by an amide bond to L;

(c) L consists essentially, of in the following order: 1,2,3-triazole group; tetrafluorophenylene group, two amide bonds, four PEG moieties: and,

(d) CA is a chloroalkane comprising at least six carbon molecules.

In some aspects, the disclosure provides a method of removing a nucleic acid, the method comprising:

(a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is not the protein to be removed and binds a protein to be removed, referred to as a target protein (on or within a single cell or individual cells in the pool); and (2) a photochemically caged dimerizer or ligand of any one of formulas I-IV or VII-VIII, thereby producing a cell comprising SNAP-tagged protein bound to the target protein and (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged dimerizer or ligand, thereby producing

an uncaged "tagging molecule" (designated below as "TAG"), under conditions under which the uncaged tagging molecule binds to SNAP and forms a complex of Formula IX:

SNAPTAG Protein ——— TAG

(Formula IX);

(c) contacting the complex of Formula IX with a separation agent comprising either a HALO protein (if the tag has a structure corresponding to formulas I-III or VII-VIII), or if the tag has a structure corresponding to formula IV, a streptavidin, an azide, or an alkyne, under conditions under which TAG binds to the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula Va:

SNAPTAG Protein ——— TAG ——— SEPARATION AGENT

(Formula IXa); and,

(d) separating the complex of Formula IXa, which comprises SNAPTAG Protein, and therefore the target protein, from other cellular components based on binding of the complex to the separation agent. In Formula IXa, SNAPTAG Protein is a SNAP-tagged protein bound to target protein on or within a cell, TAG is an uncaged SNAP-HALO dimerizer or a SNAP ligand produced in (b) and SEPARATION AGENT is the separation agent comprising a HALO protein, streptavidin, azide, or alkyne.

In some embodiments, the disclosure also relates to a method of removing a cell component, such as a target nucleic acid, from a single cell or from individual cells in a pool of cells. In the method of removing a nucleic acid, the method comprises:

(a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that binds a nucleic acid to be removed, referred to as a target nucleic acid (within a single cell or individual cells in the pool); and (2) a photochemically caged dimerizer or ligand of any one of formulas I-IV or VII-VIII, thereby producing a cell comprising SNAP-tagged protein bound to the target nucleic acid (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged dimerizer or ligand, thereby producing

an uncaged "tagging molecule" (designated below as "TAG"), under conditions under which the uncaged tagging molecule binds to SNAP and forms a complex of Formula IX:

SNAPTAG Protein ——— TAG

(Formula IX); and,

(c) contacting the complex of Formula IX with a separation agent comprising either 1) A HALO protein (if the tag has a structure corresponding to formulas I-III or VII-VIII), or 2) a streptavidin (if the tag has a structure corresponding to formula IV), 3) an azide (if the tag has a structure corresponding to formula IV), or (4) an alkyne (if the tag has a structure corresponding to formula IV), under conditions under which TAG binds to the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula IXa:

SNAPTAG Protein ——— TAG ——— SEPARATION AGENT

(Formula IXa); and,

(d) separating the complex of Formula IXa, which comprises SNAPTAG Protein, and therefore the target nucleic acid, from other cellular components based on binding of the complex to the separation agent. In Formula IXa, SNAPTAG Protein is a SNAP-tagged protein bound to target nucleic acid on or within a cell, TAG is an uncaged SNAP-HALO dimerizer or a SNAP ligand produced in (b) and SEPARATION AGENT is the separation agent comprising a HALO protein, streptavidin, azide, or alkyne.

Activation of photocaging groups can be obtained either through regular irradiation or through two-photon excitation. The latter mode allows activation of the molecule deep within biological tissue in an axially constrained way, meaning no activation is produced above and below the focal point. For example, the dimerizers described herein may be activated within a single cell in a complex organ, such as the brain.

In some embodiments, activation of photocaging groups (also referred to as "uncaging" or "photoactivation") is performed by delivery of light. In some embodiments, light is delivered by laser. In some embodiments, only photocaging groups within a particular region of interest, for example a defined volume, are uncaged. In some embodiments, the region of interest is a whole cell, cell membrane

or subcellular compartment. In some embodiments, the volume of the region of interest ranges from between about 1 μm^3 to about 1 cm^3. In some embodiments, light is delivered for between about 1 second and about 1 minute.

In some embodiments, the light delivered during photoactivation can be measured. In some embodiments, the light is measured by wavelength. In some embodiments, the light is measured by energy (joules, J). In some embodiments, the wavelength of the light delivered is from about 300 nm to about 800 nm. In some embodiments, the wavelength of the light delivered is about 365 nm. In some embodiments, the wavelength of the light delivered is about 405 nm. In some embodiments, the wavelength of the light delivered is about 488 nm. In some embodiments, the wavelength of the light delivered is between about 700 nm to about 800 nm. In some embodiments, the light is delivered by laser and the energy of the laser ranges from about 0.1 $\mu J/\mu m^2$ to 5 $\mu J/\mu m^2$.

The power of the light may affect the mechanism by which the photocaging group is photoactivated. In some embodiments, the power of the light (*e.g.*, the power of a laser) ranges from about 0.1 $mW/\mu m^2$ to about 5 $mW/\mu m^2$. In some embodiments, the delivery of light results in two-photon excitation and photoactivation. In some embodiments, the delivery of light results in one-photon excitation and photoactivation.

In some embodiments, the photochemically caged ligands can be functionalized with a variety of chemical groups allowing the production of affinity purification tags (i.e. alkyne, biotin), fluorescent reporters (rhodamine, fluorescein), intracellular protein-protein dimerizers (chloroalkane, allowing SNAP-to-HALO crosslinking) and so on. A key advantage of this technology is that cells can be selected based on any combination of parameters. Currently, cells can be specifically manipulated and isolated only on the basis of the expression of cell-type specific promoters and markers. Cell-specific promoters, however, are unable to isolate gene expression to a specific single cell, and many cell populations can only be described by their morphology, position, physiological attributes and other factors which cannot be efficiently used to either capture them or manipulate them. The disclosure is related, in part, to the surprising discovery that the photochemically caged SNAP-HALO dimerizers described herein allow highly accurate spatial and temporal

manipulation of cells.

The disclosure also relates to method of removing a cell component, such as a target protein or a target nucleic acid, from a single cell or from individual cells in a pool of cells. In the method of removing a protein, the method comprises (a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is not the protein to be removed and binds a protein to be removed, referred to as a target protein (on or within a single cell or individual cells in the pool); and (2) a photochemically caged SNAP-HALO dimerizer of any one of formulas I, II, III, VII, or VIII thereby producing a cell comprising SNAP-tagged protein bound to the target protein and (2); (b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged SNAP-HALO dimerizer, thereby producing an uncaged SNAP-HALO dimerizer (BG-L-CA, designated below as DIMERIZER), under conditions under which the uncaged dimerizer binds to SNAP and forms a complex of Formula V:

SNAPTAG Protein ——— **DIMERIZER**

(Formula V);

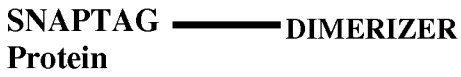
(c) contacting the complex of Formula V with a separation agent comprising HALO protein, under conditions under which the complex of Formula V binds to HALO protein of the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula Va:

SNAPTAG Protein ——— **DIMERIZER** ——— **HALOTAG**

(Formula Va); and,

(d) separating SNAPTAG Protein of Formula Va from other cellular components based on binding of the complex to the HALO protein. In Formula Va, SNAPTAG Protein is a SNAP-tagged protein bound to target protein on or within a cell, DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b) and HALOTAG is the separation agent comprising HALO protein.

In the method of removing a protein, the method comprises (a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is the protein to be removed; and (2) a photochemically caged SNAP-HALO dimerizer of any of formulas I, II, III, VII, or VIII thereby producing at least one cell comprising SNAP-tagged protein and (2); (b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged SNAP-HALO dimerizer, thereby producing an uncaged SNAP-HALO dimerizer (BG-L-CA, designated below as DIMERIZER), under conditions under which the uncaged dimerizer binds to SNAP and forms a complex of Formula V:



(Formula V); and,

(c) contacting the complex of Formula V with a separation agent comprising HALO protein, under conditions under which the complex of Formula V binds to HALO protein of the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula Va:



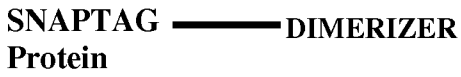
(Formula Va); and,

(d) separating SNAPTAG Protein of Formula Va from other cellular components based on binding of the complex to the HALO protein. In Formula Va, SNAPTAG Protein is a SNAP-tagged protein to be removed, DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b) and HALOTAG is the separation agent comprising HALO protein.

In some embodiments, the disclosure also relates to a method of removing a cell component, such as a target nucleic acid, from a single cell or from individual cells in a pool of cells. In the method of removing a nucleic acid, the method comprises (a) delivering to a single cell or a pool of cells (1) at least one SNAP-

tagged protein that binds a nucleic acid to be removed, referred to as a target nucleic acid (within a single cell or individual cells in the pool); and (2) a photochemically caged SNAP-HALO dimerizer of any one of formulas I, II, III, VII, or VIII thereby producing a cell comprising SNAP-tagged protein bound to the target nucleic acid (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged SNAP-HALO dimerizer, thereby producing an uncaged SNAP-HALO dimerizer (BG-L-CA, designated DIMERIZER), under conditions under which the uncaged dimerizer binds to SNAP and forms a complex of Formula V:



(Formula V); and,

(c) contacting the complex of Formula V with a separation agent comprising HALO protein, under conditions under which the complex of Formula V binds to HALO protein of the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula Va:



(Formula Va); and,

(d) separating SNAPTAG Protein of Formula Va from other cellular components based on binding of the complex to the HALO protein. In Formula Va, SNAPTAG Protein is a SNAP-tagged protein bound to target nucleic acid on or within a cell, DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b) and HALOTAG is the separation agent comprising HALO protein.

In some embodiments, individual cells in a pool of cells comprising (1) and (2) are identified and light is delivered to those cells and not to other cells in the pool.

In some embodiments of the method, the region of interest is a whole cell, cell membrane or subcellular compartment. In some embodiments, light is delivered for

between about 1 second and about 1 minute. In some embodiments, the wavelength, power, and length of exposure of the delivered light results in two-photon excitation and uncaging of the dimerizer. In some embodiments, the wavelength, power and length of exposure of the delivered light results in one-photon excitation and uncaging of the dimerizer.

In some embodiments, the wavelength is from about 300 nm to about 800 nm. In some embodiments, the wavelength is about 365 nm. In some embodiments, the wavelength is about 405 nm. In some embodiments, the wavelength is about 488 nm. In some embodiments, the wavelength is from about 700 nm to about 800 nm. In some embodiments, the light is delivered by laser and the energy of the laser ranges from about 0.1 $\mu J/\mu m^2$ to 5 $\mu J/\mu m$. In some embodiments, the light is delivered by laser and the power of the laser ranges from about 0.1mW/μm^2 to about 5 mW/μm^2.

Separation of the target protein or nucleic acid is carried out by methods that rely on the binding of the complex of Formula V (which includes the target protein or nucleic acid to be separated) to the separation agent comprising HALO protein. In some embodiments, the cell or pool of cells are lysed before the separation step (c). In some embodiments, the separation agent comprising HALO protein is a bead. In some embodiments, the separation agent comprising HALO protein is a resin. In some embodiments, the separation step is performed by affinity chromatography, immunoprecipitation, or flow cytometry.

In some embodiments of the method, the SNAP-tagged protein binds a target on or inside the cell. In some embodiments, the target on or within the cell is a protein or nucleic acid.

In some embodiments of the method, the region of interest is a whole cell, cell membrane or subcellular compartment. Examples of subcellular compartments include but are not limited to the nucleus, cytoplasm and subcellular structures (e.g. nucleolus, mitrochondria, chloroplast, Golgi apparatus, endoplasmic reticulum, lysosomes, endosomes, vesicle, vacuole, centrosome, cytosol and cytoskeleton). In some embodiments, the region of interest is defined by a volume on the surface or within a cell or cells. In some embodiments, the volume of the region of interest ranges between about 1 μm^3 and 1 cm^3.

Example 1 – Materials and Methods

Production of caged Benzyl-Guanine derivatives

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich. BG-biotin, DMNB-BG-biotin, DMNB-BG-alkyne, BG-fluorescein diacetate were custom synthesized.

To synthesize these new caged benzyl guanidine complexes the initial protocol described by Keppler et al., 2004 was modified and is shown in FIG. 2.

Reactive benzylguanine was produced by combining 2,2,2-Trifluoro-N-(4-hydroxymethyl-benzyl)- acetamide (**2** in Fig. 2) with 1-(2-Amino-7H-purin-6-yl)-1-methyl-pyrrolidinium chloride (**1** in Fig. 2) in DMF to form N-[4-(2-Amino-9H-purin-6-yloxymethyl)-benzyl] 2,2,2-trifluoro-acetamide (**3** in Fig. 2). Following that, in order to improve the synthesis and allow variability in the side chain used to extract these complexes, the dimethoxynitro benzyl photochemical protecting group was installed. Reagent (**3** in Fig. 2) was treated with 3,4-dimethoxy-6-nitro-benzylbromide (**4** in Fig. 2) in order to produce a 2:1 mixture of the N9 (**5** in Fig. 2) and N7 isomers (**6** in Fig. 2) that were easily separable by flash column chromatography. Separating the isomers at this point prevents having to use HPLC purification during the last step of the synthesis, which makes it much easier to produce larger amounts of these compounds.

The trifluoroacetamide group on the N7 isomer (**6** in Fig. 2) was then removed in refluxing methylamine to produce the N7-DMNB-BG amine (**7** in Fig. 2). This compound was then substituted with a variety of side chains through amide coupling using the desired N-hydroxy succinimidyl ester of various acids. As an example of this reaction a solution 7-DMNB-BG amine (**7** in Fig. 2) and Biotin N-hydroxysuccinimide ester (**8c** in Fig. 2) in DMF and triethylamine was stirred for 2 hours and subsequently concentrated. The resulting residue was triturated with methanol to produce the desired pure compounds (**9a-d** in Fig. 2) as white solids which could be collected by filtration. This sequence not only allows a variety of side chains to be used but the final compounds do not require chromatography making a library of compounds much easier to produce.

An azide derivative (**8b** in Fig. 2) and a fluorescein diacetate derivative were produced through reaction of (**7** in Fig. 2) with a corresponding succinimidyl ester derivative.

Compounds shown in Fig. 2 were fully characterized chemically and had the following NMR and Mass sprectral properties.

2.0 g (5.46 mmol) of *N*-[4-(2-Amino-*9H*-purin-6-yloxymethyl)-benzyl]-2,2,2- trifluoro-acetamide **3** was dissolved in DMF (20 mL) and treated with K_2CO_3 (2.26 g, 16.4 mmol).

After 5 minutes dimethoxylnitrobenzyl bromide (1.81 g, 6.55 mmol) was added and the reaction was stirred for 3 hours at room temperature. The reaction was poured into water and extracted into EtOAc. The combined organics were washed with brine, dried over $MgSO_4$, then filtered and concentrated to obtain the crude product. The crude material was purified by flash column chromatography (9:1 CH_2Cl_2/ MeOH) to separate the two isomers of the product. The desired **6** isomer was isolated in 30% yield as a white solid. ^{1}H NMR (300 MHz, CD_3OD) δ: 8.16 (s, 1H), 7.69 (s, 1H), 7.16 (d, 2H, J = 8.1 Hz), 7.04 (d, 2H, J = 8.1 Hz), 6.00 (s, 1H), 5.82 (s, 2H), 5.33 (s, 2H), 4.44 (s, 2H), 3.91(s, 3H), 3.43 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ: 164.7, 160.4, 156.9, 153.9, 148.40, 146.7, 139.9, 137.7, 135.9, 128.3, 128.1, 127.9, 110.5, 108.9, 106.4, 67.2, 56.8, 56.6, 48.2, 43.1; LCMS (ESI) m/z calc'd for $C_{24}H_{22}F_3N_7O_6$ $[M+H]^+$ 562.1617, found 562.1657. 0.91 g of **6** was suspended in methanol (5 ml) and treated with NH_2Me (15 mL). The reaction was heated to reflux for 4 hours, then cooled to 0°C to produce a yellow solid. The resulting solid was filtered and dried to obtain amine **7** in 56% yield. ^{1}H NMR (300 MHz, CD_3OD) δ: 8.26 (s, 1H), 7.78 (s, 1H), 7.26 (d, 2H, J = 7.8 Hz), 7.12 (d, 2H, J = 7.8 Hz), 6.30 (m, 3H), 5.84 (s, 2H), 5.39 (s, 2H), 3.97 (s, 3H), 3.77(s, 2H), 3.57 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ: 164.7, 160.4, 156.9, 153.9, 148.4, 146.7, 139.9, 134.5, 128.1, 127.4, 110.5, 108.9, 106.5, 67.4, 56.9, 56.6, 48.2, 46.2; LCMS (ESI) m/z calc'd for $C_{27}H_{30}N_{10}O_6$ $[M+H]^+$ 591.2383, found 591.2415.

Compound **7** (0.050 g, 0.107 mmol) and N-Hydroxysuccinimideazidopentanoic acid, **8b**, (0.036 g, 0.150 mmol) was dissolved in DMF and treated with triethylamine (0.030 mL, 0.214 mmol). The reaction was stirred for 2 hours, concentrated to obtain the crude product. The solid was triturated with methanol and filtered to obtain **9b** as a white solid in 49% yield (31 mg,). ^{1}H NMR (300 MHz, CD_3OD) δ: 8.14 (s, 1H), 7.67 (s, 1H), 7.05 (dd, 4H, J = 8.1 Hz), 6.22 (m, 3H), 5.72 (s, 2H), 5.27 (s, 2H), 4.12 (s, 1H), 3.85 (s, 3H), 3.48 (s, 3H), 2.48 (m, 2H), 2.15 (t, 2H), 1.53 (m, 4H); ^{13}C NMR (75 MHz, DMSO) δ: 172.3, 164.7, 160.4, 156.9, 153.9, 148.4, 146.7, 139.9, 135.2, 128.2, 128.0, 127.7, 110.6, 108.9, 106.4, 67.2, 56.9, 56.6, 51.2, 48.2, 42.5, 35.5, 28.7, 23.3; LCMS (ESI) m/z calc'd for $C_{27}H_{30}N_{10}O_6$ $[M+H]^+$ 591.2383, found 591.2415.

Compound **7** (0.050 g, 0.107 mmol) and N-Hydroxysuccinimidepenyt-4-ynoic acid, **8a**, (0.029 g, 0.150 mmol) was dissolved in DMF and treated with triethylamine (0.030 mL, 0.214 mmol). The reaction was stirred for 2 hours, then concentrated to obtain the crude product. The solid was triturated with methanol and filtered to obtain **9a** as a white solid in

83% yield (48 mg,). ^{1}H NMR (300 MHz,_DMSO) δ: 8.39 (m, 1H), 8.17 (s, 1H), 7.69 (s, 1H), 7.13 (d, 2H, J = 6.4 Hz), 7.06 (d, 2H, J = 3.8 Hz), 6.23 (m, 3H), 5.75 (s, 2H), 5.31 (s, 2H), 4.24 (d, 2H, J = 4.75 Hz), 3.88 (s, 3H), 3.50 (s, 3H), 2.51 (m, 1H), 2.36 (m, 4H); ^{13}C NMR (75 MHz, DMSO) δ: 172.4, 164.6, 163.1, 160.2, 156.8, 153.8, 148.3, 146.6, 139.9, 135.0, 127.9, 127.5, 110.4, 108.7, 106.2, 66.9, 61.5, 59.6, 56.6, 56.3, 55.9, 47.8, 42.2, 35.6, 28.7, 28.5, 25.7; LCMS (ESI) m/z calc'd for $C_{27}H_{27}N_7O_6$ $[M+H]^+$ 546.2056, found 546.2081.

Compound **7** (0.050 g, 0.107 mmol) and N-Hydroxysuccinimidebiotin acid, **8c**, (0.051 g, 0.150 mmol) was dissolved in DMF and treated with triethylamine (0.030 mL, 0.214 mmol). The reaction was stirred for 2 hours, then concentrated to obtain the crude product. The solid was triturated with methanol and filtered to obtain **9c** as a white solid in 72% yield (53 mg,). ^{1}H NMR (300 MHz,_DMSO) δ: 8.27 (s, 1H), 8.18 (s, 1H), 7.70 (s, 1H), 7.08 (m, 4H), 6.40 (d, 2H, J = 30.1 Hz), 6.26 (m, 3H), 5.75 (s, 2H), 5.31 (s, 2H), 4.23 (t, 4H), 3.88 (s, 3H), 3.51 (s, 3H), 3.18 (m, 2H), 2.83 (s, 1H), 2.59 (m, 3H), 2.15 (s, 2H), 1.55 (t, 2H), 1.33 (m, 2H); ^{13}C NMR (75 MHz, DMSO) δ: 172.4, 164.6, 163.1, 160.2, 156.8, 153.8, 148.3, 146.6, 139.9, 135.0, 127.9, 127.5, 110.4, 108.7, 106.2, 66.9, 61.5, 59.6, 56.6, 56.3, 55.9, 47.8, 42.2, 35.6, 28.7, 28.5, 25.7; LCMS (ESI) m/z calc'd for $C_{32}H_{37}N_9O_7S$ $[M+H]^+$ 692.2570, found 692.2596.

Non-caged BG derivatives were produced in the same way, omitting the step in which the DMNB cage is installed and moving immediately from the synthesis of benzylguanine to the removal of the trifluoroacetamide protecting group, followed by derivatization through amide coupling.

