(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
08 March 2018 (08.03.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/044540 A1

(51) International Patent Classification:
G01N 33/543 (2006.01) *G01N 21/31* (2006.01)
G01N 33/68 (2006.01) *C07K 7/06* (2006.01)
G01N 33/58 (2006.01) *A61K 47/68* (2017.01)
G01N 33/53 (2006.01)

(21) International Application Number:
PCT/US2017/046771

(22) International Filing Date:
14 August 2017 (14.08.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/381,709 31 August 2016 (31.08.2016) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: COMPOSITIONS AND METHODS FOR DETECTING PROTEIN TRAFFICKING

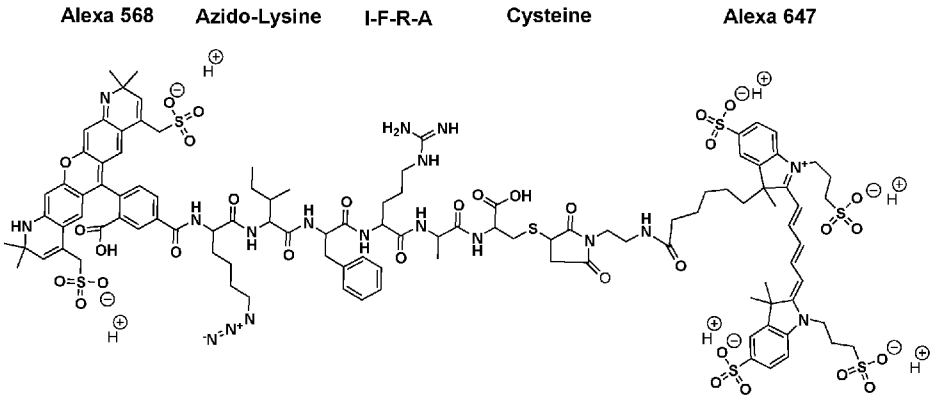

FIG. 1

(57) Abstract: Disclosed herein are compositions comprising dual labeled peptides. The dual labeled peptide can include a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid and a cleavage site. Also, disclosed herein are methods of using the dual labeled peptides.

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COMPOSITIONS AND METHODS FOR DETECTING PROTEIN TRAFFICKING

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. Provisional Application No. 62/381,709, which was filed on August 31, 2016. The content of this earlier filed application is hereby incorporated by reference herein in their entirety.

SEQUENCE LISTING

The sequence listing submitted August 14, 2017 as a text file named "37595_0011P1_SequenceListing," created on August 9, 2017, and having a size of 4,096 bytes is hereby incorporated by reference pursuant to 37 C.F.R. § 1.52(e)(5).

BACKGROUND

Cellular uptake of therapeutic polypeptides can be important to the activity of the therapeutic polypeptide. Antibodies and antibody-dug conjugates (ADCs) are non-limiting examples of therapeutic polypeptides for which intracellular uptake can be important. In addition, intracellular trafficking of a therapeutic polypeptide to the lysosome can also be important to the activity of the therapeutic polypeptide.

Antibody drug conjugates represent an established therapeutic approach in oncology. The activity of ADCs is dependent on a complex series of steps including cell surface binding, internalization, trafficking to appropriate intracellular compartments, and subsequent release of the cytotoxic payload. As with any drug, the efficacy and toxicity of a therapeutic polypeptide, such as an ADC, can be affected by the concentration of the polypeptide at the cell surface. However, existing methods of monitoring polypeptide uptake and trafficking to intracellular compartments are inadequate. Better materials and methods are required to evaluate cell surface binding, internalization, trafficking, and the kinetic profile.

In addition, better materials and methods are required, in order to evaluate whether or not polypeptides of interest, including, but not limited to, antibodies, bind to cells or cellular receptors, are internalized by a cell, and are trafficked to cellular compartments, such as the lysosome.

Using the constructs and the methods of the present invention, one can determine, in real-time, an analysis of polypeptide binding, intracellular uptake and cellular trafficking of a polypeptide of interest, in live cells.

SUMMARY

Tumor cells have been reported to have elevated levels of proteolytic enzymes, such as proteases, including cathepsins. The increased level of proteases in tumor cells often occurs in the early stage of tumor development likely due to increased cell cycling as well as for secretion to sustain invasion, metastasis and angiogenesis. The type of protease and its level can be associated with a specific cell type, physiological or pathological process. The compositions and methods described herein can be useful for tracking and monitoring, for example, antibodies in live cells, either *in vitro* or *in vivo*.

Disclosed herein are dual labeled peptides, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid and a cleavage site. In one aspect, the at least one amino acid to which the reactive handle is linked is a peptide or polypeptide.

Disclosed herein are methods of monitoring intracellular peptide degradation, the methods comprising: (a) exposing cultured cells to a dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid and a cleavage site; (b) applying fluorescent light to the cultured cells; and (c) detecting fluorescence of the cultured cells in (b). In one aspect, the at least one amino acid to which the reactive handle is linked is a peptide or polypeptide.

Disclosed herein are methods of monitoring the intracellular uptake of a polypeptide of interest in cultured cells, the methods comprising: (a) exposing cultured cells to a dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, and a cleavage site, and at least one modified amino acid or at least one reactive handle linked to a polypeptide; (b) applying fluorescent light to the cultured cells; and (c) detecting fluorescence of the cultured cells in (b). In one aspect, a change in the wavelength

of fluorescent light that is detected indicates that the polypeptide of interest has been trafficked to the lysosome.

Disclosed herein are dual labeled peptides for detecting the presence of antibody binding in a sample, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid, and a cleavage site, and wherein the antibody is conjugated to the at least one modified amino acid or at least one reactive handle linked to at least one amino acid.

Disclosed herein are dual labeled peptides for detecting the presence of enzyme binding in a sample, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid, and a cleavage site.

Disclosed herein are methods of screening carcinoma cultures for an antibody of interest, wherein the antibody of interest is conjugated to a dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid, and a cleavage site, the method comprising: (a) providing a plurality of primary carcinoma cultures with one or more antigens; (b) performing a first screen to determine specificity of a test antibody to the antigen; (c) performing a second screen of the test antibody by exposing the test antibody to a mixture of cells, wherein the mixture comprises some cells having the antigen in labeled form and some cells having the antigen in non-labeled form; (d) assaying for in vivo internalization of co-localized test antibody with the antigen; and (e) assaying for the absence of signal from the label in the cells that lack the antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several aspects and together with the description serve to explain the principles of the invention.

Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or can be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the

elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

FIG. 1 shows an example of a dual labeled peptide.

FIG. 2 shows the cleavage of the dual labeled peptide upon exposure to cathepsin b.

FIG. 3 shows the kinetics of the cleavage of the dual labeled peptide upon exposure to varying concentrations of cathepsin b.

FIGS. 4A-D shows simultaneous measurement of dual labeled peptide binding, internalization, low pH localization, and cleavage in live cells. Unbound dual labeled peptides were washed and 63x confocal images (Zeiss LSM 710) were acquired at time 0 (FIG. 4A) or following incubations at 37 °C for 30 minutes (FIG. 4B), 1 h (FIG. 4C), or 4 h (FIG. 4D).

FIGS. 5A-B shows that anti-PRLR dual labeled peptide co-localizes with lysotracker in T47D cells. Select image regions from FIG. 4 were enlarged. FIG. 5A shows the total anti-PRLR dual labeled peptide (Alexa 647, red) is localized to vesicles within 30 min. at 37°C, but cleavage (Alexa 568, green) and co-localization to the low pH compartments (lysotracker blue + Alexa 568 merge) are minimal. FIG. 5B shows that after 2h incubation at 37°C, most of the dual labeled peptide is in internalized (red), cleaved (green) and co-localized to the low pH compartment (blue) in the merged images (cyan). Yellow arrows indicate vesicles with co-localization of cleaved mAb and lysotracker blue. White arrows designate vesicles with internalized mAb in the absence of cleavage or lysotracker co-localization.

FIGS. 6A-C shows that total anti-PRLR dual labeled peptide cleavage is greater than anti-Her2 dual labeled peptide cleavage in T47D cells. FIG. 6A shows the cleavage kinetics of T47D after pre-incubation in the absence of bafilomycin A1, followed by incubation with anti-PRLR and anti-Her2 dual labeled peptides. FIG. 6B shows the cleavage kinetics of T47D after pre-incubation in the presence of bafilomycin A1, followed by incubation with anti-PRLR dual labeled peptides. FIG. 6C shows the cleavage kinetics of T47D after pre-incubation in the presence of bafilomycin A1, followed by incubation with anti-Her2 dual labeled peptides.

FIGS. 7A-C shows that anti-Her2 dual labeled peptide and antibody drug conjugate activity increased in combination with bispecific Her2 x PRLR or Her2 x Her2 antibodies. FIG. 7A shows T47D or T47D cells engineered to overexpress Her2 (T47D/Her2) were incubated with the anti-Her2 dual labeled peptide (ABC) (10 μg/ml) in the absence or presence of Her2 x

PRLR or Her2 x Her2 bispecific antibodies (10 μg/ml). FIG. 7B shows anti-Her2 mAb conjugated to the toxin DM1 via a non-cleavable linker, and serial dilutions of the antibody drug conjugates were combined with 10 μg/ml of unconjugated bispecific antibodies. FIG. 7C shows representative images of cleaved dual labeled peptides in T47D cells at 2 h.

FIGS. 8A-E shows that bispecific Her2 x PRLR dual labeled peptides and antibody drug conjugates more potent than single mAbs or combinations. FIG. 8A shows the relative cell surface Her2 and PRLR levels determined via flow cytometry; the histograms show the cell binding distribution and the table reports the fold change in mean fluorescent intensity above unstained levels for each mAb. FIG. 8B shows cells incubated with bispecific Her2 x PRLR, anti-PRLR, anti-Her2, or anti-C. diff dual labeled peptides (10 μg/ml), and cleavage was quantitated. FIG. 8C shows cells incubated with serially diluted bispecific Her2 x PRLR, anti-PRLR, anti-Her2, or anti-C. diff antibody drug conjugates conjugated to DM1. FIG. 8D shows cells incubated with the bispecific Her2 x PRLR ABC, anti-Her2 dual labeled peptides, or anti-Her2 ABC in combination with unconjugated Her2 x PRLR bispecific antibody (all mAbs at 10 μg/ml). FIG. 8E shows cells incubated with serial dilutions of bispecific Her2 x PRLR antibody drug conjugates, anti-Her2 ADC, or anti-Her2 antibody drug conjugates in combination with a constant amount (10 μg/ml) of unconjugated Her2 x PRLR bispecific antibody.

FIGS. 9A-B shows anti-PD-1 dual labeled peptide cleavage. FIG. 9A shows total Alexa 568 fluorescence for an anti-PD1 dual labeled peptide and an isotype control. FIG. 9B shows representative images of cleaved (green) and total (red) anti-PD1 dual labeled peptide levels at 2 h is shown (yellow indicates co-localization of total and cleaved dual labeled peptide). DAPI labeled nuclei shown in blue.

FIG. 10 is an example of the process for generating dual labeled peptides.

FIG. 11 is a schematic of lysosomal targeting by dual labeled peptides. ADC as used in this Figure represents the dual labeled antibody(s).

FIG. 12 is a schematic illustration of antibody conjugation to the dual labeled peptides.

DETAILED DESCRIPTION

The present invention can be understood more readily by reference to the following detailed description of the invention and the Examples included therein.

Unless otherwise expressly stated, it is in no way intended that any method or aspect set forth herein be construed as requiring that its steps be performed in a specific order. Accordingly, where a method claim does not specifically state in the claims or descriptions that the steps are to be limited to a specific order, it is in no way intended that an order be inferred, in any respect. This holds for any possible non-express basis for interpretation, including matters of logic with respect to arrangement of steps or operational flow, plain meaning derived from grammatical organization or punctuation, or the number or type of aspects described in the specification.

Definitions

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values described herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10" as well as "greater than or equal to 10" is also disclosed. It is also understood that throughout the application, data are provided in a number of different formats, and that these data, represent endpoints, starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point "15" are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each unit between

two particular units is also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur and that the description includes instances where said event or circumstance occurs and instances where it does not.

The word "or" as used herein means any one member of a particular list and also includes any combination of members of that list.

Compositions

Described herein are dual labeled peptides. The dual labeled peptide can include a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid; and a cleavage site. The first and second labels can be fluorescence resonance energy transfer (FRET) fluorophores. Examples of FRET fluorophores include, but are not limited to, Alexa Fluor® 568 (A568), Alexa Fluor® 647 (A647), or Alexa Fluor® 546 (A546). In an aspect, the first label can be a donor and the second label can be an acceptor. In an aspect, the first label can be an acceptor and the second label can be a donor. The fluorophores disclosed herein can be conjugated or bound to the peptide (e.g., N terminus or C terminus portion of the protein) described herein.

Attachment of the one or more labels (e.g., donor or acceptor) can be performed by conjugation. For example, the labels can be conjugated by chemically associating two or more chemical moieties. Conjugation can occur by the generation of a covalent bond.

In an aspect, the one or more labels can be attached directly or indirectly to the dual labeled peptides described herein. For example, the one or more labels can be attached to the dual labeled peptides via a spacer or other type of linker known to one of ordinary skill in the art. In an aspect, the spacer or linker do not increase the distance between the labels such that the label can no longer be substantially quenched. In other words, in some aspects the indirect attachment of the one or more labels can be a spacer or linker of a particular length that allows one label to be substantially quenched by a second label of a dual labeled peptide.

The fluorophore serves as a marker (or dye or tag). In an aspect, the first label and second label can be pH stable. The fluorophores used herein can be stable in a pH range of 2-10. For example, alexa fluorodyes are stable at a pH of 4-10 and can be best cleaved at a pH of 5 if

the linker is a cathepsin b linker. One of ordinary skill in the art would appreciate that fluorophores absorb light energy of a specific wavelength and can re-emit light at a longer or different wavelength and the time before emission can depend on the structure of the fluorophore as well as the environment (e.g., chemical environment).

The first and second labels described herein can be referred to as a pair. In an aspect, the pair can be a FRET pair. A FRET pair comprises a donor fluorophore and an acceptor fluorophore. The excitation of the donor fluorophore or molecule can result in emission of an acceptor fluorophore or molecule. Emission properties can be modulated in one or more of the followings approaches, including but not limited to distance, spectral overlap, quantum yields and orientation.

In an aspect, the distance between the first and second labels can be between about 10 Å and 120 Å. In an aspect, the distance between the first and second labels can be 47 Å. The distance between the first and second labels described herein can be 10, 15, 20, 25, 30, 35, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115 or 120 Å, or distance (e.g., Å) in between.

The labels disclosed herein can be excitable by exposure to a wavelength spectrum in any spectral range. In an aspect, the labels disclosed herein can be excitable by exposure to a wavelength spectrum in a spectral range of from about 500 nm to about 700 nm.

In some aspects, the first and second label can be a detection agent, which may or may not be a protein. Non-limiting examples of the detection agent, include, but are not limited to a fluorophore, a fluorescent protein, a radioisotope, or a dye.

Non-limiting methods of detecting fluorescence in live cells include, but are not limited to, confocal microscopy and wide-field microscopy.

The dual labeled peptide disclosed herein can comprise a peptide sequence. The peptide sequence can comprise a cleavable sequence. The peptide sequence can include a non-cleavable sequence. In an aspect, the peptide sequences described herein can be 3, 4, 5, 6, or more amino acids in length. In an aspect, the peptide sequence can be IFRA (SEQ ID NO: 1), KIFRAC (SEQ ID NO: 2), K(N3)IFRAC (SEQ ID NO: 3), K(N3)-X-X-X-X-C (SEQ ID NO: 4), FRA, FRFF (SEQ ID NO: 5), YKFF (SEQ ID NO: 6), YRFF (SEQ ID NO: 7), FKFF (SEQ ID NO: 8), XXFXX (SEQ ID NO: 9), XXLXX (SEQ ID NO: 10), VRA, YRA, or VLA. Generally, "X" can be any amino acid. In some aspects, "X" is not a proline if "X" is adjacent to the cleavage site.

In an aspect, "X" can be a proline when at least two sequential amino acids of the dual labeled peptide are not proline. In an aspect, the cleavable sequence can be KIFRAC (SEQ ID NO: 2).

With respect to preparing the peptide sequences as well as the dual labeled peptides described herein, they can be generated by any method known in the art, including synthetic methods and recombinant techniques to produce proteins from nucleic acids. The peptide sequences can be stored in an unpurified or in an isolated or substantially purified form until further use.

The term "dual labeled peptide" as used herein can refer to an amino acid-based compound formed of molecularly coupled (e.g., covalently bonded) parts. In an aspect, the parts include an amino acid sequence or peptide, a first label on the N-terminus of the peptide, a second label on the C-terminus of the peptide, at least one modified amino acid or other type of moiety that can serve as an attachment site or carrier for an antibody, protein, nucleic acid or virus capsid. The antibody, protein, nucleic acid or virus capsid can bind to a target. The amino acid sequence or peptide can comprise a protease cleavage site. An "amino acid-based compound" is one that includes primarily, but not necessarily exclusively, amino acid residues.