Production of BG-HALO, DMNB-BG-TFP-HALO and BG-SS-HALO

BG-HALO was synthesized from an amine derivative of benzylguanine (BG-PEG-NH2, obtained from New England Biolabs) and a succinimidyl ester derivative of the halotag ligand (Halotag O4 succinimidyl ester, Promega). 2 mg of BG-PEG-NH2 were dissolved in ±200 ul N-N dimethylformamide in presence of 1 equivalent of halotag O4 succinimidyl ester and 1.5 equivalents of triethylamine. The reaction was incubated at 30 C overnight, quenched with 10 mM glycine, and unreacted BG-PEG-NH2 was removed by adding 1mg of epoxide beads (dynabeads M270 epoxy, Life Technologies). The solution was dried *in vacuo* to produce the final product.

BG-TFP-HALOTAG was produced from DMNB-BG-azide (described above), 4-ethynyl-2,3,5,6-tetrafluoroaniline (Santa Cruz) and Halotag O4 Succinimidyl ester. 2 mg DMNB-BG-AZIDE were resuspended in 20ul DMSO and further diluted in 500ul phosphate-buffered saline. CuSO4 was added to a final concentration of 500 uM, in presence of 500 uM TBTA ligand and 5 mM Ascorbic Acid. The reaction was incubated under argon for 12 h at 30C, and the product ("product 1") dried under vacuum.

The resulting product was resuspended in 200ul DMF and additioned with 1 equivalent of halotag-O4-succinimidyl ester and 1.5 equivalents of triethylamine. The reaction was incubated at 30C overnight. The unreacted product 1 was removed by incubating the mixture with 1 mg of epoxide beads (dynabeads M270 epoxy, Life Technologies). The solution was dried *in vacuo* to produce the final product.

BG-SS-HALO was synthesized in one step by using an amine-to-thiol bifunctional crosslinker, SPDP (FIG 3B, compound **4**, Pierce, cat. 21857) to link an amine derivative of benzylguanine (BG-NH2, FIG. 3B, compound **3**, NEB, cat. S9148s) to a thiol derivative of the halotag linker (Halotag-Thiol-O4, FIG. 3B, compound **5**, Promega, cat. P6761).

BG-NH2 and Halotag-Thiol-O4 were dissolved in DMF to a concentration of 100 mM, while SPDP was dissolved (still in DMF) to a concentration of 500 mM. Equal volumes (60ml) of the three chemicals were mixed, and the resulting solution diluted to 1230 µl with 20 mM phosphate buffer at pH 7.5. The reaction was incubated for 24h at room temperature with mild shaking. 10 µl aliquots were taken at the beginning of the reaction, after 1.5h and at the end to quantify free amine content.

After incubation, the reaction was quenched by adding 100 ml of a 10% solution of BSA and incubating 30' at room temperature. The final product was used without further purification and stored (divided in 100 ml aliquots) at -20° C.

Ninhydrin staining

The quantity of free amine groups was used a proxy for the reaction progress (since the NH2 group on the benzylguanine compound gets crosslinked to SPDP as the reaction proceeds). To measure amines the popular Ninhydrin reaction was used. Ninhydrin (2,2-Dihydroxyindane-1,3-dione) is a chemical which produces a strong blue color (Ruhemann's purple) when it reacts with primary or secondary amines. To measure amines in our sample, the aliquots taken over the course of the reaction, as well as 4 scalar dilutions of BG-NH2,

were diluted in 500 ml Ethanol. 100 ml of a 3.5 mg/ml solution of Ninhydrin in ethanol were added, and the samples were incubated at 100°C for 5'. Absorbance was measured at 570 nm through an Ultrospec 7000 spectrophotometer (GE Healthcare).

Testing of BG-HALO derivatives

To test whether BG-HALO reacted with both the SNAP-tag and Halotag proteins, a purified SNAP-TEV-HALO protein, (see section 7.5 for details on the production process) which can be cleaved by the Tobacco Etch Virus protease enzyme (TEV) resulting in two subunits, was produced. The ability of the BG-HALO linker to re-form a full-length protein by attaching SNAP to Halotag was tested. The TEV enzyme used is a mutated version with enhanced properties commercialized by Promega (ProTEV plus enzyme, cat. V6101)

40 µg of SNAP-TEV-HALO protein were resuspended in 20 µl 1X proTEV buffer in presence of 2 µl of proTEV enzyme. 1 µl of a 1:4 dilution of BG-HALO ligand, or 1 µl of undiluted BG-SS-HALO ligand, were then added to some samples (one sample was left without ligand as control), and the tubes were incubated for 1 hour at 37°C. HALO-modified BG derivatives were estimated to have a final concentration of approximately 500 µM. The final protein concentration was approximately 40 µM.

After the incubation DTT was added to a final concentration of 100 µM to one of the BG-SS- HALO samples, to cleave the disulfide bond, and incubated for an additional 30' at the same temperature. All the samples were then mixed with 10ul 3X SDS "red" loading buffer, run on a 12% SDS-PAGE gel and analyzed by western blotting.

Plasmids

All cloning has been performed according to (Sambrook et al., 1989). Unless otherwise specified, enzymes were purchased from New England Biolabs (NEB) and oligonucleotides from Integrated DNA Technologies (IDT) The bacterial strains used for the propagation of plasmids (obtained from Invitrogen) were:

1. DH5α chemically competent cells(non-viral plasmids) (genotype: F- Φ 80lacZΔM15 Δ(lacZYAargF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 D-thi-1 gyrA96 relA1)

2. Stbl3 chemically competent cells (viral plasmids) (genotype: F-mcrB mrrhsdS20(rB-, mB-) recA13

supE44 ara-14 galK2 lacY1 proA2 rpsL20(StrR) xyl-5 l-leumtl-1)

pCMV-SNAPf

This plasmid expresses a mutated version of O6-alkylguanine-DNA-alkyltransferase (SNAP tag) with increased affinity for O6-benzylguanine over other alkylated nucleobases and improved kinetics under the control of the strong cytomegalovirus (CMV) promoter. The plasmid also includes a multiple cloning site upstream and downstream of SNAPf and a Neomycin resistance gene suitable for eukaryotic selection with G418, as well as an ampicillin resistance gene for propagation in bacterial strains. It is available commercially from New England Biolabs (cat. N9183S)

pCMV-H2B-SNAP

pCMV-H2B-SNAP includes the coding sequence of histone protein H2B fused in-frame with an enhanced version of SNAP-tag (SNAPf). The plasmid can be obtained from New England Biolabs (cat. N9186S).

pCMV-Rpl10a-SNAP

The coding sequence for the ribosomal protein L10a was amplified from a human cDNA library according to standard molecular cloning protocols. Briefly, HEK 293T cells were lysed and their RNA extracted using Trizol reagent (Ambion) according to the suggested protocol. cDNA was produced using the superscript III reverse transcriptase enzyme (Invitrogen) and oligo dT(20) primers. The Rpl10a cDNA was amplified using touch-down PCR (annealing temperature decreasing from 68°C to 58°Cover the first 10 cycles) using primers adding the BamHI and NotI restriction sites upstream and downstream of the gene. The PCR product was purified, digested with BamHI and NotI and cloned downstream of the SNAP tag in a similarly cut and purified pSNAPf plasmid. The identity of the plasmid was confirmed by diagnostic restriction and sequencing of the insert.

pGIPZ-H2B-SNAP

In order to produce a lentiviral vector suitable for stable transduction of JIMT-1 cells the GIPZ system was used. pGIPZ is a second generation HIV-1 based lentiviral vector optimized for the expression of short hairpin RNAs (shRNA) for the knock-down of genes in cells (Paddison et al., 2004; Silva et al., 2005).

GIPZ was chosen as a background plasmid. To produce GIPZ-H2B-SNAP, PCR was used to amplify the CMV-H2B-SNAP region of the homonymous plasmid adding XbaI and

NotI restriction sites at the 5' and 3' of the fragment. Empty (devoid of any shRNA sequence) GIPZ vector was digested with the same enzymes, removing the original promoter and turboGFP sequences. The two fragments were ligated, inserting CMV-H2B-SNAP immediately before the IRES region of GIPZ. The plasmid was propagated in stbl3 cells at 30°C to reduce the chances of recombination among the two LTRs. Success of the cloning was verified by sequencing.

pGIPZ-Rpl10a-SNAP

The CMV-SNAP-Rpl10 fragment was amplified by PCR and cloned using XbaI and NotI in an empty GIPZ vector. Plasmid was propagated at 30°C in stbl3 cells and confirmed success of the cloning by sequencing.

pT7-SNAP-TEV-HALO-6His

This plasmid includes the coding sequences of the enhanced SNAP tag (SNAPf) and Halotag proteins fused in frame, separated by a Tobacco Etch Virus (TEV) protease site and followed by a hexa-histidine tag for affinity purification. The entire complex can be expressed under the control of a T7 polymerase promoter.

The backbone vector used for cloning was the plasmid pH6HTC (His6-Halotag), available from Promega (cat. G8031). Plasmid was digested with the restriction enzymes XhoI and EcoRI, both situated upstream of the TEV cleavage site on the 5' end of the protein coding sequence. The SNAP-tag sequence was obtained from the pSNAPtag-T7(2) plasmid (NEB, cat. N9181S) through digestion with the same enzymes. The two fragments were ligated and initially transformed in DH5a cells to isolate a clone bearing the correct sequence (validated through sequencing). The plasmid was then retransformed in BL21(DE3) cells for protein expression (Invitrogen, genotype: F-ompT hsdSB (rB-, mB-) galdcmrne131 (DE3)).

psPAX2, pMD2.G

These plasmids were used in the production of lenviral particles. psPAX2 is an HIV-1 second-generation packaging vector including the HIV factors gag, pol, rev, cPPT and tat under the control of a hybrid CAG promoter (CMV early enhancer + first intron of chicken beta-actin + splice acceptor of rabbit beta globin). pMD2.G expresses the G envelope protein of the vesicular stomatitis virus (VSV-G) under the control of the CAG promoter, and allows

the production of a pseudotyped lentivirus capable of infecting essentially every cell type (VSV-G tends to bind phospholipids and general membrane components).

Cell Lines

All cells, unless otherwise noted, were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, purchased from Gibco) in presence of 10% v/v fetal bovine serum (Hyclone) and 1% v/v Pennicillin/Streptomycin antibiotic mix (Gibco). Cultures were kept in a humidified incubator at 37°C and 5% CO2.

JIMT-1

JIMT-1 is an epithelial breast carcinoma cell line derived from a pleural metastasis of a 62-year old woman in 2004 (Tanner et al., 2004). The cells grow well under standard culture condition (DMEM + 10% FBS, 5% CO2, 37°C) and are readily transfected (both by calcium phosphate and cationic compounds) and transduced by retroviruses and lentiviruses. JIMT-1 cells, as well as all the cell lines derived from them, were routinely split 1:5 every 48h or 1:3 every 24h.

HEK 293 / 293T

HEK 293 and HEK 293T cells (Graham et al., 1977) were received from the CSHL tissue culture facility and cultured according to standard protocols. HEK 293T cells were used to package lentiviruses to produce stable lines through transduction. 293 cells were split 1:4 every 48 hours, 293T cells 1:5 every 48 hours. This cell line expresses the green fluorescent protein Venus as a stably integrated transgene under puromycin selection. ERT cells have a fibroblast-like morphology.

JIMT-1(H2B-SNAP)

A 10cm cell culture plate of HEK 293T cells at approximately 60% confluence was transfected (using the calcium phosphate technique) with 17 µg pGIPZ-H2B-SNAP, 10 µg psPAX2 and 3 µg pMD2.G. After 12h the culture medium was changed and cells were incubated in standard conditions for 24 hours to allow production of the virus. After that time the culture medium containing the viral particles was collected, filtered through a 0.45 µm size filter and used as-is to transduce JIMT-1 cells. Viral titers were measured by infecting

HEK 293 cells with decreasing amount of virus, staining the cells with BG-TMR and measuring the amount of stained cells through cytofluorimetry (using a MACSQuant instrument, Miltenyi Biotec). Titers were measured to be around 104 PFU/ml.

1 ml of virus supernatant was used (diluted in 10ml of culture medium) to infect a 10cm plate of JIMT-1 cells. Infection was allowed to proceed for 12h, after which the medium was changed and cells incubated for 48h-72h to allow integration and expression of the transgene. After that time, cells were selected for 1 week with 10 mg/ml of puromycin, producing a stable population. Expression of the transgene was measure through staining with BG-TMR and Western Blot against SNAP.

HEK 293(Rpl10a-SNAP)

HEK 293 cells were stably transfected with pCMV-Rpl10a-SNAP using the following procedure: a 50% confluent plate of 293 cells was transfected with 15 µg pCMV-Rpl10a-SNAP. Cells were incubated 48h after the transfection to allow expression of the transgene. At that point, cells incorporating the plasmid in the genome were selected with 1 mg/ml G418 over the course of two weeks. Clonal selection was not performed, as the experiments that had to be carried out using these cells only required a population stably expressing the transgene, even if heterogeneous.

Production of fluorescent Halotag protein (TMR-Halotag)

In order to stain cells incorporating BG-HALO derivatives, a fluorescent Halotag protein was produced. SNAP-TEV-Halotag was diluted to 3mg/ml (approx 60 µM) in "SNAP buffer" (PBS + 1 mM DTT) and reacted for 1h at 37°C with a 2:1 molar excess (120 µM) of BG-TMR (NEB). The unbound fluorophore was removed by two consecutive round of microfiltration through a 10 MWCO cellulose spin filter (Amicon Ultra 4, Millipore), each time diluting with 4ml of PBS.

Incorporation of the fluorophore in the protein was measured by running it on a 12% SDS-PAGE gel and detecting the presence of a fluorescent band of the correct size using a FLA5000 phosphorimager (Fuji photo film Co.)

Example 2 - In vitro and cell culture testing of BG derivatives

Instruments used for uncaging

Three different optical systems were used to uncage DMNB-modified BG derivatives, depending on the experimental context.

The first system was simply a beam expander for a 405 nm laser light source: a 150 mW 405 nm laser diode module (Aixiz LLC, cat. AIX-405-150T) was mounted on a vertical column aligned with a 60mm focal convex lens. The expanded beam was projected on the base of the column. The vertical position of the lens could be varied to change the size of the projected spot. An iris ring placed after the lens allowed to "clean" the laser beam excluding the irregular peripheral areas. Using a radiometer, the final power (post-lens) was measured to be approximately 100 mW, which corresponded to ~1 mW/mm^2 when the beam was enlarged to cover a 1 cm^2 area. This instrument was used for bulk uncaging of the compounds in vitro and for the uncaging of large areas.

The second system was a commercial Zeiss LSM 710 confocal laser scanning microscope equipped with a 405 nm 30 mW diode laser line. The uncaging mask was obtained by scanning the focused laser beam across the sample and switching it on and off in different positions. For most experiments, a 512 x 512 pixel grid was used in the scan. The LSM 710 instrument was used in the experiments aimed at testing one-photon uncaging and its resolution limits.

The third instrument, used to test multiphoton uncaging, was a two-photon imaging system. The light source used was a Chameleon mode-locked Ti:Sapphire laser (Coherent Inc.) providing tunable illumination in the 680-1080 nm range. Light intensity was tuned using a pockel cell (which varies the polarization of a passing beam in response to a current) coupled with a polarizer. The final power delivered to the sample depended on the pockel cell settings. Using a radiometer the actual power delivered to the focal point when different voltages were supplied to the pockel cell control circuit was measured. The maximum power used was 60 mW for 0.3 V at 720 nm.

The beam was steered with a couple of galvanometric mirrors and focused on the sample plane through a 10X objective. Excitation light coming from the objective was reflected by a first dichroic mirror, separated in two components (with $l<500$ nm or > 500 nm) by a second dichroic and detected through two photomultiplier tubes (PMT) placed behind a bandpass filter (allowing red and green light respectively).

Since this instrument doesn't allow to switch on or off the laser beam at each pixel, the size of the irradiated area was increased or decreased by changing the magnitude of the

driving signal sent to the galvanometric mirrors. A signal between +1 and -1 volt produced a smaller scan pattern centered around the optical axis (used for uncaging), while a signal ranging between +4 and -4 V produced a wider scan suitable to image at the same time the uncaged area and the surrounding cells.

In vitro validation of uncaging

Thin layer chromatography

N7-DMNB-BG-Biotin and BG-Biotin were dissolved in DMSO at a 2 mM concentration. Small aliquots of the two compounds were loaded on a 5x10cm silica gel TLC plate impregnated with a fluorescent indicator (Sigma Aldrich, cat. 91835) and let dry completely. The plate was developed in a mobile phase mixture composed of Chroloform, Methanol, water and acetic acid in proportions 50:33:16.5:0.5 until the front reached to upper margin, and imaged by illumination at 254 nm using an agarose gel transilluminator.

To verify uncaging, a solution of N7-DMNB-BG-Biotin in DMSO was irradiated using an expanded laser beam for 30 minutes and run on a TLC membrane alongside a non-irradiated sample. Development was executed in the same way.

SNAP pull-down assay

The ability of caged and uncaged BG-biotin to bind the SNAP protein in vitro was measured by pulling down tagged proteins using streptavidin beads. 0.5 nmol of N7-DMNB-BG-Biotin, N9-DMNB-BGBiotin and BG-biotin (not caged) were incubated with 0.25 nmol of purified SNAP protein (from NEB, cat. P9312) in 50 ml phosphate buffered saline + 1 mM DTT for 1h at 37° C. After incubation, the samples were diluted to 500 ml with PBS and incubated with 0.5mg streptavidin magnetic beads (M280 streptavidin dynabeads, Invitrogen) for 2h at 4C. The beads were washed 5 times in PBS+0.1% v/v Tween 20 (PBST) and resuspended in 1X SDS loading buffer (20 mM Tris-HCl pH 6.8, 0.6% (w/v) SDS, 3% glycerol, 30 mM DTT, phenol red) before loading on a SDS-page gel. Protein pull-down was detected by western blot against SNAP

Staining of BG derivatives in cultured cells

Staining of BG-biotin

JIMT-1(H2B-SNAP), HEK 293 (Rpl10a-SNAP) or JIMT-1 cells were washed in PBS buffer and subject to fixation and permeabilization protocols.

After the permeabilization step, cells were washed in PBS again and incubated in a sufficient volume of complete growth medium (DMEM + 10% FBS + antibiotics) containing a 5 mM dilution of the benzylguanine derivative (BG-Biotin or DMNB-BG-Biotin). Incubation was carried out for 30 minutes in the tissue culture incubator (37°C, 5% CO2). After that, cells were washed 3 times for 5' and once for 30' in complete culture medium to remove unbound BG. Aspecific binding sites were blocked by incubating the sample with 2% w/v BSA in PBS for 15 minutes at room temperature.. A streptavidin-fluorophore conjugate (Neutravidin-tetramethylrhodamine, Invitrogen, cat. A-6373) was then diluted to a final concentration of 4 mg/ml in PBS and finally added to the samples for 30' at room temperature. The sample were imaged through the previously described Nikon Eclipse Ti microscope.

After the initial permeability assay, and for all the following experiments, cells were fixed for 10 minutes in 10% v/v PFA/PBS and permeabilized for 8 minutes in 0.2% v/v triton X-100 in PBS. Figure 14 shows a micrograph of HEK293 cells stained with BG-Biotin and BG-alkyne. Figure 14A shows that, unlike fluorescent BG derivatives (BG-TMR), BG-biotin does not penetrate cells unless they have been permeabilized. Figure 14B shows that BG-Alkyne can penetrate living cells even if they are not permeabilized.

Staining of BG-alkyne

Live JIMT-1 or JIMT-1(H2B-SNAP) cells were directly incubated with 5 mM BG-Alkyne (diluted in complete growth medium) for 30 minutes at 37°C, followed by three 5' and one 30' washes in the same medium without BG.

Following the SNAP reaction, cells were washed once in PBS, fixed for 10' in 10% PFA/PBS, washed again twice in PBS and permeabilized for 8 minutes in 0.2% Triton X-100 in PBS.

Alkyne was detected through the copper-catalyzed alkyne-azide click reaction (CuAAC) with tetramethylrhodamine-azide (TAMRA-Azide, Invitrogen, cat. T10182) using the Click-IT cell reaction buffer system (Invitrogen, cat. C10269) according to instructions. Briefly, a "click staining solution" was prepared by mixing the following reagents in H2O: 1/11th volumes Click reaction buffer 10X (solution A), 2 mM CuSO4 (solution B), 1/10th

volume click reaction additive (solution C), 5 uM TAMRA-Azide. The staining solution was incubated on the permeabilized cells for 30 minutes at room temperature in the dark, followed by three washes in PBS+0.1% Tween 20 (PBST). Samples were imaged as described above. Figure 14B shows that BG-Alkyne can penetrate living cells even if they are not permeabilized.

Staining of BG-HALO and BG-SS-HALO

Live JIMT-1 or JIMT-1(H2B-SNAP) cells were incubated for 2h with 2.5 mM SNAP-HALO r 12.5 mM SNAP-SS-HALO. Cells were washed, fixed and permeabilized as described above for BG-Alkyne. Presence of an immobilized HALO group was accomplished by incubating cells for 1h at 37°C in 0.3 mg/ml TMR-Halotag protein diluted in PBS + 1 mM DTT. The samples were washed twice for 10 minutes in PBS and imaged as described above.

One photon uncaging

For bulk uncaging experiments the same protocol indicated above was followed. DBMB-BG-Biotin was diluted in complete growth medium to a 5 mM concentration and uncaged under an expanded laser beam for 30' before adding it to the fixed and permeabilized cells.

For confocal uncaging experiments an LSM 710 laser scanning microscope (Zeiss) was used. Cells were cultured in a microfluidic chamber build from a four-wells imaging slide (LAB-TEK) which allowed to independently change buffers on two samples, performing all the steps of the reaction while maintaining the cells in focus under the microscope.

To test uncaging of DMNB-BG-biotin, cells grown in the microfluidic chamber were fixed and permeabilized. Nuclei were counterstained with a 1:1000 dilution of the DNA intercalating agent DRAQ5 (Cell Signaling Technology Inc.) in PBS, and cells were incubated in caged BG-Biotin for 15' before irradiation. The signal from the nuclear counterstain was detected through a 630 nm laser line and a Cy5 emission filter and used to focus the cells. Uncaging was produced through the 405 nm laser line using the "regions" function of the control software (ZEN 2011, Zeiss) to define an uncaging mask. Both the duration and power of irradiation were varied depending on the experiment.

After uncaging the sample was incubated in diluted DMNB-BG-Biotin for an additional 10 minutes to allow completion of the labeling reaction. Cells were then washed three times 5' and once 30' in growth medium to remove unreacted BG. Aspecific binding sites were blocked through a 15' incubation in 2% w/v BSA in PBS, after which biotin was stained with 4 mg/ml Neutravidin-TMR for 30'. Images showing the stained areas were acquired using the 560nm laser line and a rhodamine emission filter.

For DMNB-BG-Alkyne uncaging, the protocol was modified. Cells were counter-stained in DRAQ5, incubated with the 5 μM caged BG-alkyne before fixation and uncaged as described for BG-Biotin. After uncaging, cells were fixed, permeabilized and stained with TAMRA-Azide as previously described.

Figure 15 shows the uncaging of DMNB caged BG derivatives. Figure 15A shows a thin layer chromatography differentiating non-caged BG-biotin and DMNB-caged BG-biotin in function of their hydrophobicity. Figure 15B shows that caged DMNB-BG-Biotin can be converted into non-caged DMNB-BG-Biotin following light irradiation. Figure 15C shows a micrograph of HEK293 cells incubated with caged DMNB-BG-Biotin in which uncaging was produced in a small area (yellow signs). Biotin was then stained with streptavidin-tetramethylrhodamine. The image indicates that DMNB-BG-biotin can be uncaged through light irradiation in a spatially restricted way with high resolution. Figure 15D shows a micrograph analog to Figure 15C, in which uncaging was instead produced by two-photon excitation.

Two photon uncaging

For two-photon uncaging experiments, cells were grown on round coverslips (RD GERMAN COVERSLIP, 12MM,#1, Bellco Glass, cat. 1943-10012). The glass surface was treated with 100ug/ml Poly-D-Lysine (Sigma) for >3h, washed 5 times in water and completely dried before cells were seeded.

A microfluidic imaging chamber was produced from the lid of a 30mm cell culture plate by attaching to it two blunt needles used as "inlet" and "outlet" port and connected to syringes through silicone tubing and luer-lock adapters. The coverslips with the cells was transferred in this chamber before each experiment, and all the processing steps after uncaging were done under the microscope.

Cells were fixed and permeabilized and counter-stained in DRAQ5 as previously described. 5μM DMNB-BG-Biotin was then added and the cells incubated for 30' at 37°C. The coverslip was at this point placed in the imaging chamber under the microscope. The Ti:Sapphire laser emission was set to 680nm, sufficient to excite DRAQ5 in one-photon mode, and the galvanometric mirror scaling factor set to 0.5 (narrow field of view). The far-red fluorescent emission from DRAQ5 was used to focus the cells.

The laser was then switched to 720 nm (corresponding to the 2p excitation peak of DMNB), and the sample was irradiated for a defined time (regulated by opening and closing a beam shutter) and with a defined intensity (regulated by changing the pockel cell voltage).

After uncaging, cells were incubated for 10 extra minutes, washed and blocked as described above, and stained with fluorescent streptavidin (Neutravidin). Staining was done using Neutravidin-Alexa 488 instead of Neutravidin-TMR since the Alexa-488 dye is better suited for 2p excitation. Green fluorescence was detected by switching the laser to 810 nm and setting the galvanometric mirrors to 4 (wide field of view) in order to detect the neighboring non-uncaged area.

Figure 15D shows a micrograph analog to Figure 15C, in which uncaging was instead produced by two-photon excitation.

Example 3 - BG-HALO mediated pull-down

Production of Halotag protein beads

Halotag functionalized magnetic beads were produced by reacting the SNAP side of the purified SNAP-TEV- Halotag protein with benzylguanine modified beads (SNAP-capture magnetic beads, NEB, cat. S9145S).

The beads (100 μl of suspension) were washed once in 500 μl of PBS+0.1% v/v Tween 20 (PBST) and reacted with 100 mg of SNAP-TEV-Halotag protein in "SNAP reaction buffer" (PBST+1 mM DTT) for 3h at room temperature with end-to-end rotation. After this incubation, the beads were washed three times for 10 minutes in PBST, transferred to a new tube, and stored at 4°C until they were used in the pull-down protocol. Beads were never stored for more than 24h.

Chromatin pull-down protocol for BG-HALO and BG-SS-HALO

Different dilutions of BG-HALO and BG-SS-HALO were made in complete growth medium as detailed in the results, and incubated on JIMT-1(H2B-SNAP) and JIMT-1 cells overnight. The following day, cells were washed three times for 10 minutes and once for 30 minutes in complete growth medium, washed once in PBS, fixed in 1% formaldehyde/PBS, quenched in 0.125M glycine and washed twice more in PBS, as previously described.

After fixation, cells were lysed in 1 ml of a lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM DTT, 1 mM PMSF protease inhibitor, 1:50 Baculogold protease inhibitor cocktail (BD Biosciences), 5 mM EDTA, 1% Triton and 100 mg/ml RNAse A. The baculogold protease inhibitor mix was used to replace the complete MINI EDTA-free mix, which contains a compound which inhibits the Halotag reaction.

After lysis, nuclei were enriched by centrifugation, SDS was added and chromatin was fragmented as previously described. The samples were then centrifuged twice through a 10 k MWCO microfiltration device to remove SDS and replace the buffer with one more suitable for the halotag reaction (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.1% Tween 20). The resulting material was diluted to 1ml with the same buffer and, after a 100 ml input aliquot was taken, incubated on magnetic Halotag beads for 3h at room temperature with end-to-end rotation.

The samples were washed, processed and purified according to the same protocol described for the BG-Biotin and the BG-alkyne compounds.

Example 4 - Ribosome profiling through BG-HALO

Polysome fractionation

A linear gradient of sucrose in polysome buffer (10 mM HEPES pH 7.4, 15 mM MgCl2, 100 mM KCl, 2 mM DTT, 100 mg/ml cycloheximide) ranging from 50% to 15% was prepared in 13 ml polyallomer ultracentrifuge tubes with a Hoefer SG Gradient maker (Fisher Scientific) and stored at 4°C until use.

All buffers contained 100 mg/ml cycloheximide, necessary to prevent ribosome dissociation.

A plate of approximately 70% confluent HEK 293(Rpl10a-SNAP) cells was incubated in 100 mg/ml cycloheximide (dissolved in complete growth medium) for 15 minutes, washed in PBS + cycloheximide, scraped from the cell culture vessel, pelleted through centrifugation (five minutes at 600 RCF) and resuspended in 1 ml of a polysome

lysis buffer containing 10 mM HEPES pH 7.4, 10 mM KCl, 15 mM MgCl2, 3ml/ml RQ1 Rnase-Free DNAse (Promega), 2 mM DTT, 1 pill/10ml Complete MINI EDTA-Free protease inhibitor, 2 ml/ml RNASin RNAse inhibitor cocktail (Promega) and 100 mg/ml cycloheximide.

Lysis was produced by adding 50 ml of a 10% w/v solution of Sodium Deoxycholate and 50ul of a 10% w/v solution of Trion X-100 (final concentration 0.5% for both). The lysate was incubated for 10' on ice with occasional vortexing to complete the lysis process and spun for 10' at 900 RCF to remove the insoluble fraction.

The absorbance of the sample at 260 nm (indicative of its nucleic acid content) was measured, and a volume of sample corresponding to approximately 20 absorbance units was loaded on top of the pre-made sucrose gradient. The samples were then centrifuged for 2h at 38000 RPM using a SW 41 Ti rotor in a Beckman-Coulter ultracentrifuge to obtain isopycnic separation of polysomes.

The centrifuged samples were fractionated and analyzed using an ISCO UA-6 fluorescent detector connected to a fraction collector and a peristaltic pump, and 1ml fractions were collected corresponding to the cytosolic, monosomal and polysomal fractions. A 20 ml aliquot was taken from each fraction and analyzed through western blot both for presence of the SNAP protein and for the endogenous Rpl10a protein (using a polyclonal antibody from Abcam, cat. ab102079, at a concentration of 1 mg/ml)

Ribosome pull-down

Ribosome purification from HEK 293(Rpl10a-SNAP) or regular HEK 293 cells was performed using as input one 10cm plate containing approximately 15 $x10^6$ cells for each sample.

Cells were incubated overnight (at 37°C and 5% CO2) in presence of BG-HALO or BG-SSHALO (diluted in complete growth medium), at a calculated concentration of 2.5 mM for BG-HALO and 12.5 mM for BG-SS-HALO. The following days, cells were washed three times for 5' and once for 30' in complete medium.

After the last wash, 100 mg/ml were added to the cell culture medium, and ribosomes were stalled for 15' at 37°C, followed by a wash in PBS+cycloheximide. The cells were then lysed in 1 ml of polysome lysis buffer (20 mM Phosphate buffer pH 7.4, 150 mM KCl, 5 mM MgCl2, 1:50 Baculogold protease inhibitor cocktail, 1 mM PMSF protease inhibitor, 100

mg/ml cycloheximide, 1 mM DTT, 10 ml/ml RQ1 Rnase-Free DNAse, 1 ml/ml RNASin Rnase inhibitor cocktail, 0.5% w/v Sodium Deoxycholate, 0.5 v/v Triton) and incubated for 10' on ice with periodic vortexing. The cells were further disrupted by rapidly aspiring them through a gauge 26 sterile needle (10 strokes), followed by an additional incubation on ice for five minutes.

The lysate wash then cleared by centrifugation (2000 RCF, 5', 4°C), followed by detergent removal through microfiltration (using 10 MWCO microfiltration devices as previously described). Samples were diluted to a total volume of 500 ml with polysome dilution buffer (20 mM Phosphate buffer pH 7.4, 150 mM KCl, 5 mM MgCl2, 1:50 Baculogold protease inhibitor cocktail, 1 mM PMSF protease inhibitor, 100 mg/ml cycloheximide, 1 mM DTT) and a 50 ml aliquot removed and used as input sample.

The cleared lysate was applied to 100 ml of halotag magnetic beads (prepared as previously described) and incubated for 3h at room temperature with end-to-end rotation to allow pull-down of ribosomes. Five washes were performed (5 minutes each) in "Polysome wash buffer" (20 mM Phosphate Buffer pH 7.4, 250 mM KCl, 10 mM MgCl2, 100 mg/ml cycloheximide, 0.5% Sodium Deoxycholate, 0.5% Triton), with a tube change after the third wash. The sample was then washed four times quickly in PBS and resuspended in 100 ml PBS.

RNA was eluted by the addition of SDS (to a 0.5% w/v final concentration) and proteinase K (1 mg/ml) followed by a 2 hour incubation at 37°C to degrade protein and release the immobilized ribosomes. The protein fragments were then removed by organic extraction (using acid phenol followed by chloroform and ethanol precipitation at -80°C overnight in presence of 300 mM NaCl) yielding pure RNA.

The RNA amount was measured using a qubit spectrophotometer and SYBR dye incorporation using the Qubit RNA HS assay kit (Invitrogen, cat. Q32852)

Example 5 - A caged version of BG-HALO

Production of caged compounds

The LaserTAG technique is based on conferring light-dependency to the reaction between the SNAP-tag protein and benzylguanine. The reaction mechanism of SNAP is known in detail and the structure of human O6-alkylDNAalkyltransferase, the enzyme from which it derives, has been obtained (Wibley et al., 2000). The reaction process involves the

docking of benzylguanine to a cleft on the protein surface near the active site LIP(C)HRVI, followed by a one-step nucleophilic substitution between the SH group of Cys145 and the O6 position of BG. As it can be noted from the structure, the nitrogen in position 7 on the nucleobase (N7) is facing towards the protein surface, forming hydrogen bonds with residues in the active site, while the nitrogen in position 9 (N9) is facing away from the protein.

Since the nature of the functional group to be caged prevented the use of high-efficiency nitroindole or coumarin-based cages, a dimethoxynitrobenzene group (DMNB) was chosen due to its excellent reactivity, relatively long excitation wavelength (350 nm maximum, but still acceptable at 405nm) and sufficient two photon cross-section.

Synthesis of BG-Halo derivatives

The Halotag protein is notable, among self-labeling protein tags, for having a reaction rate (~ $10^6 M^{-1}s^{-1}$, SNAP in contrast has a rate of ~$10^4 M^{-1}s^{-1}$,), almost as high as the K_{on} of streptavidin-biotin. The bond formed between halotag and its ligand, a long-chain chloroalkane, is covalent and specific and the reaction is efficient over a wide range of pH and buffer compositions. As a consequence, Halotag may be used, when attached on a solid support, to purify biomolecules previously conjugated to a chloroalkane with an efficiency superior to click chemistry and similar to that of streptavidin, while retaining the advantages of covalent binding. In addition to this, the halotag ligand is cell permeable.

Synthesis of BG and halotag building blocks exploited the commercially available succinimidyl ester - amine reaction to produce the first reagent, BG-HALO, in one synthetic step (FIG. 3). The reaction proceeded overnight with sufficient yield to use the compound without further purification.

The caged version of the non-cleavable BG-HALO compound was produced by reacting a shorter succinimidyl ester Halo building block (with just two oxygen molecules) with the caged compound DMNB-BG-NH2. The resulting molecule is shown as compound **6** in FIG 4., which also depicts additional molecules produced in this study.

In-vitro validation of caged BG-HALO

Protein precipitation assay

The activity of the BG-HALO and BG-SS-HALO were validated towards both the SNAPtag and the Halotag proteins. To do so, a hybrid protein that includes both the domains

separated by a peptide sequence specifically recognized by the tobacco etch virus protease (TEV) was cloned and expressed in E. coli. Upon incubation with the TEV enzyme, the protein is split in its two constituents, having a molecular weight of 20 kD and 30 kD respectively. If both functional groups of BG-HALO are active, addition of the compound to the mix should result in crosslinking of the two proteins and reconstitution of the full-size complex (50 kD).

Indeed this is the case, as shown in FIG. 5. Incubation of approximately equimolar amounts of linker and protein resulted in crosslinking of at least 50% of the SNAP and HALO subunits that were present in solution.

Example 6 - Cell culture validation of uncaging

Permeability of Benzylguanine derivatives

As a first step towards the validation of LaserTAG in cell culture, the ability of the compounds to cross the plasma membrane and reach the inside of the cell was assayed. Biological membranes are normally permeable only to small and non-polar compounds, a category that does not include most of the affinity probes commonly used (antibody being a clear example). Using a probe on living samples, however, may require the probe to cross the cellular membrane or even the nuclear membrane (if the target protein is localized in the nucleus, as it is the case for DNA-interacting proteins). Though this can be achieved by transfection methods (liposomes, cationic molecules, dendrimers etc) or permeabilization, spontaneous diffusion of a cell-permeable compound can provide a simpler approach..

Many of the benzyl-guanine derivatives produced so far are able to cross the membrane (Keppler et al., 2004). Such is the case, for instance, of BG-fluorescein or BG-rhodamine (which can be obtained commercially), or BG-tetramethylrhodamine (BG-TMR) from NEB, which was used in this study. . In order to test the BG-HALO compounds, detection of the immobilized molecule had to be done using a fluorescent Halotag protein, which is not commercially available. To produce fluorescent Halotag protein, the SNAP::TEV::Halotag protein that was previously produced and already used for the in vitro validation of the compound was used. To make this protein fluorescent, the SNAP moiety was labeled with a BG-rhodamine derivative, effectively obtaining a red fluorescent Halotag derivative. Then, the permeability assay on BG-HALO and its cleavable analog BG-SS-HALO were performed. Both compounds readily entered the cells without permeabilization

(over the course of ~1h incubation) and were detected by the fluorescent Halotag protein (FIG. 6), indicating that they could be used for LaserTAG on live cells.

Example 7 - Chromatin pull-down from live cells using BG-Alkyne and BG-HALO

BG-Biotin is better suited for use on fixed and permeabilized cells, but may aspecifically bind and stick to non-tagged cells when irradiated by a 405 nm light. The benzylguanine derivatives, BG-Alkyne and BG-Halotag, have superior features, since they can be used on intact, living cells and they, also, provide a covalent linkage (even stronger than streptavidin-biotin) between the SNAP-tagged protein and the affinity precipitation beads.

BG-HALO chromatin purification

Production of SNAP-TEV-HALO recombinant protein

A Halotag protein may also be used as a specific probe for affinity purification in the same fashion as streptavidin. A BG haloalkane (BG-HALO) ligand is particularly suited for this approach. Compared to steptavidin, it has the additional advantage of forming a covalent bond that can withstand stronger washes and allow (potentially) higher specificity. Additionally, it allows use of the cell-permeable ligand in the first step of a pull-down experiment. A general schematic of a this pull-down assay is shown in FIG. 7, where BG-HALO may be substituted for BG-Alkyne.

Several options are available for the immobilization of proteins on beads, mostly based on reactive groups (epoxide, succinimidyl esters, iodoacetyl) that are able to bind amines and/or sulfhydryl on the side chain of amino-acids.

Another approach is site-specific binding: a specific domain, or short peptide, is attached to the protein, and a chemical reaction specifically forms a covalent bond between it and reactive group on the solid support. Several strategies exist to achieve this: they may be based on either chemical reactions (indeed, click chemistry itself can be used) or "protein ligase" enzymes (transpeptidases, split inteins) (Rusmini et al., 2007).

In addition to creating a form of the Halotag protein suitable for the site-specific immobilization on beads, a hybrid SNAP-Halotag protein was designed to validate the reactivity of the bifunctional BG-HALO compound. This also provides a practical way to obtain site-specific attachment; specifically, a BG-modified bead (commercially available

from NEB) can be used to bind the protein through the SNAP side, leaving the Halotag site free to react, and immobilize, haloalkane-modified molecules in a lysate. In this configuration, the SNAP protein acts as a "spacer" between the bead and the Halotag domain, increasing its "degrees of freedom" and potentially its reactivity. Furthermore, the addition of a protease cleavage site (TEV) in between the SNAP and Halotag groups allows specific elution of the captured targets.

A bacterial expression system was used to produce a SNAP-TEV-Halotag protein. A T7-TEV-Halo-His6 plasmid (available from Promega) was modified by adding the coding sequence for SNAP at the 5' of the TEV site. The plasmid was then introduced in the BL21 strain of E.coli, which expresses T7 polymerase under the b-galactosidase promoter, induced protein production with IPTG, and purified the recombinant protein using Nickel-NTA resin (which specifically binds the exahistidine tail). The final product was a ~50 kD protein retaining both the SNAP-tag and Halotag functions (FIG. 8A and FIG. 8B). Protein expression was validated by reacting it with both BG-rhodamine and haloalkane-rhodamine. The Halotag-functionalized surface was produced by reacting the protein with BG-modified beads (FIG. 8C).

Chromatin pull-down from through BG-halotag

The main change to the original pull-down protocol used for BG-biotin was the introduction of a buffer exchange step after lysis and sonication to prevent loss of activity of the Halotag protein, by removing the detergents (mostly SDS) used to solubilize chromatin and dissolve the nuclear membrane. The fragmented chromatin was filtered several times through a 10000 MWCO size exclusion filter, completely replacing the lysis buffer (containing SDS and Triton) with a physiological buffer (PBS) supplemented with only a small amount of reducing agents. As with BG-alkyne, BG-HALO readily crossed the cell membrane without fixation, allowing the entire first part of the protocol to be conducted on living cells (until just before lysis).

Two different BG-HALO derivatives were produced: a shorter version, where the two ligand groups are directly linked, and a longer one, where a disulfide linker is added between them. The results of the initial chromatin pull-down experiments indeed confirmed the function of these derivatives. Specific recovery of DNA occurred only when both the tagged histone and the ligand were present, as shown in FIG. 9.

Overall, the results validate use of the Halotag protein as an affinity probe for pull-down of HALO-tagged molecules.

Example 8 - RNA can be selectively purified through BG-HALO

The adapted LaserTAG protocol for ribosome precipitation, described below and outlined in FIG. 10, begins with cycloheximide treatment of live cells expressing SNAPRpl10a (or control cells). Once ribosomes are stalled, cells are incubated with the BG-HALO linker, which covalently binds to the ribosomal protein. This is the stage in which the uncaging can be applied. The unbound BG derivative is then removed by repeated washes.

The lysis step is conceptually similar to the one used in chromatin recovery, though strong detergents like SDS, are not used in favor of milder agents that preserve ribosomes integrity while still removing the cell membrane. Since sonication is not present, cells are mechanically destroyed (by crushing them through a fine gauge needle), and the lysate is cleared by centrifugation. Pull-down is then executed using BG-Halotag beads, and the unbound molecules are removed though a series of washes. As last step of the protocol, RNA is eluted from the beads through proteinase K treatment and organic extraction (acid phenol/chloroform) while residual DNA is removed by treatment with DNAse; the eluate is then measured by SYBR incorporation (using dyes specific for RNA) or electrophoresis.

As an initial test of the technique, the protocol was performed on Rpl10a-SNAP and JIMT-1 cells, using both the SNAP-HALO and the SNAP-SS-HALO. RNA was specifically purified only when both the ligand and the tagged protein were present, a result similar to the ones obtained in chromatin pull-down experiments.

RNA recovery was ~18-20 fold higher in the positive sample than in controls where BG-HALO was not provided (FIG. 11) or where SNAP was not present on the ribosome (not shown). The pull-down eluate was remarkably clean, indicating that good enrichment ratios can be achieved using the technique.