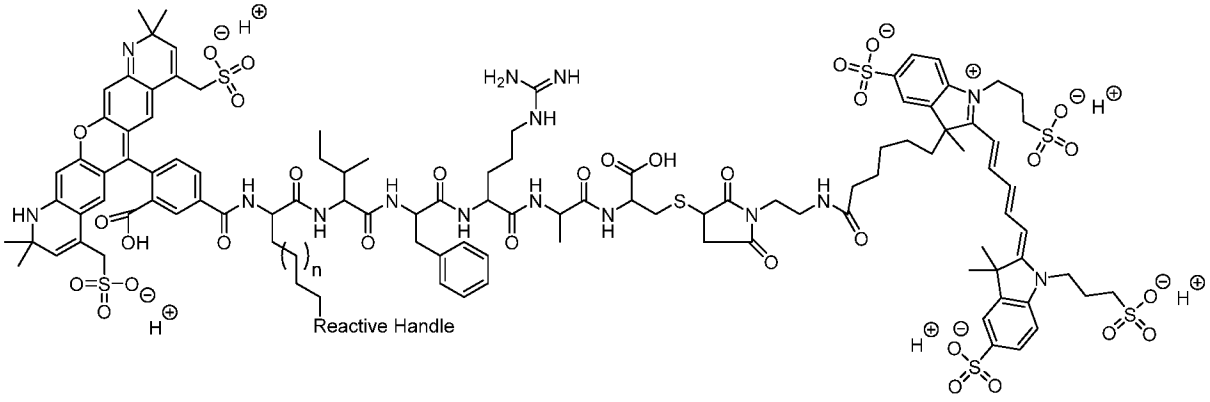
In an aspect, the dual labeled peptides described herein can be a hexapeptide comprising a cleavable sequence. The cleavable sequence can be KIFRAC (SEQ ID NO: 2). In an aspect, the N-terminal K can be replaced with an azido-lysine for chemoselective, "click" conjugation to an alkyne. In an aspect, Alexa 568 can be conjugated to the N-terminal amine. In an aspect, Alexa 647 can be conjugated to the C-terminal cysteine side chain sulfhydryl. In an aspect, the dual labeled peptide comprises an azido moiety on the N-terminal lysine. In an aspect, the maximum extended distance between Alexa 568 and Alexa 647 is 47 Å.

The dual labeled peptide disclosed herein can include at least one modified amino acid or at least one Reactive Handle linked to at least one amino acid. In an aspect, the at least one modified amino acid can be an azide, azido-lysine, azido-alanine, cyclooctyne-alanine, or cyclooctyne-lysine. In an aspect, the at least one modified amino acid can include at least one reactive handle. The site of attachment can also be an azido group of the azido-lysine. The at least one modified amino acid and/or the at least one reactive handle can serve as a site for attachment (e.g., conjugation or linkage site) for a protein, peptide, polypeptide or antibody.

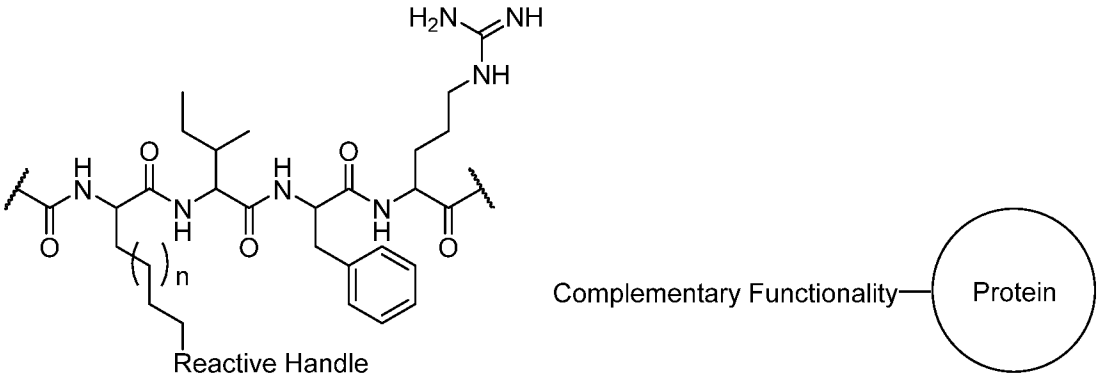
In an aspect, the dual labeled peptide disclosed herein can be attached to a protein via the at least one modified amino acid and/or the at least one reactive handle reagent by means

of native or non-native routes. Examples of a native route include but are not limited to conjugating directly to one or ore native lysine residues using an activated ester handle (e.g., N-Hydroxysuccinimide) or through a free sulfhydryl by way of maleimide. An example of a non-native route can be through a bioorthogonal handle including but not limited to an azido or strained cyclooctyne. The dual labeled peptides can comprise a group that is reactive with a primary amine on the peptide sequence or modified amino acid to which an antibody, for example, can be conjugated, linked or otherwise attached. Examples of reactive handle reagents or "linkers" include but are not limited to monofluoro cyclooctyne (MFCO), bicyclo[6.1.0]nonyne (BCN), N-succinimidyl-Sacetylthioacetate (SATA), N-succinimidyl-S-acetylthiopropionate (SATP), maleimido and dibenzocyclooctyne ester (a DBCO ester). Useful cyclooctynes, within a given linker, include OCT, ALO, MOFO, DIFO, DIBO, BARAC, DIBAC, and DIMAC. Conjugation agents useful in the present dual labeled peptides are available from many commercial sources. Conjugation can proceed between primary amine groups (e.g., on a lysine residue) and sulfhydryl groups (e.g., on a cysteine residue).

In various aspects, the reactive handle can be provided at a side-chain of one or more of the amino acid residues. For example, the dual labeled peptide can be provided as shown below.

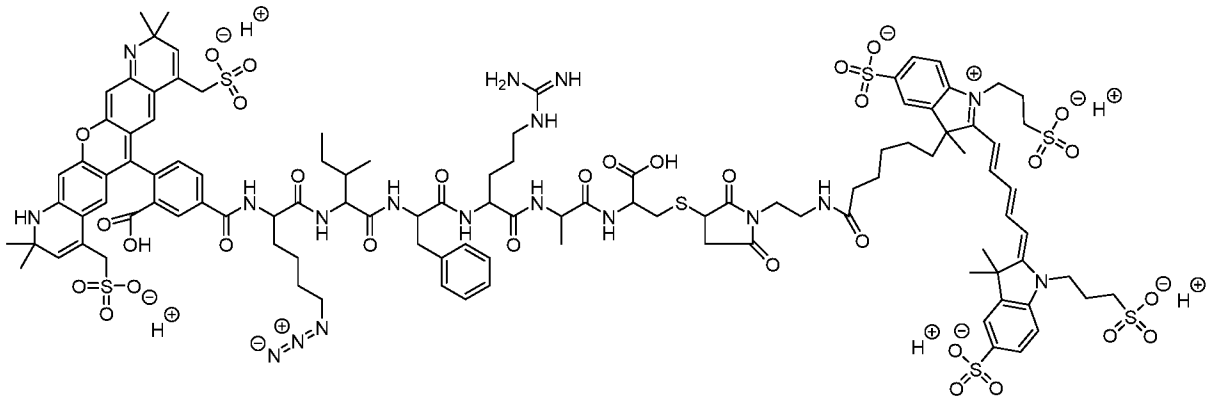


For simplicity, a portion of the structure of the dual labeled peptide can be represented as shown below.

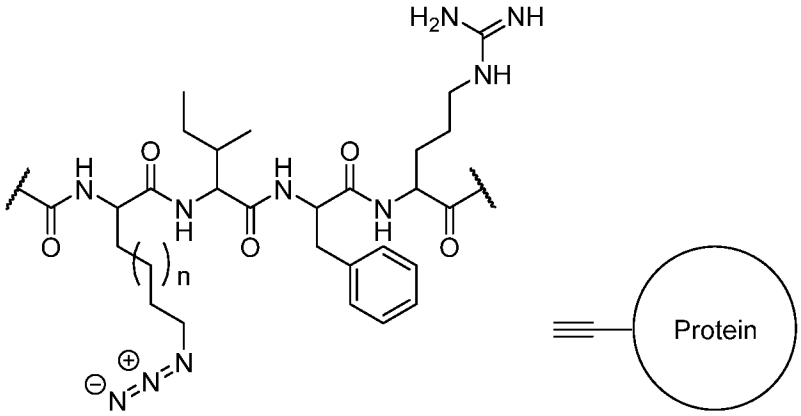


It is appreciated that the dual labeled peptide can be selectively functionalized via the Reactive Handle so as to be capable of conjugation to another agent, e.g., a protein, as shown above. It is also appreciated that such a protein can be functionalized with a Complementary Functionality selected for reaction with the Reactive Handle, as shown above.

In further aspects, the reactive handle can be selected from among chemical functionalities disclosed herein or known in the art. For example, the Reactive Handle can be selected to be an azide, as shown below.

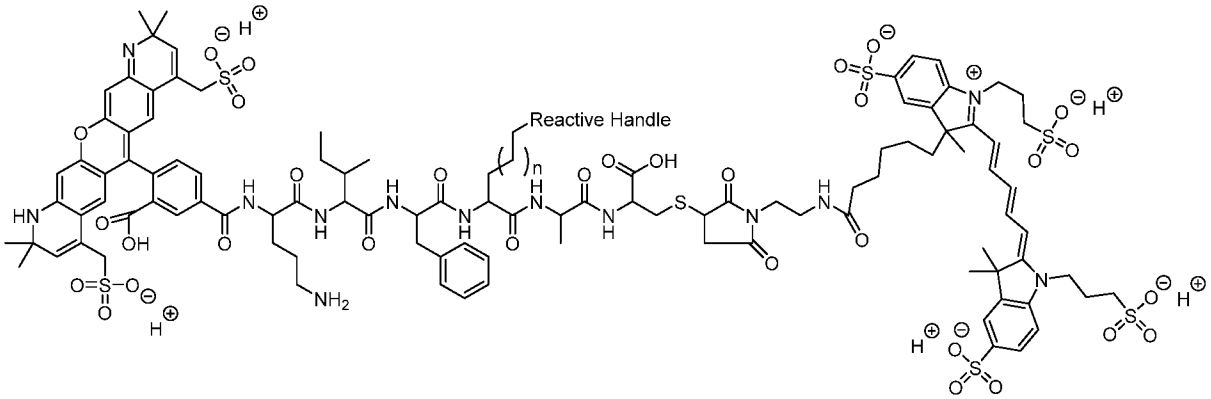


Again, for simplicity, a portion of the structure of the dual labeled peptide can be represented as shown below.

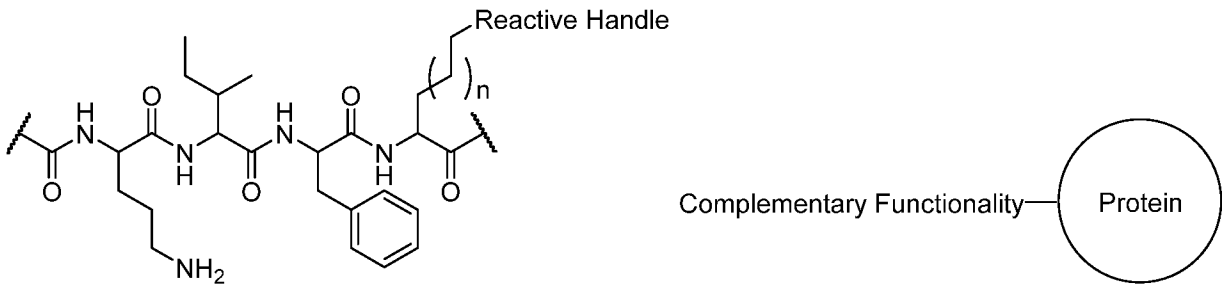


It is appreciated that a dual labeled peptide can be functionalized with a Reactive Handle selected to be an azide, capable of conjugation to another agent, e.g., a protein, via Complementary Functionality selected to be an alkyne, as shown above. It is also appreciated that the reaction between the azide and the alkyne can be copper promoted or strain promoted.

In various aspects, the Reactive Handle can be provided at a different side-chain. For example, the dual labeled peptide can be provided as shown below.



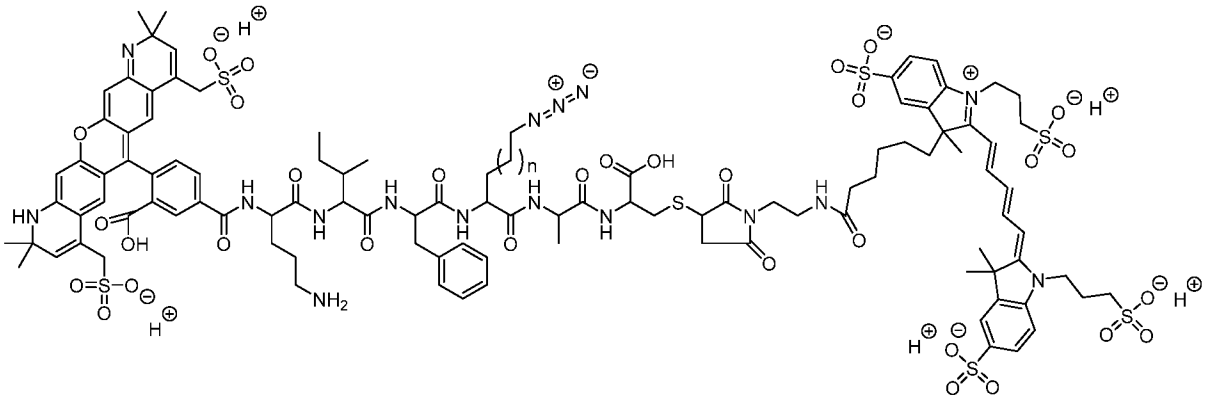
For simplicity, a portion of the structure of the dual labeled peptide can be represented as shown below.



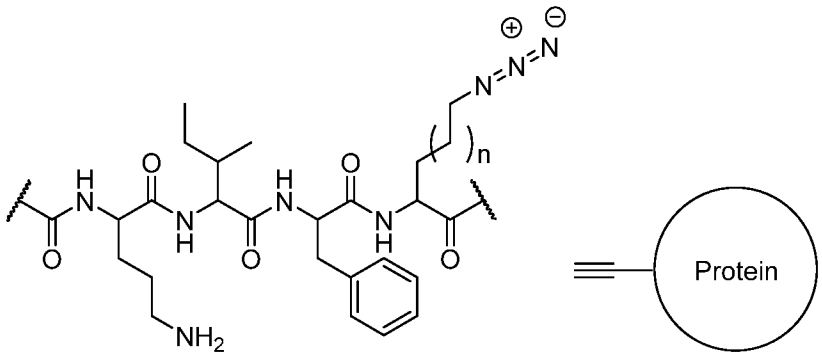
It is appreciated that the dual labeled peptide can be selectively functionalized via the Reactive Handle so as to be capable of conjugation to another agent, e.g., a protein, as shown

above. It is also appreciated that such a protein can be functionalized with a Complementary Functionality selected for reaction with the Reactive Handle, as shown above.

In further aspects, the Reactive Handle can be selected from among chemical functionalities disclosed herein or known in the art. For Example, the Reactive Handle can be selected to be an azide, as shown below.

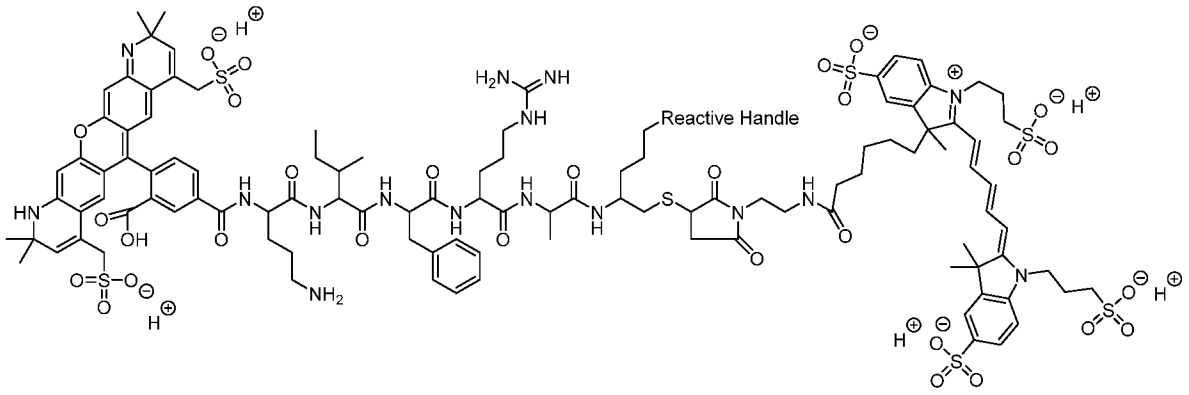


Again, for simplicity, a portion of the structure of the dual labeled peptide can be represented as shown below.

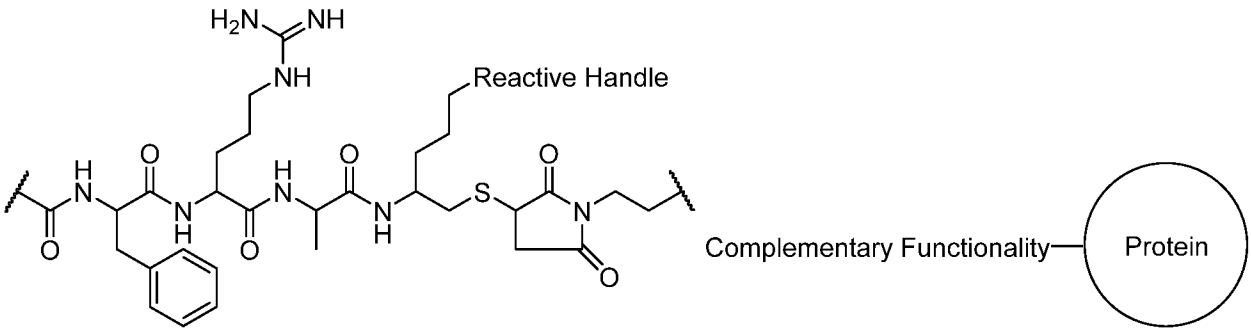


It is appreciated that a dual labeled peptide can be functionalized with a Reactive Handle selected to be an azide, capable of conjugation to another agent, e.g., a protein, via Complementary Functionality selected to be an alkyne, as shown above. It is also appreciated that the reaction between the azide and the alkyne can be copper promoted or strain promoted.

In various aspects, the Reactive Handle can be provided at a different side-chain and with a different chemical functionality. For example, the dual labeled peptide can be provided as shown below.