At the end of the pull-down protocol, samples are ready for analysis. High-throughput sequencing of short-length RNA samples (as in the case of most samples derived from ribosome purification) requires prior amplification and reverse transcription steps in order to produce a dsDNA library suitable for processing. Moreover, at this point, it is necessary to remove all the ribosomal RNA (which composes the vast majority of the sample) in order to detect the fainter signal from messenger RNAs.

Several protocols exist for this purpose. Here, single primer isothermal amplification (SPIA) embedded in a kit (Ovation RNA-seq, Nugen) was used.

Example 9- Viral delivery of genetic material by formation of SNAP-HALO heterodimers

FIG. 12A shows the proposed structure of a pseudotyped lentiviral vector for use with the SNAP/HALO heterodimer system. A fusagen protein lacking the cell-binding function will be co-expressed with the HALO-tag protein on the surface of a virus particle. The binding and fusion functions will be completely independent.

A schematic depiction of light-inducible viral delivery of genetic material is shown in FIG. 12B to 12D. Briefly, an extracellular SNAP fusion protein is be expressed on the surface of the cell or cells to which the genetic material is to be delivered (FIG. 12B). A HALO-tag pseudotyped lentivirus is then be delivered to the cells, followed by a photochemically caged SNAP-HALO dimerizer. Delivery of light uncages the SNAP-HALO dimerizer and forms a SNAP-HALO dimer complex, thus tethering the virus to the membrane of a target cell (FIG. 12C). The fusagenic protein on the viral envelope then mediates infection and the virus is endocytosed, thus delivering the genetic cargo (FIG. 12D).

CLAIMS

What is claimed is:

1. A photochemically caged dimerizer of Formula I:

P — BG — L — CA

(Formula I),

wherein:

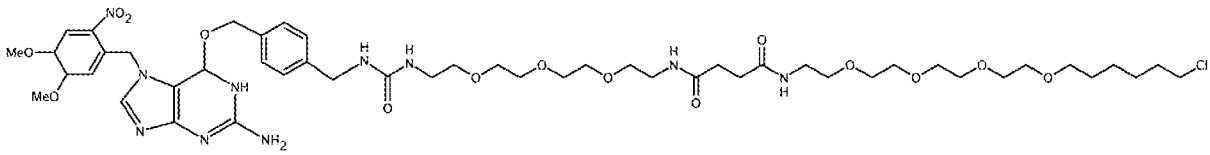
(a) P is a photocaging moiety selected from the group consisting of alpha-carboxy-2-nitrobenzyl; 4,5 dimethoxy 2-nitrobenzyl; 4,5, dimethoxy 2-nitrophenyl; and 6-nitroveratryloxycarbonyl; and bound to BG at position N_7, optionally by an ester bond;

(b) BG is O6-Benzylguanine;

(c) L is a linker that comprises at least one amide bond; at least one polyethylene glycol (PEG) moiety; or at least one amide bond and at least one PEG moiety; or at least one disulfide bond or a single tetrafluorobenzene group, and,

(d) CA is a chloroalkane.

2. The photochemically caged dimerizer of claim 1, represented by Formula II:



(Formula II)

wherein:

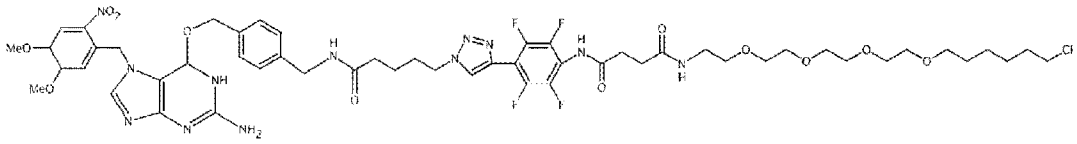
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by a carbonyl bond to L;

(c) L consists essentially of, in the following order: three PEG moieties, two amide bonds, and four PEG moieties; and

(d) CA is a chloroalkane comprising at least six carbon molecules.

3. The photochemically caged dimerizer of claim 1, represented by Formula III:



(Formula III)

wherein:

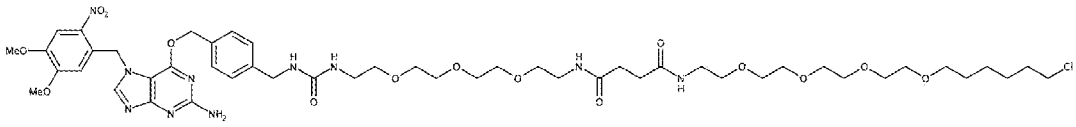
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by an amide bond to L;

(c) L consists essentially of in the following order: 1,2,3-triazole group, tetrafluorophenylene group, two amide bonds, four PEG moieties, and

(d) CA is a chloroalkane comprising at least six carbon molecules.

4. A photochemically caged dimerizer represented by Formula VII:



(Formula VII),

wherein:

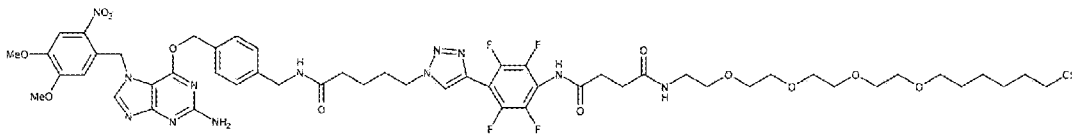
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by a carbonyl bond to L;

(c) L consists essentially of, in the following order: three PEG moieties; two amide bonds, and four PEG moieties; and,

(d) CA is a chloroalkane comprising at least six carbon molecules.

5. A photochemically caged dimerizer is represented by Formula VIII:



(Formula VIII)

wherein:

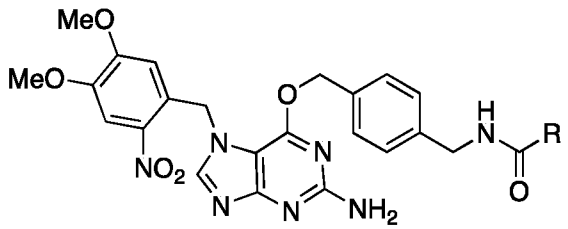
(a) P is 4,5 dimethoxy 2-nitrobenzyl bound at position N_7 of BG;

(b) BG is O6-Benzylguanine bound by an amide bond to L;

(c) L consists essentially, of in the following order: 1,2,3-triazole group; tetrafluorophenylene group, two amide bonds: and,

(d) CA is a chloroalkane comprising at least six carbon molecules.

6. A photochemically caged ligand represented by Formula IV:



(Formula IV)

wherein R is selected from the group consisting of an alkyne moiety, an azide moiety, biotin, and a fluorescent moiety.

7. A method of removing a target protein from a single cell or from individual cells in a pool of cells, comprising:

(a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is not the protein to be removed and binds the target protein to be removed (on or within a single cell or individual cells in the pool); and (2) a photochemically caged SNAP-HALO dimerizer of any of formulas I, II, III, VII, or VIII thereby producing a cell comprising SNAP-tagged protein bound to the target protein and (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged SNAP-HALO dimerizer, thereby producing an uncaged SNAP-HALO dimerizer (BG-L-CA, designated DIMERIZER), under conditions under which the uncaged dimerizer binds to SNAP and forms a complex of Formula V:

SNAPTAG Protein ——— **DIMERIZER**

(Formula V),

wherein:

SNAPTAG Protein is a SNAP-tagged protein bound to a target protein on or within a cell; DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b);

(c) contacting the complex of Formula V with a separation agent comprising HALO protein, under conditions under which the complex of Formula V binds to the HALO protein of the separation agent via uncaged dimerizer, thereby producing a complex of formula Va:

SNAPTAG Protein ——— **DIMERIZER** ——— **HALOTAG**

(Formula Va)

wherein

SNAPTAG Protein is a SNAP-tagged protein bound to target nucleic acid on or within a cell; DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b); HALOTAG is the separation agent comprising HALO protein; and,

(d) separating SNAPTAG Protein of Formula Va from other cellular components based on binding of the complex to the HALO protein.

8. A method of removing a target nucleic acid from a single cell or from individual cells in a pool of cells, comprising:

(a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is not the protein to be removed and binds the target protein to be removed (on or within a single cell or individual cells in the pool); and (2) a photochemically caged SNAP-HALO dimerizer of any of formulas I, II, III, VI, or VIII thereby producing a cell comprising SNAP-tagged protein bound to the target nucleic acid and (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient

to uncage (that uncages) the photochemically caged SNAP-HALO dimerizer, thereby producing an uncaged SNAP-HALO dimerizer (BG-L-CA, designated DIMERIZER), under conditions under which the uncaged dimerizer binds to SNAP and forms a complex of Formula V:

SNAPTAG Protein ——— DIMERIZER

(Formula V),

wherein:

SNAPTAG Protein is a SNAP-tagged protein bound to a target nucleic acid within a cell; and,

D is an uncaged SNAP-HALO dimerizer produced in (b); and,

(c) contacting the complex of Formula V with a separation agent comprising HALO protein, under conditions under which the complex of Formula V binds to the HALO protein of the separation agent via the uncaged dimerizer, thereby producing a complex of formula Va:

SNAPTAG Protein ——— DIMERIZER ——— HALOTAG

(Formula Va),

wherein:

SNAPTAG Protein is a SNAP-tagged protein bound to target nucleic acid on or within a cell; DIMERIZER is an uncaged SNAP-HALO dimerizer produced in (b); HALOTAG is the separation agent comprising HALO protein; and,

(d) separating SNAPTAG Protein of Formula Va from other cellular components based on binding of the complex to the HALO protein.

9. The method of claim 7, wherein the SNAP-tagged protein binds a target on or inside the cell.

10. The method of any one of claims 7 to 9, wherein the region of interest is a whole cell, cell membrane or subcellular compartment.

11. The method of any one of claims 7 to 10, wherein light is delivered for between about 1 second and about 1 minute.

12. The method of any one of claims 7 to 11, wherein delivery of light in (b) results in two-photon excitation and uncaging of the dimerizer.

13. The method of any one of claims 7 to 11, wherein delivery of light in (b) results in one-photon excitation and uncaging of the dimerizer.

14. The method of any one of claims 7 to 13, wherein the wavelength is from about 300 nm to about 800 nm.

15. The method of any one of claims 7 to 14, wherein the wavelength is about 365 nm.

16. The method of any one of claims 7 to 14, wherein the wavelength is about 405 nm.

17. The method of any one of claims 7 to 14, wherein the wavelength is about 488 nm.

18. The method of any one of claims 7 to 14, wherein the wavelength is between about 700 nm to about 800 nm.

19. The method of any one of claims 7 to 18, wherein the light is delivered by laser and the power of the laser ranges from about 0.1 $mW/\mu m^2$ to 5 $mW/\mu m^2$.

20. The method of any one of claims 7 to 13, further comprising lysing the cell or pool of cells is lysed prior to performing step (c) of the method.

21. The method of any one of claims 7 to 20, wherein the separation agent is a bead.

22 The method of any one of claims 7 to 20, wherein the separation agent is a resin.

23. The method of any one of claims 7 to 22, wherein (c) is performed by affinity chromatography, immunoprecipitation, or flow cytometry.

24. A method for removing a protein, the method comprising:

(a) delivering to a single cell or a pool of cells (1) at least one SNAP-tagged protein that is not the protein to be removed and binds a protein to be removed, referred to as a target protein (on or within a single cell or individual cells in the pool); and (2) a photochemically caged ligand of Formula IV thereby producing a cell comprising SNAP-tagged protein bound to the target protein and (2);

(b) delivering to a region of interest on or within the single cell or on or within individual cells in the population of cells light at a wavelength and photon density sufficient to uncage (that uncages) the photochemically caged ligand, thereby producing an uncaged "tagging molecule" (designated below as "TAG"), under conditions under which the uncaged tagging molecule binds to SNAP and forms a complex of Formula IX:

SNAPTAG Protein ——— TAG

(Formula IX);

(c) contacting the complex of Formula IX with a separation agent comprising a streptavidin, or an azide, under conditions under which TAG binds to the separation agent via the uncaged dimerizer (via the uncaged dimerizer portion of the complex), thereby producing a complex of formula IXa:

SNAPTAG Protein ——— TAG ——— SEPARATION AGENT

(Formula IXa); and,

(d) separating the SNAPTAG Protein of Formula IXa, and therefore the target protein, from other cellular components based on binding of the complex to the separation agent.

25. The method of claim 24, wherein the SNAP-tagged protein binds a target on or inside the cell.

26. The method of claim 24 or 25, wherein the region of interest is a whole cell, cell membrane or subcellular compartment.

27. The method of any one of claims 24 to 26, wherein light is delivered for between about 1 second and about 1 minute.

28. The method of any one of claims 24 to 27, wherein delivery of light in (b) results in two-photon excitation and uncaging of the dimerizer.

29. The method of any one of claims 24 to 28, wherein delivery of light in (b) results in one-photon excitation and uncaging of the dimerizer.

30. The method of any one of claims 24 to 29, wherein the wavelength is from about 300 nm to about 800 nm.

31. The method of any one of claims 24 to 30, wherein the wavelength is about 365 nm.

32. The method of any one of claims 24 to 30, wherein the wavelength is about 405 nm.

33. The method of any one of claims 24 to 30, wherein the wavelength is about 488 nm.

34. The method of any one of claims 24 to 30, wherein the wavelength is between about 700 nm to about 800 nm.

35. The method of any one of claims 24 to 34, wherein the light is delivered by laser and the power of the laser ranges from about 0.1 $mW/\mu m^2$ to 5 $mW/\mu m^2$.

36. The method of any one of claims 24 to 35, further comprising lysing the cell or pool of cells is lysed prior to performing step (c) of the method.

37. The method of any one of claims 24 to 36, wherein the separation agent is a bead.

38 The method of any one of claims 24 to 36, wherein the separation agent is a resin.

39. The method of any one of claims 24 to 38, wherein (c) is performed by affinity chromatography, immunoprecipitation, or flow cytometry.

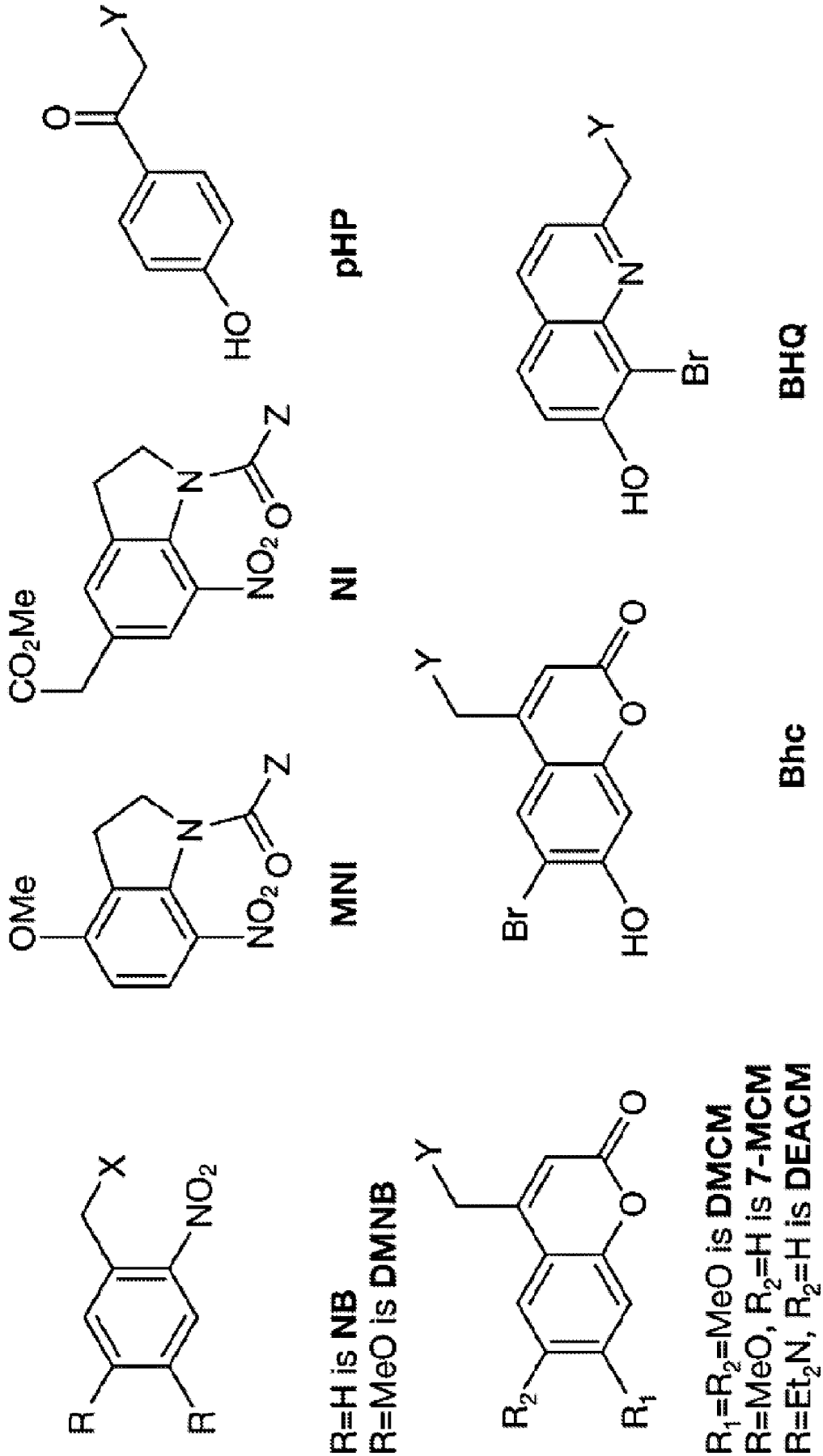

pHP
NI
MNI
Bhc
BHQ
R=H is NB
R=MeO is DMNB
R1=R2=MeO is DMCM
R=MeO, R2=H is 7-MCM
R=Et2N, R2=H is DEACM

FIG. 1

Applicability of the cage groups:

Cage	*Functional group(s)*
Nitrobenzyl NB, DMNB NPE, DMNPE CNB	*Phosphate, thiophosphate, carboxylate, amine, phenol, alcohol, thiol, carbamate, and amide*
Nitroindole MNI, NI	*Alkyl*
Coumarinyl pHP DMCM 7-MCM DEACM Bhc Bhq	*Acidic (i.e. phosphate or carboxylate)*

FIG. 1 cont.

Cage	Absorption max (nm) (extinction/mM^{-1} cm^{-1})	Fluorescence	QY (chem)	QY (fluo)	2PCS (GM)	Rate (sec^{-1})
NB[a,b]	(~0.9 at 350 nm)	None	0.04-0.7	None	0.001	$100\text{-}10^6$
DMNB[b]	350 (5.2)	None	0.01-0.23	None	0.01	140-80,000
7-MCM	328 (13.3)	400	0.1	0.03	n.d.	10^8
DEACM	399 (19.5)	484	0.22	0.0058	n.d.	10^8
DMCM	349 (11.3)	444	0.04	0.022	n.d.	10^8
pHP[a,c]	Very pH-dependent	n.d.	0.1-0.7	n.d.	n.d.	$10^6\text{-}10^8$
Bhc[c]	370 (15.0)	530	0.012-0.04	0.2-0.4	0.95	n.d.[d]
BHQ[c]	369 (2.6)	510	0.29	0.04	0.54	n.d.[d]
MNI	335 (3.6)	None	0.085	None	0.06	$>10^5$

FIG. 1 cont.

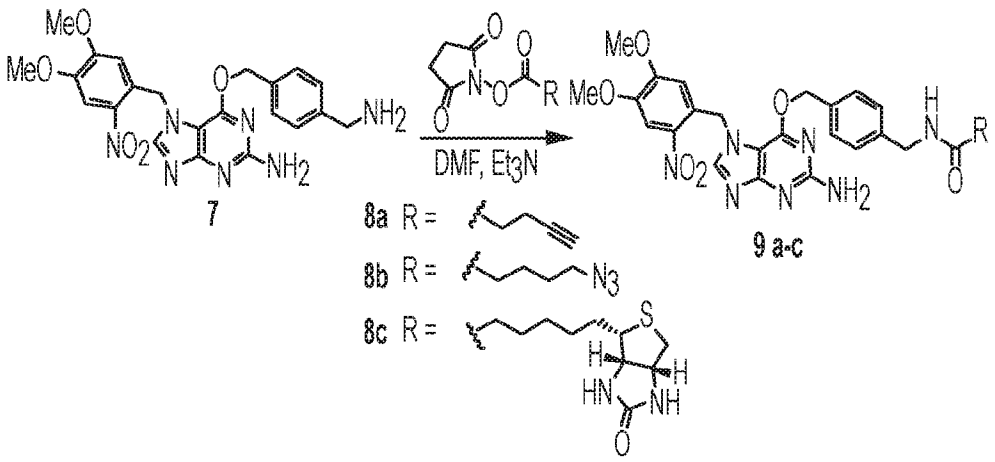


FIG. 2

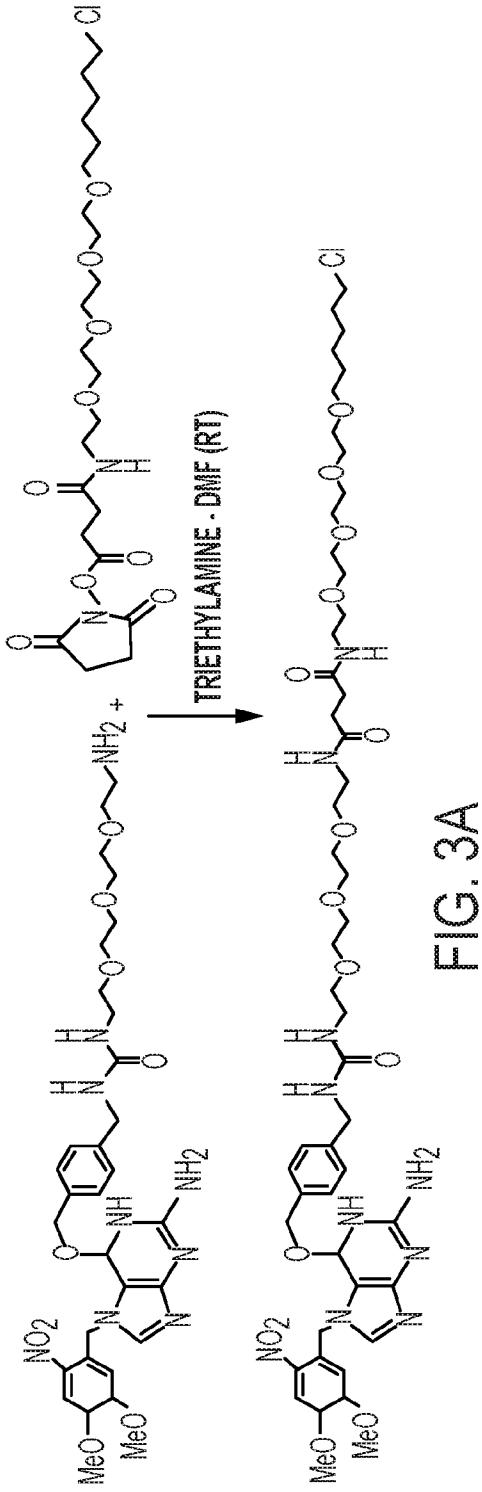

TRIETHYLAMINE - DMF (RT)

FIG. 3A

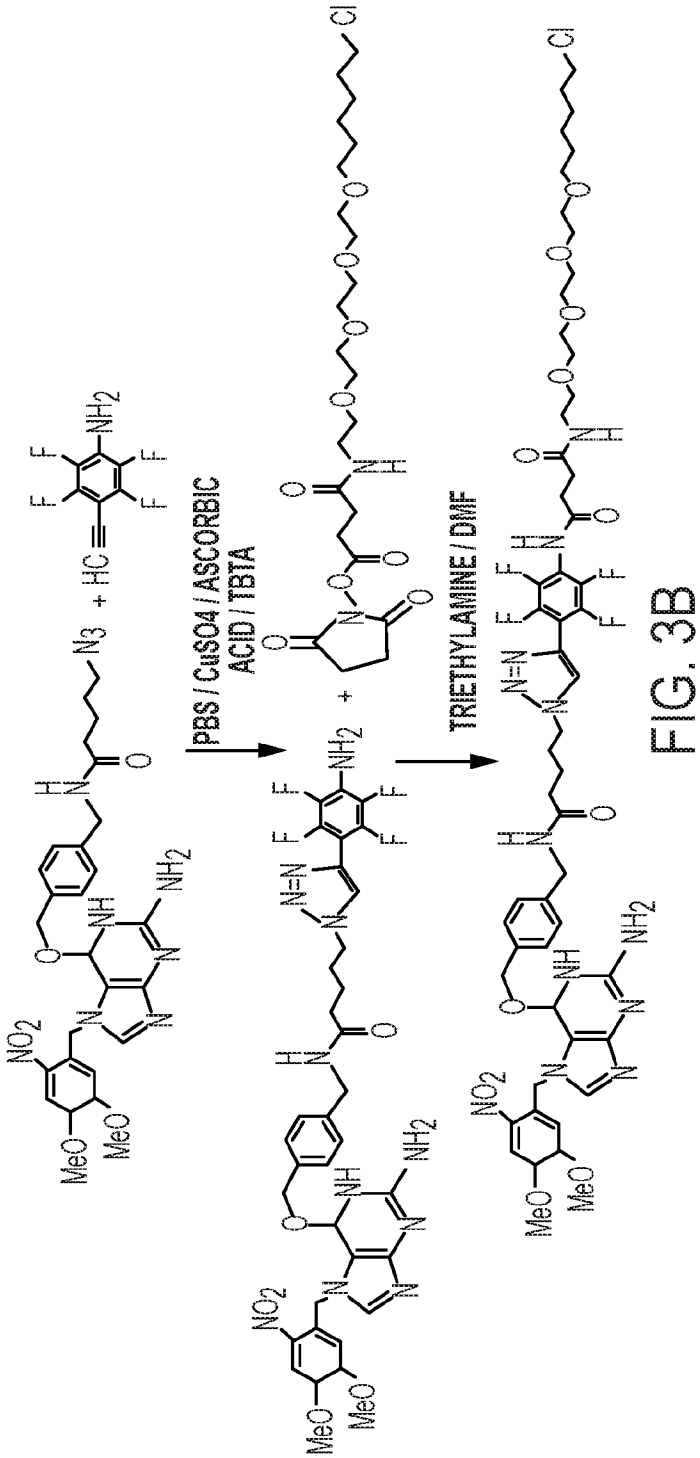

PBS / CuSO4 / ASCORBIC ACID / TBTA
TRIETHYLAMINE / DMF

FIG. 3B

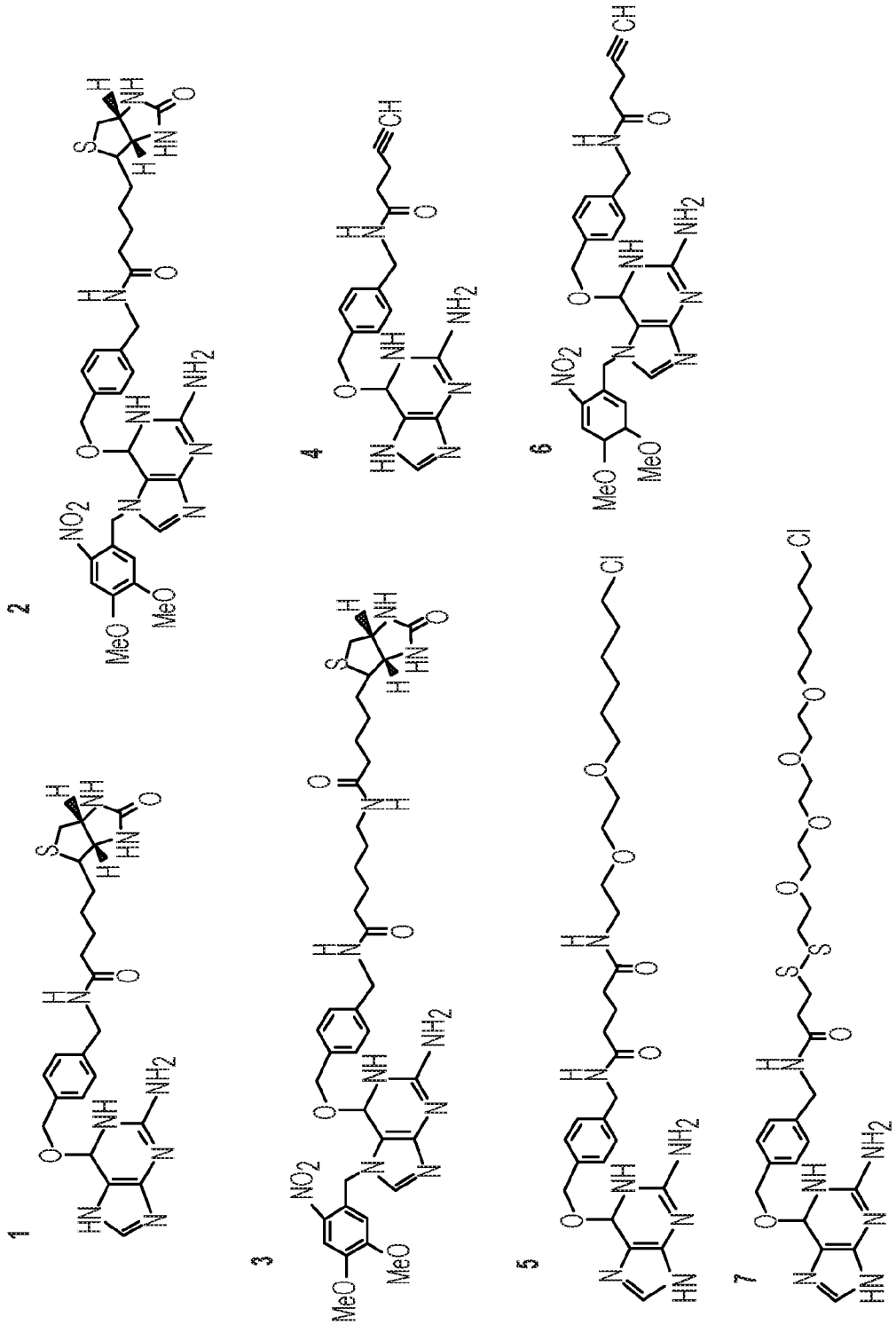


FIG. 4

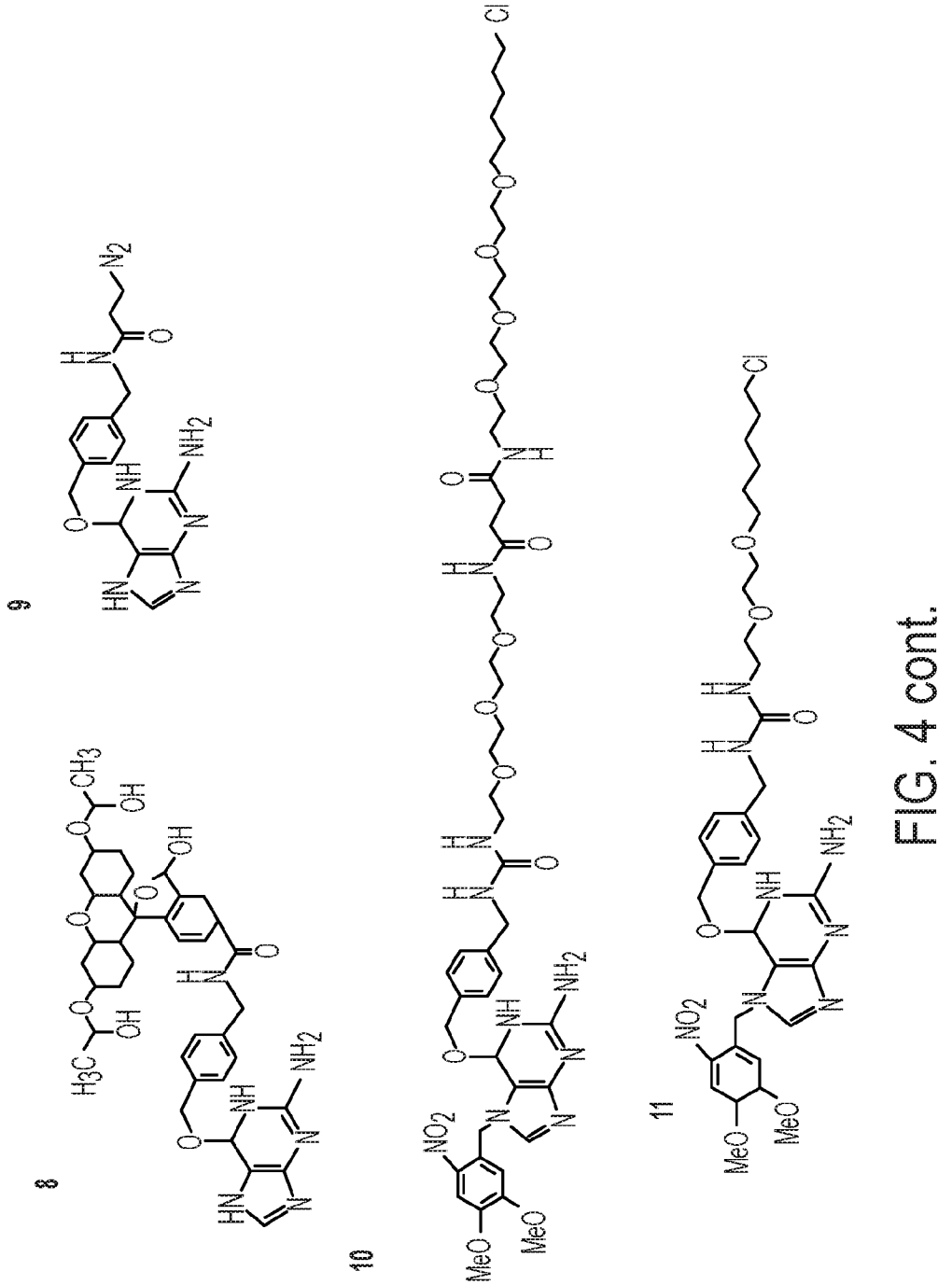


FIG. 4 cont.

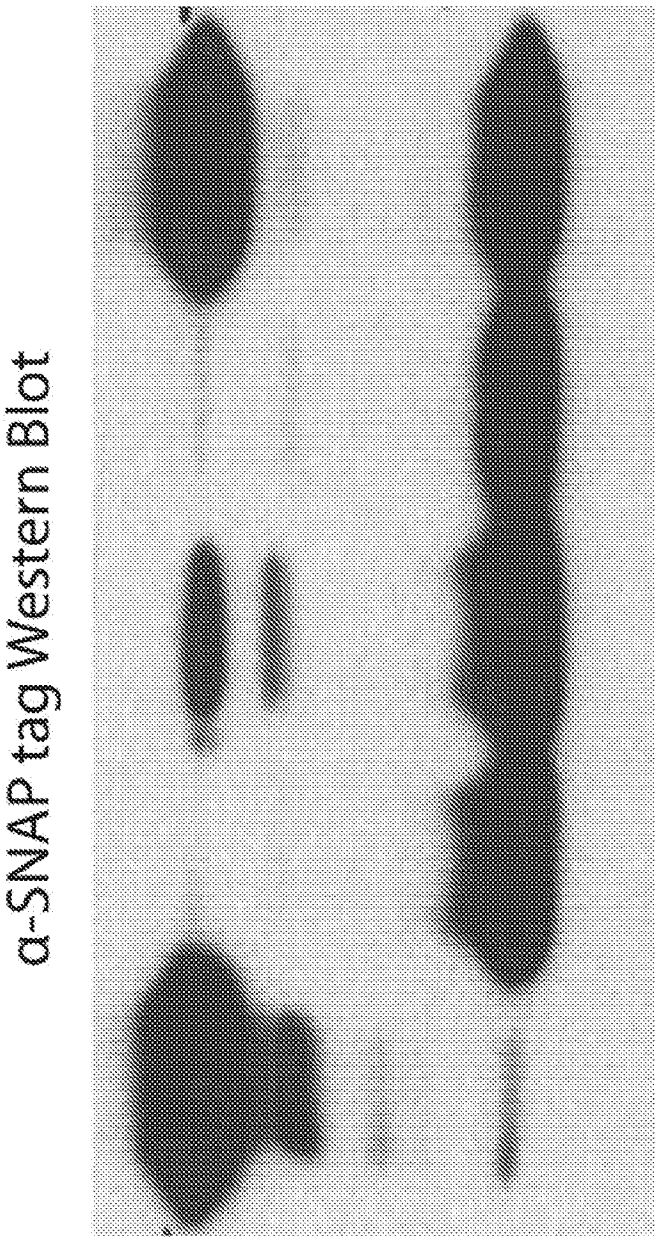

α-SNAP tag Western Blot
50 kD
~20 kD
SNAP-HALO Protein + + + + -
TEV protease cleavage - + + + +
BG-HALO linker - - BG-SS-HALO BG-SS-HALO BG-HALO
Disulfide cleavage - - - + -

FIG. 5

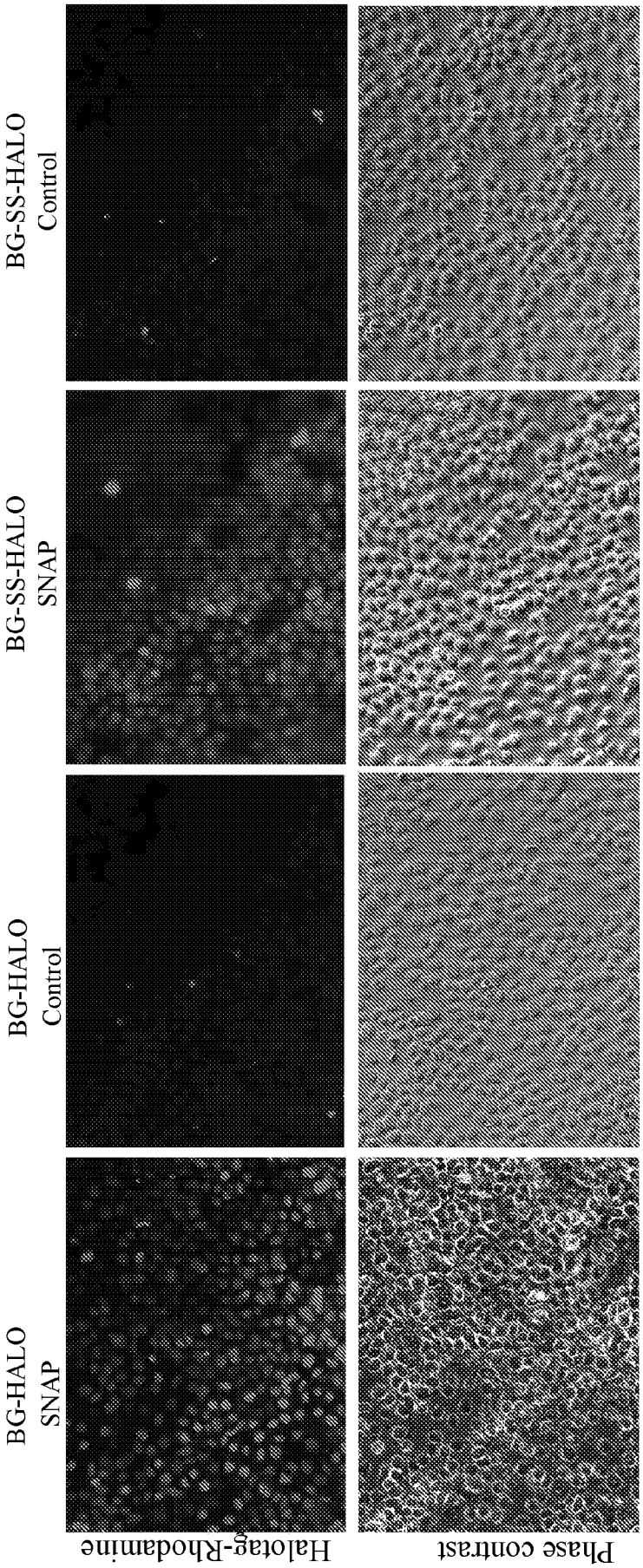

BG-HALO SNAP
BG-HALO Control
BG-SS-HALO SNAP
BG-SS-HALO Control
Halotag-Rhodamine
Phase contrast

FIG. 6.