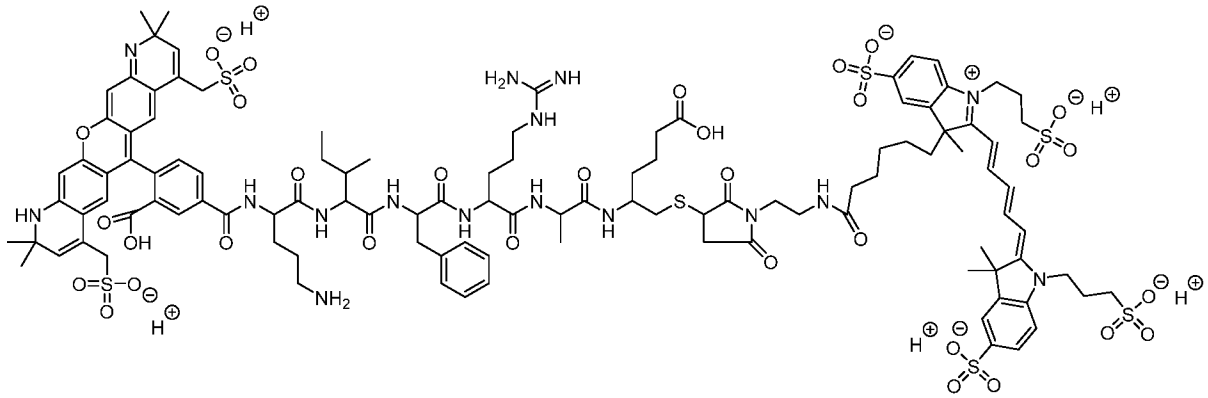


For simplicity, a portion of the structure of the dual labeled peptide can be represented as shown below.

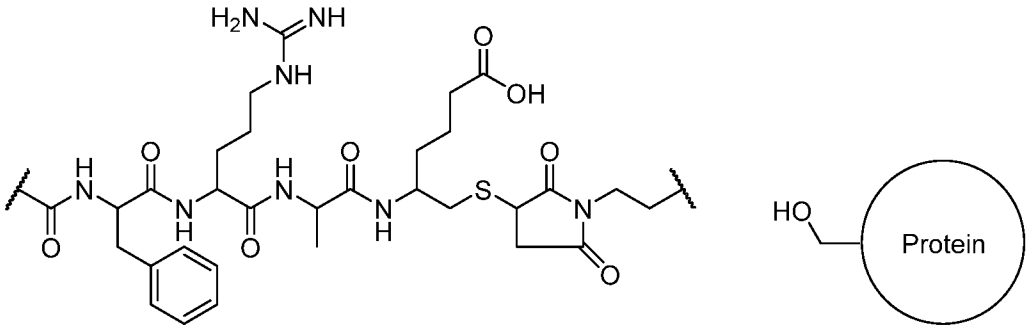


It is appreciated that the dual labeled peptide can be selectively functionalized via the Reactive Handle so as to be capable of conjugation to another agent, e.g., a protein, as shown above. It is also appreciated that such a protein can be functionalized with a Complementary Functionality selected for reaction with the Reactive Handle, as shown above.

In further aspects, the Reactive handle can be selected from among chemical functionalities disclosed herein or known in the art. For Example, the Reactive Handle can be selected to be a carboxylic acid, as shown below.



Again, for simplicity, a portion of the structure of the dual labeled peptide can be represented as shown below.



It is appreciated that a dual labeled peptide can be functionalized with a Reactive Handle selected to be a carboxylic acid, capable of conjugation to another agent, e.g., a protein, via Complementary Functionality selected to be a hydroxyl, as shown above. It is also appreciated that the reaction between the carboxylic acid and the hydroxyl can be promoted with known reagents such as carbodiimides.

Sites available for conjugating antibody, protein, nucleic acid, or virus capsid can be found on the peptide sequence of the dual labeled peptide. The linker or attachment group can be or can comprise a flexible arm having, for example, 2, 3, 4, 5, 10, 15, or 20 carbon atoms in, for instance, an aliphatic chain. An example of a linker or attachment group is an azido functional group.

Any of the amino acids incorporated in the dual labeled peptides can be modified to chemically interact with, or to include, a linker or attachment group. For example, the peptide sequence can be modified to include an N_3 azide group. In other aspects, the peptide sequence can be modified to include a cysteine residue or other thiol-bearing moiety (e.g., C-SH) at the N-terminus, the C-terminus, for reaction with, for example, a maleimmide-containing linker.

Conjugation techniques include the application of click chemistry. Generally, click chemistry is a modular reaction that is widely applicable and capable of producing high yields of products under physiological conditions. Click chemistry encompasses four classes of chemical transformations. The first are non-aldol type carbonyl chemical reactions, such as those that form ureas, thioureas, oxime ethers, hydrazone, amides, and aromatic heterocycles. The second transformations are nucleophilic substitution reactions in which a ring within a

strained heterocyclic electrophile (e.g., epoxides, aziridines and aziridinium ions) is opened. In the third, addition reactions to C-C multiples bonds, such as Michael addition, epoxidation, aziridation, and dihydroxylation occurs, and in the fourth, are cycloaddition reactions, such as 1,3-dipolar cycloaddition and Diels-Alder reactions. 1,3-dipolar cycloaddition (1,3-Huisgen reaction) of an alkyne and an azide to form five membered triazole is a particular example of a click reaction.

In an aspect, the dual labeled peptide disclosed herein can comprise a cleavage site. In an aspect, the cleavage site is a cathepsin b cleavage site. In an aspect, the cleavage site can be cathepsin l, cathepsin h or cathepsin s. Cathepsins are known in general to become activated at low pH in lysosomes with a few exceptions (e.g., cathepsin K). In an aspect, the first and second label can be separated by the dual labeled peptide disclosed herein (e.g., via the cleavage site). Cathepsin b is a lysosomal cysteine protease. It is a glycoprotein containing two N-linked oligosaccharide chains. The dual labeled peptide disclosed herein can be cleaved by endogenous cathepsin b resulting in the separation of the first and second label (e.g., a FRET pair).

The cleavage site can also be a cleavage site cleavable by proteases, acid hydrolases or acid lipases. Proteases are a type of acid hydrolase. Acid hydrolases also referred to as acid lipases are commonly located in lysosomes. Examples of acid hydrolases include but are not limited to galactosidase, glucosidase, glucuronidase, hexosaminidase and mannosidase.

In an aspect, the dual labeled peptides described herein can comprise a carrier. In an aspect, the carrier is an antibody, a protein, a polypeptide, a nucleic acid, a virus or a capsid. The carrier can be conjugated, bound or otherwise linked to the dual labeled peptide. In an aspect, the carrier is an antibody. In an aspect, the antibody, or a fragment thereof that specifically binds the same antigen to which the antibody specifically binds, is attached to the dual labeled peptide through at least one modified amino acid or at least one reactive handle. In an aspect, the carrier can be attached to the dual labeled peptide by way of strained cyclooctyne copper-free click chemistry.

In an aspect, the carrier targets a receptor or an enzyme or a gene. In an aspect, the target (e.g., receptor or an enzyme) can include but not limited to metalloreductase STEAP1, metalloreductase STEAP2, prostate-specific membrane antigen (PSMA), programmed cell death protein 1 receptor (PD1), tumor markers or other biomarkers, such as cancer antigen 125 (e.g., CA-125 or mucin 16 or MUC16), and human epidermal growth factor receptor 2 (HER2).

The dual labeled peptides disclosed herein can specifically bind an antigen that is expressed on the cell surface of a dysplasic cell, a healthy or normal cell, a tumor cell or a malignant cell.

One of ordinary skill in the art would understand that the component parts need to be associated in a compatible manner. The number of polypeptide (e.g., antibody) carriers, for instance, can be one or more. In some aspects, the ratio of dual labeled peptide to carrier or antibody can be 1:1. The ratio of dual labeled peptide to carrier can be 1:2, 1:3, 1:4, 1:5, 1:6 or any combination thereof.

In some aspects, the antibody can be a single chain antibody (scFv) or a Fab fragment; a human, chimeric or humanized antibody or a biologically active variant thereof; and/or can be (or can be derived from) a monoclonal or polyclonal antibody. The antibody can be a naturally expressed antibody (e.g., a tetrameric antibody) or a biologically variant thereof.

In some aspects, the antibody can be a non-naturally occurring antibody (e.g., a single chain antibody or diabody) or a biologically active variant thereof. As noted above, the variants include, without limitation, a fragment of a naturally occurring antibody (e.g., a Fab fragment), a fragment of a scFv or diabody, or a variant of a tetrameric antibody, an scFv, a diabody, or fragments thereof that differ by an addition and/or substitution of one or more amino acid residues. The antibody can also be further engineered.

In an aspect, the antibody can be a single chain antibody (scFv) or a Fab fragment. The antibody can be human, chimeric or humanized or biologically active variant thereof. In an aspect, the antibody can be a monoclonal antibody. In an aspect, the antibody can be a polyclonal antibody. In an aspect, the antibody can be a therapeutic agent. The antibody or therapeutic agent can be an anti-cancer agent. In an aspect, the antibody can be trastuzumab, panitumumab or cetuximab or biologically active variant thereof. In an aspect, the scFv or a Fab fragment can bind to a member of the epidermal growth factor receptor (EGFR) family or prolactin receptor. In an aspect, the antibody can be an anti-influenza antibody (e.g., anti-influenza A or anti-influenza B or a broad spectrum anti-influenza A and B antibody).

As noted above, the polypeptide to which a dual labeled peptide as disclosed herein can include an antibody or a fragment thereof that specifically binds the same antigen to which the antibody specifically binds. As is well known in the art, monoclonal antibodies can be made by recombinant DNA. DNA encoding monoclonal antibodies can be readily isolated and sequenced

using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). Libraries of antibodies or active antibody fragments can also be generated and screened using phage display techniques.

In vitro methods are also suitable for preparing monovalent antibodies. As it is well known in the art, some types of antibody fragments can be produced through enzymatic treatment of a full-length antibody. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields a fragment that has two antigen combining sites and is still capable of cross-linking antigen. Antibodies incorporated into the present dual labeled peptides can be generated by digestion with these enzymes or produced by other methods.

The antibody fragments, whether attached to other sequences or not, can also include insertions, deletions, substitutions, or other selected modifications of particular regions or specific amino acids residues, provided the activity of the antibody or antibody fragment is not significantly altered or impaired compared to the non-modified antibody or antibody fragment. These modifications can provide for some additional property, such as to remove/add amino acids capable of disulfide bonding, to increase its bio-longevity, to alter its secretory characteristics, etc. In any case, the antibody or antibody fragment must possess a bioactive property, such as specific binding to its cognate antigen. Functional or active regions of the antibody or antibody fragment can be identified by mutagenesis of a specific region of the protein, followed by expression and testing of the expressed polypeptide. Such methods are readily apparent to a skilled practitioner in the art and can include site-specific mutagenesis of the nucleic acid encoding the antibody or antibody fragment.

As used herein, the term "antibody" or "antibodies" can also refer to a human antibody and/or a humanized antibody. Many non-human antibodies (e.g., those derived from mice, rats, or rabbits) are naturally antigenic in humans, and thus can give rise to undesirable immune responses when administered to humans. Therefore, the use of human or humanized antibodies

in the methods serves to lessen the chance that an antibody administered to a human will evoke an undesirable immune response.

Antibody humanization techniques generally involve the use of recombinant DNA technology to manipulate the DNA sequence encoding one or more polypeptide chains of an antibody molecule. Accordingly, a humanized form of a non-human antibody (or a fragment thereof) is a chimeric antibody or antibody chain (or a fragment thereof, such as an Fv, Fab, Fab', or other antigen binding portion of an antibody) which contains a portion of an antigen binding site from a non-human (donor) antibody integrated into the framework of a human (recipient) antibody.

The Fv region is a minimal fragment containing a complete antigen-recognition and binding site consisting of one heavy chain and one light chain variable domain. The three CDRs of each variable domain interact to define an antigen-binding site on the surface of the Vh-Vl dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. As well known in the art, a "single-chain" antibody or "scFv" fragment is a single chain Fv variant formed when the VH and Vl domains of an antibody are included in a single polypeptide chain that recognizes and binds an antigen. Typically, single-chain antibodies include a polypeptide linker between the Vh and Vl domains that enables the scFv to form a desired three-dimensional structure for antigen binding.

To generate a humanized antibody, residues from one or more complementarity determining regions (CDRs) of a recipient (human) antibody molecule are replaced by residues from one or more CDRs of a donor (non-human) antibody molecule that is known to have desired antigen binding characteristics (e.g., a certain level of specificity and affinity for the target antigen). In some instances, Fv framework (FR) residues of the human antibody are replaced by corresponding non-human residues. Humanized antibodies can also contain residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies. Humanized antibodies generally contain at least a portion of an antibody constant region (Fc), typically that of a human antibody.

Methods for humanizing non-human antibodies are well known in the art. For example, humanized antibodies can be generated by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Methods that can be used to produce humanized antibodies are also well known in the art.

In some aspects, the dual labeled peptides, described herein, comprise an antibody (e.g., scFv or Fab fragment) that binds to an EGFR or prolactin receptor. Examples of receptors for proteins in the EGF family include an EGF receptor (EGFR), a heparin-binding EGF-like growth factor receptor (HB-EGFR), an amphiregulin receptor (AR), an epiregulin receptor (EPR), a betacellulin receptor, and a receptor for neuregulin (e.g., a receptor for neuregulin-1, neuregulin-2, neuregulin-3, or neuregulin-4). Accordingly, in some embodiments, the antibody binds to a member of the EGFR family (e.g., HER2 (human epidermal growth factor receptor 2), sometimes called ERBB2, HER2/neu) and the antibody can be, including, but not limited to, trastuzumab, cetuximab, or panitumumab, or a biologically active variant thereof. In some embodiments, the antibody binds to a prolactin receptor. More generally, the antibody can be a therapeutic agent (e.g., an anti-cancer agent).

Attributes of the dual labeled peptides disclosed herein include but are not limited to: simultaneously measuring antibody binding, internalization, localization to low pH compartments, linker cleavage kinetics, fluorescent dyes that are not impacted by pH (e.g., pH stable), possess a binary fluorescent mechanism (e.g., "on" or "off"), for example, in the "on state," Alexa 568 is highly fluorescent when cleaved in a low pH compartment (e.g., lysosomal compartments) and in the "off" state," Alexa 568 is highly quenched (e.g, greater than 95%) when bound (not cleaved from the dual labeled peptide and while in any other cellular compartments); while minimizing any intermediate fluorescent state; and real-time live cell cleavage kinetics that can be measured and amendable to high throughput screening. Further, the dual labeled peptides disclosed herein can be traceable from cell-surface binding to internalization to the lysosome, detectable in single cells at a low copy number. One of ordinary skill in the art would appreciate that the substrate concentration can be relative or absolute, and can determine the rate of cleavage. As used herein, the term "cleavage" can refer to "linker cleavage," "enzyme cleavage" or "linker release."

Methods

Disclosed herein are methods of monitoring intracellular peptide degradation. The method can include the following steps. First, exposing cultured cells to a dual labeled peptide as disclosed herein, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid; and a cleavage site. Next, applying fluorescent light to the cultured cells and detecting the fluorescence of the cultured cells that were exposed to the fluorescent light.

The cells (e.g., cultured cells) can be incubated with the dual labeled peptide as disclosed herein for a sufficient period of time to allow binding of, for example, an antibody to a surface antigen (e.g., a receptor); to permit receptor mediated endocytosis followed by trafficking to an intracellular target (e.g., lysosomal compartment); and to allow for cleavage of the dual labeled peptide to occur at the cleavage site. Next, fluorescent light can be applied to the cultured cells.

During fluorescence resonance energy transfer, the donor fluorophore (e.g., a first label) becomes excited and transfers its energy to the acceptor fluorophore (e.g., a second label). The acceptor fluorophore emits light. The emitted light is detected in specific channels. The emitted light post fluorescent light exposure and cleavage of the dual labeled peptide can be monitored and recorded. Fluorescence activation of the dual labeled peptide described herein can be caused by cleavage of a bond in the dual labeled peptide by an enzyme, for example, a cathepsin. The light recorded and emitted from the donor fluorophore can change from one color to a second color upon cleavage of the dual labeled peptide by, for example, cathepsin b. Degradation can be readily distinguished form other modifications by using additional assays. In an aspect, the degradation is the result of protease degradation.

In an aspect, the first and second label can be a pair capable of fluorescence resonance energy transfer. Fluorescence is detected by exposing the sample to a light source, and detecting emitted light with a detector.

Any cell type can be used and cultured using known techniques. In an aspect, the cell is a mammalian cell. In an aspect, the mammalian cell is the subject's cell. In an aspect, the cell is an allogenic cell.

In an aspect, the methods described herein can be applied *in vivo*.

As disclosed herein, are compositions, comprising the dual labeled peptides as described above and an acceptable carrier. The compositions of the present disclosure also contain an effective amount of the dual labeled peptides as described herein. The compositions can be formulated for administration by any of a variety of routes of administration, and can include one or more physiologically acceptable excipients, which can vary depending on the route of administration. As used herein, the term "excipient" means any compound or substance, including those that can also be referred to as "carriers" or "diluents." Preparing physiologically acceptable compositions is considered routine in the art, and thus, one of ordinary skill in the art can consult numerous authorities for guidance if needed.

The compositions as disclosed herein can be prepared for oral or parenteral administration. Compositions prepared for parenteral administration include those prepared for intravenous (or intra-arterial), intramuscular, subcutaneous, intraperitoneal, transmucosal (e.g., intranasal, intravaginal, or rectal), or transdermal (e.g., topical) administration. Aerosol inhalation can also be used to deliver the dual labeled peptides. Thus, compositions can be prepared for parenteral administration that includes dual labeled peptides dissolved or suspended in an acceptable carrier, including but not limited to an aqueous carrier, such as water, buffered water, saline, buffered saline (e.g., PBS), and the like. One or more of the excipients included can help approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents, and the like. Where the compositions include a solid component (as they may for oral administration), one or more of the excipients can act as a binder or filler (e.g., for the formulation of a tablet, a capsule, and the like). Where the compositions are formulated for application to the skin or to a mucosal surface, one or more of the excipients can be a solvent or emulsifier for the formulation of a cream, an ointment, and the like.