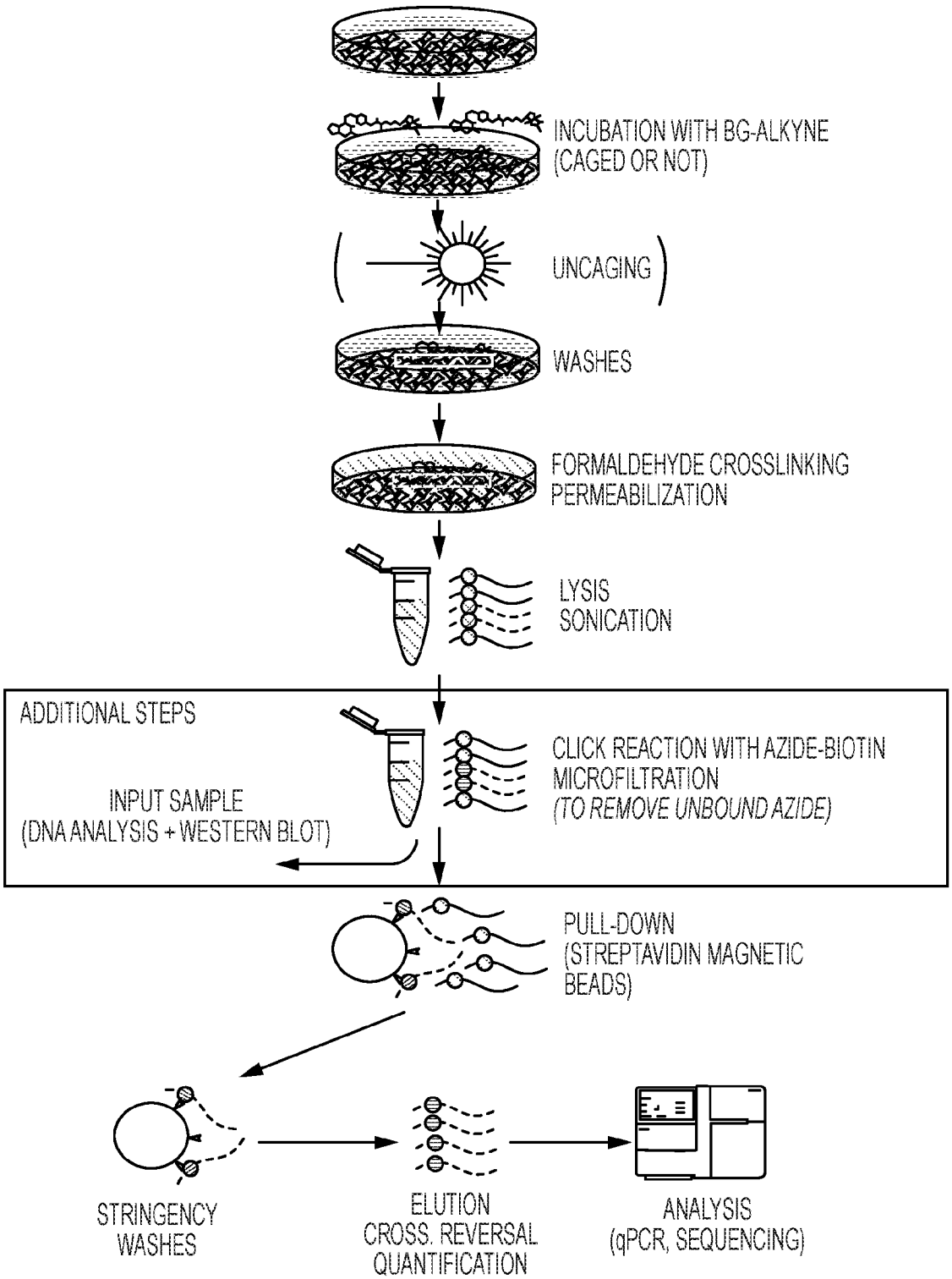

INCUBATION WITH BG-ALKYNE
(CAGED OR NOT)
UNCAGING
WASHES
FORMALDEHYDE CROSSLINKING
PERMEABILIZATION
LYSIS
SONICATION
ADDITIONAL STEPS
INPUT SAMPLE
(DNA ANALYSIS + WESTERN BLOT)
CLICK REACTION WITH AZIDE-BIOTIN
MICROFILTRATION
(TO REMOVE UNBOUND AZIDE)
PULL-DOWN
(STREPTAVIDIN MAGNETIC
BEADS)
STRINGENCY
WASHES
ELUTION
CROSS. REVERSAL
QUANTIFICATION
ANALYSIS
(qPCR, SEQUENCING)

FIG. 7



FIG. 8A

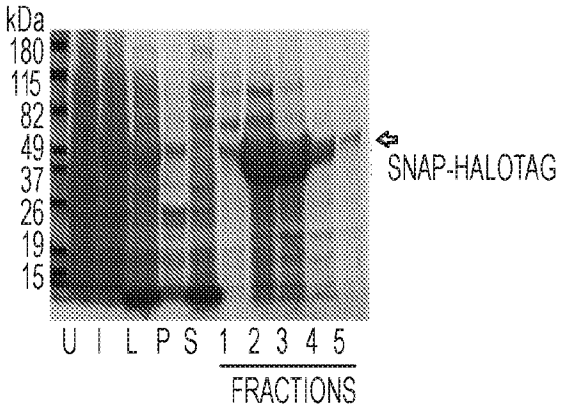


FIG. 8B

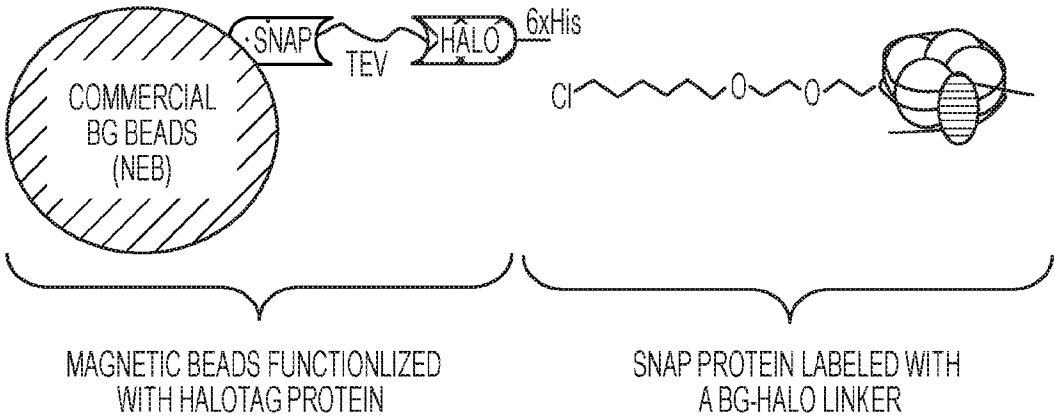


FIG. 8C

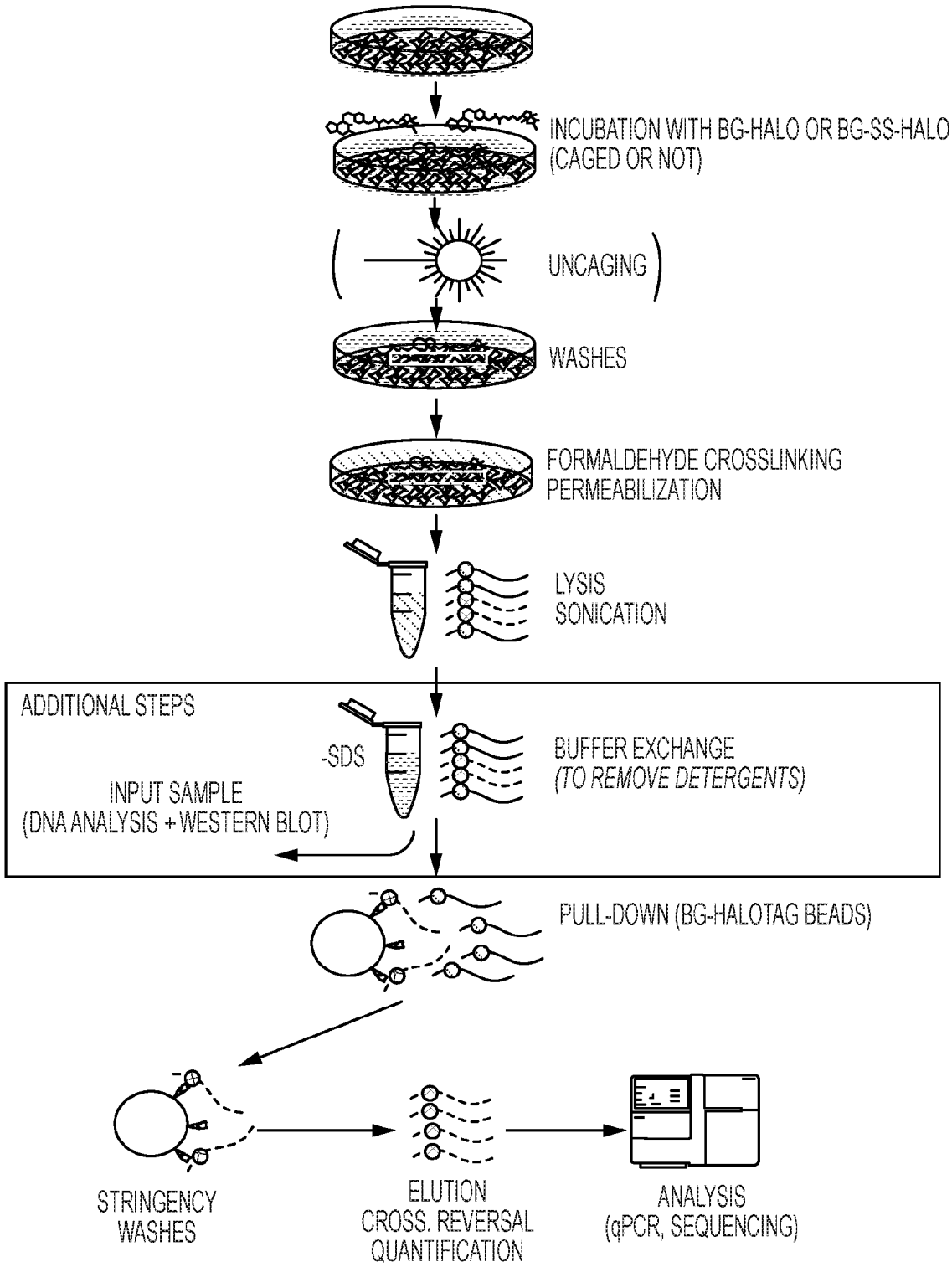

INCUBATION WITH BG-HALO OR BG-SS-HALO
(CAGED OR NOT)
UNCAGING
WASHES
FORMALDEHYDE CROSSLINKING
PERMEABILIZATION
LYSIS
SONICATION
ADDITIONAL STEPS
-SDS
BUFFER EXCHANGE
(TO REMOVE DETERGENTS)
INPUT SAMPLE
(DNA ANALYSIS + WESTERN BLOT)
PULL-DOWN (BG-HALOTAG BEADS)
STRINGENCY
WASHES
ELUTION
CROSS. REVERSAL
QUANTIFICATION
ANALYSIS
(qPCR, SEQUENCING)

FIG. 8 cont.

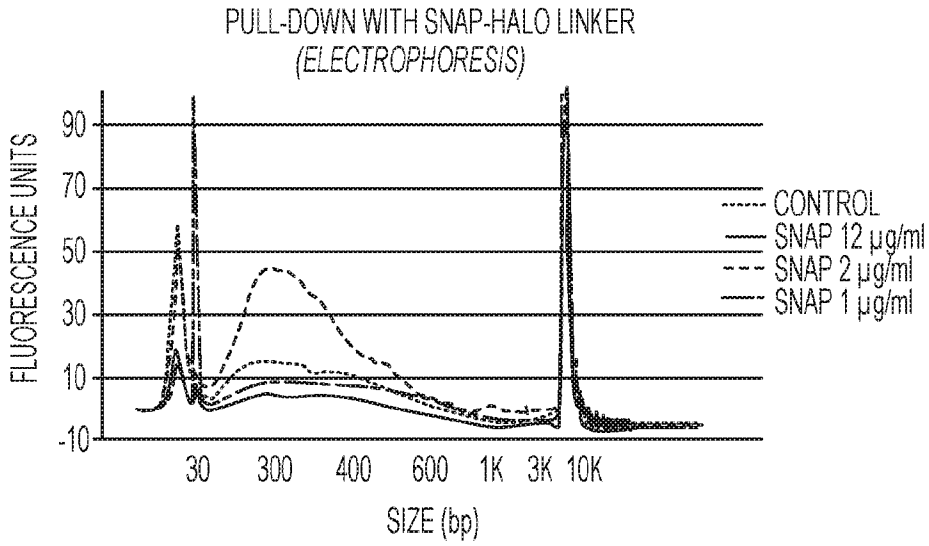


FIG. 9A

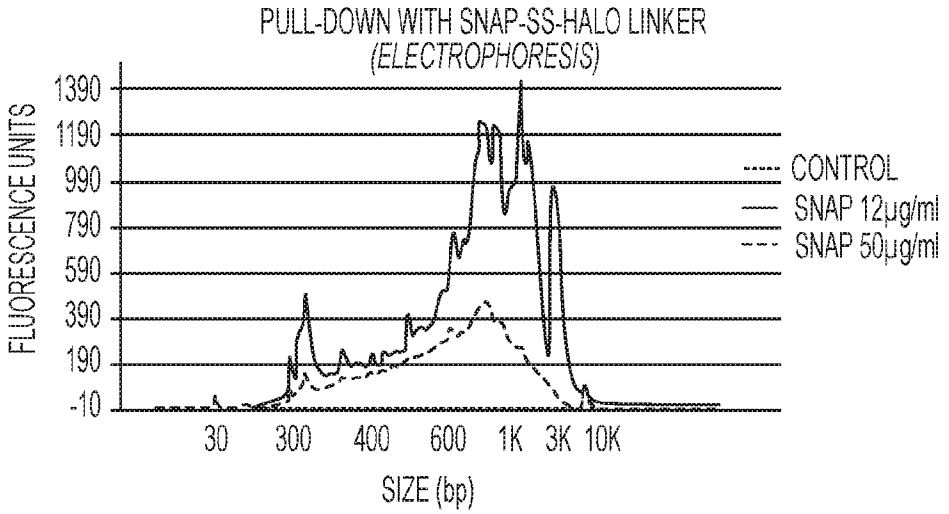


FIG. 9B

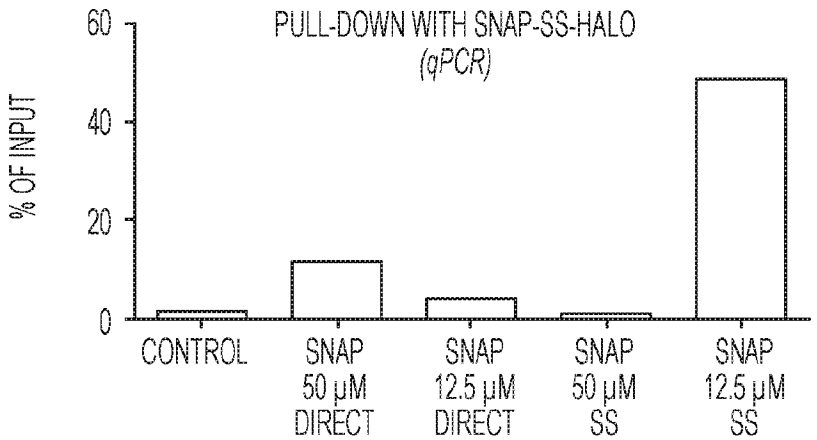


FIG. 9C

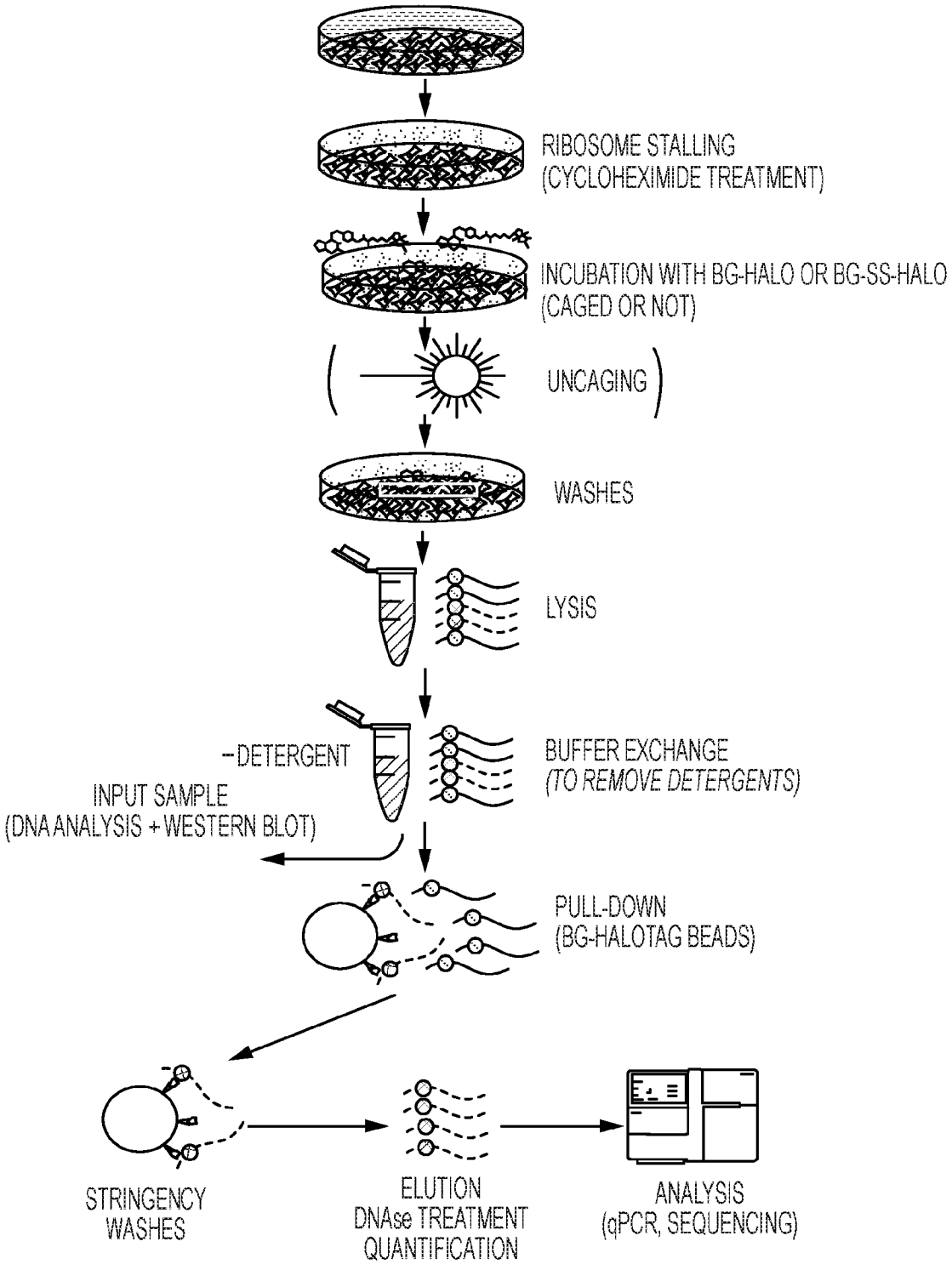

RIBOSOME STALLING
(CYCLOHEXIMIDE TREATMENT)
INCUBATION WITH BG-HALO OR BG-SS-HALO
(CAGED OR NOT)
UNCAGING
WASHES
LYSIS
-DETERGENT
INPUT SAMPLE
(DNA ANALYSIS + WESTERN BLOT)
BUFFER EXCHANGE
(TO REMOVE DETERGENTS)
PULL-DOWN
(BG-HALOTAG BEADS)
STRINGENCY
WASHES
ELUTION
DNAse TREATMENT
QUANTIFICATION
ANALYSIS
(qPCR, SEQUENCING)

FIG. 10

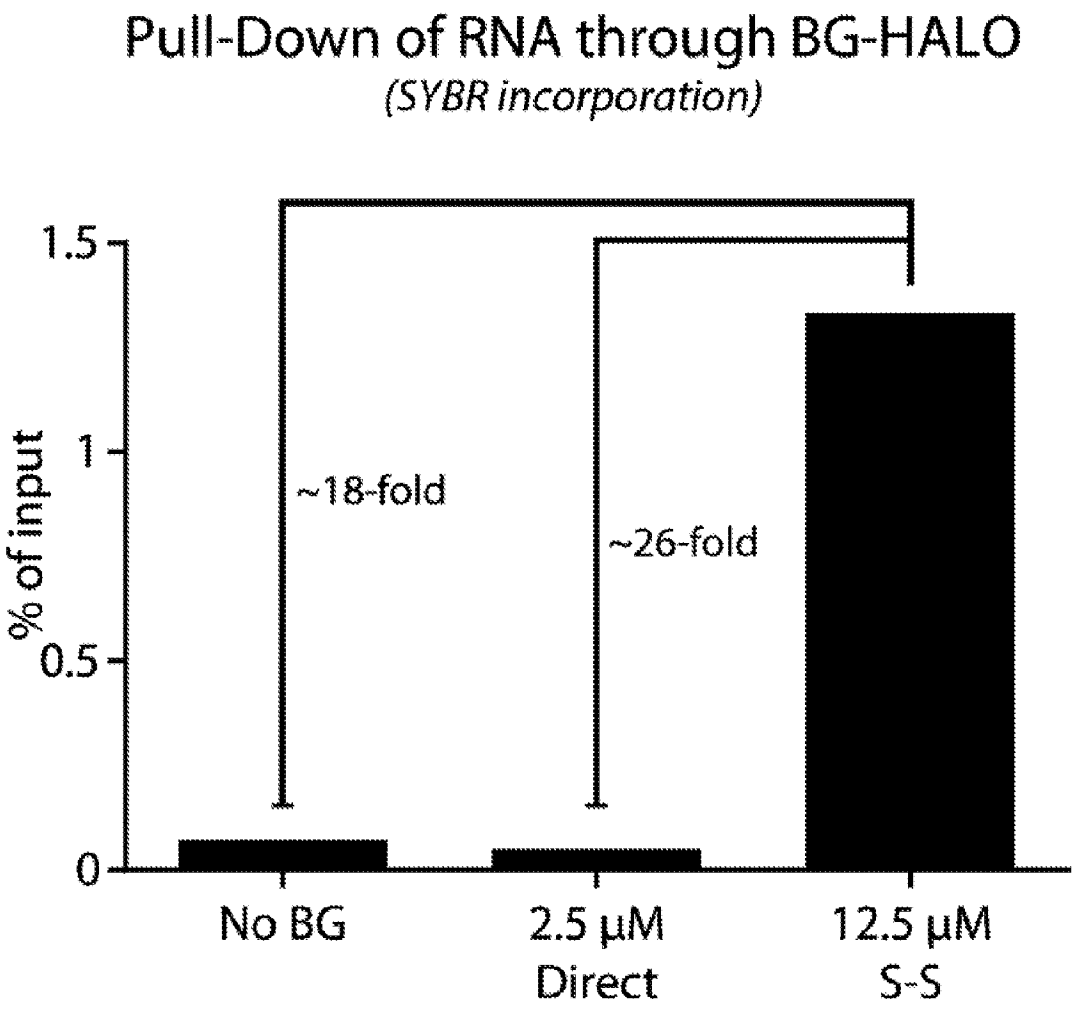

Pull-Down of RNA through BG-HALO
(SYBR incorporation)
% of input
1.5
1
0.5
0
~18-fold
~26-fold
No BG
2.5 µM Direct
12.5 µM S-S

FIG. 11

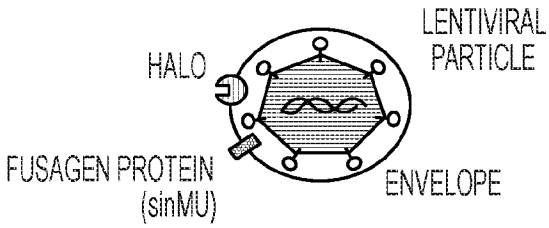


FIG. 12A

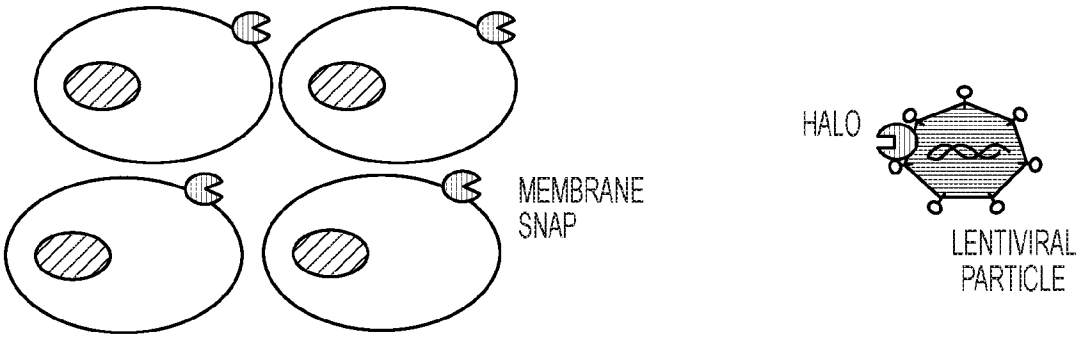


FIG. 12B

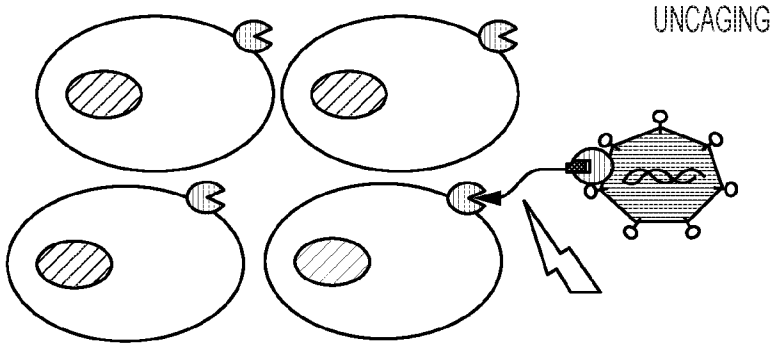


FIG. 12C

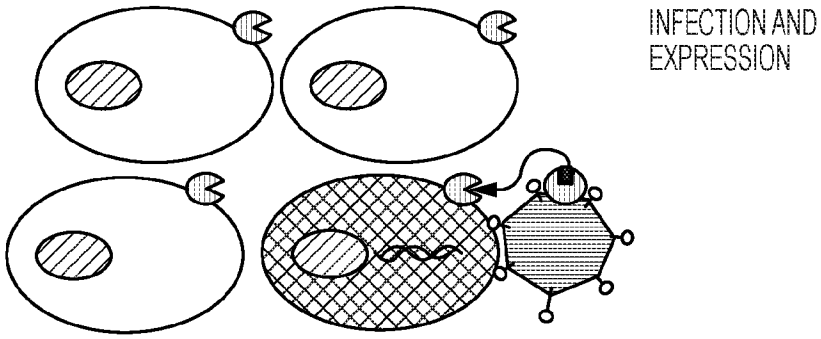


FIG. 12D

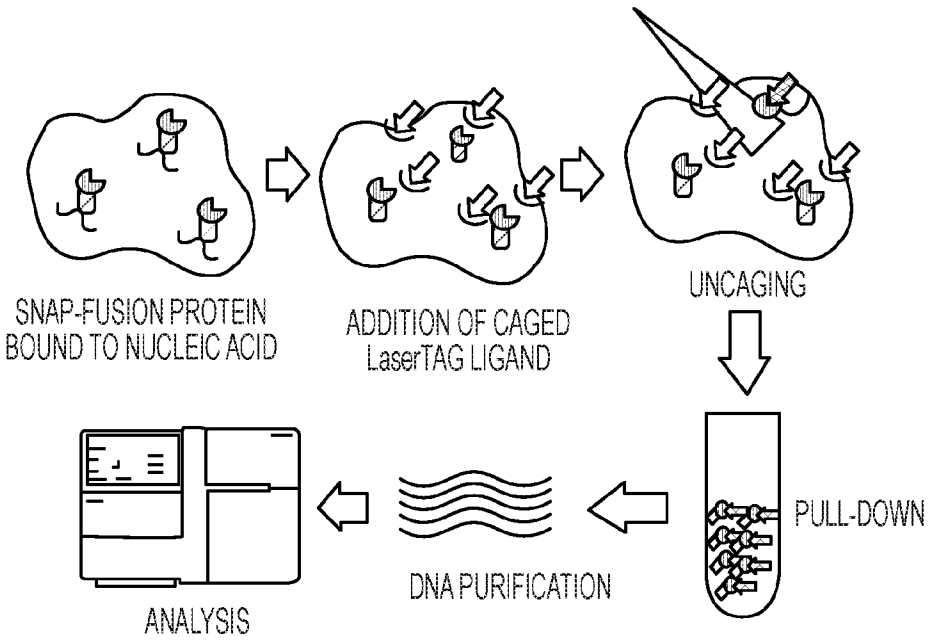

SNAP-FUSION PROTEIN
BOUND TO NUCLEIC ACID
ADDITION OF CAGED
LaserTAG LIGAND
UNCAGING
PULL-DOWN
DNA PURIFICATION
ANALYSIS

FIG. 13

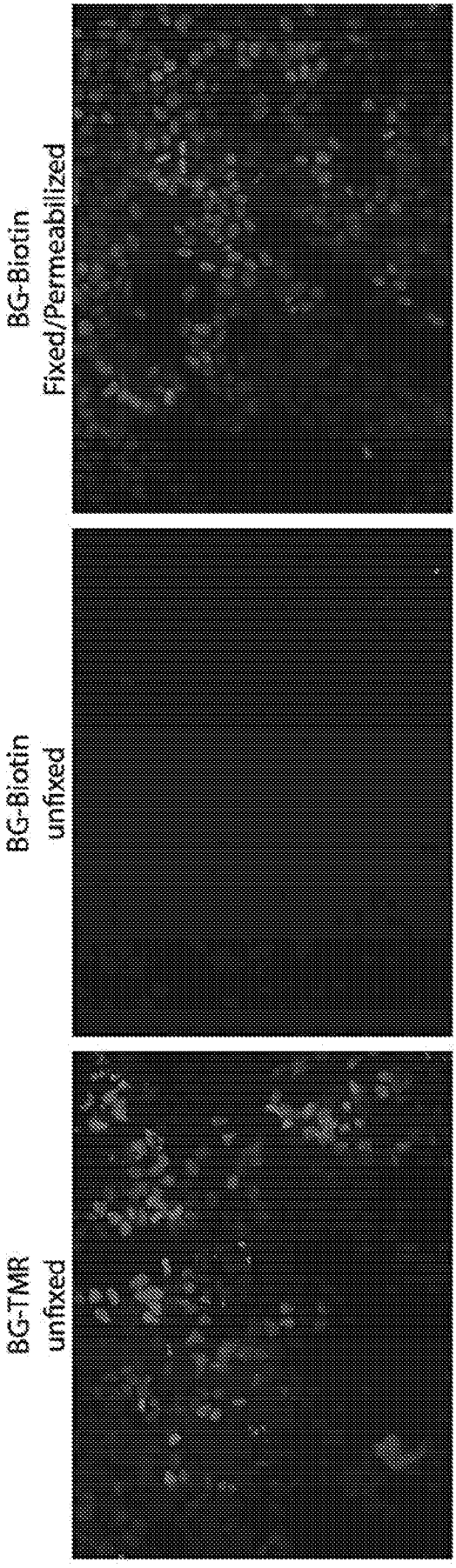

BG-Biotin
Fixed/Permeabilized
BG-Biotin
unfixed
BG-TMR
unfixed

FIG. 14A

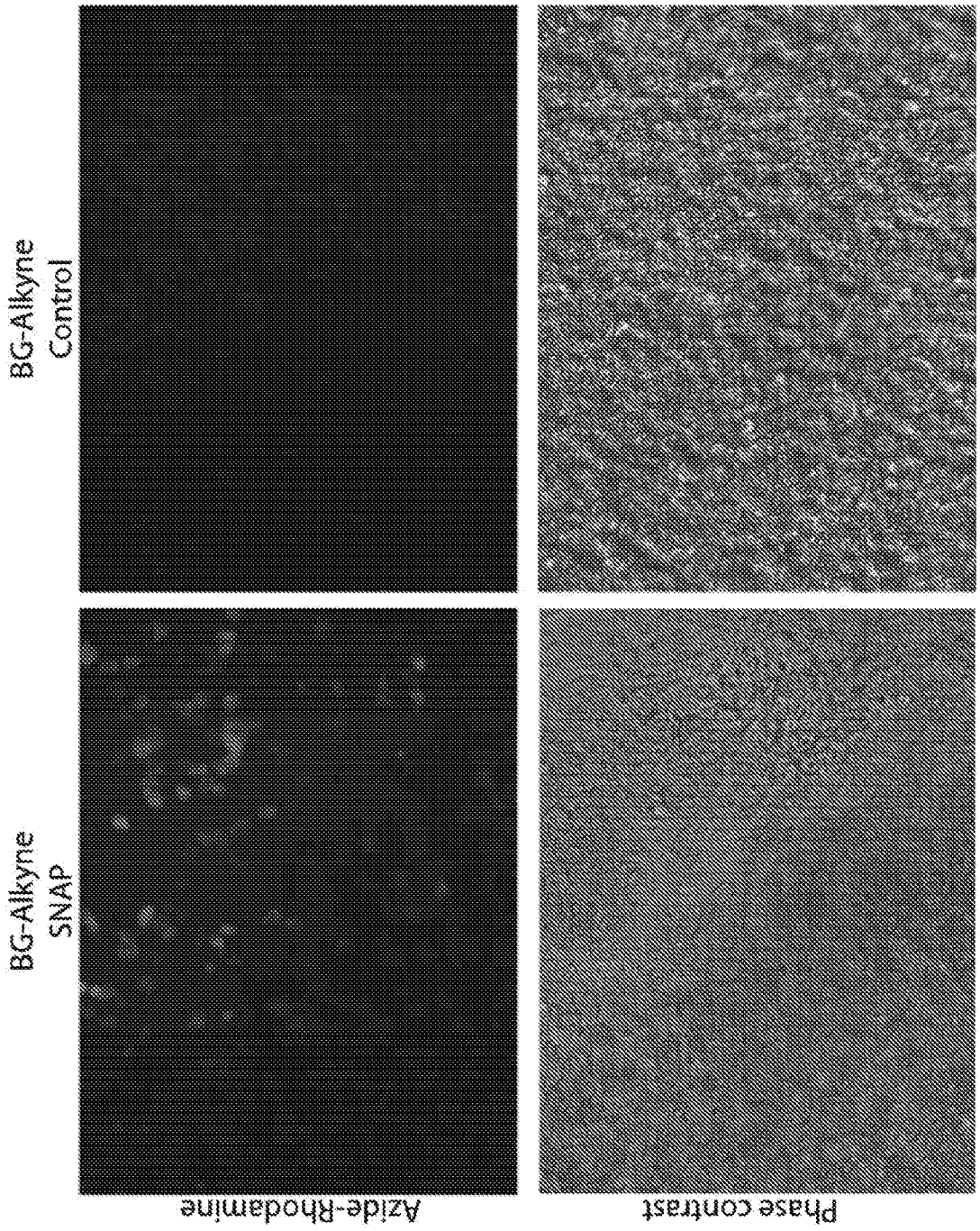

BG-Alkyne Control
BG-Alkyne SNAP
Azide-Rhodamine
Phase contrast

FIG. 14B

FIG. 15A

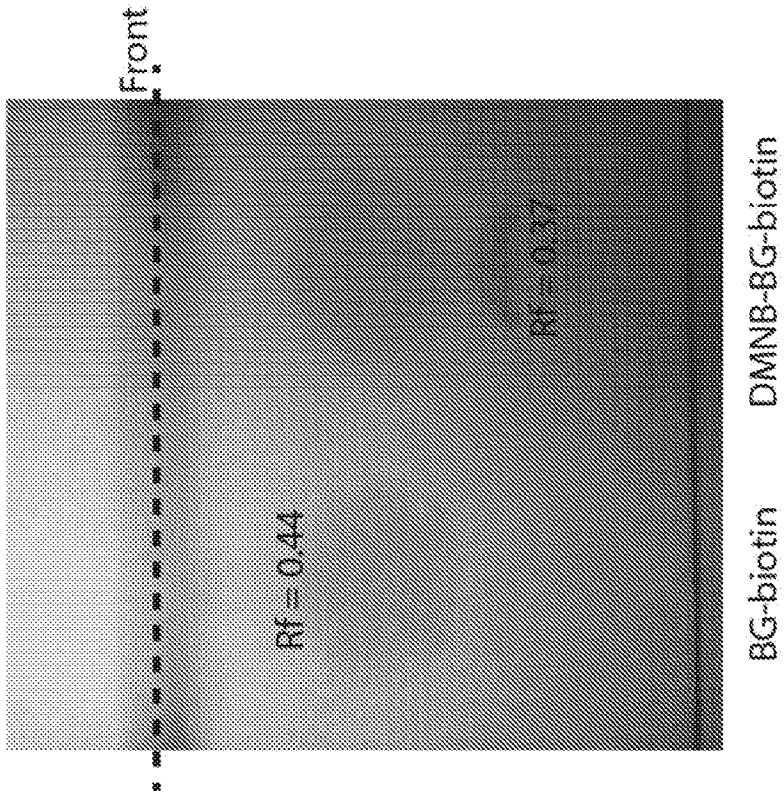
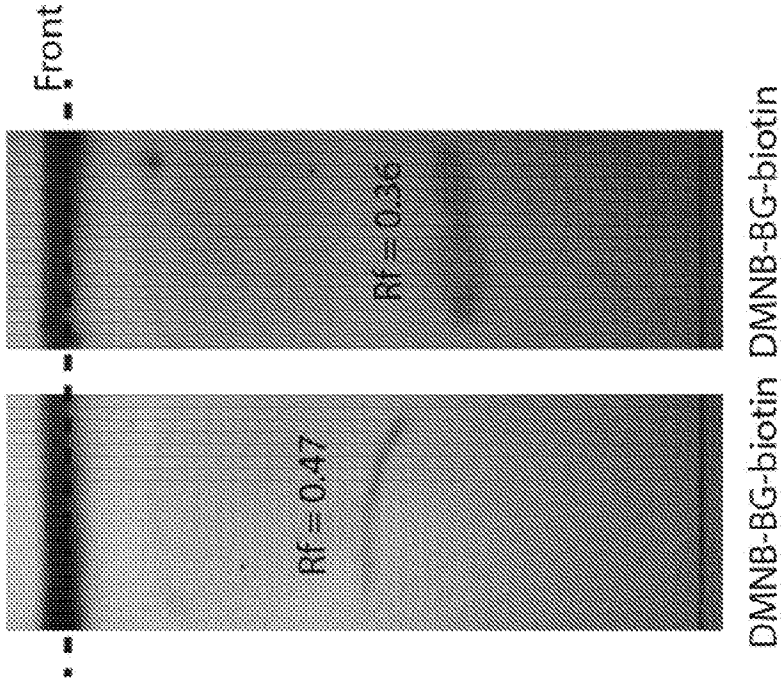


FIG. 15B



50 : 33 : 16.5 : 0.5
Chloroform : Methanol : H_2O : Acetic Acid
(Silica gel matrix on Aluminum foil)

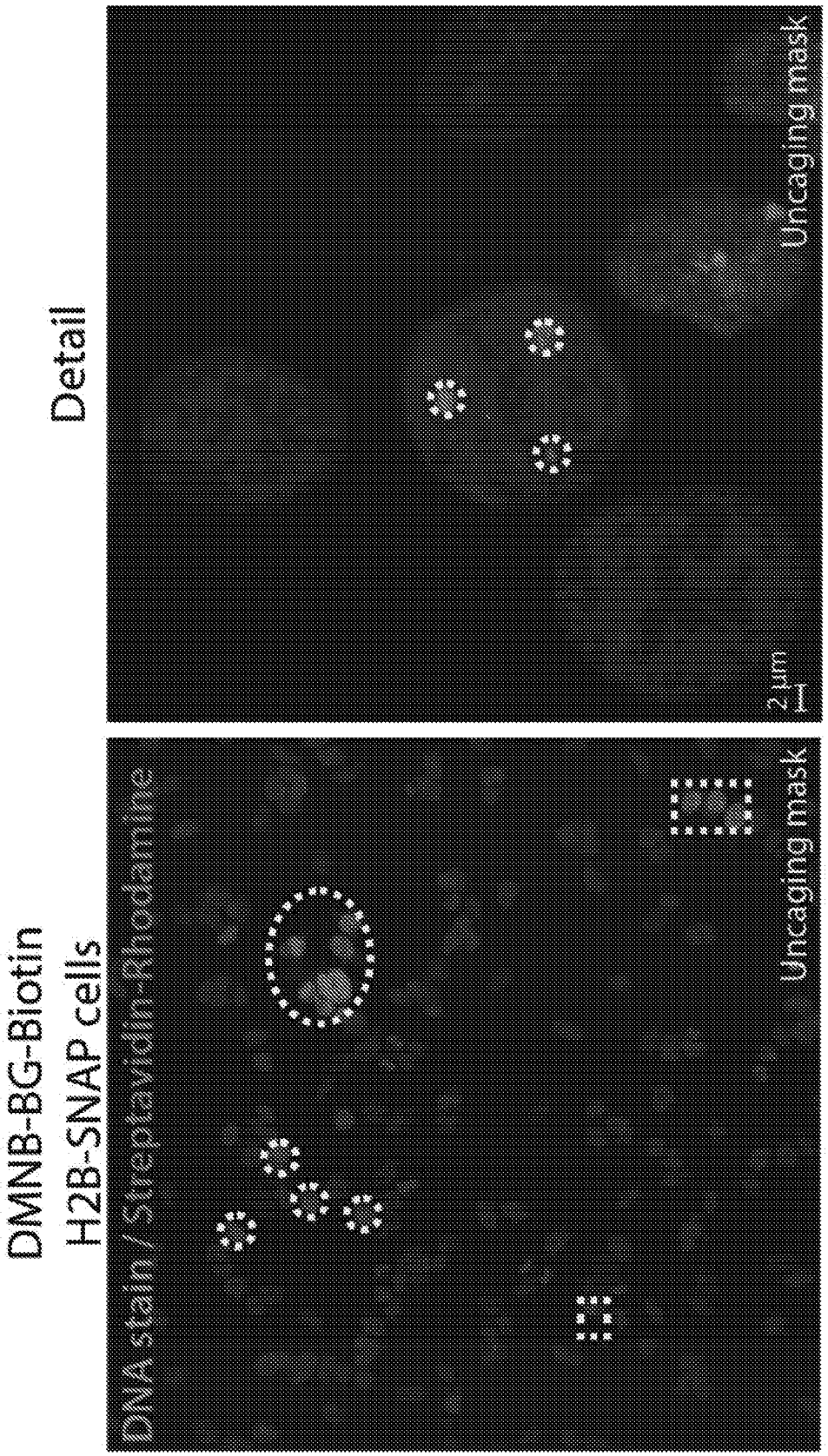

DMNB-BG-Biotin
H2B-SNAP cells
DNA stain / Streptavidin-rhodamine
Uncaging mask
Detail
Uncaging mask
2 µm

FIG. 15C

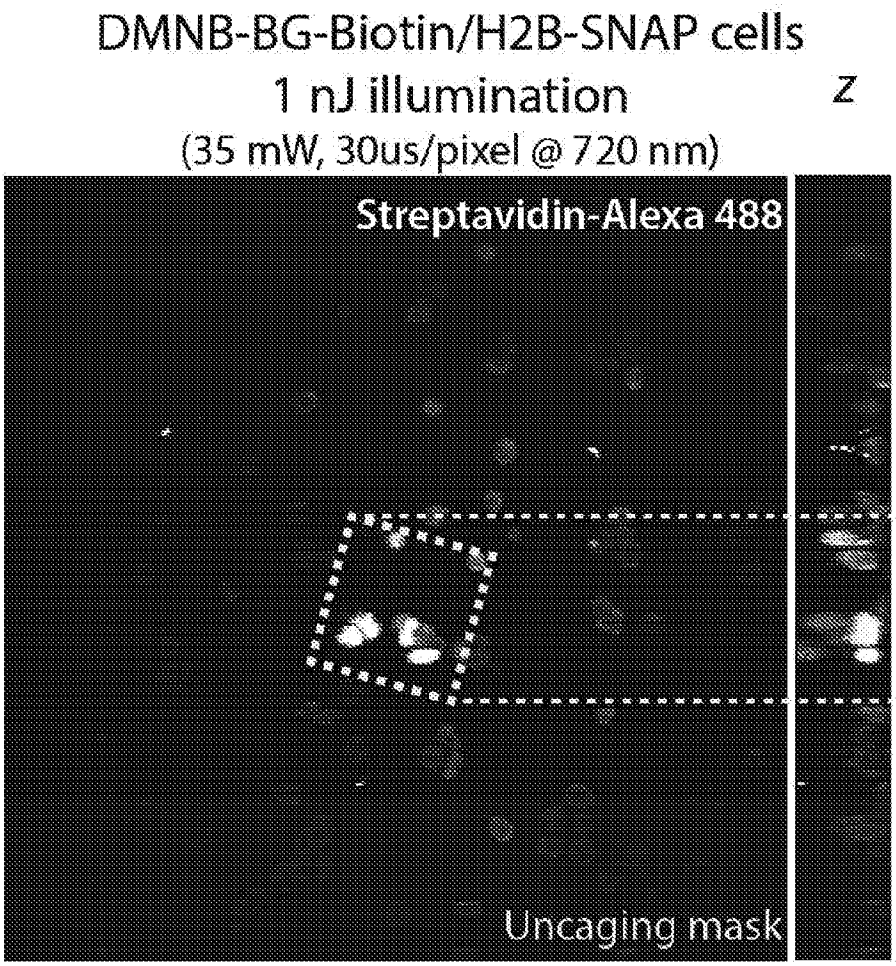

DMNB-BG-Biotin/H2B-SNAP cells
1 nJ illumination
(35 mW, 30us/pixel @ 720 nm)
z
Streptavidin-Alexa 488
Uncaging mask