The compositions as disclosed herein can be sterile and sterilized by conventional sterilization techniques or sterile filtered. Aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation, which is encompassed by the present disclosure, can be combined with a sterile aqueous carrier prior to administration. The pH of the pharmaceutical compositions typically will be between 3 and 11 (e.g., between about 5 and 9) or between 6 and 8 (e.g., between about 7 and 8). The resulting compositions in solid form can be packaged in multiple single dose units, each containing a fixed amount of the above-mentioned agent or

agents, such as in a sealed package of tablets or capsules. The composition in solid form can also be packaged in a container for a flexible quantity, such as in a squeezable tube designed for a topically applicable cream or ointment.

In an aspect, the methods described herein can be used to monitor degradation *in vitro* and *in vivo*. Non-limiting methods of detecting fluorescence include, but are not limited to, confocal microscopy and wide-field microscopy. Additional methods that can be used to monitor fluorescence using the disclosed compositions include but are not limited to fluorescence-activated cell sorting (e.g., flow cytometry).

Disclosed herein are methods of monitoring the intracellular uptake of a polypeptide of interest in cultured cells, the methods comprising: (a) exposing cultured cells to a dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, and a cleavage site, and at least one modified amino acid or at least one reactive handle linked to a polypeptide; (b) applying fluorescent light to the cultured cells; and (c) detecting fluorescence of the cultured cells in (b). In one aspect, a change in the wavelength of fluorescent light that is detected indicates that the polypeptide of interest has been trafficked to the lysosome. Applications of the method include, but are not limited to, monitoring the intracellular uptake and lysosomal trafficking of a polypeptide, such as an antibody (whether mono-specific or bi-specific), an antibody-drug conjugate (whether toxic or non-toxic). Using this method, one can determine if a polypeptide of interest merely binds to the surface of a cell, or if it is internalized, trafficked to the lysosome, and degraded. For example, when a dual labeled peptide is labeled with Alexa 568 and Alexa 647, the cells will fluoresce red if the polypeptide of interest is not internalized, trafficked to the lysosome, and degraded. On the other hand, the cells will fluoresce green if the polypeptide of interest is internalized, trafficked to the lysosome, and degraded.

Also described herein are dual labeled peptides that can be used to detect the presence of antibody binding in a sample. The dual labeled peptide can comprise a first label on the N terminus, a second label on the C terminus, at least one carrier attachment site, at least one modified amino acid or at least one reactive handle linked to at least one amino acid; and a cleavage site. Further disclosed herein are methods for detecting the presence of an antibody in a sample. The method can include the following steps: adding (or exposing or mixing or incubating) to the sample the dual labeled peptide as disclosed herein and measuring the emitted

fluorescence. In an aspect, the method can further comprise exposing the sample to a lysosomal tracking dye. The lysosomal tracking dye can be added or mixed with the sample before, after or simultaneously with the dual labeled peptide of as disclosed herein. An example of a commercially available tracking dye is LysoTracker®.

The compositions and methods disclosed herein can be used for detection of enzyme binding in a sample, antibody binding in a sample, and monitoring intracellular peptide degradation in, for example, cultured cells. In an aspect, the sample is a biological sample. The biological sample may comprise tumor or malignant cells. In some aspects, the biological sample is from a patient or subject with a condition, illness, disorder or disease. The disease can be a cell proliferative disease. The proliferative disease can be any type of cancer, including but not limited to breast cancer.

In some aspects, the tumor can be a solid tumor such as head and neck cancer including brain, thyroid cancer, breast cancer, lung cancer, mesothelioma, germ cell tumors, ovarian cancer, liver cancer, gastric carcinoma, colon cancer, prostate cancer, pancreatic cancer, melanoma, bladder cancer, renal cancer, prostate cancer, testicular cancer, cervical cancer, endometrial cancer, myosarcoma, leiomyosarcoma and other soft tissue sarcomas, osteosarcoma, Ewing's sarcoma, retinoblastoma, rhabdomyosarcoma, Wilm's tumor, and neuroblastoma, sepsis, allergic diseases and disorders that include but are not limited to allergic rhinitis, allergic conjunctivitis, allergic asthma, atopic eczema, atopic dermatitis, and food allergy, immunodeficiencies including but not limited to severe combined immunodeficiency (SCID), hypereosiniphic syndrome, chronic granulomatous disease, leukocyte adhesion deficiency I and II, hyper IgE syndrome, Chediak Higashi, neutrophilias, neutropenias, aplasias, agammaglobulinemia, hyper-IgM syndromes, DiGeorge/Velocardial-facial syndromes and Interferon gamma-TH1 pathway defects, autoimmune and immune dysregulation disorders that include but are not limited to rheumatoid arthritis, diabetes, systemic lupus erythematosus, Graves' disease, Graves opthalmopathy, Crohn's disease, multiple sclerosis, psoriasis, systemic sclerosis, goiter and struma lymphomatosa (Hashimoto's thyroiditis, lymphadenoid goiter), alopecia aerata, autoimmune myocarditis, lichen sclerosis, autoimmune uveitis, Addison's disease, atrophic gastritis, myasthenia gravis, idiopathic thrombocytopenic purpura, hemolytic anemia, primary biliary cirrhosis, Wegener's granulomatosis, polyarteritis nodosa, and inflammatory bowel disease, allograft rejection and tissue destructive from allergic reactions to

infectious microorganisms or to environmental antigens, and hematopoietic conditions that include but are not limited to Non-Hodgkin Lymphoma, Hodgkin or other lymphomas, acute or chronic leukemias, polycythemias, thrombocythemias, multiple myeloma or plasma cell disorders, e.g., amyloidosis and Waldenstrom's macroglobulinemia, myelodysplastic disorders, myeloproliferative disorders, myelofibroses, or atypical immune lymphoproliferations. In some embodiments, the neoplastic or hematopoietic condition is non-B lineage derived, such as Acute myeloid leukemia (AML), Chronic Myeloid Leukemia (CML), non-B cell Acute lymphocytic leukemia (ALL), non-B cell lymphomas, myelodysplastic disorders, myeloproliferative disorders, myelofibroses, polycythemias, thrombocythemias, or non-B atypical immune lymphoproliferations, Chronic Lymphocytic Leukemia (CLL), B lymphocyte lineage leukemia, B lymphocyte lineage lymphoma, Multiple Myeloma, or plasma cell disorders, e.g., amyloidosis or Waldenstrom's macroglobulinemia.

In some aspects the tumor or malignant cells can be non-B lineage derived. Examples of non-B lineage derived tumors of cells can include, but are not limited to, cells from Acute myeloid leukemia (AML), Chronic Myeloid Leukemia (CML), non-B cell Acute lymphocytic leukemia (ALL), non-B cell lymphomas, myelodysplastic disorders, myeloproliferative disorders, myelofibroses, polycythemias, thrombocythemias, and non-B atypical immune lymphoproliferations.

In some aspects the tumor or malignant cells can be from a B-Cell or B cell lineage derived disorder. Examples of B-Cell or B cell lineage derived neoplastic or hematopoietic condition include but are not limited to Chronic Lymphocytic Leukemia (CLL), B lymphocyte lineage leukemia, B lymphocyte lineage lymphoma, Multiple Myeloma, and plasma cell disorders, including amyloidosis and Waldenstrom's macroglobulinemia.

In one aspect, the cancer cells include, but are not limited to, CCRF-CEM cells, HL-60 cells, K-562 cells, MOLT-4 cells, RPMI-8226 cells,, SR cells, A549/ATCC cells, EKVX cells, HOP-62 cells, EKVX cells, HOP-92 cells, NCI-H226 cells, NIC-H23 cells, NCIH322M cells, NCI-H460 cells, HCI-H522 cells, COLO 205 cells, HCC-2998 cells, HCT-116 cells, HCT-15 cells, HT29 cells, KM12 cells, SW-620 cells, SF-268 cells, SF-295 cells, SF-539 cells, SNB-19 cells, SNB-75 cells, U251 cells, LOX IMVI cells, MALME-3M cells, M14 cells, MDA-MB-435 cells, SK-MEL-2 cells, SK-MEL-28 cells, SK-MEL-5 cells, UACC-257 cells, UACC-62 cells, IGR-OV1 cells, OVCAR-3 cells, OVCAR-4 cells, OVCAR-5 cells, OVCAR-8 cells, NCI/ADR-

RES cells, SK-OV-3 cells, 786-0 cells, A498 cells, ACHN cells, CAKI-1 cells, RXR 393 cells, SN12C cells, TK-10 cells, UO-31 cells, PC-3 cells, DU-145 cells, MCF7 cells, MDA-MB-468 cells, MDA-MB-231/ATCC cells, HS 578T cells, MDA-N cells, BT-549 cells, T-47/D cells, LXFL 529 cells, DMS 114 cells, SHP-77 cells, DLD-1 cells, KM20L2 cells, SNB-78 cells, XF 498 cells, RPMI-7951 cells, M19-MEL cells, RXR-631 cells, SN12K1 cells, P388 cells, and P388/ADR cells.

In some aspects, the disease can be an infectious disease, including influenza.

In an aspect, the antibody detected can be an antibody that binds to an epidermal growth factor receptor (EGFR) family or prolactin receptor. In an aspect, the antibody detected can be a HER2 receptor antibody. In an aspect, the HER2 receptor antibody can be trastuzumab, panitumumab or cetuximab.

Described herein are dual labeled peptides that can be used to detect the presence of enzyme binding in a sample. The dual labeled peptide can comprise a first label on the N terminus, a second label on the C terminus, at least one carrier attachment site, at least one modified amino acid or at least one reactive handle linked to at least one amino acid; and a cleavage site. In an aspect, the enzyme detected is a protease. The protease can be a cysteine protease. The protease can be a cathepsin. In an aspect, the enzyme detected can be cathepsin b.

The methods can include the step of separating the test sample from the bound enzyme by routine means (e.g., washing). This step can be followed by detection of the intrinsic enzymatic activity of the bound enzyme. The presence of intrinsic enzymatic activity can be indicative of the presence of the enzyme in the test sample. The absence of intrinsic enzymatic activity is indicative of the absence of the enzyme of interest (e.g., a cysteine protease) in the test sample.

Described herein are methods of detecting the presence of an enzyme in a sample. The method can comprise adding the sample to the dual labeled peptide as disclosed herein and measuring the emitted fluorescence. In an aspect, the enzyme detected can be a protease. The protease can be a cysteine protease. The protease can be a cathepsin. In an aspect, the enzyme detected can be cathepsin b.

Disclosed herein are methods of screening carcinoma cultures for an antibody of interest, wherein the antibody of interest is conjugated to a dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C

terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid, and a cleavage site, the method comprising: (a) providing a plurality of primary carcinoma cultures with one or more antigens; (b) performing a first screen to determine specificity of a test antibody to the antigen; (c) performing a second screen of the test antibody by exposing the test antibody to a mixture of cells, wherein the mixture comprises some cells having the antigen in labeled form and some cells having the antigen in non-labeled form; (d) assaying for in vivo internalization of co-localized test antibody with the antigen; and (e) assaying for the absence of signal from the label in the cells that lack the antigen.

Disclosed herein are methods of screening cancer cell lines (e.g., cancer cell cultures) for an antibody of interest. The antibody of interest can be conjugated to the dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid, and a cleavage site. The method can comprise providing a plurality of cancer cell cultures with one or more antigens. In an aspect, the cell line is a carcinoma cell line.

In an aspect, the carcinoma cells can be from a breast cancer cell line. In an aspect, the breast cancer cell line can be from any known breast cancer cell line. In an aspect, the breast cancer cell line is SKBR3, Hs5781, BT-474, T47D or MCF-7. In an aspect, the plurality of cultures can be SKBR3, Hs5781, BT-474, T47D or MCF-7.

Next, the method can include the step of performing a first screen to determine the specificity of a test antibody to the antigen. The method disclosed herein can also include the step of performing a second screen of the test antibody by exposing the test antibody to a mixture of cells. The mixture of cells can comprise some cells having the antigen in labeled form and some cells having the antigen in non-labeled form. The method can comprise the step of assaying for in vivo internalization of co-localized test antibody with the antigen. The method can also comprise the step of assaying for the absence of a signal from the label in the cells that lack the antigen. In an aspect, the first screen and second screen as described above can be carried out in the presence of cathepsin b. In an aspect, the dual labeled peptide further comprises the sequence: FRA (SEQ ID NO: 1), KIFRAC (SEQ ID NO: 2), K(N3)IFRAC (SEQ ID NO: 3), K(N3)-X-X-X-X-C (SEQ ID NO: 4), FRA, FRFF (SEQ ID NO: 5), YKFF (SEQ ID NO: 6), YRFF (SEQ ID NO: 7), FKFF (SEQ ID NO: 8), XXFXX (SEQ ID NO: 9), XXLXX

(SEQ ID NO: 10), VRA, FRA, YRA, or VLA. In an aspect, the antibody of interest is an anti-cancer antibody. In some aspects, the ratio of the mixture of cells wherein some cells have the antigen in labeled form and some cells have the antigen in non-labeled form is not important. In some aspects, the ratio can be important such as in cases wherein statistical analysis is to be carried out, then substantially equal numbers can be useful; where possible, this is done in the first screen; assaying for in vivo co-localization of the test monoclonal antibody with the antigen; and assaying for the absence of signal from the label in the cells that lack the antigen. In some aspects, the antigen is a protein, a protein fragment, peptide, cellular extract, an organelle, subcellular structure or mixture thereof. In some aspects, one or more steps are performed concomitantly. In some aspects, the first screen comprises ELISA, western or a combination thereof. In an aspect, the label is capable of detection by being fluorescent or having color. The antibodies can be labeled with a detectable moiety. The detectable moiety can be any moiety that is capable of producing either directly or indirectly a detectable signal. For example, the detectable moiety can be a radioisotope or fluorescent compound or radioactive isotopic label.

EXAMPLES

Example 1: Cathepsin B Dependent Dual labeled Peptide to Track Antibody Trafficking and Cleavage

Described herein is a method for simultaneously measuring antibody binding, internalization, lysosomal localization, and the kinetics of linker cleavage in live cells utilizing a fluorescence resonance energy transfer (FRET)-based biosensor reagent (also referred to herein as a dual labeled peptide, see FIG. 1). The biosensor (dual labeled peptide) comprises through conjugation an antibody of interest to generate an antibody-biosensor conjugate (ABC; or dual labeled peptide). The biosensor (dual labeled peptide) contains an Alexa Fluor® 568 (A568) - Alexa Fluor® 647 (A647) FRET pair, separated by a peptide sequence (KIFRAC; SEQ ID NO: 2) that is susceptible to cleavage by lysosomal cathepsin b. Prior to cleavage, greater than 95% of A568 emission is quenched, due to highly efficient energy transfer to A647. When cleavage occurs, the energy transfer is broken, and A568 is detectable at 100% emission. A647 can be detected in both cleaved and uncleaved states, providing a continuous measure of total antibody levels at both the cell surface and intracellularly.

It has been determined that anti-PRLR (prolactin receptor) mAbs are rapidly internalized and PRLR ADCs have potent cytotoxic activity in T47D (human ductal breast carcinoma) cells. In contrast, anti-Her2 mAbs are inefficiently internalized and Her2 ADCs do not kill T47D cells despite Her2 expression at or above PRLR levels. These same mAbs were selected for proof of concept conjugation to the FRET-based biosensor (dual labeled peptides described herein). In this study, the following are demonstrated: the conjugation of each of several mAbs to the dual labeled peptide, cathepsin b dependent cleavage of the dual labeled peptide in a cell free system, and intracellular release of the A568 quenched fluorophore in live T47D cells.

Consistent with prior trafficking data, rapid intracellular cleavage of the anti-PRLR conjugate was detected within 1 h in T47D cells, as measured by the appearance of A568 fluorescence. This cleavage occurred selectively in lysotracker labeled low pH compartments, and was blocked by bafilomycin A1 (an inhibitor of lysosomal acidification and protein degradation). In contrast to PRLR, detectable cleavage of the dual labeled peptide comprising a Her2 antibody was observed after 3 h.

Next, this technology was applied to measure the intracellular cleavage of a novel PRLR x Her2 bispecific antibody. Maximum cleavage of the dual labeled peptide comprising a PRLR x Her2 bispecific antibody was greater than the bivalent anti-PRLR antibody or anti-HER2 antibody alone. Surprisingly, linker release (e.g., cleavage) from the dual labeled peptide comprising a PRLR x Her2 bispecific antibody was greater than release observed from an anti-Her2 probe when combined with the unconjugated PRLR x Her2 mAb (Her2 epitopes do not cross-compete). These data suggest that Her2 clustering could impede cleavage of the PRLR x Her2 antibody.

The cleavage results were validated in a cell viability assay in which PRLR x Her2 conjugated to the maytansinoid DM1 killed cells more potently than the combination of PRLR x Her2 and an anti-Her2 conjugated to DM1. This biosensor (e.g., dual labeled peptide) technology can also be applicable to the fields of immuno-oncology and immuno-PET, where an understanding of target and antibody trafficking is important. Utilizing the dual labeled peptides as disclosed herein, it is shown that a dual labeled peptide comprising an anti-PD1 antibody is rapidly cleaved in MC38 cells expressing human PD-1, which may suggest that PD-1 and bound antibodies may be rapidly degraded. Described herein are dual labeled peptides comprising an

antibody that is capable of simultaneously measuring cell binding, internalization, and linker cleavage (e.g., linker release or enzyme cleavage) in live cells.

Cathepsin b cleaves the dual labeled peptide in a cell-free assay. FIG. 2 shows an example of the cleavage of the dual labeled peptide upon exposure to cathepsin b and the resulting fluorescence. To study the kinetics of the cleavage of the dual labeled peptide upon varying concentrations of cathepsin b, the following experiment was carried out. Pre-activated cathepsin b was incubated with a dual labeled peptide without a carrier (e.g., antibody), and A568 emission was measured over time. The increase in A568 emission measures the loss of FRET quenching following cathepsin b-dependent cleavage of the dual labeled peptide. The kcat/Km is estimated by the initial velocities to be 1100 mM-1 s-1 and is comparable to literature values for other cathepsin substrates. The overall fluorescence intensity increase of A568 is ~ 30-fold over background (left axis) and approaches the theoretical maximum emission further suggesting very efficient cleavage kinetics (FIG. 3).