FIG. 15D

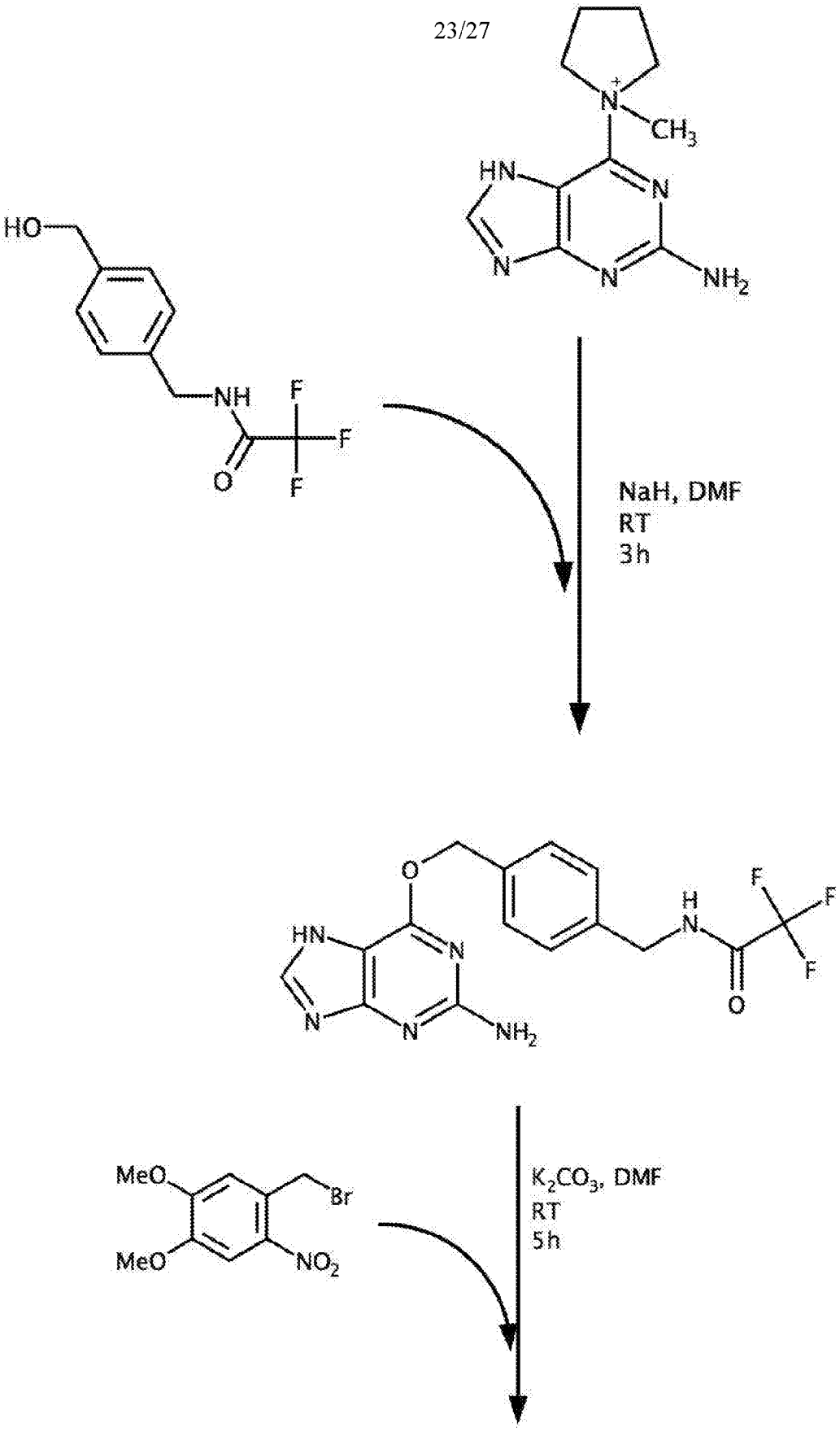

HO
NH
F
F
F
O
HN
N
N
N
N
CH_3
NH_2
NaH, DMF
RT
3h
O
HN
N
N
N
NH_2
H
N
F
F
F
O
MeO
MeO
Br
NO_2
K_2CO_3, DMF
RT
5h

FIG. 16

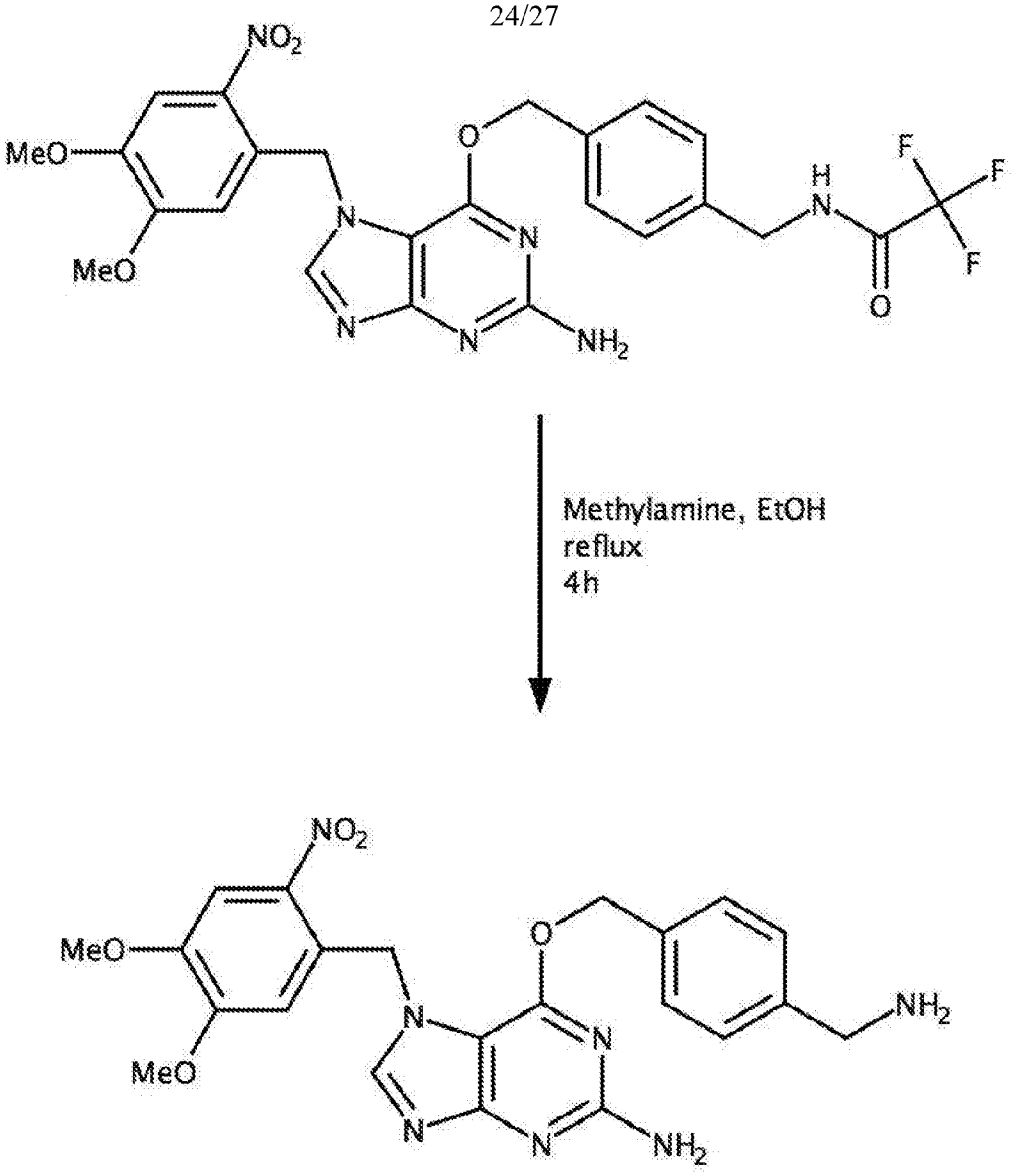

NO2
MeO
MeO
O
N
N
N
N
NH2
H
N
F
F
F
O
Methylamine, EtOH
reflux
4h
NO2
MeO
MeO
O
N
N
N
N
NH2
NH2

FIG. 16 cont.

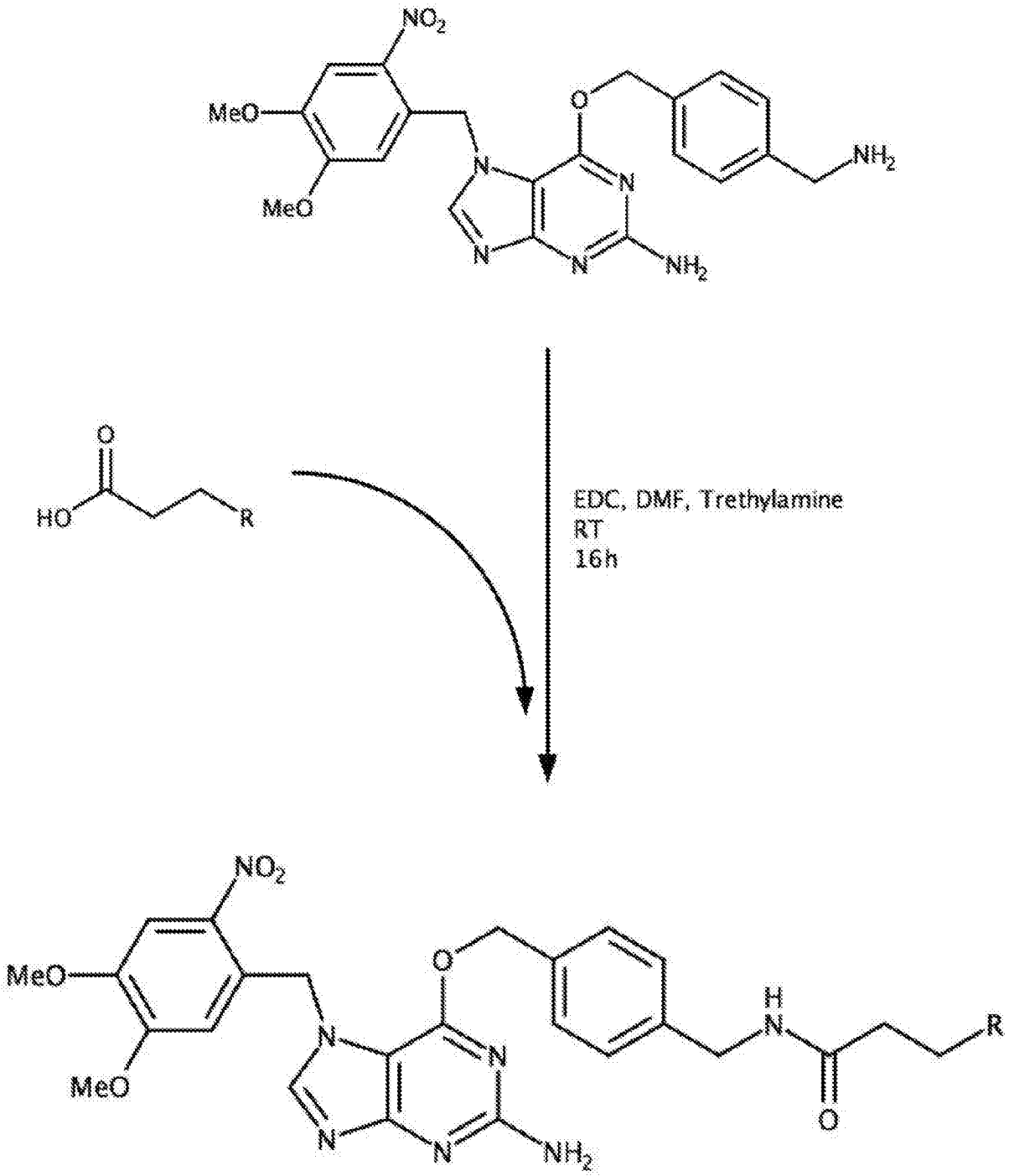

NO2
MeO
MeO
O
N
N
N
N
NH2
NH2
O
HO
R
EDC, DMF, Trethylamine
RT
16h
NO2
MeO
MeO
O
H
N
N
N
N
NH2
O
R

FIG. 16 cont.

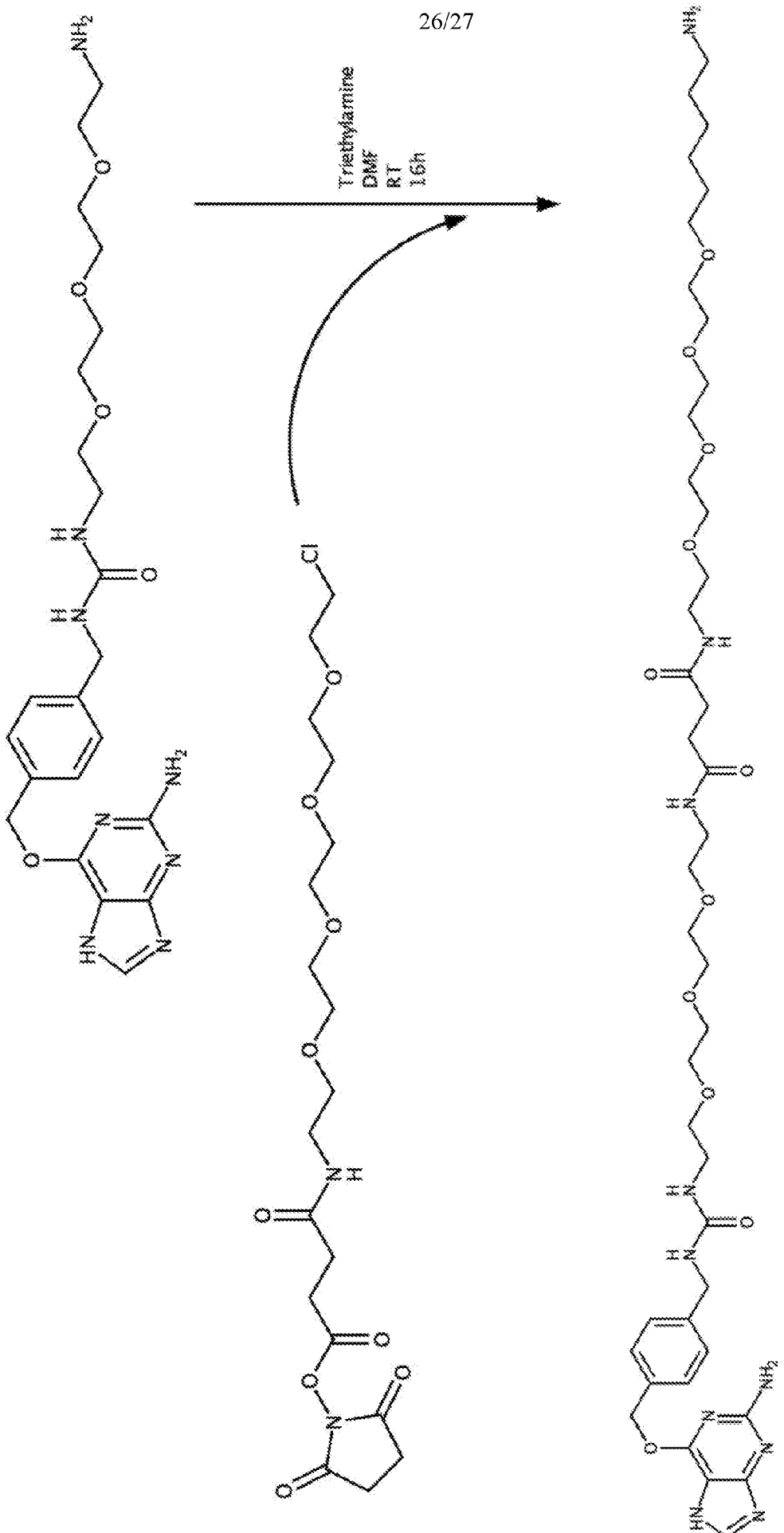


FIG. 17A

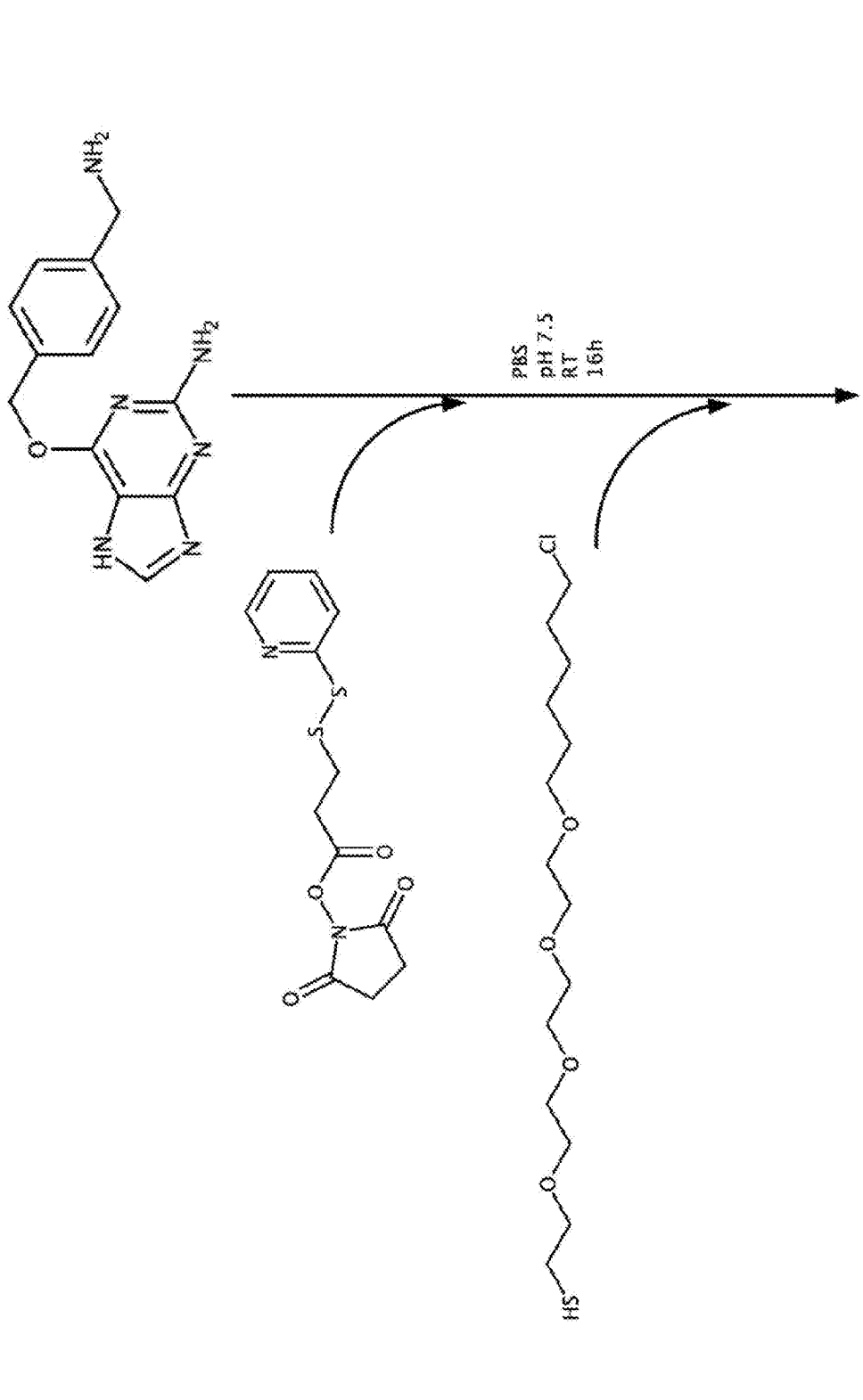


FIG. 17B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/061682

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/02(2006.01)i, C12Q 1/68(2006.01)i, C07K 1/14(2006.01)i, C12N 5/00(2006.01)i, C07C 211/43(2006.01)i, C07C 233/49(2006.01)i, C07H 21/04(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)
C12Q 1/02; G01N 33/543; C12Q 1/68; C07K 1/14; C12N 5/00; C07C 211/43; C07C 233/49; C07H 21/04

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: photochemically caged dimerizer, benzylguanine, chloroalkane, SNAP tag, HALO tag, linker

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ERHART et al., 'Chemical development of intracellular protein heterodimerizers' Chemistry & Biology, Vol.20, pp.549-557 (2013) See summary; and figures 1-2.	1-6
A		7-10,24-26
Y	LEMKE et al., 'Control of protein phosphorylation with a genetically encoded photocaged amino acid' Nature Chemical Biology, Vol.3, 12, pp.769-772 (2007) See pages 769-771.	1-6
A	SHAO et al., 'Photoactive molecules for applications in molecular imaging and cell biology' Chemical Society Reviews, Vol.39, pp.2835-2846 (2010) See the whole document.	1-10,24-26
A	ZIMMERMANN et al., 'Cell□permeant and photocleavable chemical inducer of dimerization' Angewandte Chemie International Edition, Vol.53, pp.4717-4720 (e-pub. 26 March 2014) See the whole document.	1-10,24-26

☒ 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	Date of mailing of the international search report
29 February 2016 (29.02.2016)	**29 February 2016 (29.02.2016)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/061682

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2009-060065 A1 (COVALYS BIOSCIENCES AG) 14 May 2009 See the whole document.	1-10,24-26
A	CHIDLEY et al., 'A designed protein for the specific and covalent heteroconjugation of biomolecules' Bioconjugate Chemistry, Vol.19, pp.1753-1756 (2008) See the whole document.	1-10,24-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/061682

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.:
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:

3. ☒ Claims Nos.: 11-23, 27-39
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 addtional 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/US2015/061682

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009-060065 A1	14/05/2009	None	