Next, the dual labeled peptides are tested for their ability to simultaneously measure antibody total binding, internalization, low pH localization, and cleavage. The results show that the cleavage of the anti-PRLR dual labeled peptides is more rapid than anti-HER2 dual labeled peptides.

T47D cells were incubated with 100 nM Lysotracker blue (Invitrogen) plus 10 μg/ml of anti-PRLR or anti-Her2 dual labeled peptide with ~ 1.3 dual labeled peptides per mAb (DAR). Unbound dual labeled peptides (e.g., dual labeled peptides without a carrier or antibody attached) were washed and 63x confocal images (Zeiss LSM 710) were acquired at time 0 (FIG. 4A) or following incubations at 37°C for 30 minutes (FIG. 4B), 1 h (FIG. 4C), or 4 h (FIG. 4D). Lysotracker blue marks the low pH compartments (blue), Alexa 568 (A568, green) indicates dual labeled peptide cleavage, and the Alexa 647 (red) indicates total dual labeled peptide. Merged images of cleaved dual labeled peptide and lysotracker blue (Lysotracker blue & A568 merge) and merged images of the cleaved dual labeled peptide, total biosensor, and lysotracker blue (Lysotracker blue + A568 + A647) are also shown. Total binding (A647, red) was detected for both dual labeled peptides at 4°C. The anti-PRLR dual labeled peptide internalized rapidly at 37°C with detectable cleavage by 1h (A568, green). In contrast, the anti-HER2 dual labeled peptide internalized slowly and cleavage (green) was not detectable until 4 hours. FIGS. 5A-B show that cleavage of the dual labeled peptides disclosed herein occurs in low pH compartments.

The next set of experiments shows the results of dual labeled peptide cleavage kinetics in live cells. T47D cells were pre-incubated for 2 h in the absence (FIG. 6A) or presence (FIG. 6B and FIG. 6C) of bafilomycin A1 (an inhibitor of lysosomal acidification and protein degradation). Cells were then incubated with anti-PRLR (FIGS. 6A and 6B) or anti-Her2 (FIGS. 6A and 6C) dual labeled peptides on ice and washed. Confocal 40X images (PerkinElmer Opera Phenix) of dual labeled peptide cleavage (A568) were acquired at time 0, every 10 minutes for the first 2 h, and then every 30 minutes thereafter. The A568 relative fluorescence units (RFU) are plotted for each time point. Total cleavage of the anti-PRLR dual labeled peptide (red triangle) is significantly greater than anti-HER2 cleavage (blue circle), and cleavage of both dual labeled peptides is blocked by bafilomycin A1.

Anti-Her2 dual labeled peptides and antibody drug conjugate activity increased in combination with bispecific Her2 x PRLR or Her2 x Her2 antibodies (FIG. 7). T47D or T47D cells engineered to overexpress Her2 (T47D/Her2) were incubated with the anti-Her2 dual labeled peptides (10 μg/ml) in the absence or presence of Her2 x PRLR or Her2 x Her2 bispecific antibodies (10 μg/ml). The bispecific Her2 x Her2 antibody was added to promote Her2 surface clustering and the bispecific Her2 x PRLR antibody was added to promote clustering between Her2 and the rapidly internalizing PRLR. The dual labeled peptide cleavage kinetics was determined similarly to that described in FIG. 6, and A568 RFUs (relative fluorescence units) are normalized for cell numbers and DAR. The anti-Her2 mAb was conjugated to the toxin DM1 via a non-cleavable linker, and serial dilutions of the antibody drug conjugates were combined with 10 μg/ml of unconjugated bispecific antibodies. Following a 3-day incubation, viability was determined from nuclear counts of Hoechst-stained cells. The results further demonstrate that anti-Her2 dual labeled peptide cleavage across the three tested cell lines was slightly enhanced in the presence of the bispecific antibodies. Bispecific antibodies also increased cell killing over the anti-Her2 antibody drug conjugates alone in T47D/Her2 cells. Antibody drug conjugate cell killing in T47D cells was not detectable, whereas weak dual labeled peptide cleavage was detected suggesting that cleavage does occur but not enough payload is released from the ADCs to mediate killing (FIG. 7C).

Bispecific Her2 x PRLR ABC dual labeled peptide cleavage and Her2 x PRLR antibody drug conjugates killing in engineered T47D cells is more potent than PRLR or Her2 bivalent mAbs alone or in combination (FIG. 8). The dual labeled peptide cleavage and ADC activity of

a directly conjugated Her2 x PRLR bispecific antibody was also tested in T47D and T47D/Her2 cells. Relative cell surface Her2 and PRLR levels were determined via flow cytometry (FIG. 8A). Cells were incubated on ice with 10 μg/ml mAbs and binding was detected with a PE-conjugated anti-hIgG secondary antibody. Histograms show the cell binding distribution and the table reports the fold change in mean fluorescent intensity above unstained levels for each mAb.

The dual labeled peptide cleavage and antibody drug conjugate activity of a directly conjugated Her2 x PRLR bispecific antibody was also tested in T47D and T47D/Her2 cells. Cells were incubated with bispecific Her2 x PRLR, anti-PRLR, anti-Her2, or anti-C. diff antibody drug conjugates (10 μg/ml), and cleavage was quantitated as described in FIG. 7 (FIG. 8B). The dual labeled peptide cleavage and antibody drug conjugate activity of a directly conjugated Her2 x PRLR bispecific antibody was also tested in T47D and T47D/Her2 cells. Cells were incubated with serially diluted bispecific Her2 x PRLR, anti-PRLR, anti-Her2, or anti-C. diff antibody drug conjugates conjugated to DM1 as described in FIG. 7 (FIG. 8C).

The dual labeled peptide cleavage and antibody drug conjugate activity of a directly conjugated Her2 x PRLR bispecific antibody was also tested in T47D and T47D/Her2 cells. Cells were incubated with the bispecific Her2 x PRLR ABC, anti-Her2 ABC, or anti-Her2 dual labeled peptide in combination with unconjugated Her2 x PRLR bispecific antibody (all mAbs at 10 μg/ml) (FIG. 8D). Imaging and quantitation was performed as described in panel B. Cells were incubated with serial dilutions of bispecific Her2 x PRLR antibody drug conjugate, anti-Her2 antibody drug conjugate, or anti-Her2 antibody drug conjugate in combination with a constant amount (10 μg/ml) of unconjugated Her2 x PRLR bispecific antibody (FIG. 8E). Cell viability was determined as described in panel C.

The Her2 x PRLR bispecific dual labeled peptide cleavage and antibody drug conjugate cytotoxicity was higher than the PRLR and Her2 bivalent antibodies in T47D/Her2 cells. Surprisingly, a directly conjugated Her2 x PRLR dual labeled peptide and antibody drug conjugate showed more potent activity than the combination of anti-Her2 conjugate + unbound Her2 x PRLR in T47D/Her2 cells. In T47D cells, the PRLR x Her2 antibody drug conjugate was less potent than the PRLR antibody drug conjugate despite more robust cleavage by the Her2 x PRLR dual labeled peptides.

The next set of experiments evaluated the total fluorescence as a measure of cleavage of a label (e.g., Alexa 568) from the dual labeled peptides described herein. The results show rapid

and robust anti-PD-1 dual labeled peptide cleavage in HEK293/hPD-1 and MC38/hPD-1 cells (FIG. 9). For instance, the anti-PD-1 dual labeled peptide is rapidly cleaved and reaches maximum levels in MC38/PD-1 cells around 10 hours (see, FIG. 9B). Total cleavage is significantly above isotype control levels and is comparable to levels achieved by the Her2 x PRLR bispecific in T47D/Her2 cells.

In summary, these experiments show that the dual labeled peptide comprising an antibody can simultaneously measure antibody cell binding, internalization, low pH localization, and cleavage in live cells. These results also show that anti-Her2 dual labeled peptide and antibody drug conjugate activity is increased when combined with bispecific Her2 x PRLR or Her2 x Her2 antibodies. Bispecific Her2 x PRLR dual labeled peptide and antibody drug conjugate activity is more potent than anti-Her2 or anti-PRLR bivalent conjugates alone. Bispecific Her2 x PRLR dual labeled peptide and antibody drug conjugate activity is more robust than the combination of anti-Her2 conjugate + unconjugated Her2 x PRLR. Lastly, these results demonstrate that an anti-PD-1 dual labeled peptide is rapidly cleaved in HEK293 and MC38 cells expressing human PD-1.

Example 2: Peptide Conjugation Procedures

The following provides the procedure for preparing the dual labeled peptides disclosed herein. For example, a lyophilized peptide can be reconstituted with 20 HEPES, pH 6.8, 1 mM EDTA and added to 2.5-fold XS of Mal-A647 (Alexafluor 647 maleimide), and rotated at 750 rpm for 4 hours, at room temperature. Next, add equal peptide volumes of 100 mM HEPES, pH 8.3, 1 mM EDTA, followed by the addition of 2.5-fold XS NHS-A568. Hold 36 hours at 4°C, rotating slowly. The estimated total volume is 865 uL. Next, the dual labeled peptide is passed through a G-10 column. Next, the dual labeled peptide can be purified by SEC peptide column. The center fractions as well as the fringe fractions can be collected using a speed vac. The fringe fractions are then re-purified and combined with the center fractions. The center fractions are then re-purified. Fluorescence quenching can be confirmed and the concentration can be determined by UV/VIS spectra. Additional techniques were carried out including electrospray ionization mass spectrometry (ESI-M and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). Fluorescence properties can also be assessed. FIG. 10 shows an example of the peptide conjugation procedure.

FIG. 11 provides a schematic example of using the dual labeled peptides as prepared above as a tool to target lysosomes.

The conjugation of antibody to the dual labeled peptide is provided below. For DIBO-mAb conjugation (see, FIG. 12), the following steps include: (1) 9 mg of REGN579, REGN1932 for conjugation; (2) 1 mg of DIBO-NHS was reconstituted with dry DMSO to 10 mM; (3) 6:1 fold DIBO-NHS to mAb (mol:mol), in 50 mM HBS, pH 8.0, 15% DMSO, for 3 hours at room temperature; (4) desalted by Nap 5 column, 200 uL fractions collected, fractions 2 & 3 pooled based on OD; (5) concentration estimated by UV/VIS; (6) degree of conjugation was determined by MALDI-TOF-MS; (7) H1H6765P (an anti-prolactin receptor antibody) was not added to the original list but was added later; (8) 9 mg of H1H6765P was conjugated in similar fashion, except the 15% DMSO was not inclued, resulting in a low conjugation ratio (MALDI); the sample was re-introduced to NHS-DIBO in presence of 15% DMSO at a ratio of 4:1, resulting in a better conjugation ratio.

For peptide-mAb conjugation, the following steps include: (1) 0.85 mg of REGN579, REGN1932, H1H6765P for conjugation; (2) two-fold peptide was added, 4 hours at room temperature; (3) final protein concentration of reaction mixture: 2.0 mg/mL; and (4) samples were desalted by Nap 5 column.

CLAIMS

WHAT IS CLAIMED IS:

1. A dual labeled peptide, wherein the dual labeled peptide comprises:
    a. a first label on the N terminus;
    b. a second label on the C terminus;
    c. at least one modified amino acid or at least one reactive handle linked to at least one amino acid; and
    d. a cleavage site.
2. The dual labeled peptide of claim 1, wherein the dual labeled peptide comprises the sequence IFRA (SEQ ID NO: 1), KIFRAC (SEQ ID NO: 2), K(N3)IFRAC (SEQ ID NO: 3), K(N3)-X-X-X-X-C (SEQ ID NO: 4), FRA, FRFF (SEQ ID NO: 5), YKFF (SEQ ID NO: 6), YRFF (SEQ ID NO: 7), FKFF (SEQ ID NO: 8), XXFXX (SEQ ID NO: 9), XXLXX (SEQ ID NO: 10), VRA, FRA, YRA, or VLA.
3. The dual labeled peptide of claim 1, wherein the at least one modified amino acid is an azido-lysine, azido-alanine, cyclooctyne-alanine, or cyclooctyne-lysine.
4. The dual labeled peptide of claim 1, further comprising a carrier.
5. The dual labeled peptide of claim 4, wherein the carrier is an antibody, protein, nucleic acid or virus capsid.
6. The dual labeled peptide of claim 4, wherein the carrier is conjugated to the dual labeled peptide.
7. The dual labeled peptide of claim 4, wherein the carrier is an antibody,
8. The composition of claim 7, wherein the antibody is a single chain antibody (scFv) or a Fab fragment.
9. The composition of claim 7, wherein the antibody is human, chimeric or humanized or a biologically active variant thereof.

10. The composition of claim 7, wherein the antibody is a monoclonal antibody or a polyclonal antibody.

11. The composition of claim 7, wherein the antibody is trastuzumab, panitumumab or cetuximab or biologically active variant thereof.

12. The composition of claim 7, wherein the antibody is an anti-cancer agent or an anti-influenza agent.

13. The dual labeled peptide of claim 1, wherein the at least one reactive handle is attached to the dual labeled peptide via strained cyclooctyne copper-free click chemistry.

14. The dual labeled peptide of claim 1, wherein a distance between the first and second labels is between about 10 Å and 120 Å.

15. The dual labeled peptide of claim 1, wherein the first label and the second label are fluorescence resonance energy transfer (FRET) fluorophores.

16. The dual labeled peptide of claim 1, wherein the first label and the second label are Alexa Fluor® 568 (A568), Alexa Fluor® 647 (A647), or Alexa Fluor® 546 (A546).

17. The dual labeled peptide of claim 1, wherein the first label is a donor and the second label is an acceptor or the first label is an acceptor and the second label is a donor.

18. The dual labeled peptide of claim 1, wherein the first label is Alexa Fluor® 568 (A568) and the second label is Alexa Fluor® 647 (A647) FRET.

19. The dual labeled peptide of claim 18, wherein the labels are excitable by exposure to a wavelength spectrum in a spectral range of from about 500 nm to about 700 nm.

20. The dual labeled peptide of claim 18, wherein the distance is 47 Å.

21. A method of monitoring intracellular peptide degradation, the method comprising:
(a) exposing cultured cells to a dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, a cleavage site, and at least one modified amino acid or at least one reactive handle linked to at least one amino acid;
(b) applying fluorescent light to the cultured cells; and

(c) detecting fluorescence of the cultured cells in (b).

22. The method of claim 21, wherein the dual labeled peptide comprises the sequence IFRA (SEQ ID NO: 1), KIFRAC (SEQ ID NO: 2), K(N3)IFRAC (SEQ ID NO: 3), K(N3)-X-X-X-X-C (SEQ ID NO: 4), FRA, FRFF (SEQ ID NO: 5), YKFF (SEQ ID NO: 6), YRFF (SEQ ID NO: 7), FKFF (SEQ ID NO: 8), XXFXX (SEQ ID NO: 9), XXLXX (SEQ ID NO: 10), VRA, FRA, YRA, or VLA.

23. The method of claim 21, wherein the at least one modified amino acid is an azido-lysine, azido-alanine, cyclooctyne-alanine, or cyclooctyne-lysine.

24. The method of claim 21, further comprising a carrier.

25. The method of claim 24 wherein the carrier is an antibody, protein, polypeptide, nucleic acid, or virus capsid.

26. The method of claim 25 wherein the carrier is conjugated to the dual labeled peptide.

27. The method of claim 25, wherein the antibody is a monclonal antibody or polyclonal antibody.

28. The method of claim 27, wherein the antibody is trastuzumab, panitumumab or cetuximab or a biologically active variant thereof.

29. The method of claim 28, wherein the antibody is an anti-cancer agent or an anti-influenza agent.

30. A dual labeled peptide for detecting presence of antibody binding in a sample, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid, and a cleavage site, and wherein the antibody is conjugated to the at least one modified amino acid or at least one reactive handle linked to at least one amino acid.

31. The dual labeled peptide of claim 30, wherein the first label and second label are pH stable.

32. The dual labeled peptide of claim 30, further comprising the sequence IFRA (SEQ ID NO: 1), KIFRAC (SEQ ID NO: 2), K(N3)IFRAC (SEQ ID NO: 3), K(N3)-X-X-X-X-C (SEQ ID NO: 4), FRA, FRFF (SEQ ID NO: 5), YKFF (SEQ ID NO: 6), YRFF

(SEQ ID NO: 7), FKFF (SEQ ID NO: 8), XXFXX (SEQ ID NO: 9), XXLXX (SEQ ID NO: 10), VRA, FRA, YRA, or VLA.

33. The dual labeled peptide of claim 30, wherein at least one modified amino acid is an azido-lysine.

34. The dual labeled peptide of claim 30, wherein the antibody is trastuzumab, panitumumab or cetuximab or biologically active variant thereof.

35. The dual labeled peptide of claim 34, wherein the antibody is an anti-cancer agent or an anti-influenza agent.

36. The dual labeled peptide of claim 30, wherein a distance between the first and second labels is between about 10 Å and 120 Å.

37. The dual labeled peptide of claim 30, wherein the labels are excitable by exposure to a wavelength spectrum in a spectral range of from about 500 nm to about 700 nm.

38. A method of detecting presence of an antibody in a sample, the method comprising:

(a) adding the sample to the dual labeled peptide of claim 31; and

(b) measuring emitted fluorescence.

39. The method of claim 38, wherein the antibody detected is a HER2 receptor antibody.

40. The method of claim 39, wherein the HER2 receptor antibody is trastuzumab or cetuximab or biologically active variant thereof.

41. The method of claim 30, further comprising a lysosomal tracking dye.

42. A dual labeled peptide for detecting presence of enzyme binding in a sample, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, at least one modified amino acid or at least one reactive handle linked to at least one amino acid, and a cleavage site.

43. The dual labeled peptide of claim 42, further comprising the sequence IFRA (SEQ ID NO: 1), KIFRAC (SEQ ID NO: 2), K(N3)IFRAC (SEQ ID NO: 3), K(N3)-X-X-X-X-C (SEQ ID NO: 4), FRA, FRFF (SEQ ID NO: 5), YKFF (SEQ ID NO: 6), YRFF (SEQ ID NO: 7), FKFF (SEQ ID NO: 8), XXFXX (SEQ ID NO: 9), XXLXX (SEQ ID NO: 10), VRA, FRA, YRA, or VLA.

44. The dual labeled peptide of claim 42, wherein the cleavage site is a cathepsin b cleavage site.

45. The dual labeled peptide of claim 42, wherein at least one modified amino acid is an azido-lysine, azido-alanine, cyclooctyne-alanine, or cyclooctyne-lysine.

46. The method of claim 42, wherein the enzyme detected is a protease.

47. The method of claim 42, wherein the protease is a cathepsin.

48. The method of claim 47, wherein the cathepsin is cathepsin b.

49. A method of monitoring the intracellular uptake of a polypeptide of interest in cultured cells, the methods comprising: (a) exposing cultured cells to a dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, and a cleavage site, and at least one modified amino acid or at least one reactive handle linked to a polypeptide; (b) applying fluorescent light to the cultured cells; and (c) detecting fluorescence of the cultured cells in (b), wherein a change in the wavelength of fluorescent light that is detected indicates that the polypeptide of interest has been trafficked to the lysosome.

50. A method of monitoring the intracellular uptake of a polypeptide of interest in cultured cells, the methods comprising: (a) exposing cultured cells to a dual labeled peptide, wherein the dual labeled peptide comprises a first label on the N terminus, a second label on the C terminus, and a cleavage site, and at least one modified amino acid or at least one reactive handle linked to a polypeptide; (b) applying fluorescent light to the cultured cells; and (c) detecting fluorescence of the cultured cells in (b).

51. The method of claim 50, wherein a change in the wavelength of fluorescent light that is detected indicates that the polypeptide of interest has been trafficked to the lysosome.

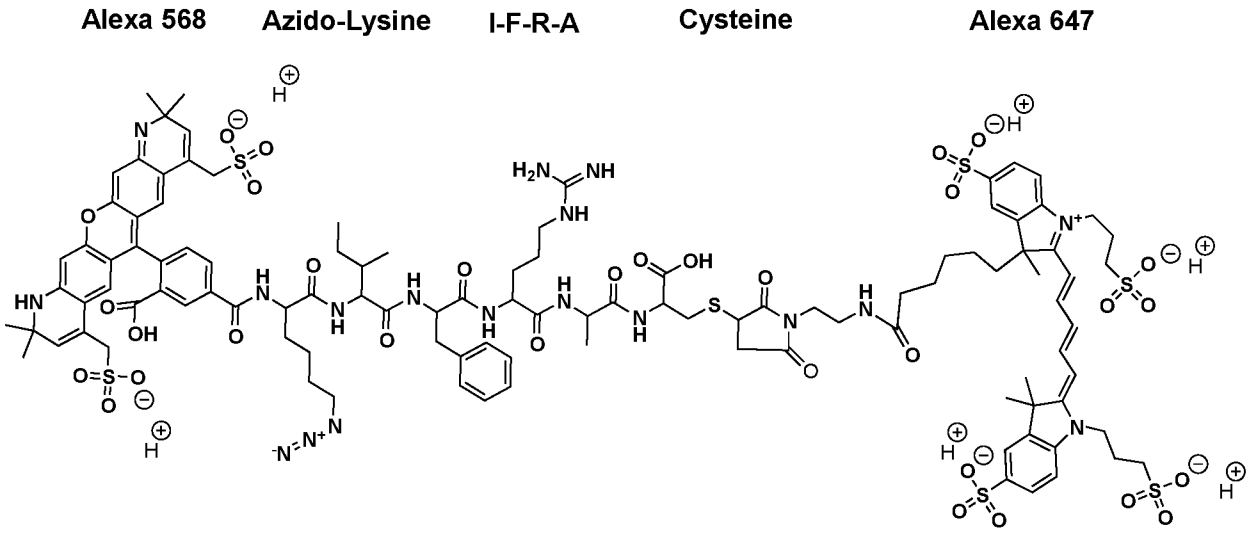


FIG. 1

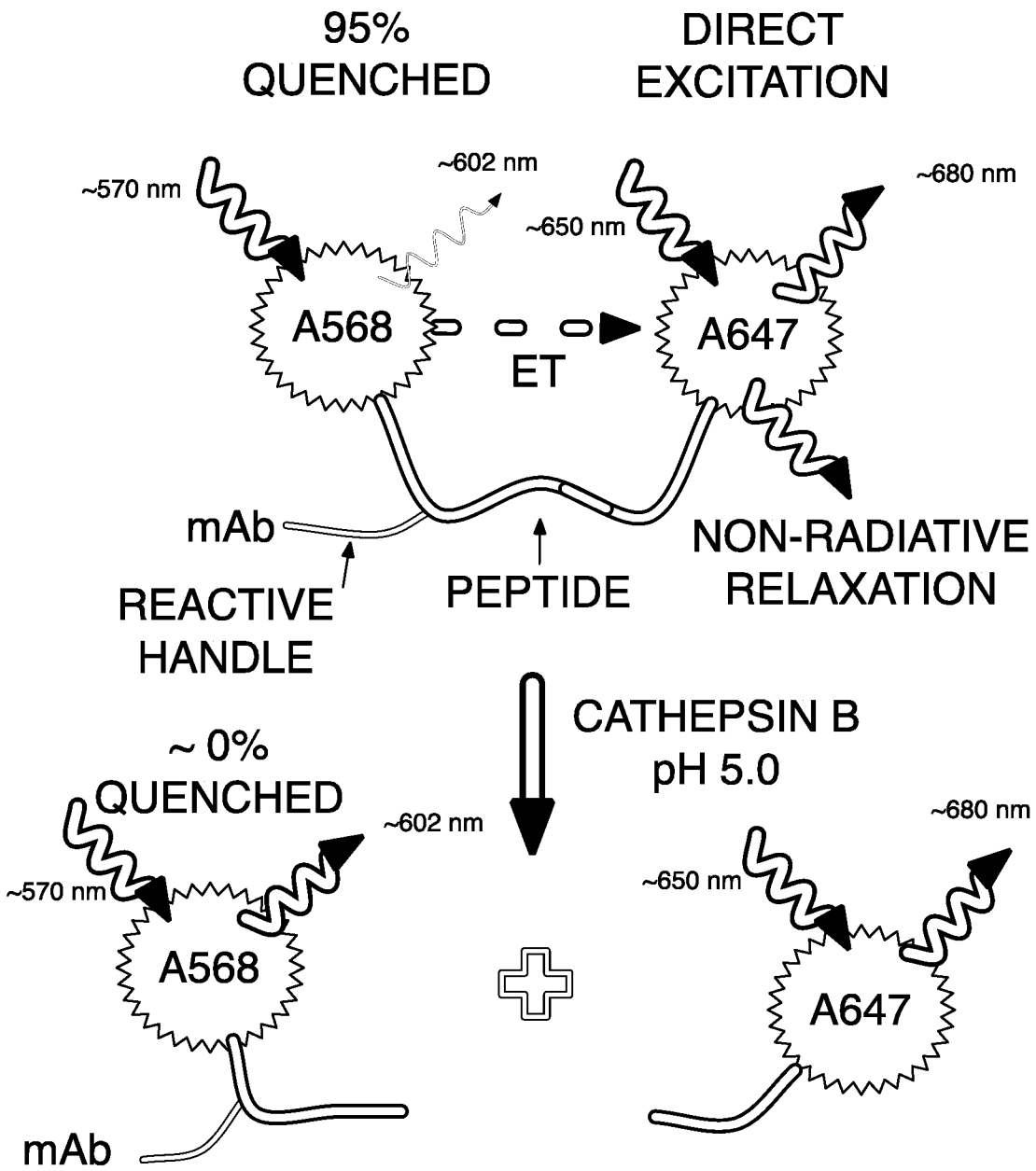

95%
QUENCHED
DIRECT
EXCITATION
~570 nm
~602 nm
~650 nm
~680 nm
A568
ET
A647
mAb
REACTIVE
HANDLE
PEPTIDE
NON-RADIATIVE
RELAXATION
CATHEPSIN B
pH 5.0
~ 0%
QUENCHED
~602 nm
~570 nm
~650 nm
~680 nm
A568
A647
mAb

FIG. 2

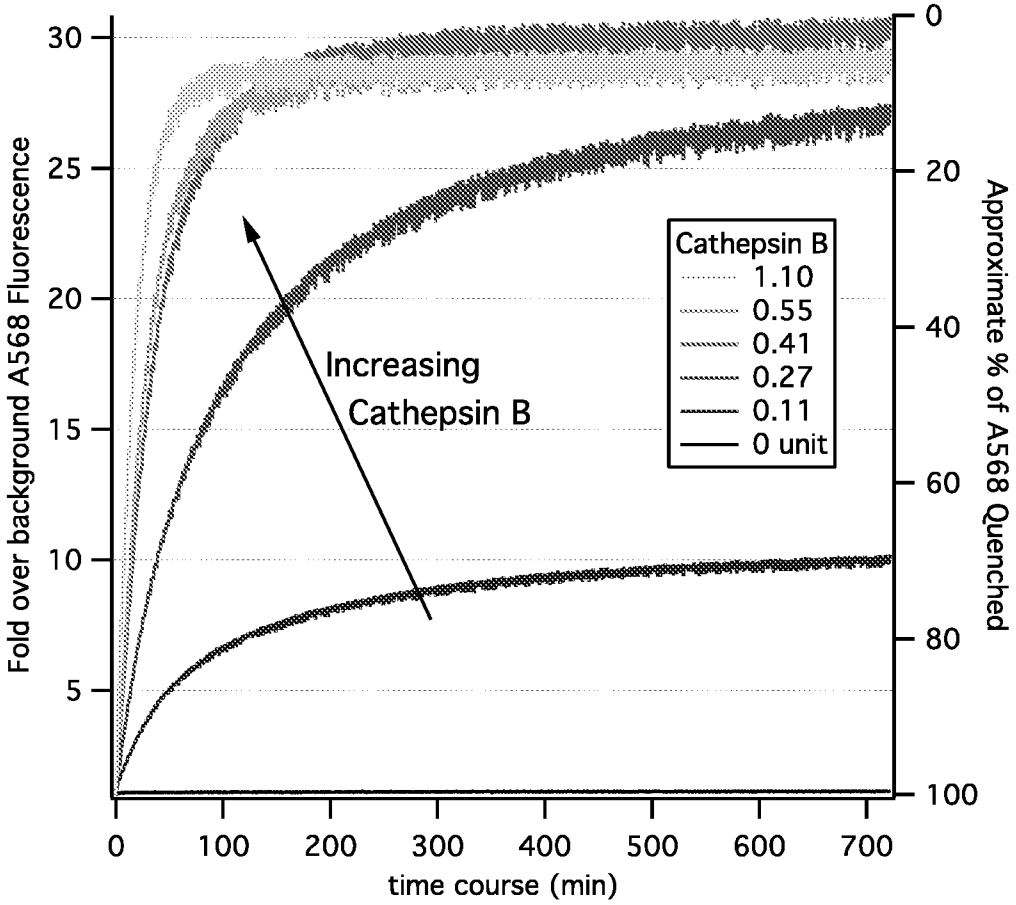

Fold over background A568 Fluorescence
Approximate % of A568 Quenched
time course (min)
Increasing
Cathepsin B
Cathepsin B
1.10
0.55
0.41
0.27
0.11
0 unit
30
25
20
15
10
5
0
20
40
60
80
100
0
100
200
300
400
500
600
700

FIG. 3

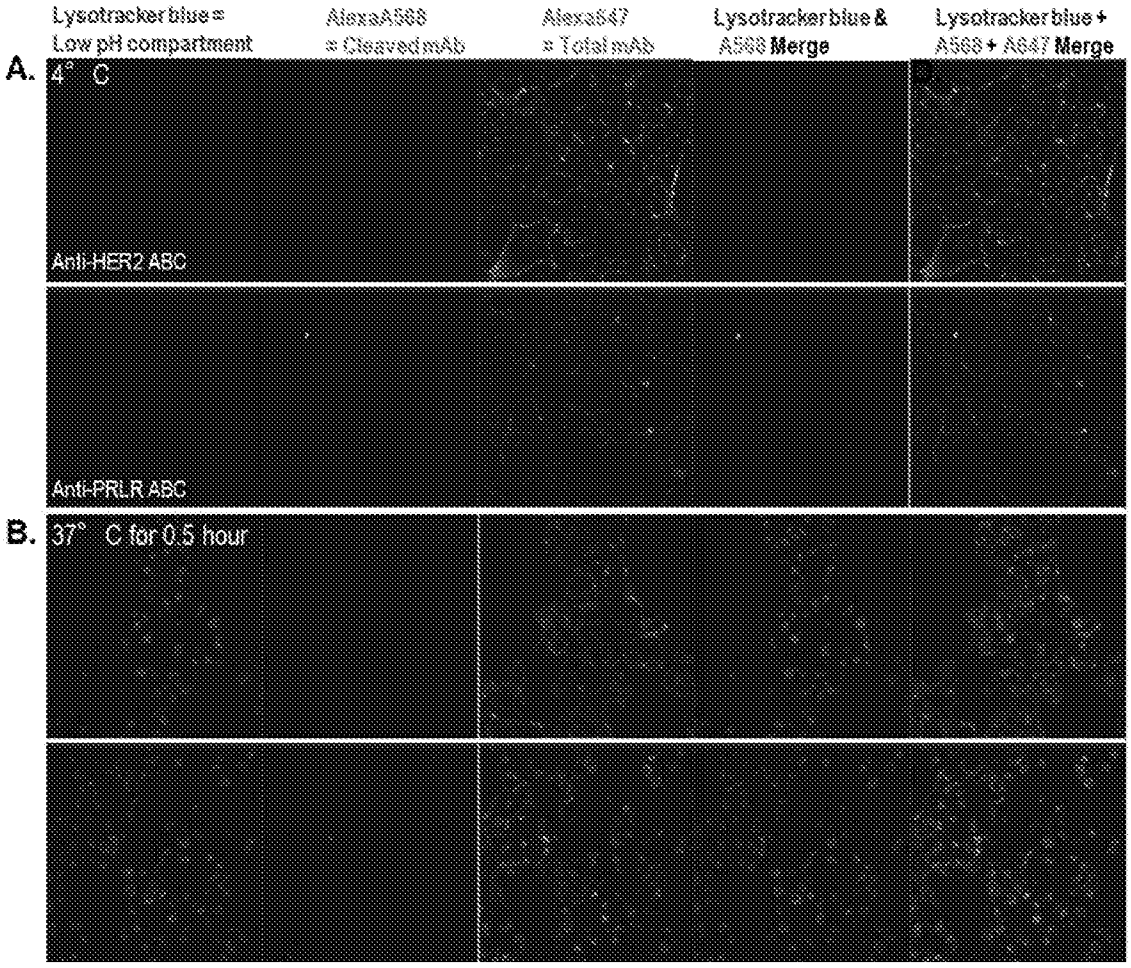

Lysotracker blue =
Low pH compartment
AlexaA568
= Cleaved mAb
Alexa647
= Total mAb
Lysotracker blue &
A568 Merge
Lysotracker blue +
A568 + A647 Merge
A.
4° C
Anti-HER2 ABC
Anti-PRLR ABC
B.
37° C for 0.5 hour

FIG. 4A, FIG. 4B

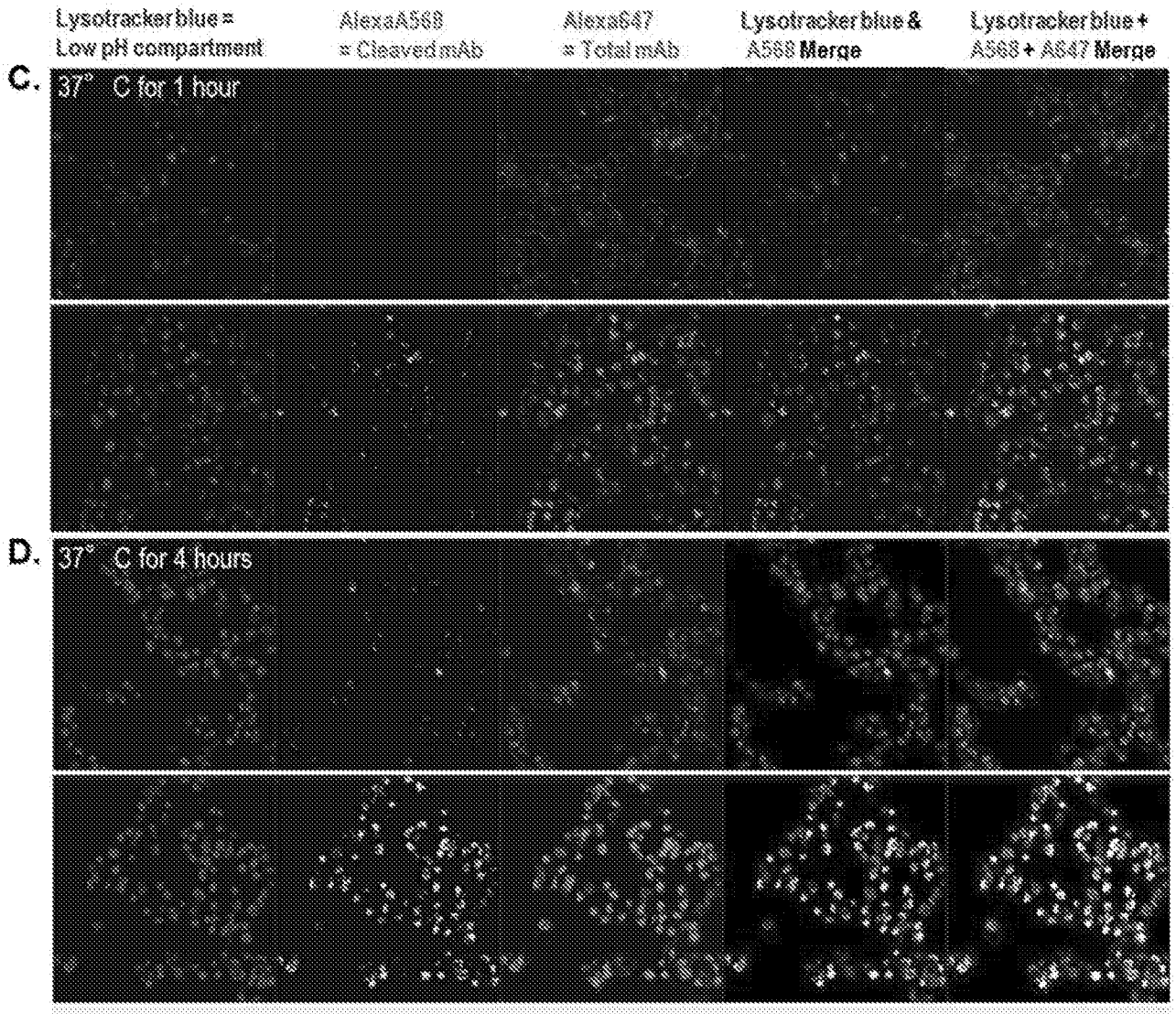

Lysotracker blue = Low pH compartment
AlexaA568 = Cleaved mAb
Alexa647 = Total mAb
Lysotracker blue & A568 Merge
Lysotracker blue + A568 + A647 Merge
C.
37° C for 1 hour
D.
37° C for 4 hours

FIG. 4C, FIG. 4D

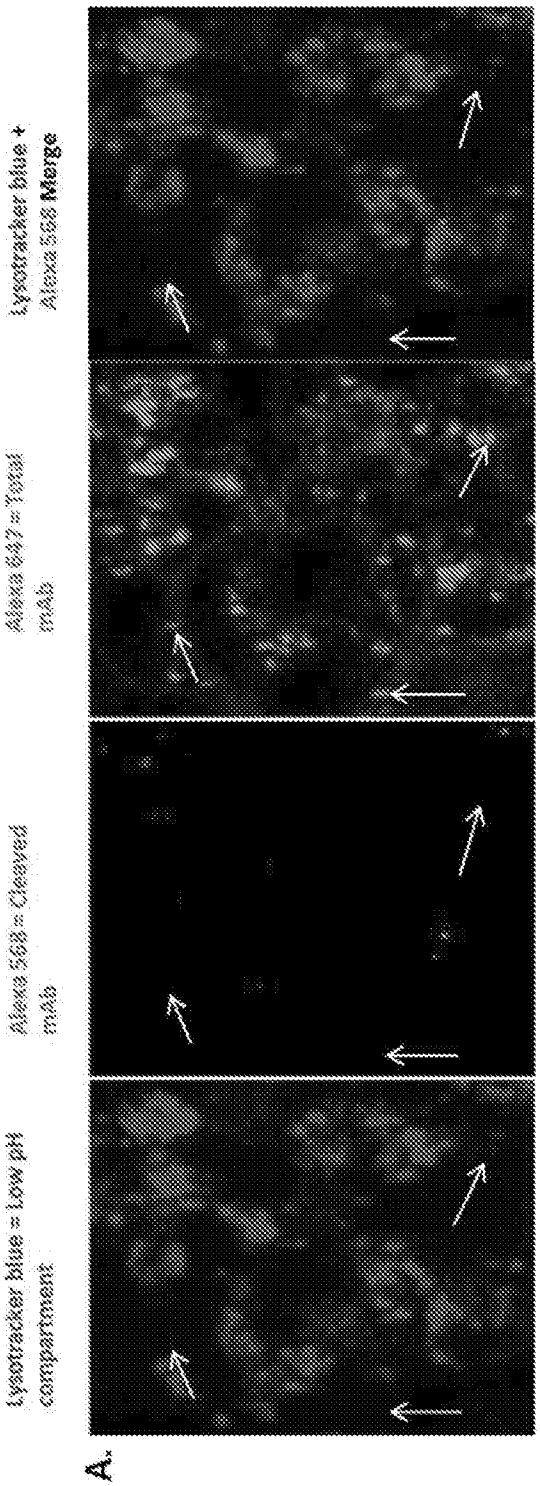

A.
Lysotracker blue = Low pH compartment
Alexa 568 = Cleaved mAb
Alexa 647 = Total mAb
Lysotracker blue + Alexa 568 Merge

FIG. 5A

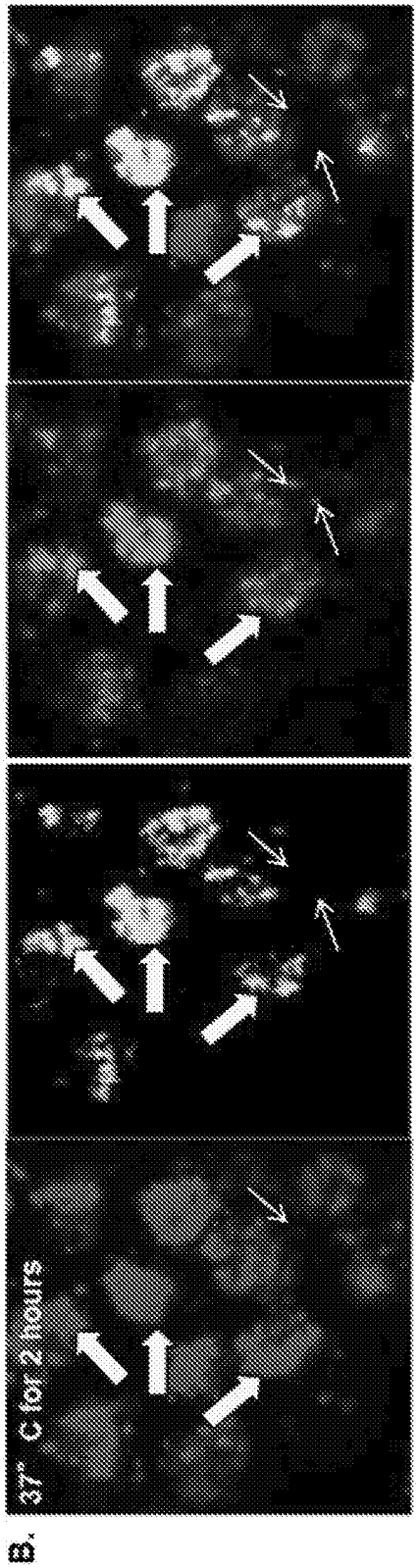

B.
37° C for 2 hours
Lysotracker blue = Low pH compartment
Alexa 568 = Cleaved mAb
Alexa 647 = Total mAb
Lysotracker blue + Alexa 568 Merge

FIG. 5B

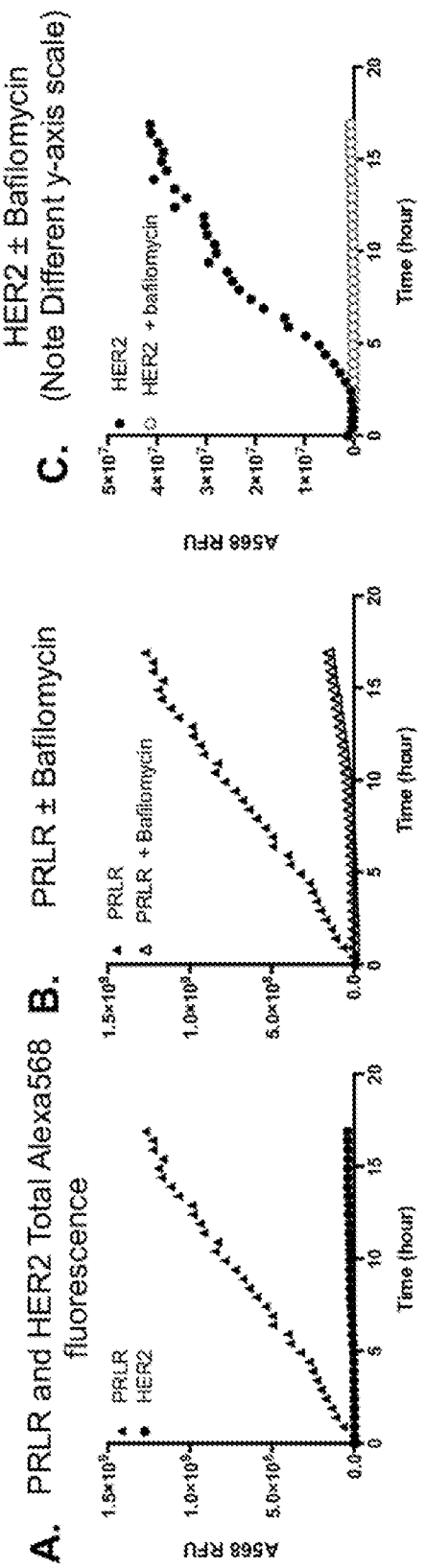
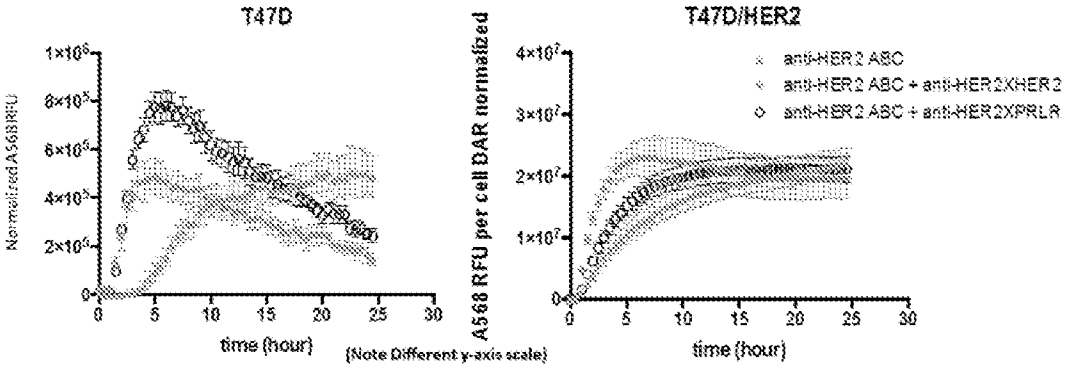
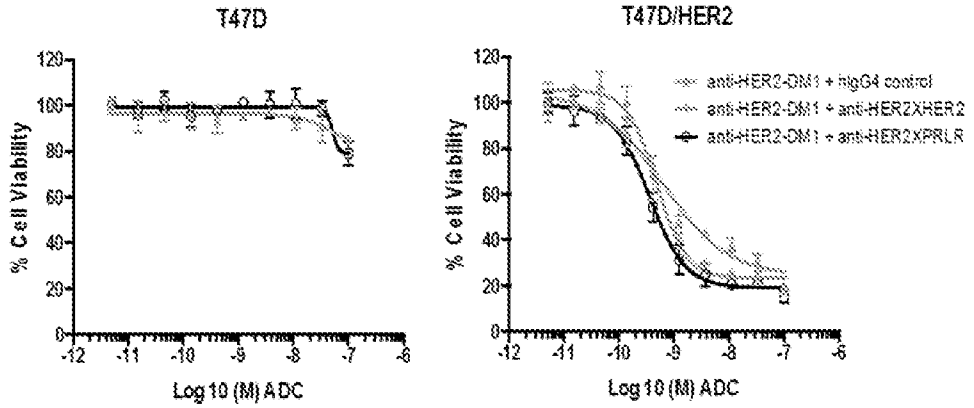


FIG. 6A, FIG. 6B, FIG. 6C

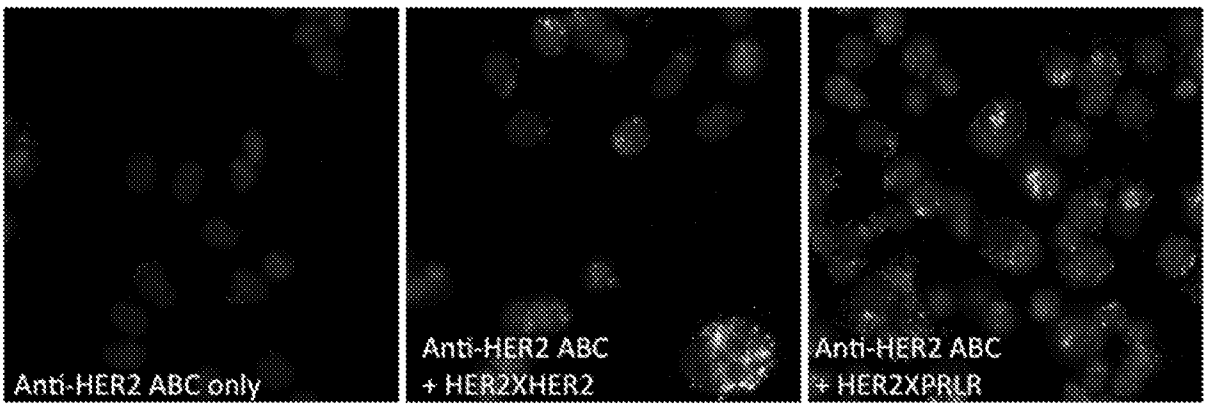
A.



B.



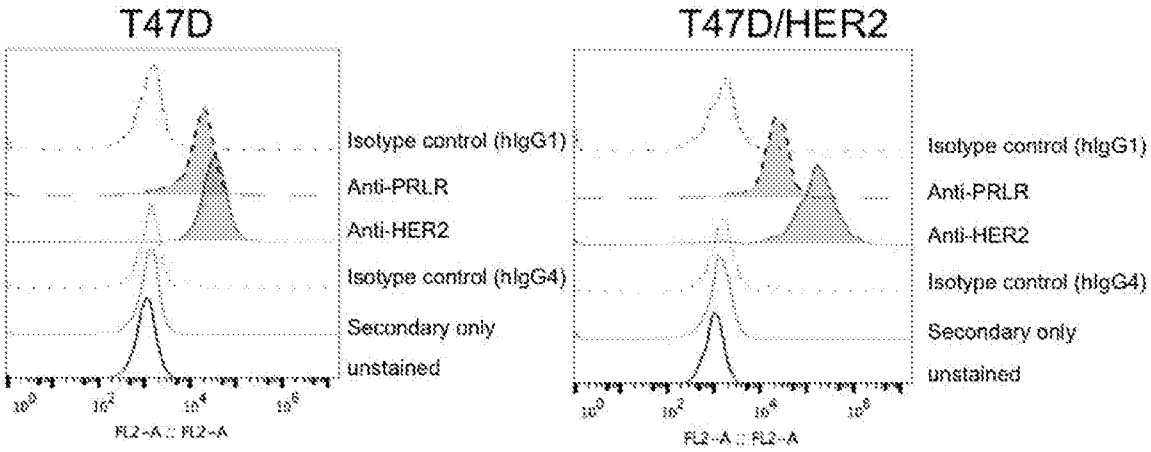
C.



T47D cells (2h at 37°C)
Cleaved anti-HER2-biosensor (Green)
Nucleus (Blue)

FIG. 7A, FIG. 7B, FIG. 7C

A



	T47D	T47D/HER2
Isotype control (hIgG1)	1	1
Anti-PRLR	15	16
Anti-HER2	33	87
Isotype control (hIgG4)	1	1
Secondary only	1	1
unstained	1	1

FIG. 8A

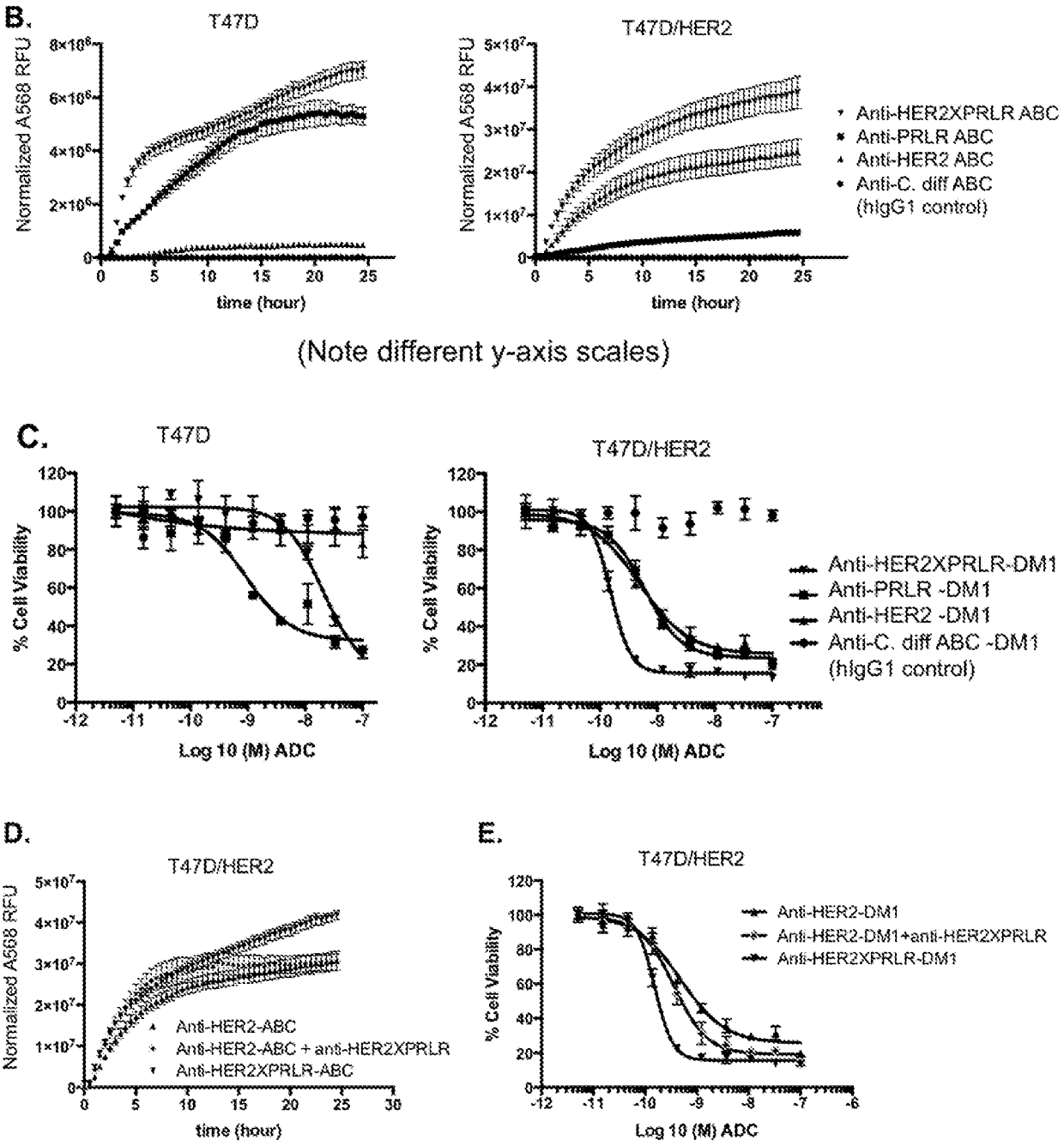
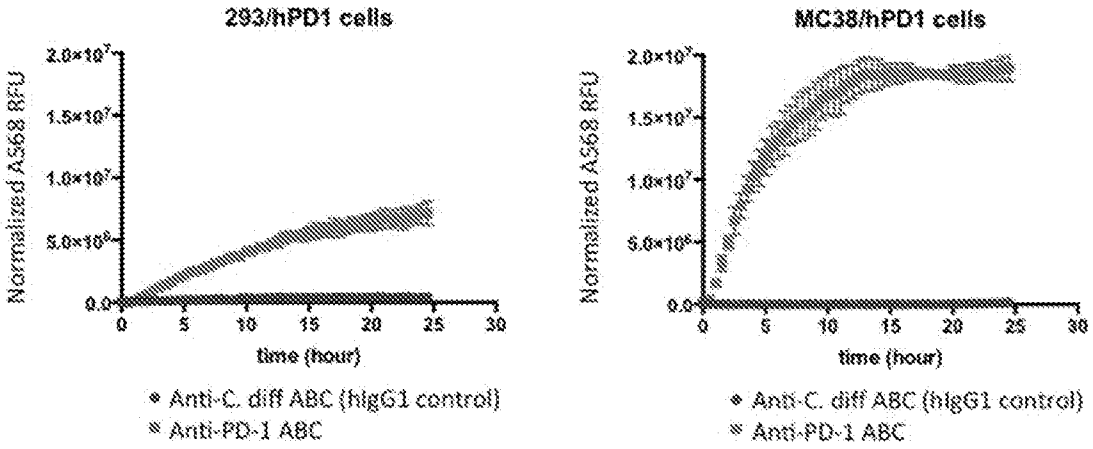
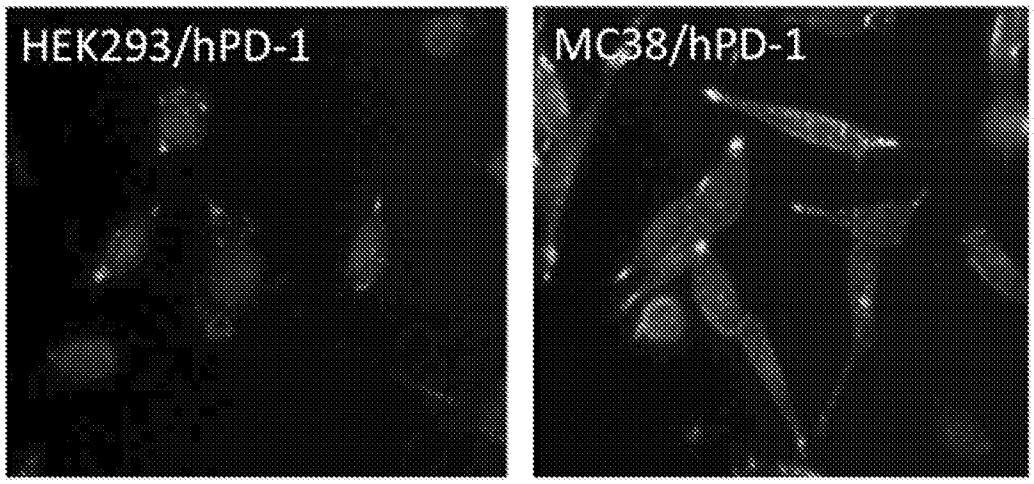


FIG. 8B, FIG. 8C, FIG. 8D, FIG. 8E

A.



B.



Incubate on 37°C for 2 hours
Cleaved anti-PD1-biosensor (Green) (Green + Red = Yellow)
Total anti-PD1-biosensor (Red), Nucleus (Blue)

FIG. 9A, FIG. 9B

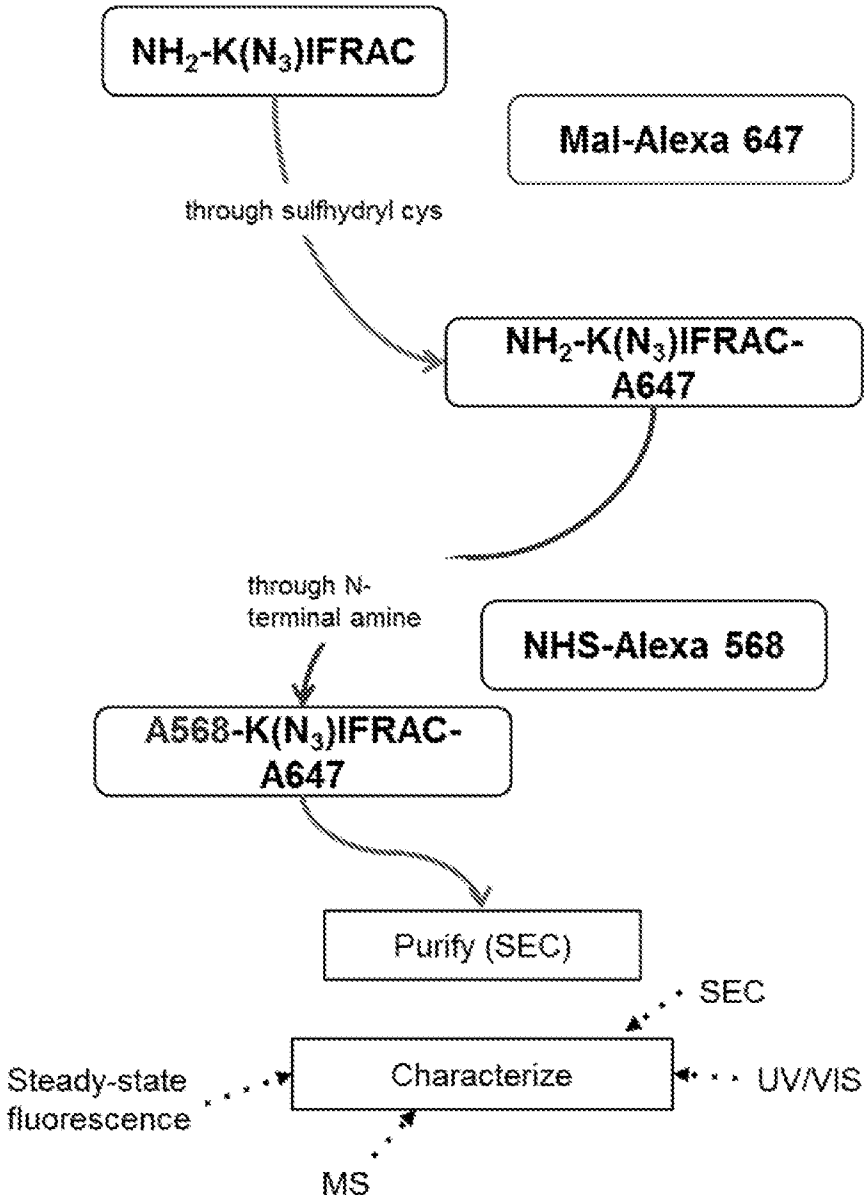

NH2-K(N3)IFRAC
Mal-Alexa 647
through sulfhydryl cys
NH2-K(N3)IFRAC-A647
through N-terminal amine
NHS-Alexa 568
A568-K(N3)IFRAC-A647
Purify (SEC)
SEC
Characterize
Steady-state fluorescence
UV/VIS
MS

FIG. 10

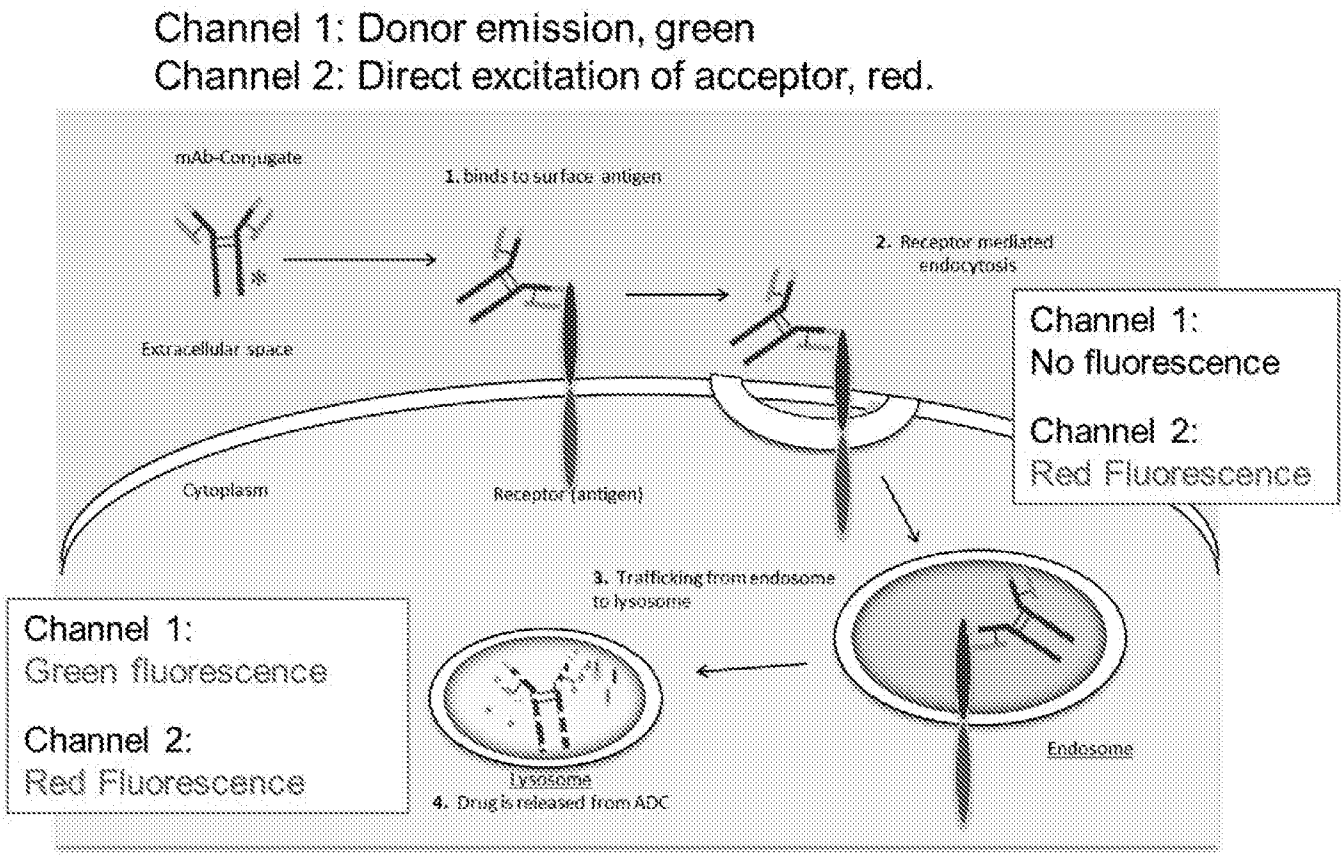

Channel 1: Donor emission, green
Channel 2: Direct excitation of acceptor, red.
mAb-Conjugate
1. binds to surface antigen
2. Receptor mediated endocytosis
Extracellular space
Cytoplasm
Receptor (antigen)
Channel 1:
No fluorescence
Channel 2:
Red Fluorescence
3. Trafficking from endosome to lysosome
Channel 1:
Green fluorescence
Channel 2:
Red Fluorescence
Endosome
Lysosome
4. Drug is released from ADC

FIG. 11

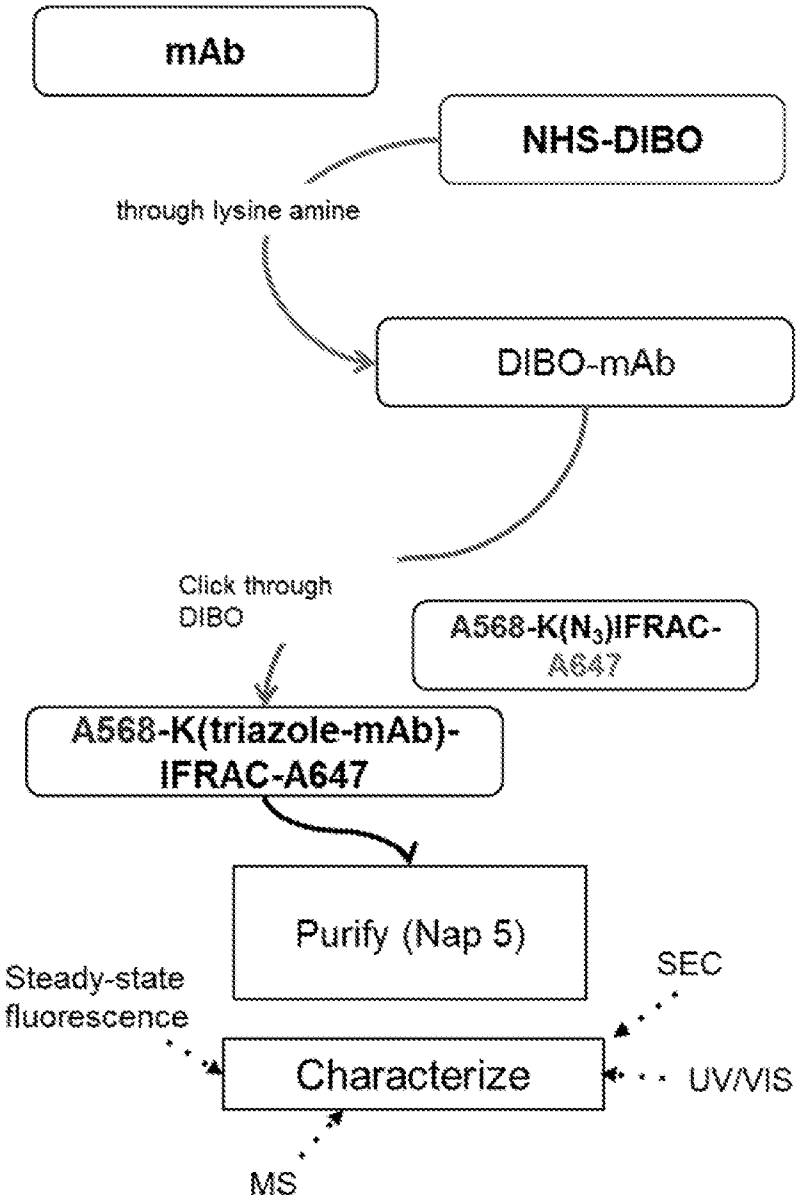

mAb
NHS-DIBO
through lysine amine
DIBO-mAb
Click through DIBO
A568-K(N3)IFRAC-A647
A568-K(triazole-mAb)-IFRAC-A647
Purify (Nap 5)
Steady-state fluorescence
SEC
Characterize
UV/VIS
MS

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/046771

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/543(2006.01)i, G01N 33/68(2006.01)i, G01N 33/58(2006.01)i, G01N 33/53(2006.01)i, G01N 21/31(2006.01)i, C07K 7/06(2006.01)i, A61K 47/68(2017.01)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)
G01N 33/543; C07K 7/06; G01N 33/58; G01N 033/569; C08F 112/08; G01N 033/554; G01N 33/68; G01N 33/53; G01N 21/31; A61K 47/68

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: dual labeled peptide, cleavage site, modification, cathepsin cleavage site, azido-lysine, conjugating antibody, protease, trafficking lysosome

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010-0273943 A1 (YAN, XIONGWEI et al.) 28 October 2010 See paragraphs [0009], [0017], [0023], [0037]-[0038], [0084], [0101]-[0112]; and figure 1.	1,4,14-21,24,42,46,50
Y		2-3,5-13,22-23,25-41,43-45,47-49,51
Y	MENARD, ROBERT et al., 'Cathepsins X and B display distinct activity profiles that can be exploited for inhibitor design', Biological Chemistry, May 2001, Vol. 382, pp. 839-845 See page 839; and table 1.	2,22,32,43-44,47-48
Y	US 2016-0041157 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 11 February 2016 See paragraphs [0069], [0077], [0094]-[0095], [0129], [0149]; and claims 13-14.	3,5-13,23,25-41,45
Y	MOODY, PAUL R. et al., 'Receptor crosslinking: a general method to trigger internalization and lysosomal targeting of therapeutic receptor: ligand complexes', Molecular Therapy, December 2015, Vol. 23, No. 12, pp. 1888-1898 See pages 1888, 1892.	39-41,49,51

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

* Special categories of cited documents:
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"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
20 December 2017 (20.12.2017)	**20 December 2017 (20.12.2017)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/046771

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2003-0166028 A1 (BURROUGHS-TENCZA, SARAH) 04 September 2003 See paragraphs [0100], [0121]; and claims 1, 5-19.	1-51

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2017/046771

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2003-0186214 A1	02/10/2003
		US 2006-0252109 A1	09/11/2006
		US 2007-0009440 A1	11/01/2007
		US 2011-0097758 A1	28/04/2011
		US 2014-0179901 A1	26/06/2014
		US 6979530 B2	27/12/2005
		US 8623995 B2	07/01/2014
		WO 02-094778 A2	28/11/2002
US 2016-0041157 A1	11/02/2016	WO 2014-145654 A1	18/09/2014
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		CA 2398583 A1	16/08/2001
		EP 1354201 A2	22/10/2003
		US 2002-0076741 A1	20/06/2002
		WO 01-59149 A2	16/08/2001